METHODS IN MOLECULAR BIOLOGY™ 461

Molecular Embryology

Methods and Protocols

Second Edition

Edited by

Paul Sharpe

Ivor Mason

Humana Press

Molecular Embryology

Second Edition

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Paul T. Sharpe

Department of Craniofacial Development, King's College London, London, UK

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Ivor Mason

MRC Centre for Developmental Neurobiology, King's College London, UK

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Editors
Paul T. Sharpe
Department of Craniofacial Development
King's College London
London, UK

Ivor Mason
MRC Centre for Developmental Neurobiology
King's College London
London, UK

Series Editor
John M. Walker
School of Life Sciences
University of Hertfordshire
Hatfield
Hertfordshire A110 9 AB, UK

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Preface

Most people have some interest in embryos; this probably results, in part, from their interest in understanding the biological origins of themselves and their offspring and, increasingly, concerns about how environmental changes such as pollution might affect human development. Obviously, ethical considerations preclude experimental studies of human embryos and, consequently, the developmental biologist has turned to other species to examine this process. Fortunately, the most significant conclusion to be drawn from the experimental embryology of the last two decades is the manner in which orthologous or closely related molecules are deployed to mediate similar developmental processes in both vertebrates and invertebrates. The molecular mechanisms regulating processes fundamental to most animals, such as axial patterning or axon guidance, are frequently conserved during evolution. (It is now widely believed that the differences between phyla and classes are the result of new genes, arising mostly by duplication and divergence of extant sequences, regulating the appearance of derived characters.)

Other vertebrates are obviously most likely to use the same developmental mechanisms as humans and, within the vertebrate subphylum, the apparent degree of conservation of developmental mechanism is considerable. It has long been recognized that particular vertebrate species offer either distinct advantages in investigating particular stages of development or are especially amenable to particular manipulations. No single animal can provide all the answers because not all types of experiments can be carried out on a single species. Traditionally, developmental biologists have worked on their particular experimental favorite, working, for example, solely on *Drosophila,* or *Xenopus,* or the mouse. In the last few years, this has started to change, and there are now increasing numbers of laboratories that have acquired the expertise to work on several different animals and are thus able to harness the experimental advantages of different developmental systems to address specific developmental questions. Alternatively, Developmental Biology departments are becoming organized so that they have expertise in several model organisms. It is the increasing necessity to be able to move between embryos of different vertebrate classes as a project progresses that prompted us to assemble *Molecular Embryology: Methods and Protocols, Second Edition.* We hope that it will allow researchers to familiarize

themselves with the various commonly studied vertebrate embryos, to make informed choices about which might be best suited to their investigations, and to understand the techniques by which they might be manipulated.

Sadly, while this book was going to press, Nigel Holder, one of its contributors, passed away. Nigel was an excellent developmental biologist, a founder of the Developmental Biology Research Group at King's College, and had recently been appointed to the Chair of Anatomy and Human Biology at University College London. He was both a colleague and friend to us and to many of the other contributors to this volume. He is greatly missed.

Paul T. Sharpe
Ivor Mason

Preface to Second Edition

The five years or so between the current and first editions of this volume have seen perhaps the greatest period in growth and productivity in the field of Developmental Biology. This is reflected in the addition of new Chapters detailsing techniques that have arisen during the intervening period including RNA interference, electroporation, "EC culture" of chick embryos, electroporation, new approaches for efficient production of transgenic zebrafish and microarrays.

We also thank the authors of other Chapters for updating their contributions since the last Edition. Some Chapters remain entirely unchanged, reflecting one of the great delights of this field, namely that "classical" techniques, unchanged for decades, are routinely employed alongside and to complement "state-of-the-art" approaches.

Finally, we would like to express our gratitude to The MRC, The Wellcome Trust and The BBSRC for supporting the research in our own laboratories. In addition, IM would like to thank the Leverhulme Trust for providing him with a Research Fellowship, which greatly facilitated the completion of this volume.

Paul Sharpe and Ivor Mason
London, June 2006

Contents

Contributors

Lúcia E. Alvares • *Department of Craniofacial Development, King's College London, London, UK*

Enrique Amaya • *The Healing Foundation Centre, Michael Smith Building, Faculty of Life Sciences, University of Manchester, Manchester, UK*

Bruce Blumberg • *Gene Expression Laboratory, Stem Cell Research Center, and Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, California, USA*

Victoria Bostock • *School of Biological Sciences, University of Manchester, Manchester, UK*

Gerard Brady • *School of Biological Sciences, University of Manchester, Manchester, UK*

Jane Brennan • *Centre for Integrative Physiology, University of Edinburgh, Edinburgh, UK*

Clare Brunet • *School of Biological Sciences, University of Manchester, Manchester, UK*

David Chambers • *MRC Centre for Developmental Neurobiology, King's College London, London, UK*

Jonathan D. W. Clarke • *Department of Anatomy and Developmental Biology, University College, London, UK*

David L. Cockroft • *Formerly Imperial Cancer Research Fund, Oxford, UK*

Peter D. Currie • *Muscle Development Laboratory, Victor Chang Cardiac Research Institute, Sydney, Australia*

Michael J. Depew • *Department of Craniofacial Development, King's College London, London, UK*

Clive Dickson • *Retired, Cancer Research UK, London, UK*

Susanne Dietrich • *Department of Craniofacial Development, King's College London, London, UK*

Amira El-Hanfy • *Department of Craniofacial Development, King's College London, London, UK*

Tamira Elul • *Department of Biology, University of Virginia, Charlottesville, Virginia, USA*

Philippa Francis-West • *Department of Craniofacial Development, King's College London, London, UK*

Jun-ichi Funahashi • *Department of Molecular Neurobiology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan*
Monica Gordon • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*
Clemens Grabher • *Developmental Biology Program, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany*
Anthony Graham • *MRC Centre for Developmental Neurobiology, King's College London, London, UK*
Andy Greenfield • *MRC Mammalian Genetics Unit, Harwell, UK*
Sarah Guthrie • *MRC Centre for Developmental Neurobiology, King's College London, London, UK*
Anna-Katerina Hadjantonakis • *Memorial Sloan Kettering Cancer Center, New York, USA*
Brigid L. M. Hogan • *Cell and Molecular Biology Program, Duke University, Durham, North Carolina, USA*
Nigel Holder • *Deceased, Department of Anatomy and Developmental Biology, University College, London, UK*
Peter W. H. Holland • *Department of Zoology, University of Oxford, Oxford, UK*
Amata Hornbruch • *Retired, MRC Centre for Developmental Neurobiology, King's College London, London, UK*
Philip W. Ingham • *Centre for Developmental Genetics, University of Sheffield, Sheffield, UK*
Carol Irving • *Department of Anatomy and Developmental Biology, University College, London, UK*
Shoko Ishibashi • *The Healing Foundation Centre, Michael Smith Building, Faculty of Life Sciences, University of Manchester, Manchester, UK*
Juan Carlos Izpisúa Belmonte • *Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla, California, USA and The Center of Regenerative Medicine in Barcelona, Barcelona, Spain*
C. Michael Jones • *Centre for Molecular Medicine, Singapore*
Katherine Joubin • *National Institute for Medical Research, Mill Hill, London, UK*
Ray Keller • *Department of Biology, University of Virginia, Charlottesville, Virginia, USA*
Andrew Kent • *School of Biomedical and Health Sciences, King's College, University of London, London, UK*
Poh-Lynn Khoo • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*

Kristen L. Kroll • *Washington University School of Medicine, Department of Molecular Biology and Pharmacology, St. Louis, Missouri, USA*
Tilo Kunath • *Institute for Stem Cell Research, University of Edinburgh, Edinburgh, UK*
Patricia A. Labosky • *Center for Stem Cell Biology, Department of Cell and Developmental Biology, Vanderbilt University, Nashville, Tennessee, USA*
Nicole M. Le Douarin • *Instituit d'Embryologie Cellulaire et Moleculaire, College de France, Nogent-sur-Marne, France*
Cairine Logan • *Department of Cell Biology and Anatomy, University of Calgary, Calgary, Canada*
Andrew Lumsden • *MRC Centre for Developmental Neurobiology, King's College London, London, UK*
Corinne Lours • *Department of Craniofacial Development, King's College London, London, UK*
Marla B. Luskin • *Department of Cell Biology, Emory University, Atlanta, Georgia, USA*
Malcolm Maden • *MRC Centre for Developmental Neurobiology, King's College London, London, UK*
Radma Mahmood • *Department of Pathology, Albert Einstein College of Medicine, New York, USA*
Paul Martin • *Departments of Physiology and Biochemistry, School of Medical Sciences, University of Bristol, Bristol, UK*
Ivor Mason • *MRC Centre for Developmental Neurobiology, King's College London, London, UK*
Gillian M. Morris-Kay • *Retired, Department of Human Anatomy and Genetics, University of Oxford, Oxford, UK*
David Murphy • *Laboratories of Integrative Neurosciences and Endocrinology, University of Bristol, Bristol, UK*
Harukazu Nakamura • *Department of Molecular Neurobiology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan*
András Nagy • *Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada*
César Núñez • *School of Biological Sciences, University of Manchester, Manchester, UK*
Maala Parameswaran • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*
Gregory Pelka • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*
Melinda Pirity • *Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada*

Marysia Placzek • *Centre for Developmental Genetics, University of Sheffield, Sheffield, UK*
Ann Poznanski • *Department of Biology, University of Virginia, Charlottesville, Virginia, USA*
Clare Pritchard • *MRC Mammalian Genetics Unit, Harwell, UK*
Gabriel A. Quinlan • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*
Tania Radziewic • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*
Maria Rex • *Institute of Genetics, Queen's Medical Centre, University of Nottingham, Nottingham, UK*
Carin Sahlberg • *Institute of Biotechnology, University of Helsinki, Helsinki, Finland*
Ebrahim Sakhinia • *School of Biological Sciences, University of Manchester, Manchester, UK*
Thomas F. Schilling • *Department of Developmental and Cell Biology, University of California, Irvine, California, USA*
Paul J. Scotting • *Institute of Genetics, Queen's Medical Centre, University of Nottingham, Nottingham, UK*
Paul T. Sharpe • *Department of Craniofacial Development, King's College London, London, UK*
William C. Skarnes • *Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK*
James C. Smith • *CRC/Wellcome Trust Gurdon Institute, University of Cambridge, Cambridge, UK*
Derek Stemple • *Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK*
Claudio D. Stern • *Department of Anatomy and Developmental Biology, University College, London, UK*
Andrea Streit • *Department of Craniofacial Development, King's College London, London, UK*
Karin Sturm • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*
Patrick P. L. Tam • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*
Marie-Aimée Teillet • *Instituit d'Embryologie Cellulaire et Moleculaire, College de France, Nogent-sur-Marne, France*
Irma Thesleff • *Institute of Biotechnology, University of Helsinki, Helsinki, Finland*
Cheryl Tickle • *Division of Cell and Developmental Biology, School of Life Sciences, University of Dundee, Dundee, UK*

Paul A. Trainor • *Stowers Institute, Kansas City, Kansas, USA*
Antonio Tugores • *Gene Expression Laboratory, Stem Cell Research Center, and Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, California, USA*
Peter Underhill • *MRC Mammalian Genetics Unit, Harwell, UK*
Hiroshi Wada • *Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan*
Catherine M. Watson • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*
Damian L. Weaver • *School of Biological Sciences, University of Manchester, Manchester, UK*
Richard J. T. Wingate • *MRC Centre for Developmental Neurobiology, King's College London, London, UK*
Joachim Wittbrodt • *Developmental Biology Program, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany*
Nicole Wong • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*
Qiling Xu • *National Institute for Medical Research, Mill Hill, London, UK*
Sheila X. Zhou • *Embryology Unit, Children's Medical Research Institute and University of Sydney, Sydney, Australia*
Catherine Ziller • *Instituit d'Embryologie Cellulaire et Moleculaire, College de France, Nogent-sur-Marne, France*

1

The Mouse as a Developmental Model

Paul T. Sharpe

The laboratory mouse, *Mus musculus*, is the developmental biologist's mammal of choice for studies of development. Its embryology and genetics have been extensively studied for over 100 years. However, the advent of *in vivo* gene manipulation in the last few years has established the mouse as probably the single most powerful animal system in vertebrate biology.

Mouse developmental biology, as it exists today, has its origins in genetics and embryology. These days it is hard to separate mouse development from mouse genetics, the two having become intertwined as genetics provides increasingly more powerful tools for studying development.

By the early 1980s, progress in understanding mouse embryology had started to stagnate. Molecular biology had provided the tools to clone genes, but identification of important genes in mouse development from the estimated 80,000–100,000 genes in the genome seemed almost intractable. One breakthrough that was to change the course of developmental studies came in 1984 with the discovery of the homeobox in flies, and the realization that mice and other vertebrates had the same genes ***(1–3)***. It seems hard to believe now, when evolutionary conservation of genes and functions between in vertebrates and mammals is the norm, that in the 1980s, many developmental biologists believed there would be no such homologies, and even if certain gene (protein) sequences, such as the homeobox, were conserved, it was believed they would have completely different functions in mammals. Evolution of developmental mechanisms has provided developmental biologists with their most powerful tool: conservation of gene function.

The ability to clone potentially important developmental genes by screening mouse libraries with *Drosophila* gene probes, together with the advent of in situ hybridization to study their spatial expression in embryos, provided the impetus for the explosion in mouse developmental studies over the last 15 years.

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Armed with potentially interesting developmental genes, mouse embryologists were able to begin to utilize transgenesis to investigate their functions and regulation. The first transgenic mice were produced using pronuclear injection in 1980–1981 in the laboratories of Frank Ruddle at Yale ***(4)*** and Frank Costantini and Elizabeth Lacy in Oxford ***(5)***, and the methodology they described is now widely used. The limitations of studying developmental gene function using transgenic animals produced by pronuclear injection soon became apparent, since it allowed only gain-of-function gene manipulations, which were not always informative. Concurrent with the progress in identifying important regulatory genes in mouse development, groups working on the pluripotentiality of mouse embryo cells produced the first embryonic stem cells (ES cells) ***(6,7)***. The isolation of ES cells and the subsequent development of gene targeting thus came at the perfect time. Mouse developmental biologists had the genes and their expression patterns but could only surmise the functions.

Gene knockout provided the missing tool in the bag, enabling gene function to be assayed directly *in vivo*. Although perhaps not fully appreciated at the time, these two strands were to come together in the most dramatic way to provide the basis for understanding mouse development and to start to approach that of *Drosophila*. ES cells also provided a way of identifying important developmental genes based on function rather than homology by gene trapping, and large-scale gene traps have been undertaken in several laboratories (Chapter 9).

The prospect of using large-scale mutagenesis to identify mouse developmental genes, as used in *Drosophila* and more recently in zebrafish, was not considered viable for many years. Although mouse developmental mutants, generated by traditional mutagenesis methods, over the years, proved a valuable resource, the advent of targeted mutation techniques greatly reduced the need for traditionally generated mutants. However, more recently, the possibility of large-scale mutagenesis screens for developmental genes has been revisited by several groups using *N*ethyl-*N*-nitrosourea (ENU) to generate point mutations. Such screens, in conjunction with the mouse genome sequence, can now provide an important contribution to mouse developmental biology in the future. More recently, use of conditonal mutant mice with Cre-expressing lines and targeted "floxed" alleles has become the technique of choice for studying mouse gene function. As more Cre mice are produced, including inducible lines, thus form of mouse genetics to study gene function in specific tissues and cells will undoubtedly increase.

Basic development of mouse embryos has been more than adequately described in several landmark texts, such as *The Mouse* by Roberts Rugh ***(8)***, *The House Mouse* by Karl Theiler ***(9)***, *The Atlas of Mouse Development* by M.H. Kaufman ***(10)***, and *Mouse Development* by Janet Rossant and Patrick Tam ***(11)***; therefore, we did not consider it necessary for this to be duplicated in this text. The chapters in this section on the mouse as a developmental model provide the background

and detail to some of the latest manipulation techniques. We recommend that readers consult *Manipulating the Mouse Embryo* by Hogan et al. ***(12)***, which provides considerable detail on the production of transgenic mice.

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2

Culture of Postimplantation Mouse Embryos

Paul Martin and David L. Cockroft

1. Introduction

A major disadvantage of working with postimplantation mammalian embryos is their relative inaccessibility to experimentation while they develop within the maternal uterus. Two techniques allow us to get around this problem to a large extent. The first, which is the subject of this chapter, can be used for mouse embryos explanted between 7.5 d of gestation (E7) and 12.5 d of gestation (E12), and involves dissecting embryos from the uterus and culturing them in roller bottles ***(1)***. In this way, embryos can be surgically or chemically manipulated or labeled and will develop quite normally in culture for periods of 12–60 h, depending on the stage at explantation ***(2,3)***. The second technique, which allows experimentation on more advanced stages, is that of *exo utero* or open uterus surgery, in which fetuses are suspended in the fluid-filled abdominal cavity of the female mouse while retaining their placental attachment to the uterine wall ***(4)***. This procedure is suitable for fetuses at 12.5 d of gestation (E12) and beyond. This chapter will focus on the techniques for culturing E11 mouse embryos with open yolk sacs at limb-bud stages ***(5)*** and will also include protocols for culturing earlier stage embryos. We will describe studies in the mouse, but similar manipulations are possible with rat embryos, bearing in mind that they are generally 1 or 2 d behind mouse development; for example, an E11 rat embryo closely resembles a mouse embryo between E9 and E10.

2. Materials

1. Microscope: A good-quality dissecting microscope is essential, preferably with both transmitted (bright- and dark-field) and incident (e.g., fiber-optic) illumination—we use Wild microscopes with an overall magnification range of 6–50x.
2. Instruments: **Table 1** shows the tools you will need and what they should be used for.

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Table 1
Tools for the Job

Items	Use	Remarks
1 Pair coarse scissors, 1 pair coarse forceps	Opening the abdominal cavity, when explanting embryos or bleeding rats	Can also be used for removing uterus
1 Pair fine scissors	Removing uterus	
2 Pairs fine serrated, or watchmaker forceps	Removing uterus, opening uterus, exposing aorta during bleeding	
2 Pairs watchmaker's forceps (no. 5), carefully ground to a tip diameter of 0.05–0.1 mm (*see* **Note 1**)	Removing decidua of E7–E9 embryos, opening Reichert's membrane	Do not use for opening uterus and so on, since they are too delicate and easily damaged by such heavy work
Iridectomy scissors (large)	Removing decidua/Reichert's membrane of E10–E11 embryos	Can use watchmaker's forceps instead
Iridectomy scissors (small)	Opening yolk sac of E10–E11 embryos	
Hemostat (Spencer-Wells forceps)	Holding colon out of the way during bleeding	

3. Dishes: 5-cm plastic Petri dishes (Sterilin) are suitable for all the operations of explanting embryos at all ages described here. You will need 3–6/litter (more for older embryos); always transfer embryos to a fresh dish if the medium becomes cloudy with blood, and so forth, and ensure that dishes contain enough medium to submerge embryos completely.
4. Saline recipes—*see* **Table 2**—we use a 1:1 mix of Tyrode's and Earle's salts (=explanting saline) for explanting open yolk sac embryos. To this, we add extra bicarbonate and glucose (=culture saline) when it is used in the culture medium, in order to equilibrate it with the CO_2 in the gas mixture used during culture and to increase the energy sources.

Embryos cultured with closed yolk sacs are explanted in PB1, and cultured in pure rat serum. The pH of all media should be about 7.2.

5. Rat serum: This is the basic culture medium for all postimplantation embryo culture, and is used undiluted for embryos cultured with closed yolk sacs (E7–E10 mouse) ***(6)***, or diluted to 25% for culture with open yolk sacs (E10–E11 mouse) ***(5)***. Rat serum is available commercially, at a price, but because of the method of preparation, it is inferior, particularly for the culture of the earlier stages ***(7,8)***, to what you can prepare yourself (*see* **Subheading 3.1.3.**).

Table 2
Media

	Explanting saline, g/L	Culture saline, g/L	PB1 medium, g/L
NaCl	6.9	6.9	8.0
KCl	0.3	0.3	0.2
$MgSO_4 \cdot 7H_2O$	0.1	0.1	–
$MgCl_2 \cdot 6H_2O$	0.05	0.05	0.1
$NaH_2PO_4 \cdot 2H_2O$	0.1	0.1	–
$Na_2HPO_4 \cdot 12H_2O$	–	–	2.88
KH_2PO_4	–	–	0.2
$CaCl_2 \cdot 2H_2O$	0.265	0.265	0.13
Glucose	1.5	2.0	1.0
Na pyruvate	–	–	0.036
$NaHCO_3$	0.5	2.0	–
Fetal calf serum	–	–	10% v/v

6. Sterility: Provided all dishes, instruments, and media used are sterile, we find that explantations and experimental manipulations can be performed on the open bench without problems of infection.

3. Methods

3.1. General

3.1.1. Obtaining Timed Pregnant Animals

The best way to obtain embryos at precisely the stage of development needed is to oversee the mouse mating procedure yourself. If only small numbers of litters are required (1–10 litters/wk), then a colony of 5 studs, with virgin females bought in as needed (say 20 females/2 wk) is quite satisfactory. For larger numbers of litters, it is probably more economical to set up your own breeding colony, with weaned newborns grown up to replace the female stock. We usually mate female mice at 8–12 wk old, and retire our breeding studs before they are a year old. With albino varieties of mice, it is relatively easy to detect the one day in the cycle of 4 or 5 d that females are in estrus, because the vagina appears pink and swollen by comparison with nonestrus females. In nonalbino strains it is harder to distinguish estrus, and if this cannot be judged, then three or four times as many breeding pairs should be set up to make allowance for the nonestrus females. We introduce females individually to studs late in the afternoon, and check for vaginal plugs the following morning. The hard whitish plug occluding the vagina is usually very obvious, but occasionally it is located deep, and this

can be checked using a round-ended probe. If you have too few studs for paired matings, then it is possible to put up to three females with one stud, but plugging efficiency and litter number will sometimes be compromised. Generally, we expect 30–80% of our females judged to be in estrus to become pregnant. Depending on strain of mouse, the litters will contain from 5–15 embryos.

3.1.2. Killing Pregnant Animals

In the United Kingdom there are "Home Office" (HO) regulations stipulating methods of killing animals. The most satisfactory HO Schedule 1 method for killing a pregnant mouse is by cervical dislocation. This is done humanely by placing the fingers of one hand behind the animal's neck and stretching it by pulling the mouse's tail with your other hand. Alternative methods of killing include overdoses of anesthetic, but this may have adverse effects on the later health of the embryos.

3.1.3. Making Rat Serum

Rats (preferably fasted overnight) should be anesthetized with halothane or (if permitted) ether. Lay the anesthetized animal on its back, douse it with 70% ethanol, and open the abdominal cavity. Displace the small intestines to the left, pull the colon to the right, and hold it there with a hemostat (Spencer-Wells forceps). Expose the dorsal aorta with fine forceps (it is generally smaller and paler than the adjacent vein), and insert, bevel down, a 19- or 21-gage needle connected to a 10- or 20-mL syringe, with all air excluded. Gently withdraw blood until the rat stops breathing, after which only a further milliliter or two can be obtained. Remove the needle and transfer the blood to a 12- or 15-mL centrifuge tube, running it gently down the side of the tube. Immediately spin the tube for at least 5 min at 2000*g*. This will precipitate the blood cells, and a whitish clot will form in the supernatant serum. When all rats have been bled (20–30 is a reasonable number for a single session), squeeze the clots with long slender forceps to expel serum, spin again as above, and decant the serum into 50-ml centrifuge tubes with a pipet. Spin again to bring down any persisting blood cells, and then pool the day's serum in a suitable container (e.g., a 250-mL tissue-culture flask). Add antibiotics if desired (e.g., 100 µg/mL Streptomycin, 100 IU/mL penicillin), and aliquot the serum in quantities of 5–20 mL before freezing. It will keep for several months at −20°C, or years at −70°C (freeze first at −20°C, and then transfer to −70°C; otherwise tubes may crack). *See* **refs. *3*** and ***9*** for further information on rat bleeding.

3.1.4. Preparing Rat Serum for Culture

Thaw the serum at room temperature or in a 37°C water bath, then heat-inactivate it for 30 min at 56°C. Gassing the serum while hot will help drive off any persisting

anesthetic dissolved in it. A residual clot often appears at this stage, and can be removed by centrifugation or, more effectively, by filtration through a 0.45-µm syringe filter (Sigma). Serum is then ready for use, either pure or diluted, according to embryonic age.

3.2. Explanting E11 Embryos (Cultured with Opened Yolk Sacs)

1. Although the procedure for explanting with open yolk sacs described and illustrated here is for E11 embryos, it is equally applicable to E10 embryos. The freshly killed pregnant animal is laid on its back and the abdomen doused with 70% alcohol. The abdominal skin can be pinched between thumb and forefinger of both hands, and the skin torn back towards head and tail to reveal a clean peritoneal surface, which can then be opened with scissors and forceps. This method avoids contaminating the abdominal cavity with hair. Alternatively, all abdominal layers can be opened at once with a large U-shaped scissor incision, with the two ends of the U at the hindlimbs.
2. The uterus with embryos is then lifted clear of the abdominal cavity. It can be held with forceps midway along one horn at a site between two embryos and needs to be severed with scissors at the tip of that horn (near the ovary), then where it communicates with the cervix (without separating the two horns), and finally at the tip of the second horn. As this is being done, the uterus can also be trimmed free of mesentery and fat.
3. The whole uterus should then be rinsed in PBS and remaining mesentery cleared away before transfer to a fresh dish of PBS. The uterus can now be opened to expose the embryos. This is done by carefully tearing along the antimesometrial wall of the uterus with fine forceps (**Fig. 1**). It is easy to damage embryos at this stage, and the best way to avoid this is to keep the two pairs of forceps close to one another, so that as they tug apart, all the effort goes into tearing the uterus and not squashing the embryos. Once the uterus is open, the embryos (conceptuses) appear like peas in a pod attached to the uterus only in their placental regions. The easiest way to free this attachment is to hold the uterus tightly with one pair of forceps, slip the other pair of forceps on either side of the uterine sheet, and then drag them across each placental attachment in turn, gently teasing the embryos free (**Fig. 2**).
4. The individual conceptuses can now be transferred by pipet to a fresh dish of explanting saline, and their decidua removed. The decidua of an E11 embryo is thin and is best peeled off, beginning on the side opposite the placenta, by shallow pinching with two pairs of fine forceps, which allows a gentle tearing action. Often the very thin and transparent Reichert's membrane, which sits beneath the decidua and clings to the yolk sac, will rupture during this operation, in which case it is easy to remove it with the decidua. When the decidua has been torn back to about the level of the placenta, it can be tidied up by trimming off with a pair of iridectomy scissors (**Fig. 3**). If Reichert's membrane is still intact, it must be torn open and trimmed back to the placenta also.
5. Next, the yolk sac must be cut with fine iridectomy scissors, close to where it abuts the placenta, taking care to avoid damaging any of the larger yolk sac vessels (**Fig. 4**).

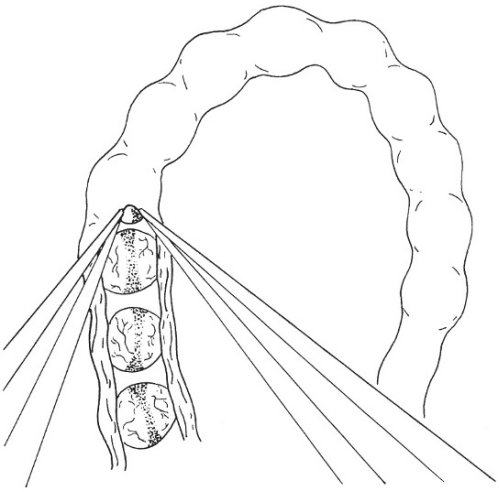

Fig. 1. The uterus (Ell) is gently torn open along its antimesometrial wall using fine forceps to expose the conceptuses.

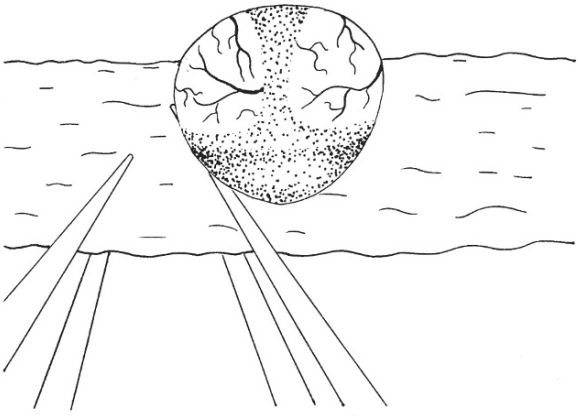

Fig. 2. Conceptuses are scraped free of their placental attachment to the uterus by drawing a fine pair of forceps between conceptus and uterus.

First use two pairs of watchmaker's forceps to make a small hole in the yolk sac adjacent to the placenta, to allow access for the iridectomy scissors. The yolk sac should not be cut completely free of the placenta—rather cut about 4/5 of the way around, creating enough of an opening for the embryo to be pulled out of the yolk sac head first. The region of yolk sac left uncut should be that adjacent to the tail of the embryo—otherwise delivery is made significantly more difficult. The embryo is drawn out of the yolk sac by pulling on the amniotic membrane overlying

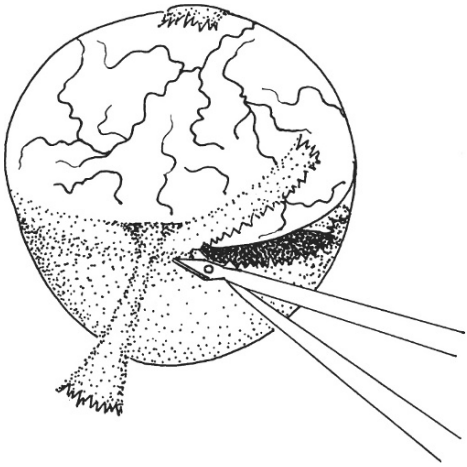

Fig. 3. The decidua and thin Reichert's membrane overlying the E11 yolk sac are trimmed back level with the placenta.

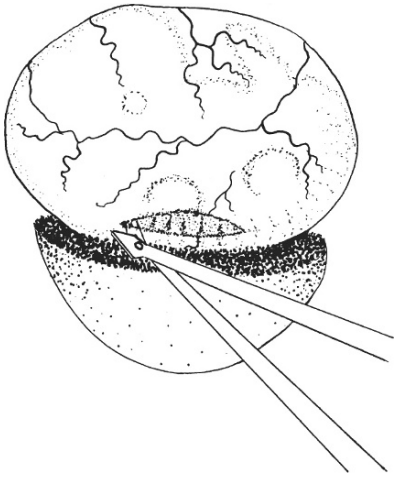

Fig. 4. A small hole is made in the yolk sac close to where it abuts the placenta and adjacent to the forelimb bud. Iridectomy scissors are then used to cut around 4/5 of the base of the yolk sac.

the embryo's head with one pair of forceps, while holding the mouth of the yolk-sac incision with a second pair of forceps (**Fig. 5**). After the head is outside the yolk sac, it is necessary to rupture the amniotic membrane in order to exteriorize the rest of the embryo. Last of all, the yolk sac and amniotic membranes are flipped under the tail, and the embryo is now available for manipulations or immediate culture.

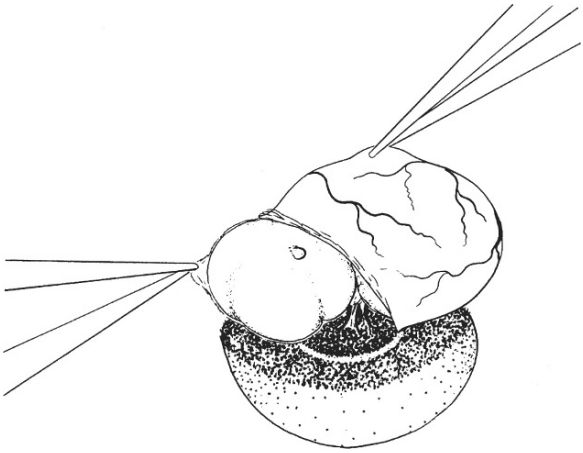

Fig. 5. The E11 embryo is delivered from the yolk sac by tugging on the amniotic membrane overlying the embryo's head.

6. If the developmental stage of the embryos is critical for your experiment, you should stage them now (*see* **Note 2**).
7. All of the above procedures and any planned subsequent manipulations (*see* **Note 5**) should take no more than about 2 h, or subsequent development in culture will be compromised. This generally imposes a limit of no more than 2 litters of embryos/culture session. When embryos are ready for culture, they are individually transferred into 50-mL Falcon tubes containing 5 mL of 25% (v/v) rat serum in culture saline (*see* **Table 2** for recipes). To ensure a gaseous seal, we apply a thin coat of silicone vacuum grease (Dow-Corning) to the rim of the tube. The tube is then gassed with 95% O_2, 5% CO_2 (for 1 min at a gas flow rate sufficient to ruffle the surface of the medium gently—**Fig. 6**). The Falcon lid is then screwed tight and the tube is placed in a 37°C incubator containing rollers-rotating at 30 rpm. We find that the custom-made BTC roller-incubators (*see* **Note 9**) are excellent for this purpose. By carefully stacking Falcon tubes in the incubator, it is possible to culture up to 18 tubes (embryos) at one time.
8. The health of embryos can be determined at any stage during culture by brief removal of the Falcon tube from the incubator and viewing under a dissecting microscope with transmitted light. Because the walls of the tube are translucent, it is easy to check how well the heart is beating, and usually whether there is good circulation in the larger vessels of the yolk sac and over the brain. Also, check whether there is any swelling of the pericardium or blistering of distal limb ectoderm (usually signs that the embryo is faring badly). In any case, embryos should be regassed every 12 h or so. Only badly damaged embryos will fail to culture successfully for 12 h and, of these, 90% will successfully make it through to 24 h: From this stage on, survival rates get steadily worse with only 1 in 3 embryos

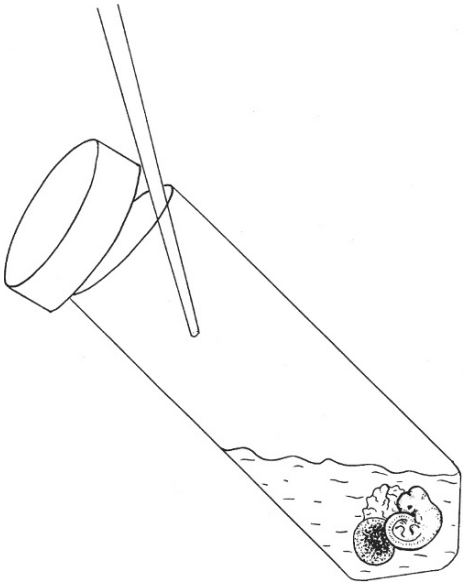

Fig. 6. Embryos are transferred to a roller bottle containing culture medium and gassed with 95% oxygen before culture begins.

healthy at 36 h and 1 in 6 or 7 making it through to 48 h. **Figure 7A** shows the appearance of E11 embryos before and after culture for 24 h.

3.3. Explanting E9 Embryos (Cultured with Closed Yolk Sacs)

1. Embryos younger than E10 at explantation are cultured with the visceral yolk sac intact. The initial stages of killing the mouse, opening the uterus, and separating the conceptuses are the same as for the E11 embryos, though of course the conceptuses are smaller.
2. Removal of the decidua of E9 embryos is similar to the E11 procedure, since it already forms a relatively thin layer over the conceptus. It is advisable to remove all of the decidua, starting at the equator and tearing toward and over the placenta, but being careful not to damage the latter, since it has an extensive blood circulation at this stage.
3. Next, open Reichert's membrane; although this is thin and transparent, it is mostly overlain with a layer of trophoblast and blood cells, and it may be necessary to pick through this layer before rupturing Reichert's membrane, after which removal is straightforward, trimming it up to, but not beyond, the placental border (**Fig. 8**).
4. If the visceral yolk sac or placenta is damaged, causing deflation or bleeding, the embryo should be discarded; otherwise, it is ready for culture.

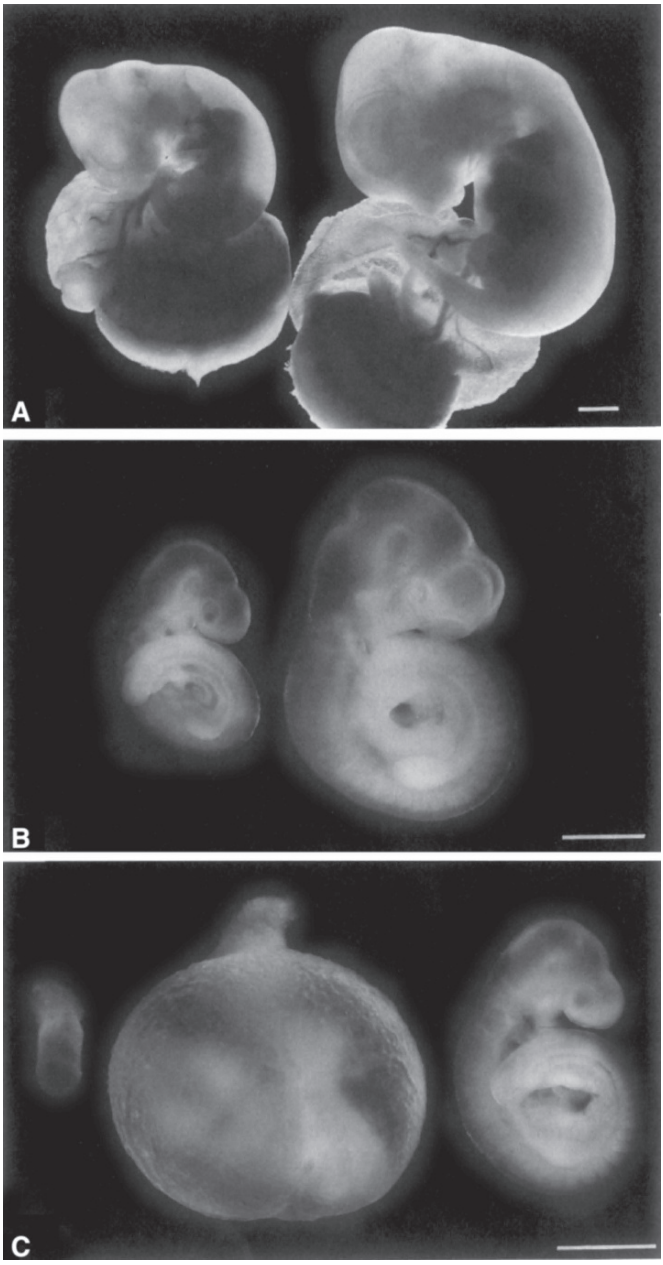

Fig. 7. **(A)** Two mouse embryos taken from the same uterus at E11. The one on the left was refrigerated at explantation. The one on the right was cultured with open yolk sac as described for 24 h before photography. **(B)** E9 embryos before and after culture. The embryo on the left was refrigerated at explantation; on the right is a littermate cultured for 48 h.

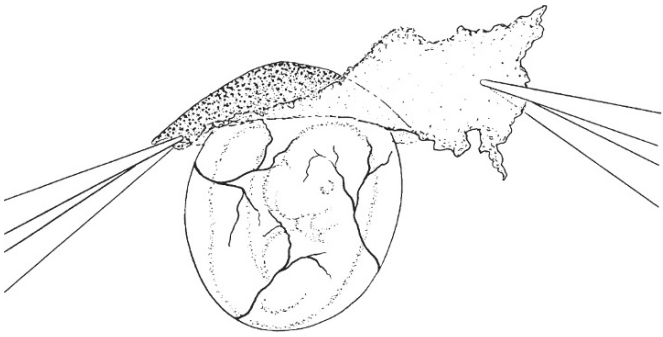

Fig. 8. The Reichert's membrane of the E9 embryo is opened using watchmaker's forceps, and the surplus is trimmed to the border of the placenta.

5. E9 embryos may be cultured in 50-mL Falcon tubes as above or 30-mL Universal containers (Nunc, with a smear of silicone grease round the internal rim of the lid to provide a gas-tight seal) with 1–1.5 mL pure serum/embryo, and 2–5 embryos/tube, depending on size. Initially, the embryos should be gassed with 5% CO_2 in air (i.e., 20% O_2, 75% N_2, 5% CO_2), which is replaced with 40% O_2, 55% N_2, 5% CO_2 after 18–24 h of culture. **Figure 7B** shows the extent of development of E9 embryos after 48 h in culture, though 18–24 h are a more realistic period if you need the majority of embryos to be healthy at termination.

3.4. Explanting E7 Embryos (Cultured with Closed Yolk Sacs)

1. The initial stages are as above, but with E7 embryos, removal of the decidua requires a rather different strategy, since the conceptus is embedded in a relatively thick mass of decidua. Start by impaling the decidual mass parallel with its long axis, but off-center, with one arm of a sharpened slender pair of forceps. Then squeeze the forceps together to form an incision in the decidua.
2. Repeat on the other side of the decidua, then unite the two incisions around the base of the conceptus (usually the thicker end).
3. Now grasp the two flaps of decidua with two pairs of watchmaker forceps, and pull apart (**Fig. 9**), when the conceptus should be exposed, and usually it will remain on one of the decidual halves as they are separated. If the embryo sticks to both

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Fig. 7. (*continued*) The embryos are shown dissected free of the extraembryonic membranes with which they are cultured. **(C)** E7 embryos before and after culture. An E7 conceptus (including the visceral yolk sac and ectoplacental cone with, which it would be cultured) is shown on the left. This was refrigerated while littermates (center—with membranes as cultured; right—dissected free of membranes) were cultured for 48 h. Scale bars for A, B, and C are all 1 mm.

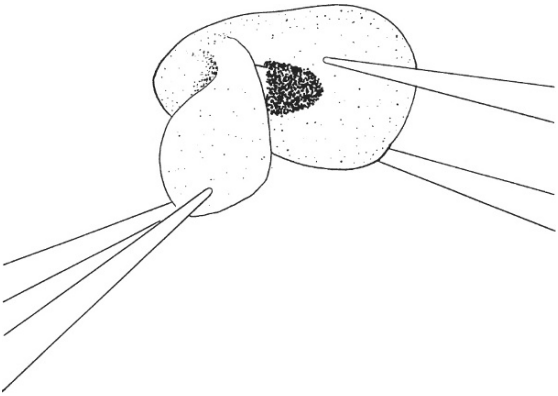

Fig. 9. As the decidua of the E7 conceptus is pulled apart, the ectoplacental cone of the embryo within can be seen.

decidual halves, so that separating them further might damage it, either hold the flaps apart with one pair of forceps, whilst teasing the embryo free on one side with the apposed tips of the other pair of forceps, or make a further incision in one of the decidual halves, so that only a quarter is removed initially, followed by the second quarter.

4. Once this has been accomplished, further divide the base of the decidua containing the embryo along the long axis, and peel apart along the length of the conceptus (**Fig. 10**). Repeat if necessary until the embryo is attached only to a thin sliver of decidua, like a segment of an orange.
5. Impale the sliver of decidua on either side of the embryo with the points of one pair of forceps, stretching it slightly, and tease the embryo free with the apposed tips of the other pair of forceps (**Fig. 11**).
6. All that remains is to open Reichert's membrane (**Fig. 12**); as with the E9 embryos, this may be overlaid with blood and trophoblast, though the membrane itself is thin and transparent. Sometimes Reichert's membrane stands clear over the embryo (at the end opposite the reddish ectoplacental cone), where it can be grasped and pulled apart. Otherwise, it will be necessary to use two pairs of watchmaker forceps to rupture the membrane midway along the length of the conceptus, in a region overlying the visceral yolk sac, where unseen damage to the underlying tissue will be less serious than damage to the embryo itself. Once opened, Reichert's membrane is trimmed off to the border of the ectoplacental cone, which is left intact.
7. The E7 embryo is now ready for culture. These embryos are cultured with 1 mL pure serum/embryo, with three embryos per 30-mL Nunc tube, or up to five embryos per 50-mL Falcon tube. They are gassed initially with 5% O_2, 90% N_2, 5% CO_2, and then with 5% CO_2 in air (i.e., 20% O_2) after 24–36 h of culture (i.e., when the heart beat and visceral yolk sac circulation are established). **Figure 7C** shows the extent of development of E7 embryos after 48 h in culture.

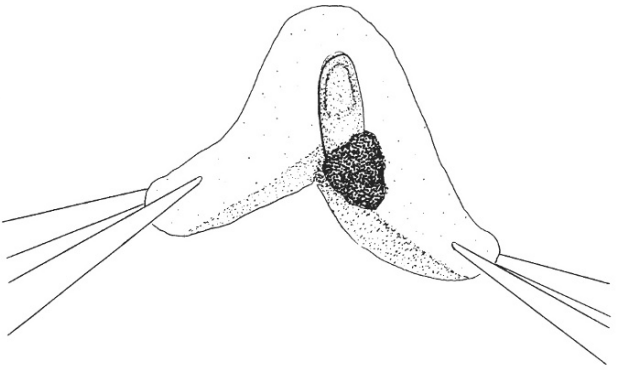

Fig. 10. The decidua is removed by successively peeling off strips along the long axis of the conceptus.

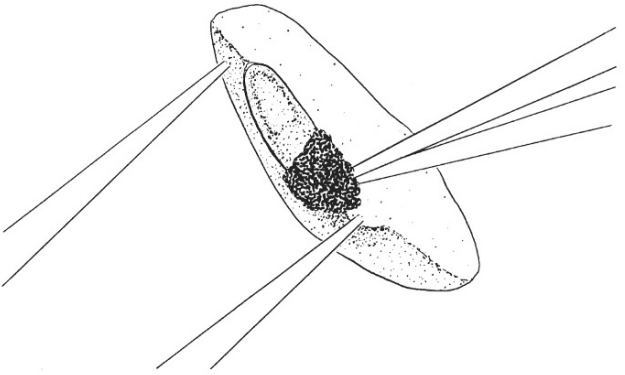

Fig. 11. The conceptus is teased free from a narrow sliver of decidua.

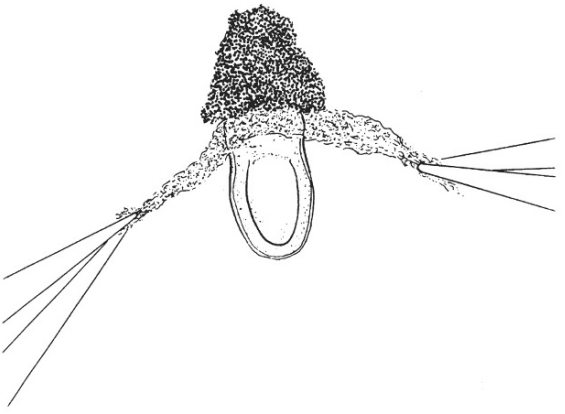

Fig. 12. Reichert's membrane is opened with finely ground watchmaker's forceps.

4. Notes

1. Sharpening forceps: A combination Japanese waterstone (1000/6000 grit, e.g., King brand) is ideal. Wet it with distilled water and grind the forceps along their length until a tip diameter of 0.05–0.1 mm is achieved. It is very important that the tips meet precisely, without crossing or one protruding beyond the other. Then round off all but the innermost (mating) edges, so that when apposed, the tips together form a blunt-ended probe.
2. Within and between litters of embryos of the same age, the range of developmental stages can vary dramatically. There are a number of established staging guides: **ref. *10*** covers all stages from fertilization to birth, **ref. *11*** from fertilization to 4 wk postpartum, **ref. *12*** from 8–16 d, **ref. *13*** from 7.5–10.5 d, **ref. *14*** from 6.75–8.0 d, and **ref. *15*** from 9 d to post natal stages. Between E9 and E14 a useful method of accurately staging embryos is to use the shape of the fore- and hindlimbs ***(16)***. These publications will also be useful in assessing the stage of development reached after culture and how it compares with growth *in vivo*. **References *10*** and ***12*** also provide a wealth of additional information, including sectioned embryos, which can help determine the success of your experiment.
3. An alternative method of transferring embryos from dish to dish while they are surrounded by their decidua is to pick them up with watchmaker forceps where the decidua is thickest, thus minimizing carryover of medium from one dish to another.
4. When the time comes to harvest your cultured embryos, they should be gently slipped into ice-cold PBS, and trimmed free of their yolk sac and placenta by cutting the umbilical vessels. Embryos can then be transferred to the fixative of your choice. We favor half-strength Karnovsky's fixative ***(17)*** if embryos are to be subsequently processed for scanning electron microscopy or resin histology, or 4% paraformaldehyde if they are for whole-mount or sectioned immunocytochemistry or *in situ* hybridization studies. For general wax histology, good results are obtained with Bouin's fixative followed by staining with Hematoxylin and Eosin after sectioning ***(10)***.
5. Manipulations: We have successfully performed a number of manipulations on the E11 embryo after it has been delivered from its yolk sac and prior to culture. It is possible to inject reagents into various epithelial lumens—for example, we have injected the marker dye Monastral Blue into the otic vesicle ***(18)***. Similarly, it is possible to perform simple surgical operations ***(19–21)*** and to label groups of cells with the lipophilic dye, DiI ***(21)***. Even if the embryos bleed a little after surgical manipulations, they generally survive and can be successfully cultured. The two biggest problems with any such manipulations are being able to see well enough what you are doing and keeping the embryo from moving around the dish as you operate on it. The first of these problems is easily resolved with good lighting (both incident and transmitted) and the second requires gentle holding or supporting of the embryo with forceps.
6. Transgenic mice: It is possible to culture transgenic mice, but these will generally be derived from heterozygote crosses, so only a fraction of the embryos cultured

from each litter will be of the required genotype. It is sometimes possible to take a small piece of tissue (tail tip or a small piece of yolk sac) at the time of explant, so that embryos can be genotyped by PCR during the culture period. Otherwise, this can be done with spare embryonic tissue taken after the culture period, but before fixation.

7. Synthetic medium: We have recently begun to culture E11 embryos with open yolk sacs in a serum-free medium made by Gibco BRL. This medium is excellent for E11 cultures up to 24 h, but we have not tried it for longer culture periods, or its efficacy for the more sensitive younger embryo stages. Good results can also be obtained over a range of stages with partially defined medium, made by extensively dialyzing rat serum, and then supplementing it with glucose, vitamins, and amino acids ***(22)***.
8. Adding glucose to serum: If you have fasted your rats before bleeding, they will have lowered blood glucose levels. Addition of 8 µL/mL of a 50 mg/mL stock solution of glucose to the serum will restore the glucose levels to that in serum obtained from fed rats.
9. Address for BTC: BTC Engineering, 12 Shirley Close, Milton, Cambridge CB4 4BG, UK.

Acknowledgments

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3

Organ Culture in the Analysis of Tissue Interactions

Irma Thesleff and Carin Sahlberg

1. Introduction

Interactions between epithelial and mesenchymal tissues constitute a central mechanism regulating the development of most embryonic organs. Studies on the nature of such interactions require the separation of the interacting tissues from each other and the follow-up of their advancing development in various types of recombined explants. The tissues can be either transplanted and their development followed *in vivo* or cultured as explants *in vitro*.

Although the transplantation methods offer certain advantages, including a physiological environment and the possibility for long-term follow-up, organ culture techniques are superior in many other aspects. The cultured tissues can be manipulated in multiple ways, and their development can be continuously monitored. The culture conditions are reproducible, and the composition of the medium is known exactly and can be modified. Furthermore, the *in vitro* culture conditions allow analyses of the nature of the inductive signals.

Many types of organ culture systems have been used over the years for studies on embryonic organ development. The Trowell method ***(1)*** has been widely applied, and it has proven to be suitable for the analysis of the morphogenesis of many different organs ***(2–6)***. In this system, the explants are cultured *in vitro* at the medium/gas interface on thin membrane filters supported by a metal grid. We used the Trowell technique as modified by Saxén ***(7)***. The embryonic tooth is a typical example of an organ in which reciprocal epithelial–mesenchymal interactions regulate morphogenesis and cell differentiation ***(8)***. We used the Trowell-type organ culture method to analyze the mechanisms of tissue interactions at various stages of tooth development ***(9–11)***. In the following, we describe the protocols for the separation and culture of dental epithelial and mesenchymal tissues and for bead experiments in which the local effects of growth factors and other diffusible molecules can be analyzed (**Fig. 1**) ***(12–15)***.

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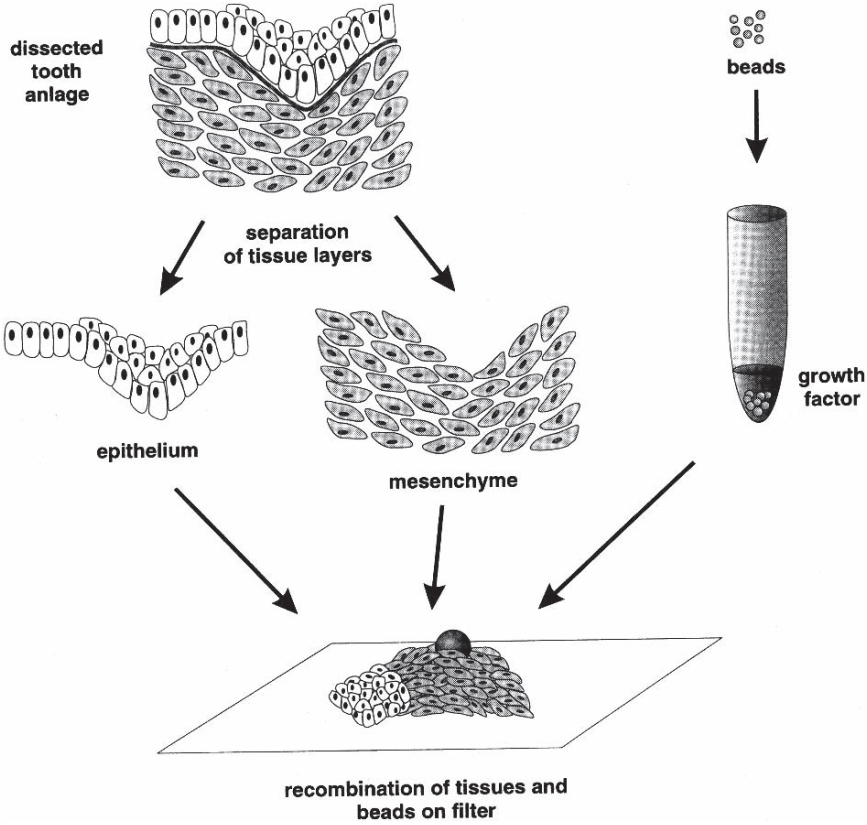

Fig. 1. Schematic representation of the method.

2. Materials

All solutions and equipment should be sterile. The glassware and metal instruments should be autoclaved and solutions filtered or autoclaved. All work should be done in a laminar flow hood; also, the dissection microscope should be placed in hood.

2.1. Salt Solutions, Enzymes, and Culture Media

1. Phosphate-buffered saline (PBS), pH 7.4: Dulbecco's phosphate-buffered saline (D-PBS, Life Technologies) supplemented with penicillin-streptomycin (PS), 20 IU/mL. (Penicillin 10.000 IU/ml, streptomycin 10.000 µg/mL, Life Technologies).
2. Enzyme solution for tissue separation: trypsin (Difco, cat. no. 0152-15); stock solution of pancreatin (Life Technologies, cat. no.45720-018), 1.25 g/mL; Tyrode's solution: NaCl (8.0 g), KCl (0.2 g), NaH_2PO_4 (0.05 g), glucose (1.0 g), $NaHCO_3$

(1.0 g). Adjust pH to 7.2, make up to 1,000 ml with distilled H_2O, and sterile filter. Store at +4°C. Dissolve 0.225 g trypsin in 6 mL Tyrode's solution on ice, using a magnetic stirrer. Add 1 mL pancreatin and 20 µL PS. Adjust pH to 7.4 with NaOH. Make up to the final volume 10 mL with Tyrode's solution and sterile filter. Aliquot 1 mL in Eppendorf tubes and store at −20°C. The enzyme solution can be stored at −20°C for 1 wk.

3. Medium for tissue dissection and culture: Dulbecco's Modified Eagle Medium with glutaMAX-1 (DMEM, Sigma, cat. no.07777), supplemented with 10% heat-inactivated fetal calf serum (FCS) and PS (20 IU/mL). Store at +4°C (*see* **Note 6**).

2.2. Dissection and Culture

1. For tissue dissection, 10-cm diameter plastic bacteriological petri dishes and 4–10- cm diameter glass petri dishes, small scissors and forceps, disposable 20- and 26-gauge needles attached to 1-mL plastic syringes (*see* **Note 1**).
2. Culture dishes: 3.5-cm diameter plastic petri dishes (bacteriological or cell culture dishes).
3. Metal grids: Prepared from stainless-steel mesh (corrosion-resistant, size of mesh 0.7 mm) by cutting approximately 3-cm diameter disks and bending the edges on a cutting form to give a 3-mm height (the height of the metal grids can be altered to allow the use of more or less culture media). Holes in the grid are produced either by nails in the cutting form or by punching. The holes facilitate the examination and photography of the explants (and they are handy when transfilter cultures are prepared) (**Note 5**) (**Fig. 2**). Organ culture dishes featuring a central well in which a metal grid (even without bended edges) can be placed are commercially available (Falcon 3037, Becton Dickinson Ltd., Oxford, UK).
4. Filters: Nuclepore polycarbonate filters (Nuclepore,Track-GTCN Membrane, Whatman cat no. 110 GOS). The pore size routinely used is 0.1 µm (*see* **Note 4**). The filters are cut in half, washed in detergent, rinsed under running water for 2 h and 10°—in distilled water, and stored in 70% ethanol
5. Glass Pasteur pipets are used for transferring tissues. They are siliconized (to prevent sticking of tissues), stuffed with cotton wool, and autoclaved. Before use, they are drawn by heating to adjust to the size of the tissues. Ideally, the diameter should be the minimal to allow free passage of the tissue.
6. Beads for growth factors: Affi-Gel blue agarose beads (Bio-Rad Laboratories, Hercules, CA) or heparin-coated acrylic beads (Sigma, St. Louis, MO) are divided into aliquots and stored at +4°C.

3. Methods

3.1. Treatment of Beads

Pipet agarose beads or heparin-coated acrylic beads to PBS in a petri dish. Count 100–200 beads under the microscope and transfer to an Eppendorf tube. Spin down the beads, and remove PBS. Add growth factors in a small volume (10–50 µL) of 0.1% bovine serum albumin (BSA) in PBS. (In general, high

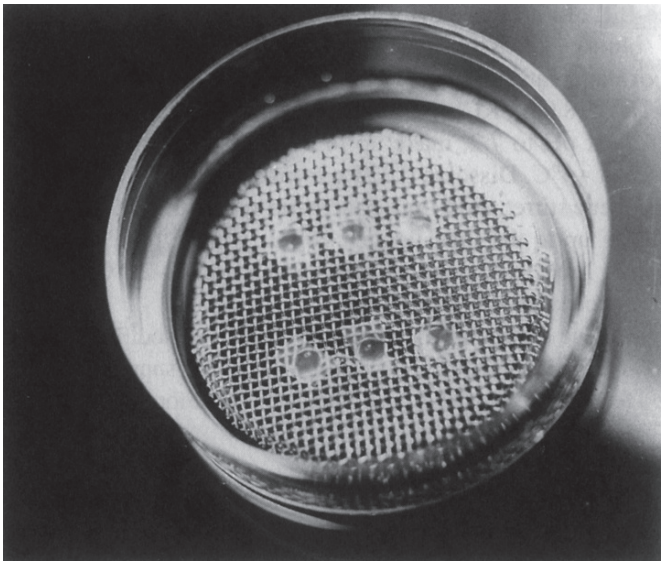

Fig. 2. The Trowell-type organ culture dish.

concentrations of proteins are used. We use FGF-4 at 20 ng/μL and TGFβ)-1 at 1 ng/μL.) An equal amount of 0.1% BSA in PBS is pipeted to control beads. Incubate for 30 min at +37°C and store at +4°C. The beads can be used at least for 14 d (depending on the stability of the protein).

3.2. Preparation of Culture Dishes

1. Take a sheet of Nuclepore filter from ethanol and rinse in PBS in a plastic 10-cm diameter petri dish. Cut the filter, using small scissors and watchmaker forceps, in approximately 3°- 3-mm pieces, and leave in PBS.
2. Place metal grids in 3.5-cm plastic culture dishes. Add approximately 2 mL culture medium (D-MEM +10% FCS, *see* **Note 6**) by pipeting through the grid. The surface of the medium should contact the plane of the grid but not cover it (excess medium results in floating of the filters and tissues). No air bubbles should remain under the grid (if present, they can be sucked empty with a thin Pasteur pipet). Using forceps, transfer the Nuclepore filter pieces on the grids placing them over the holes.

3.3. Dissection of Tissues

1. Place the mouse uterus (E 12) in a 10-cm plastic petri dish containing D-PBS and cut open the uterine wall using small scissors and forceps. Continue the work. The metal grid supports six pieces of filter. The cultured explants (like the one in the

bottom of **Fig. 1**) are seen on the filters over the holes of the grid under the stereomicroscope with transmitted light. Remove the embryos from fetal membranes and transfer them to a fresh dish of D-PBS. Cut off the heads using disposable needles as "knives." The needles are used during all subsequent steps of dissection. Transfer the heads to a glass petri dish containing D-PBS and dissect out the lower jaw. Dissect out the tooth germs of the first mandibular molar with some surrounding tissue left in place (*see* **Notes 1 and 2**).

2. With a drawn Pasteur pipet (preferably mouth controlled), transfer the tooth germs to a small culture dish. Remove most of the liquid. Melt an aliquot of pancreatin–trypsin, and spin immediately for 30 sec at 8000 g. Add cold supernatant on the tooth germs and incubate 2–10 min at room temperature or 33°C. Remove most of the liquid, add the culture medium, mix, and transfer the tooth germs to a glass petri dish containing culture medium. Leave the tissues for 30 min at room temperature.
3. Gently separate the epithelia from the mesenchymes using needles and remove excess surrounding nondental tissue (*see* **Note 3**). Transfer the tissues on the Nuclepore filters in culture dishes that have been prepared in advance. Avoid air bubbles in the pipet, and avoid sucking the tissue beyond the capillary part of the pipet. Ideally, the tissues should be placed directly in their final position, but if needed, they can be gently pushed with needles.
4. Wash the beads quickly in culture medium in a glass Petri dish (they tend to stick on plastic dishes). Under the microscope, transfer the beads one at a time to the tissues.

3.4. Culture and Fixation

1. Culture the tissues in a standard incubator at 37°C, in an atmosphere of 5% CO in air and 100% humidity for 24 h.
2. Photograph the explants before fixation (the translucent zone cannot be seen after fixation). To avoid detachment of the tissues from the filters, prefix the explants in ice-cold methanol on the grids as follows: Remove the culture medium by sucking, and pipet methanol gently on the tissues. Leave for 5 min, and transfer filters by watchmaker forceps to Eppendorf tubes for subsequent treatments (*see* **Notes 8** and **9**). Typical explants are shown in **Fig. 3**.

4. Notes

1. For tissue dissection, the disposable needles are superior to other instruments, such as scalpels or iris knives, because they need no sharpening or sterilization. The size of the needles can be chosen according to the size of the tissues. The syringes need not be absolutely sterile and can be used many times. For best preservation of tissue vitality, dissecting should be done by determined cuts, avoiding tearing. Glass Petri dishes are preferable to plastic ones during dissection of the tissues, because cutting with the needles tends to scrape the bottom of the plastic dish and loosen pieces of it.
2. The preparation and dissection of tissues should be done as quickly as possible to promote survival of the tissues. One uterus at a time should be prepared and the

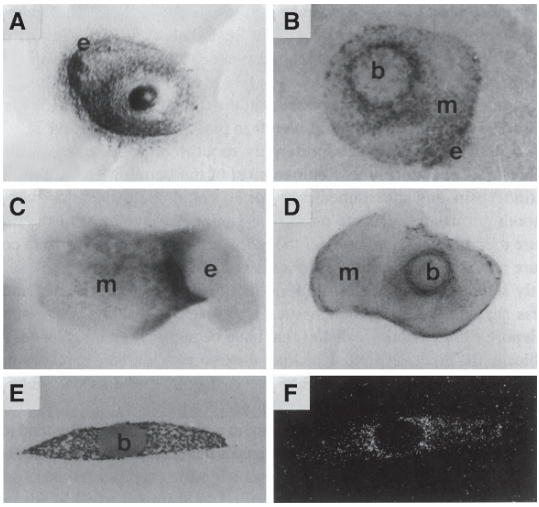

Fig. 3. Examples of the analysis of cultured explants. **A** Appearance of an explant in the stereomicroscope. The epithelium as well as the TGFβ-1-releasing bead have induced a translucent zone in dental mesenchyme. **B** Localization of cell proliferation with BrdU incorporation under the epithelium as well as around an FGF-4-releasing bead. **C** Whole-mount in-situ hybridization analysis of Msx-1 gene expression indicating induction by the epithelium in the mesenchyme. **D** Whole-mount immunohistochemical staining showing stimulation of tenascin expression in the mesenchyme around an FGF-4-releasing bead. **E** Section of an explant of dental mesenchyme and a TGFβ-1-releasing bead (the filter has been detached during processing). **F** Dark-field illumination of the explant in E showing the induction of tenascin-C transcripts by in-situ hybridization analysis: e, dental epithelium; m, dental mesenchyme; b, bead.

rest stored in D-PBS at +4°C. The dissected tissues should not be stored for long times (2–3 h max) before transfer to the culture dishes and incubator.

3. The dissection and culture techniques are basically similar when different organs or different developmental stages of tooth germs are studied. Separation of the epithelium and mesenchyme can be accomplished in young tissues, after enzyme treatment, even without dissection, by briefly vortexing the tissues. On the other hand, more advanced tissues require a longer incubation in the enzyme solution (up to 10 min). The time needed for best separation depends also on the batches of enzymes; therefore, the optimal time must always be checked for new batches of enzyme and for different tissues.
4. Different supporting materials can be used for the cultured explants. Lens paper may be used for large tissue pieces. The supporting material must allow good diffusion of the medium to the tissue; therefore, Millipore filters of 100-µm thickness

are not suitable. (The thickness of Nuclepore filters is approximately 10 µm.) Different pore size Nuclepore filters may be used. Small pores (0.05–0.2 µm) allow better examination of the explants in the stereomicroscope using transmitted light, but the tissues tend to detach from these filters more readily during fixation and other treatments after culture. Therefore, larger pore sizes (up to 1 µm) may be preferable, depending on the experiment.

5. The Trowell-type organ culture can be used for a variety of other organ culture designs. One example is the transfilter culture, where the interacting tissues are cultured on opposite sides of the filter ***(4,9)***. The tissue to be grown below the filter is glued by heated 1% agar, after which the filter is turned upside down, and the other tissue is placed on top of the filter.
6. The composition of the optimal culture medium depends on the tissues. The medium in this protocol is good for a number of different organs at early stages of development, but during more advanced stages, different organs may have special requirements. Chemically defined media with varying compositions have been designed. For cultures of whole tooth germs, we routinely use chemically defined medium composed of D-MEM and F12 (Ham's Nutrient Mixture, Gibco-BRL) 1:1, supplemented with 50 µg/mL transferrin (Sigma, T-2252,10 mg/mL, 20-µL aliquots, stored at −20°C). For more advanced stages of tooth development, ascorbic acid is added at 150 µg/mL to allow deposition of dentin collagen ***(16)***. During prolonged culture, the medium should be changed at two- to three-3 day intervals.
7. Isolated epithelial tissue does not survive as well as the mesenchymal tissue, when cultured alone. The growth of the dental epithelium (as well as epithelium from other organs) is significantly improved by culture on extracellular matrix material. Collagen has been used, but by far the best results are obtained with the basement membrane matrix, Matrigel (Collaborative Biomedical Products, Bedford, MA, cat. no. 40234), which also promotes epithelial morphogenesis ***(17)***. Matrigel is kept on ice and pipeted on the filters. Dishes are transferred to the incubator for 30 min, allowing gelling of Matrigel. At room temperature, the tissue is placed on Matrigel. Covering of the tissue with a drop of Matrigel further improves epithelial growth.
8. *Bromodeoxyuridine* (BrdU) incorporation is commonly used for the analysis of cell proliferation (**Fig. 3B**). The explants are labeled by adding BrdU 0.5–3 h before fixation (we use cell proliferation kits from Amersham International, Little Chalfont, UK, or Boehringer-Mannheim, Mannheim, Germany). After fixation in ice-cold methanol, the explants are washed in PBS and immunostained as whole mounts using antibodies against BrdU ***(12)***.
9. Usually, the tissues are analyzed after culture either as whole mounts (**Fig. 3B–D**) or they are paraffin-embedded and serially sectioned (**Fig. 3E,F**). For most purposes, they are fixed for 1 h in 4% paraformaldehyde in PBS (PFA) (after 5 min prefixation in ice-cold methanol). PFA should be fairly freshly made (no more than seven-days old). The procedure used for whole-mount immunostaining is described in ***(11)***, and that for in-situ hybridization in ***(12)***.

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4

Treatment of Mice with Retinoids In Vivo and In Vitro

Gillian M. Morriss-Kay

1. Introduction

The retinoids comprise a large group of natural and synthetic compounds related to vitamin A (retinol). The family name is derived from the early observation of the necessity of vitamin A for normal vision and the association of vitamin A deficiency with night blindness ***(1)***. With the exception of the visual cycle in the rod photoreceptor cells of the retina, in which protein-bound 11-*cis*-retinal is reversibly isomerized to free all-trans-retinal, retinoic acid is the active retinoid for biological processes. However, the visual cycle illustrates two characteristics of retinoids that are relevant to the use of this family of molecules as tools in experimental embryology and for the interpretation of results: (1) Retinoids are light-sensitive, and will undergo isomerization or degradation if exposed to light; ***(2)*** in nature, their activity and their metabolism are associated with binding to specific proteins.

The most important natural retinoids are retinyl esters, retinol, and retinoic acid (RA). In the all-*trans* form, RA is the ligand for the nuclear RA receptors RAR-£\, RAR-£], and RAR-£^; in the 9-*cis* form, it is the ligand for the retinoid X receptors (RXRs), which form heterodimers with RARs to form the transcriptionally active complex that binds to RA response elements of target genes, as well as with the RARs (*see* ***(2)*** for details and references). In the past, retinyl esters and retinol have been used for treating embryos ***(3,4)***; their effects on the developmental pattern probably depend on conversion to RA, although some other natural metabolites, such as 4-oxo-RA, may also be directly active ***(5)***. Most recent studies have used RA (mainly in the all-*trans* form) for experimental purposes.

The disadvantage of using RA, both in vivo and in vitro, is that it is highly toxic. Although it is the principal biologically active retinoid, it is not normally

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transported long distances from its source of synthesis. Retinoids are stored in the liver as esters and transported in the bloodstream as retinol bound to retinol binding protein (RBP). In early pregnancy in the mouse (i.e., at stages appropriate for studies on hindbrain and limb bud development), the maternal blood is in direct contact with the parietal yolk sac (Reichert's membrane), a thick basement-membrane-like structure with dispersed attached cells.

Immediately internal to this, the visceral yolk sac endoderm is rich in transcripts of cellular retinol binding protein (CRBP I) ***(6)***. The mechanism of transfer involves receptor-mediated uptake of retinol from maternal RBP-retinol by the visceral yolk sac endoderm, where it binds to CRBP I and interacts with the enzymes mediating RA synthesis ***(7)***. The assumption that retinol is retained specifically in CRBP I-expressing embryonic tissues has been verified by using 14C-labeled retinyl acetate (delivered intravenously to the pregnant dam) as a source of retinol ***(8)***.

RA administered to pregnant rats and mice is rapidly transferred to the embryos, where it remains at high levels for only a few hours. In the mouse, embryonic all-*trans*-RA levels are raised within 30 min of maternal oral administration, reaching a peak at 2 h, then falling rapidly to low levels by 8 h ***(9)***. In the rat, the embryonic peak for RA is also at 2 h, falling even more rapidly than in the mouse to a low level at 4 h, then more gradually to undetectable levels ***(10)***. These pharmacokinetic profiles indicate that administration of RA to pregnant rats or mice results in exposure of the embryos to RA for relatively short periods of time; if it is desired to allow development to continue for 24 h or more, we can be confident that they are not exposed to raised RA levels for more than the first few hours after the time of administration, with a peak at 1–2 h. It should also be noted that RA administered in vivo or in vitro undergoes isomerization to other active retinoids (e.g., 13-*cis*-RA and 9-*cis*-RA) both within the maternal tissues and in the embryo ***(9–11)***.

The following protocols cover only administration with RA. Retinol can be used in exactly the same way, but higher concentrations are required to induce an equivalent effect [6]. It may be more appropriate to use retinol than RA in vitamin A deficiency studies, in which much lower (physiological) concentrations should be used than in studies using RA to alter gene expression. Retinyl esters must be made up as an emulsion; they are only useful in in vivo studies, being inactive in vitro, where the necessary conversion enzymes are not available.

In addition to the protocols described later (**section 3**), RA has been applied on a bead directly to the mouse limb bud using the ex utero technique; the effects on limb development were reduction defects resembling the effects of maternal administration ***(12)***.

2. Materials

1. Retinoic acid is available in crystalline form from Sigma (UK). Once open, it must be protected from light and oxygen. Protection from oxygen can be achieved by replacing the air with nitrogen or argon, as available, immediately before sealing the container. All solutions and suspensions must be made up fresh, protected from light, and used within 24 h.
2. For oral administration (gavage) of pregnant mice, use a 1–5 in. stainless-steel 18-gauge dosing cannula with a luer fitting and a bulbous tip (available from Harvard Apparatus Ltd., Kent, UK), fitted to a standard 1 mL disposable syringe. For intraperitoneal (ip) injection, use a 25-gauge 5/8-in. needle.

3. Methods

The following examples of retinoic acid treatments are from our own studies. The timing is critical. Our timings are included to illustrate what has worked for us in the past. For each new study and repeats of previous studies, even in the same laboratory, it is essential to invest some time in establishing the time period during which the desired developmental process occurs. Timed matings, in which the male is removed after 2–4 h, minimize but do not abolish the spread of developmental stages between litters; there is also a range of stages within each litter.

It is likely that implantation time is more significant than the time of mating for determining the precise developmental stage later on. Time of year, even in an air-conditioned, light- and temperature-controlled animal house, can affect developmental timing in the mouse.

3.1. Production of Hindbrain Abnormalities

Embryos exposed to RA prior to the onset of somitic segmentation fail to develop rhombomeres, and *Hoxb*-1 is expressed throughout the preotic hindbrain instead of being specific to rhombomere 4; embryos exposed to RA after the onset of somitic segmentation form rhombomeres of variable regularity, with *Hoxb*-1 expression in rhombomere 2 as well as rhombomere 4, and rhombomeres 1, 2, and 5 showing a normal pattern of gene expression with respect to *HoxB* genes and *Krox*-20 ***(13)***. These abnormalities were induced by oral dosing with RA on days 7.75 and 8.25, respectively (dosing on day 8.0 gave mixed litters), giving 10 or 12 mg RA/kg (a mouse weighs 20–25 g).

Make up the crystalline RA as a 1 mg/mL suspension in peanut oil. Mix very thoroughly, using a magnetic stirrer, and use immediately. If kept overnight for using again, the suspension should be gassed, sealed, stored in the dark at 4°C, and mixed thoroughly again immediately before reuse. For 10 mg/kg, give 0.25 mL to a 25 g mouse. For gavage (right-handed person), proceed as follows: Place the mouse on the cage lid, holding its tail with your right hand; it will grip

the bars of the lid. Pick up the mouse with your left hand so that the skin of its neck and back is held firmly between the thumb and first finger; head movement is restricted and the snout tilted upward. Crook your little finger around the tail if necessary. The mouse should be comfortable but immobile; if it struggles the hold is wrong, and you should start again. Introduce the bulb of the cannula between the jaws at the diastema (the gap between the incisors and molars), place it on the tongue, and then use it to tilt the head into a position so that the cannula is in line with the esophagus. Slide the cannula gently down the esophagus so that the tip enters the stomach. This should be easy. If, at any stage, it is not easy, stop. Deliver the measured amount of RA suspension, and remove the cannula. The mouse should be lively immediately on replacing into the cage, and there should be no sign of oil around the mouth. This is a skilled procedure and should be learned from an experienced user. *See* **section 4**.

3.2. Production of Limb Abnormalities

Limb abnormalities can be induced by RA treatment on day 11 or early on day 12. Like all RA-induced effects, the resulting abnormalities of skeletal pattern are stage related. In our experiments, administration on day 11.0 resulted in partial or complete fusion of digits 4 and 5, administration on day 11.5 to loss of digits 1 and 5, and administration on day 12.0 to partial or complete loss of digit 1; skeletal differentiation was delayed by all of these timed exposures to RA ***(14)***.

Make up a 10 mg/mL suspension in peanut oil, and give 100–120 mg/kg (0.25–0.3 mL for a 25-g mouse) by gavage. Limb abnormalities can be analyzed at late fetal stages (day 17 or 18) by double skeletal staining for cartilage and bone.

3.3. Intraperitoneal Administration

We used ip injection for low-dose RA supplementation to *curly tail* mutant mice (15). This treatment results in a decrease in the incidence of neural tube defects and is a good example of the use of RA in studying genotype–phenotype interactions. A thick, oily suspension is unsuitable for delivery through a small-gauge needle, so the RA is first dissolved in 5–10% ethanol (this takes 15–20 min), then a small amount of oil, then mixed with the remainder of the oil. Thorough mixing at each stage is essential; for example, for administration of 5 mg/kg to a 25 g mouse, dissolve 5 mg RA in 0.8 mL Analar ethanol, add 1.2 mL peanut oil, and mix until dissolved (at least 10 min); add 8 mL peanut oil and mix again.

3.4. Treatment of Embryos and Tissues In Vitro

Exposure of embryos to RA in vitro has the advantage that the precise developmental stage at the time of exposure is known, the concentration in the medium can be precisely calculated, and the duration of the exposure controlled.

An exposure time of 2 h is sufficient to induce an effect on morphogenesis and gene expression and avoids the complication that deleterious effects on the yolk sac placenta may affect development if RA is present throughout the whole culture period. The required concentration and exposure time need to be worked out by using a range of concentrations and times for each new experiment. Hindbrain defects can be induced by a concentration of 0.25 µg/mL medium. For this, dissolve 1 mg RA in 0.8 mL Analar ethanol and add 1 µL of this solution to 5 mL culture medium. To control cultures, 1 mL ethanol should be added. This amount of ethanol is insufficient to cause abnormalities and probably evaporates rapidly at 38°C. Some laboratories use DMSO as a solvent for RA (11). RA should always be added to the medium before the embryos are added. After exposure, embryos should be washed in Tyrode saline (do not use PBS, because they require calcium for normal morphogenesis) and replaced in fresh culture medium. Both RA and retinol can similarly be introduced to the tissue-culture medium of embryonic cell, tissue, and organ cultures.

4. Notes

In the Untied Kingdom, all administration procedures to pregnant mice require Home Office personal and project licenses. Embryo culture beyond midgestation (day 9 in the mouse) and removal of tissues from embryos and fetuses also require Home Office permission. Licenses are granted only after attendance at a course on laboratory animal management and welfare (Home Office Training Modules 1–3). Readers from other countries should seek advice from the appropriate authorities.

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5

Analysis of Skeletal Ontogenesis through Differential Staining of Bone and Cartilage

Michael J. Depew

1. Introduction

The evaluation of the ontogeny of the skeletodontal system of vertebrates is a critical endeavor for many evolutionary and developmental biologists for numerous reasons, including ***(1)*** the bones, cartilage, and teeth of vertebrates are clearly essential to their life; ***(2)*** historically, the primary structural source of evidence for the evolution of vertebrate organisms resides in the past preservation of these tissues; and ***(3)*** bones, cartilage, and teeth normally develop with great fidelity to the possessor's developmental blueprint, and they therefore provide an excellent three-dimensional readout of both normal and experimentally challenged developmental patterning mechanisms. The sections that follow are constructed to facilitate such an endeavor by providing protocols to achieve differential staining of bone (via Alizarin red) and cartilage (via Alcian blue) and resources for an anatomical context in which to interpret the results.

2. Materials

2.1. Materials for the Basic Ethanol–KOH:Glycerol Alizarin Red–Alcian Blue Staining Protocol

1. Ethanol (EtOH).
2. Acetone.
3. Alizarin red S.
4. Alcian blue 8GS.
5. Distilled H_2O.
6. Glacial acetic acid.
7. Aqueous potassium hydroxide (KOH).
8. Glycerol.

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9. Clear receptacles of suitable dimensions for fixing, staining, clearing, and storage of specimens (preferably screw top).
10. Instruments for skinning (or scaling) and evisceration, including forceps and petri dishes.

2.2. Materials for the Basic Formalin–Trypsin–KOH: Glycerol Alizarin Red–Alcian Blue Staining Protocol

1. 10% formalin.
2. Distilled H_2O.
3. Alcian blue 8GS.
4. Alizarin red S.
5. Ethanol.
6. Trypsin.
7. Saturated sodium borate (NaB_4O_7).
8. Aqueous KOH.
9. Glycerol.
10. Instruments for skinning (or scaling) and evisceration, including forceps and petri dishes.
11. Clear receptacles of suitable dimensions for fixing, staining, clearing, and storage (preferably screw top).

3. Methods

3.1. Basic Ethanol–KOH:Glycerol Alizarin Red–Alcian Blue Staining Protocol

This derivative protocol ***(1,2)*** has its origins in the investigations of late 19th and early 20th century anatomists and embryologists examining the number and nature of embryonic and fetal centers of ossification ***(3–19)***. It can be universally applied to organisms of all sizes and ages (though it is best with later fetal to adult animals) and is often the protocol of choice for studies of avian and mammalian skeletogenesis and skeletodontal patterning. Each step is augmented by its corresponding Notes and Caveats portion in **section 4**.

1. Fix and dehydrate in 95% EtOH (at least 20× volume) for at least 5 d (*see* **step 2**). Agitate occasionally.
2. After 2 h in 95% EtOH, skin and eviscerate specimens (in 95% EtOH). Return to fixing solution.
3. Place samples in acetone (at least 20× volume) for at least 2 d. Agitate occasionally.
4. Decant the acetone, allowing some drying in the process, then place for 3 d in freshly made staining solution (agitating occasionally), prepared as follows:
   a. 1 volume — 0.3% Alcian blue 8GS in 70% EtOH
   b. 1 volume — 0.1% Alizarin red S in 95% EtOH
   c. 1 volume — Glacial acetic acid
   d. 17 volume — 70% EtOH

5. Wash in distilled H_2O.
6. Begin maceration and clearing by placing in 1% KOH (in distilled water) for 12 h to 2 d. Gently agitate occasionally. Proceed to next step if the specimen's bones and cartilages are clearly visible through the gelatinous superficial tissues.
7. Decant and continue clearing in increasing concentrations of glycerol in 1% KOH over the next 3 d to 3 wk. Start with 1 volume of glycerol in 4 volumes 1%KOH, carry through 1:1 and 4:1 glycerol:KOH solutions, and end in 100% glycerol.
8. Photograph and store.

3.2. Basic Formalin–Trypsin–KOH:Glycerol Alizarin Red–Alcian Blue Staining Protocol

The enzymatic treatment of formalin (or alcohol) fixed specimens is a more recent development in staining protocols. Versions of this staining protocol have generally been the choice with more recent investigations of piscine and amphibian skeletogenesis and skeletodontal patterning (20–24). Each step is augmented by its corresponding Notes and Caveats portion in **section 4**.

1. Fix in 10% formalin for 1–2 d.
2. Wash in distilled water for 3 d, changing H_2O frequently.
3. Skin and eviscerate the specimens.
4. Begin cartilage staining by placing, for 1–2 d, in Alcian blue solution. For 100 mL of solution, add 10 mg of Alcian blue 8GS to 80 mL of 95% EtOH and 20 mL glacial acetic acid.
5. Rinse in a solution of 95% EtOH for 3–6 hs.
6. Rehydrate through a graded EtOH series of 75%, 40%, and 15%.
7. Wash in distilled H_2O for several hours.
8. Begin maceration and clearing by transferring to an enzymatic solution of 1% trypsin in 30% saturated sodium borate. Check every day, changing the solution each time.
9. Begin ossification staining by transferring for 1 d to a saturated Alizarin red S solution in 0.5% KOH.
10. Wash in 0.5% KOH several times for 1 d.
11. Continue macerating and clearing over the next several days by carrying through a 0.5% KOH–glycerol series (3:1, 1:1, 1:3) to pure glycerol.
12. Photograph and store.

3.3. Context and Interpretation

A detailed ontogenetic atlas of the normal skeletal development of the organism under consideration is indispensable to the interpretation of the results of one's differential staining. This is particularly true of the complex skeleton of the vertebrate skull. The **Table 1** anatomical resources (and the references therein) provide useful anatomical narratives and figures for the craniogenic development of the most common modern model vertebrate organisms:

Table 1
Sources for Anatomical Narratives and Figures of Craniogenic Development

Class Type	General References	Model Organism	Specific References
Mammalian	25–29	*Mus musculus*	***30,31***
Avian	25–27, 32, 33	*Gallus gallus*	***34–36***
Amphibian	25–27, 37, 38	*Xenopus laevis*	***39,40***
Piscine	25–27, 41, 42	*Danio rerio*	***43–45***

4. Notes

4.1. Notes and Caveats: Basic Ethanol–KOH:Glycerol Alizarin Red–Alcian Blue Staining Protocol

1. If necessary, specimens can be stored for months in 95% EtOH. Specimens fixed in formalin can be stained as well, provided they are first washed for several days in multiple changes of distilled H_2O and gradually brought up to 95% EtOH through a graded series of EtOH.
2. Occasionally changing the EtOH can be efficacious. With many organisms, including mice from embryonic day 16 onward, it is important, after several hours in the ethanol, to remove with care the skin, viscera, fat (especially the brown fat between the scapulae), glands, eyes, and most of the muscle mass. Skinning and eviscerating after longer fixation times in EtOH is possible, but the sample will be more brittle and the skin and organs do not remove as easily. In places where its removal might include underlying bone, such as with the phalanges, the skin may be peeled back or pricked without actual removal. With rhinocentric organisms, such as mice, the vibrissae pads should not be left.
3. The acetone helps dehydrate, adds firmness to the sample, and removes residual fat. More time in acetone will only augment the staining. Cheaper plastic tubes may melt in the acetone, so be sure that the receptacle can withstand the active properties of the acetone.
4. It is usually best to make and cure stock solutions of the Alcian blue and Alizarin ahead of time, but as the Alizarin is especially light sensitive, they should be wrapped in foil or otherwise kept in the dark. The solutions may be filtered but this is not essential. The volume of the staining solution should be 20× or more the volume of the specimen. Placing the samples at room temperature is usually sufficient but increased temperature (best at 37°C or below) promotes staining, maceration, and clearing.
5. With the addition of the H_2O, specimens usually begin to turn a shade of purple and float.
6. Do not overly agitate, especially if the samples are very small and young. Check frequently and change the solution if it begins to dramatically color. This is a critical step as an appropriate balance of maceration and skeletal integrity is usually

desirable. This step can be sped up by heating the KOH. Occasionally, the residual tissue (e.g., muscle) fails to decolor, remaining blue after prolonged maceration and clearing; if this happens, one can postfix for 2 h in 10% formalin then briefly treat with trypsin (1% trypsin in 30% saturated sodium borate).
7. The time course is particularly dependant on specimen size and temperature. It is also often best to change the 100% glycerol several times; once swirling the sample in the glycerol leaves no schlieren of the glycerol one is ready to photograph.
8. The viscosity of the glycerol helps when positioning a specimen to photograph. Some investigators add a few crystals of thymol to the specimens in glycerol, as this is thought to inhibit the growth of mold and bacteria. Specimens are generally stored at 4°C.

4.2. Notes and Caveats: Basic Formalin–Trypsin–KOH: Glycerol Alizarin Red–Alcian Blue Staining Protocol

1. May fix in 4% paraformaldehyde or 95% EtOH. The formalin may also be buffered.
2. Some protocols suggest that postfixation in 70% EtOH is justifiable, particularly as the specimen subsequently can be stored indefinitely.
3. If working with larger fish, they need to be scaled.
4. This solution must be made fresh.
5. Change the solution several times.
6. The specimen usually floats initially then sinks.
7. Change the solution several times.
8. Continue to the next step once the specimen is limp and the cartilage within can be clearly seen.
9. The Alizarin solution is saturated when the solution has turned a distinctly dark purple. Staining is done when the bones are clearly red.
10. Change the solution several times.
11. Further bleaching of pigmentation can be achieved with the addition of several drops of 3% H_2O_2. It is often best to change the glycerol several times. Once swirling the sample in the glycerol leaves no schlieren of the glycerol one is ready to photograph.
12. The viscosity of the glycerol helps when positioning the specimens to photograph. Some investigators add a few crystals of thymol to the specimens in glycerol, as this is thought to inhibit the growth of mold and bacteria. Specimens are generally stored at 4°C.

4.3. General: Purpose and Preparation of Sample (Fixative and Evisceration)

It is, of course, axiomatic that one's specific purpose in staining bone and cartilage, the organism's type, size, and age all direct the choice of protocol and how closely to follow it. A number of fixatives for differential staining have generally been employed, most frequently 95% EtOH or 10% formalin

but also iodine–alcohol and paraformaldehyde. Various cartilage-only staining protocols call for Bouin's fixative generally followed by washing in ammoniated alcohol (70% EtOH containing 1% ammonia) ***(18)***. Fixation in 95% EtOH allows quicker maceration and clearing, while a formalin-fixed specimen takes longer to clear but less care must be taken to keep the specimen intact ***(1, 8, 11, 16)***; quicker yet are protocols that call for no fixation ***(19)***. As a compromise between the properties of EtOH and formalin fixatives, a combined formalin–aceto–alcohol 1:1:8(80%) solution has been utilized ***(16)***. Calcium salts are also less soluble in alcohol than in formalin. Fixation, maceration, and clearing can be done simultaneously but with mixed results ***(11)***.

Interference with the staining often occurs with older and larger samples as skin, muscle, subcutaneous fat pads and glands act either to prevent stain penetration or stain copiously themselves ***(1, 15)***. This is less of a problem with early fetal samples (e.g., earlier than embryonic day 16 in mice), as these other tissues are less well developed. Dehydration of the sample is an important step and is often combined with fixation. Acetone is best employed, as it helps with both dehydration and the removal of residual fats ***(1, 15)***. Be sure, however, to decant well the acetone and allow the specimens to quickly air dry.

4.4. General: Staining

The vital staining of bone by the consumption of madder root has been documented for over 400 years ***(6, 45, 46)***. In 1826, the French chemists Colin and Robiquet identified Alizarin and Purpurin as the active staining ingredients of madder root. Although a number of Alizarin compounds with differential dying capacities have been synthesized ***(6),*** its efficiency in staining mineralized hard tissues has made Alizarin red S the ubiquitous postvital embryonic and fetal skeletal stain. Specimens are, however, susceptible to decolorization of the Alizarin under strong and prolonged sunlight and UV light ***(7)***. Staining can also commence during or after maceration has begun ***(11)***. Other skeletal stains include indigo-carmine, alum carmine, and Bordeaux red ***(6)***.

Staining agents of cartilage vary more and include methylene blue, toluidine blue, and Alcian blue ***(1, 10, 16)***. Alcian blue, the most frequently employed of these, interacts with the plentiful sulfated proteoglycans endemic to cartilage. However, it also is a great stain for other tissues, in particular cephalic glands, and well-keratinized skin acts as a barrier; therefore, skinning, evisceration, and removal of the glands is advantageous.

4.5. General: Maceration, Clearing, and Storage

Steps for macerating and clearing are often combined. The choice of fixative directs the nature of the maceration method. KOH is commonly employed for both EtOH and formalin-fixed specimen (the length of time needed is obviously greater

if formalin fixed). Heating or increasing the concentration of the KOH quickens the pace of maceration. When heating, however, it is recommended that the specimen starts out in room-temperature KOH then is heated only gradually ***(11,13)***.

Methyl salicylate (wintergreen oil) with benzol, combined with H_2O_2, can be used for clearing but has the tendency to turn specimens brown over time and specimens shrink a bit ***(5,10,47)***; it can also be utilized with benzyl benzoate (3:1) for storage of the specimen. Toluol followed by maintenance in anise oil saturated with naphthalene has also been used for clearing ***(10,48)***. Decolorization of the surrounding soft tissue can be achieved by ethanol–glycerol solutions, methyl benzoate, strong sunlight, direct UV light, sodium hydroxide–glycerol, benzyl alcohol–EtOH–glycerol, or acidic alcohol (1% sulfuric acid in 95% EtOH) ***(8,9,11,12,15,17)***.

Stained samples are best keep in 100% glycerol in the dark at 4°C. Thymol can be added to the glycerol for storage ***(15)***.

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6

Cell Grafting and Labeling in Postimplantation Mouse Embryos

Gabriel A. Quinlan, Poh-Lynn Khoo, Nicole Wong, Paul A. Trainor, and Patrick P.L. Tam

1. Introduction

Fate mapping experiments provide direct information on the differentiation pathways normally taken by cells or tissues during embryogenesis. Systematic analyses of the developmental fate of cell populations localized in different parts of the embryo enables the construction of fate maps. A comparison of the expression pattern of lineage-specific genes and the fate map allows the identification of precursor tissue for cell lineages well before definitive histogenesis takes place. The ability to trace the early lineage history of cells greatly facilitates the elucidation of the forces and processes that lead to the specification of cell lineages and the determination (or commitment) of cell fate. The knowledge of cell fate may also assist the interpretation of the phenotype of mutant embryos produced by either spontaneous mutation or gene knockout experiments.

This chapter describes the technical aspect of fate mapping the mouse embryo during gastrulation (6.5 days post coitum = 6.5 d) ***(1–3)*** and organogenesis (8.5 d) ***(4–8)***. Two experimental strategies are used to study the developmental fate of cells. First, a specific population of cells can be marked by labeling with vital carbocyanine dyes *in situ* or by introducing genetic markers by electroporation ***(9)***, and second, the same population of cells can be isolated from a transgenic embryo followed by transplantation (grafting) to a host embryo. The pattern of tissue colonization and differentiation of the descendants of these marked or transplanted cells is then analyzed after a period of *in vitro* development to assay their development fate. This can be done by fluorescence imaging of live whole embryos under the dissecting microscope.

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Cell labeling, electroporation, and grafting procedures have their special advantages. When grafting a genetically identifiable population of cells or electroporating a gene-expression construct, there is no dilution of the label owing to cell proliferation, so the contribution of transgenic cells to every available lineage can be assessed. Cell transplantation techniques can also be applied to the study of the developmental potency of a population of cells, by confronting the cells with novel tissue environmental or inductive signals ***(10)***. The usefulness of the cell grafting approach depends critically on the ability to isolate a defined cell population for transplantation and to place these cells at the appropriate site in the host. In contrast to cell transplantations, *in situ* labeling experiments do not require tedious dissection of tissue fragments. Fate mapping studies can be carried out directly after *in situ* marking of the cells with minimal disruption of the existing tissue architecture. However, the label must be noncytotoxic and should remain only among the descendants of the labeled cells. Similar to *in situ* labeling, embryo electroporation does not require dissection. By a series of electrical pulses that permeate the cell membrane, an expression plasmid encoding a genetic marker can be introduced to cells. A major advantage is that the marker is expressed by all the descendants that inherit the electroporated gene, thereby allowing the complete lineage to be tracked over a substantial length of time or number of cell divisions with no significant diminution of signal intensity. Genetic markers that have been used successfully include green fluorescent protein (GFP) and its spectral variants, β-galactosidase, and alkaline phophatase. Electroporation works most effectively on tissue with an epithelial architecture (such as the endoderm and the ectoderm of the embryo), since the basal lamina can prevent DNA from spreading to other tissue layers during labeling. Furthermore, electroporation enables marking of cells in a thin epithelium (such as the gut endoderm, surface ectoderm, or endothelium) when the conventional technique of transplantation of cells for fate mapping is not feasible.

2. Materials

2.1. Culturing 6.5–8.5-d Embryos

1. Roller/rotator bottle culture apparatus (B.T.C. Engineering, Milton, Cambridge, UK).
2. Water-jacketed CO_2 incubator (Forma Scientific, Marietta, OH, Model 3336).
3. Four-well chamber slides (NUNC, Naperville, IL).
4. Glass culture bottles, thin-walled,15-mL, 30-mL capacity (B.T.C. Engineering).
5. Refrigerated benchtop centrifuge, (CENTRA-7R, International Equipment Company, Needingham Heights, MA).
6. 15-mL sterile centrifuge tubes (Corning, Cambridge, MA, cat. no. 25310-15).
7. Penicillin–streptomycin, 5,000 µg/mL (Trace Biosciences, Sydney, NSW, Australia).
8. Glutamine 200 µM (Trace Bioscience).

9. Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY, cat. no. 12100-046, glucose 4.5 g/L).
10. Rat serum (RS) (*see* **section 3, step 1**).
11. Human cord serum (HCS) (*see* **section 3, step 1**).

2.2. Isolation of Tissue Fragments for Grafting

1. Alloy metal needles are made by electrolytically sharpening orthodontic wire (Rocky Mountain Orthodontics, Denver, CO) with a wire polishing unit (Dental Corporation of America, Hagerstown, MD).
2. Glass needles are made from "Microcaps" micropipets (Drummond Scientific Co., USA) using a vertical micropipette puller (David Kopf Instruments Model 720).
3. Dissecting microscope (Wild M3Z or MDG 17).
4. Tissue culture dishes (60 mm, Falcon).
5. Fetal calf serum (FCS; Trace Biosciences): The FCS is thawed and inactivated by heating at 56°C for 30 min immediately before use.
6. Polyvinylpyrrolidone (PVP; Sigma, St. Louis, MO). Dialyze a 0.5% aqueous PVP solution against water at 4°C overnight followed by lyophilization.
7. Enzymatic solution contains 0.5% trypsin (Trace Biosciences), 2.5% pancreatin (Boehringer Mannheim, Indianapolis, IN), 0.2% glucose (Sigma), and 0.1% PVP dissolved in calcium–magnesium-free phosphate-buffered saline (PBS; Flow Laboratory, Costa Mesa, CA).
8. Sodium pyruvate (Sigma): Dissolve 85 mg of sodium pyruvate in 10 mL of 0.9% NaCl. Dilute 1:50 in 0.9% (w/v) NaCl before use. It can keep for 2 wk at 4°C.
9. Phenol red: Add 129 mg $NaHCO_3$ (Sigma) to 7.4 mL super quality H_2O (Millipore) and add 2.6 ml of 0.5% phenol red solution (Sigma).
10. Penicillin (Sigma): Add 599 mg to 100 mL of 0.9% (w/v) NaCl. Sterilize with a 0.22-µm Millipore filter. Dilute 1:100 before use. Aliquot and store at −20°C.
11. PB1 is prepared according to the formulation in **Table 1**. Then, 130 mg of glucose and 520 mg of bovine serum albumin (crystallized, ICN Biomedicals Inc., USA) are added, and the solution is sterilized with a 0.22-µm Millipore filter. The solution is stored in 50-mL aliquots at 4°C.

2.3. Labeling and Grafting of Cell in 6.5–8.5-d Embryos

1. Embryo culture requirement as in **section 2.1**.
2. Horizontal micropipette puller (Sutter Instrument Co., Novato, CA, Model P-79).
3. Microforge (Narishige Scientific Instrument Laboratory, Greenvale, NY, MF79).
4. Holding, injection, and grafting pipets. Holding pipets are made from thick-walled glass capillaries (Leica, cat. no. 520-119). Injection and grafting pipets are made from thin-walled glass capillaries (outer diameter, 1 mm; inner diameter, 0.75 mm; Drummond).
5. Microburner connected to a butane gas cylinder.

Table 1
The Composition of PB1 Medium for Handling Embryos and Tissue Fragments

Stock solution (g/500 mL)	Volume (mL) to add to make 100 mL solution
NaCl (4.5)	65.8
KCl (5.75)	1.8
Na_2HPO_4 (10.93)	5.2
KH_2PO_4 (10.5)	0.9
$CaCL_2 \cdot 2H_2O$ (8.1)	0.8
$MgCl_2 \cdot 6H_2O$ (16.55)	0.3
Na pyruvate[a]	21.4
Phenol red[a]	0.8
Penicillin[a]	0.3
Distilled H_2O	0.27

[a] *See text* for method of preparation.

6. Transfer pipets made by pulling Pasteur pipets on a Bunsen flame.
7. Diamond glass-cutter (Thomas).
8. Micromanipulation apparatus (**Fig. 1**): base plate with fixing elements for both manipulators (Leitz, now Leica, cat. no. 335 520 139); manipulators (Leitz, cat. no. 335 520 137 and 335 520 138); instrument holders (Leitz, cat. no. 335 520 142 and 335 520 143), and instrument sleeves.
9. Laborlux S microscope with fixed mechanical stage (Leitz).
10. Dissecting microscope (Wild, MDG 17).
11. De Fonbrune syringe (Alcatel, Malakoff, France).
12. Micrometer syringe (Wellcome, London, UK).
13. Manipulation chamber (*see* **Fig. 5** later).
14. Tissue-culture dishes (60 mm, Falcon).
15. Coverslips (24 × 50 mm, Mediglass, Sydney, NSW, Australia).
16. Light and heavy paraffin oil (BDH Chemicals).
17. 1,1-Dioctadecyl-3,3,3′3′ -tetramethylindocarbocyanine percholate (DiI; Molecular Probes, Eugene, OR) and 3,3′-dioctadecyloxacarbobyanide percholate (DiO; molecular probes): Stock dye solutions (0.5% w/v) were prepared by dissolving the crystals in 100% ethanol. Dilute 1:10 (by volume) for DiI and 1:5 (by volume) for DiO in 0.3 M sucrose (BDH Chemicals) immediately before use for cell labeling.
18. Platinum wire (similar to that from gel electrophoresis tank).
19. Tungsten needle.
20. Banana plugs and sockets
21. Plastic tubing (to fit around the platinum needle).
22. Positioner (World Precision Instruments Inc., FL, USA. Model Taurus-R).

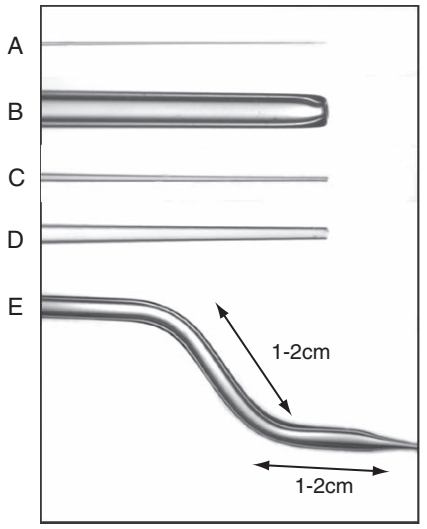

Fig. 1. Tools used for micromanipulation of 6.5–8.5-d mouse embryos. **A** The glass needle, **B** the holding pipet, **C** the injection pipet used for delivering dye or DNA solution, **D** the pipet used for grafting cell clumps, and **E** the two bends produced in the pipet (shown in B–D) to enable the tip of the pipet to reach over the wall and access the medium drops sitting on the bottom of the culture dish during micromanipulation (for setting up, see Fig. 5B).

Table 2
The composition of Tyrodes ringer saline for electroporation of embryos

Compound	g/500 mL
NaCl	4.0
KCl	0.15
$NaH_2PO_4 \cdot 2H_2O$	0.0465
KH_2PO_4	0.0125
$NaHCO_3$	0.5
Glucose	1.0

23. Electro Square Porator (Genetronics Inc.Model, BTX, USA. Model ECM 830).
24. Petri dish (200 mm).
25. Tyrodes ringer saline is prepared according to the formulation in **Table 2**. After preparation the pH of the solution is adjusted to pH 7.3 with 1 M NaOH. The solution is sterilized with a 0.22-µm Millipore filter. The solution is stored at 4°C.

3. Methods

3.1. Culturing 6.5–8.5-d Embryos

3.1.1. Preparation of Culture Media

3.1.1.1. Preparation of DMEM

1. Dissolve the contents of one packet of powdered formula in 0.95 L super quality water (less 5% of the final volume required).
2. Stir gently at room temperature until the contents dissolve.
3. Add 2.2 g of $NaHCO_3$.
4. Bring the volume up to 1 L.
5. Sterilize immediately with a 0.22-µm Millipore filter using positive pressure.
6. Test sterility of a 5-mL aliquot from each bottle by incubating at 37°C for 3–5 d.
7. Before DMEM is used for culture, add 400 µL each of glutamine and penicillin–streptomycin solution to 40 mL of DMEM. Fresh glutamine and penicillin–streptomycin should be added to the DMEM working solution after 2 wk. The working solution is good for 4 wk after preparation when kept at 4°C.

3.1.1.2. Collection of Rat Serum (RS)

1. Anesthetize the rat using 5% halothane in 1–1.5 L of O_2.
2. Blood is collected from the anesthetized rat by drawing blood from the aorta into a nonheparinized syringe using a G20 hypodermic needle.
3. Dispense the freshly collected blood in 15-mL centrifuge tubes and centrifuge at 3,000 rpm for 10 min (**Notes 1** and **2**).
4. Grasp the fibrin clot with a flame-sterilized forceps, and spool it around the shaft of the forceps to squeeze out the serum trapped in the clot.
5. Transfer the serum to a new centrifuge tube using an autoclaved Pasteur pipet, and spin again at 3,000 rpm for 10 min.
6. Collect the serum from the second spin aseptically and store at −20°C.

3.1.1.3. Collection of Human Cord Serum (HCS)

1. Collect cord blood from the placenta obtained following Caesarean delivery, and keep the blood on ice.
2. Centrifuge the blood as soon as possible in 15-mL centrifuge tubes at 3,000 rpm for 10 min at 4°C.
3. Remove the serum and store at −20°C (*see* **Notes 1** and **2**).

3.1.2. Static Culture in Four-Well Chamber Slides at 37°C in 5% CO_2 in Air

1. Thaw the required volume of HCS and RS, and inactivate the sera on day of use by heating for 30 min in a water bath at 56°C, 3.2 mL of culture medium is required for one chamber slide, and this is made up of 0.8 mL HCS, 1.6 mL RS, and 0.8 mL DMEM (1:2:1 by volume). Alternatively, use 100% RS to culture 6.5–7-d embryos.
2. Put 0.5 mL in the first well (**Note 3**) and 0.9 mL in the remaining three wells. This volume is sufficient for culturing groups of 8–10 6.5-d embryos/well (**Note 4**) for

up to 48 h (**Note 5**). The 7.5-d embryos can be cultured for up to 24 h by the static culture method (**Note 6**)

3.1.3. Roller Bottle Culture (with Continuous Gassing) for 7.5–8.5-d Embryos

1. The 7.5- or 8.5-d embryos are cultured for 24 h in a medium of DMEM:RS (1:3 by volume). Use 1 mL and 3 mL of culture medium per 7.5-d or 8.5-d embryo, respectively. Four to five 7.5-d and two to three 8.5-d embryos can be cultured in one 30-mL bottle. Bottles are maintained in a BTC embryo culture chamber at 37°C in 5% O_2, 5% CO_2, and 90% N_2, and are rotated on a roller/rotator at 30 rpm.
2. Embryos are transferred to a fresh medium of DMEM:RS (1:3 by volume) if culturing beyond the first 24 h. The gas phase remains the same for 7.5-d + 24-h embryos. For 8.5-d + 24-h embryos, the gas phase is changed to 20% O_2, 5% CO_2, and 75% N_2 (11).

3.2. Isolation of Tissue Fragments for Grafting

3.2.1. Making Glass Needles (**Fig. 1A**)

1. Attach a hypodermic syringe with a G20 needle to a bunsen burner connected to a butane gas cylinder. Adjust the gas flow by a regulator to produce a small flame.
2. Heat the "Microcaps" micropipets in the flame until the glass melts into a short solid segment.
3. Pull the fused segment of the capillary in a vertical pipet puller to produce two thinly drawn needles.
4. Coat the needle with Repelcote (BDH Chemicals, Poole, UK) to prevent the tissue from adhering during microdissection.

3.2.2. Isolation of Epiblast Fragment from 6.5-d Embryos

1. Position the embryo for easiest access to the epiblast cells required for transplantation. For example, if anterior or posterior epiblast cells are to be isolated, the embryo could be positioned with the sagittal plane in view (**Note 7**). However, to dissect cells from the lateral epiblast, the embryo is best oriented with the frontal view in sight.
2. Pin the embryo to the petri dish using a sharp metal needle. Hold it at the site immediately adjacent to the tissue fragment required for grafting. Another tungsten needle is used to slice through the epiblast in a scissorlike action against the first needle. Another cut is then made at an angle to the first, so that the tissue fragment is released from the epiblast. The dissection is shown in **Fig. 2**.
3. The endoderm usually remains attached to the epiblast. Make several scratch marks on the bottom of the petri dish with a metal needle. To remove the endoderm, place the tissue fragment, endoderm side down, on the grid and nudge the fragment onto the scratched surface. When the endoderm sticks to the surface, the epiblast layer can be torn away with metal or glass needles.
4. Cut the epiblast fragments into clumps of 5–10 cells for grafting using glass needles.

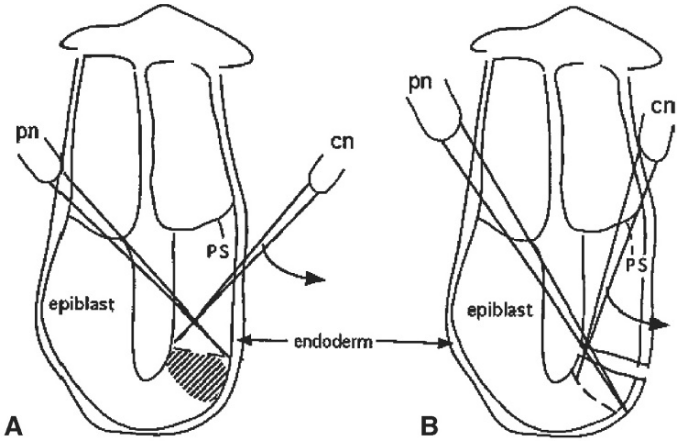

Fig. 2. The steps taken to isolate epiblast fragments from sites adjacent to the distal cap region on the posterior side of a 6.5-d embryo. **A** Position of the pinning needle (pn) and the line of cut that will be made by the cutting needle (cn) just proximal to the required tissues (shaded area). **B** Second cut made to isolate the tissue fragment. Similar cutting actions are employed to isolate tissue fragments from other regions of the embryo. Curved arrows indicate the direction of slicing made by the cutting needle. Abbreviation: ps, primitive streak.

3.2.3. Separation of the Germ Layers of 7.0–7.5-d Embryos

1. Cut the embryo at the junction between the embryonic and extraembryonic tissue (**Fig. 3A**).
2. Incubate the embryonic tissue in a trypsin–pancreatin solution (*see* **section 2.2**) for 5–10 min or until the endoderm is loosened from the mesoderm.
3. Transfer the embryonic fragment to three changes of PB1 + 10% FCS to stop the enzyme digestion.
4. Separate the germ layers as shown in **Fig. 3B–D**.
5. Cut the isolated germ layers into clumps of 5–10 cells using glass needles.

3.2.4. Isolation of Mesoderm and Premigratory Neural Crest Cells of 8.5-d Embryos

1. Remove the yolk sac and the amnion using fine watchmaker forceps.
2. Bisect the embryo using tungsten needles along its midline.
3. Make transverse cuts (using metal needles) along the neuromeric junctions to isolate wedge-shaped fragments containing the tissue to be transplanted by (7, 8). For example, to isolate somitomere IV and the middle hindbrain neural crest cells, transverse cuts should be made at the preotic sulcus and the otic sulcus.
4. Incubate the embryonic fragments in the trypsin-pancreatin solution for 20 min at 37°C. When the mesoderm and ectoderm layers are loosened, separate the tissue layers using glass needles.

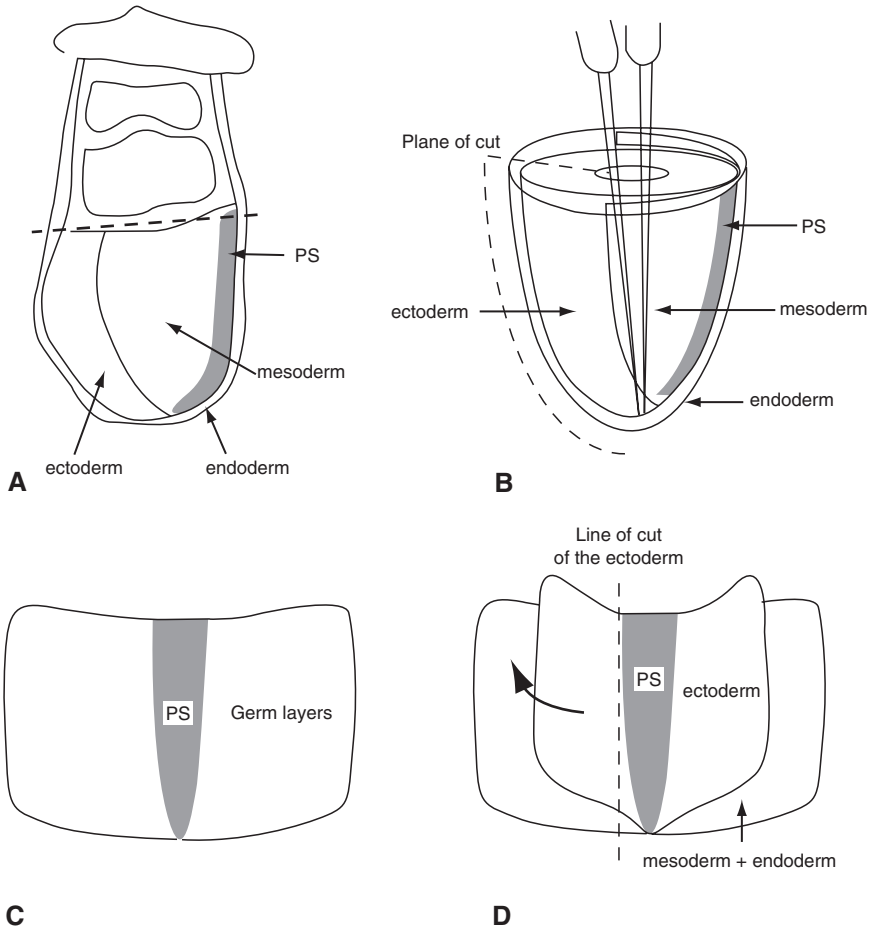

Fig. 3. The steps of germ layer separation of 7.0–7.5-d embryos. **A** Tissue organization of the 7.5-d embryo. The dashed line marks the position of the first cut at the junction between the embryonic and extraembryonic parts of the egg cylinder. **B** The embryonic portion of the embryo that reveals the anatomical relationship of the three germ layers. A metal needle is pushed into the amniotic cavity and pins the egg cylinder in the upright position. The second metal needle is then brought inside the amniotic cavity, and the embryo is cut along the anterior side (indicated by the dashed line) by slicing the second needle through all germ layers. **C** The embryo opened out flat and ready for enzyme digestion. **D** The ectoderm layer that has been loosened by enzymatic digestion. The ectoderm can be lifted up and torn away from the mesoderm using needles. It is not possible to separate the germ layers at the site of the primitive streak. The ectoderm is therefore cut along the dashed line, where no further separation from the underlying primitive streak could be made. The mesoderm layer can be separated from the endoderm in the same manner, followed again by cutting them close to the primitive streak. The remaining tissue is the primitive streak (PS).

5. Dissect the isolated mesoderm or neuroectoderm using glass needles into small clumps of five to ten cells (*see* **Note 8**).

3.3. Labeling and Grafting of Cells in 6.5–7.5-d Embryos

3.3.1. Making Pipets

3.3.1.1. Making Holding Pipets

The holding pipets are made using Leitz thick-wall glass capillaries. The internal diameter of holding pipets should be 10–50 µm, depending on the size of the embryo used for the experiment.

1. Attach a hypodermic syringe with a G20 needle to a bunsen burner connected to butane gas cylinder. Adjust the gas flow by a regulator to produce a small flame.
2. Hold the middle portion of the capillary over the flame until the glass starts to melt.
3. Take the capillary away from the flame, and pull from both ends to produce a thin segment of capillary.
4. Break the drawn capillary in the middle with a diamond pencil.
5. Polish the holding pipets on a microforge. A small glass bead is melted onto the platinum wire of the microforge. Heat the glass bead by increasing the electric current until the platinum wire glows red hot. Bring the tip of the pipet as close as possible to the glowing glass until it melts under the heat of the glass bead. Retract the pipet from the glass bead when a polished tip is produced (**Fig. 1B**; *see* **Note 9**).

3.3.1.2. Making Injection and Grafting Pipets

Injection pipets with an inner diameter of about 1–2 µm are made from thin-walled glass capillaries. The internal diameter of grafting pipets is larger and varies according to the size of the clumps of cells to be grafted.

1. Pull glass capillaries on a vertical or horizontal pipet puller to produce pipets with a fine tip, a long shaft, and a short shoulder (**Note 10**).
2. Break off the tip of the injection pipet by touching it against the cold glass bead on the filament of a microforge. This will produce a pipet for dye labeling (**Fig. 1C**).
3. To make grafting pipets (**Fig. 1D**), pull the glass capillary as in step 1. Determine a position on the shaft of the pipet by measuring with a ocular micrometer on the eyepiece of the microscope of the microforge, where the glass capillary has an inner diameter appropriate for the size of the cell clumps. Break the shaft of the pipet (**Fig. 4**) by (1) bringing the pipet to the heated glass bead on the microforge filament so that the site of intended break just touches the bead; or (2) as the capillary begins to fuse with the glass bead, turn off the power supply to the filament to cool down the filament instantly. The retraction of the filament as it cools down will snap the pipet precisely at where the capillary fuses to the bead.
4. Beveled pipets are made from the grafting pipet made in step 3 (**Fig. 3D; Notes 10 and 11**). Touch one side of the pipet with the heated glass bead. When the pipet tip fuses with the bead, slowly withdraw the pipet to pull a sharp bevel of about 5 µm in length.

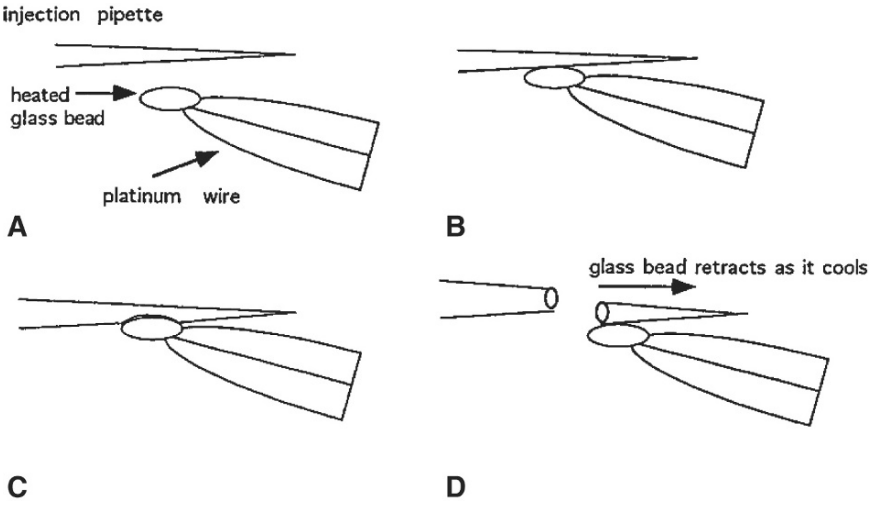

Fig. 4. Making grafting pipets. A glass bead is attached to the tip of the heating filament of the microforge. The bead can be heated and cooled instantly by switching on and off the current of the heating filament. The current is adjusted to yield the optimal heating appropriate to the thickness and the thermal property of the glass capillary. **A, B** The heated glass bead contacts the shaft of the pipet at the position that gives the desired internal diameter. **C** Melting the capillary wall into the glass bead. **D** The resultant break in the pipet after the bead cools down and retracts when the current is switched off. This produces a pipet of the desired internal diameter and an even tip.

3.3.1.3. Angled Pipets

1. Make holding, grafting, and injection pipets as described in **sections 3.3.1.1** and **3.3.1.2**.
2. Heat the pipet, over a flame, 1–2 cm from the tip of the injection pipet. It is heated until it is bent to an angle of approx 100°.
3. Turn the pipet 180°, and heat the pipet at a position 1–2 cm further proximal of the first bend. Again the pipet is bent at an angle of approx 100°. (**Fig. 1E**).

3.3.1.4. Making Transfer Pipets for 6.5–7.5-d Embryos

1. Heat the shaft of the Pasteur pipet over a flame.
2. When the glass begins to melt, take the pipet away from the flame and hand-pull the pipet to produce a segment of thin capillary.
3. Break the capillary by bending the pipet. Check the size of the pipet tip under the dissecting microscope (0.5 mm for 6.5-d embryos and 1–1.5 mm for 7.5-d embryos).
4. Connect the pipet with a flexible plastic tube to a mouthpiece. The movement of fluid and embryo in the pipet is controlled by suction or blowing into the mouthpiece.

3.3.2. Labeling the Germ Layers of 6.5–7.5-d Embryos with Carbocyanine Dye

In labeling the germ layers, various techniques are employed, depending on the layer of interest. Carbocyanine dye is microinjected when labeling the epiblast or ectoderm and the mesoderm. This is done by pushing the dye-filled injection pipet into the appropriate layer of the embryo and expelling the dye. For labeling the endoderm, carbocyanine dye is painted onto the surface of the embryo.

1. Embryos are manipulated in a hanging drop under a Laborlux S microscope. Set up the micromanipulation chamber as in **Fig. 5A**.
2. Place a 5-µL drop of media (10% FCS in PB1) for holding the embryos during manipulation on a coverslip using a transfer pipet. A second 5-µL drop of dye is placed approximately 1 cm from the first drop (**Note 12**; **Fig. 5A**).
3. Invert the coverslip, and place it across the two glass strips of the chamber.
4. Fill the chamber with light paraffin oil.
5. To transfer embryos into the manipulation chamber, place the chamber under a dissecting microscope with the drops in focus. Pick up the embryos using a mouth-controlled transfer pipet. Pass the pipet through the oil to reach the drop and gently expel the embryo with a small volume of medium.

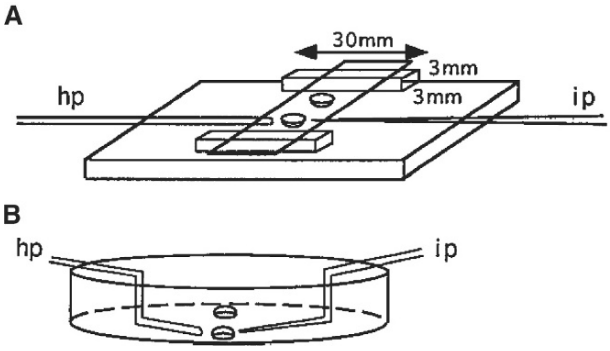

Fig. 5. **A** The manipulation chamber used for manipulating 6.5-d embryos in hanging drops. The base of the manipulation chamber is made of glass of good optical quality supplied by an optometrist. The dimension of the glass strips supporting the cover slip is 3 × 3 × 30 mm. They are set between 4 and 6 mm from the edge. Two drops of solution are placed on the cover slip approximately 1 cm apart, then the coverslip is inverted over the paraffin oil-filled chamber: hp, holding pipet; ip, injection pipet. **B** The petri dish setup used for manipulating 7.5- and 8.5-d embryos. Two drops are placed approximately 1 cm apart in the center of the dish then covered with paraffin oil. Angled pipets are used: hp, holding pipet; ip, injection pipet.

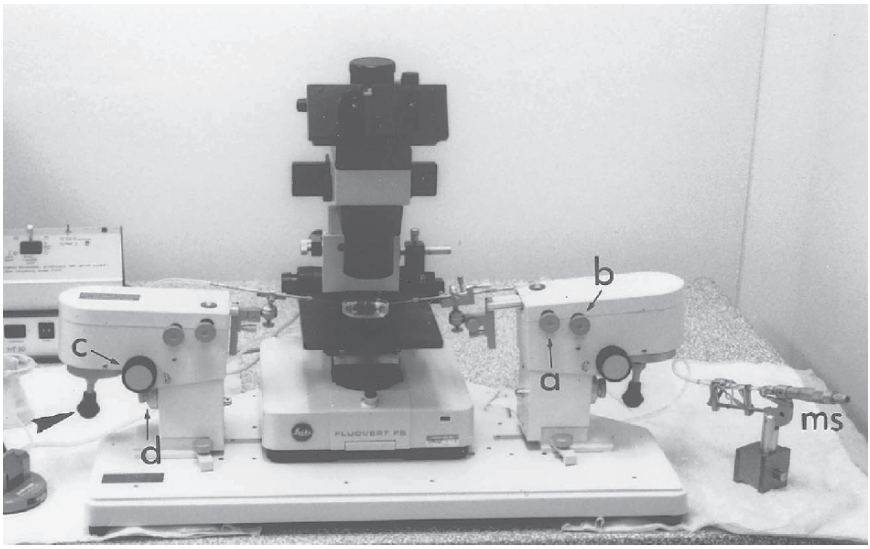

Fig. 6. The micromanipulation assembly. The manipulators are clamped to the base plate. Each manipulator can hold up to two instrument holders. Movement of the instruments is controlled by screw-knobs (**a** and **b**) that allow positioning of the manipulator in the *X-Y* horizontal plane. There is a third control (**c**) by which the angle of inclination of the manipulator can be adjusted. Coarse and fine adjustment of the position of the manipulator in the *Z*-vertical plane is controlled by the fourth knob (**d**). Fine *X-Y* movement of the instrument under the microscope is controlled by joysticks (arrowhead). The holding instrument is on the left-hand side of the assembly and the suction and expulsion of fluid are controlled by the micrometer syringe (ms) on the right-hand side. Conversely, the manipulating (labeling or grafting) instruments are on the right-hand side, controlled by the de Fonbrune syringe (fs) on the left-hand side. This setup enables the positioning of the instruments and the control of syringe to be accomplished simultaneously.

6. Set up the manipulation apparatus as shown in **Fig. 6**. Attach the holding pipet to the instrument holder on the left manipulator. Attach injection pipet to the instrument holder on the right manipulator. The holding pipet is controlled by the micrometer syringe on the right, whereas the injection pipet is controlled by the de Fonbrune syringe on the left. With this configuration, the positioning of the manipulator and the control of the syringe can be performed simultaneously.
7. Back fill the holding and injection pipets with heavy paraffin oil by adjusting the syringes.
8. Position the manipulation chamber, and adjust the microscope to focus on the medium drop containing the embryos.

9. Bring the holding pipet into the field of view by pushing it through the oil into the drop. Draw a small amount of medium into the holding pipet to create an oil medium meniscus.
10. Bring the injection pipet into view. Make sure that no medium is taken into the pipet, because contact with an aqueous solution causes precipitation of the carbocyanine dye (**Note 12**).
11. Retract the holding and injection pipets from the medium drop (**Note 13**). Bring the dye drop into the field of view by moving the stage of the microscope (**Note 14**).
12. Dip the tip of the injection pipet into the dye drop, and draw a small amount of dye into the pipet using the de Fonbrune syringe. Always keep the dye–oil meniscus in view, since this offers the only means for monitoring the flow of dye during labeling. Take the injection pipet out of the dye when the dye–oil meniscus stops moving.
13. Bring the medium drop containing the embryos back into the field of view.
14. Position the embryo by pushing it with the pipets and rotating it by lifting the pipet from beneath the embryo.
15. After the embryo is oriented correctly, bring the embryo into focus using the focusing control of the microscope, then bring the injection and holding pipets into focus using the control knob on the manipulators (**Fig. 6**). Touch the injection and holding pipets lightly against the embryo to confirm that they are in the same plane.
16. Bring the holding pipet into contact with the endoderm of the embryo next to the epiblast to be labeled. Apply a suction force using the micrometer syringe to draw the embryo against the holding pipet. Increase the suction so that a small area of the endoderm layer is partly drawn into the holding pipet (**Fig. 7**).
17. Push the injection pipet through the extraembryonic tissue into the amniotic cavity (**Note 15**). Then push the pipet tip into the epiblast layer (**Fig. 7**). When labeling the endoderm, the dye is partially expelled from the tip of the micropipette and the bolus of dye is then brought into contact with the apical surface of the endodermal layer and the cells are "painted" by moving the dye bolus against the cell surface.
18. Apply pressure via the de Fonbrune syringe to expel a small volume of dye into the epiblast. It is important to monitor the movement of the dye front to avoid injecting any oil into the embryo (**Note 16**).
19. Once the dye front has stopped advancing, retract the injection pipet.
20. Release the embryo from the holding pipet by applying a positive pressure via the micrometer syringe. Return the embryo to the culture medium (**section 3.1**).

3.3.3. Electroporating Gene Expression Construct into Germ Layer of 7–7.5-d Embryos

3.3.3.1. Setup of Electroporation Apparatus

1. Flatten the end of a 3-cm platinum wire into a plate and solder the other end of the wire onto a banana socket.
2. Solder a tungsten wire onto a banana plug. Sharpen the end of the wire by dipping it in a 3-M NaOH bath connected to a 6-V lantern battery. Attach the banana socket fused to the platinum plate onto a 150-mm petri dish with plasticine. Make a hole on side of lid of a 60-mm petri dish, insert the platinum plate through

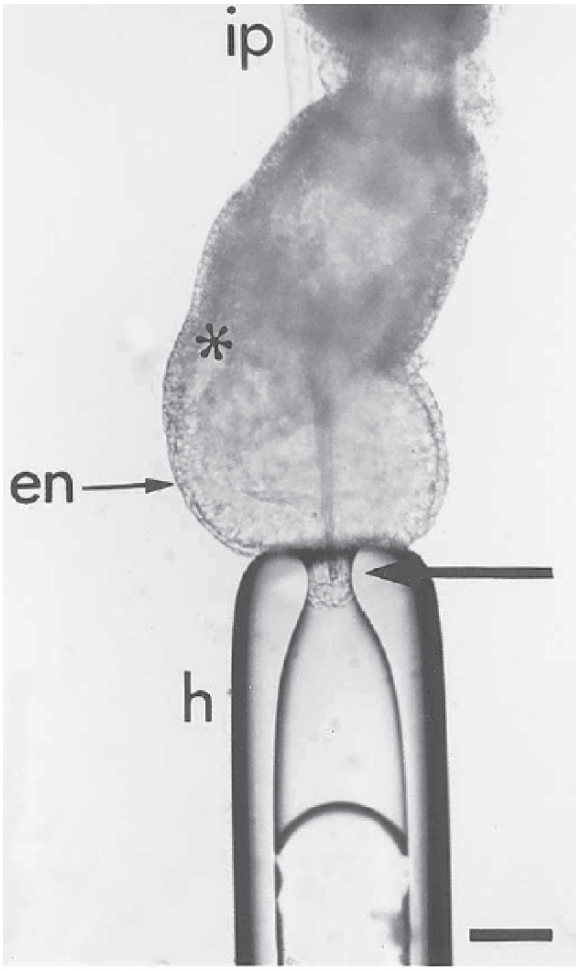

Fig. 7. Labeling of the epiblast by microinjecting DiI into the distal cap of the embryo. To inject in the midline, the embryo is held at the distal tip of the egg cylinder by gentle suction with the holding pipet (h). The injection pipet (ip) is passed through the extraembryonic tissues of the egg cylinder. The injection pipet is brought to the site of labeling from within the pro-amniotic cavity to avoid inadvertent labeling of other embryonic germ layers. The arrow points to the tip of the labeling pipet in the epiblast layer: en, primitive endoderm; *primitive streak. Bar = 20 µm.

hole, and attach a thin piece of plastic tubing covering the wire, leaving the plate exposed (**Note 17**).

3. Connect the platinum plate and tungsten wire to an Electro Square Porator, so that the platinum plate is the positive pole and the wire is the negative pole (this arrangement may have to be reversed with different electroporation experiments).

4. Set up the micromanipulation apparatus and attach the holding pipet as described in **section 3.3.2**, **steps 6–9**, except that an injection pipet and light paraffin oil is not used.
5. Place a drop of Tyrode's Ringer saline over the platinum plate until it is completely immersed. Place a separate drop of PB1 on the petri dish.
6. Attach the banana plug holding the tungsten wire to a positioner. Bring holding pipet, platinum plate, and tungsten wire into the field of view on the micromanipulator apparatus. Position the tungsten wire until it is at same focal plane under the microscope as the platinum plate, but leave a small gap between them.

3.3.3.2. Electroporating Expression DNA Construct into a Cell of the Endoderm of 7–7.5-d Embryos

1. Using a mouth-controlled transfer pipet place about seven embryos in PB1 in smallest drop possible (5–10 µL) on the petri dish.
2. Add (5 µL) concentrated plasmid DNA solution (aqueous, concentration ranges from 2–5 µg/µL) onto the pool of embryos and pipet the solution up and down to mix the two solutions together so that the final DNA concentration is 1–2 µg/µL Incubate the embryos for at least 5 min.
3. Using a mouth-controlled pipet carefully pick up one embryo from the drop with minimal amount of DNA solution and transfer the embryo to the electroporation drop of Tyrode's Ringer saline (**Note 18**).
4. Bring the electrodes into the field of view around the embryo
5. Position the embryo between the two electrodes using the holding pipet as in **section 3.3.2**, **steps 14–16**, except that the embryo is held on the side opposite the site of electroporation (**Note 19**).
6. Position the embryo between the platinum plate and tungsten wire so that the embryo touches both the plate and the wire (the latter with the endoderm at the site of electroporation).
7. Move the wire and the plate slightly apart and reposition the embryo so that the embryo stays in the gap between the wire and the plate but not touching either electrode (**Fig. 8A; Note 20**). Set the Electro Square Porator to deliver five pulses at 15 V at 50 ms apart. Switch on the electroporator.
8. Release the embryo from the holding pipet by applying a positive pressure via the micrometer syringe. Pick up the embryo using a Pasteur pipet and place it in the drop of PB1 (**Note 21**).
9. Repeat steps 1–7 for each embryo until all are electroporated (**Note 22**).
10. Culture the embryos initially for 3 h in static culture medium as in **section 3.1.2** in 100% RS (for 7.0-d embryos) and DMEM:RS (1:3 by vol; for 7.5-d embryos) (**Note 23**).
11. After 3 h, the embryos are photographed as in **section 3.5.3** to visualize the location of the electroporated cells if fluorescent constructs are employed for labeling (**Fig. 8B; Note 24**)
12. Transfer embryos to the roller culture incubator as in **section 3.1.3** and culture for the required duration of the specific experiment.

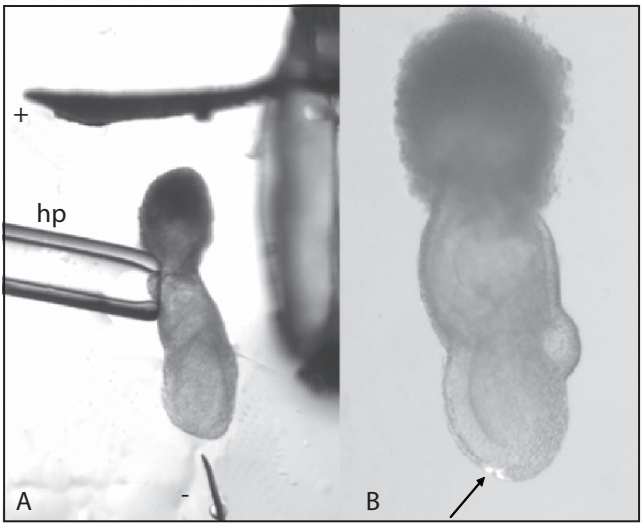

Fig. 8. Labeling the endoderm of 7.0-d embryo by electroporation. **A** The embryo is first bathed in plasmid DNA solution before transfer to the electroporation drop on the culture dish. To electroporate the distal tip, the embryo is held at the extraembryonic ectoderm by suction using the holding pipet (hp) and positioned between the platinum plate (cathode) and tungsten needle (anode). The electrodes are connected to an Electro Square Porator and the electroporation is focused at the tissue closest to the needle tip. **B** Fluorescent cells in the distal region of the embryo following electroporation with a β-actin-eGFP construct and cultured for 3 h.

3.3.3.3. Electroporating Gene Construct into a Cell of the Epiblast or Ectoderm in 7–7.5-d Embryos

1. Assemble the electroporation apparatus and the micromanipulation apparatus as in **section 3.3.3.1**, **steps 1–6**, except that the plate is set as the negative pole and the wire is set as the positive pole. Targeting the epiblast or ectoderm requires that the polarity arrangement is reversed, so that the DNA enters via the apical surface of the epiblast or ectoderm as it is driven toward the needle electrode.
2. Inject plasmid DNA into the amniotic cavity as in **section 3.3.2**, **steps 1–20**, except that the plasmid DNA solution is injected instead of carbocyanine dye. It is easier if another manipulator is used for injecting, so the injecting pipet does not interfere with the electroporation apparatus if they are set up on the same micromanipulator.
3. Pick up the embryo using mouth-controlled transfer pipet and place into the drop containing Tyrodes ringer saline.
4. Position, electroporate, and culture the embryo as in **section 3.3.3.2**, **steps 4–9**, and take images of fluorescent embryo (if a fluorescent gene constructs are used) at 3 h and other appropriate times as in **section 3.5.3**.

3.3.4. Grafting Epiblast Fragments to 6.5-d Embryos

1. Place two drops of PB1 (+10% FCS) medium approximately 1 cm apart on the coverslip, and set up the manipulation chamber as **section 3.3.2, steps 1–4**.
2. Set up the manipulation apparatus, and position the pipets as described in **section 3.3.2, steps 5–9**.
3. Position and hold the embryo as in **section 3.3.2, steps 14–16**, except that the embryos is held on the side opposite to the site of grafting (**Fig. 9**).
4. Pick up the cell clumps into the grafting pipet with a small amount of medium. Draw the cell clumps about 20 µm into the grafting pipet.

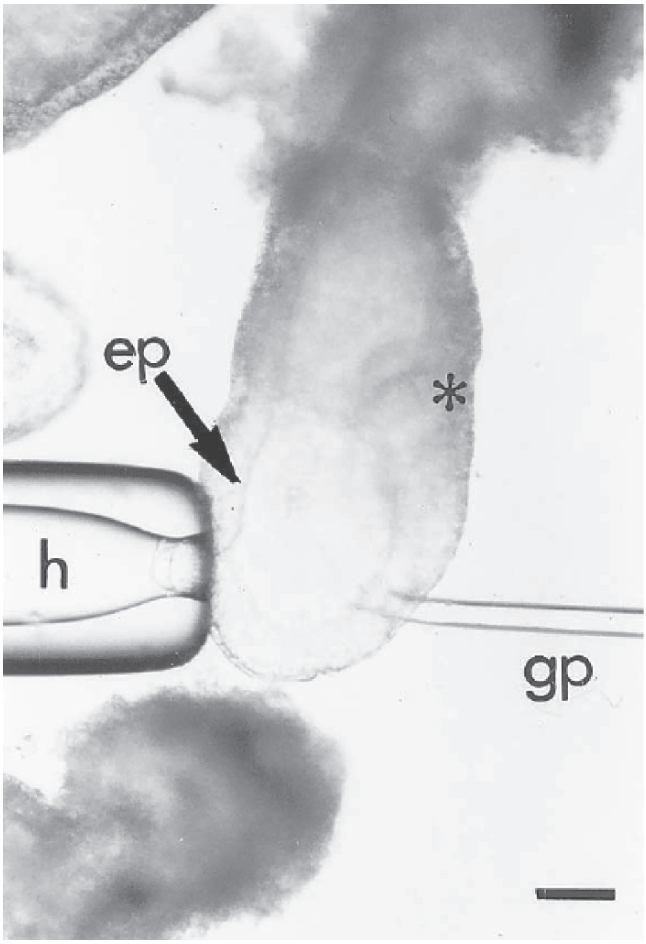

Fig. 9. Grafting of cells to a 6.5-d embryo. The embryo is held on the anterior side opposite the intended site of grafting by the holding pipet (h). The cells are grafted into the epiblast (ep) on the posterior side of the embryo (*marks the position of the primitive streak) immediately proximal to the margin of the distal cap. Bar = 20 µm.

5. Insert the grafting pipette by a sharp jabbing action through all tissue layers into the proamniotic cavity at the site of transplantation. If necessary, a beveled pipet may be used.
6. Apply a positive pressure to the de Fonbrune syringe to expel the graft from the pipet as the pipet is slowly withdrawn from the embryo (**Notes 25–27**). A coordinated movement of the pipet and the expulsion of the graft is critical to the precise positioning of the graft in the epiblast.
7. Return the embryo to the culture medium (**section 3.1**).

3.3.5. Grafting of Cells

1. Place a 50-µL drop of medium (the manipulation drop) slightly off-center in the lid of a petri dish. Place another drop of medium (for holding cells for grafting) next to the first drop (**Fig. 5B**).
2. Fill the petri dish with light paraffin oil to cover the drops.
3. Transfer embryos from the culture to the manipulation drop in the petri dish by mouth pipeting.
4. Angled pipets are used for holding and grafting cells into the embryos. Place the pipets in the instrument holders of the manipulator. Tilt the manipulators so that the tips of the pipets are immersed in the paraffin oil and point to the bottom of the petri dish. Figure 6 shows the setup for manipulating 7.5-d embryos.
5. Focus the microscope on the embryos in the manipulation drop. Lower the pipets into the drop. Manipulation is carried out according to **section 3.3.2, steps 7–20; section 3.3.4, steps 3–7;** and **Note 28**.

3.4. Transplanting and Labeling Cells in the Cranial Region of 8.5-d Embryos

3.4.1. Making Transfer Pipets

Embryos that are 8.5 d old are transferred using Pasteur pipets that are cut at the shaft to give an internal diameter of about 3–4 mm. This allows the embryos to be transferred without damage to the yolk sac.

3.4.2. Manipulation

1. Embryos that are 8.5 d old are manipulated in a petri dish in the same way as described in **section 3.3.5, steps 1–4**.
2. Orient the embryo using fine watchmaker forceps.
3. Hold the embryo by the yolk sac with the holding pipet by applying negative pressure to the micrometer syringe. The embryo is positioned so that a clear silhouette of the cranial neural fold is visible.
4. Insert the injection and grafting pipet through the yolk sac then the amnion into the amniotic cavity.
5. Push the pipet gently into the cranial neural fold or further into the cranial mesoderm.
6. Expel the cell clumps or dye by applying a positive pressure to the de Fonbrune syringe. Expulsion of the cell clumps is accompanied by the simultaneous withdrawal of the pipet.

7. For dye delivery, the pipet remains in position until the dye front has stopped moving toward the tip.
8. Return the embryo to culture (**section 3.1.3**).

3.5. Analysis of Labeled Embryos

Dye-labeled embryos are analyzed by fluorescence microscopy. Fluorescent detection indicates the location of the labeled cells within the embryos. More detailed analysis can be carried out by confocal microscopy.

3.5.1. Preparation of Embryos for Confocal Microscopy

1. For confocal microscopy, embryos are analyzed in whole mount. Embryos that are 8.5 d old and older need to have the extraembryonic tissues dissected away. Embryos that are 6.5 and 7.5 d old are analyzed intact.
2. Embryos are mounted flat under a coverslip (cover glass number 1½). To allow sufficient space for the embryos between the slide and the coverslip, small bluetac feet are placed at the four corners of the coverslip (**Note 29**).
3. The slide is filled with PBS and sealed with nail polish.

3.5.2. Confocal Analysis

A confocal microscope allows one to obtain an image of the fluorescent label and a transmission image of the embryo. The two images can be overlaid to identify where along the anterior posterior axis of the embryo the labeled cells are located. The depth of the sections of the fluorescent label gives some indication of which tissue layer contains the labeled cells.

3.5.3. Imaging Live Embryos Using Fluorescence Microscopy

As indicated previously, expression of fluorescent protein can be visualized by fluorescence microscopy.

1. Live or fixed embryos are examined using a fluorescence stereomicroscope system (Leica MZ FLIII) using excitation filter GFP-1 filter set, 425 nm.
2. Photographs are taken using a SPOT Advanced digital camera (version 3.5.9.1).
3. During photography, live embryos are kept in warmed PB1(~37°C) while fixed embryos (in 4% paraformaldehyde) are in PBS.
4. For each specimen, both bright field and fluorescent images are captured.
5. From the bright field image, a red-color scan is extracted by selecting for the red channel capture. This red-channel image is merged with the true-color fluorescent image by Adobe Photoshop 7.0 for showing the position of the fluorescent cells in the embryonic structures (**Note 30**).

3.5.4. Analysis of Grafted Embryos

The distribution of graft-derived cells may be analyzed in whole mount or on serial sections of the embryo if the cells are marked by *lacZ* transgene. For protocols used to visualize the transgenic cells, *see* **Chapter 10**.

4. Notes

1. It is important that the rat and cord blood are spun as soon as possible after collection, preferably before clotting commences. The serum obtained by squeezing the fibrin clot is known as the *immediately centrifuged serum*.
2. Serum used as medium supplement should have a low lipid content (clear instead of cloudy). Hemolyzed serum does not support normal culture of mouse embryos.
3. The medium in the first well of the NUNC slide is used to wash the embryo once before transferring to the culture wells.
4. Growth rates decline if the embryos are overcrowded and, likewise, if they are cultured alone.
5. Best development for 48 h is achieved with embryos explanted shortly after the appearance of the primitive streak.
6. The successful culturing of 5.5-d embryos was recently described using similar culture medium. Using a micromanipulator, hold the 5.5-d embryo with a holding pipet and remove the Reichardt membrane a glass needle. Culture in a medium of DMEM:RS (1:1 and 1:3 by volume) for up to 48 h ***(11)***.
7. The appearance of the primitive streak provides an unequivocal indication of the anterior–posterior axis and helps with the orientation during dissection. Early gastrulation embryos are therefore used in experiments that demand knowledge of the source of cells in the anterior–posterior and medial–lateral axis. However, if only a distinction between proximal and distal epiblast is required, both pre- (without the primitive streak) and early gastrulation embryos can be used.
8. The choice of developmental stage is important to avoid contamination of the cranial mesoderm by migrating neural crest cells. Previous studies have established that the first population of neural crest cells to leave the neural plate are those of the mesencephalon at the 5–6 somite stage ***(12–16).*** Therefore, in experiments involving the cranial mesoderm, only embryos having ≤5 somites are used, so that the mesodermal explants are free of any migrating neural crest cells. To obtain an enriched neural crest cell population for cell fate analysis, premigratory neural crest cells are isolated from the lateral region of the neural plate of these embryos for transplantation experiments.
9. It is important that the holding pipette has a smooth surface so that the embryo is not damaged when it is held against the tip by suction.
10. If the internal diameter of the grafting pipet is large relative to the size of the embryo, it may be difficult to puncture the tissue layers with the pipet during grafting. Beveled pipets can be used in this situation. The sharp point of the beveled pipet slices the tissue layers to create a passage for the grafting pipet.
11. Do not overheat the glass bead, since this may cause distortion and constriction of the pipet tip. It is useful to mark the side of the pipet with the bevel for later orientation.
12. The drops should be placed far enough apart to avoid mixing of contents as the pipets are moved around in the chamber.
13. When moving between the dye drop and the media drop, the holding pipet should be retracted, so that its tip does not become contaminated with the dye.

14. When drawing up the dye solution, it is best to keep the edge of the dye drop in sight, so that only the tip of the pipet is dipped into the dye drop. Pushing the pipet too far into the drop results in an excessive amount of dye adhering to the outer surface of the pipet.
15. Labeling of the embryonic tissue is best accomplished through the extraembryonic route, because it reduces the chance of accidentally labeling other germ layers. Even if dye is leaking from the pipet or coming off the surface of the pipet during the passage of the pipet, only the extraembryonic tissues are inadvertently labeled.
16. The pipets may clog with precipitate of the dye after repeated uses. The clogged pipet can be recovered by breaking its tip by brushing against the holding pipet while in the chamber or by wiping it gently with a lint-free tissue when it has been taken out of the chamber.
17. The lids of petri dishes are used because the rim has a lower clearance, which gives better accessibility of the angled pipets to the embryos and tissues in the dish.
18. Fill the mouth-controlled pipet with Tyrode's ringer saline first, so that the embryo and only a small amount of DNA solution enter the pipet by capillary action. Also release the embryo into the electroporation drop with minimal DNA solution.
19. Holding the embryo at the ectoplacental cone or the extrambryonic ectoderm is preferred to holding the embryo proper, so the embryo's outer germ layers are not disturbed. The holding pipet need not be as close on the opposite side of the embryo as for injecting dye or cells. However, during pulses, the current flowing can cause the embryo to swing, which could be overcome by holding the embryo closer to the site of electroporation.
20. Air bubbles emit from the tungsten wire, indicating the current has been generated. It is essential that the embryo is correctly spaced between the plate and wire such that large bubbles emitting from the plate and wire do not touch and burn the embryo and also that the targeted embryo site is close to the wire to allow focused point of electroporation.
21. A separate pipet is used, so the mouth controlled pipet is reserved for transferring embryos from DNA solution, thus reducing dilution of DNA solution.
22. Electroporation has to be done fairly swiftly to minimize the time spent by the embryo in DNA solution or Tyrode's solution.
23. Initial culture in static culture may improve the viability of the embryo, which may have sustained damage by electroporation.
24. Our data show that it requires 2–3 h for sufficient protein to be synthesized to enable detection of fluorescence.
25. If the cell clumps become sticky, they will follow the grafting pipet out of the embryo. Leave the pipet with the graft partly out of the tip at the transplantation site for 10–30 s to allow the graft to adhere to the surrounding tissue. Following that, a snappy retraction of the grafting pipet or tapping gently on the base plate of the manipulator may dislodge the graft from the pipet tip. Siliconizing the injection glassware also stops the cell clumps from adhering to the injection pipets.
26. Occasionally, the host embryos collapse because of fluid leakage through the wound made to accommodate the graft. Inflating the embryonic cavity by injecting

a small amount of medium to replenish the loss of fluid during transplantation may improve the development of the embryo.

27. A small graft is readily incorporated into the host embryo. Larger grafts may be squeezed out of the embryo as the wound closes. Pushing the tip of the grafting pipet against the graft that has been lodged in the germ layer for approximately 10–30 s helps the retention of the graft.
28. The principle for manipulating 7.5-d embryos is the same as for 6.5-d embryos. However, manipulations are carried out in a petri dish and therefore require the use of the fluovert FS inverted microscope.
29. Despite the blue-tac feet, the embryos are squashed to some extent. With 6.5- and 7.5-d embryos, it can be difficult to orient the embryos once they have been squashed. For ease of analysis, mount the embryos in the sagittal plane, and mark the direction of the anterior–posterior axis on the slide.
30. A red bright field image allows for better visualization of the fluorescent cells by providing a stronger contrast between the cells and the embryos

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7

Production of Transgenic Rodents by the Microinjection of Cloned DNA into Fertilized One-Celled Eggs

David Murphy

1. Introduction

A pioneering experiment in the early 1980s demonstrated that microinjection of a recombinant growth hormone gene into the pronuclei of fertilized one-celled mouse embryos resulted in inheritable changes in the growth of the resulting "transgenic" mice and their offspring ***(1)***. Mammalian transgenic experiments have since contributed tremendously to our understanding of numerous complex biological processes. The power of the technique lies in that it allows the function, and the developmental and physiological regulation, of almost any gene or protein to be studied within the context of the normal processes occurring in the whole animal.

How DNA integrates into the host chromosome is not known, but some insights can be inferred from a study made on the state and organization of the inserts found in transgenic mice ***(2)***. Approximately 70% of the mice carry exogenous DNA in all their somatic and germ cells, implying that integration took place prior to the first round of DNA replication. The remaining 30% of the mice show some degree of mosaicism, indicating that the transgene DNA must have integrated after this first round of replication. Integration events have been observed on many different autosomes ***(3)***, on the X-chromosome ***(4)***, and on the Y-chromosome (Murphy, unpublished observations). The transgene copy number varies (one to several hundred), but within a transgenic animal, there is usually only one integration site where, if in multiple copies, the transgene is arranged in a head-to-tail tandem array. Once incorporated into the host DNA, the transgene transmits in a classic Mendelian

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fashion. Appropriate tissue-specific and physiologically regulated expression of the transgene is a prerequisite in many transgenic experiments and can be obtained only by including the appropriate genetic elements in the structure. Once appropriate expression is obtained, gain-of-function (overexpressing the gene of interest) or loss-of-function (cell-specific expression of cytotoxic or dominant negative proteins) approaches can be employed to derive information about almost any gene or cell type. Studies of the nervous and immune systems and oncogenesis *(5–7)* are some examples where transgenic animal studies have contributed significantly to our knowledge.

This chapter details the procedures necessary for the generation of transgenic mice and rats by the injection of cloned DNA into the pronuclei of fertilized one-celled eggs. The technique demands precise technical skill and expensive equipment, but its reliability and speed make it an efficient way of generating transgenic mice and rats.

1.1. Summary of the Microinjection Method

1. Immature donor females are superovulated and mated with stud males (**section 5**).
2. Approximately 12-h post coitus (pc), the oviducts from donor females are collected and eggs are harvested and placed into culture (**section 8**.).
3. The eggs are microinjected (**section 10**) with specially purified cloned DNA fragments (**section 9**).
4. Eggs that survive microinjection are returned to the natural environment by implantation into pseudopregnant surrogate mothers (**section 12**) produced by mating sexually mature females with vasectomized males (**section 4**).
5. A percentage of the transplanted eggs survive to full term and are delivered either naturally or by Caesarean section (**section 13**).
6. Transgenic animals are identified by Southern Blot or PCR analysis of genomic DNA isolate from the tail tissue.
7. Transgenic animals are bred to produce a line and analyzed for transgene expression and are cryopreserved for posterity (**section 15**).

The efficiency of producing transgenic mice or rats varies considerably among experiments. Mice have been more often used in transgenic experiments, and the efficiencies of each stage are well recorded. Under optimal conditions, 60–80% of the eggs survive injection and 10–30% implanted in the pseudopregnant recipient proceed through normal development and are born. Of the pups, 10–30% are transgenic. In rats, the available data are that 50–75% survive injection, 10–30% survive implant and are born as pups, of which 10–33% are transgenic (8).

2. Animal Welfare

The conduct of scientific experiments involving animals is strictly regulated by most governments and institutions. Scientists must consult with the relevant regulatory bodies before commencing on any experiment involving genetic

engineering or live animals. Apart from the legalities, the scientist must always abide by the basic principle that the animals under his or her care should not suffer any avoidable distress. Guidelines for such care are published by the National Institutes of Health and the National Academy of Sciences ***(9)***. The techniques using live animals detailed in this chapter are only a guide for the novice. All manipulations involving animals must be taught to a novice by a skilled, experienced operator. For the surgical procedures (vasectomy and oviduct transfer), it is advised that the inexperienced novice first practice on cadavers until sufficient skill and confidence are gained to operate on live animals. If an animal, at any point of the experiment, appears to be suffering from undue distress, kill it immediately by decapitation or cervical dislocation.

2.1. Humane Killing of Mice and Rats

The recommended way of killing mice is by breaking the neck (cervical dislocation). This is quick, and causes the animal the minimum of distress. Pick the mouse up by the base of the tail, and place it on the top of the cage. Allow it to run away such that the animal is stretched out with its hindlegs almost in the air and its forelimbs gripping the cage bars firmly. Using a blunt instrument or your fingers, press down firmly on the base of the skull and pull on its tail. The stretching action breaks the neck, the pressure at the base of the skull defining the point of dislocation.

Kill rats by either cervical dislocation or decapitation with a rodent guillotine. The greater size and strength of rats requires that they are stunned before either procedure. Hold the animal firmly by the base of its tail, and swing it to hit the back of the head firmly on a hard surface (e.g., a solid benchtop). While the animal is stunned, dislocate its neck using a blunt instrument. Alternatively, a guillotine (e.g., Harvard Apparatus Ltd., Edenbridge, UK) can be used to decapitate the animal after it is stunned.

2.2. Anesthesia

Inhalation anesthesia using isoflurane is encouraged for both rats and mice. The requisite equipment is available from O'Neill Medicalia (Liverpool, UK). Alternatively, injection anesthesia can be employed.

2.2.1. Materials

2.2.1.1. Mice

1. Avertin: A 100% (v/v) stock is made by mixing 10 g of tribromoethylalcohol with 10 mL of tertiary amyl alcohol. The 2.5% (v/v) working solution is diluted from stock in sterile water. Both are stored at 4°C, protected from light.
2. Sterile 1-mL disposable syringe.
3. Sterile 0.5 × 16-mm disposable needles.
4. Animal balance

2.2.1.2. Rat

1. CRC: Mix one part Dormicum™ (5-mg/mL midazolam; Hoffmann-La Roche, Basel, Switzerland), one part Hypnorm™ (l0-mg/mL fluanisone and 0.2-mg fentanyl; Janssen Pharmaceuticals, Beerse, Belgium), and two parts sterile water. The made-up solution is stable at room temperature for 1 wk.
2. Sterile 1-mL disposable syringe.
3. Sterile 0.5 × 16-mm disposable needles.
4. Animal balance.

2.2.2. Methods

2.2.2.1. Mice

1. Weigh the mouse to determine the dose of Avertin to administer (15–17-µL 2.5% (v/v) Avertin/g body wt).
2. Draw up the appropriate volume into a sterile 1-mL disposable syringe. Exclude any air bubbles; with the needle of the syringe facing up, flick gently to dislodge any bubbles, and push the barrel of the piston until the bubbles are expelled.
3. Restrain the animal with one hand. Grip the scruff of the neck between thumb and forefinger and secure the tail and hence the rear of the animal with the third finger. With the other hand, introduce the needle into the abdomen of the mouse (i.e., intraperitoneally, ip), avoiding both the diaphragm and the bladder. Withdraw the barrel of the syringe slightly—a small air bubble is observed if the needle has been inserted correctly into the intraperitoneal space. Inject the anesthetic and wait awhile before withdrawing the needle. Accidental subcutaneous injection is revealed by back-leakage of drops of Avertin through the skin. The mouse will remain fully anesthetized for 0.5–1 h.
4. An adequate depth of anesthesia is indicated by an absence of a blinking reflex while blowing on the eyes and the maintenance of rapid breathing. Following surgery, the animal is observed to have regained mobility before being left to recover in a quiet, warm place.

2.2.2.2. Rats

1. Weigh the rat to determine the dose of CRC to administer (0.275-µL CRC/g body wt). Inject the rat ip as described for the mouse (**section 2.2.2.1**). If the rat is too big to be restrained with one hand, use one of the many commercially available plastic animal restrainers or ask another person to assist in restraining the rat.
2. The rat will remain fully anesthetized for 20–30 min. An adequate depth of anesthesia is indicated by an absence of a jerk reflex on pinching the animal's paw and the maintenance of rapid breathing.
3. Following surgery, the animal is observed to have regained some mobility before being left to recover in a quiet, warm place.

2.2.3. Notes

1. If an adequate level of anesthesia does not develop, inject an additional 25–50% of the initial volume of anesthesia.

2. Two other anesthetics that combine a suitably deep level of anesthesia with rapid reliable recovery in rats are
   a. 1:1 Mixture of Vetalar (100 mg/mL ketamine hydrochloride; Parke Davis Veterinary, Morris Plains, NJ) and Rompun (2% [v/v] solution equivalent to 65 mg/mL xylazine; Bayer, Leverkusen, Germany): 0.6 mL of the 1:1 Vetalar/Rompun mixture being administered to rats weighing between 250–300 g.
   b. Sagittal (60 mg/mL sodium pentobarbitone; Rhone-Merieux, Rhone-Merieux, Lyon, France): the recommended dose being 26 mg/kg.

3. Animal Stocks and Their Maintenance

3.1. Introduction

To generate and maintain transgenic animals, a scientist must have access to and be responsible for hundreds if not thousands of rodents. Therefore, only those scientists working in institutions equipped with the necessary facilities are able to undertake transgenic experiments. Practically, a suitable animal facility should be able to provide

1. Spacious caging and a regular change of clean, comfortable bedding for the animals. Cage-washing facilities should also be available.
2. A food and water supply.
3. Environmental control to regulate the lighting, ventilation, temperature, and humidity.
4. Access to veterinary care.

A variety of animals are necessary to produce a regular supply of fertilized one-cell eggs for microinjection. In addition, many more animals are produced in the process of generating and maintaining transgenic lines. The investigator and his or her staff must be prepared to devote much time to the maintenance and care of the animals if they wish to engage in transgenic experiments.

3.1.1. Mice Stocks Required for the Production of Transgenics

Fertilized one-cell eggs for microinjection are produced by mating a donor female and a stud male. Choosing the right strain of mouse for egg production is pivotal, since the ease and efficacy of generating transgenic mice is highly strain dependent. Brinster et al. ***(10)*** compared C57BL/6J inbred eggs and C57BL/6J × CBA/J hybrid eggs in terms of parameters, such as egg yield and survival after injection. Overall, the experiments on hybrid eggs were eightfold more efficient than inbred eggs. We generally use F1 animals generated from matings between CBA/J and C57BL/6J. An inbred strain called FVB can also be used. These mice are easy to superovulate, their embryos are very easy to inject, and the transgenic mice are derived on an inbred background ***(11)***.

3.1.1.1. Production of Fertilized One-Celled Eggs

Immature (12–14 g, 4–5 wk old) F1 hybrid females are superovulated and mated with F1 hybrid males. Ten immature superovulated females will yield

at least 250 viable eggs (F2 zygotes). Fifty breeding pairs (CBA/J male and C57BL/6J female) can provide 30 F1 females/wk. Alternatively, immature F1 hybrid females can be purchased from a reputable commercial and institutional supplier. Twenty F1 hybrid studs caged individually are required to mate with the immature females and are replaced every 8 mo to a year.

3.1.1.2. Recipients of the Microinjected Eggs

Estrous females (0.5 d pc) are used as surrogate mothers to nurture surviving eggs to birth. Females can be of any strain with good maternal characteristics but need to be sexually mature and >19 g in weight. We use C57BL/6J × CBA/J F1 females. Estrous females are made pseudopregnant by mating with vasectomized males.

3.1.1.3. Vasectomized Male Mice

Sexually mature males are sterilized by vasectomy and used to engender pseudopregnancy in sexually mature females. Any strain of mouse can be used, but Parkes and Swiss males are particularly suitable because they perform well. Twenty to 30 vasectomized males, mated on alternate days with females analyzed for their stage in the estrous cycle, should be able to provide at least five pseudopregnant females a day. Sexually mature male mice (about 2 mo old) are used. The vasectomy procedure is described in **section 4** of this chapter.

3.1.1.4. Foster Mothers

If only a very low number of fetuses develop in the pseudopregnant recipient, the resultant fetuses may be too large for natural delivery and a Caesarean section must be performed. The pups are fostered to mothers with natural pups of a similar age.

3.1.2. Rat Stocks Required for the Production of Transgenics

The generation of transgenic rats is a more recent development. As such, less research has been done on the efficiency of the technique using different rat strains. In our laboratory, we use only the Sprague-Dawley (SD) strain of rats.

3.1.2.1. Production of Fertilized One-Celled Eggs

Fertilized eggs are obtained from immature (4–5 wk old) superovulated females mated with mature stud males. These females yield 100–200 injectable eggs. To supply 20 immature females/week, 30 breeding pairs should be more than sufficient. At least 20 stud males caged individually are required for mating with the immature females and should be replaced every 8 mo to a year. An individual male should be presented with a female only every alternate day.

3.1.2.2. Recipients of the Microinjected Eggs

Estrous exbreeder females (females that have successfully pupped at least one litter) of between 8 and 16 wk are mated with vasectomized males the night before they are implanted with the microinjected eggs. Although virgins tend to produce larger litters than exbreeders, they often eat the resulting pups.

3.1.2.3. Vasectomized Male Rats

Vasectomized males (**section 4**) are needed to engender pseudopregnancy in the exbreeder females. Experienced males are preferred and, once vasectomized, have to be replaced every 6–8 mo as they grow quickly to a large size.

3.1.2.4. Foster Mothers

Foster mothers (with pups of about the same age) are occasionally required for the rare litter that is delivered by Caesarean section (**section 13**).

3.2. Notes

1. Following the production of transgenic founders, mate the animals as soon as possible to establish a transgenic line. Analysis of the founder phenotype should not begin until the transgene has passed successfully to a subsequent generation. In-vitro fertilization may be necessary if the founders do not mate (**section 14**).
2. Unless the animals are kept in an SPF unit, sporadic microbial infections will occur. A six-monthly check of the colony's health status by a veterinarian is advisable.

4. Vasectomy of Mice and Rats

4.1. Materials

1. Sexually mature male mice (2 mo old) or rats (5 wk old).
2. Dissection instruments (e.g., from InterFocus Ltd., Haverhill, UK).
3. Dissection scissors (large and small).
4. Fine blunt forceps (one pair curved, one pair straight).
5. Sharp curved forceps.
6. Watchmaker's forceps (size 5).
7. Curved surgical needle (size 10, triangular, pointed).
8. Surgical silk sutures (size 5).
9. Autoclips and applicator.
10. Anaesthetics: Avertin for mice, CRC for rats.
11. Bunsen burner.
12. 70% (v/v) Ethanol in squeeze bottle.

4.2. Methods

4.2.1. Mice

1. Anesthetize the mouse as in **section 2.2.** Place the animal ventral (abdomen) side up on the lid of a 9-cm glass or plastic petri dish.

2. Spray the lower abdomen with 70%, (v/v) ethanol. Comb the hair away from the proposed site of incision (*see* **Fig. 1A**) with a pair of fine forceps.
3. Lift the skin away from the body wall with a pair of fine, blunt forceps. Make a 1-cm transverse cut—level with the top of the hindlimbs (**Fig. 1A**)—with a pair of fine dissecting scissors. To reduce bleeding, stretch the incision to about 1.5 cm with the outer edges of the opened scissors.
4. Cut the body wall parallel to the skin cut. Again, stretch the incision.
5. Introduce a single stitch into one side of the body wall wound and leave in place.
6. Pull out the fat pad on one side of the animal and with it the testis, epididymis, and vas deferens (**Fig. 1B**) using blunt forceps.

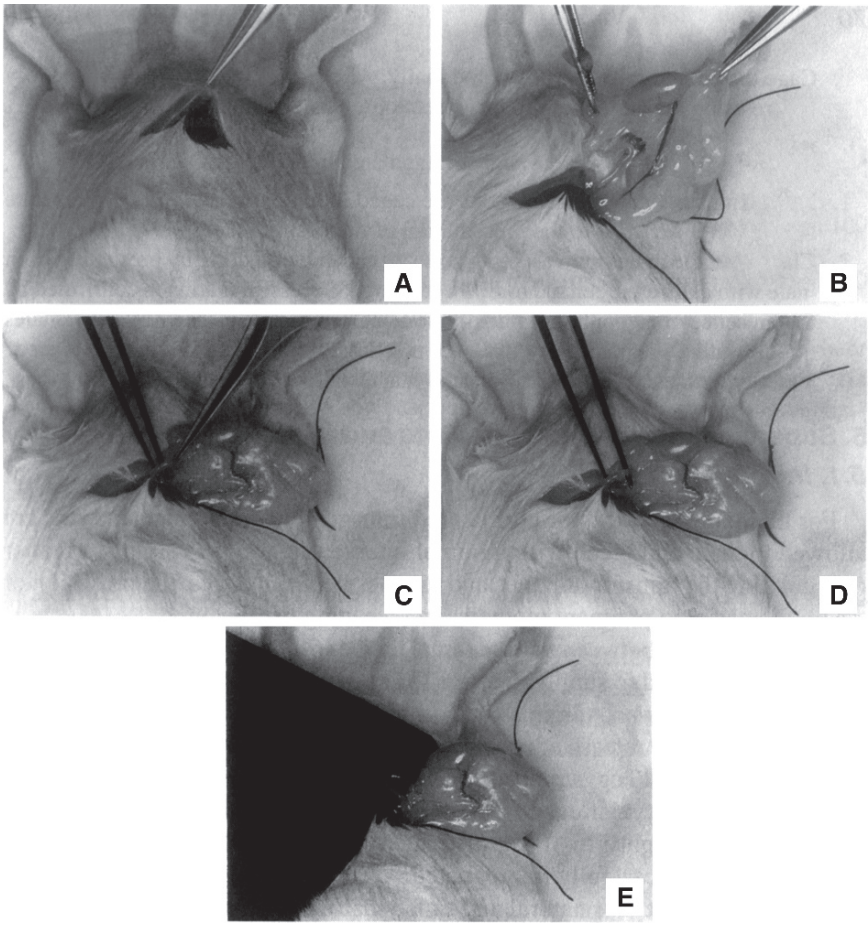

Fig. 1. Steps in the vasectomy of a male mouse. See the text for details.

7. Identify the vas deferens (**Fig. 7.1B**; held by left forceps). The vas deferens links the epididymis to the penis. It is rigid, with a blood vessel running alongside it. Free the vas deferens from the surrounding membranes using fine forceps and scissors (**Fig. 1C and 1D**). The vas deferens is clearly seen against the black background (**Fig. 1E**).
8. Hold the vas deferens in a loop with a pair of forceps. Heat a pair of blunt forceps until glowing red. Grip the vas deferens loop with the hot forceps to burn away the tube and cauterize the ends (**Fig. 2**).
9. Separate the cauterized ends.
10. Return the organs into the body cavity with a pair of blunt forceps.
11. Repeat with the other side of the reproductive tract.
12. Sew up the body wall with two or more stitches.
13. Clip the skin with two wound clips.
14. Allow the animal to recover in a warm, quiet place.
15. Cage the vasectomized males individually. Allow a few weeks for full recovery before using the animal to produce pseudopregnant recipient females.

4.2.2. Rats

1. Anesthetize the rat as in section 7.2.2.
2. Place the rat abdomen side up.
3. Swab the scrotal area with 70% (v/v) ethanol.
4. Lift the scrotal skin of one testis with a pair of fine forceps, and make a 1-cm longitudinal cut on the more anterior outer portion of the scrotum.
5. Lift the body wall (which is extremely thin) with the fine forceps, and make a parallel cut.
6. Place a single stitch into the body wall wound and leave in position.
7. Pull out the fat pad, and with it the testis, epididymis, and vas deferens.
8. Identify the vas deferens located underneath the testis. Tie off each end of the tube with cotton thread, and then cut the tube between the two ties with a pair of fine scissors.
9. Return the organs into the body cavity using a pair of blunt forceps. It is often hard to relocate the body wall—use the single stitch placed in the body wall at the start of the surgery as a marker.

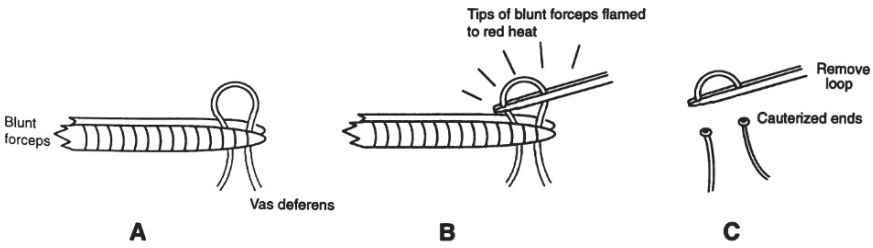

Fig. 2. Cauterization of the vas deferens.

10. Stitch up the body wall with two or more sutures.
11. Stitch up the scrotal skin.
12. Repeat with the other side of the rat.
13. Make sure the animal is regaining consciousness with some semblance of mobility before leaving it to recover in a warm, quiet place.
14. Cage the vasectomized males individually. Allow a few weeks for full recovery before using the animal to produce pseudopregnant recipient females.

4.3. Notes

1. Following surgery, the mice or rats are caged individually and left to recuperate for at least 3 wk prior to the presentation of the first female.
2. Check that the males are vasectomized but capable of mating by presenting each male with a different female for 2 wk running. Successful mating is confirmed by the presence of a copulatory plug in the female and male sterility by the females not becoming pregnant. Any male not meeting both criteria should be replaced.
3. Vasectomized males should be presented with females only once every 2 d at the most.

5. Superovulation of Immature Rats and Mice

5.1. Introduction

For maintaining stocks of normal and transgenic strains, mice and rats are allowed to mate and breed with minimal intervention. This is simply a matter of regulating the transfer of males and females between cages, the provision of nesting material to pregnant mothers, and weaning off the pups.

Natural matings between mature female mice (6–7 wk) and stud males (over 7–8 wk) can be used to supply the eggs for microinjection but yield around 10 F2 hybrid eggs/mouse. Administering gonadotrophins to immature females to induce an increased release of ova (superovulation) and mating these with male studs can yield 30–40 eggs/mouse depending on the strain used. In superovulation, the timing of ovulation, copulation, and fertilization is controlled to optimize egg yield. Eighty to 100% of treated mice and 60–100% of similarly treated rats are successfully impregnated.

It is essential both for natural and timed matings that the animals are maintained on a constant light–dark cycle. Simple on–off regimens are adequate (e.g., 1700–0600 dark; 0600–1700 light), but sophisticated regimens that simulate natural conditions are preferable (e.g., 1830–0630 0% light; 0630–0830 25% light; 0830–1130 75% light; 1130–1300 100% light; 1300–1600 75% light; 1600–1830 25% light).

5.2. Materials

1. Follicle-stimulating hormone (FSH): Either pregnant mare's serum (PMS from the National Hormone and Pituitary Program, www.humc.edu/hormones) or pregnant

mare's serum gonadotrophins (PMSG; Sigma-Aldrich, St. Louis, MO; G4527) can be used. Working solution: 50 IU/mL made up with sterile water or 0.9% (w/v) NaCl. Store frozen at 4°C in 1-mL aliquots.
2. Human chorionic gonadotrophin (hCG): Either Chorulon (Intervet Laboratories Ltd., Cambridge, UK) or hCG (Sigma-Aldrich, St Louis, MO; C8554) can be used. Working solution: 50 IU/mL made up with sterile water or 0.9% (w/v) NaCl. Store frozen at 4°C in 1-mL aliquots.
3. Sterile 1-mL disposable syringes and 25 gauge needles.

5.3. Methods

5.3.1. Mice

1. To generate approximately 200 viable eggs for microinjection, use 10 F1 hybrid (e.g., CBA/J X C57BL/6J) females (12–14 g, 4–5 wk old).
2. Inject each mouse intraperitoneally with 100 µL of 50-IU/mL FSH (5 IU/mouse) between 1000 and 1200 h.
3. Forty-six to 48 h later, inject the same mice ip with 100 µL of 50-IU/mL hCG (5 IU/ mouse). Immediately place each female with a male stud.
4. On the following morning, check each female for the presence of a copulatory plug. The plug is a creamy white mass of coagulated sperm and protein blocking the vagina. It is normally obvious, but is sometimes deep within the vagina; examine the mouse carefully with a smooth blunt probe.

5.3.2. Rats

1. To generate between 100 and 200 viable eggs for microinjection, use ten females (4 and 5 wk old).
2. Inject each rat ip with 400 µL of 50-IU/mL FSH (20 IU/rat) at 0900 h.
3. Two days later, ip inject the same rat with 200 µL of 50-IU/mL hCG (10 IU/rat) at 1400 h. Immediately place each female with a male stud.
4. On the following morning, check each female for the presence of a copulatory plug. In rats, the plug has often fallen out by the morning. A vaginal smear should be taken from any animal in which it is not detected (see section 11.) and the contents examined under 400× magnification for sperm.

5.4. Notes

1. In the light–dark cycle described, males copulate with females at around 0030 h. Fertilization occurs 30 min to 2 h later. Harvesting the eggs between 1000 h and 1130 h of the same day gives a subsequent window of 12 h during which the eggs can be injected. After this time, the fertilized eggs start to undergo the first cleavage division to give two-cell embryos; injections near this time-point may result in chimeric animals.
2. Pseudopregnant females 0.5 d pc can be implanted with microinjected eggs anytime during the day following an infertile mating.
3. For superovulating mice, we routinely inject at 1030 h for both FSH and hCG.

4. Occasionally, a high proportion of eggs from superovulated rats are unhealthy or unfertilized. In this instance, consider switching FSH and hCG injection times to 1100 h and 1200 h, respectively.

6. Culture of Fertilized One-Celled Eggs

6.1. Introduction

To make transgenic rats and mice, fertilized one-cell embryos have to be maintained outside the natural environment for up to 36 h. Fertilized eggs are collected at approximately 12 h pc (coitus normally occurs at about 0030 h) and are injected at some point during the following 12 h. The eggs are then returned to the natural environment of the pseudopregnant surrogate mother. This procedure occurs either immediately after microinjection, while the eggs are still in the one-celled stage, or after overnight culture, when the eggs are transferred in the two-celled stage.

6.1.1. Preparation of Culture Media for Fertilized One-Celled Eggs

Two types of media are required for the in-vitro manipulations of the eggs:

1. M16 for maintaining the eggs in microdrop cultures in a 37°C incubator gassed with 5% (v/v) CO_2. M16 is buffered with bicarbonate alone and unsuitable for maintaining the eggs outside the incubator, since the eggs are very susceptible to pH changes.
2. M2 essentially similar to M16, except that the bicarbonate is partially replaced by HEPES buffer to facilitate the survival of the eggs outside the CO_2 incubator. Eggs should not be in M2 longer than 30 min.

Both M2 and M16 contain bovine serum albumin (BSA), which reduces the stickiness of the eggs and absorbs low-level poisons. Some batches of BSA are toxic to the eggs, so it is crucial that each stock is tested for its ability to sustain mouse embryo development through to the blastocyst stage (e.g., BSA; Sigma-Aldrich, St Louis, MO; A4161; in Waller, Ho, and Murphy ***(8)***)

Media preparation:

1. Use Sigma-Aldrich (St Louis, MO) tissue-grade or embryo-tested chemicals throughout.
2. Make up all stocks using sterile disposable plastic containers and pipets. Washed glass items can be contaminated with detergents that are toxic to the eggs. Filter all concentrated stocks through 0.45-µm Millipore filters into sterile plastic tubes. Store frozen at 20°C.
3. Make up the M2 and M16 culture media as follows:
   a. Concentrated stocks:
      i. 100X A: Weigh out the following reagents in a 50-mL sterile tube: 2.767 g NaCl (Sigma-Aldrich S5886), 0.178 g KCl (Sigma-Aldrich P5405), 0.081 g KH_2PO_4 (Sigma-Aldrich P5655), 0.1465 g $MgSO_4$ $7H_2O$

(Sigma-Aldrich M2643), 0.5 g glucose (Sigma-Aldrich G6152), 0.03 g penicillin (Sigma-Aldrich P4687), 0.025 g streptomycin (Sigma-Aldrich S1277). Weigh out 1.305-g sodium lactate (Sigma-Aldrich L4263) into a microcentrifuge tube and add this to the 50-mL tube. Rinse the microcentrifuge tube with double-distilled water and use the rinsings to make 10× A to 50 mL final volume.

ii. 10X B: Dissolve 1.0505 g $NaHCO_3$ (Sigma-Aldrich S5761) and 0.005 g phenol red (Sigma-Aldrich P5530) in water and make up to 50 mL final volume.

iii. 100× C: 0.18 g Na pyruvate (Sigma-Aldrich P5280) in 50 mL water.

iv. 100× D: 1.26-g $CaCl_2$ $2H_2O$ (Sigma-Aldrich C7902) in 50 mL water.

v. 100× E: 2.979 g HEPES (Sigma-Aldrich H6147), 0.005 g phenol red (Sigma-Aldrich P5530) into a sterile 50-mL tube and dissolve in 25 mL double-distilled water. Adjust to pH 7.4 with 0.2 M NaOH, then make up to 50 mL final volume.

b. Preparation of M2 and M16 media from concentrated stock: To prepare M2 and M16, mix the stock solutions in the appropriate volumes as detailed next. Measure the double-distilled water in a sterile 50-mL plastic tube. Aliquot the concentrated stocks into the water, then carefully rinse the pipet by sucking the liquid up and down. Add the BSA and mix gently until dissolved. Pass the mixed solutions through a 0.45-µm Millipore filter using a large sterile 60-mL disposable syringe, aliquoting into sterile containers. Store at 4°C. Prepare fresh every 2 wk.

Stock	M2	M16
10× A	5.0 mL	5.0 mL
10× B	0.8 mL	5.0 mL
100× C	0.5 mL	0.5 mL
100× D	0.5 mL	0.5 mL
100× E	4.2 mL	—
Double-distilled water	39.0 mL	39.0 mL
BSA (Sigma-Aldrich A4161)	0.2 g	0.2 g
Total volume	50.0 mL	50.0 mL

6.2. Notes

1. The osmolarity of the medium does not have to be routinely checked; but for reference, the values should be M2 (285–287 mosM/L) and M16 (288–292 mosM/L).
2. Sigma-Aldrich supply M2 (M7167) and M16 (M7292) embryo tested liquid media. Alternatively, KSOM (Specialty Media, Phillipsburg, NJ and Metachem Diagnostics Ltd., Northampton, UK; MR-020P-5D) can be used instead of M16.

7. Preparation of Egg-Transfer Pipets

7.1. Introduction

Two types of egg-transfer pipets need to be prepared in advance; general transfer pipets and oviduct-transfer pipets. These are made from hard glass capillaries and are assembled into a mouth-operated system made up of a mouthpiece, rubber tube (approximately 60 cm), and a pipet holder (components available from Sigma-Aldrich, A5177).

7.2. Materials

1. Hard glass capillaries (e.g., Harvard Apparatus Ltd., Edenbridge, UK; 30-0036).
2. Diamond pencil.
3. Microforge (e.g., Narishige MF-9, Tokyo, Japan).
4. Gas flame supplier.

7.3. Method

7.3.1. General Transfer Pipets

1. Using a small gas flame, soften the middle of a hard glass capillary tube by rotating it slowly over the flame. As soon as the glass softens, withdraw it from the heat, and simultaneously pull the ends apart sharply but not until the two halves snap.
2. Score the capillary with a diamond pen and snap it into two. If the pulled portion is too long (>5 cm) or too narrow (<200 µm), score and snap again. The internal diameter of the pipet should be about 300 µm, and the end should be flush with no jagged ends.

7.3.2 Oviduct Transfer Pipets

1. Follow the preceding procedure for making general-transfer pipets, but aim for pipets with internal diameters of around 120–150 µm to permit easy passage of a mouse or rat egg.
2. Flame polish the end in a small gas flame or a microforge.

8. Collection of Fertilized One-Celled Eggs

8.1. Materials

1. M2 and M16 media (**section 6**).
2. 35-mm sterile tissue-culture dishes.
3. Hyaluronidase (Sigma-Aldrich H1136): 10 mg/mL in M2 medium. Store in 1-mL aliquots at −20°C (stable for several months).
4. Light paraffin oil (Fluka, Buchs, Switzerland, cat. no. 76235).
5. Egg transfer pipet and mouthpiece (**section 3.1**).
6. Dissecting scissors, one fine and one regular.
7. Watchmaker's forceps, 2× #5.
8. Stereomicroscope with understage illumination (e.g., Leica, Wetxlor, Germany, model L2).
9. Fiberoptic cold light source (e.g., Schott, Meinz, Germany; KL1500 LCD).
10. 37°C incubator gassed with 5% (v/v) CO_2.

8.2. Methods

1. At least 1 h before collecting the eggs, set up four 35-mm tissue-culture dishes containing 2–3 mL M16 medium and two 35-mm culture dishes containing M16 microdrops (40 µL) covered with liquid paraffin. Allow these to equilibrate in a 37°C incubator gassed with 5% (v/v) CO_2. At the same time, prepare five 35-mm tissue culture dishes containing 2–3 mL M2 medium and leave at room temperature.
2. Kill the plugged donor females as described in section 5.
3. Lay the animal on its back, and soak the abdomen with 70% (v/v) ethanol.
4. Pinch up the skin with fingers, cut into the midline, and skin the animal. Cut the body wall, and enter the abdominal tract. Push aside the coils of the gut to reveal one arm of the reproductive tract, which is associated with a fat pad. Identify the coiled oviduct lying between the uterus and the ovary.
5. Gripping the uterus with a watchmaker's forceps, lift up the reproductive tract. Use a pair of fine forceps to puncture the membrane (mesometrium) that joins the reproductive tract to the body wall. Trim the membrane away from the oviduct.
6. Still gripping the uterus, cut between the ovary and the oviduct (cut A in **Fig. 3**).
7. Transfer the grip to the oviduct, and cut between the uterus and the oviduct (cut B in **Fig. 3**). The cuts should be made as close to the oviduct as possible.
8. Place the oviduct into one of the dishes of M2 prepared earlier.
9. Dissect the oviduct from the other horn of the reproductive tract, and then proceed with the rest of the female donors. All the oviducts should be placed in the same dish of M2 medium.

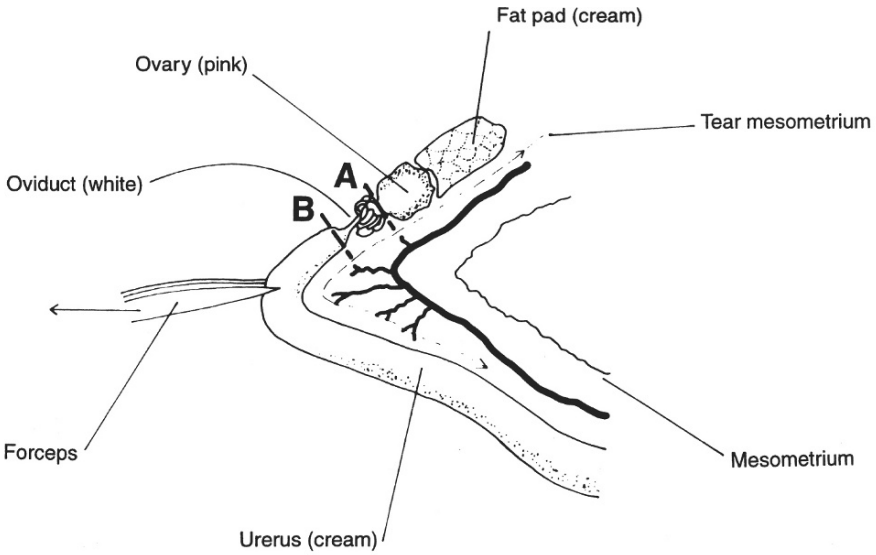

Fig. 3. Dissection of a female reproductive tract of a mouse illustrating the procedure for removal of the oviduct prior to egg collection.

10. View the oviducts under the 10–20× magnification of the dissecting stereomicroscope. The oviduct should appear as mass of opaque coils with a single transparent swollen region, the ampulla (**Fig. 4**). The ampulla is the target of the egg collection, since at this stage, it contains the cumulus mass (numerous eggs surrounded by cumulus cells). Eggs may be visible through the walls of the ampulla.
11. Use one pair of sharp watchmaker's forceps to hold down the oviduct and another to tear the ampulla. The cumulus mass should spill out of the hole. Sometimes, it is necessary to tease the eggs out of the ampulla with forceps. Discard the empty oviduct, and repeat the procedure with the rest of the oviducts.
12. Mix the cumulus mass with 50–100 µL of 10 mg/mL hyaluronidase. Enzymatic digestion is required to separate the eggs from the cumulus cells, a few minutes of treatment is sufficient; the eggs should not be in contact with the enzyme for a longer period. Gently pipeting the cells up and down with a general transfer pipet facilitates the process.
13. Using the general transfer pipet, transfer the eggs to a fresh dish of M2 medium to wash away traces of the hyaluronidase. Repeat in another dish of M2. In each wash, try to leave behind the cumulus cells.
14. Wash the eggs twice in two of the prewarmed dishes of M16 medium and, finally, transfer the eggs to the microdrop culture (20–30 eggs/drop).
15. Incubate the eggs at 37°C with 5% (v/v) CO_2 until required for microinjection. It is best to leave the eggs for at least an hour before microinjection.

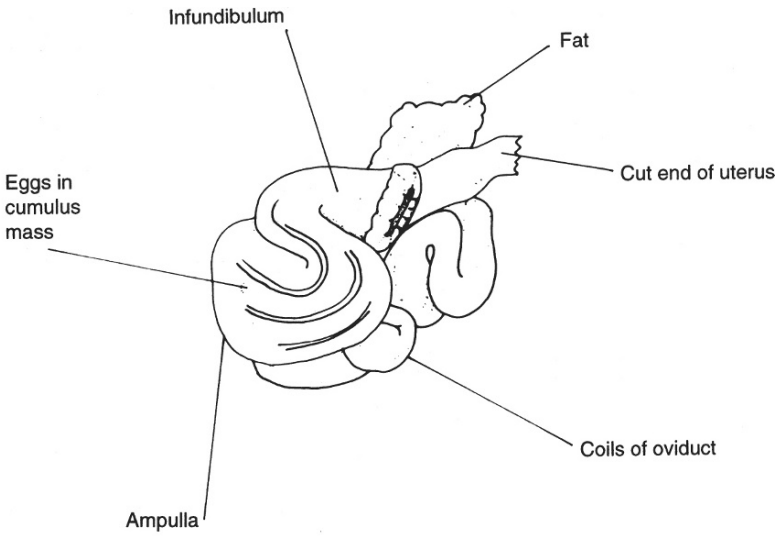

Fig. 4. The mouse oviduct showing the position of eggs in the swollen ampulla.

8.3. Note

The paraffin oil used to prevent evaporation of the media is often a source of toxins that may kill the eggs. Paraffin oil from Fluka (cat. no. 76235) allows more than 50% of the eggs to develop to the blastocyst stage in culture.

9. Purification of Microinjection DNA from Agarose Gels

9.1. Introduction

DNA for microinjection can be prepared from the plasmid or the cosmid clones by any of the standard techniques of lysozyme/Triton X-100 lysis or lysozyme/alkaline lysis followed by banding of supercoiled molecules in ethidium bromide/CsCl gradients. The many commercially available plasmid preparation kits, using alkaline lysis followed by anion-exchange column purification of DNA, provide DNA of a suitable quality with minimum time and effort (e.g., Qiagen). Vector-free DNA is isolated by restriction enzyme digestion followed by preparative agarose-gel electrophoresis. This latter step is very important. DNA fragments for microinjection must be free from all contaminants that may be toxic to the eggs and all particulate matter that could clog up the injection pipet. Isolation of DNA from agarose gels by binding to glass beads (12) followed by passage through a Sephadex G-50 column and filtration though a 0.45-µm filter provides such DNA. The method is described in the following protocol.

9.2. Materials

1. Glass beads: These can be purchased or prepared in the laboratory as here. The preparation involves boiling nitric acid, so take full safety precautions. Mix 250 mL of powdered glass flint, available from glass supply companies (e.g., Eagle Ceramics Inc., 12267 Wilkins Avenue, Rockville, MD 20852), with 500 mL sterile water. Allow the heavier particles to settle over 1 h. Decant the fines into centrifugation tubes, and spin for 5 min at 1,000 *g*. Resuspend the pelleted fines in 200 mL sterile water. Add 200 mL of concentrated nitric acid, and bring to boil. Carry out this step in an efficient fume hood. Allow to cool then centrifuge (1000 *g*, 5 min) to recover the glass beads. Discard the nitric acid carefully. Wash several times in distilled water (i.e., cycles of resuspension and spinning) until the pH of the suspension is neutral. Store the glass bead slurry as a 50% (w/v) slurry at –20°C, working stocks at 4°C.
2. Gel electrophoresis equipment.
3. 50× TAE buffer stock: 2.0 M Tris base, 1.0 M sodium acetate, 0.05 M EDTA.
4. Ethidium bromide (10 mg/mL) in sterile water.
5. Long-wave (365 nM) UV light transilluminator.
6. 6 M NaI: Dissolve 90.8 g of NaI and 1.5 g Na_2SO_3 in water to a final volume of 100 mL. Filter through a 0.45 µm Nalgene filter or Whatman no. 1 paper. Add 0.5 g Na_2SO_3 to saturate. The Na_2SO_3 crystals do not dissolve but prevent the oxidation of the NaI. Store at 4°C protected from light.

7. Ethanol wash solution: 50% (v/v) ethanol, 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Store at –20°C.
8. Sterile (autoclaved) filtered (0.2 µm) microinjection TE (MITE): 10 mM Tris-HCl, pH 7.4, and 0.2 mM EDTA.
9. Sephadex G50 slurry: Prepared by swelling G-50 powder in water followed by autoclaving.
10. 0.45-µm Millipore filter attached to a 1-mL disposable syringes.
11. Baked glass wool: Bake at 250°C for at least 3 h to destroy all contaminating nucleases.

9.3. Method

1. Digest the DNA with the appropriate restriction enzymes according to the recommendations of the manufacturer.
2. Fractionate the digested DNA in an agarose gel prepared in 1× TAE buffer containing 0.5 µg/mL ethidium bromide.
3. Visualize the DNA on the long-wave UV light transilluminator. Use a fresh scalpel blade to excise the fragment of interest carefully in as small a volume as possible. Transfer the excised fragment to a preweighed Eppendorf tube.
4. Add as much NaI as possible, the minimum ratio being 3:1 (v:w).
5. Incubate at 55–65°C with occasional agitation until the agarose has completely dissolving.
6. Add 1 µL of glass beads (fully dissolved and mixed) for every 2 µg of DNA. Mix well, and incubate on ice for 45 min inverting every 10–15 min.
7. Pellet the glass milk by centrifugation (pulse to over 12,000 *g* and release). Discard the supernatant.
8. Wash with 500 µL of NaI twice more, spinning briefly (1–2 sec) each time to recover the glass milk.
9. Wash with 500 µL of ethanol wash twice.
10. After discarding the supernatant, vortex the glass milk in the small volume of ethanol that remains and respin. Carefully remove as much of the supernatant as possible, but do not let the pellet dry out.
11. Add more than 50 µL of MITE immediately, and incubate at 55–65°C for 15 min to elute the DNA.
12. Pellet the glass beads by spinning at full speed for 2 min. Transfer the supernatant containing the DNA to a fresh tube. *After this point, all tubes and tips must be prerinsed with filtered water before use*.
13. Prepare a spun Sephadex G-50 column. Block the end of a 1-mL disposable syringe with glass wool. Fill with Sephadex G-50 until all the excess fluid drains out and the beads are packed. Position the column in a disposable 15-mL plastic conical centrifuge tube, and centrifuge at 4,000 *g* for 3 min. Equilibrate this column with 100 µL MITE by spinning as before. Do this five times.
14. Pass the eluted DNA (maximum volume 100 µL) through the equilibrated Sephadex G-50 column, collecting the purified DNA in a fresh 1.5-mL Eppendorf tube (with the lid cut off) placed at the bottom of a fresh 15-mL centrifuge tube.

15. Filter the elute through a small 0.45-µm Millipore filter attached to a 1-mL syringe.
16. Determine the DNA concentration by spectrophotometry at 260 nm or comparative ethidium staining with DNA standards of known concentration.

9.4. Notes

1. Many manipulations of DNA and RNA leave the nucleic acid in a solution containing unwanted salts, nucleotides, or radioactive moieties. In most cases, ethanol precipitation does not entirely remove these or adds further salts. Sephadex gel-exclusion chromatography is often used to purify DNA fragments. Sephadex is a bead-formed, cross-linked dextran. The cross-linking is carefully controlled to give beads consisting of a network of holes of uniform size and shape. A molecule passing through a Sephadex column passes through a volume dependent on the size of the molecule. A molecule too large to enter a pore passes through the volume of the column not occupied by beads. A molecule small enough to enter the pores does so, passes though a much larger volume, and takes longer to pass through the column. The exclusion limit is the size limit of the molecule that can just enter the pore. Molecules of intermediate size are fractionated by their molecular weight. This matrix can thus fractionate a mixture of DNA and salts cleanly, the DNA being excluded from the pores and passing straight through and the salts and nucleic acids being retained within the pores. Additionally, the sample does not become appreciably diluted.
2. Slurry DNA isolation kits are available (e.g., Bio 101 Geneclean Kits by Qbiogene, Carlsbad, CA).
3. The recovery of DNA fragments between 500 and 800 bp by glass bead isolation is relatively low. Also, there is a risk that large DNA fragments (>15 kb) may be broken during the wash cycles. If a large DNA fragment binds to two or more beads, then on washing and separation of the beads, the DNA strand may be broken.

10. Microinjection

10.1. Introduction

Central to the process of making transgenic mice and rats is the physical introduction of the cloned DNA fragments into one-celled eggs. First described by a number of investigators (1), microinjection remains a popular method of generating transgenic animals because the advantages of speed and reliability.

10.2. Materials

1. Inverted microscope (e.g., Nikon Eclipse series, Tokyo, Japan, or the Leica DM IRB or DM IRE2, Wetzlor, Germany) with the following features:
   a. Image-erected optics.
   b. A condenser with a long working distance.
   c. A fixed stage (the objective lens rather than the stage moves when focusing).
   d. 10× Magnification eyepieces.
   e. 4× Objective for low-magnification work.

f. 40× Objective for microinjection.
g. Suitable objectives: Nomarski differential interference contrast (DIC) optics are best for visualizing the internal structures of the egg. DIC optics are expensive, and glass-injection systems are required. Hoffman modulation contrast optics are compatible with plastic injection chambers but give inferior resolution to DIC. If neither is available, eggs can be viewed under bright field. Phase-contrast microscopy is not compatible with any microinjection system.

2. Micromanipulators: Two are required—for the holding pipet, which holds the egg in place, and for the microinjection pipet. The excellent Leica mechanical micromanipulator (Wetzlor, Germany), with joystick control of the horizontal movement in two planes, is most commonly used. The micromanipulators and the microscope must be positioned on a purpose-built base plate (**Fig. 5**), which must be custom engineered. Narishige (Tokyo, Japan) produces economically priced micromanipulation systems compatible with Nikon microscopes. These are very flexible and do not require a custom-built base plate.
3. Micropipet holders: Leica single-instrument holders fitted with Leica single-instrument tubes if Leica mechanical micromanipulators are used.

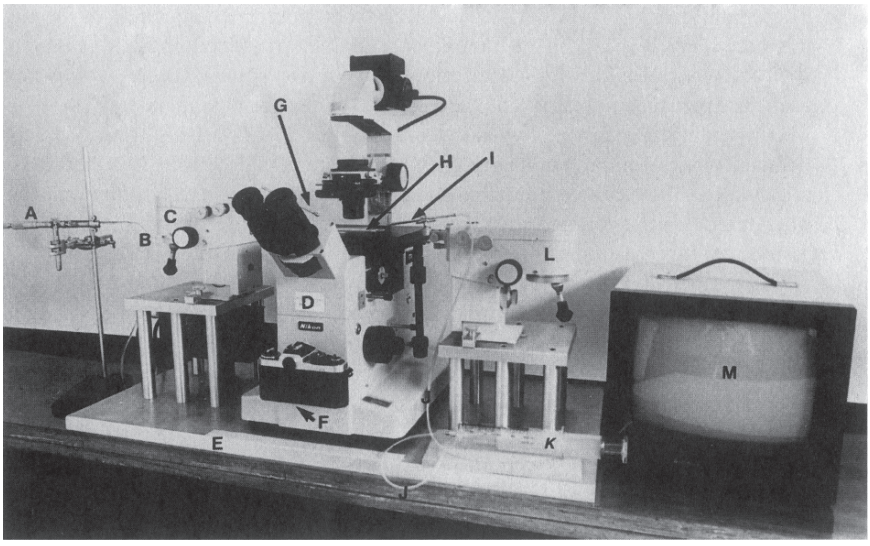

Fig. 5. A typical arrangement of the equipment needed for the microinjection of fertilized one-cell eggs. **A** Agla micrometer syringe. **B** Liquid-paraffin-filled tube. **C** Left-hand micromanipulator. **D** Inverted microscope. **E** Base plate. **F** Camera (optional). **G** Left-hand instrument tube for holding pipet. **H** Microinjection chamber (depression slide) sitting on fixed stage. **I** Right-hand instrument tube for injection pipet. **J** Air-filled tube. **K** Glass 50-mL syringe. **L** Right-hand micromanipulator. **M** Video system (optional).

4. Holding pipet (**Fig. 6**).
   a. Draw a hard-glass capillary (e.g., Harvard Apparatus Ltd., Edenbridge, UK; 1.0 mm OD, 0.78 mm ID; 30-0036) in a gas flame. Grip the ends of the capillary, and turn the middle of the capillary in the hottest part of the flame until the glass softens. Quickly withdraw the pipet from the flame, and simultaneously pull on both ends.
   b. Score the drawn section 2-cm from the shoulder with a diamond pen, and break the capillary at this point.
   c. Mount the capillary vertically in a microforge (e.g., Narishige MF900). Focus on the tip of the capillary with the 4× objective. The capillary must be straight and its tip perfectly flush with no jagged edges and an external diameter of 100 µm (±20 µm). Bring the tip close to, but not touching, the microforge filament. Heat the filament, and melt the tip until its internal diameter reaches 10–15 µm. The size of the hole is extremely important: too small a hole makes controlling the holding pipet difficult; too large a hole may allow eggs to be sucked into the pipet.
   d. Move the capillary to a horizontal position. Position the filament 1–2 mm below the capillary tip. Move the filament close to the pipet, and heat to bend the capillary by about 15° to the horizontal. One holding pipet lasts one microinjection session and is not reused. Large numbers can be made in advance and stored in a sterile plastic tube.
5. Microinjection pipets for physically introducing the DNA fragment into the nucleus of the one-celled egg: Microinjection pipets are made from thin-walled glass capillaries with an external diameter of 1 mm. Capillaries with an internal filament (Harvard Apparatus Ltd., Edenbridge, UK; 30-0020) are useful, since they can be backfilled by capillary action from the distal end to the injection tip. Pipets

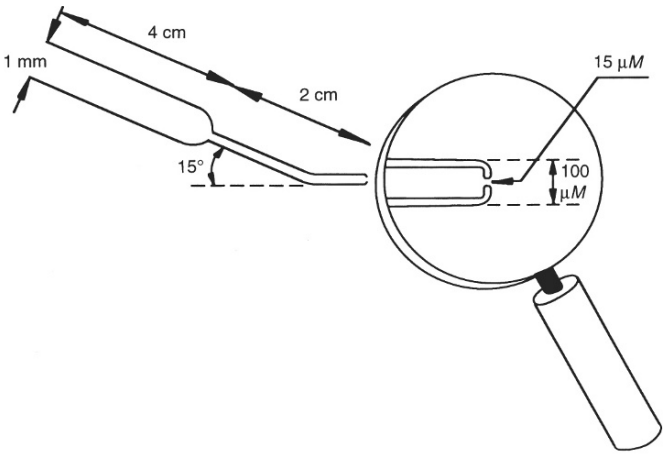

Fig. 6. Construction of a typical holding pipet.

are drawn on a commercially available pipet puller (e.g., David Kopf Instruments, Tujunga, CA; Vertical Pipet Puller Models 720, 730 or 750, or the Narishige PN-10 or PN-30). Injection tips should have a 1 µm opening. Larger tips burst eggs, and smaller ones become blocked easily. Microinjection pipets are pulled when needed.

6. The injection chamber: Glass depression slides are compatible with inverted microscopes fitted with any optic system. Siliconize slides by rinsing in a 3% (v/v) solution of dichloromethyl silane in chloroform. The slides are rinsed thoroughly with water and a standard household detergent. Prior to use, rinse the slide in ethanol, and dry with a paper tissue. It must be devoid of dust particles.
7. Micrometer syringe (e.g. Burkard Manufacturing Co. Ltd., Rickmansworth,UK, or equivalent).
8. There are two possible microinjection systems:
   a. Manual injections involve the use of a 50-mL syringe with a ground-glass plunger connected by an air-filled tube to the microinjection needle. Manual squeezing of the syringe squeezes DNA from the pipet into the egg.
   b. An automatic injection system uses compressed air to expel the DNA, triggered by a foot-operated pedal (e.g., Narishige, Tokyo, Japan: IM-300). Although more expensive than manual systems, the advantages are
      i. The foot-operated injection trigger leaves both hands free to control the micromanipulators.
      ii. A constant low positive pressure applied to the microinjection pipet produces a continual outflow of the DNA, preventing the backflow of M2 medium and clogging of the pipets.

The assembly of an automatic injection system should be done by a trained professional. The injection pressure must be determined empirically.

9. M2 and M16 culture media (section 6).
10. Tissue-culture incubator (37°C, 5% [v/v) CO_2).
11. Light liquid paraffin (Fluka 76235).
12. 26-Gauge needles.
13. Tygon (Akron, OH) tubing ($^3/_{32}$ in. ID; $^5/_{32}$ in. OD).
14. One clamp stand.
15. Diamond pen.
16. Disposable 1-mL syringes.
17. Fluorinert electronic liquid (3 M Company, St. Paul, MN; cat. no. FC77).
18. Dissecting microscope.

10.3. Methods

10.3.1. The Microinjection Chamber

1. Place 100 µL of M2 medium in the well of an ethanol-washed, dried depression slide.
2. Cover the M2 drop with light paraffin oil (Fluka) to prevent evaporation.

3. Place the slide on the stage of the microscope, and using a 4× objective, focus on the bottom of the M2 drop.

10.3.2. The Holding Pipet

1. Connect a Leica instrument tube to the micrometer syringe with 1-m Tygon tubing. Fill the system with light paraffin oil making sure that all the air bubbles have been excluded. Position the micrometer syringe in a clamp stand in a convenient position close to the left-hand manipulator.
2. Fill the holding pipet with Flourinert using a needle (26 gauge, 5 cm) attached to a 1-mL disposable syringe. Insert the holding pipet into the oil-filled Leica instrument tube, and tighten the ring to hold it in place.
3. Clamp the instrument tube into the instrument tube holder of the left-hand micromanipulator (if one is right handed). Adjust the micrometer syringe until fluid stops flowing out of the holding pipet. Do not allow air to flow back into the holding pipet.
4. Position the holding pipet 2 cm above the center of the microinjection chamber making the necessary adjustments, such that the tip of the holding pipet is horizontal and straight. Monitoring under the 4× magnification, use the fine controls of the micromanipulator to lower the holding pipet into the injection chamber. Adjust the manipulator such that the tip of the holding pipet is just above the floor of the chamber. The holding pipet should move freely in the horizontal plane throughout the field of vision and not catch on the floor of the chamber.
5. Using the micrometer syringe, draw M2 medium into the holding pipet until the meniscus between the M2 and Fluorinert is just at the shoulder of the holding pipet.

10.3.3. The Microinjection Pipet

1. Connect a Leica instrument tube holder via 1 m of Tygon tubing to either a 50-mL syringe with ground-glass plunger (lubricated with liquid paraffin) or an automatic injection system. The system is air filled.
2. Backfill a freshly drawn microinjection pipet with DNA. Place the end distal to the tip into the DNA solution, allow the liquid to ride into the tip by capillary action and withdraw when liquid can be seen in the tip. Avoid contaminating the DNA stock solution, for example, with glove powder or enzymes from the exposed hand.
3. Assemble the microinjection pipet into the instrument tube. Clamp the instrument tube onto the instrument tube holder of the micromanipulator.
4. Position the microinjection pipet until the tip is 2 cm above the center of the microinjection chamber and 15–20° to the horizontal. Using the fine vertical control of the micromanipulator, lower the tip into the injection chamber until it is just above the chamber floor. Monitor the position of the tip throughout using the 4× objective.
5. Using the horizontal controls of the micromanipulators, bring both the injection pipet and the holding pipet to the center of the field of view.

6. Switch to the 40× Normarski objective, and focus on the holding pipet. Using the fine controls of the micromanipulator, bring the tip of the microinjection pipet into the same focal plane as the holding pipet. The microinjection pipet should move freely in the horizontal plane when operated by the joystick control.

10.3.4. Microinjection of Fertilized One-Celled Mouse and Rat Eggs

1. Remove about 20 one-celled eggs from storage in M16 medium at 37°C using a general-transfer pipet.
2. Wash the eggs twice in M2 medium. Load them in as small a volume as possible in the transfer pipet, and discharge them into the injection chamber. Observe the entry of the eggs into the chamber using a 4× objective. Try to keep the eggs in a group positioned below the holding and injection pipets. Avoid releasing any air bubbles into the chamber; eggs become obscured or even lost, necessitating the reassembly of the injection chamber.
3. Readjust the vertical positions of the holding and injection pipets so they are in the same plane as the eggs.
4. Bring the tip of the holding pipet close to an egg, and by adjusting the micrometer syringe, apply light pressure such that the egg is held onto the tip of the pipet.
5. Bring the egg to the center of the field of vision. Switch to the 40× DIC objective and focus on the egg. Focus up and down to locate the two egg pronuclei. The larger (male) pronucleus is the target and must be positioned for convenient injection. The egg can be moved by gentle expulsion and resuction and rolling it with the holding pipet.
6. Ensure that the egg is tightly held by applying slightly more suction. The zona pellucida can be mildly distorted without harming the egg.
7. Focus on the target pronucleus. Position the microinjection pipet close beneath the egg so that its tip is vertically below the pronucleus. Use the fine vertical micromanipulator control to bring the tip into the same focal plane as the pronucleus. Without changing its vertical plane, bring the tip up to the zona pellucida at a level horizontal with the pronucleus.
8. Squeeze hard on the 50-mL syringe or, if using the automated system, clear the microinjection pipet. It is possible to see DNA solution being ejected by its mixing with the M2 medium, a slight movement of the egg, or the presence of contaminating particulate matter in the medium.
9. Inject the egg. The zona pellucida is easily pierced. When the tip appears to be within the pronucleus, squeeze on the injection syringe. Three things may be observed:
   a. The pronucleus swells. This is a successful injection. Apply pressure until the pronucleus is roughly twice its original volume, then withdraw the pipet in a single smooth rapid movement (**Fig. 7**).
   b. A small clear bubble appears at the tip of the microinjection pipet, and the perivitelline space may swell. The extremely elastic egg membrane has not been pierced. To penetrate the membrane, continue to push the microinjection pipet as far as the holding pipet, then pull the tip back into the pronucleus before injecting again. Experienced operators can “feel” the egg membrane give way.

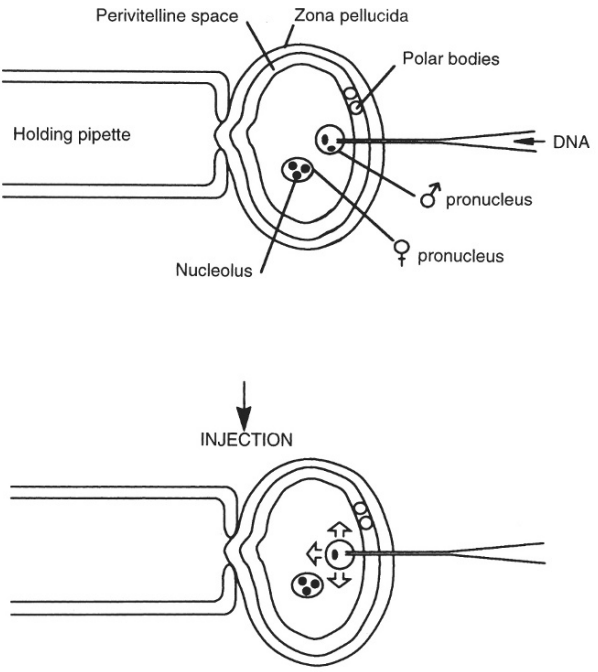

Fig. 7. Injection of fertilized one-cell eggs. High-magnification (400×) microscopic view.

c. Nothing. The injection pipet is probably blocked and should be changed. Alternatively, the pipet puller may be producing microinjection pipet with sealed tips or tips with excessively small openings—adjust the pipet puller. If particulate matter in the DNA stock is suspected, change the DNA stock.

10. Following injection, cytoplasmic granules may flow out into the perivitelline space, indicating egg lysis. If eggs lyse on two or three successive occasions, change the microinjection pipet.
11. Switch back to the 4× objective, and place the injected egg above the holding and microinjection pipet. Injected eggs should be divided into two groups: eggs that have survived injection and those that have not.
12. Eggs should be maintained in M2 medium for a maximum of 15 min. A skilled operator can inject 15–40 eggs in this period. Once all the eggs in a batch have been injected, return the survivors to M16 microdrop culture at 37°C in a 5% (v/v) CO_2 incubator through two washes of M16 equilibrated at 37°C, 5% (v/v) CO_2.
13. Eggs that have survived injection are returned by oviduct transfer to the natural environment afforded by a recipient pseudopregnant female. Eggs can be transferred on the same day as microinjection or transferred following overnight culture.

10.4. Notes

1. The quality of the DNA is crucial to the survival of the eggs. DNA should be purified as described in section 9. The composition of the solvent in which the DNA is dissolved is very important. Minute amounts of Mg^{2+} kill an injected egg as does an overly high concentration of EDTA. The optimal buffer is 10 mM Tris-HCl, pH 4, containing 0.1–0.25 mM EDTA (microinjection TE or MITE; (10)).
2. There is no correlation between the concentration of DNA and the resultant copy number of the transgene. However, excessively high concentrations of DNA are toxic to the egg ***(10)***.
3. An estimated 1–2 pL of DNA solution enters the egg pronucleus in each microinjection. Most investigators use concentrations of 1–5 µg/mL and are thus injecting about 500 copies of the DNA fragment, depending on the size of the fragment (10). For different fragments, the DNA concentration combining the optimum efficiency of integration and egg survival varies. One can make up different dilutions of the DNA (1, 2, and 5 µg/mL) and rotate between these dilutions during a microinjection session.
4. The physical state of the DNA has little bearing on the success of the experiment. Cosmid clones of 50-kb can be introduced as easily as DNA of smaller sizes ***(13)*** Linearized DNA integrates with a fivefold greater efficiency than circularized DNA (10). The structure of the DNA ends created by different restriction enzymes has no effect on the efficiency of integration or the organization of the resulting transgene ***(10)***.
5. Avoid the sticky nucleolus on microinjecting. If this attaches to the injection pipet, it may be drawn out of the egg, thus killing it.

11. Identification of Ovulating Mice and Rats

11.1. Introduction

Pseudopregnant recipient females are used as surrogate mothers to nurture surviving microinjected eggs to birth. Females are made pseudopregnant by mating them with vasectomized males. To increase the chances of a successful mating, only females in estrous are coupled with the males. Female rats and mice maintained on the light–dark cycle described here will ovulate roughly every 4 d. The stage of the estrous cycle for mice can be determined by the appearance of the vagina as detailed in **Table 1**. For rats, a vaginal smear is required (*see* **sections 11.2** and **11.3**). On the afternoon before the day of implantation, estrous females are paired with a vasectomized male. On the following morning, female mice are examined for the presence of a copulatory plug, evidence that a successful mating has occurred (50–80% of females that are visually determined to be in estrous will be impregnated). In rats, the copulatory plug will often have fallen out by the morning, and there will be no sperm in the vagina to indicate that a successful mating has taken place. However, vaginal stimulation can induce pseudopregnancy, so as a backup procedure, female rats

Table 1
Identification of Ovulating Mice

Stage of the estrus cycle	Vaginal characteristics
Diestrus	Opening small, tissues small and moist
Proestrus	Opening gaping, tissues red-pink and moist; dorsal and ventral folds
Estrus (ovulating)	Opening gaping, tissues pink and moist; pronounced folds
Metestrus	Tissues pale and dry, white cell debris

are gently prodded with a smooth blunt probe for 1 min before being placed with the vasectomized males.

11.2. Materials

1. Phosphate-buffered saline (PBS): 20 mM sodium phosphate buffer, pH 7.4, containing 0.8% (w/v) NaCl or 0.9% (w/v) NaCl alone.
2. 1-mL disposable syringe (without needle).
3. Petri dish.
4. Light microscope with 40× magnification (and 400× magnification if checking for the presence of sperm).
5. Female rats and mice (preferably experienced mothers).

11.3. Method

1. To screen for potential surrogate mothers, use female rats that have given birth to at least one litter and weaned for more than a week.
2. Mark rats on tail for identification.
3. Fill a 1-mL syringe with 0.2–0.3 mL PBS or 0.9% (w/v) saline.
4. Expose the rat's vagina by lifting its tail.
5. Gently insert the syringe into the vaginal opening until resistance is felt. Expel the PBS or 0.9% NaCl into the vagina, then draw the fluid back into the syringe to collect vaginal cells into the fluid.
6. Transfer the contents onto a suitably labeled petri dish. Repeat with several other rats.
7. Examine the cells under a light microscope with 40× magnification, and identify ovulating rats from the characteristics detailed in **Table 2**.
8. If checking for the presence of sperm, examine the vaginal smear under 400× magnification.

11.4. Notes

1. Although virgin female rats can make suitable surrogate mothers, they tend to consume a part of their litters. Experienced mothers tolerate the traumas associated

Table 2
Identification of Ovulating Rats

Stage of the estrus cycle	Cell characteristic
Diestrus	Few epithelial cells and leukocytes
Proestrus(pair rats at this stage)	Many nucleated epithelial cells, yellow appearance
Estrus	Many large cornified epithelial cells, brownish appearance
Metestrus	Fewer cornified epithelial cells, many leukocytes

with the experimental procedures better to care for the unusually large or small litters that may eventually be produced.

2. Some female rats are anestrous, remaining always in a particular phase of the estrous cycle. These are not suitable for mating.

12. Delivery of Microinjected Eggs to Surrogate Mothers by Oviduct Transfer

12.1. Introduction

In the oviduct transfer, microinjected embryos are implanted into the ampulla of recipient pseudopregnant females. It is preferably performed on the same day as the microinjections to minimize the time the injected eggs are in culture. Alternatively, it can be performed on the following day, when it is possible to observe if the injected eggs have developed to the two-cell stage. Such eggs may have a better long-term survival as well. Fifteen to 20 eggs delivered to each oviduct (30–40 eggs overall) should give an ideal litter size of five to ten pups. In practice, the number of eggs needed to achieve this number depends on the competence of the experimenter. Oviduct transfer is not an easy technique, and novice experimenters are advised to practice with cadavers until they are happy with the procedure. A convenient dye can be used in place of eggs to visualize correct delivery of the pipet contents into the ampulla.

12.2. Materials

1. Microinjected eggs in microdrop culture.
2. M2 medium.
3. 3.5-mm sterile tissue-culture dishes.
4. Pseudopregnant recipient mouse or rat.
5. CRC (for rats) or Avertin (for mice) anesthetic.
6. Dissection instruments (as in vasectomies).
7. Artery clip (InterFocus Ltd., Haverhill, UK; 18050-35).
8. Fiberoptic cold light source (e.g., Schott, Meinz, Germany; KL1500 LCD).

9. Surgical microscope with optional assistant's viewing head (e.g., OPMI series by Carl Zeiss, Welwyn Garden City, UK).
10. Oviduct transfer pipets and mouthpiece (**section 3.2**).
11. Epinephrine (Sigma-Aldrich E1635). Prepare 0.5% (w/v) epinephrine in 0.1 M HCl, and dilute to 0.1% (v/v) in PBS for a working solution.
12. Ampicillin (Binotal from Bayer).
13. Paraffin oil (Fluka, Buchs, Switzerland; cat. no. 76235).
14. 70% (v/v) ethanol in squeeze bottle.

12.3. Methods

Preliminary procedure, loading the oviduct transfer pipet (**Fig. 8**): Fill the pipet with liquid paraffin oil to the shoulder. The viscosity of the oil affords a greater degree of control over the movement of the eggs. Take up a small amount of air, then a small amount of M2, then another bubble. The eggs are then collected, preferably in a stacked rank with a minimum of medium. A third air bubble is taken up followed by a final column of M2. The total length of the eggs–bubbles–medium should not exceed 2 cm. The loaded pipet can be conveniently stuck on a piece of plasticene on the surgical microscope.

1. Anesthetize a 0.5 d pc pseudopregnant recipient female as in **section 2.2**.
2. Place the animal ventral side down on the lid of a 9-cm petri dish, if operating on a mouse, but directly onto the stage of the dissecting stereomicroscope, if using a rat. Spray the back with 70% (v/v) ethanol.
3. Comb the hair away from the incision site using a pair of fine forceps. Make a 1-cm transverse cut in the skin about 1-cm left of the spinal cord, at the level of the last rib, using large sharp scissors.
4. For mice, locate the orange-colored ovary beneath the body wall. A 3–5-mm cut should be made through the body wall at a point a few millimeters away from the ovary, using fine scissors. Stretch the cut to prevent bleeding.
5. For rats, the ovary is not normally visible through the body wall. Make a parallel 1-cm cut into the body wall using a pair of fine scissors and stretch to prevent bleeding.

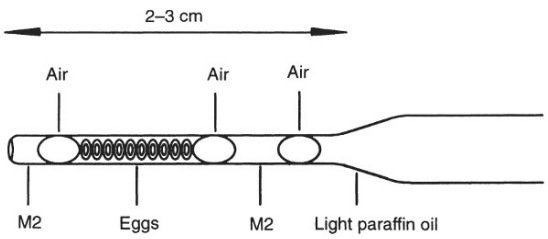

Fig. 8. The tip of an oviduct transfer pipet enlarged to show the arrangement of eggs, air bubbles, and media.

6. Introduce a single stitch into the body wall on one side of the incision, and leave the silk suture in place.
7. Pull out the fat pad joined to the ovary using a pair of fine, blunt forceps. The ovary, uterus, and oviduct will be pulled out as well.
8. Attach an artery clip to the fat pad (avoid the ovary), and position the reproductive tract over the back of the animal such that the coils of the oviduct are exposed and the ovary is to the left.
9. The mouse should be moved (while on the petri dish) to the stage of the dissecting stereomicroscope. Illuminate the oviduct with a fiberoptic light source and view under the 10–20× magnification.
10. Orient the oviduct coils to reveal the cavity (**Fig. 9**) that lies below the ovary and behind the coils of the oviduct. The opening of the oviduct (the infundibulum, target of the transfer process) is located within the cavity behind a transparent membrane (the bursa), which covers the cavity, oviduct, and ovary. Find an area of the membrane, preferably above the infundibulum, that is free of capillaries. Tear it gently with watchmaker's forceps. If the infundibulum cannot be seen through the bursa, just rip the membrane at a convenient point, and continue the search. The infundibulum may be lifted out of the cavity when gently gripped with watchmaker's forceps. Excessive bleeding can be halted with an application of epinephrine (*see* **section 12.4**).
11. Prepare the eggs for transfer. Remove a maximum of 20 microinjected eggs from the microdrop culture, and wash them in M2 medium. Load the eggs into an oviduct transfer pipet (**Fig. 8**), as described in the preliminary procedure.

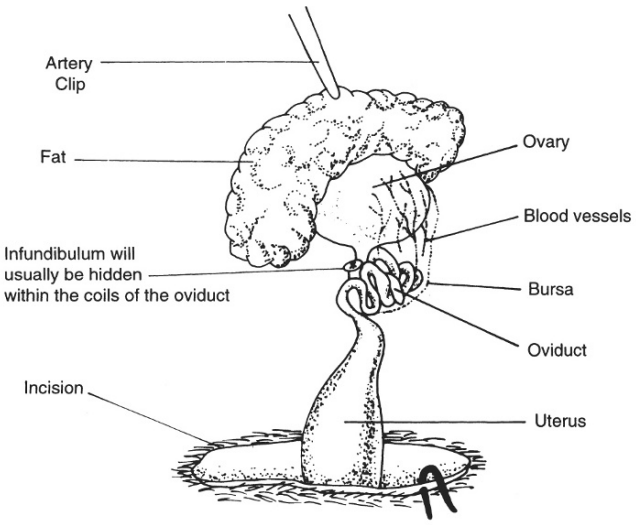

Fig. 9. Schematic diagram of the mouse ovary and oviduct prepared for oviduct transfer. The infundibulum is located within the coils of the oviduct and may be accessed by penetrating the transparent membrane that covers the oviduct.

12. Return to the animal. Use small screws of tissue paper held in the watchmaker's forceps to mop up any excess blood.
13. Grip the tip of the infundibulum with a sharp pair of watchmaker's forceps such that the opening can be accessed by the oviduct transfer pipets. Push the tip of the oviduct transfer pipet into the mouth of the infundibulum (the opening is not visible until penetrated and may be located by gentle prodding with the tip of the pipet). Push the pipet into the infundibulum until it has entered the ampulla. The pipet tip must be far enough into the infundibulum that it does not fall out when the eggs are expelled but not so far in that the opening is against the wall of the ampulla, so restricting the escape of the eggs.
14. Expel the contents of the oviduct transfer pipete into the ampulla, and monitor delivery of the eggs by the appearance of the bubbles. When three bubbles have appeared, one can be certain that the eggs have been deposited into the ampulla.
15. Withdraw the pipet. Remove the artery clip, grip the fat pad with a pair of blunt forceps, and return the reproductive tract into the body cavity. Sew up the body wall with one or two stitches, then clip the skin together with an autoclip.
16. Repeat with the other side of the reproductive tract, if the availability of the microinjected eggs allow, and the animal is sufficiently anesthesized.
17. If the recipient is a rat, inject it with 50-mg ampicillin (Binotal) ip. This prevents low-grade postsurgery infections and can optimize the number of pups subsequently delivered. This step is not necessary for mice.
18. After confirming that the animal has regained some degree of consciousness and mobility, leave it to recover in a warm, quiet place, then transfer to an individual cage.
19. Examine the animals regularly during the days after the operation to ensure that they do not pick up any infections. Mouse pups should be delivered 19–20 d after the operation and rats 21–22 d.

12.4. Note

Excessive bleeding can be prevented by a prior application of epinephrine (20–50 µL of 0.1% (v/v) solution diluted in 0.01 M HCl) made through the bursa at the top of the oviduct using a fine needle and a 1-mL disposable syringe.

13. Caesarean Section and Fostering

13.1. Introduction

The survival rate of microinjected eggs implanted into the pseudopregnant recipient female varies tremendously from 0 to 50%, resulting in abnormally small or large litters. In the former case, the few eggs that do develop tend to be overnourished. Fetuses grow too large to be delivered normally and risk dying in utero. If pregnancy proceeds for 2–3 d beyond the normal gestation period of 19–21 d, the pups can be rescued by Caesarean section and fostering. It may also

be necessary to foster pups born normally to a mother that dies unexpectedly or pups in a large litter that are not fed sufficiently.

13.2. Materials

1. 70% (v/v) Ethanol in a squeeze bottle.
2. Sharp, fine dissecting scissors.
3. Sharp watchmaker's forceps (size 5).
4. Blunt forceps.

13.3. Methods

13.3.1. Caesarean Section

1. Kill the mother by cervical dislocation.
2. Soak the abdomen with 70% (v/v) ethanol.
3. Skin the lower half of the animal. Cut open the body wall to reveal the pregnant uterus. Dissect the uterus carefully by cutting at the oviduct and the cervix and tearing away at the attached membranes.
4. Cut the uterus into sections, each containing a single pup. Gently squeeze each pup out with a blunt forceps.
5. Dissect away the membranes that surround the pup, and cut the umbilical cord. Wipe away any fluid from the mouth and nose. Rhythmically and gently squeeze the chest with blunt forceps to simulate breathing.
6. Place the pups on warm tissue, and keep warm until fostered.

13.3.2. Fostering

1. Remove the foster mother from her pups.
2. Mix the foster pups with the natural pups.
3. Try to make the foster mother urinate on the mixed litter. The animal often urinates on being picked up and restrained with one hand. SD rats often foster pups without this stimulus.
4. Leave the mother undisturbed with the mixed litter for at least 3 h.
5. Remove some of the natural pups to leave a litter of 12. The mother will be unable to care for a larger litter.

13.4. Notes

1. Foster mothers should have natural litters born within 1–2 d of the pups they are fostering.
2. The foster mother should be of a strain with good maternal characteristics (e.g., Swiss or Parkes mice, SD rats). Preferably, the coat color of the natural pups can be used to differentiate them from the fostered pups. Transgenic mice derived from (C57BL/6J × CBA/J) F2 eggs will be black or agouti, readily distinguishable from the albino pups of the Swiss 3T3 strain.

14. In-Vitro Fertilization

14.1. Introduction

Some transgenic animals may be infertile owing to an inability to mate or rear a litter. With mice, in-vitro fertilization (IVF) can be used to continue the line. The technique involves superovulating females and fertilizing the eggs with sperm taken from the epididymis of males. IVF eggs are then transferred to a 0.5-d pc pseudopregnant recipient female. The strain of mouse used should be the same as those used for the original transgenic mouse production (e.g., C57BL/6J × CBA/J F1). Males should be more than 6 wk of age and preferably studs rested the previous night. Females should be at least 4 wk of age. The described protocol employs a single male and 10 females.

14.2. Materials

1. Fertilization medium (FM): All chemicals should be Sigma-Aldrich tissue culture or embryo-tested grade. Prepare the following concentrated stocks and filter each through a 0.4-µm Millipore filter into a sterile plastic tube. Store frozen at −20°C.
   a. 10× A stock: Dissolve 7.013 g NaCl (Sigma-Aldrich S5886), 1.0 g glucose (Sigma-Aldrich G6152), 0.201 g KCl (Sigma-Aldrich P5405), 21.3 mg Na_2HPO_4 (Sigma-Aldrich S5136), 0.102 g $MgCl_2$ $6H_2O$ (Sigma-Aldrich M2393) in double-distilled or Milli-Q water. Bring to 100 mL final volume.
   b. 10× B stock: Dissolve 2.106 g $NaHCO_3$ (Sigma-Aldrich S5761) and 1.0 g phenol red (Sigma P5530) in water. Bring to 100 mL final volume.
   c. 100× C stock: Dissolve 55 mg sodium pyruvate (Sigma P5280) in 10 mL water.
   d. 100× D stock: Dissolve 0.264 g $CaC1_2$ $2H_20$ (Sigma C7902) in 10 mL water.

To prepare fertilization medium, mix 10 mL of 10× A, 10 mL of 10× B, 1 mL of 100× C, and 1 mL of 100× D. Make up to 100 mL by adding 78 mL double-distilled water. Add 3.0 g BSA (Sigma A4161), and mix gently until dissolved. Pass through a 0.45-µm Millipore filter using a large disposable syringe. Store in sterile plastic containers at −20°C.

2. M16 medium.
3. 70% (v/v) Ethanol in a squeeze bottle.
4. Light paraffin oil (Fluka, Buchs, Switzerland; cat. no. 76235).
5. 35-mm Sterile tissue-culture dishes.
6. Micropipet and sterile tips (e.g., Gilson, Villiers-le-Bel, France, Pipetman, P200).
7. Egg transfer and mouth-operated pipet system.
8. Dissecting scissors: 1 regular, 1 fine.
9. Watchmaker's forceps: 2× #5.
10. Stereomicroscope with understage illumination (e.g., Leica, Wetxlor, Germany, model L2).
11. Fiberoptic cold light source (e.g., Schott, Meinz, Germany; KL1500 LCD).
12. 37°C incubator gassed with 5% (v/v) CO_2.

14.3. Methods

1. Two days before the IVF procedure, start the superovulation of the donor females as described in section 4.
2. On the day before the IVF or at least 3 h before, preincubate one 35-mm sterile tissue-culture dish containing 1 mL FM without paraffin and six dishes containing 0.5 mL FM under paraffin oil at 37°C with 5% CO_2.
3. Early on the morning of the IVF, prepare three tissue-culture dishes containing 2–3 mL M16 medium and one tissue-culture dish containing M16 microdrop cultures. Incubate at 37°C with 5% (v/v) CO_2.
4. At 0645 h, kill the male donor by cervical dislocation. Place it abdomen side up, and soak with 70% (v/v) ethanol. Open up the body cavity. Pull out the testes with a pair of watchmaker's forceps. Dissect out the epididymis (a white mass of coils at the base of the testes) with a pair of fine scissors, and immediately transfer to the pregassed tissue culture dish containing 0.5 mL FM under paraffin. Repeat for the other epididymis.
5. Rapidly tease away the fine membrane with a pair of fine forceps, and squeeze the sperm out of the epididymis. The sperm should exit in a continuous stream. Do this fast since the sperm should be at room temperature for the minimum possible time.
6. Incubate the sperm at 37°C with 5% (v/v) CO_2 for 30 min.
7. To each preincubated tissue-culture dish containing 0.5 mL FM under paraffin, add 50 µL of sperm. The final concentration of sperm should be approx $1–2 \times 10^6$ sperm/mL.
8. Incubate the diluted sperm mixture for 2 h at 37°C with 5% (v/v) CO_2.
9. At 0940–l000 h, kill the superovulated females by cervical dislocation, and dissect out the oviducts from both sides (**section 13.3**). Collect all the oviducts into a pregassed dish containing 1 mL of FM without oil.
10. Tease away the ampulla to release the cumulus mass from the oviducts. Transfer the cumulus masses from four oviducts to each pregassed dish containing 0.5 mL FM plus sperm.
11. Incubate the sperm–egg mixture for 4 h at 37°C with 5% (v/v) CO_2.
12. At 1500 h, wash the eggs three times in pregassed M16 medium to remove excess sperm. Transfer the eggs to the M16 microdrops and culture overnight.
13. On the next day, surgically transfer the two-celled embryos to the oviducts of 0.5-d pc pseudopregnant recipient females (**section 12**).

14.4. Note

Should sperm prove difficult to obtain from the epididymis of one male, it is advisable to kill another male quickly and repeat the dissection.

15. Cryopreservation of Transgenic Rodent Lines

15.1. Introduction

Valuable transgenic lines can be frozen and preserved indefinitely by cryopreservation. Cryopreservation protects the transgene line from environmental catastrophes but is also an economical and labor-saving method of preserving lines for future detailed analysis.

Here, we describe equilibrium methods (slow cooling) for both rats and mice. These methods use a programmable cooling machine; embryos are exposed to moderate cryoprotectant concentrations and are cooled slowly at 0.3–2°C/min. Embryos are dehydrated during this slow-cooling process.

Taking into account the survival rate of the embryos as they undergo the different manipulations (e.g., from 100 frozen embryos, 88 are recovered from straws, of which 77 survive freeze–thawing, and 7 or 8 pups are born after oviduct transfers of which 4 are transgenic), at least 400 embryos must be frozen in two sessions to be sure of obtaining 15 or 16 transgenic animals.

15.2. Two-Step Method of Freezing Cleavage-Stage Mouse Embryos

This procedure is used for cryopreservation of 8–32-cell morula stage embryos (14). Mouse cleavage-stage embryos can be harvested from the oviducts of 2–3-d pc pregnant mothers or generated by culturing fertilized one-celled embryos in M16 medium for 2–3 d.

15.2.1. Materials

1. Controlled-rate liquid nitrogen freezer (e.g., Planer, Sunbury, UK; Kryo 150-1.2 or Kryo 3201.7).
2. Small Dewar flask filled with liquid nitrogen.
3. Forceps.
4. Embryo straws (Planer, Sunbury, UK; 0.25-mL sterile, FZA475 and 0.5-mL non-sterile FZA-101).
5. 1-mL syringes for loading straws.
6. Straw labels and permanent marker pen.
7. Heat sealer (Philip Harris Scientific, Nottingham, UK; B10-800).
8. Egg-transfer pipets and mouthpiece (**section 3.1**).
9. Stereomicroscope with understage illumination (e.g., Leica, Wetxlor, Germany; model L2).
10. PBS.
11. Cryoprotectant solution: 1.5 M glycerol (Sigma-Aldrich G2025) in PBS containing 3 mg/mL BSA (Sigma-Aldrich A4161), sterile filtered.
12. Diluent solution: 1.0 M sucrose (Sigma-Aldrich S1888) in PBS containing 3 mg/mL BSA (Sigma-Aldrich A4161), sterile filtered.
13. Liquid nitrogen storage tank.
14. 35-mm Tissue-culture dishes.
15. Rack.
16. 35°C Water bath.
17. 20°C Water bath.
18. Timer.
19. Alcohol for disinfecting.
20. Scissors.

21. Prepared 35-mm tissue culture dish containing M16 medium (**section 6.1.1**) under light paraffin oil.
22. M2 medium (**section 6.1.1**).

15.2.2. Methods

15.2.2.1. Freezing Procedure

1. Mark 0.25-mL straws 1 cm and 7 cm from open end.
2. Push tricolored plugs in 0.25-mL straws down 1 cm from the end to allow space for sealing the straws.
3. Label both 0.25-mL and 0.5-mL straws appropriately (0.5-mL straws to serve as "handles" for the smaller straws, which actually contain the embryos).
4. Fit a 1-mL syringe to each straw.
5. Turn on the freezer to cool it down.
6. Preload the straws. Aspirate 1.0 M sucrose diluent into a straw until it reaches the 7-cm mark. Follow this with air to the 1-cm mark, then wipe the end of the straw. Aspirate 1.5 M glycerol cryoprotectant up to the 1-cm mark, then air up to about 0.5 cm from the end of the straw, and wipe again. Keeping the syringe attached to the straw, gently lay the straw down so as not to disturb the fluid columns and making sure the open end is not touching the bench.
7. Arrange the embryos in a group in a dish of sterile PBS ready on the microscope.
8. Prepare another dish with about 3 mL of 1.5 M glycerol cryoprotectant.
9. Using the egg transfer pipet assembly, aspirate a little PBS, then pick up all the embryos in a minimal volume of PBS and transfer to the cryoprotectant. Do not expel bubbles into the dish. Allow to settle to the bottom for a minute or two.
10. Distribute the embryos in groups of ten around the periphery of the dish.
11. Pick up a group of ten and carefully pipette into the 1-cm column of cryoprotectant in a straw.
12. Aspirate another 0.5-cm column of cryoprotectant into the straw, followed by a small air gap, wipe the end, then seal it on the heat sealer for approximately 3 sec. Handle the straws very carefully to ensure that the fluid columns are not disturbed.
13. Remove the straw from the syringe and heat-seal the plugged end as well.
14. Attach a "handle"(a 0.5-cm straw) and gently lay the straw down.
15. Repeat steps 11–14 until all embryos are loaded. The total time taken to do this should be between 10 and 15 min, necessary for the embryos to equilibrate in the cryoprotectant, but no longer, as cryoprotectant is toxic over longer times at room temperature.
16. Place the loaded straws into the chamber of the controlled-rate freezer.
17. The controlled-rate freezer is preprogrammed to cool the embryos from −7°C to −35°C. At the start temperature of −7°C the embryos must be "seeded" to induce ice nucleation, which helps prevent lethal intracellular ice formation. This is simply done by using forceps precooled in liquid nitrogen (contained in the small Dewar flask) to touch each straw briefly (1–2 sec) until a small ice "nucleus" can

be seen. Do not touch the forceps on that part of the straw containing the embryos. Once all straws are seeded, start the freezer. It is programmed to cool as follows:

Hold at −7°C for 10 min
Cool to −35°C at −0.6°C/min
Hold at −35°C for 10 min

18. At the end of this time (approximately 75 min), the straws are quickly removed and immediately plunged into the liquid nitrogen contained in the small Dewar flask. The straws must not be held at room temperature or the embryos will not survive. Straws are then transferred quickly into prelabeled storage vessels in the large tanks.

15.2.2.2. Thawing Procedure

1. Remove straws from main storage tanks into liquid nitrogen in small Dewar flask and double check their identity.
2. Thaw each straw individually by placing it horizontally on a rack at room temperature, ensuring the fluid column with embryos is not touching any solid surface, for 2 min. Remove the handle and wipe the straw to remove condensation.
3. Shake the straw vigorously toward the plugged end (like a thermometer), three or four times until the fluid forms a single column. This is to mix the cryoprotectant and the diluent as quickly as possible.
4. Incubate the straw, plug end up, in the 35°C waterbath for 3 min to allow efflux of the cryoprotectant from the embryos.
5. Transfer to the 20°C water bath, plug end down, for 1 min.
6. Disinfect the outside of the straw with alcohol then rinse in sterile PBS.
7. Using disinfected scissors, cut off the heat-sealed *unplugged* end of the straw, then holding the now open end close to a dish on the microscope and the plugged end up, proceed to cut off the plugged end. The contents should flow out into the dish.
8. Locate the embryos, then wash them through several changes of PBS to remove residual freezing solutions before placing into a dish of culture medium and incubating until transfer into pseudopregnant recipient mothers (**section 12**).

15.3. Cryopreservation of Rat Embryos

Rat cryopreservation is less well researched. This protocol describes a method for freezing one-cell rat embryos ***(15)***.

15.3.1. Materials

These are the same as for **section 15.2.1**, except

1. Cryopreservant (Sol A): 1.5-M Ethylene glycol (Sigma-Aldrich E9129) in PBS containing 4 mg/mL BSA (Sigma-Aldrich A4161).
2. Thaw diluent (Sol B): 0.5 M Sucrose (Sigma-Aldrich S1888) in PBS containing 4 mg/mL BSA (Sigma-Aldrich A4161).

15.3.2. Method

15.3.2.1. Freeezing Procedure

1. Collect fertilized one-cell rat embryos (**section 8**) from females mated the previous evening into M2, transfer into M16, and incubate at 37°C in 5% (v/v) CO_2 if holding them for any length of time.
2. Just prior to commencing the freezing protocol, transfer the embryos into M2 and count the number.
3. Work out the number of straws required. Push plugs down 1 cm and label both the straws and the "handles." Mark straws at 0.5 cm and 7 cm from open end; fit syringe fillers and preload straws as follows:
   a. 7 cm Sol B.
   b. 0.5 cm air.
   c. 0.5 cm Sol A.
   d. 0.5 cm air.
   e. 0.5 cm Sol A.
   f. 0.5 cm air.
   g. 0.5 cm Sol A.

   Air to wet the plug.
4. Gently lay down the straws.
5. Wash embryos two or three times through PBS and divide into groups of 20.
6. Transfer one group of embryos into Sol A. Leave for 12 min.
7. Start the controlled- rate freezer.
8. Load each group of 20 into its straw, placing in middle column of Sol A.
9. Remove syringe and seal both ends, fit the handle and lay down gently.
10. Repeat for other groups of 20. All straws must be loaded within 12–15 min.
11. Place straws into the controlled-rate freezer at −7°C.
12. Repeat for remaining groups of embryos in the batch, until all have been equilibrated in Sol A and loaded into straws. Begin freezing run.
13. Freezer program:
    a. Start temperature −7°C.
    b. Hold 5 min (soak).
    c. Seed manually (**section 15.3.2.1**).
    d. Hold 10 min.
    e. Cool at −0.5°C/min to −30°C.
    f. Hold 3 min, while removing the straws to liquid nitrogen storage.

    Do not allow to warm at this stage.

15.3.2.2. Thawing One-Cell Rat Embryos

1. Transfer the required number of straws into liquid nitrogen in a small Dewar flask. Then handle only one straw at a time.
2. Quickly immerse the straw in a 37°C water bath for 20 sec.
3. Dry on tissue, then cut ends and expel all of the contents into a 35-mm tissue-culture dish, ensuring sucrose diluent in the straw (Sol B) is mixed with the Sol A containing the embryos. Leave for 5 min.

4. Wash through PBS, then M2, and finally place into M16 for further culture until needed for embryo transfer (**section 12**). This may be done the same day as the thaw or on the following day at the two-cell stage.

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8

Cre Recombinase Mediated Alterations of the Mouse Genome Using Embryonic Stem Cells

Anna-Katerina Hadjantonakis, Melinda Pirity, and András Nagy

1. Introduction

The introduction and establishment of transgenic, and in particular embryonic stem (ES) cell-based gene "knockout" technologies have made the mouse a key player in studying embryonic development and disease ***(1,2)***. In recent years, methods for the production of more complex genomic alterations have become increasingly widespread, hinting at an ability to manipulate and study a mammalian genome to an extent never previously thought possible. Such methodologies often partner homologous recombination-mediated gene targeting or random integration with site-specific recombination events.

This chapter is concerned with the utilization of the bacteriophage P1 derived site-specific recombinase protein Cre ***(3–5)***, and its employment as a means to catalyze modifications in homologously recombined and randomly integrated target sites within the mouse genome.

Cre is a 38-kDa protein that recombines DNA between two loxP target sites. loxP sequences are 34 basepairs (bp) long comprising two 13-bp inverted repeats flanking an asymmetric 8 bp core sequence. The recombination between two loxP sites with same orientation on the same DNA leaves two products each containing a single loxP site ***(6)*** (**Fig. 1**). This type of site-specific recombination, of which there are several other well-characterized systems in addition to the Cre/loxP, generates precise rearrangements of DNA but dispenses with the requirement for extensive homology between DNA partaking in the recombination. Recombination occurs through the recognition of the target sites by the recombinase, which then catalyzes strand exchange between them by precise breakage and rejoining events that are restricted to an internal region of identical sequence contained within the specific sites ***(6)***.

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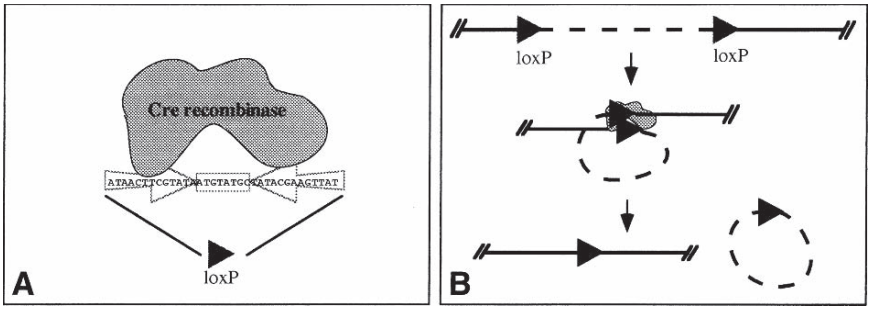

Fig. 1. The Cre recombinase has a 34-bp recognition site comprising two inverted repeats and a core sequence **(A)**. It can catalyze a site-specific recombination event leading to the deletion of the intervening DNA **(B)**.

In addition to the Cre/loxP system, another one of the many recombinase that does not require *cis*-elements, but utilizes short recognition sites for recombination is the yeast FLP/FRT system. This system has also been widely used and applied to genome alterations ***(7–10)***, though to date the Cre protein has been shown to be more amenable to use in mammalian cells, and is therefore currently favored by most laboratories for use in ES cells and transgenic mice ***(11)***. As a consequence, we will solely refer to the Cre/loxP system in the strategies we present, though it should be noted that if the FLP/FRT were to be as efficient as the Cre/loxP system it could be substituted in all methodologies. Additionally, it might also be anticipated that future experiments may require a multistep, site-specific recombination strategy, thereby requiring the use of two separate recombination systems.

This chapter will illustrate and provide the methodologies for some of the applications of such a site-specific recombination system to experiments aimed at analyzing mouse embryonic development and/or disease conditions, from single-gene alterations, lineage-restricted and/or conditional gene ablations or ectopic expression through to chromosome engineering, and finally the use of such a system for lineage analysis.

1.1. Combining Homologous and Site-Specific Recombination

Homologous recombination in ES cells allows the precise disruption (knockout) of a target gene. Many new approaches require a defined alteration of a gene or the genome. By combining the homologous and site-specific recombinations, we are now in the position of creating most desired alterations in the mouse genome ***(12,13)***. In the following section, we will briefly introduce some of the most important current applications. The list will not be complete, since novel applications for the use of this system are continuously being reported.

1.1.1. Eliminiating Any Regional Effect of a Knockout: Removal of a Selectable Marker

To identify targeted events, an introduction of a positive selectable marker, usually neomycin, into the targeted locus is required. Recently, there has been an increasing concern regarding the repressor effect of the selectable marker cassette on the genes in the vicinity of its insertion. Therefore, removal of the marker from all targeted genes is advisable. This can most easily be performed by flanking (floxing[flanking with loxP]) the selectable marker cassette by loxP sites, which on introduction of the Cre recombinase will result in the removal of neomycin.

1.1.2. Introducing Subtle Changes in a Gene of Interest

Cre/loxP type approaches can also be used to introduce subtle changes into any gene, including point mutations ***(13a)*** or small deletions into genes of interest, particularly if domain deletions ***(14)*** or domain swaps within the protein coding regions of a particular gene are desired. Such an approach is illustrated in **Fig. 2**.

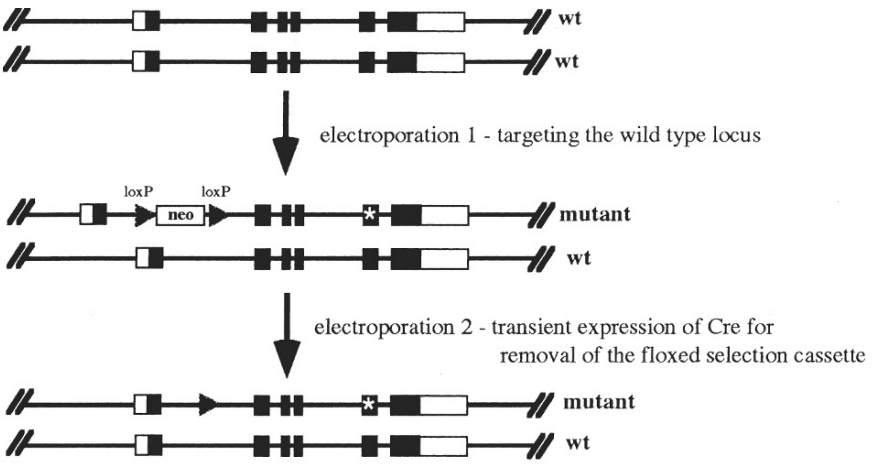

Fig. 2. Simple gene alterations—introducing a subtle change into a gene of interest. In this example, a point mutation, small deletion, or domain swap (*) is introduced into an exon of a gene using a relacement type vector, containing a floxed neomycin resistance cassette. Transient expression of Cre results in removal of the region between the loxP sites, and as a result the neo selection cassette is removed, thereby rendering the genomic structure identical to wt with the exception of the introducted change and the single loxP site remaining in the first intron.

1.1.3. Introducing Specific Chromosomal Changes

Over the past few years, strategies have been developed for chromosome engineering in ES cells. Such approaches have been used to design novel chromosomal variants, or to mimic altered chromosomes associated with human disease or metastasis ***(15)***. Such approaches rely on the sequential targeting of two loxP sites, either in *trans* (i.e., to different chromosomes) or in *cis* (some distance apart on the same chromosome), followed by the transient expression of the Cre protein in order to mediate the site-specific recombination event between the loxP sites, leading to the formation of the new chromosomal variant ***(15,16)*** (**Fig. 3**). In this case, usually three ES cell electroporations and resulting screening for the alteration are required: the two end points are targeted separately, and are followed by the introduction of the recombinase, which will mediate the recombination event between them.

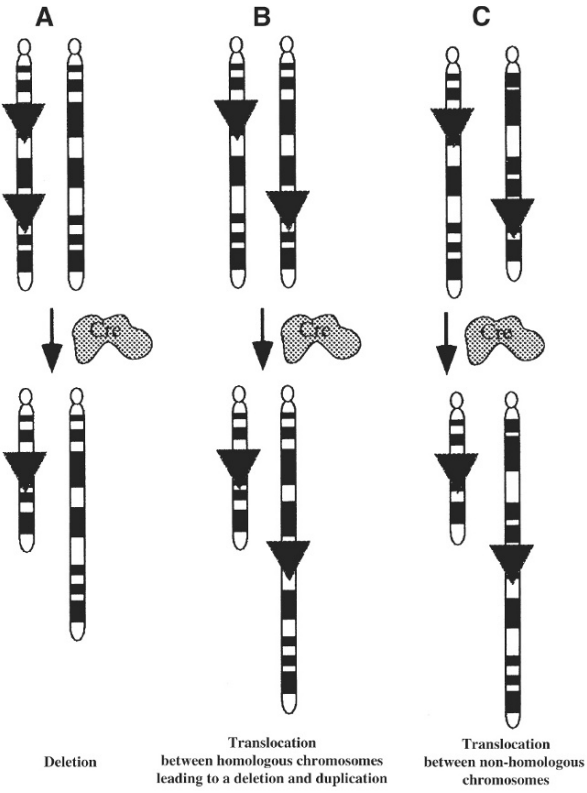

Fig. 3. Chromosomal modifications. Targeting of the two loxP sites some distance apart, either in *cis* (A) or in *trans*, in the same (B), or different chromosomes (C) can give rise to various chromosomal modifications.

This type of strategy can be used to create almost all cataloged forms of chromosomal aberration. Additionally, application of this technology could allow the creation of multiple large-scale chromosomal alterations, for example, a set of nested hemizygous deletions (also known as deficiencies) covering an entire chromosome. These could then be used to reveal novel tumor suppressor genes or functional haploinsufficiencies mapping within the deleted DNA. If a panel of deficiencies is available, screens for interesting phenotypes can be carried out either in culture, or in mice, the latter being particularly amenable to ES cell ↔ tetraploid embryo aggregation ***(17,18)*** as a means of creating completely ES cell-derived embryos, therefore bypassing the germline for accessing embryonic phenotypes ***(19)***.

Since a recombination event between two loxP sites some distance apart or positioned on different chromosomes, is relatively rare, presumably because of physical constraints, a reflection of chromosome architecture and decreased proximity, such strategies are designed incorporating a binary positive selection system that is only activated after a successful recombination recreates the cassette. Thus, the desired recombination event will reconstruct the selectable genetic marker, from two silent portions placed adjacent to each of the loxP sites. The most commonly used selection systems include the reconstitution of a human hypoxanthine phosphoribosyltransferase (HPRT) minigene ***(16–20)***, or the juxtaposition of a strong promoter upstream of a selectable marker.

1.1.4. Creating Lineage and/or Inducible Gene Alteration

1.1.4.1. Lineage-Specific Gene Knockouts

The combination of a lineage-restricted promoter and the Cre/loxP system can be used to create a modified locus that is restricted to a certain spatiotemporal domain within the mouse. This has recently been demonstrated using a keratin 5 promoter-driven Cre to ablate the X-linked pig-a gene in skin ***(21)*** and αCamkinase II promoter-driven Cre to ablate the NMDAR1 gene in a subset of postnatal cells of the CNS ***(22)***. Combinations of conditional and inducible Cre/loxP gene targeting regimes can be utilized for a more sophisticated assessment of gene function in the developing embryo and adult animal.

1.1.4.2. Lineage-Specific Gene Repair

This approach allows one to study specific cellular phenotypes over restricted time-points or spatial locations during development or adult life. Here, the targeted allele should contain the original gene structure designed to be silent or compromised ***(13a)*** owing to the interruption by the loxP-flanked "stop" sequence (resulting in no or comprimised gene expression). The Cre recombinase will be expressed from a transgene in a restricted set of cells, thereby resulting in the excision of the loxP-flanked region in these cells (resulting

in normal gene expression). Consequently, the original gene structure will be restored solely in cells expressing the Cre transgene, but the remaining population will still be deficient.

If the reparable allele phenotype is characterized, and found to be embryonically lethal, then the primary responsible lineage/organ can be identified, and a proper Cre transgenic line made (or selected from the existing lines), which expresses the recombinase only in the primarily affected lineage. When this lineage-specific Cre-expressing line is crossed over the homozygous mutant genotype, the Cre recombinase repairs the mutant allele in the primary lineage, rescuing the primary deficiency, therefore allowing for the manifestation of secondary defects. This approach is expected to be less sensitive to a possible mosaic action of the Cre recombinase, since in many cases, a mosaic repair is sufficient for complete rescue. On the other hand, in almost all cases, high-fidelity lineage-specific deletion is necessary for lineage-specific knockout.

1.1.4.3. Inducible Gene Knockout

Conditional lineage-restricted ablations can be obtained through the incorporation of an inducible system into a transgenic regime. Here the Cre protein can be induced where and when appropriate. This can either be achieved by placing the Cre gene under the control of an inducible promoter (either ubiquitous or lineage-specific) or to construct the Cre cassette as an inducible fusion protein.

Several approaches have been utilized for inducible gene expression in both experimental animals and in culture. Initially, inducible systems involved the use of heat shock, isopropylithio-β-D-galactoside (IPTG), and heavy metals as inducing agents ***(23,24)***, but owing to their lack of specificity and toxic side effects, these systems are primarily restricted to use in prokaryotes, yeast, and *Drosophila*. Unfortunately, at present there is no totally satisfactory inducible system available for use in transgenic mice, though recently several laboratories have reported the successful use of drug- and hormone-inducible systems in mammalian cell culture ***(25,26)***. A common aspect of these various approaches is that the majority comprise binary systems involving the use of chimeric transcription factors that can reversibly bind target gene sequences in response to the administered drug or hormone. Modifications of the bacterial tetracycline system ***(27)***, the *Drosophila* ecdysone receptor system ***(26)***, and molecular dimerizer systems based on FK506 or its analog rapamycin ***(28)*** have been shown to work in cells in culture and are presently being developed for use in transgenic mice.

1.1.4.4. Requirement for Lineage-Specific or Inducible Cre Transgenic Lines: A Cre Transgenic Mouse Database

All of the above technologies rely on the availability of properly working lineage-specific or inducible Cre transgenic lines. To this end, we are coordinating the

assimilation of information concerning the available and planned Cre transgenic lines, and have compiled them into a continually updated database, which can be accessed through the World Wide Web at www.mshri.on.ca/develop/nagy/nagy.htm.

1.2. Future Directions

1.2.1. Fate Mapping

A great deal of interest has centered around the fate of individual cells within the developing embryo ***(29,30)***. In lower organisms, following the fate and genesis of cells is less complex than in a mammal such as the mouse embryo, where marking a single cell by injection or transplantation and following its descendants during the course of development is technically demanding, requiring expensive equipment and expertise.

ES cell-mediated transgenic technologies utilizing the Cre/loxP system may be able to facilitate fate mapping the embryo greatly. Here a lineage-restricted promoter is used to drive the Cre recombinase. A second transgene containing a reporter gene flanked by the loxP sites is also required. The second transgene should contain a "stop" sequence between the ubiquitous promoter and the marker gene to keep the gene silent. Double transgenic animals will neither express Cre nor the marker gene until the specific developmental stage permitting Cre expression and subsequent recombination, resulting in marker gene activation. This will result in activation of the recombinase, resulting in the excision of the "stop" sequence, and expression of the marker in all the progenitors cells, regardless of the later expression status of the Cre-driving promoter. If conditions are optimized, then the Cre can be used for noncomplete excision and, thus, result in excision in a very limited number of cells or even in single cell. Another possible improvement would be the utilization of an inducible Cre recombinase, allowing the precise regulation of excision frequency and timing, thereby making it relatively straightforward to follow the fate of individual cells and their descendants based on the expression pattern of the marker.

1.2.2. Gene Trap

It is feasible that gene insertion strategies utilizing loxP sites may gain popularity in certain gene-trap experiments, the goal of such experiments being to identify novel ubiquitous and/or lineage-restricted promoter/enhancer elements or genes.

Using a vector carrying a splice acceptor sequence placed upstream of a reporter gene (such as *lacZ* or GFP [green fluorescent protein]), different types of regulatory or gene sequences can be trapped ***(31–33)***. ES cell-chimeric embryos are stained for the histochemical marker to reveal expression domains of the trapped elements ***(31–33)***. On the basis of the expression pattern information

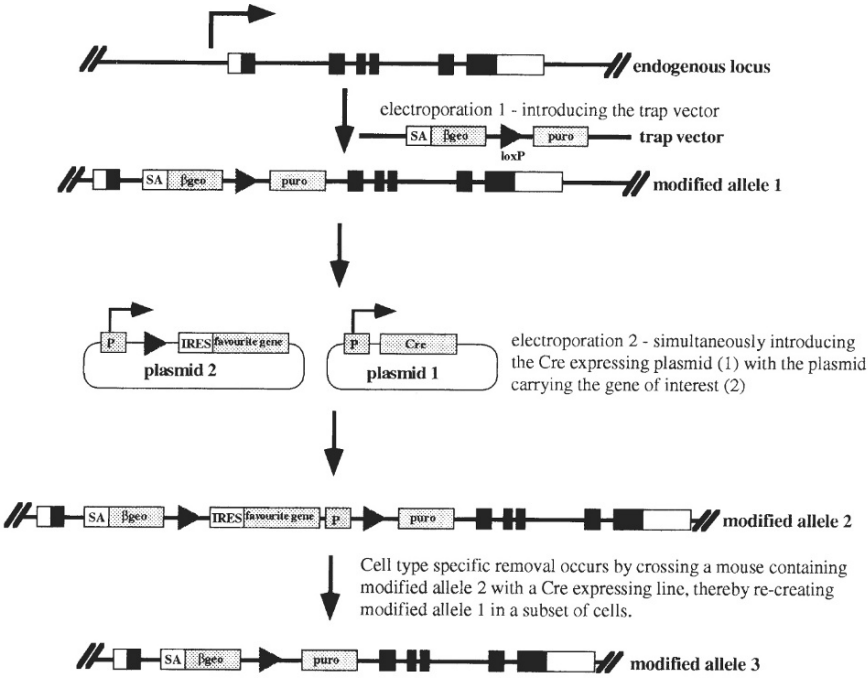

Fig. 4. Trapping an endogenous locus with the option of introducing a new gene. Here two electroporation steps are required. The first is used to identify a locus of interest, and the second is used to introduce the new gene into that locus. Two alternative alleles are created on the second electroporation, one doubly drug-resistant, the other singly. Thus, the drug selection will determine which clones can grow. P, promoter driving gene expression in ES cells; SA, splice acceptor; βgeo, βgal-neo gene fusion; puro, puromycin resistance cassettes; IRES, internal ribosome entry site.

gained on the trap cell lines, a subset is chosen for further study. If a specially designed trapping vector is used, such as that illustrated in **Fig. 4**, the trapped locus can be retargeted via loxP sites, and different transgenes can be knocked-in leading to their spatiotemporal expression being governed by the trapped element. Later, the expression can also be abolished by introducing the Cre recombinase into the system.

2. Materials

For all procedures, solutions should be made to the standard required for molecular biology using molecular biology-grade and/or "tissue-culture-tested" reagents. All solutions should be made using sterile double-distilled or MilliQ water, and where appropriate, autoclaved or filter-sterilized.

2.1. ES Cell-Culture Media and Solutions

1. DMEM+: For all procedures, we use Dulbecco's Modified Eagle's Medium (DMEM—Flow Labs [Finland], powder, cat. no. 430-1600) supplemented with the following:
   a. 0.1 m*M* nonessential amino acids (100X stock, Gibco, Grand Island, NY, cat. no. 320-1140AG; *see* **Note 1**).
   b. 1 m*M* sodium pyruvate (100X stock, Gibco cat. no. 320-1360).
   c. 100 m*M* β-mercaptoethanol (100X stock stored as aliquots at −20°C, Sigma, St. Louis, MO, cat. no. 600564AG).
   d. 2 m*M* L-glutamine (100X stock, stored as aliquots at −20°C, Gibco cat. no. 320-5030AG).
   e. 15% Fetal calf serum (FCS) (*see* **Note 2**).
   f. Penicillin and streptomycin (final concentration 50 µg/mL each, Gibco cat. no. 600-564AG).
   g. Leukemia inhibitory factor (different sources, for example, Gibco) 1000 U/mL. The supplemented DMEM used for propagation of ES cells is referred to as DMEM+ (*see* **Notes 1–3**).
2. 0.1% Gelatin: 1 g (w/v) gelatin is 0.1% gelatin (Sigma or BDH) made up in 1 L water, autoclaved and stored at 4°C (*see* **Note 4**).
3. 2X ES cell-freezing medium: 2X ES cell-freezing medium should be made up fresh each time it is to be used, and should comprise freshly prepared 60% DMEM+, 20% FCS, and 20% DMSO (Sigma, cat. no. D-5879).
4. Phosphate-buffered saline (PBS): For all tissue-culture work, we use PBS without calcium and magnesium. This is made from 10 g NaCl, 0.25 g KCl, 1.5 g Na_2HPO_4, 0.25 g KH_2PO_4, pH 7.2. The solution is autoclaved and stored at 4°C.
5. Trypsin (0.1%): Dissolve 0.5 g trypsin powder (Gibco, cat. no. 0153-61-1) in 500 mL saline/EDTA solution. Adjust the pH to 7.6, sterilize through a 0.22-µm filter, and store at −20°C. This constitutes a 5% stock, which needs to be diluted to 0.1% on defrosting.
6. Saline/EDTA solution: 1 L of saline/EDTA solution comprises 0.2 g EDTA, 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.01 g phenol red, 0.2 g glucose, pH 7.2. The solution should be sterilized through a 0.22-µm filter, and stored at room temperature.
7. Tissue-culture-treated plasticware: We routinely use NUNC, Corning, and Falcon plasticware.
8. Humidified incubator: This is maintained at 37°C and 5% CO_2.
9. Electroporation apparatus: Use apparatus, such as a Bio-Rad GenePulser, and appropriate cuvets (Bio-Rad, Hercules, CA, cat. no. 165-2090).
10. Selection reagents: There are drugs, such as G418 (Gibco, cat. no. 11811-031), gancyclovir (Syntex, cat. no. 00865516), puromycin (Sigma, cat. no. P8833), and 6-thioguanine (Sigma, cat. no. A4660) or HAT (Gibco, cat. no. 31062-037) for HPRT-negative or positive selection, and mitomycin C (Sigma, cat. no. M-0563).
11. Cre recombinase-expression vectors: Cre recombinase-expressing vectors are described in detail in a number of publications ***(34–36)***.

3. Methods

3.1. Preparation of Tissue Culture Plates

Tissue-culture plates require special treatment prior to the plating of ES cells. This can either be by coating them with gelatin or mitotically inactivated fibroblast cells.

3.1.1. Gelatinized Plates

In our experience, R1 ES cells ***(17)*** can be propagated on both types of plates without losing their totipotency or their ability to contribute to the germline (*see* **Note 7**).

1. To prepare gelatinized plates rinse the surface of tissue-culture dishes with a 0.1% gelatin solution (approx 100 µL/well of 96-well plates, or 3 mL for 6-cm plates, 5 mL for 10-cm plates).
2. Aspirate off the excess gelatin, and then allow the surface of the plates to dry a little (2–4 min).
3. Add fresh medium to the plates, and place them into the incubator until required.

3.1.2. Plates with Feeder Cells

Primary mouse embryonic fibroblast (EMFI) cells ***(37)*** or the STO fibroblast cell line ***(38)*** is the most commonly used feeder layers. Details for the preparation of a stock of EMFI or STO cells are described elsewhere ***(39)***. A brief protocol for the preparation of EMFI feeder layers will be given here.

1. Quickly defrost a vial of EMFI or STO cells, and then transfer the cell suspension to a sterile 15-mL tube containing 10 mL prewarmed feeder cell media (DMEM supplemented with 10% FCS). Then spin at 1000 *g* for 5 min, at room temperature.
2. Aspirate the supernatant, and then gently resuspend the cell pellet in 10 mL media.
3. Plate the cell suspension onto five 15-cm plates each containing 25 mL media, and place in an incubator.
4. When the cells form a confluent monolayer (usually takes 3 d) they are ready to be treated with mitomicin C.
5. Briefly, medium is aspirated from the confluent plates and replaced with 10 mL media containing 100 µL of 1 mg/mL mitomycin C, and then placed in an incubator for 2–2.5 h.
6. The medium is aspirated, and the plates washed twice each with 10 mL PBS, followed by the addition of 10 mL of trypsin/EDTA/dish.
7. Plates are placed in an incubator until the cells begin to detach.
8. Ten milliliters of media are added to each plate, and the cell suspension broken up by gentle pipeting.

9. The cell density is determined (hemocytometer) and adjusted to 2×10^5 cells/mL. Then the cells are plated directly onto dishes suitable for ES cell culture. We routinely plate approx 1×10^6 R1 cells per 10-cm dish (*see* **Notes 5–7**).

3.2. Passaging ES Cells

Optimally ES cells should be fed every day and split every second day (by which time they should be 70–80% confluent). It is important not to let them overgrow, since this may induce them to differentiate.

1. If the cells are split into gelatinized plate, the plates should be prepared (as detailed previously) before starting the trypsinization.
2. Trypsinize the cells by first aspirating the medium off the dishes and then rinsing twice with PBS.
3. Aspirate off any remaining PBS, and add trypsin (0.1%) to the cells. For 10-cm plates, we use 2.5 mL trypsin. This volume should be scaled according to the size of plate used.
4. Place the dish containing the cells in trypsin in a 37°C incubator for 3–6 min.
5. Check under an inverted microscope to see if the cells have detached. When they have, add 5 mL medium to the dish.
6. Resuspend the cells and transfer them to a 12-mL tube.
7. Spin down at 1000 *g* for 5 min at room temperature.
8. Aspirate the medium, and then add 1 drop of PBS to the pellet.
9. Flick the tube hard in order to resuspend the cells.
10. Add 5 mL of medium to the tube, pipet again to mix, and split the contents at 1:5 or 1:7 ratio in new plates containing sufficient volume of medium.

3.3. Electroporation of ES Cells

Cells are routinely passaged two days prior to electroporating. Cells are ready for electroporating when their density is optimal. Usually one 10-cm plate at approx 80% confluency will provide enough cells for 1–2 electroporations. Our standard electroporation protocol is given below.

1. Gelatinize 10-cm plates, and then add 10 mL medium to each.
2. Place them in a 37°C incubator until they are required.
3. Switch on the electroporation apparatus.
4. Harvest the cells by trypsinization.
5. Resuspend the cell pellet in ice-cold PBS (1 mL for each 10-cm plate).
6. Determine the cell density (hemocytometer), and dilute with PBS to the required density for electroporation. We regularly electroporate at a relatively high cell density: 7×10^6 cells/mL (this number varies between different labs).
7. For each electroporation, mix together 20–40 µg vector DNA (for an approx 10-kb vector; *see* **Notes 8** and **9**) and 0.8 mL of the ES cell suspension in an electroporation cuvet (Bio-Rad, cat. no. 165-2088) (*see* **Note 8**).
8. Set up the electroporation conditions prior to placing the cuvet into the electroporation chamber. We routinely use 250 V, 500 µF for the Bio-Rad GenePulser (*see* **Note 9**).

9. Zap the cuvet, then place it on ice for 20 min to 1 h.
10. Transfer the cells from the cuvet into the prewarmed medium containing dishes. (The contents of one cuvette are routinely seeded into two 10-cm dishes).
11. Change medium daily.
12. If drug selection is required, start this on the second day after electroporation (*see* **Note 10**).
13. Continue the selection until colonies become apparent, and grow to a size that is amenable to picking (usually takes 7–10 d) (*see* **Note 10**).

3.4. Picking Colonies After Selection

This can either be done with the naked eye or by placing a dissecting microscope (such as a Lietz M3B) into the laminar flow hood.

1. Colonies are ready for picking if they are large and well separated (usually 7–9 d after electroporation). For picking start by gently washing the plate twice with PBS.
2. After the second rinsing, leave a little of the PBS behind in the dish (about 1 mL) in order to keep the surface of the plate wet, therefore preventing the colonies from drying out.
3. Choose a colony to pick. The colony is optimal if it is neither too small nor too big, and contains nondifferentiated ES cells with characteristic morphology. Fill a P200 pipeter with approx 20 µL PBS, and gently pour this over the colony thereby rinsing it. Retain about 5–10 µmL of the PBS in the pipet, and with this, try to "suck up" the colony from the bottom of the plate. Hold the pipet perpendicular (if picking with the naked eye) or at 45° (if picking under the dissecting microscope) to the surface of the plate, since this facilitates the lifting of the colony from the plastic.
4. Using the P200, transfer each individual colony into separate wells of a 96-well plate containing 50 µL trypsin in each well. In doing so, pipet the colony up and down several times in order to dissociate the cells. Be careful to avoid creating too many air bubbles.
5. When all the colonies from a plate have been picked and transferred to the trypsin, the 96-well plate is placed in a 37°C incubator for 5–10 min.
6. During this time a new gelatinized 96-well flat-bottom plate with medium (200 µL/well, containing the selection agent) is prepared.
7. Working row by row with a multichannel pipeter the cell-trypsin solution is transferred to the gelatinized plate.
8. Pipet thoroughly, without creating too many air bubbles, so as to promote the formation of a single cell suspension.
9. Return the plate to the 37°C incubator.
10. Change the media daily until the cells are ready to passage (80% confluency).
11. When passaging the cells, split them into two or three new plates. These can each be used for the preparation of DNA for genotype screening and for creating frozen stocks, which can then be used for thawing the required clones.

3.5. Passaging Cells in 96-Well Plates

Optimally 3 or 4 d after picking colonies into the 96-well plates, the cells are at a density required for splitting. Since cells in different wells generally exhibit different growth potential, they will not grow at a synchronous rate. Therefore the optimal time for splitting the whole plate needs to be determined. It is best to choose a time when the majority of the cells have reached 80–90% confluency. Another more laborious alternative is to passage the clones at different stages (pooling them into groups depending on their growth rate) and replating them into different 96-well plates.

1. To passage cells in 96-well plates, first prepare several gelatinized 96-well plates.
2. Add 200 µL medium/well, and place plate in a 37°C incubator.
3. Aspirate the medium from the plate to be split, and then wash with PBS by multipipeting 200 µL of PBS into each well followed by aspiration.
4. Remove all traces of PBS, and then add 50 µL trypsin per well.
5. Incubate at 37°C for 5–10 min. The cells should detached with gentle tapping on the plate.
6. Multipipet 50 µL medium/well into each of the wells. Pipet up and down about five times so as to resuspend completely (*see* **Note 11**). Then split them (working row by row) into two or three newly gelatinized plates.
7. Return these plates to the 37°C incubator (*see* **Notes 12** and **13**).

3.6. ES Cell Freezing

3.6.1. In Cryovials

The general protocol for freezing cells grown in a standard 10-cm dish at 70% confluency is given below (*see* **Notes 14** and **15**):

1. Change media 2–3 h before freezing the cells.
2. Freshly prepare 2X freezing media.
3. Harvest the cells in a 15-mL tube containing DMEM+ after trypsinization (*see* **Note 15**).
4. Spin down at 1000 *g* for 5 min at room temperature.
5. Remove the supernatant, and then add one or two drops of DMEM+ to the tube. Shake gently, but thoroughly to disperse the cells.
6. Add an additional DMEM+ medium to a total volume of 1.5 mL, and disperse the cells carefully so that they comprise a single-cell suspension.
7. Add an equal volume (1.5 mL) of 2X freezing medium, and mix by pipeting several times.
8. Quickly aliquot the cell suspension into three vials, and immediately place them in a styrofoam box (this will allow them to cool down gradually). Alternatively, special boxes dedicated to this task can be purchased from a number of manufacturers (for example, Stratagene) (*see* **Note 16**).
9. Place the box in a −70°C freezer for 1–2 d, and then transfer the individual cryovials into a liquid nitrogen container for long-term storage (*see* **Note 16**).

3.6.2. In 96-Well Plates

1. Working one row at a time using a multichannel pipetter, change the medium 2–3 h prior to freezing.
2. Freshly prepare 2X cell-freezing media.
3. Aspirate the medium from each well, and wash the cells with PBS (approx 200 µL).
4. Add 50 µL trypsin to each well, and then place plate in an incubator for 5–10 min.
5. Working on ice, preferably in a wide, flat container, aliquot 50 µL of DMEM+ into each well. Pipet the cells several times in order to get them into a homogenous suspension.
6. Then add 100 µL 2X cell freezing media to the wells, and again pipet to mix.
7. Finally add 80–100 µL sterile mineral oil (Sigma, cat. no. M-8410) to cover the cell/freezing medium mixture.
8. Wrap the plates in parafilm, place in a styrofoam box, and store in a −70°C freezer until such time as the desired clones have been identified and need to be recovered (*see* **Note 17**).

3.7. Thawing ES Cells

1. Prepare the appropriate-sized feeder layer containing plates for the cells that are to be recovered (24-well plates for 96-well frozen plates or 6 cm plates for cryovials containing approx 5–10 × 10^6 cells).
2. Add medium to the plates, and prewarm them in the incubator at 37°C.
3. The following steps vary with the type of tissue-culture plates used (*see* **Note 18**).

3.7.1. From 96-Well Plates

1. Remove the sample from the freezer, and as it begins to melt, carefully aspirate the overlying oil from the cell/medium mixture.
2. Using a multichannel pipetter and working one row at a time, quickly, but gently multipipet the cells twice in order to resuspend them thoroughly.
3. Change the pipet to a P200 (set to 200 µL), and then quickly transfer the well contents one at a time to the individual wells of a 24-well plate. Pipet quickly to resuspend the cells in the 96-well plate. Then transfer. Once the sample is transferred to the 24-well plate, pipet again to resuspend the cells and distribute them evenly in the prewarmed fresh media.
4. Aliquot another 200 µL into the now empty well of the 96-well plate to rinse it, and remove any remaining cells.
5. Transfer this additional 200 µL to the equivalent well of the 24-well plate.
6. Repeat the above for each required well of the 96-well plate.
7. Place the 24-well plate containing the newly transferred cells in a humidified CO_2 incubator at 37°C.
8. Change the media after 8 h or the following morning (if defrosting is carried out late in the evening), and then daily until the cells are ready to passage.

3.7.2. From Cryovials

1. Defrost the cells quickly, then transfer the cell suspension to a sterile 15-mL tube containing prewarmed media (approx 10 mL), and then spin at 1000 *g* for 5 min at room temperature.
2. Aspirate the supernatant, and then add 1 drop of PBS.
3. Resuspend the cells by either flicking the bottom of the tube or by gently pipeting up and down.
4. Add few milliliters of media to the tube, and again gently pipet up and down to dissociate the cells.
5. Plate the cell suspension onto a 6-cm plate, and place in an incubator.
6. Change the media after 8 h or the following morning (if defrosting is carried out late in the evening), and then daily until passaging is required.
7. When the cells reach approx 70% confluence (usually taking 2 d), they are ready to passage.

3.8. Removal of loxP-Flanked Short Genomic Segment in ES Cells by Transient Expression of Cre Recombinase

1. Electroporate the correctly targeted ES cell line with 50 µg/mL of circular plasmid containing the Cre recombinase gene driven by an ES cell transcriptionally active promoter, for example, the pBS185 plasmid, which contains Cre under the control of the hCMV promoter ***(35)***.
2. After electroporation, plate the cells very sparsely (approx 1000 cells/10-cm dish) onto gelatinized 10 cm plates each containing 10 mL of DMEM+.
3. Change media daily until the colonies have attained a size that is ready to pick (usually takes 7 d).
4. Pick colonies into 96-well plates, and expand into two or more plates. One plate is frozen, and the others are used for PCR or Southern screening to detect the required recombination event. A subset of the colonies will be mosaic for the Cre-mediated excision, therefore requiring further subcloning for the derivation of pure lines (*see* **Notes 19** and **20**).

3.9. Deletion of loxP-Flanked Large Genomic Segment or Selection for Site-Specific Chromosomal Alteration in ES Cells by Transient Expression of Cre Recombinase

This is carried out in much the same manner as in **Subheading 3.8.**, except that cells are usually subjected to selection, since a bipartite cassette that will be reconstituted after the desired recombination is routinely used (for example, HAT for the reconstitution of the HPRT minigene). Routine selection is applied 2 d after the electroporation. As a consequence, the cells need to be plated at regular density (one cuvet into one or two 10-cm plates) after the electroporation.

3.10. Introducing ES Cells into Mice Preparation of Cells for Aggregation with and Injection into Embryos

When genetically altered ES cell lines are identified, thaw the 96-well plates, or cells from vials, onto feeders, and grow them up. Avoid taking them through too many passages, since with increasing passage number, the possibility of cells loosing their germline-transmitting ability may increase. Our standard protocol for preparing cells for aggregation is given below. Preparation of cells for blastocyst injection is performed in a similar manner, except that for this procedure, the cells need to be seeded at the usual density on d 3, trypsinized slightly longer, spun down and washed in PBS to obtain single-cell suspension on d 5 *(below)*.

Day 1: Thaw cells 4 d prior to aggregation on a feeder cell layer containing plate.
Day 2: Change the medium.
Day 3: Split cells onto gelatinized plates, but instead of the usual 1:5 ratio, pass them 1:50 or even more dilute (*see* **Notes 21** and **22**).
Day 4: Change the medium.
Day 5: Trypsinize the cells briefly, until the colonies lift up as a loosely connected clump of cells. Stop trypsin by adding DMEM+ to the plate. Select clumps directly form the plate for aggregation (*see* **Note 23**).

Protocols and descriptions detailing the introduction of cells into mice by aggregation of ES cells with preimplantation embryos are provided elsewhere ***(40)***. Our own lab protocols can be obtained through the World Wide Web at http://www.mshri.on.ca/develop/nagy/nagy.htm.

3.11. Removal of loxP-Flanked Short Genomic Segment In Situ by Crossing Germline-Transmitter Chimeras with a Stable Cre Transgenic Line

This approach requires the availability of a Cre-expressing transgenic line. In our laboratory, we have established a Cre recombinase-expressing transgenic mouse line (tgCre-1) by pronuclear injection of the hCMV Cre gene. Briefly, the insert purified from pBS 185 plasmid ***(34)*** was directly injected at a concentration of 5 ng/mL into the pronucleus of zygotes fertilized by germline-transmitting males. This line is now routinely used to remove the loxP-flanked genomic piece efficiently from a targeted locus, by crossing this line with the targeted mouse ***(13a)***. This and other lines with similar properties ***(41)*** can be found in the Cre transgenic database mentioned previously (**Subheading 1.1.4.4.**).

1. Males homozygous or heterozygous for the loxP-flanked DNA sequence are crossed with Cre transgenic females.
2. Offspring are screened for the required recombination event by PCR (from ear punch-derived sample DNA) or Southern analysis (from tail biopsy sample-derived DNA).

3.12. Removal of loxP-Flanked Genomic Segment In Situ by Transiently Expressing the Cre Recombinase in F1 Preimplantation Stage

An alternative method for excising loxP-flanked DNA is to express the Cre recombinase in an early embryo transiently ***(42)***.

1. Briefly, germline transmission of the primary loxP-flanked DNA sequence is achieved.
2. Then a Cre-expressing plasmid is injected in circular form into the pronucleus of zygotes produced by a cross between males carrying the loxP-flanked sequence and wild-type females.
3. Injected embryos are transferred into pseudopregnant recipients.
4. Offspring are screened for the required excision event.

4. Notes

4.1. Cell-Culture Media and Solutions

1. Because glutamine and LIF are unstable, DMEM+ that is kept at 4°C for a period exceeding 2 wk needs to be supplemented with a new aliquot of 100X glutamine stock and LIF.
2. The quality of the FCS is critical to the propagation and maintenance of ES cells. We recommend that several batches be tested for plating efficiency and toxicity from different suppliers for their ability to support growth of pluripotent ES cells, and that then a bulk order of the best (to last approx 1 yr) be purchased, and the bottles stored at −20°C for up to 2 yr.
3. LIF helps maintain ES cells in an undifferentiated state especially when they are growing on gelatinized plates in the absence of feeder cell layers. However, it should be noted that too high a concentration of LIF can be deleterious for the cells. We suggest using twice the lowest concentration in which the cells stay undifferentiated. For our cell line, R1 it is 1000 U/mL.

4.2. Preparation of Tissue-Culture Plates

1. Gelatinized Plates: Using R1 ES cells, it is possible to carry out all ES cell manipulations on gelatinized plates from the initial passaging through to the introduction into mice. After replating cells from feeder containing plates onto gelatin plates, their characteristic morphology may be seen to change. This is usually only transient, and in time, they will revert to their usual appearance.
2. After mitomycin C treatment, the feeders are plated onto dishes for ES cell culture, the cells usually take an overnight incubation to attach they are then ready for use.
3. The medium should be changed from feeder cell medium (DMEM+ 10% FCS) to ES cell medium (DMEM+) before the addition of the ES cells.
4. An alternative to mitomycin C treatment is to treat the cells with 6000–10,000 rad of γ-irradiation.

4.3. Electroporation of ES Cells

1. The targeting vector needs to be linearized for electroporation. The DNA is prepared by standard "maxiprep" procedures, for example, cesium chloride gradient centrifugation, or popular kits (such as Qiaex, Promega Wizard, Geneclean). Ethanol-precipitate the digested DNA before the electroporation, and dissolve the pellet in sterile TE. The concentration of the DNA should be around 1 µg/µL.
2. For the transient Cre electroporation, plasmid DNA can be used straight after "maxiprep" purification. In this case, the electroporation procedure is carried out according to the standard protocol provided. The Cre expressing vector can be coelectroporated along with a second selectable marker containing vector, or can be introduced alone ***(35)***. If a selection-based coelectroporating strategy for identifying cells taking up the Cre plasmid is used, then the cells should be plated at normal density (one cuvet into one or two 10-cm plates). Transient expression of the Cre recombinase in ES cell culture results in almost 100% excision between loxP sites placed a few kilobases apart ***(14)***.
3. When selecting for loss or gain of HPRT function, the cells should be maintained in the positive or negative selection prior to the selection switch that will assay the altered HPRT activity. The transiently expressed Cre-mediated excision is often mosaic. Therefore, a PCR screen should be carefully designed to detect such situations. Additionally, Southern blot analyses should be performed as a final check on candidate clones identified by PCR.

4.4. Passaging ES Cells

1. The cell pellet should be carefully resuspended. If this is not carried out, the ES cells will grow as large clumps, containing necrotic centers and differentiated cells at the periphery.
2. ES cells are growing optimally if they are ready for passing on each alternate day, and if their morphology does not change during this time. The typical ES cell morphology is when single cells are not visible, and they grow as characteristically shaped small clumps.
3. The number of cells to be plated at each passage and length of growth between two passages is critical. Cells should not be left to overgrow, and care should be taken not to split cells too diluted or too dense.

4.5. ES Cell Freezing

1. We usually freeze ES cells in cryovials at a density of 5–10 × 10^6 cells/mL of freezing medium. A single 10-cm dish usually gives approx three cryovials each containing 1 mL of cells.
2. To freeze cells from different-sized dishes, proportionally altered volumes of cell medium are used (for example, a 6-cm dish requires 1 mL, and a 3.5-cm dish requires 0.5 mL).
3. It is important to note that the cells should be frozen down gradually (in a styrofoam box or isopropanol container), and not to be kept at −70°C for too long a period of time. Cryovials should be transferred to liquid Nitrogen for long-term storage.

4. Unfortunately, 96-well plates can only be kept at −70°C. We do not recommend keeping the plates for more than 2 mo at this temperature. Therefore, all screening for the required alleles needs to be performed within this time period.

4.6. Thawing ES Cells

1. It is important to thaw cells as rapidly as possible in order to avoid long crystal formation as the frozen vial passing through critical temperatures. Immediately after removal from frozen storage, the cell-containing vials (cryovials or 96-well plates) are placed at 37°C in a water bath until they are almost completely defrosted (1–3 min).

4.7. Removal of loxP-Flanked Short Genomic Segment in ES Cells by Transient Expression of Cre Recombinase

1. No selectable marker is needed for this Cre-mediated excision, since 2–5% of cells are picking up the Cre expressing DNA. Practically in almost all these cells excision occurs between loxP sites placed a few kilobases apart. As a consequence, transient expression of the Cre recombinase in ES cells results in a 1 in 30 average frequency of excision.
2. If the region of DNA to be deleted contains a drug selection marker, then the newly acquired sensitivity to the marker can be tested when replica 96-well plates containing cells are available. Here, however, one should be aware of the frequent mosaic type of excision. Therefore this step does not replace the PCR or Southern blot screening.

4.8. Introducing ES Cells into Mice

1. In general cells should be replated on the appropriate size plate (96- or 24-well plate when passaging from 96-well plate, 6-cm dish when passaging from a cryovial), and grown up so that there are enough for freezing into cryovials and introducing into mice.
2. A separate plate should be prepared for aggregation as was described in the protocol. The reason for highly diluted single-cell plating is to produce clumps of 10–25 loosely connected cells prior to aggregation. Then the required-size colonies are predominantly found on the plate. Care should also be taken in order not to disaggregate the cell clumps (by pipeting the cells too vigorously or overtrypsinization).
3. For blastocyst injection purposes, cells do not need to be maintained as clumps. Therefore they should be completely dissaggregated.

4.9. Removal of loxP-Flanked Short Genomic Segment In Situ by Crossing Germline-Transmitter Chimeras with a Stable Cre Transgenic Line

In our experience, not all offspring that inherit the Cre transgene will undergo excision as shown in **Fig. 4,** indicating that although the efficacy of such a transgene is very high (complete excision in 90% of animals), it is not active in all embryos that inherit it.

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9

Gene Trapping in Mouse Embryonic Stem Cells

Jane Brennan and William C. Skarnes

1. Introduction

Gene trapping in mouse embryonic stem (ES) cells offers a method to create random developmental mutants with a direct route to cloning and defining the expression pattern of the disrupted gene ***(1)***. Gene trapping involves the use of reporter gene constructs that are activated following insertion into endogenous transcription units. A number of plasmid- and retroviral-based vectors have been developed, which differ in their requirements for reporter gene activation (reviewed in **refs.** ***2*** and ***3***). "Promoter trap" vectors simply consist of a promoterless reporter gene that is activated following insertions in exons of genes. In contrast, "gene trap" vectors contain a splice acceptor sequence upstream of a reporter and are activated following insertions into introns of genes. Both promoter and gene trap insertions create a fusion transcript from which a portion of the endogenous gene may be readily cloned ***(4,5)***. The pattern of reporter gene activity can be monitored in ES-cell derived chimeric embryos ***(6)*** or in transgenic embryos following germline transmission ***(7)***. With two plasmid-based vectors, reporter gene expression has been shown to reflect accurately that of the endogenous gene ***(5,8)***. Ultimately, the function of the trapped gene can be tested following germline transmission. Using this approach, a number of embryonic lethal mutations and visible adult phenotypes have been isolated ***(4,5,7,9–11)***.

Before initiating a screen, it is important to consider carefully the design of individual vectors, their efficiency in detecting gene trap events, and their potential biases (discussed in **refs.** ***2*** and ***3***). For example, although gene trap vectors are more efficient at detecting insertions in genes than promoter trap vectors,

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they will favor the detection of genes composed of large intronic regions, and are likely to miss genes possessing few or no introns. Gene trap vectors are also constrained by the reading frame imposed by the splice acceptor sequence. One solution has been to incorporate the splice acceptor derived from Moloney murine leukemia virus (MoMuLv) *env* gene that is capable of splicing in all three reading frames simultaneously ***(12)***. However, splice acceptors with this property may be weak, so they may fail to mutate the gene at the site of insertion effectively. Alternatively, an internal ribosome entry site (IRES) may be used to initiate translation of the reporter gene independent of the upstream open reading frame ***(13)***. Our own answer to this problem has been to construct separate vectors in each of the three reading frames. Given that individual vector designs each have their own inherent biases, we recommend using a combination of vectors to ensure the most representative sampling of the genome.

Perhaps the most useful vectors are those based on the βgeo reporter system ***(7)***. βgeo encodes a polypeptide fusion possessing both β-galactosidase and neomycin phosphotransferase (*neo*) activities, thereby providing direct drug selection for gene trap events and obviating the need to screen through a large background of nongene trap events. However, there is some debate over whether *neo* driven by its own promoter may be better suited to trap genes activated at later stages of development. In this regard, the sensitivity of the drug selection marker becomes an important consideration. It has been shown, for example, that the original βgeo reporter contained a mutation in *neo* and thus tended to preselect for genes expressed at high levels ***(8)***. Correction of this mutation has enabled the isolation of genes expressed at low levels in ES cells that are activated on differentiation.

The isolation of so-called white colonies (cells expressing less than detectable levels of β-galactosidase [βgal] activity) does not necessarily indicate that the trapped gene is expressed at a low level, but rather may reflect inactivation of the βgal enzyme in the resulting fusion. One important class of genes that produce inactive βgal fusion products are those that encode N-terminal signal sequences ***(8)***. To capture specifically this class of genes, which include secreted and membrane-spanning proteins, we modified the conventional gene trap design by adding a type II transmembrane domain upstream of βgeo. With the secretory trap vector pGT1.8TM, only fusions that acquire an N-terminal signal sequence will produce an active βgal fusion. One surprising result to emerge from this study was the isolation of two independent insertions in the same gene in a sample of six cell lines, suggesting that gene trapping may be far less random than originally anticipated. This finding further emphasizes the need to recognize and ultimately overcome inherent biases imposed by individual vector designs.

This chapter will focus on the use of βgeo-based plasmid vectors in mouse ES cells to screen for developmentally regulated genes in the mouse. Methods for the maintenance and electroporation of a feeder-independent ES cell line are described. To identify βgal-positive clones, we employ a screening protocol that simply involves staining one set of duplicate wells with X-gal. This procedure can also be used to screen for genes induced or repressed in response to specific growth factors or other inducers of ES cell differentiation. Several methods have been used to clone a portion of the endogenous gene associated with gene trap and promoter trap insertions. These include the construction of cDNA libraries ***(10)***, inverse PCR ***(4)***, ligation-mediated PCR ***(11)***, and 5′ rapid amplification of cDNA ends (RACE) ***(5)***. This chapter, provides a detailed method for 5′ RACE cloning, used routinely in the authors' laboratory, in which a number of improvements on previously published protocols have been added ***(3,14)***. The generation of ES cell-derived chimeras by blastocyst injection or morulae aggregation is used to monitor expression patterns in embryos and to transmit the insertions to the germline of mice. These methods are not covered in this chapter, since they have been described extensively elsewhere ***(15–18)***. Finally, we have included a rapid dot-blot method for genotyping transgenic mice carrying gene trap insertions. This method can be used to analyze as many as 400 tail biopsies in a single day, and can reliably distinguish between heterozygous and homozygous animals based on signal intensity.

2. Materials

2.1. Maintenance of Mouse ES Cells

1. Cell culture medium (1X): store up to 1 mo at 4°C:
   a. 10X Glasgow MEM/BHK12 (Gibco, Paisley, UK): store at 4°C — 40 mL
   b. 7.5% sodium bicarbonate (Gibco): store at 4°C — 13.2 mL
   c. 1X MEM nonessential amino acids (Gibco): store at 4°C — 4 mL
   d. 200 m*M* glutamine, 100 m*M* sodium pyruvate (Gibco): store at −20°C — 8 mL
   e. 0.1 *M* 2-mercaptoethanol (Sigma, Dorset, UK): store at 4°C for 1 mo — 0.4 mL
   f. Fetal calf serum (Globepharm, Surrey, UK), batch-tested (*see* **Note 1**), store at −20°C — 40 mL
   g. Sterile, deionized water (dH_2O) — 340 mL
   h. Differentiation inhibiting activity (DIA) (*see* **Note 1**)
2. Phosphate-buffered saline (PBS), filter-sterilized.
3. Trypsin solution: Dissolve 250 mg of trypsin (Difco, Surrey, UK) and 372 mg EDTA disodium salt (Sigma) in 1 L of PBS. Add 10 mL of chicken serum (Flow Labs, Herts, UK and filter sterilize. Store in 20-mL aliquots at −20°C.
4. 1% (w/v) Gelatin: add 1 g gelatin (Type A from bovine skin, Sigma) to 100 mL dH_2O, autoclave, and store in 20-mL aliquots at 40°C. For a working solution of 0.1%, add 10 mL of 1% gelatin to 90 mL of PBS.

5. 0.1 *M* 2-mercaptoethanol: Add 100 mL 2-mercaptoethanol (Sigma) to 14.1 mL PBS. Store up to 1 mo at 4°C.
6. Geneticin (G 418; Boehringer-Mannheim, Sussex, UK): 200 mg/mL dissolved in dH_2O.
7. Freezing solution: 1 mL of DMSO to 9 mL of cell-culture medium (made fresh).

2.2. Vector Preparation

1. Restriction endonuclease (*Hin*dIII for pGT1.8βgeo and pGT1.8TM) to linearize the gene trapping vector and a 10X concentration of the appropriate digestion buffer.
2. Absolute ethanol.
3. 70% Ethanol.
4. Sterile PBS.

2.3. X-Gal Staining Colonies

1. Phosphate buffer (0.1 *M*) made by adding 21 parts of 0.1 *M* Na_2HPO_4 (Sigma) to 4 parts 0.1 *M* NaH_2PO_4 (Sigma) to give a final pH of 7.5.
2. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Boerhinger-Mannheim) stock solution, 50 mg/mL, dissolved in dimethyl formamide (Sigma). Store in the dark at −20°C.
3. Fix buffer: 0.1 *M* Phosphate buffer, pH 7.3, 2 m*M* $MgCl_2$, 0.2% Gluteraldehyde (Sigma), 0.5 m*M* EGTA (pH 8.0), Store at 4°C for up to 1 mo.
4. Wash buffer: 0.1 *M* phosphate buffer, 2 m*M* $MgCl_2$, 0.1% sodium deoxycholate (Sigma), 0.02% Nonidet P-40 (Sigma). Store at 4°C.
5. X-gal staining solution:
   0.106 g Potassium ferrocyanide (Sigma).
   0.082 g Potassium ferricyanide (Sigma).
   1 mL X-gal (50 mg/mL).
   Filter to remove crystals, and store in the dark at 4°C. Add to 50 mL of X-gal wash buffer.

2.4. RNA Preparation from ES Cells

1. Guanidinium lysis buffer: 4 *M* guanidinium thiocyanate dissolved in 0.1 *M* Tris-HCl (pH 7.5), filtered through Whatman 3MM paper. Just prior to use, add 2-mercaptoethanol to a final concentration of 1% and sodium lauryl sarcosinate to 0.5% (w/v).
2. 5.7 *M* cesium chloride (CsCl) dissolved in 10 m*M* EDTA, pH 7.5, filtered, DEPC-treated, and autoclaved.
3. 8 *M* urea dissolved in 10 m*M* HEPES, pH 7.0.
4. DEPC H_2O prepared in a fume hood by adding 1 mL of diethyl pyrocarbonate (Sigma) to 1 L of dH_2O, shake thoroughly and leave overnight before autoclaving.
5. PBS, sterile.
6. 70% Ethanol.
7. 0.4 *M* NaCl, DEPC-treated.
8. 1:1 Phenol/chloroform.

2.5. RNA Dot Blot

1. Formamide (Gibco-BRL).
2. Formaldehyde, 37% solution (BDH, Leicester, UK).
3. 1 *M* Sodium phosphate buffer: Dissolve 70.5 g of anhydrous Na_2HPO_4 in dH_2O, add 4 mL of phosphoric acid and make up to 1 L with H_2O. Filter sterilize.
4. Hybond N membrane (Amersham, Buckhinghamshire, UK).
5. Prehybridization/hybridization buffer (40 mL, made fresh): weigh out 0.4 g of bovine serum albumin (Sigma), and dissolve in 14 mL of 1 *M* phosphate buffer. Add 12 mL of formamide and 14 mL 20% SDS.

2.6. Rapid Amplification of cDNA Ends (RACE)

1. Primers used for 5 RACE cloning are shown in **Table 1**.
2. DEPC-treated dH_2O (*see* **Subheading 2.4., item 4**).
3. Superscript II reverse transcriptase (200 U/mL), 0.1 *M* dithiothreitol (DTT) and 5X buffer: 250 *M* Tris-HCl (pH 8.3), 0.375 *M* KCl, 15 mM $MgCl_2$ (Gibco-BRL 18064-014).
4. 1 *M* Sodium hydroxide.
5. 1 *M* Hydrochloric acid.
6. Microdialysis filters: 0.025 and 0.1 µm pore size (Millipore, Watford, UK VSWP02500 and VCWP02500).
7. TE buffer: 10 m*M* Tris-HCl, pH 8.0 (20°C), 1 m*M* EDTA, pH 8.0.
8. Terminal deoxynucleotidyl transferase (TdT): 15 U/µL and 5X reaction buffer: 0.5 *M* potassium cacodylate (pH 7.2), 10 m*M* cobalt chloride, 1 m*M* DTT (Gibco-BRL 18008-011).
9. dATP for tailing reaction: 2 m*M* dATP.
10. Restriction buffer M: 10 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 1 m*M* DTT (Boehringer-Mannheim 1417 983).
11. dNTP mix: dNTPs each at a final concentration of 10 m*M* (Pharmacia, St. Albans, UK 27203501).
12. Klenow enzyme, 2 U/µL (Boehringer-Mannheim).
13. Amplitaq (5 U/µL) and 10X buffer: 100 m*M* Tris-HCl (pH 8.3), 500 m*M* KCl (Applied Biosystems N8080161).
14. 25 m*M* Magnesium chloride.
15. Restriction endonucleases *XbaI* and *KpnI* for cloning PCR products.
16. Glycogen, 5 mg/mL (Boehringer-Mannheim).
17. 10 *M* Ammonium acetate.
18. Absolute ethanol.
19. 70% Ethanol.
20. pBluescript SKII$^+$ plasmid DNA (Stratagene, Cambridge, UK).
21. T4 DNA ligase (1 U/µL) and 10X ligation buffer (Boehringer-Mannheim).
22. Electrocompetent bacteria (e.g., DH5α).
23. LB agar: 1% Bacto tryptone (w/v) (Difco, Detroit, MI), 0.5% Bacto-yeast extract (w/v), 1 % (w/v) sodium chloride (w/v), and 50 mg/mL ampicillin.

2.7. Tail DNA Dot Blot

1. Tail buffer (made up fresh): to 10 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 50 m*M* EDTA, 0.5% SDS. Add proteinase K (Sigma) to a final concentration of 0.5 mg/mL.
2. 5 *M* NaCl.
3. Chloroform.
4. 0.53 *M* NaOH.
5. Hybond N^+ membrane.
6. Prehybridization/hybridization buffer (40 mL, made fresh): Weigh out 0.2 g of Marvel milk powder, and dissolve in 6 mL water. Add 20 mL of phosphate buffer and 14 mL 20% SDS.

3. Methods

3.1. Maintenance of ES Cells

Careful maintenance of ES cells is crucial for successful germline transmission of gene trap cell lines. We generally change the medium every day, and do not allow the cells to grow to confluence. Several previously published protocols describe procedures for maintaining ES cells on fibroblast feeder layers ***(15–18)***. The conditions outlined below apply to the maintenance of a feeder-independent line of ES cells, CGR8, which rely on an exogenous source of DIA (also known as leukocyte inhibitory factor [LIF]). These cells are karyo-typically male and were derived from the 129/Ola strain of mice as described ***(19)***. Feeder-independent cell lines are more convenient to work with and contribute as efficiently to the germline of mice as feeder-dependent cell lines. We find that 80% of our gene trap cell lines contribute to the germline at an average rate of 1 germline male/10 C57B1/6 blastocysts injected (Skarnes, unpublished results).

All ES cell manipulations should be carried out in a laminar flow hood. All solutions should be warmed in a 37°C waterbath prior to use.

3.1.1. Thawing ES Cells

1. Coat a 25-cm^2 tissue-culture flask with 0.1% gelatin and aspirate off.
2. Quickly thaw ES cells (one-half of a confluent 25-cm^2 flask or approx 5×10^6 cells) in a 37°C water bath, and transfer them to a disposable centrifuge tube containing 10 mL of prewarmed medium.
3. Spin the cells down in a bench-top centrifuge at 260 *g* for 3 min.
4. Aspirate medium off and gently resuspend cells in 10 mL of pre-warmed medium.
5. Transfer to 25-cm^2 tissue-culture flask and grow in a humidified 37°C/6% CO_2 incubator.
6. Change medium after about 8 h of growth to remove dead cells and any remaining DMSO (an inducer of ES cell differentiation).
7. Medium should be changed every day.

3.1.2. Passage and Expansion of ES Cells

1. ES cells are passed once the have nearly reached confluence. For a 25-cm^2 flask, aspirate medium off, and add 5–10 mL of PBS down the opposite side of the flask to where the cells are growing. Rock the flask gently and aspirate off. Repeat.
2. Cover cells with 1 mL of trypsin, and incubate at 37°C for 1–2 min. Incubate for longer if the cells are not in a uniform suspension.
3. Add 9 mL of medium to stop trypsinization.
4. Count cells, and add 10^6 (approx 1/10 of a 25-cm^2 flask) to a freshly gelatinized flask.
5. If expanding ES cells for an electroporation, where a total of 10^8 cells are needed, plate 3×10^6 in a 80-cm^2 flask containing 30 mL of medium. Feed cells the following day with an additional 20 mL of medium. Once the cells reach confluence, trypsinize and plate 5×10^6 into three 175-cm^2 flasks containing 50 mL of medium each. On the next day, add an additional 40 mL of medium. Each confluent flask should yield about 8×10^7 cells.

3.1.3. Freezing ES Cells

1. Trypsinize 25-cm^2 flask as in **Subheading 3.1.2., steps 2** and **3**.
2. Collect trypsinized cells in 9 mL of medium and spin down at 260 *g* for 3 min.
3. Resuspend cell pellet in 1 mL of freezing solution, and dispense 0.5 mL of suspension into two 1-mL cryotubes.
4. Freeze cells at −80°C overnight and transfer to liquid nitrogen for long-term storage.

3.2. Electorporation of Gene Trap Vector into ES Cells and Selection of Colonies

3.2.1. Vector Preparation

1. In a volume of 0.3 mL, linearize 150 µg of vector DNA with the appropriate restriction enzyme. (Plasmid DNA for electroporation is prepared by alkaline hydrolysis and banded on a cesium chloride gradient as described in **ref. *20***).
2. Precipitate the digested DNA sample in 2 vol of absolute ethanol on ice for 5 min. Spin in microfuge, wash pellet several times with 70% ethanol, and drain off as much of the ethanol as possible.
3. Evaporate off the remaining ethanol in the hood by keeping the lid of the tube open (approx 1 h). Resuspend DNA pellet in 100 µL of sterile PBS, and vortex sample occasionally over a period of at least 4 h to ensure that the DNA is completely dissolved.

3.2.2. Electroporation of ES Cells and Picking G418-Resistant Colonies

1. Trypsinize three 175-cm^2 flasks using 5 mL trypsin/flask as described above. Add 8 mL of medium to each flask, and combine cells in a 50-mL disposable centrifuge tube. Spin for 5 min at 260 *g*, and resuspend in 20 mL of PBS.
2. Count cells, and resuspend at a concentration of 10^8 cells in 0.7 mL of PBS.

3. Add cells to the tube containing the 150 µg of linearized plasmid, and transfer immediately to a 0.4-cm electroporation cuvet. Electroporate in Bio-Rad Gene Pulsar unit set at 3 mF/800 V (time constant = 0.1 ms).
4. Leave cells to recover in the cuvet for 20 min, and then transfer them to 200 mL of medium. Plate 10 mL (5×10^6 cells) of the cell suspension onto 20 10-cm diameter gelatinized tissue-culture dishes.
5. After 24 h, aspirate medium, and replace with medium containing 200 µg/mL of G418. For the first 5 d, change medium daily. Once G418-resistant colonies appear, the medium may be changed every other day (*see* **Note 2**).
6. After about 10–12 d of growth, colonies should be about 1 mm in diameter. Circle colonies with a marker pen on the bottom of the dish.
7. Gelatinize the required number of 24-well plates.
8. Aspirate medium, and add 10 mL of PBS to each dish. Ideally, colonies should be picked in a tissue-culture hood. However, it is possible to pick colonies on the bench, resulting in minimal or no contamination as long as procedures are performed swiftly. Using a P200 Pipetman set at 100 µL and sterile tips, break up the colony, pipet the cells up in a volume of 100 µL PBS, and transfer to a 24-well dish. Once 24 colonies have been picked, add 100 µL of trypsin to each well, and incubate at 37°C for 10 min. Tap the dish several times to disperse the cells, and then add 2 mL of medium to each well.
9. To identify βgal-positive colonies, we normally split four-fifths of a nearly confluent well into a new 24-well plate (Experimental) for staining with X-gal, and split the remaining one-fifth into a second 24-well plate (Master) for maintenance and subsequent expansion of selected cell lines (*see* **Note 3**). Since the colonies grow at different rates, the cells are split in batches over a period of several days. Cells in the Experimental plates are stained with X-gal the following day, and βgal activity is scored after an overnight incubation. By this time, the cells on the Master plate should be ready to expand for further analysis.

3.2.4. Staining Cells for β-Galactosidase Activity

1. Aspirate medium and wash cells once with PBS.
2. Fix cells for 10 min at room temp.
3. Wash twice for 5 min.
4. Stain with X-gal overnight in a humidified chamber at 37°C. Colonies expressing low levels of β-gal may appear slightly discolored compared to β-gal negative cell lines. βgal staining is difficult to see using phase contrast. We recommend using either bright field illumination or Nomarski optics.

3.3. RACE Cloning

*3.3.1. RNA Preparation from ES Cells (*see ***Note 4****)*

1. Wash cells grown to confluence in a 25-cm^2 flask twice with PBS.
2. Add 1.4 mL of guanidinium lysis buffer/10^7 cells (approx a confluent 25-cm^2 flask) swirl flask for about 30 s and collect lysate.

3. Rinse the required number of SW50.1 centrifuge tubes with DEPC-treated water and dry. Add 3.6 mL of CsCl solution to each tube. Draw lysate through a 23-gage needle several times to shear genomic DNA, and carefully layer on top of the CsCl cushion.
4. Spin at 15,000 *g* in a SW50.1 rotor for 16 h at 20°C.
5. Remove the top 4 mL of the gradient, and wash the sides of the tube twice with guanidinium lysis buffer. Invert tube, and drain off remainder of gradient. Cut the bottom off the tube with a scalpel, and rinse RNA pellet twice with 70% ethanol. RNA pellet should become visible in 70% ethanol.
6. Resuspend pellet in 0.3 mL 8 *M* urea buffer, transfer to new tube, and vortex occasionally over a period of 1 h.
7. Add 0.1 mL 0.4 *M* NaCl. Extract sample twice with an equal volume of phenol/chloroform followed by one chloroform extraction.
8. Precipitate RNA with 2 vol of absolute ethanol and store RNA as a precipitate at –20°C.
9. To determine the concentration of RNA in the sample, vortex to produce a uniform suspension, and spin down 0.1 mL in a microfuge. Wash RNA pellet in 70% ethanol, and resuspend in 0.1 mL water. The $OD_{260\,nm}$ of this sample gives a reasonably accurate measure of the concentration of RNA in the precipitated sample.

3.3.2. RNA Dot Blot to Eliminate Intron-Containing Lines

We have observed that a significant proportion of gene trap insertions fail to utilize properly the splice acceptor of the vector and produce fusion transcripts that hybridize to intron sequences of the vector. Inefficient splicing is predicted to occur if the vector inserts either into exons of genes or in non-pol II transcription units. We have characterized two such insertions and found that both occurred within rRNA genes transcribed by RNA pol I (Sleeman and Skarnes, unpublished results). To eliminate these nongene trap events prior to RACE cloning, we use an RNA dot-blot method to detect cell lines producing intron-containing transcripts.

1. Spin down and resuspend 10 µg of RNA in 20 mL of DEPC-treated water. Add 40 mL of formamide, 14 mL of formaldehyde, and 88 mL of NaP buffer.
2. Heat samples to 55°C for 15 min, and apply one-half of each sample to duplicate sets of wells in the dot-blot apparatus. Leave for 30 min before applying a vacuum.
3. To assemble the dot-blot apparatus, and cut a piece of Hybond N membrane and a piece of Whatman paper to fit the dot-blot apparatus. Prewet the membrane in water, and then soak it in 0.5 *M* NaP buffer for 10 min. Prewet the Whatman in 0.5 *M* NaP buffer, and place it underneath the membrane.
4. Draw samples through with a gentle vacuum, and wash wells once with 0.5 *M* NaP buffer. Disassemble apparatus, remove membrane with paper attached, and crosslink RNA to membrane using Stratalinker (Stratagene) set on autocrosslink. Probe duplicate sets of membrane with intron and reporter gene probes. Results from a typical dot-blot experiment are shown in **Fig. 1.**

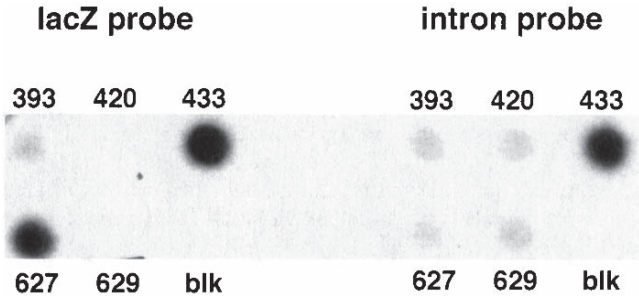

Fig. 1. RNA dot blots used to eliminate cell lines that produce intron-containing fusion transcripts. Duplicate RNA dot blots containing 10 µg total RNA were hybridized with lacZ and intron probes of the gene trap vector. 433 is a typical example of an intron-containing cell line.

3.3.3. RACE Cloning

The following protocol has been streamlined and contains a number of modifications to the original method described by Frohman, et al. (**ref. *14***). The most important of these are:

1. Alkaline hydrolysis of the RNA (required for efficient T-tailing with terminal deoxytransferase);
2. Synthesis of second-strand cDNA with Klenow instead of *Taq* polymerase; and
3. The use of microdialysis filters, which serves a dual role of removing primers/buffers between steps and size-selecting informative cDNA fragments above 300 bp in length.

By way of example, **Table 1** lists the oligos used for RACE cloning from cell lines obtained with the pGT1.8βgeo and pGT1.8TM vectors.

1. Spin down 5–10 µg of total RNA in an Eppendorf tube, wash RNA pellet twice with 70% ethanol, and partially dry pellet in Speed Vac. Repeat this step, since it is important to remove any residual urea. Resuspend RNA sample on ice in 10 µL of DEPC-treated water. Add 1 µL of 10 ng/µL primer 1, and heat for 5 min at 70°C. Cool on ice and spin briefly.
2. Set up first-strand reaction by adding:
   a. 4 µL of first-strand buffer (5X).
   b. 2 µL of 0.1 *M* DTT.
   c. 1 µL of Superscript II RT.
   d. Incubate first-strand reaction at 37°C for 1 h.
3. To improve the efficiency of the tailing reaction, hydrolyze RNA by adding 2.2 µL of **1** *M* sodium hydroxide for 20 min at 65°C. Neutralize with 2.2 µL of 1 *M* hydrochloric acid.

Table 1
Primer Combinations Used in 5' RACE[a]

Primer	Gene trap vector, pGT1.8geo	Secretory trap vector, pGT1.8tm
1	5'TAATGGGATAGGTTACG	5'CCAGAACCAGCAAACTGAAGGG
2	5'GGTTGTGAGCTCTTCTAGATG $G(T)_{17}$	5'GGTTGTGAGCTCTTCTAGATG $G(T)_{17}$
3	5'GGTTGTGAGCTCTTCTAGATGG	5'GGTTGTGAGCTCTTCTAGATGG
4	5'ATTCAGGCTGCGCAACTGTTGG	5'AGTAGACTTCTGCACAGACACC
5	5'TGCTCTGTCAGGTACCTGTTGG	5'TGCTCTGTCAGGTACCTGTTGG

[a]*Xba*I and *Kpn*I sites used in cloning the RACE products are underlined.

4. Microdialyze sample on a 0.025-µm filter floating in a Petri dish of TE for 4 h. Transfer the remainder of the sample to an Eppendorf tube, and wash the filter with water to bring the final volume to 20 µL.
5. Set up tailing reaction by adding 6 µL of TdT buffer (5X) and 2 µL of 2 m*M* dATP. Incubate for 2 min at 37°C. Add 2 µL of TdT enzyme, and incubate for a further 5 min. Stop the reaction by heating to 70°C for 2 min.
6. Carry out second-strand synthesis by adding the following to 15 µL of tailed cDNA:
   a. 2 µL restriction buffer M (10X).
   b. 1 µL dNTPs (10 m*M*).
   c. 1 µL primer 2 (10 ng/mL).
   d. 1 µL Klenow enzyme.

   Incubate for 30 min at room temperature, 30 min at 37°C, and 5 min at 70°C.
7. Microdialyze for 4 h on a 0.1-µm filter. Recover cDNA from filter into a final volume of 37 µL of H_2O.
8. For the first-round PCR reaction, add the following to 37 µL of sample:
   a. 5 µL 10X AmpliTaq buffer
   b. 4 µL 25 m*M* $MgCl_2$
   c. 1 µL dNTP mix
   d. 1 µL primer 3 (100 ng)
   e. 1 µL primer 4 (100 ng)
   f. 1 µL AmpliTaq

   Carry out 30 cycles using the following parameters:
   a. Denature 94°C for 1.5 min
   b. Anneal 60°C for 1.5 min
   c. Extend 72°C for 3.0 min
9. Microdialyze for 4 h on a 0.1-µm filter to remove smaller, uninformative PCR products and excess primers. Recover sample from filter.
10. Perform second-round PCR using 5 µL of the first-round PCR reaction and:
    a. 5 µL PCR buffer
    b. 4 µL 25 m*M* $MgCl_2$

c. 1 µL dNTP mix
d. 1 µL primer 3 (100 ng/mL)
e. 1 µL primer 5 (100 ng/mL)
f. 1 µL AmpliTaq
g. 37 µL H_2O

Use same cycle parameters as first-round PCR, but use a hot start. After the final cycle, polish the second-round PCR products by adding 50 ng of each primer, 0.5 µL of dNTPs, and 0.5 µL of Amplitaq. Perform one cycle using the following conditions:

a. Denature 94°C for 1.5 min
b. Anneal 60°C for 1.5 min
c. Extend 72°C for 20.0 min

11. Microdialyze for 4 h on a 0.1-µm filter, and recover sample from filter. Analyze 5 µL of sample by gel electrophoresis and Southern blot hybridization (*see* **Note 5**). Digest the remainder of the sample with *Xba*I and *Kpn*I.
12. Following digestion, extract the sample twice with phenol/chloroform, extract once with chloroform, and precipitate the RACE products on ice for 10 min by adding 5 µg of glycogen, 25 µL of 10 *M* ammonium acetate, and 300 µL of ethanol. Microfuge for 10 min, wash pellet with 70% ethanol, partially dry pellet in Speed Vac and resuspend in 20 µL of TE.
13. Ligate 100–200 ng of *Xba*I/*Kpn*I-digested RACE products with 50–100 ng *Xba*I/*Kpn*I-digested plasmid DNA in a final volume of 15 mL at room temperature for at least 2 h:
    a. 4–8 µL Digested PCR products (100–200 ng)
    b. 1 µL Digested plasmid DNA (100 ng)
    c. 1.5 µL Ligation buffer (10X)
    d. 1 µL T4 DNA ligase
    e. 3.5–7.5 µL H_2O
14. Transform into competent bacteria, and screen colonies for desired inserts (*see* **Note 6**).

3.4. Genotyping Animals

The following is a one-tube method for preparing genomic DNA from tail biopsies. To obtain uniform signals, it is important to apply enough DNA to saturate the membrane. This was determined empirically using a Bio-Rad 96-well dot-blot apparatus and Amersham Hybond N^+ membrane. **Figure 2** shows the results of a typical dot blot from intercrosses of a line of mice carrying a single-copy insertion. Homozygous mice, which in this case carry two copies of the vector, are easily distinguishable from their heterozygous litter mates.

1. Tail biopsies (1.5 cm in length) are taken at weaning age and digested overnight at 55–65°C in 0.4 mL of tail buffer.
2. While tubes are still warm, add 0.1 mL 5 *M* NaCl and vortex at high speed for 5–10 s. Add 0.5 mL of chloroform, and vortex again for 5–10 s. Spin in microfuge for 5 min.

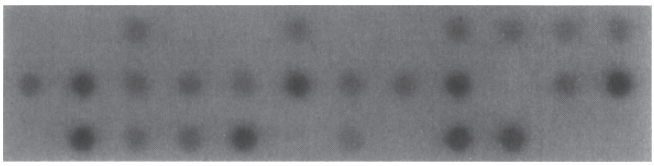

Fig. 2. Genotyping mice by dot-blot analysis. Tail DNA from intercross litters carrying a single-copy gene trap insertion were hybridized to a lacZ probe. Dots showing weak signals represent heterozygous animals, whereas those showing signals of twice the intensity represent homozygotes.

3. Transfer 50 µL of the top aqueous phase to a 96-well plate. Denature DNA by adding 150 µL of 0.53 *M* NaOH and incubating at 37°C for 30 min.
4. Apply samples to dot-blot apparatus, and leave for 30 min before applying a vacuum.
5. Prepare the dot-blot apparatus by cutting a piece of Hybond N^+ membrane and a piece of Whatman paper to fit the apparatus. Prewet the membrane in H_2O, and then soak in 0.4 *M* NaOH for 10 min. Prewet the Whatman in 0.4 *M* NaOH, and place it underneath the membrane on the apparatus.
6. Draw samples through with gentle vacuum. Disassemble apparatus and wash membrane with 30 m*M* NaP/0.1% SDS buffer. Hybridize membrane with reporter gene probe.

4. Notes

1. DIA is produced by transient expression in COS cells using the method described in **ref. *21***. Serial dilutions of the medium are tested on ES cells plated in 24-well plates. A 100-fold higher dilution than the minimal dilution required to keep ES cells undifferentiated is typically used. Serum batches are tested for their ability to sustain the growth, differentiation, and viability of ES cells grown at clonal density in the presence and absence of DIA.
2. Electroporation of 10^8 ES cells normally generates between 200 and 400 $G418^R$ colonies. Using the gene trap vector pGT1.8geo ***(8)***, we find 50% of colonies stain for βgal activity. A proportion of the white colonies represents genes expressed at low levels in ES cells, which can be induced on differentiation. The remainder probably represents inactive βgal fusion proteins, such as secretory molecules, and events where sequences from the 5 -end of βgal have been removed by endogenous exonucleases prior to insertion into the genome. Using the secretory trap vector pGT1.8TM, we find approx 25% of colonies stain positive for βgal. Almost all show the secretory pattern of staining in the peri-nuclear compartment of ES cells and in multiple cytoplasmic inclusions, and represent fusions to genes encoding a 5 -signal sequence.
3. To screen for genes induced or repressed under various growth conditions, more than one Experimental plate can be seeded. If the assays are to be conducted over a

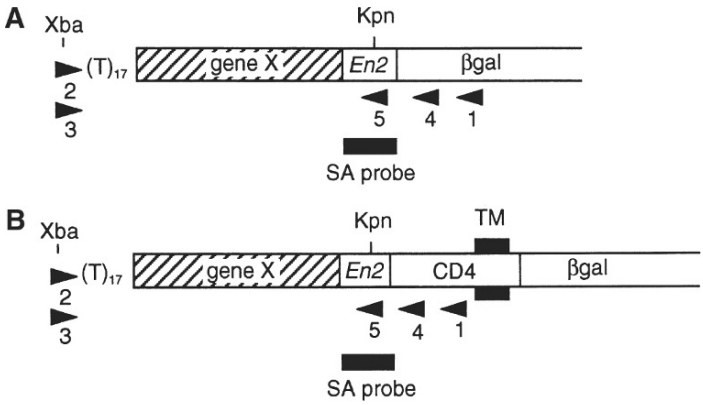

Fig. 3. Arrangement of oligos used to RACE clone genes disrupted by gene trap vector pGT1.8geo **(A)** and the secretory trap vector pGT1.8TM **(B)**. Both vectors contain the mouse Engrailed-2 (En2) splice acceptor (SA) linked to a βgeo reporter containing βgal and neomycin phosphotransferase activities. The secretory trap vector contains a fragment of CD4 that encodes the transmembrane domain (TM). Oligo 1, primer for first-strand cDNA synthesis; oligo 2, primer for second-strand cDNA synthesis; oligos 3 and 4, first-round PCR primers and oligos 3 and 5, second-round PCR primers (*see* **Table 1**).

period of days, the Master plate can be frozen at −80°C ***(22)***, allowing the recovery of selected colonies at a later date.

4. Other methods of RNA preparation have recently been shown to work well for RACE cloning. These include acid phenol (RNazol B, Biogenesis Ltd.) and poly dT-based bead selection (Dynabeads, Dynal Ltd.) protocols.
5. Before digesting second-round products, it is advisable to check the fidelity of the preceding reactions. This is done by running 5 µL of second-round PCR products on a 1.5% agarose gel and carrying out Southern blot analysis using a vector probe that includes sequences contained in the RACE products (*see* **Fig. 3**). If the reactions have worked, the autoradiograph will require less than a 15-min exposure. In most cases, we see a heterogeneous smear of amplified products. However, if the fusion transcripts are small, a band may be seen.
6. Colonies are selected by digesting plasmid minipreps with the enzymes used for cloning the RACE products and carrying out Southern analysis using the SA probe (*see* **Fig. 3**). Colonies can also be screened using a PCR strategy employing primer 5 and a primer in the pBluescript polylinker. Only clones containing gene trap vector sequences should be amplified. We normally recover products that contain between 100 and 700-bp of sequence from the disrupted gene. To confirm that the RACE clone truly represents the disrupted gene, Northern blot analysis should be performed using the RACE clone as a probe. Alternatively, if the RACE clones are small, RNase protection experiments can be used.

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Application of *lacZ* Transgenic Mice to Cell Lineage Studies

Catherine M. Watson, Paul A. Trainor, Tania Radziewic, Gregory J. Pelka, Sheila X. Zhou, Maala Parameswaran, Gabriel A. Quinlan, Monica Gordon, Karin Sturm, and Patrick P. L. Tam

1. Introduction

Cell lineage analyses trace the hierarchy of cell types derived from a progenitor population. Critical to these analyses is the ability to track reliably all or defined subsets of the clonal descendants of the progenitor population. This necessitates marking the cells with a heritable and cell autonomous marker. Transgenes encoding molecules that can be visualized directly *in situ* without compromising cell differentiation, such as the reporter, β-galactosidase encoded by *lacZ* and chloramphenicol acetyltransferase, encoded by the CAT gene are the most widely used. The *lacZ* can be readily detected using a sensitive histochemical assay such that cells in which the β-galactosidase gene is transcriptionally active produce a blue stain in tissue sections or in whole mounts ***(1)***. Some lineage studies demand the simultaneous detection of the *lacZ* product and other tissue-specific proteins or transcripts. In this chapter, we discuss the experimental strategies in which the *lacZ* transgene can be utilized in the analysis of cell lineages, and we detail assays for detecting β-galactosidase by X-gal histochemistry or immunological localization of the enzyme in combination with mRNA *in situ* hybridization, immunochemistry, and histochemical procedures, such as alkaline phosphatase staining.

1.1. Choosing the Appropriate Transgenic Animals for Lineage Analysis

The expression of the *lacZ* transgene varies according to the nature of the regulatory elements (promoter and enhancer) that drive gene expression, the

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number of active copies, and the chromosomal domain where the transgene is localized. The choice of transgene is dictated by the specific questions to be addressed. Several examples of transgenes give ubiquitous nonlineage biased expression. For example, in transgenic mice where the *lacZ* gene is regulated by the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase promoter, the transgene is expressed in all lineages at high levels during development ***(2)***. Similarly, the ROSA-β-geo transgene produced by site-directed mutagenesis (3) and the *lacZ* reporter driven by rat β-actin promoter (4) also provide ubiquitous tagging of multiple cell lineages. Ubiquitously expressed transgenes provide the most ideal marker for unbiased cell lineage analyses, provided the lineage progenitors or founder cells to be studied can be isolated as a pure cell population.

Integration of the transgene into a specific chromosome may be useful in some cases for tracing cell lineages. Transgenes that are expressed in the same pattern as neighboring genes at the site of integration provide a ready marker for any tissue-specific pattern of expression. For example, transgenes integrated into the X chromosome may behave like the endogenous X-linked gene; however, the fact that the expression pattern is due to a transgene, not an endogenous gene, must be taken into account when interpreting the results ***(5, 6)***. In female mice that carry the *lacZ* transgene on only one X chromosome, X-inactivation during embryonic development generates two cell populations, one that expresses the X-linked transgene and another that does not. Since the status of X-inactivation is heritable, descendants of either population are stable for the transgene expression. Several studies on the lineage relationship of cells in tissues, such as the retina, brain, and tongue, have been performed using the mosaicism of transgene expression generated by random X-chromosome inactivation ***(7–10)***.

Finally, some transgenes display lineage and stage-specific expression through use of a tissue-specific promoter or as a consequence of their unique integration sites. For example, in *Wnt1-lacZ* transgenic mice, the transgene is regulated by the *Wnt1* 3'-*cis*-acting enhancer element, which directs specific expression to the dorsal part of the neural tube and subsequently in the neural crest cells derived from this region ***(11)***. Another example is R197 transgenic mice, in which the *lacZ* gene is expressed principally in the muscle lineage from early organogenesis stages onward as a consequence of its integration site ***(12)***. Provided that the expression of the transgene is tissue specific for a defined period of development, such transgenes offer an excellent tool for tracing the differentiation of cell lineages. A prerequisite for using these mice and any other transgenic lines is to establish the pattern of transmission and ascertain the tissue and temporal specificity of transgene expression.

1.2. Examples of Three Distinct Patterns of Transgenic Reporter Expression

1.2.1. Ubiquitous Expression

The H253 transgenic line ***(2)*** is an example of ubiquitous expression of an X-linked transgene. Crossing homozygous X^*X^* females with X^*Y males (where X^* represents the transgene-bearing X chromosome) produces transgenic embryos (X^*X^*, X^*Y), in which β-galactosidase is expressed at high levels in all tissues and progeny during all stages of embryonic development. This series of matings produced a ubiquitous non-lineage-bias transgenic line that has been used successfully in several lineage studies in postimplantation mouse embryos ***(2,13–15)***.

1.2.2. X-Inactivation Mosaicism

The H253 transgenic line ***(2)*** can also be used to produce a non-lineage-specific mosaic pattern of expression that reflects the pattern of X-inactivation. Crossing homozygous transgenic H253 female and wild-type (C57BL/6) male mice produces hemizygous H253 female mice (X^*X) and hemizygous male mice (X^*Y). Two distinct patterns of staining are observed, uniform staining (X^*Y) and patchy staining (X^*X). The patchy staining of X^*X hemizygous female mice is due to random X chromosome inactivation. This results in the expression of the *lacZ* marker in only about half the cells. The ability to recognize clonal populations means that these two cell populations (one that does express *lacZ* and the other that does not) have been used in cell lineage analyses to reveal the ancestral history of spatially related clones ***(7–10)***.

The mosaicism of transgene expression as a consequence of X-inactivation in females has been used extensively in the analysis of specific cell lineages ***(7–10, 16)***. The *lacZ*-expressing cells can be distinguished from non-expressing cells by X-gal staining. The composition of the sorted populations may be examined for the expression of cell-specific markers by staining with appropriate antibodies. This system therefore provides an assay for the measurement of the relative proportion of specific cell types at different stages of maturation.

1.2.3. Restricted Expression

The R197 transgenic line is an example of a transgenic marker for lineage- and stage-specific expression patterns ***(12)***. The *lacZ* transgene is segmentally expressed primarily in the myogenic lineage from about 8.5 d onward. Transgenic animals can be crossed with non-transgenic (C57BL/6 X DBA) hybrids or other transgenic mice to produce transgenic embryos for the experiment. A reduction in litter size of F1 hemizygous matings and the absence of the homozygotes after genotyping indicated that homozygotes do not survive to

term (*see* **Note 1**). In this transgenic line, homozygous embryos die during the immediate postimplantation period.

2. Materials

All reagents and solutions used in these protocols should be of molecular biology grade and prepared with sterile distilled water. Reagents for some of the protocols are available commercially as kits, and these are indicated.

2.1. LacZ Expression by β-Galactosidase Histochemistry

1. PBS (Ca^{2+}/Mg^{2+}-free; 1×).
2. Paraformaldehyde (Sigma-Aldrich, St. Louis, MO, P6148). Prepare as a 4% solution in Ca^{2+}/Mg^{2+}-free PBS by heating to dissolve, but do not boil. Add two to three drops of 0.05 M NaOH to clear the solution (pH 7.5), which can be stored at 4°C for 2 wk. Ideally however, it should be freshly prepared. (Caution: Paraformaldehyde is toxic and readily absorbed through the skin. It is very destructive to the skin, mucous membranes, eyes, and upper respiratory tract. Therefore, glasses, gloves, and a mask should be worn to avoid contact with the dust and work should be performed in a fume hood.)
3. Glutaraldehyde (25% [w/v]); Sigma-Aldrich G5882).
4. X-gal (4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside; Progen, Richlands, QLD, 2000190). X-gal is prepared as a 4% (40 mg/mL) stock solution in dimethylformamide (DMF: Sigma-Aldrich D4551). Store at −20°C and protect from light.
5. Xylene.
6. Sorensen's phosphate buffer (PB), 0.1 M, pH 7.4: 0.02 M NaH_2PO_4, 0.08 *M* Na_2HPO_4.
7. X-gal staining solution: 0.1% X-gal, 2 mM $MgCl_2$, 5 mM EGTA, 0.01% (w/v) sodium deoxycholate, 0.02% (w/v) Igepal CA-630 (Sigma-Aldrich, I-3021), 5 mM $K_3Fe(CN)_6$ (potassium ferricyanide), and 5 mM $K_4Fe(CN)_6$ (potassium ferrocyanide) in 0.1 M pH 7.4, Sorensen's phosphate buffer. Potassium ferricyanide and potassium ferrocyanide can be made up as 500-mM stock solutions and should be stored at room temperature, protected from light for a maximum of 2 wk.
8. Ethanol (70, 80, 90, 100%).
9. Histolene.
10. Paraffin wax.
11. Aluminium sulfate (Sigma-Aldrich, A7523).
12. Nuclear fast red (0.1% C.I. 60760 Certistain; Merck KGaA,): Prepare by adding 500 mg of nuclear fast red and 25 g of aluminum sulfate to 500 mL of distilled water, heat or stir to dissolve, and filter when cooled to room temperature.
13. Sucrose (0.2 M).
14. Sucrose 30%.
15. OCT (optimal cutting temperature) compound (Tissue-Tek, ProSciTech, Thuringowa).
16. StarFrost glass slides (ProSciTech G311SF).

17. Isopentane (methyl-2-butane).
18. Canada balsam (in Xylene, Merck KGaA).
19. Glycol methacrylate (2-hydroxyethyl methacrylate; Sigma-Aldrich, H8633).
20. Cresyl violet (Sigma-Aldrich).
21. Acetic acid.

2.2. lacZ *Expression by β-Galactosidase Immunostaining*

1. PBS (Ca^{2+} and Mg^{2+}-free).
2. Paraformaldehyde (4%) in Ca^{2+} and Mg^{2+}-free PBS (*see* **section 10.2.1,** items **1** and **2**, for preparation).
3. Sucrose (0.2 M).
4. OCT compound (Tissue Tek, ProSciTech, IA018).
5. Liquid nitrogen.
6. Avertin: Prepare 100% stock solution by mixing 10 g of 2,2,2 tribromoethyl alcohol (Sigma-Aldrich, T4,840-2) with 10 mL of tert-amyl alcohol ([2-methyl-2-butanol] Sigma-Aldrich 24,048-6). To use, dilute 100% stock to 2.5% in water. Both the 100 and 2.5% stocks are stored wrapped in foil at 4°C. The correct dose of avertin varies with each preparation. Therefore, several mice should be test injected within the range of 0.014–0.018 mL/g body wt.
7. Sodium nitrate (Sigma-Aldrich S5506): Make up as 0.5% solution in 0.5 × PBS.
8. Vibratome (Campden Instruments, Loughborough, Leicestershire, UK).
9. Sodium azide (Sigma-Aldrich S2002).
10. Normal horse serum (Sigma-Aldrich, H0146).
11. Triton X-100.
12. *Escherichia coli* β-galactosidase antibody rabbit IgG (1° antibody; Chemicon International Ltd. Temecula CA, AB1211-5MG).
13. Biotinylated goat antirabbit IgG (2° antibody: Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, PK6101).
14. Texas red conjugated donkey anti-rabbit IgG (2° antibody; Jackson Immuno Research Laboratories Inc., West Grove, PA, 711-075-152).
15. Streptavidin–peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories; Immunodiagnostic, Burlingame, CA, PK6101).
16. Diaminobenzedine (DAB; DakoCytomation, Carpinteria, CA, S3000). Store at –20°C.
17. Hydrogen peroxide (H_2O_2).
18. Streptavidin–FITC complex.
19. Nuclear fast red (0.1%; see **section 10.2.1,** item **12**).

2.3. Visualizing lacZ *by Immunostaining or Histochemistry in Combination with Alkaline Phosphatase Histochemistry*

1. PBS (Ca^{2+} and Mg^{2+}-free).
2. X-gal staining solution (see **section 7.2.4,** item **7**).
3. Glutaraldehyde (25%; Sigma-Aldrich G5882).
4. Ethanol (25% [w/v]), 50, 70, 80, 90, 100%).

5. Polyester wax.
6. Xylene.
7. Hydrogen peroxide (H_2O_2).
8. Methanol.
9. Normal goat serum (Sigma-Aldrich G9023).
10. *E. coli* β-galactosidase antibody rabbit IgG (1° antibody; Chemicon International, AB1211-5MG).
11. Bovine serum albumin (Sigma-Aldrich Fraction V A9647).
12. Biotinylated goat anti-rabbit IgG (2° antibody; Vector Laboratories, PK6101): Store at 4°C.
13. Streptavidin–peroxidase complex (Vectastain Elite ABC reagent): Add two drops of reagent A to 5 mL PBS (with Ca^{2+} and Mg^{2+}), mix well, then add two drops of reagent B, mixing immediately, and allow the ABC reagent to stand for about 30 min before use (Vector Laboratories, PK6101).
14. Diaminobenzedine (DakoCytomation, S3000): Store at −20°C.
15. Peroxidase substrate solution: Dissolve one DAB tablet in 10 mL PBS to give 1 mg/mL DAB solution. Mix 2 mL of DAB solution with 15 mL of 3% H_2O_2 (or 16 mL of 30% H_2O_2). The substrate solution is stable for 2 h at room temperature. Unused stock DAB solution can be stored in the dark (wrapped in foil) for up to 5 d at 2–8°C, or longer at −20°C.
16. Alkaline phosphatase (ALP) buffer: 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5. Dissolve 12.1 g/L TRIS-base and 5.84 g NaCl in dH_2O, adjust pH to 9.5 with concentrated HCl, add 50 mL 1 M $MgCl_2$, and make up volume to 1 L.
17. NBT/BCIP substrate: Store at −20°C. Prepare NBT/BCIP substrate immediately prior to staining. Add 44 µL NBT to 10 mL alkaline phosphatase buffer, mix gently, then add 33 µL BCIP, mix gently (Invitrogen Corporation, Carlsbad, CA: 18280-016).
18. Canada balsam (in Xylene, Merck KGaA; 101693).
19. 4% (w/v) paraformaldehyde (see **section 7.2.4,** item **2**).
20. X-gal staining solution: 0.1% (w/v) X-gal, 2 mM $MgCl_2$, 5 mM EGTA, 0.01% (w/v) sodium deoxycholate, 0.02% (w/v) Igepal CA-630 (Sigma-Aldrich I-3021), 5 mM $K_3Fe(CN)_6$ (potassium ferricyanide) and 5 M K4Fe(CN)6 (potassium ferrocyanide) in 0.1 M pH 7.4 Sorenson's PB.

2.4. Visualizing lacZ by Histochemistry in Combination with In-Situ Hybridization

1. PBS (without Ca^{2+}- and Mg^{2+}).
2. 4% Paraformaldehyde (Sigma-Aldrich: *see* **section 7.2.1**, item **2,** for preparation).
3. Ethanol (50, 70, 80, 90, 100% v/v).
4. Xylene.
5. Paraffin wax.
6. TESPA- ([3-aminopropyl]triethoxysilane; Sigma-Aldrich A3648) coated slides are prepared as follows. Wash slides in 10% HCl/70% ethanol, followed by 95%

ethanol for 1 min each, then air dry. Dip the slides in 2% TESPA in acetone for 10 sec. Wash twice with acetone and then with distilled water before drying at 37°C.

7. DIG RNA labeling kit (Roche 1175025).
8. Triton X-100.
9. Proteinase K (20 mg/mL; 6 µg/mL).
10. SSC (20 X).
11. SDS (10% w/v).
12. Hybridization buffer: 5 × SSC, 50% (v/v) deionized formamide, 0.4% (w/v) SDS, 0.1% (w/v) N-lauroylsarcosine, 2% (w/v) blocking reagent (Roche Blocking Reagent 1096176).
13. Heparin sodium salt from porcine intestinal mucosa (50 µg/mL or 7U; Sigma-Aldrich H9399).
14. Yeast tRNA (20 µg/mL).
15. NaCl (1 M).
16. Maleic acid (1 M).
17. Sheep serum (Sigma-Aldrich S2263).
18. DIG antibody-sheep conjugated alkaline phosphatase (dilute 1:200 in 150 mM NaCl/100 mM maleic acid/10% sheep serum; Roche 1093274).
19. Alkaline phosphatase buffer (ALP; see **section 7.2.3**, item **16**).
20. NBT/BCIP substrate (see **section 7.2.3,** item **17**).
21. *E. coli* β-galactosidase antibody rabbit IgG (5 µg/mL in PBS/20% normal goat serum; Chemicon International AB1211-5MG).
22. Normal goat serum (Sigma-Aldrich G9023).
23. Biotinylated goat anti-rabbit IgG (2° antibody; diluted 1:200 in PBS/0.2%BSA; Vector Laboratories, PK6101) for 60 min at room temperature.
24. Streptavidin–β-galactosidase conjugate (diluted 1:200 in PBS/0.2% BSA; Roche 1112481).
25. X-gal washing buffer (X-gal staining solution without X-gal; *see* **section 7.2.1**, item **7**).
26. X-gal staining solution (*see* **section 7.2.1**, item 7).
27. CHAPS (3-[{3-cholamidopropyl}dimethyl-ammonio]-1-propanesulfonate zwitterionic; Roche 810118).
28. PBT (0.1% Tween-20 in PBS).
29. Methanol (25, 50, 70, l00% v/v).
30. Hydrogen peroxide (H_2O_2).
31. Dimethylsulfoxide (DMSO; Sigma-Aldrich D2650).
32. Glycine.
33. Glutaraldehyde (25% w/v; Sigma-Aldrich G5882).
34. Tween-20.
35. Prehybridization solution consists of the following and should be prepared on the day of use: 50% deionized formamide, 5×SSC, 2% blocking powder, 0.1% Tween-20, 0.5% CHAPS, 50 µg/mL yeast RNA, 5 mM EDTA, 50 µg/mL heparin.
36. Bovine serum albumin (Fraction V; Sigma-Aldrich A9467).

37. Mouse embryo powder: This is prepared by homogenizing 12.5–14.5-d mouse embryos in a minimum volume of PBS. Add 4 volumes of ice-cold acetone, mix, and incubate on ice for 30 min. Spin at 10,000 *g* for 10 min and remove the supernatant. Wash the pellet with ice-cold acetone, and spin again. Spread the pellet out, and grind into a fine powder on a sheet of filter paper. Allow the powder to air dry, and store in an airtight tube at 4°C.
38. NTMT: 100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20.
39. Levamisole hydrochloride (2 mM in NTMT; Sigma-Aldrich L9756).
40. Color reaction mix consists of the following and should be prepared immediately before use: 4.5 mL NBT, 3.5 µL BCIP (Invitrogen Corporation: 18280-016) in 1.0 mL NTMT.

3. Methods

3.1. Histochemical Staining for lacZ *with X-Gal*

3.1.1. Small Embryos (7.5–11.5 D pc) and Fragments of (12.5–18.5 D pc) Embryos

1. Fix small embryos or fragments of older embryos in 4% paraformaldehyde/0.2% glutaraldehyde for 5–15 min.
2. Wash the embryos in PBS (2 × 5 min), and stain overnight in X-gal staining solution at 37°C in the dark.
3. Wash the embryos in 70% (10 min) ethanol and dehydrate in 70, 80, and 90% ethanol (10 min each), then 100% ethanol (3 × 10 min).
4. Clear the embryos in xylene or histolene (3 × 10 min) and impregnate in paraffin wax (3 × 20 min) before embedding in fresh wax.
5. Cut 5-10 µm sections and counterstain with nuclear fast red (*see* **Notes 2–6**).

3.1.2. Older Embryos and Adult Organs

Larger specimens can be handled in two ways, depending on the thickness of the tissue. Frozen sections can be stained or, alternatively, very small tissue pieces can be stained then embedded in paraffin wax and sectioned. Paraffin embedding is described in **section 7.3.1.1** (*see* **Note 7**).

1. Fix dissected adult tissues or embryos in fresh 4% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer. Determine the time required by the size of the tissue to be fixed based on penetration rates of 1 mm/h at room temperature. (Perfusion can also be carried out if desired, *see* **section 7.3.2.2**).
2. Wash (3 × 2 min) in phosphate buffered saline (PBS).
3. Leave in 30% sucrose at room temperature overnight.
4. Fill Tissue-Tek cryomolds with OCT medium and place a piece of tissue in each labeled cryomold. Minimize the number of bubbles and avoid bubbles in contact with the tissue.
5. Fill a beaker to about one third full with isopentane and float the beaker in liquid nitrogen. When a crust of frozen isopentane is visible, lower the cryomolds into

the isopentane one at a time to freeze (1–2 min) using long forceps. Place samples on dry ice until all are processed then store samples at −80°C. (*see* **Note 8**)
6. Section samples on cryostat to desired thickness e.g.: 8–10 µm onto StarFrost coated slides.
7. Rinse slides very carefully with PBS to remove Tissue-Tek. Place on the smallest box or tray that will fit the number of slides to be stained.
8. Very gently rinse in washing buffer 3 × 20 mins.
9. Put a few drops of *lacZ* substrate on each slide and add coverslips. Seal box with parafilm and aluminium foil and incubate at 37°C overnight.
10. Gently rinse off *lacZ* substrate with PBS and follow with 2 × 10 min PBS washes.
11. Counterstain with nuclear fast red 1–3 minutes.
12. Quickly dip in water.
13. Dip in 70% ethanol.
14. Dip in 90% ethanol.
15. Immerse for 1 min in 100% ethanol, then repeat in fresh ethanol.
16. Immerse for 2 min in histolene, then repeat in fresh histolene.
17. Coverslip using Canada balsam mounting media.

3.2. Immunostaining for lacZ

3.2.1. Embryos (7.5–18.5 D pc)

1. Dissect embryos in 4% paraformaldehyde (in Ca^{2+} and Mg^{2+}-free PB) and fix for 4 h.
2. Wash the embryos in 0.2 M sucrose (1 h), then immerse in OTC briefly and immediately before freezing slowly in liquid nitrogen vapor, followed by immersion in liquid nitrogen.
3. Cut 60-µm thick sections using a cryostat and store at 4°C until required, then go to **section 3.2.3**, step 1.

3.2.2. Adult Mice and Body Organs

1. Anesthetize adult mice with 2.5% avertin (0.017 mL/g of body weight) and perfuse via the left ventricle with 0.5% w/v sodium nitrate in half-strength phosphate-buffered saline.
2. Dissect the required organ, and postfix with the same sodium nitrate fixative (2 h).
3. Section the tissue with a vibratome at 50–100-µm thickness.
4. Store sections for immunohistochemistry in PBS/0.02% sodium azide at 4°C until required. Then go to **section 3.2.3**, step 1.

3.2.3. Detection

1. Wash sections from **sections 3.2.1**. and **3.2.2**. selected for detection of β-galactosidase in PBS/10% normal horse serum/0.02% Triton X-100 at room temperature (12 h).

2. Incubate sections with *E. coli* β-galactosidase antibody (diluted 1:1000 in PBS/10% normal horse serum/0.02% Triton X-100) overnight at room temperature.
3. Wash sections in PBS (3 × 10 min), and then incubate with 2° antibodies either biotinylated goat anti-rabbit IgG (diluted 1:400 in 0.02% Triton X-100 in PBS) or Texas red donkey anti-rabbit IgG (diluted 1:500 in 0.02% triton X-100 in PBS) for 2 h.
4. Remove unbound antibody by washing in PBS (10 min).
5. If using biotinylated goat anti-rabbit IgG, incubate the sections in streptavidinperoxidase complex (diluted 1:100 in 0.2% Triton X-100) for 2 h.
6. Visualize the immunochemical reaction by washing sections with diaminobenzidine (0.5 mg/mL) in the presence of 0.01% H_2O_2 until the brown color is apparent.
7. If Texas red–conjugated donkey antirabbit IgG is used, then incubate the sections in streptavidin–FITC (diluted to 1:100 in 0.02% Triton X-100 in PBS) for 2 h.
8. Wash sections in PBS (3 × 10 min), and view either by light or fluorescence microscopy. Sections may be counterstained with 0.1% nuclear fast red.

3.3. Visualizing lacZ *by Immunostaining or Histochemistry in Combination with Alkaline Phosphatase Histochemistry*

3.3.1. Simultaneous Immunological Detection of lacZ *and Alkaline Phosphatase on Sections*

This protocol allows for the staining of *E. coli* β-galactosidase antibody and NBT-BCIP histochemical detection of alkaline phosphatase. It is designed to detect alkaline phosphatase in primordial germ cells in *lacZ*-expressing embryos. All staining should be done at room temperature unless otherwise indicated and on 6–8 μm sections.

1. Dissect out embryos in PBS medium and stain either the tail, head, yolk sac, or a nonessential body part with X-gal staining solution to screen for transgenic embryos.
2. Fix embryos in 0.5% glutaraldehyde in PBS for 5–15 min, depending on the size of the embryos. As a general rule:
   a. <8.5 d pc — 5 min
   b. 9.5 d pc — 5–10 min
   c. 10.5–12.5 d pc — 10–15 min
   d. 13.5–15.5 d pc — 15 min
   e. >16.5 d pc — 20–30 min
3. Wash embryos 2 × 10 min with PBS. The embryos then can be either stored in 70% ethanol for a short period of time or dehydrated and embedded as described in **section 7.3.3.1**, step **4**.
4. Dehydrate embryos through 70, 80, 90, and 100% (× 3) ethanol according to the following times:
   a. <8.5 d pc — 2–5 min
   b. 9.5 d pc — 5 min

c. 10.5–12.5 d pc 10 min
d. 13.5–15.5 d pc 10–15 min
e. >16.5 d pc 20–30 min

5. Transfer embryos to fresh polyester wax for 10–15 min. Repeat with fresh wax 2 × 10 min each. Embed in fresh polyester wax at room temperature, and section with either a cryostat or a microtome kept at 15°C (*see* **Note 9**).
6. Dewax sections in xylene (5 min), and rehydrate the sections by incubating them in a 37°C oven for 30 min followed by 2-min washes in 100% ethanol (2 × at 37°C), 90, 70, 50, and 25% ethanol. Finally, wash the sections for 5 min in dH_2O.
7. Quench the endogenous peroxidase activity with 0.3% H_2O_2 in 40% methanol for 20 min or 3% H_2O_2 in H_2O for 5 min.
8. Wash the sections with PBS (3 × 3 min).
9. Block non-specific binding by overlaying sections with 5–10% normal goat serum for 20 min.
10. Drain excess serum and incubate slides with *E. coli* β-galactosidase antibody (diluted 1–5 µg/mL in PBS) for 60–75 min.
11. Wash sections in PBS + 0.2% BSA (3 × 10 min).
12. Incubate sections with biotinylated goat anti-rabbit IgG (2° Ab diluted 1:200 in PBS, 0.2% BSA, 3% normal goat serum) for 30 min.
13. Wash sections with PBS + 0.2%, BSA (3 × 10 min).
14. Incubate sections for 30 min with streptavidin peroxidase complex.
15. Wash sections with PBS + 0.2% BSA (3 × 10 min).
16. Incubate sections with peroxidase substrate solution (1 mg/mL DAB + 0.0225% H_2O_2) for 2–7 min or until desired staining intensity develops.
17. Wash sections for 5 min with dH_2O.
18. Incubate sections with PBS (10 min).
19. Incubate sections with alkaline phosphatase buffer, pH 9.5 (2 × 10 min).
20. Incubate sections in NBT/BCIP substrate for 10–15 min in the dark at 37°C, or until desired intensity develops.
21. Wash the sections with dH_2O (5 min).
22. Dehydrate and mount in Canada balsam.

3.3.2. Simultaneous Whole-Mount Histochemical Staining for β-Galactosidase and Alkaline Phosphatase

1. Fix 7.5–11.5 d mouse embryos or older embryo (12.5–18.5 d) fragments and organs in 4% paraformaldehyde, 0.5% glutaraldehyde in PBS for 5–10 min.
2. Wash embryos in PBS (2 × 10 min). Incubate embryos for 10 min in alkaline phosphatase buffer.
3. Incubate embryos in NBT/BCIP substrate at 37°C for 10–20 min. Check intensity every 5 min.
4. Wash embryos extensively in X-gal washing buffer (staining solution without X-gal; 2 × 10 min).
5. Incubate embryos overnight in X-gal staining solution at 37°C.

3.4 Visualizing lacZ by Histochemistry in Combination with In-Situ Hybridization

3.4.1. Simultaneous Detection of Oct 4 mRNA and β-Galactosidase Protein on Paraffin Sections

1. Fix 7.5–11.5-d embryos or fragments and organs of older embryos (12.5–18.5 d) in 4% paraformaldehyde overnight at 4°C, then wash in PBS (2 × 10 min). Dehydrate the embryos according to the schedule given in section 7.3.3.1, step 4; wash in xylene (3 × 10 min) followed by paraffin wax (3 × 15 min); then embed the embryos in fresh paraffin wax before storing at 4°C. Cut sections at 8 µm, and mount on TESPA-coated slides.
2. A 600 bp Oct4 RNA digoxigenin probe was generated by in-vitro transcription with T3 (antisense strand) and T7 (sense strand) RNA polymerase (DIG RNA labeling kit, Roche, cat no. 1175025).
3. Dewax the embryos in xylene (5 min), rehydrate (100% × 2, 90, 80, 70, 50, dH_2O; for 1 min each), and then wash the sections in PBS (2 min).
4. Refix the sections in 4% paraformaldehyde (10 min), then wash with PBS (2 × 5 min).
5. Treat the sections with 0.3% Triton X-100 in PBS (15 min), then wash in PBS (2 × 5 min).
6. Incubate the sections in 37°C prewarmed proteinase K (20 mg/mL; 5 min), wash in PBS (3 × 10 min), then postfix the sections in 4% paraformaldehyde (15 min).
7. Prehybridize the sections for 3 h in hybridization buffer (5 × SSC, 50% deionized formamide, 0.4% SDS, 0.1% N-lauroylsarcosine, 2% blocking reagent.
8. Dilute the probe in hybridization buffer containing 50 µg/mL heparin and 20 mg/mL yeast tRNA to a final concentration of 0.05 ng/mL. Heat the probe at 80°C (10 min) and immediately store on ice until addition to the slides. Incubate with sections with 500 µL of probe at 55°C in a humidified chamber overnight.
9. Wash the sections at room temperature in 2 × SSC/0.2% SDS (2 × 10 min); then at 58°C in 0.5 × SSC/0.2% SDS (2 × 30 min) followed by 150 mM NaCl/100 mM maleic acid (pH 7.5; 10 min).
10. Block the section in 150 mM NaCl/100 mM maleic acid (pH 7.5) containing 10% sheep serum (30 min), then incubate with diluted alkaline phosphatase-conjugated sheep anti-DIG antibody (1:200 dilution) for 2 h.
11. Wash the sections in 150 mM NaCl/100 mM maleic acid (pH 7.5; 2 × 15 min), then in alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5, 10 min), and incubate with NBT/BCIP substrate. Allow the color to develop in the dark for 90 min at room temperature.
12. Wash the sections in PBS (3 × 10 min), block with 20% normal goat serum in PBS (30 min), then incubate with 5 µg/mL *E. coli* anti-β-galactosidase antibody overnight at 4°C.
13. Wash the sections in PBS containing 0.2% BSA (3 ×in), then incubate with biotinylated goat anti-rabbit IgG (2° antibody; diluted 1:200 in PBS/0.2%BSA) for 60 min at room temperature.
14. Wash the sections in PBS containing 0.2% BSA (3 × 10 min), then incubate with streptavidin-β-galactosidase conjugate for 60 min (1:200 dilution).

15. Wash the sections in PBS (2 × 10 min), then in X-gal washing buffer (10 min), and incubate in X-gal solution at 37°C (20 min) in the dark.
16. Dehydrate the sections, and mount in Canada balsam.

3.4.2. Whole-Mount In-Situ Hybridization Combined with X-Gal Staining

All steps are performed at room temperature unless otherwise stated.

1. Fix embryos in 4% paraformaldehyde for 5 min.
2. Stain embryos in X-gal staining solution for 1 h.
3. Refix embryos in 4% paraformaldehyde overnight.
4. Wash embryos in PBT (2 × 10 min).
5. Wash embryos in 25, 50, and 75% methanol/PBT (5 min), then in 100% methanol (2 × 10 min). Embryos can be stored in 100% methanol at −20°C.
6. Fix the embryos in methanol:DMSO (4:1) for 1 h, then bleach the embryos in methanol:DMSO:30%H_2O_2 (4:1:1) for 1 h.
7. Rehydrate embryos in 75, 50, and 25% methanol/PBT (5 min), then wash the embryos in PBT (2 × 5 min). It is important to poke a few holes with a small needle into these embryos prior to washing to facilitate the flow of solutions.
8. Treat the embryos with proteinase K (6 mg/mL; 5 min).
9. Wash the embryos in 0.2% glycine in PBT (2 × 5 min).
10. Refix the embryos in 4% paraformaldehyde/0.2% glutaraldehyde in PBS (30 min).
11. Wash embryos in PBT (2 × 5 min).
12. Wash embryos in 1 mL of prehybridization solution.
13. Prehybridize embryos in 1 mL of prehybridisation solution at 65°C (3 h). Embryos can be stored at this point at −20°C in prehybridization solution.
14. Incubate embryos in 1 mL of hybridization solution at 60°C overnight.
15. Posthybridize wash with each of the following for 5 min at 60°C:
    a. 100% Prehybridization solution.
    b. 75% Prehybridization solution: 25% 2 × SSC.
    c. 50% Prehybridization solution: 50% 2 × SSC.
    d. 25% Prehybridization solution: 75% 2 × SSC.
16. Wash with each of the following for 30 min at 60°C:
    a. 2 × SSC: 0.1% CHAPS.
    b. 0.2 ×X SSC: 0.1% CHAPS (×2).
17. Wash embryos in PBT (2 × 10 min).
18. Preblock embryos in 10% normal sheep serum/1% BSA in PBT at 4°C (3 h).
19. Preabsorb the antibody at 4°C (3 h) with 3 mg mouse embryo powder, 500 µL of 10% normal sheep serum, 1% BSA in PBT, and 1 µL of anti-DIG antibody, rocking the mixture gently. Spin the solution at 2,000 *g* for 5 min and remove the supernatant (preabsorbed antibody).
20. Incubate the embryos in preabsorbed antibody overnight at 4°C with constant rocking.
21. Wash embryos in 0.1% BSA in PBT (5 × 1 h).
22. Wash embryos in 2 mM levamisole in NTMT (2 × 10 min).

23. Wash embryos in NTMT (10 min).
24. Incubate embryos in the color reaction mixture until sufficient coloration is visible in the specimen.
25. Refix embryos in 4% paraformaldehyde overnight, wash in PBS, and examine (*see* **Notes 10** and **11**).

4. Notes

1. The possibility of embryonic lethality should be examined as an essential step for using transgenic mice, particularly when a significant reduction in litter size is observed. To ascertain lethality, pregnant mice can be sacrificed at different ages of gestation so that the litter size and the genotype of the embryos or fetuses can be analyzed.
2. Enzyme activity in embryos from preimplantation up to about 11.5 d pc can be directly analyzed in whole mounts. However, for embryos older than 11.5 d, penetration of the substrate in whole embryos becomes limiting; and this poses a problem for accessibility of staining reagent to internal tissues, especially after a substantial amount of insoluble reaction product has been deposited in more superficial *lacZ*-expressing tissues. Dissection of older embryos (>11.5 d) into smaller fragments facilitates penetration of the substrate and greatly improves the staining of deeper tissues.
3. To reduce the background associated with endogenous β-galactosidase activity normally found in bone, kidney, and brain, the histochemical reaction should be carried out at pH 7.4, which is optimal for bacterial β-galactosidase in contrast to the mammalian enzyme, which is most active at a more acidic pH value of 4.0.
4. Prolonged exposure of embryonic tissues to the fixative diminishes β-galactosidase activity, and enzyme activity becomes undetectable after more than 60 min in paraformaldehyde. When coverslipping sections, use Canada balsam, as other mounting media can cause the blue stain to fade.
5. If the fixation of the embryo fragment or specimen is insufficient, leakage of the reaction product stains the reaction mixture blue. It is advisable not to leave stained samples in alcohols and solvents longer than necessary, since this may leach out some of the reaction product. When sections are viewed by dark-field microscopy, the *lacZ* stain appears pink and contrasts with surrounding tissues.
6. To enhance the discrimination between expressing cells, non-expressing cells, and cells expressing endogenous β-galactosidase-like activity, it is useful to target the *lacZ* product to a particular cellular compartment, such as the nucleus. The nuclear localization signal (nls) from the early region of the simian virus (SV40) genome has been used to provide effective nuclear localization of β- galactosidase (nls*LacZ*; ***[17, 18]***).
7. For more detailed histological studies, organs can be cut by a scalpel blade into small parts, which are then dehydrated in alcohol and embedded either in paraffin wax or glycol methacrylate. Wax sections are counterstained in 0.1% nuclear fast red. Methacrylate sections are counterstained in 0.1% cresyl violet in 1% acetic acid (pH 3.3) for 50 min at 40°C.

8. Specimens go white in about 30 s when freezing them in isopentane.
9. When infiltrating with polyester wax, the specimens may float, which is normal, but they sink as they are infiltrated with wax. If, after the first 15 min of incubation in wax, the specimens have not sunk, it is still advisable to go to the next wax change.
10. It is important that the embryos are not overfixed with paraformaldehyde for X-gal staining prior to the immunohistochemistry, because it may result in some loss of antigenicity.
11. Each of these procedures contains multiple wash steps that require the transfer of embryos. If the dish containing the embryos is placed on a black background, it is easier to see the embryos during transfer.

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11

Transgenic RNA Interference to Investigate Gene Function in the Mouse

Tilo Kunath

1. Introduction

The necessity of a gene for a particular biological function is often investigated by loss-of-function studies. Although this may be accomplished by a number of means, the most common and def initive strategy is to physically disrupt the genetic locus in a way that results in the production of a nonfunctional protein or prevents transcription, without affecting the activity of nearby genes. In the mouse, this can be performed in several ways. Chemical mutagenesis and gene trap approaches have been very useful to obtain randomly induced mutations ***(1,2)***, while homologous recombination in embryonic stem (ES) cells has been the traditional method to disrupt genes in a directed manner ***(3,4)***.

A less definitive, but often effective and rapid method to investigate the necessity of a protein-encoding gene is by posttranscriptional suppression. Several experimental strategies exist that either promote mRNA degradation or inhibit translation, including antisense, ribozyme, and morpholino technologies ***(5–7)***. An exciting alternative to these methods was introduced by the discovery of the molecular trigger for RNA interference (RNAi). Although RNAi has been observed for some time in plants ***(8,9)***, it was work in *C. elegans* that showed double-stranded RNA (dsRNA) could specifically mediate this phenomenon ***(10)***. Since these seminal observations were made, an explosion of work has further elucidated the mechanisms of RNAi via experimentally introduced dsRNA or naturally occurring microRNAs (miRNAs) ***(11–15)***. This knowledge has been exploited to generate systems and protocols that rapidly and efficiently disrupt gene function in a wide range of species, including mouse and human,

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and promises to speed up analysis of gene function and provide a new therapeutic technology.

The mouse is an ideal organism to study mammalian gene function *in vivo* by RNAi because of the availability and developmental potential of mouse ES cells ***(16, 17)***. These remarkable cells can differentiate into most tissues in culture, are capable of participating in chimeras, and can give rise to entirely ES-cell-derived embryos and mice in the right conditions ***(18, 19)***. Although the technology is well described, this chapter illustrates how it can be merged with RNAi strategies to rapidly perform reverse genetics in the mouse.

1.1. RNAi Transgenes

The first and most important step in this procedure is to design, construct, and test RNAi transgenes that target the genes of interest. Studies on the molecular mechanisms that mediate RNAi helped greatly in the design of experimental systems for targeted gene knockdown. A key finding was that long dsRNA is processed by the RNaseIII enzyme, Dicer, into short dsRNA fragments of 21–22 nt in length ***(20, 21)***. These fragments have unphosphorylated 3 2-nt overhangs and 5 phosphate groups ***(22)***. Synthesis and introduction of these small (or short) interfering RNAs (siRNAs) is sufficient to elicit RNAi in all systems tested thus far ***(23–27)***. Furthermore, the introduction of long dsRNA into vertebrate organisms causes an antiviral interferonlike response that leads to a global reduction in translation ***(28, 29)***, a wholly undesirable response. Therefore, all mammalian RNAi protocols currently use siRNAs to knock down gene expression. Several plasmid-based systems have been devised to produce short hairpin RNA (shRNA) from RNA polymerase III (RNA pol III) promoters ***(30–32)***. These hairpin molecules are processed into siRNA by Dicer to mediate RNAi. Such plasmids are used here to drive shRNA expression in ES cells and embryos. Alternatively, two separate RNA pol III promoters can be used to drive two single-stranded RNA (ssRNA) molecules that subsequently anneal ***(33)***, but RNAi was less effective when directly compared to short hairpin RNA ***(34)***.

1.1.1. Choosing the Best siRNAs

Not all siRNAs are created equal and a major effort has been made to determine the best siRNAs to knockdown a gene of interest. Understanding the next step in the RNAi pathway downstream of Dicer has proven very helpful in this respect. The siRNA molecules generated by Dicer become incorporated with the RNA-induced silencing complex (RISC) that uses the short RNA as a template to target an mRNA species for cleavage and eventual degradation ***(35)***. However, each RISC complex contains only one strand of ssRNA from the siRNA duplex ***(36)***; therefore, to produce an effective knockdown, it must incorporate

the antisense strand at the expense of the sense strand. The current model for active RISC formation states that unactivated RISC can enter at either end of the siRNA duplex and incorporates the strand that it reads in a 5′ to 3′ direction (**Fig. 1**), while the other strand is simply degraded ***(37)***. Although, for some siRNA molecules, RISC may incorporate either strand with equal probability,

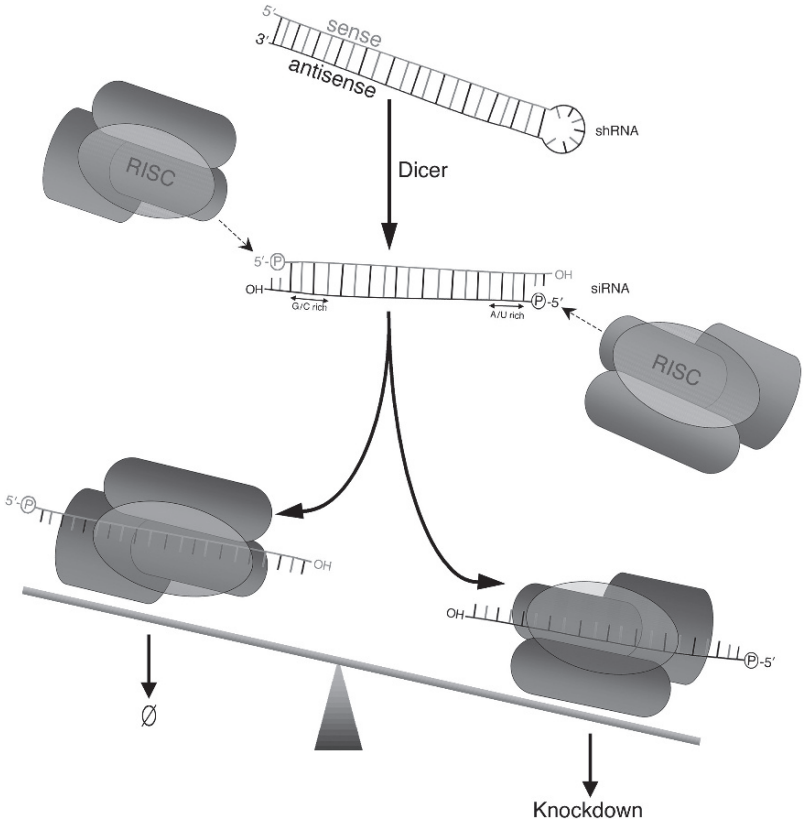

Fig. 1. Model of asymmetric RISC activation. RNAi transgenes produce shRNA molecules as shown. The strand antisense to the target mRNA is black, and the sense strand is gray. Dicer processes shRNA to siRNA, a duplex with 5′ phosphorylated ends and 2-nt 3′ overhangs. RISC may interact with siRNA at either end, but incorporates the strand it reads only in a 5′ to 3′ direction. For effective RNAi, RISC should be biased to acquire the antisense strand. This can be accomplished by designing the 5′ end of the antisense strand to be A/U rich, while simultaneously making the other end of the siRNA molecule G/C rich. This tips the balance toward preferential uptake of the antisense strand and results in knockdown of the targeted gene.

this is not normally the case. A strand bias has been observed for most siRNAs–miRNAs and a molecular explanation has been put forth and experimentally tested *(37,38)*. The simplified model states that RISC, which possesses helicase activity, preferentially enters the terminus of the short dsRNA that is less tightly paired, that is, the side that is more A/U rich (**Fig. 1**). In terms of choosing the best siRNAs, this means that the 5 end of the antisense strand should be A/U rich while the 5 end of the sense strand should be G/C rich. This encourages the RISC to enter the duplex from the less stable side and acquire the antisense strand, which mediates effective gene knockdown.

1.1.2. RNA Polymerase III Promoters

RNA pol III promoters are used to transcribe endogenous small nuclear and cytoplasmic noncoding RNAs. They are more desirable than RNA pol II promoters to drive shRNA expression, since they are not linked to processes typical of mRNA production, such as splicing and polyadenylation *(39)*. Two RNA pol III promoters are most commonly used for shRNA expression: the promoter from the human H1 gene (encoding the RNA component of nuclear RNase P) and the U6 small nuclear RNA promoter *(40,41)*. Both the U6 and H1 promoters have been effectively used to express shRNAs that mediate RNAi *(30–32)*.

1.2. Transgenic ES Cells

Plasmids, such as RNAi transgenes, can be introduced into ES cells by several methods, including electroporation *(42)*. A drug-selectable marker is convenient to have on the same plasmid as the U6 or H1 promoter-driven shRNA. This allows for selection of stable, clonal ES cell lines that may be assayed for levels of knockdown and the generation of embryos and mice. An important consideration for RNAi transgenesis in ES cells is the dominant nature of the technique. The disruption of genes required for ES cell self-renewal or survival, by definition, does not produce ES cell clones with significant knockdown. The ES cells will either differentiate or die, but if clones are recovered, they likely have mild to moderate levels of knockdown. In these situations, an inducible RNAi system is required to circumvent this problem, as will be discussed next.

1.3. Embryos Derived Directly from ES Cells

Once ES cell clones with a knockdown of the gene of interest have been identified, several experimental routes can be taken. Their ability to differentiate into a particular cell lineage can be assayed in culture, as many ES cell differentiation protocols have been described *(43)*. To investigate the role of the targeted gene in development, the knockdown ES cells can be used to directly

produce an entire embryo by combining them with tetraploid host embryos ***(18, 19,44)***. In these chimeras, the tetraploid host cells form the placenta and endoderm component of the yolk sac, while the diploid ES cells produce the embryo proper, amnion, and mesoderm component of the yolk sac ***(18)*** (**Fig. 2**). This allows for the direct analysis of the knockdown phenotype *in vivo*, without the need for germline transmission and mating of heterozygous mutants ***(45)***. An additional advantage is that all the embryos in the litter express the RNAi transgene, so genotyping is unnecessary.

1.3.1. ES Cell Lines with High Developmental Potential

The developmental potential of the parental ES cell line is an important consideration before introducing RNAi transgenes. Since ES cells vary widely in their ability to support fetal developmental, a suitable starting clone is essential. The R1 ES cell line at early passages was able to support fetal development to term and into adulthood, while later passages could not ***(19)***. Although, development to term is not necessary for the analysis of phenotypes at midgestation, it is best to use ES cell lines with the greatest developmental potential as possible. Comparison of ES cell lines derived from inbred or F1 hybrid embryos showed drastic differences in their developmental potential, with the hybrid lines exhibiting superior results ***(46)***. However, all lines, whether hybrid or inbred, should be tested alongside the RNAi transgenic ES cell clones to ensure phenotypes are due to a knockdown of the gene and not due to compromised ES cell potential.

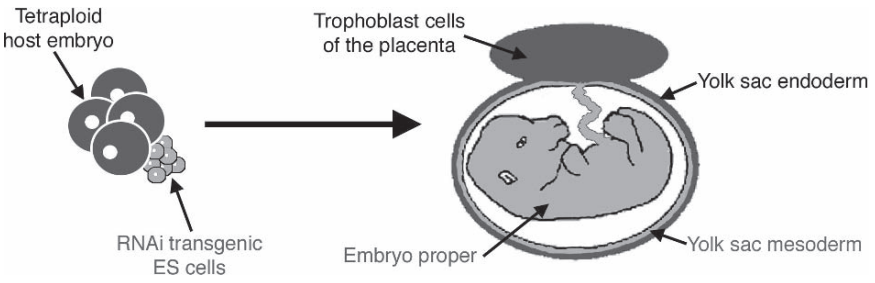

Fig. 2. ES cell-derived embryos by tetraploid complementation. RNAi transgenic ES cells (light gray) are injected into tetraploid host blastocysts (not shown) or aggregated with tetraploid morula embryos (dark gray). The chimeric embryos are allowed to develop in utero until the time of phenotypic analysis. The tetraploid cells contribute to the trophoblast portion of the placenta and the yolk sac endoderm. The knockdown ES cells develop into the entire embryo proper, as well as yolk sac mesoderm and amnion (not shown).

1.4. Future Directions

1.4.1. Retroviral-Based shRNA

Since electroporation and other transfection methods can be inefficient, several retroviral-based shRNA systems have been developed ***(47–51)***. Furthermore, lentiviral-based systems expressing shRNA have been used to successfully infect mouse zygotes and two-cell stage embryos ***(52, 53)***. This bypasses the need for ES cells and should, in theory, target all cell types including extraembryonic ones to which ES cells do not give rise. However, each embryo will likely have a different level of knockdown, depending of the number of integrated viral genomes present, and prescreening may not be as easily performed as in ES cells.

1.4.2. Inducible shRNA Transgenes

The introduction of effective shRNA transgenes into cells will result in a dominant disruption of the targeted gene's function. Therefore, genes essential for viability or maintenance of a cellular state, such as pluripotency of ES cells, are difficult to knock down. Although such results may point to the importance of the gene being targeted and yield some insights into its function, it may be desirable to use conditional shRNA strategies in these situations. Two classes of inducible shRNA systems have been reported: a Cre-loxP recombination system ***(54–56)*** and several drug-inducible or -repressible systems ***(57–59)***. Cre-loxP systems are less leaky but tend to be irreversible. The drug-regulatable RNAi systems have the advantages of being dose dependent and reversible.

1.4.3. Validated siRNAs

Excellent algorithms are now available to predict which siRNA sequence will produce an effective knockdown for a gene of interest ***(60,61)***. These are described in detail in the "Methods" section. However, concerted effort is undeerway to make available to the scientific community validated and tested siRNA sequences for all mouse and human genes ***(62)***. This resource may also be used to identify different siRNAs for the same genes that elicit different levels of knockdown, making it feasible to mimic an allelic series of mutations. Some validated siRNA sequences are already available from companies such as Imgenex, Ambion, and Oligoengine.

1.4.4. Multigene Knockdown

Due to the small size of RNA pol III promoters as well as inserts encoding for shRNA, it should be relatively easy to perform simultaneous RNAi against several genes with a single plasmid or cotransfection of multiple plasmids ***(63)***. It should also be possible to knock down several members of a gene family that share a region of exact homology with a single shRNA transgene. Transposon-

based transgenic RNAi will be useful for complete genome integration of a multiple-shRNA transgene, since partial loss of the cassette is avoided ***(64)***.

1.4.5. RNAi Libraries

Large-scale RNAi screens have been performed in *C. elegans* and *Drosophila* with dsRNA libraries ***(65–68)***. Several large-scale human and mouse RNAi libraries have been constructed from predesigned sequences and used to screen various biological problems ***(62,69,70)***. As an alternative and less laborious method, complex RNAi libraries have been enzymatically produced from mRNA or cDNA ***(71–73)***. This has the advantage of incorporating unknown genes and can be used in species where genomic and EST data are not yet available.

1.4.6. Beyond the Mouse

RNAi is a universal phenomenon and may be used to disrupt gene function in any experimental plant or animal model. Since technologies for DNA injection and retroviral infection of embryos exist for several other mammals ***(74–77)***, RNAi transgenes may be introduced to assess gene function in these species. A promising extension of this technology is to the rat model. This species has been an excellent model for physiological, neurological, and psychological studies for decades. However, rat ES cells have been elusive to derive ***(78)***, so the generation of genetic knockouts has not been possible. The completion of the rat genome sequence will make RNAi in this species more amenable ***(79)***. It is encouraging that an shRNA transgene effectively suppressed expression of EGFP in transgenic rats ***(80)***. This strongly suggested that knockdown of endogenous rat genes will be possible and in-vivo loss-of-function studies can now be performed in this species ***(81)***.

2. Materials

The methods described in this chapter require the standard materials used in molecular biology, cell culture, and mouse embryo manipulation. The one nonstandard piece of equipment is a cell–embryo fusion instrument to generate tetraploid embryos. This device is sold by BLS Ltd, Hungary (www.bls-ltd.com/fusion.html) under the name CF-150B.

2.1. RNAi Transgenes

Several options are available when choosing an RNAi transgene. Many are commercially available, but they can also be easily constructed or obtained from published sources.

2.1.1. Commercially Available RNAi Transgenes

Ambion (www.ambion.com) has a series of RNAi plasmids under the general name of pSilencer™. They consist of an H1 or U6 RNA pol III promoter downstream of which the DNA encoding for the shRNA can be cloned. A

drug-resistance gene to puromycin, neomycin, or hygromycin driven by the SV40 promoter is also on the plasmid for stable selection of transfected cells. Many other companies also provide plasmid-based siRNA systems based on this theme (*see* **Note 1**). Some of them offer inducible siRNA transgenes and retroviral variants.

2.1.2. Constructing RNA Pol III Promoters for RNAi Transgenesis

The human H1 promoter is very small ***(41)*** and can be synthesized as a pair of approximately 100-nt oligonucleotides and cloned into your vector of interest ***(45)***. Based on the published pSUPER plasmid ***(32)***, oligos encoding a modified H1 promoter (**Fig. 3**) can be ordered, annealed, and cloned into the *Bgl*II

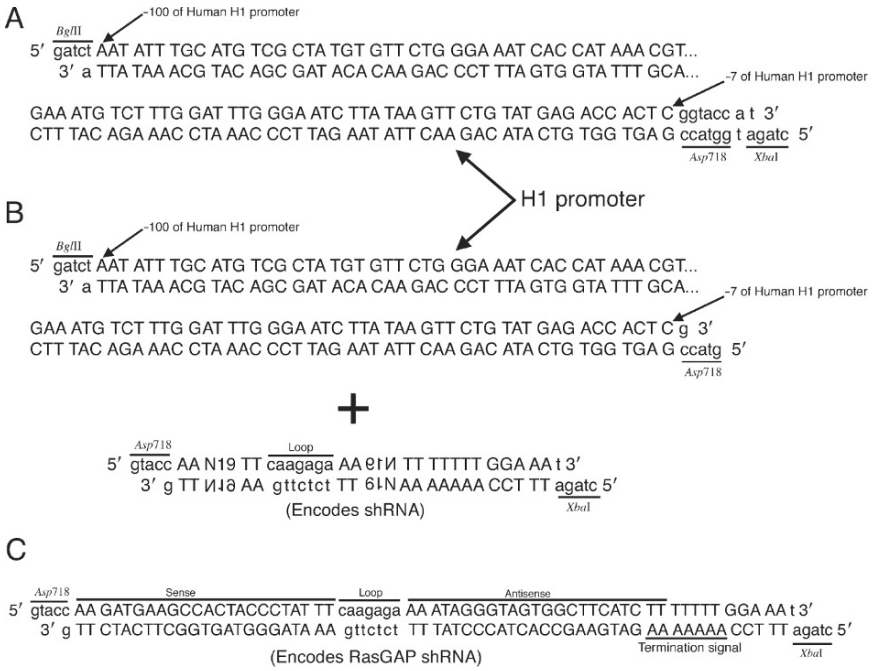

Fig. 3. Oligonucleotides used to make RNAi transgenes. A The nucleotide sequence of two oligonucleotides (107-mers) that, when annealed and cloned into a plasmid, encode for the human H1 promoter. The first six bases of the promoter were replaced with an Asp718 site and the entire product is flanked by overhanging BglII and XbaI sites for ease of cloning. B Two oligos (100-mers) encoding for an alternative H1 promoter construct lacking the 3 XbaI. This can be simultaneously ligated into the BglII-XbaI sites of pcDNA3.1(+) with annealed oligos (two 69-mers) encoding for shRNA. C The exact sequence of two oligos that encode for shRNA that mediates effective RNAi against human and mouse RasGAP. The Asp718 and XbaI linkers, loop, and transcriptional terminal signal are indicated.

and *Xba*I sites of pcDNA3.1(+) (Invitrogen) replacing the CMV promoter (the *Bgl*II site is 5′ to the CMV promoter and *Xba*I is in the multiple cloning site). Sequencing of the promoter and any subsequent shRNA-encoding inserts are essential, as long oligonucleotides often contain synthesis errors.

The U6 RNA pol III promoter (265 bp) may be amplified from human genomic DNA with the following primers, sense 5′ AAGGTCGGGCAGGAAGAGGGCCTA 3′ and antisense 5′ GGTGTTTCGTCCTTTCCACAAGA 3′, and cloned into your vector of interest.

2.1.3. DNA Inserts Encoding shRNA Molecules

The next step is to design and order oligonucleotides against the gene of interest. The design algorithms are presented in detail later (**section 3.1**). Once the oligonucleotides are designed and obtained, an annealing buffer is required. To prepare 10× annealing buffer (1 mL):

Stock	Volume (μL)	Final concentration
1 M Tris.HCl, pH7.5	100	100 mM
0.5 M EDTA, pH8.0	20	10 mM
5 M NaCl	200	1 M
ddH_2O	680	
Total	1 mL	

If the H1 promoter just described is used, the DNA encoding the shRNA molecules can be inserted into the *Asp*718 and *Xba*I sites of the plasmid, or it may be simultaneously inserted with the promoter in a three-way ligation (**Fig. 3B**).

2.2. ES Cell Lines

The R1 ES cell line from the laboratory of Andras Nagy ***(19)*** can support fetal development beyond midgestation and is suitable for analyzing phenotypes up to embryonic day 10.5 (E10.5) of development. The F1 hybrid ES cell lines from the laboratory of Rudolf Jaenisch have excellent developmental potential and, therefore, are good cell lines with which to starth. In particular, the V6.5 hybrid ES cell line (C57BL/6 × 129/Sv) appears especially potent ***(46, 82,83)***.

2.3. Embryo and Mouse Manipulation

A mouse facility and standard equipment to manipulate preimplantation embryos are required ***(84)***. However, a micromanipulator and expertise in blastocyst injections are not required, as all the procedures can be performed by embryo-ES cell aggregations ***(85,86)***.

3. Methods

3.1. Choosing Sequences for Effective siRNAs

Several rules should be considered when choosing the sequence to target. Randomly chosen targets work less than 50% of the time. Following our rules should give one greater than 90% success at knocking down target genes to less than 20% of their endogenous levels and, depending on the gene, sometimes down to undetectable levels. These guidelines have been taken from several publications ***(37, 38, 60, 61, 87)*** and not all need to be satisfied in a single target sequence. The main objective is to bias RISC uptake of the antisense strand at the expense of the sense strand (**Fig. 1**).

3.1.1. The Desired siRNA Product

After Dicer processes shRNA, the final siRNA product is a duplex of at least 21-nt including 2-nt 3′ overhangs (**Fig. 4**). For orientation purposes, $N_{1–19}$ represent nucleotides of the sense strand starting from the 5′ phosphate group and are the complementary nucleotides on the antisense strand. The 3′ end of the antisense strand contains two uracils, since RNA pol III transcribes two thymidines of the polythymidine termination signal. The 3′ end of the sense strand also has two uracils, but this is not essential, as described later. The rules that follow refer to the nucleotides in the sense strand and are not necessarily in order of importance (**Fig. 4**).

1. N_1 is G or C and N_{19} is A or U.
2. G/C content of $N_{1–4}$ is greater than G/C content of $N_{16–19}$.
3. At least three A/U bases in $N_{15–19}$.
4. N_{10} is U.

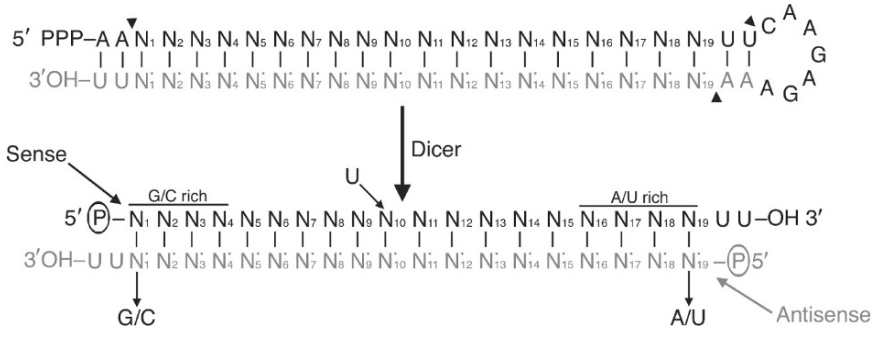

Fig. 4. An shRNA and siRNA molecule. The shRNA has a 7-nt loop and the hypothetical points of Dicer cleavage are indicated by arrow heads. The mature siRNA is a duplex of 21 nt with 5′ monophosphate groups and unphosphorylated 3′ 2-nt overhangs. Nucleotide preferences for effective RNAi are indicated and described in detail in the text.

Satisfying the first three rules should ensure that the free energy at the 5′ end of the antisense strand is higher than the 5′ end of the sense strand, which is important for strand bias in RISC uptake ***(37, 38)*** (*see* **Note 2**). The fourth rule may reflect a preference of RISC endonuclease activity, since it preferentially cleaves target mRNA between the bases complementary to N_{10} and N_{11} ***(88)***. The following rules can be used to *exclude* potential target sequences:

5. A stretch of four or more As or Us.
6. A stretch of four or more Gs or Cs.
7. A GC stretch of more than 9 nt.

The fifth rule ensures that the RNA pol III does not prematurely terminate, and the sixth rule avoids formation of G-quartet structures. The final rule fits with previous observations that high G/C content is detrimental, and it should be between 31 and 52% ($^{6}/_{19}$ and $^{10}/_{19}$) ***(87)***.

3.1.2. Target the Open Reading Frame

Regions of mRNA likely to be bound to regulatory proteins should be avoided. This includes the 5′ and 3′ untranslated regions (UTRs) and about 50–100 bp downstream of the initiation codon. However, targeting the 3′ UTR has worked ***(63)*** and may be useful when trying to rescue a phenotype by reintroducing the targeted gene with a heterologous 3′ UTR.

3.1.3. BLAST

A BLAST search (www.ncbi.nlm.nih.gov/BLAST) should be performed for each sequence selected to make sure only a single gene is targeted. Choose the algorithm "Search for short, nearly exact matches." Three or more mismatches to the closest neighbor are preferable. If only one mismatch is present to another gene, there is a good chance of off-target translation inhibition similar to the activity of miRNAs ***(89)***.

3.1.4. siRNA Design Websites and Programs

Several websites offer to perform many of the preceding activities for you. A very useful one was designed at the Whitehead Institute. The siRNA prediction program can be found under "Tools" at http://jura.wi.mit.edu/bio/. Similar programs are offered by companies (*see* **Note 3**).

For MacIntosh computer users, the iRNAi program is useful for scanning mRNA for potential target sequences. This freeware can be downloaded at www.mekentosj.com. Alternatively, any DNA analysis program with a robust "Search" function can be used to scan the target mRNA/cDNA. Examples of search terms are

A. $AAN_{19}TT$
B. AAN_{21}
C. $AASN_{17}WTT$
D. $AASN_8TN_8WTT$

where *S* is G or C and *W* is A or T. Search term C satisfies rule 1 and search term D satisfies rules 1 and 4. Search term B lacks the final pair of Ts and allows for more flexibility if $AAN_{19}TT$ sequences are rare or not present (*see* **Note 4**). The retrieved sequences can then be scanned for overall G/C content, relative G/C content at each end, and stretches of four or more of the same nucleotide.

3.1.5. Control siRNAs

Scrambling the nucleotides of an siRNA and performing a BLAST search to ensure no genes are targeted was a popular control. Now that siRNA is known to work very well with few, if any, deleterious effects, a better control may be to use an RNAi transgene that knocks down a nonessential or transgenic gene, such as GFP. Alternatively, a target sequence could be chosen that purposefully disobeys these design rules ***(1–4)***. In this control, siRNA is expressed that exactly matches the target sequence but is ineffective at mediating RNAi.

3.1.6. Adding Linkers and the Loop

Once a target region has been decided on, the last procedure is to design the long oligonucleotides with linkers, a loop connecting the sense and antisense strands, and a polythymidine transcriptional termination signal. The size of the loop is not very important, and the linkers depend on into which RNAi transgene the sequence will be cloned.

3.1.7. Working Example: RNAi Transgene Targeting RasGAP

The human *RasGAP* gene (also known as *Rasa1*) is used as an example of going through the preceding rules and designing the final oligos that would be ordered ***(45)***.

1. Obtain the sequence of the human *RasGAP* gene from NCBI (accession number: NM_002890).
2. Using a DNA analysis program, search for sequences that match $AASN_{17}WTT$ and exclude ones that are outside of the open reading frame (bases 119 to 3262). The following eight sequences should be obtained (5 is to the left and the position of the first nucleotide is indicated):

   a. 1581 AA CGTTGGAAAAATTTATATT TT
   b. 1767 AA GAACATTACATCTTTTACT TT
   c. 1798 AA CTCCAGAACAAGCAGAGGA TT
   d. 2460 AA GATGAAGCCACTACCCTAT TT
   e. 2642 AA CACTAATTTAACACACCTA TT

f. 2653 AA CACACCTATTGAACATACT TT
g. 2797 AA GAGTTGTTAGTGGTTTTGT TT
h. 2933 AA CTTAGCAAATCTTGTGGAA TT

The 1581, 1767, and 2797 sequences can be excluded because they have a stretch of four or more As or Ts (rule 5). Sequences 2642 and 2933 can be excluded because the G/C content of $N_{1–4}$ is no greater than the G/C content of $N_{15–19}$ (rule 2). Sequence 1798 is excluded because it does not have at least three A/T bases in $N_{15–19}$ (rule 3). That leaves the following two sequences:

i. 2460 AA GATGAAGCCACTACCCTAT TT
j. 2653 AA CACACCTATTGAACATACT TT

Both fall within the range of an acceptable overall G/C content ($^{9}/_{19}$ for 2460 and $^{7}/_{19}$ for 2653). Sequence 2653 also satisfies rule 4, which states that N_{10} is T/U. A BLAST search revealed that neither sequence has significant homology to other genes. Each of the two sequences is worth testing for efficient knockdown activity. The 2460 sequence exactly matches the mouse *RasGAP* and has been successfully used to knockdown RasGAP to undetectable levels in both human and mouse cells ***(45)***. Although this sequence was chosen using only the $AAN_{19}TT$ criterion at the time, it is satisfying to see that is adheres to rules that were subsequently published.

3. The next step is to add the linkers, loop, complementary strand, and polythymidine termination signal. The example shown here is based on the pSUPER plasmid [32]. A 7-nt loop of CAAGAGA and *Asp*718 and *Xba*I linkers can be added for cloning into the RNAi transgene described previously (**section 2.1.2.** and **Fig. 3**). The final pair of oligonucleotides that would be ordered for the 2460 *RasGAP* target sequence are illustrated in Fig. 3C.

3.2. Constructing the siRNA Transgene

3.2.1. Preparing the Host RNAi Transgene

The host RNAi transgene should be digested with the appropriate restriction enzymes to allow cloning of the annealed oligos encoding for the shRNA. In the preceding example, they would be *Asp*718 and *Xba*I. Some commercial vectors are available only predigested. If an insert is already in the RNAi vector, the plasmid backbone should be gel purified.

3.2.2. Annealing oligos for shRNA

1. Resuspend lyophilized oligos in ddH_2O to a concentration of 100 µM.
2. Set up annealing reaction (20 µL):
   a. 100 µM oligo1 9 µL
   b. 100 µM oligo2 9 µL
   c. 10× annealing buffer 2 µL

3. Put in heat-block at 95°C for 10 min.
4. Transfer to a beaker of microwaved water (500 mL) at 65°C and allow to cool to 30°C or room temperature (2 h).
5. The annealed oligos may be stored at −20°C or directly used for ligation.

3.2.3. Ligation and Transformation

Annealed oligos (1 µL of te preceding sample) and prepared RNAi transgene (50 ng) can be ligated under standard conditions with T4 DNA ligase and transformed into host *E. coli*, such as DH5α. Transformants can be grown up and the RNAi transgene isolated in large quantities with standard plasmid preparation kits.

3.2.4. Sequencing

A restriction digest of the RNAi transgene can be performed to determine if the insert is present. If the insert contains unique restriction enzyme sites, they may also be used for diagnostic screening. However, it is essential to sequence the entire insert and its junctions to make sure no errors in synthesis of the long oligonucleotides have occurred. Since the insert is designed to produce a hairpin structure, there is considerable secondary structure, making it difficult to sequence. Incorporating 5% DMSO into the sequencing reaction can alleviate these problems. The primer used for the modified pcDNA3.1(+) transgene (**section 2.1.2**) was BGH reverse: 5 TAG AAG GCT CAG TCG AGG 3 .

3.3. Generating RNAi Transgenic ES Cells

The procedure to generate RNAi transgenic ES cells is identical to that of any other transgene and has been well described ***(90)***. For the pcDNA3.1(+)-derived RNAi transgene described previously, linearization with *Sca*I before electroporation works well. Other transfection procedures or even targeting the RNAi transgene to a particular locus (e.g., ROSA26) is also acceptable. Unlike homologous recombination to create genetic knockouts, multiple integrations of an RNAi transgene is fine, and may even enhance the knockdown effect.

3.4. Assaying for Knockdown in ES Cell Clones

Since the procedure described here will yield clones with random integrations of the RNAi transgene, there will be varying levels of shRNA expression, due to position effect variegation. It is difficult and unnecessary to quantitatively measure the levels of shRNA in each ES cell clone. The most definitive way to screen ES cell clones for knockdown is by Western analysis of equivalent amounts of protein lysate. This requires the availability of a good antibody that recognizes your protein of interest and that the gene is expressed in ES

cells. FACS analysis can also be used to rapidly screen through ES cell clones. Immunostaining of cultured cells may be performed but may be less definitive. If an antibody is not available, the levels of RNA can be checked by Northern analysis, quantitative real-time RT-PCR, or semi-quantitative RT-PCR. However, a steady-state level of RNA may be present in clones that express no protein, since the transcripts must be synthesized before they are targeted for degradation. The gene of interest may also be epitope tagged and expressed to assay the effectiveness of an shRNA transgene at the protein level ***(91)***.

If the targeted gene is not expressed in undifferentiated ES cells, then it will be necessary to differentiate the parental and RNAi transgenic ES cell clones to a lineage where the gene is expressed to assay for knockdown.

These assays for protein or mRNA expression may be complemented by functional differentiation assays in culture. For example, if the targeted gene has a suspected role in cardiomyocyte specification, differentiation toward this lineage may yield a lower frequency of beating cultures.

3.5. Production of ES Cell-Derived Embryos by Tetraploid Complementation

Once ES cell clones with defined levels of knockdown have been identified, they can be used to directly produce embryos by tetraploid aggregation (**Fig. 2**). This is the most direct and rapid way to assess the function of the gene *in vivo* (*see* **Notes 5** and **6**). This procedure requires expertise in preimplantation embryo manipulation and has been thoroughly described in several publications ***(19,86)***; detailed protocols can be found in ***(84)*** and at www.mshri.on.ca/nagy/Tetraploid/Tetra.htm. The major difference from routine blastocyst injections or morula aggregations is the production of tetraploid embryos. This is most often performed by electrofusion of two-cell stage embryos. The fused embryos should be allowed to develop to the eight-cell stage before aggregation with ES cells or to the blastocyst stage before ES cell injection.

3.6. Phenotypic Analysis of Knockdown Embryos

This analysis varies from gene to gene. It is important to know when and where the gene of interest is expressed to determine at which stage to dissect embryos. A good starting point is to dissect knockdown embryos approximately one or two days later than the earliest detectable expression in the embryo proper. If the gene is expressed in the trophoblast or extraembryonic endoderm, these lineages are no affected, as ES cells do not contribute to them. This is often beneficial, since the possibility of knockdown embryos dying of placental failure is removed.

4. Notes

1. For other sources of commercial plasmid-based siRNA systems see Upstate Cell Signaling Solutions (www.upstate.com/sirna), GenScript Corporation (www.genscript.com/rnai), Invivogen (www.invivogen.com), Imgenex (www.imgenex.com), BD Biosciences (www.bdbiosciences.com), Promega (www.promega.com), or Oligoengine (www.oligoengine.com); other sites may be found by using the key words "plasmid based siRNA" in an Internet search engine.
2. The free energy at the 5 end of the antisense strand can be increased by introducing a less stable G:U wobble. A C in the last four bases ($N_{16–19}$) of the sense strand may be substituted for a U. The antisense strand should not be altered, since it will no longer exactly match its target mRNA. Other ways to increase the instability of the 3 sense strand, such as mismatches, can also be experimented with ***(37)***.
3. Web-based siRNA search engines are available from Ambion (www.ambion.com/techlib/misc/siRNA_finder.html); and Oligoengine (www.oligoengine.com) offers design tools as well, but registration is required.
4. If the order of the transgene elements are sense-loop-antisense, then the initial two As will still be part of the poly-T termination signal and produce a 2-U 3 overhang. The 2-nt overhang at the other will not be Us, but this is not important for effective knockdown.
5. Knockdown ES cells can also be used for chimeric analysis ***(92)***. For such experiments, the ES cells can be combined with diploid embryos and their ability to contribute to particular lineages can be investigated. As with all chimera studies, either the ES cell or the host embryos (or both) must carry a ubiquitous genetic marker just as EGFP or *lacZ* ***(84)***.
6. Knockdown ES cells can also be used to make chimeric mice and bred for germline transmission ***(93)***. Since effective RNAi transgenes behave as dominant mutations, the targeted gene must not be essential for embryonic development to obtain live pups and mice. If germ-line transmission is obtained for a moderately effective RNAi transgene, it may be bred to homozygosity in an attempt to further reduce the levels of targeted mRNA. A phenotypic difference between one and two copies of an RNAi transgene has been observed in the *Drosophila* eye ***(94)***. Distinguishing the hemi- and homozygous animals in these crosses is complicated and may require out-crossing. Also, weakly effective RNAi transgenes may not show an enhancement in the homozygous state.

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12

Mouse Primordial Germ Cells

Isolation and In Vitro Culture

Patricia A. Labosky and Brigid L. M. Hogan

1. Introduction

Since the original publication of this chapter on the manipulation of primordial germ cells (PGCs) into embryonic germ (EG) cell lines, the field has progressed considerably. Perhaps the most exciting advance has been the derivation of pluripotent human EG cell lines, using methods similar to those outlined here ***(1)***. Cells derived from these human EG lines can stimulate partial motor recovery in rats paralyzed by viral-induced motor neuron injury ***(2)***. This provides an exciting example of the therapeutic potential of human stem cell research. However, it is likely that the effect is indirect, due to the enhancement of host neuron survival and function by growth factors produced by the grafted cells. Another advance has been the use of EG lines to follow the process of erasure of genetic imprinting that normally occurs during the migration of PGCs to the gonad. However, caution is again needed, since EG cells grown *in vitro* may not accurately reflect all the imprinting changes taking place *in vivo* ***(3)*** and techniques are not yet available to assess the imprinted status of genes in single PGCs. Recent successes in generating gene expression profiles of single cells in the region of the mouse embryo where PGCs are specified have identified PGC-specific mRNAs ***(4)***. Comparative transcriptome analysis of early mouse embryos, ES cells, and EG cells highlighted differences and similarities in their global gene expression arrays and identified "signature" genes for each category ***(5)***. Finally, porcine EG cell lines have been derived and used for the generation of chimeras. However, their

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contribution to chimeras is not extensive and efforts are ongoing to improve the culture conditions used to derive the EG cell lines ***(6–9)***.

Primordial germ cells in the embryo give rise to functional gametes in the adult animal. Considering their importance in the continuation of the species, it is no wonder that there is much interest in understanding the biology of these highly specialized cells. Much has been learned from the analysis of mouse mutants that are defective in germ-cell proliferation and survival. However, given the relative inaccessibility of PGCs in the embryo, the ability to culture these cells *in vitro* has led to a greater understanding of the mechanism by which growth factors control their proliferation, migration, and differentiation *in vivo*. This chapter outlines methods to obtain PGCs from various embryonic stages, culture them *in vitro*, stain them for endogenous alkaline phosphatase activity, and finally generate embryonic germ cell lines.

Successful *in vitro* culture systems for PGCs include the use of a mitotically inactivated feeder layer of somatic cells. However, even under these conditions, the number of surviving PGCs in culture decreases dramatically after 5 d. The addition of various growth factors to the culture medium can greatly increase the survival and proliferation of PGCs *in vitro* and, in some cases, lead to the generation of cell lines resembling blastocyst-derived embryonic stem cells (ES cells). These growth factors include stem cell factor (SCF), leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF). SCF, which is also known as *mast cell growth factor*, *kit ligand*, and *Steel factor* is encoded at the *Steel* locus. Embryos mutant for SCF have PGCs, but they fail to divide and resulting *Steel* mutant mice are sterile. SCF is a transmembrane protein that can be alternatively spliced to produce a soluble form. When PGCs are cultured on feeder cells that express the membrane-bound form of SCF, their survival and proliferation are greatly enhanced ***(10–12)***. Another factor made by the feeders is LIF, also known as *differentiation inhibiting activity* (DIA). Researchers have shown that, by using LIF alone, it is possible to establish and maintain pluripotent blastocyst-derived ES cell lines ***(13–15)*** and soluble LIF stimulates PGC proliferation in the presence of other factors ***(11, 12)***. The receptor for LIF is made up of two subunits, one that binds LIF directly and the other that is a signal transducer, gp130. The gp130 subunit is shared by other cytokines, such as oncostatin M (OSM) and ciliary neurotropic factor (CNTF). OSM and CNTF can substitute for LIF in PGC culture, and neutralizing antibodies against gp130 blocks PGC survival in culture ***(16, 17)***, suggesting a role for this receptor in PGC survival and proliferation *in vivo*. Tumor necrosis factor-α (TNF-α) selectively stimulates the proliferation of early PGCs ***(18)***, interleukin-4 (IL-4) is a survival factor ***(19)***, and retinoic acid is a growth activator of PGCs ***(20)***. Also agents, such as dibutyryl cAMP and forskolin, that raise the intercellular concentrations of cAMP also stimulate the proliferation of PGCs *in vitro* ***(21)***. The *in vivo* functional significance of these growth factors is not yet known.

Long-term proliferation of PGCs was demonstrated by the addition of bFGF to a mixture of SCF and LIF in the *in vitro* culture system outlined in this chapter ***(22–25)***. These growth factors act directly on PGCs and do not indirectly affect the feeder layers to produce more or different growth factors ***(16)***. EG cell lines derived from PGCs have many characteristics of ES cells. They can form embryoid bodies *in vitro*, produce teratomas in nude mice, and even contribute to the germ line of chimeric mice ***(22–26)***. However, EG cells are not identical to ES cells with regard to their genomic imprinting. These cell lines may be used in the future to study genomic imprinting, the potency of PGCs at various stages during embryogenesis, and finally as a route to generate pluripotent stem cells from species other than mice.

2. Materials

2.1. Isolation of Primordial Germ Cells

1. Mice: ICR mice are an outbred strain that can be used to generate large numbers of PGCs. These can be obtained from a number of suppliers, including Taconic Farms (Germantown, NY, 1-888-822-6642) or Harlan Sprague-Dawley (Indianapolis, IN, 1-800-793-7287). Noon on the day of plug is 0.5 d postcoitum (dpc). *See* **Note 1** for discussion of different mouse strains.
2. Dissecting tools: Fine scissors and forceps (watchmaker's #5) for the dissections. Diamond pen for cutting glass pipets. These can be ordered through Fisher (Atlanta, GA, 1-800-766-7000) or VWR (Marietta, GA, 1-800-932-5000). More specialized dissection equipment is available from Roboz Surgical Instruments (Rockville, MD, 1-800-424-2984) or Fine Science Tools (Foster City, CA, 1-800-521-2109).
3. Tissue-culture equipment, dishes, pipets, and so on can be obtained through Fisher or VWR. Manufacturers, include Corning, Falcon, and Nunc.
4. Tissue-culture reagents can be purchased from Invitrogen or Grand Island Biological Company (1-800-828-6686). Trypsinization solution is 0.25% trypsin, 1 mM EDTA in Hank's balanced salts (cat. no. 25200-056). Dulbecco's phosphate-buffered saline (PBS) is 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8 g/L NaCl, 1.15 g/L Na_2HPO_4, and 2.16 g/L Na_2HPO_4 $7H_2O$. (cat. no. 14190). Medium is Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose (cat. no. 11965-084). For the culture of PGCs, this medium is further supplemented with 0.01 mM nonessential amino acids (cat. no. 11140-019), 2 mM glutamine (cat. no. 25030-016), 50 µg/mL gentamycin (cat. no. 15750-011), 0.1 mM 2-mercaptoethanol (cat. no. 21985-023), and 15% fetal bovine serum (ES cell tested, from HyClone Laboratories, Logan, UT, 1-800-492-5663). For the routine culture of fibroblast feeder cells, the DMEM with glucose is supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 µg/mL penicillin–streptomycin (Gibco cat. no. 15070-014). Collagenase–dispase can be purchased from Sigma (1-800-325-3010) (cat. no. C3180), made up to 1% (1 g/100 mL) in either DMEM or PBS, and stored in aliquots at −20°C. This is a 10× concentration. Dimethyl sulfoxide (DMSO) for

freezing cell lines is available from Fisher Scientific (cat. no. BP231-1). Bovine serum albumin (BSA) for dissections is available from Sigma (cat. no. A 9647).

5. Feeders for primary cultures are Sl4-m220 cells (from David Williams, Indiana University School of Medicine), whereas those for the secondary culture are generated from mouse embryos (*see* **Notes 2** and **3** for more details about feeder cells). Sl4-m220 is a cell line isolated from homozygous Steel mutant embryos and stably transfected with the membrane-associated form of SCF. Growth factors necessary for the generation of EG cell lines are LIF (ESGRO™, Gibco cat. no. 13275-011), bFGF (also called FGF2) (Gibco cat. no. 13256-029), and SCF (also known as *Steel factor*, *mast cell growth factor*, and *c-kit ligand*; R&D Systems, Minneapolis, MN, 1-800-343-7475, cat. no. 455-MC. *See* **Note 3**.
6. Feeder layers are mitotically inactivated by γ-irradiation or by mitomycin treatment. Mitomycin can be obtained from Sigma (cat. no. M0503), and a 1 mg/mL stock is stored frozen in PBS in a dark container. Mitomycin C is very toxic, and care should be used when handling (gloves, mask, and so on) (*see* **Note 4**).
7. Dissecting microscope with low-power objectives (10×–80×).
8. A mouth-controlled pipet should be used for most of the manipulations. They can be purchased from Sigma cat. no. A5177.

2.2. Staining for Endogenous Alkaline Phosphatase Activity

1. Fixative (4% paraformaldehyde in PBS, Fisher cat. no. 04042-500) (*see* **Note 5**).
2. Alkaline phosphatase staining solution: 25 mM Tris-malate, pH 9.0, 0.4 mg/mL Na-α-napthyl phosphate (Sigma cat. no. N7255), and 1 mg/mL fast red TR salt (Sigma cat. no. F2768), 8 mM MgCl2. This staining solution must be made up fresh before use (<30 min). The color reaction is dependent on the high pH of the staining solution.
3. PBS (same as previously).

2.3. Determination of the Sex of Embryos or EG Cell Lines by PCR for Zfy Gene

1. Lysis buffer, 100 mM EDTA, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% SDS. Proteinase K should be added at 0.5 mg/mL immediately before use. Proteinase K (Sigma cat. no. P2308) is made up in water at 10 mg/mL, self-digested at 37°C for 60 min and stored in aliquots at −20°C.
2. Oligonucleotide primers. 5′-primer AAGATAAGCTTACATAATCACATGGA, 3′-primer CCTATGAAATCCTTTGCTGCACATGT. A stock solution of these primers is 100 µM and should be kept frozen in aliquots at −20°C.
3. 10× PCR buffer, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 25 mM $MgCl_2$, 0.1% gelatin. Store frozen at −20°C.
4. dNTPs (Promega 1-608-274-4330, cat. no. C1141). The purchased dNTP stock is 100 mM and should be kept frozen at −20°C. 10× working solution 10 mM each dNTP) is made by combining 10 µL of each of the four stocks and adding 60 µL of water.

5. *Taq* polymerase (Stratagene 1-800-424-5444, TaqPlus™ DNA Polymerase cat. no. 600203). As with any enzyme, *Taq* should be stored at −20°C in a nonfrost-free freezer.

3. Methods

3.1. Isolation of Primordial Germ Cells

Primordial germ cells can be obtained from different stages of embryos. **Section 3.1.1** describes how to dissect early embryos (8.5 dpc) to obtain early migratory PGCs, whereas **section 3.1.2** describes how to isolate the genital ridge from later embryos (11.5 dpc and 15.5 dpc) to obtain later PGCs. **Section 3.1.3** describes how these isolated tissues should be processed for *in vitro* culture. Further information about the numbers of PGCs and their location in the embryo at various times can be found in ***(27,28)***.

Since the ultimate goal of these dissections is to generate PGCs for *in vitro* culture, care should be taken to maintain sterility at all times. Dissections are done using sterile PBS with 0.1% BSA (0.1 g/100 mL) and sterilized instruments.

3.1.1. Dissection of 5–8 Somite Stage Embryos

In the 8.5 dpc embryo, most of the PGCs are located in the posterior third of the embryo at the base of the allantois. After the embryo is removed from the uterus, decidual tissue, and yolk sac, it will appear as in **Fig. 1A**. Details of this dissection can be found in ***(29)***. Briefly, the embryos are removed from the uterus, and the broader end of the decidua is cut away. The embryo must then be gently shelled out of the remaining decidual tissue and yolk sac. To isolate large numbers of PGCs, the anterior two thirds of the embryo and the allantois are discarded, as indicated in **Fig. 1A** and **1B**. The remaining tissue is then processed as in **section 3.1.3**.

3.1.2. Dissection of Genital Ridges and Embryonic Gonads

Alternatively, if later PGCs are desired, they can be obtained from the developing genital ridge. After the 8.5 dpc stage, the PGCs become incorporated into the hindgut of the embryo. They subsequently migrate into the dorsal mesentery and then the genital ridge by 10.5–11.5 dpc. By this stage of embryogenesis, the genital ridges are attached to the dorsal body wall on either side of the spinal column. Their position inside the embryo is illustrated in **Fig. 1C**. At 10.5 dpc, it is often difficult to differentiate between a developing testis and a developing ovary in the light microscope as shown in **Fig. 1D**. To remove the genital ridges, the embryo is dissected out of the uterus and the extraembryonic tissues are removed. The embryo should be euthanized by pinching off the head with forceps. Next, the intestines and liver are removed from the body cavity.

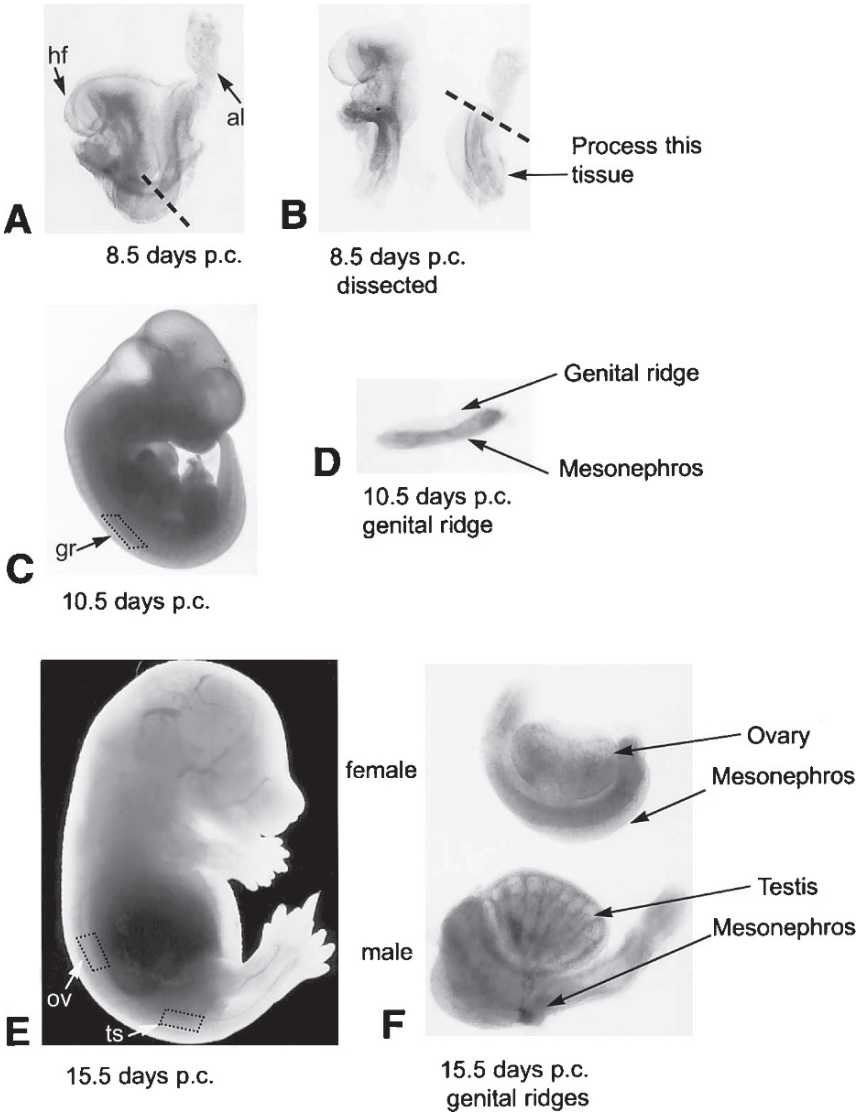

Fig. 1. (**A,B**) A 8.5 dpc embryo dissected free of the uterus, decidual tissue, and extra-embryonic membranes. At this stage, the majority of the PGCs are located at the base of the allantois. In order to isolate the PGC-containing tissue, the embryo is cut at the position of the dashed lines and the indicated tissue processed further. (**C**) An embryo at 10.5 dpc with the position of the genital ridges indicated by the box. (**D**) An isolated 10.5 dpc genital ridge showing the position of the menonephros relative to the developing gonad. (**E**) An embryo at 15.5 dpc with the positions of the developing ovary or testis (depending on the sex of the embryo) illustrated by the boxes. (**F**) Isolated gonads from 15.5 dpc embryos. Note the well-differentiated tubules in the developing testes. Abbreviations: al, allantois; gr, genital ridge; hf, head folds; ov, ovary; ts, testes.

The genital ridges lie attached to the dorsal wall of the body cavity, adhered tightly to the developing mesonephros. A genital ridge from a 10.5 dpc embryo is shown in **Fig. 1D.** The genital ridge and the mesonephros are gently teased apart with forceps, and the genital ridge is processed as in **section 3.1.3.** Be careful to avoid disrupting the integrity of the genital ridge, since PGCs can flow out if it is poked or torn.

Using a similar approach, germ cells can be isolated from later developing gonads. Shown in **Fig. 1F** are testes and ovaries from 15.5 dpc embryos. At this stage, it is now easy to distinguish between a testis and an ovary, since the tubules in the testes are well differentiated. Again, the embryo should be sacrificed by removing the head, and then the intestines and liver are removed to isolate the gonads. Testes are located in the caudal region of the pelvis (above the hips), but ovaries are located further rostrally, near the kidneys. Their location is illustrated in **Fig. 1E.**

3.1.3. Isolating Germ Cells for Culture

Depending on the experiment, PGC-containing tissues are either pooled at this stage or treated individually. Isolated tissues are kept on ice while the remaining dissections are completed. Using a mouth pipet (*see* **Note 6**), the isolated tissue is rinsed through at least two 30-µL drops of PBS, then placed into a drop of trypsin, being careful to minimize the amount of PBS transferred so as not to dilute the trypsin. The tissue is mechanically disrupted by tearing with forceps, and the sample is incubated at 37°C for 5 min. Then, the tissue is again mechanically disrupted using a finely pulled Pasteur pipet. The internal diameter of the pipet should be approximately 75% smaller than the tissue being treated. It is often necessary to use a series of pipets, decreasing in diameter, as the tissue is disrupted. The internal diameter of the pipets range between 200 µm for the largest genital ridges down to 30–50 µm for single cells. Depending on how well the cells are disrupted, the plate can be returned to the 37°C incubator for an additional 5 min. This is repeated until the tissue has been reduced to a single-cell suspension. This should be fairly easy for a 8.5 dpc embryo and a 11.5 dpc genital ridge. However, the size and extent of development of a 15.5 dpc genital ridge makes more vigorous enzymatic treatment necessary. In this case, a mixture of collagenase and dispase can be used at 37°C. Again, it is best to disrupt the tissue mechanically as much as possible before enzymatic treatment and to check the tissue at 5-min intervals, incubating for up to 30 min total. Despite these efforts, it is often difficult to dissociate the testes tubules, and any remaining large clumps of intact tissue should not be plated out in the next step. However, plenty of germ cells are released from the tubules and can be collected with a mouth pipet (*see* **Note 7**).

Once the tissue is primarily a single cell suspension of somatic cells and PGCs, the cells are plated onto mitotically inactivated feeder layers. One kind

of feeder layer that can be used is Sl4m220 fibroblasts. Alternatively, a mouse embryonic fibroblast line, STO, can also be used (*see* **Note 3**). Feeder layer are mitotically inactivated with either mitomycin C or irradiation (*see* **Note 4**) and plated onto gelatin coated tissue culture dishes at a density of 1.5µ 10^5 cells for each well of a 24-well dish (1 cm in diameter). For convenience, feeder layers are usually prepared the day prior to the dissection. However, most feeder cells have adhered by 2–4 h after plating, so the feeder plates could be prepared the same day as the dissection.

At this stage of the procedure, cells from the equivalent of one 8.5 dpc embryo are plated into each well. Similarly, one tenth of a 11.5 dpc or 15.5 dpc genital ridge is placed into each well. Growth factors are added as desired. If the goal is to produce EG cell lines, the growth factors should be added in the following concentrations: 20 ng/mL LIF, 60 ng/mL SCF, and 20 ng/mL bFGF. All cultures are maintained in a humidified incubator at 37°C with 5% CO_2. Cultures are fed every day with fresh medium and growth factors and monitored for possible yeast or bacterial contamination. It beyond the scope of this chapter to describe the effects of all tested growth factors and cytokines on the growth and differentiation of PGCs. An excellent review of these experiments is contained in ***(30)*** and the references therein.

If the generation of EG cell lines is desired, a secondary culture is generated from the primary culture after 10 d. It is a good idea to stain some portion of the primary cultures for alkaline phosphatase activity to be sure that the PGCs are present and growing before proceeding further. If there are no alkaline-positive cells in the primary culture, the experiment should be stopped at this time. To begin a secondary culture, primary cultures are washed in PBS and trypsinized for 5 min at 37°C. Add an equal volume of serum-containing culture medium to inactivate the trypsin, and pipet up and down 5–10 times to obtain a single-cell suspension. This suspension is then distributed onto fresh feeder plates at a 1:4–1:10 dilution. Secondary cultures need to be monitored daily for the appearance of EG colonies. These colonies can then be isolated and grown as cell lines, although it is not clear that they are clonal and will undergo changes in culture ***(24)***. Once EG cell lines are generated, they can be grown in the same manner as ES cell lines on feeder layers from either primary mouse embryo fibroblasts or STO fibroblasts, with only LIF added to the medium, since bFGF and SCF are no longer necessary. Single-cell suspensions of individual cell lines should be frozen at low passage numbers in a freezing medium of 10% DMSO, 30% fetal bovine serum in culture medium, and stored indefinitely under liquid nitrogen. It should be noted that, despite many attempts by ourselves and others ***(24)***, EG cell lines have not been generated with PGCs from late genital ridges (over 12.5 dpc).

3.2. Staining for Endogenous Alkaline Phosphatase Activity

The yield and growth of PGCs can be measured by staining the cultures for endogenous alkaline phosphatase (AP) activity. Unfortunately, the cells must be fixed to stain for AP; it is a good idea to have plenty of starting cultures so that a few wells can be stained every day to monitor PGC growth.

1. Wash cultures twice in PBS. Washing is done by adding fresh PBS, swirling the plate gently, removing the PBS, and adding the fresh PBS. A long incubation is not necessary.
2. Fix cultures in paraformaldehyde for 20 min at room temperature (*see* **Note 5**).
3. Wash twice in PBS.
4. Wash once in 25 mM Tris-malate, pH 9.0.
5. Stain for 15–20 min at room temperature, or until PGCs are a reddish-brown color. This can be monitored in the microscope.
6. Wash twice in PBS, and leave cultures in liquid to photograph.

3.3. Determination of the Sex of Embryos or EG Cell Lines by PCR for Zfy Gene

Depending on the nature of the experiments, it may be desirable to know the sex of either the embryos used to culture PGCs or of any EG cell lines generated. This can be done easily by using PCR to amplify sequences found only on the Y chromosome ***(24, 31)*** (*see* **Note 8**).

1. To generate DNA samples from embryos or cell lines, tissue is incubated in lysis buffer with 0.5 mg/mL proteinase K overnight at 56°C. On the next day, samples are extracted once with phenol, once with phenol:chloroform:isoamyl alcohol (25:24:1), and once with chloroform:isoamyl alcohol (24:1), and then ethanol precipitated. References for these molecular biology techniques can be found elsewhere in this volume or in ***(32)***.
2. PCR reactions are performed in a temperature cycler with 400 ng of genomic DNA as a template. Reactions are set up as follows:
   a. X µL DNA (400 ng).
   b. 5 µL dNTPs (10 mM each nucleotide).
   c. 5 µL 10× PCR buffer.
   d. 1 µL each primer.
   e. 1 µL *Taq* (diluted 1:5 in 1× PCR buffer).
   Water to 50 µL.
3. Reactions are cycled at 95°C for 45 s, 62°C for 25 s, and 72°C for 1 min for a total of 30 cycles.
4. Run one tenth or more of the completed reaction on a 1% agarose gel to detect the presence of a 600-bp reaction product indicating the presence of a Y chromosome.

4. Notes

1. ICR or CD-1 mice have been used for many PGC studies. However, depending on the objective of the experiment and especially if EG cell lines are to be generated,

it may be desirable to use inbred mouse strains, such as C57BL/6 or 129/Sv, to be assured of a uniform genetic background. Additionally, both strains have been used to generate EG cell lines that will contribute to the germ line of chimeras ***(24–26)***. Inbred mouse lines, such as C57BL/6 and 129/Sv, are available from Taconic Farms, Harlan Sprague-Dawley (section 12.2.1, item 1), and The Jackson Laboratories (Bar Harbor, MA, 1-800-422-6423).

2. Primary mouse embryonic fibroblasts (mefs) can be generated from embryos between 12.5 and 14.5 dpc, but with 13.5 dpc embryos giving the highest yield of cells. Embryos are dissected free of their extraembryonic membranes and the heads, limbs, and visceral organs removed. The carcasses are then passed five times through an 18-gauge needle (approximately 5 embryos/syringe) in approximately 3 mL of PBS, and the resulting cell mixture is plated into a 150-mm tissue-culture dish with fibroblast culture medium. After 3 d, the cultures are trypsinized and replated at a dilution of 1:4, and this secondary culture can then be mitotically inactivated or cultured further to generate more cells. These feeders can be frozen in 10% DMSO/20% fetal bovine serum in DMEM and stored indefinitely in liquid nitrogen. See section 2.1, item 4 for a description of tissue-culture reagents and suppliers.
3. It is absolutely essential to use feeder cells that are producing SCF on their cell surface. If Sl4m220 cells are not available, STO fibroblasts (American Type Culture Collection (ATTC)) can be used ***(23)***. STO cells are available from the ATTC (1-800-638-6597, cat. no. ATCC CRL 1503). Primary mefs do not make sufficient amounts of SCF to allow the PGCs to survive and divide ***(25)***. It also may be important to use species-specific growth factors. For example, rat SCF can be used to promote the survival mouse PGCs, but human SCF will not affect the growth of mouse PGCs. This may be a limiting factor for the generation of EG cell lines in species other than mouse, but this information is primarily anecdotal.
4. To inactivate feeder cells mitotically, use >5000 rad of γ irradiation. Alternatively, mitomycin C can be added to the medium at 10 µg/mL and incubated in the 37°C incubator for 2–3 h. The cells are then washed very well with PBS (five changes of PBS), and trypsinized and plated as usual. Both methods are equivalent, but irradiation requires an expensive and specialized machine, so most investigators use mitomycin C. It is important to take safety precautions with mitomycin C (wear gloves and a mask), since it is very toxic.
5. Paraformaldehyde must be dissolved in PBS and heated to 65°C before it will go into solution. Care should be used when handling paraformaldehyde to avoid contact with fumes as well as the liquid or powder form.
6. For many manipulations of the starting cell cultures, a finely pulled Pasteur pipet is used with mouth control. It generally takes some practice both to use the mouth pipet and to pull the Pasteur pipets. The long, thin end of the pipet is broken off the wider part by using a diamond pen to score the glass. Then, the center of the long, thin part is softened in the small flame of a microburner or alcohol burner (a traditional bunsen burner produces too violent a flame for this purpose), removed quickly from the flame, and pulled in one quick motion. Score the thin glass between the two ends with a diamond pen and break the glass, generating blunt ends. If necessary, the ends can be flame polished to make a smoother opening.

Glass capillary tubes can also be used for this. The internal diameter of the pipet varies with the task, so it is often a good idea to pull many pipets of various sizes (from 30 to 200 µm internal diameter). It is also important that these pipets be kept sterile so that the resulting cultures do not become contaminated. This is most easily accomplished by making the pipets shortly before use and keeping the pipets stored in a 150-mm tissue-culture dish during the dissection.

7. Most of the dissections are done with bright-field illumination, but at the end of the tissue dissociation, the use of dark-field illumination makes it easiest to see single cells. This is especially important to retrieve single cells from dissociated genital ridges.
8. PCR for the *Zfy* gene detects the presence of a Y chromosome. However, it should be noted that an EG cell line that is negative for this PCR product may actually be an XO cell line, not a normal XX female cell line. It would be necessary to perform karyotype analysis to determine the difference between an XO and an XX cell line accurately.

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13

Gene Transfer to the Rodent Embryo by Retroviral Vectors

Marla B. Luskin

1. Introduction

Over the last 20 years investigators studying vertebrate development have capitalized on the use of retroviral-mediated gene transfer to determine the lineage relationships of diverse cell types, particularly in many regions of the mammalian central nervous system (for review, *see* **ref.** ***1***). Whereas an intraperitoneal or intrauterine injection of cell proliferation markers such as tritiated thymidine or bromodeoxyuridine suffices to examine the birthdates of cells, many fundamental questions dealing with the formation of a structure in the mammalian embryo often necessitate performing intrauterine surgery to introduce genes by retroviral or other viral vectors. As retroviral vectors can be used not only to study lineage ***(2)***, but also to introduce genes to perturb development ***(3–5)***, the methods for delivering retroviruses or other viruses into the developing mammalian embryo will be in increasing demand. This chapter describes a set of procedures to generate and introduce retroviral vectors into rodent embryos.

Retroviruses only integrate into the DNA of replicating cells ***(6)***. As a result of this chromosomal integration, the progeny of the infected cell inherit the retroviral DNA. Recombinant retroviruses have been engineered to eliminate the viral structural genes. Thus, unlike wild-type retroviruses, these recombinant retroviruses deliver exogenous genes to target cells, but cannot replicate on their own and infect unrelated cells. These attributes make replication-defective recombinant retroviruses ideal for use as tracers of cell lineage during development. In addition, a replication-defective recombinant retrovirus encoding an exogenous marker gene is a useful tool because it can be introduced into dividing cells at virtually any stage of development and, unlike many other cell

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proliferation markers, no dilution of the exogenous gene occurs as a function of increasing numbers of cell division.

To generate infectious retroviral particles for use in experiments, retroviral producing cell lines are utilized. These cell lines are made by introducing the retroviral plasmid DNA into a packaging cell line which has been engineered to synthesize all the proteins required for viral assembly ***(7)***. Packaging cell lines are either ecotropic or amphotropic depending on the type of viral envelope proteins they produce. The envelope proteins are critical for determining the host range of cells which a retrovirus can infect; ecotropic retroviruses infect only rat and mouse cells, whereas amphotropic retroviruses infect cells from a broader variety of species. To generate retroviral particles, one can either use a stable producer cell line or utilize a transient transfection technique of highly transfectable packaging cells ***(8)***. A stable producer cell line offers the simplicity of thawing identical cell aliquots, but generates infectious retroviruses with relatively low titers (10^4–10^6 infectious particles/mL). The transient technique generates short-lasting producer cells, but production of higher titers (>10^7 infectious particles/mL). In either case, ecotropic packaging cell lines are used for the techniques described in this chapter.

The first retroviral lineage tracers to be used contained the reporter gene *Escherichia coli* β-galactosidase (*lacZ*), whose expression was detected histochemically at the light microscopic level ***(9,10)***. The subsequent detection of the lacZ histochemical reaction product at the ultrastructural level offered several advantages ***(11)***. Among them, it could be used to determine more definitively cell phenotype. The detection of *lacZ* at the ultrastructural level in combination with the use of other antibodies further expanded the types of questions that could be addressed ***(12)***. Furthermore, as better antibodies to *lacZ* became available, the expression of *lacZ* could be routinely detected immunohistochemically at the light microscopic level ***(13)***, opening up the possibility of using it in conjunction with other antibodies in double- and triple-labeling procedures ***(14)***. To facilitate the use of retroviral vectors encoding the gene for *lacZ* to answer more questions, highlights of procedures used to detect *lacZ* histochemically and immunohistochemically, at the light and ultrastructural level, are provided. These techniques can be applied to studies *in vivo* as well as *in vitro*.

2. Materials

All solutions should be prepared to either tissue culture or molecular biology standards. Prepare solutions using double-distilled water unless otherwise noted. For cell culture, all solutions and materials need to be sterile. Wherever possible, reagents that have been tissue culture or molecular biology tested by the manufacturer are recommended. The use of sterile solutions when performing surgical procedures is also advantageous.

2.1. Production of Supernatant Containing Infectious Retroviral Particles

2.1.1. If Using A Stable Producer Cell Line

1. Producer cell line for generation of ecotropic retrovirus encoding *lacZ:* CRE BAG 2 (American Type Culture Collection, Accession Number CRL 1858).
2. Growth media (GM, store at 4°C): The following are added to DMEM (formulation containing 4500 mg/L glucose and 2 m*M* L-glutamine) to give the final indicated concentrations: 10% fetal bovine serum (FBS); 100 U/mL penicillin; 1000 µg/mL streptomycin.
3. Passaging cells: Phosphate-buffered saline-calcium and magnesium free (PBS-CMF), pH 7.4, Trypsin (0.05% trypsin/0.53 m*M* EDTA).
4. Freezing media: 90% calf serum (*see* **Note 1**) and 10% dimethylsulfoxide (DMSO).

2.1.2. If Using Transient Producer Cells

1. Purified retroviral vector DNA: A number of retroviral vectors are available. Appropriate vectors should be chosen according to the needs of the individual investigator. These vectors differ in the promoter driving the gene of interest, and the presence or absence of drug resistance genes. Similarly, different reporter genes are available, such as *lacZ* and alkaline phosphatase. The use of a *lacZ* reporter gene is detailed here.
2. Ecotropic envelope-expressing packaging cell line: Bosc 23 (American Type Culture Collection, Accession Number CCRL 11270). For culture of these cells, the same reagents as detailed in **Subheading 2.1.1., items 2–4** are needed.
3. Transfection reagents:
   a. 25 m*M* Chloroquine stock solution in PBS-CMF (*see* **Note 2**). Filter through a 0.2-µm disposable sterile filter unit and store at −20°C.
   b. 2X *N*,*N*-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid (BES): 50 m*M* BES, 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4. The final pH should be 7.0 ± 0.05. Filter through a 0.2-µm disposable sterile filter unit and store at 4°C.
   c. 2 *M* $CaCl_2$: Filter through a 0.2-µm disposable sterile filter unit and store at −20°C.
   d. 8 mg/mL polybrene stock solution in PBS. Filter through a 0.2-µm disposable sterile filter unit and store at −20°C.

2.1.3. For Either Stable or Transient Methods

1. Harvest of supernatant containing retrovirus:
   a. 0.45 µm Disposable sterile syringe filters with cellulose acetate membrane.
   b. Cryogenic vials with gasket seal closure.
2. Enzymatic stain for *lacZ* activity:
   a. 0.1 *M* sodium phosphate buffer, pH 7.2.
   b. Fixative: 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 *M* sodium phosphate buffer, pH 7.2. For 400 mL: Heat 200 mL of 0.1 *M* sodium phosphate buffer and 16 g of paraformaldehyde in a fume hood with stirring until

dissolved (*see* **Note 3**). Add 8 mL of 25% glutaraldehyde stock and the remaining 192 mL of 0.1 *M* sodium phosphate buffer. Store at 4°C.

c. 40 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in DMSO. Store at −20°C.

d. Staining buffer (*see* **Note 4**): 5 m*M* $K_3Fe(CN)_6$; 5 m*M* $K_4Fe(CN)_6$ $3H_2O$; 2 m*M* $MgCl_2$ in PBS, pH 7.2–7.4 (*see* **Note 5**). Store at 4°C.

2.1.4. Test For Wild-Type Retrovirus

Occasionally DNA recombination occurs between the replication-defective retroviral DNA construct and the genome of the packaging cell line that encodes viral proteins resulting in the generation of wild-type retrovirus. Wildtype retrovirus is capable of replicating on its own and infecting unrelated cells making lineage experiments impossible to interpret.

1. 3T3 Fibroblast cells (American Type Culture Collection, Accession Number CCL 92).
2. 3T3 Growth Media (store at 4°C): The following are added to DMEM (formulation containing 1000 mg/L glucose and 2 m*M* L-glutamine) to give the final indicated concentrations: 10% FBS; 100 U/mL penicillin; 1000 µg/mL streptomycin.
3. 8 mg/mL Polybrene stock solution in PBS.
4. 0.45-µm Disposable sterile syringe filters with cellulose acetate membrane.

2.2. Injection of Retrovirus into Fetuses

Accurately establish the gestational age of the embryonic animals to be injected with retrovirus. The question to be addressed will dictate at which gestational age the retrovirus should be injected, and will be related to the developmental time course of the organ or structure under examination. The time point under analysis should coincide with the period of cell genesis for the structure under examination since the retroviral vectors only infect dividing cells.

Pregnant dams of a known gestational age can be obtained from a supplier. Alternatively, the investigator can establish a breeding colony and determine when insemination has occurred according to routinely used procedures. By convention, the day a vaginal plug is detected is designated embryonic d 0 (E0). Gestation usually lasts 20 d for mice and 22 d for rats.

1. A long-acting anesthetic, such as chloral hydrate should be used (*see* **Note 6**). The effective intraperitoneal dose of chloral hydrate is approx 400 mg/kg for rodents. Dissolve chloral hydrate in 0.1 *M* PBS or 0.9% NaCl. A 3-cc syringe and 25 gage needle are needed to administer the chloral hydrate.
2. Electric shaver.
3. A bifurcated fiber optic light source to transilluminate the uterine swellings (*see* **Note 7**).
4. Pneumatic picopump for controlled delivery of retrovirus. There are several pneumatic picopumps available from various vendors, including one manufactured by

World Precision Instruments (Sarasota, FL) (Model # PV 820) that is suitable for injections of small volumes. The operation of a pneumatic picopump also requires a dedicated nitrogen gas tank and vacuum line.

5. Supplies for the laparoscopic surgery and intraventricular retroviral injections:
   a. 3 × 3-in. Sterile gauze pads.
   b. Sterile PBS.
   c. 5 cc Syringe.
   d. Capillary tubing (Omega dot borosilicate glass tubing, # 30-30-1, FHC, ME).
   e. Tape.
6. Surgical platform and collecting tray for supporting the experimental animal above the reservoir of irrigation fluids during surgery. Any acrylic or metal tray large enough to accommodate the mouse or rat with extended limbs and that is raised in some fashion 1–3 cm above the base can serve as a platform. The designated platform should fit inside a tray, of slightly larger dimensions than the platform, so that the irrigation fluid (PBS) will not run all over the operating surface. A shallow metal tray will work well for this purpose.
7. Surgical instruments for laparoscopic surgery (*see* **Note 8**):
   a. Small scissors.
   b. 2 Hemostats (rat surgery only).
   c. 2 Dog ear clips (mouse surgery only).
   d. Small toothed forceps.
   e. Forceps (# 5 or 3).
   f. # 10 Scalpel holder.
   g. #10 Blades.
   h. Needle holder.
   i. #5 (rat) or # 6 (mouse) suture thread and needles (Ethicon Inc., Somerville, NJ).
8. Retroviral supernatant
   a. Ice bucket filled with ice.
   b. 1% Fast green dye (F7252, Sigma, St. Louis, MO).
   c. 1 mg/mL polybrene, prepare as in **Subheading 2.1.2., item 3d**.
   d. Aliquot of frozen viral supernatant.

The viral supernatant, fast green dye, and polybrene should all be kept on ice when not in use.

9. An infrared heat lamp should be used to warm the pregnant dam while recovering from surgery (*see* **Note 9**). The infrared light source, with shield, can be bought at a hardware store, and attached on an adjustable stand, if desired.

2.3. Detection of lacZ Positive Cells at the Light Microscopic Level

1. Perfusion instruments:
   a. Cannula for directing the flow of fixative into the left ventricle.
   b. 2 Hemostats.
   c. Scissors.
   d. Small toothed forceps.

e. # 10 Scalpel blade holder.
f. # 10 Blades.
g. Two # 5 forceps.

2. Peristaltic pump for delivery of the fixative (Masterflex Pump #7520-24, Cole-Parmer Instrument Co., Vernon Hills, IL).
3. Ether chamber: Any container with a lid large enough to accommodate the experimental animal during the anesthetization procedure and resistant to ether fumes is suitable.
4. Fixative: 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 *M* sodium phosphate buffer, pH 7.2 prepared as in **Subheading 2.1.3., item 2b**.
5. Histochemical detection of *lacZ*-positive cells: In addition to the chemicals listed in **Subheading 2.1.3., item 2:**
   a. Sodium deoxycholate.
   b. Nonidet P-40 (NP-40).
   c. 24-Well tissue culture plates.
   d. Vibratome (*see* **Note 10**).
   e. Superfrost slides (*see* **Note 11**, #12-550-15, Fisher Scientific, Pittsburgh, PA).
6. Immunohistochemical detection of *lacZ*-positive cells:
   a. Blocking serum: 10% normal goat serum, 0.2% Triton X-100 in PBS, pH 7.4.
   b. Primary antibody: mouse anti-β-galactosidase (Five Prime-Three Prime, Inc., CO), diluted 1:500 in blocking serum.
   c. Secondary antibody: Anti-mouse IgG conjugated to fluorophore, diluted in blocking serum according to manufacturer's recommendations.
7. Detection of the phenotype of *lacZ*-positive cells: The investigator can double- or triple- label tissue sections to detect the expression of *lacZ* immunohistochemically, in conjunction with other cell-type specific antibodies of choice ***(14)*** (*see* **Note 12**).

2.4. Detection of lacZ Positive Cells at the Ultrastructural Level

1. Fixative: 4% paraformaldehyde, 0.5% electron microscopy grade glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.2–7.4.
2. Post fixation solution: 4% paraformaldehyde, 2% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.4.
3. Staining and dehydration of tissue samples:
   a. 1% Osmium tetroxide in 0.1 *M* phosphate buffer, pH 7.4.
   b. 6- and 24-Well plastic tissue culture plates.
   c. 0.1 *M* Sodium acetate.
   d. 1% Uranyl acetate.
   e. 0.1 *N* Sodium acetate.
   f. 25, 50, 70, 95, and 100% ethyl alcohol.
4. Embedding compounds:
   a. Araldite.
   b. 3 × 2-in. glass slides.
   c. High-quality acetate paper (*see* **Note 13**).
   d. Hole-punch.
   e. Small weights.

3. Methods

3.1. Safety Procedures for Working with Retroviruses

These procedures are carried out at Biosafety Level 2. The proper institutional guidelines should be followed before using retroviruses. When handling retroviruses observe the following safety precautions:

1. Wear a lab coat and gloves. Minimize touching surfaces with gloved hands.
2. All work is performed in a biological safety cabinet.
3. If using pipets to transfer small volumes of retroviral supernatant, use disposable tips which contain aerosol resistant barriers.
4. Liquid waste is aspirated into a flask which contains approx 100 mL of liquid bleach at full strength.
5. All the solid waste which is generated (pipets, tips, gloves, dishes, and so forth) must be disinfected by bleaching or autoclaving before disposal. When beginning, put a small autoclavable bag in the hood and place solid waste into it as it is generated. When finished, tape the bag shut in the hood and autoclave.
6. Clean any spills of supernatant with 10% bleach.
7. When finished working with retroviral producer cells, wipe the biological safety cabinet and pipetor with 70% ethanol.
8. Expose the biological safety cabinet to UV light for a minimum of 30 min after working with retroviruses in the biosafety hood.

3.2. Production of Retroviral Supernatant Using Stable Producer Cell Line

3.2.1. Growth of CRE BAG2 Producer Cells

CRE BAG 2 producer cells are grown in Growth Media (GM, *see* **Subheading 2.1.1., item 2**) in a humidified 37°C incubator containing 5–7% CO_2.

1. Remove a cryogenic vial from liquid nitrogen storage and rapidly thaw it in a 37°C water bath.
2. Transfer the contents of the vial to a 100-mm dish containing 10 mL of GM.
3. On the following day, aspirate the spent media and refeed the cells with 10 mL of fresh GM.
4. Feed the cultures with fresh GM every 2–3 d, subculture the cells 1:4 using trypsin/EDTA when cells reach approx 80% confluence (*see* **Note 14**).

3.2.2. Subculturing CRE BAG 2 Cells

1. Aspirate the spent media and gently rinse the cells twice with PBS-CMF.
2. Add 1 mL of trypsin (0.05% trypsin/0.53 m*M* EDTA)/100 mm dish. Incubate for a few min at room temperature until the cells round up or lift off the dish.
3. Add 9 mL of GM to the dish and gently pipet the cells to disperse.
4. Seed 1/5 of the cells into each of five 100-mm dishes in 10 mL of GM.

3.2.3. Collection of Supernatant Containing Retroviral Particles

1. When the cells in the desired number of 100-mm dishes are 70–80% confluent, refeed the cultures with 5 mL of GM (*see* **Note 15**).
2. Leave the GM on the CRE BAG 2 cells for 6–12 h (*see* **Note 16**).
3. Collect the supernatant, pool, filter through 0.45-µm syringe filters, aliquot into cryogenic vials at 50 µL and 1 mL each, and freeze in a liquid nitrogen cryogenic tank.

3.3. Production of Retroviral Supernatant Using Transient Transfection of Producer Cells

The method given is modified from that of **ref. *8***.

1. Plate 7×10^6 Bosc23 cells/100-mm tissue culture dish the day prior to transfection. Cells are grown in a humidified 37°C incubator with 5% CO_2.
2. On the day of transfection: Refeed Bosc23 cells with 8 mL GM + 10 µL of 25 m*M* chloroquine (*see* **Note 17**).
3. In a 5 mL tube, mix 35–40 µg of retroviral plasmid DNA with water to give a final volume of 875 µL.
4. Add 125 µL 2 *M* $CaCl_2$ and mix well.
5. Add 1 mL of 2X BES solution to the DNA/ $CaCl_2$ mixture dropwise with bubbling or vortexing.
6. Gently add the DNA mixture to the Bosc23 cells.
7. Return the cells to the 37°C incubator at 5% CO_2 for 6–12 h.
8. Aspirate the media and refeed with 27 mL of fresh GM.
9. Twenty-four h after the start of transfection, refeed the Bosc cells with 9 mL of GM and transfer to a humidified 32°C incubator at 5% CO_2 (*see* **Note 18**).
10. Forty-eight hours after the start of transfection, collect the supernatant and filter through a 0.45-µm filter. Aliquot the supernatant to cryogenic vials (50 µL and 1 mL) and rapidly freeze using dry ice or liquid N_2. Store in a liquid nitrogen cryogenic tank.
11. Refeed the dish with 9 mL of GM and keep at 32°C.
12. Sixty hours after the start of transfection, collect the supernatant again. Filter, aliquot, and freeze as above.
13. Refeed the dish with 9 mL of GM and keep at 32°C.
14. At 72 h after the start of transfection, collect the supernatant for the last time (*see* **Note 19**). Filter, aliquot, and freeze as before.

3.3.1. Determining the Efficiency of Transfection if Using a lacZ Expressing Retrovirus

1. Fix the dish for 4 min in cold fixative (*see* **Note 21**).
2. Wash the dish, two times for 5 min, each in a large volume of PBS.
3. Dilute the X-gal to 1 mg/mL in staining buffer and incubate the cells for 3 h overnight at either room temperature or 37°C (*see* **Note 22**).
4. Determine the percentage of *lacZ*-positive cells.

3.4. Determination of Retroviral Titer

1. On the day before the retroviral infection is to be done: Seed a 6-well tissue culture plate with 3T3 fibroblasts at a density of 2×10^4 cells/well.
2. On the day of infection add 100, 200, or 400 µL of the retroviral supernatant in a final volume of 2 mL GM to different duplicate wells. Add 8 µg/mL polybrene. The control well receives 2 mL of GM + 8 µg/mL polybrene.
3. Incubate in a humidified, 5–7% CO_2 incubator for 2 d.
4. Fix and stain with X-gal as in **Subheading 3.3.1**.
5. Count the number of *lacZ*-positive cells in each well.
6. Plot volume vs number of *lacZ*-positive cells. The *y*-intercept yields the approximate number of infectious retroviral particles per microliter.

3.5. Test for Presence of Wild-Type Retrovirus

Retroviral supernatants should always be checked for the presence of wild-type retrovirus. Lineage studies can only be properly interpreted if replication-defective retroviruses are used. If replication competent wild-type virus is present, throw away all of the stored supernatant and the batch of retroviral producer cells used and obtain a new lot of cells.

1. On the day before the infection is to be done: Seed a 6-well tissue culture plate with 3T3 fibroblasts at a density of 2×10^4 cells/well.
2. On the day of infection: Infect the 3T3 cells with 2 mL of the test supernatant using 8 µg/mL polybrene (*see* **Note 23**). The negative control dish receives fresh 3T3 GM + polybrene.
3. After allowing 3–4 h for absorption, aspirate the media (*see* **Note 24**).
4. Refeed the cultures with fresh 3T3 GM + 2 µg/mL polybrene (*see* **Note 25**).
5. The day that the 3T3 cells from **step 2** become 90% confluent, seed a new 6-well plate with 3T3 fibroblasts at a density of 2×10^4 cells/well for infection the following day. Also, replace the media on the initial set of 3T3 cells infected with test supernatant with half the volume of fresh 3T3 GM.
6. Harvest the supernatant from the 3T3 cells 6–12 h later. Filter the supernatant through a 0.45 µm filter before doing the infection of the second set of 3T3 cells (*see* **Note 26**).
7. Infect the second set of 3T3 cells with 2 mL of the filtered supernatant from the original 3T3 cells using 8 µg/mL polybrene. The negative control dish receives fresh 3T3 GM + polybrene.
8. After allowing 3–4 h for absorption, aspirate media as before.
9. Refeed cultures with fresh 3T3 media + 2 µg/mL polybrene.
10. For testing the presence of a wild-type retrovirus expressing the *lacZ* gene (*see* **Note 27**): Stain with X-gal when the 2nd batch of 3T3 cells become confluent. If the test supernatant contains only replication defective virus, there should be no *lacZ*-positive cells in the 2nd batch of 3T3 cells.

3.6. Intraventricular Injection of Retrovirus

The procedure for making injections of retrovirus into the telencephalic ventricles of embryonic mice and rats will be described. The basic procedures can be adapted to inject retrovirus into other structures, such as the eye ***(15)*** or olfactory epithelium ***(16,17)***. Intraventricular injections of retrovirus can be made most readily between embryonic d 11 and 15 of mice and between embryonic d 13 and 17 of rats. Injections earlier and later than the stated times are difficult because the uterine membranes are somewhat opaque, making it difficult to resolve the enclosed embryo. Furthermore, at earlier times the embryo, as well as its brain is very small and difficult to aim for with the tip of a micropipet (*see* **Note 28**). Nevertheless, the particular question under investigation will determine the precise developmental age at which the rodent embryo is injected with recombinant retroviruses.

1. Sterilize the instruments and work area: Place the instruments in a tray or beaker with 70% alcohol for approx 30 min; subsequently allow them to air-dry on sterile gauze or paper. Clean the surgical work space with 70% alcohol and allow the surface to air-dry.
2. The use of a pneumatic picopump simplifies the procedure for delivering retrovirus into the chosen location of the developing embryo, and lateral ventricles of the forebrain in particular (*see* **Note 29**). Turn on the picopump and set the flow rates for the vacuum and the nitrogen gas tank. Attach a pipet to the electrode holder connected to the picopump. The pipet tip should be broken with forceps; the optimum diameter will need to be determined by the investigator. However, the pipet tip needs to be rigid enough to penetrate the uterine membranes and fetal skull without breaking, but sufficiently thin to prevent untoward tissue damage. Pipets that gradually taper to a sharp point usually work the best. To check the rate of intake, outflow, and hold pressure of the picopump before filling the pipet with retrovirus, first draw up colored water through pipet. Adjust rates if necessary, according to manufacturers suggestions (*see* **Note 30**).
3. Anesthetize the pregnant rat or mouse by an intraperitoneal injection of chloral hydrate (or another suitable anesthetic) (*see* **Note 31**). After the pregnant dam is completely anesthetized and no longer responds to forelimb pinch or displacement of the eyelid, immobilize the dam with limbs extended on the surgery platform. The limbs should be loosely extended and taped in a fixed position on the platform or secured in some other comparable way (*see* **Note 32**).
4. Shave the abdomen of the immobilized dam from the pubic bone to the xiphoid process (*see* **Note 33**). Clean the region of the shaved skin with 70% ethyl alcohol followed by sterile PBS. In circular movements, wipe from the center of the surgical field to the margins with both the alcohol and PBS.
5. After illuminating the abdomen with the fiber optic light, gently lift the skin at the midline of the anesthetized pregnant dam using dull or small toothed forceps. Incise the superficial skin layer of the abdomen (*see* **Note 34**), using a #10 scalpel blade, along the linea alba, which will be apparent as a longitudinally running

white line where the external and internal oblique muscles of each side of the body meet. Extend the opening anteriorly and posteriorly; do not extend the opening rostral to the rib cage or more caudal than the position of the bladder. The smaller the abdominal opening, the better.

6. Use dull or small toothed forceps to lift the exposed abdominal muscles away from body, and then with care use scissors to make a small midline, longitudinal opening in the muscle wall. Use an index finger or blunt instrument to ensure that the abdominal organs are not adhered to the muscle wall before extending the incision rostrally and caudally about the same extent as the overlying skin layer was opened.
7. Surround the entire surgical field with a bed of gauze. Open 3 × 3 in. gauze pads so that they become 3 × 6 in., and lay the strips longitudinally on both sides of the incision. Place multiple layers of gauze along the opening.
8. Constantly use sterile PBS or physiological saline to keep the area of the incision and the exposed abdomen moist. The abdominal tissue and uterine membranes must not be allowed to dry out (*see* **Note 35**).
9. Use hemostats (for rats) or dog ear clips (for mice) to retract the skin and abdominal muscle wall. The two layers can be retracted together, or separately.
10. Mice and rats have both a right and left uterine horn, each comprised of several uterine swellings, that meet at the cervix (*see* **Fig. 1**). Gently remove the uterine

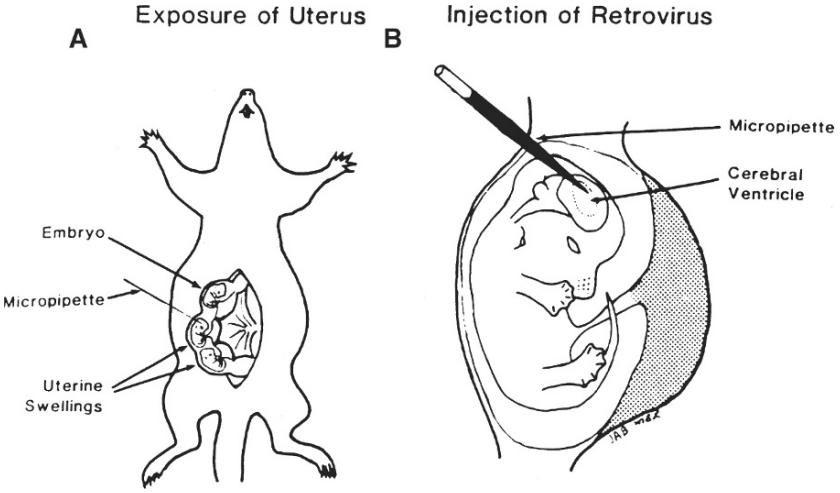

Fig. 1. Method for introducing recombinant retrovirus into the mid-gestation rat embryo. **(A)** To expose the uterine horns a midline incision is made through the skin and muscle layers of the abdomen of an anesthetized rat. In this figure three uterine swellings of the right uterine horn are diagrammed. *In utero* the orientation of the embryos is variable. **(B)** An enlargement of one uterine swelling illustrating the placement of a micropipet in the right lateral ventricle of an embryonic d 16 rat. Shaded area represents the placenta. (Reproduced with permission from Luskin, 1992.)

horns from the abdominal cavity, and accurately count all the uterine swellings on each side (*see* **Note 36**). Dull forceps can be used to manipulate the uterine swellings. Make sure that all uterine swellings are accurately counted by identifying the rostral to the uppermost swelling, the ovary rostral to the uppermost swelling on each side. Diagram the arrangement of the uterine swellings and give each a number. By visual inspection, determine if all swellings contain a viable embryo (*see* **Note 37**).

11. Tuck the uterine swellings of one side back inside the abdominal cavity, and cover all the remaining uterine swellings with moist gauze to prevent them from becoming dry (*see* **Note 38**).
12. Thaw an aliquot of the retroviral supernatant. To every 50 µL of supernatant, add 2.5 µL of 1 mg/mL polybrene and 2.5 µL of 1% fast green dye; the final concentration of the polybrene and fast green dye is 0.5 mg/mL and 0.5%, respectively (*see* **Note 39**).
13. With careful positioning of the fiber optic light guides the uterine membranes are somewhat transparent. The embryo's body and head should be detectable. The embryonic sagittal and transverse dural sinuses demarcating the lateral ventricles should be recognizable. Using dull forceps, or some other comparable instrument, the position of the embryo and its head within the uterine swelling can be maneuvered (*see* **Note 40**). Use one motion, if possible, to penetrate the uterine membranes, skull and telencephalon (*see* **Note 41**). Once the pipet tip is in the lateral ventricle, the virus can be slowly released. The presence of the blue dye can be used to verify the placement of the pipet tip. The volume of the retrovirus to be injected will depend on the design of the experiment. Volumes of up to 5 µL can be safely injected.
14. After each embryo has been injected, replace all the uterine swellings back into the abdominal cavity. Suture closed the muscle and skin layers separately. First, close the inner muscle wall using silk suture thread; be sure that the fascial coverings of muscles do not slip into the abdominal cavity. Use interrupted sutures spaced about 1/4–3/8 in. apart. Similarly, suture closed the outer skin layer.
15. Place the pregnant mouse or rat in its cage under the infrared heat lamp. Cover half of the cage with aluminum foil to give the pregnant dam a choice of environmental temperature upon arousal and recovery; the tin foil will reflect the heat.
16. Discard all items that touched retrovirus in a dilute solution of bleach.

3.7. Localization of β-Galactosidase Positive Cells in the Central Nervous System

3.7.1. Histochemical and Immunohistochemical Detection of lacZ-Positive Cells at the Light Microscopic Level

The injected embryos can be examined for the presence and distribution of *lacZ*-positive cells at a later embryonic age or at some perinatal or postnatal time point. Since it is beyond the scope of this chapter to describe individual perfusion protocols for animals of every developmental age, a protocol that can be followed for adult animals will be given in the greatest detail.

The total number of pups should be counted after the pregnant dam has given birth to the injected embryos. Often, fewer pups are born than the number of embryos that were injected. Moreover, one can not correlate individual pups with the number they were assigned prenatally at the time of surgery unless the injection site was substantially different (i.e., right hemisphere vs left hemisphere, or big differences in the volume of retrovirus injected). If the injected animals are retrieved at a later embryonic time point, individual injected animals can be distinguished by their position along each uterine horn.

3.7.1.1. Perfusion

1. Anesthetize the experimental animal by placing it in a chamber lined with gauze that has been treated with ether.
2. Extend and immobilize the limbs of the fully anesthetized animal and follow standard intracardiac perfusion procedures. Make a longitudinal incision over the sternum from neck to xiphoid process (*see* **Note 42**).
3. Lift the skin and fur over the xiphoid process and cut laterally with scissors several centimeters in both directions.
4. Grasp the xiphoid process with toothed forceps or a hemostat and then free the lower margins of the ribs from any attachments to the abdominal organs. Carefully snip the diaphragm at its midline attachment to the sternum. Free the diaphragm from the lower margin of the rib cage.
5. Free the rib cage by cutting along the axillary line on each side; begin at the lateral margins of the abdominal incision. Make sure the mediastinal structures underlying the rib cage are detached from the ribs.
6. Retract the severed rib cage; be careful not to put any tension or pressure on the structures in the neck which will interfere with the flow of fixative to the brain (*see* **Note 43**).
7. In rapid succession, snip the right atrium so the animal begins to exsanguinate and insert a cannula or syringe needle, connected to tubing of the peristaltic pump, into the left ventricle (*see* **Note 44**).
8. Turn on the peristaltic pump and perfuse the anesthetized rodent with 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 *M* phosphate buffer (*see* **Note 45**).
9. After completion of the perfusion, remove the brain from the skull and transfer it to a container with fixative for not more than 1 h (*see* **Note 46**).
10. Section the brain or part of brain of interest on a Vibratome at a chosen thickness (e.g., usually 50–100 µm). Collect the sections in 0.1 *M* phosphate buffer in a 24-well culture dish or some other partitioned tray.

3.7.1.2. For Histochemical Detection of *lacZ*

1. Incubate the sections overnight at room temperature in staining buffer containing 1 mg/mL X-gal, 0.02% sodium deoxycholate and 0.01% Nonidet P-40 (NP-40)
2. Rinse the sections in 0.1 *M* phosphate buffer and mount on Superfrost slides for examination. Alternatively, the sections can be processed for the ultrastructural visualization of the *lacZ* histochemical reaction product (*see* **Subheading 3.7.2.**). **Fig. 2A** demonstrates the appearance of two histochemically stained *lacZ*-positive cells.

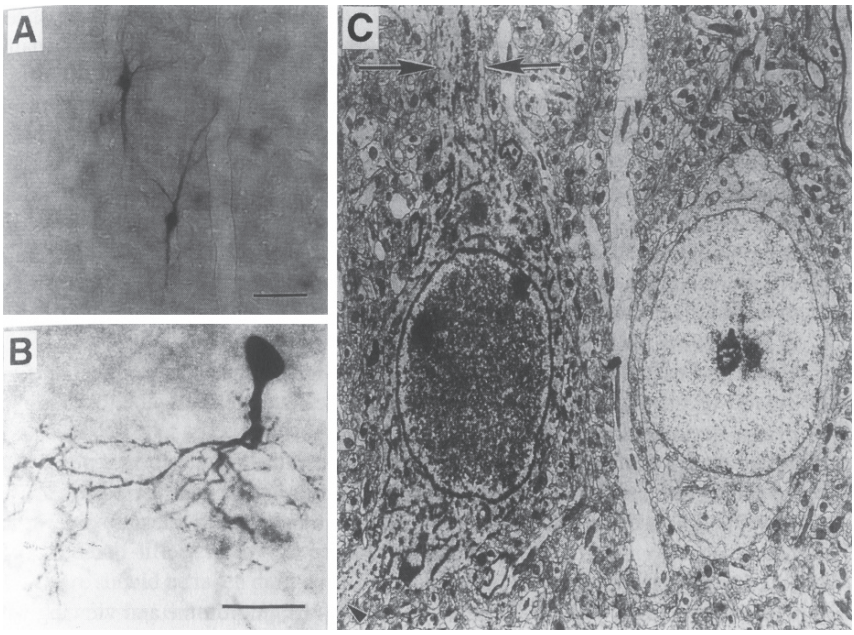

Fig. 2. Representative examples of the appearance of *lacZ*-positive cells in the central nervous system at the light and electron microscopic levels. **(A)** Representative examples of histochemically stained *lacZ*-positive neurons in the rat cerebral cortex resulting from an injection of retrovirus into the cerebral ventricles of the embryonic d 16 rat telencephalon. Note that the cell bodies and proximal dendrites are intensely stained (modified with permission from Luskin et al., 1993). **(B)** Representative example of an immunohistochemically stained neuron in the olfactory bulb resulting from a perinatal injection of retrovirus. The *lacZ*-positive cell was revealed using a primary antibody to β-galactosidase; diaminobenzidine was used as a chromogen to visualize the secondary antibody conjugated to horseradish peroxidase. In most instances immunohistochemistry can be used to label the fine processes of *lacZ*-positive cells (modified with permission from Luskin, 1993). **(C)** An electron micrograph showing a *lacZ*-positive (left) and unlabeled (right) neuron in the visual cortex from a rat that received an intraventricular injection of retrovirus at E16. The nucleus and nuclear membrane are conspicuously stained and in the cytoplasm the reaction product is associated preferentially with the endoplasmic reticulum, which extends into the apical dendrite (large arrows) and basal dendrite (arrowhead) of the labeled cell (modified with permission from Luskin et al., 1993).

3.7.1.3. For Immunohistochemical Detection of *lacZ* (After Collection of the Sections in 0.1 *M* Phosphate Buffer)

1. Incubate the sections in blocking serum for 1–2 h at room temperature.
2. Incubate the sections in the anti-β-galactosidase antibody diluted in blocking serum overnight on a rotator at 4°C.
3. Rinse the sections three times in PBS.

4. Incubate the sections in the secondary antibody conjugated to the desired fluorochrome (*see* **Note 47**) and proceed with standard immunohistochemical procedures. **Fig. 2B** demonstrates the appearance of an immunohistochemically stained *lacZ*-positive cell.

3.7.2. Histochemical Detection of lacZ*-Positive Cells at the Ultrastructural Level*

1. Day 1: As described in **Subheading 3.7.1.1.**, perfuse the experimental animals, and remove and fix the brains. Collect 100 µm tissue sections and carry out the X-gal reaction.
2. Day 2: Aspirate the X-gal reaction mixture from each well, rinse the sections with 0.1 *M* phosphate buffer and post-fix the tissue sections for a few hours in 4% paraformaldehyde/2% glutaraldehyde in 0.1 *M* phosphate buffer.
3. Rinse the tissue sections three times in 0.1 *M* phosphate buffer, incubating for 5 min each time
4. Transfer the sections from each well of the 24-well plate to a fresh well of a 6-well tissue culture plate (*see* **Note 48**).
5. Aspirate the phosphate buffer and add 0.5 mL of 1% OsO_4 in 0.1 *M* phosphate buffer (*see* **Note 49**) to each well (*see* **Note 50**). Place the 6-well plate containing the tissue sections on a rotator in the fume hood for 30 min.
6. Carefully remove the OsO_4 to a toxic waste container and add the following solutions to counterstain (uranyl acetate) and dehydrate (ethyl alcohol) the tissue sections in each well for the stated times:
   a. 0.1 *N* sodium acetate — 5 min, 2×
   b. 1% aqueous uranyl acetate — 30 min
   c. 0.1 *N* sodium acetate — 5 min
   d. 25% ethyl alcohol — 2 min
   e. 50% ethyl alcohol — 2 min
   f. 70% ethyl alcohol — 10 min
   g. 95% ethyl alcohol — 15 min, 2×
   h. 100% ethyl alcohol — 15 min, 2×
7. Aspirate the alcohol from the wells and replace with a 1:1 mixture of 100% ethyl alcohol:Araldite resin. Leave overnight on a rotator at room temperature.
8. Day 3: Remove the alcohol/Araldite mixture and replace with 100% Araldite. Change resin every 2 h so that the sections incubate in the resin at room temperature for at least 6 h.
9. Embed the tissue sections in Araldite resin as follows: Trim two acetate sheets for each slide. One piece should be the same size as the slide or slightly smaller and the other should be slightly larger.
10. Using a paper punch, make small holes in the corners of each piece of the smaller acetate sheets. These will become the bottom sheets of the microscope slide sandwich.
11. Place the lower acetate sheet on a microscope slide and place one small drop of plastic in each corner hole. These plastic drops will secure the acetate film to the slide. Place

6 small drops of embedding plastic on the acetate film (two rows of 3 drops evenly spaced) or one for each section that will be embedded. Carefully scoop the section out of the dish where it has been processed and place it on top of one of the drops of plastic. Cover each tissue section with one more small drop of embedding plastic.

12. Cover the tissue sections with a piece of the larger sheets of cut acetate film. Place weights or additional slides on top of each acetate sandwich to flatten the plastic during baking.
13. When ready to bake the tissue embedded between the acetate sheets, place them on cardboard and then place the cardboard on the oven rack for backing. This way the microscope slides will not stick to the metal oven rack if some of the plastic oozes out from between the slides.
14. Bake in the oven at 60°C 48–60 h.
15. The tissue is ready for trimming, cutting on an ultramicrotome and viewing with the electron microscope. **Fig. 2C** demonstrates the appearance of a *lacZ*-positive cell at the ultrastructural level.

4. Notes

1. All sera (fetal bovine, calf, horse) are suitable for use in freezing media.
2. Chloroquine increases the retroviral titer *(8)*.
3. Paraformaldehyde will dissolve in approx 20 min with heating and stirring.
4. $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ are commonly used at concentrations between 5 and 20 m*M*. The higher concentrations may result in increased staining intensity.
5. This pH range is optimal for detection of the bacterial *lacZ* activity. The use of higher pHs should be avoided so as to minimize the endogenous mammalian β-galactosidase activity.
6. The chloral hydrate should be made fresh on the day of surgery; stored chloral hydrate can be used for anesthetizing rats or mice prior to perfusion. Supplementary doses of chloral hydrate should be avoided because death of the experimental animal may occur. Before giving any additional chloral hydrate allow additional time for the chloral hydrate to achieve its effect. As an alternative to using chloral hydrate, equithesin can be used.
7. A heat-absorbing lens should be attached to the end of each fiber optic light guide to reduce the desiccation of exposed tissue, and in particular the uterine swellings, during surgery.
8. Different instruments should be used for surgical and perfusion procedures; traces of fixative may be deleterious to living tissue.
9. The experimental animals must be kept warm post surgery so that they do not succumb to the temperature lowering effects of anesthesia. Cover half of the cage with aluminum foil to reduce the temperature generated by the infrared heat source. Therefore, upon awakening from anesthesia the experimental animal can choose to remain under the heat source or to go under the aluminum foil heat shield. Typically the anesthetized pregnant dams awaken within 30–45 min.
10. As an alternative to sectioning with a Vibratome, the tissue can be sectioned on a cryostat and mounted on glass slides.
11. The use of Superfrost slides eliminates the need to sub the slides with albumin.

12. A retrovirus with a nuclear localization signal can be used instead so that *lacZ* expression is restricted to the nucleus ***(18)***. This allows the cytoplasmic expression of other antigens to be detected more easily.
13. Choose the acetate sheets carefully. Baking will ruin many kinds of acetate and make then useless for embedding. Avoid acetate sheets that become opaque when baked or that stick permanently to the polymerized Araldite.
14. Growing cells to confluence results in a number of unwanted changes in the producer cells, among them aneuploidy and increased frequency of DNA recombination events.
15. The volume is decreased in order to increase the relative retroviral titer.
16. The half-life of retroviral particles at 37°C is approx 4 h.
17. Care should be taken during media changes as the Bosc23 cells are loosely adherent.
18. Decreasing the temperature to 32°C during collection of the supernatant increases the retroviral titer 5- to 15-fold ***(19)***.
19. After 72 h from the start of transfection the titer of infectious retrovirus greatly drops.
20. The efficiency of transfection should approach 50%.
21. The length of fixation is critical. *LacZ* activity drops dramatically if the cells are fixed for long periods.
22. The diluted X-gal in staining buffer can be reused several times.
23. The cells should be subconfluent before infecting with virus. Retroviruses only infect replicating cells.
24. Use a new pipet to aspirate each well so as not to transfer any retrovirus into neighboring wells.
25. If the titer of wild-type retrovirus in the test supernatant is low, very few 3T3 cells will become infected. Continued growth of the cultures allows time for the wild-type retrovirus to infect other 3T3 cells in the dish increasing the likelihood of detecting its presence. Low levels of polybrene in the media help the viral infection of neighboring cells.
26. If the test supernatant contained wild-type retrovirus, then the 3T3 cultures now produce the wild-type retrovirus themselves after several days. Thus, the supernatant from the 3T3 cells is capable of infecting a naive set of 3T3 cells.
27. For testing the presence of a wild-type retrovirus expressing a neomycin resistance gene, after 2–3 d, split the second set of 3T3 cells at a 1:20 dilution into GM with 1 mg/mL G418. Change the media after 3 d and count colonies after 10 d. If the retroviral producer cells express only replication defective virus, there should be no colonies.
28. Retrovirus can also be injected into the amniotic cavity to label structures that can not be seen, but are in direct contact with the amniotic fluid, such as skin.
29. If a pneumatic picopump is not available, then alternatively, a Hamilton syringe can be loaded in the usual way, and used to deliver retrovirus.
30. The pipet tip can be backfilled with the colored water or viral supernatant. Alternatively, 5–10 μL of the viral supernatant can be deposited on a small plastic boat or piece of parafilm, and then with extreme care, the pipet tip gently placed in the center of the droplet at an angle in order to load the pipet by vacuum.

31. Work as quickly as possible once the animal has been anesthetized because the anesthesia can deleteriously lower body temperature. The investigator should strive to finish the surgery within 1.5 h.
32. The surgery platform serves two principal purposes. First, it aids in restricting the pregnant dam in a position in which her abdomen is accessible to surgery. Second, it raises the anesthetized dam above the base or tray, where the irrigation fluids will drain. This helps to keep the dam from laying in a pool of fluid, which would adversely lower the body temperature and curtail recovery.
33. While shaving the fur off the ventral surface of the abdomen, carefully shave around the nipples so that they are not injured. If the nipples are malfunctioning because of surgical trauma, the neonatal animals cannot suckle.
34. Be sure the alcohol has dried before incising the abdomen because alcohol can be irritating to an open wound.
35. If the uterine membranes dry out, they become opaque and resistant to penetration by the pipet tip. If the area of the abdominal incision dries out, a clean closure of the tissue is difficult to achieve, and subsequently the pregnant dam is more at risk of infection.
36. The number of uterine swellings present varies from animal to animal. Mice usually have between 7 and 14, whereas rats usually have between 11 and 17. Be careful not to mistake the bladder for a uterine swelling or to maneuver it any more than necessary.
37. A uterine swelling containing a dead fetus or one that is being resorbed is usually gray rather than pink, and considerably smaller than the healthy looking uterine swellings. The vascularization of the healthy uterine swellings are more prominent. Commonly one or more of the uterine swellings may contain a dead or resorbing fetus.
38. The end of the fiber optic light source can emit heat and surprisingly quickly dry out the tissues in its proximity if the tissues are not repeatedly moistened.
39. When not in use, immerse the vial of retrovirus in the ice bucket. The virus will only remain viable for a couple of hours if maintained on ice.
40. While tilting or raising the embryo within the uterine swelling, be careful not to puncture the uterine membranes or to apply excessive pressure to the embryo.
41. Do not apply excess force to enable the pipet tip to penetrate the uterine wall. If the tip will not go through easily, greater pressure will just cause the pipet tip to snap off. Instead, try to puncture the uterine wall in a slightly different place, at a slightly different angle, or with a faster motion.
42. The following procedure can be modified to perfuse injected embryos by first anesthetizing the pregnant dam and making a midline abdominal incision, similar to that done in advance of the laparoscopic surgery for retroviral injections. Expose the uterine horns. Incise the uterine membranes and remove the injected embryo by disconnecting it from placental circulation. The injected embryos can be perfused as described below or fixed by immersion in the fixative. Remove an embryo and fix it before recovering and fixing additional embryos.
43. If desired, the descending aorta can be clamped.

44. If perfusing embryos, then use a small syringe needle to deliver the fixative to the left ventricle. Butterfly needles of various gages connected to the tubing of the peristaltic pump work well to perfuse embryos as young as E12 mice and E13 rats.
45. The peristaltic pump can be turned on in advance of snipping the right atrium so that the fixative is flowing at the time the cannula is inserted into the left ventricle.
46. If the brain is left in the fixative for more than 1–1.5 h, the *lacZ* may start to decay thereby reducing the intensity of the histochemical reaction product.
47. Alternatively, a secondary antibody conjugated to an enzyme such as horseradish peroxidase or alkaline phosphatase followed by incubation in the appropriate substrate can be substituted.
48. Multiple sections can be placed in the same well.
49. Check to make sure that the osmium tetroxide is fully dissolved. Osmium crystals must be left overnight in distilled H_2O to dissolve, and then mixed the following morning with 0.2 *M* phosphate buffer to make a working solution of 1% OsO_4. Remember to wear gloves and work in the hood when handling osmium tetroxide.
50. Make sure the sections are flat and not overlapping. The OsO_4 solution will turn the tissue sections a dark color and make them somewhat brittle.
51. All experimental procedures must comply with legal restriction of the appropriate country (*see also* Notes in Chapter 4).

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14

The Avian Embryo

An Overview

Ivor Mason

1. Origins of Avian Embryology

The major advantage of the avian embryo for the embryologist is its accessibility for manipulation and observation. Indeed, it is for this reason that, historically, detailed descriptions of normal development were first available for avian embryos, generally chick embryos ***(1)***. Artificial incubation and hatching of chicken eggs date to the time of the 18th dynasty in Egypt (ca. 1400 BC) and possibly even earlier in ancient China. The Egyptian practice of egg incubation is well documented in Roman literature, including references by Pliny, Diodorus Siculus, and in the letters of Emperor Hadrian. However, artificial incubation was lost throughout the Middle Ages and revived only during the 18th century. The first recorded observations of avian embryos are included in the works attributed to Hippocrates (ca. 430 BC), although it was Aristotle who provided the first significant observations (*Historia Animalium and De Generatione Animalium*, ca. 350 BC). Detailed and illustrated accounts were published between the late 16th and 18th centuries, written by Aldrovanus (*Ornithologia*, 1597) Fabricius (*De Formatione Ovi et Pulli*, 1604), Harvey *(De Generatione Animalium*, 1651), Shrader (*Observations et Historiae*, 1674), Malpighi (*De Ovo Incubato and De Formatione Pulli in Ovo*, 1672), Mayow (*De Respiratione Foetus in Utero et Ovo*, 1674), Maître-Jan (*Observations sur la formation du poulet*, 1722), and Haller (*Sur la formation du coeur dans le poulet*, 1767). The first attempts to incubate eggs with part of the shell removed to form a "window" were described by Beguelin (*Memoir sur l'art de couver les oeufs overts*, 1749). From the 19th century, there was an explosion of publications relating to the anatomy, physiology, and biochemistry of the chick embryo to be followed by more invasive procedures during the present century ***(1,2)***.

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2. Advantages of the Avian Embryo for Experimental Embryology

The avian embryo offers a number of distinct advantages for embryonic investigation. The legacy of its long history of descriptive studies, coupled with physiological and biochemical studies of the 19th century and the experimental embryology of the 20th century, provides an enormous bibliography concerning system and organ development. Major concepts in developmental biology were established in avian embryos during the first half of the last century; Waddington extended the organizer concept to avian embryos and went on to establish the concept of competence ***(3)***, Spratt introduced the concept of regulation, while Hamburger identified naturally occurring cell death as a general feature of neural development ***(4)***.

It is readily accessible between laying (blastoderm stage) and hatching and the comparatively large size of the embryo, even at gastrulation stages, greatly facilitates microsurgical manipulation. Accessibility can be further improved by in-vitro culture methods pioneered by New ***(5)*** (see Chapter 16) that allow early embryos to be maintained for 3 d on culture rings and a simpler procedure, allowing culture for shorter periods recently was developed ***(6)*** (Chapter 17). Older embryos can be cultured by other methods ***(7)***, and organ culture approaches have been established for more than half a century. Birds, like mammals, are amniotes but are much cheaper to purchase and generally have no associated animal housing costs. Microsurgical manipulations are readily performed until about embryonic day 8, by which time all the major patterning events are complete and all the organ systems established. Tissue grafting facilitated the determination of instructive and permissive tissue interactions important in sculpting the embryo and facilitated studies of position effects, lineage, commitment, and determination (see, e.g., Chapters 18–22, 24). Many lineage-tracing studies have been undertaken in the chick embryo using engrafted quail tissue, which is distinguished cytologically or immunochemically (see Chapter 24), or by application of fluorescent tracers (see Chapter 25). The development of avian retroviral vectors first facilitated the ectopic expression of genes (see Chapter 26) and the exciting recent development of electroporation ***(8)*** (Chapter 27) added a simpler approach. Both techniques can also be used to inhibit gene function when used to introduce genes encoding dominant negative proteins or RNAi ***(9, 10)*** (Chapter 27). Electroporation can also be used to rapidly characterize gene regulatory sequences ***(11)***.

The field owes a considerable debt to Hamburger and Hamilton ((12–14), who provided a detailed staging regime using definitive criteria for the chick embryo; a similarly detailed staged series is not available for the mouse embryo. For stages between fertilization and gastrulation, Eyal-Giladi and Kochav ***(15)*** provide a further staging regime. *The Atlas of Chick Development* ***(16)***

is recommended to those undertaking histological studies and also provides a good first reference source for descriptions of early development and organogenesis. Descriptions and tables of normal early development are also available for the following avian embryos: duck ***(17, 18)***, quail ***(19)***, and turkey ***(20, 21)***.

A major recent development is the sequencing of the chicken genome ***(22, 23)*** (http://poultry.mph.msu.edu/), the first draft of which has been deposited in public databases (www.ncbi.nih.gov/Genbank). To facilitate comparative analysis, this draft was aligned to the human genome sequence (http://genome.ucsc.edu/cgi-bin/hgGateway or www.ensembl.org). In addition, an international consortium created a map of genetic variation for three different strains of domestic chickens. The strains were a broiler strain from the United Kingdom, a layer strain from Sweden, and a Silkie strain from China. To make the map, about 2 million genetic variation sites, mostly single nucleotide polymorphisms (SNPs), were identified and analyzed. The genetic variation data will soon be deposited into GenBank.

An additional valuable resource has been the isolation and sequencing of nearly 500,000 chicken ESTs, mostly from embryonic tissues. These have been organized into a searchable database ***(24)*** (www.chick.umist.ac.uk) and the sequences also deposited in Genbank. This EST resource has facilitated the preparation of the first 13 K chicken cDNA microarray ***(25)*** (www.ark-genomics.org/) and a more extensive array is now available from Affymetrix (www.affymetrix.com).

As with other vertebrate model organisms, considerable effort is being invested in high throughput analyses of gene expression patterns in the chick embryo, with the results being deposited in public databases (e.g., http://geisha.biosci.arizona.edu/ ***[26]***).

However, other vertebrate embryos offer advantages over avian embryos in some areas. It is now clear that transgenic chickens can be generated ***(27)*** and pleuripotent stem cells ***(28)*** have been isolated for that purpose, but the methods are more laborious than for mice and the costs of maintaining transgenic flocks may preclude widespread use of this technology except for agricultural purposes. In addition, the very earliest stages of development, from fertilization to formation of the blastoderm, occur prior to laying and are therefore not readily accessible.

3. A Brief Overview of Early Chick Development

The following is a brief descriptive account of the early development of the chick embryo. Excellent photographs of embryos at gastrulation and later stages are included in Chapter 16.

3.1. Fertilization to Laying

During the first reduction division involved in generation of the oocyte, the latter expands from a diameter of about 1–3.5 cm. Most of the content of this

extremely large single cell is yolk surrounded by the plasma membrane. The egg nucleus and associated cytoplasm are located peripherally in a region called the *germinal vesicle*. The "yolk" beneath this structure is clear and less dense than the remaining yellow yolk, causing the germinal vesicle to lie on the uppermost surface of the oocyte. Follicle cells surround the oocyte and pump yolk, synthesized in the maternal liver, from the blood into the oocyte. The follicle ruptures to release the mature oocyte, but the innermost part of the follicle, which is acellular, remains attached to the oocyte and forms the inner layer of the vitelline membrane. When the follicle-derived vitelline membrane is in place, the plasma membrane of the oocyte breaks down.

Fertilization occurs during the time between release of the oocyte and its entry into the end of the oviduct. Sperm penetrate the follicle-derived vitelline membrane to fertilize the oocyte and the second reduction division occurs. The resultant one-cell stage embryo is called a *blastodisc*. The requirement for rapid fertilization following follicle rupture is met by the female chicken's ability to store sperm in viable form for a number of weeks.

Peristaltic movements carry the egg down the oviduct, a journey that takes about 22 h and during which the egg is subject to a number of modifications. First, a thickened outer layer is applied to the vitelline membrane, which has two extensions, chalazae, which act as stabilizers for the yolk. Albumen is then applied to the outer surface providing a source of water, protein, and antibiotic agents. Next, the double-layered shell membrane is applied, the layers of which are closely apposed to one another, except at the blunt end of the egg, where the gap between them eventually becomes the air space. Finally, in the shell gland, calcite crystals are deposited in and over the outer shell membrane layer to form the eggshell.

Cleavage of the blastodisc is rapid. The first two cleavage divisions occur within a few hours of fertilization and are not related to future body axes. The third cleavage coincides with application of shell membrane. The chick is telolecithal (yolk is concentrated at one end of the egg), and cleavage is meroblastic (incomplete). The first three divisions are radial and incomplete with the cells being opened to the yolk ventrally, forming a syncytial blastoderm. The fourth cleavage division is horizontal, producing a bilayered blastodisc and occurs as the shell is being applied. Further divisions increase the thickness of the blastoderm, but the diameter of the embryo remains roughly constant at 3 mm during this period and zygotic transcription is activated.

Subsequently, the blastoderm begins to expand over the yolk and marginal cells of the outer region, known as the *area opaca*, become specialized to engulf the underlying yolk. The more central region, area pellucida, appears dark, owing to the underlying translucent "yolk." The area pellucida comprises an upper layer, known as the *epiblast*, from which the embryonic tissues derive and

a lower layer of large, yolky cells, the hypoblast, which constitutes the extraembryonic endoderm. These two layers are separated by a narrow fissure, which is equivalent to the blastocoel. The hypoblast derives in part from ingression of cells from the overlying blastoderm and in part from the posterior marginal zone. The hypoblast forms a triangle posteriorly, the embryonic shield or posterior marginal zone, and is generated particularly from an adjacent region of epiblast known as *Koller's sickle*. At this stage, the egg is laid (Hamburger and Hamilton, or HH, stage 1) and comprises about 60,000 cells.

3.2. Gastrulation (HH Stages 2–4)

A major function of hypoblast is to initiate gastrulation through formation of the primitive streak in the overlying epiblast through which epiblast cells migrate to form prospective endoderm and mesoderm. Thus, the initial position of the hypoblast determines the body axis. At the onset of streak formation, the blastoderm is 5–6 mm in diameter. The primitive streak begins to extend forward from the posterior of the area pellucida and ingressing epiblast cells, which mostly have an endodermal fate, migrate anteriorly and centrifugally displacing the hypoblast. Extension of the streak (between HH stages 3 and 4) is by recruitment of anterior cells rather than by cell movement from the existing streak. Hypoblast cells driven to the anterior of the area pellucida form the primary germ cells. As gastrulation proceeds, the area pellucida changes from being round to becoming pear-shaped with the expanded end anterior.

Lateral epiblast cells converge toward the streak, invaginate, extend, and diverge ventrally. Those cells of the epiblast that do not involute are fated to form the ectoderm and neuroectoderm, and divide to compensate for the loss of ingressing cells. At intermediate streak stages (HH stage 3, 12 h post laying), prospective mesoderm begins to ingress through caudally, whereas endoderm is still ingressing through the rostral streak. At this stage, the anterior end of the streak broadens to form Hensen's node, a structure roughly equivalent to the organizer of Xenopus embryos and the shield of zebrafish. As the streak reaches its longest extent (HH stage 4, 16–20 h), prospective mesoderm cells fated to contribute to lateral plate, somites (posterior streak levels), heart (midstreak), and notochord (head process, derived form the deep node) are migrating through the streak.

3.3. Neural Induction and Neurulation

Studies with molecular markers suggest that a neural plate is established by HH stage 5 (20–22 h). After this stage, the streak and node begin to regress posteriorly, a process that continues until about the 20 somite stage, after which they are no longer visible and their remnants become incorporated into the tail bud.

At stage 5, the first formation of the "head process" becomes apparent. It is a short aggregation of mesoderm directly anterior to the node and continuous with prechordal mesoderm, and it derives from the deep part of the node (primitive pit). The head process condenses to form the notochord, which is readily apparent anterior to the node, as the latter starts to regress at subsequent stages. The first pair of somites condense either side of the notochord at HH stage 7; and these, and the subsequent four pairs, lie beneath part of the neural plate fated to form the hindbrain. The remainder underlie prospective spinal cord. The cardiac primordium begins to form at the anterior end of the embryo at HH stage 8 (four somites).

The neural plate is a pseudostratified epithelium. Between its caudal tip and the prospective infundibulum, its midline overlies the notochord, which induces the overlying neuroepithelial cells to become the floor plate. Anterior to the infundibulum the neural plate overlies prechordal mesoderm. Lateral to the midline, the neural plate overlies paraxial mesoderm: the segmented somitic mesoderm posteriorly (behind the prospective otocyst) and unsegmented cranial paraxial mesoderm anteriorly.

Neural tube closure (neurulation) begins at stage 8 at the level of the midbrain and extends both anteriorly and posteriorly. Closure at the rostral extremity (anterior neuropore) is complete by stage 10, whereas the posterior neuropore remains open until the tail bud develops. Soon after closure, neural crest emerges from the midbrain and hindbrain (HH stages 10–12; 36 h) and, later, from the spinal cord.

Simultaneously with neurulation, the embryo also folds ventrally to enclose the gut and bring the two heart primordia together to fuse.

3.4. Later Development

At or before 36 h (HH stage 10), the three germ layers are present, the body axis is established and anteroposterior patterning is well under way, left–right asymmetry is established early during gastrulation, and dorsoventral patterning is ongoing. Somites are being continuously generated and give rise to the dermis, musculature, and axial skeleton. Neural crest migration commenced to give rise to derivatives that include the nonplacodal parts of the peripheral nervous system, cranial skeletal elements, some smooth muscle, and melanocytes.

During the third day of development (HH stages 13–19), the head begins to rotate to the left, and the optic vesicle, otic vesicle, nasal pits, branchial arches, and pituitary begin to develop. The limb buds begin to form and project from the trunk during this period, and the amnion extends over the embryo and closes. During the following 48 h or so, the majority of the organ systems have been established such that, between the sixth day and hatching, much of development is concerned

largely with increase in the size of existing organs, although in the case of the nervous system, this is accompanied by considerable increase in complexity.

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15

Chick Embryos

Incubation and Isolation

Ivor Mason

1. Introduction

The following methods constitute a brief description of chick egg incubation and embryo harvesting. For further details concerning optimizing embryo viability during incubation, the reader is referred to the excellent work of New ***(1)***. Embryos are staged according to the staging series devised by Hamburger and Hamilton ***(2)***, which has recently been reproduced ***(3)***. The reader is also referred to Chapter 14. A further, detailed staging series for very young embryos has also been devised ***(4)***.

2. Materials

1. Howard's ringer. (0.12 M NaCl, 0.0015 M $CaCl_2$, 0.005 M KCl (per liter: 7.2 g NaCl, 0.17 g $CaCl_2$, 0.37 g KCl, pH 7.2, with very dilute HCl) containing penecillin–streptomycin (AAM, Gibco, Paisley, Scotland).
2. Pair of curved, sharp-pointed scissors.
3. Fine watchmakers' forceps (#5 or 55).
4. Pair of spring scissors (Vannas scissors).
5. Stainless-steel spatula, 5- to 8-mm wide tip.
6. Dissecting microscope with magnification to 50× (minimum), preferably greater and transmitted, and incident illumination, preferably from a cold light source.
7. Whatman 3 MM filter paper (Millipore, Southampton, UK).
8. Glass (9-cm) and plastic (3-cm) petri dishes.

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3. Methods

3.1. Storage of Unincubated Eggs

At the time an egg is laid, the embryo has already undergone cleavage, and gastrulation has just started; it comprises a bilaminar blastoderm of about 60,000 cells. Conveniently, however, if the egg is not incubated, further development is virtually arrested at this stage, but normal development is resumed when the temperature is raised. Unincubated eggs can remain viable for about 7–10 d if stored at room temperature, although the proportion of nonviable embryos will increase during this time. The number of viable embryos is increased by storage of unincubated eggs at 12°C with the air space (blunt end) upward.

3.2. Incubation

1. Take eggs from the cool store, and leave on bench to reach room temperature before setting in the incubator.
2. When embryos are being collected and fixed directly or used to provide material for culture, the eggs can be incubated vertically. Lay eggs on their sides when incubating for manipulation (see Chapters 18–22 and 24), since during incubation, the yolk rotates such that its least dense region, the embryonic blastoderm, rotates to the highest point, the upper side of the egg, which is where a window later is cut through the shell. This property of rotation is retained in older embryos.
3. For best viability, incubate eggs at high relative humidity (>50%) and in the range of 37.5–39°C. Times to reach a specific developmental stage depend on the exact temperature (which varies with position within the incubator) and duration of previous storage (old eggs have an appreciable lag phase before they resume development). If older embryos (more than 9 d of incubation) are required, rocking the eggs during incubation improves viability by preventing adhesion between extraembryonic membranes and the shell (egg incubators are commercially available for this purpose). However, rocking does not appreciably increase viability of younger embryos, and these can be incubated in conventional ovens or incubators that function with high internal humidity.

3.3. Opening Eggs for Embryo Harvest

3.3.1. Rapid Method

1. Crack the egg on the edge of, and deposit contents into, a 100-mm glass petri dish, keeping the yolk unbroken. The embryo usually lies on the top of the yolk, but if it does not, pour the egg from one dish to another to rotate the yolk until the embryo is visible (a small white patch at 1–1.5 d, later surrounded by blood islands and then by blood vessels).
2. Vital stain as in **section 15.3.3.3** (*see* **Note 1**), observe under a microscope, and stage.
3. Cut around the perimeter of the *area opaca* with Vannas scissors, lift out the embryo with a prewetted spatula, and place into fix (usually 4% w/v paraformaldehyde in PBS, but this varies with application).

3.3.2. Method for Preserving Shape and Orientation of Young Embryos (up to Stage 14)

This is the preferred method for embryo isolation among members of my group.

1. Crack the egg into a petri dish as in **section 15.3.3.1.** above.
2. Prepare a square frame from thick filter paper (Whatman 3 MM), with external dimensions of about 1.4 × 1.4 cm and with an internal "window" of about 1 cm^2.
3. Lay down the frame on the surface of the yolk such that the embryo is central within the window and allow the filter paper to become wet; this causes the vitelline membrane to adhere to the paper.
4. Cut around the outside of the frame with spring scissors, and lift the frame gently using forceps. The embryo will be stretched out within the frame and remain so throughout processing (*see* **Note 2**).
5. Wash in a dish of Howard's Ringer solution to remove the adherent yolk, cut the embryo from the window, peel away the overlying vitelline membrane, and transfer the embryo to fix.

3.3.3. A Careful Method for Observation of Living Embryos

1. Incubate eggs to desired developmental stage ***(2)***. Crack eggs against the side of a bowl filled with warm (37°C) Howard's Ringer solution.
2. Hold the egg with the crack submerged, and gently ease the two halves of the shell apart.
3. Release the contents into the solution. The yolk will float to the surface, and the blastoderm (the least dense region) should be uppermost. If not, carefully rotate the yolk with a spatula.
4. Stain young stage embryos (pre stage 15) by applying a drop of neutral red (1% v/v aqueous solution) from the tip of a fine glass rod. The stain will rapidly permeate the vitelline membrane and stain the blastoderm beneath. Stage the embryo ***(2–4)***.
5. Carefully cut around the perimeter of the *area opaca* with spring scissors, without rupturing the yolk and clouding the Ringer' solution.
6. Pull the embryo (plus overlying vitelline membrane) away with a #5 watchmaker's forceps to a clear region of the bowl, and lift by immersing a 30-mm petri dish beneath it and withdrawing it slowly.
7. Grip the *area opaca* with a pair of forceps, and gently shake the embryo free of the vitelline membrane and adherent yolk.
8. Transfer to another petri dish containing Howard's Ringer solution.

4. Notes

1. With only a little experience, early embryos can be staged by observation through a dissecting microscope using transmitted light alone. At stages following the onset of somitogenesis, staining is not required for staging, which requires only observation with reference to ***(2)*** and ***(3)***.

2. This works well for embryos with up to 3 d of incubation, but the filter paper cannot hold the weight of older embryos—these should be simply cut out using the method in section 15.3.3.1, hand washed, and transferred to fix using a spatula.

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16

New Culture

Amata Hornbruch

1. Introduction

As the title indicates, this culture method was developed by Denis New and first described in 1955 ***(1)***. It enables the observer to study the events of gastrulation in the chick embryo in much greater detail than possible until then. It also opens the way to microsurgery without the problem that yolk and the vitelline membrane cause. Studying the effects of treatment with compound affecting morphogenesis and development became meaningful, because precise concentrations and volumes could be administered and successfully washed out again.

Most of the early work on the mechanics of gastrulation was performed on the egg of *Xenopus laevis*, because of its accessibility. New Culture offered a chance to emulate and refine some of those experiments in the chick embryo.

Several attempts have been made to culture blastoderms or fragments thereof *in vitro*, but none have been very successful. The chorioallantoic membrane (CAM grafts) has been used as a substratum to study self-differentiation, but fragments do not develop consistently true to origin. Spratt ***(2,3)*** grew blastoderms on a bed of a mixture of yolk and agar. Some of the observations from this culture method resulted in misleading conclusions being drawn because morphogenetic movements were inhibited in the layer that was in contact with the agar.

Gallera and Nicolet ***(4)*** tried a more fluid culture medium and were slightly more successful. They also tried a modification of New Culture with a double-ring setup in which the vitelline membrane was sandwiched between the two rings, one of which was just small enough to fit into the larger one. This was to facilitate operations to the dorsal aspect of the embryo. The explanted embryo could be turned over without slipping off.

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The most successful method was pioneered by New ***(1)***. Blastoderms can be explanted unincubated or incubated for up to 30 h. This culture system not only allows observation of morphogenetic movements, which are so important for the normal development of the embryo, but also lends itself to a variety of minor and major transplantation experiments, ablations, treatment with compounds in solution or bound on beads, or other carriers and many other experiments. There are two main restrictions to the use of New Culture. First, only the ventral aspect of the blastoderm is accessible to immediate and direct manipulations, although there is no reason not to invade the epiblast as well. However, this can only be done by cutting through hypoblast and mesoderm, and causing perhaps unnecessary damage to these tissues. Second, the incubation time in culture is limited to about 48 h. Consequently, experiments have to be short term.

1.1. Embryo Development

At the time of laying, the chick embryo is a flat disk consisting of about 60,000 cells. It is made up of two layers, the hypoblast ventrally, lying in close association with the yolk, and the epiblast dorsally, facing the vitelline membrane, the membrane which surrounds the yolk. The disk has a translucent inner core, the area pellucida, from which the embryo will form, and a denser outer ring of mainly yolk-containing cells, the area opaca. Only the cells at the periphery of the blastoderm are attached to the vitelline membrane, and as they migrate radially, the blastoderm expands.

After about 8–10 h of incubation, the embryonic shield or posterior marginal zone has formed, and the position from which the primitive streak will arise is determined. The disk now has polarity dorso/ventrally and antero/posteriorly as well as left/right.

2. Materials

All glassware and instruments should be clean to tissue-culture standards and autoclaved or heat-sterilized the day before use. AnalaR® reagents are recommended for the solutions, which should be made up and autoclaved the day before use. If the technique is used as a classroom exercise, it is quite sufficient to use Howard's Ringer. However, if it is employed as a tool to do serious research, the use of a phosphate-buffered salt solution, such as the one described later, is advised.

Explantation of the cultures may be performed in a quiet draft-free place on the laboratory bench if a tissue culture hood is not available.

2.1. Nonsterile Equipment

1. Humidity incubator with fan for egg incubation, preculture.
2. Humidity incubator with fan for new culture incubation.
3. Stereoscopic binocular dissecting microscope—transmitted light is preferable.
4. Container for egg disposal.

2.2. Sterile Equipment

1. Large Pyrex pie dish about 5 cm deep, 20–25 cm in diameter.
2. Watch glasses, 50–55 mm diameter, preferably with flat bottoms, for stability.
3. Glass rings, optimal 25/26 mm inner, 29 mm outer diameter ***(1)***.
4. Glass Pasteur pipets, 145-mm, flamed tips and plugged.
5. Disposable Petri dishes, 90 mm—bacteriological grade is sufficient.
6. Kleenex tissue strips for humidity retention in culture dish.

2.3. Sterile Instruments

1. A pair of coarse forceps with blunt ends, 12 cm (anatomical).
2. A pair of straight scissors, 11–12 cm.
3. A pair of straight fine scissors, 9–10 cm.
4. A pair of straight no. 5 watchmaker's forceps ***(2)***.
5. A pair of curved no. 7 watchmaker's forceps ***(2)*** (**Fig. 1**).

2.4. Balanced Salt Solutions (BSS) and Other Solutions

1. Pannett and Compton ***(5)*** buffered salt solution or Howard's Ringer, autoclaved.
2. Antibiotic/antimycotic solution (100X) (Gibco)
3. Howard's Ringer: 7.2 g NaCl, 0.23 g $CaCl \cdot 2H_2O$, and 0.37 g KCl. Dissolve in 1000 mL double-distilled water. Adjust to pH 7.2 with one or two drops of 0.5 *M* NaOH. Fill in suitable bottles or flasks, autoclave the day before, and keep refrigerated.

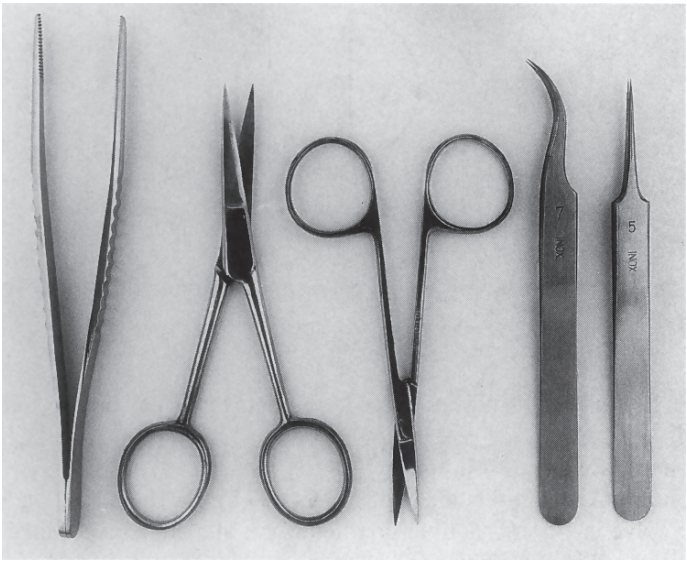

Fig. 1. Set of recommended instruments for New Culture.

4. Pannett and Compton solution:
   a. Solution A: 12.11 g NaCl, 1.55 g KCl, 0.77 g CaCl, and 1.27 g $MgCl_2$ $6H_2O$. Dissolve in 100 mL double-distilled water and autoclave. Make up multiples if required in large amounts, keep in aliquots of 40 mL, and autoclave.
   b. Solution B: 0.189 g Na_2HPO_4 $2H_2O$ and 0.019 g NaH_2PO_4 $2H_2O$. Dissolve in 100 mL double-distilled water and autoclave. Make up multiples if required in large amounts, keep in aliquots of 60 mL, and autoclave.
   c. Working solution: To make up working solution, autoclave 900 mL double-distilled water with 0.9 g glucose in a 1000-mL Erlenmeyer flask the day before. When cool, add 40 mL of solution A and 60 mL of solution B in that order, and keep at 4°C overnight. Working solution can be kept at 4°C for several days if not used up on the day of transplantation.

3. Methods

1. Incubate eggs on their sides for 18–20 h at 38 ±1°C for stages 4 and 5 ***(3)***.
2. Wipe or spray eggs with 70% alcohol.
3. Pour cold BSS in pie dish, about 4-cm depth (6–700 mL).
4. Add 1 mL antibiotic/antimycotic solution/100 mL BSS ***(4)***.
5. Open eggs with coarse forceps at the blunt end, i. e., air sack.
6. Remove shell to make opening large enough to let yolk out.
7. Pour off thick albumin (into a waste bucket nearby), and save a little of thin albumin in a 90-mm Petri dish or sterile beaker ***(5)***.
8. Pour the yolk quickly into BSS ***(6)***.
9. Put up to six yolks into the dish, and top up with BSS if they are not completely covered.
10. Remove remaining albumin from all yolks in the dish with coarse forceps or large-mouthed well-flamed pipet.

3.1. Cutting the Vitelline Membrane

For right-handed operators (reverse for left-handers) (**Fig. 2**):

1. Place yolk with blastoderm facing east.

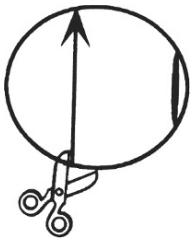

Fig. 2. Cartoon of an egg yolk being cut around the equator to remove the vitelline membrane.

2. Hold yolk with coarse forceps at west pole, and cut with larger pair of scissors around the equator or just below it, starting south and moving north, keeping an eye on the position of the blastoderm at all times.
3. When cut right around, take both pairs of watchmaker's forceps and lift vitelline membrane lightly at the cut edge. Position yolk so that blastoderm is on top, facing the operator.
4. Hold the edge of the vitelline membrane with both pairs of forceps, and pull **very slowly** by inverting it over the yolk, keeping the membrane low over the yolk, and pulling towards the bottom of the dish (*see* **Note 7**). Stage 4 and 5 blastoderms adhere fairly well to the vitelline membrane and are easy to handle. Older and younger blastoderms need more care.
5. When the vitelline membrane is freed from the yolk and the blastoderm still attached to it, immerse a watch glass into the BSS and pull the membrane onto it (*see* **Note 8**). As you raise the watch glass above the BSS level, hold onto the membrane with tweezers or it will float off.
6. Place the watch glass with the blastoderm in a 90-mm Petri dish.

3.2. Arranging Vitelline Membrane on the Ring

1. Use a stereoscopic, binocular dissecting microscope.
2. Stretch vitelline membrane, with the blastoderm in the center, well out on the watch glass. Remember the vitelline membrane ought to be inside-out at this stage.
3. Wet a glass ring in BSS (*see* **Note 9**), and lay it on the vitelline membrane and fold the membrane over the ring, holding ring with straight forceps and pulling membrane with curved tweezers, working all the way around until all the membrane is securely draped over the ring. Hold the membrane **only** at the cut edge to avoid injury to it and the blastoderm (*see* **Note 10**).
4. Pipet off most of the liquid and the adhering yolk. With fine scissors, cut away the surplus membrane close to the inside of the ring.
5. Wash all yolk from under the ring with BSS, and pipet 1 mL of thin albumin onto the watch glass beneath the ring to supply the developing embryo with a source of water and protect it from bacterial infections. Albumin is rich in bactericidal proteins. Little or no liquid should cover the embryo.
6. Lay one or two strips of Kleenex tissue in Petri dish, and wet with BSS to create a moist chamber.
7. Now the embryo is ready for surgery or the designed treatment. If surgery is performed, leave the embryo to heal at room temperature for a couple of hours before further incubation. This helps to close the wound while the blastoderm is not expanding, and there is less tension in the fragile tissues. It may be appropriate to photograph embryos at this stage.
9. Incubate at 38 (±) 1°C in a tissue-culture incubator. Facilities for an air mix of 95% air and 5% CO_2 are not essential.
10. Observe the development of the embryos the next day. They should have reached stage 10–12 (**Fig. 3**) and may be photographed again.

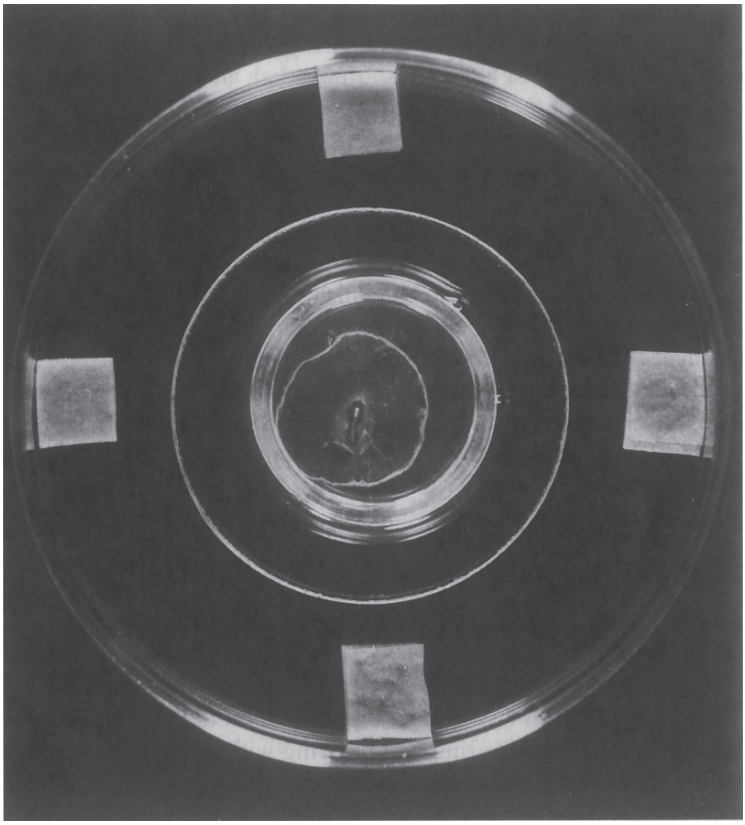

Fig. 3. New Culture after 24 h of incubation.

3.3. Analysis of Results

At this stage, the embryos can be fixed to be processed for many different purposes, including whole mounts, histology, immunocytochemistry, *in situ* hybridization, and others. The type of fixative used will depend on the further treatment. It is important **not** to bring the rings and the watch glasses into contact with any fixative to be used. It saves lengthy cleansing processes, which are unpleasant, time-consuming, and not always a great success.

Place the ring with the embryo in a Petri dish of leftover Howard's Ringer or Pannett and Compton solution from the day before, and wash off unwanted albumin and yolk particles. Either peel the blastoderm away from the vitelline membrane along its peripheral edge and stretch out on a piece of thin plastic (Melinex or overhead projection foil is very good) cut into small squares before immersing in fixative, or just cut the embryo out and transport on a spatula to fixative. Embryos not supported tend to curl up, fold, or distort, and are much more difficult to analyze.

4. Notes

1. Any glassblower will produce glass rings from glass rods but the best rings are cut from glass tubes with the desired diameter, anything from 22–28 mm inner diameter, 3–4 mm thick. They are oblong when viewed from the side, and are either left rough or better still baked to give a smooth finish on the cut surface.

   Here is a word of warning: If any kind of treatment with compounds is envisaged, rings with a rough cut edge should not be used. Despite thorough cleaning they will retain traces of compound and contaminate the next experiment. Not all egg yolks are the same size. Particularly eggs from very young hens are small and may not fit onto rings 26 mm in diameter. A few smaller rings with an inner diameter of 22 or 23 mm are recommended. Smaller rings are also easier to handle for the novice operator, and a few larger rings (28 mm inner diameter) come in handy if the eggs happen to be on the generous side.
2. Watchmaker's forceps may be straight or curved to the individual's preference. Curved forceps are handy for the release of the membrane when folded under the ring and in tricky situations.
3. The incubation time depends on the developmental stage required for the intended experiment (*see* **ref. *6***). Incubating the eggs on their sides is preferable to on their points, but it is not necessary. The blastoderm will always rise to the top of the yolk because of its lower specific gravity compared to the yolk. If the eggs are incubated on their tips, the blastoderm will be directly under the air sack and is more likely to be damaged when the egg is opened.
4. Antibiotic/antimycotic solution (100X) from (Gibco) contains
   a. 10,000 U of penicillin (base).
   b. 10,000 µg of streptomycin (base).
   c. 25 µg of amphotericin B/mL.
   d. Use 1% (1 mL in 100 mL BSS).
5. When separating the albumin from the yolk, **do not pull on the chalazae too hard or the vitelline membrane will break.** The chalazae are two thick gycoprotein threads by which the yolk is tied to the vitelline membrane and which allow it to rotate when the egg is turned so that the blastoderm may rise to the top.
6. Do not try to pour the yolk slowly and carefully from the shell into the BSS. That will certainly tear the yolk.
7. Keep the pie dish on a black surface to show up contours of the yolk, adhering albumin, and so forth.
8. Hold on to the vitelline membrane while lifting the watch glass out of the liquid.
9. A dry ring will instantly stick to the vitelline membrane when put down, and make it difficult to rearrange.
10. As mentioned before, just the cells on the periphery of the blastoderm adhere to the vitelline membrane. It is important not to destroy these delicate contacts.

    Carefully pipet small amounts of BSS at the time against the inner edge of the ring to wash away yolk particles, and suck liquid up by slowly moving the pipet backward along the ring. Embryos younger than stage 4 have **very** delicate contacts to the vitelline membrane, and extra care is needed to transplant these stages successfully.

Best results are achieved after incubation times of 20–36 h. Embryos will develop normally and can be compared to embryos of the same stage *in ovo,* though not in the same time. New Culture embryos will be between one and two stages younger having their development interrupted by the explanting procedure and the following manipulation. Longer incubation times than 36 h may be tolerated, but the development will slow down and the incidence of abnormalities increases dramatically. Lack of nutrients and space to expand for the embryo may all play a part.

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Appendix

Here are a few helpful notes and figures for the novice New Culture operator to facilitate easy apprehension of the most commonly applied early stages in the development of the chick embryo for this method. This summary cannot justify the diversity of nuances for all the stages that the operator will recognize with increasing awareness and experience, but serve as a guide only. The stages described here span from an unincubated blastoderm to a range of stages found after 24–30 h in culture.

All stated times of incubation are very tentative. They will depend on the time an egg took to pass down from the fimbriated edge of the oviduct, where it was fertilized, to the vagina, this can take up to 22 h, depending on how long the eggs were stored before incubation commenced and the temperature at which they were stored. A further variable is that most laboratory incubators are set to

Fig. 4. **(A)** A disk with virtually no landmarks to indicate the future polarity of the embryo. **(B)** Stage 1 after Hamburger and Hamilton (HH), the prestreak, incubation for 4–8 h. After several hours of incubation, the formation of the hypoblast appears as an area of higher density roughly shaped as a triangle rising from the posterior marginal wall. This is called the embryonic shield or Koller's sickle. **(C)** Stage 2 (HH), the initial streak (6–10 h), a short, squat, thickening of the blastoderm at the posterior marginal zone.

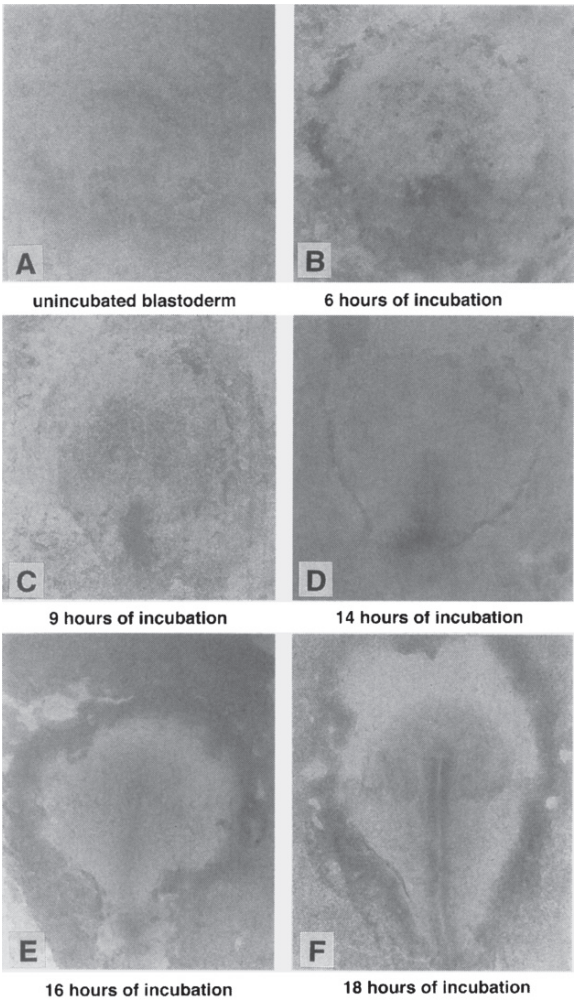

Fig. 4. *(continued)* **(D)** Stage 3 (HH), the intermediate streak (12–16 h), the streak is now extending to one-third of the area pellucida. Invagination of prospective mesoderm proceeds at the posterior part of the primitive streak and ingression of endoderm at more anterior levels of the primitive streak. (**E,F**) Stage 4– and stage 4 (HH), respectively, the definitive streak (15–20 h). The elongation of the primitive streak proceeds over several hours until it has reached its full length extending to two-thirds of the area pellucida from the posterior marginal zone. The average length of the fully extended primitive streak is about 2 mm, but varies enormously from embryo to embryo. It consists of the primitive groove flanked on either side by the primitive folds. The most anterior part of the primitive streak is broadened into a bulb, and is known as Hensen's node, the organizer region of the avian embryo, the homolog to the dorsal lip of the blastopore in amphibian embryos. Somitic mesoderm invaginates through the posterior primitive streak and heart mesoderm through the midstreak regions. The area pellucida is pear-shaped.

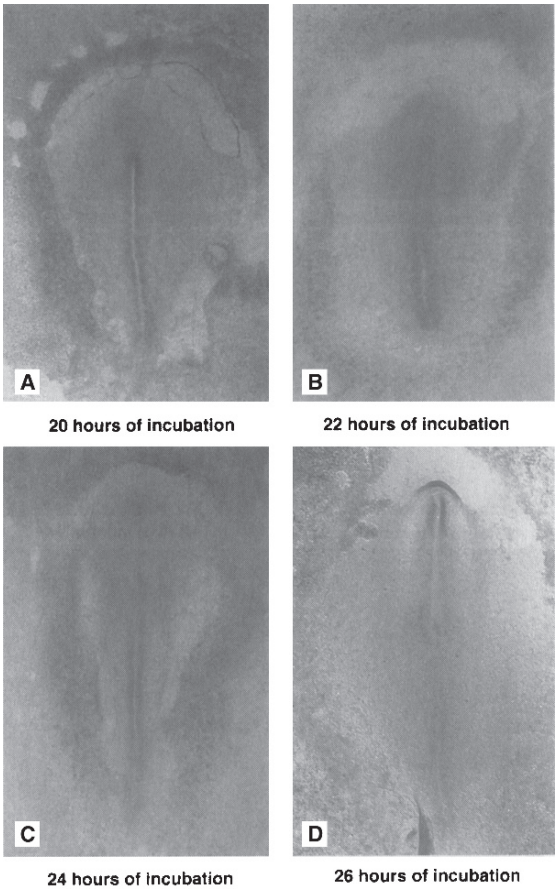

Fig. 5. **(A)** Stage 4+ (HH) (18–22 h) shows the first sign of the head process extending cranially from Hensen's node to give rise to the head notochord. (**B,C**) Stage 5– and stage 5 (HH), respectively, the head process (20–24 h), shows the condensation of mesodermal cells of the head process more clearly. **(D)** Stage 6 (HH), the head fold (23–26 h). The anterior rim of the medullary plate is pushed forward by the extending head process until it folds over under the tension, forming the subcephalic pocket ventrally. The ensuing fold in the endoderm is to give rise to the foregut. The duration for this stage is very short. The head fold can flip over within 1 h. Hensen's node is now beginning to regress leaving in its wake the notochord. Mesenchyme cells form isolated blood islands in the extraembryonic mesoderm.

slightly different temperatures, which will be reflected in the stage of development of the embryo. Many investigators make the additional distinction between winter and summer eggs.

All embryos were fixed in 3.5% paraformaldehyde in phosphate-buffered saline (PBS) for at least 4 h and stained in saturated carmine red in 4% Borax

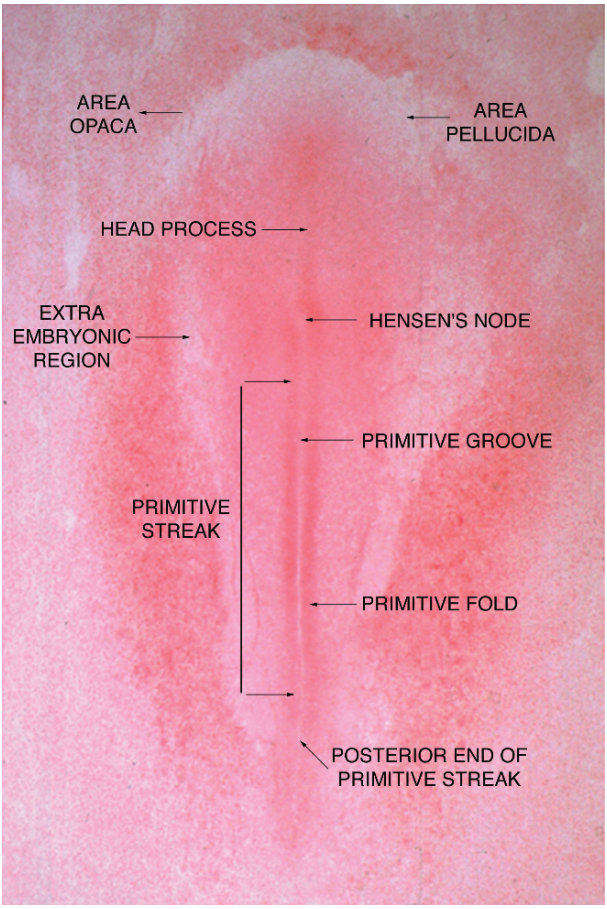

Fig. 6. Stage 5 (HH), the head process has reached its most cranial position and laid down the prospective head territory. The cells of the epiblast overlying the head process form a pseudostratified epithelium, the medullary plate, and the presumptive neural plate. Hensen's node is clearly asymmetrical. The bilateral heart primodia lie lateral and anterior to the node. (*See Color Plate*)

and diluted 50/50 in 70% alcohol until they reached the required intensity, differentiated in 70% acid alcohol, dehydrated, cleared and mounted in DPX, and photographed on a Zeiss Axiophot (mag. ×40) or on a Zeiss Stemi SV6 (mag. ×20–30) on Fujichrome 64 T slide film. All embryos were photographed from the dorsal aspect with their antero–posterior polarity running north to south.

When the egg is laid, it has little resemblance to an embryo. The unincubated blastoderm is a flat disk made up of two layers (*see* **Subheading 1.1.**).

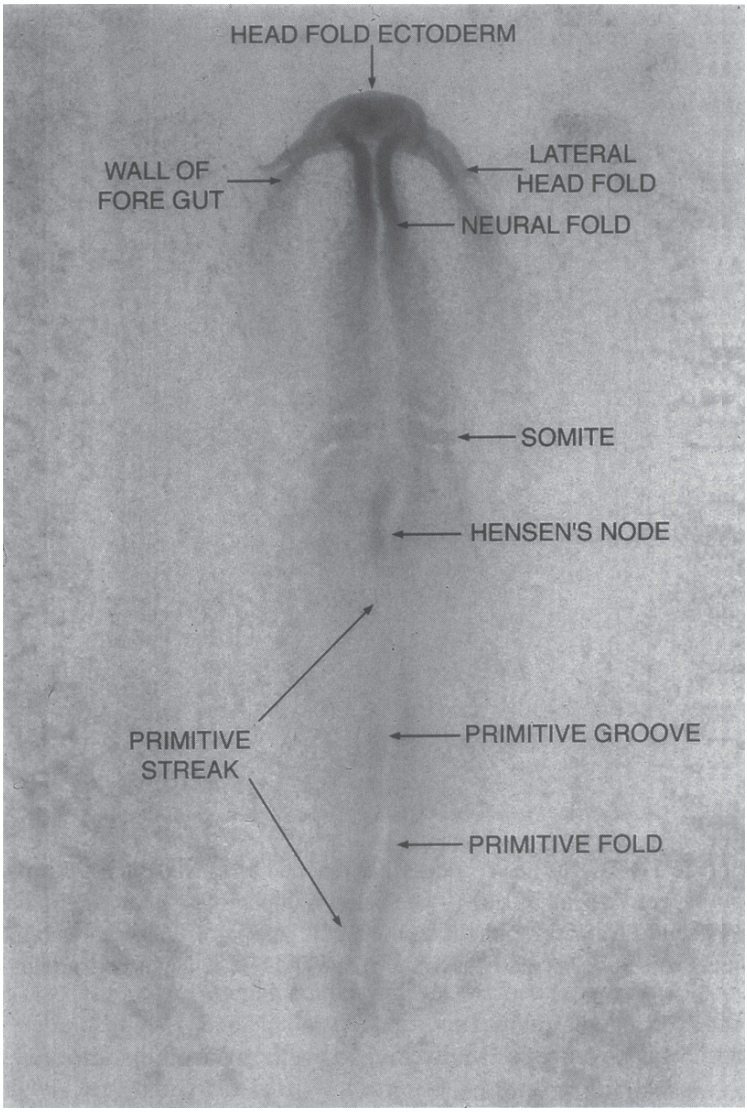

Fig. 7. Stage 7 (HH) (26–30 h) shows the segmentation of the first pair of somites from the *para*-notochrodal mesoderm. This is in fact the second pair of somites. The first somite is a phantom somite. It never forms fully and will disperse again in the next few hours of development. It is a slight mesodermal condensation anterior to the cleavage for the first somite proper. Not all embryos have a visible first somite. Subsequent pairs of somites will segment in two hourly intervals from the *para*-axial mesoderm. Neural folds reach to the mesencephalon. The wall of the foregut emerges close to the lateral head fold.

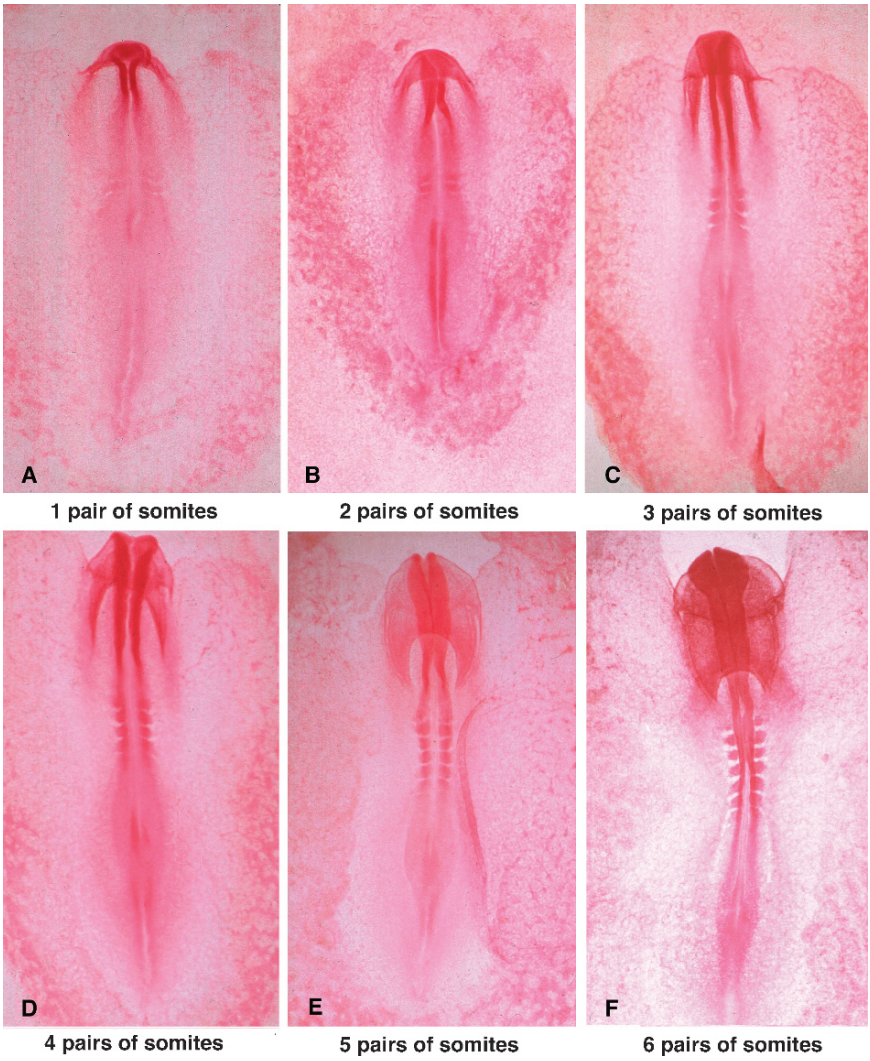

Fig. 8. Comprises stages 7 and 8. **(A)** Stage 7 (HH), 1 pair of somites. **(B)** Stage 7+ (HH), 2 pairs of somites. **(C)** Stage 8− (HH), 3 pairs of somites. **(D)** Stage 8 (HH) 4 pairs of somites. **(E)** Stage 8+ (HH), 5 pairs of somites. **(F)** Stage 9− (HH). 6 pairs of somites, dorsally the first cleft between rhomobomere 5 and 6 can be detected. (*See Color Plate*)

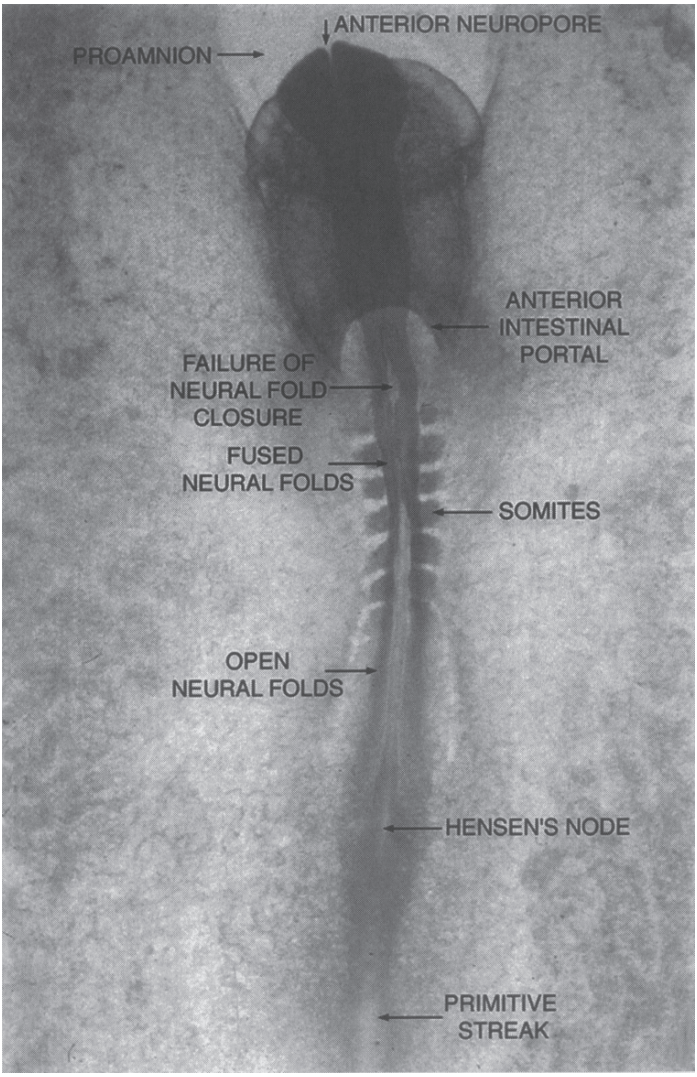

Fig. 9. Stage 8 (HH) (28–34 h), five pairs of somites. The head is raised above the proamnion. The proamnion is a region free of mesoderm between ectoderm and endoderm at the anterior edge of the area pellucida flanked by the lateral horns meeting cranially. Open neuropore, neural folds are beginning to close in the region of the mesencephalon (failure to close will lead to spina bifida in the adult, as seen here rostral to the first somite). Ventrally, the notochord can be seen through the open folds of the neural tube as the node regresses along the primitive streak. Bilaterally heart primodia are developing from the amnio-cardiac vesicle level with the midbrain. More blood islands are developing in the area opaca.

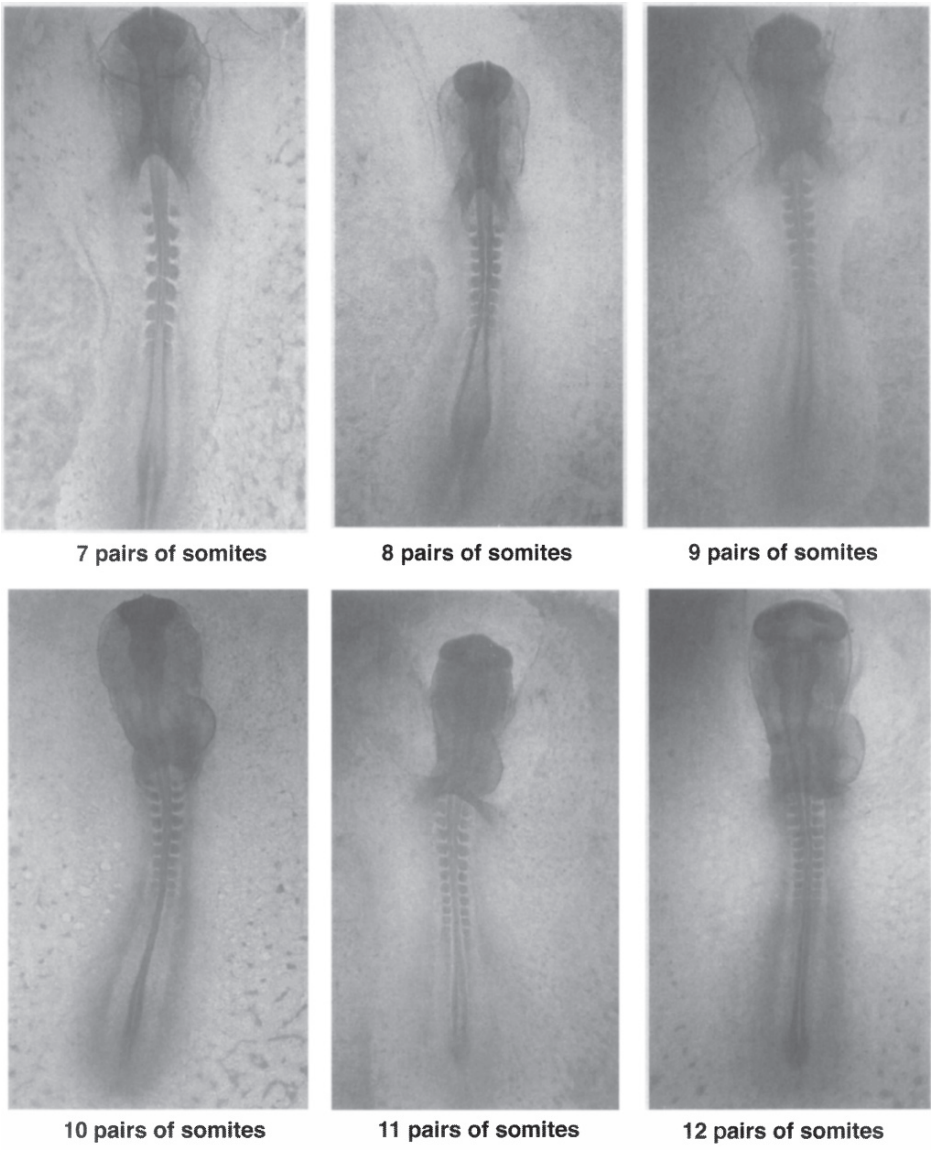

Fig. 10. Comprises stages 9 and 10. **(A)** Stage 9 (HH), (32–38 h), seven pairs of somites, dorsally bilateral primodia of the optic vesicle appearing. Mesencephalon is clearly demarcated from rhombencephalon at the isthmus. Segmentation of rhombomeres continues with boundaries 2/3 and 3/4 appearing. Ventrally the heart primodia fusing medially into a tube. Margin of anterior intestinal portal is level with vitelline vein which is connecting with heart, and splaying out laterally to area opaca. **(B)** Stage 9+ (HH), 8 pairs of somites. **(C)** Stage 10– (HH), 9 pairs of somites. **(D)** Stage 10 (HH) 10 pairs of somites. **(E)** Stage 10+ (HH) 11 pairs of somites. **(F)** Stage 11– (HH), 12 pairs of somites.

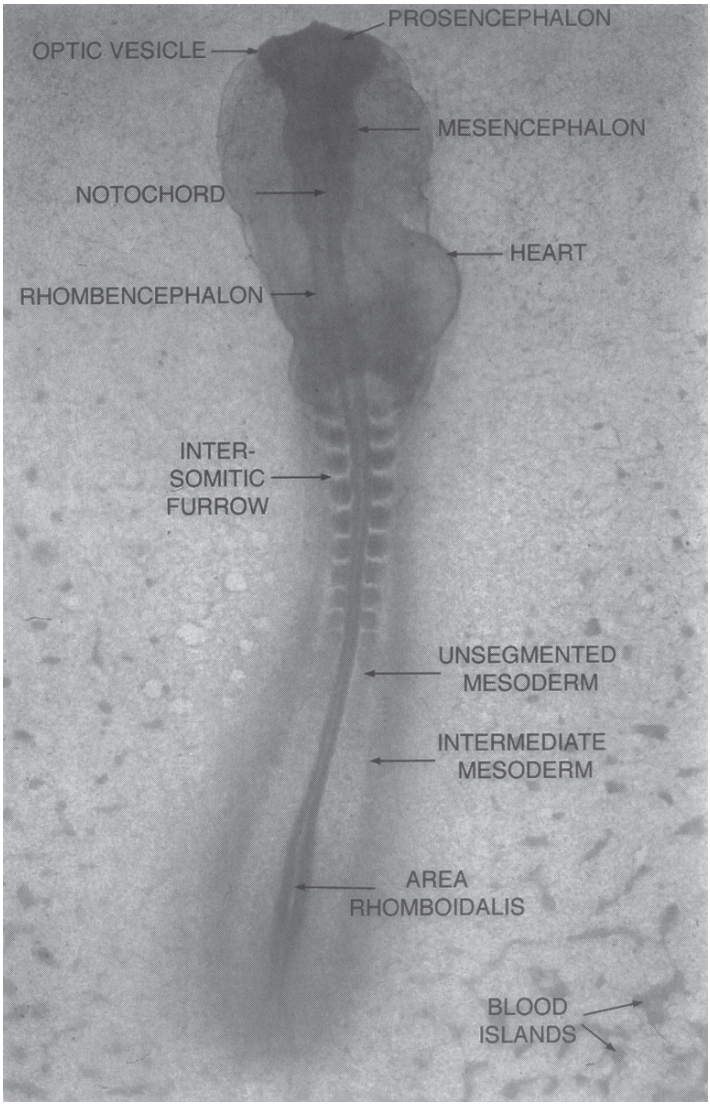

Fig. 11. Stage 10 (HH), 10 pairs of somites (36–42 h) dorsally anterior neuropore closed, prominent optic vesicles, rhombomere boundary 4/5 formed, and neural folds are closed to almost the level of the node. Ventrally, Hensen's node has regressed almost to the end of the primitive streak (the 10th pair of somites has not fully segmented caudally in this illustration), pronephric tubules develop between somites 6 and 10, heart tube turns asymmetrical bulging out to the right and contractions can be seen, and bilateral vitelline veins fan out toward the area opaca, which shows large blood islands to establish circulation.

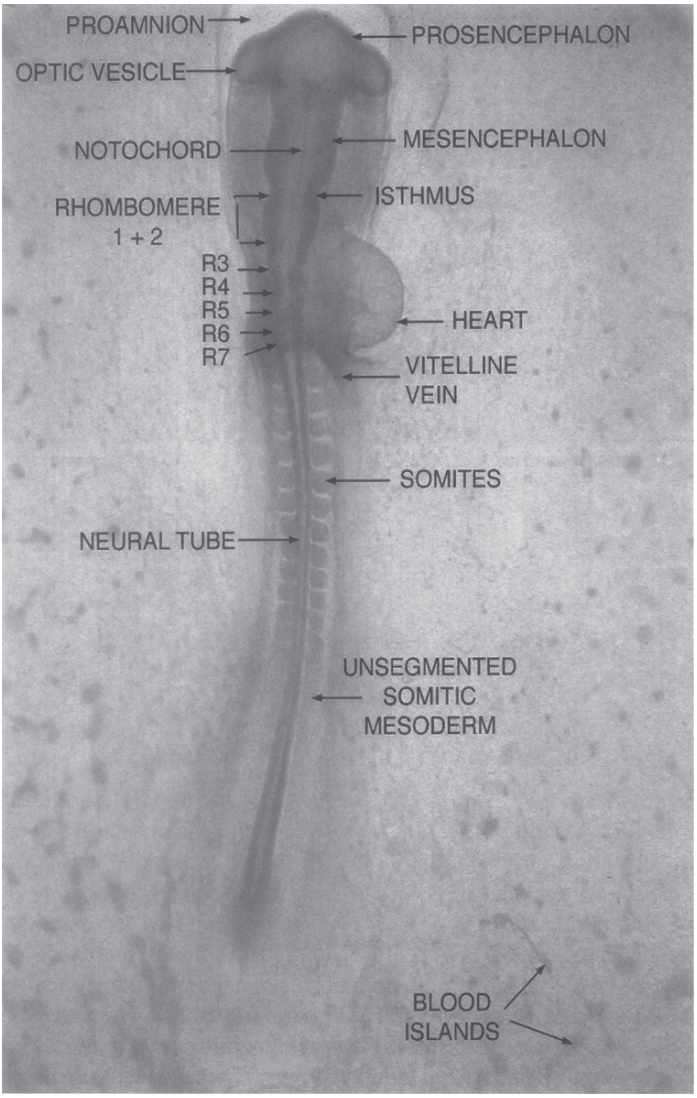

Fig. 12. Stage 11 (HH), 13 pairs of somites (40–46 h), slight flexure of the head, prominent optic vesicles with slight constriction at their base form the lateral parts of the prosencephalon, mesencephalon has clear boundaries, and all rhombomeres can be distinguished. The neural tube is virtually closed along its entire length. On the ventral side, the rostral part of the heart forms the ventricle, whereas the caudal part gives rise to the atrium and the vitelline vein leading from it. The heart beat is rhythmical, but the circulation of blood is not yet fully connected up to all peripheral blood islands. Anterior somites are beginning to differentiate.

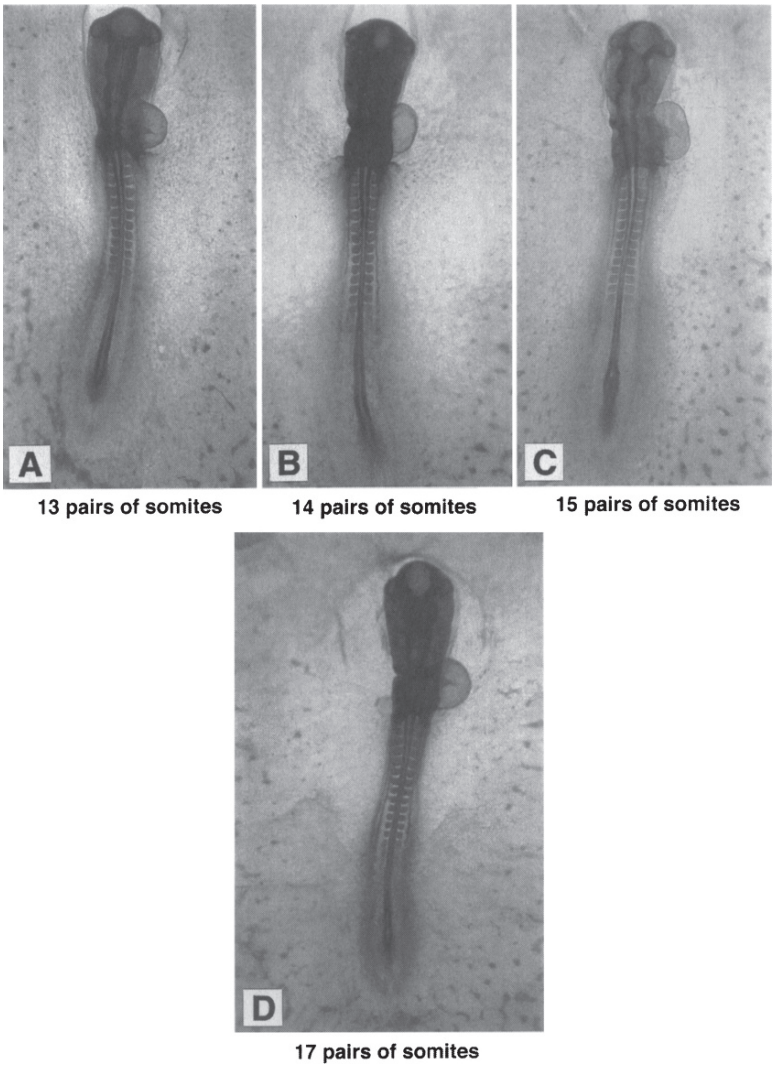

Fig. 13. **(A)** Stage 11 (HH), (40–46 h), 13 pairs of somites. **(B)** Stage 11+ (HH), 14 pairs of somites. **(C)** Stage 12– (HH), 15 pairs of somites. **(D)** Stage 12 (HH) (48–54 h), 17 pairs of somites. Embryos in New Culture will rarely develop well beyond this stage. It is possible in exceptional circumstances to maintain embryos for up to 48 h in culture. For example, embryos on rings can be left on semipermeable membranes instead of watch glasses and given culture medium containing serum instead of albumin, but development is slowed down and abnormalities increase. The eyes are budding off from the prosencephalon, the bilateral otic vesicles are clearly visible as round indentations adjacent to rhombomere 5. Ventrally the circulation of blood from the heart to the periphery is nearly complete. Somites are segmenting off at about one every 2–3 h. The notochord has regressed to the end of the primitive streak.

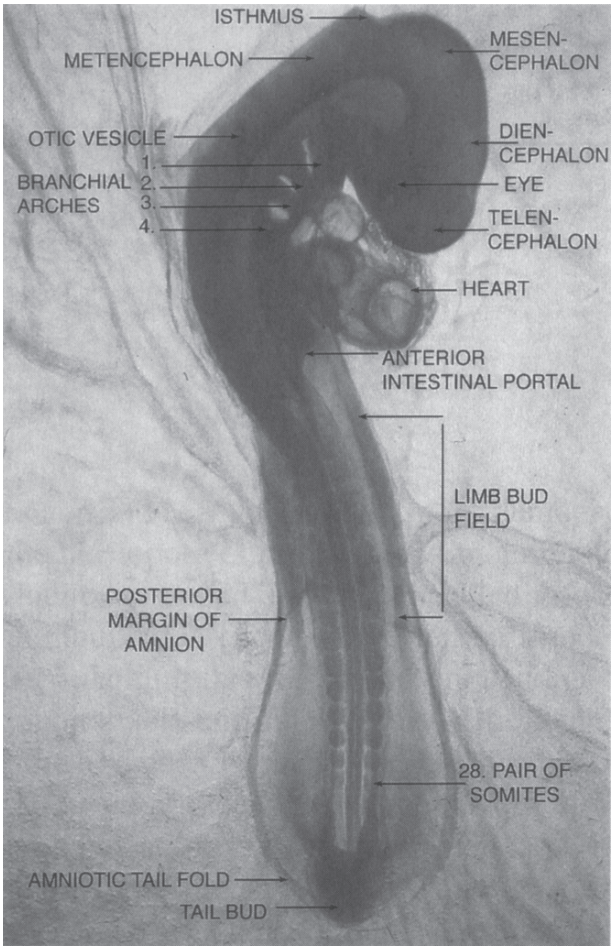

Fig. 14. Stage 16 (HH) (about 72 h), 28 pairs of somites. Very few embryos may get to this stage in development. The vascular system is ill-equipped to function in a two-dimensional space. When the cranial flexure of the embryo reaches 90° or more the main blood vessels fold under the weight of the trunk and can no longer work efficiently. The heartbeat will increase without fullfilling a better function. The embryo fills up with fluid and development slows right down. The lens of the eye has separated from the eye cup, there is a clear demarcation between the telen- and the diencephalon, the isthmus divides the mesencephalon from the metencephalon, and the otic vesicle is a shallow, almost round indentation with a raised epithelial rim. All four branchial arches are clearly structured. The flexure of the body extents to the anterior intestinal portal and the body folds curve ventrally to the tail bud. The wing bud primodia can clearly be seen in a slight thickening of the flank, but there is no such indication for the leg bud yet. The posterior margin of the amnion extends to about the 20 pair of somite. Somite numbers are no longer a reliable aid to stage embryos of this stage or older. The most cranial somites are starting to be incorporated into the base of the scull, and the increase of the flexure of the body right down to the tail bud makes it difficult to give an accurate account. There are many other landmarks for accurate staging. The shape and structure of the branchial arches, the color of the eye, the angle of the head, and the shape and size of the limb buds are just some.

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EC Culture

A Method to Culture Early Chick Embryos

Andrea Streit

1. Introduction

Avian embryos have been used as a model system to study development for more than two centuries and have proven to be particularly useful to study events that occur at early developmental stages, when other amniote species are difficult to access and maintain *in vitro*. They lend themselves to embryological manipulation, such as interspecies tissue transplantation or cell lineage analysis as well as observation of cellular behavior in the living embryo. At the time of egg laying, the embryo is a large, translucent disc of approximately 50,000 cells that is easily accessible and develops well *in vitro* from before primitive streak formation. Two well-defined staging systems are available that describe preprimitive streak stages (using Roman numbers I–XIV ***[1]***) and the stages after primitive streak formation (in Arabic numbers 2–46 ***[2]***). With recent technical advances, like introduction of expression constructs, antisense RNA (morpholinos), and dsRNA by electroporation, the chick now offers all the advantages of a well-studied system for embryological studies, which can be combined effectively with modern molecular methods. In particular, loss- and gain-of-function experiments can be performed in a temporally and spatially controlled manner to target specific cell populations. This is particularly useful when investigating the late functions of genes that are vital during early stages of development.

While McWorther and Whipple are reported to be the first to culture primitive streak stage chick embryos *in vitro* successfully ***(3)***, the most important advances in culture conditions were made between the 1930s and 1950s. Waddington ***(4)*** modified a method previously designed for organ and tissue culture, using a plasma clot as support for the blastoderm. Spratt ***(5–8)*** and

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de Haan ***(9)*** improved his technique by replacing the plasma clot with a mixture of agar and albumen or egg-yolk dialysate. Although Waddington and Spratt made important findings relating to inductive interactions and the regulative behavior of the embryo, the cultures were difficult to set up, required sterile conditions, and in general produced specimens of poor morphology.

The most significant improvement came with the introduction of a culture method by Denis New ***(10)*** that aimed to mimic conditions similar to those in the egg. As a substrate for the blastoderm, New used the vitelline membrane, which was stretched around a glass ring then placed on the albumen, with the embryo facing ventral side up. This allowed culture of embryos from prestreak stages as well as the development of embryos with excellent morphology up to stage 15 or 16, on average. Albumen as a culture medium is optically clear and therefore well suited for observation and filming. In addition, it contains nutrients as well as lysozyme, which as a bacteriostatic prevents bacterial growth. A detailed description of the New culture is found in Chapter 16 of this book and a simpler, modified version is described by Stern ***(11)***. The parameters that influence embryo survival and morphology in the New culture have been reviewed in ***(12)***.

Following New, several authors have described modifications of his method introducing, for example, double rings and inverted culture to make the dorsal epiblast accessible for manipulation and observation as well as to permit development of normal amniotic membranes ***(13)***. Other methods ***(14–16)*** were less successful for a number of reasons, including difficulties in observing details of the embryo, the requirement for complicated chambers, and sometimes intricate setting up procedures, which renders them unsuitable for culture of a large number of embryos. A more recent modification uses nonpolished glass rings to make the vitelline membrane adhere firmly to the ring, which simplifies setting up of cultures considerably, and 35-mm dishes as culture containers, allowing space-saving incubation of the cultures ***(11,17)***.

A method inspired by mouse embryo cultures in roller bottles was termed the *cornish pasty* culture ***(18)***: Embryos are maintained in a liquid medium without supporting substrates, allowing easy exposure to antisense RNA, soluble factors, and the like. However, this prevents observation and, especially, time-lapse filming during the culture period. Using this method, embryos can be cultured for long periods and develop fairly normally (except for a severe microcephaly) until at least stage HH 16.

New ***(19)*** already reported the importance of the vitelline membrane for normal growth of the embryo, as well as the special properties of its inner surface for the attachment of the edge of the extraembryonic region (*area opaca*) and, therefore, for expansion of the blastoderm. Similarly, inclusion of the vitelline membrane in agar cultures reduced the developmental anomalies often observed when the embryo was placed directly on agar ***(20)***. Chapman and colleagues

exploited these findings and recently described a modification of Spratt's agar culture, which they termed *EC* (early chick) *culture* ***(21)***. This method is useful to culture chick embryos form primitive streak and early somites stages to approximately HH stage 15 but produces less good results for the growth of embryos isolated at preprimitive streak stages.

This chapter describes the protocol to set up chick embryo cultures according to the method of Chapman and colleagues with some modifications and points out some of its advantages and disadvantages in comparison to New culture.

2. Materials

2.1. Equipment

1. Humidified 38°C incubator for egg and culture incubation.
2. Dissecting stereo microscope with transmitted light base.
3. Water bath.
4. Automatic pipetor.
5. Paper hole punch.

2.2. Materials, Chemicals, and Solutions to Prepare Culture Dishes

1. One dozen unincubated chick eggs.
2. 35 mm petri dishes (bacteriological grade).
3. 50 ml Falcon tubes.
4. 10 ml pipets.
5. 100 ml measuring cylinder (sterile).
6. 500 ml Erlenmeyer flask (sterile).
7. Plastic container for storage of agar–albumen dishes.
8. Plastic container or bag for egg waste.
9. Large pair of forceps to open eggs.
10. Bacto-agar (Difco) or AGAR select (Sigma).
11. 100× antibiotic–antimycotic (penicillin–streptomycin–amphotericin B) solution (Sigma A5955).
12. Saline: 0.719 % NaCl in distilled H_2O, autoclaved.

2.3. Materials and Solutions to Set Up Cultures

1. Whatman filter paper (no. 1–5 are all suitable).
2. 10 cm glass Petri dishes, nonsterile.
3. Soft tissue paper (Kleenex, Kimwipe).
4. Plastic container or plastic bag for egg waste.
5. Plastic container to incubate cultures.
6. 1,000 ml measuring cylinder.
7. Small beaker to collect thin albumen.
8. One pair of large forceps to open eggs.
9. Two pairs of fine forceps (watchmakers no. 4 or 5).
10. One pair of small scissors.

11. Fire-polished Pasteur pipets.
12. Pannett-Compton saline (22):
    Solution A: 121 g NaCl, 15.5 g KCl, 10.42 g $CaCl_2$ $2H_2O$ (or 7.7 g $CaCl_2$), 12.7. $MgCl_2$ 6 H_2O to 1,000 ml distilled H_2O, autoclaved.
    Solution B: 2.365 g Na_2HPO_4 $2H_2O$, 0.188 g NaH_2PO_4 $2H_2O$ to 1,000 ml distilled H_2O, autoclaved.

Both solutions can be stored at room temperature or at 4°C; before use, mix in the following order: 40 ml A, 900 ml distilled H_2O, and 60 ml B. Do not mix stock solutions A and B as it causes precipitation. Note: The original method ***(21)*** uses simple saline; however, we find that embryos are more robust when cleaned and collected in Pannett-Compton saline, in particular if they are kept in saline before culture (see later).

3. Methods

3.1. Preparation of Culture Plates

Culture plates can be prepared in advance and can be stored for up to 4 wk in a humidified, airtight container at 4°C. The following recipe is sufficient to make about 100 culture plates.

1. Heat the water bath to 49°C.
2. Bring to a boil 120 ml saline in the sterile Erlenmeyer flask and add 0.72 g agar to dissolve under constant stirring.
3. Place the flask into a water bath to equilibrate to 49°C.
4. Meanwhile, using a pair of large forceps, open eggs at the blunt end and remove shell to open a hole large enough to collect albumen.
5. Collect 120 ml of thin albumen into a 50 ml Falcon tubes; be careful not to include thick albumen. Place into the water bath to warm up; do not keep at 49°C for too long.
6. Meanwhile, arrange 100 35 mm petri dishes on a flat surface.
7. When the agar has cooled down, add the albumen to the agar solution and swirl gently to mix; add 2.4 ml of the 100× antibiotic–antimycotic stock while continuing to mix.
8. Using a sterile 10 ml pipet and pipet aid, distribute 2.5 ml agar–albumen into each petri dish, avoiding the formation of bubbles. Do not dispense more than 2.5 ml, since a thicker substrate will make observation of the embryos more difficult.
9. Allow the agar–albumen mixture in the dishes to solidify and dry for a few hours or overnight at room temperature.
10. Store dishes in a humidified, air tight container at 4°C for up to four weeks. Before use check the dishes to ensure the absence of bacterial or fungal growth.

3.2. Setting Up Cultures

Incubate fertile hens' eggs prior to the experiment in a humidified 38°C incubator to reach the desired stage ***(2)***. Whatman filter papers that serve as support to recover the embryos attached to the vitelline membrane can be prepared in advance. Cut filter paper into approximately 2 × 2 cm squares; then, using the

paper hole punch, make four overlapping holes into the center of each square in the shape of a four-leaved clover. Filter papers can be sterilized; however, we have not encountered infection problems with nonsterile paper. Before starting, prepare saline, set up a waste container and glass petri dish to collect yolks and, if required, another petri dish to wash embryos; remove agar/albumen plates from 4°C to warm up to room temperature.

1. Remove eggs from the incubator; eggs can be kept at room temperature for a few hours before proceeding. To open, hold the egg blunt end up and use a coarse forceps to crack the shell. Remove a large cap of the shell with the tips of the forceps, taking care not to damage the vitelline membrane and yolk. Collect some thin albumen into a small beaker and allow the remaining albumen to pour into the waste container. At this stage, with the help of the forceps, remove the thick albumen as much as possible, especially directly above the embryo. Make sure that the embryo is positioned uppermost; if not, use the closed forceps gently to turn the yolk until the embryo is visible. Gently slide the yolk into a 10 cm glass petri dish. The embryo should be on top facing you (**Fig. 1A**); if not, again use closed forceps to turn the yolk or turn it with the help of the chalazae (the spirals of white material protruding from two points at the equator of the yolk).
2. Remove all the remaining thick albumen from the vitelline membrane surrounding or covering the embryo. To do this, fold a piece of soft tissue, lower it onto the albumen at the edge of the embryo, and gently pull away from it (**Fig. 1B**). Repeat this process until no albumen is left. This is an important step, since albumen prevents the vitelline membrane from attaching to the filter paper. Without sufficient contact, the vitelline membrane loses its tension, and it is difficult to recover the embryo intact.
3. With small forceps, lower one of the paper rings onto the vitelline membrane such that the embryo is in the center of the clover-shaped opening (**Fig. 1C**). The paper will absorb liquid from the membrane surface and tightly adhere to the membrane itself.
4. Using a small pair of scissors, cut through the vitelline membrane around the filter paper (**Fig. 1D**). Hold the paper with a small forceps and gently pull it diagonally away from the yolk (**Fig. 1E**). Do not pull vertically since this may lead to damage of the embryo or its detachment form the vitelline membrane; in particular, primitive streak stage embryos are fairly fragile and the tissue layers separate easily under tension.
5. The next step frees the embryo from the remaining yolk and debris, which otherwise may obscure vision as well as lead to poor development of the embryo. This can be achieved in two ways. Place the embryo onto the agar–albumen dish with the vitelline membrane facing the substrate (dorsal side down) avoiding air bubbles (**Fig. 1H**). Use a fire-polished Pasteur pipete to blow gentle streams of Pannett-Compton saline onto the embryo and wash off the yolk; remove the liquid by slightly tilting the dish. Repeat this process until the embryo is sufficiently clean.

   Alternatively, immerse the filter paper with embryo into a petri dish containing Pannett-Compton saline at a slanted angle (**Fig. 1F**). Remove the yolk either by gentle swirling or using a fire-polished Pasteur pipet to blow streams of saline around

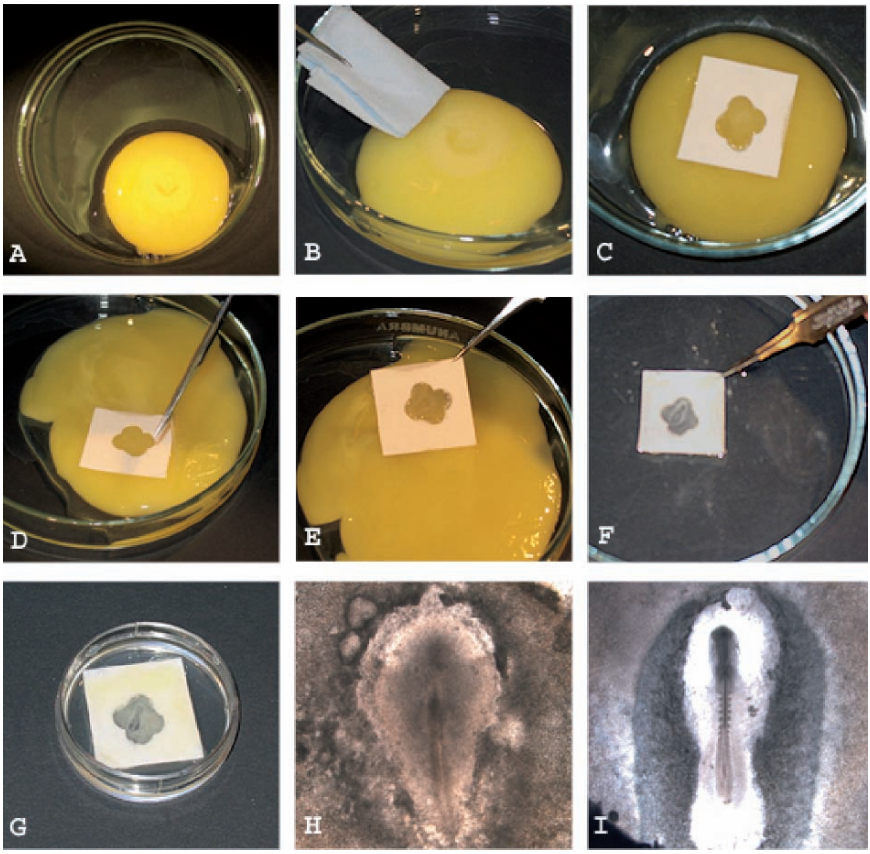

Fig. 1. Individual steps to set up an EC culture. **A** The yolk is placed into a petri dish with the embryo facing upward. **B** Albumen is removed from the area surrounding the embryo using a soft tissue. **C** A paper ring is placed onto the vitelline membrane with the embryo in the center of the clover-shaped hole. **D** Using a pair of small scissors, the vitelline membrane is cut around the paper ring. **E** The paper ring with the embryo attached to the vitelline membrane is lifted off the yolk at a slanted angle. **F** The embryo is freed from yolk and debris in Pannett-Compton saline. **G** The embryo is placed ventral side up onto the agar–albumen dish. **H** Stage HH 5 embryo at the start of EC-culture. **I** The same embryo after overnight culture has reached stage HH 9. (*See Color Plate*)

and onto the embryo. Be careful not to detach the embryo from the vitelline membrane. Once cleaned remove the embryo including the paper ring from the saline, tap off the residual liquid on a piece of tissue, and place it ventral side up onto a culture dish (**Fig. 1G**). Be careful not to trap any air bubbles between the vitelline membrane and the agar–albumen substrate. Tilt the plate gently to remove any remaining liquid with a Pasteur pipet.

The second alternative can be used to collect many embryos in a large dish of Pannett-Compton saline, where they can be kept for a few hours before proceeding. This is particularly useful if embryos are used afterwards for the same experimental procedure, like electroporation.

6. Once the embryos have been cleaned and positioned onto the agar–albumen, wet the lid of a petri dish with a thin film of albumen, replace it onto the bottom of the dish, and press down to seal. Sealing the dish with albumen prevents condensation to form on the lid, which may otherwise drop onto the embryo. If embryos are used for filming avoid albumen in the center of the lid. Stack several dishes in a plastic container containing moist tissue and incubate at 38°C for the desired time. In general, embryos grow well for 24–36 hours (**Fig. 1I**), longer incubation times can be successful but may result in a diminished survival rate and developmental abnormalities.

3.3. Variations of the Culture Method

Like most of the other culture systems described in the introduction, embryos in EC culture are normally grown ventral side up. Occasionally, however, it may be useful to have access to and to observe the dorsal surface of the embryo. From stage 8 onwards (four or five somites) cultures can be set up using the same protocol as just described but placing the embryo ventral side down onto the agar–albumen dish after washing. Younger embryos develop very poorly under these conditions. However, they can be kept ventral side up until they have reached stage 8; they are then removed from the dish by floating in Pannett-Compton saline, turned dorsal side up, and replaced on the same dish. Remove any residual liquid and continue to incubate until the desired stage is reached.

4. Notes

We made a few modifications to the method originally described by Chapman and colleagues ***(21)***. First, instead of using simple saline to clean or store the embryos, we use Pannett-Compton saline, in which the embryos remain more robust and healthy. Second, we seal the petri dishes used for culture with albumen to prevent condensation on its lid; condensation may drop onto the embryo and disturb its growth.

One of the main advantages of EC culture is that most beginners find it easier and faster to set up than, for example, the New culture described in Chapter 16 of this book. It is fairly straightforward and can usually be performed without demonstration from an expert or used in practicals for larger student groups. In addition, there is no requirement for glass rings, which can be difficult to obtain, and (unlike for the New culture) small holes in the vitelline membrane do not

result in a failure of growth of the embryo. However, just like the New culture, a certain amount of practice is required to obtain embryos of perfect morphology and development. Some of the most common problems are failure of head turning due to the semi-solid substrate, malformations in the anterior brain as well as shortening of the anterior–posterior axis (probably due to slow regression of the node), and lateral expansion of the somites after long-term culture (see also ***(20)***).

While EC culture is useful for some applications such as video-time-lapse filming of the living embryo using an upright microscope and experimental procedures like electroporation into primitive streak stage embryos followed by short culture periods (up to 24 h), it is less suitable for grafting tissues, certain kinds of beads (e.g., coated AffiGel blue beads), or cells into the embryo. The reason for this is that the embryo in the center of the filter paper opening lies slightly lower than the rest of the vitelline membrane, which can easily lead to the accumulation of liquid. It may therefore prove difficult for grafts to integrate properly (which they do best when the embryo surface is dry) or to position grafts without the risk that they will move later. In contrast, embryos in the New culture are positioned on a dome shaped vitelline membrane that allows all liquid to drain off efficiently and hence allows better integration of grafts.

Before primitive streak formation, chick embryos are particularly fragile and easily destroyed when explanted, requiring extra care to be cultured successfully. Unfortunately, the survival rates and normal development of these young embryos in EC culture is poor. One problem is that the embryos are not as firmly attached to the vitelline membrane as they are at older stages and therefore tend to break easily or come off the membrane when lifted off the yolk using the method described. A second problem is that, even when set up successfully in EC culture, development often arrests before primitive streak formation. In contrast, in our hands, the New culture offers the method of choice for prestreak embryos with survival rates close to 100%; removal of the embryo from the yolk can be performed more gently and with greater care. Even if the embryo detaches from the vitelline membrane during this procedure, it can be transferred back onto the membrane wrapped around a glass ring after removal of the subembryonic yolky "plug." Careful removal of all liquid allows the edges of the extraembryonic region to reattach to the membrane and for the embryo to grow successfully.

In summary, EC culture combines two long established techniques: paper rings as a support for collecting embryos and Spratt's agar culture technique with the vitelline membrane as a substrate. The result is a relatively simple technique that can be learned rapidly by a less experienced researcher and is useful for producing large numbers of embryo cultures and observation or filming of the embryo over a period of 24–36 hours.

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Grafting Hensen's Node

Claudio D. Stern

1. Introduction

Soon after Spemann and Mangold's ***(1)*** famous demonstration, in 1924, that the dorsal lip of the blastopore of the gastrulating amphibian embryo has the unique ability to induce a second axis when grafted into an ectopic site in a host embryo, Waddington ***(2, 3)*** showed that Hensen's node is its equivalent in amniotes. After transplanting this region into an ectopic site in interspecific combinations of rabbit, duck, and chick embryos, he found that a second axis developed, where the nervous system was derived from the host ectoderm ***(4)***. Hensen's node is situated at the anterior (cranial) tip of the primitive streak during gastrulation and, in chick embryos, appears as a bulbous thickening, some 100 μm in diameter, centered around a depression, the primitive pit. At this point, the three germ layers of the embryo are in very close apposition.

In the avian embryo, operations involving Hensen's node at the primitive streak stage (10–20 h incubation, Hamburger and Hamilton (HH stages 3–5 ***(5)***) are most easily performed in whole embryo culture, as described by New ***(6, 7)***. Even though slightly more involved than "EC culture" (*see* Chapter 17), embryos develop better in New culture: growth is less stunted and head abnormalities are less common. For assays of induction, it is essential to be able to distinguish donor from host cells because the change of fate of the host cells is central to the definition of induction. This can be achieved most easily using interspecies chimaeras, for example, quail donors and chick hosts, whose cells can be distinguished by either the Feulgen-Rossenbeck technique or using anti-quail-cell antibodies (e.g., QCPN) or species-specific riboprobes in in-situ hybridization analysis. Another way to trace the fate of the grafted cells is to label the transplanted node with a cell autonomous vital dye, such as the carbocyanine dye DiI ***(8, 9)***. In a study using these techniques in combination with tissue- and region-specific markers, Storey et al. ***(10)*** were able to determine

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that Hensen's node is at the peak of its inducing ability at the primitive streak stage, but this ability quickly declines as soon as the head process begins to emerge (HH stage 5).

One problem with the way in which Waddington originally performed his grafting experiments ***(2,3)*** is that he placed the transplanted node into a region now known to be fated to form neural plate, and therefore, although he demonstrated that this was able to initiate the formation of a second axis in the host, it is impossible to conclude from his experiment that the host cells underwent a change in fate. One way to overcome this is to place the grafts into a peripheral ring of the avian blastoderm, the inner third of the *area opaca*. During normal development, this region contributes only to extraembryonic tissues but is nevertheless able to respond to a graft of Hensen's node by generating a complete embryonic axis, where the host epiblast changes its fate from extraembryonic ectoderm to neural tissue ***(10,11)***. The competence of this region to respond to such a graft declines rapidly, such that, by HH stage 5, it is no longer able to respond to grafts of nodes derived from donors of any stage ***(10–12)***.

Manipulating Hensen's node in its normal position in the embryo is technically difficult, as are most other microsurgical operations on chick embryos at the primitive streak stage. This is particularly true when the manipulation involves all three germ layers (ectoderm, mesoderm, and endoderm), because cutting through the whole thickness of the embryo often leads to holes that expand greatly and eventually destroy the embryo. The main reason for this is that, at these stages, the embryo develops well only when it is under some tension. This tension is maintained by the migration of cells at the peripheral edge of the *area opaca* on the vitelline membrane, to which they are attached. There are several ways to overcome this problem, at least in part. One is to remove the embryo from its vitelline membrane and to culture it, epiblast side down, on the surface of agar-albumen or agar-egg extract, as described by Spratt ***(13)***. But, under these conditions, growth of the embryo is stunted and abnormalities of the development of the axis are the rule rather than the exception. Another way is to excise the most peripheral edge of the *area opaca* but leave the embryo on its vitelline membrane. The excised cells slowly appear to regenerate, while the hole has time to heal and the embryo gradually develops tension once again in time for normal axial development to occur. In my experience, this is a very successful way to proceed. A third way to prevent large holes from expanding is to keep the newly operated embryo at room temperature for 2–3 h, followed by a period (3–5 h) at 30°C before placing it at 38°C. The low temperature appears to slow down expansion of the *area opaca* while allowing healing to occur. This is also a good approach. Whatever the course of action chosen, it is important to consider that the extent of the healing process probably determines the outcome of the experiment. Healing after excision of a large portion of the embryo brings new cells into contact with one another, and the result may therefore be different than when these cells are prevented from interacting.

In the following sections, I consider two examples of operations on Hensen's node: excision from a donor quail embryo and transplantation to the inner ring of the *area opaca* of a host chick embryo to demonstrate embryonic induction, as done by Storey et al. ***(10)***, and rotation of the node about its rostrocaudal axis in situ, to demonstrate embryonic regulation as done by Abercrombie ***(14)***.

2. Demonstration of Embryonic Induction by Transplantation of Hensen's Node

2.1. Materials (Fig. 1A)

1. Dissecting microscope with trasmitted light base.
2. Pannett-Compton saline:

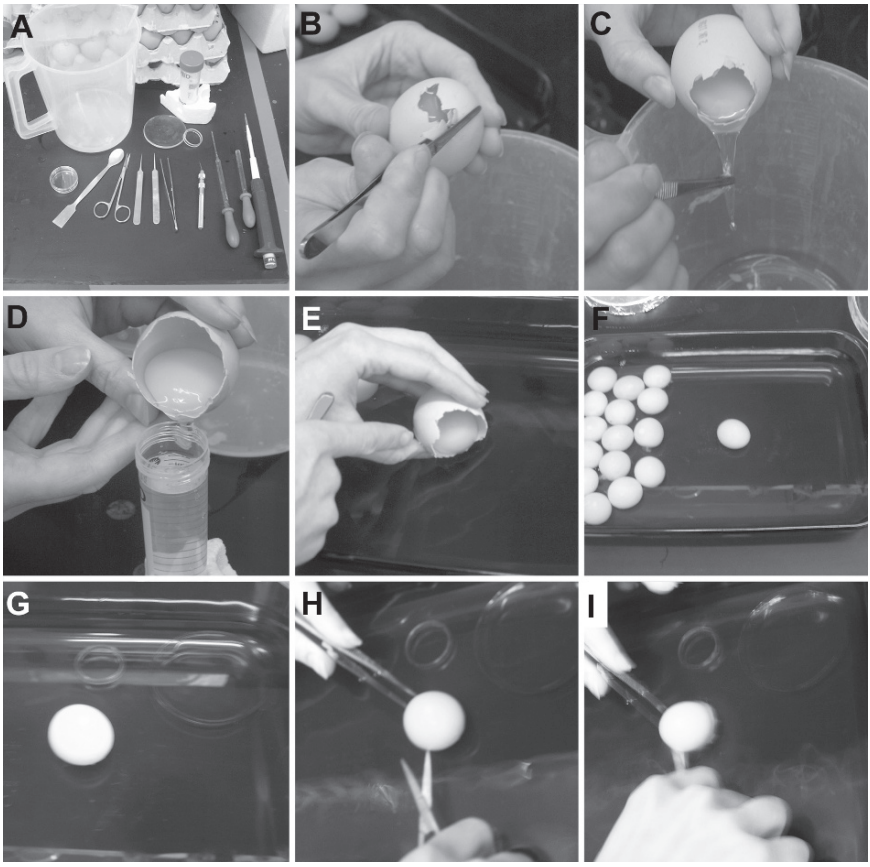

Fig. 1. Preparation of host and donor embryos for a Hensen's node graft in New culture. **A** Materials. **B–F**. Clean the yolk and place it into the dish with saline. **G–I**. Cut the vitelline membrane around the yolk.

Solution A: 121 g NaCl, 15.5 g KCl, 10.42 g $CaCl_2 \cdot 2H_2O$, 12.7 g $MgCl_2 \cdot 6H_2O$, H_2O to 1 L.
Solution B: 2.365 g $Na_2HPO_4 \cdot 2H_2O$, 0.188 g $NaH_2PO_4 \cdot 2H_2O$, H_2O to 1 L; before use, mix (in order): 120 ml A, 2700 ml H_2O, and 180 ml B.

3. Two pairs of watchmaker's forceps, number 4 or 5.
4. One pair of coarse forceps, about 15 cm (6") long.
5. One pair of small, fine scissors, with straight blades about 2 cm ($^3/_4$") long.
6. Spoon or spatula (or small teaspoon).
7. Petri dish (about 10–15-cm diameter) to collect embryos.
8. Container for egg waste.
9. Pasteur pipet with the end cut off at the shoulder, stump flamed to remove sharp edges; rubber teat.
10. Pasteur pipet (short form), end *lightly* flamed to remove sharp edges; rubber teat.
11. Pyrex baking dish about 2" (5 cm) deep, 2 L capacity.
12. 35-mm Plastic dishes with lids.
13. Watch glasses, about 5–7-cm diameter.
14. Rings cut from glass tubing, approximately 27-mm outer diameter, 24-mm inner diameter, 3–4-mm deep.
15. Very fine needles (e.g., entomological size A1 or D1) or sharpened tungsten wire mounted by melting the fine end of a Pasteur pipet (to act as a handle) or into a metal needle holder.
16. Small beaker (50–100 ml) or 50-ml centrifuge tube.
17. Plastic lunch box with lid for incubating culture dishes.
18. 38°C Incubator.
19. Hens' eggs incubated 12–18 h (depending on stage needed).
20. Quails' eggs incubated 12–18 h (depending on stage needed).

2.2. Method

2.2.1. Preparation of Donor (Quail) Embryo

1. Remove quail eggs from incubator. With the scissors, gently tap near the blunt end of an egg to penetrate the shell. Use the tip of the scissors to cut off a small cap of shell near this end, carefully, to avoid damaging the yolk.
2. Allow egg white to pour into waste bucket, assisted by the scissors, taking care to avoid damage to the yolk. You may need occasionally to cut through the rather thick albumen using the scissors.
3. Once most of the albumen has been poured off, make sure the embryo is uppermost; if not, turn the yolk by stroking it very gently with the *sides* of the scissors.
4. Make four cuts into the vitelline membrane around the embryo with scissors. If the embryo does not lie exactly in the center of the egg, make the first cut on the side of the embryo nearest the shell, and proceed in this way until all four cuts have been made. Make sure all the cuts meet each other.
5. Pick up the square of embryo and membrane with the spoon or spatula, trying to collect only a minimal amount of yolk.

6. Transfer the yolk, embryo, and membrane with the spoon into the large petri dish with Pannett-Compton saline under a dissecting microscope. With fine forceps, turn the square of yolk–membrane–embryo so that the embryo is uppermost.
7. After the desired number of donor embryos have been placed in the petri dish, use two pairs of forceps to separate the embryo from adhering yolk. Working at low magnification; pick up a corner of the square of vitelline membrane with one pair of forceps and slowly but steadily fold it back, steadying the yolk with the other pair of forceps. During the whole procedure the membrane and embryo should remain totally submerged in saline. The embryo should be attached to the membrane. If not, peel away the membrane completely then use the foceps gently to remove the embryo from the underlying yolk.
8. Pick up the embryo, with or without adhering membrane, with the widemouth Pasteur pipet, and transfer it to a 35-mm dish with clean saline for final cleaning and dissection. The edges of the extraembryonic membranes are perfectly circular, provided the embryo has not been damaged during the explantation procedure. Put this aside while preparing the host embryo.

2.2.2. Preparation of Host (Chick) Embryo

The following description is for preparing cultures based on the method of New ***(6)*** but with some modifications as described in Stern and Ireland (15). The procedure has been adapted from ***(16,17)***. The main difference between this method and that originally described by New ***(6)*** is the use of rings cut from glass tubing, rather than bent from a glass rod with a circular cross section. The advantage of these rings, with a rectangular profile, is that they grip the vitelline membrane tightly and therefore allow transfer of the assembly to a flat plastic dish. Previously, I recommended rings of 27-mm outer diameter, because it is easier to wrap the membrane around these for a novice. However, if larger (c. 30-mm diameter) rings are used, the embryos develop up to 6–9 hours longer. The longevity of the cultured embryo appears to depend both on the amount of thin albumen under the ring and the length of time for which it can be cultured before the edges of the *area opaca* reach the ring (see ***(8)***).

1. Fill the large Pyrex dish about three-quarters full with saline (about 1.5 liters).
2. Open an incubated hen's egg by tapping the blunt egg with coarse forceps, and carefully remove pieces of shell (**Fig. 1B**). Discard the thicker albumen, assisted with the coarse forceps (**Fig. 1C**). The thin albumen should be collected separately in a small beaker or 50-mL tube (**Fig. 1D**). Try to remove as much albumen as possible, which simplifies the later steps.
3. When yolk is clean and free from adhering albumen, carefully tip it into the saline container (**Fig. 1E** and **1F**), taking care not to damage the vitelline membrane on the edges of the broken shell. The blastoderm should face upward. If not, carefully turn the yolk with the *side* of the coarse forceps. Now lower a watch glass and a glass ring into the container (**Fig. 1G**).
4. Using small scissors, cut into the vitelline membrane enveloping the yolk just *below* the equator (**Fig. 1H**). Continue to cut all the way around the circumference of the yolk (**Fig. 1I**).

5. With two pairs of fine forceps (one to pull the edge of the membrane and the other to hold the yolk down as it becomes exposed; **Fig. 2A**), slowly but *steadily* "peel" the North Pole of the vitelline membrane all the way off the yolk (**Fig. 2A** and **2B**). It is best to pull slightly upward (about a 25–30° angle from the yolk surface) rather than tangentially along the yolk, since the latter tends to detach the embryo from the membrane. Do not stop during this process. The embryo should come off with the membrane (**Fig. 2B**, arrow). Let the membrane rest on the bottom of the dish, inner face (containing the embryo) pointing upward.
6. Slide the vitelline membrane, preserving its orientation, onto the watch glass, and arrange the ring over it (**Fig. 2C**) so that membrane protrudes around the ring (**Fig. 2D**). Pull out the assembly from the saline (**Fig. 2E**).
7. With fine forceps, work carefully to fold the cut edges of the vitelline membrane over the edge of the ring, all the way around its circumference (**Fig. 2F**). Do not

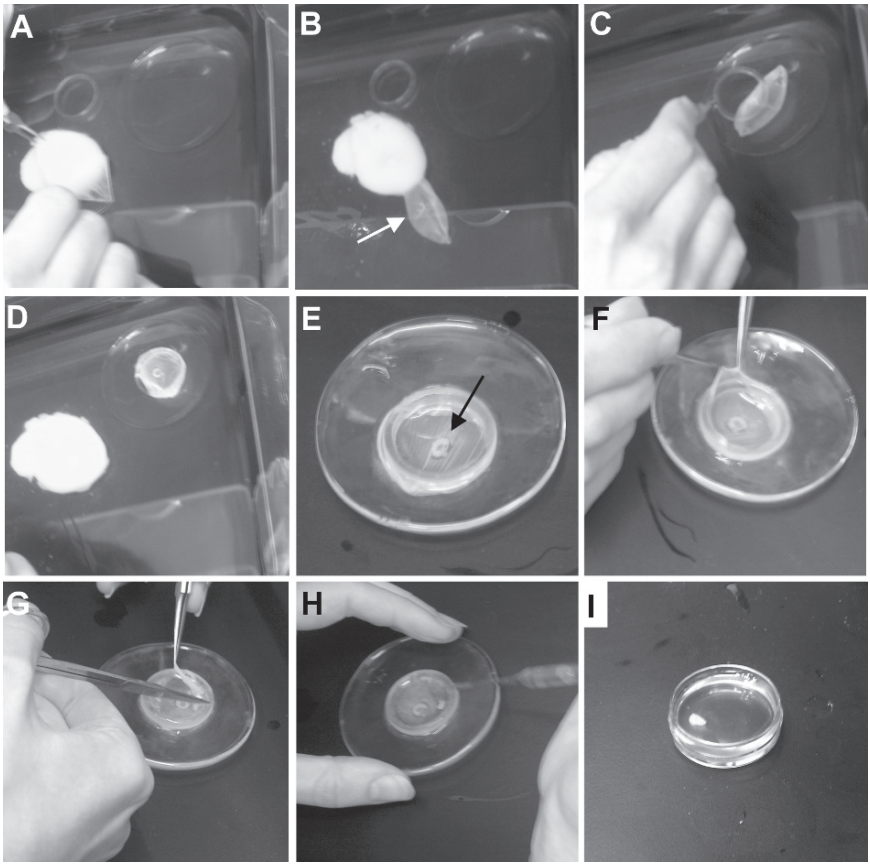

Fig. 2. **A** and **B** Peel the vitelline membrane with the embryo attached. **C–H** Complete the process by wrapping the membrane around the glass ring. The arrows in **B** and **E** point to the embryo on its membrane. **I** Donor embryo in a dish.

pull too tightly, but ensure that the bottom of the membrane is smooth and free from any large wrinkles as you work around the circumference.

8. Place the watch glass over a black surface. Suck off as much fluid as possible from the outside of the ring with the flamed Pasteur pipet. If there is much yolk remaining over or around the embryo, wash it carefully with clean saline. Discard any embryos in which the vitelline membrane inside the ring has been damaged. If there is too much vitelline membrane collecting inside the ring, trim the excess by lifting the edges with fine forceps as you trim with small scissors (**Fig. 18**.2G). Leave the host submerged in saline for the operation (**Fig. 18**.2H).

2.2.3. Grafting and Incubation

1. Having prepared both donor quail (**Fig. .2I**) and host chick embryos (**Fig. 1**, **Fig. 2A–2H**), you are ready for the operation. Place the watch glass with the host chick embryo under the microscope (Fig.3A). Using a fine needle, carefully lift up a portion of the flap of yolky cells (germ wall margin) that covers the inner margin of the *area opaca*, working outward from the *area pellucida* and taking care not to penetrate the ectoderm underneath, which is only one cell thick. This will produce a pocket into which the graft can be inserted. Alternatively, just remove a small region of yolky endoderm at the future site of grafting, using the needle (**Fig. 3B–3D**).
2. Now remove the watch glass with the host and bring the dish with the donor quail embryo under the microscope, arranging it so that its ventral (endoderm) surface is uppermost (**Fig.3E**). Using a fine needle, carefully cut out the very tip of the primitive streak, cutting through the whole thickness of the embryo. It is best to cut the two sides first, working as close as possible to the primitive streak edges (**Fig. 3F** and **3G**), then free the anterior tip (**Fig. 3H**) and finally detach the node (which is quite small, about as long as it is wide) from the rest of the streak (**Fig. 3I** and**3J**).
3. Lower the magnification of the microscope, keeping track of the excised node, and pick this up with a Gilson P20 fitted with a yellow tip, set to 1–2 µl (**Fig. 3K** and3**L**).
4. Move the dish with donor embryos away and bring back the watch glass with the host into the field. While looking down the microscope, insert the tip of the Gilson under the saline covering the host embryo and gently expel the quail node onto its surface, keeping track of it at all times.
5. Use fine needles to manipulate the donor node to close to the desired grafting site (**Fig. 3M**). Slide the quail node into the pocket, pushing it as deep as possible so that, when the flap of germ wall margin is replaced, it will cover the graft completely. Alternatively, if you have only cleaned some yolky endoderm, manipulate the node to the denuded site (**Fig. 3N** and **3O**).
6. Working under the microscope, carefully remove any remaining saline, both inside and outside the ring (**Fig. 4A**). During this process, keep watching the graft to make sure that it does not become dislodged. It is important that the embryo and the inside of the ring remain completely dry during incubation.

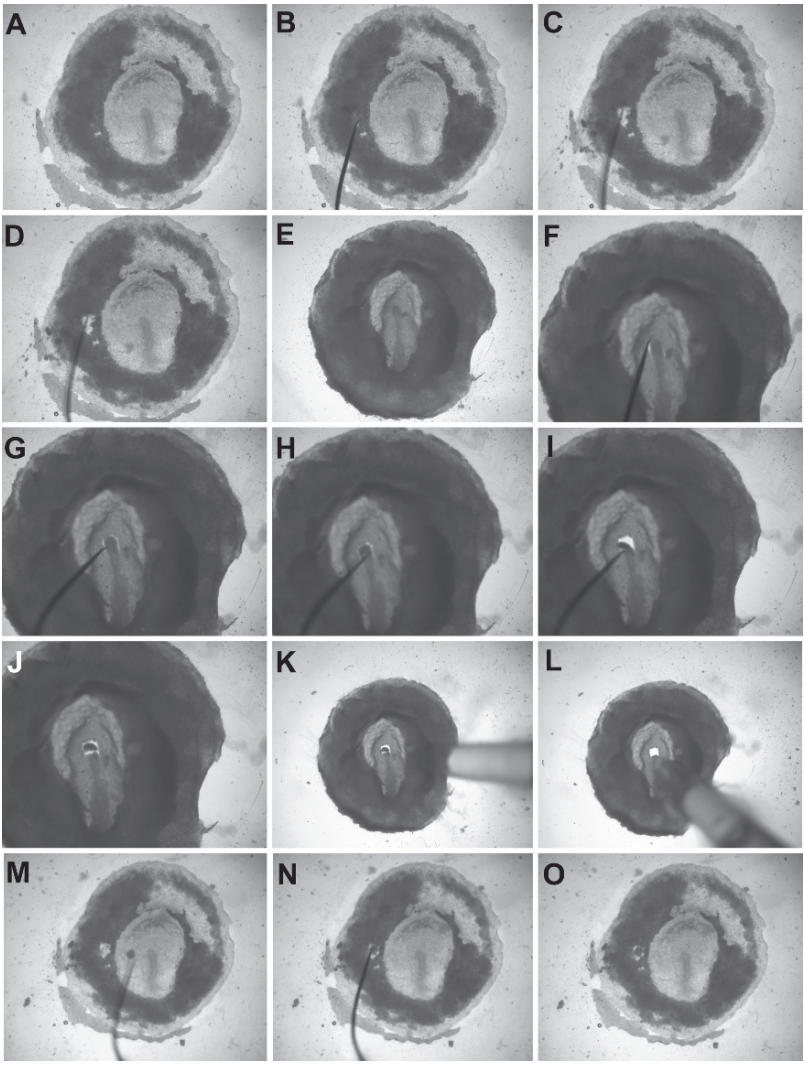

Fig. 3. Manipulations under the microscope. **A** Host embryo (stage 3+). **B–D** Clear yolky endoderm to expose the epiblast at the future grafting site. **E** Donor embryo, ventral side uppermost (stage 4). **F–J** Cut out Hensen's node from the donor. **K** and **L** Pick up the excised node with a Gilson pipet. **M–N** Manipulate the node to the graft site. **O** The finished operation.

7. Now pour some thin albumen (about a 2–3-mm thick layer) on the bottom of a 35-mm plastic dish (**Fig. 4B**). Slide the ring with vitelline membrane off the watch glass (**Fig. 4C** and **4D**), and transfer it to the dish, lowering (insert it slightly obliquely to make

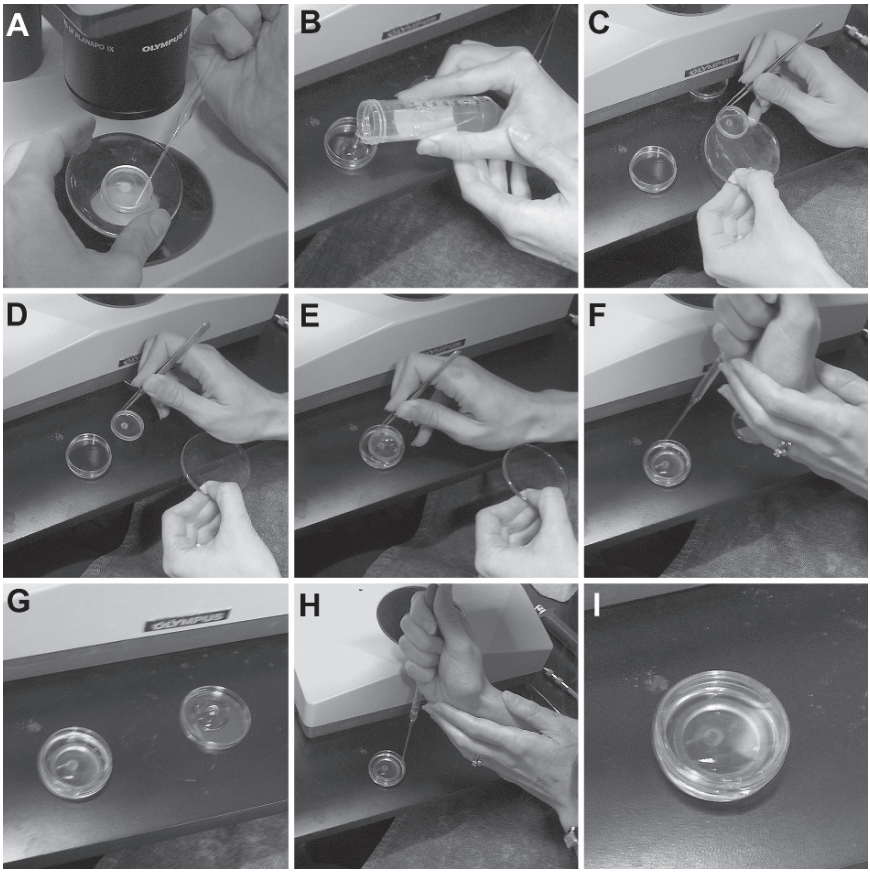

Fig. 4. Finishing touches. **A** Remove excess saline after the operation. **B-E**. Make a pool of albumen in a dish and transfer the ring with the grafted embryo onto it. **F–-H**. Remove excess albumen (which is used to wet the inside of the lid, **G**). **I** The finished assembly.

sure no air is trapped underneath) over the pool of egg albumen (**Fig. 4E**). Press lightly on the ring with two forceps to allow it to adhere to the dish.

8. If the level of albumen comes close to the edge of the ring, remove the excess (**Fig. 4F**). Also aspirate any remaining fluid from inside the ring. It is best if the vitelline membrane bulges upward above a good pool of albumen (**Fig. 4G**). This also helps drain off further fluid accumulated during culture to the edges of the ring.
9. Wet the inside of the lid of the plastic dish with albumen (**Fig. 4G**, right) and spread it all over. Discard the excess. Now check once more that there is not too much albumen in the dish (i.e., the ring should not be floating), otherwise remove some (**Fig. 4H**), and finally use the wetted lid to seal the dish (**Fig. 4I**).

10. Place the dish in a plastic box containing a piece of tissue paper or cotton wool wetted in distilled water, seal the box, and place it in an incubator at 38°C.

2.3. Analysis of Results

After the desired period of incubation, fix the embryo by flooding with methanol (for most immunological detection procedures), Zenker's fixative (for Feulgen-Rossenbeck staining), or in 4% formaldehyde in PBS (for in-situ hybridization and most other procedures). In the case of methanol or Zenker's, which are "rapid" fixatives, it is advantageous to submerge the cultured embryo in saline, then detach it from the vitelline membrane and transfer it to a clean dish with saline prior to fixation. Otherwise, the embryo adheres permanently to the membrane and the fixative denatures the albumen, generating threads of protein that tend to stick to the embryo. Formaldehyde can be poured directly onto the embryo, provided that the embryo is detached from the membrane within a few minutes.

Whatever the fixative and subsequent method of processing chosen, it is advantageous to ensure that, at the time of fixation, the embryo is as flat as possible. If it has been detached from the membrane prior to fixation, place it in a small drop of saline on a plastic surface, suck off most of the saline with a fine Pasteur pipet so that the embryo flattens on the plastic, then place the first drop of fixative directly onto the surface of the embryo, taking care not to break it. After this, it is safe simply to submerge the embryo in fixative, perhaps transferring it to a glass vial. Embryos fixed in this way usually remain flat through all subsequent manipulations.

Depending on the purpose of the experiment to be performed, embryos operated and cultured as described can be subjected to histochemistry, immunological procedures (as whole mounts), or whole-mount in-situ hybridization. In many cases, it is possible to combine two or more of these methods. For example, it is possible to fix the embryos in formaldehyde, process them as whole mounts for in-situ hybridization, postfix in formaldehyde, then perform whole-mount immunoperoxidase histochemistry with QCPN antibody to detect the quail cells. After this, they can be embedded in wax and sectioned. Methods for this have been published elsewhere in some detail ***(18)***.

3. Rotation of the Node In Situ

The procedure outlined here is "generic", and similar manipulations can be done to operate on smaller or larger portions of embryo. For example, whole, large sections of the primitive streak may be rotated as done by Abercrombie ***(14)*** and others or very small subsectors of Hensen's node transplanted as described by Selleck and Stern ***(19)*** and Storey et al. ***(9)***.

3.1. Materials

The materials required are the same as listed in **section 2.1**. In addition, you need fine capillary glass connected to an aspirator (mouth) tube. The former can be manufactured by pulling the thin end of a Pasteur pipet over a very hot bunsen flame and pulling rapidly. The latter can be purchased from Sigma (A5177).

3.2. Method

1. Follow the method in **section 18.2.2.2** to place a chick embryo in modified New ***(6)*** culture as if to receive a graft.
2. Use fine needles to cut out the node, involving the whole thickness of the embryo but being very careful to avoid damaging the vitelline membrane underneath. Even a small hole causes leakage of albumen and prevents healing or even displaces the graft. It is best to work in steps: First, make a very superficial cut of the shape required. Then deepen the cut, a little at a time, until the node is finally freed all around.
3. It is of advantage to mark one edge of the node with fine carbon (e.g., pencil lead shavings) or carmine (Sigma C1022) particles, using a fine needle. This allows the orientation of the node to be controlled through subsequent manipulations.
4. Maneuver the excised node, using the fine needles, to the desired orientation, again taking care not to damage the vitelline membrane, which is now exposed.
5. Still observing under the microscope, carefully and slowly withdraw as much saline as possible from inside and outside the ring, as described for grafting Hensen's nodes. In this experiment, where the manipulated node is not secured under a flap of tissue, it is much easier to lose it while sucking off the fluid. If necessary, replace it in position as required, using the fine needles.
6. Once all the fluid has been removed, use the fine capillary and mouth tube (if necessary) to remove *all* remaining fluid from the site of the graft. It is likely that the excised piece will appear to have shrunk. Sucking the fluid off in this way closes the gap and "knits" the pieces together, if performed with care.
7. Now, slide the ring from the watch glass and set up the culture as described in **section 18.2.2.3**. Finally, if necessary, suck off more fluid with the mouth capillary assembly to ensure that the graft site is totally dry and appears closed.
8. Before placing it in the incubator at 38°C, it is advantageous to keep the operated embryo at room temperature (in the sealed petri dish) for 2–3 h. After this, place it at 30°C for 3–5 h. Finally, transfer the dishes to an incubator at 38°C. These periods at lower temperature help the healing process, as described previously.

3.3. Analysis of Results

Operated embryos may be analyzed by histology, whole-mount immunohistochemistry, and in-situ hybridization with appropriate probes, as described for Hensen's node grafts.

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19

Grafting of Somites

Claudio D. Stern

1. Introduction

The somites are an intriguing invention of vertebrate embryos. They represent the most overtly segmented structures of the body plan, but they give rise to both obviously segmental (e.g., the axial skeleton) as well as not-so-obviously metameric (dermis and skeletal muscle) elements. In addition, they play a key role in controlling several aspects of the organization of the central and peripheral nervous system of the trunk and participate in several different types of inductive interactions, both within themselves and with neighboring tissues, like the neural tube, the notochord, the metanephric and lateral plate mesoderm, and the ectoderm and endoderm (*see **[1, 2]*** for reviews).

Questions that can be addressed by manipulating somites range from investigations on the mechanisms by which metameric pattern is established, to their influence on the segmental outgrowth and differentiation of precursors of the peripheral nervous system (neural crest cells, motor axon growth cones), to the control of myogenesis and patterning, to the establishment of regional identities of cells that contribute to the dermis, limbs, and axial skeleton. Previous experiments ***(1, 2)*** suggested that, although many aspects of somite development are controlled by surrounding tissues, many others appear to be remarkably autonomous.

Somites form at the posterior end of the embryo, such that a pair of somites is added every 100 minutes or so (***[1, 2]*** for reviews). Therefore, at any particular stage of development, the embryo contains younger (more recently formed) somites at its caudal end and older (at more advanced stages following their formation) more rostrally. To indicate this, Ordahl and his colleagues introduced a "somite stage" numbering system, using Roman numerals to indicate the position of the somite being referred to with respect to its neighbors (*see **[2]***).

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In this system, somites are counted upward from the most recently formed one, which is designated I. The most caudal four to six somites are usually epithelial spheres. Stages V, VI, and higher designate somites whose dorsolateral surfaces still remain epithelial (the dermomyotome) but whose ventromedial parts have become mesenchymal once more, to form the sclerotome ***(1, 2)***. The neural tube, notochord, ectoderm, and endoderm all play a role in determining the dorsoventral polarity of the somites with respect to their ability to form a dermomyotome and a sclerotome.

Despite the simplicity of this numbering system, it is important to remember when investigating somite development that overlapped with this age–structure is also a position-dependent address (reviewed in ***(1, 2)***). This can be demonstrated by transplanting somitic mesoderm from the thoracic level to the neck, where they go on to develop ribs as if they had not been transplanted. However, if a similar experiment is conducted to investigate the nature of the muscles that develop, it is found that any somite will give rise to muscles appropriate for their new position. Therefore, some somitic derivatives behave in a cell-autonomous way concerning their positional information, while others are subject to cues from their environment. In addition, the most rostral five or so somites ("occipital somites") have a different fate from the rest (they do not contribute to the vertebral column and some of their cells appear to contribute to the tongue) and, in addition, do not support the development of dorsal root ganglia from neural crest cells migrating in them.

Experiments in which the somitic mesoderm is manipulated can be done either *in ovo* or in whole embryo (***(3, 4)***; Chapter 18 in this volume) culture. The main advantage of the former method is that it allows embryos to develop for a long time, even up to hatching, but embryos younger than about the four somite stage (Hamburger and Hamilton, or HH, stage 8 ***(5)***) are very difficult to manipulate in this way and their survival is poor. The latter technique allows very young embryos (even before incubation, at preprimitive streak stages) to be operated on, but they survive only for 36–48 hours, even in the most expert of hands. In the following sections, I describe two examples. The first is a detailed method for operations on somitic mesoderm *in ovo*, in which the anterior half of the segmental plate (unsegmented paraxial mesoderm) of a quail embryo is grafted into the same position in a host chick embryo. The procedure is "generic" and can be adapted easily for manipulation of newly formed or older somites at stages 9–15 as well as for manipulations of the notochord, neural tube, and other tissues at these stages. The second example, to be used in conjunction with the instructions in the chapter on grafting Hensen's node (Chapter 18), gives advice on manipulating younger embryos to investigate the mechanism of segmentation from the primitive streak stage onward (*see* ***[6, 7]***). The procedures given here have been adapted from those in ***(8)***.

2. Grafting Paraxial Mesoderm and Somites in ovo

2.1. Materials (Fig. 1A)

1. Dissecting kit: two pairs small forceps (watchmaker's number 4 or 5), one pair small scissors (about 2-cm straight blades), one scalpel (No. 3 handle, No. 11 blade), a Gilson micropipet for 3-µL yellow tip(s).

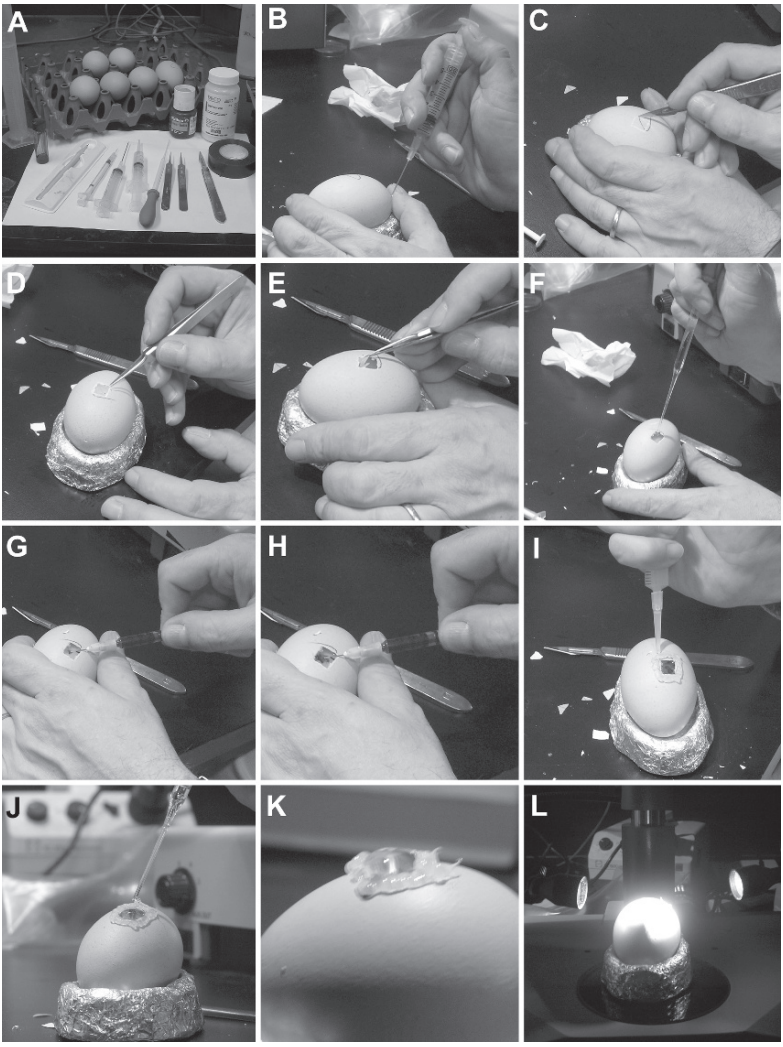

Fig. 1. Preparatory manipulations for a graft or rotation of a portion of segmental plate (presomitic mesoderm). **A** Materials. **B** Remove the albumen. **C** Score a 1-cm window in the shell. **D** and **E** Remove the shell membrane. **F** Fill with CMF saline. **G** and **H** Inject Indian ink. **I** Grease the window. **J** and **K** Make a bubble chamber with trypsin solution. **L** Shine fiberoptics light tangentially onto the window.

2. Eye surgeon's microknife, 15° angle. Suitable microknives are micro-feather microsurgery scalpels for eye surgery, 15° blade angle, Catalog number 715, manufactured by Feather (Japan) and marketed by pfm GmbH. Sold in boxes of five—not cheap!
3. Two entomological pins, size A1 or D1, or sharpened tungsten needles, each mounted by melting the end of a short Pasteur pipet (to act as handle) or inserted into a metal needle holder.
4. Plasticine (modeling clay) to make ring for resting egg on its side.
5. 100 mL calcium–magnesium-free (CMF) Tyrode's saline. To prepare, dissolve 80 g NaCl, 2 g KCl, 0.5 g NaH_2PO_4 $2H_2O$ and 10 g glucose in 1 L H_2O. Autoclave for storage. On day of use, dilute 1:10 with distilled water. The working solution may be buffered with bicarbonate, but we usually omit this.
6. Antibiotic–antimycotic solution, 100× concentrate (e.g., Sigma A9909).
7. 70% Ethanol to wipe shell.
8. Thin PVC tape (electrical insulating tape) to seal egg.
9. Two Pasteur pipets and rubber teats.
10. Container for egg waste.
11. 10-mL Plastic syringe with Gilson yellow tip stuck into the end, filled with high-vacuum silicon grease or vaseline.
12. 1-mL Syringe, 27G (or finer) × 3/4′ needle (for ink injection).
13. 1-mL Syringe, 21G needle (for antibiotics).
14. 5-mL Syringe, 21G needle (for withdrawing albumen).
15. Paper tissues.
16. 35-mm Plastic dish coated with Sylgard and steel insect pins, size A1 or D1. Sylgard 184 (Dow Corning) is clear silicone rubber polymerized by mixing two components (9 parts rubber solution:1 part of accelerator–catalyst). Mix the two well and pour to the desired depth (2–5 mm) into the plastic petri dishes. Allow the dishes to stand for about 1 h at room temperature for air bubbles to leave, then cure at about 55°C until polymerized (3 h to overnight). The dishes can be stored indefinitely. Black Sylgard is also available.
17. Indian ink (Pelikan Fount India is best; most other makes are toxic), diluted 1:10 with CMF and loaded in the ink injection syringe.
18. 50 mL Trypsin (DIFCO, 1:250), freshly made up to 0.12% (w/v) in CMF.
    Hens' and quails' eggs incubated 40–44 h so that they are at stage 10–12. The hens' eggs (hosts) should have been resting on their sides at least 30 min.
20. Dissecting microscope.
21. Fiberoptic incident illumination.

2.2. Method

Here the host embryo is operated *in ovo*, which is suitable with ease only for embryos older than about 36–48 h incubation. As an example, the operation described here consists of a graft of presomitic mesoderm (segmental plate) with a quail embryo as the donor and a chick embryo as the host. An identical procedure can be followed to transplant paraxial mesoderm that has already formed somites, but it should be remembered that this is much easier when it

involves the youngest (most caudal) five or six pairs of somites at stages 10–14. Somites situated more anteriorly in the embryo have already separated into dermomyotome and sclerotome, and special care is needed both to keep these components together during grafting and to separate them cleanly. Whatever the operation to be performed, it is generally a good idea to fix for histological analysis some of the pieces like those to be transplanted, as well as some host embryos immediately after the operation, to confirm that the graft involves the tissues of interest and no contaminating cells.

2.2.1. Preparation of Donor Quail Embryo

1. Remove quail eggs from the incubator. With the scissors, gently tap near the blunt end of an egg to penetrate the shell. Use the tip of the scissors to cut off a small cap of shell near this end, carefully, to avoid damaging the yolk.
2. Allow the egg white to pour into waste bucket, assisted by the scissors, taking care to avoid damage to the yolk. You may need occasionally to cut through the rather thick albumen using the scissors.
3. Once most of the albumen has been poured off, make sure the embryo is uppermost; if not, turn the yolk by stroking it very gently with the *sides* of the scissors.
4. Use the scissors to make four cuts into the vitelline membrane around the embryo. If the embryo does not lie exactly in the center of the egg, make the first cut on the side of the embryo nearest the shell, and proceed in this way until all four cuts have been made. Make sure all the cuts meet each other.
5. Pick up the square of embryo and membrane by grasping one corner with fine forceps and transfer it immediately to the Sylgard-coated 35-mm dish containing about 5 mL CMF–Tyrode's solution.
6. Using two pairs of fine forceps, separate the vitelline membrane from the embryo, but leave the extraembryonic membranes attached to the embryo.
7. Stretch out the embryo (either side up) by placing four to six entomological pins (size A1 or D1) through the corners of extraembryonic membranes, into the Sylgard rubber. The embryo should be under some tension. Put the dish aside while preparing the host.

2.2.2. Preparation of Chick Host Embryo

The procedure described here differs from that in grafting the notochord, neural tube, AER/ZPA (Chapter 22), and neural crest. In the method described here, a small window is cut into the shell and the embryo floated up to the level of this window for the operation. The advantages of this technique are many: (a) the embryo lies very close to the operator's hands; (b) it is completely submerged in liquid at all times (which avoids drying out and simplifies some of the manipulations); (c) the liquid above the embryo can be changed several times, for example, to wash the embryo; (d) the light illuminating it can be shone tangentially to its surface (the bubble of liquid above acts as a lens that greatly enhances the optical clarity); (e) the tension generated by floating the embryo

seems to aid the action of the trypsin, such that tissues almost appear to dissect themselves; (f) it is very easy to notice if the endoderm has been punctured accidentally, because ink fountains out very quickly; and (g) after the operation, the small hole can be closed very tightly with plastic (PVC or electrical) tape, which allows the egg to be incubated with the window downward and turned—both of these greatly enhance survival.

1. Shape plasticine (modeling clay) into a ring about 2 in. (5 cm) in diameter and place it on the stage of the microscope. Place a hens' egg (host) onto the plasticine ring, being careful that it is not rotated it with respect to its resting position (*see* **Fig. 1**).
2. Using the 5-mL syringe with a 21G needle, held nearly vertical, insert needle into blunt end of egg until the shell is felt at the bottom surface. Withdraw 0.5–1 mL egg albumen, which should come up easily (**Fig. 1B**).
3. Score a shallow 1-cm × 1-cm square on the top of the shell with the scalpel and lift up the square of shell (**Fig. 1C**).
4. With a pair of watchmaker's forceps, pierce and remove the underlying shell membrane, after wetting it with CMF. Avoid damage to the embryo underneath: Before air is allowed into the egg, the embryo lies very close to the membrane (**Fig. 1D** and **1E**).
5. Fill the cavity with CMF so that the embryo floats just to the level of the window (**Fig. 1F**).
6. After ensuring that there are no air bubbles in the syringe with Indian ink or the needle, insert the needle under the vitelline membrane, tangentially, at a position as far away from the embryo proper as possible (**Fig. 1G** and **1H**). Point toward and slightly below the embryo, and inject about 20–50 µL. It is important to minimize movement of the needle after penetrating the vitelline membrane, or the hole will be very large and yolk–ink leak out. Introduce and withdraw the needle with one clean, decisive movement, and do not stir the needle inside the yolk.
7. Draw a shallow, continuous border of silicon grease around the window (**Fig. 1I**). This contains a standing drop in which the operation is done. Now, fill this chamber with CMF saline (**Fig. 1J**) until there is a standing drop (**Fig. 1K**), and adjust the fiber-optic light to shine tangentially to the surface of the egg (**Fig. 1L**), so that the embryo can be seen very clearly with minimal light intensity from the light source (**Fig. 2A**).

2.2.3. Grafting Procedure

1. Break the vitelline membrane over the region to be operated on using several nicks of the tip of a fine hypodermic needle (**Fig. 2B–D**). The segmental plates are the rodlike structures lying on either side of the neural tube at the tail end of the embryo, just behind the last somite. The portion to be rotated in this example is the most anterior third of the plate.
2. Replace the bubble of CMF with trypsin–CMF. Increase the magnification of the microscope as much as possible.
3. Operating in the drop of trypsin, use the microknife to make initially very shallow cuts in the ectoderm next to the neural tube in the region of the operation (**Fig. 2E**). Repeat the same just lateral to the segmental plate (**Fig. 2F**).

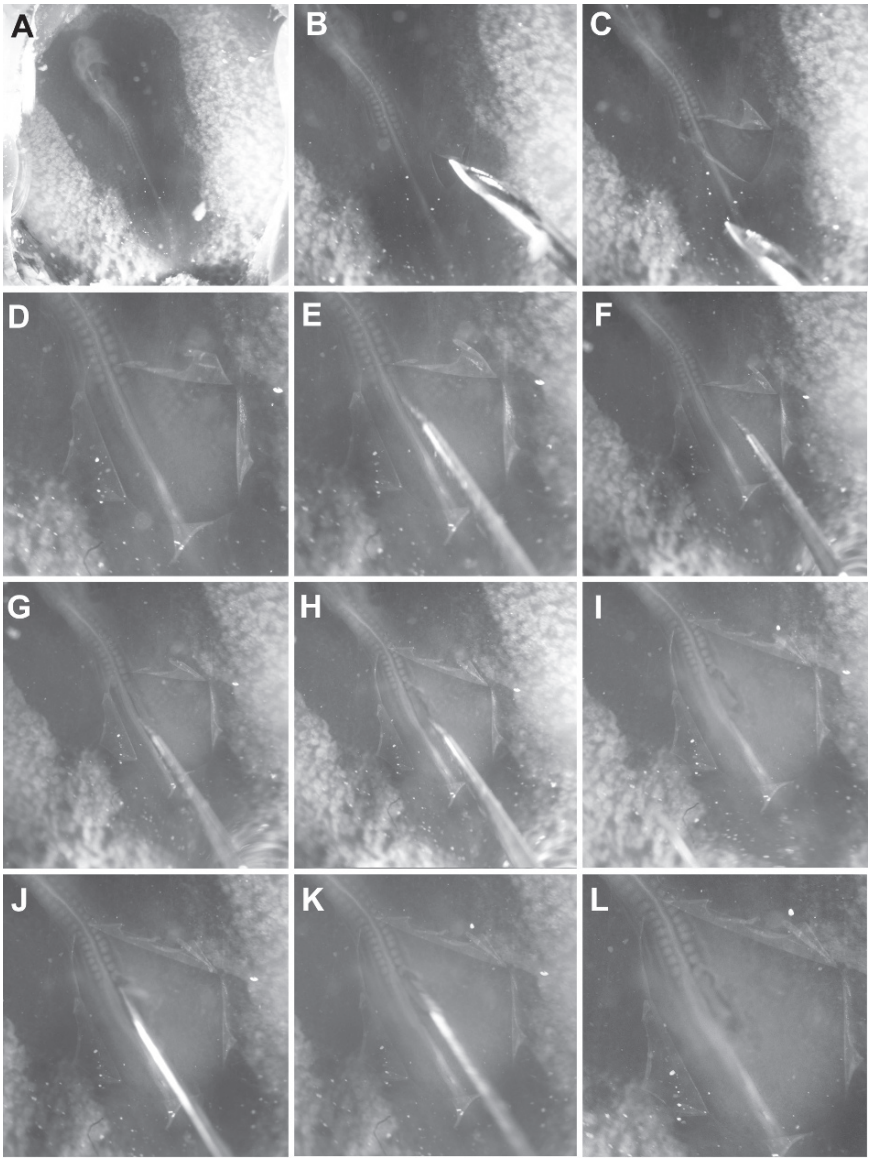

Fig. 2. Under the microscope. **A** View of the embryo; note the edges of the window. **B–D** Starting with a small nick (**B**) with the tip of a hypodermic needle, make a hole in the vitelline membrane near the region to be operated on and extend it to expose the desired region (**D**). **E** and **F** Use a microscalpel to cut through the ectoderm between neural tube and presomitic mesoderm (**E**) and lateral to the segmental plate (**F**). **G** Gently push the neural tube away to allow the trypsin to enter; this will separate neural tube from presomitic mesoderm without cutting. **H** Gently score the lateral border of the segmental plate to separate it from the intermediate mesoderm. **I** Now, free the segmental plate portion to be rotated or removed by cutting at the desired caudal position. **J** Rotate the excised portion o, alternatively, use a graft of a similar piece of tissue from a quail donor (see the text). **K** Introduce the graft into the hole with the microscalpel. **L** The finished operation. The gaps close as you remove the saline–trypsin.

4. Gradually deepen the cuts using the knife blade more as a spatula, allowing penetration of the trypsin, than as a sharp cutting edge. Once the ectoderm has been penetrated with the tip of the blade, the trypsin does the rest (**Fig. 2G**). Do the same at the lateral edge of the semgental plate: A shallow cut is necessary first, then separate the segmental plate from the adjacent intermediate mesoderm gradually by pushing the segmental plate toward the midline (**Fig. 2H**). In both cases, make sure you do not penetrate the endoderm (which is one-cell thick) or ink will pour out. If this happens, discard the egg and start again. Finally, free the posterior end of the piece and loosen the graft (**Fig. 2I**).
5. Remove the piece of segmental plate with a Gilson micropipet set to 1–2 µL. Replace the bubble over the embryo with fresh CMF twice to remove the trypsin solution. Make a new bubble of CMF while obtaining the graft from the donor.
6. Turn to the donor embryo in the Sylgard dish. Replace almost all the CMF in which it was submerged with trypsin solution. Repeat the trypsin wash and perform the dissection in this solution at room temperature.
7. Cut out an equivalent piece of segmental plate as the one removed from the host, using the same technique.
8. Pick up the graft with the Gilson. With the other hand, place the host under the microscope and, observing under low magnification, carefully place the graft into the CMF bubble over the embryo.
9. Use the knife and work at low magnification to manipulate the graft into the gap made by removal of the host piece of segmental plate (**Fig. 2J** and **2K**).
10. When the graft is in position (approximately) (**Fig. 2L**), very carefully remove most of the fluid from above the embryo with a Pasteur pipet while watching under low power (**Fig. 3A**). If necessary, reposition the graft with a fine mounted needle (but this is quite difficult if there is no liquid above, as the graft tends to stick to the needle).
11. Insert the 5 mL syringe with a 21G needle into the original hole in the blunt part of the eggshell, vertically, and carefully withdraw 2.5–3 mL thin egg albumen (**Fig. 3B**). This lowers the operated-on embryo back to its original position. Be careful when you insert the needle, since the pressure could make the graft come out of its site.
12. Wipe the edges of the shell with tissue paper moistened lightly in 70% alcohol to remove the silicon grease (**Fig. 3C**).
13. Add about 100 µL of antibiotic–antimycotic concentrate (away from the graft site) with a Gilson (**Fig. 3D**) or Pasteur pipet (1–2 drops).
14. Cut a piece of PVC tape about 6 cm long. Stretch it slightly, then let it relax. Place it over the window (**Fig. 3E**), smoothing out any unevenness carefully (**Fig. 3F** and **3G**) and avoid breaking the shell or applying too much pressure on the window.
15. Keeping the egg on its side, place it (window down; **Fig. 3H** and **3I**) into an egg tray in a humidified incubator at 38°C. If you are worried about the graft falling out, it is a good idea to incubate the embryo with the window upward until the next day, then to turn it.
16. Incubate 1–3 d. Embryo survival 2 d after this operation should be 80–100%.

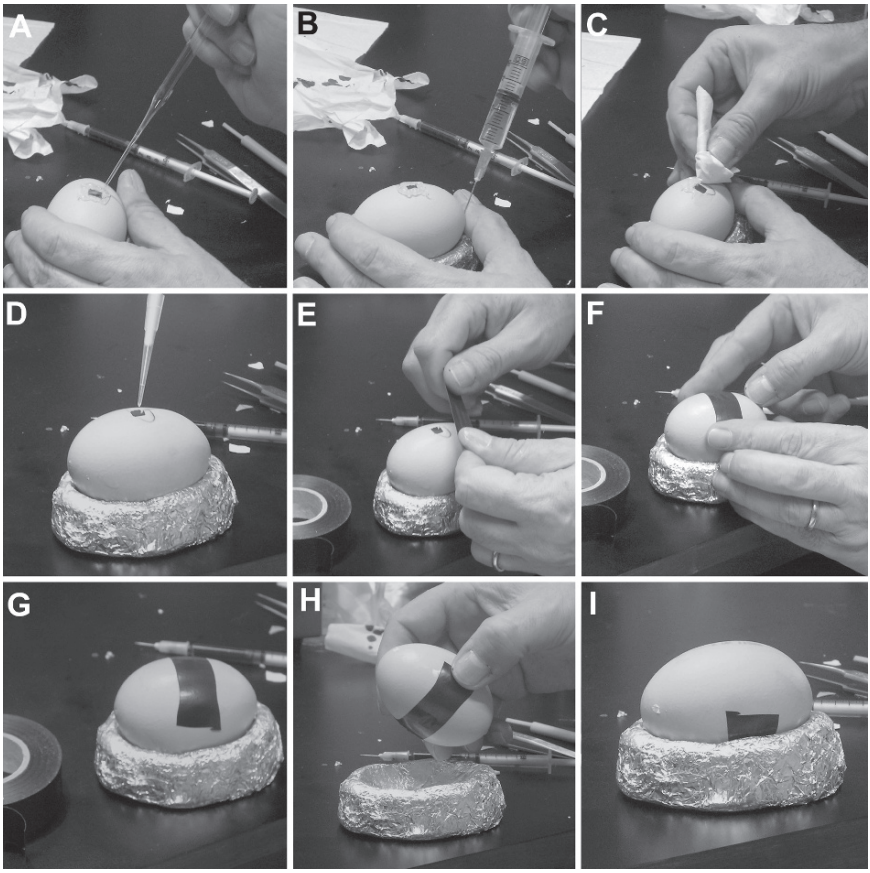

Fig. 3. Finishing touches. **A** Remove excess trypsin–CMF from the bubble above the embryo. **B** Remove 5 mL albumen through the side hole. **C** Wipe the shell. **D** Add the antibiotic. **E** Seal the egg with PVC tape. **F** Smooth out the tape. **G** and **H**. If desired, invert the egg to expose the embryo to clean shell and membrane before incubating

3. Operations on Paraxial Mesoderm of Embryos in New Culture

When the aim of the experiments is to investigate the mechanisms that set up the paraxial mesoderm or the fates and movements of presumptive somitic cells at or shortly after gastrulation are to be investigated, operations in the egg are very difficult. It is therefore generally necessary to resort to a method of whole embryo culture. For this, set up cultures of embryos at the desired stage (between HH stage 3, mid-primitive streak, and HH stage 8, four somites), following the protocols in Chapter 18 in this volume (*see* also ***(4)***). Leave the embryo completely submerged in saline during the operation.

The region of the primitive streak embryo (stages 3–4) that contributes to the somites lies in the most anterior (cranial) two thirds or so of the primitive streak and in the ectoderm to either side of this. By stage 4+–5, some of the somite progenitors have already left the streak and lie in the middle layer next to the anterior streak and gradually migrate cranially as more cells are added from the streak and node regions.

To operate on these cultured embryos, it is usually better to use fine mounted needles (entomological or sharpened tungsten) rather than knives. Trypsin is generally not necessary, but may be used (at 0.1% w/v) to facilitate separation of tissues if the specific manipulation desired turns out to be difficult due to adherence of the tissues to one another. Operations on the mesoderm are usually easier in the absence of trypsin, because the middle layer readily separates into layers in the presence of enzyme. However, for each type of operation, the sequence in which the cuts are made is very important. In some regions of the embryo it is easier to begin with a medial cut and proceed laterally, while for some other regions an anterior cut, proceeding caudally is more effective. You should investigate the relative merits of different ways to dissect the tissue of interest before starting a set of experiments.

As described for operations on Hensen's node, it is important to avoid damaging the vitelline membrane at all costs. Any leakage of the albumen culture fluid to the inside of the ring diminishes survival and may cause the grafted tissue to fall out of its site.

After the operation, follow the steps described for Hensen's node grafts (Chapter 18). Transfer the ring with the embryo to a plastic dish, seal it, place it in a humid chamber, and incubate 38°C for 24–42 hours. If cultures are set up using large glass rings (about 30 mm; *see* Chapter 18) or other methods for extended culture (*see* ***[9]***), embryos operated at stages 3–6 should survive to stage 15 or even longer.

4. Analysis of Results

Embryos operated on as just described can be studied by a variety of methods, according to the question being addressed. Fix the embryo in methanol (for most immunological detection procedures), Zenker's fixative (for Feulgen-Rossenbeck staining), or 4% formaldehyde in PBS (for in-situ hybridization and most other procedures). Details on these procedures may be found elsewhere in this volume and in ***(10)***.

For embryos operated *in ovo*, it is easiest to crack the egg into a large Petri dish first, cut the membranes around the embryo with scissors, and lift a corner of these membranes with fine forceps (as described in **section 2.2.1**). Then, immediately transfer this to a small dish with saline to clean off any adhering yolk. Finally transfer it to a Sylgard dish (*see* previous sections) containing

CMF and pin the embryo to straighten out the head and trunk, but avoid the region close to the operation. Remove the CMF and replace it with the fixative of choice. In this way, the embryos are perfectly straight, which simplifies subsequent histological sectioning, and they are more photogenic if stained as whole mounts.

For embryos operated in New culture (***[3, 4]***; Chapter 18), the glass ring should first be flooded with CMF, and the edges of the *area opaca* detached from the vitelline membrane. Then, pick up the embryo with a wide-mouthed pipet or with fine forceps (to grasp the extraembryonic membranes) and transfer it to a Sylgard dish for pinning and fixing as just described.

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20

Microsurgical Manipulation of the Notochord

Lúcia E. Alvares, Corinne Lours, Amira El-Hanfy, and Susanne Dietrich

1. Introduction

Microsurgical manipulations, such as ablation or heterotopic grafting of embryonic tissues, are powerful tools to study tissue interactions that control development. Likewise, microsurgical approaches are useful when candidate signaling molecules applied to beads or expressed by genetically engineered cells lines are being tested for their ability to replace signaling tissues *in vivo*. Finally, once signaling from particular embryonic tissues is established, these tissues can be used at various sites in the embryo as a reliable source of signaling molecules, expressed at physiological levels.

Among the structures studied in embryological experiments, the notochord, the name-giving structure shared by all animals in the chordate phylum, has received much attention, since its presence is crucial for the development of surrounding tissues. In the overlying neural tube, the notochord induces the ventral midline structure, the floor plate, which subsequently specifies neuronal cell types in the ventral half of the neural tube, and represses neuronal fates associated with the dorsal neural tube ***(1)***. In the paraxial mesoderm that flanks the neural tube and notochord, the latter induces the sclerotome, the anlage of vertebral column and ribs ***(2)***. Moreover, signals from the notochord synergize signals from the dorsal neural tube to induce the medial–paxial dermomyotomal precursor pool, from which are generated the medial–epaxial part of the myotome that gives rise to the deep muscles of the back and the dorsal dermis ***(2, 3)***. Studies on zebrafish notochord mutants established the notochord as also key regulator of myogenesis in the fish, crucial for the formation of slow-twitch muscles and the horizontal myoseptum ***(4)***. These studies revealed a further role for the notochord in dorsal aorta formation ***(5)***. Finally, the notochord has been shown to regulate the formation of the roof of the gut ***(5)***.

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Much of the biological functions of the notochord have been attributed to the signaling molecule sonic hedgehog (Shh; ***(5, 6)***). However, in mice lacking Shh, notochord-dependent structures develop to some degree, suggesting that the molecular mechanism of notochord signaling is still not fully understood ***(7)***. Moreover, the formation of the notochord itself by convergence–extension movements of the gastrulating axial mesoderm has recently received quite some attention ***(8)***. Therefore, the notochord will remain the subject of embryological and molecular studies in the future. Consequently, notochord ablation, heterotopic notochord transplantation, and the transplantation of protein-loaded beads or protein-secreting cells into the chick embryo described here remain important approaches to study notochord function *in vivo* ***(9, 3)***. It should be noted, however, that many other tissues in the chick embryo can be microsurgically manipulated using similar methods (***(9)***, and *see* Chapters 18, 19, and 21). Similarly, corresponding tissues from quail embryos may be grafted into chick hosts, allowing the detection of quail cells with quail-specific markers (***(10)***; see Chapter 24). The same applies to chick tissues electroporated to express fluorescent dyes, which can be visualized by fluorescent microscopy ***(11)***. Finally, tissues derived from mouse embryos were successfully transplanted into chick hosts using this approach ***(12)***, but not all mouse tissues develop well within a chick host (Gabrielle Kardon, personal communication).

2. Materials

2.1. General Requirements

1. Stereomicroscope with 50× magnification, light source with fiber optics, and heat filter.
2. Egg incubator set to 38–39°C with water reservoir to keep air humid (≥90% humidity).
3. Egg trays; cardboard trays the eggs are delivered on can be used.
4. Double-lined bucket to collect egg waste, specialized containers for sharps, disposal according to the local rules in the institute.
5. Egg stools, cut from foamed-plastic sleeve used for insulating water pipes.
6. Roll of clear adhesive tape (Sellotape) and roll of thick plasticized canvas tape, both 25 mm wide and on a dispenser.
7. 26-gauge × 21-mm and 19-gauge × 35-mm hypodermic needles, 1-mL and 10-mL syringes.
8. 5-cm and 3-cm bacterial petri dishes.
9. Autoclaved physiological saline solution. Traditionally, Howard's Ringer solution is used (0.12 M NaCl, 0.0015 M $CaCl_2$, 0.005 M KCl; pH adjusted to 7.2 with very diluted HCl), but saline (0.123 M NaCl; pH 7.0) or phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4) suffice. Note, when "saline" is quoted in the text, any of the three types of solutions can be used.
10. Penicillin–streptomycin (Sigma); 500-µl aliquots, store at −20°C.
11. Dispase (Sigma), 1 mg/mL in tissue culture medium, such as F12 or L15; 500- and 10-µl aliquots, store at −20°C.

12. Shellac-free, nontoxic India ink, such as. Pelican Fount India. This brand is now increasingly hard to find, so ink of different suppliers may have to be tested for their toxicity.
13. Domestic egg pricker.
14. Pair of curved, sharp-pointed scissors.
15. Pair of Vannas spring scissors.
16. Pair of ultrafine scissors or spring scissors.
17. Pair of strong, but pointed forceps, such as watchmaker's forceps No.3.
18. Two pairs of fine watchmaker's forceps (No. 5 or 55).
19. Stainless-steel spatula, 5–8 mm wide, bent to obtain 135° angle.
20. Pure tungsten wire, 100-µm diameter.
21. 6 × 120-mm glass capillary rods as needle holders.
22. Sealing wax.
23. Sterile pipet tips for 20-µL automatic pipet.
24. Long glass Pasteur pipets.
25. Thin silicone tubing plus adapter.
26. Bunsen burner.

2.2. Notochord Grafting

1. Sterile fetal calf serum or goat serum (Sigma) for coating pipet tips of automatic pipets and for setting up the 10% serum–saline dish according to **section 3.3.1.2**: 50-µl aliquots, store at −20°C.
2. For the microdissection of grafts according to **sections 3.3.1.2** and **3.3.1.3**: Sylgard-bottomed 5-cm petri dishes. Sylgard (Dow Corning, Wiesbaden, Germany) is a silicone resin that comes in two parts. When mixed and poured into petri dishes, it sets to produce a near-transparent rubbery matrix, ideal for pinning down embryos. Sylgard dishes can be prepared in advance and stored at room temperature. Cleaned with water and 70% ethanol, they can be reused.
3. To pin down embryos in Sylgard dish: stainless steel minuten pins (Watkins & Doncaster).
4. For the cleaning of enzymatically prepared notochord grafts according to **section 3.3.1.2:** DNaseI (Roche), 1 mg/mL in the saline solution, 100-µL aliquots, store at −20°C, L15 tissue culture medium (Invitrogen).

2.3. Grafting of Shh-Loaded Beads

1. Shh protein diluted to the desired concentration, following the manufacturer's instructions, 1-µL aliquots. Store at −70°C.
2. Affigel beads (Biorad) washed a couple of times in autoclaved PBS by spinning down and replacing the supernatant with fresh PBS, store in PBS at 4°C.

2.4. Grafting of Tissue Culture Cells

1. Culture of protein-secreting cells: For the materials used to raise or engineer tissue culture cells, please refer to appropriate handbooks on cell biology.
2. CellTracker orange CMTMR or CellTracker green CMFDA (molecular probes) for cell staining according to **section 3.6.2.1**, diluted to 10 mM final concentration in DMSO (Sigma), 1-µl aliquots, store at −20°C.

3. To dilute the stock-solution of fluorescent dyes, DMEM (Sigma) or other tissue culture media, depending on the particular requirements of cell lines.
4. 3-cm bacterial petri dishes for cell aggregation, according to **section 3.6.2.1**.
5. Sterile PBS (Sigma) to store cell aggregates prior to grafting.
6. Benchtop centrifuge.
7. Sterile 15-mL conical centrifuge tubes and disposable plastic pipettes.
8. Sterile fetal calf serum or goat serum for coating pipete tips.
9. Fluorescence microscope and appropriate filter sets to visualize fluorescent dyes.

3. Microsurgical Methods

3.1. Getting Started

1. Determine appropriate stage of host embryos for the planned operation. This stage depends on the type and preferred axial level of the operation. In general, Hamburger-Hamilton HH 8–13 stages ***(13)*** can be used for both notochord ablation and heterotopic grafting (***(9)***; **Fig.1A** and **1D**). Take into account that, once the floor plate of the neural tube is specified, this tissue can substitute for the notochord (***(9)***, **Fig. 1B** and **1E**). Therefore, in ablation experiments, either the posterior notochord plus notochord precursors from the node have to be removed before, or at HH10 (***(9)***; **Fig.11C** and **1F**) or later stages, notochord plus overlying floor plate have to be ablated ***(9)***.
2. Incubate eggs in a humidified incubator to desired stage (for storage of eggs, follow guidelines elsewhere in this book). If you plan to use the mechanical method of notochord preparation, incubate some eggs to HH 13–16 as donors. Lay the eggs to the side, mark the top with pencil. The developing embryo is at this site underneath the shell.
3. Set up a clean working space, preferably with lower benching. Have an adjustable chair, set to a height such that you can look comfortably down the microscope with the back straight, but at the same time rest your arms on the bench or on specialized handrest, as this steadies the hands.
4. Set up a stereomicroscope with fiber optics. Check that light source has a heat filter. Have a bunsen burner within reach, and appropriate containers for your waste.
5. Thaw one aliquot of PS, Dispase solution and serum. If you are using enzymatic method I to prepare grafts, also thaw an aliquot of DNaseI.
6. Make up 50 mL of saline solution with 1:100 diluted PS.
7. Prepare the contrasting medium, 1–5 mL of India ink, diluted 1:5 in saline–PS. Draw into a 1-mL syringe, attach 26-gauge × 21-mm needle, expel any air. You may find it handy to have the last cm of the syringe bent to a 60° angle.
8. Prepare a 1-mL syringe with saline–PS to add on top of the embryo when eggs are opened (keeps embryo moist).
9. Set up a 10-mL syringe with a 19-gauge × 35-mm needle to withdraw albumen from eggs.
10. Prepare a 5-cm petri dish with saline–PS to clean the donor embryos and a 3-cm petri dish with saline–PS on ice to collect the grafts. If enzymatic methods of graft

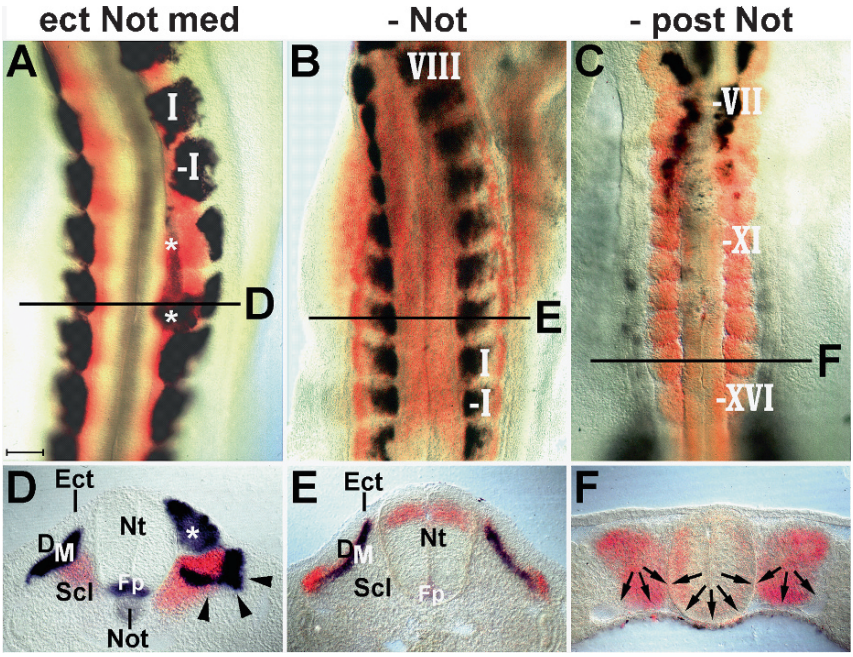

Fig. 1. **A** and **D** Ectopic transplantation of notochord (ect Not med; asterisks) medially between neural tube and paraxial mesoderm: **A** dorsal view, anterior to the top; **D** cross section, dorsal to the top; the axial level of the section is indicated in **A**. The operation has been performed at the level of the anterior segmental plate; after 24 h reincubation, the embryo was stained for the expression of the sclerotome marker *Pax1* (gray), the myotome marker *MyoD* (black), and the notochord—floor plate marker *Shh* (black). Note the ectopic notochord induced ectopic expression of *Pax1* and MyoD (arrowheads).

B and **E** Ablation of the notochord (-Not) at the level of the anterior segmental plate, by removing neural tube plus notochord and back-grafting the neural tube alone (details in (9)); reincubation time and views as in A and D. Note that the dermomyotomal marker *Pax3* (gray) and the myotomal marker *MyoD* (black) are correctly expressed due to the remaining expression of *Shh* in the floor plate of the neural tube (black, Fp).

C and **F** Ablation of the posterior notochord–notochord precursors (-post Not), reincubation and views as in A and D. Note that, in the operated region, *Shh* (black) and *MyoD* (black) are not expressed. In contrast, the expression of *Pax3* (gray) is not restricted to its usual dorsal domains any more, labeling the neural tube and somite throughout (arrows).

Abbreviations: D, dermomyotome; Ect, surface ectoderm; FP, floor plate; M, myotome; Not, notochord; Nt, neural tube; Scl, sclerotome. The maturation age of the somites at the time of operation is indicated in roman numerals. The scale bar in **A** represents 67 µm in **A–C**, 50 µm in **D** and **E**, and 25 µm in **F**.

Source: Dietrich, Schubert, and Lumsden ***(9)***; originals: blue-red double stainings.

preparation are used, prepare one Sylgard dish with saline–PS. If you are using enzymatic method I to prepare grafts, also prepare a 3-cm petri dish with 1 mg/mL Dispase, 5 µg/mL DNaseI in L15, and a 3-cm petri dish with 10% serum in saline–PS.

11. Prepare operation needles: Cut 2–3 cm of tungsten wire off the reel, straighten out the curve and put to the side. Warm up one end of the glass rod with the bunsen burner, then touch the sealing wax such that the end of the glass rod is covered. Hold the tungsten wire with the coarse forceps and maneuver it through the molten wax into the capillary of the glass rod, such that about 1–1.5 cm of the wire protrudes from the glass. Let the wax solidify, then bend the last 2–4 mm of the wire to form a 135° elbow. Hold the tip of the wire into the hottest (blue) part of the bunsen flame until the very tip flies off (do not set fire to the wax). Withdraw the needle immediately—it will have a very fine, sharp tip. Make sure you do not touch anything hard with this needle; it will ruin the tip.
12. Prepare thin glass capillary. Hold on to the extreme ends of the glass Pasteur pipet, then place its capillary over the bunsen flame. When the capillary gets soft, gently bend an angle in the middle of it. Warm the portion between this angle and the main part of the pipet, then pull to obtain a very thin capillary. Reduce the capillary to 2 cm in length by breaking off the rest of the pipet. The coarse end of this capillary can be attached to the adapter of the silicone tubing to form an aspirator (use the pipet tip with a filter as mouth part; store the mouth end in a sterile petri dish to avoid contamination). Note that the remainder of the Pasteur pipet (and, at the end of the operation session, the capillary) needs to be disposed of in a container for glass or sharps. Also note that these capillaries cannot be used if the local rules forbid use of an aspirator.

3.2. Opening Eggs for Manipulation (Windowing)

1. Wear protective clothes, in case eggs break. Wear gloves when you have an egg allergy or if you have a history of allergic reactions.
2. Disinfect eggs by spraying with 70% v/v ethanol, allow to dry, then, using the egg pricker or the blunt forceps, pierce the blunt end of the egg (**Note 1**). You may prepare one tray of eggs at a time.
3. Insert the 19-gauge needle on the 10-mL syringe into the hole, direct the tip of the needle toward the bottom of the egg, avoiding the thick albumen that surrounds the yolk. Slowly withdraw 1–2 mL of the thin albumen.
4. With the blunt forceps or curved scissors, drill a small hole into the middle to the upper side of the egg, then carefully nick the shell's membrane underneath. Let the egg sit aside for a moment while the embryo, which is immediately beneath the shell, subsides. A drop of saline over the hole may accelerate this process.
5. Cover the top of the egg with clear tape, press down firmly, smooth out creases.
6. Use the curved scissors to make a spiral cut, starting with the small hole in the middle of the upper side of the egg. Cut open a circle about 1.5 cm wide over the blastoderm (*see* **Note 2**).

7. Examine and roughly stage the embryo under the stereomicroscope; you may use neutral red to improve visibility of the embryo (*see* Chapter 16). If precise staging is required, inject some ink (*see* **section 3.3.2.1** and **Note 3**) and count somites. Select those eggs in which the embryo lies centrally on top of the yolk as hosts, the others can be used as donors if undertaking isochronic grafts.
8. Hosts: Apply a drop of saline–PS from your 1-mL syringe, cover the egg loosely with clear tape, and leave aside (they are happy to be out of the incubator for several hours; their development will pause).
9. Donors: When performing isochronic grafts, you can economize by using as donors those embryos that are found, on windowing, less accessible. Cut around the perimeter of the *area opaca* with Vannas scissors, taking care not to rupture the yolk. Lift embryo with the prewetted spatula and transfer into petri dish with saline–PS. Float off the vitelline membrane, shake the blastoderm to free it from adhering yolk, and transfer it to fresh petri dish or the Sylgard dish. Subdissect as required (*see* **section 3.3.1**).

3.3. Heterotopic Grafting of Notochord

3.3.1. Dissection of Donor Notochords

After cleaning the donor embryos from adhering yolk, the notochord is explanted, using one of the three methods that follow. The mechanical method is the most rapid but may leave traces of other tissues adhering to the graft. In methods 2 and 3, the grafts are cleaned from adhering tissues with proteolytic enzymes (Dispase). The best results are obtained with method 3. Here, the embryos are treated very gently to maintain their overall structural integrity and recover additional tissues for further grafting experiments. Note that, to transfer the grafts, serum-coated pipet tips, obtained by pipeting up and down some serum, are required to prevent the graft from adhering to the plastic.

3.3.1.1. Mechanical Method

1. Cut off and discard the body from HH 13–16 embryos below the level of the last somites.
2. Grip the embryo with two pairs of forceps, one holding onto the area of the first pharyngeal arch, the other gripping the upper trunk. Pull apart. The notochord stays attached to the head and slides out intact from the trunk.
3. Trim off the notochord and set aside in a dish of fresh saline–PS on ice (*see* **Note 4**).

3.3.1.2. Enzymatic Method I

1. Transfer the embryo to a Sylgard dish containing saline–PS. Submerge and pin out through the membranes with stainless steel minuten pins. Subdissect the axial tissues of the embryo, using ultrafine scissors under transmitted illumination.
2. Using a serum-coated 20-µl pipet tip, transfer tissues to a solution of 1 mg/mL Dispase and 5 µg/mL DNaseI in L15 medium.

3. Treat for 20–25 min at room temperature, then transfer to fresh saline with 10% serum. Reflux tissues through pipet tip until free of adhering tissues (check under microscope).
4. Transfer to saline–PS on ice.

3.3.1.3. Enzymatic Method II

1. Pin down the embryo as in **section 3.3.1.2** but ventral side up.
2. Carefully open the endoderm on both sides of the notochord, using the flame-sharpened tungsten needles.
3. Overlay the embryo with approximately 20–50 µL Dispase solution. Leave at room temperature until the notochord comes free. Use the operation needles to help the separation of tissues and to tear off any adhering endoderm.
4. Cut the notochord with the operation needles or the ultrafine scissors.
5. Transfer to saline–PS on ice.

3.3.2. Preparation of Host Embryos

3.3.2.1. Improving the Visibility of the Embryo

Push the needle of the ink-filled syringe into the yolk outside the *area pellucida*, and move its tip, as horizontally as possible, into the subblastodermal space. Be careful not to scrape the underside of the embryo and, for older stages, not to rupture any blood vessels. Expel about 0.1 mL of ink, taking care not to introduce air bubbles (*see* **Note 3**).

3.3.2.2. Grafting the Donor Notochord into the Host

3.3.2.2.1. General Remarks

1. Illuminate the embryo with one or two fiber-optic lenses on an oblique path. Use around 50× magnification, hold egg with one hand, gently pressing down on the egg stool to keep the embryo in focus. Support wrists on handrests or lower arm on bench, and for the operating hand, rest the third to fifth fingers on the side of the egg—this enables you to steady the operating needle. Avoid large movements.
2. Under the dissecting microscope, place a drop of saline–PS onto the host embryo. Nick the vitelline membrane with the fine forceps and expose the area to be operated on; make sure that both layers of the vitelline membrane are opened. As the vitelline membrane ruptures, the drop of saline flows in and prevents the albumen from covering the embryo (if this should happen, both host and donor tissues become sticky and grafting becomes very difficult).

3.3.2.2.2. Transplantation of the Notochord

1. The manipulation described here results in an ectopic induction of the sclerotome, as shown by Dietrich et al. (***(9)***; **Fig. 1A** and **1D**), and floor plate, as shown by Goulding, Lumsden, and Gruss ***(14)***, since the graft faces the dorsoventrally uncommitted neural plate and paraxial mesoderm. For this operation, the host embryos need to be at HH 9–10 and the site of operation is the open neural plate

at the posterior end of the embryo (open posterior neuropore; *see* also **Note 5**). Using the flame-sharpened tungsten needle, make a longitudinal slit (about 500–600 µm long) through the ectoderm at its junction with the neural plate. This is best achieved by inserting the tip of the operation needle into the ectodermal layer (do not aim deeper), then flicking the needle upward. Periodically clean the needle of adhering tissues or albumen, holding it briefly into the flame of the bunsen burner.

2. If permitted, use the aspirator to suck up a small drop of Dispase into the fine glass capillary, then expel this drop over the cut ectoderm. If aspiration is not possible, then a small amount of Dispase has to be pipeted on top of the embryo with an automatic pipet.
3. Use the tungsten needle to gently separate the neural plate from the paraxial mesoderm: Roll off or push away the neural plate as it comes loose (**Note 6**). Rinse the operated area with saline–PS from the 1-mL syringe to wash off the Dispase. The aim of this procedure is to make a pocket beneath the lateral part of the neural plate to house the notochord implant in close apposition with the basal surface of the neuroepithelium. Therefore, do not fully separate the neural plate and the paraxial mesoderm, as the graft will then settle down too deeply next to the host notochord.
4. Cut donor notochords into pieces of about 500–1,000 µm, transfer one piece in as little volume as possible to a serum-coated 20-µL pipet tip.
5. Deposit the graft over the posterior end of the embryo, avoiding the albumen.
6. Maneuver the graft into the prepared slit: Open the slit by pulling on the lateral ectoderm with the operation needle. The notochord should drop into the hole. Push it in more deeply with the point of the needle.

3.3.2.3. Reincubation of Embryos

1. After the operation, place another drop of saline over the embryo (**Note 7**). Check that the graft has not been dislodged.
2. Close the egg, either with clear tape or, better, with canvas tape. Stretch the tape before applying to fit the double curvature of the egg and smooth it down carefully such that it does not admit any air. High humidity inside the egg is essential after the operation: Drying, even slightly, is the major cause of mortality.
3. Mark the shell with name and number or stage of the operation using a pencil and place in the recovery incubator. Do not open the egg until you want to harvest the embryo; checking up on progress causes drying and reduces survival.

3.4. Notochord Ablations

As the notochord is crucial for the development of the surrounding tissues but can be replaced by the floor plate of the neural tube (**Fig. 1B** and **1E**), once this structure is established, the effect of notochord ablations varies depending on the type and timing of the operation. It is therefore advisable to analyze the embryos for the process of interest, but in addition for a known phenotype of the particular manipulation. This can be achieved, for instance, by double whole mount in-situ hybridization (*see* **Fig. 1**), employing molecular markers

for motor neurons in the neural tube ***(1)*** or markers for the sclerotome ***(2,9)***: on complete notochord (and floor plate) removal at the level of the posterior (i.e., developmentally immature) neural tube–paraxial mesoderm in embryos up to HH 13, these markers are absent. Likewise, markers for the dorsal neural tube or somitic dermomyotome can be used, which will be expressed in an ectopic, ventral location ***(1,2,9,14)***.

Note that, when performing notochord ablations, the remarks on improving the visibility of the embryo (**section 3.3.2.1**) and reincubation of operated embryos (**section 3.3.2.3**) apply.

3.4.1. Ablation of Posterior Notochord from Early Embryos

3.4.1.1. General Remarks

In embryos at or before HH 10, the floor plate in the posterior neural plate is not yet established ***(14)***. Therefore, when in this area the notochord is removed, the floor plate fails to develop, preventing any compensatory signaling from this tissue ***(9, 14)***. Take into account that the notochord can regenerate to some extent; therefore, it is advisable to also remove as much of the notochord precursor pool in the node as possible. Note that this operation leads to a notochord–floor-plate-less stretch in the middle of the embryo at the level of the fore limbs and upper flank (***(9, 14)***; Fig. 1C and 1F).

3.4.1.2. Method of Posterior Notochord Ablation

1. Follow the instructions in **section 3.3.2.2.2**, item1 to make a unilateral, longitudinal cut in the ectoderm along the border of the posterior neural plate, and apply a drop of Dispase as described in **section 3.3.2.2.2**, item 2.
2. Roll off the neural plate from the paraxial mesoderm as described in **section 3.3.2.2.2**, item 3, this time fully separating the neural plate from the paraxial mesoderm on the side of entry into the embryo.
3. Gently push the neural plate upward to free it from the notochord, and pull the notochord toward the paraxial mesoderm to free it from the underlying endoderm.
4. Make the anterior cut through the notochord at a position close to the somitic paraxial mesoderm. Then, ease the notochord between the cut and the node out of the embryo.
5. For the final cut, pierce the tip of the needle into the flattened posterior end of the notochord and the node, such that eventually these tissues are dug out from underneath the neural plate. Keep the needle horizontal to not rupture the endoderm.
6. Move the notochord explant away from the embryo, flip back the neural plate into its original position, rinse the embryo with saline to wash away the Dispase, then seal and reincubate the embryo as described in **section 3.3.2.3**.

3.4.2. Ablation of the Notochord and Floor Plate from Late Embryos

3.4.2.1. General Remarks

When the signaling of ventral midline tissues needs to be studied at embryonic stages later than HH 10 or at axial levels different from fore limb–flank levels, this is the method of choice. Note that the ventral midline tissues are accessed through the neural tube. Therefore, great care needs to be taken not to accidentally rip out dorsal neural tube tissues, which are a further signaling center in the embryo ***(1, 2)***. If this can be achieved, then the cut dorsal edges of the neural tube heal without trace ***(9)***.

3.4.2.2. Procedure of Notochord–Floor Plate Ablation

1. Window and ink the eggs as described before. Then, make a longitudinal cut in the middle of the dorsal neural tube, using the fine, flicking movements described earlier.
2. Move the cut dorsal edges of the neural tube apart such that the ventral midline becomes visible. Follow the wall of the neural tube ventrally and make a cut before the neural tissue bends toward the ventral midline, aiming the cut in a ventral–lateral direction. Perform this cut on both sides of the ventral midline.
3. Perform the anterior and posterior cut through the floor plate and notochord, aiming for an explant corresponding to about three somites in length. For this purpose, apply the same movements as before, gradually making the cut deeper. Do not cut through the endoderm, or the ink will rise though the cut obstructing the view.
4. Apply a small drop of Dispase to the cuts.
5. Gently move the floor plate–notochord complex from side to side to ease it off the endoderm, then insert the tip of the operating needle into either the anterior or posterior end of the cut floor plate–notochord fragment and pull upward. The tissues should lift off the endoderm and out of the embryo.
6. Rinse the operated area with saline–PS and reincubate as described before.

3.5. Replacement of the Notochord by Shh-Loaded Beads

3.5.1. General Remarks

1. To replace the ablated tissue with Shh-loaded beads, follow the instructions for notochord ablation and continue with **section 3.5.2.** However, the beads may be implanted anywhere in the embryo.
2. When grafting beads into previously Dispase-treated areas, make sure that the area has been well-rinsed with saline.
3. The beads have buoyancy in the embryo and tend to float out of the operated site. To prevent this, either keep the hole through which the bead is inserted into the embryo small, break the bead in half (the broken surfaces are slightly more sticky), or cover the operated area with a piece of egg shell membrane or tantalum foil to weigh the bead down.

4. If protein other than Shh is to be used, clarify whether Affigel beads or rather another type of bead has to be used.

3.5.2. Loading Beads with Shh Protein

1. The day before the planned grafting experiment, spin down beads and remove supernatant.
2. Thaw one aliquot of Shh protein, transfer some 50–100 beads in as little volume as possible into the protein suspension. Leave at 4°C over night. Cover the stock of beads again with PBS.
3. At the day of operation, prepare a 3-cm petri dish with a 100-µL drop of saline on ice and transfer the estimated amount of beads required for the experiment in as little volume as possible into the saline. Store on ice.
4. The remaining protein-loaded beads can be stored at 4°C for at least a week.
5. Remember to perform control grafting with beads loaded only with, for example, bovine serum albumin (BSA).

3.5.3. Bead Implantation

1. Prepare the host embryo as described earlier (**sections 3.3.2.1** and **3.4**).
2. Set the automatic pipet to 1–2 µL, lift up the petri dish containing the beads into the optical plane used to manipulate the embryo, and suck up a single bead.
3. Place the petri dish back on ice, move the egg under the microscope and expel the bead over the embryo.
4. Move bead into the desired position with the operation needle, weighing it down if required.
5. Add saline, controlling that the bead is not washed out of its slot, then reincubate egg as before (**section 3.3.2.3**).

3.6. Replacement of the Notochord by Cells Expressing Signaling Molecules

3.6.1. General Remarks

1. Any avian or mammalian cells that perform at 38–39°C can be grafted into the chick embryo. It is advisable to use adhering cell lines to prevent the dispersal of the grafts. As for the protein-loaded beads, the cells can be placed anywhere in the embryo.
2. Remember to also perform control grafting of the cells not expressing the molecule of interest. Consider that the engineering of the cells may have triggered the expression or suppression of further signaling molecules.
3. The dye suggested here to stain the cell pellets remains visible after standard whole mount in-situ hybridization.
4. For the raising of tissue culture cells, please refer to handbooks on cell biology.

3.6.2. Preparation and Grafting of Tissue Culture Cells

3.6.2.1. Staining and Aggregation of Cells

1. Thaw one vial of CellTracker stock-solution and dilute it to a final concentration of 10 to 25 µM in prewarmed DMEM or other tissue culture medium. Avoid a medium containing high concentrations of serum, since it can reduce cell staining. Perform CellTracker dilution immediately before use and keep the dye protected from light.
2. Trypsinize cells following standard cell culture protocols. Carefully wash cells in PBS to remove all traces of trypsin. Centrifuge the cell suspension and aspirate the supernatant from the pellet.
3. Gently resuspend the pellet in the previously diluted CellTracker.
4. Transfer the resuspended cells to a 3-cm bacterial petri dish and incubate them for 30 min under the conditions normally used for the selected cell line.
5. After incubation, add 3–5 mL of medium to the petri dish and transfer the cells to a 15-mL conical centrifuge tube. Centrifuge and aspirate the supernatant. Repeat washing twice.
6. Resuspend the pellet in a small volume of medium and transfer the cell suspension to the center of a bacterial petri dish. Incubate overnight to allow aggregates to form.
7. In the following morning, rinse the aggregates twice in sterile PBS.
8. Store the cell aggregates for grafting in PBS, set on ice.

3.6.2.2. Grafting of Cell Aggregates

1. Prepare the host embryos as described under **sections 3.3.2.1** and **3.4**.
2. Collect cell pellet with a serum-coated pipet tip in the same fashion as described for the beads (**sections 3.5.2.2** and **3.5.2.3**). If pellets are large, cut to size with an operation needle. If they are small, transplant several.
3. Expel cell pellet over embryo, maneuver into position with operation needle.
4. Add saline–PS, seal and reincubate egg as described before (**section 3.3.2.3**).

4. Harvesting and Analysis of the Operated Embryos

1. After the desired period of reincubation, remove eggs from incubator.
2. Cut open the tape covering the egg's window, using the curved scissors.
3. Using the curved scissors or Vannas scissors, cut around the embryo, not rupturing the yolk.
4. Prewet a spatula with PBS, move underneath the embryo, and with fine forceps, pull the embryo onto the spatula, transferring as little yolk as possible.
5. Submerge the embryo in PBS, remove the adhering yolk and cut away the extraembryonic membranes with the ultrafine scissors. If the following analyses depend on the penetration of the embryo by in-situ probes or antibodies, it is advisable to pierce the hollow structures of the embryo, such as brain vesicle and heart, using the ultrafine scissors.

6. Maneuver the embryo onto the spatula in a flat position, dorsal side up, and transfer into fixative. Most commonly, 4% paraformaldehyde in PBS is used. Embryos up to HH 18–20 are lightweight enough to float on top of the fixative. If this is done for 5 min, then the embryo is prefixed in the same flat, stretched-out position it had in the egg. Eventually, the embryo has to be submerged to thoroughly fix all tissues.
7. If the operation or expected outcome varies from embryo to embryo, collect embryos separately in plastic multiwell plates and keep a record for each.
8. The embryos can be analyzed by standard in-situ hybridization and immunohistochemistry techniques. The site of tissue culture cell implantation can easily be identified using fluorescent microscopy.

5. Notes

1. Egg albumen is bactericidal, so instruments need not be sterilized, but they should be thoroughly cleaned after use and may be dipped in 70% v/v ethanol and allowed to air dry before use. *On no account should instruments (other than the tungsten needles) be flamed. It destroys the temper of the steel.*
2. Some workers prefer to cut an incomplete circular aperture into the egg's shell, tipping the cut shell backward and out of the way (like a trapdoor), then closing and sealing it with tape on completing the operation. No matter which way of closing the egg is used, keep the outside of the egg dry, as the tape will not stick to a wet surface.
3. Do not inject the contrast medium until the egg is ready for grafting—it can disperse quite quickly.
4. Instead of storing the explanted notochord fragments on ice, you may directly graft one into a readily prepared host. However, do not keep the grafts at room temperature for long, as they will coil up and become intractable.
5. To induce ectopic sclerotome formation, the procedure in **section 3.3.2.2.2** may slightly be varied: The paraxial mesoderm gains competence to respond to notochordal signals at the time of epithelial somite formation 10. In addition, newly formed somites remain labile for some time with respect for their dorsoventral and mediolateral pattern. Therefore, neither using HH 9–10 donor embryos nor operating at the level of the posterior neural plate is critical. You may find it convenient to insert the graft at the level of the anterior segmental plate.
6. During operations, make sure the needle is kept clean of adherent albumen and tissue by briefly passing it through the flame of the bunsen burner. Keep the needle in the hot flame for longer to remove and resharpen a bent tip.
7. Prevent the embryo from drying during the operation by adding saline–PS.

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21

Transplantation of Avian Neural Tissue

Sarah Guthrie

1. Introduction

The transplantation of neural tissue provides a means of addressing many questions in developmental neurobiology and regeneration of the nervous system. Although this technique has been used in fish, amphibia, avian, and mammalian species, this chapter focuses on neural transplantation in avia, which has several advantages. The development of the nervous system provides a close parallel to that of mammalian embryos in many of its aspects, and embryos can be accessed at developmental stages impossible in mammals. In addition, avian eggs can be obtained at low cost, and minimal equipment and facilities are required for these types of grafting experiments. Transplantation is possible at both the cranial and spinal levels of the neuraxis, but the accessibility of these regions varies with stage. For example, transplantation of brain regions (e.g., ***(1)***) is relatively easy at stages in which the neural tube has yet to develop the local expansions of the brain vesicles and has not yet become extensively vascularized, that is, before E3 in the chick, but becomes more difficult after this time. Stages at which transplantation into the spinal cord (e.g., ***(2)***) can be performed depend on the axial level to be investigated owing to the rostro–caudal order of its generation, but transplants into spinal regions remain feasible at later stages.

Examples of the application of neural grafting in chick embryos have been to explore the role of a signaling region by transplanting it to an ectopic site ***(3)*** or to investigate the mechanisms of hindbrain segmentation ***(4)***. In some experimental paradigms, it is desirable to follow the position and fate of the transplanted tissue in the host environment. For this purpose, chick–quail chimeras have been used extensively to explore a number of developmental questions (*see* Chapter 24 for detailed discussion). Quail tissues transplanted into a chick host can later be recognized using quail cell-specific or axon-specific antibodies.

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This allows the fate mapping of cells derived from the grafted tissue or axon projections arising from the graft. In chick-to-chick transplants, it is also possible to track grafted tissues that have been labeled using Hoechst or other fluorescent dyes (e.g., ***(5)***). Transplantation of retrovirally infected avian tissue into a host embryo resistant to viral infection is a possibility to analyze the fate of cells carrying a transgene ***(6)***. Grafting of mouse tissue into a chick host has also been used to analyze developmental interactions between tissues of transgenic animals and that of the host embryo ***(7)***.

This chapter describes the procedure for grafting neural tissues in general, and provides diagrams depicting grafting of rhombomeres as an example of this technique. Learning neural transplantation is a long, frustrating business, which requires many repetitions and probably weekly practice to achieve any success. However, many of the trivial problems of its execution can be overcome by the strategies outlined here. Grafting experiments can often provide a valuable adjunct to other studies, such as molecular biological or tissue-culture experiments, in exploring the role or interactions of a molecule or tissue region. In addition, they may be the method of choice for addressing some developmental problems, such as the state of determination of a tissue, its inductive influence, or to construct a fate map. This technique therefore continues to provide a valuable tool for experimental embryologists.

2. Materials

2.1. Microscopes

It is important to use a good-quality stereomicroscope with focusing eyepieces and provision for both epiillumination and transillumination. Bear in mind that, for much of the day, you will probably need to monopolize two microscopes, one for the grafting itself (epiillumination) and one with the dish of tissue pieces for transfer (transillumination). A good light source (preferably a cold light source) is also essential, particularly to locate grafted pieces once you have transferred them into the egg. You also need a comfortable chair of adjustable height and armrests, so that both forearms are supported while you dissect and perform the transplantions. It is virtually impossible to accomplish these types of grafting experiments with quaking hands suspended over the egg. If you are not "sitting comfortably" at the microscope (for what may be many hours), then transplantation becomes a frustrating and ultimately fruitless business.

2.2. Dissecting Tools for Preparation of Donor Embryo Fragments and Host Embryos

1. Tungsten needles: These can be made by mounting 2–3-cm pieces of 100-µm pure tungsten wire (Goodfellow) on needle holders made of aluminum or wood of diameter 30–40 mm. Pieces of wire can be mounted using either sealing wax

or adhesive (e.g., Araldite). The end of the needle should be sharpened to a tapering point in the hottest part of the bunsen flame either before or after mounting. The end of the needle can then be bent over to an angle of 90–135° using forceps. Needles tend to get bent and should be kept straight and sharpened in the flame throughout the operation to remove attached tissues and prevent snagging of the tissues being dissected.
2. Spring scissors and fine scissors for dissection of donor embryos.
3. Small spatula for removal of embryos from donor eggs.
4. Curved scissors or scalpel for opening host eggs.
5. Fine forceps (Dumont 5) for dissection of donor embryos.
6. Coarse- and fine-gauge needles for withdrawing albumen from eggs and injecting ink.
7. Sellotape and opaque egg tape (Beiersdorf).
8. Carmine dye particles (Sigma) for labeling transplanted tissue fragments.
9. Sterile plastic pipets or fire-polished Pasteur pipets for transferring tissue pieces.

Note: Sterilize all tools that are to contact the embryos by spraying with 70% ethanol and allowing them to air dry. Do not flame fine dissection tools.

2.3. Solutions

1. Chick Ringer's solution: 7.2 g/L NaCl, 0.23 g/L CaCl, 0.37 g/L KCl, pH 7.2. Immediately before use, add 1 in 50 of penicillin–streptomycin solution (pen/strep; Gibco, Grand Island, NY).
2. 1× Hanks' Balanced Salt Solution (HBSS) (Gibco).
3. Dispase (Grade 1; Boehringer Mannheim, Mannheim, Germany). Dilute at 1 mg/mL in HBSS containing 50 µg/mL deoxyribonuclease (Sigma, St. Louis, MO).
4. Ink solution for subblastodermal injection to visualize embryos. Ink is Pelikan drawing ink A, shade 17 (black). Dilute at 1 in 15 to 1 in 20 in Ringer solution containing penecillin–streptomycin.

3. Methods

3.1. Preparation of Donor Tissues

1. Remove donor embryos from eggs. This may be done by cracking eggs into a petri dish or by windowing them (*see* Chapter 15). If you are dealing with embryos of stage 9 or younger, it is more reliable to window eggs to avoid losing embryos by accidentally puncturing the blastoderm.
2. Remove donor embryos by cutting through the vitelline membrane and blastoderm and around the embryo using spring scissors.
3. Slip a small spatula under the embryo and transfer into a dish of Ringer's solution.
4. Dissect away extraneous tissues to isolate the region of interest. In the case of rhombomere transplants, for example, the entire hindbrain from E2 embryos is dissected away from spinal cord, midbrain, and forebrain and the heart and gut removed (**Fig. 1**).
5. Place the isolated regions in a small volume of Dispase (1 mL) in a 35-mm petri dish, and leave for 5 min.

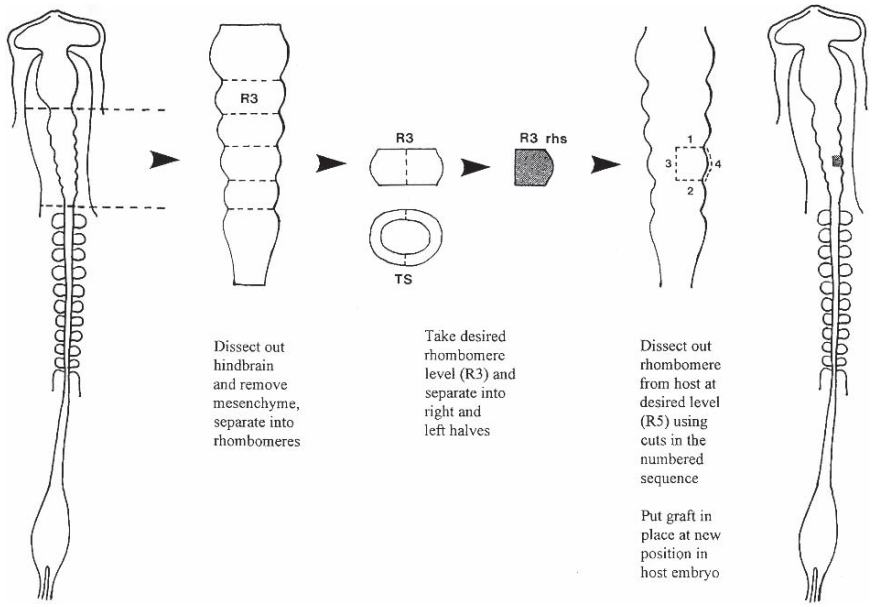

Fig. 1. This figure illustrates diagrammatically the procedure for grafting a rhombomere unilaterally from a donor stage 10 chick embryo into an isochronic host embryo. Once the embryo has been removed from the egg, the entire hindbrain region is dissected free of adjacent tissues, and the adhering mesenchyme cells are removed by exposure to Dispase. Then individual rhombomeres are separated from each other, and at the desired axial level (rhombomere 3 in this case), the right and left halves are separated from each other. Meanwhile the region that will receive the graft in the host embryo is prepared by removing the host rhombomere 5 using 4 cuts in the numbered sequence. The graft tissue can then be transferred into the egg and put in place in the ectopic location. The right-hand side piece of the rhombomere has been grafted such that the anteroposterior and dorsoventral polarity have been maintained.

6. After this time, use tungsten needles to test whether adjacent tissues are dissociating from the neural tubes. If so, transfer immediately to a fresh dish of HBSS. If not, leave for a minute or so longer—the exact time depends on the age of embryo and region to be dissected. In any case, it is crucial that embryos are not exposed to Dispase for too long. Otherwise, neural tissue becomes irreversibly damaged.
7. Adjacent mesodermal tissues can be teased away from the neural tubes using tungsten needles, then clean the neural tubes transferred into fresh HBSS. Cut away undesired tissues by pressing down on the plastic petri dish with the tungsten needle. When pulling away mesenchymal or other tissues, avoid contacting the neural tissue directly with the needles.
8. From now on, keep tissues as far as possible on ice, since this prevents deterioration.

9. Transfer individual neural tubes from the dish on ice to the microscope for further subdissection, such as dissection of individual rhombomeres (**Fig. 1A**), but keep the rest cold. At this stage or previous stages of the dissection, a few carmine particles can be brushed against the surface of the neural tissue using a needle to label its polarity, if this is required.
10. Different tissue pieces may be stored in individual wells in multiwell plates, and chick tissues can be kept on ice for up to 8 h without detriment. For rhombomere transplantation in which unilateral grafts are to be performed, the bilateral rhombomere fragments can be kept intact until just before grafting then separated into right and left halves (**Fig. 1**).

3.2. Preparation of Host Embryos

1. The outer surface of eggs should be sterilized with alcohol and 1 mL of albumen removed via the blunt end of the egg using a syringe and 21-gauge needle.
2. Eggs can then be windowed by cutting a hole in the top using a scalpel or curved scissors (*see* Chapters 15, 16 and 27). Avoid pointing the blades of the scissors down into the egg, since this may damage the embryo.
3. Embryos can then be visualized by subblastodermal injection of ink (about 100 µL) using a 25-gauge needle. Inject the ink outside the *area opaca* at a shallow angle and minimize the size of the hole.
4. Eggs can be resealed with pieces of Sellotape and left at room temperature (or in the incubator) until they are to be used (*see* **Note 1**).

3.3. Grafting of Neural Tissue Pieces

1. Take a host egg of the desired stage, remove the sealing tape, and remoisten the embryo with a few drops of Ringer's solution.
2. Using a tungsten needle, lift up the vitelline membrane, and cut a flap above the embryo; keep this to the minimum size required for access to the region of interest.
3. Using one tungsten needle make cuts around the tissue to be removed to receive the graft (**Fig. 1**). To do this, insert the needle to a depth of 100 µm or so, and pull gently upward and separate tissues. Then, gradually deepen the cut with successive strokes of the needle (*see* **Note 2**). It is preferable to make the hole slightly bigger than the piece you intend to put into it.
4. Then either remove the tissue piece by hooking it out with the needle or leave it in the egg. If you do the latter and are using unlabeled donor tissue, you should be confident that you can tell the grafted tissue piece apart from the host tissue you have just removed.
5. Retrieve the neural tissue piece to be grafted from the petri dish using a P20 pipetman and yellow tip set on 10–20 µL. If there are problems with the tissue getting stuck to the plastic pipet, you can coat the inside of the yellow tip with sterile serum before use.
6. Draw some fluid into the pipet before drawing up the tissue piece. Otherwise the tissue may hit the meniscus and disintegrate. Then transfer the piece into the egg as quickly as possible. Do this on low magnification, and try to track the piece with your eye to avoid losing it.

7. Maneuver the tissue piece into place adjacent to the grafting site using a tungsten needle (**Fig. 1**).
8. Orient the piece as required, and check for several seconds to see that it does not move out of position. It is possible to place small fragments of sterile drawn glass or other small objects on top of the neural tissue fragment to keep it in place, but this is not normally necessary (*see* **Note 3**).
9. Seal the egg with a piece of opaque egg tape (preferably stretchy). If you intend to incubate the embryo for more than a few days, extra Sellotape around the edges is also a good idea.
10. Eggs should be replaced in the incubator and left until the desired stage for harvesting (*see* **Note 4**). Opening up to check the stage of development and resealing for further incubation is not recommended, as it leads to certain death. When calculating the stage for harvesting, assume a day's delay in the development of embryos.
11. To open the eggs, cut a slightly bigger hole than before in the top of the egg using curved scissors.
12. Cut and lift the embryo out using spring scissors and forceps or a spatula, transfer to Ringer's solution, and wash off excess yolk before further processing.

4. Notes

1. For grafting of very young embryos (younger than stage 9), it may be desirable to avoid opening the eggs until immediately before grafting, since dehydration is a major cause of mortality. Replacing the cut piece of shell over the hole and securing with tape may also improve survival rates.
2. Always keep the motion of the needle toward you, rather than down into the egg, since this guards against damage to the embryo. Use more smaller cuts rather than fewer larger ones to free the region from its surrounding tissues. For rhombomere grafting, this involves cuts at the rostral and caudal ends, the lateral edge where the neural tube abuts the mesenchyme, in the dorsal midline, and the ventral midline (where the ventral neural tube abuts the floor plate).
3. If you have been struggling for many minutes with the graft, you may notice that the surface of the embryo has dried out, and needs the addition of Ringer's solution. If so, do this very carefully to avoid dislodging the graft.
4. The major problem of all neural grafting experiments is the low survival rates of the grafted embryos. Even if the operations have been performed flawlessly, mortality can be about 50% after several days. The causes of this are unclear, but the major factors are likely to be dehydration of the embryo or contamination. Dehydration can be prevented by addition of Ringer's solution, performing the operation in the shortest possible time, and making sure the egg is well sealed at the end. Contamination should not be a problem provided sterile plastics, solutions, and tools are used throughout; and the only tool used inside the egg is a tungsten needle, which is frequently flamed. Excessive damage to the embryo, surrounding membranes and yolk, and the developing vasculature are also obvious problems, which should diminish with practice. Low survival

rates produce the related problem that sample sizes in any one experimental category are very small. It may take a long time to obtain data that are amenable to statistical analysis. Furthermore, experimental results obtained within these categories may sometimes be heterogeneous owing to differences in the grafting procedure. For this reason, it may be desirable to label the grafted tissue in some way (*see* **section 1**).

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22

Grafting of Apical Ridge and Polarizing Region

Cheryll Tickle

1. Introduction

The apical ectodermal ridge (AER; also known as the apical ridge) and the zone of polarizing activity (ZPA; also known as the polarizing region) are two major signaling regions in developing vertebrate limbs. Limbs arise as small buds of undifferentiated mesenchyme cells encased in ectoderm. The apical ridge and the polarizing region were first identified in limb buds of chick embryos, and functions of these regions were explored by traditional "cut and paste" experiments by Saunders (reviewed in **ref.** *1*). Similar signaling regions are also found in embryonic limb buds of other vertebrate species, including mice, rats, snapping turtles, and humans ***(2)***. Signals from apical ridge and polarizing region act together with signal(s) from the ectoderm in a region of undifferentiated cells called the progress zone ***(3)***. The progress zone is found at the tip of the bud as it grows out and is maintained by the apical ridge. As cells leave the progress zone, they lay down the structures of the limb in sequence with proximal structures being formed first and distal structures later ***(4)***. Cellular responses to polarizing and ectoderm signals in the progress zone establish the limb plan, and subsequently cartilage, bone, muscle, tendon, and other specialized cell types differentiate and become organized into tissues.

The apical ridge is the thickened rim of epithelium at the tip of the limb bud and is required for bud outgrowth (**Fig. 1**). Apical ridge cells are elongated, tightly packed, and linked by extensive gap junctions. When the apical ridge is removed, bud outgrowth ceases and a truncated limb develops; in contrast, grafting a second apical ridge to the surface of a limb bud induces a second outgrowth ***(1,5)***. The signal from the apical ridge can be substituted by beads soaked in fibroblast growth factors (FGF-2, FGF-4, FGF-8; ***6,7***). Several

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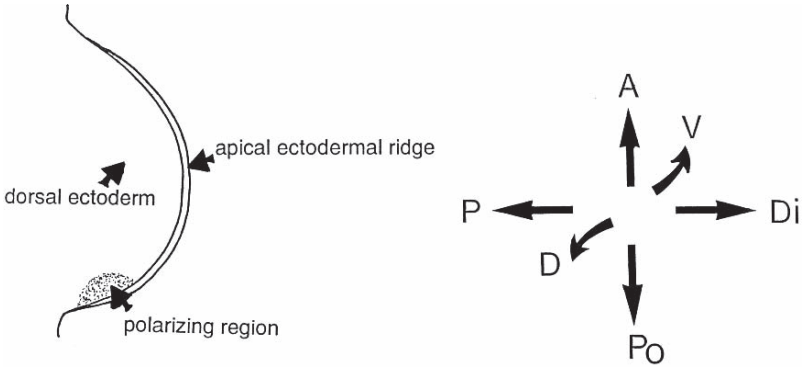

Fig. 1. Diagram to show early chick limb with signaling tissues and the three axes, A-Po (anterior–posterior), P-Di (proximal–distal), D-V (dorsal–ventral).

members of the FGF family are expressed in tissues at the tip of the limb bud, including apical ridge (reviewed in **ref.** ***8***). The polarizing region is a group of mesenchyme cells at the posterior edge of the bud (**Fig. 1**; the posterior edge is that nearest the tail end of the embryo). Signaling by the polarizing region controls antero-posterior limb pattern (reviewed in **refs.** ***1*** and ***5***). Cells of the polarizing region of chick wing buds cannot be distinguished morphologically, but have a remarkable effect on the pattern when grafted to the anterior margin of a second wing bud. The normal chick wing has three digits arranged from anterior to posterior 2 3 4 (**Fig. 2**). The limb develops six digits following a polarizing region graft, and these are arranged in the mirror-image symmetrical pattern 4 3 2 2 3 4 (**Fig. 2**). The additional digits arise from anterior mesenchyme in response to a signal from the graft. Tissues from different regions of the limb bud and from other regions of embryos have been assayed for polarizing activity. This has led to construction of maps showing distribution of cells with polarizing activity or potential polarizing activity (***9–12***; **Table 1**).

The extent of digit duplication depends on both the number of polarizing region cells in the graft and the length of time that the graft is left in place (reviewed in **ref.** ***5***). With small numbers of polarizing region cells (around 30),only an additional 2 are produced giving the pattern 2234; with more polarizing region cells (60 cells) an additional digit 3 is formed (patterns, such as 32234, 3234, 334) and about 120 cells are required to give an additional digit 4 (**ref.** ***21***). Signaling by the polarizing region can be attenuated by X-irradiation and various chemicals. All of these treatments probably effectively reduce the number of signaling cells. When the graft is left in place for short periods of time (14 h) and then removed, only an additional digit 2 is formed, but when the graft is left for longer, then the formation of more posterior digits, such as digit 3,

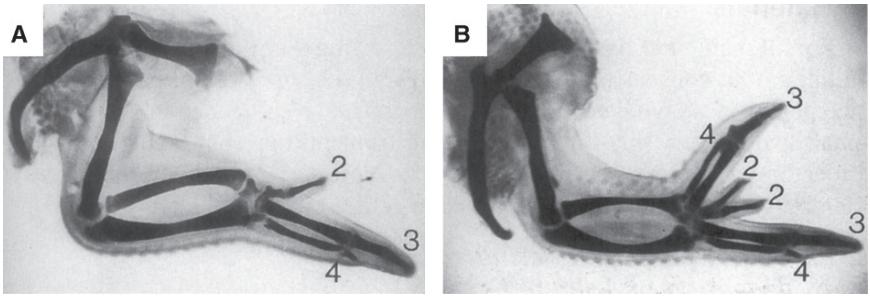

Fig. 2. **(A)** Normal chick wing at 10 d development stained with alcian green to show skeleton laid down in cartilage. Digit pattern 234. **(B)** Chick wing with mirror-image duplication following a polarizing region graft to anterior margin. Digit pattern 432234.

Table 1
Tissues with Polarizing Activity

Posterior margin of limb bud, chicken, quail, mouse, snapping turtle, human ***(1,2,12)***
Hensen's node, chicken, mouse ***(13,14)***
Presumptive flank tissue, chicken ***(11,15)***
Floor plate of neural tube, chicken, mouse ***(14,16)***
Amnion, chicken ***(17)***
Mesonephros, chicken ***(1)***
Tail bud mesenchyme, chicken ***(18)***
Genital tubercle, mouse ***(19)***
Gut epithelium, chick ***(20)***

results ***(22)***. Polarizing signaling can be reproduced by beads soaked in either retinoic acid or sonic hedgehog protein ***(23,24)***. Many of the tissues that have polarizing activity have been shown to be able to generate retinoic acid and to express *sonic hedgehog* (reviewed in **ref. *5***).

Limb duplications can be obtained when just the mesenchyme cells from the limb posterior margin are grafted without the normally associated ectoderm and/or apical ridge, provided that grafts are placed in contact with the apical ridge of the host limb. In the normal limb bud, apical ridge and ectoderm maintain polarizing activity of posterior mesenchyme ***(25)***. *Sonic hedgehog* transcripts are found in posterior mesenchyme, and the distribution of these transcripts closely follows maps of polarizing activity in developing limbs ***(26)***. The posterior part of the apical ridge expresses *Fgf-4*, and FGF-4 maintains *sonic hedgehog* expression in posterior mesenchyme ***(27)***.

2. Materials

For all manipulations, a good set of dissecting instruments is required. The minimum kit consists of: a pair of small fine scissors, a blunt pair of forceps, two pairs of watchmaker forceps (nos. 4 or 5), two sharpened tunsten needles, small spatula, very small spatula. Other instruments that could be useful include iridectomy scissors and a small spoon (*see* **Note 1**). Also needed are a series of Hamburger/Hamilton stages (reprinted in **ref.** ***28***).

2.1. Separation of Ectoderm/Mesoderm and Dissection of Apical Ridge

1. Sterile phosphate-buffered saline (PBS).
2. Sterile ice-cold tissue-culture medium containing serum (for example, Minimal Essential medium + Hank's salts buffered with HEPES + antibiotics + fetal calf serum).
3. Sterile ice-cold 2% trypsin. (Recently, we have been using crude type II from porcine pancreas made up in calcium-magnesium free saline; pH to 7.5) store in aliquots at −20°C.
4. Ice-cold slab (a freezer block covered with black plastic will do).
5. Sterile pipets for transferring tissue.

2.2. Apical Ridge Grafts and Ectoderm-Mesenchyme Recombinations

1. Platinum wire pins. (These can be made by cutting short lengths of platinum wire [0.025 µm in diameter; Goodfellow metals] under 70% alcohol in a Petri dish and bending one end into a hook. The pins are then washed in tissue culture medium to rinse away the alcohol before use.)

2.3. Polarizing Region Grafts

1. Sterile trypsin (*see* **Subheading 2.1., item 3**).
2. Sterile ice-cold medium (*see* **Subheading 2.1., item 2**).
3. Ice-cold slab.
4. Sterile pipets or gilson pipet tips.

3. Methods

3.1. Separation of Ectoderm and Mesoderm Components of Limb Buds (Fig. 3A)

This can be accomplished for limb buds over a range of developmental stages from stage 19 to 29 ***(29)***.

1. Remove chicken embryo from egg through a window made in the shell by cutting a circle into the yolk through the vitelline and chorionic membranes around the embryo, and then lift out embryo on a small spatula.
2. Place embryo in Petri dish containing PBS or tissue-culture medium (that equilibrates with air), and pull away the membranes surrounding embryo with a fine pair of forceps.

3. Remove limb buds from embryo using fine forceps to pinch through tissue where buds attach to body wall.
4. Prick ectoderm of limb buds with fine needles, so that trypsin will penetrate easily to bud tip.
5. Place limb buds in ice-cold 2% trypsin, and leave on ice for between 30 min and 1 h (precise length of time will depend on size of limb bud and batch of trypsin; **ref. *30***).
6. Transfer limb buds to ice-cold tissue-culture medium containing serum and leave on ice for 5 min. (Serum contains trypsin inhibitor and will "stop" trypsin)
7. Place Petri dish containing limbs on a cold slab, and ease loosened ectoderm from mesenchyme by inserting a needle between the tissues and working from cut edge of ectoderm. It should be possible to remove the ectoderm, which looks like a diaphanous mitten, with attached basement membrane (***31***).

3.2. Dissection of Apical Ridge and Grafting to Limb Bud (Fig. 3A)

1–3. As in **Subheading 3.1.** (*see* **Note 3**).

4. Dissect apical ridge of the limb bud away from underlying mesenchyme using fine needles.
5. Place apical ridge with small amount of adherent mesenchyme into ice-cold trypsin (*see* separation section) for 5–10 min. This should be sufficent time to loosen tissues.
6. Transfer tissue to ice-cold tissue-culture medium containing serum and peel apical ridge away from mesenchyme in ice-cold tissue-culture medium.
7. To graft apical ridge to the dorsal surface of a wing bud, cut a shallow slit running from anterior to posterior in the dorsal surface near the tip of right wing bud (good stages are 20–21) with a sharp needle.
8. Transfer isolated ridge into host egg, maneuver over slit, and poke into slit with a blunt needle.

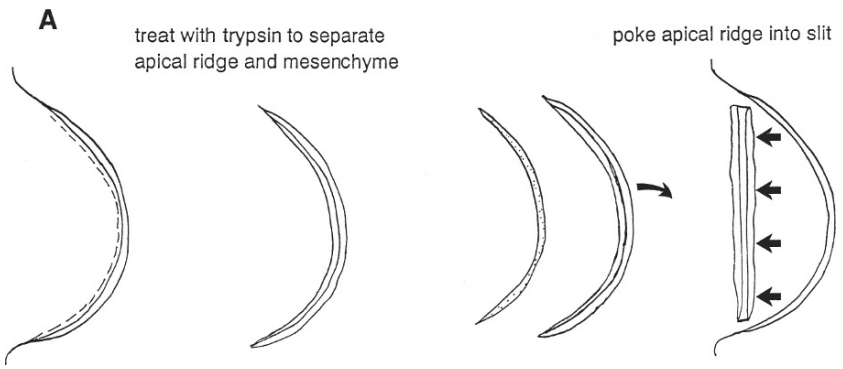

Fig. 3. (**A**) (**Subheading 3.2.**) Diagrams to show how an apical ridge is isolated and grafted to the dorsal surface of a limb bud. Remove thin sliver of donor limb bud tip, and soak in 2% trypsin at 4°C for 5–10 min. Separate apical ectodermal ridge, and keep in ice-cold medium. Graft ridge to slit made in dorsal surface of host limb bud, and poke into place with blunt needle.

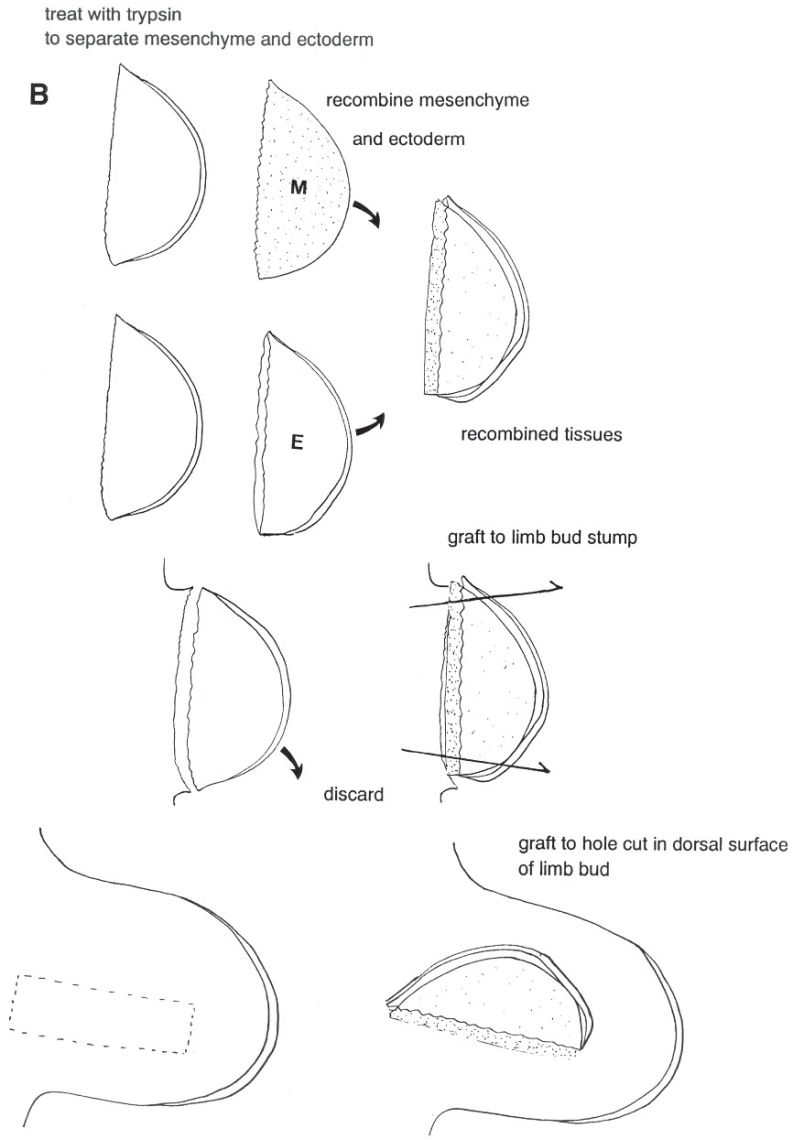

Fig. 3. *(continued)* **(B)** Diagrams to illustrate how mesenchymal and ectodermal components of limb buds are separated, recombined and grafted to allow further development (**Subheadings 3.1. and 3.3.**). Separate ectoderm and mesenchyme of chick limb buds by soaking in 2% trypsin for 1 h at 4°C and then placing in tissue culture containing serum. Recombine mesenchymal core (M) from one limb bud with ectodermal hull (E) from another bud. Allow tissues to reanneal at 37°C for 1 h, and then graft recombined tissues either to wing bud stump and pin in place or to dorsal surface of older wing bud.

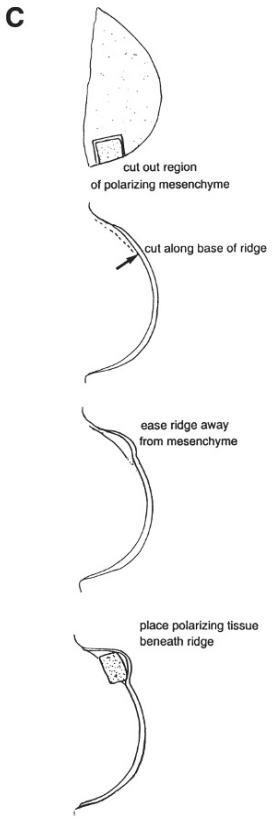

Fig. 3. *(continued)* **(C)** (**Subheading 3.4.**) Diagram to show how a polarizing region is grafted beneath the apical ridge at the anterior margin of a chick limb bud. Cube of posterior mesenchyme cut from donor limb bud. Site for graft prepared in host limb bud by cutting along base of apical ridge, starting at arrow, and then easing ectoderm away from mesenchyme. Cube of posterior mesenchyme placed beneath anterior loop of apical ridge.

3.3. Apical Ridge Recombinations with Limb Mesenchyme (Fig. 3B)

1. First remove the ectoderm from a limb bud that will be used to provide the mesenchyme core, and remove this ectoderm from the dish.
2. Now transfer limb bud that will provide the ectodermal jacket for the recombination into the dish containing the isolated mesenchyme core.
3. Separate ectodermal jacket, and immediately push mesenchymal core inside. **Steps 1–3** are to be carried out in ice-cold medium on a cold slab.
4. Take the dish with recombined tissues off cold slab, and leave at room temperature for 10 min. Then place at 38°C for about 1 h to allow the ectodermal jacket to shrink on to the mesenchyme.
5. Graft recombined tissues to prepared site in a new embryo to continue development.

a. Pin to limb bud stump. The embryo normally lies on its left side, so that dorsal surfaces of right limbs are uppermost. It is therefore easy to manipulate the right limb bud and leave the left as a control. Cut off right wing bud to give a stump (stages 20–22 would be suitable). Pin recombined tissues to stump. It is best to push one pin through one edge of the tissue recombination in a dish so that the hooked end marks the ectoderm surface and the pointed end without a hook protrudes from the naked mesenchyme surface. Then transfer speared tissue into host egg and pin tissue loosely to stump, so that the bare mesenchyme surface abutts the mesenchyme of the stump. Then take a second pin and secure other edge of graft. (Pins can be removed the next day.)
b. Place recombination in trench on dorsal surface of wing bud. Cut a square away from the tip on dorsal surface of right wing bud (developmental stages 22–24). Lift up ectoderm and a layer of mesenchyme to create a shallow trench, and remove tissue from the egg on a small spatula. (The bleeding will soon stop.) Transfer tissue recombination into host egg and stand tissue recombination in trench with mesenchyme surface downward.

3.4. Polarizing Region Grafts (Fig. 3C) (see Note 4)

1. Remove chick embryo from egg, and place in medium (as in **Subheading 3.1.**).
2. Treat chick limb buds (stages 19–21) with cold trypsin and remove ectoderm.
3. Polarizing region in chick wing buds has been mapped from stages 17–29, and with reference to these maps of polarizing activity ***(9–11),*** cut appropriate region out of mesenchymal core of limb bud with sharp needles. A piece of tissue about $200 \times 200\,\mu m^2$ from the thickness of the limb bud is easy to handle, although smaller pieces can be used.
4. Prepare host embryo by peeling away vitelline membranes and amnion over right wing bud. (Host embryos can be used between stages 18 and 24 but graft must be placed in contact with the ridge; to obtain complete digit duplications do not use stages later than 20/21; **ref.** ***32***).
5. Make site for graft by cutting along base of apical ridge over anterior margin of right wing bud with sharpened needle. First make a series a short superficial cuts through the dorsal ectoderm and into the underlying mesenchyme. The cuts are made just below the junction between the transparent ridge and opaque mesenchyme working from the bud apex toward the anterior edge of the bud (**Fig. 3C**). Then push needle through to the ventral side of wing bud, and repeat process working along slit. Pull loosened apical ridge away from mesenchyme to make a loop. The apical ridge can be stretched, but it will gradually shrink back against the mesenchyme.
6. Transfer polarizing tissue graft into host egg using either a glass or gilson pipet or a very small spatula.
7. Maneuver graft under apical ridge loop using the end of a needle. The apical ridge will hold graft in place.
8. Seal egg with sellotape.
9. It is often useful to cut a small hole in the sellotape window the next day, so that graft and limb bud can be inspected. It is usually possible to see whether the graft has remained in place.

4. Notes

1. Dissecting instruments need to be kept clean to guard against infection. Wipe instruments before use and before putting away with 70% alcohol. Another useful tip that can cut down on infection, as well as preventing the embryo from drying under the bright lights used to illuminate it, is to add a small drop (up to 10 µL) of medium containing antibiotics to the embryo during or after the operation.
2. For separation of mesenchyme and ectoderm, it is very important that all solutions and the tissue be kept very cold. Otherwise the tissue can become sticky. In early days of tissue disaggregation, the sticky gel associated with cells following trypsin treatment was thought to be glue that held cells together—later it was shown that this glue could be digested by DNase! Batches of trypsin may vary so it is wise to test out new batches in a trial run.
3. Until one gets used to looking at the embryo in the egg, it can be difficult to make out the apical ridge and to find tissue/cells that have been transferred into the egg. One way of increasing contrast is to use a green filter in the light path, but this reduces the light intensity. It is also possible to use a very weak solution of a vital dye, such as nile blue sulfate. Add a few drops of 0.1–0.2% nile blue sulfate in PBS to the embryo in the egg, or place tissue in a similar solution for a few seconds so that it is stained a very light blue. Be aware that nile blue sulfate at high concentrations can be toxic. We have found that if polarizing regions are stained, their signaling ability is reduced in parallel with the deepness of the shade of blue.
4. For polarizing region grafts to work most effectively, they must be placed in contact with the apical ridge. It is therefore important that the apical ridge is separated as cleanly as possible from the underlying mesenchyme. If the wing bud bleeds while you are lifting the ridge, you are cutting too far away from the apical ridge and into the marginal sinus that runs about 100 µm away from the chick wing ectoderm ***(33)***.

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23

Tissue Recombinations in Collagen Gels

Marysia Placzek

1. Introduction

The characterization of molecular and antigenic markers of cells in many vertebrate tissues has increased dramatically in recent years, allowing cell differentiation and organogenesis to be followed *in vivo* in a way that was unprecedented even two decades ago. In-vitro systems that allow tissues and organs to be isolated and cultured can be used to great effect in parallel with in-vivo studies, to dissect the tissue interactions, signaling and transcription factors that direct tissue patterning, cell differentiation, and organogenesis. Three-dimensional collagen gels provide an excellent in-vitro culture environment. First, they provide a scaffold that supports tissue architecture, a requirement vital for proper development of any three-dimensional tissue or organ system. Second, they stabilize diffusible gradients. Initially developed as a means to culture embryonic neuronal tissue and examine the effect of trophic factors ***(1)***, such gels were then initially used to examine inductive ***(2–4)***, chemotropic ***(5–9)***, and chemorepulsive ***(10)*** interactions. More recently, however, the value of such gels has been more widely appreciated, and they have been powerfully deployed in investigating a wide range of processes, including organogenesis ***(11)***, branching morphogenesis ***(12)***, tissue engineering ***(13)***, and invasion systems ***(14)***. When used in conjunction with in-vivo assays, results obtained from such three-dimensional in-vitro assays are especially compelling and can be used to extend and evaluate rapidly an observation made initially in an in-vivo bioassay.

The ability to assay specific tissues in isolation has many advantages. Large numbers of experiments can be set up in a single day, and the effects of tissues and proteins can be examined in the absence of other tissue that would normally be adjacent and potentially interfering *in vivo*. Cell-autonomous versus non-cell-autonomous effects can easily be distinguished. The ability to dissect,

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interfere with, and assay tissues at a particular time provides the opportunity to effectively perform "conditional" experiments. The isolation and culture of appropriate pieces of tissue enables one to understand the mechanism underlying an observed effect; thus, for instance, one can distinguish trophic from tropic interactions and inductive vs. migratory effects in a manner that is impossible in an in-vivo assay.

Although other types of material, such as fibrin clots or matrigel, can be used in place of collagen, the collagen matrix is less costly and is easy to manipulate. The collagen, which is prepared and maintained under conditions of low pH and low temperature, begins to gel as the temperature and the pH are increased. These two paramenters can be altered to establish optimal conditions in which the collagen sets sufficiently slowly to allow tissues to be manipulated within it. Matrices such as agar or agarose cannot be used as substitutes for collagen: The high density of such gels appears to suffocate the explanted tissue, whereas collagen fibrils are sufficiently spaced to allow the diffusion of nutrients from the culture fluid through the collagen gel to reach the cultured tissue.

The protocol presented here describes how to prepare collagen suitable for culturing embryonic and postnatal tissues and how to process it to make a three-dimensional gel in which tissue explants can be manipulated and embedded. The techniques used to isolate and embed tissue are described, as are the ways in which growth and differentiation factors can be presented to tissue explants within these gels. In addition, a summary is provided of the manner in which such cultures can be manipulated to process by immunohistochemical or in-situ hybridization techniques to examine cell differentiation within tissue explants.

2. Materials

All solutions are made using molecular-biology-grade reagents and sterile distilled water.

2.1. Tissue Dissection

1. Liebovitz L15-air (Gibco-BRL) (***1***).
2. Dispase (Grade 1, Boehringer-Mannheim, Mannheim, Germany), 1 mg/mL in L15-air, made fresh.
3. Serum.

2.2. Collagen Preparation (2)

1. Hemostats and scissors, sterilized with ethanol (***3***).
2. Four to six adolescent rat tails.
3. Glacial acetic acid.
4. 0.1×DMEM (Gibco-BRL), pH 4.0 (without bicarbonate, with phenol red, filtered, sterile).
5. Dialysis tubing, sterile.

2.3. Tissue Embedding in Three-Dimensional Collagen Gel

1. Collagen.
2. 10× DMEM (without bicarbonate, with phenol red, filtered, sterile).
3. 0.8 M $NaHCO_3$, sterile.
4. Four-well (16-mm) multidishes (Nunc cat. no. 134673).

2.4. Assaying Growth and Differentiation Factors

1. LipofectAMINE (Gibco-BRL).
2. DMEM with fetal calf serum (FCS).

2.5. Immunohistochemical or In-Situ Analysis of Cultures

1. 0.2 M Phosphate buffer, pH 7.4 (PB): 6 g/L NaH_2PO_4, 21.8 g/L Na_2HPO_4.
2. 4% Paraformaldehyde in 0.12 M PB, pH 7.4: Heat 20 mL H_2O to 80°C. Add 2 g paraformaldehyde, invert, and add two to three drops 1 M NaOH. Invert or stir until solution clears. Make up to 50 mL with 0.2 M PB and filter.
3. 30% sucrose in 0.1 M PB.
4. OCT (TissueTek) and gelatin-subbed slides or Superfrost Plus slides (Fisher).
5. Glycergel (DAKO).

3. Methods

3.1. Tissue Dissection

Tissues from mouse, rat, and chick embryos have been cultured successfully in three-dimensional collagen gels.

1. Dissect embryos from the decidua or chick embryos from the eggshell into ice-cold L15 air (**Note 1**). Dissect embryos away from membranes and adhering yolk and assess developmental stage.
2. To isolate a specific portion of tissue, first dissect out that tissue surrounded by adjacent tissues, using either electrolytically sharpened tungsten needles or small Vannas scissors. Treatment with an enzyme such as Dispase, frequently is necessary to free the required explant from adjacent tissues. Place the tissue in a small volume (approximately 1 mL) of Dispase at room temperature for 5–15 min. Once the reaction has occurred (**Note 4**), remove tissues into cold L15 air (**Note 5**) with a drop of serum included (**Note 6**), and allow to rest on ice for approximately 15–30 min before beginning to dissect out the appropriate portion of tissue using tungsten needles. Collect tissues into L15 air with a drop of serum, and keep on ice.

3.2. Collagen Preparation

1. Collect tails from adolescent rats and rinse in 95% ethanol. Keep frozen or use fresh.
2. Sterilize frozen tails by rinsing in 95% ethanol, and remove the tendons as follows: Clamp the tail 2 cm from its distal end using a hemostat and clamp the second hemostat immediately adjacent and proximal. Fracture the tail by bending it sharply with the hemostats; the distal portion of the tail will now

be held only by the tendons. Slide this piece of tail off the tendons slowly. Cut the pieces of tendon that dangle from the remainder of the tail, so that they drop into a sterile petri dish. Repeat, working up the tail, until the tendons are completely removed.

3. Wash tendons three times with sterile water. Tease apart using blunt #5 forceps, so they form a fine mesh of fibers. Collect 3 g of wet tendons.
4. Dissolve for 24–36 h, but no longer (**Note 7**), in 300 mL of 3% (v/v) glacial acetic acid at 4°C in a 200 mL sterile conical flask, stirring as slowly as possible (**Note 7**) (note that the stir bar will stop stirring as the solution becomes viscous; check a few times during first few hours).
5. When most of the tendons have dissolved, spin at 20,000 *g* for 60 min to pellet the remainder.
6. Transfer supernatant to dialysis tubing (boiled in EDTA and washed extensively in sterile water), and dialyze at 4°C against 3 L of sterile 0.1× DMEM, pH 4.0, without bicarbonate. Dialyze for three days, changing the medium once a day. Dialysis removes excess acid while keeping the pH low (the collagen gels when alkalinized).
7. Store in 15–50-mL aliquots at 4°C. Do not freeze. Keeps at least 6–12 mo.

3.3. Tissue Embedding in Three-Dimensional Collagen Gel

Explants are transferred to a base of gelled collagen, then overlaid with a cover of gelling collagen. Explants are manipulated and positioned appropriately as the top cushion sets.

1. Prepare a desired volume (100–500 µL) of 90% collagen and 10% of 10× DMEM (**Note 8**) in an Eppendorf tube. Vortex. Add enough 0.8 M $NaHCO_3$ to make the solution turn straw yellow after vortexing. For each batch of collagen, the $NaHCO_3$ must be titered: Typically 0.7–1.2 µL $NaHCO_3$ is added to 100 µL of collagen and 10× DMEM (**Note 9**). Collagen can be used within 10–15 min if kept on ice. At room temperature, it will start to set within minutes.
2. In a four-well multidish (**Note 10**), prepare a collagen cushion by pipeting 20–25 µL of collagen mix onto the bottom of the dish. Stir around to spread and flatten the drop (**Note 11**), and let it set at room temperature for 20 min.
3. Transfer explant(s) from the medium onto the cushion, using either a Pasteur pipet or a fine-drawn capillary tube with a mouth attachment (**Note 12**).
4. Remove excess medium with a fine-drawn 25-µL Drummond microdispenser capillary tube with a mouth attachment. Leave on a tiny amount of medium so that the explants do not dry out.
5. Overlay with 25–30 µL of gelling collagen (**Note 13**) prepared in the same way as the bottom cushion, position explants using an electrophoretically sharpened tungsten needle (**Note 14**) (with blunted tip), and allow to set for 30–60 min at room temperature (*see* **Fig. 1**). Cover collagen moulds with approximately 0.4–0.5 mL medium (**Note 15**), and transfer to incubator for the desired time of incubation (**Note 16**).

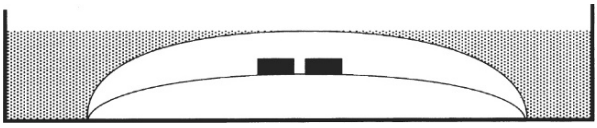

Fig. 1. Schematic diagram of a three-dimensional collagen gel culture. Side view, showing explants positioned at the interface between the collagen bilayer. The explants should lie in the center of the gel and rest on the bottom collagen cushion. The bottom layer of collagen is flat. The top layer of collagen overlays the bottom cusion precisely. Medium is added just to cover the gel.

3.4. Assaying Growth and Differentiation Factors

The effect of growth and differentiation factors on explanted tissue can be assayed in different ways. Factors that are available as purified proteins can be assayed simply by adding them to the culture medium (**Note 17**) or by delivering them to the appropriate beads then implanting the beads within the collagen. Alternatively, if purified protein is not available, but full-length clones encoding a protein are, attempts can be made to produce protein in COS cell aggregates transiently, which are then implanted in the collagen. The technique is as follows:

1. Seed COS-1 cells at approximately 2.5×10^5 cells/35-mm dish on d 1.
2. On d 2, transfect expression vector containing the clone under examination into COS-1 cells, using standard techniques (e.g., lipofection, electroporation, DEAE transfection).
3. On d 3, clump transfected cells. Treat the cell layers with either trypsin or enzyme-free dissociation buffer. Wash the cells twice with DMEM with 10% HIFCS, and resuspend in DMEM with 1% HIFCS (0.25 mL/35-mm dish; **Note 18**).
4. Place 20-µL drops of the cell suspension onto the lids of 60-mm culture dishes, and invert over dishes containing 5 mL of DMEM. Incubate the hanging drop cultures for 14–16 h.
5. Harvest cell aggregates into L15-air medium, and trim as required with tungsten needles for use in explant culture.

3.5. Analysis of Cultures

Subsequent to culture, the patterns of cell differentiation within explants can be examined either by immunohistochemical or in-situ hybridization techniques (**Fig. 2**). In either case, explants can be sectioned directly in the gel then analyzed or processed for whole-mount labeling. The decision whether to section or process for whole-mount labeling is largely probe dependent. With a strong probe or antibody, whole-mount labeling techniques work well (**Fig. 2C**). With a weaker probe or antibody, sectioning is more appropriate (**Fig. 2D**).

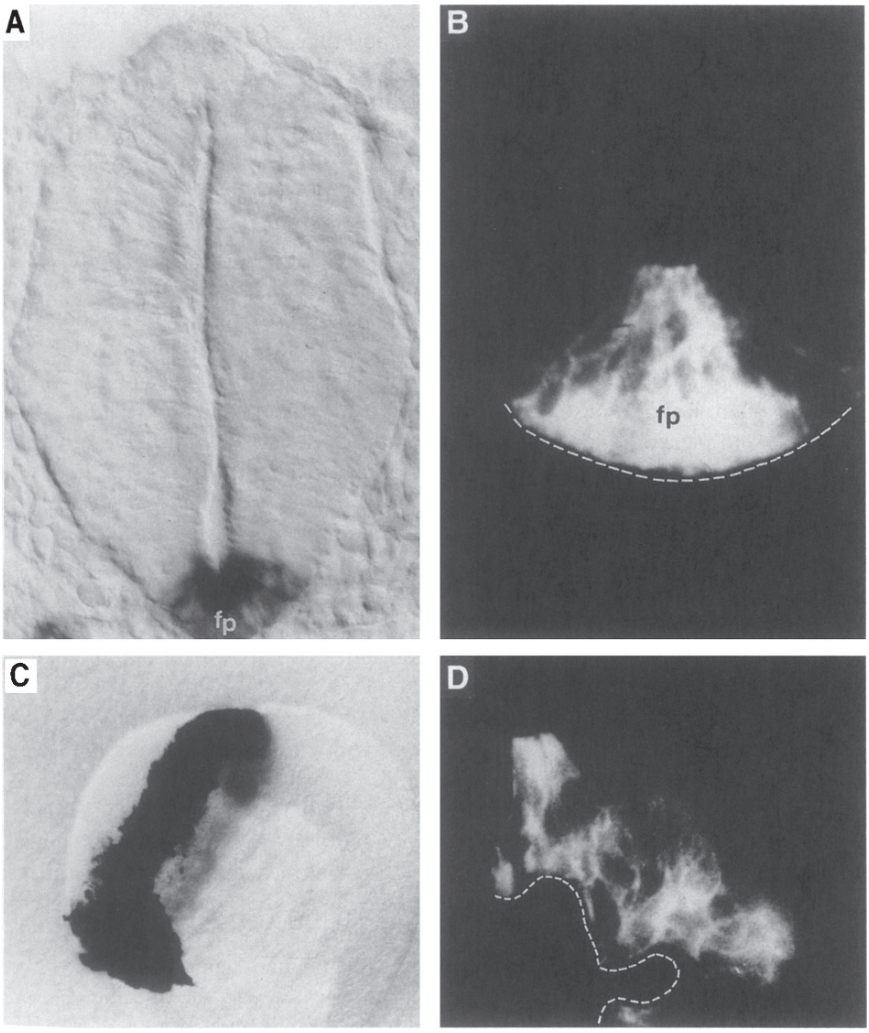

Fig. 2. Analysis of cell differentiation within neural explants cultured in three-dimensional collagen gels. **(A,B)** Transverse sections through an embryonic rat, showing expression of sonic hedgehog mRNA (A) and expression of the FP3 antigen (B) by floor plate (fp) cells of the spinal cord. **(C,D)** Floor plate cells that differentiate within explanted neural tissue in collagen gels can be detected through *in situ* hybridization techniques (C) or immunolabeling techniques (D). (C) Shows a neural explant that has been cultured and processed by *in situ* hybridization with antisense probe to sonic hedgehog. Floor plate cells that express the gene extend in a stripe within the explanted neural tissue. (D) Shows a section through a cultured rat neural explant, labeled to detect FP3. The dotted line in **(B)** marks the edge of the spinal cord, and in (D), marks the edge of the neural tissue.

3.5.1. Immunohistochemical Analysis of Cultures

3.5.1.1. Cryostat Sectioning Explants for Immunohistochemical Analysis

1. Remove culture medium, and replace with 0.12 M PB containing 4% paraformaldehyde, so that explants are immersed in the fixative. Leave at 4°C for 2 h, then replace fixative with 30% sucrose in 0.1 M phosphate buffer for 24–48 h at 4°C.
2. Using blunt forceps, tease around the edges of the collagen bilayer containing the explant until it lifts off the base of the four-well dish. Transfer onto a glass plate. Trim the gel, so that the explant is contained in a square of collagen, approximately 0.6 cm^2. Freeze this in OCT (**Note 19**) (TissueTek).
3. Collect 12–20-µm frozen sections (**Note 20**) onto gelatin-subbed slides or Superfrost Plus slides, and process according to standard techniques.

3.5.1.2. Whole-Mount Immunohistochemical Analysis of Explant Cultures

1. Remove culture medium, and replace with 0.12 M PB containing 4% paraformaldehyde, so that explants are immersed in the fixative. Leave at 4°C for 2 h.
2. Using blunt forceps, etch around the edges of the collagen containing the explant until it lifts off the base of the four-well dish and transfer to an Eppendorf tube. Process according to standard whole-mount techniques.

3.5.2. In-Situ Hybridization of Cultures

3.5.2.1. Sectioning Explants for In-Situ Hybridization

1. Remove culture medium, and replace with 0.12 M PB containing 4% paraformaldehyde so that explants are immersed in the fixative. Leave at 4°C for 2 h.
2. Transfer explants in collagen to 30% sucrose in 0.1 M PB, leave at 4°C overnight, embed in OCT, and process according to standard techniques.

3.5.2.2. Whole-Mount In-Situ Analysis of Explant Cultures

1. Remove culture medium, and replace with 0.12 M PB containing 4% paraformaldehyde so that explants are immersed in the fixative. Leave at 4°C for 2 h.
2. Using blunt forceps, tease the collagen containing the explant off the base of the four-well dish, transfer to a sterile Eppendorf tube, then process according to standard techniques (**Note 21**).
3. Explants can subsequently be viewed directly as whole mounts or be sectioned. If sectioning, refix in 4% paraformaldehyde 1–2 h at 4°C, then transfer to 30% sucrose, and process as described previously. An optimal signal is maintained on thicker sections (20 µm). Mount sections in Glycergel.

4. Notes

1. Although dissections can be performed in PBS, tissues isolated from very young embryos survive far better if dissected into an air-buffered culture medium, such as L15 air. It is of utmost importance to keep tissue ice-cold at all stages (with the exception of the enzymatic treatment step) prior to positioning on the collagen

bed. Rat or mouse embryos should be removed from the decidua immediately and placed on ice. Never allow embryos or explanted tissue to remain off ice for more than approximately 5–10 min. If working to isolate a specific piece of tissue from an embryo, all the other embryos from the litter should remain on ice. When dissecting small portions of tissue, relax and remember to breathe.

2. Collagen is also available commercially (e.g., Vitrogen). However, the gelling properties of collagen isolated from rat tails are somewhat better than those of commercially obtained samples. Furthermore, the currently tested whole-mount in-situ hybridization techniques do not appear to work well on commercially obtained collagen.
3. Collagen cannot be sterilized once made. It is therefore critical to prepare it under sterile conditions. Swab instruments well with ethanol, and during the isolation of the collagen, work in an air-filter hood.
4. The optimal time needed for Dispase to work varies according to the age and tissue and is best assessed empirically. As a rule, the Dispase can be judged to have been effective when sheets of tissue, such as mesoderm and ectoderm, begin to separate. Tissues can be microdissected easily after an acute treatment with Dispase. Therefore, material can be more easily dissected after a 5-min incubation in Dispase at 1 mg/mL than after a 10-min incubation at 0.5 mg/mL. Tissues become difficult to dissect after prolonged exposure to the enzyme and die if overexposed. Do not place too many explanted tissues together in a small volume of Dispase: They will become intertwined, sticky, and difficult to separate. Dispase is made up fresh, but the same batch can be used throughout the day.
5. Take care when transferring the tissues from Dispase to cold L15 air: The tissues coming out of Dispase are relatively warm, that is, room temperature, and may rise to the top of the colder, denser L15 air and burst at the meniscus. This can be avoided if the tissues are transferred slowly and into a deep amount of L15 air. Small tissues are especially fragile after being incubated in Dispase and must be transferred with care. To prevent the tissue explants sticking to the transfer pipet, use a glass Pasteur pipet or a pulled glass capillary (*see* **Note 12**), and do not draw the tissue explants too far up the pipet. Siliconize the glass pipets if necessary. Always use the microscope to visualize the tissues as they are transferred between vials.
6. Treatment with Dispase results in the tissue becoming sticky, as cells begin to break down. Further dissection is facilitated by the inclusion of a small quantity of serum into medium to which tissue is transfered (1 drop in 5 mL). Serum inhibits further action of the Dispase. In addition, nonspecific binding by the serum to the tissue results in it becoming less sticky.
7. If stirred too vigorously or too long, tendon proteins other than collagen start dissolving.
8. The purpose of the 10× DMEM is twofold. First, it provides a nutritious medium, enhancing the condition of the explanted tissue. Second, it serves as a color indicator, facilitating titration of the bicarbonate: The solution will turn pink if too much bicarbonate is added.
9. If insufficient bicarbonate is added, the collagen will not set; if too much is added, it will set too quickly and unevenly.

10. The four-well dishes are convenient to use; and in these wells, gels can be covered with as little as 0.4 mL of medium (important for testing growth factors and conditioned medium).
11. The beds must be flat, since explants tend to slide off a convex bed.
12. Fragile tissues can be manipulated far better using a pulled capillary tube than a Pasteur pipet. When picking up and transferring small amounts of tissue, always work through the stereo–dissecting microscope. When transferring tissue in a pulled capillary tube, allow the tissue to enter the tube by gentle sucking, then remove the mouth attachment from the mouth. The tissue remains in position. Otherwise, tissues are frequently either expelled or swallowed as the dissector suddenly remembers to breathe. To place the tissue on the collagen bed, it should be sufficient to touch down the capillary tip onto the collagen bed. Otherwise, place the mouth attachment back in mouth and expel very gently. Do not blow hard—the tissue will fragment. Sufficient medium should be transferred with the explant(s), so that they do not dry out. However, the medium should stay on the collagen bed: If too much medium is added and flows off the bed, the second layer of collagen also flows off the bed and explants cannot be appropriately positioned.
13. Keep the gelling collagen on ice, so it does not set too quickly. When adding the top layer of collagen, explants often float to the top of the drop. This is minimized if explants are left in a tiny amount of medium and are not allowed to dry out. Nevertheless, even when explants do float to the top of the drop, they can and must be pushed back down onto the collagen and repositioned. With practice,this can be accomplished with the tungsten needle without tearing even fragile tissue. It is important that the tissue comes to lie on the bottom collagen bed, rather than within the top cushion. Also note that the collagen cushion and top collagen become alkaline (pink) while setting. This has never been found to affect experiments, although it is possible that some tissues may be sensitive to the alkalinization. The top layer of collagen should overlap the bottom collagen bed precisely (*see* **Fig. 1**).
14. When establishing collagen gel cultures, first practice manipulating only a single explant within a gel. With practice, many (up to about ten) explants (approximately 100–200 μm^2) can be positioned within a single bed. Explants can be positioned in experimental configurations relative to each other as the collagen sets. For instance, explants can be intertwined if contact is required between them or positioned apart. Manipulation of the explant(s) is facilitated if it lies in the center of the collagen bed.
15. The type of medium used varies according to the explants being cultured. Neural tissue survives well in Opti-MEM (Gibco-BRL), especially if supplemented with small quantities of sera. Serum screening should be performed, since certain batches adversely affect embryonic tissue in an apparently species-dependent fashion. Medium should be added to the top of the collagen, rather than to one side, to prevent the two layers from separating.
16. Neural explants survive well within collagen cultures for at least 5 d. Often, necrosis is observed early on in culture around the cut edges of the explanted tissue.

17. The collagen gels are composed of fibers that are not so dense that they preclude the diffusion of small growth and differentiation factors. Nevertheless, the isolation of collagen is sufficiently crude that the cultures may be contaminated with extracellular matrix components. Although there are no documented examples, it remains possible that these may affect the properties or presentation of growth factors within the cultures.
18. The resuspension volume can vary considerably. In different experiments, the number of cells per hanging drop (20 µL) has varied between 500 and 20,000 ***(4,9)***.
19. To freeze in OCT, place a small amount of OCT on a chuck and place on dry ice. As soon as the OCT begins to freeze, remove the chuck from the dry ice and add a small drop of OCT. Quickly place the collagen in the center of this drop and transfer back to dry ice to freeze. Often, it is unecessary to push the collagen into the OCT, sufficient OCT flows over it by capillary action.
20. If the tissue explants are very small (around 50–200 μm^2), the entire explant is collected in only a few sections. Appropriate care must therefore be taken to ensure every section is collected.
21. The variables that must be considered are the same as those considered for standard in-situ hybridization of embryos: Temperature, probe concentration, and washing stringency must all be optimized. For some probes, better results are obtained if the explants are removed from either one half or all of the collagen after fixation and processed in small vials.

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Quail–Chick Chimeras

Marie-Aimée Teillet, Catherine Ziller, and Nicole M. Le Douarin

1. Introduction

The understanding of several mechanisms that are essential for embryonic development has greatly benefited from cell-marking techniques that allow tracing of definite cells and their progeny, and thus, the study of their behavior and fate. A cell marker must be precise and stable; it must not interfere with normal development. The quail–chick labeling technique meets these requirements perfectly.

The principle of the method ***(1)*** is based on the observation that in all embryonic and adult cells of the quail (*Coturnix coturnix japonica*), the heterochromatin is condensed in one (sometimes two or more, depending on the cell types) large mass(es) associated with the nucleolus, thus making this organelle strongly stained after DNA staining, e.g., the Feulgen and Rossenbeck staining ***(2)***. When quail cells are combined with cells of the chick (*Gallus gallus*) which possess, like most of the animal cells, only small chromocenters dispersed in the nucleoplasm, they are readily recognizable by the structure of their nucleus, which thus provides a permanent natural marker (**Fig. 1A**).

The main purpose of constructing quail–chick chimeras is to follow the fate of definite embryonic territories during development and, in this way, to discover the place and time of origin of the different groups of cells constituting certain organs. The investigations carried out on the neural crest ***(3)*** and the mapping of the neural primordium at different stages (***4,5***) provide good examples of the possible uses of the quail–chick chimera system for studying developmental problems. This type of study implies that the developmental processes unfold in the chimeras as they do in normal embryos. To achieve this, transplantations of quail tissues into chick embryos (or vice versa) are performed *in ovo* and do not consist of adding the grafts to normal embryos, but of removing a given territory

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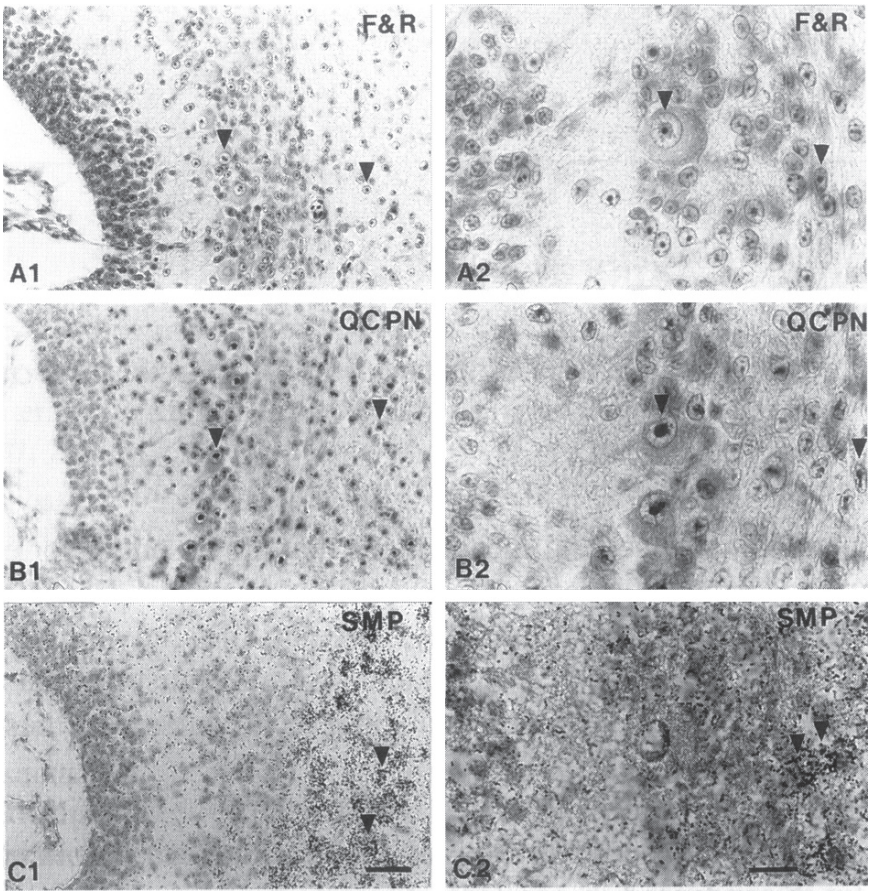

Fig. 1. Quail cells are differentially identified in 5-µm paraffin sections of a chimeric cerebellum (*see* **refs. *16,21,22***). (**A**) With the Feulgen and Rossenbeck staining (F&R), quail neuronal and glial cells show a strongly stained nucleolus (*see* arrowheads), whereas chick cells present almost homogeneously stained nuclei. (**B**) With the QCPN MAb, nucleolus of quail neurons and glial cells are more violently stained, but chick nuclei are not labeled. (**C**) The SMP probe hybridizes specifically with the quail oligodendrocytes. Medial and high magnifications of each staining are presented. A1, B1, C1: bar = 70 µm; A2, B2, C2: bar = 30 µm.

in the host and replacing it as precisely as possible by the equivalent region of the donor, which must be at the same developmental stage. Quail and chick are closely related in taxonomy, although they differ by the duration of their incubation time (17 d for the quail, and 21 d for the chick) and their size at birth (about 10 g for the quail and 30 g for the chick). However, during the first days

of incubation, when most of the important events in embryogenesis take place, the size of the quail and chick embryos and the chronology of their development differ only slightly. Obviously, it is safe to carry out the grafts not only from quail to chick, but also from chick to quail, and to perform control chronological studies in order to discard any bias owing to differential development processes between chick and quail embryos.

Quail–chick chimeras constructed according to the above principles can hatch and survive in good health for certain time; time of survival is limited by the appearance of an immunological reaction developed by the host. Although no immune reaction against the graft takes place during embryogenesis, when the immune system is immature, the transplant triggers its own rejection, which occurs at various times after birth. For strictly neural grafts, a long delay (1–2 mo) is observed between the onset of immune maturity and the rejection ***(6)***. For neural grafts associated with other tissue grafts and for grafts of any nonneural tissue, rejection occurs as soon as maturation of the immune system is achieved ***(7,8)***.

Isochronic and isotopic grafts are not the only type of grafts used to construct quail–chick embryonic chimeras. Certain developmental processes can be studied by heterotopic, and heterochronic grafts with or without previous ablation of tissues. This was instrumental in testing the degree of determination of the neural crest cells and their derivatives ***(3,9)*** and in demonstrating the precise periodicity of the colonization of the primary lymphoid organ rudiments (thymus and bursa of Fabricius) by hemopoietic cells in birds ***(10)***.

For many years, the analysis of the quail–chick chimeras was based on the differential staining of the nucleus by either the Feulgen and Rossenbeck nucleal reaction (**Fig. 1A**) or any other method revealing specifically the DNA profiles in light or electron microscopy. Recently, significant progress was accomplished when species-specific antibodies recognizing either quail or chick cells of one or several types were prepared (*see* **Subheading 3.4.3.** and **Fig. 1B**). Nowadays, several specific quail or chick nuclear probes are also available, allowing, at the single-cell level, specific gene activities to be distinguished (*see* **Subheading 3.4.4.** and **Fig. 1C**).

This chapter will describe the protocols of several representative examples of neural quail–chick chimeras. These particular protocols can be adapted to the graft of any other type of tissue.

2. Materials

2.1. Egg Incubation

1. Fertilized chick and quail eggs (*see* **Note 1**).
2. Humidified and ventilated incubators equipped with time programmers. Time programmers are useful in obtaining very precise stages of development (*see* **Note 2**).
3. Egg holders (*see* **Note 3**).

4. Developmental tables of Hamburger and Hamilton for chick embryo development ***(11)*** and of Zacchei for quail embryo development ***(12)*** (*see* **Note 4**).

2.2. Preparation of Host and Donor Embryos and Grafts

1. 70% Alcohol for sterilization.
2. Disposable syringes (1- or 2-mL) and needles (0.6–0.8 mm in diameter).
3. Transparent Scotch tape (5 cm in width) (*see* **Note 5**).
4. Paraffin 60°C and a thin paint-brush.
5. Physiological liquids: PBS or Tyrode solution.
6. Antibiotics: penicillin and streptomycin (*see* **Note 7**).
7. Proteolytic enzymes (pancreatin) (*see* **Note 7**).
8. Bovine serum (*see* **Note 7**).
9. Pasteur pipets.
10. Glass micropipets hand-drawn from Pasteur pipets and equipped with plastic tubes for mouth use (Tygon) (*see* **Note 8**).
11. Small glass dishes (salières) normal or containing a plastic base for dissections (rhodorsyl, Rhône-Poulenc, France) (*see* **Note 9**) and insect pins.
12. Indian ink (Pelikan for drawing) (*see* **Note 10**).
13. Microscalpels and their holders (*see* **Note 11**).
14. Small curved scissors, iridectomy (Pascheff-Wolff) scissors, and thin forceps (Swiss Dumont no. 5 forceps) (Moria Instruments, Paris).
15. Optical equipment: stereomicroscope equipped with a zoom from ×6 to ×50.
16. Optic fibers for object illumination (*see* **Note 12**).

2.3. Histological Analysis of the Chimeric Embryo Tissues and Organs

1. Carnoy fluid or 4% paraformaldehyde for fixation of the tissues (*see* **Note 13**).
2. Materials for paraffin histology.
3. Reagents for Feulgen and Rossenbeck staining ***(2,13)*** (*see* **Note 14**).
4. Reagents for immunohistochemistry with species-specific and/or cell type-specific antibodies.
5. Reagents for *in situ* hybridization with species-specific nuclear probes.

3. Methods

3.1. Preparation of the Host and Donor Embryos

1. Incubations: Quail and chick eggs are incubated with their long axis horizontal during the time necessary to obtain the stages adequate for the experiments: 36–48 h for the experiments described here (*see* **Note 4**).
2. Environmental conditions of the experiments: The experiments have to be made under relatively sterile conditions. Eggshells are cleaned with 70% alcohol. Instruments are sterilized in a dry oven (1.5 h at 120°C). Sterile physiological liquids are supplemented with antibiotics (10–20 IU/mL). Experiments are performed in a clean separate room, but never under forced-air apparatus (*see* **Notes 6** and **12**).

3. Opening the eggs: The blastoderm develops on the top of the yolk against the shell membrane. Before opening a window in the shell, a small quantity of albumen (about 0.3 mL) is removed at the small end of the egg, using a syringe in order to separate the blastoderm from the shell membrane. Another method consists of perforating the eggshell at the level of the air chamber and then rolling the egg horizontally several times. These manipulations are sufficient to loosen the blastoderm from the shell membrane and allow a window through the shell without damaging the embryo. The small holes through the eggshell are obturated with a piece of tape or a drop of paraffin (*see* **Note 5**).
4. Contrasting the embryos *in ovo*: India ink, diluted 1/1 in PBS or Tyrode supplemented with antibiotics, is injected under the blastoderm (donor or recipient) using a glass micropipet mounted with a plastic tube for mouth use (*see* **Note 10**).
5. Gaining access to the embryos *in ovo*: The vitelline membrane, which covers the embryo, is slitted out with a microscalpel just at the place where the microsurgery will be made.
6. Explanting the donor blastoderms: In certain cases, the donor blastoderm is cut out from the egg with Pascheff-Wolff scissors, washed free of vitellus in PBS or Tyrode supplemented with antibiotics, tranferred onto a dish with a black plastic base, and pinned out before dissection.

3.2. The Grafts

3.2.1. Neural Tube Transplantations (Fig. 2)

Orthotopic transplantations of fragments of neural tube have allowed the construction of a neural crest fate map ***(3)*** and the detection of definite crest cell migration pathways ***(14)***. The rules of this operation are based on the fact that neural crest cells start migrating first in the cephalic region and then progressively from rostral to caudal when neural tube forms. The interspecific graft is performed at a level where crest cells are still inside the apex of the neural tube, i.e., in the neural folds in the cephalic area, at the level of the last formed somites in the cervical and thoracic regions, and at the level of the segmental plate in the lumbo-sacral region. The operation has to be made on the length of no more than five to six somites to take into account the rostro-caudal differential state of evolution of the neural crest. Donor and host embryos are strictly stage-matched (*see* **Note 4**).

1. Excision of the host neural tube: The selected neural tube fragment is excised from the host embryo by microsurgery *in ovo*. A longitudinal slit through the ectoderm and between the neural tube and the adjacent paraxial mesoderm, at the chosen level, is made bilaterally using a microscalpel. The neural tube is then gently separated from the neighboring mesoderm and cut out transversally, rostrally, and caudally without damaging the underlying notochord and endoderm. The fragment of neural tube is then progressively severed from the notochord and finally sucked out using a calibrated glass micropipet (*see* **Note 8**).

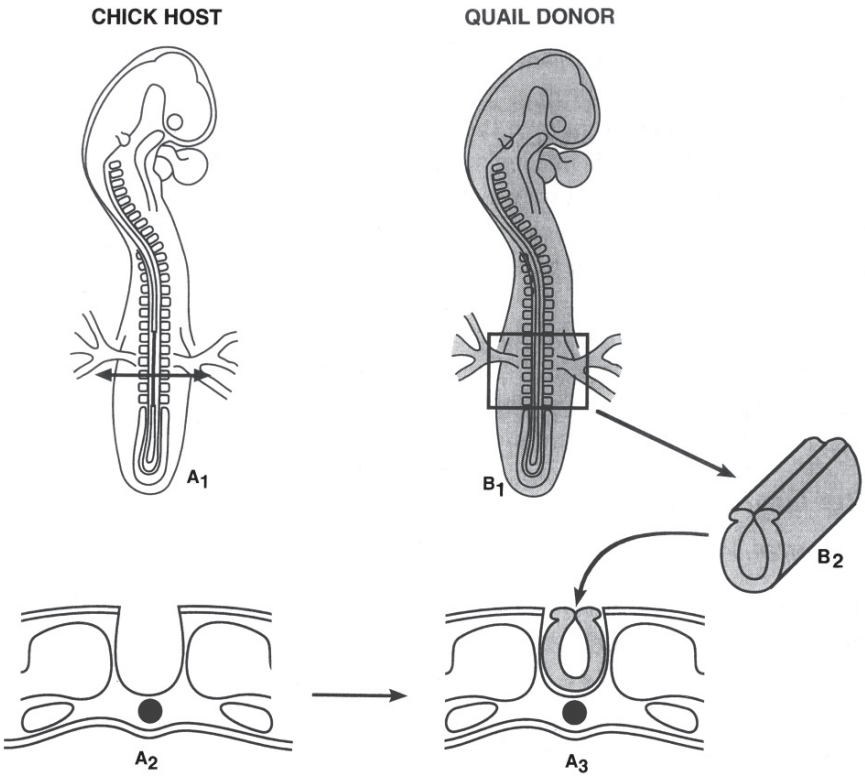

Fig. 2. Scheme of the orthotopic quail/chick neural tube transplantation. A chick embryo *in ovo* (**A1**) is microsurgically deprived of its neural tube (**A2**) at the level of the last formed somites. The corresponding fragment of blastoderm of a quail at the same stage (**B1**) is enzymatically dissociated. The isolated quail neural tube (**B2**) is orthotopically grafted into the chick embryo (**A3**).

2. Preparation of the graft: The transverse region of the stage-matched donor embryo comprising the equivalent fragment of neural tube plus surrounding tissues (ectoderm, endoderm, and mesoderm) is retrieved with iridectomy scissors and subjected *in vitro* to enzymatic digestion (pancreatin, Gibco, one-third in PBS or Tyrode) for 5–10 min on ice or at room temperature according to the stage of the embryo (*see* **Note 7**). Then tissues are dissociated using two smooth microscalpels (*see* **Note 11**), and finally, the isolated neural tube fragment is rinsed with PBS or Tyrode supplemented with bovine serum to inhibit the action of the proteolytic enzymes. It is then ready to be grafted.
3. Grafting procedure: The donor neural tube is transferred to the host embryo using a calibrated glass micropipet and placed in the groove produced by the excision, in the normal rostro-caudal and dorso-ventral orientation (*see* **Note 15**).

Heterotopic graftings were instrumental to study whether the fate of the neural crest is specified when the operation is carried out ***(9)***. The graft is taken at a more rostral or more caudal level than the acceptor level. Depending on the latter, the donor embryo is older or younger than the recipient (*see* **Note 16**).

Partial dorso-ventral orthotopic graftings have also been made in order to localize possible early segregation of precursors in the neural tube ***(15)***.

3.2.2. Orthotopic Transplantations of Brain Vesicles (Fig. 3)

This operation has been devised to label defined regions of the neuroepithelium and thus to study cell migrations and morphogenetic movements during brain development ***(4,16,17)***. It was also applied to the transfer of a genetic behavioral or functional trait from donor to recipient in either xenogeneic or isogeneic combinations ***(8,18,19)***.

1. Excision of brain vesicles from donor and host embryos: Equivalent brain vesicles are excised microsurgically in the same way in stage-matched donors and recipients (*see* **Note 17**). The dorsal ectoderm is slit precisely at the limit between the neural tissue and the cephalic mesenchyme on each side of the selected part of the brain. The neural epithelium is loosened from the cephalic mesenchyme, then cut out transversally at the chosen rostral and caudal levels, and finally severed from the underlying notochord.
2. Exchange of brain vesicles: The transfer of brain vesicles from the quail to the chick and vice versa (or from a mutant to a normal chick embryo) is made using a calibrated glass micropipet. The piece of neural tissue is inserted into the groove made by the excision, with the normal rostro-caudal and dorso-ventral orientation, and then adjusted (*see* **Note 18**).
3. Modifications of the technique consist of orthotopic partial dorsal or dorsolateral grafts of brain vesicles ***(17,20–22)***. Heterotopic grafts have also been performed to study specific problems ***(20,23–25)***.

3.2.3. Neural Fold and Neural Plate Transplantations

Orthotopic and isochronic grafts have been made in order to map the early rostral or caudal neural primordium ***(5,26–29)***.

1. Excision of precise pieces of the neural fold or neural plate in the chick host: Very thin microscalpels (made up from insect pins or steel needles sharpened on an oil stone) are used to excise precise fragments of the folds and neural plate in 0- to 5-somite stage chick embryos *in ovo*. An ocular micrometer is used to measure the pieces of tissue to be removed.
2. Excision of equivalent pieces of tissue from the quail donor: The grafts are excised from stage-matched quail *in vitro* using the same method. They are not subjected to enzymatic treatment.
3. Graft: Pieces of quail tissue are grafted orthotopically into the chick host.

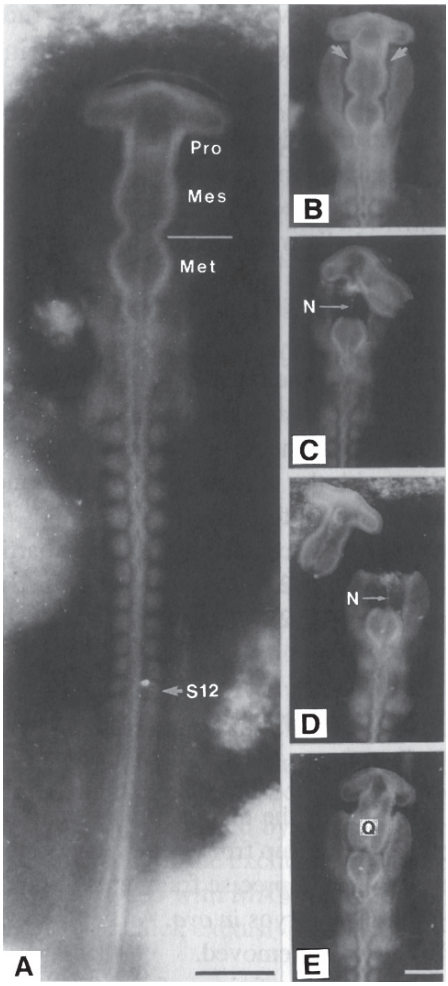

Fig. 3. Scenario of the orthotopic graft of brain vesicles. (**A**) 12-somite chick embryo *in ovo* after injection of a solution of Indian ink under the blastoderm. Brain vesicles are well delineated. (**B**) Longitudinal incisions are made between the cephalic neural tube and the head mesenchyme to delimit the brain excision (arrows). (**C**) After a transversal section at the level of the mesencephalo-metencephalic constriction, the prosencephalon and the mesencephalon are separated from the head mesoderm and endoderm. The notochord (N) is then visible. (**D**) The excised chick brain vesicles are discarded. (**E**) The equivalent quail brain vesicles (Q) are grafted into the chick host. Pro: prosencephalon; Mes: mesencephalon; Met: metencephalon; S12: somite 12. A bar = 0.05 mm; B, C, D, E bar = 0.05 mm.

Heterotopic grafts have also been made to establish the degree of autonomy of precise territories of the rhombencephalon ***(30,31)***.

Simple excisions combined with adjacent orthotopic grafts have been performed in order to identify neural crest cells differentiating at the level of the excision ***(32)***.

3.3. Sealing the Eggs and Postincubation

When the grafting operations are achieved, the window in the eggshell is sealed with a piece of tape and the eggs reincubated in a horizontal stable position (*see* **Notes 5** and **19**).

3.4. Analysis of the Grafts

3.4.1. Fixation of the Experimental Embryos

Host embryos can be fixed from several hours after the operation to several days after hatching according to the experimental design (*see* **Note 20**). Carnoy fluid is one of the best fixatives utilizable at once for Feulgen and Rossenbeck staining ***(13)***, immunohistochemistry, and *in situ* hybridization on paraffin sections. Fixation with 4% paraformadehyde is used for whole-mount immunohistochemistry and *in situ* hybridization, and all treatments on cryostat sections.

3.4.2. Feulgen and Rossenbeck Staining

The Feulgen and Rossenbeck nucleal reaction ***(2,13)*** (*see* **Note 21**) is applied on 5-µm serial sections.

3.4.3. Immunohistochemistry

1. Two antibodies recognize virtually all cell types in the quail and no one in the chick: the polyclonal antibody raised by Lance-Jones and Lagenaur ***(33)*** and the monoclonal antibody (MAb) QCPN prepared by B. M. Carlson and J. A. Carlson, which is available at the Developmental Studies Hybridoma Bank (Department of Biology, University of Iowa, 436BB, Iowa City, IA 52242). The use of QCPN is easy and can be combined with other antibodies like HNK1 ***(34)***, which recognizes neural crest cells or 13F4 ***(35)***, which marks muscle cells and their precursors.
2. Other MAbs are species- and cell type-specific: MB1 and QH1 ***(36,37)***, which recognize a glycosylated epitope carried by surface proteins expressed in quail leukocytes and endothelial cells at the exclusion of any cell type of the chick.
3. Neural chimeras can be analyzed with MAbs that recognize either neuronal cell bodies or neurites of quail or chick exclusively ***(38,39)***.

3.4.4. In Situ *Hybridization*

A growing number of species-specific nucleic probes can be used on sections or whole-mount preparations. As examples, the use of a chick probe of the homeobox gene *goosecoid* has demonstrated the induction of this gene in a chick

in which goosecoid-producing tissues had been grafted ***(40)***. The quail specific SMP (Schwann cell myelin protein) probe ***(41)*** allows quail oligodendrocytes in chimeric spinal cord to be distinguished ***(15)*** (**Fig. 1C**). Chick *Wnt1* and quail *Wnt1* probes have been combined to demonstrate the induction of *Wnt1* in quail–chick chimeras ***(42)***.

4. Notes

1. If possible, select a rapidly growing strain of chickens in which early stages of development will be in phase with the ones of quail embryos. A nonpigmented strain of chickens can be chosen in order to use the heavily pigmented quail melanocytes as a second marker. Freshly laid eggs are stored no more than 1 Wk at 15°C.
2. Ideal conditions for chick and quail incubation are 38°C, 45% humidity (first two-thirds of incubation time), 75% (last one-third of incubation time and hatching). Small incubators equipped with time programmers can be placed in a 15°C room in order to obtain very precise stages of develoment.
3. Multiple wire tongs, individual egg holders, and hollowed-out wooden slats can be used for preincubation, microsurgical experiment, and postincubation, respectivley, of the chicken eggs.
4. The operations described here are performed at embryonic d 2 (E2) when somites, which can be easily counted, can serve to stage the embryos.
5. In order to perturb the gas exchanges at the level of the shell as little as possible, avoid using too large pieces of tape, and stick it carefully against the shell without folds, which would cause air entry and consequently progressive drying of the egg content.
6. PBS and Tyrode solutions supplemented with antibiotics (10–20 IU/mL) directly deposited on the blastoderm are commonly used to humidify it at any time during the experiment.
7. Pancreatin (Gibco) is diluted one-third to one-sixth with PBS or Tyrode solution. In this way, the tissue dissociation can be easily controlled. Titer and temperature are adapted to the stage of development of the tissues. The younger the tissues, the lower the titer and temperature; for example, tissues from 10-somite stage embryos will be treated with 20% pancreatin in Tyrode on ice, whereas tissues from 20-somite stage embryos will be treated with 30% pancreatin at room temperature. Tyrode solution supplemented with bovine serum will serve to stop enzyme action.
8. Glass micropipets hand-drawn from Pasteur pipets are curved and calibrated according to use: injection of liquids or transfer of pieces of tissues. Calibration of the micropipet according to the size of the rudiment to be transplanted (for instance, neural tube vs brain vesicle) is an important requirement.
9. The Rhodorsyl base is now preferred over the paraffin base. It can be either black or perfectly transparent depending on whether animal carbon is added to the commercial preparation. Moreover, it can be sterilized as often as necessary in dry oven and supports insect pins without damage.
10. Indian ink has to be tested for toxicity before use and must be used without excess.
11. Microscalpels have to be perfectly adapted to each use. Microscalpels, manufactured by stropping and honing steel needles (sewing needles) on an Arkansas oil

stone, are the most convenient for excising fragments of neural tube or brain vesicles, because they can be both extremely thin and resistant. For dissociating tissues after enzymatic treatment, they must have a smooth tip. Tungsten microscalpels ***(43)*** or microscalpels made up from insect pins are also useful for dissecting very small pieces of tissues. They are quicker to prepare, but more fragile.

12. Formerly used conventional light bulbs with a condensor tend to radiate heat and cause traumatic drying to the embryos during the surgery, so that defects in amnios formation and subsequent death are often observed.
13. Zenker fluids like Carnoy fluid are good fixatives for Feulgen and Rossenbeck staining ***(13)*** but they do not allow immunohistochemistry or *in situ* hybridization to be made.
14. A modification of the Feulgen and Rossenbeck classical protocol consists of performing the hydrolysis in 5 *N* HCl for 20–30 min at room temperature after Carnoy fixation, instead of 1 *N* HCl for 4–8 min at 60°C as previously recommended in Gabe ***(13)***.
15. Dorso-ventral and rostro-caudal orientations of the graft are recognized either by morphological characters or by various labelings, for instance, a minute precisely localized slit.
16. Differences in stage and caudo-rostral level implicate difference of size. If the fragment of neural tube to be grafted is much bigger than the one that has been removed, it should be ressected before grafting.
17. Transplantations of brain vesicles are made at the 12- to 14-somite stages, which are favorable for the following reasons: Brain vesicles, still uncovered by the amnion, are clearly demarcated by constrictions in the absence of brain curvature; the notochord is no longer strongly adherent to the ventral part of the neural epithelium at this level; the neuroepithelium is not yet vascularized. Some neural crest cells and cephalic mesoderm are transferred along with the brain vesicles. Their presence does not interfere with the development of the brain, and presence of melanocytes in the head feathers of the chimera indicates the level of the brain graft.
18. A good adhesion of the graft to the host tissues is favored by sucking out with a micropipet the excess of physiological liquid added during the operation.
19. Daily gentle manual rocking of the operated eggs can enhance embryo survival. Incubator humidity must grow from 45–75% on d 18 of incubation if hatching of the operated embryos is expected.
20. E3–E4 chimeric embryos are fixed as a whole, for 1–3 h at room temperature. Older embryos have to be fixed as fragments and maintained in vacuum, during a growing time according to the stage and the size of the tissue pieces or organs. The same conditions will be applied for dehydratation and paraffin impregnation of the samples.

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25

Using Fluorescent Dyes for Fate Mapping, Lineage Analysis, and Axon Tracing in the Chick Embryo

Jonathan D. W. Clarke

1. Introduction

This chapter deals largely with the use of fluorescent dyes in the investigation of the development of the chick embryo. It covers three issues; generating fate maps, lineage (or clonal) analysis from single-cell injections, and axonal tracing techniques to uncover the neuronal organization of the early nervous system. The construction of fate maps in the early embryo is an important step in the process of understanding how an embryo is built. Fate maps tell us about the origin of particular cell groups, the morphogenetic movements that occur as the embryo takes shape, and can reveal the potential for signaling between cells whose proximity may be transient and obscured by subsequent cell rearrangements. Fate maps can be constructed by analyzing the fate of several neighboring cells labeled simultaneously (here the tracking dyes are usually applied to cell surfaces by extracellular injection) or more precisely by following the fate of individual cells (where the tracking dyes are usually injected intracellularly).

2. Materials

2.1. Fate Mapping with Lipophilic Membrane Dyes

1. DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine) perchlorate, cat. no. D-282 from Molecular Probes (Eugene, OR).
2. Steromicroscope and fiber-optic light source.
3. Micromanipulator attached to base plate or directly to stereomicroscope.
4. Pressure injector and micropipet holder.
5. Micropipet puller.
6. 9-V battery and microelectrode holder.
7. 90% Glycerol in PBS.
8. DABCO (and antiquenching agent from Sigma, St. Louis, MO).

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9. Paraformaldehyde.
10. Dimethyl formamide (DMF).
11. Dimethyl sulfoxide (DMSO).
12. 1 *M* lithium chloride.

2.2. Lineage Analysis by Single-Cell Injection of Fluorescent Dextran

1. A fixed-stage microscope (i.e., one that focuses by moving the objective lens rather than the specimen platform) with an epifluorescent attachment and an extra-long working distance ×20 objective lens—a condenser and normal microscope stage are not required as a platform to hold the egg, since it can be attached to the fitment designed to hold the condenser. The microscope does not need its own light source. The embryo will be illuminated from the side with a fiber-optic.
2. A high-resolution micromanipulator, for example, a Huxley design. It is helpful to have one that has a fine axial drive on it, i.e., a drive that moves the electrode along the axis of the electrode.
3. An antivibration table or equivalent antivibration device.
4. An oscilloscope.
5. An amplifier suitable for intracellular DC recording and a current injection facility.
6. A fiber-optic light source.
7. A good micropipet-puller (e.g., Flaming/Brown Model P-87 from Sutter Instruments, Novarto, CA).

2.3. Neuronal Trancing Using Lipophilic Membrane Dyes, Fluorescent Dextrans, and Horseradish Peroxidase (HRP)

1. Paraformaldehyde.
2. Phosphate-buffered saline (PBS).
3. DiI (*see above*).
4. DMF.
5. Glycerol.
6. DABCO.
7. Physiological saline (137 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 1 m*M* NaH_2PO_4, 5 m*M* HEPES, 11 m*M* glucose, pH 7.4).
8. Fluorescent dextran, Molecular Probes product numbers D-3308, D-3306.
9. HRP, Boehringer Grade 1 lyophilized (Boehringer Mannheim, Indianapolis, IN).
10. 2.5% gluteraldehyde in PBS (pH 7.4).
11. Diaminobenzidine (DAB).
12. Hydrogen peroxide.

3. Methods

3.1. Fate Mapping with Lipophilic Membrane Dyes

The lipophilic membrane dye DiI is the first-choice dye for following the fate of relatively small groups of neighboring cells *in ovo*. It is expensive at first glance ($190 for 100 mg), but 100 mg does go a long way.

DiI, like the other carbocyanine membrane dyes, such as DiO and DiA (Molecular Probes D-275 and D-291), is lipophilic, and thus following application to a tissue, it readily and preferentially diffuses into cell membranes rather than remaining in and diffusing through the aqueous extracellular spaces.

There are a number of ways of applying DiI to tissues, most of which initially involve dissolving the dye in a suitable solvent. Using 3 mg DiI in 1 mL of DMF in the first instance is suggested. DMSO or alcohol can be used as an alternative solvent if you suspect DMF is toxic for your cells.

3.1.1. Preparation of Embryos

Incubate eggs on their sides. Punch a small pinhole through the blunt end of the shell, and window eggs. The window needs to be large enough to enable easy access of a micropipet to the embryo. Inject ink beneath the embryo. Make a small hole in the vitelline membrane directly over the part of the embryo to be labeled, and carefully add a small drop of saline onto the embryo to prevent it from drying out. Use alcohol-cleaned instruments and sterile solutions.

3.1.2. Pressure Injection

This is the most traditional method of applying DiI to tissues in the chick embryo. It is a good way to label many cells in a small region of the embryo, but is not as suitable for analyzing the fate of small numbers of neighboring cells. Although you can use a mouthpiece to control the application of the dye, it is best to use a pressure injection device, such as the Picospritzer II, made by General Valve Corporation (Fairfield, NJ). You will also need a micromanipulator, which is firmly attached either to a base plate or directly to the microscope being used to visualize the embryo. A good stereomicroscope is essential to target accurately the embryonic tissue. To check the accuracy of application, it is a good idea to use an epifluorescent microscope fitted with a long working distance objective. If the dye deposits are not checked each time at the time of application, then it is essential that some embryos be sacrificed immediately after injection so that the initial spread of the dye can be assessed in fixed and cleared preparations. Dye is delivered via micropipets manufactured on a micropipet puller. The author uses 1-mm diameter, thin-walled borosilicate capillary glass with internal filament and pulls these capillaries to make pipets with a tip opening of approx 1.5 µm. Small aliquots of DiI can be delivered from these pipets using a pressure of 50 psi and a pulse duration of about 5 ms (*see* **Notes 1–4**).

3.1.3. Iontophoresis

This is the easiest method for labeling very small numbers of adjacent cells. It can be used to label between 1 and 10 cells. It is not recommended as a way of labeling many cells. DiI is a charged molecule and can thus be driven out of a micropipet by applying a potential difference across the pipet and using it as a

microelectrode. In addition to a micropipet puller, good micromanipulator, and stereomicroscope, all you need is a 9-V battery. The negative pole of the battery should be connected to the albumin in the egg. This is easily achieved by a flexible cable attached to a thin silver wire, which is inserted into the hole in the shell created at the time of windowing the egg. The positive pole is attached to the back of the microelectrode via an electrode holder. Contact with the DiI in the electrode tip is acheived via a 1 *M* lithium chloride solution, which is backfilled into the electrode. The author routinely uses an electrode with a 1.5-µm tip diameter to label a few cells in the neural plate although electrodes with submicron tips also work well. The author uses 1.2-mm diameter, thin-walled borosilicate glass with internal filament. First insert the negative silver wire electrode into the albumin, and then manipulate the dye-laden microelectrode tip onto the cells to be labeled before completing the electrical circuit (*see* **Note 4**). Between 1 and 5 s of current are sufficient to label a few cells brightly (*see* **Notes 5** and **6**). The amount of dye expelled is very small and may not be visible without the aid of an epifluorescent microscope, but this technique is very effective and so can be confidently used without epifluorescence. Of course, if you want to check or measure exactly where the dye is deposited, an epifluorescent microscope with long working distance objective becomes essential.

3.1.4. Application as a Solid

To label superficial cells exclusively (e.g., ectoderm), rather than deep cells is difficult using micropipets and microelectrodes, because their sharp tips can readily slip through superficial cell layers. A better approach is to manipulate small crystals of DiI directly onto the surface cells. This can be acheived by first recrystalizing the dissolved DiI onto the blunt tip of a tiny glass rod and then touching this glass rod onto the surface to be labeled. Tiny glass rods can be made from micropipets by carefully melting and sealing their tips in a small flame or using a microforge. To recrystalize the dye onto the tip, place a small drop of DiI dissolved in either DMF of alcohol onto a clean plastic surface and wait for most of the solvent to evaporate off. As the solution becomes increasingly more concentrated and sticky, it will simply adhere to the tip of a glass rod when one is dipped into it. Gently manipulate the DiI-loaded glass rod onto the cells, and the dye will redissolve into the cell membranes.

3.1.5. Fixation, Mounting, and Viewing

The maximum survival time for the embryo and the dye will depend on the rate of dye dilution by cell division. The fate of rapidly dividing cells can readily be studied for up to 3 d, and less rapidly dividing systems for at least a week. Embryos should then be fixed in 3.5% paraformaldehyde and stored in this solution in the refrigerator until viewing. The material can be viewed as a whole-mount

if the fluorescent cells are close to the surface, or it can be sectioned either on a cryostat or vibratome. To increase the transparency of the tissue, the material can be cleared in 90% glycerol in PBS containing 2.5% DABCO. Observe and photograph the material as soon as possible for the best results.

3.1.6. Two Color Fate Mapping

If you want to examine the relative movements of two cell populations directly in the same embryo, then each population should be labeled with a differently colored dye. A good combination is to label one set with DiI and the other with DiA (Molecular Probes, cat. no. D-291). DiA fluoresces over a broad range of wavelengths when it is incorporated into cell membranes, but is most intense as a green emission. Using appropriate filter sets, it is thus readily distinguished from the red/orange fluorescence of DiI (**Fig. 1**). DiA performs better than the other commonly used green-fluorescing dye DiO (Molecular Probes, cat. no. D-275), because it is more soluble, it iontophoreses more efficiently, and it fluoresces more intensely.

3.2. Lineage Analysis by Single-Cell Injection of Fluorescent Dextran

Labeling single cells *in ovo* and then studying their fate during subsequent development can be a powerful tool in the investigation of how embryos develop. It can be used to construct fate maps at the single-cell level, analyze

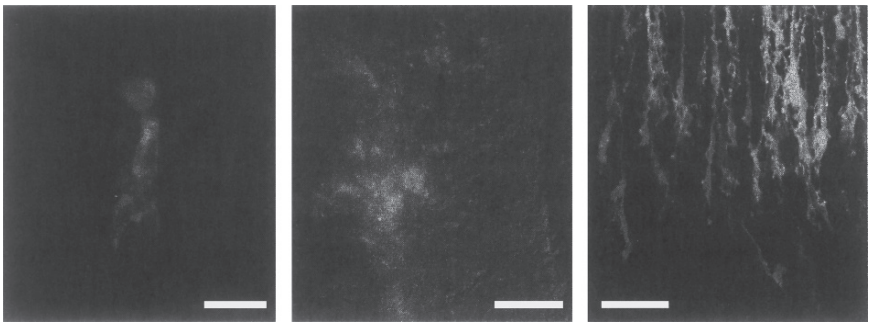

Fig. 1. **(A)** Cells labeled with DiI and DiA in the chick embryo neural plate and viewed immediately after iontophoretic applications. About four cells are labeled with DiI (red) and one cell with DiA (green). Bar is 10 µm. **(B)** After 48 h of development, the descendants of cells labeled as in (A) are still visible and their two colors distinct. Bar is 50 µm. **(C)** Motor neurons retrogradely labeled from their peripheral nerves in fixed tissue. One nerve was labeled with DiI, and the adjacent nerve with DiA. Bar is 40 µm. (*See Color Plate*)

morphogenetic movements, address issues of tissue specification, and assess the importance of lineage in the determination of regional or cellular identity. One way of labeling single cells is by infection with replication-deficient retroviral vectors. This method allows cells and their descendants to be permanently labeled with a genetic tag, thus allowing for the long-term analysis of clonally related cells. A second method is to inject single cells with tracer dyes using intracellular microelectrodes. The advantages of using the single-cell injection technique are that you can accurately target particular areas of the embryo, there are no doubts about clonality because each injection can be checked visually the time, and by using fluorescent tracers, the sequential analysis of the same clone at different times of development becomes possible. The disadvantage of the single-cell injection technique is that the marker is diluted by cell division and increased cell volume. This effectively limits its usefulness to between about 6 and 9 rounds of cell division, depending on the quality of the initial injection.

Labeling single cells *in ovo* by intracellular injection is not the sort of technique that can easily be learned by simply reading about it. It is much better to see the technique in action. This account will assume a basic understanding of intracellular recording technology and electrophysiological technique. If you do not have this, then make friends with a pharmacologist or neurophysiologist.

3.2.1. Method

1. Make microelectrodes—we use 1.2-mm diameter, thin-walled aluminosilicate glass with internal filament (A-M Systems, Everett, WA). Electrode tip should have a nice constant taper and be fine but not wispy. When back-filled with dextran and 1 *M* potassium chloride, they should have a resistance of between 50 and 150 MΩ. In practice, the precise electrode resistance does not matter; if the electrode penetrates a cell, records a stable membrane potential, and passes sufficient dye, then it is a good electrode (*see* **Notes 7** and **8**). Electrodes should first be back-filled with approx 0.5 µL of fluorescent dextran (100 mg/mL in distilled water, Molecular Probes cat. no. D-3308 and D-3306 for tetramethylrhodamine and fluorescein fluorescence, respectively) and then with a little 1 *M* potassium chloride. Don't worry about air bubbles. The capillary action of the internal filament will deal with them.
2. Window egg, inject ink subblastodermally, make a small hole in vitelline membrane over target area, and carefully drop a little saline solution onto the embryo to decrease the risk of drying out and to improve visibility. Do all this under a stereomicroscope.
3. Transfer egg to platform on fixed-stage injection microscope and insert silver/silver chloride reference electrode into albumin via air hole in shell.
4. Illuminate embryo with fiber-optic directed into the windowed egg. Find and focus embryo under ×20 objective.
5. Manipulate microelectrode tip down into the saline covering the embryo.

6. Switch on amplifier, and check that you are recording a stable baseline potential. You need to work with a "gain" of 10 mV/division and a time base set to 1 s/division. The oscilloscope must be set to monitor DC potentials.
7. Gently manipulate electrode tip down onto the surface of the cells (*see* **Notes 9** and **10**). You probably will not be able to see individual cells. As the electrode tip touches a cell membrane, there will be small change in the appearance of the trace on the oscilloscope. This may be an increase in the thickness of the trace or more usually a small positive deflection. This change is the best indication that the electrode has just touched a cell membrane; it is better than trying to see it happen down the microscope.
8. "Ring," "buzz," or "zap" the capacitance-compensation button or knob on the amplifier to penetrate the cell membrane. If successful, a small but stable negative deflection should be seen on the oscilloscope (anywhere between −5 and −30 mV is common for neural plate cells). This is the most efficient method of getting the electrode tip inside a cell, but it does have one big disadvantage. When you "ring," "buzz," or "zap" an electrode, you simultaneously squirt dye out of the end of the electrode. With a successful penetration, most if not all of this dye will go straight into the cell to be labeled. However, quite often some dye will also be sprayed out onto adjacent cells, and if these have been damaged (by the electrode scraping past them for instance), they will often take up significant amounts of the dye. If this happens, this is the end of your lineage analysis, because you will have labeled more than one cell. It is therefore essential to check briefly each injection visually with epifluorescence in order to ensure only one cell has been labeled. Do not admire your cell for too long, since phototoxicity can kill it. Cell penetration in the absence of simultaneous dye injection can be effected with a piezo-electrode stepper device, but these are not as efficient at getting into small cells as "ringing." No matter how you penetrate your cells, you should then iontophorese more dye into them by using the amplifier's current injection facility (for lysinated rhodamine dextran use positive current pulses of about 4 nA and 250-ms duration for about 30 s).
9. Rapidly withdraw the microelectrode using the axial drive of the manipulator. The oscilloscope trace should spring back up to its original baseline level.
10. Carefully drop a little more saline onto the embryo, and then reseal the egg with tape and reincubate to the appropriate stage.
11. Fix embryo in a solution containing 3.5% paraformaldehyde.
12. Observe results in either sectioned material or whole mounts using conventional epifluorescence or confocal microscopy.

3.3. Neuronal Tracing Using Lipophilic Membrane Dyes, Fluorescent Dextrans, and Horseradish Peroxidase (HRP)

The organization of neurons and their axons in the CNS and peripheral nervous system can be examined using anterograde and retrograde tracing techniques with fluorescent or nonfluorescent dyes. The lipophilic membrane dyes, such as

DiI, have the advantage that they may be used on fixed as well as living tissue, whereas the intracellular dyes, such as fluorescent dextrans and HRP, rely to some extent on the mechanisms of axonal transport and thus work best on living tissue. The principle is the same for all the dyes; if you are interested in seeing the neuronal cell bodies, then apply the dyes to their distal axons, and if you are interested in the axonal projections, terminations, or growth cones, then apply the dyes to the neuronal cell bodies. The techniques described below work well on chick embryos up to at least Hamburger and Hamilton stage 25.

3.3.1. Lipophilic Membrane Dyes

1. Fix embryos in 3.5% paraformaldehyde, and store them in this solution in the fridge until you are ready to label them.
2. Dissect in PBS to reveal the part of the CNS or PNS to be labeled. Do not be too rough with the tissue, since this techniques relies on the dye diffusing through intact cell membranes. If you break the membranes it will not work.
3. Back-fill a micropipet (tip opening approx 2–5 µm) with a small quantity of DiI or DiA (D-282 and D-291 from Molecular Probes, respectively). Use a concentration of 3 mg/mL in DMF. DiI fluoresces intensely red and DiA fluoresces also in the red, but more intensely green, and with appropriate filter sets, can readily be used for double-labeling experiments (**Fig. 1**).
4. Micromanipulate the tip of the micropipet into the tissue, and use a pressure injector (e.g., a Picospritzer II) to deposit the dye. Injections are often made more easily if the pipette is fractionally withdrawn from the full depth of the penetration. If you want to label the cut end of a peripheral nerve, depositing the dye onto the cut surface will efficiently label the axons within.
5. Gently blow away with a Pasteur pipet any excess dye that floats up from the targeted area. If these are allowed to settle onto the embryo, they will label inappropriate areas.
6. Return the specimen to 3.5% paraformaldehyde at room temperature in the dark for 12–48 h.
7. Observe on an epifluorescent microscope either as a whole mount or after sectioning on a cryostat or vibratome. Tissues can be cleared in 90% glycerol in PBS containing 2.5% DABCO (Sigma). The signal in these specimens is not permanent, so they should be analyzed and photographed as soon as possible.

3.3.2. Fluorescent Dextrans

Fluorescent dextrans may in some circumstances have advantages over lipophilic membrane dyes. Those that have a lysine residue can be fixed into the cytoplasm and thus form a nice stable signal. Because the fluorescence is in the cytoplasm they may work better in double-staining procedures, which use cell-surface antibodies. The author finds that for labeling axons within the CNS, dextrans often give more intense staining of neuronal projections than the membrane dyes. For efficient labeling of axons in the PNS, the membrane dyes are, however, the best.

1. Carefully remove embryo from egg and transfer to a Sylgard Dow Corning, Midland, MI)-covered dish containing a physiological saline solution.
2. Free embryo of membranes, and pin it down to dissect and reveal the appropriate area of neural tissue.
3. Mix up a small aliquot of fluorescent dextran. Use Molecular Probes product numbers D-3308 and D-3306 for tetramethylrhodamine and fluorescein fluorescence, respectively. The author keeps a small frozen stock of dextran made up at a concentration of 100 mg/mL in distilled water from which he transfer a very small drop onto a Sylgard surface. As the water evaporates off, the dextran becomes sticky and is easily picked up on to the tip of a stainless-steel or tungsten micropin or onto the tips of watchmaker's forceps. Simply add more water to the drop of dextran if it dries out too much.
4. To label axons within the CNS, push a micropin laden with dextran into the region of the axon tract. The dextran is taken up into the axons damaged by the pin, so you can regulate the number of axons labeled by controlling the size of the damage. To label axons in peripheral nerves, the author finds it is more efficient to crush the nerve between the tips of dextran-laden watchmaker's forceps.
5. The cut ends of the axons will seal over in about 30 min to 1 h. Thus, if you wait this long and blow away the excess dextran from the first application, a second application with a differently colored dextran can be made nearby without risking contamination of the first axons.
6. Place the embryo in fresh aerated physiological saline at room temperature for up to 12 h (small embryos will need considerably less time than this, about 3–4 h).
7. Fix tissue in 3.5% paraformaldehyde for at least 2 h, and then observe either as a whole mount or after sectioning. Tissue can be sectioned using a cryostat, vibrotome, or by following conventional wax embedding.

3.3.3. HRP

HRP is not a fluorescent tracer (it generates a dense brown reaction product as outlined below), but has the advantage of producing permanent preparations and can be used in combination with whole-mount *in situ* hybridization techniques.

1–4. Follow the procedure outlined for labeling with dextrans, but substitute a thick HRP solution for the dextran mixture. For HRP, the author mixes a small pile of crystals with a small drop of distilled water, and wait for it to evaporate to a sticky consistency.
5. Place the embryo in fresh aerated physiological saline at room temperature for up to 12 h (small embryos will need considerably less time than this, about 3–4 h).
6. Fix tissue in 2.5% gluteraldehyde in PBS (pH 7.4) for 1–2 h.
7. Wash thoroughly in several changes of PBS over a period of at least 2 h.
8. Incubate in diaminobenzidine (DAB) (5 mg in 10 mL of PBS) for at least 1 h. DAB is thought to be carcinogenic and should be handled with care. Wear gloves, and use it only in a fume hood.
9. Add hydrogen peroxide to a concentration 0.003% in the DAB solution for about 5–15 min. Carefully look at the reaction in a covered Petri-dish under a dissecting

microscope to check its not going too fast. The reaction can be slowed down by reducing the concentration of hydrogen peroxidase. Stop reaction by washing in excess PBS with azide.
10. Discard DAB solutions into an excess of potassium permanganate before disposal.
11. Observe as a whole mount or after sectioning by vibrotome, cryostat, or after wax embedding. Specimens can be cleared in 90% glycerol in PBS or in nonaqueous mountant.

4. Notes

1. Micropipet tips easily become blocked when the DiI solution comes into contact with aqueous solutions and/or is covered in cellular debris. The easiest way to unblock the pipet is to stroke the pipet tip very gently with a small wisp of tissue paper soaked in alcohol. Do this under a dissecting microscope. When the pipet is unblocked, a small deposit of DiI will be drawn out onto the tissue paper.
2. Minimize the time the pipet tip spends in the embryo. The longer it is in contact with embryo or aqueous solution, the more likely it is to block up.
3. If the targeted tissue has sufficient depth, then a small withdrawal of the pipet tip after its initial insertion into the tissue will often facilitate deposition of the dye.
4. If your pipet does not readily push through superficial cell layers to reach deeper cells, then you can first make a small superficial hole with a sharpened tungsten needle and follow this with your DiI pipet.
5. If a small blob of DiI forms on the tip of the electrode as you are trying to inject, first disconnect the battery to stop current flow and wait a short while (30 s or so). The DiI blob may simply dissolve in to the surrounding cells. If it does not dissolve, it will probably stick to the electrode as it is withdrawn from the tissue. Do not worry. You will still have labeled plenty of cells. Use a fresh electrode for the next injection.
6. Your electrode should only ever touch the cells you want to label. Try not to have to reposition it. Its amazing how easily you can inadvertently label cells simply by touching them with the electrode even when the battery is disconnected. This is especially true if the electrode is not a new one.
7. Use a new electrode at least every three embryos. The best electrode is a new one.
8. Poor electrode design is the most likely reason for lack of success.
9. Vibration must be eliminated. Make sure microscope and manipulator are solidly attached to the base plate and that the manipulator only moves when you want it to move, not for instance, when you grab the manipulator, but have yet to turn any knobs.
10. Watch out for embryos that float very slowly across the field of view. This means they are not perfectly balanced within the egg and the egg should be slightly rotated in an appropriate direction to eliminate this drift. You cannot microinject a moving target (well, the author cannot anyway).

Acknowledgments

The author became familiar with several of these techniques while working with Andrew Lumsden and the single-cell injection procedure is largely inherited from Scott Fraser. They could both have written this chapter better than the author.

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Gene Transfer in Avian Embryos Using Replication-Competent Retroviruses

Cairine Logan and Philippa Francis-West

1. Introduction

A series of replication-competent, avian-specific retroviral vectors, known as RCAS or RCAN, were developed by Hughes et al. ***(1)*** and used successfully by a rapidly expanding number of groups to assess gene function directly in ovo (e.g.,***(2–14)***). These proviral vectors are derived from the Rous sarcoma virus (RSV) and contain a unique *Cla*I restriction site in place of the region normally encoding the *src* oncogene, into which foreign DNA fragments of up to approximately 2.4 kb can be inserted. An *E. coli* plasmid backbone allows the gene of choice to be introduced by standard subcloning techniques, while retention of the viral long terminal repeat (LTR) sequences together with sequences encoding the viral *gag*, *pol*, and *env* genes facilitates viral replication and transmission. RCAN is a variant of RCAS, from which the splice acceptor immediately upstream of the *Cla*I site has been removed, preventing translation of the inserted gene, and acts as a control for nonspecific effects owing to viral infection.

These proviral vectors can be efficiently transfected into cultured chick embryo fibroblasts (CEFs) using standard techniques, and supernatant containing infectious virions is easily collected and concentrated to yield high-titer viral stocks, which can be used to infect chick embryos of susceptible strains. Alternatively, transfected CEF cells can be grafted directly into host embryos. To increase their host range and make it possible to introduce two vectors carrying different genes into the same cell, vectors containing different subgroup *env* genes have been constructed. The viral surface glycoprotein encoded by the *env* gene primarily determines the host range specificity

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of the virus. Avian-specific vectors are currently available that contain *env* genes derived from subgroups A, B, D ***(1)*** avian leucosis viral (AVL) glycoproteins as well as subgroup E ***(15)***. Modified RCAS vectors containing *env* genes derived from murine leukemia viral glycoproteins have also been generated that can efficiently infect (but not replicate in) mammalian cells, thus making it possible to also use the RCAS system for gene transfer in mice ***(16)***. In addition, transgenic mouse lines expressing the tva coding region (i.e. encodes for the subgroup A ALV receptor) under the control of either ubiquitous or tissue-specific promoters were also developed (e.g., ***(17, 18)***). Such transgenic lines can be efficiently infected using RCAS vectors encoding subgroup A ALV *env* glycoproteins.

The accessibility of the avian embryo, however, coupled with its amenability to microsurgical manipulation, means that expression of the retrovirally introduced gene can be easily manipulated in ovo simply by varying the site and time of infection or limited to a specific region by transplantation of infected tissue or CEF cells into a host not susceptible to infection. Specific examples of such manipulations are to be found in the literature cited herein. Detailed protocols for the numerous steps involved in the production of high-titer viral stocks and transfected CEF cells that can subsequently be used to assess gene function directly within the developing chick or mouse embryo are outlined. A protocol for the grafting of transfected cell pellets is also included. Detailed information and protocols for injection of high-titer viral stocks can be found in an excellent review by Morgan and Fekete ***(19)***.

2. Materials

All solutions should be made to the standard required for molecular biology or tissue culture using appropriate molecular-biology- or tissue-culture-grade reagents and sterile distilled water. For tissue culture, all solutions should be sterilized by autoclaving (where possible) or filtering and sterile technique used throughout.

2.1. Construction of Retroviral Vectors

1. Adaptor plasmids:
   a. pCla12Nco (contact S. Hughes; National Cancer Institute, Frederick, MD).
   b. pSlax13 (contact B. Morgan; Harvard Medical School and Massachusetts General Hospital, Chestnut Hill, MA).
2. Viral vectors:
   a. RCASBP (Subgroups A, B, and D) (contact S. Hughes; as earlier).
   b. RCANBP (Subgroups A, B and D) (contact S. Hughes; as earlier).
   c. RCASBP (Subgroup E) (contact D. Fekete; Depart of Biological Sciences, Purdue University, West Lafayette, IN).

2.2. Preparation of Primary Cultures of CEFs

1. One dozen eggs (appropriate strain), incubated for 10 d. Remember to turn the eggs a quarter turn every 2 d to increase viability.
2. Sterilized surgical instruments (scissors, watchmaker's forceps, razor blade).
3. Sterilized 250-mL Erlenmeyer flask.
4. Sterile 10-mL wide-mouth pipets.
5. Sterile 50-mL plastic centrifuge tubes.
6. Sterile 1.5-mL cryovials.
7. 10-cm petri and tissue-culture dishes.
8. 70% v/v Ethanol.
9. 1× Trypsin/EDTA (0.5 mg/mL trypsin, 0.2 mg/mL EDTA), available as 1× stock in modified Puck's saline A from Invitrogen.
10. Fetal calf serum (FCS), available from Invitrogen.
11. Chicken serum (CS), available from Invitrogen.
12. Penicillin–streptomycin (pen–strep), available from Invitrogen.
13. Dulbeco's modified Eagle's medium (DMEM; high-glucose, L-glutamine, sodium pyruvate), available from Invitrogen.
14. Dimethyl sulfoxide (DMSO), available from Sigma. DMSO is an irritant requiring adequate safety precautions.

2.3. Transfection of Proviral Constructs

1. 6- and 10-cm Tissue-culture dishes.
2. Sterile 4-mL polypropylene round-bottom tubes.
3. TE pH 8.0 (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).
4. 70% v/v Ethanol.
5. 2× HEPES buffered saline (HBS): 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, 50 mM HEPES. Adjust pH to 7.05 with 0.5 N NaOH and filter–sterilize. Aliquot and store at −20°C.
6. 2 M $CaCl_2$: Filter–sterilize, and store in aliquots at −20°C.
7. CEF media (*see* **section 3.2**).
8. 15% (v/v) Glycerol in HBS: Filter-sterilize.
9. Stock solution of 2 mg/mL polybrene (Sigma, St. Louis, MO) in H_2O: filter-sterilize, and store in aliquots at −20°C. Polybrene is harmful by inhalation. Therefore, adequate safety precautions should be taken.

2.4. Collection and Concentration of Viral Stocks

1. 10-cm Tissue-culture dishes.
2. Sterile 50-mL plastic centrifuge tubes.
3. Sterile 1.5-mL microfuge tubes.
4. 40 mL Polyallomer ultracentrifuge tubes (Beckman, Fullerton, CA): sterilize by rinsing well in 70% ethanol and air drying in the tissue-culture hood.
5. Phosphate-buffered saline (PBS). PBS tablets are available from Sigma.
6. CEF media (*see* **section 3.2**).
7. Reduced serum CEF media (DMEM + 5% FCS + 1% CS).

2.5. Preparation of Infected Cells for Grafting

1. 250-mL Tissue-culture flasks.
2. Sterile 15- or 50-mL plastic centrifuge tube.
3. Sterile 1.5-mL Eppendorf tubes.
4. Sterile scissors.
5. Sterile 3.5-cm petri dish.
6. Sterilized tungsten needle.
7. 1× Trypsin/EDTA (0.5 mg/mL trypsin, 0.2 mg/mL EDTA). Available as 1× stock in modified Puck's saline A from Invitrogen.
8. CEF media (*see* **section 3.2**).

2.6. 3C2 Immunostaining of Virally Infected CEF, DF1, or QT6 Cells

1. PBS: PBS tablets are available from Sigma.
2. Triton X-100, available from Sigma.
3. Goat serum: available from Invitrogen.
4. 3C2 hybridoma supernatant: available from the Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA.
5. Peroxidase-conjugated antimouse IgG, IgM secondary antibody, available from Jackson Laboratories, West Grove, PA.
6. 30% v/v Hydrogen peroxide, available from Sigma. Hydrogen peroxide can cause burns and is harmful by inhalation. Therefore, adequate safety precautions should be taken.
7. 3.5% w/v Paraformaldehyde: Dissolve 3.5 g paraformaldehyde in 100 mL of PBS (pH 7.4) by gently heating in a fume hood. Aliquot and store at −20°C. Paraformaldehyde is extremely toxic. Therefore adequate safety precautions should be taken.
8. 0.5 mg/mL 3,3 -Diaminobenzidine tetraaminobiphenyl (DAB) in PBS. DAB tablets (10 mg) are available from Sigma. Activate by adding 1 µL/mL of 30% (v/v) H_2O_2 to DAB just prior to use. DAB is extremely toxic. Therefore, adequate safety precautions should be taken.
9. Nickel chloride, available from Sigma. Nickel chloride is a known mutagen and is extremely toxic. Therefore adequate safety precautions should be taken.

3. Methods

3.1. Construction of Retroviral Vectors

For optimum expression of the inserted gene, there are several important points to consider when cloning:

First, the level of expression of the inserted gene is influenced not only by proviral sequences within the LTR but also by sequences within the *pol* region. RCAS vectors containing the *pol* region from the Bryan high-titer strain of RSV, known as RCASBP (BP stands for Bryan *pol*), express introduced genes at higher levels ***(20)*** than do the original RCAS vectors and may be preferable for cloning (*see* also **Note 1**).

Second, the gene of interest should be cloned into the unique *Cla*I restriction site of the RCASBP retroviral vector via an intermediate cloning step using the adapter plasmids pCla12Nco ***(1)*** or pSLAX13 ***(19)***. The pSLAX13 was made by transferring the *Cla*I to a *Cla*I fragment from pCla12Nco into a pBluescript (Stratagene) vector and has the increased advantage of a higher copy number than pCla12Nco and recombinants can be identified via standard blue/white selection. In addition, commercially available T3 and T7 primers can readily be used for sequencing pSLAX13 subclones. Both adapter plasmids contain several restriction sites flanked by two *Cla*I sites to facilitate cloning as well as part of the 5′-untranslated region of the *src* oncogene, the inclusion of which has been found to enhance considerably the level of expression of the inserted gene.

Third, the coding sequences of the gene of interest should be inserted in frame with the ATG of the *Nco*I site within the adapter polylinker to avoid altering the N-terminus of the encoded protein. Inclusion of untranslated 5′ and 3′ regions of the gene of interest should be avoided, since they can adversely affect subsequent transcriptional or translational efficiency.

1. Appropriate insert fragments to be subcloned can be generated by either restriction digests or PCR and subcloned into the adapter plasmid by standard cloning procedures. When using pCla12Nco, recombinants must be identified by radioactive screening or PCR as *LacZ* blue-white selection is not possible. If the insert fragment is generated by PCR, restriction sites can be added to the oligos to facilitate cloning. In the latter strategy, an *Nco*I site can be conveniently introduced (if not already present) at the initiator ATG site, thus allowing the fragment to be directly cloned in frame into the *Nco*I site of the adapter polylinker. Provided that the second codon begins with a guanine, such mutagenesis does not alter the encoded sequence. If the second codon does not begin with a guanine, a more complicated cloning strategy, such as that outlined by Morgan and Fekete ***(19)*** is required. For efficient cutting of the PCR product, at least two bases (preferably four for *Nco*I) should be included in the oligo 5′ to the restriction sites. To avoid errors in the PCR product, the oligos should be very pure and a thermostable polymerase, which has proofreading ability (e.g., PFU polymerase, Promega), should be used. Finally, once subcloned, the sequence of the PCR product or inserted fragment should be thoroughly checked to ensure that no mutations have been introduced (*see* **Note 2**).
2. The *Cla*I to *Cla*I fragment from the adaptor plasmid containing the inserted coding sequences is then isolated by partial or complete restriction enzyme digestion and subcloned into the appropriate RCASBP or RCANBP vectors by standard cloning procedures. Recombinants must be identified by radioactive screening or PCR analysis. Orientation of the insert may be determined by restriction digest analysis. Alternatively, the inserts may be sequenced using appropriate oligonucleotide primers ***(19)*** (*see* **Note 3**).

3.2. Preparation of Primary Cultures of CEFs

Primary cultures of CEFs or cultures of the spontaneously immortalized avian fibroblast cell line, DF1 (*see* **Note 4**; available from the American Type Culture Collection), compatible with the retroviral subtype being used are required for the production of high-titer viral stocks and infected cells for grafting. All retroviral subtypes, except E, can be grown on either CEFs made from line 0 embryos or DF1 cells. Neither contains any endogenous retroviruses (*see* **Note 5**). CEFs made from line $15b_1$ embryos are susceptible to infection by E subgroup viruses. **Sections 3.2.1–3.2.2** describe the preparation of primary CEFs.

All instruments should be sterilized by autoclaving (where possible) or rinsing in alcohol and sterile technique used throughout. Wear gloves (ethanol sterilized).

3.2.1. Day 1

1. Wash eggs well with 70% (v/v) ethanol.
2. Open the eggs by cutting a hole in the shell. Pluck out embryos with forceps and place in a sterile 10-cm petri dish.
3. Cut off and discard the head and limbs, and remove and discard the viscera.
4. Mince the torso into small fragments with a sterile razor blade.
5. Collect minced embryos using a wide-mouth 10-mL pipet. Place in a sterile 250-mL Erlenmeyer flask with 10 mL of 1× trypsin/EDTA for every four embryos.
6. Place flask on rotator, and gently swirl for 12–15 min at room temperature. You should see small clumps disappear, but you should not wait until they all disappear or the cells will start to die.
7. Take the flask off the rotator and allow large clumps to settle. Transfer supernatant (i.e., leave clumps behind) to a sterile 50-mL plastic centrifuge tube.
8. Add an equal volume of fetal calf serum (FCS) to inhibit trypsin. Mix gently, and let stand for approx 5 min again to allow any big clumps to settle. Decant supernatant to a new tube.
9. Spin at low speed (approximately 1,000–2,000 *g*) for 5 min at room temperature to pellet cells.
10. Discard supernatant, and resuspend pellet in 25 mL of FCS. Count cell number to obtain an approximate idea of the number of cells/mL.
11. Pellet cells again (5 min at low speed as earlier).
12. Discard supernatant and resuspend cell pellet in 20 mL of CEF media (DMEM + 10% v/v FCS + 2% v/v CS + 1X pen/strep).
13. Plate on 10-cm tissue-culture dishes (*see* **Note 6**) in CEF media using a range of concentrations: for example, 10^7 cells/dish, 3×10^6 cells/dish, 10^6 cells/dish, and 3×10^5 cells/dish. Incubate at 37°C in a 5% CO_2 incubator.

3.2.2. Day 2

Change media to remove cell debris. It is somewhat usual to see many floating or dead cells at this point.

3.2.3. Day 3

Cells plated at the appropriate density should be reaching confluence. You now have two choices: You can freeze them immediately (P0) or pass the cells 1:3 or 1:4 and then freeze them 1–2 d later when they look almost confluent (P1). Following trypsinization, cells are frozen in 1-mL aliquots in CEF media (without the pen/strep) + 12% v/v DMSO at a ratio of one 10-cm tissue-culture dish in three 1.5-mL cryovials. The cells are frozen overnight at −70°C before transferring to liquid nitrogen for long-term storage.

3.3. Transfection of Proviral Constructs

Being replication competent, virus rapidly spreads horizontally throughout the culture of primary CEF or immortalized DF1 cells, and therefore, almost any transfection protocol suffices. The following protocol (i.e., **sections 3.3.1–3.3.3**) works well and minimizes cell death.

3.3.1. Day 1

1. Plate cells at 10–20% confluence in a 6-cm (or 10-cm) dish. This can be achieved by splitting a newly confluent dish 1:4 or 1:5. Cells plated at this density should be approximately 50–60% confluent by the following day.
2. Precipitate DNA, rinse in 70% (v/v) ethanol, air dry in the tissue-culture hood, and resuspend in sterile TE, pH 8.0, at an appropriate concentration (e.g., 0.3 µg/µL).

3.3.2. Day 2

1. For each 6-cm (or 10-cm) dish to be transfected, place 0.3 mL (or 0.6 mL) of 2× HBS into a sterile 4-mL polypropylene tube.
2. Add 3–6 µg (10–20 µg) plasmid DNA and mix well.
3. Add 18 µL (32 µL) of filter-sterilized 2 M $CaCl_2$ dropwise while gently mixing (i.e., tapping the tube) or slowly vortexing.
4. Flick or slowly vortex tube for 20 sec.
5. Let stand at room temperature for 45 min to allow a precipitate to form.
6. Remove media from each dish to be transfected by thorough aspiration. Alternatively, this media can be saved and replaced later.
7. Add DNA precipitate to the center of the dish and rock gently by hand. Incubate at room temperature in the tissue-culture hood for 20 min (rock gently after 10 min).
8. Add 5 mL CEF media (or replace original medium) and incubate at 37°C for 3–4 h.
9. Remove media, and aspirate well. Carefully add 1 mL (2.5 mL) of sterile 15% (v/v) glycerol in HBS and rock gently. Incubate at room temperature for exactly 90 sec.
10. Wash with 5 mL of CEF media, rocking gently to mix.
11. Remove media, and repeat the same wash once or twice.
12. Remove final wash, add fresh CEF media, and incubate at 37°C.

3.3.3. Day 3

The media should be changed, and if appropriate, the cells should be split. The cells should be passaged as usual, making sure they are split at least every other day. For all viral subtypes except A, 2 µg/mL of polybrene should be included in the media at this stage to enhance infection. Viral supernatant should be collected (*see* **section 3.4.**) 7–10 d following transfection. Similarly, transfected cells can be used for grafting experiments (*see* **section 3.5.**) after 7–10 d in culture *(see* **Note 7**).

3.4. Collection and Concentration of High-Titer Viral Stock

Prior to viral collection, transfected cells should be checked by immunostaining with the 3C2 monoclonal anti-GAG antibody ***(21)*** to ensure that the retrovirus has spread throughout the culture (*see* **section 3.6.**). To optimize retroviral titers, it is important that the supernatant is collected from newly confluent or near-confluent dishes of dividing cells. Viral titers can be enhanced by minimizing both the volume and serum concentration of the media used for collection. Collection of supernatant containing infectious viral particles using reduced serum CEF media facilitates resuspension of the viral particles after concentration by centrifugation and does not adversely affect viral titer.

3.4.1. Collection of Viral Supernatant

1. Split transfected CEFs/DF1s 1:5 from newly confluent plates into an appropriate number of 10-cm tissue-culture dishes. Incubate cells until they are 80–90% confluent (1 or 2 d).
2. Discard medium and rinse once with sterile PBS.
3. Replace with 5 mL of reduced serum CEF media containing 5% FCS and 1% CS. Do not include pen/strep or polybrene in the culture media. Incubate cells overnight.
4. Collect supernatant and put into sterile 50 mL plastic centrifuge tubes. Supernatant can be stored at −70°C at this point.
5. If the monolayer of cells is still intact, collect a second aliquot of supernatant by placing an additional 5 mL of reduced-serum CEF media on plates and incubating for a further 4–24 h (*see* **Note 8**).

3.4.2. Concentration of Viral Stock

1. Thaw viral supernatant on ice (or in cold water). Be careful to keep thawed supernatant on ice at all times.
2. Spin supernatant at approximately 2 K for 10 min at 4°C to pellet nonviral, cellular debris.
3. Carefully decant supernatant into 40 mL polyallomer ultracentrifuge tubes (Beckmann), which have been rinsed well with 70% ethanol and left to air dry in the tissue-culture hood.
4. Spin at 20 K for 2 h at 4°C in a SW28 rotor to pellet viral particles.

5. Pour off supernatant and aspirate well.
6. Allow the tubes to stand upright on ice for a few minutes.
7. Carefully resuspend the pellet in the supernatant left in the tube (usually about 50–100 µL) by gently pipeting up and down. Avoid making bubbles, since this can denature the viral proteins. Resuspension may take up to 2 h if the pellet is fairly sticky. It is convenient to leave the tubes in an ice bucket in the tissue-culture hood and pipet the suspension approximately once every 15 min.
8. Pool identical samples. To avoid repeated freeze–thawing, concentrated viral supernatant should be aliquoted into an appropriate volume convenient for injections (e.g., 20 µL) and stored at −70°C (*see* **Note 9**).
9. To determine the number of infectious virions/mL, concentrated viral supernatant stocks can then be titered by infecting either CEFs/DF1s or the chemically transformed quail embryonic fibroblast cell line, QT6 ***(22)***. Viral titers are determined by infecting cells with serial dilutions of concentrated viral supernatant. Following an 18–48 h incubation, the cells are fixed using 3.5% paraformaldehyde, and the number of infected cells is determined by immunostaining using the monoclonal 3C2 anti-GAG antibody ***(21)*** (*see* **section 3.6.**). Titers of concentrated viral stocks should be between 5×10^7 and 10^9 infectious virions/mL (*see* **Note 10**). Where possible, QT6 cells are used in preference to CEFs/DF1s, since although they are initially infected as well as CEFs/DF1s, the virus does not spread as rapidly from cell to cell, thus making it easier to identify clonal isolates. The QT6 cells can be infected with viruses of all subtypes except B and E.

3.5. Preparation of Infected Cells for Grafting

Transfected CEF/DF1 cells expressing the gene of interest can be easily grafted into developing chick embryos (e.g., ***(3)***). By using a host embryo from an infection-resistant strain, this technique also provides an effective method to analyze the local effect of secreted factors whereby infection can easily be limited to the implanted cells. Although it saves having to prepare high-titer viral stocks, infected cells must be cultured for each experiment.

1. To obtain enough cells for grafting, cells should be grown to confluence in a 250-mL tissue-culture flask. This can be achieved within 5 d after transferring cells from a confluent 10-cm tissue-culture dish.
2. Trypsinize cells and transfer to a sterile 15- or 50-mL plastic centrifuge tube. Spin at approximately 3 K for 5 min. Remove supernatant, and resuspend cell pellet in 0.5 mL of CEF media.
3. Transfer the cells to a 1.5-mL Eppendorf tube, and pulse briefly (20 s) in a microfuge to pellet the cells.
4. Dislodge the cells carefully using a sterile tungsten needle, keeping the cell pellet as intact as possible (*see* **Note 11**). Alternatively, the cell pellet can be resuspended in approximately 50 µL media (**Note 12**) and cells can be directly micropipetted into the developing embryo.

5. Using a micropipet (*see* **Note 13**), transfer the cell pellet to a sterile 3.5-cm petri dish containing 2 mL of CEF media. To avoid fragmenting the cell pellet, cut the end off the yellow or blue tip using sterile scissors. If the cell pellet does fragment while attempting to transfer it, respin and try again.
6. Place the petri dish containing the cells in a 5% CO_2 tissue-culture incubator at 37°C, and allow cells to consolidate for at least 1 h.
7. For grafting, dissect the cell pellet into suitable-size pieces under a dissecting microscope. Transfer the pellet to be grafted onto the embryo (*see* **Note 14**) using a micropipet, and position pellet appropriately using sterile tungsten needles (*see* **Notes 15 and 16**).

3.6. 3C2 Immunostaining of Virally Infected CEF, DF1, or QT6 Cells

The monoclonal antibody (MAb) 3C2 ***(21)*** recognizes the viral GAG protein and can be used to assess the extent of infection following transfection of line 0 CEF/DF1cells (**section 3.3.**) or to determine the viral titer of concentrated supernatant (**section 3.4.**).

1. Rinse cells twice with 1× PBS.
2. Fix for 15–30 min at room temperature with 3.5% (v/v) paraformaldehyde in PBS.
3. Rinse cells twice with 1× PBS.
4. Block 3× 30′ at room temperature in CEF media containing 0.05% Triton X-100.
5. Incubate in 3C2 1° antibody (diluted 1:4 in CEF plus 0.05% Triton X-100) overnight at 4°C. The 1° antibody can be reused several times and between uses should be kept at 4°C in the presence of 0.02% (w/v) sodium azide.
6. Rinse 3× 30′ with 1× PBS containing 10% serum (e.g., goat serum).
7. Incubate in 2° antibody (peroxidase-conjugated antimouse IgG, IgM diluted 1:200 in 1× PBS plus 10% serum) for at least 1 h at room temperature.
8. Rinse 3× 15 in 1× PBS.
9. Develop with activated DAB. To enhance the signal, 0.5 mg/mL nickel chloride can be added to the DAB solution, giving a black precipitate, which is easier to visualize (*see* **Fig. 1**).
10. The reaction is stopped by rinsing several times with 1× PBS.

4. Notes

1. The choice of viral subgroup should also be considered. Although subgroup A retroviral vectors have been routinely used for limb studies (e.g., ***(2, 3, 5–7, 12–14)***), subgroup B vectors have been found to infect developing neural tissue more efficiently ***(23)***. However, subtypes B and C have been found to be more cytotoxic to cells than subtype A.
2. Before starting the cloning procedure, it is also important to consider how expression of the inserted gene will be determined. If antibodies are not available with which to detect the encoded protein, it may be appropriate to label the protein with an expression tag, such as myc ***(24)*** or FLAG ***(25)***, provided such a tag does not

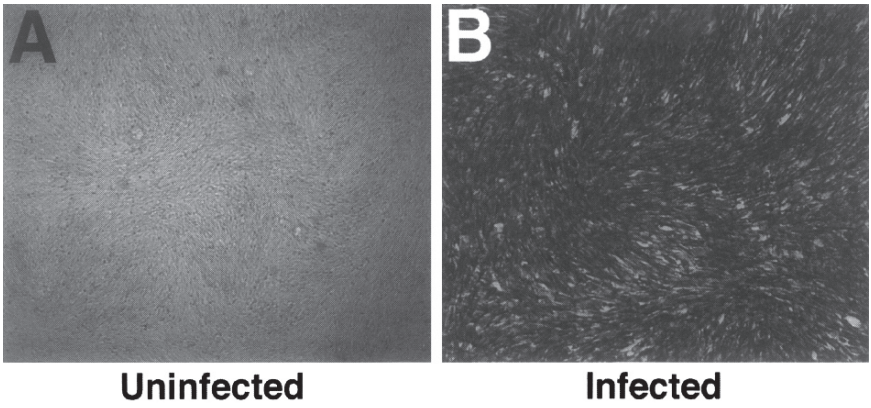

Fig. 1. Immunohistochemical analysis using the 3C2 anti-GAG monoclonal antibody ***(18)*** of (**A**) uninfected and (**B**) RCASBP(A)-infected CEF cells (courtesy of I. Campbell).

interfere with protein function. However, if such expression tags are used, it is important first to ensure that the antibody against the tag does not cross-react with endogenous chick proteins.

3. Where possible, expression of the encoded protein should be assayed immunohistochemically via Western blots of total protein extracts or supernatant (if secreted) from transfected CEF cells. Protein function should also be assayed if possible. Alternatively, Northern blot analysis of mRNA from transfected CEF cells could be used to determine if the inserted sequences are being properly spliced to produce the transcript required for translation of the exogenous gene. Three alternatively spliced mRNA viral transcripts should be produced in transfected cells. It is the smallest splice variant, whose abundance should be >10% of the total viral RNA, used for translation of the exogenous gene. In addition, the pSLAX13 adapter plasmid containing the inserted sequences could be used in an in-vitro transcription or translation assay to determine if protein of the correct size can be translated. Kits are readily available from several manufacturers. We routinely use the TNT-coupled wheat germ extract system (L4120) and tRNAnscend nonradioactive translation detection system (L5080) from Promega.
4. The DF1 cell line is maintained and transfected as for primary CEFs ***(26,27)***. However, cells are cultured at 39°C in 10% CO_2. This cell line has the advantage that it, unlike CEFs that can be passaged only 25–30 times, can be grown indefinitely. In addition, it can also be grown at lower densities, as it does not require cell-cell contact and, therefore, can be routinely split 1:5 or 1:10. Furthermore, up to twofold higher viral titers have been obtained for RCAS(BP) subtype A using this cell line ***(27)***. Viral titers obtained with B and C subgroups are similar when grown on DF1 and CEF cells ***(27)***. However, DF1 cells have been reported to be more susceptible to the cytotoxic effects of subtype C ***(26)***.

5. The lack of endogenous retroviruses makes line 0 CEFs/DF1s desirable for growing viral stocks, since the possibility of recombination between the exogenous virus being introduced and endogenous viruses is minimized and viral spread can be readily determined by immunostaining using the monoclonal 3C2 anti-GAG antibody ***(21)***.
6. CEF cells are very sensitive to the type of plasticware used. In our experience, they preferentially adhere to and grow best on tissue-culture dishes supplied by Griener, Corning, and Nunc.
7. To introduce two vectors carrying different genes into the same CEF cells, separate transfections (using vectors containing different subgroup *env* genes) should be done sequentially. The CEF/DF1 cells should be passaged two or three times between transfections.
8. The titers obtained after 4–6 h of incubation are almost as high as those obtained after 16 h (i.e., O/N). Therefore, multiple collections of supernatant could also be done at 4–6 h intervals.
9. Concentrated viral stocks can maintain their titer for months or years when stored at −70°C.
10. The titer of concentrated viral stocks varies considerably, depending on the virus, confluence, and fitness of the cells used for collection, and the volume of supernatant used to resuspend the viral pellet. In general, titers below 10^8 infectious virions/mL do not produce high-efficiency infection and would thus not be considered useful.
11. The cell pellet should be compact. If it is too dispersed, the cells cannot be used for grafting, since the cell pellet will disintegrate during transfer or subsequent manipulations.
12. The density of cells is critical: If they are too dense, the capillary becomes blocked; but if they are too dilute, the cells are not transferred effectively into the embryo when injected.
13. Plastic disposable pipets should not be used to transfer the cell pellet, since we have found that the pellet often sticks to the side of the pipet.
14. When adding the pellet to the embryo, it is very important to watch where the pellet goes, since it is often invisible on the embryo.
15. Manipulations, such as the grafting of cell pellets, can sometimes delay development.
16. Although compact cell pellets can be implanted into a specific region of the embryo, cells often disperse within 24–48 h (e.g., *see* ***(3)***).

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27

Electroporation in Avian Embryos

Jun-ichi Funahashi and Harukazu Nakamura

1. Introduction

Manipulating the gene expression is a powerful approach to study its function. Here, we introduce in-ovo electroporation as a very effective gene transfer method. By this method, one can test gene function quickly and easily. Gain-of-function study of the gene of interest by this method greatly contributed to understand the molecular mechanisms of development ***(1,2,3)***. Gene silencing by electroporating the siRNA vector is now possible (***(4)*** *see* also **Note 1**). We have to construct only an appropriate expression vector, locally apply this plasmid to an avian embryo, and charge electric pulses. The translated product is observed only 2 h after electroporation (**Fig. 1**). The area of transfection can be localized or spread by choice of electrode. There are also drawbacks, when it is compared with the retrovirus-mediated gene transfer. The expression with this method is transient, and not all of the cells facing to the electrode can be transfected as far as when the conventional expression vector is used. However, these problems can be overcome by a combination of provirus vector, such as RCAS ***(5)***, and virus-competent eggs (**Note 2**).

2. Materials

1. DNA: Super-coiled plasmid, made through an ion-exchange chromatographical purification system, such as QIAGEN plasmid kits, produces sufficient efficiency. It is redissolved in a concentration of 1–5 μg/μL in TE. A higher concentration gives better transfection, although it can be hard to inject due to its viscosity. Some researchers use PBS instead of TE, although we could not find any significant difference in efficiency between them.
2. Electroporator: An apparatus that can generate a low-voltage (5–50 V) square pulse is used. Several models are available from BTX Instrument Division, Holliston, MA, or NEPA GENE Company, Ltd, Japan.

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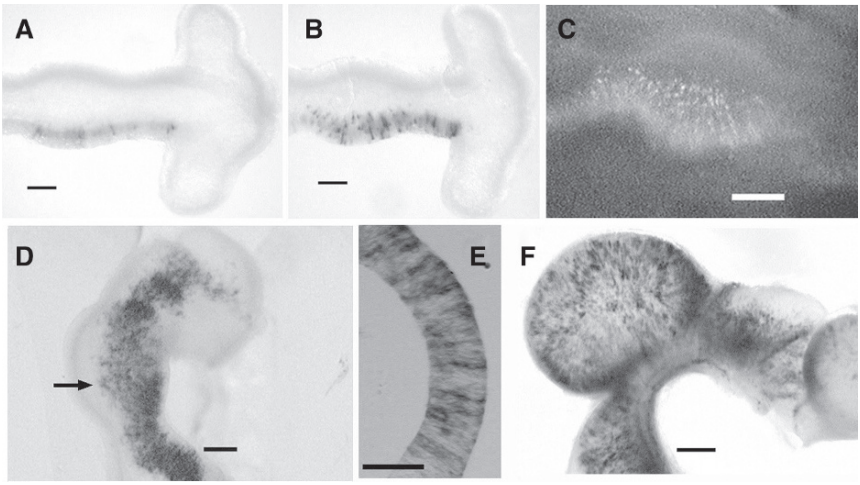

Fig. 27.1. Efficiency of electroporation. The efficiency of in-ovo electroporation was checked by injecting the *lacZ* expression vector (pMiwZ) or the *GFP* expression vector (pEGFP-N1) at stage 10. The *lacZ* expression is already recognizable 2 h after the electroporation (**A**) and becomes strong 3 h after the electroporation (**B**). Nine hours after the electroporation with *GFP* vector, one has the image in **C**. The efficiency of transfection can be checked in ovo with the *GF*P vector. At the transfection zone 24 h after the electroporation (**D** and **E**), more than half of the cells express *lacZ*. The expression is transient, but *lacZ* expression is still strong 72 h after the electroporation (**F**). The *lacZ* transfection exerts no morphological effects. The arrow in **D** indicates the section in **E**. Bars are 200 μm (**A**, **B**, **C**, **D**, **F**); 50 μm (**E**). Source: ***(3)***.

3. Electrodes: To introduce DNA in a broad domain of tissue, we recommend using platinum electrodes, as gold-plated ones wear off easily. These can be hand made,although it is hard to solder platinum to other metals, alternatively they are commercially available from several companies: NEPA GENE Company, Ltd in Japan, Protech International Inc. in the United States. To restrict the transfected area, a tungsten needle electrode and large platinum electrode are used. These are available from the companies just listed.
4. Three-axis micromanipulator: A coarse one will do, as very fine movement control is not required (e.g., Narishige Corporation).
5. Microscopes: For electroporation, a binocular dissection microscope and fiber-optic light source. For monitoring GFP fluorescence, a fluorescence dissection microscope.
6. Buffered saline or serum-free medium: Hanks' buffered saline, Tyrode's solution, or L15 medium (Life Technologies).
7. Indian ink (Rotring or Pelican) for visualizing embryos: About one fifth dilution with buffered saline.

8. Micropipet puller (e.g., Sutter Instruments).
9. Glass micropipets prepared by pulling glass capillaries (e.g., GD-1 Narishige).
10. Pressure injector or aspirator tube with cylinder (e.g., Eppendorf picospritzer).
11. Small toothbrush to clean electrodes.

3. Methods

3.1. Preparation of the Embryos

3.1.1. In-Ovo Electroporation

1. Incubate eggs on their sides at 38°C. Remove 2–4 mL albumin by a syringe with 16–18-gauge needle from a small hole made at the acute pole of the egg with a scissors.
2. Seal the hole with Scotch tape.
3. Open a window of about 2 cm in diameter by a scissors (**Fig. 2A and 2B**).
4. Put several drops of buffered saline or serum-free medium on the embryo to avoid drying. Injection of diluted Indian ink under the yolk sac helps to see the embryos well (**Fig. 2C**).
5. You might be required to cut the vitelline membrane and amnion with a microscalpel, a tungsten needle, or miroscissors. *See* **section 2** for the manipulation of embryos for more detail.

3.1.2. Ex-Ovo Electroporation

For the specific applications, shell-less culture or New culture is used, *see* Chapters 16 and 17.

3.2. Positioning the Electrodes

3.2.1. Parallel Electrodes (Fig. 2C and 2E)

To transfect a wide area,

1. Hold parallel electrodes with a micromanipulator, and place them on the embryo. The embryo should lie between electrodes (**Fig. 2A–2C**).
2. Apply slight pressure with the electrodes to make shallow dome of the vitelline membrane.

3.2.2. Needle Electrodes

To restrict transfection to a relatively small number of cells,

1. Use a needle electrode as a cathode and a large platinum electrode (as used in **section 3.2.1**) as an anode.
2. Put the needle electrode as close as possible to the tissue of interest, but do not touch it. To transfect the neural tube, insert the needle in the tube and put the anode outside the embryo.
3. Place the anode 1–2 mm away from cathode.

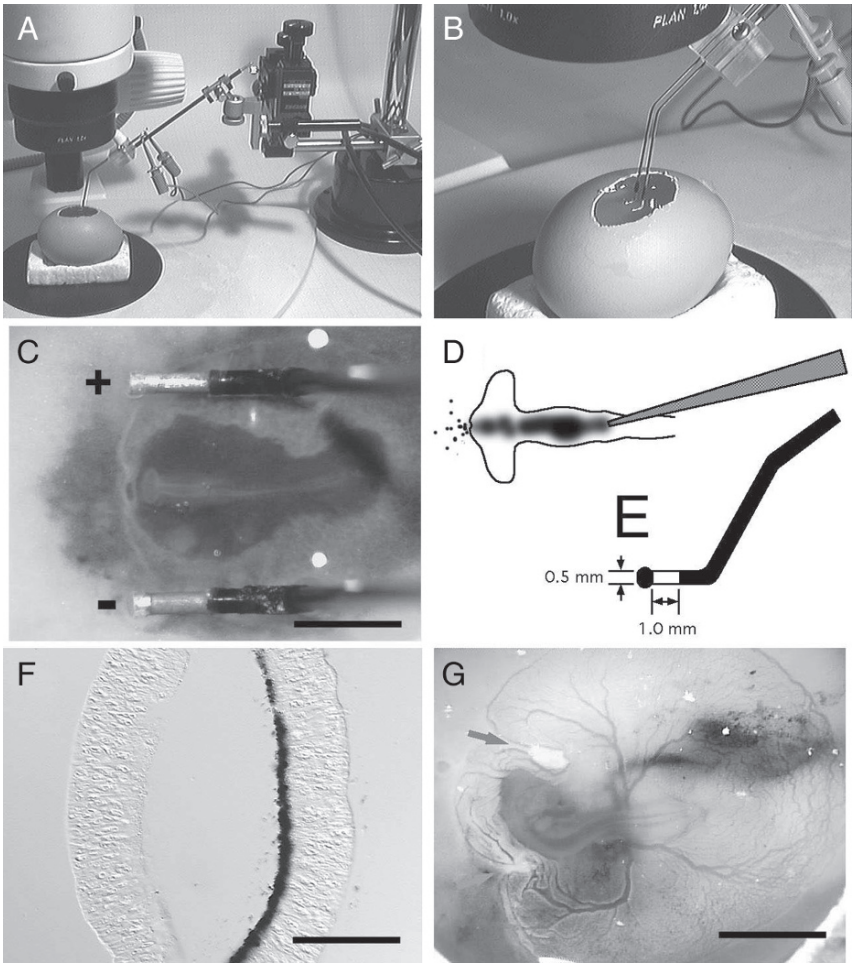

Fig. 2. In-ovo electroporation. A pair of electrodes held by a manipulator (**A**) is inserted intom a window opened on the shell (**B**). The electrode is placed on the vitelline membrane overlying the embryo (**C**), and a 25-V 50-ms pulse is charged five times. The entire procedure is monitored under a dissection microscope. Plasmid solution is injected to the E2 (HH stage 10) chick neural tube (**D**) prior to the pulse charge.The dimensions of the electrode are shown in **E**. Most of the electrode is insulated (black in the figure) so that only the tip is exposed (white area). One hour after electroporation, some embryos were fixed, processed for paraffin sectioning, and observed with a Nomarski interference microscope (**F**). The right-hand side of the figure corresponds to the right of the embryo, where injected plasmid was transfected. The morphology of the cells and the structure of neural tube were almost normal. The blue deposit inside the neural tube is a complex of plasmid and the color substrates not removed by washing in dimethylformamide after whole-mount in-situ hybridization. Twenty-four hours after electroporation, the development of yolk sac plexus, vitelline veins, and vitelline arteries are retarded in the area contacted on the electrodes (arrows in **G**). Bar is 2 mm (**C**); 50 µm (**F**); 4 mm (**G**). Source: ***(3)***.

3.3. Injecting DNA

If the tissue of interest is a closed canal or cyst, such as neural tube or eye primordium, fill it by injecting DNA solution (**Fig. 2D**). The injection raises the pressure inside the canal, and the injected solution sometimes comes out. Make a tiny hole at a possible place or suck out the inside solution before injection of DNA solution to keep the plasmid solution inside. Adding dye such as 0.1% fast green to the plasmid solution helps this procedure by making it possible to visualize the injected DNA solution. To transfect the surface ectoderm, place concentrated plasmid solution on it.

DNA moves from cathode to anode; therefore, the order of materials is: cathode | plasmid solution | tissue of interest | anode (**Fig. 2C**). If you co-electroporate a *GFP* expression vector, you can monitor the efficiency and site of transfection under a fluorescence dissection microscope or by subsequent immunohistochemistry (see Chapter 46).

4. Electroporation

4.1. Electroporation Settings

4.1.1. Large Parallel Electrodes

1. Voltage: 25 V.
2. Length of the pulse: 50 msec.
3. Frequency: Four or five times.
4. Pulses are generated every 1 sec so that the pulse of 50 msec is followed by a 950-msec rest phase.

4.1.2. Needle Electrodes

1. Voltage: 5–15 V; it is necessary to optimize the conditions for each tissue and electrode combination.
2. Length of the pulse: 50 msec.
3. Frequency: Two or three times.
4. Pulses are generated every 1 sec so that the pulse of 50 msec is followed by a 950-msec rest phase.

When pulses are charged, bubbles form on both of the electrodes. Remember that the cathode produces more bubbles than the anode. Only the anode side of the tissue is transfected (**Fig. 1**, **Fig. 2F**). Under these conditions, damage to the embryo is usually minimal (**Fig. 2G**).

4.1.3. Cleaning the Electrodes

When a parallel electrode is used, clean it every three embryos. This is done with small toothblush in buffered saline under the binocular.

4. Notes

1. *Gain of function and loss of function*. This method can be used for both the gain-of-function and loss-of-function approaches. For gain-of-function analysis, eukariotic expression vectors such as pMiw ***(6)*** and pCMV-*Tag* (Stratagene) is used. For loss-of-function analysis, one can use the siRNA expression vector or dominant–-negative protein expression vector. For the siRNA, the plasmid vector, such as pSilencer-U6 (Ambion), is used. Short hairpin RNA transcribed by RNA polymerase III from the vector can work as siRNA ***(4)***.
2. *Use of a provirus vector such as RCAS*. For prolonged overexpression, a combination of a provirus vector and virus-sensitive egg may be used. One can directly electroporate provirus vectors to avoid preparing virus particles. When a lineage trace is needed, virus-insensitive eggs are useful, since the expression of the gene of interest is restricted to the descendants of the transfected cell in such virus-insensitive embryos ***(7)***. Transfected virus-sensitive tissue transplanted to an insensitive host produces a tissue-specific transfection.

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28

An Overview of *Xenopus* Development

C. Michael Jones and James C. Smith

1. Introduction

Embryos of the amphibian *Xenopus laevis* have been used as a model system for the analysis of developmental mechanisms since the 1950s. As described by Gurdon ***(1)***, one of the reasons for the popularity of *Xenopus* is that it is easy to obtain large numbers of embryos. This is illustrated by the fact that until the mid-1950s, *Xenopus* was used as a pregnancy test in humans: injection of urine from a pregnant woman into the dorsal lymph sac of a female *Xenopus* causes the frog to lay eggs. This simple assay can readily be applied only to *Xenopus,* for as Gurdon points out, to persuade *Rana* species to lay eggs, it is necessary to inject homogenized pituitary glands, a procedure that, furthermore, only works at certain times of the year. Thus, *Xenopus* is the easiest amphib'ian species from which to obtain embryos, especially as it is now possible to buy human chorionic gonadotrophin from Sigma (St. Louis, MO), thus circumventing the requirement, in a busy developmental biology laboratory, for a constant supply of pregnant colleagues.

However, there are other reasons for working on *Xenopus*. The animal is completely aquatic, and therefore much easier to keep than other amphibians, which have a tendency to hop away. It is also rather robust, and only rarely succumbs to disease or infection. The early embryo is relatively large in size and, like all amphibian embryos, is readily accessible to the investigator because it develops outside the mother. Dissection and microinjection are therefore easily performed, and because each embryonic cell is provided with yolk reserves to serve as an energy source, tissues can be isolated and cultured for several days in simple salt solutions without the need for poorly characterized serum components. Finally, development is rapid. Together, these virtues have made the *Xenopus* embryo a favorite of vertebrate developmental biologists.

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This chapter provides a brief overview of *Xenopus* embryogenesis from egg to the formation of the definitive body plan. It concentrates on the stages that are most relevant to the molecular embryologist; other, more detailed, descriptions have recently been published in **refs. *2*** and ***3***. In particular, the movements of gastrulation have been extensively described by Keller and colleagues ***(3–5)***. The staging series is that of Nieuwkoop and Faber ***(6)***.

2. The Egg and Fertilization

The *Xenopus* egg is 1.2–1.4 mm in diameter, and consists of a darkly pigmented "animal hemisphere" and a lighter yolky "vegetal hemisphere." When laid, the eggs are oriented randomly with respect to gravity, and held in position by a transparent vitelline membrane inside a jelly coat (**Fig. 1**), but after fertilization, granules located just below the surface of the egg fuse with the plasma membrane and release their contents into the space between the vitelline membrane and the egg. This material provides some lubrication, allowing the egg to rotate such that the less dense animal hemisphere is uppermost. This rotation usually occurs within 20 min of fertilization.

Xenopus is monospermic, and the successful sperm enters the egg in the animal hemisphere. The site of sperm entry (the "sperm entry point," or SEP) is often visible as a small aggregation of pigment. The position of sperm entry defines the future dorsal–ventral axis of the embryo: the future "dorsal" side of embryo forms from the side of the egg opposite the SEP. (Dorsal is in quotation marks in the previous sentence, because the dorso–ventral axis of the egg is not directly translated into the dorso–ventral axis of the tadpole; *see below*.) The SEP defines the dorso–ventral axis of the embryo by determining the direction of rotation of a cortical layer of cytoplasm, just beneath the plasma membrane. This rotation, of about 30°, is driven by a transiently aligned microtubule array in the vegetal hemisphere of the egg and begins about 40 min after fertilization. Through mechanisms that are still completely unclear, the rotation establishes a signaling center, often referred to as the "Nieuwkoop Center," which directs the development of the dorso–anterior region of the embryo (*see below*).

3. Early Cleavage Stages

It is important to note that early stages of *Xenopus* development rely completely on maternal stores of RNA and protein; transcription does not begin until the so-called midblastula transition, about 7 h after fertilization and when there are 4096 cells (that is, after 12 cleavages) ***(7)***.

The first *Xenopus* cell cycle occupies about 90 min at 21°C; subsequent cycles last about 30 min. One of the greatest advantages of *Xenopus* as a tool for developmental biology is that cleavage planes are regular and result in the formation of identifiable blastomeres whose fates may be predicted. It is important

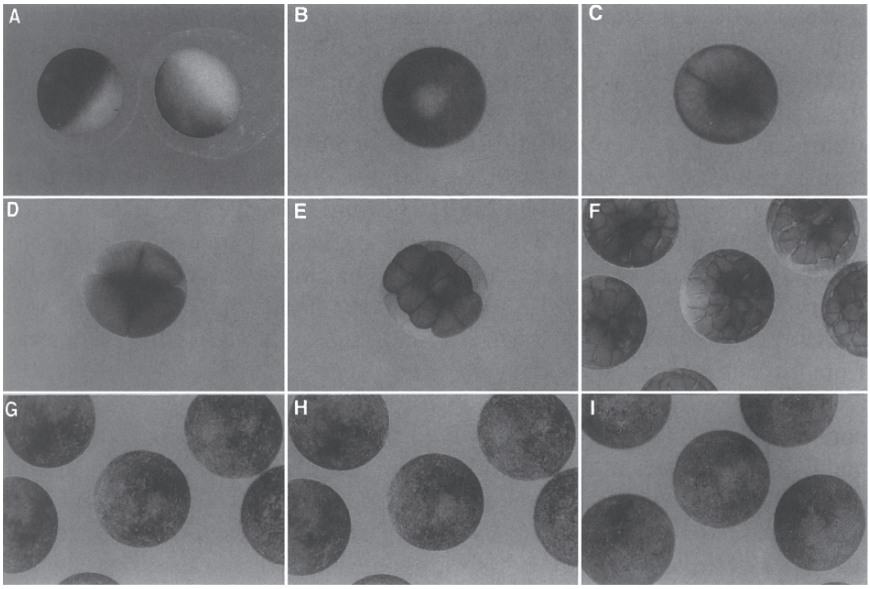

Fig. 1. *Xenopus* eggs and embryos at cleavage and blastula stages. **(A)** Unfertilized eggs. The darkly pigmented animal hemisphere is easily distinguishable from the lighter vegetal hemisphere. The swollen translucent jelly coat is evident around each. **(B)** Animal view of a one-cell embryo after removal of the jelly coat. **(C)** A two-cell embryo. The cleavage plane divides the embryo into the future right and left halves along the animal–vegetal axis. **(D)** Animal view of a four-cell stage embryo. Note the pigmentation differences between the smaller, more lightly pigmented presumptive dorsal blastomeres (the upper two) and the presumptive ventral blastomeres. **(E)** A 16-cell embryo. The smaller animal blastomeres are resting on the larger vegetal cells. **(F)** Animal view of 32- to 64-cell embryos. The lightly pigmented presumptive dorsal side (to the left) is easily recognized in the embryo in the center. **(G)** The same embryos as in (F) at a midblastula stage (stage 8). The embryos consist of approx 4000 cells. **(H)** The same embryos at a later blastula stage (stage 9). Note the smaller size of the cells than in (F) and (G). **(I)** Embryos at a very late blastula stage, just prior to the beginning of gastrulation. The images in (B)–(E) are photographed at the same magnification. The embryos are approx 1.3 mm in diameter.

to emphasize, however, that the fate maps described here, and by other workers, apply only to embryos in which the cleavages are archetypical. Sometimes (indeed, quite often), cleavages are irregular, and although the embryo develops perifectly normally, it is not possible in these cases to predict the fates of particular cells.

Bearing these provisos in mind, the first cleavage in *Xenopus* separates the future leftand right-hand sides of the embryo. The second, 30 min later, is at right-angles to the first and separates the future dorsal and ventral halves. At

this four-cell stage, it is often possible to distinguish the two dorsal blastomeres from their ventral counterparts: if the first two cleavages have occurred in a regular pattern, the dorsal blastomeres are usually slightly smaller and more lightly pigmented than the ventral cells. The third cleavage, which occurs after another 30 min, is orthogonal to the first two, and separates the animal and vegetal poles. Unlike the first two divisions, however, which divide the embryos into (roughly) equal pieces, the plane of the third cleavage is well above the equator of the embryo, reflecting a rule that all cells cleave away from the side with more yolky cytoplasm.

After the fourth (meridional) and fifth (equatorial) cleavages, the embryo comprises 32 cells, arranged in four tiers of eight cells (stage 6 of Nieuwkoop and Faber). The fates of these cells have been determined by microinjection of cell lineage markers ***(8,9)***, and the fate map of Dale and Slack is shown in **Fig. 2**. Notice that individual cells at this stage are far from restricted to a single fate, and that there may be considerable variation from embryo to embryo.

During the early cleavage stages, and beginning with the first, a small space forms becomes larger as cleavages proceed, and eventually becomes the blastocoel.

4. Blastula Stages

This rapid series of cleavage divisions continues beyond the 32-cell stage, resulting in the formation of progressively smaller cells. Blastomeres are larger at the vegetal pole than the animal pole, a consequence of the rule that cleavages occur away from the more yolky region of cells. The first tangential cleavage occurs at the early blastula stage (stage 7), and this changes the previously single-cell-layered morula into a double-layered embryo. What was the cleavage cavity is now called the blastocoel, and this enlarges by osmotic uptake of water (*see* **ref. *10***).

This pattern of development—one cleavage every 30 min—lasts until cycle 13, when division becomes asynchronous, and it slows down significantly ***(7)***. This point, the midblastula transition (MBT), is also marked by the onset of cell motility and zygotic transcription. It corresponds to stage 8 of ***(6)*** and presages the next important stage in *Xenopus* development gastrulation.

5. Gastrulation

During gastrulation, the blastula, a hollow ball of cells with radial symmetry, is converted into a three-layered structure with a central midline and bilateral symmetry. The highly dramatic movements of gastrulation are preceded, during late blastula stages, by pregastrulation movements of which the most obvious is epiboly, a vegetally directed movement of animal hemisphere cells. This results in a thinning of the blastocoel roof and the accumulation of prospective mesodermal cells from a position above the equator of the embryo to a subequatorial location.

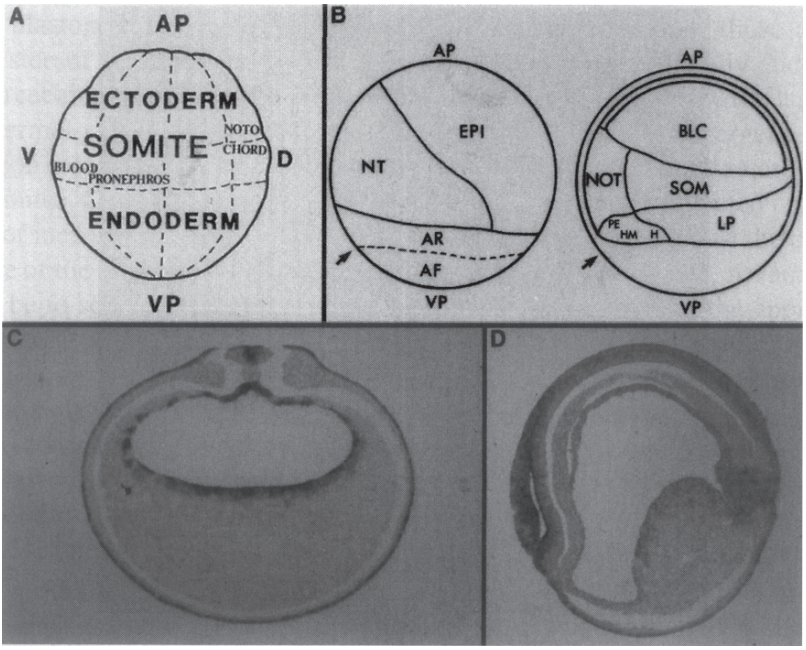

Fig. 2. Fate maps of *Xenopus* embryos, including tracing the outer layer of late blastula embryos by marking the cells with Bolton-Hunter Reagent ***(11)***. **(A)** Fate map of the 32-cell embryo ***(8)***. **(B)** Fate maps of the superficial (left) and deep layers (right) of an early gastrula embryo. Reproduced from Keller ***(12,13)***. **(C)** Transverse section of a neurula stage embryo that was dipped in Bolton- Hunter Reagent at a blastula stage to mark the outer layer of cells. The darkly marked cells have migrated during gastrula stages and can be seen lining the archenteron, in the neural tube and in the epidermis. Dorsal is to the top. **(D)** A sagittal section of a similarly marked embryo showing darkly marked cells in the same tissues. Anterior is to the left and dorsal upward in this photo. AP, animal pole; VP, vegetal pole; V, ventral; D, dorsal; NT, neural tissue; AR, archenteron roof; AF, archenteron floor; EPI, epidermis; BLC, blastocoel; NOT, notochord; SOM, somite; LP, lateral plate; PE, pharyngeal endorlerm; HM, head mesoderm; H, heart.

The first sign of gastrulation proper is the appearance of a pigmented depression in the dorsal—vegetal quadrant of the embryo—the dorsal lip of the blastopore (**Fig. 3**). Formation of this blastopore lip, and the associated line of pigment, reflects the formation of the so-called bottle cells. These are a group of superficial cells that undergo dramatic changes in shape in which their apices contract and cytoplasm is forced away from the surface of the embryo. This causes the cells to adopt their eponymous bottle-like shape, and the accumulation of pigment granules in the apices of the cells results in the formation of the blastoporal pigment line. The first bottle cells to form do so on the dorsal side

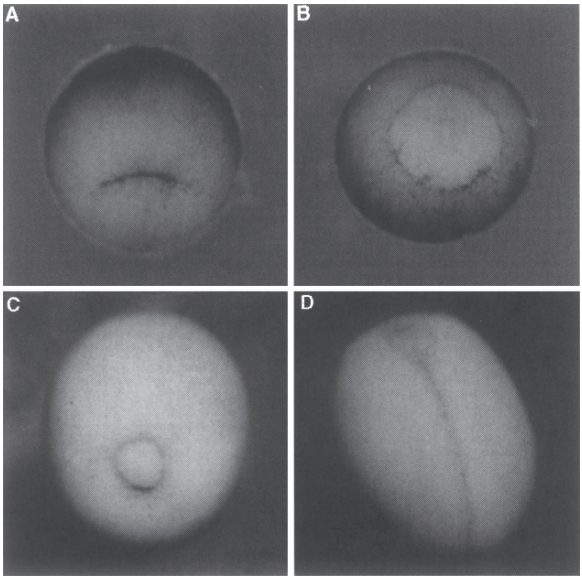

Fig. 3. Blastopore regions of gastrulae and a dorsal view of an early neurula stage embryo. **(A)** Dorso–vegetal view of an early gastrula (stage 10.5). Note the darkly pigmented, crescent-shaped dorsal blastopore lip. The animal hemisphere is upward. **(B)** Vegetal view of a midgastrula (stage 11). The blastopore is now circular. Dorsal is to the top. **(C)** A postero–ventral view of a late gastrula embryo (stage 12.5). The closing circular blastopore is now smaller than at earlier stages. **(D)** Dorsal view of an early neurula (stage 15). The more darkly pigmented closing neural tube is evident in the midline. Anterior is to the upper left.

of the embryo, but they are soon joined by more lateral and ventral cells, so that the lip becomes an arc, then a semicircle, and finally a complete circle. As development proceeds, the bottle cells are propelled to the interior of the embryo by the mesodermal cells. Eventually, by the midgastrula stage, they respread on the roof of the blastocoel and form part of the archenteron, or gut.

At the same time that the blastopore lip becomes visible on the outside of the embryo, prospective mesodermal cells on the inside ***(11)*** begin to migrate across the roof of the blastocoel toward the animal pole. As with the formation of the blastopore lip, the first mesodermal cells to migrate are those at the dorsal side of the embryo, and the movement then spreads laterally and ventrally, reaching the ventral side by the late gastrula stage. It is only the leading mesodermal cells that migrate; those that follow undergo convergence and extension, processes that occur most vigorously in the prospective notochord and somites. Convergence and extension both result from the directed intercalation of mesodermal cells in such a way that they converge toward the dorsal midline of the embryo and in doing so

cause the embryo to extend. An analogy would be to squeeze a tube of toothpaste (not from the bottom, the approach we prefer in a domestic situation, but rather by gripping the whole tube in one's fist). As the toothpaste converges toward the center of the tube, it extends from the hole at the end.

As convergence and extension proceed, the circumference of the blastopore becomes smaller, and eventually is reduced to a slit. The cells of the animal hemisphere now cover the entire embryo, and constitute the ectoderm. The mesoderm lies beneath the ectoderm, having reached this position through active movements, such as migration, convergence, and extension. Finally, the endoderm lies within the mesoderm. The endoderm occupies this position partly by default, because the yolk mass is dragged passively into the middle of the embryo by the mesoderm. However, the superficial cells of the early gastrula that go on to form the endoderm (remember that in *Xenopus*, all the mesoderm is formed from deep tissue ***[11]***) also undergo convergent extension, and these foim the roof and walls of the archenteron, or primitive gut (**Fig. 2**).

The closure of the blastopore marks the end of gastrulation proper, and by this stage the three germ layers have reached their definitive positions. The animal–vegetal and dorso–ventral axes of the blastula-stage embryo are no more, and although at first sight it might seem that they can be translated directly into the antero–posterior and dorso–ventral axes of the late gastrula/early neurula stage embryo, this is not so. The earlier, and more dramatic, gastrulation movements on the "dorsal" side of the early gastrula ensure that these cells form most of the anterior tissues of the embryo as well as the entire notochord and much of the somites. The "ventral" side of the early gastrula forms much of the posterior of the embryo, and, especially toward the tail, gives rise even to substantial amounts of somitic tissue.

Although many region-specific markers are expressed during gastrula stages (which can be used in experiments on inductive interactions), little obvious cytodifferentiation has occurred. The most obvious tissue at this stage is the notochord, which has physically separated from the somites that flank it, and has acquired a "stack-of-coins" appearance.

6. Neurulation and Beyond

Early in neurulation, the posterior neural plate, like the mesoderm, undergoes convergence and extension, thus assisting in blastopore closure. This movement is autonomous and does not require the mesoderm to be underneath the neural plate. At later stages, the process of neurulation in *Xenopus* is much less dramatic than in urodele amphibia, such as *Ambystoma mexicanum*; the neural folds, in particular, are not as obvious, and they do not form the classic, well-defined "keyhole" shape. That said, the first sign of neurulation in *Xenopus* is the thickening of the inner layer of dorsal ectoderm, the so-called censorial

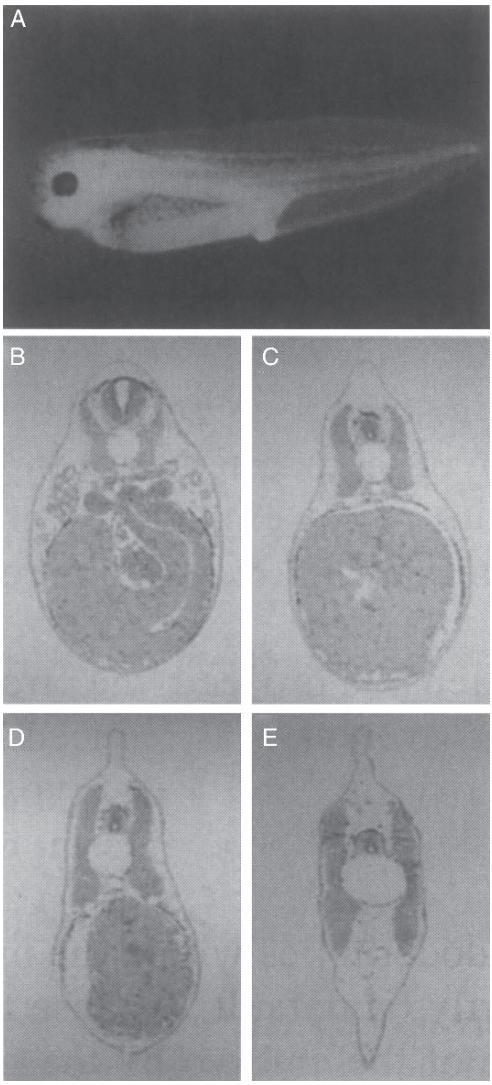

Fig. 4. A late stage tadpole and sections of a similar stage highlighting the tissue organization that results after gastrulation and neurulation are complete. **(A)** A tadpole (stage 40). Anterior is to the left and dorsal to the top. **(B)** A section through the anterior region of the tadpole. The neural tube is stained a magenta color owing to the high concentration of nuclei and is the uppermost structure. Beneath the neural tube is the vacuolated notochord, which is flanked on each side by somitic tissue (stained green). Nephtic tubules are evident lateral and ventral to the somites, and the large yolk-filed gut (endoderm) is stained yellow. **(C)** A slightly more posterior section than (B). The neural tube, notochord, somites, and endoderrn are evident. **(D)** An even more posterior section than (B) or (C). **(E)** A section through the tail region. Note the proportionally smaller neural tube and larger somites than in more anterior regions. The notochord is a similar size along the anterior–posterior axis. The magnification in (E) is slightly greater than in (B)–(D).

layer. This is followed, at the midneurula stage, by the formation of a dark pigment line along the dorsal midline of the embryo. This neural groove arises through the formation of bottle-like cells in the neural midline, which, like the bottle cells of the blastopore lip, contract their apices and concentrate the pigment granules in a smaller area. As neurulation proceeds, the neural groove deepens, and the lateral neural folds converge on the dorsal midline, where they eventually fuse to form the neural tube (**Fig. 3D**).

The most lateral cells of the neural plate do not participate in neural tube closure and are not recruited into the neural tube. These are the future neural crest cells, which in the truck go on to form pigment cells and neural derivatives, such as the dorsal root ganglion, and in the head form a wide variety of structures, such as the cephalic ganglia, the mandibular, hyoid, and branchial arches, and the head mesenchyme.

Finally, during and after neurulation, the mesoderm becomes subdivided into different tissues along the dorso–lventral axis (**Fig. 4**). The most dorsal mesodermal cell type is the notochord, a rod of vacuolated cells running the length of the embryo. Lateral to the notochord are the cells of the somites, which in *Xenopus* form predominantly muscle, and lateral and ventral to the somites are the cells of the pronephros. The lateral mesoderm goes on eventually to form structures such as the limbs, and the most ventral mesoderm forms blood.

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29

Mesoderm Induction Assays

C. Michael Jones and James C. Smith

1. Introduction

Inductive interactions play a major role in early development, and one of the earliest such interactions in amphibian development, and perhaps the in development of all vertebrates, is mesoderm induction ***(1–5)***. Mesoderm induction occurs at blastula stages, when a signal from the vegetal hemisphere of the embryo acts on overlying equatorial cells, causing them to form mesoderm rather than ectoderm. This interaction was first discovered in experiments in which prospective ectodermal tissue of the embryo (the so-called "animal cap") is juxtaposed with future endoderm from the vegetal hemisphere (**Fig. 1**). When cultured alone, the animal caps form epidermis; when cultured adjacent to vegetal pole blastomeres, they form mesoderm.

In the last decade, great advances have been made in coming to understand the signals involved in mesoderm induction as well as in identifying the intracellular signal transduction pathways used by these factors. Progress has also been made in identifying target genes of mesoderm-inducing factors, and even in studying how the transcription of these genes is regulated. This chapter describes three assays for mesoderm induction. The first is the original assay described above, in which animal pole and vegetal pole tissue is juxtaposed. In the second, the inducing factor is supplied to the target tissue not from vegetal pole cells, but as a soluble protein, and in the third, the inducing agent is supplied to the responding cells by microinjecting RNA encoding the protein in question into the developing embryo.

This last method has proven particularly useful for studying proteins that cannot yet be obtained in purified, soluble form ***(6,7)*** and for studying the activities of intracellular proteins, such as components of the MAP kinase pathway ***(8–10)***. The technique can also be adapted to identify novel mesoderm-inducing agents

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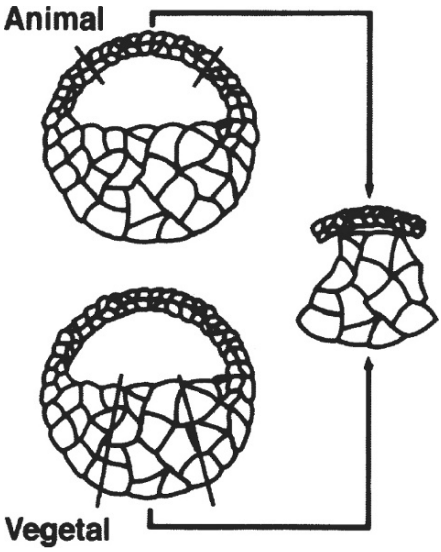

Fig. 1. Mesoderm induction. Cells from the animal hemisphere of the embryo are juxtaposed with cells from the vegetal hemisphere.

by "expression-cloning" ***(11)***. Finally, mesoderm induction can also be used as a convenient read-out for ligand–receptor interactions, which have nothing to do with the *in vivo* process. For example coexpression of GDNF and the receptor tyrosine kinase Ret in *Xenopus* animal caps causes the formation of mesoderm, whereas expression of either protein alone has no effect, thus suggesting that GDNF signals through Ret ***(12)***.

2. Materials

All solutions used for culture of *Xenopus* tissue should use distilled water, and should be sterilized by autoclaving or filtration as indicated below.

2.1. Obtaining Xenopus *Embryos by Artificial Fertilization*

1. *Xenopus laevis* adult females and males: These may be obtained from a variety of sources; up-to-date details of suppliers throughout the world are available on the *Xenopus* molecular marker resource: URL http://vize222.zo.utexas.edu/. Instructions for keeping *Xenopus* are beyond the scope of this chapter; interested readers may refer to **ref. *13***.
2. Pregnant mare serum gonadotrophin (PMSG; Sigma, St. Louis, MO).
3. Human chorionic gonadotrophin (HCG; Sigma).
4. 2-mL Syringe and 23- to 25-gage needles.

2.2. Dissection Dishes and Instruments

1. 35- or 60 mm Petri dishes coated with a thin layer of 1% agarose in water, previously sterilized by autoclaving.
2. Two pairs of no. 5 Dumont watchmaker's forceps, sharpened using an "Arkansas" sharpening stone.
3. Tungsten needles are made by mounting about 5 cm of tungsten wire (0.5-mm diameter. 99.95% purity, Goodfellow metals) in a 7 cm piece of capillary tubing. The needle is sharpened by electrolysis in 1 *M* NaOH using a potential difference of 9–12 V AC. The simplest procedure is to place a small piece of modeling clay on the end of the wire and to dip about 2 mm of naked wire into the NaOH. When the clay falls off, the needle is sharp enough.

2.3. Culture Media

1. Embryos and explants are cultured in dilutions of Normal Amphibian Medium (NAM; ***14***): 110 m*M* NaCl; 2 m*M* KCl; 1 m*M* $Ca(NO_3)_2$; 1 m*M* $MgSO_4$; 0.1 m*M* Na_2EDTA; 2 m*M* sodium phosphate pH 7.5; 1 m*M* $NaHCO_3$; 50 µg/mL gentamycin. It is convenient to prepare a 10X stock solution of all the components of NAM with the exception of the phosphate buffer, the $NaHCO_3$, and the gentamycin. This 10X "NAM salts" stock may be autoclaved. We then prepare:
   a. 0.1 *M* Sodium phosphate pH 7.5, which may be autoclaved and serves as a 50X stock.
   b. 0.1 *M* $NaHCO_3$, which is filter-sterilized and stored frozen and serves as a 100X stock.
   c. 10 mg/mL gentamycin which is also filter-sterilized and stored frozen and serves as a 200X stock.

   Dilutions of NAM refer only to the "NAM stocks" and $NaHCO_3$; levels of phosphate buffer and gentamycin are held constant.
2. 4% Ficoll in 75% NAM.
3. 0.1% Bovine serum albumin (BSA) in 75% NAM.
4. 60% Leibovitz L-15 medium (for storage of *Xenopus* testes).
5. 2% Cysteine hydrochloride, adjusted to pH 7.9–8.1 with NaOH.
6. Sterile mineral oil.

2.4. Microinjection Equipment

As discussed previously ***(13)***, there are several different techniques for injecting RNA into *Xenopus* eggs, and it is not possible to describe them all. We therefore describe the methods used in our own laboratory.

1. Microinjection needles are prepared from capillary tubing, such as GC120F-15 made by Clark Electromedical Instruments (Reading, England). We have used two types of needle puller. One consists of nothing more than a heated platinum coil. The capillary is clamped at its top, passed down through the coil, and a piece of modeling clay (about 7 g) is attached to the bottom. The current is then switched on. The shape of the needle and the diameter of the tip depend on the amount of current passed through the coil, the exact weight of the modeling clay, and on whether the investigator is able to catch the tubing before it hits the

bench. The second option is to use more expensive pullers, such as those made by Campden Instruments or Kopf. Whatever kind of puller is used, the diameter of the needle tip should not exceed 15 µm. Needles should be heated to 180°C to destroy RNAase activity, and they should be stored in a dust-free atmosphere.
2. Singer Instrument Company Micromanipulator, Mark I.
3. Inject + Matic air-driven injector (Supplied by Micro Instruments Ltd., Long Hanborough, Oxford, UK).

2.5. Lineage Labeling

This is performed with fluorescein-lysine-dextran (Fluoro-emerald: Molecular Probes, Eugene, OR).

2.6. RNA Transcription

Stable transcripts and high yields of protein are produced from cDNAs cloned into the vector pSP64T ***(15)*** or pCS2+ ***(16,17)***. The original version of pSP64T is slightly inconvenient, because although it has several sites for linearization, the only cloning site is *Bgl*II. Several laboratories have, however, produced more user-friendly versions of pSP64T, which include a multiple cloning site, and two such vectors are available from our colleague Masazumi Tada. Plasmids should be linearized before transcription with a restriction enzyme that generates a blunt end or a 5′-overhang.

1. Linearized plasmid at 1 mg/mL.
2. 10X Transcription buffer: 400 m*M* Tris (pH 7.5), 60 m*M* $MgCl_2$, 20 m*M* spermidine HCl, 50 m*M* NaCl.
3. 0.25 *M* DTT.
4. 10 m*M* ATP.
5. 10 m*M* CTP.
6. 10 m*M* UTP.
7. 1 m*M* GTP, 10 m*M* GTP.
8. 2.5 m*M* GpppG.
9. RNasin.
10. SP6 RNA polymerase.
11. RNase-free water.
12. RQ1 DNase I (Promega).
13. Phenol/chloroform.
14. Ethanol.
15. 1% Agarose TBE gel (RNase-free).

3. Methods

3.1. Artificial Fertilization of Xenopus Eggs

Xenopus females are induced to lay eggs by the injection of 500–1000 IU human chorionic gonadotrophin (HCG) into their dorsal lymph sacs. This technique is

defined as a "procedure" under the UK Animals (Scientific Procedures) Act of 1986, and requires a Home Office license. Yields of eggs may be improved if the frogs are "primed" a few days before HCG injection with 30 IU of pregnant mare serum gonadotrophin (PMSG). It is prudent to inject two or three frogs in order to be sure of obtaining enough embryos for an experiment. In what follows, it is assumed that the investigator is right-handed.

1. Dissolve 30 IU PMSG in 0.5 mL water and load a 2-mL syringe.
2. Select a female frog (fat ones are usually good). Hold her still on a flat surface. We use the left hand to hold the frog, with the heel of the hand pressed firmly against the bench in front of the frog's head, and with the middle and index fingers between the frog's back legs. If the muscles of the hand are tensed, as if making a "bridge" for a snooker or pool cue, the hand forms a "cage," which restrains the frog without exerting significant pressure on her.
3. Injections are made into the dorsal lymph sac of the animal. Using the right hand, the injection needle should be inserted just beneath the skin on the dorsal surface of the frog's right hindleg. The needle tip should then be moved beneath the row of stitches of the lateral line organs toward the dorsal midline. After injection, the needle tip should be withdrawn slowly along the path of insertion. There should be little or no bleeding, and the frog should not experience any discomfort. It should be returned gently to a marked tank.
4. Two or 3 d after injection of PMSG, frogs should receive injections of 500–1000 IU HCG, using the same technique. They should be kept overnight in the dark, at 18–22°C.
5. To obtain testes, male *Xenopus* should be killed by heavy anesthesia followed by decapitation and rostral and caudal pithing. The testes are pale, curved structures about 1 cm long, which are positioned on either side of the spine. They may be removed by making an incision in the ventral surface of the animal, and they can be stored in 60% Leibovitz L-15 medium at 4°C for up to 1 wk.
6. Female *Xenopus* should start to lay eggs the morning after injection. Fresh eggs can be "squeezed" from the frogs by gentle peristalsis of their ventro-lateral surfaces. The eggs should be transferred to a Petri dish and rinsed with distilled water. Then, using a Pasteur pipet, as much liquid as possible should be removed from the eggs.
7. Fertilize the eggs by rubbing them lightly with a piece of dissected testis.
8. Wait 5 min, and flood the eggs with 10% NAM. After about 15 min, the eggs should rotate so that their heavily pigmented animal hemisphere is uppermost. This is a reliable sign of successful fertilization.
9. "Dejelly" the fertilized eggs by incubating them for about 5 min in 2% cysteine hydrochloride. Rinse thoroughly in 10% NAM. Transfer to an agarose-coated Petri dish. Embryos should begin to cleave about 90 min after fertilization. They can be cultured at temperatures between 14 and 23°C. At the warmer temperature, it takes about 5 h for embryos to reach stage 8 (the midblastula stage), which is when mesoderm induction assays are usually carried out.

3.2. RNA Synthesis

1. Set up transcription reaction at room temperature:
   a. Linearized DNA (1 mg/mL) 5 µL
   b. 10X transcription buffer 5 µL
   c. 0.25 *M* DTT 2.5 µL
   d. 10 m*M* ATP 5 µL
   e. 10 m*M* CTP 5 µL
   f. 10 m*M* UTP 5 µL
   g. 1 m*M* GTP 5 µL
   h. 2.5 m*M* GpppG 10 µL
   i. RNasin 2.5 µL
   j. SP6 RNA polymerase 2.5 µL
   k. RNase-free water 2.5 µL
2. Mix gently, spin briefly to get the components to the bottom of the tube, and incubate at 37°C for 30 min.
3. Add 2.5 µL 10 m*M* GTP, and incubate for an additional 1 h.
4. Add 5 µL RQ1 DNase I, and incubate at 37°C for 30 min.
5. Extract twice with phenol/chloroform, and ethanol-precipitate twice. Wash the pellet twice with 75% ethanol.
6. Redissolve RNA in 30 µL DEPC-treated water. Measure A_{280}, and adjust RNA concentration to 1 mg/mL.

RNA can be tested for integrity by taking 1 µL of the reaction after **step 4** and running it on a 1% RNaase-free TBE gel. There should be a strong RNA band of the appropriate size.

3.3. Microinjection

Microinjection of RNA and of the lineage tracer fluorescein-lysine-dextran uses the same technique, except that it is essential to bake injection needles used for RNA to destroy RNase activity. As stated above, there are several different kinds of injection apparatus, and our description is therefore rather general.

1. Transfer embryos to be injected to 4% Ficoll in 75% NAM.
2. Attach the injection needle to the micromanipulator and injection system. Dispense about 2 µL of water onto a small piece of parafilm, and suck it up into the pipet. This rinses the pipet and allows one to calibrate it.
3. Calibrate the injector/needle combination by injecting into a dish of sterile mineral oil. Calculate the volume injected by the formula $V = 4/3\pi r^3$. A volume of about 10 nL can be safely injected into a fertilized egg.
4. Expel water, and load the injection sample.
5. Inject embryos. This is most easily done by supporting the embryo with a pair of forceps held in the left hand (this is for a right-hander again) and using the right hand to control the micromanipulator. Injections can be made into any part of the egg, but for uniform distribution of the injected material, it is best to aim for the

equatorial region, where the pigmented animal hemisphere meets the paler vegetal hemisphere. A few hundred embryos can be injected in an hour.
6. Culture embryos in 4% Ficoll in 75% NAM until stage 8.

3.4. Animal/Vegetal Conjugates

1. When they reach stage 8, remove the vitelline membranes from a group of embryos using sharpened forceps. Half the embryos should have been lineage-labeled by injection of fluorescein-lysine-dextran injection, and half should be uninjected. Transfer embryos to 75% NAM.
2. Dissect animal pole regions from the center of the pigmented regions of the labeled embryos. This can either be done using a pair of forceps as "scissors" or by using a "picking" motion with a tungsten needle. During dissection, the embryo can be kept still using a pair of forceps held in the other hand.
3. Dissect vegetal pole regions from uninjected embryos as described above for animal pole regions.
4. Place an animal pole region with its originally outer surface down, and position a vegetal pole region on top of it such that its originally outer surface is up. The two pieces of tissue will adhere to each other quickly, but care should be taken not to disturb the conjugate for at least 10 min.
5. Culture in 75% NAM at 18–22°C.

3.5. Animal Cap Assay with Soluble Inducer

1. Dissect animal pole regions from Xenopus embryos as described above.
2. Transfer animal caps to 75% NAM containing 0.1% BSA and the putative inducer. It is sensible to use a range of different inducing factor concentrations. Molecules like activin are active at 0.1–10 ng/mL.
3. Culture in 75% NAM at 18–22°C.

3.6. Animal Cap Assay Following RNA Injection

1. Dissect animal pole regions from *Xenopus* embryos, which have been injected with RNA encoding the putative inducer.
2. Culture in 75% NAM at 18–22°C.

3.7. Scoring the Results

The result of a mesoderm induction assay may be score in several ways. In increasing order of complexity, these are:

1. Observation of gastrulation-like movements. Normally, isolated animal pole tissue "rounds up" after being dissected from the embryo, and it forms a sphere. Treatment with a mesoderm-inducing factor causes the animal pole cells to undergo gastrulation-like movements. However, and the animal caps elongate in a characteristic fashion (***18***; **Fig. 2**). Activin and other members of the TGF-β family cause more dramatic gastrulation movements than do members of the FGF family ***(6,18,19)***. Elongation movements are visible after approx 4 h of culture. When

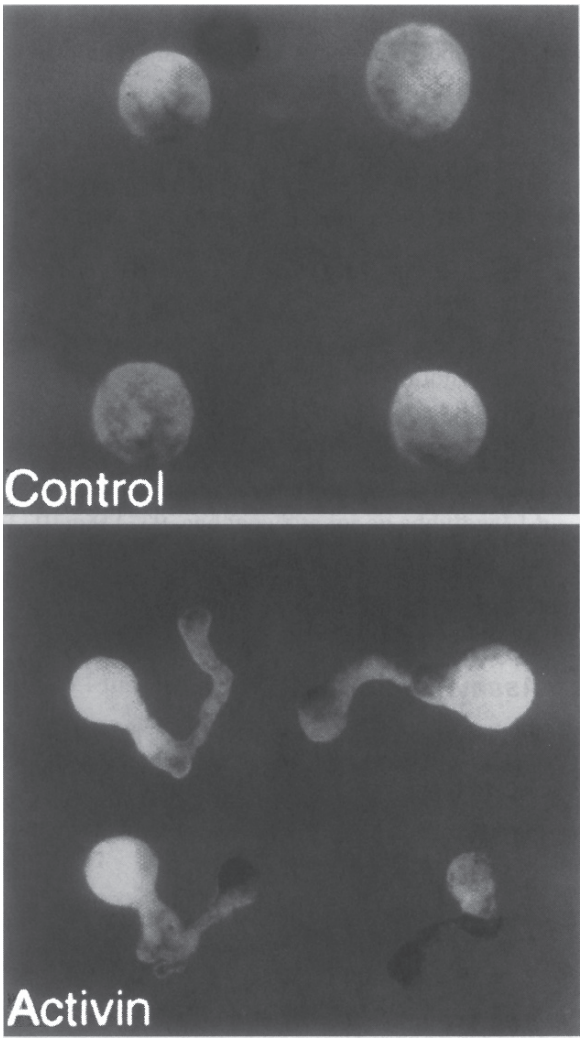

Fig. 2. Gastrulation movements induced by activin treatment of animal pole cells. Control explants on the top form spheres; those treated with activin, on the bottom, elongate.

animal pole–vegetal pole conjugates have been made, observation with a fluorescent microscope should reveal that the cells undergoing gastrulation movements are those derived from the animal cap.

2. Observation of later morphology: After 3 d of culture, when sibling embryos are at tadpole stages, animal caps treated with mesoderm-inducing factors form characteristic structures. In the case of activin, these have been termed "embryoids," because they resemble miniature embryos ***(20)***. Treatment with FGF causes the

formation of translucent balloon-like structures with a layer of smooth muscle beneath epidermis. Uninduced animal caps remain as opaque darkly pigmented spheres.

3. Numerous molecular markers are available that allow the identification not only of mesoderm, but also of which region of the mesoderm has been induced. Suitable markers are listed on the *Xenopus* molecular marker resource: URL http://vize222.zo.utexas.edu/. Expression of these marker genes can be studied by RNase protection or by reverse-transcription PCR (*see* Chapter 43).

4. Notes

Mesoderm induction assays are usually very reliable. However:

1. If fertilization of eggs is unsuccessful, it is usually the fault of the eggs rather than the sperm. The sperm can be checked by observation under an inverted phase-contrast microscope. If they are healthy, they should wiggle.
2. Sometimes, animal caps just seem to fall apart into their constituent cells for no reason. There is nothing you can do about this—just repeat the experiment.

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Experimental Embryological Methods for Analysis of Neural Induction in the Amphibian

Ray Keller, Ann Poznanski, and Tamira Elul

1. Introduction

Our objective is to describe and critique some of the experimental embryological preparations used to analyze tissue interactions involved in neural induction in amphibians. The molecular basis of neural induction and the tissue interactions that carry the inductive signals are areas of active research, stimulated by the recent identification of several potential neural inducers ***(1–6)***, availability of regional molecular markers easily visualized with a good whole-mount RNA *in situ* hybridization method ***(7)***, and the work on Hox genes that may have a role in specifying regional differentiation of the vertebrate nervous system ***(8)***. These advances demand more of and make more useful the classical embryological manipulations used to characterize the tissue interactions involved in neural induction.

We will first describe the location and movements of the inducing and induced tissues, since misunderstanding of these aspects remains the major source of confusion in experimental design and interpretation in this area of research. Then we will describe several classical embryological methods that have been useful to us and to others in studying neural induction and development, pointing out the problems, difficulties, and liabilities of each of these methods.

1.1. The Origins and Movements of the Inducer and the Induced

A detailed description of the early embryological development of *Xenopus laevis*, with illustrations of anatomical features and key landmarks as seen under the stereomicroscope, has been presented elsewhere ***(9)***. Here we describe those features particularly relevant to analysis of neural induction.

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1.2. Fate Maps of Organizer and Neural Tissue in X. laevis

The fate map of the prospective neural tissue and of the Spemann Organizer, which is thought to be the major neural-inducing tissue, are shown as they appear in the very early gastrula stage of *X. laevis* (**Fig. 1**, stage 10–) ***(10)***. The prospective nervous system forms a crescent on the dorsal side, lying mostly above the equator at this stage. It consists of prospective fore- and midbrain (F, M), hindbrain or rhombencephalon (RH), and spinal cord (SC). The prospective RH and SC are very wide in the mediolateral direction and very short in the anterior–posterior direction, extending about five to seven cells along the dorsal midline ***(10)*** (**Fig. 1**). These regions converge (narrow) mediolaterally, and extend (lengthen) anterior–posteriorly greatly during gastrulation and neurulation, described in detail below. This equatorial–subequatorial annulus of neural tissue undergoing convergent extension closes around the blastopore (**Fig. 1**, stage 12) and is often called the "noninvoluting marginal zone" (NIMZ) ***(11)***.

The "involuting marginal zone" (IMZ) lies vegetal to the prospective neural tissue, and its dorsal sector (about 30°) contains the "Spemann Organizer" ***(12)***. The Organizer contains much of the neural-inducing activity, based on the experiments of Spemann and Mangold in urodeles ***(13)***, as well as others since on *Xenopus* ***(14)***, in which this tissue was grafted to the ventral side of another embryo where it induced the formation of a second axis, including a nervous system. For simplicity, only the tissues of the Organizer lying in the dorsal midline are shown in **Fig. 1**. These tissues are prospective prechordal mesoderm (PM) or "head mesoderm," and prospective notochord (N), both in the deep, nonepithelial region (**Fig. 1**, stage 10–). The superficial epithelial layer of the Organizer consists of prospective endoderm (E) (**Fig. 1**, stage 10–), a fact that is often overlooked. This "suprablastoporal" endoderm lies above the site of blastopore formation and involutes to form the archenteron roof (**Fig. 1**, stages 10– to 17). It is distinct from the "subblastoporal" or vegetal prospective endoderm (VE), which lies below the site of blastopore formation. During gastrulation, the VE is covered over by the IMZ, including the archenteron roof, and becomes the archenteron floor (**Fig. 1**, stage 17). The vegetal end of the suprablastoporal epithelium consists of specialized endodermal cells, prospective bottle cells (BC), which play a role in initiating gastrulation (*see* **Sub-heading 1.3.1.**). At its lateral regions, the organizer includes some prospective anterior somitic mesoderm (not shown in **Fig. 1**).

1.3. Movements of the Inducing and Induced Tissues

1.3.1. Early Events

Gastrulation nominally begins as the cuboidal prospective BC constrict their apices and elongate in the apical-basal direction, acquiring their definitive shape ***(15, 16)*** (**Fig. 1**, stage 10–, 10+). This process begins about 30 min before

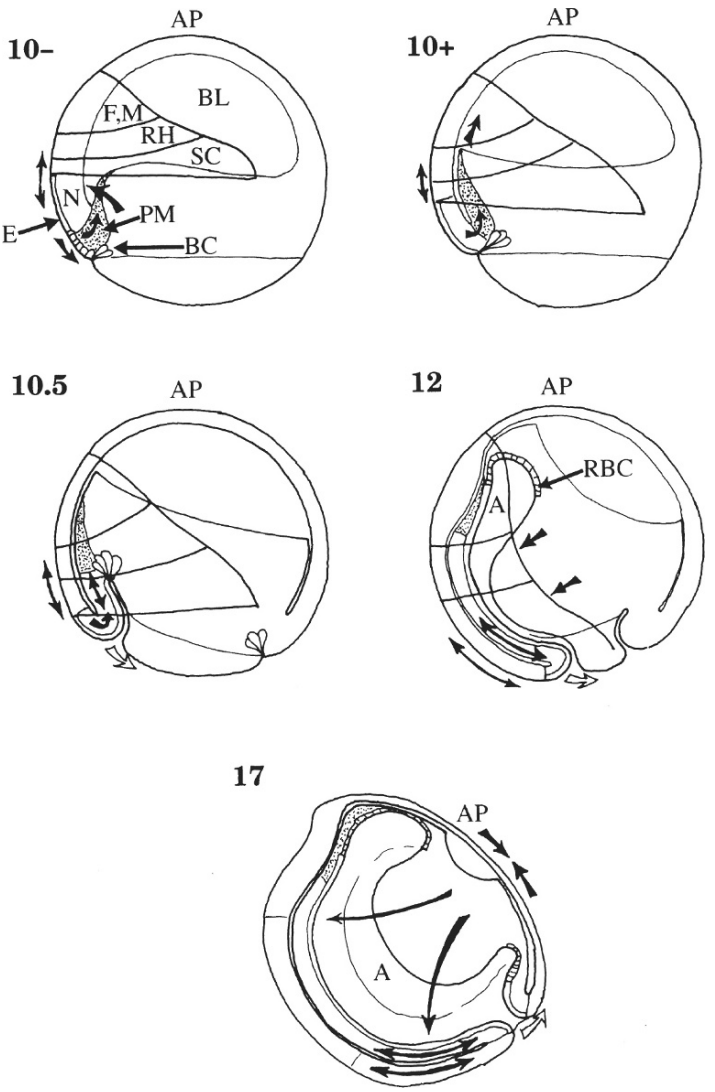

Fig. 1. These diagrams show important aspects of the fates and movements of the prospective neural tissue and the neural-inducing or "Organizer" tissue during gastrulation and neurulation. The embryos are viewed from the right sides such that dorsal is to the left, the AP is at the top, and vegetal pole is at the bottom. The Organizer occupies the dorsal sector of the involuting marginal zone, spreading laterally about 30° on either side of the midline, but for simplicity, only the prospective tissues at the dorsal midline are shown, including prospective prechordal (PM) and prospective notochord (N), covered on the outside by the prospective endoderm (E) of the archenteron roof. Both the midline and the lateral aspects of the prospective neural plate, including the prospective fore and midbrain (F, M), rhombencephalon (RH), and spinal cord (SC) are shown. Movements are indicated by arrows. Also indicated are the BC, the blastocoel (BL), the archenteron (A), and the respread bottle cells (RBC).

stage 10, as described by Nieuwkoop and Faber ***(17)***, and is progressive. The number of cells with constricted apices and the degree of constriction of individual cells increas, resulting in an increasing concentration of black pigment in the apices of these cells, transforming them from yellow to gray, and finally to black (*see* **Fig. 2** of **ref. *16***). As a result, IMZ rotates, the outside being pulled vegetally by the constricting BC, and the deep prospective PM, formerly associated with the deep ends of the prospective BC, is displaced upward ***(16)*** (**Fig. 1**, stages 10–, 10+). At this time, radial intercalation of cells occurs in the dorsal region of the gastrula, including both the prospective nervous system and the Organizer, and these regions become thinner and extend vegetally (*see* ***18,19***) (*see* arrows in **Fig. 1**, stages 10–, 10+). This vegetal extension and the formation of BC act together to rotate the vegetal edge of the IMZ inward and upward, such that it comes to lie beneath and in contact with the overlying prospective neural ectoderm (**Fig. 1**, stage 10+). This process of early, precocious movement of the deep prospective mesoderm was described in detail by Nieuwkoop and Florshutz ***(20)***.

1.3.2. A Stage of Rapid Transition

Because this early rapid movement of PM beneath the prospective neural ectoderm is a critical event in neural induction (*see* **Subheading 4.1.**), we distinguish among and characterize several forms of this stage. Stage 10– is characterized by a small area of prospective BC initiating constriction, their apices darkening slightly, to gray (**Fig. 2**, stage 10–, top). In most 10– embryos, the PM has neither rotated completely nor reached the overlying prospective neural ectoderm. The dorsal blastocoel wall is connected to the blastocoel floor by a smooth concave surface (**Fig. 2**, stage 10–, bottom). In the transition from stage 10– to stage 10, the BC apices constrict and darken further to form the blastoporal pigment line. Initially, this line is straight and extends about one-fifth to one-quarter the diameter of the embryo (stage 10 of Nieuwkoop and Faber, **ref. *17***) (**Fig. 2**, stage 10, top). The interior of the embryo at this stage is variable (**Fig. 2**, stage 10, bottom). It may be identical to that of the previous or the following stages, or intermediate between the two. As more BC are recruited, and those that have already formed constrict more, the field of BC widens and a shallow groove forms (**Fig. 2**, stage 10+, top; cf **Fig. 1**, stage 10+). Inside, the involuting cells have rotated upward, contacting the inner surface of the prospective neural tissue (arrows, **Fig. 2**, stage 10+, bottom). What was previously the smooth concave surface at the margin of the blastocoel has become a cleft along which the PM and the overlying neural tissue are in contact, a fissure called the "cleft of Brachet" (pointer, **Fig. 2**, stage 10+, bottom). Thus the involuted tissue may come into initial apposition with the overlying neural tissue in stage 10 embryos, and this has almost always occurred by stage 10+. Thereafter,

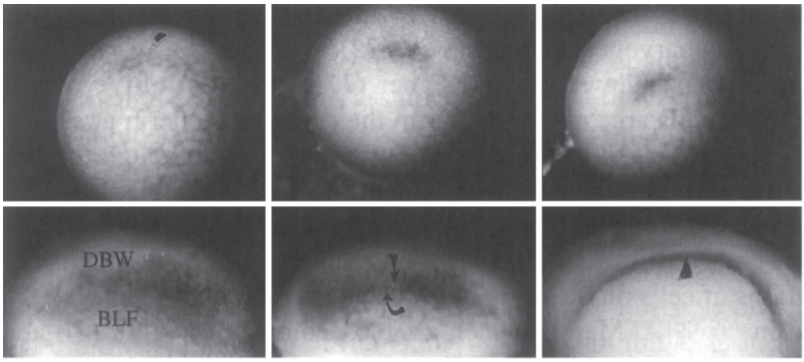

Fig. 2. Video frames taken through the stereomicroscope during dissections to make explants show the external view of the progressive formation of BC (top panels) and the involution of the leading edge of the Organizer mesoderm beneath the prospective neural tissue, on the inside (bottom panels). At stage 10–, only a few of the prospective BC have begun to undergo apical constriction, and these appear in a broad area of gray cells (pointer, stage 10–, top). Internally, the dorsal blastocoel wall (DBW) is continuous with the blastocoel floor (BLF) across a smooth curvature at the lateral edge of the blastocoel (stage 10–, bottom). By stage 10, more BC have formed, and the ones that have formed are darker, indicating greater contraction of their apices (stage 10, top panel). Internally, the curvature of the lateral margin of the blastocoel decreases in radius as the blastocoel floor rises and the blastocoel wall is pulled vegetally (arrows, stage 10, bottom). By stage 10+, more BC have formed, the constriction of those already formed is greater, and a shallow invagination has developed (stage 10+, top). Internally, the leading edge of the Organizer has rotated upward and outward, contacting the overlying neural tissue across the cleft of Brachet (pointer, stage 10+, bottom).

the cells spread on, and begin to migrate anteriorly on, the inner surface of the prospective neural tissue.

The prospective fate of this early involuting tissue is heterogeneous. A tongue of smaller, grayish or brownish PM cells extends animally from the bottle cells along the inner surface of the neural ectoderm (stippled, **Fig. 1**, stage 10+). By stage 10+ to 10.25, this tongue of PM cells reaches the leading edge of the involuted material in some embryos. In others, the tongue falls short of the leading edge. Central to these PM cells, and anterior to them as well (in embryos in which they do not reach the leading edge of the involuted tissue), larger, light-colored cells with coarse yolk platelets are found. These larger cells are indistinguishable from the rest of the central endodermal cells extending upward from the vegetal pole. Because cells in this region map to the pharyngeal region of the embryo, or anterior to the pharynx, they are thus referred to as pharyngeal endoderm ***(20–22)***.

According to Nieuwkoop and Faber ***(17)***, these definitive mesodermal mantle (PM) cells are "delimited" from the central endoderm at approx stage 10.25. This appears to be the case in our experience, although the timing of their appearance and the ease with which they can be distinguished from the remaining central endodermal cells varies between spawnings and sometimes between embryos in a single spawning. Nakatsuji ***(23)*** distinguished these populations of cells on the basis of the size distribution of their yolk platelets, as seen in sections. Markers, such as goosecoid ***(24)***, noggin ***(4)***, and Otx2 ***(25)***, are expressed in this PM or "head" mesoderm, although on the basis of location, the "pharyngeal endoderm" may also express these markers (*see also* **ref.** ***25a***). Vodicka and Gerhart ***(26)*** correlated the early regions and movements of the organizer tissues with the expression of molecular markers, specifically, Xbra, noggin, goosecoid, and XNR3, in fate maps (*also see* the fate map in **ref.** ***27***). Whether the PM, the pharyngeal endoderm, or both, contribute to the inductive activity of the anterior organizer, and whether their contributions are the same or different from one another is a matter of speculation, since no assays of induction have clearly distinguished between them.

Since stage 10 involves rapid internal transitions, the external staging criteria by which it is defined is not a reliable predictor of internal events. Nieuwkoop and Faber ***(17)*** describe the dorsal gastrula wall of this stage as having one epithelial layer from two or three to five or six layers of deep cell. This range reflects the rapid radial intercalation (*see* ***19***) and vegetal extension (*see* **ref.** ***18***) in the dorsal gastrula wall from stage 10– through stage 10+, a process that appears not to be strictly correlated with the BC formation on which external staging is based. Thus, in cases where the presence or absence of this early contact with the neural tissue appears to be important, we directly determine the amount of involution that has occurred at a given external stage (*see* **Subheading 4.2.3.**), particularly during the transitional stage 10, which is less consistent internally, than stage 10– or 10+.

1.3.3. Convergent Extension: a Good Way to Make an Axis (and a Good Way to Fool the Investigator!)

The posterior regions of both the neural ectoderm and the dorsal mesoderm are originally very wide and very short, and they acquire their final form by extreme convergence (narrowing) and extension (lengthening) during gastrulation and neurulation ***(10)***. One can be seriously misled in designing and interpreting neural induction experiments if these convergent extension movements and their contribution to embryonic development are misunderstood. The common impression of events following BC formation (**Fig. 1**, stage 10.5) is that the length of the dorsal axial structures (the nervous system, the notochord, and the archenteron) is generated as the involuted mesoderm crawls anteriorly a long distance, and finally

comes to rest beneath the appropriate part of the prospective neural ectoderm. It is assumed that at that time, or perhaps before, neural induction occurs as signals are passed from the mesoderm to the neural ectoderm.

In fact, this is not what occurs. After BC formation, the mesoderm contacts the neural ectoderm at or slightly above the equator (**Fig. 1**, stage 10+), and it only migrates approximately to the animal pole (**Fig. 1**, stages 12–17). Thus the leading edge of the involuted mesoderm actually migrates only about a quarter of the circumference of the embryo. Although this migration is very important, it contributes little to the elongation of the dorsal aspect of the embryo. The length of the dorsal embryonic tissues is generated as the nervous system extends posteriorly, across the VE, and converges transversely (mediolaterally), squeezing the blastopore shut over the ventral aspect of the yolk plug (see arrows, **Fig. 1; Fig. 3A**). As this occurs, the prospective PM/endodermal tissues of the IMZ involute and converge, and extend on the inside, more or less in concert with the overlying posterior neural tissue (*see* **refs.** ***28*** or ***29***). As the dorsal sector of the embryo elongates through neurulation, the ventral sector shortens. The prospective ventral epidermis diverges around both sides of the ventral midline and moves dorsally with the rise of the neural folds (*see* arrows, **Fig. 1**, stages 12–17) ***(21)***. These shortening movements on the ventral side contribute to the overall dominance of the sagittal profile at the end of neurulation by the dorsal, extending tissues (**Fig. 1**, stage 17). The powerful and continuing role of convergent extension in elongating the posterior axis means that in the early fate maps, the prospective RH and SC, the two neural regions undergoing most of the convergent extension, appear very broad and very short (**Fig. 3B**). They elongate and narrow (converge and extend) greatly in the course of gastrulation and neurulation ***(10)***.

In summary, we present a list of facts important for designing and interpreting experiments on neural induction:

> The potential inducing tissues of the Organizer involute and make contact with the inner surface of the potential responding tissues earlier than previously thought, usually during stage 10, and nearly always by 10+.
>
> Because the prospective neural tissues are very short in the animal–vegetal axis at the early gastrula stage, the first, early contact of the inducing tissues on the undersurface of the ectoderm is in the anterior neural region.
>
> Inducing and responding tissues shear relatively little, since the first contact of the two is relatively anterior, and because their posterior regions extend more or less together. Thus, corresponding anterior–posterior regions spend more time together than previously thought.
>
> Since the prospective nervous system is initially very short, most of it, particularly its posterior region, is very close to the inducing, Organizer tissue. Inducing signals passing through the plane of the tissue do not have to travel very far.

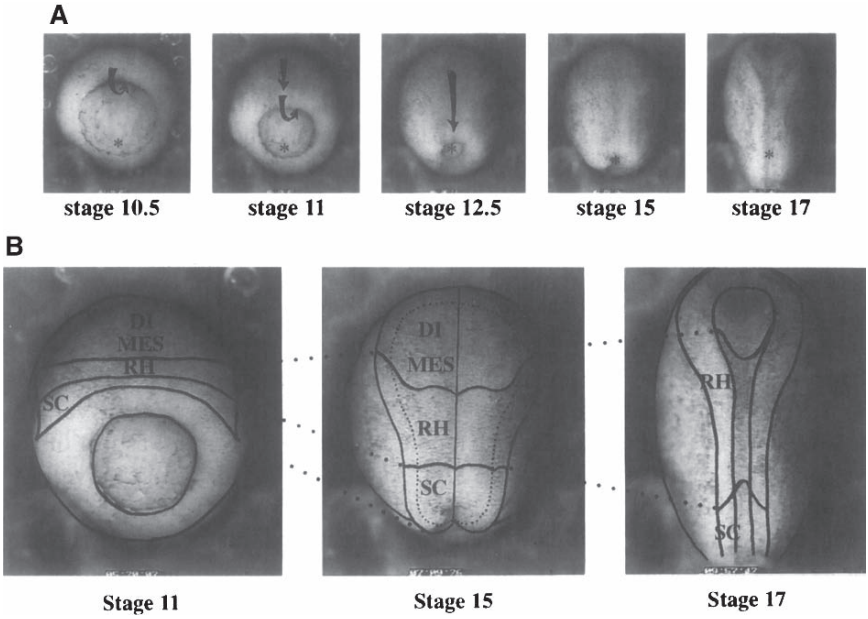

Fig. 3. Video frames from a time-lapse recording of the vegetal view of an embryo during gastrulation and neurulation show the extreme extension of the neural tissue across the yolk plug of the embryo. This embryo was held in clay with its AC fixed. Dorsal is at the top of the frames. In **(A)**, the Spemann Organizer involutes (curved arrows, stage 10.5–11), whereas the prospective neural plate converges and extends across the yolk plug (straight arrows, stage 10.5–12.5), to close over the ventral part of the VE (asterisk). After blastopore closure, the neural tissue continues to extend posteriorly, pushing the blastopore posteriorly (stages 12–17). The involuted PM converges and extends coordinately with the overlying neural plate. In **(B)**, the fate map of the stage 15 neural plate, redrawn from Eagleson and Harris ***(91)*** and projected onto the video image of the stage 15 neural plate, was mapped backward to stage 11 and forward to stage 17 by tracing individual cells at the junctions of map coordinates. Shown are the diencephalon (DI), mesencephalon (MES), RH, SC. This is the same embryo shown in panel (A).

1.4. Regionalization of the Inducing Tissue

There is evidence that different regions of the mesoderm produce different inducing signals, which induce the regional differences that pattern the neural tissue (*see* **refs. *30–37***). Accordingly, it is useful to know how to identify different regions of the mesoderm. The detailed correspondence of mesodermal and neural fates throughout their relative movements, in both anterior–posterior and mediolateral axes, is complex and best visualized with color diagrams (consult **Fig. 4** in **ref. *9***). However, the archenteron cavity and the BC at its edge are

useful landmarks for identifying prospective mesodermal regions until they take on a recognizable character of their own. The leading edge of the mesodermal mantle, the first tissue to involute, consists of migratory mesoderm that spreads across the blastocoel roof in dorsal, lateral, and ventral sectors of the gastrula. It consists of prospective PM dorsally, heart mesoderm dorsolaterally, lateral plate mesoderm laterally, and ventral (blood island) mesoderm ventrally. In general, these early involuting tissues lie ahead of the BC, located at the periphery of the archenteric cavity. The late involuting tissues, the prospective notochord in the dorsal sector and the prospective somites in the lateral and ventral sectors lie behind the BC, immediately above the archenteron roof. For example, in the dorsal midline of the early gastrula, the PM lies ahead of the dorsal BC, found at the tip of the archenteron, and the notochordal and somitic mesoderm lie behind the bottle cells (**Fig. 1**, stages 10–, 10+). However, the dorsal BC respread in late gastrulation, when they expand the archenteron anteriorly, beneath the PM ***(15, 16)***, changing this relationship (**Fig. 1**, stages 11.5–17). At the late gastrula and neurula stages, the PM, notochordal, and somitic mesoderm lie dorsal to and approximately coincident with the roof of the archenteron, and the neural plate lies dorsal to and approximately coincident with this mesoderm. If one cuts through the body wall at the peripheral aspect of the archenteron at the late gastrula through neurula stages, the explant contains the entire neural plate, underlaid with PM, somitic, and notochordal mesoderm, and archenteron roof endoderm.

As one cuts and manipulates different regions of the mesoderm, one should be aware of the dramatic differences in their properties. The posterior, dorsal (notochordal and somitic) mesoderm differs in behavior from the anterior leading edge (PM, heart, lateral plate, and ventral mesoderm) in that the former undergo convergent extension, whereas the latter spread and migrate ***(38)***. The cell motility in the posterior mesoderm consists of an intrinsic bipolar, mediolaterally directed protrusive activity ***(39)***, whereas in the anterior mesoderm, it consists of monopolar, animally directed, substrate-dependent protrusive activity ***(40,41)***. These two types of mesoderm also behave differently with respect to extracellular matrix ***(40,41),*** and they express different molecular markers. Goosecoid ***(24)***, Otx2 ***(25)***, and noggin ***(4)*** are expressed in the leading edge, PM/pharyngeal endoderm, whereas brachyury is expressed in the postinvolution notochord ***(42)***. Other useful markers for regions of mesoderm include the antibody tor-70 ***(43)***, which marks notochord from stage 17 onward (*see* **ref. *44***), and 12–101, a monoclonal antibody (MAb) ***(45)*** that marks somitic mesoderm from stage 18 onward.

The regional character of the mesoderm is not fixed at the onset of gastrulation, but is patterned during gastrulation. The precise anterior to posterior progression of the cell behaviors driving mediolateral cell intercalation ***(46)*** is

actually organized during gastrulation ***(47)***. Likewise, regional neural-inducing properties appear in the early neurula ***(36)***.

1.5. Regionalization of the Animal Cap (AC)

By the onset of gastrulation, if not before, the AC shows several dorsal-ventral differences relevant to neural induction. Early in the blastula stage, only the ventral half of the AC expresses the epidermal-specific antigen, Epi-1 ***(48,49)***, possibly owing to early signals acting through the plane of the tissue ***(50).*** After treatment with activin, the dorsal half of the AC is more likely than the ventral to form mesodermal tissue ***(51).*** The dorsal half can be induced to converge and extend ***(52)*** and to express neural molecular markers ***(53)*** when placed in planar apposition to the Organizer, whereas the ventral side shows little or no response. Dorsal and ventral halves differ in expression of protein kinase C isozymes, which is thought to have a role in biasing the dorsal half to form neural tissue ***(54).*** Bone morphogenetic protein (BMP) signaling between ventral cells (prospective epidermal) may prevent neuralization and ensure epidermalization, while blocking such signaling results in neuralization (***55***; *see also* **ref. *56***). Thus, the dorsal side of the AC is biased in the direction of neural development by the early gastrula stage. Later ventrally derived epidermal tissue takes an active role in patterning the dorsal aspects of the neural tube in amphibians ***(57–59)*** and in other vertebrates ***(60,61).*** Using ACs from UV "ventralized" embryos, which lack an Organizer and a nervous system ***(62),*** would presumably avoid this dorso–ventral bias of the AC.

2. Materials

2.1. Solutions

A variety of saline solutions are commonly used to culture amphibian embryonic tissues ***(63).*** Our favorites are modified Barth's solution (MBS) ***(64),*** a general-purpose saline, and modified Danilchik's solution (DFA), a saline specialized for supporting the normal motility and behavior of deep, nonepithelial cells ***(65).***

MBS (modified Barth's solution, from Gurdon, **ref. *64***):

Stock	Per 1 L	Final concentration, m*M*
4 *M* NaCl	22.0 mL	88.0
50 m*M* $MgSO_4$	16.4 mL	0.82
0.8 *M* $NaHCO_3$	3.0 mL	2.40
0.1 *M* KCl	10.0 mL	0.01
33 m*M* $Ca(NO_3)_2$	10.0 mL	0.03
1 *M* $CaCl_2$	0.41 mL	0.41
Hepes	2.38 g/L	5.00

Adjust pH to 7.4 with NaOH.

DFA ("Danilchik's for Amy" from Sater et al., **ref. *65***):

Stock	In 500 mL	In 1 L	Final concentration
4 *M* NaCl	6.625 mL	13.25 mL	53.0 m*M*
1 *M* Na_2CO_3	2.5 mL	5.0 mL	5.0 m*M*
K gluconate	0.525 g	1.05 g	4.5 m*M*
Na gluconate	3.49 g	6.93 g	32.0 m*M*
1 *M* $CaCl_2$	0.5 mL	1.0 mL	1.0 m*M*
1 *M* $MgSO_4$	0.5 mL	1.0 mL	1.0 m*M*

Adjust pH to 8.3 with 1 *M* bicine.

The original Danilchik's ***(66,67)*** as well as subsequent versions (*see* **refs. *39,65***) were developed to mimic the ionic composition of the blastocoel fluid of *X. laevis* ***(68)***.

Embryos are kept in third-strength saline, and then transferred to full-strength for microsurgery. DFA is the appropriate solution for "open-faced" explants, which contain exposed deep cells. Although DFA allows more normal behavior of deep cells, it interferes with some functions of the epithelial cells, such as neural fold fusion (*see* **refs. 11,66,67**). MBS or an equivalent should be used for all other microsurgery. After healing, the embryos should be transferred to 3rd- or 10th-strength saline. We make two types of full-strength salines, one plain and one with 0.1% bovine serum albumin (BSA). BSA reduces adhesion of the cells to the surfaces of the dish and coverslips. Embryos should be transferred to plain saline before fixation, or the BSA may be fixed or precipitated on the surface of the specimens, making them appear dirty in a number of staining procedures. Solutions are filtered through a 0.22-µm Millipore filter, and aliquoted into 50-mL plastic centrifuge tubes, and frozen at −20°C until needed. We also freeze 1.0-mL aliquots of 100X antibiotic/antimycotic solution (10,000 U penicillin, 10 mg streptomycin, 25 µg amphotericin B, per mL, in 0.9% NaCl, Sigma Chemical, St. Louis, catalog #A9909) at −20°C. These are thawed and added at 0.5 mL/50 mL tube of solution at the time of use. Culture solutions are replenished every 12 h. Embryos are kept at 16–24°C. We try to avoid temperatures below or above this range, though two more degrees in either direction may be fine with good spawnings.

2.2. Tools and Dishes

The vitelline envelops are removed with Dumont #5 watchmakers forceps. For cutting embryos, we use eyebrow hairs embedded in wax in the tips of pulled, disposable "Pasteur" pipets. Hairloops, which are used to position and hold embryonic tissues, are also embedded in pipets. Pipets are pulled to a diameter

several times that of a hair and scored with a diamond pencil; the vibration of scoring will usually fracture the pipet transversely. An eyebrow hair with a fine, evenly tapered point is placed in the pipet, butt first, such that its natural curvature brings the distal third to an angle of about 120° with respect to the pipet. In order to make a hairloop, both ends of a long hair are placed into the pipet until the smallest possible loop is formed. (It is important to use a long piece of hair, since the friction of the hair on the side of the pipet, a key factor in making a small loop, is increased with length.) Pipets are then dipped in wax, which has been melted in a spoon. The excess wax is removed by heating a steel spatula in an alcohol burner until warm, covering it with small piece of laboratory tissue, and transiently touching the eyebrow hair or hairloop to the tissue. Once melted, the excess wax will soak into the tissue without releasing the eyebrow hair or hairloop from the pipet. These instruments are sterilized by dipping them in 70% ethanol for a few minutes. We have found that these "low-tech" instruments outperform more pretentious instruments; they are sharp, flexible, and relatively nonadhesive to cells.

For microsurgery, a plastic disposable 60-mm Petri dish is used. Grafts between embryos are most easily made in a dish containing black modeling clay (Pastalina, Certified Nontoxic, Van Aken International, Rancho Cucamonga, CA 91730). The clay, available from many toy and art stores, is pressed into the bottom of the dish to a depth of 2–3 mm. To sterilize the clay, the dish is flooded for 20–30 s with 70% alcohol and rinsed in five changes of sterile saline.

To hold grafts or explants in place, small fragments of coverslips are cut ahead of time in rectangular shapes, about 2–4 mm by 10–15 mm, with a diamond pencil, and kept in plastic Petri dishes. These coverslip fragments are handled with watchmaker's forceps (*see* **Subheading 3.6.**).

2.3. Microscopes

Proper setup of a stereo-dissecting-type microscope is important for good microsurgery. In addition to to good posture, eyepiece height, and working height of the bench, it is very important to adjust the eyepieces according to the manufactuer's instructions, such that both eyes focus at the same level and have stereoptic vision. We work on a large, flat, cooled surface, rather than on a raised stage. We buy the microscope with a boom stand or without a stand, and mount the microscope to a large cooling plate, of dimensions 40 × 80 cm. The cooling plate is made by milling a continuous, 2-cm-wide channel in a sheet of Plexiglas of this size, about a centimeter thick, beginning at the back right hand corner, coming forward, turning towards the back again, and so on, in a continuous, sinuous pattern, staying about 3 cm from the edge, and ending at the back left. This channeled plate is glued to a second Plexiglas plate. Finally the pair of plates are glued and screwed, with machine screws, to the aluminum plate.

The screw heads are recessed into the bottom Plexiglas plate, forming a smooth surface, and are screwed into tapped holes on the underside of an aluminum plate. The aluminum plate is then drilled and tapped for a water inlet and outlet. These are located over the ends of the channels in the Plexiglas at the back right and left of the plate in order to maximize water flow and to be out of the way. The inlet is made smaller (3/8 in.) than the outlet (1/2 in.) to reduce buildup of internal pressure. Water is circulated through the plate from a large water bath/chiller, and adjusted to 16–18°C. A boom stereoscope is swung out over the plate, or a pillar matching the diameter of that required for the stereoscope is mounted on the aluminum plate, using a 3/8 in. machine screw-tapped into the base of the pillar and into the top of the plate. Illumination is by fiber optic illuminator, which is cool and does not heat the embryos.

3. Methods

All the operations described below, and others as well, can be done with a combination of a few basic manipulations.

3.1. Cutting Tissues

Large pieces that are to be cut along straight lines from an intact embryo are cut out by pressing an eyebrow hair directly through the embryo, along the line of the cut until the substratum is reached. Explants are trimmed to size with the same type of movement (**Fig. 4A**). It is best to use a long, stiff eyebrow hair for this type of operation. In order to remove only a superficial part of the embryo without damaging deeper tissues, a type of "stitching motion" is used. The eyebrow hair is inserted into the wall of the embryo at an angle of 45° with the surface until the tip is at the desired depth; the hairloop is placed next to the eyebrow hair, along the line of cut on the surface of the embryo, and the tissue is cut by quickly raising the tip of the eyebrow hair (**Fig. 4B**). The operation is repeated very quickly, advancing the eyebrow hair and the retaining hairloop only a small distance at each cycle, never cutting through more than a few cells at a time, to avoid undue strain in the tissue (**Fig. 4B**). By varying the depth of insertion of the eyebrow hair, a cut of any depth can be made, ranging from the epithelial layer alone (about 10–15 μ thick) to the epithelial and deep region together (about 25–100 μm, depending on the stage and region). A small, sharp-tipped eyebrow hair is best for this type of stitching cut. An alternative method for cutting tissue at a limited depth is to insert a long, stiff eyebrow hair along a line of tissue that is to be cut, and then rub the hairloop back and forth against it, on the outside, cutting the intervening tissue. This method is good for making straight cuts, but is not conducive to making curved cuts, and it does not allow precise control of depth below the curved surface of the embryo.

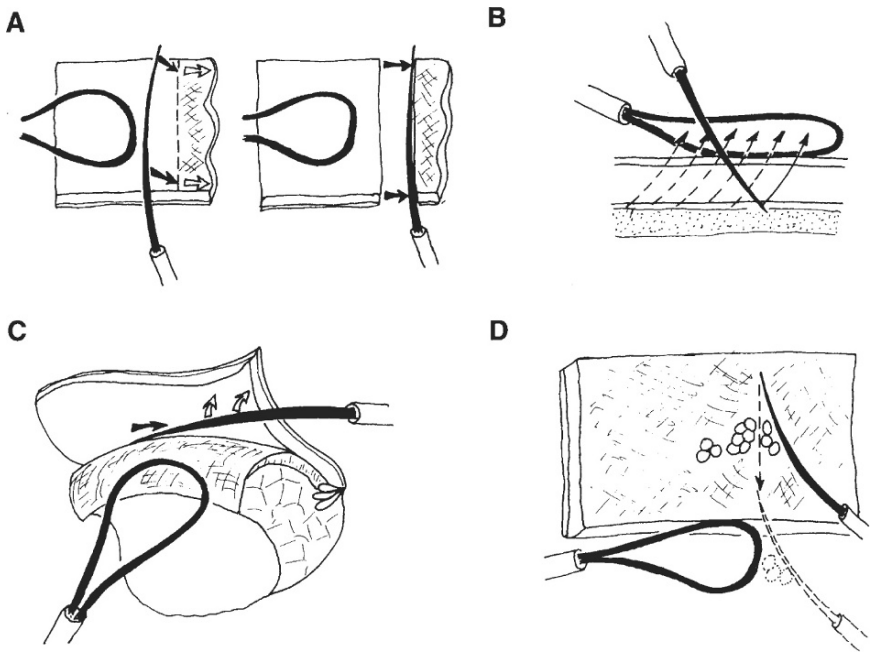

Fig. 4. Methods of cutting **(A,B)**, peeling **(C)**, and shearing cells off layers **(D)** are shown with diagrams. In order to trim an explant usin a long, straight type of cut (A), the tissue is held lightly with a hairloop, while a long, stiff eyebrow hair knife is brought down on the explant along the desired line of the cut (dashed line, A, left), and pressed through the tissue until it hits the substratum. The eyebrow hair is then quickly moved to the right, cleanly separating the two pieces of tissue (A, right). A method of cutting through a limited and controlled depth of tissue is shown in a face-on view of the cutting surface, as if the tissue on the viewer's side of the cut had been removed (B). The hairloop is used to hold the tissue along the line of the cut, and the eyebrow hair tip is inserted to the desired depth and lifted repeatedly and quickly (arrows, B), cutting only a few cells at a time. A method of peeling tissue from underlying tissue is shown (C). In this diagram, the postinvolution PM (below restraining hairloop) and the neural ectoderm (above the eyebrow hair) of an early gastrula are being separated. The tip of the eyebrow hair is moved along the interface between the tissues (solid arrow), whereas the heel of the curved hair is rocked against the prospective neural tissue (open arrows). Adherent mesodermal cells are sheared off from the inner surface of an explant of the neural tissue (D). The hairloop is placed at the bottom of the tissue to prevent the tissue from moving toward the operator. The eyebrow hair is then swept at low angles straight toward the operator, just above the surface of the explant, shearing off the adherent cells. For clarity, the size of the eyebrow hair and hairloop is reduced relative to the size of the tissues.

3.2. Peeling Layers Apart

One will often want to peel layers of tissue apart. Examples include removing the epithelium from the underlying deep cells ***(69)*** or removing the outer wall of the gastrula from the underlying involuted cells (*see* **Subheadings 4.2.** and **4.2.2.**). The "peel" is begun by probing with the tip of an eyebrow hair for the interface between the tissues to be separated; the interface reveals itself as a line of easier separation. Once the interface is discovered, the background tissue is held with the side of the hairloop. The tissue to be peeled off is pulled away by running the tip of the eyebrow hair along the interface, and angling the butt or heel of the eyebrow hair against the surface of the tissue (**Fig. 4C**). Initiation of a peel at the proper interface is difficult if the cut at the edge of the peel has gone too deep. In this case, the area of the underlying tissue beneath the peel will be more likely to come along with the peeled tissue.

3.3. Shearing Cells Off Layers

In order to remove remaining unwanted cells on layers that were peeled apart, one can perform a shearing operation. For example, when making sandwich explants from older gastrula-stage embryos, mesodermal cells will often adhere to the inner surface of the blastocoel wall. The following method can be used to remove them. The explant is positioned with its deep surface uppermost. The eyebrow hair is turned such that a considerable length of its tip lies parallel to the surface and just above the explant, to the right of the area of contamination (**Fig. 4C**). The tip of the eyebrow hair should be inclined at an angle of about 20–30° off a line running vertically from the bottom to the top of the field of view. The hairloop is then placed against the near edge of the explant, just to the left of the eyebrow hair; this will prevent the explant from moving toward the experimenter. The eyebrow hair is repeatedly and rapidly brought down until it just touches the surface of the explant, and back towards the experimenter, always remaining in its original plane. The restraining hairloop is moved slowly to the left and followed with the eyebrow hair, shearing off any recalcitrant, adherent cells. The eyebrow hair should be oriented at a low angle of attack, since a low attack angle does not exert large forces on the explant (**Fig. 4C**). The eyebrow hair and hairloop can be switched right to left, and the process begun at the other edge of the explant, if one prefers.

3.4. Open-Faced Explants

"Open-faced" explants are ones having the inner, deep cells exposed to the medium, often for the purpose of studying deep cell motility ***(39,46,66,67,70)***, and thus Danilchik's (DFA) medium is used. A culture chamber for these explants is made by drilling a 20-mm hole in a 60-mm plastic Petri dish and gluing a #1.5, 24-mm coverslip over the hole with silicone high vacuum grease

(Dow Corning, Midland, MI). The tissue is placed with the inner or deep surface down on the coverslip, and restrained with another coverslip, supported at each end with silicone high vacuum grease. This allows high-resolution optics to be used with an inverted microscope. An upright microscope can be used by overfilling the dish with media, covering the top with a large glass slide, and then inverting it, such that the coverslip and the deep surface of the explant are at the top, facing the objective.

3.5. Sandwich Explants

Sandwiches consist of abutting the deep, inner surfaces of two identical tissues together, such that the epithelial sheets covering each half heal across the exposed edges, forming a physiological barrier surrounding the deep cells. The two components are excised and their inner surfaces apposed, the quicker the better, since delay will result in curling of the two halves, which makes apposition difficult. Dabs of silicone high vacuum grease are placed on both ends of a precut rectangular coverslip, considerably larger than the explant, and the coverslip is rested on the grease, straddling the explant and some distance above it. Then, the coverslip is tapped with a forceps, bringing it down on top of the sandwich, pressing the two components together lightly. Healing should occur in 15–20 min, and then the explant should be removed. The high vacuum grease does not appear to be overtly toxic, but the less time the explant spends near it, the better.

3.6. Grafting Tissues

The easiest method of grafting from embryo to embryo is to use a clay-bottomed dish. A hole the size of the embryo is made in black modeling clay with a ball tip, formed at the end of a disposable pipet by holding it in a flame until the correct size molten ball is formed. The host embryo is placed in the hole, graft site uppermost, if possible. If the embryo rotates, small nibs of clay can be pushed against it with forceps, holding it in position. The donor embryo is placed alongside the host, also in a depression (necessary only if one wants to keep the same levels of both embryos in focus at higher magnifications). A graft is cut out of the donor and a graft site cut in the host; which operation is done first depends on which is the most troublesome to do, and which preparation gives the most trouble when left alone for a time. Speed is crucial. If left alone too long, the graft will curl, and the hole made to accept it will first gape and then heal. The boundaries of graft and graft site should be closely matched, since healing will occur fastest under these conditions. If the graft is slightly larger than the site, it is important to tuck the edges of the graft into the graft site. If the edges of the graft overlap the host epithelium, healing will be delayed. The graft epithelium will turn back on itself and ultimately find the edge of the host epithelium, but this will take a while.

In order to hold the graft in place, forceps are used to push up two long ridges of clay on each side of the embryo, some distance (2–4 mm) away. A small coverslip fragment, preferably a precut rectangle, is bridged across the two ridges, above the embryo. The coverslip is pushed down on the graft with forceps tips, taking care to align the surface of the glass exactly perpendicular to the graft surface, pushing it directly into the graft site. If the embryo or graft moves one way or the other as pressure is applied, the glass bridge can be tilted to counter these movements. Healing should occur in 15–20 min at which time the coverslip is removed and embryo is removed from the clay. Most batches of clay appear to be somewhat deleterious to the embryo over long periods, although embryos from many spawnings will develop normally to advanced stages on clay. We prefer black clay for contrast, but other colors can be used according to the experimenter's taste. Some colors appear to affect the embryos more than others.

The method described above is the easiest, but not necessarily the best way to graft tissues. With practice, one can make grafts between free, unconstrained embryos lying in a dish. This is the least disruptive and yields the best results, if done correctly. It is important that the edges of grafts are mated precisely. The grafts should be relatively small, and they must stick in the graft site without external pressure, all of which require much practice.

3.7. Tricks

Many tricks can be used to facilitate grafting and explanting embryonic tissues. Although these tricks provide advantages in some situations, they usually come with liabilities. High pH and or low calcium makes tissues easier to separate or cut, but also retards healing. Hypertonicity and hypotonicity may facilitate separation of layers of cells, by shrinking or swelling the cells, but may retard healing, and kill or damage cells. In cutting through the epithelial surface of the embryo, either the epithelial or the deep cell populations will be at a disadvantage, depending on what solution is used. The deep cells and basolateral surfaces of the epithelial cells require high salt, whereas the outer surface of the epithelium is normally exposed to low salt. External solutions of high pH and/or high salt cause lesions in the epithelial layer and increased cell motility at these lesions, particularly in the case of high pH. Conversely, low pH and low salt cause sluggish or abnormal motility or swelling of deep cells, respectively. These facts, discovered by Holtfreter (*see* **refs. *71–73***), make any grafting or explantation operation suboptimal for the health and function of one cell population or the other.

3.8. Handling Albinos

It is advantagous to use albino embryos for experiments involving whole mount, RNA *in situ* hybridization, as well as for some antibody staining of tissues. However, because these embryos have low contrast, they are difficult to

stage and orient. Recognition of BC and other pigment-dependent landmarks is very difficult. Albino embryos can be staged and oriented more easily if are stained a pale, "baby" blue, achieved by soaking the embryos for a few minutes in third-strength saline containing a few drops of 1.0% Nile blue. With this light background stain, the constricting BC apices will appear darker, much as they would in normal embryos. Staging and orientation during gastrulation can be done much like that of normal embryos, and at later stages, the staining will enhance the neural plate and the neural folds.

3.9. Tipping and Marking

"Tipping and marking" embryos *(74)* facilitate identification of the dorsal, lateral, or ventral sides of the embryo at early cleavage and late blastula stages, which is essential for regional injection of dyes, RNAs, or plasmids. This procedure makes use of the fact that movement of the cortex of the egg relative to the deep cytoplasm during the first cell cycle determines where the dorsal side will form *(75)*. If the equator is rotated uppermost at any meridian early in the first cell cycle and left there, the dorsal side will form at that meridian, overriding the influence of the sperm entry site on specifying the dorsal side. The embryos are first placed on a Nitex grid (about 1 mm mesh size) in 6% ficol in saline. After fetilization, but before first cleavage, the equator is tilted upper-most until after first cleavage. The site that is uppermost is marked with a wand bearing vital dye. The wand is made by pulling a disposable pipet to a small diameter and then melting a small glass ball at the end. Dye is precipitated by placing a bit of Nile blue sulfate (1% in water) and 100 m*M* sodium carbonate on a microscope slide, and mixing the two with the wand. A precipitate of dye will form, which is picked up on the tip of the wand and placed against the embryo for a couple of seconds. Care should be taken, since the dye will appear darker later, and it is easy to overstain. The dorsal side will develop at the stained site.

4. Issues and Specific Experimental Preparations

We will describe in detail several methods of analyzing some current and difficult problems in neural induction, including the relative roles of planar and vertical signaling in neural induction and patterning. However, many of the issues, problems, and solutions described below are generic ones, applicable to other experiments as well.

4.1. Planar and Vertical Signaling

The traditional view of neural induction is that the mesoderm comes to lie beneath the prospective dorsal neural ectoderm and sends signals vertically, inducing the ectoderm to realize its neural fate (**Fig. 5A**). In the past 10 years, there has been a revival of interest in edgewise or "planar" induction. Planar

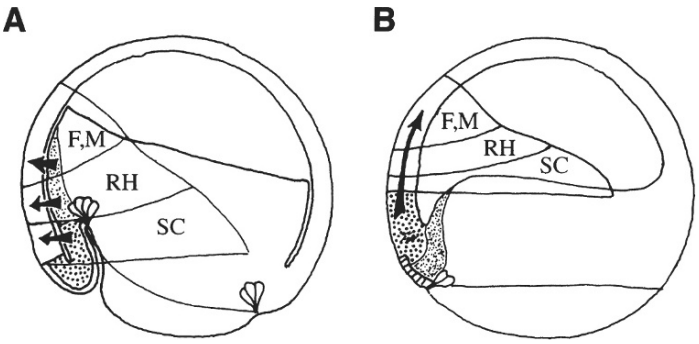

Fig. 5. Diagrams illustrate the concepts of vertical induction **(A)** and planar induction **(B)**. In vertical induction, the organizer mesoderm (stippled) first involutes beneath the prospective neural tissue, and then sends signals radially (outward or vertically) to the overlying neural tissue (arrows, A). In planar induction, the Organizer mesoderm sends signals animally, from its posterior edge, into the prospective neural tissue (arrow, B). One difference is that vertical signaling requires involution of the Organizer and thus must occur at a relatively older stage (stage 10.5 shown in A), whereas planar signaling can occur early (stage 10– shown in B). The prospective fore- and midbrain (F, M), rhombencephalon (RH), and spinal cord (SC) are shown. The components of the organizer in the sagittal plane, the prospective prechordal mesoderm (PM) and notochordal mesoderm, are indicated with fine and course stippling, respectively.

induction is thought to involve a signal passing from the posterior edge of the Organizer, through the plane of the tissue, into the prospective neural region (*see* **refs.** ***25,50,52,53,65,76–78***) (**Fig. 4B**). This idea is not new, originally favored by Spemann, but discarded because of Holtfreter's work ***(79)***, demonstrating that exogastrulae, which supposedly have planar, but not vertical apposition of the inducing and responding tissues, underwent no obvious neural development. With the revival of interest in planar induction, the traditional route of neural induction is now called "vertical" induction.

Several experimental preparations have been used to study the relative roles of planar and vertical signaling. We will describe these preparations and examine their usefulness and limitations, including difficulties in making them, problems of interpretation, and unknown factors that might make them misleading.

4.2. The Sandwich Explant of the Dorsal Marginal Zone and Prospective Neural Tissue

This explant is made by sandwiching the inner deep surfaces of two dorsal sectors, taken from two individual gastrulae, together (**Fig. 6**). Both the involuting (PM/endodermal) and noninvoluting (prospective neural) regions of this explant converge and extend, whereas the animal-most ectodermal region, corresponding to

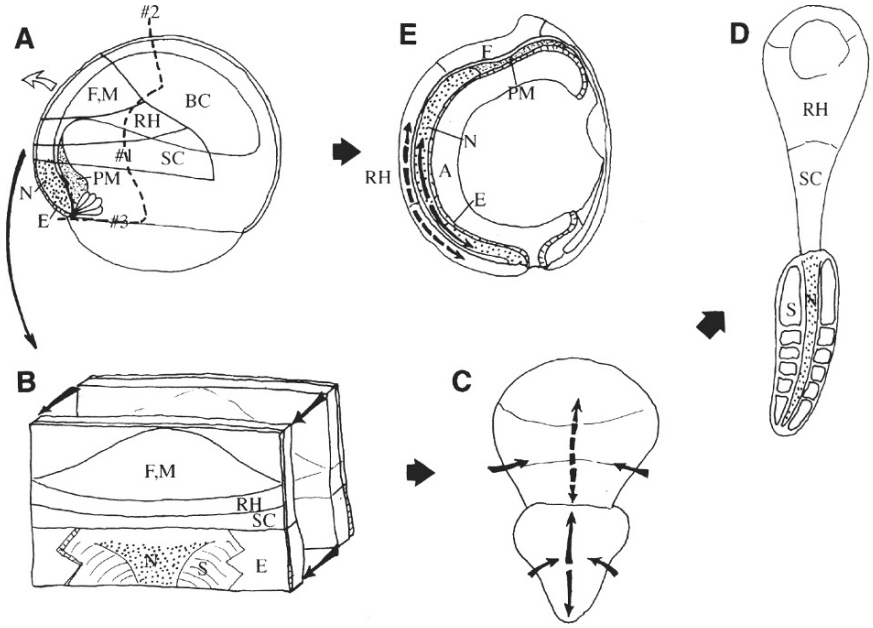

Fig. 6. The method of making a sandwich ("Keller") explant of the dorsal sector of the gastrula is shown **(A,B)**. The dorsal sector of the early gastrula (A) is excised by cutting 45–60° on both sides of the dorsal midline, and across the AC (heavy dashed lines, #1 and #2, respectively). This flap of tissue is then peeled outward, away from any involuted material (open arrow), and cut off where it joins the VE, above or below the BC (#3). Two such explants are trimmed at the edges to match one another, and sandwiched, with their inner, deep surfaces together (B) by placing them between a coverslip fragment and the bottom of the dish. The prospective areas of such an explant are shown, including prospective fore- and midbrain (F, M), hindbrain (RH), SC, endoderm (E), notochord (N), and somite (S). Prospective PM may or may not be included, depending on whether the explant was made early or late in stage 10. After 15 min or so, of healing, the explant is taken from under the coverslip and allowed to develop. Such an explant undergoes convergent extension **(C,D)** of both the involuting marginal zone (solid arrows) and the prospective posterior neural tissue (broken arrows). The involuting marginal zone differentiates into notochord and somitic tissue, whereas the neural region differentiates into regions that express early markers of the SC, RH, and F and M ***(12,25,78)*** (D). If the explant is large enough in the animal direction, cement gland is also formed. In the intact embryo (E), the neural and mesodermal tissues converge and extend in parallel (arrows, E), the later beneath the former, rather than serially, as in the explant (C).

the prospective mid- and forebrain, remains bulbous ***(52,66,67)*** (**Fig. 6**). Since Organizer tissues of this explant are in edgewise or planar apposition to the ectoderm, it is assumed the planar signals must induce the ectoderm to acquire

a neural fate. However, some thought on the requirements for demonstrating planar induction suggests that the interpretation of the behavior of this sandwich explant is more complex than might first appear.

4.2.1. Is the Assayed Behavior Already Autonomous When the Test for Induction Is Done?

The neural convergent extension or marker expression in sandwich explants of early gastrulae is not necessarily induced by planar signals from the Organizer. They may have been induced previously or patterned by cytoplasmic localizations, and may be autonomous behaviors by gastrulation. Testing for autonomous expression at the early gastrula is not a trivial problem, particularly in the case of the SC and hindbrain, since these regions are short and difficult to manipulate ***(52)***.

4.2.2. Do Contaminating Cells Provide Vertical Signaling?

The second problem is that mesodermal cells may contaminate the interior of the noninvoluting, prospective neural tissue, providing vertical signals in a preparation supposedly devoid of vertical signals. In sandwiches made at stage 10 to 10+, the leading edge of the mesodermal mantle likely has begun involution, as described in **Subheading 1.3.2.** and **Fig. 2**. If these mesoderm cells are not removed from the inner surface of the explant, they have access to the fibronectin-rich inner surface of the blastocoel roof. Under these conditions, these cells are highly invasive ***(41)*** and will invade the core of sandwich and provide vertical signals (**Fig. 7A**). If sandwich explants are made early, at stage 10–, PM will be found at the vegetal end of the explant. These PM cells can also migrate animally beneath the neural tissue, both as individuals and in streams, and even lead the more posterior cells in an invasion of the core of the explant (**Fig. 7B**) (*see also* **Fig. 10** in **ref. *9***). As few as four or five notochordal cells can change the expression of neural genes in the overlying ectoderm (*see* **ref. *80***).

There are several methods one can use to solve the problem of invasion. The best method, of course, is to remove all the potential invading cells from the inner surface of the vegetal end of the explant, as well as adherent cells higher in the marginal zone. They are easy to identify (**Fig. 7A**; *see also* Fig. 10 in **ref. *9***). One could also monitor such invasions with markers expressed by the potentially invading tissue. However, one must know what markers this tissue should express, and the test for marker expression must be sufficiently sensitive at the appropriate stage.

A better way to document the absence of such invasion and to demonstrate convincingly that only planar induction has occurred is to appose an Organizer from a fluorescently labeled embryo next to an unlabeled sandwich of ectoderm that, in isolation, does not show neural development. Any labeled cells from the

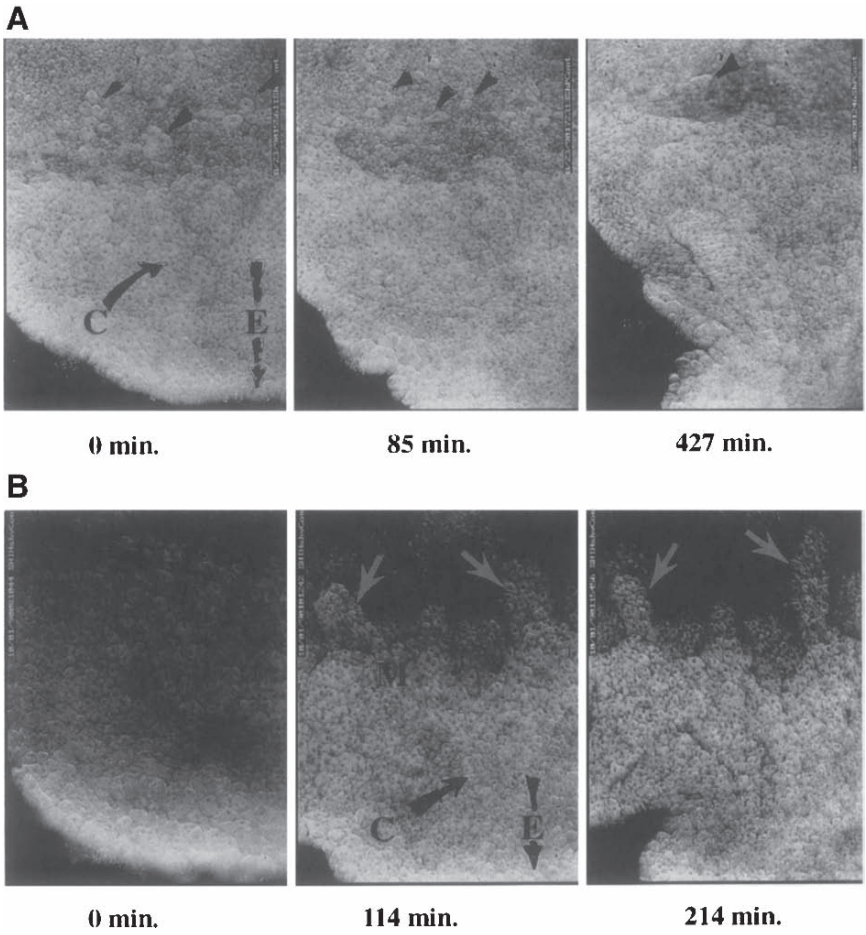

Fig. 7. Time-lapse video microscopy of the inner surface of an open-faced explant shows the tendency of mesodermal cells to migrate animally on the inner surface of the prospective neural tissue. As the bulk of the mesoderm converges and extends vegetally (arrows, **A**), a number of individual mesodermal cells break off from the posterior edge of the mesoderm and migrate on the inner surface of the prospective neural ectoderm (pointers, A). In some cases, large tongues of mesodermal cells may migrate animally (white arrows, **B**) while the rest of the mesoderm converges (C) and extends (E) vegetally (black arrows, B). Photos courtesy of John Shih.

inducing tissue that invade, regardless of phenotype, will be noticed (*see* **Fig. 7C** in **ref.** ***52***).

Finally, a positive control for the effect of contamination can be done. Contamination is allowed, both a little of it and a lot of it, to determine if it makes a difference in neural convergent extension or marker expression, relative to

cases thought to have no contamination. For example, neither a little nor a lot of contamination increased the amount of convergence and extension of the neural region, and thus we concluded that vertical signals had no detectable effect on convergent extension induced by planar signals alone ***(52)***.

4.2.3. Has Vertical Signaling Already Occurred? Use of Skewered Sandwiches

One must also be certain that vertical signals have not occurred prior to testing the effect of planar signals alone. As pointed out above, the prospective nervous system is very wide and very short, and *Xenopus* shows early, cryptic movement of the leading edge mesoderm inside. This means that the distance the leading edge of the organizer must move to come beneath a good part of the prospective nervous system, and the time it takes to do so are very short. If staging is not absolutely precise, or too late a stage is used, some opportunity for vertical signaling will already have occurred prior to experimental manipulations used to study solely planar signals (*see* **refs. *52,80***).

We use the following method to determine how far beneath the prospective neural ectoderm the Organizer has reached at the time of making explants. Explants are made at stages 10– through 10+, Also, as the dorsal sector is dissected, the highest point of contact of any involuted mesoderm/endoderm is marked by piercing the prospective neural ectoderm with an eyebrow hair (**Fig. 8A**, **A**). Two similarly marked explants are selected and sandwiched together. A small glass bead (glass beads, acid-washed, 106 µ and finer, US Sive 140, cat. no. G4649, Sigma Chemical Co.) is inserted into the hole made by the eyebrow hair (**Fig. 8B**, **B**), marking the upper limit of mesodermal/endodermal contact. The two halves are then held together beneath a glass coverslip for healing. After healing, the coverslip and glass bead are removed and the explant is skewered to a bed of 2% agarose, with a hair pushed through the hole left by the bead. The hair permanently marks the site of mesodermal/endodermal contact (**Fig. 8C**, **C**). Video microscopy shows convergent extension is not affected by the presence of the bead and hair. Convergent extension occurs, moving the explant with respect to the anchor point provided by the skewer. Using this method, we have found that expression of the homeobox gene *Hoxb-1* is affected by the early vertical contact of PM with neural tissue, whereas convergent extension is not ***(80)***. Expression of other neural properties and genes may or may not be affected by these early, transient signals.

4.3. The Edgewise (Planar) Apposition of Sandwiched Tissues

The best way to demonstrate planar induction is to place the Organizer in edgewise apposition to a responding tissue that normally does not undergo neural development. We have done such an experiment to demonstrate the planar

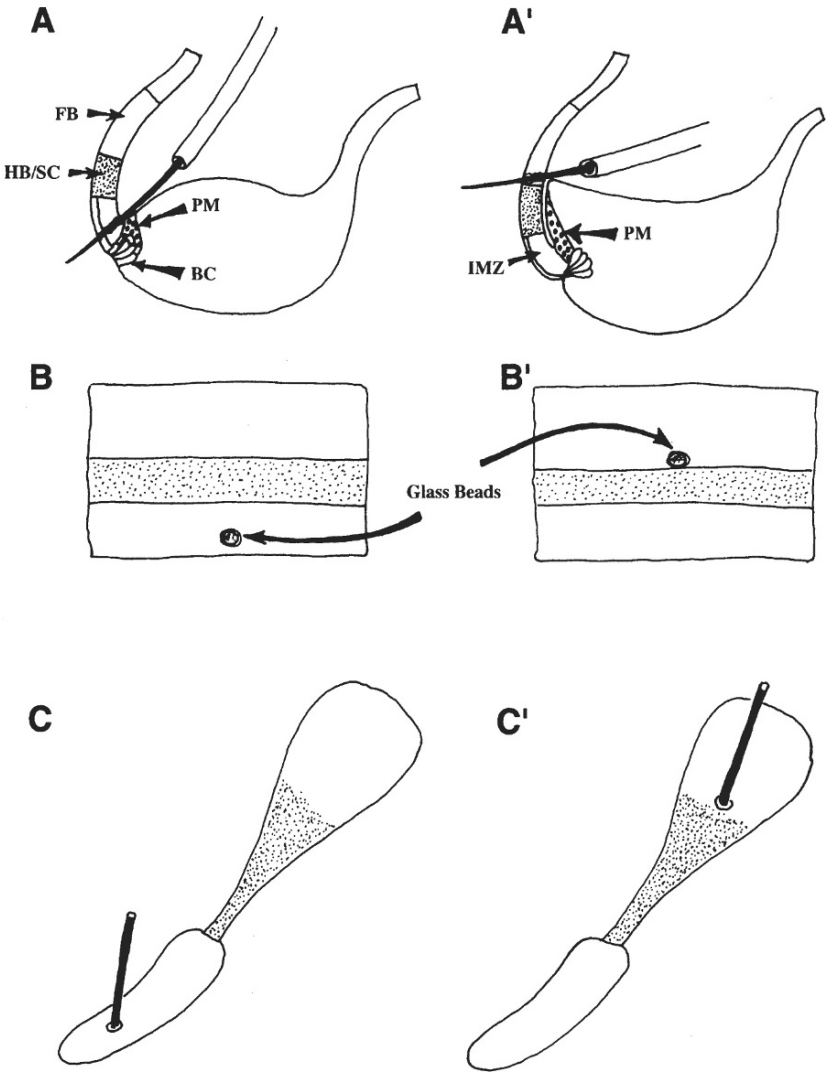

Fig. 8. The skewer method of marking the anterior limit of contact of the involuting mesoderm with the overlying neural ectoderm is shown in diagrams in cases of no contact (A–C) and substantial, anterior contact (A′-C′). When the AC is cut open, in preparation for making the explant, an eyebrow hair knife is used to puncture the outer gastrula wall at the point of inflection of the VE to gastrula wall at early stages (A), or at the top edge of contact of the involuted material with the gastrula wall (A′). Two explants with holes in matched or nearly matched positions are then sandwiched and the holes marked with small glass beads (**B**, B′) before pressing them together under a coverslip. After the coverslip is removed, the beads are removed and the explants skewered to the bottom of a dish covered with 2% agarose. As the explants converge and extend, the position of the original contact is marked by the eyebrow hairs (C, C′).

induction of convergent extension ***(52)*** by grafting a labeled piece of Organizer to the edge of a piece of dorsal ectoderm, taken from far above the blastoporal lip, so that it did not include the original converging and extending neural tissue (**Fig. 9A**). In another experiment, a second Organizer was abutted to the animal end of the dorsal sector of the gastrula, inducing a second extension of polarity opposite that of the original (**Fig. 9B**). These explants can be made as sandwiches of two full thickness of tissue, grafted with their inner surfaces together, as shown above (**Fig. 6**). In this case, the explant consists of the outer epithelium, the apposed deep layers of each, and the outer epithelium of the second.

A somewhat easier way to make these explants is to excise one-half of such an explant from one embryo and graft to its inner, deep surface the corresponding epithelium from a second embryo, as shown in **Fig. 9A,B**. This provides a complete epithelial covering, maintaining the advantage of a controlled internal environment. In addition, these thinner explants are somewhat easier to abut edgewise than their thicker counterparts.

Multistep preparations such as these are easier done if several rules are followed. They should be done fast. Otherwise, problems will arise, such as rolling of the epithelial sheets, migration of the epithelium over the cut edges to be apposed, and "bowing" of the tissues, which results in tissues "saddling" crosswise to one another when the sandwich is made. Also, the preparations should be done in stages, at least until one develops expertise. For example, this experiment is easier if two sandwiches, one of the Organizer and one of the responding ectoderm, are made first. Each of these sandwiches consist of an explant and the corresponding epithelium from another embryo (steps #1 and #2, **Fig. 9**). When these sandwiches are slightly healed, they are removed from beneath their coverslips and abutted to one another edgewise (step #3, **Fig. 9**). A fresh wound surface should be made at the surfaces to be mated so that they will join and heal.

In any case, these "blunt-end ligations" of tissue are difficult. Epithelial sheets abhor a free edge, and immediately after being cut, their margins will migrate across the exposed deep cells at the ends of the explants, until they meet another epithelium. If the edges of the explants do not match precisely and a gap appears where the deep cells are abutted, the epithelial sheets will not span the breach. Rather, each epithelium will migrate down along its own deep cells to the bottom of the gap, where it will meet and heal with its counterpart. At this point, the continuous epithelium will pop up out of the groove and form a smooth surface. This takes some time, and such slow or incomplete healing could block any induction that might have occurred.

To avoid these problems, we often make a slight lap joint, or "sticky-end ligation", in which the cut edges of the deep cells and epithelial cells do not coincide. One end of an explant is cut off squarely, and several rows of epithelial cells are lifted off the deep region at the edge, with the eyebrow hair (#1, **Fig. 9C**). This operation is repeated on the opposite side. A cut is then

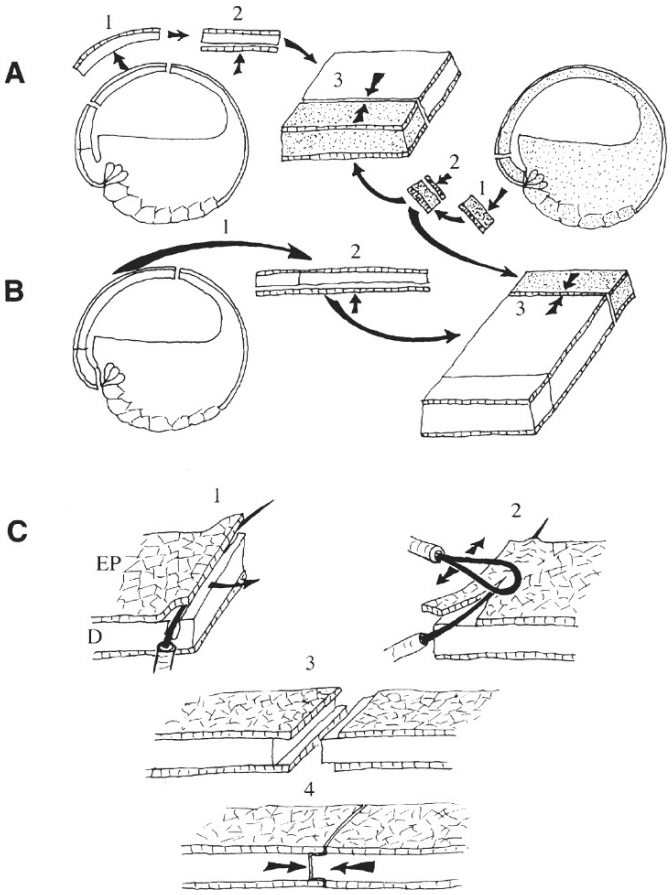

Fig. 9. A method for edgewise apposition of inducing and responding tissue is shown. In the first case, Organizer tissue is placed in edgewise (planar) contact with AC ectoderm at the early gastrula **(A)**. In the second case, a second organizer is grafted in planar apposition to the opposite end of the AC ectoderm, whereas the original Organizer is left in place **(B)**. In both cases, the inducing and responding tissues are excised and sandwiched with an epithelial layer from the corresponding region of another embryo (steps #1 and #2, A and B), to provide a protective covering. The inducing and responding tissues are then abutted edgewise (step #3, A and B). A method of making a slight lap joint, which will aid in abutment and healing of tissues joined edgewise, is shown **(C)**. One explant is cut such that flaps of epithelial tissue are exposed (#1, C), and the other is cut such that the deep nonepithelial cells are exposed (#2, C). The two are laid facing one another (#3, C) and then pushed together and held there with a coverslip until healed (#4, C).

made downward, through most of the deep layer; the operation is repeated from the opposite side, removing a short section of deep layer (#3, **Fig. 9C**). On the other explant, the opposite operation is performed; an eyebrow hair is pushed back under the epithelium a couple of cells, and a hairloop is rubbed across the top, cutting off a short piece of epithelium (#2, **Fig. 9C**); the operation is repeated on the other side, making a notched explant (#3, **Fig. 9C**). If one works slowly, it is best to reverse the order of these operations, since the free epithelial sheets in the second type of explant will curl faster than their counterparts will advance across the deep cells in the first type of explant. Next, the edges of the free epithelial sheets are teased outward and the exposed deep cells of the other explant are stuffed in between them (#4, **Fig. 9C**). Finally, the entire preparation is pressed lightly between a coverslip and the bottom of the dish for the duration of healing, about 10–15 min.

4.4. Exogastrulae

The last, and the least useful, of the experimental preparations for analyzing planar induction in *Xenopus* is the exogastrula. Holtfreter ***(79)*** found no obvious sign of neural development in the ectoderm in exogastrulae, in which the endomesoderm was extruded outward and thus made only planar or edgewise contact with the ectoderm. In *Xenopus*, exogastrulae can be made by culturing the blastulae in hypertonic salt solution, usually 1.3–2.0 × normal culture medium. Under these conditions, the endoderm and mesoderm "evaginate" rather than involute, resulting in a specimen in which the notochord and somites, covered by endoderm, are connected by a narrow stalk to an ectodermal sack. *Xenopus* exogastrulae have been used to argue that planar signals induce neural tissue properties, on the assumption that they, like the exogastrulae used by Holtfreter, have no vertical signaling ***(81,82)***.

Holtfreter, however, worked with the axolotl and several species of anurans, other than *Xenopus* (*Rana fusca* and *Hyla arborea*), for which he described exogastrulation in some detail. In contrast, the movements and mechanism of exogastrulation in *Xenopus* have never been described in adequate detail. What we do know about normal gastrulation and exogastrulation in *Xenopus* suggests that its exogastrula is unsuitable for studies of neural induction. Video microscopy of *Xenopus* "total exogastrulae" shows that they form BC and progress as far as the equivalent of normal stage 10.25–10.5 before exogastrulating (R. Keller, unpublished observations). This fact, together with the fact that head mesoderm undergoes early cryptic movement in *Xenopus*, and the fact that much of the prospective neural tissue is very close to the mesoderm in the early gastrula, make it very likely that early and transient, and perhaps permanent vertical neural-inducing signals occur in *Xenopus*

exogastrulae. In addition, the blastocoel shrinks and the blastocoel roof often collapses on the VE in high-salt solutions, making it likely that both the morphogenetic movements and inductive interactions in the blastula stages are abnormal (*see* **refs.** ***83,84***). Unless more work is done on the movements and anatomy of the *Xenopus* exogastrulae, it is useless for the study of neural (and mesodermal) induction. It may be very useful in situations where the possible early vertical contact of mesoderm with ectoderm is not an issue.

Interestingly, Holtfreter's work on the axolotl may not suffer from any of these weaknesses. Urodele embryos of the type he studied have a very long prospective neural plate and Organizer, and do not have cryptic, early involution of mesoderm (*see* **ref.** ***85***), thus negating the problem of early vertical induction at anterior levels. Moreover, these urodele embryos have a large blastocoel, which tends to wrinkle on itself rather than against the VE (R. Keller, unpublished observations), making fortuitous mesoderm inductions less likely.

5. Planar Signals in the Context of Prospective Neural and Epidermal Interactions

The sandwich explants described above consist of the dorsal 90–120° of the embryo, which includes little or none of the prospective epidermal ectoderm. Thus, in these explants, planar signals from the Organizer act on the prospective neural tissue in the absence of any influences from the prospective epidermis. Since there is evidence that epidermal–neural interactions pattern the neural tissue and regulate its morphogenesis ***(57–61)***, we have developed several types of explants that allow planar signals from the Organizer to act in the context of also having planar neural–epidermal interactions.

5.1. The "Giant" Sandwich

In the giant explant, the entire AC, NIMZ, and IMZ are excised and sandwiched (**Fig. 10**). The vegetal region of the embryo is turned uppermost, and single-stroke cut is made (**Fig. 4A**), from the center of vegetal region toward the midventral line, through the entire embryo all the way to the AC (cut #1, **Fig. 10A**). The embryo is then turned over, and the AC, NIMZ and IMZ are pulled away from any mesoderm/endoderm that has involuted (**Fig. 10A**; *see* **Subheading 3.2.** and **Fig. 4C**). These tissues are separated from the involuted tissue, as well as the VE by a second cut at the boundary of the VE (cut #2, **Fig. 10A**). The explant is then laid out, inner surface uppermost. Any adherent, postinvolution cells are removed from its inner surface (*see* **Subheading 3.3.** and **Fig. 4D**), and it is trimmed at the animal end to make a clean rectangle. The explant consists of the entire array of prospective tissues of the gastrula, its free edges at the ventral midline (**Fig. 10B**). Two of these giant explants are

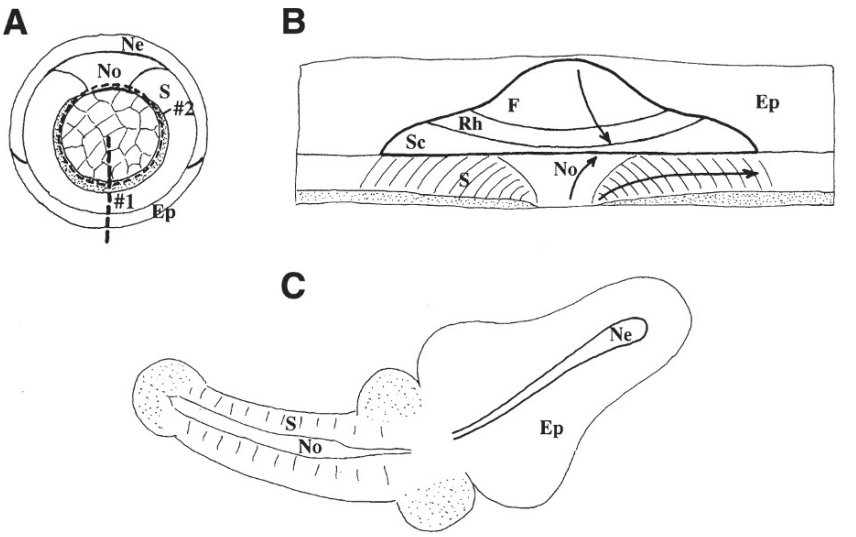

Fig. 10. To test the results of planar signaling in the context of the entire AC, a giant sandwich is used. A giant sandwich is made by cutting through the entire gastrula, from the ventral pole animally, along a line that extends from the center of the vegetal pole along the ventral midline (step #1, A). The embryo is then turned over, the entire gastrula wall peeled off from any involuting material, and the entire involuting marginal zone and AC cut away from the VE (step #2, A). This large piece, spanning the entire dorsoventral extent of the embryo, is then trimmed at the animal end to form a rectangle and sandwiched with a similar piece from another embryo to form a giant explant **(B)**. As is the case with the standard sandwich explant, this explant shows convergent extension of both the neural and mesodermal regions. It differentiates neural and epidermal regions in the former, and notochord and somitic mesoderm in the latter **(C)**. Prospective areas or the corresponding differentiated tissues are shown, including notochord (N), somitic mesoderm (S), epidermis (Ep), forebrain/midbrain (F), RH, SC, and neural tissue (Ne). The mesodermal prospective fates are shown as they would appear if the overlying epithelial endoderm were removed. The arrows in A and B show the anterior to posterior polarity of the neural and mesodermal tissues.

sandwiched together and allowed to develop. Both the neural and mesodermal/endodermal regions of the explant converge and extend (**Figs. 10C, 11A,B**). Moreover, the cells of the neural ectoderm columnarize, wedge, and attempt to roll the neural plate into a neural tube. Generally, a neural groove, rather than a tube, forms on both sides of the explant (pointers, **Fig. 11B**). Patterning in such explants can be visualized by RNA whole-mount *in situ* hybridization according to the method of Harland (*7*). Otx2, a marker for forebrain and head mesoderm (**Fig. 11C**), and staining with tor 70 antibody, a marker for notochord (**Fig. 11D**), shows the locations of these tissues in the giant sandwich explant.

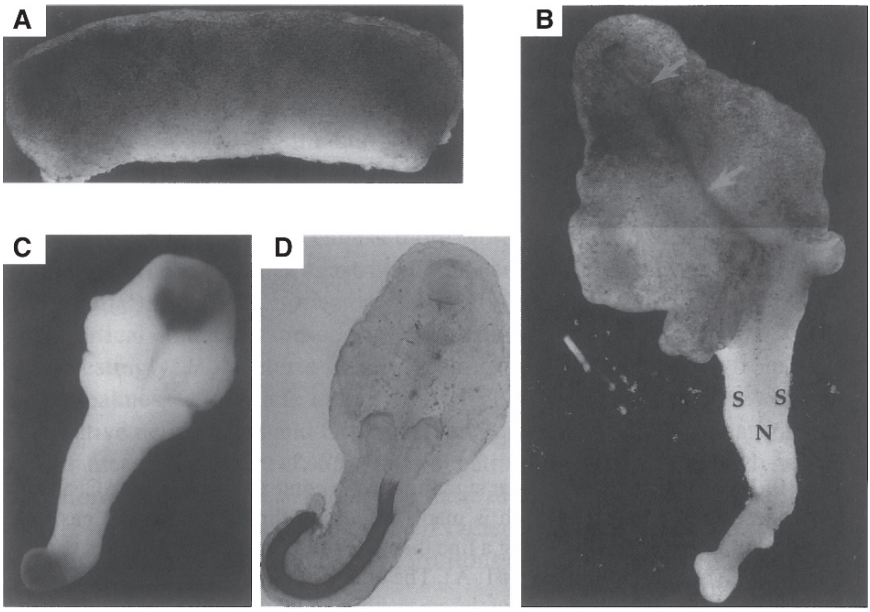

Fig. 11. Giant sandwiches are shown shortly after construction **(A)** and at stage 27 **(B)**. The vegetal end at the bottom and the AC is at the top of all figures. The neural region has converged, extended, and rolled into a neural trough (pointers, B), whereas the lateral regions form epidermis. The mesodermal/endodermal region converges and extends in the opposite direction, and consists of notochord (N) centrally and somitic mesoderm (S) on both sides **(B)**. A whole-mount RNA *in situ* hybridization **(C)** shows an explant stained for Otx-2, expressed in the forebrain region of the giant explant (top of explant) and in the prechordal mesoderm (bottom of explant). An explant stained with the antibody tor 70 shows the notochord in the deep region of the mesodermal/endodermal component of the explant **(D)**.

5.2. The "Pita" Sandwich

The giant explant preserves the normal dorsoventral planar tissue relationships in the gastrula, except at the ventral midline, where it has a free edge. Moreover, the corresponding inner surfaces of the explant are apposed, and the rolling of the neural tubes occurs opposite one another, both abnormal configurations that might affect morphogenesis. Another type of explant, the "pita" sandwich, both retains the normal planar tissue relationships of the gastrula by keeping the midventral line intact and avoids the problem of basal surface apposition. The pita sandwich is made by cutting through the IMZ, along the blastoporal lip, as described above (**Fig. 4B**). The IMZ is teased outward and an eyebrow hair inserted into the blastocoel, toward the animal pole (**Fig. 12A**). The IMZ-NIMZ-AC is then pulled away from the involuted tissue (**Fig. 12B,C**). By gently stretching the tissue, the entire IMZ-NIMZ-AC can be removed from the involuted

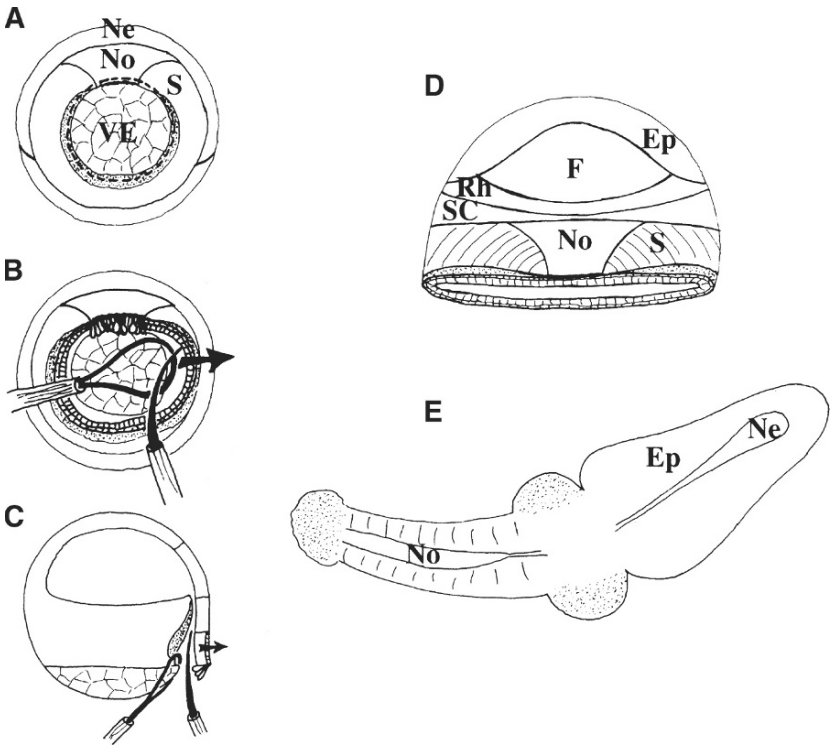

Fig. 12. The "pita" sandwich is similar to the giant sandwich, but consists of tissues taken from only one embryo, sandwiched together in the dorsoventral direction. This explant is made by cutting around the VE (dashed lines, **A**), and then sticking the eyebrow hair animally, along the interface between postinvolution and preinvolution tissues. The hairloop is used to hold the VE and postinvolution material, and the eyebrow hair is moved outward, pulling the gastrula wall away from the postinvolution tissues **(B,C)**. The hairloop is then used to extract all the postinvolution material and VE out through this artificial "blastopore," leaving a pita-like affair, which is compressed in the dorsal-ventral direction **(D)**. This explant develops much like the giant sandwich, but has a neural groove only on one side **(E)**. Prospective areas or the corresponding differentiated tissues are shown, including notochord (N), somitic mesoderm (S), epidermis (Ep), forebrain/midbrain (F), rhombencephalon (RH), spinal cord (SC), and neural tissue (Ne). The mesodermal prospective fates are shown as they would appear if the overlying epithelial endoderm were removed.

mesodermal tissues at any stage from 10 to 11.5, and flattened or cultured as a vesicle (**Fig. 12D**). The pita sandwich converges, extends, and differentiates dorsal neural and mesodermal tissues as well as epidermis and other tissues (**Fig. 12E**). This explant, though difficult to make, preserves some relationships of the normal embryo not maintained in the giant or standard explant.

5.3. Variations of Age, of Amount of Involution, and of Prospective Areas of the Organizer Included

Varying amounts of Organizer tissue (prospective PM and pharyngeal endoderm) can be included in the giant, pita, and standard sandwich explants. The amount of Organizer tissue included is determined by the age of the embryos from which the explants are made and where, with respect to the blastoporal lip, the anterior end of the explant is trimmed. For example, when explants are made from stage 10 or 10+ embryos, trimming off the vegetal end of the explant just above the VE, at the level of the BC, will leave behind with the embryo most of the prospective PM, which has already involuted (**Fig. 13A**). In order to include prechordal mesoderm in explants at this stage, the following method should be used. The wall of the gastrula (the preinvolution IMZ-NIMZ-AC) should be peeled outward, as described above, and the postinvolution prospective PM cut away from the central endoderm of the gastrula (**Fig. 13B**). The explant should

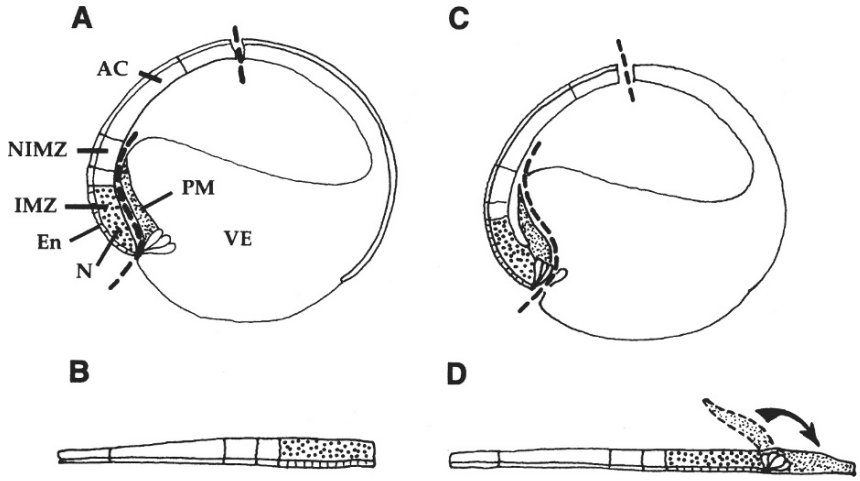

Fig. 13. Two methods of dissecting a stage 10+ gastrula for the purpose of making Organizer-prospective neural explants are shown. On the left **(A)**, the involuting marginal zone, noninvoluting marginal zone, and animal cap (IMZ-N IMZ-AC) is separated from the postinvolution PM and cut from the VE at the level of the BC (heavy dashed line), such that the PM is left with the embryo. In this case, the vegetal end of the explant **(B)** consists of prospective notochord (N, coarse stippling) and the overlying superficial endodermal layer (En, cuboidal layer). On the right **(C)**, the IMZ-NIMZ-AC is peeled away from the underlying involuted prechordal mesoderm along the interface (Cleft of Brachet) between the two, but instead of cutting this flap of tissue off the VE at the BC, a cut is made between the PM and the VE (heavy dashed line, C). The involuted, PM is folded vegetally to make the explant (arrow, **D**).

be bent at the point of involution such that the AC-NIMZ-preinvolution IMZ and postinvolution IMZ material are spread open and apart, like the leaves of a strap hinge (**Fig. 13C**). Finally, two such explants are flattened and sandwiched together. The PM or head mesoderm will form a ball of tissue that does not converge and extend at the anterior end of the IMZ portion of the explant. It should stain with PM markers, such as Otx2 (**Fig. 11C**).

6. Vertical Signaling

Analysis of vertical signaling might appear simple, because it is relatively simple to combine parts of the dorsal organizer mesoderm with ectoderm. Unfortunately, it is not very straightforward to evaluate or control the amount of planar signaling that has happened or is continuing along with the vertical signaling. One can explant the prospective neural plate at the early through late gastrula stages, using the fate maps as guides. At progressively later stages, a number of properties of the neural plate become established and can be studied in such an explant. If one makes the explant from late gastrula stages, the dorsal mesoderm can be included with the prospective neural plate. The development of the neural plate with its corresponding mesodermal tissues can be compared with development of the neural plate alone. Although these types of explants have not been used much to date in *Xenopus*, they were used in the pioneering experiments of Jacobson and his associates on the axolotl and the newt, work that defined many of the morphogenetic issues involved in constructing a neural plate and neural tube ***(86,87)***. To study regionalization of the neural plate, various parts of the neural plate and mesoderm can be recombined (*see* **ref. 36**).

6.1. Neural Plate Explants

The neural plate can be excised cleanly from the underlying dorsal mesoderm as diagrammed in **Fig. 14**. The tip of the eyebrow hair should be pushed only through the neural plate, which consists of an epithelium and one layer of deep cells at these stages (stages 11–12.5), using the method described above (**Fig. 4B**). The depth of the neural plate is determined by experience, judging at what depth the eyebrow hair is two cells deep, staying on the shallow side. The eyebrow hair tip is used to tease away the edges of the two layers of the neural plate, searching for the easily separable interface that lies between the neural ectoderm and the underlying mesoderm. If only one layer comes up, the cut is too shallow. If the edge of the patch is lifted and more than two layers come up, the vertical cut likely was too deep, traversing into the mesoderm. In this case, the underlying mesoderm on the neural plate side of the cut will come along with the neural plate. If this occurs, one should use the tip of the eyebrow hair to start a cleft at the interface of neural and mesodermal tissues. The neural cells are usually smaller and grayer than those of the mesoderm. However, once the

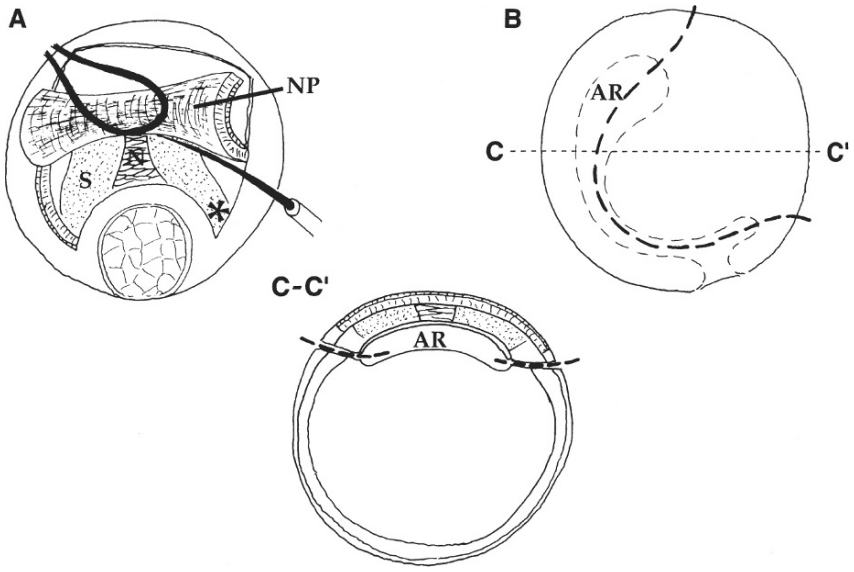

Fig. 14. The neural plate can be removed from later-stage gastrulae by first cutting through the two layers of the neural plate (NP) completely surrounding the area to be removed, using method diagrammed in **Fig. 4B**. A separation is then begun at the interface of the neural plate and underlying mesoderm, using the tip of the eyebrow hair. We prefer to begin at the posterior, right corner (asterisk). Once a sufficiently large area is lifted, the hairloop is used to deflect the neural plate animally, and the tip of the eyebrow hair is run along the interface, separating it from the underlying mesoderm. From stage 11.5 onward, the notochordal mesoderm (N) can be distinguished from somitic mesoderm (S) lying on both sides. The neural plate and underlying dorsal mesoderm can be removed together at late gastrula stages (**B**), by cutting (heavy dashed lines) through the body wall, into the archenteron (AR, outlined with light dashed lines), from its lateral aspect. A cross-section (**C**-C′) shows the line of cut (dashed lines) through the body wall, into the archenteron (AR).

integrity of the mesoderm has been violated, the forces of peeling off the neural plate may tear it further; if so, one must start over with a new embryo.

This procedure is easy to do at stages 10.5–11.5, but at stage 11.5 onward, the region lying above the notochord, the notoplate *(87)*, progressively adheres more tightly to the underlying notochord. As one peels off the neural plate, the notoplate cells will tend to stay with the notochord, or vice versa. When the undersurface of the notoplate is inspected, gaps will appear, reflecting notoplate cells that adhered to the notochord. Conversely, cells sitting above the general surface of the notoplate are notochord cells that have adhered to the notoplate and were pulled out of the notochord. These cells must be stripped off with an eyebrow hair, one by one (*see* **Fig. 4D**) to assure a pure neural plate cell

population. It is best to check the explant after fixation with tor 70 staining for notochord cells, since just a very few notochord cells can affect development of the neural plate (*see* **ref.** ***80***). Laterally, contamination of the neural plate with the underlying somitic cells is less of a problem, since these cells adhere weakly to the neural plate.

Another difficulty is controlling whether or not mesoderm is left in planar apposition with the posterior neural plate. This requires knowing precisely where the posterior boundary of the neural plate lies. If one desires mesoderm to be present, the explant should be cut at the blastoporal lip. If not, one must err on the neural side of the boundary to be assured that no mesoderm will be included. This presents a dilemma. Although considerable convergent extension has occurred at the mid- to late-gastrula stages, the posterior neural tissue is still relatively short and wide. Cutting a little too far animally will remove all the prospective posterior neural tissue that would have converged, extended, and made the spinal cord. Likewise, lateral epidermis can be included in the explant, or not, depending on where the lateral cuts isolating the explant are made with respect to the boundaries shown in the fate maps. This type of explant has been used to study the effect of the lateral epidermis on morphogenesis of the neural tube in the newt and the axolotl ***(59)***.

6.2. Neural Plate/Dorsal Mesoderm Explants

The neural plate can be explanted together with the underlying dorsal mesoderm/endoderm at the same stages by cutting completely through the anterior, lateral, and ventral walls of the archenteron ***(88)***. This type of explant was first used to analyze *Xenopus* mesodermal cell behavior by stripping off the endoderm of the archenteron roof, exposing the deep somitic and notochordal cells (*see* **ref.** ***88***). However, it can also be used to compare autonomous development of the neural plate isolated and cultured alone, with development under the continued influence of the underlying mesoderm. In this explant, the ventral mesoderm that is destined to contribute to posterior somitic tissue moves around both sides of the blastopore, which is pushed posteriorly by the extending notochord (*see* **refs.** ***88,89***). This is the only explant preparation that allows the posterior somitic mesoderm, which lies at the ventrolateral and even ventral IMZs of the gastrula, to move beneath the neural plate in a relatively normal fashion (*see* **Fig. 5** in **ref.** ***29***).

6.3. Recombination of Neural Plate and Organizer Mesoderm

A major issue in neural development is determining to what degree detailed regional patterning of the nervous system is owing to self-organizing processes within the neural plate, elicited by general signals from the mesoderm, and what is owing to direct induction by pre-existing regionalized pattern within the mesoderm. This issue can be addressed by recombining parts of the prospective

neural plate and Organizer mesoderm in vertical apposition (*see* **ref. *36***). In this type of preparation, as in the neural plate isolates described above, care should be taken to separate mesodermal and neural areas cleanly before recombining, so that one does not have crosscontamination of regional tissues. One must also carefully define the regions involved, which is not a trivial task, considering the dramatic movements occurring in both neural and mesodermal tissues. Although the published fate maps can serve as a guide, it is best to check the prospective fates with control marking of tissues. Similar concerns apply to recombination of mediolateral components of the neural and mesodermal tissues.

6.4. Visualizing Cell Behavior in the Neural Plate

High-resolution video microscopy of neural plate explants similar to the type described above can be used to characterize the cell behaviors driving neural convergent extension. Protrusive activity of cells in a tissue is best visualized if a scattered population of the cells is labeled with a vital dye. To achieve such a labeling in the neural plate, we inject 20–30 nL of rhodamine dextran amine (RDA, Molecular Probes Inc., Eugene, OR) into the "A" or "B" dorsal tiers of 32 cell-stage embryos. Dorsal is identified by the tipping and marking method described above. By the midgastrula stage (stage 11.5), cell rearrangement associated with convergence and extension has occurred among the derivatives of the injected blastomere, so that labeled cells are scattered along the length of the neural plate. At stage 12.5, the outer epithelial layer of the neural plate is removed and discarded, exposing the labeled deep cells of the neural plate. The remaining layer of neural plate deep cells, with or without the underlying mesoderm, is removed and cultured under a restraining coverslip, oriented with the neural deep cells facing the bottom of the culture chamber. In this configuration, the neural deep cells can be visualized with time-lapse video recording, using fluorescence illumination, a low-light camera, and image processing, as described previously ***(39)***. We have used this method to study the cell behavior underlying convergence extension of the posterior neural plate ***(89)***.

7. Combination of Vertical and Planar Signaling: The "Kintner" Sandwich Explant

If the sandwich explant is properly made, it should allow only planar signaling. Dixon and Kintner ***(77)*** tested the effect of vertical signals in addition to planar signals by making standard "Keller" explants and adding head/PM to their inner, anterior surfaces (*see* **Fig. 5** in **ref. *77***). In this case, they found that neural tissue, assayed by expression of several markers, was induced poorly when only vertical apposition of tissues was allowed. Planar apposition was more effective, but the strongest response came when vertical apposition was coupled with planar apposition, implying that a synergistic effect of the two signaling routes exists.

8. Other Preparations

A large number of other preparations have been used to study induction and neural development, including classical Organizer grafts, pioneered by Mangold and Spemann in urodeles ***(13,30)***, and used more recently in studies of *Xenopus* neural development ***(14)***, and placing the Organizer into the blastocoel (the "Einsteck") ***(90)***. The general rules that can be taken from the above discussion for any embryological manipulation of these types are:

- Know the fates and locations of the tissues being used.
- Understand the intrinsic morphogenetic movements of the tissues involved.
- Mark the tissues involved and follow their movements, instead of assuming what they will do.
- Do the operations quickly so that healing is rapid.

Acknowledgments

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31

A Method for Generating Transgenic Frog Embryos

Shoko Ishibashi, Kristen L. Kroll, and Enrique Amaya

1. Introduction

1.1. Summary

The amphibian embryo has classically been one of the best systems for elucidating the molecular mechanisms of early development, in particular for studies of mesodermal and neural induction. Amphibian embryos develop externally and are large and robust. Therefore, tissues can be dissected, isolated, or transplanted with high precision and ease in these embryos. In addition, it is relatively easy to manipulate the expression of gene products by injecting in-vitro transcribed RNAs into developing embryos. However, since RNAs are translated soon after injection, this method has been used mainly for studying early stages of development. Manipulating genes specifically during later stages of development requires fine control over the time and place of expression, which can be achieved only through transgenic technology. In this chapter, we describe a very efficient method of transgenesis developed for *Xenopus laevis* and *Xenopus tropicalis*.

1.2. Background

Understanding the molecular basis of pattern formation and differentiation in frog embryos was previously hindered by the lack of a system for temporal and tissue-specific expression of wild-type and mutant forms of developmentally important genes. RNA injection, the most common transient expression method in *Xenopus*, has been effectively used to study maternally expressed genes. However, since RNAs are translated immediately after injection, this method is unfavorable for the study of zygotic gene products that are expressed only after the midblastula transition. Direct injection of DNA can be used to express genes behind temporal and tissue-specific promoters after the midblastula transition.

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However, in frog embryos, this approach has been only marginally successful for two reasons: ***(1)*** injected DNA does not integrate into the frog chromosomes during early cell cycles, and therefore, the embryo expresses the genes in a highly mosaic pattern; and ***(2)*** many promoters lack adequate temporal fidelity and tissue specificity of expression when the DNA is not integrated into the genome of the frog. To overcome these technical problems, we developed a nuclear transplantation–based approach to transgenesis ***(1)***. The approach enables stable expression of cloned gene products in *Xenopus* embryos, allowing a broader range of feasible experimentation than that previously possible by transient expression methods.

This technique has been used for many applications. For example, transgenesis has been used to express wild-type and mutant forms of genes in specific regions of the embryo or at distinct times of development (for examples, see ***(1–7)***). In addition, transgenic lines that express fluorescent proteins ubiquitously have been established, and these have proven useful for lineage studies ***(8–12)***. Transgenic lines that express fluorescent proteins in a tissue-specific manner have been used to visualize an inductive response ***(13–15)***. Transgenesis can also be used to study the regulation of genes *in vivo* (for examples, *see* ***[16–27]***). Regulatory elements from various species can drive appropriate expression in transgenic *Xenopus* embryos ***(28–34)***. By comparison with mouse transgenesis, transgenic *Xenopus* embryos can be obtained rapidly, at low cost, and in large numbers. Therefore, transge-nesis is useful for large-scale functional analysis of *cis*-regulatory regions of genes ***(33)***.

The transgenic technique has enormous potential for addressing the function of genes in late development and organogenesis. However, in cases where the effect of misexpression is subtle, it may be difficult to rely solely on F0 transgenic embryos for the analysis. The reason for this is that each F0 animal is unique; that is, each carries a different copy number of transgenes and distinct sites of integration. The net result is that F0 embryos have an inherent variability that complicates the analysis of misexpression experiments. In addition, integration of plasmids into the embryonic genome occurs through chromosomal damage with this transgenic technique; therefore, a subset of the embryos develop with abnormalities. For these reasons, strategies for doing misexpression studies using established transgenic lines were developed. One approach is to use the GAL4-UAS system, which has been very useful for misexpression studies in the fruitfly, *Drosophila melanogaster* ***(35,36,6)***. Another approach is the use of the site-specific recombination system, FLP/FRT (and CRE/LOX) to "FLP ON" genes of interest in restricted temporal and spatial patterns in established transgenic lines ***(37)***. This system also offers the potential for cell-lineage studies in living embryos ***(38)***.

1.3. Overview of Transgenesis Procedure

The transgenesis protocol described here can be divided into three parts: preparation of egg extracts, sperm nuclei preparation, and nuclear transplantation (**Fig. 1**). A crude egg extract is prepared using a low-speed centrifugation step. These extracts are driven into the interphase stage of the cell cycle by addition of calcium. A high-speed centrifugation is then performed to generate an interphase cytosolic fraction containing proteins required for the efficient decondensation of the sperm nuclei (**Fig. 1A**). In addition, sperm nuclei are prepared from isolated sperm by treatment with lysolecithin, which causes a gentle permeabilization of the sperm plasma membrane (**Fig. 1B**). The nuclear transplantation procedure involves incubation of linearized plasmid DNA with sperm nuclei, decondensation of sperm nuclei by addition of a high-speed egg extract containing a small amount of the restriction enzyme, and dilution of the reaction mix and a dilute suspension of the treated nuclei for transplantation into unfertilized eggs (**Fig. 1C**). The egg extract partially decondenses sperm chromatin and the restriction enzyme stimulates recombination by creating double-strand breaks, facilitating integration of DNA into the genome ***(1, 39–41)***. Since the transgene integrates into the genome prior to fertilization, the resulting transgenic embryos are not chimeric and there is no need to breed to the next generation to obtain nonmosaic transgenic animals, as is the case with transgenesis procedures in mice and zebrafish.

1.4. The Efficiency of the Transgenesis Procedure

One person can transplant sperm nuclei into several hundred to thousands of eggs in a typical experiment. About 30–40% of these transplanted eggs develop into normally cleaving 4-cell stage embryos. About 60–80% of these embryos proceed through gastrulation normally, while the other 20–40% exhibit gastrulation abnormalities resulting from chromosomal damage to the sperm nuclei or physical damage to the egg during transplantation. Thus, approximately 20–30% of the eggs initially injected with nuclei often proceed to postgastrula stages in an experiment. Approximately 10–50% of these normally developing embryos show stable expression of transgenes.

A main factor affecting the efficiency of transgenesis is the amount of chromosomal damage sustained by sperm nuclei prior to transplantation. Chromosomal damage can occur during sperm nuclear preparation or be deliberately induced by addition of restriction enzyme to the sperm, extract, and plasmid reaction mixture used for transplantations. Nuclei with very little chromosomal damage promote a much higher frequency of normal embryonic development to late stages. However, nuclei that have sustained more chromosomal damage give rise to transgenic embryos at higher frequencies. Therefore, a balance must be achieved between attaining normal development of embryos versus promoting

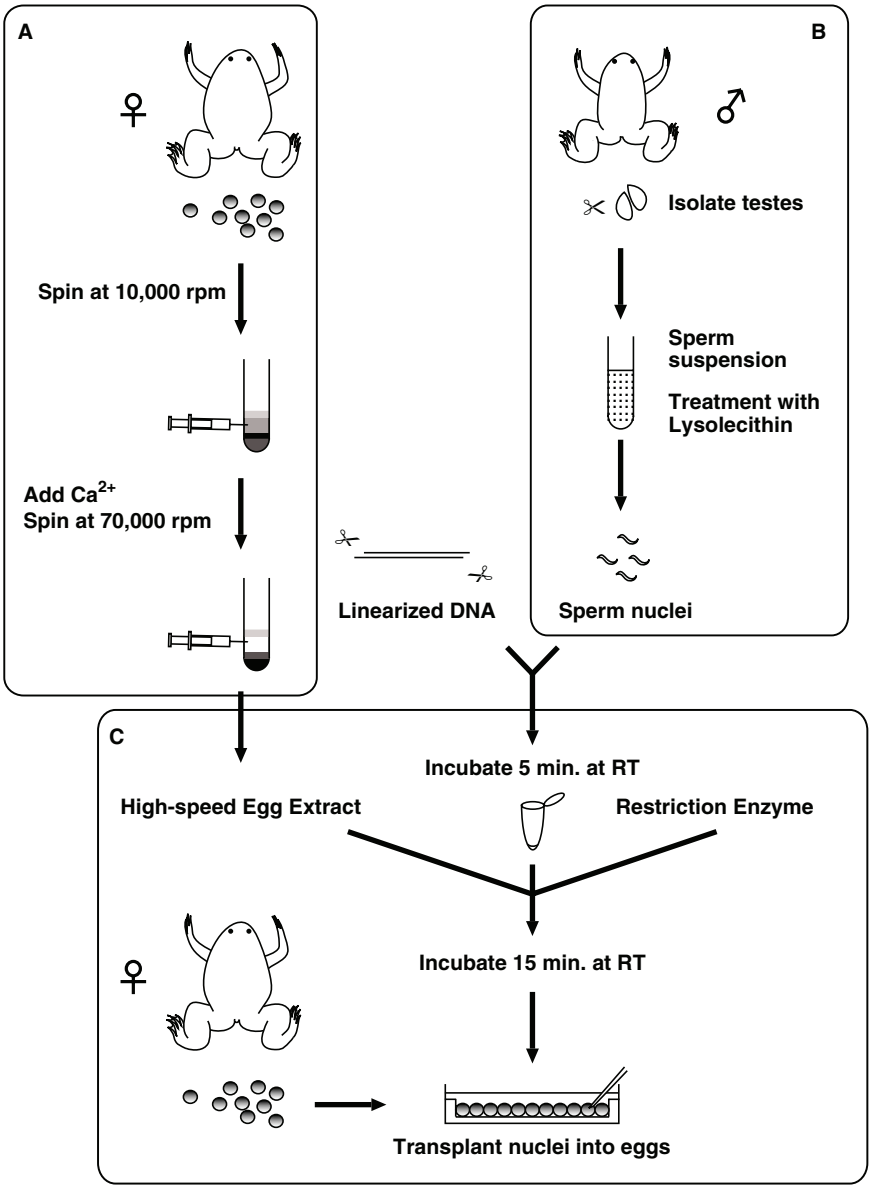

Fig. 1. The transgenesis procedure includes (**A**) preparation of egg extracts, (**B**) sperm nuclei preparation, and (**C**) nuclear transplantation. The egg extracts and sperm nuclei can be stored at −80°C. **A** Calcium is added to allow the crude egg extract (which are held in meiotic arrest) to progress to interphase, and a high-speed centrifugation is performed to obtain the cytosolic fraction. **B** Testes are macerated and filtered, then the sperm suspension is treated with lysolecithin to disrupt the plasma membrane of the cells.

high-frequency transgenesis. We generally use a procedure that results in a lower (10–30%) efficiency of transgenesis but with improved overall survival of the embryos. Another variable that can affect efficiency is egg quality. Needles for nuclear transplantation are much larger than those used for RNA injections, to not damage swollen nuclei. Therefore, eggs need to be of high quality to recover after injection. Eggs used for nuclear transplantation should, in particular, have a firm cortex and maintain a regular round shape.

In this transgenesis method, the transgene integrates into the genome as a concatemer (*see* **section 31.1.5**). Therefore, two constructs mixed in the same reaction cointegrate into the same site of the genome at high frequency (i.e., 80–90%) [4]. To distinguish transgenic embryos, a marker gene, for example, γ-crystallin promoter driving *GFP*, can be cointegrated with a desired transgene. The γ-crystallin promoter drives the expression specifically in the lens, making it very easy to detect, even in adult animals ***(42, 13, 9)***.

Generally, the same restriction enzyme used to linearize the transgene is used in the transgenic reaction before the nuclear transplantation. However, an enzyme, that was not used to linearize the transgene can still be used for the reaction, as long as it does not cut within the transgene. To cointegrate two constructs into the genome, both DNAs can be digested with either the same enzyme or different enzymes, without loss of cointegration efficiency. Although it is possible to omit the restriction enzyme from the transgenic reaction, this usually results in a lower efficiency of transgenesis ***(1)***. This is desirable for some applications, such as establishment of transgenic lines, as embryos generated from reactions that do not include restriction enzyme tend to develop to postmetamorphic and adult stages with fewer anomalies.

1.5. Analysis of DNA Integration in Transgenic Embryos

We analyzed genomic DNA from transplantation-derived tadpoles to determine whether early integration of introduced plasmids into sperm or egg chromosomes is responsible for the nonmosaic expression observed ***(1)***. The pCARGFP plasmid was introduced into embryos by transplantation of sperm nuclei, and tadpoles 2.5 wk to 1 mo old were scored for nonmosaic expression of *GFP* in the somites; the presence and arrangement of pCAR*GFP* were then determined by probing Southern blots of genomic DNA from these tadpoles

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Fig. 1. (*continued*) **C** Sperm nuclei are incubated with linearized DNA for a brief period of time. High-speed egg extracts and a restriction enzyme are added. The egg extracts partially decondenses chromosomes and the restriction enzyme stimulates recombination by creating double-strand breaks, facilitating integration of DNA into the genome. Diluted nuclei are transplanted into an unfertilized egg.

with a 1-kb probe consisting of *GFP* sequences from one end of the linearized pCAR*GFP* plasmid. We found that transplantation-derived tadpoles that did not express pCAR*GFP* did not contain the plasmid, whereas each tadpole that expressed *GFP* contained between 5 and 35 copies of the plasmid–cell. The probe recognized four to eight bands in each *GFP*-expressing tadpole that were of unique sizes and were not found in other *GFP*-expressing tadpoles. These fragments represent putative junction points at which pCAR*GFP* was integrated into the genome of each tadpole. Additionally, the probe recognized two common bands in all the *GFP*-expressing tadpoles, corresponding to products formed by tandem and back-to-back concatemerization of the pCAR*GFP* plasmid. By comparing the intensity of these bands relative to the putative junction fragments, we estimate that pCAR*GFP* was integrated into the genome as single copies in some instances and as short (two to six copies) concatemers in other instances. Since the plasmid is expressed in all expected cells in the embryo, it is likely that most of these integrations occurred prior to the first cleavage division, assuring that all cells of the embryo would inherit several copies of the plasmid.

2. Materials

2.1. High-Speed Egg Extract Preparation

1. 1× Marc's modified Ringer (MMR): 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.5. Prepare a 10× stock, and adjust the pH with NaOH to 7.5. Sterilize 10× solutions by autoclaving.
2. 20× Extract buffer (XB) salt stock: 2 M KCl, 20 mM $MgCl_2$, 2 mM $CaCl_2$, filter–sterilize and store at 4°C.
3. Extract buffer: 1× XB salts (100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$; from 20× XB salts stock solution), 50 mM sucrose (1.5 M stock; filter–sterilize and store in aliquots at −20°C), 10 mM HEPES (1 M stock, titrated with KOH so that pH is 7.7 when diluted to 15 mM, should require about 5.5 ml of 10 N KOH for 100 mL, dilution drastically changes the pH of HEPES, so pH must be monitored after dilution; filter–sterilize, and store in aliquots at −20°C). Prepare about 100 mL.
4. 2% (w/v) L-Cysteine hydrochloride 1-hydrate (BDH, 370555X): Made up in 1× XB salts before use and titrated to pH 7.8 with NaOH. Prepare about 300 mL.
5. CSF-XB: 1× XB salts (100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$), 1 mM $MgCl_2$ (in addition to $MgCl_2$ present in XB salts; final concentration 2 mM), 10 mM HEPES, pH 7.7, 50 mM sucrose, 5 mM EGTA, pH 7.7. Prepare 50 mL.
6. Protease inhibitors: Mixture of leupeptin (Roche, 1 017 101), chymostatin (Roche, 1004 638), and pepstatin (Roche 253 286), each dissolved to a final concentration of 10 mg/mL in dimethyl sulfoxide (DMSO). Store in small aliquots at −20°C.
7. 1 M $CaCl_2$ (filter–sterilize and store at 4°C).
8. Energy mix (store in aliquots at −20°C): 150 mM creatine phosphate (Roche, 621 714), 20 mM ATP (Roche, 519 979), 20 mM $MgCl_2$, Store in 0.1 mL aliquots at −20°C.

9. Pregnant mare serum gonadotropin (PMSG): 100 U/mL PMSG (P.G.600®, Intervet, Inc., 021825). Dissolve in water and store at −20°C.
10. Human chorionic gonadotropin (HCG): 1000 U/mL HCG (Chorulon®, Intervet, Inc., 057176). Dissolve in water and stored at 4°C.

2.2. Sperm Nuclei Preparation

1. 1× MMR. Prepare as described in section 31.2.1., item 1.
2. 0.1% Tricaine methanesulfonate (MS222, aminobenzoic acid ethyl ester, Sigma A-5040), 0.1% sodium bicarbonate. Dissolve in water.
3. 2× Nuclear preparation butter (NPB): 500 mM sucrose (1.5 M stock; filter–sterilize and store aliquots at −20°C), 30 mM HEPES (1 M stock; titrate with KOH so that pH 7.7 is at 15 mM, filter–sterilize, and store aliquots at −20°C), 1 mM spermidine trihydrochloride (Sigma S-2501; 10 mM stock; filter–sterilize and store aliquots at −20°C), 0.4 mM spermine tetrahydrochloride (Sigma S-1141; 10 mM stock; filter–sterilize and store aliquots at −20°C), 2 mM dithiothreitol (Sigma D-0632; 100 mM stock; filter–sterilize and store aliquots at −20°C), 2 mM EDTA (500 mM EDTA, pH 8.0 stock; autoclave and store at room temperature). On the day of the sperm nuclei preparation, make up 25 ml of 2× NPB for one or two males from the stock solutions.
4. 1× NPB: Make up 30 mL of 1× NPB by mixing 15 ml of 2× NPB with 15 mL of water and store on ice.
5. Lysolecithin: 100 µL of 10 mg/mL L-α-lysolecithin, egg yolk (Calbiochem, 440154); dissolve at room temperature just before use. Store solid stock at −20°C. Discard the stock powder if it becomes sticky. Digitonin can be used instead of lysolecitin. Digitonin is more specific for the plasma membrane leaving the nuclear membranes intact.
6. Bovine serum albumin (BSA): 10% (w/v) BSA (fraction V, Sigma A-7906) Make up 5 mL in water on the day of the sperm nuclei preparation.
7. 1× NPB, 3% BSA: Mix 5 mL 2× NPB, 3 mL 10% BSA, and 2 mL water and store on ice.
8. 1× NPB, 0.3% BSA: Mix 2.5 mL 2× NPB, 0.15 mL 10% BSA, and 2.35 mL water and store on ice.
9. 100% Glycerol.
10. Sperm storage buffer: 1× NPB, 30% glycerol, 0.3% BSA. Make up by mixing the following solutions, 250 µL of 2× NPB, 15 µL of 10 % BSA, 150 µL of 100% glycerol, and 85 µL water.
11. Sperm dilution buffer: 250 mM sucrose, 75 mM KCl, 0.5 mM spermidine trihydrochloride, and 0.2 mM spermine tetrahydrochloride. Add about 80 µL of 0.1 N NaOH per 20 mL solution to titrate to pH 7.3–7.5 and store 0.5-1 mL aliquots at −20°C.
12. Hoechst No. 33342 (Sigma B-2261): 10 mg/mL stock in dH_2O, store in a light-tight vessel at −20°C.

2.3. Nuclear Transplantation Reagents and Equipment

1. 2.5% Cysteine hydrochloride 1-hydrate in 1× MMR (titrate to pH 8.0 with NaOH). Make up freshly.

2. 100 mM $MgCl_2$.
3. Sperm dilution buffer. Prepare as described in **section 2.1.**, item 11.
4. 0.4× MMR, 6% (w/v) Ficoll (Sigma, F-4375), 10 µg/mL gentamycin (a 10 mg/mL stock solution is purchased from Gibco-BRL 15710-015). Sterilize by filtration.
5. 0.1× MMR, 6% (w/v) Ficoll, 10 µg/mL gentamycin. Sterilize by filtration.
6. 0.1× MMR, 10 µg/mL gentamycin.
7. Linearized plasmid (100 ng/µL in water): Any enzyme can be used for linearization of plasmid. Digest DNA using standard conditions, and purify by phenol–chloroform extraction and ethanol precipitation. There is no need to gel purify the plasmid.
8. Restriction enzyme: Dilute in water before adding to the transplantation reaction. Although any enzyme can be used, do not use an enzyme that digests within regulatory or protein coding regions of the construct. We usually use enzymes, NotI, SalI, or SfiI purchased from Roche. Some calibration may be required to determine the optimal amount of enzyme to add to each reaction, as too much enzyme may adversely affect the development of embryos derived from nuclear transplantations.
9. Agarose-coated injection dishes: 1.0% agarose in 0.1× MMR is poured into 60-mm petri dishes. Before the agarose solidifies, a template is laid onto it. After the agarose has solidified, the templates are removed and the dishes are wrapped in parafilm and stored at 4°C until use. As a template, we routinely use a 35-mm × 35-mm square, small weighing boat, which holds about 400 *X. laevis* eggs.
10. Transplantation needles: 30-µL Drummond MICROCAPS® (Cat. No. 1-000-0300) are pulled to produce large needles with long, gently sloping tips (**Fig. 2**). We use a Flaming/Brown micropipet puller Model P-87 (Sutter Instruments Co.) for pulling needles using a condition, $p = 50$, $v = 100$, and $t = 5$. Needles are clipped with a forceps to produce a beveled tip of 80–100 µm diameter (40–60 µm for *X. tropicalis*), using the ocular micrometer of a dissecting microscope for measurement (**Fig. 2**).

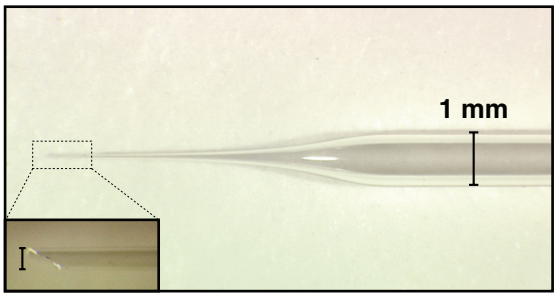

Fig. 2. Transplantation needle has a gently sloping tip and is clipped with forceps to produce a beveled, 80–100 µm wide tip for *X. laevis* and a 40–60 µm wide tip for *X. tropicalis*.

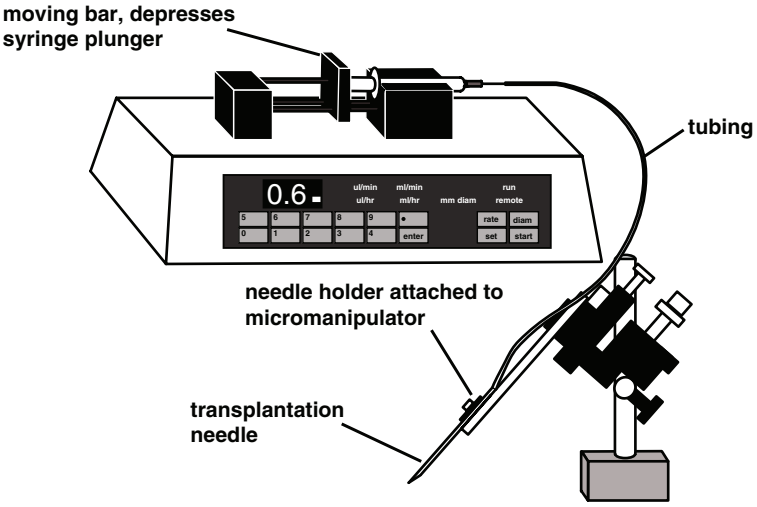

Fig. 3. We use an oil-filled injection system for the nuclear transplantations. The syringe and tubing are filled with mineral oil and the infusion pump depresses the syringe plunger, resulting in a constant, desirable flow rate.

11. Transplantation apparatus: Most commercial injection apparatus used for RNA and DNA injections, which are based on air pressure, are unsuitable for nuclear transplantation, due to the difference in needle tip size. Therefore, we use an oil-filled injection system, Harvard apparatus 22 syringe pump (NP 55-2222) with two 2.5 mL Hamilton gas tight syringes (two 0.1 mL Hamilton gas tight syringes for *X. tropicalis*) and plastic tubing (ID = 0.7 mm, OD = 2.4 mm) (**Fig. 3**). Two people can transplant nuclei at the same time. We use mineral oil (Sigma M-8410) in the system. The infusion pump allows us to control flow rate easily. We set the speed of flow at 0.6 µL/min (0.2 µL/min *for X. tropicalis*).

3. Methods

3.1. Transgenesis Method

3.1.1. High-Speed Extract Preparation

This protocol is an adaptation of Murray ***(39)***. Briefly, a crude cytostatic factor (CSF) arrested egg extract (cytoplasm arrested in meiotic metaphase) is prepared. Calcium is then added to allow the extract to progress into interphase, and a high-speed spin is performed to obtain a purer cytoplasmic fraction. Cytochalasin is omitted from the protocol, since the carryover of cytochalasin into the final extract used for sperm incubations interferes with the normal development of transplant embryos. Use of high-speed rather than crude

cytoplasmic extracts is advantageous, because high-speed extracts promote swelling of added sperm nuclei (and some chromatin decondensation) but do not promote DNA replication. Replication of sperm DNA incubated in these extracts occurs after transplantation of the nucleus into the egg rather than in the extract. High-speed extract can be stored frozen in small aliquots (at −80°C) and thawed before use.

1. Prime 8–12 female adult *X. laevis* about 3–5 d prior to HCG injection by injecting 50 U PMSG into the dorsal lymph sac. The evening before the extract, preparation begins, inject each frog with 500 U HCG and place two frogs/container into 2 L 1× MMR. Since one frog with lysing or activating eggs can compromise the whole extract preparation, we prefer to separate the frogs into pairs for the ovulation. The frogs are then placed at 15–18°C overnight (12–14 h). On the next morning, the egg quality from each container is screened before mixing all the eggs and starting the extract preparation. All the eggs released in a container with mottled, lysing, or dying eggs are left out of the extract preparation.
2. All solutions should be prepared before beginning the extract preparation, since the procedure should be carried through all steps promptly once it is initiated; optimally, the high-speed spin should begin within 45–60 min of dejellying the eggs.
3. Gently, manually expel eggs from each frog into large beakers containing 1× MMR, and collect unbroken eggs with even pigmentation. Good eggs can also be collected from the 1× MMR in the frog buckets. The total volume of eggs should be 100 mL or greater before dejellying.
4. Remove as much MMR as possible from the eggs. Dejelly eggs in 2% cysteine in XB salts (no HEPES–sucrose). Add a small amount at a time, swirl the eggs, and partially replace with fresh cysteine several times during dejellying. Remove broken eggs with a pipet during dejellying. Dejellying should be initiated separately for different batches of eggs, and batches that show breakage or egg activation are discarded. The rest of the eggs can then be combined.
5. Wash eggs in XB (with HEPES–sucrose). We use about 35 mL for each wash, and do four washes.
6. Wash eggs in CSF-XB with protease inhibitors. We do two 25 mL washes.
7. Using a wide-bore Pasteur pipet, transfer eggs into Beckman ultraclear tubes. For these volumes, we typically use 14 × 95-mm tubes (Beckman, 344060). If multiple tubes are used, try to transfer an equal volume of eggs per tube. Allow the eggs to settle and remove as much CSF-XB as possible.
8. Spin for about 60 sec at 1,000 rpm (150 *g*) in a Beckman SW 40 Ti Rotor (or similar rotor) in an ultracentrifuge. Remove the excess CSF-XB and then balance the tubes.
9. Spin the tubes for 10 min at 16,000 *g* (10,000 rpm) at 2°C in a Beckman SW 40 Ti Rotor (or similar rotor) in an ultracentrifuge to crush the eggs. The eggs should be separated into three layers: lipid (top), cytoplasm (center), and yolk (bottom). Collect the cytoplasmic layer from each tube with an 18-gauge needle by inserting the needle at the base of the cytoplasmic layer and withdrawing slowly. Transfer cytoplasm to a fresh Beckman tube on ice. If large volumes of darkly pigmented

eggs are used, the cytoplasmic layer may be grayish rather than golden at this step. After a second spin to clarify this extract, it should be golden.

10. Add protease inhibitors to the isolated cytoplasm (do not add cytochalasin); recentrifuge the cytoplasm in the Beckman tubes for an additional 10 min at 16,000 *g* to clarify, again using a swinging bucket rotor. Collect the clarified cytoplasm as before. Expect to get obtain 0.75–1.00 mL cytoplasm/batch of eggs collected from one frog.
11. Add 1/20 vol of the ATP-regenerating system (energy mix). Transfer the clarified cytoplasm into TL100.3 thick-wall polycarbonate tubes (Beckman, 349622). Tubes hold about 3 mL each and should be at least half full.
12. Add $CaCl_2$ to each tube to a final concentration of 0.4 mM; this inactivates CSF and pushes the extract into interphase. Incubate at room temperature for 15 min then balance for the high-speed spin.
13. Spin tubes in a Beckman tabletop TL-100 ultracentrifuge in a TL100.3 rotor (gold top; fixed angle) at 70,000 rpm for 1.5 h at 4°C.
14. The cytoplasm fractionates into four layers, top to bottom: lipid, cytosol, membranes–mitochondria, and glycogen–ribosomes. Remove the cytosolic layer from each tube (about 1 mL if 2–3 mL were loaded into the tube) by inserting a syringe into the top of the tube through the lipid layer. Transfer this fraction to fresh TL-100 tubes, and spin again at 70,000 rpm for 20 min at 4°C.
15. Aliquot the high-speed cytosol supernatant into 25 µL aliquots in 0.5-mL Eppendorf tubes. Quick-freeze aliquots in liquid nitrogen, and store at −80°C until use. We typically obtain 1–2 mL of high-speed cytosol from preparations of this scale. Sperm nuclei should be incubated in an aliquot of extract and stained with Hoechst, as previously described, to determine whether extract is effective. If active, interphase extract should cause nuclei to swell visibly (thicken and lengthen) within 10 min of addition at room temperature.

3.1.2. Sperm Nuclei Preparation

We generally follow the standard protocol of Murray ***(39)***, but omit the protease inhibitors leupeptin and phenylmethylsulfonyl fluoride from all steps to avoid transfer into the final mixture, which is diluted for egg injections. We always obtain better sperm nuclei when males are injected with hormones.

1. Prime one or two males about 3–5 d prior to HCG injection with 50 U PMSG.
2. Inject the males 12–15 h before the preparation with 500 U HCG.
3. Dissect and isolate the testes from the male:
   a. Anesthetize a male by immersion in 0.1% tricaine methanesulfonate/0.1% sodium bicarbonate for at least 20 min (immersion of the animal in ice water for 20 min may also be used), and pith it.
   b. Cut through the ventral body wall and musculature, and lift the yellow fat bodies to isolate the two testes, which are attached to the base of the fat bodies, one on each side of the midline.
   c. Remove the testes with dissecting scissors, and roll them on a paper towel to remove the blood, blood vessels, and fat body.

   d. Wash the testes briefly in a 60-mm petri dish containing cold 1× MMR, removing any attached pieces of fat body or debris with a forceps. Take care not to puncture the testes, as this releases the sperm. This is particularly true with testes from *X. tropicalis*.
   e. Rinse the testes in cold 1× NPB.
4. Move the cleaned testes to a dry 60-mm petri dish, and macerate the testes well (until clumps are no longer visible to the naked eye) with a pair of clean forceps.
5. Add 2 mL cold 1× NPB and mix well by pipeting the solution up and down with a 10 mL plastic pipet.
6. Squirt the sperm suspension through four thicknesses of cheesecloth placed into a funnel, and collect the solution in a 14-mL tube (we use Falcon 2059).
7. Rinse the dish with an additional 3 mL of cold 1× NPB, and force this through the cheesecloth into the 14-mL tube.
8. Add 5 mL of cold 1× NPB and squeeze the cheesecloth by hand, wearing gloves, to get any remaining liquid through the funnel into the 14-mL tube.
9. Pellet the sperm by centrifugation at 3,000 rpm for 10 min at 4°C (we use a Sorvall HB-4 or similar swinging bucket rotor with the appropriate adapters). During the spin, allow 1 mL of 1× NPB to equilibrate to room temperature.
10. Decant the supernatant and resuspend the sperm in 9 mL 1× NPB using a 10-mL plastic pipet and repellet by centrifugation at 3,000 rpm for 10 min at 4°C.
11. Decant the supernatant and resuspend the pellet with a 1 mL blue tip in the 1 mL 1× NPB that has equilibrated at room temperature.
12. Add 50 µL of 10 mg/mL lysolecithin. Mix gently and incubate for 5 min at room temperature.
13. Add 10 mL of cold 3% BSA/1× NPB to the suspension to stop the reaction, and centrifuge at 3,000 rpm for 10 min at 4°C.
14. Decant the supernatant and resuspend the pellet in 5 mL cold 0.3% BSA/1× NPB. Mix well by pipeting with a 5-mL plastic pipet and centrifuge at 3,000 rpm for 10 min at 4°C.
15. Take supernatant carefully and resuspend the pellet in 500 µL of sperm storage buffer, and transfer suspension into a 1.5-mL Eppendorf tube.
16. Count the number of sperm nuclei using a hemacytometer: Cut off the end of a yellow tip with a razor blade and mix the sperm nuclei well by pipeting. Dilute 1 µL of the sperm nuclei with 100 µL of sperm dilution buffer, and add 1 µL of 1:100 diluted Hoechst stock to visualize the sperm nuclei under a fluorescence microscope. For a 1:100 dilution of our sperm stock, we typically obtain counts of 100–200 ($\times 10^4$ nuclei/mL) in a 1-mm × 1-mm × 0.1-mm square of an improved Neubauer hemacytometer. At this concentration, the undiluted stock contains 1–2 $\times 10^5$ nuclei/µL. If your sperm stock is substantially less concentrated (i.e., a count of <50 for a 1:100 dilution), let the sperm settle for a few hours or overnight, and remove some of supernatant. We leave fresh nuclei overnight at 4°C to allow the penetration of glycerol for best cryopreservation of the sperm. The next day the sperm is aliquoted (20 µL/aliquot) and fast frozen in liquid nitrogen. The frozen aliquots are then stored at −80°C.

3.1.3. Transgenesis by Sperm Nuclear Transplantation into Unfertilized Eggs

1. Prime two females 3–5 d before HCG injection with 50 U PMSG.
2. Inject the females with 500 U HCG, 12–15 hours before they are needed.
3. Allow an aliquot of sperm dilution buffer (SDB) to equilibrate to room temperature, and make up 2.5% cysteine in 1× MMR, pH 8.0.
4. Turn on the switch of the Harvard apparatus and start the infusion pump to get the flow to stabilize before injection.
5. Set up a reaction using a clipped yellow tip: Mix 4 µL sperm stock (~4–8 × 10^5 nuclei) and 1–2 µL linearized plasmid (100 ng/µL), and incubate for 5 min.
6. Dilute 0.5 µL of a restriction enzyme in 4.5 µL of water, and mix 1 µL of the diluted enzyme with 18 µL of SDB, 2 µL of 100 mM $MgCl_2$ and 2 µL of high-speed egg extract.
7. Add the mixture to the sperm–DNA and mix well by gentle pipeting (using a clipped yellow tip). Incubate for 15 min at room temperature; sperm nuclei should visibly swell if diluted into Hoechst as before and observed with a 10–20× objective.
8. During the reaction, collect eggs in a beaker by squeezing frogs and dejelly them in 2.5% cysteine/1× MMR, pH 8.0. This usually takes about 10 min, so by the time the eggs are ready, the reaction is nearly complete. Squeeze eggs directly into a dry beaker and add cysteine immediately to keep egg quality. You may need to dejelly eggs from different females separately, as dejellying times vary among females and poor quality eggs make the solution dirty.
9. Wash the dejellied eggs with 1× MMR at least three times, and transfer the eggs to injection dishes containing 0.4% MMR/6% Ficoll using a wide-bore Pasteur pipet. We generally fill the square space with eggs so that no gap is left between the eggs. After about 5 min in 0.4× MMR, 6% Ficoll, the eggs pierce easily. Transplantation should be performed at around 16°C. To achieve this we place the injection dish on the plastic box half-filled with ice and place the box and eggs under the injection microscope.
10. After the incubation with extracts, mix the sperm nuclei gently by pipeting with a clipped yellow tip. Then transfer 5 µL of the reaction into 150 µL of SDB that has equilibrated at room temperature.
11. Mix well but avoid making bubbles, using a clipped yellow tip with a piece of plastic tube attached (**Fig. 4A**). Fill the clipped yellow tip with the diluted sperm suspension, carefully detach the clipped yellow tip, keeping the tip horizontal and backfill a transplantation needle by attaching it to the tube (**Fig. 4B**). You can keep the yellow tip with the remaining nuclei, by placing it horizontally, in case you need to load another needle. Keep decondensed sperm nuclei at room temperature and transplant them within an hour, but preferably within 30 min.
12. Attach the needle to the tube filled with mineral oil that is connected to the syringe on the Harvard apparatus.
13. Check the flow and start injecting. Keep the needle inside each egg for approximately 0.5 sec, and move the needle fairy rapidly from egg to egg, piercing the plasma membrane of each egg with single, sharp motion. We usually transplant for

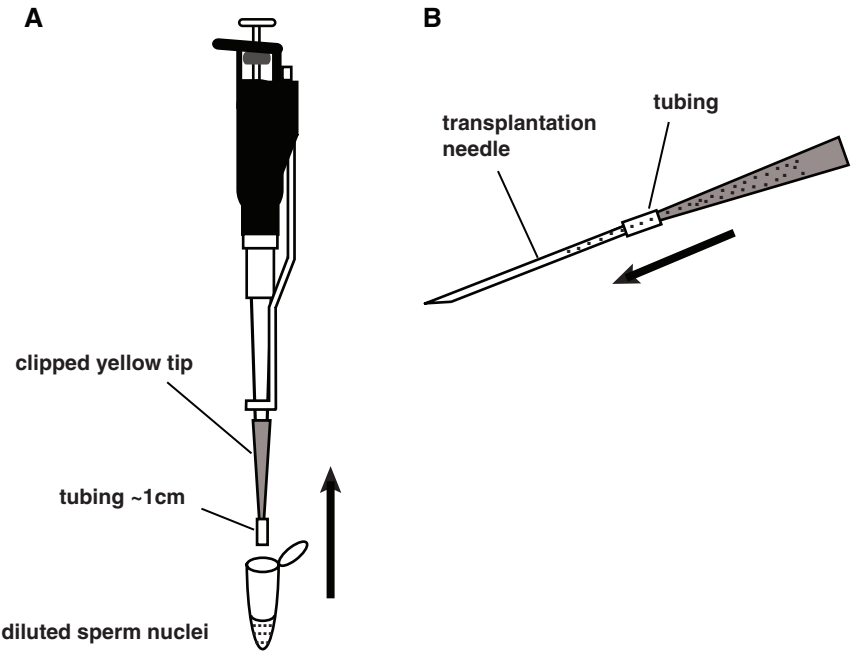

Fig. 4. Method for backfilling nuclei into a transplantation needle. **A** The diluted reaction mix is drawn into a clipped yellow tip containing about 1 cm of Tygon tubing. **B** The yellow tip containing the Tygon tubing and dilute sperm nuclei is carefully detached from the pipetman and connected to the back of needle using the tubing. The needle is gently loaded with the dilute sperm nuclear reaction by gravity. This is done by slowly increasing the angle of the yellow tip–tubing–needle so that the mixture flows gently into the needle. Once the needle is complete filled, the needle is detached from the yellow tip and ready to connect to the infusion pump. The remaining sperm mixture can be set aside horizontally and used to reload another needle, if two people are injecting simultaneously or if a needle is accidentally damaged or blocked.

about 15–20 min. If a needle is blocked by debris during transplantation, change needle or try to fix it by pinching the tube or cutting the tip of needle using forceps.

You can determine whether the sperm dilution and the flow rate used for injections were appropriate by watching the first cleavage of the transplanted eggs. If few of the eggs received a nucleus, the frequency of cleavage will be low; one third of our transplantations typically result in normally cleaving embryos. Eggs that were injected with more than one nucleus divide at the time of first cleavage abnormally into three or four (or more) cells. Many of these embryos develop to blastula stages, but most fail during gastrulation; in some, a region of the embryo fails to cellularize and dies. Eggs injected with multiple nuclei that do gastrulate usually do so abnormally; typically, blastopore closure is incomplete,

resulting in embryos that form two wings of somites and neural tissue on each side of the exposed yolky tissue lying in the center of the trunk. This type of gastrulation failure is common to stressed or unhealthy embryos (particularly embryos derived from "soft" eggs).

14. After injection, incubate embryos at 16°C.
15. When the embryos reach the four-cell stage (about 3–4 h after injection at 16°C), gently transfer normally dividing embryos to a 10-cm petri dish containing 0.1× MMR, 6% Ficoll, 10 µg/mL gentamycin using a wide-bore Pasteur pipet.
16. The next day, when embryos are around stage 12, transfer healthy embryos to a new 10-cm petri dish containing 0.1× MMR, 10 µg/mL gentamycin without Ficoll. Because of the large needle tip used for transplantations, embryos often develop large blebs at the site of injection. These blebs occur when cells are forced out of the hole left in the vitelline membrane at the injection site, but they generally do not affect development. The blebs usually fall off at the neurula or tailbud stages.
17. Incubate embryos until they reach the stage that you need to analyze at 14–22°C.

3.2. Transgenesis Method for Xenopus tropicalis

Xenopus tropicalis has a diploid genome and shorter generation time than *X. laevis*, but retains many of the advantages of *X. laevis* ***(43, 44)***. In *X. tropicalis*, transgenesis can be combined with genetic experiments. The basic protocol for transgenesis in *X. tropicalis* is very similar to the procedure used for *X. laevis*. In this section, we point out the differences in the *X. tropicalis* procedure relative to the procedure already describe for *X. laevis*. We use a high-speed egg extract from *X. laevis* in the protocol for *X. tropicalis*.

3.2.1. Reagents and Equipment

X. tropicalis eggs and embryos appear to be more sensitive to salt concentration than *X. laevis*. Therefore, we use less concentrated MMR solutions for dejellying and culture and MOH buffer instead of SDB for the nuclear transplantation ***(13)***. We also use L-cysteine (free base) for dejellying *X. tropicalis* eggs instead of the L-cysteine hydrochloride 1-hydrate used to dejelly *X. laevis* eggs. The reason for this is the higher sensitivity of *X. tropicalis* eggs and embryos to high salt concentrations. The acidic version of cysteine requires considerable amounts of NaOH to bring the pH up to 8.0, thus bringing the NaCl concentration to a high level. The free-base version of cysteine requires much less NaOH to bring the pH up to 8.0 and thus results in lower final concentration of NaCl.

1. 2% Cysteine 0.1× MMR (titrate to pH 8.0 with NaOH). Make up freshly.
2. 0.1× MMR, 6% (w/v) Ficoll, 10 µg/mL gentamycin. Sterilize by filtration.
3. 0.01× MMR, 6% (w/v) Ficoll, 10 µg/mL gentamycin. Sterilize by filtration.
4. 0.01× MMR without gentamycin. Since the development of *X. tropicalis* embryos in later stages seems to be affected by gentamycin, we do not use gentamycin for long-term culture.

5. Sperm injection buffer modified for *Xenopus tropicalis* (MOH): 10 mM KPO_4, pH 7.2, 125 mM potassium D-gluconate (Sigma, G-4500), 5 mM NaCl, 0.5 mM $MgCl_2$, 250 mM sucrose, 0.25 mM spermidine trihydrochloride, 0.125 mM spermine tetrahydrochloride.
6. Agarose-coated 24 well dishes: 1.0% agarose in 0.1× MMR is poured into 24-well dishes. We use agarose-coated dishes to culture *X. tropicalis* embryos until they hatch at around stage 28, because their vitellin membrane is quite sticky. We also use 24-well dishes in which one or two embryos are cultured per well until the neurula stage.
7. Transplantation needles: Needles are clipped with a forceps to produce a beveled tip of 40–60 µm diameter.
8. Transplantation apparatus: 0.1 mL Hamilton gas tight syringes on the system. Set the flow rate at 0.2 µL/min.

3.2.2. Sperm Nuclei Preparation

1. Amount of hormone: Prime two males the day before the sperm nuclei preparation with 15 U PMSG. Inject the males 3 h before the nuclei preparation with 150 U HCG.
2. Concentration of lysolecithin: Dilute 20 µL of 10 mg/mL lysolecithin with 980 µL of water (0.2 mg/mL) and add 50 µL of that diluted lysolecithin to the sperm suspension (thus, 50 times less lysolecithin than used for *X. laevis*). Mix gently and incubate for 5 min at room temperature.
3. Count of the sperm nuclei: After the final spin, resuspend the pellet in 100 µL of sperm storage buffer. For a 1:100 dilution of our sperm stock, we typically obtain counts of ~80 (×10^4 nuclei/mL) in 1-mm × 1-mm × 0.1-mm square of an improved Neubauer hemacytometer. At this concentration, the undiluted stock contains 8 × 10^4 nuclei/µL, and we use 10 µL of that for a reaction. If the sperm stock is substantially less concentrated, let the sperm settle for a few hours or overnight, and remove some of supernatant. If the count is still low, use up to 30 µL of the sperm nuclei per reaction. Sperm nuclei can be frozen in aliquots at −80°C.

3.2.3. Nuclear Transplantations

1. Amount of hormone: Prime two females the day before the nuclear transplanations with 15 U PMSG. Inject the females 3–4 h before the nuclear transplantation with 150 U HCG and leave frogs at 22–24°C. We use two females for two rounds of the nuclear transplantation a day. If more rounds of transgenesis are needed in the same day, inject another two females 1 or 2 h after the first pair. This will allow for four rounds of transgenics, each around 1 h apart.
2. Reaction of the sperm nuclei: Use 10 µL of sperm nuclei (~8 × 10^5 nuclei) per reaction. After the incubation with egg extract, dilute all the reaction mix with 130 µL of MOH that has equilibrated at room temperature. Mix well and backfill a needle using a clipped yellow tip with a piece of tube. If you use more than 10 µL of the sperm (up to 30 µL), the amount of MOH buffer should be reduced. The concentration of nuclei must be considerably higher with *X. tropicalis*, since the volume injected into each egg is about one fifth the amount used for *X. laevis*.

3. Injection: After dejellying eggs in 2% cysteine/0.1× MMR, pH 8.0, transfer the eggs to injection dishes containing 0.1% MMR/6% Ficoll. We generally fill half of the square space with eggs, because we need to inject eggs within 30 min. Transplantation is performed at room temperature (22–24°C).
4. Screening: Embryos reach the four-cell stage approximately 1.5 h after injection at 22–24 °C, therefore it may be necessary for one person to sort the embryos while others continue later rounds of injections (i.e., the third and fourth rounds of injections). Collect normally dividing embryos and transfer them to agarose-coated 24-well dishes (one or two embryo/well) containing 0.01× MMR/6% Ficoll/10 µg/mL gentamycin. Culture embryos overnight at 22°C.
5. The next day transfer healthy embryos to an agarose-coated 10-cm petri dish containing 0.01× MMR without Ficoll or gentamycin, and culture embryos at 22°C.

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32

Axolotl/Newt

Malcolm Maden

1. Overview

Limb regeneration is one of the oldest topics within developmental biology, since the first experiments were reported in 1768 by Spallanzani, a Roman Catholic priest and Professor of Natural History at Padua, Italy. In his work *Prodromo di un opera da imprimersi sopra la riproduzioni animali*, he showed that regenerative ability was widespread throughout the animal kingdom, and that the legs and tails of newts could regenerate perfectly time after time. These studies provided much fuel to the contemporary debate between the preformationists and epigeneticists, and won for Spallanzani his election as a foreign correspondent of the Royal Society.

The Italian connection was crucial to the early development of regeneration research, because in 1823 Todd ***(1)*** showed that the regeneration of newt limbs was completely inhibited when the nerve supply to the limb was cut. Todd was a physician who served in the Royal Navy and apparently performed his experiments while at the British naval base in Naples. This was the first demonstration of the neurotrophic requirement for limb regeneration. As the 19th century progressed, the pace of regeneration research heated up, and by the turn of the century, there were hundreds of papers being published each year, mostly in German. Limb regeneration, along with studies on frog and newt eggs, were the driving forces in vertebrate developmental biology at that time. This pace continued throughout the first half of this century and from the 1930s researchers in the US made dominant contributions. In the last 20 yr, the pace has considerably slackened, since very few researchers have entered what has become an unfashionable field. Modern developmental biology is now dominated by *Xenopus* eggs and mouse genetics.

Nevertheless, virtually all of the fundamental and fascinating questions that have intrigued students of limb regeneration for more than a century remain

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unanswered and are still there to inspire today's enquiring minds. Why can newts regenerate limbs and mammals cannot? What factors do the nerves supply? What factors does the epidermis supply? How do cells dedifferentiate and undergo transformation into another cell type? Are the mechanisms by which redifferentiation takes place the same as those that were used to develop the limb in the first place? Is the newly regenerated limb a different age from its contralateral unregenerated partner, and if so, is this a way to reverse the deleterious processes of aging?

Imagine what benefit the answers to these questions would bring to humankind. Indeed, there is already one good example of how limb regeneration studies have been of direct practical benefit to medicine. After limb amputation, the epidermis migrates over the stump to close the wound resulting in epidermal/mesenchymal interactions, which are crucial to the induction of regeneration (*see* **Subheading 2.2.**). It has been known since 1906 ***(2)*** that if full thickness skin is sewn over the amputation plane regeneration is inhibited. This knowledge led to a change in surgical procedure in dealing with the amputated fingertips of children. Prior to Illingworth's report ***(3)***, such cases were dealt with by suturing the wound to make a cosmetically acceptable product, but one which was missing the terminal phalanx. Illingworth showed that if such amputations are simply covered and left to heal normally, then the terminal phalanx and nail will regenerate perfectly. Thus young children up to about the age of 11 yr have considerable capacity for regeneration of fingertips, provided the principles of regeneration are followed.

For more details on the subject of limb regeneration than can be included here, the reader is referred to a book by Wallace ***(4)***, which also contains much information on the older literature.

1.1. Which Species Regenerate?

Limb regeneration occurs after amputation of most larval and adult urodeles (newts and salamanders) and in the tadpole stages of anurans (frogs and toads). After metamorphosis in frogs and toads, regeneration is inhibited with the maximal response being the growth of a long spike. After metamorphosis in urodeles, regeneration is noticeably retarded, but still occurs. It is often said that all urodeles can regenerate their limbs, but this is not so ***(5)***, and in general, postmetamorphic regenerative ability declines with age and size.

A host of different species have been used over the past century for limb regeneration studies, including the European crested newt, *Triturus cristatus*, which is now a protected species. Today, however, four species predominate. One is the eastern spotted newt of North America, *Notophthalmus viridescens*, which is used as an adult. The adults of this species are common, adaptable to

laboratory conditions, and can be purchased from commercial suppliers or collected in the wild. The spotted salamander, *Ambystoma maculatum*, is used at larval stages having been collected from ponds in the spring. The other two species, the ribbed newt, *Pleurodeles waltl*, and the axolotl, *Ambystoma mexicanum*, breed readily in captivity, and there are several established breeding colonies. These two offer several advantages—they breed more than once a year, the eggs can also be used for studies of early development, the larvae regenerate limbs remarkably rapidly, they are available in large numbers, and comparative studies can be performed between limb development and limb regeneration not only in the same species, but in the same animal. In the latter case, because the hindlimbs develop many days after the forelimbs, grafts can be exchanged between forelimb regeneration blastemas and hindlimb developing limb buds ***(6)***. The axolotl offers the peculiar advantage in having suppressed metamorphosis to retain its larval form and aquatic habit throughout life. Consequently, they have become worldwide curiosities not only for the very large size of the elderly adults, but also because by adding thyroxine to the water, they can be induced to metamorphose and transform into a different species! In addition, there is a very large axolotl colony established at Indiana University, Bloomington, IN (run by Susan T. Duhon, IU Axolotl Colony, Jordan Hall 407, Bloomington, IN 47405), which will supply eggs and larvae to anywhere in the world. They also keep all the available mutant lines, some of which are useful for regeneration research, e.g., Short toes ***(7)***.

2. Limb Regeneration

Young axolotl larvae will regenerate their limbs in 2–3 wk, whereas adult newts take 2–3 mo, but the processes they go through are identical.

2.1. Amputation of Limbs

1. Anesthetize the animals by placing them in a solution of 3-aminobenzoic acid ethyl ester (MS222) (Sigma). A concentration of 1 in 10,000 is required for young larvae, but older animals need a stronger solution, 1 in 1000. The acidic solution should be returned to neutrality with NaOH. Five to 10 min in this solution are usually enough.
2. Remove the animals from the anesthetic, place them on a wet paper towel under a dissecting microscope, and amputate the limbs at the desired level with a scalpel, razor blade, or scissors for larger animals.
3. Limb bones will protrude almost immediately owing to retraction of the skin and muscles from the wound surface. Trim these protruding bones, since they interfere with wound healing.
4. Return the animals to their tanks. It is better to keep experimental animals in individual bowls, since this prevents cannibalism, and they can be individually identified.

2.2. Stages and Major Features of Limb Regeneration

Figure 1 shows a series of drawings following amputation either through the midradius and ulna (the zeugopodium level) or the midhumerus (stylopodium level) of an adult newt limb. This emphasizes several principles—whatever the level of amputation, the regenerate is a perfect copy of what was removed; the stages are the same whatever the level of amputation; regeneration from the lower arm is completed more quickly than from the upper arm. The latter occurs because there is less tissue to be replaced from lower arm amputations and the rate of cell division of blastemal cells is the same at lower arm levels as at upper arm levels ***(9)***.

Staging systems for various species have been published: adult *N. viridescens* ***(10)***, large axolotls ***(11)***, and larval *A. maculatum*, also valid for small larval

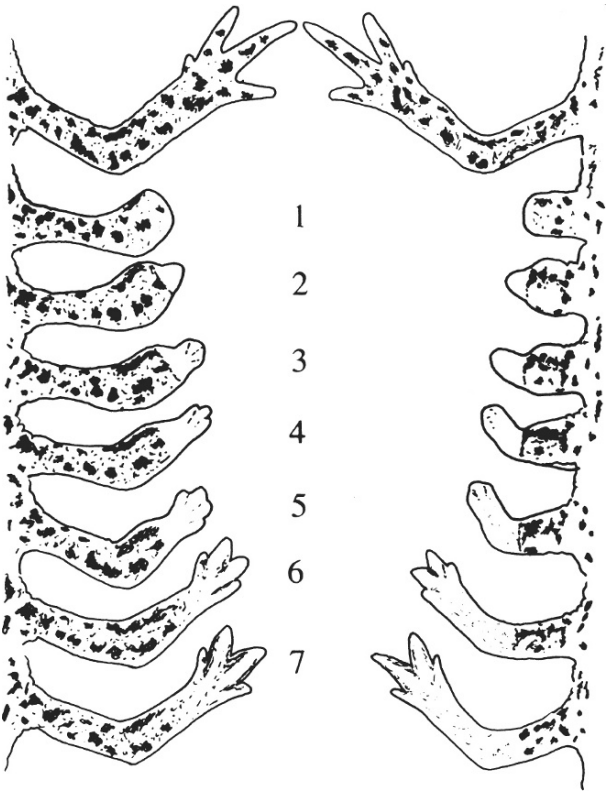

Fig. 1. Stages in the regeneration of adult newt limbs following amputation through the midlower arm (left side) or the midupper arm (right side). At the top are the original limbs. Stage 1–7 d; stage 2–21 d, stage 3–25 d, stage 4–28 d, stage 5–32 d, stage 6–42 d; stage 7–70 d after amputation. Redrawn from **ref. *8***.

axolotls ***(12)***. In the following description, the major events of regeneration are described rather than a precise staging system, which can be obtained from the above references. Also, times are not included, since this depends on the ambient temperature, age of the animal, and species. As mentioned above, in small larval axolotls, this is a very rapid process, being completed in 2–3 wk.

1. Wound healing (**Fig. 1**, stage 1; **Fig. 2A**): This is achieved in a matter of hours after amputation by migration of the epidermis from the cut edges of the stump. It is slower if the protruding bones are not trimmed away. There are large numbers of damaged mesodermal cells and blood clots that the wound epidermis has covered, and during the first few days after amputation, phagocytes accumulate to remove this cell debris. This debris can also be seen between the cells of the wound epithelium, so some must be ejected via this route. Fluid often accumulates at the tip, but a circulating blood supply is rapidly re-established. Mitosis in the wound epithelium is inhibited for several days after amputation.
2. Dedifferentiation (**Fig. 2B,C**): Once the local damage has been repaired, the net effect of wound healing can be appreciated—the juxtaposition of a naked epidermis and mesodermal tissues. Normally the dermis and basal lamina are present to prevent interactions. Indeed, if full thickness skin is sewn over an amputated stump, then limb regeneration is inhibited, as described above ***(2,13)***. The wound epithelium seems to be active in inducing histolysis of the mesodermal tissues it is now in contact with and the process of dedifferentiation begins. The cytoplasm of the myotubes fragments and surrounds individual nuclei, and osteocytes appear along the cut ends of the bones breaking down the matrix and releasing individual chondrocytes (**Fig. 2B,C**). This process is clearly an organized one unrelated to simple phagocytic breakdown, because the result of dedifferentiaion is the appearance of embryonic cells with large pale nuclei and active cytoplasms rich in rough endoplasmic reticulum. These dedifferentiated cells now begin cell division and accumulate under the apical cap.
3. Apical cap (**Fig. 2D**): After closure of the wound, the epidermis continues to migrate and piles up at the tip forming a very thick apical epidermis or apical cap. This structure has been likened to the apical ectodermal ridge (AER) of the chick limb bud, since it is responsible for the accumulation of dedifferentiated cells beneath it, for the direction of outgrowth of the blastema, and it behaves like the AER in inducing an accessory outgrowth when transplanted ***(14)***. Perhaps it also generates fibroblast growth factor ***(15)***, as the AER does.
4. Blastema: The accumulation of dedifferentiated cells beneath the apical cap results in the appearance of a small, conical structure known as the early bud blastema (**Fig. 2D**). The blastemal cells now begin to divide rapidly and increase in number by proliferation rather than by continued dedifferentiation, the latter now ceasing. As more and more cells are generated, the blastema increases in size, forming a larger and larger cone at the limb apex (stage 2 in **Fig. 1**) and is known as the medium bud blastema (**Fig. 2E**). Nerve fibers are present throughout the blastema, but the vasculature appears sparse.

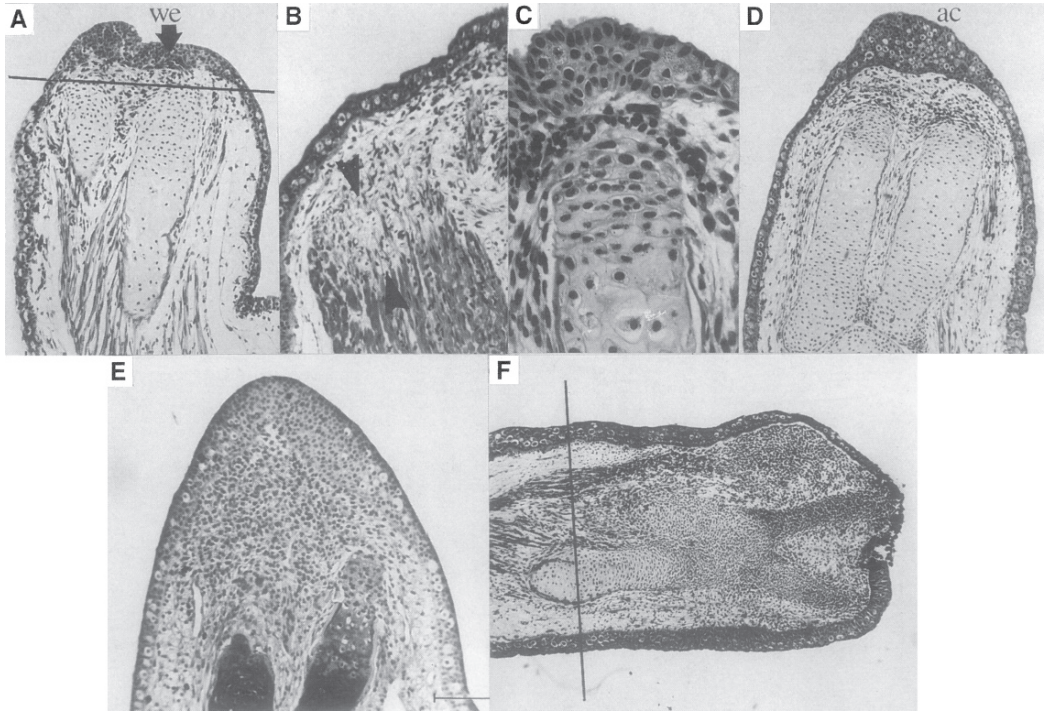

Fig. 2. Stages of limb regeneration. (**A**) Wound healing. Within a few hours after amputation a wound epithelium (we) migrates over the stump to heal the wound completely. Solid line marks the level of amputation through the radius and ulna. (**B,C**) Dedifferentiation. In B, muscle dedifferentiation is shown. The loss of myofiber structure can clearly be seen stretching back a considerable distance from the amputation plane, between the two arrowheads. In C, cartilage dedifferentiation is shown. At the bottom of the micrograph are typical small, darkly staining chondrocytes. In the middle of the micrograph, the nuclei of the chondrocytes can be seen to be enlarging and staining less intensely. At the cut tip of the cartilage, cells are released into the blastema as the cartilage matrix has been degraded. (**D**) Apical cap. As dedifferentiation progresses and an early bud blastema begins to accumulate, the wound epithelium piles up thickly, and the apical cap (ac) forms at the tip of the stump.

5. Redifferentiation (**Fig. 1**, stage 3; **Fig. 2F**): As the blastema enlarges and becomes an elongated cone, the cells in the proximal region adjacent to the stump begin to segregate into precartilage and premuscle masses. This stage is known as the late bud or palette stage. As redifferentiation commences, the first new cartilage element (e.g., the distal half of the humerus if amputation was through the midhumerus level) appears as a continuation from the stump, as if the remaining piece of humerus serves as a model. The same applies to the newly differentiating muscles. Redifferentiation is occurring proximally, while distally, proliferation of the blastemal cells continues. Clearly then, redifferentiation progresses in a generally proximal-to-distal fashion (except for a blip in the wrist), since the appearance of the humerus is soon followed by the appearance of a completely new radius and ulna, followed by the digits, followed by the wrist elements. This sequence is a repeat of the sequence of appearance of elements during development, even down to the wrist elements and digits, which develop and regenerate in an anterior to posterior sequence. When the digits first appear (**Fig. 1**, stage 4 on the left and stage 5 on the right), the regenerate is at the early digit stage, and when all the digits have appeared (**Fig. 1**, stage 5 on the left and stage 6 on the right), the regenerate is at the late digit stage.

2.3. Control of Limb Regeneration

1. Nerves: Since the pioneering experiments of Todd ***(1)***, it has been known that a denervated limb will not regenerate. The incredibly detailed series of experiments by Singer and colleagues in the 1940s ***(16)*** in which he partially denervated limbs, counted the remaining nerve fibre numbers at various limb levels and then recorded the resulting frequency of regeneration, led to the hypothesis that a threshold number (between 30 and 50% of normal) was required for regeneration to occur. Either nerve type, motor or sensory, will suffice provided they are present in sufficient quantity. It was subsequently assumed that the nerves provide a neurotrophic factor and that it was concentration of this factor that Singer had been quantitating. The neurotrophic factor is responsible for stimulating blastema cell division. A strong candidate for the neurotrophic factor is glial growth factor ***(17)***, although no one has managed to replace completely the function of the nerves by a defined compound. This is an extremely difficult experiment to perform because of the problem of administering minute quantities of a test substance over a prolonged period of many weeks and the problem of keeping the limb denervated as amphibian nerves readily regrow after crushing or severing.

Fig. 2. (*continued*) Cartilage dedifferentiation can be clearly seen from the cut ends of the radius and ulna. (**E**) Blastema. Dedifferentiation followed by proliferation of the released cells generates a blastema consisting of a mass of rapidly dividing, embryonic cells covered by an epithelium. This is at the medium bud blastema stage. (**F**) Redifferentiation. After the blastema has reached a certain size, redifferentiation begins proximally and spreads distally until all the elements that were removed by amputation are replaced. Here the solid line marks the amputation plane, and the new distal radius can be seen to be fused perfectly with the remaining proximal radius in the stump. The first two digits can clearly be seen as well as a large cartilage mass between the radius and the digits, which will form the wrist elements. This is at the early digit stage.

This well-founded neurotrophic theory also encompasses a fascinating paradox. Limbs that have never been innervated, so-called aneurogenic limbs, regenerate perfectly in the absence of nerves. When nerves are allowed to enter the aneurogenic limb, they gradually become dependent on innervation—the blastemas cells are thought to have become "addicted" to the neurotrophic factor. More recent experiments have cast some light on these rather vague concepts by showing that the nerve controls the molecular phenotype of blastemal cells ***(18)***.

2. Hormones: It is generally taken for granted that the appropriate "hormonal milieu" is required for regeneration. This mileau includes the adrenal corticosterioids, somatotrophin, thyroxine, insulin, and prolactin. However, it has been particularly difficult to demonstrate specific requirements for several reasons. First, removal of the gland under consideration is usually so severe an operation either physically or physiologically that the animals do not survive. Second, the successful removal of an organ, such as the pituitary, has such a profound effect on many other hormonal systems that it is impossible to dissect out individual requirements for regeneration. With the advent of cloned products, however, specific requirements are being demonstrated, e.g., for growth hormone ***(19)***. Third, removal of a gland followed by replacement therapy has either involved mammalian preparations whose similarity of action in amphibia is unknown, or impure preparations have been used. Nevertheless, we would expect that hormones, such as growth hormone or prolactin, which have such an important role in the control of basic cell metabolism, should be involved in regeneration, but not nesessarily in a controlling capacity.
3. Origin of blastemal cells: The cells that form the blastema arise from within 1–2 mm of the amputation plane by the process of dedifferentiation of the mesodermal tissues as described above. The epidermis cannot contribute to any internal tissues, but only forms the apical cap. Meticulous studies recording cell and mitotic counts have concluded that all mesodermal tissues contribute to the blastema (dermis, muscle, connective tissue, periosteum, bone and even Schwann cells) roughly in proportion to the number of cells in each tissue in a cross-section of the stump (***20***, although *see* ***21***). There is a tendency to assume that the majority of cells revert back to their former differentiated state, but this is not necesarily so, as two recent studies have conclusively demonstrated. In the first, cells were marked by grafting between diploid and triploid animals followed by meticulous cell counting. It transpired that there was an overrepresentative contribution of dermis from the stump and an underrepresentative contribution from the cartilage ***(21)***. In the second study, cultured myotubes were labeled with lysinated dextran, implanted into blastemas, and when redifferentiation began, labeled cells were occasionally seen in the cartilage of the regenerate ***(22)***. Thus, a proportion of cells seem to undergo a metaplastic transformation during normal regeneration.

Another type of experiment in which cells are forced to do more than they naturally would has also demonstrated the metaplastic potential of blastemal cells. In these experiments, limbs are irradiated with X-rays, which permanently prevents cell division, and then a graft of unirradiated tissue is provided, which supplies new cells with regenerative potential. If the graft is from a white axolotl and the

host is a black axolotl, then the regenerate will be white confirming the origin of the tissues of the regenerate. In these experiments, it was shown that grafts of muscle could provide all the tissues of the regenerate, including cartilage (as revealed in the labeling experiment described above). However, grafts of dermis and grafts of cartilage could provide all the tissues of the regenerate, except muscle. Thus, it seems that most metaplastic transformations are possible, except that only myoblasts can generate new myoblasts.

This type of tissue transformation is readily demonstrable in a simple experiment where the cartilage of the stump is removed prior to amputation. Even though there is no cartilage at the amputation plane and no chondrocytes dedifferentiate to supply the blastema, perfect cartilage elements are produced distal to the amputation plane (**Fig. 3A**).

4. Regional and axial determination: That individual blastemal cells remember the region of the body from which they come is the general conclusion from a long history of grafting studies. Tail tissue grafted to limbs or vice versa or forelimb tissues grafted to hindlimbs or vice versa results in the regeneration of organs specific to the graft type, not the host type. Until recently, it had never been possible to change the organ specificity of cells, but this has now been done. The regenerating tail blastema of frogs can be homeotically transformed into tails by treatment with retinoids prior to metamorphosis ***(23,24)***. The same general conclusion is also true of axial determination. From the very beginning, blastemal cells carry a knowledge of their axial position. This has been demonstrated many times in experiments in which blastemas are cut off the stump and then either rotated 180° and put back on or grafted from left to right (or vice versa). The former manipulation reverses both anteroposterior and dorsoventral axes and results in the appearance of supernumerary limbs (**Fig. 3B**). The same is true if either the anteroposterior or dorsoventral axis is reversed (**Fig. 3C**). Thus, axes cannot be respecified. The interaction between cells whose axes conflict results in the generation of extra tissue to resolve the conflict.

   In the proximodistal axis level-specific information is similarly present within the blastemal cells. Clearly, it must be or the limb would not know how much of itself to regenerate. If a proximal blastema is grafted onto a distal amputation stump, then the result will be a limb that has serially duplicated elements (**Fig. 3D**). In this case, there seems to be no interaction between stump and blastema in reaction to the disparity in level-specific information. When the converse experiment is performed, a distal blastema grafted onto a proximal stump, then interaction does take place because the disparity is recognized and the gap filled in to generate a normal limb (**Fig. 3E**). The gap is filled in by proximal cells, not by distal cells, and these observations led to the formulation of the "law of distal transformation" which only allows cells within the limb field to become more distal, never more proximal. Significantly, just like the respecification of regional determination by retinoids, the only instance where the law of distal transformation has been broken is when distal blastemas are treated with retinoids. In this case, a complete limb, including proximal elements, can be regenerated from a distal level amputation after retinoid treatment (**Fig. 3F**) ***(25)***.

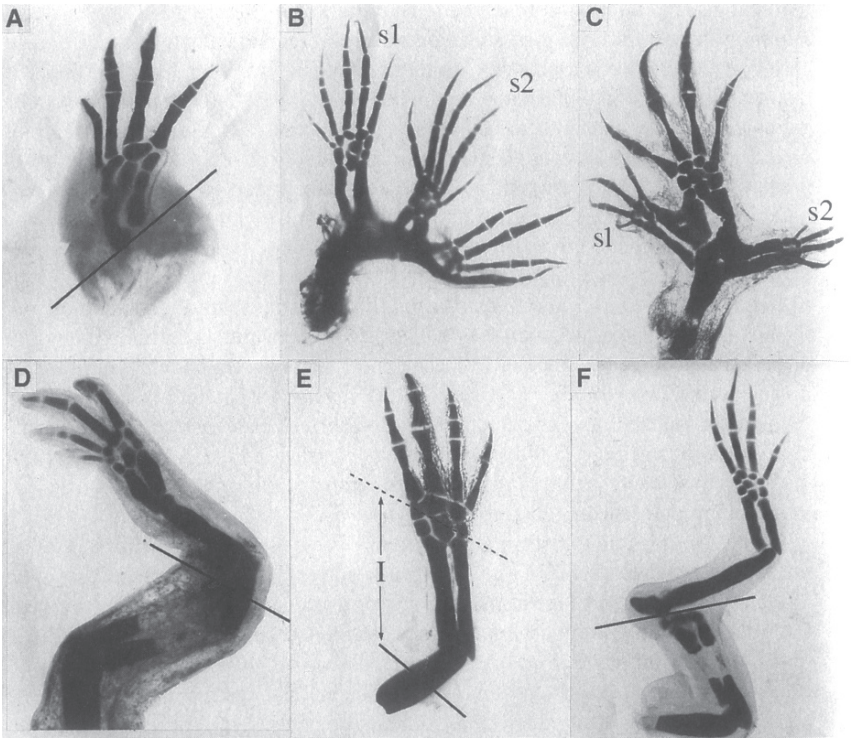

Fig. 3. Victoria blue-stained limb regenerates to show the cartilage patterns after various treatments. **(A)** The structure of the regenerate after removing all the cartilage and bone from a forelimb and then amputating through the lower arm level. A solid line marks the amputation plane. Proximal to the amputation plane, there is only muscle, which does not stain with Victoria blue. Distal to the amputation plane, the ends of the radius and ulna, wrist elements, and digits have regenerated perfectly despite the absence of cartilage in the stump from which the dedifferentiated tissues of the blastema were derived. This is a simple demonstration of tissue metaplasia. **(B)** The result of cutting off a blastema from a regenerating hindlimb, rotating it 180° and putting it back on the stump. Two supernumerary limbs (S1 and S2) have been produced in addition to the original limb. **(C)** The result of putting a left forelimb blastema on a right stump to reverse the anteroposterior axis. As in B, two supernumerary limbs are generated (S1 and S2) in addition to the original limb. **(D)** The result of grafting a proximal blastema (from the shoulder level) onto a distal amputation stump (through the ends of the radius and ulna). The result is two limbs in tandem. A solid line marks the amputation plane. **(E)** The result of grafting a distal blastema (from the wrist level) onto a proximal stump (the midupper arm level). The dotted line marks the level of the grafted distal blastema and the solid line marks the amputation plane through the mid upper arm. The result is a complete limb as the missing portion, the intercalary regenerate represented by the gap between the solid line and the dotted line (I), is filled in by cells from the proximal level stump according to the law of distal transformation. This phenomenon is also shown here because the distal blastema was from a black animal (melanophores can be seen in the digits and wrist) and it was grafted to a white animal.

2.4. Recent Techniques

Throughout this century, many techniques have been used in an attempt to discover the mechanisms of limb regeneration—denervation, transplantation of blastemas to ectopic sites, organ culture, radiolabeling, gel electrophoresis, administration of compounds, such as retinoic acid, and so forth. However, recently three technical advances have been made that are certain to have an important influence on future discoveries.

One is the advent of whole-mount *in situ* hybridization for use with probes to genes expressed at very low levels, such as the homeobox genes ***(26)***. Gardiner et al. have cloned 17 homeobox genes from the axolotl, and work such as this is already beginning to answer some question, such as: Are these genes expressed in the same domain and in the same sequence during regeneration as they were during development?

The second advance is the establishment of long-term cultures of blastemas cells, allowing for their genetic manipulation by transfection and grafting back into the blastema. In contrast to normal, untransformed cells from mammalian tissues, both blastemal cells and a muscle cell line established from a dissociated limb have generated permanent cell lines, showing no senescence ***(27)***. This again emphasizes a unique feature of blastemal cells, and the ability of limb tissues to dedifferentiate and turn into blastemal cells.

The third advance has to some degree obviated the need for cultures, because it is a remarkable technique by which cells can be transfected both *in vitro* and *in vivo*. It involves the use of a biolistic gun ***(28)***, which fires 1.6-μm gold particles coated with the DNA of one's choice into cells in a Petri dish or into the cells of the regenerate (**Fig. 4**). If the gun is fired at the external surface of the blastema then cells in the epidermis are transfected at a frequency of about 10%, and their altered behavior can be assessed in various ways. If the blastema is cut off the limb stump and the cut edge exposed to the gold particles, then the mesenchymal cells of the blastema are transfected. Transfection with constructs, such as chimeric retinoic acid receptors (RARs), has provided a wealth of valuable data on which receptors perform which functions ***(29)***. At least six different receptors are expressed in the limb blastema and in addition to the positional respecification referred to above, RA induces many other phenotypic

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Fig. 3. (*continued*) The intercalary regenerate (I) is white, since it has no melanophores showing that it came from stump tissue. **(F)** The result of treating a distal level regenerate (solid line marks the amputation plane through the wrist) with retinoids. Instead of just regenerating the missing elements, a complete limb has been produced from distal level blastemal cells. Retinoids respecify blastemal cells in a manner that breaks the law of distal transformation.

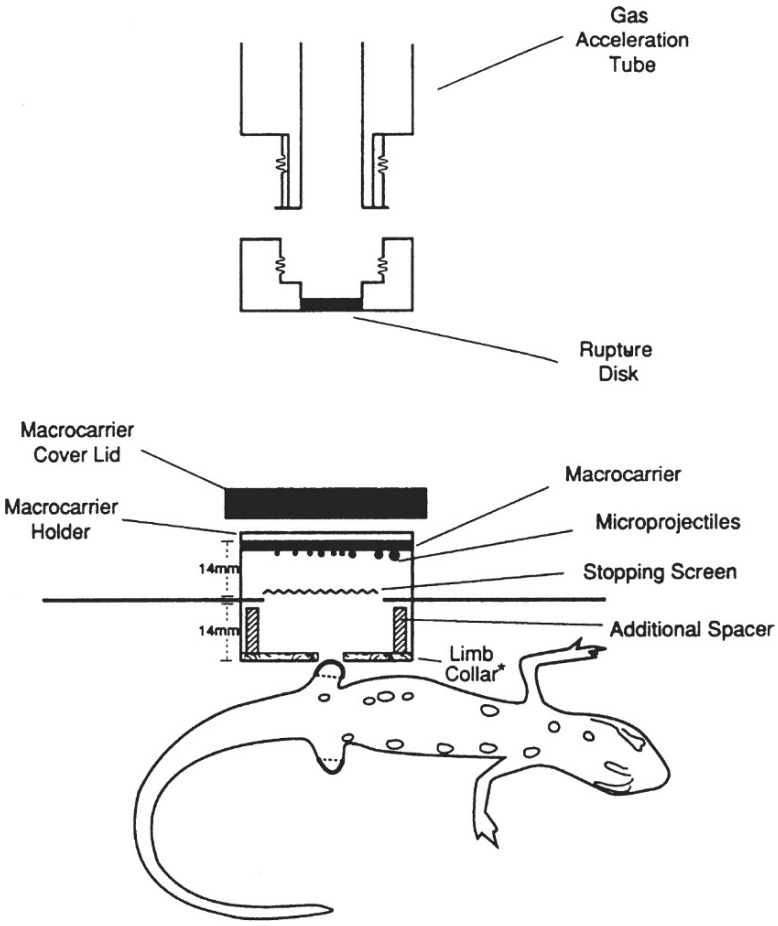

Fig. 4. Drawing of a Biolistics Particle Delivery System for transfecting a regenerating limb. Helium at high pressure is passed into the gas acceleration tube, and the ruptured disk breaks. This releases a shock wave, which propels the macrocarrier disk and DNA-coated gold microprojectiles downward. The screen is halted by a stopping screen, but the gold particles continue on toward the target. The anesthetized animal is supported against the bottom of the assembly and the regenerate inserted into the hole, so that it can serve as a target (from **ref. 29**).

changes in the cells. In order to examine which receptor mediates which effect, Schilthuis et al. ***(30)*** constructed chimeric receptors by exchanging each of the newt RAR ligand binding domains with that from the *Xenopus* thyroid hormone receptor α. The genes activated by each RAR then became responsive, not to the RA, but to thyroid hormone. In this way, they showed, for example, that the RARα was responsible for growth inhibition, one of the established effects of

RA on blastemal cells. It is hard to imagine how such a result would have been obtained without these important technical advances.

Hopefully, these and further advances will ultimately provide answers to those fundamental questions posed at the beginning of this chapter, the most profound of which is: Why can urodeles regenerate and mammals cannot and will we ever be able to stimulate regeneration in humans?

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33

The Zebrafish

An Overview of Its Early Development

Nigel Holder and Qiling Xu

1. The Emergence of the Zebrafish as a Model System for the Study of Vertebrate Development

During the past 10 yr, the zebrafish has emerged as an important model system for the study of vertebrate development. This is primarily because of the promise of the system for developmental genetic studies. but, in addition to the necessary features of an animal that can be used for genetics, there are a range of experimental approaches that have proven successful in studies of tissue interactions, gene function, and early neural development. Such methods include embryonic cell transplantation, the analysis of gene function by injection of RNA or antibodies into the fertilized egg, and the analysis of identified neurons in the developing central nervous system. The object of this chapter is to outline the main features of early zebrafish development and to provide details of the methods for injecting the fertilized egg with nucleic acid or protein.

It is important to point out that analysis of gene function in the zebrafish, whether by mutational screens or DNA/RNA injection, is carried out against an increasingly extensive knowledge of basic embryology. Thus, the transparency and rapid development of the embryo have been exploited to great effect in establishing fate maps ***(1–3)*** produced using cell marking experiments in which fluorescent dyes are injected into single or small groups of cells. This kind of analysis has also led to an understanding of the lineage relationships of cells in the blastula and an assessment of the timing of commitment. It is now clear, despite some data to the contrary ***(4,5)***, that there is no clear restriction of cell fate in the zebrafish until gastrulation begins ***(6–8)***. Consistent with this

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is the demonstration that the dorsoventral axis, despite being established during blastula stages, is positioned randomly with respect to the initial blastomere divisions ***(9)***.

1.1. Developmental Genetics in the Zebrafish

The zebrafish owes its elevation from common pet shop aquarium fish to one of the few model systems for the study of vertebrate development largely to the "Oregon School." George Streisinger, at the University of Oregon at Eugene, first settled on the zebrafish for his pioneering genetic studies. The medaka may have some advantages over the zebrafish for certain procedures and is still used for developmental studies; for example, it has been used recently for the analysis of mesoderm formation ***(10)***.

Streisinger established a number of methods for studying zebrafish, including the generation of isogenic homozygous diploid lines and the screening of haploid embryos for developmentally interesting mutations ***(11)***. The haploid zebrafish embryo develops for several days and eventually dies; a screening program based on haploid embryos has the advantage that recessive mutations present in the female can be revealed in a single generation. This method has been used effectively by Kimmel's laboratory at the University of Oregon to reveal several mutants either induced or present in the genetic background of the zebrafish stock held in Eugene. Such mutants include *spadetail* ***(12)***, *cyclops* ***(13)***, and *no-tail* ***(14)***; lines that have subsequently been extensively studied, the last two being important for studies of patterning of the axial mid-line of the embryonic axis. Mapping of candidate genes has allowed a number of mutants to be identified. These include *spadetail* ***(15)***, cyclops ***(16)***, and other important regulatory genes, such as fleating head ***(17)*** and FgF8 ***(18)***.

Since the first attempts to establish zebrafish for developmental genetics the single most important step has been the selection of this system for a number of major screens using chemical mutagenesis and analysis of sib-crosses in the F2 generation to reveal mutations originally induced into male germ cells by chemical mutagenesis using ethyl nitrosourea (ENU) ***(19)***. The strategy, methodology, and results from these screens have recently been published by these two laboratories, and are the subject of Chapter 34. Together with the continuing screening in Oregon and elsewhere, these two large-scale screens are now at the stage where mutations are being characterized genetically and morphologically. The screens have revealed many mutants of developmental interest affecting the major organ systems and structures of the embryo ***(19)***.

Considerable progress has been made in generating a genetic recombination map for genes relative to the chromosomes. Methods based on polymerase chain reaction (PCR) (so-called RAPD method) have been used to identifiy polymorphic short sequences which, in conjunction with generation of haploids, have

been applied by John Postlethwait's laboratory ***(20–22)*** to generate a recombination map ***(21,22)***. Genes newly isolated by mutation and genes cloned by homology to those in other species are now being added to the map. It is clear the map will have many markers within a short time and will be a valuable resource for molecular identification of novel mutations either by mapping of cloned genes to the same site as an identified mutation ***(15–17)*** or by positional cloning ***(23)***.

1.2. The Presence of Identified Neurons Allows Analysis of Neural Differentiation at the Single-Cell Level

In addition to genetic studies, there are other advantages to using the zebrafish for studies of early development. Principal among these is the presence in the developing nervous system of identified neurons, cells that differentiate in the same place and at the same time in every embryo ***(24)***. The identified neurons that have been most heavily studied, in terms of their time of origin, the mechanisms leading to their determination, and subsequent differentiation are the primary motor neurons of the spinal cord ***(25,26)***, and the reticulospinal neurons of the

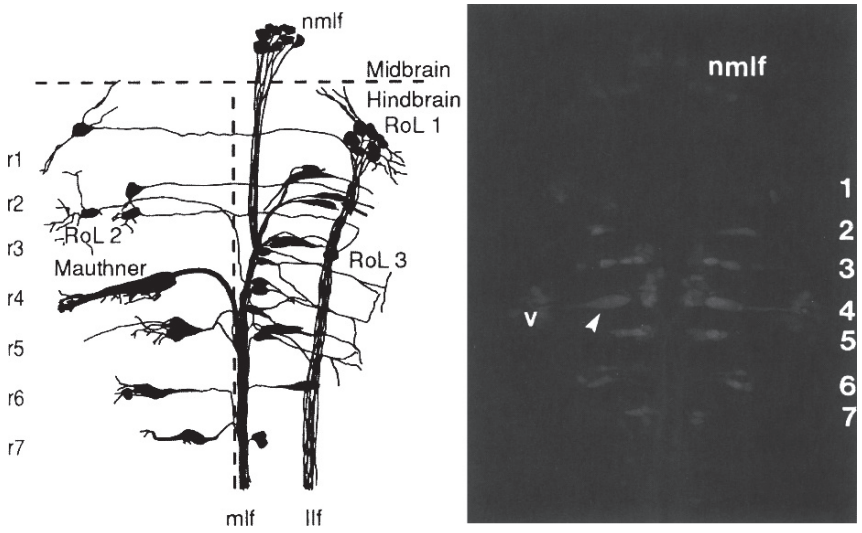

Fig. 1. The reticulospinal complex of the zebrafish. **(A)** The cells of the complex indicating rhombomeres (rl-7) and the principal identified cells (based on **ref. *25***). **(B)** Backfilled reticulospinal neurons in a normal embryo. The Mauthner cell is arrowed, v indicates the vestibulospinal neurons, and nmlf indicates the midbrain nucleus of the longitudinal vesiculus. (*See Color Plate*)

hindbrain *(27–29)*. The presence of identified neurons within the central nervous system allows analysis at the level of the single cell and provides a level of analysis not possible with higher vertebrates. An example of such a study is the identification of rhombomere respecification in the hindbrain following retinoic acid treatment of the gastrula *(30)*. In this study, the reticulospinal complex, which is illustrated in **Fig. 1**, *(shown on p. 433)* was used to identify respecification of the Mauthner cell, normally present in rhombomere 4, in rhombomere 2 *(30)*.

1.3. What the Future Holds

The near future holds great promise for work on the zebrafish because of the flood of interesting mutations that emerged from the large-scale screens (*Development*, vol. **123**, 1996). The continued generation of a genetic map is a crucial development and has allowed the first positional cloning projects to be started *(23)*. Other techniques are being tackled, such as the establishment of a mouse ES cell equivalent, which is yet to be obtained in any other vertebrate, and the use of viruses for transgenesis and insertional mutagenesis. The future is becoming increasingly more promising for the studies of developmentally regulated genes in this organism.

2. The Zebrafish Is a Rapidly Developing Embryo: Stages and Key Events

A detailed description of the stages of zebrafish development has recently been published *(32)* and the reader who is interested in a detailed look at the key features of this developmental series should read this paper and refer to the *Zebrafish Book (33)*. What follows is a brief description of the key stages of zebrafish development over the first 2-s period—all of the terms and stages are consistent with those used by Kimmel et al. *(32)* and relate to embryos growing at 28.5°C.

2.1. The First 5 H

This includes the zygote, cleavage, and blastula periods *(32)*. Within 10 min of the egg being fertilized, streaming cytoplasmic movements cause a cap of clear cytoplasm to emerge at the animal pole of the otherwise yolky cell. After 45 min, the first cleavage occurs incompletely separating the clear cytoplasmic region (**Fig. 2A**). Subsequent cleavages are more rapid, occurring at 15-min intervals, generating the 64-cell stage by 2 h. The cells adjacent to the yolk cell communicate by intracellular bridges. The blastula stage (**Fig. 2B**) begins with the generation of 128 cells. Divisions initially remain largely synchronous, but this synchrony begins to be lost at the time of the midblastula transition *(34)*, which begins at the 10th cell cycle (512-cell stage). Other key events that occur during the blastula stage are the formation of the yolk syncytial layer (YSL) and the beginning of the process of epiboly. The YSL is formed as the marginal

blastomeres sink into the yolk cell distributing their cytoplasm and nuclei as a layer across its superior edge. Epiboly is the spreading of the blastodisc and the YSL over the surface of the yolk cell. By the end of gastrulation, the yolk cell is completely engulfed. In addition to the YSL and the deep cells (DEL) of the blastodisc, during the blastula stage, the single outer layer of cells flattens to form the enveloping layer (EVL).

2.2. The Second 5 H

To be accurate, gastrulation begins at 5¼ h; this process comprises the cell movements of involution, convergence and extension ***(35)***, which are initiated when epiboly has reached 50% (50% coverage of the yolk cell; **Fig. 2C**). At the onset of gastrulation, the marginal zone becomes thickened to form the germ ring, one region of which, at the site of dorsal axial involution, becomes distinctively thickened. This localized thickening, caused by convergent movement of cells to the axial (dorsal) location, is called the shield (**Fig. 2D**). Gastrulation movements lead initially to the formation of two layers, the inner hypoblast (mesendoderm), and the epiblast (ectoderm) (**Fig. 2E**). Understanding the cell movements of gastrulation is aided by appreciation of the fate maps that are available for this period ***(1,3)***.

During gastrulation, cells in the axial midline extend anteriorly to create the notochord and the most anteriorly located prechordal plate (**Fig. 2F**). This anterior region produces the hatching gland and the pharyngeal endoderm. More laterally, the rostral hypoblast forms the muscles of the head and, more caudally, the somites. By the end of gastrulation epiboly is complete and the tail bud has formed (**Fig. 2F**); in addition, the ectodermally derived neural plate is beginning to be formed on the dorsal side of the gastrula ***(36)***.

2.3. The Segmentation Period Runs from Gastrulation Until 24 H

One of the striking features of this time period is the growth of the tail, which extends to give the embryo its characteristic shape. In addition to tail elongation, the major organ systems, including the somites, the pronephros, the heart, and the central nervous system, form. Segmentation is seen in the forming hindbrain and in the mesodermally derived somites (**Fig. 2G**). The number of formed somites is a good check on the exact stage that the embryo has reached. Eventually there will be over 30, but, for example, 20 have formed by 19 h of development (**Fig. 2H**). Spontaneous contractions of the somites begin at the 17 somite stage as the axons from the primary motor neurons first reach the differentiating muscle fibers. Within the central nervous system, 10 distinctive brain regions or neuromeres are evident by 18 h (18 somites). The most anterior of these is the telencephalon, which lies adjacent to the diencephalon. These two regions form the forebrain. The midbrain or mesencephalon separates these from the more

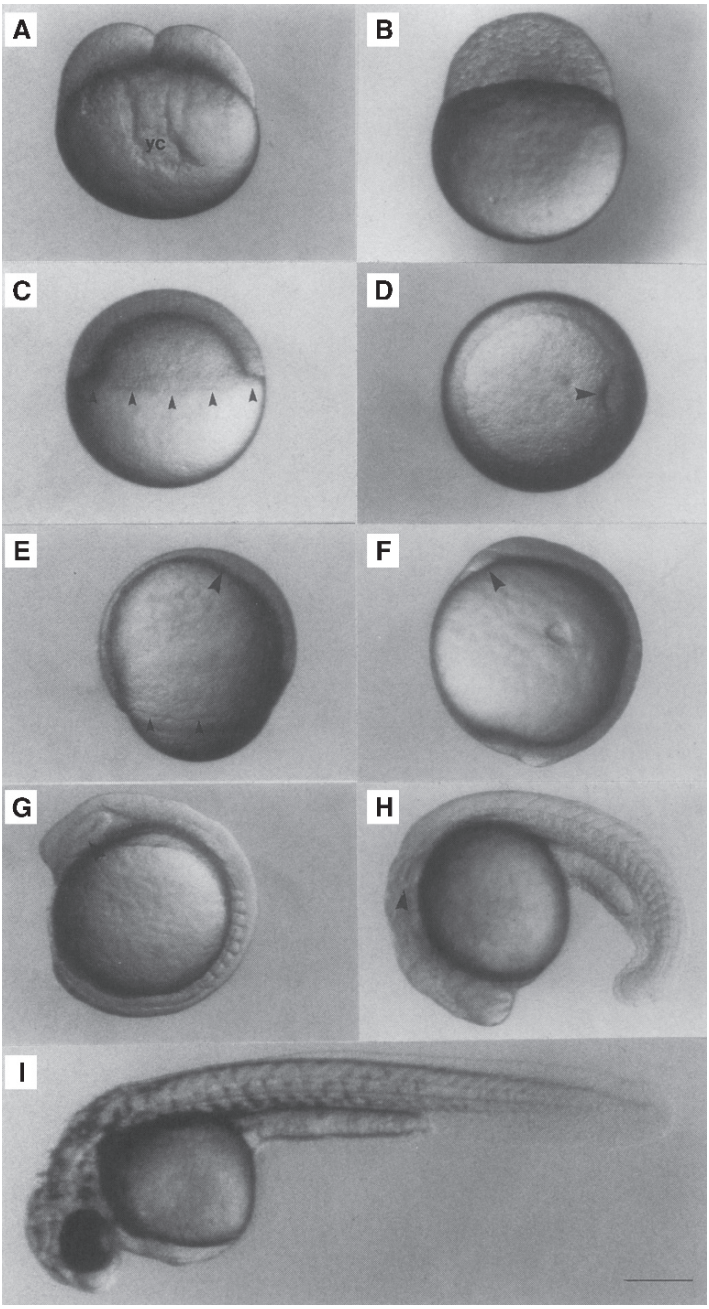

Fig. 33.2. Stages in the development of the zebrafish. This figure is adapted, with permission of the authors, from the definitive zebrafish staging series published by Kimmel et al. *(32)*. **(A)** Two-cell staged embryo showing the large yolk cell as an incomplete cleavage. **(B)** The blastula stage illustrated with an embryo at 3.3 h of development. The ever-smaller blastomeres are piled up on the yolk cell. **(C)** Gastrulation begins at 50% epiboly (5.25 h)

clearly segmented hindbrain or rhombencephalon. The hindbrain segments are termed rhombomeres of which seven clearly form in the zebrafish.

2.4. The Second Day: The Pharyngula Period

During this period, the body continues to lengthen and the pharyngeal arches (after which the stage is named) become evident. The arches are a series of structures located in the head ventral to the midhindbrain region, beneath the otic vesicle (the structure that makes the inner ear). The first arch forms the mandible, the second the hyoid cartilage, and the subsequent arches the gills. An accurate way to stage the embryo during this period is to use Nomarski optics to locate the tip of the caudally migrating lateral line primordium ***(37)***. This has reached a precise somite location by a specific time. In addition, the fins begin to grow, and the brain undergoes a compression such that the otic vesicle lies closer to the eye. The heart begins to beat at the beginning of the period, and the vascular system develops. Finally, pigment cells begin to differentiate and are evident over the body and in particular sites, such as the pigmented retinal epithelium (**Fig. 2I**).

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Fig. 33.2 (*continued*) by which time the blastomeres have moved down over the yolk cell to cover half its extent (arrows). **(D)** Animal pole view of the shield stage (6 h) at which the position of axial involution is evident (arrow). **(E)** At 70% epiboly, the edge of blastomeres (small arrows) has spread further round the yolk cell. The anterior extent of the axially involuting hypoblast can be seen approaching the animal pole (large arrow). **(F)** The bud stage (10 h) is reached at the end of gastrulation. The yolk cell is completely covered by cells and the anterior extent of the axial hypoblast can be seen as the polster (arrow). **(G)** The eighth somite stage (13 h). The arrow marks the eye. **(H)** 22 somite stage (19 h). The arrow marks the otic vesicle. **(I)** The prim 20 stage (33 h). Pigmentation is now clear as melanocytes spread over the body and the retinal pigment epithelium of the eye differentiates. Scale bar = 250 μ.

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34

Small-Scale Marker-Based Screening for Mutations in Zebrafish Development

Peter D. Currie, Thomas F. Schilling, and Philip W. Ingham

1. Introduction

We describe a standardized mutagenic protocol and a methodology for small-scale directed screening of the zebrafish genome for mutations in specific developmental processes. The methods are based primarily on those developed for large-scale screens in Tubingen, Germany; Boston, MA; and Eugene, OR as well as our experiences with a smaller facility. By combining a marker-based screening protocol with both haploid and diploid screening methods, one can efficiently recover mutants in specific processes.

Random mutagenesis provides the ability to survey the genome of an organism, without bias, for genes that function in particular processes. For many years, geneticists have been reaping the rich harvest of mutations produced by such a mutagenic approach, directed against particular developmental processes of the fruit fly *Drosophila melanogaster* ***(1)***. Analysis of the genes uncovered by this approach has revolutionized our understanding of the genetic control of animal development.

Researchers eager to see a similar mutagenic approach applied to the vertebrate genome have been stymied by the genetic intractability of classical vertebrate developmental models. The mouse is the only vertebrate organism in which large-scale screens for mutations have been performed. These screens have, in the main, been limited to identification of defects in visible morphological traits after birth, since screening for embryonic mutant phenotypes is difficult because development occurs *in utero* ***(2)***. Biologists interested in using a mutagenic approach to study early aspects of vertebrate development have been forced to search for an alternative.

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The fishes represent the largest group of vertebrate taxa and perhaps the largest uncharted waters in terms of vertebrate developmental studies. Until recently, debates on the molecular mechanisms that govern vertebrate development have largely ignored fish. However, with the rediscovery that certain teleosts allow the application of both sophisticated embryological manipulations and classical genetic analysis, the stock of fish as a developmental system has risen. Specifically, studies on the zebrafish, *Brachydanio rerio*, have indicated its promise as both a genetic and an embryological model ***(3,4)***.

Zebrafish are small and have a short life cycle, reaching sexual maturity in three months. Females produce a large brood size, typically hundreds of eggs, which are fertilized externally. They are inexpensive to maintain and can be bred to great numbers easily. Embryonic stages are completely transparent, allowing most structures of the developing fish to be viewed without the aid of sophisticated microscopy. This last attribute has allowed researchers to gain an intricate knowledge of cell movements and behavior during early development. This information can now be coupled with detailed fate maps and a precise staging series, making zebrafish a sophisticated embryological model ***(5–7)***. As with some other teleosts, zebrafish can be induced to undergo either gyno-genetic or haploid development, abilities that greatly enhance its stock as a genetically manipulable model and make it unique among animal developmental systems. These attributes have attracted a number of laboratories to undertake large-scale mutagenic screens in an attempt to reach saturation for lethal mutations within the zebrafish genome (***8,9,*** and **Note 1**).

The data published by these laboratories describe the general classes of mutations found using this approach ***(8,9)***. These include general necrosis, edema, brain necrosis, and general retardation. Mutations of these common classes account for up to two-thirds of the mutations uncovered and have, for the most part, been discarded by the large screens, since they represent an unwieldy task in the determination of individual complementation groups. Small-scale directed screening may reveal that a significant number of such mutations disrupt in specific developmental processes that can only be revealed by the use of gene or protein-specific markers, that is, marker-based screening.

The efficiency of different mutagenic agents in inducing mutations within the germline of zebrafish has also been assessed. These studies have relied largely on the vast literature concerning the efficient induction of mutations in mice by ethyl nitrosourea (ENU). Similarly, Mullins et al. ***(8)*** and Solnica-Krezel et al. ***(9)*** demonstrated that ENU induces mutations within male spermatogonial cells at rates between 1/450 and 1/1000 for specific pigmentation genes in zebrafish. This range is typical for mutagenic rates at given loci for a number of mutations,

and the rate is significantly higher than that produced in similar treatments with the other most frequently used alkylating agent, EMS ***(8,9)***. Large-scale mutagenic screens have tended to focus on the use of chemical mutagens as their action is not site-specific and they usually induce lesions that are limited to single genes. However X-and γ-rays are also efficient in inducing mutations within the zebrafish germline and are more applicable to other screening rationales (*see* **ref. *10***).

The two large scale mutagenic screens have both used a similar general methodology (*see* **Fig. 1**). Mutations are induced by ENU in G_0 males and detected in the diploid offspring of the intercrossed F2, where the mutation has been driven to homozygosity. A "family" of F2 fish, half of which is heterozygous for a given mutation, is a significant advantage of a F2 diploid screen. It provides an immediate working stock from which to rescreen and recover mutants. Its major disadvantage is the large numbers of fish that have to be maintained and screened. The large screens have chosen not to use F1 screening via the induction of haploid or gynogenetic diploids because: (1) some strains do not consistently produce eggs for fertilization *in vitro*, and (2) the manipulations produce a high background of developmental defects.

Although these disadvantages may preclude the detection of every mutable locus within the zebrafish genome via F1 screening, those laboratories not concerned with such a goal can still effectively screen for mutations in a given developmental process.

Large-scale diploid screens require facilities and support beyond the scope of most laboratories. The flexibility of zebrafish as a genetic model makes it suitable for use in small-scale mutagenic screens directed to specific developmental processes. In particular, these attributes include:

1. The ability to screen for defects in haploid or gynogenetic diploid embryos. As outlined below, this greatly reduces the number of fish needed, since the offspring of F1 founders can be screened directly. Thus, even small facilities can survey a significant amount of the genome for particular types of mutations ***(3)***.
2. The optical transparency of the embryo allows the detection of gene-specific protein or mRNA markers in whole mounts and, therefore, allows a greater efficiency of screening by the direct detection of mutations that are involved in a given developmental process. The three major advantages of such an approach are:
   a. The detection of subtle defects not visible by morphological screening.
   b. The ability to direct a screen toward mutations that affect a process of interest. Thus, the use of tank space can be maximized by immediately discarding mutations in which phenotype is unrevealing, and there are no alterations to a given marker's distribution.

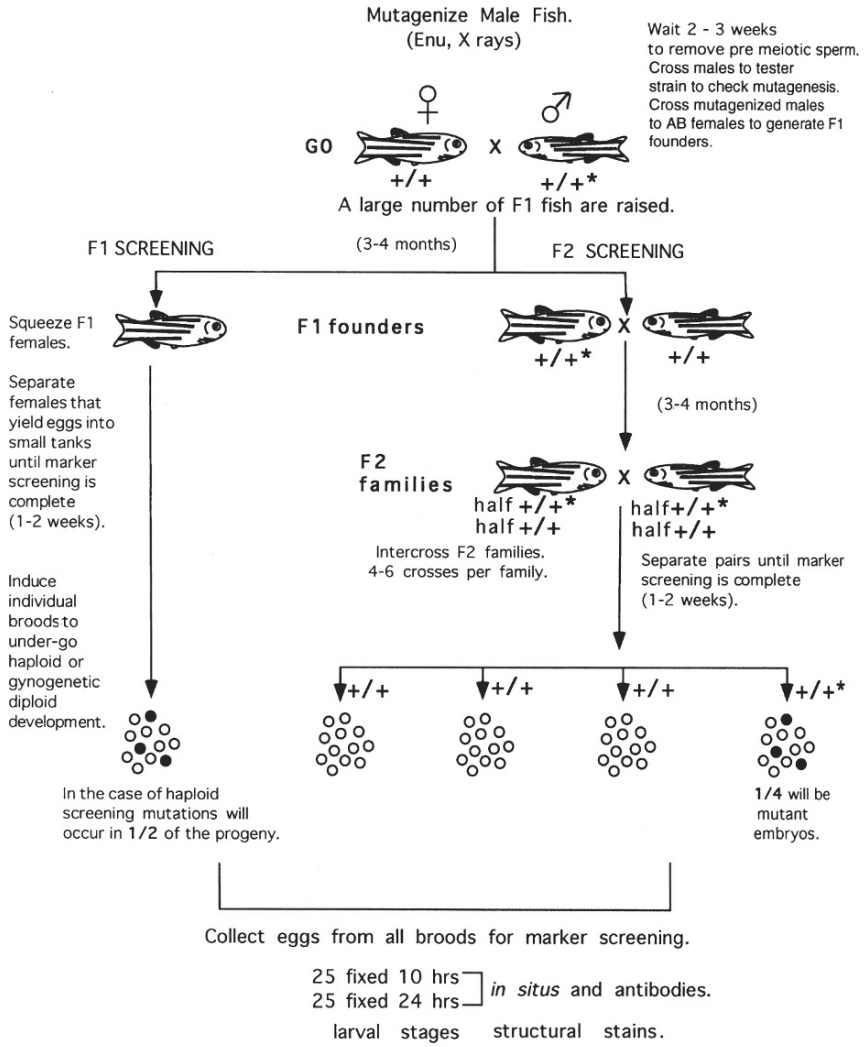

Fig. 1. Flowchart of procedures for F1- and F2-based screening. Screening the progeny of F1 females saves the researcher one generation time and the space required for large families of F2 fish in diploid screening. Single mutations (*) are generated in the spermatogonia of G_0 males, and these are mated **(X)** to wild-type females. Similar pairwise matings of F1 or F2 offspring are used to identify fish, male or female, carrying the mutation, and these fish must be kept separate during the screening. Time intervals are indicated alongside arrows, which are not drawn to scale.

c. The ability to interpret better mutants identified by morphological inspection in which phenotype is unrevealing regarding gene function, but developmental markers are affected.

2. Materials

2.1. Mutagenesis

1. Fish strains (*see* **Notes 3** and **4**): Strains are available from laboratories on request. The choice of strain to mutagenize is extremely important, since particular strains have been used for mapping and some are more amenable to genetic manipulations. The inbred strain recommended for mutagenesis is one derived originally by the late George Streisinger (University of Oregon) and is designated AB, although other laboratories have developed their own inbred strains ***(8,9)***. The advantages of AB over other strains are to be found primarily in haploid screening. AB fish have been selected over many generations for their ability to provide eggs for fertilization *in vitro* and for the lack of embryonic lethal mutations within the genetic background. Further, the most extensive genetic map for zebrafish, using polymorphic variation in the PCR products generated by random primers, has used AB as its inbred strain ***(11)***. In the absence of information about the amount of polymorphic variation between other strains and AB, it would seem useful to induce mutations in this strain to aid in mapping.
2. Mutagens: The choice of mutagen will determine the type and number of mutants you find. ENU (Sigma, St. Louis, MO) has proven to be the most efficient mutagen for the induction of mutations within the zebrafish germline ***(8,9)***. It is highly mutagenic and carcinogenic, and must be handled with extreme care. Whenever possible, dedicated sets of equipment and tanks must be used, and all mutagenic procedures carried out in a suitable fume hood. All solutions and equipment contaminated with ENU are inactivated by incubation in a 10% solution of sodium thiosulfate, adjusted to pH 10.0 with sodium hydroxide, for at least 24 h at room temperature. To create the mutagenic solution, ENU is dissolved directly in the ampule in 10 m*M* acetic acid to a concentration of 100 m*M* and stored at −20°C as a stock solution (ENU activity is highly pH-dependent; it is higher with increasing pH and a correspondingly higher lethality). Immediately prior to use, the stock solution is thawed and diluted to 3–10 m*M* sodium phosphate buffer (pH 6.6) to make the final mutagenic ENU solution (*see* **Notes 5** and **6**).

2.2. Fish Raising and Embryo Manipulation

1. Tanks and water (*see* **Note 2**): Systems for rearing and maintaining zebrafish range from commercially available modular systems to small-scale, self-built facilities ***(8,12)***. What is of most importance, however, is water quality. To manage a diploid screen efficiently, fish must lay in a high percentage of pair matings. This seems to relate, at least in part, to the water quality. Tap water quality can be greatly improved by the use of filtration systems. Charcoal filters seem to be neccesary with sand filters proving a useful second-step filtration, although neither of these can buffer against regional differences in water quality, such as heavy metal content. Water quality should be assayed before facility designs are too advanced to accommodate any specialized needs. One valuable addition to consider is a UV sterilizer, which can greatly aid in keeping stocks disease-free. It is also important

that pH is kept neutral or slightly high, since zebrafish are sensitive to an acidic environment, but seem to tolerate mild alkaline water.

There are many varieties of tank designs. In a diploid screen where many pair matings are performed, it is vital to have an efficient system to set up breeding pairs to collect embryos. We use small plastic boxes with wire mesh replacing the bottom of one box and stacked inside another. Eggs fall through the mesh, and parents are restrained from eating them. Pairs or individual females are kept separate in small plastic boxes. Larger 4-L mouse cages are used for rearing of juvenile fish, and adult fish are kept in larger permanent glass tanks of the facility. A serial system of glass tanks with a commom water-flowthrough is the most efficent design for facilities with space constraints (*see* **Note 7**).

2. Food: There are also many diets that can be used. Adults do well on most commercial flake diets, and we use Tetramin flakes finely ground with a mortar and pestle to a powder. Juveniles from about 3 wk of age are fed hatched live brine shrimp, and these can be fed to the adults also. Care must be taken to remove all unhatched brine shrimp, since these are not easily digested. Fry from a week old are fed alternatively filtered paramecium cultures and a commercial protein diet of liquifry (Tetramin) and Tetramin fry food mixed. Fish of all stages are fed twice a day. Sexual maturity can be achieved much more quickly if the number of feedings and food density is kept high to juveniles. However, adult fish fed in this manner may have a shorter life cycle and stop breeding more quickly. This can be a major problem at the end of a screen lasting a year or so, since tanks of females identified as carrying mutations may have stopped breeding before they can be out-crossed or sperm collected for freezing, in the case of the males.
3. Hank's solution: Full-strength Hank's solution is 0.137 *M* NaCl, 5.4 m*M* KCl, 0.25 m*M* Na_2HPO_4, 0.44 m*M* KH_2PO_4, 1.3 m*M* $CaCl_2$, 1.0 m*M* $MgSO_4$, 4.2 m*M* $NaHCO_3$. Bicarbonate is made fresh.
4. Embryo medium: This is 10% (v/v) Hank's with magnesium and calcium at full strength.
5. Methylene blue: A weak solution of methylene blue acts as an antifungal agent. A stock solution is made by diluting methylthione chloride powder (Sigma) to 2% (w/v) in dH_2O. One or 2 drops are added/400–500 mL just until water turns pale blue. Higher concentrations result in uptake of the blue dye by developing embryos.
6. 1-Phenyl-2-thiourea (PTU): An active solution of PTU is made by dissolving the powder to 0.003% (w/v) in 10% Hank's. Fish must be raised in this solution from fertilization to inhibit pigment synthesis. PTU is also neurotoxic, and solutions and powder should be handled with care. Fish raised in this solution should be separated from the rest of the fish to avoid the possibility of PTU being placed into the system.
7. Tricaine: This is the most readily used fish anesthetic and, if applied correctly, seems to have no adverse side effects. A stock solution of tricaine is made by dissolving 3-amino benzoic acidethylester (Sigma) powder to 0.4% (w/v) in 20 m*M* Tris (pH 9.0)

and adjusting the pH to 7.0. This solution is stored at −20°C and diluted to approx 0.015–0.020% (v/v) in water to provide a working solution. The stock solution loses its efficacy over time and should be made up monthly. Tricaine is also a mild carcinogen. Although it is impossible to avoid contact with the solution in manipulations involving its use, care should be taken to avoid overexposure.

8. Methyl cellulose: A 3% (w/v) solution of methyl cellulose can be used to mount and photograph live anesthetized embryos. Powder is dissolved in water via gentle heating to a viscous solution. The solution is refrigerated to remove air bubbles and then brought to room temperature before use. Fish can simply be removed from the methyl cellulose by sequential rinses in water.
9. UV source: Any source of shortwave UV light can induce haploidization in zebrafish embryos as long as this effect is calibrated. The source used in our laboratory is Ultraviolet Products Incorporated UVL-56.
10. Fish Ringers. Fish Ringers is 0.11 *M* NaCl, 0.0034 *M* KCl, $CaCl_2(H_2O)_2$, and 0.0024 *M* $NaHCO_3$. Bicarbonate is added last and immediately before use.

2.3. Detection of Mutant Phenotypes

1. Microscopy: All morphological and marker screening utilizes simple light microscopy via a dissecting scope. We use Zeiss Stemi SR microscopes. However, any microscope with the capability of translucent illumination and up to 5X objectives would be adequate. Translucent lighting is essential for clear optical inspection of structures of the embryo.
2. *In situ* hybridizations: *In situ* probes and protocols use standard methods (*see* Chapter 31 and **refs. *12,13***).
3. Antibodies: Antibodies are either bought commercially or are available from different laboratories on request. We utilize standard methods for protein detection ***(12)***.
4. Alcian blue: A stock solution of alcian blue stain is made by dissolving powder to 0.1% (w/v) in 70% (v/v) EtOH/30% (v/v) glacial acetic acid.

3. Methods

Many of the methods listed below are derived from **refs. *8,9,12***.

3.1. Mutagenic Methods: ENU Mutagenesis

Tests of different mutagenic regimes in zebrafish favor mutagenizing premeiotic germ cells in adult males (**Fig. 1**). This is because, in a classical diploid screen, use of sperm derived from postmitotic stages of spermatogenesis results in a mosaic germline in F1 founder fish and increases the number of crosses required between F2 siblings to drive mutations to homozygosity. Mutagenizing G_0 males involves the following procedures:

1. Pretest males (4–8 mo optimal) for high fertility by single pair matings. Place males directly in ENU solution.

2. Mutagenize in ENU solution for 1 h at room temperature in a darkened fume hood. The fish are stressed and tend to leap out of containers. A darkened quiet environment seems to minimize this behavior.
3. Transfer to a similar volume tank containing water from the fish facility at room temperature. Allow to recover for 6–8 h at this temperature.
4. Finally, transfer to a large 5-L tank in the fish facility.
5. Following a 1-wk recovery period, the treatment is repeated two further times, each followed by 1 wk of recovery. This recovery period may be shortened, but may lead to an increase in lethality. In a typical mutagenesis, only 30–50% of mutagenized fish survive all three treatments with increasing lethality per treatment.
6. Fish are then mated several times in a 4–6 wk period to remove mutant, mosaic postmeiotic sperm cells.
7. To test the efficacy of the mutagenesis, these G_0 males (**Fig. 1**) are then crossed to a tester strain female, usually a strain carrying a homozygous viable pigment mutant, such as *golden* (*gol*). An effective mutagenesis should reveal a pigmentless embryo at a specific locus frequency of approx 1/500 ***(8,9)***.
8. These embryos may be raised as F1 founder fish or their G_0 fathers crossed to wild-type females of a required strain to generate F1 founders.

3.2. Screening Methods

The ability to generate haploid or gynogenetic diploid development provides the researcher with a number of different options for screening mutagenized F1 founder fish. More than one of these can be performed in parallel, and each of these will be discussed in turn.

3.2.1. Haploid-Based Screen

This type of screen saves one generation by screening F1 fish directly and reduces the number of fish required to survey a given number of haploid genomes. Haploid development is initiated by inactivating sperm by UV irradiation and using this sperm to fertilize eggs *in vitro*. Haploid embryos develop essentially as normal for the first few days, but have a few consistent defects, including short tails, deformed ear capsules, and edema, by early larval stages, after which they die. Therefore, a haploid-based screen for mutations in organogenesis and late differentiating structures would be inadvisable. However, mutations that alter the basic structure of the zebrafish body plan are easily identifiable, and most of the mutations previously identified in zebrafish have been identified by haploid screening of X-ray-induced mutations ***(14–16)***.

The ease with which haploids may be produced depends entirely on the ability of mutagenized F1 females to yield their stored eggs, a trait that seems highly strain-dependent. Typically, in a strain considered accessible to this manipulation, sexually mature gravid females will yield eggs in at least 20–30% of squeeze attempts. Each wild-type strain should be assessed for its ability to undergo this

procedure. Ideally, fish of the AB strain should be used, since they have been selected over several generations for the ability to allow *in vitro* fertilization in this manner. Tricks that seem to lead to a more consistent yield from females are:

1. The separation of males from females the night before squeezing to prevent the males from inducing spawning in the females.
2. Squeezing females early in the morning, since they are induced to spawn when the lights come on. Since sperm has to be collected and inactivated prior to females being squeezed, placing female fish in an enclosed light cycle that comes on later than the main facility may help this procedure.

Production of haploid embryos requires little or no specialized equipment ***(4)***. A shortwave UV source and anesthetic are all that is required. Sperm may be collected from males and used to fertilize eggs *in vitro* in the following way:

1. Anesthetize sexually mature males by placing them in a 0.002% (w/v) tricaine solution until gill movement has stopped. Rinse anesthetized fish in water.
2. Place ventral side up in a moist sponge bed designed to keep the fish wedged upright. The genital opening is located between the pelvic fins and should be wiped dry before sperm collection.
3. With Millipore forceps, gently squeeze this area while collecting sperm in a glass capillary pipet attached by thin-gage tubing to a mouth pipet or other suction device.
4. Place sperm in a solution of full-strength Hank's on ice, and store until enough has been collected in the vial to turn the solution opaque and slightly milky. Approximately 0.5 mL are needed for 20 fertilizations.
5. Transfer the Hank's/sperm solution on a watch glass or small plastic Petri dish, and UV-irradiate. Inactivated sperm should be stored on ice. Since individual sources vary in their output of UV irradiation, a time-course of irradiation should be performed. However, as a reference point, we find that irradiation for 5 min at a distance of 20 cm by our UV shortwave source produces inactivated sperm still able to fertilize. Shorter periods fail to inactivate and lead to diploid embryos, easily discernable by their longer tails. Longer periods highly reduced fertility.
6. Anesthetize females, and then dry initially by flipping the fish on a clean paper towel.
7. Place in a clean, small plastic Petri dish. Apply even pressure to the belly, pushing toward the genital opening. Hold the back of the fish gently with the other hand. Undue pressure will lead to internal hemorrhaging and death, so a desire to yield eggs must be tempered against the need to keep the fish alive, no matter how gravid a female may appear. Increased pressure will never lead to an increased percentage of fish that yield eggs. Also, the success of a haploid-based screen relies on the female surviving, since she carries the only recoverable chromosomes in the screen. This risk can be circumvented by separating expressed eggs into dishes, fertilizing one with wild-type, unirradiated sperm, and keeping these out-crossed embryos until the results of a screen are known.
8. Females often yield eggs that cannot be fertilized. These eggs appear milky and opaque when they are squeezed into the Petri dish. Fertile eggs appear shiny yellow

and complete in appearance. Separate eggs from the female using a clean spatula, and add 50–100 µL of the Hank's/sperm solution, followed immediately with 0.5 mL of water. Eggs must not be left to dry and should be covered. Sperm solution should be added as quickly as possible after the eggs have been expressed.

9. After 1 min add 3–4 mL of water, and swirl the fertilized eggs gently to avoid clumping.
10. Monitor eggs to see if the chorions raise. Separate from infertiles, and allow to develop.

3.2.2. Gynogenetic-Based Screen

This type of screening provides the space and time-saving aspects of haploid screening, but has the added advantages of allowing the observation of the diploid phenotype combined with viable resultant embryos. Gynogenetic diploid embryos can be produced by subjecting eggs fertilized with UV-inactivated sperm to either heat-shock or high hydrostatic pressure. Heat-shock administered 13 min after fertilization suppresses the first mitotic division and, therefore, induces gynogenesis. However, a large percentage of heat-shocked embryos exhibit atypical development possibly owing to structural damage to the fertilized eggs, limiting its effectiveness as a methodology for random screening. High hydrostatic pressure delivered just after fertilization restricts the second meiotic anaphase. A significant number of early pressure- (EP) induced gynogenetic embryos also develop abnormally owing to damage to the eggs, but this is seen at a lower rate than in heat-shock treated embryos. Since crossingover occurs at the first meiotic division, only mutations that occur close to the centromere will exhibit a high rate of homozygosity in the gynogenetic embryos. This somewhat hampers the use of this technique, since the percentage of mutant embryos is directly proportional to the distance that a mutation maps from the centromere and, therefore, is unpredictable. It has consequently been used as a measure of gene distance from the centromere of known mutations. This technique also has the drawback of requiring the use of controlled pressure equipment.

3.2.2.1. Production of Gynogenetic Embryos via Heat-Shock

1. Collect and UV-inactivate sperm as described in **Subheading 3.2.1**.
2. Collect eggs and fertilize *in vitro* as described in **Subheading 3.2.1**. Keep fertilized eggs at 28°C.
3. Approximately 10 min after fertilization, transfer the developing eggs to a 42°C water bath for 2 min.
4. Return embryos to 28°C water, and allow to develop normally.

3.2.2.2. Production of Gynogenetic Embryos via Early Pressure

This requires a hydraulic press or French press. These are not easily located but perhaps one place to look is amongst the discarded equipment of a microbiology department.

1. Collect and UV-inactivate sperm as described in **Subheading 3.2.1**.
2. Collect eggs and fertilize *in vitro* as in **Subheading 3.2.1**.
3. Place fertilized eggs in embryo water in pressure vials within pressure cylinder.
4. Apply pressure to fertilised embryo 1.5 min after fertilization to 8000 lbs./sq. in. for 4.5 min.
5. Remove embryos from vials, distribute into dishes, and allow to develop normally. With both gynogenetic diploid and haploid screening, we find it useful to separate embryos with mechanically induced defects from normal developing embryos as soon as possible. All embryos are kept and screened, but are also ranked by quality. Any consistent defect that occurs in a percentage of the embryos predicted by the screening rationale is considered mutant and rescreened. Researchers learn defects that are induced by manipulating the embryos and those that reflect a true genetic lesion.

3.2.3. Classical Diploid Screening

Alternatively, those researchers that possess a large enough facility to generate and maintain large numbers of mutagenized families may opt for a classical diploid screen approach. In such a screen, mutations are homozygosed in the F3 generation. Advantages include a very low incidence of abnormal development per a given embryo in a brood, and mutations typically show Mendelian segregation. Recessive mutations will be a quarter of the progeny of the F2 intercross of the raised family (**Fig. 1**). Another drawback of haploid screens is that two generations are required before you recover the mutation as a diploid, because the squeezed female must be out-crossed and then the resulting progeny intercrossed before homozygous mutant embryos are produced. The F2 families raised in the diploid screen also provide a large number of fish immediately for analysis, since 50% of these fish should be heterozygous for the induced mutation (**Fig. 1**).

Diploid screening simply involves pair-mating fish of the F2 families, so that the statistical likelihood that two heterozygous fish for an induced mutation have been crossed is high. It is important to raise families of sufficient size and numbers of males and females to achieve this. Ideally 30–50 fish should be raised. Small families should be discarded as soon as it is realized that insufficient fish are present in the brood, since they will take up tank space and not provide sufficient fish to ensure detection of any mutations. Because 50% of fish should carry the mutation as heterozygotes in a given family, four to six crosses are usually sufficient to uncover mutations within a family. If the number of families is limited, more can be crossed to increase the certainty of detecting mutations. Pairs need to be kept separate until all embryo screening methods have been performed and, if no mutation is detected, can be returned to original tanks. Once it has been determined which mutations are to be kept, fish carrying those mutations must be identified from individual families by crossing to already identified fish. Noncarriers can be discarded, and tanks used for new F2 families.

3.3. Marker-Based Detection of Mutant Phenotypes

Irrespective of how mutant embryos are produced, a similar protocol can be employed for the use of markers to detect mutant phenotypes. Zebrafish offer some unique alternatives in the detection of specific phenotypes. The ease with which embryos can be manipulated, the large brood size, optically clear embryos and reliability of procedures to detect mRNA or protein in fixed embryos allow the use of "markers" of specific developmental processes. Several markers may be used in combination. A typical protocol for marker-based screening is outlined below. Obviously, the stage of embryos fixed will vary between those markers used (*see* **Note 8**).

3.3.1. Strategy for Marker-Based Screening

1. Collect and sort 100 fertile embryos from each pair mating. Separate into two dishes of 50 embryos. Individual pairs or F1 females must be labeled and kept separately. Space constraints will determine the number of pairs or females that can be screened at any given time, since the fish must be kept separate until marker screening is done and it may take a number of weeks until enough individual crosses or squeezes are generated to perform an *en masse* marker-based screen.
2. Allow the embryos to develop to the stages to be screened morphologically (*see* **Subheading 4.** for specific details on morphological screening). One dish of 25–50 embryos is fixed for use in a marker-based screen. At least half of the embryos are kept for morphological screening and structural stains at later stages. We fix 25 embryos at bud and early pharyngula stages for use in an *in situ*-based screen. Embryos of different stages from the same parent(s) are combined in small baskets and incubated through the *in situ* protocol in 24-well tissue-culture plates. A similar approach can be used for screening with antibodies to specific antigens. We routinely screen embryos from 30–50 individual broods at a time. It is possible to scale up the *in situ* protocol to use larger well number microtiter plates, such as 96-well plates, moved through common solution baths. However, it is difficult to keep large numbers of pairs or individual females separate for any length of time in a small facility. Thus, the number of broods screened at any given time will be dictated by this space constraint.
3. Allow to develop to 3 d, and screen for defects in organogenesis if a diploid screen is being employed. Collect embryos for structural stains at appropriate stages. If no phenotype has been detected at any stages, pairs or females should be returned to original tanks in diploid screens, or to a separate tank in haploid-or gynogenetic-based screens.

   Choosing markers: A large number of cloned genes and cell-type-specific antibodies have been generated that allow visualization of specific regions or molecular processes within the zebrafish embryo. These markers can often reveal subtle changes in the molecular phenotype of a given mutation that may not be

detectable morphologically. They may also implicate a mutation that has an otherwise uninteresting phenotype in a specific molecular process. Some examples are given in **Table 1**.

Table 1
***In Situ* and Antibodies Availiable for Marker Based Screening of Early Segmentation and Pharyngula Stages in Zebrafish**[a]

Germ layer	Marker	Region expressed	Ref.
Neuroectoderm	*zash 1a* and *1b*	tel. and di., r1–6	***18***
	emx 1 and *2*	tel. and di.	***19***
	otx 1, 2, and *3*	tel., di., and mes	***20***
	pax 2	optic stalk, mes/rhomb, and dorsal spinal neurons	***21***
	pax 6	retina, lens, and dorsal spinal neurons	***22***
	nk 2.2	di.	***23***
	shh	fp, ventral di.	***24***
	dlx2 and *4*	tel. and di.	***25***
	wnt 1	mes/rhomb	***26***
	eng 1, 2, 3	mes/rhomb	***27***
	krx 20	r3 and 5	***28***
	rtk 1	r2 and 4	***29***
	pou 2	r2 and 4	***30***
	axial	fp, ventral di	***31***
	collagen type 2	fp	***32***
	ZN1*	1° motor neurons, most differentiating neurons	***33***
	ZN12*	reticulospinal and Rohon-Beard neurons	***33***
Mesoderm	*axial*	noto., pcp	***31***
	znot	noto	***34***
	gsc	pcp	***35***
	shh	noto., pcp	***24***
	Brachyury	noto	***36***
	snail 1	paraxial	***37***
	twist	noto., somite	***38***

[a] This is by no means an exhaustive list and is meant to provide the reader with a starting point for designing marker screens. Abbreviations: di—diencephalon, fp—floorplate, mes—mesencephalon, noto—notochord, pcp—prechordal plate mesoderm, r—rhombomere, rhomb—rhombencephalon, tel—telencephalon.

3.3.2. Selection of Markers for Use in Screens

The techniques for whole-mount *in situ* hybridization and antibody staining have been comprehensively reviewed elsewhere and, therefore, will not be discussed (Chapter 31; **refs. *12,13***). However, some general pointers and pitfalls for the selection of markers can be outlined.

1. Care must be taken to use *in situ* probes or antibodies that are robust in their application. If this is the case, most protocols can be modified and considerably shortened without much loss of sensitivity.
2. Reagents can often be reused many times without loss of sensitivity of detection, and this sensitivity can actually increase with use. Since cost is a major factor in designing such a screen, with dioxygenin and antibodies being the major expense, this is an important consideration.
3. Since it is envisaged that a screen based on revealing gene patterns or specific proteins would utilize several markers at a time, care must be used to select individual markers that are within each other's range for sensitivity of detection. Also, each individual marker should mark distinct subpopulations of cells that do not obscure visualization via other markers.
4. For antibody stainings, fixation times for individual antigens may be incompatible. These parameters can often only be determined empirically.
5. Importantly, detection must be via simple inspection utilizing light microscopy, without the need for sophisticated mounting and visualization techniques, since this is preclusive to the ability to screen large numbers of individual broods.
6. Choose markers that reveal some aspect of developmental regulation that is not obvious on inspection. For example, somite formation is clearly visible within the transparent embryo, and markers that stain the whole of the developing somite may reveal less than simple inspection of the developing somite. However, a marker, such as *krox 20*, that stains specific rhombomeres in the hindbrain of the developing embryo that are difficult to visualize under the dissecting microscope may be more useful in a marker-based screen.

A large number of genes and proteins have been identified in zebrafish that fit these basic criteria. Examples of markers that can be utilized in such a screen are given in **Table 1.** This is by no means an exhaustive list, with the number of possible markers growing fast, and it is meant only as a guide to demonstrate the possibilities of this approach. Although the number of mutations that directly affect the expression pattern or distribution of gene transcript and proteins of interest will only be a small subset of the mutations revealed, this type of approach immediately allows the assessment of a particular mutation and its possible involvement in a process of interest.

3.3.3. Use of Stains in Screening

Structural stains: A number of stains have been described in vertebrate systems that bind particular cellular or extracellular components. These types of stains can be used to reveal particular aspects of fish anatomy that may not

be visible on inspection, such as the vertebral column and head skeleton ***(17)***. A number of fluorescent stains are available that stain nuclear or cell membrane components that are detectable by fluorescent microscopy. These are included for reference, but clearly may not be accessible techniques for every lab and may not be suitable for use in a larger screen.

3.3.3.1. Alcian Blue

This stain binds to a matrix component of cartilage that begins to develop during the hatching period. Staining in embryos and early larvae reveals the pattern of the developing fin cartilage and head skeleton. Subtle changes in the patterning of these structures can be revealed by using simple light microscopy without the need for dissection.

1. Fix early larvae (3–5 d) in 10% buffered formalin (pH 7.0) for 3 h to overnight.
2. Rinse once in 50% EtOH/50% (v/v 1X) PBS.
3. Place in 0.1% (w/v) alcian blue overnight at room temperature.
4. Rinse in EtOH and rehydrate with PBS.
5. To clear tissue, place in dilute trypsin solution (~0.05% w/v) and leave at room temperature until embryos become soft and transparent. The eyes can then be removed, and the embryos mounted in glycerol. (This step is optional and required only when looking in detail at cartilage.)
6. Pigmentation may be bleached by placing stained specimens in 0.35% H_2O_2 dissolved in 0.1 *M* KOH.

3.3.3.2. Alizarin Red

This stain binds to forming bone ***(17)***. Although most bone develops relatively late, researchers may wish to analyze the affects of mutation on early ossifications.

1. Fix larvae in 3% (w/v) KOH for 24 h.
2. Stain in 0.001% (w/v) alizarin red/1% (w/v) KOH for 3 h.
3. Rinse and store in glycerol.

3.3.3.3. Acridine Orange

This stain can be used to access the amount of cell death in a given mutation.

1. Soak live embryos in 0.1 mg/mL solution for 2–3 min.
2. Examine immediately with fluorescein filter set.

4. Notes

1. An in-depth guide to zebrafish raising and techniques has been provided in *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish* (Brachydanio rerio), University of Oregon Press ***(12)***. This book provides an invaluable reference for most experimental manipulations in zebrafish.
2. Raising of zebrafish embryos has traditionally been done in a defined salt solution or embryo medium. We find that this is not necessary as long as an antifungal agent,

such as methylene blue, is added to system's water. This may of course vary with the quality of individual water sources.

3. It may be advantageous for some researchers to screen for mutations in pigmentless embryos. In wild-type embryos, pigment develops during the early pharyngula period of development. By early hatching, pigment can significantly reduce the information gained from optical inspection of the embryo. Although a number of fully viable pigment mutations exist in the appropriate genetic background, the researcher has the option of using PTU to inhibit pigment synthesis (*see* **Subheading 2.**). This may be of use when rescreening mutations to determine effects later in development.
4. It is important to realize that many different regulations govern the importation of fish into different countries, and obtaining import licences can often be costly and time-consuming. It is important to investigate these procedures to avoid fish being impounded and delays that could lead to death of transported fish.
5. Many regulations also govern the mutagenesis of vertebrate animals. In many instances mutagenesis of zebrafish requires a licence from the appropriate authority. Mutagenesis without this licence may be illegal.
6. X- or γ-ray mutagenesis and PCR-based screening: A number of studies have indicated the efficacy of X- and γ-rays in inducing mutations within the germline of zebrafish, although at a significantly lower efficiency than the above-described treatment with ENU ***(8)***. X- and γ-rays induce mutation by producing double-strand breaks within the DNA, and often result in deletion or rearrangement of large regions of the chromosome. This complexity may reduce overall viability of F1 embryos or affect the segregation of mutations in the adult germline. This may explain the lower frequency of recovered mutations. Inducing translocations and deletions, however, is desirable in screens that utilize PCR to detect gene-specific lesions or as a reference point for cloning the gene represented by a given mutation. Mutations can be easily induced in adult fish or in collected sperm, and a brief method for mutagenesis is described below. It is important to use a calibrated X- or γ-ray source with a wide beam to ensure whole-sample irradiation.
   a. Sperm can be collected as outlined above, irradiated while being kept on ice and resuspended in 10% Hank's solution in small glass vials. Alternatively, adult male fish, pretested for high fertility, can be anesthetized in tricane and placed on a moist sponge bed to constrain the fish within the path of the beam.
   b. The ability of a given source to induce mutations should ideally be calibrated, but 200–300 rads seem to be sufficient to induce mutations.
   c. Sperm after irradiation can be added directly to eggs obtained from squeezed females, or irradiated fish can be treated similarly to those treated with ENU to produce F1 founder fish.

   It is theoretically possible to search for specific DNA lesions generated by X- or γ-rays via the use of PCR. Primers that span a region of interest can be used to amplify target sequences from haploid embryos derived from F1 founder fish generated from X or γ-rays mutagenized fish or sperm. Although the chances of locating a rearrangement that breaks specifically in a known gene are small, it is

possible to identify deletions that span a gene region seemingly with fairly high frequency. This is dependent on the dose of radiation that is used to induce such rearrangements, and it is quite likely that complex rearrangements are induced by high doses of X- or γ-rays. Such complex rearrangements invariably led to complex phenotypes, and it is difficult to say with certainty that a given phenotype results from rearrangement in a specific gene region. It is thus yet to be determined if PCR can be used to identify gene-specific lesions efficiently by this method. It can be used to check quickly if an identified phenotype results from the deletion of a gene of interest, and it may be in this application that PCR-based screening is most useful, rather than its systematic application to every brood of a random screen.

The ability to generate a number of gene-specific PCR markers in any one PCR reaction allows multiple genes to be screened at any one time for a given phenotype. The screen procedure first involves the preparation of DNA from fish demonstrating a given phenotype. PCR is performed on this DNA and scored for the presence or absence of a DNA marker. PCR can also be used to map quickly the limits of a deletion and assess its suitability for further genetic analysis, such as the saturation mutagenesis of a specific region.

7. Stock maintenance: The protocols required for general fish raising and maintenance have been listed in detail elsewhere ***(12)***, but it is worthwhile to mention a few tips that specifically impinge on the efficiency of a small-scale mutagenic screen. Mutations will be identified in an ENU screen on average once per one to two haploid genomes screened ***(8,9)***. There is a great temptation to keep every mutation that is identified from such a screen. However, the maintenance of these mutations as stocks can quickly cut into available tank space in a small facility. It is far more efficient to make a decision about mutations when they are identified. Alternatively, mutations can be stored as frozen sperm and can be recovered by *in vitro* fertilization of squeezed wild-type eggs with the thawed sperm. Care should be taken to assay the protocol for the ability of thawed sperm to fertilize by initially utilizing wild-type sperm in test fertilizations.
   Freezing sperm:
   a. Sperm is collected using the methods outlined above. Sperm from at least four males identified as heterozygotes for the mutation of interest should be used.
   b. Sperm is added to 4–5 vol of fish Ringers containing 10% methanol and 15% powdered nonfat milk.
   c. The solution is frozen in capillary tubes placed in 10-mL plastic centrifuge tubes (Sorvall) by incubation in dry ice for 20 min.
   d. Capillary tubes are stored under liquid nitrogen after freezing.
8. Morphological screening: Marker-based screening is most efficient when coupled with a morphological screen. Zebrafish embryos are amenable to screening at most stages of development. If a researcher is interested in processes during organogenesis at stages later than 2 d it may be advisable to perform mutagenesis in a strain mutant for pigment synthesis, such as *golden* or *albino*, since this greatly increases the ability to detect subtle defects in organogenesis. The rapid development of

the zebrafish embryo can often make screening at some stages inconvenient. The ability to screen some stages may rely on placing the fish "off cycle" by utilizing time-regulated light sources for tanks so that fish are induced to lay at specific times. Researchers should make a checklist of structures and processes that are of interest and screen accordingly. After early pharyngula stages embryos should be anesthetized in tricaine to stop twitching and movement to best reveal structure. A subset of embryos should be dechorinated, but the dorsal aspects of the embryo are best revealed by rolling the embryo within the chorion to present the dorsal surface. Later stages require anesthetizing also, since by 3 d fry are able to swim rapidly and can be frustrating to screen.

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Microinjection and Cell Transplantation in Zebrafish Embryos

Qiling Xu, Derek Stemple, and Katherine Joubin

1. Introduction

The use of microinjection to study gene function in zebrafish has become widespread. Its applications include ectopic expression of genes by introducing DNA ***(1)*** or RNA into embryos and perturbation of gene function by injecting RNA-encoding truncated proteins ***(2, 3)***, blocking antibodies ***(4)***, or antisense morpholino oligonucleotides ***(5)***. The method involves the injection of DNA, RNA, or morpholino into the yolk or cytoplasm of one-cell-stage embryos using a pressure microinjector and micromanipulator as described here.

The ability to transplant cells and tissue allows one to assess the interactions and behaviors of cells and tissues when placed in ectopic locations or different genetic backgrounds. The combination of microinjection with transplantation complements the genetic tools offered by zebrafish, facilitates studies of inductive interactions and cell behaviors, and enables the generation of germ-line chimeras.

2. Materials

The following general equipment is required for these techniques:

1. Breeding pairs of adult zebrafish are maintained at 26°C with a 14-h light, 10-h dark cycle. Embryos are obtained by natural spawning or in-vitro fertilization as described in *The Zebrafish Book* ***(6)***.
2. The microinjection system consists of a stereomicroscope, a three-dimensional micromanipulator, and an automatic pressure microinjector.
3. For embryos with their chorions, an injection chamber is made by placing a glass microscope slide into an 85-mm diameter disposable plastic petri dish. Flood the injection chamber with embryo medium ***(6)***, and allow a thin film of liquid to form between the slide and the dish. Remove the excess liquid using a glass pipet.

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4. For dechorionated embryos, injection ramps are prepared as follows: Pour 15 mL of hot 1.5% agarose in embryo medium into an 85-mm petri dish, and leave to set. Add another 15 mL of the agarose solution to the solidified dish. Place a glass slide into the liquid agarose at an angle such that a trough is created. Remove the slide after it is set, and flood the chamber with embryo medium. Alternatively, store the injection chamber at 4°C for later use.
5. Microinjection pipets are prepared as follows: Pull fine glass capillaries with inner filament (Clark Electromedical Instruments, Reading, UK; 1.0-mm outside diameter and 0.58-mm inside diameter) with a micropipet puller (Model P-87, Sutter Instrument Co. Novato, CA, or any equivalent). The tip of the micropipet should be about 0.05 mm. If the tip is too thin, it will lack the tensile strength to penetrate the chorion. Thicker pipets do not easily withdraw from the chorion without dragging the embryo out of the chamber after injection. Just before microinjection, break the micropipet tip under a microscope with a pair of blunt forceps or a razor blade to produce a sharp end.
6. A transplant chamber is made using an acrylic mould containing 1.0-mm × 1.0-mm ramps placed in 2% agarose in 1× Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6) ***(7, 8)***.
7. Transplant needles are made from pulled capillaries without inner filaments. For cell transplants at the sphere stage, the tip of the needle should be cut at an angle using a razor blade and the inside diameter of the tip should be 15–20 µm. For shield transplants, use thick-walled capillaries (World Precision Instruments, 1B100–4), which are pulled then cut with a diamond pencil orthogonal to the long axis of the needle to obtain an inner diameter of 200 µm. Transplant needles can be washed with ethanol after use and reused.
8. The transplant rig consists of an oil-filled needle holder connected to a Hamilton syringe (25 µL for cell transplants, 10 µL for shield transplants) regulated by a micrometer-controlled syringe pump (Stoelting, 51218).
9. Molecular-biology-grade reagents should be used to prepare the solutions, DNA, RNA, and morpholino oligonucleotide samples.

2.1. Preparation of DNA

1. Agarose (electrophoresis grade, e.g., Roche).
2. 0.2 M KCl.
3. 2.5% Phenol red (e.g., Life Technologies).
4. Microspin columns (Amersham Biosciences, Piscataway, NJ).
5. Restriction enzymes and buffers (e.g., Roche).

2.2. Preparation of RNA

1. In-vitro transcription kit (e.g., Message Machine, Ambion).
2. RNase-free water (Sigma, W-4502).
3. Restriction enzymes and buffers (e.g., Roche).
4. Agarose (electrophoresis grade, e.g.. Roche).
5. RNase-free DNase1 (e.g., Roche).
6. Phenol–chloroform (1:1; Roche).
7. Microspin columns (Amersham Biosciences, Piscataway, NJ).

2.3. Preparation of Morpholino Oligonucleotides

1. Morpholino. These can be designed for the investigator by the manufacturer (GeneTools Inc).
2. RNase-free water (Sigma, W-4502).
3. 200 mM KCl, 5 mM HEPES, pH7.0, 0.5% phenol red.

3. Methods

3.1. Preparation of DNA

DNA injections are carried out to get transient expression or to aim for chromosomal integration and germ-line transmission. It has been shown that, if a transgene of interest is flanked by two I-SceI meganuclease recognition sites and microinjected with the I-SceI enzyme, its buffer, and BSA into one-cell-stage fish embryos, the transient promoter-dependent expression and germ-line transmission rate of the transgene are dramatically increased ***(9)***. The other advantage of this method is the low copy number of integration, since integration loci are found to contain only one or a few copies of the transgene in tandem. For this approach, it is important to inject into the cell of one-cell-stage embryos as opposed to the yolk.

If one cannot include I-SceI in the strategy for making a transgenic, the DNA should be linearized or the insert should be isolated from the plasmid sequences. Supercoiled DNA, however, can be used for transient gene expression work. After phenol–chloroform extraction to remove the restriction enzyme, the DNA is further purified on a MicroSpin column (MicroSpin S-300 HR, Amersham Biosciences, Piscataway, NJ) as follows:

1. Loosen the cap one-fourth turn and snap off the bottom tip.
2. Place the column in a decapitated 1.5-mL Eppendorf tube for support and spin in a microcentrifuge at 3,000 rpm (735 *g*) for 1 min.
3. Place the column in a fresh 1.5-mL tube, load the DNA solution into the column without disturbing the resin bed, and spin at 3,000 rpm for 2 min. The purified sample is collected in the bottom of the tube.
4. Estimate the DNA concentration by running an aliquot of the purified DNA on an agarose gel and comparing with a known amount of DNA, and store the DNA at −20°C. Alternatively, check the concentration by reading the OD at A_{260}.
5. Before microinjection, dilute the DNA in 0.2 M KCl, 0.25% phenol red. The dye facilitates the estimation of the injection volume.

3.2. Preparation of RNA

The RNA for microinjection is synthesized by in-vitro transcription. The structure of the 5′- and 3′- termini of synthetic mRNA can affect its stability and the efficiency of translation. Capping the 5′- terminus with GpppG and adding the poly(A) tail improve the stability of synthetic mRNAs. The constructs used

to generate the DNA templates for in-vitro transcription should be cloned into the pSP64T and pCS2 vectors ***(10–13)***. The synthetic RNA can be tested in an in-vitro translation system to ascertain that a protein product is made.

1. Prepare 2 µg of template DNA by digestion with a suitable restriction enzyme. Make sure the reaction is completed by analyzing an aliquot of the DNA digest in an agarose gel.
2. Purify the linear DNA by extraction with phenol–chloroform and precipitation with ethanol. Redissolve the DNA at 0.5 mg/mL in RNase-free water (Sigma, W-4502) or DEPC-treated water.
3. Mix the following components at room temperature in the order shown:
   a. 4.0 µL DNA template.
   b. 19.5 µL RNase-free water.
   c. 2.0 µL 0.1 M dithiothreitol.
   d. 4.0 µL 10× transcription buffer.
   e. 4.0 µL each of 50 mM rATP, rUTP, rCTP.
   f. 2.0 µL 50 mM rGTP.
   g. 2.5 µL 40 mM cap analog (GpppG).
   h. 2.0 µL placental RNase inhibitor (10 U/mL).
   i. 2.0 µL DNA-dependent RNA polymerase.
4. Incubate for 2 h at 37°C. Analyze an aliquot of the reaction (1–2 µL) on an agarose gel to check the transcription efficiency.
5. Add 1 µL of RNase-free DNase I (1 mg/mL), mix, and continue the reaction for 30 min at 37°C.
6. Add 60–160 µL of RNase-free water and purify the RNA by phenol–chloroform extraction twice.
7. Further purify the RNA by MicroSpin columns as described previously.
8. Check the size and quality of RNA by gel electrophoresis. Aliquot the RNA, and store at −80°C.
9. The amount of RNA for microinjection varies according to the nature of its encoded protein. It is therefore necessary to titrate the RNA.

3.3. Preparation of Morpholino Oligonucleotides

Gene specific and control morpholino oligonucleotides are available commercially and supplied in powdered form (Gene Tools, Philomath, OR; Open Biosystems, Huntsville, AL). Morpholino oligonucleotides are solubilized in RNase-free water. The resulting stock solution can be stored at −20°C. A working solution is prepared by diluting the stock solution to an appropriate concentration in morpholino dilution buffer (200 mM KCl, 5 mM HEPES, pH7.0, 5 µg/mL phenol red). Read the OD at A_{265} to determine the concentration of stock solution accurately. The working solution can be stored at room temperature. Because morpholinos can precipitate, before injecting. the morpholino should be heated to 65°C for at least 5 min and vortexed. If necessary, morpholinos can be further purified on a MicroSpin G-25 column following the manufacturer's instructions.

3.4. Microinjection

Injections can be done on embryos with or without their chorions. It is not necessary to dechorionate embryos for microinjection. Dechorionation is time consuming, and dechorionated embryos are much more fragile and susceptible to infection. Dechorionation can be done manually with watchmaker's forceps or with 1 mg/mL pronase (Sigma), which is added to embryos until their chorions collapse when poked, followed by several washes in embryo medium. If one has to work with dechorionated embryos for microinjection, 1.5% (w/v) agarose-coated dishes or glass dishes should be used to incubate embryos.

1. Fill the micropipet with the DNA, RNA, or morpholino using a microloader (Eppendorf, Germany). The capillary action of the inner microfilament should direct the solution to the very tip of the micropipet.
2. Attach the micropipet to the needle holder connected with the micromanipulator (Narishige, Tokyo, Japan). The system is operated using compressed air, which delivers pulses controlled by a microinjector (Picospritzer II, General Valve Corp., USA) or any equivalent.
3. Precalibrate the amount injected by counting the number of pulses to expel 1 µL of solution under fixed pressure and duration (e.g., 50 psi and 30 ms). For 200 pulses, to deliver 1 µL of solution requires 5 nL injection volume. Alternatively, injection volume can be measured accurately using a graticule (Graticules Ltd, Tonbridge, Kent, UK; Ward's Natural Sciences). Inject about 500 pL–2 nL DNA, RNA, or morpholino at the one-cell stage and 100 pL if injecting into one cell at the 128–256-cell stage.
4. For embryos with their chorions, use a wide-mouth glass pipet to transfer fertilized embryos into the injection chamber and align them (30–40 embryos) along the trough created between the slide and the petri dish. Tilt the injection chamber slightly to collect and remove the excess liquid as much as possible. With a pair of blunt forceps, orient the embryos such that the germinal disk faces the trough.
5. Injections can be done through the chorion and directly into the yolk or cell. To prevent the embryo from rotating away from the micropipet tip, it is important to have a relatively steep angle (about 45°) between the pipet and the embryo. Use the micromanipulator to move the micropipet vertically to penetrate the chorion and the cell membrane.
6. Following injection, tilt the injection chamber, and add embryo medium to the embryos. Transfer the embryos into a clean dish, and incubate them in a humidified incubator at 28.5°C. Later, remove all dead or uncleaved embryos.
7. For dechorionated embryos, transfer embryos to agarose ramp using a flamed glass Pasteur pipet. The angle of injection is not important if done at the one-cell stage. However, when injecting into a single cell at the 128–256-cell stage, the angle should be about 30°.

3.5. Cell Transplantation

Cell transplants are easiest when done at the sphere stage. They can be done later; however, the enveloping layer becomes tougher to pierce and epiboly makes penetration of the yolk much more likely. Embryos should be dechorionated,

either with pronase or manually, then placed on their sides in individual 1-mm wells made of 2% agarose in 1× Danieau solution with 5% penicillin–streptomycin (Gibco-BRL). Ensure that no bubbles are in the transplant setup, then place a medium-filled transplant needle into the pipet holder filled with mineral oil. Use a micromanipulator to pierce the EVL with the transplant needle, then use the syringe pump to gently suck up 1–50 cells from the embryo. The cells should be visible as they enter the needle. If the needle is too narrow, the cells break and are not discernible. Remove the needle from the donor embryo and pierce the host embryo. When the tip is just under the EVL, expel the cells. Allow embryos to recover for about 20 min before moving them to an agarose dish containing 0.3× Danieau's solution and 5% penicillin–streptomycin. If embryos will be reared for several days, grow them up individually to minimize infection.

3.6. Shield Transplantation

For shield transplants *(7)* experiments should be performed at 19–21°C in 1× Danieau's solution containing 5% penicillin–streptomycin. Host embryos should be manually dechorionated, as pronase makes the yolk slightly more fragile. Donors can be treated with pronase only if they will not be reared after transplantation. Host and donor embryos should be placed in individual wells on their sides with the tissue to be transplanted or insertion site facing the transplant needle tip. With the micromanipulator, the tip is first placed directly onto the donor embryo's shield, which should be slowly drawn into the needle by the syringe pump. Although the yolk is also initially pulled into the transplant needle, the shield tissue separates from the yolk, which recoils back toward the embryo. With the shield still in the transplant pipet, the pipet is placed onto the insertion site of the host embryo. In a similar fashion to the shield, the host tissue is pulled off and discarded into the medium. The shield is then pushed to the tip of the pipet using the syringe pump, and the pipet is placed onto the hole left at the insertion site. The pipet is pushed into the gap and the shield is pushed out. The host blastoderm initially inflates but deflates after a few seconds, leaving the donor tissue in the gap. Embryos should be allowed to recover for 30 min before being transferred to agarose plated dishes containing 0.3× Danieau's solution and 5% penicillin–streptomycin.

4. Notes

1. Regulated expression of the gene of interest both in space and time can be achieved through the use of gene-specific promoters and inducible systems. One of the best characterized inducible systems is the heat shock promoter of the *hsp70* gene in zebrafish ***(14–16)***. The *hsp70* driven transgene is not expressed under the normal temperature but is activated at the ambient temperature. It is interesting that the *hsp70* promoter can also be activated by the laser beam, and this technique allows targeted expression at single-cell level.

2. Recent reports suggest that the *tol2* transposable element system is a very efficient method for generation of transgenic zebrafish lines. Use of *tol2* requires the coinjection of mRNA and appropriate transposable element DNA constructs. This transposable element system has the advantage of not forming concatomers on integration into the genome ***(17,18)***.
3. Morpholino oligonucleotides have been used to investigate the function of different isoforms, since in addition to the ability to block translation, they can inhibit pre-mRNA splicing. For example, antisense morpholino oligonucleotides effectively block *fgf8* pre-mRNA splicing in zebrafish embryos (19) and generate phenotypes similar to the ace mutation of *fgf8* ***(20)***. Splice-blocking morpholino oligonucleotides have the advantages that the efficacy of gene knockdown can be quantified by RT-PCR and can selectively prevent the production of an alternative splice variant.

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Recent Advances in Meganuclease- and Transposon-Mediated Transgenesis of Medaka and Zebrafish

Clemens Grabher and Joachim Wittbrodt

1. Introduction

Transgenesis, the introduction of transgenes into the genome of an organism and its appropriate expression in subsequent generations, represents a major technological advance in modern biology. Ectopic expression of transgenes (gain of function) and disruption of endogenous genes (loss of function) in transgenic animals have proven to be highly valuable in extending our knowledge of mechanisms of development and developmental gene regulation, the action of oncogenes, and intricate cell interactions within the immune and nervous systems. By employing reporter genes under the control of specific regulatory sequences, transgenic techniques enable the functional dissection of *cis*-acting elements responsible for spatial and temporal gene expression patterns. In addition, tissues or cells expressing a reporter transgene can be used in cell lineage analysis and transplantation experiments. Furthermore, the transgenic technology offers exciting possibilities for generating precise animal models for human genetic diseases.

Fish with their extrauterine development of transparent embryos are excellent candidates for the production of transgenics, as they represent the largest and most diverse group of vertebrates and provide an ideal system for in-vivo studies of developmental processes during embryology. Two teleost fish species, medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), represent the most advanced fish model systems today. Despite many similarities, the evolutionary distance between medaka and zebrafish to their last common ancestor of about 160 million years is reflected in many aspects of their biology. These species

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provide ideal resources for comparative studies, allowing the identification of conserved and species-specific molecular mechanisms underlying development and evolution.

Both model organisms are similarly amenable to transgenesis protocols that can be broadly classified as viral and nonviral. Pseudotyped retrovirus infection has been used to generate single copy insertions of transgenes ***(1–3)***. Present protocols of this method are highly efficient for insertional mutagenesis and enhancer trapping (T. Becker, personal communication). However, construction, packaging, titering, and infection are laborious processes requiring considerable expertise and safety precautions. Small laboratories with the intention to generate only few transgenic fish may therefore want to use simpler approaches. Nonviral technologies include a variety of different approaches, such as electroporation, particle bombardment, liposomes, stem cell transfection, nuclear transfer, and microinjection ***(4–18)***.

Presently, microinjection provides the fastest and simplest means for germ-line transgenesis and transient expression studies in fish ***(19–22)***. For more detail concerning microinjection procedures, see Chapter 35 of this volume.

1.1. Transgenesis by Microinjection

The first transgene to be delivered into medaka embryos was the δ-crystalline gene of chicken ***(10)*** resulting in transient mosaic expression of the transgene. In 1988, Stuart McMurray, and Westerfield reported the first successful germ-line transmission and subsequent expression of a transgene on microinjection in a teleost genetic model system (zebrafish; ***(12)***).

For microinjection experiments in fish, a glass needle loaded with DNA solution is used to penetrate the plasma membrane of a one-cell-stage embryo. The injection procedure is performed with the aid of a micromanipulator under a standard dissecting microscope. Once the tip of the needle has entered the cytoplasm, approximately 1–2 nL of DNA solution containing 10^5 to 10^7 DNA molecules is injected.

Unfortunately, the direct injection of DNA is accompanied by some drawbacks, as it often results in mosaic transgene expression in G0, low integration frequency (often in large tandem arrays), and mosaic germ-line distribution; so, techniques widely used in mouse and *Drosophila*, such as enhancer and gene trapping ***(23–27)*** are rarely used due to the low frequency of vector integration into the fish genome ***(28)***. Transgenesis frequencies on conventional DNA microinjection range within 1–10% ***(12,29–33)***. Transient expression of a transgene in G0 is invariably mosaic and may occur in 10–50% of injected fish ***(19,34)***. Consequently, technological improvements on transgenesis by microinjection are on demand.

To develop strategies that overcome the drawbacks of DNA microinjection, one has to consider the fate of injected DNA inside a cell. Plasmid DNA, injected

into fish embryos, may (a) persist and replicate in an extrachromosomal state within a cell and its descendants for several cell divisions or (b) integrate into the chromosomal DNA of the cell.

In most cases, either of the two fates results in embryos that are mosaics with respect to the presence of plasmid DNA. Uneven distribution and replication of the episomal DNA among daughter cells (a) result in mosaic expression in injected fish (G0). The earlier an integration event occurs in a given cell (b), the more descendents of this transgenic cell also contain and express the transgene. Consequently, early genomic insertion yields a transgenic animal exerting broad transgene expression in G0, while transgene integration at a later time point affects fewer cells and thus results in mosaic expression in G0. Moreover, genomic integration at later time points during embryogenesis (at best) results in only few primordial germ cells containing an insertion of injected DNA leading to a mosaic germ line. Thus, the proportion of transgenic F1 progeny depends on the degree of mosaicism and only genomic integration at the one-cell stage leads to a fully transgenic germ line, where 50% of the F1 offspring inherit the transgene.

Disappointingly, integration of transgenes has been found to affect its expression pattern in many unpredictable ways. For example, it has been frequently observed that neighboring sequences influences transgenes. In addition, there have also been problems with the silencing of transgenes as a result of mechanisms such as DNA methylation and heterochromatin formation. It is notable that transgene integration as an intact single copy, as opposed to a tandem array of multiple copies, may be desirable, since tandem arrays may be more readily inactivated by DNA methylation or heterochromatin formation ***(35–37)***. To avoid position effects, DNA methylation or changes of chromatin state affecting transgene expression, it is desirable to supply the transgene with regulatory sequences. The importance of the inclusion of enhancer elements, introns, and appropriate polyadenylation (pA) signals is generally accepted ***(38)***. Moreover, genes are thought to be organized on chromosomes as contiguous but independent units, known as *expression domains* ***(39)***. These expression domains are believed to remain insulated from neighboring sequences by boundary regions ***(40)***.

In this chapter, we review two recently developed approaches for transgenesis in medaka and zebrafish that significantly enhance the potential of DNA microinjection. Detailed protocols for their use are also presented. Both are based on the use of enzymes that bind to DNA, mediate entry into the nucleus, and facilitate early integration of foreign DNA into the host genome. In combination with regulatory sequences or insulators, both techniques also guarantee stable transgene expression in subsequent generations. One technique involves the coinjection of the meganuclease I-SceI ***(20,22)***, while the second is based on transposons ***(41–46)***.

1.2. I-SceI Meganuclease-Mediated Transgenesis

I-SceI is a homing endonuclease encoded by the mobile group I intron of the large rRNA gene of *S. cerevisiae* ***(47,48)***. This family of enzymes mediates the propagation of the intron by cutting intronless genes at the site of the intron insertion. Like restriction enzymes, homing endonucleases cleave double-stranded DNA with high specificity in the presence of divalent metal ions. However, they differ from restriction endonucleases in their recognition properties and structures, as well as in their genomic location ***(49)***. In particular, whereas restriction enzymes have short recognition sequences (3–8 bp), homing endonucleases, despite their small size, recognize long DNA sequences (12–40 bp). They have been classified into four families based on both their sequence motifs and DNA cleavage mechanism ***(50)***. The protein I-SceI is a member of the largest class of homing enzymes, characterized by the presence of either one or two conserved amino acid residue sequence motifs (LAGLIDADG). I-SceI acts as a monomeric endonuclease of 235 amino acids containing two conserved motifs ***(51)***. Its catalytic activity depends on the presence of Mg^{2+} or Mn^{2+}. Cleavage of its recognition sequence (TAGGGATAACAGGGTAAT) results in a 4-bp overhang presenting a 3′-hydroxyl terminus ***(51)***. The enzyme displays a low turnover because of a strong affinity for one of the products of the cleavage reaction ***(52)***. The recently resolved crystal structure of I-SceI bound to DNA attributes the high sequence specificity of I-SceI to its recognition sequence to numerous direct phosphate and base-specific contacts and water-mediated interactions ***(53)***. Theoretically, an 18-bp recognition sequence appears once in 70 billion bases of random sequence. Vertebrate genomes analyzed to date, including medaka and zebrafish, are free of I-SceI sites, abolishing the risk of genome fractionation by the meganuclease.

Application of I-SceI in mammalian cells efficiently led to stably transfected cell lines with single copy integrations ***(22)***. Based on this, a simple, fast, and efficient technique was established in medaka, allowing the generation of stable transgenic lines by coinjection of the I-SceI protein with reporter vectors that are flanked at both ends by the corresponding recognition sites (**Fig. 1**). In the meantime, the meganuclease approach has been used successfully in several fish species (medaka, stickleback, zebrafish), amphibia (axolotl, *Xenopus*), and ascidians (*Ciona*). Application of the meganuclease protocol highly improves the number of fish expressing a transgene in a promoter-dependent manner. In addition, the level of mosaicism is strongly reduced and stable, promoter-dependent expression is achieved already in G0. Ultimately, the frequency of resulting transgenic F1 offspring is enhanced by a factor of 3 compared to conventional DNA microinjection in fish. Uniform G0 expression in combination with an elevated germ-line transmission frequency resulting from I-SceI/DNA coinjections is a good indication for an early insertion event. Meganuclease

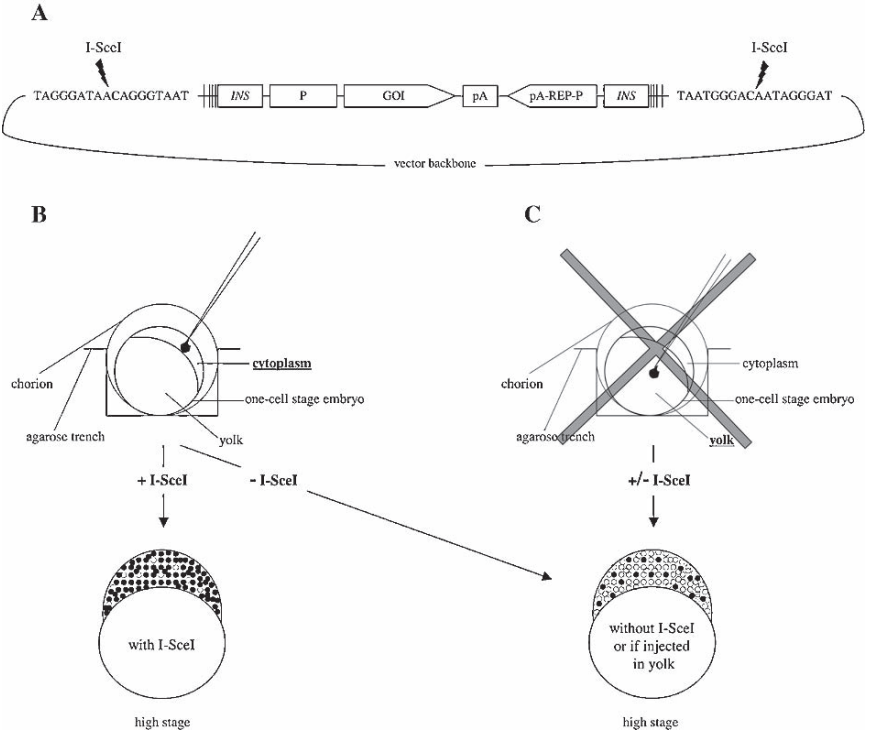

Fig. 1. I-SceI vector microinjection. Microinjection for meganuclease-mediated transgenesis should be performed as depicted. Embryos should be oriented as indicated. The injection volume must not exceed 10% of the cell volume. **A** An I-SceI vector. The insertion cassette contains an expression cassette including the gene of interest and a reporter cassette. Two insulator sequences and two inverted I-SceI recognition sites flank the entire insertion cassette. **B** Injection is performed directly into the cytoplasm of the cell. Upon coinjection of DNA with I-SceI, this procedure significantly enhances transient transgene expression and transgenesis frequency. Injection without I-SceI results in highly mosaic transient transgene expression and low transgenesis frequency, even if injected into the cytoplasm. **C** Injection into the yolk of a one-cell stage zebrafish reduces the enhancing effect of I-SceI resulting in transgene expression and transgenesis frequency similar to conventional microinjection. Therefore, injection into the yolk should be avoided. Abbreviations: GOI, gene of interest; INS, insulator; REP, reporter gene; P, promoter; pA, poly adenylation signal. Source: Modified from ***(20)***.

transgenesis in medaka and zebrafish have resulted in germ-line transmission rates of 25–50% that, together with enhanced G0 expression, show that I-SceI efficiently facilitates early functional integration of the transgene (one- to two-cell stage).

Transgenic fish generated with I-SceI show Mendelian segregation of transgene expression in F2, indicating that meganuclease-mediated transgenesis

preferentially yields transgene integration at a single genomic locus. Moreover, transgenes insert as single copy or as head-to-tail tandem arrays of low copy number (1–10 copies; see ***(22)***). Therefore, the size of tandem arrays is considerably lower than observed in standard plasmid injections (up to 2,000 copies) and transgene expression has been found to be stable for many generations.

How does I-SceI improve transgenesis? During the process of intron homing, I-SceI creates double-strand breaks (DSB). Does meganuclease create DSB in the host genome and the injected plasmid DNA to promote integration by a repair mechanism? This consideration is in contrast to the observation of independent insertion sites in several medaka and *Ciona* transgenic lines as the existence of multiple endogenous sites is highly unlikely to occur due to its length (18 bp). Recognition-site mutation and deletion experiments in medaka revealed that nuclear localization mediated by I-SceI is not sufficient to improve transgenesis frequency. Enhanced transgenesis frequency requires cleavage of injected DNA ***(22)***. I-SceI may counteract endogenous ligase and replicase activity, which is thought to be responsible for the strong concatemerization of conventionally injected DNA. Thereby, the transgenes remain short fragments exposing more recombinogenic ends. Through association with one of its cleavage products, I-SceI may accomplish "inhibition" of endogenous ligases or replicases by both cleavage of generated concatemeres and protection of cleaved recombinogenic ends from degradation and ligation. I-SceI-induced DSBs allow recombination in the mammalian system at high frequency ***(54)***. Additionally, the natural homing process is thought to involve the host DSB repair system. Enhanced integration frequency is initiated by DSBs within the injected DNA and may involve direct interaction of I-SceI with the double-strand break repair machinery. Future experiments to identify interaction partners of I-SceI will help elucidate the function of the meganuclease in transgenesis and to further increase its efficiency.

1.3. Transposon-Mediated Transgenesis

Transposable elements are discrete segments of DNA capable of moving from one locus to another in their host genome or between genomes. They are distributed across the living world and play a fundamental role as motors of genome plasticity. DNA transposons contain inverted repeats (IRs) at either end flanking a central region encoding for a transposase, which catalyzes transposition. Transposase-deficient elements can be mobilized if the transposase is provided *in trans* by means of an inducible transposase source or coinjection of transposon and tranposase mRNA.

Eukaryotic transposons move in a nonreplicative manner via excision and integration (cut and paste). For transposition, the transposase binds to the IRs and cleaves the DNA, thereby precisely excising the transposon. Additionally, the transposase binds to the target DNA, introducing a staggered cut, leading to

protruding single strands. The DNA repair machinery accomplishes integration into the host genome, resulting in target site duplications directly adjacent to the transposon ends.

DNA transposons provide stable gene expression from a single-copy insertion and simultaneously tag the integration locus to facilitate subsequent molecular analyses. A variety of different transposable elements have been utilized successfully as tools for insertional mutagenesis and gene delivery in *Drosophila* ***(55,56)***, *C. elegans* ***(57,58)***, and plants ***(59)***. Until recently, they have not been used for investigation of vertebrate genomes because most transposon systems active in a certain species cannot simply be transferred to vertebrates, due to the requirement of species-specific cofactors for transposition. Second, only few well-defined DNA transposons have been identified in vertebrate species.

The P-element of *Drosophila melanogaster* is the most famous DNA transposon, used for generating P-element insertion lines. P-element insertions can lead to regulated reporter gene expression through an adjacent enhancer ***(26,60)*** and cause mutations ***(61,62)***. Genomic regions flanking the insertion can be cloned by plasmid rescue ***(60)***. In the *Drosophila* P-element system, other factors than the transposase are involved in the transposition event. Therefore, attempts to use the P-element for transgenesis of nondrosophilid insects, zebrafish, and mammalian cells have been unsuccessful ***(63–65)***.

1.3.1. The Tol*2 Transposable Element*

One of the few active DNA transposons was discovered in medaka. *Tol*2 is a member of the hAT superfamily (hobo of *Drosophila melanogaster*, Ac of maize, and *Tam*3 of the snapdragon ***(66)***. This transposon has inserted into the tyrosinase gene of the *albino* mutant and has been shown to be active during medaka embryogenesis ***(67,68)***. Due to its endogenous activity, it is not a candidate tool for transgenesis in medaka. However, it has been successfully applied in zebrafish ***(45)***. Coinjection of *tol*2 transposase mRNA with plasmid DNA containing a nonautonomous *tol*2 element into zebrafish embryos results in transposon-mediated genomic integration of the *tol*2 element. Genomic insertions are submitted to the F1 generation, yielding true transgenic zebrafish. In contrast to the P-element, *tol*2 transposition is independent of host specific factors and has been successfully applied in mouse embryonic stem cells ***(69)***. Thus, *tol*2 is a promising transgenesis vector for vertebrates other than its original host.

1.3.2. Tc*1/Mariner-like Transposable Elements*

Homologs of the *Tc*1 element in *C. elegans* and those of the mariner transposon in *D. mauritiana*, are probably the most widespread DNA transposons in nature ***(70)***. The presence of *Tc*1*/mariner*-like elements in many different species indicates that, in contrast to P-elements, they are independent of any host factors and

therefore might be used for germ-line transformation in many different species. This hypothesis is corroborated by the fact that recombinant *Tc*1 and *mariner* transposase are able to catalyze transposition *in vitro* without help of any other factor ***(71,72)***. Furthermore, vectors based on *minos*, a *Tc*-like element from *Drosophila hydei*, were successfully used for germ-line transformation of the fly *Ceratis capitata* ***(73)***, and the *mariner* element from *Drosophila mauritiana* was capable of undergoing transposition in the protozoan *Leishmania* ***(74)***.

Raz and colleagues used *Tc*3 from *C. elegans* to stably introduce a reporter construct containing GFP into zebrafish embryos by coinjection of the *Tc*3 transposase mRNA together with the reporter flanked by inverted repeats ***(46)***. In one line, they could show transposon-mediated integration, expression of the reporter construct and germline transmission.

*Tc*1/*mariner*-like elements have been found in many vertebrate genomes ***(75–79)***. However, unlike *tol*2 in medaka, all the transposon copies isolated to date are inactive remnants of once active transposons. Inspired by the potential for a vertebrate transposon Ivics and colleagues engineered a functional *Tc*1/*mariner*-like transposon from inactive copies of several fish species and named it *Sleeping Beauty* (SB; ***(80)***). The SB transposase has been shown to efficiently mediate transposition in cells from fish, mouse, and human in culture ***(80)*** and has been used as a genetic tool in the mouse *in vivo* ***(81–84)***. Within the last several years SB has been developed into an efficient gene transfer tool also in medaka and zebrafish ***(41,43,44)***.

1.3.3. The SB Transposon System

The SB transposase is a *Tc*1/*mariner*-like transposase reconstructed from a salmonid genome by stepwise correction of the accumulated mutations. The SB system consists of two parts: the SB transposon, containing inverted repeats flanking the DNA element anticipated for mobilization and the SB transposase. Each IR contains two direct repeats (DRs), one at either end, constituting the core of the binding sites for the transposase. The transposase binds to both DRs and adjacent sequences, but only the outer DRs are utilized for site-specific cleavage and excision of the transposon. The left and right IRs are imperfect, with a match less than 80% at the center, but perfect in the DRs. The SB transposase harbors a N-terminal DNA binding domain that provides specificity for salmonid-type IRs and DRs and includes a bipartite nuclear localization signal (NLS). The C-terminal half comprises a glycine-rich sequence of unknown function and the DD ***(34)*** E domain that catalyzes transposition, termed after the highly conserved amino acid residues Asp, Asp, Glu, in which the latter are separated by 34 amino acids. Transposons of the *Tc*1/*mariner* superfamily, including SB, integrate into TpA target di-nucleotides occurring approximately every 20 bp in a vertebrate genome. Accordingly, SB appears to insert in a random manner ***(85)***.

In medaka and zebrafish, the SB transposon system has been applied successfully to generate transgenic animals by coinjection of transposon DNA together with transposase encoding mRNA (**Fig. 2**). Transgenesis frequencies (germ-line integration and expression in subsequent generations) were enhanced to more than 30% of the injected embryos, equaling I-SceI transgenesis rates. Also, the numbers of embryos expressing the transgene in a promoter dependent fashion in G0 was improved fourfold compared to control injections in medaka. Using a second-generation SB system, exhibiting improved transposition efficiency ***(86)***, genomic insertions were clearly mediated by the transposase in zebrafish ***(41)***. In medaka, on the other hand, the original SB system ***(80)*** did not prevent concatemerization of plasmid DNA and resulted in genomic insertions of plasmid tandem arrays ***(43)***. However, tandem array insertions also yielded robust and invariable expression during subsequent generations. Notably, by using a moderate-strength ubiquitous promoter to drive *GFP* expression, efficient enhancer trapping yielded in 12% of the transgenic medaka spatially and temporally restricted transgene expression. We hypothesize that, in these cases, the SB transposase directed transgene insertion to AT-rich scaffold attachment regions or matrix-attached regions. Such insertion lines provide useful tools for cell biology, cell lineage and other developmental biology applications.

In all SB generated transgenic medaka and zebrafish lines, transgene expression was stable for more than eight generations, indicating that even concatemerization

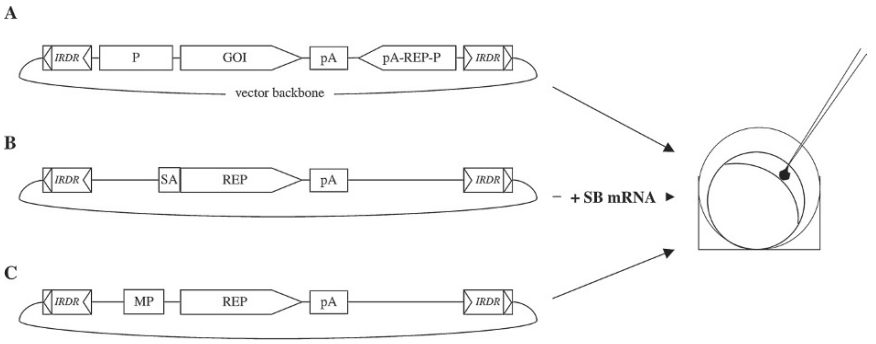

Fig. 2. SB vector microinjection. **A** SB transgenesis vector. The insertion cassette containing an expression cassette including the gene of interest and a reporter cassette is flanked by two SB IRDRs. **B** SB gene-trap vector containing a splice acceptor site preceding a reporter gene. **C** SB enhancer-trap vector. A minimal promoter drives expression of a reporter gene. SB vectors are coinjected with SB mRNA into one-cell-stage embryos. Abbreviations: GOI, gene of interest; IRDR, inverted/direct repeat; MP, minimal promoter; P, promoter; pA, polyadenylation signal; REP, reporter gene; SA, splice acceptor site.

of transgenes did not negatively affect gene expression. Growing evidence suggests an insulating activity of inverted repeats that may account for stabilized gene expression by shielding from heterochromatin spreading ***(40,87,88)***. Most, but not all transgenics yielded insertions at a single genomic locus. Therefore, the SB transposon system provides flexibility. It can be adjusted for robust transgene delivery and expression at a single genomic loci or for transposase-mediated single-copy insertions at multiple loci, favorable for insertional mutagenesis and gene or enhancer trapping (**Fig. 2**).

1.4. Perspectives

Similar applications may be envisaged for both, meganuclease- and transposon-mediated transgenesis. Nonetheless, due to the different biological characteristics of the two technologies, certain applications appear more suitable to one or the other.

1.4.1. I-SceI Meganuclease

The meganuclease may be used in fish for comparative studies of *cis*-acting regulatory elements and homologous recombination (HR).

Direct comparison of regulatory elements is often hampered by effects on transgene expression mediated by the specific locus of insertion or distribution of episomal plasmid DNA. A meganuclease site, artificially engineered into a host genome may serve as a unique entrance site for transgene integration. Thus, a suitable expression locus that is unaffected by the host genomic environment can be used for any kind of transgenesis and, in particular, to perform comparative studies on regulatory elements under controlled conditions. Further improvements on meganuclease transgenesis include insulators upstream and downstream of the DNA of interest to protect the transgene from influences of heterochromatin and epigenetic control of surrounding genomic sequences.

Gene targeting in *Drosophila* has been achieved by using I-SceI, although at low efficiencies ***(89)***. These low efficiencies have been coupled with a highly efficient repair of chromosomal I-SceI-mediated DSBs. Direct interactions of meganucleases with components of the host DSB repair are conceivable as association of homing endonucleases with DSB repair is evolutionary ancient. Indeed, meganuclease-induced DSBs are not sufficient to promote integration into the genome as injection of in-vitro linearized DNA fragments resulted in lower transgenesis frequencies. This is indicative of an additional function performed by the meganuclease. Enhanced integration frequency may be initiated by DSBs and direct interactions of I-SceI with the double-strand break repair machinery, thereby I-SceI meganuclease could provide potential to be used for gene targeting also in fish.

1.4.2. Transposons

The efficiency of SB-mediated gene transfer is inversely proportional to transposon size. This is not an issue for insertional mutagenesis, in which the sole purpose of the transposon is to inactivate a gene and provide a short sequence tag for recovery of the insertion. For transgenesis, however, it is desirable that a transposon vector can mobilize long genes. Recent advances have been achieved in the development of hyperactive SB vectors, including the use of more efficient repeat sequences, transposases of higher activity, and application of "sandwich" transposons that allow mobilization of large transgenes ***(86,90)***. Furthermore, CpG methylation of the SB transposon enhanced transposition in mammalian cells a 100-fold ***(91)***. It is to expect that these improvements in transposition efficacy also hold true in fish, which is especially important for applications that require high insertion rates, such as insertional mutagenesis and enhancer- or gene-trap experiments.

A major advantage of highly efficient transposon-mediated transgenesis for mutagenesis and trapping experiments is the possibility to remobilize transposon insertions by classical breeding of transgenic animals. One transgenic line would express the transposase, while the other would harbor the transposon to be mobilized. In vertebrates, such experimental approaches have been successfully applied in mice ***(81–84)***. Ideally, the transposon would contain a gene-trap vector (**Fig. 2**) to directly select for transposon insertions into genes and to reveal their expression pattern.

Adding to the potential of the SB system, another artificially reconstructed transposon, *Frog Prince* (FP), with high transpositional activity in vertebrate cells has been described ***(92)***. Pilot experiments in medaka confirmed the activity of FP in fish embryos (Wittbrodt unpublished), providing the community with yet another promising tool.

2. Materials

2.1. Preparation of Plasmid DNA

Plasmid purification kit (e.g., Quiagen).

2.2. Microinjection Plates

1. Agarose.
2. Petri dishes (9 cm).
3. Injection mold.
4. Yamamoto's media: 10× 7.5% (w/v) NaCl, 0.2% KCl, 0.2% $CaCl_2$, 0.002% $NaHCO_3$, pH 7.3.
5. Danieau's solution.

2.3. I-SceI Microinjection

1. I-SceI enzyme and buffer (e.g,. New England Biolabs).
2. Yamamoto's buffer (*see* ***section 2.2***).

3. Protocols

3.1. Preparation of Plasmid DNA

The I-SceI vector to be injected should be designed such that the expression cassette of interest (e.g., including promoter, transgene, and polyadenylation signal) is flanked by two I-SceI recognition sites (**Fig. 1**). The expression cassette of the SB vector is flanked by two SB IRs and DRs (**Fig. 2**). The plasmid DNA should be prepared and purified using a high-purity plasmid preparation kit. DNA concentration and purity can be checked by spectrometry. The ratio of A_{260}/A_{280} should be between 1.8–2.0.

3.2. Preparation of Microinjection Plates

Several types of microinjection plates may be used (e.g., 30). An example is shown in **Fig. 3**. The 1.5% agarose is prepared with tap water. Warm agarose solution is poured into a petri dish, and a plastic injection mold (**Fig. 3**) is put on top of the agarose solution (floating) and left at room temperature until the agarose solidifies. Prior to injection the mold is removed from the solid agarose, which is overlaid with ddH_2O and stored in a refrigerator. On the day of injection, the plate is equilibrated to room temperature for 1 h and the water is exchanged with 1× Yamamoto's embryo rearing medium (medaka) or 0.3× Danieau's solution (zebrafish; ***(93,94)***).

3.3. Preparation of Embryos

Medaka and zebrafish matings are set up as described ***(93,94)***. Embryos are collected approximately 20 min after allowing a female and a male zebrafish to mate (or as soon as eggs are laid and fertilized). Single embryos are transferred and

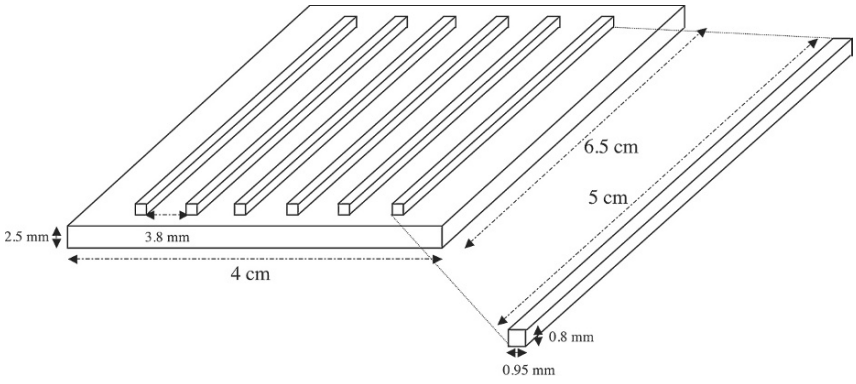

Fig. 3. An injection mold. The structure and dimensions of a type of injection mold suitable for zebrafish embryos are indicated. The mold should be made of thermo-resistant plastics Source: After ***(20)***.

aligned into the trenches of the injection plate using a Pasteur pipet (approximately 20 embryos/trench). Aiming for transient assays or the generation of stable transgenic lines, the embryos must be at the one-cell stage for consistent results. Medaka embryos may be injected in Yamamoto's embryo rearing medium chilled to 4°C to slow development.

3.4. I-SceI Microinjection (20)

Due to the low stability of the meganuclease, aliquots of enzyme solution should be prepared (e.g., 2 µL) on arrival and stored at −80°C. The microinjection solution should be prepared shortly before injection and kept on ice. Medaka and zebrafish may be injected using identical composition of injection solution: DNA: 10–30 ng/µL, I-SceI buffer: 0.5×, (Yamamoto's buffer: 0.5×), I-SceI enzyme: 0.3 U/µL, ddH_2O, and 30 µL. However, results in medaka are improved by adding Yamamoto's buffer: 10× 7.5% (w/v) NaCl, 0.2% KCl, 0.2% $CaCl_2$, 0.002%$NaHCO_3$, pH 7.3.

Microinjection needles are prepared as described ***(95)***, backfilled with injection solution (4 µl), mounted to a micromanipulator, and connected to a microinjector. To inject, the chorion and membrane of the cell are penetrated with the open tip of the needle. The injection volume should not exceed 10% of the total cell volume. Larger volumes result in increased mortality rates of injected embryos. For consistent results using the meganuclease approach, it is mandatory to inject directly into the cytoplasm of the cell. The concentration window of 10–30 ng/µL is a good starting point but the DNA concentration resulting in best transient expression and highest transgenesis rates may depend on the type of DNA (promoter, regulatory elements, transgene, etc.) and should be optimized empirically in case of unsatisfactory results. Embryos should be raised at the appropriate temperature after injection.

3.5. SB Microinjection (41,43,44)

The injection procedure is as described for I-SceI. SB mRNA is transcribed *in vitro* from a transcription vector containing the SB open reading frame ***(96,97)***. Transposase mRNA should be used at 100 ng/µL. The transposon DNA concentration for best results depends on the specific vector and needs to be evaluated empirically within a window of 5–50 ng/µL.The injection solution for SB microinjection is identical for medaka and zebrafish, with exception of Yamamoto's buffer: DNA: 5–50 ng/µL, RNA: 100 ng/µL, (Yamamoto buffer: 1×), ddH_2O.

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Retinoids in Nonmammalian Embryos

Malcolm Maden

1. Introduction

The family of retinoids comprise an enormous number of compounds related to vitamin A. Many of these compounds are naturally occurring substances generated during the biological functioning of retinoids: the conversion of dietary sources of retinoids (β-carotenes, retinyl esters) to those that can be taken up by the absorptive epithelium of the gut (retinol); the conversion of absorbed forms to stored forms in the liver (retinyl esters); the conversion of stored forms to active forms as mediators of vision (retinals), skin differentiation (retinoic acids), and general cell differentiation and proliferation (retinoic acids). However, more of these compounds are synthetic, for example, the retinobenzoic acids, and have been generated in the desire to find more potent and less teratogenic retinoids for pharmaceutical use. This chapter will be concerned only with a very few retinoids, ones that have been used in an embryological context.

The original definition of a retinoid was based on its chemical structure, but this soon became obsolete when these new compounds were synthesized. The current definition of a retinoid is a substance that can elicit specific biological responses by binding to and activating specific receptors or a set of receptors. This perceptive definition was formulated by Sporn and Roberts ***(1)*** before the discovery of the retinoid receptors and remains pertinent today with our knowledge of the retinoic acid receptors (RARs), whose ligand is all-trans-retinoic acid (tRA), and the retinoid X receptors (RXRs), whose ligand is 9-cis-retinoic acid (9-cis-RA) ***(2)***. In **Table 1,** the structure of tRA and 9-cis-RA is shown, as well as several other retinoids of embryological interest, and in the following section are a few comments on each of the compounds.

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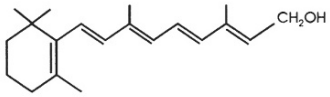
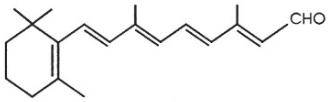
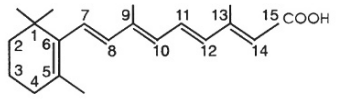
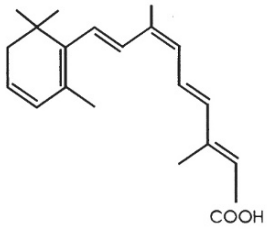
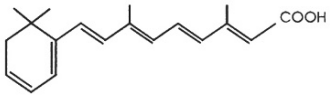
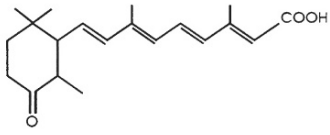
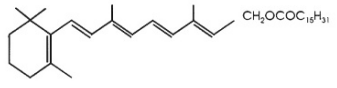
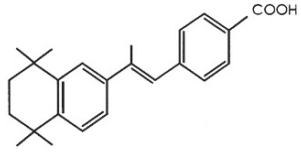
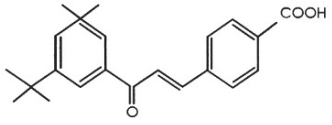
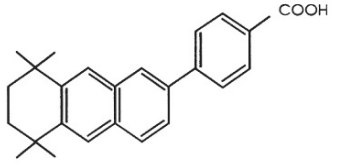
Table 1
Chemical Formulae of the Retinoids that Are of Embryological Interest[a]

Retinol	
Retinal	
All-trans-Retinoic Acid	
9-cis-Retinoic Acid	
3,4-Didehydro-Retinoic Acid	
4-oxo-Retinoic Acid	
Retinyl Palmitate	
TTNPB	
Ch 55	
CD 367	

[a] The numbering of the carbon atoms is shown only on the tRA.

1.1. Embryologically Interesting Retinoids

1.1.1. Retinol

This is the "parent" vitamin A molecule from which tRA is derived. All-trans-retinol is first converted to retinal and then tRA by two dehydrogenase enzymes, presumably specific. It is found at high levels endogenously in mammalian and chick embryos ***(3–5)***. Usually it is about 10-fold less potent than tRA in most biological assays ***(6)***, including mammalian teratogenicity ***(7)***, but it is inactive in inducing pattern duplication when applied locally to the regenerating amphibian limb ***(8)***, the chick limb bud ***(9)***, or developing chick skin ***(10)***.

1.1.2. Retinal

This is the intermediate metabolite between retinol and tRA and is used in the visual cycle where 11-cis-retinal binds with the protein opsin to form rhodopsin, the visual pigment. It is undetectable in mammalian and chick embryos, but present at very high levels in amphibian ***(11,12)*** and zebrafish eggs ***(13)***. It is not active in inducing pattern duplication in the regenerating amphibian limb ***(8)***.

1.1.3. tRA

This was formerly the most active naturally occurring metabolite of *t*-retinol in biological assays, including pattern respectfication in embryos, although perhaps it is now superseded by 9-cis-RA, at least in some assays ***(14)***. It is the ligand for the RARs and is present endogenously in varying concentrations throughout the mammalian and chick embryo ***(3–5,15)***, in the regenerating amphibian limb ***(16)***, and the *Xenopus* and zebrafish embryo ***(12,13)***.

1.1.4. 9-cis-RA

This is the ligand for the RXRs. Presumably, it is generated from tRA via a specific isomerase, although this is not known. It is present in adult mouse liver and kidney ***(17)***, but undetectable endogenously in chick and mouse limb buds ***(4)***. It is present in *Xenopus* embryos ***(12)*** and the regenerating amphibian wound epidermis generates 9-cis ***(18)***. It is 25 times more potent than tRA in inducing duplications of the chick wing bud ***(14)***.

1.1.5. 3,4-Didehydroretinoic Acid

This is found in the chick limb bud at 5–6 times higher amounts than tRA ***(4,19)***. It is generated by a parallel pathway to tRA from 3,4-didehydroretinol via 3,4-didehydroretinal. It is equipotent with tRA at inducing duplications in the chick wing bud ***(19)***.

1.1.6. 4-oxo-RA

This is a more polar metabolite of tRA, originally thought to be a breakdown product, but present in *Xenopus* embryos ***(20)***. It is more potent than tRA in inducing neural defects in *Xenopus* and a more potent mammalian teratogen ***(21)***. It binds and activates RARβ, but only weakly activates RXRα, resembling tRA in this respect ***(20)***.

1.1.7. Retinyl Palmitate

This is a typical esterified form of retinol used as storage in the liver. It is convenient for administration to amphibians, since it is available in a soluble form attached to corn starch and can be simply added to the tank water. It is eightfold less potent than tRA at inducing limb duplications during regeneration as assayed by this method of administration ***(22)***.

1.1.8. TTNPB, Ch-55, CD-367

These are examples of synthetic derivatives of tRA, which usually have far higher potencies in biological assays than tRA, including embryological assays, such as duplication of the chick limb bud ***(23–25)***, the regenerating amphibian limb ***(8,25)***, and feather transformation in chick skin ***(10)***. This may be because they are much more stable in cells than tRA and are not broken down by the tRA metabolism pathway.

2. Materials

2.1. Preparation of Retinoid Solutions and Stability

Retinoids are insoluble in water, but soluble in organic solvents, such as ethanol and dimethylsulfoxide (DMSO), which are the most commonly used solvents for administration of tRA. Although one always performs control experiments, it is important to be aware of the fact that DMSO acts as a differentiating agent to embryonal carcinoma cell cultures in the same way that tRA does, so perhaps ethanol is a "better" solvent. The maximum solubility of tRA in DMSO is about 50 mg/mL.

Retinoids are stable in powder form for many months at −20°C. Light and oxygen cause isomerization and breakdown, so they are often stored in brown vials in an atmosphere of nitrogen. Solutions of retinoids are unstable, especially at room temperature, and tRA in DMSO begins to isomerize to 13-cis-RA, 11-cis-RA and 9-cis-RA in a few minutes when left on the laboratory bench. This can be very useful for generating standards for HPLC, but a problem when administering retinoids to animals over periods of several hours! The latter tends to negate the emphasis on absolute purity that many retinoid researchers insist on—as soon as one administers these compounds to the embryo, either the light

or the embryonic cells will change the nature of thesubstance you have given it. Nevertheless, for experimental purposes it is better to make up one solution and aliquot it as follows;

1. Prepare 1 mL of a 10 mg/mL solution of tRA (Sigma, Dorset, UK) in DMSO (Spectrosol or Analar-Grade, BDH, Poole, UK) or ethanol.
2. Split the 1 mL into 5-µL aliquots in capped tubes.
3. Freeze at −20°C.
4. Use one aliquot for each experiment, never refreeze and use again.

2.2. Preparation as a Solid

An alternative to administering retinoids as a solution is to administer them as neat powder in a depot. In this case, a rubber compound called Silastic 382 Medical Elastomer (Dow Corning Corporation, Midland, MI) acts as the depot. tRA is released from this compound at a constant rate over the first 24 h and then the release plateaus by which time about 65–70% of the total content has been released ***(26)***.

1. Measure 1 mL of Silastic, either as a volume from a syringe or by weight, into a small Petri dish.
2. Add the required amount of tRA, e.g., 10 mg to the Silastic.
3. Mix thoroughly for 10 min until an even consistency is obtained—easy to judge with a yellow compound like tRA.
4. Add one drop of catalyst, stannous octoate, mix, and leave to set for an hour at room temperature.
5. The silastic can now be cut up into any shape and size for administering to the embryo. This is performed down a dissecting microscope with a graticule on the stage plate. The required size is normally determined empirically by the dose or by the size of the embryo or embryonic field to be treated. The amount of retinoid administered can be calculated from the size of the block, e.g., a 200-µm cube has a volume of 8×10^6 μm^3. If the concentration of the mixture is 10 mg/mL = 10 mg/10^{12} μm^3 then this block will have $(8 \times 10^6)/10^{12} \times 10$ mg = 80 ng of RA in it.

2.3. Preparation on a Bead

This method was designed for adminstering RA to the anterior side of the chick limb bud ***(27,28)***.

1. Use AG 1-X2 chromatography beads, formate form, 100–200 mesh (Bio-Rad, Hemel Hempstead, UK).
2. Select a number of uniformly sized beads (approx 200 µm) in a small Petri dish and add an aliquot of tRA in DMSO at a concentration of, for example, 1 mg/mL. Leave for 20 min. The beads are very difficult to see once in this solution, and the angle of the light source has to be altered to be able to visualize them.

3. Rinse the beads by removing the RA solution and putting on a few drops of medium, e.g., MEM. The beads will take up phenol red from the medium and become perfectly visible.
4. Repeat the rinse.
5. Incubate the beads in medium for 20 min at 37°C. They are then ready for implantation.

3. Methods

3.1. Administration to Chick Embryos

Chicks have been treated with RA administered in solution to cultured embryos, with RA on beads, with RA in Silastic, or directly injected into the embryo.

3.1.1. Administration in Silastic

1. Cut a block of the appropriate size.
2. Window eggs at the appropriate stage.
3. Tear the vitelline membrane adjacent to the part of the embryo to be treated with a tungsten needle.
4. Place the block adjacent to the embryo.
5. Seal the egg and replace in the incubator.

This method is useful for early embryos, e.g., treating a stage 9/10 hindbrain where the embryo is relatively immobile, but not good for late embryos, which will turn and begin to move. In this case, the block is displaced and the experiment fails.

3.1.2. Administration on Beads

These can be placed over the embryo or adjacent to the embryo exactly as described in **Subheading 3.1.1.**, but beads are also ideal for inserting into the embryo and were originally used for inserting into the anterior margin of the chick limb bud *(27)*. In this case:

1. Window the eggs at the appropriate stage, e.g., stage 20.
2. Tear all the membranes above the limb bud. This is usually the right limb bud owing to the turning of the embryo, but not always.
3. With a tungsten needle, make a slit between the AER and the mesenchyme from the apex of the bud to the anterior margin.
4. Stretch the AER away from the mesenchyme to expand it, but do not break it.
5. With the AER pulled away with a needle, take a bead with a pair of fine forceps, place it into the gap between the AER and the mesenchyme, and release the AER. The bead will stay in place as the AER contracts.
6. Seal the eggs and replace in the incubator.

3.1.3. Administration of RA on Paper

An alternative to beads is to use the RA soaked onto paper *(29)*.

1. A piece of absorbent paper (blotting paper, newsprint, and so forth) is placed into a solution of RA in DMSO (*see* **Subheading 2.1.**) for 10 min and then washed in medium to remove excess RA.

2. Cut the paper into small pieces (500-μm squares).
3. Insert a square into a slit made in the anterior margin of the limb bud with a tungsten needle. The slit is made perpendicular to the base of the limb opposite intersomite 16/17.

3.1.4. Exposure of Embryos in New Culture to RA

Chick embryos in culture have been treated with RA in several instances ***(30,31)***. RA is dissolved in DMSO or ethanol and added to the culture medium at the required concentration for a particular period of time. At the end of that time, embryos are washed and fresh medium without RA is added.

3.1.5. Injection of RA into the Yolk

A further variation on the theme of administering retinoids to the chick embryo is to inject them directly into the yolk through the air sac. In this case the required amount of tRA dissolved in DMSO is taken up into a syringe and injected after making a hole in the egg at the end with the air sac with a 20-gage needle.

3.2. Administration to Amphibians

As in the case of chicks, various methods have been used to treat amphibians with retinoids and for two experimental purposes: first, to study early development, and second, to study limb development and regeneration. Obviously, these two types of experiments involve treatment at widely differing stages, and a large number of different species of amphibians have been used for limb regeneration studies.

3.2.1. Early Development

Treatment with retinoids at gastrulation stages results in the progressive loss of head structures ***(11)***. In this case, stage 10 embryos are treated by taking an aliquot of tRA in DMSO and simply adding it to the medium in which the embryos are cultured to produce a final concentration of 10^{-5} *M* or 10^{-6} *M*. A typical treatment time would be for 30 min, at the end of which the medium is changed.

Virtually all studies on early development of amphibians have used this technique because of its speed and simplicity. One experiment, however, involved the injection of RA suspended in corn oil ***(32)***. Stage 10–11 embryos had their fertilization envelope manually removed, and were injected with a 1-nL droplet between the surface and deep ectodermal layers by means of an air pressure injection system. The droplet also contained a fluorescent dye, DiI, so its presence could be traced throughout further development of the embryo. The additional unique feature of this system was that the effects of RA were asymmetric because only one side of the embryo was injected.

3.2.2. Limb Regeneration Studies

Adult newts, or axolotls, or *Pleurodeles* at various stages of larval growth are usually used for these studies. The limbs are amputated, and then the animals are treated with retinoids in various ways. One method of administration is simply to add 10–60 mg retinyl palmitate (Sigma, complexed with corn starch) to 1 L of water in which the animals are kept. The water turns cloudy because of the corn starch and should be changed every day. Treatment times vary from 1–14 d ***(22)***. It is also possible to treat animals in this way with tRA and other retinoids. In this case the appropriate amount of retinoid is used dissolved in DMSO (e.g., 300 µL of a 1 mg/mL solution of tRA), and this is put into the water. The retinoid immediately precipitates out, being insoluble in water, forming a yellow scum on the surface. Nevertheless, this method works perfectly well.

It is also common to administer retinoids by ip injection (e.g., ***33***). Animals are anesthetized and placed on their backs on a wet towel. A 27-gage needle is used to make a puncture through the skin and abdominal muscles into the ip cavity of each animal, a few millimeters anterior to one hindlimb and lateral to the midline. The volume of solution containing the desired dose to tRA is then injected into the abdomen with a Hamilton microsyringe using the previously made puncture hole. tRA percipitates at the injection site, being visible through the skin as a yellow mass, and gradually dissipates over the next 24–48 h. Animals are returned to their water as soon as possible. Control animals are injected with an equal amount of DMSO. This method tends to induce some mortality among the animals, since DMSO injected into the peritoneum is very unpleasant for the liver.

A very reliable and nontoxic method of administration is to use retinoids in Silastic as described above. Pieces of a uniform size of Silastic, e.g., 1 mm^3 containing tRA at the appropriate dose, e.g., 100 mg/mL, are prepared. Animals are anesthetized and a tunnel made in the limb under the skin by inserting the points of a pair of watchmaker forceps through the skin and pushing them along the limb between the skin and the underlying musculature. In this way, the piece of Silastic can be placed directly adjacent to the regeneration blastema on either the dorsal, ventral, anterior, or posterior side. Animals are returned to the water, and the Silastic remains in place for several weeks. With a colored compound, such as tRA, one can see the color gradually disappear from the Silastic. The diffusion characteristics of tRA from Silastic have been described *above* ***(26)***. Only very rarely are the blocks ejected from the limb. One example of this we have found is when the Siastic contains retinal ***(22)***. Uniquely, this retinoid induced the epidermis to eject the block.

Finally, gastric intubation has been used to administer retinoids to adult newts ***(34)***. In this case, the retinoid solution in DMSO is squirted into the stomach of the anesthetized animal through a thin plastic tube attached to a syringe.

3.3. Administration to Fish

Zebrafish have been used in studies on the effects of CNS development ***(35)***, heart development ***(36)***, eye development ***(37)***, and fin regeneration ***(38)***. In all these cases, tRA is administered to the animals in the tank water. A tRA in DMSO aliquot is used at an appropriate concentration to give a final concentration of 10^{-6} *M* or 10^{-7} *M* when diluted in, say, 5 mL of zebrafish water. Many embryos can be treated at once in a 5-mL Petri dish. For studies of CNS and heart development, embryos are treated for 1 h at 50% epiboly; for eye development for 4 h at 10.5 h of development, and for fin regeneration, adults are treated for 4 or 7 d.

3.4. Reporter Cells

Several stably transfected F-9 or L-cell lines have been generated that can be used as reporter cells for the presence of RA when embryonic tissue is placed on top of them. The line produced by Wagner, et al. ***(39)*** contains a retinoic acid-response element (RARE) from the human β-retinoic acid receptor gene, which functions as an inducible enhancer that responds to the α, β, and γ RAR subtypes. A single copy of this RARE was placed upstream of the *Escherichia coli lacZ* or firefly *luciferase* genes, conferring retinoid responsiveness to these genes. The *lacZ* gene is used for histochemical detection of retinoid responsive cells and quantitation by cell counting, and the *luciferase* gene is used to provide a direct quantitative assay. The inclusion of the NEO^r gene permits the establishment of transfected cell lines that stably maintain the reporter gene constructs.

3.4.1. Preparation of Media and Solutions

1. Medium: 10 mL 10X DMEM with NEAA without L-glutamine, sodium pyruvate, or $NaHCO_3$ (Gibco-BRL, Paisley, Scotland, UK, no. 12501-011): 7 mL 5.3% $NaHCO_3$, 1 mL penicillin/streptomycin, 58.4 mg L-gluamine (Sigma), 82 mL autoclaved milli-Q H_2O. Filter-sterilize and adjust pH to 7.4 with 800 µL 1 *N* NaOH.
2. Medium with serum: 15 mL medium (*see* **item 1**), 3 mL heat-inactivated fetal bovine serum, 2 mL heat-inactivated horse serum.
3. Growth medium: Medium with serum, add 0.8 mg/mL G418 (Geneticin G-418 sulphate; Gibco-BRL no. 11811-01E), filter-sterilize.
4. Coated dishes: Make fresh a 0.1% gelatin solution (Sigma) in PBS by gentle heating and shaking. Filter-sterilize while still warm. Completely cover the base of tissue-culture dishes. Leave for 2 h at room temperature, and then aspirate off and wash 1X with sterile PBS. Plate cells or cover with fresh PBS and store at 4°C (maximum 2 wk).
5. Serum-free medium: 10 mL media, 20 µL N3 (serum supplement—*see* **item 6**).
6. N3 serum supplement: 246 µL Hank's balanced salt solution (HBSS) without calcium and magnesium, 50µL 10 mg/mL bovine serum albumin in HBSS (store at 4°C), 100 µL 100 mg/mL human transferrin in HBSS (store at −20°C), 20 µL 80 mg/mL putrescine hydrochloride in HBSS (store at −20°C), 50 µL 10 m*M* sodium selenate in

HBSS (store at −20°C), 5 µL 20 mg/100 mL triodothyronine, sodium salt in 0.01 *M* NaOH (store at −20°C), 20 µL 25 mg/mL bovine pancreatic insulin in 20 m*M* HC1 (store in plastic at −20°C), 5 µL 12.5 mg progesterone in absolute alcohol (store in glass at −20°C), 1 µL 2 mg/mL corticosterone in absolute alcohol (store in glass at −20°C).

7. X-gal stain: 3.08 mL 0.5 *M* Na_2PO_4 0.46 mL 1 *M* NaH_2PO_4, 0.026 mL 1 *M* $MgCl_2$ 1.2 mL 50 m*M* $K_3Fe(CN)_6$ 1.2 mL 50 m*M* $K_4Fe(CN)_6$, 1.0 mL X-gal stock = a 2% solution of 5 bromo-4 chloro-3-indoxyl-β-D galactopyranoside (Novabiochem) made up in *N*-*N*-dimethylformamide (Sigma), 13 mL milli-Q H_2O.

3.4.2. Preparation of Cells from a Frozen Aliquot

1. Thaw the frozen cells in 20 mL of medium with serum to 37°C.
2. Plate in a gelatin-coated 80-cm^2 tissue-culture flask (Nuclon) with 20 mL of growth medium. The latter is added to enrich the plated culture in transfected F-9 cells; the transfected cells; are geneticin-resistant, but untransfected F-9 cells are not.
3. Grow the culture at 37°C 5%CO_2 for 3–5 d until just confluent.

3.4.3. Preparation of Cells for Explant Coculturing

1. For culturing to assay explants, cells are grown on gelatin-coated 35-mm diameter tissue-culture dishes.
2. Starting with a just confluent flask, wash the cells with PBS, and then loosen the cells by covering with with 5 mL trypsin/EDTA (Gibco-BRL no. 610-5300AG) (store at −20°C in 5-mL aliquots) for 3 min.
3. Triturate the cell suspension thoroughly with a plastic pipet to give a complete single cell suspension. F-9 cells are tough and will tolerate much up and down pipeting.
4. Transfer the cell suspension to a 15-mL tube and add 5 mL of medium with serum. Add 40–50 drops of this suspension to 20 mL of growth medium. Put 1 mL of this suspension into each of 20 35-mm dishes. This gives a cell concentration which 5% of which will give near confluence in 2 d when incubated at 37°C.

3.4.4. Explant Coculturing

The cell plating and the incubations of embryonic tissue must be synchronized to give confluent F-9 cells on the day embryonic tissue is ready for explanting. Superconfluent F-9 cultures should be avoided, since the cells pile up and this makes it more difficult to keep the explant attached to the monolayer of cells.

1. Dissect the tissue explants in a balanced salt solution. If the ectoderm is trypsinized off, for example, or any other enzymes used to separate tissues, it is very important to wash the tissue and neutralize the enzymes with medium with serum prior to explanting, since any remaining trypsin will destroy the monolayer.

2. Immediately before placing the explant on the cell monolayer, remove the growth medium from the F-9 cells and replace with 1 mL serum-free medium. Explants are always cultured in serum-free medium, since serum has retinoids in it.
3. Transfer the explants onto the cell monolayer with a pipet.
4. The dishes should be moved as little as possible to allow the explants to settle and attach to the cell monolayer. If attachment proves to be a problem, minimizing the medium over the cells when first introducing the explant can increase cell contact. After a hour or so, gently add more medium to these cultures to ensure that they do not become dry overnight.
5. Culture at 37°C, in a 5% CO_2 atmosphere overnight.
6. Wash the cultures twice very gently (in order not to disturb the F-9 cells) with room temperature PBS. Then fix for 5 min at 4°C with 2% paraformaldehyde, 0.2% glutaraldehyde in PBS.
7. Rinse gently twice more with PBS, then cover with 1 mL of X-gal stain/dish, and incubate at 37°C overnight.
8. Count the number of blue cells around the explant as a percentage of all cells.

Each time an experiment is performed with tissue explants, a series of control RA dilutions need to be run in parallel, which is why one needs about 20 dishes. A typical series would be 10^{-6}–10^{-11} *M* in steps of 10-fold dilutions. In the case of F-9 cells with the *lacZ* gene, the results are determined by taking a field of view down an inverted microscope and counting the number of blue cells as a percentage of the total cells. The plate is then moved to another field and the counts repeated. The result of such an experiment is shown in **Fig. 1A**. When the explants are counted, then an approximation of the amount of retinoid present can be made by comparing % blue cells with the control curve.

Several explants of the same tissue type can be placed in one Petri dish. Usually, the explants remain in place all through the β-gal staining process, but if they do not, that is rarely a problem because a ring of blue cells marks the place where they were and counts can still be performed. Counts are made of the percent blue cells within a fixed distance from the explant.

Obviously, 50 cell diameters away from the explant, none of the cells are blue, whereas the majority of those directly touching the explant will probably be blue, so a compromise in distance much be reached and adhered to for all explants. A typical result for chick limb buds cut into two halves is shown in **Fig. 1B**. By comparison with the standards in **Fig. 1A** it is possible to suggest that the posterior halves of chick limb buds contain in the region of 10^{-9} M RA and that the anterior halves contain considerably less. This result fits well with the original HPLC data of Thaller and Eichele ***(3)***.

Finally, it is important to remember that this assay does not measure tRA alone because several retinoids are equally efficient at activating the RARs, including didehydroretinoic acid and 9-cis. Therefore, what one measures here is the combined concentration of the retinoic acids.

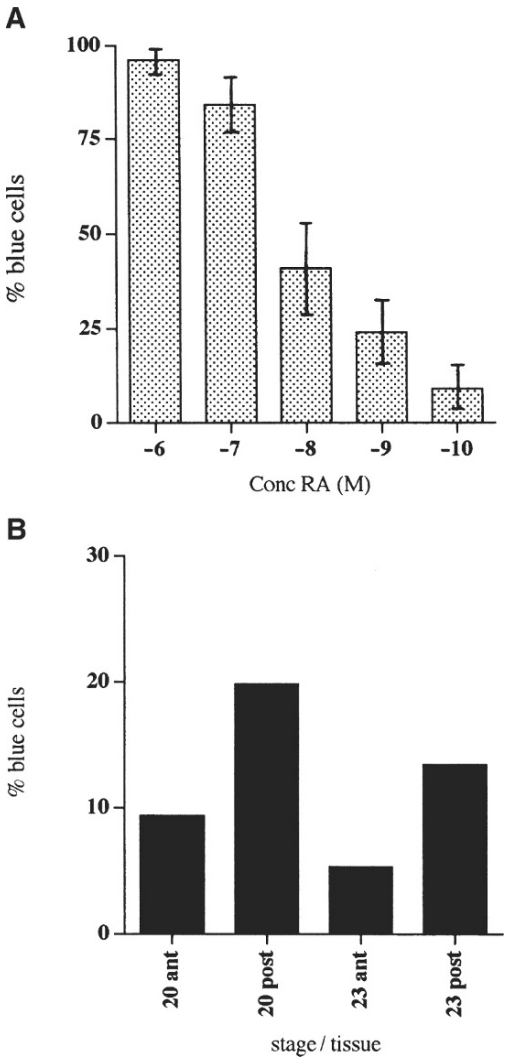


Fig. 1. **(A)** Calibration curve generated by counting the number of F-9 reporter cells that have turned blue after treatment with increasing concentrations of tRA. A straight line is usually obtained. From these data, an approximation of the concentration of RA present in explanted tissues can be made from the % blue cells recorded in the explant dishes. Bars mark standard deviations. **(B)** Example of data obtained after explanting anterior (ant) and posterior (post) half chick limb buds at two different stages of development, stage 20 and stage 23. It is clear that posterior halves contain more RA than anterior halves. A value of 20% blue cells from stage 20 posterior halves gives a concentration of close to 10^{-9} *M* RA from the standard curve in A.

3.5. High Pressure Liquid Chromatography (HPLC)

The use of HPLC to identify precisely individual retinoids is a methodology that is subject to infinite variability (*see*, for example, **ref. *40***). The variables include normal-phase or reverse-phase, type of column, composition of buffers, mix of buffers, single-step or multistep elution, step or gradient elution, and rates of flow. The idea is to use a system such that each of the retinoids one is interested in will come off at a different and highly repeatable elution time. Reverse-phase is generally preferred over normal-phase chromatography, the latter suffering from difficulty in reproducing precise elution times. Therefore, which method should one use?

The method that was used to identify tRA in the chick limb bud by Thaller and Eichele ***(3)*** is described below with only minor modifications, since it has been successfully used by several groups, including ourselves, to identify retinoids in the mouse embryo ***(5)***. An example of this type of chromatography is given in **Fig. 2. Figure 2A** shows the separation of a mixture of standards and **Fig. 2B**, the retinoids present in a mouse embryo. However, this method was developed before the discovery of 9-cis-RA, and it fails to separate this isomer because 9-cis coelutes with retinol, so this is by no means a perfect method. To identify 9-cis, we have developed a method ***(18)*** that involves first running the sample on a normal-phase system with an NH_2 column that retains only the acids and then running the acid fractions on a reverse-phase system as described below.

Because there is no contaminating retinol left, all the tRA isomers can be detected, but one does not obtain any quantitation for retinol by this method. However, Kraft et al. ***(12)*** describe a method involving a gradient elution of a mixture of ammonium acetate and methanol whereby 9-cis *is* identifiable as a separate peak.

Most embryological interest has been centered on the retinoic acids and retinol, but there are other retinoids of biological interest, such as the retinyl esters, that these methods do not identify, because they come off the column after very long elution times. Another method that was designed to separate 14 different retinoids, including those of greatest interest, such as 4-oxo-RA, tRA, and retinol, as well as retinyl acetate, palmitate, and stearate, was described by Cullum and Zile ***(41)***. This involves a multistep, three solvent (water, methanol, chloroform) gradient system eluting for 50 min. Thus, the choice of method is dependent on which retinoids one wants to identify.

3.5.1. Extraction Procedure

Ideally this should be done in red or gold light, but at the very least it should be done in subdued lighting (turn the lab lights off) and in lightproof (brown) microcentrifuge vials. All chemicals should be of the highest quality.

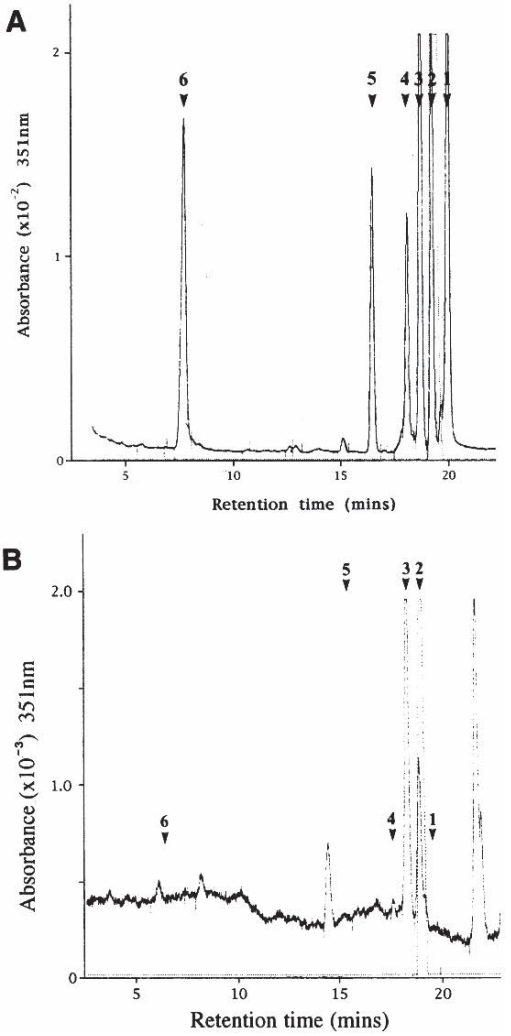


Fig. 2. Examples of HPLC chromatograms using the methods described in the text. Solid lines show the UV absorbance at 351 nm, and dotted lines the cpm measured with an on-line radioactivity detector. **(A)** A mixture of six retinoid standards separated out into individual peaks with different elusion times. Peak 1 = retinal, peak 2 = tRA, peak 3 = retinol, peak 4 = 13-cis-RA, peak 5 = didehydroretinol, peak 6 = 4-oxo-RA. The dotted line marks the cpm of [^{3}H] tRA, which was also added to the mixture and this coeluted with the cold tRA. **(B)** The retinoid extracted from a whole 10.5-d mouse embryo. The same six peaks of known standards are marked. Only retinol (peak 3) and tRA (peak 4) are clearly identifiable. Peak 2 coelutes with authentic [^{3}H] tRA, suggesting that the mouse embryo contains significant quantities of tRA and a good deal of retinol. The peak on the extreme right of the chromatogram is an unknown, and the peak to the left of arrow 5 is butylated hydroxy-toluene, the antioxidant added to the extraction solvent.

1. Collect batches of embryonic tissue on ice. Not <500 mg can usually be used. They can be stored for short periods at −70°C to allow the collection of enough material.
2. Add an equal volume of ice-cold stabilizing buffer and sonicate. Stabilizing buffer = 5 mg/mL ascorbic acid + 5 mg/mL Na_3 EDTA dissolved in PBS, with pH adjusted to 7.3 with NaOH.
3. Take 10 µL of homogenate for protein estimation, so that at the end the amount of retinoid can be given in ng/mg protein. Alternatively, use this sample for DNA determination, and express the data in ng/µg of DNA.
4. Add a known amount, 1–2 nC, of [^{3}H] tRA, so that the recovery ratio can be determined and the tRA peak identified on the chromatograph.
5. Extract in 2 vol of extraction solvent. Extraction solvent = ethyl acetate:methyl acetate 8:1 + 50 µg/mL butylated hydroxytoluene as an antioxidant. Extract for 20 min while continuously mixing on a vibromax.
6. Separate the solvent phase by microcentrifugation at low speed. Keep the solvent phase.
7. Repeat the extraction on the homogenate, and separate again by centrifugation. Remove the solvent phase, and pool it with that from the first extraction.
8. Dry down the combined solvent phases under a stream of nitrogen.
9. Resuspend the extract in 100 µL methanol, and microcentrifuge at high speed to remove the particulate matter. This is now ready to be injected onto the HPLC column.

3.5.2. Chromatography

The ideal HPLC hardware is one that measures both the UV absorbance of the eluate and the radioactivity with an on-line isotope detector. The two sets of data are then superimposed by the computer to allow for the identification of individual peaks (**Fig. 2**). Chemicals should be HPLC grade and buffers degassed before use.

1. Use a 5-µm encapped C_{18} column (Lichrospher, Merck, Darmstadt, Germany) with an equivalent precolumn. The precolumn prevents any particulate matter, such as phospholipids, from entering the column and is changed at regular intervals.
2. Inject the sample onto the column.
3. Elute at 1 mL/min using the following mobile phases. Solvent A, 1% acetic acid in MilliQ water. Solvent B, acetonitrile/methanol 3:1. The initial conditions are 60% solvent B rising linearly to 100% solvent B over 25 min.
4. Monitor the eluent at 351 nm with a UV detector and the radioactivity with an isotope detector.

3.5.3. Quantitation and Identification of Peaks

The radioisotope detector will give a readout of the cpm of the tRA, which was added to the initial tissue prior to extraction. By knowing how much was added, one can thus work out a recovery ratio that will allow a correction factor to be introduced when working out the endogenous levels of retinoids. Typical

values are in the 60–80% range. The UV detector will give a readout of the peak areas and the amount in nanograms for each peak it has recorded. It can do this, because when setting up the machine, one establishes a calibration curve for each retinoid, which the machine uses to give a value in nanograms for each of the UV peaks.

The identification of individual peaks can be done in several ways. First, an indication is given simply by comparing the elusion time of each peak with the known elution time of each standard. This one has done when setting up the machine to establish calibration curves as described above. Thus, in **Fig. 2A**, the elution profile of a set of retinoid standards is shown. The elution time of peak 2 (IRA) is 19.25 min. In **Fig. 2B,** the retinoids extracted from a 10.5-d mouse embryo is shown, and in this chromatograph, a peak is present at exactly the same elution time (peak 2) suggesting that tRA is present in mouse embryos. By comparing the standards with the mouse embryo extract, it can be seen seen that some peaks are present (2 and 3) and the others are absent. Second, a further identification is provided by coelution of the radioactivity in the spiked sample with an endogenous UV peak (**Fig. 2B**). This is shown here for tRA, but can clearly be done for all the other retinoids of interest, or a cocktail of radioactive retinoids can be added to the sample. Third, the sample can be spilt into two, and a spike of one cold retinoid, e.g., tRA is added to one of the samples. They are then run on the HPLC, one after the other, and in the spiked case, only one peak should increase in height, i.e., the tRA one. Fourth, the individual peaks can be collected in a fraction collector and then peaks of interest can be rechromatographed on a different system. For example, if this was done with the retinol peak obtained from the reverse-phase chromatograph in **Fig. 2B** (peak 3) and the sample was then run on a normal-phase column, this would reveal whether there really were two peaks here (retinol and 9-cis), since the elution times of each retinoid changes on a different chromatography system. Fifth, the peaks can be collected and then derivatized. This involves methylating the material in the peak, e.g., the suspected tRA and then running it on a normal-phase system to confirm that it now coeluted with authentic tRA methyl ester or performing gas chromatography/mass spectorscopy to the same end ***(3)***.

Amounts as low as 1 ng of tRA can readily be detected by the method described above. However, it is to be emphasized that the figures one generates after quantitation of retinoids in tissue are only estimates. In addition to the corrections described above (recovery ratio, comparison with calibration curves), there is the further problem of UV detection. The suggested wave-length of 351 nm is an average value for several retinoids. In fact, each retinoid has a different absorption maximum, e.g., all-trans-retinol = 325 nm; all-trans-retinal = 383 nm; tRA = 350 nm; 9-cis- RA = 345 nm; ddRA = 370 nm; 4-oxo-RA = 360

nm. So were the wavelength to be set differently then a different value for the quantitation of each retinoid would be obtained. The use of a scanning wavelength detector would solve this problem, but such detectors are an order of magnitue less sensitive than fixed-wavelength detectors. Nevertheless, within these limitations, good results have been obtained, particularly where differences in retinoid content between different parts of the embryo have been identified.

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38

Protochordates

Peter W. H. Holland and Hiroshi Wada

1. Introduction

The protochordates (amphioxus and tunicates) occupy a pivotal position in chordate phylogeny, being the closest living invertebrates to the vertebrates. In spite of their evolutionary significance, these animals do not feature commonly in modern developmental biology research. This has not always been the case; indeed, amphioxus ranked as one of the principal animals for embryological description in the early part of this century. The ascidia (one group of tunicates) have received intensive study as a model for determinative development, and considerable experimental and molecular data have been accumulated over the past few decades ***(1)***.

The realization that many genes playing key roles in early development have been widely conserved in animal evolution has helped bring protochordates back toward the mainstream of developmental biology research. The existence of homologous control genes in divergent species is a starting point for investigating evolutionary changes in developmental control; ascidia and amphioxus are a natural choice for inclusion in such studies, since they occupy such important phylogenetic positions.

Here we give protocols for obtaining embryos and larvae of one ascidian species, *Ciona intestinalis*, and one amphioxus species, *Branchiostoma floridae.*

2. Materials

2.1. Spawning Ascidia

1. Fine scissors and watchmakers forceps.
2. Dechorionating solution: 1% sodium thioglycolate, 0.05% protease type I (Sigma) in filtered seawater; adjust to pH 10.0–11.0 using NaOH.
3. Plastic dishes coated with 1% agar (dissolved in filtered seawater).

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2.2. Spawning Amphioxus

1. Strong shovels with 1–2 m handles.
2. 40-cm Sieves with 1 mm mesh size.
3. Electrical stimulator (e.g., Grass) fitted with platinum electrodes.

3. Methods

3.1. In Vitro Fertilization of Ciona intestinalis (Ascidia)

Many species of ascidian have been studied by developmental biologists; here we cover only *C. intestinalis.* This species is cosmopolitan and common; in the United Kingdom it is abundant in the vicinity of several major marine biology laboratories, including Plymouth, Southampton, and Millport. As with many other ascidian species, if adults are kept in constant illumination, they can be induced to spawn by moving them to the dark ***(2)***; animals can also be kept in the dark, and spawned by moving to the light. An alternative procedure to obtain embryos involves *in vitro* fertilization using gametes obtained by dissecting gonoducts. This method is convenient, since embryos can be obtained immediately after collection of animals. They can be spawned throughout the year or throughout summer in northern Europe.

1. Adults should be kept in seawater aquaria (12–18°C or temperature at collection site), in constant dark or constant illumination to prevent spontaneous spawning.
2. Select several mature adults; these can be recognized by the white sperm duct visible through the body wall. The yellow or brown oviduct is also sometimes visible.
3. Using sharp scissors, cut the test and body wall open longitudinally. The white sperm duct is usually obvious in mature animals; the oviduct, which lies parallel, is sometimes less clear. A dissecting microscope is sometimes required to see eggs within the oviduct.
4. Carefully cut through the oviduct, and squeeze eggs out using fine forceps. Transfer eggs to a Petri dish using a Pasteur pipet. Only use eggs from the oviduct; do not dissect from the ovary.
5. Now cut through the sperm duct and squeeze out sperm in a similar manner. Transfer sperm to a separate Petri dish, before it becomes dilute.
6. Repeat **steps 3–5** for at least one further animal.
7. Add a drop of sperm from one animal to several hundred eggs collected from another. After 10 min, wash away excess sperm with several changes of filtered seawater.
8. At 16°C, first cleavage occurs after 1 h, gastrulation at 5 h, and hatching after about 24 h. *Ciona* has a nonfeeding tadpole larva that swims for 12–24 h before settling and metamorphosis.
9. If prehatching stages are required, e.g., for *in situ* hybridization to embryos, the chorion must be removed before fixation. This membrane surrounding the egg is associated with large follicle cells projecting from its outer surface and test cells on its inner surface. The chorion and associated cells can be removed manually using sharp tungsten needles; however, this is difficult and time-consuming owing to the small perivitelline space.

10. An alternative method is to place fertilized eggs, prior to first cleavage, into dechorionating solution. Observe under a dissecting microscope until most eggs are dechorionated (10–20 min), and then gently wash with several changes of filtered seawater. This procedure can also be performed prior to fertilization.
11. After dechorionation, embryos must be cultured in agar-coated Petri dishes.

3.2. In Vitro Fertilization of B. floridae *(Amphioxus)*

Several species of amphioxus can be found in temperate and tropical seas; the protocol given is designed for *B. floridae*. These can be collected in large numbers from Old Tampa Bay, Florida, or other sandy bays around the Gulf of Mexico. They can only be spawned between July and September; even then, successful spawning is obtained only once every 10–14 d.

1. Locate a suitable population of adult amphioxus, by sieving sand from subtidal regions. Sites around Old Tampa Bay include south of the Courtney Campbell Causeway and St. Petersburg beach—in each case, in around 1 m of water.
2. After 4 PM, collect several hundred amphioxus by digging sand and sieving. Transfer animals to clean seawater for transport and keep in the shade. Collect seawater from the same site.
3. Transfer ripe animals into beakers containing 50 mL seawater (up to 5 animals/beaker). Keep males and females separate; the serially repeated gonads are white in ripe males, and yellow in females. Keep in the light at 25°C.
4. Attempts to collect eggs and sperm should be made between 9 PM and 1 AM. Either place beakers of animals in the dark, or (more reliably) use a stimulator to give a brief (2-s) nonlethal shock of direct current (50 V in 10-ms pulses) to a beaker of animals.
5. If gametes are released from the gonopore, they should be transferred immediately to Petri dishes (eggs) or microfuge tubes (sperm) using clean Pasteur pipets. Use separate pipets for eggs and sperm, and keep sperm as concentrated as possible. Not every animal will spawn, even on a successful spawning night.
6. If no gametes are obtained within 30 min after electrical stimulation of 50–100 animals, it is likely that spawning cannot be induced on that night. In this case, return animals to the collecting site on the next day, and repeat **steps 1–5.**
7. *In vitro* fertilization should be set up with freshly obtained eggs and sperm. Add one drop of sperm suspension to several hundred eggs in a 9-cm Petri dish of seawater filtered through Whatman No. 1 paper. After 5 min, observation under a dissecting microscope should reveal the presence of an elevated membrane around each fertilized egg.

   Change the water twice to flush away excess sperm.
8. At 25°C, first cleavage occurs after 45 min, second cleavage after 75 min, and gastrulation after 5 h.
9. Hatching from the fertilization membrane occurs at 10–12 h; at this stage, the embryo is a bean-shaped neurula actively swimming using epidermal cilia. Pour hatched cultures into 50-mL centrifuge tubes and direct an angle-poise lamp at the water surface for 20 min to attract swimming neurula away from debris. Transfer

active neurulae from the top 10 mL water to fresh Petri dishes, using a P1000 Gilson pipetman (Gilson Co., Worthington, OH) fitted with a cut-off disposable tip.
10. Embryos are readily raised from 12 h (5 somite stage neurula) to 60 h in Petri dishes of filtered seawater, with little attention.
11. At 36 h, the larval mouth opens; feeding commences around 60 h. Beyond this stage, culture is considerably more difficult; **ref. *2*** should be consulted.
12. If embryos are to be fixed, e.g., for *in situ* hybridization, the embryo cultures may need to be concentrated prior to fixation. Pour cultures into 15-mL centrifuge tubes, spin at low speed (2000*g*) for 5 min, and dispose of all except the bottom 1 mL of water. The embryos in 1 mL seawater are then transferred to a microfuge tube and concentrated further by centrifugation at 6000*g*.

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Subtractive Hybridization and Construction of cDNA Libraries

Bruce Blumberg and Juan Carlos Izpisúa Belmonte

1. Introduction

Genes that are differentially expressed both in time and space are the basis for how single cells, through the process of embryonic development, give rise to animals with an extraordinary diversity of cell types. As a first step in understanding differential gene expression, many researchers seek to identify those genes whose transcripts are temporally or spatially restricted to particular cells, tissues, or embryonic stages. Although there are a variety of methods suitable for identifying moderately to highly expressed genes, the isolation of the most interesting class of mRNAs, those that are not abundant, but that may be cell-or tissue-specific, remains the most difficult task.

Several basic types of methods have been employed to identify low-abundance, tissue-specific transcripts. The more classical differential hybridization techniques (e.g., ***1***) are mostly limited to the detection of moderately abundant transcripts representing >0.05% of the mRNA population ***(2)***. Subtractive hybridization techniques can increase the detection sensitivity by 10- to 100-fold and make the identification of quite rare genes possible ***(2)***. A specialized form of subtractive hybridization, the "Gene Expression Screen" ***(3)***, can detect both upregulated and downregulated transcripts. A number of protocols have been devised in recent years to simplify and expedite the process of transcript identification by subtractive hybridization ***(4–8)***. Here we present a comprehensive set of methods that have proven quite successful in our laboratories and that may serve as an entry point for future refinement.

In the following protocol, we utilize the commonly available and widely used phage vector λZAPII, since one can produce oriented libraries in phage or phagemids, and subsequently utilize the libraries to produce essentially unlimited

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quantities of sense or antisense RNAs for subtraction and screening. Furthermore, Stratagene (San Diego, CA) and other suppliers provide a number of high-quality, premade cDNA libraries that can save considerable time if one happens to exist for the tissue of interest. Recent advances in vector technology and hybridization allow one to produce subtracted probes and libraries even when starting material is quite limited.

2. Materials

2.1. cDNA Synthesis

1. 10X First-strand buffer: 0.5 *M* Tris-HCl, pH 8.9 (this will be 8.3 at 42°C), 0.1 *M* $MgCl_2$, 0.2 *M* KCl, 50 m*M* dithiothreitol (DTT).
2. 10X Second-strand buffer: 0.2 *M* Tris-HCl, pH 7.5, 0.05 *M* $MgCl_2$, 0.1 *M* $(NH_4)_2SO_4$, 1.0 *M* KCl.
3. 10X *Eco*RI methylase buffer: 1.0 *M* Tris-HCl, pH 8.0, 1.0 *M* NaCl, 0.01 *M* EDTA. Make as a stock solution and then add S-adenosyl methionine to 0.8 m*M* to a small aliquot of this stock before use.
4. 10X T4 polynucleotide kinase buffer: 0.5 *M* Tris-HCl, pH 8.0, 0.1 *M* $MgCl_2$, 50 m*M* DTT.
5. 10X T4 ligase buffer: 0.3 *M* Tris-HCl, pH 7.4, 0.04 *M* $MgCl_2$, 0.1 *M* DTT, 2 m*M* ATP. Make ~10 mL of this buffer and store in 100-µL aliquots at −20°C. Repeated freeze-thaw cycles rapidly deplete the buffer of both DTT and ATP.
6. dNTP mix with 5-methyl-dCTP: 20 m*M* dATP, 20 m*M* 5-methyl-dCTP, 20 m*M* dGTP, 20 m*M* dTTP.
7. dNTP mix: 20 m*M* dATP, 20 m*M* dCTP, 20 m*M* dGTP, 20 m*M* dTTP.
8. 10X *Eco*RI buffer—supplied by the manufacturer: 0.5 *M* Tris-HCl, pH 7.5, 0.1 *M* $MgCl_2$, 1.0 *M* NaCl, 10 m*M* DTT.
9. Column buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 0.15 *M* NaCl.
10. TBE for electrophoresis (10X): 0.89 *M* Tris base (Boehringer Mannheim [BMB], Indianapolis, IN), 0.89 *M* boric acid, 0.02 *M* EDTA. Filter and autoclave (prevents precipitate from forming with time).

2.2. Enzymes for cDNA Library Construction

1. AMV reverse transcriptase (Seikagaku America #120248-1 [St. Petersburg, FL]).
2. DNA polymerase I, endonuclease-free (BMB # 642-711).
3. RNase H (Pharmacia #27-0894-02 [Milwaukee, WI]).
4. T4 DNA polymerase (Pharmacia #27-0718-02).
5. T4 polynucleotide kinase (Pharmacia #27-0734-01).
6. T4 DNA ligase (Pharmacia #27-0870-01).
7. T4 RNA ligase (Pharmacia #27-0883-01).
8. *Escherichia coli* DNA ligase (BMB #862 509).
9. *Eco*RI methylase (New England Biolabs, NEB #211S [Tozier, MA], S-adenosyl methionine is supplied with the enzyme.
10. Human placental ribonuclease inhibitor (Pharmacia RNA guard #27-0815-01).

11. *Eco*RI high concentration (BMB #200-310).
12. *Xho*I—high concentration (BMB #703-788).

2.3. Vectors and Packaging Extracts

1. UniZAP-XR—ZAPII, digested with *Xho*I and *Eco*RI and dephosphorylated (Stratagene #237211).
2. *E. coli* (ER-1647 NEB #401-N).
3. Gigapack II Gold packaging extract (Stratagene #200216).

2.4. Miscellaneous Reagents and Supplies for cDNA Synthesis

1. A primer consisting of ~15 Ts followed by at least an *Xho*I site and preferably several other rare sites. The one we use is 5′ (ACTAGTGCGGCCGCCTAGGCCTCGAGTTTTTTTTTTTTTT)3′.

   This has the following restriction sites (in 5′ -> 3′ order): *Spe*I, *Not*I, *Eag*I, *Sfi*I, *Avr*II, *Stu*I, *Xho*I.

2. *Eco*RI linkers—octamer (GGAATTCC) (Pharmacia 5′-OH, #27-7726-01, 5′-PO_4, #27-7428-01).
3. 5-methyl-dCTP (Pharmacia #27-4225-01). Store as a 100-m*M* stock.
4. dNTPs (Pharmacia #27-2035-01). 100 m*M* solutions of each.
5. 10 m*M* ATP (Pharmacia #27-2056-01) (dilute from 100 mM stock).
6. 0.1 *M* CH_3HgOH (ALFA products #89691).
7. 5.8 *M* 2-mercaptoethanol (BME; Sigma M6250 [St. Louis, MO]).
8. 100% Ethanol (Rossvile Gold Shield or equivalent).
9. α[^{32}P]-dATP 800 Ci/m*M* (New England Nuclear, Wilmington, DE, #NEG-012A).
10. γ[^{32}P]ATP 6000 Ci/m*M* (New England Nuclear #NEG-002Z).
11. 1-kb Ladder (BRL #5615SA).
12. Agarose (Pharmacia #17-0554-02 or Bio-Rad, Hercules, CA, #162-0126).
13. 40% acrylamide (19:1 acrylamide:bisacrylamide) acrylamide (Bio-Rad #161-0101), bis-acrylamide (Bio-Rad #161-0201).
14. β-NAD (BMB #775-7). The stock solution is 0.045 *M* in H_2O.
15. Sepharose Cl-4B (Pharmacia #17-0150-01) equilibrated in column buffer.
16. Tris-base (BMB #604-205).
17. X-gal (BMB #745-710).
18. IPTG (BMB #724-815).
19. Ultrapure, recrystallized phenol (BMB #100-300).
20. DTT (BMB #100-032).
21. Sodium dodecyl sulfate (SDS) (BMB #100-155).
22. Guanidine HCl (optional) (BMB #100-173 or BRL #5502UA).
23. Guanidine thiocyanate (BMB #100-175 or BRL #5535UA).
24. Ultrapure urea (BMB #100-164).
25. LiCl (Sigma L-0505).
26. Sephadex G-50 spun columns, equilibrated in TE Sephadex G-50 medium (Pharmacia #17-0043-01), or Sephadex G-50 spun columns (Pharmacia #17-0855-01).

27. LB media and plates.
28. Minimal media and plates (use maltose as carbon source).
29. 20% Maltose (Difco).
30. All other chemicals and reagents should be at least ACS-reagent grade. You cannot go wrong by buying small quantities of ultrapure chemicals (e.g., from Aldrich) and reserving them for making cDNA buffers and solutions.

2.5. Reagents for Biotinylation and Subtraction

1. Photobiotin acetate (Clontech K1012-1 [Palo Alto, CA]).
2. Reflector sunlamp (Clontech 1131-3).
3. Biotin-21-UTP (Clontech 5024-1).
4. Streptavidin (BRL Life Technologies #15532-013 [Gaithersburg, MD]).

2.6. Reagents for Isolation of Total RNA

1. Guanidine thiocyanate solution (GuSCN): 4.0 *M* guanidine thiocyanate (BMB #100-175), 0.01 *M* Na-acetate, pH 7.0, 0.1 *M* 2-mercaptoethanol (Sigma), 0.1% (w/v) *n*-lauryl sarcosine (Sigma), 0.5% (v/v) antifoam C (Sigma).
2. CsCl-EDTA cushion: 6.0 *M* CsCl (BMB), 0.1 *M* EDTA, pH 7.0.
3. Phenol:chloroform:isoamyl alcohol: 25 parts ultrapure phenol (BMB #100-300), 24 parts chloroform (Fisher ACS grade, Fisher Scientific, Pittsburgh, PA), 1 part isoamyl alcohol (Fisher ACS grade). Prepared as described in **ref. *9***.
4. Chloroform:isoamyl alcohol: 24 parts chloroform, 1 part isoamyl alcohol, store at room temperature in a dark bottle.
5. Ethanol-sodium acetate: 0.04 *M* Na-acetate, 60% v/v ethanol, prepare by mixing 3 parts of 80% ethanol with 1 part of 0.15 *M* Na-acetate, pH 7.0.
6. Ammonium acetate for precipitations: 7.5 *M* NH4-acetate pH 7.0—treat with 0.2% DEPC and autoclave. Store at −20°C.

2.7. Reagents for Poly A$^+$ Selection

1. 2X Loading buffer: 40 m*M* Tris-HCl, pH 7.6, 1 *M* NaCl, 2 m*M* EDTA, 0.2% SDS.
2. 0.1 *N* NaOH.
3. Loading buffer (low-salt): 40 m*M* Tris-HCl, pH 7.6, 0.1 *M* NaCl, 2 m*M* EDTA, 0.2% SDS.
4. Elution buffer: 10 m*M* Tris-HCI, pH 7.5, 1 m*M* EDTA, 0.05% SDS.
5. 3 *M* sodium acetate, pH 5.2.
6. 100% Ethanol (reserved for RNA use).
7. 70% Ethanol (reserved for RNA use).
8. Oligo (dT) cellulose, Type 2 (Collaborative Research, Los Altos, CA, cat. # 20002).
9. Quik-Sep columns (Isolab, Akron, OH, cat. # QS-Q).

2.8. Reagents for In Vitro Transcription (Optional)

1. Megascript T7 kit (Ambion, Austin, TX, #1334).
2. Megascript T3 kit (Ambion #1338).

3. Methods

Whether to produce subtracted cDNA libraries or screen standard libraries with subtracted probes is the first consideration one must address. We advocate the construction of representative cDNA libraries that can later be screened with any desired probe. Indeed, such libraries can also be used to produce either synthetic driver or target mRNAs in large quantities. This approach has the advantage that only one or two libraries must be constructed for each target/driver pair, and any type of probe can be used as may later be required. It has the disadvantage that more clones must be screened to ensure the representation of the rarest mRNAs.

The choice of subtraction protocol to be followed depends on the availability of mRNA from both target and driver cells or tissues. If the driver mRNA is not limiting (50 µg available) then one can begin with **Subheading 3.7**., photobiotinylation of driver mRNA, otherwise one should first construct a library which may then be used to generate driver RNA. Similarly, the abundance of target mRNA and the desire to produce a representative or subtracted library will dictate whether the target will be oligodT-primed first strand cDNA or random primed cDNA produced after *in vitro* transcription of the library (**Fig. 1**).

3.1. Preparation of Total RNA (Based on Ref. 10*)*

1. There are a number of quite good procedures for preparing high-quality RNA. In addition, several commercial kits are available for this purpose that work reasonably well, but are rather expensive. The method appropriate for your cells or tissue depends largely on the amount of endogenous RNases present. The following method is somewhat tedious but always gives high-quality RNA.
2. Homogenization—the tissue is homogenized in 5 vol of ice-cold GuSCN solution on ice with two 30-s bursts of the polytron at maximum speed. Be sure that the tissue is completely dispersed. The size of polytron generator used depends on the quantity of tissue, but for most cases, the 1-cm type is adequate. For hard tissues, such as bone, a generator with blades should be used. Cultured cells are first trypsinized, rinsed with PBS, and resuspended in a minimum volume of PBS. Each 1 mL of densely suspended cells is homogenized in 10 mL of ice-cold GuSCN solution on ice as above. It is convenient to use sterile, disposable 50-mL polypropylene tubes for both homogenization and extractions.
3. Extract the homogenate with phenol:chloroform once and centrifuge to separate the layers for 5 min at 3000*g*. The upper, aqueous layer will appear somewhat milky, and is removed to a fresh tube. If you just use phenol instead of phenol chloroform, there will be no phase separation.
4. Extract the aqueous layer once with an equal volume of chloroform:isoamyl alcohol and centrifuge as above.
5. Centrifugation—the homogenate is layered onto 2.2-mL cushions of CsCl-EDTA solution prepared in SW-41 tubes. Centrifugation is for 18 h at 28,000 rpm at 20°C in an SW-41 rotor.

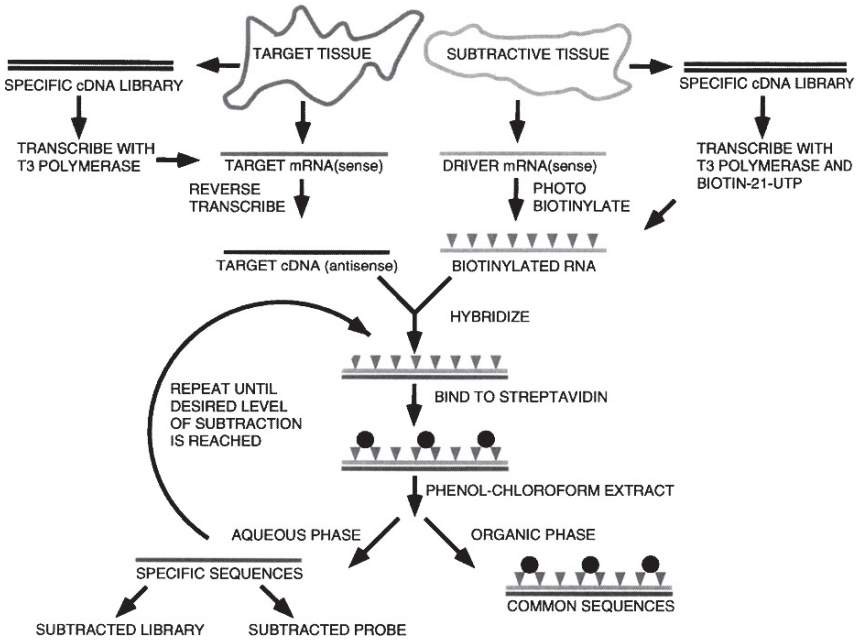

Fig. 1. Schematic view of the steps in constructing a subtracted cDNA library or subtracted probe. (*See* color plate 1 appearing after p. 368.)

If an SW-28 or SW-27 rotor is used, then the centrifugation speed should be decreased to 22,000 rpm. If other rotors are used, the volume of the cushion should be one-fifth of the total tube volume. Be sure to check the rotor manual to determine the maximum rotor speed with concentrated CsCl solutions.

6. Collection of the RNA—the tubes are removed from the centrifuge and placed in a rack.
7. The GuSCN layer is aspirated down to and including 0.5 mL of the cushion. The tube is carefully filled with DEPC containing H_2O, allowed to stand 2–5 min, and then the water is aspirated. This is repeated twice.
8. After the final rinse, the tube is quickly inverted and allowed to drain. The bottom 2 cm of the tube are cut off with a fresh scalpel, and the tube placed upright in a rack. The RNA appears as a glistening button in the center of the tube. Small amounts of RNA may not be visible. The pellet is carefully rinsed with 0.5 mL of DEPC-treated H_2O, and the tube inverted to dry.
9. The pellet is then macerated with, and taken up into a tip containing 100 µL of DEPC-treated H_2O (or an appropriate amount for the RNA yield you end up with). The RNA is pipeted up and down and transferred to an Eppendorf tube. Be sure you transfer all the RNA. The RNA is then heated at 70°C for 2–30 min until dissolved. Large amounts of RNA need longer times and require more H_2O. The

insoluble debris is removed by brief centrifugation, and the supernatant removed to a fresh Eppendorf tube.

10. Add 0.5 vol of NH_4-acetate and 2 vol (i.e., 2X the volume of the RNA + acetate solution) of absolute ethanol. Precipitate for 15 min at −70°C or several hours to overnight at −20°C (preferred method). Centrifuge for 15–30 min at 4°C, and then drain the supernatant. Rinse the pellet three times with the ethanol-sodium acetate solution, centrifuging briefly between rinses.
11. Rinse the pellet once with 80% ethanol, once with 100% ethanol, and air-dry. Resuspend the RNA in an appropriate volume of H_2O, and store at −70°C.

3.2. Evaluation of RNA Quality

1. Quantitate the RNA by spectrophotometry. $OD_{260:280}$ ratios should be 1.8 or greater, although tissues containing significant quantities of proteoglycans may give lower ratios. If the ratio is too low, phenol-extract, chloroform-extract, and ethanol-precipitate the RNA one or more times.
2. Evaluate the integrity of the RNA by gel electrophoresis, either denaturing or nondenaturing.
3. Nondenaturing agarose gels: The simplest method is to run a 0.8% agarose gel containing 100 µg/mL ethidium bromide (EtBr), using TBE as the running buffer, just as for a normal DNA gel.
4. Denaturing gel—use a 0.8% formaldehyde-agarose gel run in MOPS-acetate buffer as described in **ref.** ***(9).*** Stain with ethidium bromide for 30 , and view under UV.
5. The 28S and 18S rRNA bands should be sharp and in a 2:1 ratio. Some organisms, e.g., *Drosophila melanogaster* and *Alligator mississipiensis*, have 28S rRNAs, which are nicked and migrate with the 18S bands in denaturing gels. Use nondenaturing gels with these organisms and if you do not see the 28S band in denaturing gels.
6. Storage of RNA—store at −70°C in H_2O or as an ethanol precipitate at −20°C (preferred).

3.3. Preparation of Poly A^+ RNA

1. Once the isolation of total RNA is complete, isolation of mRNA can be performed by affinity chromatography on oligo (dT) cellulose, since the vast majority of mRNAs of mammalian cells carry tracts of poly (A) at their 3 -termini. Several companies make kits for preparing poly A^+ RNA. We have had success with those by Qiagen (75022 or 70042 [Los Angeles, CA]) and Invitrogen (K1520-02 [San Diego, CA]). We also describe here a reliable protocol that we have been routinely using before changing to the commercially available kit.
2. Equilibrate the oligo (dT) cellulose in sterile loading buffer. Binding affinity is batch-specific. However, a good rule of thumb is that 1 g of resin will bind 2–2.5 mg of poly (A)+ RNA.
3. Pour 1.0 mL packed volume of oligo (dT) cellulose in the Quik-Sep column.

4. Wash the column with, successively, three column volumes of sterile DEPC-treated H_2O, 0.1 *N* NaOH, sterile DEPC-treated H_2O. Continue the last wash step until the pH of the column effluent is <8.0.
5. Wash the column with 5 vol of sterile loading buffer.
6. Dissolve the total RNA in sterile water. Heat to 65°C for 5 min. Add an equal amount of 2X loading buffer, and cool the sample to room temperature.
7. Apply the sample to the column, and allow to flow through by gravity. Collect the flow-through.
8. Heat the flow-through to 65°C, cool, and reapply to the column as above.
9. Wash the column with 5–10 column volumes of loading buffer, followed by four column volumes of low-salt loading buffer. The first RNA to elute off the column will be the poly (A)− fraction. The poly (A)+ fraction will elute with the no-salt elution buffer.
10. Elute the poly (A)+RNA with two to three column volumes of sterile elution buffer. The eluted poly (A)+ RNA can be selected again on oligo (dT)-cellulose by adjusting the NaCl concentration of the eluted RNA to 0.5 *M* and repeating **steps 6–10.** We generally elute into Falcon 2059 polypropylene tubes (17 × 100-mm).
11. Add sodium acetate (3 *M*, pH 5.2) to a final concentration of 0.3 *M*. Precipitate the RNA with 2.5 vol of 100% ethanol at −20°C overnight.
12. Spin the precipitated RNA at 10,000 rpm (12,000*g*) for 30 min. Carefully aspirate the supernatant, rinse the pellet with 70% EtOH, and air-dry. Resuspend the RNA pellet in an appropriate volume of DEPC-treated H_2O. Determine the RNA concentration spectrophotometrically. Reprecipitate and store as an ethanol precipitate at −20°C. RNA is most stable when stored under ethanol at −70°C.

3.4. Preparation of a Directional cDNA Library (Ref. 11)

3.4.1. First-Strand Synthesis

1. Use 20–400 µg of total cellular RNA (150 µg seems sufficient) or 1–5 µg of poly (A)+ RNA. Precipitate the RNA if necessary such that the final volume of the RNA will be 28 µL.
2. Denature the RNA by adding 5 µL of 0.1 *M* methylmercuric hydroxide (*see* **Note 1**) and incubating for 5 min at room temperature. Quench the reaction with 0.5 µL of 5.8 *M* 2-mercaptoethanol, and incubate for 5 min at room temperature.
3. The reaction mixture is then assembled as follows:
   a. 33.5 µL RNA mixture (from **Subheading 3.4.1., step 2**);
   b. 6.0 µL 10X first strand buffer;
   c. 6.0 µL 20 m*M* dNTPs containing 5-methyl-dCTP;
   d. 3.0 µL 80 m*M* Na-pyrophosphate;
   e. 2.0 µL primer (5 mg/mL);
   f. 2.0 µL ^{32}P-dATP;
   g. 3.0 µL placental RNAse inhibitor (30–100 U);
   h. 5.0 µL AMV reverse transcriptase (50–100 U);

   Incubate at 42°C for 1 h. Add 3 µL reverse transcriptase and incubate for 1 h more.

4. Determine the percent incorporation by TCA precipitation. Remove 2 µL of the reaction mix. Add 8 µL of DEPC-treated H_2O, and spot 5 µL onto a Whatman GFC filter and reserve it. To the other 5 µL, add 25 µL of 2 mg/mL bovine serum albumin (BSA) and 100 µL of 20% trichloroacetic acid (TCA). Incubate on ice for 30 min, and then filter through GFC in a vacuum filtration device. Wash with 20 mL of 5% TCA, and then dry the filter for 20 min at 60°C or under a heat lamp. Transfer both filters to scintillation vials, add scintillation fluid, and count. Determine the percent incorporation of the trace label into cDNA. The yield in nanograms of cDNA is incorporation ×120 (nmol each nucleotide) ×4 (nucleotides) ×330 (g/mol of nucleotide).
5. Dilute the cDNA to 150 µL and load onto a 1 mL Sephadex G-50 spun column in TE. Spin for 3 min at 1000 rpm, and collect the flowthrough. Measure the volume by weighing the liquid and assuming 1 g/mL for H_2O. This step is necessary to remove 5-methyl-dCTP. Any remaining will be incorporated into the second strand and prevent cleavage at the 3 *Xho*I site.

3.4.2. Second-Strand Synthesis

1. The reaction mix is assembled as follows:
   a. xx µL first-strand reaction (~125–150 µL);
   b. 30 µL 10X second-strand buffer;
   c. 30 µL 20 m*M* dNTPs;
   d. 3 µL RNase H (~2.5 U);
   e. 1 µL 45 m*M* β-NAD;
   f. 1 µL *E. coli* DNA ligase (~250 ng);
   g. 12 µL DNA polymerase I (~80 U);
   h. xxx µL H_2O—to a final volume of 300 µL;
2. Incubate overnight at 14°C.
3. Heat at 80°C for 15 min, cool on ice, and spin briefly to collect the liquid.
4. Add ~20 U of T4 DNA polymerase, and incubate 1 h at 37°C (*see* **Note 2**).
5. Phenol extract, ethanol precipitate, rinse, and dry.

3.4.3. EcoRI Methylase Treatment

1. Resuspend the cDNA in 85 µL of H_2O. The reaction mixture is as follows:
   a. 85 µL cDNA;
   b. 11 µL 10X methylase buffer;
   c. 10 µL *Eco*RI methylase (100–400 U).
   Incubate for 30 min at 37°C. Add 4 µL more methylase and incubate for 30 min longer.
2. Heat 80°C for 15 min, then cool on ice
3. Add 1 µL of 20 m*M* dNTP's and 1 U of T4 DNA polymerase. Incubate for 30 min at 37°C (*see* **Note 3**).
4. Phenol-extract, ethanol-precipitate, rinse, and dry the cDNA.

3.4.4. Kinase Linkers

1. 0.8 µg of ^{32}P-labeled linkers is needed for each cDNA preparation (*see* **Note 4**). An example reaction is the following:

a. 8 µL linkers = 4 µg;
b. 2 µL 10 kinase buffer;
c. 1 µL α-^{32}P-ATP;
d. 1 µL T4 polynucleotide kinase (~5 U);
e. 8 µL H_2O.

Incubate for 15 min at 37°C.

2. Add:
   a. 1 µL 10X kinase buffer;
   b. 4 µL 10 m*M* ATP;
   c. 1 µL T4 polynucleotide kinase;
   d. 4 µL ddH_2O.

   Incubate for 45 min at 37°C.
3. Heat-kill the enzyme at 80°C for 15 min, and then cool on ice.

3.4.5. Linker Ligation (see **Note 5**)

1. Resuspend cDNA in 27 µL ddH_2O. An example reaction is the following:
   a. 27 µL cDNA;
   b. 5 µL 10X ligase buffer;
   c. 5 µL 10 m*M* ATP;
   d. 6 µL ^{32}P-labeled linkers—0.8 µg;
   e. 3 µL unlabeled linkers—3 µg;
   f. 2 µL T4 DNA ligase—10–20 Weiss U;
   g. 2 µL T4 RNA ligase—12–18 U (*see* **Note 6**).

   Incubate for 1 h at room temperature then 14°C overnight.
2. Heat-kill the enzyme at 80°C for 15 min, and then cool on ice.
3. Remove 1 µL of the ligation mix to test for ligation (*see* **Note 7**).

3.4.6. EcoRI Digestion

1. An example reaction is the following:
   a. 49 µL ligation mix.
   b. 20 µL 10X buffer H.
   c. 126 µL ddH_2O.
   d. 5 µL *Eco*RI (450 U).

   Incubate for 1 h at 37°C.
2. Add 2 µL more enzyme and incubate 1 h more.
3. Remove 4 µL to check for digestion (*see* **Note 7**).
4. Optionally, save a portion for cloning into *Eco*RI cut vector.

3.4.7. XhoI Digestion (see **Note 8**)

1. Make the reaction mix 150 m*M* in NaCl by adding NaCl and H_2O. *Xho*I cuts better in this increased salt concentration.
2. Add 5 µL of *Xho*I (250–500 U), and incubate 2 h at 37°C.
3. Save a 5-µL aliquot to check the digestion.
4. Phenol-extract, ethanol-precipitate, rinse, and dry the cDNA.

3.4.8. Removal of Linkers and Small cDNAs

1. Pour a column in a 1-mL disposable pipet plugged with sterile polyester wool (*see* **Note 9**).
2. Make a reservoir with a 2-mL disposable Pasteur pipet cutting the bottom off at the appropriate level and slipping over the end of the column. Now cut the very top of the pipet off, fill, and rinse with 10 mL of column buffer.
3. Resuspend the cDNA in 50 µL of column buffer, and apply to the column (*see* **Note 10**).
4. Monitor the progress of the radioactivity into the column with a Geiger counter. When it gets one-third of the way (~250 µL), start collecting two-drop fractions into microfuge tubes. Continue collecting fractions until the major ^{32}P peak (linkers) reaches the bottom.
5. Count the all of the fractions by Cerenkov counting, and plot the results. You should see two distinct peaks.
6. Run aliquots of alternate column fractions on a 1% agarose gel with labeled 1-kb ladder and unlabeled 1-kb ladder.
7. Dry the gel and expose to film. Pool the peaks containing cDNA from about 500 bp to the beginning of the column (*see* **Note 11**).

3.4.9. Ligation to Vector

1. Add an appropriate amount of vector to the pooled cDNA and ethanol-precipitate (overnight is best), rinse carefully, and dry (*see* **Note 12**). Try to use about 10X the weight in cDNA of λ arms. The use of less will result in a significant proportion of clones containing multiple inserts, although the total number of clones will increase.
2. Resuspend the cDNA/vector pellet in 5 µL of ligation cocktail, which contains the following:
   a. 0.5 µL 10X ligation buffer;
   b. 0.5 µL 10 m*M* ATP;
   c. 2.0 µL T4 ligase (10–12 Weiss units);
   d. 2.0 µL ddH_2O.
3. Be sure the pellet is completely dissolved, and centrifuge to put everything into the bottom of the tube.
4. Ligate at 14°C overnight.

3.4.10. Packaging and Titering the Library

1. Package the ligation mix as directed in the instructions accompanying the packaging mix (**Note 13**).
2. Dilute the packaged phage with 500 µL of SM, and add 20 µL of chloroform (**not** chloroform:isoamyl alcohol). Store in the dark at 4°C.
3. Plate 1, 10, and 100 µL of a 10^{-3} dilution on 300 µL of ER-1647 plating cells grown as described below. Adsorb the phage to the plating cells for 15–30 min at room temperature, and then transfer to 37°C for 5 min. Phage can adsorb to the receptors, but cannot inject their DNA at room temperature. The transfer to 37°C produces a

relatively synchronous infection. Incubate at 37°C for 8 h to overnight. It is essential that the strain used be deficient in methylcytosine restriction (mcrA⁻, mcrBC⁻).

4. Determining the fraction of recombinant clones in the library. Since ER-1647 is lac-, the library must be plated on an appropriate strain that allows a complementation and is McrABC⁻, like YS-1, or amplified first on ER-1647 and then plated on XL1-Blue. After amplification, the fraction of recombinants can be determined by plating on XL1-Blue with X-gal and IPTG as described above. Using UniZap-XR, a typical yield is 95+% recombinant phages.

3.4.11. Preparation of E. coli for Plating Libraries (see **Note 14**)

1. Maintain strains for growth on minimal plates, using maltose as the carbon source and supplemented with the appropriate auxotrophic requirements. ER-1647 requires histidine, methionine, and tryptophane.
2. Grow a single colony overnight in an appropriate volume of minimal medium with the required supplements.
3. Spin down the overnight cells and resuspend in 0.5 vol of 10 m*M* $MgSO_4$. The cells are stable for about a month when resuspended in 10 m*M* $MgSO_4$, but only a few days if not. Of course, fresh cells work better.
4. Use 200–300 µL of cells for a 100-mm plate or 600–800 for a 150-mm plate. *In vitro* packaging mixes contain a large excess of phage tails, so use the larger volume for them. Maltose-grown cells also help a lot.

3.4.12. Amplification of Libraries

1. It is beneficial to screen unamplified libraries, since one needs to screen fewer clones; however, it is better to amplify the library for permanent storage. A good practice is to reserve a portion of the library unamplified and amplify the remainder for permanent storage.
2. It is convenient to plate $100–150 \times 10^3$ phage/fresh 150-mm plate. We prefer to plate in the morning and observe phage growth during the day. When plaques are touching, overlay each plate with 15 mL of SM, and incubate at 4°C overnight with shaking if possible. Plaques grown this way will typically be only 1–2 mm in diameter.
3. Harvest the liquid and make it 5.0% in chloroform. Be sure to use fresh chloroform that has been stored in the dark, because photodegradation products of chloroform are reportedly toxic to phage.
4. Spin out the debris, and transfer the lysate to an appropriate container. For storage at 4°C make the lysate 5% chloroform and store in the dark. A foil-covered Erlenmeyer flask, or media bottle is a good choice. The chloroform inhibits the growth of molds, which cause the library titer to drop rapidly.
5. It is probably a good idea to store aliquots of the library at −70°C for permanent storage. Bring the lysate to 7% DMSO, and freeze conveniently sized aliquots.

3.5. λ Phage Minipreps

1. In order to prepare synthetic RNA from a λZAPII library, one must first prepare a sufficient quantity of phage DNA. With current RNA preparation technology, ~10 µg should be sufficient.

2. A high titer library (10^{10} PFU/mL) will yield about 500 ng of phage DNA/mL, so 20–50 mL is about the right amount.
3. Add DNase I and RNase A to 50 µg/mL. Incubate at 37°C for 1 h with gentle shaking.
4. Transfer to a centrifuge tube, and spin at 12,000g for 10 min. Carefully decant the supernatant to a fresh tube.
5. Add 1/4 vol of 20% PEG 8000, 2.5 *M* NaCl, mix well, and incubate on ice for 1 h. Spin at 12,000g for 10 min at 4°C. Carefully remove the supernatant taking care not to dislodge the phage pellet.
6. Resuspend the phage in a minimum volume of proteinase K reaction buffer (10 m*M* Tris, pH 8.0, 5 m*M* EDTA, 0.5% SDS). Take care to resuspend well at this point for maximum yields.
7. Transfer to a 1.5-mL microfuge tube, and add proteinase K to a final concentration of at least 200 µg/mL, and preferably 1 mg/mL. Incubate at 60°C for >30 min or 37°C overnight (*see* **Note 15**). Be sure that the phage do not clump up during the digestion. Pipet up and down if they do, or you will lose them in the phenol extraction.
8. Phenol:chloroform:isoamyl alcohol-extract and back-extract the organic phase with 20% of the original volume of TE. Pool the two aqueous phases.
9. Repeat the phenol extraction.
10. Transfer the aqueous phase to a fresh tube, and add 2 vol of EtOH. Now add 0.5 of the original volume of 7.5 *M* NH_4-acetate and mix well (*see* **Note 16**). Mix well and store on ice for 30 min. Spin for 20 min at 4°C. Rinse the pellet well and dry.
11. Resuspend the pellet in a minimum volume of TE, quantitate, and check a small aliquot for purity by agarose gel electrophoresis.

3.6. In Vitro Transcription of Phage DNA

1. Digest 10 µg of phage DNA in a final volume of 100 µL with *Not*I or *Xho*I, depending on whether sense or antisense transcripts are desired.
2. Phenol-extract, ethanol precipitate, rinse, and dry the digested DNA. Resuspend at 1 mg/mL.
3. Prepare sense (T3 polymerase and *Xho*I digest) or antisense (T7 polymerase and *Not*I digest) RNA using the Ambion Megascript kit following the instructions with the kit. This kit reproducibly gives yields of 70–100 molecules of RNA/molecule of template. We typically use 2 µg of template and scale the reaction up 2X.
4. If desired, one can incorporate biotin-21-UTP during the transcription reaction for the production of driver RNA. Add biotin-21-UTP (Clontech) at 1/20 the final concentration of UTP in the transcription reaction.
5. Recover the RNA after transcription by adding an equal volume of 5 *M* LiCI and incubating at −20°C overnight.
6. Spin the precipitate for 20 min at 4°C, rinse well, and dry. Resuspend in DEPC-treated H_2O and quantitate.
7. Add 0.5 volume 7.5 *M* NH4-acetate and 2.5 vol of ethanol, mix well, and store at −20°C. Calculate the new concentration of RNA, and determine the volume/µg of RNA.

3.7. Photobiotinylation of RNA (12, 13)

1. How much RNA is needed? A maximum of about 60-fold molar excess of driver RNA is required for a proper subtraction. If necessary, this can be reduced by limiting the short hybridizations to about 4X molar excess and the long hybridizations to 10-fold excess. If one takes the trouble to calculate the amount of cDNA remaining after each subtractive hybridization, the total amount may be significantly reduced.
2. Mix poly (A)+ RNA or *in vitro* synthesized RNA (10–30 µg/reaction) with 50 µg of photoactivatable biotin acetate (Clontech). With the tube tops open and the lamp 6 in. from the sample, irradiate on ice for 15 min. Be sure to support the tube in a water bath rack, since the sunlamp rapidly melts the ice.
3. Add 1/10 vol 1 *M* Tris-HCl, pH 9.0, and extract repeatedly with TE-saturated 2-butanol to remove unreacted photobiotin (until the butanol phase is clear).
4. $CHCl_3$ extract the RNA, ethanol-precipitate, rinse, and dry. Resuspend in DEPC-treated H_2O and repeat the photobiotinylation. At this stage, the pellet should be reddish if the biotinylation has been successful.
5. Pool identical RNAs, and store as ethanol precipitates at −20°C.

3.8. Subtractive Hybridization

1. The subtraction involves two different types of hybridization. We and others ***(3,14)*** have found that the typical long hybridizations are inefficient at removing abundant mRNAs, and yield libraries and probes containing significant quantities of housekeeping genes.
2. Prepare first-stranded target cDNA (*see* **Note 17**) from poly (A)+ mRNA as described above (preferred) or *in vitro* sense RNA derived from a target cDNA library. In the latter case one should use 5 µg of random hexamers as primers, since one cannot be sure that the poly A tail is present after *Xho*I digestion. Remove the RNA by adding 10 µL of 0.25 *M* EDTA and 30 µL 0.15 *N* NaOH, and incubating for 60 min at 65°C. Neutralize with 30 µL of 0.15 *N* HCl, ethanol-precipitate, rinse, and dry.
3. Sequence of hybridization—perform one short hybridization (**steps 4–10**) and then one long hybridization (**steps 11–17**) overnight. Repeat.
4. Short hybridization: spin an appropriate amount of biotinylated driver RNA precipitate (10-fold molar excess to the cDNA) rinse and dry it. Resuspend the RNA in 20 µL of hybridization buffer (HBS = 50 m*M* HEPES, pH 7.6, 0.2% SDS, 2 m*M* EDTA, 500 m*M* NaCl).
5. Transfer the resuspended RNA to the tube with the precipitated cDNA, and resuspend it as well.
6. Transfer the mixture to a 500 µL microfuge tube, and overlay with mineral oil. Boil for 3 min, and snap-cool on ice. Incubate for 30 min at 55°C.
7. Transfer the aqueous phase to 100 µL of hybridization buffer (HB = HBS minus SDS), and add 5 µg streptavidin. Mix well, and incubate at room temperature for 5 min.
8. Extract with 100 µL of phenol:$CHCl_3$ and centrifuge to separate the phases. Back-extract the organic phase with 20 µL of HB, and pool the aqueous phases.

9. Repeat the streptavidin and phenol extraction procedure twice.
10. $CHCl_3$-extract the pooled aqueous phases, ethanol-precipitate, rinse, and dry the cDNA.
11. Long hybridization: Spin an appropriate amount of biotinylated driver RNA precipitate (10-fold molar excess to the cDNA), rinse, and dry it. Resuspend the RNA in 20 µL of HBS.
12. Transfer the resuspended RNA to the tube with the precipitated cDNA, and resuspend it as well.
13. Carefully draw the mixture into the center of a baked, siliconized, 20-µL capillary tube, and seal the ends with a Bunsen burner. Transfer the sealed tube to a beaker of boiling water and heat for 5 min. Transfer the beaker containing the capillary to a 65°C water bath, and incubate for 24 h.
14. Remove the beaker from the water bath and slow cool to room temperature. Remove the capillary, and score both ends. Break one, and and transfer the capillary (cut end down) to an Eppendorf tube. While inverted, break the other end, and blow the mixture into the Eppendorf tube. Rinse the capillary with 20 µL of HB, and add another 80 µL of HB.
15. Add 5 µg streptavidin. Mix well and incubate at room temperature for 5 min. Extract with 100 µL of phenol:$CHCl_3$, and centrifuge to separate the phases. Back-extract the organic phase with 20 µL of HB, and pool the aqueous phases.
16. Repeat the streptavidin and phenol extraction procedure twice.
17. $CHCl_3$-extract the pooled aqueous phases, ethanol-precipitate, rinse, and dry the cDNA.
18. Calculate the yield of subtracted cDNA based on the amount of counts remaining compared with the starting material. One should expect to subtract >95% of the starting material.

3.9. Use of the Subtracted cDNA

1. Construction of a subtracted cDNA library. Begin with **Subheading 3.4.2., step 2**—second-strand synthesis.
2. Preparation of a subtracted cDNA probe. Divide the cDNA into three portions. Reagents for random priming are prepared as follows: 1 *M* HEPES, pH 6.6, DTM (0.1 m*M* each of dGTP, dTTP in 0.25 *M* Tris-HCl, pH 8.0, 0.025 m*M* $MgCl_2$, 0.05 m*M* BME), OL (1 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA containing 90 OD U of random hexanucleotides [Pharmacia]/mL), LS (1 *M* HEPES:DTM:OL [25:25:7] [v/v]) ***(15)*** (*see* **Note 18**).
3. The reaction mix contains 11.4 µL solution LS, 1 µL BSA (Sigma) (10 mg/mL), one-third of the subtracted cDNA fragment and H_2O to 37.5 µL.
4. Boil this reaction for 5 min, and then snap-cool on ice.
5. Add 5 µL each of α-[^{32}-P]dATP and dCTP (3000 Ci/m*M*) and 2.5 U of the Klenow fragment of DNA polymerase I (Pharmacia).
6. Incubate for 2 h to overnight at room temperature.
7. Remove the unincorporated nucleotides by Sephadex G-50 spun-column chromatography as above.

3.10. Library Screening

1. Plate the library to be screened at relatively low density (5–10,000 phage/150-mm plate) to minimize the number of purification steps required. Concerning the amplification step described above, it is best to plate the phage in the morning, observe their growth during the day, and remove the plates to 4°C when plaques are relatively large (2-mm), but still well isolated from each other.
2. Lift duplicate filters from each plate, the first for 3 min and the second for 6 min (*see* **Note 19**). Store the filters plaque side up on Whatman 3MM paper until all lifts are completed. Be sure to position registration marks carefully for accuracy in aligning the autoradiograms later.
3. Denature the phage by placing the filters (plaque side up) on pads of Whatman 3MM paper saturated with the following solutions for 3 each (10): Solution 1—0.5 *N* NaOH, 1.5 *M* NaCl; Solution—2 1.0 *M* Tris-HCl, pH 7.5, 1.5 *M* NaCl; Solution 3—2X SSC. Next, transfer the filters to 3MM paper, and allow to air-dry.
4. Interleave the filters between circles of filter paper, and secure the entire stack with tape. Bake at 80°C for 30 min.
5. After baking, transfer the filters to a dish of 50 m*M* NaOH, and incubate for 15 min with shaking. This step reduces later background. Rinse with at least four changes of distilled H_2O for a total of 15 min.
6. Transfer the filters to a hybridization bag, and add 2 mL/filter of Church's buffer (7% SDS, 0.5 *M* $NaPO_4$, pH 7.2) ***(16)***. Prehybridize at 65°C for a convenient time, usually 15 min to several hours. Up to 20 filters (132 mm) can be processed/bag.
7. Remove the prehybridization solution and add fresh Church's buffer containing 5% w/v Dextran sulfate (Pharmacia) at 750 µL/filter. Denature the probe by adding 0.1 vol 2 *N* NaOH and incubating at room temperature for 5 min. Add the denatured probe directly to the bag, seal, and mix well. Hybridize at 65°C overnight with shaking if possible.
8. Remove and save the hybridization solution at −20°C (add fresh probe to it for subsequent screening). Wash the filters 3 × 20′ at 65°C in 0.5× SSC, 0.1% SDS. Expose to X-ray film with two intensifying screens overnight or longer.
9. Align the duplicate autoradiographs, and pick plaques with the wide end of a Pasteur pipet, which appear on both filters to 1 mL of SM buffer.
10. Plate several dilutions of each plaque stock, and incubate overnight at 37°C. Select a plate with 50 plaques for the next round of screening. After this round, the individual plaques should be pure and can be picked with the narrow end of a Pasteur pipet.
11. Convert the phage to plasmids according to the Stratagene protocol provided with the ZAPII vector or library.

4. Notes

1. Be sure to use the appropriate laboratory technique when handling radioactive and toxic materials. Consult a laboratory safety manual if you are in doubt regarding what proper practices are. Methyl mercuric hydroxide is quite toxic and should be handled with extreme care in a fume hood. This toxicity is balanced by its

extremely potent and reversible denaturing activity. Each new batch of methyl mercuric hydroxide should be tested for performance in denaturing gel electrophoresis as described in **ref. *9***. If sharp bands are not observed, purify the methyl mercuric hydroxide by stirring for 2 h at room temperature with a mixed-bed resin, such as Amberlite MB-1 or equivalent. Remove the resin and other debris by passing through a 0.2-µm syringe filter. Store in small aliquots at −70°C. Be sure to dispose of mercury waste appropriately.

2. This step is required to repair the cDNA and render it blunt-ended prior to linker ligation.
3. This additional blunting is required to repair any damage caused by *Eco*RI methylase. We have noticed variable amounts of nuclease activity in methylases and think this extra step is prudent.
4. We usually buy phosphorylated linkers as well as unphosphorylated ones, since it is more efficient to synthesize the linker with the phosphate on than to add it later enzymatically.
5. One should be careful that the linkers are in sufficient excess in this reaction. This, of course, depends on the yield of cDNA. Assume an average cDNA length of 1 kb in the first strand reaction and calculate the number of picomoles of ends. Be sure that the linkers are in 50- to 100-fold molar excess to ensure that cDNAs are not artifactually ligated to each other.
6. The addition of T4 RNA ligase stimulates blunt-end ligation up to 10-fold. Furthermore, RNA ligase is capable of ligating linker molecules to RNA remaining at the 5′-end of cDNA. It is quite probable that some of the longest cDNA molecules will have a few nucleotides of RNA left at the 5′-end, which RNase h cannot remove. If RNA ligase is not added, then linkers can not be added to this end and these cDNAs will be lost from the library.
7. Check for ligation and digestion by running an 8% polyacrylamide gel in TBE before and after digestion samples. A typical protein gel apparatus is appropriate. Run the gel at 300–400 V, and stop it when the bromophenol blue goes half way down. Expose to film for several hours, or dry the gel down and expose for an appropriate time. Expect to see a ladder of linkers in the undigested sample, it should be gone in the *Eco*RI-digested sample, and there should be two bands near the bottom of the gel. If the *Xho*I digest was done separately, expect to see one additional band larger than the two in the *Eco*RI digest. If the ligation or either digestion did not work, then go back to the blunt-ending step, and repeat carefully checking each step individually.
8. Alternatively, the *Eco*RI and *Xho*I digests can be performed together using buffer H, but this does not permit checking that each step has worked. We usually do the digestions separately when testing new batches of enzymes and reagents, but otherwise do them together to save time.
9. Use a sterile, plugged, individually wrapped plastic pipet suitable for tissue culture. Score the pipet in the middle of the cotton. Break off and remove the cotton. Make a tiny ball of polyester wool, push into the column top with forceps, and blast into the bottom with compressed air or gas (available at most lab benches). Polyester wool used for aquarium filtration and is available at most pet shops.

10. Proper technique is critical here for good separation. Allow the liquid in the column to drain to the top of the bed. Apply 50 µL of cDNA carefully, and allow to run in. While this is going on, reattach the reservoir, and carefully add column buffer when the cDNA has fully entered the column. Of course, the column should not be allowed to run dry at any point or else resolution will be compromised.
11. Using Sepharose C1-4B and the column system described above, the first fraction following the cDNA peak is about 500 bp. Pool the fractions from here to the beginning.
12. Take care not to overdry the DNA, or it will be quite difficult to resuspend. One to 2 min in the Speed-Vac are sufficient.
13. Stratagene recommends a 2-h incubation at room temperature. We have used up to 6 h with equally good results.
14. Although it is slightly more trouble to maintain cells on minimal plates and grow them in minimal media, the increased plating efficiency and reproducibility are worth it. Some strains, most notably C600 HflA[150] and Y1090, throw off resistant mutants at a high rate. Growth on media with maltose as the carbon source minimizes this phenomenon.
15. Proteinase K works well at elevated temperatures, but is denatured at temperatures above 65°C. This is a good step to let go overnight if desired.
16. When precipitating large amounts of DNA, you will get cleaner precipitates by adding the alcohol first, mixing well, and then adding the salt and mixing well again. Mix well, and store on ice, −20°C, or −70°C for 10+ min. For reasonable amounts of DNA, these three are about equivalent. The centrifugation time is much more critical. We typically use ice for 30 min followed by a 20 min spin at room temperature. For small quantities of DNA, overnight at −20°C gives superior recoveries.
17. If the production of a subtracted cDNA library is desired, then one is practically limited to using poly (A)+ RNA from the target tissue as the source of material. If a subtracted probe is being prepared, then either poly (A)+ RNA or RNA synthesized *in vitro* from an existing library is adequate. Whenever there is sufficient poly (A)+ RNA available, its use is preferred to minimize changes in complexity of the target RNA.
18. There are a number of high-quality kits available for labeling DNA by random priming. If you choose a commercial kit, be sure that it can be adapted to use two radionucleotides, since the probe must be of very high-specific activity to detect rare clones.
19. The choice of nitrocellulose or nylon membranes depends on the number of times the library will be screened. For one to three screenings we prefer to use supported nitrocellulose (e.g., BAS/NC, Schleicher and Schuell, Keene, NH) owing to its inherent high signal-to-noise ratio. If the library may be screened three or more times, them it makes sense to use a nylon membrane such as Nytram (Schleicher and Schuell). We have has intermittent difficulties with nylon membranes from other manufacturers and recommend each batch prior to using it.

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Differential Display of Eukaryotic mRNA

Antonio Tugores and Juan Carlos Izpísua Belmonte

1. Introduction

Arbitrarily primed PCR is a method that was initially developed to analyze and compare genome complexity ***(1,2)***. By using arbitrary primers, an array of stochastic sequences can be amplified using the polymerase chain reaction (PCR) and resolved by denaturing acrylamide gels, generating a characteristic pattern or fingerprint. This technique can be readily applied for the analysis of differentially expressed genes in two different cell types by simply converting the mRNA into cDNA ***(3,4)***. A different fingerprint pattern will then reflect differential gene expression between the cell types analyzed. The number and intensity of the fingerprints depend largely on two parameters: the abundance of the original RNAs, and how well the primers match the primary sequence so that efficient amplification can take place. Taking this into consideration, the method appears to be more suitable for the comparison of cell types where a large number of differentially expressed genes are anticipated. A major advantage of this method when compared to other strategies used for the isolation of differentially expressed genes is that a very small amount of RNA is required. This makes the method very suitable for embryological studies, where sometimes the quantity of tissue that can be collected is limiting.

At least two major approaches can be taken to generate and amplify cDNA (**Fig. 1**). We will designate them as (1) arbitrarily primed PCR of RNA (RAP-PCR ***[3]***), and (2) differential display of mRNA 5' ends ***(4)***.

1.1. Arbitrarily Primed PCR of RNA

If a single primer is to be used as both upstream and downstream primer for amplification, cDNA can be synthesized with this single primer or with an oligodT primer to select for polyadenylated transcripts. Using the same primer for both the cDNA synthesis and the PCR is very convenient, since only one primer

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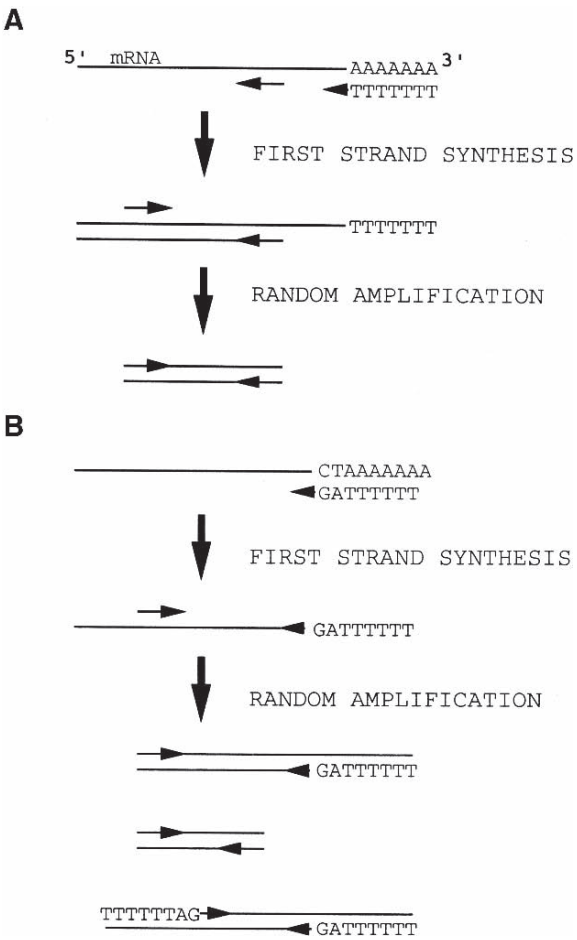

Fig. 1. The two major approaches that can be taken for the random amplification of RNA. In the first case **(A)**, synthesis of the first strand can be driven with either oligodT or the same arbitrary primer that will be used for amplification. After random amplification, the product obtained should be identical in both cases. In the second case **(B)**, the downstream (3') and upstream (5') primers are different. The first strand of cDNA is synthesized with a tailed oligodT that will only recognize a fraction of the mRNA population. Random amplification is then performed with a second arbitrary primer. Note that three different products can be obtained depending on the primer combination (*see* **ref. *16***).

is required and good matches with the primer will be already selected during cDNA synthesis. On the other hand, priming with oligodT will select for polyadenylated transcripts, and thus, cDNA will not be made efficiently from ribosomal or heterogeneous nuclear RNAs, which are extremely abundant as compared to mRNA. A disadvantage of the latter is that nonpolyadenylated mRNAs will not

be represented among the cDNA population. After oligodT-driven synthesis of cDNA, a single primer might then be used for the amplifications. This method also allows the use of nested primers that will select for population of PCR products that are in low abundance and thus underrepresented when a single primer is used ***(5,6)***. The basis for this approach is discussed below.

1.2. Differential Display of mRNA 3′ Ends

If different primers are to be used for the upstream and downstream positions, synthesis of cDNA should be driven with the downstrean primer. A strategy has been devised to generate different populations of cDNAs by using "tailed" oligodT primers for both the synthesis of cDNA and the PCR process. This method, developed by Liang and Pardee ***(4)***, relies on the use of several downstream primers in combination with multiple upstream primers. This approach allows a myriad of combinations. Furthermore, additional products can form as a result of the priming of the individual primers with themselves. The cost of synthesizing and purifying the multiple primers used in this method can be considerable, and the authors have now commercialized a kit (GenHunter Corporation, 50 Boylston St., Brookline, MA 02146) that is definitively worth trying. This method has been proven successful in a variety of systems and could be a good choice. The major disadvantage is that this method will select preferentially for abundant mRNAs, and that the products will belong preferentially to the 3 noncoding regions that are the least conserved among related genes in different species. This can make data base searching less informative.

Both methods have been proven succesful in a variety of biological systems ***(4,6–9)***. Unfortunately, it is impossible to predict what will be the result of choosing one strategy over the others until the experiments are actually done and the products analyzed. Once an authentic product has been isolated, isolation of a full-length cDNA will require the screening of a suitable cDNA library, although other PCR-based methods can also be used for this purpose ***(10)***.

Here, we provide a protocol that can be followed among many others. This protocol is based on the RAP-PCR approach, as described originally by Welsh et al. ***(3)***. A very detailed protocol of the second method has been described ***(4)***, or can be obtained from GenHunter Corporation. Overall, what is really needed is not many protocols but a great deal of good luck.

2. Materials

All solutions listed should be made with the highest quality reagents available and with sterile double-distilled water.

2.1. RNA Extraction and Synthesis of cDNA

1. RNA STAT-60 (TEL-TEST"B", Friendswood, TX).
2. Chloroform.

3. Isopropanol.
4. 100% Ethanol.
5. Nuclease-free water.
6. Electrophoresis-grade agarose (Bio-Rad Laboratories, San Francisco, CA).
7. 10X TBE buffer, 0.89 *M* Tris base, 0.89 *M* boric acid, 0.02 *M* ethylene diamino tetraacetate, disodium salt.
8. 10 mg/mL ethidium bromide (*see* **Note 1**).
9. DNAse I (RNase-free) (Boehringer Mannheim, Indianapolis, IN).
10. Human placental ribonuclease inhibitor: RNA guard (Pharmacia, Milwaukee, WI).
11. 2X Proteinase K (PK) buffer; 200 m*M* Tris-HCl, pH 8.0, 350 m*M* NaCl (5 *M* stock), 25 m*M* EDTA (0.5 *M* stock), 1% SDS (20% stock), 10 mg/mL PK (Boehringer). Store at −20°C.
12. Phenol-SEVAG reagent: a mixture of 25 parts of ultrapure buffer-equilibrated recrystallized phenol, 24 parts of chloroform and 1 part of isoamylalcohol (*see* **Note 2**).
13. 10X First-strand buffer; 100 m*M* Tris-HCl, pH 8.3 at 37°C, 40 m*M* $MgCl_2$ 500 m*M* KCl.
14. 100 m*M* deoxynucleotide triphosphate (dNTP) stocks (Pharmacia).
15. Cloned MuTLV reverse trancriptase (Stratagene, San Diego, CA) (*see* **Note 3**).

2.2. Amplification of cDNA and Electrophoretic Analysis

1. Oligonucleotides. A various number of synthetic oligonucleotides will be required depending on the method of choice. These will be indicated in the methods sections where appropriate. The quality of the primers is of great importance, and purification is strongly recommended (*see* **Note 4**) for a brief protocol on how to purify synthetic oligonucleotides by using denaturing acrylamide gels).
2. 10X Second strand buffer; 100 m*M* Tris-HCl, pH 8.3 at 37°C, 20 m*M* $MgCl_2$, 250 m*M* KCl.
3. α^{32}P-dCTP (3000 Ci/mmol, 10 mCi/mL) (*see* **Note 5**).
4. Recombinant Ampli*Taq* DNA polymerase (Perkin Elmer, Vaterstetten, Germany).
5. 5 *M* Ammonium acetate (*see* **Note 6**).
6. 10 mg/mL Oyster glycogen.
7. Urea (Boehringer Mannheim).
8. A 5% (w/v) solution of acrylamide-methylen bis-acrylamide (29:1) (Boehringer Mannheim) in 1X TBE, 50% urea.
9. Formamide loading dye: 95% deionized formamide (Gibco-BRL, Gaithersburg, MD) (v/v), 20 m*M* EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v), xylene cyanol (w/v).
10. Talcum baby powder.
11. Single-sided emulsioned autoradiographic film (Kodak BioMax, Rochester, NY).
12. TE buffer: 10 m*M* Tris-HCl, pH 7.6, 1 m*M* EDTA.

2.3. Analysis of Differential Clones

1. Random oligonucleotide labeling solutions (*see* **Note 7**).
2. Deionized formamide (Gibco-BRL).

3. 37% Formaldehyde (Sigma-Aldrich Chemicals, St. Louis, MO).
4. 10X MOPS buffer; 0.4 *M* MOPS pH 7.0, 0.1 *M* sodium acetate, 10 m*M* EDTA.
5. 6X gel loading buffer: 25% (w/v) Ficoll 400, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 1 m*M* EDTA.
6. 0.5 *M* Na_2HPO_4.
7. 0.5 *M* $NaH_2 PO_4$.
8. MAGNA NT nylon hybridization membranes (Micron Separations, Inc., Westboro, MA) or equivalent.
9. 20X SSC: 0.3 *M* tri-sodium citrate, 3.0 *M* NaCl.
10. UV crosslinker or 80°C oven.
11. 50X Denhardt's solution: 1% (w/v) bovine serum albumin, 1% (w/v) polyvinyl pirrolidone, 1% (w/v) ficoll 400. Filter-sterilize and store in aliquots at −20°C. Thaw at room temperature when needed. Can be temporarily stored at 4°C.
12. 10 mg/mL Salmon sperm DNA (*see* **Note 8**).
13. 20% Sodium dodecyl sulfate (SDS).
14. TA Cloning kit (Invitrogen) (*see* **Note 9**).
15. DNA sequencing kit (USB, Cleveland, OH).

2.4. Additional Equipment Required

1. Polytron (KINEMATICA AG, Lucerne, Switzerland).
2. Microcentrifuge.
3. UV spectrophotometer and quartz cuvet.
4. Thermal cycler (MJ Research or equivalent).
5. Thin-wall PCR tubes.
6. Sequencing gel setup.
7. Autoradiography equipment.

3. Methods

3.1. Preparation of RNA

For the preparation of embryo tissue RNA, we routinely use a commercial reagent called RNA STAT-60. This procedure is based on the method described by Chomczynski and Sacchi ***(11)***, but the RNA STAT-60 reagent contains the guanidine thiocyanate together with the phenol for easier handling. Other manufacturers supply similar reagents yielding identical results (*see* **Note 10**).

1. Embryonic tissue should be collected and washed in ice-cold PBS before addition of the extraction reagent. If the tissue of interest has to be collected over a long period of time, the tissue should be rinsed in PBS and flash-frozen into a tube inserted in a tank containing liquid nitrogen. Transferring of PBS to the freezing tube is not desirable. Samples can be stored frozen at −80°C.
2. Once a desired amount of tissue has been collected, the extraction solution should be added directly to the frozen tissue. Typically 4 vol of RNA STAT reagent are added/vol of sample in a 15-mL conical polypropylene graduated tube. Homogenization of the tissue with the chaotropic solution should be done as fast as

possible. For this purpose, a Polytron is essential. The isolation of intact RNA greatly depends on the speed of this step. For small amounts of tissue, the homogeneization can be performed in a microcentrifuge tube: add the solution and shear the sample passing all the material through a 21-gage needle three or four times, until the material easily flows through the needle.

3. After homogeneization, 0.2 mL of chloroform are added/mL of RNA STAT solution used. The tubes are left at room temperature for 5 min.
4. Spin down the tubes at maximum speed in a tabletop centrifuge for 10 min. Alternatively, the mix can be transferred to 1.5-mL microfuge tubes that can be spun in a microcentrifuge.
5. Transfer the aqueous phase to 1.5-mL tubes (a maximum of 700 µL/tube), and add 700 µL of isopropanol. Precipitate for 1 h at room temperature. These tubes can also be stored at −20°C until needed.
6. Spin for 10 min, discard the supernatant, and rinse the RNA pellet with 70% ethanol to eliminate traces of salts and phenol. Once the ethanol has been completely removed (spin the tube shortly and remove the remaining liquid with a yellow tip), the pellet is resuspended in a minimal volume of nuclease-free water. Do not let the pellet dry, or resuspension will be very difficult.
7. Once resuspended, take a small volume of the sample, dilute it in a volume of water according to the size of the spectrophotometer quartz cuvet available, and measure the absorbance at 260 nm. Consider that 1 OD = 40 mg/mL of RNA. Absorbance at 280 nm should indicate contamination with proteins and/or phenol. A good-quality RNA should have a ratio of OD_{260}/OD_{280} = 1.8–2.0.
8. Load an amount equivalent to 1 µg of total RNA in a regular freshly made 0.8% agarose-1X TBE gel containing 0.2 µg/mL ethidium bromide to check the integrity of the RNA. Two major bands corresponding to the 28S and 18S ribosomal RNAs should appear clear and sharp.
9. Once the RNA has been checked, DNase treatment is required to remove DNA that will interfere with the PCR process, giving rise to bands that do not correspond to cDNA. Resuspend part of the RNA solution (5–50 µg) in 42 µL of nuclease-free water and add 5 µL of any clean 10X restriction enzyme buffer, 2 µL of DNase I RNase-free, and 2 µL of RNase guard. Incubate at 37°C for 30 min. Store the rest of the RNA at −80°C.
10. Add 50 mL of 2X PK buffer and 5 mL of 10 mg/mL PK. Incubate at 37°C for 30 min.
11. Extract twice with 100 mL of Phenol-SEVAG, recover the aqueous phase, and add 250 mL of 100% ethanol. Precipitate at least 30 min at room temperature. Do not precipitate at a cold temperature, since an excess of SDS and salt can also precipitate and interfere with subsequent steps.
12. Spin down the precipitate, rinse with 70% ethanol, remove all the remaining liquid after a 5-s spin, and resuspend the pellet in a small volume of nuclease-free water. Measure the absorbance at 260 nm, and resuspend the RNA at 100 ng/mL. Integrity of the RNA should be checked at this point as mentioned above. To minimize repeated freezing and thawing, the RNA should be stored in small aliquots at −80°C. The integrity of the RNA is crucial for the success of the whole procedure.

3.2. Synthesis of cDNA and PCR

For cleaner results, polyadenylated RNA can be isolated as mentioned in the libraries chapter (Chapter 39). However, large amounts of RNA are required to obtain polyadenylated mRNA, so this choice is limited by the availability of the sample. Also, the time and effort employed in the extraction of more material do not necessarily correlate with an equal increase in performance. Therefore, total RNA will be the starting material used in these protocols.

The choice of the arbitrary primer to be used for amplification is of great importance. Primers should not form secondary structures with themselves. Therefore, internal hybridization sequences and palindromes should be avoided. The length of the primer for which the protocol below is designed should be 17–20 bases. For a trial experiment, either the M13 universal or reverse primers could be used.

General considerations must be taken when the first-strand synthesis is performed. First, cDNA should be made from three different dilutions of RNA from the same sample. This is especially important, since a particular band that does not appear with the same intensity in all three dilutions has a good chance of being nonspecific. Second, at least the first time a particular RNA is used with a particular primer, a similar reaction for cDNA synthesis should be performed in the absence of reverse transcriptase. This will be an indicator of the nonspecific background created by the PCR process on a particular sample.

1. Prepare dilutions of the RNA samples at concentrations of 100, 25, and 5 ng/mL in nuclease-free water. Mix in a sterile thin-walled PCR tube (amounts are in µL):
   a. Nuclease-free water 7.0
   b. 10X first strand buffer 2.0
   c. 0.1 *M* DTT 4.0
   d. 1 m*M* dNTPs 2.0
   e. 10 pmol/mL primer* 2.0
   f. RNA 2.0
2. Heat at 65°C for 5 min and chill on ice.
3. Add 5 U (1 µL) of MuTLV reverse transcriptase.
4. Incubate at 37°C for 1 h.
5. Heat at 94°C for 5 min to kill the room temperature. For convenience, these temperature steps can be programmed in a thermocycler. After first-strand synthesis, reactions can be stored at −20°C.
6. Add to each tube 20 µL of a cocktail containing (in µL):
   a. Nuclease-free water 14.5
   b. 10X second strand buffer 2.0
   c. 10 pmol/mL primer** 2.0
   d. α^{32}P dCTP (*see* **Note 11**) 1.0
   e. *Taq* DNA polymerase 0.5

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*Use arbitrary primer or oligodT.

Same arbitrary primer used for first-strand synthesis (*see* **Note 12).

Subject the samples to a low-stringency amplification step: 94 °C/5 min; 40 °C/5 min; 72 °C/5 min, followed by 30 high-stringency cycles: 94 °C/1 min; 60 °C/1 min; 72 °C/2 min. At the end, add an additional step of 5 min at 72 °C to allow the polymerase to elongate unfinished products. Transfer tubes to ice or store at −20 °C if they are not going to be used immediately.

7. Transfer the reaction to a 1.5 µL microfuge tube containing 2 µL of 0.2 *M* EDTA, 1 µL of 10 mg/mL oyster glycogen, 43 µL of 5 *M* ammonium acetate, and add 260 µL of 100% ethanol. Precipitate at room temperature for at least 5 min. Spin down for 15 min at 12,000 rpm in a microcentrifuge, carefully discard the supernatant, and resuspend in 8 µL of formamide loading dye (*see* **Note 13**).
8. Together with the samples, a labeled size marker should be also loaded. Refer to **Note 14** for preparation of a size marker. Heat the samples at 100 °C for 5 min, chill on ice, and load 3 µL on a 50% (w/v) urea, 1X TBE, 5% (w/v) acrylamide: bis-acrylamide (29:1), 50 cm long, 0.4-mm thick gel (*see* **Note 15**). Run at 55–60 W constant power until the xylene cyanol (the slowest migrating dye) band is about 5 cm from the bottom. In this gel percentage, xylene cyanol comigrates with DNA fragments of approx 160 bases.
9. Lift the gel with a piece of Whatman 3MM paper, and dry immediately without fixing. After drying, a little bit of baby powder might be applied to the surface of the gel to prevent sticking to the film during autoradiography. Expose the gel to a single-sided emulsioned autoradiography film (Kodak BioMax) at room temperature without intensifying screens. Be sure to place position markers, e.g., fluorescent dots, so that the gel can be perfectly aligned with the film after autoradiography.
10. A 5-h to overnight exposure should give enough signal to visualize the fingerprint pattern generated. Sometimes a difference in the footprint is not really obvious, and all lanes should be carefully checked. Bona fide products should appear in all three different dilutions (**Fig. 2**). If no differential bands are observed at this point, another oligonucleotide might be used (*see also* **Subheading 3.4.**).
11. Once convincing differentially expressed bands have been identified, punch holes at both sides of the band. Align the gel with the autoradiogram, and mark the position with a sharp pencil through the holes. Cut the region delimited by the pencil marks with a razor blade. and place the insert in a microfuge tube containing 100 µL of TE buffer. Close the tube firmly, heat the sample at 65 °C for 1 h, and store at −20 °C. After cutting the bands, the gel should be re-exposed to verify that the selected band has been accurately removed.
12. Once identity of the bands has been checked, thaw the sample, mix by vortexing, spin down the solid for 10 min, and place 5 µL of supernatant in a tube containing (in µL):

    a. Nuclease-free water 18.5
    b. 10X Second-strand buffer 4.0
    c. 10 pmol/mL same primer 4.0
    d. 0.1 *M* DTT 4.0
    e. 1 m*M* dNTP 4.0
    f. *Taq* DNA polymerase 0.5

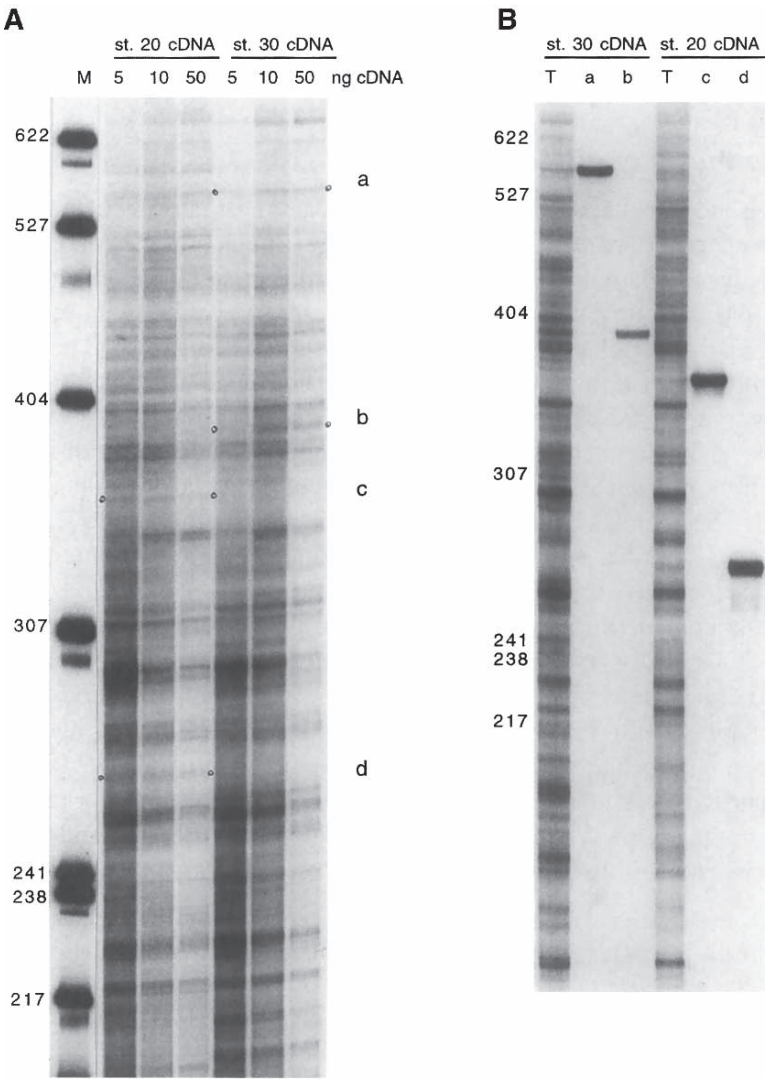

Fig. 2. Examples of a differential display analysis. Total RNA was isolated from chicken embryos at two different embryonic stages. Five, 10, and 50 ng of total RNA were subjected to reverse transcription, and afterward to amplification for 35 cycles using the M13 reverse primer, under the conditions described in the text. The amplification products were then separated on a denaturing polyacrylamide gel **(A)**. Several bands were selected (observe the holes punched to recover the products from the gel), and after selection, the gel was re-exposed to verify recovery (not shown). The recovered material was subjected again to PCR under the conditions described in **Subheading 3.2.** As markers, 50 ng of cDNA were amplified independently as described in (A), to check again for the appearance of the products selected. These products are now cloned, sequenced, and the major product will be considered as the "real" differentially expressed gene. Until Northern blot analysis is performed, there is no previous clue about the authenticity of the products.

Subject the sample to 35 cycles of amplification: 94 °C/1 min; 50 °C/1 min; 72 °C/2 min. Take 5 µL at the end of the amplification, and repeat as above.

13. Load 20 µL of each sample on a 1.2% (w/v) agarose, 1X TBE gel containing 0.2 µg/mL ethidium bromide. Bands can be seen after the first round of amplification and should definitely be seen after the second one.

3.3. Analysis of the Products

The product of the amplification may contain several bands that migrate at the same position in the sequencing gel. The best way to assess the identity of the bands in the first place is to clone the PCR product into a suitable vector for cloning PCR products. Several clones should be sequenced to verify homogeneity of the initial product. Once a major clone has been identified, Northern blot hybridization should be performed using the cloned major PCR product as a probe. We do not recommend either dot-blot or Southern blot hybridization of the PCR products with a hot cDNA probe to verify positive clones, since repetitive sequences and labeled ribosomal RNA can give rise to nonspecific positive signals.

3.3.1. Cloning and Sequencing of the PCR Products

Because a single size band may contain several DNA species, several clones should be sequenced so that the real differentially displayed clones are not missed. If several sequences appear, individual clones should be tested as probes in Northern blot analysis, as described below. An alternative strategy has been described to clone specific products after Northern blot hybridization. This procedure involves the capturing and the reamplification of the hybridized cDNA from the membrane ***(13)***.

Several kits are available to clone PCR products. The TA cloning kit from Invitrogen (San Diego, CA) allows direct cloning of homogeneous PCR products without purification and/or blunting of the DNA ends. If this kit is used, take 2 or 3 µL of the PCR mixture (after verification that a sufficient amount of the desired band is present) for the ligation reaction. Do not dry concentrate the product since dNTPs can inhibit the activity of T4 DNA ligase. An alternative kit form Stratagene requires blunt-ending of the cDNA prior to ligation. Both manufacturers provide excellent step-by-step protocols for the use of their products, so we will not provide a protocol for this procedure.

After cloning, clones should be sequenced for further analysis. This can be performed by using the dideoxy chain termination method using primers flanking the insert sequence (T3 and/or T7 primers) as initiators for the polymerase. All reagents required for sequencing can be purchased from USB (Cleveland, OH) as a kit, accompanied by instructions from the manufacturer.

3.3.2. Northern Hybridization

3.3.2.1. Labeling of the Probe

Approximately 25 ng of the PCR product should be labeled with random hexanucleotides according to the procedure of Feinberg and Vogelstein ***(12)***. A detailed procedure including the solutions needed, can be found in Chapter 39 (**Subheading 3.9.**), and also **Note 7** in this chapter. Adding 10 pmol of the random oligonucleotide used for amplification to the labeling mix containing the random hexamers will help to reach a better specific activity.

3.3.3. Electrophoresis of RNA

1. Preparation of the gel: Add 1 g of agarose to 72.6 mL dH_2O. Melt in a microwave oven. Once the flask can be handled (55–60 °C), add 10 mL of 10X MOPS buffer and 17.4 mL of 37% formaldehyde. Assemble a horizontal gel cast in a fume hood, because formaldehyde vapors are toxic. Allow the gel to solidify.
2. Preparation and running of samples: From the original RNA solutions made, resuspend 10–20 mg of RNA in 7 µL of nuclease free water. Then add 15 µL deionized formamide, 3 µL 10X MOPS, 5 µL 37% formaldehyde, and 1 µL of a 1 mg/mL ethidium bromide solution. Heat at 65 °C for 5–10 min. Chill on ice, add 7 µL of 6X gel loading buffer, and load on the gel that should be in a gel apparatus in 1X MOPS running buffer. Run at 5.6 V/cm (distance between electrodes), until the bromophenol blue is at 3 cm from the bottom of the gel.

3.3.4. Blotting and Hybridization

Wash the gel twice for 30 min each time in 10 m*M* phosphate buffer, pH 6.8. To make this solution, mix 51 mL of 0.5 *M* NaH_2PO_4 with 49 mL of 0.5 *M* Na_2HPO_4. Dilute the resulting 0.5 *M* solution to 10 m*M*. Place the gel in a blotting apparatus, and transfer to a nylon membrane in 20X SSC. Once transfer is completed, check the position of the ribosomal markers with a handheld UV lamp (sometimes they are visible without it), and mark their position with a soft pencil. Also mark the orientation of the gel and the position of the wells. Wash the membrane in 2X SSC and fix the RNA in a UV crosslinker (Stratalinker or similar) or in an 80 °C oven for 1–2 h. Prehybridize the gel in a solution containing 50% formamide, 5X Denhardt's solution, 0.1% SDS, 20 m*M* phosphate buffer, pH 7.0, 5X SSC, and 250 µg/mL of denatured salmon sperm DNA ***(16)*** at 42 °C for at least 1 h. To denature the DNA, heat it at 100 °C for 10 min, and immediately chill on ice. After prehybridization, discard the solution, and add fresh solution containing the same ingredients plus 1–5 × 10^6 cpm/mL of probe. Remember that the probe has to be denatured prior to adding it to the hybridization solution. Leave hybridizing overnight at 42 °C.

3.3.5. Washings and Autoradiography

Wash the membrane twice in the following solutions: 2X SSC/0.1% SDS at room temperature for at least 15 min each; 0.1X SSC/0.1% SDS at 42 °C for at least 30 min each; same solution at 50 °C for at least 30 min each. Check the extent of washing with a Geiger counter. If the background is high on the membrane (high counts where no RNA is expected), repeat washing with the same solution at 55 °C for at least 30 min each. If the background is still high, wash briefly several times in the same solution at 60 °C. Once washed, wrap the wet membrane in Saran Wrap, and expose to double-side emulsioned autoradiography film at −80 °C using an intensifying screen. Sometimes bands require several days of exposure to be seen ***(15)***. Once bands are detected, the size of the mRNAs can be roughly calculated as they migrate according to the logarithm of their molecular weight. The known sizes to draw a curve should be the 28 and 18S ribosomal RNAs whose position was marked with a pencil ***(16)***.

3.4. Alternative Priming Methods

1. Nested priming. This method was developed to normalize the PCR relative to mRNA abundance. If two different messages have equally good matches with the first arbitrary primer, but their abundance differs by 10-fold, the differentially displayed bands will differ in intensity by 10-fold. The introduction of a nested primer with a new overhanging nucleotide at the 3'-end may give a chance to the least abundant message to be amplified if it happens that the new nucleotide matches the sequence. Therefore, by adding one nucleotide at the 3' end, 1/16 of the previously amplified molecules will be further amplified. If two nucleotides overhang, then only 1/256 parts will be amplified and so forth. A practical example of nested oligonucleotide design and their use are extensively discussed in **ref. *6***.
2. For nested primer amplification, proceed as in cDNA synthesis. Subject the sample to amplification as described earlier except that only 10 high stringency cycles are to be performed. After this, transfer 3.5 µL of each reaction to tubes containing 36.5 µL of: dH_2O, 22.0 µL, 10X second-strand buffer 4.0 µL, 10 pmol/mL nested primer 4.0 µL, 1 *M* DTT 4.0 µL, 1 m*M* dNTP 2.0 µL, *Taq* DNA polymerase 0.5 µL. Subject the sample to 40 high stringency cycles and procceed as above (**Subheading 3.2.1., step 7**).

3.4.1. Use of Shorter Oligonucleotides

Alternatively, shorter oligonucleotides could be used for cDNA production and amplification. If, for example, 10 mers are used, the temperature of the high-stringency cycles should be dropped to as low as 37 °C, and a slope period of 15 s should be allowed for the 37–72 °C transition to prevent denaturation of the primer from the template. Also, shorter primers will have the tendency to generate less products than longer primers. The use of Stoffel fragment for amplification instead of Ampli*Taq* DNA polymerase will help to minimize this

problem ***(14)***. Except for these considerations, the protocol to follow is the same as that already described for longer primers.

4. Notes

1. Ethidium bromide is a suspected mutagen, and should be handled and disposed accordingly.
2. The preparation of Phenol-SEVAG is described in detail in **ref. *15***. For convenience, this reagent can also be purchased ready-to-use from USB, 0.1% 8-hydroxiquinolin (w/v) can be added to prevent oxidation of phenol and for easy visualization of the organic phase.
3. Owing to the large amounts of reverse transcriptase used, cloned MuLTV RT, is preferred over AMV RT because the former is considerably cheaper, although considered of lower performance when extending long products (i.e., when synthesizing cDNA to construct a library).
4. Purification of synthetic oligonucleotides: If the oligonucleotide has the trityl group on, detritylate it by resuspending the dry pellet in 200 µL 80% acetic acid. Leave at room temperature for 30 min. Dry in a Speed-Vac. Resuspend with 50 µL dH_2O, 50 µL, deionized formamide, and 25 µL of formamide loading dye. For a 15- to 25-mer, prepare a 2-mm thick 15% sequencing acrylamide, 1X TBE gel in a protein gel cast. Use a wide well (5 cm), or use several smaller wells. Heat the sample at 100 °C for 2 min and load. Run at 250 V until bromophenol blue (the fastest migrating dye) is at 2 cm of the bottom (if in doubt, check **ref. *15***, for the relative position of fragments in denaturing acrylamide gels according to the migration of the dyes). Disassemble the cast, place the gel on Saran Wrap and on a silica gel TLC plate with fluorescence indicator (Sigma, T-6270), and illuminate the gel with a handheld UV lamp. Wear appropiate eye protection when using short wave UV light. The DNA will appear as a shade, since it absorbs the UV light that stimulates fluorescence on the TLC plate. Degradation and unfinished synthesis products will also appear, but the bona fide band should represent over 60% of the total. Cut the gel containing the DNA with a scalpel, and mince the gel piece thoroughly. Place the puree in a 15-mL conical tube, and add 2 mL of dH_2O and 1 mL of TE. Shake the tube overnight at 37 °C. On the next day, recover the liquid by centrifugation, and concentrate with *n*-butanol. This is done by adding equal volumes of *n*-butanol to the DNA solution, mixing well, and spinning in a tabletop centrifuge. Discard the top butanol layer, and repeat until the final volume is 1 mL. Make sure now that you are using a polypropylene tube. Add 5 mL of chloroform, mix, spin, and recover the top layer. Adjust the volume to 1 mL, and apply the sample to a Sephadex G25 NAP-10 column (Pharmacia) previously equilibrated with H_2O. Elute the sample with 1.5 mL of dH_2O, and dry in the Speed-Vac. Resuspend the pellet in 200 µL of dH_2O, and measure the absorbance of 2 µL in 1 mL of H_2O at 260 nm (1 OD_{260} = 30 µg/mL oligo). Calculate the concentration in mol/mL (the average molecular weight of a nucleotide is 330 g/mol).
5. Special care should be taken when handling radioisotopes. Refer to a laboratory safety manual for proper handling and disposal of ^{32}P.

6. Never autoclave solutions containing ammonia. This solution should be filter-sterilized through a 0.2-µ filter.
7. (*See also* **Subheading 3.9.** and Chaper 37) This is also available as a commercial kit. Make sure the primers contained in the kit are random hexamers (i.e., Boehringer), since longer primers will label short DNA fragments less efficiently.
8. After preparation, sonicate extensively to shear the DNA and store at −20 °C. DNA can also be sheared by passing multiple times through a 21-gage needle until the material flows easily through the needle.
9. This kit includes vector, T4 DNA ligase, 10X ligation buffer, and competent cells in order to clone the PCR clones obtained. Although expensive, its use greatly facilitates the cloning of PCR products.
10. RNA can be rapidly degraded by contaminating ribonucleases. For this reason, RNase-free material must be used, and gloves should be worn throughout the whole procedure.
11. ^{35}S dATP can also be used.
12. If oligodT was used for cDNA synthesis, then add the arbitrary primer at this point. The optimal primer concentration in the PCR reaction ranges from 0.3–10 µ*M*. This parameter will depend on the primer sequence and on the particular primer preparation used. To minimize effects of the latter, purification of oligonucleotides through denaturing acrylamide gels is recommended. In a typical reaction, we use the primer at 1 µ*M*.
13. This precipitation step is added to remove most of the unincorporated nucleotide that would contaminate the lower buffer during electrophoresis. It also concentrates the product so that autoradiography can be performed in a shorter period of time.
14. Preparation of a sequence marker. Digest pBR322 with *Msp*I. After digestion, heat-inactivate the enzyme at 65 °C for 20 min. Remove an aliquot containing approx 100 ng and label in 20 µL containing: 2 µL 10X First strand buffer, 2 µL 0.1 *M*DTT, 3 µL of α^{32}P dCTP, and 1 µL of MuTLV RT. Bring the volume to 20 µL with dH_2O. Incubate at 37 °C for 1 h. Add 1 µL of 0.2 *M* EDTA, 1 µL 10 mg/mL oyster glycogen, 22 µL of 5 *M* ammonium acetate, and 132 µL of 100% ethanol. Precipitate 5 min at room temperature, spin for 15 min at 12,000 rpm, carefully discard the supernatant, and resuspend the pellet in 50 µL of formamide loading dye. Place 1 mL in a tube and count it without scintilation fluid in a scintillation counter. Load 3000–5000 cpm/gel. The bands generated are (in bp): 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160 + 160, 147 + 147, 123, 110, 90, 76, and 67. Some double bands may appear if the marker has not been well denatured. This marker will be stable for a long time at −20 °C, but the half-life of the isotope has to be considered (14.3 d).
15. Instructions for the preparation and casting of this type of gels are provided in **ref. *15***.
16. Several bands may appear if the original PCR product is not homogeneous. Also, a single gene may give rise to several mRNAs.
17. The size of ribosomal RNAs varies among species.

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Using DNA Microarrays

Clare Pritchard, Peter Underhill, and Andy Greenfield

1. Introduction

Expression profiling using DNA microarrays offers a high-throughput, semi-quantitative method for studying the transcriptional basis of development in metazoans and, at the same time, affords a novel approach to functional annotation of the genome. The increasingly inventive use of microarrays to be found in the literature is testimony to their growing impact in the biosciences. However, for developmental biologists their exploitation (reviewed in ***(1)***) has some well-characterized impediments, perhaps paramount among these being the limiting amounts of RNA available and the paucity of affordable, high-quality, genomewide microarrays. Notwithstanding these obstacles, which are gradually becoming less significant, we describe a series of protocols that have been used in our laboratory in a wide range of experimental systems, including developmental models. The description of these protocols rests on the following assumptions and points:

1. You want to use glass-slide microarrays and they are available. We do not describe any protocols used with Affymetrix GeneChips (*see* **Chapter 42**). Glass-slide microarrays are now widely available for most organisms. (Many are freely available, such as the mouse and human arrays produced at the HGMP Resource Centre, Hinxton, UK). In our opinion, high-quality, glass-slide microarrays are best produced by people who care about little else than making DNA microarrays. Several reviews and websites dealg with robotic printing of DNA probes and attachment chemistry, should you need to produce your own (*see* Appendix). Choosing microarrays with the appropriate content is important. When whole-genome arrays become widely available, there will be little point in using anything else. Until such time, specialized cDNA libraries (or oligonucleotides designed from sequencing thereof) may be a valuable source of microarray production. It is also important that arrays contain crucial control spots (see the comments on nomenclature at the end of this section),

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such as known reference genes, "housekeeping" genes (if required), repetitive elements, homopolymeric sequences, "alien" genes from unrelated species, and empty (blank) spots. Such sets of control spots are now commercially available.

2. Equipment and software are available. The most obvious requirements are a scanner and analysis software. Again, several websites exist that discuss differences between commercially available scanners and software or even offer freeware (*see* Appendix).

   One topic that cannot be omitted from this preamble is that of experimental design. Unless the experiment in question is the simplest pairwise comparison aimed merely at identifying differentially expressed genes, it is likely that a microarray user requires varying degrees of analytical sophistication. Such analyses may require lesser or greater degrees of sensitivity to variation or confidence in the variation detected. The extent to which such analyses can be performed depends to a large degree on the experimental design adopted. Familiar experimental design factors include isolating the biological process in question by careful sample selection, the incorporation of experimental and biological replicates, sample pooling, the use of appropriate reference-target RNA, independent validation, and crucially, thorough annotation at all stages of the experimental process. To maximize the informativeness of the data generated, the design factors selected are usually matched with an appropriate series of analysis options, for example, the use of particular statistical methodologies. The details of microarray data mining fall outside the scope of this chapter, although **section 3.6** deals with the important process of normalization. The simplest recommendation is this: Do not wait until the experiment is completed before deciding on which sources of variation are most important to you.

   Note on nomenclature: probes (spots, features) are simple and are immobilized on the microarray; target (RNA) is complex and fluorescently labeled and hybridized to the array.

2. Materials

2.1. RNA Preparation

2.1.1. Homogenization

1. RNAlater: Tissue storage solution (Ambion #7020).
2. QIAshredder (50) (Qiagen #79654; www1.qiagen.com/Products/Accessories/QIAshredder.aspx).
3. Rotor–stator homogenizer (Wheaton #358007).

2.1.2. RNA Extraction

1. Trizol (Invitrogen #15596018 or #10296010).
2. Chloroform (Sigma #C2432).
3. Isopropanol (propan-2-ol) BDH (Merck) #296946H.
4. Rneasy Mini Kit (Qiagen #74104).
5. Ethanol (Merck).

6. Nuclease-free water (Eppendorf molecular biology grade water, supplied by Fisher #E0032006159; www1.qiagen.com/Products/RnaStabilizationPurification/RNeasySystem/RNeasyMini.aspx).

2.1.3. RNA Quality Control

1. Agilent 2100 bioanalyzer (Agilent Technologies).
2. Nano Labchip kit (Agilent Technologies #5065-4476).
3. RNA ladder for use with Agilent nanochip (Ambion #7152).
4. Agarose (Amresco #0710-500G).
5. Orange G dye (Sigma # O7252).
6. Loading dye (1× TAE, 20% glycerol).

2.2. Target Generation

2.2.1. Direct Incorporation

1. Oligo dT (T_{15}) primer (Promega #c1101).
2. dNTP (ABgene Technology #0315/B).
3. Rnasin (RNAse inhibitor) (Promega #N2511).
4. 5× RT buffer, 0.1 M DTT (Invitrogen #18064-014).
5. M-MLV Superscript II (Invitrogen #18064-016).
6. FluoroLink™ Cy™ 5-dCTP, 25 µL (Amersham #PA55021).
7. FluoroLink Cy 3-dCTP, 25 µL (Amersham #PA53021).
8. 0.5 M EDTA solution pH 8.0.
9. Ethanol (Merck).
10. 3.0 M NaOAc (pH 5).
11. Microcentaur (Sanyo).
12. Rnase A (Sigma # R-4875).

2.2.2. Single Primer Amplification (SPA)

1. Roche cDNA synthesis kit (# 1117 831).
2. SPA first-strand RT primer 5′AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGCGCTTTTTTTTTTTTTTTV-3′. This primer should be PAGE purified. (*V* denotes A, C, or G).
3. SPA amplification (heel) primer 5′-CGGCCAGTGAATTGTAATACGACTCACTATAGGCG-3′.
4. We order oligonucleotides from Sigma Genosys and find that these are RNAse free.

2.2.3. SMART™ PCR (All Clontech, BD Biosciences, Unless Stated)

1. Advantage® two PCR kits (#PT3281-2).
2. SMART PCR cDNA synthesis kit (#K1052-1).
3. SMART PCR cDNA synthesis kit (#PT3041-1).
4. 3′ SMART CDS primer II A (10 mM) 5′-dAAGCAGTGGTATCAACGCAGAGTACT$_{30}$VN-3 (*N* = A, C, G, or T).

5. SMART II A oligonucleotide (10 mM) 5′-dAAGCAGTGGTATCAACGCAGAG-TACGC-rGGG-3′.
6. 5′ PCR primer II A (10 mM) 5′-dAAGCAGTGGTATCAACGCAGAGT-3′.
7. TE buffer: 10 mM Tris (pH 7.6), 1 mM EDTA.
8. QIAquick PCR purification kit (50) (Qiagen #28104).

2.2.4. Random-Primed Klenow Labeling

1. Bioprime kit (Invitrogen # 18094-011). Contains Klenow enzyme (50 U/μL), 2.5× random primer–reaction buffer mix: 125 mM Tris (pH 6.8), 12.5 mM $MgCl_2$, 25 mM β-mercaptoethanol and random octamers (750 μg/mL).
2. Safelock 1.5-mL tubes (amber for protection of sample from light) (Eppendorf #0030 120.191).
3. 10× low dCTP dNTP mix: 1.2 mM dATP, dGTP, and dT 0.6 mM dCTP (dNTPs AB gene technology #0315/B).

2.3. Hybridization and Washing

1. Formamide (Fluka puriss #47670).
2. 20× Saline sodium citrate solution (SSC).
3. Mouse cot 1 DNA (Invitrogen #18440-016).
4. 20% Sodium dodecyl sulphate (SDS) Amresco (NBS Biologicals, UK, #0837).
5. Poly(dA) (Amersham #27-7836-02).
6. Probequant columns (Sephadex G50; Amersham #27-5335-01).
7. 5× Denhardt's solution (Sigma #D2532).
8. 20% SDS Amresco (NBS Biologicals, UK. #0837).
9. Hybridization chambers (Corning #2551).
10. Coverslips (BDH #406/0188/52).
11. Bovine serum albumin (BSA; Sigma # A-2153).

2.3.1. Hybridization Buffers

1. All hybridization buffers and water are filtered through 0.2 μm nylon filters (Sartorius Minisart #16534) before use.
2. 1.3× cDNA hybridization buffer (6.6 × SSC, 66% formamide, 0.13% SDS): 8 mL formamide, 4 mL 20× SSC, 80 μL 20% SDS solution. When diluted to 1× gives final concentration of 5× SSC, 50% formamide, 0.1%SDS).
3. 1.3× Oligonucleotide hybridization buffer (6.6 × SSC, 55% formamide, 0.13% SDS): 6.5 mL formamide, 4 mL 20× SSC, 80 μL 20 % SDS solution, 1.5 mL water.
4. 1× Alternative hybridization buffer: 40% deionized formamide (Sigma # F-9037), 5× Denhardt's solution, 5× SSC, 10 μg/mL Poly A (Sigma # P-9403), 1 mM Na pyrophosphate, 50 mM Tris, pH 7.4, 0.1% SDS. It is possible to omit poly A with oligonucleotide arrays because they do not contain any homopolymeric sequences.
5. Hybridization oven (Techne HB-2D).
6. Water bath (Grant).

2.3.2. Slide Wash Solutions

1. All wash solutions are filtered through a 0.2 µM nylon filter (Nalgene; Fisher Scientific #TKV-230-060H)) before use.
2. Wash solution A: 2× SSC.
3. Wash solution B: 0.1× SSC, 0.1% SDS.
4. Wash solution C: 0.1× SSC.

3. Methods

The relationship between the different protocols described here and their place in the overall experimental workflow is shown in **Fig. 1**.

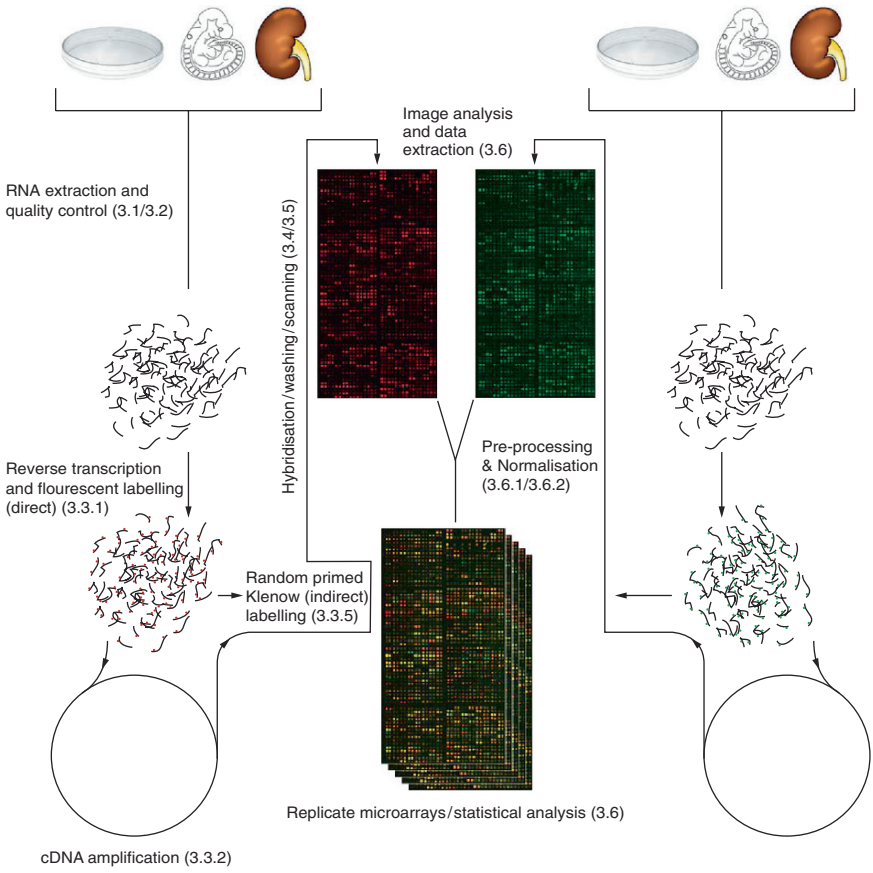

Fig. 1. Experimental overview. (*See* color plate)

3.1. Preparation of Total RNA

The quality of RNA used in a microarray experiment is probably the single most important determinant of success. We always isolate, and subsequently work with, total RNA in preference to polyA+ RNA. The protocols that follow have been used to extract RNA from embryonic material, adult tissue, and cell lines. It is important to wear gloves and eye protection during the homogenization steps.

3.1.1. Homogenization Method

3.1.1.1. Needle or Syringe and QIAshredder Method

Add the chosen disruption buffer (RLT, supplied with RNeasy kit, or Trizol) to the tissue or cells in a clean tube. Use the needle tip to begin the disruption of the tissue, then carefully draw tissue suspension up and down the syringe at least five times or until it appears smooth. Apply this suspension to a QIAshredder column placed in a 2-mL collection tube, and centrifuge at maximum speed for 2 min or until all homogenate has passed through the Qiashredder column.

3.1.1.2. Electric Homogenization

Place tissue in the tube and add homogenization buffer. Homogenize at top speed for 2 min or until sample is uniformly homogeneous. This is our method of choice for fibrous or large tissue pieces, such as adult kidney, muscle, or liver.

3.1.1.3. Rotor–Stator Homogenization

If you lack access to an electronic homogenizer, rotor–stator homogenization is a cost-effective, efficient alternative. Place the weighed (fresh, frozen, or RNAlater-stabilized) tissue in the vessel of the homogenizer. Add the appropriate volume of homogenization buffer (RLT or Trizol depending on RNA extraction protocol). Homogenize immediately using a conventional rotor–stator homogenizer until the sample is uniformly homogeneous (usually 20–40 sec). Rotor–stator homogenization simultaneously disrupts and homogenizes the sample.

3.1.1.4. Mortar and Pestle with QIAshredder Homogenization

1. Place the weighed, frozen tissue in a dry-ice-cooled mortar, add extra dry ice and grind with a precooled pestle, to a powdery consistency. Decant tissue and dry ice powder into a cooled, RNase-free microcentrifuge tube. Allow the dry ice to evaporate without allowing the tissue to thaw. Add the appropriate volume of lysis buffer (RLT). Pipet the lysate directly onto a QIAshredder spin column placed in 2 mL collection tube, and centrifuge for 2 min at maximum speed.
2. Grinding the sample using a mortar and pestle disrupts the sample but does not homogenize it. This method is useful if RNA is to be extracted from a large piece of frozen tissue, where internal areas may thaw and degrade before contacting the homogenization buffer.

3.1.2. RNA Extraction

3.1.2.1. Trizol Method

1. Homogenize samples in Trizol (1 mL per 100 mg of tissue).
2. Incubate for 5 min at room temperature.
3. Add 200 µL (sometimes 400 µL in the case of brain or muscle) of chloroform to each 1 mL of Trizol–tissue solution and shake tube for 15 sec.
4. Incubate for 3 min at room temperature.
5. Centrifuge at 5°C for 15 min at 10,000–12,000 *g* (no more than 12,000 *g*).
6. Remove the top layer of liquid containing the RNA and place in a new tube.
7. Add 700 µL (or equal volume) chilled (5°C) isopropanol to precipitate the RNA in the solution.
8. Incubate at room temperature for 10 min.
9. Centrifuge at 5°C for 10 min at 10,000–12,000 *g* (no more than 12,000 *g*).
10. Remove the supernatant. The RNA should have formed a pellet.
11. Wash the pellet with 70% ethanol (1 mL for each 1 mL of Trizol).
12. Vortex to break up the pellet.
13. Centrifuge at 5°C for 5 min at 7,000 *g* (not more than 7500 *g*).
14. Remove ethanol and air dry the pellet briefly.
15. Dissolve in Rnase-free water.

3.1.2.2. RNA Extraction Using Qiagen Rneasy Mini Columns

1. We follow these protocols and find they routinely yield high-quality RNA, usually at approximately 2 µg/µL when the columns are fully loaded. It is important to carry all steps of the RNeasy protocol at temperatures of between 20 and 25°C. Below 20°C, column membranes become clogged and the extraction may fail.
2. Weigh the tissue or measure the amount of cells. Do not use more than 30 mg tissue per RNeasy mini column.
3. Disrupt tissue or cells and homogenize lysate in 600 µL RLT buffer.
4. Centrifuge the tissue lysate for 3 min at maximum speed in a microcentrifuge.
5. Carefully transfer the supernatant to a new microcentrifuge tube by pipeting and use this sample in all subsequent steps.
6. Add 1 volume (600 µL) of 70% ethanol to the cleared lysate and mix by pipeting. Do not centrifuge and continue with the protocol immediately.
7. Apply the sample, including any precipitate that may have formed, to an RNeasy mini column placed in a 2-mL collection tube. With the tube closed, centrifuge for 15 sec at 17,900 *g*. Discard the flow-through. If the volume exceeds 700 µL, load and centrifuge aliquots successively onto the RNeasy column. After the RNA has been loaded onto the Qiagen column, it is ready for DNase treatment.
8. We routinely remove genomic DNA from our RNA preparations using DNAse I. This is to prevent random priming from the genomic DNA target when the Klenow method of label incorporation is employed (*see* **section 3.3.5**). DNase I is especially sensitive to physical denaturation. Mixing should be carried out only by gently inverting the tube. Do not vortex.

a. Pipet 350 µL buffer RW1 into the RNeasy mini column, and centrifuge for 15 sec at 17,900 *g* to wash. Discard the flow-through. Reuse the collection tube in step c.
b. Add 10 µL DNase I stock solution to 70 µL buffer RDD per mini column. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
c. Pipet the DNase I incubation mix (80 µL) directly onto the RNeasy silica–gel membrane, and incubate at room temperature for 15 min.
d. Pipet 350 µL buffer RW1 into the RNeasy column, incubate at room temperature for 5 min, then centrifuge for 15 sec at 17,900 *g*. Discard the flow-through.
e. Transfer the mini column into a new 2 mL collection tube. Pipet 500 µL of prepared buffer RPE onto the column and centrifuge for 15 sec at 17,900 *g* to wash the column. Discard the flow-through.

9. Add another 500 µL buffer RPE to the RNeasy column and centrifuge for 2 min 17,900 *g*.
10. To dry the RNeasy membrane, place the column in a clean microfuge tube and centrifuge at full speed for 1 min.
11. To elute, transfer the RNeasy column to a new 1.5 mL microfuge tube and pipet 30 µL RNase-free water directly onto the RNeasy silica–gel membrane. Centrifuge the sample for 1 min at 17,900 *g*.
12. Repeat the elution step. Add RNase-free water to the eluant from **step 9** until it reaches a volume of 30 µL, then pass this solution through the column a second time.

3.1.2.3. Trizol Followed by RNeasy Cleanup

Occasionally, a combination of Trizol and RNeasy technology proves to be most convenient. This is sometimes the case when large amounts of RNA are to be extracted. The isopropanol-precipitated RNA can be redissolved and aliquoted. Individual aliquots can then be bound to the Qiagen columns and cleaned or Dnase I-treated using the manufacturer's protocols (available at www1.qiagen.com/literature/Handbooks/PDF/RNA/INT/RNY_Mini/1016272HBRNY_062001WW.pdf).

3.1.3. RNA Quantitation

When the quantity of RNA is not limiting, a simple spectrophotometric measurement of RNA yield can be performed using the estimation that 1 absorbance unit (A_{260} unit) contains approximately 40 µg of RNA. Thus, to measure RNA yield using a quartz cell of path length 1 cm at 260 nm wavelength: [RNA yield (µg/µL) = (absorbance × dilution × 40)/1,000]

3.2. RNA Quality Control

Assessing the quality of RNA prior to labeling is vital. We analyze RNA quality in two ways: using atragose gels or using the Agilent bioanalyzer.

3.2.1. Agarose Gels

Using 0.2 µm-filtered solutions, it is possible to run a standard 1% agarose gel:

1. Melt 1 g agarose in 90 mL sterile water and add 10 mL 10× TAE (0.2 micron filtered); pour the gel.
2. Run 2 µg total RNA per track for 20 min (or as long as required to visualize the 18S and 28S ribosomal RNA bands).
3. All the equipment used in this procedure can be cleaned with 10% peroxide solution and washed with copious amounts of sterile water to avoid RNase contamination.
4. Except where the gel will form part of a Northern assay, we do not use denaturing agarose gels for RNA quality control. Gels produced by this method tend to show less discrete bands than nondenaturing agarose gels

3.2.2. Analysis Using the Agilent Bioanalyzer

Where the quantity of RNA is limiting, the Agilent bioanalyzer is an extremely useful tool for assessing both the quality and quantity of total RNA. The system is based on capilliary electrophoresis and can be used on samples down to 50 ng mass (for the nano assay) or 25 ng (for the pico assay).

1. 1 µL RNA solution in water (at concentration between 50 ng per µL and 500 ng per µL) is loaded onto a prepared Labchip.
2. The prepared chip is electrophoresed by the bioanalyzer, and traces for each sample can be viewed and analyzed on the attached computer.
3. The bioanalyzer measures concentration and purity. Remembered that the quantitation is performed by comparison to the RNA ladder loaded onto that chip and so is only as accurate as the ladder quantitation and pipeting accuracy at 1 µL volume. Also, the bioanalyzer does not give an immediate view of any genomic DNA contaminating the sample (unlike agarose gel electrophoresis).

3.3. Fluorescent Target Generation

In all the protocols, we use the fluorescent target produced and hybridized to the microarray is cDNA. In general, the aim is to make an accurate copy of the extracted messenger RNA, containing a quantifiable label.

The protocols that follow employ the cyanine (Cy) dyes produced by Amersham Biosciences. These molecules are closely related compounds that absorb and fluoresce in the visible light range. Their relatedness means that they have similar properties (although not exactly identical), and the resulting fluorescence can be directly compared after a dual-target hybridization.

The methods for preparing fluorescent targets can be divided into two main classes: unamplified methods and methods where the amount of target is increased, after reverse transcription, by some form of amplification procedure. (*See* **Note 1** for discussion of labeling protocols not described here.) In general,

if RNA yield is not limiting, it is probably best to avoid amplification and any artifacts that might arise from it.

3.3.1. Unamplified Methods

3.3.1.1. Direct Incorporation of Fluorescent Nucleotides during Reverse Transcription Reaction (Based on ***(2)***), Suitable for 50-100 µG Total RNA

1. When preparing fluorescent targets for microarray analysis it is important to use powder-free gloves—all dust and particulate matter is fluorescent.
2. Mix the following in a *lightproof* 1.5 mL Eppendorf tube (or cover tube in foil): 16 µL (100 µg) total RNA, 8 µL (4 µg) oligo (dT) primer (T_{15}).
3. Heat to 70°C for 10 min. Snap cool on ice for 5 min.
4. While on ice, prepare the following cocktail and add it to the RNA–oligo (dT) mix: 10 µL 5× RT buffer, 5 µL 0.1 M DTT, 1 µL 25 mM dATP/dTTP/dGTP, 2 µL 2.5 mM dCTP, 4 µL 1 mM Cy3- or Cy5-dCTP, 2 µL RNase inhibitor, 2 µL Superscript II.
5. Mix thoroughly by pipeting and spin down (10 sec pulse).v
6. Incubate at 42°C for 1 h.
7. Add 1.0 µL Superscript II every hour for a further 3 h incubation.
8. The half life of Superscript II is 30–60 min at 37°C; therefore, long incubations require extra enzyme to be added periodically. But it is advisable to keep the enzyme volume to less than 10% of the total reaction volume, as higher glycerol contents may interfere with reaction efficiency.
9. Add 2 µL Rnase A (10 mg/mL) and incubate at 37°C for 20 min.
10. Stop reactions with 5 µL 0.5 M EDTA.

3.3.1.2. Reverse Transcription (to Be Followed by Random-Primed Klenow Labeling)

1. In a clean RNase-free tube, mix the following: 50–100 µg total RNA, 8 µL (4 µg) oligonucleotide primer T_{23}VN, and RNase-free water to a total volume of 24 µL.
2. Incubate at 70°C for 5 min and snap cool on ice for 1 min.
3. With the samples on ice, prepare the following mixture: 10 µL (5×) RT buffer, 5 µL 0.1 M DTT, 2 µL 25 mM dNTPs, 1 µL RNasin, 6 µL RNase-free H2O.
4. Add the above mixture to the RNA/primer solution and mix. Add 2.0 µL of M-MLV Superscript II and incubate at 42°C. Add a further 1 µL of enzyme every 60 min.
5. After 4 h, add 1 µL of Rnase A (10 mg/mL) and incubate at 37°C for 20 min.
6. Purify the first-strand cDNA product on a Qiagen PCR purification column, eluting with 50 µL water.
7. Run a 1% agarose gel to assess cDNA quality (*see* **section 3.3.4**). Label the cDNA by the random-primed Klenow method (**section 3.3.5**).

3.3.1.3. Reverse Transcription Using Roche cDNA Synthesis Kit

This is a further method for producing cDNA (double-stranded in this case) suitable for labeling by the random-primed Klenow method.

1. Mix 17 µL (20 µg) of total RNA with 4 µL oligo-dT primer (supplied with the kit) in a clean thin-walled 200-µL PCR tube.
2. Incubate the samples at 70°C for 10 min in a PCR block (with heated lid).
3. Snap chill the samples on ice.
4. Add the remaining first-strand ingredients, supplied with the kit, mix thoroughly and incubate for 1 h at 42°C. Use the PCR block again.
5. Place samples on ice to stop the first-strand reaction.
6. Add reagents for the second-strand synthesis, mix by pipeting, and return the tube to the PCR block at 16°C (do not use heated lid at this point) for a further 2 h.
7. Add T4 polymerase to fill any gaps in the cDNA and continue to incubate at 16°C for 5 min.
8. Add 0.5 M EDTA pH 8 (5 µL) to stop the reaction.
9. Add RNase I (1 µL) and incubate sample at 37°C for 20 min.
10. Purify resulting double-stranded cDNA using Qiagen Qiaquick PCR purification column, eluting with 50 µL water (*see* **section 3.3.3**).
11. Run a 1% agarose gel to assess cDNA quality (*see* **section 3.3.4**).
12. The cost per sample of each of these last two methods is similar. It is perhaps preferable to produce double-stranded cDNA and thus have targets produced in the subsequent labeling reaction recognizing both sense and antisense probes.

3.3.2. Methods Incorporating a cDNA Amplification Step

We routinely use methods employing a *Taq*-based amplification. For references to other methodologies, *see* **Note 1**.

3.3.2.1. Single Primer Amplification (SPA; Based on *[3, 4]*)

This protocol has been used by us to allow microarray data to be generated with the equivalent of 50 ng of total RNA, although we would recommend starting with 1–5 µg. Comparison of microarray data produced pre and post amplification indicate that it does not alter significantly the ability to detect differential gene expression. It comprises a double-stranded cDNA synthesis step using a modified oligo-dT primer, common to other amplification methods, followed by a variant of a "cycle-sequencing" reaction using a heel primer and *Taq* polymerase. The cycling reaction has the effect of amplifying cDNA in a nonlinear but also nonexponential fashion.

1. Reverse transcription: Follow Roche kit protocol (**section 3.3.1.3**) to produce the first- and second-strand cDNA using the SPA first-strand RT primer instead of the primer supplied with the kit.
2. After RNase I digestion, apply samples to Qiaquick PCR purification columns (*see* **section 3.3.3.1**) and elute with 50 µL water.
3. Amplification: Use 25 µL of this double-stranded cDNA product in a 50 µL SPA amplification reaction as follows (shown are the final concentrations):

a. Buffer 1.5 mM Mg
b. dNTP 0.2 mM
c. T7 amplification primer 1 µM
d. Taq polymerase 3 µL per 50 µL reaction
e. ABgene buffer IV (ammonium-based buffer, 15 mM Mg) is the buffer used here.
f. Cycling conditions are 94°C for 3 min, 35× (1 min at 94°C, 1 min at 59°C, 2 min at 72°C), 4°C soak.

4. PCR products are then purified on Qiaquick PCR purification columns and eluted with water (*See* **section 3.3.3.1**).
5. This amplified cDNA is then run on a 1% agarose gel for quality control and labeled by random primed Cy-dye incorporation using Klenow fragment (*see* **section 3.3.5**).

3.3.2.2. SMART PCR Procedure

This protocol exploits the familiar Clontech Capfinder methodology utilized for RACE-PCR to perform global cDNA amplification. **Figure 2** shows an overview of this procedure.

1. First-strand cDNA synthesis: For each sample and control, combine the following reagents in a sterile thin-walled 200-µL PCR tube: 1–3 µL RNA sample (0.05–1 µg of total RNA), 1 µL 3′ SMART CDS primer II A (10 µM), 1 µL SMART II A oligonucleotide (10 µM), and RNase-free water to a total volume of 5 µL.
2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Incubate the tube at 70°C for 2 min in a PCR block with heated lid.
4. Cool the tube on ice for 2 min.
5. Spin the tube briefly in a microcentrifuge to collect contents at the bottom of the tube.
6. Add the following to each reaction tube: 2 µL 5× first-strand buffer, 1 µL DTT (20 mM), 1 µL 50× dNTP (10 mM), 1 µL PowerScript reverse transcriptase.
7. Mix by gently pipeting and spin the tubes briefly.
8. Incubate the tubes at 42°C for 1 h in an air incubator.
9. Dilute the first-strand reaction product by adding 40 µL TE buffer.
10. Heat tubes at 72°C for 7 min.
11. Samples can be stored at –20°C for up to 3 mo.
12. A negative control reaction should be performed alongside the first-strand synthesis reaction using 3 µL sterile water in the place of RNA starting material. Positive controls can also be included in the reaction (placental RNA is supplied with the kit).
13. Amplification of cDNA: Prepare three amplification reactions for each first-strand cDNA sample. One of these tubes is used to determine an ideal cycle number for optimal amplification, while the other two are used to generate amplified product. For each amplification reaction, place in a clean thin-walled 200-µL PCR tube 10 µL of first-strand cDNA solution (or dilution thereof in water). *See* **Note 2** for recommended dilutions.

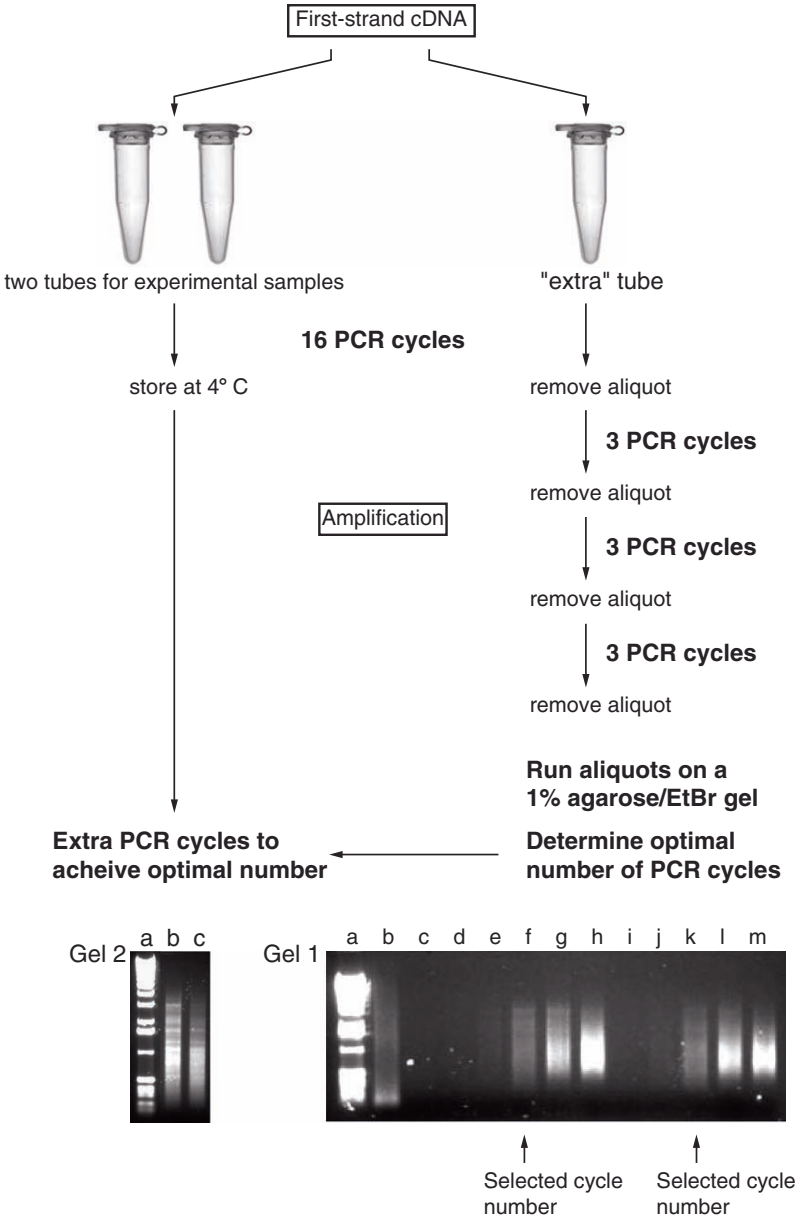

Fig. 2. SMART PCR amplification of cDNA target. For details *see* **section 3.3.2.2**. The appearance of an ideal amplification is indicated by arrows on Gel1. Gel 1: lane a, DNA ladder; lane b, cDNA control 9unamplified0; lane c, SMART negative control; lane d, PCR negative control; lane e, 16-PCR cycle aliquot (sample 1); lane f, 19 PCR cycle aliquot; lane g, 22-cycle aliquot; lane h, 25-cycle aliquot; lane I, blank lane; lane j, 16-cycle aliquot (sample 2); lane k, 19-cycle aliquot; lane l, 22-cycle aliquot; lane m, 25-cycle aliquot. Gel 2: lane a, DNA ladder; lane b, amplified and purified sample 1; lane c, amplified and purified sample 2.

14. Prepare a master mix for all reaction tubes, including additional tubes for negative controls. Run a PCR negative control (water) and a SMART negative control using a portion of the first-strand reaction negative (no RNA) control. (Take aliquots at all cycle stages—all these samples should have no visible DNA products.) For each reaction combine the following components in the order shown: 74 µL PCR-grade water, 10 µL 10× Advantage 2 PCR buffer, 2 µL 50× dNTP (10 mM), 2 µL 5′ PCR primer II A (10 µM), 2 µL 50× Advantage 2 polymerase mix (90 µL total volume).
15. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
16. Aliquot 90 µL of the PCR master mix into each amplification reaction tube containing 10 µL cDNA solution. Mix thoroughly and spin briefly.
17. Commence thermal cycling using the following parameters: 1 min 95°C followed by 16 cycles of (95°C 30 sec, 65°C 30 sec, 68°C 6 min).
18. At this stage, store two of the tubes from each amplification reaction at 4°C. Use the remaining tube from each sample, and the negative control samples, to determine the optimal number of PCR cycles. To do this, transfer 5 µL PCR sample from the reaction tube to a clean microcentrifuge tube for subsequent agarose gel electrophoresis (*see* **Fig. 2**).
19. With the remaining 95 µL of the PCR mixture, run three additional individual PCR cycles of (95°C 1 min, 65°C 1 min, 68°C 6 min), giving a total of 19 cycles. Transfer 5 µL from this effective 19-cycle PCR to a clean microcentrifuge tube (for agarose gel electrophoresis). Use the remaining PCR mixture to repeat this procedure three times to collect 5 µL aliquots at cycle numbers of 22, 25, and 28.
20. Electrophorese the five 5-µL aliquots of each PCR reaction alongside 0.1 mg of 1-kb DNA ladder on a 1% agarose gel in 1× TAE buffer. Determine the optimal number of cycles required for each experimental sample and control sample. (*See* **Fig. 2**)
21. Retrieve the 16-cycle PCR tubes from 4°C, return them to the thermal cycler, and subject them to the relevant number of additional cycles. When the cycling is completed, analyze a 5-µL sample of each PCR product alongside 0.1 µg of 1 kb DNA ladder on a 1% agarose gel in 1× TAE buffer (to confirm contents of tube).

3.3.3. Purification and Concentration of PCR Products and cDNA

Any good quality purification or concentration method should be sufficient to produce cDNA ready for the next stage in target preparation. We use the Qiagen PCR purification protocol in our standard protocol for purification of cDNA products of reverse transcription and cDNA products from amplification reactions.

1. Add 5 volumes of buffer PB to 1 volume of the PCR sample and mix.
2. Place a QIAquick spin column in a provided 2-mL collection tube.
3. To bind the DNA, apply the sample to the QIAquick column and centrifuge for 60 sec at 13,000 rpm. (all centrifugation steps are at same speed).
4. Discard flow-through. Place the QIAquick column back into the same tube.

5. To wash, add 0.75 mL buffer PE to the QIAquick column and centrifuge for 60 sec.
6. Discard flow-through.
7. Place the QIAquick column in a clean tube and centrifuge the column for an additional 1 min at maximum speed.
8. Place QIAquick column in a further clean 1.5-mL microcentrifuge tube.
9. Carefully place 50 µL water (minimum pH 7) directly on the silica membrane of the column.
10. Allow sample to rest at room temperature for 1 min.
11. Elute cDNA from column by centrifugation for 1 min at maximum speed (17,000 *g*).

3.3.4. cDNA Quality Control

3.3.4.1. Agarose Gel Analysis of cDNA

1. Samples are electrophoresed in a 1% agarose gel in 1× TAE
2. One tenth of the sample is run out in each case, that is, 5 µL of 50 µL eluted volume.
3. Run 10 µL of 1-kb ladder (0.1 mg) beside the samples to assess the size range of the cDNA smear. This should usually be in the range of 0.5–2.5 kb.

3.3.4.2. 1-kb Ladder Preparation

Ladders are prepared in our laboratory in a batch method—adding the same volume of the same batch of ladder to each gel allows some reasonable comparisons between gels.

1. To a clean 15-mL tube add 1 mL ladder (as received from Invitrogen), 6 mL water, 2 mL glycerol, and 1 mL 10× TAE solution. Fast orange G dye is added until the mixture has enough color to be easily visible in the gel wells and during the gel run. (This dye runs, as its name suggests, quickly on agarose and therefore shows the gel front without obscuring the cDNA smears.)
2. Aliquots of prepared ladders can be stored at −20°C for at least a year.

3.3.5. Random-Primed Klenow Labeling **(5)**

We use this protocol to label cDNA from most sources, including basic reverse transcriptions (20–50 µg total RNA) and a variety of amplified cDNA products. We also use it successfully to label subtracted cDNA samples (*see* **Note 1**). Random primers and Klenow are from the Bioprime kit (Invitrogen).

1. Mix the following in a lightproof 1.5-mL Eppendorf tube (or cover tube in foil): 20 µL target cDNA, 20 µL (2.5×) random primer or reaction buffer mix.
2. Boil for 5 min and snap cool on ice.
3. While on ice, add the following: 5 µL (10×) dNTP mix (low dCTP), 3 µL 1 mM Cy5-dCTP or Cy3-dCTP, 1.5 µL Klenow (high concentration, e.g., 50 U/µL).
4. Incubate the samples at 37°C for between 1 and 24 h, usually 2.5 h.
5. Stop reaction with 5 µL 0.5 M EDTA (pH 8.0).

3.3.6. Measuring the Amount of Labeled cDNA Present in Sample

Although we do not routinely measure the amounts of labeled target used in each hybridization, spectrophotometry can be used to determine the amount of Cy-dye incorporated into labeled nucleic acid (*see* **Note 3**).

3.4. Microarray Hybridization

3.4.1. Slide Preparation

Rehydration without prehybridization: Place array slide into hybridization cassette and put 15 µL of water in each end of the chamber to ensure high humidity. Seal the chamber and place in oven at 42°C for 30–60 min.

3.4.2. Prehybridization Method (Amino-Silane Slides Such as CMT GAPS)

1. Immerse the slide to be hybridized in 50 mL 1× prehybridization solution. A 50-mL falcon tube is a convenient container for this procedure.
2. Incubate the slide in this prehybridization solution at 42°C for 30 min.
3. Wash the slide by transferring it to a falcon tube containing 50 mL filtered deionized water (0.2 µM filtered milliQ water) and shake gently for 2 min.
4. Further wash the slide by transferring it to a falcon tube containing 50 mL of propan-2-ol (isopropanol).
5. Dry the slide by centrifugation. This is simply done by placing the slide, handled side down, into a dry 50-mL falcon tube and spinning at 500 g for 3 min. The excess isopropanol is spun off the end before it has chance to dry and leave solvent marks on the slide.
6. Place the slide into a humid slide chamber, seal, and incubate at 42°C until ready to apply target solution.

3.4.3. Target Preparation

3.4.3.1. Precipitation Method, cDNA Slides

1. Combine the contents of the tubes containing Cy3- and Cy5-labeled targets together in a single, clean tube.
2. Add 12 µL Cot 1 (1 µg/µL) and any blocking oligonucleotides required (e.g., poly dA).
3. Blocking agents are added to mask, by competitive hybridization, any sequences common in the target or probe set. Without the addition of blocking sequences, there would be a risk of indiscriminate hybridization to every probe (on cDNA arrays) or hybridization of the target to itself in preference to the probe (solution–solution hybridization tending to take place faster than solution–solid phase hybridization). Further packing or blocking reagents, such as tRNA, are sometimes added to microarray hybridizations for similar reasons.
4. Precipitate products by addition of 13 µL 3 M sodium acetate, to produce a final concentration of 0.3 M in the aqueous sample, and 450 µL of ethanol (3× volume). Incubate on ice for 20 min, and spin down to a pellet by centrifugation at 17,900 g for 20 min at 5°C.

5. Wash pellet twice with 450 µL cold 70% ethanol. Spin samples for 5 min at 17,900 *g* between washes.
6. Dry the pellet briefly and resuspend pellet in 12.5 µL water, then add 37.5 µL of 1.3× hybridization solution.

3.4.3.2. Target Preparation, Sephadex Purification

1. Resuspend the resin in a G-50 sephadex column by vortexing gently
2. Snap off bottom closure, loosen cap a quarter turn, and place the column in a 1.5-mL microfuge tube.
3. Prespin column at 2,000 *g* for 1 min to remove buffer. Blot tip of column dry on a clean paper towel.
4. Remove top cap and place tube in a fresh 1.5-mL tube.
5. Pipet the sample onto the center of the angled surface of the resin bed, being careful not to disturb the resin (purify the Cy3- and Cy5-labeled target separately).
6. Centrifuge at 2,000 *g* for 2 min and discard the column.
7. Pool the purified Cy3- and Cy5-labeled products in a clean 1.5-mL tube and add 12 µL Cot 1 DNA and, if hybridizing to cDNA arrays, 2 µL poly dA.
8. Dry the sample using a "speedvac" or similar lyophilization method. (samples can be precipitated with sodium acetate and ethanol at this stage).
9. Redissolve the sample in 12.5 µL filtered water.
10. Add 37.5 µL 1.3× hybridization solution.
11. Mix thoroughly and incubate at 85°C for 5 min.
12. Incubate at 42°C for 30–60 min.
13. Centrifuge the sample at full speed for 4 min.
14. Apply to microarray slide.

3.4.4. Manual Hybridization Method

In the case of manual hybridization, there are two common methods for applying the target solution to microarray on the slide. In all cases, good results can be achieved if the target, slide, and coverslip are all warm, that is, approximately at hybridization temperature.

3.4.4.1. Capillary Action Method

1. Open the hybridization chamber and check that the chamber and slide are moist. If the chamber appears dry, it is unlikely that it is sealed properly and it may allow the chamber to flood when placed in the water bath overnight. Remove the water from the chamber reservoirs and replace it with 1× hybridization buffer. Place the coverslip over the array and pass the target solution underneath the coverslip using capilliary action to draw the solution from the edge of the coverslip over the entire slide.
2. Close the chamber and immerse the chamber in a water bath at 48°C overnight.

3.4.4.2. "Splat" Method

Carefully pipet the target solution onto the slide surface, taking care not to leave any bubbles on the slide surface. Then drop the coverslip into place.

A third method used is to place the target on the coverslip, then use the slide to pick up the coverslip by touching the target solution puddle.

3.4.5. Washing

1. Place slide rack in beaker of wash solution A (1 L) and quickly transfer slides from hybridization chambers into the slide rack. Agitate rack until all coverslips have fallen off.
2. Transfer the rack of slides to a similar container of wash solution B and mix vigorously for 3 min.
3. Transfer rapidly to container with 1 L wash solution C and mix vigorously for 1 minute.
4. Quickly move slides into 50-mL Falcon tube using slide forceps. Place slides in tube with the end touched by the forceps at the bottom of the Falcon tube, so that any debris from the forceps is spun away from the array, not over it.
5. Spin for 3 min at 500 *g* at room temperature.
6. Move slides to fresh, dry 50-mL tubes. Inspect slides immediately for liquid and spin again if required (then move to another dry tube). Do not expose any portion of the slides to the air. If liquid dries on the slide, the quality of the array suffers.

3.5. Slide Scanning

Many instruments are available for reading the fluorescent signals on a microarray surface (*see* Appendix). Scanners are difficult to compare, and so the only recommendation we can give is to try your scanner with a known target on the array of your choosing. In general, the things to consider when choosing a scanner are as follows:

1. *Are the images produced in a format from which data can be extracted?* The majority of data extraction software requires tagged image format files (tiffs) as input. Often, the easiest option is to use the data extraction program recommended by the scanner manufacturer.
2. *Is the resolution good enough to accurately quantify the array signals?* Each feature on the array surface should be covered by enough measurements to get a reasonable average reading (e.g., a 100-µm spot with a 10-µm resolution scanner returns 100 pixels per spot).
3. *Asses scanner performance*. The easiest way to test the scanner function is to make up a test slide with Cy dye spots. Make a dilution series of Cy5 and Cy3 spots and apply the Cy dye spots with a spotter. Homemade spotters are easily prepared by drawing a glass capillary. When scanned, these should show that both fluorescent channels are being read. It is also useful to scan slides in both orientations (provided the first scan has not bleached the entire slide). Any spatial variation should be reproducibly at the same position on the slide surface regardless of scan direction; that is, ratios should be the same with each scan.

3.6. Image Analysis and Data Extraction

Many sources for microarray analysis software are available, both commercial and academic or freeware. Several reviews describe the software packages available, and websites compare the functionality of these packages (*see* Appendix). Generally speaking, the freeware versions are slightly less user-friendly and, in some cases, require more time-consuming manual adjustment than the more advanced commercial packages. The general principle of microarray image analysis is common to most of these packages: two images (one for each dye used on an array) are superimposed (overlaid) and given a false color (red for Cy5 and green for Cy3). These are the commonly seen images of microarrays (**Fig. 1**), with red and green spots representing over- and underexpression of genes in each sample and yellow indicating identical (or near identical) levels. Next, the position of each feature (spot) has to be established. A grid or segmentation algorithm is fitted to the superimposed images to define the features and the background. Depending on the software package, the degree of user input at this stage can differ from starting an automatic algorithm to manually positioning every grid, subgrid, or ring. Once the features have been identified, a gene-tracking file that links each position to a unique cDNA or oligonucleotide is loaded and the data for each feature is calculated.

The signal is calculated for both the features and an area outside of each feature for use as a background measurement. This background signal represents the natural fluorescence of the glass and any coating or impurities in the hybridization. Once each feature's position has been established, the pixels to be used in the calculation of that feature's signal must be defined. The method used to define these pixels differs among software packages. Another important feature of the image analysis process is flagging and quality control. Features that do not meet certain quality-control criteria can be "flagged" and discarded or downweighted in further analysis. The quality-control criteria available differ, depending on the software employed, and the settings need to be adjusted to suit individual requirements. However, on occasion, visual inspection of a feature reveasl that the quantified signal is not related to the hybridization for that feature (e.g., those caused by dust, scratches, surface abnormalities, and the like). In these cases, the user can manually flag this feature and ignore the data from further analysis.

As a summary, the important differences among software packages are

1. Amount of manual alignment or intervention required. Total manual adjustment is impractical and time consuming, while no manual adjustment is prone to misplacing and misidentifying features. Manual placement of a grid and automatic alignment with manual adjustment available is a good compromise.
2. Grid-fitting algorithm used. The efficiency of automatic grid-alignment algorithms depends greatly on the regularity and quality of the arrays used. In most cases, it is necessary to try each package with slides of the type and standard intended for use.

3. Segmentation algorithm used. Fixed circle and adaptive circle segmentation provide less accurate measurements of signal than histogram and seeded region growing methods.
4. Background area sampled. Different packages sample slightly different areas for the background; as long as there is an individual measurement for each feature and the feature signal does not overlap into the background region, the measurements are fairly comparable.
5. Number and type of measurements available per feature. All the software packages give a signal measurement; however, some packages provide better quality-control data and a wider range of spot measurements. While these are not currently used in most analyses, they may have uses in reducing variability in the future.

3.6.1. Data Preprocessing

As already stated, it is common to have both signal and background measurements for an individual feature, with many possible options for those measurements. Generally speaking, the background measurement is subtracted from the feature signal measurement, as background is thought to represent an additive error within the signal. However, on occasion, the background fluorescence is higher than the feature (leaving dark spots or black holes), and subtracting the background signal from the feature signal would lead to a negative number. In these instances, either a method for estimating a correct background signal must be used or the data should be flagged as unreliable.

Flagged data can be either removed from the data se, or, in some analysis packages, given a weight and included this in the analysis. Depending on the reason for the flag, the data may still be of value; for instance, a feature flagged for low signal might be of interest while a spot contaminated by a scratch or dust is of no use. In some cases, it is feasible to return to the image and visually inspect each flagged feature to decide whether to include the feature in a data set. However, in most large-scale analyses, this is an unrealistic option and would be too time consuming. In such cases, it is important to have the options and criteria for the flags set to a level that includes the data you are willing to accept.

Further microarray analysis requires the data to have certain properties, including constant variability at all expression levels and an expression distribution that is approximately bell shaped. To achieve this, it is necessary to transform the data. The most common transformation is logarithmic, although other more complex transformations can be used (see the online article by Cui, Kerr, and Churchill listed in the appendix).

3.6.2 Normalization

Normalization is a collection of procedures intended to remove the many sources of systematic variation in microarray experiments that affect the measured gene expression levels, resulting in an accurate measure of gene expression.

Several sources of variation may be found both within a single array and across multiple arrays. Within a single two-color hybridization, variation arises from differences in the behavior of the two Cy dyes, including their incorporation into the target, their excitation and emission response, and their detection efficiency. For two-color hybridization, the central assumption is that most genes on an array are not going to be expressed differentially between the two samples. Normalization procedures usually provide a better estimate of gene expression only if this assumption is correct. The most common procedure to deal with the differences caused by the two dyes is described as global scaling. This requires transforming the data such that the mean or median of each expression intensity distribution (Cy5 and Cy3) is equal. Other methods involve linear or nonlinear (Lowess) regression. (The Terence Speed website is particularly useful here, *see* Appendix.)

Another possible source of variation within slides is known as *spatial bias*. This describes any effect that influences the intensity of a spot based on its position on a slide, such as variations in hybridization efficiency over the slide surface, variations in slide flatness, and scanner focal depth across a slide as well as print-tip-dependent effects. Currently, two methods are used to deal with these: The first is to divide the slide into subsections, typically subgrids from individual pins, and normalize each subset separately. Or, when the variation is not restricted to a regular subgrid, such as with slide flatness problems, a two-dimensional Lowess technique can be used (see the Lorenze Wernisch website in the appendix). This is a more complex form of the Lowess regression, where a polynomial surface is fitted to the data based on slide coordinates and the surface is then "smoothed." The procedure can be run on either the log ratio, the signal intensity, or the background intensities.

If data from multiple slides are to be compared, they need to be normalized "across slides" as well as "within a slide." These methods assume that the variation between the slides derives from the experimental processes and is not biologically relevant; that is, all the distributions should be the same. The simplest method of across-slide normalization is a simple mean scaling, whereby all the slides are centered to a mean ratio of zero. Centering of the distributions is a similar method, where all the means are zero and the standard deviation of each distribution is one.

3.6.3. Differential Expression

In most microarray experiments, the main aim is to detect differentially expressed genes between two or more samples. Unfortunately, this is an area where the use of distinct analyses still has to provide a concrete method of choice. The best option is to use a statistical test. However, the degree of replication needed for most statistical tests is prohibitive for most microarray experiments.

Of the available options, ratio-based cutoffs are the simplest and most widely used but also probably the least meaningful, due to the arbitrary nature of the cutoff and the insensitivity to noise at low signal levels. Better methods include bootstrapped *t*-tests, Bayesian probability methods, and ANOVA analysis. However, all these depend on the design of the experiment. Generally speaking, outliers identified in replicate hybridizations provide a good starting point for further validation ***(6)***.

3.6.4. Advanced Analysis

Depending on the design of the experiment and its rationale, many forms of advanced analysis are available, ranging from hierarchical clustering, to network and pathway reconstruction, to tissue and disease classification. The details and use of these techniques go far beyond the scope of this chapter, but see the appendix for useful links.

3.6.5. Sample Data Analysis Schema

1. Load Cy5 and Cy3 image from scanner.
2. Overlay and align images.
3. Create, align, and adjust grid.
4. Load gene-tracking file.
5. Calculate the signal background and quality-control measures.
6. Flag "poor"-quality features.
7. Save raw extracted data.
8. Preprocess raw data: Remove flags, subtract background, and log transform data.
9. Plot Cy5 versus Cy3 as a scatter plot.
10. Plot the MA scatter plot.
11. Normalize appropriately using scaling, linear, or nonlinear regression.
12. If analyzing multiple slides, scale or center the data sets across slides.
13. Select outliers.

4. Notes

1. Other indirect methodologies for the production of labeled targets include aminoallyl labeling and the dendrimer labeling system of Genisphere (www.genisphere.com). These are compared to the direct labeling method in ***(7)***.

   For amplification of the target, methodologies involving production of antisense RNA (aRNA) from a RNA polymerase T7 promoter engineered at the 3′ end of the cDNA are widely used (*see* ***(8)***). A popular commercial use of this technique is the MessageAmp™ aRNA kit from Ambion (www.ambion.com). Multiple rounds of amplification may be employed.

   Recently, PCR-based methods permitting massive amounts of amplification have been shown to allow good preservation of mRNA representation, even from an individual cell's worth of transcript ***(9, 10)***. In a similar fashion, we successfully

labeled (Klenow method) and hybridized two distinct subtracted cDNA libraries to identify differentially expressed genes (unpublished data). Because of the relatively simple nature of the cDNA samples labeled, such a microarray experiment ("A minus B" library hybridized with "B minus A" library) is sensitive enough to detect even rare differentially expressed transcripts.

2. The amount of first-strand product (solution in TE) required for SMART amplification depends on the amount of starting RNA:

RNA starting material (µg)	Volume of cDNA used in PCR (µL)
~1.0	1
~0.5	2
~0.25	4
~0.1	10
~0.05	10

3. Although we do not routinely measure the amounts of labeled target for each hybridization, spectrophotometry can be used to determine the amount of Cy dye incorporated into labeled nucleic acid. This can be achieved by measuring the absorbance of the solution containing the nucleic acid at the absorption maximum for Cy3 and Cy5. These wavelengths are 550 nm for Cy3 and 650 nm for Cy5. From the known extinction coefficients corresponding to these wavelengths, the concentration and amount of Cy dye in the sample can be calculated. The amount of Cy dye in the purified sample can be used as a guide to optimize the amount of probe in the hybridization. Best results are achieved when the amounts of Cy3 and Cy5 in dual color hybridization are equal.

Extinction coefficients:
150,000 M–1 cm–1 at 550 nm for Cy3 and
250,000 M–1 cm–1 at 650 nm for Cy5

Calculation equations:
pMol Cy3 in purified sample = $(A_{550}/150{,}000) \times$ dilution factor $\times Z \times W \times 1012$
pMol Cy5 in purified sample = $(A_{650}/250{,}000) \times$ dilution factor $\times Z \times W \times 1012$
where A_{550} = absorbance at 550 nm, A_{650} = absorbance at 650 nm, Z = the volume of sample after purification in µL, and W = optical path of cuvette in cm

Appendix

We included this list of websites because we found them useful in the past: It is not meant to be comprehensive. Some contain many links to other sites that, no doubt, will help you find a solution to your microarray-related problem.

General Interest

The Institute for Genomic Research microarray resources: www.tigr.org/tdb/microarray.

Resources and protocols from the NHGRI microarray project: http:research.nhgri.nih.gov/microarray/main.html

Stanford microarray protocols: http://cmgm.stanford.edu/pbrown/protocols/index.html.

Pat Brown's Lab home page: http://cmgm.stanford.edu/pbrown.

Stanford microarray database, expression data already collected: http://genome-www5.stanford.edu/MicroArray/SMD.

European Bioinformatics Institute: www.ebi.ac.uk/microarray.

Lots of free software available and home of the Arrayexpress microarray expression database: www.ebi.ac.uk/arrayexpress.

Telechem International, all sorts of microarray resources: http://arrayit.com.

Microarray Gene Expression Data Society (MGED): www.mged.org.

MIAME, (minimal information about a microarray experiment), a set of guidelines about experimental annotation aimed at developing microarray repositories and data analysis tools: www.mged.org/Workgroups/MIAME/miame.html.

Data Extraction, Manipulation, and Analysis

Genepix Pro (found with most Axon scanners): www.axon.com/GN_GenePixSoftware.html.

Imagene, from Biodiscovery: www.biodiscovery.com/imagene.asp.

Mike Eisen s home page, by a pioneer in microarray data analysis: http://rana.lbl.gov.

Scanalyze, cluster, and treeview, useful, free pieces of software for microarray analysis: http://rana.lbl.gov/EisenSoftware.htm.

Gary Churchill's statistical genetics website: www.jax.org/staff/churchill/labsite.

Cui, Kerr, and Churchill, data transformations for cDNA microarray data: www.jax.org/staff/churchill/labsite/pubs/index.html.

Y.F. Leung's functional genomics website: http://ihome.cuhk.edu.hk/~b400559.

A comparison of image analysis software: http://ihome.cuhk.edu.hk/~b400559/arraysoft_image.html.

Terry Speed's pages on statistical analyses of microarray data and much more: www.stat.berkeley.edu/users/terry/Group/index.html.

Yee Hwa Yang and Terence Speed (tech report 584), a comparison of methods for image analysis on cDNA microarray data: www.stat.berkeley.edu/users/terry/zarray/Html/papersindex.html.

Lorenze Wernisch YASMA (yet another statistical microarray analysis) R package, instructions and source code: http://people.cryst.bbk.ac.uk/~wernisch/yasma.html.

Two websites listing many useful references: http://bioinformatics.upmc.edu/Help/MicroarrayReferences.html and www.nslij-genetics.org/microarray/

Genespring: www.silicongenetics.com/cgi/SiG.cgi/Products/GeneSpring/index.smf.

Informax: www.informaxinc.com/solutions/xpression/main.html.

The R project for statistical computing www.r-project.org.
Bioconductor: www.bioconductor.org.

Robotic Spotters and Arrayers

Genetix (Q array): www.genetix.co.uk.
Biorobotics (TAS): www.biorobotics.com.
Amersham (Lucidea): www5.amershambiosciences.com/APTRIX/upp01077.nsf/Content/microarrays_deposition?OpenDocument&hometitle=microarrays.

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Profiling Gene Transcription in the Developing Embryo

Microarray Analysis on Gene Chips

David Chambers and Andrew Lumsden

1. Introduction

As a result of whole-genome sequencing and other genetic approaches, microarray-based screening strategies for the first time have allowed genome-wide insight into the transcriptional regulatory events of many model organisms. The capacity of current oligonucleotide arrays (i.e., the number of individual transcripts represented on a single chip) along with developments in scanning resolution now mean that entire transcriptomes can be read on a single array. Therefore, it is likely that, as remaining genes are uncovered, annotated, and incorporated into ever-more-comprehensive arrays, microarray screening approaches will become the de facto standard for the interrogation of transcriptional regulation. Considering this, the application of the microarray screening to address fundamental questions of cellular specification or patterning in the developing embryo has been relatively slow to emerge. However, recent studies have demonstrated the usefulness of microarray screening and shown how transferable the technology is to molecular embryology (e.g., ***[1]***). The following procedures describe the approaches required to generate array data from either discrete embryo tissues or isolated cell populations (e.g., fluorescent activated cell sorting methodologies), including RNA preparation, cDNA generation, in-vitro translation, labeled extract preparation, fragmentation, hybridization to arrays, and subsequent washing. The protocols presented are modified from the manufacturer's originals and adapted for use with Affymetrix GeneChips (expression arrays) using single-channel fluorescence, although total RNA and labeled extract preparation are applicable to other oligonucleotide-based microarray

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systems. Issues such as experimental design, number of replicates, bioinformatic analysis, and data validation are dealt with elsewhere (e.g., *see* ***(2)***).

2. Materials

2.1. General

1. EDTA (Sigma E7889).
2. Tween 20 (Sigma P-1379).
3. 5 M NaCl (Ambion 9760G).
4. Benchtop microfuge (preferably cooled; e.g., Sigma 1K15 Philip Harris, UK)
5. Horizontal gel electrophoresis tank and power supply (e.g., Life Technologies, Scotland).
6. 10× TBE: 0.89 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA; per liter 108 g Tris base, 55 g boric acid, 9.4 g EDTA.
7. Ethidium bromide (10 mg/mL in water,stored at 4°C in the dark; Invitrogen).
8. 1 kb Plus DNA LADDER (10488-085 Invitrogen).
9. Microarray platform: For protocols used here, computer workstation Type I with GeneChip® operating software (a Windows XP-based unit with pre-installed specialized controller boards to control the GeneChip Scanner 3000 and Fluidics Station 450) 00-0180, hybridization oven 640 800139, GeneChip Fluidics Station 450 00-0079, GeneChip Scanner 3000 7G System 00-0213.
10. Spectrophotometer (e.g., Beckman DU 530 ; *see* **Note 14**).

2.2. Total RNA Extraction

1. DNase- RNase-free water (Ambion 9930).
2. TRIzol reagent (Invitrogen 15596-018).
3. Chloroform (BDH).
4. Optional: Phase-lock gel tubes (Merck 427366 U).
5. 70% ethanol (prepared with DNase- RNase-free water).
6. 100% isopropanol (BDH).
7. Glycogen (5 mg/mL; Ambion 9510).
8. Optional: RNeasy mini kit (Qiagen 74104).
9. Absolutely RNA miniprep kit (Stratagene 400800).

2.3. Labeled Extract Preparation and Microarray Hybridization: BioArray Highyield RNA Transcript Labeling System (Enzo)

1. Superscript II cDNA synthesis kit containing RT (200 U/µL), 5× first-strand reaction buffer, 0.1 M DTT, 10 mM dNTP mix, SuperScript™ II RT, 5× second-strand reaction buffer, *E. coli* DNA ligase, *E. coli* DNA polymerase I, *E. coli* RNase H, T4 DNA polymerase, and DEPC-treated water. (Invitrogen 11917-010).
2. Poly-A RNA control kit (Affymetrix 900433).
3. T_7–T_{24} primer (GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(T)_{24}$; Affymetrix 900375; *see* **Note 17**).
4. DNA ligase (10 U/µL; Invitrogen 18052-019).

5. BioArray high-yield RNA transcript labeling kit (Enzo Diagnostics 42655-10).
6. GeneChip sample cleanup module (Affymetrix 900371).
7. Test 3 arrays (Affymetrix 900341).
8. Eukaryotic hybridization controls (Affymetrix 900454).
9. B2 control oligonucleotide (Affymetrix 900301).
10. Expression arrays (e.g., MOE430v2; Affymetrix 900495).
11. MES (2-[N-morpholino]ethanesulfonic acid) hydrate (Sigma M-5287).
12. MES (2-[N-morpholino]ethanesulfonic acid) sodium salt (Sigma M-5057).
13. 5 M NaCl (Ambion 9760G).
14. 0.5 M EDTA (Sigma E7889).
15. Herring sperm DNA (e.g., Sigma D7290; preferably molecular biology grade).

2.4. Labeled Extract Preparation and Microarray Hybridization: Ovation Biotin System

1. Ovation Biotin System (NuGEN Technologies 2300-12; 12 reactions).
2. DyeEx columns (Qiagen 63204).

3. Methods

3.1. General

The protocol for extraction of total RNA depends on the expected yield. Where >1 µg has been calculated (*see* **Notes 1–7**), the TRIzol procedure is recommended. For amounts <1 µg we routinely use a column-based strategy (e.g., Absolutely RNA miniprep kit, Stratagene). All steps are performed at room temperature unless otherwise stated. Throughout the whole procedure, good practice for working with RNA should be adopted, including wearing gloves and using sterile plastic and glassware (*see* **Note 4**).

3.2. Total RNA Extraction: TRIzol Protocol

1. Homogenize specific tissue or cell population from the embryo in 1 mL of TRIzol reagent per 100 mg tissue or per 10^6 cells in suspension. Where chunks of tissue are directly added to the TRI reagent, they should be immediately homogenized (or thoroughly vortexed). Homogenized samples are left at 37°C for 10 minutes to ensure complete dissociation of nucleoprotein complexes. If multiple samples are being processed simultaneously, TRIzol aliquots can be stored at −80°C prior to further processing. Frozen aliquots must be thawed at room temperature for 5 min before proceeding to **step 2**.
2. Add 0.2 mL of chloroform per 1 mL TRIzol, cap the tubes and vortex for 15 sec. Following incubation at room temperature for 2–3 min, centrifuge the samples at maximum speed (>10,000 *g*) in a microfuge at 4°C for 20 min. Following phase separation, carefully remove the colorless upper aqueous phase containing the total RNA (approximately 3× volume of chloroform used), being sure to completely avoid disturbing the interface (containing genomic DNA) and organic phase (containing cellular proteins etc.) and transfer to a fresh RNase-free tube.

3. To obtain purer RNA, we introduce one of two additional steps. Either add fresh TRIzol back to the aqueous phase and repeat **step 2** or centrifuge the aqueous phase for a further 5 min and check for the presence of any organic solution. If found, recover the aqueous phase exclusively. If RNA amounts are limiting, a double extraction procedure may not be desirable.
4. To precipitate the total RNA, add 0.5 mL isopropanol per mL of TRIzol used, vortex briefly incubate at room temperature for 10 min. To enhance the yield of RNA, add 1 µL of glycogen to the reaction and leave for a further 5 min. Recover the RNA by centrifugation at 12,000 *g* for 15 min. The RNA should appear as a small gellike pellet at the bottom of the tube. Alternatively, at this point, the RNA can be recovered from solution by passing the mixture through a RNA binding column (e.g., from the RNeasy mini kit) and following manufacturer's instructions (*see* **Notes 8** and **9**)
5. Carefully remove the supernatant without disturbing the pellet and wash with 0.5 mL of ice cold 70% ethanol. If the pellet is dislodged during this process, the tubes can be centrifuged (12,000 *g*) for 2–3 mins. Repeat the washing step and remove the final 70% ethanol solution.
6. Briefly air dry the pellet and resuspend the RNA in an appropriate volume of ice-cold RNAse- Dnase-free water. Allow the pellet to sit in the water for at least 10 min on ice before resuspending by pipeting. If the pellet does not dissolve fully, incubate at 37°C for 5 min. It is important that the final concentration is sufficiently high to continue with the labeling procedures. Using this method, it is not necessary to add RNase inhibitors to ensure viability.
7. Check the RNA quantity and quality using spectrophotometry, as described elsewhere. If the final concentration is very low, it may be necessary to use specialized equipment, such as a nanodrop spectrophotometer (www.nanodrop.com), for accurate measurement. The integrity of the RNA should be established by running at least 50 ng on a denaturing gel and checking for the characteristic 18 and 28s rRNA bands (*see* **Note 10** and **Fig. 42.2A**, later). Where available, the exact profile of the total RNA can be determined using a Bioanalyser (www.chem.agilent.com) and comparison well established criteria for intact RNA (*see* manufacturer's instructions and details therein).

3.3. Total RNA Extraction: Column Protocol (Absolutely RNA Miniprep Kit)

1. Prepare the lysis and low and high salt-wash buffers as per manufacturer's instructions. Add β-ME to the appropriate amount of lysis buffer on the day of extraction and do not use beyond that day.
2. Calculate the correct volume of lysis buffer for the amount of tissue to be processed. The values we successfully used for FACS-isolated cells (*see* **Note 11**) follow:
    a. 100–10^4 cells 100 µL lysis buffer (0.7 µL β-ME).
    b. 10,000–10^5 cells 250 µL lysis buffer (1.75 µL β-ME).
    c. 10,000–10^6 cells 350 µL lysis buffer (2.5 µL β-ME).

3. Pellet the cells by centrifugation (1,000 *g* for 5 min), aspirate the supernatant, and resuspend in the appropriate volume of lysis buffer- β-ME mixture.
4. Immediately homogenize or triturate the cell pellet to ensure rapid and complete disruption of all cell membranes. If the mixture is particularly resistant to mixing, it can be passed through a 19-gauge syringe needle several times. We store the lysate mixture for several months at this point.
5. Transfer the homogenate (up to 700 µL) to a prefilter cup (placed in a 2-mL collection tube), cap, and centrifuge (12,000 *g*) for 5 min. This step is vital to remove any cellular debris before proceeding.
6. Remove the spin cup and discard. To precipitate the RNA, add an equal volume of 70% ethanol to the filtrate (i.e., 100, 250, or 350 µL) and vortex for 15 sec. Allow this reaction to stand for 10 min at room temperature before the next step.
7. Transfer this entire mixture (up to 700 µL) to a RNA binding spin cup that has been placed in a fresh 2-mL tube (supplied with kit). To maximize yields, we allow the RNA at least 10 min at room temperature to fully adsorb to the column.
8. To facilitate maximum RNA binding, spin the mixture through the column 30 sec (12,000 *g*). Recover the filtrate and immediately pass back through the column to promote full recovery of total RNA.
9. Wash the immobilized RNA on the column by adding 600 µL of 1× low-salt wash buffer followed by spinning at maximum speed (12,000 *g*) for 30 sec. Transfer the spin cup to a fresh tube and spin for a further 2 min to make sure that column is adequately dry prior to **step 10**.
10. For each spin cup in use, add 55 µL of DNase solution (made as per manufacturer's instructions) directly to the center of the column and leave for 15 min at room temperature. It is implicit that the solution is added to the center of the column and subsequently distributed evenly (*see* **Note 12**)
11. Wash by the addition of 600 µL of 1× high-salt wash buffer, cap, and spin at 12,000 *g* for 30 sec.
12. Wash by the addition of 600 µL of 1× low-salt wash buffer, cap, and spin at 12,000 *g* for 30 sec.
13. Wash by the addition of 300 µL of 1× high-salt wash buffer, cap, and spin at 12,000 *g* for 30 sec.
14. Transfer the spin cup to a sterile 1.5 mL tube and add 30 µL of elution buffer directly to the center of the column. Leave the column to sit at room temperature for 15–30 min (*see* **Note 13**) before **step 15**.
15. Recover the RNA by spinning (12,000 *g*) for 2 min. Collect the eluate and pass through the column once more.
16. The purified RNA is quantified and its integrity validated as described previously (*see* **Note 14** and **Fig. 42.3A** later).

3.4. Labeled Extract Synthesis: General

Along with the isolation of a high-quality representative RNA population, the synthesis of labeled extracts for hybridization to a microarray is a crucial step in determining the overall success of a microarray experiment. At this point, factors

such as fidelity (e.g., 3 /5 ratio), representation (e.g., % present call), and background (e.g., noise/raw Q) levels (*see* www.Affymetrix.com for an explanation of these terms and their relevance to a informative microarray experiment) can be dramatically affected by the methodological approach. Many protocols and kits are available for processing total RNA into biotin-labeled cRNA. The kits vary not only in the technology employed but also in the amount of total RNA required. Here, we present two protocols that cover both low and high starting amounts of total RNA (10 ng to 2 µg, respectively) and have proven robust and reproducible in our hands (*see* **Note 15**).

3.4.1. Labeled Extract Synthesis: BioArray High-Yield RNA Transcript Labeling System (for 2 µg or Total RNA and Above)

The BioArray Highyield RNA transcript labeling system can be subdivided into discrete steps (*see* **Fig. 1**)

1. First-strand cDNA synthesis.
2. Second-strand cDNA (ss cDNA) synthesis.
3. Clean up of ss cDNA.
4. In-vitro transcription reaction (production of biotin labeled cRNA).
5. Clean up of biotin labeled cRNA.
6. Fragmentation of labeled cRNA.
7. Hybridization of fragmented cRNA to microarray.

3.4.2. BioArray High-Yield RNA Transcript Labeling System: First-Strand cDNA Synthesis

1. Prepare the following reagents, mix by trituration, and spin briefly to collect the contents (*see* **Notes 16** and **17**):
   a. X µL (2 µg) total RNA in nuclease free dH_2O.
   b. Y µL (100 pmol) T_7–$(T)_{24}$ primer (*see* **Note 18**).
   c. 1 µL Poly A^+ controls (*see* **Note 19**).

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total RNA (see **section 3.4**). If the poly A RNA contained within the total RNA sample is successfully converted to biotin labeled cRNA it forms a characteristic smear as shown (see **section 3.4.5.**). The presence of labeled cRNA >3-4kb is indicative of efficient conversion. (**C**) An example of a scan generated by hybridizing one of the labeled extracts from (**B**) to an Affymetrix Mouse 430 2 GeneChip. Using the protocols detailed in **sections 3.4.1-8**, expression data was obtained that meets the requisite quality control data (i.e. performance of poly A controls, hybridization controls, 3' /5' ratios, number present and noise etc [See www.Affymetrix.com for further information regarding quality control indicators). The edge of the microarray and data label can be seen due to the hybridization of the B2 oligo (*see* **note 36**). (*See* color plate)

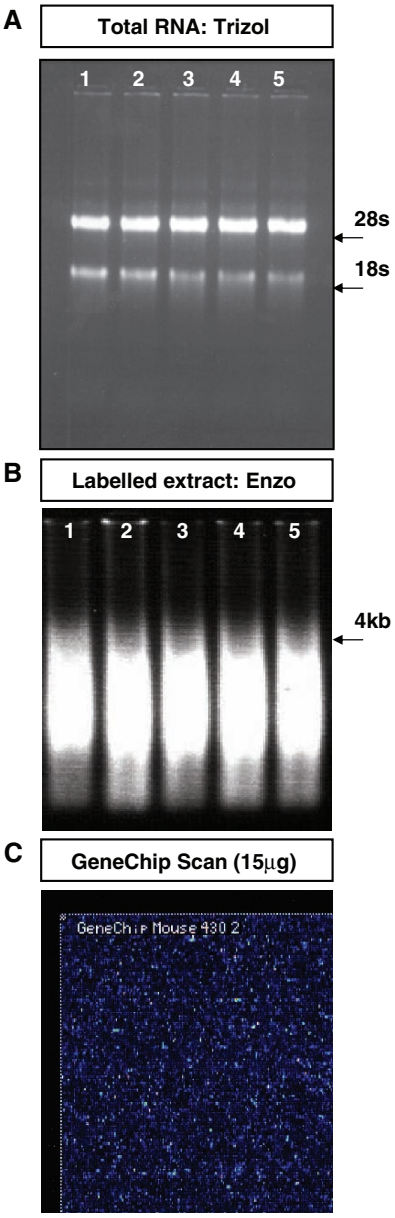


Fig. 1. (**A**) Agarose gel electrophoresis of total RNA (500ng/lane) extracted from five different regions (1-5) of the neural tube of E9.5 CD1 mouse embryos using the Trizol protocol detailed in **section 3.2**. The arrows point to the characteristic 28s and 18s rRNA bands. For intact total RNA, the 28s band should be approximately double the intensity of the 18s band (*see* **Notes 1-11**). (**B**) Agarose gel electrophoresis of the biotin labeled extracts generated by the Enzo BioArray Highyield RNA Transcript Labelling System from 2μg of starting

d. Z µL nuclease-free dH_2O.
e. 11 µL final volume or reaction.

2. Incubate at 70°C for 10 min to denature any secondary structure in the RNA transcripts, then place on ice (*see* **Note 20**).
3. Prepare the following reaction master mix on ice. The amounts are for individual reactions. Mix well by pipeting and spin to collect if necessary.
   a. 4 µL 5× first-strand buffer (thaw at 37°C prior to use, *see* **Note 21**).
   b. 2 µL DTT (0.1 M) (supplied).
   c. 1 µL dNTPs (10 mM).
   d. 7 µL final volume or reaction.
4. Transfer 7 µL of master mix to each of the heat denatured RNAs on ice and incubate at 42°C for 2 min.
5. Add 2 µL (400 U) Superscript II reverse transcriptase and mix gently by pipeting while still on ice.
6. Incubate at 42°C for 1 h in either a water bath or a hot block.
7. Spin to collect any evaporation and place tubes on ice. Here, the reactions can be frozen on dry ice and stored at −80°C. However, it is recommended to proceed directly to second-strand cDNA synthesis.

3.4.3. BioArray High-Yield RNA Transcript Labeling System: Second-Strand cDNA Synthesis

1. Prepare the reagents in the following order, mix by triturating, and spin briefly to collect the contents:
   a. 30 µL 5× second-strand buffer (supplied).
   b. 3 µL 10 mM dNTPs.
   c. 1 µL DNA ligase (10 U).
   d. 4 µL DNA Pol I (40 U) (supplied).
   e. X µL RNase H (2 U) (see Note 22).
   f. Y µL nuclease-free dH2O (not DEPC treated).
   g. 130 µL final volume or reaction.
2. On ice, add 130 µL of the master mix to each of the first-strand reaction tubes. Mix well by triturating, and spin briefly to collect the contents (*see* **Note 23**).
3. Incubate at 16°C for 2 h in either a water bath in a cold room or a chilled block with a lid.
4. Pulse the tubes to collect any condensation and place the reactions on ice. The samples may be frozen on dry ice and stored at −80°C prior to further processing.

3.4.4. BioArray High-Yield RNA Transcript Labeling System Cleanup of Second-Strand cDNA: GeneChip Sample Cleanup Module

1. The cDNA synthesis reaction can be cleaned up using either a phenol chloroform or column-based approach (i.e., GeneChip sample cleanup module, Affymetrix). We preferentially use the latter, as it is rapid and avoids any loss of material associated with precipitation and centrifugation. (*see* **Note 24**).
2. Prepare of the buffers as per manufacturer's instructions (i.e., add 24 mL of 100% ethanol to cDNA wash buffer concentrate).

3. Transfer the cDNA synthesis reactions to a sterile 1.5-mL tube on ice and add 600 µL of cDNA binding buffer to the 150 µL final double-stranded cDNA synthesis preparation. Mix by vortexing for 3 sec and pulse in a centrifuge to collect.
4. Apply 500 µL of the sample to the cDNA cleanup spin column sitting in a collection tube, and centrifuge for 1 min at 8,000 *g* (approximately 10,000 rpm in a standard benchtop microfuge). Discard the flow-through. (*see* **Note 25**).
5. Apply the remaining 250 µL to the column and repeat the preceding spin. Discard the flow-through.
6. Transfer spin column into a new 2-mL collection tube and add 750 µL cDNA wash buffer (diluted with ethanol as described) onto the spin column. Centrifuge for 1 min at 8,000 *g*. Discard the flow-through.
7. Place the column a new 2-mL collection tube, add 750 µL cDNA wash buffer, and spin 1 min at 8,000 *g*. Discard the flow-through but retain the 2-mL collection tube.
8. Open the cap of the spin column and centrifuge for 5 min at maximum speed (12,000 *g*). Discard the flow-through and collection tube.
9. Transfer spin column into a 1.5-mL collection tube, and add 14 µL of room temperature cDNA elution buffer directly onto the spin column membrane. Incubate for 5 min at room temperature and centrifuge for 1 min at maximum speed (12,000 *g*) to elute. Ensure that the cDNA elution buffer is dispensed directly onto the membrane. The average volume of eluate is 12 µL from 14 µL elution buffer (*see* **Note 26**).

3.4.5. BioArray High-Yield RNA Transcript Labeling System: RNA Labeling In-Vitro Transcription Reaction

1. The protocol described here is an adaptation from that supplied with the BioArray high-yield RNA transcript labeling kit (*see* **Note 27**). Thaw the high-yield 10× reaction buffer and DTT at 37°C and keep at room temperature prior to use. Thaw all other reagents at room temperature and place the enzymes on ice. It is essential that samples and reactions constituting the same microarray study be derived from the same master mix.
2. Prepare the reagents in the following order, mix by triturating, and spin briefly to collect the contents:
   a. 12 µL double-stranded cDNA (eluate from cleanup) (see Note 28).
   b. 10 µL nuclease-free dH2O.
   c. 4 µL 10× high-yield reaction buffer (supplied).
   d. 4 µL biotin-labeled ribonucleotides (supplied).
   e. 4 µL DTT (supplied).
   f. 4 µL RNase inhibitor mix.
   g. 2 µL T7 RNA polymerase (supplied).
   h. 40 µL final volume or reaction.
3. Immediately place the tube in a 37°C water bath, hybridization oven, or thermocycler in which the heated lid parallels the block temperature. Incubate for 16 h. Periodically mix the components by gentle pipeting and pulse to collect condensation. We generally leave this reaction to run overnight in a 37°C water bath with a sealed lid.

4. Pulse the tubes to collect condensation and place on ice. The samples may be frozen at −80°C prior to further processing. Remove a 4-µL aliquot from the unpurified reaction and analyze by running (1 h, 120 V, constant current) the entire sample on a 0.8% (w/v) agarose gel in 1× TBE containing ethidium bromide (0.1 µg/mL). (*See* **Notes 29–31** and **Fig. 2B**).
5. Proceed to the cleanup protocol.

3.4.6. BioArray High-Yield RNA Transcript Labeling System: RNA Labeling In-Vitro Transcription Reaction: Clean Up

1. Before hybridization to a microarray, the biotin-labeled RNA must be purified away from unincorporated nucleotides. It is inadvisable to perform this operation using a phenol chloroform approach because the biotin causes some of the RNA to be partitioned into the organic phase, thus lowering the yield. Instead, use the same cleanup columns used to purify the cDNA reaction (GeneChip sample cleanup module, Affymetrix). Before proceeding, ensure that buffers have been diluted with ethanol where appropriate. This protocol is supplied by the manufacturer and is described here with minor modification
2. Add 60 µL of RNase-free water to the in-vitro transcription reaction and mix by vortexing for 3 sec.
3. Add 350 µL IVT cRNA binding buffer to the sample and mix by vortexing for 3 sec.
4. Add 250 µL ethanol (100%) to the lysate, and mix well by pipeting. Do not centrifuge.
5. Apply the sample (700 µL) to the IVT cRNA cleanup spin column sitting in a 2-mL collection tube. Centrifuge for 15 sec at ≥8,000 *g* (≥10,000 rpm). Discard the flow-through and collection tube.
6. Transfer the spin column into a new 2-mL collection tube. Pipet 500 µL IVT cRNA wash buffer onto the spin column. Centrifuge for 15 sec at ≥8,000 *g* (≥10,000 rpm) to wash. Discard flow-through.
7. Pipet 500 µL 80% (v/v) ethanol onto the spin column and centrifuge for 15 sec at 8,000 *g* (≥10,000 rpm). Discard the flow-through.
8. Open the cap of the spin column and centrifuge for 5 min at maximum speed. Discard the flow-through and collection tube (*see* **Note 25**).

10ng of starting total RNA (see **section 3.5**). If the poly A RNA contained within the total RNA sample is successfully converted to biotin labeled cDNA it forms a characteristic smear as shown (see **section 3.5.4.**). The presence of labeled cRNA >0.4kb is indicative of efficient conversion (see http://www.nugeninc.com/html/02_technology1.ht for further information). (**C**) An example (control panel) of a scan generated by hybridizing one of the labeled extracts from (**B**) to an Affymetrix Mouse 430 2 GeneChip. Using the protocols detailed in **sections 3.5.1-8**, expression data was obtained that meets the requisite quality control data (i.e. performance of poly A controls, hybridization controls, 3' /5' ratios, number present and noise etc [See http://www.nugeninc.com/pdfs/WT_Ovation_UserGuide.pdf for further information regarding quality control indicators). (*See* color plate)

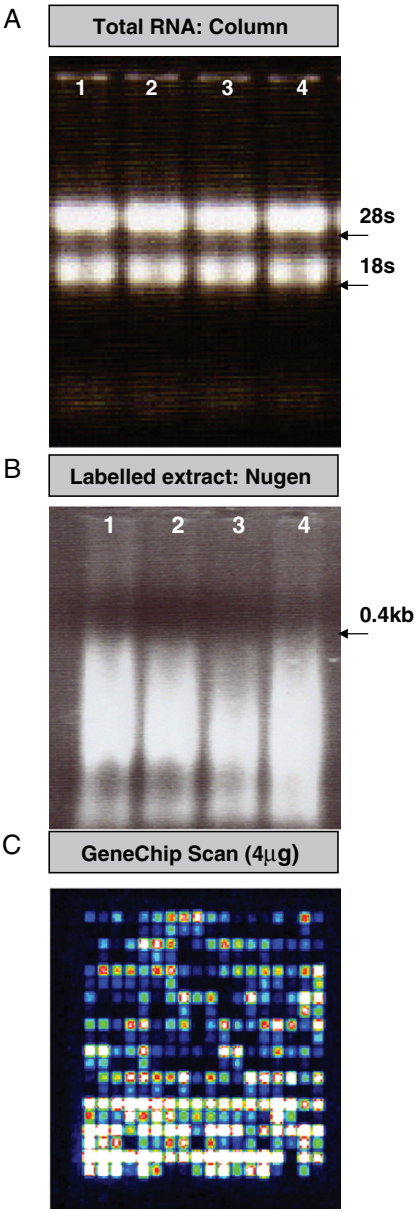


Fig. 2. (**A**) Agarose gel electrophoresis of total RNA (500ng/lane) extracted from four different regions (1-4) of the neural tube of E9.5 CD1 mouse embryos using the column protocol detailed in **section 3.3**. The arrows point to the characteristic 28s and 18s rRNA bands. For intact total RNA, the 28s band should be approximately double the intensity of the 18s band (*see* **Notes 1-11**). (**B**) Agarose gel electrophoresis of the biotin labeled extracts generated by the Ovation Biotin RNA Amplification and Labelling System (Enzo) from

9. Transfer spin column into a new 1.5-mL tube and pipet 11 µL of RNase-free water directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane and leave it to stand for 5 min at room temperature. Centrifuge for 1 min at maximum speed (12,000 *g*) to elute. To increase the yield, recover the eluate and place it back onto the membrane. Leave to stand for a further 2 min at room temperature. Centrifuge for 1 min at maximum speed (12,000 *g*) to elute.
10. For subsequent photometric quantification of the purified cRNA, we perform a 1:100-fold dilution of the eluate. Using these protocols we generate in the region of 15–20 µg of cRNA per 2 µg of total RNA (*see* **Note 32**). The labeled cRNA can be stored for several months at −80°C prior to further processing.

3.4.7. BioArray High-Yield RNA Transcript Labeling System: RNA Labeling In-Vitro Transcription Reaction—Fragmentation of the IVT Reaction

1. For hybridization to Affymetrix oligonucleotide-based microarrays the biotin-labeled cRNA must be first fragmented. Fragmentation shears the cRNA molecules to a size of approximately 100 bases. The smaller size is necessary to facilitate efficient and faithful binding to the complementary oligonucleotide on the microarray. The protocol described here is for sufficient cRNA to hybridize to one array (15 µg) (*see* **Notes 30** and **32**)
2. Prepare the reagents in the following order at room temperature, mix by triturating, and spin briefly to collect the contents:
   a. X µL 20 µg cRNA (minimum concentration 1.25 µg/µL).
   b. 4 µL 5× fragmentation buffer (supplied).
   c. Y µL RNase-free water.
   d. 20 µL final volume or reaction.
3. Place the reactions at 95°C for 35 min, preferably in a hot block or thermal cycler with a heated lid.
4. Pulse the reactions briefly to collect any condensation and place on ice.
5. Transfer 15 µL (15 µg) to a clean tube and either proceed to hybridization or freeze immediately at −80°C. Use the remaining 5 µL to assess the efficacy of the fragmentation process (*see* **Note 33**)

3.4.8. BioArray High-Yield RNA Transcript Labeling System: Hybridization of the Fragmented cRNA to a Microarray

1. Prior to hybridization to an expression array, it is prudent to assess the representation of the fragmented cRNA by first applying to a test array (*see* **Note 34**).
2. The following protocol is for hybridization to a full expression array. Prepare 2× hybridization buffer. This can be prepared in advance and then stored away from light at 4°C for up to 12 mo.

a. 8.3 mL 12× MES stock.
b. 17.7 mL 5 M Nacl.
c. 4.0 mL 0.5 M EDTA.
d. 0.1 mL 10% (v/v Tween 20).
e. 19.9 mL nuclease-free water.
f. 50 mL final volume or reaction.
g. The 12× MES stock consists of
h. 70.4 g MES free-acid monohydrate.
i. 193.3 g MES sodium salt.
j. 800 mL molecular-biology-grade water.
k. Mix and adjust volume to 1,000 mL and pH to 6.5. Filter the solution through 0.2 µM mesh (do not autoclave).

3. Following successful IVT and fragmentation reactions, prepare a master mix and for each sample add:
   a. 150 µL 2× hybridization buffer.
   b. 15 µL 20× eukaryotic hybridization controls (*see* **Note 35**).
   c. 5 µL control oligonucleotide B2 (3 nM) (*see* **Note 36**).
   d. 3 µL herring sperm DNA (10 mg/mL).
   e. 3 µL acetylated BSA (50 mg/mL).
   f. 15 µL fragmented cRNA (15 µg).
   g. 109 µL nuclease-free water.
   h. 300 µL final volume or reaction.
4. Thoroughly mix the reagents and pulse briefly to collect the contents before either proceeding with hybridization or freezing on dry ice and storing at −80°C.
5. Equilibrate the GeneChips (stored at 4°C) to room temperature. This is essential to prevent subsequent rupturing of seals and septa (approximately 20 min). Set the hybridixation oven (Affymetrix GeneChip hybridization oven 640, 800138).
6. Heat the hybridization cocktail containing the fragmented cRNA at 95°C for 5 minutes in a hot block or thermal cylcer with a heated lid to fully denature the transcripts. Pulse briefly to collect condensation.
7. Cool the heat denatured cRNA at 45°C for 5 min and centrifuge for 5 min at maximum speed (12,000 *g*) in a microfuge.
8. During this incubation, remove the liquid from inside chamber of GeneChip (approximately 200 µL) and replace with 200 µL of 1× hybridization mix. Incubate in the hybridization oven at 45°C, 60 rpm for 10 min (*see* **Notes 37** and **38**). The remaining mix can be stored at −20°C.
9. Remove the 1× hybridization mix from the array (remembering to insert the additional pipet tip into the other septa).
10. Carefully draw off 200 µL from the top (avoiding any deposits at base of tube) of the denatured, spun hybridization–cRNA mix and pipet into the appropriately matched and labeled GeneChip. Check the septa for leaks. If these are found to be persistently damaged by filling and draining, additional covers can be purchased from Affymetrix.

11. Incubate at 45°C, 60 rpm for 16 h.
12. After this incubation (generally overnight), recover the hybridization mix from the array and store at −20°C, spin briefly to collect the contents in a fresh tube. Where necessary, hybridization–cRNA mixes can be reused for other arrays, although not as a biological replicate. Using this system, technical replicates are not necessary (*see* **Note 39**).
13. Replace the hybridization–cRNA mix with 200 µL of wash buffer A.

    Following hybridization, the Affymetrix GeneChip must be washed, stained, and scanned to obtain a quantitative measure of hybridization to each of the probes immobilized on the chip (*see* **Fig. 2C**). However, the washing and staining protocols used are specific to the type or version of chip used and the steps therein are dictated by the default GeneChip operating software driving these processes. Manual alterations to these protocols can be made, but the subsequent effects on the efficacy of staining should be carefully monitored. In general, these protocols work well and should be followed as closely as possible to ensure reproducibility and possible comparisons with other, similarly produced data sets. Where appropriate or relevant, all reagents should be divided and stored in rational amounts. For older Affymetrix systems, it is also imperative to ensure that the scanner is capable of recognizing the resolution of the proposed GeneChip to be used (i.e., recent version 2 incarnations of the GeneChips; *see* www.affymetrix.com).

3.5. Labeled Extract Synthesis: Ovation Biotin RNA Amplification and Labeling System (for 10 ng Total RNA)

The need to analyze gene expression in homogenous cell populations by microarray often dictates that relatively small quantities of total RNA are available for processing. Although some PCR-based protocols generate labeled extracts (e.g., ***[3, 4]***) the ability to robustly and reproducibly synthesize these from very small amounts of total RNA has only recently become possible with advances in RNA amplification procedures. In our hands, the Ribo-SPIA system developed by Nugen (www.nugeninc.com) has proven an extremely efficient, robust method for working with as little as 10 ng total RNA starting material. This amplification process uses a chimeric primer, RNase H, and DNA polymerase to initiate replication of a transcript that is repeated up to 10,000 times, resulting in a linear accumulation of each transcript species in the total RNA pool. The amplified product is antisense cDNA that can be fragmented, biotin labeled, and hybridized to oligonucleotide arrays, as described previously. Data provided by the manufacturer and obtained by us have shown this system to be highly technically reproducible and to yield comparable results (e.g., present calls) to the T_7 polymerase-based systems described earlier and elsewhere. A further description of the technology underlying this process can be found at www.nugeninc.com/html/02_technology1.html.

The BioArray high-yield RNA transcript labeling system can be subdivided into discrete steps:

1. First-strand cDNA synthesis.
2. Second-strand cDNA synthesis.
3. SPIA amplification reaction.
4. Purification of SPIA amplification reaction.
5. Fragmentation of SPIA amplification reaction.
6. Biotin labeling of SPIA amplification reaction.
7. Purification of biotin-labeled SPIA cDNA.

3.5.1. Ovation Biotin RNA Amplification and Labeling System: First-Strand cDNA Synthesis

1. Thaw the nuclease-free water and first-strand reagents at room temperature and ensure that they are thoroughly mixed. (The first-strand primer mix is stored at −80°C, while the other reagents are stored at −20°C).
2. Combine 5 µL of total RNA (10–100 ng) with 2 µL of first-strand primer mix (labeled A1 by NuGEN) in a thin-walled 0.2-mL PCR tube, mix gently by triturating, spin briefly to collect the contents (*see* **Note 40**).
3. Heat at 65°C for 5 min and snap cool on ice (*see* **Note 20**).
4. Prepare a master mix of the following reagents, mix by triturating, and spin briefly to collect the contents:
   a. 12 µL first-strand buffer buffer (supplied).
   b. 1 µL first-strand enzyme mix (reverse transcriptase) (supplied).
   c. 13 µL final volume or reaction.
5. Add 13 µL of this mix to 7 µL RNA–primer mix and incubate for the following (ensure the heated lid is operational; *see* **Note 41**):
   a. 48°C for 60 min.
   b. 70°C for 15 min.
   c. 4°C forever.
6. Briefly spin the tubes to collect any condensation, place on ice, and proceed directly to second-strand reaction

3.5.2. Ovation Biotin RNA Amplification and Labeling System: Second-Strand cDNA Synthesis

1. Thaw the second-strand buffer at room temperature and ensure it is thoroughly mixed. Place the second-strand enzyme mix on ice after gently mixing.
2. Prepare a master mix of the following reagents, mix by triturating, and spin briefly to collect the contents:
   18 µL second-strand buffer (supplied).
   2 µL second-strand enzyme mix (DNA polymerase) (supplied).
   20 µL final volume or reaction.
3. Add 20 µL of second-strand master mix to each first strand reaction, mix, and spin.

4. Place the tubes in a thermal cycler set to run the following program (ensure the heated lid is operational):
   37°C for 60 min.
   75°C for 15 min.
   4°C forever.
5. Once the reactions have been cooled to 4°C, pulse in a centrifuge, and place on ice.
6. The reactions can be stored at −80°C here if necessary.

3.5.3. Ovation Biotin RNA Amplification and Labeling System: SPIA Amplification

1. Thaw the SPIA amplification reagents at room temperature and ensure they are thoroughly mixed. Place the SPIA enzyme mix on ice after gentle mixing.
2. Prepare a master mix of the following reagents on ice, mix by triturating, and spin briefly to collect the contents:
   a. 72 µL SPIA buffer mix (supplied).
   b. 4 µL SPIA primer mix (supplied).
   c. 4 µL nuclease-free water.
   d. 40 µL SPIA enzyme mix (DNA polymerase, RNase H) (supplied).
   e. 120 µL final volume or reaction.
3. Add 120 µL of SPIA amplification master mix to each second-strand reaction (40 µL), mix, and spin
4. Split the 160 µL reaction into two separate 0.2-mL thin-walled tubes to ensure thermal coverage of the reaction by the block of the PCR machine (*see* **Note 42**).
5. Place the tubes in a thermal cycler set to run the following program (ensure the heated lid is operational):
   a. 48°C for 60 min.
   b. 95°C for 15 min.
   c. 4°C forever.
6. Once the reactions have been cooled to 4°C, pulse in a centrifuge, recombine the two halves of each reaction (to give final volume of 160 µL), place on ice, and proceed directly to quality-control step.
7. The efficacy of the SPIA amplification should be checked at this point by running a small amount of the reaction (3 µL of 160 µL) on a 1% agarose gel containing ethidium bromide and using molecular weight markers (e.g., 1 kb plus ladder). The amplified product should appear as a smear ranging between 200 bp and approximately 0.4 kb (*see* **Note 42** and **Fig. 42.2B**). To increase accuracy, the concentration of the amplified cDNA should be checked after purification.
8. The reactions can be stored at −20°C here if necessary or proceed directly to the purification step.

3.5.4. Ovation Biotin RNA Amplification and Labeling System: Purification of SPIA cDNA

1. The SPIA amplification product can be cleaned up using the GeneChip sample module as described previously, with only the few modifications that follow.

2. Prepare of the buffers as per manufacturer's instructions (i.e., add 24 mL of 100% ethanol to cDNA wash buffer concentrate).
3. Transfer each SPIA reaction to a sterile 1.5-mL tube on ice and add 600 µL of cDNA binding buffer to 150 µL of the SPIA amplified cDNA reaction. Mix by vortexing for 3 sec and pulse in a centrifuge to collect.
4. Apply 500 µL of the sample to the cDNA cleanup spin column sitting in a collection tube, and centrifuge for 1 min at 8,000 *g* (approximately 10,000 rpm in standard benchtop microfuge). Discard the flow-through.
5. Apply the remaining 250 µL to the column and repeat the preceding spin. Discard the flow-through.
6. Transfer spin column into a new 2-mL collection tube and add 750 µL cDNA wash buffer (diluted with ethanol as described) onto the spin column. Centrifuge for 1 min at 8,000 *g*. Discard the flow-through.
7. Place the column a new 2-mL collection tube, add 750 µL cDNA wash buffer and spin 1 min at 8,000 *g*. Discard the flow-through but retain the 2-mL collection tube.
8. Open the cap of the spin column and centrifuge for 5 min at maximum speed (12,000 *g*). Discard flow-through and collection tube.
9. Transfer spin column into a 1.5-mL collection tube, and add 34 µL of room-temperature cDNA elution buffer directly onto the spin column membrane. Incubate for 5 min at room temperature and centrifuge for 1 min at maximum speed (12,000 *g*) to elute. Ensure that the cDNA elution buffer is dispensed directly onto the membrane. The average volume of eluate is 33 µL from 34 µL elution buffer.
10. The efficacy of the cleanup process can be checked by running 1 µL of the eluate on a 1% agarose gel containing ethidium bromide (*see* **Fig. 42.3B**).
11. The yield of the SPIA amplification process can be determined using spectrophotometry. The spectrophometer should be set to calculate the concentration based on the absorbance of single-stranded DNA (the SPIA product is antisense single-stranded cDNA). For yields around 7 µg eluted in 34 µL of buffer the concentration would be 200 ng cDNA/µL.
12. Proceed to fragmentation of SPIA cDNA.

3.5.5. Ovation Biotin RNA Amplification and Labeling System: Fragmentation of SPIA cDNA

1. Thaw the fragmentation reagents at room temperature and ensure they are thoroughly mixed by vortexing. Place the fragmentation enzyme on ice after gentle mixing by triturating.
2. Pipet 25 µL of the cleaned SPIA cDNA into a clean 0.2-mL tube.
3. Add 5 µL of fragmentation buffer and mix by triturating.
4. Add 5 µL of fragmentation enzyme and mix by triturating (final volume 35 µL).
5. Place the tubes in a thermal cycler and run the following program: 50°C for 30 min, 4°C forever.
6. Once the reactions have been cooled to 4°C, pulse in a centrifuge, place on ice.
7. Proceed to biotin-labeling reaction.

8. The efficacy of the fragmentation reaction can be assessed by electrophoresing 1 µL of the reaction on a 1% agarose gel containing ethidium bromide and the appropriate molecular weight markers (e.g., 1 kb plus ladder).

3.5.6. Ovation Biotin RNA Amplification and Labeling System: Biotin Labeling of SPIA cDNA

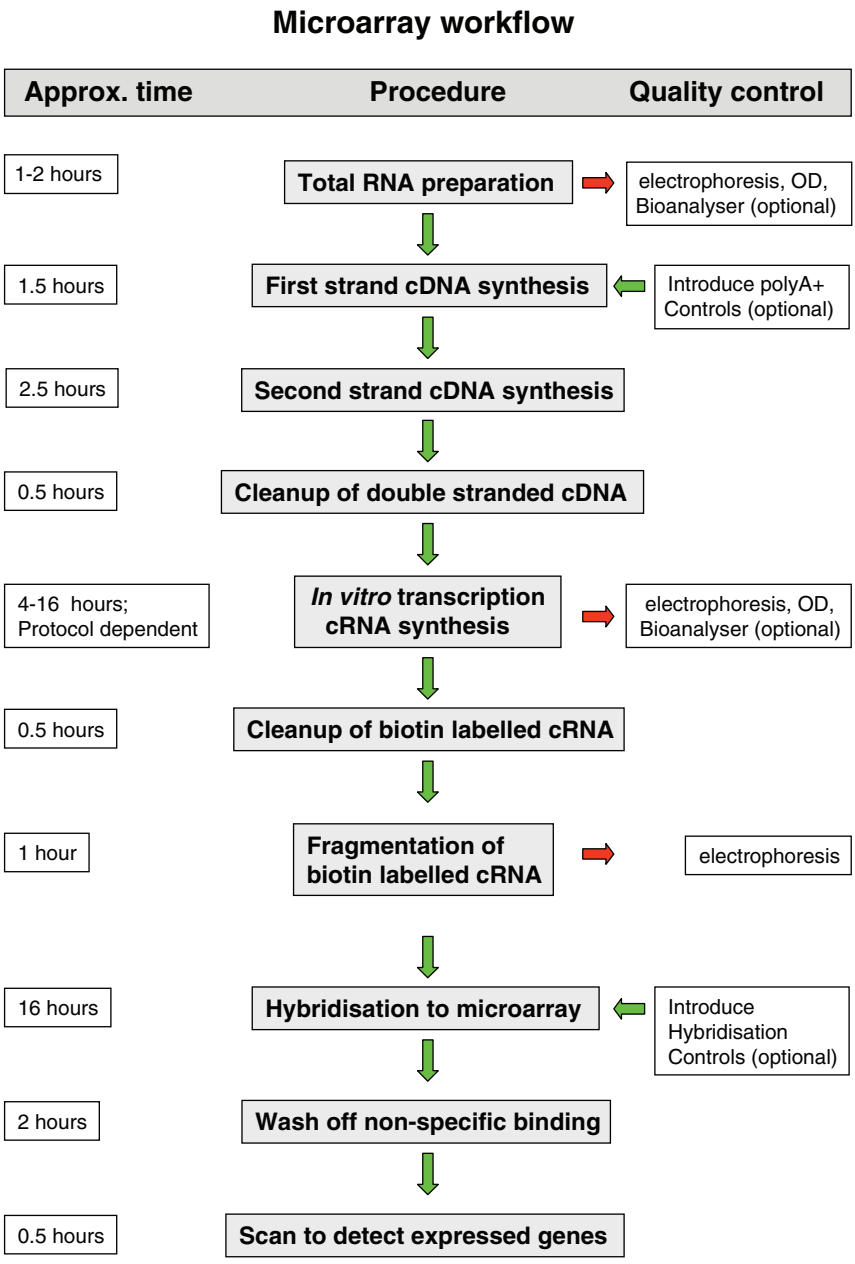
1. Thaw the labeling buffer, biotin reagent and stop solutions at room temp and mix by vortexing
2. Add 5 µL of labeling buffer to each fragmented SPIA cDNA reaction and mix by triturating.
3. Add 2.5 µL of biotin reagent to each reaction and mix by triturating.
4. Place the tubes in a thermal cycler and run the following program: 50°C for 30 min, 4°C forever.
5. Once the reactions have been cooled to 4°C, pulse in a centrifuge, place on ice.
6. Add 7.5 µL of stop solution to each reaction, mix by triturating, and pulse briefly to collect (final volume is 50 µL).

3.5.7. Ovation Biotin RNA Amplification and Labeling System: Purification of Biotin-Labeled SPIA cDNA

1. For each sample, use one DyeEx column (*see* **Note 43**).
2. Briefly vortex the column to resuspend the resin, then loosen the cap one quarter to avoid forming a vacuum.
3. Snap off the bottom of the column and place it in a 2-mL collection tube.
4. To establish the resin column, centrifuge thiss for 3 min at 750 *g* in a benchtop microfuge. Discard the flow-through and place the column in a clean 1.5-mL collection tube.
5. Carefully spot the labeled SPIA cDNA (final volume is 50 µL) onto the center of the column being careful not to touch the resin (*see* **Note 44**).
6. Centrifuge for 3 min at 750 *g* in a benchtop microfuge.
7. Remove and discard the spin column. The eluate (final volume is 50–52 µL) contains the labeled SPIA cDNA.
8. To ensure complete removal of unincorporated biotin, the whole process can be repeated with a new column.
9. The yield and purity is determined using spectrophotometry at $OD_{260/280}$. The spectrophometer should be set to calculate the concentration based on the absorbance of single-stranded DNA (the SPIA product is antisense single-stranded cDNA). For yields around 7 µg eluted in 50 µL the concentration ise 140 ng cDNA/µL.
10. The biotin-labeled SPIA cDNA can be stored at –20°C.

3.5.8. Ovation Biotin RNA Amplification and Labeling System: Hybridization of Biotin-Labeled SPIA cDNA to a GeneChip

The purified biotin-labeled SPIA cDNA is now ready to be hybridized to a GeneChip. The labeled extract generated here can now be treated in exactly the same manner as that described for the ENZO-generated product, with the excep-



tion that we routinely use 4 µg SPIA cDNA to hybridize to a chip rather than 15 µg (*see* **Fig. 3C**). Our data, as well as that provided by the manufacturer (*see* www.nugeninc.com/html/02_technology1.html and associated web pages) have shown that the Ovation biotin system provides similar signal intensities, repro-

ducibly, and expression calls (present, marginal, absent) as either the ENZO or Affymetrix standard protocols (*see* **Note 45**). As with the previous protocol, it is advisable to determine the efficacy of the whole process and representation of the SPIA cDNA by first hybridizing to a test 3 array and checking all the appropriate metrics (*see* **Note 34**).

3.6. Notes on Interpretation and Data Analysis

Once data have been acquired from a hybridized GeneChip experiment, several quality-control criteria must be checked to assess to the overall success of the process. The details of these and how to obtain them can be found in the appropriate section of the GCOS user manual. If the data obtained are deemed satisfactory, then they can be used in the algorithms most suited to the original biological question. A very broad range of bioinformatic methodologies can be applied to microarray data, with each operating under particular assumptions and thus having particular pros and cons. In general, the whole process can be divided into two. First is how the expression values are extracted from the microarray; for example, the Microarray Suite 5 (MAS5; Affymetrix default), dChip (http://biosun1.harvard.edu/complab/dchip), and Robust Multichip Average (RMA; http://stat-www.berkeley.edu/users/bolstad/RMAExpress/RMAExpress.html) methods. Second is how the gene expression data are manipulated; for example, to obtain differential gene expression between groups, software packages include Genespring (www.silicongenetics.com), Rosetta Revolver (www.rosettabio.com/products/resolver/default.htm), and Bioconductor (www.bioconductor.org). However, several other bioinforamtic packages are available, both freeware and commercial, that should be investigated for their applicability, and the input of astatistician familiar with the biological question is invaluable. For further reading, the series of *The Chipping Forecast* supplements ***(2)*** and references therein are a useful source of information.

4. Notes

1. It is important to keep detailed records of all the procedures undertaken for a microarray experiment, so that they can submitted alongside the final data for publication in compliance with the MIAME (minimum information about microarray experiment) standard (*see* www.mged.org/Workgroups/MIAME/miame.html).
2. While harvesting tissue from developing embryos, where possible ensure that manipulations are performed in a solution or tissue culture medium that maintains the intrinsic cellular identity and does not promote transdifferentiation, cell death or division, or other responses that initiate transcriptional change. If cells are being harvested from a culture, it is preferable to lyse them directly from the dish with TRIzol rather than first releasing them with proteases or otherwise. The tissue volume should not exceed 10% of the volume of TRIzol used.

3. Where small amounts of tissue or few cells are recovered per embryo, a pooling strategy can be adopted but care must be taken to establish appropriate biological replicates. To minimize variability, pooled samples or individual biological replicates must come from developmentally stage-matched embryos.
4. Although there are many other single-step solutions (e.g. RLT buffer Qiagen) for extraction of total RNA from cells or tissues, TRIzol reagent has consistently produced RNA of high quality from tissues that have been stored up to 5 years at −70°C. Regardless of which RNA isolation protocol is used, standard precautions for avoiding degradation should be followed. A thorough understanding of the precautions to be taken when working with RNA can be obtained from several sources (e.g., www.ambion.com/techlib/tb/tb_159.html). Total RNA isolated by double extraction through TRIzol has been shown to be stable for >1 year at −70°C in the absence of RNase inhibitors. Alternatively, for longer-term storage, we have placed total RNAs in nuclease-free water containing 20 U/mL RNAse inhibitor. As a general rule, total RNAs should be prepared by the same protocol for each microarray experiment.
5. Whenever possible, RNA from different conditions, tissues, or treatment should be harvested at the same time to reduce technical variability. For large studies, it is prudent to randomize the order in which the reagents are added at each step (e.g., for making labeled extracts) to minimize any possible grouping effects.
6. Due to large variations in the extent of cellular transcription between developmental time points, specific tissues, or states, it is useful to perform a titration experiment to determine the likely amount of total RNA expected per mg of tissue or per cell.
7. All RNA pellets are preferentially air dried rather than vacuum dried, which can sometimes make the RNA difficult to resuspend.
8. As an alternative to precipitation, the aqueous layer from the phase separation can be recovered using a phase-lock gel tube and following the manufacturer's instructions. In either circumstance, the collection of RNA free from genomic DNA is critical to prevent the synthesis of amplification products that can mimic the target expected from the RNA.
9. Where RNA is purified with the use of a binding column, the RNA should be eluted with room-temperature Dnase- RNase-free water (not DEPC-treated water). The recovered eluate should be passed over the column again to maximize yield.
10. If the integrity of the total RNA is determined using gel electrophoresis, a good indicator of wholly intact RNA is that the 28s rRNA band should be approximately twice as intense as the 18s rRNA band. However, the exact ratio of these rRNA transcripts depends on the method of purification (precipitation versus column), so it is only an indicator. Also, if there is evidence of enrichment of transcripts at a very low molecular weight, this can be indicative of degradation.
11. This protocol has also been used to successfully isolate total RNA from small pieces of tissue derived from developing embryos, but additional physical homogenization or vortexing may be required. If the cells are grown in culture, they can be directly harvested in lysis buffer on the dish following aspiration of culture

medium. We have found that, on average, 20 ng of the total RNA can be recovered from 1,000 neuronal cells of an E9.5 mouse embryo or equivalent.

12. For use in microarray applications, we always include the DNase treatment step when purifying total RNA by column-based protocols.
13. For maximal yield, we incubate the column for 15–30 min at room temperature prior to centrifugation rather than the 2 min quoted by the manufacturer. If yields are lower than expected, the elution buffer can be prewarmed to 37°C or 60°C prior to use.
14. The final concentration of RNA from the column purification may be extremely low, due to low starting cell numbers, and thus difficult to accurately quantify on a traditional spectrophotometer. Here, it is extremely important to get an accurate measurement by other, more-sensitive approaches (i.e., nanodrop spectrophotometer; www.nanodrop.com). In the absence of such equipment, the amount, and thus concentration, can be estimated by extrapolation from quantities achieved from larger number of cells. However, this is not an advisable approach, due to such factors as the potential nonlinear performance of the column to binding RNAs of varying concentrations.
15. The protocols for generating labeled RNA for hybridization to microarrays are constantly being updated by manufacturers to increase representation and yield. As such, they should be checked for modifications that are not detailed here.
16. For all the reactions listed, a master mix containing sufficient reagents for all the samples should be prepared and thoroughly mixed. To increase reproducibility, all the reagents used must be consistent from one experiment to another (e.g., suppliers of reverse transcriptase, dNTPs, polymerises).
17. The T_7 primer supplied by Affymetrix is an expensive option. Several manufacturers can supply the primer, as long as it is HPLC purified and shown not to be degraded to any degree. In this case, the primer must be diluted to the appropriate working concentration between batches.
18. For the starting values of 2 µg of total RNA, the amount of T_7 primer used can be substantially reduced (i.e., ten up to tenfold less than the amount stated, 100 pM) with out any noticeable loss of signal. This 100 pm of primer is the amount recommended by Affymetrix as being suitable for this type of protocol.
19. The poly-A RNA controls are used to monitor the entire target-labeling process and evaluate assay sensitivity, consistency, and dynamic range. They are exogenous spike-in poly A transcripts that act as positive controls to monitor the success of the entire labeling process independent from the starting material quality. Four independent poly-A RNA controls are used (*lys*, *phe*, *thr*, *dap*) at staggered concentrations, and the resultant signal intensities for each of them serve as sensitive indicators of the efficiency of the labeling reaction. The concentrations are such that, at the end of the process, they should span the spectrum of absent, marginal, and present.
20. If yields are lower than expected but starting RNA has met all quality-control criteria, it is possible that it has not been sufficiently denatured at the outset. In these cases, the denaturation temperature can be raised to 95°C for minutes before being placed immediately on ice.

21. Where appropriate, all buffers must be thoroughly thawed before use to ensure that all components are fully dissolved.
22. It is important to check the concentration and use only exactly 2 U of RNase H per reaction.
23. When 2 µg of total RNA is used as a starting amount, the whole first-strand reaction (20 µL) is used as a template in the second-strand synthesis. If >10 µg is used, then this volume is reduced to 10 µL.
24. Data from Affymetrix suggests that highly concordant (e.g., overall signal intensity and qualitative calls) results have been obtained by comparing global array hybridization results obtained from samples cleaned up by either column or phenol chloroform methods. However, it is recognized that cDNA cleanup columns reduce the recovery of fragments of 100 nucleotides or less, whereas these fragments are retained in the phenol–chloroform method. The implications of this should be borne in mind for the system under study.
25. To avoid damage to the caps during this process, place columns into the centrifuge using every second bucket. Then position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation; that is, if the microcentrifuge rotates in a clockwise direction, orient the caps in a counterclockwise direction. It is essential to centrifuge the columns with the caps open to fully dry the membrane and allow maximal elution and purity.
26. We generally do not quantifying the amount of double-stranded cDNA by absorbance at 260 nm, as the T_7 primer can contribute significantly to the absorbance.
27. Using T_7 polymerase and biotin-labeled nucleotides, large amounts of single-stranded RNA molecules are produced. The chemistry of the reactions is such that many copies of RNA are produced from a single cDNA template, resulting in an amplification of the material to be hybridized to the chip. Labeled RNA transcripts are used because RNA–DNA hybrids (Affymetrix microarrays are composed of DNA oligonucleotides) have a higher melting temperature than corresponding DNA–DNA hybrids, thus allowing for higher stringency and fidelity.
28. If >8 µg of total RNA was used originally, the volume of cDNA synthesis reaction can be reduced by 50%.
29. The efficacy of conversion of the double-stranded cDNA to biotin-labeled cRNA can be checked by agarose gel electrophoresis. The biotin-labeled cRNA should form a smear ranging in size from approximately 100 bases to >6 kb. The smear is an accumulation of cRNAs of many different sizes, reflecting the broad range in sizes of polyadenylated messenger RNAs. The rRNA bands do not participate in this process, because they lack a polyA tail. Characteristically, we *see* the most intense region between 1–4 kb. The size of the smear extending above 6 kb is a good indication that the IVT reaction (as well as those reactions preceding it) has been successful and truly representative (*see* **Fig. 2B**).
30. The most likely cause of a low yield in the IVT reaction is poor-quality starting RNA.
31. If there is to be a long duration between synthesis of the cRNA and hybridization to the microarray, the biotin-labeled cRNA should be stored prior to fragmentation.
32. The 15 µg is the final amount required for hybridization to a GeneChip. A small amount of cRNA (approximately 250 ng) is required for quality control of the

reaction. However, we have obtained good signal levels and reproducibility with 10 µg, although the amount used must be consistent across the experiment.

33. Following fragmentation, a small amount of cRNA (between 250 ng and 1 µg) should be run on a 1% agarose gel containing EtBr (1 h, 120 V, constant current) with appropriate molecular weight markers (e.g., 1-kb ladder, Gibco). All the cRNA should appear at 200 bp or less. If the cRNA has not been suitably fragmented, the process can be repeated. Alternatively, the efficacy of the reaction can be more accurately assessed on a bioanalyzer.
34. A test array provides an accurate method to determine the quality of labeled cRNA prior to its analysis on the GeneChip expression arrays. This array contains probe sets representing a subset of characterized genes from various organisms, including mammals, plants, and eubacteria. Additionally, the GeneChip test array contains a subset of human and mouse housekeeping genes, so their representation can be validated prior to further study. Lack of signal, high background or noise, and poor 3 /5 ratios are good indicators of poor cRNA quality.
35. Eukaryotic hybridization controls are premixed biotin-labeled bioB, bioC, bioD, and cre cRNAs present in staggered concentrations and added directly to the hybridization cocktail. These controls facilitate monitoring of the hybridization by giving a characteristic signal levels following optimal hybridization and scanning.
36. The control oligo B2 is used to provide alignment signals for image analysis (e.g., accurate determination of the edges and corners of the GeneChip by the scanner).
37. The GeneChip chambers are filled and emptied via two septa on the rear of the casing. While filling using one septa, a second pipete tip must be inserted into the other septa to allow passage of air and similarly for evacuation of the chamber. Care must be taken to avoid tearing the septa and allowing hybridization cocktail to escape during the 16-h incubation.
38. A standard Affymetrix GeneChip is filled with 200 µL of hybridization mix. The chambers have a maximum volume of approximately 250 µL. As such, a small bubble will be seen in the chamber after filling. This may actually be beneficial, as it is thought to contribute to mixing during the subsequent 16 h of rotation at 60 rpm.
39. If hybridization–cRNA mixes are to be used again, then they must be denatured and spun as described for original use. We routinely use hybridization cocktails twice with no discernable loss of signal on hybridization controls and the like.
40. It is imperative that the concentration of the RNA be accurate and consistent between samples. If higher concentrations of total RNA are not available prior to dilution, then the amounts must be measured using specialized spectrophotmetric methods (*see* **Note 14**).
41. All the incubations in this protocol can be preprogrammed into a PCR machine with a heated lid and allowed to run autonomously.
42. The expected yield of the SPIA amplification reaction is approximately 7 µg (although can be anywhere between 5 and 10 µg) from 10 ng of total RNA in an initial volume of 160 µL. The analysis of 4 µL of the SPIA reaction volume should provide >200 ng amplified cDNA product to be visualized on an agarose gel. If an amplification product cannot be seen from this volume of the reaction, it is likely

that the process generated insufficient product for further use. The upper size limit of around 0.4 kb of the amplified cDNA is a good indicator of reaction success.

43. To prevent high background readings that effectively reduce representation, it is essential that labeled SPIA cDNAs are purified from unincorporated biotin.
44. When loading the column, the labeled SPIA cDNA must be placed directly on the center and not allowed to pass down the side and into the collection tube.
45. Data supplied by NuGEN demonstrate that there is 90% concordance of gene calls between the Ovation Biotin System and Affymetrix standard protocol.

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RT-PCR on Embryos Using Degenerate Oligonucleotide Primers

Anthony Graham

1. Introduction

The polymerase chain reaction (PCR) is an incredibly versatile technique that has made a profound impact on many areas of biology. PCR is based on the use of oligonucleotides, which flank the region of DNA of interest and which are complementary to sequences on either DNA strand, to prime the replication of each strand by *Taq* DNA polymerase. This enzyme is thermostable, and consequently, one can go through cycles of template denaturation and renaturation after each round of replication and exponentionally amplify the sequence of interest. This procedure is very sensitive, and as such, it allows one to amplify, isolate, and utilize specific DNA sequences from vanishingly small quantities of starting material using either genomic or cDNA as a substrate.

PCR can also be used to facilitate the isolation of genes that are evolutionarily related, such as cognate genes from different species or multiple members of given gene families. In these cases, one is often trying to isolate DNA fragments whose sequence is not actually known. This can be done by taking advantage of the fact that the conservation of molecules is most evident at the protein level. In these cases, the sequence of the primers to be used is derived from conserved amino acid sequences that exist between the related genes. Since usually more than one codon encodes most amino acids (**Fig. 1**), and also since the amino acid sequence conservation between proteins may not be 100%, if the primers are to cover all of the possible combinations of DNA sequence that could encode a given amino acid motif, they will necessarily be degenerate. This chapter will focus on the use of degenerate oligonucleotide primers in PCR on cDNA synthesized from embryonic tissue. Using cDNA as a substrate with degenerate oligonucleotides rather than genomic DNA has

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Amino acid		Codons
Alanine	Ala A	GCA GCC GCG GCU
Cysteine	Cys C	UGC UGU
Aspartic acid	Asp D	GAC GAU
Glutamic acid	Glu E	GAA GAG
Phenylalanine	Phe F	UUC UUU
Glycine	Gly G	GGA GGC GGG GGU
Histidine	His H	CAC CAU
Isoleucine	Isl I	AUA AUC AUU
Lysine	Lys K	AAA AAG
Leucine	Leu L	UUA UUG CUA CUC CUG
CUU		
Methionine	Met M	AUG
Asparagine	Asn N	AAC AAU
Proline	Pro P	CCA CCC CCG CCU
Glutamine	Glu Q	CAA CAG
Arginine	Arg R	AGA AGG CGA CGC CGG CG
Serine	Ser S	AGC AGU UCA UCC UC
UCU		
Threonine	Thr T	ACA ACC ACG ACU
Valine	Val V	GUA GUC GUG GUU
Tryptophan	Trp W	UGG
Tyrosine	Tyr Y	UAC UAU

Fig. 1. The genetic code. Each of the amino acids is shown along with its corresponding RNA codons.

the advantage that one does not need to worry about introns and also that if the appropriate fragment is amplified from a cDNA sample, one can therefore conclude that this gene is expressed in the tissue from which the RNA was isolated. This allows one to amplify cDNA fragments of genes of interest that have not already been isolated from that organism, and also to survey what members of a given gene family, be they previously identified or not, are expressed in a given tissue at any particular stage of interest and to isolate cDNA fragments for each gene.

2. Materials

2.1. RNA Preparation

1. All of the reagents should sterile and where possible treated with diethylpyrocarbonate to inactivate any RNases ***(1)***. Solution D: 25 g guanidinium thiocyanate, 29.3 mL water, 1.76 mL 0.75 *M* Na citrate, pH 7, 2.64 mL 10% sarkosyl, 38 mL β-mercaptoethanol. Store at −70°C.
2. Na acetate, pH 4.0.
3. Acid phenol: phenol that is water-saturated, but unbuffered.
4. Chloroform.
5. Glycogen.
6. Ethanol.

2.2. cDNA Synthesis

Use cDNA synthesis kit—Boehringer Mannheim (# 1 483 188), Promega (# A3500), or Pharmacia (# 27-9261-01).

2.3. Primers

There are a number of companies that will synthesize oligonucleotides to order.

2.4. PCR Reaction

1. *Taq* DNA polymerase, including 10X buffer (Mg-free or not) and $MgCl_2$ available from a number of suppliers, such as Promega.
2. Deoxynucleotide triphosphates (Boehringer Mannheim) or equivalent.
3. Paraffin oil.

3. Methods

3.1. RNA Preparation

This method is basically that of Chomczynski and Sacchi ***(2)***—it works well from as little as 100–10^6 cells and it can be scaled up if necessary.

1. Add 100 µL of solution D to 10^6 cells or 10 mg tissue in a microcentrifuge tube, and vortex. Most tissues break up almost immediately, but tough tissues may need to be syringed.
2. Add 10 µL of 2 *M* Na acetate, pH 4.0, and mix.
3. Add 100 µL of water saturated unbuffered phenol, and mix.
4. Add 20 mL of chloroform, and mix very well. The sample should become cloudy at this stage. If it does not, add extra chloroform.
5. Chill the sample on ice for 15 min, and then spin in a microfuge for 15 min.
6. Take the top phase into a new tube. If one is dealing with a small amount of tissue, one can add 1 µL of RNase-free glycogen at 20 mg/mL to aid precipitation.
7. Add 240 µL of ethanol, and put at −20°C for 30 min.

8. Spin for 15 min in a microcentrifuge.
9. Wash the pellet with 70% ethanol, and drain.
10. Redissolve the RNA in 20 µL H_2O and store at −20 or −70°C with the latter being better for long-term storage.

3.2. cDNA Synthesis

For PCR, only the first cDNA strand needs to be synthesized, since the second strand is copied in the first cycle of the PCR reaction. Many companies sell first-strand synthesis kits, and these are recommended. This reaction uses the enzyme reverse transcriptase, which is an RNA-dependent DNA polymerase, i.e., it copies RNA into DNA. The two most commonly used enzymes are avian myeloblastosis virus (AMV) reverse transcriptase and Moloney murine leukemia virus (M-MuLV) reverse transcriptase. These enzymes have slightly different activities, but both function adequately. It should also be noted that AMV is used at 42°C, whereas M-MuLV works best at 37°C. The RNA extraction procedure outlined above yields a sample of total RNA and, therefore in addition to including mRNA, it also contains rRNA and tRNA. To copy mRNA preferentially in the reverse transcription reaction, the primer that is used is an oligo dT oligomer. This will bind specifically to the poly (A) tail of mRNA molecules and ensure that they are replicated in preference to the other RNAs in the sample. The other reagents include the appropriate buffer for the reverse transcriptase enzyme, deoxynucleotide triphosphates from which the DNA will be synthesized, RNase inhibitor, and water to make up the volume. The procedure is carried out according to the instructions of the manufacturer of the cDNA kit. In a first-strand synthesis reaction, I would use between 2 and 5 µL of the RNA extracted as above, depending on the amount of starting material.

3.3. Primer Design

There are a number of factors that should be taken into consideration when designing degenerate oligonucleotide primers. These will be illustrated using the isolation of chick activin type II receptors by degenerate RT-PCR as an example. The amino acid sequences of activin type II receptors from both mouse and *Drosophila* are shown in **Fig. 2** ***(3)***. There are a number of regions that are well-conserved between these family members, and the amino acid sequences that were chosen to make primers against are highlighted, and their corresponding DNA sequences shown in **Fig. 2**. In this case, nested primers were employed, two external and one internal. The utilization of nested primers increases the specificity of the reaction owing to the fact that the substrate for the second and final round of PCR is considerably less complex than a total cDNA sample, since it has already

been selectively amplified in the first round of PCR with the external primers. The two external primers were used in the first round of PCR, and then a small aliquot was taken, and in the second round this was subjected to further amplification using the internal primer and the 5′ -external primer. The primers are spaced such that the final amplified sequence will be around 500 bp, which is a useful size, because it also allows one to use this fragment for other purposes, such as whole-mount *in situ* hybridization immediately after cloning it. The sequence of the primers to be used is dependent on the available amino acid sequences. The primers should be as nondegenerate as possible. This in effect means trying to avoid amino acid sequences that are comprised of amino acids encoded by multiple codons, such as arginine, serine, or leucine (**Fig. 1**). That having been said, an overriding consideration in primer design is that the 3′ of the primers end be as close as possible to being unique. This in turn means looking for methionines or tryptophans in any peptide sequence, and since each of these amino acids is only encoded by one codon, making the 3′ -end of the oligo correspond to these positions. If neither of these amino acids is conveniently located, then the next best option is to scan for amino acids that are only encoded by two codons, both of which will only vary at the third base. Therefore, in the case of the oligonucleotide which will prime the replication of the sense strand, to ensure that there is an exact match at the 3 -end the oligonucleotide should terminate at the second base of that codon. This is not a problem for the oligonucleotide that primes the replication of the antisense strand, since the two most 3 -bases of this oligo will be complementary to the first two positions of the codon, which are often invariant. As can be seen from **Fig. 2,** the activin receptor type II primers are all anchored on methionine and tryptophan codons and they cover sequence encoding six amino acids in all and should therefore be long enough to be stable at 72°C, which is the optimal temperature for *Taq* polymerase. These oligonucleotides also have restriction enzyme sites added at their 5′ -ends—*Eco*RI in the case of the 5′ -external oligo and *Xba*I for both the 3′ -oligos—to aid the subcloning of the amplified fragment. To ensure that the restriction enzymes cut efficiently, two extra bases have been added to the 5′ -end. The choice of the enzymes that are used and bases added at the 5′ -end to enhance digestion are based on available information, such as that in the appendices of the New England Bioloabs catalog. It is of course possible that the amplified sequence may also contain an internal sites for one of the restriction enzymes that is chosen. If this is the case, then the PCR product can be cloned without prior digestion in the standard manner for blunt-end ligation, or it may be directionally cloned by only digesting with the enzyme that does not cleave internally and using the other end as a blunt end.

Fig. 2. (**A**) portion of the amino acid sequences of the *Drosophila* Act-R gene and the mouse Type II Activin receptors ***(3)***. The motifs shown in bold are those on which the PCR primers are based.

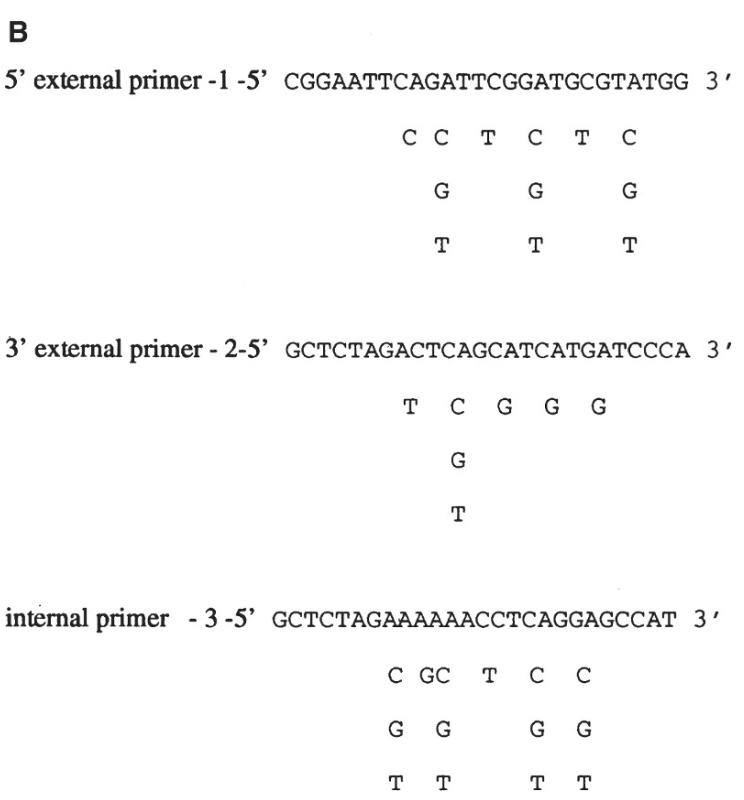

Fig. 2. *(continued)* **(B)** The sequences of the primers are shown underneath. Note that the 5′ -primer also has an added *Eco*RI site and the two 3′ -primers an *Xba*I site.

3.4. PCR Reaction

Because the oligonucleotides that are used are degenerate, there are no "standard" reaction conditions that will invariably work. The reaction parameters must be empirically determined for each primer pair. The two variables that are most frequently altered are the concentration of $MgCl_2$ in the reaction and the temperature at which the oligonucleotides anneal to the substrate. In a typical reaction, the author would initially try three final concentrations of $MgCl_2$, which span the normal range that is usually effective—1, 1.5, and 2 m*M*. If these concentrations are not effective, then concentrations from 0.5–5 m*M* can also be tested. In the case of exact match primers, one tends to use an annealing temperature that is 10°C below the melting temperature of the oligo. This is obviously not applicable to degenerate oligos, which are a mix of oligos with a range of melting temperatures. Ideally, the higher the annealing temperature that can be used,

the greater the specificity of the reaction but the temperature should also be low enough to allow the oligos to anneal to all of the potential target sites of interest. A useful annealing temperature to start with is 50°C. If at this temperature no sequence is amplified then the temperature can be dropped even to as low as 37°C, although reactions using these lower temperatures will invariably produce spurious amplification products at a much higher frequency. If annealing at 50°C produces multiple amplified products, then the annealing temperature should be raised. Some of the background may be owing to the primers annealing and *Taq* extending at lower temperatures as the reaction mix warms up. One way to reduce this sort of background amplification is to "hot start" the reaction. This is achieved by adding the *Taq* polymerase to reaction mix that has been denatured at 94°C and then cooled to 55°C or less effectively, but more simply by making up the complete PCR reaction mix, including Taq, on ice and then transferring the tubes straight to a block that has been prewarmed to 94°C.

The PCR reaction consists of the following components: *Taq* polymerase buffer, $MgCl_2$, deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) at a final concentration of 0.2 m*M*, oligonucleotide primers that should be in excess and are often used at concentrations of 0.5–1 m*M*, 1 U of *Taq* polymerase, target cDNA, and H_2O to make up the volume.

A typical PCR reaction of 100 µL using *Taq* polymerase purchased from Promega would be as follows:

1. Add the following to a microcentrifuge tube:
   a. 10X Reaction buffer (Mg^{2+} free) 10 µL.
   b. 25 m*M* $MgCl_2$ 4 µL (1 m*M*), or 6 µL (1.5 m*M*), or 8 µL (2 m*M*).
   c. dNTPs (20 m*M*) stock 1µLl.
   d External primers (at a concentration of 0.5 µg/µL approx 50 µ*M*) 1 µL of each.
   e. *Taq* polymerase (5 U/mL) 0.2 µL.
   f. H_2O 78, 76, or 74 µL.
   g. cDNA 5 µL.
2. Overlay with 50 µL of paraffin oil to prevent evaporation. This step is not necessary if one is using a thermal cycler with a heated lid.
3. Place the sample in the thermal cycler and carry out the amplification. A typical amplification protocol is:
   a. First cycle (X1)—denature at 94°C for 2 min, ×1
   b. Subsequent cycles (×30)—denature, 94°C for 30 s.
   c. anneal 50°C for 2 min.
   d. extend 72°C for 2 min.
   e. Final cycle (×1)–extend 72°C for 10 min.
4. For the second round of amplification, use the internal primer and the appropriate external primer, and use 5 µL of the completed first-round PCR reaction mix as the DNA substrate. Mix the reagents and carry out the amplification as for the first round again with the range of Mg concentrations. One other factor that can be varied is the

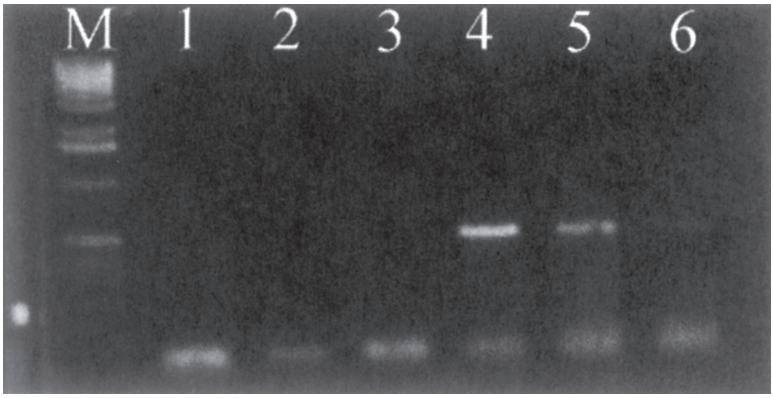

Fig. 3. Ethidium bromide-stained agaraose gel electrophoresis of the products PCR amplification. M is the marker track (1-kb ladder from Gibco-BRL), 1–3 are from the first round of amplification and 4–6 are from the second. The reactions run in lanes 1 and 4 were at 1 m*M* $MgCl_2$, 2 and 5 at 1.5 m*M*, and 3 and 6 at 1.5 m*M*.

amount of first-round reaction that is added as a substrate for the second round. In some instance, the second round may be a lot cleaner if considerably < 5 µL is added.

5. At the end of the second round, run a 10- µL aliquot of all samples, i.e., all Mg concentrations from both the first and second rounds, on an agarose gel. **Figure 3** shows the results obtained with the activin type II receptor primers. As can be seen the first round of amplification with the external primers did not produce a band at any of the Mg concentrations used, but in the second round with the internal primer and the 5′-external primer, a fragment of the predicted size was amplified under all Mg concentrations, although the 1-m*M* concentration was clearly more efficient.

If the amplification has worked and an appropriate-sized fragment is present, the PCR reaction mix should then be cleaned up by phenol/chloroform extraction, especially if using oil, then ethanol-precipitated and redissolved in a small volume, and then digested with the appropriate restriction enzymes. The digest should then be run on a gel, the fragment isolated and subcloned into an appropriate vector, and the products sequenced. In some cases, all of the resulting clones will contain fragments of members of the gene family of interest, but in a number of cases, one will also find that other unrelated products have been amplified. This last point merely serves to stress the importance of sequencing the products of the amplification.

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Single-Cell RT-PCR cDNA Subtraction

Ebrahim Sakhinia, Damian L. Weaver, César Núñez, Clare Brunet, Victoria Bostock, and Gerard Brady

1. Introduction

A major problem in trying to understand complex developmental processes is heterogeneity at both the cellular and molecular levels. At the cellular level, it is often difficult to identify cells that are undergoing developmental changes and establish the stage of differentiation they have reached. At the molecular level, there is then a problem in establishing which the many thousands of expressed genes are playing a role in regulating development. Several approaches for identifying expressed candidate developmental regulatory genes are based on comparing the mRNA expression patterns in cells before and after developmental transition points. Differential screening of cDNA libraries with labeled total cDNA probes from contrasting cell samples ***(1)*** provides a simple means of identifying genes that are expressed at high levels in one of the samples. The cDNA subtraction protocols ***(2,3)*** increased the sensitivity of this type of approach by removing sequences expressed in both samples. Often, the major limitation of these approaches lies in the large amounts of starting material necessary for cDNA preparation and subtraction. With the advent of the polymerase chain reaction (PCR; ***(4)***) and the development of techniques that allow amplification of target sequences expressed in single cells ***(5)***, the limitations of applying cDNA subtraction and differential screening have been removed.

Here, we describe the details of an improved cDNA subtraction method based on a cDNA subtraction approach previously applied to single cells ***(6)***, which in turn is based on a global cDNA amplification protocol (*PolyAPCR*) used to produce amplified cDNAs, representing all the mRNAs present in the samples as small as a single cell ***(7,8)***. *PolyAPCR* has been applied successfully to a wide range of samples, including single micromanipulated cells ***(6,7,9,10)***, antibody fractionated populations ***(11)***, and fixed tissues ***(12)***.

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The current subtraction method described here is based on a genomic DNA subtraction protocol utilizing biotin addition and organic extraction ***(13)***. To simplify the original method, we reduced the overall time required for subtraction and utilized dUTP incorporation in the driver cDNA, allowing removal of the residual driver by UNG treatment following subtraction ***(14)***. The final enriched probes can be used to screen full-length cDNA libraries or DNA arrays. The advantage of this procedure compared to the related methods of differential displays is primarily that all differences between the starting samples are present after subtraction. Furthermore, the flexibility of the initial *PolyAPCR* procedure enables the subtraction to be applied to a wide range of samples. Potential disadvantages of the *PolyAcDNA* subtraction are that it can be applied to only relatively few samples and clones containing repetitive sequences may be lost.

2. Materials

All reagents are of molecular biology grade and obtained from Sigma (St. Louis, MO) unless otherwise stated.

2.1. Preparation of PCR Material

1. 10× *Taq* buffer: 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3.
2. 25 mM dNTP mix: 1:1:1:1 mix of 100 mM dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim, Mannheim, Germany), store in 50-µL aliquots at −20°C.
3. Oligonucleotides (Pharmacia, Piscataway, NJ):
   *Not*I 40: 5′cat ctc gag cgg ccg ctt ttt ttt ttt ttt ttt ttt ttt t 3′: Primary PCR only.
   *Not*I 24: 5′gcg gcc gct ttt ttt ttt ttt ttt 3′: Amplification of driver.
4. dH_2O.
5. *Taq* polymerase (Roche).
6. PCR reaction mix: 10× *Taq* polymerase reaction buffer, 0.25 mM dNTPs, 0.5 OD260/mL (1.5 mM) oligonucleotide, 1.2 U/100 µΛ *Taq* polymerase and dH_2O.
7. 1.7% (w/v) agarose gel (SeaKem, FMC BioProducts, Rockland, ME).
8. 1XTBE: 0.9 M Tris-borate, 0.002 M EDTA, pH 8.0.
9. Ethidium bromide (10 mg/mL) (as a possible carcinogen, adequate safety precautions must be taken).
10. Known concentration *of PolyAcDNAs*.
11. GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech).
12. HE buffer, pH 8.0: 10 mM HEPES, 1 mM EDTA.

2.2. Photobiotinylation of Purified Driver cDNA

1. Photobiotin: 1 mg/mL in water, store in 80-µL aliquots at −20°C in lightproof tubes.
2. UV source: 240-V AC, 300-W ultra vitalux bulb (Osram, Sylvania, Danvers, MA). (Do not allow exposure of skin to UV.)
3. 200 mM Tris-HCl, pH 9.0.

4. 7.5 M ammonium acetate.
5. Ethanol: 100, 80, and 70%.
6. 1 M EPPS, pH 8.5 and 8.25, autoclave and filter sterilize.
7. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
8. Extraction buffer: 50 mM EPPS, pH 8.5, 0.5 M NaCl, 5 mM EDTA.
9. Streptavidin: 4 µg/µL in 50 mM EPPS, pH 8.25, store at −20°C in 5-µL aliquots to avoid freeze–thaw problems.
10. TE-saturated phenol–chloroform (1:1). (Prepare fresh immediately prior to use do not store.) Add 700 µL phenol–chloroform (Gibco-BRL, Gaithersburg, MD) to 700 µL TE, pH 8.0, vortex mixture, centrifuge, decant aqueous phase, and repeat three times.

2.3. Hybridization of Driver and Tracer

1. 5 mg/mL tRNA: Store at −20°C in small aliquots.
2. 3 M NaAc, pH 8.0
3. 3× Hybridization buffer: 50 mM EPPS, pH 8.5, 10 mM EDTA, pH 8.0, 0.3% SDS. Store at −20°C in aliquots.
4. 40% (w/v) PEG 8000, in water.
5. 5 M NaCl.
6. Subtractive hybridization buffer (prepare fresh): 3 µL 3× hybridization buffer, 3 µL 5 M NaCl, and 3 µL 40% PEG-8000 in water.

2.4. Assessing the Enrichment of Probes Generated by Subtraction

1. 32P dCTP 3000 Ci/mmol (Amersham, Amersham, UK).
2. dNTP labeling mix: 27 mM dATP, 27 mM dTTP, 27 mM dGTP, 16 mM dCTP.
3. Southern hybridization buffer: 50% deionized formamide (BDH), 1 M NaCl, 1%SDS, 10% dextran sulfate.

3. Methods

3.1. Preparation of PCR Material

When designing the X-dT oligonucleotides (e.g., *Not*I 40), it was found that oligonucleotides with an X component of 15–36 bases gave good amplification, but it was found that shorter oligonucleotides generally gave a lower yield.

1. Amplification of tracer: Add 1 ng of material previously amplified by *Not*I 24 to 100 µL PCR reaction (10 µL of 10× *Taq* polymerase reaction buffer, 1 µL of 25 mM dNTPs, 1 µL of *Not*I 24 oligonucleotide, 86 µL of dH_2O and 1 U/100 µL *Taq* polymerase). Thermal profile: 94°C for 1 min, 42°C for 1 min, 72°C for 2 min, × 25 cycles.
2. Amplification of driver: Add 1 ng of material previously amplified by *Not*I 24 to a 100 µL PCR reaction (10 µL of 10× *Taq* polymerase reaction buffer, 1 µL

of 25 mM dNTPs, 1 µL 25 mM dUTP, 1 µL of *Not*I 24 oligonucleotide, 86 µL of dH_2O, and 1 U/100 µL *Taq* polymerase). Thermal profile: 94°C for 1 min, 42°C for 1 min, 72°C for 2 min, × 25 cycles.

3. Purify the tracer and driver-amplified materials using a GFX *PCR DNA* and gel band purification kit (Amersham Pharmacia Biotech) as recommended by the manufacturer.
4. Concentrate the eluted material (final vol 0.8 µL) by isopropanol precipitation (80 µL 3 M NaAc and 650 µL isopropanol). For efficient recovery, chill on wet ice for 20 min and spin at full speed in a refrigerated centrifuge for 15 min. Wash the resultant pellet with 70% ethanol, centrifuge again for 7 min at 4°C, air dry 15 min, and resuspend the pellet in 50 µL HE buffer.
5. Quantitate the recovery of PCR material by gel electrophoresis and comparison to a dilution series of a well-characterized *PolyAPCR* sample.

3.2. Photobiotinylation of Purified Driver cDNA

We have generally found that photobiotinylation is an efficient and reliable method of biotin incorporation. However, despite the reliability of photobiotinylation, it is recommended that the efficiency of biotin incorporation into the driver should be tested functionally as described prior to its use in the subtractive hybridization reaction.

1. Boil 40 µL (20–100 µg) driver cDNA for 2 minute, then snap-cool on ice prior to adding 40 µL of photobiotin (1 mg/mL), and mix well (by flicking the tube). Place tube with the lid open 10 cm from the UV source, and irradiate for 5 minute. Leaving the UV source on (to preserve the bulb), take out the sample, and mix by flicking the tube and replace under the UV for an additional 5 minute.
2. Remove the sample, add a further 40 µL of photobiotin, mix well (by flicking the tube) and replace under UV for a further 5 min. Then add an equal volume of 200 mM Tris-Cl, pH 9.0, to stop the reaction.
3. Free photobiotin may be removed by ethanol precipitation (100 µL 7.5 M ammonium acetate, 20 µL 1 M $MgCl_2$ and 750 µL 100% ethanol). For efficient recovery chill on ice for 15 minute and spin at full speed in a refrigerated centrifuge for 15 minute. Discard the supernatant and wash the pellet with 80% ethanol. Following 15 minutes air drying a dry orange/brown pellet will be visible which should be resuspended in 50 µL HE.
4. Efficient biotinylation may be assessed by adding 50 µL of fresh extraction buffer and 5 mL of 4 µg/µL streptavidin to 5 µg biotinylated driver. After 7 minutes incubation at room temperature remove and set aside 10 µL for gel analysis and add 50 µL TE-saturated phenol–chloroform to the remaining sample. Vortex for 30 seconds and then centrifuge at full speed for 3 minutes in a microcentrifuge. Remove the top three quarters of the aqueous phase (~25–30 µL) avoiding the interphase and transfer it to a fresh tube. Compare 10 µL of this extracted aqueous phase to the 10 µL removed before subtraction by agarose gel electrophoresis.

3.3. Subtractive Hybridization

Common tracer sequences are removed by solution hybridization with the biotinylated driver, followed by the removal of all biotin-containing hybrids after an addition of streptavidin and an organic extraction.

1. Combine in a 0.5 mL microtube 200 ng tracer, 4 µg biotinylated driver and 5 µg carrier tRNA. Adjust the total volume to 80 µL with HE buffer, mix well, boil for 2 min, then place on ice. To precipitate, add 8 µL 3 M NaAC and 200 µL 100% ethanol. Vortex briefly, chill on ice for on ice for 15 min and spin at full speed in a refrigerated centrifuge for 15 min. Wash the resultant pellet with 70% ethanol and air dry.
2. Carefully resuspend the pellet in 9 µL subtractive hybridization buffer, cover with 70 µL mineral oil, and boil for 5 min (Note: Ensure the lid is secured using a suitable cap lock).
3. Place the sample into a PCR machine preheated to 80°C and run the following thermal profile: 5 min at 98°C, 5 min at 80°C, (optional ramp 80°C–68°C over 15 min), 60 min at 68°C, then hold at 68°C.
4. To the sample held at 68°C *immediately* add 90 µL of extraction buffer (preheated to 80°C), centrifuge briefly to ensure all the aqueous layers have combined, and remove as much mineral oil from the top as possible. Centrifuge briefly again, transfer the bottom 75 µL of hybridization–extraction mixture to a fresh tube containing 21 µL of fresh extraction buffer plus 4 µL of 4 µg/µL streptavidin, mix, and incubate at room temperature for 5 min.
5. Add 100 µL TE-saturated phenol–chloroform, vortex for 30 sec, and spin at full speed at room temperature for 5 min. Remove top three quarters of the aqueous phase (~75 µL) avoiding the interphase and transfer to a fresh tube.
6. Extract the transferred aqueous phase with 50 µL of chloroform vortex for 30 sec, spin at full speed at room temperature for 5 min, and transfer as much of the aqueous layer as possible to a fresh tube.
7. To carry out sequential rounds of subtraction, combine the 60 µL of extracted tracer removed in **step 6** with 4 µg biotinylated driver and repeat **steps 1** to **6**.
8. Following steps 2–4 extraction cycles, add 20 µg carrier glycogen (Roche) to the ~60 µL of chloroform extracted material (step 6) and precipitate the combination ethanol as described in step 1. The resultant air-dried pellet is resuspended in 10 µl TE. 9. 2 µL of the subtracted material is amplified in a 100 µL PCR reaction (10 µL of 10× buffer [Roche],1 µL oligo *NotI* 24, 1 µL 25 mM dNTPs, 85 µL dH_2O, 1 µL *Taq* [Roche], 1 U UDG). The product is incubated for 10 min at 20°C and heated to 95°C for 2 min to inactivate the UDG, then amplified using the following thermal profile: 5 min at 95°C × 1 cycle and linked to 30 sec at 94°C, 30 sec at 42°C, 60 sec at 72°C × 30 cycles. The resultant amplified material is purified using a GFX gel band purification kit (Pharmacia Biotech) and eluted in 50 µl TE buffer.

3.4. Verification of Subtractive Hybridization

Southern and colony hybridization with labeled subtracted material can be used to assess the efficiency of the subtraction and obtain full-length cDNA clones of differentially expressed genes (**Figs. 1** and **2**).

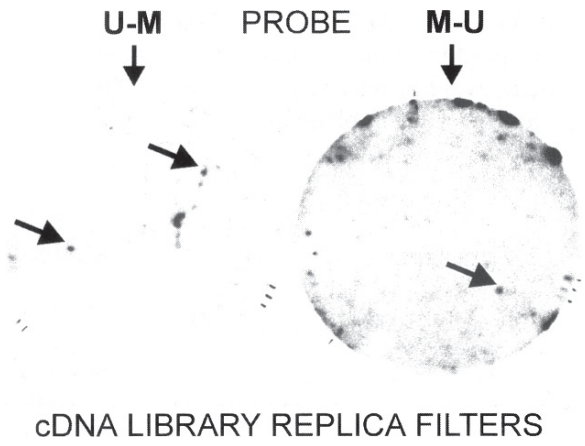

Fig. 1. Differential screening of a human cDNA library with subtracted *PolyA cDNA* probes. A human cDNA library is prepared using the lambda cloning vector Lambda ZAP® (Stratagene). Two replica filters are produced from a single petri dish containing approximately 2,000 recombinant clones. One filter is hybridized with a probe enriched for sequences expressed in unilineage cells (U–M), and the other hybridized with a probe enriched for sequences expressed in multilineage cells (U–M). The arrows indicate clones that were hybridized strongly with one probe and not the other.

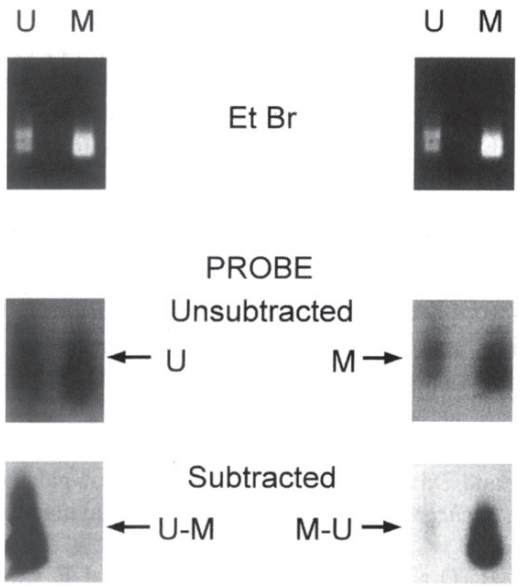

Fig. 2. Southern hybridization of starting *PolyAcDNA* with enriched and nonenriched probes. The starting *PolyAcDNA* samples U and M are pools of *PolyAcDNAs* derived from 20 unilineage mouse hematopoietic precursors (U) and 5 multilineage mouse hematopoietic precursors (M) ***(6)***. Replica filters of the starting samples are hybridized with unsubtracted (probes U and M) and with *PolyAcDNA* remaining after four sequential rounds of subtraction (probes U–M and M–U).

1. Prepare Southern filters of the starting samples and replica filters of cDNA libraries using standard protocols ***(15)***.
2. Label *PolyAcDNA* probes either by random priming or incorporating the radioactive nucleotide by PCR using the following conditions: 1× *Taq* buffer, 1:100 dNTP labeling mix, 0.5 OD260/mL of the oligo used to generate the tracer and driver, 1.2 U *Taq* polymerase, 5 µL 32P dCTP (final vol 100 µL). Thermal profile: 30 sec at 94°C, 30 sec at 60°C, 90 sec at 72°C × 20 cycles.

4. Notes

1. Hybridization conditions were optimized after a series of test subtractions in which tracer cDNAs were radioactively labeled and the removal of shared sequences monitored by measuring the amount of radioactivity remaining in the extracted aqueous phase. Over 80% of tracer sequences could reproducibly be removed in one round using these hybridization conditions, leading to 99% removal of common sequences after four sequential rounds of subtraction.
2. When washing Southern filters containing *PolyAcDNAs* hybridized with a *PolyAcDNA* probe, the probe will anneal to the polyA/T ends present in all *PolyAcDNA* molecules making it necessary to wash at high stringency (0.2× SSC) at temperatures up to 73°C.
3. The hybridization and washing conditions for library screening should be carried out at a lower stringency, particularly when the probe and library are derived from different species. In the example in **Fig. 1**, a mouse was used to screen a human library, and washing stringency was kept low (45°C and 2× SSC).

Acknowledgments

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In-Situ Hybridization of Radioactive Riboprobes to RNA in Tissue Sections

Radma Mahmood and Ivor Mason

1 Introduction

The development of such techniques as transgenesis, saturation mutagenesis, and the polymerase chain reaction (PCR), all of which are described in detail elsewhere in this volume, revolutionized experimental embryology. However, no single procedure has been applied more broadly across the field than that of in-situ hybridization to RNA. This allowed researchers to determine rapidly the spatial and temporal expression of their gene of interest without having to resort to more tedious and less certain approaches requiring the production of antisera against the protein product. As such, in-situ hybridization has become the standard first step toward the characterization of the developmental significance of a newly identified gene. Of course, in-situ hybridization tells you nothing regarding the translation of the mRNA into protein or about protein's subsequent localization, modification, or stability. However, in-situ hybridization remains an extremely powerful tool, enabling the researcher to predict likely developmental functions for gene products rapidly.

Historically, this technique was pioneered by the Angerers working with sea urchin embryos ***(1)***. It was refined by several groups, notably by McMahon and Wilkinson in studies of gene expression in sections of mouse embryos ***(2, 3)***. During the past decade or so, the use of radioactive in-situ hybridization of tissue sections has been largely superseded by the application of nonradioactive approaches on whole embryos at early developmental stages (*see* Chapters 46–48) and, to a lesser extent, on sections of older embryos and adults (*see* Chapter 49). The latter approaches offer considerable advantages in terms of their speed and, in the case of in-situ hybridization to whole embryos, further advantages in the ease of spatial interpretation of results. However, the radioactive

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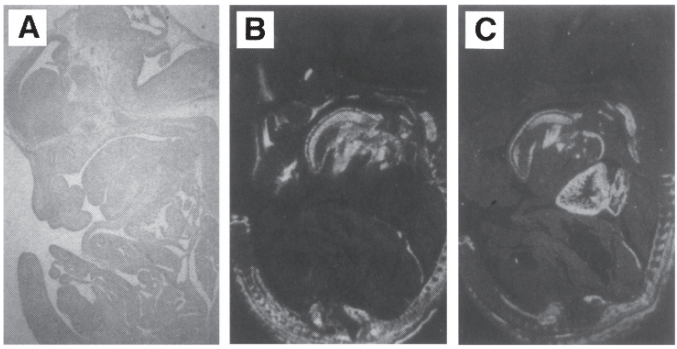

Fig. 1. Adjacent sagittal sections of an E14.5 mouse embryo. **(A)** Bright-field view of toluidine blue-stained section. **(B)** Same field viewed under dark ground optics to show hybridization of FGF-7 probe to a number of tissues, including a subset of muscles, nasal epithelium, cartilage capsules, and lung. Section was exposed for 14 d **(C)**. Adjacent section hybridized with a cardiac α-actin probe showing hybridization to all skeletal and cardiac muscle. Section was exposed for 24 h.

approach remains more sensitive and is the only method by which certain "low-abundance" transcripts are detectable. Furthermore, for these reasons, it is likely that many studies of developmental gene expression undertaken by nonradioactive, whole-embryo approaches are incomplete. As such, radioactive in-situ hybridization remains a valid approach in embryological and other fields. The following protocol ***(4)*** is that used in our laboratory (*see* **Fig. 1**) and is a modification of an original method of Wilkinson and Green ***(5)***.

2. Materials

2.1. Preparation of Material for Sectioning

1. Diethylpyrocarbonate (DEPC)-treated water. DEPC (Sigma, Poole, UK) is added to double-distilled water to a concentration of 0.1% (v/v). The solutions are shaken then autoclaved. All solutions used for steps between sectioning tissue and the first posthybridization wash are prepared in DEPC water. All subsequent steps use solutions prepared in double-distilled water.
2. 10× PBS: 1.3 M NaCl, 70 mM Na_2PO_4, 30 mM NaH_2PO_4. Check that pH is 7.0 (if required adjust slightly with HCl or NaOH as appropriate). Add DEPC to 0.1% and autoclave.
3. 4% (w/v) Paraformaldehyde in PBS freshly prepared on the day of use for pretreatment of sections but can be stored for fixation of embryos. 16 g Paraformaldehyde (Fluka no. 76240, Gillingham, UK) weighed out in a fume hood—wear mask and gloves. This is added to 400 mL of 1× PBS in distilled water and heated at 65°C in a water bath with occasional swirling until dissolved (about 90 min). It is cooled to 4°C prior to use or stored in 20-mL aliquots at −20°C.
4. 10× Saline: 8.3% (w/v) NaCl. Add DEPC to 0.1% and autoclave.

5. Howard's Ringer (0.12 M NaCl, 0.0015 M $CaCl_2$, 0.005 M KCl. Per liter: 7.2 g NaCl, 0.17 g $CaCl_2$, 0.37 g KCl, pH 7.2, with very dilute HCl).
6. Absolute (100%) ethanol and graded ethanols 70, 80, and 95% (v/v) prepared with DEPC water.
7. Xylene (AnalaR, Merck BDH, Poole, UK).
8. Histoclear (National Diagnostics, Hull, UK).
9. Wax (Paraplast Plus, Sherwood, St. Louis, MO).
10. Plastic molds for wax (e.g., R.A.Lamb, London, UK).

2.2. Preparation of Slides

1. Washed glass microscope slides and coverslips (Merck BDH).
2. 25-Place metal slide racks (R.A. Lamb).
3. 400-mL Capacity glass troughs with lids suitable to hold slide racks (R.A. Lamb).
4. Concentrated hydrochloric acid (Merck BDH).
5. Acetone (AnalaR, Merck BDH).
6. TESPA (3-aminopropyltriethoxysilane; Sigma) used in the fume hood.

2.3. Cutting and Mounting Sections

1. Suitable rocking microtome, disposable blades and holder (reduces chance of contamination with RNase from a generally used blade), bath for sections (well-cleaned with detergent, rinsed well with distilled water, and filled with DEPC water), and hot plate.
2. Plastic slide boxes suitable for storing slides with sections such that slides are well separated (e.g., R.A. Lamb).
3. Silica gel (Sigma).

2.4. Preparation of Template for Riboprobe Synthesis

1. Appropriate restriction endonucleases and manufacturer's buffers (e.g., Boehringer Mannheim, Lewes, UK).
2. Agarose (Life Technologies, Paisley, Scotland).
3. 10× TBE (0.89 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA; per liter 108 g Tris base, 55 g boric acid, 9.4 g EDTA).
4. Ethidium bromide (10 mg/mL). Stored at 4°C in the dark. Caution: Ethidium bromide is a potent carcinogen.
5. Phenol (molecular biology-grade; Life Technologies). Melted at 65°C in a fume cupboard and equilibrated with 0.1 M Tris. HCl, pH 7.0, followed by addition of a few crystals of 8-hydroxyquinoline, which will color the solution yellow. Stored in the dark at 4°C and discarded if the solution begins to turn orange.
6. Chloroform (AnalaR; Merck BDH).
7. 3 M Sodium acetate, pH 7.0 (with acetic acid).
8. 100% (v/v) Ethanol and 70% (v/v) ethanol.

2.5. Synthesis of ^{35}S-UTP-Labeled Riboprobes

1. RNA polymerases, manufacturer's buffers, and 200 mM DTT (Promega, Southampton, UK and Boehringer Mannheim).
2. ^{35}S-UTP (>1,000 Ci/mM, Amersham International, Amersham, UK).

3. RNasin (Boehringer Mannheim).
4. RNase-free DNase 1 (Boehringer Mannheim).
5. Yeast total RNA (Sigma) 10 mg/mL dissolved in DEPC water, and stored as aliquots at −20°C.
6. 5 M Ammonium acetate (Sigma) dissolved in DEPC water.
7. Aqueous scintillant, such as Aquasol (Dupont NEN, Hounslow, UK).

2.6. Hybridization to Tissue Sections

1. Proteinase K (Boehringer Mannheim) 20 mg/mL in water stored in single-use (20-μL) aliquots at −20°C.
2. TE pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) diluted in DEPC water and prepared from concentrated stocks dissolved in DEPC water.
3. Triethanolamine (Merck BDH).
4. Acetic anhydride (Merck BDH).
5. 20× SCC (3 M NaCl, 0.3 M sodium citrate).
6. Hybridization buffer: 50% (v/v) deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 10% (w/v) dextran sulfate, 1× Denhardt's solution, 0.5 μg/mL yeast tRNA. Store in aliquots at −70°C.

2.7. Posthybridization Washes

1. TNE (0.5 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 8.0).
2. High-stringency buffer: 50% v/v formamide, 2× SSC, 10 mM DTT prepared fresh on day of use using solid DTT or a 100-mM stock of DTT stored at −20°C.
3. RNase A (10 mg/mL) in water. Store at −20°C.
4. Graded (30, 50, 70, and 95% v/v) ethanols containing 0.3 M (final) ammonium acetate. The presence of the ammonium acetate ensures that probe-target RNA duplexes remain intact during the dehydration process. Duplexes are stable in 100% (v/v) ethanol.

2.8. Autoradiography

1. Emulsion (K-S no. 1355127, Ilford Photographic, Mobbesley, UK).
2. Glycerol (Merck, BDH).
3. Two-place plastic slide mailers (R.A. Lamb).

2.9. Developing and Mounting Slides

1. D19 developer (Kodak, Paris, France): Dissolve 64 g D19 developer in 400 mL water. This can be used several times on the day of preparation, but discard after use.
2. Stop solution: 350 mL of 1% (v/v) acetic acid/1% (v/v) glycerol.
3. Fixing solution: 350 mL of 30% (w/v) sodium thiosulfate.
4. 0.25% Toluidine blue (Sigma) filtered through a Whatman general-purpose filter.
5. Graded ethanols (30, 50, 80, 95, 100% v/v).
6. Depex mounting medium (Merck BDH).

3. Methods

Between **sections 3.1., step 2** and **3.6, step 20,** there must be no chance of contamination with RNases. Wear gloves, use disposable plastics instead of glass, and where possible, also use pipetmen designated for RNA use only, also DEPC-treat all solutions. If the presence of RNases is still suspected, then glassware can be treated as well.

3.1. Preparation of Material for Sectioning

1. Dissect embryos in 1× PBS (mice) or Howard's Ringer (chicks). Fix in a large volume (at least five times the volume of embryonic tissue) of 4% (w/v) paraformaldehyde in PBS at 4°C overnight in a vessel that adequately contains the fumes (**see section 2.3**). Smaller embryos can be fixed for shorter periods of time (4 h minimum), and it is convenient to dechorionate zebrafish after fixation. Embryos can be fixed pinned out in petri dishes, if required, in the designated refrigerator. Fixation times vary with the size of the embryo, from a few hours (mice up to embryonic [E] 9.5, chicks to stage 18) to 12 h (E18 mice) or even longer (newborn mice or chicks from stage 38; see Note 1). In our experience fixation for 2–3 d does not adversely affect results.
2. Incubate in 1× PBS on ice for 30 min (small embryos, i.e., chicks <E6.5, mice <E13.5) or 1 h (larger material). Use glass containers, since later treatments involve high temperatures and organic solvents.
3. Further dissect embryos if required, such as cut embryos away from surrounding membranes. Stage according to Theiler ***(6)*** or Hamburger and Hamilton ***(7)*** if required.
4. Remove PBS and replace with 1× saline. Incubate for 30 min (small embryos) or 1 h (larger material) on ice.
5. Replace saline with saline–ethanol (1:1), and incubate for 30 min (small embryos) or 1 h (larger material) on ice.
6. Replace saline–ethanol with 70% (v/v) ethanol (prepared with DEPC-treated water), and incubate for 30 min (small embryos) or 1 h (larger material) on ice.
7. Repeat **step 6**.
8. Replace 70% (v/v) ethanol with 80% (v/v) ethanol and incubate on ice 30 min.
9. Replace with 95% (v/v) ethanol and incubate on ice for 30 min (small embryos) or 1 h (larger material) on ice.
10. Replace with 100% ethanol and incubate on ice for 30 min (small embryos) or 1 h (larger material) on ice (*see* **Note 2**).
11. Place embryos in 100% ethanol for 30 min (small embryos) or 1 h (older embryos).
12. Replace with 100% ethanol for 30 min (small embryos) or 2 h (older embryos).
13. Replace 100% ethanol with xylene or (preferably) histoclear in glass bottles and incubate on ice 30 min (small embryos) or 1 h (older embryos).
14. Repeat **step 13** with fresh xylene or histoclear.

15. Pour off and half-fill bottles with fresh molten wax (sections cut from blocks prepared with freshly melted wax have better elasticity than from wax that has remained molten for days), and place at 52°C for 45 min (small embryos) or 90 min (large embryos).
16. Change wax twice, and incubate each time for 60 min (small embryos) or 90 min (large embryos).
17. Embed tissue: Fill molds with wax first (if possible, do this on a warmed plate to keep the wax molten), carefully transfer the embryo to the mold, orient with a hot mounted needle or warm forceps, and move to a cooled plate or onto the bench. Label one end of the block for reference after the wax is set and the embryo's orientation is no longer visible (e.g., by inserting a piece of card on which details of the specimen and its orientation are written) and allow the wax to set completely (1–2 h).
18. Blocks may be stored at 4°C for several years. Any degradation of RNA in such material is most likely owing to incomplete penetration of reagents during the preceding processing steps.

3.2. Preparation of Slides

Microscope slides need to be washed and silanized prior to collection of sections. Alternatively, pretreated slides (e.g., Superfrost, Merck BDH) are now commercially available.

1. Place metal racks of 25 slides for 10 sec each in 400 mL of 70% (v/v) ethanol, 10% (v/v) concentrated HCl, then in distilled water, and finally in 95% (v/v) ethanol.
2. Wrap in baking foil to keep free from dust, and place the slides at 150°C for 30 min.
3. Cool on bench wrapped in foil.
4. Place the rack of slides for 10 min each first in acetone containing 2% (v/v) TESPA, then in two changes of acetone, and finally in distilled water (not DEPC-treated). Do this step in the fume cupboard.
5. Dry at 37°C overnight wrapped in foil.
6. Slides are stored individually in racks (to avoid scratches) at room temperature for up to 3 mo.

3.3. Cutting and Mounting Sections

1. Cut 7- to 10-µm sections as ribbons on a rotary microtome using a disposable blade.
2. Ribbons are placed on a pool of 20% (v/v) ethanol in DEPC-treated water on a slide and cut into smaller strips with a clean razor blade if required.
3. The sections are floated out onto DEPC-treated water in a clean section bath at 45°C until creases in them disappear.
4. Sections are then collected onto TESPA-treated slides and dried on a 48°C hot plate for 2 h or overnight at 37°C. It is useful to collect at least two adjacent sections per slide; this aids the subsequent identification of hybridizing tissues, since these should give identical results on both sections.

5. Store slides desiccated at 4°C such that they are free from moisture and sections still give acceptable results months after being cut. Before use, allow slides to warm to room temperature. If some slides are to be returned to storage, place them in the 37°C oven for 30 min prior to storage to reduce the moisture and store sealed with fresh desiccant.

3.4. Preparation of Template for Riboprobe Synthesis

1. The DNA fragment should have been previously cloned into a plasmid vector, such that prokaryotic RNA polymerase promoters flank it. Suitable vectors include the pGem series (Promega, Madison, WI) and the pKS or pBluescript series (Stratagene, La Jolla, CA). In our experience, T_7 and T_3 RNA polymerases give the best overall performance on a wide range of templates, whereas SP6 polymerase has problems transcribing certain templates.
2. Linearize plasmid (usually 10–20 µg) with a suitable restriction endonuclease using manufacturer's buffer and conditions (*see* **Note 3**).
3. Check that digestion is complete by analyzing 0.25 µg on a 1% (w/v) agarose gel in 1× TBE containing ethidium bromide (0.1 µg/mL) alongside some uncut plasmid. If the digestion is incomplete, add more enzyme, incubate for a further period, and reanalyze by gel electrophoresis.
4. Extract the remainder of the reaction mixture once with phenol, once with phenol–chloroform (1:1), and twice with chloroform.
5. Precipitate the plasmid DNA by the addition of 0.1 vol of 3 M sodium acetate (pH 7.0) and 2.2 vol of ethanol followed by incubation on ice for 15 min.
6. Collect the precipitate by centrifugation at 10,000 *g* in a microfuge for 10 min, aspirate off the supernatant, and wash the pellet with 70% (v/v) ethanol.
7. After allowing the last traces of ethanol to evaporate, the pellet is resuspended in DEPC-treated water to give a final concentration of 0.5 µg/µL and stored at –20°C.

3.5. Synthesis of ^{35}S-Labeled Riboprobes

1. Warm buffers, nucleotides, DTT, and templates to room temperature.
2. Assemble the following reaction mixture in this order: 10 µL ^{35}S-UTP, 2 µL 10× transcription buffer, 1 µL 200 mM DTT, 1 µL RNase inhibitor, 2 µL 2.5 mM NTP, pH 8.O (a mix of CTP, GTP, and ATP to produce 2.5 mM final of each), 1 µL DEPC water. Mix by gently pipeting up and down.
3. Add 1 µL of the appropriate RNA polymerase, and mix gently by pipeting (do not vortex).
4. Add 2 µL (1 µg) of DNA template, mix, and spin for 5 s in microfuge to collect all liquid in the bottom of the tube.
5. Incubate at 37°C for 1–2 h.
6. Add 10 µL DEPC water, 2 µL yeast RNA, and 2 µL RNase-free DNase, and incubate at 37°C for 15 min.
7. Add 50 µL 5 M ammonium acetate (prepared with DEPC water) and 200 µL ethanol. Incubate on ice for 30 min and spin in a microfuge for 15 min.

8. Remove supernatant, and immediately dissolve pellet in 30 µL 10 mM DTT.
9. Repeat **step 7** of **section 3.5**.
10. Remove the supernatant, immediately redissolve probe in 20 µL 100 mM DTT over 15–20 min with occasional vortexing, and count 1 µL in an aqueous scintillant. Calculate probe specific activity in dpm/µL/kb probe length: Acceptable probes must be $>10^6$ dpm/µL/kb, i.e., specific activity of 1 µL 8 length of labeled RNA transcript in kb. Store probes at −70°C for up to 2 mo.

3.6. Hybridization to Tissue Sections

1. Early in the day, make up 400 mL fresh 4% paraformaldehyde in PBS, and store on ice until required. Also ensure that you have 3.5 L of DEPC-treated water for dilution of salines and the like. Use designated RNase-free glassware and racks for all the following procedures, wear gloves throughout, and use DEPC-treated water and solutions.
2. Dewax slides in xylene twice each for 10 min in a fume cupboard. For this and all subsequent steps, use 350 mL of each solution for each incubation; sufficient to cover the slides completely. Slides are processed in metal racks holding up to 25 individual slides in appropriately sized staining troughs. Do not agitate the slides during incubations, since it may cause sections to detach.
3. Place in 100% ethanol for 2 min to remove most of the xylene. Meanwhile make up 1.6 L 1× PBS and 800 mL 1× saline.
4. Rehydrate sections by "dipping" through the following graded alcohol series: 100% ethanol twice, 95% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, and 30% ethanol. (Keep and reuse ethanol up to ten times).
5. Place in 1×saline for 5 min then in 1× PBS for 5 min. Discard used saline and PBS.
6. Place in the freshly made, cold 4% paraformaldehyde in PBS for 20 min in the fume hood. At the end of this, do not discard the fix, but keep for **step 11** of **section 3.6.**
7. Meanwhile, for 25 slides, prepare 15 mL of DEPC-treated TE, pH 8.0, mixed with 20 µL of proteinase K (20 mg/mL). Also wash sufficient coverslips of appropriate size in ethanol and leave to air dry in a rack covered in foil.
8. Place slides in 1× PBS twice each for 5 min, then lay flat on a sheet of foil on the bench with the sections uppermost.
9. Cover sections with 300 µL or more of proteinase K (sufficient to cover the sections) and incubate for exactly 7 min at room temperature.
10. Tip off the proteinase K and place slides in a rack in fresh 1× PBS for 5 min.
11. Return the slides to the cold paraformaldehyde (**section 3.6., step 6**) for 5 min in the fume hood.
12. Meanwhile, fill two troughs with DEPC water and place in the fume hood. To the second (acetylation bath), add 6 mL triethanolamine and 1 mL acetic anhydride and mix with a small stir bar on a stirrer.
13. Dip slides briefly into the DEPC water, then place in the acetylation bath for 10 min with stirring. (A small stir bar or "flea" fits beneath standard slide racks and allows

stirring in the presence of the latter. This step acetylates slides and sections and helps prevent nonspecific binding of probe).

14. Place slides in fresh 1× PBS for 5 min then fresh 1× saline for 5 min.
15. Dehydrate by "dipping" through the same ethanol series used in **section 3.6, step 4,** starting with 30% ethanol and increasing to the two 100% ethanols.
16. Place slides section side up to air dry on foil. Add probe to them the same day.
17. Dilute probe in hybridization buffer to give 10^5 dpm/μL/Kb.
18. Mix probe by vortexing and briefly spin in a centrifuge. Heat to 80°C for 3 min to remove secondary structures owing to internal hybridization of the probe. Allow to cool briefly on ice and then mix again.
19. Place 15–30 μL of probe solution on slide adjacent to sections, spread over sections with a piece of parafilm, and cover with a clean coverslip (**section 3.6, step 7**) taking care not to introduce any bubbles.
20. Slides are collected on their sides in a rack place inside a suitable box with a tightly sealed lid. Include a tissue soaked in 5× SSC, 50% formamide to prevent dehydration. Seal the box with suitable tape and hybridize at 55°C overnight in an oven.

3.7. Posthybridization Washes

For all procedures after hybridization, we use separate glassware and graded ethanol solutions from those used prior to hybridization, because RNase A is used during the washing procedures and could contaminate all glassware and reused solutions.

1. Place slides in 4× SSC at room temperature until coverslips fall off (about 20 min).
2. Remove coverslips and place slides in fresh 4× SSC for 1 h.
3. Meanwhile, make up 1.6 L of TNE buffer and 80 mL of high-stringency buffer/25 slides, prewarming the latter to 50°C or 65°C as appropriate (*see* **step 4**).
4. Place slides back to back in Coplin jars containing high-stringency buffer in a 65°C water bath (high stringency) or 50°C (low stringency) for 30 min (*see* **Note 4**).
5. Transfer slides to a rack, and place in 350 mL of TNE buffer for 10 min at 37°C. Repeat this wash once more.
6. Place slides in a third trough containing 400 mL prewarmed TNE buffer and 800 μL of 10 mg/mL RNase A for 30 min at 37°C.
7. Wash slides for 10 min in fresh TNE buffer at 37°C.
8. Wash slides for 30 min in Coplin jars containing prewarmed high-stringency buffer at 65°C (or appropriate temperature).
9. Wash for 15 min in 2× SSC, then for 15 min in 0.1× SSC both at room temperature.
10. Dehydrate through graded ethanols containing 2.5 M ammonium acetate (the latter to prevent "melting" of the probe from target RNAs) from 30 to 100% by dipping as before.

11. Air dry in a rack loosely wrapped in foil for 60 min minimum. The slides may be left overnight to dry.

3.8. Autoradiography

All steps are performed in a darkroom using a red safe light.

1. Put a foil-wrapped glass sheet onto a tray of ice to cool in the darkroom.
2. Place about 15 mL of emulsion ribbons into a 50-mL Falcon tube and incubate in a 42°C water bath to melt them.
3. Decant 8 mL of the melted emulsion into a slide mailer, and replace at 42°C.
4. Prepare 8.8 mL water containing 180 µL glycerol and add to the melted emulsion. Mix gently with a glass pipet to expel bubbles and leave in water bath for 10 min to warm to 42°C.
5. Dip slides briefly and with a uniform speed into the emulsion, wipe the back (nonsection side) of each, and lay section side up on cold tray for 10 min to set. If bubbles or streaks are visible, then redip the section.
6. Place slides in a box for emulsion to harden in complete darkness for 2 h, then transfer to a sealed, lighttight box containing silica gel packets, and store at 4°C for a few days to 2 wk depending on abundance of target transcript.

3.9. Developing and Mounting Slides

Unless otherwise stated, all procedures are carried out in the darkroom using a red safe light.

1. Make up troughs containing 350 mL fresh developer, stop solution, and fixing solution. Allow or adjust the temperature of all solutions to reach 20°C and place in the darkroom.
2. Remove slides from the refrigerator and allow them to equilibrate to room temperature.
3. Place a rack of slides in the developer for exactly 2 min and 30 sec, then directly into the stop solution for 1 min and into the fixing solution for 5 min.
4. Wash in troughs of distilled water twice, each for 10 min. Slides can now be removed from the darkroom.
5. For counterstaining, place each slide in 50 mL of 0.25% (w/v) fresh filtered toluidine blue for exactly 1 min. Dehydrate by rapid dipping through a graded series of ethanols (30–100%) washing twice in 100% ethanol.
6. Then, clear with xylene (histoclear cannot be used here, since it leaves an oily film on the slide) and mount with coverslips under Depex.

4. Notes

1. It is crucial, during fixation, processing, and embedding in wax prior to sectioning, that all the reagents completely penetrate and equilibrate within the embryonic tissue. Times vary according to the size of the embryo or tissue being prepared. The following illustrates modifications that have been used successfully on larger tissues (e.g., heads of E10 chick embryos and whole E18 mouse embryos).

On d 1, fix in 4% paraformaldhyde in 1× PBS overnight at 4°C.
On d 2, wash twice in PBS each for 30 min, wash twice in saline each for 30 min, then incubate in 30% ethanol for 1 h, 50% ethanol for 1 h, 70% ethanol for 1 h, 100% ethanol for 3 h, and in fresh 100% ethanol overnight.
On d 3, incubate in fresh 100% ethanol for a further 5 h. Then incubate in three changes of histoclear (or xylene) the first for 1 h, the second for 3 h, and the last overnight.
On d 4, replace the histoclear with freshly melted (52°C) wax, and incubate at 52°C in an oven for 3 h, replace the wax with fresh and incubate at 52°C for 5 h, then replace once more and incubate at 52°C overnight.
On d 5, incubate with two more changes of wax each for 3 h, then embed the tissue.

2. Embryos can be stored at −20°C for up to a week at this stage.
3. Restriction enzyme sites should be selected to produce a transcript of 300–1,000 bp. If any larger, we find that nonspecific background signal increases. Also, if possible, select enzymes that produce a blunt end or a 5′- overhang, because 3′ -overhanging ends facilitate "wraparound" transcripts, resulting from the polymerase transcribing back along the complementary DNA strand. If there is no alternative to using an enzyme producing a 3′ -overhanging end, the following procedure can be used after digestion to "end polish," generating a blunt end. After digestion, add Klenow polymerase to a final concentration of 5 U/μg DNA template, and incubate at 22°C for 15 min prior to proceeding with the rest of the transcription protocol.
4. Coplin jars containing 30–40 mL of solution are used at this stage and in **section 3.7, step 8,** to reduce the volume of high-stringency buffer used; the large amounts of DTT required would otherwise prove very expensive.

Acknowledgments

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In-Situ Hybridization and Immunohistochemistry in Whole Embryos

Carol Irving

1. Introduction

The ability to visualize zones of active gene expression in whole embryos or tissues is a powerful tool toward understanding spatial and temporal relationships among molecules during development. Combining simultaneously a number of RNA probes with immunohistochemical detection of proteins allows direct comparison between transcription and translation of any given gene, a direct spatiotemporal comparison of a number of molecules, or can be used to identify exactly their expression domain within the embryo with relation to stained "tissue landmarks."

Performing in-situ hybridization and immunohistochemistry before sectioning is advantageous, as larger sample numbers can be processed with speed. Sites of specific interest can subsequently be sectioned for visualization at a single-cell resolution.

A number of protocols are currently available, each with modifications for different organisms. Here, we describe a protocol that can be used for a variety of species and is suitable for hybridization to embryos with probes derived from DNA sequences from both the same species and other species, modified from those of Wilkinson ***(1)*** and Henrique et al. ***(2)***.

We also include a method for "two-color" in-situ hybridization, which allows the simultaneous expression of two genes to be examined (Fig. 1 A,B). In addition, these protocols can be coupled with a protocol we describe for whole-mount immunohistochemistry following in-situ hybridization (Fig. 1 C,D).

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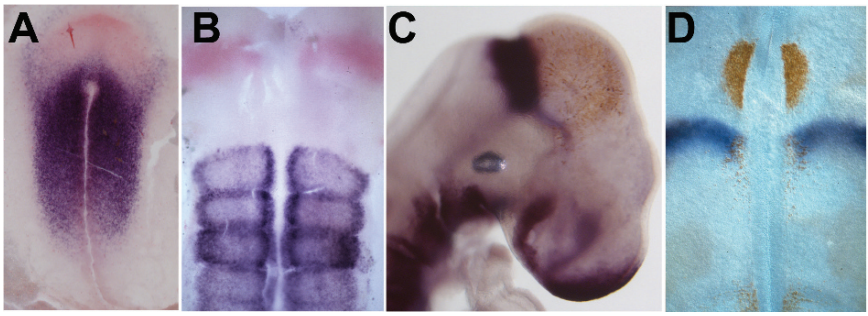

Fig. 1. **A** Two-color in-situ hybridization of *Otx2* (red) and *Gbx2* (blue) to a stage 5 chick embryo showing expression in adjacent domains. **B** Two-color in-situ hybridization of *Fgf8* (red) and *Hoxa2* (blue) to a stage 14 chick embryo, flat mounted. **C** Dual in-situ hybridization and immunohistochemistry to *Fgf8* mRNA (blue) and ectopic GFP protein (brown) in a stage 15 chick embryo showing the advantage of dual detection to reveal the location of ectopically introduced proteins and their effect on endogenous gene expression. **D** Dual in-situ hybridization and immunohistochemistry to *Fgf8* mRNA (blue) and Isl2 protein (brown) to a 4-d chick embryo, from which the neural tube has been flat mounted. Detection of motor neurons by Isl2 protein expression shows the advantage of dual detection for spatiotemporal comparison of gene and protein expression. (*See Color Plate*)

2. Materials

2.1. General

The following equipment is required: a dissection microscope offering up to at least 65× magnification (e.g., Nikon, Kingston upon Thames, UK or Zeiss,Welwyn, Garden City, UK); transmitted (Nikon, Zeiss) and fiberoptic (Schott, Zeiss, Leica, Milton Keynes, UK) light sources; heated water bath and microfuge (e.g., Eppendorf, Heraeus) for enzyme digests and riboprobe synthesis (e.g., Grant, Merck, Poole, UK); horizontal gel electrophoresis tank and power supply (e.g., Life Technologies, Paisley, Scotland); oven (e.g., Heraeus, Merck), heated block (e.g. Grant QBT2) for hybridizations and washes; shaker at 4°C and at room temperature for incubations with antibodies and subsequent washes; suitable photomicroscopes and equipment for histological analysis.

2.2. Fixation, Processing, and Hybridization

1. Phosphate-buffered saline (PBS; 10× concentrated): 1.3 M NaCl, 70 mM Na_2HPO_4, 30 mM NaH_2PO_4. Check that the pH is 7.0 (if required, adjust slightly with HCl or NaOH as appropriate). Add DEPC to 0.1% and autoclave.
2. Howard's Ringer: 0.12 M NaCl, 0.0015 M $CaCl_2$, 0.005 M KCl. Per liter: 7.2 g NaCl, 0.17 g $CaCl_2$, 0.37 g KCl, pH 7.2, with very dilute HCl.

3. MEMFA fixative: 1× MEM, 4% formaldehyde 10× stock MEM kept in dark at 4°C:1 M MOPS, 20 mM EGTA, 10 mM $MgSO_4$ pH 7.8, filter sterilize (do not autoclave).
4. PBT is 1× PBS containing 0.1% (v/v) Tween 20 (Sigma).
5. Methanol (AnalaR; Merck BDH).
6. Hydrogen peroxide (30% w/w; Sigma, replace every 4 mo).
7. Proteinase K (Roche, UK) 20 mg/mL and stored in single use (15–20-µL) aliquots at −20°C.
8. 25% (w/v) Glutaraldehyde (electron microscopy grade; BDH, Poole, UK). Glutaraldehyde is carcinogenic and should be used in a fume cupboard.
9. 2 Hybridization buffer: 2% BBR, 0.1% Tween20, 0.5% CHAPS, 1 mg/mL yeast tRNA, 50 µg/mL heparin.
10. Prehybridization–hybridization solution: 50% (v/v) deionized formamide (Fisons, Loughborogh, UK), 5× SSC, pH 4.5 (20× SSC stock is 3 M NaCl, 0.3 M sodium citrate; use citric acid to pH), 2% Boehringer blocking reagent (BBR) (stock is 10% in MAB: heat to dissolve, autoclave, store at −20°C in aliquots), 50 mg/mL yeast RNA (Sigma), 50 mg/mL heparin (Sigma). Heparin and yeast RNA are stored as concentrated 50 mg/mL solutions at −20°C.

2.3. Preparation of Template for Riboprobe Synthesis

1. Appropriate restriction endonucleases and manufacturer's buffers (e.g., Roche).
2. Agarose (Life Technologies).
3. 10× TBE: 0.89 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA; per liter 108 g Tris base, 55 g boric acid, 9.4 g EDTA.
4. Ethidium bromide (10 mg/mL). Stored at 4°C in the dark. Caution: Ethidium bromide is a potent carcinogen.
5. Qiagen Qiaquick gel extraction kit (cat no. 28706).
6. 3 M sodium acetate, pH 7.0 (with acetic acid).
7. Absolute (100% v/v) ethanol and 70% (v/v) ethanol.

2.4. Synthesis of Digoxygenin (DIG) or Fluorescein-12 UTP (FITC) Labeled Riboprobes

1. RNA polymerases, manufacturer's buffers, and 200 mM DTT (Promega, Southampton, UK, and Roche).
2. 10× DIG or fluorescein RNA labeling nucleotide mixes (Roche).
3. RNasin (Roche).
4. RNase-free DNase-1 (Roche).
5. TE, pH 7.0: 10 mM Tris-HCl, pH 7.0, 1 mM ethylenediamine tetraacetic acid, disodium salt (EDTA), pH 8.0, diluted from stocks dissolved in RNAse-free water.
6. 4 M Lithium chloride.
7. 70% (v/v) Ethanol.

2.5. Posthybridization Washes

1. Wash solution: 50% formamide, 1× SSC, 0.1%Tween-20.
2. MABT: 100 mM maleic acid, 150 mM NaCl, 0.1% Tween20, pH 7.5 (autoclave prior to addition of Tween20).
3. Goat or sheep serum (Sigma) heat-inactivated at 55°C for 30 min and stored in 1–2-mL aliquots at −20°C.
4. Anti-DIG or anti-FITC Fab fragments conjugated to alkaline phosphatase (Roche).

2.6. Postantibody Washes and Histochemistry

1. NTMT, pH 9.5: 100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20 (prepared freshly on day of use from concentrated stock solutions of the individual components).
2. Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP/x-phosphate) (Roche).
3. PBT containing 20 mM EDTA.
4. NTMT, pH 8.0: 100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl2, 0.1% Tween 20 (prepared freshly on day of use from concentrated stock solutions of the individual components).
5. Fast red TR/naphthol AS-MX (Sigma).

2.7. Immunohistochemistry

1. Hydrogen peroxide (30% w/w; Sigma, replace every 4 mo).
2. Triton X-100 (Sigma, Poole, UK).
3. Sodium azide (Sigma).

3. Methods

3.1. General

1. Perform all steps at room temperature for 5 min. with rocking or slow rotation unless otherwise stated. For larger tissues, increase times to 10–15 min.
2. To avoid mechanical damage to embryos, leave a small quantity of liquid over them between each step.
3. To avoid degradation of target mRNAs, all reagents must be free from RNase contamination until after hybridization. Wear gloves, use disposable plastics, and use autoclaved solutions where possible.

3.2. Preparation of Template for Riboprobe Synthesis

Probes are generated by in-vitro transcription of a DNA template, cloned into a vector containing an appropriate polymerase initiation site. Probes are complementary to the target mRNA (i.e., an antisense strand is synthesized) and are labeled by the incorporation of hapten-conjugated nucleotides (typically DIG gives the best results).

We find the quality of probe preparation is the most important determinant for strong signals with low background. Plasmids should be linearized to completion at the 5 end of the cDNA as inclusion of plasmid sequences in the probe increases nonspecific binding. The ideal probe length is between 300 and 1,000 bp. Larger transcripts give a nonspecific background signal (probably due to trapping of the probe). The linear DNA is column purified (e.g., Qiagen Qiaquick gel extraction kit, cat no. 28706, following *Qiaquick Spin Handbook* protocol for PCR purification). DNA is eluted in 30 µL superclean H_2O to a final concentration of 1 µg/µL.

3.3. Transcription of Riboprobe

1. Assemble on ice in the following order:

Component	20-µL mix
H_2O	7
100 mM DTT (vortex before use)	4
5× transcription buffer (vortex)	4
Ribonucleotide mix (DIG or FITC)	2
RNAsin RNAse inhibitor	1
RNA pol (T_7 or T_3 or SP6)	1
Linear DNA template (1 µg/µL)	1

2. Mix by gently pipeting up and down and incubate at 37°C for 2 h. For SP6 probes, use 2× DNA template and 2 µL SP6 polymerase and incubate at 40°C.
3. Check that probe has been synthesized by analyzing 1 µL of product on a 1% (w/v) agarose gel in 1× TBE containing ethidium bromide (0.1 µg/mL). A DNA band (1 µg) and RNA band (10 µg) of the same intensity should be seen under ultraviolet (ethidium bromide intercalates poorly into RNA).
4. Add 2 µL RNase-free DNase and incubate at 37°C for 15 min (optional).
5. Purification: Riboprobes are column purified to obtain maximum yield (Qiagen Qiaquick gel extraction kit, cat. no. 28706) following *Qiaquick Spin Handbook* protocols for PCR purification (p. 18). Elute RNA probe in 100 µL RNASe-free H_2O to give about 1 µg/10 µL as estimated from the gel, add 2 µL RNasin and store at −20°C.

3.4. Fixation and Prehybridization

1. Dissect out embryos in ice-cold PBS (mouse, chick) or Howard's Ringer (chick) and open cavities.
2. Fix in a large volume (for this and subsequent procedures up to the hybridization step, use an excess, e.g., 20-fold by volume, of solution over embryo tissue) of MEMFA fix at 4°C for 4 h–overnight. Tissue can be stored at this point for a week. It is convenient to dechorionate zebrafish after fixation.

3. Wash twice in PBT (PBS, 0.1%Tween20).
4. Dehydrate embryos by washing with a 25%, 50%, 75% methanol series in PBT. Finally wash with 100% methanol twice each for 20 min (second wash at −20°C). Embryos can be stored at this point at −20°C for up to a month.
5. Rehydrate embryos by washing with the methanol series in PBT for 10 min (75%, 50%, 25% methanol). Finally wash twice with PBT each for 10 min.
6. Treat with 10 µg/mL proteinase K. The optimum time for proteinase K treatment varies for each new batch of protease, and the length of treatment should be optimized depending on the size and species of embryo. Treatment for 7 min is a good starting point for small embryos: mouse 8.5 dpc; chick 10 somites; *Xenopus* up to stage 14. Incubation times can be increased for larger embryos: for example, for chick embryos, 7 min for HH stage 10, 10 min for HH stage 17, and 20 min for HH stage 22 and above ***(3)***. Zebrafish require shorter incubation times (5–10 min). Overexposure to proteinase K results in poor signals or disintegration of embryos.
7. Rinse with PBT (gently as embryos are fragile until fixed).
8. Refix embryos using 0.2% (w/v) gluteraldehyde, 4% (w/v) paraformaldehyde in PBT for 20 min or 0.2% (w/v) gluteraldehyde in MEMFA.
9. Wash three times with PBT.

3.5. Hybridization

1. Remove PBT and rinse embryos once with prewarmed prehybridization solution.
2. Replace with fresh prehybridization solution and incubate at 65–70°C for 1 h–overnight. We place samples in Eppendorfs in a hot block where possible, to minimize probe volume (0.5 mL is sufficient). Large samples can be placed in multiwell dishes in an oven. At this point, samples can be stored at −20°C for a period of months.
3. Incubate embryos with prewarmed hybridization solution containing approximately 1 µg/mL of riboprobe (usually 10 µL probe to 1 mL prehybridization solution). For double in situs, add both DIG- and FITC-labeled probes simultaneously. Incubate at 65–70°C overnight.

3.6. Posthybridization

1. Wash embryos once with prewarmed hybridization buffer for 30 min at 65–70°C. For all washes, use an excess of wash solution (e.g., a 20-fold excess) over the volume of the embryonic tissue.
2. Wash once with prewarmed wash solution for 30 min at 65–70°C.
3. Wash once in 50%wash/50%MABT for 30 min at 65–70°C.
4. Wash twice in MABT (room temperature) for 10 min.
5. Block embryos for at least 2 h in MABT, 2% BBR, 20% goat serum. At the same time as embryos are blocking, preincubate anti-DIG antibody in MABT, BBR, GS on ice at 1:2,000 dilution.
6. Replace block solution with antibody solution and rock overnight at 4°C.

7. Remove antibody solution and store at 4°C for reuse up to five times (add azide to 0.1% to prevent contamination).
8. Wash at least six times for 1 h each in MABT, then overnight (overnight washes are not necessary for explants or zebrafish).

3.7. Color Detection

1. Rinse in NTMT pH 9.5.
2. Wash twice for 20 min each in NTMT.
3. Incubate in NTMT containing NBT/BCIP in the dark until the color develops (NBT: 4 µL per mL, BCIP: 3.5 µL per mL).
4. For in-situ hybridization using two probes, proceed directly to **step 6.**
5. For in-situ hybridization using a single probe, stop the reaction by washing thoroughly in MABT. Refix in 4% paraformaldehyde for 1 h prior to storing or sectioning.
6. For in-situ hybridization using two probes, rinse several times with MABT, then inactivate alkaline phosphatases conjugated to anti-DIG antibody by incubating embryos in MABT at 70°C for 30–40 min.
7. Wash three times with MABT.
8. Preblock embryos again with MABT + BBR + 20% GS for 60–90 mins.
9. Incubate with 1/2,000 dilution of anti-FITC.AP antibody in MABT, 2% BBR, 20%GS overnight at 4°C with rocking.
10. Repeat **steps 1–7**, but use fast-red color detection in **step 6** in NTMT pH 8. This color reaction takes longer to develop: Embryos can be left overnight at 4°C.

3.8. Storage and Photography

1. If overstained, wash in MABT overnight or for 2–3 d. If understained, leave in NTMT or NTMT plus color reaction overnight or for 2–3 d. Note: Fast red color washes out in TritonX100—beware if followed in situ with immunohistochemistry.
2. Fix in MEMFA for 20 min at room temperature.
3. Rinse with MABT.
4. Wash 5 min MABT.
5. Wash 80% glycerol/PBT (embryos take at least 20 min to equilibrate before photography).
6. Photograph on 1% agarose beds made in petri dishes with coverslip.
7. Store at 4°C in 1.5-mL Eppendorfs.
8. For sectioning, wash out glycerol with repeated washes in MABT.

3.9. Double In-Situ Hybridization and Immunohistochemistry on Whole Embryos

To simultaneously compare both mRNA and protein expression patterns, it is possible to follow in-situ hybridization with immunohistochemistry. The suitability of individual antibodies should be tested, however, as some antibodies are not able to detect their antigen following in-situ hybridization.

We use a modified in-situ hybridization method to increase the subsequent performance of the antibody:

1. Fixation: Antibodies requiring TCA or methanol fixation do not combine well with in-situ hybridization. For other antibodies, MEMFA or 4% paraformaldehyde should first be optimized, as either can be used for in-situ hybridization.
2. Proteinase K: We find that a shorter incubation time (5 min) or an alternative permeablization method often improves the signal of the subsequent antibody (the antigen may be destroyed by proteinase K). We routinely substitute the proteinase K with two 20-min washes in a detergent mix (1% IGEPAL, 1% SDS, 0.5% deoxycholate, 50 mM Tris-HCL pH 8, 1 mM EDTA, 150 mM NaCl).
3. Hybridization: Lowering the hybridization temperature to 60°C may increase the viability of the antigen.
4. Detection of mRNA probe: For double in-situs where fast-red TR/naphthol AS-MX is used as a stain, we found that this substrate is not permanent and the intensity of color fades, due to subsequent washing steps or immunohistochemical methods. We therefore use less detergent in washing steps. It should be noted, however, that for some antibodies, this may increase nonspecific binding of the antibody.

Fast red can be advantageous however, as it flouresces at a 568-nm wavelength and can be detected at a different wavelength from a FITC-conjugated secondary antibody by fluorescence microscopy. This allows simultaneous fluorescence detection of both mRNA and protein.

5. Following in-situ hybridization, refix embryos for 20 min in either MEMFA or 4% paraformaldehyde.
6. Wash twice in PBT for 5 min.
7. For older embryos (post HH stage 22 chick), block endogenous peroxidase enzymes with PBS containing 0.05% (v/v) hydrogen peroxide, 1% (v/v) Triton X-100 at 4°C for 6 h on a rocker. Wash in PBS containing 1% (v/v) Triton X-100 three times, each for 10 min.
8. Incubate in PBT, 20% sheep serum for 1–2 h at room temperature.
9. Incubate in the appropriate dilution of primary antibody in PBT, 10% sheep serum overnight (or up to 4 d) at 4°C with rocking.
10. Wash for 3 h in PBT, 5% sheep serum (six times, 30 min).
11. Incubate in the appropriate dilution of secondary antibody appropriate for the primary antibody used in **step 4** (e.g., FITC-conjugated anti-rabbit for polyclonal antibodies raised in rabbits) in PBT, 10% sheep serum overnight at 4°C with rocking.
12. Wash for 3 h in PBS (six times, 30 min).
13. For fluorescent-conjugated antibodies, equilibrate in DABCO glycerol and mount.
14. For horseradish peroxidase (HRP)-conjugated antibodies, wash twice in 100 mM Tris (pH 7.4) for 5 min.
15. Incubate in 0.5 mg/mL DAB for 10 min at room temerature (DAB is stored as a 50-mg/mL stock in 100 mM Tris pH 7.4, in aliquots at −20°C).

16. Add H_2O_2 to 0.1 µL/mL of 30% stock and develop color until brown—this may take up to 45 min.
17. Wash five times with PBS, fix in MEMFA or 4% paraformaldehyde for 20 min at room temperature.
    Note: All plastics and solutions that have been in contact with DAB should be decontaminated in 50% bleach or in accordance with manufacturer's SDS.

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Wholemount *In Situ* Hybridization to *Xenopus* Embryos

C. Michael Jones and James C. Smith

1. Introduction

Whole-mount *in situ* hybridization was first used to detect gene expression in *Drosophila* embryos ***(1)***. Various methods are now used to localize mRNAs in most species used for biological studies, and the methods have proven particularly useful when applied to vertebrate species. Here we provide a protocol commonly used for localizing transcripts in *Xenopus* embryos. The method is applicable to whole embryos and intact tissue explants. The methods are essentially as originally described by Hemmati-Brivanlou et al. ***(2)*** and then modified by Harland ***(3)***.

Standard molecular biology techniques not described in detail here can be found in Sambrook et al. ***(4)***.

2. Materials

Digoxygenin-11-UTP (Boehringer-Mannheim 1209 256)
Ribonucleotides
1 *M* DTT
RNasin
RNA Polymerase (SP6,T3, T7)
DNase I (RNase-free)
MOPS
EGTA
$MgSO_4$
Formaldehyde
Methanol
Formamide
Torula RNA
Heparin
Tween-20
CHAPS
EDTA
Maleic acid (optional)
NaCl
Lamb serum
MgCl
Tris, pH 9.5
Levamisol
BSA

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20X SSC
Nitro blue tetrazolium (NBT)
5-bromo-4-chloro-3-indolyl-phosphate (BCIP)
Dimethyl formamide
Triton X-100

Other reagents and optional equipment are listed in the text where appropriate. Working concentrations and storage conditions for stock solutions are also detailed.

3. Methods

3.1. Probe Synthesis

A standard *in vitro* transcription reaction ***(5)*** incorporating digoxygenin-11-UTP is performed. SP6, T7, or T3 RNA polymerase may be used. Temperature sensitive reagents should be kept on ice, but the reaction should be mixed at room temperature to avoid precipitation of the DNA template. Reaction:

a.	5X Transcription buffer	10.0 µL
b.	Linearized DNA template (1 µg/µL)	2.5 µL
c.	1 *M* DTT	0.5 µL
d.	2.5 m*M* NTP mix	10.0 µL
e.	RNasin	1.0 µL
f.	H_2O	24.0 µL
g.	RNA polymerase (20 U/µL)	2.0 µL

1. Incubate reaction at 37°C for 1–2 h.
2. Check synthesis by running 1 µL of the reaction on an agarose gel, and estimate the amount of RNA by comparing the intensities of the RNA and DNA template.
3. Add 2 µL of RNase-free DNase I, and incubate at 37°C for 10 min.
4. Purify the probe from unincorporated nucleotides by passing the reaction through a G50 spin column. These columns can be homemade. Alternatively, disposable cartridges can be purchased from IBI (R50 Spun Columns, cat. no. 06080).
5. Precipitate the flowthrough from the spin column.
6. Redissolve the purified pellet in H_2O. Probes can be reduced in size by limited alkaline hydrolysis if necessary (*see* **Note 1**). Store probe stock at −20°C. Probes are stable indefinitely.
7. Most probes work well when used at approx 1 µg/mL in hybridization buffer.

3.2. Fixation of Embryos

Albino embryos are preferred, because pigmentation in wild-type embryos can obscure the hybridization signal. If pigmented embryos or explants are to be used, they can be taken through the procedure and then bleached afterwards by incubating in 10% hydrogen peroxide in methanol. Satisfactory results can be obtained in this manner. The bleaching can take from several hours to several days depending on the pigmentation of the embryos.

Just before fixation, embryos should be removed from their vitelline membranes using sharpened watchmaker's forceps. For blastula and gastrula stage

embryos, a hole should be placed in the blastocoel to prevent nonspecific trapping of the probe and/or antibody. For neurula stages, the archenteron should be pierced. Embryos are fixed in small glass vials or scintillation vials if a large number are to be processed simultaneously. The preferred fixative is MEMFA (0.1 *M* MOPS, pH 7.4, 2 m*M* EGTA, 1 m*M* $MgSO_4$, 3.7% formaldehyde). A 10X stock solution of the salts can be kept at 4°C. To make fresh fixative, add 1/10 vol 37% formaldehyde and 1/10 vol 10X salts and water to make the volume needed. Embryos should be fixed for approx 1 h at room temperature with constant rotation. After fixation, remove the fixative, and add 100% ethanol or methanol to the embryos. Make two changes of the 100% alcohol, and store the embryos at −20°C. Embryos can be stored for several months or longer.

3.3. Hybridization

Embryos can be processed either in small glass vials or in mesh-bottomed baskets. Using glass vials is more labor-intensive, since each vial must be handled individually and solution changes require aspiration and refilling of each. Baskets can be processed in mass, and only involves lifting the baskets from solution to solution. Additionally, the risk of damaging or losing the tissues is reduced when baskets are used. Baskets can be made by attaching small mesh to an Eppendorf tube from which the bottom has been removed. Alternatively, baskets are available commercially from Costar (15-mm Netwell, 74-µm mesh, cat. no. 3477) and fit easily into 12-well plastic dishes (Costar 12-well cluster, cat. no. 3513), which makes transferring the baskets to different solutions easy. A large number of embryos, from different stages, can be processed simultaneously for each probe of interest. A sense control hybridization should be included to control for nonspecific signal and overall level of background staining. Embryos can be transferred by using a Pasteur pipet from which the end has been cut to increase the size of the opening. Each set of embryos is then processed as follows:

1. Rehydrate embryos by incubating for 2–5 min in 100% Methanol (MeOH), 75% MeOH/25% H_2O, 50% MeOH/50% H_2O, 25% MeOH/75% 1X PBS + 0.1% Tween-20 (PBST), and 100% PBST.
2. Wash three times for 5 min in PBST.
3. Incubate embryos in 10 µg/mL Proteinase K in PBST. The time must be determined for each batch of Proteinase K, but a good starting point is approx 30 min at room temperature. This treatment is optional, but it probably increases sensitivity to some degree.
4. Rinse twice for 5 min in 0.1 *M* triethanolamine (pH 7.0–8.0).
5. Add 12.5 µL acetic anhydride/5 mL of triethanolamine and incubate for 5 min. Then, add another 12.5 µL of acetic anhydride and incubate for an additional 5 min. These steps block positively charged groups within the tissues ***(6)***.
6. Wash twice for 5 min in PBST.

7. Refix embryos by incubating in 4% paraformaldehyde in PBST for 20 min.
8. Wash five times for 5 min in PBST.
9. Replace PBST with hybridization buffer (listed below). If 5-mL glass vials are used, 500 µL are a sufficient volume to cover a large number of embryos. If baskets are used, an appropriate volume to cover all embryos must be used. For the Costar netwells, use 2–2.5 mL. Hybridization buffer:
   50% Formamide, 5X SSC, 1 mg/mL Torula RNA, 100 µg/mL Heparin, 1X Denhardt's, 0.1% Tween-20, 0.1% CHAPS, and 5 m*M* EDTA.
10. After embryos settle through the dense hybridization solution, replace the buffer with fresh hybridization buffer and incubate at 60°C. Prehybridize embryos 5–6 h.
11. After prehybridization, replace the solution with hybridization buffer containing the probe (1 µg/mL). Hybridize overnight at 60°C.

3.4. Washing

1. Keep the Hybridization Buffer containing the probe. It can be reused up to two times. Replace hybridization buffer with a solution of 50% formamide, 5X SSC (Mock-Hyb Buffer). Hybridization buffer can be used here, but it is more economical to use the Mock-Hyb Buffer. Wash at 60°C for 10 min.
2. Wash in 50% Mock-Hyb buffer/50% 2X SSC 10 min at 60°C.
3. Wash in 25% Mock-Hyb buffer/75% 2X SSC for 10 min at 60°C.
4. Wash twice for 20 min in 2X SSC/0.3% CHAPS at 60°C.
5. RNAse treat by incubating for 30 min at 37°C in 2X SSC/0.3% CHAPS containing 20 µg/mL RNase A and 10 U/mL RNase T_1.
6. Rinse in 2X SSC/0.3% CHAPS for 10 min at room temperature.
7. Wash twice for 30 min in 0.2X SSC/0.3% CHAPS at 60°C.
8. Wash twice for 10 min in PBST/0.3% CHAPS at 60°C.
9. Wash three times for 5 min in PBST at room temperature.

3.5. Antibody Incubation

Antibody incubations are performed in one of two buffer systems. One buffer consists of 1X PBS containing 2 mg/mL BSA and 0.1% Triton X-100 (PBT). The other is a maleic acid buffer developed by Tabitha Doniach (MAb = 100 m*M* maleic acid, 150 m*M* NaCl, pH 7.5). If background staining becomes a problem, the MAb is recommended. After the final wash in PBST, proceed as follows:

1. Wash three times for 5 min with PBT or MAb at room temperature.
2. Block tissues by incubating one hour at room temperature in PBT + 20% Heat-treated lamb serum. Lamb serum is heat-treated by placing it in a water bath at 55–60°C for 30 min. The serum is then stored frozen in aliquots. Alternatively, MAb containing 2% blocking reagent from Boehringer Mannheim (cat. no. 1096 176) and 20% serum can be used.
3. After blocking, replace the blocking buffer with the same solution containing a 1:2000 dilution of antidigoxygenin antibody (Fab fragments) coupled to alkaline phosphatase (BMB cat. no. 1093 274). Incubate overnight at 4°C, or at least 4 h at room temperature with constant shaking.

3.6. Antibody Washes

Wash tissues at least five times each for 1 h at room temperature in PBT or MAb. It is best if the tissues are moving gently within the wash solutions. Washes can be carried out overnight (or over the weekend) at 4°C if convenient.

3.7. Chromogenic Reaction

1. After antibody washes, rinse tissues twice for 5 min in alkaline phosphatase buffer (APB): 100 m*M* Tris, pH 9.5, 50 m*M* $MgCl_2$, 100 m*M* NaCl, 0.1% Tween-20, and 5 m*M* Levamisol. APB should be made fresh just before use, since the levamisol is not stable. Levamisol can be made as a 1 *M* stock and stored at −20°C.
2. For color development, replace the last wash with APB containing 4.5 µL NBT (nitro blue tetrazolium, 75 mg/mL in 70% dimethyl formamide) and 3.5 µL BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 50 mg/mL in 100% dimethyl formamide)/mL. Alternatively, the color reaction can be carried out by placing the tissues directly into BM purple AP substrate, precipitating (BMB cat. no. 1442 074). The BM purple substrate tends to give less background staining than the NBT/BCIP system, and is recommended if long reaction times are required. The appearance of staining is dependent on the level of gene expression and the probe that is used. Positive staining can become visible after several minutes, or can take up to 1 d.
3. When satisfied with the color development, stop the chromogenic reaction by placing the tissues in MEMFA. This postfixation stops the color reaction and stabilizes the stain. Postfix the tissues for at least 1 h, or they can be left overnight.

3.8. Alternative Protocol

An alternative to the processing steps listed in **Subheading 3.4.** can be used. For most markers that are routinely assayed, this shortened procedure works well and requires much less effort. After pretreatment and hybridization as described above, process the samples as follows:

1. Replace hybridization solution with 50% formamide/5X SSC for 10 min at 60°C.
2. Wash once in 25% formamide/2X SSC for 10 min at 60°C.
3. Wash twice for 30 min with 2X SSC/0.1% CHAPS at 60°C.
4. Wash twice for 30 min with 0.2X SSC/0.1% CHAPS at 60°C.
5. After the final wash at 60°C, wash twice at room temperature with PBT or MAb, and then proceed to the blocking step and antibody incubations as described above (**Subheading 3.5.**).

4. Notes

1. Limited alkaline hydrolysis of the probe to reduce its overall length to 200–300 bp is a consideration. Most probes work well when used as full length (2–3 kb probes have worked well), but others may function better if their length is reduced. To hydrolyze a probe, resuspend the precipitated probe in 50 µL of a solution containing 40 m*M* sodium bicarbonate, and 60 m*M* sodium carbonate. Heat at 60°C for 30 min to 1 h. After hydrolysis, increase the volume to 200 µL with water and

precipitate. Redissolve the pellet in water, and use at a final concentration of 1 µg/mL for hybridization.

2. If persistent background is a problem, consider preincubating the anti-digoxygenin antibody with embryonic acetone powder prior to use. Acetone powder is easy to make and the protocol can be found in *Antibodies: A Laboratory Manual (7)*. Additionally, background is usually reduced when BM purple AP substrate is used for the color development instead of NBT/BCIP in APB.
3. Whole-mount specimens can be processed for histological sectioning, which allows more detailed analysis. If sectioning is desired, it is a good idea to develop the color as intensely as possible (overstain), monitoring to ensure that background does not become a problem. It is essential to postfix the tissue to stabilize the stain before processing for histology. To prepare the tissues for sectioning, dehydrate through a series of methanol (30, 50, 70, 90, and 100%), and clear with xylene or histoclear. Embed tissues in wax, and section at 10–20 µm. Thicker sections provide more intense signal. Tissues can be counterstained with eosin, which provides a nice contrast to the purple color reaction product.

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Wholemount *In Situ* Hybridization to Amphioxus Embryos

Peter W. H. Holland

1. Introduction

Once a gene has been cloned the spatial distribution of its transcripts may be determined by *in situ* hybridization. This involves applying a labeled antisense RNA probe, with complimentary nucleotide sequence to the mRNA, either to tissue sections or to whole fixed specimens. The cephalochordate amphioxus has small embryos (100–300 µm in length) that are particularly well suited to whole mount *in situ* hybridization. The method has been successfully used to examine expression of many developmental expressed genes in amphioxus embryos, giving insight into the evolutionary conservation and divergence of gene expression between amphioxus and vertebrates.

2. Materials

1. MOPS buffer: 0.5 *M* NaCl, 1 m*M* $MgSO_4$, 2 m*M* EGTA, 0.1 *M* morpholinopropanesulfonic acid buffer, pH 7.5. Sterile.
2. Paraformaldehyde: electron microscopy-grade (TAAB, Aldermastan, UK).
3. Nunclon four-well multiwell dishes (Available from Sigma-Aldrich, catalogue number D6789. Nunclon is a trademark of Nalge Nunc International).
4. PBS: 0.9 % (w/v) NaCl, 20 m*M* sodium phosphate buffer, pH 7.4. Sterile.
5. PBT: PBS plus 0.1% Tween 20.
6. Proteinase K (10 mg/mL stock). Store frozen.
7. 0.1 *M* Triethanolamine, pH 8.0. Sterile.
8. Acetic anhydride.
9. Hybridization buffer: 50% formamide (Fluka, Gillingham, UK), 1.3X SSC, pH 4.5, 5 m*M* EDTA, pH 8.0, 500 µg/mL yeast RNA, 0.2% (v/v) Tween 20, 0.5% CHAPS, 100 µg/mL heparin, in DEPC-treated sterile water.
10. Oven with shaking platform, e.g., Hybaid Midi-Dual oven.

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11. Wash solution A: 50% formamide (Fluka), 4X SSC, 0.1% Tween-20.
12. Wash solution B: 50% formamide (Fluka), 2X SSC, 0.1% Tween-20.
13. Wash solution C: 50% formamide (Fluka), 1X SSC, 0.1% Tween-20.
14. Wash solution D: 0.2X SSC, 0.1% Tween-20.
15. Blocking buffer: 5% (w/v) Boehringer Mannheim (Lewes, UK) digoxigenin blocking reagent, 2 mg/mL BSA in PBT. Takes over 1 h to dissolve at 70 °C. Store frozen.
16. Amphioxus powder: Grind frozen adult amphioxus under liquid nitrogen, or homogenize in PBS. Then incubate in ice-cold acetone for 30 min. Recover powder by centrifugation, and grind further in a pestle and mortar. Store dry powder frozen.
17. Sheep serum: Heat to 55 °C for 30 min. Store frozen.
18. Preabsorbed 1:3000 anti-DIG fab fragments: To 1.5 mg of amphioxus powder, add 400 µL PBT, and heat to 70 °C for 10 min. Add 50 µL 20 mg/mL BSA, 50 µL pretreated sheep serum, 0.5 µL anti-DIG fab fragments (Boehringer Mannheim). Mix and incubate at 37 °C for 1 h, and then overnight at 4 °C. Centrifuge for 5 min to remove powder; to the supernatant, add 1 mL PBT containing 2 mg/mL BSA. Add 50 µL pretreated sheep serum. Store at −20 °C in 200-µL aliquots.
19. APT buffer: 100 m*M* NaCl, 100 m*M* Tris-HCl, pH9.5, 50 m*M* $MgCl_2$, 1% Tween-20.
20. NBT/BCIP staining mix: Just before use, add 4.5 µL stock nitro blue tetrazolium salt (NBT) and 3.5 µL stock 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to 1 mL APT buffer containing 2 m*M* levamisole. Stock NBT is 75 mg/mL in 70% dimethyl formamide, and stock BCIP is 50 mg/mL in 100% dimethyl formamide; both stocks are supplied by Gibco-BRL as Immunoselect kit.
21. 37–40% Formaldehyde stock solution.

3. Methods

The protocol given is designed for amphioxus embryos and larvae, obtained as described in Chapter 38. The method is modified from **ref. *1*** and has been used to visualize RNA from several developmentally expressed genes of amphioxus (*see* **Note 1**). With modification, it has also been successfully applied to embryos of other marine invertebrates, notably ascidia (e.g., **ref. *2***).

1. Fix amphioxus embryos and larvae (up to 3 d postfertilization) in freshly made 4% paraformaldehyde in MOPS buffer, at 4 °C for 12 h.
2. After fixation, transfer specimens through two changes of 70% ethanol, and store at −20 °C. Do not allow to freeze.
3. Digoxigenin-(DIG) labeled riboprobes are made by standard protocols (Chapter 46); redissolve probes in 50% formamide at 100 ng/µL, and store at −20 °C. (*see* **Notes 2** and **5**).
4. Transfer selected embryos into one or more wells of a disposable Nunclon four-well dish, and add 0.5 mL PBT. All subsequent incubation, wash, and hybridization steps are performed in the same Nunclon dish at room temperature, unless otherwise stated. Each change of solution is performed using a Gilson pipetman, while observing under a dissecting microscope to ensure that embryos are not lost.
5. Change solution for fresh PBT.

6. Change solution for 7.5 µg/mL proteinase K in PBT; incubate without agitation for 10 min.
7. Quickly wash with two changes of PBT. Leave second wash for 5 min.
8. Change solution for 4% paraformaldehyde in PBT; incubate without agitation for 1 h.
9. Wash with two changes of 0.1 *M* triethanolamine, 2–5 min each.
10. Remove solution from embryos. Quickly add 2.5 µL acetic anhydride to 1 mL 0.1 *M* triethanolamine in a microfuge tube, vortex, and add 300 µL onto embryos. Incubate without agitation for 5 min.
11. Repeat **step 10.**
12. Wash with two changes of PBT, 2–5 min each.
13. Change solution for a 1:1 mix of PBT and hybridization buffer; incubate for 5 min.
14. Change solution for hybridization buffer; incubate for 10 min.
15. Change solution for hybridization buffer prewarmed to 60 °C; incubate at 60 °C for at least 1 h, with horizontal rotation. A hybridization oven is useful.
16. Add 2 µL of DIG-labeled riboprobe from **step 3** to 200 µL hybridization buffer. Heat to 75 °C for 2 min (*see* **Note 3**).
17. Remove solution on embryos, and replace with the heated and diluted probe. Seal sides of the multiwell dish with Parafilm, and place on foam microfuge rack floating in 70 °C water bath for 2 min.
18. Move the dish to the shaking oven, and incubate overnight at 60 °C with horizontal rotation.
19. Wash embryos in prewarmed solution A, 2 × 30 min at 60 °C with agitation.
20. Wash embryos in prewarmed solution B, 2 × 30 min at 60 °C with agitation.
21. Wash embryos in prewarmed solution C, 2 × 30 min at 60 °C with agitation.
22. For more stringent washes, wash with solution D, 30 min at room temperature.
23. Wash with two changes of PBT, 5–10 min at room temperature (*see* **Note 4**).
24. Change solution for blocking buffer; incubate for 1 h at room temperature.
25. Change solution for preabsorbed 1:3000 anti-DIG fab fragments. Place on a rotating platform at room temperature for 10 min, and then incubate overnight at 4 °C. Agitation is not necessary.
26. Carefully remove antibody solution from embryos. Store antibody frozen for reuse.
27. Wash embryos in PBT, 2× for 10 min at room temperature.
28. Wash embryos in PBT, 3× for 30 min at 37 °C.
29. Wash embryos in APT buffer, 2× for 10 min at room temperature.
30. Wash embryos in APT buffer, 2× for 20 min at room temperature.
31. Replace solution with NBT/BCIP staining mix, and place in dark (e.g., cover in foil) until color develops (10 min to 48 h). If staining requires longer than overnight, change for fresh NBT/BCIP mix each day. Some recommend that this step be performed in wash glasses; we find no difference between glass and plastic dishes.
32. When all embryos have developed a consistent staining pattern, wash with two changes of PBT and fix in 4% formaldehyde in PBT for 1 h at room temperature.
33. Mount embryos on slides in 80% glycerol in PBT. For amphioxus embryos, support the coverslip with strips of autoclave tape stuck onto the slide.

4. Notes

1. Recent improvements to this protocol, incorporated above, include the high concentration of nonspecific RNA in the hybridization buffer (500 µg/mL), and long posthybridization and postantibody washing steps.
2. It is essential to use high-purity formamide in the hybridization buffer and wash solutions A, B, and C. Fluka formamide gives consistent results.
3. After prehybridization (**Subheading 3., step 15**), embryos can be stored at −20 °C for later use.
4. In contrast to some other protocols, we omit an RNase step in the posthybridization washes; in amphioxus, it can reduce signal without enhancing specificity. Different results may be found in other species.
5. In our experience, the most critical variant is the quality of the probe. After synthesis, probes must be checked by agarose electrophoresis to assess concentration and degradation; storage in 50% formamide (**step 3**) greatly improves probe stability and dissolution.

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In-Situ Hybridization to Sections (Nonradioactive)

Maria Rex and Paul J. Scotting

1. Introduction

In-situ hybridization (ISH) takes advantage of the ability of mRNA within a cell to hybridize with exogenously applied complementary RNA (riboprobes) or DNA molecules. This interaction is visualized by labeling the applied nucleic acid probe with a detectable molecule (radioactive, such as 35S, or nonradioactive, such as digoxygenin [DIG]). The technique allows patterns of gene expression to be visualized in many tissues or cell types simultaneously. Nonradioactive ISH has several advantages over radioactive ISH. It lacks the biohazards associated with the use of radioisotopes, it takes days rather than weeks to get a visible signal, and it is cheaper. Using nonradioactive detection, the degree of cellular resolution is significantly improved over that achieved with autoradiography. Another advantage of nonradioactive ISH is that it can be performed in combination with other assays with relative ease, to compare mRNA and protein distribution in the same tissues ***(1)***, as described in this chapter. The procedures presented here are for ISH of DIG-labeled riboprobes to tissue sections from frozen and wax embedded tissues. The use of tissue sections rather than whole mount embryos is preferable where the size of the embryo or the density of tissues leads to incomplete penetration of probe in whole mounts. The use of sections also allows a number of probes (for mRNA or proteins) to be used on the same or adjacent sections, an approach that is less straightforward in whole mounts. Both whole-mount and section in-situ hybridization protocols can therefore be used in a complementary way to give optimum results. The procedures involved are described next.

1.1. Preparation of Probe

DIG-labeled riboprobes are synthesized by the transcription of sequences of interest cloned into a vector such that they are flanked by two different

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RNA polymerase binding sites. The vector is first linearized with a restriction enzyme such that transcription produces "run-off" transcripts, that is, transcripts derived from the insert sequence alone. The restriction enzyme is chosen such that transcription yields an RNA probe that is complementary (anti-sense) to the target mRNA. The probe transcribed from the opposite strand (sense probe) can be used as a negative control. The length of the probe is important. Probes of up to 1.5 kb are optimal. Longer probes penetrate the tissue less efficiently but can be partially degraded by alkaline hydrolysis to a more suitable size *(2)*.

1.2. Preparation of Tissue

To preserve morphology, the biological material must be fixed. Cross-linking fixatives, such as paraformaldehyde, can give greater accessibility and retention of cellular RNA than precipitating fixatives. Tissue sections can be cut either on a cryostat (frozen sections) or after embedding in a matrix, such as paraffin wax, on a microtome. Tissue morphology is generally better preserved in wax embedded tissues.

1.3. Prehybridization Treatment of Tissues

When using wax embedded tissues, the wax is removed from the sections by dissolving in organic solvent. Proteinase treatment is used to render the target RNA more accessible to the probe by digestion of cellular protein. The optimal extent of proteinase K treatment should be determined empirically, since it varies among tissues and between frozen and wax embedded samples. Sections must be refixed after proteinase treatment.

1.4. Hybridization of Probe to Tissue and Posthybridization Washes

The temperature for hybridization can be varied according to the specificity of interaction of the probe and target required. For a homologous probe and target, as used in most cases, we routinely use 70°C, use of lower temperatures may lead to nonspecific artifacts. Following hybridization, slides are washed at high stringency to remove unbound probe and probe that has bound to sequences similar to but distinct from the target sequence.

1.5. Color Detection

Bound probe is visualized by incubation of sections with alkaline-phosphatase conjugated antibody against DIG and subsequent addition of substrates that yield an insoluble colored product. This reaction can be carried out for several days to get a stronger signal (though a strong signal can sometimes be obtained in a few hours). The limiting factor is the amount of nonspecific background staining obtained.

1.6. Immunohistochemistry

Slides are processed for immunohistochemistry to detect other markers immediately following color detection of the ISH signal. The immunohistochemical probe can be visualized with an alternative color or fluorescent label (**Fig. 1**). One potential problem of using this protocol described here is residual alkaline phosphatase activity from the ISH, which may produce a product with the fast-red substrate. Controls in which no primary antibody was used during immunohistochemistry show that this is not generally the case. If residual alkaline phosphatase activity is a concern with other probes or reagents, it can be inactivated by treatment with 0.1 M glycine HCl, pH 2.2, prior to immunohistochemistry. Occasionally, the proteinase K step used in the ISH protocol may destroy the antigen for subsequent immunohistochemical staining. In such cases, a different method of antigen retrieval, such as pressure cooking or microwaving, can be used ***(3)***.

2. Materials

All solutions should be made to the standard required for molecular biology using molecular-biology-grade reagents and sterile distilled water.

2.1. Preparation of DIG-Labeled Single-Stranded RNA Probe (Riboprobe) by In-Vitro Transcription

1. Riboprobe Gemini system ii buffers kit (Promega, Southampton, UK) includes unlabeled ribonucleotides, DTT, transcription-optimized buffer. Store at −20°C. NTPs go off, so kits to suit level of use should be bought.
2. T_3 and T_7 RNA polymerase (Promega, Southampton, UK). Store at −20°C.
3. DIG-UTP (Roche Diagnostics Ltd., Lewes, UK). Store at −20°C.
4. RNasin ribonuclease inhibitor (Promega, Southampton, UK). Store at −20°C.
5. Restriction enzymes (single cut 5′ and 3′ of insert).
6. Phenol and chloroform.
7. Minispin G50 columns (Amersham Biosciences) or 7.8 M ammonium acetate and 4 M lithium chloride.

2.2. Preparation of Tissue (Fixation, Embedding, and Sectioning)

1. Phosphate-buffered saline (PBS) (see Note 1).
2. 4% Paraformaldehyde is prepared on the day of use by adding 4 g paraformaldehyde (BDH/Merk Ltd, Poole, UK)/100 mL PBS, heating at 65°C until the paraformaldehyde has dissolved and cooling on ice (aliquots can frozen but only freeze–thaw once). Paraformaldehyde is toxic, handle solutions within a fume cupboard.
3. Saline (0.83% sodium chloride).
4. 100% Ethanol.

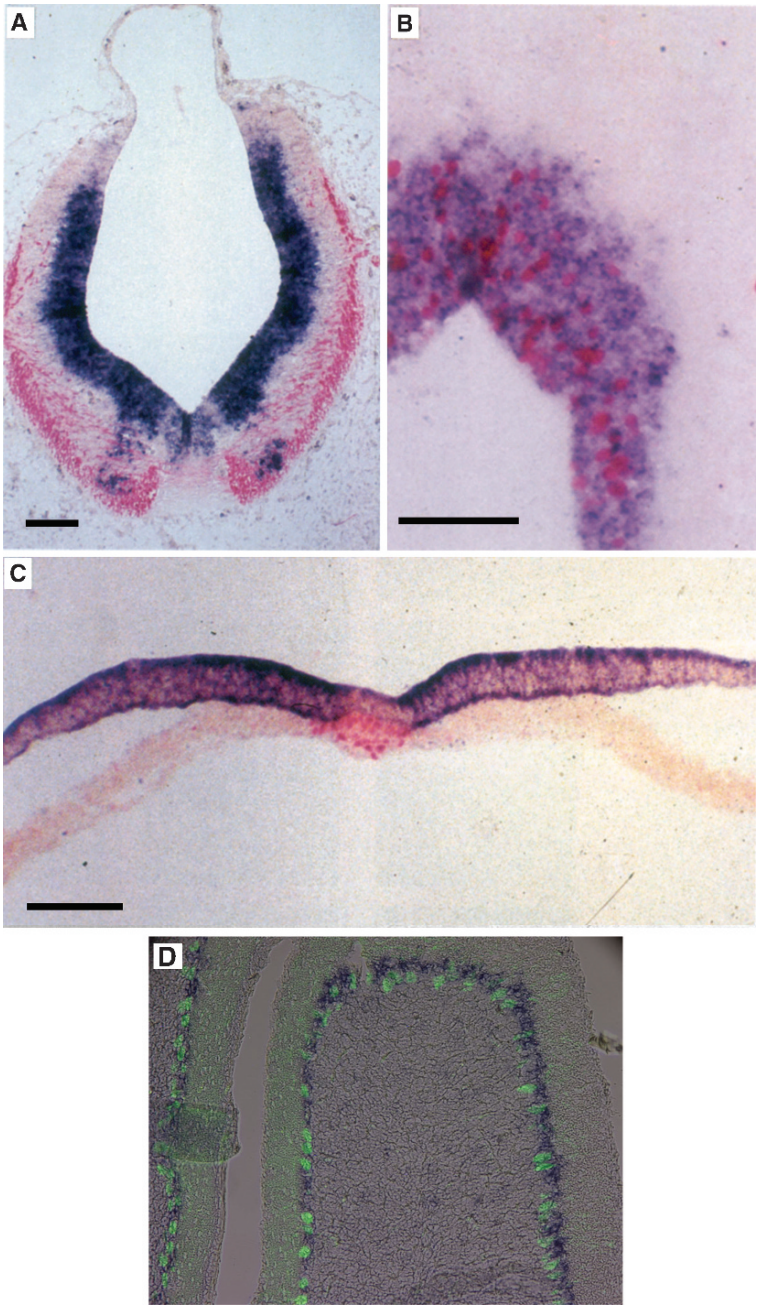

Fig. 1. (*opposite page*) Nonradioactive in-situ hybridization on wax-embedded tissues (**A–C**) and frozen tissue (**D**) in combination with immunohistochemical staining. Blue staining represents in-situ hybridization signal and red/green is the reaction following immunohistochemical

5. Histolene (Cellpath plc, Hemel Hempstead, UK), a less toxic alternative to xylene.
6. Paraffin wax, Tissue Tek pastilated.
7. 30% Sucrose made up in RNAse free PBS and filter sterilized.
8. OCT compound (BDH, Poole, UK).
9. Superfrost® plus slides (VWR international)

2.3. Prehybridization Treatment and Hybridization

1. Proteinase K is prepared as a 10 mg/mL stock and is stored at −20°C in small aliquots. On day of use dilute with PBS.
2. PBT: 0.1% Tween-20 in PBS (pH 7.3).
3. 4% Paraformaldehyde prepared as in section 2.2.
4. Methanol.
5. Hybridization solution: 50% deionized formamide (BDH/Merk Ltd, Poole, UK molecular biology grade); 5× SSC (pH 4.5 with citric acid [BDH]); 50µg/mL yeast tRNA (Roche); 1% SDS; 50µg/mL heparin (Sigma). Store at room temperature for up to 1 mo.
6. Histolene.
7. 20× SSC: stock solution of 0.3 M NaCl and 0.3 M sodium citrate.

2.4. Posthybridization Washes and Color Detection

1. Formamide-GPR (BDH/Merk, Poole, UK). Formamide is toxic. Avoid contact with the skin and use within a fume cupboard.
2. 20× SSC (pH 4.5 with citric acid).
3. 10% SDS.
4. Heat inactivated (60°C, 30 min) sheep serum (Sigma); store in aliquots at −20°C.
5. Alkaline-phosphatase anti-digoxygenin Fab fragments (Roche, Lewes, UK). Store at 4°C.
6. TBST: 10× TBS-135 m*M* NaCl, 250 m*M* Tris-HCl, pH 7.5.
7. On day of use, dilute to 1× and where necessary (see Method 3.5), add 0.024 g levamisole and 0.5 mL Tween-20 to each 50 mL of 1× TBST.
8. Alkaline phosphatase buffer: 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl pH 9.5, 1% Tween-20. Make on the day of use from stock solutions.

Fig. 1. *(continued)* detection of other antigens. **A** In-situ hybridization for *cSox21* (a transcription factor, (4]) plus immunohostochemical staining for neurofilament (antineurofilament monoclonal (Dako Ltd). **B** In situ hybridization for *cSox2* ***(5)*** plus immunohistochemical detection of BrdU to identify proliferating cells (*see* ***(6)*** for further details). **C** In-situ hybridization for *cSox3* (***(7)*** plus immunohistochemical detection of Brachyury (a nuclear transcription factor. **(D)** In-situ hybridization for *mSox9* plus immunohistochemical detection of calbindin (using a flouroscein-labeled secondary antibody) in 14-d postnatal mouse cerebellum. (*See Color Plate*)

9. Color solution is alkaline phosphatase buffer plus 45 µL/100 mL NBT (75 mg/mL, 4-nitro blue tetrazolium chloride, Roche) and 35 µL/100 mL of BCIP (50 mg/mL, 5 Bromo-4-chloro-3-indolyl-phosphate, Roche), and 2 mM levamisole (Sigma). Levamisole is made fresh from solid, but may be stored for up to 2 wk at 4°C.

2.5. Immunohistochemistry

1. Sheep serum (Sigma). store in aliquots at −20°C.
2. Primary antibody–antiserum and secondary antibody raised in the appropriate animal. The dilution of antibodies is determined empirically in each case. Store at 4°C or −20°C long term.
3. PBS.
4. Alkaline phosphatase streptavidin (Vector Laboratories).
5. Fast red (Sigma). Prepare according to suppliers instructions.
6. Mowiol 4-88 (Calbiochem-Novabiochem Corp). Mowiol is prepared by adding 2.4 g of Mowiol to 6 g of glycerol and 6 mL of water. The mixture is left at room temperature for several hours before the addition of 12 mL of 0.2 M Tris, pH 8.5. Heat to 50°C for 10 min. Store as aliquots at −20°C.

3. Methods

3.1. Preparation of DIG-Labeled Single-Stranded RNA Probe (Riboprobe) by In-Vitro Transcription

1. Linearize DNA with an appropriate restriction enzyme (*see* **Note 2**).
2. Extract with phenol–chloroform and precipitate. Resuspend in sterile water at 1 mg/mL.
3. Warm buffers, nucleotides, DTT, and templates to room temperature (*see* **Note 3**).
4. Add the following, in the order shown, at room temperature:
   a. X µL RNase-free-water (to final volume of 20 µL).
   b. 4 µL 5× transcription buffer.
   c. 2 µL 100 mM DTT.
   d. 2 µL rATP(10 mM).
   e. 2 µL rCTP (10 mM).
   f. 2 µL rGTP (10 mM).
   g. 1.3 µL rUTP (10 mM).
   h. 0.7 µL DIG-UTP (10 mM).
   i. 0.5 µL RNasin.
   j. 1 µL RNA polymerase (T_3 or T_7).
   k. X µL (~1 µg) template.
5. Mix gently, do not vortex, pulse in microfuge.
6. Incubate at 37°C for 2–6 h.
7. Check 1 µL on a gel, and in the meantime, purify the probe using one of the following methods:
   a. Minispin G50 column (Amersham Biosciences) Follow manufacturers instructions to purify probe by binding and subsequent elusion from G50 column.

b. Precipitation: Add 100 µL water, 10 µL 4 M LiCl, 300 µL ethanol, 1 µL glycogen (4 mg/mL). Place at −80°C for at least 1 h. Spin and resuspend pellet in 30 µL of water; add 15 µL 7.8 M ammonium acetate, 1 µL glycogen and 100 µL ethanol. Place at −80°C for at least 1 h. Spin and resuspend pellet in 30 µL of water. Store at −80°C.

3.2. Preparation of Tissue (Fixation, Embedding, and Sectioning)

3.2.1. Wax Embedding

1. Fix tissue in 4% paraformaldehyde in PBS at 4°C for 1–24 h, depending on tissue size and type.
2. Successively replace solution with the following (at least 30 min each with occasional agitation, at room temperature except where stated).
   a. Saline, twice at 4°C.
   b. 1:1 Saline–ethanol mix at 4°C.
   c. 70% Ethanol twice.
   d. 85% Ethanol.
   e. 95% Ethanol.
   f. 100% Ethanol twice.
   g. Histolene twice.
   h. 1:1 Histolene–melted paraffin wax for 20 min at 60°C.
   i. Wax, three times (20 min each at 60°C).
3. Transfer tissues to a mold at 60°C, orientate, and allow wax to set and cool before removing from mold.
4. Cut ribbons of 6-µm sections on a microtome. Float the ribbons of sections on a bath of distilled water at 40°C until free from creases. Collect on Superfrost® plus slides. Dry slides at 37°C overnight.

3.2.2. Frozen Sectioning

1. Fix tissue in 4% paraformaldehyde in PBS at 4°C for 1–24 h, depending on tissue size and type.
2. Wash twice in PBS.
3. Immerse in 30% sucrose in PBS until tissue sinks
4. Embed in OCT compound and freeze by submerging container in isopentane precooled in liquid nitrogen.
5. Cut section on a cryostat onto Superfrost® plus slides
6. Dry slides at 45°C for at least 3 h.

3.3. Prehybridization Treatment and Hybridization

3.3.1. Wax Sections

1. Dip slides in Histolene two times for 10 min to remove wax (*see* **Note 4**).
2. Rehydrate through graded alcohols (1 min in each): 100% MeOH (twice), 75% MeOH/25% PBT, 50% MeOH/50% PBT, 25% MeOH/75% PBT.

3. Immerse slides in PBT for 5 min.
4. Overlay the slides with proteinase K (10 µg/mL, prewarmed to 37°C) and leave for 15 min at room temperature or 37°C (time and temperature may need to be established empirically for different tissues).
5. Wash three times for 3 min with PBT.
6. Immerse the slides in ice-cold 4% cold paraformaldehyde in PBS and leave for 20 min at room temperature.
7. Wash the slides with PBT, three times for 5 min.
8. Add probe to hybridization solution (10–200 ng/slide—actual amount determined empirically). Heat to 70–80°C for 5 min to ensure uniform solution and quench in ice.
9. Apply the hybridization mix (approximately 70 µL/slide) to the sections and cover with a coverslip (we prefer plastic hybrid slips, Sigma, which tend to stick to tissues less). Incubate overnight in a box humidified with 50% formamide, 5× SSC. We routinely use 70°C for intact riboprobes) (see Note 5).

3.3.2. Frozen Sections

1. Fix slides in fresh 4% paraformaldehyde at room temperature for 10 min.
2. Wash slides in PBST, three times 5 min.
3. Overlay the slides with proteinase K, (1 µg/mL, prewarmed to 37°C) and leave for 15 min at room temperature or 37°C (time and temperature may need to be established empirically for different tissues).
4. Follow steps 5–9 in the preceding section.

3.4. Posthybridization Washes and Color Detection

1. Remove slides from box and submerge in 50% formamide, 5× SSC pH 4.5, 1%
2. SDS at 65°C until the coverslips fall off (about 15 min).
3. Wash in 50% formamide 5× SSC pH 4.5, 1% SDS at 65°C for 30 min.
4. Wash twice in 50% formamide, 2× SSC at 65°C; 30 min each.
5. Wash three times for 5 min in TBST at room temperature.
6. Block for 30 min at room temperature in 10% heat inactivated (65°C for 30 min) sheep serum in TBST.
7. Immerse (more reliable for uniform results) or overlay slides in alkaline-phosphatase anti-DIG Fab fragments diluted 1/5,000 –10,000 in TBST plus 1% sheep serum and incubate at 4°C overnight or for 2–4 h at room temperature.
8. Wash three times for 20 min in TBST with a rotating stir bar.
9. Wash two times for 5 min in alkaline phosphatase buffer.
10. Incubate in color solution in the dark until desired reaction product intensity is achieved (between 4 h and 10 d).
11. Stop color reaction by incubating for 5 min in 10 mM Tris-HCl pH 7.8, 10 mM EDTA.

3.5. Immunohistochemistry

Most standard protocols should work fine after in-situ hybridization. Here, we describe one protocol we use. Fluorescent antibodies can also work well (*see* **Fig. 1**).

1. Block for 30 min in 20% sheep serum in PBS at room temperature.
2. Incubate with primary antibody–antiserum at the desired dilution in PBS plus 5% sheep serum for 1–2 h at room temperature.
3. Wash several times with PBS.
4. Incubate in biotinylated secondary antibody at appropriate dilution in PBS.
5. Wash in PBS.
6. Incubate in alkaline phosphatase streptavidin (1/500) in PBS for 30 min at room temperature.
7. Wash in PBS.
8. Incubate in fast-red in the dark until desired reaction product intensity is achieved (15 min–2 h). Stop the reaction by washing in water.
9. Mount in Mowiol with a coverslip.

4. Notes

1. It is essential to avoid the degradation of the target of ISH, cellular RNA, prior to hybridization. Ribonuclease contamination should be avoided by autoclaving solutions; baking glassware and consumable supplies and reagents should be kept exclusively for ISH. We found that PBS tablets can be contaminated with RNAse and so should be treated with DEPC and autoclaved prior to use according to manufactures instructions. Disposable gloves should be worn.
2. Extraneous transcripts have been reported to occur during the preparation of the riboprobe when the template contains 3′ protruding ends; therefore, do not use restriction enzymes that generate 3′ overhangs. If there is no alternative restriction site, the 3′ overhang should be converted to a blunt end using Klenow DNA polymerase.
3. The mixture for the in-vitro transcription protocol should be kept at room temperature during the addition of each successive component, since DNA can precipitate in the presence of spermidine (present in the transcription buffer) if kept at 4°C.
4. Histolene used for the initial dewaxing of sections can be kept for use in mounting slides in DePex.
5. The hybridization temperature may need to be altered. Oligonucleotide probes are hybridized at 30–37°C overnight ***(4,5)***. The cRNA probes are hybridized at 40–70°C ***(3,5)***. Also bubbles may form under the coverslip during hybridization. This can be avoided by "degassing" the hybridization solution for 30 min under vacuum prior to use.

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Immunohistochemistry Using Polyester Wax

Andrew Kent

1. Introduction

For the preparation of sections for immunohistochemistry at the level of the light microscope, the choice of sectioning medium usually lies between frozen (fresh or fixed-cryoprotected) or paraffin wax. If the antigen survives fixation and processing at 60°C, paraffin wax is used. If the antigen survives fixation, but not heating to 60°C, frozen sections from fixed cryoprotected tissue are prepared, but if the antigen does not survive chemical fixation, sections are prepared from fresh, rapid-frozen tissue, and a trade-off in morphological preservation is accepted. However, if the antigen survives chemical fixation, but is sensitive to temperatures above 40°C (as many are to some degree) or is soluble in the clearing agent, polyester wax offers an opportunity for immunostaining without having to resort to frozen sections.

Steedman ***(1,2)*** introduced polyester wax as an alternative sectioning medium to paraffin wax for histology, claiming improved morphological preservation. Polyester wax is alcohol-soluble (i.e., negates the need for an additional clearing reagent, e.g., xylene or chloroform) and has a melting point of 37°C. These two properties contribute to milder solvent conditions, causing less extraction and thereby improve morphological preservation ***(3)***. By analogy, polyester wax should be inherently more suited to antigen preservation and therefore immunohistochemistry than paraffin wax.

The use of this wax for immunohistochemistry has previously been reported in combination with both formalin fixation ***(4)*** and acid-ethanol fixation ***(5)***. Its routine use for immunohistochemistry was described in 1991 ***(6)***, and it has been used in my laboratory as the method of preference for immunohistochemistry for 11 yr (e.g., ***7,8***). It can be used for any immunostaining procedure, e.g., immunofluorescence or immunoperoxidase, with or without counterstaining, or combined with *in situ* hybridization (ISH) ***(9)***.

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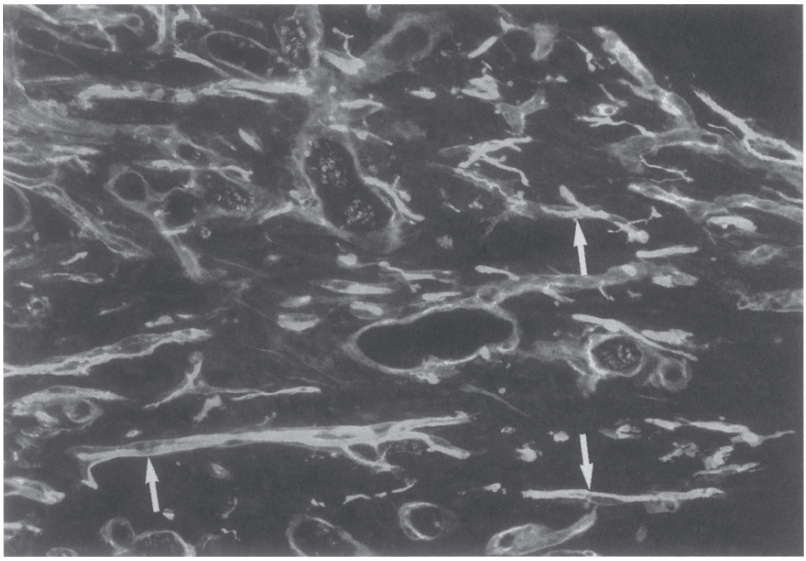

Fig. 1. Regrowing axons, sprouting from a transected peripheral nerve, are always seen in association with Schwann cells (®). Migrating Schwann cells are labeled with antilaminin and detected with antirabbit IgG-FITC. Axons are labeled with anti-(-tubulin isoform III and detected using biotinylated antimouse IgG and Extravidin-TRITC (×220). (*See Color Plate*)

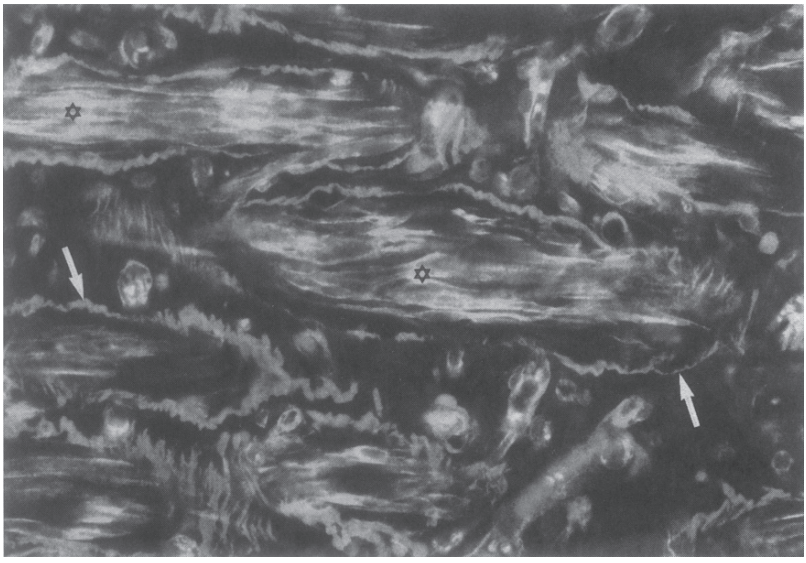

Fig. 2. Schwann cells from the proximal segment of a transected peripheral nerve invade the myotubes of an acellular muscle graft. The sarcolemmal basement membrane (®) is labeled with antilaminin and detected with antirabbit IgG TRITC. The Schwann cells (*) are labeled with anti-S100β and detected using biotinylated antimouse IgG and Extravidin-FITC (×450). Both micrographs were taken with dual excitation on an Olympus Provis photomicroscope using Fujichrome Provia (1600 ASA). (*See Color Plate*)

Many antigens benefit from processing in polyester wax. In general, immunostaining is much improved in polyester wax than paraffin wax and a strong signal can usually be obtained by an indirect method rather than a more lengthy amplification method. In some cases, immunostaining in polyester wax sections can be achieved where a result is only possible in paraffin wax sections after antigen retrieval (trypsinization or pressure cooking), e.g., labeling of Schwann cells with antibody to S100, clone SH-B1 (Sigma-Aldrich, Poole Dorset, UK) (*see* **Figs. 1** and **2**).

2. Materials

2.1. Processing and Sectioning

1. Fixative, either:
   a. 4% paraformaldehyde (PF) in phosphate-buffered saline (PBS), pH 7.4. For 1 L dissolve 40 g of PF (Taab laboratories Ltd., Aldermasto Berks., UK) in 900 mL of distilled water at 60°C with two to three drops of 1 NaOH. Add NaCl (8 g), KCl (0.2 g), anhydrous Na_2HPO_4 (1.15 g) and KH_2PO (0.2 g). Make up to 1 L with distilled water.
   b. Ethanol-acetic acid: For 100 mL, add 5 mL of glacial acetic acid to 95 mL of pure ethanol. **NB**. Other aldehyde or alcohol-based fixatives can be used, e.g., Bouin's fixative.
2. Industrial methylated spirit (IMS) for dehydration of tissue and dewaxing sections.
3. Polyester wax. For 500 g stock, melt 480 g of 400-polyethylene glycol distearate (PEGD) (Aldrich Chemical Co.) at 40°C overnight. Melt 20 g cetyl alcohol (Merck, Poole, UK) at 60°C. Warm up the PEGD to 60°C, add the cetyl alcohol, mix well, pour into convenient size trays, and allow to set. Store at 4°C.
4. Rotary microtome.
5. Pelcool unit or equivalent (Bright Instrument Co. Ltd., Huntington, Cambs., UK) with associated cooled chuck.
6. Glass Ralph knifemaker (e.g., Taab Histoknifemaker).
7. Glass strips (16 in. × 1.5 in. × 6 mm, Taab Superglass) or disposable, low-profile Feather blades (Raymond Lamb, London, UK).
8. Ralph knife holder or disposable blade holder (Raymond Lamb).
9. PTFE aerosol spray, Sprayflon Plus 3 (Filcris, Royston, Herts, UK).
10. Section adhesive, 1% aqueous gelatin or either Biobond (British BioCell International, Cardiff, Wales, UK) or Vectabond (Vector Labs Ltd., Bretton, Peterborough, UK).

2.2. Immunostaining

1. Humidified staining box either an adapted "sandwich" box or purpose-made black perspex box with transparent perspex lid (Genex Ltd., Coolsdon, Surrey, UK).
2. Blocking solution, either bovine serum albumin (BSA) (0.1–1%), or normal serum (1–5%) from the species in which the secondary antibody was raised, diluted in PBS.

3. Primary antibody, diluted appropriately with blocking solution.
4. Secondary antibody diluted with blocking solution (usual range 1 in 100 to 1 in 200).
5. Tertiary antibody, e.g., Extravidin-FITC (Sigma) or ABC-peroxidase (Vector Labs. Ltd.) at the recommended dilution in PBS.
6. For immunoperoxidase:
   a. Suitable substrate, e.g., diaminobenzidine (DAB). For 10 mL, dissolve 6 mg DAB in 10 mL PBS and, immediately before use, add 3 µL of 30% H_2O_2, or a DAB alternative, e.g., Vector VIP.
   b. Suitable counterstain, e.g., for DAB, 0.1% toluidine blue.
   c. A setting mountant, e.g., DPX (Merck).
7. For immunofluorescence:
   a. An antiquenching mountant. For 100 mL, dissolve 2.5 g 1,4-diazabicyclo [2.2.2] octane (DABCO) (Sigma) in 90 mL of glycerol at 37°C, and 0.1 g sodium azide in 10 mL of PBS. Then combine, mix well, and store in a dark bottle at 4°C.
   b. Nail varnish for sealing cover slips onto slides.

3. Method

3.1. Tissue Processing

1. Tissue is excised and immersed in fixative at 4°C as quickly as possible. If tissues have a minimum dimension > 5 mm, then slices of this width are prepared. This ensures that over the fixation period of 4–24 h (time dependent on the size of the tissue and on the antigen) all regions of the tissue are adequately fixed, and the antigen is retained uniformly.
2. Following fixation with paraformaldehyde, the tissue is washed in PBS prior to dehydration with IMS using a series of 50, 70, and 90%, and three absolute IM changes (30 min each).
3. If the tissue is fixed with acid-ethanol, it can be immediately transferred to absolute alcohol.
4. From the final alcohol change, tissues are transferred to a mixture of polyester wax and alcohol (50:50) at 40°C and subsequently to a 75:25 mixture before changing to 100% wax. Infiltration times are dependent on the size of the tissue, but the size recommended above, 2 h in each of the mixtures and 4 h (2 changes) in wax should suffice. For larger pieces of tissue, the infiltration times should be extend accordingly. However, it should be remembered that the longer the tissue remains at 40°C, the greater is the risk of antigen loss. Extended infiltration (>24 h) also encourages hardening of tissue, which makes sectioning more difficult.
5. Following infiltration, tissue is blocked out and allowed to set at room temperature.
6. Blocks are best stored at 4°C.

3.2. Sectioning

1. For sectioning, blocks are mounted on a cooled chuck connected to a rheostat (e.g., Pelcool Unit) for temperature control. Sections can be cut in the range of

2 ***(5)*** to 30 µm ***(3)*** thick, with 7 µm being suitable for most applications. The temperature of the block face is maintained at 10–15°C and room temperature below 22°C. Blocks can be cut on low-profile disposable metal knives, e.g., Feather blades, but glass Ralph knives, thinly coated with PTFE, are preferable. Ribbons can be stored at 4°C.

2. Sections intended for routine immunohistochemistry are mounted on clean glass slides, and sufficient adherence is obtained by allowing sections to decompress on a pool of 1% (w/v) aqueous gelatin with careful warming. (Placing the slide on a tissue-culture flat flask filled with warm water [30°C] is ideal).
3. Excess gelatin is removed, and sections are dried on overnight at room temperature. If trypsinization is necessary to expose the antigen, then sections should be mounted on Biobond- or Vectabond-coated slides. For ISH, sections are also mounted on Biobond or Vectabond coated slides, but additionally, following removal of wax in 100% IMS, the slides are removed and dried for 30 min at 37°C ***(9)*** prior to equilibration in PBS (*see* **Notes 1–6**).

3.3. Immunostaining

3.3.1. Quick Method for Abundant Antigens

Following dewaxing in a descending series of alcohols (100 to 50% v/v) and equilibration in PBS, the following routine protocols are recommended. All incubations are carried out in an humidified chamber to prevent drying out at any stage during immunostaining.

1. Incubate for 2 h in primary antibody, diluted in 0.1% (w/v) BSA/PBS, at room temperature.
2. Wash in PBS (twice for 15 min).
3. Incubate for 1 h in species-specific anti-IgG antibody conjugated to FITC, and diluted in 0.1% (w/v) BSA/PBS (1 in 100 recommended).
4. Wash in PBS (twice for 15 min).
5. Mount in glycerol-based antiquench mountant, and seal cover slip with nail varnish.

3.3.2. Amplification Method for Less Abundant Antigens

1. Preblock in 1% (v/v) normal serum (from the species in which the intended secondary antibody is raised), 0.1% (w/v) BSA in PBS for 30 min.
2. Incubate overnight at 4°C in primary antibody, diluted in the blocking solution.
3. Wash in PBS (twice for 15 min).
4. Incubate for 1–2 h at room temperature in biotinylated secondary antibody diluted in the blocking solution (1 in 200 recommended).
5. Wash in PBS (twice for 15 min).
6. Incubate for 1 h at room temperature in Extravidin-FITC diluted in PBS (1 in 100 recommended).
7. Wash in PBS (twice for 15 min).

8. Mount in glycerol-based antiquench mountant, and seal cover slip with nail varnish (*see* **Notes 7–10**).

3.3.3. Immunoperoxidase Method for Less Abundant Antigen

1. Follow **steps 1–5** in **Subheading 3.3.2**.
2. Incubate for 1 h at room temperature in Vectastain Elite ABC reagent (prepared 30 min before use).
3. Wash in PBS (2 × 15 min).
4. Incubate with appropriate substrate, either DAB (brown) or Vector SG (black) or Vector VIP (deep purple).
5. Wash in distilled water (5 min).
6. Counterstain, dehydrate in IMS, clear in xylene, and mount in DPX.

4. Notes

4.1. Problems with Processing and Sectioning

1. Sections fragment during decompression. Inadequate fixation, dehydration, or infiltration—reassess size of block or processing times. Section left to decompress for too long or at too high a temperature—take more care when sections decompress.
2. Sections compress too much, and do not regain original block dimensions. Knife blunt—shift to new area of knife or change knife. Block temperature too cold or too warm—alter Pelcool setting.
3. Section exhibits chatter. Knife angle wrong—check angle is set to 5/6°. Loose component—check security of knife holder, knife, and block.
4. Examination of stained sections reveals wrinkles. Decompression inadequate—increase time or temperature to allow sections to decompress optimally.
5. Sections fail to ribbon. Room temperature too high—turn on air conditioning or seek alternative venue. Block poorly trimmed—use a sharp blade to retrim and align sides of the block.
6. Sections do not adhere to slide. Sections dried onto slide too briefly—dry sections overnight. Sections not mounted on either an adhesive solution or a coated slide—check mounting solution is 1% gelatin or that slides are coated as intended. Proteinase is contaminating the diluent—use fresh diluent (bacteria grow readily in warm diluent!).

4.2. Problems with Immunostaining

7. No positive immunostaining obtained. Antigen does not survive processing—check antigen's sensitivity to fixation and dehydration using fresh frozen, and fixed, cryoprotected, frozen sections. Antigen is not present in tissue—use a positive control. Primary antibody is at fault—use a positive control and a new aliquot or batch. Secondary antibody or detection system is at fault—check potency of secondary antibody using an alternative primary antibody. If using an indirect method of detection, this may not be sensitive enough—try an amplification system.
8. Patchy immunostaining obtained. Gelatin solution crept over surface of section during decompression—remount new sections with additional care. Nonuniform fixa-

tion—reassess fixation protocol. Sections allowed to dry out during staining—restain using an adequate volume of immunoreagent and ensure humidity of chamber.

9. Background staining too high. Concentration of primary antibody too high—reduce titer to minimum. Secondary antibody is interacting with the tissue–e.g., for mouse monoclonals used on rat tissue, use rat adsorbed antimouse IgG, or use control lacking primary antibody. Tissue has (a) autofluorescence or (b) endogenous enzyme, e.g., peroxidase—(a) View section without antibodies or (b) react section with enzyme substrate alone. Antibodies binding via ionic interaction—increase pH from 7.4–8.2., increase salt concentration, use detergent, e.g., 0.1% Tween-20.
10. Immunofluorescent stain is rapidly lost from the section during microscopy. Antibody (probably primary) is labile in mountant—refix sections in 4% PF for 5 min after immunostaining and prior to mounting in glycerol-based mountant.

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Immunohistochemistry on Whole Embryos

Ivor Mason

1. Introduction

Immunohistochemical studies on intact embryonic material offers the same advantages as similar in situ hybridization techniques (Chapters 46–48), namely, that spatial relationships of tissues expressing the antigen of interest are more readily appreciated (*see* **Fig. 1**). In addition, the lack of prior processing and sectioning of material considerably reduces the time required for such analyses, and material can still be sectioned subsequently if required. However, it should be noted that relatively few antibodies or antisera are suitable for this technique, owing to cross-reactivity with other antigens, low affinity, or low titer. Cross-reactivity, even with low affinity, produces unacceptable levels of nonspecific or "background" staining and is most commonly a feature of polyclonal antisera. Thus, although relatively few monoclonal antibodies (MAbs) are high affinity and high titer, this type of reagent is most frequently suitable for use on intact embryos. The procedure described here is slightly modified from that in ***(1,2)***.

2. Materials

1. Phosphate-buffered saline (PBS; 10× concentrated, pH 7.2)/L: 2.1 g KH_2PO_4, 90 g NaCl, 7.26 g Na_2HPO_4 $7H_2O$. Autoclaved.
2. Paraformaldehyde (Fluka, Gillingham, UK).
3. Hydrogen peroxide (30% w/w; Sigma, replace every 4 mo).
4. Triton X-100 (Sigma, Poole, UK).
5. Sodium azide (Sigma).
6. Basal medium (Eagle) containing 25 mM HEPES (Life Technologies, Paisley, Scotland).
7. Calf serum (Life Technologies).
8. Normal goat or donkey sera (Sigma).
9. Peroxidase-conjugated secondary antibodies (Jackson Laboratories).
10. 0.1 M Tris-HCl, pH 7.2.
11. Diaminobenzidine (DAB; 10 mg tablets from Sigma).

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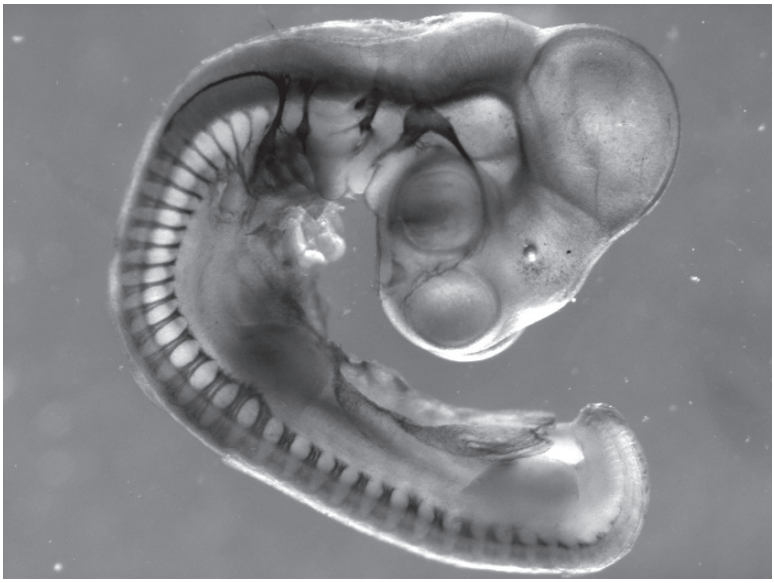

Fig. 1. Whole-stage 22 chick embryo stained with a monoclonal antibody against neurofilament, which detects axons within both the central and peripheral nervous systems.

3. Method

1. Fix embryos overnight in 4% w/v paraformaldehyde in PBS at 4°C (*see* **Notes 1** and **2**) using a large (20- to 50-fold by volume) excess of fixative.
2. Wash in PBS three times, each for 30 min.
3. Block endogenous peroxidase enzymes with PBS containing 0.05% (v/v) hydrogen peroxide, 1% (v/v) Triton X-100 at 4°C overnight on a rocker or slowly rotating wheel (*see* **Note 3**).
4. Wash in PBS containing 1% (v/v) Triton X-100 three times each for 1 h.
5. Dilute antibody (*see* **Note 4**) in basal Eagle's medium containing 10% calf serum, 1% (v/v) Triton X-100 and 0.02% (w/v) sodium azide (*see* **Note 5**). Incubate for 2–4 d at 4°C with rocking.
6. Wash in PBS containing 1% v/v normal serum of same species as peroxidase-conjugated secondary antibody (e.g., normal goat serum for goat antimouse secondary antibodies) and 1% (v/v) Triton X-100, three times each for 1 h at 4°C.
7. Incubate with peroxidase-conjugated secondary antibody appropriate for the primary antibody used in step 5 (e.g., peroxidase-conjugated goat antimouse IgG or IgM for mouse MAbs). The secondary antibody is diluted in PBS containing 1% (v/v) Triton X-100 and 1% (v/v) normal serum as in step 6 at 4°C overnight. The precise dilution of secondary antibody should be determined empirically, but we generally find that dilutions of 1:100 are about right.
8. Wash as step 6.

9. Wash in 0.1 M Tris-HCl, pH 7.2, twice for 30 min.
10. Incubate in 0.5 mg/mL DAB in 0.1 M Tris-HCl, pH 7.2, for 3 h at 4°C in the dark with rocking (*see* **Note 6**).
11. Replace with 0.5 mg/mL DAB in 0.1 M Tris-HCl, pH 7.2, containing 1 µL/mL hydrogen peroxide, and allow color to develop (usually 5–15 min; longer may be required if embryo is to be sectioned subsequently).
12. Reaction is stopped by several quick changes of tap water followed by several changes of PBS over a few hours.
13. Embryos can be further dissected at this point, then cleared in 90% (v/v) glycerol, 1% PBS, 0.02% (w/v) sodium azide for storage or mounting for photography.
14. For sectioning, embryos are dehydrated through graded ethanols, equilibrated in xylene, then embedded in wax, as described in Chapter 45, followed by sectioning at thicknesses of 7–15 µm.

4. Notes

1. This protocol works well on intact embryos up to at least mouse embryonic d 12, chick Hamburger and Hamilton ***(3)*** stage 24, and zebrafish to 72 h (*see* also **Note 7** for zebrafish modifications). For older embryos, it is likely that some block dissection is required prior to fixation to keep background levels low. Alternatively, some tissues, such as the neural tube, can be dissected after performing the procedure on intact older embryos.
2. It is worth trying a range of fixatives before ruling out the suitability of an antibody or antiserum for this procedure. In addition to standard paraformaldehyde fixation (**section 3, step 1**), it is worth trying 0.5% (w/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde; 4% (w/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde; 5% (v/v) acetone, 95% (v/v) ethanol; perfix (Fisher Scientific, Loughborough, UK); dry AnalaR acetone. The last three fixatives are precipitating (rather than cross- linking) fixatives and, therefore, are probably not worth trying if the antigen is a small soluble protein (e.g., $M_r < 20{,}000$). For a detailed consideration of antibody–antigen interactions including fixation, *see* ***(4)***.
3. Some antibodies may bind more strongly at 37°C, and for these, the use of a combined rotating wheel and incubator, such as a "Spin 'n' Stack" (Hybaid, Teddington, UK), is recommended.
4. Required dilution must be determined empirically and depends on titer and affinity. We used dilutions varying between 1:2 and 1:500 for MAbs raised against different antigens.
5. The diluted antibody can be frequently reused one or more times. It should be retrieved and stored at 4°C between uses.
6. DAB is carcinogenic. All materials in contact with it and all waste solutions should be treated overnight with excess bleach to break it down.
7. Zebrafish older than 36 h require permeabilization with 2.5% (w/v) trypsin between steps 2 and 3. The precise time must be determined empirically for each batch of enzyme, but a rough guide is 36 h of development, 3 min.; 48 h, 5–6 min.; 72 h, 6–7 min. Embryos should be rinsed five times each for 5 min in PBS before proceeding.

Acknowledgments

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Whole Embryo Assays for Programmed Cell Death

Anthony Graham

1. Introduction

Cell death occurs by two processes that are cytologically and biochemically distinct. Pathological cell death, or necrosis, is characterized by early cell swelling and the lysis of intracellular organelles with nuclear breakdown following at a later stage. In contrast, nonpathological cell death or apoptosis is associated with early nuclear fragmentation, cytoplasmic shrinkage, and preservation of intracellular organelles ***(1)***. Necrosis is usually associated with cell injury; ATP levels fall early, and consequently, protein synthesis is inhibited. On the other hand, in apoptosis, ATP levels remain near normal, and new protein synthesis is required to initiate the process. One result that stems from the early nuclear fragmentation that is associated with apoptosis is that DNA degradation, resulting in the formation of a DNA ladder, is frequently observed.

During development, programmed cell death plays a vital role in eliminating unwanted cells, and it is invariably apoptotic in nature with the dying cells displaying the hallmarks of this process ***(2)***. Programmed cell death will act to prune cells that have been produced in excess, such as neurons. It is also involved in the eradication of cells of structures that are no longer necessary, such as the tail of tadpoles or the Müllerian duct in males ***(2)***. The apoptotic elimination of cells is also important in morphogenesis, and in both the limb and the cranial neural crest, it functions to ablate populations of cells locally and, as such, spatially separates the surviving cell populations from each other ***(2,3)***.

In developing systems, one can often not obtain enough material to analyze programmed cell death by biochemical means, such as assaying for DNA laddering, and therefore one must use alternative methods. The most definitive method of determining whether or not apoptosis, as opposed to necrosis, is occurring in a group of cells is to analyze the cells ultrastructurally using electron microscopy (EM). This is a powerful tool because it will allow one to identify unequivocally

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a cell as being apoptotic or necrotic. The problem with using EM to analyze cell death in developing embryos is that it is a cumbersome technique that cannot be used practically to detect apoptosis or to gain an impression of a spatiotemporal profile of apoptosis. This chapter will describe two methods for detecting apoptosis in whole embryos, both of which will give a complete picture of apoptosis in any system. Both of these techniques are based on detection of the DNA fragmentation that occurs early in the apoptotic program. One point that should be noted is that although both of these techniques will pick out apoptotic sites in the embryo, the fact that the cells are actually dying by apoptosis should also then be confirmed independently by EM analysis.

1.1. Visualization of Programmed Cell Death in Whole Embryos Using Acridine Orange

Vital staining with the dye, acridine orange, has been previously used to analyze patterns of apoptosis in both vertebrate and invertebrate embryos ***(3,4)***. During apoptosis, cells fragment into a number of apoptotic bodies that are chromatin-rich and are intensely stained by acridine orange. In contrast, healthy cells exclude this dye. Thus, when used vitally, i.e., on living specimens, it can only pick out dead cells. The method described below will work, with slight modifications, on any type of embryo.

1.2. Visualization of Programmed Cell Death in Whole Embryos Using TUNEL

The terminal transferase-mediated dUTP-biotin Nick End Labeling (TUNEL) method to detect apoptotic cells is based on the fact that terminal transferase will catalyze a template-independent addition of dNTPs to the 3′-OH ends of DNA strands and that one of the early events during apoptosis is the cleavage of a cell's DNA ***(5,6)***. Thus, cells undergoing programmed cell death are detected by incubating the embryo, which has been fixed, with terminal transferase and a labeled nucleotide, dUTP-biotin conjugated, with the latter being added to the nicked ends of the DNA fragments and subsequently detected with a streptavidin conjugate. Again, this method will work with slight modification for any type of embryo (*see* **Note 2**).

2. Materials

2.1. Acridine Orange Staining

1. Phosphate-buffered saline (PBS): 0.13 *M* NaCl, 7 m*M* Na_2HPO_4, 3 m*M* NaH_2PO_4. Adjust pH to pH 7.0 and autoclave.
2. Howard's Ringer: 125 m*M* NaCl, 1.5 m*M* $CaCl_2$ $2H_2O$, 5 m*M* KCl adjust to pH 7.2 with 1 *M* NaOH and autoclave.
3. Acridine orange: (Sigma, Poole, UK) or equivalent—Make a 5 mg/mL stock in H_2O, and dilute from that, stepwise, into the physiological salt solution.

2.2. TUNEL

1. 4% Paraformaldehyde, (w/v), in PBS: *see* **Subheading 2.1., item 1** for PBS recipe.
2. Triton X-100.
3. Terminal transferase (Boehringer Mannheim, Mannheim, Germany), which comes with the terminal transferase buffer as a 5X stock and 25 m*M* $CoCl_2$.
4. Deoxy uridine triphosphate (dUTP) and biotin-16-deoxy uridine triphosphate (Biotin-16-dUTP) (Boehringer Mannheim).
5. Streptavidin–fluorescein reagent (Amersham, Little Chalfont, UK).
6. Streptavidin–horseradish peroxidase conjugate reagent (Amersham).
7. 1,4-Diazabicyclo [2,2,2] octane (DABCO) (Sigma).
8. 3′,3′-Diaminobenzidine (DAB) (Sigma).
9. Hydrogen peroxide (30% w/v) (Sigma).

3. Methods

3.1. Acridine Orange Staining

1. Dissect out the embryos into an appropriate physiological salt solution, such as PBS, for mouse embryos or Howard's Ringer for chick embryos, and process directly.
2. Incubate embryos at 37 °C in PBS, or Ringer's, containing acridine orange at a concentration of 5 µg/mL for 15 min.
3. Wash the embryos twice, with shaking, for 1 min each in PBS.
4. Mount the specimens under a glass coverslip in either PBS or Ringer's and analyze the specimens immediately under rhodamine epifluoresence.
5. Photograph the results.

Results obtained with acridine orange staining of whole chick embryos are shown in **Fig. 1**. The images shown here were taken using a Bio-Rad MRC 600 (Bio-Rad, Hemel Hempstead, UK) confocal microscope, and the rhodamine and phase images have been merged to give a clear picture of the relationship between areas of acridine orange staining and embryonic anatomy. During the early phase of neural crest production in the chick hindbrain, a focus of acridine orange staining cells is observed over rhombomere 3 (**Fig. 1A**), whereas at slightly later stages, the focus over this segment is enlarged, and there is also prominent staining over rhombomere 5 (**Fig. 1B**). The importance of apoptosis in this system is that by clearing these two territories (rhombomeres 3 and 5) of neural crest cells, the neural crests that emerge from the adjacent regions and that are spatially preprogrammed are kept separate from each other ***(3)***.

Acridine orange staining has the advantages that it is extremely rapid, cheap, and easy to perform. On the other hand, it has a number of disadvantages. Firstly, because it is used vitally, and since the dye is not fixable and consequently the stain is transient, the embryos cannot be processed for other detection procedures,

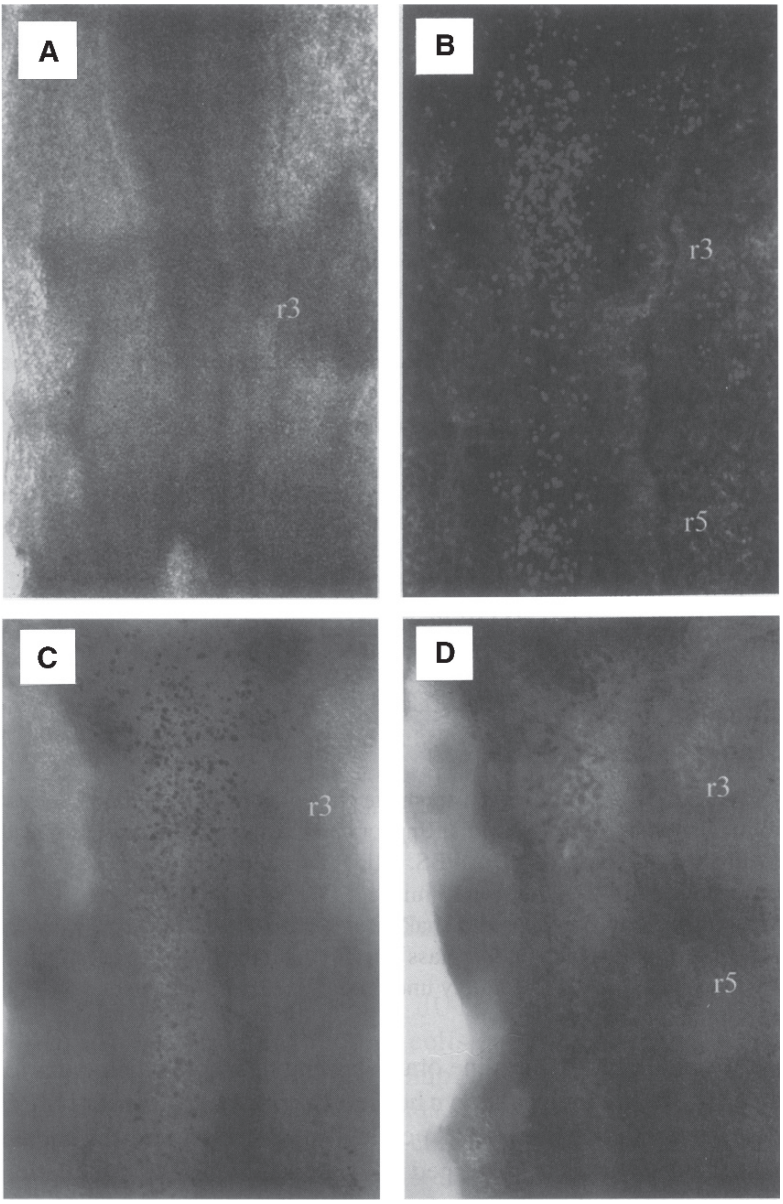

Fig. 1. Apoptosis in the rhombencephalic neural crest of the chick embryo as revealed by acridine orange staining **(A,B)** and TUNEL **(C,D)**. The embryos shown in (A) and (C) are at stage 10, but those in (B) and (D) are older, stage 11 (*see* **Note 1** and color plate 3 appearing after p. 368). (*See Color Plate*)

such as immunohistochemistry. Acridine orange staining also has the drawback that the embryos must be processed swiftly and viewed almost immediately, at least within 1 h of staining, since the tissue dies and the intense staining of the apoptotic bodies is lost. It should also be pointed out that although the signal may be more intense under flourescein epifluoresecne, necrotic cells will also be picked up under this illumination. This method also has the disadvantage that since there is no permeabilization step, apoptotic cells that lie deep within tissues may not be detected. One way of improving this is to cut the embryo prior to staining so that there is greater access to the tissue of interest. For example, with the central nervous system, one can slit the neural tube dorsally.

3.2. TUNEL

1. Fix the embryos in 4% paraformaldehyde in PBS either for 2 h at room temperature or overnight at 4 °C.
2. Wash the embryos in PBS containing 1% Triton X-100 three times for 30 min each.
3. Wash the embryos once in 1X terminal transferase buffer, 2.5 m*M* $CoCl_2$, 1% (v/v) Triton X-100.
4. Incubate the embryo for 3 h at 37 °C in 1X terminal transferase buffer, 2.5 m*M* $CoCl_2$, 0.5 µ/mL terminal transferase, 10 m*M* dUTP (2:1 dUTP:dUTP-biotin), 1% (v/v) Triton X-100.
5. Wash the embryo three times in PBS, 1% (v/v) Triton X-100 for 30 min.
6. Incubate overnight at 4 °C with rocking in an appropriate streptavidin conjugate, such as strepevidin-fluorescein or streptevidin-horseradish peroxidase.
7. Wash the embryos three times in PBS, 1% (v/v) Triton X-100 for 1 h each. Then proceed either according to **step 8** or **steps 9–12** as appropriate.
8. In the case of streptavidin-fluorescein detection, mount the specimens under 90% (v/v) glycerol, 1X PBS containing the antiquenching agent DABCO at 2.5% (v/v), and view under fluorescent optics.
9. Wash the embryos twice in PBS for 30 min each.
10. Incubate the embryos in DAB in PBS (5 mg/10 mL PBS) for between 1 and 3 h, depending on the stage of the embryo at 4 °C with rocking in the dark.
11. Exchange the above solution for the same, but this time add hydrogen peroxide (60% or 200 vol), 10 µL/10 mL DAB in PBS, and develop in the dark for 5–15 min.
12. When the background staining starts to appear, stop the reaction by washing several times with PBS.
13. Wash in PBS twice each for 30 min, and then mount the specimens under a glass coverslip in 90% glycerol/10% PBS and view under bright-field optics.

4. Notes

1. **Fig. 1C,D** shows the results obtained using TUNEL, and streptavidin-horseradish peroxidase detection, on whole chick embryos during the period of hindbrain neural crest apoptosis. As was found with the acridine orange staining, during the early phase of crest production, there is staining over rhombomere 3 (**Fig. 1C**), and

at a slightly later stage, this staining is more pronounced and extensive, and also apoptotic cells are also found over rhombomere 5 (**Fig. 1D**).

2. The disadvantages of using TUNEL are that it is relatively expensive and takes somewhat longer than the acridine orange staining, but it has many advantages that together make it the method of choice. One big advantage is that this method uses embryos that are fixed. This means that one can accumulate specimens at 4 °C and that they can be processed at leisure. The fixation also allows one to permeabilize the embryos using detergent, Triton X-100, and therefore even apoptotic cells deep within the embryo can be detected. The fact that this method can use fluorescein or horseradish peroxidase detection also means that this technique can be coupled with other procedures, such as immunohistochemistry or axonal tracing, and at least when using horseradish peroxidase detection, the preparations are permanent. The horseradish peroxidase-reacted embryos can also be sectioned afterward to allow a more complete analysis of the distribution and the cell type of the apoptotic cells.

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Protein Techniques

Immunoprecipitation, In Vitro Kinase Assays, and Western Blotting

Clive Dickson

1. Introduction

The regulation of complex developmental pathways involves the spatial and temporal interaction of specific gene products. Several powerful genetic approaches, including the tagging of gene products or cells, have allowed their distribution in space and time to be determined. The advent of procedures to create transgenic animals with acquired or abrogated gene functions has allowed the phenotypic consequences of genetic alteration to be analyzed by design rather than chance mutation. For example, the introduction of a portion of the Y chromosome into a female mouse can change its sex, demonstrating the presence of a sex-determining gene ***(1)***. In combination with a range of molecular biology techniques, such as RNA protection, PCR amplification, *in situ* hybridization, immunohistochemistry, and the use of reporter genes, to indicate how, when, and where a gene is expressed. This avalanche of information has started to provide a real insight into the molecular mechanisms that regulate developmental processes. However, studies on the gene products are still crucial for an understanding at the cellular level processes, such as pattern formation and organogenesis.

Proteins may be modified by glycosylation, phosphorylation, proteolysis, or myristylation, and each of these modifications affects the properties of the protein. For example, specific changes in the level of phosphorylation of certain proteins have been observed during the compaction of the mouse embryo, and it is a central paradigm for cell:cell signaling by tyrosine kinase receptors ***(2–5)***. To determine the presence of a particular protein or to investigate possible post-translational modifications, such as glycosylation, phosphorylation, proteolytic

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processing, and protein:protein interactions, there are several basic protein separation techniques that can be applied, and some general recipes for these are described ***(6–9)***.

2. Materials

All solutions should be made of Analar-quality reagents and distilled water. Stock solutions of 20% (w/v) SDS, phosphate-buffered saline (PBS), 5 *M* NaCl, 0.5 *M* EDTA pH 8.0, 1 *M* dithithrietol, 1 *M* Tris HCI at pH 8.8, 8, 7.5, and 6.8 are required.

1. Lysis buffers:
   a. RIPA lysis buffer: 150 m*M* NaCl, 1% (v/v) NP-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 50 m*M* Tris-HCl, pH 8.0, and protease inhibitors. NP-40 lysis buffer: 150 m*M* NaCl, 1% (v/v) NP-40, 50 m*M* Tris, pH 7.5, and protease inhibitors.
   b. Protease inhibitors: A mixture of 1 m*M* phenylmethylsulfonyl fluoride (PMSF), (the 1 *M* concentrated stock dissolved in acetone), and 1 mg/mL each of antipapain, chymostatin, leupeptin, and pepstatin (*see* **Note 1**).
   c. 2X dissociation buffer: 4% (w/v) SDS, 120 m*M* Tris-HCl, pH 6.8, 0.002% (w/v).
   d. Bromophenol blue, 20% (v/v) glycerol 0.1 *M* dithiothreitol (*see* **Note 2**).
2. Antibody coupling solutions: 0.1 *M* borate buffer pH 8.2, 20 m*M* ethanolamine pH 8.2, 0.2 *M* triethylamine, pH 8.2, dimethyl pimelimidate, and protein A Sepharose beads (Amersham Pharmacia Biotech, Little Chalfont, UK).
3. Kinase buffer: 100 m*M* NaCl, 25 m*M* HEPES pH 7.5, 5 m*M* $MnCl_2$, 10 m*M* $MgCl_2$, 1 m*M* Vanadate, 10 µ*M* Mg-ATP (Sigma), γ^{32}P-ATP (3000 Ci/mmol).
4. SDS polyacrylamide gel electrophoresis (PAGE) solutions.
   a. Resolving gel: 12.5 mL 30% (29:1) acylamide/*bis*-acrylamide mix, 7.5 mL 1.5 *M* Tris-HCl, pH 8.8, 0.3 mL 10% (w/v) SDS, 9.9 mL H_2O, 0.3 mL 10% (w/v), ammonium persulfate (made fresh), 12 µL TEMED.
   b. Stacking gel: 0.83 mL of 30% (29:1) acylamide/*bis*-acrylamide mix, 0.63 mL 1 *M* Tris-HCl, pH 6.8, 50 µL 10% (w/v) SDS, 3.4 mL H_2O, 50 µL 10% (w/v) ammonium persulfate, 5 µL TEMED.

   Running buffer: 25 m*M* Tris base, 192 m*M* glycine, 0.1% (w/v) SDS.
5. Blotting solutions:
   a. Semidry blotting buffer: 48 m*M* Tris base, 39 m*M* glycine, 0.037% (w/v) SDS, 20% (v/v) methanol.
   b. Western blocking buffer: PBS 5%(w/v), BSA 0.05% (v/v), Tween-20, or PBS, 5% (w/v) nonfat milk powder.
   c. Western washing buffer: PBS 0.05% Tween-20.
   d. ECL reagent commercially available from Amersham Pharmacia Biotech.
6. Antibody-labeling solutions:
   25 m*M* KI, Iodogen (Pierce, Warriner, UK), $CHCl_3$, Sephadex G-25 (Amersham Pharmacia Biotech), and ^{125}I (100 mCi/mL).

7. Gel-staining solutions:
    a. Gel-fixing solution: 45% (v/v) methanol, 9% (v/v) acetic acid.
    b. Coomassie stain: 45% (v/v) methanol, 9% (v/v) acetic acid containing 0.25% (w/v) Coomassie blue R250.

3. Methods

The embryonic material can be used in several ways as indicated in **Fig. 1**; the various techniques are described in order below.

3.1. Radiolabeling Cell/Tissue Cultures

1. The cultures or tissue explants are rinsed in culture medium missing the appropriate substrate. Thus, the medium must be methionine-free or phosphate-free if these are to be the radiolabeled compounds.
2. The cultures are then incubated in the same medium containing 1 mCi/mL of the radiolabeled compound, at 37°C, in tissue-culture conditions for 2–3 h.
3. The radiolabeled medium is removed, and the embryonic tissue is washed once in "cold" medium before being dissociated in either 2X dissociation buffer or RIPA/NP40 lysis buffer.

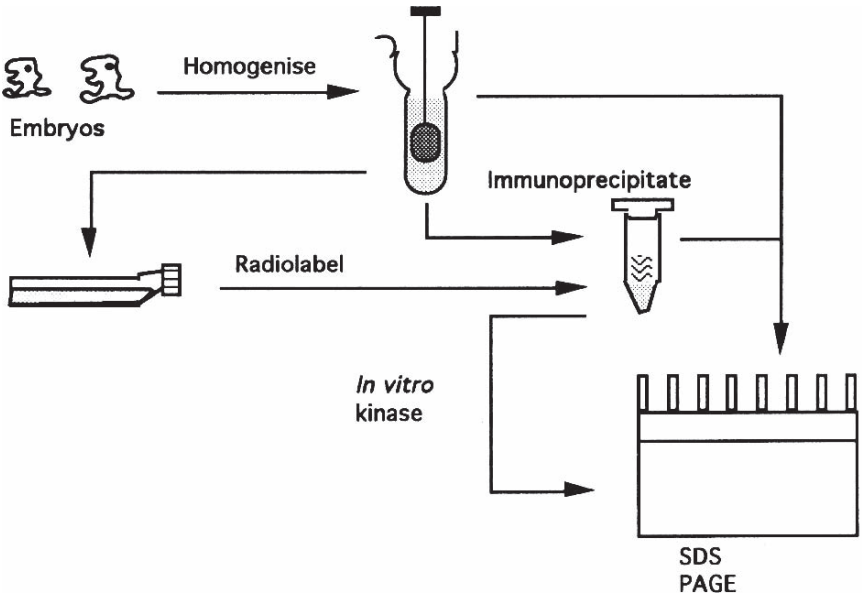

Fig. 1. Schematic diagram of the relationship of the protocols described in this chapter.

3.2. Preparation of Extracts

To immunoprecipitate proteins from embryonic tissue or cell culture, the material has to be disrupted under conditions that solubilize the proteins (*see* **Notes 3** and **4**).

1. Embryos or cell cultures are collected and washed in PBS prior to homogenization using a dounce homogenizer, or other commercial disruption device, with ice-cold RIPA or NP40 lysis buffer.
2. Centrifuge the lysate at 12,000*g* for 10 min at 4°C to remove the insoluble material; in the case of NP-40 lysates, this will also cause the unlysed nuclei to sediment.

3.3. Coupling of Antibodies to Protein A Beads

In order to immunoprecipate antigens, the revelant antibody is preferably coupled to the solid support (protein A-sepharose beads), but alternatives are mentioned in **Note 5**.

1. Wash the protein A-sepharose beads with PBS, and then add the monoclonal antibody (MAb) (*see* **Note 6**) to 2 mg/mL of packed protein A-sepharose beads in PBS for 2 h at 4°C. Fifty millimeters of ascites or 1 mL of polyclonal antiserum or tissue-culture supernatants/mL of packed beads can also be used.
2. Wash in 0.1 *M* borate buffer, pH 8.2, three times, and then once with 0.2 *M* triethylamine, pH 8.2.
3. Add 5 vol of 0.2 *M* triethylamine, pH 8.2, containing 20 m*M* dimethyl pimelimidate for 45 min to crosslink the protein to the beads.
4. Spin down the beads, and add 20 m*M* ethanolamine, pH 8.2, to stop the reaction. After 5 min, wash three times with 0.1 *M* borate buffer, and finally resuspend the coupled beads in RIPA or NP40 lysis buffer (**Note 7**).

3.4. Immunoprecipitation

Immunoprecipitation is a technique that allows the biochemical characterization of protein from relatively few cells, and is an important technique where material may be a limiting factor. It can also be used to study various types of posttranslational modification (*see* **Note 8**). The amount of material required will depend on the abundance of the antigen (*see* **Note 9**).

1. The lysates are precleared twice for 1 h at 4°C with 25 µL of protein A-Sepharose beads (*see* **Note 10**).
2. The precleared lysates are then immunoprecipitated with 10 µL of 2 mg/mL antibody protein A-Sepharose beads for 4 h to overnight at 4°C.
3. The beads are washed six to eight times in lysis buffer for 1 min with as much of the solution as possible being removed at each wash (**Note 11**).

At this stage, the immunoprecipitate can be analyzed by standard fractionation techniques, such as SDS-PAGE, or assessed for enzyme activity (e.g., kinase activity).

3.5. In Vitro Kinase Assay

1. The beads are resuspended in 30 µL of kinase buffer.
2. 10 µCi of γ^{32}P-ATP are added to the immune precipitates and incubated at ambient temperature for 15 min.
3. 0.7 mL of lysis buffer containing 30 m*M* EDTA, pH 8.0, is added to stop the reaction, and the beads are then washed three times in kinase buffer. The samples are then run on an SDS polyacrylamide gel as described below.

3.6. SDS Polyacrylamide Gel Electrophoresis (PAGE)

The basic SDS-PAGE method is based on that described by Laemmli ***(10, 11)*** and as an aid the 12.5% polyacylamide gel, is described in **Subheading 2.4.** (*see also* **Note 12**). SDS polyacrylamide gels are electrophoresed in running buffer (**Note 13**). Gels containing SDS separate on the basis of molecular weight, and it is possible to calibrate the gel using a sample of appropriate marker proteins; these are commercially available as prestained, or ^{14}C-labeled markers, which allow them to be visualized by autoradiography or on immunoblots.

1. The resolving gel is poured (adding the TEMED and ammonium persulfate last), and the surface is flattened by overlaying with a small amount of butan-1-ol.
2. After polymerization the butan-1-ol is drained off, and the stacking gel poured on top of the resolving gel.
3. To the sample, an equal volume of 2X dissociation buffer is added and boiled for 3 min prior to loading onto the gel (*see* **Fig. 1**).

After electrophoresis, the gels can be fixed and stained with Coomassie blue to visualize the proteins if the gel is not to be immunoblotted (*see* **Note 14**). The gels are fixed for 30 min, and then stained with Coomassie blue for 4 h, followed by destaining in "fixing solution," which can be accelerated at 50°C.

3.7. Immunoblotting

1. After electophoresis, the proteins within the gel are transferred onto a nitrocellullose membrane using a semidry electroblotter in semidry blotting buffer at 0.3 A for 40 min (*see* **Subheading 2.5.** and **Note 15**).
2. The remaining protein bindings sites on the membrane are blocked for 1 h in Western blocking buffer.
3. Rinse once in PBS and twice in Western washing buffer.
4. Incubate in 0.1–2 µg/mL of specific antibody in Western washing buffer containing 0.5% (w/v) milk powder for 1 h at room temperature. The sensitivity may be increased by incubating overnight at 4°C. However the background may also increase.
5. Wash three times for 5 min in Western washing buffer.
6. Incubate with the second antibody (e.g., antimouse HRP-labeled secondary antibody for primary MAbs) in Western washing buffer containing 0.5% (w/v) milk powder for 1 h at room temperature.

7. Wash six times for 5 min in Western washing buffer.
8. Develop in ECL reagent for 1 min, and expose to X-ray film (*see* **Note 16** for alternative methods).

3.8. Antibody-Labeling Method

An alternative to ECL is the use of radiolabeled probes, such as ^{125}I labeled protein A or second antibody raised against the detecting antibody, which are also commercially available (*see* **Note 17**).

1. Two holes are made in the bottom of a 1.5-mL Eppendorf tube with a 19-gage needle, and the bottom is plugged with a small piece of nylon wool. The Eppendorf is then filled with 1 mL of swollen G25 Sepharose resin.
2. The "column" is washed with 10 mL of PBS containing 10 mg/mL BSA. The column is finally washed with 2 mL of PBS containing 25 m*M* KI, which has been freshly prepared. Dissolve 1 mg Iodogen in 1 mL of $CHCl_3$ and pipet 4 µL of the solution into a 1.5-mL Eppendorf tube, and evaporate to dryness.
3. Add about 30 µg of antibody in 200 µL PBS to the Iodogen-coated tube with 400 µCi of ^{125}I at room temperature for 10 min.
4. 200 µL of the labeled antibody are then loaded onto the G25 Eppendorf "column" and centrifuged at 170*g* for 1 min in a Falcon 2063 test tube containing 20 µL of 10 mg/mL BSA. The labeled antibody can be stored at 4°C for up to 3 wk, and is used at 5×10^5 cpm/mL in Western blocking buffer containing 1 mg/mL of unlabeled antibody.

Prior to autoradiography, the gel must be dried onto 3MM paper under vacuum at 60°C for 2 h, after fixing for 30 min. The gel is then exposed to X-ray film with an intensifying screen at −70°C (**Notes 18** and **19**).

4. Notes

1. A 20-µmol aqueous solution of PMSF at pH 8.0 has a half-life of 35 min, and therefore, this inhibitor must be added just prior to use ***(12)***. The inclusion of 10 m*M* iodoacetamide is also useful to prevent crosslinking of proteins owing to the presence of free sulfhydryl groups during immunoprecipitation.
2. The dissociation buffer is usually made reducing by the addition of 0.1 *M* dithiothreitol or 1.4 *M* β-mercaptoethanol prior to boiling, under which conditions disulfide-linked chains will become dissociated. Such reagents maybe omitted, and this may be useful in immunoprecipitation techniques where the immunoreactive Ig chains in their reduced form (25 and 50 kDa) obscure the antigen in question.
3. The way cells are lysed has a critical effect on the composition of the extract ***(13,14)***. Nonionic detergents, such as Nonident P-40 (NP-40), solubilize antigens in their native state and preserve the nuclei in an intact state, so that they may be removed by centrifugation. Mild detergents such as CHAPS, digitonin, and Brij 96, enable macromolecular complexes to be immunoprecipitated allowing protein associations to be investigated. However, the most widely used and versatile

immunoprecipitation buffer is RIPA. This lysis buffer is often the best to use when little is known about the solubilization behavior of the antigen in question.

4. Strong ionic detergents, such as SDS, denature the antigen, and so can be used with antibodies that are considered to be "nonimmunoprecipitating" antibodies. Such antibodies often include antipeptide antibodies, since they are usually generated against linear epitopes, which may be masked by the tertiary structure of the native protein. Thus, SDS denaturation of the protein allows the masked epitope to be exposed and accessible to the antibody: to do this, lyse the cells in 10 vol of 2% SDS, 50 m*M* Tris, pH 7.5, and place in a boiling water bath for 10 min. The DNA is then sheared by passing through a 21-gage needle several times, or by using a probe sonicator with several short bursts. Pellet the insoluble material by centrifugation at 10,000g for 10 min, and then dilute the supernatant 20-fold in PBS containing 2% (w/v) BSA.
5. Antibodies or antiserum can be added directly to the cell lysate at 4°C for 1 h or overnight. It is often worth trying both procedures, since the former usually leads to cleaner immunoprecipitates, but if the antibody is of low affinity, the latter may be necessary to achieve optimum binding to antigen. The antibody, including the antibody/antigen complexes; is then recovered using a second antibody against the first, although it is far more usual to use protein A or protein G linked to Sepharose beads. These reagents provide high-affinity binding to the Fc component of the antibody, and make washing and recovery of the antigen very easy (*see* **Note 11**).
6. Most MAbs or polyclonal antibodies used in immunodetection procedures are either mouse or rabbit antibodies. With the exception of mouse IgG subclass 1, all these antibodies can be coupled to protein A-sepharose, allowing maximal correct orientation of the antibody for immunoprecipitation. The use of the crosslinker dimethyl pimelimidate ***(15)*** also means that the antibody remains attached to the protein A beads during electrophoresis producing "cleaner" Western blots.
7. The percentage attachment of the antibody can be determined by measuring the optical density at 280 nm before and after coupling. If the coupling is low, then either protein G-sepharose or high-salt coupling can be used; for high-salt coupling, the first step of the low-salt coupling procedure is replaced by washing the protein A-sepharose beads three times in PBS, 3 *M* NaCl, pH 9.0, and then add the antibody for 2 h at 2 mg/mL in the same buffer.
8. Immunoprecipitation can also be used in combination with radiolabeled compounds in order to study protein modifications, such as phosphorylation, or the study of newly synthesized proteins (by ^{35}S-methionine incorporation). The addition of radioisotopes to cell-culture media to follow protein modification of embryonic cell lines is routinely performed, and could be adapted to incubating, over short time spans, early embryos or dissected tissues from fetuses. Experiments have been performed on early embryos for ^{32}P ***(16)***, and ^{35}S-methionine labeling of proteins ***(17)***. Microinjection of radiolabeled compounds into embryos has also been tried, for example, the labeling of proteoglycans with [^{35}S] sulfate ***(6)***.
9. From early stage embryos, moderately abundant proteins can be detcted by immunoprecipitation, but not by simple staining procedures, such as with Coomassie

blue or the more sensitive silver-staining technique. As a guideline, immunoprecipitation has been used to detect C-cadherin using two 48-cell embryos and subsequent separation by SDS-PAGE and immunoblotting using an ECL detection system (***18,*** and *see* **Subheadings 3.6.** and **3.7.** for details of techniques). However, between 230 and 310 eight-cell mouse embryos were needed to detect E-cadherin by the same method ***(16)***. In general, the amount of material needed for this type of experiment is really a matter of trial and error. The affinity of the antibody is also a major factor with affinities of > 10^7/mol required to immunoprecipitate antigens efficiently.

10. A preclearing step is not essential, but usually leads to more specific immunoprecipitation. For clean immunoprecipitates, which is especially important if radio-labeled antigens are being immunoprecipitated, an extra preclearing step is required. This is usually with nonspecific antibody coupled to protein A-sepharose for 1 h at 4°C, i.e., mouse immunoglobulins in the case of MAbs.
11. The use of a 100 mL Hamilton syringe with a very narrow bore and blunt end (point style 3) allows the liquid to be removed without any beads.
12. This can only be considered to be a very brief description, and more comprehensive reviews are available elsewhere ***(19)***. A 12.5% polyacrylamide gel is suitable for proteins between 12 and 45 kDa, whereas a 7.5% gel is suitable for 50–150 kDa proteins. Gradient gels allow the separation of most proteins of interest, and can be poured by mixing equal volumes of 5 and 15% acrylamide solutions in a gradient maker.
13. As guide to running conditions that will depend on the apparatus used: 4 h at 140 V, or 16 h at 35 V. These conditions will also vary depending on the size and percentage of the gel.
14. A more sensitive staining method is to use silver, and there are commercial kits available based on the method of Sammons et al. ***(20)***. However, staining is not usually appropriate for immunoprecipitates that invariably are associated with radiolabeling or immunoblotting detection procedures.
15. The semidry blotting technique gives a higher yield of transfer and is quicker. However, the small buffer volumes used can sometimes lead to asymmetric transfer of proteins across the gel, and it is also possible to "overblot" where the protein passes through the blotting membrane. For semidry blotting, the membrane can either be nitrocellulose or specially developed membranes, such as Immobilon P (PVDF, Millipore). In the case of PVDF membranes, they should be "activated" for a few seconds in methanol and then washed in transfer buffer, whereas nitrocellulose membranes should just be washed in transfer buffer. Wet blotting is often more effective when a PVDF membrane is used with a transfer buffer composed of 10 m*M* CAPS (Sigma), pH 11.0, and the transfer is carried out at 0.3 A, 60 V at 4°C overnight.
16. Colorimetric development of immunoblots probed with phosphatase and peroxidase-labeled antibodies is possible. However, these procedures are relatively insensitive, the color can fade, and they are generally less versatile than other techniques. Electrochemi-illuminescence (ECL) is just as easy to perform and gives a

permanent record in the form of an autoradiograph, the exposure time is relatively short, and the length of exposure can be varied in order to produce the correct intensity of band. ECL reagents are readily available in kits, and therefore, the technique will not be described here.

17. Immunoprecipitation is a sensitive technique, especially when it is combined with detection by ^{125}I-labeled antibodies. Labeling the first antibody with ^{125}I produces a more specific reagent and, therefore, cleaner immunoblot, although it may be less sensitive, since it does not have the amplification of signal (and background), which occurs with methods incorporating another layer in the detection system.
18. The autoradiograph produced may be intensified by the use of fluorography (^{3}H by 1000-fold, and ^{35}S by 5- to 10-fold), and can be performed using commercially available products, such as Amplify (Amersham), or by the following method: after fixing, the gel is rinsed twice in DMSO for 45 min, and then in DMSO plus 22% (w/v) diphenyloxazole (PPO) for 45 min. The gel is finally rinsed under a stream of water for 1 h and dried before autoradiography.
19. In recent years, the general availability of phosphoimagers has replaced autoradiography and fluorography as a detection system for radiolabeled proteins. It has several advantages, including a linear response over a wide range of activities, and in conjunction with the appropriate software, can be used for easy quantitation for comparative studies.

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Microscopy and Photomicrography Techniques

Richard J.T. Wingate

1. Introduction

The study of embryology relies heavily on visual analysis by microscopy. Many experimental procedures are carried out under either dissection or compound microscopes, and photomicrographs may often constitute the principle data in a given study. Most researchers are therefore familiar with achieving an adequate illumination, resolution, and contrast in observing or manipulating specimens. However, when faced with a choice of objectives, contrast enhancement optics, film types, and image processing options, high image quality cannot be realized by trial and error. Achieving the highest optical performance for photomicrography requires that a variety of options within the microscope are optimized with respect to the preparation of the specimen. Photomicrographic reliability and quality also rely on knowledge of either digital image acquisition or the fundamental properties of film.

This chapter is not intended as an academic consideration of how image quality is limited by optical and photographic constraints, but rather as a pragmatic guide to basic microscopy for embryologists. It can only supplement the essential technical information contained in every microscope instruction manual.

1.1. Stereo Dissection Microscopes

Stereo dissection microscopes have low-magnification lenses with long working distances, which give good depth of field but poor resolution. Each eyepiece is effectively views the specimen through a discrete microscope tube to give a three-dimensional quality to the observed specimen. When a camera is attached, the image is collected through only one eyepiece. For photomicrography, achieving adequate illumination is often more difficult than for slide mounted specimens. Nevertheless, when dealing with whole embryos and live specimens, stereo dissection microscopes equipped with a digital or

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film camera and, increasingly, with epifluorecent light sources for observing fluorochromes have become an important tool for data collection. For general observation, there are no rigid principles. Lighting is a matter of personal preference and depends on the specimen. However, where live specimens are being observed, "cold" light sources (where the lamp is remote and the light transmitted to the specimen by fiber optics) are preferred to lamps integrated into the microscope stand (which generate heat). Many stereo dissection microscopes are also equipped for transillumination (light shining through the specimen) and transparent Sylgard- (*see* **section 2.1**) coated dishes have therefore generally replaced wax as a substrate for pinning out specimens. For photomicrography, translucent agarose-filled dishes provide a less rigid support for the specimen, but give a more diffuse background light (*see* **section 2.2**). Optimizing photomicrography usually requires maximizing illumination, and in many respects, the principles applied for compound microscopes are identical for dissection microscopes (*see* **section 4**).

1.2. Compound Microscopes

Modern research compound microscopes can appear to be bewilderingly complex, and yet the principles underlying their use are constant, despite various configurations of optical components. The most familiar form of the compound microscope is the upright configuration, where objectives are sited above the specimen stage and transillumination originates beneath the slide. Inverted compound microscopes reverse this arrangement to allow, for example, wet preparations to be viewed on a stable specimen stage. Both inverted microscopes and "fixed-stage" upright microscopes are common platforms for experimental procedures carried out under high magnification. For photomicrography, the standard-format compound microscopes may have an integrated camera system or a connecting tube mounted on a trinocular head with fittings for video, digital cameras, or a film camera body.

Various factors affect the quality of image that can be achieved. The way in which the specimen is prepared and mounted influences the type of objectives that can be used and which type of contrast enhancement filters are appropriate.

2. Materials

1. Sylgard (Dow Corning, Barry, South Glamorgan, UK): This is made up nine parts to one with a hardener. Sylgard dissolves in solvents, such as histoclear and xylene.
2. Agarose-filled dishes: Use 1% agarose in distilled water. Heat to >60°C to dissolve and cool to set.
3. Cleaned coverslips: Wash in 70% ethanol/1% concentrated HCl and then 2% (TESPA) 3-aminopropyl- triethoxysilane/3-(triethoxysilyde)-propylamine for 10 min. Rinse in acetone for 10 min, then distilled water before drying.

4. Gelvatol (Monsanto Chemicals, Knowsley, UK)–Mowiol (Hoechst, AG, Frankfurt, Germany): Use 2.4 g Mowiol to 6 g of glycerol. Stir to mix. Add 6 mL water and leave for several hours at room temperature. Add 12 mL of 0.2 M Tris buffer (pH 8.5), and heat to 50°C for 10 min to dissolve. For fluorescence, add 1,4-diazobicyclo-(2.2.2)-octane (DABCO).

3. Methods

3.1. Specimen Preparation

Different techniques require that tissue be fixed and processed in a variety of different ways. This, in turn, influences how slides are prepared for compound micoroscopy. For example, different kinds of mounting media require different optical considerations. In particular, the refractive index of the mountant affects the spatial resolution that can be achieved for a given objective.

3.1.1. Whole-Mount Specimens

Particular care must be taken in mounting relatively large, intact or partially dissected embryos under a coverslip. Although such material may be photographed through a dissection microscope, mounting a specimen under cover glass and in a mountant with a high refractive index gives better spatial resolution. It may be desirable to clean slides and cover glass prior to use (*see* **section 2.3**) and ensure that the thickness of cover glass is exactly 0.17 mm, where optimal resolution or transmission of, in particular, fluorescent light is required (*see* **section 3.2.6**). Whole-mount specimens are mounted under raised coverslips, which use small drops of silicon grease at each corner of the coverslip as supports. The coverslip is thus held above the specimen, and its final height can be adjusted by carefully applying downward pressure. Without silicon grease supports, delicate embryonic tissue is easily flattened. Excess mountant can be removed using absorbent Postlip paper (Hollingsworth & Vose Co., Winchombe Glos., UK). If a nonsetting mountant (e.g., glycerol) is used, then the edges of the coverslip can be sealed by painting a heavy layer of clear nail varnish around the edges of the glass.

Even with the support of a silicon grease plinth, applying too much downward pressure to the specimen is potentially disastrous. Therefore, cleaning the cover glass above a whole-mount preparation requires care. Dust and grease can be removed by dragging a piece of lens tissue, which has been moistened with a drop of 70–100% alcohol, across its surface.

3.1.2. Mountants

The choice of mountant is influenced by whether the specimen is dehydrated and affects not only the permanence of the preparation but also the clarity of the image. Embryos that are small and virtually transparent can be fixed whole or sectioned and cleared with glycerol–PBS, which itself can be used as a mountant. Glycerol remains liquid and cannot be regarded as a reliable

permanent mounting medium. By contrast, mountants that set to form a solid matrix are not only more stable over time but also allow the use of oil-immersion objective lenses, which give optimal visual resolution. Oil-immersion lenses should not be used on anything other than permanent preparations (*see* **section 3.2.4**).

1. Nonpermanent preparations: A convenient mountant, which also clears delicate whole mounts, it is a mixture of 90% glycerol/0.02% azide in PBS (*see* **Note 1**). For fluorescent specimens, 2.5% DABCO (1,4-diazobicyclo-[2.2.2]-octane) can be added to this mixture to prevent fading. Stains that use FITC as a fluorochrome should be mounted in a medium with a pH of at least 8.5. For a more durable preparation, the cover glass can be sealed with nail varnish.
2. Permanent preparations: Water-soluble permanent mountants (i.e., which set) usually suffer from shrinkage, which can ruin a specimen. The more viscous they are, the less of a problem this is likely to be. However, viscosity makes the mountant hard to handle. Probably the best aqueous, setting mountant is Gelvatol (Monsanto)/Mowiol 4–88 (Hoechst), which hardens overnight and can be stored as frozen aliquots (*see* **section 2.4**).

Some tissues can be cleared adequately only by xylene or its less toxic equivalent, histoclear (National Diagnostics, Hessle Hull, UK), having first been dehydrated through an alcohol series. Some counterstains, such as cresyl violet (Nissl), require that the tissue is dehydrated to xylene or histoclear. Tissue cleared in this way can be mounted in DePex or, if fluorescent, in Fluoromount (Sigma), both of which set solid over time.

3.2. Choice of Objectives

A typical compound microscope is set up with a range of objectives of increasing magnification, all of which are approximately parfocal (*see* **Note 2**) (a standard range would be ×2.5, ×10, ×25, ×40, ×60, or ×100). *Parfocality* means that it is possible to switch objectives without adjustment to the focus. Where eyepieces have an adjustable focus collar (diopter adjustment), parfocality also depends on whether the diopter of the eyepieces has been correctly set (*see* **Note 3**). Low-power objectives usually have a longer working distance between the specimen stage and objective lens. An exception being long-working distance (LWD or ELWD) high-power objectives, which may be used to monitor intracellular dye injection on upright microscopes. Therefore, it is always advisable to bring the specimen into focus with a low-power objective first, moving subsequently to higher magnifications and shorter working distances. Apart from magnification, lenses vary in a number of optical parameters, which are engraved on the side of the objective (**Fig. 1**). It is important to ensure that an appropriate objective is being used with respect to the microscope and the specimen preparation. The following features are usually described.

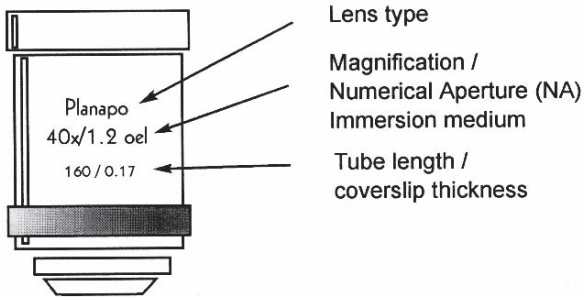

Fig. 1. Objective lenses have information engraved on their casing that gives details of their correct use. The various features that might be described are explained in **Subheadings 3.2.1.–3.2.6**.

3.2.1. Lens Type

Lenses vary in the degree to which they are corrected for spherical aberration and wavelength transmission. With lower degrees of correction and, therefore, a lower quality, color fringes may appear at the edges of the image under certain lighting conditions (reflecting residual spherical aberration), and some anomaly may be perceived in the depth of objects of different colors within the sample.

1. Achromatic: Spherical aberration corrected for one color only, whereas chromatic aberration is corrected for two colors. Green or yellow/green illumination reduces this residual aberration. Because of the incomplete color correction, photomicrographic images may not match the visually perceived image.
2. FL (semiapochromatic–fluorite): Well corrected for chromatic aberration, but may have a slight curvature of field.
3. Apo (apochromatic): Corrected for red, green, and blue spectra. This allows higher numerical apertures (NA) to be achieved, optimizing resolution and color transmission for photomicrography.
4. Plan (plan achromatic): Corrected for spherical aberration and, therefore, producing a flat visual field but with minimal color correction.
5. Plan Apo (plan apochromatic): Corrected for spherical aberration and spectral continuity. These objectives may contain up to 15 separate lenses, as reflected in their price. For black-and-white photography and under the correct lighting conditions, a Plan lens may equal a Plan Apo lens in resolution. However, for color photomicrographs, the latter is preferred.
6. Fluor–neofluar: Optimized for transmission of UV epifluorescence. Unless lenses are corrected within the UV range of the spectrum, the fluorescent image and light image are out of focal register. Fluorescent samples usually look significantly worse with any other kind of objective.
7. Other markings; for example, LWD, ELWD: Long and extra-long working distance. Ph indicates an objective with a phase plate, which is used for

phase-contrast microscopy (*see* **section 3.5.1**). The code matches a particular phase setting on the substage condenser.

3.2.2. Magnification

The magnification engraved on the side of the lens is an approximation. The discrepancy between the nominal magnification and the actual power of the lens may be up to ±×0.3. This variability may be important in the calibration of some computer-based imaging systems (such as confocal microscopes), where images are usually scaled according to the nominal magnification factor, unless otherwise corrected.

3.2.3. Numerical Apertures

The numerical aperture (NA) of the lens is engraved beside or beneath the magnification and reflects the optical quality of the lens and its working distance. The NA depends on the refractive index of the medium between the lens and the specimen (*see* **Note 4**) and, therefore, is greater for immersion lenses (*see* section 3.2.4.). Higher values also entail shorter working distances, greater light-gathering capacity, and give a higher spatial resolution (*see* **Note 5**).

Depth of field is inversely proportional to the square of the NA of the objective. Therefore, for a given magnification, if a greater depth of field is required, for example, when scanning a specimen, high-power eyepiece lenses can be combined with a lower-power objective, which have a smaller NA. Greater depth of field can also be produced by closing (stopping down) the aperture diaphragm of the condenser lens (*see* section 3.4.1.).

3.2.4. Immersion Medium

Unless otherwise marked, objective lenses require no immersion medium. Immersion media improve the NA of the lens, specifically allowing values >1 to be achieved. Immersion lenses are usually optimized for one particular immersion medium (immersion oil [oil, oel, H.I.], glycerol or glycerin, or water [W.I., WAS]), a drop of which is place between the objective front lens and the coverslip. Some objectives have a rotating collar, which compensates for the refractive index of different media (air = 1.00, water = 1.33, glycerol = 1.47, oil = 1.52).

The density of the immersion fluid should be as closely matched as possible to the density of the mountant. For example, tissue mounted in a permanent mountant, such as DePex, should be viewed using oil immersion lenses *(see* **Note 6**). Because the working distance and depth of field of such lenses are small, considerable care must be taken in advancing the objective toward the specimen. It may not be possible to resolve through the depth of some particularly thick specimens. Many lenses are spring mounted to cushion any impact, but contact with the coverslip should be avoided. It is always advisable to focus on the specimen with a parfocal low-power objective before changing to a high-power objective.

3.2.5. Tube Length

Most modern microscope systems have an effective infinite mechanical tube length ("infinity corrected optics" as designated by ∞). These are more comfortable for observation and also allow optical components to be inserted into the light path (such as fluorescence filter blocks) without disturbing the positioning of the final image. Older microscopes have an overall tube length that is conventionally 160 mm (occasionally 170 mm). Appropriate objectives for a given mechanical tube length should be used.

3.2.6. Coverslip Thickness

Most lenses are corrected for a cover-glass thickness of 0.17 mm (coverslip # 1½), and many objectives have this figure engraved on their casing. Some objectives (such as those used for metallurgy) may be marked with a 0 or NCG, designating no cover glass, or a dash (-), indicating either with or without a cover glass. For high-quality dry objectives, a correction collar may be included to compensate for variations in glass thickness (as much as ±0.3 mm). This collar can be adjusted to maximize the contrast in the specimen. For fluorescence, coverslip thickness should fall between 0.15 and 0.19 mm.

3.3. Light Source and Field Diaphragm

The lamps for both bright-field (tungsten or halogen light) and fluorescent light sources must be correctly centered and focused to achieve optimal imaging. For the most basic of microscopes, with a reflecting mirror under the substage condenser, the light source is focused directly onto the specimen ("critical illumination"). For the majority of research microscopes, which are designed for Köhler illumination (*see* **section 3.4.2.**), the filament image is focused onto the aperture diaphragm of the substage condenser by a collector lens system, which produces a homogeneous illuminated field. Apart from a variable control on the voltage passing to the light source, the field of view illuminated by the lamp is regulated by the field diaphragm, which is situated before the substage condenser in the light path (**Fig. 2**).

For epifluorescence, the image of the filament is focused directly onto the specimen (critical illumination). On some fluorescence microscopes, the images of the filament and a second reflected, virtual image can be viewed and aligned on a focusing window. On others, an image of the lamp can be focused by placing a piece of paper where the slide would be and observing the lamp directly. As with tungsten–halogen light sources, the field of illumination can be regulated via a field diaphragm. Since fluorescence may fade rapidly, it may be desirable to use the field diaphragm to reduce the scatter of light outside the area of interest. Although lamps vary, mercury vapor bulbs, once lit, should burn for at least 30 min and should not be reignited for 30 min after they are switched

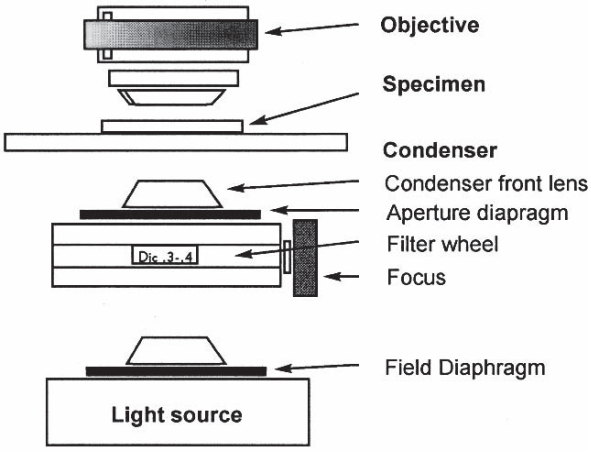

Fig. 2. The standard arrangement of light source, substage condenser, and specimen stage on a microscope designed for Kohler illumination. Two separate diaphragms, at the light source (field diaphragm) and on the condenser (aperture diaphragm), regulate the passage of light to the specimen.

off. Each time the lamp is switched on and off, approximately 2 h are lost from the life-span of the lamp.

3.4. Substage Condenser

The substage condenser contains a lens complex, an aperture diaphragm, and may also house prisms and filters used in contrast enhancement. The condenser can be focused to optimize the illumination of the specimen. The aperture diaphragm works independently of the field diaphragm to optimize contrast, depth of field, and resolution by altering the NA of the condenser lens (**Fig. 2**). The maximal NA of the condenser lens is usually engraved on its side. On some condensers, a swung-in lens can be used to alter the power of the condenser lens according to the choice of objective lens. Stopping down the aperture diaphragm reduces the amount of light reaching the specimen but, unlike the field diaphragm, does not produce vignetting when the specimen is viewed through the microscope eyepieces.

As with objectives, the lenses in the condenser can be optically corrected in various ways, although this is not of primary importance for the resolution of the final image. Similarly, although it is possible to use immersion oil on virtually any high NA condenser lens, gains in image quality may be negligible.

3.4.1. Focusing the Condenser–Köhler Illumination

Bringing the condenser into focus is simply a matter of bringing the edges of the field diaphragm into sharp focus (**Fig. 3A**).

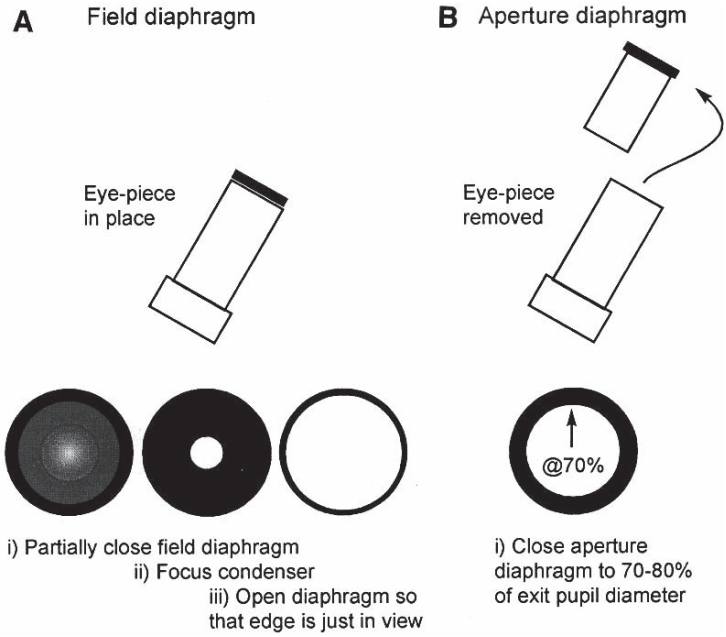

Fig. 3. The steps involved in focusing the condenser, optimizing the field diaphragm (A) and the condenser aperture diaphragm (B) (*see* **Subheading 3.4.1.** and **3.4.2.**). Schematic views through the eyepiece for each step are shown below.

1. Focus the objective lens so that the image of the specimen is sharp through the eyepiece.
2. Where the condenser has a range of front lenses and, particularly, if it has a range of differential interference contrast (DIC) prisms, check that the NA marked on the condenser lens and on the DIC adjustment collar matches that of the objective.
3. Close the field diaphragm so that its edges are in view, and center the circle of illumination with adjuster screws on the substage condenser.
4. Adjust the focus of the condenser such that the edges of the field diaphragm are sharp.
5. Open the field diaphragm to illuminate the field of interest but minimize the amount of stray light. The edges of the field diaphragm should remain just inside the field of view.
6. Repeat this procedure every time an objective is changed.

3.4.2. Aperture Diaphragm Adjustment

This adjustment matches the NA of the condenser to that of the objective, optimizing the cone of light that passes through the specimen (**Fig. 3B**).

1. Focus the condenser as previously (*see* **section 3.4.1**).
2. Remove the eyepiece and ensure that the field diaphragm is sufficiently open to fully illuminate the field of view.

3. Close the aperture diaphragm on the substage condenser so that its radius is 70–80% of the illuminated field (the "exit pupil" of the objective). Screening off the outer 20–30% of the exit pupil gives an approximate match between the NAs of condenser lens and objective lens.
4. Replace the eyepiece.
5. Repeat this procedure every time an objective is changed.

The 20–30% screening of the exit pupil is a "rule of thumb," and the final adjustment of the aperture diaphragm alters image quality in various ways. As the aperture diaphragm is closed, resolution decreases, but both the contrast and the depth of field increase (*see* **Note 7**). As stated already, there should be no vignetting when the aperture diaphragm is adjusted, only a uniform variation in lighting intensity.

3.5. Contrast Enhancement: Phase Contrast, DIC (Nomarski), Dark Field

The principle of all contrast enhancement systems is to turn otherwise invisible variations in material (density, polarizing ability, and so forth) into differences in perceived light intensity. For embryological specimens, some kind of contrast enhancement is usually extremely useful. For example, with fluorescent preparations, standard counterstains may either quench fluorescence or be autofluorescent themselves. On occasion, however, a particular contrast enhancement may reduce the information within an image; for example, Nomarski optics may make small labeled objects (such as cell nuclei) less easy to see. It is always worth viewing a specimen both with and without contrast enhancement before photographing. The use of three common image enhancement systems is described next.

3.5.1. Phase Contrast

Phase contrast converts differences in the refractive index or thickness within an otherwise transparent specimen into differences in light intensity. This is achieved by illuminating the specimen with a ring of light by means of a phase "annulus" within the substage condenser. The ring of illumination is aligned with a complementary phase plate within the back of the objective. Since these elements must be in register, only objectives of the same geometrical characteristics can be used with a given phase annulus. The phase condenser may therefore contain a number of different annuli for different objectives. The phase annulus (in the condenser) and phase plate (in the objective) are aligned by means of either a focusing telescope (which replaces the eyepiece) or an Amici-Bertrand lens that can be swung into the light path. Phase-contrast optics should be used with thin specimens.

3.5.2. Differential Interference or Nomarski Contrast

Differential interference contrast (DIC) can be used with both thin and thicker specimens to generate a "pseudorelief" image of the preparation. DIC filters enhance local gradients in optical density by prismatically splitting then recombining polarized light. From the lamp, light passes first through a polarizing filter, then a "Wollaston" prism within or beneath the condenser. Having passed through the specimen, light is refracted by a second Wollaston prism (usually mounted just behind the objective) and filtered by a second polarizing filter, the "analyzer" filter situated closest to the eyepieces. The analyzer is easily swung in or out and should always be removed for fluorescence imaging, since it reduces the amount of light passing to and from the slide. The polarizer can be rotated to optimize the DIC effect. For good DIC, the field and aperture diaphragms should be optimized for Köhler illumination (*see* **sections 3.4.1. and 3.4.2 and Fig. 3A and 3B**).

Since this system utilizes polarized light to generate changes in light amplitude, naturally polarizing media, such as mica and Plexiglas, may generate optical artifacts when placed in the light path. In addition, some biological materials, for example, structures consisting of parallel oriented fibers, are also inherently polarizing or "birefringent." With only the polarizer and analyzer filters of the DIC system present, birefringent structures therefore appear as either very bright or very dark, if oriented at 90° to their inherent optical axis. Birefringence may be useful in identifying particular kinds of structures within a specimen.

Digital photomicrography with polarized light requires additional care. The chip in the camera comprises a gridded array of detectors, which effectively impose a polarizing filter on the image. With the analyzer removed and only the polarizer in place, an observer does not detect a DIC/Nomarski effect through the eyepieces. However, a digital camera may still act as an analyzer and generate a pseudo-relief image (until the polarizer is removed).

3.5.3. Dark Field

Dark-field or oblique illumination is used to observe dark, particulate staining or, for example, darkly labeled axons against a homogenous bright-field background. By producing what is effectively a negative image of the specimen, such features become clearer as bright objects against a dark background. The condenser directs an oblique illumination through the specimen, and the objective lens receives light only if it is reflected or refracted by opaque objects in the illuminated field. Where parts of the specimen are transparent, light passes uninterrupted through object space and does not enter the objective. Darkly stained particles or fibers within the specimen interrupt the light path and appear as bright objects.

Certain stains are considerably clearer under dark-field illumination, even though there is no real gain in spatial resolution. However, specimens have to be carefully prepared: The image is impaired if tissue is not well cleared (the background does not appear black) or if the slide is contaminated with grease or dust (which is visible as very bright points of light).

3.6. Fluorescence Microscopy

Fluorescent labeling of specimens offers numerous advantages, not least of which is the use of multiple labels linked to different fluorochromes on the same preparation. Fluorescent specimens are conventionally viewed on microscopes equipped with an epifluorescent light source (for epi-illumnation, light is both projected and detected through the objective lens) and an appropriate system of excitation and emission filters and dichroic beam-splitting mirrors. These ensure that a given fluorochrome is excited at a wavelength that is distinct from the resulting fluorescence emission. Where two or more fluorochromes are used, filtering can be optimized to ensure a minimal overlap between the emission spectra of each label.

3.6.1. Choice of Fluorochromes

In most experimental situations, a choice of fluorochromes is available. Where two fluorochromes are used in the same specimen, it is important that their fluorescence signals be clearly distinguished (for example, different populations of dye-labeled axons or different immunolabels). It is important to compare the specifications of the excitation and emission filters with the absorption and emission spectra of the fluorochromes chosen. Other factors that should be taken into account are the brightness and photostability of a particular fluorochrome. Some fluorescent dyes survive fixation better than others. For example, for intracellular dye injection, the highly fluorescent 5,6-carboxyfluorescein cannot be fixed, whereas the relatively less bright lucifer yellow can be cross-linked by paraformaldehyde fixation.

3.6.2. Sample Preparation

Some special conditions should be noted for fluorescent samples. Glutaraldehyde is autofluorescent, and so its use should be avoided. If there is no alternative to this fixative, its autofluorescence can be countered by treatment with $NaBH_4$. Dyes may be pH sensitive; for example, FITC fluorescence yield is significantly reduced if the pH of the mountant is <8.5–9.0. Some counterstains are autofluorescent (e.g., Feulgen and Nissl stains). Fluorescent latex beads, which can be used as axonal tracers or as implants for the slow release of a variety of soluble factors, are dissolved by alcohol and xylene (*see* **Note 8**). In general, background fluorescence increases with time stored. Background can

be reduced by keeping tissue at 4°C, but it is advisable to view and, in particular, photograph fluorescent material as soon as possible. Permanent, nonaqueous mountants tend to transmit more light than glycerol-based mountants, decreasing photographic exposure times and hence the risk of photobleaching.

3.7. Digital Photmicrography

Since the first edition of this book, digital cameras have largely superceded film photography for reasons of both cost and convenience. The advantages of "what you see is what you get" offered by a digital approach are self-evident, and the ease of achieving a good digital photograph depends on the idiosyncrasies of software packages. A reasonably pragmatic approach when encountering a new digital camera or software package is to start with settings restored to their default values, rather than accept definitions carried over from other users. A few general practical considerations can be applied across most formats of digital camera. These foolow.

3.7.1. Optimize the Microscope to What the Camera Sees

Digital camera software allows a "live" view of the specimen that may be of a lower resolution and higher refresh rate than a captured still image. The microscope must be aligned and focused with respect to the camera rather than the eyepieces. This includes condenser adjustments for Köhler illumination (see **section 3.4.2**) and special considerations with polarized illumination (*see* **section 3.5.2**).

3.7.2. Noise and Blur Increase at Low Light Levels

Digital cameras are subject to a background noise at the level of the detector array and its readout. Electronic noise becomes an issue at lower light levels, as the background "flutter" of pixel activation impinges on the real signal. This is a significant consideration in fluorescence photomicrosocpy but can also be a problem with bright-field illumination. Pragmatically, if the incident light is too low, captured images begin to appear grainy, as the camera struggles to amplify a small incoming signal confounded by background noise. Because, on many digital cameras, image brightness is automatically adjusted, the deterioration in quality may be the only clue that a specimen under a dissection microscope, for example, is not lit brightly enough.

3.7.3. Color Changes with Illumination Levels

Bright-field illumination uses white light to illuminate the specimen. Changing the level of illumination alters the color temperature of white light. While this is indistinguishable to our relatively sophisticated visual system, a digital color camera registers the orange hue of dimmer illuminations. As the brightness of

a lamp is increased, its color becomes more blue. For consistent color between images, it is therefore vital that color is calibrated against an empirically determined white background using the software each time the voltage passing through the tungsten lamp providing bright-field illumination is altered.

3.7.4. Pseudocolor Can Be Used to Indicate Saturation

Beyond the optical parameters dictated by the microscope, the sensitivity of the camera must be adjusted so that an image falls between the minimum and maximum detection thresholds of the device. Camera sensitivity is regulated by exposure time and gain. Unlike conventional film, exposure times are not a limiting factor on image capture unless the specimen is moving, unstable, or photosensitive. Gain can be used to compensate when exposure times are too short to capture sufficient light. Increases in gain amplify digital noise and can lead to a more blurred image. To set image brightness, many software packages indicate when a pixel has reached maximum or minimum brightness by indicating areas of saturation by pseudocolors. In addition, commonly, a histogram of pixel values shows whether the range of incident light falls neatly within the detection range of the camera. Maximizing the information content of the image involves ensuring that the image is mapped onto the full range of intensity values with minimal areas of saturation.

3.7.5. Gamma Adjustments Can Enhance Image Capture

In addition to defining a maximum and minimum brightness levels, the way in which intensity levels are mapped between these limits can be adjusted to enhance the image. By default there is a linear relationship between brightness and resultant pixel intensity (gamma = 1). Most software packages allow this relationship to be made nonlinear (for example, gamma adjustments, square root functions). This can boost the intensity of dimmer parts of the image with respect to bright maxima (gamma <1) or increase the contrast between bright and dark (gamma >1). In either case, gamma adjustments do not alter the degree of saturation in the image.

3.7.6. File Format Can Affect Image Quality

Digital images can be saved in a variety of different formats with differing amount of data compression. Although digital storage capacity is increasingly a negligible problem, data compression may still be an important factor in the electronic transmission of images. Reductions in file size using the “lossless” file formats (GIFF, TIFF, or PICT) do not result in information loss. The “lossy” compression algorithm used in JPEG formats results very effective compression of photomicrographs but degrades edge information within the image and so should be avoided in archiving primary data.

3.8. Confocal Laser Scanning Microscopy

The majority of confocal microscopes use a scanning laser beam light to excite fluorochromes within the specimen. Resultant emissions are recorded through a variable sized pinhole by a digital photomultiplier tube (PMT). This confocal optical arrangement ensures that light outside a precise plane of focus is deflected wide of the detection pinhole and does not pass to the collection array (an important practical consideration is that an out-of-focus image is simply invisible as opposed to blurred). The image collected thus represents a thin optical slice of the fluorescently labeled sample in perfect focus. Smaller detection pinholes give thinner optical sections (which may be consequently fainter). Importantly, thinner optical sections have a greater spatial resolution. While a single optical slice may represent a useful data set in itself., a complete image can also be assembled out of multiple optical slices in a "stack" or "z series," each of which is in perfect focus. A projected two-dimensional image of the stack contains no out-of-focus blur and has a close to optimal sharpness when the pinhole size is reduced at collection.

Confocal microscopy represents a specialized form of black and white (monochrome) digital photomicroscopy. Optical sections may be very faint and assessing appropriate pixel intensity levels relies on pseudocolor indicators of saturation maxima and minima. Most software packages allow user-defined pseudocolor lookup tables (LUTs) to be applied to confocal images and include a preset LUT that returns maximum (white) and minimum (black) intensity values as colors. As with other digital image capture, gamma adjustment is an important tool in enhancing confocal image (*see* **section 3.7.4.**).

A practical consideration in using laser illumination is the damage that illumination can cause in terms of photobleaching the sample or even phototoxicity in living preparations. For this reason, it is often advantageous to capture images with a camera set at high sensitivity and gain, thus allowing epi-illumination with a minimal amount of laser output. High-gain settings result in noisy images (*see* **section 3.7.2.**), but all software packages allow repeated confocal scans (of the same focal plane) to be averaged, thus eliminating random pixel fluctuations between scans (Kalman filtering). The number of averages required to achieve a "clean" image can be determined empirically by simply watching the live filtering of the scan. In terms of specimen preservation, a setting of low laser power–high gain–increased Kalman filtering is better than high laser power–low gain–less Kalman filtering.

3.9. Photomicrography with Film

Cameras used for photomicrography generally consist of either a standard single-lens-reflex (SLR) 35-mm camera body mounted via a tube to the microscope (which uses its own integral exposure meter) or a specially designed unit integrated into a dedicated photomicroscope (which generally has more sophisticated

exposure controls and may use either a 35-mm or larger format film). Despite the rise of digital photography, the resolution of film and the tonal range that it can represent may still be superior. Nevertheless, film has become marginalized because of the near inevitability that film images ultimately are digitized for publication and presentation, together with the cost and complexity of its use in microscopy.

One obvious difference between film and digital imaging is the lag between image capture and development. Film must be processed to reveal an image, and in the absence of an immediate what-you-see-is-what-you-get feedback, there are a number of additional considerations in photographic optimization when using film.

3.9.1. Focusing a Film Camera

Light from the specimen must be directed to the camera and focused onto the surface of the film. This usually involves redirecting or splitting the light path from the eyepieces to the camera tube and ensuring that what is in focus for the eyepiece is also in focus on the camera. Such parfocality cannot always be assumed for a microscope with a 35-mm camera attachment via a camera tube, and it is advisable to check that the image is correctly focused for the camera through the eyepiece of the SLR camera body. Photomicroscopes are designed so that camera and eyepieces are parfocal when the diopter of each eyepiece is correctly adjusted for the observer (*see* **Note 3**). This is usually achieved by focusing the crosshairs of a reticule placed or projected onto the eyepiece diaphragm. The diopter ring is adjusted such that the reticule image is sharp when the eyes of the observer are relaxed. The simplest way to relax eye muscles is to glance briefly at a distant object before focusing the crosshairs. When the specimen is focused, crosshairs should also remain sharply in focus. Particular care must be taken with objectives of a low magnification and especially with dissection microscopes, where the increased depth of field allows the observer's eyes to accommodate across a greater depth within the specimen. In such situations, a "focus telescope" may be useful to focus accurately on a specific optical plane.

3.9.2. Black and White Photography

Black and white offers certain advantages over color photography. Monochrome films are less sensitive to variations in exposure times and, in particular, are unlikely to suffer reciprocity law failure where exposure times are under 1 min (*see* **section 3.9.1**). Films vary in their speed (measured by ASA or DIN), reflecting their sensitivity to light: Generally, faster film speeds result in a coarser-grain image. Films may also vary in their gradation or contrast, which can be characterized by the γ of their emulsion. Within a reasonable exposure time, the response of

an emulsion to increasing exposure is linear. Gamma (γ) is defined as the tangent of the angle of the linear portion of this response curve (α) and is a useful guide to the contrast of the film. If $\gamma > 1$ ($\alpha > 45°$), then the emulsion has an enhanced reaction to changes in intensity and, therefore, a "hard" contrast. If $\gamma < 1$ ($\alpha < 45°$), then the film has a "soft" contrast. Emulsion may also react slightly differently to different colors of light. A panchromatic film hase an approximately even sensitivity across all wavelengths but may still be less responsive to deep reds. A selection of films used in black and white photomicrography follows:

T-Max 100 ASA (soft contrast).
Ilford FP4 (intermediate contrast).
Kodak Technical Pan 125 ASA (high contrast).
T-Max 400 ASA (for fluorescence).
AGFA Scala 200 ASA (reversal film for black and white transparencies).

Black and white photomicrography allows the use of color filters to optimize the contrast of objects within the specimen. Color filtering can be used to convert differences in color contrast into differences in perceived intensity. An absorption filter of one color removes wavelengths of the complementary color, deepening the contrast between such objects and the background. For example, a blue-green or cyan filter darkens the reddish-brown (DAB) product used in histological peroxidase reactions. The effects of different colors of contrast filters are shown in the following table (adapted from ***(1)***).

	An object stained:			
Filter used	Blue	Green	Yellow	Red
		becomes:		
Red	Very dark	Dark	Light	Very light
Green	Dark	Very dark	Light	Very dark
Blue	Very dark	Dark	Dark	Very dark
Cyan	Light	Light	Dark	Vert dark
Magenta	Light	Vert dark	Light	Light
Yellow	Vert dark	Light	Vert dark	Light

3.10. Color Photography

The most frequent problem encountered in color photomicrography is matching the colors recorded on film to those perceived by the observer through the eyepiece. In particular, the color of the photographic image depends on the color temperature of the light source. Tungsten filaments give off a reddish light, which is not obvious to the observer but is clear in photomicrographs taken with a standard "daylight" film. Films designed

for tungsten light sources (e.g., Fujichrome 64T) are therefore adjusted for a color temperature of 3,200 K. Some microscope lamps have a "PHOTO" or "3200 K" setting, which produces a standardized bright illumination for photography. Manufacturers may alternatively supply a color temperature versus voltage curve, which enables lamp intensity to be adjusted accordingly. The high color temperatures required for color constancy (usually equivalent to a lamp burning at its brightest) may demand impossibly rapid shutter speeds. Since altering the brightness of the lamp also changes the color temperature of the illumination, light intensity can be reduced only by placing neutral density filters in the light path. These absorb energy equally across the spectrum and can be used additively to achieve a reasonable photographic exposure time without changing color temperature.

Daylight films are adjusted for a color temperature of 5,500–6,000 K, and when used with tungsten illumination, a blue filter (Kodak Wratten 80A) can compensate for the orange cast of artificial lighting conditions (**Fig. 4A**). Daylight films should be used for color fluorescence photomicrographs (UV lamps have a higher color temperature), although unless two different fluorochromes are being photographed, black and white film generally gives better resolution and exposure reliability than color film (*see* **section 3.9.1.**). Typical films follow, all are color-reversal films (producing transparencies):

Fuji 64 T (for standard tungsten illumination).
Ektachrome 400 ASA (fluorescence).
Kodak P1600X (fluorescence).

When exposure times exceed much more than maybe 2 sec, the sensitivity of color-reversal films begins to change. Effectively the film's speed decreases with time, a phenomenon known as *failure of reciprocity* (the relationship between exposure and sensitivity). This can be corrected for by multiplying exposure times by the appropriate factor, and most photomicroscopes have a built-in facility to correct for reciprocity failure. By entering the appropriate reciprocity index for a given film, exposure times are automatically adjusted for long exposures. If necessary, reciprocity failure can be determined by trial and error for a given film type (increasing exposure 2× on successive exposures). Between 2 and 8 sec, there is also a shift in the sensitivity of the different color pigments within the emulsion. For example, as the green pigment

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Fig. 4. (*opposite page*) **(A)** Color temperature: The effect of a blue filter when a daylight film is used to photograph a specimen under tungsten illumination. The filter compensates for the pinkness of the illumination and increases the contrast. **(B)** Pseudocolor: Three examples of the use of pseudocolor with black-and-white digital images. (Top) A pseudocolor LUT is used to convert the gray scales within a "sharpened" confocal microscope image of a DiI-labeld

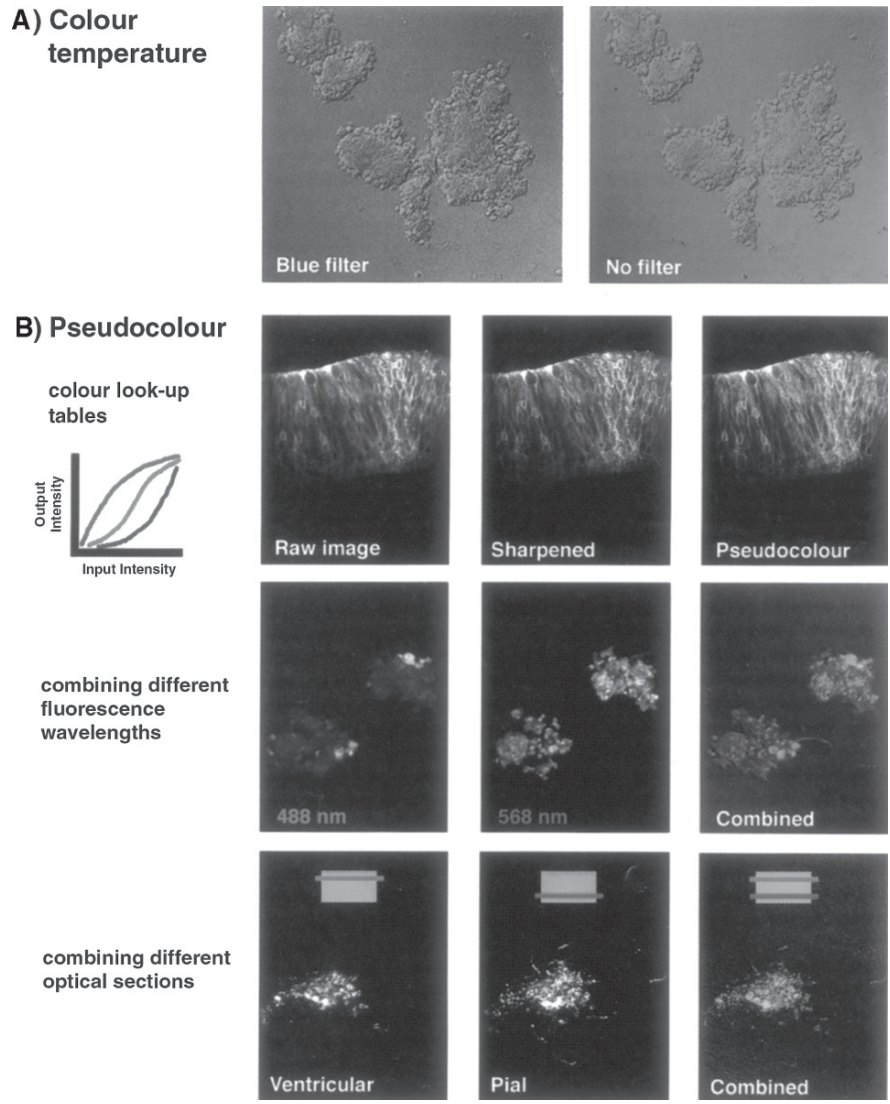

Fig.4. *(continued)* embryonic brain into changes in color complements. (Middle) Black- and white images of the same specimen illuminated for different fluorescent dyes are color-coded and merged to produce an image equivalent to a photographic double exposure. This image is then merged with a DIC picture of the same specimen. (Bottom) Confocal microscope optical sections are color-coded and merged to show the differences in cell dispersal at different depths in the tissue. Pseudocolor pictures were prepared within Adobe Photoshop from 8-bit 768×512 pixel image produced on a Bio-Rad MRC-600 confocal system mounted on a Nikon Diaphot inverted compound microscope. (*See Color Plate*)

becomes less sensitive, the film takes on a purplish tinge. Film manufacturers may supply details of appropriate color compensation filters within this exposure range, which can be used to correct shifts in color sensitivity. Beyond 8 sec of exposure, it is unlikely that any color filters can compensate entirely for the shifts in color balance ***(2)***.

3.11. Digital Image Manipulation

Having produced the optimal photomicrograph, computer software packages allow a range of further image manipulation. The superimposition of, for example, a fluorescence image and a DIC illuminated view of the same specimen, which might normally be achieved by a photographic double exposure, can be routinely composed within software programs, such as Adobe Photoshop (Adobe Systems Inc., Mountain View, CA). Further manipulation can involve spatial filtering to reduce noise or sharpen contrast; and perhaps most frequently, the pseudocoloring of black and white digital images. These techniques also raise the possibility of fairly sophisticated "touching up" of data. It is self-evident that any manipulation of data using digital tools should be avoided. In addition to commercial packages, there are excellent public domain resources: NIH image (http://rsb.info.nih.gov/nih-image/) developed for the Mackintosh computer and Image/J (http://rsb.info.nih.gov/ij/), a cross-platform Java-language-based program, can manipulate, process and analyze a wide range of digital images and confocal stacks from different sources.

3.11.1. Spatial Filtering

Even with a picture that has been optimized for contrast and illumination, it is sometimes desirable to sharpen local contrast gradients or smooth an image to remove random noise. A common filtering strategy, called a *kernel operation*, is to compare the intensity value of a pixel with that of its immediate neighbors and use any differences to increase or reduce the intensity of this central pixel by a computed factor. The degree of filtering depends both on the algorithm used by the kernel operation and the area over which pixel values are sampled.

1. Sharpening filters (*see* **Fig. 4**) produce a crisper image by accentuating differences in intensity between a given pixel and its neighbors. For example, a Laplacian filter equalizes the intensity over areas of low contrast and accentuates the intensity changes where gradients in pixel intensity are sharp. This produces an image composed largely of edge information (effectively a first-order derivative of the original), which when subtracted from the original pixel values, produces an image in which boundaries and the contrast of fine structures are enhanced.
2. Noise reduction filters remove the random pixel values that may be generated when, for example, a digital camera is working at maximum gain. Such noise is usually reduced during digital image collection by averaging successive images of the same field. If such real-time filtering is not practicable or

still leaves a noisy image, random intensity fluctuations can be filtered post hoc. Noise reduction filters take a block of pixels of a predetermined size and replace the intensity value of the central pixel with a value based on the average or median pixel intensity within the block. This has the effect of eliminating rogue, high or low single-pixel values. Algorithms based on averages may result in an unacceptable loss of contrast or even the appearance of pseudoresolution artifacts ***(3)***. However, such artifacts can be avoided using filters that "rank" rather than average pixel values, such as the "median" filter included in many software packages.

3.11.2. Pseudocolor

Pseudocolor is used to accentuate the information in black and white images by translating differences in intensity into differences in color. Pseudocolor allows the information in monochrome images to be enhanced but has a wider and more important role in identifying different data sets within a composite image. Most commonly it allows monochrome images of different fluorochromes to be color coded and recombined. Figure 4 gives examples of the use of pseudocolor in interpreting a confocal stak of images.

In the first example of pseudocolor rendering, a confocal image of cells within a chick hindbrain stained with the fluorescent dye DiI is first sharpened then intensity values converted into color differences. The color conversion is achieved by differentially adjusting the relationship between the original intensity of the black and white image (input) and the intensity of red, green, and blue brightness (output) for a given input value. This relationship is shown on the graph to the left where input is on the *x*-axis and output is on the *y*-axis. This kind of plot, known as a *color* or *output lookup table* (LUT), can usually be user-defined within a given software package.

In the second example, an aggregate of heterogeneously labeled chick hindbrain cells was scanned with two different excitation wavelengths (488 nm for green/yellow and 568 nm for red/yellow fluorescence). This reveals two populations of cells. The black and white images have been pseudocolored then recombined. In the 488-nm image, red and blue were completely removed from the image leaving a green intensity spectrum. In the 568-nm image, all green and blue were removed from the image, leaving red. The combined green and red fluorescence images were then superimposed onto a DIC filtered view of the same preparation. Although this figure adopts a conventional red and green coding to identify different data sets, magenta and green are a more audience-friendly pairing given the high frequency of red–green color blindness in the population (further information is available at http://jfly.iam.u-tokyo.ac.jp/color/).

In the third example, pseudocolor coding is used to identify populations of similarly labeled cells at different depths through a preparation. A cluster of cells, all labeled with DiI (which emits a red–orange fluorescence), were

scanned at two depths through the thickness of a chick hindbrain using a confocal microscope. The two black and white optical sections were then color coded in red and green according to depth and recombined. This allows the relative dispersal of labeled cells at different layers of the developing brain to be contrasted. This kind of image could not be produced by conventional double-exposure film photomicrography.

3.11.3. Excessive Digital Manipulation

Information in a digitized image can be altered, almost seamlessly, to produce "cleaner" results. A digital paintbox can remove dust and scratches from a scanned 35-mm transparency, compensate for uneven illumination, or produce a uniform background color. Although this may appear similar to the traditional "dodging and burning" used during black and white printing, the ease and extent to which a digital image can be altered are considerably greater. Similarly, since in many laboratories images are routinely generated on computer for publication, the opportunities for excessive image manipulation have substantially increased. Ultimately, in such an environment, producing reliable data is a matter of personal responsibility. Some self-evident principles are

1. Use sharpening, edge detection, or noise reduction filters globally across the image.
2. Preserve the data intact. Never digitally paint over areas that show data in an attempt to "clean up," for example, staining patterns. Never move or "clone" areas of the image where data are represented.

3.12. Cleaning Optical Components

Simple precautions reduce the need for cleaning optical components within the microscope. Most obviously, dust covers should be used for all microscopes. Glass components should be handled with great care and should never be in contact with bare skin. When cleaning objective lenses, immersion oil can be drawn off by applying lens tissue to the edge of the front lens. It should be necessary to apply only very light, if any, pressure to the lens itself. If the lens is to be cleaned directly with lens tissue, then movements should always be outward from the center of the lens. It is important to use fresh lens tissue for every action. If a cleaning solvent is needed, then it is important to refer to the recommendations of the lens manufacturer: Some solvents dissolve the glue that holds the front lens in place. Dry objectives should never be used with immersion fluid. Other coated-glass components, such as fluorescence filters, should never be handled and should not be cleaned with lens tissue or solvents. Pressurized air in containers contains a solvent that may condense on surfaces that are held too close to the jet of air.

3.13. Troubleshooting

Related section numbers are given in parentheses.

1. You cannot see an image or the image is vignetted.
   a. Check that the light path to specimen is clear. Is the specimen evenly illuminated?
      i. Is a condenser lens of the correct maximum NA in place? For lower magnifications, it might be necessary to remove "swung-in" lenses—objectives of ×1 magnification are normally used without a substage condenser.
      ii. Is the dark-field condenser prism out of the light path?
      iii. Are neutral density or color filters fully out of the light path?
      iv. Is the field diaphragm sufficiently open?
      v. Is the aperture diaphragm sufficiently open?
      vi. Is the DIC analyzer filter swung out (particularly for fluorescence illumination)?
   b. Check that light path to eyepieces is clear and, in particular, the beam is being directed to the eyepieces and not to the camera.
   c. For fluorescent specimens, check that the appropriate filter combination is in place and the fluorescent lamp is correctly aligned and focused.
2. You cannot bring the specimen into focus at a high power.
   a. Is the thickness of the specimen too great for the NA of the objective lens (**section 3.2.3**)? Specifically, is the objective touching the slide?
   b. Is a dry objective being used incorrectly with immersion oil or vice versa (**section 3.2.4.**)?
   c. Is the immersion medium matched to the refractive index of the mountant (**section 3.2.4.**)?
   d. For inverted compound microscopes only—is the slide placed correctly with cover glass facing downward?
3. The contrast is insufficient.
   a. Focus condenser to achieve "Köhler illumination" (**section 3.4.1**).
   b. Adjust the aperture diaphragm of the substage condenser to the correct NA (**section 3.4.2**).
4. The DIC is not producing a "pseudorelief" image (**section 3.5.2**).
   a. Is the analyzer filter in place?
   b. Are the DIC prisms in the substage condenser matched to the NA of the objective (in more sophisticated microscope systems)?
   c. Are the aperture and field diaphragms correctly adjusted (*see* **Note 3**)?
   d. Is the polarizer rotated to achieve optimal illumination?
4. The digital image is granular or blurred.
   a. Is sufficient light being directed to the specimen (**section 3.7.2**)?
   b. Is the gain set too high to compensate for a low camera sensitivity?
   c. (Confocal) Is the Kalman filter activated and a sufficient number of scans specified (**section 3.8.**)?
5. Parts of the digital image are too dark when brightest pixels have reached saturation? Is the gamma optimally defined for this image? Start with a setting of gamma = 1, then monitor the image as the setting is varied (**section 3.7.5**).

6. The photomicrograph exposure is seemingly infinite.
   a. Is all light (particularly for fluorescence) directed to the camera (i.e., no image should be seen through the eyepieces)?
   b. Is the film speed set correctly?
   c. Are the camera batteries exhausted (where a separate 35-mm camera body is attached via a tube)?
7. Color photomicrographs have variable color balance and brightness.
   a. Was the camera voltage supply set to PHOTO, 3200 K, or a constant voltage?
   b. Was an artificial (tungsten) light source film used?
   c. If using "daylight" film (*see* **section 3.10**), was a compensating blue filter (Kodak Wratten 80A) in place?
   d. Were exposure times longer than 2 sec? If so,
      i. Was the correct reciprocity index programmed into the camera exposure mechanism with respect to film type?
      ii. Were the correct color compensation filters used?

4. Notes

1. Specimens that are to be further processed for in-situ hybridization with RNA probes or are labeled with a volatile dye, such as DiI, can be mounted in fixative in the same way.
2. A general exception is ×1 objectives, which are highly unlikely to be parfocal with any other objective lens.
3. Each eyepiece has a diopter adjustment ring, which can be aligned with engraved markings on the eyepiece tube. Observers who would normally wear glasses should adjust the diopter with respect to each eye. With the ring aligned to the zero position, the specimen should be brought into focus at high power. To achieve parfocality, the same field should be viewed at low magnification and the specimen focused again using the diopter adjustment ring on the eyepiece. This should be repeated for each of the lower-power objective lenses and separately for each eyepiece.
4. The NA is calculated as the product of the refractive index of the medium between the objective lens and the specimen multiplied by the sine of the half angle of the cone of light entering the objective. This cone becomes narrower (and therefore the NA smaller) as the working distance increases ***(4)***.
5. Spatial resolution is proportional to the wavelength of transmitted light divided by the NA.
6. For an oil-immersion lens, using a mounting medium with a refractive index lower than glass or oil results in a corresponding loss in numerical aperture (effectively that achievable with the less dense medium). Any area of low refractive index in the object space, such as a water or air bubble, has this effect.
7. Stopping the aperture diaphragm right down can produce "false resolution," a diffraction image that gives the impression of a fine ultrastructure within the specimen.
8. If dehydrating through an alcohol series, reduce the overall exposure to approximately 2 min.

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