Methods in Molecular Biology 770

Springer Protocols

Francisco J. Pelegri *Editor*

Vertebrate Embryogenesis

Embryological, Cellular, and Genetic Methods

Humana Press

METHODS IN MOLECULAR BIOLOGY™

Series Editor
John M. Walker
School of Life Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For other titles published in this series, go to
www.springer.com/series/7651

Vertebrate Embryogenesis

Embryological, Cellular, and Genetic Methods

Edited by

Francisco J. Pelegri

Department of Genetics, University of Wisconsin, Madison, WI, USA

Humana Press

Editor
Francisco J. Pelegri
Department of Genetics
University of Wisconsin
Madison, WI, USA
fjpelegri@wisc.edu

ISSN 1064-3745 e-ISSN 1940-6029
ISBN 978-1-61779-209-0 e-ISBN 978-1-61779-210-6
DOI 10.1007/978-1-61779-210-6
Springer New York Dordrecht Heidelberg London

Library of Congress Control Number: 2011933224

Printed on acid-free paper

Humana Press is part of Springer Science+Business Media (www.springer.com)

Preface

One of the striking findings of modern developmental biology has been the high degree of conservation of signaling and developmental mechanisms among different animal species. Such conservation allows information learned from a given organism to be applicable to other species, including humans, and has validated the use of a few model systems to deduce general biological principles. In spite of this underlying conservation, however, each species has unique characteristics arising from its evolutionary history. The picture emerging from the ongoing research on a limited number of model organisms is thus a patchwork of knowledge that reflects those unique systems. The important contribution of other less-studied, emerging model systems will be invaluable to fill these gaps. The modern developmental biologist strives to fill this currently fragmented picture in search of a more refined, fuller view, which better reflects both the underlying biological principles and the genetic continuum between species.

While studying the same process in various types of organisms may highlight common developmental mechanisms, individual features of species differ, each providing advantages for analyzing various aspects of a given process. With regard to laboratory research, some approaches may be more feasible in some species than in others; for example, the ease of embryological manipulations in amphibians and chicken set them apart as premier embryological models in the last century. Differences in underlying developmental genetic circuitries may also give differential access to analyzing a given process, such as when different types of mutant phenotypes affecting the same organ are found in different organisms. In addition, some laboratory systems may be more closely related to the species in which specific knowledge is needed, for example, mammalian species for medical applications or fish species for aquaculture. Studies in other systems may also be desired to better understand the process in an evolutionary context. Thus, for a number of reasons an optimal overarching research strategy may require the analysis of multiple species.

It is in this dual context, to better define the landscape in which development operates in nature and to maximize the output of biological research, that modern biological research is increasingly crossing model system boundaries. In addition, studies involving multiple species are the focus of new research areas, such as the use of hybrids to understand mechanisms of speciation or the use of interspecies nuclear/oocyte transfer in bioregenerative medicine and conservation genetics. This volume attempts to address the increasingly important need of straddling species boundaries in the context of a single research program by compiling research protocols used in a wide range of vertebrate species. These protocols include not only embryological methods but also cellular and genetic approaches that have complemented and expanded our understanding of embryonic development. Undoubtedly, assembling in full detail the entire set of methods available for a laboratory model system in a single or a couple of chapters is beyond feasibility. This volume does not pretend to turn a blind eye to this reality, but rather strives to provide a platform to facilitate the exchange of ideas and protocols between scientists studying different vertebrate species. In fact, this volume has been designed so that readers can readily find information on species *other* than the one with which they may be most familiar.

Keeping in mind this purpose, contributors have attempted to emphasize the advantages and challenges of research in each particular organism, as well as unique features of the system. It is our hope that this will allow comparisons between protocols and facilitate transposing experimental strategies from one research organism to another, as well as help readers make informed decisions on the feasibility of using an alternative model system to analyze a specific biological question. In addition, we hope that this volume will also be useful as a compilation of methods for educators leading advanced laboratory courses.

While a number of chapters are dedicated to the most popular model systems, this volume also incorporates other emerging systems spanning the vertebrate subphylum. Underlining that not even a group as diverse as vertebrates can be considered in isolation and that comparative studies in more ancestral forms may be key to the understanding of the basis of vertebrate development, we also include protocols in closely related invertebrate chordates such as ascidians and amphioxus. In addition, a number of chapters highlight a specific method that is in principle applicable to multiple species, such as TILLING and ZFN-mediated mutagenesis, the generation of embryonic stem (ES) cell lines, and nuclear/oocyte transfer. Opening this volume, an account of the use of various species in the field of developmental biology places the rest of the chapters in a historical context. Another chapter highlights important insights from an investigator with a multi-species research program. Reflecting that animal research is a privilege that needs to minimize discomfort to the organism experimented upon, the volume closes with a chapter on animal care guidelines applicable to vertebrates.

We hope this compilation of protocols will be of use to the molecular, cell, and developmental biology community to accelerate the pace of its research. While reading and editing these chapters, I have already found a number of ways in which approaches in other model systems could help resolve hurdles present in my own laboratory. We hope the reader will similarly benefit from this compilation. May this volume contribute to the ongoing collective effort toward a better understanding of the beauty and logic of vertebrate development.

Madison, Wisconsin *Francisco J. Pelegri*

Contents

Contributors

SHARON L. AMACHER • *Department of Molecular and Cell Biology, Center for Integrative Genomics, University of California, Berkeley, CA, USA*

MICHAEL BRAND • *Dresden TILLING Project, c/o Biotec TU Dresden, Dresden, Germany*

TAMARA CASPARY • *Department of Human Genetics, Emory University School of Medicine, Atlanta, GA, USA*

ANTHONY W.S. CHAN • *Department of Human Genetics, Yerkes National Primate Research Center, Emory University School of Medicine, Atlanta, GA, USA*

TIEN-CHENG ARTHUR CHANG • *Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, School of Medicine, University of Texas Health Science Center, San Antonio, TX, USA*

JANET CHENEVERT • *Biologie du Développement, UMR 7009 CNRS/UPMC, Observatoire Océanologique, Villefranche sur Mer, 06230, France*

SHOTA CHIBA • *Department of Molecular, Cell and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA, USA*

JAN L. CHRISTIAN • *Department of Cell and Developmental Biology, Oregon Health and Science University, School of Medicine, Portland, OR, USA*

MARCELO COUTO • *Executive Director and Campus Veterinarian, UCLA School of Medicine—DLAM, Los Angeles, CA, USA*

MICHAEL V. DANILCHIK • *Department of Integrative Biosciences, Oregon Health & Science University, Portland, OR, USA*

SCOTT T. DOUGAN • *Department of Cellular Biology, The University of Georgia, Athens, GA, USA*

YANNICK DOYON • *Sangamo BioSciences, Inc., Pt. Richmond Tech Center, Richmond, CA, USA*

RÉMI DUMOLLARD • *Biologie du Développement, UMR 7009 CNRS/UPMC, Observatoire Océanologique, Villefranche sur Mer, 06230, France*

MAKOTO FURUTANI-SEIKI • *Department of Biology and Biochemistry, Centre for Regenerative Medicine, The University of Bath, Bath, UK*

LAURA S. GAMMILL • *Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, USA*

TIMOTHY J. GEACH • *National Institute for Medical Research, London, UK*

ANNE E. GRIEP • *Department of Cell and Regenerative Biology, University of Wisconsin—Madison, Madison, WI, USA*

NICOLA GSCHEIDEL • *Dresden TILLING Project, c/o MPI CBG, Dresden, Germany*

ANNA-KATERINA HADJANTONAKIS • *Developmental Biology Program, Sloan-Kettering Institute, New York, NY, USA*

CELINE HEBRAS • *Biologie du Développement, UMR 7009 CNRS/UPMC, Observatoire Océanologique, Villefranche sur Mer, 06230, France*

LINDA Z. HOLLAND • *Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA, USA*

NICK HOPWOOD • *Department of History and Philosophy of Science, University of Cambridge, Cambridge, UK*

VANESSA L. HORNER • *Department of Human Genetics, Emory University School of Medicine, Atlanta, GA, USA*
CLARE HUDSON • *Biologie du Développement, UMR 7009 CNRS/UPMC, Observatoire Océanologique, Villefranche sur Mer, 06230, France*
AKIHIRO IKEDA • *Department of Medical Genetics, University of Wisconsin – Madison, Madison, WI, USA*
SAKAE IKEDA • *Department of Medical Genetics, University of Wisconsin – Madison, Madison, WI, USA*
MANORAMA C. JOHN • *Biotechnology Center, University of Wisconsin—Madison, Madison, WI, USA*
CATHERINE E. KRULL • *University of Michigan, Biologic and Materials Sciences, Ann Arbor, MI, USA*
GLORIA S. KWON • *Developmental Biology Program, Sloan-Kettering Institute, New York, NY, USA*
JENNIFER H. MANSFIELD • *Department of Biological Sciences, Barnard College, Columbia University, New York, NY, USA*
JASMINE M. MCCAMMON • *Department of Molecular and Cell Biology, Center for Integrative Genomics, University of California, Berkeley, CA, USA*
ALEX MCDOUGALL • *Biologie du Développement, UMR 7009 CNRS/UPMC, Observatoire Océanologique, Villefranche sur Mer, 06230, France*
EDWINA MCGLINN • *Department of Genetics, Harvard Medical School, Boston, MA, USA*
KATHERINE E. MCLEAN • *Department of Biomedical Sciences, University of Guelph, Guelph, ON Canada, N1G-2W1*
MIZUHO S. MIMOTO • *Department of Cell and Developmental Biology, Oregon Health and Science University, School of Medicine, Portland, OR, USA*
SREELAJA NAIR • *Laboratory of Genetics, University of Wisconsin Madison, Madison, WI, USA*
NGAN LE NGUYEN • *Biologie du Développement, UMR 7009 CNRS/UPMC, Observatoire Océanologique, Villefranche sur Mer, 06230, France*
TAKAYUKI ONAI • *Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA, USA*
ALEXANDRE PAIX • *Biologie du Développement, UMR 7009 CNRS/UPMC, Observatoire Océanologique, Villefranche sur Mer, 06230, France; Univ. Nice-Sophia Antipolis, Nice 0600, France*
ANDREW J. PASK • *Department of Zoology, The University of Melbourne, Melbourne, VIC 3010, Australia; Department of Molecular and Cellular Biology, University of Connecticut, Storrs, CT, USA*
FRANCISCO J. PELEGRI • *Department of Genetics, University of Wisconsin Madison, Madison, WI, USA*
ANNA PILISZEK • *Developmental Biology Program, Sloan-Kettering Institute, New York, NY, USA*
SEAN R. PORAZINSKI • *Department of Biology and Biochemistry, Centre for Regenerative Medicine, The University of Bath, Bath, UK*
GÉRARD PRULIERE • *Biologie du Développement, UMR 7009 CNRS/UPMC, Observatoire Océanologique, Villefranche sur Mer, 06230, France*
MARILYN B. RENFREE • *Department of Zoology, The University of Melbourne, Melbourne, VIC 3010, Australia*

CHRISTIAN SARDET • *Biologie du Développement, UMR 7009 CNRS/UPMC, Observatoire Océanologique, Villefranche sur Mer, 06230, France*
HAZEL SIVE • *Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology, Cambridge, MA, USA*
WILLIAM C. SMITH • *Department of Molecular, Cell and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA, USA*
YUHUA SUN • *Department of Cellular Biology, The University of Georgia, Athens, GA, USA*
MICHAEL T. VEEMAN • *Department of Molecular, Cell and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA, USA*
MATTHEW K. VICKARYOUS • *Department of Biomedical Sciences, University of Guelph, Guelph, ON, Canada*
HUIJIA WANG • *Department of Biology and Biochemistry, Centre for Regenerative Medicine, The University of Bath, Bath, UK*
ZHONGDE WANG • *Department of Epigenetics and Embryo Development, Hematech, Inc., Sioux Falls, SD, USA*
SYLKE WINKLER • *Dresden TILLING Project, c/o MPI CBG, Dresden, Germany*
DOROTA WLOGA • *Nencki Institute of Experimental Biology, Polish Academy of Science, Warsaw, Poland*
HITOYOSHI YASUO • *Biologie du Développement, UMR 7009 CNRS/UPMC, Observatoire Océanologique, Villefranche sur Mer, 06230, France*
LYLE B. ZIMMERMAN • *National Institute for Medical Research, London, UK*

Chapter 1

Approaches and Species in the History of Vertebrate Embryology

Nick Hopwood

Abstract

Recent debates about model organisms echo far into the past; taking a longer view adds perspective to present concerns. The major approaches in the history of research on vertebrate embryos have tended to exploit different species, though there are long-term continuities too. Early nineteenth-century embryologists worked on surrogates for humans and began to explore the range of vertebrate embryogenesis; late nineteenth-century Darwinists hunted exotic ontogenies; around 1900 experimentalists favored living embryos in which they could easily intervene; reproductive scientists tackled farm animals and human beings; after World War II developmental biologists increasingly engineered species for laboratory life; and proponents of evo-devo have recently challenged the resulting dominance of a few models. Decisions about species have depended on research questions, biological properties, supply lines, and, not least, on methods. Nor are species simply chosen; embryology has transformed them even as they have profoundly shaped the science.

Key words: Developmental biology, embryology, evo-devo, history, methods, model organisms, species choice.

1. Species Choice

Species choice has recently become prominent and controversial in debates over the pros and cons of the dominant "model organisms" in developmental biology (1). New systems seem to be announced almost monthly and laboratories are now more likely to cross species boundaries too. While this volume aims to promote that shift, this chapter puts these changes into historical perspective.

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_1,

Embryologists have chosen organisms for their medical, agricultural, fisheries, sporting or other practical importance, or because they were considered biologically special. They have worked on surrogates for the species of most interest, especially humans, and on convenient representatives of groups (2). Different kinds of embryology have exploited various vertebrates in contrasting ways. Late nineteenth-century evolutionists, for example, risked life and limb on expeditions to hunt phylogenetically strategic embryos for histology. Twentieth-century experimentalists chose accessible organisms that would provide abundant living, easily analyzed embryos on demand.

The histories of such models as chick, *Xenopus*, mouse, and zebrafish show that species selection never simply matches research questions and biological properties. It is also about a community's values, institutions, networks, and techniques: the kind of research it admires, the supply lines it can set up, the methods it can develop, and, increasingly, the features it can engineer (3–9). So species are not simply chosen for embryology. Complex experiments need elaborate infrastructures around highly domesticated organisms, but even to produce the most basic description embryos have to be seen within a developmental frame. It is easy to take this for granted today; historically, it was necessary to set up standard series and to challenge competing interpretations by other people (10, 11).

Species choice creates opportunities and sets limits that strongly shape research (1). Competing research programmes invest in rival organisms (12, 13); scientists bet on which organism–problem combination will prove most productive and agencies fund one rather than another. This is now clear for particular organisms and episodes, especially in the later twentieth and early twenty-first centuries, but the overall pattern is only starting to come into view. The chapter introduces the major approaches in the history of research on vertebrate embryos (14) and shows, in broad outline, why and how they have exploited different organisms. It begins to survey the long-term politics of species choice in embryology (*see* **Note 1**).

2. Histories of Development

Philosophers and physicians had for centuries investigated the generation of various animals and especially the chick, because its large eggs were abundantly available as food. But only in the age of revolutions around 1800 was embryology made a separate science. Developing embryos were framed as the objects of interest by rejecting older views, for example, of the acquisition

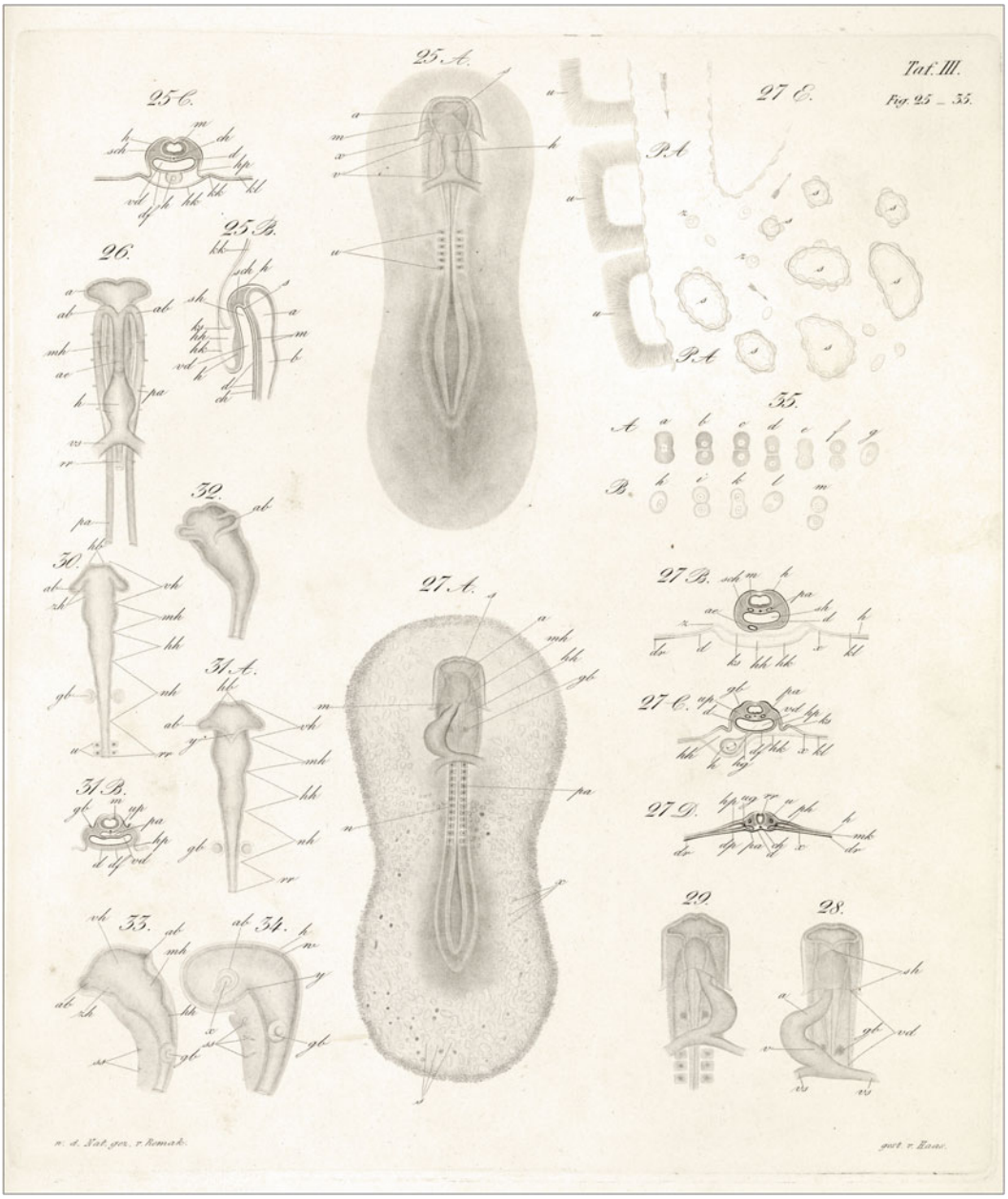

Fig. 1.1. Copper plate, showing whole views, organs, sections, and (blood) cells of second- and third-day chick embryos, from the 1855 book by the Berlin microscopist Robert Remak that refined the germ-layer doctrine and linked it to the cell theory. He argued that division of the egg cell produced layers composed of cells that each divided and differentiated to produce specific tissues and organs. Engraving by Haas after Remak's own drawings, reproduced from (15) by kind permission of the Syndics of Cambridge University Library. Printed surface 30 cm × 26 cm.

of a rational soul as the crucial event in human pregnancy, and by using new techniques. Especially in German university institutes of anatomy and physiology, microscopists explored how complex bodies develop from simple beginnings. Through the mid-1800s they collected and dissected specimens, preserved them in spirits of wine, and observed and drew them through increasingly effective microscopes. They set up developmental series, correcting times for temperature where they could, and selecting representatives against which to assess new finds. They analyzed embryos into germ layers and cells. Copper plates or lithographs accompanied the most prestigious publications (**Fig. 1.1**).

Medical and anthropological interests focused on humans, but anatomists had to rely on encounters with aborting women and the occasional postmortem. So embryos were inaccessible for about the first fortnight and rare for the next few weeks. Suspicions of abnormality made it hard to have confidence in accounts of normal development. Conveniently then, the most exciting comparative discoveries, such as the 1825 announcement of "gills in mammals" (16), reinforced the assumption that, across all the vertebrates, early development was fundamentally the same. So researchers fished amphibian spawn out of ponds, warmed hens' eggs in artificial incubators, and bought and bred rabbits and dogs. Physiologists criticized those who concentrated on human material they saw as uninformative. "[T]he history of the bird embryo is … the ground on which we march forward," while "that of the mammalian fetus is the guiding star, which promises us safety on our route towards the development of man" (17).

Yet embryologists also hoped that embryos would reveal the true relations between groups more clearly than in later life, and thus help comparative anatomy to produce a natural classification. To explore the play of difference within the underlying unity, they collected viper eggs, acquired deer from hunters, and obtained the conveniently transparent teleost embryos by artificial fertilization (5, 18, 19). Dealers supplied occasional exotics, and when Louis Agassiz emigrated from Switzerland to the United States in 1846 he opened up the American fauna, notably fishes and turtles, for comparative embryology (20). Embryologists were few, though, and the biological, geographical, and social obstacles were large. A major survey of 1881 still identified huge gaps (21)—but by then things were beginning to change.

3. Ontogeny, Phylogeny, and Histology

Darwinism drew on embryology for some of the strongest and most detailed evidence for common descent. From the late 1860s, with the slogan "ontogeny recapitulates phylogeny," the

German zoologist Ernst Haeckel raised its profile in the universities and among the general public (22, 23). He also changed its species politics. Nothing had been so damaging, he controversially declared, as concentration on the development of the chick. This had suffered such major changes from the ancestral form of the vertebrates—it was, in Haeckel's terminology, so "cenogenetic," as to give a wholly misleading view. Embryology should start again from the acraniate amphioxus and systematically pursue comparative research (24). While teaching focused on a few types, usually including the chick (25), he encouraged embryologists to discover the origins of the vertebrates, of tetrapods, and especially of human beings.

Land-locked European researchers, most of them pursuing careers as professors of anatomy or of zoology, created new institutions and exploited imperial networks to gain access to the rest of the world (26). Marine stations made it possible to utilize the sea more efficiently. Haeckel's student Anton Dohrn founded the most important in 1872 at Naples, where the Russian Alexander Kovalevsky had already influentially explored the development of ascidians and amphioxus and significant work on elasmobranchs would be done (27–29). Embryologists took advantage of an increasingly global web of collectors, for example, to establish a breeding colony of opossums, an American marsupial, in Bavaria (30).

The most intrepid scientists set sail to bring home "living fossils" and "missing links." They expected to find evidence of the major transitions most faithfully preserved in the early embryos of these groups. They caught lungfish spawn and other documents of tetrapod origins in South America, West Africa, and the Australian bush (11, 31), which also provided embryos of monotremes (egg-laying mammals): the platypus (26) and the spiny anteater or echidna. Colonial officials and settler farmers gave another Haeckel student Richard Semon access to echidna country and helped recruit native Australians. They staffed his camp and collected the nocturnal anteaters that lived, shyly and quietly, in the most impenetrable bush. Many settlers had never seen one, but the "incomparable nose and hawk's eye" of "the blacks" could follow the slight and complex tracks over difficult terrain to the hollows where the animals slept by day (**Fig. 1.2**). So they were cross when Semon paid little or nothing for the more numerous males (32). Many females were also sacrificed in vain. He had to preserve specimens on the spot, and because the aborigines returned at dusk, often ended up dissecting uterine embryos out of their tight-fitting shells "by the light of a flickering candle" (33).

The explorers valorized their own derring-do and excused the gaps in their collections by presenting rabbit breeding as tame (33). Yet another of Haeckel's students, Willy Kükenthal,

Fig. 1.2. The German zoologist Richard Semon's echidna hunters and their prey. (**A**) Semon's "particular friends," Ada and her husband Jimmy, in the camp on the River Boyne, a tributary of the Burnett, in Queensland. Though Semon

accompanied whalers in the Northern seas, but found it hard to intervene during the freezing storms on the ships, where everything had to happen fast. He did better at the processing stations in Spitsbergen (34). The Erlangen Darwinist Emil Selenka's hunting trips to the East Indies laid the foundations of the embryology of apes. But he lost rare treasures in a boat collision and was so sick with malaria that his wife Lenore had to make good the loss (35). The most arduous, and among the least successful, embryological collecting was of emperor penguin eggs during the fateful "winter journey" of Robert Falcon Scott's Antarctic expedition in 1911. Working out the embryology of "the nearest approach to a primitive form not only of a penguin, but of a bird" had seemed "a matter of the greatest possible importance," and cost biologist Edward A. Wilson his life, but sadly, no one much cared about the three fairly late-stage eggs that made it back (36, 37).

Collecting worked profound intellectual transformations. This is because it framed materials as embryos that the suppliers had often interpreted in other terms. The aborigines knew how to track the echidna, or "cauara," because it was a prized delicacy; they also told Semon of its origin from a bad man who was filled with spears. He impressed "the bushmen" by showing that the young were not "conceived on the teat," as they had believed, but began, like other mammals, in the womb (32). Some of the deepest transformations went on closest to home. Even women who knew they were pregnant—and in the early stages, especially before hormonal tests, many did not—rarely interpreted the blood clots they passed in embryological terms. Depending on whether or not a woman desired a pregnancy, she might think in terms of a child to come or of waste material that had to be removed. Anatomists appropriated bleeds that had been experienced variously as unremarkable late periods, distressing miscarriages or desired restorations of menstrual flow (10).

Embryos of different species were then made equivalent by analyzing them in comparable ways. The great innovation of the 1870s was routine serial sectioning with microtomes to give more detailed access to internal forms than dissection could achieve. Though embryos were sometimes observed fresh using low-power microscopes and drawing apparatus, sectioning became central to embryological technique. Once obtained, and sometimes cultured, the material was fixed and stained, embedded and cut by methods adapted to each taxonomic group and stage

Fig. 1.2. (continued) regarded the aborigines as "one of the lowest human races," he admired their skill in the noble art of hunting. Jimmy, once a famous warrior, was Semon's "best huntsman" and a fine raconteur. The bottles on the table were likely for preserving and staining the embryos. (**B**) An echidna or spiny anteater. Reproduced from (32) by kind permission of the Syndics of Cambridge University Library.

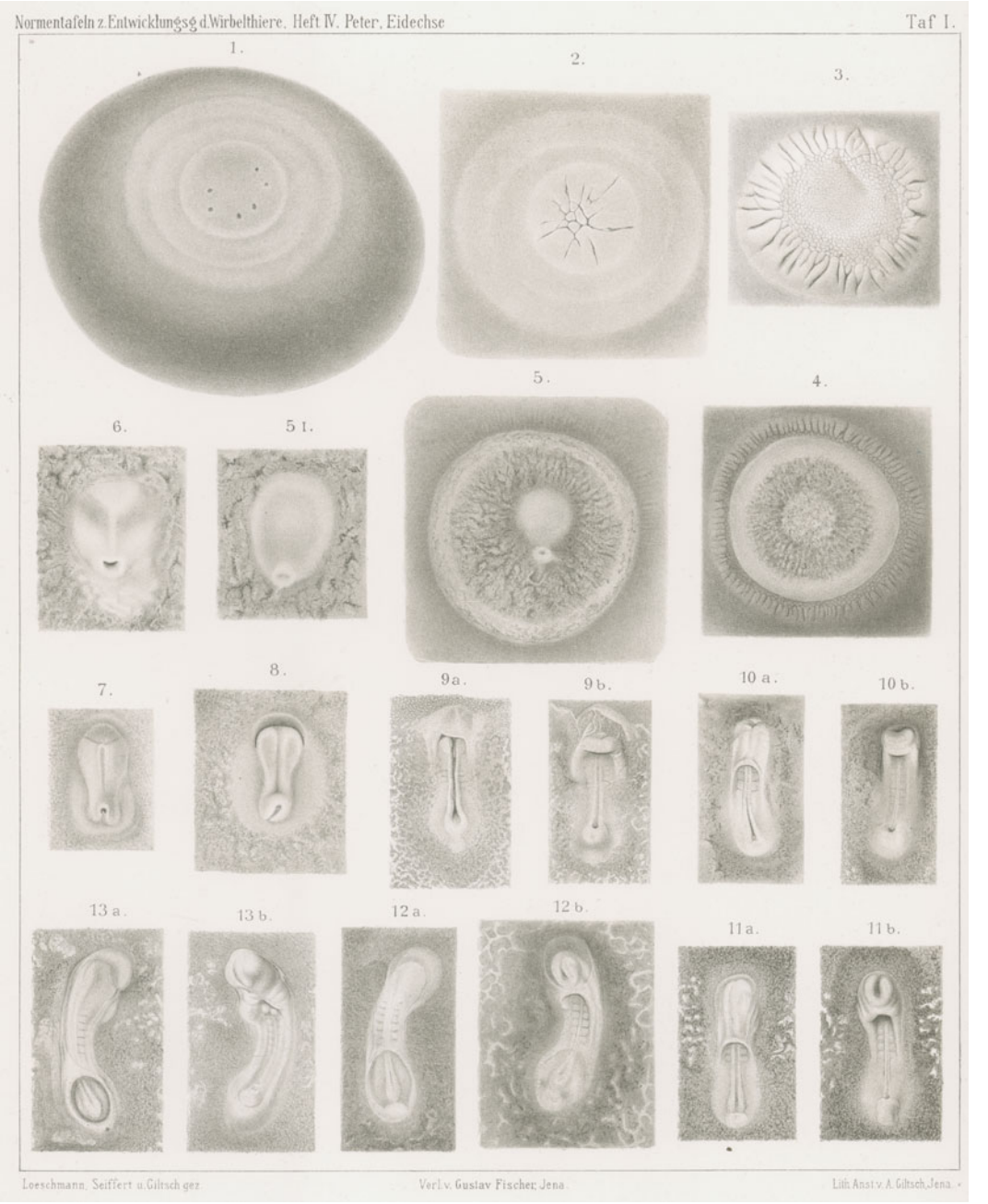

Fig. 1.3. The normal development of the sand lizard, *Lacerta agilis*. The fourth (1904) volume in Franz Keibel's *Normal Plates on the Development of the Vertebrates* is the only one devoted to a reptile, though he had hoped to include turtles too (11) and snakes and crocodilians had also been studied before. The author, the Breslau (now Wrocław) anatomist Karl Peter, raised the lizards in terraria over six summers. This first of four plates covers development from an uncleaved egg; magnification is 10 × (1–5) or 20 × (5^{I}–11); embryos 9–11 are shown from (a) dorsal and (b) ventral sides. Specimens were drawn unstained, with reference also to stained specimens and photographs, each drawing combining features that could be seen in nature only by illuminating from various angles. Lithograph by Adolf Giltsch, after drawings by Emil Loeschmann, a Mr Seifert and Giltsch, from (46). Original dimensions of border 26.5 cm × 21.9 cm.

(38, 39). For particularly complex forms it became common to reconstruct three-dimensional views from the sections, either graphically or in wax (40).

Debates over evolution made degrees of similarity and difference so contested that other vertebrates could no longer stand in for human embryos. Haeckel's leading critic, the Swiss anatomist Wilhelm His, reformed the field by applying the microtome to a rich supply of precious human specimens from the third week to the end of the second month. Since he could not set up rigorous stages for this scarce and variable material, he invented a "normal plate" that simply arranged representative specimens in series (10).

Anatomists now prided themselves on studying human embryos directly. In 1914 they established this non-evolutionary human embryology, primarily using material recovered during surgery, by founding the Carnegie Institution of Washington Department of Embryology at the Johns Hopkins University (41–43). A primate colony was installed there in the 1920s (44). (Today the human embryo collection is at the National Museum of Health and Medicine in Washington, DC.)

Meanwhile, as evolutionists increasingly questioned Haeckel's doctrine of recapitulation, high-profile disagreements sent the field into crisis (11, 22, 45). To reassess the relations between ontogeny and phylogeny, the German anatomist Franz Keibel organized an international series of 16 vertebrate normal plates (11) (**Fig. 1.3**). The revived comparative studies were institutionalized in 1911 in the International Institute of Embryology. Constituted through a series of meetings in different locations, this club promoted "salvage" embryology: collecting endangered colonial mammals for what became the Central Embryological Collection at the Hubrecht Laboratory in Utrecht (47). (It was transferred in 2004 to the Natural History Museum in Berlin.) Evolutionary embryology nevertheless declined after World War I, and experimentalists disparaged comparative work as merely "descriptive."

4. Experimental Cultures

From the 1880s, some embryologists took a radically different approach, reconstructing embryology not as a historical science but on the model of the new experimental physiology with its ideal of controlling life. Occasional earlier experiments had generated additional forms to anatomize and taxonomize, but now the focus was less on evolutionary questions than on how, in the present, one stage produced the next.

The anatomist Wilhelm Roux and other exponents of "developmental physiology" or "developmental mechanics" employed a range of interventions, mechanical (shaking, cutting, constricting, pressure, gravity, centrifugal force), thermal, chemical, and electrical. The pioneers tended to use small metal scissors, needles, and knives; in the next generation zoologist Hans Spemann's microsurgery relied on hair loops and much finer glass instruments that he made himself (48) (**Fig. 1.4**). The new stereomicroscopes allowed finer manipulations (50), but careful culture was at least as important as fancy apparatus, especially since antibiotics came in, for the more challenging cultures, only after World War II. Keibel's elaborate normal plates were condensed into diagnostic "normal stages" (11). "Fate maps" used vital dyes to show what early regions would become (51). Grafts were also marked by species differences in pigmentation.

Species here mattered little for their own sakes. So fishes tended to lose out, because researchers no longer much cared about either their extraordinary diversity or their position as basal vertebrates, while other classes provided living embryos that were more easily cultured and manipulated in large numbers (5). Among the vertebrates the freely accessible, large and extremely

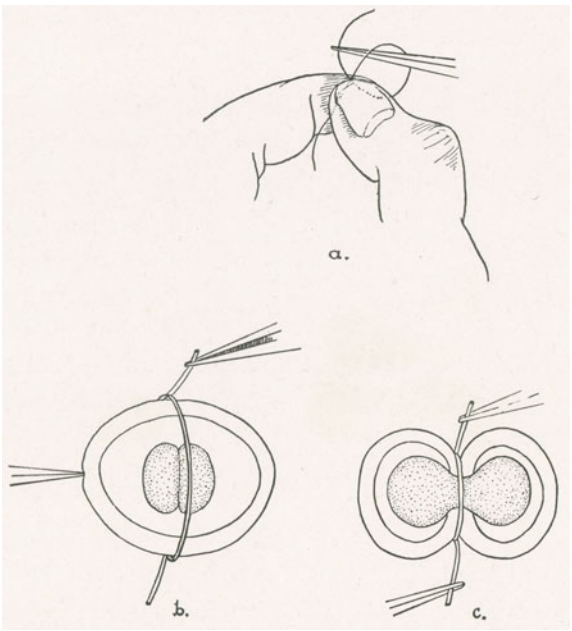

Fig. 1.4. "The production of twin embryos and of duplications in Urodela by constriction. After Spemann." Diagram from a manual for student practicals by Spemann's former colleague, Viktor Hamburger. Constricting only slightly produces conjoined anterior duplications. Spemann had used his daughter's hair. Hamburger recommended the Eastern newt, *Triturus* (now *Notophthalmus*) *viridescens*. Reproduced, by permission of the University of Chicago Press, from (49).

resilient eggs of local amphibia were much the most popular for extirpation, explantation, and transplantation, with chicks in second place (49, 52, 53). Relevant work on mammals went on in the new field of reproductive science (54). The pig was used in teaching alongside the chick.

Embryologists had always specialized in certain groups, but never as much as Spemann, co-discoverer of the organizer. He arranged his career and those of almost all his students and collaborators around microsurgical work on species of the salamander *Triton* (now mostly *Triturus*). This concentration shows the shape of things to come, but the breeding season still limited the experiments to the spring (55, 56).

5. Model Organisms

After World War II, massively expanded government funding allowed biological and especially biomedical research to expand and intensify. Seeking the most productive experimental systems, biologists and especially geneticists focused on a few readily available model organisms. With their short generation times, small adult sizes, and general suitability for laboratory domestication, these species would dominate research on development.

Evolution was sidelined as the new "developmental biology" studied cellular, molecular, and genetic processes, and increasingly patterns and mechanisms of gene expression, in the most convenient organisms. Comparative research continued in traditional departments, museums, marine stations, and fisheries labs (57), and experiments used a wide variety of embryos (*see* **Note 2**). But just a few species account for most of the big growth in developmental biology (58, 59). The fruitfly *Drosophila melanogaster* and later the nematode *Caenorhabditis elegans* bid most strongly to become the embryological *Escherichia coli*, but three and then four vertebrates were among the top half-dozen species, in part because of their medical relevance, in part because they were more suitable for experimental embryology and biochemistry.

The most venerable, the chick, was used in the postwar era especially to explore the development of limbs and nerves (60). Much research on the neural crest has employed chick–quail chimeras, with their histologically distinguishable nuclei as intrinsic markers (61). Chick eggs may have been exploited for embryology before other sciences, but more often developmental biologists adopted species that had already entered laboratories. Introduced in the 1930s as a test animal for pregnancy diagnosis, the South African clawed frog *Xenopus laevis* has large eggs and—the

basis of the test—an injection of chorionic gonadotrophin will induce laying almost at will. By releasing experimenters from the seasonality of indigenous amphibian spawning, this increased productivity and marginalized other species. *Xenopus* was soon favored for combining experimental embryology with biochemistry and later molecular biology, but the genetic possibilities of this pseudotetraploid species were limited (7).

Most significant for medicine and agriculture was the opening up in the 1960s of preimplantation mouse embryos for culture and manipulation (62, 63). While the larger rabbit had been preferred for work on fertilization and embryo transfer before World War II, the more general establishment of inbred mice as standard genetic models for human beings (8) gave them a decisive advantage (**Fig. 1.5**). By the 1980s more articles in developmental biology journals were devoted to mice than any other species (59).

"The mammalian embryo" tended to mean "the mouse," but researchers had strong practical incentives to cross species barriers. Embryo transfer in livestock was made a major industry in the 1970s (65), and human embryology and reproductive

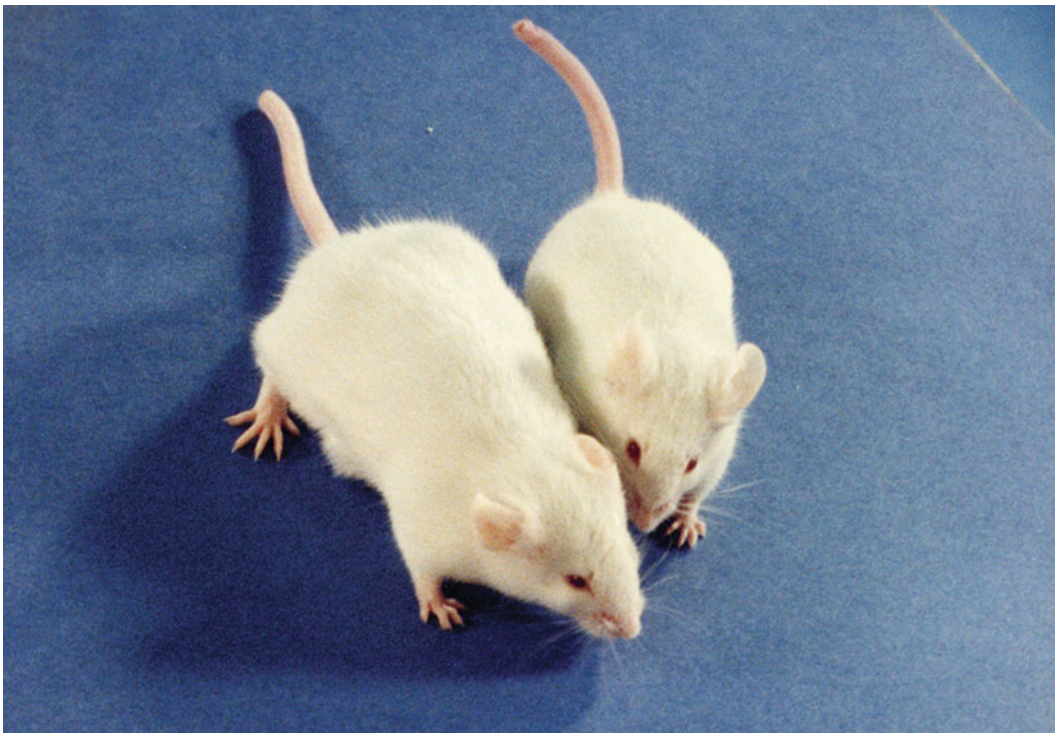

Fig. 1.5. A transgenic mouse. This image appeared on the cover of *Nature* in 1982 with the caption "Gigantic mouse—from eggs injected with growth hormone genes" (64). One littermate had a body weight almost twice that of its sibling because it carried a hybrid gene containing the mouse metallothionein-1 promoter fused to the rat growth hormone gene. Courtesy of Ralph L. Brinster, School of Veterinary Medicine, University of Pennsylvania.

medicine were revolutionized with the 1978 achievement of a live birth following in vitro fertilization (66, 67). Some innovations, notably freezing and cloning by nuclear transplantation, were first achieved with the larger embryos of sheep (62, 68). Interest in exotics was initially rare, but vets and zoos have been engaged in reproductive science for several decades (69, 70). With echoes of the International Institute of Embryology, cloning is being controversially applied to conserving endangered species (71, 72).

Organisms had long needed work to adapt them for embryology, if only in the form of normal plates and/or special methods for culture or histology. As scientific objects, they were always made as well as found. Now they were increasingly heavily engineered for research in developmental genetics and cell biology, with mutant stocks and a panoply of sophisticated techniques for following and manipulating cells and gene expression. Most revolutionary was the combination since the 1970s of the new molecular cloning with older methods of genetic screening and embryo manipulation (4). The investments of individuals and groups combined with the laboriously built-up advantages of resources, techniques, and colleagues to entrench model systems. These powerfully channeled research to the questions they were best suited to answer. Distinct communities specialized in different organisms, procedures, and phenomena.

A new model could be successfully launched only with the prospect of greater productivity and high-level support to achieve it within a reasonable time. This happened in the 1980s for the zebrafish *Danio rerio*. A pet-shop staple had in the 1970s been turned into an effective genetic organism that could be screened much faster than mice and developed in full view. By the 1980s its potential for combining genetics, experimental embryology, neuroanatomy, and cell-lineage analysis was clear. A research community was becoming established, when in the late 1980s senior *Drosophila* developmental geneticists alighted on the zebrafish as the most suitable vertebrate for the mass mutagenesis that had proved so transformative in flies. The results of a "big screen" in Tübingen and Boston, published in 1996, stimulated major investment by the NIH (9, 73).

Model organisms were never the whole story. Some developmental biologists insisted through the 1970s and 1980s on studying unfashionably difficult vertebrates, such as various fish, urodeles, turtles, crocodilians, and marsupials; some models were only locally important, for example, the teleost medaka in Japan. Things changed more profoundly when new approaches generated new questions and new methods made innovation easier.

6. Beyond Models?

From the 1990s the dominance of the few big embryological species was challenged in various ways, but these remain firmly ensconced and have in some ways become even more attractive. New organisms are emerging, while the old survive by being re-engineered and reconceived.

The discovery of deep molecular homologies across phyla breathed new life into studies of development and evolution that had continued through the twentieth century but most developmental biologists had scorned. Evolutionary developmental biology ("evo-devo") claims to revive and revise Haeckel's questions at the molecular level (74). In evo-devo and "eco-devo" or ecological developmental biology, species politics are more explicit than ever. Proponents critiqued over-reliance on model organisms on the grounds that precisely the qualities that had led to their selection, notably rapid, strongly canalized development that was resistant to environmental effects, made them unrepresentative of their own taxa, not to mention life beyond the laboratory walls (75). Funding the old models would just privilege the old reductionism, leaving evolution and ecology out of account. Conveniently, whole-genome sequencing and powerful new methods of functional analysis lowered the barrier to comparative studies.

The stakes are high as the NIH favors established models and the NSF promotes new ones (1). Defenders of old systems fight for continued recognition—one even wrote of "'anti-chick' racism" (60)—while reformers advertize their favorite organisms and debate selection criteria. The dog, with its enormous selected within-species variation, has been proposed as a model for studying evolutionary changes in regulatory genes. The contrast between eyed surface and eyeless cave-dwelling forms of the Mexican tetra *Astyanax mexicanus* is advocated as a model for evolutionary response to environmental variation (**Fig. 1.6**). These choices highlight conceptual themes, rather than simply picking diverse leaves from the phylogenetic tree (77).

Evo-devo and eco-devo were initially critical of models, but may accept them if reframed as organisms in their evolutionary and environmental contexts. Established models are even being repositioned not as sufficient surrogates for the rest of the animal kingdom, but as beachheads from which to explore phylogeny and ecology (1). Whether primarily oriented toward physiology, evolution or ecology, or trying to integrate all three perspectives, developmental biologists today share key methods.

In laboratories devoted to physiological mechanisms of development, the traditional models, with their better developed genome databases and stock centers, are also being enriched. On the one hand, more can now be done in any one species.

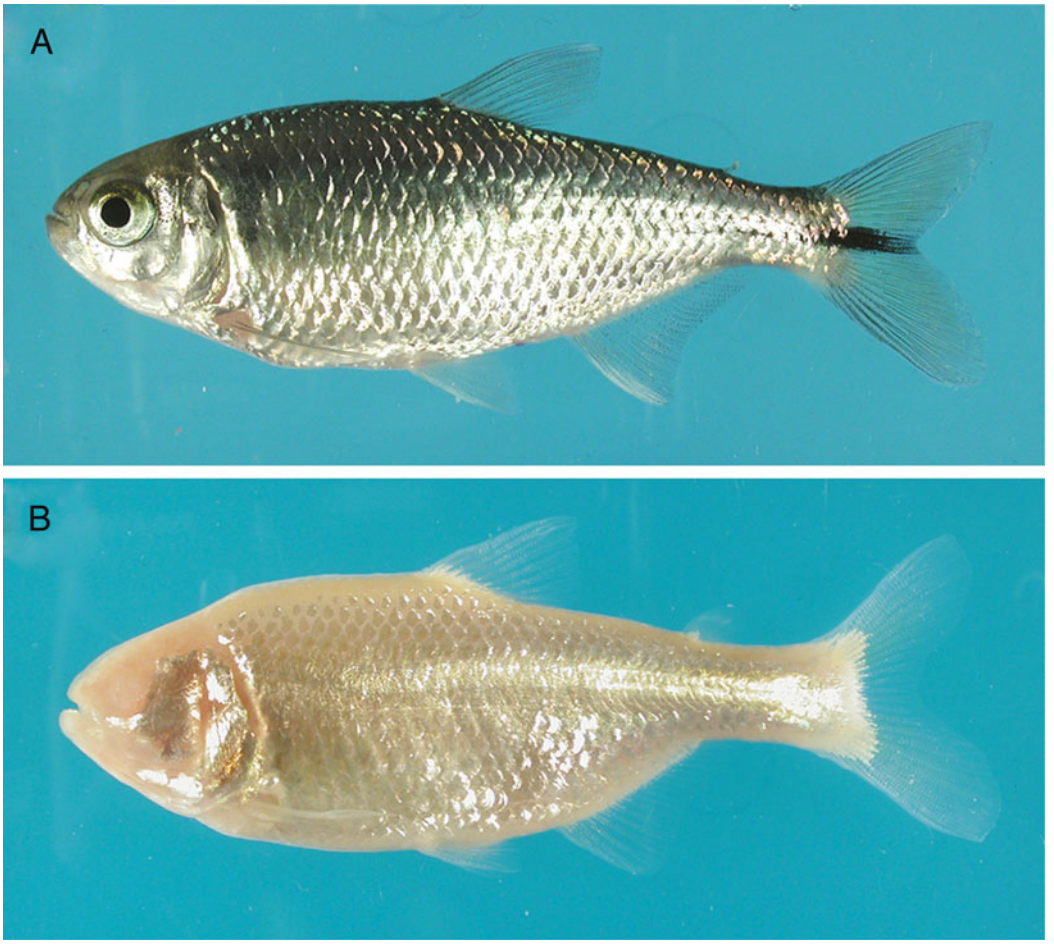

Fig. 1.6. The Mexican tetra, *Astyanax mexicanus*, a model for evolutionary response to environmental variation (*see* (76)). (**A**) Eyed surface fish, and (**B**) blind cavefish. Courtesy of William Jeffery, Department of Biology, University of Maryland.

Transgenic technology, for example, which initially only increased the genetic advantages of the mouse, has finally made it easier to do reverse genetics in frogs and chicks. For the former this has involved international cooperation to build resources for the previously little-used *Xenopus tropicalis*, a close relative of *X. laevis*, with a shorter generation time and smaller diploid genome (78). On the other hand, as several chapters in this volume show, researchers have in the last decade become more flexible and adventurous about using multiple species in any one project.

7. Conclusion

Scientists with different approaches have adapted different species for embryology. The most dramatic contrast is around 1900. Comparative evolutionary embryologists still traveled the world

to obtain lungfish, echidna, and apes, while developmental physiologists already devoted whole careers to experimenting systematically on the local amphibians. What is convenient for one kind of work may also suit another; Darwinists had previously dissected, sectioned, and modeled those same frogs and newts. But though existing knowledge and arrangements favor continuity, when much else is in flux long traditions are as remarkable as change. They depend on finding fresh advantages and withstanding new competition. Take the grand old man of embryological species, the chick. In 1835 Valentin advocated its use in preference to rare and often abnormal human specimens, but 40 years later Haeckel rejected it as phylogenetically misleading; it still played a significant role in teaching and as an experimental species, but recent defenders have had to fight for its privileged place in developmental biology.

The history of human embryos and their substitutes shows particularly clearly the play of continuity and change. Early and mid-nineteenth-century embryologists mostly studied chicks and domestic mammals as surrogates and also as more general representatives of vertebrate development. By contrast, post-Darwinists prided themselves on researching human embryos directly, exploited the rise of operative gynecology to investigate ever earlier stages, and even modeled studies of other mammals on the human work. Early developmental biology tended to ignore human embryos as experimentally intractable, while engineering the mouse as the principal "model for man." Experiments with this and other laboratory species made possible in vitro fertilization, which brought human embryos into laboratory and clinic. For some techniques they again led the way.

The range of actively researched species has varied a good deal. So has the rate at which new organisms have been domesticated for embryology and the height of the barriers between them. The chances of taxonomic innovation and of transfer between species, into as much as within embryology, depend on the perceived balance between difficulty and rewards. Obtaining scarce material from distant lands presented nineteenth-century comparative embryologists with a major challenge, even as improved transportation shrank the globe. But it could make a reputation and a little tinkering was usually enough to adapt standard histological methods. From the 1930s, pregnancy testing and genetics provided experimentalists with improved frogs, mice, and later fish, which developmental biologists then customized with specific methods and resources. By the 1970s and 1980s, problems, techniques, and resources seemed so segregated that the vast majority stuck to the model in which they had trained. Exemplary work on *Drosophila* and the universalizing effects of molecular cloning brought the field together. In the 1990s, more transferable methods and the prospect of tackling

new (and old) questions opened things up. But species preference is no simple cost–benefit calculation; it has an aesthetic dimension too: with what animals, and what other humans, does an embryologist wish to spend time?

8. Notes

1. For general references on the history of embryology, *see* (14); those given here are limited to the historical writing, or, where this is unavailable, selected primary sources, most relevant to questions of species and methods. The chapter does not attempt to explore the effects of species choice on embryological knowledge.
2. For the range, see the research topics and the "'Supply and demand' service for laboratory animals" listed in the Hubrecht Laboratory's *General Embryological Information Service*, which ran from 1949 to 1980.

Acknowledgments

For comments on a draft I thank Scott Gilbert, Martin Johnson, and Francisco Pelegri. Research was supported by the Wellcome Trust [074298].

References

1. Gilbert, S. F. (2009) The adequacy of model systems for evo-devo: modeling the formation of organisms/modeling the formation of society. In *Mapping the Future of Biology: Evolving Concepts and Theories,* vol. 266: *Boston Studies in the Philosophy of Science,* Barberousse, A., Morange, M. and Pradeu, T., Eds. Dordrecht: Springer Netherlands, 57–68.
2. Bolker, J. A. (2009) Exemplary and surrogate models: two modes of representation in biology. *Perspect. Biol. Med.* **52**, 485–499.
3. Kohler, R. E. (1994) *Lords of the Fly*: Drosophila *Genetics and the Experimental Life*. Chicago: University of Chicago Press.
4. Keller, E. F. (1996) *Drosophila* embryos as transitional objects: the work of Donald Poulson and Christiane Nüsslein-Volhard. *Hist. Stud. Phys. Biol. Sci.* **26**, 313–346.
5. Wourms, J. P. (1997) The rise of fish embryology in the nineteenth century. *Am. Zool.* **37**, 269–310.
6. de Chadarevian, S. (1998) Of worms and programmes: *Caenorhabditis elegans* and the study of development. *Stud. Hist. Phil. Biol. Biomed. Sci.* **29**, 81–105.
7. Gurdon, J. B. and Hopwood, N. (2000) The introduction of *Xenopus laevis* into developmental biology: of empire, pregnancy testing and ribosomal genes. *Int. J. Dev. Biol.* **44**, 43–50.
8. Rader, K. A. (2004) *Making Mice: Standardizing Animals for American Biomedical Research, 1900–1955.* Princeton: Princeton University Press.
9. Endersby, J. (2007) *A Guinea-Pig's History of Biology: The Plants and Animals Who Taught Us the Facts of Life*. London: Heinemann.

10. Hopwood, N. (2000) Producing development: the anatomy of human embryos and the norms of Wilhelm His. *Bull. Hist. Med.* **74**, 29–79.
11. Hopwood, N. (2007) A history of normal plates, tables and stages in vertebrate embryology. *Int. J. Dev. Biol.* **51**, 1–26.
12. Mitman, G. and Fausto-Sterling, A. (1992) Whatever happened to Planaria? C. M. Child and the physiology of inheritance. In *The Right Tools for the Job: At Work in Twentieth-Century Life Sciences,* Clarke, A. E. and Fujimura, J. H., Eds. Princeton: Princeton University Press, 172–197.
13. Newmark, P. A. and Alvarado, A. S. (2002) Not your father's planarian: a classic model enters the era of functional genomics. *Nat. Rev. Genet.* **3**, 210–219.
14. Hopwood, N. (2009) Embryology. In *The Cambridge History of Science,* vol. 6: *The Modern Biological and Earth Sciences,* Bowler, P. J. and Pickstone, J. V., Eds. Cambridge: Cambridge University Press, 285–315.
15. Remak, R. (1855) *Untersuchungen über die Entwickelung der Wirbelthiere.* Berlin: Reimer.
16. Rathke, H. (1825) Kiemen bey Säugethieren. *Isis*, 747–749.
17. Valentin, G. G. (1835) Foetus. In *Encyclopädisches Wörterbuch der medicinischen Wissenschaften,* vol. 12, Busch, D. W. H. et al., Eds. Berlin: Veit, 355–389.
18. Rathke, H. (1839) *Entwickelungsgeschichte der Natter* (Coluber natrix). Königsberg: Gebrüder Borntträger.
19. Bischoff, T. L. W. (1854) *Entwicklungsgeschichte des Rehes.* Giessen: Ricker.
20. Lurie, E. (1988) *Louis Agassiz: A Life in Science.* Baltimore, MD: Johns Hopkins University Press.
21. Balfour, F. M. (1881) *A Treatise on Comparative Embryology*, vol. 2. London: Macmillan.
22. Gould, S. J. (1977) *Ontogeny and Phylogeny.* Cambridge, MA: Harvard University Press, Belknap Press.
23. Buklijas, T. and Hopwood, N. (2008) *Making Visible Embryos*, an online exhibition, http://www.hps.cam.ac.uk/visibleembryos/.
24. Haeckel, E. (1875) *Ziele und Wege der heutigen Entwickelungsgeschichte.* Jena: Dufft.
25. Foster, M. and Balfour, F. M. (1883) *The Elements of Embryology,* 2nd edition, edited by Sedgwick, A. and Heape, W. London: Macmillan.
26. MacLeod, R. (1994) Embryology and empire: the Balfour Students and the quest for intermediate forms in the laboratory of the Pacific. In *Darwin's Laboratory: Evolutionary Theory and Natural History in the Pacific,* MacLeod, R. and Rehbock, P. F., Eds. Honolulu: University of Hawai'i Press, 140–165.
27. Bowler, P. J. (1996) *Life's Splendid Drama: Evolutionary Biology and the Reconstruction of Life's Ancestry, 1860–1940.* Chicago: University of Chicago Press.
28. Blackman, H. (2007) Lampreys, lungfish and elasmobranchs: Cambridge zoology and the politics of animal selection. *Brit. J. Hist. Sci.* **40**, 413–437.
29. Hall, B. K. (2009) Embryos in evolution: evo-devo at the Naples Zoological Station in 1874. *Theory Biosci.* **128**, 7–18.
30. Selenka, E. (1887) *Studien über Entwickelungsgeschichte der Thiere*, no 4: *Das Opossum* (Didelphys virginiana). Wiesbaden: Kreidel.
31. Hall, B. K. (2001) John Samuel Budgett (1872–1904): in pursuit of *Polypterus. BioScience* **51**, 399–407.
32. Semon, R. (1896) *Im australischen Busch und an den Küsten des Korallenmeeres. Reiseerlebnisse und Beobachtungen eines Naturforschers in Australien, Neu-Guinea und den Molukken.* Engelmann: Leipzig.
33. Semon, R. (1894) *Zoologische Forschungsreisen in Australien und dem Malayischen Archipel,* vol. 2: *Monotremen und Marsupalier I* (*Denkschriften der Medicinisch-naturwissenschaftlichen Gesellschaft zu Jena*, vol. 5). Jena: Fischer.
34. Kükenthal, W. (1893) *Vergleichend-anatomische und entwickelungsgeschichtliche Untersuchungen an Walthieren (Denkschriften der Medicinisch-naturwissenschaftlichen Gesellschaft zu Jena*, vol. 3). Jena: Fischer.
35. Selenka, E. (1903). *Studien über Entwickelungsgeschichte der Tiere*, no. 10 *(Menschenaffen [Anthropomorphae]. Studien über Entwickelung und Schädelbau*, no. 5): *Zur vergleichenden Keimesgeschichte der Primaten*, Keibel, F., Ed. Wiesbaden: Kreidel.
36. Wilson, E. A. (1907) Aves. In *National Antarctic Expedition, 1901–1904*, section 1: *Natural History*, vol. 2: *Zoology (Vertebrata: Mollusca: Crustacea)*. London: British Museum.
37. Raff, R. A. (1996) *The Shape of Life: Genes, Development, and the Evolution of Animal Form.* Chicago: University of Chicago Press.
38. Whitman, C. O. (1885) *Methods of Research in Microscopical Anatomy and Embryology.* Boston: Cassino.
39. Röthig, P. (1904) *Handbuch der embryologischen Technik.* Wiesbaden: Bergmann.

40. Hopwood, N. (2002) *Embryos in Wax: Models from the Ziegler Studio, with a Reprint of "Embryological Wax Models" by Friedrich Ziegler*. Cambridge: Whipple Museum of the History of Science, Bern: Institute of the History of Medicine.
41. Clarke, A. E. (1987) Research materials and reproductive science in the United States, 1910–1940. In *Physiology in the American Context, 1850–1940,* Geison, G. L., Ed. Bethesda, MD: American Physiological Society, 323–350.
42. Maienschein, J., Glitz, M. and Allen, G. E. Eds. (2004) *Centennial History of the Carnegie Institution of Washington*, vol. 5: *The Department of Embryology.* Cambridge: Cambridge University Press.
43. Morgan, L. M. (2009) *Icons of Life: A Cultural History of Human Embryos.* Berkeley: University of California Press.
44. Hanson, E. (2004) How rhesus monkeys became laboratory animals. In *Centennial History of the Carnegie Institution of Washington,* vol. 5: *The Department of Embryology,* Maienschein, J., Glitz, M. and Allen, G. E., Eds. Cambridge: Cambridge University Press, 63–81.
45. Nyhart, L. K. (1995) *Biology Takes Form: Animal Morphology and the German Universities, 1800–1900.* Chicago: University of Chicago Press.
46. Peter, K. (1904) *Normentafel zur Entwicklungsgeschichte der Zauneidechse* (Lacerta agilis). Jena: Fischer.
47. Richardson, M. K. and Narraway, J. (1999) A treasure house of comparative embryology. *Int. J. Dev. Biol.* **43**, 591–602.
48. Abderhalden, E. Ed. (1923) *Handbuch der biologischen Arbeitsmethoden*, section V: *Methoden zum Studium der Funktionen der einzelnen Organe im tierischen Organismus*, part 3, A: *Methodik der Entwicklungsmechanik*. Berlin and Vienna: Urban & Schwarzenberg.
49. Hamburger, V. (1942) *A Manual of Experimental Embryology.* Chicago: University of Chicago Press.
50. Sander, K. (1994) An American in Paris and the origins of the stereomicroscope. *Roux Arch. Dev. Biol.* **203**, 235–242.
51. Gilbert, S. F. (2007) Fate maps, gene expression maps, and the evidentiary structure of evolutionary developmental biology. In *From Embryology to Evo-Devo: A History of Developmental Evolution,* Laubichler, M. D. and Maienschein, J., Eds. Cambridge, MA: MIT Press, 357–374.
52. Rugh, R. (1948) *Experimental Embryology: A Manual of Techniques and Procedures*, revised edition. Minneapolis, MN: Burgess.
53. Willier, B. H., Weiss, P. A. and Hamburger, V. Eds. (1955) *Analysis of Development.* Philadelphia: Saunders.
54. Clarke, A. E. (1998) *Disciplining Reproduction: American Life Sciences and "The Problems of Sex."* Berkeley: University of California Press.
55. Churchill, F. B. (1997) Life before model systems: general zoology at August Weismann's institute. *Am. Zool.* **37**, 260–268.
56. Fäßler, P. E. (1997) *Hans Spemann 1869–1941. Experimentelle Forschung im Spannungsfeld von Empirie und Theorie. Ein Beitrag zur Geschichte der Entwicklungsphysiologie zu Beginn des 20. Jahrhunderts.* Berlin: Springer.
57. Wourms, J. P. (2007) The relations between comparative embryology, morphology, and systematics: an American perspective. In *From Embryology to Evo-Devo: A History of Developmental Evolution,* Laubichler, M. D. and Maienschein, J., Eds. Cambridge, MA: MIT Press, 215–266.
58. Wilt, F. H. and Wessells, N. K. Eds. (1967) *Methods in Developmental Biology.* New York: Crowell.
59. Davies, J. A. (2007) Developmental biologists' choice of subjects approximates to a power law, with no evidence for the existence of a special group of "model organisms." *BMC Dev. Biol.* **7**, 40.
60. Stern, C. D. (2004) The chick embryo: past, present and future as a model system in developmental biology. *Mech. Dev.* **121**, 1011–1013.
61. Le Douarin, N. M. (2004) The avian embryo as a model to study the development of the neural crest: a long and still ongoing story. *Mech. Dev.* **121**, 1089–1102.
62. Graham, C. (2000) Mammalian development in the UK (1950–1995). *Int. J. Dev. Biol.* **44**, 51–55.
63. Alexandre, H. (2001) A history of mammalian embryological research. *Int. J. Dev. Biol.* **45**, 457–467.
64. Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. G., Birnberg, N. C. and Evans, R. M. (1982) Dramatic growth of mice that develop from eggs microinjected with metallothionein–growth hormone fusion genes. *Nature* **300**, 611–615.
65. Betteridge, K. J. (2003) A history of farm animal embryo transfer and some associated techniques. *Anim. Reprod. Sci.* **79**, 203–244.
66. Biggers, J. D. (1984) In vitro fertilization and embryo transfer in historical perspective. In *In Vitro Fertilization and Embryo*

Transfer, Trounson, A. O. and Wood, C., Eds. London: Churchill Livingstone, 3–15.
67. Henig, R. M. (2004) *Pandora's Baby: How the First Test Tube Babies Sparked the Reproductive Revolution*. Boston: Houghton Mifflin.
68. Franklin, S. (2007) *Dolly Mixtures: The Remaking of Genealogy*. Durham, NC: Duke University Press.
69. Short, R. V. (1972) Species differences. In *Reproduction in Mammals,* book 4: *Reproductive Patterns,* Austin, C. R. and Short, R. V., Eds. Cambridge: Cambridge University Press, 1–33.
70. Wildt, D. E. and Wemmer, C. (1999) Sex and wildlife: the role of reproductive science in conservation. *Biodivers. Conserv.* **8**, 965–976.
71. Holt, W. V., Pickard, A. R. and Prather, R. S. (2004) Wildlife conservation and reproductive cloning. *Reproduction* **127**, 317–324.
72. Friese, C. (2009) Models of cloning, models for the zoo: rethinking the sociological significance of cloned animals. *BioSocieties* **4**, 367–390.
73. Grunwald, D. J. and Eisen, J. S. (2002) Headwaters of the zebrafish: emergence of a new model vertebrate. *Nat. Rev. Genet.* **3**, 717–724.
74. Laubichler, M. D. and Maienschein, J. Eds. (2007) *From Embryology to Evo-Devo: A History of Developmental Evolution*. Cambridge, MA: MIT Press.
75. Bolker, J. A. (1995) Model systems in developmental biology. *BioEssays* **17**, 451–455.
76. Jeffery, W. R. (2001) Cavefish as a model system in evolutionary developmental biology. *Dev. Biol.* **231**, 1–12.
77. Jenner, R. A. and Wills, M. A. (2007) The choice of model organisms in evo-devo. *Nat. Rev. Genet.* **8**, 311–319.
78. Smith, J. C. (2005) *Xenopus* genetics and genomics. *Mech. Dev.* **122**, 259–262.

Chapter 2

Manipulating and Imaging the Early *Xenopus laevis* Embryo

Michael V. Danilchik

Abstract

Over the past half century, the *Xenopus laevis* embryo has become a popular model system for studying vertebrate early development at molecular, cellular, and multicellular levels. The year-round availability of easily fertilized eggs, the embryo's large size and rapid development, and the hardiness of both adults and offspring against a wide range of laboratory conditions provide unmatched advantages for a variety of approaches, particularly "cutting and pasting" experiments, to explore embryogenesis. There is, however, a common perception that the *Xenopus* embryo is intractable for microscope work, due to its store of large, refractile yolk platelets and abundant cortical pigmentation. This chapter presents easily adapted protocols to surmount, and in some cases take advantage of, these optical properties to facilitate live-cell microscopic analysis of commonly used experimental manipulations of early *Xenopus* embryos.

Key words: *Xenopus laevis*, embryo, dorsal–ventral axis, cytoskeleton, time-lapse microscopy, live-cell confocal imaging.

1. Introduction

Xenopus laevis offers numerous attractions as an experimental model for early vertebrate development (1). The adults are easy to maintain in the laboratory. Unlike many amphibians, gravid *Xenopus* females can be induced by hormone injection to spawn at any time of year, and they produce several thousands of eggs at each spawning. Eggs are easily fertilized, and embryos can be cultured under non-sterile, table-top, room-temperature conditions to feeding-stage tadpoles in less than a week. With a little care, tadpoles can be reared through metamorphosis to produce froglets in 6 or 7 weeks. A well-documented normal table provides an anatomic description of the entire range of embryonic

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_2,

stages from fertilization through metamorphosis (2) (*see* also http://www.xenbase.org/anatomy/).

This chapter describes basic methods and tools used for culturing, experimentally manipulating, and imaging early *Xenopus* embryos. Several methods are presented for live-embryo analysis of experimental manipulations, emphasizing some of the main experimental advantages of the early *Xenopus* embryo: its great size, relatively consistent cleavage pattern, and tolerance to a wide variety of experimental perturbations. These properties facilitate the microdissection and explantation of specific cells or tissue layers and make possible the introduction—via external exposure or targeted microinjection—of various reagents, including small molecules, antibodies, mRNAs, and morpholinos, to interfere with various signaling pathways important for tissue specification and body axis formation. Because tissue specification, early morphogenesis, and body axis formation are integrated within a brief developmental window, perturbations of many of the relevant pathways result in a characteristic array of axial defects that become evident by early tadpole stage (3). Thus, the impact of a perturbation—or the effectiveness of its rescue—can be determined by visual assay within 2 days.

Although the size and opacity of the *Xenopus* embryo present some unique optical challenges, the results of many experimental perturbations are easiest to analyze via direct observation using a stereomicroscope. Incipient body axes can be recognized from very early stages because of consistent regional pigmentation differences that develop shortly after fertilization. This natural marking has facilitated the development of comprehensive fate maps (4–6) as well as maps of the prospective movements of both deep and superficial tissues during gastrulation (7, 8) that make possible lineage-specific perturbations. The cytoplasm of *Xenopus* eggs is filled with yolk platelets—large (2–15 μm) membrane-bound inclusions that provide each cell with its own nutrient and energy store that can sustain isolated blastomeres and tissue explants in culture for days. Yolk platelets are highly refractile, and their collective light scattering render the cytoplasm essentially opaque. While this property—as well as the embryo's great size—makes it difficult to view the deep contents of most cells, the opacity itself makes possible direct visualization of live cells without the need for fluorochromes or vital dyes (9, 10). Autofluorescence of yolk in fixed embryos provides an excellent fluorescent-cytoplasm background for confocal analysis of embryos subjected to axis-perturbing treatments (11, 12).

Despite their opacity, early *Xenopus* cells and tissues make excellent subjects for live-cell imaging, particularly via confocal microscopy. Many of the morphogenetically dynamic events of cleavage, gastrulation, and neurulation, including membrane protrusive activity (10, 13–15); cytoskeletal rearrangements during

wound healing (16); cortical rotation (17) and cleavage furrow formation (18–20); and microtubule-dependent localization of cortical determinants (21, 22), happen to occur near the cell surface. These events are easily captured via confocal time lapse in embryos expressing GFP constructs or labeled with fluorescent lipid dyes.

2. Materials

2.1. Experimental Tools and Equipment

1. Stereomicroscope (*see* **Note 1**).
2. Fiber-optic illuminator (*see* **Note 2**).
3. Temperature control (*see* **Note 3**).
4. Watchmaker's forceps (e.g., Dumont #5, Fine Science Tools) (*see* **Note 4**).
5. X-Y-Z micromanipulator (Narishige M-152 or equivalent) on magnetic stand.
6. Borosilicate glass Pasteur pipets and latex rubber bulbs.
7. Wax: a small block of any food-grade wax, such as dental wax, Gouda cheese covering, or beeswax.
8. A few thin, straight-shafted human hairs and full-length eyebrow hairs for hair loops and eyebrow knives.
9. Alcohol lamp and lighter.
10. Diamond-tipped pencil (e.g., Ted Pella).
11. Agarose, high gelling temperature Agarose Type V (Sigma): 1% w/v in MMR/3.
12. Polystyrene dishes, 60 mm × 15 mm and 35 mm × 10 mm.
13. Nylon snap caps from 2 dram shell vials.
14. Glass coverslips, 22 mm × 22 mm #1 and 24 mm × 40 mm #1.5.
15. Glass depression slides, 3.2 mm thick (Ward's).
16. Fraction collector tube rack (Gilson "Code 1" rack).

2.2. Fertilization and Embryo Culture

1. MMR (Marc's modified Ringers): 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, and 5 mM HEPES (pH 7.4). The original recipe for this medium (23) included 0.1 mM EDTA, now usually omitted. MMR is used at full strength (1× MMR) as a tissue isotonic medium for organ (e.g., testis or oocyte) culture and in diluted form (e.g., MMR/3) as a pondwater substitute for fertilizing eggs, culturing early embryos, and rearing early tadpoles.

2. Testis solution: 10% fetal bovine serum and 0.25 μg/mL gentamycin in 1× MMR.
3. Dejellying solution: 2.5% cysteine, pH 8.0–8.5 in MMR/3. In a 50 mL beaker with a magnetic stir bar, dissolve 1 g of L-cysteine in 40 mL MMR/3. Add 6 drops of 10 N NaOH. 40 mL dejellying solution should be sufficient to dejelly as many as 5 or 6 large spawnings. Cover tightly with Parafilm^{TM} and use within ~8 h. Discard any remaining cysteine solution at the end of the day, as prolonged exposure to atmospheric oxygen produces a tough-to-remove precipitate.
4. DeBoer's solution: 110 mM NaCl, 1.3 mM KCl, and 0.44 mM $CaCl_2$, pH 7.2–7.4, adjusted with $NaHCO_3$. Diluted to 1/20× for rinsing away cysteine at the end of the dejellying procedure.
5. Blastocoel buffer (24): 53 mM NaCl, 15 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 3.6 mM Na_2CO_3, 4.5 mM potassium gluconate, 23.4 mM sodium isethionate, 1 mg/mL bovine serum albumin, and 5 mM bicine, pH 8.3. Filter sterilize and store in 50 mL aliquots.
6. Cell dissociation medium (calcium- and magnesium-free medium (25)): 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, and 7.5 mM Tris–HCl, pH 7.6.
7. Human chorionic gonadotropin (hCG; Sigma). Lyophilized hCG is reconstituted to 4,000 U/mL with sterile water and stored at 4°C for up to 1 month.
8. Pyrex petri dishes, 60 mm × 15 mm.
9. Stopwatch.
10. Pasteur pipets.
11. Sample pestle and matching 1.5 mL Eppendorf tube.

2.3. Experimental Manipulations

In addition to embryos, general materials, tools, and equipment listed above, specific items required for particular experimental procedures are listed below.

2.3.1. Ventralizing Embryos with UV Irradiation

1. UV lamp (UVP, Inc. Mineralite; or Cole-Parmer 254/365 nm 4 watt; or equivalent).
2. Eye protection goggles (rated for short-wave UV).

2.3.2. Dorsalizing Embryos by Exposure to LiCl

1. Lithium chloride solution for external exposure: 300 mM LiCl in MMR/3.
2. Lithium chloride solution for microinjection: 300 mM LiCl in water.

2.3.3. Vitelline Envelope Removal

1. Two good pairs of watchmaker's forceps.
2. Agarose-coated polystyrene dishes with melted 1 mm wells, constructed as described in **Section 3.3.3**.

2.3.4. Microinjection

1. Microinjection gas pressure delivery system (Medical Systems PL1-100 Pico-injector® or equivalent).
2. X-Y-Z micromanipulator (Narishige M-152 or equivalent) on magnetic stand.
3. Micropipet puller (Sutter P-97 or equivalent).
4. Pulled micropipets from ~1 mm capillary tubing (Narishige, Drummond, and Sutter all supply good-quality borosilicate glass). Pulled micropipets should taper gradually over about 1 cm length to a tip whose outer diameter is about 10 μm. Tips will be clipped off during injection calibration, so it is not important whether they are initially open or closed.
5. A good pair of watchmaker's forceps.
6. Polystyrene dish.
7. 2 cm × 4 mm strip of glass cut from a microscope slide.
8. Vacuum grease (e.g., Dow-Corning, high vacuum).
9. Ficoll solution: Ficoll 400-L, MW 40,000 (Sigma): 6% w/v in MMR/3.
10. Stage micrometer, ruled with 10 μm intervals (Graticules Ltd. PS8, 100 × 0.01 1 mm, or equivalent).

2.3.5. Dorsal Marginal Zone ("Keller") Explants

1. Agarose-coated polystyrene dishes with melted 1 mm wells, constructed as described in **Section 3.1**, Step 5.
2. Watchmaker's forceps.
3. Braking pipet (**Section 3.1**, Step 1).
4. Hair loop (**Section 3.1**, Step 2).
5. Eyebrow knife (**Section 3.1**, Step 3).
6. Blastocoel buffer (**Section 2.2**).
7. Pasteur pipet.
8. Depression slide.
9. Modeling clay.
10. 2 mm × 4 mm strip cut from #1 coverslip.
11. Vacuum grease.
12. Stereomicroscope or confocal microscope (10, 26).

2.4. Time-Lapse Stereomicroscopy

1. Time-lapse image capture system (*see* **Note 5**).
2. Depression slides.

3. Coverslips.
4. Modeling clay.
5. ImageJ software (available gratis at http://rsbweb.nih.gov/ij/).
6. Quicktime Pro (v. 7.x.x) or equivalent authoring software (upgrade available for both Mac and PC). Note that Quicktime X (bundled with Mac OS Snow Leopard) cannot presently be upgraded to Pro to enable authoring/editing functions: reinstall v. 7.x, which can be registered and upgraded.

2.5. Whole-Mount Confocal Microscopy

2.5.1. Fixation and Staining for Microtubules

1. Formaldehyde/glutaraldehyde fixative ("FG fix") buffer: 80 mM K-PIPES, pH 6.8, 5 mM EGTA, and 1 mM $MgCl_2$. Avoid sodium: use KOH to adjust pH (adapted from (27)).
2. Formaldehyde (37% stock; commercial formalin).
3. Glutaraldehyde (sealed ampules of 25% stock, EM grade; Ted Pella).
4. Tris-buffered saline (1× NTBS): 155 mM NaCl, 10 mM Tris–HCl, pH 7.4, and 0.1% Nonidet P-40.
5. Phosphate-buffered saline (1× PBS): 128 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.2.
6. Bleaching solution: 10% HOOH in 67% MeOH. Add 1 vol fresh 30% HOOH to 2 vol MeOH.
7. Borohydride solution: 100 mM $NaBH_4$.
8. Primary antibody working solution: 1× NTBS, 10% fetal bovine serum (FBS), 5% DMSO, and mouse anti-α-tubulin (Sigma; DM1A), diluted 1:1,000.
9. Secondary antibody working solution: 1× NTBS, 10% fetal bovine serum, 5% DMSO, and Alexa-conjugated anti-mouse IgG (Sigma), diluted 1:100 in FBS solution.
10. Rocking platform or nutator.
11. Absolute methanol.
12. Murray's Clear (benzyl benzoate:benzyl alcohol::2:1 v/v).
13. Upright or inverted confocal microscope.
14. Observation chambers, constructed as described in **Section 3.1**, Steps 6–7.

2.5.2. Fixation and Staining for Microfilaments

1. FG fix buffer. *See* **Section 2.5.1**, Step 1 (Adapted from (27)).
2. Formaldehyde (8% stock solution). Commercial formalin is not used for this solution; better results are obtained with freshly made paraformaldehyde. For 200 mL of stock, heat

180 mL distilled H_2O to 55°C—do not exceed 60°C! Using a fume hood, weigh 16.84 g paraformaldehyde (EMS). Add to the heated water, cover beaker with Parafilm, and stir for 10 min. Add drops of 1 M NaOH until the solution clears. Adjust pH to 7.40 with HCl. Filter through Whatman #2 filter paper. Add dH_2O to 200 mL. Store at –80°C in 50 mL tubes—25 mL per tube.

3. Glutaraldehyde (sealed ampules of 25% stock, EM grade; Ted Pella).
4. Tris-buffered saline (1× NTBS): 155 mM NaCl, 10 mM Tris–HCl, pH 7.4, and 0.1% Nonidet P-40.
5. Phalloidin working solution: NTBS containing 5% DMSO and 2 units/mL Alexa-543 phalloidin (Invitrogen). Because Alexa-phalloidin is supplied as a methanol stock, it must be dried under vacuum in a spin-vac and then reconstituted at desired concentrations in NTBS/DMSO.
6. Rocking platform or nutator.
7. Upright or inverted confocal microscope.
8. Observation chambers, constructed as described in **Section 3.1**, Steps 6–7.

2.6. Live-Cell Confocal Microscopy

2.6.1. Time Lapse of Aggregating Germ Plasm

1. Inverted confocal microscope.
2. $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide; Invitrogen or Kodak): 1 mg/mL in anhydrous ethanol, frozen at –20°C in 10 μL aliquots.
3. Coverslip-bottomed observation chamber (**Section 3.1**, Step 6).

2.6.2. Time Lapse of Cortical Vesicle Translocation at the Embryo's Equator

1. Upright confocal microscope.
2. $DiOC_6(3)$: 1 mg/mL in anhydrous ethanol, frozen at –20°C in 10 μL aliquots.
3. Polystyrene dish.
4. Right-angle prism (Newport or Melles Griot right-angle prism, A = B = C = 5.0 mm with aluminized hypotenuse).
5. Modeling clay.

2.6.3. Imaging Filopodia in the Blastocoel of Embryos Expressing GFP-Mem

1. GFP construct mRNA (~1 μg/μL), frozen at –20°C in 3–5 μL aliquots. Protocols for transcribing and capping synthetic mRNAs from pCS2 plasmids via mMessage mMachine (Ambion) are provided in **Chapter 3**, this volume.
2. Microinjection gear, as listed in **Section 2.3.4**.
3. Watchmaker's forceps.
4. Braking pipet (**Section 3.1**, Step 1).
5. Hair loop (**Section 3.1**, Step 2).

6. Blastocoel buffer (**Section 2.2**).
7. Depression slide.
8. 22 mm × 22 mm coverslip.
9. Modeling clay.
10. Upright confocal microscope.

2.6.4. Time Lapse of FM1-43-Stained Filopodia Within Blastocoel

1. Polystyrene petri dish with agarose bottom: 1% agarose is dissolved in calcium-free cell dissociation medium.
2. Cell dissociation medium.
3. Watchmaker's forceps.
4. Braking pipet (**Section 3.1**, Step 1).
5. Hair loop (**Section 3.1**, Step 2).
6. Blastocoel buffer (**Section 2.2**).
7. FM1-43 (Invitrogen): 40 mM in water, frozen at –20°C in 10 μL aliquots.
8. Depression slide.
9. 22 mm × 22 mm coverslip.
10. Modeling clay.
11. Upright confocal microscope.

3. Methods

3.1. Experimental Tools and Equipment

In addition to a stereomicroscope and accessory gear for illumination, temperature control, microinjection, and image capture, some handbuilt tools are needed for a wide range of experimental protocols. These items include (a) tools for pipetting, nudging, and cutting embryo or tissue explants and (b) various kinds of observation chambers to accommodate different kinds of microscopes. A collection of these easily built tools and chambers should be on hand in advance of most experiments.

1. Braking pipet. A braking pipet is a transfer pipet with a narrow aperture or constriction to permit slow-volume fluid transfer in the vicinity of easily damaged explants or devitellinated embryos. Melt a Pasteur pipet near its tip with an alcohol lamp to provide an ergonomic 20 or 30° bend (**Fig. 2.1a**). The bent tip is then remelted, drawn out with a pair of blunt forceps, and cut with a diamond pencil to produce a taper (**Fig. 2.1b**) which can be fire-polished to close down its aperture to desired size.
2. Hair loop. Prepare a tapered, bent Pasteur pipet handle as above. Heat the tip again and insert it briefly into a block of wax to melt and draw up a few microliters of molten

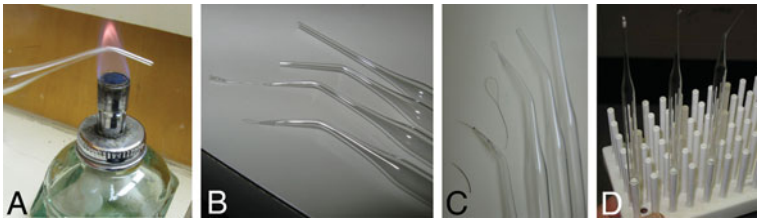

Fig. 2.1. Preparing and storing hair loops and eyebrow knives. (**a**) Bending the pipet shaft; (**b**) shaft is pulled out and cut with a diamond pencil to produce a tapered end; (**c**) hair loop or eyebrow hair is inserted into tapered end and immobilized with molten wax; (**d**) tools are stored upright on a pegged tube rack.

wax. Prepare a hair loop (**Fig. 2.1c**) by folding a hair in half and then twisting the ends between thumb and forefinger. Finally, remelt the wax and insert the twisted ends of the hair into the tapered tip and hold it in place a few seconds until the wax hardens.

3. Eyebrow knife. Prepare a tapered, bent Pasteur pipet handle as above and insert into it a long, relatively straight, uncut eyebrow hair. Leave about 3 mm of its length protruding from the end of the pipet.
4. Tool rack. Tools are stored upright on a pegged fraction-collector rack (**Fig. 2.1d**).
5. Agarose-coated culture dishes. Clean polystyrene is too sticky for culturing small tissue explants or dissociated cells. A nearly frictionless surface suitable for explant culture is generated by pouring 1 or 2 mL of molten 1% (w/v) agarose in MMR/3 into polystyrene dishes. Round-bottomed depressions of approximately 1 mm diameter are then melted into the agarose surface with the fire-polished tip of a 100 μL glass capillary tube to provide support for explants and devitellinated embryos (**Fig. 2.2**). Agarose dishes can be stored at 4°C for up to several weeks if covered and wrapped tightly with a strip of Parafilm.
6. Observation chambers for inverted compound microscopy. An inexpensive, disposable observation chamber is quickly constructed from a nylon shell-vial snap cap and coverslip (**Fig. 2.3**). Cap is carefully cut with a fresh razor blade to produce a thin-walled cylinder. The cylinder is dipped briefly in molten wax and placed on a clean #1.5 coverslip. The coverslip is then quickly passed through an alcohol lamp flame to reheat the wax, which flows around the lip of the cylinder, sealing it to the glass. The chamber is deep enough to accommodate forceps and hair loops, so last minute manipulations of live specimens can be performed in situ. The wax seal resists Murray Clear for several hours; this disposable chamber is therefore ideal for observing cleared

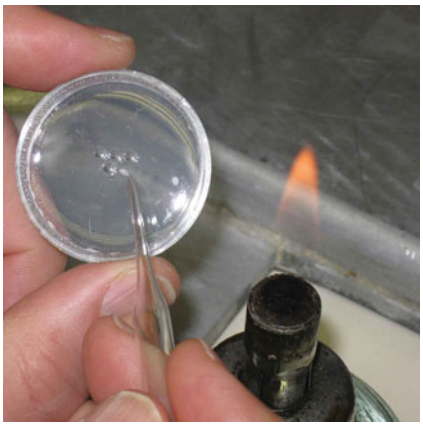

Fig. 2.2. Melting rounded depressions into an agarose surface.

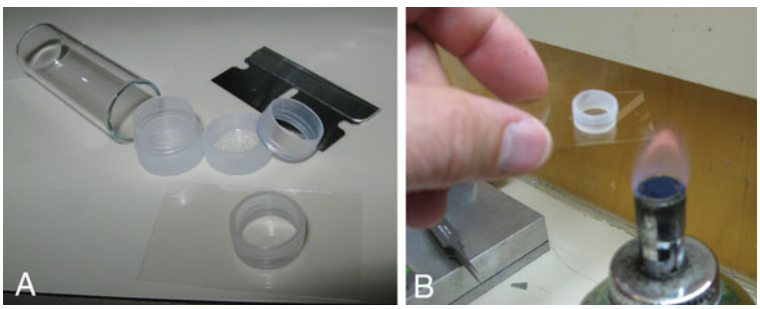

Fig. 2.3. Constructing an inverted-microscope observation chamber. (**a**) Nylon cap from shell vial is cut with razor. Cylinder is dipped briefly in molten wax and placed on clean coverslip. (**b**) Coverslip is briefly flamed to reheat wax which seals lip of cap to the glass. The wax seal is suitable for all aqueous media as well as Murray Clear.

specimens via inverted epifluorescence compound or confocal microscopy.

7. Observation chambers for upright compound microscopy. Cleared, whole-mount specimens, or cut fragments thereof, can be observed via a hanging drop method. A specimen, for example a cleared embryo half-stained via whole-mount immunocytochemistry (**Section 3.5**), is placed on a 22 mm × 22 mm coverslip, cut surface facing the glass, with a small droplet of Murray Clear surrounding the specimen. The coverslip is then inverted with the sample hanging by surface tension and placed over the well of a deep depression slide (**Fig. 2.4b**). This method works well for high-magnification, high-NA objectives because the surface tension of the drop (**Fig. 2.4c**) holds the specimen closely and stably near the optical surface of the coverslip.

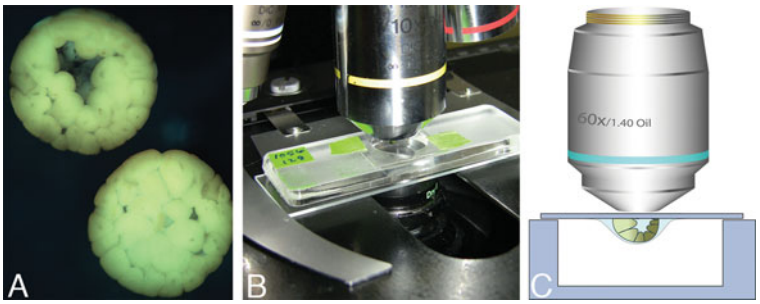

Fig. 2.4. Hanging drop method for upright confocal analysis of whole-mount specimens prepared for immunofluorescence detection. (**a**) Fixed embryo is cut in half with razor blade prior to staining, washing, and clearing. (**b**) Cleared embryo half is placed in a drop of Murray Clear on coverslip and flipped so that the cut surface is facing glass. The coverslip is inverted over a deep depression slide for confocal observation. (**c**) The surface tension of Murray Clear stably immobilizes specimen against the coverslip.

3.2. Fertilization and Embryo Culture

Collection of gametes, fertilization, and dejellying are described in extensive detail in **Chapter 3**, this volume. The abbreviated protocol below includes some variations on those techniques, reflecting the inherent tolerance of the *Xenopus* embryo to a wide variety of laboratory conditions.

1. Adult female *X. laevis* are induced to spawn by injecting 150 μL of 4,000 U/mL hCG solution (600 IU) into the dorsal lymph sac. Frogs are kept at 16–21°C overnight in their usual colony tank water in 1 gallon Tupperware® food containers with snap-secured lids which are perforated to allow adequate air exchange. At 16°C, spawning usually commences 12–14 h post-injection; at 21°C, spawning takes only about 8 h.
2. Spawning frogs are gently squeezed around the torso, mimicking the action of an amplexing male (*see* **Chapter 3**, this volume, for details on how to hold a frog). Eggs are extruded into a dry petri dish. Depending on the experiment, one may choose to collect as few as a dozen to as many as several thousand eggs for synchronous development from a single fertilization.
3. An ~1 mm thick fragment sliced off the end of the cultured testis (*see* **Chapter 3** for details of how to obtain testes) is macerated with a conical tissue grinder in a 1.5 mL Eppendorf tube containing ~1 mL MMR/3. This sperm suspension is immediately poured over the eggs, and a stopwatch is started to keep track of time elapsed since fertilization. The dish is gently tipped back and forth for about 30 s to ensure that all eggs are in contact with the sperm suspension. The dish is then filled with about 8 mL MMR/3 and allowed to stand for 20 min. During this time, eggs will exhibit signs of activation first by contracting the pigmented cap toward

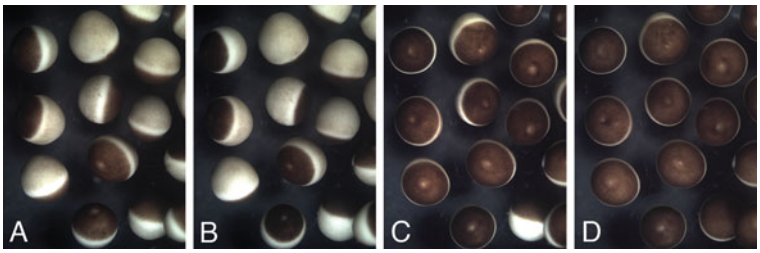

Fig. 2.5. Activation and righting of fertilized *Xenopus laevis* eggs. (**a**) Unfertilized eggs deposited in random orientations on substrate; (**b**) 8 min post-fertilization. Pigmented animal caps have contracted around their animal poles; (**c**) 16 min post-fertilization. Embryos have almost fully rotated within their fertilization (vitelline) envelopes; (**d**) 24 min post-fertilization. Animal caps have relaxed to 50% of the egg surface, and eggs have fully righted themselves.

the animal pole (**Fig. 2.5a, b**) and then by rotating within their vitelline envelope until the animal cap points upward (**Fig. 2.5c, d**).

4. Twenty minutes post-fertilization, dejelly the eggs by replacing the MMR/3 with fresh dejellying solution. Gently rock or agitate the dish for approximately 5 min until the jelly coat fully dissolves and embryos can settle closely together. Thoroughly rinse the dish of embryos with four or five exchanges of DeBoer's solution (1/20×) and then two exchanges of MMR/3. Embryos should be carefully inspected under the stereomicroscope at this point. Any broken, unfertilized, or abnormal eggs should be culled.
5. Embryos can be continuously cultured in MMR/3 to desired stages at temperatures ranging from 14 to 22°C (*see* **Note 6**). Because developmental rate is strictly dependent on temperature, one can use different incubation temperatures to manipulate the time at which embryos develop to given stages (*see* **Table 2.1**). For long-term culture, they should be kept at a density of fewer than 40 embryos per 10 mL dish and the MMR/3 should be replaced about twice a day. It is important to quickly remove any unfertilized, dead, or ruptured eggs to minimize bacterial infection.

3.3. Experimental Manipulations

In *Xenopus*, the point of sperm entry (SEP) normally defines the orientation of the embryo's dorsal–ventral axis (**Fig. 2.6a**). The fertilizing sperm contacts the egg at a random position around the animal pole, and the orientation of the dorsal–ventral axis is subsequently specified by development of an extensive array of microtubules that emanates unidirectionally across the vegetal cortex away from the eccentrically located sperm centrosome toward the prospective dorsal side (28). A 30° rotation of the

Table 2.1
Approximate time (hours) for *Xenopus laevis* embryos raised at different temperatures to reach developmental landmarks

	Stage 6	Stage 8	Stage 10	Stage 12	Stage 14
14°C	7	12	22	33	46
16°C	6	9	16	23	35
19°C	5	8	12	18	28
22°C	4	7	11	16	22
25°C[a]	3[a]	5	8	12	17

[a]25°C is too warm for the earliest cleavage stages: keep below 22°C until about stage 6.

inner cytoplasm relative to this microtubule array displaces vegetal pole determinants toward the equator on the side opposite to the SEP (22). This "vegetal cortical rotation" (29) results in the localized suppression of β-catenin degradation (30) and the initiation of a dorsoanterior-specific gene expression program (31). The pigment accumulation at the SEP normally persists through early cleavage stages, making possible the provisional identification of ventral and dorsal tiers of blastomeres for lineage-specific manipulations (**Fig. 2.6b**, **c**).

A classical demonstration of the dependence of dorsal–ventral axis formation on the vegetal cortical rotation is to irradiate the vegetal pole with UV light before the rotation has begun (32). This treatment results in the formation of ventralized embryos that display a characteristic spectrum of mild, moderate, or severe loss of dorsal anterior structures consistent with loss of dorsal mesodermal tissues. Ventralization produced by a treatment may

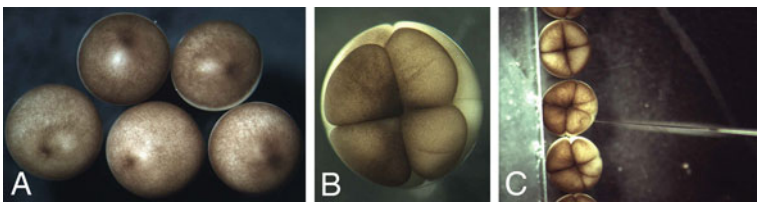

Fig. 2.6. Recognizing and using prospective dorsal–ventral pigmentation differences. (**a**) Fertilization occurs at a random location in the animal hemisphere. The sperm entry site (SEP) can be seen about 40 min after fertilization as a dark accumulation of pigment at a single site, marking ventral side of embryo. Pigmentation differences are retained during cleavage: in an eight-cell embryo (**b**), darker prospective ventral blastomeres (*left pair*) can be easily distinguished from lighter prospective dorsal (*right pair*) blastomeres. (**c**) Pigmentation differences permit identification of particular lineages during microinjection.

Fig. 2.7. Dorsoanterior index (DAI). Embryos 0–4 were ventralized via 55 s UV irradiation during the first cell cycle (**Section 3.3.1**). Control embryo 5 was not treated. Embryos 6–10 were dorsalized via 10 min exposure to LiCl during first cleavage (**Section 3.3.2**). At stage 36, they were arranged according to DAI criteria established by (3).

be scored using a nonparametric "dorsoanterior index" or DAI (3) (**Fig. 2.7**). Irradiated embryos can be rescued by various means, including tipping the egg 90° off-axis for the duration of the first cell cycle, using gravity to produce an internal cytoplasmic displacement similar to that produced by the normal rotation (33). The UV-irradiated embryo is highly responsive to localized injection of lithium chloride (34). Similarly, exogenous mRNAs coding for dorsalizing factors can be used to elicit nearly complete axis rescue (35); this sensitivity has served as a valuable assay for screening for new organizer genes (36).

3.3.1. Ventralizing Embryos with UV Irradiation

Ventralized embryos are easy to generate, requiring little more than a handheld UV light source (short wave, ~254 nm) and a stopwatch. Although one can go to the trouble of constructing quartz-bottomed cuvettes, we have found that it is just as effective to deposit embryos directly onto the UV lens after it has been waterproofed to prevent leakage of culture medium inside the lamp housing (*see* **Note 7** for waterproofing directions).

1. Fertilize and dejelly embryos as described in **Section 3.2**. In order to begin irradiation before the vegetal cortical rotation begins (approximately 30–35 min post-fertilization), the dejellying procedure should be initiated no later than 20 min post-fertilization.
2. With the UV lamp facing up and power *off*, pipet about 1 mL of MMR/3 directly onto the UV lens to form a pool about 2 mm high.
3. Pipet as many as 100 recently dejellied embryos directly into the MMR/3 pool on the UV lens. Gently nudge any embryos that are not facing animal pole into an upright orientation.
4. Remember to set aside an appropriate number of no-treatment control embryos.
5. *Caution:* don UV-protective goggles and warn nearby colleagues to avert eyes.
6. Turn the UV lamp *on* to irradiate embryos for desired number of seconds (*see* **Note 8** for calibration instructions), turn

it *off*, and immediately pipet the embryos to a new petri dish containing MMR/3 for culture to desired stages.

7. Severely irradiated embryos cannot hatch, since they lack dorsoanterior hatching glands (37). Their survival beyond hatching stage (stages 28–29) requires manual devitellination (**Section 3.3.3**) and frequent changes of fresh MMR/3.
8. Score embryos at stages 35–40 for dorsoanterior defects using the criteria of the DAI scale (3) (**Fig. 2.7**).

3.3.2. Dorsalizing Embryos by Exposure to LiCl

Embryos treated externally with lithium chloride (LiCl) during the first few cleavage cycles develop with a full range of phenotypes consistent with overproduction of dorsal anterior tissues. The resulting phenotypes at tadpole stage include macrocephaly, various degrees of twinning, and fully radialized dorsal structures (3) (**Fig. 2.7**). Because external treatment is only effective during the early cleavage stages, it is likely that external lithium gains access to the blastocoel along advancing cleavage furrows before tight junctions have fully sealed. Ventralized UV-irradiated embryos can be rescued for normal dorsal axial development by microinjecting single vegetal blastomeres at the 32-cell stage with LiCl (34). Similarly, secondary body axes can be generated in normal embryos via microinjecting lithium into single ventral blastomeres (38).

1. Fertilize and dejelly embryos as described in **Section 3.2**. Maintain in MMR/3 until after cleavage begins.
2. Do not forget to set aside an appropriate number of no-treatment controls in a separate dish of MMR/3.
3. Transfer cleaving embryos to a dish containing 0.3 M LiCl in MMR/3. Swirl the dish briefly to expose embryos thoroughly to the new medium.
4. Incubate in LiCl solution for 10 min. This interval may be varied to obtain different ranges of dorsalized phenotypes.
5. Transfer embryos to a fresh dish containing MMR/3. Thoroughly rinse out the LiCl via three 1 min exchanges of fresh MMR/3.
6. Culture embryos at room temperature to desired stages.
7. Perturbations will first become evident at gastrulation with the appearance of a uniformly circular blastopore lip. Depending on the severity of the phenotype, neurulation may or may not occur.
8. As with UV treatment, severely dorsalized embryos will not be able to hatch and require manual devitellination (**Section 3.3.3**) with frequent changes of fresh MMR/3.
9. Score embryos at stages 35–40 for dorsoanterior defects using the criteria of the DAI scale (3) (**Fig. 2.7**).

10. UV-ventralized embryos can also be rescued via microinjection of 0.2–1 nL of LiCl solution (**Section 3.3.4** for microinjection procedures).

3.3.3. Vitelline Envelope Removal

In addition to jelly coat layers, fertilized embryos are enclosed in a thin, transparent, extracellular coat, the vitelline envelope, which must be removed before isolating blastomeres or making explants. Also, as mentioned in **Section 3.3.1**, UV-irradiated embryos require manual devitellination since they cannot hatch. The vitelline envelope normally lifts away from the egg surface during egg activation at fertilization (*see* **Note 9**). Because the perivitelline fluid is hypertonic relative to pondwater (or MMR/3), the semipermeable vitelline envelope becomes turgid and adopts a nearly perfect spherical shape. Because there is very little loft between the vitelline envelope and the enclosed embryo, it is often a challenge to grasp the former without damaging the latter (*see* **Note 10** for two methods which can help with difficult batches or stages of embryos).

1. Fertilize and dejelly embryos as described in **Section 3.2**.
2. The plasma membranes of devitellinated embryos are very delicate and tend to stick to both glass and plastic. To avoid mechanical damage, devitellination should be done on an agarose surface, prepared by coating the bottoms of plastic petri dishes with 2 or 3 mL of molten agarose. For long-term culture, cleavage-stage embryos require mechanical support which can be provided by placing them in rounded pits of ~1 mm diameter melted into the agarose layer with the hot tip of a flamed capillary pipet (**Section 3.1**, Step 5 and **Fig. 2.2**).
3. While viewing under the stereomicroscope, grasp the vitelline envelope at the animal pole without pinching the embryo's plasma membrane, using a pair of fine, flat-tipped watchmaker's forceps (*see* **Note 4**). Do not let go.
4. With the other hand, use a pair of sharper, point-tipped watchmaker's forceps to grasp the pleat in the vitelline envelope formed by the first pair.
5. With a smooth, rapid movement, tear the vitelline envelope downward toward the vegetal pole. Because there is considerable hydrostatic pressure within the perivitelline space, it is important to rapidly tear a relatively large opening in the vitelline envelope: an embryo may undergo rupture if it is forced to extrude through a too small opening.

3.3.4. Microinjection

1. Fertilize and dejelly embryos as described in **Section 3.2**.
2. Prepare an injection chamber: cut a 3 or 4 mm wide strip of glass from a microscope slide. Immobilize the strip in the bottom of a clean polystyrene dish with a thin smear

Table 2.2
Calibration of microinjected volumes

Droplet diameter (μm)	volume (nL)
100	0.5
124	1.0
157	2.0
197	4.0
226	6.0
248	8.0
267	10.0

of vacuum grease. The lightly polished original edge of the slide provides a bumper against which embryos can be lined up (**Fig. 2.6c**).

3. Back-fill a microelectrode with injectate and attach it to the XYZ micromanipulator. Connect microelectrode to the pressure injection system via polyethylene tubing (Intramedic PE-100, 1.52 mm O.D., or equivalent).
4. Clip the tip of the microelectrode with watchmaker's forceps. Tip outer diameter should be about 10 μm.
5. Using a stage micrometer to measure drop size, calibrate the microinjection system to deliver pulses of desired volume of injectate (*see* **Table 2.2**). Useful volumes are typically in the range of 1–10 nL. With a pressure injection system, one can vary the pulse length and/or the pulse pressure to adjust to the variable tip diameter.
6. Transfer embryos of desired stage to the injection chamber.
7. It may be difficult to tip eggs off-axis to inject vegetal or marginal zone cells. If necessary, one can transfer 6% Ficoll to the injection dish. Ficoll will dehydrate the perivitelline fluid, causing the vitelline envelope to shrink a little, after which embryos can be rolled perpendicularly to expose vegetal tier cells for injecting.
8. Inject blastomeres as required.
9. Leave injected embryos in Ficoll for a few hours to minimize leakage through the small injection hole in the vitelline envelope. Transfer embryos out of Ficoll before gastrulation begins to avoid exogastrulation.

3.3.5. Dorsal Marginal Zone ("Keller") Explants

For several years Keller and colleagues have been studying explants of the dorsal marginal zone to investigate the cellular

basis of the dramatic morphogenetic movements of gastrulation (10, 26, 39, 40). Initially, these explants were viewed with low-magnification, long working distance optics, illuminated by obliquely directed white light, and filmed in black and white with 16 mm Bolex cameras. Now, a variety of refined optical and image capture techniques are available to analyze the role of protrusive activity in the control of cell polarity (14), the relationship of extracellular fibers to cell movements (10), and assessing the role of cytoskeletal components in morphogenesis (41). The procedure below describes construction of the basic Keller open-faced explant, which can be used as a starting point for various kinds of analysis, e.g., at low magnification for basic time-lapse analysis of cellular movements or with confocal microscopy and GFP-construct expression to study protrusive activities during particular morphogenetic movements under control or experimentally abrogated conditions.

1. Culture dejellied embryos in MMR/3 to stage 10. Embryos may be prepared beforehand, for example by injecting with mRNAs coding for various GFP-cytoskeletal protein or GFP-membrane tag constructs during early cleavage stages.
2. When the dorsal lip just begins to appear, transfer a few embryos, as needed, to an agarose-coated dish containing blastocoel buffer (**Fig. 2.8a**).
3. Devitellinate embryos (**Fig. 2.8b**). Stage 10 embryos are difficult to devitellinate without puncturing the underlying tissue. Since Keller explants are made with dorsal tissues, begin the vitelline envelope removal from the ventral side, where tissue damage will not matter.
4. Use a hair loop to flip the embryo upside down.
5. Orient the embryo so that the dorsal lip can be approached with the eyebrow knife.
6. Make two vertical cuts—one on each side of the dorsal lip—by forcing the tip of the eyebrow knife downward through the yolky vegetal cells toward the animal pole (**Fig. 2.8c**, **d**).
7. Use the hair loop to flip the embryo right side up (**Fig. 2.8e**).
8. Insert the eyebrow knife through the two slits up near the animal pole. Quickly flick outward so the eyebrow shaft cuts through the ectodermal layer, severing a wedge-shaped flap of dorsal equatorial tissue which is then free to fall away from the animal pole (**Fig. 2.8f**).
9. As it falls downward, this flap will peel away from the dorsal endodermal mass and expose the inner face of the dorsal

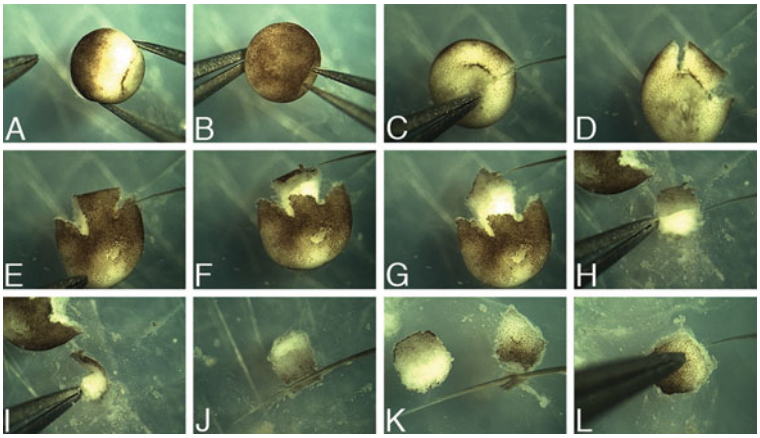

Fig. 2.8. Preparing a Keller sandwich. (**a**) Stage 10 embryo is placed on agarose surface; (**b**) embryo is devitellinated; (**c**) embryo is placed upside down and an eyebrow knife used to cut a slit from right edge of dorsal lip toward animal pole; (**d**) a similar cut is made on left edge of dorsal lip; (**e**) embryo is reoriented animal pole up; (**f**) eyebrow knife cuts dorsal flap away from animal pole; (**g**) dorsal flap falls away from remainder of embryo; (**h**) dorsal flap is cut away from remainder, using eyebrow knife; (**i**) dorsal tissue is turned on side to reveal large amount of yolky vegetal tissue which must be removed by carefully carving with eyebrow knife; (**j**) edges of dorsal tissue fragment are trimmed to produce rectangular explant; (**k**) a second dorsal explant is prepared identically to the first; (**l**) for a double-faced sandwich, two dorsal explants are sandwiched together and held in place manually for about a minute until they begin to adhere. Entire operation requires only 4 or 5 min to complete.

marginal zone. The flap remains connected to the remainder of the embryo at its vegetal end by bottle cells of the blastopore lip (**Fig. 2.8g**).

10. Use the hair loop to gently push downward and gradually peel the preinvolution surface away from any already involuted head mesoderm.
11. At this point, the flap of tissue should lie relatively flat on the substrate, inner side up and still attached by bottle cells to the rest of the embryo. Use the eyebrow knife to sever this connection.
12. Trim the explant to desired shape and dimensions (**Fig. 2.8i**, **j**). *See* (42) for ideas and experimental rationales for subdividing the basic explant shown here.
13. Observe carefully whether any already involuted head mesodermal cells remain attached. These can be carefully flicked off one by one with the tip of the eyebrow knife. To make a double-faced explant, repeat Steps 1–13 and then gently push the two explants together (**Fig. 2.8k**, **l**).
14. To observe cellular activities in an open-faced explant, gently transfer the explant from the dissection dish, with a minimum of broken cell debris and yolk, into a depression slide

containing a drop of fresh blastocoel buffer. Orient the tissue with its inner, blastocoel surface cells facing upward.

15. Aspirate gently with a braking pipet to finish cleaning up debris along the explant's margins.
16. For time-lapse imaging of cell movements, the explant is immobilized and kept flat with a small rectangular glass bridge cut from a coverslip. A 1 mm bead of vacuum grease is placed at either end of the glass strip. Grasp the strip with watchmaker's forceps and place it atop the explant. Gradually tap the glass downward until it just makes contact with the explant.
17. Explants may be cultured in blastocoel buffer for several hours to overnight to score for extent of convergent extension or to image via low-magnification or compound microscopy.
18. Unlabeled specimens are imaged via time-lapse stereomicroscopy (*see* **Section 3.4**).
19. Specimens expressing GFP-construct mRNAs are imaged via time-lapse confocal microscopy (**Section 3.6.3**).

3.4. Time-Lapse Stereomicroscopy

Time lapse is an indispensible tool for recording and analyzing morphogenetic movements and quickly learning the results of experimental manipulations. Low-magnification time-lapse work with *Xenopus* embryos or various kinds of tissue explants is typically done at a stereomicroscope-based work station equipped with ready-at-hand gear for illumination, temperature control, micromanipulation, microinjection, and digital image capture. One example of many possible configurations is shown in **Fig. 2.9** (*see* **Notes 1–3**).

1. Place embryo or explant in appropriate observation chamber or petri dish.
2. Arrange lighting to accommodate constraints of the image capture system.
3. Calculate number of frames and frame capture rate to effectively record the event of interest (*see* **Note 11**).
4. Save captured frames to their own file folder. Use a filename convention that includes an unambiguous numerical date-and-time stamp as part of the filename structure, e.g., "20080403_094837_a.tif".
5. Check on the progress of the movie while it is being generated: open the sequence via ImageJ ("File/Import/Image Sequence...") or Quicktime Pro ("File/Open/Image Sequence...").
6. If on-the-fly adjustments of framing, light intensity, or focus become necessary, they should be made gradually, i.e.,

Fig. 2.9. Live-embryo workstation. Stereomicroscope with digital camera mounted from side port. Microscope rests on a custom-built aluminum cooling stage which was channeled to circulate coolant from the temperature-controlled water bath located below the table. Note arrangement of fiber lights, micromanipulator, and embryo dishes on stage.

distributed over a series of five or more frames, to avoid visual discontinuities during playback.

7. For use in Powerpoint or Keynote presentations or web pages, open the movie sequence via Quicktime Pro ("File/Open/Image Sequence…") and export as a .mov movie via "File/Export/…."
8. For image analysis, open the image sequence as a .tiff stack via ImageJ ("File/Import/Image Sequence…"). Save the stack as ("File/SaveAs/Tiff…").
9. Archive the original, untouched file sequence in its own folder.

3.5. Whole-Mount Confocal Microscopy

Different cytoskeletal structures require different fixation protocols. Excellent protocols for fixing and visualizing microtubules in *Xenopus* oocytes and egg embryos have been optimized by David Gard and colleagues (*see* (27) and references within) and later adapted by others for embryo work (13, 20, 42, 43). In the sections below, two different fixation protocols are presented: one protocol for microtubules, in which specimens are optically cleared following immunostaining, and the other protocol for microfilaments, in which the specimens, while hydrated and non-transparent, still provide excellent imaging of microfilamentous structures.

3.5.1. Fixation and Staining for Microtubules

1. Dejellied embryos are cultured to desired stages and then fixed in FG fix buffer containing 3.7% formaldehyde (from commercial formalin), 0.25% glutaraldehyde, and 0.2% Triton X-100 for 2–4 h on a gently rocking nutator at room temperature (not overnight; bad for epitopes). Fixation is done in 4 mL glass shell vials with nylon snap caps, containing 1–2 mL fixative. Fix no more than 20 embryos/mL fixative. Post-fixation is overnight or longer in anhydrous MeOH at –20°C.
2. Pigment is bleached in bleaching solution. Bleaching is done under strong white-light illumination for 1 h or more and stopped when animal hemisphere pigment has faded to desired degree. Vials are laid on their side on a white fluorescent light table with a piece of aluminum foil placed over them. Use gloves to avoid contact with the peroxide solution.
3. Rehydrate embryos in PBS via three consecutive rinses for 10 min each in
   50% MeOH/50% PBS.
   25% MeOH/75% PBS.
   100% PBS.
   (possibly do devitellinating/bisecting at this point; *see* **Note 12**).
4. Reduce autofluorescence: Incubate embryos for 6–16 h (e.g., overnight) in freshly made borohydride solution (Caution: hydrogen gas! *see* **Notes 13** and **14**).
5. NTBS wash: Wash embryos via five 30 min exchanges of 1× NTBS (possibly do devitellinating/bisecting at this point, *see* **Note 12**).
6. Primary antibody: Incubate embryos in primary antibody working solution or in FBS solution alone (no primary control). 250 μL is sufficient for 10 embryos or embryo

fragments. Use slow, gentle rocking overnight at 4°C. We sometimes leave embryos over weekend at this step.

7. Wash embryos with five 45 min exchanges with 1× NTBS. Extensive washing is necessary to reduce nonspecific staining.
8. Secondary antibody: Incubate embryos in secondary antibody working solution. Avoid unnecessary exposure to light from this point on, e.g., by wrapping vials in foil. Use slow, gentle rocking overnight at 4°C. We do *not* extend this incubation period past overnight, to avoid nonspecific background fluorescence.
9. Wash embryos with five 45 min exchanges with 1× NTBS. Again, extensive washing reduces nonspecific staining.
10. Dehydrate embryos via two consecutive rinses for 15–30 min each in anhydrous methanol.
11. Embryos are cleared via two 15–30 min changes of Murray's Clear. They are ready for confocal analysis when they have sunk to the bottom of vial and no opacity remains.

3.5.2. Fixation and Staining for Microfilaments

Xenopus oocytes (44), cleavage-stage embryos (13), blastulae (45), and gastrulae (10) are excellent targets for observing actin filament dynamics in both live cells expressing fluorescent-protein constructs (18) and fixed specimens stained with fluorochrome-coupled phalloidin. Since phalloidin does not remain bound to microfilaments during specimen dehydration, embryos must be examined while fully hydrated, and therefore opaque. Nevertheless, since most microfilaments, e.g., those of the contractile ring and filopodia, exist in the cell cortex, excellent visualization is possible via confocal microscopy.

1. Fix embryos in room temperature FG fix buffer containing 4% formaldehyde (from freshly made stock, not commercial formalin), 0.25% glutaraldehyde, and 0.2% Triton X-100. Store overnight at 4°C.
2. Rinse samples in NTBS.
3. Devitellinate manually.
4. Incubate overnight at 4°C in phalloidin working solution on a slow rocker or nutator.
5. Rinse thoroughly in NTBS.
6. For inverted microscopy, place NTBS-washed samples in coverslip-bottomed observation chambers (**Section 3.1**, Step 6). For upright microscopy, use the hanging drop method described in **Section 3.1**, Step 7.
7. Note that these hydrated specimens are opaque: staining will only be detected near the surface (*see* (13) for examples of phalloidin-stained contractile rings and apical filopodia).

3.6. Live-Cell Confocal Microscopy

Embryos are readily prepared for live-cell fluorescence microscopy by directly staining target membranes or vesicles with vital dyes, such as $DiOC_6(3)$ (21, 45) or FM1-43 (13). Alternatively, one can express GFP or YFP constructs encoding proteins of interest by microinjecting mRNAs into specific blastomeres or the intact embryo. The following four experiments are given as examples of different live-cell approaches focused on different kinds of target regions, cells, or developmental phenomena.

3.6.1. Time Lapse of Aggregating Germ Plasm

Germ plasm is a complex aggregation of vesicles, mitochondria, intermediate filaments (46), mRNAs (47), and other exotic maternal transcripts (48). Germ plasm is found initially dispersed across the vegetal cortex in the form of hundreds of ~10 μm islands. During the first two cell cycles, via a process related to surface contraction waves (49), these islands undergo a dramatic microtubule- and Xklp1-dependent relocalization (50) to produce a few large aggregates that will be inherited by a limited number of blastomeres committed to the germ lineage.

1. Dejellied embryos are transferred from MMR/3 to a dish containing 1 μg/mL $DiOC_6(3)$, made by 1:1,000 dilution of the stock $DiOC_6(3)$ solution in MMR/3. To ensure exposure of the vegetal surface to the dye, the dish is rocked slowly (1–2 Hz) on a nutator or rocking platform.
2. After 3 min, transfer embryos to fresh MMR/3 and rinse thoroughly.
3. $DiOC_6(3)$-stained embryos are very light-sensitive and should be kept shaded from fluorescent room lights whenever possible.
4. To detect germ plasm on the vegetal pole, place stained embryo upright in glass-bottomed observation chamber.
5. Record motions of $DiOC_6(3)$-stained accumulations of germ plasm-associated mitochondria and endoplasmic reticulum via confocal microscope using FITC filters (488 nm excitation, >520 nm emission).

3.6.2. Time Lapse of Cortical Vesicle Translocations at the Embryo's Equator

Embryos quickly right themselves within their vitelline envelopes with respect to gravity shortly after fertilization (**Fig. 2.5**). They will not develop normally if this orientation is perturbed during the first few cleavage cycles. Because most microscopes are in either an upright or an inverted configuration, the equatorial region (aka the marginal zone) of live embryos is generally not available for viewing. However, use of a 45° mirrored prism (**Fig. 2.10a**) provides direct access to this region via long working distance compound microscope objectives (**Fig. 2.10b**) or stereomicroscopy (**Fig. 2.10c**).

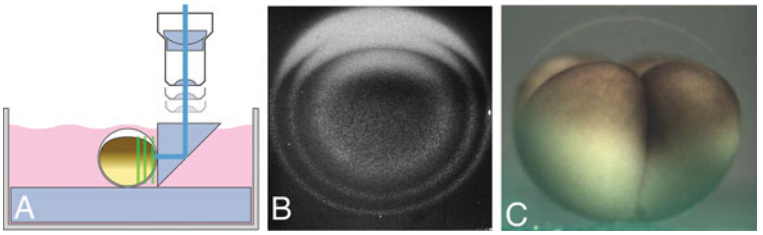

Fig. 2.10. Right-angle prism for imaging equatorial region of live embryos. Adaptable for low-magnification confocal (**b**) or stereomicroscopy (**c**). (**a**) Diagram shows arrangement of culture dish, embryo, prism, and long working distance objective (e.g., 4×, 0.3 NA). Image focus is subject to same working distance constraints as a conventional top-down arrangement, although the prism thickness may produce some spherical aberration. (**b**) Three superimposed confocal image planes trained on the equator of a *Xenopus* embryo stained with lipid dye $DiOC_6(3)$. This image is a single frame from a 3-D time-lapse movie to detect motions of cortical vesicles and yolk platelets near the equator during the first cell-cycle rotation. (**c**) Side view of a normal living *Xenopus* embryo at second cleavage, observed through a stereomicroscope.

1. A side-viewing chamber is prepared by mounting a 5 mm right-angle aluminized hypotenuse prism on a lump of modeling clay in a polystyrene culture dish. The upper horizontal surface of the prism is not submerged in culture medium (**Fig. 2.10a**).
2. Stain embryos with $DiOC_6(3)$ as in **Section 3.6.1**.
3. Using a hair loop, gently press embryo in upright orientation against the submerged vertical prism face.
4. Use low-magnification objective with at least 5 mm working distance on confocal microscope to observe or record through the horizontal prism surface (**Fig. 2.10b**).

3.6.3. Imaging Filopodia in the Blastocoel of Embryos Expressing GFP-Mem

Expression of fluorescent protein (FP) constructs has greatly advanced the analysis of cytoskeletal and membrane dynamics in early *Xenopus* embryos (10, 13, 14, 16, 18, 51, 52). One straightforward practice is to microinject synthetic mRNAs coding for FP-containing constructs into the fertilized egg. Expression of detectable levels of fluorescence generally develops within 2 h (four cleavage cycles). The example below uses an mRNA coding for a membrane-anchored GFP (GFP-mem) (14) to visualize filopodia and protrusive activity in the cleavage-stage blastocoel.

1. Microinject fertilized, dejellied eggs with 7 nL of mRNA (approximately 1 μg/μL) late in the first cell cycle.
2. Culture to desired stages in MMR/3.
3. Transfer embryo to a depression slide containing a drop of blastocoel buffer.
4. Devitellinate embryo, being careful to avoid puncturing any blastomeres.

5. Use a hair loop to gently immobilize the embryo. Grasp a single vegetal-tier blastomere with a pair of watchmaker's forceps and remove it.
6. Use braking pipet to very gently remove any broken cell debris, leaving a clean opening to the blastocoel (**Fig. 2.11a**). This opening will gradually sag open, affording a nearly unperturbed view of blastocoel contents.
7. Scrape each corner of a 22 mm × 22 mm coverslip across the surface of a ball of modeling clay to build up ~1 mm clay feet.
8. Apply a small drop of blastocoel buffer to the coverslip, invert it, and carefully lower it directly onto the depression slide. With practice, this operation can be performed smoothly without introducing bubbles or disturbing the exposed blastomeres.
9. Press down each corner of the coverslip to bring it into close proximity to the opening between blastomeres.
10. Focus a high-magnification objective of the confocal microscope on the contacting surfaces of two blastomeres.

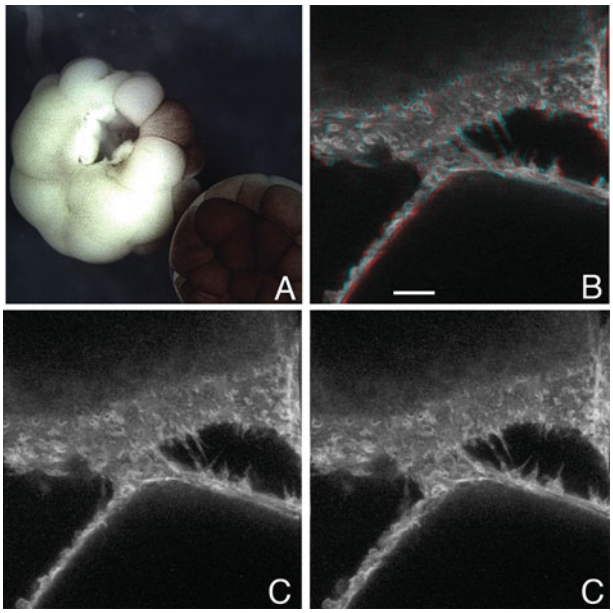

Fig. 2.11. (**a**) Live 32-cell embryo expressing eGFP-mem with single blastomere removed to permit contents of blastocoel to be imaged via 4-D confocal microscopy. Eleven confocal images were captured at 2-μ intervals in a region where filopodia extend between two blastomeres that are coming into contact. Image stack of a single time point was projected to produce a pair of images with ±7° of virtual rotation about the Y-axis. Confocal projections are presented as a red-blue anaglyph which may be viewed in color in the online version of this chapter (**b**) and as a stereo pair (the two panels may be viewed in stereo by fusing the two C′s via either crossed or diverged eyes). Bar = 10 μm.

11. Use manual focus and stage manipulation to locate a region of interest.
12. Record a preliminary "xyzt" sequence by specifying the capture of four or five optical slices 1.5 μm apart, repeating every 20 s, for 30 time points.
13. Modify the optical depth, step size, and time point interval as needed to capture events of interest.
14. Image stacks can be projected as stereo pairs using commercial software or ImageJ to produce red-blue anaglyphs (**Fig. 2.11b**) or side-by-side pairs (**Fig. 2.11c**).

3.6.4. Time Lapse of FM1-43-Stained Filopodia of Dissociated Blastomeres

Early embryos display a lot of membrane protrusive activity along their basolateral (blastocoel-facing) surfaces that appears to be related to adhesion and shaping of the blastocoel. The water-soluble styryl dye FM1-43 becomes fluorescent when associated with plasma membranes and can be used to visualize membrane protrusive activity in living embryos. To record protrusive activity, blastomeres are first dissociated in low-calcium medium and then allowed to reassociate in the presence of FM1-43. The resulting fluorescence is sufficiently bright to enable 3-D time-lapse confocal imaging. The procedure below outlines a demonstration of membrane protrusive activity on the basolateral surfaces of 32-cell blastomeres that are reestablishing cell–cell contacts following low-calcium cell dissociation. Results and figures from a similar experiment can be found in (13).

1. Embryos undergoing first or second cleavage are placed in agarose-bottomed dish containing cell dissociation buffer and manually devitellinated, with care taken not to puncture any blastomeres.
2. At 10 min intervals over the next three cleavage cycles (about 90 min at room temperature), tip the dish back and forth gently a few times to gradually dissociate blastomeres from each other. Do not use a nutator, since regular agitation may cause incompletely-cleaved cells to drift apart too rapidly.
3. Use a braking pipet to gently clean up any broken-cell debris.
4. Prepare a depression slide by placing a 400 μL drop of blastocoel buffer containing 10 μM FM1-43.
5. Use a braking pipet to gently transfer (one at a time) two unbroken blastomeres to the culture dish.
6. Under the stereomicroscope, gently nudge the two cells together such that basolateral surfaces are in close contact.
7. Follow Steps 8–13 in **Section 3.6.3**.

4. Notes

1. High-quality stereomicroscopes suitable for work with *Xenopus* embryos are available from major optical suppliers (Leica, Nikon, Olympus, Zeiss, etc., *see* **Fig. 2.9** for an example setup.) A stereoscope should be capable of continuous zoom from about 5× to at least 60× and be equipped with a beamsplitter, a camera port (or a trinocular head), and C-mount adapter to accommodate image recording. Most modern systems are modular and can be customized to include not only a digital imaging system but also epifluorescence illumination and even motorized focus.
2. Because most tissues of *Xenopus* embryos are opaque, substage illumination is useless and so may be omitted from the purchase of a new modular stereomicroscope system. Overhead illumination is generally provided by fiber-optic light systems. A variety of fiber-optic illuminators using halogen bulbs or LEDs are available from standard scientific equipment vendors. Flexible, dual-arm, focusable fiber-optic systems may be preferred over annular illuminators mounted on the objective ("ring lights"), because *Xenopus* tissues change quite drastically in their coloration (pigment distribution) during early development: different intensities or directions of illumination may be desired for adequate imaging. On the other hand, ring illuminators are sometimes useful to eliminate shadows that may be bothersome at particular stages.
3. For laboratory setups in rooms without adequate air-temperature regulation, seasonal fluctuations can be accommodated with a custom-built, water-cooled aluminum base (**Fig. 2.9**). Temperature of the base is maintained by a cooled, circulating water bath. For overnight culture at regulated temperatures, upright laboratory incubators are useful.
4. Although there is wide preference for forceps tips that are sharpened to fine points (e.g., Dumont's "Biologie" tip), sharp tips are easily bent and can inadvertently puncture a Xenopus egg's vitelline envelope before a firm grasp has been made. New users may find the more flattened (Dumont's "Standard") forceps tip to be just as effective as the pointed tip and better able to resist accidental bending. For some demanding operations, such as defolliculating early vitellogenic oocytes, forceps can be tuned by bending tips toward each other slightly for "tips first" grasping, but for most applications, the shanks and tips should

close flatly, nearly simultaneously, along their entire length. Sharpening of dulled or bent forceps is relatively easy to accomplish with a fine Arkansas stone or jeweler's-grade crocus (rouge) cloth. Progress should be followed under a stereomicroscope, with care taken that tips will meet precisely at their ends, simultaneous with contact along the shanks.

5. At a minimum, a digital camera system should be capable of capturing and displaying 8-bit, RGB, megapixel-range images both as still frames and as sequences of frames for time-lapse work. Although SLR and other handheld cameras can be used for capturing single frames at high resolution, time-lapse work typically requires a computer to store and play back movie files and requires software control to specify frame capture rate. Choose a digital capture system that saves frames as individual files. One then has random access to the image sequence while it is being generated, making possible minor on-the-fly adjustments or the decision to scrub a session altogether if something does not look right.
6. Later stage *X. laevis* embryos develop comfortably within a broad temperature range (~14–25°C). However, the earliest cleavage stages should be kept cooler than 22°C.
7. Handheld UV lights should be waterproofed by building up an ~2 mm wall of dental wax or silicone rubber cement along the joint between the UV filter and instrument housing.
8. The appropriate dose of UV is determined empirically by irradiating groups of ~25 embryos as in **Section 3.3.1** for durations varying by 5 or 10 s increments from 30 to 90 s and scoring for DAI (3) when the embryos have reached stages 35–40. A useful target dose will yield highly ventralized embryos (DAIs of 0–2; **Fig. 2.7**) that are not moribund and capable of responding to rescue, e.g., via LiCl or *Siamois* injected into vegetal blastomeres (34, 35).
9. The vitelline envelope undergoes progressive crosslinking and gradually hardens over an ~30 min period following fertilization (53). Thus, when embryos are dejellied after 30 min post-fertilization, the fully hardened vitelline envelope retains its original diameter, just slightly greater than that of the embryo itself. However, vitelline envelope hardening can be blocked by earlier dejellying treatment: when embryos are dejellied within ~5 min post-fertilization, the vitelline envelope remains distensible and swells greatly, resulting in a flattened embryo (*see* Supplemental Figure S1 in (13)). This flattening forces normally horizontal cleavage

planes (e.g., 3rd and 5th) to a more vertical orientation, but usually has no impact on later development.

10. It is relatively easy to remove the vitelline envelope before first cleavage. However, the amount of loft between it and the embryo steadily diminishes with each cleavage cycle, making removal progressively more difficult. Two methods make the vitelline envelope easier to remove, particularly at later stages. One method is to dejelly eggs within about 10 min after fertilization: cysteine blocks the hardening of the vitelline envelope which normally takes about 30 min to complete. The softened vitelline envelopes of early dejellied embryos expand greatly and are easier to remove mechanically than fully hardened ones. A second method is to place embryos in 6% Ficoll solution which, by dehydrating the perivitelline fluid, causes the vitelline envelope to deflate slightly, providing a deformable, wrinkly surface, easier to grasp with forceps.
11. To effectively compress a developmental event, one should know in advance something about its duration. Some events (e.g., fertilization waves), occur in only minutes, while others (e.g., neurulation) take several hours. Whatever an event's realtime duration, its playback for presentation purposes (e.g., Powerpoint, Keynote) should last only 15 s or so. Assuming one uses a standard 24 frame-per-second (fps) playback, then the number of frames generally needed to record is $24 \times 15 = 360$ frames. As an example, to record a single 30 min cleavage cycle in 360 frames, one would set the realtime capture rate to 12 frames per minute (360 frames/30 min = 12 fpm). In contrast, the 6 h of neurulation would be recorded at a capture rate of 360 frames/360 min = 1 fpm. For motion analysis work, e.g., of morphogenetic movements, lamellipodial motion, or exocytotic events, the frame capture rate should be increased as needed.
12. At some point vitelline envelopes need to be removed, and desired surfaces exposed for enhancing antibody penetration. In principle, this could occur at any step following fixation, but the earlier it is done, the more beat up the cut surfaces become. The borohydride treatment is particularly damaging to cut surfaces. On the other hand, devitellination and cutting happen to be particularly easy while embryos are still in 50% methanol; they are somewhat more brittle.

    Devitellination can be done manually at some stages, embryo by embryo, using watchmaker's forceps, as described in **Section 3.3.3**. However, if the vitelline envelopes are very closely applied to the embryo surface,

as happens from about stage 8 onward, this may be time consuming, tedious, and potentially damaging to the specimen. An alternative approach is to cut a Pasteur pipet to a diameter just able to accommodate the fixed embryo itself. Do not fire-polish the pipet; the sharp edge will actually be helpful here. Simply pipet up and down (somewhat violently) a few times, and the embryos will be freed of their vitelline envelopes.

Bisection: Transfer devitellinated embryos into a plastic Falcon dish in current medium (you are either at Step 3 or Step 5 of the immunostaining procedure of **Section 3.5.1**). Grasp a small, triangular-shaped razor fragment (broken from a NEW razor blade with pliers; wear goggles while breaking razor), and *slice*—do not push—in desired plane. Straight cuts will generally result if one begins the cut on the vegetal surface. Tissue should pop cleanly into two fragments. Sometimes it is helpful to crack along older cleavage planes. Some cleavage stages, such as eight-cell embryos, are particularly difficult to cut except along established cleavage planes.

13. Since borohydride emits hydrogen gas, the solution will bubble vigorously at first (avoid nearby flame or spark!). Vial caps should be punctured with a needle to prevent them from popping off. Embryos will tumble and float to surface at first. Vials may be shaken occasionally to ensure that embryos stay submerged.
14. Expanding H_2 bubbles in the blastocoel sometimes blow large holes in stages 8–11 embryos. Two possible solutions: (a) use the borohydride solution after its effervescence has subsided somewhat; (b) bisect embryos to open up the blastocoel prior to this step.

Acknowledgments

Danilchik's research is supported by the National Science Foundation (IOS-0921415).

References

1. Wallingford, J. B., Liu, K. J., and Zheng, Y. (2010) Xenopus. *Curr. Biol.* **20**, R263–R264.
2. Nieuwkoop, P. D. and Faber, J. (1994) *Normal Table of Xenopus laevis (Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg Till the End of Metamorphosis*. Garland Pub, New York, NY.
3. Kao, K. R. and Elinson, R. P. (1988) The Entire Mesodermal Mantle Behaves as Spemann's Organizer in Dorsoanterior

Enhanced Xenopus laevis Embryos. *Dev. Biol.* **127**, 64–77.
4. Moody, S. A. (1987) Fates of the Blastomeres of the 16-Cell Stage Xenopus Embryo. *Dev. Biol.* **119**, 560–578.
5. Moody, S. A. (1987) Fates of the Blastomeres of the 32-Cell-Stage Xenopus Embryo. *Dev. Biol.* **122**, 300–319.
6. Dale, L. and Slack, J. M. W. (1987) Fate Map for the 32-Cell Stage of Xenopus laevis. *Development* **99**, 527–551.
7. Keller, R. E. (1975) Vital Dye Mapping of the Gastrula and Neurula of Xenopus laevis. I. Prospective Areas and Morphogenetic Movements of the Superficial Layer. *Dev. Biol.* **42**, 222–241.
8. Keller, R. E. (1976) Vital Dye Mapping of the Gastrula and Neurula of Xenopus laevis. II. Prospective Areas and Morphogenetic Movements of the Deep Layer. *Dev. Biol.* **51**, 118–137.
9. Keller, R. and Danilchik, M. V. (1988) Regional Expression, Pattern and Timing of Convergence and Extension During Gastrulation of Xenopus laevis. *Development* **103**, 193–209.
10. Davidson, L. A., Dzamba, B. D., Keller, R., and Desimone, D. W. (2008) Live Imaging of Cell Protrusive Activity, and Extracellular Matrix Assembly and Remodeling During Morphogenesis in the Frog, Xenopus laevis. *Dev. Dyn.* **237**, 2684–2692.
11. Wallingford, J. B., Sater, A. K., Uzman, J. A., and Danilchik, M. V. (1997) Inhibition of Morphogenetic Movement During Xenopus Gastrulation by Injected Sulfatase: Implications for Anteroposterior and Dorsoventral Axis Formation. *Dev. Biol.* **187**, 224–235.
12. Fredieu, J. R., Cui, Y., Maier, D., Danilchik, M. V., and Christian, J. L. (1997) Xwnt-8 and Lithium Can Act Upon Either Dorsal Mesodermal or Neurectodermal Cells to Cause a Loss of Forebrain in Xenopus Embryos. *Dev. Biol.* **186**, 100–114.
13. Danilchik, M. V. and Brown, E. E. (2008) Membrane Dynamics of Cleavage Furrow Closure in Xenopus laevis. *Dev. Dyn.* **237**, 565–579.
14. Wallingford, J. B., Rowning, B. A., Vogeli, K. M., Rothbacher, U., Fraser, S. E., and Harland, R. M. (2000) Dishevelled Controls Cell Polarity During Xenopus Gastrulation. *Nature* **405**, 81–85.
15. Park, T. J., Gray, R. S., Sato, A., Habas, R., and Wallingford, J. B. (2005) Subcellular Localization and Signaling Properties of Dishevelled in Developing Vertebrate Embryos. *Curr. Biol.* **15**, 1039–1044.
16. Bement, W. M., Sokac, A. M., and Mandato, C. A. (2003) Four-Dimensional Imaging of Cytoskeletal Dynamics in Xenopus Oocytes and Eggs. *Differentiation* **71**, 518–527.
17. Houliston, E. and Elinson, R. P. (1991) Patterns of Microtubule Polymerization Relating to Cortical Rotation in Xenopus laevis Eggs. *Development* **112**, 107–117.
18. Burkel, B. M., Dassow, G. V., and Bement, W. M. (2007) Versatile Fluorescent Probes for Actin Filaments Based on the Actin-Binding Domain of Utrophin. *Cell Motil. Cytoskeleton* **64**, 822–832.
19. Noguchi, T. and Mabuchi, I. (2001) Reorganization of Actin Cytoskeleton at the Growing End of the Cleavage Furrow of Xenopus Egg During Cytokinesis. *J. Cell. Sci.* **114**, 401–412.
20. Danilchik, M. V., Funk, W. C., Brown, E. E., and Larkin, K. (1998) Requirement for Microtubules in New Membrane Formation During Cytokinesis of Xenopus Embryos. *Dev. Biol.* **194**, 47–60.
21. Savage, R. M. and Danilchik, M. V. (1993) Dynamics of Germ Plasm Localization and Its Inhibition by Ultraviolet Irradiation in Early Cleavage Xenopus Embryos. *Dev. Biol.* **157**, 371–382.
22. Weaver, C. and Kimelman, D. (2004) Move It or Lose It: Axis Specification in Xenopus. *Development* **131**, 3491–3499.
23. Ubbels, G. A., Hara, K., Koster, C. H., and Kirschner, M. W. (1983) Evidence for a Functional Role of the Cytoskeleton in Determination of the Dorsoventral Axis in Xenopus laevis Eggs. *J. Embryol. Exp. Morphol.* **77**, 15–37.
24. Keller, R. E., Danilchik, M. V., Gimlich, R., and Shih, J. (1985) The Function and Mechanism of Convergent Extension During Gastrulation of Xenopus laevis. *J. Embryol. Exp. Morphol.* **89**, 185–209.
25. Sargent, T. D., Jamrich, M., and Dawid, I. B. (1986) Cell Interactions and the Control of Gene Activity During Early Development of Xenopus laevis. *Dev. Biol.* **114**, 238–246.
26. Doniach, T., Phillips, C. R., and Gerhart, J. C. (1992) Planar Induction of Anteroposterior Pattern in the Developing Central Nervous System of Xenopus laevis. *Science* **257**, 542–545.
27. Becker, B. E. and Gard, D. L. (2006) Visualization of the Cytoskeleton in Xenopus Oocytes and Eggs by Confocal Immunofluorescence Microscopy. *Methods Mol. Biol.* **322**, 69–86.
28. Elinson, R. P. and Rowning, B. (1988) A Transient Array of Parallel Microtubules in Frog Eggs: Potential Tracks for a

Cytoplasmic Rotation That Specifies the Dorso-Ventral Axis. *Dev. Biol.* **128**, 185–197.
29. Gerhart, J. C., Vincent, J. P., Scharf, S. R., Black, S. D., Gimlich, R. L., and Danilchik, M. V. (1984) Localization and Induction in Early Development of Xenopus. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **307**, 319–330.
30. Miller, J. R., Rowning, B. A., Larabell, C. A., Yang-Snyder, J. A., Bates, R. L., and Moon, R. T. (1999) Establishment of the Dorsal-Ventral Axis in Xenopus Embryos Coincides with the Dorsal Enrichment of Dishevelled That Is Dependent on Cortical Rotation. *J. Cell Biol.* **146**, 427–437.
31. Rowning, B. A., Wells, J., Wu, M., Gerhart, J. C., Moon, R. T., and Larabell, C. A. (1997) Microtubule-Mediated Transport of Organelles and Localization of β-Catenin to the Future Dorsal Side of Xenopus Eggs. *Proc. Natl. Acad. Sci. USA* **94**, 1224–1229.
32. Scharf, S. R. and Gerhart, J. C. (1983) Axis Determination in Eggs of Xenopus laevis: A Critical Period Before First Cleavage, Identified by the Common Effects of Cold, Pressure and Ultraviolet Irradiation. *Dev. Biol.* **99**, 75–87.
33. Black, S. D. and Gerhart, J. C. (1986) High-Frequency Twinning of Xenopus laevis Embryos From Eggs Centrifuged Before First Cleavage. *Dev. Biol.* **116**, 228–240.
34. Kao, K. R., Masui, Y., and Elinson, R. P. (1986) Lithium-Induced Respecification of Pattern in Xenopus laevis Embryos. *Nature* **322**, 371–373.
35. Sokol, S., Christian, J. L., Moon, R. T., and Melton, D. A. (1991) Injected Wnt RNA Induces a Complete Body Axis in Xenopus Embryos. *Cell* **67**, 741–752.
36. Smith, W. C. and Harland, R. M. (1992) Expression Cloning of Noggin, a New Dorsalizing Factor Localized to the Spemann Organizer in Xenopus Embryos. *Cell* **70**, 829–840.
37. Drysdale, T. A. and Elinson, R. P. (1991) Development of the Xenopus laevis Hatching Gland and Its Relationship to Surface Ectoderm Patterning. *Development* **111**, 469–478.
38. Busa, W. B. and Gimlich, R. L. (1989) Lithium-Induced Teratogenesis in Frog Embryos Prevented by a Polyphosphoinositide Cycle Intermediate or a Diacylglycerol Analog. *Dev. Biol.* **132**, 315–324.
39. Keller, R. and Danilchik, M. V. (1988) Regional Expression, Pattern and Timing of Convergence and Extension During Gastrulation of Xenopus laevis. *Development* **103**, 193–209.
40. Keller, R. and Shook, D. (2008) Dynamic Determinations: Patterning the Cell Behaviours That Close the Amphibian Blastopore. *Philos. Trans. R. Soc., B, Biol. Sci.* **363**, 1317–1332.
41. Rolo, A., Skoglund, P., and Keller, R. (2009) Morphogenetic Movements Driving Neural Tube Closure in Xenopus Require Myosin IIB. *Dev. Biol.* **327**, 327–338.
42. Davidson, L. A., Hoffstrom, B. G., Keller, R., and Desimone, D. W. (2002) Mesendoderm Extension and Mantle Closure in Xenopus laevis Gastrulation: Combined Roles for Integrin, Fibronectin, and Tissue Geometry. *Dev. Biol.* **242**, 109–129.
43. Lee, C., Scherr, H. M., and Wallingford, J. B. (2007) Shroom Family Proteins Regulate Gamma-Tubulin Distribution and Microtubule Architecture During Epithelial Cell Shape Change. *Development* **134**, 1431–1441.
44. Mandato, C. A. and Bement, W. M. (2001) Contraction and Polymerization Cooperate to Assemble and Close Actomyosin Rings Around Xenopus Oocyte Wounds. *J. Cell Biol.* **154**, 785–797.
45. Nandadasa, S., Tao, Q., Menon, N. R., Heasman, J., and Wylie, C. C. (2009) N- and E-Cadherins in Xenopus Are Specifically Required in the Neural and Non-Neural Ectoderm, Respectively, for F-Actin Assembly and Morphogenetic Movements. *Development* **136**, 1327–1338.
46. Wylie, C. C., Heasman, J., Parke, J. M., Anderton, B., and Tang, P. (1986) Cytoskeletal Changes During Oogenesis and Early Development of Xenopus laevis. *J. Cell Sci. Suppl.* **5**, 329–341.
47. Houston, D. W. and King, M. L. (2000) A Critical Role for Xdazl, a Germ Plasm-Localized RNA, in the Differentiation of Primordial Germ Cells in Xenopus. *Development* **127**, 447–456.
48. Kloc, M., Wilk, K., Vargas, D., Shirato, Y., Bilinski, S., and Etkin, L. D. (2005) Potential Structural Role of Non-Coding and Coding RNAs in the Organization of the Cytoskeleton at the Vegetal Cortex of Xenopus Oocytes. *Development* **132**, 3445–3457.
49. Quaas, J. and Wylie, C. (2002) Surface Contraction Waves (Scws) in the Xenopus Egg Are Required for the Localization of the Germ Plasm and Are Dependent Upon Maternal Stores of the Kinesin-Like Protein xklp1. *Dev. Biol.* **243**, 272–280.
50. Robb, D. L., Heasman, J., Raats, J., and Wylie, C. (1996) A Kinesin-Like Protein Is Required for Germ Plasm Aggregation in Xenopus. *Cell* **87**, 823–831.

51. Wallingford, J. B., Ewald, A. J., Harland, R. M., and Fraser, S. E. (2001) Calcium Signaling During Convergent Extension in Xenopus. *Curr. Biol.* **11**, 652–661.
52. Weaver, C., Farr, G. H., Pan, W., Rowning, B. A., Wang, J., Mao, J., Wu, D., Li, L., Larabell, C. A., and Kimelman, D. (2003) GBP Binds Kinesin Light Chain and Translocates During Cortical Rotation in Xenopus Eggs. *Development* **130**, 5425–5436.
53. Larabell, C. A. and Chandler, D. E. (1990) Stepwise Transformation of the Vitelline Envelope of Xenopus Eggs at Activation: a Quick-Freeze, Deep-Etch Analysis. *Dev. Biol.* **139**, 263–268.

Chapter 3

Manipulation of Gene Function in *Xenopus laevis*

Mizuho S. Mimoto and Jan L. Christian

Abstract

Xenopus laevis embryos are particularly well suited to address questions requiring either knockdown or overexpression of genes in a tissue-specific fashion during vertebrate embryonic development. These manipulations are achieved by targeted injection of either antisense morpholino oligonucleotides or synthetic mRNAs, respectively, into the early embryo. Herein we offer detailed protocols describing how to design and perform these experiments successfully, as well as a brief discussion of considerations for performing a microarray analysis in this organism.

Key words: *Xenopus laevis*, embryogenesis, microinjection, morpholinos, gene knockdown, microarray.

1. Introduction

Among the many advantages of working with *Xenopus laevis* embryos as compared with other vertebrate embryos are that they are very large (approximately 1 mm in diameter at the one-cell stage), and they develop rapidly and externally. Their size and accessibility allow for microdissection and manipulation of specific tissues at even the earliest stages of development. In addition, their characteristic pigmentation and cleavage patterns together with extensive lineage tracing studies (1–3) facilitate targeted injection of constructs in order to manipulate early gene expression in a tissue-specific fashion (**Fig. 3.1**). Individual blastomeres may be targeted reasonably well up to the 32-cell stage. For later stage targeting, it is preferable to use an alternate strategy, such as transgenesis employing the appropriate DNA promoter, to achieve overexpression in a regulated fashion. This technique

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_3,

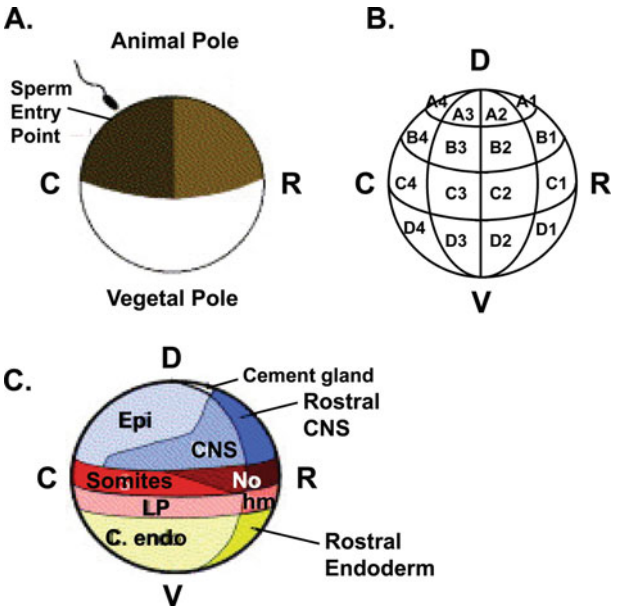

Fig. 3.1. Fate map of a 32-cell *X. laevis* embryo. (**a**) Following fertilization and cortical rotation, cells derived from the lightly pigmented side of the embryo will form rostral (R) structures and the darkly pigmented side will give rise to more caudal (C) structures. This schematic represents a 90° rotation from the traditional dorsal/ventral (D/V) axis. It should be noted that neither the original fate map nor the updated version is strictly accurate in assigning the D/V axis since dorsal and rostral fates are closely aligned, as are ventral and caudal fates. (**b**) Blastomere nomenclature of a 32-cell (stage 6) embryo (only cells in one of two sides with respect to the *left–right axis* are shown). **(c)** Prospective adult tissues derived from regions of a 32-cell embryo, as seen from a side view. c—Adapted with permission from (21). D = dorsal, V = ventral, R = rostral, C= caudal, Epi = epidermis, CNS = central nervous system, No = notochord, LP = lateral plate mesoderm, hm = head mesoderm, endo = endoderm.

is described in an accompanying chapter, as is the use of chemicals in order to induce competency in specific tissue types (e.g., mesoderm induction by animal cap exposure to activin (4)), to perturb specific signaling pathways (e.g., inhibition of FGF signaling by incubation in SU5402 (5)), or to alter cell fate (e.g., UV or LiCl treatment to ventralize or dorsalize embryos, respectively (6, 7)), all of which are common approaches used to study changes in gene expression in *X. laevis*.

In this chapter we describe methods to generate and culture *Xenopus* embryos and to perform targeted injection of antisense morpholino oligonucleotides (MOs) and capped mRNAs in order to manipulate gene expression in various tissues. It is worth noting that the microinjection techniques described herein are not limited to straightforward overexpression and knockdown of gene function and are best applied in the context of established *Xenopus* resources for optimum utility. For example, many signaling pathways responsible for early vertebrate axis formation and

tissue patterning (such as the bone morphogenetic protein (BMP) or the Wnt pathway) have been very well studied in *Xenopus*. The defects specific to alterations in these pathways may therefore be used as readouts for perturbations at particular steps of the given pathway. This phenomenon is well illustrated by studies that take advantage of the BMP signaling pathway. During *Xenopus* development, BMP is expressed in a caudal to rostral gradient across the early embryo. High BMP expression on the caudal side of the embryo is required to specify ventral and caudal fates, and misexpression of BMPs or molecules that are BMP downstream effectors on the rostral side of the early embryo causes characteristic ventralization of dorsal structures and caudalization of anterior structures (8, 9). Conversely, injection of BMP inhibitors, or proteins that have a dominant negative effect on BMP signaling, on the caudal side of the embryo results in inappropriate dorsalization and rostralization in this region and produces a characteristic axis duplication (10, 11). Overexpression or misexpression of genes in tissues where they are not normally expressed may thus provide clues to their normal function, depending on the effect that they have on an established molecular pathway or patterning event by changing downstream gene expression or morphology. Indeed, many genes of unknown function have been identified as naturally occurring agonists or antagonists of BMP function based on the dorsalized or ventralized phenotype that is observed when they are ectopically expressed.

A disadvantage of using injection of mRNAs is that there is poor control of transcriptional timing. Injected mRNAs are immediately translated and persist for many hours, and up to several days in some cases, in the embryo. Injection of cDNA expression constructs with tissue- or temporal-specific promoters is an alternative method for overexpression. cDNAs containing ubiquitously expressed viral promoters, such as the cytomegalovirus promoter, will not be transcribed until zygotic transcription initiates during the mid-blastula transition, so this technique may be useful for determining the effect of later gene induction. However, this approach has the major drawback that expression of cDNA constructs is of variable efficiency and results in highly mosaic expression (12, 13).

While different antisense technologies continue to be developed in a variety of systems, morpholinos have proven the most effective method for attaining reproducible knockdown of a specific gene (14). Blocking a known signaling pathway may also be accomplished by injection of a dominant negative protein (as described above for BMP). It is important to note, however, that dominant negative proteins (as is the case with a dominant negative BMP receptor) may affect other related proteins and family members and thus phenotypic effects may not be a result of inhibiting a single target.

Once gene expression has been altered by injection or by chemical exposure, tissues within individual treatment groups can be easily pooled, subject to microarray analysis (as discussed below), and analyzed for changes in global gene expression due to a specific perturbation. Although lack of a sequenced genome still poses a significant challenge when working with *X. laevis*, the availability of genetic resources continues to be improved.

1.1. Considerations When Performing a Microarray Analysis in X. laevis

X. laevis is an excellent system for controlling early gene expression (i.e., knockdown, upregulation, and/or mis-regulation of specific genes) in a whole animal system and for gaining access to tissues at very early time points. Because many embryos are obtained in a single spawning event, a large amount of material may be generated in a relatively short period of time. Experiments can thus be performed, and adequate quantities of sample for a microarray can be collected rapidly without relying on amplification techniques. However, because the genomic resources are scarce there tends to be very little or poor annotation for most genes on commercial *X. laevis* chips. It is therefore important to consider the annotation status for a particular chip before embarking on a resource-intensive study such as a microarray project. One may want to consider using a chip from the related organism *Xenopus tropicalis*, which shares a high-degree of homology with *X. laevis*. The *X. tropicalis* genome is not duplicated and it has thus been fully sequenced. Despite these drawbacks, a number of genetic resources have been developed lately (*see* (15) for a good review and comprehensive list) and progress continues to be made in understanding the *X. laevis* genome. Finally, because *X. laevis* is not a clonal species, it is also important to recognize that there will be a high degree of background variability between individual frogs. It is therefore important to have enough biological replicates to identify significant targets above the background and to have a good statistician on hand. In spite of these genomic limitations, its large degree of genetic conservation with higher organisms, short developmental timeline, and overall accessibility make it a very useful model system with which to efficiently study early events in vertebrate development.

2. Materials

Unless otherwise indicated, solutions should be prepared and stored at room temperature.

2.1. Generation and Testing of Morpholino Oligonucleotides

1. Morpholino oligonucleotides (GeneTools, LLC; see **Section 3** for considerations regarding morpholino ordering and appropriate control morpholinos).

2.2. Generation of Synthetic mRNA for Microinjection

1. mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Ambion). Polymerase-specific kits are available and selection depends on which RNA polymerase promoter is upstream of the gene of interest.
2. Equilibrated phenol, pH 8.0 (USB).
3. Sevag [Chloroform:isoamylalcohol (24:1)].
4. 10 M ammonium acetate.
5. Ethanol.
6. Isopropanol.
7. Sephadex G-50 spin column (IBI Scientific). Optional but recommended.
8. Linearized template cDNA.

2.3. Collection of Testes

1. Tricaine: 0.2% (w/v) dissolved in dH_2O (prepare fresh).
2. Modified Barth's Saline (MBS): for 10× stock, 880 mM NaCl, 10 mM KCl, 25 mM $NaHCO_3$, 100 mM HEPES (pH 7.5), 10 mM $MgSO_4$, 0.14 mM $Ca(NO_3)_2$, and 0.41 mM $CaCl_2$. Adjust to pH 7.5 with NaOH, filter sterilize, and store at 4°C.
3. Testis buffer: 10% fetal bovine serum, 1% Pen/Strep (100 U/mL penicillin and 100 μg/mL streptomycin; Sigma) in 1× MBS. Divide into 10 mL aliquots and store at –20°C.

2.4. Collection and Fertilization of Embryos

1. Powder-free, latex-free vinyl gloves for handling frogs (*see* **Note 1**).
2. 1 mL sterile syringe.
3. 27 and 20 gauge sterile syringe needles.
4. Human chorionic gonadotropin (Sigma): 4,000 U/mL dissolved in sterile dH_2O; store at 4°C.
5. Holtfreter's frog water: for 200× stock, 3 M NaCl, 34 mM KCl, 12.5 mM $NaHCO_3$, and 33.7 mM $CaCl_2$. For 4 L, dissolve NaCl, KCl, $NaHCO_3$ in 3 L dH_2O. Dissolve 19.8 g $CaCl_2 \cdot 2H_2O$ in 500 mL dH_2O, add slowly to above and bring to 4 L.
6. Conical Tissue Grinder (Research Products International).
7. Aged tap water (tap water, allowed to sit for at least 24 h for chlorine to evaporate; optional as 0.1× MBS may be used instead).
8. Dejellying solution (prepare fresh): 2% (w/v) cysteine dissolved in dH_2O. Adjust to pH 7.8–8.0 with NaOH.

9. DeBoer's Pond Water: for 20× stock, 100 mM NaCl, 1.3 mM KCl, 0.44 mM $CaCl_2$. Adjust to pH 7.4 with $NaHCO_3$ and store at 4°C.
10. Glass petri dish (100 × 15 mm).

2.5. Targeted Embryo Injection and Culture

1. Dissecting microscope and transmitted light source with double gooseneck arms. A manually operated X–Y stage is optional but recommended, particularly if homemade injection trays (Step 7) are to be used (**Fig. 3.2a**, **b**).
2. Microinjector that can accurately deliver 10–100 nL volumes. Narishige and Drummond make two good options. For this protocol, we describe injections using the Drummond Nanoject II.
3. Pulled glass capillaries (micropipettes; injection needles) used for sample injection, obtainable from companies that make microinjectors (Narishige $L = 90$ mm, OD = 1 mm, and ID = 0.6 mm; Drummond $L = 3.5$ in., OD = 1.14 mm, and ID = 0.53 mm).
4. Micropipette puller (Sutter).
5. 26 gauge Hamilton syringe to be used for backfilling the micropipette needle with mineral oil (for use with Drummond Nanoject).
6. Low-temperature (14–25°C) bioincubator is useful but not required.

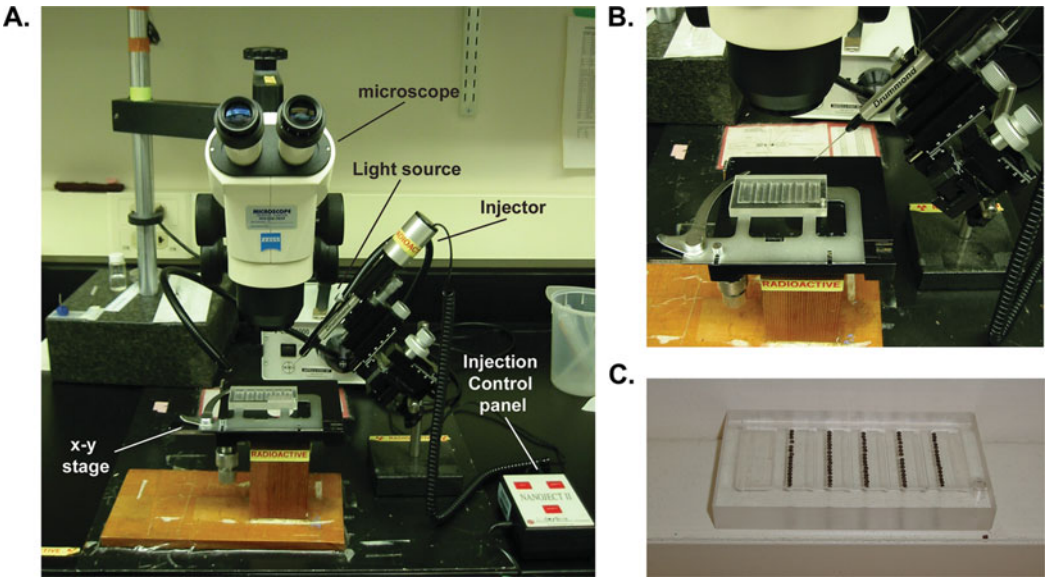

Fig. 3.2. Microinjection apparatus. (**a**) Example of a microinjection setup using a dissecting microscope, fiber optic light source, and an X–Y stage. (**b**) Close-up view of the X–Y stage with injection tray and injection apparatus (*right*). (**c**) Homemade injection tray with embryos prepared for injection in each of the five lanes.

7. Homemade injection trays: These are optional but highly recommended to facilitate injection (*see* **Note 2** and **Fig. 3.2c**).
8. Wooden applicator sticks for manipulating and sorting embryos. Sticks can be shaped to a point using a box cutter and then blunted slightly to avoid puncturing embryos during manipulation.
9. Sterile mineral oil (Sigma; for use with Drummond Nanoject).
10. Ficoll (type 400 DL; Sigma) solution: 5% w/v in 0.1× MBS. Adjust to pH 7.5.
11. Gentamicin, 50 mg/mL (Gibco).

3. Methods

3.1. Selection and Testing of Morpholino Oligonucleotides

Antisense morpholino oligonucleotides (MOs) block gene function by either binding to sequence near the ATG start codon of an mRNA and inhibiting its translation or binding to the splice junction of a gene and preventing splicing. Because complete sequence information, including splice junctions, is not available for *X. laevis*, translation blocking morpholinos are more commonly used in this species. *X. laevis* is pseudotetraploid, having undergone a partial duplication of the genome and thus it is important to search available databases [e.g., Xenbase (http://xenbase.org/genomes/blast.do?); Entrez (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene); The Gene Index Project (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi)] to determine whether there are multiple copies of your gene of interest and, if so, to identify sequences corresponding to both before designing MOs. MOs are purchased from GeneTools. The company provides instructions for optimal MO design on their Website (http://www.gene-tools.com/node/18), or customers can upload relevant sequence information and GeneTools will identify the most appropriate target sequence. In most cases, it is necessary either to design one oligo that will block both copies of your gene of interest or, if this is not possible, to use a mixture of two oligos that will target both copies of the gene. A non-overlapping oligo may be used as a positive control, for additional verification of phenotype specificity. A standard negative control MO is available from GeneTools. Alternatively, mismatched MOs containing five mismatched bases distributed throughout the oligo sequence are a more stringent test of specificity. The appropriate dose is dependent upon the individual MO and must be determined

empirically. To minimize potential toxic effects, we recommend starting at a low dose (1–5 ng) and increasing as necessary. We have found that high doses of MO may be tolerated reasonably well (up to 100 ng), depending on the particular MO.

1. MO solubility is an ongoing issue and appears to be dependent upon the individual oligonucleotide (*see* **Note 3**). MOs are shipped as lyophilized pellets. The MO is then resuspended, at a concentration appropriate to the particular use, in sterile (not DEPC-treated) dH_2O and heated at 65°C for 10 min to bring it into solution. For *Xenopus* injections, we have found that 1 mM stock dilutions are sufficiently concentrated for all required applications. Create appropriate working dilutions and store in individual aliquots at –80°C (*see* **Note 3**). Prior to use, heat aliquots at 65°C for 10 min and vortex briefly to ensure that all MO is in solution to obtain an accurate concentration. Spin tubes at top speed for 5 min in a microcentrifuge to remove insoluble material that may clog the injection needle.
2. There are several methods outlined below to verify that MOs specifically knockdown the intended target.
   i. Demonstrate reduction in protein synthesized by immunohistochemistry (IHC) or Western blotting in morphants compared to control embryos.
   ii. In the absence of an adequate gene-specific antibody, demonstrate reduction of an overexpressed epitope-tagged protein in morphants compared to control embryos by IHC or Western blotting.
   iii. If splice-blocking morpholinos are used, demonstrate that the targeted splicing event is inhibited via polymerase chain reaction (PCR) to detect loss of the spliced form in morphants.
   iv. Ultimately the best test of specificity is to demonstrate that phenotype(s) attributed to knockdown of a particular gene are rescued by co-injection of a gene-specific mRNA that harbors silent mutations that prevent morpholino recognition.

3.2. Generation of Synthetic RNA for Microinjection

mRNAs require a 5′ 7-methylguanosine cap and a 3′ polyadenylate [poly(A)] tail to prevent degradation and to promote efficient translation in vivo. For in vitro synthesized RNAs, the former is accomplished by addition of cap analog [m7G(5′)ppp(5′)G] to the synthesis reaction. A poly(A) tail can be added following the RNA synthesis reaction using a commercially available kit (e.g., Ambion poly(A) tailing kit) but is more commonly encoded in the cDNA or in the expression vector (e.g., pSP64T, obtainable from http://faculty.washington.edu/rtmoon/XE40.html (16)).

Alternatively, polyadenylation can be accomplished in vivo by the inclusion of an SV40 polyadenylation signal in the expression vector. pCS2+ is a commonly used multipurpose expression vector that includes an SP6 promoter upstream and an SV40 poly(A) signal downstream of the multiple cloning site, as well as a second polylinker with unique restriction sites for linearizing the template DNA downstream of the SV40 poly(A) signal (17, 18). A number of pCS2+ derivatives have been constructed that allow fusions to epitope tags or reporter proteins such as ß-galactosidase to facilitate detection in downstream applications. As with MOs, the dose for a particular mRNA must also be empirically determined. RNA tends to be more toxic than MOs, thus much lower amounts (10 pg–1 ng) should be used initially when determining the appropriate dose.

1. Linearized DNA template should be prepared in advance and resuspended in H_2O or TE at 0.5 μg/μL. An example protocol is as follows: Cut 20 μg of template DNA in 100 μL reaction volume with the appropriate restriction enzyme. Run 5 μL on an agarose gel to verify that cutting is complete. Extract once with Phenol:Sevag (1:1) and once with Sevag alone. Add ammonium acetate to 0.4 M and 2 volumes 100% EtOH. Precipitate overnight at –20°C or for 15 min at –80°C. Spin 10 min at 4°C at top speed and rinse with 70% EtOH. Vacuum dry DNA pellet and resuspend in 36 μL DEPC dH_2O. This will give a concentration of roughly 0.5 μg/μL, assuming a 10% loss of input.
2. Capped mRNAs for overexpression and/or morpholino rescue are generated using Ambion's mMESSAGE mMACHINE High Yield Capped RNA transcription kit.
3. To prepare capped RNAs, add the following ingredients, in the order listed below, to a sterile 1.5 mL tube at room temperature (if tube is kept on ice, the spermidine in the buffer can precipitate the template DNA).
   4 μL DEPC-treated dH_2O
   10 μL 2× rNTP Mix [includes m7G(5′)ppp(5′)G cap analog]
   2 μL 10× transcription buffer
   2 μL linearized template DNA (0.5 μg/μL)
   2 μL T7 or T3 RNA polymerase for a total volume of 20 μL.
4. Tap tube to mix and spin briefly to collect contents at the bottom of the tube.
5. Incubate at 37°C for 2–4 h (*see* **Note 4**).
6. Add 1 μL DNase (from kit) and incubate at 37°C for 15 min.

7. Before use in injection, the capped mRNA must be purified from the reaction components, particularly from the cap analog, which will compete with transcription of full-length product (*see* **Note 5**). Bring up reaction volume to 50 μL with 30 μL DEPC dH_2O to minimize loss during the purification and do a single Phenol:Sevag (1:1) extraction.
8. During centrifugation for the Phenol:Sevag extraction, prepare sephadex G-50 spin columns according to the manufacturer's instructions. Briefly, invert column to resuspend the gel. Remove caps on both ends of the column, place in a collection tube (provided) and spin at 1,100×*g* for 1 min in a swinging bucket centrifuge to drain excess buffer. Empty collection tube and repeat. Place column in new collection tube.
9. Following the Phenol:Sevag extraction, transfer the aqueous phase of the extraction (~50 μL) to the center of the gel bed of the sephadex G-50 column and spin at 1,100×*g* for 4 min.
10. Transfer the G-50 column eluate to a new 1.5 mL tube and precipitate the capped mRNA with 0.2 M ammonium acetate and two volumes of 100% EtOH at –80°C for 15 min. Spin at top speed in a microcentrifuge at 4°C for 10 min. Rinse pellet with ice-cold 70% EtOH and vacuum dry briefly. Do not over dry.
11. Resuspend the small RNA pellet in 25 μL DEPC dH_2O and determine mRNA concentration by UV spectroscopy. Run 300 ng of capped mRNA on a 1× MOPS gel to determine that a single full-length transcript has been synthesized and to verify expected concentration (*see* **Note 6**).

3.3. Collection of Testes

1. To harvest testes for fertilization, a male is anesthetized in 0.2% Tricaine for 20–40 min until unresponsive when its claw is pinched firmly between your thumb and forefinger.
2. Use a pair of blunt forceps to pull the skin up from the body wall. A tissue placed over the prospective incision site to remove excess moisture may aid in grasping the slippery skin. Using either a surgical knife or a pair of fine-pointed scissors, make a horizontal incision in the skin across the abdomen (**Fig. 3.3a**). Make a parallel incision in the underlying body wall, being careful not to damage the underlying organs. Make a third, this time vertical incision along the length of the abdomen, beginning at the midpoint of the horizontal incision and continuing anteriorly up the midline through both layers (**Fig. 3.3a**). Fold back the resulting flaps like the pages of a book (**Fig. 3.3b**). Clip ventricle of

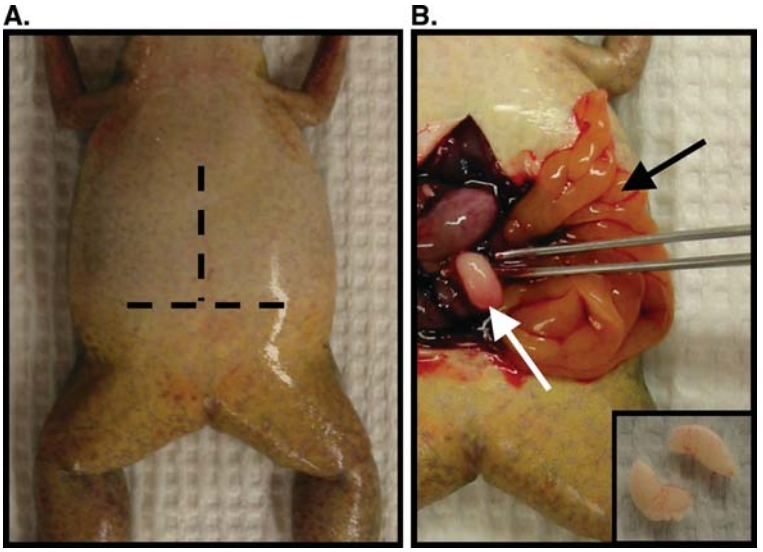

Fig. 3.3. Harvesting testes from a male frog. (**a**) Ventral view of an anesthetized male frog. *Dashed lines* represent the approximate location of horizontal and vertical incisions to be made. (**b**) Testis removal following incisions in *panel A* and exsanguination (described in **Section 3**). The testis (*white arrow* and *inset*) is located at the end of the fat bodies (*black arrow*) on the left and right side of the abdominal cavity.

the heart with the scissors to exsanguinate and ensure death. To prevent contamination of the testes, take particular care to avoid perforating the gut during the dissection.

3. Using a blunt pair of forceps, move the overlying organs aside and pull the fat bodies out (**Fig. 3.3b**, black arrow), following them back to either side to find the testes (**Fig. 3.3b**, white arrow). Remove each testis by snipping it away from the fat bodies with scissors.
4. Once the testes are removed, carefully snip away any adherent fat bodies and vasculature and rinse in a petri dish filled with cold 1× MBS to remove remaining blood before storing in cold Testis buffer.
5. For optimum results, use freshly isolated testes. Remaining testes may be stored at 4°C and used for up to 2 weeks, although sperm viability will decrease with time (*see* **Note 7**).
6. Dispose of the carcass appropriately according to IACUC regulations.

3.4. Collection and Fertilization of Embryos

1. To induce spawning, prime three mature females with 1,200 U human chorionic gonadotropin (HCG) solution by injecting 0.3 mL of 400 U/mL HCG per frog into the dorsal lymph sac (**Fig. 3.4a**, *see* **Note 8**). Injected females are housed in plastic drawers or buckets in a 15°C bioincubator in 1× Holtfreter's Frog Bath overnight (*see* **Note 9**).

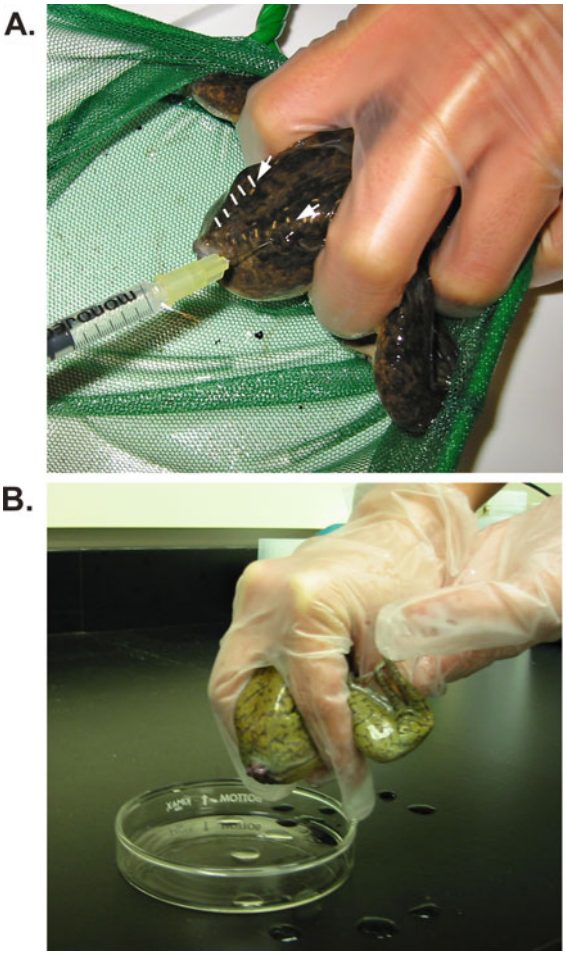

Fig. 3.4. Spawning eggs from a female frog. (**a**) Priming a mature female by injection of human chorionic gonadotropin into the dorsal lymph sac on the posterior aspect of the frog. The injection needle is inserted just lateral to the V-shaped lateral line sutures (*white arrows*; *dashed white lines* demarcate the sutures on the *left side* of the frog's back). The head and eyes of the frog are covered with the palm of the hand (out of view) to reduce stress, and the frog is immobilized by using a net to prevent forward motion and by holding the legs forward with two fingers. (**b)** Spawning of a female frog. The frog is held as described above, using the opposite hand, instead of the net, to provide additional stability.

Spawning will begin 12–14 h later if they are kept at 15°C. It may be necessary to exchange the Frog Bath to bring the frogs up to room temperature more quickly if spawning does not begin. Alternatively, females can be injected the day eggs will be used, housed at room temperature, and will begin spawning within approximately 6 h.

2. Collect eggs in a glass petri dish containing 1× MBS (*see* **Note 10**). To induce spawning, pick up the female in one hand, holding the rear legs anteriorly against her body

with the index and middle fingers on either side of her torso (**Fig. 3.4b**). To keep the frog calm, cover the eyes and head with the palm of the same hand. *Gently* apply pressure to the abdomen with thumb. The other hand may be used to stabilize the animal on the ventral side and to apply gentle pressure laterally (*see* **Note 11**). More than one dish may be used if necessary to distinguish the eggs from individual frogs.

3. Just prior to fertilization, cut a small piece of testis (~1/5, from **Section 3.3**) and place it into a clean 1.5 mL tube containing ~1 mL 1× MBS. Crush with a conical tissue grinder to make a testis slurry and store on ice until used up (~three fertilizations). Alternatively, a small piece of whole testis may be used, as described below.
4. Remove as much of the 1× MBS as possible from the dish of eggs with a plastic transfer pipet, taking care to avoid touching the eggs, which may damage them as they are fragile at this stage. The jelly-like consistency of the egg mass will protect the eggs from excess surface tension until they are fully dejellied (step 5). Add ~300 μL testis slurry and ~1 mL 0.1× MBS and agitate dish to ensure that solution reaches all eggs. The lower salt solution facilitates fertilization by increasing sperm activity (*see* **Note 12**). Allow eggs and sperm to incubate together for 2–3 min in the ~1 mL of 0.1× MBS to permit fertilization to take place. After fertilization, fill the dish with aged tap water or 0.1× MBS and let stand for about 20 min. During this time, the jelly coat will form such that the eggs adhere to each other and to the dish. Eggs that have been fertilized will rotate within the fertilization membrane such that the pigmented animal pole faces upward. From this time on, embryos may be kept at varying temperatures, ranging from 14 to 25°C to control the rate of development as necessary (*see* **Note 13**). An approximation of the changes in developmental timing based on temperature is as follows: 25°C = 100%, 20°C ≈ 75%, 16°C ≈ 50%, and 14°C ≈ 25% normal developmental rate. Timing of normal development is based on staging by Nieuwkoop and Faber (19).
5. To prevent the eggs from adhering to one another and to the needle during injection, it is important to fully dejelly the eggs. Once rotation is complete (~20 min) and prior to injection, embryos may be dejellied at any time. It should be noted, however, that empirical evidence suggests that cleavage patterns may be more regular if they are dejellied after they have cleaved at least once. Pour off the aged tap water or 0.1× MBS and fill the dish with 2% cysteine solution (pH 7.8–8.0). Very gently agitate on an orbital shaking plat-

form for 3–5 min until the jelly coat is completely removed and the eggs are clustered tightly together in the center of the dish.

6. Once the eggs are dejellied, remove the cysteine solution using a plastic transfer pipet or by tilting the dish and pouring into a waste container. The eggs are free-floating and fragile at this point and thus it is important to tilt the dish in order to keep the eggs covered with a small volume of fluid at all times to reduce damage from surface tension. Using the same technique, rinse the eggs two to three times with 1× De Boer's solution and then two times with 0.1× MBS. Embryos can be cultured in 0.1× MBS throughout early development.

3.5. Targeted Embryo Injection and Culture

There are many different small volume microinjectors available, but two commonly used apparati for injection of *Xenopus* eggs and oocytes are the Pico-injector (Medical Systems) and the Nanoject (Drummond Scientific). The Pico-injector uses compressed gas to reliably deliver nanoliter volumes through micropipettes by applying a regulated pressure for a digitally set period of time. For this method, the tip diameter of the micropipette is irrelevant, and thus finely tapered micropipettes with very small tip openings can be used to minimize damage to embryos, which is especially important when injecting single cells at late cleavage stages. A disadvantage is that injection volume must be calibrated for each new micropipette by measuring the size of the drop delivered using a stage micrometer. The Nanoject uses positive volume displacement to dispense preset volumes. This instrument is less costly and has the advantage that one does not need to calculate drop size for each micropipette. Micropipettes must exceed a certain minimal tip diameter, however, and must also be back-filled with mineral oil prior to use. Below, we describe a protocol for use of the mineral oil-based volume displacement method. For a detailed description of the gas pressure-based injector *see* (20).

1. To generate needles for microinjection, glass capillaries are heated and pulled to a fine point using a needle puller. Specific settings will vary between needle pullers, and some experimentation with the various parameters (i.e., heat, pull, velocity) is required. However, once the appropriate settings for a given capillary have been established, many needles may be pulled quickly in case replacement is required during the injection day or for future experiments.
2. A good needle is sharp with a small bore at an angle of approximately 45°. Under a dissecting microscope, clip the tip of the needle using a pair of sharp forceps. The needle

should be sharp but not so thin that the tip is flexible and will bend when it comes in contact with the embryo.

3. Backfill the needle with mineral oil using a Hamilton syringe. Insert the syringe all the way into the tip of the needle. Applying constant pressure to the plunger, eject the oil while slowly withdrawing the syringe to avoid introducing bubbles, which will compromise the accuracy of the volume displacement method used by the injector (*see* **Note 14**).
4. Load the needle onto the injector taking care not to introduce bubbles. Be sure that the needle is loaded straight to avoid bending the recessed plunger on the injector and apply gentle but firm pressure until the needle pops snugly into the rubber o-ring.
5. The injection control box has three buttons labeled "empty," "fill," and "inject." The inject button may also be operated using a foot pedal, allowing for hands-free injection. Lay a piece of parafilm on the injection stage, clean side up. Depress the "empty" button to eject one-third to one-half the volume of oil onto the parafilm in order to make room for the injection solution (*see* **Note 15**). Pipet a few microliters of the sample solution onto the parafilm and depress the fill button to draw sample into the needle, taking care to avoid air bubbles by keeping the needle submerged (*see* **Note 16**). When preparing to inject, depress the "inject" button to eject several droplets into air to determine if the injector is yielding droplets of roughly equivalent size.
6. Mount the injection tray on the injection stage (**Fig. 3.2b**) and fill with 5% Ficoll solution. Check that the injector is aligned properly with the stage such that the needle is positioned over the tray and tracks appropriately along each lane.
7. Using a disposable plastic transfer pipet, select and transfer embryos to the injection tray. When selecting embryos be sure to choose ones that look healthy (i.e., cleavage and pigmentation patterns are regular).
8. Arrange embryos of the desired stage in the injection tray, with the appropriate side facing the needle (e.g., if dorsal injection is desired, align the embryos with the dorsal side facing the injector).
9. The injection volume and speed are controlled by the orientation of a panel of dipsticks located on the right side of the injection control box. Find the key for dipstick settings and their corresponding volumes and speeds on the bottom of the control box and set them appropriately.

10. Proceed with injection, periodically injecting into air to ensure that the needle is not clogged and droplets are of expected size.
11. Once injections are complete, culture the embryos in Ficoll solution for several hours to overnight to allow them to heal (*see* **Note 17**).
12. Rinse two to three times in 0.1× MBS and remove any unhealthy or dying embryos as they will compromise the health of the others in the dish, then culture embryos in 0.1× MBS. 50 μg/mL gentamicin may be added in order to inhibit bacterial growth (optional).
13. Continue to culture embryos in 0.1× MBS (± gentamicin) until desired stage is reached. Culture solution should remain clear and be kept free of debris.

4. Notes

1. Frogs may be handled with clean hands or with vinyl gloves. Gloves made from other materials (e.g., latex) will damage the frogs' fragile skin. If gloves are not used, hands should be washed thoroughly before and after contact with the frogs to ensure that they are free from lotions and trace detergent, which can be damaging to the frog, and to remove bacteria that may have transferred from the skin of the frogs.
2. Though not required, we have found it extremely useful to use homemade injection trays (**Fig. 3.2c**) to hold embryos and oocytes for injection. These trays can be ordered from any custom acrylic fabricator and will be manufactured to specification. Our trays consist of a 7.5 cm × 4.0 cm × 1.0 cm acrylic block that is hollowed out to a depth of 4 mm. Narrow strips of acrylic (4 mm wide and either 1 or 2 mm high) are placed 2 mm apart in an alternating fashion to generate a trough in which one side is taller than the other; this allows the embryo to rest against the taller side while being injected on the opposite side (**Fig. 3.2b**, **c**). Embryos can be lined up in recessed lanes, allowing them to be completely submerged in Ficoll solution without floating about. This ensures that they are not subject to excess surface tension, yet are still held in place so that keeping track of which embryos have been injected is not an issue. A number of other techniques have also been used to hold embryos in place during injection. In the most basic setting, embryos can be placed in a petri dish and, using a

pair of blunt forceps, positioned and stabilized appropriately for injection. Following injection, the embryos are placed to one side of the dish to keep track of which ones have been injected. Alternatively, a plastic petri dish may be fitted with a nylon mesh and used to hold embryos in place (20).

3. GeneTools recommends storing MO stocks and working dilutions at room temperature and, if necessary, heating them at 65°C for 10 min prior to use (http://www.gene-tools.com/files/Essential%20INFO%2002-09.pdf). We have found that while some oligos are stable (i.e., they generate reproducible results) when stored at room temperature, we have also found that both the more concentrated stocks and the working dilutions of certain oligos have given more reproducible results when stored at –80°C and heated at 65°C for 10 min prior to use.
4. It is necessary to determine the appropriate cap analog:GTP ratio for your particular transcript. Increasing cap analog increases the fraction of capped transcripts but reduces yield. Yield is also dependent on the efficiency of the particular transcription enzyme (i.e., SP6, T7, or T3). We bias the reaction toward increasing the amount of capped transcript, as uncapped mRNA is rapidly degraded in the embryo. Ambion provides a very detailed protocol entitled "Synthesis of Capped RNA Transcripts" in the mMESSAGE mMACHINE manual which describes the potential benefits to varying cap analog:GTP ratio and incubation time which may be useful.
5. As an alternative to purification of capped mRNA on a sephadex column, nucleotide removal may also be accomplished by isopropanol precipitation after DNase treatment as follows: Add 115 μL RNase-free water and 15 μL ammonium acetate stop solution (from kit). In a fume hood, add 75 μL Tris-buffered phenol and 75 μL Sevag. Vortex well to mix. Microcentrifuge at 4°C for 5 min. Remove the aqueous top layer to a new 1.5 mL tube. Discard the bottom organic layer into an appropriate organic waste container. Add 150 μL 100% isopropanol, mix by inverting tube, and allow RNA to precipitate at –20°C for 15 min to 1 h. Spin at 4°C in a microcentrifuge for 15 min at top speed. Remove and discard the supernatant. Rinse pellet with 70% ethanol, spin briefly, remove all supernatant, and resuspend in 25 μL DEPC-treated dH_2O.
6. The expected yield for a single reaction starting with 0.5 μg cDNA template is 15–20 μg capped mRNA. The Ambion manual provided with the mMESSAGE mMACHINE kit

provides several useful sections on troubleshooting various issues with capped RNA synthesis. Low RNA yield may be due to several factors. Increasing the incubation time to 4–6 h, the amount of template, and/or the amount of polymerase are simple first steps to try. We have found that the quality and purity of the cDNA template is very important. Preparing new plasmid DNA or repurifying the cut template by phenol/chloroform extraction and ethanol precipitation often resolves yield issues for established transcripts. For transcripts that are being used for the first time, it may be necessary to empirically determine the appropriate ratio of cap analog:GTP as addition of the cap analog dramatically reduces yield. Some reactions may yield multiple products. Multiple bands on the gel may be due to persistent mRNA secondary structure from incomplete denaturation on the gel or to an artifact of electrophoresis or to premature termination of the mRNA synthesis reaction. In the case of the latter, the incomplete product will lack a polyA tail and will likely be degraded once injected. It is, however, important to adjust the concentration for injection appropriately so that it reflects only the full-length product.

7. To check sperm viability, the testis may be homogenized in 1× MBS, and a small amount of sperm may be diluted in 0.1× MBS, transferred to a glass slide, and examined for motility under a light microscope using a 10× objective.
8. We prime three females per experiment day to ensure that enough eggs are produced for a particular day's experiments. We recommend priming at least two frogs, as there is significant variability between individual frogs as to how well they spawn and the quality of eggs laid. When performing an experiment (such as a microarray analysis) in which minimizing background variability is essential, we recommend using the eggs from a single female.
9. If older females are used, ovary development may be stimulated by pre-priming frogs 3–10 days prior to desired injection day with 50 U Pregnant Mare Serum Gonadotropin (PMSG; Sigma) injected into the dorsal lymph sac. Females will need to be temporarily kept in a separate holding tank to distinguish them from females that have not been pre-primed.
10. In the wild, eggs are spawned into fresh water which induces formation of a jelly coat around the embryos. The jelly coat serves several functions, including providing mechanical support, acting as a block to polyspermy, and activating the acrosomal reaction required for fertilization. To facilitate in vitro fertilization, eggs are spawned into a

high salt solution (1× MBS) to prevent formation of the jelly coat. Eggs may also be spawned into a dry petri dish but care must be taken to avoid splashing water into the dish as this will induce instantaneous formation of a jelly coat and inhibit subsequent fertilization.

11. The cloaca of a female that is spawning will be dark red or pink in color. Females that are not spawning should not be squeezed. To induce spawning it may necessary to gently squeeze the frog as described in the methods. However, it is absolutely necessary to be gentle when squeezing and one must be sure not to press on the back of the animal as this may cause injury. Alternatively, the frog may push out the eggs when you pick her up. In this case, it is only necessary to stabilize her, using the same technique described above, while holding her over the egg collection dish.
12. Alternatively, cut testes may be used to directly fertilize the eggs rather than first homogenizing it in buffer. Remove 1× MBS, add ~1 mL 0.1× MBS and use a small piece of testis to touch each of the eggs. Proceed as described.
13. Embryos may be allowed to develop at a range of temperatures, from 14 to 23°C. This allows for control of the rate of development to facilitate injections at a particular stage or to produce embryos at varying stages simultaneously. While changing the incubation temperature does not cause problems for most applications, it is important to include the appropriate controls to ensure that your particular experimental output is not sensitive to changes in temperature.
14. Introduction of air bubbles into the injection needle is a common issue with this apparatus. Some tips to avoid introducing bubbles are as follows: (1) When backfilling the needle with mineral oil, be sure that the tip of the syringe is inserted as far as possible into the tip of the needle. Begin ejecting oil firmly *before* beginning to withdraw the syringe and continue ejecting oil until the syringe is completely withdrawn. Excess oil spilling out of the back of the needle can be blotted off with a tissue later. (2) When loading the needle onto the injector use a single upward movement. Avoid moving the needle up and down onto the plunger as this will also introduce unwanted bubbles. When seating the needle in the o-rings, take care not to chip the back of the needle as this will compromise the seal between the needle and the rubber o-ring.
15. A very small tip on the needle may prevent the mineral oil from being ejected effectively, if at all. If this is the case, carefully clip the needle back with sharp forceps to a 45°

angle (this can be done on the injector if you're careful) and attempt to eject the mineral oil. Repeat until the mineral oil can be ejected consistently.

16. When filling the needle with sample, keep the tip submerged in the sample and fill slowly. The plunger withdraws more quickly than either the solution or the mineral oil can pass through the needle, particularly when the tip is very small. This creates a vacuum, which can result in bubbles if the sample is depleted too quickly during filling. Pausing occasionally to allow the mineral oil and sample to catch up prevents this issue. A very small bubble or two may be unavoidable. If this is the case, be sure that the size of the bubble is minimized by depressing the eject button until sample starts to exit the tip of the needle. This will ensure that compression of the gas in the bubble does not affect the volume of oil displaced by the plunger during injection. A few small bubbles may not pose a significant problem, but if they become too numerous or too large, the needle will have to be reloaded.
17. Prolonged culture in Ficoll, while not detrimental, may affect the rate of development. Therefore, it may be necessary to also incubate controls in Ficoll. We recommend prompt removal from Ficoll solution once embryos have healed for several hours.

Acknowledgments

This work was supported by grants from the NIH (RO3HD058841 and RO1HD37976) and Shriners' Hospital Research Foundation to JLC. MM was supported by NIH grant T32HD049309.

References

1. Dale, L. and Slack, J. M. (1987) Fate map for the 32-cell stage of Xenopus laevis. *Development* **99**, 527–551.
2. Moody, S. A. (1987) Fates of the blastomeres of the 32-cell-stage Xenopus embryo. *Dev. Biol.* **122**, 300–319.
3. Moody, S. A. (1987) Fates of the blastomeres of the 16-cell stage Xenopus embryo. *Dev. Biol.* **119**, 560–578.
4. Green, J. B., Smith, J. C., and Gerhart, J. C. (1994) Slow emergence of a multithreshold response to activin requires cell-contact-dependent sharpening but not prepattern. *Development* **120**, 2271–2278.
5. Galli, A., Roure, A., Zeller, R., and Dono, R. (2003) Glypican 4 modulates FGF signalling and regulates dorsoventral forebrain patterning in Xenopus embryos. *Development* **130**, 4919–4929.
6. Scharf, S. R. and Gerhart, J. C. (1983) Axis determination in eggs of Xenopus laevis: a critical period before first cleavage, identified

by the common effects of cold, pressure and ultraviolet irradiation. *Dev. Biol.* **99**, 75–87.
7. Kao, K. R., Masui, Y., and Elinson, R. P. (1986) Lithium-induced respecification of pattern in Xenopus laevis embryos. *Nature* **322**, 371–373.
8. Dale, L., Howes, G., Price, B. M., and Smith, J. C. (1992) Bone morphogenetic protein 4: a ventralizing factor in early Xenopus development. *Development* **115**, 573–585.
9. Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V., and Hogan, B. L. (1992) DVR-4 (bone morphogenetic protein-4) as a posterior-ventralizing factor in Xenopus mesoderm induction. *Development* **115**, 639–647.
10. Maeno, M., Ong, R. C., Suzuki, A., Ueno, N., and Kung, H. F. (1994) A truncated bone morphogenetic protein 4 receptor alters the fate of ventral mesoderm to dorsal mesoderm: roles of animal pole tissue in the development of ventral mesoderm. *Proc. Natl. Acad. Sci. USA* **91**, 10260–10264.
11. Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K., and Ueno, N. (1994) A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* **91**, 10255–10259.
12. Sargent, T. D. and Mathers, P. H. (1991) Analysis of class II gene regulation. *Methods Cell Biol.* **36**, 347–365.
13. Vize, P. D., Melton, D. A., Hemmati-Brivanlou, A., and Harland, R. M. (1991) Assays for gene function in developing Xenopus embryos. *Methods Cell Biol.* **36**, 367–387.
14. Heasman, J. (2002) Morpholino oligos: making sense of antisense? *Dev. Biol.* **243**, 209–214.
15. Ogino, H. and Ochi, H. (2009) Resources and transgenesis techniques for functional genomics in Xenopus. *Dev. Growth Differ.* **51**, 387–401.
16. Zelus, B. D., Giebelhaus, D. H., Eib, D. W., Kenner, K. A., and Moon, R. T. (1989) Expression of the poly(A)-binding protein during development of Xenopus laevis. *Mol. Cell. Biol.* **9**, 2756–2760.
17. Rupp, R. A., Snider, L., and Weintraub, H. (1994) Xenopus embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8**, 1311–1323.
18. Turner, D. L. and Weintraub, H. (1994) Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434–1447.
19. Nieuwkoop, P. D. and Faber, J., and Hubrecht-Laboratory (1967) *Normal table of Xenopus laevis (Daudin). A systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis*, 2 ed. North-Holland Pub. Co., Amsterdam.
20. Sive, H. L., Grainger, R. M., and Harland, R. M. (2000) *Early Development of Xenopus laevis: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. Lane, M. C. and Sheets, M. D. (2006) Heading in a new direction: implications of the revised fate map for understanding Xenopus laevis development. *Dev. Biol.* **296**, 12–28.

Chapter 4

Developmental Genetics in *Xenopus tropicalis*

Timothy J. Geach and Lyle B. Zimmerman

Abstract

The diploid pipid frog *Xenopus tropicalis* has recently emerged as a powerful new model system for combining genetic and genomic analysis of tetrapod development with embryological and biochemical assays. Its early development closely resembles that of its well-understood tetraploid relative *Xenopus laevis*, from which techniques and reagents can be readily transferred, but its compact genome is highly syntenic with those of amniotes. Genetic approaches are facilitated by the large number of embryos produced and the ease of haploid genetics and gynogenesis.

Key words: *Xenopus tropicalis*, genetics, development, organogenesis, gynogenesis, genetic screens, genetic mapping, mutagenesis.

1. Introduction

Xenopus embryos have been remarkably productive models for developmental biologists for over 70 years (1). The dominant laboratory species *Xenopus laevis* provides an outstanding platform for embryological manipulations and gain-of-function gene assays, but its tetraploid genome and long generation time preclude many genetic and genomic approaches. The related diploid species *Xenopus* (*Silurana*) *tropicalis* shares many advantages of *X. laevis* for experimental embryology and has been adopted to combine these with genetic and genomic strategies.

Unlike the genome of teleost fish, derived from an ancient duplication, or that of *X. laevis*, from hybridization of two separate species, the genome of *X. tropicalis* is that of a canonical diploid vertebrate with a simple evolutionary history. At $\sim 1.5 \times 10^9$ bp, about the same size as zebrafish, it is one of

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_4,

the smallest tetrapod genomes and shows remarkable synteny with those of amniotes (2), simplifying orthology assignment and analysis of non-coding regulatory elements. Pilot forward genetic screens have already yielded a number of heritable mutants (3–5), some of which have now been mapped to specific genes (6). Genetic studies in *X. tropicalis* are facilitated by the production of up to >5,000 embryos from a single mating; meioses are thus rarely limiting for mapping studies. Extensive genomic resources are available, including a high-quality draft genome assembly and more than one million ESTs. Gain-of-function, molecular and embryological assays are readily transferred from the well-characterized *X. laevis* system. Combining these with loss-of-function genetics greatly enhances the range of analysis of vertebrate gene function in a single in vivo system.

This chapter offers a survey of methods that are specifically useful for genetic analysis of *X. tropicalis* development, including basic husbandry, breeding, genome manipulations (gynogenesis), mutagenesis, screening protocols, mapping strategies and analysis of mutant phenotypes.

2. Materials

2.1. General Embryological Materials

1. Frog/tadpole water: 0.55 g/L Sea salt in distilled H_2O (dH_2O) gives conductivity of ~1,000 μS.
2. Marc's modified Ringer's (MMR) 20× stock: 2 M NaCl, 40 mM KCl, 20 mM $MgCl_2$, 40 mM $CaCl_2$, 100 mM HEPES, pH 7.5. Dilute to 1× or 0.05× with dH_2O. Add gentamicin (GibcoBRL) as required at 50 μg/mL prior to use; adjust pH to 7.7–7.9 with 1N NaOH.
3. 0.05× MMR + BSA: 1 mg/mL Bovine serum albumin (BSA) in 0.05× MMR; adjust 0.05× MMR to pH 8.3 prior to adding BSA to prevent protein accumulation on pH probe. BSA is slightly acidic and will readjust pH to approximately 7.7.
4. 2.2% Cysteine: Cysteine hydrochloride (Sigma) in 0.05× MMR, adjust pH to 7.7–7.9 with 10 N NaOH. Use within 2 h.
5. 0.4% MS-222: Add 4 g of ethyl 3-aminobenzoate methanesulphonate (MS-222) to 1 L 0.05× MMR. Adjust pH to 7.7 with 1N NaOH. Store at 4°C and reuse up to 10 times.
6. L15/CS: Leibovitz-15 (L-15) media (GibcoBRL) supplemented with 10% calf serum (CS) (GibcoBRL). Store 10 mL aliquots at –20°C.

These materials are used in a variety of different protocols. Materials for specific procedures are outlined below. All chemicals obtained from Sigma, Poole, UK, unless otherwise specified.

2.2. Husbandry and Obtaining Embryos

1. Human chorionic gonadotrophin (HCG) (Sigma): Make stock of 1,000 U/mL in sterile H_2O and dilute accordingly. Store at 4°C. Sterilize seal with ethanol before and after each use.
2. Sera Micron Powder (Sera, Heisenberg, Germany).
3. ReptoMin sticks (Tetra, Melle, Germany).
4. Tropical fish flake (Sinclair Animal & Household Care, Gainsborough, UK) or equivalent.
5. Sorting tools. Manual pipette pump and stock of glass Pasteur transfer pipettes (*X. tropicalis* embryos tend to stick to plastic transfer pipettes). Notch glass Pasteur pipettes with a diamond pen, break off and blunt edges with a Bunsen burner flame.

2.3. Karyotyping

1. 27 g hypodermic needles.
2. Microscope slides (e.g. positively charged Superfrost Plus from Fisher) and large coverslips.
3. Paper towels.
4. Distilled H_2O.
5. 60% Acetic acid in distilled H_2O.
6. Hoechst 33342 stain (Sigma, Poole, UK), working stock 0.1 mg/mL in distilled H_2O.
7. 70% Glycerol in phosphate buffered saline (PBS).

2.4. Mutagenesis

1. *N*-Nitroso-*N*-ethylurea (ENU). 1 g Isopac (Sigma, Poole, UK).
2. 2-(*N*-Morpholino)ethanesulphonic acid (MES) (Sigma, Poole, UK): Make two 100 mM stocks in dH_2O. Adjust one to pH 6.0 and one to pH 6.2 with 1N NaOH, store at 4°C.
3. Lab coat, plastic wrist guards, gloves and facemask.
4. Decontamination bath: 10% Sodium thiosulphate, 1% sodium hydroxide in H_2O.
5. Nutator or roller.

2.5. Mapping

1. Embryo lysis buffer: 50 mM Tris–HCl, pH 8.8, 1 mM EDTA, 0.5% Tween-20. Store stock at RT and add 200 μg/mL proteinase K (Roche) immediately prior to use.

2. PCR-compatible 96-well plates.
3. Standard PCR reagents and equipment.
4. Super Fine Resolution (SFR) agarose (Amresco, Solon, USA).

2.6. Acrylamide Gels and Silver Staining Materials

1. Sequencing gel apparatus; two large glass plates, one larger than the other (Thistle Scientific—Model 2). Spacer thickness 0.4 mm.
2. Shark tooth combs.
3. One photographic developing dish, large enough to fit glass sequencing plate.
4. Denaturing DNA-loading buffer: 50 mL Stock = 49 mL formamide, 1 mL 0.5M EDTA, 0.1 g bromophenol blue, 0.1 g xylene cyanol.
5. 1× Tris borate EDTA (TBE) buffer.
6. 3-(Trimethoxysilyl)propyl methacrylate.
7. 100 and 70% Ethanol.
8. Acrylease (Stratagene).
9. 1 L of 10% Ethanol.
10. 1 L of 1% Nitric acid.
11. 1 L of 2 g/L silver nitrate.
12. 1 L Developing solution: 29.6 g sodium carbonate, 450 μL of 37% formaldehyde. Prepare in advance and keep on ice.
13. 1 L of 10% Acetic acid.

2.7. Genotyping

1. 0.07% MS-222: Add 0.7 g of ethyl 3-aminobenzoate methanesulphonate (MS-222) to 1 L 0.05× MMR. Adjust pH to 7.7 with NaOH.
2. Stock of fresh scalpels/razor blades (fresh one for each frog).
3. Lysis buffer: 100 mM Tris–HCl, pH 8–8.5, 200 mM NaCl, 0.2% SDS, 5 mM EDTA; 100 μg/mL proteinase K added just before use (Roche).
4. Isopropanol.
5. 70% Ethanol.

2.8. Sperm Freezing

1. Cryoprotectant: Disperse one egg yolk (about 15 mL) in an equal volume of distilled water; dilute to 20% (v/v) in 0.4 M sucrose, 10 mM sodium bicarbonate, 2 mM pentoxifylline solution. Centrifuge for 20 min at 13,000 rpm and use the supernatant (can be stored at –20°C for 1 month).
2. Styrofoam box small enough to fit into –80°C freezer.

3. Methods

3.1. Husbandry

Multigeneration genetic studies are critically dependent on minimizing generation time and maximizing egg quality and fertilization success. *Xenopus tropicalis* frogs will not thrive in same conditions as *X. laevis*, and the two species should *never* be housed in shared water systems, even with filtration, due to the risk of trans-species infection:

1. Housing for adults: Frogs may be kept in biofiltered recirculating systems or dump-and-fill standing water tanks. Temperature should be between 24 and 26°C, and conductivity adjusted to approximately 1,000 μS with sea salt, with pH 7–8. Take care to avoid sudden changes in temperature during water changes.
2. Diet for adults: Adult frogs are fed a mix of Tetra ReptoMin and fish flake three times a week.
3. Housing for larvae tadpoles are kept in standing water (0.55 g/L sea salt in distilled water) or very slow flow in a recirculating system. Change approx. half the water once or twice daily, taking care to avoid sudden changes in temperature during water changes. After the second week, an air bubbler is used to gently oxygenate standing water. Metamorphosing froglets benefit from floating platforms where they can rest out of the water.
4. Diet for larvae: Sera Micron powder is an excellent nutrition for filter-feeding *X. tropicalis* tadpoles in standing water. Feed very small amounts several times daily for the first 2 weeks, enough to produce transparent faintly green water. If tadpoles are kept in bright light, this diet can produce a healthy green bloom. Older tadpoles should be fed enough Sera Micron for the water to maintain a deep green colour. Older tadpoles in flow-through systems can be fed finely ground fish flake. Metamorphosing froglets to subadults are transferred to flow-through systems and fed whole fish flake and crushed ReptoMin daily.

(*see* **Note 1**).

3.1.1. Strains

Animals which differ at many genetic loci (polymorphic strains) are essential for genetic mapping. Two strains (*IC* (Ivory Coast) and *N* (Nigerian)) have been inbred for >11 generations and successfully used for mapping mutations (6, 7). An inbred *N* animal was the basis of the draft genome assembly (2); this strain may thus be more effectively targeted by sequence-based interventions such as morpholino oligonucleotides. Wild caught animals are occasionally available, but extreme care must be taken to

prevent disease introduction and taxonomic misidentification, as morphologically identical species are known to occur in overlapping range.

3.1.2. Sexing

Sexual dimorphism in *X. tropicalis* can be subtle, particularly in immature animals. Females are usually larger and plumper than males, with a more prominent cloaca, especially following hormone treatment. Males are slimmer, significantly more nervous and 'jumpier' than females, and have nuptial pads on the interior forelimb that may appear as dark streaks that are rough to the touch.

3.1.3. Obtaining Embryos and Early Embryo Care

Hormone injections are used to stimulate ovulation and breeding behaviour:

1. Animals may be grasped in hand for injection, with an index finger between the hind legs, or animals can be immobilized by swaddling with wet paper towels, with a hole torn over the posterior for injection.
2. Two injections of HCG are used to induce mating behaviour or ovulation. An optional 'priming' injection of 10 units of hormone in a volume of 0.1 mL of sterile water (i.e. dilute stock 10:1) is followed 12–72 h later by a second 'boosting' injection of 100 units in 0.1 mL (undiluted stock). Injections are made into the dorsal lymph sac by inserting a 27 g needle subcutaneously between the dorsal lateral line stripes (*see* **Note 2**).

3.1.4. Natural Mating

1. After the boosting injection, sexed pairs are placed in a fresh container with >10 cm deep clean frogwater (or 0.05× MMR, pH 7.6–8.0 + gentamicin) at 22–26°C in a quiet dark place.
2. The mating embrace (amplexus) should begin 1–3 h after boosting, with egg deposition beginning 1 or 2 h later, and continuing for up to 6 h. Water must be deep enough (>10 cm) to accommodate 'somersaulting' behaviour during egg deposition. Eggs can be collected using a plastic transfer pipette, taking care not to disturb the amplexed pair. Alternatively, the tank can be emptied of frogs and water and eggs stuck to the tank collected directly by cysteine treatment (**Section 3.1.6**).

3.1.5. In Vitro Fertilization

1. After the boosting injection, females are returned to containers at 25°C.
2. Male frogs are killed by terminal anaesthesia in 0.4% MS-222 followed by decapitation, and testes dissected into L15/CS at 16°C. Use testes from two males for up to four females, adding another male for every five additional females.

3. Three to five hours after the boosting injection, squeeze eggs from females into dishes containing a few drops of 1× MMR (high salt prevents premature egg activation). Discard batches containing lysing or stringy eggs.
4. Macerate testes with an Eppendorf pestle in an Eppendorf tube containing 0.5 mL L15/CS (or macerate in the dish with eggs using a scalpel), then distribute testis suspension over eggs and mix by shaking or stirring with a pipette tip.
5. After ~5 min for sperm binding, activate development by flooding with low-salt medium (i.e. 0.05× MMR or distilled water).
6. Eggs may be dejellied after 15–20 min (earlier dejellying can affect cortical rotation). First cleavage begins after ~45 min to 1 h at 23°C.
(*see* **Note 3**).

3.1.6. Dejellying and Early Embryo Care

No dejellying is necessary if >90% of embryos are cleaving normally and no early manipulations (such as microinjection) are required; embryos with jelly coats can be transferred to fresh dishes with 0.05× MMR + gentamicin. However, if >10% of the eggs are unfertilized or dead, viable cleaving embryos must be dejellied and sorted into separate dishes with fresh media:

1. More than 20 min after flooding, remove medium from eggs and replace with 2.2% cysteine in 0.05× MMR, pH 7.6–8.0; swirl intermittently for 4–8 min until the jelly is completely removed and eggs are touching each other.
2. Wash with 3–5 changes of 0.05× MMR.
3. Using a flame-polished glass Pasteur pipette fitted to a manual pipette pump, sort the cleaving embryos into fresh BSA-coated dishes containing 0.05× MMR + gentamicin, pH 7.6–8.0. Embryos should be plated at low density (~30/6-cm dish, 100/10-cm dish, 300/15-cm dish) and spread so that they are not touching each other. Culture at 25°C (22–28°C are tolerated).
4. On successive days, remove dead/dying embryos and replace with fresh 0.05× MMR + gentamicin daily. At 4 days, replace with 0.05× MMR without gentamicin (**Section 3.1**).
(*see* **Note 4**).

3.2. Genome Manipulations: Haploid Genetics and Gynogenesis

Simple and efficient procedures exist for generating both haploid *X. tropicalis* embryos, which can undergo several days of development, and viable gynogenetic embryos derived solely from the maternal genome. These manipulations are extremely useful for a wide range of applications including identifying polymorphisms, high-throughput forward genetic screens, rapid low-resolution

genetic mapping, homozygosing transgenes and generating completely homozygous isogenic strains.

Xenopus eggs, like those of many other lower vertebrates, are deposited prior to completion of second meiosis; extrusion of the second polar body normally begins approximately 5 min post-activation. If sperm suspensions are UV irradiated prior to use for in vitro fertilization, egg activation, polar body formation and cleavage can occur normally, but the cross-linked paternal genome cannot contribute to the zygote, resulting in the formation of haploid embryos (**Fig. 4.1**). Haploid embryos are not viable beyond feeding stages, with a high level of gastrulation defects and posterior abnormalities, but form anterior structures well enough for many phenotypes to be scored. If the mother is a heterozygous carrier of a recessive mutation, the phenotype may be visible in 50% of the haploid progeny.

In gynogenesis, embryos fertilized with UV-irradiated sperm are rescued to diploidy using one of the two basic methods. Polar body formation can be blocked with a simple cold shock shortly after fertilization (early cold shock, ECS), leading to the retention of both sister chromatid products of meiosis II. This method rescues haploids to viable diploidy with high efficiency and is extremely useful for uncovering recessive phenotypes in the progeny of carrier females. Critically, Mendelian phenotypic ratios are *not* expected in ECS embryos (gynogenotes), but the observed ratio can provide useful low-resolution map information (*see* **Fig. 4.1** and **Section 3.7**).

Alternatively, haploid embryos can be allowed to undergo the first round of DNA duplication, then rescued to diploidy by blocking the first cell division with late cold shock (LCS). This procedure can be less efficient than ECS but produces completely homozygous isogenic embryos and uncovers recessive phenotypes, regardless of chromosomal location in 50% of the progeny of heterozygous carriers. Both of these procedures were originally developed in *X. laevis* using pressure treatments (8, 9) but have been modified for the simpler cold shock technique by Rob Grainger's group (University of Virginia).

3.2.1. Production of Haploid Embryos

1. Twelve to seventy-two hours prior to procedure, prime two or more adult female *X. tropicalis* (as described in **Section 3.1.3**).
2. On the day of procedure, boost primed females.
3. Approximately 3 h after boosting females, kill two *male X. tropicalis* and dissect testes into L15/CS.
4. Label two 90-mm culture dishes, 'haploid' or 'diploid'.
5. Place a few drops of 1× MMR into 'haploid' dish for each female.

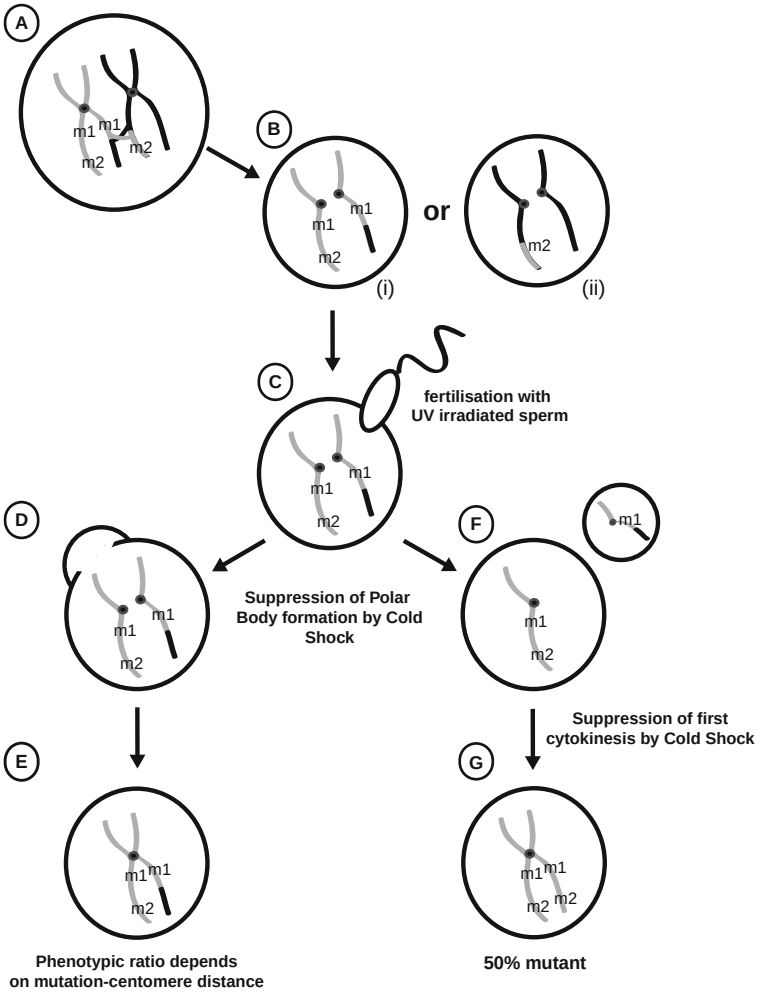

Fig. 4.1. Formation of haploid and gynogenetic embryos. (**a**) Diplotene oocyte in a female hybrid for mutagenized grey strain chromosomes and polymorphic black strain, showing a crossover event between mutant loci m1 and m2. (**b**) Unfertilized eggs showing segregation of sister chromatids after meiosis I. Note that regions where centromeres hold sister chromatids together are homozygous. (**c**) UV-irradiated sperm activates development without paternal genetic contribution, forming haploid embryos (**f**) following polar body extrusion. (**d**) Early cold shock suppresses formation of the second polar body, with the resulting gynogenote (**e**) rescued to diploidy and retaining both sets of sister chromatids. Recessive phenotypes at loci closer to centromeres (m1) are more likely to be uncovered than those at distal loci (m2), where recombination produces heterozygous wild type. (**g**) Late cold shock of haploid embryos following DNA replication prevents first cytokinesis, rescuing haploid to completely homozygous diploids.

6. Express eggs into media; avoid getting tank water from the frogs onto the eggs, this affects egg activation and fertilization efficiency. Discard dead, lysing or stringy eggs.
7. Transfer small number of eggs with pipette to 'diploid' control dish.

8. Place testes in an Eppendorf tube containing 500 μL L15/CS, macerate with Eppendorf pestle, add another 500 μL L15/CS and mix.
9. Allow testis fragments to settle and place sperm suspension onto glass Petri dish; save large chunks of testis for diploid control.
10. UV-irradiate sperm suspension in Stratalinker (Stratagene) or equivalent with 50,000 μJ ('energy' setting 500).
11. Add irradiated sperm to 'haploid' dish (*see* **Note 5**).
12. Add 500 μL fresh L15/CS to the non-irradiated testis fragments in the Eppendorf tube, mix and use to fertilize 'diploid' plate.
13. Gently shake dish to mix eggs and sperms, wait for 5 min and flood with 0.05× MMR.
14. Dejelly (**Section 3.1.6**), sort evenly cleaving embryos and culture overnight at 25°C.

3.2.2. Early Cold Shock (Gynogenesis by Suppression of Polar Body Formation)

As in haploid production (**Section 3.2.1**). In addition, add the following steps:

1. At least 1 h before in vitro fertilization, for each female being screened, chill ~50 mL 0.05× MMR in a slushy ice bucket (slushy ice gives better cold transfer).
   For each female, label two 10-cm dishes, 'diploid' and 'haploid', plus one 6-cm 'ECS' (*e*arly *c*old *s*hock) dish.
2. Carry out Steps 1–9 in **Section 3.2.1**.
3. Set timer for 5 min, flood embryos with 0.05× MMR and start timer.
4. Transfer ~90% of embryos from flooded 'haploid' dish to ECS dish and remove the media.
5. At 5 min, add ice-cold 0.05× MMR to ECS dish and place in slushy ice bucket for 7 min and 30 s.
6. After 7 min and 30 s, remove ECS dishes from slush bucket and replace media with RT 0.05× MMR.
7. Wait for >20 before dejellying and sorting. ECS cleavage will be delayed by 15–20 min relative to haploid and diploid controls (*see* **Note 6**).

3.2.3. Late Cold Shock (Gynogenesis by Suppression of First Cleavage)

Suppressing cytokinesis after the first round of DNA replication in the fertilized embryo can also rescue *Xenopus* haploids to completely homozygous diploids.

As in haploid production (**Section 3.2.1**); in addition, the following steps are followed:

1. At least 1 h before squeezing females, chill ~50 mL of 0.05× MMR per female in ice bucket as described in **Section 3.2.2**.

2. For each female, label three 10-cm dishes, 'diploid', 'haploid' and 'LCS' (*l*ate *c*old *s*hock).
3. Carry out Steps 1–9 in **Section 3.2.1**.
4. Set timer for 48 min (for RT of 22–23°C), flood embryos with 0.05× MMR and start the timer.
5. Transfer ~90% of embryos from flooded 'haploid' dish to LCS dish.
6. After 47 min, remove media from the LCS dish.
7. At 48 min, add ice-cold 0.05× MMR to the LCS dish and place in slushy ice for 5 min.
8. After 5 min, remove LCS dishes from ice bucket and replace media with RT 0.05× MMR.
9. Wait for more than 20 min before dejellying and sorting (*see* **Note 7**).

3.2.4. Karyotyping

Karyotyping may be used to confirm ploidy status and distinguish between *X. tropicalis* and similar non-diploid species. This protocol was developed by the Grainger lab (University of Virginia) and modified by M. Khokha (Yale University):

1. Place 10-stage 24–34 tadpoles into a dish of deionized water.
2. With a scalpel or a 27 g needle, remove the yolky ventral portion of the tadpole and discard; allow remaining dorsal portions to stand for 20 min.
3. Pipette the dorsal halves with as little water as possible into an Eppendorf tube containing 0.2 mL of 60% acetic acid in water; let stand for 5 min.
4. Pipette all of the tissue (with minimal acetic acid) and place on a positively charged slide (e.g. Superfrost Plus from Fisher); blot away excess acetic acid.
5. Place a large coverslip on the slide. Fold a paper towel to the size of the coverslip and place it on top. Apply heavy pressure on top of the paper towel/coverslip for about 5 min using a lead brick or by pressing forcefully with a thumb, being careful not to move around (*see* **Note 8**).
6. After 5 min, carefully remove the lead brick and paper towel.
7. Place the slide on dry ice for 5 min, then remove from dry ice and use a razor blade to gently pry the coverslip from the still-frozen slide.
8. Place the slide on a paper towel and stain the nuclei/chromosomes with Hoechst 33342 (1 μL Hoechst 33342 (stock: 0.1 mg/mL) in 1 mL distilled water) for 5 min. Wear gloves when working with Hoechst.

9. Tip the slide to allow stain to run off onto the paper towel.
10. Mount by placing a drop of 70% glycerol/PBS on the slide, add large coverslip and seal edges with clear nail polish.
11. Examine the slide for stained chromosomes by UV fluorescence using a high-power (63× or higher) objective.

3.3. Mutagenesis Strategies

A number of methods may be used to obtain mutations in *X. tropicalis*. Chemical mutagenesis seems to have the highest efficiency of mutation induction but requires a significant investment in positional cloning to identify the responsible gene (**Sections 3.4**, **3.7** and **3.8**). Chemical mutagens may be applied in vitro to mature sperm or in vivo to target spermatogonia. In vitro mutagenesis efficiently induces sequence lesions but has the complication that it results in a mosaic F1 generation, as typically the chemical adducts (usually produced by alkylating agents such as *N*-nitroso-*N*-ethylurea (ENU)) on a single strand of the sperm DNA double helix are not repaired and fixed on the complementary strand until the first somatic DNA replication or later.

Insertional mutagenesis is an attractive strategy, since known transgene sequences greatly ease identification of genomic integration sites and reduce reliance on positional cloning. A number of protocols have been described for mediating stable transgenesis in *Xenopus*, including transfer of sperm nuclei (10), various transposable elements (11), I-*Sce*I meganuclease (12) and phiC31 integrase (13). However, the relative inefficiency of transgenesis in *X. tropicalis* has thus far precluded large-scale screens for insertional mutants. Genetic manipulation of transgenic lines is also potentially powerful. Many reporter lines have been established in *X. tropicalis* (14), which may be useful substrates for genetic screens focusing on specific tissues or processes, and binary and inducible systems are available for experimental manipulation of gene function (15, 16). Cre recombinase has also been shown to be functional in *Xenopus* for lineage analysis (17) but has not yet been used to introduce conditional knockout alleles, since this also depends on obtaining a null background for the gene in question. As more mutant strains become available, this may become a viable strategy.

3.3.1. Reverse Genetic Strategies in Xenopus tropicalis

In vertebrates, reverse genetics, or mutation of known sequences in order to study phenotypic outcomes, is commonly mediated by homologous recombination in mouse ES cells. Equivalent procedures do not currently exist for *X. tropicalis*, although intriguingly, *Xenopus* oocytes and extracts efficiently perform extrachromosomal homologous recombination (18). More recently, chimeric zinc finger nucleases have shown promise for gene targeting in zebrafish (19).

As the cost of high-throughput sequencing falls, approaches based on scanning large populations of randomly mutagenized animals for specific sequence lesions have become more attractive. One such strategy, *t*argeting-*i*nduced *l*ocal *l*esions *i*n *g*enomes (TILLING), shows promise in *X. tropicalis* (3, 20). Genomic DNA samples are obtained from a large population of mutagenized animals, from which coding regions of target genes are amplified and sequenced. *Xenopus tropicalis* is particularly suited for this strategy since the mutagenized population can be archived as either frozen sperm (**Section 3.15**, (21)) or living stocks, as these frogs are much more long-lived than are other vertebrate genetic models. A *X. tropicalis* TILLING resource is currently under construction.

3.4. Chemical Mutagenesis in Xenopus tropicalis

ENU is highly carcinogenic and must be treated with extreme caution; all manipulations should take place in a fume hood, wearing lab coat, double gloves and plastic wrist guards. All materials that come into contact with ENU solutions should go into decontamination bath for 24 h. ENU solutions are also highly labile, and biologically effective dosage can be difficult to control. It is recommended that titration series are performed for each batch prepared.

3.4.1. ENU Mutagenesis

3.4.1.1. Preparation of ENU Stock Solution

1. Prepare fume hood by lining with absorbent bench coat and placing the decontamination bath and waste container/burn bin within.
2. Prepare 100 mL of 5 mM MES solution from 100 mM, pH 6.0, stock in dH_2O. MES buffer (unlike many other common buffers such as Tris) does not contain amine groups that react with ENU.
3. Remove ENU isopac bottle from protective canister (save can).
4. Using a 50-cm^3 syringe with 18-g needle, inject 85.4 mL of 5 mM MES, pH 6.0, into the ENU isopac bottle, carefully withdrawing air from the bottle into syringe while adding medium to avoid overpressurizing the bottle.
5. Return the bottle to shipping canister (or cover with aluminium foil) and place on nutator or roller shaker in hood for several hours, occasionally monitoring.
6. When powder is all (or nearly) in solution, swirl, allow to settle and freeze 1 mL aliquots at –80°C. Retain 20 μL for spectrophotometric determination of concentration.

3.4.1.2. Determining ENU Concentration by Spectrophotometry

1. 100 mM ENU solution should be approximately 11.7 mg/mL (1 g/85.4 mL).

2. Dilute 20 μL ENU solution to 1 mL with 5 mM MES, pH 6.0 (i.e. 1:50 dilution).
3. Using a disposable plastic cuvette, determine OD_{398}.
4. 1 mg/mL solution of ENU gives OD_{398} =0.72:
   a. Therefore, [ENU](mg/mL) = $(OD_{398})(50)/0.72$
   b. or [ENU](mg/mL) = $(OD_{398})(69.4)$.

3.4.1.3. In Vitro ENU Mutagenesis of Mature Sperm

1. Prime and boost five adult female frogs.
2. Make 10 mL of 3 mM MES, pH 6.2, in L15 (without calf serum) (add 0.3 mL of 100 mM MES, pH 6.2, stock to 9.7 mL L15).
3. Thaw an aliquot of L15/10% CS.
4. Kill five males and dissect testes into L15/CS media.
5. Prepare four 15-mL tubes with L15/3 mM MES (do not add ENU stock or sperm suspension until the last moment). MES buffer (unlike many other common buffers such as Tris) does not contain amine groups that react with ENU.

Sperm (mL)	ENU, 100 mM stock (mL)	L15/3 mM MES (mL)	f.c. (mM)
0.1	0	0.9	0
1	0.1	0.9	5
1	0.15	0.85	7.5
1	0.2	0.8	10

6. Thaw an aliquot of 100 mM ENU.
7. Macerate all of the testes from the five males in 0.5 mL L15/3 mM MES, pH 6.2 (no CS), using Eppendorf and pestle, then transfer to a 15-mL conical tube and bring volume to 3.5 mL with L15/3 mM MES, swirl to mix.
8. Add 0.1 mL sperm solution to 0 mM ENU control and 1 mL to the 15-mL tubes with L15/3 mM MES corresponding to each of the ENU treatments.
9. Add ENU as indicated in Step 5 and swirl to mix.
10. Place at 18°C for 1 h. Swirl to mix every 15 min.
11. Add 10 mL L15 to each tube and spin down sperm for 5 min at 1,000 rpm at RT in benchtop centrifuge.

12. While sperm solutions are spinning, squeeze eggs from females into drop of 1× MMR (**Section 3.1.5**); discard lysing/dead eggs, pool eggs from good females, mix and split into three 15-cm dishes for the ENU doses and a smaller aliquot of eggs in a 10-cm dish for no-ENU control.
13. At conclusion of spin, carefully pipette as much of the supernatant as possible to decontamination bath without disturbing the sperm pellet, then resuspend the sperm pellet in residual liquid by flicking. Repeat Steps 11 and 13 two times, then gently resuspend in 1 mL L15/CS.
14. Remove the remaining MMR from eggs, add treated sperm solution to eggs and mix by shaking briefly.
15. After 5 min, flood with 0.05× MMR twice (removing first rinse to decontamination bath) and dejelly after 20 min. Eggs may now be treated as safe to handle normally.
16. *Important:* Sort control and ENU-treated dishes at *4–8 cell* stages, making sure to make comparable dishes of *regularly cleaving* embryos from all doses. Dominant effects relative to controls will be scorable only if equivalent regularly cleaving embryos are compared.
17. The following 3 days, sort viable embryos and score control and mutagen-treated samples for dominant effects on gastrulation, death and other abnormalities:

	Wt(%)	*Gastrulation defect*	*Oedema*	*Other*	*Dead*
Control					
ENU 5 mM					
ENU 7.5 mM					
ENU 10 mM					

18. At feeding stage, select the dose(s) that result in a population of viable embryos, but also showed clear dominant effects compared to controls. If desired, expand population of animals treated at this dose.

3.4.2. Spermatogonial Mutagenesis

Mitotic spermatogonia, rather than mature sperm, can be targeted for in vivo mutagenesis by injecting adult male frogs with ENU. Replication in the spermatogonial lineage then fixes mutations in the germline, avoiding mosaicism in the F1 generation. Animals usually need several months to recover after an injection series, during which time the mutagenized mature sperm

(which would contribute to an unwanted mosaic F1) will be cleared:

1. Obtain five or more adult male frogs.
2. Weigh individual frogs (males typically weigh between 6 and 9 g).
3. Record weight and calculate the amount of 100 mM ENU stock needed for injection (0.1 mg ENU/g frog). Dose per frog (in mL) = [frog weight] × [0.1 mg/g] × [1 mL/11.7 mg ENU], or 0.006333 × frog weight.
4. Immobilize frogs by immersing for 2–5 min in a fresh stock of 0.07% MS222 at RT until they visibly begin to slow down. Immobilizing frogs with anaesthetic during injection reduces the risk of accidents with ENU-contaminated needles.
5. Inject the volume calculated in Step 3 to contain 0.1 mg ENU/g of frog weight subcutaneously into dorsal lymph sac.
6. Allow frogs to recover on wet paper towels in observation tank. They are usually awake and active in ~15–20 min.
7. Transfer frogs to observation tank with fresh water. Make sure to discard the paper towels and liquid in ENU waste and treat appropriately as ENU waste material.
8. After several hours, discard the frog water in ENU liquid waste and replace with fresh water.
9. The next day, discard the frog water in ENU liquid waste and replace with fresh water. Do this throughout the day for two to three more water changes.
10. Return frogs to colony.
11. Re-inject once a week for a total of three doses.
12. Allow frogs to recover for >3 months before breeding.

3.5. Genetic Screens

3.5.1. Forward Genetic Screens

Conventional three-generation breeding schemes to uncover recessive phenotypes are compatible with spermatogonial mutagenesis (**Fig. 4.2**). However, in vitro mutagenesis of mature sperm results in a mosaic F1 generation, making recovery of homozygotes by incrossing in subsequent generations very inefficient.

Populations derived from in vitro mutagenesis can still be efficiently screened by gynogenesis. Gynogenesis by polar body suppression ('early cold shock') will bias towards recovery of centromere-linked alleles, as these loci will be uncovered in a higher proportion of gynogenetic progeny ('gynogenotes') compared to those produced by more distal loci (**Section 3.7**). If females are in good condition laying good-quality eggs, sufficient

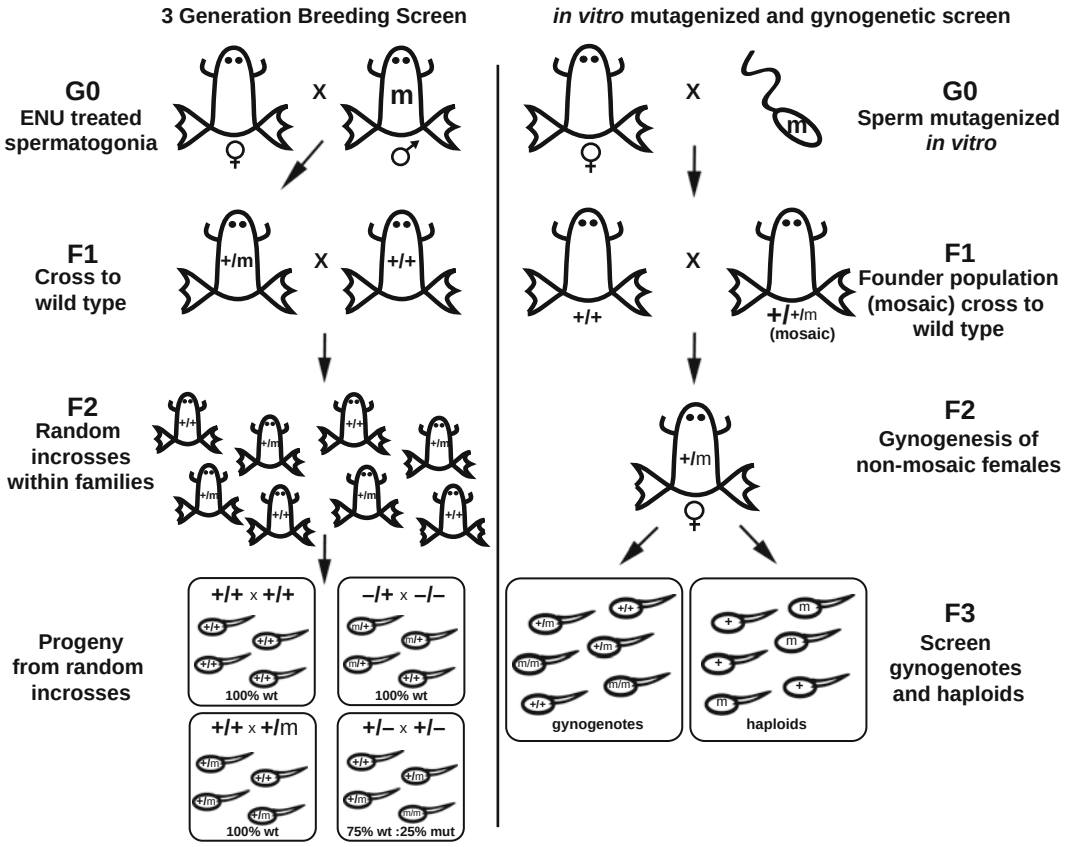

Fig. 4.2. Screening of mutagenized populations. *Left*: Three-generation breeding screen. In vivo mutagenized males are crossed to wild-type females (G_0), with the resulting F1 individuals outcrossed again to wild type to create F2 families. Progeny of random incrosses within families are then analysed for mutant phenotypes. *Right*: In vitro mutagenesis and gynogenetic screen. Eggs are fertilized with mutagenized mature sperm, creating a mosaic F1 population, which is crossed again to create a population of non-mosaic F2 candidate carrier animals. F2 females are then screened by gynogenesis to uncover recessive mutations.

numbers of gynogenotes can be produced to reveal mutations in much of the genome.

3.5.2. Early Cold Shock Gynogenetic Screen for Recessive Phenotypes

Gynogenetic screens are primarily used to identify carrier females that are heterozygous for recessive mutations. Ideally, non-mosaic animals are screened, as a greater proportion of mutant progeny will be produced and more readily detected. Females being screened must be identified individually or housed separately for the duration of the screen.

Early cold shock gynogenesis (**Section 3.2.2**) should include haploid and diploid outcross (non-irradiated sperm) controls. The diploid outcross control serves to assess sperm and egg quality and reveals dominant effects, and if a compelling phenotype is observed, these can be raised as the next generation of the line. Haploid controls help evaluate efficiency of sperm irradiation

(**Section 3.2.1**) and are useful for identifying polymorphisms (**Section 3.6.2**). Some anterior phenotypes may also be scorable on the haploid background; these are expected to be observed in ~50% of the embryos.

The fraction of ECS gynogenotes in which a given recessive phenotype is observed (*Fm*) is not Mendelian. Loci that are close to centromeres will be uncovered in up to 50% of the gynogenetic progeny of heterozygous females. More distal loci will tend to be observed with progressively lower frequency, bottoming out at ~10–15% due to presence of multiple crossovers.

In most cases, ECS does not result in quantitative rescue of all haploid embryos to diploidy. Background abnormalities from remaining haploid and aneuploid embryos can make it difficult to identify pre-neurulation phenotypes. Post-neurulation phenotypes can be screened efficiently by selecting morphologically perfect wild-type embryos from ECS and diploid control dishes on the morning after fertilization (st. 18–22) and monitoring these for subsequent appearance of abnormalities.

3.5.3. Morphological Screening Checklist

1. Compare sibling outcrossed diploid with ECS and haploid embryos for stage-specific developmental processes and to establish a baseline of egg-based, non-heritable abnormalities and/or dominant phenotypes. Specific phenotypes that are uncovered in multiple embryos within a clutch are particularly convincing. If you see a phenotype in ECS or haploid dishes, separate those embryos and record specific defect(s) and number of phenotypically mutant and wild-type embryos. The phenotype might be lethal; isolating those embryos will make it easier to score the following day. Phenotypes that are scorable in haploids are expected at 50%. Single-gene phenotypes in a clutch of ECS embryos are expected at a maximum of 50% for centromere-linked loci, decreasing to ~10% for distal loci. Record all abnormalities on a score sheet (*see* example in **Fig. 4.3**). Collect both mutant and wild-type ECS embryos in 96-well plates for use in low-resolution mapping and assignment of linkage group (**Sections 3.6.1** and **3.7**). Also collect a small set (6–12) of haploid embryos for identifying polymorphic markers (**Section 3.6.2**).

   Day 0: gynogenesis and cleaving embryo sorting

2. Perform gynogenesis on potential mutant carriers as outlined in **Section 3.2.2**.
3. Sort regularly cleaving embryos from unfertilized embryos at 4–16 cell stages. Irregularly cleaving embryos will gastrulate poorly, increasing background 'noise' and making it more difficult to detect specific phenotypes. Likewise, treat embryos with optimum care to minimize

Gyno Scoresheet Your Initials: MateDate:
Mother Stock No: Genotype: Frog No.

A: Axis	B: dwarf	C:Circulation	D: Ear	E: Oedema	F: Eye
G: Gut	H: Heart	I: Head	J: pronephr	K: Cilia	L: lethal/dead
M: Motility	N: Neural	O: Other/notes	P: Pigment	Q: blastop.	R: pre-gast

ECS

Phenotype	Day1	Day2	Day3	Day4
Wild type				
Total				

Haploid

Wild type hap				
Diploid-like				
Total				

Diploid outcross

Wild type				
Total				

Notes:

Conclusion:

Fig. 4.3. Gynogenetic screening checklist. Sample form for scoring phenotypes during a gynogenetic screen.

abnormalities caused by overcrowding or other mistreatment (**Section 3.1.6**).

Day 1: Tailbud stage sort (~16–20 hpf; St. 18–24)

4. As early as possible, sort normal from dead/abnormal embryos in all dishes, again to obtain a low background of early defects upon which to recognize later-developing phenotypes. Record number of dead embryos and remove them. Remove abnormally developing embryos from ECS dish to fresh plate noting phenotype.
5. Check ECS dishes for any obvious axial or dorsoventral polarity defects. If these are seen in >50% of embryos, the

defect is likely due to imperfect gynogenetic rescue or poor egg quality.

6. Sort haploid dishes and discard embryos that fail to develop reasonable heads, then score those with good heads for percentage of 'diploid-looking haploids'. True haploids typically display posterior truncations, failure of blastopore closure and raised neural folds. Appearance of diploids can be due to either spontaneous polar body failure (in which case both diploid-appearing haploids and ECS can be scored for recessive phenotypes) or failure to inactivate sperm DNA, resulting in diploid embryos in the haploid control and triploid embryos in the ECS dish, reducing the proportion of embryos in which recessive phenotypes may be detected.

   Day 2: 48 hpf; St. 35–40

   Check for the following:

   a. *Axial defects:* Size/shape of embryos, truncation/kinking and gross tissue defects.

   b. *Mobility phenotypes:* Swirl embryos to the centre of the dish and gently poke with forceps tip. Wild-type embryos respond by twitching or swimming away.

   c. Next add a few drops of 1:1,000 MS-222 to the dish, swirl and repeat until embryos are immobilized.
   Score the embryos for the following defects:

   d. *Cilia:* Anaesthetized wild-type embryos 'glide' forwards due to coordinated beating of epidermal cilia.

   e. *Heartbeat:* Is heartbeat present/regular, speed of beat normal? Note that anaesthesia can affect heart rate.

   f. *Circulation:* Look at the tail above, below and in-between the somites for blood movement.

   g. *Kidney:* Is pronephros forming/looping?

   h. *Somites:* Are the somites patterned properly, chevron-shaped and numbered?

   i. *Pigmentation:* Retinal pigment epithelium (RPE) defects? Have melanocytes formed and taken on the spreading star shape? Is there an increase in or strange patterns of pigmentation?

   j. O*edema:* Check for oedema in unusual or interesting places. Nonspecific oedema often forms around the ventral abdomen or heart, but can also be associated with specific phenotypes, e.g. heart defects, and should not necessarily be disregarded.

After screening, transfer embryos back to fresh media without anaesthetic.

Day 3: 72 hpf; St. 40–43

Check for the following:

Repeat day 2 checks. Embryos without heartbeat will probably display oedema by now. If not, check for blood flow and note any accumulation of blood in the body cavity.

Gut defects: Check for correct coiling of the gut. Does coiling occur, is it always in the same direction? Stage comparison is important.

Otolith/Otic vesicle: Is the size and shape of otic vesicle correct? Are there differences in the otoliths?

Day 4: 96 hpf; St. 43–46

Repeat previous checks. Saccular and utricular otoliths will be much clearer today.

Head morphology: By day 5 of development, the head will have flattened and cleared. Compare jaw morphology and hindbrain segmentation with diploid controls.

3.6. Mapping Mutations

Many of the mapping strategies developed in other genetic systems (22) can be applied directly to mapping in *X. tropicalis*. *Xenopus tropicalis* has several unique advantages for positional cloning. While the *X. tropicalis* genome assembly is currently fragmented (2), a meiotic map of simple sequence length polymorphisms (SSLPs) has been organized into 10 linkage groups corresponding to the 10 *tropicalis* chromosomes (http://tropmap.biology.uh.edu). Many phenotypes uncovered by gynogenesis can be rapidly assigned to one of the 10 chromosomes using a small set of centromere markers (**Section 3.7** and (7)). *Fm*, the fraction of phenotypic gynogenotes, also provides an estimate of the gene–centromere distance. Such low-resolution map information is useful for evaluating candidate genes. Higher resolution mapping is accelerated by the large numbers of embryos produced; upwards of 5,000 meioses can be scored routinely from a single cross. **Figure 4.4** shows a flowchart with mapping strategies.

3.6.1. Embryo Genomic DNA Prep

Genomic DNA for mapping is readily obtained from whole embryos using a proteinase K-based lysis buffer:

1. When embryos are at least 3 days old, sort phenotypic mutants into a separate dish using a flamed Pasteur pipette.
2. Place mutant embryos individually in wells of 96-well plates.
3. Collect ~12–24 wild-type embryos from the same breeding into clearly marked wells.
4. Remove excess media from each well and freeze at –80°C unless prepping genomic DNA immediately.
5. Add 50 μL of lysis buffer with proteinase K.
6. Incubate in PCR machine at 56°C for 4 h followed by 5 min at 95°C.
7. Use directly in PCR; no clean-up required for most mapping applications (*see* **Note 9**).

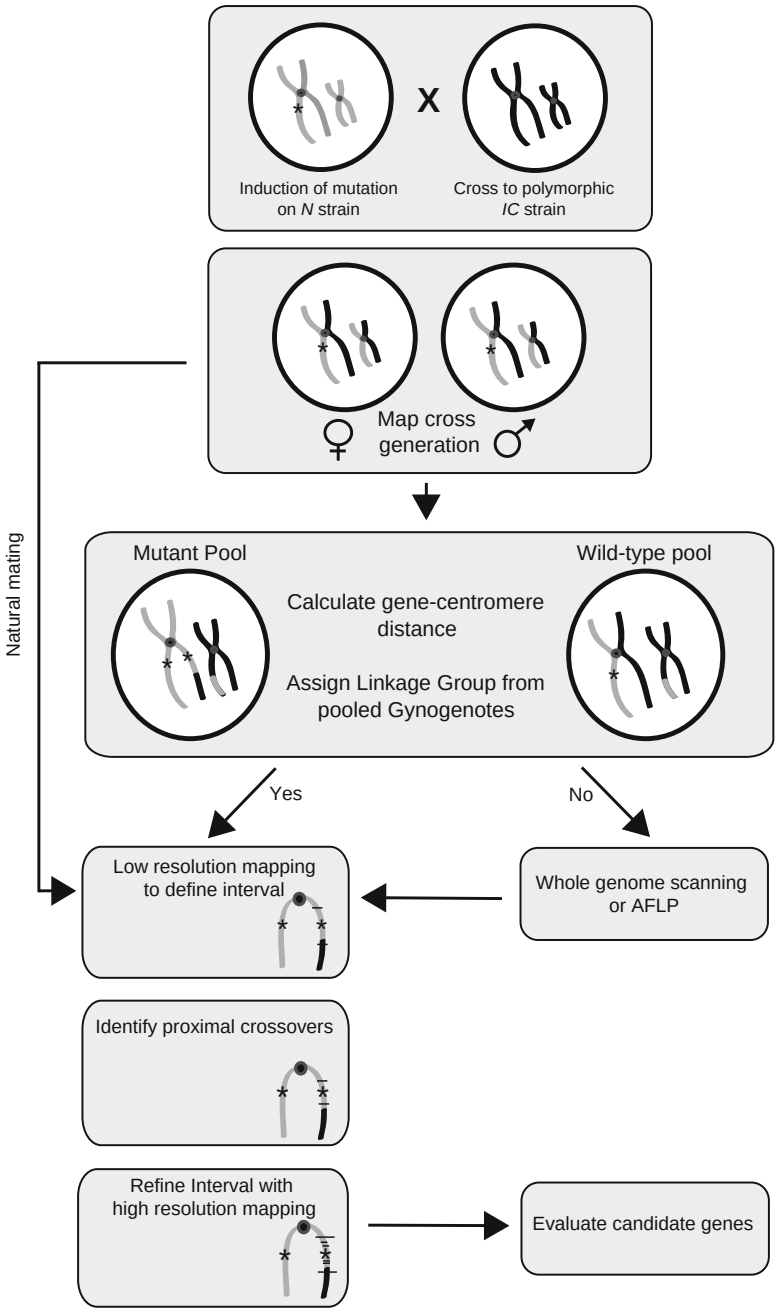

Fig. 4.4. Flowchart for genetic mapping in *X. tropicalis*. A recessive mutation induced on one strain (*grey*) is crossed to a polymorphic mapping strain (*black*) to obtain hybrid map cross carrier animals. Gynogenetic embryos are obtained from map cross females to calculate gene–centromere distance and for bulk segregant analysis with centromere markers to identify linked chromosome. Conventional crosses between map cross carriers are performed for subsequent analysis. If chromosomal linkage cannot be assigned by bulk segregant analysis, whole genome scanning with polymorphic markers, or amplified fragment length polymorphism (AFLP) analysis, can be used. Low-resolution mapping with a small number of mutant embryos is used to identify markers ~3–10 cM apart flanking the mutation. These two flanking markers are then used to type large numbers (>500) of mutant embryos to identify those with crossover events between the flanking marker and the mutation. Small sets of recombinants can then be analysed with further markers to refine the interval

3.6.2. Identifying Polymorphic Markers

Simple sequence length polymorphisms (SSLPs) are abundant between *N* and *IC* strain animals, so ideally mapping is conducted on the offspring of hybrid *N/IC* 'mapcross' animals to detect sequence polymorphisms that are linked to the phenotype. However, in all but the most inbred stocks, sufficient polymorphisms for low-resolution mapping are still likely to be present. It may be necessary to test several candidate SSLPs in a region to identify those that are polymorphic in a given cross:

1. First prepare the following:
   a. Genomic DNA extracted from haploid embryos from a mapcross hybrid female (**Section 3.6.1**).
   b. PCR master mixes with range of potential polymorphic markers.
2. Transfer 2 μL of DNA from six individual haploid embryos to fresh tubes.
3. Set up PCR master mix with primers for marker to test.
4. Add 8 μL of master mix to each individual haploid embryo DNA.
5. Run PCR under the following conditions: 94°C for 2 min, 35 cycles of (94°C for 30 s, 58°C for 30 s, 72°C for 1 min), 72°C for 5 min, 4°C hold.
6. Run 5 μL on 3% Super Fine Resolution (SFR) agarose gel or polyacrylamide gel and silver stain (**Section 3.6.3**).
7. If individual haploids produce different molecular weight PCR bands at ~1:1 ratio, the marker will be polymorphic in the female parent and can be used for mapping.
8. Repeat for each candidate marker.

3.6.3. Polyacrylamide Gels and Silver Staining

While agarose gels are quick and convenient, resolution is limited and subtle polymorphisms may be missed. Single-base resolution can be obtained using standard sequencing-style denaturing 6% polyacrylamide/8% urea gels, visualizing DNA bands with silver nitrate. This protocol was adapted from (23):

1. Thoroughly clean and dry both glass plates.
2. Coat small glass plate with mixture of 5 mL of 100% EtOH, 75 μL of 10% acetic acid and 5 μL of 3-(trimethoxysilyl)propyl methacrylate.
3. Wash with dH_2O followed by 70% EtOH, wipe and allow to dry.

Fig. 4.4. (continued) and number of genes contained within it. Candidate genes are then evaluated by changes in gene expression, spatial expression of transcripts and cDNA sequence. Functional confirmation of any mutation found is accomplished by morpholino phenocopy and rescue with mRNA.

4. Spray large glass plate with Acrylease (Stratagene). Wait for 5 min, then wipe with clean wet tissue.
5. Pour 6% acrylamide gel (containing 8 M urea) using shark tooth combs to make wells.
6. Pre-run gel in 1× TBE for 30 min at 80 W.
7. Dilute PCR 1:2 with denaturing DNA-loading buffer and heat to 95°C for 3 min in a thermocycler.
8. Load 5 μL of sample onto gel and run at 55 W. Run is complete when the buffer front passes through the bottom of the gel.
9. Split the glass plates apart with a razor blade.
10. Transfer glass plate containing gel to a large photographic developing dish, with the gel side up.
11. Fix gel in 1 L of 10% EtOH (this can be reused up to six times) for 10 min.
12. Wash in 1 L of 1% nitric acid for 3 min (this can be reused twice).
13. Rinse twice in dH_2O 3 min for each wash.
14. Stain for 20 min in 1 L silver nitrate.
15. Rinse twice in dH_2O 3 min for each wash.
16. Add 1 L developing solution and agitate gently until bands appear. This is usually within 5 min depending on the temperature of the solution.
17. Stop the reaction in 10% acetic acid for 5 min.
18. Wash gel in dH_2O for 10 min.
19. Transfer to a light box to photograph with a standard digital camera.

3.7. Low-Resolution Mapping with Centromere Markers and Gynogenesis

The initial step in positional cloning usually entails defining the chromosome or genetic linkage group that contains the mutation. In many cases, this can be accomplished rapidly by analysing pools of mutant and wild-type gynogenetic embryos with polymorphic markers located near each of the 10 *X. tropicalis* centromeres to identify one which segregates with the mutant phenotype (*see* (7)). Examination of DNA from pools of mutant and wild type, known as 'bulk segregant' analysis, simplifies rapid identification of markers linked to the mutant phenotype.

As outlined in **Section 3.2**, gynogenesis prevents second polar body extrusion allowing the post-recombination sister products of meiosis II to be retained. The genome of a gynogenote is therefore completely maternally derived, but not completely homozygous, analogous to half of a yeast tetrad (**Fig. 4.1** and (24). Polymorphic markers at the centromeres, where each pair

of sister chromatids is held together during recombination, will be homozygous, with the different alleles segregating into different individual gynogenotes (**Fig. 4.1**). Gynogenetic embryos that are phenotypically mutant for a recessive allele are also by definition homozygous at this mutant locus. If the mutation is located reasonably close to a centromere, a pool of mutant gynogenotes will also appear homozygous for the cognate centromeric marker derived from the mutagenized strain, while the wild-type pool will contain the alternative allele (**Fig. 4.5**). For the chromosomes that do not contain the mutation, both centromere alleles will contribute equally to mutant and wild-type pools. In this fashion, the linked chromosome can be identified using only the small set of 10 centromeric markers, corresponding to the 10 different chromosomes, and two pools of mutant and wild-type gynogenetic DNAs.

Conveniently, this apparent centromere–mutation linkage extends to much more distal mutant loci. Consider a recessive mutation *m1* induced on the *N* background and crossed onto the polymorphic *IC* strain to create a heterozygous N^*/IC carrier female (**Fig. 4.1**). The gynogenetic offspring of such a hybrid will thus each be homozygous *N/N* or *IC/IC* at all centromeres, and the mutant embryos will be N^{m1}/N^{m1} at the mutant locus. For a mutation *m2* further from its centromere, recombination events are more likely in the interval, resulting in gradual accumulation of wild-type N^{m1}/IC heterozygotes and decreasing the fraction of mutant gynogenotes. The wild-type pool will thus contain both *IC/IC* (from the original parental allele) and *N/N* centromeres (from single crossovers producing heterozygotes at the mutant locus). However, the reduced fraction of N^{m1}/N^{m1} mutant embryos is still likely to be homozygous *N/N* at the corresponding centromere. The exceptions derive from multiple crossover events; half of double crossovers will return linkage to the original centromere allele, while half may switch to the alternative 'non-parental' allele (*see* (7)). Only when the mutant locus is so distal that the majority of gynogenetic embryos contain multiple intervening crossovers will the mutation no longer appear linked to its centromere.

In addition, a rough estimate of the gene–centromere distance can be obtained from the proportion of mutant gynogenotes observed. If we assume that only single crossovers are present, then

$$\text{gene–centromere distance (cM)} = 50(1–(2 \times Fm))$$

where *Fm* is the mutant/total number of embryos

In practice, this formula provides useful information for loci less than ~30 cM from centromeres ($Fm > 0.2$), where single crossovers predominate. If $Fm < 0.2$, the gene–centromere

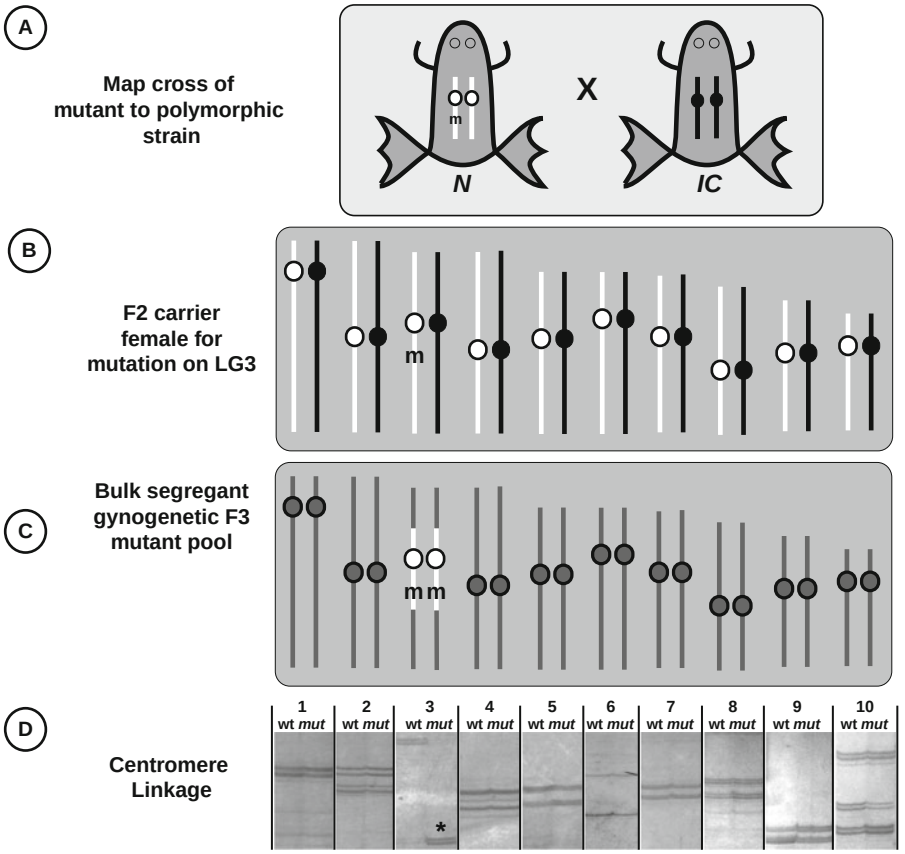

Fig. 4.5. Assigning chromosome linkage by bulk segregant analysis. (**a**) A frog carrying a recessive mutation m on the *N* strain (*white chromosomes*) is crossed to a polymorphic *IC* strain (*black chromosomes*). (**b**) 'Mapcross' hybrid F2 carrier inherits one chromosome from each parent. (**c**) Pools of ~20 phenotypically mutant and wild-type gynogenetic embryos are collected (mutant pool represented). Unlinked chromosomes show equal contribution from white *N* and black *IC* alleles (*grey chromosomes*) in both mutant and wild-type pools. However, on the chromosome containing the mutation, the mutant pool is greatly enriched for the white *N* centromeric allele; the wild-type pool may contain either the *IC* allele or both *N* and *IC*. Centromere linkage can often be detected over large genetic distances in gynogenetic embryos. (**d**) Silver-stained gel showing pools of mutant and wild-type embryos scored with polymorphisms at the 10 *X. tropicalis* chromosomes. Linkage is detected to chromosome 3.

distance calculation only establishes that the locus is further than 30 cM from the centromere, as multiple crossovers are common in the longer chromosome arms. This rough map information can be used to refine candidate gene sets and to select markers for higher resolution linkage analysis.

3.7.1. Assigning Genetic Linkage Group by Bulk Segregant Analysis

1. First prepare the following:
   a. Two pools of DNA from 10 to 20 phenotypically mutant gynogenotes (5 μL from each). Single pools and smaller numbers of embryos can be used, but risk of false positives increases.

   b. Two pools of DNA from 10 to 20 phenotypically wild-type gynogenotes (5 μL from each).
   c. Primer stocks for SSLP markers closely linked to the centromere (*see* genetic locations of centromeres in (7)).
2. Identify polymorphic markers within 1.5 cM of *X. tropicalis* centromeres (**Section 3.9**).
3. Aliquot 2 μL of DNA from the two mutant and wild-type pools into PCR reaction tubes for each polymorphic centromere marker being tested.
4. Add 8 μL of a standard PCR master mix for polymorphic centromere marker to each tube.
5. Run PCR under standard conditions for 35 cycles with an annealing temperature of 58°C (for all http://tropmap.biology.uh.edu SSLPs).
6. Run 5 μL on 3% SFR agarose gel. Some polymorphisms are scorable only using higher resolution 6% polyacrylamide sequencing gels followed by a silver stain (**Section 3.6.3**).
7. If a mutation is linked to a given centromere, one band will predominate in the mutant lane; the corresponding wild-type lane will show either the other band (consistent with a tightly linked locus) or both species (consistent with a more distal locus). Unlinked centromeres will display identical mutant and wild-type bands (**Fig. 4.5**).
8. Repeat until linkage is observed or polymorphisms at all 10 centromeres have been tested (if no linkage is detected, *see* **Section 3.7.2**).
9. Confirm by testing individual embryos with linked centromere marker and determine linked chromosome arm by testing markers ~5 cM on either side of centromere.

3.7.2. Alternate Strategies

Distal loci may show weak linkage or appear unlinked to centromere markers in bulk segregant analysis, where a small minority of embryos with multiple crossovers can obscure linkage in pools. Weak linkage can sometimes be confirmed by scoring >20 individual mutant gynogenotes for centromere markers. Alternatively, the larger chromosome arms, or indeed the entire genome, can be scanned using more polymorphisms 10–20 cM apart. Scanning can be performed using bulk segregant analysis with either gynogenetic embryos or the progeny of conventional crosses. Scanning strategies can often be efficiently combined with candidate gene approaches. If a number of related phenotypes have been characterized in other systems, the scaffolds containing their *X. tropicalis* orthologs may be located on the genomic sequence assembly and possibly the meiotic map. Nearby SSLP markers may be used for scanning if the scaffolds have been mapped. If not, it is straightforward to identify microsatellite repeat regions and

generate homemade 'bespoke' markers (**Section 3.9**). Linkage analysis can detect unrelated mutations in the vicinity as well as mutations in the candidate genes themselves.

*A*mplified *f*ragment *l*ength *p*olymorphism (AFLP) analysis can provide a more direct route to obtaining linked sequences but is somewhat laborious. In brief, mutant and wild-type pooled genomic DNAs are digested and randomly amplified in a fashion that allows control over complexity of products (25). The maximum number of bands that can be analysed on sequencing gels can then be inspected for differences between mutant and wild type. Bands that are present in wild-type but not mutant lanes are candidate linked wild-type alleles. These can be cut out of the gel, re-amplified, sequenced and placed on the genome assembly. Additional nearby bespoke SSLPs are then obtained from the identified sequence scaffold and tested to confirm linkage. Convenient kits are available to facilitate AFLP analysis, for example, Invitrogen AFLP Analysis System I.

3.8. Higher Resolution Mapping

Most of the considerations for subsequent steps in positional cloning are not specific to *X. tropicalis*. Gynogenetic embryos, which have fewer crossovers on the centromere side of the mutant locus, are less suitable for fine mapping than are those derived from a conventional mating, which have useful crossover events on both sides. Conventional crosses can also provide larger numbers of embryos compared to gynogenesis; for successful positional cloning, at least 1,000 mutant embryos are often required.

After placing a mutation on a linkage group, the next step involves locating the mutation between two easily scorable flanking markers <10 cM apart. Initially, the linked chromosome can be scanned with markers spaced at ~10 cM intervals, using bulk segregant analysis of mutant and wild-type pools of ~20 embryos. Polymorphisms showing strong linkage are then evaluated using ~24–48 individual mutant embryos and 6–12 wild-type siblings, and other nearby markers from the meiotic map are tested. Markers further from the mutation will yield more recombinants (heterozygotes) compared to closer markers. Importantly, markers on opposite flanks of the mutation give non-overlapping sets of recombinants, and markers on the same side share recombinants. Flanking markers should be less than ~10 cM apart and should be relatively easy to score, i.e. simple two-allele systems, preferably distinguishable on agarose gels, as these will be used to genotype large numbers of embryos.

After flanking markers have been obtained, genotype the available mutant embryos with them to identify those that are recombinant. Mutant embryos that are heterozygous at a flanking marker will have informative crossovers near the mutation. Once a set of >20 recombinants has been obtained, these can be typed with additional markers distributed evenly between the

flanking markers to narrow the interval. The available resolution is determined by the number of meioses scored. In practical terms, the first goal is to place the mutation between two markers on a single sequence scaffold. Even large scaffolds can be inspected for candidate genes easily using Ensembl BioMart (**Section 3.10.1**). Scaffolds can be usually subdivided rapidly with additional markers from the meiotic map, or bespoke SSLP markers can be generated (**Section 3.9**) to refine the interval.

In some regions, microsatellite repeat polymorphisms may be difficult to find. Single-nucleotide polymorphisms (SNPs) will be abundant in polymorphic crosses. These can be usually identified by simply sequencing random amplicons from intergenic regions from several mutant and wild-type individuals. SNPs detected by sequencing can be converted into a variety of high-throughput assays. Many SNPs destroy or create restriction sites, so alleles can be distinguished by digesting an amplification product (cleaved amplified polymorphic sequence, CAPS). A variation based on introducing a mismatched base in primers, 'dCAPS' (*d*erived *c*leaved *a*mplified *p*olymorphic *s*equence) (26), can produce differentially cleavable alleles starting from any SNP sequence.

An alternative to identifying SNPs by sequencing is *s*ingle-*s*trand *c*onformation *p*olymorphism (SSCP) analysis. Many allelic variations, including SNPs, affect the mobility of single-stranded DNA under certain electrophoresis conditions. This strategy simply involves generating amplicons to non-coding regions where positive selection is lower than in coding and variations may accumulate. The amplification products are denatured but then run on non-denaturing polyacrylamide gels (**Section 3.9.7**).

3.9. Identification of Bespoke Mapping Markers

The *X. tropicalis* meiotic map (http://tropmap.biology.uh.edu) currently arranges more than 1,600 SSLPs in 10 linkage groups corresponding to the 10 *X. tropicalis* chromosomes. While this provides ample markers for linkage assignment and rough mapping, higher resolution can require identification of bespoke markers. SSLP markers are easy to identify and score, but high-resolution mapping may exhaust SSLP candidates in a region, and other types of bespoke polymorphisms can be pursued. This section outlines how to identify potential polymorphisms for use in high-resolution analysis.

3.9.1. Obtaining Bespoke SSLPs

1. From a genome browser (e.g. JGI, UCSD, Xenbase G-Browse or Ensembl), download a scaffold of interest in FASTA format.
2. Go to Tandem Repeat finder website (http://tandem.bu.edu/trf/trf.html).
3. Click 'Submit a Sequence for Analysis'.
4. Click 'Basic'.

5. Copy and paste or upload FASTA format sequence to website.
6. Click 'Submit Sequence' button (this step may take 2–3 min).
7. On the following page, click 'Tandem Repeats Report'.
8. Look through second column of table (Period Size) for 2, 3 or 4 (di, tri or quad repeat).
9. Next look through third column (Copy Number) of copy numbers for 2, 3 or 4 Period repeats and identify those with a copy number in region of 10–30 copies.
10. Click on the link for these repeats in first column (Indices).
11. The following pages give the repeat and sequence flanking.
12. Search the original FASTA format.txt file for the repeat plus 20–30 bp of flanking sequence.
13. Go to Primer3 website (e.g. http://frodo.wi.mit.edu/).
14. Copy and paste the identified repeat plus flanking sequence into text box on Primer3 (leave parameters unchanged).
15. Click 'Pick Primers' button.
16. Chose primers flanking the repeated sequence.
17. Repeat Steps 12–16 for all Period Sizes of 2, 3 or 4 with a copy number of 10–30.

3.9.2. Cleaved Amplified Polymorphic Sequence (CAPS)

CAPS (27) describes the detection of restriction fragment length polymorphisms in PCR amplicons. Scanning non-coding amplicons from mutant and wild type with four-cutter enzymes chosen at random often reveal variations. Non-coding regions under relaxed selection are likely to harbour higher density of SNPs and other polymorphisms:

1. Design primers to amplify several 300–800 bp products from non-coding regions >5 kb from exons.
2. Using PCR, amplify these regions from at least six individual haploid embryos.
3. Digest PCR products with 3–5 different four-cutter restriction enzymes (e.g. *Rsa*I, *Mbo*I and *Hpa*II).
4. Run on gel to identify differences in restriction fragments from individual haploids.

3.9.3. Obtaining Candidate SNPs From Extant Genome Sequence

1. On the JGI genome browser, go to a scaffold of interest.
2. Scroll down to the *Xenopus* EST tracks highlighted in green.
3. Expand (if not already done so) the 'Gurdon *Xenopus tropicalis* clusters' track by clicking the + on the left-hand menu.
4. The list of EST clusters available from the Gurdon collection will now be present on the left-hand column.

5. Click one of the 10 digit numbers in this column (normally starting 100xxxxxx).
6. On the following page, click the link in the top table called 'Defline'.
7. On the next page (Gurdon Institute *Xenopus tropicalis* EST Database), scroll down to the alignment of ESTs.
8. Any SNPs detected are highlighted in brown 'SNP'.
9. Scroll to the right to find positions of the SNP.

3.9.4. Snip-SNP Markers

Many SNPs identified by sequence result in RFLPs, or snip-SNPs, that are simpler to score than are by sequencing multiple embryo DNAs:

1. To find/determine snip-SNPs, select 30 bp of sequence flanking the SNP for both alleles.
2. Copy each sequence to a restriction enzyme site prediction program (e.g. NEBcutter V2.0, http://tools.neb.com/NEBcutter2/index.php).
3. Compare the predicted restriction sites between the two for any sites unique to one allele.
4. If there is a difference, then design primers to amplify this sequence
5. PCR up this sequence from individual haploids.
6. Clean PCR product for each and cut with the enzyme unique for one allele to test for polymorphic status.

3.9.5. Bespoke SNPs

SNPs are also abundant in intergenic non-coding regions and may be identified directly by sequencing PCR products (amplified with a high-fidelity polymerase) from mutant and wild-type embryos:

1. Design PCR primers to amplify ~ 400 bp from non-coding regions >5 kb away from exons.
2. Amplify from three or more individual wild-type and mutant embryos, homozygous for other markers, or six or more unsorted haploids, using a proofreading polymerase.
3. Sequence all six fragments and compare, looking for consistent single-nucleotide changes between wild-type and mutant embryos.
4. Analyse for snip-SNP or by sequencing amplicons from individual mutant and wild-type embryos.

3.9.6. Derived Cleaved Amplified Polymorphic Sequence Analysis (dCAPS)

In this variation on the CAPS procedure, virtually any identified SNP can be converted into a snip-SNP for high-throughput analysis (26) by generating PCR primer sequences in which a mismatch is introduced, converting one of the SNPs into a specific cleavable polymorphism. The PCR products can then be digested

and compared. The most time-consuming part of this process can be generating mismatched primer sequences creating snip-SNPs; the authors have thoughtfully generated an online 'dCAPS Finder' at http://helix.wustl.edu/dcaps/dcaps.html

3.9.7. Single-Strand Conformation Polymorphism (SSCP) Analysis

SSCPs are sequence differences that cause a change in the secondary structure of a short length of one strand of amplified DNA. These can often be detected in PCR products without sequencing, by denaturation followed by cooling to allow secondary structure to form, then resolved and visualized on silver-stained, non-denaturing polyacrylamide gels:

1. First prepare the following:
   a. 8–10% Non-denaturing polyacrylamide gel depending on PCR product size.
   b. SSCP denaturing buffer (100 mM NaOH, 5 mM EDTA (pH 8.0), 0.25% bromophenol blue).
   c. 0.5× TBE.
2. Perform a standard PCR to amplify 100–500 bp sequences from a region of interest from more than four individual haploid embryos to assess the presence of polymorphisms (*see* **Note 10**).
3. Prepare a 8–10% non-denaturing acrylamide gel.
4. Pre-run non-denaturing gel in 0.5× TBE for 10–15 min.
5. Mix 3 μL of PCR product with 8 μL SSCP denaturing buffer.
6. Heat PCR product to 94°C for 10 min to denature.
7. Cool on ice (keep on ice until loading).
8. Load onto gel (all 11 μL).
9. Run gel at constant 8–10 W for 10–12 h at RT (do not let gel heat to above RT).
10. Silver stain gel (**Section 3.6.3**).
11. Check for products which are polymorphic between haploids.

3.10. Evaluation of Candidate Genes in Mapped Interval

Scaffold gene lists, with GO and protein domain information, are easily downloaded using the BioMart tool in Ensembl (www.ensembl.org). These are very useful for rapid inspection for candidate genes in mutation-containing intervals:

3.10.1. Ensembl BioMart

1. Go to www.ensembl.org/Xenopus_tropicalis/Info/Index
2. Click on the BioMart link (top right).
3. Choose database (a high-number Ensembl or Vega).
4. Choose dataset *X. tropicalis* genes.

5. Under 'Filters' in the left-hand menu, select 'Region' and input scaffold information under 'Multiple Chromosomal Regions' in the format 'Scaffold_Number:base-base' (e.g. 'scaffold_1:xxxxx-yyyyy' to obtain genes on the scaffold between polymorphisms flanking a mutation at base xx,xxx and yy,yyy). GO terms and external references can be added under the 'Attributes' section on the left under the 'GENE' subsection. Clicking 'count' at the top left gives the number of Ensembl genes in the set.
6. Clicking Results on the top left will generate a spreadsheet with all the genes and transcripts.

3.10.2. Synteny Walking

When mapping with the current fragmented *X. tropicalis* genome assembly, it is often helpful to generate a hypothetical *in silico* local assembly in order to obtain additional unmapped scaffolds within a region or to confirm that no unmapped scaffolds are likely to intervene between two mapped ones. *Xenopus tropicalis* retains a high degree of synteny, or shared chromosomal gene order, with other vertebrate genomes such as mouse and human, whose assemblies feature far better long-range contiguity. Synteny analysis has been used to obtain large hypothetical assemblies of scaffolds, or synteny linkage groups, from which bespoke markers may be obtained to confirm the assembly genetically or by physical means (2).

Alternatively, synteny analysis may be performed manually using the Metazome (www.metazome.net), which is also linked to the JGI *X. tropicalis* browser. To identify candidate neighbouring scaffolds, find a gene at or near one end of an identified scaffold, then follow the following steps:

1. Go to www.metazome.net
2. Select the Vertebrate or Tetrapod nodes:
   a. Either enter 'Keyword' search with gene name or Ensembl ID, or select Blast and enter peptide sequence.
   b. This brings up a multi-species alignment centred on the searched gene with five neighbouring 5′ and 3′ genes, colour coded for shared orthologs. If synteny is conserved, the well-assembled human or mouse genomes can be used as a path to move beyond the end of one *X. tropicalis* scaffold and identify a candidate neighbouring scaffold.
   c. Candidate neighbouring unmapped scaffolds should be confirmed by genetic linkage analysis using bespoke markers.

3.11. Analysis of Candidate cDNAs

Compelling candidate genes in the mutation-containing interval can be evaluated in a number of ways. RT-PCR or in situ

hybridization with 3′ probes may be used to detect changes in expression levels in mutant embryos. These are not necessarily the result of changes in transcription level; mutations which introduce stops are frequently degraded by nonsense-mediated decay (28). Likewise, immunostaining or Western blot analysis is useful where antibodies are available. Sequencing specific cDNAs from the mutant is often an informative and relatively inexpensive option unless the gene is very large.

3.12. Confirmation of Candidate Genes and Phenotypic Analysis

Many mutagenesis procedures will introduce multiple lesions per genome; induced base changes from chemical mutagenesis are detected as frequently as 1/50,000 bases. Even if a sequence lesion is identified in a coding region within the genetically defined interval, other mutations may also be present, and independent evidence is usually required to show that one gene is responsible for the mutant phenotype. Ideally, the phenotype can be rescued by a wild-type allele delivered by mRNA injection or as a transgene. Obtaining a specific phenocopy by morpholino oligonucleotide knockdown of the wild-type allele is also compelling. Microinjection techniques are similar to those used for *laevis*, with volumes and dosages adjusted for smaller *X. tropicalis* embryo. While all mRNA and morpholino oligonucleotides should be titrated, a starting point of 1/10th the dose used for *laevis* is useful, in an injection volume of up to 2 nL in one cell of a two-cell embryo in filter-sterilized 3% Ficoll/0.05× MMR.

3.13. Phenotypic Analysis

Techniques appropriate for characterization of the effects of different mutations vary with each phenotype, and a full review is beyond the scope of this chapter. Generally, after-effects on external morphology are described and mutant embryos are processed for histology and fixed for whole-mount in situ hybridization or staining with specific antibodies. Many published protocols for *X. laevis* are directly transferrable to *X. tropicalis*.

3.14. Genotyping Adult Frogs

Once a mutation has been identified or mapped to a narrow region, it may be simpler to identify carriers using a PCR or an SNP-based approach using genomic DNA from an adult frog (rather than breeding to known carriers).

3.14.1. Toe Clip Sampling for Genomic DNA

Large quantities of genomic DNA can be harvested non-lethally from adult *X. tropicalis* toes, which regenerate after about a month:

1. Anaesthetize frog in 0.07% MS222 for 4–6 min. Time for this can vary, so continually observe until frogs begin to slow their swimming motions, being careful not to over-anaesthetize.
2. Remove the frog immediately and briefly rinse in fresh water.

3. Place the frog on damp paper towel and remove a single toe with a fresh scalpel/razor blade. Place the toe in an Eppendorf tube containing 400 μL lysis buffer.
4. Rinse the frog in fresh water and place in clean tank containing damp paper towels. Cover the frog in a further damp paper towel. The frog should completely recover in approximately 1 h.
5. Clipped toes can be frozen at –80°C until required or genomic DNA extracted immediately.
6. Incubate the clipped toe in 400 μL lysis buffer at 55°C overnight with agitation.
7. Add 300 μL isopropanol.
8. Spin at 4,000 rpm for 20 min.
9. Wash the pellet in 500 μL of 70% ethanol.
10. Resuspend the pellet in 100 μL nuclease-free water. Usually yields ~10 μg genomic DNA, depending on the size of the toe per frog.

3.14.2. Back Swab for Genomic DNA

Taking a small sample from the surface of the frog is an alternative non-invasive method for obtaining genomic DNA. However, DNA yield is sufficient only for a few reactions and usually requires amplification with nested primers:

1. Hold frog with gloved hand (to prevent DNA contamination).
2. Using a sterile bacterial inoculation stick or pipette tip, wipe across the skin on the frog's back approximately 10 times.
3. Shake tip or bacterial stick in 500 μL of lysis buffer.
4. Incubate at 55°C for 2 h.
5. Add 1 mL of 100% EtOH.
6. Spin at 13,000 rpm at 4°C for 10 min.
7. Wash the pellet in 70% EtOH and spin for a further 5 min.
8. Resuspend in 14.3 μL nuclease-free water ready for PCR.
9. Add a master mix of primary PCR to this tube and cycle in a thermocycler under primer-specific conditions for 35 cycles.
10. Use 0.2 μL of this PCR as template for a second reaction with nested primers.

3.15. Xenopus tropicalis Sperm Freezing

Storage of frozen sperm at –80°C facilitates archiving of specific stocks and strains; shipping frozen sperm on dry ice is often simpler and more reliable than shipping adult animals. These protocols have been adapted from (21).

3.15.1. Sperm Freezing

1. Prepare cryoprotectant.
2. Inject males with 100 μL HCG 12–24 h before harvesting testes.
3. The next day, kill males and dissect out testes, rolling on clean paper towel to remove traces of blood.
4. Macerate both testes with Eppendorf pestle in a single 1.5-mL Eppendorf tube with 500 μL L15/CS.
5. Add 500 μL cryoprotectant.
6. Divide evenly into 4–10 separate tubes.
7. Place tubes into small styrofoam box. Wrap lid with foil.
8. Place the box with the tubes of sperm in –80°C for at least 24 h, then transfer tubes to rack or box for long-term storage at –80°C (*see* **Note 11**).

3.15.2. Using Frozen Sperm for In Vitro Fertilization

1. Prepare 25°C water bath and express eggs from females into a dry dish, discarding poor-quality eggs.
2. Thaw frozen sperm in 25°C water bath. Remove immediately when thawed (<30 s).
3. Dilute sperm with 2 mL distilled water and add to eggs.
4. Gently mix the sperm and eggs with a pipette tip.
5. After 2 min, flood with distilled water.
6. Fertilization rates of 10–15% are usually observed with a half testis frozen aliquot on ~1,000 eggs.

3.16. Conclusion

We hope the reader has obtained an outline of some of the strategies and protocols for developmental genetics using *X. tropicalis*. Amphibian embryos have historically proven to be a highly useful system for understanding vertebrate development, using a broad range of embryological, molecular and, more recently, genomic tools. With its compact genome and short generation time, *X. tropicalis* offers the unique prospect of combining genomics and precision loss-of-function genetic approaches with the conventional *Xenopus* toolkit to provide a unique range of analysis of gene function in a single in vivo vertebrate model system.

4. Notes

1. Adult females housed at temperatures over ~26°C may suffer from poor egg quality, but tadpoles and subadults may be grown at temperatures up to 30°C. Lower than 23°C is likely to depress immune function and increase illness. A variety of diets work well for adults as long as they are

relatively protein rich and small enough for frogs of a given size to swallow easily, but large enough to grasp with their forelimbs. Live foods such as insect larvae or nonprocessed offal have some risk of transmitting parasites. Powdered fish flake can supplement the diet of larger tadpoles in flow-through systems but rapidly fouls standing water.

2. Injections are made into the dorsal lymph sac by inserting a 27 g needle subcutaneously between the dorsal–lateral line stripes. For optimal egg quality, females may be re-ovulated every 6 weeks to 6 months. Some groups re-use males as often as once a week, and females once a month, although it is likely that ovulating females more than 5–6 times per year may be stressful in the long term.
3. Always handle amphibians with wet hands to prevent damage to their skin. To squeeze eggs from frogs, grasp the female dorsally, with her left leg between the index and middle finger of your right hand, and her left leg in your left hand. Gently massage her abdomen in an anterior-to-posterior direction with your thumb to express eggs. Eggs will activate prematurely if they contact low-salt solutions, so avoid dripping water from the frog while squeezing. Good-quality eggs will be spherical, surrounded by clear jelly, with uniform pigmentation in the animal ('Northern') hemisphere except for a lighter polar patch overlying the germinal vesicle; poor-quality eggs may be lysing (clouding the jelly) and in linear strings. *Xenopus tropicalis* in vitro fertilization is typically less efficient than with *laevis*; testes are smaller, contain less sperm and appear to be more salt sensitive. *Xenopus tropicalis* embryos do not consistently undergo upward reorientation of the animal hemisphere as *laevis* do upon activation; cortical pigment contraction and germinal vesicle breakdown are more reliable indicators. When sacrificing frogs, use RT anaesthetic solution; chilled solutions may immobilize animals without anaesthetic effect.
4. Wait at least 15 min after activation to allow completion of cortical rotation; dejellying during this early period can produce axial defects. BSA-coating dish prevents embryos from sticking to the plates, which can affect gastrulation.
5. If available, testes from males bearing fluorescent transgenes can be used to assess haploid formation; efficient UV irradiation will block paternal transgene transmission. Development of haploid embryos is strongly affected by both egg quality and genetic background. Haploid development typically includes deficits in axis elongation and posterior structures, incomplete blastopore closure and

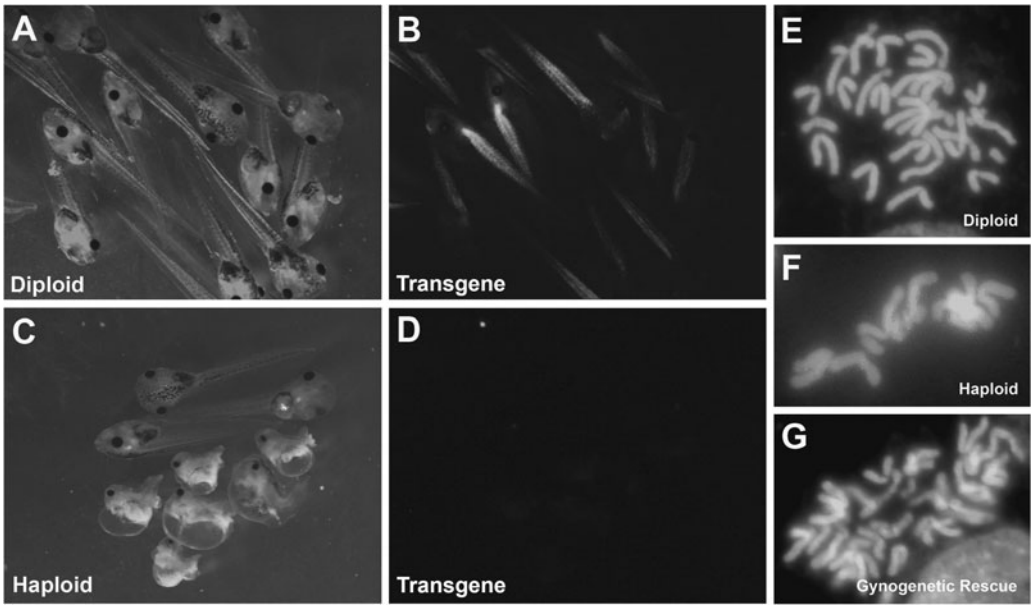

Fig. 4.6. Haploid and gynogenetic embryos. Wild-type eggs were fertilized using homozygous cardiac actin–RFP transgenic sperm, either untreated to form conventional diploid embryos (**a**) or UV irradiated to form haploids (**c**). Paternal transgene transmission is visible in the diploid clutch (**b**) but absent in the haploids (**d**). Haploids can form anterior structures well, but posterior structures are truncated. Note that three spontaneously diploidized embryos without paternal transgene appear in the haploid clutch. *Panels e–g* show the karyotype of outcrossed diploid (**e**), haploid (**f**) and early cold shock gynogenetic diploid embryos (**g**).

other gastrulation defects, but anterior structures are often well formed (compare **Fig. 4.6a** with **c**). Diploid-appearing embryos are also observed in some batches of haploids. These may result from incomplete UV irradiation of sperm, but spontaneous diploidization of haploids has also been observed, probably due to failure of polar body formation (**Fig. 4.6a–d**).

6. Depending on egg quality and genetic background, this procedure typically rescues 25% or more of the cleaving haploid embryos to viable diploids.
7. Haploid and outcrossed diploid controls should be reaching the four-cell stage when LCS embryos undergo the first cell division; LCS embryos cleaving in sync with controls should be discarded. Rescue efficiency is strongly dependent on egg quality and genetic background. Use of transgenic sperm is recommended to control for efficiency of UV inactivation.
8. Heavy compression is important for getting good spreads, but the coverslip should not slide around. This technique can produce hundreds of stained nuclei, but finding those with complete countable spreads requires patience.

9. Provided the proteinase K is adequately denatured (by the 5 min, 95°C step), this DNA can be immediately used in PCR reactions without the need for precipitation or clean-up. For mapping purposes, collect as many mutant embryos as are available. Assignment of mutant loci to specific chromosomes by centromere linkage can be accomplished with as few as 12 mutant and wild-type gynogenotes (*see* **Section 3.7.1**). Analysis of 1,000 mutant embryos from conventional crosses (~2,000 parental meioses) provides a theoretical resolution of 0.05 cM (*see* **Section 3.8**), which is usually sufficient to define the mutation-containing interval on a single sequence scaffold.
10. Polymorphisms can produce changes in secondary structure of short DNA fragments. These can be detected by denaturing short DNA fragments, allowing them to refold into a secondary structure conformation, and analysing on a non-denaturing gel.
11. It is advisable to test-fertilize some eggs with aliquot of fresh sperm from each male; if fresh sperm is not capable of fertilization, frozen testis from that male should be discarded. This freezing method seems to work as well as more elaborate methods described in Sargent and Mohun (21).

Acknowledgements

A large number of colleagues have adapted and developed the protocols outlined in this chapter. In addition to all past and present members of the Zimmerman laboratory, the authors would particularly like to thank Rob Grainger (University of Virginia), Richard Harland (UC Berkeley) and Mustafa Khokha (Yale). T.J.G. and L.B.Z. are funded by the Medical Research Council, UK.

References

1. Gurdon, J. B. and Hopwood, N. (2000) The introduction of *Xenopus laevis* into developmental biology: of empire, pregnancy testing and ribosomal genes. *Int. J. Dev. Biol.* **44**, 43–50.
2. Hellsten, U., Harland, R. M., Gilchrist, M. J., Hendrix, D., Jurka, J., Kapitonov, V., Ovcharenko, I., Putnam, N. H., Shu, S., Taher, L., Blitz, I. L., Blumberg, B., Dichmann, D. S., Dubchak, I., Amaya, E., Detter, J. C., Fletcher, R., Gerhard, D. S., Goodstein, D., Graves, T., Grigoriev, I. V., Grimwood, J., Kawashima, T., Lindquist, E., Lucas, S. M., Mead, P. E., Mitros, T., Ogino, H., Ohta, Y., Poliakov, A. V., Pollet, N., Robert, J., Salamov, A., Sater, A. K.,

Schmutz, J., Terry, A., Vize, P. D., Warren, W. C., Wells, D., Wills, A., Wilson, R. K., Zimmerman, L. B., Zorn, A. M., Grainger, R., Grammer, T., Khokha, M. K., Richardson, P. M., and Rokhsar, D. S. (2010) The genome of the Western clawed frog *Xenopus tropicalis. Science* **328**, 633–6.

3. Goda, T., Abu-Daya, A., Carruthers, S., Clark, M. D., Stemple, D. L., and Zimmerman, L. B. (2006) Genetic screens for mutations affecting development of *Xenopus tropicalis. PLoS Genet.* **2**, e91.
4. Grammer, T. C., Khokha, M. K., Lane, M. A., Lam, K., and Harland, R. M. (2005) Identification of mutants in inbred *Xenopus tropicalis. Mech. Dev.* **122**, 263–72.
5. Noramly, S., Zimmerman, L., Cox, A., Aloise, R., Fisher, M., and Grainger, R. M. (2005) A gynogenetic screen to isolate naturally occurring recessive mutations in *Xenopus tropicalis. Mech. Dev.* **122**, 273–87.
6. Abu-Daya, A., Sater, A. K., Wells, D. E., Mohun, T. J., and Zimmerman, L. B. (2009) Absence of heartbeat in the *Xenopus tropicalis* mutation muzak is caused by a nonsense mutation in cardiac myosin myh6. *Dev. Biol.* **336**, 20–9.
7. Khokha, M. K., Krylov, V., Reilly, M. J., Gall, J. G., Bhattacharya, D., Cheung, C. Y. J., Kaufman, S., Lam, D. K., Macha, J., Ngo, C., Prakash, N., Schmidt, P., Tlapakova, T., Trivedi, T., Tumova, L., Abu-Daya, A., Geach, T., Vendrell, E., Ironfield, H., Sinzelle, L., Sater, A. K., Wells, D. E., Harland, R. M., and Zimmerman, L. B. (2009) Rapid gynogenetic mapping of *Xenopus tropicalis* mutations to chromosomes. *Dev. Dyn.* **238**, 1398–46.
8. Reinschmidt, D., Friedman, J., Hauth, J., Ratner, E., Cohen, M., Miller, M., Krotoski, D., and Tompkins, R. (1985) Gene–centromere mapping in *Xenopus laevis. J. Hered.* **76**, 345–7.
9. Tompkins, R. and Reinschmidt, D. (1991) Experimentally induced homozygosity in *Xenopus laevis. Methods Cell Biol.* **36**, 35–44.
10. Kroll, K. L. and Amaya, E. (1996) Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* **122**, 3173–83.
11. Yergeau, D. A. and Mead, P. E. (2007) Manipulating the *Xenopus* genome with transposable elements. *Genome Biol.* **8** Suppl 1, S11.
12. Ogino, H., McConnell, W. B., and Grainger, R. M. (2006) Highly efficient transgenesis in *Xenopus tropicalis* using I-SceI meganuclease. *Mech. Dev.* **123**, 103–13.
13. Allen, B. G. and Weeks, D. L. (2005) Transgenic *Xenopus laevis* embryos can be generated using phiC31 integrase. *Nat. Methods* **2**, 975–9.
14. Hirsch, N., Zimmerman, L. B., Gray, J., Chae, J., Curran, K. L., Fisher, M., Ogino, H., and Grainger, R. M. (2002) *Xenopus tropicalis* transgenic lines and their use in the study of embryonic induction. *Dev. Dyn.* **225**, 522–35.
15. Chae, J., Zimmerman, L. B., and Grainger, R. M. (2002) Inducible control of tissue-specific transgene expression in *Xenopus tropicalis* transgenic lines. *Mech. Dev.* **117**, 235–41.
16. Hartley, K. O., Nutt, S. L., and Amaya, E. (2002) Targeted gene expression in transgenic *Xenopus* using the binary Gal4-UAS system. *Proc. Natl. Acad. Sci. USA* **99**, 1377–82.
17. Ryffel, G. U., Werdien, D., Turan, G., Gerhards, A., Goosses, S., and Senkel, S. (2003) Tagging muscle cell lineages in development and tail regeneration using Cre recombinase in transgenic *Xenopus. Nucleic Acids Res.* **31**, e44.
18. Lehman, C. W. and Carroll, D. (1991) Homologous recombination catalyzed by a nuclear extract from *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **88**, 10840–4.
19. Doyon, Y., McCammon, J. M., Miller, J. C., Faraji, F., Ngo, C., Katibah, G. E., Amora, R., Hocking, T. D., Zhang, L., Rebar, E. J., Gregory, P. D., Urnov, F. D., and Amacher, S. L. (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.* **26**, 702–8.
20. Carruthers, S. and Stemple, D. L. (2006) Genetic and genomic prospects for *Xenopus tropicalis* research. *Sem. Cell Dev. Biol.* **17**, 146–53.
21. Sargent, M. G. and Mohun, T. J. (2005) Cryopreservation of sperm of *Xenopus laevis* and *Xenopus tropicalis. Genesis* **41**, 41–6.
22. Bahary, N., Davidson, A., Ransom, D., Shepard, J., Stern, H., Trede, N., Zhou, Y., Barut, B., and Zon, L. I. (2004) The Zon laboratory guide to positional cloning in zebrafish. *Methods Cell Biol.* **77**, 305–29.
23. Bassam, B. J., Caetano-Anolles, G., and Gresshoff, P. M. (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* **196**, 80–3.
24. Streisinger, G., Singer, F., Walker, C., Knauber, D., and Dower, N. (1986) Segregation analyses and gene–centromere distances in zebrafish. *Genetics* **112**, 311–19.
25. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A.,

Pot, J., Peleman, J., Kuiper, M., et al. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**, 4407–14.
26. Neff, M. M., Neff, J. D., Chory, J., and Pepper, A. E. (1998) dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. *Plant J. Cell Mol. Biol.* **14**, 387–92.
27. Konieczny, A. and Ausubel, F. M. (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. *Plant J. Cell Mol. Biol.* **4**, 403–10.
28. Chang, Y. -F., Imam, J. S., and Wilkinson, M. F. (2007) The nonsense-mediated decay RNA surveillance pathway. *Annu. Rev. Biochem.* **76**, 51–74.

Chapter 5

Embryological and Genetic Manipulation of Chick Development

Laura S. Gammill and Catherine E. Krull

Abstract

The ability to combine embryological manipulations with gene function analysis makes the chick a valuable system for the vertebrate developmental biologist. We describe methods for those unfamiliar with the chick wishing to initiate chick experiments in their lab. After outlining how to prepare chick embryos, we provide protocols for introducing beads or cells expressing secreted factors into the embryo and for culturing tissue explants as a means of assessing development in vitro. Chick gain-of-function and loss-of-function (RNAi and morpholino oligonucleotide) approaches are outlined, and methods for introducing these reagents by electroporation are detailed.

Key words: Chick, embryology, electroporation, RNAi, morpholino oligonucleotide.

1. Introduction

Chick embryos have captivated developmental biologists since the days of Aristotle. Large and externally developing, chick embryos are amenable to in vivo and in vitro manipulations that assay fate, inductive signaling interactions, and, more recently, gene function. Long a mainstay of limb, somite, neural crest, and spinal cord developmental studies, the newfound ability to combine chick embryology with gain- or loss-of-function approaches has greatly increased the utility and popularity of the chick system. In addition to enabling a wider array of developmental inquiry in the chick, this new era makes possible comparative studies between the chick and other vertebrate model organisms. For example, when used in conjunction with a genetic system such as the mouse (particularly useful as both are amniotes and thus evolutionarily

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_5,

close), new experiments become feasible. The conservation of gene function can be evaluated, and chick embryological manipulations that are impossible in the mouse can be used to further investigate mechanisms suggested by genetic analysis. As a result, a more complete and comprehensive view of a developmental process unfolds.

This chapter serves as an introduction to chick embryological and gene manipulation methods. Basic protocols for preparing embryos, introducing sources of exogenous factors, culturing chick embryo explants, and electroporating embryos with gain-of-function and loss-of-function reagents are described.

2. Materials

2.1. Solutions

1. Ringer's saline (1 L): 7.2 g NaCl, 0.17 g $CaCl_2$, 0.37 g KCl, 0.115 g Na_2HPO_4, and 0.02 g KH_2PO_4. Adjust pH to 7.4 and add H_2O up to 1 L. Filter sterilize.
2. PBS (1 L): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4. Adjust pH to 7.4 and add H_2O up to 1 L. Autoclave.
3. Trypsin–EDTA (Invitrogen).
4. DMEM (Invitrogen).
5. DMEM-F12 (Invitrogen).
6. N2 supplement (Invitrogen).
7. Fetal bovine serum (Hyclone).
8. CellTracker CM-DiI (Invitrogen C7000): immediately before use, resuspend one 50 μg aliquot in 25 μL 100% ethanol and then dilute in 500 μL of fresh, sterile 10% sucrose. Spin at full speed for 10 min in a microcentrifuge and transfer to a fresh microcentrifuge tube. Add 1 mL of Ringer's saline and mix. Make fresh before each use.
9. Dispase II (Roche): Add 5 mL sterile 1 M Hepes, pH 7.5, to 500 mL DMEM. To 50 mL of this medium (*see* **Note 1**), add 0.075 g of dispase. Freeze in 3 mL aliquots.
10. Ca^{2+}-, Mg^{2+}-free Tyrode's saline (10×): 80 g NaCl, 2 g KCl, 0.5 g NaH_2PO_4, and 10 g glucose. Add water to 1 L, filter sterilize. Dilute 1× fresh before use and dissolve 0.05% solid trypsin (Sigma).
11. Collagen: Mix 90 μL rat tail collagen (BD Biosciences) with 10 μL 10× DMEM (Invitrogen). Add 4.5 μL of 7.5% $NaHCO_3$ and vortex well. Can be stored for short periods on ice. Solution should be light pink and set in 15–20 min

at room temperature. More (or less) $NaHCO_3$ may need to be added if this is not the case.

12. Albumen–agar plates: For 30 plates, mix 30 mL of sterile 0.12 M NaCl with 0.153 g of agar. Heat to boiling and equilibrate to 55°C in a water bath. Collect 30 mL of thin albumen from unincubated chicken eggs by breaking a 1 in. slit 1/3 of the way from the blunt end of the egg and tipping albumen into a 50 mL conical tube. Add 60 μL of penicillin–streptomycin (10,000 units penicillin and 10,000 μg streptomycin per mL; Invitrogen) to the albumen and warm to 55°C in a water bath. Combine the albumen and agar and swirl to mix. Pour 2 mL per 35 mm tissue culture plate in a tissue culture hood. Let plates cool for 40–60 min and then store in a sealed plastic tub at 4°C for up to 1 week.
13. 4% paraformaldehyde in PBS (or other fixative of choice). May be stored in aliquots at –20°C.
14. 70% ethanol prepared with water.

2.2. Equipment

1. Needle puller (e.g., Sutter, Narishige, or Stoelting).
2. Forced air injection apparatus (e.g., General Valve Corporation Picospritzer or Harvard Apparatus PLI-100).
3. Electroporation chamber, custom made by local machine shop (**Fig. 5.1**).
4. Electroporation electrodes (*see* (1) for fabrication instructions).

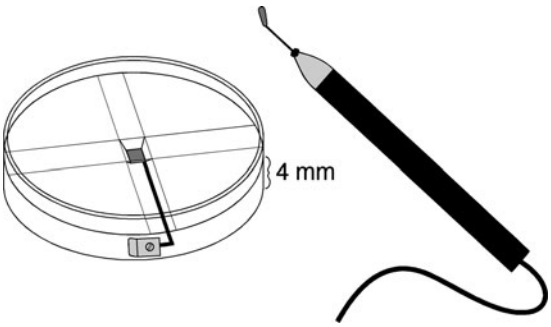

Fig. 5.1. Early embryo electroporation apparatus. A platinum plate is embedded at the bottom of a 4 mm deep divet in a plastic base. Lines are etched onto the base to mark the position of the electrode plate. A wire connects the plate through the base to a flap on the side, onto which a micro-alligator clip attaches the bottom electrode wire. A plastic ring is attached to the top of the base, creating a dish to hold Ringer's saline. The top electrode is fashioned from an empty ball point pen casing by stringing a second wire through the casing and soldering to a 1 mm platinum wire with an end that is bent and flattened into a paddle. The wire is fixed in place with epoxy at the pen tip. Note that the bottom and top electrodes are not to scale. *See* (11) for additional details.

5. Square pulse electroporator (*see* (1) for recommendations).
6. Egg incubator set to 100°F/38°C.
7. Air incubator or tissue culture incubator set to 100°F/38°C.

2.3. Other Materials

1. Fertile chicken eggs (obtain from a nearby farm or order SPAFAS-specific pathogen-free eggs from Charles River, *see* **Note 2**).
2. Fine, pointed scissors (Fine Science Tools).
3. Watchmakers forceps, #5 and #2 (Fine Science Tools and World Precision Instruments).
4. Blunt forceps (Fine Science Tools or similar).
5. Pin holder, to hold tungsten needles (Fine Science Tools).
6. Pin vise, to hold glass needles (Fine Science Tools).
7. Minutien insect pins (Fine Science Tools).
8. 3 and 1 mL syringes.
9. 25 gauge, 5/8 in., hypodermic needles.
10. 18 gauge, 1.5 in., hypodermic needles.
11. Type A India Ink (Pelikan. Note that other brands can be deleterious to embryos).
12. $^{3}/_{4}''$ wide Scotch magic tape (3 M).
13. Parafilm squares (Pechiney Plastic Packaging).
14. Gauze squares.
15. Beveled-edge watch glass (Fisher).
16. Sharpened tungsten wire: 1 in. lengths of 0.01″ tungsten wire sharpened to a point by dipping the wire tip into 1.0 N NaOH in an alkaline electrolysis bath (2).
17. Glass capillary tubes (we prefer 0.8–1.1 mm diameter, 100 mm long), pulled into needles.
18. 35 mm tissue culture plates.
19. Petri dishes.
20. Clear packing tape (3 M; hand-tearable or 3710 packing tape. Note that some tapes are deleterious to embryos).
21. Bovine serum albumin, fraction V (Sigma).
22. Heparin acrylic beads (Sigma H-5263).
23. Affigel blue beads (Bio-Rad 153-7301 or 7302).
24. Microloader pipette tips (Eppendorf).
25. Mouth aspirator assembly (Sigma, A5177-5EA).
26. Square glass baking dish (Pyrex or similar).
27. Sylgard (Dow Corning)-coated 35 mm petri dish (prepare sylgard according to the manufacturer's instructions).

28. Whatman 3 mm filter paper, cut into $^{1}/_{2}'' \times 5/8''$ rectangles with two overlapping holes punched in the center using a standard hole punch.

3. Methods

3.1. Embryology

3.1.1. Preparing Embryos for In Ovo Manipulation

Eggs can be manipulated in ovo by opening a hole in the shell to access the embryo (**Fig. 5.2**). When this hole is sealed with tape to maintain a humid, sterile environment, the embryo can be incubated further in order to determine the consequences of any manipulations.

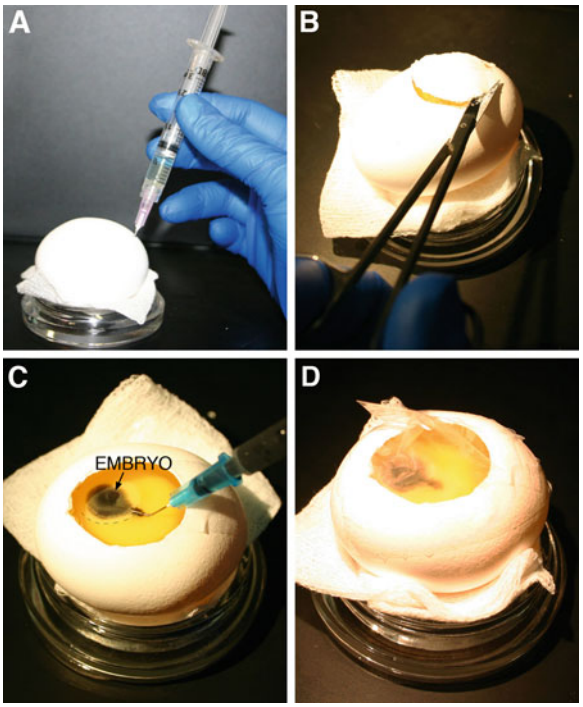

Fig. 5.2. Opening chicken eggs. (**a**) A 3 mL syringe outfitted with an 18 gauge needle is carefully introduced into the top side of the blunt end of the egg. The needle tip is inserted along the curvature of the shell to avoid puncturing the yolk and 3 mL of albumin is withdrawn from the bottom of the egg. (**b**) One or two pieces of scotch tape are laid across the top of the egg, and a hole 1.5–2 cm diameter is cut in the shell. (**c**) India ink diluted in Ringer's saline is injected under the embryo using a 1 mL syringe outfitted with a 25 gauge needle bent at a 45° angle to enhance contrast. The needle tip is inserted just outside the perimeter of the lighter-colored blastoderm (indicated with a *dotted line* along its lower edge in the photograph) and brought up under the embryo, where ink is expelled. (**d**) Following manipulation, eggs are sealed well with tape and placed back into a 38°C/100°F incubator.

1. Use the Hamburger and Hamilton staging guide to determine the approximate time needed to incubate fertile chicken eggs to the stage of interest (3). Set eggs on their sides (3 rows on a 2.5 dozen egg crate) and place into a humidified 100°F/38°C incubator for the desired number of hours (*see* **Note 3**).
2. Swab the incubated eggs with 70% ethanol, taking care to maintain the egg in its original orientation as the embryo gradually floats to the top of the yolk. Once dry, place one or two 3 cm long pieces of Scotch tape across the top of the egg so that the shell may be cut without shattering.
3. Select an egg and place on a bed of gauze on a watch glass (again, keep the position of the egg exactly as it was in the crate). Carefully insert an 18 gauge needle placed on a 3 mL syringe into the top side of the blunt end of the egg (**Fig. 5.2a**). Run the needle tip down along the blunt end of the egg, taking care not to puncture the yolk. Remove 3 mL albumen from the lowest part of the egg to drop the embryo away from the shell (*see* **Notes 4** and **5**).
4. Use scissors to cut a 1.5–2 cm diameter window into the top-most surface of the egg, being careful to keep the lower scissor blade close to the shell so as not to disrupt the yolk (**Fig. 5.2b**).
5. Dilute three drops of India ink into 10 mL of Ringer's saline (about 5%). Fill a 1 mL syringe with diluted ink and fit with a 25 gauge needle bent to a 45° angle with the bevel pointed up. Flush out air bubbles.
6. Insert the needle just outside the edge of the blastoderm (the lighter yellow, circular area consisting of the extraembryonic area opaca surrounding the clear, central area pellucida, which contains the embryo) and bring the needle tip up under the embryo. Dispense a small amount of ink to enhance embryo visibility (**Fig. 5.2c**). Gently shake the egg to disperse the ink, if necessary (*see* **Note 6**).
7. With the egg still sitting on the gauze/watch glass, look at the embryo under a microscope and determine the stage (3). Mark the stage on the shell of good eggs and discard eggs that have not developed properly (*see* **Note 4**). Add a few drops of Ringer's saline and stretch a small piece of parafilm or apply two strips of Scotch magic transparent tape across the window to seal the eggshell and to keep the embryo from drying out until ready to proceed.

3.1.2. Implanting Cell Pellets

It is possible to assay the developmental consequences of a secreted protein by implanting a pellet of cells that produce the factor of interest (as in (4)). This creates a localized and

continuously replenished source of the protein. Implanted cells are fluorescently labeled to identify the source at later stages. The effects on neighboring cells (changes in gene expression, altered morphology, and diverted migration) are assayed after a period of incubation.

1. Prepare a confluent 10 cm plate of a stable cell line or cells transfected with an expression construct using standard tissue culture protocols. Remember to prepare control cells as well.
2. Remove the media and wash the plate with 10 mL of PBS. Aspirate well. Add 1 mL of trypsin–EDTA and rock/rotate the plate to cover the entire surface. Aspirate excess trypsin. Incubate 3–5 min, until cells break free (bang the plate edge to encourage detachment).
3. Collect the cells by rinsing with 10 mL of media + 20% fetal bovine serum. Transfer cells to a 50 mL conical tube. Pipette in and out to achieve a single-cell suspension and then allow cells to recover for 30 min in the tissue culture hood, swirling occasionally to keep in suspension.
4. Bring the volume to 50 mL with PBS or Ringer's saline. Gently collect cells by centrifugation at 1,250 rpm for 5 min.
5. Carefully aspirate the supernatant and resuspend cells in 300 μL of DiI solution. Incubate for 15 min in the hood at room temperature.
6. Bring the volume to 50 mL with PBS or Ringer's. Gently collect cells by centrifugation at 1,250 rpm for 5 min. Aspirate supernatant.
7. If cells will be injected, resuspend cells in 1 mL of Ringer's saline + 0.1% BSA. Transfer to a 1.7 mL microcentrifuge tube, centrifuge at 300×*g*, and aspirate supernatant. Resuspend pellet in an equal volume of Ringer's saline + 0.1% BSA by tapping and flicking the tube. Store tube on ice.
8. Back load 2 μL of thoroughly resuspended cells into a pulled glass capillary needle using microloader pipette tips. Air-pressure inject groups of cells into a region of interest (e.g., the lumen of the neural tube or the head mesenchyme).
9. If cells will be surgically grafted, resuspend cells from step 6 in an equal volume of complete culture medium by tapping and flicking. Pipette 35–50 μL drops of cell suspension onto bacterial plates. Invert the plate and incubate at 37°C in a CO_2 incubator for 1 h. Groups of cells can then be grafted into host embryos through incisions cut with sharpened tungsten wire.

10. Add several drops of Ringer's saline to the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (**Fig. 5.2d**). Make sure the egg is well sealed or the embryo will dry out.
11. Incubate to the desired stage in a humidified 100°F/38°C incubator.

3.1.3. Implanting Beads

If purified protein is available, beads soaked in a factor of interest may also be implanted (as in (5)). Instead of bathing a whole embryo or tissue in the purified factor, the bead gradually releases the protein, creating a localized source easily identified by the presence of the bead using a microscope equipped with bright-field optics. The consequences on neighboring cells (changes in gene expression, altered morphology, and diverted migration) are assayed after a period of incubation.

1. Obtain heparin acrylic or Affigel blue beads according to the application (*see* **Note 7**).
2. Wash the beads three times with PBS.
3. Place a drop of protein diluted in PBS + 0.1% BSA at the center of a 35 mm tissue culture dish (*see* **Note 8**). Place additional drops of PBS around the outside of the dish for humidity.
4. Select a bead and place it on a dry area of the plate. Move the bead back and forth to draw out excess PBS. This step is important so that you do not transfer PBS along with the bead and dilute the protein.
5. Place the dried bead into the protein drop and incubate 2 h at room temperature to 4°C overnight.
6. Wash the bead by moving it through two or three drops of PBS.
7. Implant the bead into a region of interest in the embryo. Typically a slit must be prepared with a tungsten needle and the bead pushed into the slit with forceps.
8. Add several drops of Ringer's saline to the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (**Fig. 5.2d**). Make sure the egg is well sealed or the embryo will dry out.
9. Incubate to the desired stage in a humidified 100°F/38°C incubator.

3.1.4. Collagen Explant Culture

Chick tissues can be dissected from the embryo and cultured in a three-dimensional collagen matrix (as in (6)). The collagen supports the tissue and retains its normal conformation, in contrast

to culturing tissue directly on a culture dish, which causes tissues to spread and adopt an artificial two-dimensional arrangement. Explants in collagen allow the researcher to test specification (revealing the instructions the tissue had received before it was removed), induction (by bathing the tissue in a factor, providing a pellet of inducer-secreting cells, or co-culturing with a neighboring tissue), and chemoattraction or repulsion (of migratory cells, such as neural crest cells, or growing axons of neurons).

1. Incubate several dozen eggs to the stage at which the tissue of interest is present. Use the Hamburger and Hamilton staging guide to estimate the length of incubation required (3). The eggs may be incubated vertically.
2. Without breaking the yolks, carefully and gently crack the eggs into a square glass baking dish a dozen at a time (*see* **Note 9**).
3. Identify the embryo. In embryos stage 12 and older, the plexus of blood vessels that surrounds the embryo marks its location. In younger embryos, find a light yellow circle with a clear, hourglass center. Using a pair of scissors, cut around the embryo to free it from the yolk (*see* **Note 10**). With the scissor blades closed, scoop under the embryo and lift, so that the embryo drapes over the closed blades (*see* **Note 11**).
4. Quickly submerge the embryo/scissor blades into a petri dish full of Ringer's saline and swish back and forth to release the embryo. Repeat until all embryos are in the dish of Ringer's.
5. Lift off the vitelline membrane using a pair of forceps or tear it with a tungsten needle and rinse the embryos using a cut-off transfer pipette. Transfer the embryos to a clean dish of Ringer's saline (*see* **Note 12**).
6. Tissues may be isolated through a variety of methods. One way is to use a bent tungsten needle as a knife (by pressing against the bottom of the dish) to cut out a small block of tissue that includes the tissue of interest. This tissue block can then be incubated in dispase to dissociate the connections between tissues (*see* **Note 13**). Pipette dispase-treated tissue in and out against the side of the dish with a fire-polished (to remove sharp edges) Pasteur pipette to gently dissociate adhering layers.
7. Alternatively, minutien insect pins may be used to fasten embryos to a sylgard-coated dish in calcium- and magnesium-free Tyrodes saline + 0.05% trypsin. Over time, this solution causes tissues to release from one another and allows the tissue of interest to be dissected free with pulled glass capillary needles or sharpened tungsten wire.

8. Enzymatically treated tissue should be rinsed immediately in several changes of ice-cold media (DMEM or DMEM-F12) + 10% fetal bovine serum (*see* **Notes 14** and **15**).
9. Spread 6 μL of collagen onto the bottom of a tissue culture dish (*see* **Note 16**). Cover the dish and allow the collagen to set (it will become opaque), but do not let it dry out.
10. Place the tissue/cells to be cultured on the collagen bed (*see* **Note 15**). Transfer as little solution as possible. Add 4 μL more collagen over the tissue and allow to set.
11. Cover the collagen-embedded tissue with culture medium (we use DMEM-F12 + N2 supplement) and culture at 37°C with CO_2.

3.2. Manipulating Gene Function

Chick biologists achieve transient knockdown of endogenous protein by two means: RNA interference (RNAi) or antisense morpholino oligonucleotides (MOs).

3.2.1. Techniques to Achieve Knockdown

3.2.1.1. RNAi

To achieve RNAi, short hairpin RNAs (shRNAs) are expressed from RNA polymerase III (pol III) promoters in plasmid vectors. Plasmid-based RNAi allows continuous production of shRNAs and is inexpensive. Good results have been obtained with Ambion's pSilencer and Promega's siSTRIKE vectors that utilize mammalian pol III promoters (7–9). A vector with a chick U6 promoter has been described (10), and additional RNAi vectors that are optimized for chick are on the horizon. Electroporated cells are traced by co-electroporating green fluorescent protein (GFP; e.g., pSilencer) or by inclusion of a bicistronic GFP cassette in the RNAi plasmid (e.g., siSTRIKE).

Tips for the use of RNAi vectors:

1. Follow the guidelines for designing the shRNA constructs in (12). Each vendor has information regarding inserting that construct into the vector (*see* **Note 17**).
2. Purchase of siRNAs from companies is not recommended if you must disrupt the translation of a protein for 1 day or longer. If your experiments require that you disrupt the translation of a protein for 1 day or less, then siRNAs purchased from companies are recommended (Dharmacon).
3. Antibodies against your protein of interest are highly recommended to determine that you have knocked it down using shRNAs. Refer to (12) for information regarding the determination of whether shRNAs against your protein of interest really influence it by reducing the protein levels.

3.2.1.2. Morpholinos

Morpholino oligonucleotides (MOs) are modified nucleic acids in which the sugar backbone has been replaced with morpholine rings. Very stable, sequence specific, and typically eliciting minimal off-target effects in the chick, MOs can be designed to block pre-mRNA splicing or translation initiation (11, 13) (*see* **Note 18**). However, MOs are diluted by cell division and so are ineffective over long-term culture. MOs are produced exclusively by Gene Tools LLC and are expensive, and multiple MOs sometimes must be tried to identify one that knocks down protein expression efficiently. Electroporated cells are traced by including a fluorescein modification on the 3′ end. This modification also provides a negative charge on the uncharged MO, allowing it to be electroporated (*see* **Note 19**).

Tips for the use of morpholinos:

1. Based upon recent guidelines from Gene Tools, resuspended MOs should be stored at room temperature in the original vial (i.e., in glass and in the dark). Storage in plastic and at cold temperatures may result in precipitation. If aliquots need to be made, they should be freeze dried according to the protocol on the Gene Tools Website (www.gene-tools.com) and individually resuspended before each use.
2. MOs are usually resuspended and electroporated at 1.0 mM. Resuspend in high-quality, nuclease-free water according to Gene Tools recommendations. Although the fluorescein modification gives the solution color, 2% vegetable dye (FD&C Blue, Spectra Colors Corporation) can be added to the MO at 1:10–1:20. Fast Green is not recommended because it can inhibit uptake of the MO (14).
3. Adding 0.3 μg/μL DNA (any non-biologically active DNA will do) to the MO can vastly improve electroporation efficiency by acting as a carrier for the minimally charged MOs.
4. While the immediate effect of a splice-blocking MO can be monitored by RT-PCR, an antibody against the target protein is the best way to document knockdown, which also depends upon protein turnover (13) (*see* **Note 20**).

3.2.2. Gain of Function

Chick biologists increase endogenous gene expression levels or express genes at ectopic times or locations, by introducing promoter-driven DNA constructs into the embryo. A variety of vectors are available for this purpose, including pCIG (15), pCAβ (16), and pMES (17). All contain the chick beta actin promoter, an internal ribosome entry site, and GFP as a lineage tracer in a bicistronic message. While these vectors vary in their ease of use at the subcloning stage, all work effectively in the embryo. The most important consideration for generating an overexpression construct is to limit the amount of untranslated sequence that

is included with the gene of interest, as untranslated sequence causes genes to be expressed less efficiently.

3.2.3. Ex Ovo Early Embryo Electroporation

Gain- and loss-of-function reagents must be introduced into chick embryos by electroporation. In the case of knockdown, because either approach inhibits synthesis of the target protein, perdurance of stable proteins translated prior to electroporation can mask the consequences of protein knockdown. To circumvent this problem, we electroporate at least 10–12 h before the stage to be assayed, to allow for protein turnover. Thus, in order to analyze gene function at stage 10, we electroporate at stage 4 (3). Because stage 4 embryos are flat, they must be removed from the yolk in order to create an electric field perpendicular to the blastoderm to achieve electroporation.

1. Using published fate maps, determine the location of the precursors of the cell type to be electroporated in a stage 4+ embryo (18–21).
2. Incubate eggs to stage 4+, usually about 25–26 h. Wipe eggs with 70% ethanol and let cool to room temperature for 30 min before use.
3. Have ready agar–albumen plates equilibrated to room temperature (22) (*see* **Note 21**).
3. Holding the egg pointed end down and using a pair of blunt forceps, tap/crack/cut a 1.5–2 in. slit in the side of the egg shell about half way from the rounded end. Carefully lift off the egg shell top and pour the yolk from the bottom shell into the palm of your gloved hand.
4. With the gloved finger of your other hand or a pair of blunt forceps, wipe and pinch off the thick albumen that adheres to the yolk and rotate the yolk until the embryo is on top (*see* **Note 22**). Use the edge of a pair of blunt forceps to wipe off the yolk to ensure there is no thick albumen remaining attached (*see* **Note 23**).
5. Place a Whatman filter square onto the yolk so that the hole reveals the embryo. The paper will adhere to the vitelline membrane that covers the embryo. Use open scissor blades to press the paper onto the yolk to ensure it adheres well.
6. Insert a scissor blade into the yolk and cut the yolk membranes all around the paper. Grasp the embryo from one side with a #2 forceps and gently lift one side of the embryo up away from the yolk.
7. Place the embryo paper (dorsal) side down/yolky (ventral) side up into a dish of Ringer's saline. Gently and slowly pull the embryo back and forth through the Ringer's saline to rinse (*see* **Note 24**).

8. Place the embryo paper (dorsal) side down/yolky (ventral) side up onto an agar–albumen plate and replace the cover to maintain humidity until use (*see* **Note 25**).
9. When ready to electroporate, tilt the dish toward you at a 45° angle. Use a transfer pipette to drip Ringer's saline onto the elevated side of the plate (avoiding dripping directly onto the embryo) so that Ringer's saline washes over the embryo. Tip the dish back and forth to further clean yolk from the embryo. Use the transfer pipette to aspirate the Ringer's wash.
10. Using a pair of forceps, lift the embryo from the plate and place paper (dorsal) side down/yolky (ventral) side up in an electroporation cuvette containing clean Ringer's saline (**Fig. 5.1**). Use the cuvette alignment lines to orient the embryo so that the region to be electroporated lies over the bottom electrode (*see* **Note 26**).
11. Use a microloader tip to back-fill a glass needle with 2 μL plasmid or MO. Place the needle in the needle holder of a forced air injection apparatus. Break off the very tip of the needle and expel any air remaining in the tip. Test and adjust the injection time until only a small drop is produced (*see* **Note 27**).
12. Gently insert the needle through the embryo from the ventral side into the subvitelline space of the region to be electroporated and expel a puddle of plasmid or MO (*see* **Note 28**).
13. Before the injected solution has a chance to disperse, place the upper electrode directly over (but not touching) the region of the embryo containing the injected solution. If the paddle of the top electrode is not covered with Ringer's saline, add more.
14. Apply five, square wave 7 V 50 ms pulses with 100 ms gaps between pulses (*see* **Note 29**).
15. Transfer the embryo from the electroporation cuvette back to the agar–albumen plate. Culture in a humidified chamber at 38°C to the desired stage.

3.2.4. In Ovo Electroporation

1. Incubate and window eggs as in **Section 3.1.1**.
2. Use a microloader tip to back-fill a glass needle with 2 μL of plasmid or MO. Place the needle in the needle holder of a forced air injection apparatus. Break off the very tip of the needle and expel any air remaining in the tip. Test and adjust the injection time until only a small drop is produced (*see* **Note 27**).

3. Inject plasmid or MO into the tissue to be electroporated (*see* **Note 28**). Best results will be obtained by injecting into a lumen (e.g., the interior of the neural tube) that will act as a reservoir to contain the injected material and limit diffusion. Keep in mind that negatively charged DNA and the negative charge of the fluorescein modification on the MO (MOs themselves are uncharged) will enter cells in the direction of the positive electrode. *See* (1) for additional pointers.
4. Moisten the electrodes with Ringer's saline and place them 4 mm apart on either side of the embryo, with the tissue to be electroporated located between the injected material and the positive electrode. The degree to which the electrode contacts the embryo surface and the angle between the electrodes will determine the extent and direction of electroporation (*see* **Note 30**).
5. Apply five, square wave 17 V, 50 ms pulses with 100 ms intervals between pulses (*see* **Note 29**).
6. Let the embryo recover for 1–2 min and then gently place a few drops of Ringer's saline onto the embryo, wipe any albumen from the egg shell, and seal the window with a rectangle of clear packing tape, starting at one end and sealing around the hole (**Fig. 5.2d**). Make sure the egg is well sealed or the embryo will dry out.
7. Incubate to the desired stage in a humidified 100°F/38°C incubator.

3.3. Post-incubation

3.3.1. In Ovo

1. Cut tape from the window, add a few drops of Ringer's saline to the embryo, and cut around the embryo. Lift from the yolk by grasping a cut edge with a pair of forceps or by scooping and lifting under the embryo with a pair of closed scissor blades.
2. Place the embryo in a petri dish with Ringer's saline. Rinse yolk from the embryo, remove the vitelline membrane, and trim extraembryonic tissues.
3. Fix embryos according to standard protocols for immunocytochemistry or in situ hybridization (23) (*see* **Note 31**).

3.3.2. Ex Ovo

1. Embryos may be rinsed, fixed, and photographed while still attached to the paper (*see* **Note 32**).
2. Alternatively, to remove from the filter paper, place into a petri dish of Ringer's saline with the paper side down. Find a place where the membranes are rolling up or detaching. With a pair of forceps, gently peel the embryo away. The vitelline membrane will remain attached to the paper.
3. Fix embryos according to standard protocols for immunocytochemistry or in situ hybridization (23) (*see* **Note 31**).

3.3.3. Explants

1. After incubation, tissue embedded in collagen can be fixed and processed for immunocytochemistry or in situ hybridization using standard protocols (23). The collagen may be left attached to the culture dish for staining or may be gently lifted off with a pair of forceps and processed as a single mass (*see* **Note 33**).

3.4. Conclusion: Direction of Methodologies

In the past decade, a variety of new techniques have become available to the chick biologist, including electroporation, RNAi, morpholino oligonucleotides, and genomic approaches. While the methods we discussed in this chapter have greatly expanded the lines of inquiry possible in the chick, the techniques are still evolving. For example, ex ovo electroporation protocols are continually being improved to increase survival and decrease the incidence of electroporation-induced defects. Meanwhile, RNAi vectors that are optimized for chick are in development and may produce more robust effects. As these techniques become established, we expect that the combined use of chick loss-of-function and embryological manipulations will become more commonplace.

4. Notes

1. Excess DMEM plus HEPES may be stored in 50 mL aliquots at –20°C for later use.
2. Eggs can be stored at 13–20°C for up to 1 week before use, although fertility will be highest when eggs first arrive. An old refrigerator outfitted with a WINE-STAT (Walnut Creek, CA) makes a good egg-storage cooler. Eggs may also be placed in a cool corner of the lab.
3. Incubators may be placed on timers in order to start eggs at inconvenient times (e.g., the middle of the night).
4. Before starting, have ready an egg waste bag and a beaker to discard albumen. Egg waste should be disposed as required by your institution.
5. It is typically not necessary to apply scotch tape over the syringe needle hole as the egg usually seals itself; however, tape should be applied if the hole is extensive. Alternatively, if the syringe needle hole is far enough toward the top of the egg, it may be used as a starting point to cut the access hole in the next step (**Section 3.1.1**, step 5).
6. Over time the ink may disperse within the yolk. To re-ink, try to reinsert the needle through the same hole next to the blastoderm.

7. Affigel blue beads work for a variety of proteins, while heparin acrylic beads work well for growth factors that bind heparin. Affigel blue beads are "stickier," while heparin acrylic beads can be easier to manipulate. If your heparin acrylic beads do not stay in the intended tissue, try chipping the bead and using a small fragment instead.
8. The protein concentration used depends upon the factor of interest and must be determined empirically. Generally, FGFs, Shh, and Noggin are typically used at 1 mg/mL, while BMPs can be used at lower concentrations, for example, 0.1 mg/mL.
9. Let the eggs cool at least 30 min at room temperature before collecting them into the baking dish. The yolks are fragile and break easily when hot.
10. When cutting the yolk membranes, make your first cuts along the side of the embryo that is lowest on the yolk. The contents of the yolk will spill out once it is broken, thus if you cut the top side first, the embryo will be pulled under the yolk.
11. Embryos stage 10 and younger are very fragile when harvested in this manner. Be very gentle. Paper discs (as described in **Section 3.2.2**) may be used to isolate young embryos from the yolk.
12. Embryos will stick to non-tissue culture-coated plastic. Bacteriological plates and plastic transfer pipettes can be used if they are first coated with yolky Ringer's saline (Ringer's that is milky with egg yolk. For example, Ringer's saline in which embryos have been rinsed or into which egg yolk has been added) and rinsed with fresh Ringer's saline before embryos are added.
13. To isolate trunk neural tubes, we incubate stage 14–15 caudal trunks in dispase for 15 min on ice and then 10 min at 37°C, until tissues begin to fall apart. Dispase incubation conditions will need to be optimized for each tissue type.
14. Neural crest cell cultures grow best in DMEM-F12 media. However, to rinse away enzyme, either DMEM or DMEM-F12 is acceptable.
15. With practice, a Pasteur pipette or capillary tube that has been pulled in a flame (we usually pull ours at a 90° angle) fitted onto an aspirator assembly provides a controlled means to move small tissue explants from one dish to another while transferring very little liquid, making rinsing more efficient.
16. The collagen should be spread only a little. It should not be too thin nor should it be significantly domed. More collagen can be used if larger explants are cultured.

17. shRNA target sequences are designed using the Whitehead Institute's siRNA Selection Program (24) and Ambion's pSilencer siRNA Converter Program. *See* (12) for details.
18. MO target sequences are selected using Gene Tools free design service.
19. Note that MOs can also be modified with lissamine, which is more stable than fluorescein. However, because lissamine is positively charged, it cannot be coelectroporated with DNA in order to assess rescue of MO effects, and so it is not recommended.
20. Comprehensive guidelines for performing controlled morpholino experiments are provided in (13).
21. Before and during culture, we place our culture plates in covered square BioAssay dishes (such as Corning 431272) containing wet paper towels to ensure the plates and embryos do not dry out.
22. Make sure the gloved finger you use to wipe the yolk is wet with albumen and not dry or you will break the yolk.
23. If albumen remains on the yolk, the paper will not stick well, the embryo will detach, and development will arrest.
24. Be gentle and patient. Do not wash the embryo vigorously and be sure the embryo does not detach from the paper. Both over- and underwashing lead to viability problems.
25. Embryos are stable on agar–albumen plates at room temperature for several hours. Depending on the size of your embryo and filter paper, two embryos may be cultured on a single plate.
26. Ensure the embryo is clean and that large amounts of yolk are not present over the lower electrode or above the embryo.
27. If a forced air injection system is not available, you can fit the needle onto a mouth aspirator assembly to deliver DNA to the embryo. Expel air about three to five times with the mouth aspirator.
28. We generally inject by hand, but a micromanipulator can be used. If the injected solution dissipates rapidly (typically not a problem with MOs), sterile 10% sucrose can be added at 1:4 or 1:1 to make the solution more viscous.
29. The number of pulses and voltage can be adjusted to optimize survival and targeting efficiency for your tissue and age of embryo. Generally speaking, younger embryos must be electroporated with lower voltages.
30. The tips, bends, or arms of the electrodes may be placed on the embryo for electroporation. The angle of

electroporation is changed by placing more pressure on one electrode than the other.

31. It is a good idea to image fluorescein-modified MO-electroporated embryos before subsequent staining protocols as the fluorescence quickly fades. Keep the embryos in the dark at all times to minimize this.
32. Ex ovo culture can result in non-specific midbrain neural tube closure defects. Because electroporation is performed unilaterally, phenotypes can be interpreted as long as appropriate controls are included.
33. Occasionally the collagen gives high background staining, in which case it is necessary to dissect the pieces of tissue from the collagen and stain them individually.

References

1. Krull, C. E. (2004) A primer on using in ovo electroporation to analyze gene function. *Dev. Dyn.* **229**, 433–439.
2. Conrad, G. W., Bee, J. A., Roche, S. M., and Teillet, M. A. (1993) Fabrication of microscalpels by electrolysis of tungsten wire in a meniscus. *J. Neurosci. Methods* **50**, 123–127.
3. Hamburger, V. and Hamilton, H. (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92. Republished in (1992) *Dev. Dyn.* **195**, 231–272.
4. Garcia-Castro, M. I., Marcelle, C., and Bronner-Fraser, M. (2002) Ectodermal Wnt function as a neural crest inducer. *Science* **297**, 848–851.
5. Crossley, P. H., Martinez, S., and Martin, G. R. (1996) Midbrain development induced by FGF8 in the chick embryo. *Nature* **380**, 66–68.
6. Dickinson, M., Selleck, M., McMahon, A., and Bronner-Fraser, M. (1995) Dorsalization of the neural tube by the non-neural ectoderm. *Development* **121**, 2099–2106.
7. Chesnutt, C. and Niswander, L. (2004) Plasmid-based short-hairpin RNA interference in the chicken embryo. *Genesis* **39**, 73–78.
8. Dai, F., Yusuf, F., Farjah, G. H., and Brand-Saberi, B. (2005) RNAi-induced targeted silencing of developmental control genes during chicken embryogenesis. *Dev. Biol.* **285**, 80–90.
9. Harris, M. L., Hall, R., and Erickson, C. A. (2008) Directing path finding along the dorsolateral path—the role of EDNRB2 and EphB2 in overcoming inhibition. *Development* **135**, 4113–4122.
10. Das, R. M., Van Hateren, N. J., Howell, G. R., Farrell, E. R., Bangs, F. K., Porteous, V. C., Manning, E. M., McGrew, M. J., Ohyama, K., Sacco, M. A., Halley, P. A., Sang, H. M., Storey, K. G., Placzek, M., Tickle, C., Nair, V. K., and Wilson, S. A. (2006) A robust system for RNA interference in the chicken using a modified microRNA operon. *Dev. Biol.* **294**, 554–563.
11. Sauka-Spengler, T. and Barembaum, M. (2008) Gain- and loss-of-function approaches in the chick embryo. *Methods Cell. Biol.* **87**, 237–256.
12. Sahin, M., Greer, P. L., Lin, M. Z., Poucher, H., Eberhart, J., Schmidt, S., Wright, T. M., Shamah, S. M., O'Connell, S., Cowan, C. W., Hu, L., Goldberg, J. L., Debant, A., Corfas, G., Krull, C. E., and Greenberg, M. E. (2005) Eph-dependent tyrosine phosphorylation of ephexin1 modulates growth cone collapse. *Neuron* **46**, 191–204.
13. Eisen, J. and Smith, J. (2008) Controlling morpholino experiments: don't stop making antisense. *Development* **135**, 1735–1743.
14. Kos, R., Reedy, M., Johnson, R., and Erickson, C. (2001) The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. *Development* **128**, 1467–1479.
15. Megason, S. G. and McMahon, A. P. (2002) A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087–2098.
16. McLarren, K., Litsiou, A., and Streit, A. (2003) DLX5 positions the neural crest and

preplacode region at the border of the neural plate. *Dev. Biol.* **259**, 34–47.
17. Swartz, M., Eberhart, J., Mastick, G., and Krull, C. (2001) Sparking new frontiers: using in vivo electroporation for genetic manipulations. *Dev. Biol.* **233**, 13–21.
18. Cui, C., Cheuvront, T. J., Lansford, R. D., Moreno-Rodriguez, R. A., Schultheiss, T. M., and Rongish, B. J. (2009) Dynamic positional fate map of the primary heart-forming region. *Dev. Biol.* **332**, 212–222.
19. Ezin, A. M., Fraser, S. E., and Bronner-Fraser, M. (2009) Fate map and morphogenesis of presumptive neural crest and dorsal neural tube. *Dev. Biol.* **330**, 221–236.
20. Fernandez-Garre, P., Rodriguez-Gallardo, L., Gallego-Diaz, V., Alvarez, I. S., and Puelles, L. (2002) Fate map of the chicken neural plate at stage 4. *Development* **129**, 2807–2822.
21. Lopez-Sanchez, C., Garcia-Martinez, V., and Schoenwolf, G. C. (2001) Localization of cells of the prospective neural plate, heart and somites within the primitive streak and epiblast of avian embryos at intermediate primitive-streak stages. *Cells Tissues Organs* **169**, 334–346.
22. Chapman, S., Collignon, J., Schoenwolf, C., and Lumsden, A. (2001) Improved method for chick whole-embryo culture using a filter paper carrier. *Dev. Dyn.* **220**, 284–289.
23. Nieto, M., Patel, K., and Wilkinson, D. (1996) In situ hybridization analysis of chick embryos in whole mount and tissue sections. *Methods Cell Biol.* **51**, 219–235.
24. Yuan, B., Latek, R., Hossbach, M., Tuschl, T., and Lewitter, F. (2004) siRNA Selection Server: an automated siRNA oligonucleotide prediction server. *Nucleic Acids Res.* **32**, W130–134.

Chapter 6

Embryological Manipulations in Zebrafish

Yuhua Sun, Dorota Wloga, and Scott T. Dougan

Abstract

Due to the powerful combination of genetic and embryological techniques, the teleost fish *Danio rerio* has emerged in the last decade as an important model organism for the study of embryonic development. It is relatively easy to inject material such as mRNA or synthetic oligonucleotides to reduce or increase the expression of a gene product. Changes in gene expression can be analyzed at the level of mRNA, by whole-mount in situ hybridization, or at the level of protein, by immunofluorescence. It is also possible to quantitatively analyze protein levels by Western and immunoprecipitation. Cell behavior can be analyzed in detail by cell transplantation and by fate mapping. Because a large number of mutations have been identified in recent years, these methods can be applied in a variety of contexts to provide a deep understanding of gene function that is often more difficult to achieve in other vertebrate model systems.

Key words: Zebrafish, *Danio rerio*, methods, Western, immunofluorescence, cell transplants, two-color in situ hybridization, microinjection.

1. Introduction

The teleost *Danio rerio* is a uniquely powerful experimental system due to the ability to use a combination of both embryological and genetic techniques. Virtually the entire developmental program is accessible to experimental analysis because fertilization is external and the embryos develop quickly (1). Between 100 and 500 embryos per week can be obtained from a single female, by either natural mating or in vitro fertilization (IVF). This provides a rich source of material. The eggs are large (0.7 mm in diameter) and amenable to manipulations such as cell and tissue transplantation (1). These techniques can address fundamental embryological questions about cell fate commitment, cell autonomy, and the inductive capacity of embryonic tissues (2–4). They

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_6,

can also be used to determine the regenerative capacity of potential stem cells (5). To study gene function, it is often desirable to increase or decrease its activity. This can be easily accomplished in zebrafish by injecting early stage embryos with solutions containing in vitro-synthesized mRNA, chemically modified oligonucleotides, or purified protein. In addition, small molecule inhibitors are a relatively new tool in the zebrafish arsenal and are increasingly being used to conditionally inactivate key signal transduction pathways (6, 7).

Analysis of zebrafish embryos is facilitated by a number of features. First, the embryos are optically clear because the yolk platelets are segregated to the extra-embryonic yolk cell (1). This makes possible high-resolution analysis of live tissues, including deep tissues, by DIC microscopy. In addition, the behavior of individual cells can be monitored in vivo from the earliest stages by time-lapse microscopy. Second, the expression patterns of more than 8,000 genes have been determined by large-scale in situ hybridization screens and the expression of 3,000 more are reported in the literature (8). Images from the majority of these patterns have been cataloged on ZFIN, the official database for the zebrafish model organism (http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg). This database provides a rich source of anatomical markers that can be used at every developmental stage. A small but growing number of antibodies are also cataloged on ZFIN.

1.1. Embryo Injection

The activity of a gene product can be increased in zebrafish by injecting in vitro-synthesized mRNA into embryos before the eight-cell stage (1.25 h postfertilization, hpf). The activity of nearly any gene can be reduced by injection of a morpholino oligonucleotide (MO) complimentary to the translation start site (TLMO) or the splice junction (SPMO) of the transcript (9, 10). These oligonucleotides are designed and synthesized by Gene Tools, Inc. (Philomath, OR). Since no endogenous enzymes recognize their artificial morpholine–sugar backbone, they have a very long half-life in cells. RNAi and siRNA are commonly used to reduce gene function in worms, flies, and mammals but do not work reliably in zebrafish despite a functional Dicer pathway (11–14).

The architecture of the early zebrafish embryo ensures the even distribution of most injected material, except for plasmid DNA. The cells that produce the entire fish are situated on top of an extra-embryonic yolk cell. During the first three divisions, the cells divide meroblastically, separating from each other but maintaining open connections to the yolk mass (1). Maternal gene products located in the yolk are delivered to the cells through these connections via an array of microtubules (15–17). Similarly, compounds injected into the yolk during the first

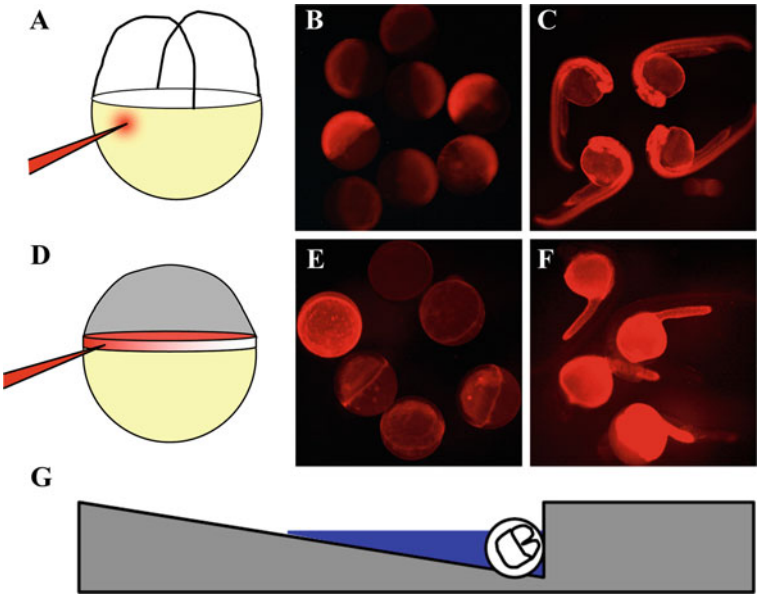

Fig. 6.1. Embryo injection. (**a**) To inject embryos at the 1–4 cell stage, the injection needle should penetrate the embryo through the yolk, preferably near the margin, just underneath the large blastomeres. At this stage, similar results are obtained if the material is injected into a single blastomere. (**b**) Material injected into the yolk cell at this stage is actively transported to all blastomeres. Except for DNA, the injected material is evenly distributed in all blastomeres at 5 hpf and absent from the yolk. Plasmid DNA is inherited unevenly with each cell division, resulting in mosaic embryos. (**c**) Cells remain fluorescent at 24 hpf. (**d**) To target the YSL, the injection needle should penetrate the yolk at the margin but must not poke into the blastoderm. (**e**) Injected material remains in the YSL at 5 hpf and is still localized in the YSL at 24 hpf (**f**). (**g**) To inject, embryos in the chorions are placed in the injection ramp containing egg water. Prior to injection, some egg water is removed to expose the tops of the chorions.

hour of development are transported to the cells and are segregated evenly during subsequent cell divisions (**Fig. 6.1a–c**). After the eight-cell stage, microtubule-based transport stops and injected compounds remain in the yolk. Therefore, you must inject embryos prior to this stage in order to increase or decrease the level of a gene product in all cells.

In contrast to vertebrates like *Xenopus*, the zebrafish embryo is also a good model for studying vertebrate extra-embryonic tissues. During the tenth cell cycle, the cells in direct contact with the yolk fuse with each other, forming the extra-embryonic yolk syncytial layer (YSL) (1). The YSL expresses many of the same genes as the mammalian visceral endoderm, and some of these genes perform similar functions in the two species (18, 19). Injections can be targeted directly to the YSL (**Fig. 6.1d**). mRNA, DNA, and MOs remain in the YSL for days after injection (**Fig. 6.1f**), but certain proteins may be actively transported out of the YSL (18, 20–22). There are reports that the YSL is resistant

to apoptosis (23). Consistent with this, we have observed that the YSL can tolerate higher concentrations of injected DNA or artificial oligonucleotides compared to embryonic cells. Finally, since the YSL is accessible to injection from the time it forms, at 2.75 hpf through at least 24 hpf, it is possible to conditionally activate or inactivate extra-embryonic gene function.

1.2. Cell Transplant

Chimeric animals provide information about gene function that is not revealed by standard mutant analysis. In zebrafish, cell transplantation is the method of choice to generate chimeras. This technique has been used to generate fish with chimeric germ lines that produce embryos lacking maternally provided maternal gene products (24). In embryos, cell transplants permit the analysis of individual mutant cells at a level of detail not possible in the context of a mutant embryo, in which all cells lack gene function. For example, these experiments permit the analysis of gene function at the level of specific tissues, or of individual cells. By determining whether a gene is required cell autonomously, cell transplants can be used to dissect the molecular mechanisms that mediate cell–cell communication. In these experiments, donor embryos from a cross of heterozygous parents are permanently marked with a fluorescent lineage-tracing molecule (**Fig. 6.2b**). During the blastula stages, cells are extracted from the donor embryos and inserted into unlabeled embryos from wild-type parents (**Fig. 6.2d**, **e**, **g**, **h**). The genotype of the donor embryos is determined retrospectively, by either PCR genotyping or embryo morphology. Alternately, two groups of donor embryos, obtained from parents of different genotypes, can be injected with different fluorophores (**Fig. 6.2c**). Both groups of cells are then transplanted into unlabeled, wild-type host embryos (**Fig. 6.2f**, **i**). Normally, one set of labeled donor embryos is from wild-type parents, and cells from these embryos act as an internal control to compare to mutant cells.

Cell transplants can also be used to determine when wild-type or mutant cells become committed to their fates (4, 25). In these experiments, donor cells from a cross of heterozygous parents are transplanted into a novel location in the host embryo (heterotypic transplant) (**Fig. 6.2d**, **e**, **g**, **h**) or into a host embryo that is at a different stage than the donor (heterochronic transplant). In both cases, the transplanted cells are exposed to different embryonic environments, which may or may not influence the identity of the descendants of the transplanted cells.

1.3. Fate Mapping and Lineage Analysis

Many gain- and loss-of-function phenotypes in zebrafish are characterized by missing or expanded tissues, changes in morphology, or defective organogenesis. These defects could result from a transformation in cell fate or changes in cell proliferation rates or viability. Cell lineage-tracing experiments are the only way to

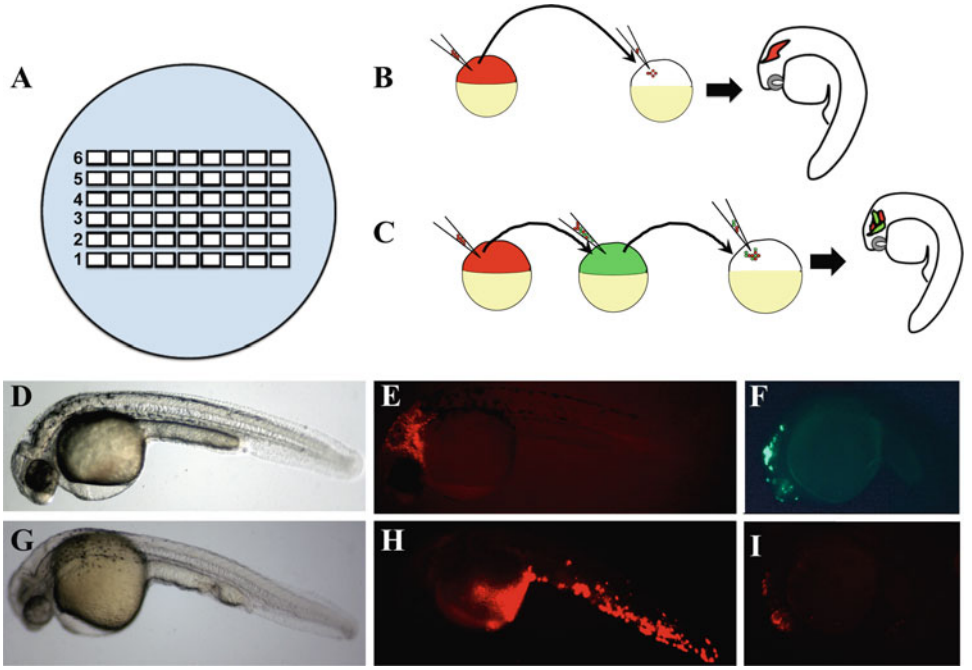

Fig. 6.2. Cell transplants. (**a**) Cell transplant plate. Dechorionated donor and host embryos are placed in alternate rows. (**b**) In a standard cell transplant, cells are removed from an embryo labeled with a lineage tracer dye and inserted into an unlabeled host embryo. At later stages, the fates of the descendants of the transplanted cells can be determined by cell morphology and location. (**c**) An alternate method is to label two sets of donor embryos with different lineage tracer dyes. Cells are removed from each donor embryo, mixed in the same needle, and inserted into an unlabeled host. At later stages, the fates of the descendants of the transplanted cells can be determined by cell morphology and location. (**d**) Bright-field image of a chimeric embryo in which donor cells were inserted into the animal pole of the host. (**e**) Fluorescent image of embryo in *d*. (**g**) Bright-field image of a chimeric embryo in which donor cells were inserted into the margin of the host. (**h**) Fluorescent image of embryo in *g*. (**f**) Green-fluorescent image of a chimeric embryo containing cells from two differently labeled host embryos, as shown in **c**. (**i**) Red-fluorescent image of the embryo in *f*.

formally distinguish between these possibilities. A progenitor cell is labeled at early stages by an indelible marker, and the fates of the resulting clone of cells are observed at later stages. Typically in zebrafish, lineage markers are fluorescent molecules that are unable to permeate cell membranes and are inherited by daughter cells during mitosis. These molecules are introduced directly into the progenitor cell by injection. Since the fate map of zebrafish is well described, shifts in the fate map can be readily detected in mutants (26–28). Due to extensive cell mixing, the cell lineage of zebrafish is indeterminate during the cleavage and early blastula stages, and labeling at this stage does not lead to a reproducible fate map (29, 30). This is in striking contrast to organisms like *Xenopus laevis*, in which the fate map can be determined as soon as the early cleavage stages (31). Thus, by the time it is possible to generate a reliable fate map in zebrafish, the cells are too small to accommodate standard injection needles. One way to circumvent this technical challenge is to introduce the lineage marker by

iontophoresis. In this technique, an electrode filled with a fluorescent dye is brought into contact with the plasma membrane of the target cell. The membrane is then depolarized, and the dye flows freely into the cell (for details, *see* (32)). This method is non-invasive and can be applied to the very small cells present in late stage embryos. Thus, it is possible to generate fate maps at single-cell resolution of embryos at a wide range of embryonic stages. Iontophoresis, however, requires specialized electrophysiology equipment that may not be readily available to all laboratories.

Other methods of labeling cells rely upon the use of photoactivatable fluorescent compounds. These compounds are injected into early embryos when the cells are large, and their fluorescence is activated at a later stage when cells are small. This technique is widely used because it does not require specialized equipment but is limited by the inability to label a single progenitor cell. Since the photoactivatable compounds are sensitive to exposure to light, they are not suitable for time-lapse imaging of the labeled cells.

The Kaede protein from stony coral, *Trachyphyllia geoffroyi*, has been used in zebrafish fate-mapping experiments (33, 34). In response to excitation with light at 488 nm, Kaede protein emits light in the green spectrum, with a peak at 518 nm. Exposure to ultraviolet light (i.e., 405 nm) cleaves the protein into a shorter form that emits in the red spectrum, with a peak at 582 nm, in response to excitation at 543 nm (33). The conversion from the green form to the red form is irreversible, and the red form is highly stable. In our hands, we have observed red-fluorescent Kaede in embryos as long as 4 days after the photoconversion reaction. Thus, the red-fluorescent Kaede isoform indelibly marks the descendents of the group of progenitor cells that were initially photoconverted. The identities of labeled cells are determined by their locations and morphologies.

1.4. Analysis of Gene Expression

In order to understand the underlying basis for an embryonic phenotype, it is important to determine which tissues and organs are affected. These defects are usually reflected by changes in gene expression that can be easily detected by whole-mount in situ hybridization (WISH) (8). This technique relies upon the ability of RNA polymerase to incorporate versions of UTP that have been chemically modified to contain an antigen. The most common epitopes are digoxigenin, biotin, and fluorescein. Although WISH using a single probe can generate important information about the time and place a gene is expressed during normal and abnormal development, WISH with two probes demonstrates the spatial relationship between two genes (**Fig. 6.3g**, **h**). For two-color WISH, the embryos are hybridized to two probes made by incorporating different epitopes. WISH can also be followed by immunohistochemistry.

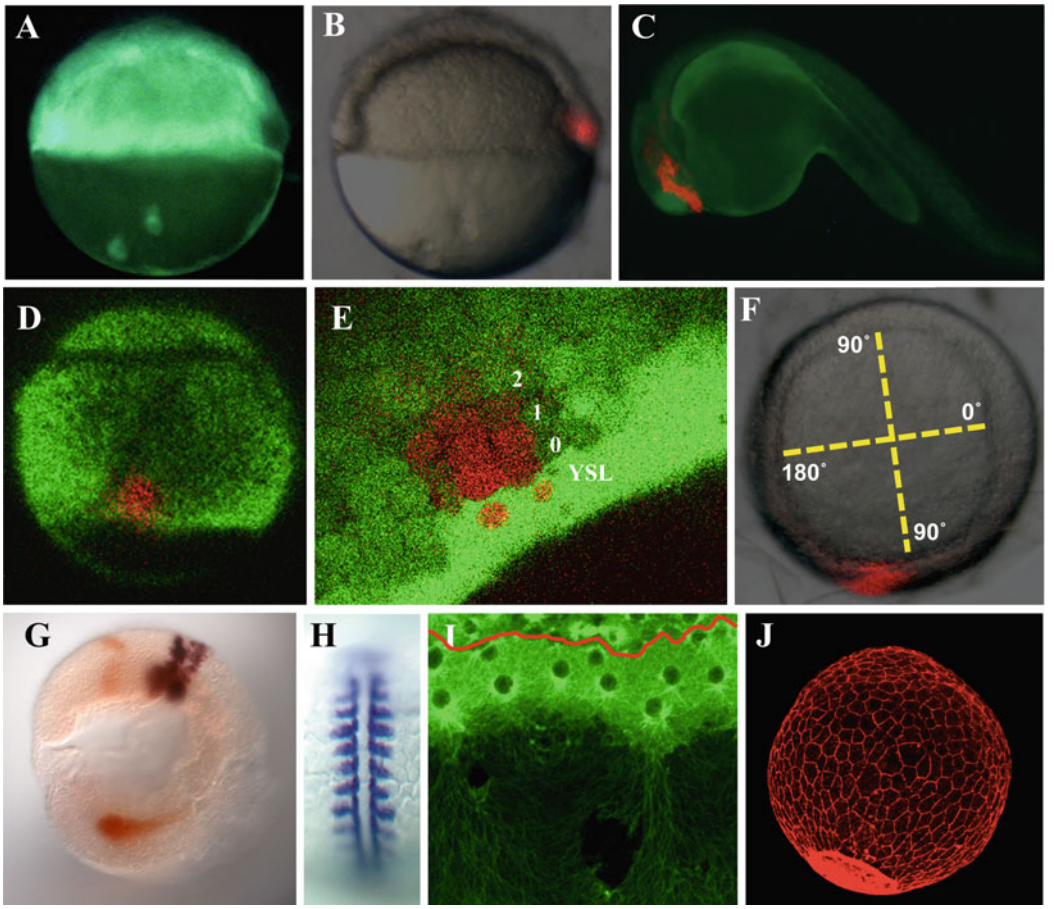

Fig. 6.3. Photoconversion of Kaede protein. (**a**) Image of an embryo injected with 2 ng Kaede protein immediately after photoconversion taken with a fluorescent dissecting microscope. There is no green fluorescence in the region targeted for photoconversion. (**b**) Overlay of bright-field image of a photoconverted embryo, and an image of the same embryo using epifluorescence under the TRITC channel. (**c**) Image of the embryo depicted in (**a**, **b**) at 24 hpf. Overlay of images taken under the FITC and TRITC channels. (**d**) Low-magnification, confocal image of an embryo immediately after photoconversion. (**e**) High-magnification image of the embryo shown in **d**. The row of cells juxtaposed to the YSL is numbered 0. Row 1 encompasses the cells one cell diameter away from the YSL, row 2 cells are two cell diameters from the YSL, and so on. (**f**) Animal pole images of a 6-hpf embryo, taken with a fluorescent dissecting microscope. The bright-field image reveals the position of the shield (marked at 0°). A fluorescent image of the embryo is overlaid, showing the position of the labeled cells along the dorsoventral axis. In this case, the labeled cells are about midway between the dorsal (0°) and the ventral (180°). (**g**) Two-color WISH of a two-somite embryo, using probes against *krox-20* (*purple*) and *floating head* (*brownish red*) (48, 49). The embryo was mounted in glycerol and the yolk dissected. (**h**) One-color WISH of a 10-somite embryo, using a probe against the somite marker *MyoD* (50). The embryo was dehydrated, cleared in benzyl benzoate:benzyl alcohol, and mounted in Canada balsam:methyl salicylate. (**i**) Confocal image of a 4-hpf embryo processed for immunofluorescence using the 12G10 antibody against α-tubulin. The *red line* indicates the boundary between the YSL (*below*) and the blastomeres that contribute to the embryo (*above*). Nuclei are indicated by *dark holes*. (**j**) Confocal image of an embryo at 90% epiboly stained with rhodamine–phalloidin.

1.5. Immunofluorescence

Many developmental phenotypes are associated with changes in protein distribution or with changes in the protein subcellular localization. Analysis of the cytoskeleton can be particularly revealing, since defects in cell shape, movement, and tissue morphology can be traced to problems with actin filaments or microtubule-based organelles. These changes can be detected by immunolocalization in whole-mount embryos using primary antibodies specific to the protein of interest and a secondary antibody conjugated to a fluorophore. The fluorophore emits a photon of light at one wavelength in response to excitation by light at a different wavelength. Alternately, the secondary antibody can be conjugated to an enzyme and visualized by a colorimetric assay (immunohistochemistry, IHC). At the concentrations typically used, immunofluorescence is the more quantitative and sensitive method of detection. Because of the higher resolution, immunofluorescence is used to determine the subcellular distribution of antigens. Due to the development of fluorophores with numerous different excitation and emission wavelengths, embryos can be processed for several antigens simultaneously. This permits the simultaneous visualization of two or more proteins in co-localization studies.

The main disadvantage of IF is that the fluorophores are unstable and the embryos cannot be stored for long periods of time. In addition, continued excitation of the fluorophore will lead to its irreversible destruction, a process called photobleaching. Fluorescein is particularly sensitive to photobleaching, and more stable derivatives, such as Alexa Fluor 488 and Oregon Green, are available from Molecular Probes. Because of these problems, embryos processed for IF should be kept in the dark and the results documented as quickly as possible. The longevity of the fluorophores can be extended by the addition of chemicals such as 1,4-diazabicyclo[2,2,2]octane (DABCO) in the mounting medium.

Although relatively few antibodies have been made against zebrafish antigens, antibodies against highly conserved mammalian proteins are likely to cross-react with zebrafish proteins. The 12G10 antibody against α-tubulin (Developmental Studies Hybridoma Bank) works well to reveal microtubules in zebrafish (**Fig. 6.3i**) (35, 36). Actin filaments in zebrafish are recognized by a fluorescently tagged version of the mushroom toxin, phalloidin (**Fig. 6.3j**) (36, 37).

1.6. Embryo Lysate

It is often necessary to make protein extracts in order to better understand an embryonic phenotype or to understand the mechanism by which a protein affects embryonic development. Western blots are important controls in experiments in which gene function is reduced by injection of artificial

oligonucleotides, such as morpholino oligonucleotides (MOs) (38). Co-immunoprecipitation experiments are a key tool to understand endogenous protein–protein interactions. These experiments are complicated in zebrafish by the large, yolky egg cell. Because of this cell, vitellogenin is by far the most abundant protein in zebrafish embryos and often competes out much less abundant but developmentally more interesting proteins on immunoblots. Protocols for removing the yolk are difficult to find in the published literature, leaving researchers on their own to figure out the best methodology. We present here a protocol for making embryo lysates free of yolk that was adapted from the proteomic work in the Heisenberg laboratory (39). These lysates can then be used for Western blots and immunoprecipitation experiments.

2. Materials

2.1. Injection

2.1.1. Equipment

1. Zeiss Stemi DV4 or similar dissecting microscope with a reflected light base.
2. Fluorescent dissecting microscope (optional).
3. Pipette puller (Sutter Instrument Co. P-97 Flaming/Brown Micropipette Puller).
4. Pneumatic PicoPump (World Precision Instruments, WPI).
5. Micromanipulator and stand (WPI M3301).
6. Glass Pasteur pipette. Cut off the first 1.5 in. of the tip and flame the tip to smooth the edges.
7. Kimax glass Petri dish, 60 mm diameter (VWR), or a 24-well Costar plate, coated with 2% agarose for use with dechorionated embryos.
8. Stage micrometer, 1 mm × 0.01 mm increments (Ward's Natural Sciences).
9. Scienceware Pipette Pump Filler/Dispensers, 10 mL (Fisher).
10. Quick Spin RNA G-50 column (fine) (Roche).
11. Injection plate: To make the plate, pour 25 mL of 2% agarose in water into a 100-mm-diameter Petri dish. Place the injection mold (Adaptive Science Tools) upside down into the agarose before it cools. The mold will make a series of troughs in the agarose, which are deep enough to hold chorionated embryos (**Fig. 6.1g**). If a mold is not available, place a glass slide on one lip of the Petri dish and let the other settle into the middle of the agarose.

12. Injection needles: To make injection needles, place a glass capillary tube (4.0 in. × 1 mm) with filament (WPI) in a pipette puller. The shape of the needle depends upon the kind of injection you are performing. For injecting dechorionated embryos, the needles should be tapered to come to a fine point. If the needle is too fine, however, the tip may be prone to clogging. For injecting chorionated embryos, the tips should be tapered more sharply so that the needle is strong enough to penetrate the chorion. If the taper is too sharp, it may be impossible to make an opening at the tip small enough to deliver a small enough volume. The needles can be stored embedded in soft clay stuck to the bottom of a 150-mm-diameter polystyrene Petri dish. Prior to the injection, the needle tips should be broken to an external diameter of no greater than 10 μm. Place the needle on a strip of parafilm on the transmitted light stage, orient the tip perpendicular to an optical graticule in the eyepiece, and slice the tip at the appropriate width using a clean razor blade.

2.1.2. Reagents and Solutions

1. Methylene blue.
2. Phenol red.
3. Instant Ocean salts (Aquatic Ecosystems).
4. Ambion mMessage mMachine Kit, SP6, T7, T3 (Ambion, Inc.).
5. Morpholino oligonucleotides, custom synthesis (Gene Tools, Inc).
6. Mineral oil (MP Biomedicals).
7. Methylcellulose (ICN Biomedicals).
8. 3% Methylcellulose in E2 medium.
9. 0.4 Wt% Phenol red in water or 0.2 M KCl.
10. 1 mg/mL Methylene blue in water.
11. Egg water: 60 μg/mL Instant Ocean sea salts, 50 μg/mL methylene blue.
12. Morpholino oligonucleotides (MOs): MOs are provided as a lyophilized powder and should be reconstituted by adding sufficient water to a concentration of 30 mg/mL (or alternately, 1 mM.) They should be stored at room temperature in a container sealed with parafilm to prevent evaporation.

2.2. Cell Transplant

2.2.1. Equipment

1. Embryo injection equipment (**Section 2.1.1**).
2. Synthetic hair paint brush (round size 0 or round size 1) (ArtistSupplySource.com, SKU SN-3103-R-O).

3. Transplant rig. Hydraulic transplant apparatus such as the Narishige IM microinjector series, or the Eppendorf CellTram or a homemade rig.
4. Transplant needles: Transplant needles are made from thin wall glass capillary tubes (4.0 in. × 1 mm) *without* a filament (WPI). The capillary tubes should be pulled in a pipette puller so that they are long and thin.
5. Prior to use, the needle tip should be broken under a dissecting stereomicroscope. Since younger embryos have larger cells than do older embryos, the diameter of the tip needed depends upon the age of the embryos you wish to transplant. For blastula stage embryos (4–5 hpf), we break our needles to an outer diameter of 20–25 μm. Place the needle on a strip of parafilm on the transmitted light stage, orient the tip perpendicular to an optical graticule in the eyepiece, and slice the tip at the appropriate width using a clean razor blade. The needles should be stored separately from injection needles, in a clearly labeled container.
6. Transplant plate: To make a transplant plate, pour 10 mL of 2% agarose in water into a 100-mm-diameter Petri dish. Place the injection mold (Adaptive Science Tools, PT-1) upside down into the agarose before it cools. The mold will make a series of wells in the agarose big enough to hold a single, dechorionated embryo (**Fig. 6.3a**).
7. Quick Spin RNA G-50 columns (Roche).

2.2.2. Reagents and Solutions

1. Penicillin/streptomycin stock (Invitrogen): 60 mg/mL Penicillin/100 mg/mL streptomycin.
2. Texas Red-conjugated dextran, fixable (Molecular Probes).
3. Alexa Fluor 488-conjugated dextran, fixable (Molecular Probes).
4. Pronase stock (Roche) (10 mg/mL).
5. Methylcellulose (ICN Biomedicals).
6. 3% Methylcellulose in 1× E2 medium.
7. Mineral oil (MP Biomedicals).
8. 1× E2 medium: 15 mM NaCl, 0.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 7.0 mM $NaHCO_3$ (freshly added), adjust pH to 7.1–7.4.
9. 50 μg/mL Fixable Texas Red-conjugated dextran in 0.2 M KCl.
10. 50 μg/mL Fixable Alexa Fluor 488-conjugated dextran in 0.2 M KCl.
11. 10 mg/mL Pronase stock in water.

2.3. Fate Mapping

2.3.1. Equipment

1. Embryo injection equipment (**Section 2.1**).
2. Glass depression slides and coverslips.
3. Fluorescent dissecting microscope.
4. Confocal microscope or a compound microscope equipped with epifluorescence and a Micropoint laser.

2.3.2. Reagents and Solutions

1. Methylcellulose (ICN Biomedicals).
2. Low-melting point agarose (Sigma).
3. CoralHue pKaede-S1 Fluorescent Protein Vector (MBL International).
4. Mineral oil (MP Biomedicals).
5. 3% Methylcellulose in 1× E2 medium.
6. Mounting medium B: 0.1% Low-melting point agarose in 1× E2 medium.
7. 2 mg/mL Kaede protein: Bacterially expressed Kaede protein should be purified by column chromatography as described (40). Store purified protein at 2 mg/mL in aliquots in 5 mM HEPES (pH 7) and 0.2 M KCl buffer at –80°C.

2.4. Whole-Mount In Situ Hybridization (WISH)

2.4.1. Equipment

1. Zeiss Stemi DV4, Leica S6E, or similar dissecting microscope with illumination from above.
2. Upright compound microscope with 10× objective, equipped with DIC optics.
3. Microcentrifuge tubes and rack.
4. Pyrex spot plates, nine cavity (85 mm ×100 mm) (Fisher).
5. Cut glass Pasteur pipettes.
6. Scienceware Pipette Pump (Fisher).
7. BD Adams Nutator Mixer (optional).
8. Dry bath with thermometer.
9. Dumont forceps (Fine Science Tools).
10. Quick Spin RNA G-50 columns, fine (Roche).
11. Vacuum apparatus: Place a rubber stopper in the top of a 1-L Erlenmeyer flask and insert broken plastic, 5-mL serological pipettes into the stopper holes. Attach one pipette to the house vacuum with plastic Tygon tubing. Use Tygon tubing to connect the arm of the Erlenmeyer flask to a glass Pasteur pipette and place a yellow pipette tip to the end of the Pasteur pipette.
12. Make slides with raised coverslips. Fix two 22-mm × 6-mm coverslips (Fisher, custom order) to the slide with superglue, approximately 22 mm apart. Superglue two

more 22-mm × 6-mm coverslips to each original coverslip so that each stack is three coverslips high.

2.4.2. Reagents and Solutions

1. Digoxigenin (DIG) RNA Labeling Kit (Roche).
2. PCR DIG Probe Synthesis Kit (Roche) (optional).
3. Fluorescein RNA Labeling Kit (Roche) (optional).
4. Methyl salicylate.
5. Benzyl benzoate.
6. Benzyl alcohol.
7. Permount (Fisher).
8. Paraformaldehyde powder (Sigma).
9. Canada balsam (Sigma).
10. Proteinase K.
11. Bovine serum albumin, fraction V protease free (Sigma).
12. Anti-DIG, alkaline phosphatase conjugated Fab fragment (Roche).
13. Anti-fluorescein, alkaline phosphatase conjugated Fab fragment (Roche).
14. Normal sheep serum (Jackson Immunoresearch).
15. Torula (yeast) RNA type VI (Sigma).
16. Glycine.
17. Tween-20.
18. Heparin.
19. Formamide.
20. INT/BCIP reaction solution (Roche).
21. NBT/BCIP ready-to-use tablets (Roche).
22. Methanol.
23. Goat anti-mouse IgG–HRP conjugate (Sigma).
24. Goat anti-rabbit IgG–HRP conjugate (Sigma).
25. 3,3′-Diaminobenzidine tetrahydrochloride, 10 mg tablet (Sigma).
26. 30% Hydrogen peroxide (H_2O_2).
27. 10× PBS: 1.38 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 17.6 mM KH_2PO_4, pH 7.4.
28. 100 mM Glycine, pH 2.2.
29. 4% Paraformaldehyde.
30. 10% Tween-20 in water.
31. 10% NaN_3 (wt%) in water.
32. PBST: 1× PBS, 0.1% Tween-20, 0.02% NaN_3.
33. 20× SSC: 3 M NaCl, 300 mM sodium citrate dihydrate, pH 7.0–7.2.

34. Hybridization buffer: 50% Formamide, 5× SSC, 500 μg/mL torula (yeast) RNA type VI, 50 μg/mL heparin, 0.1% Tween-20, adjust to pH 6.0–6.5.
35. Benzyl benzoate:benzyl alcohol (2:1 ratio).
36. Canada balsam:methyl salicylate (10:1 ratio).
37. Methanol dilution series (75, 50, 25% in PBST) (made fresh).
38. 2× SSC (made fresh).
39. 0.2× SSC (made fresh).
40. Antibody-blocking solution: 2% BSA, 5% normal sheep serum (may be replaced with other serum species) in PBST (made fresh).
41. Hybridization buffer dilution series (75, 50, 25% in 2× SSC) (made fresh).
42. 0.2× SSC dilution series (75, 50, 25% in PBST) (made fresh).
43. NTMT staining buffer: 100 mM Tris–HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20 (made fresh).
44. NBT/BCIP reaction solution: One ready-to-use NBT/BCIP tablet in 10 mL NTMT staining buffer (made fresh).
45. INT/BCIP reaction solution: 75 μL INB/BCIP stock solution in 10 mL NTMT staining buffer (made fresh).
46. DAB solution: 1 DAB tablet, 10 mg in 20 mL 1× PBS (made fresh).
47. Hydrogen peroxidase solution (50 μg of 3% H_2O_2) (made fresh).

2.5. Immunofluorescence

2.5.1. Equipment

1. Wheaton Conical Bottom V-Vials (5 mL) (Daigger).
2. Cut glass Pasteur pipettes: Cut off the first 1.5 in. of the tip and flame the tip to smooth the edges.
3. Scienceware Pipette Pump Filler/Dispensers, 10 mL (Fisher).
4. Zeiss Stemi DV4, Leica S6E, or similar dissecting microscope with illumination from below.
5. Compound microscope equipped with epifluorescence or access to a confocal microscope.
6. Pyrex spot plates, nine cavity (85 mm × 100 mm) (Fisher).
7. BD Adams Nutator Mixer (optional).
8. Dumont forceps (Fine Science Tools).
9. Raised slide coverslips: Fix two 22-mm × 6-mm coverslips (Fisher, custom order) to a slide with superglue,

approximately 22 mm apart. Superglue two more 22-mm × 6-mm coverslips to each original coverslip so that each stack is three coverslips high.

2.5.2. Reagents and Solutions

1. Dimethyl sulfoxide (DMSO).
2. 1,4-Diazabicyclo[2,2,2]octane (DABCO).
3. Normal sheep serum (Jackson Immunoresearch).
4. Bovine serum albumin, fraction V protease free (Sigma).
5. 50% Glutaraldehyde (Fisher Scientific).
6. Paraformaldehyde powder (Sigma).
7. Sodium borohydride ($NaBH_4$).
8. Methanol.
9. Benzyl benzoate.
10. Benzyl alcohol.
11. Methyl salicylate.
12. Canada balsam (Sigma).
13. Primary antibodies (made in mouse or rabbit).
14. Secondary antibodies, conjugated to a fluorophore (Alexa Fluor 488, fluorescein).
15. Rhodamine–phalloidin (Invitrogen): 1.5 Methanol to 300 U; final concentration is 200 U/mL or 6.6 μM.
16. Glycerol.
17. Egg water (**Section 2.1.2**).
18. 10× PBS (**Section 2.4.2**).
19. PBST (**Section 2.4.2**).
20. PBSTX: 0.5% Triton X-100, 0.02% NaN_3 in PBS.
21. Gard's fixative in microtubule-stabilizing buffer (41): 3.7% Paraformaldehyde, 0.25% glutaraldehyde, 0.5% Triton-X-100 in 800 mM PIPES, pH 6.8, 5 mM EGTA, 1 mM $MgCl_2$. Optional: add 10 μM Paxlitacel just prior to use.
22. DABCO mounting medium: 100 mg/mL DABCO in 90% glycerol.
23. Optional mounting medium: 70% glycerol.
24. Methanol dilution series (75, 50, 25% in PBST) (made fresh).
25. Sodium borohydride ($NaBH_4$): 3 mg/mL wt% in PBST (made fresh).
26. Antibody-blocking solution: 2% Bovine serum albumin, 0.5% normal sheep serum, 1% dimethyl sulfoxide in PBSTX (made fresh).

2.6. Embryo Lysates

2.6.1. Equipment

1. Microcentrifuge tubes.
2. Microcentrifuge.
3. 20–200-μL Pipette and yellow tips.
4. 100-mm-Diameter Petri dish, agarose coated.
5. Dumont forceps (Fine Science Tools).
6. Zeiss Stemi DV4 or similar dissecting microscope with a reflected light base.

2.6.2. Reagents and Solutions

1. Pronase (Roche): 10 mg/mL in water. Prior to use, dilute to 2 mg/mL in egg water.
2. RIPA buffer (Sigma).
3. Cell lysis buffer (Cell Signaling Technology).
4. 0.1 M Phenylmethylsulfonyl fluoride (PMSF) (Sigma).
5. Protease inhibitor (Roche).
6. Bicinchoninic acid (BCA) assay (Thermo Scientific).
7. E2 medium (**Section 2.2.2**).
8. RIPA buffer: 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate.
9. Deyolking buffer (Ringer's calcium-free medium): 116 mM NaCl, 2.9 mM KCl, 5.0 mM HEPES, pH 7.2.
10. Western lysis buffer: 1 mM PMSF, 1× protease inhibitor in RIPA buffer.
11. Cell lysis buffer (non-denaturing): 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, pH 8.0, 1 mM EGTA, pH 8.0, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin. Solution is stable for 2 weeks at 4°C.
12. 2× Sample buffer: 3.55 mL H_2O, 1.25 mL 0.5 M Tris–HCl, pH 6.8, 2.5 mL glycerol, 2 mL 10% SDS, 0.5% bromophenol blue, 0.5 mL β-mercaptoethanol.

3. Methods

3.1. Injecting Embryos

3.1.1. mRNA Synthesis

1. Linearize 20 μg of plasmid DNA with a restriction enzyme that cuts downstream of the SV40 polyadenylation signal (*see* **Note 1**). After two phenol/chloroform extractions, a chloroform extraction, and precipitate DNA, dissolve in 20 μL

distilled water to a concentration of 1 μg/μL and check on gel to verify DNA is cut. Store at –20°C.

2. At room temperature, add 2 μL (2 μg) linearized template DNA, 10 μL of 2× NTP/CAP buffer, 2 μL of 10× reaction buffer, 1 μL RNasin, and bring the volume up to 18 μL with RNase-free water (provided in the Ambion mMessage mMachine). Add 2 μL enzyme, mix thoroughly, and incubate for 2 h at 37°C.
3. Add 1 μL of RNase-free DNase I, mix, and incubate at 37°C for 15–30 min. Remove aliquot and store at 4°C to run on a gel.
4. To stop the reaction, bring the volume up to 50 μL in RNase-free water and phenol–chloroform extract. Purify the transcript on a mini Quick Spin RNA G-50 column (fine). Remove a 1 μL aliquot to run on gel. Determine concentration (*see* below), add 6.25 μL of 4 M LiCl and 188 μL chilled ethanol. Incubate for 30 min at –80°C and centrifuge at 14,000×*g* for 15 min and suspend at a concentration of 500 ng/μL.
5. Quantification of transcript: The most accurate method to quantify the amount of mRNA synthesized is to incorporate trace amounts of [α-^{32}P]UTP into the reaction. Measure the total radioactivity at the beginning of the reaction and after the reaction in the column eluate, by the Cerenkov method (42). Alternate methods, including measuring OD_{260} or gel quantification, are less reliable, but avoid the potential for trace amounts of radioactivity contaminating the injection setup. The maximum mRNA yield afforded by the mMessage mMachine Kit is 39.6 μg using T7 or T3 polymerases and 26.4 μg using SP6 polymerases. If your yield is significantly lower than this, try increasing the amount of template DNA. For longer transcripts, add an extra 1 μL of 20 mM GTP to the reaction mix and increase the reaction time to 4 h. Note that increasing the concentration of GTP may decrease the fraction of capped transcripts. We have successfully employed these modifications to generate functional transcripts >9 kb in length. The mRNA should be suspended to a concentration of 1 mg/mL and stored at –80°C in 1 or 2 μL aliquots until use.
6. Prior to injection, the mRNA should be diluted to the desired concentration in a solution of 0.2 M KCl and 0.1 volume phenol red stock. This working stock of mRNA should be kept on ice throughout the entire injection procedure, except while it is in the needle.
7. Morpholino oligonucleotides should be diluted to the desired concentration in a solution of 0.2 M KCl and

0.1 volume phenol red stock and stored at room temperature (*see* **Note 2**).

3.1.2. Microinjection

1. Collect embryos (*see* **Note 3**).
2. Load the needle by backfilling. Place the back of a broken needle into 10 μL of injection solution in a microcentrifuge tube. The solution will be drawn toward the tip along the filament by capillary action. If the solution is not drawn to the tip, try loading the needle into the injection apparatus.
3. Alternately, load the needle by frontloading. Take up 4–5 μL of the injection solution into a pipette. Partially expel the solution and touch the tip of the injection needle. The solution will be drawn into the tip of the needle by capillary action. Once the needle is full, insert the needle into its holder and twist the clamp tight to hold the needle in place. Load a fresh needle to use as a backup in case the first needle becomes clogged.
4. Turn on the Pneumatic PicoPump and open the attached nitrogen gas tank. Set the injection pressure to 30 psi and turn the hold pressure off.
5. Transfer embryos to the injection ramp with a glass pipette and align them in single file.
6. Remove embryo medium from the plate, exposing the tops of all the chorions. This holds the embryos in place and facilitates penetration of the chorion by the needle. It is important to only leave the tops of the embryos exposed, because removing too much medium can result in dehydration and decrease 24 h survival rates.
7. Move the micromanipulator to bring the needle tip into the field of view of the microscope and orient the needle at roughly 45° with respect to the plane of the injection plate.
8. Submerge the needle tip under the surface of the remaining embryo medium. Slowly increase the hold pressure until there is a constant but low rate of flow of injection fluid from the needle (*see* **Note 4**). Be careful at this step because there is usually a slight delay between turning the hold pressure knob and observing increased pressure in the needle. Set the apparatus for timed rather than gated injections.
9. Carefully raise the needle out of the embryo medium and above the injection plate. Remove the injection plate from stage and place an objective micrometer (Ward's Natural Science), with a drop of mineral oil on top of the scale. Eject a drop into the mineral oil and measure diameter of drop as it falls toward the scale. The total injection

volume should be between 0.5 and 1 nL. The volume can be adjusted by increasing or decreasing the length of the injection pulse. Steps 7 through 9 need to be repeated for each new needle.

10. Once the injection settings are established, raise the needle and replace the injection plate on the microscope stage. Bring the needle tip and embryos into the same field of view, focusing on the embryos.
11. Hold the injection plate with one hand and the micromanipulator in the other. Turn the micromanipulator knob clockwise to push the needle forward in order to insert needle through the chorion. It is best to enter the embryo either through the vegetal pole or from the side. Bring the needle tip to a position just underneath the cells. Press the foot pedal to release a pulse of fluid into the embryo. Injections into one of the blastomeres before the eight-cell stage are also permissible, since the injected material will freely diffuse into all cells. But inserting a needle through the top of the blastomeres increases the amount of physical damage done to the embryo during the procedure. A successful injection is indicated by a concentrated ball of phenol red remaining in the embryo. The phenol red should diffuse slowly over a period of time. If the phenol red disappears immediately, it indicates the needle tip was not inserted into the embryo.
12. Remove needle quickly and slide the injection plate over so that the next embryo lies underneath the needle tip. Repeat the procedure with next embryo. Monitor the size of the ball of phenol red in each embryo to make sure the injection volume remains constant throughout the experiment (*see* **Note 5** for strategies to deal with clogged needles).
13. When all embryos are injected, transfer them to a fresh Petri dish filled with 25 mL embryo medium. Remove the injection needle and turn off the gas flow to the injection apparatus. Turn up the hold pressure to flush out all air remaining in the system. Turn off the apparatus.
14. After 3–4 h, the injected embryos should be apparent by a phenol red tinge to the cells. If injecting a fluorescent substance, the embryos can also be checked under a fluorescent dissecting microscope. Transfer fertilized, injected, and undamaged embryos into a fresh plate and incubate at 28°C.
15. Record injection and results on an injection record form (**Fig. 6.4**). This form can be modified for cell transplants.

Date of Experiment_____

Stock: _____

Purpose:

Ramp	Parent Geno.	Inject	Conc.	drop dia.	Inj. Vol.	Qty inj.	stage inj.	site of inj.	stage fixed	# embs.
1										
2										
3										
4										
5										
6										
7										

NOTES/RESULTS:

Fig. 6.4. Sample microinjection score sheet.

3.1.3. Special Modifications for Injecting into YSL

1. To inject into the YSL, use embryos older than 2.75 hpf. It is also important to include a lineage tracer dye, such as Texas Red-conjugated dextran in the injection solution. This will permit you to verify the correct targeting of the injection fluid simply by examining the distribution of fluorescence 1–2 h post-injection. The exact concentration of lineage tracer is unimportant, as long as it is visible.

3.2. Cell Transplants (See Note 6)

3.2.1. Transplants with One Set of Donor Embryos

1. Collect donor and host embryos, which may be of different genotypes.
2. Dechorionate both donor and host embryos (*see* **Note 7**).
3. Add 1 mL penicillin/streptomycin stock to 1 L 1× E2 medium. The entire procedure and subsequent culture should be performed in this medium. For optimal embryo survival, the transplants must be performed in sterilized media at the proper pH and ionic strength. The addition of antibiotics to the medium also improves survival.

4. Transfer dechorionated embryos into an injection plate using a glass Pasteur pipette. Avoid letting them contact the surface of the medium.
5. Inject 1 nL of 50 μg/mL (50 pg) Texas Red-conjugated dextran (MW 10,000) into 1–4 cell stage donor embryos. Other fluorophores may also be used (*see* **Note 8**).
6. Use a glass Pasteur pipette to transfer the embryos to a Kimax glass Petri dish (60 mm diameter) containing 10 mL of 1× E2 medium. Visualize the embryos under a fluorescent dissecting microscope and select those with the most intense fluorescence. Incubate the embryos at 28°C, keeping them in the dark as much as possible. Wrapping the dishes in aluminum foil during transport is a convenient way to protect the embryos from ambient light that could bleach the fluorescence.
7. When the embryos reach the desired stage, add 25 mL 1× E2 medium containing penicillin/streptomycin to the injection plate. Transfer the donor embryos into the transplant plate using a glass Pasteur pipette, placing one donor embryo in each well of the odd numbered rows (**Fig. 6.2a**).
8. Next, transfer the host embryos into the transplant plate. Each host embryo should be placed in a well directly behind a donor embryo (**Fig. 6.2a**).
9. It is often necessary to remove cells from a specific region of the donor embryos and to insert them into a different region of the host. In this case, it is important that the donor and hosts are oriented to facilitate easy access of the needle to the regions of interest. Since dechorionated embryos are so delicate, they are prone to breakage when touched. The best way to orient the embryos is to move them with a fine paintbrush, with all but a few hairs cut. Alternately, pumping E2 medium over the embryos can rotate the embryos to the correct orientation. This method is gentler, but less precise.
10. Insert the transplant needle into the holder. Turn the knob on the microinjector in the clockwise direction to expel some oil from the needle.
11. During the procedure, turn the micromanipulator knob A (**Fig. 6.5b**) to move the injection needle into and out of embryos. Turn the microinjector knob (**Fig. 6.5a**) to remove cells from the donor embryos and insert them into host embryos.
12. Hold the transplant plate with one hand and the micromanipulator knob A (**Fig. 6.5b**) with the other. Orient

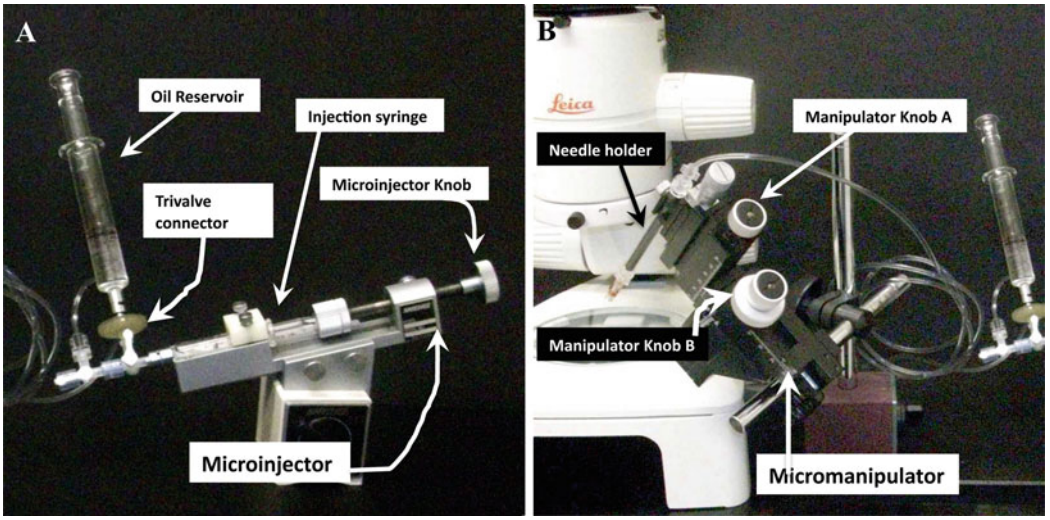

Fig. 6.5. Cell transplant apparatus. (**a**) Image shows the arrangement of the microinjector, glass injection syringe filled with mineral oil, and the glass oil reservoir syringe. The microinjector knob is turned to push or pull the injection syringe, permitting the uptake or the expulsion of cells into the transplant needle. (**b**) Image depicts the arrangement of the micromanipulator and transplant needle holder. The micromanipulator knob A is turned to move the transplant needle in and out of the embryo.

the transplant plate and micromanipulator so that a donor embryo is underneath the needle tip.

13. To fill transplant needle with E2 medium, submerge the tip of the needle under the E2 medium, and twist the microinjector knob counterclockwise to generate negative pressure. This will draw the aqueous medium into the needle, with no air bubbles.
14. Move the transplant plate to place a donor embryo underneath the needle tip. Turn the micromanipulator knob A clockwise to bring the needle forward, inserting the tip into the donor embryo.
15. Turn the microinjector knob in the counterclockwise direction to apply negative pressure within the needle. This will draw cells into the needle, which can be observed under normal bright-field light conditions or under the fluorescent channel of a fluorescent dissecting microscope.
16. Once the cells are in the needle, turn the micromanipulator knob A counterclockwise to remove the needle tip from the donor.
17. Move the transplant slide back to bring the host embryo underneath the needle tip.
18. Turn the micromanipulator knob A clockwise to bring the transplant needle forward, inserting it into the host embryo.

19. Turn the microinjector knob clockwise to eject the cells into the host embryo. Mineral oil is lethal to embryos, so carefully monitor the interface between the E2 medium and mineral oil during the transplant.
20. Once all the cells are expelled from the needle, turn the micromanipulator knob 3 counterclockwise to remove the needle from the host embryo. If the donor embryos are from a cross of heterozygous parents, it is important to expel all the cells from the needle before inserting the needle into a new donor embryo to prevent cross-contamination.
21. Once the transplants have been completed, incubate each donor–host pair separately in 1× E2 medium. The embryos can be incubated in 60-mm glass dishes or in Costar 24-well plates coated with 2% agarose at 28°C. Number each pair in anticipation of determining the genotype of the donor embryos.
22. After 24 hpf, determine the genotype of the donor embryos by examining their morphology or by PCR.
23. Assign cell fates based on their location in the embryo and the morphology of the cells under high magnification. To confirm cell fates, fix embryos and process for in situ hybridization for the expression of tissue-specific marker genes (*see* below). The lineage label can be visualized under bright-field microscopy by using antibodies against the fluorophore (antibodies against fluorescein and Texas Red are available). Some fluorophores, such as GFP, survive fixation and can be used to mark transplanted cells in whole mounts.

3.2.2. Transplants with Two Sets of Donor Embryos

1. The above protocol can be used with minor modifications for experiments involving the transplantation of two sets of donor embryos (**Fig. 6.2c**). The first set of donor embryos should be placed in the wells of rows 1 and 4 in the transplant plate (**Fig. 6.2a**).
2. The second set of donor embryos should be placed in the wells of rows 2 and 5 in the transplant plate.
3. The unlabeled host embryos should be placed in the wells of rows 3 and 6 in the transplant plate.
4. After extracting cells from donor embryo 1, insert the transplant needle into donor embryo 2 and extract more cells (**Fig. 6.2c**).
5. Insert the transplant needle into the host embryos and expel cells from both donor embryos. Monitor the expulsion of cells under bright-field or fluorescent microscopy.

6. After transplantation, incubate the pair of donor embryos with the host embryo in a coated 24-well plate at 28°C in 1× E2 medium containing penicillin/streptomycin.
7. The location of the descendants of the transplanted cells can be visualized at later stages under fluorescent dissecting microscope using the proper channels (**Fig. 6.2f, i**).

3.3. Fate Mapping

1. Thaw aliquot of 2 mg/mL Kaede protein and add 0.1 volume of phenol red stock (*see* **Notes 9** and **10**).
2. Inject 1 nL (roughly 2 ng protein) Kaede protein into chorionated, 1–4 cell stage embryos, as described above. Alternately, inject 100–500 pg in vitro-synthesized, capped mRNA encoding Kaede, diluted in a solution of 0.2 M KCl and phenol red.
3. Transfer embryos into a Petri dish containing 25 mL egg water. Cover with lid and wrap with aluminum foil. Incubate at 28°C until the desired stage.
4. Prior to photoconversion, examine embryos under a fluorescent dissecting microscope. Remove the physically damaged embryos and those that are only weakly fluorescent.
5. Add 3% methylcellulose to a depression slide. Transfer one embryo to the depression slide for photoconversion and cover with a coverslip.
6. Orient the embryo under a dissecting microscope by sliding around the coverslip until the region to be targeted by the laser is visible. This method of orienting the embryos in this manner can cause severe damage if they are dechorionated prior to the photoconversion step.
7. Alternately, embryos can be dechorionated embryos prior to this point (*see* **Note 7**) and mounted in 0.1% agarose in 1× E2 Medium. (To make, add 0.1 g agarose to 100 mL of 1× E2 and microwave. Wait until solution cools to below 30°C). Draw a circle of Vaseline on a slide and pour agarose into the center of circle. Quickly pipette the embryos in E2 medium into the agarose and orient them using a paintbrush. Place a coverslip over the slide.
8. Place the slide in the confocal microscope with coverslip facing the laser source (i.e., place the slide with the coverslip down if the microscope is inverted).
9. Image the embryo under bright field with a 10× objective. Once the embryo is in the field of view, scan it with the argon laser (488 nm) to excite the full-length Kaede protein. View emission in the green (GFP or fluorescein) channel (for alternate method of photoconversion, *see* **Note 11**).

10. Select region of interest and zoom in. Scan region of interest with the diode laser (405 nm) to photoconvert Kaede protein. Switch to live viewing mode for roughly 2 min (the exact duration of the exposure needs to be determined empirically for each injection).
11. After the photoconversion, switch to the HeNe laser (543 nm) to excite short form of Kaede. View emission in the red (rhodamine) channel. If the photoconversion is incomplete, scan the region again with the diode laser for 30 s to 1 min.
12. When photoconversion is complete, all cells in the region of interest will emit at 582 nm (red), and none will emit at 518 nm (green) (**Fig. 6.3a–f**).
13. For fate mapping, the position of the labeled cells with respect to the animal–vegetal and dorsoventral body axes must be recorded soon after photoconversion on a graphic representation of a blastoderm stage embryo (**Fig. 6.6**). The embryos should be photographed from a lateral view that encompasses both the yolk and the labeled cells. Both green and red isoforms should be visualized using the 488 and 543 nm excitation wavelengths, respectively. The position along the animal–vegetal axis is measured by the distance of the labeled cells from the embryo margin (juncture of the cells with the yolk) (**Fig. 6.3d**, **e**). This can be counted in absolute distance (μm) or in the number of cell diameters (or tiers of cells from the yolk). If the latter, it is helpful to take the images at a high enough magnification that the individual cells are visible. After the embryo is documented, assign it a number, remove it from the depression slide, and incubate it at 28°C in the dark in a Costar 24-well plate in egg water.
14. To document the position of the cells along the dorsoventral axis, the embryo must be permitted to develop until

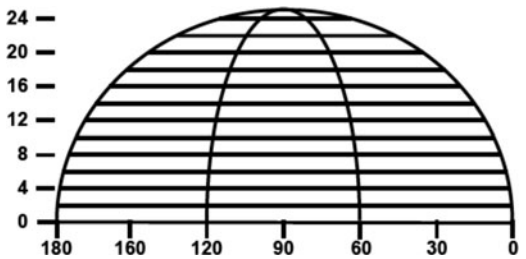

Fig. 6.6. Graphical representation of a blastula stage embryo for fate-mapping experiments. *x*-Axis represents position along the dorsoventral axis, with 0° representing the dorsalmost position. *y*-Axis represents position along the animal–vegetal axis, with 0° representing the vegetal-most tier of cells.

6 hpf when the embryonic shield forms (**Fig. 6.3f**). This is the first morphological manifestation of the dorsoventral axis in zebrafish (1). Photograph the embryo in bright-field and fluorescent channels under a dissecting microscope, from the animal pole. Focus on the embryonic shield in the bright-field optics and the labeled cells in the fluorescent optics. The distance from the embryonic shield is measured in degrees, from 0° to 180°, with the shield marking 0° (**Fig. 6.3f**). In some mutant contexts, the embryonic shield does not form. In these cases, the assignment of position along the dorsoventral axis must be delayed until the dorsoventral axis becomes apparent.

15. Once the positions of the labeled progenitor cells have been determined, return the embryo to its place in the 24-well dish and incubate in the dark for 1 or 2 days. Examine the embryos under bright-field and fluorescent optics to determine where are the daughters of the labeled progenitors. Detailed analysis should be performed under a compound microscope, which will provide greater resolution and easier identification of the morphology of the labeled cells.

3.4. One- or Two-Color, Whole-Mount In Situ Hybridization (See Note 12)

3.4.1. Synthesis of DIG-Labeled RNA Probe

1. Linearize 20 μg of plasmid template in 100 μL volume. Check the digest by running a 2% agarose gel. For antisense probe, cut plasmid at the 5′-end of the transcript; for sense (control) probe, cut plasmid at the 3′-end of the transcript.
2. Phenol/chloroform extract: Add 1/10 volume of 3 M sodium acetate and 2.5 volume of ice-cold 100% ethanol. Centrifuge at 14,000×*g* for 10 min. Wash the pellet once in ice-cold 70% ethanol. Air-dry the pellet and resuspend in 20 μL H_2O to a concentration of 1 μg/μL. Store at –20°C.
3. Mix 2 μL plasmid, linearized to transcribe antisense transcript (2 μg of template DNA), 2 μL of 10× transcription buffer, 2 μL of 10× NTP DIG-labeling mix, 1 μL of RNase inhibitor, 2 μL SP6 or T7 RNA polymerase, 11 μL RNase-free H_2O for a final reaction volume of 20 μL.
4. Incubate for 2 h at 37°C.
5. Add 2 μL DNase I and incubate for 15 min at 37°C to remove plasmid DNA.
6. Stop the reaction by adding 2 μL of 0.2 M EDTA.
7. Use Quick Spin columns (Roche) to purify the labeled RNA (*see* **Note 13**). Bring final volume to 100 μL in RNase-free water.
8. Run 5 μL on a 2% agarose gel to check quality and quantity of probe. As a standard, 5 μL from tube #5 is 500 ng on a gel (*see* **Note 14**).
9. Add 900 μL hybridization buffer and store at –20°C.

3.4.2. Synthesis of Fluorescein-Labeled RNA Probe

1. Mix 2 μL linearized plasmid (1 μg of template DNA), 2 μL of 10× fluorescein RNA-labeling mix, 2 μL of 10× transcription buffer, 1 μL of RNase inhibitor, 2 μL SP6 RNA polymerase, and 11 μL RNase-free H_2O for a final reaction volume of 20 μL.
2. Incubate for 2 h at 37°C.
3. Add 2 μL DNase I and incubate for 15 min at 37°C to remove plasmid DNA.
4. Stop the reaction by adding 2 μL 0.2 M EDTA.
5. Purify the probe with Quick Spin columns for RNA. RNA probe can be stored at –80°C. Bring final volume to 100 μL in RNase-free water.
6. Run 5 μL on a 2% agarose gel to check quality and quantity of probe. As a standard, 5 μL from tube #5 is 500 ng on a gel.
7. Add 900 μL hybridization buffer and store at –20°C.

3.4.3. Embryo Preparation

1. To fix embryos, transfer them to a microcentrifuge tube with a glass Pasteur pipette.
2. Remove all egg water and replace with 4% PFA for 3 h at RT or overnight at 4°C.
3. Dechorionate embryos manually with forceps while they are still in fix. Transfer embryos to a nine-cavity glass spot plate, and under a dissecting microscope, grab the chorion with two sharp Dumont forceps and pull. In fix, the embryos often pop out with a simple push. If the embryos are dehydrated, they are very delicate and should be handled with care during dechorionation.
4. After dechorionation, transfer embryos into a microcentrifuge tube. Remove fix and add 1 mL of 100% methanol. Store embryos at –20°C. Some protocols suggest dehydrating embryos gradually by washing in a series of solutions with increasing concentrations of methanol. We have not noticed a difference between gradual and rapid dehydration for WISH.
5. Preabsorb antibodies: Dilute anti-DIG Fab to 1:400 in 1 mL antibody-blocking solution. Add 50–100 embryos to 500 μL antibody solution. Incubate at room temperature for 3 h or overnight at 4°C. Bring final volume to 10 mL in antibody-blocking solution, for final working antibody dilution of 1:4,000. Anti-FITC Fab should be preabsorbed at a dilution of 1:100 and used at 1:2,000. Do not mix anti-DIG Fab with anti-FITC Fab if they are both conjugated to alkaline phosphatase. Store at 4°C until ready to use.

Diluted antibody can be reused multiple times, usually with improved results.

3.4.4. Two-Color In Situ Hybridization (Day 1)

1. Gradually rehydrate embryos (*see* **Note 15**). Wash embryos once with 500 μL of 75% methanol in PBST, 5 min at room temperature.
2. Wash embryos once with 500 μL of 50% methanol in PBST, 5 min at room temperature.
3. Wash embryos once with 500 μL of 25% methanol in PBST, 5 min at room temperature. At this step, make sure that each tube contains between 10 and 30 embryos. Label the top of each tube, with the stage of the embryos, and the probes to be used.
4. Wash embryos five times with 500 μL of 100% PBST, 5 min each, at room temperature.
5. Embryos older than the one-somite stage need to be treated with 10 μg/mL proteinase K in PBST, 5 min for 1-somite-to-24 hpf, 10 min for 24–36 hpf, and 15 min for 36–48 hpf. If embryos are younger than 1-somite stage, go directly to Step 8 (washes before pre-hybridization).
6. After proteinase K digestion, rapidly transfer embryos to 500 μL of 4% PFA. Fix for 20 min at room temperature.
7. Wash five times in 500 μL of 100% PBST, 5 min each wash.
8. Remove PBST and add 500 μL hybridization buffer. Incubate for 3 h to overnight at 70°C in a dry bath.
9. Make working dilution of probes. For one tube, add 15 μL DIG probe and 15 μL fluoresceinated probe to 200 μL hybridization buffer. Make cocktail if the probes are to be used on more than one tube of embryos.
10. After pre-hybridization, remove pre-hybridization solution and add the working dilution of probe. Gently tilt the tube to the side and back again to ensure thorough mixing. Carefully check to make sure no embryos remain stuck to the side of the tube.
11. Incubate overnight at 70°C in a dry bath.

3.4.5. Two-Color In Situ Hybridization (Day 2)

1. Preheat the hybridization buffer dilutions in 2× SSC and the 0.2× SSC dilution series in PBST to 70°C.
2. After incubation, remove the probe and store at –20°C. Many probes improve in quality with repeated usage, but the exact number of times a probe can be reused is variable.
3. Add 500 μL preheated 75% hybridization buffer in 2× SSC to each tube. After the hybridization step, embryos

are especially brittle. Therefore, extra care should be taken to wash gently. Incubate for 10 min at 70°C.

4. Remove the solution and add 500 μL preheated 50% hybridization buffer in 2× SSC to each tube. Incubate for 10 min at 70°C.
5. Remove the solution and add 500 μL preheated 25% hybridization buffer in 2× SSC to each tube. Incubate for 10 min at 70°C.
6. Wash twice in 500 μL preheated 100% 2× SSC to each tube at 70°C, 10 min each wash.
7. Wash twice in 500 μL preheated 100% 0.2× SSC to each tube at 70°C, 30 min each wash.
8. Wash in 500 μL of 75% 0.2× SSC in PBST, 10 min at room temperature.
9. Wash in 500 μL 50% 0.2× SSC in PBST, 10 min at room temperature.
10. Wash in 500 μL of 25% 0.2× SSC in PBST, 10 min at room temperature.
11. Wash in 500 μL of 100% PBST, 10 min at room temperature.
12. Incubate in 500 μL antibody-blocking solution for at least 2 h at room temperature.
13. Remove the blocking solution and add 500 μL anti-DIG Fab, conjugated to alkaline phosphatase (AP) (1:4,000 final dilution) in PBST, 2 mg/mL BSA, and 5% sheep serum in PBST.
14. Incubate overnight at 4°C.

3.4.6. First-Color Reaction

1. Remove and store anti-DIG Fab for reuse.
2. Wash embryos six times in 1 mL antibody-blocking solution.
3. Wash three times in 500 μL freshly made NTMT staining buffer, 5 min each wash.
4. Remove NTMT staining buffer and add 750 μL NBT/BCIP reaction solution. The reaction will generate a dark blue/purple precipitate.
5. Keep embryos in the dark during the reaction, removing them at intervals to check on the progress of the reaction.
6. When the reaction is complete, wash the embryos twice in 500 μL PBST (*see* **Note 16**). Skip washes and go to **Section 3.4.9** if embryos were hybridized only to one probe.

7. Wash the embryos with 1 mL of 100 mM glycine for 30 min to destroy the AP enzyme associated with the anti-DIG Fab.
8. To remove glycine, wash three times in PBST, 5 min each wash.
9. Incubate in antibody-blocking solution for at least 2 h.
10. Remove antibody-blocking solution and add 500 μL anti-FITC, conjugated to AP Fab (1:2,000 final) in PBST, 2 mg/mL BSA, and 5% sheep serum.
11. Incubate overnight at 4°C.

3.4.7. Second-Color Reaction

1. Remove antibody and store at 4°C for reuse.
2. Wash six times in 500 μL PBST, 15 min each wash.
3. Wash three times in 500 μL freshly made NTMT, 5 min each.
4. Remove NTMT and add 500 μL INT/BCIP reaction solution. The reaction will generate a brownish-red precipitate.
5. Keep embryos in the dark during the reaction, removing them at intervals to check on the progress of the reaction (*see* **Note 17**).
6. To stop the reaction, fix the embryos in 500 μL of 4% PFA for 20 min.
7. Store embryos processed for two-color in situ hybridization in PBST at 4°C. Store embryos processed for one-color in situ hybridization at –20°C in 100% methanol. The INT/BCIP precipitate is soluble in methanol and ethanol, so embryos with this precipitate should not be dehydrated. (For alternate protocol using an HRP-conjugated antibody to visualize the second transcript, *see* **Notes 18** and **19**; for tips on how to decrease background, *see* **Note 20**.)

3.4.8. Photography (Two-Color WISH)

1. Because the INT/BCIP precipitate is unstable, these embryos should be documented as soon as possible. Remove PBST and add 80% glycerol to embryos.
2. Let embryos settle to bottom of microcentrifuge tube.
3. Remove the embryos and place them in a well of Pyrex nine-cavity spot plate. Place a spot of glycerol on a slide.
4. Place a single embryo in the spot of glycerol on the slide.
5. The yolk is highly refractive in glycerol and must be removed. The embryo is very flexible in glycerol and highly tolerant of manipulation. Drag the embryo to the edge of the glycerol and poke yolk with sharp forceps. Continue poking yolk to remove all granules, monitoring progress

under a dissecting microscope. Occasionally move embryo to fresh area of the slide.

6. Once all the granules are removed, transfer embryo to a fresh slide with a drop (10–20 μL) of 80% glycerol. Orient embryo under a dissecting microscope, add coverslip, and photograph under a compound microscope using DIC optics. Raised coverslips are not necessary when mounting filleted embryos in glycerol.

3.4.9. Photography (One-Color WISH)

1. Embryos processed for one-color WISH using the NBT/BCIP substrate should be stored in 100% methanol at –20°C.
2. Remove methanol and add 1 mL benzyl benzoate:benzoic acid (2:1) (*see* **Note 21**).
3. Once embryos settle to the bottom of the microcentrifuge tube, transfer them to a well of Pyrex nine-cavity spot plate. The embryos will be transparent and visible only by their stain.
4. Choose an embryo to photograph and transfer to a slide with raised coverslips.
5. Remove excess benzyl benzoate:benzyl alcohol with a Kimwipe.
6. Fill the chamber between two raised coverslips with Canada balsam:methyl salicylate (11:1). Avoid air bubbles under the coverslip, which can cause the embryo to drift, making photography difficult.
7. Place a 22-mm × 22-mm square coverslip over the embryo, in a diamond orientation.
8. Orient embryo under the dissecting microscope moving the coverslip in order to roll the embryo. Dehydrated embryos are very brittle, and they break easily if rolled too vigorously.
9. Photograph under a compound microscope (*see* **Note 22**).

3.4.10. WISH Followed by Immunohistochemistry

1. Follow steps outlined above for one-color WISH, except mix the alkaline phosphatase-conjugated, anti-DIG antibody (1:4,000) with the primary antibody against the antigen of interest, at the appropriate dilution.
2. When alkaline phosphatase reaction is complete, remove NBT/BCIP reaction solution and add 4% PFA. Fix for 20 min at room temperature.
3. Wash two times in PBSTX (without NaN_3), 1 min each.
4. Wash six times in PBSTX (without NaN_3), 15 min each.
5. Incubate embryos in antibody-blocking solution (without NaN_3), 2 h at room temperature.

6. Dilute secondary antibody (goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP) 1:100 in antibody-blocking solution (without NaN_3).
7. Incubate embryos in goat anti-mouse (or rabbit) IgG–HRP conjugate for 4–6 h at room temperature or overnight at 4°C.
8. Remove and discard the secondary antibody.
9. Wash embryos four times in PBSTX (without NaN_3), 10 min each.
10. Rinse three times in 1× PBS, 5 min each.
11. Incubate embryos in 1.5 mL of DAB solution for 20–30 min.
12. Add 75 μL hydrogen peroxidase solution to the embryos in the DAB solution.
13. Transfer the embryos to a nine-spot cavity glass plate.
14. Monitor the reaction under a dissecting microscope, with illumination from above. The peroxidase reaction can last from 5 to 15 min.
15. As soon as the signal reaches maximum intensity, before background appears, stop the reaction by removing the DAB solution and adding PBST with NaN_3.
16. Wash three in PBST (with NaN_3), 10 min each.
17. Clear and photograph as described above.

3.5. Immunofluorescence

This protocol is optimized for visualization of the actin and microtubule cytoskeleton but works for other antigens as well. If you are not examining microtubules, then omit the paclitaxel from Gard's fixative. All solutions are always carefully added on the side of the tube (tube wall) to avoid damaging of embryos.

3.5.1. Embryo Preparation

1. Collect embryos in a 100-mm-diameter Petri dish with 25 mL egg water. Incubate at 28°C to the desired age.
2. Prior to fixation, transfer embryos to a 100-mm Petri dish with 2% agarose and sufficient egg water to cover submerged embryos.
3. Manually dechorionate the living embryos under a dissecting microscope (illumination from below) using Dumont forceps. Before gastrulation, embryos are extremely delicate. After gastrulation, live embryos get progressively easier to dechorionate. Place the tips of both pairs of forceps close to each other on the chorion surface and pinch. Slowly pull forceps away from each other, until a small hole appears in the chorion. Open the forceps and grab the edges of the chorion hole with both pairs of forceps. Grab the edges of

the hole in a new location and gently widen the hole, trying not to deform the embryo (although the embryos can tolerate slight deformation). If the yolk breaks, discard the embryo. When the hole is large enough, the embryo will fall out of the chorion. Collect the chorion debris and remove from the dish by wiping forceps on a Kimwipe.

4. Enzymatic dechorionation is a convenient alternative to manual dechorionation (*see* **Note 7**), but care must be taken to completely wash out all pronase.
5. Using a glass Pasteur pipette, transfer 15 embryos into a 3–5-mL glass vial containing 1 mL Gard's fixative (*see* **Note 23**). Live, dechorionated zebrafish embryos are very sensitive to surface tension, and pregastrula stage embryos burst when they come in contact with the air/liquid interface. To avoid exposing dechorionated embryos to the surface, fill the glass tube with 1 mL Gard's fixative. Next, hold the Pasteur pipette at a steep angle and aspirate the embryos from the coated Petri dish. Once the embryos are inside the pipette, tilt the pipette horizontal to prevent the embryos from touching the liquid surface and raise the pipette tip out of the egg water. While holding the pipette tip in the horizontal position, bring the embryos over the glass vial containing the fix. Insert the pipette tip under the surface of the fix and raise the pipette to a vertical position. Gravity will pull the embryos into the tube, so there is no need to expel any egg water into the fix. The main advantage of this technique is that it is very gentle and embryos are rarely damaged.
6. Alternately, transfer the embryos in egg water to the glass vial. Remove the egg water, leaving a minimal amount covering the embryos. Next, carefully add the Gard's fixative to the embryos, by tilting the tube sideways and adding drops of fixative to the side of the tube. The buffer is denser than egg water, so unless added very carefully, it can easily swoop the embryos up toward the surface and cause their breakage. This method is easier than the one described above but is rougher on the embryos. To ensure there is no yolk leakage, check the embryos inside the tube after fixation, by visualizing them under the dissecting scope. If the yolks are leaking, fix a new batch of embryos.
7. Incubate for 2–4 h at room temperature.

3.5.2. Antibody Incubation

1. Remove fix and wash five times in 500 μL PBST (for special notes for visualizing the cytoskeleton, *see* **Note 24**).
2. Wash the embryos once with 500 μL of 25% methanol in PBST, 5 min at room temperature.

3. Wash the embryos once with 500 μL of 50% methanol in PBST, 5 min at room temperature.
4. Wash the embryos once with 500 μL of 75% methanol in PBST, 5 min at room temperature.
5. Wash twice in 500 μL of 100% methanol. The embryos can be stored at –20°C indefinitely.
6. Gradually rehydrate embryos.
7. Wash the embryos once with 500 μL of 75% methanol in PBST, 5 min at room temperature.
8. Wash the embryos once with 500 μL of 50% methanol in PBST, 5 min at room temperature.
9. Wash the embryos once with 500 μL of 25% methanol in PBST, 5 min at room temperature.
10. Wash the embryos twice in 500 μL of 100% PBST, 5 min at room temperature (for antigens expressed in deep tissues, *see* **Note 25**).
11. Remove PBST and wash once in 500 μL of 3 mg/mL $NaBH_4$, 5 min.
12. Incubate in 500 μL of 3 mg/mL $NaBH_4$ (3 mg/mL) solution, 6 h at room temperature (*see* **Note 26**).
13. Wash six times in 500 μL PBST, 5 min each.
14. Wash once in 500 μL antibody-blocking solution, 5 min at room temperature.
15. Incubate for 1 h in antibody-blocking solution, at least 2 h.
16. Make appropriate dilution of primary antibody in antibody-blocking solution. For the monoclonal anti-α tubulin antibody 12G10 (Developmental Studies Hybridoma Bank, University of Iowa), use at a final dilution of 1:50.
17. Transfer the embryos to plastic microcentrifuge tubes.
18. Incubate the embryos at 4°C overnight. Position the tube so that it is lying on its side so that all embryos are evenly distributed in the solution.
19. Wash three times in 500 μL PBSTX, 10 min each.
20. Dilute secondary antibodies in 500 μL antibody-blocking solution.
21. Incubate for 2–4 h at room temperature in the dark (tubes can be wrapped in aluminum foil). Position the tube so that it is lying on its side so that all embryos are evenly distributed in the solution.
22. Wash five times in 500 μL PBSTX, 10 min each.
23. Wash five times in 500 μL PBS, 10 min each.

24. Mount one embryo at a time in DABCO on a slide with a raised coverslip and analyze under an epifluorescent compound microscope or a confocal microscope (*see* **Note 27**). For tips on reducing background fluorescence and specificity controls for your antibody, *see* **Notes 28, 29,** and **30**.

3.6. Embryo Lysate

1. Collect embryos at the appropriate stage and dechorionate them (*see* **Note 2**).
2. Transfer dechorionated embryos to 1.5-mL microcentrifuge tubes. In contact with plastic, dechorionated embryos slowly lose an exposed yolk membrane. Therefore, one must proceed immediately to the subsequent steps if plastic tubes are used. Otherwise, it is better to use glass vials.
3. Remove egg water and add 0.1 mL ice-cold deyolking buffer/100 embryos.
4. Break the yolks by poking them with a yellow tip on a 20–200-μL pipette.
5. Vortex for 1 min at 1,100 rpm.
6. Centrifuge at 300×*g* for 40 s.
7. Discard the supernatant, which contains the yolk granules.
8. *For Western*: Add 0.1 mL Western lysis buffer/100 embryos.
9. *For IP:* Add 0.1 mL of 1× cell lysis buffer/100 embryos.
10. Incubate on ice for 10 min.
11. Centrifuge at 13,000×*g* for 10 min at 4°C.
12. Collect the supernatant and determine the protein concentration by colorimetric assays such as the Bradford or bicinchoninic acid (BCA) assays. In the Bradford assay, binding of protein to a dye results in an increase in absorbance at 595 nm that is proportional to the amount of protein in solution within a concentration range of 0.1–1.4 mg/mL (43). The BCA assay relies on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline solution. Cu^{1+} is chelated by two molecules of bicinchoninic acid, resulting in a light blue complex (44). Unlike the BCA assay, the Bradford assay is tolerant of reducing agents and intolerant of detergent. The BCA assay is more sensitive than the Bradford assay and can be used to detect protein concentrations as low as 0.5 μg/mL, according to Sigma-Aldrich.
13. This supernatant can be used in co-immunoprecipitation experiments.
14. For Western blot, add 2× sample buffer and boil at 94°C for 4 min.

4. Notes

1. To make capped transcripts suitable for injecting into mRNA, the cDNA of interest should be inserted into a suitable vector, such as pCS2 and its derivatives (http://sitemaker.umich.edu/dlturner.vectors). These vectors contain convenient SP6, T3, and T7 promoters flanking the cDNA insertion site. Some versions of pCS2 in circulation have a mutated form of the T7 promoter that is inactive. This was corrected in the subsequent derivative, pCS107.
2. It is important to determine the dosage at which the MO specifically and effectively targets the correct transcript (for review, *see* (38)). Prepare a dilution series of the MO and the corresponding 5-bp mismatch MO and compare the effects of the two MOs in side-by-side injections. The optimal window for effective and specific effects is defined as the lowest dose at which the target MO shows a measurable activity, and the highest dose at which the 5-bp mismatched MO has no measurable activity. If the MO is targeted to a high copy transcript, and the 5-bp mismatch MO interacts with a low-copy, off-target transcript, it is possible that the mispaired oligonucleotide produces a phenotype below the effective concentration of the target MO.

   There are two other methods to determine MO specificity. The first alternative relies on comparing the activities of two non-overlapping oligonucleotides directed against the 5′-UTR of the transcript. The two MOs should have the same effect on embryos when injected individually and should be effective at lower doses when injected together than when injected separately. This approach can also be used with two different splice-blocking MOs, or with a combination of translation and a splice-blocking MOs. The second alternative is to rescue the morphant by mRNA injection. This method is not always possible, since some transcripts cause dominant phenotypes when overexpressed. The oligonucleotides and mRNA should be injected with two separate needles to prevent them from associating in the injection needle, which would neutralize the MO. Alternatively, mutations could be introduced into the region of the transcript targeted by the MO.

   Scrambled sequence MOs, or a collection of random 25-mers, can be used to control for chemical toxicity.
3. Embryonic development in zebrafish is temperature dependent. It takes 1.25 h for the embryos to reach the eight-cell stage at 28.5°C but about 2 h at room temperature

(25°C) (1). Thus, the investigator can extend the window for injection simply by keeping the embryos at room temperature until after the injection. Cooling the embryos to temperatures lower than 22°C is not recommended, as this may disrupt microtubules and cause developmental defects (16). The preparations for the injections should be done the previous day in order to maximize injection time.

4. Injections are performed under positive pressure in order to prevent backflow into the needle before or during the injection. This helps maintain a consistent size and concentration of the injection fluid, and prevents the needle from taking up cytoplasmic material that can cause clogs. Positive pressure injections require an apparatus with regulated hold and ejection pressures, such as WPI's Pneumatic PicoPump. When the machine is set for "timed," as opposed to "gated," injections, the size of the injection drop can be regulated by controlling the duration of the injection pulse.
5. If the needle gets clogged, try moving it out of the embryo medium and resubmerging it. Press the foot pedal to expel the fluid into the embryo medium. Second, press the foot pedal rapidly a few times to try to force out the clogging material. If this does not work, increase the duration of the pulse and repeat. If all of these tricks fail, replace clogged needle with a fresh one.
6. Homemade rigs are cheap and easy to make, and consist of a microinjector that holds a glass syringe for injection, plastic tubing and needle holder, and a second syringe that serves as a reservoir for mineral oil (**Fig. 6.5a**, **b**). A trivalve connector links the injection syringe to the oil reservoir and the needle holder. To fill the rig, open the valves connecting the oil reservoir and the injection needle and push the plunger until drops of oil appear at the end of the injection needle. It is very important to flush out all air bubbles from the tubing. The injection syringe should also be filled with oil.
7. Zebrafish embryos are surrounded by a clear protective shell called a chorion that must be removed prior to transplantation. Although it can be removed manually with a pair of sharp forceps, it is faster to digest them enzymatically:
    1. Transfer the embryos into a 10-mm glass dish and remove as much egg water as possible. Add 2 mg/mL pronase solution in egg water and monitor embryos under microscope. Monitor chorion strength by pinching them with forceps. A strong chorion will retain its

shape after a gentle pinch as long as it is not punctured, whereas a weak chorion remains deformed.

2. When the chorions are weak, transfer the embryos into a 1-L beaker filled with 500 mL egg water. Let embryos settle to bottom of the beaker and swirl. Tilt the beaker so that embryos settle together and pour out the majority of the egg water. Add 500 mL fresh egg water and repeat the process at least five times, until all embryos are freed from chorions.

8. Cell transplants and lineage-tracing experiments require the use of molecules that visually mark specific cells and their progeny. The ideal molecule to use for these experiments should be easy to visualize without perturbing embryonic development. It should be non-toxic and have no discernable effect on cellular behavior, cell fate choice, or gene expression. Finally, the molecule should not be membrane permeable so that it is retained in the cell in which it is injected and is passed to its descendants during mitosis. Genetic markers, such as a transgene-expressing eGFP or β-galactosidase, fit all these criteria and are ideal for cell transplants and some lineage-tracing experiments. Since expression of transgenes may depend upon cell identity, not all transgenic lines are suitable for use in experiments addressing questions of cell fate. Other lineage-tracing molecules, such as fluorophore-conjugated dextran, are more versatile. These markers act independently of cell fate but must be injected into the cell to be studied.

9. As an alternative to injecting Kaede protein, it is also possible to inject capped, full-length mRNA into embryos (250–500 pg). For this, the Kaede cDNA should be subcloned into a vector derived from pCS2. The template should be transcribed as described in **Section 3.4.1**. There are two main disadvantages to this approach. First, there is a significant lag between the time of mRNA injection and the appearance of fluorescence. This is because the protein needs to be synthesized and form active tetramers. Thus, it is not possible to photoconvert cells during the mid-blastula stages even when the maximum amount of mRNA is injected (40). Second, since new Kaede protein continues to be synthesized after photoconversion, the labeled cells will contain both green-fluorescent Kaede and red-fluorescent Kaede proteins. In photographic overlays, the cells will appear yellow or yellow-green depending on the relative amounts of the two isoforms.

10. Until recently, the most commonly used photoactivatable lineage-tracing molecule in zebrafish was DMNB-caged

fluorescein dextran (45, 46). This compound has the advantage that anti-FITC antibodies recognize uncaged but not caged fluorescein, permitting the visualization of labeled cells in fixed tissue (46). Thus, it is possible to confirm cell identity assignments made in living embryos by examining the genes expressed in the transplanted cells, using WISH followed by immunohistochemistry. Unfortunately, this compound has been discontinued by Molecular Probes (Invitrogen, Inc). Since no antibodies have yet been reported that can distinguish the long and short forms of Kaede protein by immunofluorescence, it is not yet possible to confirm the fates of the Kaede-labeled cells in whole mount.

11. Photoconversion can also be performed with a compound microscope equipped with epifluorescence and a Micropoint laser (Photonic Instruments). The cells to be labeled are targeted by focusing on them under bright-field optics. The laser pulse is activated by a foot pedal. The length of the pulse and the strength of the beam should be calibrated before the experiment so that the laser does not kill the targeted cells. This method provides the same high-level resolution as the confocal, but without having to use a common facility. The main disadvantage is the cost of the Micropoint laser system.
12. Because WISH is a complicated procedure that extends over many days, it is useful to generate and print out a checklist each time the experiment is done.
13. Phenol/chloroform should never be used to purify digoxigenin-labeled probes, since the digoxigenin moiety segregates to the non-aqueous phase. Since nucleic acids segregate to the aqueous phase, the probe lies at the interface.
14. Probe quantification by gel electrophoresis or by OD is unreliable. Therefore, each newly synthesized probe should be tested by titration on embryos.
15. All washes should be performed gently in order to avoid damaging embryos. Use suction from the vacuum apparatus to remove liquid from each tube and slowly add fresh liquid to the side of the microcentrifuge tube.
16. The alkaline phosphatase reaction can continue as long as no background appears. To a large extent, this depends upon the expression level of the targeted transcript, the amount of probe in the hybridization buffer, and the temperature of the hybridization. For the NBT/BCIP reaction, the background is recognizable as a purple-bluish tinge to the parts of the embryo that do not stain with

the probe. This is different from the pinkish tinge embryos often take on when they are submerged in the NBT/BCIP reaction buffer.

If the probe is to a particularly weakly expressed transcript, the alkaline phosphatase reaction could take several hours. In some cases, it may be necessary to temporarily stop the reaction and continue the reaction the following day. Stop the reaction by washing five times in 500 μL PBST and store overnight at 4°C. The next day, wash three times in 500 μL NTMT and add 750 μL NBT/BCIP staining solution. If yellow crystals form during this process, they can be removed by rinsing three times in 100% methanol. Although this is not problematic for one-color WISH performed with NBT/BCIP, the crystals and subsequent methanol treatment will inhibit the second-color reaction in two-color WISH.

17. For the INT/BCIP reaction, the background is reddish brown. Since the background is typically higher for the second reaction than the first, the order of the reactions is important. Since the NBT/BCIP reaction produces a stronger signal, this reaction should be used to visualize the weaker probe and should be carried out first. If it is not clear which of the two probes is weakest from previous single-color WISH experiments, then it is advisable to determine the order empirically. The INT/BCIP precipitate is soluble in methanol and ethanol, so embryos with this precipitate should not be dehydrated.

18. NaN_3 is a preservative that increases the shelf life of PBST, PBSTX, and the antibody-blocking solutions. It is also a poison for the horseradish peroxidase (HRP) enzyme. Therefore, the embryos should be thoroughly washed in solutions lacking NaN_3 prior to addition of secondary antibodies containing HRP. For immunohistochemistry with a secondary antibody conjugated to horseradish peroxidase, it is important to remove NaN_3 from all the solutions.

19. Instead of using two alkaline phosphatase-conjugated antibodies to visualize different probes, it is possible to substitute an HRP-conjugated, anti-FITC antibody (Thermo Scientific). In this case, follow the protocol for two-color WISH until after the NBT/BCIP color reaction (**Section 3.4.6**, Step 6). Next, wash embryos four times in 500 μL PBST (without NaN_3), 10 min each wash, and three times in 500 μL of 1× PBS, 5 min each wash. Incubate the embryos in 1.5 mL of DAB solution for 20–30 min. Initiate the reaction by adding 75 μL hydrogen peroxidase solution and monitor the reaction under a dissecting microscope. Stop the reaction by transferring

the embryos into a tube with PBST (with NaN_3 to stop the HRP reaction). The embryos can be stored in methanol at –20°C. The HRP reaction can be intensified by adding 8 μL of 8% $NiCl_2$ and 8 μL of 8% $CoCl_2$ to the DAB solution just before the addition of H_2O_2.

The substrates for alkaline phosphatase are non-toxic and can be disposed of accordingly. On the other hand, DAB is a possible carcinogen, and protective clothing and gloves should be worn when handling this reagent. Solid waste, including pipette tips and plastic tubes, should be disposed of in a glass beaker, which serves as a temporary hazardous waste container. After the reaction, neutralize the used (and extra) DAB staining solution by adding an equal volume of DAB neutralization solution (3% $KMnO_4$, 2% Na_2CO_3 in water). Rinse all solid wastes in DAB neutralization solution before disposal. Neutralized solutions and solid wastes are non-toxic and can be disposed of accordingly.

20. High background can be caused by problems in any of several steps. First, a low signal-to-noise ratio could result from problems with the probe, such as non-specific hybridization or a low concentration. In our experience, most weak probes can be improved simply by increasing the amount of probe in the hybridization buffer. Alternatively, non-specific binding can be decreased by raising the formamide concentration in the hybridization buffer to 60% (47). Subtle variations in the temperature of the dry bath can affect your results. In addition, the optimal temperature may vary for different probes, depending on the sequence, the length, or the composition. DNA:RNA hybrids are less stable than RNA:RNA hybrids, so if a DNA probe is used, the hybridization temperature should be reduced. Non-specific binding increases at lower temperatures, however, which can increase background stain. Many protocols include 1 mM levamisole (Sigma) freshly added to the NBT/BCIP or INT/BCIP reaction solution in order to block endogenous phosphatase activity. We routinely omit this reagent to no ill effect, but it may decrease the background in some cases.

    Increasing the number or the duration of the washes could also decrease the background. The post-hybridization washes are particularly important in reducing background. Although the protocol only calls for 30-min washes at this point, these washes can be extended to 1 h each, or even more. To reduce background, it is more efficient to increase the number of washes, than simply to rely on increasing their duration. Finally, the embryos in NBT/BCIP reaction buffer should be kept in the dark as

much as possible, and the buffer should be replaced immediately if it turns purple during the reaction. The alkaline phosphatase reaction continues for some time after the substrate is diluted. Therefore, background is decreased by stopping the enzymatic activity immediately by placing the embryos in 4% PFA.

21. Embryos should not be stored for long periods of time in benzyl benzoate:benzyl alcohol, as the precipitate is soluble in this medium. After photography in Canada balsam:methyl salicylate, embryos can washed twice in 500 μL benzyl benzoate:benzyl alcohol and twice in 1 mL of 100% methanol for long-term storage.
22. During WISH, the substrate penetrates the egg yolk and remains. These substrates react under conditions of high light to turn the yolk red. To minimize this problem, the embryos should be oriented in the compound microscope and the focal plane adjusted under low light conditions. Expose the embryo to bright light just prior to recording the image and immediately decrease light levels again. Alternatively, the substrate can be washed out of the yolk by successive washes in 100% methanol or by prolonged storage in 100% methanol. This method cannot be used for two-color WISH, in which INT/BCIP was used in one of the reactions, due to the solubility of the INT/BCIP substrate in methanol.
23. Glutaraldehyde is a strong fixative, and overfixation can destroy epitopes and lead to difficulty for the antibody to penetrate the embryo. 4% Paraformaldehyde in PBS can be used as an alternative fixative.
24. Methanol disrupts the phalloidin-binding sites on actin, so fixatives containing methanol should be avoided for visualization of actin filaments. To preserve microtubules, the embryos must be dechorionated prior to fixation. This also improves results for the actin cytoskeleton. Most other antigens, however, tolerate fixation in the chorion. For visualizing the actin cytoskeleton, dilute rhodamine:phalloidin 1:40 in antibody-blocking solution. Instead of incubating embryos with the primary antibody in **Section 3.5.2**, Step 16, incubate the embryos with rhodamine–phalloidin for 2 h at room temperature in the dark. Continue protocol in **Section 3.5.2**, Step 17.
25. For antigens that are expressed in deep tissues, it may be necessary to employ alternate methods to permeabilize the embryo to permit greater penetration of the antibodies. For obvious reasons, proteinase K should not be used when analyzing the protein distribution in whole embryos.

Instead, permeabilize the embryos by washing them in acetone at –20°C for 7 min after rehydration (**Section 3.5.2**, Step 10). This will not disrupt actin filaments, but we have not tested it for microtubules.

26. Sodium borohydride ($NaBH_4$) reduces free aldehyde groups to alcohol. This decreases the autofluorescence associated with unreacted glutaraldehyde in fixed tissue.
27. Stained embryos can be stored at 4°C in PBST (including NaN_3) but should be shielded from light. Embryos processed for immunofluorescence should be mounted in an aqueous solution. The chemicals used for clearing and mounting dehydrated embryos increase the autofluorescence of the embryo. The mounting medium should contain DABCO, which is a water-soluble compound that extends the life of most fluorophores. DAPI or TOPRO-3 may be added to the DABCO mounting medium to visualize DNA. The embryos should be oriented under a dissecting microscope prior to visualization in the confocal. Alternately, the embryos can be mounted in 70% glycerol, after washing in a series of 30% glycerol:70% PBS; 50% glycerol:50% PBS; and 70% glycerol:30% PBS. If a flat mount is desired, dissect the yolk with a pair of forceps as described in **Section 3.4.8**, steps 5–6 of the WISH protocol.
28. Antibodies that work in immunoprecipitation or in Western blots do not necessarily work in immunofluorescence. Therefore, it is important to test antibodies that have not been previously characterized on whole-mount embryos. First, a dilution series should be performed to determine the optimal concentration of the primary immune serum. In parallel, perform a dilution series using pre-immune serum, if available, as a control for non-specific binding of other antigens in the serum. To control for non-specific binding of the secondary antibody, treat embryos with the secondary antibody alone.
29. Several steps can be taken to reduce high background levels. First, the primary immune serum should be cleaned up by affinity purification, if the antigen is known. The primary and secondary antibodies should be preabsorbed at a high concentration in 20–50 embryos (2 h at room temperature). The blocking serum in the antibody-blocking solution should be from the species in which the secondary antibody was generated. For example, when using a secondary antibody made in donkey, it is advisable to block embryos with normal donkey serum instead of normal sheep serum.
30. When visualized with epifluorescence, fluorescence from tissue layers above and below the focal plane contaminates

the image and decreases the resolution. Therefore, it is recommended to visualize the cytoskeleton (or any widely expressed antigen) using a confocal microscope. Typically, the entire zebrafish embryo can be visualized only under low (10×) magnification objectives, under which the fluorescent signal from a confocal optical section is weak. To boost the signal, one should make a Z-stack series of the entire embryo.

Acknowledgments

We thank Ben Feldman for providing Kaede protein, Joeseph Frankel for providing the 12G10 antibody (available from the Developmental Studies Hybridoma Bank, developed under the auspices of NICHD and maintained by the University of Iowa), Jim Lauderdale for advice on the protocol for WISH followed by immunohistochemistry, and Rebecca Ball for helpful comments on the manuscript. S.T.D. is a Georgia Cancer Coalition distinguished investigator. Work in the Dougan lab is supported by the American Cancer Society (RSGDDC-112979).

References

1. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995) Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310.
2. Zhang, Y., McCulloch, K., and Malicki, J. (2009) Lens transplantation in zebrafish and its application in the analysis of eye mutants. *J. Vis. Exp.* **28**, pii:.1258.
3. Mizuno, T., Shinya, M., and Takeda, H. (1999) Cell and tissue transplantation in zebrafish embryos. *Methods Mol. Biol.* **127**, 15–28.
4. David, N. B. and Rosa, F. M. (2001) Cell autonomous commitment to an endodermal fate and behaviour by activation of Nodal signalling. *Development* **128**, 3937–3947.
5. Dovey, M. C. and Zon, L. I. (2009) Defining cancer stem cells by xenotransplantation in zebrafish. *Methods Mol. Biol.* **568**, 1–5.
6. Hagos, E. G. and Dougan, S. T. (2007) Time-dependent patterning of the mesoderm and endoderm by Nodal signals in zebrafish. *BMC Dev. Biol.* **7**, 22.
7. Hagos, E. G., Fan, X., and Dougan, S. T. (2007) The role of maternal Activin-like signals in zebrafish embryos. *Dev. Biol.* **309**, 245–258.
8. Thisse, B., Heyer, V., Lux, A., Alunni, V., Degrave, A., Seiliez, I., Kirchner, J., Parkhill, J. P., and Thisse, C. (2004) Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. *Methods Cell. Biol.* **77**, 505–519.
9. Ekker, S. C. (2000) Morphants: a new systematic vertebrate functional genomics approach. *Yeast* **17**, 302–306.
10. Nasevicius, A. and Ekker, S. C. (2000) Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216–220.
11. Fjose, A., Ellingsen, S., Wargelius, A., and Seo, H. C. (2001) RNA interference: mechanisms and applications. *Biotechnol. Annu. Rev.* **7**, 31–57.
12. Schier, A. F. and Giraldez, A. J. (2006) MicroRNA function and mechanism: insights from zebra fish. *Cold Spring Harb. Symp. Quant. Biol.* **71**, 195–203.
13. Liu, W. Y., Wang, Y., Sun, Y. H., Wang, Y. P., Chen, S. P., and Zhu, Z. Y. (2005) Efficient RNA interference in zebrafish embryos using siRNA synthesized with SP6 RNA polymerase. *Dev. Growth Differ.* **47**, 323–331.

14. Zhao, Z., Cao, Y., Li, M., and Meng, A. (2001) Double-stranded RNA injection produces nonspecific defects in zebrafish. *Dev. Biol.* **229**, 215–223.
15. Pelegri, F. (2003) Maternal factors in zebrafish development. *Dev. Dyn.* **228**, 535–554.
16. Jesuthasan, S. and Stahle, U. (1997) Dynamic microtubules and specification of the zebrafish embryonic axis. *Curr. Biol.* **7**, 31–42.
17. Solnica-Krezel, L. and Driever, W. (1994) Microtubule arrays of the zebrafish yolk cell: organization and function during epiboly. *Development* **120**, 2443–2455.
18. Fan, X., Hagos, E. G., Xu, B., Sias, C., Kawakami, K., Burdine, R. D., and Dougan, S. T. (2007) Nodal signals mediate interactions between the extra-embryonic and embryonic tissues in zebrafish. *Dev. Biol.* **310**, 363–378.
19. Ho, C. Y., Houart, C., Wilson, S. W., and Stainier, D. Y. (1999) A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the hex gene. *Curr. Biol.* **9**, 1131–1134.
20. Erter, C. E., Solnica-Krezel, L., and Wright, C. V. (1998) Zebrafish nodal-related 2 encodes an early mesendodermal inducer signaling from the extraembryonic yolk syncytial layer. *Dev. Biol.* **204**, 361–372.
21. Feldman, B., Gates, M. A., Egan, E. S., Dougan, S. T., Rennebeck, G., Sirotkin, H. I., Schier, A. F., and Talbot, W. S. (1998) Zebrafish organizer development and germ-layer formation require Nodal- related signals. *Nature* **395**, 181–185.
22. Little, S. C. and Mullins, M. C. (2009) Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis. *Nat. Cell Biol.* **11**, 637–643.
23. Ikegami, R., Hunter, P., and Yager, T. D. (1999) Developmental activation of the capability to undergo checkpoint-induced apoptosis in the early zebrafish embryo. *Dev. Biol.* **209**, 409–433.
24. Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E., and Schier, A. F. (2002) Production of maternal-zygotic mutant zebrafish by germ-line replacement. *Proc. Natl. Acad. Sci. USA* **99**, 14919–14924.
25. Ho, R. K. and Kimmel, C. B. (1993) Commitment of cell fate in the early zebrafish embryo. *Science* **261**, 109–111.
26. Kimmel, C. B., Warga, R. M., and Schilling, T. F. (1990) Origin and organization of the zebrafish fate map. *Development* **108**, 581–594.
27. Woo, K., Shih, J., and Fraser, S. E. (1995) Fate maps of the zebrafish embryo. *Curr. Opin. Genet. Dev.* **5**, 439–443.
28. Warga, R. M. and Nusslein-Volhard, C. (1999) Origin and development of the zebrafish endoderm. *Development* **126**, 827–838.
29. Kimmel, C. B. and Law, R. D. (1985) Cell lineage of zebrafish blastomeres. III. Clonal analyses of the blastula and gastrula stages. *Dev. Biol.* **108**, 94–101.
30. Kimmel, C. B. and Warga, R. M. (1988) Cell lineage and developmental potential of cells in the zebrafish embryo. *Trends Genet.* **4**, 68–74.
31. Dale, L. and Slack, J. M. (1987) Fate map for the 32-cell stage of *Xenopus laevis*. *Development* **99**, 527–551.
32. Fraser, S. E. (1996) Iontophoretic dye labeling of embryonic cells. *Methods Cell Biol.* **51**, 147–160.
33. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. (2002) An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 12651–12656.
34. Hatta, K., Tsujii, H., and Omura, T. (2006) Cell tracking using a photoconvertible fluorescent protein. *Nat. Protoc.* **1**, 960–967.
35. Thazhath, R., Liu, C., and Gaertig, J. (2002) Polyglycylation domain of beta-tubulin maintains axonemal architecture and affects cytokinesis in Tetrahymena. *Nat. Cell Biol.* **4**, 256–259.
36. Webster, D. M., Teo, C. F., Sun, Y., Wloga, D., Gay, S., Klonowski, K. D., Wells, L., and Dougan, S. T. (2009) O-GlcNAc modifications regulate cell survival and epiboly during zebrafish development. *BMC Dev. Biol.* **9**, 28.
37. Zalik, S. E., Lewandowski, E., Kam, Z., and Geiger, B. (1999) Cell adhesion and the actin cytoskeleton of the enveloping layer in the zebrafish embryo during epiboly. *Biochem. Cell Biol.* **77**, 527–542.
38. Eisen, J. S. and Smith, J. C. (2008) Controlling morpholino experiments: don't stop making antisense. *Development* **135**, 1735–1743.
39. Link, V., Shevchenko, A., and Heisenberg, C. P. (2006) Proteomics of early zebrafish embryos. *BMC Dev. Biol.* **6**, 1.
40. Brown, J. L., Snir, M., Noushmehr, H., Kirby, M., Hong, S. K., Elkahloun, A. G., and Feldman, B. (2008) Transcriptional profiling of endogenous germ layer precursor cells identifies dusp4 as an essential gene in

zebrafish endoderm specification. *Proc. Natl. Acad. Sci. USA* **105**, 12337–12342.
41. Schroeder, M. M. and Gard, D. L. (1992) Organization and regulation of cortical microtubules during the first cell cycle of *Xenopus* eggs. *Development* **114**, 699–709.
42. Gelsema, W. J., de Ligny, C. L., Luten, J. B., and Vossenberg, F. G. (1975) The use of the Cerenkov effect in the counting of beta- and gamma- emitting radionuclides. *Int. J. Appl. Radiat. Isot.* **26**, 443–450.
43. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254.
44. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85.
45. Kozlowski, D. J., Murakami, T., Ho, R. K., and Weinberg, E. S. (1997) Regional cell movement and tissue patterning in the zebrafish embryo revealed by fate mapping with caged fluorescein. *Biochem. Cell Biol.* **75**, 551–562.
46. Feldman, B., Dougan, S. T., Schier, A. F., and Talbot, W. S. (2000) Nodal-related signals establish mesendodermal fate and trunk neural identity in zebrafish. *Curr. Biol.* **10**, 531–534.
47. Thisse, C. and Thisse, B. (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protoc.* **3**, 59–69.
48. Oxtoby, E. and Jowett, T. (1993) Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. *Nucleic Acids Res.* **21**, 1087–1095.
49. Talbot, W. S., Trevarrow, B., Halpern, M. E., Melby, A. E., Farr, G., Postlethwait, J. H., Jowett, T., Kimmel, C. B., and Kimelman, D. (1995) A homeobox gene essential for zebrafish notochord development. *Nature* **378**, 150–157.
50. Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. G., Grunwald, D. J., and Riggleman, B. (1996) Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. *Development* **122**, 271–280.

Chapter 7

Practical Approaches for Implementing Forward Genetic Strategies in Zebrafish

Sreelaja Nair and Francisco J. Pelegri

Abstract

The tropical fresh water minnow, *Danio rerio*, more commonly known as zebrafish, has emerged rapidly over the last decade as a powerful tool for developmental geneticists. External fertilization, high fecundity, a short generation time, and optical transparency of embryos during early development combined with the amenability to a variety of genetic manipulations constitute in the zebrafish the convergence of several unique advantages for a vertebrate model system. Traditional forward genetic screens, which employ the use of a chemical mutagen such as *N*-ethyl-*N*-nitrosourea to induce mutations in the male genome, have also proven to be highly successful in the zebrafish. This chapter provides experimental approaches to successfully induce pre-meiotic mutations in the male zebrafish germline and genetic strategies to recover and maintain such mutations in subsequent generations (**Section 3.1**). Though discussed specifically in the context of zebrafish research in this chapter, many of these genetic approaches may also be broadly applicable in other model systems. We also discuss experimental techniques to manipulate the ploidy of zebrafish embryos, which when used in combination with the standard mutagenesis protocol significantly expedite the identification of the induced mutations (**Section 3.2**). Additional stand-alone procedures are provided in **Section 3.3**, which are also required for the execution of the experiments discussed in its preceding sections.

Key words: ENU, zebrafish, mutagenesis, ploidy, haploid, gynogenesis, genetic screen, in vitro fertilization.

1. Introduction

1.1. Induction and Recovery of Recessive Mutations in Zebrafish

N-ethyl-*N*-nitrosourea (ENU) is a very potent mutagen, which functions by alkylating a single strand of DNA resulting in point mutations in the genome (1–4). In zebrafish, ENU has been successfully used to directly mutagenize mature spermatozoa in vitro (2, 5). However, because of the absence of DNA repair

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_7,

mechanisms in mature spermatozoa, the derived progeny are frequently genetically mosaic, making it difficult to carry out screens for lethal mutations in any gene or process (2, 5, 6). This can be overcome by mutagenizing adult zebrafish males by soaking them in a solution of ENU at sub-lethal doses repeatedly over time to induce point mutations in the spermatogonia (1, 2). The DNA replication rounds prior to differentiation fix a given mutation in the mature spermatozoa, which eliminates genetic mosaicism in the founder progeny. A standard inbreeding strategy can then be employed to isolate the mutations by screening for the molecular or physical phenotypes of interest (1, 2, 7, 8).

1.2. Expedited Identification of Phenotypes of Interest Through Ploidy Manipulation

As described in **Section 3.1.4** and **Fig. 7.1**, in a conventional screen using diploids, the identification and recovery of a mutation of interest is a labor-intensive, time-consuming, and expensive process. Using such a strategy, the earliest a recessive zygotic or parental-specific mutant can be identified is by testing the 3rd and 4th generations after mutagenesis, respectively, which would be about a year from the date of the start of ENU mutagenesis. This is sometimes prohibitive in terms of the infrastructure required for carrying out saturation genetic screens wherein the goal is to isolate at least one mutant allele for every single gene. For a successful saturation screen in zebrafish, beginning in the F2 generation, multiple crosses need to be raised to uncover homozygosity for mutations in all the 25 chromosomes, which significantly increases the number of required holding tanks. Thus, a reduction in the number of generations has the collateral benefit of decreasing the amount of labor and infrastructure required for the mutagenesis screen. This can be achieved by screening either F2 gynogenetic haploids (**Section 3.2.1**, **Fig. 7.2**, (9)) or gynogenetic diploids (**Sections 3.2.2**, **3.2.3**, and **3.2.4**, **Fig. 7.3**, (10, 11)), which are obtained by manipulating the ploidy of zebrafish embryos.

1.3. Mechanisms of Ploidy Manipulation in Vertebrates

There are several instances of successful experimental manipulation of ploidy in vertebrates. In mice, gynogenetic haploids are produced by exposing mature, unfertilized eggs to ethanol or by microsurgical removal of the male pronucleus (12, 13). In other vertebrates such as zebrafish (14), frogs (15), and Rainbow trout (16), gynogenetic haploids have been generated by in vitro fertilization of eggs with UV-irradiated spermatozoa, which destroys the sperm nucleus.

The process of producing diploid gynogenotes or androgenotes in mammals involves surgical removal of the male or female pronucleus after fertilization and subsequent treatment of the zygote with anti-mitotic chemicals (17). In non-mammalian vertebrates such as the zebrafish, diploid gynogenotes are obtained by first in vitro fertilizing eggs using

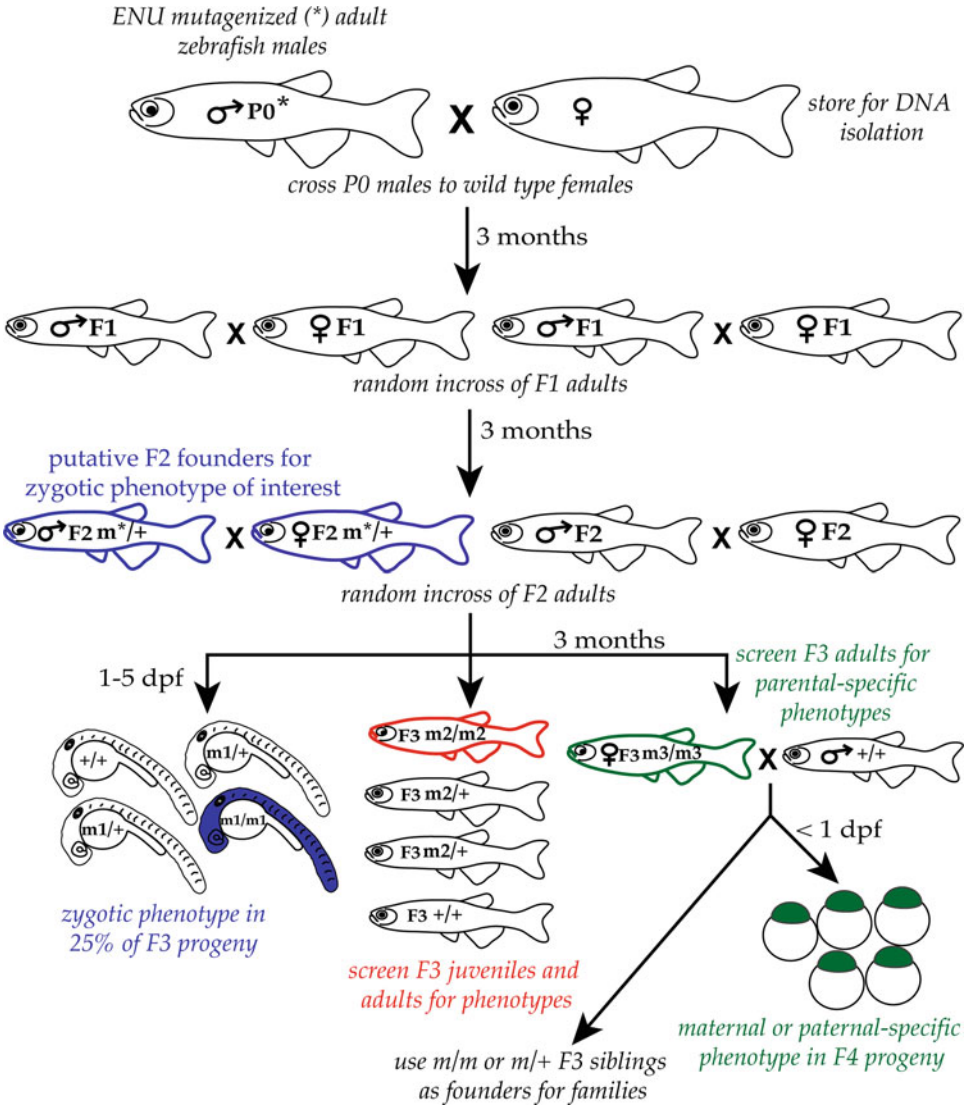

Fig. 7.1. Schematized strategy for a standard genetic screen. ENU-mutagenized P0 males are used to generate F1 progeny, which are randomly incrossed to generate F2 heterozygotes. Random incrossing of F2 heterozygotes for any given mutation (m*) results in identification of the phenotype in a quarter of the F3 progeny. An example of a zygotic phenotype in the F3 progeny during larval stages is indicated by the notation m1. A juvenile or adult phenotype in the F3 generation is indicated by the notation m2. A subset of the adult phenotypes can be parental-specific ones (m3). In this schematic, a maternal-effect gene (m3) is isolated by crossing a homozygous F3 adult female with any wild-type male. Such a maternal-effect phenotype should manifest in most if not all (100%) of the F4 progeny. Zygotic embryonic and juvenile mutations can be propagated from the F2 heterozygotes, whereas parental-specific mutations can be propagated from the F3 siblings.

spermatozoa in which the male genome has been destroyed by UV irradiation. Subsequently, shortly after fertilization, depending upon the method employed, cytokinesis associated either with the completion of meiosis II or the first mitotic division is inhibited. This effectively restores zygotic ploidy to the normal

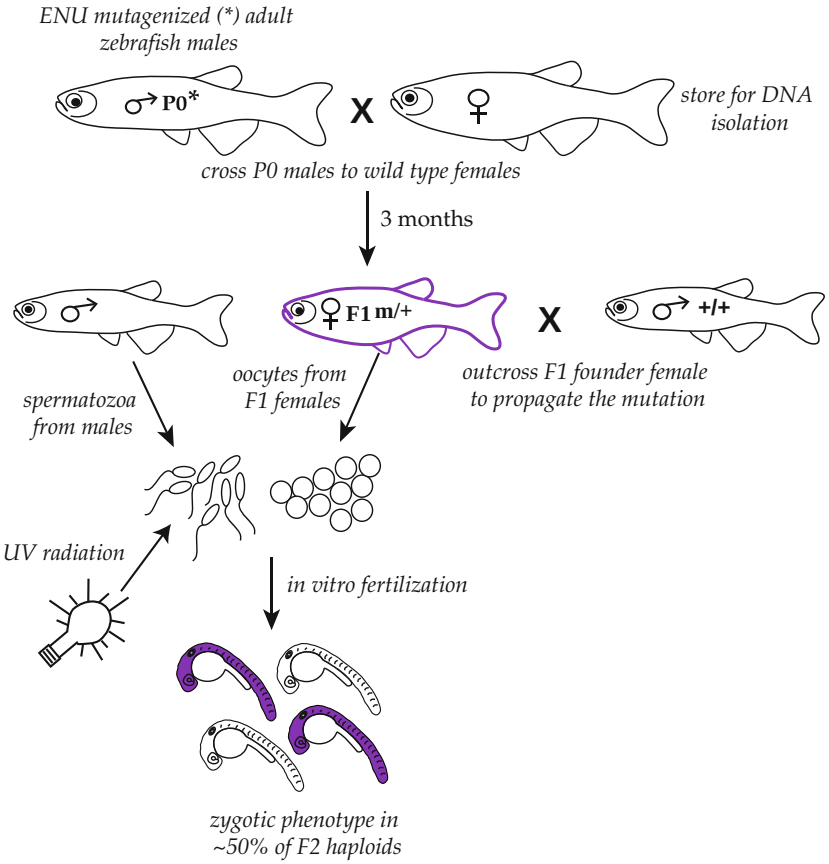

Fig. 7.2. Schematized strategy for a haploid-based genetic screen. ENU-mutagenized P0 males are used to generate F1 progeny that are heterozygous for several new mutations. Eggs are manually stripped from the F1 females and in vitro fertilized with UV-irradiated spermatozoa. The destruction of the male genome by irradiation results in the F2 progeny becoming gynogenetic haploids. Zygotic phenotypes can be expected in 50% of such F2 progeny since the source of eggs was a heterozygous female. Identified mutations can be recovered by crossing the corresponding F1 heterozygous female to a male and by incrossing siblings from the F2 generation.

diploid state, though its genome is entirely maternal in origin. In non-mammalian vertebrates where this has been achieved, including the zebrafish, the preferred methods to inhibit these early cytokinesis events are early pressure or late heat shock. Similar methods have been employed to produce gynogenetically diploid Mexican axolotls (18), rainbow trouts (16), *Xenopus laevis* (19), *Xenopus tropicalis* ((20), **Chapter 4**, this volume), and zebrafish (14), which highlight the commonality in the principle behind the techniques across diverse vertebrates.

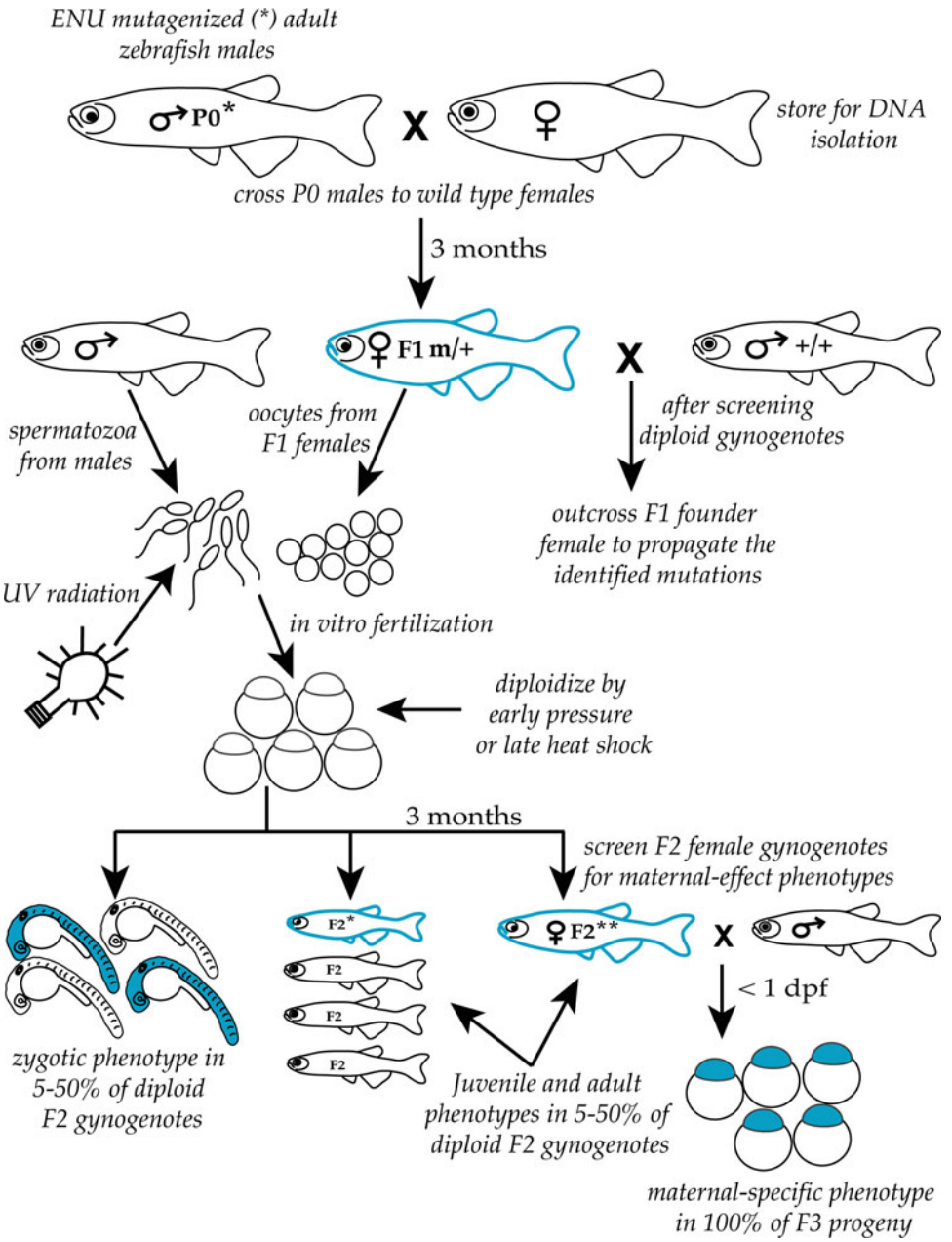

Fig. 7.3. Schematized strategy for a diploid gynogenote-based genetic screen. ENU-mutagenized P0 males are used to generate F1 females, which are manually stripped for eggs. These eggs are then in vitro fertilized with UV-irradiated spermatozoa, which would normally result in gynogenetic haploids. Instead, diploidization is achieved by subjecting the zygote to early pressure or late heat shock immediately after in vitro fertilization. Such F2 gynogenetic haploids can manifest zygotic larval, juvenile, and adult phenotypes at an expected frequency of 5–50% (indicated by F2* for juvenile and adult phenotypes). A subset of the adult phenotypes in the F2 diploid gynogenotes could be parental specific (F2**). In this schematic, crossing an F2 diploid gynogenote female to wild-type males can reveal a maternal-effect mutation in the female, which results in a phenotype in most if not all (100%) of the F3 progeny. Isolated mutations are propagated using progeny of the F1 female obtained by crossing to a male.

2. Materials

2.1. Induction and Recovery of Recessive Mutations in Zebrafish

1. *N*-ethyl-*N*-nitrosourea (ENU)—1 g.
2. 10 mM acetic acid—100 mL.
3. 1 M NaOH (to decontaminate ENU-exposed materials)—3 L.

2.1.1. Preparation of N-Ethyl-N-Nitrosourea

4. 18G surgical needles—2.
5. 25 mL syringe.

2.1.2. Mutagenizing Zebrafish Males Using N-Ethyl-N-Nitrosourea

1. ENU solution in 10 mM acetic acid.
2. 1 L round plastic containers—9.
3. Cylinders with a fine mesh bottom that fit inside the plastic containers—3.
4. Petri dish lids which fit on top of the containers—3.
5. 5 L fish tank.
6. 0.5 M Na-phosphate buffer, pH 6.0—50 mL.
7. 10 mM Na-phosphate buffer, pH 6.6: 40 mL 0.5 M Na-phosphate buffer, pH 6.0 + 1,960 mL fish water.
8. MESAB stock: 2 g/L ethyl-*m*-aminobenzoate methanesulfonate, pH 7.0, adjusted with 1 M Tris–HCl, pH 9.0. Store at 4°C.
9. 1 M NaOH (to decontaminate ENU-exposed materials)—1 L.

2.2. Expedited Identification of Phenotypes of Interest Through Ploidy Manipulation

2.2.1. Generating F2 Gynogenetic Haploids to Screen for Recessive Mutations

1. UV-irradiated spermatozoa (**Section 3.3.4**).
2. Freshly stripped eggs from F1 females (**Section 3.3.5**).
3. Embryo medium (**Section 2.3.6**, step 3).

2.2.2. Generating Gynogenetic Diploids by Early Pressure

1. UV-irradiated spermatozoa (**Section 3.3.4**).
2. Freshly stripped eggs from F1 females (**Section 3.3.5**).
3. Embryo medium (**Section 2.3.6**, step 3).
4. Glass scintillation vials with perforated caps.
5. French press cell (40 mL volume).
6. French pressure cell press.

2.2.3. Generating F2 Gynogenetic Diploids by Late Heat Shock

1. UV-irradiated spermatozoa (**Section 3.3.4**).
2. Freshly stripped eggs from F1 females (**Section 3.3.5**).
3. Embryo medium (**Section 2.3.6**, step 3).
4. Ultracentrifuge tubes with mesh bottoms (made by cutting the bottom of the tubes and heat sealing a fine mesh to the base).
5. Embryo medium water bath at 28°C and 41.4°C.
6. Immersible racks or floaters that can hold the ultracentrifuge tubes.

2.3. Additional Experimental Procedures

2.3.1. Cryopreservation of Zebrafish to Isolate DNA

1. MESAB stock (**Section 2.1.2**, step 8).
2. MESAB working solution: 30 mL MESAB + 200 mL fish water, prepare fresh.
3. 1.8 mL cryotubes.
4. Liquid nitrogen.
5. A spoon.
6. Long (4–8 in.) forceps.

2.3.2. Spermatozoa Collection from Dissected Testes

1. Adult male zebrafish.
2. MESAB stock (**Section 2.1.2**, step 8).
3. MESAB working solution: 30 mL MESAB + 200 mL fish water, prepare fresh.
4. Hank's solution 1: 8.0 g NaCl + 0.4 g KCl in 100 mL dH_2O. Store at 4°C.
5. Hank's solution 2: 0.358 g Na_2HPO_4 anhydrous + 0.6 g KH_2PO_4 in 100 mL dH_2O. Store at 4°C.
6. Hank's solution 4: 0.72 g $CaCl_2$ in 50 mL dH_2O. Store at 4°C.
7. Hank's solution 5: 1.23 g $MgSO_4 \cdot 7H_2O$ in 50 mL dH_2O. Store at 4°C.
8. Hank's solution 6: 0.35 g $NaHCO_3$ in 10 mL dH_2O, prepare fresh.
9. Hank's Premix: combine Hank's solutions (steps 4–7) in this order: 10 mL of solution 1 + 1 mL of 2, 1 mL of 4 + 86 mL dH_2O + 1 mL of 5. Store at 4°C.
10. Hank's buffer: 900 μL Hank's Premix (step 9) + 10 μL of Hank's solution 6 (step 8), prepare fresh.
11. A spoon.
12. An incident light source.

2.3.3. Spermatozoa Collection from Live Zebrafish

1. Adult male zebrafish.
2. MESAB stock (**Section 2.1.2**, step 8).

3. MESAB working solution: 30 mL MESAB stock + 200 mL fish water, prepare fresh.
4. Hank's buffer—1 mL (**Section 2.3.2**, step 10).
5. Disposable glass micropipette 10–20 μL.
6. Sponge with a 1.5 in. slit cut into it.
7. A pair of flat forceps.
8. A spoon.
9. An incident light source.

2.3.4. Inactivating Male Pronuclei by UV Irradiation of Spermatozoa

1. Spermatozoa supernatant—0.5–1 mL (**Section 3.3.2** or **3.3.3**).
2. Clean, dry watch glass.
3. UV lamp (115 V, 60 Hz).

2.3.5. Stripping Eggs from Female Zebrafish

1. Adult female zebrafish.
2. MESAB stock (**Section 2.1.2**, step 8).
3. MESAB working solution: 30 mL MESAB + 200 mL fish water, prepare fresh.
4. Hank's buffer—10 mL (**Section 2.3.2**, step 10).
5. 10% bovine serum albumin (BSA)—1 mL.
6. A spatula.
7. A spoon.

2.3.6. In Vitro Fertilization of Zebrafish Eggs

1. Spermatozoa supernatant—100 μL (**Section 3.3.2** or **3.3.3**).
2. Freshly stripped eggs (**Section 3.3.5**).
3. Embryo medium—500 mL: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% methylene blue.

3. Methods

3.1. Induction and Recovery of Recessive Mutations in Zebrafish

3.1.1. Preparation of N-Ethyl-N-Nitrosourea

1. Wear protective lab coats, closed shoes, and double glove at all times during the experiment. Treat all labwares and disposable materials that come in contact with ENU for decontamination with 1 M NaOH prior to disposal (*see* **Note 1**).
2. Line the surface of a chemical hood at 21–22.5°C with disposable protective bench paper. Place a 4 L plastic container half filled with 1 M NaOH for use as ENU decontaminant near the work area.

3. Remove the small circle of aluminum from the ENU bottle cap. Do not remove the rubber cap or the remaining foil that holds it in place.
4. Stuff the base opening of an 18G needle with cotton and insert the needle into the rubber cap of the ENU bottle. This serves to relieve pressure from the subsequent introduction of solvent while preventing the dispersal of ENU powder or solution.
5. Using a 25 mL syringe and a second 18G needle, inject 10 mL of 10 mM acetic acid through the rubber cap and swirl gently to wet the ENU powder.
6. Leaving the needle in place, withdraw the syringe and inject the remaining 90 mL of 10 mM acetic acid in two to three installments.
7. Discard the needles and syringe into 1 M NaOH decontaminant solution (*see* **Note 1**).
8. Wrap the neck of the ENU bottle with parafilm and shake well until ENU dissolves completely. Shake the bottle manually several times in a period of 30 min as it is difficult to get ENU into solution. The resulting solution should be clear and light yellow in color.
9. After the first round of mutagenesis, aliquot the remaining ENU in equal volumes into two 50 mL falcon tubes, wrap individually in parafilm, and store inside plastic bags for secondary containment. Store tubes upright at –20°C.
10. If frozen, ENU solution should be thawed for 1–2 h at room temperature prior to use.

3.1.2. Mutagenizing Zebrafish Males Using N-Ethyl-N-Nitrosourea

1. A week before the mutagenesis, preselect 15 healthy, male zebrafish for fertility by setting them up in pair matings with females (*see* **Note 2**).
2. Line the surface of a chemical hood at 21–22.5°C with disposable bench paper and arrange the nine plastic containers in 3 × 3 rows. Decontaminate the plastic containers with 1 M NaOH after completion of each round of ENU treatment (*see* **Note 3**).
3. Fill cylinders in the first and second rows with 300 mL of 10 mM Na-phosphate buffer, pH 6.6, and the third row with 300 mL of clean fish water.
4. Place the three mesh-bottom cylinders inside the third row containers and transfer five male zebrafish into each third row cylinder (*see* **Note 4**). Transferring males prior to adding the ENU in step 5 prevents possible ENU contamination of the fish nets.

5. Add 10.9 mL of ENU to each first row container with the 300 mL of 10 mM Na-phosphate buffer, pH 6.6, to obtain a final concentration of 3 mM ENU (*see* **Note 5**). Transfer the mesh-bottom cylinders containing the males from row three into row one.
6. Leave fish undisturbed in the ENU for 1 h. Turn off the chemical hood lights and keep activity around the hood to a minimum to avoid startling the fish.
7. Add 1.5 mL of MESAB into row two containers and transfer the fish from row one into row two. Leave fish undisturbed for 1 h (*see* **Note 6**).
8. Add 1.5 mL MESAB into third row containers and transfer the fish from row two into row three. Again, leave fish undisturbed for 1 h (*see* **Note 6**).
9. Add 7.5 mL of MESAB into 3 L of fish water in a 5 L fish tank (*see* **Note 6**). Transfer fish from row three out of the mesh-bottom cylinders and into the fish tank.
10. Move the fish tank into the fish room but do not transfer males into tanks with circulating water yet. Feed the males with an appropriate amount of live brine shrimp such that residual food does not accumulate in the tank.
11. Add an additional 7.5 mL of MESAB to the 3 L of fish water if the ENU-treated fish continue to display symptoms of distress (*see* **Note 6**). Monitor for amelioration of symptoms at regular intervals for 3–4 h after the ENU treatment.
12. Leave fish overnight on the bench. In the morning, transfer the males into 3 L of fresh fish water without MESAB and again monitor for any signs of distress. If fish appear fidgety, add 3 mL of MESAB and leave for 1–2 h on the bench (*see* **Note 6**).
13. Transfer fish into fish water without MESAB and if fish appear to behave normally, place them back into the system in a labeled tank and feed well.
14. After the fish have rested and recovered for 1–1.5 weeks, repeat the ENU treatment (steps 2–13) for a second time (*see* **Note 5**).
15. Let the fish recover again for 1–1.5 weeks and repeat ENU treatment (steps 2–13) for the third and final time (*see* **Note 5**).

3.1.3. Performing a Specific-Locus Test to Analyze Efficiency of Mutagenesis

1. One week after the final round of mutagenesis, set up the males in pair matings with females homozygous for a recessive, visible, homozygous viable embryonic mutation, such as the pigment mutation, *albino* (*see* **Notes 2, 7,** and **8**).

2. Set up matings between non-mutagenized males and *albino* females to control for spontaneous infertility and background phenotypes.
3. The resulting clutches from mutagenized males should exhibit a 30–40% reduction in fertilization rates and yield only about 1% of phenotypically normal embryos at 24 h post-fertilization (hpf) when compared to the control clutches. Additionally, by 36–72 hpf, when pigmentation becomes obvious, a fraction of progeny from the mutagenized males should be pigment mosaics. These defects in fertility and the mosaicism in progeny embryos reflect the effects of ENU in post-meiotic, mature spermatozoa (*see* **Note 8**).
4. Repeat pair matings after a 1-week interval. Fertility rates and phenotypic characteristics should approach normal levels and pigment mosaicism should decrease, reflecting now the presence of spermatozoa that were in pre-meiotic stages during the mutagenic treatments. Repeat pair matings at 1-week intervals if reduction in fertility rates and abnormal morphology of embryos continue to persist.
5. Provide each mutagenized male with an identifier number and house in individual tanks. These are the parent (P0) males for raising mutagenized families.
6. Perform specific-locus test by mating each P0 male with homozygous *albino* females and recording the number of non-mosaic albino embryos exhibiting a normal morphology (*see* **Note 7**). Score at least 3,000 embryos, as the expected frequency for such a mutation is $1–3 \times 10^{-3}$ (1, 2).

3.1.4. Identifying Recessive Mutations in a Standard Genetic Inbreeding Screen

1. After performing the specific-locus test and recovery to normal fertilization rates and development, mate each mutagenized parent male (P0) with wild-type females. A schematic of the genetic screen strategy to identify and recover recessive mutations is outlined in **Fig. 7.1**.
2. The embryos obtained from such matings are F1 carriers of several different mutations. Raise about 500 F1 progeny from each mutagenized P0 male (*see* **Note 9**).
3. Raise to adulthood each F1 family derived from an individual P0 male separately. After obtaining the necessary numbers of F1 families, euthanize and store the P0 fish at –80°C as described in **Section 3.3.1**, to be used as a source of DNA for future genotyping and sequencing experiments.
4. Randomly incross F1 males and females to raise F2 families. These F2 fish will be heterozygous carriers for newly induced mutations. F1 individuals can also be outcrossed to

wild-type fish at this point, but incrossing them allows testing two mutagenized genomes in a single F2 family.

5. Randomly incross F2 males and females and examine the F3 clutches for physical and/or molecular phenotypes of interest. If a mutation behaves as a true zygotic recessive, one fourth of the embryos of an F3 clutch should exhibit the phenotype of interest.
6. To maintain a recessive zygotic mutation, F2 heterozygotes known to carry the mutation (because of a phenotype in their F3 offspring) can be either crossed to wild-type fish or to another heterozygous carrier. Crossing the F2 fish to a strain with DNA markers polymorphic with respect to the strain in which the mutations were induced allows linkage mapping of the mutation (*see* **Notes 10** and **11**).
7. To identify mutations in adult traits, including maternal- and paternal-effect mutations, raise the F3 generation to adulthood and assay for the trait. In the case of maternal- and paternal-effect mutations, set up the F3 adults against wild-type fish in pair matings and assay for a specific phenotype in most if not all of the resulting F4 embryos (*see* **Note 12**). This step has been simplified by pooling F3 families or through ploidy manipulation ((21), **Sections 3.2.2**, **3.2.3**, and **3.2.4**).

3.2. Expedited Identification of Phenotypes of Interest Through Ploidy Manipulation

In this section, we describe experimental strategies that can be employed to significantly reduce the number of generations needed to identify mutations by screening either F2 gynogenetic haploids (**Section 3.2.1**, **Fig. 7.2** (9)) or gynogenetic diploids (**Sections 3.2.2**, **3.2.3**, and **3.2.4**, **Fig. 7.3** (10, 11)). Both strategies have the time saver advantage over the inbreeding screen outlined in **Section 3.1.4** and **Fig. 7.1**; however, depending on the developmental time point or phenotype of interest and particular needs of the researcher, one approach may be better suited than the other. In this chapter, we discuss only the use of gynogenetic haploids and diploids for genetic screens. It is also possible to generate androgenetic haploids (22) and diploids (23) for similar screens; however, androgenesis is less effective than gynogenesis. The basic methodologies for androgenote production are variations of the procedures used in gynogenote production and are discussed in that context in the following sections.

In the following sections, we provide the experimental details required to produce gynogenetic diploid zebrafish by using both early pressure and late heat shock. A distinct advantage of the F2 gynogenetic diploid screen strategy compared to the haploid screen is the possibility to isolate genes essential for development past 72 hpf. These include genes required for organogenesis, skeletal development, and adult traits such as pigment

patterns, in addition to genes that have maternal or paternal effects (11, 24). Although early pressure and late heat shock both yield gynogenotes, the degree of homozygosity in the resulting gynogenotes and expected phenotypic ratios in the presence of recessive mutations varies between these methods. Due to recombination during meiosis, early pressure results in partial heterozygosity of the genome, where regions near centromeres exhibit a higher frequency of homozygosity than more distal chromosomal regions. When the mother is a heterozygous carrier for a mutation, this partial heterozygosity results in a variable fraction of the embryos exhibiting a given phenotype, ranging from near 0 to 50% depending on the locus to centromere distance (14, 25–27). On the other hand, inhibition of the first mitosis through late heat shock results in embryos homozygous across the genome, where mutations, regardless of their chromosomal location, lead to phenotypes at an invariant frequency of 50% (14, 21). In spite of the theoretical advantages of heat shock over early pressure, early pressure typically results in better survival rates than heat shock (14, 24) and has therefore been more frequently used in gynogenesis-based genetic screens (10, 11, 14).

3.2.1. Generating and Screening F2 Gynogenetic Haploids for Recessive Mutations

In such gynogenetic haploid zebrafish, a mutant allele present in the female genome manifests its phenotype in a haploid state (2, 5). However, haploid embryos exhibit a variety of systemic developmental defects, such as a shortened and broadened body axis as well as generalized edema and are typically inviable past 48–72 hpf (9). Thus, it is prudent to limit a haploid-based screen strategy to phenotypes that do not overlap with those characteristics of the haploid syndrome and which occur prior to 48 h of embryonic development (*see* **Notes 13** and **14**).

1. After ENU mutagenesis of the males as described in **Section 3.1.2**, perform specific-locus tests and obtain F1 families as described in **Sections 3.1.3** and **3.1.4**, respectively. The females from the F1 generation are heterozygous for newly induced mutations and will be the source of eggs for haploid production and screening. A schematic for a haploid-based screen strategy is provided in **Fig. 7.2**.
2. The evening before the experiment, set up F1 females in pair matings with wild-type males (*see* **Note 2**). Strip eggs from the females as described in **Section 3.3.5**.
3. Label both the females and their eggs with an identifying number and return females to small individual tanks.
4. In vitro fertilize a clutch of eggs from one female with 30 μL of UV-irradiated spermatozoa (*see* **Note 15**).
5. Since the embryos will only carry the maternal genome, they will develop as gynogenetic haploids. If a given mutation is

a true recessive, in a haploid clutch, the phenotype should manifest in 50% of the haploid progeny (*see* **Notes 16** and **17**).

6. Once a phenotype of interest is identified in the haploids, outcross the corresponding F1 female to a wild-type male to raise F2 families. At this point if linkage mapping of the mutation is desired, males from a wild-type polymorphic strain may also be used to raise F2 mapping families (*see* **Note 10**).
7. Randomly incross the F2 generation to identify heterozygous carriers of the mutation to generate mutant embryos for analysis and to propagate the mutation.

3.2.2. Generating Gynogenetic Diploids by Early Pressure

Mature zebrafish oocytes are arrested in development at meiosis II, which is completed within the first 2 min post-fertilization (mpf) resulting in extrusion of the second polar body (28, 29). To inhibit cytokinesis associated with meiosis II, zebrafish eggs fertilized with UV-irradiated sperm can be subjected to high pressure immediately after fertilization (early pressure or EP) resulting in diploid gynogenotes (10, 11, 14). Diploid gynogenotes can be screened for developmental phenotypes beyond 24 hpf including adult traits (*see* **Note 18**).

1. Follow steps 1–3 as described in **Section 3.2.1**.
2. In vitro fertilize a clutch of eggs from one female as described in steps 1–3 in **Section 3.3.6**, using UV-irradiated spermatozoa (**Section 3.3.4**, *see* **Note 19**). Transfer the fertilized eggs into the glass scintillation vials and fill the vial with embryo medium.
3. Cap the vials and place inside the pressure cell. Depending on the size of the scintillation vials, between two and four vials can be placed at a time inside the pressure cell.
4. Fill the pressure cell with embryo medium taking care to avoid introducing air bubbles. Close the pressure cell allowing excess embryo medium to drain out of the side valve before closing it.
5. Insert the pressure cell into a French Pressure Cell Press and apply pressure of 8,000 psi for 6 min, beginning 1 min 20 s after egg activation (step 2). If multiple clutches are being pressure treated, keep track of the position of each clutch inside the pressure cell.
6. After 6 min (at time 7 min 20 s post-activation), release the pressure and remove the vials from the pressure cell. Label the vials and incubate at 28°C.
7. After 45 min, but not longer than 4 h, transfer the embryos into a petri dish. Remove inviable or lysed embryos at regular intervals (6, 24, 36 h, etc.).

8. Assay the survivors for phenotypes of interest at the appropriate time point.

3.2.3. Generating F2 Gynogenetic Diploids by Late Heat Shock

Diploid gynogenotes can also be obtained by the relatively simple method of heat shock treatment of embryos at 13–15 mpf (14). Similar to early pressure, diploid gynogenotes obtained by heat shock can also be screened for post-24 h phenotypes, including adult or parental traits. However, the late heat shock method is less effective at producing gynogenetic diploid progeny than early pressure.

1. Follow steps 1–3 as described in **Section 3.2.1**.
2. In vitro fertilize a clutch of eggs from one female with 30 μL of UV-irradiated spermatozoa (**Section 3.3.6**, steps 1–3) and note down to the second the time when you first add embryo medium to the eggs as time t_0.
3. Transfer approximately 30 embryos into a mesh-bottom ultracentrifuge tube and place it onto the floaters. At t_{13} minutes, begin the heat shock: remove excess embryo medium by briefly blotting the tube bottom against a short stack of paper towels and place the tube into the embryo medium bath held at 41.4°C.
4. At t_{15} minutes, conclude the heat shock: remove the tube from the 41.4°C bath, briefly blot excess embryo medium away from the tube bottom, and transfer into the embryo medium bath held at 28°C.
5. Keep the embryos in the ultracentrifuge tubes in the 28°C bath for 45 min. Transfer embryos from each tube into a clean petri dish and incubate at 28°C.
6. Remove inviable or lysed embryos at regular intervals (6, 24, 36 h, etc.) and analyze the surviving embryos for phenotypes of interest at the appropriate time point.

3.2.4. Screening F2 Gynogenetic Diploids and Recovery of Identified Mutations

F2 gynogenetic diploids obtained by either early pressure or late heat shock can be screened in an identical manner and are hence discussed under a common section. A schematic for the screen is presented in **Fig. 7.3**.

1. Diploid F2 gynogenotes can be screened for developmental phenotypes at all stages of embryonic development including during larval stages.
2. Since diploid F2 gynogenotes survive to adulthood and are fertile, adult traits including parental-effect phenotypes can be assayed for starting within 3 months from the date of the early pressure or heat shock treatment ((21), *see* **Note 20**).
3. Once a F1 female carrying the mutation of interest is identified, outcross the fish to a wild-type or a mapping strain

to raise F2 generations. Randomly incross F2 fish to recover and maintain the mutation (*see* **Note 21**).

4. If the homozygous mutant progeny are viable and fertile, mutations can be recovered by incrossing the homozygous mutant individuals. If the mutation is inviable or infertile, mutations can also be recovered through multiple random crosses from siblings of the affected individuals.

3.3. Additional Experimental Procedures

The following are essential satellite protocols required for executing the experiments detailed in the preceding sections.

3.3.1. Cryopreservation of Zebrafish to Isolate DNA

1. Euthanize fish in MESAB working solution in a 250 mL glass beaker until no obvious gill movements are detectable.
2. Scoop fish out with a spoon, rinse in fish water briefly, and blot dry on a paper towel.
3. Insert the whole fish into a 1.8 mL cryotube and flash-freeze in liquid nitrogen.
4. Recover tubes from liquid nitrogen with long forceps and store at –80°C.
5. To obtain tissue for DNA extraction, use a surgical blade to excise a small piece of tissue from the frozen fish and proceed with standard DNA extraction protocols.

3.3.2. Spermatozoa Collection from Dissected Testes

1. Aliquot 100 μL of freshly made Hank's buffer into a 1.5 mL Eppendorf tube and keep on ice.
2. Euthanize males in MESAB until there are no detectable gill movements. Male zebrafish need not be set up in pair matings prior to testes dissection.
3. Using a spoon, scoop out the fish onto a short stack of paper towels and pat dry to remove excess water. Decapitate and make a longitudinal cut along the abdomen of fish.
4. Using a dissecting scope equipped with an incident light source, remove the swim bladder and gut located in the body cavity. Testes are elongated, whitish, semi-translucent structures located along each side of the body cavity.
5. Remove testes with dissecting forceps and place them directly into Hank's buffer.
6. Shear the testes to release spermatozoa by pipetting repeatedly through a 200 μL pipette tip without generating air bubbles.
7. Return the tube to ice and allow the pieces of testes to settle for 5–10 min. Pipette the supernatant into a fresh Eppendorf tube and use the resulting spermatozoa solution for UV inactivation and/or in vitro fertilization. Spermatozoa can be used for up to 2 h if kept on ice.

8. If required, additional spermatozoa can be harvested from the remaining testes tissue by adding an additional 100 μL of Hank's buffer and repeating steps 6–7.
9. A pair of testes can yield approximately 200 μL of spermatozoa supernatant, which is sufficient to fertilize 6–7 clutches or about 1,000 eggs (*see* **Note 22**).

3.3.3. Spermatozoa Collection from Live Zebrafish

1. Aliquot 100 μL of freshly made Hank's buffer into a 1.5 mL Eppendorf and keep on ice.
2. Anesthetize males in MESAB for 2–4 min until gill movements are reduced, scoop out with a spoon, and rinse briefly with fish water. Transfer onto a short stack of paper towels and pat dry.
3. Place the male ventral-side up into the slit cut in the sponge and place the sponge under a dissecting scope equipped with an incident light source.
4. Part the anal fins to expose the cloaca using a pair of flat forceps and dry the area with paper towels. Keep a 10–20 μL micropipettor ready in one hand, with the plunger in.
5. Apply gentle pressure to the anterior of the anal fin base while simultaneously withdrawing the plunger of the micropipettor to collect the sperm as it is squeezed out of the fish.
6. Each male can yield approximately 5 μL of extruded spermatozoa, which should be milky white in appearance (*see* **Note 22**).
7. Transfer the collected spermatozoa into the Hank's buffer on ice. Mix well by gently pipetting the solution up and down while avoiding generating air bubbles. Spermatozoa can remain effective for about 2 h after collection when kept on ice. Use approximately 30 μL of spermatozoa to fertilize a clutch of about 150 eggs.
8. Place fish back into fresh water and allow recovery for at least 1 week in individual tanks.

3.3.4. Inactivating Male Pronuclei by UV Irradiation of Spermatozoa

1. Place the watch glass on ice and transfer the spermatozoa supernatant onto it. If the testes isolation method was used, avoid testes debris, which may shield spermatozoa from the UV light and result in incompletely inactivated sperm solution.
2. Place the watch glass on ice directly under the UV lamp at a distance of 30 cm from the UV bulb and irradiate for 2.5–3 min, gently swirling the solution at 30 s intervals (*see* **Note 23**).

3. While irradiation is in progress, place a clean 1.5 mL Eppendorf on ice and transfer the spermatozoa solution into this tube once irradiation is complete.
4. The UV-irradiated spermatozoa can be used for up to 2 h after UV exposure if kept on ice.

3.3.5. Stripping Eggs from Female Zebrafish

1. Set up female zebrafish in pair matings the evening before the day of the experiment (*see* **Notes 2** and **24**).
2. Immediately after the light cycle begins, check for potential overnight layers. Replace the fish water in tanks with pair matings where there may be eggs present in the medium (*see* **Note 25**).
3. Check the pair matings for eggs every several minutes. As soon as the pair begins to lay eggs, separate the female from the male (*see* **Note 26**). Separation of the female immediately after initiation of egg laying is necessary to ensure that not all mature eggs present are released naturally.
4. Anesthetize the female in MESAB in a 250 mL glass beaker for 2–3 min until gill movement slows down. To ensure recovery of the fish, exposure should be limited to less than 1 min once gill movement stops.
5. Using a spoon, scoop out the fish from the MESAB solution and dip the spoon briefly in fish water to rinse the fish. Place the female onto a short stack of paper towels to remove excess water (water can prematurely activate eggs prior to fertilization).
6. Gently transfer the female onto a petri dish and further dry the area near the anal fin with a soft tissue.
7. Support the back of the fish with the index finger of one hand while simultaneously applying gentle pressure to the abdomen with a clean, dry spatula. If the female has mature eggs, they will be released immediately as a translucent, viscous mass (*see* **Notes 27** and **28**).
8. Separate the eggs from the fish using the spatula and transfer the female into fresh fish water for recovery. If necessary (e.g., during a genetic screen), place the females in a separately labeled tank in the system. Allow recovery for 1 week before use in natural matings and about 4 weeks prior to additional egg stripping.
9. The eggs should be used within the first 2 min of harvesting to avoid their drying due to loss of moisture. If a delay is anticipated in using the eggs, they can be maintained in an inactivated state for up to 1 h in Hank's buffer supplemented with 0.5% BSA (*see* **Note 29**).

3.3.6. In Vitro Fertilization of Zebrafish Eggs

1. Add 30 μL of spermatozoa directly onto a pool of approximately 150 freshly stripped eggs in a petri dish (*see* **Note 30**).
2. Swirl the petri dish gently once to mix, add 0.5 mL of embryo medium onto the pool of eggs to activate them, mix gently again and wait for 1 min.
3. After 1 min, flood the plate with 25 mL of embryo medium.
4. Incubate at 28°C, checking the plate after 45–60 min to ensure that post-fertilization cellular cleavages are occurring normally. Embryos not undergoing a regular cleavage pattern are unfertilized and should be discarded (*see* **Note 31**).

4. Notes

1. To decontaminate ENU-exposed materials, immerse all disposable items such as gloves, falcon tubes, syringes, needles, and pipettes that come in contact with ENU in a large beaker containing 1 M NaOH and leave overnight in the hood. Remove all solid disposable waste and treat as regular autoclave waste. Drain the liquids into the sink and wash all reusable items thoroughly before using again.
2. Zebrafish pair matings are carried out in 1 L acrylic boxes (30). The fish are placed in an insert with a mesh bottom, which allows the eggs to fall through into the box. This safeguards the eggs from being ingested by the fish in such close quarters.
3. Add 100–200 mL of 1 M NaOH into each container and the mesh-bottom cylinders used for mutagenesis and leave overnight in the hood prior to washing with water. Wash thoroughly to remove all traces of NaOH before use in subsequent rounds of mutagenesis.
4. To ensure high post-mutagenic survival rates, it is critical to limit the number of males per ENU treatment in a container to between 5 and 7.
5. While treating fish for the second and third time, if volumes in the frozen aliquots significantly fall short of the required 10.9 mL of ENU, adjust the volume of 10 mM Na-phosphate buffer, pH 6.6, proportionally.
6. Mutagenized fish exhibit exaggerated nervousness upon removal from the ENU solution and display distress symptoms such as swimming rapidly in the tanks, going into shock, swimming belly-side up, and sudden death. The

addition of MESAB in the post-mutagenic washes ameliorates the nervousness of the fish and leads to a significantly higher survival rate (31). Remove dead ENU-treated fish immediately and place in a beaker containing 1 M NaOH for 30 min prior to disposing the carcass.

7. Specific-locus tests can in principle be performed for any recessive phenotype. However, if a recessive, homozygous-lethal developmental trait is opted for, the test will assay only half as many genomes as scored embryos, since the tester female would be a heterozygous carrier. Performing specific-locus tests with pigment mutants (*albino*, *golden*, or *sparse*) provides an easy visual readout of a phenotype that is homozygous viable and consequently tests as many genomes as scored embryos.
8. In addition to or instead of *albino* females, wild-type females can be used to purge the mutagenized males of spermatozoa exposed to ENU during post-meiotic stages. An advantage of using *albino* females is that the induction of new mutations in post-meiotic spermatozoa (and thus the success of the mutagenic treatment) can be readily assessed by the presence of embryos mosaic for the *albino* phenotype, in addition to inviable or abnormal progeny (32).
9. It is advisable to limit the number of F1 progeny produced to 500 per mutagenized parent P0 male as the number of spermatogonial stem cells in an adult male is estimated to be about 500–1,000 (1). Raising larger numbers of F1 progeny per P0 male may increase the possibility of isolating clonal copies of the same newly induced mutation.
10. Genetic linkage of isolated mutations is established by positional cloning, a multi-step strategy that involves segregation analysis to link the mutation to a contiguous stretch of DNA (i.e., contig) and DNA markers flanking the mutation, which eventually leads to the identification of the affected gene within the contig (33).
11. Mutagenized P0 males can be directly crossed with females of a polymorphic strain and genetic linkage information can be immediately obtained from the pools of F2 mutant and wild-type individuals (21, 34).
12. Genes that are required maternally as well as zygotically (maternal–zygotic genes (35, 36)) for embryonic development can be uncovered at this stage by incrossing F3 siblings (21). A maternal–zygotic dominant interaction will manifest as an additive phenotype in mutant F4 progeny lacking both types of genetic contribution.

13. In general, the phenotype in a haploid is almost identical to the homozygous diploid, although in some cases the phenotype may be more severe (9). It is advisable to test the robustness of the phenotype in non-mutagenized sibling females especially when morphological appearance is the screening criteria, since subtle defects such as changes in size and shape of organs and tissues may otherwise be missed.
14. An F2 gynogenetic haploid screen can only identify zygotic genes essential for development. Recessive maternal-effect genes cannot be identified in such a screen as the source of the eggs is a F1 heterozygous female.
15. The presence of day 5 embryos with inflated swim bladders is indicative of incomplete UV irradiation of spermatozoa resulting in some viable diploid embryos. Such embryos can be easily visually identified if the F1 females used to obtain eggs are homozygous for a recessive mutant allele in a pigmentation gene (e.g., *albino*), and the source of spermatozoa is males wild type for the same gene. Incomplete UV irradiation of spermatozoa will manifest as either mosaic or wild-type pigmentation in the resulting progeny and such embryos can be discounted from further analysis.
16. The haploid progeny of heterozygous F1 females could in theory manifest both recessive and dominant mutations. However, a dominant mutation in a gene essential for embryonic development would lead to inviability of the heterozygous F1 fish themselves, precluding haploid production and hence the possibility of isolating such mutations in a haploid-based screen.
17. The remaining 50% of the gynogenetic haploids that do not exhibit the phenotype of interest should exhibit the short, broad body axis, generalized edema, and lethality at 48–72 hpf typically associated with haploidy (9).
18. A relatively high fraction of EP-derived embryos exhibit embryonic lysis or developmental abnormalities, likely due to the physical perturbation associated with the technique. These abnormalities occur primarily during the first 24 h of development, precluding the use of this method for screening during this early period of development.
19. Because of the time constraint of placing the fertilized eggs under hydrostatic pressure by 1 min 20 s after egg activation, flooding of the plate with embryonic medium (normally recommended at 1 min after egg activation, Step 3 in **Section 3.3.6**) can be carried out as early as 30 s post-activation.

20. An advantage of screening diploid gynogenotes over haploids is the possibility to identify juvenile or adult traits including parental-specific phenotypes. However, in such instances, the F1 females will need to be kept in labeled individual tanks until the corresponding diploid F2 gynogenotes are screened.
21. Genetic linkage mapping of mutations can in principle be initiated concomitant with diploid gynogenote production if the P0 mutagenized males are crossed to females from a polymorphic strain (21). Mutant and wild-type pools of F2 diploid gynogenotes can then be used immediately to obtain genetic linkage information for the mutation of interest. However, unambiguous determination of linkage requires about 10–12 fish in each pool. Therefore, prior to initiating this approach, the investigator should ensure that the gynogenetic method used yields sufficient offspring to justify such a coordinated screening/mapping strategy. On the other hand, in the absence of a sufficient yield to allow such a direct mapping strategy, the introduction of polymorphic markers during the screen itself should be avoided, as it may limit the ability to perform linkage mapping subsequent to identification of the mutation.
22. Dissecting the testes out of a male is the most reliable method of obtaining spermatozoa from zebrafish. However, it has the disadvantage that the males need to be sacrificed. Conversely, though spermatozoa can be collected from live males, the disadvantage is that sperm may not always be obtained from a given male.
23. If incomplete UV inactivation of sperm is observed (*see* **Note 14**), increase the UV exposure time by 30 s increments until genetic mosaicism is no longer detectable. Overexposure to UV light, however, will result in the inability of sperm to fertilize the eggs.
24. Although females from certain zebrafish strains such as the AB star yield eggs readily even without being set up overnight in pair matings, most strains are less amenable to manual stripping, even if gravid. However, regardless of strain type, we have found that females that begin the process of egg-laying naturally, after having been set up overnight with a male, consistently yield good quality eggs through manual stripping.
25. Although egg-laying behavior is most common in the first hours of the light cycle, on occasion pairs of fish lay eggs prior to the initiation of the light cycle. Replacing the water in tanks to exclude such eggs allows to more easily visualize tanks with newly laid eggs, which will contain females ready for manual egg stripping.

26. During a natural pair mating, eggs are typically released in multiple waves, spaced between 5 and 30 min apart. Therefore, if the tanks are closely watched, it is possible to separate females when most of the eggs have not yet been released through natural mating.
27. Dry fish and human skin can be sticky upon contact. To avoid stickiness, wear gloves or moisten fingertips slightly (but not excessively, as it may result in premature egg activation) with water.
28. Healthy, mature, inactive eggs will be yellowish, translucent, flaccid, and evenly granular. Egg activation will lead to an increase in turgor in the eggs, the separation of yolk granules to the vegetal region of the egg, and the egg cytoplasm to the animal pole. On occasion, released eggs appear white and opaque immediately after their extrusion from the female. These eggs are undergoing resorption, cannot be fertilized, and should be discarded.
29. Additional Hank's buffer supplemented with 0.5% BSA can be added in 30 μL increments to compensate for loss of the buffer by evaporation during the 1 h delay period.
30. The average clutch size of the eggs obtained after manual stripping of eggs from a gravid zebrafish female is about 150. Thus, using 30 μL of sperm solution per clutch, the 200 μL of spermatozoa obtained either from live males or from testes dissection should be sufficient to fertilize approximately 1,000 eggs.
31. Unfertilized eggs will undergo pseudocleavages, which result in cells of uneven shapes and sizes. Such eggs can be easily distinguished from fertilized embryos, which exhibit a stereotypic pattern of regular cell cleavages resulting in equal sized blastomeres (*37*).

Acknowledgments

Research in our laboratory is funded by NIH RO1 GM065303.

References

1. Mullins, M. C., Hammerschmidt, M., Haffter, P., and Nusslein-Volhard, C. (1994) Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr. Biol.* **4**, 189–202.
2. Solnica-Krezel, L., Schier, A. F., and Driever, W. (1994) Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* **136**, 1401–1420.

3. Justice, M. J., Noveroske, J. K., Weber, J. S., Zheng, B., and Bradley, A. (1999) Mouse ENU mutagenesis. *Hum. Mol. Genet.* **8**, 1955–1963.
4. Shibuya, T. and Morimoto, K. (1993) A review of the genotoxicity of 1-ethyl-1-nitrosourea. *Mutat. Res.* **297**, 3–38.
5. Grunwald, D. J. and Streisinger, G. (1992) Induction of recessive lethal and specific locus mutations in the zebrafish with ethyl nitrosourea. *Genet. Res.* **59**, 103–116.
6. Inoue, M., Kurihara, T., Yamashita, M., and Tatsumi, K. (1993) Effects of treatment with methyl methanesulfonate during meiotic and postmeiotic stages and maturation of spermatozoa in mice. *Mutat. Res.* **294**, 179–186.
7. Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J., Jiang, Y. J., Heisenberg, C. P., Kelsh, R. N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., and Nusslein-Volhard, C. (1996) The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **123**, 1–36.
8. Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J., and Boggs, C. (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123**, 37–46.
9. Walker, C. (1999) Haploid screens and gamma-ray mutagenesis. *Methods Cell Biol.* **60**, 43–70.
10. Beattie, C. E., Raible, D. W., Henion, P. D., and Eisen, J. S. (1999) Early pressure screens. *Methods Cell Biol.* **60**, 71–86.
11. Pelegri, F., Dekens, M. P., Schulte-Merker, S., Maischein, H. M., Weiler, C., and Nusslein-Volhard, C. (2004) Identification of recessive maternal-effect mutations in the zebrafish using a gynogenesis-based method. *Dev. Dyn.* **231**, 324–335.
12. Kaufman, M. H. (1982) The chromosome complement of single-pronuclear haploid mouse embryos following activation by ethanol treatment. *J. Embryol. Exp. Morphol.* **71**, 139–154.
13. Modlinski, J. A. (1975) Haploid mouse embryos obtained by microsurgical removal of one pronucleus. *J. Embryol. Exp. Morphol.* **33**, 897–905.
14. Streisinger, G., Walker, C., Dower, N., Knauber, D., and Singer, F. (1981) Production of clones of homozygous diploid zebra fish (Brachydanio rerio). *Nature* **291**, 293–296.
15. Pogany, G. C. (1971) Effects of sperm ultraviolet irradiation on the embryonic development of Rana pipiens. *Dev. Biol.* **26**, 336–345.
16. Chourrout, D. (1982) Gynogenesis caused by ultraviolet irradiation of salmonid sperm. *J. Exp. Zool.* **223**, 175–181.
17. Hoppe, P. C. and Illmensee, K. (1977) Microsurgically produced homozygous-diploid uniparental mice. *Proc. Natl. Acad. Sci. USA* **74**, 5657–5661.
18. Trottier, T. M. and Armstrong, J. B. (1976) Diploid gynogenesis in the Mexican axolotl. *Genetics* **83**, 783–792.
19. Thiebaud, C. H., Colombelli, B., and Muller, W. P. (1984) Diploid gynogenesis in Xenopus laevis and the localization with respect to the centromere of the gene for periodic albinism ap. *J. Embryol. Exp. Morphol.* **83**, 33–42.
20. Noramly, S., Zimmerman, L., Cox, A., Aloise, R., Fisher, M., and Grainger, R. M. (2005) A gynogenetic screen to isolate naturally occurring recessive mutations in Xenopus tropicalis. *Mech. Dev.* **122**, 273–287.
21. Pelegri, F. and Mullins, M. C. (2004) Genetic screens for maternal-effect mutations. *Methods Cell Biol.* **77**, 21–51.
22. Ungar, A. R., Helde, K. A., and Moon, R. T. (1998) Production of androgenetic haploids in zebrafish with ultraviolet light. *Mol. Mar. Biol. Biotechnol.* **7**, 320–326.
23. Corley-Smith, G. E., Lim, C. J., and Brandhorst, B. P. (1996) Production of androgenetic zebrafish (Danio rerio). *Genetics* **142**, 1265–1276.
24. Pelegri, F. and Schulte-Merker, S. (1999) A gynogenesis-based screen for maternal-effect genes in the zebrafish, Danio rerio. *Methods Cell Biol.* **60**, 1–20.
25. Grunwald, D. J., Kimmel, C. B., Westerfield, M., Walker, C., and Streisinger, G. (1988) A neural degeneration mutation that spares primary neurons in the zebrafish. *Dev. Biol.* **126**, 115–128.
26. Streisinger, G., Singer, F., Walker, C., Knauber, D., and Dower, N. (1986) Segregation analyses and gene-centromere distances in zebrafish. *Genetics* **112**, 311–319.
27. Talbot, W. S., Trevarrow, B., Halpern, M. E., Melby, A. E., Farr, G., Postlethwait, J. H., Jowett, T., Kimmel, C. B., and Kimelman, D. (1995) A homeobox gene essential for zebrafish notochord development. *Nature* **378**, 150–157.
28. Dekens, M. P., Pelegri, F. J., Maischein, H. M., and Nusslein-Volhard, C. (2003) The maternal-effect gene futile cycle is essential for pronuclear congression and mitotic

spindle assembly in the zebrafish zygote. *Development* **130**, 3907–3916.
29. Selman, K., Wallace, R. A., Sarka, A., and Qi, X. (1993) Stages of oocyte development in the zebrafish, *Brachydanio rerio. J. Morphol.* **218**, 203–224.
30. Brand, M., Granato, M., and Nusslein-Volhard, C. (2002) Keeping and raising zebrafish. In *Zebrafish—A Practical Approach*, Nüsslein-Volhard C. and Dahm R., eds. Oxford University Press, Oxford, Vol. **261,** pp. 7–37.
31. Trevarrow, B. (2004) Reduced fish death during ENU (N-ethyl-N-nitrosourea mutagenesis), 6th International Conference on Zebrafish Development and Genetics, p. 629 (Abstract).
32. Pelegri, F. (2002) Mutagenesis. In *Zebrafish—A Practical Approach*, Nüsslein-Volhard C. and Dahm R., eds. Oxford University Press, Oxford, Vol. **261**, pp. 145–174.
33. Talbot, W. S. and Schier, A. F. (1999) Positional cloning of mutated zebrafish genes. *Methods Cell Biol.* **60**, 259–286.
34. Rawls, J. F., Frieda, M. R., McAdow, A. R., Gross, J. P., Clayton, C. M., Heyen, C. K., and Johnson, S. L. (2003) Coupled mutagenesis screens and genetic mapping in zebrafish. *Genetics* **163**, 997–1009.
35. Kane, D. A., Hammerschmidt, M., Mullins, M. C., Maischein, H. M., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C. P., Jiang, Y. J., Kelsh, R. N., Odenthal, J., Warga, R. M., and Nusslein-Volhard, C. (1996) The zebrafish epiboly mutants. *Development* **123**, 47–55.
36. Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C. P., Jiang, Y. J., Kelsh, R. N., and Nusslein-Volhard, C. (1996) Genes establishing dorsoventral pattern formation in the zebrafish embryo: the ventral specifying genes. *Development* **123**, 81–93.
37. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995) Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310.

Chapter 8

Essential Techniques for Introducing Medaka to a Zebrafish Laboratory—Towards the Combined Use of Medaka and Zebrafish for Further Genetic Dissection of the Function of the Vertebrate Genome

Sean R. Porazinski, Huijia Wang, and Makoto Furutani-Seiki

Abstract

The medaka, *Oryzias latipes*, a small egg-laying freshwater fish, is one of the three vertebrate model organisms in which genome-wide phenotype-driven mutant screens have been carried out. Despite a number of large-scale screens in zebrafish, a substantial number of mutants with new distinct phenotypes were identified in similar large-scale screens in the medaka. This observed difference in phenotype is due to the two species having a unique combination of genetic, biological and evolutional properties. The two genetic models share a whole-genome duplication event over that of tetrapods; however, each has independently specialized or lost the function of one of the two paralogues. The two fish species complement each other as genetic systems as straightforward comparison of phenotypes, ease of side-by-side analysis using the same techniques and simple and inexpensive husbandry of mutants make these small teleosts quite powerful in combination. Furthermore, both have draft genome sequences and bioinformatic tools available that facilitate further genetic dissection including whole-genome approaches. Together with the gene-driven approach to generate gene knockout mutants of the fish models, the two fish models complement the mouse in genetically dissecting vertebrate genome functions. The external embryogenesis and transparent embryos of the fish allow systematic isolation of embryonic lethal mutations, the most difficult targets in mammalian mutant screens. This chapter will describe how to work with both medaka and zebrafish almost as one species in a lab, focusing on medaka and highlighting the differences between the medaka and zebrafish systems.

Key words: Medaka, zebrafish, vertebrate, genome function, evolution, mutant.

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_8,

1. Introduction

Genetically defined alterations in gene loci, or mutants, especially those with phenotypic outcomes, are one of the most important tools for obtaining insights into the function of genes at the whole-organism level. Systematic phenotype-driven mutant screening involves generation of mutants by random mutagenesis of the genome and screening for the phenotype affecting the process of interest. Thus, the phenotype-driven screen is an unbiased approach to understand gene function. In contrast, a gene-driven approach to remove function of particular genes (e.g. knockout) provides a complementary method. In systematic unbiased mutant screens, mutants are classified according to the phenotypes, and the genes required for each class of mutants are defined by genetic complementation analysis. The value of phenotype-oriented mutant screening is that it systematically dissects the biological process into genetically defined steps, i.e. phenotypic classes, and the genes required for each step can be identified. In vertebrates, systematic mutant screens have been carried out in two teleost fish species, zebrafish (1) and medaka (2), as well as the mouse (3–5).

In vertebrates, the use of a single species to address gene loss-of-function analysis will not be sufficient to uncover all gene functions. This is due to (i) functional overlap among related genes; (ii) the manner of development for the analysis of phenotypes, e.g. accessibility of an embryo that allows identification of dynamic phenotypes; (iii) repeated usage of the same gene during development—lethality due to the early requirement of a gene makes it difficult to detect its later phenotypes; (iv) species-specific differences in gene function due to changes in the nature of its interacting partners such as in signalling cascades (6).

1.1. Benefits of Working on Both Medaka and Zebrafish

Following the genome-wide large-scale mutagenesis screens in medaka and zebrafish, there is accumulating evidence that the use of two fish species facilitates discovery of new phenotypes (2, 6, 7).

A duplication of the genome occurred in the ancestor of teleosts 350 million years ago. Medaka and zebrafish were separated from their last common ancestor between 110 and 160 million years ago, a distance that roughly corresponds to the distance between human and chicken (**Fig. 8.1**) (8). Generation of two functionally equivalent genes (paralogues) by the genome duplication facilitated rapid evolution resulting in three fates during re-diploidization according to the duplication–degeneration–complementation (DDC) model (9) (**Fig. 8.2**): (i) one of the paralogues lost its function (dis-functionalization); (ii) acquired

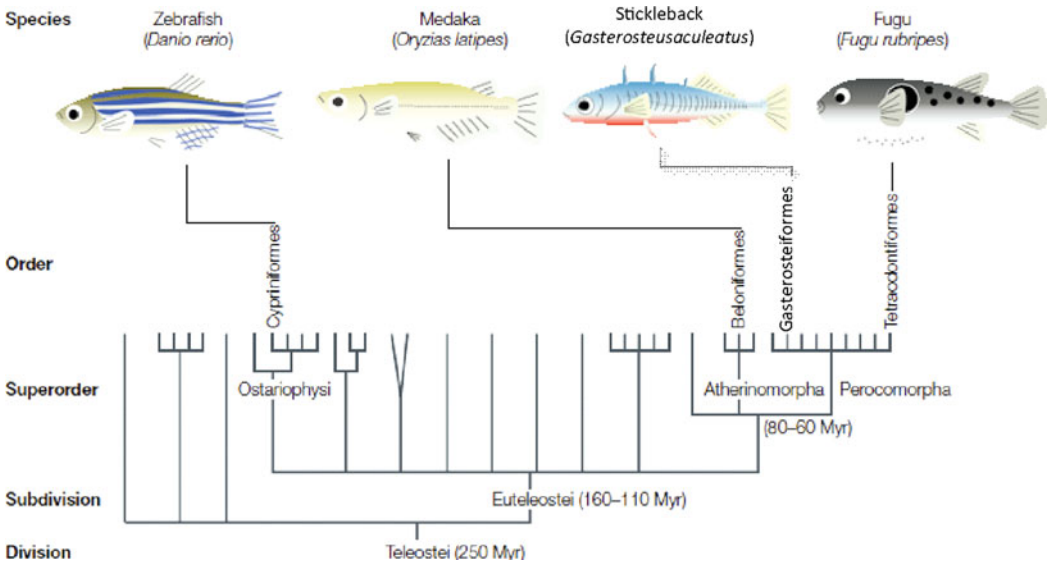

Fig. 8.1. Evolutional relationship of medaka and other fish models. Medaka and zebrafish lineages became separated from their common ancestor between 110 and 160 million years ago (modified from (8)). Stickleback (Platy) is most related to medaka and fugu is more related to medaka than to zebrafish. Therefore, the genome sequence of stickleback and fugu is useful for cloning medaka genes.

a new function (neo-functionalization); or (iii) the paralogues divided the function of the ancestral gene (sub-functionalization) (10, 11). Thus, the analysis of the two fish species allows functions unidentifiable in one species to be uncovered in the other: (i) Phenotypes masked by the two redundant genes can be visible in another species in the case of dis-functionalization. (ii) Mutations in the individual fish paralogues can represent a

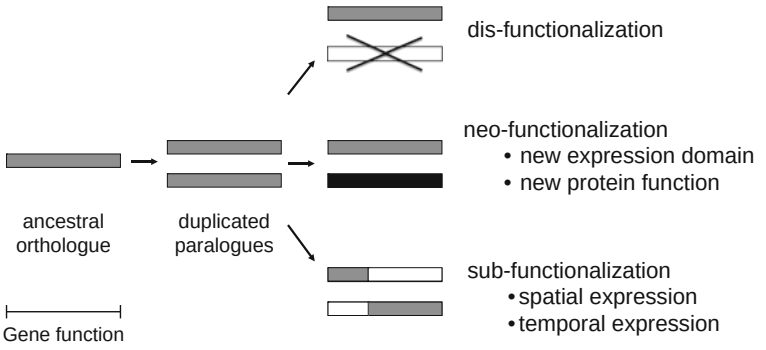

Fig. 8.2. Conceptual representation of rapid evolution of duplicated paralogous genes between medaka and zebrafish. Each bar represents the functions of a given gene according to the duplication–degeneration–complementation (DDC) model (9). In the presence of two paralogues, one of the paralogues is free from evolutional constraints and can evolve into three cases. One of the paralogues can lose its function (dis-functionalization) or gain new functions (neo-functionalization). The two paralogues can divide up the functions of their ancestral orthologue.

subset of the features of the complex mouse phenotype, due to sub-functionalization as a consequence of the genome duplication. Phenotypes of later functions masked by lethality of the embryo due to early gene function can be seen if early and late functions have been divided between the paralogues by sub-functionalization. This sub-functionalization is likely to be different in different teleost species. (iii) New phenotypes can be detected for the gene generated by neo-functionalization. The evolutionary distance between medaka and zebrafish seems ideal for such comparisons (12).

Analysis of mutant phenotypes in the two fish species as well as functional and evolutionary interpretation is relatively straightforward, since medaka and zebrafish embryos develop in a similar manner. Divergent phenotypes between the two species are also due to differences in phenotype manifestation, such as visibility of the target organ and physiology: (i) In live embryos, the liver is more conspicuous in medaka than in zebrafish whereas the opposite is true of the notochord. (ii) Medaka and zebrafish have several species-specific features that are amenable to the genetic studies. Two particularly good examples of these features are sex determination (13) and adult pigment patterning (14), which are best studied in medaka and zebrafish, respectively.

1.2. The Two Fish Models Complement the Mouse

Among the vertebrate model organisms, medaka and zebrafish complement the mouse in genetically dissecting vertebrate development and organogenesis: (i) close observation of development at multiple points facilitates isolation of mutants, since manifestation of phenotypes is dynamic. Transparent bodies and external development of fish embryos allow observation of dynamic cellular behaviours underlying development; (ii) unlike mouse embryos, longer survival of fish mutant embryos with compromised circulation or aberrant development allows isolation of mutants, as exemplified by a large number of zebrafish heart mutants (15, 16); (iii) embryos with weaker phenotypes than the null mutant survive longer and often allow detection of later phenotypes. Chemical mutagens that induce point mutations or anti-sense morpholino oligonucleotides can generate weaker phenotypes. Interspecies differences in lethal mutants between medaka and zebrafish mutants (see sub-functionalization as discussed above) also contribute to identification of later phenotypes (17); (iv) presence of maternal RNA in fish embryos also prevents early lethality of the mutants during gastrulation. Zygotic mutant embryos survive longer in the presence of normal maternal RNA provided by the mother, even if zygotic transcription that starts after the 1,000-cell stage of blastula is compromised.

1.3. Advantages of Medaka as a Model System to Study Development and Organogenesis

Medaka has been established as an experimental animal that allows both genetic and embryological analysis (8, 18) since 1913 when it was used to demonstrate Mendelian inheritance in vertebrates (19). The characteristics of medaka as a model system in comparison to those of zebrafish are summarized in **Table 8.1**. Its long history has embraced rich insights into vertebrate biology, physiology and genetics (18), as well as experimental technologies and useful resources. The former includes sex determination (20) that led to the discovery of the second sex determination gene DMY (21, 22), radiation response (23), tumorigenesis (24, 25) and evolution and development. Development of technologies and resources in medaka allows comparable analyses to those of zebrafish as summarized in **Table 8.1**. These include inbred strains (26), multi-locus tester strains (27), collections of natural populations of wild-type strains with diverse genetic polymorphisms (28), spontaneous mutants (29), the first transgenics in fish (30), a see-through strain that has a transparent body in

Table 8.1
Comparison of zebrafish and medaka as model systems

Characteristics	Zebrafish	Medaka
Generation time	10 weeks	8 weeks
Pair mating in a box	1–2 days	No limit
Eggs	ca. 100/1–2 weeks Fall to the ground	ca. 30/day Attached to belly by filaments
Temp. range for development	24–34°C	4–35°C
Development	Fast	Slower
Rhythmic yolk contraction	No	Occurs at stages 14–28; can be blocked by heptanol
Hatching	2 days	7 days
Swimming	5 days	7 days
Chorion	Soft	Tough with filaments
Embryo	Hardy	Softer
Sex determination	Unknown	XY chromosomes
Inbred strains	No	15 strains
Genome size	1,700 Mb	800 Mb
Genetic polymorphisms	Low (<1/1,000 bp)	High (1/100 bp)
Transgenesis	Yes	Yes
Cryopreservation of sperm	Yes	Yes
Morpholino knockdown	Yes	Yes
Manipulation of embryos	Simple	More involved

adulthood (31), a fine-detail genetic map with polymorphic markers (32), ESTs (33, 34), BAC contigs (35), ES-like cells (36), nuclear transplantation (37), pilot mutagenesis screens (38, 39), BAC library (40), the systematic large-scale mutagenesis screen (2, 41) and the TILLING mutant library for generating gene knockout medaka (42). A draft genome sequence became available owing to inbred strains and a small genome size (800 Mb, one-half that of zebrafish and only twice that of fugu) (43).

Medaka not only complements zebrafish for further genetic dissection of vertebrate genome function but also has unique features facilitating genetic studies: (i) endogenous transposons (44) used for transgenesis in zebrafish (45); (ii) development at low temperatures for identification of temperature-sensitive alleles; (iii) inbred strains (26) that give low phenotypic variation and allow cell transplantation analysis in adults, for example, to validate carcinoma cells; and (iv) reliable storage of frozen sperm for maintaining mutant strains.

2. Materials

2.1. Development of the Embryos and Stock Maintenance

1. Embryo medium: 200 mL 50× stock solution, 1 mL 1% methylene blue in H_2O/10 L reverse osmosis (RO) water (50× stock solution: NaCl 14.7 g, KCl 0.6 g, $CaCl_2 \cdot 2H_2O$ 2.4 g and $MgSO_4 \cdot 7H_2O$ 4.0 g/1 L RO water).
2. High-salt fish medium: 0.3% sea salts in RO water.
3. ZM-100 Small Premium Granular food (ZM Systems).

2.2. Microscopic Observations

2.2.1. Cleaning and Mounting Embryos

1. Heat-polished wide-opening glass Pasteur pipette attached to a pipette pump (Scienceware, USA).
2. Fine waterproof sandpaper—p2000 grit size placed in the lid of a 9 cm Petri dish.
3. 3% methylcellulose in sterile 1× BSS.
4. 1× balanced salt solution (BSS): 20× BSS: 130 g NaCl, 8 g KCl, 4 g $MgSO_4 \cdot 7H_2O$, 4 g $CaCl_2 \cdot 2H_2O$ and 10 mg phenol red in 1 L MilliQ water and autoclave; 500 mM HEPES in MilliQ water autoclaved.

Add 25 mL 20× BSS and 15 mL 500 mL HEPES, pH 7.0, fill up to 500 mL and filter sterilize before use.

2.2.2. Removing the Chorion

All tools should be sterilized with 70% ethanol followed by rinsing with 1× BSS.

1. Sandpaper (p2000 grit size, waterproof).
2. Heat-polished wide-opening glass Pasteur pipette attached to a pipette pump.

3. 20 mg/mL pronase, aliquot (1 mL) and store at –20°C.
4. Hatching enzyme, store at –80°C (**Section 2.2.3**).
5. Embryo medium (**Section 2.1**).
6. 1× BSS (**Section 2.2.1**).
7. Micro-dissecting forceps (55 INOX A, Dumont & Fils, Switzerland).

2.2.3. Making Hatching Enzyme

1. Microhomogenizer with disposable plastic pestles, battery driven (Kleinfeld Labortechnik, Germany).

2.2.4. Mounting Dechorionated Embryos

All tools should be sterilized with 70% ethanol followed by rinsing with 1× BSS.

1. Sterilized 1× BSS.
2. 3% ultra-low gelling temperature agarose solution (Type IX-A, Sigma) in 1× BSS. Store at 4°C.
3. Hair loop.
4. Heat-polished wide-opening glass Pasteur pipette attached to a pipette pump.
5. Petri dish culture chamber, glass base dish 35 mm with 12 mm window (Iwaki, Asahi Techno Glass).
6. 14 cm diameter Petri dish.
7. Tricaine (TMS): 400 mg tricaine in 97.9 mL distilled water and 2.1 mL 1 M Tris–HCl, pH adjusted to 7; 4.2 mL of this solution in 100 mL clean tank water. Should be kept in dark/covered as TMS is light sensitive forming toxic by-products upon light exposure. Store at 4°C.

2.3. Microinjection

1. Agarose injection plate. This plate is made using a plexiglass mould with ridges. When the mould is placed face down into a Petri dish containing 0.5 cm depth of molten agarose the ridges produce troughs in the agarose upon setting. The plexiglass mould can then be removed and the plate covered with embryo medium and stored at 4°C until needed.
2. Microinjection needle (GC100-10, Harvard Apparatus).
3. Micro-needle puller (PP-830, Narishige).
4. Microinjector apparatus (5242, Eppendorf, Germany, or equivalent with the function to clear a clogged needle tip).
5. Embryo medium (**Section 2.1.5**).
6. RNA/DNA mixture.
7. Micro-forceps.
8. Dissecting microscope (MZ12.5, Leica).
9. Micromanipulator (MN151, Narishige).

2.4. Whole-Mount In Situ Hybridization (WISH)

1. Straight-walled polished glass tubes with screw caps (allows easy tracking of embryos) (4 mL sample vial, Wheaton).
2. Heat-polished wide-opening glass Pasteur pipette attached to a pipette pump.
3. 1× phosphate-buffered saline (PBS): 800 mL of distilled H_2O, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4, topped up to 1 L with additional distilled H_2O. pH adjusted to 7.4 and sterilized by autoclaving.
4. Phosphate-buffered saline with 0.1% Tween-20 (0.1% PBST).
5. 4% PFA/PBST.
6. 100% methanol.
7. 6% H_2O_2/PBST.
8. 20 mg/mL Proteinase K, diluted to 10 μg/mL in PBS.
9. Hyb+ buffer: 50% formamide, 5× SSC, 0.1% Tween-20, 50 mg/mL heparin and 5 mg/mL torula (yeast) RNA. Store at –20°C.
10. Probes of choice (see the manufacturer's protocol for the DIG RNA Labeling Mix, Roche).
11. 2× SSC.
12. Anti-DIG-AP Fab fragments (Roche).
13. Blocking solution: 49 mL PBST, 1 mL 2% sheep serum, 0.1 g BSA and 10% sodium azide diluted to 0.02% final concentration in solution.
14. Alkaline-phosphatase (AP) buffer: 100 mM Tris–HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl and 0.01% Tween-20. Make fresh before colour development.
15. NBT/BCIP solution: 4.5 μL NBT (75 mg/mL in 70% DMF/30% H_2O) and 3.5 μL BCIP (50 mg/mL in 100% DMF) in 1 mL AP buffer.
16. 100% ethanol.
17. Glycerol.

2.5. Immunohistochemistry (IHC)

2.5.1. Cryosectioning of Medaka Embryos

1. 4% PFA/PBS.
2. 100% methanol.
3. 100% dimethyl sulphoxide (DMSO).
4. PBS.
5. 30 mM glycine.
6. 10% sucrose with 0.02% azide.
7. 20% sucrose with 0.02% azide.

8. 15% and 25% fish gelatin (Sigma), both with 15% sucrose.
9. Cryostat (CM1850, Leica).
10. Rubber flat embedding moulds (G3690, Agar Scientific).
11. Hair loop.
12. Razorblade.
13. OCT mounting medium (Tissue-Tek).
14. Superfrosted glass slides.

2.5.2. Immunostaining of Medaka Embryo Sections

1. Pap pen (Super PAP PEN mini, Daido Sangyo).
2. 100% acetone.
3. PBS.
4. Blocking solution (**Section 2.4**).
5. Antibodies of choice (primary diluted 1:100–1:2,000 and secondary around 1:250).
6. Mounting medium (VECTASHIELD, Vector Laboratories) and coverslips.

2.5.3. Whole-Mount Immunostaining of Embryos

1. 4% PFA/PBS.
2. 100% methanol.
3. 100% dimethyl sulphoxide (DMSO).
4. PBS.
5. PBST.
6. 0.5% Triton X-100/PBS.
7. Antibodies of choice (primary diluted 1:100–1:2,000 and secondary around 1:250).

2.6. Cell Transplantation

1. Heat-polished wide-opening glass Pasteur pipette attached to a pipette pump.
2. 70% ethanol.
3. 1× BSS.
4. Manual microinjector with 12 mL syringe (Sutter instrument).
5. Depression microscope slides.
6. Petri dish, 9 cm diameter.
7. 3% methylcellulose in sterile 1× BSS.
8. Hair loop.
9. Penicillin–streptomycin premixed stock solution: 10,000 units/mL and 10,000 μg/mL, respectively (Invitrogen)

3. Methods

The best entry point to work with the two species is when you cannot see your expected phenotype deduced from gene expression patterns in either the mutant or morpholino anti-sense oligonucleotide injected embryos. In such cases, one can inject morpholino anti-sense oligonucleotides into the other species with the hope of uncovering the phenotype. A typical workflow and corresponding sections are shown:

1. Data mining and cloning cDNA of gene of interest (two paralogues from each of medaka and zebrafish) (**Section 1**). Stickleback is most related to medaka and fugu is more related to medaka than to zebrafish. Therefore, the genome sequence of stickleback and fugu is often useful for cloning medaka genes (**Section 3.7**).
2. Comparison of expression patterns by in situ hybridization (**Section 3.4**).
3. Gene knockdown or knockout by morpholino injection or targeted mutagenesis (**Section 3.3**).
4. Phenotype analysis of live embryo/fixed embryos by in situ hybridization and antibody staining (**Sections 3.4** and **3.5**).
5. Transplantation to analyse cell autonomy (**Section 3.6**).
6. Identification of additional gene functions of the gene of interest.

Some modifications are necessary to adapt zebrafish experimental techniques (46, 47) to medaka.

3.1. Keeping and Raising Medaka

Medaka can be maintained together with zebrafish in standard zebrafish aquaria with recirculation for maintaining high density of fish. Medaka can also be maintained in still water at low density with weekly exchange of water.

3.1.1. Aquaria System

3.1.2. Water and Room Conditions

1. The water condition of our fish facility is pH 6.8–7.5, 200–450 mS/cm, 26–28°C, NH_4 <0.2 mg/L, NO_2 <0.05 mg/L and NO_3 <20 mg/L. While medaka is hardy and more tolerant to varying water quality than zebrafish, weak inbred strains, such as *HNI* and *Hd-rR*, prefer soft water (300 μS/cm) compared to zebrafish (700 μS/cm). As the natural habitats of medaka are rice fields and streams with little water flow, medaka are stressed in a strong water flow such as that in which zebrafish are kept. Thus, water flow to tanks needs to be adjusted to a minimum.
2. Since medaka is native to East Asia with four seasons, medaka tolerates wide ranges of temperature (4–38°C) and

salinity. The fish room is kept at 25–28°C, with 14 h of light and 10 h of darkness. Medaka spawns better in relatively strong light (at least 100 lux).

3.1.3. Choice of Strains

1. Medaka has two genetically distinct, highly polymorphic populations (Northern and Southern) that are useful for genetic mapping of mutations. The *b* mutant strains of Southern background that have colourless melanophores are used as the standard wild-type strains for most studies since they remain relatively transparent after the pigmentation stage. The *b* strain is strong and highly productive unlike the zebrafish albino strain. Among the *b* strains, the *Cab* strain performs very well in the hard water conditions in European/American countries. The *Kyoto-Cab* line that was established from the *Cab* line for carrying out mutagenesis screens has much fewer background malformation mutations. The isogenic strains were developed for validating tumorigenicity by transplanting into an isogenic host that does not exhibit allogenic immune response (26). However, isogenic strains are less robust and fecund than the *Cab* line.
2. For mapping mutations induced in the Southern strain, crossing with Northern strains such as *HNI* and *Kaga* is necessary. Since the *HNI* strain is isogenic, mapping of mutations is more straightforward and does not suffer from polymorphisms between individual fish used for the mapping cross. However, the *HNI* strain is difficult to maintain in countries with hard water.

3.1.4. Mating and Collecting Eggs

1. Unlike zebrafish, sexually mature female and male medaka can easily be distinguished by the size and shape of the anal and dorsal fins (**Fig. 8.3**). The anal fins of the males are larger and parallelogram shaped compared to that of the

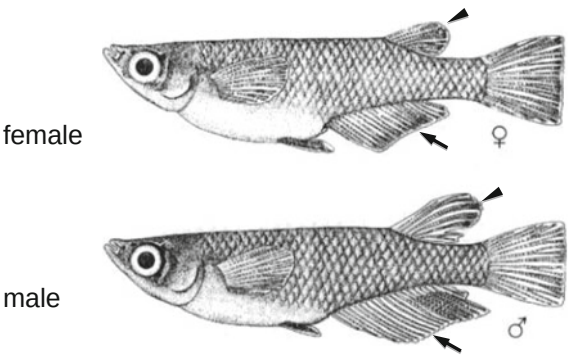

Fig. 8.3. Female and male of the medaka (modified from (14)). The anal or dorsal fin is indicated by an *arrow* or an *arrowhead*, respectively.

females. The female anal fin is smaller and triangle shaped. The male dorsal fin has a clearly visible deep notch between the last two rays.

2. Medaka lay up to 40 eggs every day whereas zebrafish lay up to 100 eggs once in 1 or 2 weeks. Unlike zebrafish, medaka females carry eggs clustered at their belly by attachment filaments for several hours before they are stripped off at plants in the tank.
3. Females can be caught in a net and held inside gently from both sides by one hand. Eggs can then be gently teased from the belly with the index finger of the other hand. The female can then be returned to the tank. Eggs should be transferred to a 6 cm Petri dish filled with embryo media by blunt forceps or a finger. Since medaka eggs adhere to the nets and fingers, care should be taken to avoid contamination of eggs from different sources (e.g. by changing nets).
4. Unlike zebrafish, medaka pairs rarely fight when they are kept in a small mating box. Therefore, they can be kept in one small tank with water flow so that they can be fed and eggs can be collected from the same pair every day for a couple of months.
5. It is important that female fish should not be kept without males, since ovulation occurs every day in medaka females. Otherwise, females cannot deliver eggs and become unwell.

3.1.5. Development of the Embryos

1. When they are laid, eggs are clustered because of attachment filaments on the chorion. To let embryos develop normally, it is necessary to separate eggs. Tangle and cut attachment filaments by holding the attachment filaments with two forceps.
2. After unclustering, eggs are separated from faeces and algae and transferred to fresh embryo medium at a maximum density of 40 eggs per 6 cm Petri dish.
3. Development of medaka is staged according to Iwamatsu's staging (48). Medaka embryos develop slightly slower than zebrafish at 27°C. The timing of hatching is different between the two species; medaka embryos hatch from the chorion in 7 days and immediately start to swim and eat, whereas zebrafish embryos hatch in 2 days but start to swim and eat in 5–6 days.
4. The timing of appearance of organs/tissues is slightly different in medaka compared with zebrafish, i.e. in medaka somitogenesis occurs after the onset of brain development whereas in zebrafish somitogenesis precedes brain development (*see* tables 1 and 2 in 12).

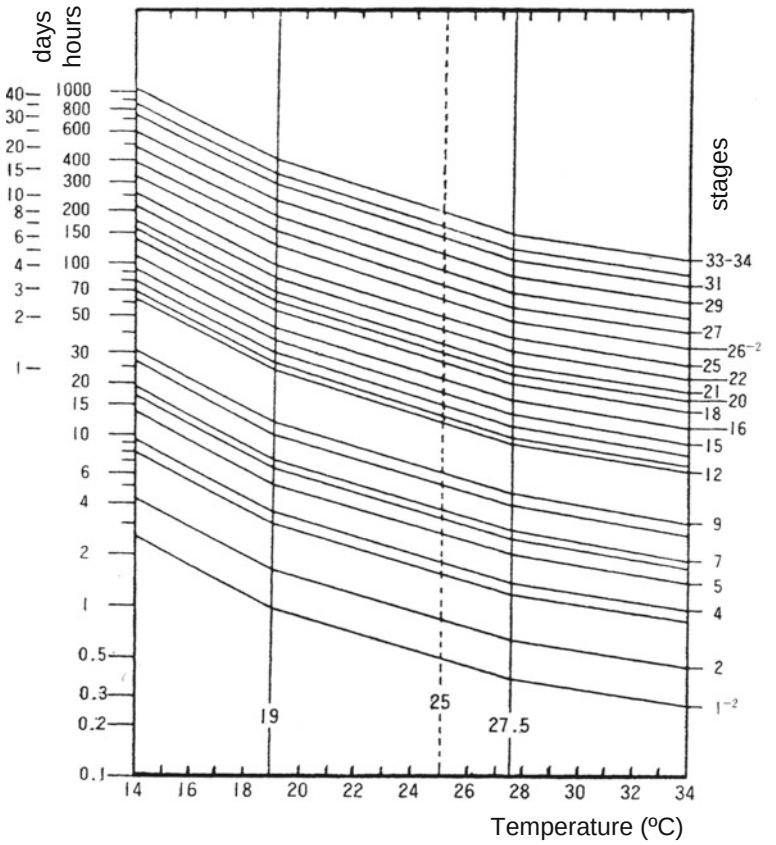

Fig. 8.4. This diagram allows estimation of the duration of time for the development of embryos at the given temperatures (adapted from (53)).

5. The development of medaka embryos can be conveniently adjusted to experimental plans by selecting the appropriate temperature (**Fig. 8.4**). Development of medaka embryos can be stopped at 4°C in early development for a couple of days without an effect on viability. After stage 24 when heartbeat starts, development can be slowed using a minimum temperature of 18°C.

3.1.6. Raising Medaka Larvae

1. When embryos hatch from the chorion, they are transferred to a tank filled with 3 cm depth of high-salt fish medium, which contributes to a higher survival rate.
2. Hatched larvae should be fed with finely powdered dry food, i.e. ZM-100, for 1 week. One week after hatching, larvae are fed with brine shrimp for 1–2 weeks. No water changes are necessary (only compensate for evaporation by adding reverse osmosis (RO) water).

3. Place this fish tank in the fish system with dripping water. Fish are fed with brine shrimp two times a day and once a day with powdered TetraMin (*see* **Note 1**).

3.1.7. Maintaining Stocks

1. Medaka strains can be maintained for a longer period of time if they are kept at 20°C with a 10 h light cycle mimicking winter climate.
2. While snails (Florida freshwater snails) are known to be useful for avoiding algae and maintaining water quality, we do not add snails to the system, as they can become a source of infection. To avoid algae, we minimize the time for bright light in the morning and keep the aquarium at a lower light intensity the rest of the time.
3. We strictly avoid putting in live fish/using live fish food from pet shops (or other fish facilities), since they might be contaminated with various infections. Fish obtained from other fish facilities should be kept for one generation in a separate room to monitor for disease. Eggs are bleached before being transferred to the main aquaria (46).
4. Sperm can be frozen for long-term storage very reliably in medaka (*see* **Note 2**).

3.2. Microscopic Observation of Medaka Embryos

A hard chorion coupled with soft and periodically contracting embryos makes embryological manipulation/observation of medaka embryos more involved than zebrafish. Thus, establishing the following procedure is essential to work with medaka.

For simple observation of medaka development, cleaning up eggs by unclustering and removing hairs from the outer surface of the chorion is sufficient (**Section 3.2.1**). For detailed observation of embryogenesis or imaging in other protocols such as cell transplantation, in situ, or antibody staining, it is necessary to dechorionate embryos (**Section 3.2.2**).

3.2.1. Cleaning Embryos and Mounting for Simple Observation

1. Transfer unclustered eggs (**Section 3.1.5**) with a glass pipette to sandpaper placed in the lid of a 9 cm Petri dish. Remove excess medium but ensure a sufficient volume remains as to prevent drying of embryos.
2. Gently roll embryos on sandpaper using the forefinger, applying minimal pressure and keeping finger parallel to the surface of the sandpaper, for around 45–60 s to remove some of the outer surface hairs of the chorion. Do not roll more than five to seven embryos at once (*see* **Note 3**).
3. Add a small amount of 3% methylcellulose to the centre of a depression glass slide. Transfer cleaned embryos to the depression slide and orientate using forceps.
4. To recover the embryo from methylcellulose after observation, add drops of 1× BSS to loosen the methylcellulose.

3.2.2. Removing the Chorion

The chorion of medaka consists of two protective layers with a hard inner layer and a soft outer surface. Thus, a two-step protease treatment employing pronase and hatching enzyme is necessary to remove this chorion.

Once dechorionated, embryos should be kept in 1× BSS. Semi-sterile conditions will enhance the successful culture of dechorionated embryos, especially when longer periods of observation are required. These include using sterile solutions (e.g. sterilized 1× BSS with antibiotics) and tools sterilized with 70% ethanol followed by rinsing with 1× BSS.

Dechorionated medaka embryos are softer and more fragile than dechorionated zebrafish embryos. Thus, extra care must be taken to ensure they do not contact air or bubbles in the pipette, as this will cause immediate collapse. To ensure minimal damage to embryos, a wide-mouthed heat-polished glass pipette with pipette pump should be used for transferring embryos and a hair loop should be utilized to orientate embryos for observation. Non-adhesive Petri dishes should be used to prevent embryos from attaching to surfaces.

1. Prior to dechorionation it should be checked that eggs have been sufficiently separated and cleaned up (**Sections 3.1.5** and **3.2.1**).
2. Transfer eggs to p2000 sandpaper placed in the lid of a 9 cm Petri dish. Remove excess medium but ensure a sufficient volume remains as to prevent drying of embryos.
3. Gently roll embryos for around 45–60 s to remove some of the outer surface hairs and lightly score the surface of the chorion (as in **Section 3.2.1**). Transfer embryos back to the original Petri dish and examine.
4. Replace egg medium in a dish with 20 mg/mL pronase and incubate embryos for 40–60 min at 27°C (*see* **Note 4**).
5. Recover pronase for reuse and wash embryos five times in embryo medium to remove traces of pronase, as this will inactivate the hatching enzyme to be added.
6. Remove embryo medium and cover embryos with hatching enzyme (**Section 3.2.3**), ensuring embryos sit as a monolayer in the dish. If eggs sit on top of one another in the dish, those at the bottom will be crushed as the chorion dissolves (*see* **Note 5**).
7. Incubate embryos at 27°C and periodically check the progress of hatching using a stereomicroscope (*see* **Note 6**). The entire process of hatching may take 15–60 min.
8. As soon as significantly sized holes appear in the chorion, transfer embryos to a Petri dish containing 1× BSS. Ensure not to transfer hatching enzyme by touching the tip of the

glass pipette onto the surface of the 1× BSS allowing the embryos to gently roll out.

9. Once all embryos are transferred from hatching enzyme, make a final transfer to another fresh dish of 1× BSS (*see* **Note 7**).
10. If embryos need to develop following dechorionation, penicillin/streptomycin should be added to the 1× BSS to prevent bacterial growth.

3.2.3. Preparing Hatching Enzyme

Hatching enzyme is secreted from the hatching gland of medaka just before hatching and dissolves the inner layer of the chorion during development (49). Therefore, hatching enzyme is prepared by homogenizing embryos just before hatching.

1. Collect eggs (we utilize eggs leftover from experiments), clean up (**Section 3.2.1**) and incubate at 27°C until just before hatching. Change egg medium and remove dead eggs every day.
2. Transfer approximately 100 eggs to a 1.5 mL Eppendorf tube and remove as much medium as possible.
3. Grind embryos to a homogenized paste using a plastic pestle. Briefly spin tubes down (10 min at 10,000 rpm) and store at 4°C overnight (*see* **Note 8**).
4. Centrifuge tubes at 10,000 rpm for 10 min at 4°C. Supernatant is stored at –80°C for several months in aliquots in new Eppendorf tubes (up to 0.2 mL).
5. The hatching enzyme is thawed and kept on ice before use. It can be reused and frozen/thawed at –20°C until it loses its activity.

3.2.4. Mounting Dechorionated Embryos

Agarose embedding is useful for longer periods of imaging (e.g. time-lapse imaging) of live embryos as well as for detailed observations of fixed embryos.

During gastrulation and early organogenesis (stages 14–28), medaka embryos exhibit waves of rhythmic contractile movements across the periderm, a tissue layer covering both the developing embryo and the yolk (50). While embryos can be treated with 3.5 mM 1-heptanol to stop contractile movements (51), the concentration of heptanol should be optimized to avoid toxic effects.

To stop movement of embryos after stage 28 (64 hpf), embryos are anaesthetized by adding drops of tricaine (TMS) to the medium before embedding (sufficient for stopping movement but not heartbeat, usually several drops of TMS in a 6 cm dish), as well as to the agarose.

1. Thaw a small amount of 3% low gelling temperature agarose (in 1× BSS) by heating to 45°C and maintain at 30°C.

2. The following steps need to be carried out swiftly to ensure the agarose does not solidify before orientating the embryo. Using a wide-mouthed glass pipette transfer enough molten agarose to fill the depression in a cut cap of an Eppendorf tube.
3. Transfer one dechorionated embryo to the cap depression minimizing carrying over BSS with the embryo. Immediately uptake the molten agarose and embryo from the cap depression and transfer to a Petri dish culture chamber (*see* **Note 9**).
4. Transfer the 3.5 cm Petri dish into a 14 cm diameter Petri dish containing ice and water (to a level roughly one-third the total depth of the 14 cm dish). While holding the chamber down firmly on the bottom of the 14 cm Petri dish, use a hair loop to gently orientate the embryo as desired in the molten agarose. Hold the embryo while the agarose solidifies by gently raising and lowering the smaller dish to its original position in the ice-cold water.

3.3. Microinjection of Medaka Embryos

In medaka, DNA, RNA, morpholino oligonucleotides and tracer dyes are microinjected through the chorion into the cytoplasm of the 1- to 64-cell stage embryo rather than the yolk as in zebrafish. Since the cytoplasm is smaller and less visible in medaka compared to zebrafish, practice in injecting dyes such as rhodamine-dextran is recommended to develop this skill. Addition of phenol red as a visible tracer is also encouraged to allow confirmation of injections.

3.3.1. Making Agarose Plates for Holding Embryos

The embryos are held in troughs made with a plexiglass mould in 1.5% agarose. It may be necessary to adjust the concentration of agarose to hold embryos sufficiently.

1. Make a frame of agarose to hold the plexiglass mould. Pour 1.5% agarose in water to a 0.5 cm depth in a 9 cm Petri dish and wait until it completely solidifies. Cut out an area of the agarose just smaller than the size of the mould.
2. Pour 1.5% agarose to fill the cut area and place the mould (ridged side down) ensuring that no bubbles are trapped. Wait until agarose solidifies. This agarose plate can be covered and stored in the refrigerator (4°C).
3. Remove the mould to create troughs to hold medaka eggs. Add enough embryo medium to immerse the mould.

3.3.2. Injecting Embryos

1. Prepare injection solution with phenol red as a tracer and load it into the needle from the back with an Eppendorf microloader. Connect the needle to the air pump injector ready for injection.

2. Eggs must be collected and unclustered as soon as possible after fertilization to inject at the one-cell stage (*see* **Note 10**). More embryos can be injected by storing them on ice to stop cell division (maximum for 30 min).
3. Transfer eggs into the agarose plate (**Section 3.3.1**) and align them in the troughs with forceps.
4. Place the needle close to the eggs. Open the tip of the needle by gently touching the tip of the needle to the chorion or using micro-forceps.
5. Just before injection, five eggs are rotated so the needle can hit the cell membrane of the one-cell cytoplasm perpendicularly (**Fig. 8.5a**) (*see* **Note 11**).
6. Puncture through the chorion to place the tip of the needle inside of the chorion. Adjust the holding pressure of the injector to be relatively high, to ensure no reflux occurs due to the high pressure encountered after puncture through the tough chorion (settings of 80–100 hPA for holding pressure and 500–700 hPA for injection pressure).
7. Insert the tip of the needle into the cytoplasm by sharply poking the membrane of the egg; otherwise the membrane will not be punctured (*see* **Note 12**).
8. Injected embryos should be transferred to a clean dish containing embryo medium to let them develop properly.

3.4. Whole-Mount In Situ Hybridization (WISH)

This protocol detects gene expression in whole-mount medaka embryos. To detect co-expression of two genes at a single-cell resolution, fluorescent detection is recommended (52). This protocol provides a basis that can be modified for fluorescent detection in zebrafish embryos.

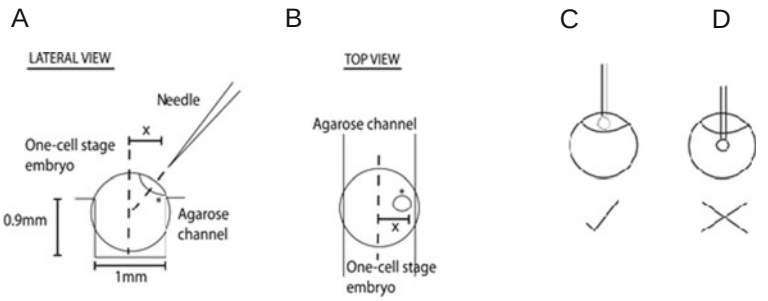

Fig. 8.5. Microinjection into medaka eggs. *Side* (**a**) and *top* (**b**) views of the egg being injected. The egg needs to be rotated so that the needle can poke the membrane of the one-cell perpendicularly (**a**). Actual view through the microscope looks as shown in (**b**). Successfully injected liquid into the cytoplasm does not have clear boundary (**c**), whereas liquid unsuccessfully injected into the cytoplasm has a distinctive border (**d**).

The concentration of the DIG-labelled probe needs to be optimized. Carry out test WISH using 1:10, 1:30 and 1:100 dilutions of the stock probe in 500 μL Hyb+ buffer. ISH involves many washes of the embryos and extra care should be taken to carry these steps out gently so as not to damage the embryos. Unless otherwise stated each stage should be carried out at room temperature and volumes of liquids used should be around three to four times the volume required to sufficiently immerse the embryos. Wash steps require gentle agitation.

Day 1—fixation of embryos:

1. Fix dechorionated embryos in 4% PFA/PBST at 4°C for 2–3 days (*see* **Note 13**).

Day 4—hybridization of probe:

1. Wash embryos 4× in PBST for 5 min each wash.
2. Dehydrate embryos with a methanol series by replacing the PBST gradually to produce solutions of 25, 33, 50, 66, 75, 90 and 100% methanol (*see* **Note 14**).
3. Rehydrate embryos with a reverse of the above methanol series by steadily increasing the PBST concentration in the tube (*see* **Note 15**).
4. Wash embryos twice with PBST for 5 min each time.
5. Embryos later than stage 26 need to be bleached in order to get rid of pigmentation in the skin. Incubate embryos in 6% H_2O_2/PBST for 1–12 h (depending on the developmental stage). Periodically observe embryos every 30 min to check if the embryos have become transparent. Follow with four rinses with PBST for 5 min each time.
6. Permeabilize embryos with 10 μg/mL Proteinase K (in PBST) at 37°C—a smaller volume that just immerses the embryos will be sufficient here (*see* **Note 16**).
7. Wash embryos five times with PBST for 5 min each wash.
8. Refix in 4% PFA/PBST for 2 h at room temperature and then wash embryos in PBST for 5 min five times.
9. Add 1 mL of prewarmed 65°C Hyb+ buffer to the embryos and incubate for 2 h at 65°C (*see* **Note 17**).
10. After 1.5 h of incubation prepare the hybridization mixture (probe diluted in Hyb+ buffer), which also needs to be prewarmed to 65°C (*see* **Note 18**).
11. Remove the prehybridization buffer and add 200 μL of hybridization mix (*see* **Note 19**).
12. Hybridize embryos overnight on a rotator at 65°C (*see* **Note 20**).

Day 5—washes and antibody staining:

13. Remove probe mixture and keep at –20°C for future reuse (*see* **Note 21**).
14. Wash embryos as follows:
    - 66% Hyb+/33% 2× SSC at 65°C for 10 min;
    - 33% Hyb+/33% 2× SSC/33% MilliQ H_2O at 65°C for 10 min;
    - 2× SSC at 65°C for 10 min;
    - Twice with 0.1% SSC/MilliQ H_2O at 65°C for 30 min to 1 h each wash.
15. Remove 0.1% SSC and wash embryos in PBST for 5 min twice.
16. Add blocking solution to embryos and incubate for 1 h. During incubation prepare the anti-DIG-AP antiserum (×5,000 dilution in blocking solution).
17. Incubate embryos overnight at 4°C or for 3–4 h at room temperature in the antibody with gentle agitation.

Day 6—washes and staining:

18. Recover antibody solution and store at 4°C as it can be reused several times.
19. Wash embryos in PBST for 5 h changing the solution every 30 min. During the last wash prepare the AP buffer (*see* **Note 22**).
20. Wash embryos in AP buffer 3× for several minutes and prepare NBT/BCIP solution.
21. Remove AP buffer and incubate the embryos in NBT/BCIP solution in ceramic wells. Monitor staining periodically using a light microscope (*see* **Note 23**).
22. Stop staining by removing NBT/BCIP and washing embryos twice in PBST for 5 min.
23. Optional clearing step to reduce background noise. Wash embryos in
    - 15% EtOH/PBST for 10 min;
    - 80% EtOH/PBST twice for 20 min per wash;
    - 100% EtOH (*see* **Note 24**);
    - 80% EtOH/PBST for 5 min;
    - 15% EtOH/PBST for 5 min.
24. Remove EtOH by rinsing embryos 3× with PBST.
25. Embryos can now be imaged after refixation with 4% PFA/PBST for 1 h at room temperature followed by transferring through a glycerol series (20, 50, 80 and 100%) and mounting on a slide (*see* **Note 25**).

3.5. Immunohistochemistry (IHC) in Medaka

Several factors need to be considered before carrying out IHC:

1. Staining of sectioned or whole-mount embryos:
   (i) Whole mount will be chosen when 3D protein expression information is important.
   (ii) Staining of sections will be chosen if staining needs to penetrate deep tissues or if higher resolution is required.
2. Detection by fluorescence or colour:
   (i) Fluorescence detection provides better resolution in 3D tissue using confocal microscopy, especially in the case of multiple colour staining.
3. Optimization of fixation for the antibody. The following two conditions give considerable differences in staining in our experience:
   (i) 4% PFA/PBS overnight at 4°C;
   (ii) cold 80% MeOH/20% DMSO for 2 h at room temperature.
4. Immunohistochemistry (IHC) can be combined with ISH provided the antibody being used can detect its epitope following Proteinase K treatment. If the epitope is both stable and abundant enough, even three-colour detections (i.e. red, blue and green) are possible, using, for example, a Roche multicolour detection set.

3.5.1. Cryosectioning of Medaka Embryos

1. One of two fixatives can be used to prepare embryos for sectioning following ISH (as outlined above).
2. Wash embryos 3× in PBS for 10 min each wash.
3. Transfer embryos to 10% sucrose with 0.02% azide until they sink. Move to 20% sucrose with 0.02% azide with continuous gentle mixing.
4. After embryos sink, transfer embryos to 15% cold water fish gelatin until they sink followed by immersion in 25% gelatin, again until sinking is seen. This is usually overnight.
5. Gelatin treatment pads out tissues to offer support during sectioning. Embryos can be stored for up to 2 weeks in gelatin at 4°C. Long incubation in sucrose helps to ensure all water is removed from tissues prior to cryosectioning at low temperatures.
6. Set the cryostat to –28°C around 1 h before sectioning is to be done.
7. Cool gelatin on ice and transfer to moulds. Orientate samples in gelatin moulds using a hair loop and freeze on dry ice (approximately 30 min).
8. Remove frozen gelatin (containing sample) from mould and trim with a razorblade as quickly as possible.

9. Quickly fix gelatin blocks to specimen discs using OCT embedding medium. Blocks should be glued to specimen discs by the end of the block not containing the sample so that cutting begins at the end containing the sample.
10. Transfer mounted gelatin blocks to cryostat for 30 min to equilibrate (block will melt at temperatures higher than –14°C).
11. Cut 15 μm sections collecting the ribbon of continual sections on superfrosted glass slides.
12. Slides can be briefly air-dried and stored at –80°C provided they are frozen within 1 h of sectioning.

3.5.2. Immunostaining of Sectioned Medaka Embryos

1. Following sectioning (or defrosting of frozen samples) dry slides at room temperature under a hood overnight.
2. Draw around sections on slide with a pap pen and allow them to dry for several minutes at room temperature.
3. Permeabilize sectioned tissue by placing slides into staining jar with acetone for 30 s followed by a 30 s wash with PBS.
4. Place slides in moist slide chamber and immerse slides in blocking solution for 1 h at room temperature.
5. Remove blocking reagent and add primary antibody. Antibody dilution is antibody dependent but usually ranges from 1:100 to 1:2,000 in blocking solution. Incubate slides at room temperature for 1–2 h.
6. Recover antibody and store at 4°C for reuse. Wash slides with PBS 3× at room temperature for 10 min each wash.
7. Add secondary antibody diluted in blocking solution. Incubate for 1–2 h at room temperature in the dark.
8. Wash slides with PBS three times at room temperature for 10 min each wash in the dark.
9. Remove as much PBS as possible and quickly add mounting medium and a coverslip. Carry out this step in the dark at room temperature. Use clear nail polish to seal the coverslip to the slide.
10. Following observation, slides can be stored at –80°C for a couple of days.

3.5.3. Whole-Mount Immunostaining of Embryos

1. Wash embryos briefly 3× in PBS at room temperature followed by three washes with PBST.
2. Permeabilize embryos in 0.5% Triton X-100/PBS overnight at 4°C with gentle shaking.
3. Carry out three washes with PBST at room temperature to remove all traces of Triton.

4. Incubate embryos in blocking solution for 2 h at room temperature.
5. Remove blocking solution and add primary antibody. Incubate at 4°C overnight.
6. Wash embryos 3× in PBST, 10 min for each wash at room temperature.
7. Add secondary antibody and incubate overnight at 4°C.
8. Wash embryos 3× in PBST, 10 min for each wash at room temperature.
9. Embryos can then be imaged following fixation with 4% PFA/PBS at room temperature for 2 h and mounting.

3.6. Cell Transplantation in Medaka Embryos

The goal of this procedure is to determine whether the gene of interest acts cell-autonomously (within a cell) or non-cell-autonomously (between cells). Preparation of the transplantation needle and assembly of the transplantation setup (**Fig. 8.6**) is described in detail for zebrafish embryos (46) (see also **Chapter 6**, this volume).

Use a wide-mouthed glass pipette with pipette pump throughout this procedure and sterilize all tools (including slides) beforehand with 70% EtOH followed by thorough rinsing with sterile 1× BSS. Recipient embryos are usually developed to around stage 12 as this allows discrimination of the ventral and dorsal poles when carrying out the transplantation.

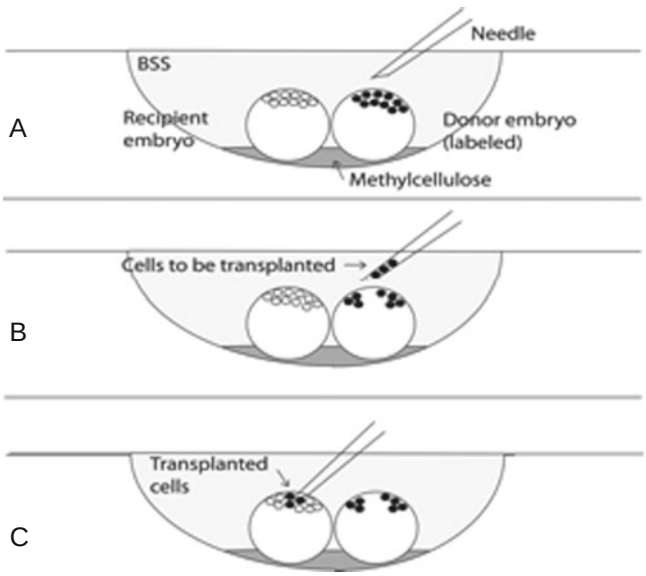

Fig. 8.6. Schematic view of the cell transplantation procedure. The depression slide coated with the 3% methylcellulose at the bottom is filled up with 1× BSS. The donor and recipient embryos are held facing up by the methylcellulose as in (**a**) and cells in the donor are sucked up by the transplantation pipette (**b**) and transferred to the recipient (**c**).

1. Commence dechorionating embryos 1.5–2 h prior to transplantation.
2. Set up injection apparatus and prepare necessary materials.
3. Place a cavity microscope slide into a 9 cm diameter Petri dish.
4. Add a small amount of 3% methylcellulose to the centre of the cavity slide using a sterile pipette tip and spread thinly.
5. Dry methylcellulose for approximately 1–2 min.
6. Add 350 μL of sterile 1× BSS to fill slide depression.
7. Transfer one donor embryo and up to three recipient embryos to the slide using the glass pipette.
8. Orientate embryos using a hair loop so the blastoderm of the embryo is upwards (*see* **Note 26**).
9. Gently insert micro-needle into donor blastoderm and slowly take up 10–20 cells (*see* **Note 27**).
10. Gently insert needle into required area of recipient embryo blastoderm and expel the cells slowly.
11. Repeat steps 9 and 10 for remaining recipient embryos that were transferred.
12. Carefully pour sterilized 1× BSS into the dish as close to the side of the dish as possible so as not to disrupt the embryos. Never pour BSS directly onto the embryos and add sufficient BSS such that the slide and embryos are immersed.
13. Add 100 μL of penicillin–streptomycin to dish and cover. Carefully transfer dish to a 27°C incubator to allow normal development.
14. Repeat steps 3–13 until all embryos are utilized.
15. Embryos can be periodically observed as desired. When using transplantation to carry out gain-of-function and/or phenotype rescue experiments, some morphological changes observed might be due to effects of transplantation. Thus multiple transplantations are necessary. After 2–3 days, melanophores should be present on the yolk sac, head, eyes and trunk. If present on transplanted embryos then a successful chimera has most likely been produced (*see* **Note 28**).

3.7. Useful Resources for Medaka

3.7.1. General Information About Medaka Fish

- Medakafish homepage (http://biol1.bio.nagoya-u.ac.jp:8000/): This site provides a wide variety of useful information about medaka as a model organism, such as strains, phylogeny, genome, genetics, embryology, physiology and ecology.
- National Bioresource project (http://www.shigen.nig.ac.jp/medaka/top/top.jsp): This site provides general

information as well as useful resources available, such as wild-type and mutant strains, cDNAs, BAC fosmid clones, genetic markers and a DNA microarray.

- Medaka Genome Initiative (http://park.itc.u-tokyo.ac.jp/K-medaka/MGI2/MGI.html).

3.7.2. Genome Sequence Database

- UT genome browser medaka (http://medaka.utgenome.org/):
  In addition to the basic information that is mirrored in the ENSEMBL and the UCSC genome browser, additional information can be found, including the genetic markers and PCR primers flanking the genetic markers, single nucleotide polymorphism information between the Hd-rR and HNI strains, BAC/fosmid end sequences anchored on the medaka chromosomes and 5′ serial analysis of gene expression (5′-SAGE) tags of transcription start sites.
- ENSEMBL Medaka (http://www.ensembl.org/Oryzias_latipes/Info/Index):
  Location of the chromosome in ENSEMBL is linked to view the corresponding chromosomal region in the UT genome browser.
- UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=147916543&clade=vertebrate&org=Medaka&db=0):
  The particularly useful feature is the track of the comparative genome information of five teleost species (medaka, stickleback, fugu, tetraodon and zebrafish) for finding conserved elements to find candidate sequences for the promoter/enhancer.
- Keio Medaka Ensembl (LG22) (http://keioensembl.dmb.med.keio.ac.jp/Medaka/):
  High-quality genome sequence of LG22 generated by sequencing of the BAC contigs.

3.7.2.1. EST and Expression Pattern Database

- DFCI medaka: http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=o_latipes
- MEPD: http://ani.embl.de:8080/mepd/

3.7.3. Other Resources

- Anatomy: http://www.shigen.nig.ac.jp/medaka/medaka_atlas/
- Phylogeny: http://www.actioforma.net/nibb/medaka/index.html
- Techniques, medaka book: http://www.shigen.nig.ac.jp/medaka/medakabook/

4. Notes

1. Generation time of medaka is around 6–8 weeks (8–10 weeks in zebrafish).
2. Detailed procedures of sperm freezing and in vitro fertilization can be found at http://www.shigen.nig.ac.jp/medaka/medakabook/index.php?3.3.1%20Cryopreservation%20of%20Medaka%20sperm.
3. This precaution will minimize the risk of crushing embryos beneath each other.
4. Ensure embryos are sufficiently covered by pronase and a lid is present on the dish. Pronase should be kept on ice during this step to minimize self-digestion and can be reused until activity is lost (approximately 1–2 weeks).
5. Since hatching enzyme is a proteinase, it should be kept on ice and exposure of embryos to this enzyme should be minimized.
6. It will be seen that a number of lunar crater-like holes begin to appear in the inner layer of the chorion, which soon dissolves leaving the soft outer layer of the chorion. This outer layer can be easily removed manually.
7. This ensures embryos are not subjected to any remnants of hatching enzyme, as remaining enzyme will damage exposed embryos. Once in this final dish any embryos still possessing the outer layer of the chorion can be manually liberated using sterilized micro-forceps while viewing under the stereomicroscope.
8. This allows separation of hatching enzyme and embryonic debris.
9. A 3.5 cm Petri dish with a glass window at the bottom is used. For imaging using an inverted microscope, embryos are orientated face down and placed close to the cover glass. For imaging using an upright microscope, the thickness of agarose is minimized.
10. Injection will be most successful if injection can be done at stage 0 among the stages that have a one-cell cytoplasm (stages 0, 1, 2a and 2b). The deep and small cytoplasm present at stage 0 ensures less chance to inject into the yolk and an even distribution of injected material. Despite the deep cytoplasm present at stage 2b, injection is less successful due to the difficulty in puncturing the cell membrane unlike at stage 0 when the membrane is easily penetrated.

11. The cytoplasm of the one-cell can be found by looking for an area devoid of small oil droplets, which is opposite to the side of the egg with densest oil droplets. The identified area can be confirmed to be the cytoplasm by viewing from the side.
12. Avoid inserting the tip of the needle into the yolk. Unlike zebrafish, material injected into the yolk will not be transferred into the cytoplasm of the cell. Injected liquids have a distinct boundary when injected into the yolk whereas the boundary is blurred when in the cytoplasm. Occasionally the needle may become blocked by agarose/debris. Forceps can be used to touch/rebreak the end of the needle and remove the blockage. If a major break occurs in the needle at any point during injection, bubbling will be seen in the embryo medium and substance will be lost. Injections should be carried out systematically from top to bottom along agarose channels and from left to right across the plate.
13. PFA fixation for 2–3 days ensures the fluid-filled yolk sac is completely fixed and hardened such that it is not damaged and lost during the subsequent steps of the protocol. If the yolk sac is lost during the procedure it becomes very difficult to see embryos and they may be lost during washes.
14. It should be ensured that embryos have sunk to the bottom of the tube for each concentration of methanol (usually 2–3 min). Gradual dehydration prevents damage to the embryos. Embryos can be stored for 1 week at –20°C in this 100% methanol. Longer storage periods will result in weakened staining.
15. Gradual rehydration prevents the formation of air bubbles within the embryos.
16. This is a critical step for good staining. Since efficacy of Proteinase K is dependent on the batch, optimization of Proteinase K treatment time needs to be carried out for each batch. A large quantity of Proteinase K stock solution can be made (e.g. 50 mL) and frozen in aliquots. Incubation time in Proteinase K is also dependent on embryonic stage at fixation.
17. Prehybridization ensures specific binding of the probe to target sites. Note that embryos can be kept in Hyb+ buffer at –20°C for several months.
18. The concentration of the probe required depends on the type and quality of the probe as well as on the stage of the embryos. It is advisable to test several different probe concentrations to find the optimal level (as outlined above).

19. This volume will sufficiently cover five to seven embryos per tube.
20. As the volume of solution in the tubes is small, extra effort should be taken to ensure the embryos do not dry out, as this will increase background staining. This can be achieved by securing the tubes on the rotator at an angle perpendicular to the direction of rotation.
21. There now follow a series of washes for which the solutions must be prewarmed to 65°C (taking approximately 15 min).
22. AP buffer needs to be made fresh every time.
23. Colour development should be adjusted for imaging in whole mount or section. For the latter, slight over-staining gives a better final signal.
24. This step can be carried out overnight at 4°C to enhance the signal but ensure embryos are not in 100% EtOH for an extended period as this will begin to weaken the signal.
25. Following refixation embryos can be stored at 4°C overnight in preparation for cryosectioning. Here additional antibody staining may be incorporated.
26. Embryos can be carefully leant against each other to further increase stability.
27. Donor cells can be labelled with a tracer dye such as rhodamine-dextran prior to transplantation (*see* **Section 3.3**) or a transgenic strain with GFP expression may be utilized, allowing transplantation to be assessed. A combination of both labelling techniques is often useful to overcome background due to auto-fluorescence. For time-lapse studies following transplantation, GFP expression is particularly useful. Great care should be taken when inserting cells into the recipient blastoderm so as not to disrupt the cell–yolk sac boundary as this will result in death of the embryo.
28. If necessary, genotype the donor embryo(s) by transferring to PCR tube(s) containing 25 μL of 20 mg/mL Proteinase K. Incubate at 55°C for 4 h followed by 10 min at 94°C and carry out PCR.

Acknowledgements

The authors would like to thank Dr. Matthew Harris and Prof. Cheryll Tickle for valuable comments.

References

1. Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., et al. (1996) The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **123**, 1–36.
2. Furutani-Seiki, M., Sasado, T., Morinaga, C., Suwa, H., Niwa, K., Yoda, H., et al. (2004) A systematic genome-wide screen for mutations affecting organogenesis in Medaka, Oryzias latipes. *Mech. Dev.* **121**, 647–658.
3. Hrabe de Angelis, M.H., Flaswinkel, H., Fuchs, H., Rathkolb, B., Soewarto, D., Marschall, S., et al. (2000) Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nat. Genet.* **25**, 444–447.
4. Nolan, P.M., Peters, J., Vizor, L., Strivens, M., Washbourne, R., Hough, T., et al. (2000) Implementation of a large-scale ENU mutagenesis program: towards increasing the mouse mutant resource. *Mamm. Genome* **11**, 500–506.
5. Nolan, P.M., Peters, J., Strivens, M., Rogers, D., Hagan, J., Spurr, N., et al. (2000) A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. *Nat. Genet.* **25**, 440–443.
6. Loosli, F., Staub, W., Finger-Baier, K.C., Ober, E.A., Verkade, H., Wittbrodt, J., et al. (2003) Loss of eyes in zebrafish caused by mutation of chokh/rx3. *EMBO Rep.* **4**, 894–899.
7. Sasado, T., Yasuoka, A., Abe, K., Mitani, H., Furutani-Seiki, M., Tanaka, M., et al. (2008) Distinct contributions of CXCR4b and CXCR7/RDC1 receptor systems in regulation of PGC migration revealed by medaka mutants kazura and yanagi. *Dev. Biol.* **320**, 328–339.
8. Wittbrodt, J., Shima, A., and Schartl, M. (2002) Medaka—A model organism from the Far East. *Nat. Rev. Genet.* **3**, 53–64.
9. Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L., and Postlethwait, J. (1999) Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* **151**, 1531–1545.
10. Ohno, S. (1970) *Evolution by Gene Duplication*. Springer, Heidelberg, Germany.
11. Amores, A., Force, A., Yan, Y.L., Joly, L., Amemiya, C., Fritz, A., et al. (1998) Zebrafish hox clusters and vertebrate genome evolution. *Science* **282**, 1711–1714.
12. Furutani-Seiki, M. and Wittbrodt, J. (2004) Medaka and zebrafish, an evolutionary twin study. *Mech. Dev.* **121**, 629–637.
13. Schartl, M. (2004) A comparative view on sex determination in medaka. *Mech. Dev.* **121**, 639–645.
14. Haffter, P., Odenthal, J., Mullins, M.C., Lin, S., Farrell, M.J., Vogelsang, E., et al. (1996) Mutations affecting pigmentation and shape of the adult zebrafish. *Dev. Gene Evol.* **206**, 260–276.
15. Chen, J.N. and Fishman, M.C. (1996) Zebrafish tinman homolog demarcates the heart field and initiates myocardial differentiation. *Development* **122**, 3809–3816.
16. Stainier, D.Y., Fouquet, B., Chen, J.N., Warren, K.S., Weinstein, B.M., Meiler, S.E., et al. (1996) Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* **123**, 285–292.
17. Rohner, N., Bercsenyi, M., Orban, L., Kolanczyk, M.E., Linke, D., Brand, M., et al. (2010) Duplication of fgfr1 permits Fgf signaling to serve as a target for selection during domestication. *Curr. Biol.* **19**, 1642–1647.
18. Yamamoto, T. (1975) *Medaka, Biology and Strains*. Yugakusya Publ, Tokyo.
19. Toyama, K. (1916) Some examples of Mendelian characters. *Nihon Ikushugaku Kaiho [in Japanese]* **1**, 1–9.
20. Aida, T. (1921) On the inheritance of color in a fresh-water fish, Aplocheilus latipes Temminck and Schlegel, with special reference to sex-linked inheritance. *Genetics* **6**, 554–573.
21. Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., et al. (2002) DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature* **417**, 559–563.
22. Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., et al. (2002) A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. *Proc. Natl. Acad. Sci. USA* **99**, 11778–11783.
23. Egami, N. (1969) Kinetics of recovery from injury after whole-body x-irradiation of the fish Oryzias latipes at different temperatures. *Radiat. Res.* **37**, 192–201.
24. Masahito, P., Aoki, K., Egami, N., Ishikawa, T., and Sugano, H. (1989) Life-span studies on spontaneous tumor development in the medaka (Oryzias latipes). *Jpn. J. Cancer Res.* **80**, 1058–1065.

25. Hyodo-Taguchi, Y. and Matsudaira, H. (1984) Induction of transplantable melanoma by treatment with N-methyl-N′-nitro-N-nitrosoguanidine in an inbred strain of the teleost Oryzias latipes. *J. Natl. Cancer Inst.* **73**, 1219–1227.
26. Hyodo-Taguchi YaE, N. (1985) Establishment of inbred strains of the medaka *Oryzias latipes* and the usefulness of the strains for biomedical research. *Zool. Sci.* **2**, 305–316.
27. Shima, A. and Shimada, A. (1991) Development of a possible nonmammalian test system for radiation-induced germ-cell mutagenesis using a fish, the Japanese medaka (Oryzias latipes). *Proc. Natl. Acad. Sci. USA* **88**, 2545–2549.
28. Sakaizumi, M. (1986) Genetic divergence in wild populations of medaka, Oryzias latipes (Pisces; Oryziatidae) from Japan and China. *Genetica* **69**, 119–125.
29. Tomita, H. (1971) The new body color mutants in the medaka (Abstract, in Japanese). *Zool. Mag.* **80**, 450–451.
30. Ozato, K., Kondoh, H., Inohara, H., Iwamatsu, T., Wakamatsu, Y., and Okada, T.S. (1986) Production of transgenic fish: introduction and expression of chicken delta-crystallin gene in medaka embryos. *Cell Differ.* **19**, 237–244.
31. Wakamatsu, Y., Pristyazhnyuk, S., Kinoshita, M., Tanaka, M., and Ozato, K. (2001) The see-through medaka: a fish model that is transparent throughout life. *Proc. Natl. Acad. Sci. USA* **98**, 10046–10050.
32. Naruse, K., Fukamachi, S., Mitani, H., Kondo, M., Matsuoka, T., Kondo, S., et al. (2000) A detailed linkage map of medaka, Oryzias latipes: comparative genomics and genome evolution. *Genetics* **154**, 1773–1784.
33. Kimura, T., Jindo, T., Narita, T., Naruse, K., Kobayashi, D., Shin-I, T., et al. (2004) Large-scale isolation of ESTs from medaka embryos and its application to medaka developmental genetics. *Mech. Dev.* **121**, 915–932.
34. Lo, L., Zhang, Z., Hong, N., Peng, J., and Hong, Y. (2008) 3640 unique EST clusters from the medaka testis and their potential use for identifying conserved testicular gene expression in fish and mammals. *PLoS ONE* **3**, e3915.
35. Khorasani, M.Z., Hennig, S., Imre, G., Asakawa, S., Palczewski, S., Berger, A., et al. (2004) A first generation physical map of the medaka genome in BACs essential for positional cloning and clone-by-clone based genomic sequencing. *Mech. Dev.* **121**, 903–913.
36. Hong, Y., Winkler, C., and Schartl, M. (1996) Pluripotency and differentiation of embryonic stem cell lines from the medakafish (Oryzias latipes). *Mech. Dev.* **60**, 33–44.
37. Wakamatsu, Y. (2008) Novel method for the nuclear transfer of adult somatic cells in medaka fish (Oryzias latipes): use of diploidized eggs as recipients. *Dev. Growth Differ.* **50**, 427–436.
38. Loosli, F., Koster, R.W., Carl, M., Kühnlein, R., Henrich, T., Mücke, M., et al. (2000) A genetic screen for mutations affecting embryonic development in medaka fish (Oryzias latipes). *Mech. Dev.* **97**, 133–139.
39. Ishikawa, Y. (2000) Medakafish as a model system for vertebrate developmental genetics. *Bioessays* **22**, 487–495.
40. Matsuda, M., Kawato, N., Asakawa, S., Shimizu, N., Nagahama, Y., Hamaguchi, S., et al. (2001) Construction of a BAC library derived from the inbred Hd-rR strain of the teleost fish, Oryzias latipes. *Genes Genet. Syst.* **76**, 61–63.
41. Watanabe, T., Asaka, S., Kitagawa, D., Saito, K., Kurashige, R., Sasado, T., et al. (2004) Mutations affecting liver development and function in Medaka, Oryzias latipes, screened by multiple criteria. *Mech. Dev.* **121**, 791–802.
42. Taniguchi, Y., Takeda, S., Furutani-Seiki, M., Kamei, Y., Todo, T., Sasado, T., et al. (2006) Generation of medaka gene knockout models by target-selected mutagenesis. *Genome Biol.* **7**, R116.
43. Kasahara, M., Naruse, K., Sasaki, S., Nakatani, Y., Qu, W., Ahsan, B., et al. (2007) The medaka draft genome and insights into vertebrate genome evolution. *Nature* **447**, 714–719.
44. Koga, A., Inagaki, H., Bessho, Y., and Hori, H. (1995) Insertion of a novel transposable element in the tyrosinase gene is responsible for an albino mutation in the medaka fish, Oryzias latipes. *Mol. Gen. Genet.* **249**, 400–405.
45. Kawakami, K., Shima, A., and Kawakami, N. (2000) Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. *Proc. Natl. Acad. Sci. USA* **97**, 11403–11408.
46. Nuesslein-Volhard, C.D. (2002) *Zebrafish.* Oxford University Press, New York, NY.
47. Westerfield, M. (1994) *The Zebrafish Book: A guide for the laboratory use of zebrafish (Brachydanio rerio).* Univ. Oregon press, Eugene, Oregon.

48. Iwamatsu, T. (1994) Stages of normal development in the medaka Oryzias latipes. *Zool. Sci.* **11**, 825–839.
49. Yasumasu, S., Iuchi, I., and Yamagami, K. (1989) Isolation and some properties of low choriolytic enzyme (LCE), a component of the hatching enzyme of the teleost, Oryzias latipes. *J. Biochem.* **105**, 212–218.
50. Cope, J., Fluck, R., Nicklas, L., Plumhoff, L.A., and Sincock, S. (1990) The stellate layer and rhythmic contractions of the Oryzias latipes embryo. *J. Exp. Zool.* **254**, 270–275.
51. Rembold, M., Loosli, F., Adams, R.J., and Wittbrodt, J. (2006) Individual cell migration serves as the driving force for optic vesicle evagination. *Science* **313**, 1130–1134.
52. Brend, T. and Holley, S.A. (2009) Zebrafish whole mount high-resolution double fluorescent in situ hybridization. *JoVE* **25**, 1–3.
53. Iwamatsu, T. (2006) *Encyclopedia of Medaka Study (in Japanese)*. University Education Press, Okayama.

Chapter 9

Ex Utero Culture and Live Imaging of Mouse Embryos

Anna Piliszek, Gloria S. Kwon, and Anna-Katerina Hadjantonakis

Abstract

Mouse genetic approaches when combined with live imaging tools have the potential to revolutionize our current understanding of mammalian biology. The availability and improvement of a wide variety of fluorescent proteins have provided indispensable tools to visualize cells in living organisms. It is now possible to generate genetically modified mouse strains expressing fluorescent proteins in a tissue-specific manner. These reporter-expressing strains make it possible to image dynamic cell behaviors in the context of a living embryo. Since mouse embryos develop within the uterus, live imaging experiments require culture conditions that closely mimic those in vivo. Over the past few decades, significant advances have been made in developing conditions for culturing both pre- and postimplantation stage embryos. In this chapter, we will discuss methods for ex utero culture of preimplantation and postimplantation stage mouse embryos. In particular, we will describe protocols for collecting embryos at various stages, setting up culture conditions for imaging and using laser scanning confocal microscopy to visualize live processes in mouse embryos expressing fluorescent reporters.

Key words: Mouse embryo, ex utero culture, live imaging, fluorescent protein, time lapse.

1. Introduction

Over the past 100 years, mouse genetics has been developed into a powerful system for understanding mammalian biology at the molecular level. The mouse is an excellent model organism to study mammalian biology due to its short gestation period, large litter size, small body size, and resistance to infection.

Unlike many other model organisms such as zebrafish and amphibians, which are readily live imaged as they undergo normal development (1), mouse embryos develop within the uterus, making it necessary to closely mimic conditions in the womb during ex utero culture. In combination with the development of

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_9,

fluorescent labeling techniques and advances in microscope technology, mouse embryos can now be live imaged to visualize developmental processes in vitro.

The characterization and cloning of green fluorescent protein (GFP), originally derived from jellyfish, was awarded the Nobel Prize in chemistry in 2008. Indeed, the discovery and popularization of fluorescent proteins combined with the power of mouse genetics provide attractive tools to follow cells in live organisms (2–5). Subcellular-localized fluorescent proteins such as the human histone H2B fusion protein (H2B–GFP) label active chromatin (**Fig. 9.1a**), thereby greatly facilitating cell tracking (6–9), while glycosylphosphatidylinositol (GPI) and other membrane-localized fusion proteins help to visualize cell morphology (10–12). The development of spectrally distinct fluorescent proteins such as the cyan and red fluorescent proteins will facilitate labeling and tracking of different cell populations within the embryo (13–16). More information can be obtained by simultaneously visualizing multiple cellular characteristics, such as cell position and cell morphology, which require the use of multiple subcellularly localized labels. To do this, cells can be dual tagged in various spectral combinations so that they express two fluorescent proteins, for example, one at the plasma membrane and a second in the nucleus (17, 18).

Lineage-specific expression of fluorescent reporters is an invaluable tool for studying mouse development both in wild-type

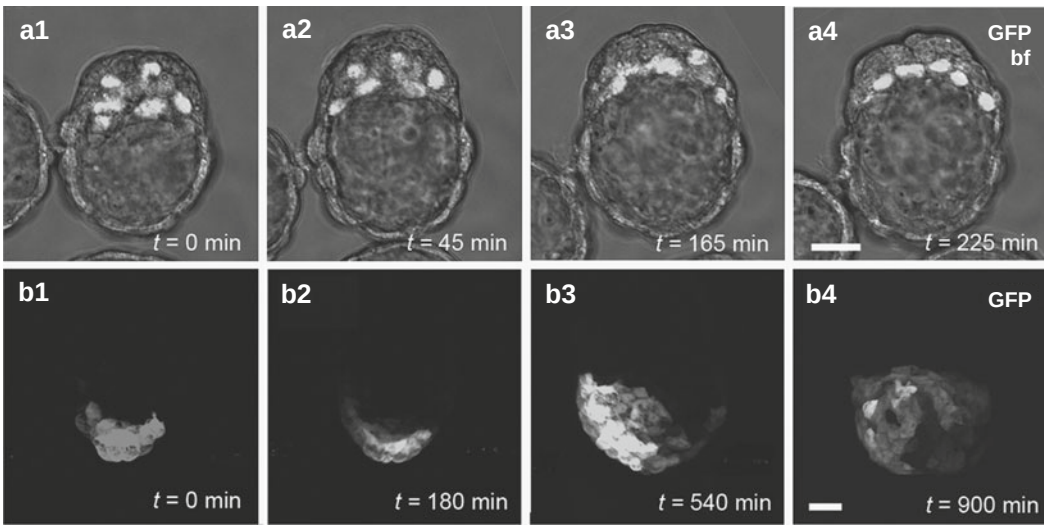

Fig. 9.1. Examples of 3D time-lapse imaging of mouse embryos. (**a1–a4**) Primitive endoderm formation in E3.5–E4.5 embryos; nuclear-localized *Pdgfrα*$^{H2B–GFP}$ labeling the primitive endoderm population provides single-cell resolution and facilitates cell tracking. (**b1–b4**) Anterior visceral endoderm (AVE) migration in E5.5 embryos; cytoplasmic localization of *Hex::GFP* in AVE cells highlights shapes of migrating cells. All panels represent 3D reconstructions of *z*-stacks taken during fluorescence time lapse. Note that anterior is to the left in panels **b1–b3**, and frontal in **b4** (as the embryo has rotated 90 degrees counterclockwise). Scale bar: 20 μm.

and mutant embryos and allows for the observation of gene expression in situ in real time (**Fig. 9.1**) (19–22). Single-cell labeling using fluorescent proteins can be achieved either by injection or electroporation of nucleic acids into individual or groups of cells (23, 24) or by using photomodulatable proteins such as KikGR and activating or converting fluorescent proteins in cells in a region of interest (25).

Imaging in bright-field differential interference contrast (DIC) has provided useful information about the timing and plane of cell division in early preimplantation embryos (26, 27) or somitogenesis in later stage embryos (28). Fluorescence microscopy, while providing a powerful tool for visualizing whole embryos and subcellular structures, introduces the problem of out-of-focus light depending on the thickness of specimens. This problem is partially resolved by image processing and deconvolution techniques. Recently, confocal microscopy has been used extensively in imaging as it optically sections specimens and eliminates out-of-focus light completely.

Laser scanning confocal microscopy excludes light outside the plane of focus making it possible to optically section a sample, which can then be reconstructed into a 3D image with the appropriate software. Laser point scanning confocal microscopes are most commonly used; however, other variants are also commercially available. Slit-scanning confocals (for example, the Zeiss LSM5LIVE) or Nipkow-type spinning disc confocals (for example, the Perkin Elmer UltraView) allow for increased scan speeds and reduced exposure times and may be preferred for high-speed imaging of rapid processes or for samples that are sensitive to phototoxicity. Multiphoton microscopes also minimize exposure times by illuminating only one focal plane at a time (3, 29). These advanced optical imaging modalities, combined with optimized ex utero embryo cultures and reporter-expressing strains of genetically modified mice, provide powerful tools to live image dynamic cell behaviors in situ in embryos.

2. Materials

2.1. Media

Culture and manipulation media are commercially available from several companies. These media can also be manually prepared in the laboratory.

1. M2—preimplantation embryo manipulation (Millipore).
2. KSOM—preimplantation embryo culture (KSOM + AA, Millipore).

3. 95% DMEM/F12 (1:1) (Invitrogen) + 5% newborn calf serum (e.g., Lonza)—postimplantation embryo dissection.
4. DR100, DR75, or DR50—postimplantation embryo culture (*see* **Fig. 9.2** for specific requirements according to the stage of development): rat serum, diluted in DMEM/F12 (1:1) with GlutaMAX (Invitrogen). (DR100 = pure rat serum, DR75 = 75% rat serum in media, DR50 = 50%).
5. Rat serum (although commercially available, the best results are achieved using homemade serum (30)).
   a. Anesthetize rats (preferably large males) with ether or other volatile gas.
   b. Make an incision in the abdomen and expose the dorsal aorta.
   c. Gently collect blood from the aorta (12–15 mL per rat), using a syringe.
   d. Place the tube with the collected blood on ice.
   e. Euthanize the rat.
   f. Centrifuge the blood for 20 min at 1,300×*g*.
   g. Collect the supernatant and remove the pellet.
   h. Centrifuge the serum for 10 min at 1,300×*g*.
   i. Collect the supernatant.
   j. Heat-inactivate the serum for 30 min at 56°C.
   k. Filter the serum with a 0.45-μm filter.
   l. Aliquot the serum and freeze at –80°C for up to 1 year.

2.2. Mice

1. Place one to two female mice in a cage with a single male (to increase efficiency, females can be inspected for estrus before mating). Embryo donors should be at least 6 weeks old.
2. Check females the following morning for the presence of a vaginal plug. The day of plug detection is counted as embryonic day 0.5 (E0.5) since mating is assumed to have occurred at the midpoint of the dark period.
3. Dissect out the oviduct or uterus to collect embryos. Embryos at E0.5–E2.5 are found in the oviduct, while later stage embryos remain in the uterus (*see* **Fig. 9.2** for detailed description) (*see* **Note 1**).
4. Stage early postimplantation embryos according to morphological landmarks (31, 32). The time of dissection should not be used as the criterion for staging of embryos, as a range of stages occur even within a single litter of any given age.

2.3. Microscopes

1. Stereomicroscope with transmitted light and both 20× and 40× magnification.

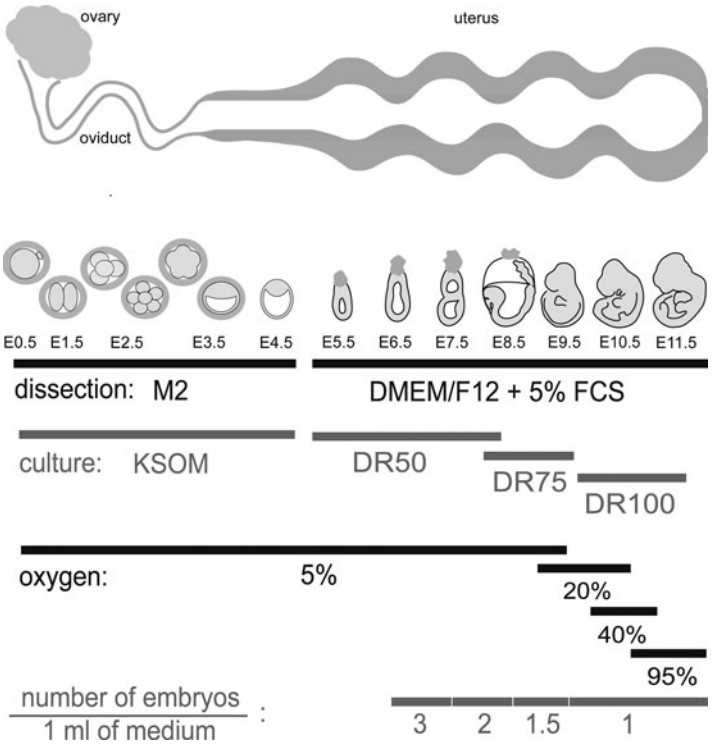

Fig. 9.2. Schematic representation of time–course of mouse embryonic development. Requirements for dissection media, culture media, and gas content for in vitro culture at each embryonic stage and approximate location of embryos in reproductive tract at each stage of development are included. Note that CO_2 concentration is 5% for all stages. Media and gas compositions are the same for both roller and static cultures. DR = rat serum: (DMEM/F12 + GlutaMAX) (*see* **Section 2**); embryos not to scale.

2. Laser scanning inverted microscope with 5×, 10×, 20×, and 40× objectives (for example, PlanApo or PlanNeo objectives). 5× and 10× objectives are usually used dry, 20× objectives are usually used either dry or multi-immersion, and 40× objectives are usually oil or multi-immersion. 5× magnification is used for scanning the field of view to identify and position samples. 10× is used for low-magnification 3D time-lapse image acquisition, and 20× and 40× are used for high-magnification 3D time-lapse imaging. Occasionally a 63× objective may be used for imaging, but in our experience this is too high a magnification for experiments on even the smallest mouse embryos or explants.
3. Computer workstation with image data acquisition and processing software.

2.4. Embryo Culture

1. Humidified CO_2 incubator.
2. Roller apparatus (rotating ~30 rpm) in an incubator chamber (37.5°C). A roller culture apparatus providing a

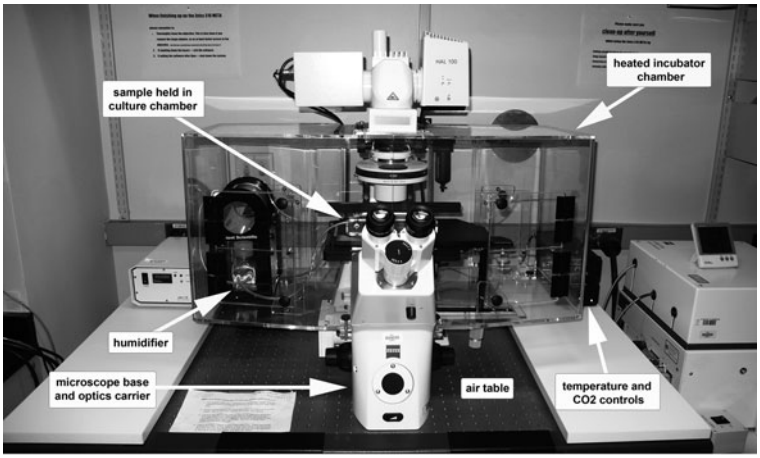

Fig. 9.3. Microscope setup for live time-lapse imaging. Inverted microscope with environmental chamber provides proper conditions for culture and live imaging of mouse embryos.

constant gas supply is recommended (BTC Engineering, Cambridge, UK).

3. On-stage environmental chamber that provides a stable temperature and gas content required for embryo culture (**Fig. 9.3**).
4. Gas mixtures ($CO_2/O_2/N_2$; consult **Section 3** for appropriate selection).
5. Watchmaker's forceps #5 (two pairs, e.g., Roboz) and small surgical scissors (e.g., Roboz) (*see* **Note 2**).
6. 35- and 60-mm plastic Petri dishes.
7. Organ culture dishes—optional (Falcon).
8. 35-mm glass bottom dishes (MatTek) or Lab-tek coverslip bottom chambers.
9. Mouth pipette: assemble from mouthpiece (HPI Hospital Products Med. Tech., 200 μL tip can be used instead), latex tubing (e.g., latex 1/8″ ID, 1/32″ wall, Fisherbrand), and finely drawn glass Pasteur pipette (e.g., Fisherbrand), using 1,000 μL tip as a connector. Pasteur pipette can be hand-pulled over the flame to a diameter of 1.5× the embryo. Pipettes can be siliconized prior to use to prevent embryos from sticking to the glass.
10. Plastic transfer pipettes for moving older embryos (e.g., Fisherbrand). These can be cut to accommodate larger embryos.
11. A 1 mL syringe, 26- or 27.5-gauge needle and blunt 30-gauge needle (cut or blunted with sandpaper or sharpening stone (e.g., Becton Dickinson)).

12. Embryo-tested lightweight mineral oil (Sigma).
13. CoverWell perfusion chamber gaskets (Invitrogen).
14. Human eyelashes, sterilized with 70% ethanol.
15. Suction holding pipette (optional; e.g., Eppendorf CellTram Air).

3. Methods

3.1. Microscope Setup for Culturing and Imaging Mouse Embryos

For live imaging, it is important to combine conditions that allow for embryonic development closely resembling those in utero with a setup that ensures the best image quality. This can be accomplished using an inverted microscope with an environmental chamber setup (**Fig. 9.3**) that provides the stable temperature and gas content required for embryo culture.

1. With the exception of some bright-field contrast microscopy, images can only be acquired through glass coverslips of not more than 1.5 μm thickness (for example, P35G-1.5-14-C MatTek dishes or Lab-Tek coverslip chambers).
2. Since ultraviolet light and laser beams are harmful to the embryos, imaging conditions should be adjusted to ensure proper development of the embryo. Reducing laser power and exposure time by decreasing the frequency of scans, increasing the size of optical sections, or increasing scan speed can help embryos develop while still allowing for the best quality images.

3.2. Culturing and Imaging Preimplantation Mouse Embryos

At the earliest stages of development, mouse embryos float freely along the mother's reproductive tract. Therefore, in vitro culture of preimplantation embryos requires the appropriate media, temperature, and gas conditions closely resembling those in the womb. These culture conditions are now largely established, allowing for the proper timing and development of preimplantation stage embryos.

3.2.1. Collection of Preimplantation Mouse Embryos

1. Before starting the dissection of embryos, equilibrate and pre-warm the KSOM culture media by placing the dish covered with mineral oil for at least 30 min in a humidified incubator at 37.5°C and 5% CO_2 in air.
2. After sacrificing a pregnant female, either (for E0.5–E2.5 embryos) dissect out the oviduct leaving a small part of the distal uterus attached or (for E3.5–E4.5 embryos) remove the entire uterus and place in a drop of pre-warmed M2 media.

3. Place the dish under a stereomicroscope and flush the oviduct/uterus with pre-warmed M2 media. Use a 1 mL syringe with a 26-gauge needle (uterus) or a blunt 30-gauge needle (for the oviduct, insert the needle in the oviduct infundibulum).
4. Collect the embryos and transfer into a previously prepared culture dish using a mouth pipette attached to a pulled Pasteur pipette.

3.2.2. Culturing Preimplantation Mouse Embryos

1. Prepare the culture dish by placing drops (10–100 μL each) of KSOM culture media in the bottom of a 35 mm plastic dish and cover with embryo-tested light mineral oil.
2. Place the dish for at least 30 min in a humidified incubator at 37.5°C and 5% CO_2 in air, to allow for equilibration.
3. Transfer the embryos into the microdrops under oil. Transfer through several drops, to rinse off residual M2 media. Ideally, culture several embryos together, as a higher density of embryos enhances development.
4. Embryos in KSOM media should only be removed from the incubator for minimal periods of time as the bicarbonate buffered media quickly changes pH in the air.
5. Under these conditions, embryos can develop from a zygote to the late blastocyst. If the dissected embryos are at an earlier stage than expected, they can be cultured in vitro until they reach the proper stage without compromising their development, and imaged afterward.

3.2.3. Live Imaging of Preimplantation Mouse Embryos

1. Preimplantation embryos are live imaged under the same conditions as those for static culture. If the levels of CO_2 cannot be reliably maintained, embryos can be imaged short-term at 37.5°C in M2 media instead.
2. Pre-warm the on-stage incubator to 37.5°C before live imaging; this can take from 30 min to several hours, depending on the incubator.
3. Prepare the culture dish by placing a drop of KSOM culture media in a glass bottom dish and covering it with embryo-tested light mineral oil (*see* **Note 3**).
4. Place the dish for at least 30 min in a humidified incubator at 37.5°C and 5% CO_2 in air to allow for equilibration.
5. Transfer the embryos into the equilibrated dish. If possible, culture several embryos together even if only one of them is to be imaged. Place the dish on the microscope stage and immediately provide CO_2.
6. Image the embryos. Minimize embryo exposure to laser light by reducing laser power and exposure time, decreasing the frequency of scans, and/or increasing the size of

optical sections and scan speed. These adjustments should be determined empirically and will depend on the individual microscope and brightness of the fluorophore. In many cases, 2 μm thick optical sections (up to total of ~100 μm, but *see* **Note 4**) taken at 15 min intervals and combined with low laser power give good results.

7. To prevent embryos from drifting out of the imaging plane, make sure the microscope stage is leveled. The amount of media (too much or too little, especially flat drops) may also affect embryo drifting. Placing several embryos together can help to keep them immobile.

3.3. Culturing and Imaging Postimplantation Mouse Embryos

Around E4.0, mouse embryos start to implant in the uterus and begin to form and expand extraembryonic structures, which provide a physical connection with the mother and help support later development. This makes dissection more difficult and creates a unique challenge for ex utero culture once the mother–embryo connection is irreversibly lost. The methods in this chapter describe protocols for embryo culture up until E9.5 (*see* **Notes 1**, **5**, and **6**).

3.3.1. Collection of Postimplantation Mouse Embryos

1. Before starting the dissection, equilibrate and pre-warm the culture media by placing the culture dish covered with mineral oil in a humidified incubator at 37.5°C and 5% CO_2 in air for at least 1 h.
2. After sacrificing the female, dissect out the uterus and place in a dish of pre-warmed (25–30°C) dissecting media (DMEM/F12 + 5% FCS; **Fig. 9.2**).
3. Place the dish under a stereomicroscope, dissect deciduae out of the uterus, and carefully remove embryos from each decidua using watchmaker's forceps. For detailed dissection instructions consult (33) (*see* **Note 1**).
4. Remove/reflect Reichert's membrane from each embryo using watchmaker's forceps. Great care should be taken to avoid damaging embryos in the process of dissection and to ensure that the ectoplacental cone is left intact. Embryos that have been damaged during dissection should not be used for further culture.
5. Immediately after dissection, carefully move the embryos into a dish of culture media with a pipette so that only the smallest amount of dissecting media is transferred.

3.3.2. Roller Culture of Postimplantation Mouse Embryos

Roller culture provides the most optimal ex utero conditions for embryonic development at early postimplantation stages. Using this method, embryos are cultured in controlled temperature and gas conditions and are kept in constant motion.

1. Pre-warm roller culture incubator to 37.5°C before onset of culture.
2. Mix culture media appropriate for the stage of the embryo (**Fig. 9.2**). The amount of media required depends on the stage of the embryo.
3. Equilibrate media with the gas mixture appropriate for the stage of the embryo (**Fig. 9.2**) at 37.5°C for at least 1 h before culture. For a roller culture apparatus that has a constant gas supply, place a small amount of media in the culture bottle within the machine. If this apparatus is not available, blow gas on the surface of the media using a Pasteur pipette and place in an open dish at 37.5°C.
4. Move embryos into roller culture bottles with a pipette. Make sure that only the smallest amount of dissecting media is transferred. If necessary, wash embryos in culture media before moving them into culture bottles.
5. Re-gas the tubes, close tightly, and place in the roller apparatus at 37.5°C.
6. Re-gas the tubes every 12 h (unless constant gas flow is being provided).
7. Replace the media with a newly equilibrated mixture after 24 h.

3.3.3. Static Culture and Imaging of Postimplantation Mouse Embryos

Although roller culture provides the best conditions for ex utero development of postimplantation mouse embryos, it is not suitable for time-lapse imaging. For live imaging, embryos are cultured statically, which allows development to proceed for up to 24 h (34). Generally, the conditions for static culture are the same as for roller culture (**Fig. 9.2**).

1. Pre-warm the on-stage incubator to 37.5°C before live imaging. This can take from 30 min to several hours, depending on the incubator.
2. Prepare culture media appropriate for the stage of the embryo.
3. Prepare the glass bottom culture dish for imaging. Early postimplantation embryos (E5.5–E8.5) are cultured in drops of media covered with embryo-tested light mineral oil (*see* **Notes 4** and **6**). Place the culture dish in a humidified incubator at 37.5°C and 5% CO_2 in air or appropriate gas mixture if available (*see* **Fig. 9.2**) for at least 1 h to pre-warm and equilibrate.
4. Move embryos into the culture dish with a pipette. Make sure that only the smallest amount of dissecting media is transferred. If necessary, wash embryos in culture media before moving them to the culture dish (*see* **Note 7**).
5. After moving the dish containing the embryos to the microscope stage, immediately provide CO_2 (*see* **Note 8**).

6. Image the embryos. Minimize embryo exposure to laser light by reducing laser power and exposure time, decreasing the frequency of scans, and/or increasing the size of optical sections and scan speed. These adjustments will depend on the microscope, brightness of the fluorophore, and developmental stage of the embryo. In many cases, 2 μm optical sections (up to total of ~100 μm, but *see* **Note 4**) taken at

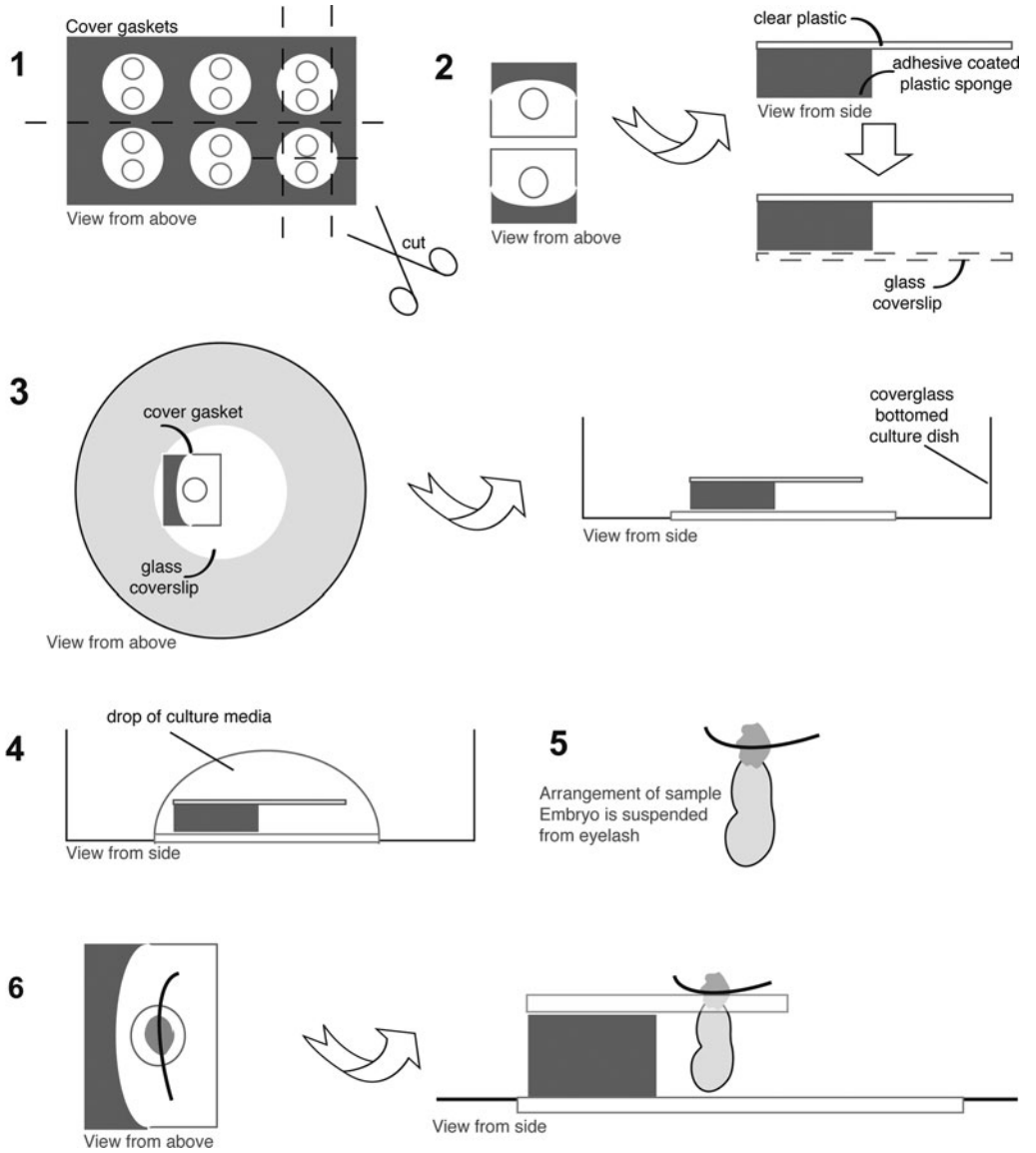

Fig. 9.4. Immobilizing postimplantation embryos using chamber gaskets. (**1**) CoverWell chamber gaskets are cut into small pieces, each containing a silicon body attached to a plastic surface with a hole. (**2**) Bottom plastic surface is removed. (**3**) Such prepared piece of the gasket is placed on a glass bottom dish, silicone part down, and placed on a hot surface for better adhesion. (**4**) Culture media added to the dish. (**5**) Ectoplacental cone of the embryo is pierced with an eyelash. (**6**) The embryo is suspended in the hole of the gasket.

15 min intervals and combined with low laser power give good results. Since embryonic development is easily perturbed by culture conditions or phototoxicity, carefully optimize the conditions on wild-type or heterozygous embryos that do not have a defect before proceeding to analyze mutants with phenotypes.

3.3.4. Immobilizing Postimplantation Embryos

For some experiments (35) postimplantation embryos may need to be imaged on their distal or ventral side. This is done by suspending them in cultures using either a suction holding pipette or a modified chamber such as a CoverWell chamber gasket (**Fig. 9.4**) (35). These gaskets are cut into fragments containing a silicon body attached to a plastic surface with a hole (gaskets of different thicknesses can be used according to embryo size, and the plastic plate can be bent to position the embryo at different angles or to accommodate smaller embryos).

1. Adhere a pre-cut piece of the gasket to a glass bottom dish and place the dish on a hot surface to facilitate adhesion by melting of the plastic (**Fig. 9.4**, steps 1–3)
2. Add culture media to the dish (**Fig. 9.4**, step 4).
3. Pierce the ectoplacental cone of the embryo with an eyelash and suspend the embryo in the hole of the gasket. The embryo should hang from the plastic plate (**Fig. 9.4**, steps 5–6).
4. Cover the media with light mineral oil.
5. Move the embryos to the microscope stage incubator set at 37.5°C and supplied with CO_2.
6. Image the embryos.

4. Notes

1. It is imperative to work quickly and efficiently. Prolonged time on the bench adversely affects embryos and compromises their subsequent culture. Therefore, a balance needs to be struck between speed and care. If you have more than one litter to dissect, sacrifice females one at a time.
2. It is recommended to use a set of coarse tools for the dissection of the uterus from the mouse, then one set of less pristine watchmaker's forceps (#5s) to remove the decidua from the uterus, and a second set of pristine watchmaker's forceps (#5s) for the dissection of embryos from the decidua. Removal of Reichert's membrane requires particularly fine forceps, which can be sharpened whenever necessary with a sharpening stone.

3. If zona-free embryos are to be cultured (for example, after embryo manipulation), coat the glass bottom dish with a small amount of 2% agarose to avoid sticking of the embryo to the dish. At E4.0 mouse embryos begin hatching from the zona pellucida and changing their shape in the process. These changes may cause the embryo to move and obscure the visualization of processes being imaged. To overcome these movements during live imaging, the zona pellucida can be removed beforehand.
4. Embryos at E9.5 can be dissected out of their yolk sac for live imaging. Although it is feasible to culture mouse embryos in vitro beyond E10.5 (36), the size of the embryo and thickness of its tissues make imaging extremely difficult. However, recent reports using multiphoton excitation for live imaging in neonatal and adult mice (37, 38) suggest that the same technique can be used in older mouse embryos to a depth of up to 1,000 μm (39).
5. Successful roller culture of midgestational mouse embryos (E10.5) free of yolk sac and amnion has been reported in serum-free media (36).
6. For imaging at later stages, it is recommended to dissect out the region of interest (such as the ureteric buds (21) or pancreas (40)) and image as an explant culture.
7. Transferring older embryos into oil-covered media may be a problem due to the surface tension of the media and a relatively large diameter of the pipette being used. To address this issue, the media can be equilibrated prior to embryo dissection in an organ culture dish in a humidified incubator. Just before starting the culture, place a drop of the equilibrated media in a glass bottom dish, transfer the embryos into the drop, and cover with mineral oil.
8. It is necessary to replace water in the incubator humidifier bottle periodically, as the water can become contaminated and affect embryo development. The bottle should be rinsed with 70% ethanol and refilled with sterile water.

Acknowledgments

We thank our laboratory colleagues for helping perfect the techniques detailed in this chapter. Work in our laboratory is supported by the National Institutes of Health (RO1-HD052115 and RO1-DK084391), NYSTEM, and The Starr Foundation.

References

1. Keller, P. J., Schmidt, A. D., Wittbrodt, J., and Stelzer, E. H. (2008) Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science* **322**, 1065–1069.
2. Megason, S. G. and Fraser, S. E. (2003) Digitizing life at the level of the cell: high-performance laser-scanning microscopy and image analysis for in toto imaging of development. *Mech. Dev.* **120**, 1407–1420.
3. Hadjantonakis, A. K., Dickinson, M. E., Fraser, S. E., and Papaioannou, V. E. (2003) Technicolour transgenics: imaging tools for functional genomics in the mouse. *Nat. Rev. Genet.* **4**, 613–625.
4. Nowotschin, S., Eakin, G. S., and Hadjantonakis, A. K. (2009) Live-imaging fluorescent proteins in mouse embryos: multi-dimensional, multi-spectral perspectives. *Trends Biotechnol.* **27**, 266–276.
5. Dickinson, M. E. (2006) Multimodal imaging of mouse development: tools for the postgenomic era. *Dev. Dyn.* **235**, 2386–2400.
6. Hadjantonakis, A. K. and Papaioannou, V. E. (2004) Dynamic in vivo imaging and cell tracking using a histone fluorescent protein fusion in mice. *BMC Biotechnol.* **4**, 33.
7. Fraser, S. T., Hadjantonakis, A. K., Sahr, K. E., Willey, S., Kelly, O. G., Jones, E. A., Dickinson, M. E., and Baron, M. H. (2005) Using a histone yellow fluorescent protein fusion for tagging and tracking endothelial cells in ES cells and mice. *Genesis* **42**, 162–171.
8. Plusa, B., Hadjantonakis, A. K., Gray, D., Piotrowska-Nitsche, K., Jedrusik, A., Papaioannou, V. E., Glover, D. M., and Zernicka-Goetz, M. (2005) The first cleavage of the mouse zygote predicts the blastocyst axis. *Nature* **434**, 391–395.
9. Plusa, B., Piliszek, A., Frankenberg, S., Artus, J., and Hadjantonakis, A. K. (2008) Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst. *Development* **135**, 3081–3091.
10. Rhee, J. M., Pirity, M. K., Lackan, C. S., Long, J. Z., Kondoh, G., Takeda, J., and Hadjantonakis, A. K. (2006) In vivo imaging and differential localization of lipid-modified GFP-variant fusions in embryonic stem cells and mice. *Genesis* **44**, 202–218.
11. Chi, X., Hadjantonakis, A. K., Wu, Z., Hyink, D., and Costantini, F. (2009) A transgenic mouse that reveals cell shape and arrangement during ureteric bud branching. *Genesis* **47**, 61–66.
12. Larina, I. V., Shen, W., Kelly, O. G., Hadjantonakis, A. K., Baron, M. H., and Dickinson, M. E. (2009) A membrane associated mCherry fluorescent reporter line for studying vascular remodeling and cardiac function during murine embryonic development. *Anat. Rec. (Hoboken)* **292**, 333–341.
13. Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A., and Tsien, R. Y. (2002) A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 7877–7882.
14. Hadjantonakis, A. K., Macmaster, S., and Nagy, A. (2002) Embryonic stem cells and mice expressing different GFP variants for multiple non-invasive reporter usage within a single animal. *BMC Biotechnol.* **2**, 11.
15. Long, J. Z., Lackan, C. S., and Hadjantonakis, A. K. (2005) Genetic and spectrally distinct in vivo imaging: embryonic stem cells and mice with widespread expression of a monomeric red fluorescent protein. *BMC Biotechnol.* **5**, 20.
16. Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., and Tsien, R. Y. (2004) Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat. Biotechnol.* **22**, 1567–1572.
17. Nowotschin, S., Eakin, G. S., and Hadjantonakis, A. K. (2009) Dual transgene strategy for live visualization of chromatin and plasma membrane dynamics in murine embryonic stem cells and embryonic tissues. *Genesis* **47**, 330–336.
18. Trichas, G., Begbie, J., and Srinivas, S. (2008) Use of the viral 2A peptide for bicistronic expression in transgenic mice. *BMC Biol.* **6**, 40.
19. Jones, E. A., Baron, M. H., Fraser, S. E., and Dickinson, M. E. (2004) Measuring hemodynamic changes during mammalian development. *Am. J. Physiol. Heart Circ. Physiol.* **287**, H1561–1569.
20. Kwon, G. S. and Hadjantonakis, A. K. (2007) Eomes::GFP—a tool for live imaging cells of the trophoblast, primitive streak, and telencephalon in the mouse embryo. *Genesis* **45**, 208–217.
21. Srinivas, S., Goldberg, M. R., Watanabe, T., D'Agati, V., al-Awqati, Q., and Costantini, F. (1999) Expression of green fluorescent protein in the ureteric bud of transgenic mice: a new tool for the analysis of ureteric bud morphogenesis. *Dev. Genet.* **24**, 241–251.
22. Kwon, G. S. and Hadjantonakis, A. K. (2009) Transthyretin mouse transgenes

direct RFP expression or Cre-mediated recombination throughout the visceral endoderm. *Genesis* **47**, 447–455.
23. Meilhac, S. M., Adams, R. J., Morris, S. A., Danckaert, A., Le Garrec, J. F., and Zernicka-Goetz, M. (2009) Active cell movements coupled to positional induction are involved in lineage segregation in the mouse blastocyst. *Dev. Biol.* **331**, 210–221.
24. Perea-Gomez, A., Meilhac, S. M., Piotrowska-Nitsche, K., Gray, D., Collignon, J., and Zernicka-Goetz, M. (2007) Regionalization of the mouse visceral endoderm as the blastocyst transforms into the egg cylinder. *BMC Dev. Biol.* **7**, 96.
25. Nowotschin, S. and Hadjantonakis, A. K. (2009) Use of KikGR a photoconvertible green-to-red fluorescent protein for cell labeling and lineage analysis in ES cells and mouse embryos. *BMC Dev. Biol.* **9**, 49.
26. Sutherland, A. E., Speed, T. P., and Calarco, P. G. (1990) Inner cell allocation in the mouse morula: the role of oriented division during fourth cleavage. *Dev. Biol.* **137**, 13–25.
27. Lehtonen, E. (1980) Changes in cell dimensions and intercellular contacts during cleavage-stage cell cycles in mouse embryonic cells. *J. Embryol. Exp. Morphol.* **58**, 231–249.
28. Flint, O. P. and Ede, D. A. (1982) Cell interactions in the developing somite: in vitro comparisons between amputated (am/am) and normal mouse embryos. *J. Embryol. Exp. Morphol.* **67**, 113–125.
29. Dickinson, M. E., Simbuerger, E., Zimmermann, B., Waters, C. W., and Fraser, S. E. (2003) Multiphoton excitation spectra in biological samples. *J. Biomed. Opt.* **8**, 329–338.
30. Copp, A. J. and Cockroft, D. L. (1990) *Postimplantation Mammalian Embryos. A Practical Approach.* IRL, Oxford.
31. Downs, K. M. and Davies, T. (1993) Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* **118**, 1255–1266.
32. Theiler, K. (1989) *The House Mouse, Atlas of Embryonic Development.* Springer, New York, NY.
33. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003) *Manipulating the Mouse Embryo, A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
34. Jones, E. A., Crotty, D., Kulesa, P. M., Waters, C. W., Baron, M. H., Fraser, S. E., and Dickinson, M. E. (2002) Dynamic in vivo imaging of postimplantation mammalian embryos using whole embryo culture. *Genesis* **34**, 228–235.
35. Yamanaka, Y., Tamplin, O. J., Beckers, A., Gossler, A., and Rossant, J. (2007) Live imaging and genetic analysis of mouse notochord formation reveals regional morphogenetic mechanisms. *Dev. Cell.* **13**, 884–896.
36. Moore-Scott, B. A., Gordon, J., Blackburn, C. C., Condie, B. G., and Manley, N. R. (2003) New serum-free in vitro culture technique for midgestation mouse embryos. *Genesis* **35**, 164–168.
37. Keller-Peck, C. R., Walsh, M. K., Gan, W. B., Feng, G., Sanes, J. R., and Lichtman, J. W. (2001) Asynchronous synapse elimination in neonatal motor units: studies using GFP transgenic mice. *Neuron* **31**, 381–394.
38. Cahalan, M. D., Parker, I., Wei, S. H., and Miller, M. J. (2003) Real-time imaging of lymphocytes in vivo. *Curr. Opin. Immunol.* **15**, 372–377.
39. Theer, P., Hasan, M. T., and Denk, W. (2003) Two-photon imaging to a depth of 1000 micron in living brains by use of a Ti:Al2O3 regenerative amplifier. *Opt. Lett.* **28**, 1022–1024.
40. Puri, S. and Hebrok, M. (2007) Dynamics of embryonic pancreas development using real-time imaging. *Dev. Biol.* **306**, 82–93.

Chapter 10

Detection of Gene Expression in Mouse Embryos and Tissue Sections

Edwina McGlinn and Jennifer H. Mansfield

Abstract

Analysis of gene expression patterns is central to the study of embryonic development. This chapter details methods for detecting gene expression in whole mouse embryos and in tissue sections. The most commonly used methods available in mouse are described and include mRNA in situ hybridization, immunohistochemistry, and detection of enzymatic and fluorescent protein reporters.

Key words: Mouse embryogenesis, in situ hybridization, immunohistochemistry, β-galactosidase, alkaline phosphatase, green fluorescent protein.

1. Introduction

Most studies of embryonic development include analyses of gene expression, either in unperturbed or experimentally manipulated systems. Gene expression patterns can be assessed in a number of ways, depending on the organism and type of information required. For precise spatial and temporal resolution, methods for detection within embryonic tissues (in situ) are most commonly performed; the two main techniques for this are mRNA in situ hybridization and protein immunohistochemistry. mRNA in situ hybridization has the advantages that it can be performed for any gene for which sequence is known and uses a relatively standardized procedure. However, it is somewhat technically challenging and further mRNA expression patterns do not always reflect protein expression and localization. Immunohistochemistry has the advantage of directly detecting protein expression; however, assays must generally be optimized for each antibody, and the

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_10,

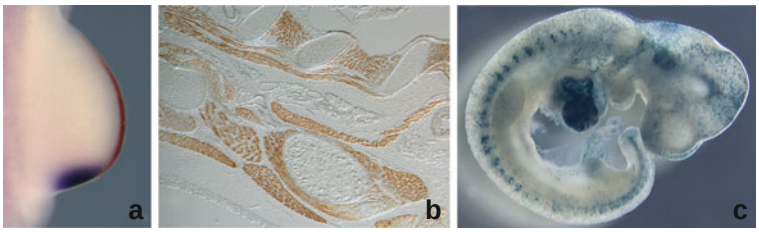

Fig. 10.1. (**a**) Dual-color whole mount in situ hybridization (**Section 3.2.1**). Stage 21 chick embryonic forelimb detecting two independent mRNA transcripts as follows: Sonic hedgehog (*purple*) using DIG-labeled riboprobe followed by NBT/BCIP color reaction; Fgf8 (*brown*) using fluorescein-labeled riboprobe followed by INT/BCIP color reaction (see text). Reproduced with permission from (15). (**b**) Immunohistochemistry in a section (**Section 3.3.2**). Transverse paraffin section in an E14.5 embryo reveals fast skeletal muscle myosin in the limb and intercostal muscles. This 10 μM paraffin section was processed with a monoclonal antibody (MY32, Sigma) followed by an HRP-conjugated secondary antibody and DAB detection. For antigen retrieval, slides were heated to 90°C for 10 min in 0.1 M sodium citrate (see text). (**c**) β-Galactosidase staining in whole mount (**Section 3.4.1**). An E9.5 mouse embryo transgenic for the broadly expressed CAGGS-lacZ transgene shows staining in various cell types and is most highly expressed in the heart and myotome.

availability of antibodies is a major limitation. In mice, well-established methods for making transgenic and targeted knock-out or knock-in animals make the detection of exogenous fluorescent or enzymatic reporter proteins another common read-out of gene expression. Illustration of each of these three types of experiments is shown in **Fig. 10.1**.

The following chapter is divided into four sections: (1) preparation of tissue sections, (2) mRNA in situ hybridization, (3) immunohistochemistry, and (4) reporter detection: β-galactosidase, alkaline phosphatase, and green fluorescent protein. **Sections 2**, **3**, and **4** are further subdivided for separate whole mount and section protocols. For additional resources about these and similar methods, please see also (1–3). Finally, although these protocols are written for mouse, they have been and can be adapted to other vertebrate embryos.

2. Materials

2.1. Preparation of Tissue Sections

2.1.1. Paraffin Sections

1. Phosphate buffered saline (PBS): Prepare as a 10× stock solution, 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4 in dH_2O. Adjust pH to 7.4. Autoclave and store at room temperature. Make 1× PBS by diluting 1 part 10× PBS in 9 parts dH_2O. Store at room temperature.

2. Phosphate buffered saline with 0.1% Tween-20 (PBT): Prepare by adding Tween-20 to a final concentration of 0.1% in PBS. Store at room temperature.
3. 4% paraformaldehyde: Prepare fresh on the day of use from a 20% (w/v) stock solution of paraformaldehyde (Fisher) in PBS. To dissolve, heat to 65–70°C with constant mixing on a stir plate. If necessary, add a drop or two at a time of 10 N NaOH, followed by several minutes of stirring, until the solution clears. Filter through Whatman paper, aliquot, and store at –20°C. Reheat stock to 65°C to redissolve before use. Caution: paraformaldehyde (*see* **Note 1**).
4. Ethanol.
5. Xylene. Caution (*see* **Note 1**).
6. Low melting paraffin wax, such as Paraplast X-Tra (McCormick).
7. Molds and plastic embedding rings (VWR).
8. Superfrost Plus (Fisher) or TESPA-treated slides (*see* **Note 2**).

2.1.2. Oct Cryosections

1. Phosphate buffered saline (PBS) (**Section 2.1.1**).
2. 5% sucrose in PBS (w/v). Dissolve and filter sterilize. Store at room temperature.
3. 30% sucrose in PBS (w/v). Dissolve and filter sterilize. Store at room temperature.
4. OCT compound (Tissue Tek).
5. Peel-away plastic embedding molds (VWR).
6. Superfrost Plus or TESPA-treated slides (*see* **Note 2**).

2.1.3. Gelatin Cryosections

1. Phosphate buffered saline (PBS) (**Section 2.1.1**).
2. 4% paraformaldehyde in PBS (**Section 2.1.1**).
3. 5% sucrose in PBS (w/v). Dissolve and filter sterilize. Store at room temperature.
4. 20% sucrose in PBS (w/v). Dissolve and filter sterilize. Store at room temperature.
5. Gelatin solution: 7.5% gelatin (porcine, Sigma) and 15% sucrose in PBS. To prepare make a 15% (w/v) gelatin solution in PBS and autoclave. Make a 30% (w/v) sucrose in PBS solution and filter sterilize. Mix together in equal proportions. Aliquot and store gelatin solution at 4°C. Heat to 37°C before use.
6. Dry ice/100% ethanol bath.
7. 2-Methylbutane. Caution (*see* **Note 1**).
8. Superfrost Plus or TESPA-treated slides (**Section 2.1.1**).

2.2. RNA In Situ Hybridization

2.2.1. Preparation of Riboprobes

1. DNA template corresponding to gene of interest (*see* **Note 3**).
2. Phenol (pH 8):chloroform:isoamylalcohol (50:49:1) can be purchased premixed or individually. Caution: phenol and chloroform (*see* **Note 1**).
3. RNA polymerase (T7, T3, or Sp6; 20 U/μL) with associated transcription buffer.
4. Digoxigenin (DIG) RNA Labeling Mix (Roche). For alternative labeled nucleotides used in double and triple in situ hybridization (*see* **Note 4**).
5. RNase inhibitor (40 U/μL, Roche).
6. RNase-free DNase (10 U/μL, Roche).
7. Diethyl pyrocarbonate (DEPC)-treated dH_2O. Caution: DEPC (*see* **Note 1**).
8. 3 M NaOAc (pH 5.2) made with DEPC-treated dH_2O.
9. 100% ethanol and 70% ethanol made up by adding 7 parts of ethanol to 3 parts of DEPC-treated dH_2O.

2.2.2. Whole Mount In Situ Hybridization

1. Phosphate buffered saline (PBS) (**Section 2.1.1**).
2. Phosphate buffered saline with 0.1% Triton X-100 (PBTX). Prepare by adding Triton X-100 to a final concentration of 0.1% to treated PBS. Store at room temperature.
3. 4% paraformaldehyde/0.2% glutaraldehyde in PBS. Prepare fresh on the day of use from 20% paraformaldehyde and 25% glutaraldehyde frozen stocks as described in **Section 2.1.1**. Glutaraldehyde can be purchased as a 25% solution.
   Caution: paraformaldehyde and glutaraldehyde (*see* **Note 1**).
4. Methanol. Caution (*see* **Note 1**).
5. H_2O_2 solution: 6% H_2O_2 in PBTX (make by diluting 30% stock H_2O_2). Prepare immediately before use.
6. Proteinase K (10 mg/mL, Roche).
7. Pre-hybridization/hybridization solution: 50% formamide, 5× SSC (pH 7.0), 2% blocking powder (Roche #1096176), 0.1% Triton X-100, 0.5% CHAPS (3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid), 1 mg/mL yeast RNA, 5 mM EDTA, and 50 mg/mL heparin made up in dH_2O. Store at –20°C and preheat to 65°C immediately prior to use. Caution: CHAPS, formamide (*see* **Note 1**).
8. Solution 1: 50% formamide, 5× SSC, 0.5% CHAPS, and 0.1% Triton X-100 made up in dH_2O. Store at RT and

preheat to 65°C immediately prior to use. Caution: formamide (*see* **Note 1**).

9. Tris-buffer with Triton X-100 (TBTX): 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Triton X-100 made up in dH_2O and store at room temperature.
10. Antibody blocking solution: 10% serum (Sigma) and 2% BSA in TBTX. We commonly use sheep, horse, or donkey serum. Prior to use, serum should be heat inactivated at 70°C for 30 min and stored at –20°C.
11. AP-conjugated anti-digoxigenin (DIG) antibody (Roche). For double and triple in situ hybridization, AP-conjugated anti-fluorescein and AP-conjugated anti-biotin antibodies are required.
12. NTMT buffer: 100 mM NaCl, 100 mM Tris-HCl pH9.5, 50 mM $MgCl_2$, and 0.1% Tween-20 in dH_2O. NTMT is prepared fresh each time; it will acidify over time.
13. Nitroblue tetrazolium chloride (NBT) stock: 100 mg/mL in 70% DMF. Store at –20°C.
14. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) stock: 50 mg/mL in dH_2O. Store at –20°C.
15. 2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride (INT) stock: 50 mg/mL in DMSO. Store at –20°C.

2.2.3. Section In Situ Hybridization

1. Phosphate buffered saline (PBS) (**Section 2.1.1**).
2. Phosphate buffered saline with 0.1% Tween-20 (PBT) (**Section 2.1.1**).
3. Hybridization solution: 10 mM Tris-HCl pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.25% SDS, 10% dextran sulfate (American Bioanalytical 50% solution), 1× Denhardt's, 200 mg/mL yeast tRNA (Gibco), 50% formamide. Store at –20°C. Caution: SDS and formamide (*see* **Note 1**).
4. Flexible plastic coverslips cut from polypropylene bags (BelArt).
5. SSC: 20X stock pH 7.0.
6. 10× triethanolamine (TEA): 1 M TEA, pH 8.0. Caution (*see* **Note 1**).
7. Acetic anhydride (Sigma). Caution (*see* **Note 1**).
8. TNE: 10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA.
9. MAB (5×): 0.5 M maleic acid, 0.75 M NaCl, bring to pH 7.5 with NaOH. Make 1× MABT by diluting 1 part 5× MAB in 4 parts dH_2O and add Tween-20 to a final concentration of 0.1%. Store at room temperature.

10. Antibody blocking solution: 20% sheep serum and 2% Boehringer Blocking Reagent (Roche) in MABT, heat to 55°C to dissolve. Prior to use, serum should be heat inactivated at 70°C for 30 min, aliquoted, and stored at –20°C.
11. NTM (pH 9.5): 100 mM NaCl, 100 mM Tris-HCl pH 9.5, and 50 mM $MgCl_2$.
12. NTM (pH 8): 100 mM NaCl, 100 mM Tris-HCl pH 8 and 50 mM $MgCl_2$.
13. Nitroblue tetrazolium chloride (NBT) stock: 100 mg/mL in 70% DMF. Store at –20°C.
14. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) stock: 50 mg/mL in dH_2O. Store at –20°C.
15. 2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride (INT) stock: 50 mg/mL in DMSO. Store at –20°C.
16. Fast red tablets (Sigma).
17. Gelvatol or other aqueous mounting media. Gelvatol is prepared essentially as described in (4): To make 1 L, dissolve 125 g PVA (AIRVOL 205 Polyvinyl Alcohol, Air products, Allentown, PA) in 500 mL 0.14 M NaCl, 10 mM phosphate buffer pH 7.2 (50× phosphate buffer stock is made by adding 0.5 M KH_2PO_4 to 0.5 M Na_2HPO_4 until pH reaches 7.2). Stir overnight, then microwave to >90°C (do not boil), then continue to stir at room temperature until solution cools. Add 500 mL glycerol and stir until completely mixed. Allow to rest at room temperature overnight, remove any solids, then aliquot, and store at 4°C. Heat to 65°C before use.

2.3. Immunohistochemistry

1. Phosphate buffered saline (PBS) (**Section 2.1.1**).
2. Phosphate buffered saline with 0.1% Tween-20 (PBT) (**Section 2.1.1**).
3. 4% paraformaldehyde (**Section 2.1.1**). Caution: paraformaldehyde (*see* **Note 1**).
4. Dent's bleach: 10% H_2O_2, 13% DMSO, in methanol. Make fresh on the day of use. H_2O_2 stock solutions (usually 30%) can be stored for several months at 4°C, protected from light. H_2O_2 can be omitted if not detecting with a peroxidase. Caution: methanol (*see* **Note 1**).
5. Blocking solution: 5% serum (Sigma) in PBT; 20% DMSO can be added to increase permeability. Optional: Add 2% Boehringer blocking reagent (Roche). Ideally, serum should be from the same species that the secondary antibody was raised in. Prior to use, serum should be heat inactivated at 70°C for 30 min, aliquoted, and stored at –20°C.

6. Primary antibody of interest.
7. HRP-conjugated secondary antibody against species primary antibody was raised in (Jackson ImmunoResearch). Alternative methods using AP or fluorophore-conjugated secondary antibodies are described in **Section 3.3.1**.
8. DAB solution: 1 mg/mL diaminobenzidine in 100 mM Tris-HCl pH 7.2. We use DAB tablets (Sigma), which eliminate the need for weighing out the powder, which is highly toxic. One tablet should be dissolved immediately before use. Caution: diaminobenzidine (*see* **Note 1**).
9. H_2O_2 solution: 0.04% H_2O_2 in water (make by diluting 30% stock H_2O_2). Prepare immediately before use.
10. Detection buffer: Immediately before use, mix equal parts of DAB solution and H_2O_2 solution.
11. BABB: Mix one part of benzyl alcohol with two parts of benzyl benzoate. Store in a fume hood at room temperature (for whole mount only). Caution: benzyl alcohol and benzyl benzoate (*see* **Note 1**).
12. Slide staining tray (*see* **Note 5**, for sections only).
13. Pap (hydrophobic) pen (Invitrogen; for sections only).
14. Gelvatol (for sections only; preparation described in **Section 2.2.3**).

2.4. β-Galactosidase Detection

1. Phosphate buffered saline (PBS) (**Section 2.1.1**).
2. Phosphate buffered saline with 0.1% Tween-20 (PBT) (**Section 2.1.1**).
3. Fixation buffer: 2% paraformaldehyde and 0.2% glutaraldehyde in 1×PBS. Make fresh on the day of use from 20% paraformaldehyde and 25% glutaraldehyde frozen stocks. Caution: paraformaldehyde and glutaraldehyde (*see* **Note 1**).
4. 1 M sodium phosphate buffer (NaP buffer). Add 1 M NaH_2PO_4 (pH 4.0) stock solution to 1 M Na_2HPO_4 (pH 8.8) stock solution until the solution reaches pH 7.4 (approximately 150 mL NaH_2PO_4 per liter Na_2HPO_4 (pH 8.8)). Autoclave and store at room temperature.
5. β-Gal washing buffer: 100 mM NaP buffer, 2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.2% nonidet P-20. Filter sterilize and store at room temperature.
6. β-Gal detection buffer: 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/mL X-gal in β-gal washing buffer. Make immediately before use from stock solutions of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (each solution is 0.5 M, in dH_2O, stored at room temperature and protected from light).
7. Gelvatol (for sections only; preparation described in **Section 2.2.3**).

2.5. Alkaline Phosphatase Detection

1. Phosphate buffered saline (PBS) (**Section 2.1.1**).
2. Phosphate buffered saline with 0.1% Tween-20 (PBT) (**Section 2.1.1**).
3. 4% paraformaldehyde in PBS (**Section 2.1.1**). Caution: paraformaldehyde (*see* **Note 1**).
4. NTM buffer: 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$. Optional: 0.1% Tween-20 can be added; Tween will not only increase the reaction rate but can also increase the rate of background color development.
5. AP detection buffer: Immediately before use, add to NTM (or NTMT) buffer: 4-nitroblue tetrazolium chloride (NBT, Roche) to a final concentration of 0.45 mg/mL and 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt (BCIP, Roche) to a final concentration of 0.175 mg/mL.
6. Gelvatol (for sections only; preparation described in **Section 2.2.3**).

2.6. EGFP Detection

1. Phosphate buffered saline (PBS) (**Section 2.1.1**).
2. Optional: 4% paraformaldehyde in PBS (**Section 2.1.1**). Caution: paraformaldehyde (*see* **Note 1**).
3. Gelvatol (for sections only; preparation described in **Section 2.2.3**).

3. Methods

3.1. Preparation of Tissue Sections

To achieve cellular resolution of gene expression with any of the following techniques, tissues can be embedded in paraffin or cryoembedded in OCT or gelatin and sectioned. Sectioning can be done before or after detecting gene expression, and different methods are best for different applications. In general, paraffin sections produce the best morphology. OCT cryosections produce the highest signal for in situ hybridizations and for some immunohistochemistry reactions. Gelatin cryosections produce relatively good morphology and are recommended for sectioning tissues stained previously in whole mount. Please see notes within each of the following protocols about which sectioning method(s) are best. The embedding protocols detailed here are courtesy of Constance Cepko and Clifford Tabin (Harvard Medical School, Boston, MA, USA).

3.1.1. Paraffin Sections

1. Dissect embryos out in ice-cold PBS and transfer to glass vials (*see* **Note 6**).

2. Incubate embryos in 4% paraformaldehyde at 4°C, rocking, overnight (*see* **Note 7**). For younger embryos (<E12.5) this step should be shorted to 1–2 h at 4°C. Over-fixation of embryos or incomplete dehydration may lead to crumpled sections.
3. Rinse embryos twice in PBS.
4. Dehydrate embryos through a graded ethanol series with 10 min washes rocking at room temperature, as follows:
   25% ethanol/PBS
   50% ethanol/PBS
   75% ethanol/dH_2O
   100% ethanol
   100% ethanol
   For older embryos (>E12.5), increased wash times and additional incremental increases between 75 and 100% are beneficial.
5. Optional: Embryos may be stored at –20°C in 100% ethanol at this stage for several months at least.
6. Clear embryos by incubating in xylene twice for 5–30 min (dependent on embryo size), rocking at room temperature. Both over- and under-treatment with xylene may lead to brittle, crumpled sections.
7. Incubate embryos in 50:50 xylene:paraffin for 15 min at 60°C.
8. Incubate embryos in 100% paraffin three to five times 1 h at 60°C.
9. Incubated embryos in paraffin, at 60°C, under a vacuum, for at least 1 h.
   Steps 8 and 9 can be extended overnight if necessary; however, if problems with in situ signal strength are observed then strict adherence to same day processing may be helpful.
10. Embed embryos in molds with embedding rings and store blocks at 4°C until needed.
11. Section and collect on Superfrost Plus or TESPA-treated slides (*see* **Note 2**).
12. Dry sections overnight at 37°C and store at 4°C until needed.

3.1.2. Oct Cryosections

1. Dissect embryos out in ice-cold PBS and transfer to glass vials (*see* **Note 6**).
2. Incubate embryos in 4% paraformaldehyde several hours to overnight, at 4°C (*see* **Note 7**).

3. Wash embryos in PBS 3 × 5 min.
4. Transfer embryos to 5% sucrose/PBS and rock at 4°C until embryos sink.
5. Incubate embryos in 30% sucrose/PBS, rocking overnight at 4°C.
6. Incubate embryos in 50:50 30% sucrose/PBS:OCT mounting media, gently rocking until solution is homogenous. Prepare 50:50 30% sucrose/PBS:OCT mounting media early.
7. Embed embryos in OCT, in peel-away plastic molds, on dry ice and store blocks at –80°C.
8. Section and collect on either Superfrost Plus or TESPA-treated slides.
9. Air dry sections 20 min to 3 h and store sections at –80°C.

3.1.3. Gelatin Cryosections

1. Dissect embryos or tissues into ice-cold PBS and transfer to glass vials (*see* **Note 6**).
2. Incubate in 4% paraformaldehyde several hours to overnight (*see* **Note 7**).
   If tissue has already been processed in whole mount, skip steps 1–2.
3. Wash embryos in PBS, once quickly, then 3 × 5 min rocking, at room temperature.
4. Incubate embryos in 5% sucrose/PBS until embryos equilibrate (until they sink, rocking at 4°C).
5. Incubate embryos in 20% sucrose/PBS until embryos equilibrate, rocking at 4°C.
6. Incubate in pre-warmed gelatin solution at 37°C for 1.5–4 h (depending on the stage of the embryo).
7. Change to fresh gelatin solution and embed embryos in peel-away plastic molds (VWR). Place molds on a flat layer of wet ice and position embryos under a dissecting microscope. It is helpful to allow a thin layer of gelatin to partially solidify in the bottom of the mold before adding the embryo, so that the embryo would not be right at the surface of the block. However, if layer becomes too solid, then block can crack at the interface during freezing.
8. Leave blocks on ice until gelatin is set, but do not allow to freeze.
9. Store blocks at 4°C, in a humidified box, to completely set the gelatin (overnight to 2 days).
10. Prepare a dry ice/ethanol bath, place a beaker of 2-methylbutane in the bath, and allow to cool.
11. Peel-away the plastic molds and cut blocks to size with a razor blade. Fold strips of thin cardboard or oak tag into

an L shape. Place gelatin block in the bottom of the L with the cutting surface up.

12. Lower blocks on cardboard handles into 2-methylbutane. Immerse until frozen (around 5–10 s) but do not overfreeze, as blocks may crack.
13. Store blocks at –80°C until use, but no longer than 2 weeks.
14. Section blocks on a cryostat, collect on Superfrost Plus or TESPA-treated slides (*see* **Note 2**), and air dry briefly.
15. Store slides at –80°C until use.

3.2. RNA In Situ Hybridization

The ability to detect RNA transcripts within the developing embryo relies on a stable hybridization reaction between a labeled anti-sense RNA probe (riboprobe) and the RNA transcript of interest. To interrogate gene expression within the context of the entire embryo, whole mount in situ hybridization is performed, and the protocol detailed here is based on early methods (5) with slight modifications (6). Multiple RNA transcripts can be detected simultaneously, as shown in **Fig. 10.1a**. To obtain cellular resolution of gene expression, section in situ hybridization is performed, and the protocol detailed here is courtesy of Constance Cepko and Clifford Tabin (Harvard Medical School, Boston, MA, USA). Both protocols utilize colorimetric detection of the riboprobe, however, can be substituted with fluorescent detection if required. The detection of small RNA species such as mature microRNAs requires protocol optimization, particularly at the level of tissue fixation, probe design, and hybridization temperature. These alterations to core in situ protocols are beyond the scope of this chapter, however, useful references include (7–9).

Before embarking on this technically challenging procedure, it is recommended to search various publically available gene expression databases in which a vast number of developmental expression patterns are catalogued. Such databases include EMAGE (http://www.emouseatlas.org/emage/), MGI (http://www.informatics.jax.org/), and Genepaint (http://www.genepaint.org/).

3.2.1. Riboprobe Generation

Considerations for choice of riboprobe template sequence are discussed (*see* **Note 3**). Generation of DNA template for riboprobe synthesis can be achieved by either plasmid linearization or PCR amplification.

3.2.1.1. Template Generation by Plasmid Linearization

1. Linearize 1 μg of plasmid with an appropriate enzyme, preferably to give a 5'-overhang (if a 3′-overhang is generated, blunt using the 3′-exonuclease activity of Klenow). For considerations related to labeling choices when using multiple probes, *see* **Note 4**.

2. Run 50 ng on a 1% agarose gel to check digest is complete. Important: All steps from now on should be carried out in RNase-free (RF) conditions (*see* **Note 8**).
3. Dilute to 200–500 μL with DEPC-treated dH_2O.
4. Add an equal volume of phenol (pH 8):chloroform: isoamylalcohol (50:49:1), vortex, and centrifuge 2 min.
5. Remove upper layer and place in a new tube.
6. Add an equal volume of chloroform:isoamylalcohol (49:1), vortex, and centrifuge 2 min.
7. Remove upper layer and place in a new tube.
8. Add 1/10 volume of RF 3 M NaOAc (pH 5.2) and 2.5 volumes of cold RF 100% ethanol.
9. Precipitate on ice for 15 min.
10. Spin down in microfuge for 15 min at maximum speed at 4°C.
11. Wash pellet once with cold RF 70% ethanol and air dry for 10 min.
12. Resuspend in 11.5 μL DEPC H_2O.
13. Run 1 μL on a 1% agarose gel to check template before proceeding to the transcription reaction.

3.2.1.2. Template Generation by PCR Amplification

1. Using 10 ng plasmid DNA as a template, amplify insert sequence by PCR.

   PCR primer sequences must be either external to or directly corresponding to the RNA polymerase binding sites.
2. Run 1 μL on a 1% agarose gel to check single PCR product generation.
3. Add DEPC H_2O to a final volume of 50 μL.
4. Add 50 μL chloroform, mix, and centrifuge at maximum speed for 2 min at room temperature.
5. Remove upper layer (approximately 40 μL) and place in a new tube. In general, 2–4 μL of purified PCR product will be sufficient for a transcription reaction.

3.2.1.3. Riboprobe Transcription

1. Add reagents to Eppendorf tubes in the following order:

2 μL	10× transcription buffer
2 μL	digoxygenin (DIG) RNA labeling mix
x μL	DNA template (approximately 1 μg)
0.5 μL	ribonuclease inhibitor (40 U/μL)
2 μL	SP6, T7, or T3 RNA polymerase (10 U/μL)

   DEPC-treated dH_2O to 20 μL

2. Incubate at 37°C (or 40°C if using SP6) for 2 h.
3. Remove a 1 μL aliquot and run on 1% agarose gel to check synthesis (*see* **Note 9**).
4. Optional: Add 1 μL DNase (10 U/μL, RNase free, Roche) and incubate at 37°C for 15 min.
5. Dilute the probe to 50 μL with DEPC H_2O and add 1/10 volume of RF 3 M NaOAc (pH 5.2) and 2.5 volumes of RF 100% ethanol. If probe yield is low, addition of 1 μL of RF glycogen (20 μg/μL) during precipitation is helpful.
6. Precipitate on ice for 30 min.
7. Centrifuge at maximum speed for 20 min at 4°C.
8. Wash pellet once with RNase-free 70% ethanol, air dry, and resuspend in 100 μL DEPC-treated dH_2O.
9. Run 5 μL on a 1% agarose gel to check probe; approximate probe concentration can be estimated by comparison to a standard DNA ladder (*see* **Note 9**).

3.2.2. Whole Mount RNA In Situ Hybridization

Important: All steps prior to and including hybridization should be carried out in RNase-free (RF) conditions (*see* **Note 8**).

3.2.2.1. Dissection, Fixation, and Dehydration of Embryos

1. Dissect embryos out in ice-cold DEPC-treated phosphate buffered saline (PBS). To prevent probe trapping, puncture the neural tube once it has closed (E9.5 onwards). If dissecting out small embryos (E9.0 or younger), use PBS containing 100 mg/L $MgCl_2$ and 100 mg/L $CaCl_2$ to maintain tissue integrity (for example: Dulbecco's modified PBS, Sigma). Transfer to glass vials (*see* **Note 6**).
2. Incubate embryos in 4% paraformaldehyde at 4°C, rocking, overnight. For younger embryos (9.5 dpc and younger) this step should be shortened to 2 h at 4°C.
3. Wash 2 ×10 min with PBTX at 4°C, rocking.
4. Dehydrate embryos through a graded methanol/PBS series with 20 min washes rocking at room temperature, as follows:
   25% methanol/PBS
   50% methanol/PBS
   75% methanol/PBS
   100% methanol
   100% methanol
5. Optional: Embryos may be stored at –20°C in 100% methanol at this stage for several months at least.
6. Optional: Bleach embryos with 6% H_2O_2 in methanol for 1 h at RT with gentle rocking.

7. Rehydrate embryos through a graded methanol/PBS series with 20 min washes rocking at room temperature, as follows:
   75% methanol/PBS
   50% methanol/PBS
   25% methanol/PBS
8. Wash 3 × 10 min in PBTX.
9. Treat with 10 μg/mL proteinase K in PBTX for 2–35 min depending on the stage of embryo.
   Each new batch of proteinase K may need to be tested for appropriate treatment times. For consistent results proteinase K stock (10 mg/mL) should be distributed into single use aliquots and stored at –20°C. Make a fresh dilution to 10 μg/mL when treating embryos.
   Approximate treatment times are as follows:

7.5 dpc	2 min
8.5 dpc	5 min
9.5 dpc	10 min
10.5 dpc	15 min
11.5 dpc	20 min
12.5 dpc	25 min
13.5 dpc	30 min
14.5 dpc	35 min

10. Wash 2 × 5 min with PBTX. Take care with washes—the embryos are fragile.
11. Refix with 0.2% glutaraldehyde/4% paraformaldehyde in PBTX with gentle rocking at room temperature for 20 min.
12. Wash 2 × 10 min with PBTX.
13. Transfer embryos to hybridization vials (*see* **Note 8**). Remove as much PBTX as possible and add enough pre-hybridization solution to completely cover the embryos. Allow embryos to sink to the bottom.

3.2.2.2. Probe Hybridization

14. Incubate at 65°C for 2 h to overnight with constant agitations (*see* **Note 10**). Pre-hybridization time can be extended.
15. Remove liquid and replace with a fresh volume of pre-hybridization solution. We commonly do not change the pre-hybridization solution but ensure that minimal amounts of PBTX are carried over when embryos are transferred to the pre-hybridization solution.
16. Add probe (0.2–1.0 μg/mL DIG-labeled probe, determined empirically) to pre-hybridization solution.
17. Incubate overnight at 65°C. Hybridization temperature can be altered (*see* **Note 11**).

3.2.2.3. Post-hybridization Washes, Antibody Incubation, and Post-antibody Washes

18. At this point RNase-free conditions are no longer necessary. Wash embryos with constant agitation for 5 min each at 65°C as follows:
    100% solution 1
    75% solution 1/25% 2× SSC
    50% solution 1/50% 2× SSC
    25% solution 1/75% 2× SSC
    These washes can be done in a heating block on a rocker or in hybaid bottles (*see* **Note 10**).
19. Wash two times with 2 × SSC, 0.1% CHAPS at 55–65°C, 10 min each wash.
20. Wash two times with 0.2×SSC, 0.1% CHAPS at 55–65°C, 15 min each wash.
21. Wash two times with TBTX at RT, 10 min each wash.
22. Incubate embryos with antibody blocking solution (freshly made) for 1 h rocking at room temperature.
23. Optional: Preadsorb anti-DIG antibody to remove nonspecific antibodies (*see* **Note 12**).
24. Remove blocking solution and replace with blocking solution plus anti-DIG antibody (Roche) diluted at 1:2,000.
25. Rock overnight at 4°C. Antibody incubation can be shortened to 4 h at room temperature.
26. Remove antibody solution. Antibody solution can be reused a number of times for up to a month as long as it is stored at 4°C and no growth is observed in the high serum solution.
27. Wash five times with TBTX plus 0.1% BSA at room temperature, 30 min each wash.
28. Wash overnight with TBTX plus 0.1% BSA at 4°C.

3.2.2.4. Histochemical Detection

29. Wash two times in TBTX at RT for 15 min each wash.
30. Wash three times in fresh NTMT for 10 min each wash.
31. Remove NTMT wash and add NTMT including 3.5 μL of 100 mg/mL NBT (Roche) and 3.5 μL 50 mg/mL BCIP (Roche) per milliliter. Alternatively, use of the premixed substrate solution BM Purple (Roche) is highly recommended as background staining is significantly reduced.
32. Keep embryos in the dark as much as possible and rock gently until the color has developed to the desired extent.
33. Wash with NTMT (5–10 min, do not leave too long) and then PBTX for 20 min. If color has not developed before it is time to leave, the embryos can be washed in NTMT, then TBTX, stored at 4°C overnight, and the color development restarted at step 30 the next day.

34. Wash several times in PBS plus 1% Triton X-100. The last wash can be done overnight at 4°C—this helps to remove background.
35. Fix stained embryos in 4% paraformaldehyde in PBTX overnight at 4°C.
36. Exchange embryos into PBS and photograph as soon as possible (*see* **Note 13** for photography suggestions). To store for extended periods embryos can be kept at 4°C in PBS + sodium azide or can be taken through a PBTX series into 100% glycerol.

3.2.3. Section RNA In Situ Hybridization

Embryonic samples can be processed by either OCT cryosection or paraffin section prior to RNA in situ hybridization analysis (**Sections 3.1.1** and **3.1.2**). Signal strength is often greater following cryosection while preservation of tissue morphology is often better following paraffin section:

1. Riboprobe Generation – *see* **Section 3.2.1**.
2. Preparation of slides for hybridization. Important: All steps prior to and including hybridization should be carried out in RNase-free (RF) conditions (*see* **Note 8**). For paraffin sections proceed to steps 3–6 and for frozen sections proceed to step 7.
3. Bake slides on hot plate at 60°C for 1 h.
4. Allow slides to come to room temperature.
5. Dewax in xylene 2 × 5 min.
6. Rehydrate sections through a graded ethanol series with 5 min washes at room temperature, as follows:
   100% ethanol
   100% ethanol
   75% ethanol/dH_2O
   50% ethanol/PBS
   25% ethanol/PBS
   PBS
   PBS
   Proceed to step 8.
7. Thaw slides and air dry for 15–20 min at room temperature. Proceed to step 8.

3.2.3.1. Hybridization

8. Incubate slides in 4% paraformaldehyde for 10 min.
9. Rinse briefly then wash twice for 5 min in PBT.
10. Treat slides with proteinase K diluted in PBS (generally 1 μg/mL for 10 min, but concentration and length of incubation can be varied).
11. Wash twice in PBT for 5 min.

12. Incubate slides in 4% paraformaldehyde for 5 min. PFA from first post-fix can be reused.
13. Rinse briefly then wash twice for 5 min in PBT.
14. Incubate slide for 10 min in acetylation reaction mix. To prepare acetylation mix, add 625 μL acetic anhydride to 250 mL 0.1 M TEA (triethanolamine) in a fume hood and shake well, use immediately.
15. Rinse briefly then wash twice for 5 min in PBT.
16. Rinse briefly in dH_2O.
17. Blot excess liquid and air dry slides for no more than 10 min, until any remaining droplets have evaporated, but do not allow tissue to become completely dry. Process 5–10 slides at a time so slides do not over-dry.
18. Combine 100 μL pre-warmed hybridization solution at 65°C and 1 μL of probe, add carefully to slides to avoid air bubbles, and add coverslip. For double or triple in situ hybridizations, add 1 μL of each probe to 100 μL hybridization solution.
19. Place slides in humidified slide box (use Whatman paper or paper towels soaked in 5×SSC/50% formamide). Alternatively, slides can be completely submerged in a slide mailer box (four slide box) containing 8 mL hybridization solution plus riboprobe. This solution can be reused multiple times if stored at –20°C.
20. Incubate overnight at 65°C.

3.2.3.2. Post-hybridization Washes

Important: Pre-warm wash solutions.

21. Remove coverslips by allowing them to float off (do not pull) in 5× SSC at 65°C.
22. Wash slides in 1× SSC/50% formamide for 30 min at 65°C.
23. Wash slides in TNE for 10 min at 37°C.
24. Incubate slides in TNE plus RNase A (20 μg/mL, Roche) for 30 min at 37°C.
25. Wash slides in TNE for 10 min at 37°C. Reuse TNE from first wash.
26. Wash slides in 2× SSC for 20 min at 65°C.
27. Wash slides in 0.2× SSC for 20 min at 65°C.
28. Wash slides in 0.2× SSC for 20 min at 65°C.

3.2.3.3. Antibody Incubation and Histochemical Detection

29. Wash slides in MABT twice for 5 min at RT.
30. Incubate in antibody blocking solution for a minimum of 1 h.
31. Add anti-DIG-AP antibody (Roche), diluted 1:2,000 in 2% sheep serum/MABT. Add 500 μL to 1 mL per slide

and incubate overnight at 4°C in humidified chamber (*see* **Note 5**). Antibody incubation can be reduced to 3 h at room temperature.

32. Rinse briefly in MABT.
33. Wash three times in MABT for 5 min.
34. Wash slides in NTM pH 9.5 for 10 min. Tween is not generally added to NTM for section in situ hybridization. Tween will speed up the reaction but causes higher background levels.
35. Incubate slides with 1 mL NTM pH 9.5 with 4.5 μL NBT (50 mg/mL) and 7.0 μL BCIP (25 mg/mL).
36. Allow color to develop for 1 h to 3 days in the dark dependent on signal intensity. Changing the solution frequently reduces background—change twice daily or as soon as detection solution becomes brown.
37. Rinse briefly in NTM pH 9.5.
38. Wash slides in PBS twice for 5 min.
39. Fix color by incubating slides in 4% paraformaldehyde for 30 min. Fix for 10 min if doing double or triple detection.
40. Wash twice in PBS for 5 min.
41. For single detection: Rinse slides in dH_2O and mount in gelvatol. Heat gelvatol to 65°C before using. For double/triple detection proceed to step 42.

3.2.3.4. Detection of Second Probe

42. Wash twice with MABT for 5 min.
43. Incubate in antibody blocking solution for a minimum of 1 h.
44. Add anti-FITC-AP antibody (Roche), diluted 1:2,500 in 5% sheep serum/MABT. Add 1 mL per slide and incubate overnight at 4°C in humidified chamber.
45. Rinse briefly in MABT.
46. Wash three times for 5 min with MABT.
47. Wash slides in NTM pH 9.5 for 10 min.
48. Incubate slides with NTM pH 9.5 + INT + BCIP.
49. Allow color to develop for 1 h to 3 days (dependent on signal intensity) in the dark. Change solution as necessary.
50. For double detection, rinse slides in dH_2O and mount in gelvatol. Heat gelvatol to 65°C before using. For triple detection, proceed to step 51.

3.2.3.5. Detection of Third Probe

51. Wash twice with MABT for 5 min.
52. Incubate in antibody blocking solution for a minimum of 1 h.

53. Add anti-biotin-AP antibody (Roche), diluted to 1:2,500 in 5% sheep serum/MABT. Add 1 mL per slide and incubate overnight at 4°C in humidified chamber.
54. Rinse briefly in MABT.
55. Wash three times for 5 min with MABT.
56. Wash slides in NTM pH 8.0 for 10 min.
57. Dissolve Sigma Fast Red TR/Naphtali AS-MX ready-made tablets (Sigma) in buffer provided and filter through 0.2–0.8 mm syringe filter. Dilute 1:10 in NTM pH 8.0 and add to slides.
58. Develop for 1 h to 3 days (dependent on signal intensity) in the dark. Change solution as necessary.
59. Rinse briefly in NTM pH 8.0.
60. Rinse slides in dH_2O and mount in gelvatol. Heat gelvatol to 65°C before using.

3.3. Immunohistochemistry

Immunohistochemistry protocols must be optimized for each antibody, primarily because the stability and accessibility of different epitopes is variable. Some are sensitive to heat or organic solvents. In addition, aldehyde-induced protein crosslinking can block an antibody's access to its epitope. In this case, epitopes must be unmasked by an antigen retrieval/permeabilization step after fixation. Many methods are available, and the best for a given antibody should be determined empirically. Finally, different fixation and antigen retrieval conditions can affect the sub-cellular distribution of antigens differently. For a full discussion of these issues, please *see* (10). See also (1) for a complete discussion of antibodies and antibody-related techniques.

The following protocols use an HRP-conjugated secondary antibody and DAB detection, which tend to give strong signal and do not require heating. An example is shown in **Fig. 10.1b**. The whole mount protocol is based on (11). Suggestions for modifying these protocols to detect with alkaline phosphatase or fluorescence are referenced throughout (*see* **Note 14**).

3.3.1. Whole Mount Immunohistochemistry

1. Dissect embryos or tissues into ice-cold PBS. For older embryos, it may be necessary to dissect away individual organs or to bisect the embryo for better antibody penetration. For stages after the neural tube has closed (E9.5 and later), puncture the neural tube to prevent trapping of reagents. Transfer to glass vials (*see* **Note 6**).
2. Incubate embryos in 4% paraformaldehyde at 4°C, rocking, overnight. For younger embryos (<E12.5) this step can be shorted to 1–2 h (*see* **Note 15**).
3. Wash embryos in PBT quickly, then 3 × 10 min.

4. Fix and permeabilize embryos in Dent's bleach overnight, 4°C rocking. This step can sometimes be skipped for smaller embryos and tissues, depending on the antibody (*see* **Notes 16** and **17**).
5. Optional: Embryos may be transferred to 100% methanol and stored at –20°C.
6. Rehydrate embryos through a graded methanol/PBS series with 10 min washes, as follows:
   75% methanol/dH_2O

   50% methanol/PBS

   25% methanol/PBS
7. Wash embryos in PBT quickly, then 2 × 10 min (*see* **Note 18** for additional step if using alkaline phosphatase detection).
8. Incubate embryos in blocking solution for one-several hours, rocking. Embryos may be left overnight in blocking reagent at 4°C. If background is a problem, extending blocking time to at least overnight can be helpful.
9. Incubate embryos in primary antibody, diluted in blocking solution, at 4°C overnight, rocking. Antibody dilution must be determined empirically (*see* **Note 19**).
10. Wash embryos in PBT 3 × 10 min, then 5 × 1 h.
11. Incubate embryos in secondary antibody, diluted in blocking solution, at 4°C overnight. The optimal dilution should be determined empirically but for many commercially available secondary antibodies (such as from Jackson Immunoresearch), 1:500–1:1,000 is a good starting point.
12. Wash embryos in PBT 3 × 10 min, then 5 × 1 h. If using a fluorophore-conjugated secondary antibody, proceed to step 20. If using alkaline phosphatase detection, proceed as described in **Section 3.4.3**, steps 5–10.
13. Incubate embryos in HRP detection buffer, observing embryos closely for color development. This can begin within seconds, but can also take minutes to hours. Embryos should be monitored to determine reaction speed (*see* **Note 20**).
14. Wash embryos in PBT, once quickly, then 3 × 10 min.
15. Post-fix embryos in 4% paraformaldehyde, 30 min to overnight.
16. Wash embryos in PBT, once quickly, then 3 × 10 min.
17. Optional: If clearing is not required, proceed directly to photography.

18. To clear, dehydrate embryos through a methanol/PBS series, incubating 10 min in each of the following solutions:
    25% methanol/PBS
    50% methanol/PBS
    75% methanol/PBS
    2 × 100% methanol
19. Incubate in 50% methanol, 50% BABB for 5 min.
20. Incubate in BABB, in a glass container, until clear (*see* **Note 21** for considerations and optional clearing protocol).
21. Photograph under a dissecting microscope (*see* **Note 13**). Signal will fade over time, so it is best to photograph immediately.

3.3.2. Section Immunohistochemistry

1. Prepare paraffin or OCT cryosections as described in **Sections 3.1.1** and **3.1.2**, respectively. Note that some antigens do not survive the paraffin embedding process (*see* **Note 22** for sectioning considerations). Steps 2–5 are only necessary for paraffin sections.
2. Bake slides on hot plate at 60°C for 1 h.
3. Allow slides to come to room temperature.
4. Dewax in xylene 2 × 5 min.
5. Wash 2 × 5 min in 100% methanol.
6. For cryosections, thaw slides and air dry for 15–20 min at room temperature.
7. Quench endogenous peroxidases by incubating 1 h in 3% H_2O_2 in methanol (for HRP detection only; *see* **Note 23** for modification if using fluorophore or alkaline phosphatase detection).
8. Rehydrate sections through a graded alcohol series with 5 min washes at room temperature, as follows:
    100% methanol
    100% methanol
    75% methanol/dH_2O
    50% methanol/PBS
    25% methanol/PBS
    PBS
    PBS
9. Optional: Permeabilize tissue by incubating 2–5 min in 1 μg/mL proteinase K in PBS. Proteinase K solution should be diluted fresh from a 10 mg/mL stock, stored

at –20°C, and optimal time should be determined (*see* **Note 24** for alternative permeabilization methods).

10. Wash in glycine/PBT once quickly, then 2 times 5 min to stop the digestion.
11. Using a pap pen, draw a line across the right edge of the slide label, to prevent fluid from covering the label. Blot slides and lay them flat inside a humidified staining tray (*see* **Note 5**).
12. Gently pipette 1 mL of blocking solution onto each slide, taking care to avoid pipetting directly onto tissue. Cover humidified tray and incubate 1 h or more. If background is a problem, increasing blocking time to at least overnight can be helpful.
13. Remove blocking solution, and incubate slides in primary antibody diluted in blocking solution for 3 h at room temperature or overnight at 4°C in covered, humidified tray (*see* **Notes 5** and **19**).
14. Return slides to staining buckets with PBT and allow coverslips (if used) to gently float off. Do not pull coverslips off.
15. Wash slides in PBT once quickly, then five times for 5 min.
16. Blot slides and lay flat on a staining tray. Incubate in 1 mL secondary antibody, diluted in blocking solution for 3 h at room temperature or overnight at 4°C in covered, humidified tray. If a fluorescent secondary antibody is used, protect slides from light for the rest of the procedure.
17. Return slides to buckets and wash in PBT once quickly, then five times for 5 min. For fluorescent detection, proceed to step 19. For AP detection, proceed as described in **Section 3.4.4**, steps 8–14.
18. Incubate slides in HRP detection buffer and observe closely to determine the rate of color development. This can not only begin within seconds, but also take minutes to several hours.
19. Wash slides in PBT, once quickly, then three times for 5 min.
20. Post-fix in 4% paraformaldehyde 30 min to overnight.
21. Wash slides in PBT, once quickly, then three times for 5 min.
22. Mount in gelvatol and photograph.

3.4. Reporter Detection

A major strength of the mouse system is its genetics, including the ability to detect gene expression using reporter proteins in transgenic or knock-in animals. Reporter detection is generally easier than the methods above, because they are stable proteins

with robust detection assays and are already present in the tissue, so less permeabilization is necessary. An enormous advantage of EGFP is that it can be observed in living tissue. EGFP signal is generally weaker than enzymatic reporters and is sensitive to fixation and organic solvents; however, GFP antibodies can be used to get around these problems in fixed tissues. Many color variants of EGFP are also available. β-Galactosidase and alkaline phosphatase are both very stable enzymatic reporters that produce strong signal and low background in most mouse tissues (*see* **Notes 25–29** and (2, 12) for further discussion). An example of a β-galactosidase detection is shown in **Fig. 10.1c**. β-Galactosidase activity is more sensitive to heat, fixation, and organic solvents than is alkaline phosphatase. Both are generally used in colorimetric assays, but fluorescent detection methods are available. Further, EGFP and β-galactosidase are available in many forms, including fusions and tags that target them to particular subcellular regions and destabilized variants with short half-lives that serve as better markers for active transcription. The following are general protocols for detecting these three reporters in whole or sectioned mouse tissues, courtesy of Constance Cepko and Clifford Tabin (Harvard Medical School, Boston, MA, USA).

3.4.1. β-Galactosidase Detection in Whole Mount

1. Dissect embryos or tissues into ice-cold PBS. For older embryos (>E12.5) it may be helpful to bisect or dissect out tissues of interest in order to keep fixation times to a minimum, as long fixes decrease enzymatic activity (*see* **Note 25**). Transfer to glass vials (*see* **Note 6**).
2. Incubate in fixation buffer, rocking, at 4°C:
   For embryos E9.5-E12.5, 30 min.
   For younger embryos, fix 15 min.
   For older embryos, fixation time can be increased to up to 2 h (13).
3. Wash embryos in β-galactosidase wash buffer once quickly, then 3 × 15 min at room temperature.
4. Incubate embryos in β-gal staining buffer at 37°C, protected from light, for several hours to overnight. Observe embryos to determine the rate of reaction.
5. Wash embryos in PBS once quickly, then 3 × 15 min. For embryos older than E12.5, more washes may be required; 15 min washes should be continued until the wash solution remains clear, not yellow.
6. Post-fix embryos in 4% paraformaldehyde in PBS, 30 min to overnight, rocking.
7. Wash embryos once quickly followed by 3 × 15 min in 1× PBS.

8. Photograph embryos (*see* **Note 13**).
9. Optional: Embryos may be cryoembedded in gelatin (recommended) or OCT (**Sections 3.1.3** and **3.1.2**, respectively) and sectioned following detection in whole mount.

3.4.2. β-Galactosidase Detection in Sections

1. Prepare OCT sections as described in **Section 3.1.2**.
2. Thaw sections and wash in PBS 5 min.
3. Post-fix 10 min in freshly made 4% paraformaldehyde at 4°C.
4. Wash slides in β-gal washing buffer once quickly, then 2 × 10 min.
5. Incubate slides in β-gal detection buffer at 37°C, protected from light, until color develops (1 h to overnight). Reaction should be closely monitored for the first several hours to determine its rate.
6. Wash slides in PBS quickly, then 3 × 10 min, or until solution stops turning yellow.
7. Mount slides in gelvatol and photograph.

3.4.3. Alkaline Phosphatase Detection in Whole Mount

1. Dissect embryos or tissues into ice-cold PBS. For stages after the neural tube has closed (E9.5 and later), puncture the neural tube to prevent trapping of reagents. Transfer to glass vials (*see* **Note 6**).
2. Incubate embryos from 2 h to overnight in 4% paraformaldehyde, rocking.
3. Wash embryos in PBT, 2× quickly followed by 3 × 10 min, rocking.
4. Incubate embryos at 65°C for one-several hours, in PBT, to inactivate endogenous phosphatases. Embryos do not need to be rocking during this step. The length of incubation depends on how highly expressed AP is (lower expression will require more inactivation of background-producing phosphatases) (*see* **Note 26**).
5. Wash embryos in NTM, 1× quickly and 1 × 10 min, rocking.
6. Incubate embryos in detection buffer (NTM-containing NBT and BCIP), rocking at room temperature, in the dark, until signal has developed. Monitor the reaction closely to determine speed, which can take between a few minutes to many hours. If reaction proceeds very slowly, it can be left at 4°C overnight. Staining is complete when the staining of interest is clearly visible, or when background begins to come up at the same rate at which the signal is developing. For whole mount, it is unlikely that improvement will be seen for embryos incubated longer than overnight at 4°C.

7. Wash embryos in PBT 1× quickly followed by 3 × 10 min, rocking.
8. Post-fix embryos in 4% paraformaldehyde for 30 min, rocking.
9. Wash embryos in PBT 1× quickly followed by 3 × 10 min, rocking.
10. Optional: If background has developed too much, embryos can be washed overnight at 4°C in PBS containing 1% Tween-20.
11. Photograph embryos (*see* **Note 13**).

3.4.4. Alkaline Phosphatase Detection in Sections

1. Prepare either paraffin or cryosections, as described in **Sections 3.1.1**, **3.1.2**, and **3.1.3**.
   Steps 2–6 are only necessary for paraffin sections.
2. Bake slides on hot plate at 60°C for 1 h.
3. Allow slides to come to room temperature.
4. Dewax in xylene 2 × 5 min.
5. Wash 2 × 5 min in 100% methanol.
6. Rehydrate sections through a graded ethanol series with 5 min washes at room temperature, as follows:
   100% methanol
   100% methanol
   75% methanol/dH_2O
   50% methanol/PBS
   25% methanol/PBS
   PBS
   PBS
   For cryosections, thaw slides and air dry for 15–20 min at room temperature.
7. Heat-inactivate endogenous phosphatases by incubating slides in PBT at 65°C for 30 min.
8. Incubate slides in NTM buffer for 10 min at room temperature.
9. Using a pap pen, draw a line across the right edge of the slide label, to prevent fluid from covering the label. Blot slides by touching the bottom edge to a paper towel and lay them flat inside a humidified staining tray (*see* **Note 5**).
10. Gently pipette 1 mL of detection buffer onto each slide, taking care to avoid pipetting directly onto tissue. Cover humidified tray and incubate until signal has developed (several minutes to hours or, rarely, days).

11. Wash slides in PBT once quickly, then three times 5 min.
12. Post-fix in 4% paraformaldehyde for 30 min to overnight.
13. Wash slides in PBT once quickly, then three times 5 min.
14. Mount slides in gelvatol and photograph.

3.4.5. Detecting EGFP in Whole Mount

1. Dissect embryos or tissues into ice-cold PBS (*see* **Note 27**).
2. If possible, observe and photograph embryos immediately, as the signal is strongest in fresh tissue (*see* **Note 28** for GFP filter sets).
3. Optional: Transfer to glass vials (*see* **Note 6**) and fix embryos in 4% paraformaldehyde for 30 min to 2 h at 4°C. It is important to keep the fix as short as possible to preserve GFP fluorescence (*see* **Note 27**).
4. Wash embryos in PBS 1× quickly, then 3 × 10 min in PBS.
5. Photograph embryos (*see* **Note 13**).

3.4.6. Detecting EGFP in Sections

1. Prepare OCT sections as described in **Section 3.1.2**. Optional: For better morphology, a method for directly visualizing GFP in paraffin sections has been reported (2).
2. Thaw slides and wash briefly in PBS.
3. Mount in gelvatol and photograph.

4. Notes

1. Hazardous chemicals
   Acetic anhydride is flammable and harmful if inhaled. It can cause burns to the respiratory tract, skin, and eyes upon contact. Wear suitable protective clothing/equipment and work with acetic anhydride in a fume hood.

   Benzyl alcohol/benzyl benzoate (BABB) is harmful if swallowed and is an irritant to the skin, respiratory tract, and eyes.

   CHAPS is an irritant to the skin, eyes, respiratory tract, and mucosal membranes. Strongly avoid breathing the dust. Wear appropriate protective clothing/equipment.

   Chloroform is highly volatile and is an irritant and carcinogen. Wear suitable protective clothing/equipment and work with chloroform in a fume hood.

   Diethyl pyrocarbonate (DEPC) produces toxic, corrosive, flammable, or explosive gases. It is an irritant to the skin, eyes, and respiratory tract. Wear appropriate protective clothing/equipment and only work with DEPC in a fume hood.

Diaminobenzidine (DAB) is a carcinogen. Wear suitable protective clothing/equipment and handle in a fume hood.

Formamide is toxic and is harmful if inhaled. It is an irritant to the skin, eyes, and respiratory tract. Wear appropriate protective clothing/equipment. Formamide-based solutions, especially those heated to 65°C, should be handled in a fume hood.

Glutaraldehyde is toxic. It is an irritant to the skin, eyes, and respiratory tract. Wear appropriate protective clothing/equipment and work with glutaraldehyde in a fume hood.

Methanol is toxic and flammable. It is an irritant to the skin and eyes and is harmful to many organ systems if ingested. Wear suitable protective clothing/equipment and handle only with sufficient ventilation.

2-methyl butanol is flammable and an irritant. Avoid breathing vapors and wear appropriate protective clothing/equipment.

Paraformaldehyde is toxic. Strongly avoid breathing the dust. It is an irritant to the skin, eyes, and respiratory tract. Wear appropriate protective clothing/equipment and work with paraformaldehyde in a fume hood.

Phenol is toxic and will cause burns if exposed to skin or eyes. Wear suitable protective clothing/equipment and work with phenol in a fume hood.

Sodium dodecyl sulfate (SDS) is an irritant to the skin, eyes, respiratory tract, and mucosal membranes. Strongly avoid breathing the dust. Wear appropriate protective clothing/equipment.

Triethanolamine (TEA) is an irritant to the skin and eyes and can cause liver and kidney damage. Wear appropriate protective clothing/equipment and work with TEA in a fume hood.

Xylene is flammable and toxic and should be handled only in a fume hood.

2. To TESPA treat: Take clean, plain glass slides through the following washes, in glass staining dishes: 1 min in 2 N HCl, 1 min in H_2O, 1 min in acetone, <30 s acetone/2% TESPA(2-aminopropyltriethoxysilane, Sigma), two times 30 s in acetone, 30 s in ethanol, then air dry overnight at room temperature, protected from dust; 2% TESPA solution should be made fresh before use. Use dedicated glass slide dishes and glass pipettes for treatment, as all will become TESPA coated.
3. Considerations for designing riboprobes: In general, probes of 400–700 bp are designed though are certainly not restricted to this size. Probes are often designed to

encompass the 3′UTR of a gene to limit cross reactivity of the probe with homologous genes. Potential alternate splicing of the target sequence should be considered.

4. Single, double and triple in situ hybridization reactions can be performed simultaneously to detect up to 3 unique RNA transcripts. Each RNA riboprobe must be differentially labeled with either digoxygenin (DIG), fluorescein (FL) or biotin-conjugated UTP and all probes are added simultaneously to the hybridization reaction. Detection of each uniquely labeled riboprobe must be performed sequentially, with a short 4% paraformaldeyhe fixation step in between each to inactivate previous AP activity. A common method for triple detections is as follows:
   a. Detect DIG using AP-conjugated anti-DIG antibody. Anti-DIG-AP then catalyses the conversion of NBT/BCIP to a purple precipitate.
   b. Detect FL using AP-conjugated anti-FL antibody. Anti-FL-AP then catalyses the conversion of INT/BCIP to a brown precipitate.
   c. Detect biotin using AP-conjugated anti-biotin antibody. Anti-biotin-AP then catalyses the conversion of Sigma Fast Red TR/Naphtali AS-MX to a pink precipitate.

   As FL and biotin-labeled riboprobes exhibit somewhat reduced signal, it is best to generate these riboprobes against genes of interest that exhibit the most robust signal.
5. For section procedures, steps using limiting/expensive reagents can be carried out on horizontal slide staining trays at lower volume. Trays can be purchased or made by fixing rails (such as plastic stripettes, cut to size) to the bottom of a flat, covered dish. We use 240 × 240 × 20 mm QTrays (Genetix). Wet paper towels or Whatman paper is placed in the bottom of the tray to keep it humidified. About 1 mL of liquid per slide is sufficient in a covered, humidified tray overnight. A volume of 100 μL can be used if slides are covered with a plastic coverslip (cut from thick polyethylene bags (Bel Art)). These should be floated off gently in buffer, rather than pulled off, to minimize tissue damage.
6. Unless otherwise noted, incubations on whole mount embryos and tissues should be done in 5–10 volumes (or more) of solution greater than the volume occupied by the tissue, on a rocking platform. We use 4 or 20 mL screw-top scintillation vials (VWR). For slides, we use 5 slide mailers (approximately 20 mL) or 25 slide buckets (approximately 200 mL) (VWR).

7. To fix embryos into positions (such as with a straight body axis), which can make sectioning easier, fixation can be carried out in Petri dishes with black dissection wax in the bottom, and embryos can be pinned out with insect pins. Embryos fixed this way will hold their position during subsequent processing.
8. To prevent degradation of RNA riboprobe, all steps up to and including hybridization should be performed under RNase-free (RF) conditions. Consumables such as Eppendorf tubes and pipette tips can be purchased RNase free. Stock solutions such as dH_2O and 10× PBS should be treated with 0.1% diethyl pyrocarbonate (DEPC), which covalently modifies and inactivates RNase. DEPC is highly toxic and should only be added in a fume hood. Once added, stir vigorously overnight. DEPC must be inactivated by autoclaving prior to use.
9. Following riboprobe synthesis, an RNA band of approximately 10-fold greater intensity than the plasmid band indicates that approximately 10 mg of probe has been synthesized. There may be more than one band due to secondary structure but there should not be a smear. A smear may indicate probe degradation (if smear is below probe band) or synthesis from uncut plasmid (if smear is above probe band).
10. Efficient hybridization requires overnight incubation at 65°C with constant gentle agitation. This is most easily achieved using screw-top scintillation vials (VWR or Wheaton; 4 or 20 mL depending on embryo size and number) placed in a heated orbital shaking water bath. If this is not available, embryos can be placed in a 2 mL round bottom RNase-free Eppendorf tube, parafilm sealed, and placed in tube holder (e.g., water bath tube holder). This holder is placed inside a hybaid bottle such that it is fixed in place, and incubation performed in a hybridization oven with constant rotation.
11. In general, a hybridization temperature of 65°C yields strong signal with minimal background. If no signal is observed, the hybridization temperature should be lowered, initially to 60°C and then 55°C if necessary. Conversely, if high background is observed, the hybridization temperature can be increased to 70°C.
12. In the past, preadsorption of anti-DIG antibody with embryo powder was performed to remove non-specific antibodies. This is no longer routinely performed but may be helpful if high background levels are observed.

Generation of Embryo Powder

a. Homogenize ~12.5–14.5 dpc mouse embryos in a minimum volume of PBS.
b. Add 4 volumes of ice-cold acetone and mix.
c. Incubate on ice for 30 min.
d. Centrifuge at 10,000 rpm for 10 min and remove supernatant.
e. Wash pellet with ice-cold acetone (taking care to resuspend the pellet well) and spin again.
f. Spread the pellet out and grind into a fine powder on a sheet of filter paper. Allow to air dry.
g. Store in an air-tight tube at 4°C.

Preadsorption of Anti-DIG Antibody with Embryo Powder

a. For each 2 mL required, weigh out 1.5 mg of embryo powder and add 0.5 mL of antibody blocking solution and 1 μL of anti-DIG antibody.
b. Rock gently at 4°C for 2 h or longer.
c. Centrifuge at maximum speed for 10 min at 4°C.
d. Dilute the supernatant to 2 mL using antibody blocking solution
e. Store at 4°C until required

13. Whole mount embryos can be photographed on a standard dissecting microscope, either in depression chambers or in Petri dishes and submerged in buffer (such as PBS). Placing 20–30 mL solidified 2% agarose/PBS in the bottom of the dish allows the embryo to be positioned, either by propping them in depressions or on blocks of agarose or by immobilizing them with insect pins.
14. Enzymatic detection (HRP or AP) gives higher signal than does fluorescence and is generally easier to optimize for whole mounts. If background from endogenous enzymes is an issue, it can be reduced somewhat by extensive washing in PBT or PBS + 1% Tween-20 or Triton X-100 (overnight or longer) after detection and can also be reduced by clearing embryos in BABB or glycerol.
15. An overnight fixation step is usually acceptable for whole mount IHC; however, over-fixation can increase epitope masking, and aldehyde fixation, especially glutaraldehyde, also increase tissue auto-fluorescence. It is therefore sometimes useful to shorten fixation to a few hours at 4°C. Alternatively, the weaker Dent's fix (1 part DMSO to 4 parts methanol) can be used in place of aldehydes by incubating for 24 h.

16. Dent's bleach can be replaced with Dent's fix (1 part DMSO to 4 parts methanol) if not using HRP detection, where it is necessary to quench endogenous peroxidases.
17. Weak proteinase digestion is an alternative to permeabilization in Dent's and can be used in place of steps 4–5 in this protocol. For HRP detection, tissue must still be bleached in 3% H_2O_2 in methanol prior to rehydration. Next, incubate embryos in 10 μg/mL proteinase K (Roche) in PBS. Make fresh from a stock solution of 10 mg/mL proteinase K, stored at –20°C. Incubate embryos for 5–10 min (time must be optimized for each antibody to balance permeabilization with antigen degradation). Stop digestion with two rapid washes in 2 mg/mL glycine in PBS. Make glycine solution fresh on the day of use. Proceed to step 7.
18. If enzymatic detection with an AP-conjugated secondary antibody is used, endogenous phosphatases should be denatured by heating the embryos to 65°C for one-several hours. Note that this will also destroy some antigens.
19. Secondary-only controls (omitting only the primary antibody) should be performed for all IHC procedures.
20. The precipitate formed by DAB is brown and can be made a more visible dark gray by adding metal salts to the detection solution (1).
21. BABB dissolves plastic, so glass dishes and tubes must be used. Additionally, BABB dissolves the precipitate formed by AP detection, so if it is used, photography must be done immediately. Glycerol is a weaker but non-toxic clearing agent and is easier to use with AP detection. Incubate embryos in 50% glycerol/PBS, rocking at room temperature, until equilibrated (until they sink), then replace with 100% glycerol and equilibrate again.
22. Section IHC often works well in paraffin-embedded tissues and these give the best morphology. However, if the heat or organic solvents required for embedding destroy an antigen then tissues must either be stained in whole mount, then embedded, or they can be stained in OCT cryosections.
23. For AP or fluorophore-coupled secondary antibodies, dehydration and bleaching steps can be skipped. For AP detection, endogenous phosphatases are inactivated by incubating slides in PBS at 65°C for 30 min before proceeding to step 5. However, heat can denature some antigens.
24. As with whole mount IHC a key consideration is usually permeabilization/antigen retrieval, although many antibodies do not require a permeabilization step. A short proteinase K treatment is often effective, but should be

optimized to find the right balance between permeabilization, epitope degradation, and increased background. Antigen retrieval with detergents or heat can be good alternatives. For detergent, 1% Tween-20 (weaker) or 0.1–1% Triton X-100 (stronger) in PBS for 30 min can be used. Heating slides to near-boiling or in a pressure cooker (5–20 min, determined empirically) effectively exposes many antigens, although it can produce poor morphology, and care should be taken to avoid excessive bubbling. Heat slides either in a microwave or by making a double boiler on a hot plate, with a PBS or 0.1 M sodium citrate (pH 6.0)-filled slide bucket suspended in a beaker of boiling water or buffer. *See* (10) for a full discussion of antigen retrieval techniques and their advantages and drawbacks.

25. Although it is stable in vivo, β-galactosidase (β-gal) is sensitive to aldehyde fixation, organic solvents, and heat. Key considerations are minimizing fixation time, keeping tissues cold, and processing them as quickly as possible. When β-gal is to be detected along with other markers (such as an AP reporter or IHC using any detection method), the β-gal detection should be done first to minimize loss of signal. Staining in whole mount followed by gelatin embedding and sectioning is recommended, but it may not be possible to develop signal deep inside larger embryos/tissues. OCT embedding is the best method if tissues are to be stained in section because it does not require organic solvents or heat and is least damaging to the signal. Paraffin-embedded sections cannot be stained for β-galactosidase.
26. Human placental alkaline phosphatase is a robust and stable reporter of gene expression that can be readily detected in whole mount or embedded and sectioned tissues. Alkaline phosphatase retains its activity following aldehyde fixation, in organic solvents including methanol and ethanol, and after extended heating. The primary consideration for detecting alkaline phosphatase reporters is inactivating endogenous phosphatases in tissues, which can lead to high background. This is accomplished by heating tissues to 65°C, a temperature at which mouse but not human placental alkaline phosphatase is inactivated.
27. Preserving and detecting GFP signal while minimizing auto-fluorescence is the key consideration for visualizing EGFP reporters. Many embryonic tissues emit auto-fluorescence, especially blood, lung, liver, and other gut tissues. Aldehyde fixation (especially glutaraldehyde) increases background fluorescence and also weakens GFP signal, so fixation, if it is required at all, should be kept to a minimum. As an alternative to direct visualization, commercial antibodies against EGFP and other GFP

variants are available and can be used with standard IHC techniques.

28. EGFP can be detected with a standard FITC filter set. If background fluorescence is a problem, a long-pass filter set specialized for EGFP excitation can be used instead (Nikon or Chroma Technologies). With this filter, background auto-fluorescence appears yellowish-green while EGFP is very bright green. The suppliers above also make a variety of GFP filter sets optimized for different color variants and combinations.
29. There have been some reports in cultured cells that the subcellular pattern of GFP fluorescence can change following fixation (14). If this is an important consideration, then GFP distribution should be examined in fresh compared with fixed tissues.

Acknowledgments

We are deeply grateful to Connie Cepko, Cliff Tabin, Carol Wicking, and to past and present members of the Cepko and Tabin labs, who developed most of the protocols as they are described here, and who have generously shared them with us. J.H.M. is supported in part by NSF IOS-0818223.

References

1. Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York, NY.
2. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003) *Manipulating the Mouse Embryo: A Laboratory Manual,* 3rd edn. Cold Spring Harbor Laboratory Press, New York, NY.
3. Tuan, R. S. and Lo, C. W. (2000) *Developmental Biology Protocols,* Vol. 135**.** Humana Press, Totowa, NJ.
4. Satoh, T. and Fekete, D. M. (2009) Lineage analysis of inner ear cells using genomic tags for clonal identification. *Methods Mol. Biol.* **493**, 47–63.
5. Wilkinson, D. G. and Nieto, M. A. (1993) Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225**, 361–373.
6. Fowles, L. F., Bennetts, J. S., Berkman, J. L., Williams, E., Koopman, P., Teasdale, R. D., and Wicking, C. (2003) Genomic screen for genes involved in mammalian craniofacial development. *Genesis* **35**, 73–87.
7. Pena, J. T., Sohn-Lee, C., Rouhanifard, S. H., Ludwig, J., Hafner, M., Mihailovic, A., Lim, C., Holoch, D., Berninger, P., Zavolan, M., and Tuschl, T. (2009) miRNA in situ hybridization in formaldehyde and EDC-fixed tissues. *Nat. Methods* **6**, 139–141.
8. Kloosterman, W. P., Wienholds, E., de Bruijn, E., Kauppinen, S., and Plasterk, R. H. (2006) In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat. Methods* **3**, 27–29.
9. Obernosterer, G., Martinez, J., and Alenius, M. (2007) Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. *Nat. Protoc.* **2**, 1508–1514.
10. D'Amico, F., Skarmoutsou, E., and Stivala, F. (2009) State of the art in antigen retrieval for

immunohistochemistry. *J. Immunol. Methods* **341**, 1–18.
11. Kardon, G. (1998) Muscle and tendon morphogenesis in the avian hind limb. *Development* **125**, 4019–4032.
12. Cepko, C. L., Ryder, E., Austin, C., Golden, J., Fields-Berry, S., and Lin, J. (2000) Lineage analysis with retroviral vectors. *Methods Enzymol.* **327**, 118–145.
13. Nakamura, E., Nguyen, M. T., and Mackem, S. (2006) Kinetics of tamoxifen-regulated Cre activity in mice using a cartilage-specific CreER(T) to assay temporal activity windows along the proximodistal limb skeleton. *Dev. Dyn.* **235**, 2603–2612.
14. Brock, R., Hamelers, I. H., and Jovin, T. M. (1999) Comparison of fixation protocols for adherent cultured cells applied to a GFP fusion protein of the epidermal growth factor receptor. *Cytometry* **35**, 353–362.
15. McGlinn, E. and Tabin, C. J. (2006) Mechanistic insight into how Shh patterns the vertebrate limb. *Curr. Opin. Genet. Dev.* **16**, 426–432.

Chapter 11

Gene Targeting in the Mouse

Anne E. Griep, Manorama C. John, Sakae Ikeda, and Akihiro Ikeda

Abstract

Establishment of methods to inactivate genes by homologous recombination in embryonic stem (ES) cells has provided great advantages to the field of mouse genetics. Using this technology, a number of null mutant mice, so-called knock-out mice, have been generated. The gene-targeting technology offers a strong tool that allows us to understand the function of a particular gene of interest in the whole animal and has contributed to studies in a wide variety of biological research areas. More recently, the original knock-out technology has been further refined to develop advanced strategies to generate conditional knock-out and knock-in mice. In this chapter, an overview of gene-targeting strategies is presented and procedures to generate these genetically engineered mice are discussed.

Key words: Gene targeting, ES cells, homologous recombination, gene-targeting vector, knock-out mice.

1. Introduction

1.1. Gene Targeting in the Mouse

The mouse has been used as a genetic model for over 100 years. During the last 30 years since the technology for manipulating the mouse embryo to knock out a particular gene was developed (1, 2), mice have become a more powerful model organism to test the functions of genes of interest in vivo. Because of genomic, anatomical, and physiological similarities between mice and humans, mice can be used to generate models for a wide variety of human genetic disorders as well.

Embryonic stem (ES) cells in mice were first established in 1981 (3, 4). ES cells are also referred to as pluripotent stem cells, based on the potential they have to differentiate into any somatic cells except for placental trophoblast cells. Establishment

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_11,

of pluripotent ES cell lines was a key for manipulating the cells and generating mice carrying the manipulated genetic allele. Through homologous recombination in ES cells and the subsequent introduction of these mutated ES cells into mouse blastocysts to generate knock-out mice, endogenous genes of the mouse can be mutated at will.

While conventional knock-out mice lack the functional gene in all cell types from the time of conception, tissue- and time-specific inactivation of genes is achieved in conditional knock-out mice. In these mice, we are able to study the functions of genes throughout the life span of mice in tissue-specific manner even in cases where complete knock-out of those genes leads to early embryonic lethality (5, 6). Using the knock-in strategy, which is another application of homologous recombination, we can place a gene of interest (reporter gene, cre recombinase gene, etc.) under the control of the *cis*-acting regulatory element of an endogenous gene (7). Alternatively, we are able to introduce a point mutation, for example, mimicking a human disease by generating a mouse model carrying a mutation corresponding to that found in humans (8).

Figure 11.1 shows the overview of generating knock-out mice. Generation of knock-out mice starts with an established pluripotent ES cell line and the generation of the targeting vector. The genome of these ES cells is modified in culture by electroporation of the targeting vector into the cells. The genetically engineered ES cells then are microinjected into blastocysts derived from wild-type mice to generate chimeric mice. Through transmission of ES cell-derived cells into the germline of chimeric mice, mice heterozygous for the knock-out allele are obtained. By intercrossing these heterozygous mice, mice homozygous for the knock-out allele can be generated to test the consequence of inactivation of the gene of interest. This conventional knock-out technology has been modified and has given rise to conditional knock-out and knock-in technologies, which will be discussed in the following sections.

1.2. Gene-Targeting Strategies and Construction of Gene-Targeting Vectors

1.2.1. Conventional Gene Knock-Out

Gene targeting is achieved by homologous recombination of the targeting vector with the corresponding endogenous DNA sequence. The basic DNA construct for generating a knock-out allele is designed to contain two DNA segments that are homologous to the genomic sequence of the gene of interest (homology arms). In the knock-out vector, these two homology arms generally flank a drug resistance gene such as neomycin phosphotransferase (*neo*^{r}), which is used as a selectable marker. The antibiotic geneticin (G418) is used to select ES cells carrying the targeting vector including the *neo*^{r} gene. The gene-targeting vector also carries a negative selection marker outside of the region between homology arms. The HSV thymidine kinase

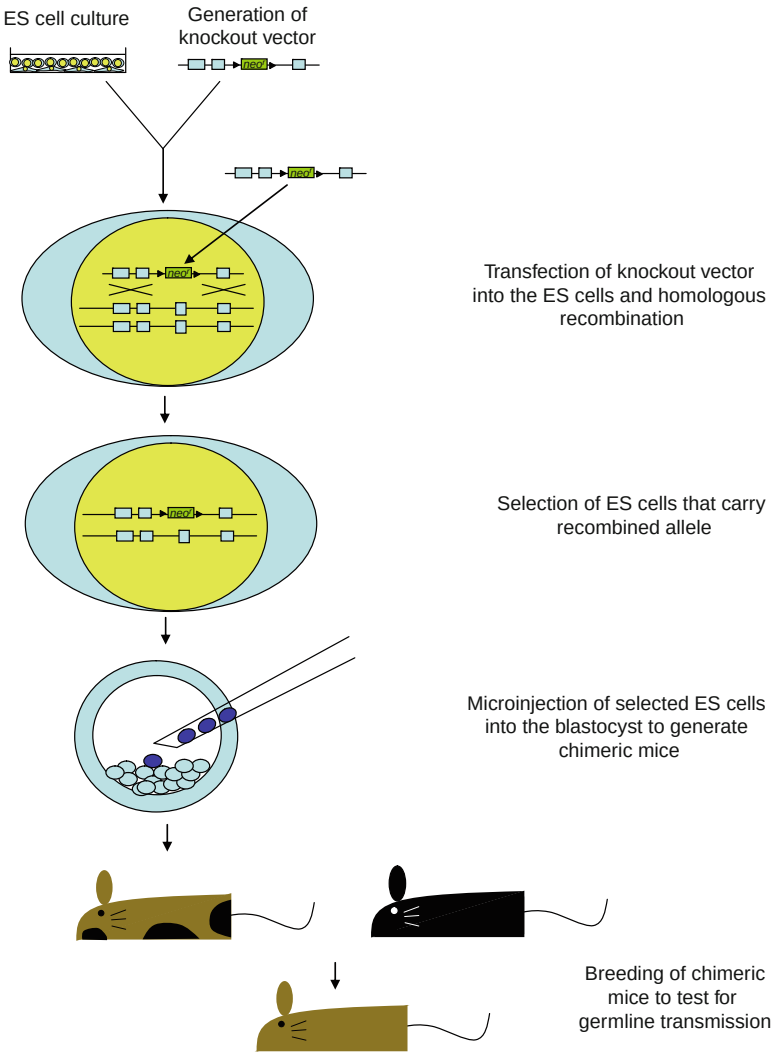

Fig. 11.1. Overview of knock-out mice generation.

(HSV-tk) gene is the most frequently used. HSV-tk phosphorylates chemicals such as ganciclovir or fialuridine (FIAU) into cytotoxic compounds. The double crossover event between two homologous regions replaces the endogenous genomic sequence flanked by homology arm sequences with the knock-out vector sequence flanked by two homology arms. In order to delete specific genomic sequences, the two homology arms are designed based on genomic sequences that flank exons that are desired to be deleted in the knock-out allele (**Fig. 11.2**).

The design of the construct is a key for successful recombination and there are several points that should be considered. First, the targeting vector should be designed so that remaining exons are not spliced in frame. This ensures that a functional null allele is

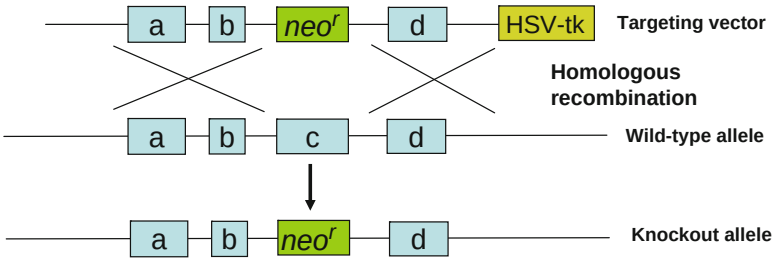

Fig. 11.2. Gene inactivation by homologous recombination. *a*, *b*, *c*, and *d* represent exons of the wild-type gene. A conventional targeting construct carries positive (*neo*^{r}) and negative (HSV-tk) selection genes. The targeting vector is aligned with the targeted wild-type genomic DNA. In this example, exon c is replaced by the *neo*^{r} gene as a result of homologous recombination, rendering the cells resistant to G418. Upon homologous recombination, HSV-tk, which is outside the region of homology, is lost, rendering the ES cells resistant to ganciclovir.

generated instead of merely deleting the exon(s) with the partial function of the encoded protein remaining. Second, two homology arms should have enough length for efficient homologous recombination to occur. It is known that the length of homologous DNA segments should be more than 2 kb for efficient and correct homologous recombination to occur (9). Typically the two homology units differ in length, for example, one arm might be 5 kb and the second would be 3 kb, as this facilitates homologous recombination. Third, attention should be paid to the homology of each arm to the genomic DNA of ES cells. Ideally, the same DNA source as ES cells should be used for the homology arms. If this is not possible, one strategy is to use exon sequences for the homology arms, since exon sequences are generally more conserved compared to intronic or regulatory sequences.

1.2.2. Conditional Knock-Out (Cre-Lox and FLP-FRT Systems)

As noted above, a major problem with conventional germline null mutant mice is embryonic lethality that precludes studying the consequences of loss-of-gene function in specific tissues in the postnatal animal. To circumvent this issue, researchers have developed ways to conditionally knock out a gene of interest in a tissue- or temporal-specific manner. The design of a targeting vector for conditional deletion has the same elements as a vector for conventional knock out. It is important to note that the normal function of the gene needs to be maintained in all tissues except for the targeted tissue. For this reason, there are some additional elements that need to be incorporated into the vector to allow tissue-specific inactivation. The conditional knock-out strategy which is widely used is the Cre-lox system (5, 6). A site-specific recombinase, Cre, is derived from bacteriophage P1. Cre recognizes specific DNA sequences, called *loxP* sites, and catalyzes

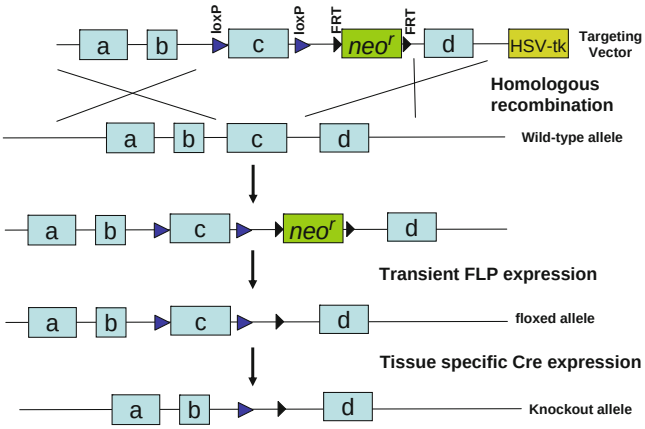

Fig. 11.3. Generation of a conditional knock-out allele. The vector for conditional knock-out is designed so that *loxP* sites are inserted flanking exon c. The *neo*^{r} gene can be flanked by FRT sequences so that the *neo*^{r} gene can be excised in ES cells transiently expressing FLP recombinase in the ES cells or by breeding the mouse to a FLP deleter strain (19). The knock-out allele is generated specifically in the targeted tissue by the tissue-specific expression of Cre recombinase.

recombination between these sites, resulting in the deletion of the intervening DNA.

The adaptation of this system for use on conditional knock-out experiments is shown in **Fig. 11.3**. The targeting vector incorporates the *loxP* sites flanking the DNA that is to be deleted when Cre is expressed. In this example, one exon of the gene of interest is flanked by two *loxP* sequences. It is important to note that deletion of the exon(s) flanked by *loxP* sites must give rise to a frame shift.

It should be also noted that the *loxP* sequences inserted should not interrupt normal splicing processes. Therefore, the *loxP* sequences are normally inserted to sites that are slightly distant from splice donor and acceptor sites. Typically, we place the *loxP* sequences about 50–100 bases away from the splice site. In order to achieve tissue-specific disruption of the gene of interest, expression of the Cre recombinase gene is regulated by a tissue-specific promoter. Upon expression of Cre, recombination between the *loxP* sites occurs and the exon is excised out only in the tissue where Cre is expressed (5, 6). Expression of Cre is provided by a transgenic mouse strain that expresses Cre in the desired tissue. Identification or generation of such a transgenic mouse line needs to be considered in the design phase of the project. More than 500 transgenic mouse lines expressing Cre recombinase have been generated in the research community and a database "Cre-X-Mice" containing the information for such transgenic lines is being developed (10).

The conditional knock-out vector can also be designed so that a selectable marker gene such as *neo*^{r} is excised by a different

site-specific recombinase. Flippase (FLP) recombinase from the 2 μm plasmid of *Saccharomyces cerevisiae* is used for such purpose (6). If the selectable marker gene is flanked by FLP recombinase target (FRT) sequences, it can be specifically excised by FLP in tissue culture either before the ES cells are microinjected into blastocysts or after the mutant mice are generated (**Fig. 11.3**).

Introduction of the conditional targeting allele into the ES cells is achieved by homologous recombination, the same process as the conventional gene targeting.

1.2.3. Gene Knock-In

Another application of homologous recombination is the knock-in strategy, with which it is possible to replace an endogenous gene with another gene of interest, insert an additional gene or insert a point mutation in a gene. There are a number of applications for the knock-in strategy; however, in this chapter, two representative applications are discussed.

(i) Replacing a gene
In this application, an endogenous gene is replaced by a gene of interest through homologous recombination. The advantage of this method is that the expression of the gene of interest can be regulated using the endogenous promoter. By introducing the internal ribosome entry site (IRES) sequence, co-expression of the endogenous gene and the inserted gene of interest under the control of a single promoter can also be achieved (**Fig. 11.4**, (7)).

(ii) Introducing a point mutation
Generating a mouse model that carries a particular point mutation observed in a human genetic disorder is a powerful approach to understand the pathogenesis of human

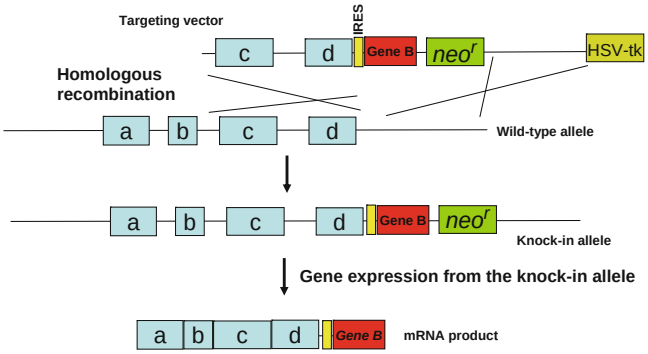

Fig. 11.4. Knock-in of cDNA by homologous recombination. In this targeting construct, one of the homology arms includes the 3′ UTR region of the targeted gene. Homologous recombination results in insertion of gene B together with the IRES sequence. In the mRNA transcribed from the knock-in allele, the original targeted gene and gene B are connected in tandem. Due to the existence of the IRES sequence, the original gene and gene B are separately translated to produce two separate proteins.

genetic diseases. Using the same homologous recombination technique, the mutation which is observed in the human genetic disorder can be introduced in mice (8). One of the homology arms of the targeting vector carries the mutation desired to be introduced. As for other regions of this arm, care should be taken so that this arm still has enough homology with the target sequence for homologous recombination to occur efficiently (**Fig. 11.5**).

For all of the above examples, once the targeting vector is designed, DNA fragments are assembled to generate a targeting vector. Previously, this process included isolation of genomic DNA fragments from the genomic DNA library, identifying restriction enzyme recognition sites, and ligation of multiple fragments into the targeting vector, which were quite time consuming. In recent years, the process of generating the targeting vector has become much easier. This is due to the development of the long PCR technique, completion of sequencing of the entire mouse genome, the availability of BACs carrying the gene of interest from commercial sources (for example, from geneservice, Source Bioscience, UK), and the use of recombination in the test tube or in bacteria instead of ligation (11). Commercially available kits for recombination are now widely used for generation of the gene-targeting vector (12).

The overall procedure for generating gene-targeted mice includes multiple steps for which numerous protocols have been developed. It is beyond the scope of this chapter to cover such enormous body of information, and readers are advised to consult the book "*Manipulating the Mouse Embryo: A Laboratory Manual*" (13) for detailed protocols for each step of the gene-targeting procedure. Two excellent resources for details on vector design are "*Gene Targeting, A Practical Approach*" (14) and "*Laboratory Protocols for Conditional Gene Targeting*" (15). In this chapter, we mainly focus on the procedures for introduction

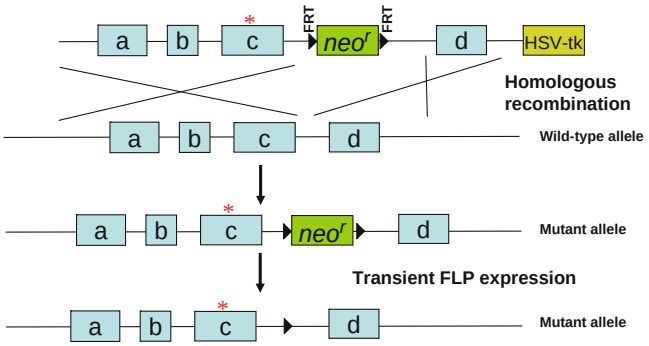

Fig. 11.5. Introduction of a mutation through the knock-in strategy. The targeting vector carries a mutation (missense, nonsense, or small deletion) in the targeted exon (exon c in this example).

of the targeting vector to ES cells and detection of the targeted allele, which are routinely performed in individual research laboratories. This information should also be beneficial to readers interested in applying them to other model organisms and cell culture systems.

2. Materials

2.1. Preparing the DNA for Electroporation

1. Targeting vector DNA.
2. QIAGEN-tip 500 column (Qiagen).
3. EtOH.
4. NaOAc, 3 M, pH 5.2.
5. TLE (TLE): 10 mM Tris–HCl pH 7.5, 0.1 mM EDTA, sterilized.
6. Spectrophotometer.
7. λHindIII molecular weight marker (Promega).

2.2. Electroporation and Selection

1. Tissue culture hood.
2. Incubator.
3. Inverted microscope.
4. ES cells. Some commonly used cell lines are: AB2.2 (16), R1 (17), E14 (18).
5. ES cell medium (for ES cells other than AB2.2 cells): 76.8% DMEM (high glucose), 19.2% FBS (heat inactivated), 1.9 mM L-glutamine, 96 μM MEM non-essential amino acids, 115 units/mL penicillin, 115 μg/mL streptomycin, 0.96% β-mercaptoethanol (100×). Filter sterilize through a 0.22 μm bottle top filter (cellulose acetate).
6. M15 medium (for AB2.2 ES cells): 82% DMEM (high glucose), 15% FBS (heat inactivated), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids, 1% β-mercaptoethanol (100×). Filter sterilize through a 0.22 μm bottle top filter (cellulose acetate).
7. Targeting vector DNA.
8. 60 and 100 mm plates with mitotically inactivated SNL feeder cells (*see* **Note 1**).
9. Dulbecco's phosphate-buffered saline (D-PBS) (Gibco): 200 mg/L KCl, 200 mg/L KH_2PO_4, 8 g/L NaCl, 2.16 g/L $Na_2HPO_4 \cdot 7H_2O$.

10. 0.25% trypsin with EDTA · 4Na, liquid (Gibco): 0.25% trypsin, 400 mg/L KCl, 60 mg/L KH_2PO_4, 350 mg/L $NaHCO_3$, 8,000 mg/L NaCl, 48 mg/L Na_2HPO_4, 1,000 mg/L D-glucose, 10 mg/L phenol red.
11. Hemacytometer.
12. Gene Pulser electroporation system (BioRad).
13. Cuvettes (0.4 mm gap).
14. Geneticin (G418) + ganciclovir (GAN) selection medium: G418 stock (300×) is 100 mg/mL, final concentration is 333 μg/mL; GAN stock (1,000×) is 2 mM, final concentration is 0.002 mM.

    For selection, up to 850 mL of medium may be needed. Dilute G418 and GAN stocks to 1× by adding to filtered ES cell medium. It is unusual to make all 850 mL at once. Instead, take the ES cell medium remaining from the targeting, remove 50 mL for G418-only medium, and use the remainder for G418 + GAN selection medium. When this runs out, only make enough selection medium to get through the remaining period because this medium should not be used if more than 2 weeks old.
15. G418-only selection medium:
    G418 stock (300×) is 100 mg/mL, final concentration is 333 μg/mL.

    For selection, make 50 mL of medium. Dilute G418 stock to 1× by adding 167 μL to 50 mL filtered ES cell medium. Do not use selection medium over 2 weeks old.

2.3. Picking Colonies

1. Tissue culture hood.
2. Incubator.
3. Inverted microscope.
4. 100 mm plate and 96-well plate with SNL feeder layer.
5. 0.5 mg/mL mitomycin C in D-PBS.
6. Gelatinized 96-well plates (for gelatinization of plates, cover the entire surface of wells with 0.1% solution of gelatin in water and then completely aspirate it off right before use).
7. 0.25% trypsin (**Section 2.2**).
8. ES cell medium (**Section 2.2**).
9. D-PBS (**Section 2.2**).
10. G418 (**Section 2.2**).
11. GAN (**Section 2.2**).
12. Hood with a dissecting microscope for picking colonies.

2.4. Duplicating and Freezing 96-Well Plate Clones

1. Tissue culture hood.
2. Incubator.
3. Inverted microscope.
4. Gelatinized 96-well plates (**Section 2.3**).
5. 96-well plate with SNL feeder layer.
6. 0.25% trypsin (**Section 2.2**).
7. ES cell medium (**Section 2.2**).
8. 0.5 mg/mL mitomycin C in D-PBS (**Section 2.3**).
9. D-PBS (**Section 2.2**).
10. DMSO.
11. Styrofoam box.
12. –80°C freezer.
13. 2× freezing medium: 60% DMEM, 20% FBS, 20% DMSO. Use sterile solution, do not filter as DMSO does not go through the 0.22 μm filter.
14. Mineral oil (0.22 μm filtered).
15. Sealing tape.

2.5. Preparing DNA from 96-Well Plates for Genotyping

1. Lysis solution: 1 mg/mL proteinase K, 0.5% sarcosyl, 10 mM Tris–HCl (pH 7.5), 10 mM EDTA, 10 mM NaCl. Prepare 6 mL of lysis solution for each 96-well plate.
2. Digest buffer: 10% restriction enzyme buffer (10×), 1 mM spermidine, 0.1 mg/mL RNase, 0.1 mg/mL BSA, 10 units/μL restriction enzyme. Prepare 3.3 mL of digest buffer for each 96-well plate.
3. EtOH.
4. NaCl.
5. TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA.
6. Agarose gel for electrophoresis.
7. Gel-loading buffer.
8. λHindIII molecular weight marker (Promega).
9. Sealing tape.
10. SealPlate film (EXCEL Scientific).

2.6. Thawing and Expanding Clones from 96-Well Plates

1. Tissue culture hood.
2. Incubator.
3. Inverted microscope.
4. 6- and 24-well plates.
5. 60 and 100 mm plates.
6. 0.25% trypsin (**Section 2.2**).

7. ES cell medium (**Section 2.2**).
8. D-PBS (**Section 2.2**).
9. 0.5 mg/mL mitomycin C in D-PBS (**Section 2.3**).

3. Methods

Once the targeting vector is completed, it is introduced into ES cells. Many ES cell lines have been used successfully in gene knock-out experiments and many are readily available. Electroporation is the most commonly used method to efficiently introduce the targeting vector to ES cells. Following electroporation, the vector DNA is integrated into the genome of ES cells (**Sections 3.1** and **3.2**).

Once electroporation is successfully performed, ES cells that went through homologous recombination need to be selected. Depending on the selectable markers inserted into the targeting vector, selection medium containing appropriate antibiotics and chemicals is prepared. ES cell colonies that grow in the selection medium are selected (**Section 3.3**), expanded (**Section 3.4**), and DNA from selected ES cells is isolated (**Section 3.5**) and further genotyped by southern hybridization or PCR (*see* **Note 2**). After genotyping, ES cell colonies that contain the correctly targeted allele are expanded (**Section 3.6**), re-genotyped, and stored frozen for further procedures such as injection into the blastocyst. Typically, six or more correctly targeted clones are expanded and frozen. Finally, several clones are karyotyped to identify clones with a normal karyotype. Typically, two to three karyotypically normal clones are then microinjected into blastocysts for the generation of chimeric mice.

3.1. Preparing the DNA for Electroporation

In this protocol you will prepare your targeting vector DNA for electroporation into ES cells.

1. Amplify and purify the knock-out DNA construct according to the manufacturer's protocol for QIAGEN-tip 500.
2. Cut 200 μg of DNA with the appropriate restriction enzyme (*see* **Note 3**).
3. Check digest by running 500 ng on an agarose minigel (*see* **Note 4**).
4. Precipitate the DNA:
   a. Add 1/10 vol. of 3 M NaOAc, pH 5.2, and mix by pipeting.
   b. Add 2.4 vol. of 100% EtOH and invert to mix.

c. Incubate at –20° for 1 h. Spin down for 15 min at max speed and decant.
d. Add 500 μL of 70% EtOH, invert once, spin 5 min at max speed (*see* **Note 5**).
e. In the tissue culture hood, decant and air dry the precipitated DNA.

5. Resuspend the DNA in TLE to a final concentration of ≈1 mg/mL. Assume a DNA loss of 50% during steps 2–4 (*see* **Note 6**).
6. Quantify the DNA using a spectrophotometer and confirm by running 100 and 200 ng of digested DNA on a gel alongside 500 ng λHindIII molecular weight marker DNA.

3.2. Electroporation and Selection

In this protocol you will prepare your ES cells for electroporation, electroporate your DNA into ES cells, and use positive–negative drug selection to grow out clones of targeted ES cells.

3.2.1. Preparing the ES Cells (See Note 7)

1. Change medium 2–4 h before starting.
2. Aspirate medium from two to four 100 mm plates of 50–80% confluent ES cells. Wash twice with D-PBS.
3. Add 2.0 mL 0.25% trypsin per plate and incubate for 5 min at 37°C.
4. Add 5.0 mL ES cell medium/plate to neutralize.
5. Resuspend vigorously to a single-cell suspension (*see* **Note 8**). Place in a 50 mL tube.
6. Take 10 μL of suspended cells, dilute to 100 μL with ES cell medium. Count the number of cells using a hemacytometer and calculate total number of cells.
7. Spin cells for 5 min at RT at 800×*g*.
8. Resuspend in 10 mL D-PBS, spin again for 5 min at RT at 800×*g*.
9. Resuspend in D-PBS at 1.1×10^7 cells/mL assuming a 10–15% loss in cell number.
10. Count the cell number again to confirm.
11. Aliquot 0.9 mL of cells into each of three 50 mL tubes.

3.2.2. Electroporation

1. Add 20–25 μg of DNA (*see* **Note 9**) to each tube. Mix well. Leave at RT for 5 min.
2. Transfer 0.9 mL of cells plus DNA to each of three cuvettes and cap.
3. Electroporate in the Bio-Rad Gene Pulser (*see* **Note 10**) at 0.230 kV, 500 μF. Record the actual volts and the time constant (should be about 6–7). Optional condition: 0.800 kV, 3 μF.

4. Incubate 5 min on ice.
5. Transfer cells from cuvettes to one 50 mL tube containing 10 mL ES cell medium, rinsing cuvettes with 1 mL fresh medium. Mix by pipeting up and down gently without causing to bubble. Transfer cells to a 250 mL bottle and bring volume to 161 mL with ES cell medium. Mix well by swirling.
6. Remove medium from mitotically inactivated SNL feeder cells (*see* **Note 1**).
7. Swirl ES cell suspension vigorously and plate 4.5 mL (≈3%) onto one 60 mm plate with SNL feeder cells, and 12 mL onto each of the twelve 100 mm plates with SNL feeder cells (*see* **Note 11**).
8. Incubate plates at 37°C for 24 h before starting "selection of resistant ES cells" (**Section 3.2.3**). The day electroporation is performed is considered to be "day 0".

3.2.3. Selection of Resistant ES Cells

1. Start selection about 24 h after electroporation.
2. Make G418+GAN and G418-only ES media.
3. Aspirate medium from plates.
4. Feed the twelve 100 mm plates with 12 mL ES cell medium with G418 + GAN.
5. Feed the 60 mm plate with 4 mL ES cell medium with G418 only.
6. Refeed plates each day for 9–10 days using the same volumes of fresh ES cell medium with the appropriate drug(s).
7. On days 9–10 begin the "picking colonies" protocol (**Section 3.3**).

3.3. Picking Colonies

In this protocol you will transfer individual drug-selected ES colonies into 96-well plates for growth, genotyping, and expansion.

3.3.1. Preparing the 96-Well Feeders

1. Prepare SNL feeder cells at least 24 h before picking.
2. Remove 8 mL of medium from two 100 mm plates with SNL feeder cells that are well confluent and then add 160 μL of 0.5 mg/mL mitomycin C in D-PBS.
3. Incubate at 37°C for 3 h.
4. Pass the SNL feeder cells from these two 100 mm plates to five gelatinized 96-well plates:
   a. Trypsinize two 100 mm plates of feeder cells (**Section 3.2.1**, steps 1–4).
   b. Resuspend the feeder cells at 0.35×10^6 cells/mL using ES cell medium.

c. Plate 150 μL of cell suspension into each well of the gelatinized 96-well plates.

d. Change to fresh ES cell medium the next day before using.

3.3.2. Picking Colonies (See Note 12)

1. Refeed the twelve 100 mm ES plates 2–4 h before picking by replacing the medium with 12 mL of fresh ES cell medium.
2. Refeed 96-well feeders by replacing the medium in each well with 100 μL ES cell medium.
3. Aliquot 50 μL of 0.25% trypsin into each well of a new 96-well plate.
4. Aspirate medium from one (or more, as needed) of the twelve 100 mm plates with ES cells under G418 + GAN selection.
5. Wash the 100 mm plate(s) once with D-PBS.
6. Add 10 mL D-PBS to the 100 mm plate(s).
7. Align a 96-tip rack with the 96-well plate containing trypsin. Pick colonies and transfer to 96-well plate, one colony per well, as follows (*see* **Note 13**).
8. a. Set the P20 pipet to 10 μL and attach a tip.
   b. Depress plunger above layer of D-PBS before proceeding under the surface.
   c. Scrape a circle around the ES colony using the tip. Use the tip as a spatula and scrape at the base of the colony.
   d. Pipet the loose colony into the tip.
   e. Transfer the single colony into the well with trypsin, in the same position as the tip originated from in the 96-tip rack, pipeting up and down 2–4 times.
   f. Repeat until all the 96 wells have been picked.
9. When the 96-well plate is filled incubate for an additional 5 min at 37°C.
10. Add 50 μL ES cell medium + G418 + GAN per well with a multi-channel pipet.
11. Pipet up and down 15–30 times at 70–80 μL setting to dissociate cells (*see* **Note 14**), then transfer to 96-well feeders.
12. Continue row by row until the 96-well plate is complete.
13. Go to step 4 and repeat until all the colonies have been picked from the 100 mm plates or until five 96-well plates have been filled.
14. Grow 3–5 days before preparation of duplicates and freezing of master plate.

3.4. Duplicating and Freezing 96-Well Plate Clones

In this protocol you will generate triplicate 96-well plates of your clones. Two plates will be used for genotyping ("plates A and B") and one plate will be frozen as the master plate ("plate C").

1. Refeed ES cells by replacing the medium in each well with 100 μL ES cell medium 2–4 h prior to trypsinization.
2. Prepare one gelatinized 96-well plate (**Section 2.3**) for each 96-well plate cultured in **Section 3.3.2**.
3. Add 100 μL ES cell medium per well in the gelatinized 96-well plate. This is plate A.
4. Wash one 96-well plate of ES colonies from **Section 3.3.2** twice with D-PBS. This is plate B.
5. Add 50 μL trypsin to each well of plate B.
6. Incubate 10 min 37°C.
7. Add 50 μL ES cell medium to each well.
8. Pipet up and down (at 70–80 μL setting) to dissociate cells, 15–30 times (*see* **Note 14**). Change setting every time between 70 and 80 μL, so that cells do not accumulate in a particular position in the pipet tip.
9. Transfer 55 μL to a new 96-well (not gelatinized) plate. This is plate C, the master plate.
10. Transfer 17.5 μL from plate B to plate A.
11. Continue row by row until the whole plate is finished.
12. Add 55 μL of 2× freezing medium (prepared fresh) to the master plate, plate C. Put the tip down to the bottom of the well and dispense the freezing medium. Pipet up and down twice to mix.
13. Add 100 μL of sterile mineral oil (0.22 μm filtered) to each well.
14. Seal plate with sealing tape and put into a styrofoam box and store it at –80°C.
15. Now add 100 μL ES cell medium per well to plate B.
16. Grow plates A and B to confluence (2–5 days), changing medium daily.
17. Identify the positive clones from the duplicate 96-well plates A and B following the protocol in **Section 3.5**.

3.5. Preparing DNA from 96-Well Plates for Genotyping

In this protocol, you will prepare DNA samples from your ES clones in the duplicate plates A and B for genotyping using Southern blotting or PCR amplification.

1. Wash cells twice with D-PBS (150 μL/well).
2. Add 50 μL lysis solution per well.

3. Seal the top of the 96-well plate with a SealPlate film. Cover plate with lid and seal sides with stretchable sealing tape.
4. Place the plate inside a plastic container containing some wet paper towels and cover with lid. This stops the wells from drying out. Incubate overnight at 60°C while rocking.
5. The next day, remove the SealPlate film, apply 100 μL EtOH + salt (10 mL 100% EtOH and 0.15 mL 5 M NaCl) to each well. Put the lid back on and make sure not to mix.
6. Leave out on the bench at RT for 60 min. Be sure not to disturb the plate (*see* **Note 15**).
7. Spin the plate for 10 min at 3,000×*g*.
8. Gently invert the plate onto a stack of paper towels.
9. Wash three times with 150 μL 70% EtOH, inverting gently each time.
10. After the last wash invert once more to remove trace of EtOH.
11. Tilt plate slightly and allow to air dry for 20 min (*see* **Note 16**).
12. Apply 30 μL of digest buffer to each well and cover the plate with SealPlate film, replace lid and seal the plate with stretchable sealing tape. Place the plate inside a plastic sealed container containing some wet paper towels to prevent drying. Incubate at 37°C overnight.
13. Add gel-loading buffer and load samples on 0.7% TBE gel. Run at 100 V for 10 min for the DNA sample to run into the gel matrix, then turn down voltage, and run until bromophenol blue, the tracking dye, reaches wells of the next row. It is typical to run gels at 20–25 V overnight or at 70–80 V for 4–6 h.

3.6. Thawing and Expanding Clones from 96-Well Plates

In this protocol, you will slowly and sequentially expand the clones from 96-well plates to 100 mm plates (*see* **Note 17**).

1. Refeed 24-well feeders with 1 mL per well fresh ES cell medium.
2. Place a frozen 96-well plate with clones (Plate C from **Section 3.4**) in 37°C incubator until thawed—about 10–15 min (*see* **Note 18**).
3. Gently pipet up and down in selected wells, then move the entire contents (including oil) to the well of 24-well feeders, spreading evenly in the well. Rinsing the 96 well is optional.

4. Refeed daily by exchanging the medium in each well with 1 mL fresh ES cell medium.
5. Grow 3–5 days, then passage to a 6-well plate with feeder cells (use the basic ES passing protocol):
   a. Change medium 2–4 h before passing.
   b. Rinse twice with D-PBS (without Ca^{2+} and Mg^{2+}).
   c. Add 200 μL 0.25% trypsin and incubate for 3–5 min at 37°C.
   d. Add 400 μL ES cell medium.
   e. Pipet up and down with a P1000 pipet 15–25 times and check for a single-cell suspension—repeat pipeting if necessary.
   f. Transfer the entire contents to a well of a 6 well plate—this is about a 1:3 split.
   g. Refeed daily by exchanging the medium with 2.5 mL fresh ES medium.
6. Grow 2–3 days, then passage to 100 and 60 mm plates as follows (per standard protocol, *see* **Note 19**).
7. Change medium 2–4 h before passing.
8. Rinse twice with D-PBS (without Ca^{2+} and Mg^{2+}).
9. Add 1.5 mL 0.25% trypsin and incubate for 3–5 min at 37°C.
10. Add 3.5 mL ES cell medium.
11. Pipet up and down with a 5 or 10 mL pipet 15–25 times and check for a single-cell suspension—repeat pipeting if necessary.
12. Split 4/5 of dish contents (4 mL) to a 100 mm plate with feeder cells—refeed daily by exchanging the medium with 4 mL of fresh ES medium.
13. Split 1/5 of dish contents (1 mL) to a gelatinized 60 mm dish—refeed daily by exchanging the medium with 1 mL of fresh ES medium.
14. Freeze down the 100 mm plate when the cells are 50–70% confluent (2–3 days).
15. Use the standard *ES Freezing* protocol (**Section 3.4**).
16. Freeze down five vials at 5×10^6 cells/vial in 0.5 mL aliquots.
17. Prepare genomic DNA from the 60 mm dish when the cells are 50–100% confluent (4–5 days) using the *Puregene DNA Isolation Kit* according to the manufacturer's protocol.

4. Notes

1. Mitotically inactivated SNL (STO-Neo-LIF) feeder cells must be prepared at least a day before electroporating. For procedures in **Section 3.2**, prepare 1–60 mm dish (control plate) and 12–100 mm (picking plates) at 100% confluence. The protocol for preparing feeder cells can be found in **Chapter 21** of this volume.
2. Since selection using selectable markers does produce false positives, it is critical to test if ES cells selected through this screening indeed have experienced homologous recombination. Negative selection can be performed for this purpose, and particularly to distinguish between random insertion of the targeting vector and homologous recombination. Southern hybridization is the preferred method for negative selection, since it is more accurate to test the occurrence of homologous recombination with lower risk of false positives compared to PCR, despite the fact that it takes more effort and time. Since the targeted allele produces different restriction enzyme recognition sites, the targeted allele can be distinguished from the wild-type allele based on the size of the positive band for the specific probe in southern hybridization. It is also important to test if both homology arms are recombined. In some cases, only one arm is recombined and the other arm is integrated into the genome by insertion. Therefore, two sets of southern hybridization experiments, using probes from each of the arms, should be designed to test if both arms are recombined. Alternatively, this can be confirmed by sequencing the targeted allele. For example, in our laboratory, we sequence across the *loxP* sites to test if a conditional targeting allele is successfully integrated into the genome by homologous recombination.
3. The DNA concentration is best at 0.5 μg/μL for digestion but can be increased to 1 μg/μL.
4. Proceed only if the digest is complete. If not complete, add more restriction enzyme (e.g., $^1/_4$ to $^1/_2$ of the original amount of enzyme added) and repeat steps 2 and 3.
5. At this point the DNA is sterile (because of the EtOH steps). Therefore, all remaining steps must be performed under sterile conditions using sterile solutions.
6. Keep in mind that it is not unusual to lose a significant amount of DNA in its preparation. Also, it is easier to dilute

the DNA to the desired concentration rather than concentrating it.

7. All steps are performed under sterile conditions.
8. It may help to pipet the cells 10–20 times using a P1000 pipet tip before neutralizing.
9. We use 25 μg of DNA.
10. Using the Bio-Rad Gene Pulser:
    1) Turn on the Gene Pulser.
    2) Set capacitance at 500 μF with dial.
    3) Set Kilovolts at 0.23 kV with raise and lower buttons.
    4) Place cuvette in tray. Slide the tray so that the electrodes touch the cuvette firmly.
    5) Press and hold the two red buttons. The tone indicates that the charging is complete. Release the button to deliver the pulse.
    6) Record actual volts and time constant.
    7) Remove the cuvette from the instrument.
    8) Manually discharge capacitor by turning the main power switch on and off two times.
11. Keep cells evenly suspended, so that each plate receives approximately the same number of cells.
12. A dissecting microscope positioned in a tissue culture hood, dedicated for picking colonies, is required for this protocol.
13. One can pick up to 100–150 colonies/h.
14. Check the first row to confirm if they are in a single-cell suspension.
15. DNA should be visible as a white filamentous network/sheet.
16. Care should be taken not to air dry the plate for excessive amount of time, since over-dried DNA may not dissolve very well in the next step.
17. We thaw *all* the positive clones in the 96-well plate and move them to the 24-well plates (**Section 3.6.3**). When it is time to passage to 6-well plates (**Section 3.6.5**), we passage 6–8 clones and freeze the rest of the clones in freezing medium in cryovials and store in liquid nitrogen. This way no clones will remain at –80°C in 96-well plates, and if needed, frozen clones can be thawed and expanded.
18. We recommend not to exceed 15 min, since longer incubation in freezing medium and mineral oil at 37°C is not optimal for the well-being of cells.

19. When passing the ES cells to the 100 mm plate, it is important that ES cells in the 6 well plate be 50–80% confluent. This will ensure that the correct density of cells will be achieved when freezing down. Therefore, it may be necessary to pass a clone one or more extra times at the 24-well or 6-well stages.

References

1. Thomas, K. R. and Capecchi, M. R. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503–512.
2. Doetschman, T., Gregg, R. G., Maeda, N., Hooper, M. L., Melton, D. W., Thompson, S., and Smithies, O. (1987) Targeted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature* **330**, 576–578.
3. Martin, G. R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638.
4. Evans, M. J. and Kaufman, M. H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.
5. Nagy, A. (2000) Cre recombinase: the universal reagent for genome tailoring. *Genesis* **26**, 99–109.
6. Cohen-Tannoudji, M. and Babinet, C. (1998) Beyond 'knock-out' mice: new perspectives for the programmed modification of the mammalian genome. *Mol. Hum. Reprod.* **4**, 929–938.
7. Chikama, T., Hayashi, Y., Liu, C. Y., Terai, N., Terai, K., Kao, C. W., Wang, L., Hayashi, M., Nishida, T., Sanford, P., Doestchman, T., and Kao, W. W. (2005) Characterization of tetracycline-inducible bitransgenic Krt12rtTA/+/tet-O-LacZ mice. *Invest. Ophthalmol. Vis. Sci.* **46**, 1966–1972.
8. Nilles, K. M. and London, B. (2007) Knockin animal models of inherited arrhythmogenic diseases: what have we learned from them? *J. Cardiovasc. Electrophysiol.* **18**, 1117–1125.
9. Melton, D. W. (2002) Gene-targeting strategies. *Methods Mol. Biol.* (Clifton, N.J) **180**, 151–173.
10. Nagy, A., Mar, L., and Watts, G. (2009) Creation and use of a cre recombinase transgenic database. *Methods Mol. Biol.* (Clifton, N.J) **530**, 365–378.
11. Liu, P., Jenkins, N. A., and Copeland, N. G. (2003) A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res.* **13**, 476–484.
12. Iiizumi, S., Nomura, Y., So, S., Uegaki, K., Aoki, K., Shibahara, K., Adachi, N., and Koyama, H. (2006) Simple one-week method to construct gene-targeting vectors: application to production of human knockout cell lines. *Biotechniques* **41**, 311–316.
13. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003) Manipulating the Mouse Embryo: A Laboratory Manual, Third Edition. Cold Spring Harbor Laboratory Press, New York.
14. Joyner, A. L. (Ed.) (2000) Gene Targeting: A Practical Approach, Second edition. Oxford University Press, New York.
15. Torres, R. M. and Kuhn, R. (1997) Laboratory Protocols for Conditional Gene Targeting. Oxford University Press, New York.
16. Matzuk, M. M., Finegold, M. J., Su, J. G., Hsueh, A. J., and Bradley, A. (1992) Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature* **360**, 313–319.
17. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **90**, 8424–8428.
18. Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature* **326**, 292–295.
19. Farley, F. W., Soriano, P., Steffen, L. S., and Dymecki, S. M. (2000) Widespread recombinase expression using FLPeR (flipper) mice. *Genesis* **28**, 106–110.

Chapter 12

Creating a "Hopeful Monster": Mouse Forward Genetic Screens

Vanessa L. Horner and Tamara Caspary

Abstract

One of the most straightforward approaches to making novel biological discoveries is the forward genetic screen. The time is ripe for forward genetic screens in the mouse since the mouse genome is sequenced, but the function of many of the genes remains unknown. Today, with careful planning, such screens are within the reach of even small individual labs. In this chapter we first discuss the types of screens in existence, as well as how to design a screen to recover mutations that are relevant to the interests of a lab. We then describe how to create mutations using the chemical *N*-ethyl-*N*-nitrosourea (ENU), including a detailed injection protocol. Next, we outline breeding schemes to establish mutant lines for each type of screen. Finally, we explain how to map mutations using recombination and how to ensure that a particular mutation causes a phenotype. Our goal is to make forward genetics in the mouse accessible to any lab with the desire to do it.

Key words: ENU, mutagenesis, mutant, phenotype-driven screen.

1. Introduction

Recent years have brought an explosion of whole-genome sequencing in a wide variety of organisms. From this explosion, comparative genomics has emerged as a powerful tool for shedding light on a range of biological processes, with the potential to reveal much about human variation, development, and disease. However, comparative genomics will not fulfill its potential until we have a more complete understanding of the functions of the individual genes in these genomes, so they can be related back to their human counterparts. For example, the function of a third of the genes in the mouse genome is still completely unknown.

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_12,

Of the approximately 26,000 genes in the mouse genome, 8,154 (31%) genes have no functional annotation (1). Perhaps more remarkably, 17,904 (68%) genes in the mouse genome have no mutant alleles (1). Several international projects are underway to produce null alleles of every gene in the mouse genome, so that gene function can be inferred from the resulting phenotype (2, 3). Such a "reverse genetics" approach will provide valuable resources to the mouse community and fill many gaps in our knowledge. Complementary to this approach is forward genetics, which begins with a mutant phenotype in a biological process of interest and then asks what gene is disrupted to produce that particular phenotype. Forward genetic screens, therefore, can give us an unbiased view of a biological process from which novel discoveries can flow. Furthermore, the nature of the allele obtained in a mutagenesis screen can tell us a great deal about a particular protein's role in a specific process in a way that deletion of the protein cannot. Finally, another benefit of alleles created via chemical mutagenesis is that they tend to mimic human disease alleles (4).

Reverse genetics has become the preferred method for individual labs studying specific mammalian genes. Recently, however, a growing number of labs are interested in forward genetics, largely for two reasons. First, the availability of the mouse genome sequence has made positional cloning much more straightforward, due in part to a denser set of markers that allows one to more easily narrow down the region in which a mutation lies. Further, we now know exactly how many genes are in any particular region. This information, combined with available gene expression data, makes it easier to prioritize which genes to sequence to find the causative mutation. Second, mutagenesis screens in the mouse have the unique ability to impartially reveal a collection of genes involved in a biological process of interest. In the current genomics era, where the focus is shifting from understanding single gene products to understanding how networks of gene products interact and influence one another, forward genetics is a particularly apt and powerful tool.

How practical is it for an individual lab to perform a forward genetic screen in the mouse? General concerns are time, breeding space required, and cost. Although the time from mutagenization to the establishment of mutant lines is about 1 year, much of this is passive time spent waiting for males to recover fertility after mutagenization and setting up crosses. The active screening time is 4 or 5 months. The amount of breeding space required reflects this passive/active time pattern, with a long period of housing relatively few mice, followed by the active screening phase, when a burst of mice are produced (**Fig. 12.1**). Once mutant lines are established, active positional cloning takes several months to about a year to complete. However, next-generation resequencing technology holds promise that we will further accelerate this

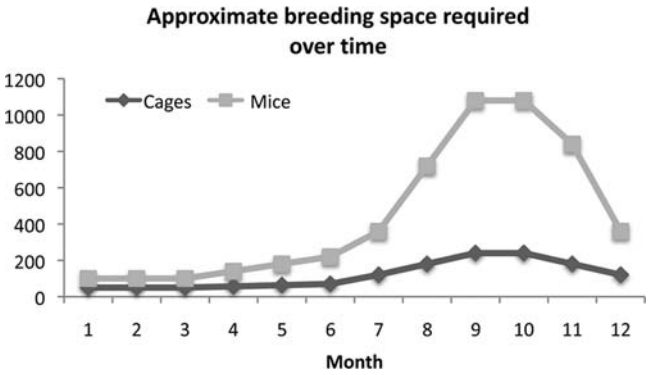

Fig. 12.1. Approximate breeding space required per month in a generic yearlong screen for recessive mutations.

step, as longer portions of a chromosome can be sequenced for less time and cost. Overall, it is quite feasible for an individual lab to carry out a mutagenesis screen, and the goal of this chapter is to provide the reader with practical considerations and instructions to do just that.

2. Materials

1. Mice: 7- to 8-week-old males of the desired strain for mutagenization (**Section 3.2**).
2. *N*-ethyl-*N*-nitrosourea (ENU).
3. 95% ethanol: make fresh each time.
4. Phosphate/citrate buffer: 0.1 M dibasic sodium phosphate and 0.05 M sodium citrate, adjust to pH 5.0 with phosphoric acid. Make fresh each time.
5. ENU inactivating solution: Use one of the following:
   0.1 M potassium hydroxide

   Alkaline sodium thiosulfate: 0.1 M sodium hydroxide and 1.3 M sodium thiosulfate.
6. Syringes/needles: For ENU dilution: 18-gauge needles, 10 mL syringes, and 30–50 mL syringes. For ENU injections: 25-gauge needles and 1 mL syringes.
7. Squirt bottles.
8. Waste containers: hazardous waste plastic bags, container for deactivated ENU, and sharps disposal box.
9. Personal protective equipment for handling ENU: disposable gowns, masks, gloves.
10. Disposable bench paper to line hood during ENU injections.

3. Methods

3.1. Designing a Screen

The initial consideration is a critical one: how to design a screen to recover mutations that suit the interests and goals of the lab? One way to approach this question is to first determine whether you are interested in a general biological process or a particular gene or region of the genome (**Fig. 12.2**). Those interested in a general biological process are best served by a genome-wide screen, since it is likely that numerous genes scattered throughout the genome control the process of interest. Those more interested in the functional content of a given region of the genome, or in generating an allelic series of a particular gene, will find a region-specific screen more appropriate. Another consideration is the time it will take to map and clone causative mutations once the screening is complete. In a genome-wide screen, the recovered mutations can be at any position on any chromosome. Positional cloning takes several months to a year to complete, because one must generate enough embryos to allow up to 1,500 opportunities for recombination, design primers to find polymorphic markers, and sequence. Since region-specific screens are limited to a defined portion of the genome, finding the causative mutation is greatly simplified, reducing the overall time and cost. We will examine several classes of both genome-wide and region-specific genetic screens below.

Having defined screening criteria is another important factor to consider when designing a screen, for ease of phenotype identification and reproducibility. For example, our lab recently completed a screen for recessive mutations that affect embryonic development. We broadly examined embryos for morphological abnormalities, but for consistency we chose nine key features to

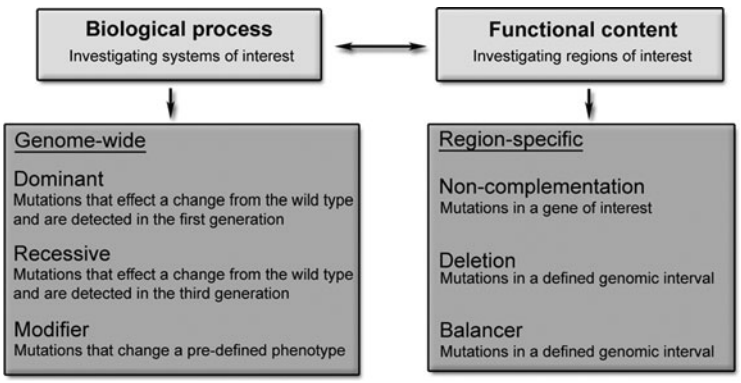

Fig. 12.2. Classes of genome-wide and region-specific forward genetic screens. Adapted with permission from Macmillan Publishers Ltd: *Nat Rev Genet* (33), copyright 2005.

score, such as brain lobes, eyes, and pharyngeal arches. Increasingly complex assays can lead to lengthy or slow screening. For instance, screens that include criteria such as serum analysis or behavioral assays may limit the number of mutant lines that can be screened. Each lab must weigh for itself the relative costs and benefits of including extra steps in a screen.

3.1.1. Genome-Wide Screens

Genome-wide screens can be designed to recover mutations that create either dominant or recessive alleles. Dominant alleles cause a phenotype that is observed in the heterozygous state, either because two normal alleles are required for normal function of the gene (haploinsufficiency), because the mutant allele disrupts the function of the normal allele (dominant negative), or because the mutant allele has new or increased activity (gain of function). One purely practical reason to screen for dominant alleles is that they can be recovered in the fewest number of crosses, thereby reducing time and cost (**Section 3.3**). Another possible rationale for performing a dominant screen is to model a human disease condition with a dominant mode of transmission (for examples, *see* (5)).

Recessive alleles can have partial or total loss of function, and both alleles must be mutant to produce a phenotype. Therefore, three crosses are required to recover mutations that create recessive alleles (**Fig. 12.3**). The additional breeding time can be justified, however, since it is easier to infer normal gene function from recessive alleles, as they are generally loss of function.

The final class of genome-wide screen is the modifier screen: recovering new genes that suppress or enhance a phenotype of

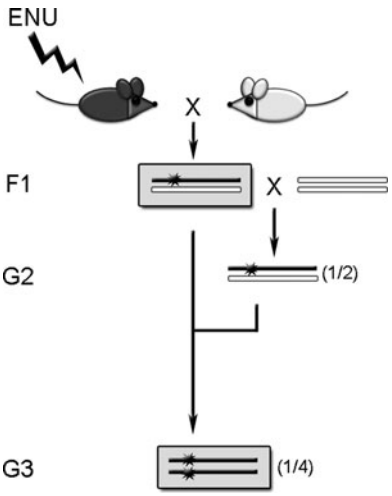

Fig. 12.3. Crossing scheme for dominant (*upper gray box*) or recessive (*lower gray box*) mutant alleles. In this and all subsequent figures, chromosomes from the mutagenized *black mouse* are represented as *black bars*; chromosomes from the *white mouse* are represented as *white bars*. Additionally, in all figures *stars* represent mutant alleles.

interest. Modifier screens are performed when at least one gene is known to be necessary for a biological process of interest, and the goal is to discover other genes in the same pathway or same process. Modifier screens can be designed to recover dominant or recessive alleles, as above. They can also be performed with known alleles that are not viable in the homozygous state, although the crossing scheme is more involved (**Fig. 12.4**).

3.1.2. Region-Specific Screens

The narrowest type of region-specific screen is the non-complementation screen. The purpose of a non-complementation screen is to find new alleles of a gene of interest, because mutations in different protein domains can reveal much about the function of those domains and/or can help to define specific interactions with other proteins. In a non-complementation screen, one crosses an animal carrying a known mutation in a particular allele with an animal carrying random mutations (**Fig. 12.5**). If the progeny of such a cross exhibit the mutant phenotype of the known allele, the newly mutagenized allele is said to "fail to complement" the original allele. It is important to note that since mutations are induced randomly in the genome, a failure to complement can be either allelic or non-allelic; if it is allelic, then the mutation will be revealed through sequencing the gene in the new mutant background. If it is non-allelic, the mutation must be mapped via meiotic recombination (**Section 3.4**).

Deletion screens incorporate mouse strains with deletions in known portions of their genome. A number of deletion strains are available in the mouse, with about half of the chromosomes having at least one "deletion complex" or collection of overlapping deletions (**Table 12.1**). The first seven deletion complexes were

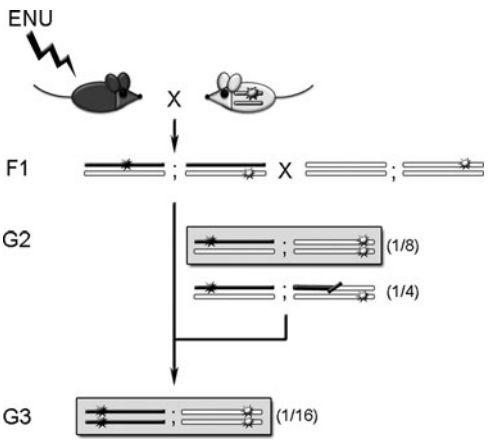

Fig. 12.4. Crossing scheme for dominant (*upper gray box*) or recessive (*lower gray box*) modifier alleles. In this crossing scheme the allele to be modified (in the *white mouse*) is assumed to be homozygous lethal or sterile. The *half-black/half-white* chromosome in the second generation indicates that either allele is acceptable in this cross.

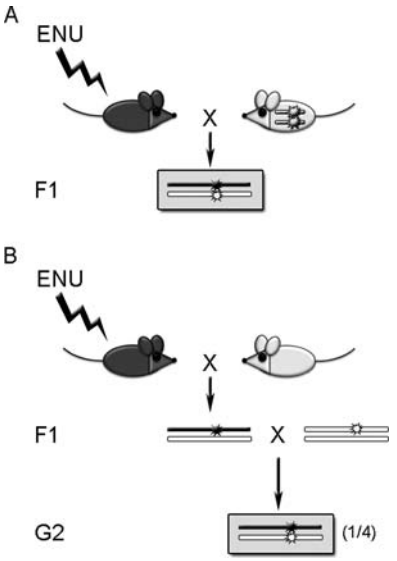

Fig. 12.5. Non-complementation crossing scheme. (**a**) The allele to be tested (in *white mouse*) is viable and fertile as a homozygote. (**b**) The allele to be tested (in *white mouse*) is lethal or sterile as a homozygote.

generated by irradiating or chemically mutating mice and then crossing them to mice with visible markers. In this way deletions could be located to the region surrounding the visible marker (specific locus test; (6)). These initial deletion complexes each contain many available mouse strains with overlapping deletions (**Table 12.1**, gray rows). More recently, deletion complexes are created in genomic areas of interest using embryonic stem cells (ES cells). Deletions are generated in ES cells through irradiation or through Cre–loxP-mediated recombination and then mice bearing the deletions are produced from the ES cells, when possible (**Table 12.1**, white rows) (7–10). In addition to simplifying the mapping process, another practical reason to perform a deletion screen is that recessive mutations can be recovered in fewer crosses than in a genome-wide recessive screen (**Fig. 12.6**). The only caveat is that the deletion strain used in the screen must be viable as a heterozygote (i.e., cannot be haploinsufficient), a fact not yet known for the deletions that only exist as ES cells.

The final region-specific screen is the balancer screen, modeled after successful screens performed in *Drosophila melanogaster* and *Caenorhabditis elegans*. A "balancer chromosome" is one that contains inversions to prevent recombination with its homolog, plus a dominant marker, so that animals carrying it can be recognized. Balancer chromosomes may contain recessive lethal mutations as well. They are called "balancers" because they prevent any lethal or sterile mutations on the homologous chromosome from being removed from a population (i.e., they maintain

Table 12.1
Mouse chromosomal deletion complexes

Chr	Deletion Complex	No. mouse/cell lines	Mode of generation	Total span of nested deletions, if known	Reference
2	Non-agouti (*a*)	~17 mouse lines	Mixed (chemical and radiation) of mouse/germ cells		(1, 6, 34, 35)
2	*Notch1*	10 cell lines	ES cell: recombination-mediated deletion	6.2–7.7 cM (10.4 Mb)	(36)
4	Brown (*Tyrp1*^{b})	~35 mouse lines	Mixed (chemical and radiation) of mouse/germ cells	~21 Mb	(1, 6, 37–39)
5	*Hdh*, *Dpp6*, *Gabrb1*	10 mouse lines	ES cell: X-ray irradiation	40 cM	(40)
7	Albino (*Tyr*^{c})	~55 mouse lines	Mixed (chemical and radiation) of mouse/germ cells	6–11 cM for 29 of the lines	(1, 6, 41, 42)
7	Pink-eyed dilution (*p*)	~65 mouse lines	Mixed (chemical and radiation) of whole mouse		(1, 6, 43–45)
9	Dilute (*Myo5a*^{d})	~16 mouse lines	Mixed (chemical and radiation) of mouse/germ cells		(1, 6, 46, 47)
9	Short ear (*Bmp5*^{se})	~4 mouse lines	Mixed (chemical and radiation) of mouse/germ cells		(1, 6, 47, 48)
9	Dilute and Short ear (*d se*)	~29 mouse lines	Mixed (chemical and radiation) of mouse/germ cells		(1, 6, 47)
9	*Ncam*	28 cell lines	ES cell: X and UV irradiation	28 cM	(8)
11	*Hsd17b1 [Del* (11) *Brd]*	8 mouse lines	ES cell: recombination-mediated deletion	8 Mb	(49)
14	Piebald (*Ednrb*^{s})	20 mouse lines	Mixed (chemical and radiation) of mouse/germ cells	15.7–18 cM	(1, 6, 50, 51)
15	*Sox10*	2 mouse lines	ES cell: X-ray irradiation	577 kb	(52)
15	*Oc90*	2 mouse lines	ES cell: X-ray irradiation	658 kb-5 Mb	(52)
15	*Cpt1b*	192 cell lines	ES cell: X-ray irradiation		(52)

Table 12.1 (continued)

Chr	Deletion Complex	No. mouse/cell lines	Mode of generation	Total span of nested deletions, if known	Reference
17	*D17Aus9*	3–7 mouse lines	ES cell: X-ray irradiation	<1–7 cM	(9)
17	*Sod2, D17Leh94*	8 mouse lines	ES cell: X-ray irradiation	~14 Mb	(53)
X	*Hprt*	4 cell lines	ES cell: recombination-mediated deletion	~1 cM	(49)
X	*Hprt*	9 cell lines	ES cell: X and UV irradiation	1–3 cM	(8)
X	*Hprt*	2 mouse lines	ES cell: X-ray irradiation	200–700 kb	(54)

The number of available mouse lines per deletion complex is estimated based on the primary literature and current information from Mouse Genome Informatics (MGI). If no mouse lines have been established, but embryonic stem (ES) cell lines have been generated, they are listed. The deletion complexes are named after the loci that served as the deletion focal point. *Rows colored dark gray* indicate deletion complexes identified in the specific locus test (6). The *light gray row* is a deletion complex that includes two closely linked loci identified in the specific locus test.

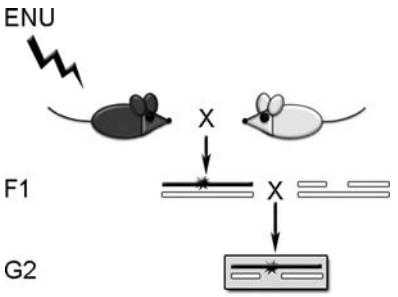

Fig. 12.6. Deletion screen crossing scheme. The chromosome with a gap indicates the region that is deleted. Mutant alleles are recovered in the second generation (*gray box*).

heterozygosity). Screens performed with balancer chromosomes therefore have several advantages: the visible marker allows one to identify and select the G2 and G3 mice that are potentially carrying mutations, in contrast to performing blind crosses, as one must in genome-wide screens (**Fig. 12.7**). In addition, the ability to genotype using visible markers is not only faster and cheaper than PCR-based methods but provides an advantage in determining whether the mutation segregates to the balancer region. Finally, if the mutant phenotype is recessive lethal or sterile, the line can be more easily maintained, since it is balanced. One drawback of performing a balancer screen is that cur-

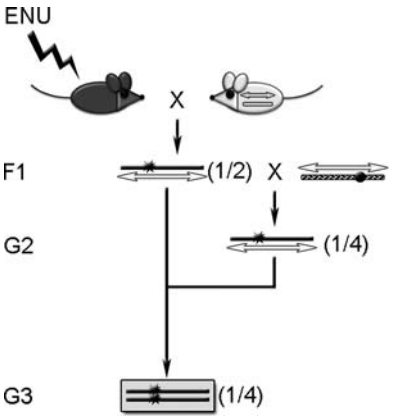

Fig. 12.7. Balancer screen crossing scheme. The *white bar with double-sided arrows* indicates the balanced chromosome. In the first generation, F1 mice are crossed to mice carrying the inversion *in trans* to a WT chromosome marked with a dominant visible mutation (*dotted bar with black circle*). Mutant alleles are recovered in the third generation (*gray box*).

rently there are not yet many balancer mouse strains available (*see* **Table 12.2**). However, they can be generated using recombination in ES cells (7, 11, 12). Furthermore, more G0 males may need to be injected, because only half of the F1 males will be subsequently used (those carrying the balancer, *see* **Fig. 12.7**). Finally, when screening for embryonic lethal phenotypes, it is best to use a balancer that is viable when homozygous to prevent confusion about the cause of lethality.

3.2. Mutagenization

There are several methods to mutagenize the mouse genome: chemicals like *N*-ethyl-*N*-nitrosourea (ENU) and chlorambucil, irradiation with X-rays or gamma rays, and transposons such as *sleeping beauty* (6, 13–17). For the purposes of this chapter we focus on the most widely used method, the chemical ENU. ENU is a powerful mutagen. Depending on the strain of mouse and the dose given, ENU induces a point mutation every 0.5–16 Mb throughout the genome (18–22), which is about 100 times higher than the spontaneous mutation rate per generation in humans (23). Further, ENU primarily affects spermatogonial stem cells, so that one male mouse will produce multiple clones of mutated sperm after completion of spermatogenesis (24). In addition to its efficient nature, another advantage of ENU is the variety of protein altercations that can result from this form of mutagenization. Since ENU is an alkylating agent that induces point mutations, nonsense (10%), missense (63%), splicing (26%), and "make-sense" (1%) mutations can all occur (reviewed in (25–27)). Therefore, in addition to null alleles, other alleles will also be generated, including hypomorphs, hypermorphs, and

Table 12.2
Mouse balancer strains (strains that are viable as homozygotes are indicated)

Chr	Name	Dominant marker phenotype (gene)	Recessive lethal? (gene, if known)	Mode of generation	Reference
4	Inv (4)Brd1$^{Mit281\text{-}Mit51}$	Coat color *(Tyrosinase* and *K14-agouti)*	No	Cre–loxP-mediated recombination	(55)
4	Inv (4)Brd1$^{Mit117\text{-}Mit281}$	Coat color *(Tyrosinase* and *K14-agouti)*	No	Cre–loxP-mediated recombination	(55)
5	Rump white *(Rw)*	Coat color *(Kit receptor tyrosine kinase)*	Yes	Irradiation	(56, 57)
10	Steel panda *(Sl^{pan})*	Coat color *(Kit ligand)*	No	Irradiation	(58)
11	Inv (11)$^{Trp53\text{-}Wnt3}$	Coat color *(K14-agouti)*	Yes (*Wnt3)*	Cre–loxP-mediated recombination	(12)
11	Inv (11)$^{Wnt3\text{-}D11Mit69}$	Coat color *(K14-agouti)*	Yes (*Wnt3)*	Cre–loxP-mediated recombination	(59)
11	Inv (11)$^{Trp53\text{-}EgfR}$	Coat color *(K14-agouti)*	No	Cre–loxP-mediated recombination	(59)
15	In (15)2R1	Short, hairy ears *(Eh)*	Yes	Chemical mutagenesis or irradiation	(60)
15	In (15)21Rk	Coat color *(K14-agouti)*	Yes	Modification of line derived from chemical mutagenesis or irradiation	(61)

dominant-negative alleles. This ability to generate an allelic series is one of the great strengths of forward genetics.

3.2.1. Inbred Strains and ENU Dose

One of the first practical considerations is which strain of mice to mutagenize. A popular choice is C57BL/6J, because the effective dose of ENU is well defined and the genome is sequenced for this strain, facilitating future mapping and analysis portions of the screen. Nevertheless, with the increased density of genetic markers and cheaper and more advanced resequencing technologies, choosing other strains has become feasible. Such advancements allow more flexibility in screen design, for instance by enabling

Table 12.3
Recommended dose of ENU for different inbred mouse strains

Inbred mouse strain	Recommended dose (mg/kg)[a]	No. days of sterility	Percent regained fertility
A/J	3 × 90	74–113	90 ($n = 10$)
BALB/cJ	3 × 100	89–154	83 ($n = 6$)
BTBR/N	1 × 150–200	70–210	50–83 (ND)
C3He/J	3 × 85	96–148	70 ($n = 10$)
C3HeB/FeJ	3 × 75	89–142	90 ($n = 10$)
C57BL/6J	3 × 100	90–105	80 ($n = 10$)

[a]The doses are recommended based on the least amount of death and shortest period of sterility. For details and alternate doses, see the original papers.
ND = no data.
Modified from (28, 29).

one to incorporate visible markers (such as GFP) that may only be available on a particular genetic background. When choosing the strain of mice to mutagenize, it is important to note that ENU affects inbred strains differently (**Table 12.3**). In all strains, ENU initially depletes all spermatogonia from the testes, leading to a period of sterility from which some males never recover. In addition, some mice may die during the sterile period due to somatic mutations that lead to cancer or increase susceptibility to pathogens. The length of the sterile period and the deaths vary with ENU dosage and each inbred strain; some strains (like BALB/cJ and C57BL/6J) can tolerate a relatively high dose, whereas others (like FVB/N) are very sensitive to ENU. For successful mutagenesis, one must balance the highest possible mutation load with the lowest rates of sterility and death. Thanks to careful analysis and experimentation by Justice *et al.* (28) and Weber *et al.* (29), the optimal ENU dose for various inbred strains can be estimated; we provide a summary in **Table 12.3**. As indicated in **Table 12.3**, a fractionated series of injections at weekly intervals is generally more effective than one single large injection, since a series maximizes the mutagenic effect and minimizes animal lethality (30). For instance, rather than a single dose of 300 mg/kg, inject 3 doses of 100 mg/kg at weekly intervals (written as 3 × 100 mg/kg).

3.2.2. Number of Mice to Inject

The number of males to inject depends on how many genes in the genome one wishes to survey. Each F1 animal is estimated to be heterozygous for about 20–30 gene-inactivating mutations, based on the specific locus test and data from other mutagenesis screens (6, 31). In a genome-wide screen, 100 F1 lines will

therefore interrogate 2,000–3,000 genes, about 8–12% of the genome. Since ENU mutagenizes spermatogonial stem cells leading to clones of mutant sperm, not more than eight F1 animals should come from any particular G0 father, to avoid rescreening the same mutation. In theory, for 100 F1 lines, a minimum of 12–13 G0 males should be injected. However, since some percentage of the G0 males will either fail to recover fertility or die (or both), it is good practice to inject about three times the minimum number of males. For example, in a recently completed genetic screen in our lab, we injected 50 C57/BL6 males with 3 × 100 mg/kg ENU. After 10–12 weeks, about half the males had died before recovering fertility. From the remaining G0 males, we recovered 122 F1 males.

3.2.3. ENU Injection Protocol

ENU is carcinogenic and must be handled with extreme care (modified from (32)). Most institutions require an IACUC safety approval justification and common FAQs on ENU. ENU can be obtained as 1 g of powder in a light-protected ISOPAC container. ENU is sensitive to light, humidity, and pH. For this reason, it should be stored at –20°C in the dark until use and then diluted not more than 3 h before injection.

3.2.3.1. Prior to Injection

1. Complete all institutional IACUC safety approval procedures (varies from institution to institution).
2. Order male mice of the strain to be injected so that they will be 7–8 weeks old at the time of injection, keeping in mind that they will need at least 1 week to adjust to their new environment after arrival.

3.2.3.2. Day of Injection

3. Make all solutions and gather all materials (see above).
4. Weigh all males to be injected and calculate the amount of ENU to inject per mouse, based on the following formula:

$$10\,\text{mg/mL ENU}\ (x\ \text{mL to inject}) = (\text{final concentration of ENU})(\text{mouse body weight})$$

For example, if you want a final concentration of 100 mg/kg ENU in a 20 g C57BL/6J mouse:

$$10\,\text{mg/mL ENU}\ (x\ \text{mL to inject}) = (0.1\,\text{mg/g})(20\,\text{g})$$

$x = 0.2$ mL of 10 mg/mL ENU, for a final concentration of 100 mg/kg ENU

5. Dissolve and dilute ENU to 10 mg/mL (*see* **Note 1**):
   a. Inject 10 mL of 95% ethanol into the ISPOAC container. Swirl gently to dissolve. When dissolved, ENU is a clear yellow liquid.

b. Vent ISOPAC with an 18-gauge needle. Inject 90 mL of phosphate/citrate buffer into container.

6. Inject mice: an experienced person familiar with intraperitoneal injections should inject each mouse with the proper volume (determined from the formula above) following standard procedures.
7. After injection, the mice will become uncoordinated from the alcohol and lose consciousness for a short time, usually about 20 min. During this time they should be monitored to ensure they recover consciousness.
8. Deactivate and dispose of ENU: ENU should be completely deactivated. Since it has a short half-life under alkaline conditions, use one of the two inactivating solutions given above to thoroughly rinse all materials that came in contact with ENU. In our experience it is best to minimize handling the materials on the day of injection; therefore, we leave all materials in the hood *with the light on* overnight to further ensure that the ENU is deactivated. Prominent signs should be displayed on the hood and room in which ENU is deactivating, alerting unknowing staff and coworkers to the presence of ENU.

3.2.3.3. After Injection

9. After the last weekly injection, let males recover for 2–3 weeks.
10. A good indication that the mutagenesis was successful is sterile males. To ensure that males are sterile, mate them with females (at this time the females can be any strain and can likely be used for other experiments if the males are indeed sterile). Males are sterile if mating plugs are observed but the females do not become pregnant.
11. Starting 2–3 weeks before the males are expected to regain fertility (*see* **Table 12.3**), set males up with 1 or 2 females of the desired strain (usually different from the G0 strain, for mapping purposes, see below).

3.3. Breeding Crosses and Establishment of Mutant Lines

Once the G0 males have recovered fertility, the more active phase of the screening process begins: breeding crosses to screen for mutant phenotypes and establish mutant lines. The class of screen dictates the series of crosses to perform; each crossing scheme is outlined below.

3.3.1. Genome-Wide Screen: Dominant Mutations

1. *1st cross*: Cross the mutagenized G0 male to one or two females of a different (preferably inbred) strain. It is advantageous to cross the G0 males to females of a different strain, as polymorphic markers between the strains permit

Table 12.4
The number of informative SNPs between common inbred strains. The polymorphic SNPs are derived from a low-density whole-genome SNP panel of 768 SNPs

	C57BL/6J	129X1/SvJ	BALB/cJ	C3H/HeJ	DBA/2J	FVB/NJ	A/J	CBA/J	C57BL/10J
C57BL/6J		508	497	598	555	539	581	562	68
129X1/SvJ			315	333	365	316	367	313	455
BALB/cJ				233	323	285	203	262	448
C3H/HeJ					241	294	226	111	552
DBA/2J						323	325	235	518
FVB/NJ							274	281	492
A/J								276	547
CBA/J									514

From the Mutation Mapping and Developmental Analysis Project (MMDAP), with permission from J. L. Moran and D. R. Beier (personal communication).

straightforward mutation mapping (**Section 3.4**). The number of polymorphisms between strains varies, so this should be taken into account when choosing the crossing strain (**Table 12.4**).

2. Dominant mutations will be recovered in the first generation (F1) (upper gray box in **Fig. 12.3**). Since the mutations occur randomly in the sperm of the G0 male, each F1 animal represents a unique suite of mutations and is thus considered a "line." However, since the G0 spermatogonial stem cells are mutated, it is best to screen not more than eight F1 animals from any one G0 male to avoid rescreening the same mutation. Collect F1 animals and screen for the phenotype of interest. Once F1 animals with an interesting phenotype are identified, they must be maintained as separate lines. If the dominant mutation is viable and fertile, it is simply a matter of breeding the F1 animal to the same inbred strain chosen in cross #1.

3.3.2. Genome-Wide Screen: Recessive Mutations

1. *1st cross*: Same as above. Collect eight F1 males per G0 male and allow them to come to breeding age. Discard F1 females (**Fig. 12.3**).

2. *2nd cross*: Breed each F1 male individually to two wild-type females of the same inbred strain used in the 1st cross. Collect G2 females only and allow them to come to breeding age; discard G2 males to save mouse room space and cost (new G2 males can be obtained later, if needed, to establish lines of interest).
3. *3rd cross*: Backcross G2 females to their F1 fathers. Mate at least six G2 females to each F1 male. When a phenotype has been observed in at least two G3 animals from two separate G2 females, it is likely genetic (*see* **Note 2**). To maintain the line, collect G2 males and mate them to their sibling G2 females to determine whether the G2 male is a carrier; carrier males are then kept for subsequent breeding and analysis (**Section 3.4**).

Alternative 3rd cross: A theoretical drawback to backcrossing the G2 females to F1 males is that it places reproductive strain on the F1 male, since he will be needed to produce many litters. In our experience, however, we have not encountered problems with this. Nonetheless, an alternative to backcrossing is intercrossing G2 male and female siblings. This method has the advantage that G2 carrier males are immediately identified; a drawback is that both G2 males and females must be weaned from the second cross, above, increasing mouse room space and cost.

3.3.3. Genome-Wide Screen: Dominant or Recessive Modifier

The breeding scheme presented here assumes that the mutation to be modified is not viable in the homozygous state (white mouse in **Fig. 12.4**).

1. *1st cross*: Cross G0 males to females of a different strain who are heterozygous for the allele to be modified. Collect F1 animals (not more than eight per G0 male, as above) and allow them to come to breeding age.
2. *2nd cross*: Cross F1 animals to animals of the same strain as the females crossed to G0 males, above. Dominant modifiers will be seen in G2 animals; collect and screen for enhancement or suppression of the phenotype of interest.
3. *3rd cross*: Backcross G2 females to their F1 fathers. Recessive modifiers will be seen in G3 animals; collect and screen for enhancement or suppression of the phenotype of interest.

3.3.4. Region-Specific Screen: Non-complementation, if the Starting Allele Is Homozygous Viable and Fertile

1. *1st cross*: Cross G0 males with females of a different inbred strain who are homozygous for the allele of interest. Collect F1 animals (not more than eight per G0 male, as above) and screen them for failure to complement the mutation (**Fig. 12.5**) (i.e., exhibit the same phenotype as animals homozygous for the starting allele (white mouse in **Fig. 12.5a**)).

3.3.5. Region-Specific Screen: Non-complementation, if the Starting Allele Is Homozygous Lethal or Sterile

1. *1st cross*: Cross G0 males with wild-type females of the same genetic background as those containing the allele of interest. Collect F1 animals (not more than eight per G0 male, as above) and allow them to come to breeding age.
2. 2nd cross: Cross F1 animals to animals that are heterozygous for the allele of interest. Screen the resulting G2 progeny for a failure to complement the allele of interest. As mentioned above, a failure to complement (**Fig. 12.5**) can be either allelic or non-allelic, and this can be determined through direct sequencing of the gene in the new mutant background (White Mouse in **Fig. 12.5b**).

3.3.6. Region-Specific Screen: Deletion Screen for Lethal or Sterile Recessive Mutations

1. *1st cross*: Cross G0 males to wild-type females from the same genetic background as the deletion strain used in the 2nd cross, below. Collect F1 animals and allow them to come to breeding age (**Fig. 12.6**).
2. *2nd cross*: Cross F1 animals to animals hemizygous for a deleted region of interest. Any recessive mutations that occur *in trans* to the deleted region will be observable in the G2 progeny.

3.3.7. Region-Specific Screen: Using Balancers

1. *1st cross*: Cross G0 males to females of a different inbred strain who are heterozygous for a balancer chromosome. Collect F1 animals carrying the balancer chromosome (one-half of the F1 progeny) and allow them to come to breeding age (**Fig. 12.7**).
2. *2nd cross*: Cross F1 animals carrying the balancer to animals carrying the balancer *in trans* to a wild-type chromosome marked with a dominant visible marker that is distinct from the visible marker on the balancer chromosome. Collect G2 animals that are heterozygous for the newly mutated chromosome over the balancer chromosome (can be distinguished based on visible markers). Discard the rest of the progeny.
3. *3rd cross*: Backcross G2 animals to their F1 parents. The G3 animals can again be distinguished by their visible markers. If a G3 animal is not carrying a balancer chromosome, then it is homozygous for the newly mutated chromosome. If such animals exhibit a phenotype, then the mutation lies in the balanced region of the genome. However, if a G3 animal has a phenotype but is heterozygous for the balancer, then the mutation lies outside the balanced region, elsewhere in the genome.

3.4. Analysis and Cloning

The excitement of establishing a new mutant line with an interesting phenotype may only be surpassed by discovering the

underlying genetic change that causes the phenotype. Traditionally, there are three main steps to accomplish this goal: recombination mapping to narrow down the genomic interval in which a mutation lies, sequencing candidate genes in this genomic interval, and confirming that a particular mutation is indeed responsible for the observed phenotype.

3.4.1. Mapping Based on Recombination

Since mice from one inbred strain *(x)* are mutagenized and then crossed to mice of another inbred strain *(y)*, the F1 generation is 50% *x* and 50% *y*. In the process of establishing and maintaining mutant lines, mice are continually crossed to the nonmutagenized (*y*) background, all the while selecting for the mutation. Over several generations, therefore, the genome of the mutant lines will largely be of the *y* background, while the region surrounding the mutation will be of the *x* background. The premise of recombination mapping is that the causative mutation will be linked to the *x* background, which can be distinguished by polymorphisms that differ between the *x* and *y* backgrounds. There are two main classes of polymorphisms used in recombination mapping: simple sequence length polymorphisms (SSLPs), which are short repeated segments that differ in length between inbred strains, and single nucleotide polymorphisms (SNPs). Both classes can be used to create polymorphic "markers." SSLP markers are created by designing PCR primers around the SSLP, so that the size of the PCR product differs between two strains. SNP markers can be created by finding SNPs that create restriction fragment length polymorphisms (RFLPs), also detectable by PCR. In addition, SNPs can be genotyped directly using array-based SNP panels (see below).

The first step of recombination mapping is to determine on which chromosome the mutation lies. This is achieved by performing a genome-wide scan using polymorphic markers that are spaced at regular intervals throughout the genome at low density. Several commercially available SNP panels have been designed for this purpose. For example, Illumina's mouse Low Density (LD) and Medium Density (MD) Linkage Panels contain 377 and 1,449 SNPs, respectively, spaced across the entire mouse genome. DNA from affected (mutant) animals is obtained, and the SNPs contained in the linkage panels are genotyped to determine which chromosome has the largest cluster of DNA from the mutagenized background. The required starting amount of DNA is low (750 ng–1.5 μg) and can be obtained from tissue from a single animal. To detect linkage, DNA from eight or nine affected animals should be SNP genotyped.

The next step is high-resolution mapping, which is essentially the same process, but using markers that are more closely spaced. In the course of mapping their own mutations, several groups have created polymorphic markers and made them available to

the public (see online resources, below). You should first determine whether any of the available markers are appropriate for your use. If there are no informative markers in the region of interest, then markers will need to be created. Step-by-step instructions are available from the Sloan-Kettering site, below. Use the markers to genotype both affected and non-affected animals from each line. Since affected animals are known to carry the mutation, and the mutation lies in a region of mutagenized background DNA (e.g., "*x*"), informative animals will be recombinants that have wild-type DNA (e.g., *y*) adjacent to mutagenized DNA. Since the portion of the chromosome containing wild-type DNA cannot contain the mutation, that portion can be ruled out. As more affected recombinant animals are genotyped, longer portions of the chromosome are eliminated. Conversely, non-affected recombinant animals are used to rule out portions of the chromosome that are homozygous for mutagenized DNA (for recessive alleles). It is important to note that, if there are any issues with penetrance of the phenotype one can easily be misled by apparently non-affected animals and may want to exclude them from analysis.

Below is a partial list of online resources to locate or design appropriate markers:

Sloan-Kettering Mouse Project Website: https://mouse.mskcc.org

1. MarkerBase: Provides a list of available Sloan-Kettering Institute (SKI) developed makers, a searchable database of Massachusetts Institute of Technology (MIT) markers, and a guide to create your own.

Mouse Genome Informatics (MGI): www.informatics.jax.org

1. Integrated Whitehead/MIT Linkage and Physical maps: Provides a list of available MIT markers by chromosome: www.informatics.jax.org/reports/mitmap
2. Strains, SNPs, and polymorphisms
   a. SNP query: search for SNPs by strain, SNP attributes, genomic position, or associated genes.
   b. Search for RFLP- or PCR-based polymorphisms by strain, locus symbol, or map position.

Ensembl Genome Browser: www.ensembl.org/Mus_musculus/Info/Index

1. Browse for SNPs by chromosome (karyotype) or enter genomic location.
   a. Genetic variation: resequencing data for nine inbred strains are compared with the C57BL/6J genomic sequence, and SNPs are highlighted.

3.4.2. Sequencing

Once the genomic interval in which a mutation lies has been narrowed sufficiently, the next step is to sequence candidate gene(s) in the interval. A number of factors influence the decision of when

and what to begin sequencing. One consideration is whether there are additional polymorphisms that could potentially narrow the interval further. However, the chance of obtaining recombinant animals decreases as the interval is narrowed. Perhaps the best indicator that the time to sequence has come is that there are a manageable number of genes in the interval, which may or may not be correlated with the physical size of the interval. What is "manageable" depends on the investigator, but larger collections of genes can be prioritized for sequencing based on expression data or any available phenotypic data. In addition, since ENU causes mutations in exons and splice sites in the vast majority of cases, sequencing entire genes is not necessary.

The availability of next-generation resequencing technologies is poised to change how investigators perceive what is a manageable number of genes to sequence. It is becoming practical to sequence very long portions of a chromosome at a time and for less money. This technology may drastically alter the balance of time spent mapping versus sequencing, to the point that, ultimately, one may only need to know which chromosome contains the mutation before beginning to sequence.

3.4.3. Confirmation

How do you know that a mutation actually causes the observed phenotype? Direct evidence includes genetic rescue or complementation. Genetic rescue occurs when a wild-type copy of the gene is introduced into the mutant background, and the mutant phenotype is no longer observed. Although direct, this method is time consuming because it involves creating transgenic mice. The other direct method is a complementation analysis, which involves creating mice that have one copy of your mutant allele and one copy of a known mutant allele in the suspected gene. If the mutant phenotype is seen in such an animal, then your allele fails to complement the phenotype and is an allele of the suspected gene. While this is faster than genetic rescue, it depends on the availability of mutant alleles in the gene of interest. There can also be indirect evidence that a mutation causes the observed phenotype, including disruption of gene expression, protein production, protein activity, or cellular/tissue localization. Other indirect evidence may be that the observed mutant phenotype is similar to other alleles of the suspected gene or is similar to the mutant phenotype of genes in the same pathway.

4. Notes

1. ENU: To spec or not to spec? The concentration of ENU can be determined by spectrophotometry after dilution in phosphate/citrate buffer. This is the best way to be

absolutely certain about the exact amount of ENU you are injecting into the mice, since it is possible that there is not exactly 1 g of ENU in the container provided by Sigma. Problems can result if the amount of ENU injected is too high (e.g., all the G0 males die or fail to recover fertility) or too low (e.g., failure to obtain relevant mutant lines). If you are experiencing one of these problems despite having taken the inbred mouse strain into consideration, you may need to spec the ENU. A good protocol can be found in (32). However, in our experience we have found that handling the ENU as little as possible is best, and following the strain guidelines and injecting a sufficient number of males yield good results.

2. It may be hard to tell if a particular phenotype is truly genetic or just a random phenomenon. A good rule of thumb is that the phenotype should be seen in multiple animals from separate litters, at a frequency of approximately 25% (for recessive alleles).

   If screening for embryonic lethal mutations, the G2 females will be dissected to view the G3 embryos. To avoid an overwhelming number of dissections on any 1 day, it is best to mate only two G2 females to the F1 male at a time. As mating plugs are observed, place the pregnant females in a separate cage and replenish the mating cage with new G2 females.

References

1. Bult, C. J., Eppig, J. T., Kadin, J. A., Richardson, J. E., and Blake, J. A. (2008) The Mouse Genome Database (MGD): mouse biology and model systems. *Nucleic Acids Res.* **36**, D724–728.
2. Collins, F. S., Finnell, R. H., Rossant, J., and Wurst, W. (2007) A new partner for the international knockout mouse consortium. *Cell* **129**, 235.
3. Collins, F. S., Rossant, J., Wurst, W., and Consortium, T. I. M. K. (2007) A mouse for all reasons. *Cell* **128**, 9–13.
4. O'Brien, T. P. and Frankel, W. N. (2004) Moving forward with chemical mutagenesis in the mouse. *J. Physiol.* **554**, 13–21.
5. Hamosh, A., Scott, A. F., Amberger, J. S., Bocchini, C. A., and McKusick, V. A. (2005) Online Mendelian Inheritance in Man (OMIM), a knowledgebase of human genes and genetic disorders. *Nucleic Acids Res.* **33**, D514–517.
6. Russell, W. L., Kelly, E. M., Hunsicker, P. R., Bangham, J. W., Maddux, S. C., and Phipps, E. L. (1979) Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse. *Proc. Natl. Acad. Sci. USA* **76**, 5818–5819.
7. Mills, A. A. and Bradley, A. (2001) From mouse to man: generating megabase chromosome rearrangements. *Trends Genet.* **17**, 331–339.
8. Thomas, J. W., LaMantia, C., and Magnuson, T. (1998) X-ray-induced mutations in mouse embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **95**, 1114–1119.
9. You, Y., Bergstrom, R., Klemm, M., Lederman, B., Nelson, H., Ticknor, C., Jaenisch, R., and Schimenti, J. (1997) Chromosomal deletion complexes in mice by radiation of embryonic stem cells. *Nat. Genet.* **15**, 285–288.
10. You, Y., Browning, V. L., and Schimenti, J. C. (1997) Generation of radiation-induced deletion complexes in the mouse genome using embryonic stem cells. *Methods* **13**, 409–421.
11. Hentges, K. E. and Justice, M. J. (2004) Checks and balancers: balancer chromosomes

to facilitate genome annotation. *Trends Genet.* **20**, 252–259.
12. Zheng, B., Sage, M., Cai, W. W., Thompson, D. M., Tavsanli, B. C., Cheah, Y. C., and Bradley, A. (1999) Engineering a mouse balancer chromosome. *Nat. Genet.* **22**, 375–378.
13. Flaherty, L., Messer, A., Russell, L. B., and Rinchik, E. M. (1992) Chlorambucil-induced mutations in mice recovered in homozygotes. *Proc. Natl. Acad. Sci. USA* **89**, 2859–2863.
14. Russell, L. B., Hunsicker, P. R., Cacheiro, N. L., Bangham, J. W., Russell, W. L., and Shelby, M. D. (1989) Chlorambucil effectively induces deletion mutations in mouse germ cells. *Proc. Natl. Acad. Sci. USA* **86**, 3704–3708.
15. Russell, L. B. and Russell, W. L. (1992) Frequency and nature of specific-locus mutations induced in female mice by radiations and chemicals: a review. *Mutat. Res.* **296**, 107–127.
16. Russell, W. L. (1951) X-ray-induced mutations in mice. *Cold Spring Harb. Symp. Quant. Biol.* **16**, 327–336.
17. Takeda, J., Keng, V. W., and Horie, K. (2007) Germline mutagenesis mediated by Sleeping Beauty transposon system in mice. *Genome Biol.* **8** Suppl. 1, S14.
18. Beier, D. R. (2000) Sequence-based analysis of mutagenized mice. *Mamm. Genome* **11**, 594–597.
19. Chen, Y., Yee, D., Dains, K., Chatterjee, A., Cavalcoli, J., Schneider, E., Om, J., Woychik, R. P., and Magnuson, T. (2000) Genotype-based screen for ENU-induced mutations in mouse embryonic stem cells. *Nat. Genet.* **24**, 314–317.
20. Coghill, E. L., Hugill, A., Parkinson, N., Davison, C., Glenister, P., Clements, S., Hunter, J., Cox, R. D., and Brown, S. D. (2002) A gene-driven approach to the identification of ENU mutants in the mouse. *Nat. Genet.* **30**, 255–256.
21. Concepcion, D., Seburn, K. L., Wen, G., Frankel, W. N., and Hamilton, B. A. (2004) Mutation rate and predicted phenotypic target sizes in ethylnitrosourea-treated mice. *Genetics* **168**, 953–959.
22. Gondo, Y., Fukumura, R., Murata, T., and Makino, S. (2009) Next-generation gene targeting in the mouse for functional genomics. *BMB Rep.* **42**, 315–323.
23. Nachman, M. W. and Crowell, S. L. (2000) Estimate of the mutation rate per nucleotide in humans. *Genetics* **156**, 297–304.
24. Russell, L. B. (2004) Effects of male germ-cell stage on the frequency, nature, and spectrum of induced specific-locus mutations in the mouse. *Genetica* **122**, 25–36.
25. Balling, R. (2001) ENU mutagenesis: analyzing gene function in mice. *Annu Rev Genomics Hum. Genet.* **2**, 463–492.
26. Justice, M. J., Noveroske, J. K., Weber, J. S., Zheng, B., and Bradley, A. (1999) Mouse ENU mutagenesis. *Hum. Mol. Genet.* **8**, 1955–1963.
27. Noveroske, J. K., Weber, J. S., and Justice, M. J. (2000) The mutagenic action of N-ethyl-N-nitrosourea in the mouse. *Mamm. Genome* **11**, 478–483.
28. Justice, M. J., Carpenter, D. A., Favor, J., Neuhauser-Klaus, A., Hrabe de Angelis, M., Soewarto, D., Moser, A., Cordes, S., Miller, D., Chapman, V., Weber, J. S., Rinchik, E. M., Hunsicker, P. R., Russell, W. L. „ and Bode, V. C. (2000) Effects of ENU dosage on mouse strains. *Mamm. Genome* **11**, 484–488.
29. Weber, J. S., Salinger, A., and Justice, M. J. (2000) Optimal N-ethyl-N-nitrosourea (ENU) doses for inbred mouse strains. *Genesis* **26**, 230–233.
30. Russell, W. L., Hunsicker, P. R., Carpenter, D. A., Cornett, C. V., and Guinn, G. M. (1982) Effect of dose fractionation on the ethylnitrosourea induction of specific-locus mutations in mouse spermatogonia. *Proc. Natl. Acad. Sci. USA* **79**, 3592–3593.
31. Wilson, L., Ching, Y. H., Farias, M., Hartford, S. A., Howell, G., Shao, H., Bucan, M., and Schimenti, J. C. (2005) Random mutagenesis of proximal mouse chromosome 5 uncovers predominantly embryonic lethal mutations. *Genome Res.* **15**, 1095–1105.
32. Salinger, A. P. and Justice, M. J. (2008) Mouse Mutagenesis Using N-Ethyl-N-Nitrosourea (ENU). Cold Spring Harbor Protocols, New York, NY.
33. Kile, B. T. and Hilton, D. J. (2005) The art and design of genetic screens: mouse. *Nat. Rev. Genet.* **6**, 557–567.
34. Barsh, G. S. and Epstein, C. J. (1989) Physical and genetic characterization of a 75-kilobase deletion associated with al, a recessive lethal allele at the mouse agouti locus. *Genetics* **121**, 811–818.
35. Miller, M. W., Duhl, D. M., Vrieling, H., Cordes, S. P., Ollmann, M. M., Winkes, B. M., and Barsh, G. S. (1993) Cloning of the mouse agouti gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal yellow mutation. *Genes Dev.* **7**, 454–467.
36. LePage, D. F., Church, D. M., Millie, E., Hassold, T. J., and Conlon, R. A. (2000) Rapid generation of nested chromosomal

deletions on mouse chromosome 2. *Proc. Natl. Acad. Sci. USA* **97**, 10471–10476.
37. Bennett, D. C., Huszar, D., Laipis, P. J., Jaenisch, R., and Jackson, I. J. (1990) Phenotypic rescue of mutant brown melanocytes by a retrovirus carrying a wild-type tyrosinase-related protein gene. *Development* **110**, 471–475.
38. Smyth, I. M., Wilming, L., Lee, A. W., Taylor, M. S., Gautier, P., Barlow, K., Wallis, J., Martin, S., Glithero, R., Phillimore, B., Pelan, S., Andrew, R., Holt, K., Taylor, R., McLaren, S., Burton, J., Bailey, J., Sims, S., Squares, J., Plumb, B., Joy, A., Gibson, R., Gilbert, J., Hart, E., Laird, G., Loveland, J., Mudge, J., Steward, C., Swarbreck, D., Harrow, J., North, P., Leaves, N., Greystrong, J., Coppola, M., Manjunath, S., Campbell, M., Smith, M., Strachan, G., Tofts, C., Boal, E., Cobley, V., Hunter, G., Kimberley, C., Thomas, D., Cave-Berry, L., Weston, P., Botcherby, M. R., White, S., Edgar, R., Cross, S. H., Irvani, M., Hummerich, H., Simpson, E. H., Johnson, D., Hunsicker, P. R., Little, P. F., Hubbard, T., Campbell, R. D., Rogers, J., and Jackson, I. J. (2006) Genomic anatomy of the Tyrp1 (brown) deletion complex. *Proc. Natl. Acad. Sci. USA* **103**, 3704–3709.
39. Rinchik, E. M. (1994) Molecular genetics of the brown (b)-locus region of mouse chromosome 4. II. Complementation analyses of lethal brown deletions. *Genetics* **137**, 855–865.
40. Schimenti, J. C., Libby, B. J., Bergstrom, R. A., Wilson, L. A., Naf, D., Tarantino, L. M., Alavizadeh, A., Lengeling, A., and Bucan, M. (2000) Interdigitated deletion complexes on mouse chromosome 5 induced by irradiation of embryonic stem cells. *Genome Res.* **10**, 1043–1050.
41. Rikke, B. A., Johnson, D. K., and Johnson, T. E. (1997) Murine albino-deletion complex: high-resolution microsatellite map and genetically anchored YAC framework map. *Genetics* **147**, 787–799.
42. Shibahara, S., Okinaga, S., Tomita, Y., Takeda, A., Yamamoto, H., Sato, M., and Takeuchi, T. (1990) A point mutation in the tyrosinase gene of BALB/c albino mouse causing the cysteine—serine substitution at position 85. *Eur. J. Biochem.* **189**, 455–461.
43. Gardner, J. M., Nakatsu, Y., Gondo, Y., Lee, S., Lyon, M. F., King, R. A., and Brilliant, M. H. (1992) The mouse pink-eyed dilution gene: association with human Prader-Willi and Angelman syndromes. *Science* **257**, 1121–1124.
44. Johnson, D. K., Stubbs, L. J., Culiat, C. T., Montgomery, C. S., Russell, L. B., and Rinchik, E. M. (1995) Molecular analysis of 36 mutations at the mouse pink-eyed dilution (p) locus. *Genetics* **141**, 1563–1571.
45. Russell, L. B., Montgomery, C. S., Cacheiro, N. L., and Johnson, D. K. (1995) Complementation analyses for 45 mutations encompassing the pink-eyed dilution (p) locus of the mouse. *Genetics* **141**, 1547–1562.
46. Mercer, J. A., Seperack, P. K., Strobel, M. C., Copeland, N. G., and Jenkins, N. A. (1991) Novel myosin heavy chain encoded by murine dilute coat colour locus. *Nature* **349**, 709–713.
47. Rinchik, E. M., Russell, L. B., Copeland, N. G., and Jenkins, N. A. (1986) Molecular genetic analysis of the dilute-short ear (d-se) region of the mouse. *Genetics* **112**, 321–342.
48. Kingsley, D. M., Bland, A. E., Grubber, J. M., Marker, P. C., Russell, L. B., Copeland, N. G., and Jenkins, N. A. (1992) The mouse short ear skeletal morphogenesis locus is associated with defects in a bone morphogenetic member of the TGF beta superfamily. *Cell* **71**, 399–410.
49. Su, H., Wang, X., and Bradley, A. (2000) Nested chromosomal deletions induced with retroviral vectors in mice. *Nat. Genet.* **24**, 92–95.
50. Roix, J. J., Hagge-Greenberg, A., Bissonnette, D. M., Rodick, S., Russell, L. B., and O'Brien, T. P. (2001) Molecular and functional mapping of the piebald deletion complex on mouse chromosome 14. *Genetics* **157**, 803–815.
51. Hosoda, K., Hammer, R. E., Richardson, J. A., Baynash, A. G., Cheung, J. C., Giaid, A., and Yanagisawa, M. (1994) Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell* **79**, 1267–1276.
52. Chick, W. S., Mentzer, S. E., Carpenter, D. A., Rinchik, E. M., Johnson, D., and You, Y. (2005) X-ray-induced deletion complexes in embryonic stem cells on mouse chromosome 15. *Mamm. Genome* **16**, 661–671.
53. Browning, V. L., Bergstrom, R. A., Daigle, S., and Schimenti, J. C. (2002) A haplolethal locus uncovered by deletions in the mouse T complex. *Genetics* **160**, 675–682.
54. Kushi, A., Edamura, K., Noguchi, M., Akiyama, K., Nishi, Y., and Sasai, H. (1998) Generation of mutant mice with large chromosomal deletion by use of irradiated ES cells--analysis of large deletion around hprt locus of ES cell. *Mamm. Genome* **9**, 269–273.

55. Nishijima, I., Mills, A., Qi, Y., Mills, M., and Bradley, A. (2003) Two new balancer chromosomes on mouse chromosome 4 to facilitate functional annotation of human chromosome 1p. *Genesis* **36**, 142–148.
56. Hough, R. B., Lengeling, A., Bedian, V., Lo, C., and Bucan, M. (1998) Rump white inversion in the mouse disrupts dipeptidyl aminopeptidase-like protein 6 and causes dysregulation of Kit expression. *Proc. Natl. Acad. Sci. USA* **95**, 13800–13805.
57. Stephenson, D. A., Lee, K. H., Nagle, D. L., Yen, C. H., Morrow, A., Miller, D., Chapman, V. M., and Bucan, M. (1994) Mouse rump-white mutation associated with an inversion of chromosome 5. *Mamm. Genome* **5**, 342–348.
58. Bedell, M. A., Brannan, C. I., Evans, E. P., Copeland, N. G., Jenkins, N. A., and Donovan, P. J. (1995) DNA rearrangements located over 100 kb 5′ of the Steel (Sl)-coding region in Steel-panda and Steel-contrasted mice deregulate Sl expression and cause female sterility by disrupting ovarian follicle development. *Genes Dev.* **9**, 455–470.
59. Klysik, J., Dinh, C., and Bradley, A. (2004) Two new mouse chromosome 11 balancers. *Genomics* **83**, 303–310.
60. Davisson, M. T., Roderick, T. H., Akeson, E. C., Hawes, N. L., and Sweet, H. O. (1990) The hairy ears (Eh) mutation is closely associated with a chromosomal rearrangement in mouse chromosome 15. *Genet. Res.* **56**, 167–178.
61. Chick, W. S., Mentzer, S. E., Carpenter, D. A., Rinchik, E. M., and You, Y. (2004) Modification of an existing chromosomal inversion to engineer a balancer for mouse chromosome 15. *Genetics* **167**, 889–895.

Chapter 13

Assisted Reproductive Technology in Nonhuman Primates

Tien-cheng Arthur Chang and Anthony W.S. Chan

Abstract

Nonhuman primates (NHP) are the closest animal species to humans and have been widely used for studying human reproductive physiology. Assisted reproductive technology (ART) in Old World NHPs provides great opportunity for studying fertilization, embryo development, embryonic stem cell (ESC) derivation for regenerative medicine, somatic cell nuclear transfer (cloning), and transgenic NHP models of inherited genetic disorders. Here we present two ART protocols developed for rhesus monkey (*Macaca mulatta*) and baboon (*Papio cynocephalus*).

Key words: Nonhuman primate, rhesus, baboon, assisted reproductive technology, IVF, embryo culture.

1. Introduction

Nonhuman primates (NHP) are the closest animal species to humans, which have been widely used for studying human reproductive physiology. Extensive effort toward development of follicle stimulation protocols, understanding the mechanism of sperm capacitation, and fertilization events in NHPs led to success in in vitro fertilization in the early 1980s (1, 2). To date, hundreds of NHP infants have been born by assisted reproductive technologies (ARTs) worldwide (3). Of over two hundred species of NHPs, only a limited number were studied extensively, with the majority of the findings in reproductive biology coming from studies of Old World monkeys including the rhesus macaque, cynomolgus macaque, squirrel monkey, baboon, and a New World primate, the common marmoset. ART in Old World NHPs provides a great opportunity for studying

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_13,

fertilization mechanisms, the development of embryo culture systems for studying early embryo development, embryonic stem cell (ESC) derivation for regenerative medicine, and the creation of identical animals by embryo splitting and nuclear transplantation (cloning). Furthermore, the combination of ART and transgenic technology has led to the development of transgenic NHP for studying human inherited genetic disorders (4–8), modeling of implantation potential and investigating the interaction between the embryo with the extracellular matrix (9–11) and the maternal endometrial environment (12–15), mitochondrial gene replacement (16), etc.

Assisted reproductive technologies have been applied in different NHP species including rhesus monkey (2, 5–7, 11, 17–67), cynomolgus monkey (68–75), African green monkey (vervet) (68, 76, 77), squirrel monkey (78–85), marmoset (86–90), great apes (91), and baboon (1, 81, 92–100). Various levels of success in these species indicated the fundamental differences in gametogenesis, preimplantation embryo development, and nutrition requirement for optimal in vitro embryo development (101, 102), which suggested a custom-designed ART protocol is needed. Directly applying human ART protocols on NHPs does not produce the same results and modifications are needed due to physiological differences between NHPs and humans. These include follicular development that affects hormonal regimen for stimulation, sperm activation and subsequent fertilization events, oocyte maturation mechanisms, and nutritional requirements for preimplantation embryo development. Thus, a thorough study to optimize assisted reproductive techniques in NHP will not only provide a unique model system for studying early embryonic development but also benefit our understanding of human embryo development, allowing for the development of an optimal embryo culture protocol for use in future infertility treatment.

The protocol for obtaining oocytes for ART starts with ovarian hyperstimulation by follicle-stimulating hormone (FSH) and luteinizing hormone (LH), followed by human chorionic gonadotropin (hCG) prior to laparoscopic follicle aspiration (*see* **Note 1**). The purpose of applying FSH and hCG in the hyperstimulation protocol is to stimulate and increase the number of follicles followed by maturation during the ovarian cycle with increasing levels of pituitary gonadotropin hormones. The first set of FSH injections stimulate a large number of antral follicles containing oocytes, and subsequent injection of hCG, an LH-like hormone with a longer circulating half-life that binds to the LH receptor, enhances follicular development and synchronizes the antral follicle pool, which improves the competence of oocytes and preimplantation development of IVF-derived embryos (2, 20, 103). However, recombinant hormones commonly used in human ART are antigenic to monkeys. As a

result of such limiting factor, repeated stimulation protocols often cause a high percentage of poor-quality oocytes in subsequent stimulations. Macaque-specific FSH and CG are not commercially available at this time, and future development is necessary for the improvement of current macaque stimulation protocols.

Although sperm samples can be collected by different methods, electrode-stimulated ejaculation is among the most commonly used methods for the recovery of high-quality semen (104, 105). Mature oocytes are inseminated by conventional IVF methods or intracytoplasmic sperm injection (ICSI).

At 17–20 h post-insemination, oocytes with male and female pronuclei (zygotes) are transferred into sequential medium and/or co-cultured with feeder cells (e.g., buffalo rat liver and green monkey kidney) (56, 92) to continue development to blastocyst stage. Co-culture methods were initially used for rhesus monkey embryo culture in vitro. Chemically defined culture media, including HECM-9 and KSOM-AA, were developed later and are widely used in rhesus monkey ART protocols (64, 106–108). Some commercially available human IVF medium systems also provide slightly higher developmental rates in baboon embryos (100).

Embryo transfer (ET) of zygotes, early stage 4- to 8-cell embryos, morula, or blastocysts, to synchronized recipient female macaques is also a critical step toward a successful ART program. Various ET methods including laparotomy or laparoscopic transfer of embryos to the oviduct, or direct cannulation of the uterine cervix, have resulted in live births with various success rates, which are largely due to the proficiency of the surgeon (50–53, 81, 109).

While we have provided a brief introduction on the development of ART in nonhuman primates and its role in human fertility, it is important to develop an improved and standardized protocol in higher primates that will optimize outcomes. Here we present two ART protocols developed for rhesus monkey (*Macaca mulatta*) and baboon (*Papio cynocephalus)*.

2. Materials

The two NHP ART protocols demonstrated in this chapter, one with rhesus monkey and the other with baboon, share many common reagents and equipment derived from years of NHP ART research and human reproductive medicine. Listed here are commonly used materials for NHP ART. Various brand names or substitutes manufactured by pharmaceutical companies may be available in different countries and regions.

2.1. Reagents and Chemicals

1. Buffalo rat liver (BRL) cells (ATCC).
2. Caffeine 1 mM (Sigma) for rhesus monkey sperm hyperactivation.
3. Connaught Medical Research Laboratories medium 1066 (CMRL-1066, Invitrogen).
4. Density gradient for semen processing ("Isolate," Irvine Scientific).
5. Dulbecco's modified Eagle medium (DMEM, Invitrogen).
6. Dibutyryl cyclic adenosine monophosphate (dbcAMP) 1 mM (Sigma) for rhesus monkey sperm hyperactivation.
7. Rhesus monkey oocyte maturation medium: Connaught Medical Research Laboratories medium 1066 (CMRL-1066, Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone), 40 μg/mL sodium pyruvate, 150 μg/mL glutamine, 550 μg/mL calcium lactate, 100 ng/mL estradiol (Sigma), and 3 μg/mL of progesterone (Sigma).
8. Gonadotropin-releasing hormone (GnRH) antagonist ("Antide," Ares Serono).
9. Hamster embryo culture medium 9 (HECM-9) with amino acids/pantothenate (AAP) stock without serum (HECM-9/AAP) (18, 57, 110, 111): NaCl 113.8 mM, KCl 3 mM, $CaCl_2 \cdot 2HCl$ 1.90 mM, $MgCl_2 \cdot 6H_2O$ 0.46 mM, $NaHCO_3$ 25 mM, Na-Lactate 4.5 mM, polyvinyl alcohol (PVA) 0.1 mg/mL, HCl 1.4 μL/mL. Adjust prepared medium to pH 7.35–7.45 *and* osmolarity 277 ± 5; add amino acid/pantothenate (AAP) on the day of use.
10. Amino acid/pantothenate (AAP, 100×): Taurine 0.5 mM, asparagine 0.01 mM, cysteine 0.01 mM, histidine 0.01 mM, lysine 0.01 mM, proline 0.01 mM, serine 0.01 mM, aspartic acid 0.01 mM, glutamic acid 0.01 mM, glutamine 0.20 mM, pantothenic acid 3 mM (Sigma). AAP is aliquoted and kept at –20°C until addition into HECM-9 before preparation of culture plate.
11. Heparin (Sigma).
12. Heat-inactivated fetal bovine serum (FBS, HyClone).
13. Human chorionic gonadotropin, recombinant (r-hCG, Serono).
14. Human follicle-stimulating hormone, recombinant (r-FSH, Ares Serono).
15. Human luteinizing hormone, recombinant (r-hLH, Ares Serono).

16. Human serum albumin (HSA) (Irvine Scientific).
17. Human tubal fluid medium, modified, Hepes buffered with gentamicin (Irvine Scientific).
18. Hyaluronidase 2 mg/mL (Sigma).
19. Mineral oil (Sigma) or light paraffin oil (Sage).
20. P1 medium for oocyte culture (Irvine Scientific).
21. Polyvinylpyrrolidone (PVP) (Irvine Scientific).
22. Sperm Washing Medium (Irvine Scientific): mHTF Hepes buffered supplemented with 5 mg/mL human serum albumin (HSA).
23. Sydney IVF cleavage and blastocyst sequential media for embryo culture (Cook Medical).
24. Trypsin (Invitrogen).
25. Tyrode's albumin-lactate-pyruvate-Hepes stock medium (TALP-Hepes stock) (17): $CaCl_2 \cdot 2H_2O$ 2.0 mM, KCl 3.2 mM, $MgCl_2 \cdot 6H_2O$ 0.5 mM, NaCl 114 mM, $NaH_2PO_4 \cdot H_2O$ 0.4 mM, lactic acid (60% syrup) 10 mM, $NaHCO_3 \cdot 2$ mM, Hepes 10 mM, penicillin 100 IU/mL, gentamycin 25 μg/mL, phenol red 0.2 mg/100 mL. To 950 mL of Milli-Q water, pH to 7.38–7.4, bring down with HCl if necessary. Bring to volume and adjust osmolarity to 290 ± 5 with Milli-Q water. The TL-Hepes stock medium can be kept at 4°C for 1 month.
26. TALP-Hepes medium: add pyruvate 0.2 mM, bovine serum albumin (BSA) 0.3%, PVA 0.1 mg/mL into TL-Hepes stock on the day of oocyte collection.

2.2. Equipment and Consumables

1. 4-well plate (Nunc).
2. Cell strainer, 70 μm (BD Falcon).
3. Embryo concentrator filter ("Em Con" filter, Immuno Systems, Spring Valley, WI, USA).
4. Embryo transfer catheter (Wallace embryo transfer catheter, Smiths, UK).
5. Gas tanks, triple gas (5–6% CO_2, 5% O_2, and 89–90% N_2) and CO_2.
6. Incubator, humidified triple gas (5–6% CO_2, 5% O_2, 89–90% N_2), or CO_2.
7. Inverted microscope for micromanipulation (with HMC or DIC optics, micromanipulators, injectors, warming plate or warmed enclosure, digital camera, and imaging software).
8. Holding pipette, O.D. 100 μm and I.D. 20 μm (Humagen).

9. Microinjection pipette, O.D. 7–8 μm and I.D. 4–5 μm, 50° beveled tip (Humagen).
10. CellTram microinjector (Eppendorf).
11. Micromanipulator (Narishige).
12. Inverted microscope (with phase contrast optics).
13. IVF workstation (K-Systems, Denmark) with heated surface and integrated light source for stereomicroscope.
14. Needle suction device with 20-gauge needle and Teflon tubing for oocyte retrieval (17, 19, 50).
15. Oocyte denudation pipette, I.D. 130–140 μm (MidAtlantic Diagnostics, USA, or Vitrolife, Sweden).
16. Oocyte denudation pipette holder "Stripper" (MidAtlantic Diagnostics, USA), or pipette holder (Vitrolife, Sweden).
17. Stereomicroscope.
18. Tissue culture flasks, T25 and T75 (Corning).
19. Ultrasonography equipment (various brands).

2.3. Other Essential ART Laboratory Equipment and Supplies

In addition, a modern assisted reproductive technology laboratory should include the items listed below.

2.3.1. Essential Equipment

1. Autoclave.
2. Precision balances.
3. Biosafety cabinet: cell and tissue culture or laminar flow hood.
4. Centrifuges and microcentrifuge.
5. Computers and printers.
6. Desiccator.
7. Dissecting and stereomicroscopes.
8. Filtration apparatus.
9. First aid kits (NHP tissue exposure).
10. Heating blocks and warming plates to maintain 37°.
11. Hemacytometer or computer-assisted sperm analyzer.
12. Ice bucket or block.
13. Lab chairs (non-porous), cabinets, and furniture (low VOC emission).
14. Liquid nitrogen Dewars.
15. Magnetic stirrer with heater.
16. Magnetic stir bars.
17. Osmometer.

18. pH meter.
19. Pipettors (single channel).
20. Refrigerator (4°C) and freezer (–20 and –80°C).
21. Timer and stopwatch.
22. Tygon tubing.
23. Vortex mixers.
24. Water bath.
25. Purified water supply (purification systems or cell culture-grade bottled water).
26. Veterinary equipment for electroejaculation and laparoscopic-assisted surgery.

2.3.2. Essential Consumables and Supplies

1. Bench protectors (e.g., "blue" pads).
2. Biohazard disposal bags and containers.
3. Centrifuge tubes and test tubes.
4. Disc filter units.
5. Gloves (non-latex).
6. Protective goggles.
7. Lab coats.
8. Laboratory glassware.
9. Liquid nitrogen tanks.
10. Microscope slides and coverslips.
11. Pipettes (2, 5, 10, 20, and 50 mL).
12. Plastic or rubber cell scrapers (e.g., "Policeman").
13. Syringes and needles.
14. Test tube racks.

3. Methods

3.1. Rhesus Monkey In Vitro Fertilization and Embryo Culture

3.1.1. Superovulation

1. Adult healthy females exhibiting regular menstrual cycles between the ages of 6- and 15-year old and with body weight of 6–10 kg are identified as oocyte donors.
2. Females with regular cycles as described are identified and enrolled in follicle stimulation schemes, which are induced with exogenous gonadotropins (17–19, 50).
3. Prior to hormonal stimulation, at least two normal menstrual cycles are confirmed by virginal bleed (*see* **Note 2**). Individual menstrual profiles are established by daily monitoring of virginal bleed.

4. Beginning on days 1–2 of menses (*see* **Note 2**), follicle stimulation is initiated by daily subcutaneous injections of gonadotropin-releasing hormone (GnRH) antagonist (Antide, 0.5 mg/kg body weight) and by twice daily intramuscular injection (i.m.) of recombinant human follicle-stimulating hormone [r-FSH; 30 IU] for 6 days, and followed by 1, 2, or 3 days of r-FSH + r-hLH (recombinant human luteinizing hormone, 30 IU each, i.m., twice daily) dependent on the size of the follicles.
5. Ultrasonography is performed on day 7 of the stimulation (*see* **Note 2**) to confirm follicular response.
6. An injection of 1,000 IU r-hCG (recombinant human chorionic gonadotropin, i.m., Serono) is administered for ovulation induction when there are follicles at 3–4 mm in diameter. For the recovery of mature metaphase II arrested oocytes, r-hCG is administered at approximately 37 h prior to the desired time of oocyte retrieval.

3.1.2. Oocyte Recovery

1. Follicular aspiration is performed 37 h after the r-hCG injection. The female monkey is anesthetized with Telazol (6 mg/kg, i.m.) and maintained by 1–2% isoflurane through a facemask during the surgery.
2. Oocytes are aspirated from follicles using a needle suction device lined with Teflon tubing modified by Bavister (17, 19, 50). In brief, a 10 mm trocar is placed through the abdominal wall and a telescope is introduced. Ovaries are visualized using a monitor attached to the telescope. Two small skin incisions facilitate the insertion of 5 mm trocars bilaterally, with an incision on each side of the abdominal wall, with one trocar in each incision. Grasping forceps are introduced through each trocar to fixate the ovary at two points.
3. Once stabilized, a 20-gauge stainless steel hypodermic needle is connected with Teflon tubing to an attached vacuum regulator for oocyte aspiration.
4. The tubing is first flushed with sterile Tyrode's albumin-lactate-pyruvate-Hepes medium (TALP-Hepes) (17), supplemented with 5 IU/mL of heparin in order to prevent blood clots in the tubing during aspiration (*see* **Note 3**).
5. Follicles are aspirated with continuous vacuum pressure adjusted at approximately 40–60 mmHg into a 15 mL conical tube containing 1 mL of TALP-Hepes supplemented with 5 IU/mL of heparin and maintained at 37°C (*see* **Note 4**).

3.1.3. Oocyte Collection and Evaluation

1. Collection tubes with follicular fluid are supplemented with 2 mg/mL hyaluronidase to loosen the cumulus complex.
2. Follicular fluid with TALP-Hepes with hyaluronidase is filtered through a 70 μm cell strainer to remove cell debris and blood clots while the oocytes trapped in the strainer are flushed with TALP-Hepes medium and collected in a 60 mm petri dish containing 5 mL of TALP-Hepes with hyaluronidase. The cell strainer is washed one more time with 5 mL of TALP-Hepes with hyaluronidase and any oocyte still trapped in the cell strainer is flushed and collected in a separate 60 mm petri dish (*see* **Notes 5** and **6**).
3. Oocytes are picked up under a dissecting microscope, and cumulus cells are transferred into a dish containing fresh TALP-Hepes with no hyaluronidase (*see* **Notes 7** and **8**).
4. Oocytes are rinsed twice in TALP-Hepes followed by one wash in maturation media before being transferred into a 50 μL drop of pre-equilibrated rhesus monkey oocyte maturation medium under mineral oil.
5. Oocytes at different maturation stages include (1) metaphase II (MII) arrested stage with distinctive first polar body (2), germinal vesicle break down (GVBD) stage without distinctive polar body, and (3) germinal vesicle (GV) stage. In general, 10–15 oocytes or embryos are cultured in a 50 μL drop of media. Metaphase II arrested oocytes, exhibiting a distinct perivitelline space and first polar body, are maintained in maturation medium before fertilization. Immature oocytes (GVBD and GV) will be matured in maturation medium for up to 24 h.

3.1.4. Preparation of Rhesus Monkey Sperm for In Vitro Fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI)

1. Rhesus males of proven fertility are trained for routine semen collection by penile electroejaculation (17, 18, 50, 112) (*see* **Note 9**).
2. Freshly collected samples are allowed to liquefy at room temperature for approximately 10 min.
3. Liquid portion of the semen is transferred into a 15 mL conical centrifuge tube followed by serial washes using 10 mL of TALP-Hepes and centrifugation at $400 \times g$ for 10 min to pellet down the sperm cells. After two washes, the supernatant is carefully removed and the pellet will be gently resuspended in 1 mL of TALP-Hepes.
4. A small sample is removed for analysis of motility and morphology. The remaining suspension is counted, diluted to a concentration of 2×10^7 sperm/mL in equilibrated TALP, and incubated at 37°C with 5% CO_2 prior to IVF or ICSI.

3.1.5. In Vitro Fertilization (IVF) Method A: Conventional Oocyte Insemination

1. Sperm suspensions are incubated at 37°C under 5% CO_2 in air for 6 h.
2. 1 mM caffeine and 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP) are added during the final hour to induce hyperactivation (*see* **Notes 10** and **11**).
3. After confirming adequate hyperactivation, 2 μL of sperm suspension is added to each 100 μL TALP drop, containing a maximum of 10 oocytes. Return the plate to incubator and culture at 37°C under 5% CO_2 (17–19, 113).

3.1.6. In Vitro Fertilization (IVF) Method B: Intracytoplasmic Sperm Injection (ICSI)

1. A holding pipette (O.D. 100 μm and I.D. 20 μm) and a microinjection needle (O.D. 7–8 μm and I.D. 4–5 μm) with a 50° beveled tip are mounted on an inverted microscope. The holding and injection pipettes are filled with mineral oil, connected to a CellTram microinjector, and held on a Narishige micromanipulator.
2. Injection is carried out in a 20 μL drop of TALP-Hepes medium covered with mineral oil on a 35 mm petri dish.
3. Sperm suspension is diluted 1:10 in 10% polyvinylpyrrolidone (PVP) in TALP-Hepes to reduce motility and placed in a separate drop on the manipulation dish.
4. A single sperm is selected and immobilized by gently squashing the mid-piece of the sperm against the bottom of the dish. Immobilized sperm is aspirated tail first from the sperm–PVP drop into the beveled injection needle and transferred to the oocyte-containing drop.
5. Oocytes are held by a holding pipette with the polar body positioned at the 6 o'clock or 12 o'clock position. The injection needle with a sperm is inserted through the zona into the cytoplasm. The oolemma is penetrated by gentle cytoplasmic aspiration and the sperm is expelled into the oocyte (*see* **Notes 12** and **13**).

3.1.7. In Vitro Culture

1. After IVF or ICSI, oocytes are washed twice in hamster embryo culture medium 9 (HECM-9) supplemented with amino acids/pantothenate (AAP) stock without serum (HECM-9/AAP) (18, 57, 110, 111) before transferring into a pre-equilibrated 4-well plate with 500 μL of HECM-9/APP, covered with 300 μL of mineral oil, and incubated at 37°C with 5% CO_2 and 90% N_2.
2. For the first 48 h post-IVF or ICSI, embryos are cultured in HECM-9/AAP without serum (18, 57, 110, 111). Fresh HECM-9/AAP supplemented with 10% fetal bovine serum (FBS) is replaced at 48 h post-IVF or ICSI, and the embryos are cultured in this medium until the blastocyst stage.

3.1.8. Confirmation of Fertilization

1. Fertilization is confirmed by the detection of a second polar body and two pronuclei the following morning after IVF or ICSI.
2. Zygotes are selected and returned to culture until reaching the 4- to 8-cell stage or 48 h post-fertilization for embryo transfer or in vitro culture is continued in a freshly pre-equilibrated HECM-9/AAP/FBS culture medium at 37°C with 5% CO_2 and 90% N_2.

3.1.9. Selection of Surrogate Females for Embryo Transfer

1. Female monkeys with prior successful pregnancy and normal menstrual cycles are selected for screening as potential embryo recipients. Screening is performed by collecting daily blood samples beginning on day 8 of the menstrual cycle (day 1 is the first day of menses).
2. Relative level of serum progesterone and estrogen is determined for at least 4–5 consecutive days in order to capture the profile of the changes. When serum estrogen increases two to four times that of basal levels, the LH surge has occurred and ovulation usually follows within 12–24 h. Timing of ovulation can be detected by a significant decrease in serum estrogen and an increase in serum progesterone to greater than 1 ng/mL.
3. Embryo transfer is performed on days 2–3 following ovulation by transferring two 4- to 8-cell embryos into the oviduct of the recipient (*see* **Notes 14, 15,** and **16**).

3.1.10. Embryo Transfer by Laparotomy

1. Surgical embryo transfers are performed by mid-ventral laparotomy.
2. A Wallace embryo transfer catheter containing one 4- to 8-cell stage embryo in TALP-Hepes-buffered medium is carefully inserted into the oviduct.
3. Embryos are slowly expelled from the catheter into the oviduct with a minimal amount of medium while the catheter is slowly withdrawn. The catheter is then flushed with medium following removal to ensure that the embryo was successfully transferred (*see* **Note 17**).
4. This procedure is repeated on the other side of the oviduct.

3.1.11. Confirming and Monitoring of Pregnancy

1. To confirm implantation, blood samples are collected bi-weekly and analyzed for serum estrogen and progesterone concentrations (33, 112).
2. If hormone levels indicate a possible pregnancy, the pregnancy is confirmed by a transabdominal ultrasound on day 60 post-transfer.
3. Ultrasound examination is performed, once more, during the second trimester to determine developmental normalcy.

3.2. Baboon In Vitro Fertilization and Embryo Culture

We have developed a baboon ART protocol including ovarian stimulation, in vitro fertilization, and embryo culture procedures, based on experience in human clinical ART and rhesus monkey ART protocols. This protocol demonstrated promoting effects on baboon embryo development through blastocyst stage (100) and consequent production of embryonic stem cell lines (114).

3.2.1. Stimulation and Follicle Retrieval from Baboon Ovaries

1. Endocrine-based superovulation: The baboon menstrual cycle lasts 32–33 days with ovulation clearly demonstrated by gradual sex skin swelling, an external indicator of menstrual cycle status in baboons (115, 116). Ultrasonography can be used to non-invasively monitor folliculogenesis.
2. Adult healthy fertile baboons will be identified as oocyte donors. Baboons at day 1 or 2 post-menses will be injected with recombinant human gonadotropin r-FSH (75 IU/day) for 7–8 days and r-FSH + r-LH (75 IU each/day) for the next 3 days followed by a single injection of 2,500 IU of hCG, 24 h after the last r-FSH + r-LH injection, and approximately 36 h prior to follicular aspiration by laparoscopic surgery. Sex skin is monitored daily prior to ovulation to identify the efficient stimulation on folliculogenesis (*see* **Notes 18** and **19**).

3.2.2. Baboon Sperm Processing

1. Semen is collected into a sterile cup or 50 mL conical tube by rectal probe electroejaculation (*see* **Note 20**) and allowed to liquefy at room temperature for approximately 30 min.
2. The liquid portion is aspirated from the coagulum and transferred to a sterile 15 mL conical tube. A small amount 1–2 mL of Sperm Washing Medium (Irvine Scientific) is used to rinse the collection cup and then transferred into the 15 mL conical tube.

   Semen specimen is processed by swim-up (steps 3–7) or density gradient (steps 8–12).
3. Semen Processing Method A (steps 3–7): Swim-up. Semen sample is mixed with 2 mL Sperm Washing Medium and centrifuged at 300×*g* for 8 min.
4. After the supernatant is removed, the sperm pellet is gently resuspended in 0.3–0.5 mL Sperm Washing Medium and divided into 3 new 15 mL conical tubes. 1 mL medium is gently added on top of the resuspended sperm solution. The tubes are incubated for 45–60 min.
5. The supernatant containing motile sperm is aspirated from all three tubes into a new 15 mL conical tube with 2 mL of Sperm Washing Medium and centrifuged at 300×*g* for 8 min. The supernatant is aspirated.

6. This washing process is repeated once.
7. The pellet is resuspended with 0.2–0.5 mL of medium depending on the size of pellet. The sample is kept at room temperature and ready for use (*see* **Note 21**).
8. Sperm Processing Method B (steps 8–12): Density gradient separation: A two layer density gradient is set up using 90% bottom layer and 40% upper layer, 1.5–2.0 mL of each density, in a 15 mL conical tube.
9. Liquefied semen specimen is gently placed into the tube on top of the 40% upper gradient.
10. The tube is centrifuged for 10–20 min at 200–300×*g*. After the centrifugation, a pellet appears at the bottom of tube contains most of the healthy sperm and the top two layers contain debris and dead sperm. Both layers of gradient are carefully aspirated to expose the pellet. The pellet is transferred into a new tube and resuspended in 2 mL Sperm Washing Medium (Irvine Scientific) for centrifugation at 200×*g*. After 8 min of centrifugation, the supernatant is removed.
11. This step is repeated once for a second wash, and the supernatant is discarded.
12. The pellet is resuspended with 0.2–0.5 mL of medium at room temperature and the resuspended solution is ready for use (*see* **Note 21**).

3.2.3. Buffalo Rat Liver (BRL) Feeder Cell Culture and Preparation

BRL cells are used in co-culture with oocytes, zygotes, and preimplantation stage embryos (*see* **Note 22**).

1. Frozen BRL cells are thawed in a 37°C water bath and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) in 25 cm^2 T25 tissue culture flasks in a 5% CO_2 incubator. Subculture is performed weekly by trypsinizing BRL cells using 0.05% trypsin.
2. To prepare monolayer BRL for embryo culture, cells are plated at a density of 1×10^4 per well in a Nunc 4-well plate in DMEM with 10% FBS in a 37°C 5% CO_2 incubator 24–36 h prior to oocyte retrieval. We usually plate 2–3 plates or more.
3. Eight to twelve hours prior to oocyte retrieval, DMEM is replaced with oocyte culture medium in one 4-well plate, and zygote/cleavage stage culture media in two to three 4-well plates. 500 μL of medium is placed in each well covered by light paraffin oil (~300–400 μL) depending on the viscosity of oil. Currently light paraffin oil is used in our laboratory.
4. New 4-well plates of BRL are set up every other day for embryo culture.

3.2.4. Oocyte Collection and Culture

1. *Oocyte Culture Medium:* P1 medium supplemented with 5% defined fetal bovine serum (FBS) was used for oocyte culture post-retrieval and prior to ICSI.

   Cumulus–oocyte complex collection and cumulus cell removal can be carried out by two approaches: traditional (steps 2–5) and simplified (steps 6–11) methods.

2. Oocyte Collection and Culture Method A (steps 2–5): a traditional and more thorough method, while more time consuming, to search for oocytes in aspirate. Several empty 60 mm petri dishes (Falcon 35-1007) and 2–3 dishes with modified Human Tubal Fluid (mHTF) Hepes-buffered medium are set up on 37°C warmed plate or IVF workstation.
3. Follicle fluid aspirate containing cumulus–oocyte complexes (COC's) is poured in 60 mm dishes and searched for COC's under a stereomicroscope. Washing medium (mHTF commonly used) is added if the aspirate is dense and difficult to search under microscope. COC is picked up and placed in a dish containing Hepes-buffered medium with addition of hyaluronidase to a final concentration of 1 mg/mL in the dish to loosen cumulus cells of the COC (*see* **Note 23**).
4. Oocyte denudation pipette, inner diameter at 130–140 μm, attached to the pipette holder, is used to remove cumulus cells.
5. Cumulus-free oocytes are moved to new dishes containing Hepes-buffered washing medium and rinsed twice. Examine the maturation stages of cumulus-free oocytes, such as MII, GVBD, GV, and record the finding. Oocytes are moved into 4-well plates of oocyte culture medium and kept in 37°C triple gas (5% CO_2, 5% O_2, and 90% N_2 or 6% CO_2, 5% O_2, and 89% N_2) incubator until ICSI on the same day or kept for in vitro maturation (IVM) attempts and later ICSI (*see* **Note 24**).
6. Oocyte Collection and Culture Method B (steps 6–11): We have adapted a simplified and reliable technique by using embryo concentrator filter ("Em Con" filter) to remove cumulus cells and obtain oocytes. Em Con filter is placed on top of a 250 or 500 mL flask.
7. Follicular fluid aspirate containing COC is poured into the Em Con filter. Oocyte wash medium (mHTF Hepes buffered) is used to rinse the aspirate tubes and then poured into the filter. The Em Con filter is gently swirled and the fluid is released to the bottom flask, while a small amount of fluid is kept in the filter to avoid drying of the medium.

8. The Em Con filter is rinsed again with oocyte wash medium containing 1 mg/mL final concentration of hyaluronidase (100 mg/mL stock aliquot in dPBS). Example: 4 mL of oocyte wash media + 40 μL hyaluronidase aliquot.
9. The medium is gently swirled and then decanted from the top of Em Con filter to a 60 mm Falcon dish. Repeat this process two to three times.
10. Under a dissecting microscope, the 60 mm petri dishes are searched thoroughly for cumulus-free oocytes. Oocytes are collected and transferred to a new 60 mm dish containing mHTF. Oocyte morphology, maturation stage, and first polar body presence are examined.
11. Oocytes are transferred into 4-well plates containing oocyte culture medium and the plates are returned into a 37°C triple gas incubator until ICSI (*see* **Note 25**).

3.2.5. In Vitro Fertilization: Intracytoplasmic Sperm Injection (ICSI) Micromanipulation Equipment Setup

1. Inverted microscope with Hoffman modulation contrast (HMC) or differential interference contrast (DIC) with heating plate and micromanipulators (Narishige, Japan) installed. The setup should be calibrated by the vendor and tested by the user.
2. An antivibration table may be required depending on the laboratory environment.
3. A holding pipette (O.D. 95–120 μm and I.D. 15–20 μm) and a microinjection pipette (O.D. 7–8 μm and I.D. 5–6 μm, with 25–35° 11–12 μm length beveled tip) will be connected to microinjectors (holding side: SAS; injection side: CellTram Vario) through tubing filled with light paraffin oil and held on Narishige micromanipulators (*see* **Note 26**).

3.2.6. In Vitro Fertilization: Microinjection of Baboon Sperm

1. Drops of 10 μL polyvinylpyrrolidone 10–15% (PVP) are placed on the center of 60 mm petri dish lid, surrounded by 6–8 drops of 5 μL mHTF Hepes-buffered medium covered with paraffin oil (*see* **Note 27**).
2. Diluted sperm suspension is placed in the PVP drop in the micromanipulation dish.
3. Select sperm with normal morphology; immobilize the sperm tail by gently pushing the tail onto the dish with the injection pipette tip. Aspirate the sperm, tail first, into the injection pipette. Transfer the injection pipette to mHTF Hepes-buffered drops containing the oocyte (*see* **Note 12**).
4. The oocyte is held by the holding pipette with the polar body in the 12 or 6 o'clock position as viewed through the microscope.

5. The injection pipette is inserted through the zona to penetrate the oolemma, and the sperm is expelled into the oocyte. Repeat the injection with other MII stage oocytes (*see* **Notes 12** and **13**).
6. After ICSI, injected oocytes are rinsed in mHTF Hepes-buffered medium twice by sequential transfer into clean wells of medium and transferred into 4-well plates of zygote/cleavage stage medium with BRL feeder cells supplemented with 10% FBS.

3.2.7. Days 1–3 Post-ICSI: Zygote and Cleavage Stage Embryo Culture

1. *Zygote and Cleavage Stage Embryo Culture Medium:* Sydney IVF Cleavage Medium, supplemented with 10% defined FBS and amino acids (*same as recipe of amino acids as in the rhesus macaque protocol*), is used for sperm-injected oocyte culture post-ICSI (*see* **Note 28**).
2. After batches of matured oocytes were injected and rinsed as in step 3.1.7, oocytes are pooled and rinsed through two wells of a 4-well plate containing Cleavage Stage Embryo Culture Medium and transferred into a pre-equilibrated 4-well plate containing a monolayer of BRL feeder cells and 500 μL of this Cleavage Stage Embryo Culture Medium covered by 300 μL paraffin oil, with 10–30 oocytes in each well.
3. The plate is moved to incubator at 37°C with triple gas (5% CO_2, 5% O_2, and 90% N_2 or 6% CO_2, 5% O_2, and 89% N_2).

3.2.8. Confirmation of Fertilization by Pronuclei Examination on Day 1 Post-ICSI

Two pronuclei and protrusion of the second polar body can be identified by 16–20 h post-ICSI. Zygotes with normal fertilization are selected and pooled in one or two wells, with up to 30 zygotes per well and returned to continuous culture (*see* **Note 29**).

3.2.9. Day 3 Post-ICSI: 8-Cell Through Blastocyst Stage Embryo Culture

1. *Blastocyst Stage Embryo Culture Medium:* Sydney IVF Blastocyst Medium with 15% FBS and amino acids is used to culture embryos starting day 3 post-ICSI through the blastocyst stage.
2. At 48 h post-ICSI, embryos at the 8-cell stage are rinsed twice in Blastocyst Stage Embryo Culture Medium, and transferred into a new pre-equilibrated 4-well plate containing pre-plated BRL cells and 500 μL of Blastocyst Culture Medium covered by 300 μL paraffin oil (*see* **Note 30**).
3. The plate is then returned to an incubator at 37°C with triple gas (5% CO_2, 5% O_2, 90% N_2, or 6% CO_2, 5% O_2, 89% N_2).
4. This process is repeated every 2 days with embryos transferred into new 4-well plates through the blastocyst stage.

3.2.10. Baboon Embryo Transfer

Numerous variables including the stage of embryos at the time of transfer, the location within the female tract into which the embryos are deposited, the number of embryos transferred per recipient female and/or per oviduct, and variations in the preparation of recipients, etc., could affect the success rate of embryo transfer (ET). Practice in most human fertility clinics favors transcervical transfer of either cleavage stage 8-cell embryos at 3 days post-fertilization or blastocysts at 5 days post-fertilization, whereas methods established on rhesus monkeys favor laparoscopic transfer of cleavage stage embryos directly into the oviduct. Currently the most effective approach for baboon embryo transfer is yet to be established, while several research groups have tested both laparoscopic and transcervical techniques.

Embryos produced in vitro and maintained in culture are transferred at the cleavage stage (steps 1–3) or blastocyst stage (steps 4–6). Prior to transfer, the quality of embryo is assessed according to stage-specific morphological criteria (*see* **Note 31**) and only high-quality embryos are chosen for transfer.

1. Cleavage Stage Embryo Transfer (steps 1–3). High-quality cleavage stage embryos are surgically transferred into the oviducts of recipients using the standard laparoscopic-assisted tubal embryo transfer procedure (*see* **Note 1**).
2. Embryos in mHTF Hepes-buffered medium are loaded into an embryo tested Wallace transfer catheter and gently introduced into the fimbria of the Fallopian tube under laparoscopic visualization. Less than 10 μL of transfer media containing the embryos is deposited approximately 1 cm into the ampulla of the Fallopian tube (*see* **Note 32**).
3. The catheter is examined under the dissection scope following transfer to ensure successful release of embryos.
4. Blastocyst Transfer (steps 4–6). Transcervical transfer of blastocysts into the uteri of recipients is accomplished non-surgically.
5. Blastocysts are loaded into a Wallace transfer catheter. Under direct visualization, the tip of the catheter is passed through the cervical canal into the endometrial cavity and less than 10 μL of mHTF Hepes-buffered media containing the embryos is expelled into the uterus (*see* **Note 32**).
6. The catheter is examined under the dissection scope following transfer to ensure successful transfer of embryos.

3.2.11. Confirming and Monitoring of Pregnancy

Following ET, pregnancy is monitored by daily examination of the sex skin and monitoring for vaginal bleeding, to confirm a lack of menses. Pregnancy is later confirmed by ultrasonography at 30 and 70 days post-transfer. ART-derived offspring can be delivered by Cesarean section (115, 116).

4. Notes

Notes are divided into two sections according to each of the two NHP ART protocols.

4.1. Rhesus Monkey In Vitro Fertilization and Embryo Culture

1. Laparoscopy is often used for follicle aspiration in Old World NHP ART (17, 83, 84, 117–119). A fiber-optic pediatric laparoscope is inserted through a trocar–cannula assembly with gas (5% CO_2 or triple gas as described later in this protocol) insufflating the abdominal wall to visualize the ovaries, and the follicles are aspirated. The surgical incisions are smaller with laparoscopy than the open-abdomen invasive surgery of laparotomy.
2. The first appearance of virginal bleed considered as day 1 of mense. Virginal bleed can be confirmed by virginal swab, blood spot on the cage pan, and trace of blood at the virginal area. Regular menstrual cycle is important for effective hormone stimulation.
3. Appropriate vacuum pressure should be applied in order not to strip off the cumulus cells surrounding the oocytes.
4. Collection tube should be kept warm before and after oocyte retrieval by a heated block or a small incubator.
5. Proper personal protective equipment such as mask, eye protection, and gloves should be used to prevent contact with biohazardous materials.
6. All liquid waste should be treated with bleach before pouring into the sink or other container for disposal.
7. Avoid carry over of too much medium during wash and subsequent transfer into culture drop.
8. To remove cumulus cells before culture, a fire polished pipette with diameter just slightly bigger than the oocyte should be used.
9. The monkey is sedated with a light dose of Telazol (0.7–1.0 mg/kg body weight), administered i.m. The penis is extended from the body. One pre-sized defibrillator gel electrode is wrapped around the base of the penis and connected to the negative lead. The second gel electrode is positioned immediately behind the glans and connected to the positive lead. It is then extended slightly and positioned over a collection tube. Turn on the ejaculator, then slowly, and steadily increase the output adjust dial until a slight erection, engorgement of the glans, and the collection of sample in a 15 mL conical tube.

10. It has been shown that, with rhesus monkey sperm, treatment with caffeine significantly increased the number of sperm bound to the zona pellucida, while treatment with dibutyryl cyclic adenosine monophosphate (dbcAMP), a cell-permeable cAMP analog that activates cAMP-dependent protein kinase (PKA), resulted in a higher percentage of acrosome-reacted sperm on the zona (72, 120).
11. Addition of dbcAMP and caffeine to baboon sperm and oocyte culture dishes in conventional IVF yields a lower percentage of fertilization and embryo development than with ICSI protocol.
12. Minimize the amount of medium injected into an oocyte during sperm injection.
13. To ensure the penetration of the injection pipette into the oolemma, aspirate a small amount of cytoplasm by applying negative pressure until feeling the "pop" confirming the breakage of the cytoplasmic membrane. Stop the aspiration action instantly after the breakage of the membrane, applying positive pressure to expel the sperm into the oocyte.
14. To enhance blastocyst hatching rate and subsequent implantation, assisted hatching by drilling holes in the zona pellucida will allow blastocysts to hatch followed by implantation at a higher rate. Zona drilling can be either using acidified Tyrode's solution, a laser, or PIEZO device.
15. To avoid multiple pregnancies, not more than two healthy embryos should be transferred into each surrogate female.
16. A surrogate female with good reproductive history is critical for successful establishment of pregnancy. Females can be trained for conscious bleeding (as the monkeys are not sedated but trained to present their legs for bleeding) which will reduce stress and improve subsequent pregnancy.
17. For tubal embryo transfer, the transfer catheter should be placed as far as possible into the oviduct. After embryos are expelled from the catheter, the latter should be removed slowly to avoid backflow of media.

4.2. Baboon In Vitro Fertilization and Embryo Culture

18. Sex skin deturgescence (121) occurs most likely 2–3 days prior to ovulation in the baboon. Premature deturgescence occurs occasionally indicating poor results of ovarian stimulation. In those cases, gonadotropin injection will be discontinued and follicle retrieval laparoscopic-assisted surgery will be cancelled.

19. Recombinant hormones commonly used in human-assisted reproductive technology may be antigenic in macaque species including rhesus monkey and baboons. Consequently, repeated stimulation may result in a low number of oocytes and a high percentage of poor-quality oocytes (122, 123). Macaque-specific FSH and CG are not commercially available at this time.
20. Rectal probe electroejaculation is one of the commonly applied methods to collect nonhuman primate semen specimen (124). Readers may apply other methods which work best for their experimental settings.
21. Sperm quality, including motility and progression, often decreases quickly after the first few hours post-processing. The quality of sperm varies with individual male monkey. Resuspending the sperm in insemination medium (P1 with 5% SSS in our protocol) and placing the vial at room temperature with tri-gas (in the incubator or isolated modular chamber) usually prolongs the storage time of processed sperm over 24 h for a potential next day ICSI.
22. Co-culture is used to improve embryo development by possible mechanisms of removing substances from the medium/microenvironment which may be toxic or inhibitory to embryo growth and/or secreting growth factors, proteins, and other stimulating effects for embryo development.
23. It is recommended that the total time of manipulating oocytes in the hyaluronidase dish not exceed 1 min.
24. Nonhuman primate oocyte IVM is not well established at this moment and we do not include IVM methods in this protocol (125, 126). ICSI on IVM oocytes usually yields poor embryo development.
25. It is recommended to pool oocytes of same maturation stage in one well. MII oocytes are ready for ICSI. Oocytes at MI/germinal vesicle breakdown (GVBD) occasionally proceed to MII later in the day, and in such case, those later matured MII oocytes can be used for ICSI. Immature oocytes with visible GV are not suitable for insemination.
26. Tubing connected to holding pipette may be filled with air; however, oil is critical for controlling the injection pipette.
27. Baboon sperm shows more progressive motility than human sperm, therefore a higher concentration of PVP is needed to slow down the sperm and enable selection of sperm with normal morphology.
28. CMRL-based complex medium conditioned by the co-cultured feeder cells supplemented with serum has

been commonly used. In other studies, methods using buffalo rat liver (BRL) and green monkey kidney (Vero) monolayers as feeder cells were used, and those trials produced high rates of nonhuman primate blastocysts (56, 92). Later studies using HECM-9 supplemented with amino acids showed higher success rates of embryo development in rhesus monkey ART, as indicated in the rhesus monkey ART protocol in this chapter. However, we have found that applying various human clinical ART embryo culture media with BRL feeder has generated a higher yield of baboon embryos in vitro, compared to protocols of CMRL and HECM-9 (100).

29. Occasionally immature oocytes from the previous day can acquire an MII morphology the next morning. ICSI on those later maturing oocytes is not recommended since the development of such embryos, even if they form zygotes and proceed to cleavage stages, is very poor and those embryos rarely form blastocysts.
30. Embryos may show slower development, e.g., 2–4 cells instead of 8 cells, on day 3. Those embryos can be kept in the first part (Cleavage Stage Embryo Culture Medium) of this sequential culture system, and then moved into the second part (Blastocyst Culture Medium) when they reach the 8-cell stage.
31. Preimplantation embryo quality is usually graded by criteria including cell number and morphology (fragmentation, symmetry, vacuoles, etc.). Blastocysts are graded by the formation of inner cell mass (ICM) and trophectoderm (TE). Further information can be found in literatures dedicated to this topic (127–129).
32. It is important to maintain stable pH and temperature conditions for the embryos in the culture petri dish and transfer catheter. Portable incubators purged with triple gas to keep the embryo culture dish at a balanced pH and at a constant temperature, as well as a minimal time of embryos staying in the transfer catheter are preferred.

References

1. Clayton, O. and Kuehl, T. J. (1984) The first successful in vitro fertilization and embryo transfer in a nonhuman primate. *Theriogenology* **21**, 228.
2. Bavister, B. D., Boatman, D. E., Collins, K., Dierschke, D. J., and Eisele, S. G. (1984) Birth of rhesus monkey infant after in vitro fertilization and nonsurgical embryo transfer. *Proc. Natl. Acad. Sci. USA* **81**, 2218–2222.
3. Wolf, D. P. (2004) Assisted reproductive technologies in rhesus macaques. *Reprod. Biol. Endocrinol.* **2**, 37.
4. Yang, S. -H., Cheng, P. -H., Banta, H., Piotrowska-Nitsche, K., Yang, J. -J., Cheng, E. C. H., Snyder, B., Larkin, K., Liu,

J., Orkin, J., Fang, Z. -H., Smith, Y., Bachevalier, J., Zola, S. M., Li, S. -H., Li, X. -J., and Chan, A. W. S. (2008) Towards a transgenic model of Huntington's disease in a non-human primate. *Nature* **453**, 924.

5. Chan, A. W., Luetjens, C. M., Dominko, T., Ramalho-Santos, J., Simerly, C. R., Hewitson, L., and Schatten, G. (2000) TransgenICSI reviewed: foreign DNA transmission by intracytoplasmic sperm injection in rhesus monkey. *Mol. Reprod. Dev.* **56**, 325–328.
6. Chan, A. W., Luetjens, C. M., Dominko, T., Ramalho-Santos, J., Simerly, C. R., Hewitson, L., and Schatten, G. (2000) Foreign DNA transmission by ICSI: injection of spermatozoa bound with exogenous DNA results in embryonic GFP expression and live rhesus monkey births. *Mol. Hum. Reprod.* **6**, 26–33.
7. Chan, A. W., Chong, K. Y., Martinovich, C., Simerly, C., and Schatten, G. (2001) Transgenic monkeys produced by retroviral gene transfer into mature oocytes. *Science* **291**, 309–312.
8. Sasaki, E., Suemizu, H., Shimada, A., Hanazawa, K., Oiwa, R., Kamioka, M., Tomioka, I., Sotomaru, Y., Hirakawa, R., Eto, T., Shiozawa, S., Maeda, T., Ito, M., Ito, R., Kito, C., Yagihashi, C., Kawai, K., Miyoshi, H., Tanioka, Y., Tamaoki, N., Habu, S., Okano, H., and Nomura, T. (2009) Generation of transgenic non-human primates with germline transmission. *Nature* **459**, 527.
9. Douglas, G. C., VandeVoort, C. A., Kumar, P., Chang, T. -C., and Golos, T. G. (2009) Trophoblast stem cells: models for investigating trophectoderm differentiation and placental development. *Endocr. Rev.* **30**, 228–240.
10. Chang, T. -C., Bondarenko, G. I., Durning, M., Vielhuber, K., Garthwaite, M. A., and Golos, T. G. (2008) A three-dimensional in vitro implantation model with nonhuman primate embryos and extracellular matrix under various culture conditions. *Reprod. Fertil. Dev.* **20**, 142–143.
11. Chang, T. -C., Bondarenko, G. I., Gerami-Naini, B., Drenzek, J. G., Durning, M., Garthwaite, M. A., and Golos, T. G. (2007) A novel three-dimensional in vitro implantation model to study primate embryo development and early pregnancy. *Reprod. Sci.* **14**, 121A.
12. Lopata, A., Kohlman, D. J., Bowes, L. G., and Watkins, W. B. (1995) Culture of marmoset blastocysts on matrigel: a model of differentiation during the implantation period. *Anatomical Record* **241**, 469–486.
13. Seshagiri, P. B. and Hearn, J. P. (1993) In-vitro development of in-vivo produced rhesus monkey morulae and blastocysts to hatched, attached, and post-attached blastocyst stages: morphology and early secretion of chorionic gonadotrophin. *Hum. Reprod.* **8**, 279–287.
14. Seshagiri, P. B., Terasawa, E., and Hearn, J. P. (1994) The secretion of gonadotrophin-releasing hormone by peri-implantation embryos of the rhesus monkey: comparison with the secretion of chorionic gonadotrophin. *Hum. Reprod.* **9**, 1300–1307.
15. Enders, A. C., Meyers, S., VandeVoort, C. A., and Douglas, G. C. (2005) Interactions of macaque blastocysts with epithelial cells in vitro. *Hum. Reprod.* **20**, 3026–3032.
16. Tachibana, M., Sparman, M., Sritanaudomchai, H., Ma, H., Clepper, L., Woodward, J., Li, Y., Ramsey, C., Kolotushkina, O., and Mitalipov, S. (2009) Mitochondrial gene replacement in primate offspring and embryonic stem cells. *Nature* **461**, 372.
17. Bavister, B. D., Boatman, D. E., Leibfried, L., Loose, M., and Vernon, M. W. (1983) Fertilization and cleavage of rhesus monkey oocytes in vitro. *Biol. Reprod.* **28**, 983–999.
18. Boatman, D. E. (1987) In Vitro growth of non-human primate pre- and peri-implantation embryos. In *The mammalian preimplantation embryo*, ed. Bavister, B. D. Plenum Press, New York, pp 273–308.
19. Bavister, B. D., Leibfried, M. L., and Lieberman, G. (1983) Development of preimplantation embryos of the golden hamster in a defined culture medium. *Biol. Reprod.* **28**, 235–247.
20. Schramm, R. D. and Paprocki, A. M. (2000) Birth of rhesus monkey infant after transfer of embryos derived from in-vitro matured oocytes. *Hum. Reprod.* **15**, 2411–2414.
21. Borman, S. M., Chaffin, C. L., Schwinof, K. M., Stouffer, R. L., and Zelinski-Wooten, M. B. (2004) Progesterone promotes oocyte maturation, but not ovulation, in nonhuman primate follicles without a gonadotropin surge. *Biol. Reprod.* **71**, 366–373.
22. Chan, A. W., Dominko, T., Luetjens, C. M., Neuber, E., Martinovich, C., Hewitson, L., Simerly, C. R., and Schatten, G. P. (2000) Clonal propagation of primate offspring by embryo splitting. *Science* **287**, 317–319.
23. Enders, A. C., Boatman, D., Morgan, P., and Bavister, B. D. (1989) Differentiation of blastocysts derived from in vitro-fertilized

rhesus monkey ova. *Biol. Reprod.* **41**, 715–727.
24. Gabriel Sanchez-Partida, L., Maginnis, G., Dominko, T., Martinovich, C., McVay, B., Fanton, J., and Schatten, G. (2000) Live rhesus offspring by artificial insemination using fresh sperm and cryopreserved sperm. *Biol. Reprod.* **63**, 1092–1097.
25. Hewitson, L. C., Simerly, C. R., Tengowski, M. W., Sutovsky, P., Navara, C. S., Haavisto, A. J., and Schatten, G. (1996) Microtubule and chromatin configurations during rhesus intracytoplasmic sperm injection: successes and failures. *Biol. Reprod.* **55**, 271–280.
26. Hewitson, L., Dominko, T., Takahashi, D., Martinovich, C., Ramalho-Santos, J., Sutovsky, P., Fanton, J., Jacob, D., Monteith, D., Neuringer, M., Battaglia, D., Simerly, C., and Schatten, G. (1999) Unique checkpoints during the first cell cycle of fertilization after intracytoplasmic sperm injection in rhesus monkeys. *Nat. Med.* **5**, 431–433.
27. Hewitson, L., Simerly, C., Dominko, T., and Schatten, G. (2000) Cellular and molecular events after in vitro fertilization and intracytoplasmic sperm injection. *Theriogenology* **53**, 95–104.
28. Hewitson, L., Simerly, C., and Schatten, G. (2000) Cytoskeletal aspects of assisted fertilization. *Semin. Reprod. Med.* **18**, 151–159.
29. Hewitson, L. and Schatten, G. (2002) The use of primates as models for assisted reproduction. *Reprod. Biomed. Online* **5**, 50–55.
30. Hewitson, L., Martinovich, C., Simerly, C., Takahashi, D., and Schatten, G. (2002) Rhesus offspring produced by intracytoplasmic injection of testicular sperm and elongated spermatids. *Fertil. Steril.* 77, 794–801.
31. Johnson, L. D., Mattson, B. A., Albertini, D. F., Sehgal, P. K., Becker, R. A., Avis, J., and Biggers, J. D. (1991) Quality of oocytes from superovulated rhesus monkeys. *Hum. Reprod.* **6**, 623–631.
32. Kubisch, H. M., Ratterree, M. S., Williams, V. M., Johnson, K. M., Davison, B. B., Phillippi-Falkenstein, K. M., and Harrison, R. M. (2005) Birth of rhesus macaque (Macaca mulatta) infants after in vitro fertilization and gestation in female rhesus or pigtailed (Macaca nemestrina) macaques. *Comp. Med.* **55**, 129–135.
33. Lanzendorf, S. E., Zelinski-Wooten, M. B., Stouffer, R. L., and Wolf, D. P. (1990) Maturity at collection and the developmental potential of rhesus monkey oocytes. *Biol. Reprod.* **42**, 703–711.
34. Meng, L. and Wolf, D. P. (1997) Sperm-induced oocyte activation in the rhesus monkey: nuclear and cytoplasmic changes following intracytoplasmic sperm injection. *Hum. Reprod.* **12**, 1062–1068.
35. Meng, L., Ely, J. J., Stouffer, R. L., and Wolf, D. P. (1997) Rhesus monkeys produced by nuclear transfer. *Biol. Reprod.* **57**, 454–459.
36. Mitalipov, S. M., Nusser, K. D., and Wolf, D. P. (2001) Parthenogenetic activation of rhesus monkey oocytes and reconstructed embryos. *Biol. Reprod.* **65**, 253–259.
37. Mitalipov, S. M., Yeoman, R. R., Kuo, H. C., and Wolf, D. P. (2002) Monozygotic twinning in rhesus monkeys by manipulation of in vitro-derived embryos. *Biol. Reprod.* **66**, 1449–1455.
38. Mitalipov, S. M., Yeoman, R. R., Nusser, K. D., and Wolf, D. P. (2002) Rhesus monkey embryos produced by nuclear transfer from embryonic blastomeres or somatic cells. *Biol. Reprod.* **66**, 1367–1373.
39. Mitalipov, S. M. and Wolf, D. P. (2006) Nuclear transfer in nonhuman primates. *Meth. Mol. Biol.* **348**, 151–168.
40. Morgan, P. M., Warikoo, P. K., and Bavister, B. D. (1991) In vitro maturation of ovarian oocytes from unstimulated rhesus monkeys: assessment of cytoplasmic maturity by embryonic development after in vitro fertilization. *Biol. Reprod.* **45**, 89–93.
41. Nusser, K. D., Mitalipov, S., Widmann, A., Gerami-Naini, B., Yeoman, R. R., and Wolf, D. P. (2001) Developmental competence of oocytes after ICSI in the rhesus monkey. *Hum. Reprod.* **16**, 130–137.
42. Schramm, R. D. and Bavister, B. D. (1996) Use of purified porcine follicle-stimulating hormone for ovarian stimulation of macaque monkeys. *Theriogenology* **45**, 727–732.
43. Schramm, R. D., Paprocki, A. M., and Watkins, D. I. (2001) Birth of MHC-defined rhesus monkeys produced by assisted reproductive technology. *Vaccine* **20**, 603–607.
44. Schramm, R. D. and Paprocki, A. M. (2004) Strategies for the production of genetically identical monkeys by embryo splitting. *Reprod. Biol. Endocrinol.* **2**, 38.
45. Schramm, R. D. and Paprocki, A. M. (2004) In vitro development and cell allocation following aggregation of split embryos with tetraploid or developmentally asynchronous blastomeres in rhesus monkeys. *Cloning Stem Cells* **6**, 302–314.
46. Simerly, C. R. and Navara, C. S. (2003) Nuclear transfer in the rhesus monkey:

opportunities and challenges. *Cloning Stem Cells* **5**, 319–331.
47. Sutovsky, P., Hewitson, L., Simerly, C. R., Tengowski, M. W., Navara, C. S., Haavisto, A., and Schatten, G. (1996) Intracytoplasmic sperm injection for Rhesus monkey fertilization results in unusual chromatin, cytoskeletal, and membrane events, but eventually leads to pronuclear development and sperm aster assembly. *Hum. Reprod.* **11**, 1703–1712.
48. VandeVoort, C. A. (2004) High quality sperm for nonhuman primate ART: production and assessment. *Reprod. Biol. Endocrinol.* **2**, 33.
49. Weston, A. M. and Wolf, D. P. (1996) Differential preimplantation development of rhesus monkey embryos in serum-supplemented media. *Mol. Reprod. Dev.* **44**, 88–92.
50. Wolf, D. P., Vandevoort, C. A., Meyer-Haas, G. R., Zelinski-Wooten, M. B., Hess, D. L., Baughman, W. L., and Stouffer, R. L. (1989) In vitro fertilization and embryo transfer in the rhesus monkey. *Biol. Reprod.* **41**, 335–346.
51. Wolf, D. P., Meng, L., Ouhibi, N., and Zelinski-Wooten, M. (1999) Nuclear transfer in the rhesus monkey: practical and basic implications. *Biol. Reprod.* **60**, 199–204.
52. Wolf, D. P., Thormahlen, S., Ramsey, C., Yeoman, R. R., Fanton, J., and Mitalipov, S. (2004) Use of assisted reproductive technologies in the propagation of rhesus macaque offspring. *Biol. Reprod.* **71**, 486–493.
53. Wolfgang, M. J., Eisele, S. G., Knowles, L., Browne, M. A., Schotzko, M. L., and Golos, T. G. (2001) Pregnancy and live birth from nonsurgical transfer of in vivo- and in vitro-produced blastocysts in the rhesus monkey. *J. Med. Primatol.* **30**, 148–155.
54. Zelinski-Wooten, M. B., Hutchison, J. S., Trinchard-Lugan, I., Hess, D. L., Wolf, D. P., and Stouffer, R. L. (1997) Initiation of periovulatory events in gonadotrophin-stimulated macaques with varying doses of recombinant human chorionic gonadotrophin. *Hum. Reprod.* **12**, 1877–1885.
55. Zelinski-Wooten, M. B., Hutchison, J. S., Hess, D. L., Wolf, D. P., and Stouffer, R. L. (1998) A bolus of recombinant human follicle stimulating hormone at midcycle induces periovulatory events following multiple follicular development in macaques. *Hum. Reprod.* **13**, 554–560.
56. Zhang, L., Weston, A. M., Denniston, R. S., Goodeaux, L. L., Godke, R. A., and Wolf, D. P. (1994) Developmental potential of rhesus monkey embryos produced by in vitro fertilization. *Biol. Reprod.* **51**, 433–440.
57. Zheng, P., Si, W., Wang, H., Zou, R., Bavister, B. D., and Ji, W. (2001) Effect of age and breeding season on the developmental capacity of oocytes from unstimulated and follicle-stimulating hormone-stimulated rhesus monkeys. *Biol. Reprod.* **64**, 1417–1421.
58. Zheng, P., Patel, B., McMenamin, M., Moran, E., Paprocki, A. M., Kihara, M., Schramm, R. D., and Latham, K. E. (2005) Effects of follicle size and oocyte maturation conditions on maternal messenger RNA regulation and gene expression in rhesus monkey oocytes and embryos. *Biol. Reprod.* **72**, 890–897.
59. Zheng, P. (2007) Effects of in vitro maturation of monkey oocytes on their developmental capacity. *Anim. Reprod. Sci.* **98**, 56–71.
60. Bavister, B. D. and Boatman, D. E. (1992) 'Test-tube' primates: the next generation. *Hum. Reprod.* **7**, 1035.
61. Lee, D. M., Yeoman, R. R., Battaglia, D. E., Stouffer, R. L., Zelinski-Wooten, M. B., Fanton, J. W., and Wolf, D. P. (2004) Live birth after ovarian tissue transplant. *Nature* **428**, 137–138.
62. Schramm, R. D. and Bavister, B. D. (1996) Development of in-vitro-fertilized primate embryos into blastocysts in a chemically defined, protein-free culture medium. *Hum. Reprod.* **11**, 1690–1697.
63. Schramm, R. D. and Bavister, B. D. (1999) A macaque model for studying mechanisms controlling oocyte development and maturation in human and non-human primates. *Hum. Reprod.* **14**, 2544–2555.
64. VandeVoort, C. A., Leibo, S. P., and Tarantal, A. F. (2003) Improved collection and developmental competence of immature macaque oocytes. *Theriogenology* **59**, 699–707.
65. VandeVoort, C. A., Hung, P. H., and Schramm, R. D. (2007) Prevention of zona hardening in non-human primate oocytes cultured in protein-free medium. *J. Med. Primatol.* **36**, 10–16.
66. Weston, A. M., Zelinski-Wooten, M. B., Hutchison, J. S., Stouffer, R. L., and Wolf, D. P. (1996) Developmental potential of embryos produced by in-vitro fertilization from gonadotrophin-releasing hormone antagonist-treated macaques stimulated with recombinant human follicle stimulating hormone alone or in combi-

nation with luteinizing hormone. *Hum. Reprod.* **11**, 608–613.
67. Zelinski-Wooten, M. B., Alexander, M., Christensen, C. L., Wolf, D. P., Hess, D. L., and Stouffer, R. L. (1994) Individualized gonadotropin regimens for follicular stimulation in macaques during in vitro fertilization (IVF) cycles. *J. Med. Primatol.* **23**, 367–374.
68. Shimozawa, N., Okada, H., Hatori, M., Yoshida, T., and Sankai, T. (2007) Comparison of methods to stimulate ovarian follicular growth in cynomolgus and African green monkeys for collection of mature oocytes. *Theriogenology* **67**, 1143–1149.
69. Ng, S. C., Martelli, P., Liow, S. L., Herbert, S., and Oh, S. H. (2002) Intracytoplasmic injection of frozen-thawed epididymal spermatozoa in a nonhuman primate model, the cynomolgus monkey (Macaca fascicularis). *Theriogenology* **58**, 1385–1397.
70. Suemori, H., Tada, T., Torii, R., Hosoi, Y., Kobayashi, K., Imahie, H., Kondo, Y., Iritani, A., and Nakatsuji, N. (2001) Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. *Dev. Dyn.* **222**, 273–279.
71. Ogonuki, N., Sankai, T., Cho, F., Sato, K., and Yoshikawa, Y. (1998) Comparison of two methods of assisted fertilization in cynomolgus monkeys (Macaca fascicularis): intracytoplasmic sperm injection and partial zona dissection followed by insemination. *Hum. Reprod.* **13**, 2555–2560.
72. Mahony, M. C., Lanzendorf, S., Gordon, K., and Hodgen, G. D. (1996) Effects of caffeine and dbcAMP on zona pellucida penetration by epididymal spermatozoa of cynomolgus monkeys (Macaca fascicularis). *Mol. Reprod. Dev.* **43**, 530–535.
73. Lanzendorf, S. E., Gordon, K., Toner, J., Mahony, M. C., Kolm, P., and Hodgen, G. D. (1995) Prediction of ovarian response to exogenous gonadotropin stimulation: utilization for collection of primate oocytes for fertilization in vitro. *Theriogenology* **44**, 641–648.
74. Lefevre, B., Gougeon, A., Nome, F., and Testart, J. (1991) Effect of a gonadotropin-releasing hormone agonist and gonadotropins on ovarian follicles in cynomolgus monkey: a model for human ovarian hyperstimulation. *Fertil. Steril.* **56**, 119–125.
75. Balmaceda, J. P., Pool, T. B., Arana, J. B., Heitman, T. S., and Asch, R. H. (1984) Successful in vitro fertilization and embryo transfer in cynomolgus monkeys. *Fertil. Steril.* **42**, 791–795.
76. Sankai, T., Cho, F., and Yoshikawa, Y. (1997) In vitro fertilization and preimplantation embryo development of African green monkeys (Cercopithecus aethiops). *Am. J. Primatol.* **43**, 43–50.
77. Sparman, M. L., Carrie, C. M. R., Thomas, M., Mitalipov, S. M., Fanton, J. W., Maginnis, G. M., Stouffer, R. L., and Wolf, D. P. (2007) Evaluation of the vervet (Clorocebus aethiops) as a model for the assisted reproductive technologies. *Am. J. Primatol.* **69**, 917–929.
78. Asakawa, T. and Dukelow, W. R. (1982) Chromosomal analyses after in vitro fertilization of squirrel monkey (Saimiri sciureus) oocytes. *Biol. Reprod.* **26**, 579–583.
79. Chan, P. J., Hutz, R. J., and Dukelow, W. R. (1982) Nonhuman primate in vitro fertilization: seasonality, cumulus cells, cyclic nucleotides, ribonucleic acid, and viability assays. *Fertil. Steril.* **38**, 609–615.
80. Dukelow, W. R., Chan, P. J., Hutz, R. J., Demayo, F. J., Dooley, V. D., Rawlins, R. G., and Ridha, M. T. (1983) Preimplantation development of the primate embryo after in vitro fertilization. *J. Exp. Zool.* **228**, 215–221.
81. Dukelow, W. R., Pierce, D. L., Roudebush, W. E., Jarosz, S. J., and Sengoku, K. (1990) In vitro fertilization in nonhuman primates. *J. Med. Primatol.* **19**, 627–639.
82. Hutz, R. J., Chan, P. J., and Dukelow, W. R. (1983) Nonhuman primate in vitro fertilization: biochemical changes associated with embryonic development. *Fertil. Steril.* **40**, 521–524.
83. Kuehl, T. J. and Dukelow, W. R. (1975) Fertilization in vitro of Saimiri sciureus follicular oocytes. *J. Med. Primatol.* **4**, 209–216.
84. Kuehl, T. J. and Dukelow, W. R. (1979) Maturation and in vitro fertilization of follicular oocytes of the squirrel monkey (Saimiri sciureus). *Biol. Reprod.* **21**, 545–556.
85. Pierce, D. L., Johnson, M. P., Kaneene, J. B., and Dukelow, W. R. (1993) In vitro fertilization analysis of squirrel monkey oocytes produced by various follicular induction regimens and the incidence of triploidy. *Am. J. Primatol.* **29**, 37–48.
86. Marshall, V. S., Browne, M. A., Knowles, L., Golos, T. G., and Thomson, J. A. (2003) Ovarian stimulation of marmoset monkeys (Callithrix jacchus) using recombinant human follicle stimulating hormone. *J. Med. Primatol.* **32**, 57–66.
87. Marshall, V. S., Wilton, L. J., and Moore, H. D. (1998) Parthenogenetic activa-

tion of marmoset (Callithrix jacchus) oocytes and the development of marmoset parthenogenones in vitro and in vivo. *Biol. Reprod.* **59**, 1491–1497.
88. Gilchrist, R. B., Nayudu, P. L., and Hodges, J. K. (1997) Maturation, fertilization, and development of marmoset monkey oocytes in vitro. *Biol. Reprod.* **56**, 238–246.
89. Wilton, L. J., Marshall, V. S., Piercy, E. C., and Moore, H. D. (1993) In vitro fertilization and embryo development in the marmoset monkey (Callithrix jacchus). *J. Reprod. Fertil.* **97**, 481–486.
90. Lopata, A., Summers, P. M., and Hearn, J. P. (1988) Births following the transfer of cultured embryos obtained by in vitro and in vivo fertilization in the marmoset monkey (Callithrix jacchus). *Fertil. Steril.* **50**, 503–509.
91. Loskutoff, N. M., Kraemer, D. C., Raphael, B. L., Huntress, S. L., and Wildt, D. E. (1991) Advances in reproduction in captive, female great apes: value of biotechniques. *Am. J. Primatol.* **24**, 151–166.
92. Pope, C., Pope, V., and Beck, L. (1982) Development of baboon preimplantation embryos to post-implantation stages in vitro. *Biol. Reprod.* **27**, 915–923.
93. D'Hooghe, T. M., Spiessens, C., Chai, D. C., Mwethera, P. G., Makokha, A. O., and Mwenda, J. M. (2004) Ovarian stimulation, egg aspiration, in vitro fertilization and embryo transfer in the baboon (Papio anubis): a pilot project at the Institute of Primate Research, Nairobi, Kenya. *Gynecol. Obstet. Invest.* **57**, 23–26.
94. Amboka, J. N. and Mwethera, P. G. (2003) Characterization of semen from olive baboons. *J. Med. Primatol.* **32**, 325–329.
95. Irsigler, U. M., Van der Merwe, J. V., and Botes, A. D. (1984) Fertilization and cleavage in vitro of baboon oocytes. *S. Afr. Med. J.* **66**, 447–450.
96. Fourie, F. R., Snyman, E., and van der Merwe, J. V. (1987) Supplementation of Ham's F10 culture medium with three different sera in the culturing of baboon oocytes. *Comp. Biochem. Physiol. A* **87**, 1103–1106.
97. Fourie, F. R., Snyman, E., van der Merwe, J. V., and Grace, A. (1987) Primate in vitro fertilization research: preliminary results on the folliculogenic effects of three different ovulatory induction agents on the chacma baboon, Papio ursinus. *Comp. Biochem. Physiol. A* **87**, 889–893.
98. Cseh, S., Corselli, J., Chan, P., and Bailey, L. (2002) Superovulation using recombinant human FSH and ultrasound-guided transabdominal follicular aspiration in baboon (Papio Anubis). *Anim. Reprod. Sci.* **70**, 287–293.
99. Nyachieo, A., Spiessens, C., Chai, D. C., Mwenda, J. M., and D'Hooghe, T. M. (2009) Menstrual cycle synchronization, ovarian stimulation, and in vitro fertilization in olive baboons (Papio anubis): a prospective randomized study. *Fertil. Steril.* **91**, 602–610.
100. Chang, T. -C., Eddy, C. A., Ying, Y., Liu, Y. -G., Holden, A. E., Brzyski, R. G., and Schenken, R. S. (2011) Ovarian stimulation, in vitro fertilization, and effects of culture conditions on baboon preimplantation embryo development. *Fertil. Steril.* **95**, 1217–1223.
101. Bavister, B. D. (2004) ARTs in action in nonhuman primates: symposium summary–advances and remaining issues. *Reprod. Biol. Endocrinol.* **2**, 43.
102. Bavister, B. D. (1995) Culture of preimplantation embryos: facts and artifacts. *Hum. Reprod. Update* **1**, 91–148.
103. Zelinski-Wooten, M., Hutchison, J., Trinchard-Lugan, I., Hess, D., Wolf, D., and Stouffer, R. (1997) Initiation of periovulatory events in gonadotrophin-stimulated macaques with varying doses of recombinant human chorionic gonadotrophin. *Hum. Reprod.* **12**, 1877–1885.
104. Lanzendorf, S. E., Gliessman, P. M., Archibong, A. E., Alexander, M., and Wolf, D. P. (1990) Collection and quality of rhesus monkey semen. *Mol. Reprod. Dev.* **25**, 61–66.
105. Sarason, R. L., VandeVoort, C. A., Mader, D. R., and Overstreet, J. W. (1991) The use of nonmetal electrodes in electroejaculation of restrained but unanesthetized macaques. *J. Med. Primatol.* **20**, 122–125.
106. McKiernan, S. H. and Bavister, B. D. (2000) Culture of one-cell hamster embryos with water soluble vitamins: pantothenate stimulates blastocyst production. *Hum. Reprod.* **15**, 157–164.
107. Schramm, R. D., Paprocki, A. M., and VandeVoort, C. A. (2003) Causes of developmental failure of in-vitro matured rhesus monkey oocytes: impairments in embryonic genome activation. *Hum. Reprod.* **18**, 826–833.
108. Weston, A. M. and Wolf, D. P. (1996) Differential preimplantation development of rhesus monkey embryos in serum-supplemented media. *Mol. Reprod. Dev.* **44**, 88–92.

109. Kraemer, D. C., Moore, G. T., and Kramen, M. A. (1976) Baboon infant produced by embryo transfer. *Science* **192**, 1246–1247.
110. Ji, W. and Bavister, B. D. (2000) Development of zona-free hamster ova to blastocysts in vitro. *Theriogenology* **54**, 827–834.
111. Zheng, P., Bavister, B. D., and Ji, W. (2001) Energy substrate requirement for in vitro maturation of oocytes from unstimulated adult rhesus monkeys. *Mol. Reprod. Dev.* **58**, 348–355.
112. Chan, A. W. S., Chong, K. Y., and Schatten, G. (2002) Production of transgenic primates. In *Transgenic animal technology*, ed. Pinkert, C. A., 2nd ed. Academic, San Diego, CA, pp 359–394.
113. Ouhibi, N., Zelinski-Wooten, M. B., Thomson, J. A., and Wolf, D. P. (2001) Assisted fertilization and nuclear transfer in nonhuman primates. In *Contemporary endocrinology*, eds. Wolf, D. P. and Zelinski-Wooten, M. B. Humana Press Inc., Totowa, NJ, pp 253–284.
114. Chang, T. -C., Liu, Y. -G., Eddy, C. A., Jacoby, E. S., Binkley, P. A., Brzyski, R. G., and Schenken, R. S. (2011) Derivation and characterization of novel nonhuman primate embryonic stem cell lines from in vitro-fertilized baboon preimplantation embryos. *Stem Cells Dev.* **20**, 1053–1062.
115. Bennett, B. T., Abee, C. R., and Henrickson, R. (1995) *Nonhuman Primates in Biomedical Research: Biology and Management*, 1st ed. Academic, New York, NY.
116. VandeBerg, J. L., Williams-Blangero, S., and Tardif, S. D. (2008) *The Baboon in Biomedical Research*. Springer, Berlin.
117. Jewett, D. A. and Dukelow, W. R. (1973) Follicular observation and laparoscopic aspiration techniques in Macaca fascicularis. *J. Med. Primatol.* **2**, 108–113.
118. Dukelow, W. R. and Ariga, S. (1976) Laparoscopic techniques for biomedical research. *J. Med. Primatol.* **5**, 82–99.
119. Dierschke, D. J. and Clark, J. R. (1976) Laparoscopy in Macaca mulatta: specialized equipment employed and initial observations. *J. Med. Primatol.* **5**, 100–110.
120. VandeVoort, C. A., Tollner, T. L., and Overstreet, J. W. (1994) Separate effects of caffeine and dbcAMP on macaque sperm motility and interaction with the zona pellucida. *Mol. Reprod. Dev.* **37**, 299–304.
121. MacLennan, A. H. and Wynn, R. M. (1971) Menstrual cycle of the baboon: I. clinical features, vaginal cytology and endometrial histology. *Obstetrics Gynecol.* **38**, 350–358.
122. McCarthy, T. J., Fortman, J. D., Boice, M. L., Fazleabas, A. T., and Verhage, H. G. (1991) Induction of multiple follicular development and superovulation in the olive baboon, Papio anubis. *J. Med. Primatol.* **20**, 308–314.
123. Stouffer, R. L. and Zelinski-Wooten, M. B. (2004) Overriding follicle selection in controlled ovarian stimulation protocols: quality vs quantity. *Reprod. Biol. Endocrinol.* **2**, 32.
124. VandeVoort, C. (2004) High quality sperm for nonhuman primate ART: production and assessment. *Reprod. Biol. Endocrinol.* **2**, 33.
125. Lee, Y. S., Latham, K. E., and VandeVoort, C. A. (2008) Effects of in vitro maturation on gene expression in rhesus monkey oocytes. *Physiol. Genomics* **35**, 145–158.
126. Dupont, C., Bavister, B. D., Armant, D. R., and Brenner, C. A. (2009) Rhesus macaque embryos derived from MI oocytes maturing after retrieval display high rates of chromosomal anomalies. *Hum. Reprod.* **24**, 929–935.
127. Veeck, L. L. (1999) *An Atlas of Human Gametes and Conceptuses*. Informa Healthcare. Parthenon Publishing Group, New York.
128. Veeck, L. L. and Zaninovic, N. (2003) *An Atlas of Human Blastocysts*. Informa Healthcare. Parthenon Publishing Group, New York.
129. Elder, K. and Cohen, J. (2007) *Human Preimplantation Embryo Selection*. Informa Healthcare. London, UK.

Chapter 14

Embryological Methods in Ascidians: The Villefranche-sur-Mer Protocols

Christian Sardet, Alex McDougall, Hitoyoshi Yasuo, Janet Chenevert, Gérard Pruliere, Rémi Dumollard, Clare Hudson, Celine Hebras, Ngan Le Nguyen, and Alexandre Paix

Abstract

Ascidians (marine invertebrates: urochordates) are thought to be the closest sister groups of vertebrates. They are particularly attractive models because of their non-duplicated genome and the fast and synchronous development of large populations of eggs into simple tadpoles made of about 3,000 cells. As a result of stereotyped asymmetric cleavage patterns all blastomeres become fate restricted between the 16- and 110 cell stage through inheritance of maternal determinants and/or cellular interactions. These advantageous features have allowed advances in our understanding of the nature and role of maternal determinants, inductive interactions, and gene networks that are involved in cell lineage specification and differentiation of embryonic tissues. Ascidians have also contributed to our understanding of fertilization, cell cycle control, self-recognition, metamorphosis, and regeneration. In this chapter we provide basic protocols routinely used at the marine station in Villefranche-sur-Mer using the cosmopolitan species of reference *Ciona intestinalis* and the European species *Phallusia mammillata*. These two models present complementary advantages with regard to molecular, functional, and imaging approaches. We describe techniques for basic culture of embryos, micro-injection, in vivo labelling, micro-manipulations, fixation, and immuno-labelling. These methods allow analysis of calcium signals, reorganizations of cytoplasmic and cortical domains, meiotic and mitotic cell cycle and cleavages as well as the roles of specific genes and cellular interactions. Ascidians eggs and embryos are also an ideal material to isolate cortical fragments and to isolate and re-associate individual blastomeres. We detail the experimental manipulations which we have used to understand the structure and role of the egg cortex and of specific blastomeres during development.

Key words: Ascidians, eggs, embryos, isolated cortex, methods, micro-injections, in vivo labelling, imaging, micro-manipulations, immuno-labelling.

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_14, © Springer Science+Business Media, LLC 2011

1. Introduction

The European tradition of studying tunicate embryos (ascidians and appendicularians) started with Kowalevsky in 1866 (1) and Fol in 1879 (2) who discovered that these marine invertebrates (**Fig. 14.1a**, **d**) developed from a simple tadpole larvae (**Fig. 14.1g**, **h**) which represented a greatly simplified chordate body plan. The first experimental manipulations separating blas-

Fig. 14.1. (continued) network (*red*) and incubated in DiO-C2(3) to label mitochondria (*green*). (**I**) Montage of the two fluorescent channels from an egg, spliced together along the animal, vegetal axes (confocal section). Note the subcortical layer rich in mitochondria (Mito) and poor in ER (*arrows*) in the vegetal hemisphere; from Prodon et al. (33). (**J**) 16 cell stage embryo, *arrows* show the myoplasm in the smaller posterior-most (P) blastomeres. (**K**) Ascidian egg fertilization calcium wave: the wave of elevated calcium (Ca^{2+}, *red*) starts from the point of sperm entry (*arrow*) and propagates through the fertilized egg. Confocal section of an egg injected with Calcium-Green dextran. (**L**) *Phallusia* egg injected with two synthetic mRNAs: one coding for a histone (RFP fusion, in *red*) and the other for a nuclear and kinetochore marker (Venus fusion, in *green*). The injected egg was then fertilized. The image shows a 4 h post-fertilization gastrula stage embryo with nuclear (*arrows*) and mitotic chromosomes plus kinetochores (*arrowheads*) labelling. (**M**) Ascidian embryo injected with mRNA encoding EGFP at the 1 cell stage (*upper* embryo) and at the 2 cell stage (one blastomere which gives rise to a half-labelled embryo, *lower* embryo). (**N**) *Phallusia* egg fixed with formaldehyde 5 min post-fertilization and labelled with rhodamine phalloidin (in *red*). Accumulations of actin microfilaments are observed in the vegetal (*v*) contraction pole (*arrowheads*) and at the animal (*a*) pole corresponding to the position of the first meiotic spindle (*arrow*). (**O**) *Phallusia* egg fixed with methanol 5 min post-fertilization and immuno-labelled for microtubules (MT, rhodamine-coupled secondary antibody, in *red*) and mitochondria (Mito, Cy5-coupled secondary antibody, in *magenta*). DNA is labelled with Hoechst (in *blue*). This view of the animal pole shows the meiotic spindle before polar body extrusion. (**P**) *Phallusia* 4 cell stage embryo fixed with methanol and immuno-labelled for aPKC and mitochondria. View of the CAB region (*arrowheads*) showing accumulation of aPKC at the posterior pole. Primary antibody against aPKC is used at 1/100 dilution and signal is amplified with biotin/streptavidin (coupled with fluorescein, in *green*). Mitochondria are labelled with a secondary antibody coupled with Cy5 (in *magenta*). (**Q**) *Phallusia* 4 cell stage embryo fixed with methanol and immuno-labelled for aPKC. *Posterior view* showing aPKC enrichment in the CAB (*arrowheads*). Primary antibody against aPKC is used at 1/500 dilution, and the signal is amplified with TSA (Alexa488, in *green*). (**R**) *Ciona* isolation/re-association of blastomeres: indicated cells were isolated or co-isolated from 8 cell stage embryos and cultured until control embryos reached the 110 cell stage. According to the cell lineage, at this developmental stage the A4.1 lineage generates four notochord, four neural, three endoderm and one trunk lateral precursors. In the A4.1-derived partial embryo (*left*), however, a notochord marker gene, *brachyury*, is expressed in eight cells (*arrow*), indicating an ectopic formation of notochord precursors. In contrast, when A4.1 was co-isolated with a4.2 (*right*), the derived partial embryo expresses *brachyury* in four cells (*arrow*), indicating that cellular interactions between A4.1 and a4.2 lineages repress the formation of ectopic notochord fates. This cell isolation experiment resulted in identification of an Ephrin ligand, which is expressed in a4.2-derived cells and acts as the signal to repress notochord fates. *See* Picco et al. (20). (**S**) *Phallusia* isolated cortices: low magnification view of a field of cortical fragments isolated from eggs and 8 cell stage embryos (*arrowheads*: CAB). ER is labelled with CM-DiI-C16(3) (in *red*). (**T**) *Phallusia* cortical fragment isolated from an egg (about 15 μm in diameter). The ER network (*arrowheads*) adhering to the plasma membrane is labelled with DiO-C6(3) after fixation (in *green*). (**U**) *Phallusia* cortex isolated from an 8 cell stage embryo. ER labelled with DiO-C6(3) after fixation (in *green*) accumulates in the CAB (*arrowheads*). (**V**) *Phallusia* cortex isolated from an 8 cell stage embryo. The cortex was prepared in presence of EGTA and taxol, then fixed and immuno-labelled for microtubules (MT, Cy5-coupled secondary antibody, in *magenta*) and aPKC (primary antibody used at 1/100 dilution). The aPKC signal was amplified with the biotin/streptavidin system (fluorescein-coupled, in *green*). MT and aPKC are retained in the CAB (*arrowheads*) after cortex isolation.

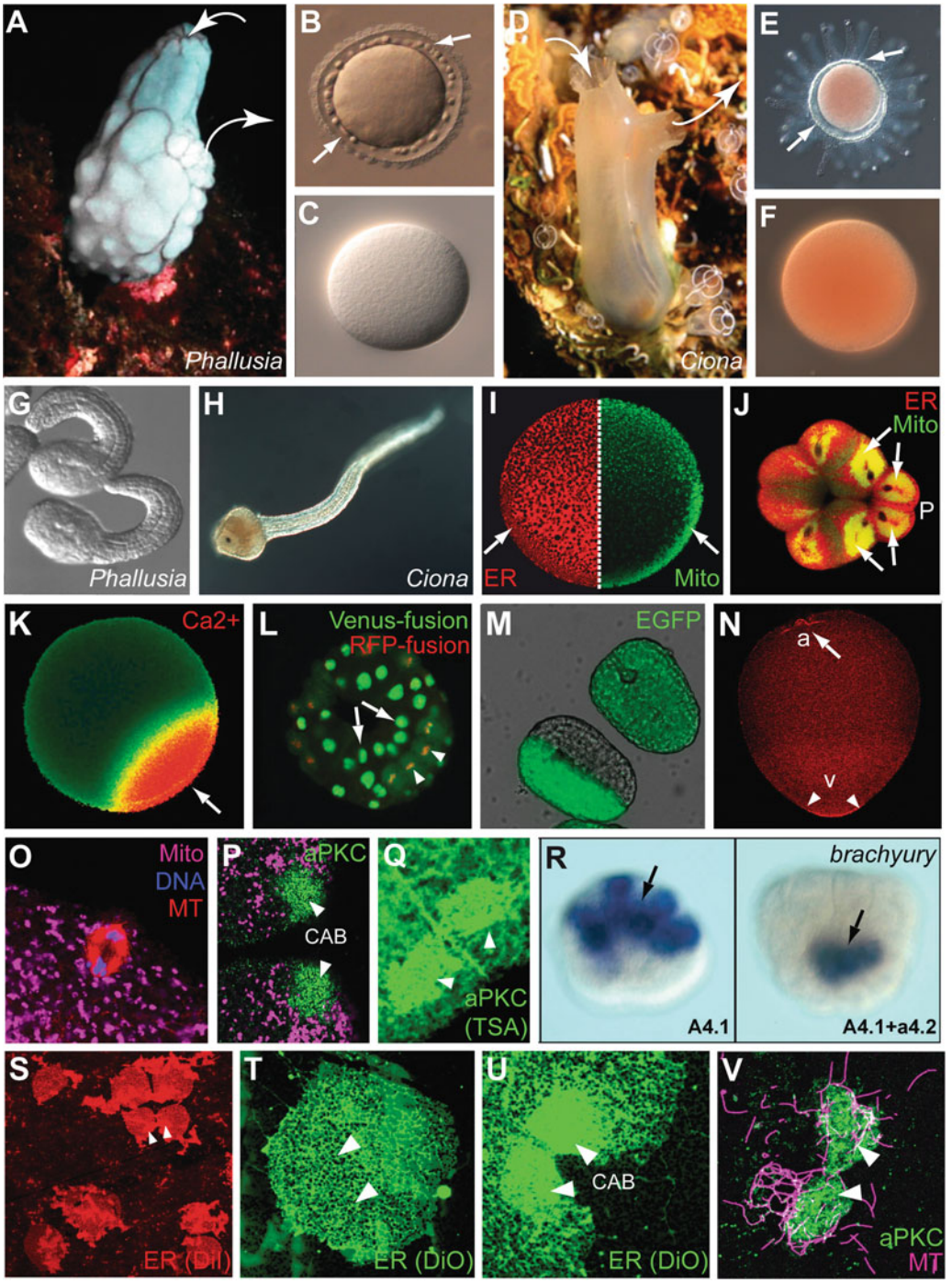

Fig. 14.1. Examples of labelled *Phallusia* and *Ciona* eggs and embryos. (**A, B, C**) *Phallusia mammillata*. (**A**) Adult animal with siphons (*arrows*); (**B**) unfertilized egg with chorion (*arrows*); (**C**) dechorionated egg (diameter is about 110–120 μm). (**D, E, F**) *Ciona intestinalis*. (**D**) Adult animal with siphons (*arrows*); (**E**) unfertilized egg with chorion (*arrows*); (**F**) dechorionated egg (diameter is about 120–130 μm). (**G**) Two *Phallusia* tadpoles whose development from fertilization has been filmed in time lapse (DIC optics) for 10 h in a micro-chamber (see whole sequence on BioMarCell web site, **Note 1**). (**H**) *Ciona* tadpole (16 h post-fertilization). (**I, J**) *Phallusia* eggs were injected with DiI-C16(3) to reveal the ER

tomeres of any embryos were performed by Chabry using the ascidian *Ascidiella aspersa* (3). More than a century ago, Conklin proposed that "organ forming substances" were located in peripheral regions of the ascidian egg and in particular that the "myoplasm", a sub-cortical domain (coloured yellow in *Styela partita*) gave rise to tail muscle cells (4). Fifty years later Italian biologists (5) and more recently Japanese investigators (6–8) showed through key ablation and transplantation experiments that determinants of axis establishment and muscle cell differentiation were situated in cortical and sub-cortical domains. Some of these determinants have now been identified as localized maternal mRNAs (9, 10) like in the fly *Drosophila*, the toad *Xenopus* and the jellyfish *Clytia* (11–13).

Recent molecular phylogeny studies suggest that ascidians (urochordates) are the closest sister groups of vertebrates (14). There is a sense that certain questions tackled on vertebrate models may be more easily addressed using the tadpole of ascidians, a relatively simple assemblage of approximately 3,000 cells whose lineages are well documented. A small set of precursor cells specified between the 16- and 110 cell stages generates the six tissues and a population of primordial germ cells making up the tadpole (8, 15, 16). Ascidians are particularly attractive to study the so-called mosaic type of development, maternal determinant segregation, and cell and tissue differentiation in a simple tadpole. They are also used to address questions of self-recognition, metamorphosis and regeneration (7, 17). In addition to partitioning maternal determinants, the stereotyped cell division patterns have enabled researchers to rapidly identify the inductive interactions that take place between blastomeres. The signalling pathways and gene networks involved in these cellular interactions are being unravelled using sophisticated micro-manipulation and gene-based strategies (18–20).

Five ascidian species *Ciona intestinalis, Ciona savignyi, Phallusia mammillata, Halocynthia roretzi, Botryllus schlosseri* and the larvacean *Oikopleura dioica* have become prominent models for research (21). A dynamic and growing scientific community (about 500 people), which meets every 2 years (International Tunicate Meetings), contributes to the development and propagation of the urochordate model systems (21, 22). Many tools and approaches have been developed for the cosmopolitan species of reference *C. intestinalis*: a sequenced genome which is small and non-duplicated (about 160 Mb and 15,000 genes) (23), micro-manipulations and injection of synthetic mRNAs (24), introduction of plasmids by electroporation (25), gene silencing using morpholino oligo-nucleotides (26), the recent mastering of culture, transgenesis as well as successes with RNAi approaches (27–31), and excellent databases (*see* **Note 1**). Some of these tools are becoming available for the other ascidian model species which present advantages complementary to those

of *C. intestinalis*. Eggs and embryos of *P. mammillata* are remarkably transparent and are well suited for live imaging and early expression of exogenous mRNA (32–35). Cell lineages and morphogenetic events can be easily observed from egg to tadpole using specific labelling methods, observation chambers and time-lapse imaging stations. The larger (280 μm) eggs and embryos of *H. roretzi* are the best suited for micro-manipulations (36). Finally *C. savignyi* is useful for comparison of gene regulatory sequences with *C. intestinalis* (37, 38). This diversity of available models is suited to evo-devo studies of genes and molecular and cellular mechanisms (19, 39).

There are several useful resources for those who may consider working with ascidians. Noriyuki Satoh's classical book "*Developmental Biology of Ascidians*" remains the reference (7). Recent advances in the ascidian field are presented in special issues (17), International Tunicate Meetings reports (32) or reviews (16, 40–42). The Tunicate portal web site regroups a large amount of information about leading laboratories and resources (*see* **Note 1**). Time tables of development, digitized representations, and videos of *C. intestinalis* embryos are available on the ANISEED and FABA sites (*see* **Note 1**). Videos of fertilization and development of *P. mammillata* can be downloaded from our BioMarCell and BioDev web sites (*see* **Note 1**). Concerning methods, a chapter by B.J. Swalla in *Methods in Cell Biology* gives a phylogenetic description of urochordates as well as basic methods of culture, fertilization, etc. (43) and a chapter by W. Smith in this issue of *Methods in Molecular Biology* covers genetic approaches in ascidians. A list of methods for labelling marine embryos can be found in the Center for Cell Dynamics (Friday Harbor, USA) web site (*see* **Note 1**). A recent Cold Sping Harbor protocole series also covers many aspects of experimentation with *Ciona*.

In this chapter, we present the basic protocols used at the Villefranche-sur-Mer marine station ("Observatoire Océanologique de Villefranche-sur-Mer") by members of the Developmental Biology research unit. Three groups in this department (McDougall, Sardet, Yasuo) work on ascidians as their main experimental models (*C. intestinalis* and *P. mammillata*). Here we detail most basic techniques of ascidian culture, embryology, and cell biology, except for in situ hybridization (*see* **Note 2**) which is described in a separate volume of *Methods in Molecular Biology* devoted to mRNA visualization (44).

2. Materials

2.1. Fertilization and Culture of Ascidian Embryos

1. Animals: ascidians are sessile marine animals easy to collect from docks. The ascidian *P. mammillata* (termed *Phallusia*,

Fig. 14.1a–c) can be obtained all year long on the Mediterranean and Atlantic coasts while *C. intestinalis* (termed *Ciona*, **Fig. 14.1d–f**) can be collected in many temperate regions of the world. Both species can be maintained in aquaria at appropriate temperatures (16–18°C for *Ciona*, 18–22°C for *Phallusia*). There are institutional suppliers such as the Ascidian Stock Center at UC Santa Barbara (*see* **Note 1**), USA, or the Station Biologique de Roscoff, France. *Ciona,* which has marked reproductive periods in the wild, has been successfully cultivated through several generations in Japan, USA and Europe (31, 45). *Phallusia* gives abundant gametes throughout the year but the quality of embryonic development is best in spring and fall. They can be kept gravid in aquaria for several months under constant light when fed artemia and micro-plankton.

2. Sea water (SW): natural SW is sterilized with a large volume 0.2 μm filter unit. Natural SW can be replaced by ASW and/or supplemented with TAPS buffer and/or BSA (see below).
3. Artificial sea water (ASW): 420 mM NaCl, 9 mM KCl, 10 mM $CaCl_2$, 24.5 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.15 mM $NaHCO_3$ and 10 mM Hepes buffer, pH 8.0. Sterilize with a 0.2 μm filter and add 0.05 g/L kanamycin sulphate. It can be stored at 4°C for several days. Note that *Ciona* development is sensitive to the SW quality and may be better with ASW.
4. TAPS buffer stock solution: 500 mM *N*-tris (hydroxymethyl)methyl-3-aminopropanesulphonic acid, pH 8.2. Store at room temperature (RT).
5. TAPS-SW, EDTA-SW and BSA-SW: although filtered natural SW or ASW works well, adding TAPS buffer at a concentration of 10 mM final or/and 1 mM EDTA can sometimes increase the quality of embryonic development for certain batches. Some batches of embryos can also be very sticky, in which case adding bovine serum albumin (BSA) at a concentration of 0.1% is helpful (rinse with SW before fixation).
6. 10× trypsin stock solution: 1% trypsin in SW and 100 mM TAPS, pH 8.2. Store at –20°C in 1 mL aliquots.
7. 1× Pronase/thioglycolate solution: 0.05% pronase and 1% thioglycolate in SW. Can be kept at 4°C and used for 1 week. For longer storage, aliquots of 20× stock solution (1% pronase, 20% thioglycolate) can be kept at –20°C.
8. GF (gelatin/formaldehyde): dissolve gelatin and paraformaldehyde each at a concentration of 0.1% in

distilled water by heating them under a fume hood at 60°C for 1 h. Store in 50 mL aliquots at 4°C.

9. Non-sticky coated dishes and glassware for *Phallusia*. To coat plastic or glass surfaces, wet with a thin layer of GF, dry, and rinse well in distilled water. After use, GF-coated dishes can be rinsed with tap water, stored at RT and re-used several times. Pasteur pipettes or glass capillaries should be similarly coated by passing GF through them a few times, allowed to dry and then rinsed.
10. Non-sticky coated dishes and glassware for *Ciona*: GF is not as effective at preventing sticking of *Ciona* eggs and embryos. It is therefore best to use agarose-coated dishes. Heat 1% agarose in SW and pour in dishes to make a thin (2 mm) layer, leave to cool and rinse in SW. For storage (up to a week), add ASW to agarose dishes and store wet at 4°C. New glassware should be soaked in tap water for 1–2 days (no detergent) to reduce stickiness. With use, pipettes and tubes become coated with egg debris which also prevents dechorionated eggs from sticking.
11. Micro-pipettes for handling eggs: a glass tube (outer diameter 5 mm; inner diameter 3 mm) is pulled under flame, so that one end becomes tapered to around 1 mm in diameter. To the large end, attach a rubber tube with its other end stapled (**Fig. 14.2b**). For pipetting very small volumes, pull a coated capillary tube (10–50 μL) under a flame to the desired diameter (just over the width of an egg) and attach it to an adaptor (Fisher Scientific 4,356 M) 30 cm long tube fitted with a mouth piece (suction by mouth pipette) or to a stapled tube as above (suction by hand pressure). Coated plastic tips for mechanical pipettes (200 μL yellow tip, small opening >200 μm diameter) may also be used.
12. GF-coated glass slides and cover-slips: apply thin layer of GF solution (about 50 μL for a slide, 20 μL for a cover-slip), dry then wash in distilled water.
13. Vaseline (local supermarket) and silicone grease (Dow Corning high vacuum grease): fill a syringe equipped with a plastic yellow tip with the grease.
14. Paper frame chamber for live imaging: this method is best for long-term observation and time-lapse acquisitions; it ensures excellent exchange of gasses (O_2/CO_2) and can even sustain development through metamorphosis (3–5 days after fertilization). Prepare small paper frames (18 mm × 18 mm) to fit under cover-slips (22 × 22, #1). Use lens cleaning paper or tissue paper which when wet will reach a thickness close to that of eggs and embryos: 120–150 μm. Deposit a paper frame on a coated slide and

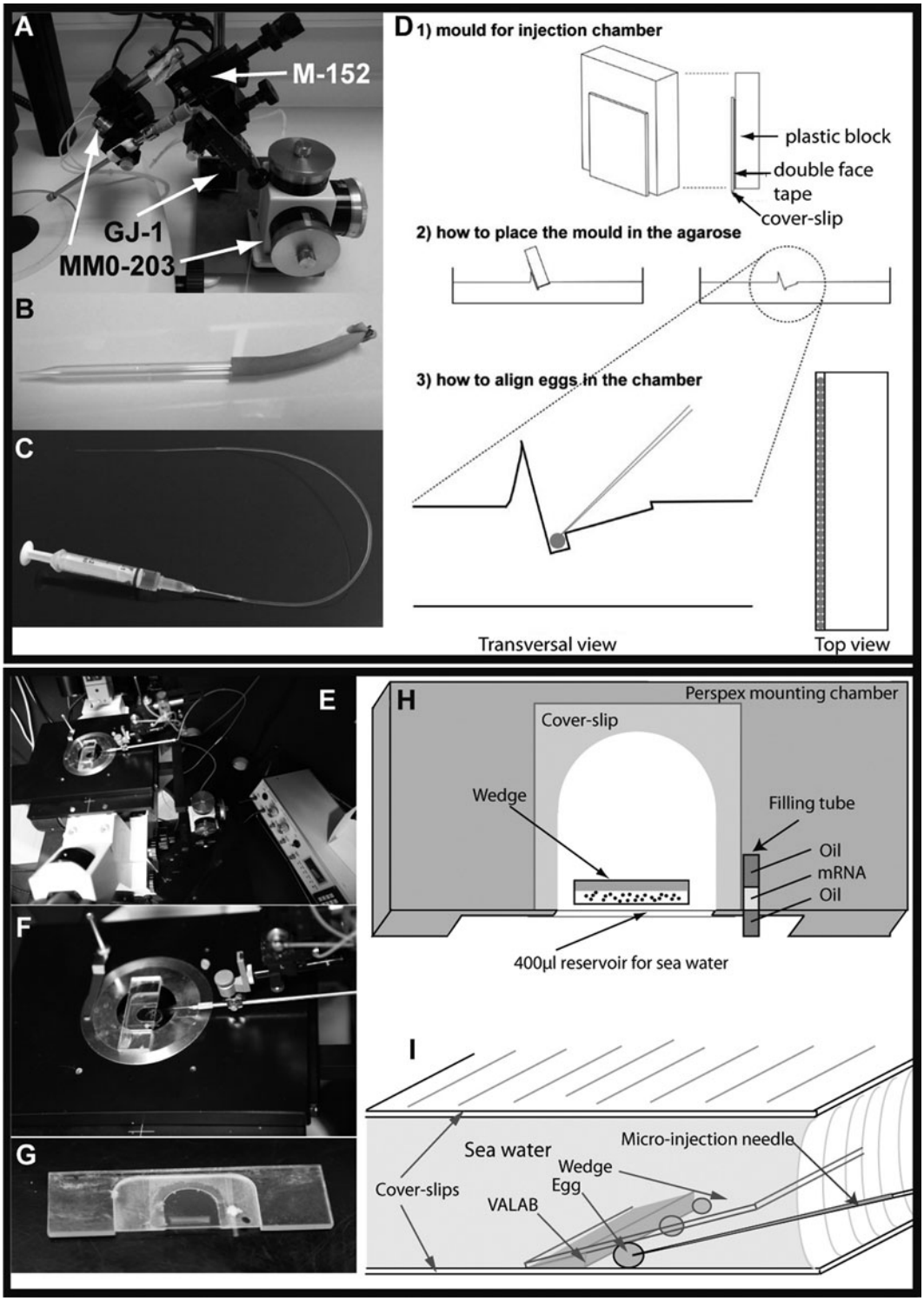

Fig. 14.2. Micro-injection setup. (**A–D**) Stereo-microscope setup/(**E–I**) inverted microscope setup. (**A**) Enlarged view of the manipulator assembly. The needle holder is connected to the glass syringe using the following Bio-Rad products: 1/16″ OD post-pump fittings and double Luer tubing adaptor. (**B**) Micro-pipette. (**C**) Mineral oil needle filler.

surround with a thin line of vaseline or silicone grease extruded from the syringe, drawn as a square (slightly smaller than the cover-slip). Deposit a tiny drop of SW with a few eggs or embryos in the centre of the frame. Pipette 30–50 μL SW onto paper frame. Cover with a coated cover-slip. Press delicately on the cover-slip to make sure there is a good seal all around and that eggs or embryos are just held in place in the micro-drop between coated slide and cover-slip. There should be an air space between the drop of SW and the moistened paper frame.

15. Vaseline/silicone chamber for live imaging: suitable for frequent or short-term observations (up to a few hours). Using the syringe, extrude two parallel lines of vaseline (or silicone) grease on a GF-coated slide. Place the embryos in a drop of SW (about 20 μL) on the slide. Cover with a GF-coated cover-slip, and gently flatten the grease lines with forceps until the embryo is slightly compressed such that it no longer moves when the cover-slip is tapped delicately. Fill the rest of the space between slide and cover-slip with SW placed on the side of the cover-slip, again checking that the embryos remain snug. This chamber can be perfused with solutions (for example, activated sperm to fertilize the eggs or a chemical inhibitor) or can be sealed with vaseline (or silicone) grease to prevent evaporation.

2.2. Injection of Eggs and Embryos

1. Set up on stereo-microscope/injecting from above: this is the most common way of injecting large numbers of eggs or embryos using a needle located above a line of eggs or embryos and approaching them at an angle (**Fig. 14.2a–d**). We use the following equipment: Leica S8APO stereo-microscope with Leica TL BFDF (brightfield–darkfield transmitted light base), Narishige MMO-203, three-axis oil hydraulic micro-manipulator, Narishige M-152 manipulator, Narishige GJ-1 magnetic stand, Narishige IMH1 injection holder assembly, glass syringe with a male Luer-Lock connection fitting, iron plate. For making injection chamber:

Fig. 14.2. (continued) A hand-pulled capillary is connected, via a Teflon tube, to a needle attached to a 2 mL plastic syringe. (**D**) Agarose injection chamber. Schematics showing: (**1**) how to make a mould for injection chamber; (**2**) how to place the mould; (**3**) how to align ascidian eggs in the chamber. (**E**) The wedge injection chamber mounted on the stage of an inverted IX70 Olympus microscope with micro-injection needle, three-way micro-manipulator and injection box shown. (**F**) Close up of the wedge injection chamber with micro-injection needle horizontal to the stage. (**G**) Close up of the wedge injection chamber with the wedge and filling tube visible. (**H**) Schematic showing the wedge injection chamber with the wedge and filling tube highlighted. In this example mRNA has been loaded into the filling tube. (**I**) Close up of the injection showing the needle filled with injection solution being inserted into an egg held in place in the wedge.

1.5% agarose in SW, plastic Petri dishes (5 cm diameter), cover-slip (#1), plastic block (about 15 mm × 15 mm × 5 mm), double face tape.

2. Setup on inverted microscope/horizontal "wedge" method: detailed articles have been published on this method (46) (**Fig. 14.2e–i**). We use the following equipment: an inverted Olympus IX70 microscope with stage control on the left to free the right hand for the micro-manipulator, MHO-103 three-axis oil micro-manipulator (Narishige, now the MMO series), type-A stage-side mounting system (mounted on the right-hand side) with a NR adaptor pillar, H1-7 type injection needle holder (IMH1 set with Teflon tubing and connector) connected to a B-8B ball joint connector, a silent air compressor (local supermarket) connected to a Narishige IM300 (we use about 60–70 psi air pressure from the compressor). Plexiglass stage mounting chambers are made by our workshop. For making wedge injection chamber, use GF-coated cover-slips (#1, 22 mm × 22 mm).
3. Morpholinos are purchased from Gene Tools. They are resuspended at 2 mM in distilled water and stored in aliquots at –80°C (note that the manufacturer recently recommended storing morpholino at RT).
4. Synthetic mRNAs are prepared as concentrated solutions (1–2 μg/μL) in distilled water and small aliquots are frozen at –80°C. We routinely use the vectors pRN3 (47) or its derivative pSPE3 (48) to synthesize mRNA for microinjection. For pRN3, the ORF of the gene of interest is PCR amplified such that it can be cloned directionally into the multi-cloning sequence at BglII, EcoRI and NotI restriction sites. For pSPE3, the ORF of interest is first cloned into a Gateway entry vector (pENTR/D-TOPO) and then inserted into the destination vector using recombination cloning technology (Invitrogen). Both pRN3 and pSPE3 constructs are linearized with SfiI restriction enzyme and used as a template for in vitro mRNA synthesis using T3 mMESSAGE MACHINE kit (Ambion). The vector pCS2+ (49) can also be used for mRNA synthesis, but the resulting mRNAs must be additionally polyadenylated using a Poly (A) Tailing Kit (Ambion) for efficient translation in ascidian embryos.
5. Fast Green (Sigma-Aldrich): 1 mg/mL in distilled water (2× stock).
6. Fluorescent dextrans, 10 kDa molecular weight (Texas-Red-coupled, fluorescein-coupled, rhodamine-coupled; Molecular Probes): 2 mM in distilled water (2× stock).

2.3. In Vivo Labelling of Eggs and Embryos

1. DiO-C2(3) (Molecular Probes): a 1,000× stock solution is made at 5 mg/mL in ethanol and kept at 4°C. Observe with fluorescein filter set.
2. MitoTracker (Molecular Probes). We have successfully used MitoTracker Red FM, MitoTracker Green FM and MitoTracker Red CM-H2XRos. For each of these, a 1,000× stock solution is made at 1 mM in DMSO and stored at –20°C.
3. Rhodamine 123 (Molecular Probes): a 1,000× stock solution is made at 1 mg/mL in ethanol and kept at 4°C. Observe with fluorescein filter set.
4. TMRM and TMRE (Molecular Probes): a 1,000× stock solution is made at 1 mM in ethanol and kept at 4°C. Observe with rhodamine filter set.
5. Hoechst 33342: powder is dissolved in distilled water at a concentration of 10 mg/mL and stored in aliquots at –20°C. An aliquot is diluted 1/50 (200 μg/mL) in distilled water and stored for several months at 4°C as a 400× stock solution for in vivo labelling of chromosomes. Observe with UV filter set.
6. Calcium-Green dextran, 10 kDa molecular weight (Molecular Probes): dissolve in injection buffer (180 mM KCl, 100 μM EGTA, 30 mM BES buffer, pH 7.1) at a concentration of 10 mM. Observe with fluorescein filter set.
7. Fura-2 dextran, 10 kDa molecular weight (Molecular Probes): dissolve as for Calcium-Green dextran. Observe with Fura-2 filter set (an excitation filter wheel is required).
8. DiI-C16(3) (1,1′-dioctadecyl-3,3,3;3′-tetramethylindocarbocyanine perchlorate; Molecular Probes). For injection and endoplasmic reticulum (ER) labelling, a saturated solution of DiI is made by mixing several crystals of DiI in 100 μL of soybean oil (Wesson oil). For plasma membrane labelling, make a 2.5 mg/mL stock solution in ethanol and store at –20°C. Observe with rhodamine filter set.
9. Succinylated concanavalin A, Alexa488 conjugate (Molecular Probes): a 1,000× stock solution is made at 5 mg/mL in 0.1 M sodium bicarbonate pH 8.3, aliquoted and stored at –20°C. Observe with fluorescein filter set.
10. FM 4-64 lipophilic dye (Molecular Probes): a stock solution is made at 10 mg/mL in DMSO, aliquoted and stored at –20°C. Observe with rhodamine filter set.
11. FITC: fluorescein isothiocyanate (Molecular Probes). Make 100× stock solution by dissolving FITC in DMSO at 10 mg/mL. Store at –20°C. Observe with fluorescein filter set.

12. Syto12 (Molecular Probes): 5 mM solution in DMSO. Store at –20°C. Observe with fluorescein filter set.
13. Nile Blue powder (Merck).

2.4. Blastomere Isolations and Ablations

1. Glass needle/knife: the tapered part of a Pasteur pipette is pulled under flame to create a very thin string of glass, which should be like a wool fibre. When first using a new glass embryo knife it is necessary to coat the knife to prevent sticking. This is done by smashing an egg or embryo and pulling the knife back and forth through the cellular debris.
2. 1.5% agarose-coated Petri dishes: make them like 1% agarose dishes (**Section 2.1**).
3. EMC (or calcium-free artificial sea water): 480 mM NaCl, 9.4 mM KCl and 23.6 mM EGTA. Equilibrate pH to 8.0 with NaOH, autoclave and store at RT.

2.5. Fixing and Immuno-labelling Eggs and Embryos

1. PBS solution: make a standard 10× PBS solution (1.37 M NaCl, 26.8 mM KCl, 100 mM Na_2HPO_4, 17.6 mM KH_2PO_4, pH 7.5), autoclave and store at RT. Dilute in distilled water to prepare 50 mL of 1× PBS solution (termed PBS) which can be stored for use at 4°C for 1–3 days.
2. 20% formaldehyde stock solution: dissolve 10 g of paraformaldehyde powder in 50 mL of distilled water and add 80 µL of 10 N NaOH. Keep at 50°C overnight (ON) and mix until completely dissolved. Pass through a 0.4 µm filter and store in aliquots at –20°C. To thaw, aliquots must be warmed at 50°C for 1 h. For convenience, formaldehyde solution can also be purchased commercially (32% stock solution without methanol, Electron Microscopy Sciences).
3. Formaldehyde fixative: dilute the formaldehyde stock solution to 4% in PBS, add NaCl to 0.5 M. Store at 4°C for 1 month maximum in 1.3 mL aliquots in screw cap tubes; for longer storage, keep at –20°C.
4. Methanol fixative: 90% methanol and 50 mM EGTA, pH 7.5. Store at –20°C in 1.3 mL aliquots in screw cap tubes.
5. PBS-Tween solution (PBS-Tw): add Tween20 to PBS at a final concentration of 0.1%. Store at 4°C for 1–3 days.
6. PBS-Triton solution (PBS-Tr): add TritonX100 to PBS at a final concentration of 0.1%. Store at 4°C for 1–3 days.
7. PBS-Tw-BSA: add Tween20 and BSA at final concentrations of 0.1 and 0.5%, respectively, in PBS.
8. PBS-BSA: dissolve BSA in PBS at a final concentration of 1%. Store at –20°C in aliquots.
9. Primary antibodies: antibodies are aliquoted and stored at –80°C. Once thawed, aliquots can be kept at 4°C for

months (*see* **Note 3**). In the example given in **Sections 3.5** and **3.6**, we use an antibody which labels aPKC (rabbit polyclonal sc216 from Santa Cruz Biotechnology) at a working dilution ranging from 1/100 (with fluorescently coupled secondary antibody or biotin/streptavidin amplification) to 1/500 (with TSA method), the NN18 antibody (mouse monoclonal N5264 from Sigma-Aldrich, *see* **Note 4**) which labels mitochondria at a working dilution of 1/400 and anti-tubulin antibody YL1/2 (rat monoclonal ab6160 from Abcam) at a working dilution of 1/500.

10. Secondary antibodies: fluorescently labelled secondary antibodies raised in goat against rabbit, mouse and rat immunoglobulin are purchased from Jackson Immuno Research laboratories. Antibodies against rabbit are pre-absorbed by the manufacturer against human serum proteins, whereas antibodies against mouse and rat are also pre-absorbed against related species (rat for antibody against mouse and inversely). All antibodies are reconstituted at the concentration recommended by the manufacturer. We add an equal volume of pure glycerol for cryo-protection and store the antibodies as small aliquots at –80°C. Once thawed, working aliquots can be kept at 4°C for several months (*see* **Note 3**). In the examples provided in the method (**Sections 3.5** and **3.6**) we use a Cy5-conjugated goat anti-mouse, and rhodamine or Cy5-conjugated goat anti-rat, each at a 1/100 dilution.
11. Biotin-conjugated antibodies and fluorescent streptavidin: purchased from Jackson Immuno Research laboratories and handled like fluorescently labelled secondary antibodies (see above). In the example provided in **Sections 3.5** and **3.6**, we used a biotin-conjugated goat anti-rabbit at 1/200 dilution and a fluorescein-conjugated streptavidin at 1/100 dilution.
12. HRP-coupled antibodies and TSA kits (Molecular Probes). In the example provided in **Section 3.5**, we use an HRP-conjugated goat anti-rabbit at 1/100 dilution. We recommend the Alexa488-TSA kit, which gives very strong green fluorescence labelling without background (for TSA kits using other fluorophores or haptens, *see* **Note 5**).
13. Fluorescent phalloidins (Sigma-Aldrich or Molecular Probes): reconstituted at a concentration of 50 μg/mL (approx. 35 μM, 1 unit/μL) in DMSO (*see* **Note 6**) and stored in aliquots at –20°C. If the fluorescent phalloidin is provided in methanol, the methanol must first be eliminated by evaporation (keep vial opened for a few days at RT in a dark chamber) before reconstituting in DMSO.

Once thawed, use phalloidin at a dilution of 1/100 and store the working aliquot at 4°C. In the examples provided (**Sections 3.5** and **3.6**), we used rhodamine–phalloidin.

14. Hoechst 33342: Use 200 μg/mL working aliquot as a 100× stock solution for chromosome labelling on fixed samples (**Section 2.3**).
15. Citifluor AF1 antifade mounting medium (Electron Microscopy Sciences). Store at –20°C in aliquots. Once thawed, store the working aliquot at 4°C.

2.6. Isolated Cortices: Preparation, Labelling and Imaging

1. Glass cover-slips (18 mm × 18 mm, #1) successively cleaned with 10% Tween20, distilled water and ethanol. Keep them dry on parafilm in closed Petri dishes.
2. EMC (**Section 2.4**).
3. Buffer X: 350 mM K-aspartate, 130 mM taurine, 170 mM betaine, 50 mM glycine, 19 mM $MgCl_2$ and 10 mM Hepes buffer. Equilibrate pH at 7.0 with KOH. Sterilize with 0.2 μm filter and store at –20°C in 10 mL aliquots.
4. CIM solution: 800 mM glucose, 100 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA and 10 mM MOPS buffer. Equilibrate pH at 7.0 with KOH. Sterilize with 0.2 μm filter and store at –20°C in 10 mL aliquots.
5. CIM fixative: add 3.7% formaldehyde (without methanol trace, **Section 2.5**) and 0.1% glutaraldehyde to CIM solution just before use.
6. CM-DiI-C16(3) ("Fixable DiI"; Molecular Probes). Add 20 μL ethanol to a vial of fixable DiI. This 2.5 mg/mL stock solution can be stored at 4°C for several days. Observe with a rhodamine filter set.
7. DiO-C6(3) (Molecular Probes). This dye labels all membranes including the ER network, the plasma membrane, and occasional vesicles and mitochondria in preparations of living or fixed isolated cortices. A stock solution at 0.2 mg/mL in ethanol is stable and can be stored at 4°C for several years. Observe with a fluorescein filter set.

3. Methods

3.1. Fertilization and Culture of Ascidian Embryos

3.1.1. Obtaining Gametes

1. Dissect the hermaphroditic animals by cutting through their tunics between the siphons and peel away the tunic (50).
2. Pierce the overlying oviduct and collect the eggs using plastic or glass pipettes, massaging the oviduct to empty all the eggs. Deposit the eggs in SW and store them at the appropriate temperature (16°C for *Ciona* and 18°C for *Phallusia*).

3. Then collect sperm from the underlying spermiduct using a pipette or a needle and syringe. Concentrated sperm can be stored several days at 4°C in small plastic tubes. *Phallusia* eggs can be efficiently fertilized by sperm from the same individual (therefore one should take care not to contaminate eggs with sperm while collecting gametes), whereas self-fertilization is inefficient in *Ciona*, so it is necessary to open at least two animals.
4. It is also possible to obtain immature oocytes from ovary tissue in order to study oocyte maturation and polarity. We have analysed this process for *Ciona* (50).

3.1.2. Egg Dechorionation

Ascidian eggs are surrounded by a chorion layer containing test cells and follicle cells (**Fig. 14.1b**, **e**) which should be removed for observation and subsequent experimental procedures (unless one wants to observe tadpoles, which emerge from the chorion by hatching). After dechorionation (**Fig. 14.1c**, **f**), eggs and embryos tend to stick, so they should be handled gently and transferred with large diameter plastic pipettes or coated glass pipettes into dishes which are specially coated. For transferring a small number of eggs or embryos, we use hand-held micro-pipettes.

1. For *Phallusia*: add 1 mL 10× trypsin stock solution to 9 mL chorionated eggs in SW, add 250 µL of 500 mM TAPS buffer and shake or rotate gently (20 rpm) for 2–4 h at 18–22°C (51). Eggs can also be treated briefly with pronase/thioglycolate (as for *Ciona*) if test cells are not completely removed.

   When most eggs have a smooth surface (**Fig. 14.1c**) and settle to the bottom of the dish, wash several times with SW by swirling eggs to the centre of the dish with a gentle wrist motion. The chorion debris, dead cells and chorionated eggs will float and can be removed with vacuum aspiration.

   Transfer dechorionated eggs to a GF-coated Petri dishes (5 or 10 cm diameter) at a low density, so that they are not touching.

   Eggs can be kept at 18°C for several hours or up to a day, awaiting fertilization, injection or fixation.
2. For *Ciona*: add 250 µL of 1 N NaOH to 10 mL of 1× pronase/thioglycolate solution and mix well. Then add 250 µL more of 1 N NaOH followed by mixing (pH will rise to around 10). Adding NaOH creates white precipitates which should vanish once the solution is mixed.

   Transfer chorionated eggs (about 5 mL) to a 15 mL glass test tube (with use these tubes become coated with debris which prevents sticking, so we simply rinse in tap water—no detergent—and re-use the same glass tubes).

Add 1 mL of the basic pronase/thioglycolate solution to the glass tube containing chorionated eggs. Mix well by gentle pipetting. Wait 5 min or so until eggs sediment to the bottom of the tube. Remove as much supernatant as possible.

Add remaining volume of the basic pronase/thioglycolate solution to the packed chorionated eggs and mix with gentle pipetting. Within 2–3 min, chorions should start to dissolve. Check occasionally under a stereo-microscope, and once the majority (2/3) eggs lose their chorion (**Fig. 14.1f**), gently add SW to fill the glass tube.

Wait for dechorionated eggs to settle to the bottom (dechorionated eggs sediment quickly) and remove supernatant. Gently add fresh SW and repeat the washing procedure three times.

Transfer eggs to agarose-coated Petri dishes (5 or 10 cm diameter) and place at 16°C.

Eggs can be kept for several hours or up to a day, awaiting fertilization, injection or fixation.

3.1.3. Sperm Activation

If one wishes to fertilize eggs rapidly and synchronously, it is best to first activate sperm and test the different sperm batches individually for their ability to fertilize (52). If synchrony is not important, one can simply add concentrated sperm to eggs in SW at a dilution of 1/1,000 and most eggs will become fertilized over a period of 10–30 min.

1. Activation by exposure to chorionated eggs: add 25 μL of concentrated sperm to 5 mL SW containing 100 μL chorionated eggs; shake gently (20 rpm) for 15–60 min. Let chorionated eggs sediment to the bottom of the tube; the activated sperm suspension can be kept at 4°C and used to fertilize for several hours.
2. Activation by alkaline SW (with NaOH): dilute 10 μL of concentrated sperm in 2 mL of SW and then add 4–12 μL of 1 N NaOH. Wait 1 min and add the activated sperm to eggs. You have to find the right concentration of NaOH (usually 8 μL work well). This activation works very well with *Phallusia* sperm but should be done each time, just before fertilization.
3. Activation by alkaline SW (with Tris–HCl buffer): this is the preferred method for *Ciona*. Add 25 μL of 1 M Tris–HCl buffer (pH 9.5) to 0.5 mL of SW. To this alkaline SW, add 2.5 μL of concentrated sperm; mix and wait 1 min before fertilizing.

3.1.4. Fertilization and Culture of Embryos

Similar methods are used to fertilize *Phallusia* and *Ciona* eggs.

1. For *Phallusia*: to fertilize eggs in a synchronous manner, add 2 mL of activated sperm to 10 mL of dechorionated eggs in SW. Mix well. You can judge if synchronous fertilization has been achieved by observing egg shape change: 80% of the eggs should lose their roundness and become pear shaped within 5 min of sperm addition.

   Once eggs are fertilized, wash extensively with SW and culture the embryos at 18–22°C, either spread in a monolayer (but not touching) in appropriately sized coated Petri dishes or keep as a suspension in a GF-coated beaker equipped with a paddle rotating at 50 rpm. For *Phallusia* maintained around 20°C, first cleavage takes place about 50 min after fertilization, gastrulation after 3 h and the embryo develops into a tadpole in 12 h. To see fertilization and development of *Phallusia* consult the BioMarCell film archive (*see* **Note 1**).

2. For *Ciona*: to fertilize eggs in a synchronous manner, add 500 μL of sperm activated with alkaline SW to 10 mL of dechorionated eggs in SW. Mix well. With an appropriate stereo-microscope, one can see active sperm moving around eggs. Eggs start to spin and, when fertilized, become pear shaped.

   After 10 min incubation, transfer eggs to another agarose-coated Petri dish containing SW and culture them at 16–18°C. Information on *Ciona* embryonic stages, morphology and cell lineages can be found on FABA and ANISEED databases (*see* **Note 1**).

3.1.5. Mounting Live Embryos for Imaging

Live ascidian eggs and embryos can be placed in glass bottom dishes (MatTek corporation) first coated with GF or cellular debris for observation with an inverted microscope. For improved optics on inverted or upright microscopes, we routinely mount live *Phallusia* eggs and embryos on coated slides using two types of observation chambers (paper frame chamber or vaseline chamber, **Section 2.1**). Live *Ciona* eggs and embryos can be similarly imaged for brief periods on GF-coated slides or on slides which have been licked to make them less sticky. During the time when slides are not being imaged, they should be kept in humid chambers made by placing wet paper towels inside appropriately sized plastic boxes or Petri dishes.

3.2. Injection of Eggs and Embryos

Two different set ups are routinely used in our laboratory and are somewhat complementary being more or less convenient for functional or imaging studies. One is based on a stereo-microscope with the needle at an angle, while the other is based on an inverted microscope with the needle horizontal (**Fig. 14.2**).

3.2.1. Injection Using the Stereo-microscope Setup

This injection method (**Fig. 14.2a–d**) is suitable for injecting large numbers of eggs and embryos, for instance, when they are to be used for functional studies followed by in situ hybridization or immuno-labelling.

1. Making the agarose injection chamber: a mould has to be prepared, a cover-slip (#1, thickness similar to the diameter of *Ciona* eggs: 120 μm) is stuck to a plastic block of about 15 mm × 15 mm × 5 mm on one of the largest surfaces using double face tape. One end of the cover-slip should protrude, ideally about 150 μm, from an edge of the plastic block. Carefully bring 1.5% agarose-containing SW just to a boil in a microwave oven. Pour the agarose SW into a plastic Petri dish (5 cm diameter) and float the Petri dish on ice-filled water.

   Place the mould in the centre of the dish as shown in **Fig. 14.2d**. Make sure that the cover glass does not touch the bottom of the dish. Let the agarose harden for about 2 min. Carefully pull out the mould and cover the agarose with SW.

   The agarose injection chamber can be stored at 4°C and be re-used for several injections.

   Using a micro-pipette, align dechorionated eggs in the well made with the protruding edge of the cover-slip of the mould.

2. Loading the needle: the injection holder assembly is connected to a glass syringe, via Teflon tubing which is filled with mineral oil (make sure that there are no air bubbles in any parts of this assembly).

   Needles are made from glass capillaries containing a thin glass filament (Harvard Apparatus GC100TF-10) using a needle puller (we use a Narishige PN-30).

   Needles are backfilled with about 0.5 μL of the solution to inject (morpholino, mRNA, etc.) by depositing the injection solution on the open large end of the needle, so that the solution will be transferred to the tip of needle along the filament by capillary action. Gently tap the needle while holding it upright to remove most of the air bubbles from the injection solution (injections solutions may be coloured using Fast Green).

   Once the injection solution is transferred to the tip of the needle, the needle should be filled completely with mineral oil using a hand-pulled capillary attached by tubing to a syringe (**Fig. 14.2c**).

   When the needle is inserted into the needle holder, special care should be taken not to introduce air bubbles. Apart from tiny air bubbles found in the injection solution, there should be no air bubbles in mineral oil from the needle to

the glass syringe. It is also important that the barrel and piston parts of the glass syringe move very smoothly.

3. To start injecting, proceed in the following way: break the tip of the needle by bringing it very slowly to a piece of cover-slip placed at a slant in the injection chamber. This is a critical step for successful injection. Make the opening of the tip as tiny as possible. The syringe piston should be pressed gently while the needle is brought towards the cover slip. This allows one to visualise when the needle is broken as small amounts of the coloured solution (Fast Green) in the injection needle can be seen leaking into the chamber.

   Place the tip of needle at the centre of egg and then apply brief suction by pulling on the syringe piston, so that a "hole" is made in the plasma membrane.

   Press the piston gently to deliver injection solution into the egg.

3.2.2. Injection Using the Inverted Microscope

We use this method (**Fig. 14.2e–i**) for mRNA injection into *Phallusia* eggs since it cuts down on the cost because 1 μL of mRNA can be used repeatedly for approx. 1 month (stored at 4°C in filling tubes, **Fig. 14.2e**). This method is also convenient for imaging live eggs and embryos under a light or confocal microscope while they are being injected or soon after (for example, to image calcium signals).

1. Making a wedge injection chamber: cut coated cover-slips into small pieces (approx. 4 mm ×10 mm) and attach to whole coated cover-slips with VALAB (composed of 1:1:1 vaseline, lanolin and bees wax) and a spacer to elevate one side of the wedge. The spacer is a cut piece of cover-slip (**Fig. 14.2h, i**).

   Heat on a hot plate until VALAB melts.

   Remove from hot plate and allow VALAB to cool (approx. 30 s or when it turns opaque).

   Carefully remove spacer with forceps (we keep spacers and re-use them); this creates the wedge, a slanted coated-glass sandwich.

   Attach the wedge to a plexiglass mounting chambers using silicone grease to seal on three sides and add dechorionated eggs or embryos using a mouth pipette (up to 100 eggs/embryos per wedge).

   Attach a second cover-slip to sandwich the wedge and fill the reservoir with approx. 400 μL SW (**Fig. 14.2h, i**) (*see* **Note 7** for alternative wedge).

2. Loading the needle: first, make a filling tube to hold the injection solution. Cut glass capillaries into pieces approx. 1 cm long. To one end add 0.5 μL mineral oil, then 0.5 μL injection solution and again 0.5 μL mineral oil.

The filling tube is fixed to the underside of the wedge using VALAB (**Fig. 14.2h**).

Needles are made from glass capillaries that do not contain a thin glass filament (Harvard Apparatus GC100T-10) using a needle puller (we use a Narishige PN-30).

The needle is inserted into the needle holder which is connected to an IM300 injection box via Teflon tubing and advanced towards the filling tube.

The needle is first broken carefully against the filling tube then front-filled with about 1 nL of injection solution (Morpholino, mRNA or fluorescent indicator) from a filling tube (**Fig. 14.2h**) by brief suction ("fill" function, approx. 30 psi). Needles that fill too fast are discarded and those that fill too slowly are broken again.

3. Injection: once the needle is filled with injection solution the balance pressure is adjusted, so that the meniscus moves slightly out towards the pipette tip.

   Needles are inserted into the centre of the egg (**Fig. 14.2i**) and suction is applied (Narishige "fill" function) to break the plasma membrane.

   Eggs are injected at approx. 5–10 psi air pressure using the foot pedal attached to the IM300.

   Filling tubes containing injection solutions can be stored at 4°C and re-used for several weeks.

3.2.3. Gene Knockdown with Morpholino

An aliquot of the morpholino to be injected is heated at 65°C for 10 min and made to the desired concentration with distilled water. A range (0.25–1.0 mM pipette concentration) is first injected to determine which concentration is best suited for the experiment. Prior to injection, the solution is spun in a micro-centrifuge at maximum speed for 5 min to sediment particles that might block the injection needle.

3.2.4. Injection of mRNAs and Plasmid DNA

Synthetic mRNAs are prepared as concentrated solutions (1–2 μg/μL) in distilled water and small aliquots are frozen at –80°C. Solutions are centrifuged in a micro-centrifuge at maximum speed for 5 min prior to loading the needle or filling tube, in order to sediment particles that might block the injection needle. For injection, different concentrations are tested after dilution in distilled water, ranging from 0.25 to 2.0 μg/μL in the pipette; generally using the lower concentrations for functional studies and the higher concentrations for visualization of fluorescent fusion proteins (**Fig. 14.1l**, **m**). For *Phallusia*, fluorescence from fluorescent protein constructs can be observed in an unfertilized egg a few hours after injection of concentrated mRNAs. Expression can be detected more rapidly in eggs that are fertilized, and fluorescent proteins continue to accumulate in dividing embryos.

For *Ciona*, unfertilized eggs do not translate exogenous mRNAs, but expression of fluorescent protein constructs can be observed few hours after fertilization (gastrula stage embryo) (35). Plasmid DNA is treated in a similar fashion for micro-injection, but at a lower concentration (generally 50 ng/μL).

3.2.5. Quantitation of Injection

The final concentration of the injected solution in the egg will be 1–10% of the pipette concentration depending on the injected volume. Injection volume is estimated by the diameter of clearing in the cytoplasm upon injection; this cleared space can be rendered more visible by the addition of an equal volume of Fast Green (0.5 mg/mL final) or fluorescent dextran (1 mM final) to the morpholino or mRNA injection solution. Centrifugation to clear the injection solution should be performed after the addition of these dyes. Typically, eggs are injected until 1/4 diameter of egg is filled with the coloured solution. The injected amount can be further quantified from the intensity of signal in the egg using a fluorescence microscope (for a precise method to quantify the amount of protein expressed from injected mRNA, *see* **Note 8**).

3.3. In Vivo Labelling of Eggs and Embryos

Dechorionated eggs of *Phallusia* can be labelled with vital fluorescent dyes to study the distribution of many organelles as well as to observe surface, cortical and cytoplasmic reorganizations (*see* **Note 9**). Dyes for mitochondria, chromosomes, plasma membrane and yolk platelets are cell permeable, whereas labelling with probes for calcium and ER requires injection.

3.3.1. Mitochondria

Incubate dechorionated eggs or embryos for 15–20 min in SW containing a 1/1,000 dilution of the stock solution of any one of the following mitochondrial dyes (**Section 2.3**): DiOC2(3), Mitotracker, TMRM, TMRE, Rhodamine123 (51, 53) (**Fig. 14.1i**, **j**). Wash once with SW before observation with the appropriate filter set.

3.3.2. Chromosomes

Incubate eggs or embryos in 0.5 μg/mL Hoechst 33342 in SW for 15 min and wash twice before observation (*see* **Note 10**). Alternatively the less soluble Hoechst 33258 (make a stock solution as for Hoechst 33342) can be injected to avoid background labelling of sperm if they cannot be washed away in time.

3.3.3. Plasma Membrane

Four different approaches can be used to label plasma membrane.

1. DiI-C16(3): add 1 μL DiI Ethanol stock solution to 1 mL SW. Pull the solution in a syringe and run through a small gauge needle several times to create micelles. Add to an equal volume of eggs or embryos suspended in SW. Incubate 2–5 min, during which some DiI micelles will fuse with the plasma membranes. Wash by transferring eggs or embryos

to a large volume of SW. Mount and observe soon after labelling.

2. FM 4-64: dilute FM 4-64 stock solution to 20 μg/mL in SW and mix this working solution 1:1 with the live embryos just prior to observation in a dish or directly on the GF-coated slide (35). Washing is not necessary (*see* **Note 11**).
3. FITC: under alkaline conditions, FITC binds to the plasma membrane and does not penetrate the egg. Incubate eggs in FITC SW pH 10 (add 10 μL FITC stock to 990 μL SW to make 0.1 mg/mL, add 50 μL NaOH 1 N) for 20 min. Wash with SW before observation.
4. Succinylated concanavalin A: centrifuge the stock solution before use to pellet insoluble particles. Add to eggs in SW at 5 μg/mL final concentration and incubate for 15 min, then wash twice with SW. Other lectins can be used (33) (*see* **Note 12**).

3.3.4. Yolk Platelets

Incubate dechorionated eggs with 1 μM Syto12 in SW for 20 min. Wash once with SW (34).

3.3.5. Analysis of Surface Movements

Place eggs or embryos in a coated Petri dish containing finely ground Nile Blue particles in SW. Particles of chalk or charcoal or a 1 μM suspension of fluospheres (Molecular Probes) can also be used (54, 55). Depending on the density of particles you want to attach on the surface, either let eggs fall on particles for a low density or gather eggs and particles to the centre of the dish using a gentle swirling motion if you want many surface particles attached. Wash several times with SW and select embryos with the desired number and position of attached particles for observation.

3.3.6. Endoplasmic Reticulum

Prepare a saturated solution of DiI-C16(3) in Wesson cooking oil (**Section 2.3**). Micro-inject a small oil droplet into the egg or embryo. Allow the dye to diffuse in the tubes and sheets of the continuous ER network for 15–30 min prior to observation (33, 56) (**Fig. 14.1i**, **j**).

3.3.7. Calcium

Calcium-sensitive dyes (34, 57) (*see* **Note 13**) that are cell permeable do not function in *Phallusia* eggs, so the free acid forms must be injected. It is best to use dextran-coupled dyes because they remain cytoplasmic unlike non-coupled dyes which enter large organelles such as yolk vesicles. Inject an amount of Calcium-Green dextran or Fura-2 dextran equivalent to approximately 0.1–0.2% egg volume to give final concentration in the egg of 10–20 μM (**Fig. 14.1k**). Wait 30 min for diffusion of the dye before imaging. Calcium signals traverse the eggs within 10–20 s and images must be acquired every few seconds in order to observe wave-front propagation.

3.3.8. Mounting Live Embryos for Imaging

See chambers for live imaging in **Sections 2.1** and **3.1**.

3.4. Blastomere Isolations and Ablations

Thanks to the stereotyped cell division pattern of ascidian embryos, it is possible to isolate or ablate a blastomere with the certainty of its identity (**Fig. 14.1r**). We find it possible to isolate individual blastomeres up to the 32 cell stage for embryos of *Ciona* and *Phallusia*.

3.4.1. Blastomere Isolations by Cutting

Isolation is carried out on a 1.5% agarose-coated Petri dish. Place an embryo at the centre of dish and identify the blastomere of interest. Place a glass knife between the blastomere of interest and a neighbouring blastomere and then press the needle to separate them. By repeating the procedure on isolated portions of embryos, a single blastomere can be isolated. One can also perform blastomere isolation on embryos mounted in a wedge as described above for **Section 3.2**, using the same glass capillary needles and hydraulic micro-manipulator as for micro-injection.

3.4.2. Embryo Dissociation Using Calcium-Free Sea Water

Rear embryos in SW as explained in **Section 3.1**. At the desired stage, pipette a few embryos into a large volume of calcium-free sea water (EMC). Transfer them a second time into fresh EMC to ensure elimination of calcium. Using a fine-coated glass capillary (**Sections 2.1**, steps 9 and 10), pipette the embryos roughly up and down until blastomeres separate. Return isolated blastomeres to SW for further divisions and observation. Embryos chosen for dissociation should be early in the division cycle (prior to nuclear envelope breakdown), since towards mitosis the lateral connections between blastomeres become tightly sealed up.

3.4.3. Blastomere Ablations

Ablation is carried out on a 1.5% agarose-coated dish in a hole made with a tungsten needle. The hole should be only slightly bigger than the ascidian embryo. Place an embryo in the hole made in the agarose. Using a stream of SW from micro-pipette, rotate the embryo, so that the blastomere of interest faces you. Ablation of a blastomere is achieved by injecting water into the blastomere of interest until it bursts. With our stereo-microscope setup, it is possible to ablate individual blastomeres from embryos up to the 64 cell stage.

3.5. Fixing and Immuno-labelling Eggs and Embryos

Fixation and permeabilization procedures followed by fluorescent labelling using antibodies can be carried out on large populations of ascidian eggs and embryos or a small number of injected eggs and embryos in order to analyse the distribution of macromolecular structures. Usually, the localization of the cytoskeleton, organelles, proteins and mRNAs can be best imaged using a confocal microscope (33, 58, 59). Use of different primary

antibodies made in rabbit, mouse and rat allows one to distinguish several different proteins or macromolecular structures in the same sample.

3.5.1. Fixation

Primary antibodies are initially tested on embryos fixed in two different ways, some antibodies will work for immuno-labelling with one of these fixatives but not the other (*see* **Note 14**).

1. Methanol fixation: collect dechorionated eggs, embryos or tadpoles in a small volume of SW (less than 100 μL) and plunge them into cold (–20°C) methanol fixative stored in screw cap tubes. Store the tube at –20°C until use.
2. Formaldehyde fixation: add a small volume (less than 100 μL) of dechorionated eggs, embryos or tadpoles into formaldehyde fixative stored in screw cap tubes. Fix for 2 h at RT or ON at 4°C with shaking (20 rpm). Place the tubes upright in order to allow fixed eggs and embryos to settle to the bottom. Wash three times in PBS and store 1–3 days at 4°C. For longer storage, one can replace the PBS with ethanol by a graded series (25, 50, 75, 100%) of consecutive washes and then place at –20°C (*see* **Note 15**).

3.5.2. Immuno-labelling

Labelling of fixed samples is typically performed in volumes of 50–100 μL in 0.5 mL tubes or in multiwell plates (Falcon, flexible plate U-bottom). For all incubations >30 min, the multiwell plate should be placed in a "humid chamber" made by lining an appropriately sized plastic box or Petri dish with wet paper towels. As examples we provide standard methods for labelling two cell structures (microtubules and mitochondria) and for determining the localization of a protein of interest (aPKC: polarity protein atypical protein kinase C) in methanol-fixed samples (**Fig. 14.1o–q**).

1. Sample rehydration, permeabilization and blocking: transfer the desired amount of fixed eggs and/or embryos to a multiwell plate. Re-hydrate samples in 1:1 ethanol/PBS-Tw solution, followed by three washes in PBS-Tw. The samples are then blocked by washing three times for 10 min in PBS-Tw-BSA with shaking (20 rpm).
2. Primary antibody labelling: dilute primary antibodies which label mitochondria (NN18, raised in mouse) and tubulin (YL1/2, raised in rat) in PBS-BSA and add to the fixed samples to give a final volume of 50–100 μL per well. Incubate ON (*see* **Note 16**) at RT with shaking (20 rpm). Resuspend the samples a few times during the incubation. Wash five times in PBS-Tw.
3. Secondary antibody labelling: dilute secondary antibodies in PBS-BSA and add to samples. Use an antibody raised against rat immunoglobulin (pre-absorbed to minimize mouse cross reaction, **Section 2.5**) coupled with rhodamine

and an antibody raised against mouse immunoglobulin (pre-absorbed to minimize rat cross reaction, see material) coupled with Cy5 (**Fig. 14.1o**). Incubate 4 h at RT with shaking (20 rpm), resuspending gently from time to time.

3.5.3. Amplification with Biotin/Streptavidin or TSA Method

To detect some non-abundant proteins, it may be necessary to amplify the signal of the secondary antibody. Amplification procedures also allow one to use less primary antibody, thus reducing background and conserving precious antibodies.

1. Amplification using biotin/streptavidin: secondary antibodies coupled to biotin bind multiple fluorescent streptavidins and therefore increase the signal compared to fluorophore-coupled secondary antibodies (but *see* **Note 17** for biotin/streptavidin limitations). As an example, to detect aPKC first incubate with a primary antibody raised in rabbit against a conserved aPKC peptide sequence (1/100 dilution). After washing as described above, incubate samples for 4 h with a biotin-conjugated anti-rabbit antibody. Then dilute the fluorescein-labelled streptavidin in PBS-BSA (**Fig. 14.1p**), add to samples and incubate for 2 h at RT with shaking (20 rpm) and occasional resuspension.
2. Amplification using tyramide system amplification (TSA): secondary antibodies coupled to peroxidase (HRP) react with a detectable substrate to give enzymatic amplification (*see* **Note 5**). In the TSA method, a labelled tyramide derivative covalently couples to amino groups of adjacent proteins when activated by peroxidase. This allows strongly amplified and high-resolution labelling, without the signal diffusion that can occur with more commonly used methods based on precipitation of coloured HRP substrates (*see* **Note 5** for TSA limitations). Incubate samples labelled with anti-aPKC antibody (1/500 dilution) with a HRP-coupled secondary antibody (1/100 dilution) for 4 h at RT. Dilute 1 μL of 30% H_2O_2 in 200 μL of amplification buffer (provided in the kit). Next, add 1 μL of the intermediate H_2O_2 dilution (in order to have a H_2O_2 final concentration of 0.0015%) and 1 μL of Alexa488-tyramide (reconstituted following the manufacturer's recommendations) to 100 μL of amplification buffer. Mix and apply immediately to the samples. Reaction time must be determined for each antibody but 20 min is a good starting point. We have observed TSA reaction times ranging from 5 min to 2 h (*see* **Note 5**) (**Fig. 14.1q**).

3.5.4. Labelling Chromosomes

Wash immuno-labelled samples in PBS-Tw. Incubate with Hoechst 33342 diluted in PBS for 15 min at RT with shaking (20 rpm) (**Fig. 14.1o**). DNA labelling can also be achieved by mounting embryos directly in Vectashield mounting medium with DAPI (Vector Laboratories).

3.5.5. Actin Microfilament Labelling with Phalloidin in Formaldehyde-Fixed Samples

Formaldehyde fixation allows one to label actin using fluorescent phalloidin (*see* **Note 6**) (**Fig. 14.1n**) in addition to immunolabelling of proteins.

1. Transfer the desired amount of fixed embryos to a multiwell plate.
2. Wash with PBS-Tw, then permeabilize with PBS-Tr (*see* **Note 6**) for 30 min with shaking (20 rpm).
3. Block with three times 10 min washes in PBS-Tw-BSA.
4. If desired, first immuno-label the protein of choice as described above.
5. Label actin with phalloidin just before staining DNA and mounting: dilute phalloidin coupled with rhodamine in PBS-BSA and add to egg or embryo samples. Place the multiwell plate in a humid chamber and incubate 2 h at RT with shaking (20 rpm), resuspending from time to time. Wash once with PBS-Tw.
6. Immediately label DNA with Hoechst as above (step 4) and mount as described below (**Section 3.5.6**). Phalloidin-labelled samples should be imaged soon after labelling as the signal decreases rapidly with time.

3.5.6. Mounting and Imaging

Wash samples three times with PBS-Tw followed by three washes with PBS. Put 20 μL of Citifluor mounting medium on a glass slide. Deposit approximately 20 μL of labelled eggs or embryos in the Citifluor drop. To create a spacer, add tiny feet to a cover-slip (22 mm × 22 mm, #1) by swiping each of the corners in modelling clay. Place the cover-slip on the drop of Citifluor containing the sample and press gently on the four clay feet with forceps in order to fix the cover-slip on the slide and immobilize eggs or embryos (*see* **Note 18**). Seal with nail polish. Observe labelled samples in a fluorescence or confocal microscope with appropriate laser settings to discriminate between fluorophores.

3.6. Isolated Cortices: Preparation, Labelling and Imaging

A special advantage of the ascidian model is that isolated cortical fragments can be prepared from eggs and early embryos (2–16 cell stages) (**Fig. 14.1s**). Because isolated cortex preparations are extremely thin (0.5–5 μm), they are ideal for fluorescent and confocal microscopy and provide very high-resolution images for determining the association of macromolecules with the major components of the cortex (plasma membrane, ER and microfilaments) (60). Furthermore, isolated cortices constitute an "open-cell preparation" which does not require permeabilization via exposure to detergents or organic solvents that have deleterious effects on cell structures (such as ER).

3.6.1. Preparing and Fixing Cortices

Isolated cortical fragments are prepared as described in our previous publications (33, 53, 61) and in the BioMarCell web site

(*see* **Note 1**) with some modifications. The procedure is best done by watching all steps under a stereo-microscope. Ascidian eggs and embryos are deposited with a mouth pipette on a glass cover-slip into a large drop of calcium-free sea water (EMC). Wait 20–30 s for eggs or embryos to settle and attach onto the cover-slip. Replace EMC by isotonic Buffer X using a gentle stream from a Pasteur pipette coming from the side of the cover-slip (do this carefully in order to avoid breaking eggs and embryos). Wash again with Buffer X. Then shear eggs and/or embryos with a vigorous stream of Buffer X using a Pasteur pipette. Wash the lawn of isolated cortical fragments quickly with Buffer X. Label the ER with DiI if necessary (see below) and then fix with CIM fixative for 30 min at RT in a humid chamber using gentle shaking (5 rpm). Wash one time with CIM solution and three times in PBS (*see* **Note 19** for microtubule preservation and immuno-labelling of unfixed cortices). ER can be labelled with DiO after fixation (see below).

3.6.2. Endoplasmic Reticulum Labelling

There are three ways to visualize the contiguous ER network on isolated cortices (**Fig. 14.1s–u**).

1. Labelling of ER with CM-DiI-C16(3) ("fixable DiI") before fixation: remove Buffer X from just-isolated cortices (see above). Dilute 1.7 μL of the CM-DiI stock solution in 0.5 mL of Buffer X and emulsify by passing the liquid through a fine gauge needle. Apply to living isolated cortices and incubate for 1 min. Wash one time with Buffer X and fix the cortices with CIM fixative.
2. Labelling of ER with DiO-C6(3) after fixation: prepare isolated cortices as described above and fix them with CIM fixative. Wash one time with CIM solution, followed by three washes in PBS. Expose fixed isolated cortices to DiO-C6(3) diluted in PBS (0.2 μg/mL) for 10 s. Wash three times with PBS. Observe the cortices and ER networks immediately. If isolated cortices are to be immuno-labelled (see below), DiO labelling should be performed after immuno-labelling just before mounting.
3. Labelling of ER with DiO-C6(3) without fixation: remove Buffer X from just-isolated cortices. Expose living isolated cortices to DiO diluted to 0.2 μg/mL in Buffer X for 10 s. Wash one time with Buffer X and observe immediately.

3.6.3. Mitochondria Labelling

Some cortices (those made at 2 cell stage particularly) retain plaques of mitochondria-rich myoplasm. It is possible to image these mitochondria by isolating cortices from embryos which were previously labelled with DiO-C2(3) as in **Section 3.3** above.

3.6.4. Actin Labelling Using Phalloidin

The best actin labelling is obtained when performed on freshly isolated cortices before fixation. Live cortices are exposed for 2 min to Buffer X containing fluorescent phalloidin (prepared as described in **Section 2.5**) followed by one wash with Buffer X and fixation with CIM fixative. Then wash one time with CIM solution, three times in PBS and observe labelling with a fluorescence or confocal microscope.

3.6.5. Immuno-labelling of Cortices

Cortices can be labelled for microtubules, ribosomes (on rough ER), mitochondria and other constituents using antibodies (33, 59, 60). As an example, we provide methods for labelling aPKC and microtubules on fixed isolated cortices (**Fig. 14.1v**).

1. Prepare and fix isolated cortices as described above (step 1). For labelling of microtubules, we recommend adding EGTA and taxol at 10 mM and 1 μg/mL, respectively, in Buffer X during the process of shearing eggs and embryos and during subsequent washes (*see* **Note 19**). Cover-slips are positioned sample-side-up on a parafilm layer in Petri dish made into a humid chamber.
2. Block with three washes of PBS-BSA for 10 min with gentle shaking (5 rpm).
3. Dilute aPKC and YL1/2 antibodies in PBS-BSA and add to samples a volume of 30–50 μL per cover-slip is sufficient. Incubate antibody-covered cover-slips in the humid chamber for 1 h at RT with gentle shaking (5 rpm).
4. Wash three times in PBS.
5. aPKC protein can be visualized using either fluorescent secondary antibodies or biotin/streptavidin amplification system. In the case of biotin/streptavidin labelling, dilute biotin-coupled secondary antibody in PBS-BSA and add to sample. Use a Cy5 conjugated secondary antibody for visualizing microtubules. Incubate with secondary antibodies for 1 h at RT with gentle shaking (5 rpm).
6. Wash three times in PBS.
7. Dilute fluorescein-conjugated streptavidin in PBS-BSA, add to sample and incubate 1 h at RT with gentle shaking (5 rpm).
8. Wash three times in PBS.

3.6.6. Mounting and Imaging

Put a 10 μL drop of Citifluor in the centre of a microscope slide. Remove excess solution from the labelled isolated cortices on the cover-slip, but do not allow it to dry out. Carefully position the cover-slip unto the Citifluor drop and remove excess liquid with absorbent paper. Seal with nail polish. Because isolated cortex preparations are extremely thin (0.5–5 μm) they can be imaged

at high resolution with a regular fluorescent microscope. Confocal microscopy allows very high resolution co-localization of ER, ribosomes, microfilaments, microtubules and/or any mRNAs and proteins of choice.

4. Notes

1. Useful links:
   - Tunicate Portal: http://www.tunicate-portal.org/index.htm
   - Ascidian News: http://depts.washington.edu/ascidian
   - ANISEED, Ascidians Network for In Situ Expression and Embryological Data: (62): http://aniseed-ibdm.univ-mrs.fr/
   - GHOST, *Ciona intestinalis* genomic and cDNA resources (63): http://ghost.zool.kyoto-u.ac.jp/
   - FABA, Four-dimensional Ascidians Body Atlas (64, 65): http://chordate.bpni.bio.keio.ac.jp/faba2/2.0/top.html
   - DBTGR, DataBase of Tunicate Gene Regulation (64): http://dbtgr.hgc.jp/
   - CIPRO: an integrated protein database of the ascidian Ciona intestinalis. http://cipro.ibio.jp/2.5/
   - CITRES Ciona Intestinalis Transgenic line RESources http://marinebio.nbrp.jp/ciona/index.jsp
   - JGI *Ciona* genome browser: http://genome.jgi-psf.org/Cioin2/Cioin2.home.html
   - BioMarCell, Ascidians Film Archive (32): http://biodev.obs-vlfr.fr/recherche/biomarcell/
   - BioDev Research Unit, Villefranche-sur-Mer marine station (McDougall/Yasuo/Sardet labs): http://biodev.obs-vlfr.fr/recherche_en.htm
   - Ascidian Stock Center at UC Santa Barbara: http://www.ascidiancenter.ucsb.edu
   - Dutch ascidians (about species worlwide): http://www.ascidians.com
   - Friday Harbor Centre for Cellular Dynamics methods: http://raven.zoology.washington.edu/celldynamics/downloads/index.html
2. mRNA detection using in situ hybridization is not described in the present chapter. Protocols can be found in previous publications (58, 66–68) and in our chapter

"Localization and anchorage of maternal mRNAs to cortical structures of ascidian eggs and embryos using high resolution in situ hybridization" in a separate volume of *Methods in Molecular Biology* (44).

3. Commercial antibodies and streptavidin are generally supplied with sodium azide as preservative (or thimerosal in case of HRP-coupled secondary antibodies and streptavidins), allowing them to be conserved for several months at 4°C. If preservatives are not included by the manufacturer, add sodium azide or thimer at 0.02% to the antibodies before aliquoting and freezing.
4. Although the monoclonal NN18 antibody was originally produced against vertebrate neuro-filaments, in ascidian embryos this antibody recognizes a mitochondrial ATP synthase subunit (personal communication of T. Nishikata).
5. Many TSA kits corresponding to various fluorophores and haptens exist. Although we generally use the Alexa488-TSA kit from Molecular Probes, we sometimes use Cy3-TSA and Cy5-TSA kits from PerkinElmer since they are cheaper and provide intense red and far red fluorescent signals. Biotin-TSA reaction followed by fluorescent streptavidin labelling does not give a much better result than direct fluorophore-TSA based reactions. Quenching of endogenous peroxidases is not necessary for ascidian eggs and early embryos when TSA method is used. TSA amplification increases with time and is not linear with respect to quantity of targeted protein in the cell; therefore it is important to limit reaction times if the purpose is meaningful comparisons of protein amount. We sometimes observe high background staining with the TSA system and it may be necessary to further dilute the primary antibodies for optimization of signal-to-noise ratio. When the confocal microscope is set to high sensitivity we have noticed the presence of nuclear background labelling using the TSA reaction.
6. Use of fluorescently coupled phalloidins is a convenient way to label actin microfilaments; however, note that phalloidin labelling should not be performed on fixed samples which have been dehydrated with alcohol (instead, a treatment with Triton aids in permeabilization). For samples which have been fixed in methanol or stored in ethanol, it is possible to use an anti-actin antibody (Calbiochem CP01) (59) to label microfilaments.
7. For horizontal injections, one can also use a "ledge" chamber as in Jaffe and Terasaki, 2004 (46). A small piece of

coated cover-slip is pressed onto double stick tape (which acts as the spacer) to give an overhanging side, so that the eggs line up under or next to this ledge.

8. Injection quantification for fluorescent proteins produced after mRNA injection can be performed as in Levasseur and McDougall, 2000 (69). A calibration curve is made by measuring the fluorescence from egg-sized droplets containing known concentrations of GFP protein. The droplets are formed by extruding different GFP solutions from a micro-injection needle under silicone onto cover-slips treated with dimethyldichlorosilicane (2% in 1,1,1-trichloroethane) to remove the surface tension, so that the egg-sized bubbles remain spherical. Accurate production of egg-sized droplets is important since the fluorescence intensity is proportional to total volume as well as to concentration. Using the same imaging parameters (filters, exposure time, binning, etc.) measure the fluorescence of the injected egg and compare this value to the calibration curve to determine the concentration of the fluorescent protein in vivo.
9. In vivo labelling techniques work best for transparent eggs and embryos of *Phallusia* (33, 55), whereas for *Ciona* eggs and embryos the opacity of yolk vesicles and intrinsic autofluorescence hinder the detection of fluorescent labelling unless it is near the surface. Fixed *Ciona* samples are suitable for fluorescent detection of proteins or mRNAs; however, signal intensity and resolution are greater in transparent eggs and embryos of *Phallusia* (58).
10. Live embryos can only be labelled for short periods (30 min) with Hoechst because it inhibits DNA replication and results in DNA bridges.
11. FM 4-64 tends to concentrate at the centrosomes if embryos are crowded on the slide and dying.
12. Concanavalin A (non-succinylated) and wheat germ agglutinin (WGA) also label membranes of fertilized eggs and embryos, but they should not be used on unfertilized eggs because they induce capping and cause eggs to be activated (70).
13. A ratiometric method should be used when one wishes to quantify calcium signals or to control for artefacts due to sample thickness, cytoplasmic domains, or dye concentration. The eggs can be injected either with the ratiometric dye Fura-2 dextran or with a mixture of Calcium-Green dextran and the calcium-insensitive dye Texas-Red dextran. Acquire fluorescent images simultaneously with appropriate excitation and emission filters.

14. Methanol treatment causes instantaneous fixation by dehydration and a good permeabilization of eggs and embryos. Microtubule structures are well preserved by methanol fixation. A high concentration of EGTA (50 mM) is added to the methanol as tubulin is one of the major targets of the potent calcium-dependant protease. Formaldehyde treatment causes progressive fixation by making intra- and inter-molecular bonds. It is well suited for maintaining the integrity of the cell cortex and in particular microfilament labelling using phalloidin. Fixations for electron microscopy of whole eggs and embryos and cortices prepared from them are not detailed here but can be found in our previous publications (33, 61).
15. The ethanol dehydration step after formaldehyde fixation further permeabilizes the samples and improves signal for many antibodies and for labelling of structures deep within the embryo (but is not compatible with phalloidin labelling, *see* **Note 6**). As for methanol-fixed embryos, ethanol-dehydrated samples should be re-hydrated into PBS before immuno-labelling.
16. Incubation with antibodies can be shortened to 2 h at RT but labelling structures situated deep within the embryo is better with ON incubation at RT or 4°C.
17. Although biotin/streptavidin gives good amplification of signals, note that streptavidin attaches to mitochondria yielding background labelling. Therefore this method is not appropriate for detection of cytoplasmic proteins.
18. It is important that the embryos be slightly compressed and snug between slide and cover-slip if an oil immersion objective is to be used. This is not necessary for an air objective which does not touch the cover-slip.
19. Calcium ions are implicated in microtubule depolymerization and we observed that adding EGTA to Buffer X helps to preserve microtubules on isolated cortices. Moreover addition of taxol, a drug which stabilizes microtubules, gives better results than EGTA alone. Also note that it is also possible to immuno-label unfixed live cortices (an open-cell preparation) by diluting primary and secondary antibodies in Buffer X instead of PBS and using brief incubation times (5 min separated by quick washes with Buffer X). Observe live-labelled cortices within 30 min of making cortices, since as time passes the ER network will start vesiculating.

Acknowledgements

We thank personnel of the Developmental Biology unit (BioDev, Villefranche-sur-Mer Marine Station, CNRS/UPMC UMR7009, France) for helpful discussions and previous members of the Villefranche lab, Fabrice Roegiers and François Prodon. We thank M. Khamla for help with graphics. The laboratories of C. Sardet, A. McDougall and H. Yasuo are supported by ANR (Agence Nationale de la Recherche), CNRS-ACI (Centre National de la Recherche Scientifique—Action Concertée Incitative), UPMC (Université Pierre et Marie Curie, Paris 06), ARC (Association pour la Recherche sur le Cancer) and AFM (Association Française contre les Myopathies).

References

1. Raff, R. A. and Love, A. C. (2004) Kowalevsky, comparative evolutionary embryology, and the intellectual lineage of evo-devo. *J. Exp. Zool. B Mol. Dev. Evol.* **302**, 19–34.
2. Fol, H. (1879) *Recherches sur la fécondation et le commencement de l'hénogénie chez divers animaux.* Mém. Soc. de Phys. et d'Hist. Nat. Genève **26**, 92–397.
3. Chabry, L. (1887) *Contribution à l'embryologie normale et tératologique des Ascidies simples.* Paris: Felix Alcan.
4. Conklin, E. G. (1905) The organization and cell lineage of the ascidian egg. *J. Acad. Sci. Philadelphia* **13**, 1–119.
5. Reverberi, G. (1956) The mitochondrial pattern in the development of the ascidian egg. *Experientia (Basel)* **12**, 55–56.
6. Nishida, H. (1997) Cell fate specification by localized cytoplasmic determinants and cell interactions in ascidian embryos. *Int. Rev. Cytol.* **176**, 245–306.
7. Satoh, N. (1994) *Developmental Biology of Ascidians.* Cambridge University Press, New York.
8. Nishida, H. (2005) Specification of embryonic axis and mosaic development in ascidians. *Dev. Dyn.* **233**, 1177–1193.
9. Nishida, H. and Sawada, K. (2001) macho-1 encodes a localized mRNA in ascidian eggs that specifies muscle fate during embryogenesis. *Nature* **409**, 724–729.
10. Negishi, T., Takada, T., Kawai, N., and Nishida, H. (2007) Localized PEM mRNA and protein are involved in cleavage-plane orientation and unequal cell divisions in ascidians. *Curr. Biol.* **17**, 1014–1025.
11. Momose, T. and Houliston, E. (2007) Two Oppositely Localised Frizzled RNAs as Axis Determinants in a Cnidarian Embryo. *PLoS Biol.* **5**, e70.
12. Kloc, M. and Etkin, L. D. (2005) RNA localization mechanisms in oocytes. *J. Cell. Sci.* **118**, 269–282.
13. St Johnston, D. (2005) Moving messages: the intracellular localization of mRNAs. *Nat. Rev. Mol. Cell. Biol.* **6**, 363–375.
14. Delsuc, F., Brinkmann, H., Chourrout, D., and Philippe, H. (2006) Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **439**, 965–968.
15. Lemaire, P., Smith, W. C., and Nishida, H. (2008) Ascidians and the plasticity of the chordate developmental program. *Curr. Biol.* **18**, R620–31.
16. Satou, Y., Satoh, N., and Imai, K. S. (2009) Gene regulatory networks in the early ascidian embryo. *Biochim. Biophys. Acta* **1789**, 268–273.
17. Satoh, N. (2007) Toward a new paradigm for studying ascidian developmental dynamics. *Dev. Dyn.* **236**, 1695–1697.
18. Imai, K. S., Levine, M., Satoh, N., and Satou, Y. (2006) Regulatory blueprint for a chordate embryo. *Science* **312**, 1183–1187.
19. Hudson, C. and Yasuo, H. (2008) Similarity and diversity in mechanisms of muscle fate induction between ascidian species. *Biol. Cell* **100**, 265–277.
20. Picco, V., Hudson, C., and Yasuo, H. (2007) Ephrin-Eph signalling drives the asymmetric division of notochord/neural precursors in Ciona embryos. *Development* **134**, 1491–1497.

21. Sardet, C., Swalla, B. J., Satoh, N., Sasakura, Y., Branno, M., Thompson, E. M., Levine, M., and Nishida, H. (2008) Euro chordates: Ascidian community swims ahead. The 4th International Tunicate meeting in Villefranche sur Mer. *Dev. Dyn.* **237**, 1207–1213.
22. Fujii, S., Nishio, T., and Nishida, H. (2008) Cleavage pattern, gastrulation, and neurulation in the appendicularian, Oikopleura dioica. *Dev. Genes Evol.* **218**, 69–79.
23. Dehal, P., Satou, Y., Campbell, R. K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D. M., Harafuji, N., Hastings, K. E., Ho, I., Hotta, K., Huang, W., Kawashima, T., Lemaire, P., Martinez, D., Meinertzhagen, I. A., Necula, S., Nonaka, M., Putnam, N., Rash, S., Saiga, H., Satake, M., Terry, A., Yamada, L., Wang, H. G., Awazu, S., Azumi, K., Boore, J., Branno, M., Chin-Bow, S., DeSantis, R., Doyle, S., Francino, P., Keys, D. N., Haga, S., Hayashi, H., Hino, K., Imai, K. S., Inaba, K., Kano, S., Kobayashi, K., Kobayashi, M., Lee, B. I., Makabe, K. W., Manohar, C., Matassi, G., Medina, M., Mochizuki, Y., Mount, S., Morishita, T., Miura, S., Nakayama, A., Nishizaka, S., Nomoto, H., Ohta, F., Oishi, K., Rigoutsos, I., Sano, M., Sasaki, A., Sasakura, Y., Shoguchi, E., Shin-i, T., Spagnuolo, A., Stainier, D., Suzuki, M. M., Tassy, O., Takatori, N., Tokuoka, M., Yagi, K., Yoshizaki, F., Wada, S., Zhang, C., Hyatt, P. D., Larimer, F., Detter, C., Doggett, N., Glavina, T., Hawkins, T., Richardson, P., Lucas, S., Kohara, Y., Levine, M., Satoh, N., and Rokhsar, D. S. (2002) The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. *Science* **298**, 2157–2167.
24. Nishida, H. (1996) Vegetal egg cytoplasm promotes gastrulation and is responsible for specification of vegetal blastomeres in embryos of the ascidian Halocynthia roretzi. *Development* **122**, 1271–1279.
25. Erives, A. and Levine, M. (2000) Characterization of a maternal T-Box gene in Ciona intestinalis. *Dev. Biol.* **225**, 169–178.
26. Hudson, C., Lotito, S., and Yasuo, H. (2007) Sequential and combinatorial inputs from Nodal, Delta2/Notch and FGF/MEK/ERK signalling pathways establish a grid-like organisation of distinct cell identities in the ascidian neural plate. *Development* **134**, 3527–3537.
27. Tiozzo, S., Voskoboynik, A., Brown, F. D., and De Tomaso, A. W. (2008) A conserved role of the VEGF pathway in angiogenesis of an ectodermally-derived vasculature. *Dev. Biol.* **315**, 243–255.
28. Nishiyama, A. and Fujiwara, S. (2008) RNA interference by expressing short hairpin RNA in the Ciona intestinalis embryo. *Dev. Growth Differ.* **50**, 521–529.
29. Sasakura, Y., Konno, A., Mizuno, K., Satoh, N., and Inaba, K. (2008) Enhancer detection in the ascidian Ciona intestinalis with transposase-expressing lines of Minos. *Dev. Dyn.* **237**, 39–50.
30. Dahlberg, C., Auger, H., Dupont, S., Sasakura, Y., Thorndyke, M., and Joly, J. S. (2009) Refining the Ciona intestinalis model of central nervous system regeneration. *PLoS ONE* **4**, e4458.
31. Joly, J. S., Kano, S., Matsuoka, T., Auger, H., Hirayama, K., Satoh, N., Awazu, S., Legendre, L., and Sasakura, Y. (2007) Culture of Ciona intestinalis in closed systems. *Dev. Dyn.* **236**, 1832–1840.
32. Sardet, C., Paix, A., Prodon, F., Dru, P., and Chenevert, J. (2007) From oocyte to 16-cell stage: cytoplasmic and cortical reorganizations that pattern the ascidian embryo. *Dev. Dyn.* **236**, 1716–1731.
33. Prodon, F., Dru, P., Roegiers, F., and Sardet, C. (2005) Polarity of the ascidian egg cortex and relocalization of cER and mRNAs in the early embryo. *J. Cell. Sci.* **118**, 2393–2404.
34. Dumollard, R. and Sardet, C. (2001) Three different calcium wave pacemakers in ascidian eggs. *J. Cell. Sci.* **114**, 2471–2481.
35. Prodon, F., Chenevert, J., Hebras, C., Dumollard, R., Faure, E., Gonzalez-Garcia, J., Nishida, H., Sardet, C., and McDougall, A. (2010) Dual mechanism controls asymmetric spindle position in ascidian germ cell precursors. *Development* **137**, 2011–2021.
36. Nishikata, T., Hibino, T., and Nishida, H. (1999) The centrosome-attracting body, microtubule system, and posterior egg cytoplasm are involved in positioning of cleavage planes in the ascidian embryo. *Dev. Biol.* **209**, 72–85.
37. Johnson, D. S., Davidson, B., Brown, C. D., Smith, W. C., and Sidow, A. (2004) Noncoding regulatory sequences of Ciona exhibit strong correspondence between evolutionary constraint and functional importance. *Genome Res.* **14**, 2448–2456.
38. Hill, M. M., Broman, K. W., Stupka, E., Smith, W. C., Jiang, D., and Sidow, A. (2008) The C. savignyi genetic map and its integration with the reference sequence facilitates insights into chordate genome evolution. *Genome Res.* **18**, 1369–1379.
39. Sobral, D., Tassy, O., and Lemaire, P. (2009) Highly Divergent Gene Expression Programs Can Lead to Similar Chordate Larval Body Plans. *Curr. Biol.* **19**, 2014–2019.

40. Corbo, J. C., Di Gregorio, A., and Levine, M. (2001) The ascidian as a model organism in developmental and evolutionary biology. *Cell* **106**, 535–538.
41. Manni, L., Zaniolo, G., Cima, F., Burighel, P., and Ballarin, L. (2007) Botryllus schlosseri: a model ascidian for the study of asexual reproduction. *Dev. Dyn.* **236**, 335–352.
42. Lamy, C. and Lemaire, P. (2008) Ascidian embryos: from the birth of experimental embryology to the analysis of gene regulatory networks. *Med. Sci. (Paris)* **24**, 263–269.
43. Swalla, B. J. (2004) Procurement and culture of ascidian embryos. *Methods Cell Biol.* **74**, 115–141.
44. Paix, A., Chenevert, J., and Sardet, C. (2011) Localization and anchorage of maternal mRNAs to cortical structures of ascidian eggs and embryos using high resolution in situ hybridization. Messages on the move: Techniques in RNA visualization. *Methods Mol Biol.* **714**, 49–70.
45. Hendrickson, C., Christiaen, L., Deschet, K., Jiang, D., Joly, J. S., Legendre, L., Nakatani, Y., Tresser, J., and Smith, W. C. (2004) Culture of adult ascidians and ascidian genetics. *Methods Cell Biol.* **74**, 143–170.
46. Jaffe, L. A. and Terasaki, M. (2004) Quantitative microinjection of oocytes, eggs, and embryos. *Methods Cell Biol.* **74**, 219–242.
47. Lemaire, P., Garrett, N., and Gurdon, J. B. (1995) Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81**, 85–94.
48. Roure, A., Rothbacher, U., Robin, F., Kalmar, E., Ferone, G., Lamy, C., Missero, C., Mueller, F., and Lemaire, P. (2007) A multicassette Gateway vector set for high throughput and comparative analyses in ciona and vertebrate embryos. *PLoS ONE* **2**, e916.
49. Turner, D. L. and Weintraub, H. (1994) Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434–1447.
50. Prodon, F., Chenevert, J., and Sardet, C. (2006) Establishment of animal-vegetal polarity during maturation in ascidian oocytes. *Dev. Biol.* **290**, 297–311.
51. Zalokar, M. and Sardet, C. (1984) Tracing of cell lineage in embryonic development of Phallusia mammillata (Ascidia) by vital staining of mitochondria. *Dev. Biol.* **102**, 195–205.
52. Speksnijder, J. E., Jaffe, L. F., and Sardet, C. (1989) Polarity of sperm entry in the ascidian egg. *Dev. Biol.* **133**, 180–184.
53. Dumollard, R., Hammar, K., Porterfield, M., Smith, P. J., Cibert, C., Rouviere, C., and Sardet, C. (2003) Mitochondrial respiration and Ca2+ waves are linked during fertilization and meiosis completion. *Development* **130**, 683–692.
54. Roegiers, F., McDougall, A., and Sardet, C. (1995) The sperm entry point defines the orientation of the calcium-induced contraction wave that directs the first phase of cytoplasmic reorganization in the ascidian egg. *Development* **121**, 3457–3466.
55. Roegiers, F., Djediat, C., Dumollard, R., Rouviere, C., and Sardet, C. (1999) Phases of cytoplasmic and cortical reorganizations of the ascidian zygote between fertilization and first division. *Development* **126**, 3101–3117.
56. Speksnijder, J. E., Terasaki, M., Hage, W. J., Jaffe, L. F., and Sardet, C. (1993) Polarity and reorganization of the endoplasmic reticulum during fertilization and ooplasmic segregation in the ascidian egg. *J. Cell Biol.* **120**, 1337–1346.
57. McDougall, A. and Sardet, C. (1995) Function and characteristics of repetitive calcium waves associated with meiosis. *Curr. Biol.* **5**, 318–328.
58. Paix, A., Yamada, L., Dru, P., Lecordier, H., Pruliere, G., Chenevert, J., Satoh, N., and Sardet, C. (2009) Cortical anchorages and cell type segregations of maternal postplasmic/PEM RNAs in ascidians. *Dev. Biol.* **336**, 96–111.
59. Patalano, S., Pruliere, G., Prodon, F., Paix, A., Dru, P., Sardet, C., and Chenevert, J. (2006) The aPKC-PAR-6-PAR-3 cell polarity complex localizes to the centrosome attracting body, a macroscopic cortical structure responsible for asymmetric divisions in the early ascidian embryo. *J. Cell. Sci.* **119**, 1592–1603.
60. Sardet, C., Prodon, F., Dumollard, R., Chang, P., and Chenevert, J. (2002) Structure and function of the egg cortex from oogenesis through fertilization. *Dev. Biol.* **241**, 1–23.
61. Sardet, C., Speksnijder, J., Terasaki, M., and Chang, P. (1992) Polarity of the ascidian egg cortex before fertilization. *Development* **115**, 221–237.
62. Tassy, O., Daian, F., Hudson, C., Bertrand, V., and Lemaire, P. (2006) A quantitative approach to the study of cell shapes and interactions during early chordate embryogenesis. *Curr. Biol.* **16**, 345–358.
63. Satou, Y., Kawashima, T., Shoguchi, E., Nakayama, A., and Satoh, N. (2005) An integrated database of the ascidian, Ciona intesti-

nalis: towards functional genomics. *Zoolog. Sci.* **22**, 837–843.
64. Sierro, N., Kusakabe, T., Park, K. J., Yamashita, R., Kinoshita, K., and Nakai, K. (2006) DBTGR: a database of tunicate promoters and their regulatory elements. *Nucleic Acids Res.* **34**, D552–5.
65. Hotta, K., Mitsuhara, K., Takahashi, H., Inaba, K., Oka, K., Gojobori, T., and Ikeo, K. (2007) A web-based interactive developmental table for the ascidian Ciona intestinalis, including 3D real-image embryo reconstructions: I. From fertilized egg to hatching larva. *Dev. Dyn.* **236**, 1790–1805.
66. Ikuta, T. and Saiga, H. (2007) Dynamic change in the expression of developmental genes in the ascidian central nervous system: revisit to the tripartite model and the origin of the midbrain-hindbrain boundary region. *Dev. Biol.* **312**, 631–643.
67. Sardet, C., Nishida, H., Prodon, F., and Sawada, K. (2003) Maternal mRNAs of PEM and macho 1, the ascidian muscle determinant, associate and move with a rough endoplasmic reticulum network in the egg cortex. *Development* **130**, 5839–5849.
68. Wada, S., Katsuyama, Y., Yasugi, S., and Saiga, H. (1995) Spatially and temporally regulated expression of the LIM class homeobox gene Hrlim suggests multiple distinct functions in development of the ascidian, Halocynthia roretzi. *Mech. Dev.* **51**, 115–126.
69. Levasseur, M. and McDougall, A. (2000) Sperm-induced calcium oscillations at fertilisation in ascidians are controlled by cyclin B1-dependent kinase activity. *Development* **127**, 631–641.
70. Speksnijder, J. E., Sardet, C., and Jaffe, L. F. (1990b) The activation wave of calcium in the ascidian egg and its role in ooplasmic segregation. *J. Cell Biol.* **110**, 1589–1598.

Chapter 15

Ciona Genetics

Michael T. Veeman, Shota Chiba, and William C. Smith

Abstract

Ascidians, such as *Ciona*, are invertebrate chordates with simple embryonic body plans and small, relatively non-redundant genomes. *Ciona* genetics is in its infancy compared to many other model systems, but it provides a powerful method for studying this important vertebrate outgroup. Here we give basic methods for genetic analysis of *Ciona*, including protocols for controlled crosses both by natural spawning and by the surgical isolation of gametes; the identification and propagation of mutant lines; and strategies for positional cloning.

Key words: *Ciona*, ascidian, genetics, invertebrate chordate.

1. Introduction

Ascidians are the closest invertebrate relatives of the vertebrates (1). Their simple though stereotypically chordate larval body plans and their small, relatively non-redundant genomes have made ascidians an increasingly popular model system for developmental and systems biology (2–4). With hermaphroditic reproduction and extremely polymorphic genomes, they are also well suited for traditional forward genetics.

Although researchers make use of a wide range of ascidian species, here we will emphasize methods for *Ciona intestinalis* and *Ciona savignyi*, the two sequenced ascidians. Both have genomes of approximately 180 MB, which is roughly 1/20 the size of most mammalian genomes, and ~15,000 predicted genes, versus ~23,000 in humans (5–8). More importantly, many developmentally important gene families show considerably less redundancy than in vertebrate genomes (9–12). This is thought to be due

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_15,

to both the ascidian lineage having diverged prior to two rounds of genome duplication in the vertebrate lineage (13) and also to extensive gene loss in the ascidians (14). This decreased redundancy is extremely valuable for genetic analysis and gives rise to a different spectrum of mutant phenotypes (15–17).

There are two unusual aspects of *Ciona* genetics that are important to keep in mind. The first is that *Ciona* are hermaphrodites that can be successfully self-crossed, which simplifies many aspects of genetic analysis. The second is that *Ciona* are astonishingly polymorphic, which has both advantages and disadvantages for the geneticist.

1.1. Self-Fertility

Ciona has been variously described as being both self-fertile and self-sterile. This is largely a matter of definition, and they are probably best described as being modestly self-fertile. Non-self-fertilization is clearly favored (18), and a genetic mechanism for this preference has been determined (19). Self-fertilization rates are extremely variable, ranging from 100% in some spawnings to 0% in others (20). It is quite common for only 5–10% of eggs to self-fertilize, which might be described as largely self-sterile by a stringent definition. However, in a typical clutch of 500 eggs, 5% self-fertilization gives 25 offspring. This degree of self-fertility thus gives more than enough sampling power to reliably detect recessive mutations.

1.2. Polymorphism

In comparison to typical vertebrate genomes, ascidian genomes are extremely polymorphic. The two haplotypes derived from sequencing the genome of a single *C. savignyi* adult show a genomewide average single nucleotide polymorphism (SNP) heterozygosity of 4.5% (21). *C. intestinalis* is somewhat less polymorphic at 1.2–1.5% (22). By comparison, the human and chimpanzee genomes differ by less than 1.5%, and humans differ from gibbons and siamangs by less than 5%. This high polymorphism rate can be advantageous in that almost any arbitrary piece of amplified DNA is likely to contain an SNP or small indel and thus be usable as a genetic marker. This is particularly valuable given that efforts to construct inbred, isogenic lines have been unsuccessful to date. The high polymorphism rate is disadvantageous, however, in that it gives a high background when detecting mutations for positional cloning.

1.3. Genomic Resources

High-quality assemblies are available for both *Ciona* genomes (6, 7, 22). Genomic scaffolds have also been placed onto linkage groups by FISH mapping in *C. intestinalis* (23) and by recombination mapping in *C. savignyi* (24). There are large collections of ESTs, particularly in *C. intestinalis* (25, 26), and a large database of *C. intestinalis* gene expression patterns by in situ hybridization (27, 28). Good web resources include the Aniseed

(http://aniseed-ibdm.univ-mrs.fr/) and Ghost (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) databases and the Ensembl genome browser (http://www.ensembl.org).

1.4. Genetic Strategies

Although ascidians have been a model system for embryonic development for more than a century, they are a comparatively new model system for genetics. *Ciona* can be chemically mutagenized (20), and transposon-based mutagenesis is being actively developed (29). Recessive mutations are sufficiently common in natural populations, however, that they can easily be identified by self-crosses of wild-caught individuals. Mapping strategies are evolving quickly, with earlier AFLP-based methods having been largely supplanted by direct mapping of SNPs. Methods based on deep sequencing are likely to be important in the near future. Here we emphasize the basic protocols for ascidian genetic analysis: controlled crosses; mutant screening; expanding mutant lines; DNA extraction; SNP mapping; and microinjection. For discussions of transient and stable transgenesis, refer to (29–31).

2. Materials

2.1. Crosses

1. Adult *Ciona*: *C. intestinalis* is widespread in temperate coastal waters (32), whereas *C. savignyi* is only found in Japan, the Pacific Coast of North America, and the Atlantic Coast of Argentina (33). Both are easily collected in marinas and harbors, particularly on pilings, buoys, and ropes that have been in the water for some time without having been cleaned. The undersides of unused dinghies and kayaks are also prime locations. There is considerable month-to-month variation in ascidian abundance at local marinas (34). There are also seasonal variations, possibly reflecting changes in water temperature (35), with summer typically the best season in North America and Europe but the worst season in Japan. Be sure to follow local rules for collecting scientific specimens. *Ciona* are also available from the Ascidian Stock Center at UCSB (http://www.ascidiancenter.ucsb.edu), the Woods Hole Marine Biological Laboratory (http://www.mbl.edu/mrc/), the Roscoff Marine Station (http://www.sb-roscoff.fr/ModBiol/), and others.
   Ciona are easily maintained in tanks or sea tables with running, unfiltered seawater. They can also be kept in recirculating systems with varying degrees of water exchange. Although not necessary in Southern California, some regions may benefit from heating or cooling mechanisms

to stabilize water temperatures. Aquaculture system design is beyond the scope of this review and readers are directed elsewhere (20, 36).

Be aware that *C. intestinalis* is divided into two partially reproductively isolated subspecies, with type A being found in the Pacific and the Mediterranean and type B found in the Atlantic (32, 37).

2. Plastic cups: Disposable 16 oz plastic cups (aka "beer cups" or "party cups") are ideal for spawning single *Ciona* (**Fig. 15.1a**). They can be washed and reused many times. Label each cup near the brim to keep track of individual crosses.
3. Egg filters: Handy filters for collecting and washing eggs and embryos can be made from 50 mL centrifuge tubes and 70 μM nylon mesh. For each tube, cut off the bottom with a band saw and drill or cut a large hole in the cap. Stretch a square of mesh over the tube threads and hold it in place with the drilled cap (**Fig. 15.1b**). It is convenient to have a large number of egg filters, so as to not have to wash them between cups when screening large numbers of animals.
4. Petri dishes: Several sizes of disposable plastic petri dish are needed: 60 mm ×15 mm for screening the embryos from a single adult; 100 mm × 15 mm for larger crosses; and 150 mm × 15 mm for settling larvae to grow until maturity.
5. Net twine: Twine or string is needed to hang plates and condos in the culture system. Lightly tarred nylon fishing net twine holds knots well and does not decay in seawater (Memphis Net and Twine Co., Memphis, TN).
6. Scintillation vials: 1 dram (3.7 mL) glass vials filled with water and capped make good weights to keep outcross plates and condos submerged in the culture system.
7. Cable ties: 4″ plastic cable ties (zip-ties) are used to attach vials, nametags, etc., to plates and condos, and to close condos when initially occupied.
8. Condos: Small cages ("condos") are needed to house individual ascidians in the culturing system after they have been detached from their substrate (**Fig. 15.1c**). Perforated plastic sheet of the type sold at craft stores as a substrate for needlepoint is a good material to build these cages. It is inexpensive, has holes of the right size, and does not foul with algae. Squares of this material can be sewn together with synthetic yarn to make boxes with one hinged side. Zip tie a scintillation vial filled with water to the bottom of the box to weight it down. Tie a loop of net

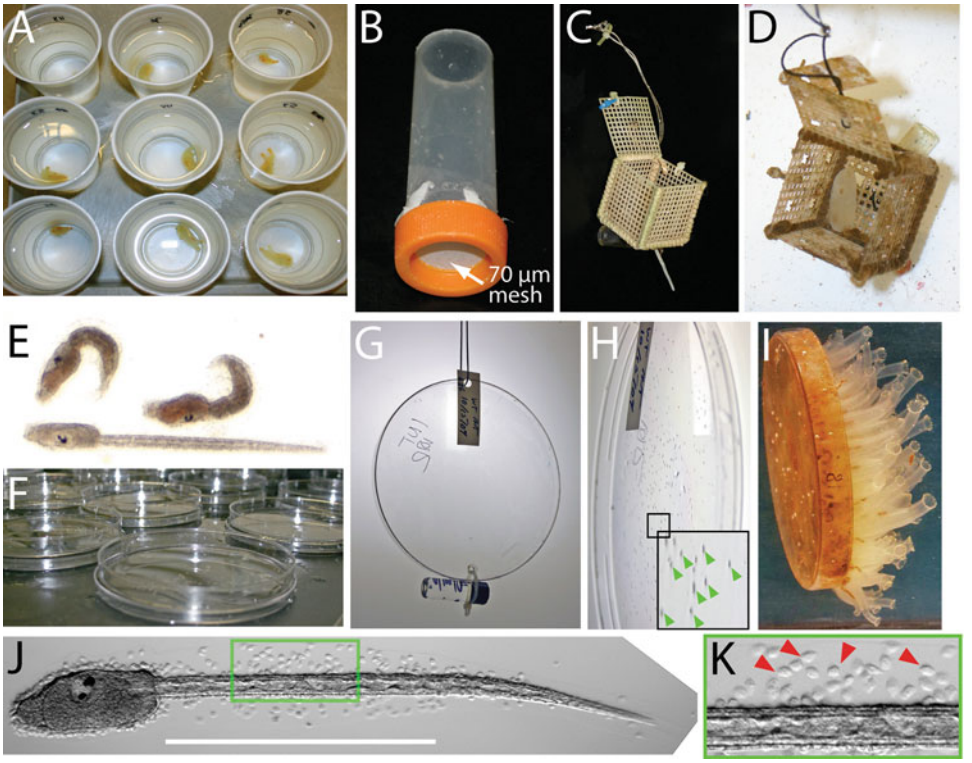

Fig. 15.1. Tools and techniques for ascidian genetics. (**a**) Disposable plastic drink cups make ideal vessels for spawning single ascidians. (**b**) Homemade egg filters with a 70 µm nylon mesh bottom are indispensable for washing eggs and embryos and for performing certain types of crosses. (**c**) ''Condos'' are used to house and label single ascidians after they have been detached from their original substrate. (**d**) An opened condo containing an attached adult. (**e**) An example of a wildtype larva (*bottom*) together with two mutant siblings. (**f**) Minimize the air–water interface when settling larvae onto petri dishes by using the lid (concave side up) as a dish to hold swimming larvae, and then float the part that is normally used as the dish on top (also concave side up). (**g**) Prepare a plate with attached juveniles for long-term culture by making two holes on opposite sides of the plate with a soldering iron. Attach a small weight, such as a scintillation vial to one hole with a zip tie, and tie a loop of string through the other hole. (**h**) Attachment density can be checked by briefly pouring off the water and viewing the plate at an angle. Juveniles are easily seen in oblique light as small bumps on the plate (indicated with arrowheads in inset). (**i**) A plate of adult *Ciona savignyi*. (**j**) A hatched, swimming larva of *C. intestinalis*. Scale bar is 0.5 mm. *Box* indicates the closeup in (**k**). (**k**) Closeup view of a segment of tail showing numerous test cells (some indicated with *arrowheads*) in the larval tunic.

twine to the lid to suspend it in the culture system. Condos can be labeled with small squares of plastic cut from disposable lab weigh boats. Write the relevant details on the plastic label with an indelible lab marker, punch a hole in the label with a paper punch, and zip tie it to the condo. When an animal is first housed in a condo, the lid needs to be zip tied shut so that it does not fall out. After 1–2 weeks, the animal will have attached to the wall of the condo and the lid should be opened to allow better water circulation (**Fig. 15.1d**).

9. Sperm freezing solution: 25% ultrapure DMSO (from a sealed glass ampule, e.g., Sigma) in seawater.

10. Sperm freezing rack: A Styrofoam tube rack that will float on the surface of a liquid nitrogen bath is needed to freeze sperm at the appropriate cooling rate.

2.2. Mapping

2.2.1. Basic Mapping Materials

1. PCR primer pairs for mapping: PCR primers designed against intergenic regions often fail to amplify due to polymorphism in the primer binding sites. Avoid this by designing primers that are anchored in exon sequences. Keep primer melting temperatures similar, so that all markers can be amplified in the same run.
2. BSA-coated pipette tips: Fixed embryos and live dechorionated embryos are extremely sticky. All pipette tips used to transfer such embryos need to first be coated with a thin film of bovine serum albumen (BSA) by briefly pipetting up and down a 10 mg/mL solution of BSA in seawater.
3. Agarose-coated dishes: Petri dishes to be used with fixed embryos or with live, dechorionated embryos should first be agarose coated. Make a melted 1% agarose solution by heating. Fill one dish with the agarose and then immediately pour it from dish to dish, coating each one with a thin film. For live, dechorionated embryos, the agarose should be dissolved in seawater, whereas for DNA extractions from fixed embryos the agarose should be dissolved in deionized water.

2.2.2. Single Tadpole/Small Pool DNA Extraction for PCR

1. 37% formaldehyde solution.
2. 2× STL (single tadpole lysis) buffer: 1% Triton X-100, 100 mM NaCl, 20 mM Tris-HCl pH 7.8, 1 mM EDTA.
3. 10 mg/mL proteinase K in ultrapure water. Store in single-use aliquots at –20°C.

2.2.3. Large-Scale Purification of Genomic DNA

Use the same materials as in **Section 2.2.2**, but also including

1. Disposable plastic pestles with matching 1.5 mL microcentrifuge tubes.
2. GuSCN buffer: 30 g guanidine thiocyanate, 2.5 mL 1 M Tris-HCl pH 6.4, 2.2 mL 0.5 M EDTA, 0.65 g Triton X-100, 25.8 mL ultrapure water.
3. Glass milk (from GENECLEAN III kit, MP Biomedicals).
4. 70% ethanol.
5. Acetone.

2.3. Injections

1. Horizontal micropipette puller (e.g., Sutter P-80).
2. 1 mm outer diameter, 100 mm long, thin wall borosilicate tubing with filling fiber (e.g., World Precision Instruments TW100F-4).
3. Handmade holding pipette.

4. Dissecting microscope (epifluorescence optics are helpful but not essential).
5. Coarse micromanipulator for holding pipette (e.g., Narishige MN-151).
6. Fine oil hydraulic micromanipulator with fourth axis axial advance for injection pipette (e.g., Narishige MO-202U + MMO-220A).
7. PicoSpritzer pneumatic microinjector (Parker Hannifin Corp.) customized with a three-way vacuum-rated pneumatic toggle switch (e.g., McMaster-Carr 8399K13). Connect the output from the switch to the input for the external solenoid valve. Connect one input to the regulated air pressure supply from the PicoSpritzer. Connect the other input to a source of regulated vacuum (e.g., house vacuum with a regulator [McMaster-Carr 41585K41] and gauge [McMaster-Carr 3935K21]).

3. Methods

3.1. Crosses

Genetic crosses in *Ciona* can be performed by either dissecting out gametes and combining them in vitro or controlling the natural circadian spawning schedule by manipulating ambient light levels. Dissected crosses typically provide large numbers of extremely clean embryos (free from the fecal matter and pieces of sloughed tunic that are often filtered out with the eggs in a natural spawning), but have the disadvantage of killing the parents.

For both dissected and naturally spawned crosses, it can be helpful to keep the parental ascidians under constant light for 2–5 days to prevent spawning and thus allow the accumulation of gametes. A gravid animal will have a full oviduct (orange for *C. savignyi* and brownish-yellow for *C. intestinalis*) and a full sperm duct (white) clearly visible through the tunic.

3.1.1. Collecting Gametes by Dissection

1. Take an adult *Ciona* and gently remove the tunic by ripping it open with your fingers or slitting it with a small pair of scissors (**Fig. 15.2a**, **b**). Take care not to rip or puncture the gonoducts.
2. Using fine forceps and a dissecting microscope, dissect through the muscle layer overlying the gonoducts (**Fig. 15.2c**).
3. Use forceps to rip a small hole in the oviduct. Be careful not to rip the sperm duct yet.

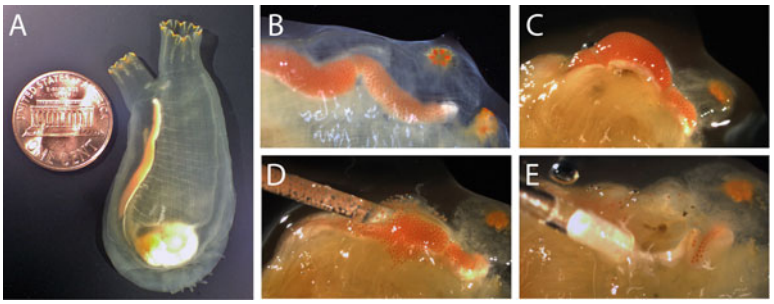

Fig. 15.2. Collecting gametes by dissection. (**a**) Gravid adult *Ciona savignyi*. Note the parallel ooduct and sperm duct. (**b**) Closeup of ooduct and sperm duct after the tunic has been removed. (**c**) The body wall muscle layer has been dissected open to expose the ooduct and sperm duct. (**d**) The ooduct has been pierced and the eggs are being collected with a Pasteur pipette. (**e**) The sperm duct has been pierced and sperm is being collected with a Pasteur pipette.

4. Collect the eggs flowing out of the punctured oviduct with a Pasteur pipette (**Fig. 15.2d**) and deposit them into an egg filter sitting in a cup of seawater.
5. Use closed forceps or the Pasteur pipette to gently stroke the oviduct toward the puncture site, forcing as many eggs as possible out of the hole. Collect them with the Pasteur pipette and move them to the egg filter.
6. Wash the collected eggs by transferring the egg filter through two cups of seawater. Eggs are best used immediately, but typically remain fertilizable for several hours. For critical experiments it is best to wait for 2 h to ensure that the eggs are not inadvertently self-fertilized.
7. Puncture the sperm duct by ripping a small hole in it with forceps.
8. Collect the sperm spilling out of the hole with a new Pasteur pipette. Try to collect the sperm as "dry" and clean as possible (**Fig. 15.2e**).
9. Transfer the sperm to a 1.5 mL microcentrifuge tube. If it is not to be used right away, store it at 4°C. Sperm can typically be stored at 4°C for up to 1 week.
10. If you are dissecting multiple individuals, be sure to clean your forceps and use fresh Pasteur pipettes between each individual. An easy way to prevent sperm cross-contamination is to rinse your forceps off with a squirt bottle of seawater, dip them into a beaker of ethanol, and then rinse them again with the squirt bottle of seawater.

3.1.2. In Vitro Fertilization

1. Place the eggs to be fertilized in an egg filter sitting in a cup half full of seawater.

2. Add a small amount of sperm. If the sperm was collected at full concentration, then 5 or 10 μL is usually enough to fertilize thousands of eggs. Polyspermy is seldom a problem, however, unless the eggs have been dechorionated.
3. Swirl the cup gently until the sperm is dispersed.
4. Wait at least 10 min for fertilization to take place, then remove the filter from the cup, invert it over a 100 mm petri dish and gently rinse the eggs into the dish with a squirt bottle of seawater.
5. Incubate embryos at 18°C. The first cleavage should occur approximately 1 h after fertilization and can easily be observed with a dissecting scope (*see* **Note 1**).

3.1.3. Natural Spawn

1. Place the ascidians to be crossed in a cup, tub, or bucket of seawater in a room or chamber with timer-controlled lighting (*see* **Note 2**).
2. *C. savignyi*: Set the timer to turn the lights *off* 6 h before the desired time of spawning and then *on* at the desired time of spawning.
   C. intestinalis: Place the animals in the dark for 30 min.
3. As the lights come on, transfer the adults to a clean container of seawater. (This step is optional, but gives cleaner eggs with less fecal matter.)
4. Spawning will typically occur roughly 15 min after the lights come *on* for *C. savignyi* and 50 min after the lights go *off* for *C. intestinalis*. Fertilization typically takes another 10 min, but may take as much as 60 min for a self-cross.
5. It is best to remove the adults after spawning to prevent them from eating their eggs.
6. At some point between fertilization and hatching, the eggs need to be moved from the spawning container to a petri dish so that they can be inspected under a dissecting microscope:
   a. Pour the water containing fertilized eggs/embryos through an egg filter.
   b. Invert the egg filter over a 100 mm petri dish and rinse the eggs/embryos into it with a squirt bottle of seawater.
7. Incubate embryos at 18°C. The first cleavage should occur approximately 1 h after fertilization and can easily be observed with a dissecting scope (*see* **Note 1**).

3.1.4. Settling Larvae onto Petri Dishes

1. At 18°C, hatching will occur approximately 17 h after fertilization (*see* **Note 1**). Rinse the hatched larvae in an egg filter by sequentially placing the filter into two cups of

fresh seawater. This removes dead sperm and fragments of chorion that may inhibit attachment.

2. Label a 150 mm petri dish on the outside rim (top and bottom) with the relevant genotype, date, etc.
3. Invert the filter over the *lid* of a large petri dish, placed concave side upward, and rinse the hatched larvae into the lid with a squirt bottle of seawater. For a typical outcross using eggs from four to six wildtypes, split the larvae between six and eight sets of dishes.
4. Fill the lid with seawater until it is nearly but not quite overflowing.
5. Float the *bottom* of the petri dish (concave side up) on top of the water in the lid (**Fig. 15.1f**). This allows larvae to attach to both surfaces and minimizes the air/water interface where larvae often become trapped.
6. Attachment and metamorphosis are somewhat variable, but most larvae will have attached and metamorphosed after 2 days.
7. There are often some healthy larvae that have, for whatever reason, failed to attach. These can often be induced to attach by collecting them in an egg filter, inverting the filter over a dry petri dish at a height of 10–12 in., and rinsing them onto the dish with a squirt bottle of seawater.
8. Some mutants are competent to undergo metamorphosis but seldom do so because they cannot swim (e.g., *aimless* (16)). These can sometimes be forced to attach by repeated dropping from a height or by manually forcing their palps against the dish with a hair loop or a fine pipette tip (e.g., gel loading tip).
9. Plates should be moved to the culture tanks 2–5 days after plating (*see* **Note 3**). Take each plate and make two holes on opposite sides of the rim with a hot soldering iron (best done in fume hood). Use a zip tie to attach a glass scintillation vial full of water to one hole. This acts as a weight to keep the plate submerged. Tie a loop of string through the other hole, to suspend the plate in the culture system (**Fig. 15.1g**). Attached juveniles can be seen as small dots on the plate when viewed at an angle (**Fig. 15.1h**).
10. Juveniles are particularly susceptible to predation by nudibranchs. If your culture system uses raw seawater, be sure to check frequently and remove any nudibranchs or their eggs.
11. The generation time depends on the temperature, feeding levels, and other environmental conditions, but sexual maturity typically takes 3–4 months (**Fig. 15.1i**).

3.1.5. Screening for Mutations in Wild Populations

1. Collect 50 or more wild *Ciona* adults.
2. Leave them under constant light for several days to accumulate gametes.
3. Take 50 adults that have both sperm and eggs and array them into 50 labeled cups of seawater. (More or fewer can be screened, but 50 is a convenient number that fits onto the top shelf of a standard lab cart and can easily be filtered in a few hours.)
4. Induce self-fertilization by natural spawning as described in **Section 3.1.3**.
5. Filter the embryos into labeled petri dishes. Be careful to ensure that the label on each dish of embryos matches the label of the cup holding the parent.
6. After hatching, examine each dish with a dissecting scope for a phenotype affecting 25% of the progeny (*see* **Note 4**) (**Fig. 15.1e**).
7. It is likely that some animals will have failed to spawn or will have spawned but the eggs will not have fertilized. If these animals still have gametes, they can be set up for another spawning cycle and retested (*see* **Note 5**). This can be done immediately for *C. savignyi* or after a 6 h wait for *C. intestinalis*.
8. Potential mutant carriers should be housed in condos and moved to the culturing system for 1–2 weeks to recover.
9. After 1 or 2 weeks, respawn the putative carriers. This second screening is important to reduce false positives due to bad clutches of eggs or other non-Mendelian causes of aberrant embryos (*see* **Notes 6** and **7**).
10. Putative carriers should be outcrossed (**Section 3.1.6**) so as to expand the line and allow testing for Mendelian inheritance in the second generation, complementation testing against previous mutations, phenotypic analysis, sperm freezing, etc. (*see* **Note 8**). Offspring from self-crosses can also be settled onto plates, but outcrosses are typically much more efficient for expanding mutant lines, and sibcrosses are better than self-crosses for testing homozygote viability.

3.1.6. Outcrossing a Mutant

1. Set up a known mutant carrier to spawn naturally.
2. Approximately 90 min before spawning, dissect eggs from several putatively wildtype *Ciona* keeping the eggs from each animal separate. Wait 1 h and check that the eggs are not cleaving. If they are cleaving, discard that batch of eggs.
3. Take a sample of eggs from each putatively wildtype animal and fertilize them with self-sperm. This is a control to

ensure that these animals are not themselves carrying any phenotypically meaningful recessive mutations.

4. When the animal starts spawning, put an egg filter (or multiple filters) containing unfertilized wildtype eggs into the container holding the known mutant carrier. As long as the top of the filter is kept above the water level, sperm can freely pass through the filter mesh to fertilize the wildtype eggs while keeping these eggs separate from the eggs spawned by the mutant adult. Alternatively, add sperm-containing seawater from the mutant (filtered through an egg filter) to dishes containing wildtype eggs.
5. After an hour, remove the egg filter and rinse the eggs into a petri dish. Check for cleavage with a dissecting scope.
6. After hatching, confirm that the control self-crosses do not show any mutant phenotype. If a phenotype is apparent, one option is to discard the outcross and try again. If the new phenotype is interesting, however, or if the outcross is particularly precious, then the outcross can be kept and screened for single and double heterozygotes.
7. After hatching, settle the outcross larvae onto petri dishes as described in **Section 3.1.4**.

3.1.7. Identifying Carriers

The simplest way to identify carriers from an outcross is to do self-crosses by spawning them individually in cups. This is reasonably effective, but there is always a variable fraction of animals that fail to self-fertilize. An efficient alternative is to add a small amount of sperm to each cup from a known carrier. This could be frozen sperm from a previous generation, fresh sperm from a previously identified carrier, or seawater in which a previously identified carrier(s) had just been naturally spawned and then strained through an egg filter to remove any eggs.

Alternatively, a small amount of sperm-containing seawater (~50 mL) can be removed from each cup 25 min after the lights come on. This can then be mixed, strained through an egg filter to remove any eggs, and redistributed to each cup. This gives the eggs in each cup an opportunity to self-fertilize, and then a second opportunity to fertilize with sperm mixed from all of the potential carriers. As there should be 50% carriers in a typical outcross, 25% of the sperm in the mixed solution should carry the mutation, giving ~1/8 mutant progeny if an animal is a carrier but failed to self-fertilize (*see* **Note 9**).

3.1.8. Obtaining Large Numbers of Mutant Embryos

Phenotypic analyses and genetic mapping benefit from having large numbers of mutant embryos. Dissected crosses between multiple carriers work well, but will quickly reduce your numbers of identified carriers. The best way to get as many embryos

as possible without killing any carriers is to do large group spawns. Five to ten gravid carriers can be placed in a tub or bucket and spawned en masse, giving rise to thousands of mutant embryos. These large group spawns typically give very good fertilization rates.

An alternative method if you do not have large numbers of known carriers is to do bulk spawns of outcrosses before they have been identified. A whole plate of animals derived from an outcross can be spawned together, giving rise to extremely large numbers of offspring with ~1/16 being mutant.

3.1.9. Freezing Sperm

1. Aliquot 80 μL sperm freezing solution into each of several 1.0 mL cryovials.
2. Collect as much clean, dry sperm as possible by dissection (**Section 3.1.1**).
3. Add 20 μL sperm to each tube, gently mixing the sperm with the sperm freezing solution.
4. Gradually freeze each tube in a Styrofoam rack floating on liquid nitrogen.
5. After 10 min, store the tubes in liquid nitrogen (*see* **Note 10**).

3.1.10. Thawing Sperm

1. Remove one tube of frozen sperm from liquid nitrogen.
2. Quickly thaw the frozen sperm by pipetting 1 mL of seawater up and down in the tube.
3. Quickly add the thawed sperm to unfertilized eggs.

3.2. Linkage Mapping and Positional Cloning

The first ascidian mutations to be positionally cloned were mapped by AFLP analysis of bulked pools of phenotypically wildtype versus mutant tadpoles (16). AFLP remains a viable option, but has largely been supplanted in our lab by mapping SNPs by direct sequencing. The *Ciona* genomes are sufficiently polymorphic that most ~500 bp PCR products are expected to contain at least one SNP.

3.2.1. SNP Mapping

Initial mapping is best performed on pools of DNA extracted from either mutant tadpoles (at least 20, with 50–100 being better) or an equivalent number of phenotypically wildtype siblings (**Fig. 15.3**). Initial mapping should use embryos derived from a single selfed individual, so that no more than two alleles are present at any marker. The two pools of DNA are then used as templates for a panel of PCR reactions using primer pairs designed to span the genome. We have had good results with a *C. intestinalis* panel consisting of two primer pairs evenly spaced on each chromosome arm and with a *C. savignyi* panel with a primer pair near the center of each of the largest 96 reftigs. Direct sequencing

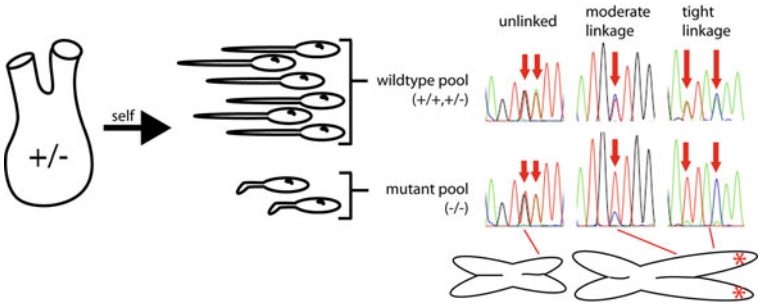

Fig. 15.3. Mapping by direct sequencing of SNPs. Initial linkage can be detected by preparing pools of DNA from phenotypically wildtype versus mutant individuals from a single selfed heterozygote. Use the two pools as templates for PCR reactions using primer pairs evenly spaced through the genome. SNPs can be detected as double peaks in the sequencing chromatogram for the wildtype pool. The ratio of these peak heights is repeatable for a given pool but varies considerably from SNP to SNP through sequence-specific variation in polymerase incorporation efficiency. Linkage can be detected as a variation in the relative peak heights in the mutant pool versus the wildtype pool. This becomes more extreme with markers increasingly close to the mutant locus (*asterisk*).

of the PCR products can then be used to look for SNPs in the wildtype pool. If the marker is linked to the mutant locus, it will be visible as a change in the relative chromatogram peak heights for the two SNP alleles in the mutant pool versus the wildtype pool. In the case of tight linkage, only one peak will be visible. This is typically the smaller of the two alleles in the wildtype pool, as the SNP allele linked in *cis* with the mutant allele will be under-represented in the wildtype pool.

A good metric for quantifying linkage in this assay is to take the ratio of the relative peak heights for the two pools $(H_{\mathrm{mutA}}/H_{\mathrm{mutB}})/(H_{\mathrm{wtA}}/H_{\mathrm{wtB}})$. This normalizes for context-dependent variation in incorporation frequency by the sequencing polymerase. This method is only semiquantitative, however, and potential weak linkage needs to be confirmed by testing other linked and unlinked markers.

Once initial linkage to a chromosome arm or genomic scaffold has been determined, a finer-grained panel of primer pairs should be designed against that region (keeping in mind that the mutant locus may still be quite far away…). This can be used to narrow the candidate region to a region of tight linkage flanked by markers showing appreciable recombination.

SNP mapping by direct sequencing of PCR products from large pools is ultimately limited by the inability to determine small differences in allele frequency as a function of chromatogram peak height (*see* **Note 11**). For finer-scale mapping, it is necessary to switch to smaller pool sizes (we have been able to detect a single recombinant in a pool of five tadpoles) or else to perform single tadpole PCR (**Section 3.2.3**).

3.2.2. Refining the Candidate Gene List

Once the critical interval has been narrowed by recombination mapping to ~500 kb, it becomes feasible to begin examining candidate genes (although if mutant tadpoles are plentiful, finer genetic mapping will always be the most unambiguous way of narrowing the field of candidates). A 500 kb interval typically contains ~50 genes. The genome browsers at Ensembl and Aniseed provide good starting points for inspecting the region and searching for obvious candidates.

The *C. intestinalis* EST and in situ collections are often helpful for tentatively narrowing the candidate list. These databases can also be helpful for *C. savignyi* after identifying the orthologous gene.

For all of the mutations we have cloned so far, the mutant gene transcript is absent or downregulated compared to wildtype (15–17). This likely reflects a nonsense-mediated decay mechanism. Although not definitive, this is another important method for narrowing the candidate list.

Once a strong candidate is apparent, the coding regions should be amplified in overlapping segments from mutant genomic DNA and sequenced to look for mutations. Keep in mind that *Ciona* are extremely polymorphic, so a polymorphism that does not cause a severe predicted truncation or interfere with a known active site, etc., will need rigorous functional confirmation by rescue or knockdown.

3.2.3. Single Tadpole/Small Pool DNA Extraction for PCR

1. Fix embryos by adding 1/10 volume of 37% formaldehyde; 10 min of fixation is sufficient, and more is potentially harmful.
2. Wash the embryos twice with deionized water. The test cells, a maternally derived cell type on the outside of the embryo, should fall off (*see* **Note 12** and **Fig. 15.1j**, **k**). If they do not, move the embryos to an egg filter and rinse them with a stream of water from a squirt bottle.
3. The fixed embryos are sticky, so move them to agarose-coated dishes and use BSA-coated pipette tips.
4. The fixed embryos can be kept at 4°C for several days. This can be helpful if large numbers of mutant and wildtype siblings need to be sorted.
5. Transfer single tadpoles to individual PCR tubes (or plate wells) in 5 μL of water. For small pools, vary the volumes accordingly.
6. Add 5 μL STL buffer and 1 μL of 10 mg/mL proteinase K.
7. Seal well and incubate for at least 12 h at 55°C.
8. Heat to 95°C for 10 min to inactivate the proteinase. Spin down and store at –20°C.

9. 1/5 of an embryo equivalent is usually sufficient for robust amplification.

3.2.4. Large-Scale Purification of Genomic DNA

Although standard phenol/chloroform preps can be used, we have obtained much better yields using a modified guanidinium/glass milk procedure:

1. Add ~1,000 embryos (fixed or unfixed) to a 1.5 mL tube (use the tube that comes with a disposable plastic pestle).
2. Centrifuge at 400×*g* to pellet embryos and remove as much seawater as possible.
3. Add 200 μL 1× STL buffer and 20 μL 10 mg/mL proteinase K. Homogenize with disposable plastic pestle.
4. Incubate lysate at 55°C for at least 12 h.
5. Add 600 μL GuSCN buffer and 10 μL resuspended glass milk to the lysate.
6. Incubate 10 min at room temperature, briefly vortexing every few minutes.
7. Centrifuge for 30 s at 12,000×*g*. Discard the supernatant to GuSCN waste.
8. Wash pellet once by adding 200 μL GuSCN, resuspend by brief vortexing, spin down for 30 s at 12,000×*g*, and discard the supernatant.
9. Wash pellet twice as above with 200 μL 70% ethanol.
10. Wash once with 200 μL acetone.
11. Dry the pellet by leaving the tube lid open for 10–15 min at 55°C.
12. Add 20 μL water. Resuspend and heat at 55°C for 10 min.
13. Vortex and centrifuge for 5 min at 12,000×*g*.
14. Remove the supernatant to a new tube and store at –20°C (*see* **Note 13**).

3.3. Injections

Although not strictly a genetic method, microinjections can be invaluable in confirming candidate loci by rescue or morpholino knockdown.

Ciona eggs are small and have a tough, elastic cortex that can be difficult to pierce. Once pierced, however, the eggs are quite delicate, and lyse easily. *C. savignyi* eggs can be injected through the chorion, whereas *C. intestinalis* eggs have a tougher chorion and are best dechorionated before injection. Here we will focus on injecting *C. savignyi* eggs through the chorion.

We pull needles from 1 mm thin wall borosilicate tubing with a filling fiber on a Sutter P-80 horizontal puller. Good needles need to be tested empirically for their ability to pierce the

egg without lysing it. Fill needles from the back with a capillary loading pipette tip and let the injection solution wick to the front of the needle by capillary action. Once the injection pipette is mounted in the injection rig, it can be broken open by gently tapping it against the wall of a scratch made in the floor of the injection petri dish using a pair of fine forceps.

A holding pipette is used to keep the egg in place for microinjection. These are pulled by hand. First roll the center of a length of capillary tubing in a Bunsen burner flame. When it starts to soften, remove it from the flame, and quickly pull it apart by ~3 cm. Cut the capillary in half, at the center of the thinned central section, by scoring and snapping. Take one half and repeatedly but briefly dip the thin end into the flame to constrict the tip. Check with a dissecting scope for the desired shape (**Fig. 15.4d**). Lastly, form a hockey stick-shaped bend in the pipette by holding it briefly in the edge of the flame until it softens and droops.

We use a dissecting scope with epifluorescence optics mounted with two micromanipulators (**Fig. 15.4a**, **b**). The left manipulator is a basic one that is only used to position the holding pipette. The right manipulator is a four-axis oil hydraulic manipulator. The holding pipette is connected to a 10 mL syringe to manually provide a slight negative holding pressure. The injection pipette is connected to a custom-modified pneumatic microinjector.

The main peculiarity about injecting *Ciona* eggs is that they have such a tough, elastic egg cortex that they cannot be injected

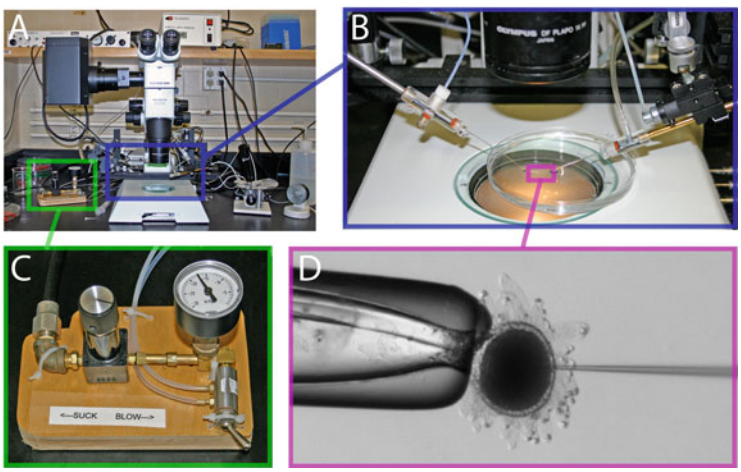

Fig. 15.4. Microinjection of *Ciona savignyi* eggs. (**a**) Overview of injection station. (**b**) Closeup showing the holding pipette (*left*) and injection pipette (*right*). (**c**) Closeup of the homemade vacuum/pressure switching device. (**d**) High-magnification view of injection, showing the relative proportions of the egg (surrounded by the chorion and an outer layer of follicle cells), the holding pipette, and the injection pipette.

by pressure alone, but require an initial pulse of vacuum to break the egg cortex. Without this pulse of vacuum, it can appear that you are injecting into the cytoplasm, but the injected material either remains confined to a membrane-bound vesicle or else leaks back out of the egg. Traditionally, both the positive and negative pressures were supplied manually with a syringe. We have obtained significantly better results using a pneumatic microinjector modified to provide pulses of vacuum as well as positive pressure.

The Picospritzer brand of microinjector uses a single external solenoid valve box that normally controls the flow of air between a pressure regulator and the needle holder. We added a toggle-controlled pneumatic switch allowing the source to the solenoid valve to be switched between the normal regulated positive pressure and a source of regulated vacuum. When the toggle is set to "Blow" the microinjector acts as a normal, pneumatic microinjector. When the toggle is set to "Suck," the microinjector delivers pulses of vacuum (**Fig. 15.4c**).

To inject an egg, position the holding pipette in the middle of the field of view and move the petri dish around until an egg is near its tip. Pull back a little on the syringe connected to the holding pipette to capture the egg against the holding pipette. Use the XYZ joystick controls of the hydraulic manipulator to move the needle tip in line with the center of the egg (this will put the tip somewhat above and to the right of the egg). Use the axial control to slowly advance the needle tip through the chorion and into the center of the egg (**Fig. 15.4d**). Set the toggle to "Suck" and apply vacuum until a small amount of egg cytoplasm can be seen moving into the needle tip. Switch the toggle to "Blow" and apply small pulses of positive pressure until the injected droplet is roughly 1/8 the diameter of the egg. Use the axial advance to rapidly remove the needle from the egg. Push forward a little on the holding pipette syringe to release the injected egg. Move it to a new dish with a P20 micropipette.

Injections are typically performed with 0.2% Fast Green (which fluoresces in the red) or labeled dextran as a marker dye. Epifluorescence optics on the injection scope are helpful for confirming injection, but not strictly necessary. Fast Green fluorescence is typically not visible while it is being injected, but appears rapidly as it diffuses into the egg cytoplasm. Injection concentrations need to be titrated individually, but DNA is typically injected at 30–100 ng/μL, RNA at 20–60 ng/μL, and morpholinos at 0.05–0.2 mM. The injected volume can be estimated as a function of the radius of the injected droplet, but as this varies with the cube of the radius it is quite imprecise and should be thought of as only semi-quantitative.

4. Notes

1. 18°C is a good default temperature for culturing *Ciona* embryos, but for experimental convenience they can also be cultured as cool as 12°C to slow down development.
2. We normally perform natural spawnings in an environmental chamber kept at 17°C. Room temperature is generally fine in a cool, air-conditioned room, but it is best to keep *Ciona* between 12 and 20°C.
3. There is considerable flexibility in when you move outcross plates to the culture facility. One can do so as soon as a sufficient number of larvae have attached to the plate, but it is sensible to wait for another 2 days or so to ensure that they undergo metamorphosis. It is best to move them within 5 days of metamorphosis, however, as they need to start eating. The water in the dish can be changed every 2–3 days.
4. Subtle phenotypes can be difficult to identify if the larvae are actively swimming. They can be anesthetized by adding a few milliliters of 0.2% MS-222 (tricaine) to the dish.
5. In our experience, it is extremely common for a second spawning to give fertilized eggs when the first spawning did not. This is potentially because both sperm and eggs are not always released with every light/dark cycle.
6. Typical mutant phenotypes include short tails, club-shaped heads, and pigmentation defects. There are several types of phenotypes that we have found to rarely show Mendelian inheritance. These include tails with multiple severe kinks, and tails that are only slightly shorter than wildtype. These phenotypes typically present at either significantly less than or more than the expected 1:3 ratio, which is another sign that they are not Mendelian.
7. An outcross can be performed simultaneously with the second screening by using filtered, sperm-containing water from the self-cross to fertilized wildtype eggs. This is sensible if the founder adult seems at all unhealthy or the putative mutation is particularly precious. If the second screening is negative, then the outcross can simply be discarded.
8. It is always a quandary whether to keep the founder animal alive as long as possible for further experimentation or whether to kill it and freeze its sperm as a precaution against losing the line. This is left to the researcher's discretion and should depend on the apparent relative robustness of the founder animal versus its outcrosses.

9. One issue with this method is that it may not reliably detect a second mutation inadvertently segregating in the animals being screened if it is present at much less than 25%. Such mutations should be unusual, however, if the wildtypes used for outcrossing are confirmed to be truly wildtype.
10. It is a good idea to test that the freezing was successful by thawing one aliquot and confirming that it can fertilize eggs.
11. The ability to detect a single recombination event in pooled DNA is limited by noise in the chromatogram and by contaminating maternal DNA (*see* **Note 12**). If the chromatogram is extremely clean and there is no maternal contamination, then it is possible to detect a minor peak that is a tenth the size of the major peak (i.e., a single recombinant in a pool of five embryos). That is probably the best-case scenario, however, and smaller pools are more robust. Single tadpole PCR provides the most unambiguous results, but requires more sequencing to examine the same number of meioses.
12. There is a population of maternally derived cells known as test cells that are found between the embryo and the chorion. After hatching, the test cells are found in the larval tunic on the outside of the larva (**Fig. 15.1j**, **k**). Unless removed, the test cells are a source of contaminating maternal DNA that may confound genotyping assays. The test cells can be removed by dechorionating fertilized eggs, but dechorionation is a relatively harsh procedure that often gives a background of perturbed embryos. Alternatively, formaldehyde fixation has the counterintuitive property of loosening the test cells so that they can be washed off the embryo.
13. This prep copurifies large amounts of RNA with the DNA. If RNA-free DNA is required, treat the sample with RNAse and then ethanol precipitate.

References

1. Delsuc, F., Brinkmann, H., Chourrout, D., and Philippe, H. (2006) Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **439**, 965–968.
2. Imai, K.S., Levine, M., Satoh, N., and Satou, Y. (2006) Regulatory blueprint for a chordate embryo. *Science* **312**, 1183–1187.
3. Passamaneck, Y.J. and Di Gregorio, A. (2005) Ciona intestinalis: chordate development made simple. *Dev. Dyn.* **233**, 1–19.
4. Lemaire, P., Smith, W.C., and Nishida, H. (2008) Ascidians and the plasticity of the chordate developmental program. *Curr. Biol.* **18**, R620–631.
5. Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D.M., et al. (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* **298**, 2157–2167.

6. Satou, Y., Mineta, K., Ogasawara, M., Sasakura, Y., Shoguchi, E., Ueno, K., Yamada, L., Matsumoto, J., Wasserscheid, J., Dewar, K., et al. (2008) Improved genome assembly and evidence-based global gene model set for the chordate *Ciona intestinalis*: new insight into intron and operon populations. *Genome Biol.* **9**, R152.
7. Small, K.S., Brudno, M., Hill, M.M., and Sidow, A. (2007) A haplome alignment and reference sequence of the highly polymorphic *Ciona savignyi* genome. *Genome Biol.* **8**, R41.
8. Vinson, J.P., Jaffe, D.B., O'Neill, K., Karlsson, E.K., Stange-Thomann, N., Anderson, S., Mesirov, J.P., Satoh, N., Satou, Y., Nusbaum, C., et al. (2005) Assembly of polymorphic genomes: algorithms and application to *Ciona savignyi*. *Genome Res.* **15**, 1127–1135.
9. Hino, K., Satou, Y., Yagi, K., and Satoh, N. (2003) A genomewide survey of developmentally relevant genes in *Ciona intestinalis*. VI. Genes for Wnt, TGFbeta, Hedgehog and JAK/STAT signaling pathways. *Dev. Genes Evol.* **213**, 264–272.
10. Sasakura, Y., Shoguchi, E., Takatori, N., Wada, S., Meinertzhagen, I.A., Satou, Y., and Satoh, N. (2003) A genomewide survey of developmentally relevant genes in *Ciona intestinalis*. X. Genes for cell junctions and extracellular matrix. *Dev. Genes Evol.* **213**, 303–313.
11. Satou, Y., Sasakura, Y., Yamada, L., Imai, K.S., Satoh, N., and Degnan, B. (2003) A genomewide survey of developmentally relevant genes in *Ciona intestinalis*. V. Genes for receptor tyrosine kinase pathway and Notch signaling pathway. *Dev. Genes Evol.* **213**, 254–263.
12. Wada, S., Tokuoka, M., Shoguchi, E., Kobayashi, K., Di Gregorio, A., Spagnuolo, A., Branno, M., Kohara, Y., Rokhsar, D., Levine, M., et al. (2003) A genomewide survey of developmentally relevant genes in *Ciona intestinalis*. II. Genes for homeobox transcription factors. *Dev. Genes Evol.* **213**, 222–234.
13. Dehal, P. and Boore, J.L. (2005) Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol* **3**, e314.
14. Hughes, A.L. and Friedman, R. (2005) Loss of ancestral genes in the genomic evolution of *Ciona intestinalis*. *Evol. Dev.* **7**, 196–200.
15. Chiba, S., Jiang, D., Satoh, N., and Smith, W.C. (2009) Brachyury null mutant-induced defects in juvenile ascidian endodermal organs. *Development* **136**, 35–39.
16. Jiang, D., Munro, E.M., and Smith, W.C. (2005) Ascidian prickle regulates both mediolateral and anterior-posterior cell polarity of notochord cells. *Curr. Biol.* **15**, 79–85.
17. Veeman, M.T., Nakatani, Y., Hendrickson, C., Ericson, V., Lin, C., and Smith, W.C. (2008) *Chongmague* reveals an essential role for laminin-mediated boundary formation in chordate convergence and extension movements. *Development* **135**, 33–41.
18. Jiang, D. and Smith, W.C. (2005) Self- and cross-fertilization in the solitary ascidian *Ciona savignyi*. *Biol. Bull.* **209**, 107–112.
19. Harada, Y., Takagaki, Y., Sunagawa, M., Saito, T., Yamada, L., Taniguchi, H., Shoguchi, E., and Sawada, H. (2008) Mechanism of self-sterility in a hermaphroditic chordate. *Science* **320**, 548–550.
20. Hendrickson, C., Christiaen, L., Deschet, K., Jiang, D., Joly, J.S., Legendre, L., Nakatani, Y., Tresser, J., and Smith, W.C. (2004) Culture of adult ascidians and ascidian genetics. *Meth. Cell Biol.* **74**, 143–170.
21. Small, K.S., Brudno, M., Hill, M.M., and Sidow, A. (2007) Extreme genomic variation in a natural population. *Proc. Natl. Acad. Sci. USA* **104**, 5698–5703.
22. Kim, J.H., Waterman, M.S., and Li, L.M. (2007) Diploid genome reconstruction of *Ciona intestinalis* and comparative analysis with *Ciona savignyi*. *Genome Res.* **17**, 1101–1110.
23. Shoguchi, E., Kawashima, T., Satou, Y., Hamaguchi, M., Sin, I.T., Kohara, Y., Putnam, N., Rokhsar, D.S., and Satoh, N. (2006) Chromosomal mapping of 170 BAC clones in the ascidian *Ciona intestinalis*. *Genome Res.* **16**, 297–303.
24. Hill, M.M., Broman, K.W., Stupka, E., Smith, W.C., Jiang, D., and Sidow, A. (2008) The *C. savignyi* genetic map and its integration with the reference sequence facilitates insights into chordate genome evolution. *Genome Res.* **18**, 1369–1379.
25. Satou, Y., Takatori, N., Fujiwara, S., Nishikata, T., Saiga, H., Kusakabe, T., Shin-i, T., Kohara, Y., and Satoh, N. (2002) *Ciona intestinalis* cDNA projects: expressed sequence tag analyses and gene expression profiles during embryogenesis. *Gene* **287**, 83–96.
26. Satou, Y., Yamada, L., Mochizuki, Y., Takatori, N., Kawashima, T., Sasaki, A., Hamaguchi, M., Awazu, S., Yagi, K., Sasakura, Y., et al. (2002) A cDNA resource from the basal chordate *Ciona intestinalis*. *Genesis* **33**, 153–154.
27. Imai, K.S., Hino, K., Yagi, K., Satoh, N., and Satou, Y. (2004) Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards

a comprehensive understanding of gene networks. *Development* **131**, 4047–4058.
28. Kusakabe, T., Yoshida, R., Kawakami, I., Kusakabe, R., Mochizuki, Y., Yamada, L., Shin-i, T., Kohara, Y., Satoh, N., Tsuda, M., et al. (2002) Gene expression profiles in tadpole larvae of *Ciona intestinalis*. *Dev. Biol.* **242**, 188–203.
29. Sasakura, Y. (2007) Germline transgenesis and insertional mutagenesis in the ascidian *Ciona intestinalis*. *Dev. Dyn.* **236**, 1758–1767.
30. Deschet, K., Nakatani, Y., and Smith, W.C. (2003) Generation of Ci-Brachyury-GFP stable transgenic lines in the ascidian *Ciona savignyi*. *Genesis* **35**, 248–259.
31. Zeller, R.W., Virata, M.J., and Cone, A.C. (2006) Predictable mosaic transgene expression in ascidian embryos produced with a simple electroporation device. *Dev. Dyn.* **235**, 1921–1932.
32. Nydam, M.L. and Harrison, R.G. (2007) Genealogical relationships within and among shallow-water *Ciona* species (Ascidiacea). *Mar. Biol.* **151**, 1839–1847.
33. Hoshino, Z. and Nishikawa, T. (1985) Taxonomic studies of *Ciona intestinalis* (L.) and its allies. *Publ. Seto Mar. Biol. Lab* **30**, 61–79.
34. Silva, N. and Smith, W.C. (2008) Inverse correlation of population similarity and introduction date for invasive ascidians. *PLoS One* **3**, e2552.
35. Dybern, B.I. (1965) The life cycle of *Ciona intestinalis* (L.) f. typica in relation to the environmental temperature. *Oikos* **16**, 109–131.
36. Joly, J.S., Kano, S., Matsuoka, T., Auger, H., Hirayama, K., Satoh, N., Awazu, S., Legendre, L., and Sasakura, Y. (2007) Culture of *Ciona intestinalis* in closed systems. *Dev. Dyn.* **236**, 1832–1840.
37. Suzuki, M.M., Nishikawa, T., and Bird, A. (2005) Genomic approaches reveal unexpected genetic divergence within *Ciona intestinalis*. *J. Mol. Evol.* **61**, 627–635.

Chapter 16

Analyses of Gene Function in Amphioxus Embryos by Microinjection of mRNAs and Morpholino Oligonucleotides

Linda Z. Holland and Takayuki Onai

Abstract

The invertebrate chordate amphioxus (*Branchiostoma*), which is the most basal living chordate, has become an accepted model for the vertebrate ancestor in studies of development and evolution. Amphioxus resembles vertebrates in regard to morphology, developmental gene expression, and gene function. In addition, the amphioxus genome has representatives of most vertebrate gene families. Although it has not undergone the two rounds of whole genome duplications that occurred early in the vertebrate lineage, the amphioxus genome has retained considerable synteny with vertebrate genomes. Thus, studies of genes and development in amphioxus embryos can reveal the fundamental genetic basis of the vertebrate body plan, giving insights into the developmental mechanisms of such organs as the somites, pharynx, kidney, and the central nervous system. Moreover, amphioxus is very useful for understanding how these characters evolved. This chapter details methods for microinjection of amphioxus eggs with mRNAs or morpholino antisense oligonucleotides to analyze gene networks operating in early development.

Key words: Amphioxus, gene network, microinjection, *Branchiostoma*, morpholino oligonucleotides, gene function.

1. Introduction

Over the last 20 years, the basal chordate amphioxus (*Branchiostoma*) has been shown to be an excellent simple model for understanding the genetic basis of chordate development. Amphioxus is a small (3–5 cm long) marine organism that lives burrowed in the sand with only its anterior end exposed for filter feeding (**Fig. 16.1**). There are about 28–30 species of *Branchiostoma* worldwide, of which 3, *B. floridae* (from the southeastern USA),

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_16,

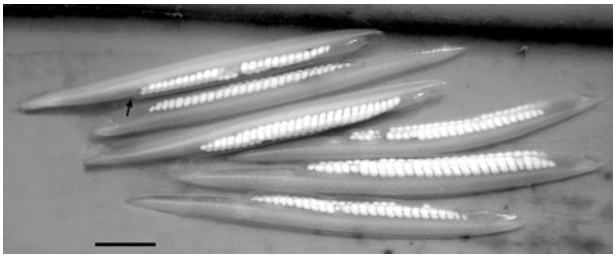

Fig. 16.1. Six ripe females of *Branchiostoma floridae*. The anterior is to the right except for the second animal from the top. Each animal has 26–28 gonads on the right side and an equal number on the left. Occasionally, one or two gonads fail to develop as in the animal at the top and the third from the bottom. The *arrow* indicates the atriopore through which the gametes are expelled at spawning. Scale bar = 0.5 cm.

B. lanceolatum (from Europe), and *B. belcheri* (from China, Taiwan and Japan) are commonly used in developmental studies. Although the amphioxus and vertebrate lineages split about 520 mya, amphioxus is evolving relatively slowly. Its genome has retained remarkable synteny with those of vertebrates (1) even though it has not undergone the two rounds of whole genome duplication that occurred early in the vertebrate lineage (2). Amphioxus has also retained a vertebrate-like body plan with a dorsal, hollow nerve cord, segmental muscles, notochord, kidney, and simple heart. Moreover, early embryonic patterning mechanisms in amphioxus are largely comparable to those of vertebrates. For example, amphioxus has a probable homolog of the vertebrate gastrular (Spemann's) organizer (3) and uses Wnt/β-catenin signaling to specify posterior identity and retinoic acid signaling to specify position along the anterior/posterior axis (4, 5). However, a major difference between amphioxus and vertebrates is that while amphioxus has in place much of the gene network for specification of premigratory neural crest, the genes specifying migration of neural crest and differentiation into many cell types were only recruited to this network at the base of the vertebrates (6). Thus, amphioxus lacks neural crest and all neural crest derivatives such as cartilage and bone, pigment cells (except for pigment cells associated with photo-receptors in the central nervous system), and the cranial ganglia (7). This lack of neural crest greatly facilitates studying the roles of the three major tissue layers—endoderm, ectoderm, and mesoderm—in patterning the early embryo.

Techniques for manipulating amphioxus embryos have been slow to be developed, in large part because amphioxus has not yet been put into continuous breeding culture in the laboratory. Therefore, embryos can only be obtained during the summer breeding season. One difficulty with amphioxus is that, like vertebrates, amphioxus stores primary oocytes, which on the day of

spawning undergo the meiotic divisions and arrest at second meiotic metaphase. In natural populations, falling light levels after sunset trigger the emergence of the ripe adults from the sand followed by the shedding of gametes. Therefore, until the last 20 years, the only way to obtain embryos from amphioxus was from natural spawnings. Although the development of isolated blastomeres of *B. lanceolatum* was studied in the late nineteenth century (8, 9) and for *B. belcheri* in the early 1960s (10–13), studies of amphioxus embryos were few until the late 1980s when it was discovered that adults of *B. floridae* collected in the afternoon of a day on which they would normally spawn, would not spawn if kept in the light but could then be induced to spawn during the night whenever desired by a mild electric shock (14). This allowed the experimenter to control the time of fertilization, which has in turn allowed the development of methods for microinjection. What is still needed is spawning of amphioxus year-round in the laboratory at the whim of the researcher. At present, several laboratories are working toward that goal for each of the three species. For *B. lanceolatum*, spawnings have been induced a month or so before the normal summer breeding season by shifting the temperature from the normal 17° to 21°C. Thirty-six hours after this shift, ripe animals spawn (15). To date, for *B. belcheri*, biologists are relying on natural spawnings. This species has been raised through two generations in the laboratory, but the animals, located near a window, only spawned in the laboratory about the same date that they would have spawned in the field (16). For warm water populations living at 30°C such as *B. floridae* or *B. belcheri* in southern China, a temperature shift cannot be done since 35°C is lethal. However, these warm water animals are the ones of choice for culturing since their development is rapid and the embryos can be raised from 22° to 30°C, the rate of development being directly proportional to the temperature (17). In the field, *B. floridae* embryos from spawnings in June can reach sexual maturity and breed in August, opening the possibility of developing genetic strains once they are brought into breeding condition in the laboratory (18). Embryos of *B. floridae* can be easily raised to adults in the laboratory in about 3–4 weeks at 30°C (17, 19). Recently, it has been shown that metamorphosis, which is gradual with retention of most larval structures in the adult, can be induced prematurely by application of T4 thyroid hormone (20). Individuals of *B. floridae* typically reproduce every 10–14 days throughout the summer, and because local populations of *B. floridae* are often out of phase with one another as long as the water temperature stays from 28° to 30°C, gametes can typically be obtained every 3 or 4 days throughout the summer. Consequently, all of the available techniques for studying the genetic basis of development in amphioxus (e.g., in situ hybridization, overexpression of genes and knockdown of gene function,

and detection of apoptosis and cell division) have been developed in *B. floridae* (19, 21–23). Techniques for in situ hybridization (23, 24) have also been applied to *B. lanceolatum* (25) and *B. belcheri* (26), and as the eggs and embryos of all species of *Branchiostoma* are nearly identical, it is likely that other techniques developed for one species will be applicable to the others.

The unfertilized egg of *B. floridae* is about 140 μm in diameter and has an egg coat about 1 μm thick (14, 27). At fertilization, cortical granules expel their contents into the perivitelline space; however, after the cortical reaction, the egg continues to secrete material. Therefore, the fertilization envelope requires about 20 min to elevate fully. As a result of the wide perivitelline space, the diameter of the zygote plus extracellular coats is about 450 μm. If the fertilization envelope is removed before it is fully elevated, the zygotes stick to one another due to the material still being secreted. Since the egg is free to rotate within the fully elevated fertilization envelope, it is not possible to inject fertilized eggs with the envelope intact. Therefore, we developed techniques for injecting unfertilized eggs. This allows the investigator ample time to inject 200–300 eggs before fertilizing them.

2. Materials

2.1. Gamete Collection

1. Sieve (**Fig. 16.2**).
2. Shovel and bucket.
3. Small dip net (available at any pet store or aquarium supply store).
4. Waterproof flashlight.
5. Dissecting microscope.
6. Fiber optic light.
7. Neurophysiology stimulator [the model is unimportant; the least expensive is the Grass model SD9 (Grass-Telefactor division of Astro-Med Inc., Astromed Industrial Park, 600 East Greenwich Avenue, West Warwick, RI 02893, USA)] fitted with stainless steel or platinum electrodes.
8. Plastic disposable cocktail glasses (available at most supermarkets).
9. Plastic disposable transfer pipettes (Fisher Scientific, Pittsburgh, PA) with the tip cut off to a diameter (about 4 mm) just larger than that of an amphioxus.
10. Seawater filtered through Whatman no. 1 paper.

Fig. 16.2. Sieve made of 1″ × 4″ boards for collecting amphioxus in fine sand. The dimensions of the sieve are 12″ wide by 18″ long. Nylon window screening [standard mesh with 18 × 16 openings/in. (2.54 cm)] is fastened over the wooden frame with a staple gun. Wire mesh with 1/4–1/3″ meshes is nailed over the window screen. Thin moldings are nailed over the edges of the screen to hold it in place and hide sharp edges.

2.2. Synthetic Messenger RNA

1. pCS2+ vector developed by D. Turner at the University of Michigan, Ann Arbor, MI, USA (http://sitemaker.umich.edu/dlturner.vectors). This vector has a strong promoter and the SV40 late polyadenylation site.
2. Clones of tdTomato, a red fluorescent protein, developed by Roger Tsien (http://www.tsienlab.ucsd.edu/Samples.htm), can be obtained from Clontech Laboratories, Mountain View, CA, USA, and subcloned into pCS2+.
3. A long-range PCR kit (e.g., the Expand Long Template PCR System from Roche Applied Sciences, Indianapolis, IN, USA).
4. mMessage mMachine High Yield Capped RNA Transcription Kit (Ambion/Applied Biosystems, Austin, TX, USA).
5. 25:24:1 phenol:chloroform:isoamyl alcohol.
6. RNase-free water (0.5 mL diethyl-pyrocarbonate/500 mL deionized water; shake and let stand for 12 h or longer;

autoclave to eliminate DEPC). Can also be purchased commercially.

7. 100% ethanol.
8. 5 M NaCl.
9. Proteinase K (10 mg/ml stock) (Sigma/Aldrich, St. Louis MO, USA).

2.3. Antisense Morpholino Oligonucleotides

1. Antisense morpholino oligonucleotides (Gene Tools, Philomath, OR, USA) are designed either against sequences near the ATG start codon to block translation or to splice junctions to interfere with splicing. Gene Tools offer a free morpholino design service.

2.4. Polylysine-Coated Injection Dishes

1. 60 mm × 15 mm tissue culture dishes (Falcon 3002; Becton Dickinson, Franklin Lakes, NJ, USA).
2. Poly-L-lysine hydrobromide (30,000–70,000 mol. wt) dissolved in distilled water at 0.25 mg/mL (Sigma/Aldrich, St. Louis, MO, USA).

2.5. Injection Needles

1. Horizontal micropipette puller (Flaming/Brown) with a 2.5 mm filament (e.g., Sutter Instruments P-87 or P-97).
2. Borosilicate glass capillary tubing with inner filament 1.0 mm OD × 0.75 mm ID (e.g., from Frederick Haer & Co., Bowdoinham, ME, USA).

2.6. Microinjection

1. Dissecting microscope.
2. Air-driven microinjector (Picospritzer; General Valve division of Parker-Hannifin, Fairfield, NJ, USA) with a foot pedal. Must be able to deliver picoliter amounts.
3. Micromanipulator (e.g., Narishige M-3333 micromanipulator, Tritech Research Inc., Los Angeles, CA, USA). A micromanipulator with a joystick may facilitate injections, but the small ones are adequate.
4. pH 8.0 water: Sterile water is adjusted to pH 8.0 with a drop of concentrated NH_4OH. Check pH by spotting a drop on pH paper.
5. Texas Red dextran 10,000 mw (Molecular Probes/Invitrogen, Eugene, OR, USA).
6. Microelectrode holder model MPHPS (E. W. Wright Co., Guilford, CT, USA)
7. Antibiotic stock solution: 100 mg/mL penicillin (Na salt) and 200 mg/mL streptomycin sulfate.
8. PFA/MOPS fixative: 4% paraformaldehyde, 0.1 M MOPS buffer (3[*N*-morpholino] propanesulfonic acid), 0.5 M NaCl, 2 mM $MgSO_4$, 1 mM EGTA [ethylene glycol-bis

(β-amino ethyl ether) *N*, *N*, *N′*, *N′*-tetraacetic acid], pH 7.4. Be sure to buy powdered paraformaldehyde, not granular, to facilitate dissolving. For 50 mL PFA/MOPA fix, dissolve 2 g paraformaldehyde in 5 mL 1 N NaOH by heating to 60°C. Add 40 mL MOPS buffer pH 7.4 and add 5 mL 1 N HCl. Check pH and adjust if necessary.

3. Methods

The methods listed here are specifically for *B. floridae* collected from Old Tampa Bay, Florida, USA, where local populations occur in shallow water in most areas with a sandy bottom. Depending on water temperature, males and females of *B. floridae* spawn at intervals of 9–17 days with an average of 14 days. Spawning does not occur if the water temperature is less than 28°C. Therefore, the breeding season typically begins sometime between late May and late June and extends into early September. Gametes spawned early in the summer can develop into breeding adults by mid-August. Local populations are labile depending on the amount of rainfall, the intensity of storms, and the population of sting rays, which eat everything burrowed in the sand including amphioxus. When the weather is hot and sunny, breeding of local populations can be out of phase, and each local population can spawn on two to three consecutive nights. Because of predation by stingrays, the highest density of amphioxus adults is generally at the edges of sea grass beds. Methods listed below should be generally applicable to other species of *Branchiostoma*. All species have similar breeding habits with the oocytes undergoing meiotic maturation in the early afternoon and spawning occurring about 30 min after sunset. Sperm acquire the ability to become motile when shed into seawater within a day or so of spawning. However, methods of collection may vary depending on the depth and the size of the sand grains.

3.1. Collecting Animals

In Old Tampa Bay, Florida, animals can be collected during the afternoon in water depths of 1.5 m or less, depending on the tide. Oocytes in females collected in the morning generally will not undergo the meiotic divisions when brought back to the laboratory even if kept at 30°C in the light. *B. floridae* has 26 gonads on each side (**Fig. 16.1**). Because the animals are transparent, the relative degree of ripeness can be estimated by eye. When the animals are ripe enough to spawn, the gonads are filled with gametes, most of them maximum size (~140 μm in diameter) as in **Fig. 16.1**. Sexes are separate, with the ovaries typically being yellow and the testes white. However, since the color of

the oocytes depends on diet, they can be relatively white. Because the animals burrow within the top few centimeters of sand, they can be dug with an ordinary garden shovel. The sand containing amphioxus is placed in a sieve made of window screening supported by 1 cm wire mesh (hardware cloth) (**Fig. 16.2**) (17). The sand is sieved out through the screening, leaving the amphioxus in the sieve. The amphioxus are then caught with a small fish net and transferred to a bucket containing about 8–10 cm of seawater. The water is refreshed as often as necessary to maintain the temperature at 28–30°C. Typically 100–200 animals can be collected in about 2 h. The animals are then returned to the laboratory and placed under a light, which serves the dual purpose of keeping them warm and in the light. If the animals are collected late in the afternoon, it is best to include a waterproof flashlight in the bucket during transportation to the laboratory in order to prevent premature spawning.

3.2. Obtaining Amphioxus Gametes

1. Females can be induced to spawn only if their oocytes have undergone the meiotic divisions. Ripe females can be observed carefully under a dissecting microscope. With practice, it is possible to determine if a nucleus is still present, as shown by a grayish zone at the animal pole of the oocytes. If nuclei can be seen in at least some oocytes, the female will not spawn that day. If nuclei cannot be seen (an indication that nuclear breakdown and meiosis have commenced), and the oocytes appear round and loosely packed in the ovaries, they are certainly ready to spawn. However, the round and loose-packed appearance is not an essential criterion for spawning.
2. After sunset (beginning about 2,100 h) males and females are placed individually into plastic cups (disposable cocktail glasses work well) in about 1 cm of filtered seawater. The cutoff transfer pipette works very well for transferring the animals out of the bucket into the cups. Spawning is induced with 2–3 s of 10 ms pulses of 50 V DC delivered by an electrical stimulator via electrodes placed near the head and tail of the animal. If the animals do not immediately spawn, the water in the cup is changed to reduce the chlorine gas generated by the electrical pulses. Spawned sperm and eggs are collected with a Pasteur pipette. To keep them as concentrated as possible, the sperm are best collected as they are expelled from the atrial pore at the posterior end of the series of gonads (**Fig. 16.2**). Concentrated sperm should be transferred immediately to a 1.5 mL eppendorf tube and kept on ice until use. They remain viable at least 24 h if kept on ice or in the refrigerator. Eggs are transferred to a Petri dish with fresh, filtered seawater. Although eggs remain viable

for several hours after spawning, the percentage of eggs fertilizing and developing normally decreases with time after spawning. It is, therefore, best to keep individual females in the light and spawn them just before eggs are needed. In that way, good eggs can be obtained throughout the night. Even so, by the next morning, the quality of eggs begins to decline and the percentage of normal development drops.

3.3. Preparation of Synthetic Messenger RNA

1. The desired cDNA is cloned into the pCS2+ vector. PCR with a long-range PCR kit is used to amplify cDNAs in other vectors. Conditions for PCR amplification vary depending on the template DNA. Typically, the entire coding region plus some of the 5′-UTR is amplified. Although sometimes it is desirable to include the 3′-UTR, it is not always necessary for overexpression in amphioxus embryos. Clone tdTomato between the *Bam*HI and *Eco*RI sites of pCS2+.
2. The cDNA in pCS2+ is linearized with an appropriate restriction enzyme. Restriction digests of cDNA (10 μg) are in 100 μL volumes. After 2 h at 37°C (some restriction enzymes have different temperatures for the best activity), it is useful to treat the restriction digest with 100 μg/ml proteinase K at 55°C for 1 h in order to remove the restriction enzyme and any contaminating RNase completely. Subject an aliquot to electrophoresis on an agarose gel to confirm that the linearization is complete.
3. Purify the DNA by extracting with 1 volume of 25:24:1 phenol:choloroform:isoamyl alcohol.
4. Vortex for 20 s and centrifuge at 13,000 rpm for 2 min. Remove the supernatant to a new 1.5 mL eppendorf tube and add 1 volume of chloroform. Vortex and centrifuge as above.
5. Remove supernatant to a new 1.5 mL eppendorf tube and precipitate the DNA with 4 μL of 5 M NaCl and 2.5 volume of 100% ethanol. Mix well and place on dry ice or in the –80°C freezer for 30 min or longer.
6. Centrifuge at 13,000 rpm for 20 min at 4°C to pellet the DNA. Wash the pellet with 70% EtOH and dry at room temperature.
7. Suspend the DNA in RNase-free water at about 1.0 μg/μL.
8. 1 μg of linearized DNA is used in a 20 μL reaction mix to synthesize capped mRNA with the mMessage mMachine kit (Applied Biosystems/Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions.

9. After precipitation of the RNA, the pellet is suspended in 10 μL RNAse-free water. The quality of the RNA is tested by electrophoresis on a 1% agarose gel.
10. Measure the RNA concentration by determining the optical density at 260 nm. For RNA an OD of 1 at 260 nm= 40 mg/mL. Store frozen at –80°C.

3.4. Preparation of Polylysine-Coated Dishes

1. 50 mL of 0.25 mg/mL polylysine solution is sufficient to coat 1 sleeve of 20 of Falcon 3002 culture dishes. The polylysine solution is stored frozen and can be reused. Fill several culture dishes to about 0.5–1 cm depth with the polylysine solution. Let sit for 5 min and then pour the solution from the dishes into another set of dishes. Dishes are dried at room temperature by placing them upside down with one edge resting on the lid and the opposite one on a paper towel. Dishes should be used within 4 or 5 days of coating (*see* **Note 1**).

3.5. Pulling Micropipettes

1. Because of the thick egg coat, it is absolutely essential to pull micropipettes on a horizontal puller with a wide (2.5 mm) filament. A single pull suffices. The tip of the microinjection needle should be about 1.5 cm long and sealed at the tip. The program for pulling the needles is determined empirically for each filament (*see* **Note 2**).

3.6. Preparation of Injection Solutions and Filling Micropipettes

For 10 μL mRNA injection solution, mix 0.5–5 μg/μL capped mRNA, 1.5 μL 50 mg/mL Texas Red dextran, 2–2.5 μL 80% glycerol, and pH 8.0 water to a final volume of 10 μL (*see* **Note 3**):

1. To control for adverse effects due to injecting too large a volume or too much mRNA, a final concentration of 0.5–1.0 μg/μL pCS2+Tdtomato capped mRNA can be used and the Texas Red dextran omitted.
2. For antisense morpholino oligonucleotides (MOs), dissolve 300 nM in 150 μL pH 8 water for a 2 mM stock. Use 5 μL stock morpholino, 1.5 μL 50 mg/mL Texas Red dextran, 2–2.5 μL 80% glycerol, and pH 8.0 water to 10 μL (*see* **Note 4**).
3. To remove any particulate material that could clog the needles, the solution is centrifuged before use for 5 min at 13,000 rpm.

Injection needles are backfilled with a pipetter fitted with an Eppendorf microloader tip (available in the USA from Fisher Scientific, Pittsburgh, PA, USA). Fill needles just before use. They can be stored at –80°C for a few days, but it is preferable to prepare a fresh mix for each day's injections.

3.7. Microinjection of Amphioxus Eggs

1. Check freshly spawned eggs under the dissecting microscope to make sure that none have partially elevated fertilization envelopes. Such eggs are very difficult to inject, although they can be fertilized and will generally develop normally. To 10–20 eggs, add 1 drop of sperm to ensure that 100% of the eggs have clearly elevated fertilization envelopes within 5 min of adding sperm. Although it requires 20 min for envelopes to fully elevate, 5 min suffices to see that the envelope is elevating. Eggs stuck to a polylysine-coated dish for over 30 min may fertilize less readily than ones not stuck down. Therefore, it is counter-productive to inject an egg batch in which many eggs fail to elevate their fertilization envelopes within a few minutes.
2. Insert a filled injection needle into the microelectrode holder (**Fig. 16.3**). Observe the tip of the needle under a dissecting microscope and use fine forceps to break the tip. Adjust the picospritzer to 20 psi and a pulse length of about 300 ms. When the tip is broken, you should see a very tiny drop. Insert the tip of the needle into a 3 cm Petri dish containing mineral oil or vegetable oil. Inject a drop into the oil and adjust the pulse length so that the drop is about 10% of the diameter of an egg. The pulse length should be >100 ms. If >450 ms, the tip should be rebroken as it will clog after injecting only a few eggs. If <~100 ms discard the needle and break a new one as eggs injected with a relatively large-bore needle, if they survive, are liable to cleave abnormally.
3. For orientation, inscribe three or four scratches or a grid on the bottom of a polylysine- coated Petri dish with a sharp

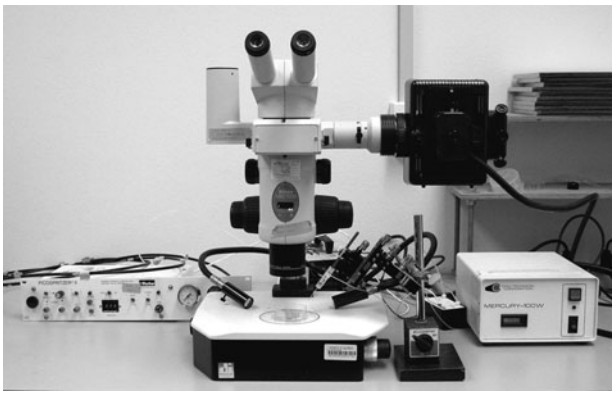

Fig. 16.3. The setup for microinjection. A picospritzer operated with a foot pedal (not shown) is at the *left*. A microscope used is shown; in this case a fluorescence microscope with 15× eyepieces, although a non-fluorescence microscope with 40× objective and 10× eyepieces is adequate. Fiber optics are adjusted so that the light reflects off eggs in the Petri dish on the stage of the microscope. The micromanipulator is adjusted so that the needle is at a 45° angle.

glass pipette or diamond scriber. Fill the dish to a depth of about 0.7 cm with filtered seawater. Row 200–300 eggs alongside the scratches. It is convenient to use a Pasteur pipette with the tip pulled out to a slightly larger diameter than of the eggs. A mouthpiece rather than rubber bulb facilitates rowing the eggs as it allows one to add the eggs to the dish very slowly.

4. Remove the needle from the dish of oil and place the dish of eggs under the dissecting microscope. The needle should be at a 45° angle to the dish. Adjust the optics to have a good view of the eggs and the tip of the needle (**Fig. 16.3**). If working with *Branchiostoma* species that develop at 20°C or less, a cooling stage is necessary. For *B. floridae* or other warm water species, this is not necessary, but care should be taken to avoid over-heating the eggs. Using the scratches on the dish as guidelines, inject eggs in order. Advance the needle with the medium and fine controls until it enters the egg. The needle should enter the uppermost surface of the egg rather than the side. The needle should penetrate just beneath the egg cortex. If it goes into the egg too deeply, the eggs will likely die. Note that the eggs are randomly in the dish with respect to which side is uppermost. Under a dissecting microscope, it is generally not possible to see the polar bodies, which are the only indication of polarity in living eggs. Using the foot pedal to repeatedly expel the solution from the needle as the needle approaches the egg may help to show when the egg has been penetrated. If the egg dies when injected, the injected volume is too large. It is advisable to vary the pulse length when injecting a dish of eggs. Eggs injected with too little survive and develop but generally have a normal phenotype, while those injected with too large an amount (but less than that which immediately kills the egg) generally do not cleave or cleave abnormally. It is the Goldilocks principle, just enough but not too much. Once all the eggs are injected, the needle can be placed into the dish of oil and used to inject a second dish of eggs.
5. Remove as much of the seawater from the dish as possible without uncovering the eggs. Add fresh filtered seawater and fertilize the injected eggs with a drop or two of sperm. If the eggs do not all fertilize, as evident from elevation of the fertilization envelopes, add more sperm. When all are fertilized, change the seawater again to remove as many of the extra sperm as possible. Once the fertilization envelopes have fully elevated, gently agitate the dish to detach the embryos from the bottom. This is best done before cleavage as the blastomeres are not tightly adherent to one another

during early cleavage and can readily dissociate if the fertilization envelope is removed. Add 5 μL antibiotic solution for final concentrations of 50 μg/mL penicillin and 100 μg/mL streptomycin. Cover the dish and allow to develop at 24–30°C.

6. Examine injected embryos under a fluorescence microscope to ensure that injections were successful. The co-injected Texas Red dextran will emit red fluorescence under a rhodamine filter (green light). The endogenous green fluorescent protein will fluoresce green under a fluorescein filter (blue light) (*see* **Note 5**).
7. Once embryos have developed to the desired stage, they can be fixed in PFA/MOPS for either antibody labeling (store at –20°C in methanol after fixation) or in situ hybridization (store at –20°C in 70% ethanol after fixation). To ensure good fixation, change the fixative once. For gastrula stages, remove the fertilization envelope by sucking embryos gently in and out of a Pasteur pipette with the tip drawn out over a flame and then broken off (score the tip with a diamond scriber to break evenly) to a diameter just greater than that of the egg (~140 μm), but smaller than that of the egg plus fertilization envelope (~450 μm). Rinse the gastrulae in fresh seawater before transferring to the fixative. This removes the hatching enzyme, which will digest eggs slowly even after fixation and storage in alcohol (*see* **Note 6**).

4. Notes

1. The use of the Falcon 3002 tissue culture dishes is essential. The polylysine will not adhere to Petri dishes that are not tissue culture dishes. The coated dishes can be used as soon as they have dried. The coated dishes lose their ability to stick down amphioxus eggs by a week or so after coating. The dishes cannot be recoated.
2. Although according to folklore, microinjection needles must be used within a few weeks of being pulled, we have found that as long as the tips are unbroken, the needles can be used a year or more after being pulled. A convenient box for storing is a 25 cm × 25 cm square Petri dish. Three strips of Styrofoam covered with tape, sticky side out, are taped across the bottom of the dish. The needles are stuck to the strip of tape, and a second strip of Styrofoam is laid across each row of needles. When the lid is taped on the dish, the needles are held in place.

3. Amphioxus eggs and embryos contain endogenous green fluorescent protein (28). Therefore, constructs encoding green fluorescent protein and dyes that fluoresce green when illuminated with blue light cannot be used.
4. Because it is not possible to inject amphioxus eggs with more than a few picoliters, it is desirable to inject as high a concentration of the morpholino oligonucleotide (MO) as possible. The control morpholino purchased from Gene Tools is a good negative control. The efficacy of an MO complementary to sequence near the ATG start codon can be tested in an in vitro translation assay. If the MO does not block translation in vitro, it is not likely to do so in vivo. However, an MO that blocks translation in vitro may not necessarily do so in vivo, especially if there is a large amount of maternal mRNA for the gene of interest. Some confidence in the specificity of the MOs is given by using two MOs against the same gene. The best control is a rescue experiment in which capped mRNA for the same gene (but lacking the MO target site) is co-injected with the antisense morpholino oligonucleotide. However, for amphioxus, limitations on the volume that can be injected generally preclude such a rescue experiment. If available, treatment of developing embryos with a chemical or protein agonist may suffice as a control. An alternative control is labeling MO-injected embryos with an antibody against the protein whose translation is being blocked. For splice-blocking morpholinos, the best control is exon to exon PCR of first-strand cDNA synthesized from RNA extracted from about 200 to 300 morpholino-injected embryos. Primers are designed to amplify across the exon that is normally spliced out. The size of the amplified band compared to controls determines the effectiveness of the morpholino. A number of additional pitfalls with morpholinos have been observed in practice (29).
5. The fluorescence from the Texas Red dextran begins to fade in living amphioxus embryos after 30 h of development at 24°C. The fluorescence survives formaldehyde fixation, but fades somewhat during storage in 70% ethanol.
6. Changing the fixative is necessary if more than 1/10 volume of seawater is transferred with the embryos into the fixative or if gastrula stages are fixed. While some antigens (e.g., β-catenin) may survive storage in 70% ethanol (30), other antigens are more labile and will be denatured more readily in 70% ethanol than in methanol.

Acknowledgments

This work was supported by grants from the National Science Foundation, USA. NSF IOS 07-43485 and MCB 06-20019 to L. Z. H. and IOB 0416292 to L. Z. H. and N. D. Holland. Onai T. was supported by JSPS Postdoctoral Fellow for Research Abroad (Japan). We thank Professors Susan Bell and John Lawrence for generously providing laboratory space at the University of South Florida during the amphioxus breeding season.

References

1. Putnam, N., Butts, T., Ferrier, D.E.K., Furlong, R.F., Hellsten, U., Kawashima, T., et al. (2008) The amphioxus genome and the evolution of the chordate karyotype. *Nature* **453**, 1064–1071.
2. Kuraku, S., Meyer, A., and Kuratani, S. (2009) Timing of genome duplications relative to the origin of the vertebrates: did cyclostomes diverge before or after? *Mol. Biol. Evol.* **26**, 47–59.
3. Yu, J.-K., Satou, Y., Holland, N.D., Shin-I, T., Kohara, Y., Satoh, N., et al. (2007) Axial patterning in cephalochordates and the evolution of the organizer. *Nature* **445**, 613–617.
4. Schubert, M., Holland, N.D., Laudet, V., and Holland, L.Z. (2006) A retinoic acid-Hox hierarchy controls both anterior/posterior patterning and neuronal specification in the developing central nervous system of the cephalochordate amphioxus. *Dev. Biol.* **296**, 190–202.
5. Holland, L.Z. (2002) Heads or tails? Amphioxus and the evolution of anterior-posterior patterning in deuterostomes. *Dev. Biol.* **241**, 209–228.
6. Yu, J.K., Meulemans, D., Mckeown, S.J., and Bronner-Fraser, M. (2008) Insights from the amphioxus genome on the origin of vertebrate neural crest. *Genome Res.* **18**, 1127–1132.
7. Knecht, A.K. and Bronner-Fraser, M. (2002) Induction of the neural crest: a multigene process. *Nat. Rev. Genet.* **3**, 453–461.
8. Wilson, E.B. (1893) Amphioxus, and the mosaic theory of development. *J. Morphol.* **8**, 579–639+ pl. XXIX–XXXVIII.
9. Morgan, T.H. (1896) The number of cells in larvae from isolated blastomeres of amphioxus. *Arch. Entwicklungsmech.* **3**, 269–294+ pl. XVII.
10. Tung, T.C., Wu, S.C., and Tung, Y.Y.F. (1958) The development of isolated blastomeres of amphioxus. *Sci. Sinica* **7**, 1280–1320.
11. Tung, T.C., Wu, S.C., and Tung, Y.Y.F. (1960) The developmental potencies of the blastomere layers in *Amphioxus* egg at the 32-cell stage. *Sci. Sinica* **9**, 119–141.
12. Tung, T.C., Wu, S.C., and Tung, Y.Y.F. (1962) The presumptive areas of the egg of amphioxus. *Sci. Sinica* **11**, 629–644.
13. Tung, T.C., Wu, S.C., and Tung, Y.Y.F. (1962) Experimental studies on the neural induction in amphioxus. *Sci. Sinica* **11**, 805–820.
14. Holland, N.D. and Holland, L.Z. (1989) Fine structural study of the cortical reaction and formation of the egg coats in a lancelet (= amphioxus), *Branchiostoma floridae* (phylum Chordata: supphylum Cephalochordata = Acrania). *Biol. Bull.* **176**, 111–122.
15. Fuentes, M., Benito, E., Bertrand, S., Paris, M., Mignardot, A., Godoy, L., et al. (2004) Insights into the spawning behavior of the European amphioxus (*Branchiostoma lanceolatum*) *J. Exp. Zool.* **302B**, 384–391.
16. Zhang, Q.J., Sun, Y., Zhong, J., Li, G., Lü, X.M., and Wang, Y.Q. (2007) Continuous culture of two lancelets and production of the second filial generations in the laboratory. *J. Exp. Zool.* **308B**, 464–472.
17. Holland, L.Z. and Yu, J.K. (2004) Cephalochordate (Amphioxus) embryos: procurement, culture, basic methods. *Methods Cell. Biol.* **74**, 195–215.
18. Stokes, M.D. (1996) Larval settlement, post-settlement growth and secondary production of the Florida lancelet (=amphioxus). *Branchiostoma floridae Mar. Ecol. Prog. Ser.* **130**, 71–84.

19. Holland, N.D., Paris, M., and Koop, D. (2009) The club-shaped gland of amphioxus: export of secretion to the pharynx in pre-metamorphic larvae and apoptosis during metamorphosis. *Acta Zool. (Stockh)* **90**, 372–379.
20. Paris, M., Escriva, H., Schubert, M., Brunet, F., Brtko, J., Ciesielski, F., et al. (2008) Amphioxus postembryonic development reveals the homology of chordate metamorphosis. *Curr. Biol.* **18**, 825–830.
21. Holland, N.D. and Holland, L.Z. (2006) Stage- and tissue-specific patterns of cell division in embryonic and larval tissues of amphioxus during normal development. *Evol. Dev.* **8**, 142–149.
22. Bayascas, J.R., Yuste, V.J., Benito, E., Garcia-Fernandez, J., and Comella, J.X. (2002) Isolation of AmphiCASP-2/7, an ancestral caspase from amphioxus (*Branchiostoma floridae*). Evolutionary considerations for vertebrate caspases. *Cell Death Diffferen.* **9**, 1078–1089.
23. Holland, L.Z., Holland, P.W.H., and Holland, N.D. (1996) Revealing homologies between body parts of distantly related animals by in situ hybridization to developmental genes: amphioxus versus vertebrates. In *Molecular Approaches to Zoology and Evolution*, ed. Ferraris, J.D. Wiley, New York, NY, pp. 267–282; pp. 473–483.
24. Yu, J.K.S. and Holland, L.Z. (2009) Cephalochordates (Amphioxus or Lancelets): a model for understanding the evolution of chordate characters. *Cold Spring Harb Protoc* **130**, pdb.emo130.
25. Dos Santos, S., Bardet, C., Bertrand, S., Escriva, H., Habert, D., and Querat, B. (2009) Distinct expression patterns of glycoprotein hormone-α2 and -β5 in a basal chordate suggest independent developmental functions. *Endocrinology* **150**, 3815–3822.
26. Li, X.Y., Zhang, W., Chen, D.Y., Lin, Y.S., Huang, X.W., Shi, D.L., et al. (2006) Expression of a novel somite-formation-related gene, *AmphiSom*, during amphioxus development. *Dev. Genes Evol.* **216**, 52–55.
27. Holland, L.Z. and Holland, N.D. (1992) Early development in the lancelet (= amphioxus) *Branchiostoma floridae* from sperm entry through pronuclear fusion: presence of vegetal pole plasm and lack of conspicuous ooplasmic segregation. *Biol. Bull.* **182**, 77–96.
28. Deheyn, D.D., Kubokawa, K., McCarthy, J.K., Murakami, A., Porrachia, M., Rouse, G.W., et al. (2007) Endogenous green fluorescent protein (GFP) in amphioxus. *Biol. Bull.* **213**, 95–100.
29. Eisen, J.S. and Smith, J.C. (2008) Controlling morpholino experiments: don't stop making antisense. *Development* **135**, 1735–1743.
30. Holland, L.Z., Panfilio, K.A., Chastain, R., Schubert, M., and Holland, N.D. (2005) Nuclear –β catenin promotes non-neural ectoderm and posterior cell fates in amphioxus embryos. *Dev. Dyn.* **233**, 1430–1443.

Chapter 17

Reptile Embryology

Matthew K. Vickaryous and Katherine E. McLean

Abstract

Reptiles (lizards, snakes, turtles and crocodylians) are becoming increasing popular as models for developmental investigations. In this review the leopard gecko, *Eublepharis macularius*, is presented as a reptilian model for embryonic studies. We provide details of husbandry, breeding and modifications to two popular histological techniques (whole-mount histochemistry and immunohistochemistry). In addition, we provide a summary of basic reptilian husbandry requirements and discuss important details of embryonic nutrition, egg anatomy and sex determination.

Key words: Reptilia, gecko, husbandry, embryo, sex determination, whole-mount histochemistry, immunohistochemistry, PCNA.

1. Introduction

Reptiles are a diverse radiation of amniotes with more than 8,700 recognized species. In the modern sense, Reptilia includes all amniotes that are not mammals (or more accurately 'the most inclusive clade containing *Lacerta agilis* Linneaus 1758 and *Crocodylus niloticus* Laurenti 1768, but not *Homo sapiens* Linneaus 1758') (1). Consequently, birds are reptiles. Historically, however, reptiles (as a group) have excluded both birds and mammals. To avoid unnecessary confusion, this contribution will focus on reptiles in the classical or structural sense: a group distinguished by keratinized scales and poikilothermy.

Modern reptiles include turtles and tortoises (Testudines), alligators, crocodiles and relatives (Crocodylia), and tuatara, snakes and lizards (Lepidosauria). Testudines are easily recognized by the unique presence of a carapace and plastron ('shell')

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_17,

and a pectoral apparatus deep to the ribcage. Crocodylians are large, semiaquatic predators with robust tails and thecodont dentition (teeth set in sockets). Lepidosauria are enormously diverse and include limbed forms, numerous unrelated species with varying degrees of limb reduction, as well as independently evolved limbless groups. Snakes (Serpentes) are the best known group of limbless lepidosaurs. Other limbless groups include many species of lizards and amphisbaenians, a group of elongate burrowing forms with robust skulls and annulated skin (giving members an earthworm-like appearance). Although superficially lizard-like, tuatara represents the outgroup to other lepidosaurs and demonstrates various primitive features including an akinetic skull (with a well-developed lower temporal bar) and the absence of hemipenes. Lepidosaurs exclusive of tuatara are known as squamates (Squamata). The term 'lizard' is typically used to denote a non-serpent, non-amphisbaenian squamate.

Until recently, species selection was often limited to locally available taxa. However, increasing numbers of reptiles are becoming widely obtainable due to their popularity in the pet trade, including Eastern corn snakes (*Pantherophis guttatus*), bearded dragons (*Pogona vitticeps*), anoles (*Anolis* spp.) and various geckos (*Eublepharis macularius*, *Paroedura pictus*). In addition, breeders of exotic species may provide unique opportunities to investigate less common reptiles (e.g. African rock pythons, *Python sebae*) (2, 3). For many reptiles, especially large, semiaquatic species (e.g. crocodylians, various turtles), it is more practical to collect eggs from wild populations or commercial farms and then artificially incubate them in a controlled environment. If considering captive husbandry, it is essential to investigate the species-specific environmental requirements (*see* **Note 1**).

Most reptiles, including all turtles, crocodylians, tuatara and the majority of squamates, produce cleidoic eggs following internal fertilization, a pattern of reproduction known as oviparity (4). As the greater part of development occurs outside the body, oviparous development is particularly sensitive to changes in environmental conditions, specifically temperature (5). Consequently, the incubation period may vary considerably both between and within species (7). Furthermore, many reptiles demonstrate temperature-dependent sex determination (*see* **Note 2**). Oviparous species are lecithotrophic, obtaining the majority of their nutrients from the yolk mass (4). Eggs produced by oviparous species are enclosed by a series of membranes and a calcareous shell that varies in structure (*see* **Note 3**). Depending on the species, the eggshell or the yolk sac may act as the primary source of calcium.

As an alternative to oviparity, various squamates have independently evolved viviparity: in utero embryonic development (6). More specifically, viviparity refers to embryonic development

that combines the nutritional features of both placentotrophy, in which the placenta provides nutrients to the developing offspring, and lecithotrophy, in which the yolk sac is responsible for providing nutrients to the developing offspring (7). Historically, the term ovoviviparity was used to describe a mode of reproduction wherein the embryos obtained a majority of their nutrients from the yolk sac but used the placenta for gas exchange (8). Ovoviviparity has since been recollected as part of the viviparous spectrum of development. The proportion of nutritional elements that are obtained from the yolk sac and placenta varies among species. For instance, in scincid, lizards members of the genera *Pseudemoia* and *Niveoscincus* rely more heavily on placentotrophy than lecithotrophy to obtain nutrients (4), whereas the species *Eulamprus tympanum* rely more heavily on lecithotrophy than placentotrophy (9).

Related to the increasing interest in reptilian development, embryonic staging tables are now available for various turtles (10–12), crocodylians (13, 14), snakes (15, 16) and lizards (17–19). Although embryos of reptiles such as tuatara and amphisbaenians remain difficult to acquire, staging tables have been published (20, 21). This chapter describes husbandry procedures for keeping and breeding leopard geckos, *E. macularius*, a tractable, laboratory-appropriate oviparous lizard. In addition, we provide details on two common techniques for use with reptile embryos: whole-mount histochemistry and serial immunohistochemistry.

1.1. Husbandry and Embryogenesis in Leopard Geckos

Leopard geckos (*E. macularius*, **Fig. 17.1a**) are one of the most popular reptiles in the pet trade. Adults are 120–140 mm in body length with a docile temperament and have minimal husbandry requirements. Males are typically more robust than females and demonstrate a conspicuous 'V'-shaped row of femoral pores (immediately cranial to the vent) and hemipenile swellings. Clutch size is small (most commonly two), but females can have multiple clutches throughout a season (19). Leopard geckos use temperature-dependent sex determination, and the temperatures for producing males and females are documented (22). Furthermore, an embryonic staging table of in ovo development has recently been published (19).

Female leopard geckos may be kept singly or in small groups (2–10) depending on enclosure size. Males should be kept singly or with one or more females for the purposes of breeding. Males may fight if housed together. A standard rat-sized Nalgene or polycarbonate enclosure (23 cm × 42 cm × 19 cm high), with a stainless steel wire lid, water bowl, two hide boxes and a layer of newspaper, is suitable for 1–2 adult individuals (**Fig. 17.1b**). A weigh boat with powdered supplement should be provided to sexually immature (and rapidly growing) subadults and gravid adult females. Although lacking subdigital adhesive pads, leopard

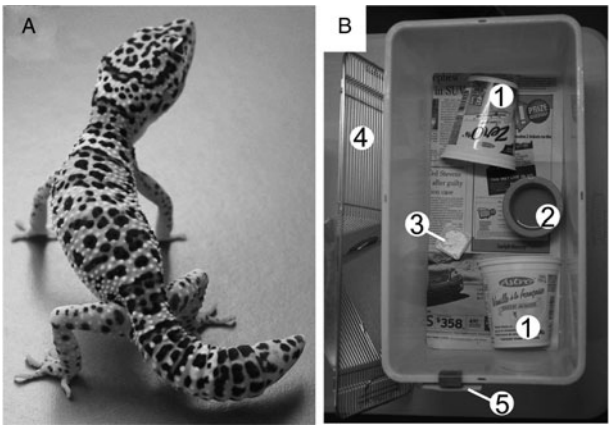

Fig. 17.1. (**a**) Adult female leopard gecko, *E. macularius*. As for all eublepharids (but unlike most geckos), leopard geckos have movable eyelids and lack adhesive toe pads. (**b**) Leopard gecko laboratory enclosure. One or two adult leopard geckos can be housed in a standard rat-sized cage lined with newspaper or paper towel. Each enclosure should include (*1*) two hide boxes, (*2*) water bowel, (*3*) a dish of powdered supplement (calcium and vitamin D_3) and (*4*) a stainless steel mouse-gauge wire top (*5*). Card holders and cage labels are useful for organizing larger colonies.

geckos are capable climbers and can squeeze through relatively small openings. Leopard geckos are adept at escaping, so it is important that mouse-gauge wire lids (distance between bars = 1 cm) be used and that hide boxes be short so as not to allow individuals to reach the wire lid. Avoid using wire lids with a built-in cradle for food and water bottle or invert these lids. Two hide boxes should be provided, one at either end of the enclosure. As for many reptiles, leopard geckos seem to prefer enclosed spaces, so hide boxes need only be large enough for the number of individuals present. Individuals are fed a diet of gut-loaded mealworms or crickets, both of which should be dusted with powdered supplement every 1–3 days. Subadult geckos will eat an average of five regular-sized mealworms (range 1–10) daily. Sexually mature adults can be fed larger numbers of mealworms less frequently. Although most individuals establish a latrine site, enclosures should be replaced weekly and given a change of newspaper, a clean water dish and new hide boxes. Ambient temperature can range between 22 and 32°C with a preferred body temperature of 25.8°C (23). The establishment of a heat gradient is important for behavioural thermoregulation. The heat cable can be placed under one end of the enclosure (below one of the hide boxes). Our colony is organized using a rack system with multiple enclosures and kept at an ambient temperature of 24°C (temperatures over the heat cable averaging 3–4°C above ambient), on a light cycle of 12:12 with an ambient humidity of 40–50%. Hatchling geckos often have difficulty removing shed skin from toes. If necessary, soak the feet in room temperature water to hydrate shed

skin and carefully remove with forceps. With regular handling, leopard geckos become tame and docile.

Females have a preference for nest sites between 28 and 29°C (24). One of the hide boxes can be converted into a nesting chamber by the addition of 1:1 vermiculite and water (or other water-absorbing material) to provide an area of elevated humidity. Once eggs are deposited, they should be collected and incubated in a commercial avian incubator. Leopard geckos use temperature-dependent sex determination (TSD). Females are produced at low temperatures (26–28°C), mostly females (75%) at high temperatures (34°C) and mostly males (~89%) at intermediate temperatures (31.5°C) (22). Between these temperatures, both males and females are produced. Humidity during the incubation period should be maintained at close to 80%.

Embryogenesis (**Fig. 17.2**) is well underway prior to laying (oviposition) and, similar to avians, absolute time offers only a rough guide to state of development. As noted previously, rate of development is strongly influenced by incubation temperature. The role of other factors, including passage time within the oviduct, egg retention and humidity of the incubator, may also influence the developmental stage at oviposition. Although details remain unclear, gastrulation and neurulation are predicted to be comparable with the events observed in avians. In leopard geckos, oviposition occurs at stage 28, characterized in part by the presence of forelimb buds (but not hind limb buds) and incomplete closure of the choroid fissure of the developing eye. This correlates with avian embryos at Hamburger and Hamilton (HH) stage 17 (19). At a constant temperature of 28°C ± 1°C, the duration of in ovo development for leopard geckos is an average of 52 days.

1.2. Whole-Mount Histochemistry: Single (Alizarin Red) and Double (Alizarin Red and Alcian Blue) Embryo-Staining Protocol

Whole-mount clearing and staining commonly involves double staining with alizarin red S for calcified structures in combination

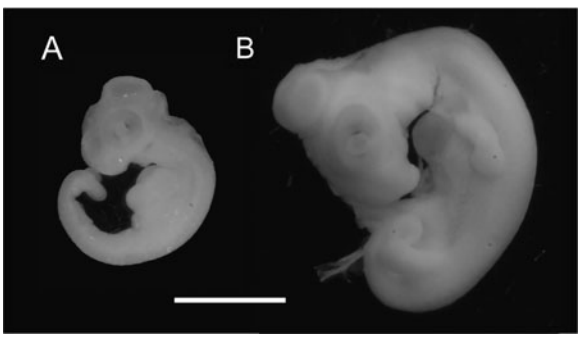

Fig. 17.2. Leopard gecko embryos. (**a**) Stage 29, at or shortly after oviposition. At this stage, both forelimb and hind limb buds are present. (**b**) Stage 31. The forelimb buds have developed a paddle-shaped autopodium. *See* (19) for details. Scale bar, 2 mm.

with Alcian blue 8GX for tissues rich in glycosaminoglycans (25). However, Alcian blue solution uses glacial acetic acid as a solvent and fixative which may decalcify early mineralized bone. The omission of Alcian blue steps results in a greater affinity for alizarin red and thus provides a more accurate indication of the earliest stages of ossification. To facilitate a more rapid penetration of reagents and reduce superimposing of skeletal elements, it is often beneficial to segment the embryo transversely into head, thorax, pelvis and tail regions. The head may be further segmented sagittally (hemisectioned) to assist in the visualization of deeper elements of the developing skull (e.g. palatine and vomer). Once hemisectioned, one half of the head can be single stained with alizarin red, while the other half is double stained with alizarin red and Alcian blue. Opaque (alizarin red negative) condensations are often easier to visualize against the Alcian blue-positive endoskeleton of double-stained materials.

1.3. Paraffin-Embedded Serial Section Immunohistochemistry (IHC)

Proliferating cell nuclear antigen (PCNA, also known as cyclin) is a 36-kDa protein found within the cell nucleus. It is an auxiliary protein of DNA polymerase delta that is expressed during S phase of the cell cycle and plays an important role in cell proliferation (26). Our studies have utilized PCNA immunohistochemistry to identify patterns of limb growth and development in reptiles. The primary focus has been on limbs and tails of American alligator embryos (*Alligator mississippiensis*), although we have also used this protocol to identify proliferating cells in the regenerating tail of the leopard gecko, *E. macularius*.

2. Materials

2.1. Materials for Leopard Gecko Husbandry and Breeding

1. Opaque Nalgene or polycarbonate rat cages, 23 cm × 42 cm × 19 cm high (Ancare).
2. Stainless steel mouse cage lid, bar spacing 1 cm (Ancare).
3. Water bowls, polycarbonate.
4. Hide boxes (polypropylene food containers cut longitudinally to create two hide boxes).
5. Newspaper, sheets.
6. Mealworms (*Tenebrio molitor*) or crickets (*Acheta domestica* or *Gryllus bimaculatus*).
7. Weigh boat, polypropylene.
8. Powdered calcium and vitamin D_3 (cholecalciferol) supplement.
9. Heat cable (Zoo Med's Repti Heat Cable).

10. Vermiculite.
11. Hova-Bator Thermal Air Flow Incubator (G.Q.F. Breeding Technology).

2.2. Materials for Whole-Mount Histochemistry: Single (Alizarin Red) and Double (Alizarin Red and Alcian Blue) Embryo-Staining Protocol

1. 10% Neutral buffered formalin (NBF) (Fisher Scientific).
2. Ethanol (EtOH).
3. Alcian blue 8GX solution (20 mg Alcian blue 8GX, 70 mL absolute EtOH, 30 mL glacial acetic acid).
4. Glacial acetic acid.
5. Alizarin red S solution (alizarin red S monohydrate saturated in 0.5% KOH; MP Biomedicals).
6. Distilled water (dH_2O).
7. Trypsin.
8. 2% Sodium borate (NaB_4O_7).
9. Aqueous potassium hydroxide (KOH).
10. Glycerol.
11. Slotted spoon.
12. Instruments for cutting and eviscerating, including forceps and scalpel.
13. Glass (or clear) receptacles for fixing, staining, digesting and storage.

2.3. Materials for Paraffin-Embedded Serial Section Immunohistochemistry (IHC)

1. American alligator embryos, *A. mississippiensis*.
2. 10% Neutral buffered formalin (NBF) (Fisher Scientific).
3. 28- or 30-gauge needles and syringes (Precision Glide).
4. Distilled water (dH_2O).
5. Cal-Ex decalcifier solution (Fisher Scientific).
6. Ventana Renaissance Tissue Processor.
7. Formulated purified paraffin synthetic polymer (Fisher Scientific).
8. Tissue cassettes.
9. Tissue forceps and scalpel.
10. Tissue-Tek thermal console, dispensing console and cryo console.
11. Rotary microtome: Microtome 2050 Supercut.
12. Feather microtome blade R35.
13. Gelatin type B (Fisher Scientific).
14. Water bath.
15. Precleaned microslides, Snow Coat X-tra (Surgipath).
16. Incubator preset to 37°C.

17. Xylene, histological grade (Fisher Scientific).
18. 2-Propanol (= isopropyl alcohol), 70% (w/v) and 100% (w/v) (Fisher Scientific).
19. 30% Hydrogen peroxide (H_2O_2).
20. Staining boat and staining dishes.
21. Phosphate-buffered saline (PBS), 10× (Sigma).
22. Coplin jars.
23. Humidity chamber.
24. Kimwipes (Kimtech).
25. 100–1,000 μL pipette (Eppendorf) and general purpose 101–1,000 μL pipette tips (Fisherbrand Redi-Tip).
26. 10–100 μL pipette (Eppendorf) and general purpose 1–200 μL pipette tips (Fisherbrand Redi-Tip).
27. 0.5–10 μL Pipette (Eppendorf) and general purpose 0.5–10 μL pipette tips (Fisherbrand Redi-Tip).
28. Blocking solution: 3% Normal goat serum (Quelab) in PBS.
29. PCNA (FL-261), rabbit polyclonal IgG 1:500 dilution in PBS (Santa Cruz Biotechnology).
30. Biotin–SP-conjugated AffiniPure F(ab')2 fragment goat anti-rabbit IgG (H+L), 1:500 dilution in PBS (Jackson ImmunoResearch Laboratories, Inc.).
31. Peroxidase-conjugated streptavidin 1:200 dilution (Jackson ImmunoResearch Laboratories, Inc.).
32. 3,3′-Diaminobenzidine (DAB) solution: 2 mL DAB, 200 mL PBS and 300 μL of 3.0% H_2O_2.
33. Harris modified haematoxylin (Fisher Scientific).
34. Ammonia water: 0.25% Ammonium hydroxide in dH_2O.
35. Cytoseal mounting medium (Richard Allan Scientific).
36. Microscope cover glass, 22 mm × 50 mm × 1 mm (Fisher Scientific).

3. Methods

3.1. Methods for Leopard Gecko Husbandry and Breeding

As for most reptiles, mating is stimulated by changes in photoperiod and temperature. Reproductive success is enhanced by cycling both males and females. Cycling involves a period of dormancy (summarized in (19)). Briefly, the photoperiod is reduced by 0.5 h every second day until it reaches 8 h light:16 h dark.

During the 'dark hours', ambient temperature is reduced to 21°C and the heat gradient is turned off. The amount of food being offered during this time should be reduced. Once the 8:16 photoperiod is reached, feeding is suspended and the ambient temperature is maintained at a near constant 21°C with no heat gradient. Fresh water should always be made available. After 2 months, the photoperiod can be reversed back to 12:12, at 0.5 h increments every second day, the ambient temperature returned to 22–32°C, and regular feedings can resume.

Following dormancy and re-acclimation to a daily feeding regime and 12:12 photoperiod, a single male can be introduced to one or more females for a period of 1–5 days. Females are capable of storing sperm (27) and may produce multiple clutches within a single year-long season. Successful mating will result in a clutch of two eggs every 21–28 days. Oviposition occurs approximately 11 days after ovulation (28).

3.2. Methods for Whole-Mount Histochemistry: Single (Alizarin Red) and Double (Alizarin Red and Alcian Blue) Embryo-Staining Protocol

1. Fix embryos (injection and emersion) in 10% NBF for 24 h (*see* **Note 4**).
2. Rinse with dH_2O.
3. Transversely segment specimen into head and one or more body regions (e.g. pectoral and pelvic regions) and eviscerate (*see* **Note 5**). Sagittally section (hemisection) head into left and right halves. Skinning embryos is usually not necessary.
4. If single staining with alizarin red S, skip to Step 7. Otherwise, rinse with dH_2O.
5. Stain with Alcian blue 8GX solution overnight.
6. Hydrate through an EtOH series for a minimum of 1 h at each step in the series. Begin with two changes of absolute EtOH, then 95, 70, 40, 15% and finally into dH_2O.
7. Macerate with either 1% trypsin in a 2% sodium borate solution (for larger specimens or those in the later stages of development) or 0.5% KOH (for smaller or early-staged specimens). Check every 2–6 h until specimen becomes limp (*see* **Note 6**). If necessary, place the specimen in dH_2O overnight and then replace into fresh maceration solution.
8. Stain with alizarin red S solution for 24 h (*see* **Note 7**).
9. Rinse and clear for 24 h at each stage of a 0.5% KOH–glycerol series beginning with 3:1, 1:1, 1:3 and then pure glycerol (*see* **Note 8**).
10. Store in glycerol (*see* **Note 9**).

3.3. Methods for Paraffin-Embedded Serial Section Immunohistochemistry (IHC)

3.3.1. Tissue Processing

1. Fix embryos (injection and emersion) in 10% NBF for 24 h (*see* **Note 10**).
2. Rinse with dH_2O.
3. Store in 70% EtOH.
4. Dissect tissue of interest (e.g. limbs) from embryos using a scalpel or fine dissection tools and place into tissue cassettes. Later-staged embryos (e.g. Ferguson stage 17 or later (13)) should be decalcified for 10 min in Cal-Ex decalcifying solution and briefly rinsed with dH_2O prior to tissue processing.
5. Process tissues in a tissue processer overnight.
6. Embed the tissues in paraffin wax and allow blocks to cool.

3.3.2. Sectioning

7. Cool tissue blocks on ice for 30 min prior to sectioning. Section blocks into 5 mm thick slices using a rotary microtome.
8. Transfer the sections to a water bath, mount on positively charged slides and dry overnight in an incubator preset to 37°C.

3.3.3. Immunostaining

9. Deparaffinize slides using three changes of xylene (2 min each).
10. Rehydrate through three changes of 100% 2-propanol, 70% 2-propanol and dH_2O (2 min each).
11. Quench slides in 1.0% H_2O_2 for 10 min, rinse in dH_2O and three changes of PBS (2 min each).
12. Remove excess fluid from slide by wiping around the tissue sample with a Kimwipe.
13. Apply 200 μL of blocking solution to each tissue sample and incubate slides for 1 h at room temperature in a humidified chamber.
14. Tip off the blocking solution and apply 200 μL of primary antibody (1:500 dilution) to each test slide. Apply sterile PBS to the negative control. Incubate slides at 4°C overnight in a humidified chamber.
15. Rinse slides in three changes of PBS (2 min each) (*see* **Note 11**). Remove excess liquid from slides using Kimwipes. Apply 200 μL biotinylated secondary antibody (1:500 dilution) to each tissue sample and incubate slides for 1 h in a humidified chamber at room temperature.
16. Rinse slides in three changes of PBS (2 min each) (*see* **Note 11**). Remove excess liquid from slides using Kimwipes. Apply 200 μL streptavidin HRP (1:200 dilution) to each tissue sample and incubate slides for 1 h in a humidified chamber at room temperature.

17. Rinse slides in three changes of PBS (2 min each) (*see* **Note 11**). Submerge slides in a DAB solution for 40 s and then rinse under running water.
18. Counterstain with Harris haematoxylin (2 dips), rinse in running water and blue in ammonia water (*see* **Note 12**).
19. Dehydrate through three changes of clean 100% 2-propanol (2 min each) and three changes of xylene (2 min each). Cover slip using cytoseal mounting solution.

4. Notes

1. Although details of reptile husbandry are taxonomically variable and often species specific (29), the basic environmental factors to consider include the following:

   Temperature—reptiles are poikilothermic and require a temperature gradient in order to thermoregulate. Daily and seasonal temperature fluctuations, and even a period of hibernation (brumation), may be necessary to stimulate breeding. Preferred temperature ranges for many common pet-trade reptiles are provided by Rossi (29). Temperature gradients for terrestrial species can be established using subsurface heating sources (e.g. heating pads), whereas arboreal species may require the use of radiant heat sources such as ceramic heaters and heat lamps. Use of a rheostat and thermometer is beneficial to maintaining constant temperatures.

   Photoperiod and ultraviolet light—photoperiod (amount of daylight) is also an important external stimulus for initiating breeding, particularly for temperate and subtropic species. In addition, many reptiles require exposure to ultraviolet B (UVB) spectrum radiation (290–320 nm) to promote vitamin D_3 synthesis. Vitamin D_3 is then used to absorb calcium. For species that are active at dawn/dusk, vitamin D_3 powdered supplement may be used instead of a UVB light source.

   Humidity—humidity requirements vary with the species. Saturated environments with poor ventilation will promote fungal and bacterial growth, and lead to disease. Artificial cover (e.g. plastic hide boxes) may be used to provide microenvironments of elevated humidity for incubating eggs or during periods of skin shedding (ecdysis). Positioning a water dish above a subsurface heat source will provide a localized increase in humidity. Well-ventilated enclosures may require daily misting.

Substrate—in laboratory settings, inexpensive and easy-to-replace substrates, such as newspaper, have obvious advantages. However, some species prefer to create burrows and/or deposit eggs in soil/sand. Therefore, details of biology and captive management should be investigated prior to establishing a breeding colony. Although granular substrates may have aesthetic appeal, and be easier to establish an elevated humidity, in many small and medium-sized species ingested particles can lead to gastrointestinal impaction and death. Aromatic substrates like cedar bark can lead to irritation and death due to secondary complications (29) and should be avoided.

Diet and water—reptiles include a large array of carnivores, insectivores, herbivores and diets in-between. Nutritional requirements based on observations in the wild should be investigated if data are available. The popularity of many reptiles as pets has made many captive-raised prey, including mice, rats and arthropods, widely accessible year round. Arthropods are typically low in calcium and should be raised on diets of calcium-rich leafy greens ('gut loaded') (30). It may also be necessary to dust arthropods with powdered calcium supplement prior to being offered. Free access to fresh water is usually necessary, although species from arid climates may prefer occasional misting. Terrestrial species typically make use of floor-based water dishes, whereas arboreal species may require misting, a drip system or an elevated water source.

Enclosure—minimal enclosure floor space and height will depend on the size and behaviour of the species (29). Consideration should be given to social factors such as intraspecific aggression and territorial behaviours. Opaque enclosures or opaque cage dividers may be necessary. Aquatic species (crocodylians and some turtles) require a terrestrial area to haul out of the water and bask. Enclosures, water bowls, hide boxes and other cage furniture must be disinfected on a regular basis using either a standard cage washing machine or a disinfection solution such as sodium hypochlorite (household bleach, 2–10% solution). It should be noted that some captive-bred and wild reptiles (including common pet-trade species such as leopard geckos and Eastern corn snakes) may be infected with *Cryptosporidium* sp., a coccidian protozoan that produces oocysts resistant to disinfection by bleach. Cryptosporidiosis can lead to chronic weight loss, diarrhoea, lethargy and death. To avoid cross-contamination, enclosures housing individuals suspected

of having cryptosporidiosis should be disinfected with ammonia water (5% solution) followed by a period of air-drying (31).

2. Sex determination in reptiles may be the result of genetic or environmental factors. In genotypic sex determination, sex chromosomes determine males from females. Genotypic sex determination includes male heterogamety, in which males are characterized by having two dissimilar sex chromosomes; female heterogamety, in which females are characterized by having two dissimilar sex chromosomes; and genetic systems that have not been linked to heteromorphic sex chromosomes (32). Various turtles and lizards, as well as most snakes, use genetic sex determination.

   Alternatively, in all crocodylians and numerous species of turtles and lizards (32), there are no sex chromosomes. Instead sexual identity is established during embryogenesis by incubation temperature (32). It is worth noting that the effects of incubation temperature are cumulative in both magnitude and duration (33, 34). The use of species that employ temperature-dependent sex determination (TSD) in experimental settings has many obvious advantages and it is often possible to produce a 1:1 sex ratio at pivotal species-specific temperatures (35). The specific timeframe during which eggs are sensitive to incubation temperature has been established for several species, typically within the middle third to half of embryonic development (34, 36). Gonadal differentiation has yet to occur when this period begins but by the conclusion of this period, sex-specific gonadal changes have become apparent (32).

   As with genotypic sex determination, there are several distinctive patterns of TSD. Crocodylians and some lizards employ female–male TSD, wherein lower incubation temperatures result in a predominately female population and higher incubation temperatures result in a predominately male population (37). Alternatively, male–female sex determination occurs in many Testudines. Another pattern of TSD is female–male–female found in some crocodylians, lizards and Testudines. In female–male–female TSD, predominately female populations are produced at lower and higher temperatures, whereas males are produced at intermediate temperatures (37). Lastly, there are some species of the gecko *Tarentola* in which males are produced at lower temperatures, females are produced at intermediate temperatures and a balanced sex ratio is found at high temperatures (38).

3. Reptile eggs are categorized either as hard-shelled or with parchment-like (leathery; soft) shells (39). Both types have

an inorganic outer calcareous layer, primarily composed of calcium carbonate (40), and an organic inner layer or shell membrane. The thickness and the continuity of the calcareous layer determine the overall structure of the eggshell. Shells with a thick and continuous calcareous layer are 'hard', whereas those with a thin and/or a discontinuous calcareous layer are parchment like (39). Discontinuous calcareous layers allow for more flexibility and expansion due to embryo growth and consequently are difficult to window for in ovo manipulations (40).

The shell membrane lies deep to the calcareous layer and consists of multiple fibrous horizons (41). Fibres within each horizon demonstrate differing orientations (39) and the exact number of fibrous horizons is taxonomically variable. The deepest horizon of the shell membrane is the amorphous layer (= inner boundary layer (42); limiting membrane (43)). This amorphous layer has many small pores but is considered to function as a barrier to pathogens during development (44).

4. Specimens for whole-mount histochemistry can be stored in 70% EtOH for months prior to staining. They will need to be rehydrated through a graded EtOH series (40%, 15%, dH_2O).
5. Segmented specimens are easier to completely eviscerate. It is worth noting that Testudines, crocodylians and *Sphenodon* have skeletal structure located superficially across the abdomen (plastral elements and gastralia). In order to maintain these elements in situ across the ventral midline, avoid opening the abdominal cavity with a sagittal incision. Alternatively, remove the viscera by making an incision in the lateral body wall (through the rib elements) on one side of the abdomen. Hemisectioned heads reduce superimposition of contralateral elements.
6. Following maceration, specimens will be very delicate. Use a slotted spoon to transfer specimens into subsequent solutions.
7. Alizarin red S stains for structures mineralized with calcium salts. Most commonly, this is bone but may also include calcified cartilage, dental tissues and calcified endolymphatic ducts. These ducts may be very prominent in the occipital area of the head among early-staged reptile embryos. Premineralized bone (osteoid) condensations will be alizarin red negative. Prior to calcification, osteoid condensations are visible using transmitted light as white or opaque web-like concentrations of tissue.

8. Make stock solutions of the 0.5% KOH—glycerol series ahead of time to ensure that they are well mixed and without bubbles.
9. Prior to photography, position the specimen and wait for 1–3 h to reduce distortions in the glycerol.
10. Embryonic specimens must be staged prior to processing using an appropriate staging table. If possible, stage prior to fixation or soon thereafter to minimize distortion of tissues and colour change. Ferguson (13) provides a detailed embryonic staging table for American alligator. Avoid overfixation (i.e. longer than 24 h). Specimens fixed with 10% NBF do not necessarily require antigen retrieval. Enzymatic digestion (e.g. pronase) and heat-induced epitope retrieval (citrate buffer at 80°C) may result in increased amounts of background staining compared with samples that did not undergo retrieval.
11. Unless treated carefully, tissue sections may lift off the slides. Avoid agitating slides and use Coplin jars, rather than a spray bottle, to wash with PBS.
12. The intensity of the background staining can be altered by either increasing or decreasing the number of dips in haematoxylin. In addition, you can decrease the intensity of the haematoxylin after you have stained by dipping in 1% acid alcohol.

Acknowledgements

We thank Pat A.D. Wise for comments on the manuscript, Helen Coates for assistance with the immunohistochemical protocol and Ilinca Lungu for assistance with the photography. This work is funded by the Natural Sciences and Engineering Research Council of Canada.

References

1. Modesto, S.P. and Anderson, J.S. (2004) The phylogenetic definition of *Reptilia*. *Syst. Biol.* **53**, 815–821.
2. Buchtová, M., Boughner, J., Fu, K., Diewert, V.M., and Richman, J.M. (2007) Embryonic development of *Python sebae*—II: Craniofacial microscopic anatomy, cell proliferation and apoptosis. *Zool.* **110**, 231–251.
3. Buchtová, M., Handrigan, G.R., Tucker, A.S., Lozanoff, S., Town, L., Fu, K., Diewert, V.M., Wicking, C., and Richman, J.M. (2008) Initiation and patterning of the snake dentition are dependent on Sonic hedgehog signaling. *Dev. Biol.* **319**, 132–145.
4. Stewart, J.R. and Thompson, M.B. (2008) Parallel evolution of placentation in

Australian scincid lizards. *J. Exp. Zool. (Mol. Dev. Evol.)* **310B**, 1–13.
5. Shine, R. (1983) Reptilian reproductive modes: the oviparity–viviparity continuum. *Herpetologica* **39** (1), 1–8.
6. Guillette, L.J., Jr (1993) The evolution of viviparity in lizards. *Bioscience* **43** (11), 742–751.
7. Blackburn, D. (1992) Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. *Am. Zool.* **32**, 313–321.
8. Blackburn, D.G. (2000) Classification of the reproductive patterns of amniotes. *Herpetol. Monogr.* **14**, 371–377.
9. Thompson, M.B., Biazik, J.B., Lui, S., Adams, S.M., and Murphy, C.R. (2006) Morphological and functional changes to the uterus of lizards with different placental complexities. *Herpetol. Monogr.* **20**, 178–185.
10. Yntema, C.L. (1968) A series of stages in the embryonic development of *Chelydra serpentina*. *J. Morphol.* **125**, 219–252.
11. Tokita, M. and Kuratani, S. (2001) Normal embryonic stages of the Chinese softshelled turtle *Pelodiscus sinesnsis*. *Zool. Sci.* **18**, 705–715.
12. Greenbaum, E. and Carr, J.L. (2002) Staging criteria for embryos of the spiny softshell turtle, *Apalone spinifera* (Testudines: Trionychidae). *J. Morphol.* **254**, 272–291.
13. Ferguson, M.W.J. (1985) Reproductive biology and embryology of the crocodilians. In *Biology of the Reptilia*, eds. Gans, C., Billett, F., and Maderson, P.F.A. Academic, New York, NY, vol. **14**, Development A, pp. 329–491.
14. Lungman, J., Pina, C.I., and Siroski, P. (2008) Embryological development of *Caiman latirostris* (Crocodylia: Alligatoridae). *Genesis* **46**, 401–417.
15. Zehr, D.R. (1962) Stages in the normal development of the common garter snake, *Thamnophis sirtalis sirtalis*. *Copeia* **1962**, 322–329.
16. Boughner, J.C., Buchtová, M., Fu, K., Diewert, V., Hallgrimsson, B., and Richman, J.M. (2007) Embryonic development of *Python sebae*—I: staging criteria and macroscopic skeletal morphogenesis of the head and limbs. *Zoology* **110**, 212–230.
17. Noro, M., Uejima, A., Abe, G., Manabe, M., and Tamura, K. (2009) Normal developmental stages of the Madagascar ground gecko *Paroedura pictus* with special reference to limb morphogenesis. *Dev. Dyn.* **238**, 100–109.
18. Sanger, T.J., Losos, J.B., and Gibson-Brown, J.J. (2008) A developmental staging series for the lizard genus *Anolis*: a new system for the integration of evolution, development, and ecology. *J. Morphol.* **269**, 129–137.
19. Wise, P.A.D., Vickaryous, M.K., and Russell, A.P. (2009) An embryonic staging table for in ovo development of *Eublepharis macularius*, the leopard gecko. *Anat. Rec.* **292**, 1198–1212.
20. Moffat, L.A. (1985) Embryonic development and aspects of reproductive biology in the Tuatara, *Sphenodon punctatus*. In *Biology of the Reptilia*, eds. Gans, C., Billett, F., and Maderson P.F.A. Academic, New York, NY, vol. **14**, Development A, pp. 495–521.
21. Montero, R., Gans, C., and Lions, M.L. (1999) Embryonic development of the skeleton of *Amphisbaena darwini heterozonata* (Squamata: Amphisbaenidae). *J. Morphol.* **239**, 1–25.
22. Viets, B.E., Tousinant, A., Ewert, M.A., Nelson, C.G., and Crews, D. (1993) Temperature-dependent sex determination in the leopard gecko, *Eublepharis macularius*. *J. Exp. Zool.* **265**, 679–683.
23. Werner, Y.L., Takahashi, H., Mautz, W.J., and Ota, H. (2005) Behavior of the terrestrial nocturnal lizards *Goniurosaurus kuroiwae kuroiwae* and *Eublepharis macularius* (Reptilia, Eublepharidae) in a thigmothermal gradient. *J. Therm. Biol.* **30**, 247–254.
24. Bragg, W.K., Fawcett, J.D., Bragg, T.B., and Viets, B.E. (2000) Nest site selection in two eublepharid geckos species with temperature-dependent sex determination and one with genotypic sex determination. *Biol. J. Linn. Soc.* **69**, 319–332.
25. Klymkowsky, M.W. and Hanken, J. (1991) Whole-mount staining of *Xenopus* and other vertebrates. In *Xenopus laevis: Practical Uses in Cell and Molecular Biology*, Methods in Cell Biology, eds. Kay, B.K. and Peng, H.B. Academic, New York, NY, vol. **36**, pp. 419–441.
26. Garcia, R.L., Coltrera, M.D., and Gown, A.M. (1989) Analysis of proliferative grade using anti-PCNA/cyclin monoclonal antibodies in fixed, embedded tissues. *Am. J. Pathol.* **134**, 733–739.
27. Thorogood, J. and Whimster, I.W. (1979) The maintenance and breeding of the leopard gecko as a laboratory animal. *Int. Zoo. Yearbook* **19**, 74–78.
28. Rhen, T., Sakata, J.T., Zeller, M., and Crews, D. (2000) Sex steroid levels across the reproductive cycle of female leopard geckos, *Eublepharis macularius*, from different incubation temperatures. *Gen. Comp. Endocrinol.* **118**, 322–331.

29. Rossi, J.V. (2006) General husbandry and management. In *Reptile Medicine and Surgery*, ed. Mader, D.R., second edition. Saunders Elsevier, St. Louis, MO, pp. 25–41.
30. Finke, M.D. (2003) Gut loading to enhance the nutrient content of insects as food for reptiles: a mathematical approach. *Zoo Biol.* **22**, 147–162.
31. Pasmans, F., Blahak, S., Martel, A., and Pantchev, N. (2008) Introducing reptiles into a captive collection: the role of the veterinarian. *Vet. J.* **175**, 53–68.
32. Wibbels, T., Bull, J.J., and Crews, D. (1994) Temperature-dependent sex determination: a mechanistic approach. *J. Exp. Zool.* **270**, 71–78.
33. Pieau, C. and Dorizzi, M. (1981) Determination of temperature sensitive stages for sexual differentiation of the gonads in the embryos of the turtles, *Emys orbicularis*. *J. Morphol.* **7**, 373–382.
34. Wibbels, T., Bull, J.J., and Crews, D. (1991) Chronology and morphology of temperature-dependent sex determination. *J. Exp. Zool.* **260**, 371–381.
35. Wibbels, T., Cowan, J., and LeBoeuf, R. (1998) Temperature-dependent sex determination in the red-eared slider turtle, *Trachemys scripta*. *J. Exp. Zool.* **281**, 409–416.
36. Webb, G.J.W., Beal, A.M., Manolis, S.C., and Dempsey, K.E. (1987) The effects of incubation temperature on sex determination and embryonic development rate in *Crocodylus johnstoni* and *C. porosus*. In *Wildlife Management: Crocodiles and Alligators,* eds. Webb, G.J.W., Manolis, S.C., and Whitehead, P.J. Surrey Beatty and Sons, Winnellie, pp. 507–531.
37. Lang, J.W. and Andrews, H.V. (1994) Temperature-dependent sex determination in crocodilians. *J. Exp. Zool.* **270**, 28–44.
38. Köhler, G. (2005) Influence of temperature on sex determination. In *Incubation of Reptile Eggs*, ed. Köhler, G. Krieger Publishing Company, Malabar, FL, pp. 35–36.
39. Packard, M.J. and DeMarco, V.G. (1991) Eggshell structure and formation in eggs of oviparious reptiles. In *Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles*, eds. Deeming, D.C. and Ferguson, M.W.J. Cambridge University Press, Cambridge, pp. 53–69.
40. Packard, G.C. (1994) Patterns of mobilization and deposition of calcium in embryos of oviparous, amniotic vertebrates. *Isr. J. Zool.* **40**, 481–492.
41. Aitken, R.N.C. and Solomon, S.E. (1976) Observations on the ultrastructure of the oviduct of the Costa Rican green turtle (*Chelonia mydas* L). *J. Exp. Mar. Bio. Ecol.* **21**, 75–90.
42. Trauth, S.E. and Fagerberg, W.R. (1984) Ultrastructure and stereology of the eggshell in *Cnemidophorus sexlineatus* (Lacertilia: Teiidae). *Copeia* **1984**, 826–832.
43. Tranter, H.S., Sparks, N.H.C., and Board, R.G. (1983) Changes in structure of the limiting membrane and in oxygen permeability of chicken egg integument during incubation. *Br. Poul. Sci.* **24**, 537–547.
44. Kern, M.D. and Ferguson, M.W.J. (1997) Gas permeability of American alligator eggs and its anatomical basis. *Physiol. Zool.* **70**, 530–546.

Chapter 18

Reproductive and Developmental Manipulation of the Marsupial, the Tammar Wallaby *Macropus eugenii*

Marilyn B. Renfree and Andrew J. Pask

Abstract

The developing marsupial is an ideal animal for use in biomedical research. Marsupials are mammals that have been separated from eutherian mammals for over 130–140 million years. They all deliver altricial young that complete their growth and development after birth usually in a pouch, but not all marsupials have a pouch. Their lactation changes dynamically throughout the period of pouch life, and the mother controls their growth via the production of milk that is tailor-made for each stage of development. The tammar wallaby, *Macropus eugenii*, has been the experimental marsupial of choice for over five decades, as it is highly amendable to handling and breeds well in captivity. The tammar is especially interesting because it has both a lactational and a seasonal control of its reproduction and embryonic diapause that normally lasts 11 months. Standard molecular techniques can be used for most manipulations in marsupials. However, there are several special techniques for treating the young for experimental surgery and for organ culture that we detail below.

Key words: Marsupial, pouch young manipulation, experimental surgery, developmental biology, manipulation of sexual differentiation.

1. Introduction

Marsupials are an emerging new model for developmental biology and genetics. Their specialized mode of reproduction coupled with their evolutionary distance from eutherian mammals makes them uniquely suited to answering specific questions of mammalian development. Marsupials are mammals that last shared a common ancestor with eutherians 148 million years ago (1). This makes evolutionary comparisons with eutherian mammals extremely powerful for the identification of key developmental

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_18,

components. Marsupials differ from eutherians primarily in their mode of reproduction. Marsupials give birth to relatively underdeveloped altricial young that complete the majority of their growth and development while external to the mother, usually in a pouch. The young spend a relatively long time depending on lactation when most organ systems complete their development and mature (2–4). This period of development is equivalent to stages of intrauterine development in most eutherians. Whilst the neonate is less developed than a neonatal mouse, the weaned young-at-foot is more akin to the precocial young of the cow, sheep and horse (**Fig. 18.1**). Thus marsupial development in the pouch provides direct access to developing young during stages of development that occur in utero and are difficult to access in eutherians.

One of the most intensively studied model marsupial species is the tammar wallaby (*Macropus eugenii*). The tammar has been chosen as model marsupial as it breeds well in captivity and its reproduction and development have been extensively characterized (2, 5, 6). The tammar is a particularly useful model for

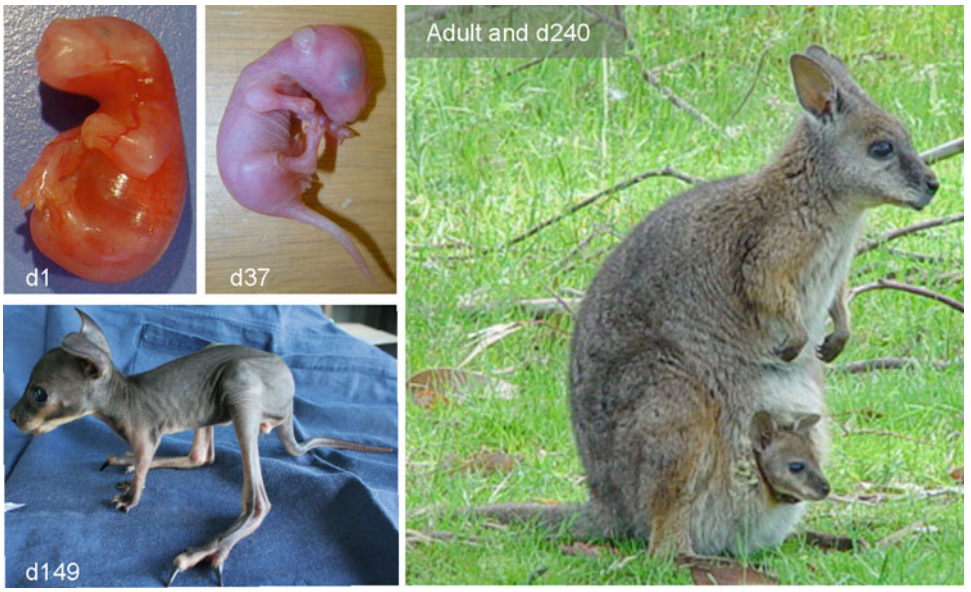

Fig. 18.1. Tammar wallaby pouch young development. The day 1 neonate (head length 7.2 mm, weight 440 mg) has poorly developed hind limbs and well-developed forelimbs to assist in climbing to the pouch. The young are heterothermic until around day 100 post-partum (pp). The gonads are indifferent at birth but will begin to form cords in XY neonates around this time point. In XX neonates, evidence of ovarian structure is not apparent until around day 8. By day 37 post-partum, the hind limbs have differentiated to a similar stage as the forelimb. The gonads are developed in males and females and the urogenital systems are also sexually dimorphic except for the phallus which becomes sexually dimorphic from around day 60 pp. By day 149 pp, the young have open eyes and a very fine covering of fur. The young remain in the pouch at this stage. By day 240 post-partum (weight around 1.5–2 kg), the young are fully furred and intermittently exit the pouch. The young will continue to drink milk for the next 40 or so days. The newly emerged young will reach sexual maturity between 18 and 24 months in males (34) and 10 months in females, immediately after they are weaned from their mothers (30).

developmental biology due to the seasonal and lactational control over embryo development (2, 5). The tammar has a post-partum estrous and the resulting embryo from that mating develops only to the blastocyst stage when it enters embryonic diapause while there is a sucking young in the pouch. If the pouch young is removed or lost, the blastocyst will reactivate and the mother will give birth 26.5 + 0.4 days later (6, 7). Using this feature, it is possible to generate cohorts of embryos or pouch young at equivalent developmental stages for experimental analyses. This chapter will restrict itself therefore to descriptions of the tammar.

The tammar wallaby genome has been sequenced and the very detailed physical maps of the genome (8) enable the study of the underlying developmental genetics of marsupial development. Genomic comparisons alone have been very useful in identifying gene promoters (9) and examining the evolution of developmental genetics and epigenetics (10).

Studies in the tammar have greatly contributed to our understanding of mammalian sexual development. At birth, the urogenital system has not yet differentiated toward a male or a female fate, this process occurs while in the pouch. Thus in marsupials, the young are readily accessible to experimental manipulations that are not possible at equivalent stages of eutherian development. Using this unique feature, studies of the tammar have shed new light on both endocrine and molecular control of sex determination and differentiation (11–15).

The handling and the husbandry of tammar wallabies have been well described elsewhere (2, 3, 6, 16), so they will not be covered in detail here. Similarly, the methods used for manipulation of their reproduction and the use of embryos, fetuses, and pouch young have been described, so we will provide only a summary of those and detail only those methods that have so far not been described (*see* **Note 1**).

1.1. Animal Husbandry

Tammar wallabies are typically wild caught and can be captive bred in large colonies (3). Wild populations of tammar wallabies can be found on several islands off the coast of Western and South Australia, as well as a small number on the mainland of southwest Western Australia. Colony animals are kept outdoors in large enclosures with access to grass, lucerne cubes, and a fresh water source. Colony animals should be vaccinated against tetanus and lumpy jaw and treated for external and internal parasites (16). Animals are caught in a large net and transferred into sacks for processing. Whilst still in the sack, the researcher can hold the animal and open the pouch to check for newborn young, administer treatments or manipulations. The young can also be removed from the pouch for surgery and replaced back on the mother's teat, even up to 12 h later to continue its development. Tammar wallabies are particularly amenable to human handling and

do not show any adverse reactions to the careful handling of their pouch young at any stage of development, making them ideal experimental animals (more details on the care and use of tammar wallabies can be found in (16)).

Embryonic stage tammar wallabies can be obtained by removing pouch young, leading to the reactivation of the diapausing blastocyst (2, 7). By counting the days after removal of the pouch young (days after RPY), precisely staged embryos can be obtained. Age of pouch young can be determined by their head length using established growth curves (17) or by counting the days from birth. Embryos can be sexed from approximately day 24 of gestation onward by the presence or the absence of scrotal bulges or mammary primordia (18, 19). Where sex is unclear or fetuses are collected earlier than day 24 of gestation, sex can be determined by PCR for the Y-linked *SRY* gene (20).

1.2. Embryological Development

One of the most fundamental differences of the marsupial versus the eutherian fetus is in its early development. The marsupial embryo forms an approximately 100-cell, hollow, single-layer blastocyst, but this embryo has no inner cell mass. Instead, the embryo develops from the unilaminar to a bilaminar blastocyst, then the embryonic disc forms on the blastocyst surface (2). This difference in embryo specification raises many interesting questions about early marsupial and mammalian development in general. In the tammar, it is still unknown how the cells are specified in the unilaminar blastocyst that will go on to form the embryo, but in the polyovular dasyurid marsupials, and also in the opossum, there appears to be cellular polarity in cleavage stages (21–23). Whether the signals that regulate specification and induction are the same or different from those that regulate the specification of the eutherian mammal inner cell mass is under investigation. After the differentiation of the embryonic area, the embryo proper develops in a planar fashion on the surface of the embryonic vesicle. This makes the study of early embryonic events and morphogenesis easier to observe and manipulate than in the complicated egg cylinder formed in the mouse. The embryo remains free in the uterus until approximately day 17–18 of gestation (mid-somitogenesis), after which the head-fold embryo becomes enclosed in the yolk sac that forms an interdigitation or attachment to the endometrium. The yolk sac forms a fully functional chorio-vitelline placenta, which is the site of maternal fetal exchanges, supports the later stages of fetal growth and metabolism, and produces hormones that influence maternal physiology (9, 24). Placental development is intimately linked with genomic imprinting in eutherian mammals. Investigation of the underlying genetics of marsupial placental development has shown that imprinting evolved in the common therian mammal ancestor. In addition to a more invasive placenta, eutherian

mammals also have more complex imprinting mechanisms that encompass more loci in placental development compared to marsupials (9, 25, 26).

1.3. Analysis of Gene Expression in Developing Marsupials

Gene expression can be examined using reverse-transcriptase PCR and quantitative PCR techniques according to standard protocols used in other mammalian species. However, whole-mount in situ techniques developed for the mouse cannot be readily used on developing tammar embryos. Due to the early developmental stage at which the fetus is born, the outer skin is thickened and keratinized early in development. In addition, the marsupial fetus is bigger at equivalent developmental stages than the mouse embryo. Both these features together hinder the penetration of in situ probes, especially in the later stages of uterine development and early pouch life. To achieve effective in situ results, we typically follow a standard mouse embryo whole-mount protocol (27) but extend the proteinase K (10 μg/mL PBT) digestion times accordingly (as outlined in **Table 18.1**).

In the later stages of fetal development (day 23 of gestation onward), the washing times after probe hybridization and antibody detection are all extended to at least three repeats of 1-h washes. Likewise, the development time with NBT/BCIP is also extended to allow the reagents to penetrate the embryo. Whole-mount in situ can also be performed on individual organs. Again, extended proteinase K digestion times as detailed above apply, due to both the integrity and the relative size of the tissue.

1.4. Experimental Manipulation of Developing Marsupials

Marsupial development can be easily manipulated at stages equivalent to intrauterine development in eutherians. These manipulations can be achieved in several ways, many of which have been previously described (16), namely gonadal

Table 18.1
Approximate proteinase K digestion times for tammar wallaby embryos prior to whole-mount in situ hybridization

Developmental stage	Proteinase K digestion time (min)
Day 19 of gestation	3
Day 22 of gestation	4–5
Day 24 of gestation	6–8
Day 26 of gestation	10
Day of birth	10–12
Day 2 post-partum	15

transplantation and hypothermic anesthesia, and so will only be summarized. Here we will detail the additional techniques for early stage castration, ovariectomy, and oral treatment of pouch young not previously described.

1.5. Surgical Manipulation of Pouch Young

A unique feature of the newborn marsupial is the late development of an active immune system. They are born with an innate immunity and depend on immunoglobulins from the maternal milk for the first 100 days or so post-partum. This makes them exceptionally amenable to tissue transplants and xenografts (2, 16). Furthermore, at early developmental stages (until approximately 100 days post-partum), the young are heterothermic, so anesthesia in very small young can be administered by inducing mild hypothermia through the cooling of the body temperature (28). This is the recommended technique for carrying out surgery on small pouch young of macropodid marsupials, because it causes the least trauma (National Health and Medical Research Council of Australia guidelines, 1990). A fall in the body temperature of the young marsupial at this early stage of development slows the heart rate, and respiration may cease. Apnea is not serious in these small pouch young (2). Interestingly, there is little or spontaneous EEG activity before day 127 and no response to noxious stimuli until their eyes are open and pelage development has started (29). Surgery is performed on a metal tray surrounded by ice (**Fig. 18.2**). Once the pouch young has ceased, movement surgery can be performed using standard techniques. Gonads transplanted under the skin just above the hind limb become readily vascularized and develop as normal even in hosts of the opposite sex. Pouch young older than day 50 are usually anesthetized using gaseous fluothane administered by a very small mask following standard doses for mammals, but as noted (30), there is no evidence of pain sensations until after day 140.

1.6. Oral Administration of Compounds to Pouch Young

Development can also be manipulated through the delivery of compounds orally. Oral administration has been particularly effective for the delivery of hormones and inhibitors. Oral treatments are given using 0.5-mm (internal diameter) soft polyethylene tubing. Steroid hormones are usually delivered in an oil vehicle such as arachis oil:

1. *Injection procedures:* Non-orally active compounds can be delivered by injection either subcutaneously or intraperitoneally. We have used a range of vehicles for compound delivery. The most effective way to administer steroid hormones is to dissolve the required amount of steroid hormone in 10% ethanol in triolein (a triglyceride formed from oleic acid and the principal component of olive oil) (31). Injections are given into the abdomen either subcutaneously or intraperitoneally at the site shown in **Fig. 18.3a** using the smallest gauge needle appropriate (e.g., 26 G). Latex gloves

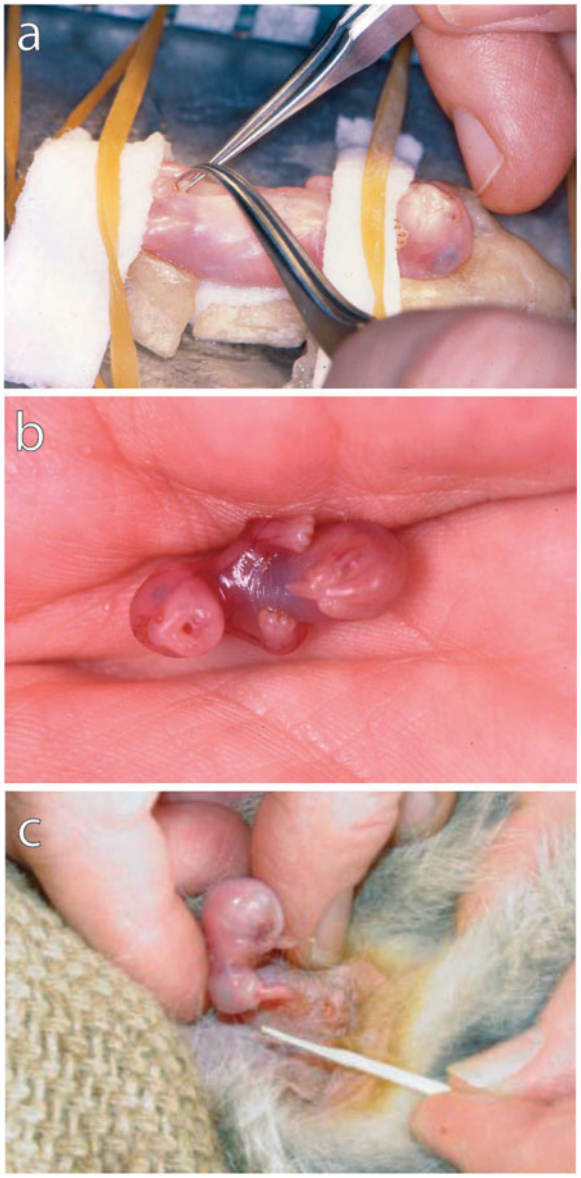

Fig. 18.2. Pouch young surgery and reattachment. (**a**) Pouch young are anesthetized for surgery by cooling. The young are placed on a wet cotton swab and held in place by a beeswax support, molded to the size of the pouch young and elastic bands covered with wet gauze where they restrain the pouch young. These are all placed on a metal tray sitting on top of an ice bath to maintain a cold temperature. Once the young have stopped moving, surgery begins. After surgery and suture placement, the young are warmed gently in the palm of the hand (**b**) and heated with warm breath until active movement resumes. The young are then transferred back into the mother pouch and reattached to the nipple (**c**). A whittled matchstick is used to assist with the placement of the nipple back in the pouch young's mouth. Once young are attached, the pouch is closed. The young are checked approximately 1 h later to ensure reattachment before the mothers are released back into housing pens.

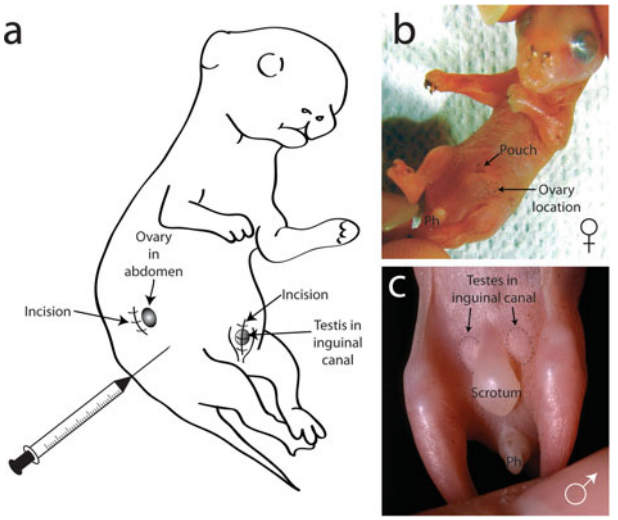

Fig. 18.3. Location of gonads and sites for surgery and subcutaneous injection. (**a**) The approximate locations of the gonads in the day 25 pouch young. At this stage, the testes are located within the inguinal canal and can be identified just below the skin surface in the lower abdomen (**c** – *dotted lines* outline the inguinal testes). The position of the incision is made directly above the inguinal testis to open the canal. The testis can be removed by cutting the Wolffian duct and gubernaculum attachments. The wound is then sutured as shown in (**a**). The ovaries are located within the abdominal cavity (**b** – *dotted lines* indicate approximate locations of the ovaries deep within the abdomen). The incision is made between the last rib and the top of the hind limb, toward the dorsal side of the abdomen. The ovary can be located through this incision and removed by lifting it up by its attachment to the Müllerian duct, ligating, and incising. The wound is then sutured as shown. Sexual development can also be altered through injection of compounds in the abdomen of the pouch young as shown in (**a**). Ph, Phallus.

should not generally be used as they catch on the fine skin of the pouch young.

1.7. Organ Cultures

Tissues can be removed and cultured in vitro to enable experimental manipulations or live imaging of differentiation events. We have used this technique for many years to examine gonadal development. We have used several protocols for organ culture but find that tissues placed on filter paper (Millipore) resting on a mesh grid suspended in an organ culture dish (Falcon) give us good morphology and reproducibility (32). We have also used hanging drop cultures (commonly used in mouse gonad culture) but due to the larger size of the indifferent tammar gonad, this system does not work as well but it has been used successfully for culturing marsupial follicles (33). We have also cultured gonads on agar blocks (16). The size of the grooves in the agar block is expanded to allow for the larger gonad size compared to those used for the mouse (grooves were approximately 3 mm wide and 1.5 mm deep). This method is effective for maintaining the structural integrity of the gonads throughout development.

2. Materials

2.1. Surgical Castration or Ovariectomy of Tammar Pouch Young Days 20–40 Post-partum

1. Saline (0.9%; sterile).
2. Tammar wallaby pouch young.
3. Dissecting microscope.
4. Ice.
5. Iridectomy 8-0 or 9-0 resorbable sutures (e.g., iridectomy sutures with attached very fine curved needle).
6. Iridectomy scissors.
7. Jewelers' forceps.
8. Curved fine forceps.
9. Matchsticks (the clean end of the match is trimmed (whittled) to a flat fine shape, then softened by chewing to allow the teat to be gently pushed into the mouth of the young without traumatizing the teat).
10. Rubber bands.
11. Scale and calipers for weighing and measuring the pouch young.
12. Stainless steel tray (Kodak processing dish or similar).
13. Restraint mold (beeswax) on a metal plate that covers Kodak dish.
14. Surgical instruments (sterilized) (watchmaker's forceps, iridectomy scissors, blunt forceps, small curved forceps, fine curved forceps).
15. Swabs (saline moistened).

2.2. Oral Administration of Compounds to Pouch Young

1. Tammar wallabies, adults with pouch young.
2. Polyethylene tubing (0.5 mm internal diameter – Dow Corning).
3. Calipers.
4. Safety glasses.
5. Otoscope.
6. Hand nets; aluminum (10-mm diameter) metal hoop that is >60 cm in diameter with fish netting ~80 cm deep. A 1.8-m-long metal handle is attached to the hoop.
7. Hessian sacks with short (30 cm) pieces of thick twine attached near the top of the open end of the sack to tie closed.
8. Oral treatment compound in solution.

2.3. Gonad Culture on Mesh Grids

1. DMEM (equilibrated and warmed to 37°C) (Dulbecco's modified Eagle's medium).
   Prepare the medium the night before and leave it in the incubator overnight to equilibrate. Add L-glutamine before use. Approximately 1.5 mL of medium is required for each organ culture dish.
2. Phosphate-buffered saline.
3. Milli-Q H_2O (autoclaved).
4. Saline (0.9%).
5. Tammar wallaby pouch young.
6. Dissecting dish (35-mm tissue culture dish filled with black wax) and 26-gauge needles for pinning.
7. Dissecting instruments (watchmaker's forceps, iridectomy scissors, blunt forceps, small curved forceps).
8. Dissecting microscope.
9. Ice (crushed).
10. Laminar flow hood.
11. Petri dishes (sterile plastic, tissue culture grade, 35-mm and BD-Falcon center-well organ culture dishes).
12. Metal grids for organ culture (stainless steel triangles with bent legs allowing the grid to sit suspended in the inner well of the organ culture dish).
13. Polycarbonate membrane (0.8-μm pore size; Costar).
14. Scalpel blade (sterile).
15. Tissue culture incubator preset to 37°C and 5% CO_2.
16. Tissue culture tubes or flasks.
17. Sterile disposable transfer pipettes.

3. Methods

3.1. Surgical Castration or Ovariectomy of Tammar Pouch Young Days 20–40 Post-partum

3.1.1. Males

1. After removal from the mother's pouch, young are anesthetized on ice (*see* **Note 2**).
2. Once movement of the young has ceased, they are placed in a beeswax mold, shaped to the size of the young. This is placed on top of a metal operating tray, over an ice bath to maintain a low temperature. The operating tray consists of an ice-filled Kodak processing dish or similar. The young are restrained with ice-cold, saline-moistened swabs and elastic bands to loosely hold the young in place for surgery (2, 30)

(**Fig. 18.2a**). The surgery should be performed under a binocular microscope.

3. At days 20–40 post–partum, the testes are located within the inguinal canal and should be identified just below the skin surface in the lower abdomen as a pale patch, slightly raised (**Fig. 18.3a**, **c**). The small incision through the skin but not through the body wall is made directly above and parallel to the inguinal canal using iridectomy scissors. Once the skin is open, a second small incision is then made into the inguinal canal itself to expose the testis and ducts within it. The testes can be removed by pulling the ducts that attach to the cranial region of the testes (the Wolffian duct) and to the caudal end of the testes (the gubernaculum, which guides the testes into the scrotum) gently upward before tying the ducts with a ligature with 6-0 -8-0 resorbable suture (both are suitable but sometimes 8-0 is hard to obtain) below the testes and removing by cutting distal to the ligature.
4. The skin is sutured with 6-0 or 8-0 sutures (e.g., Ethicon resorbable braided suture) depending on the size of the young (*see* **Note 3**).
5. The young are slowly warmed in the palm of the hand until movement resumes. At this stage, the young are placed back in the mother's pouch and reattached to the teat as described (2, 16) (**Fig. 18.2b**, **c**).

3.1.2. Females

1. Young are anesthetized on ice as described (*see* **Note 2**).
2. Once movement of the young has ceased, they are prepared for surgery as described for males above (Step 2).
3. Ovariectomy is much more difficult due to the fact that the ovaries are hidden by the gut, which easily escapes from any incision through the body wall, so care must be taken to avoid this. A lateral incision is made between each bottom rib and hind leg. The ovary should be located in the cavity by gently moving the intestines. After locating the ovary, it is lifted up with fine forceps and the connecting Müllerian duct ligated with 6-0 resorbable suture. The ovary can then be removed by holding it tightly below the fimbrium and cutting along the distal surface to the forceps under the ovary. The pressure of the forceps below the ovary is normally sufficient to prevent any bleeding. Alternatively, an 8-0 suture can be tied around the oviduct and accompanying vessels below the ovary (*see* **Note 3**), but this is difficult as the outer skin and body walal can tear, allowing the gut to escape.
4. The body wall is then closed using fine (6-0 to 8-0 depending on availability and confidence of the surgeon in tying the ultrafine) absorbable sutures.

5. The young are slowly warmed in the palm of the hand until movement resumes. At this stage, the young are placed back in the mother's pouch and reattached to the teat as described (2, 16) (**Fig. 18.2b**, **c**).

3.2. Oral Administration of Compounds to Pouch Young

1. Animals should be caught and placed in hessian wheat sacks as described (*see* **Note 4**).
2. The handler should sit down with a sacked animal held firmly between their knees. The animal should be rotated onto their backs and the sac opened to reveal the pouch. The sac should remain covering the animal's head.
3. The handler should gently but firmly restrain the back legs and open the pouch using their thumbs.
4. The person who is administering the treatment should wear safety goggles at all times as their head will be in close proximity to the strong hind legs with their long nails.
5. Newborn young can be sexed with the use of an otoscope. With the pouch open, the young should be carefully rotated on to its back and the otoscope used to examine the region immediately anterior to the phallus for the presence of scrotal bulges (males) or four whitish dots (two on each side) that indicate mammary primordia (females). Care should be taken not to pull the legs away from the curvature of the body too far or the skin will tear in the groin region.
6. Once sex is determined, the correct amount of treatment should be loaded into the polyethylene tubing. This is achieved by capillary action into the tube up to a pre-marked line.
7. The pouch young should be oriented in the pouch to gain easy access to the nipple.
8. Without moving the pouch young, the polyethylene tubing is placed down the side of the teat and into the mouth of the attached young and held in place until the liquid is gone (sucked) from the tube (**Fig. 18.4**).
9. The tube is then gently removed from the mouth being careful.

3.3. Gonad Culture on Mesh Grids

3.3.1. Preparation of Organ Culture Dishes (Use Aseptic Techniques for All Steps)

1. Grids are placed in the inner well of organ culture dishes (**Fig. 18.5**).
2. A small piece of polycarbonate membrane is placed on the grid (sufficient in size to hold the gonads to be cultured).
3. DMEM is pipetted into the inner well, sufficient to be at the level of the grid and wet the membrane but not to cover the grid or the membrane.

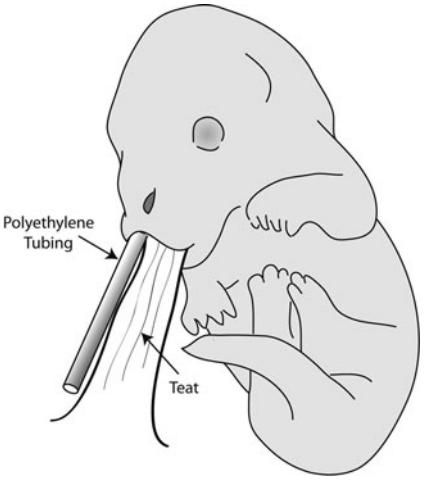

Fig. 18.4. Oral treatment of young on the teat. Compounds can be delivered directly to developing young whilst in the pouch attached to the teat. The compound (*in liquid form*) is administered through polyethylene tubing. The soft plastic tubing is inserted alongside the teat into the mouth of the pouch young being very careful not to knock it off the teat. The pouch young takes up the liquid as it drinks the milk. Once the liquid has gone, the tubing is carefully removed from the mouth and the pouch closed.

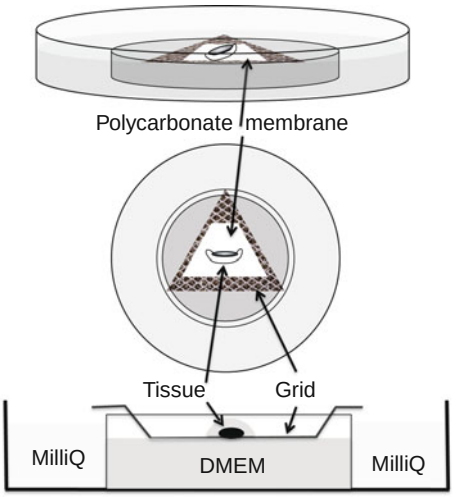

Fig. 18.5. Gonad culture setup. 3D view (*top*), top view (*middle*), and side view (*bottom*). The tissue is placed on a polycarbonate membrane (*white*) on top of a metal grid. This is suspended in the inner well of an organ culture dish filled with DMEM. A drop of DMEM was placed over the tissue to form a thin film. The outer well is filled with sterile Milli-Q.

4. The outer well is flooded with sterile Milli-Q H_2O.
5. Dishes are placed back in the incubator to equilibrate while dissection takes place.

3.3.2. Dissection of Gonads

1. Young (neonates weigh only 400 mg) are removed from the pouch and placed into a small plastic 15-mL pot with a saline-moistened swab for transport from the colony. They are preferably used within 2–4 h but can be held in this way in a warm pocket or a shirt for 8 h or more.
2. They are cooled to room temperature for 15–30 min, removed from the pot, and quickly decapitated using a sharp scalpel blade (*see* **Note 5**).
3. The body is placed dorsal side down in a wax-dissecting dish and limbs are pinned. Dissection takes place in a laminar flow hood using a dissecting microscope.
4. An incision is made from immediately above the phallus to the base of the rib cage and the body wall opened.
5. The intestines are either removed with blunt forceps or pushed aside.
6. The gonad and attached mesonephros complex is carefully dissected out as a whole and transferred to sterile saline in a 35-mm Petri dish. Whenever handling the tissues, the forceps grip the anterior tip of the mesonephros to minimize damage to the gonad – or gonads can be transferred in solution using transfer pipettes.

3.3.3. Culturing Gonads

1. The gonads are washed twice briefly in equilibrated DMEM, before transferring to the organ culture dishes.
2. Gonad–mesonephros complexes are placed on top of the polycarbonate membrane and a single drop of DMEM is placed on top of the tissue to cover it in a thin layer of media.
3. Organ cultures are placed in a tissue incubator.
4. Cultures are checked daily to ensure that the gonads are covered by a thin layer of medium. Media is changed every 2 days.
5. At the end of the culture period, tissues are rinsed in PBS and snap frozen or fixed for analyses.

4. Notes

1. Additional detailed information on the handling and husbandry of tammar wallabies, the methods used for manipulation of their reproduction, and the use of embryos, fetuses, and pouch young can be found in (2, 3, 7, 16).

2. Briefly, the PY is placed on its back on a saline-moistened swab on top of a bed of ice and regularly rotated so that all of the skin surfaces are cooled. Once movements cease, the young should be placed onto a beeswax mould, shaped to fit the PY body (16). The beeswax mould is kept cold by placing it on top of a stainless steel tray sitting on ice. The PY is held in place by rubber bands secured gently over swabs, so as not to damage the delicate skin. The heart rate slows to ~25 beats/min or less, and breathing may cease. Apnea is not serious in the very small PY, because cutaneous exchange is sufficient so long as the young is moist. For additional details, *see* (2, 16, 28).
3. Nylon sutures are not suitable as the ends when cut are too sharp for the thin pouch young skin. We typically use Ethicon resorbable braided 6-0 or 8-0 sutures for neonates up to day 20 post-partum and 8-0 or 9-0 sutures for older pouch young.
4. We use a standard size (60 cm wide × 110 cm long) hessian/burlap grain storage sacks with short (30-cm) piece of rope attached near the top of the open end of the sack. Once animals are placed in sacs, the top is folded over and secured with the attached rope. Sacs are placed on a dry surface in the shade.
5. We use a size 22 scalpel blade attached to a handle. This is the recommended method as it is quick, humane, and causes the least trauma (National Health and Medical Research Council of Australia guidelines, 1990).

Acknowledgments

We thank associate professor Geoff Shaw for his valuable expertise in all aspects of these methods and his practical contribution to the development of many of them. He also provided the excellent photography of **Figs. 18.2** and **18.3**. We acknowledge the pioneering contribution of Dr Hugh Tyndale-Biscoe in all the early work on marsupial reproduction and development. A.J.P. was supported by a National Health and Medical Research Council RD Wright fellowship and M.B.R. by an Australian Research Council Federation fellowship.

References

1. Bininda-Emonds, O.R., Cardillo, M., Jones, K.E., MacPhee, R.D., Beck, R.M., Grenyer, R., Price, S.A., Vos, R.A., Gittleman, J.L., and Purvis, A. (2007) The delayed rise of present-day mammals. *Nature* **446**, 507–512.

2. Tyndale-Biscoe, C.H. and Renfree, M.B. (1987) *Reproductive Physiology of Marsupials*. Cambridge University Press, Cambridge.
3. Tyndale-Biscoe, C.H. and Janssens, P.A. (1988) *The Developing Marsupial: Models for Biomedical Research*, eds. Tyndale-Biscoe, C.H. and Janssens, P.A. Springer, Heidelberg, pp. 1–8.
4. Renfree, M.B. (1983) Marsupial reproduction: the choice between placentation and lactation. In *Oxford Reviews of Reproductive Biology*, ed. Finn, C.A. Oxford University Press, Oxford, vol. **5**, pp. 1–29.
5. Tyndale-Biscoe, C.H., Hearn, J.P., and Renfree, M.B. (1974) Control of reproduction in macropodid marsupials. *J. Endocrinol.* **63**, 589–614.
6. Renfree, M.B. and Tyndale-Biscoe, C.H. (1973) Intrauterine development after diapause in the marsupial *Macropus eugenii*. *Dev. Biol.* **32**, 28–40.
7. Renfree, M.B., Fletcher, T.P., Blanden, D.R., Lewis, P.R., Shaw, G., Gordon, K., Short, R.V., Parer-Cook, E., and Parer, D. (1989) Physiological and behavioural events around the time of birth in macropodid marsupials. In *Kangaroos, Wallabies and Rat Kangaroos*, eds. Grigg, G., Jarman P., and Hume, I.D.. Surrey Beatty & Sons Pty. Ltd., Sydney, pp. 323–337.
8. Zenger, K.R., McKenzie, L.M., and Cooper, D.W. (2002) The first comprehensive genetic linkage map of a marsupial: the tammar wallaby (*Macropus eugenii*). *Genetics* **162**, 321–330.
9. Yu, H., Pask, A.J., Shaw, G., and Renfree, M.B. (2009) Comparative analysis of the mammalian WNT4 promoter. *BMC Genomics* **10**, 416.
10. Renfree, M.B., Hore, T.A., Shaw, G., Graves, J.A., and Pask, A.J. (2009) Evolution of genomic imprinting: insights from marsupials and monotremes. *Annu. Rev. Genomics Hum. Genet.* **10**, 241–262.
11. Renfree, M.B. and Shaw, G. (2001) Germ cells, gonads and sex reversal in marsupials. *Int. J. Dev. Biol.* **45**, 553–563.
12. Renfree, M.B., Harry, J.L., and Shaw, G. (1995) The marsupial male: a role model for sexual development. *Philos. Trans. R. Soc. Lond. B* **350**, 243–251.
13. Wilson, J.D., Leihy, M.W., Shaw, G., and Renfree, M.B. (2003) Unsolved problems in male physiology: studies in a marsupial. *Mol. Cell. Endocrinol.* **211**, 33–36.
14. Renfree, M.B., Pask, A.J., and Shaw, G. (2001) Sex down under: the differentiation of sexual dimorphisms during marsupial development. *Reprod. Fertil. Dev.* **13**, 679–690.
15. Pask, A.J. and Renfree, M.B. (2009) Marsupial reproductive and developmental genetics. In *Marsupial Genetics and Genomics*, eds. Deakin, J., Waters, P. and Graves, J. Springer, New York London Paris, in press.
16. Hickford, D., Frankenberg, S., and Renfree, M.B. (2010) The tammar wallaby, Macropus eugenii: a model kangaroo for the study of developmental and reproductive biology. In *Emerging Model Organisms: A Laboratory Manual, Volume 2*, Crotty, D.A., and Gann, A., eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 449–494.
17. Poole, W.E., Simms, N.G., Wood, J.T., and Lubulwa, M. (1991) Tables for age determination of the Kangaroo Island wallaby (Tammar) *Macropus eugenii* from body measurements, CSIRO Division of Wildlife and Ecology Technical Memorandum. No. 32. CSIRO, Collingwood, Australia.
18. O, W.S., Short, R.V., Renfree, M.B., and Shaw, G. (1988) Primary genetic control of somatic sexual differentiation in a mammal. *Nature* **331**, 716–717.
19. Renfree, M.B. and Short, R.V. (1988) Sex determination in marsupials: evidence for a marsupial-eutherian dichotomy. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **322**, 41–53.
20. Harry, J.L., Koopman, P., Brennan, F.E., Marshall Graves, J.A., and Renfree, M.B. (1995) Widespread expression of the testis-determining gene, SRY, in a marsupial. *Nat. Genet.* **11**, 347–349.
21. Selwood, L., Robinson, E.S., Pedersen, R.A., and Vandeberg, J.L. (1997) Development in vitro of marsupials: a comparative review of species and a timetable of cleavage and early blastocyst stages of development in *Monodelphis domestica*. *Int. J. Dev. Biol.* **41**, 397–410.
22. McCrady, E., Jr. (1938) The embryology of the sopossum. *Am. Anat. Mem.* **16**, 233.
23. Selwood, L. and Johnson, M.H. (2006) Trophoblast and hypoblast in the monotreme, marsupial and eutherian mammal: evolution and origins. *Bioessays* **28**, 128–145.
24. Renfree, M.B. (2000) Maternal recognition of pregnancy in marsupials. *Rev. Reprod.* **5**, 6–11.
25. Pask, A.J., Papenfuss, A.T., Ager, E.I., McColl, K.A., Speed, T.P., and Renfree, M.B. (2009) Analysis of the platypus genome suggests a transposon origin for mammalian imprinting. *Genome Biol.* **10**, R1.
26. Hore, T.A., Renfree, M.B., Pask, A.J., and Graves, J.A.M. (2009) Genomic imprinting. In *Marsupial Genetics and Genomics*, eds.

Deakin, J., Waters, P. and Graves, J. Springer, New York, London, Paris, in press.
27. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R., eds (2002) *Manipulating the Mouse Embryo, A Laboratory Manual*, 3rd Edition. Cold Spring Harbor Press, New York, NY.
28. Renfree, M.B. (2002) Hypothermic anaesthesia of early postnatal marsupial pouch young. In learning, animals and environment: changing the face of the future. *ANZCCART* **15**, 119–121.
29. Diesch, T.J., Mellor, D.J., Johnson, C.B., and Lentle, R.G. (2010) Developmental changes in the electroencephalogram and responses to a noxious stimulus in anaesthetized tammar wallaby joeys (*Macropus eugenii eugenii*). *Lab Anim.* **44**, 79–87.
30. Nurse, S.C. and Renfree, M.B. (1994) Pubertal development of the pouch and teats in a marsupial. *J. Reprod. Fert.* **101**, 279–285.
31. Shaw, G., Fenelon, J., Sichlau, M., Auchus, R.J., Wilson, J.D., and Renfree, M.B. (2006) Role of the alternate pathway of dihydrotestosterone formation in virilization of the Wolffian ducts of the tammar wallaby, *Macropus eugenii*. *Endocrinology* **147**, 2368–2373.
32. Whitworth, D.J., Shaw, G., and Renfree, M.B. (1996) Gonadal sex reversal of the developing marsupial ovary in vivo and in vitro. *Development* **122**, 4057–4063.
33. Nation, A. and Selwood, L. (2009) The production of mature oocytes from adult ovaries following primary follicle culture in a marsupial. *Reproduction* **138**, 247–255.
34. Williamson, P., Fletcher, T.P., and Renfree, M.B. (1990) Testicular development and maturation of the hypothalamic–pituitary–testicular axis in the male tammar, *Macropus eugenii*. *J. Reprod. Fertil.* **88**, 549–557.

Chapter 19

Mutant Generation in Vertebrate Model Organisms by TILLING

Sylke Winkler, Nicola Gscheidel, and Michael Brand

Abstract

TILLING (Targeting Induced Local Lesions IN Genomes) is a popular reverse genetic approach that has been successfully applied in several genetic model organisms such as zebrafish, rat, *Drosophila*, *Arabidopsis,* or medaka. In contrast to classical targeted knockout technologies that work in mice by directly targeting a gene of interest, TILLING follows an indirect strategy. The first step of the TILLING pipeline is the generation of a TILLING library that consists of large numbers of mutagenized individuals. In a second step, these individuals are screened for mutations in any gene of interest. Screening is performed by PCR amplification of specific exons from each individual of a library followed by mutation detection. This could be done, for example, by direct re-sequencing of PCR fragments or alternatively, by CEL1 endonuclease-mediated mutation discovery. Individuals carrying potentially deleterious point mutations are isolated from the library and mutant lines are established. TILLING allows the identification of a whole range of point mutations, covering nonsense, splice site, and missense mutations in only one screening round, because the generation of mutations by mutagenesis as well as the screening tools is not biased. Potential knockout mutations are initially the mutations of choice, but TILLING screens can also be used to isolate allelic series of point mutations ranging from complete null phenotypes to hypomorphic or even dominant-negative or conditional alleles. These allelic series can be helpful for a comprehensive functional analysis of a gene of interest. TILLING is applicable to any kind of genetically tractable model organism, as long as this model organism is amenable to chemical mutagenesis, and genomic sequence information for a gene of interest is available. This chapter describes the design and pipeline of a TILLING facility as we are currently operating it for zebrafish in Dresden. Protocols for mutation detection by direct re-sequencing are described in detail. However, alternatives to this pipeline do exist and will be mentioned briefly.

Key words: TILLING, reverse genetics, zebrafish, point mutations, amplicon re-sequencing.

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_19,

1. Introduction

Since 2001, when the outcome of the human genome sequence project was published, several other whole genome sequencing projects of various organisms were initiated. Currently, circa 110 (stand 1st of June 2011) different vertebrate species and their genomic sequence data are found on the NCBI Eukaryotic Genomic Sequencing Projects homepage.

With the recent development of the "next generation sequencing technologies" (NGS), whole genome sequencing has become much faster and affordable. Therefore, the number of sequenced and annotated genomes of organisms will increase further and will finally span the range of common as well as "exotic" genetic model systems. However, correlating the DNA sequence data with the actual function of the encoded gene product is lagging behind and is therefore a primary goal for current and future research.

Forward and reverse genetic tools applied to various genetic vertebrate model systems help to define these correlations. Forward genetic screens induce mutations in model organisms and analyze them for phenotypic effects followed by molecular cloning of the mutated gene. Reverse genetic technologies make use of the molecular information about a gene of interest and specifically target this gene, followed by phenotypic characterization of the resulting mutant. Targeting of genes could take place at the level of the mRNA expression or translation (e.g., antisense oligonucleotides, RNA interference, and alternative approaches). Alternatively, gene targeting could take place directly at the genomic locus. Recently, modified zinc finger nucleases have been used to target and hereditarily destroy genomic loci in several species (**Chapter 20** by Jasmine M. McCammon et al., this volume). Targeted inactivation or knockout of specific genes in embryonic stem cells and subsequent embryonic stem cell transfer back into the animal is so far limited to mice only (**Chapter 11** by Anne E. Griep et al., this volume). An alternative tool to induce mutations at the level of the genomic DNA is achieved by the TILLING (Targeting Induced Local Lesions In Genomes) technology. TILLING was first invented in *Arabidopsis* (1, 2) and later adapted to several vertebrate model systems, such as zebrafish (3, 4), rat (5), and medaka (6), reviewed in (7). TILLING screens for induced mutations in any gene of interest in libraries of mutagenized individuals. The targeted gene is amplified by PCR and mutations are detected by different technologies that are suitable for large-scale setups such as mutation detection by high-performance liquid chromatography (HPLC) (2), CEL1-endonuclease-mediated cleavage of DNA heteroduplexes (1, 8), and targeted re-sequencing of PCR fragments (4).

Zebrafish are an excellent vertebrate model system not only for human diseases but also for studying genetic networks controlling vertebrate development. Genomic sequence and annotation data are provided by the *Danio rerio* genome sequencing project performed at the Sanger Institute (http://www.sanger.ac.uk/Projects/D_rerio/). A systematic screen for point mutations in zebrafish was initiated in the context of the Sixth Framework Programme Project "Zebrafish models for Human Development and Disease, Zf Models." The targeted knockout project was the first collaborative project that took place in three different European Laboratories (Edwin Cuppen, Utrecht; Derek Stample, Hinxton; Michael Brand and Sylke Winkler, Dresden). Zebrafish TILLING libraries were established in the individual laboratories and shared among the partners. Point mutations were detected by large-scale re-sequencing of PCR fragments as shown in **Fig. 19.1**, which illustrates a general TILLING strategy for zebrafish. Over the course of 5 years (2004–2009), a

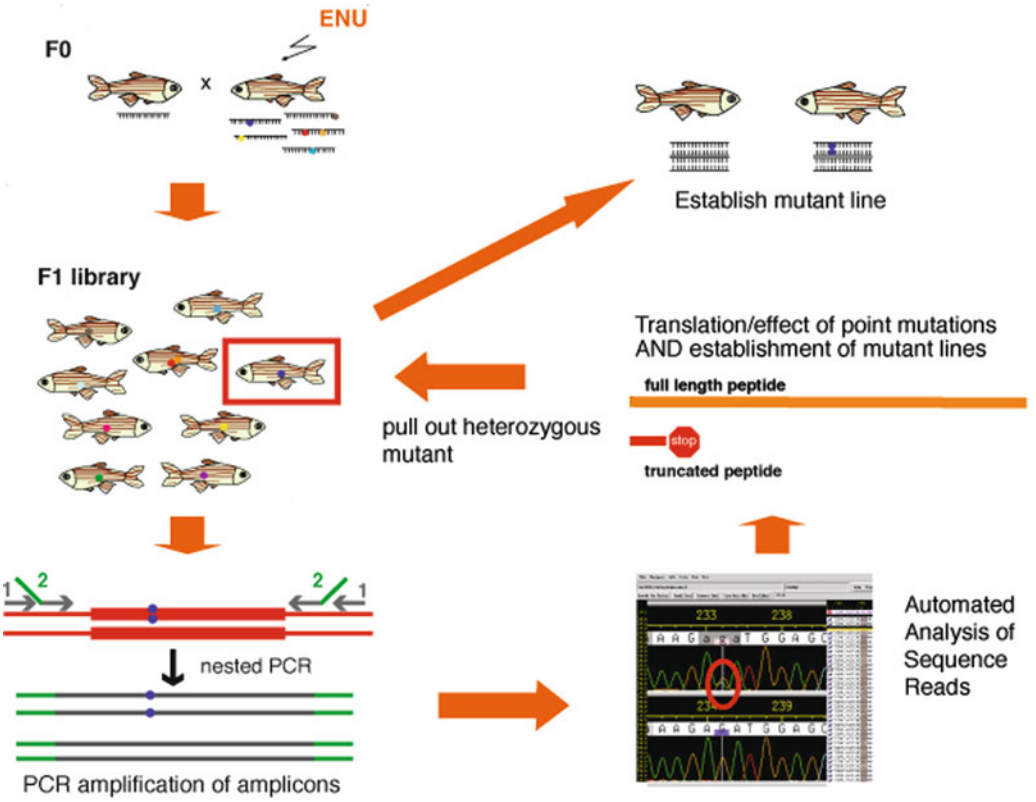

Fig. 19.1. TILLING strategy. Zebrafish males are treated with the chemical mutagen ENU to induce point mutations and subsequently crossed against wild-type zebrafish females. A TILLING library is generated from this F1 offspring, which consists of genomic DNA and living fish and sperm samples, respectively. The library is screened for a gene of interest by direct re-sequencing of PCR fragments. Sequence data are analyzed for point mutations and verified mutations are translated into peptides. Founders of potentially deleterious alleles are isolated from the TILLING library and propagated to establish a mutant line.

total of 251 potential knockout alleles covering 212 different genes have been identified from more than 20,000 mutagenized zebrafish. The targeted knockout project focussed on nonsense and splice site mutations that are expected to destroy the function of the resulting peptide. This extremely successful project is going to be continued at the Sanger Institute (http://www.sanger.ac.uk/Projects/D_rerio/mutres/) and Dresden TILLING facility (http://www.mpi-cbg.de/en/facilities/profiles/tilling.html). In addition to the European Initiatives, a consortium of three laboratories coordinates and performs TILLING screens in zebrafish in the USA (Cecilia Moens, Seattle; Lila Solnica-Krezel, Nashville; and John Postlethwait, Eugene) (9). Furthermore, the Australian Centre for Vertebrate Mutation Detection (ACVMD) has been initiated recently (Peter Curry and Joan Heath, Melbourne, and Graham Lieschke, Parkville). The actual screening strategies vary among these laboratories and new technologies for mutation detection that are based on "next generation sequencing" are being explored in individual laboratories that will finally increase screening efficiency and reduce screening costs.

1.1. Suitability of Genetic Model Organisms

The decision if a vertebrate model organism is suitable for TILLING approaches should take the following considerations into account:

- The vertebrate species allows genetic approaches (prerequisites: short generation time, large number of offspring, and laboratory handling of many individuals).
- A chemical mutagenesis can be performed (either by exposing the whole animal or by intraperitoneal injections).
- DNA sequence data for a gene of interest are available.

1.2. TILLING Libraries

One of the basic requirements of the TILLING technology is the induction of mutations at random positions in genomes by chemical mutagens such as the alkylating agent *N*-ethyl-nitrosourea (ENU). As indicated before, TILLING is applicable to various vertebrate model systems that allow chemical mutagenesis. Certainly, the specific conditions to induce mutations have to be explored systematically, since the "ideal" TILLING library should contain as many mutations as possible leading to the best possible survival rates of the treated animals.

In zebrafish, ENU mutagenesis is well established (10–12). It is based on repeated treatments of males with ENU, which acts by transferring its ethyl group to nucleophilic nitrogen or oxygen sites on each of the four deoxyribonucleotides. This event is manifested in the affected cells during subsequent cell proliferation and DNA replication events and results in heritable mutations. For TILLING libraries, mutagenized males are outcrossed against wild-type females to manifest ENU-induced mutations in

spermatogonia. Mutations are found in heterozygous mutant F1 offspring, which actually represent the library for further screens. Zebrafish ENU mutagenesis protocols were optimized for TILLING libraries recently (13) and lead to high-frequency point mutations with only minor influences on survival and fertility of mutagenized individuals.

Living libraries of zebrafish offspring (F1 generation) and their corresponding genomic DNA derived from tail fins can be kept. These fish can be screened up to the age of about 18–24 months depending on their robustness and fertility. This strategy requires regular performance of ENU mutagenesis to continuously provide TILLING libraries. Alternatively, freezing sperm from 9- to 12-month-old F1 males allows the generation of permanent TILLING libraries (14). However, both ENU mutagenesis and sperm-freezing protocols have to be established and require experience and training. Sharing libraries among different laboratories in a collaborative project is therefore a way to distribute this effort among individual laboratories.

1.3. Success of TILLING Screens

The success of a TILLING screen, which is defined as the identification and establishment of a mutant line that, for example, carries a nonsense mutation, depends on several aspects:

A. The frequency of point mutations in a library is determined by the mutagenesis protocol and the robustness of mutagenized individuals. In zebrafish, a frequency of one point mutation in 100–200 kb coding sequence can be achieved under well-established and optimized conditions.

B. The size of the library, as more mutagenized individuals increase the probability to find an interesting mutation (see also calculation below). However, this number might be restricted by available space for keeping either living individuals or for storing sperm samples.

C. The amount of coding sequence that is screened in one screening round. This number depends on the amount of coding sequence (exon size) and is limited by the technology that is used to identify point mutations. Re-sequencing of PCR fragments restricts this length to 500–750 bp. Since zebrafish genes often exist of small exons surrounded by large introns, this number should be sufficient for the majority of all zebrafish genes (3).

D. The codon composition of a defined exon: It has been shown that ENU exposure of zebrafish genomes results mainly in C/G to T/A transitions (32–40%) and to a lesser extent by A/T to T/A transversions (21–27%) (**Fig. 19.2a**). However, the exact numbers are variable when comparing different assays (detection of point mutations by re-sequencing performed at the TILLING

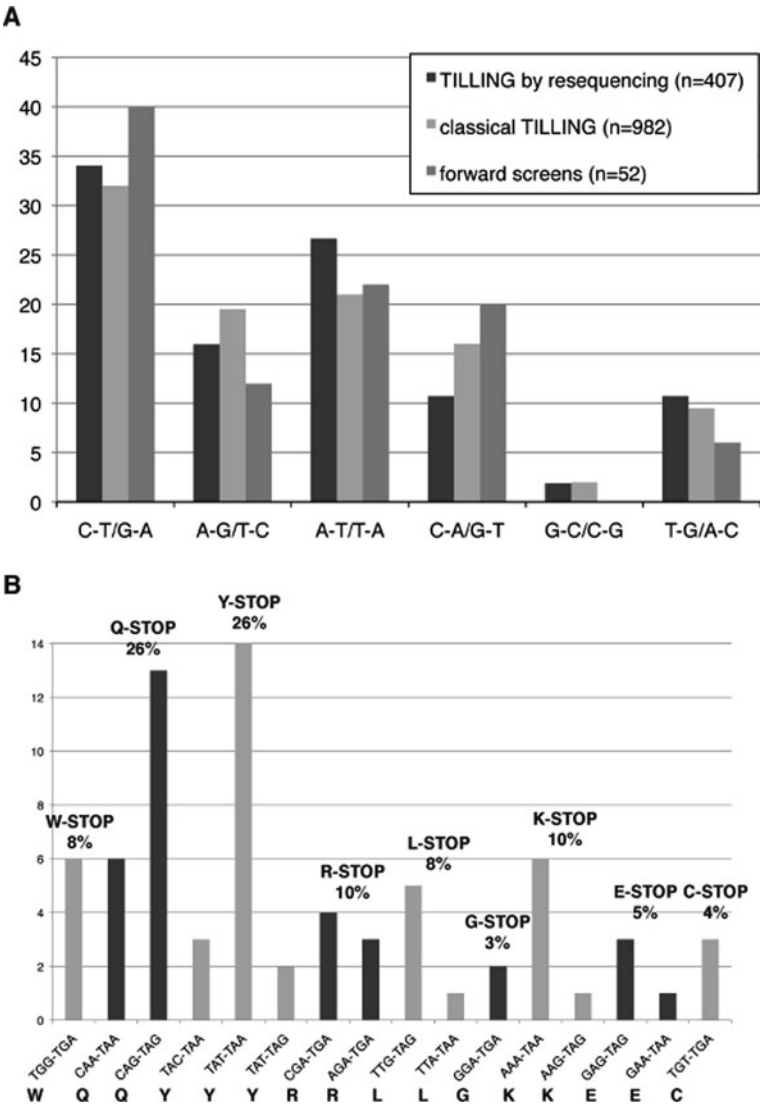

Fig. 19.2. (**a**) Distribution of six groups of nucleotide exchanges. Data were generated at the Dresden TILLING facility (mutation detection by re-sequencing of PCR fragments), in a classical TILLING approach (assaying by Cel-1 endonuclease cleavage) and based on forward screens after molecular cloning of the mutation (9). (**b**) Quantification of codons and their corresponding amino acids that were found to be mutated to stop codons ($n = 73$). Based on our data, mostly codons representing glutamate ($Q = 26\%$) and tyrosine ($Y = 26\%$) are mutated to stop codons, followed by arginine ($R = 10\%$), lysine ($K = 10\%$), tryptophane ($W = 8\%$), leucine ($L = 8\%$), glutamic acid ($E = 5\%$), cytosine ($C = 4\%$), and glycine ($G = 3\%$). Screens were performed at the Dresden TILLING facility.

facility in Dresden, by classical TILLING approaches, and positional cloning of point mutations resulting from forward genetic screens) (9). This might be due to a bias in the individual screening tools and should be experimentally determined for TILLING approaches on other vertebrate model systems.

The specific amino acids that can be mutated to nonsense codons are mostly represented by glutamate (Q) and tyrosine (Y) (52%) as shown in **Fig. 19.1b** (re-sequencing of PCR fragments of zebrafish TILLING libraries using ENU as mutagen). As a simplified rule, exons containing several codons that give rise to glutamate and tyrosine are good candidate exons to detect nonsense mutations.

Several software tools such as LIMSTILL and CODDLE were specifically designed for TILLING approaches and make use of these statistics to select the best amplicons to screen. Criteria to choose a suitable exon in a gene of interest are defined by the nature of a mutation of interest. If the mutation should knockout the function of a gene completely, a target exon should be chosen that is located in the 5′-region of the gene and shows a high chance of generating a stop codon (stop probability). If one is looking for potential missense mutations that lead to a weaker phenotype compared to an already existing null phenotype, one can specifically target functional domains of the gene. Since TILLING screens on randomly mutagenized individuals are not biased toward null phenotypes, also hypomorphic or dominant-negative mutations could be identified that ideally lead to an allelic series of mutations for a given gene.

Based on the frequency of point mutations in a given library, the size of the exon, and the expected stop probability for an exon, the total amount of individuals that have to be screened to find one nonsense mutation can be calculated:

$$\frac{\left[\left(\frac{100}{x}\%\right) \times y \text{ kb}\right]}{z \text{ kb}} = \text{Individual to be screened}$$

where x is the stop probability for an exon of interest (based on the codon composition of each exon, see above); y is the frequency of point mutations in the TILLING library (has to be determined for each TILLING library/ENU mutagenesis); and z is the screened coding sequence (usually determined by the exons that are included in the chosen amplicon). For example

$$\frac{\left[\left(\frac{100}{6.5}\%\right) \times 150 \text{ kb}\right]}{0.5 \text{ kb}} = 4{,}615 \text{ individuals}$$

where $x = 6.5\%$ stop probability for an exon of interest; $y = 1$ in 150 kb is the frequency of point mutations; and $z = 0.5$ kb coding sequence per amplicon.

This calculation gives an idea about the number of individuals that have to be screened to identify one stop mutation with a probability near 1 for one gene of interest under almost ideal conditions (full sequence read is covered by coding sequence and a very high frequency of point mutations). However, exons in

the zebrafish genome are often significantly smaller than 500 bp. Based on our experience (78 screened exons), the average size of a zebrafish exon that is suitable for TILLING screens is 320 bp (early in the gene, stop probability about 6%). Taking this number into account, 7,211 individuals have to be screened on a TILLING library (frequency 1 point mutation in 150 kb) to identify one nonsense mutation with a probability near 1. If the frequency of point mutations decreases further, e.g., to 1 in 180 kb, this number increases to more than 8,600 individuals. These examples show that it is extremely important to optimize conditions for mutagenesis to achieve a high frequency of mutations since this significantly reduces screening effort and costs especially for small exons. Alternatively and if only libraries of a lower efficiency are available, one should consider to increase the amount of coding sequence to be sequenced and screened for the gene of interest.

1.4. Detection of Point Mutations

Different strategies to detect point mutations have been reported in the literature. These include the classical TILLING approach, where heteroduplexes resulting from re-annealed and fluorescently labeled PCR fragments are cleaved by the endonuclease CEL1 (1, 8, 15–17). Cleavage fragments can be separated on high-resolution PAGE sequencers such as the LiCor DNA analyzer or alternatively on other agarose gel or PAGE-based systems. Positive candidates are validated and further defined by Sanger sequencing. In an alternative approach, point mutations can be detected by direct re-sequencing of PCR fragments (Sanger sequencing) followed by sequence alignment (4). Heterozygous positions and effect on the resulting peptide sequence can be detected, for example, with Polyphred, a non-commercial base calling software tool for large sample numbers (18, 19).

With the development of massively parallel sequencing platforms that allow the simultaneous generation of millions of short DNA sequence reads, large-scale screens for point mutations from mutagenized individuals are going to be elaborated. Pooling and barcoding strategies can be used to identify individuals in combination with high-throughput sequencing of shorter DNA fragments of a size of 50 to 200 bp, or sequencing of captured DNA fragments by customized microarrays or in-solution strategies. Some of these strategies are currently explored (pers. communication Derek Stemple and Edwin Cuppen).

1.5. Establishment and Propagation of Mutant Animals

After identification and verification of mutations, the effect of a mutation has to be determined before a mutant line is established. Mutated DNA is translated *in silico* and the resulting peptides analyzed using bioinformatics approaches. Point mutations can create nonsense codons or affect splice sites leading to

premature stop codons, which finally result in truncated peptides. These truncated/mutated versions most likely interfere with the function of the generated peptide. Missense mutations could result in deleterious effects if they change the biochemical properties of the affected amino acid (non-conservative substitution). The probability of interfering with the function of the translated peptide is high if the substituted amino acid is found in a functional domain of the peptide and/or if the affected position is conserved among different species. Individuals carrying interesting and potentially deleterious point mutations are isolated from the actual library by re-sequencing of pooled individuals and

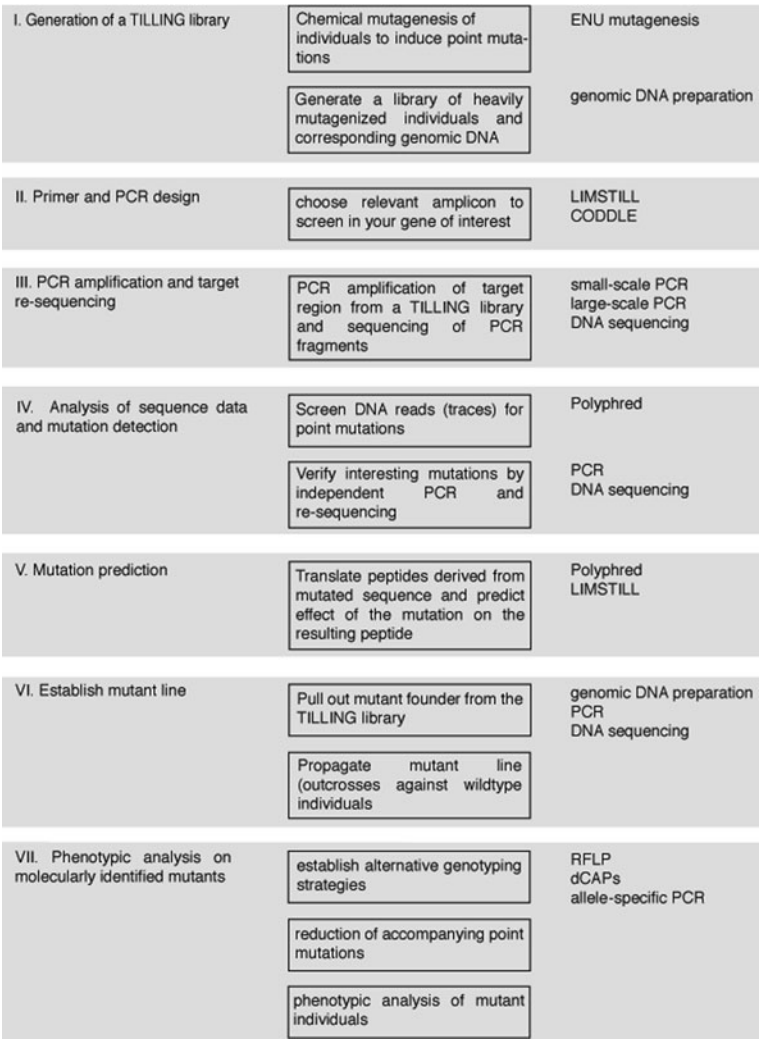

Fig. 19.3. Overview of the TILLING workflow for mutation detection by direct re-sequencing of PCR fragments.

subsequent breeding against wild-type individuals (outcross). If frozen sperm libraries exist, positive sperm samples are defrosted and used to fertilize wild-type oocytes. The resulting offspring are raised to fertility, genotyped for the identified point mutations, and individuals carrying this mutation are mated to each other (incross) to screen homozygous offspring for phenotypes.

In this chapter, we describe a TILLING setup as we are currently operating it for zebrafish libraries in Dresden. Our TILLING workflow of this pipeline is illustrated in **Fig. 19.3** and is based on direct re-sequencing of PCR fragments (Sanger sequencing) from libraries of mutagenized zebrafish. This pipeline can be established for any given genetic model in a laboratory with background in molecular biology and access to significant sequencing capacity or alternative mutation detection technologies, such as conventional TILLING by CEL1-based cleavage (for details, *see* **Note 1** and (16, 17, 20, 21)).

2. Materials

2.1. Generation of a Genomic TILLING Library from Zebrafish Tail Fins

1. Tricaine anesthetic stock solution: 0.4% ethyl 3-aminobenzoate methanesulfate (MESAB, MS-222, Tricaine) in 70 mM Na_2HPO_4 solution. Keep the stock at 4°C. Dilute freshly 25× in fish water to anesthetize adult zebrafish for fin clipping.
2. Scalpel, forceps, and two beakers with 70% ethanol and ddH_2O each to rinse tools.
3. Two deep-well plates (96-well, well volume 2 mL), kept on dry ice. Alternatively, 2 mL Eppendorf tubes can be used if less individuals are clipped (*see* **Note 2**).
4. Lysis buffer: 100 mM Tris-HCl, pH 8.0; 200 mM NaCl; 5 mM ethylenediaminetetraacetic acid (EDTA); 2% sodium dodecyl sulfate (SDS); prepare 10× stock solutions, dilute in ddH_2O for each preparation and add 100 μg/mL proteinase K for each experiment. We keep the already diluted lysis buffer without proteinase K for ca. 1–2 weeks at room temperature.
5. Water bath or incubator at 55°C.
6. 100% isopropanol, 70% ethanol, 1× Tris-EDTA solution (TE, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
7. 0.7% agarose gels and agarose gel setup.
8. *Hin*dIII-digested lambda DNA as DNA marker for quantification of genomic DNA and conventional DNA ladder.

9. ddH_2O, microplates (deep-well and PCR plates in 384-well or 96-well format), or Eppendorf tubes for dilution and aliquots of genomic DNA for PCR application.

2.2. Choice of TILLING Targets

1. CODDLE (http://www.proweb.org/coddle/).
2. LIMSTILL (http://limstill.niob.knaw.nl/index.html).

2.3. PCR Amplification and Targeting of Amplicons from a TILLING Library

1. PCR suitable multiwell plates (384 or 96 well) or other single tubes.
2. 5× PCR buffer: 125 mM tricine, 425 mM NH_4acetate, 40% glycerol (w/v) 10% dimethyl sulfoxide (DMSO) in ddH_2O (w/v), adjust to pH 8.7 with 25% ammonia, store in small aliquots at –80°C. The buffer does not like repeated freezing and thawing.
3. dNTPs mix: 10 mM each, store small aliquots at –80°C.
4. 25 mM $MgCl_2$ (molecular grade) in ddH_2O, store small aliquots at –80°C, the working solution can be kept at 4°C.
5. PCR-polymerase (e.g., Taq-polymerase, *see* **Note 3**).
6. PCR primer: *see* **Note 4** when working in small-scale format and *see* **Note 5** when working in large-scale format (≥384 samples).
7. Agarose gels (1%) and agarose gel setup for quality control steps.
8. Exonuclease I (20,000 units/mL, e.g., NEB).
9. rAPid enzyme mix and 10× rAPid buffer (rAPid, Roche).
10. Plate sealing material (adhesive foil or heat sealing foil, alternatively re-usable full plate covers, e.g., Life Technologies).

2.4. Sequencing of PCR Fragments

1. Big dye version 3.1 (Life Technologies/Applied Biosystems).
2. 5× sequencing reaction buffer (Life Technologies).
3. Sequencing primers (*see* **Note 6**).
4. DNA precipitation mix: 7.1 M ammonium acetate, 0.03 M EDTA (stable at room temperature).
5. 100% ethanol (ice cold).
6. 80% ethanol.
7. Formamide–ddH_2O mix (50% each, *see* **Note 7**).

2.5. Detection of Mutations

1. Open-source tool: Polyphred (http://droog.gs.washington.edu/polyphred/).
2. Required system: UNIX or LINUX operating system.

3. Methods

3.1. Generation of TILLING Libraries

3.1.1. Chemical Mutagenesis to Induce Point Mutations in Zebrafish

ENU is the agent of choice to induce point mutations that are randomly distributed all over the genome. Improved protocols to achieve high frequencies of point mutations in pre-meiotic germ cells of adult zebrafish males have been published recently ((13); **Chapter 7** by Sreelaja Nair and Francisco Pelegri, this volume) and will not be described in detail. The mutagenesis and subsequent breeding of about 100 males should result in 6,000–8,000 heavily mutagenized F1 individuals, which will form the TILLING library. These animals can either be kept alive or alternatively a permanent library of male sperm could be generated at this point (for details (14), *see* **Note 8** for the decision, which library format is optimal for zebrafish under defined laboratory conditions).

3.1.2. Mutagenesis of Other Vertebrate Model Systems Than Zebrafish

ENU-based mutagenesis protocols are available for mammals, such as mice (22), rat (5), and other fish species, such as medaka (6). Several of them are optimized for TILLING approaches. In general, TILLING or a systematic screen for point mutations can be adopted for every genetic vertebrate model system. However, mutagenesis conditions have to be developed and optimized for the individual cases.

3.1.3. Preparation of the Genomic DNA Library in Zebrafish

Since the preparation of genomic DNA from larger numbers of individuals is required for an adequate TILLING library all steps are described for handling 96-well plates (*see* **Note 9** for single tube preparations). Alternatively to this inexpensive protocol for DNA isolation, commercially available kits to prepare genomic DNA can be used (*see* **Note 10**):

1. Adult zebrafish (>6 months) are anesthetized for about 2 min in Tricaine working solution in batches up to five individuals. Cut some tissue of the tail fin of individual fish with a scalpel (ca 5 mm). Transfer fin tissue to a defined well in a deep-well plate that is kept on dry ice. Transfer affected fish immediately to the dedicated tank before getting the next fish. Rinse scalpel and forceps first in 70% ethanol followed by ddH_2O. Anesthetized zebrafish should wake up within a few minutes.
2. Warm deep-well plate to room temperature and immediately add 500 µL of lysis buffer. Incubate for 2 h to overnight at 55°C. Vortex plates regularly during the first 2 h.
3. Incubate samples for 10 min at 85°C to inactivate proteinase K.
4. Vortex deep-well plate extensively and immediately spin plates for 30 min at 3,761×*g* at room temperature. Carefully transfer the supernatant to a fresh plate (*see* **Note 11**).

5. To precipitate genomic DNA add 400 μL of isopropanol, seal plate, and mix by inverting the plate several times. Spin plates for 1 h at 3,761×*g* at room temperature and discard the supernatant.
6. Wash DNA pellet with 300 μL 70% ethanol, spin 20 min at 3,761×*g*, and discard supernatant.
7. Repeat washing step for long-term storage of the genomic DNA library (not necessary for genotyping experiments).
8. Dry DNA pellet for 25 min (first upside down, then turn plate upside up).
9. Carefully dissolve genomic DNA in 200 μL TE. This could be done for several hours on a shaker at room temperature.
10. Check an aliquot (ca. 2 μL) of genomic DNA for integrity and concentration on 0.7% agarose gels. An aliquot of Lambda DNA digested with *Hin*dIII can be used as reference DNA (load 5–10 μL at 50 ng/μL). The yield of the genomic DNA preparation can be estimated when comparing the intensity of the genomic DNA band with the largest Lambda band of 23 kb (concentration 24 ng/μL) (*see* **Note 12** for an example).
11. Based on the quantitation, the concentration of the genomic DNA can be normalized to 1–2 ng/μL (*see* **Note 13**).
12. The genomic DNA is stored as an undiluted stock at –20°C (*see* **Note 14**).
13. Dilute genomic stock DNA in water (e.g., 1–50) as template for PCR (required are about 2–20 ng of genomic DNA per PCR). The aliquoted and diluted DNA is kept frozen at –20°C (*see* **Note 15**).

3.2. Choice of TILLING Targets

Although the frequency of point mutations in genomes is increased by mutagenesis, the detection of nonsense mutations is still a relatively rare event. For example, one nonsense mutation should be found while screening 4,615 individuals at a given frequency of mutations of 1 in 150 kb and 500 bp coding sequence analyzed per individual (see calculation in **Section 1.3**). To keep the number of screened individuals, PCRs, and sequencing reactions as low as possible, one should determine carefully which part of a gene will be targeted.

Guidelines to choose the best region:

– Screen as much coding sequence as possible per individual per amplicon. This is actually very much influenced by the screening method that is available, e.g., direct re-sequencing of PCR fragments allows amplicon sizes up to 750 bp, whereas CEL1-based screens allow amplicons up to 1,500 bp in size.

- The stop chance calculated from the codon composition of exons. Since nonsense mutations are represented by only three different codons (TAA, TAG, and TGA) only a limited set of codons can be mutated in any of the three nucleotide positions to become a nonsense codon. Taking the mutagenic activity of ENU in a vertebrate background into account (e.g., in zebrafish 34% of nucleotide exchanges represent C to T/G to A transitions and 27% A–T, T–A transversions, numbers determined in the Dresden TILLING facility, **Fig. 19.2a**), it is possible to calculate the stop probability per codon for a given exon.
- The location of an interesting exon in the annotated gene: To isolate mutations that are expected to impair with the function of a protein, it is recommended to screen 5′ located exons. Alternatively, functional domains can be targeted, in which mutations are expected to impair the function of the mutated peptide.
- The size of an interesting exon: Ideally, large exons with a high stop probability should be screened.
- The existence of alternative translation initiation sites: This should be either determined experimentally or predicted bioinformatically. These alternative peptides can be functionally active and redundantly take over the action of the mutated version.

Available web-based tools to identify suitable amplicons:

1. CODDLE helps to identify regions in exons that have a high likelihood to harbor nonsense mutations and to identify regions that encode conserved domains where missense mutations might lead to a deleterious effect.
2. LIMSTILL allows gene annotation, primer design, and data management for several vertebrate and invertebrate species and was developed by Victor Guryev in Edwin Cuppens laboratory in Utrecht (*see* **Notes 16** and **17** and **Fig. 19.4**).

3.3. Targeting Amplicons from a TILLING Library by PCR

Several technologies have been established to screen for point mutations. Direct re-sequencing of PCR fragments amplified from a library of genomic DNA will be described in detail for two different scenarios. A small-scale protocol makes use of normalized genomic DNA as template and performs one single PCR step followed by direct re-sequencing of PCR fragments (**Section 3.3.1**). The large-scale protocol (**Section 3.3.2**) performs a nested PCR approach where normalization is achieved by an initial PCR step with gene-specific primers, followed by tailed inner primers (*see* also **Fig. 19.1**). This strategy allows usage of common sequencing primers. This large-scale approach requires liquid handling robotics and standardized PCR and sequencing

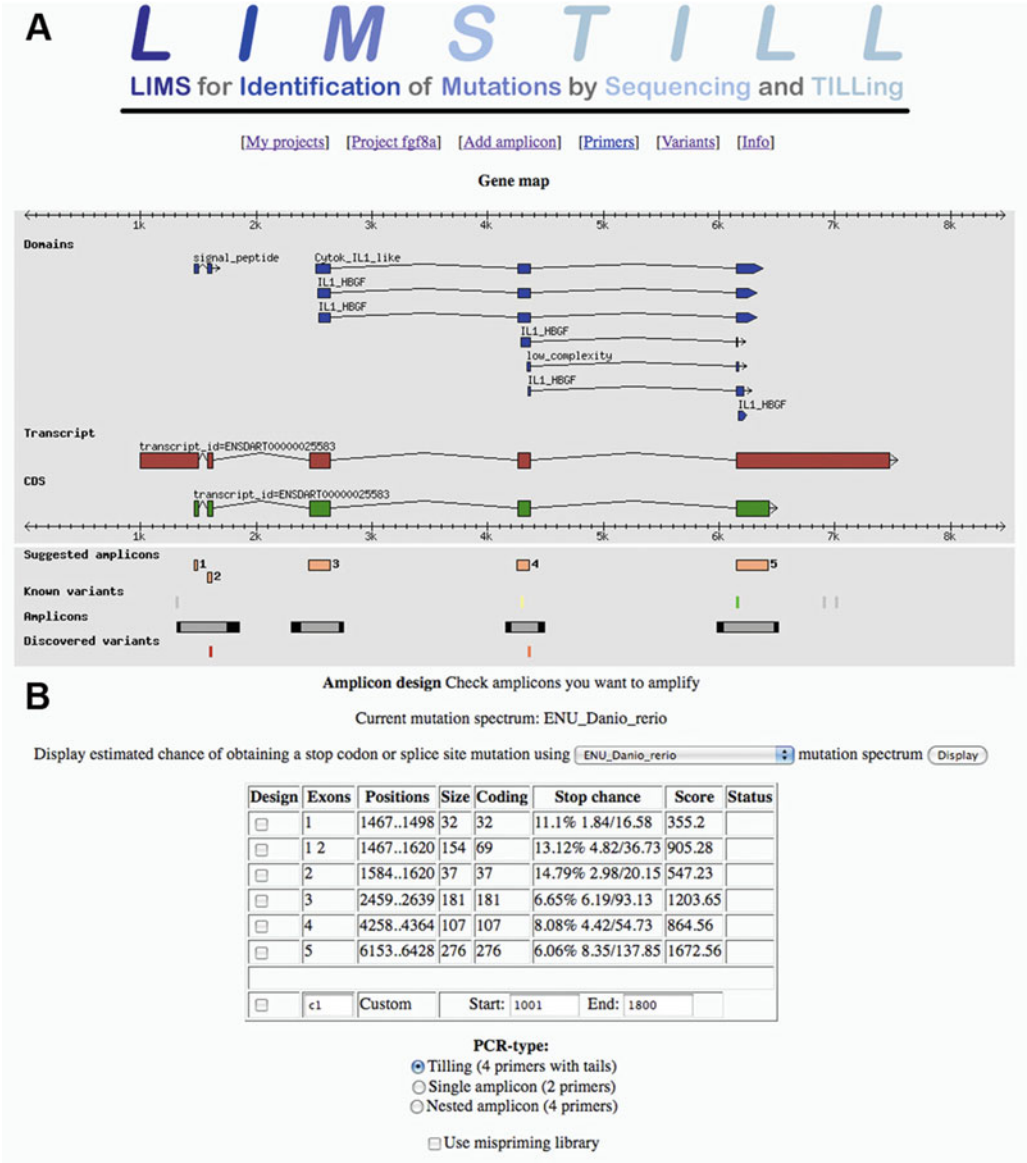

Fig. 19.4. LIMSTILL for project management, primer design, and mutant management (generated by Victor Guryev, Hubrecht Institute in Utrecht). (**a**) Overview of gene annotation, designed amplicons, known and discovered variants for an example derived from zebrafish, details are based on genome sequence and gene annotation as defined by Zebrafish Ensembl Zv 8 (Zebrafish genome sequencing project, Sanger Institute, Cambridge, November 2009). (**b**) Amplicon design tool, making use of statistics of ENU-based nucleotide exchanges in zebrafish (available also for other species).

conditions. An example of an automated setup as it is used by the Dresden TILLING facility is shown in **Fig. 19.5**.

3.3.1. Small-Scale/Single Tube TILLING PCR Protocol

PCR can be performed in single PCR tubes, PCR strips, or 96-well plates. Multichannel and multidispense pipettes should be used whenever it is possible to avoid pipetting errors:

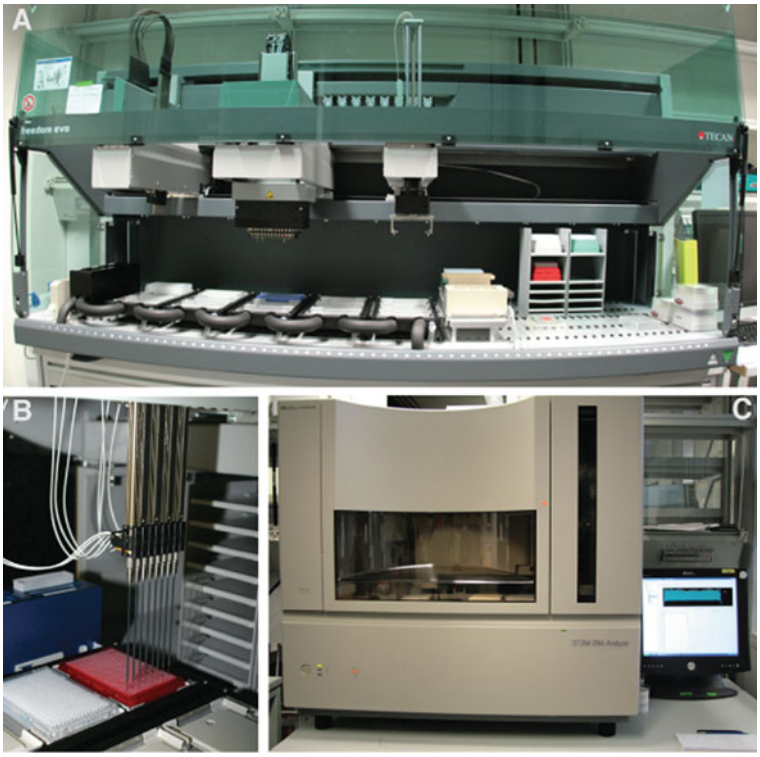

Fig. 19.5. Liquid handling setup of the Dresden TILLING facility. (**a**) The setup contains a liquid handling pipetting station (TECAN Freedom) that allows low volume (**b**) as well as multichannel pipetting (96-well liquid handling tool). All liquid handling, PCR amplification, and sequencing steps are performed in 384-well format (**b**). (**c**) 384-well plates are sequenced on an AB3730XL DNA sequencer.

1. Set up PCR master mix on ice for *n* samples (*n* = number of samples plus excess volume = 1–2 reactions)

   1× PCR setup (master mix preparation, per sample):

   0.2 μL forward primer (10 μM)
   0.2 μL reverse primer (10 μM)
   0.2 μL dNTP (10 mM each)
   0.8 μL 25 mM $MgCl_2$
   2 μL 5× PCR buffer
   x μL PCR-polymerase (*see* **Note 3**)
   x μL ddH_2O (fill up to 5 μL total volume)

   1× PCR setup (reaction preparation, per sample):

   5 μL mastermix
   5 μL genomic DNA (normalized and diluted to 1–2 ng/μL)
   10 μL total PCR volume

2. PCR conditions
   Step 1 94°C 5 min
   Step 2 94°C 30 s
   Step 3 T_{anneal} 20 s
   Step 4 72°C 60 s perform steps 2–4 for 30 cycles
   Step 5 72°C 10 min
   15°C until collected
3. For quality control and quantification load 3 μL aliquots of PCR fragments on 1% agarose gels. The concentration of amplified fragment is estimated from these agarose gels that contain marker DNA of a defined concentration (e.g., conventional DNA ladders or specific ladders for quantification, *see* **Note 12**). The PCR should result in one discrete band only (*see* **Note 18**).
4. Dilute PCR fragments for sequencing reaction (*see* **Note 19**).

3.3.2. Large-Scale Approach

The large-scale setup is based on a nested PCR approach and subsequent direct re-sequencing of the PCR fragments. In this case, normalization of the final product is achieved during the nested PCRs and therefore the labor-intensive normalization step of the genomic DNA can be avoided. We perform all steps in 384-well format making use of liquid handling robotics that takes over all liquid handling steps such as dilutions and plate copies. All PCR conditions are standardized with regard to annealing temperature and dilution steps. This setup allows processing of up to 9,000 samples per week that are optimized for inexpensive PCR and sequencing conditions (one 96 capillary sequencer). Robotic pipetting is performed with a low volume system of reusable pipetting tips for all master mixes. Samples are transferred or copied to new target plates by disposable tips that are washed between the different pipetting steps (**Fig. 19.5**, please contact us directly for further details):

1. Set up outer PCR on ice (*see* **Note 20**):

   1× PCR with gene-specific outer primers (master mix preparation, per sample):

   0.02 μL forward outer primer (100 μM)

   0.02 μL reverse outer primer (100 μM)

   0.2 μL dNTP (10 mM each)

   0.8 μL 25 mM $MgCl_2$

   2 μL 5× PCR buffer

   x μL PCR-polymerase (*see* **Note 3**)

   x μL ddH_2O (fill up to 5 μL total volume)

1× PCR setup with gene-specific outer primers (reaction preparation, per sample):

5 μL master mix

5 μL diluted genomic DNA (corresponding to 2–20 ng)

10 μL total PCR volume

2. PCR conditions
   Step 1 94°C 5 min
   Step 2 94°C 30 s
   Step 3 57°C 20 s (defined by LIMSTILL settings for primer design)
   Step 4 72°C 60 s perform steps 2–4 for 25 cycles
   Step 5 72°C 10 min
   15°C until collected
3. For quality control and quantitation take 3 μL of a subset of samples (e.g., one row or column) from each plate and run on 1% agarose gels (*see* **Note 12**).
4. Dilute PCR fragments for nested PCR setup 1:3 in ddH_2O (*see* **Note 21**).
5. Set up master mix for inner PCR on ice (*see* **Note 20**)

   1× inner PCR with tailed gene-specific primers (master mix preparation, per sample):

   0.006 μL forward inner primer (100 μM)

   0.006 μL reverse inner primer (100 μM)

   0.1 μL dNTP (10 mM each)

   0.8 μL 25 mM $MgCl_2$

   2 μL 5× PCR buffer

   x μL PCR-polymerase (*see* **Note 3**)

   x μL ddH_2O (fill up to 10 μL total volume)

   1× inner PCR with tailed gene-specific primers (reaction preparation, per sample):

   10 μL mastermix

   1 μL of diluted PCR 1

   11 μL total volume

6. PCR conditions: identical to PCR 1 (outer PCR) (**Section 3.3.2**).
7. For quality control and quantitation take 3 μL of a subset of samples (e.g., one row or column) from each plate and run on 1% agarose gels (*see* **Note 12** for quantitation and **Note 22** for quality adjustment).

8. Cleanup PCR fragments
   The removal of unused PCR primers and unincorporated dNTPs is performed in solution. This step is not absolutely essential but increases sequencing quality.

   Set up mastermix on ice (*see* **Note 20**)

   1× PCR cleanup reaction (sample mix preparation, per sample):

   0.25 μL rAPid enzyme mix

   0.05 μL exonuclease I

   1.5 μL 10× rAPid buffer

   3.2 μL ddH2O

   5 μL total

   1× PCR cleanup reaction (reaction preparation, per sample): add 5 μL rAPid/ExoI mix per well per plate.

9. Incubation:
   37°C 30 min
   75°C 2 min
   10°C until collected

3.4. Sequencing of PCR Fragments

The specific sequencing conditions have to be determined based on the DNA sequencing options that are available for the individual project. Herein, conditions for high-throughput sequencing of PCR fragments on a 96-capillary ABI DNA analyzer 3730XL are described.

3.4.1. Sequencing Reaction

1. Dilute PCR fragments for sequencing reaction (*see* **Note 23**)
2. Set up master mix for sequencing reaction on ice (*see* **Note 20**)

   1× sequencing reaction (master mix preparation, per sample)

   0.2 μL Big dye version 3.1

   0.4 μL 5× sequencing buffer (Applied Biosystems)

   0.05 μL sequencing primer (100 μM) (*see* **Note 6**)

   6.35 μL ddH_2O (to fill up to a final volume of 7 μL)

   1× sequencing reaction (reaction preparation, per sample):

   7 μL total sequencing mastermix per reaction

   1 μL diluted PCR fragment

   8 μL total volume sequencing reaction

3. Sequencing PCR
   Step 1 96°C 1 min
   Step 2 96°C 10 s

Step 3 52°C 5 s
Step 4 60°C 3 min perform 30 cycles of steps 2–4
4°C until collected

3.4.2. Purification of the Sequencing Reaction

1. Add to each sample 2 μL precipitation mix and 20 μL 100% ethanol (ice cold).
2. Spin at 4°C for 30 min at maximum speed (for multiwell plates, e.g., 3,761 × *g*).
3. Turn plates upside down on absorbent pad and spin for 1 min at 400 rpm to get rid of all ethanol.
4. Add 20 μL of 80% ethanol to each well to wash the DNA pellet.
5. Spin at room temperature for 10 min at maximum speed (for multiwell plates, e.g., 3,761 × *g*).
6. Turn plates upside down on absorbent pad and spin for 1 min at 400 rpm to get rid of all ethanol.
7. Dry DNA pellet for about 30 min at room temperature protected from light.
8. Dissolve pellet in 10 μL formamide-ddH_2O mix (1:2) (*see* **Note 7**).
9. Either sequence the plates directly, for example, on a DNA analyzer AB3730 XL or alternatively keep them frozen.

3.4.3. Load Plates Directly on the DNA Analyzer AB3730 XL

1. Load plates to DNA analyzer AB3730 XL according to the instructions of the manufacturer.
2. Injection conditions for sequencing of PCR fragments on 36 cm arrays (*see* **Note 24**): 1.5 V injection voltage, 5 s injection time, 7.5 V run voltage, run time 22–50 min for 300–750 bp fragments.
3. File format of trace files generated during the run: ab1 or scf files are generated and could be used for any further analysis (e.g., when using Polyphred for mutation discovery).

3.5. Analysis of Sequence Data and Mutation Detection

Several tools to screen sequence trace files for point mutations have been developed recently. Suitable open-source tools are, for example, Polyphred ((18, 19), see **Note 25**), SeqDoC (23), novoSNP (24), or SNPdetector (25). Furthermore, several commercial suppliers developed software tools to screen for point mutations.

3.6. Verification of Primary Hits

Primary hits carrying point mutations are verified by an additional and independent PCR amplification and sequencing reaction to rule out PCR-polymerase mistakes. This step becomes important if a non-proofreading enzyme is used and is performed in the same way as it was described for small-scale applications (**Sections 3.3.1** and **3.4** if a sequencing setup is available).

3.7. Translation of Verified Point Mutations and Effect Prediction

Translation of the affected DNA sequence can be done during the screening process, which immediately defines the effect on the resulting peptide (nonsense, splice site, missense, or silent mutation). The LIMSTILL software tool helps to predict the potential effect of the discovered point mutations and manages the identified mutations. LIMSTILL includes a matrix that defines the nature of an amino acid exchange (non-conservative versus conservative amino acid substitution) and includes a BLAST of the affected peptide region (uses a mutated amino acid with the adjacent 20 amino acids) against various peptide databases to see the conservation of this position (**Fig. 19.4a**) (*see* **Note 26**).

3.8. Establishment of Mutant Lines

If a living zebrafish library is screened, the individual has to be pulled out from the small number of F1 individuals pooled in one aquarium and verified by re-sequencing. Propagation of the line is achieved by breeding the identified F1 founder against wild-type zebrafish. When screening permanent libraries of sperm samples, the corresponding sperm sample is used for in vitro fertilization of female oocytes.

3.9. Molecular and Phenotypic Analysis of Mutants

Screened mutant founders of the F1 generation after mutagenesis carry a high number of accompanying point mutations. These mutations are typically unlinked to the identified mutation in a gene of interest and therefore distinguishable. However, accompanying mutations could also affect the expected mutant phenotype by either potentiating or reducing the expressivity of a phenotype when synergistic or interacting partners are affected by a point mutation.

Therefore is extremely important

(a) to reduce the number of genetically unlinked point mutations (*see* **Note 27** for an example) and

(b) to show that an observed phenotype is linked to the identified allele. It is recommended to work with identified carrier individuals only, which have been genotyped before. This can be either done by a molecular approach (see below) or genetically by complementation tests against alternative and phenotypically defined alleles of a gene of interest if these are available.

Fast and inexpensive genotyping strategies are required to explicitly identify mutant individuals, especially if direct re-sequencing strategies are not available. An unambiguous molecular identification of a carrier individual becomes extremely important if accompanying mutations influence an observed phenotype. Several alternative strategies to direct re-sequencing PCR fragments have been published recently that could be easily implemented into any molecular biology laboratory, and the actual strategy often depends on the individual point mutation (*see* **Note 28**).

4. Notes

1. The TILLING protocol described focuses on zebrafish as a genetic model system in combination with automated pipetting devices and access to DNA sequencing tools (an overview of the equipment is shown in **Fig. 19.5**). However, this pipeline is not limited to zebrafish but does also work for other vertebrate and invertebrate species. In addition, it is possible to screen lower sample numbers making use of multichannel pipettes, replicators, and dispensing tools for microplates.
2. Keep deep-well plate or Eppendorf tubes on dry ice during the clipping procedure to avoid any tissue and DNA degradation.
3. The required activity of the chosen PCR-polymerase has to be determined experimentally. The goal especially for large-scale screens is a careful titration of the polymerase to keep the screening costs as low as possible.
4. PCR primer small-scale format: Gene-specific forward and reverse PCR primers are designed with LIMSTILL or any other primer design software that is available. Ideally, the splice sites should be included in the chosen amplicon.
5. Primer large-scale format: Gene-specific forward and reverse PCR primers designed with LIMSTILL ("four primers for TILLING") or alternative software. The non-tailed primers are used in the first PCR. Tailed nested primers: the inner parts of these primers are gene-specific with universal tails: M13forward (TGTAAAACGACG GCCAGT linked to gene-specific 5′ primer) and M13reverse (AGGAAACAGCTATGACCAT linked to gene-specific 3′ primer) sequences. It is very important to test primer pairs, PCR conditions, and sequencing conditions on small numbers of wild-type DNA before the large-scale screen is initiated.
6. Choice of sequencing primers: For small-scale application use either forward or reverse gene-specific PCR primer as a sequencing primer. To calculate a suitable annealing temperature, please follow this rule:
   T_{melting}: 4(G+C) + 2(A+T)
   $T_{\text{annealing}}$: $T_{\text{melting}} - 5°C$
   Sequencing primers for large-scale setup are either M13forward (TGTAAAACGACGGCCAGT) or M13reverse (AGGAAACAGCTATGACCAT), which correspond to one of the tails of inner primer pairs. In addition, custom sequencing primers that are located internally to the

universal tales can be designed in case heterozygous insertions or deletions are present in the PCR fragments that result in a parallel and non-resolvable sequence read. The actual sequencing primer that is finally chosen depends on the initial tests of primer pairs on wild-type individuals and should cover as much coding information as possible.

7. Based on our experience, the fluorescently labeled dNTPs (Applied Biosystems Big Dye sequencing mix) used in the sequencing reaction are more stable in formamide–ddH_2O mix compared to ddH_2O alone.
8. The decision whether the library should be transient (living) or permanent (frozen sperm) depends on several factors:
   (A) The capacity of the fish facility: With zebrafish, 24–48 individuals can be kept pooled in a 15 L aquarium. More individuals in one tank would require more genotyping to identify and isolate the mutant of choice from your pooled population. Under optimal conditions in the fish facility, a healthy library of mutagenized F1 zebrafish can be successfully screened up to the age of 18–24 months.
   (B) The sperm-freezing capacity (pipeline and storage capacity): Ideally sperm should be frozen from males at the age of 9–12 months after these individuals had been successfully mated at least once. Therefore, a defined pipeline taking these steps into account is absolutely required. Since only half of a population (males) of mutagenized zebrafish is used, females can be kept as a living library in parallel.
   (C) The screening capacity for point mutations: This is defined by the PCR and sequencing capacity of the screening laboratory.

   Since both ENU mutagenesis in zebrafish and sperm freezing require some training and expertise, it is recommended to start with pilot experiments on smaller numbers of males. It is important to improve these procedures taking into account individual laboratory conditions as well as robustness of the chosen zebrafish background.
9. Genomic DNA preparation in single tube format: Alternative to multiwell plates, Eppendorf tubes of 2 mL volume can be used. In this case, all volumes used for DNA precipitation, ethanol washes, and final dilution of the pellet stay the same. All centrifugation steps can be performed in a suitable centrifuge and speed and duration can be adjusted:

- removal cell debris: 10 min at 15,700×*g*
- DNA precipitation: 20 min at 15,700×*g*
- salt removal from DNA pellet: 10 min at 15,700×*g*.

10. Commercially available genomic DNA preparation kits could provide optimized cell lysis conditions and might result in faster and cleaner DNA extractions, often depending on the genetic model organism.
11. This step removes cell debris and is recommended for long-term storage of genomic DNA stocks (not necessary for genotyping experiments).
12. Example of quantitation of DNA bands on agarose gels: When loading 2 μL of isolated genomic DNA (>23 kb when comparing to the largest *Hin*dIII-digested lambda marker band), a band appears double as wide compared to the largest reference band (23 kb, at 24 ng/μL) of the genomic DNA ladder. This corresponds to 48 ng/μL of loaded genomic DNA. Since 2 μL of genomic DNA was loaded, the concentration is about 24 ng/μL. Based on our experience, the overall yield of genomic DNA prepared from one fin clip is about 2–30 μg.

    For quantitation of PCR fragments choose a conventional DNA ladder spanning 100 bp to 5 kb. Please take care that at least for one band in the ladder the amount of loaded DNA is defined.

    Do not determine the concentration of genomic DNA in a spectrophotometer because residual RNA is not removed from these samples and interferes with photometric measurements.
13. Normalization of genomic DNA is required for direct resequencing of PCR fragments without a second nested PCR in small-scale TILLING screens.
14. For long-term storage of libraries, a copy of the genomic DNA to a master plate should be considered.
15. It is recommended to aliquot the diluted DNA in larger batches to avoid multiple freezing and thawing steps of the DNA stock as well as the diluted aliquots. When using a large-scale setup in 384-well format, the genomic DNA can be diluted and arrayed in 384-well deep-well plates making use of liquid handling robotics. In this case, 4 × 96-well plates are arrayed to a 384-well format. Dilutions are done in 384 deep-well plates (well volume, e.g., 100 μL) and 5 μL PCR template. DNA aliquots are transferred to 384-well PCR plates and kept frozen.
16. LIMSTILL is linked to several genomic databases (currently 24 different including zebrafish, human, mouse,

chick, rat, fugu) and these can be searched for potential target genes. Alternatively, Ensembl IDs or manually annotated genes can be uploaded. The genomic annotation and functional domains are visualized (**Fig. 19.4a**). Amplicons can be chosen directly taking exon size and the zebrafish ENU mutation spectrum into account (**Fig. 19.4b**). Primer design for interesting exons and TILLING projects could be done directly making use of primer3 algorithms. In addition, LIMSTILL allows management of identified mutations later on in the screening process (**Fig. 19.4b**).

17. LIMSTILL and TILLING screens in vertebrate model systems other than zebrafish: Whereas the number of sequenced vertebrate genomes is growing daily, annotation of gene structures is often missing. Especially for non-common vertebrate model organisms this requires manual annotation of genes by comparing cDNA and genomic sequences. Manually annotated genes or only defined exons can be uploaded into LIMSTILL according to its formatting options. In general, LIMSTILL databases can be easily adapted to "non-established" vertebrate model systems as long as an annotated genomic sequence of a gene of interest can be defined.
18. Smeared or multiple bands in a PCR should *not* be used for subsequent sequencing. Instead a nested PCR approach could be applied to increase PCR specificity.
19. The dilution of PCR fragments for Sanger sequencing depends on the chosen sequencing chemistry and DNA sequencer and has to be determined in collaboration with a provider of DNA sequencing.
20. Prepare master mix for all samples that should be processed in one batch; please take the dead volume of reservoirs used with your liquid handling setup into account. This should not exceed 5–15% of the sample volume to avoid extra costs.
21. For 384-well plates, add 20 μL water to each well. Take care: a total volume of 30–35 μL is the maximum volume that fits in 384-well plates.
22. The nested PCR should result in one discrete band. If not, optimize the nested approach or choose different primers. An unspecific PCR (manifested by multiple bands after PCR) should *not* be used for subsequent sequencing. The concentration of amplified fragments is estimated from agarose gels that contain marker DNA of defined concentration (e.g., conventional DNA ladders or specific ladders for quantification).

23. 20 μL of ddH_2O (molecular biology grade) is added to each nested PCR in a 384-well plate (1:3 dilution of the nested PCR product). This volume is limited by the maximum well capacity in a 384-well plate (30–35 μL). If single tubes or 96-well plates are used, the dilution volume could be increased up to 50–100 μL (1:5–1:11 dilution of PCR fragment).
24. In case a longer array (50 cm) is available for the AB3730 XL, these conditions have to be defined experimentally to achieve the optimal resolution.
25. PolyPhred is a program that compares fluorescence-based sequences across traces obtained from different individuals to identify sites heterozygous for single nucleotide substitutions. The recent version runs under LINUX/UNIX operating systems and is free to academic researchers (http://droog.gs.washington.edu/polyphred/). Our Polyphred settings allow the alignment and analysis of 768 traces in one step. Presumptive heterozygous as well as homozygous mutations are labeled in the alignment. The individual sequence read is directly connected to the chromatogram, where the sequence quality of the mutated context could be reviewed online (**Fig. 19.6**). All three open readings frames per strand can be translated and shown. Already during the screening process "interesting" mutations such as nonsense, splice site, or missense mutations can be kept and processed further whereas silent mutations can be ignored.
26. Whereas nonsense and splice site mutations result in truncated peptides that are expected to show a functional

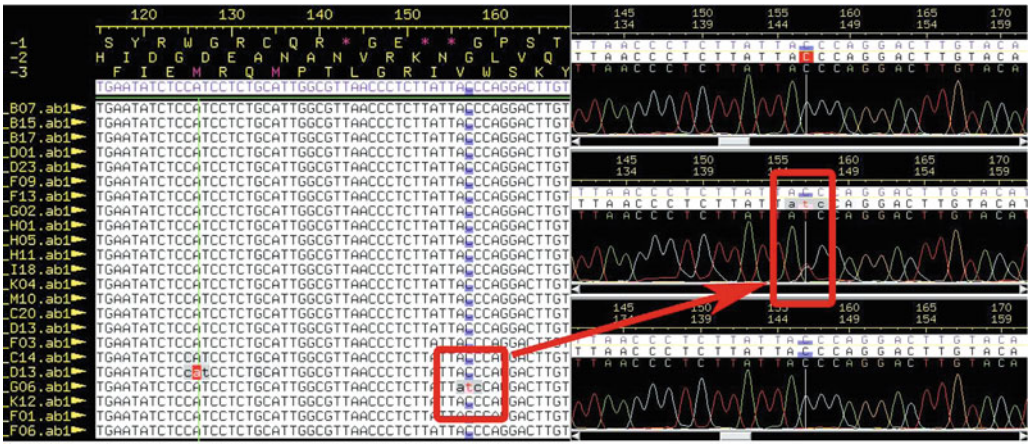

Fig. 19.6. Polyphred sequence alignment tool. Trace files are aligned and a consensus sequence as well as translation of three different frames is provided. Point mutations (homo- and heterozygous) are labeled (*squares* in alignment and chromatogram). Chromatograms of primary hits are directly linked, with "online" quality control.

defect, missense mutations might result in more subtle effects. Missense mutations that change biochemical features such as charge, polarity, or steric configurations of an affected amino acid (= non-conservative substitutions) might impair the function of the peptide. If furthermore amino acids are affected that are located in functional domains within the peptide and if they are conserved among different species, the chance of resulting in a deleterious effect is high. Only these "interesting" mutations are worth keeping and propagating; therefore, primary hits should be verified and analyzed carefully before a mutant line is established. If both criteria "non-conservative substitution and conserved position within a peptide" are fulfilled, the individual that carries the mutation should be propagated to establish a mutant line.

27. The following example illustrates the strategy for removing accompanying mutations: The size of the zebrafish genome is about 2×10^6 kb and the frequency of mutations of a given TILLING library is 1 in 150 kb. In this case, the genome of one F1 zebrafish founder of this library carries more than 13,000 different point mutations. To get rid of these, molecularly identified carriers are crossed against wild-type individuals. In each mating round about 50% of accompanying point mutations are lost, which results in only 3% accompanying point mutations (430 of 13,000) in the sixth generation after mutagenesis.
28. Since the molecular nature of a mutated allele is known, strategies such as restriction fragment polymorphisms (RFLP CAPs) that are generated by a point mutation can be applied. If no restrictions sites are directly affected, it is possible to design an artificial restriction site (derived cleaved amplified polymorphisms (dCAPs)) close to the mutation by mismatched PCR primers (26, 27). Alternatively, allele-specific PCR primers can be designed that discriminate directly between wild-type- and mutant-specific PCR products (28). The ideal genotyping strategy is defined by the molecular nature of the mutation and the available and easily accessible tools.

5. Useful Web-Based Links and Tools

Vertebrate resources for mutations:

Zebrafish: Mutation Resource Project at the Sanger Institute, England (http://www.sanger.ac.uk/Projects/D_rerio/

mutres/); MPI CBG TILLING facility (http://www.mpi-cbg.de/en/facilities/profiles/tilling.html)

Zebrafish genome sequencing (http://www.sanger.ac.uk/Projects/D_rerio/)

Mouse: Genome-wide mutant mouse library at the Riken Institute, Japan (http://www.brc.riken.go.jp/lab/mutants/RGDMSavailability.htm)

TILLING tools for primer design and organization of screens

LIMSTILL: http://limstill.niob.knaw.nl/index.html
CODDLE: http://www.proweb.org/coddle/
dCAPS: http://helix.wustl.edu/dcaps/dcaps.html
Polyphred: http://droog.gs.washington.edu/polyphred/

Acknowledgments

The authors would like to thank Sylvia Schimpke, Katja Steinberg, Claudia Seidel, and Evelin Lehmann for excellent technical support, Julia Jarrells for critical reading of the manuscript, and our collaborators Edwin Cuppen, Victor Guryev, and Ewart de Bruijn (Hubrecht Institute, Utrecht), Derek Stemple, Ross Kettleborough, and Elisabeth Busch-Nentwich (Sanger Institute, Cambridge) for sharing zebrafish TILLING libraries, materials, and protocols. The Dresden TILLING facility is supported by the MPI for Molecular Cell Biology and Genetics in Dresden, the Center for Regenerative Therapies in Dresden, the Biotechnology Center of the Technical University Dresden, and by grants from the European Union to M.B. (Zf Models and Zf Health).

References

1. Colbert, T., Till, B. J., Tompa, R., Reynolds, S., Steine, M. N., Yeung, A. T., McCallum, C. M., Comai, L., and Henikoff, S. (2001) High-throughput screening for induced point mutations. *Plant Physiol.* **126**, 480–484.
2. McCallum, C. M., Comai, L., Greene, E. A., and Henikoff, S. (2000) Targeted screening for induced mutations. *Nat Biotechnol.* **18**, 455–457.
3. Draper, B. W., McCallum, C. M., Stout, J. L., Slade, A. J., Moens, C. B., Draper, B. W., McCallum, C. M., Stout, J. L., Slade, A. J., and Moens, C. B. (2004) A high-throughput method for identifying N-ethyl-N-nitrosourea (ENU)-induced point mutations in zebrafish. *Meth. Cell Biol.* 77, 91–112.
4. Wienholds, E., Schulte-Merker, S., Walderich, B., Plasterk, R. H., Wienholds, E., Schulte-Merker, S., Walderich, B., and Plasterk, R. H. A. (2002) Target-selected inactivation of the zebrafish rag1 gene. *Science* **297**, 99–102.
5. Smits, B. M., Mudde, J., Plasterk, R. H., and Cuppen, E. (2004) Target-selected mutagenesis of the rat. *Genomics* **83**, 332–334.
6. Taniguchi, Y., Takeda, S., Furutani-Seiki, M., Kamei, Y., Todo, T., Sasado, T., Deguchi, T., Kondoh, H., Mudde, J., Yamazoe, M., Hidaka, M., Mitani, H., Toyoda, A., Sakaki, Y., Plasterk, R. H., Cuppen, E., Taniguchi, Y., Takeda, S., Furutani-Seiki, M., Kamei, Y., Todo, T., Sasado, T., Deguchi, T., Kondoh, H., Mudde, J., Yamazoe, M., Hidaka, M.,

Mitani, H., Toyoda, A., Sakaki, Y., Plasterk, R. H. A., and Cuppen, E. (2006) Generation of medaka gene knockout models by target-selected mutagenesis. *Genome Biol.* **7**, R116.
7. Stemple, D. L. and Stemple, D. L. (2004) TILLING--a high-throughput harvest for functional genomics. *Nat. Rev. Genet.* **5**, 145–150.
8. Wienholds, E., van Eeden, F., Kosters, M., Mudde, J., Plasterk, R. H., Cuppen, E., Wienholds, E., van Eeden, F., Kosters, M., Mudde, J., Plasterk, R. H. A., and Cuppen, E. (2003) Efficient target-selected mutagenesis in zebrafish. *Genome Res.* **13**, 2700–2707.
9. Moens, C. B., Donn, T. M., Wolf-Saxon, E. R., Ma, T. P., Moens, C. B., Donn, T. M., Wolf-Saxon, E. R., and Ma, T. P. (2008) Reverse genetics in zebrafish by TILLING. *Brief. Funct. Genomics Proteomic.* **7**, 454–459.
10. Grunwald, D. J. and Streisinger, G. (1992) Induction of recessive lethal and specific locus mutations in the zebrafish with ethyl nitrosourea. *Genet. Res.* **59**, 103–116.
11. Mullins, M. C., Hammerschmidt, M., Haffter, P., and Nusslein-Volhard, C. (1994) Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr. Biol.* **4**, 189–202.
12. Solnica-Krezel, L., Schier, A. F., and Driever, W. (1994) Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* **136**, 1401–1420.
13. de Bruijn, E., Cuppen, E., Feitsma, H., de Bruijn, E., Cuppen, E., and Feitsma, H. (2009) Highly efficient ENU mutagenesis in zebrafish. *Meth. Mol. Biol.* **546**, 3–12.
14. Carmichael, C., Westerfield, M., Varga, Z. M., Carmichael, C., Westerfield, M., and Varga, Z. M. (2009) Cryopreservation and in vitro fertilization at the zebrafish international resource center. *Meth. Mol. Biol.* **546**, 45–65.
15. Till, B. J., Colbert, T., Tompa, R., Enns, L. C., Codomo, C. A., Johnson, J. E., Reynolds, S. H., Henikoff, J. G., Greene, E. A., Steine, M. N., Comai, L., Henikoff, S., Till, B. J., Colbert, T., Tompa, R., Enns, L. C., Codomo, C. A., Johnson, J. E., Reynolds, S. H., Henikoff, J. G., Greene, E. A., Steine, M. N., Comai, L., and Henikoff, S. (2003) High-throughput TILLING for functional genomics. *Meth. Mol. Biol.* **236**, 205–220.
16. Winkler, S., Schwabedissen, A., Backasch, D., Bokel, C., Seidel, C., Bonisch, S., Furthauer, M., Kuhrs, A., Cobreros, L., Brand, M., Gonzalez-Gaitan, M., Winkler, S., Schwabedissen, A., Backasch, D., Bokel, C., Seidel, C., Bonisch, S., Furthauer, M., Kuhrs, A., Cobreros, L., Brand, M., and Gonzalez-Gaitan, M. (2005) Target-selected mutant screen by TILLING in Drosophila. *Genome Res.* **15**, 718–723.
17. Sood, R., English, M. A., Jones, M., Mullikin, J., Wang, D. M., Anderson, M., Wu, D., Chandrasekharappa, S. C., Yu, J., Zhang, J., and Paul Liu, P. (2006) Methods for reverse genetic screening in zebrafish by resequencing and TILLING. *Methods* **39**, 220–227.
18. Bhangale, T. R., Stephens, M., and Nickerson, D. A. (2006) Automating resequencing-based detection of insertion-deletion polymorphisms. *Nat. Genet.* **38**, 1457–1462.
19. Nickerson, D. A., Tobe, V. O., and Taylor, S. L. (1997) PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. *Nucleic Acids Res.* **25**, 2745–2751.
20. Till, B. J., Zerr, T., Comai, L., Henikoff, S., Till, B. J., Zerr, T., Comai, L., and Henikoff, S. (2006) A protocol for TILLING and Ecotilling in plants and animals. *Nat. Protoc.* **1**, 2465–2477.
21. Uauy, C., Paraiso, F., Colasuonno, P., Tran, R. K., Tsai, H., Berardi, S., Comai, L., and Dubcovsky, J. (2009) A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. *BMC Plant Biol.* **9**, 115.
22. Acevedo-Arozena, A., Wells, S., Potter, P., Kelly, M., Cox, R. D., Brown, S. D., Acevedo-Arozena, A., Wells, S., Potter, P., Kelly, M., Cox, R. D., and Brown, S. D. M. (2008) ENU mutagenesis, a way forward to understand gene function. *Annu. Rev. Genomics Hum. Genet.* **9**, 49–69.
23. Crowe, M. L. (2005) SeqDoC: rapid SNP and mutation detection by direct comparison of DNA sequence chromatograms. *BMC Bioinformatics* **6**, 133.
24. Weckx, S., Del-Favero, J., Rademakers, R., Claes, L., Cruts, M., De Jonghe, P., Van Broeckhoven, C., and De Rijk, P. (2005) novoSNP, a novel computational tool for sequence variation discovery. *Genome Res.* **15**, 436–442.
25. Zhang, J., Wheeler, D. A., Yakub, I., Wei, S., Sood, R., Rowe, W., Liu, P. P., Gibbs, R. A., and Buetow, K. H. (2005) SNPdetector: a software tool for sensitive and accurate SNP detection. *PLoS Comput. Biol.* **1**, e53.
26. Neff, M. M., Neff, J. D., Chory, J., and Pepper, A. E. (1998) dCAPS, a simple technique

for the genetic analysis of single nucleotide polymorphisms: experimental applications in Arabidopsis thaliana genetics. *Plant J.* **14**, 387–392.
27. Neff, M. M., Turk, E., and Kalishman, M. (2002) Web-based primer design for single nucleotide polymorphism analysis. *Trends Genet.* **18**, 613–615.
28. Bui, M. and Liu, Z. (2009) Simple allele-discriminating PCR for cost-effective and rapid genotyping and mapping. *Plant Methods* **5**, 1.

Chapter 20

Inducing High Rates of Targeted Mutagenesis in Zebrafish Using Zinc Finger Nucleases (ZFNs)

Jasmine M. McCammon, Yannick Doyon, and Sharon L. Amacher

Abstract

Animal models, including the zebrafish, without a reliable embryonic stem cell system are not easily amenable to targeted mutagenesis for studying gene function. Three recent publications have shown that zinc finger nucleases (ZFNs) have circumvented this shortcoming in zebrafish. Similar to restriction enzymes, ZFNs can introduce site-specific double-strand breaks (DSBs); moreover, they can be designed to recognize virtually any target sequence. Because the preferred DSB repair pathway in zebrafish embryos, non-homologous end joining, is error-prone, ZFNs can be used to create mutations in a gene of interest. Here we review the protocols for a yeast-based assay to detect effective ZFNs. Additionally, we detail the procedures for synthesis and injection of ZFN-encoding mRNA into zebrafish embryos, screening of injected embryos for induced mutations in the soma, and recovery of germline mutations.

Key words: Zinc finger nuclease, zebrafish, reverse genetics, targeted mutagenesis, golden, no tail, non-homologous end joining.

1. Introduction

The mouse has been a successful vertebrate laboratory model in part due to its established embryonic stem cell system, allowing for directed gene modification. While the zebrafish genome is amenable to many manipulations (1–4), targeted mutagenesis was previously not possible until three groups independently demonstrated that zinc finger nucleases (ZFNs) could be used for exactly this purpose (5–7).

A ZFN is a fusion protein composed of a zinc finger protein (ZFP) containing multiple zinc finger motifs that bind DNA in a sequence-specific manner and the cleavage domain of FokI

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_20,

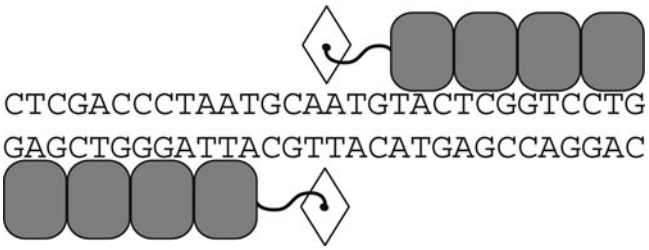

Fig. 20.1. Schematic representation of ZFN pair bound to DNA target sequence. The DNA sequence shown is the *no tail* (*ntl*) target site. Each zinc finger motif (*boxes*) essentially recognizes a 3 bp sequence. The ZFNs used in our study have four finger composite motifs, so each ZFN has a 12 bp recognition site (5). The binding sites are optimally spaced at 6 bp apart, appropriate for the length of the amino acid linker to the FokI endonuclease cleavage domain (*diamonds*). Because dimerized FokI cleavage domains cleave without sequence specificity, once the ZFN pair is bound, the FokI domains dimerize, become active, and cleave the intervening DNA sequence, creating a double-strand break (DSB) that has to be repaired.

endonuclease that cuts DNA without site specificity (**Fig. 20.1**). Although each zinc finger motif essentially recognizes a core DNA triplet, other protein:DNA interactions, as well as interactions between finger motifs, can influence binding specificity and affinity (8). A variety of zinc finger motifs, both naturally derived and engineered, have been collected, allowing for the design of composite motifs that have a 9, 12, 15, or 18 bp recognition site (5–7, 9, 10). Because the cleavage domain of FokI must dimerize to be active, two ZFNs are designed flanking a 5–6 bp spacer region (11–13). When a ZFN pair binds DNA, the FokI domains dimerize and cleave the intervening sequence to create a double-strand break (DSB) in the spacer region. In zebrafish embryos, the preferred DSB repair pathway is non-homologous end joining (NHEJ) (14), which can be mutagenic because the broken ends are often misaligned during repair, leading to the incorporation of small insertions and/or deletions at the site of the break (15). By inducing site-specific DSBs, ZFNs can be used for targeted mutagenesis.

There are a variety of ways to design ZFNs. The ZFNs used in our studies were engineered by Sangamo Biosciences using a proprietary method (5), which is available to the research community through Sigma-Aldrich. An alternative strategy to build a DNA-binding domain involves selection in bacteria using randomized or semi-randomized zinc fingers to isolate a zinc finger protein (ZFP) that most effectively recognizes the desired site. The two other zebrafish ZFN papers utilized variations of this approach (6, 7). A third method, termed modular assembly, advocates linking individual zinc fingers with known specificity for making three-finger arrays designed to bind 9-bp target sites ((16) and references therein). No matter what ZFP engineering method is

used, an essential requirement is to create two ZFP–FokI fusion proteins (a ZFN pair) that dimerize productively in vivo. We developed a reporter system in budding yeast to determine the efficiency of ZFNs to induce a DSB. Using this assay, we were able to identify the most effective ZFNs for modifying the zebrafish genome (5). In addition, this approach identified active ZFNs for genome engineering in rats, tobacco, and maize (9, 10, 17).

To show by proof of principle that ZFNs effectively mutagenize target sequences, we chose the *golden (gol)* and *no tail (ntl)* loci due to their thorough characterization and obvious mutant phenotypes. *gol* encodes a cation exchanger required for melanin deposition (thus early embryonic pigmentation) (18, 19), while Ntl is an essential T-box transcription factor necessary for posterior mesoderm development (20, 21). An experimental overview is depicted in **Fig. 20.2**. Briefly, ZFNs were designed

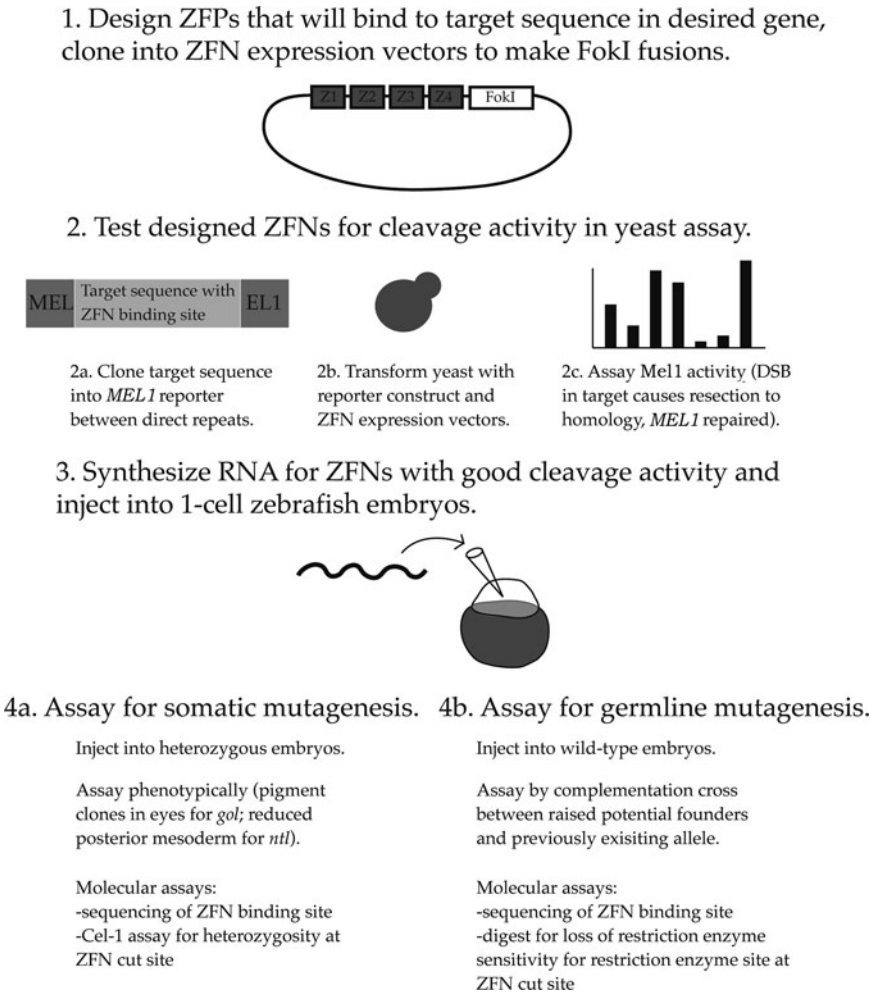

Fig. 20.2. Experimental overview.

to target the *gol* and *ntl* loci at Sangamo (step 1). These ZFNs were then tested for cleavage activity in the yeast reporter assay (step 2). In this assay, the desired target sequence containing the ZFN-binding sites is cloned between direct repeats in the *MEL1* gene, a secreted form of alpha-galactosidase. Yeast strains are generated carrying this reporter and are subsequently transformed with different ZFN pairs designed and cloned in step 1. In yeast, a DSB in such a target construct is repaired very efficiently; essentially, if a ZFN pair cleaves the target sequence, the ends will be resected until the homology of the direct repeats is detected. Subsequent DSB repair restores the *MEL1* open reading frame, whose gene product is secreted into the media and can be assayed colorimetrically. In other words, the amount of Mel1 produced is a readout of ZFN cleavage activity. To reduce the potential for off-target mutations that might occur with continuous ZFN exposure, the most active *gol* and *ntl*-targeting ZFNs identified in the yeast assay were transcribed in vitro and injected into one-cell embryos as mRNA for transient ZFN expression (step 3) (5). The mRNA injection strategy was also used by the two other zebrafish groups to target six additional loci (6, 7) and has also been used successfully in both flies and rats (9, 22). Through a combination of molecular, embryological, and genetic methods, we found that ZFNs effectively mutagenized these loci in both somatic and germline cells (step 4). Here we present the details of our published work, noting where one might alter the protocol depending upon the ZFNs utilized and the nature of the intended target locus.

2. Materials

2.1. Yeast Reporter Strain Construction

1. YPD Broth, autoclave for 20 min (Sigma).
2. YPD Agar plates, autoclave for 20 min (Sigma).
3. YPD Agar plates + 200 μg/mL G418 disulfate salt solution (Sigma), autoclave for 20 min and cool down before adding G418.
4. 1 M lithium acetate (LiOAc), filter sterilized 0.22 μm.
5. 100× TE: 1 M Tris–Cl, pH 7.5, 0.1 M EDTA, filter sterilized 0.22 μm.
6. 50% w/v PEG 3350 (polyethylene glycol average mol wt 3,350), filter sterilized 0.22 μm (Sigma).
7. Sonicated salmon sperm DNA (ssDNA) (Stratagene).
8. BY4741 yeast strain (Open Biosystems).

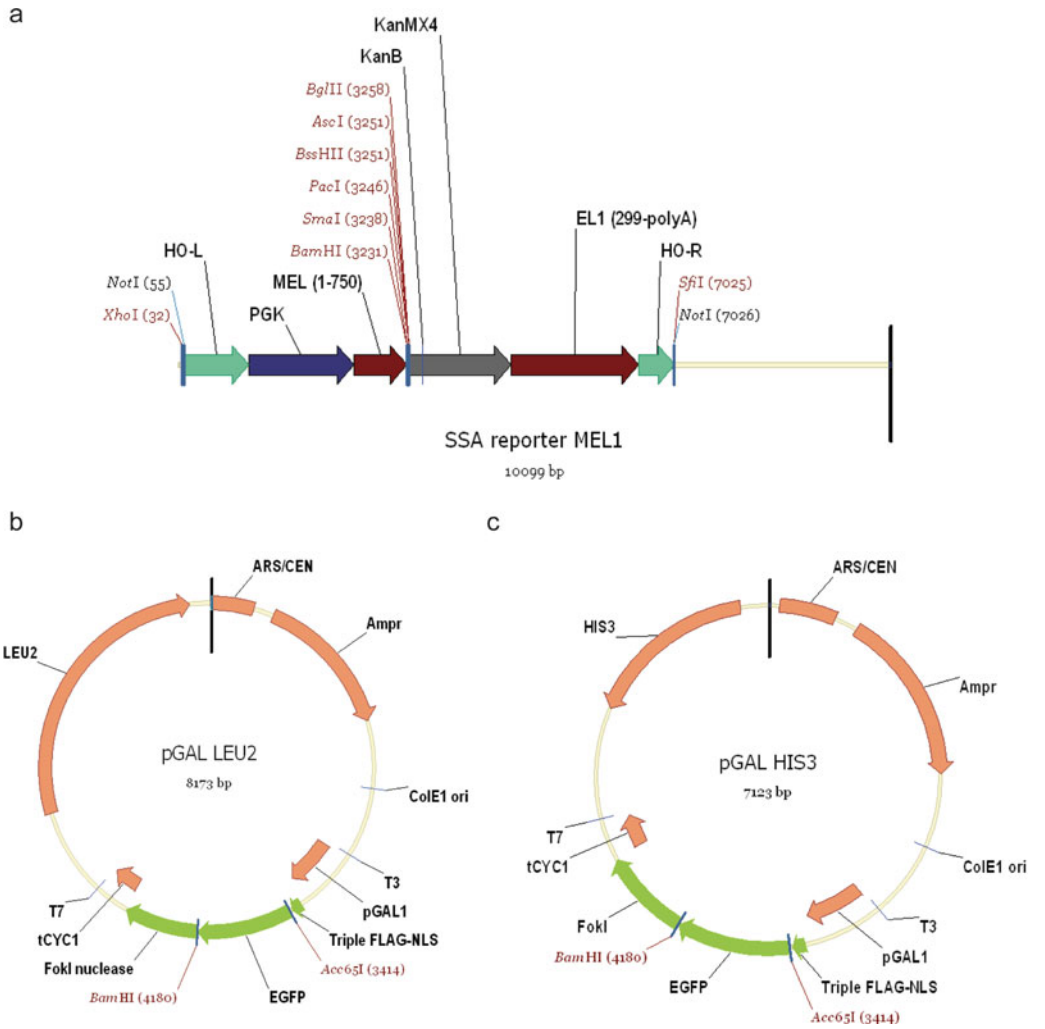

Fig. 20.3. Components of the yeast reporter system. (**a**) Vector map of the SSA reporter construct. HO-L, targeting arm for the HO locus; PGK, PGK1 promoter; MEL (1–750), nucleotides 1–750 of the *MEL1* ORF; KanB, sequencing primer-binding site; KanMX4, G418 resistance cassette; EL1 (299-polyA), 3′ end of the *MEL1* gene from nucleotide 299 of the ORF to the end of the poly A sequence; HO-R, targeting arm for the HO locus. The complete sequence of the SSA reporter can be found in supplementary material for Doyon et al. (5). (**b**) Galactose-inducible ZFN expression vectors. pGAL1, *GAL1* promoter; triple FLAG-NLS, epitope tag, and nuclear localization sequence; EGFP, to be removed during ZFP cloning; FokI nuclease, wildtype cleavage domain; tCYC1, *CYC1* transcriptional terminator sequence. *LEU2* and *HIS3* are auxotrophic markers. ARS/CEN, autonomously replicating and centromere sequences; Amp^r, *bla* gene; ColE1, prokaryotic origin of replication; T3 and T7, sequencing primer-binding sites.

9. SSA reporter *MEL1* plasmid (request from Sangamo Biosciences, **Fig. 20.3**).
10. Solution I (25 mL): 250 μL 100× TE, 2.5 mL 1 M LiOAc, 22.5 mL sterile water.
11. Solution II (25 mL): 250 μL 100× TE, 2.5 mL 1 M LiOAc, 22.5 mL 50% PEG.

2.2. Yeast Reporter Assay

1. 96-well round bottom microwell plate, 0.3 mL, with lid, sterile, polystyrene (Nunc).
2. 96 well EIA/RIA clear, flat bottom, polystyrene, non-treated microplate (Corning).
3. 96-well deep well blocks, sterile, 2 mL (Nunc).
4. AirPore tape sheets (Qiagen).
5. Tape pads (Qiagen).
6. Filter units, 0.22 μm (Nalgene).
7. Multichannel pipettor with sterile tips (10–200 μL).
8. Inoculating loops (Koch's style).
9. Bunsen burner.
10. ZFN expression vectors (pGAL *LEU2* and pGAL *HIS3*, **Fig. 20.3**) (request from Sangamo Biosciences).
11. Synthetic drop-out media with glucose lacking histidine and leucine (SC glucose H- L-): 3.35 g yeast nitrogen base (YNB, Fisher), 10 g glucose, 0.335 g complete supplement mixture His-Leu- (CSM H- L-, Fisher), 500 mL water, autoclave for 20 min.
12. Synthetic drop-out media with raffinose lacking histidine and leucine (SC raffinose H- L-): 3.35 g yeast nitrogen base (YNB, Fisher), 10 g raffinose, 0.335 g complete supplement mixture His-Leu- (CSM H- L-, Fisher), 500 mL water, autoclave for 20 min.
13. Synthetic drop-out media with galactose lacking histidine and leucine (SC Galactose H- L-): 3.35 g yeast nitrogen base (YNB, Fisher), 0.335 g complete supplement mixture His-Leu- (CSM H- L-, Fisher), 450 mL water, autoclave for 20 min. Cool down and add 50 mL 0.22 μm filtered 20% w/v galactose.
14. 20% glucose, sterile.
15. Citrate/phosphate buffer (Mel1 buffer): for 15 mL, combine 5.8 mL 0.2 M Na_2HPO_4, 9.2 mL 0.1 M citric acid and 600 μL of PNPG (*p*-nitrophenyl-alpha-D-galactoside, 50 mg/mL in methanol stock, Biosynth). Make fresh, pH should be close to 4.5.
16. 1 M Na_2CO_3.

2.3. RNA Synthesis

1. ELIMINase (Decon Labs).
2. Sharp precision barrier tips (Denville).
3. *Eag*I restriction enzyme, store at –20°C.
4. mMessage mMachine kit (Ambion), store at –20°C .

5. Nuclease-free water, e.g., UltraPure Distilled Water, DNase, RNase free (Invitrogen).
6. Phenol:chloroform:isoamyl alcohol (25:24:1) (Fisher), store at 4°C.
7. Chloroform.
8. 3 M sodium acetate.
9. Nuclease-free ethanol, store at –20°C.
10. RNasin (Roche), store at –20°C.

2.4. Zebrafish Husbandry

1. Strains: AB wild type, *golden*^{b1} homozygous adults, *no tail*^{b195} heterozygous adults.
2. Mating tanks.

2.5. Embryo Microinjection

1. Capillaries for injection needles, borosilicate with filament. O.D. 1.2 mm, I.D. 0.94 mm. 10-cm length (Sutter Instruments).
2. Sutter Instrument Co. Flaming/Brown Micropipette Puller (Model P-87) or comparable puller.
3. Phenol red, 1% stock solution.
4. Olympus SZ-60 dissecting microscope with transillumination base or comparable microscope.
5. Pressure injector (Applied Scientific Instrumentation, #MMPI-3), including back pressure unit and micropipette holder or comparable injection apparatus.
6. An apparatus to hold embryos during injection. We used plexiglass molds, 3.5 cm × 12 cm, with V-shaped grooves cut every 1 cm. The grooves were 1 mm wide and 0.5 mm deep. However, there are many other alternatives.

2.6. Phenotype Characterization: Transient Assay

1. Tricaine: for embryos add 15 drops of stock (0.4 g 3-amino benzoic acid ethylester, 0.8 g Na_2HPO_4 [anhydrous] in 100 mL RO water) to 35 mm × 10 mm culture dish of fish water. Final concentration is approximately 0.004%.
2. Pokers: 2-pound test fishing line or eyelash hairs glued into small glass capillary tubes. Poker stems can be enfolded in laboratory tape to facilitate gripping.
3. Camera/Microscope set-up: Zeiss Axioplan 2 with Axiocam digital camera or comparable microscope and camera.
4. Depression slides ("Hanging drop" slides, Fisher Scientific).
5. Methylcellulose, 3% solution.

2.7. Germline Mutagenesis Analysis

The materials used in **Section 2.7** overlap with those in **Sections 2.4** and **2.8**.

2.8. Genotyping

1. Thermopol buffer (New England Biolabs): 20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100. Store at –20°C.
2. Primers: for *golden*: 5′-ATCTGATATGGCCATGTCCAAC-ATCG-3′ and 5′-GGAACAATCCCATACGCTCCTGCAG-3′; for *ntl*: 5′-ACGAATGTTTCCCGTGCTCAGAGC-3′ and 5′-GCTGAAAGATACGGGTGCTTTCATCCAGT-GCG-3′.
3. TOPO TA cloning kit (Invitrogen).
4. Competent cells (i.e., TOP10, Invitrogen).
5. LB media: add 25 g LB Miller Broth to 1 L water, autoclave 20 min.
6. Ampicillin plates: add 25 g LB Miller Broth, 15 g agar to 1 L water, autoclave 20 min. Once cool to touch, add ampicillin (final concentration: 50 μg/mL) to liquid and pour into Petri dishes. Store poured plates upside down at 4°C.
7. Mini-prep kit (Qiagen).
8. *BsrDI*, store at –20°C, or appropriate enzyme for the designated target locus.
9. Agarose gel electrophoresis materials.

3. Methods

3.1. Yeast Reporter Strain Construction

Overview: The reporter construct consists of the desired target sequence, in our case parts of the *gol* or *ntl* genes containing the appropriate ZFN-binding site, cloned in between 452 bp direct repeats in the *MEL1* gene (**Fig. 20.3a**). The reporter plasmid is constructed in bacteria, and then integrated at the *HO* locus in yeast using the *HO* left and right targeting arms to generate the appropriate reporter strain. Subsequently (**Fig. 20.2**, step 2), different ZFN pairs are expressed in the reporter strain and assayed for their ability to cut the target sequence; this strategy reveals the efficiency of ZFN cleavage because in yeast such a DSB will be processed by single-strand annealing and result in restoration of the *MEL1* open reading frame (**Fig. 20.2**, step 2A). Repair is assayed by measuring the *MEL1* gene product, a secreted alpha-galactosidase.

1. Clone your ZFN target sequence (*see* **Note 1**) into the polylinker spanning the *BamHI—BglII* restriction sites of the SSA reporter *MEL1* plasmid to generate the reporter construct (**Fig. 20.3**). Use either ampicillin or kanamycin for selection in *Escherichia coli*. The cloned DNA can

be isolated following any standard molecular biology procedure.

2. Linearize 2 μg of your reporter construct by digesting with 2 μL of *NotI* in a 50 μL reaction and purify the DNA using a PCR purification kit. If a *NotI* site is present in your target, perform a double digest using *SfiI* and *XhoI* (**Fig. 20.3**). Store DNA at –20°C until needed.
3. Start a 5 mL preculture of BY4741 from an isolated yeast colony (streaked on YPD agar from the glycerol stock) and incubate in a shaking incubator (300 rpm) overnight at 30°C. You will need 1 mL of preculture per transformation (*see* **Note 2**).
4. Transfer 1 mL of the yeast preculture to a sterile 1.5 mL tube and spin down at 4,500 rpm for 2 min. You can prepare the competent cells in one batch if you have multiple transformations to perform. Adjust volumes accordingly.
5. Discard supernatant.
6. Wash cells by resuspending in 250 μL solution I.
7. Spin down cells (2 min at 4,500 rpm), discard supernatant.
8. Resuspend cells in 250 μL solution I.
9. Incubate 20 min at room temperature to make cells competent (this step can be extended to 1 h). Aliquot cells: 250 μL per microfuge tube at this point when performing multiple transformations.
10. Spin down cells (2 min at 4,500 rpm), discard supernatant.
11. Add 10 μL of denatured (boiled 5 min and cooled on ice) salmon sperm DNA (10 mg/mL) directly to the cells.
12. Add the purified and linearized reporter construct prepared in step 2 (50 μL).
13. Mix cells and DNA by gently pipetting up and down.
14. Add 650 μL of solution II pre-warmed at 42°C, vortex gently to disperse cells.
15. Heat shock for 30 min at 42°C, mix by inverting every 5–10 min.
16. Spin down cells (2 min at 4,500 rpm), discard supernatant.
17. Resuspend cells in 1 mL of YPD.
18. Incubate cells for 3 h in YPD at 30°C with shaking before plating on YPD + 200 μg/mL G418. Spread 10 and 90% of the cells on two different plates.
19. Incubate 2–3 days at 30°C (*see* **Note 3**).

3.2. Yeast Reporter Assay

Overview: The purpose of the yeast reporter assay is to evaluate the ability of candidate ZFN pairs to cleave the ZFN target

sequence in the reporter strain. One member of each ZFN pair is cloned into the pGAL-*LEU2* vector and the other member of the pair into the pGAL-*HIS3* vector, and then both vectors are transformed into the reporter strain (**Fig. 20.2**, step 2B). ZFN expression is under the control of galactose-inducible promoters (**Fig. 20.3b** and **c**). As mentioned previously, cleavage of the target sequence by a candidate ZFN pair will result in efficient resection of the intervening target sequence and antibiotic selection marker to the direct repeats of *MEL1* sequence, and subsequent repair will restore the *MEL1* ORF (**Fig. 20.3a**). Because Mel1 is a secreted form of α-galactosidase, supernatant from cultures of yeast reporter strains transformed and expressing ZFNs can be assayed colorimetrically using a galactosidase assay. Thus, the amount of Mel1 product directly reflects the ability of the candidate ZFN pairs to recognize and cleave their target sequence.

1. Start a preculture from a single clone of the yeast reporter strain in YPD + 200 μg/mL G418. You will need 1 mL for each transformation.
2. Incubate overnight at 30°C with shaking at 300 rpm.
3. Harvest cells at 4,000 rpm for ~3 min. Discard supernatant.
4. Wash cells with 250 μL of solution I (per mL of initial preculture). You can prepare the competent cells in one batch if you have multiple transformations to perform. Adjust volumes accordingly.
5. Spin down cells (2 min at 4,500 rpm), discard supernatant.
6. Resuspend cells in 25 μL of solution I (per milliliter of initial preculture).
7. Incubate 20 min at room temperature to make cells competent (this step can be extended to 1 h).
8. Boil salmon sperm DNA for 5 min at 95°C, then cool on ice.
9. During incubation (step 7), prepare a round bottom microwell plate at room temperature by adding the following to each well. You will need 1 well per transformation, i.e., per ZFN pair:
   a. 10 μL denatured ssDNA (10 mg/mL).
   b. 10 μL (0.5–2 μg) ZFN expression vector 1 (pGAL *LEU2*) (*see* **Note 4**).
   c. 10 μL (0.5–2 μg) ZFN expression vector 2 (pGAL *HIS3*) (*see* **Note 4**).
10. Add 25 μL of competent yeast cells, mix gently by slowly pipetting up and down.
11. Add 150 μL of solution II (heated to 42°C) to each well and mix by slowly pipetting up and down. Alternatively,

cover the plate with tape and mix on a shaking platform. We use a MixMate (Eppendorf) at 1,200 rpm.

12. Cover the plate with tape and heat shock for 30 min at 42°C.
13. Spin the microplate for 5 min at 1,500×*g*.
14. During the spin, prepare a selective media plate by adding 1 mL of glucose SC H- L- into each well of a deep well block. You will need 1 well per transformation, i.e., per ZFN pair.
15. Remove and discard 200 μL of the supernatant from each well of the spun microplate.
16. Gently resuspend the cells in each well with 100 μL of selective media (glucose SC H- L-) and transfer them to a corresponding well in the prepared deep well block. Cover block using air pore tape.
17. Incubate 48–72 h in a 30°C shaker at 300 rpm (*see* **Note 5**).
18. To enrich for transformants, resuspend cells by pipetting up and down (MixMate at 1,200 rpm) and transfer 200 μL of the culture to 1 mL of fresh glucose SC H- L- media in a deep well block.
19. Incubate overnight in a 30°C shaker at 300 rpm.
20. To derepress the *GAL1* promoter, transfer 100 μL of the glucose cultures to 1 mL of fresh raffinose SC H- L- media in a deep well block (*see* **Note 6** and **7**).
21. Incubate overnight in a 30°C shaker at 300 rpm.
22. ZFN expression is typically induced for 6 h. To induce ZFN expression, transfer 100 μL of the raffinose cultures into 1 mL of galactose SC H-L- media and incubate for 6 h in a 30°C shaker at 300 rpm. To assay ZFN activity under derepression conditions but in the absence of galactose induction, transfer 100 μL of the raffinose culture into 1 mL of glucose SC H-L- media and incubate in a 30°C shaker at 300 rpm (*see* **Notes 7** and **8**).
23. After the 6 h incubation, add 200 μL of sterile 20% glucose to stop ZFN expression.
24. Let cells recover overnight in a 30°C shaker at 300 rpm.
25. Mel1 reporter activity should be performed 16–18 h after adding 20% glucose.
26. Determine culture density by transferring 50 μL of each culture to a clear flat bottom microwell plate containing 150 μL of water, pipetting up and down, and then measuring OD_{600} nm using a spectrophotometer. The OD_{600} nm value should be within the linear range for your microplate reader.

27. Spin down the deep well block at 4,000 rpm for 5 min.
28. Transfer 10 μL of supernatant to a clear flat bottom microwell plate (*see* **Note 9**).
29. Add 90 μL Mel1 buffer pre-warmed to 30°C.
30. Cover microplate with tape and incubate for 1 h at 30°C.
31. Stop the reaction by adding 100 μL of 1 M Na_2CO_3 and mix by pipetting up and down.
32. To determine total alpha-galactosidase activity in each sample, measure OD_{405} nm using a spectrophotometer. Make sure that the OD_{405} nm value is in the linear range of both the enzymatic assay and the absorption measurement (*see* **Note 10**).
33. ZFN activity is determined based on the Mel1 (alpha-galactosidase) activity of the culture supernatants, normalized by cell density. Normalized Mel1 activity is calculated using the following formula: $\text{mU} = \text{OD}_{405} \times V_f \times 1{,}000/[(\varepsilon \times b) \times t \times V_i \times \text{OD}_{600}]$. This equation becomes simplified to $\text{mU} = \text{OD}_{405}/\text{OD}_{600} \times 7.94$, after inputing the known variables: V_f is the final volume of the assay in microliters (200 μL), V_i is the volume of culture medium supernatant added in microliters (10 μL), t is the elapsed time of incubation in minutes (60 min), OD_{600} is the optical density of the cultures (step 26), and $\varepsilon \times b$ is a constant equal to 10.5 derived from the *p*-nitrophenol molar absorptivity at OD_{405} nm multiplied by the light path.

3.3. RNA Synthesis

1. Select the zinc finger sequence and FokI nuclease variant (*see* **Note 11**) to be used in study and subclone ZFNs into appropriate plasmids to use for RNA synthesis (*see* **Note 12**).
2. Prepare an RNase-free area and RNase-free micropipettors by wiping area and pipettors down with ELIMINase.
3. Make the template for mMessage mMachine transcription reaction: linearize ~8–12 μg of plasmid DNA encoding ZFNs with appropriate enzyme. In our case, we used 2 μL *EagI*, a restriction enzyme with a unique cut site after the BGH polyA signal in the pVAX vector, in a 50 μL reaction for 4 h at 37°C.
4. Stop the reaction by purifying with phenol chloroform extraction (*see* **Note 13**): add 50 μL of nuclease-free water to digest, add 100 μL of phenol:chloroform (1:1). Mix by vortexing and centrifuge at top speed for 4 min at room temperature. Extract the top aqueous layer to a clean microcentrifuge tube. Add one volume of chloroform to the extracted layer, mix, and centrifuge as before. Extract the top aqueous layer to a clean tube.

5. Perform an ethanol precipitation to concentrate the DNA template. Add a 1/10th volume of 3 M sodium acetate and 2 volumes of ice cold 100% ethanol, then mix and freeze at –20°C for at least 20 min (can go overnight). Centrifuge at top speed for 10 min at room temperature, noting where the pellet will form, aspirate the liquid, add 100 μL of cold 70% ethanol, centrifuge for 1 min to make the pellet stick. Carefully aspirate the 70% ethanol to avoid disturbing or moving pellet. Evaporate the remaining ethanol by leaving the tube caps open on the bench top for ~5 min. Resuspend the pellet in 6 μL of nuclease free water.
6. Set up a transcription reaction: thaw the 10× reaction buffer and 2× NTP/CAP, vortex and spin down. Keep the 2× NTP/CAP on ice but leave the 10× reaction buffer at room temperature. In a clean microcentrifuge tube at room temperature, add 10 μL of 2× NTP/CAP, 2 μL of 10× reaction buffer, 5.5 μL of linearized DNA template (recommended ~1 μg), 0.5 μL of RNase inhibitor, 2 μL of T7 (or other appropriate polymerase for sense transcription) enzyme mix. Incubate for 3 h at 37°C. Add 1 μL of DNase to the transcription reaction to destroy the template and incubate at 37°C for 15 min.
7. Stop the reaction by precipitating with the LiCl solution included with the kit (*see* **Note 14**). Mix and store at –20°C for at least 20 min or overnight. Centrifuge at top speed for 15 min at 4°C, noting where the pellet of RNA will form. Aspirate the liquid, being careful not to disturb the pellet. Add 100 μL of ice cold 70% ethanol, and centrifuge for 5 min. Carefully remove the ethanol, and allow the pellet to air dry for 3–4 min. Resuspend the RNA in 15 μL of nuclease free water and store at –20°C.
8. Determine the RNA concentration with a 1:100 dilution (1 μL RNA and 99 μL water) by measuring the absorbance with a UV spectrophotometer. Multiply the 260 nm absorbance reading by 4 to determine the concentration in μg/μL.

3.4. Zebrafish Husbandry

1. Maintain adult strains at 28.5°C on a 14/10 h light/dark cycle.
2. Obtain embryos from natural spawning: The afternoon before the desired embryo collection day, separate male and female fish in holding tanks without food. Shortly after the lights turn on the next morning, combine 3 females and 3 males of the desired genotype in mating tanks with mesh bottoms to prevent the adults from consuming the embryos once they are made. Alternatively, in vitro fertilization (IVF)

can be used to generate embryos (for IVF protocols, the reader is referred to other chapters within this volume). If there is a concern that fish strains may carry single nucleotide polymorphisms (SNPs) in the loci of interest, thereby affecting subsequent analyses (i.e., **Section 3.6**, step 6), then embryos can be generated from genotyped individuals.

3.5. Embryo Microinjection

1. Prepare injection needles using a micropipette puller and borosilicate capillaries with filaments (*see* **Note 15**). The parameters for our micropipette puller to generate injection needles from the indicated capillaries are Heat 573, Pull 150, Velocity 100, Time 50, Pressure 110; however, the reader should refer to their micropipette puller manual for information on performing the appropriate ramp test for the capillary used and for determining the puller parameters to generate the appropriate needle characteristics.
2. Prepare the injection solution at fivefold serial dilutions. Dilute the RNA with RNase-free water and 0.1% phenol red as an injection tracer. Typically the range for the *gol* and *ntl* experiments was 40 pg/nL–1 ng/nL. For experiments with other ZFN mRNAs, the effective range may depend upon the number of zinc finger motifs in the ZFN, the FokI endonuclease variant used, and/or the vector into which the ZFN is cloned for in vitro transcription. Load 1 μL of injection mix into needles.
3. Inject 3–5 nL of the mRNA solution into 1–2 cell embryos (heterozygous embryos for transient assays and wild-type embryos for transient or germline assays) into a cell or the yolk (*see* **Note 16**).
4. Remove infertile embryos during the blastula stages (3–4 h post fertilization, hpf).

3.6. Phenotype Characterization: Transient Assay

To analyze gol-targeting ZFN-injected embryos:

1. At 2 days post fertilization (dpf), immobilize *gol*^{b1} heterozygous embryos injected with *gol*-targeting ZFNs in 0.004% tricaine. Carefully manipulate individual embryos using pokers and evaluate each eye for the appearance of patches of unpigmented cells, using a standard dissecting microscope with transillumination (**Fig. 20.4**).
2. Mount immobilized embryos on a drop of 3% methylcellulose in a depression slide to photograph.

To analyze Ntl-targeting ZFN-injected embryos:

3. At 1 dpf, using a standard dissecting microscope with transillumination, evaluate *ntl*^{b195} heterozygous embryos injected with *ntl*-targeting ZFNs for *ntl*-like appearance: reduced or missing posterior mesoderm, misshapen somites, and lacking notochord cells (**Fig. 20.4**).

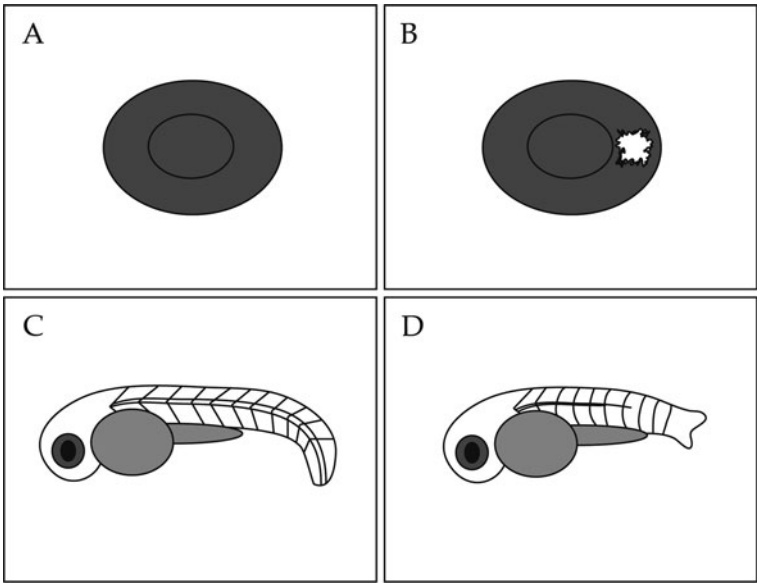

Fig. 20.4. Schematized results depicting ZFN-induced somatic phenotypes at the *gol* and *ntl* loci. (**a**) The eye of a *gol*$^{b1+/-}$ uninjected embryo is uniformly darkly pigmented at 2 days post fertilization (dpf). (**b**) The eye of a *gol*$^{b1+/-}$ embryo injected with 5 ng of *gol*-targeting ZFNs displays patches of unpigmented cells, indicative of mutagenic events occurring on the non-*gol*^{b1} mutant chromosome. (**c**) An uninjected *ntl*$^{b195+/-}$ embryo at 1 dpf has a fully formed notochord, chevron-shaped somites, and a fully extended tail. (**d**) A *ntl*$^{b195+/-}$ embryo injected with 5 ng of *ntl*-targeting ZFNs fully or partially phenocopies the *ntl* mutant phenotype, having misshapen somites and a reduced or missing notochord and tail.

4. Immobilize the embryos in 0.004% tricaine and photograph as described above (**Section 3.6**, step 2).
5. To characterize more subtle notochord defects, fix 18–22 hpf ZFN-injected *ntl*^{b195} heterozygous embryos in 4% paraformaldehyde overnight at 4°C in microcentrifuge tubes (20 embryos/tube) for a *ntl* in situ hybridization (**Fig. 20.5**). There are several published protocols for in situ hybridization; our protocol is essentially the same as described by Thisse and Thisse for the zebrafish expression screen project (detailed protocol available online at http://zfin.org/ZFIN/Methods/ThisseProtocol.html). In addition, the reader is referred to detailed protocols included in other chapters in this book.
6. To evaluate ZFN mutagenesis at the molecular level, one can assay for heterozygosity at the test locus by using a mismatch-sensitive endonuclease, Cel-1 (*see* **Note 17**).

3.7. Germline Mutagenesis Analysis

1. Inject wild-type embryos with RNA encoding *ntl*-targeting ZFNs and raise healthy larvae that develop swim bladders (scored using a dissecting microscope at 5 dpf) to adulthood.

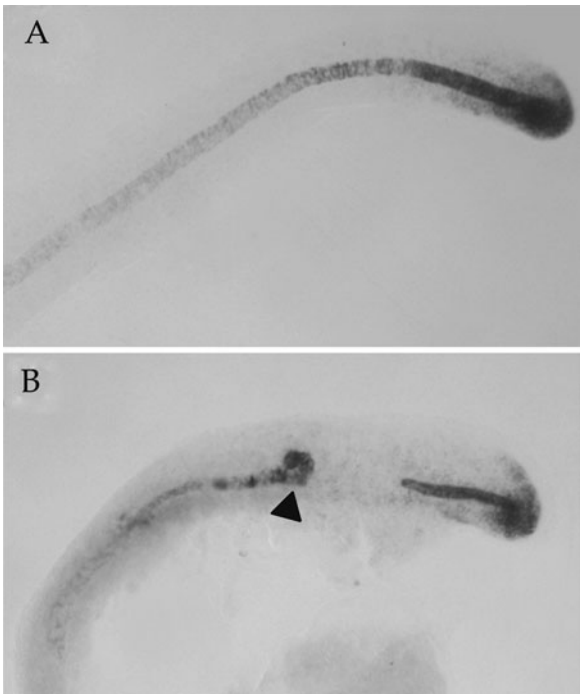

Fig. 20.5. Subtle notochord defects in *ntl* ZFN-injected embryos can be detected by in situ hybridization. (**a**) A wild-type embryo fixed at approximately 20 hpf and processed for in situ hybridization with a *ntl* probe shows that *ntl* is expressed throughout the notochord and in the tail bud. (**b**) A $ntl^{b195+/-}$ embryo injected with 5 ng of *ntl*-targeting ZFNs simultaneously processed as described in **Fig. 20.4**(d) shows that *ntl* is expressed in the tail bud. However, notochord expression is irregular and even missing in places. In addition, there are some notochord morphology defects (*arrowhead*).

2. When injected potential founders are 2–3 months old, perform complementation crosses with ntl^{b195} heterozygous fish by natural crosses or in vitro fertilization.
3. Score the resulting progeny for a *ntl* mutant phenotype (**Fig. 20.6**). To estimate the fraction of mutant gametes derived from the germline of the founder parent, double the percentage of mutant embryos observed in the complementation cross, since only half the ZFN-injected embryos inherited a ntl^{b195} allele from the tester parent. If crossing to a homozygous mutant tester parent, such as gol^{b1}, which is a viable mutation, the percentage of mutant embryos directly reflects the percentage of mutant gametes and there is no need to double this number. Genotyping of randomly selected embryos can be used to confirm the actual percentage of ZFN-induced mutant chromatids among progeny of the complementation cross (**Section 3.8**).

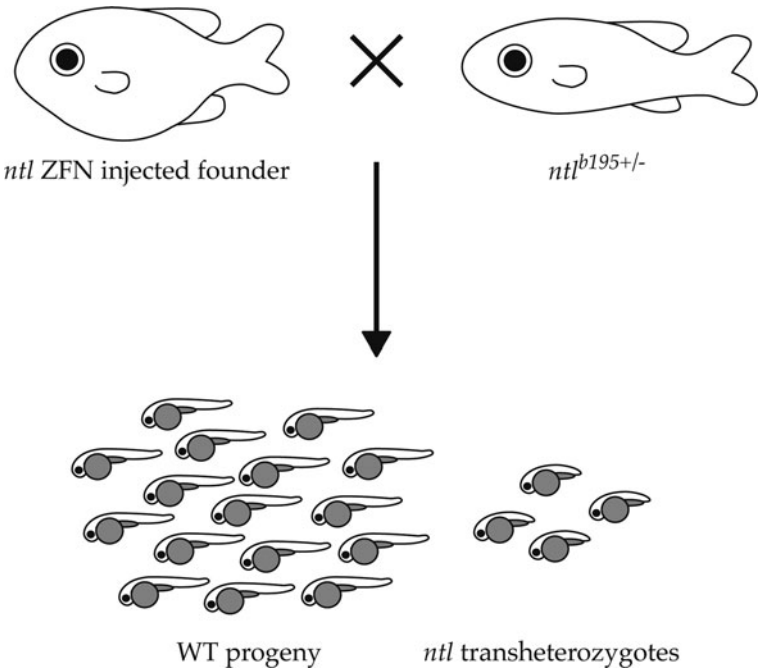

Fig. 20.6. Complementation cross identifies *ntl* ZFN-induced mutations in the germline. Potential founder fish (wild-type embryos injected with *ntl*-targeting ZFNs and raised to adulthood) are crossed to a *ntl*^{b195} heterozygous fish. The resulting progeny are analyzed for the *ntl* mutant phenotype. In this example, the percentage of mutant embryos recovered is 20% (4 of 20). The germline transmission frequency from this founder is twice that number (40%), because only half of the embryos carrying a *ntl* ZFN-induced mutant allele will inherit a mutant *ntl* allele from the *ntl*^{b195} heterozygous parent. The other half inherits a wild-type *ntl* allele from the *ntl*^{b195} heterozygous parent and thus appears phenotypically wild-type.

3.8. Genotyping

3.8.1. BsrDI (or Other Appropriate Enzyme) Digest

1. To confirm that the phenotypes observed are a result of ZFN-induced mutagenic repair by NHEJ at the desired site, one can assay for insertions and/or deletions at the cut site by restriction digestion. First, isolate genomic DNA from *ntl*-like embryos. Place individual embryos in 50 μL of 1× thermopol buffer. Heat the samples to 98°C for 10 min, then cool to 55°C. Add 5 μL of 10 mg/mL proteinase K, and incubate at 55°C for 1 h, flicking the tubes after 30 min to help break up embryos. Heat to 98°C for 10 min to inactivate proteinase K. Centrifuge the samples for 3 min at 14,000 rpm to pellet undigested tissue. Remove the supernatant containing the genomic DNA to clean tubes.
2. PCR amplify the 226 bp region for *ntl* using the primers listed in **Section 2.8**, step 2. Use 1 μL of genomic DNA in a 10-μL PCR reaction (PCR: 94°C for 2 min; 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, for 30 cycles; 72°C for 2 min, 4°C hold). Due to a 1.5 kb insertion in the *ntl*^{b195} allele,

the *ntl*^{b195} chromatids are not amplified using this protocol, allowing one to assay only the chromatid inherited from the ZFN mRNA-injected parent.

3. Digest PCR product with *BsrDI* in a 20 μL reaction at 65°C for 1 h (*see* **Note 18**).
4. Run the digest on a 2% agarose TBE gel. Expected band sizes: 226 bp for the mutant allele, 176 bp + 50 bp for the wild-type allele (*see* **Note 19**).

3.8.2. Sequencing

5. To subclone the PCR product into the TOPO TA cloning pCRII vector, combine 4 μL of the undigested PCR product (from step 2) with 1 μL of salt solution and 1 μL of vector for 10 min at room temperature (*see* **Note 20**).
6. To transform bacteria, add 1 μL of the ligation reaction to 100 μL of competent cells, and incubate the cells on ice for 20 min before heating at 42°C for 45 s. Recover the cells on ice for 2 min, add 900 μL of LB medium, and shake the tube at 37°C for 45 min.
7. Plate 50 μL of cells on selective media plates and grow overnight at 37°C.
8. Select multiple colonies for sequencing with a T7 sequencing primer. If mutations are ZFN induced, there should be short insertions, deletions, or a combination thereof at the site of ZFN binding.

4. Notes

1. The target sequence can be genomic DNA, a cDNA, or a synthetic sequence containing multiple ZFN-binding sites. Due to the high processivity of DNA resection (processing of the ends of a double-strand break) in yeast, apparent ZFN activity is insensitive to the target length or orientation. This is particularly useful when testing ZFNs targeting multiple positions within a large target sequence. We have cloned targets up to 10 kb and did not observe a decrease in overall signal. The kanB primer (5′ CTG CAG CGA GGA GCC GTA AT 3′) (**Fig. 20.3**) can be used to sequence the target.
2. Good sterile technique is the first and most important step in ensuring consistent results since antibiotics are not used for yeast culture. Use of a Bunsen burner is required and flaming should be routinely done when caps are removed

from tubes or bottles. Always clean all work areas (70% ethanol) thoroughly before and after working with yeast.

3. It is good microbiology practice to restreak large colonies on a new selection plate and perform experiments using an isolated clone. Selection on G418 media often results in a background of small colonies that are thought to arise from abortive integrations. Also, it is not uncommon to obtain "petite" mutants during yeast transformation. Always avoid working with small colonies. We have discovered that it is unnecessary to genotype a clone for correct integration since we never observed random integration; however, we recommend when establishing the system that clones be confirmed prior to use. It is also a good practice to make a glycerol stock of your reporter strain. Start an overnight culture in YPD + 200 μg/mL G418 and freeze at –80°C after adding glycerol to a final concentration of 20% (v/v).
4. ZFPs are cloned into the ZFN expression vector using Acc65I and BamHI (**Fig. 20.3**).
5. In yeast genetics experiments, transformants are usually isolated by plating cells on selective media. We have found that it is possible to select a pool of transformants in liquid media without introducing bias in the assay. This method of selection greatly simplifies the manipulations and allows us to perform the assay using liquid handling systems.
6. If yeast growth following dilution in fresh glucose media (**Section 3.2**, steps 18–19) is not vigorous (low turbidity, small cell pellet), you should transfer 200 μL instead of 100 μL. This situation happens when the transformation efficiency is low or when the initial selection time (**Section 3.2**, step 17) is reduced.
7. The presence of glucose in the media results in the repression of the promoter, and switching to raffinose releases that repression and allows for basal expression (resulting in low basal levels of expression). The subsequent addition of galactose results in the active induction of the promoter (resulting in higher levels of expression). When assaying very active ZFNs we observed that basal (leaky) transcription from the *GAL1* promoter provides sufficient ZFN expression to cleave and repair the reporter, thus restoring the *MEL1* ORF. In such situations, strong activity will be detected at $t = 0$ h. This has typically been a very good indicator for the identification of a highly active ZFN pair.
8. If you wish to monitor ZFN expression, add 2% galactose to the remainder of the raffinose cultures and incubate for 6 h at 30°C with shaking at 300 rpm before collecting cells for protein extraction. ZFN expression cassettes contain a triple FLAG epitope.

9. Note that the Mel1 enzyme is secreted into the periplasm and diffuses into the media. An easy way to greatly improve assay sensitivity is to directly use the cell suspension instead of the supernatant. We have found this to be unnecessary for most applications.
10. We established that the alpha-galactosidase assay is linear up to a OD_{405} nm value of ~2 under the assay conditions. Use less of the supernatant to perform the enzymatic assay if the value indicates saturation. This should be relatively infrequent and is indicative of a highly active ZFN pair.
11. Several FokI nuclease variants are available (23, 24). In most cases, we used an obligate-heterodimer high-fidelity version of the FokI cleavage domain. This version has point mutations at the interaction interface to ensure that only ZFN pairs binding as heterodimers can create an active version of the FokI cleavage domain, not homodimers binding at off-target sites. When we used the wild-type FokI in zebrafish, we found that, while these ZFNs induced more mutations at the target site, we also observed more embryos exhibiting developmental defects after injection with ZFNs containing wild-type FokI than with the same amount of ZFNs containing the high-fidelity FokI variant.
12. The expression vector used can vary depending upon the ease of subcloning as well as personal preference. We used pVAX for most of our experiments; however, a pCS2+-based vector was used in the study by Meng et al. (6). In most cases, pairs of ZFNs were placed in the same plasmid, spaced by a viral 2A peptide ribosomal stutter sequence, which should give equal amounts of both peptides. However, we also made singly transcribed RNA from plasmids containing only one ZFN coding sequence, quantified the amount of RNA using a UV spectrophotometer, and co-injected equal amounts of RNA for paired ZFNs. We did not observe a significant difference in either case; however, we mostly used 2A fusion plasmids for convenience.
13. Phenol:chloroform extraction is essential. We do not recommend alternatives, such as heat inactivation of the digest reaction, as this method leads to decreased transcription efficiency in our hands. Note, phenol is corrosive. Use appropriate protection.
14. We sometimes phenol:chloroform-extract RNA after LiCl precipitation, which tends to decrease RNA yield. We did not note a significant increase in toxicity when injecting embryos without this additional phenol:chloroform extraction step.

15. Needles were not specially treated to remove RNase; however, care was taken to wear gloves when pulling needles and when loading RNA into needles.
16. To obtain *ntl*-like embryos in transient assays, we injected heterozygous *ntl* embryos with RNA encoding ZFNs containing the high fidelity version of FokI. However, we could also obtain *ntl*-like embryos by injecting wild-type embryos if we used RNA encoding ZFNs containing the wild-type version of FokI (*see* **Note 11**). Many NHEJ mutagenic repair sequences were recovered from these *ntl*-like embryos (5). However, biallelic mutations were also likely induced with the obligate-heterodimer variant of FokI, as wild-type embryos injected with *ntl* ZFNs carrying this variant occasionally grew up as adult fish with tail truncations ((5), **Fig. 20.7**).
17. Note that in order for an assay with a mismatch-sensitive endonuclease to work, one will need non-polymorphic strains, at least in a 200–300 bp region containing the ZFN-binding site. Lack of existing polymorphism ensures that a PCR product from the region will be cleaved only when heterozygosity has been induced by ZFN-directed cleavage and NHEJ-mediated repair (instead of cleavage due to pre-existing SNPs). Thus, we recommend that the region surrounding the target be screened for segregating

Fig. 20.7. *ntl* targeting ZFNs can induce biallelic mutations. As many as 21% of wild-type fish injected with *ntl*-targeting ZFNs fail to develop tail fins and some posterior tail tissue (*top fish*). Because *ntl* is a strictly recessive mutation, we hypothesize that this tail phenotype represents a rare biallelic mutation in cells that would normally give rise to the tail. Although single cell analysis is not feasible, *ntl* genotyping of DNA samples prepared from posterior tissue biopsies of adult tailless fish showed that a small number of specific ZFN-induced NHEJ-type mutations represented a substantial fraction of sequence reads from any single fish, as one might expect for clonal expansion of a cell or few cells carrying two newly induced alleles (5).

SNPs before ZFNs are injected and progeny raised. Mismatch sensitive endonuclease analysis is particularly useful to detect mutagenic events in the case where the ZFN target site does not contain a restriction enzyme site (*see* **Note 18**).

18. When selecting ZFNs binding sites, it is helpful to choose one where the 5–6 bp spacer region is a recognition site for a restriction enzyme; loss of this site is a likely indicator of mutagenic NHEJ repair. One can then molecularly identify mutagenic events without needing a pre-existing mutant allele over which to genetically screen for loss of complementation.
19. Because of the high percentage of germline mutations, one does not need a pre-existing mutant allele to identify germline mutations when targeting a novel locus with ZFNs and may be able to identify founders by intercrosses. Alternatively, with careful planning of ZFN-binding sites around a restriction enzyme recognition site, one can directly genotype for germline mutations in the progeny of injected founders.
20. There is no need to clean up the PCR product before ligation when using this kit; however, be aware that some high-fidelity versions of polymerase result in blunt-ended products. The TOPO vector relies on the polyA overhangs generated by normal *Taq* polymerase for ligation, so a blunt-ended PCR product must be appropriately modified by incubation with normal *Taq* polymerase at 72°C for 10 min with dATPs.

References

1. Moens, C.B., Donn, T.M., Wolf-Saxon, E.R., and Ma, T.P. (2008) Reverse genetics in zebrafish by TILLING. *Brief. Funct. Genomic Proteomic.* **7**, 454–459.
2. Nasevicius, A. and Ekker, S.C. (2000) Effective targeted gene 'knockdown' in zebrafish. *Nat Genet.* **26**, 216–220.
3. Amsterdam, A. and Hopkins, N. (2006) Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. *Trends Genet.* **22**, 473–478.
4. Amsterdam, A. and Becker, T.S. (2005) Transgenes as screening tools to probe and manipulate the zebrafish genome. *Dev Dyn.* **234**, 255–268.
5. Doyon, Y., McCammon, J.M., Miller, J.C., Faraji, F., Ngo, C., Katibah, G.E., et al. (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.* **26**, 702–708.
6. Meng, X., Noyes, M.B., Zhu, L.J., Lawson, N.D., and Wolfe, S.A. (2008) Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat. Biotechnol.* **26**, 695–701.
7. Foley, J.E., Yeh, J.J., Maeder, M.L., Reyon, D., Sander, J.D., Peterson, R.T., and Joung, J.K. (2009) Rapid mutation of endogenous zebrafish genes using zinc finger nucleases made by oligomerized pool engineering. *PloS ONE.* **4**, e4348.
8. Isalan, M., Choo, Y., and Klug, A. (1997) Synergy between adjacent zinc fingers in sequence-specific DNA recognition. *Proc. Natl. Acad. Sci. USA* **94**, 5617–5621.
9. Geurts, A.M., Cost, G.J., Freyvert, Y., Zeitler, B., Miller, J.C., Choi, V.M., et al.

(2009) Knockout rats via embryo microinjection of zinc-finger nucleases. *Science* **325**, 433.
10. Shukla, V.K., Doyon, Y., Miller, J.C., DeKelver, R.C., Moehle, E.A., Worden, S.E., et al. (2009) Precise genome modification in the crop species *Zea mays* using zinc finger nucleases. *Nature* **459**, 437–441.
11. Kim, Y.G., Cha, J., and Chandrasegaran, S. (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc. Natl. Acad. Sci. USA* **93**, 1156–1160.
12. Pabo, C.O., Peisach, E., and Grant, R.A. (2001) Design and selection of novel Cys2His2 zinc finger proteins. *Annu. Rev. Biochem.* **70**, 313–340.
13. Smith, J., Berg, J., and Chandrasegaran, S. (1999) A detailed study of the substrate specificity of a chimeric restriction enzyme. *Nucleic Acids Res.* **27**, 674–681.
14. Hagmann, M., Bruggmann, R., Xue, L., Georgiev, O., Schaffner, W., Rungger, D., et al. (1998) Homologous recombination and DNA-end joining reactions in zygotes and early embryos of zebrafish (*Danio rerio*) and *Drosophila melanogaster*. *Biol. Chem.* **379**, 673–681.
15. Valerie, K. and Povirk, L.F. (2003) Regulation and mechanisms of mammalian double-strand break repair. *Oncogene* **22**, 5792–5812.
16. Ramirez, C.L., Foley, J.E., Wright, D.A., Müller-Lerch, F., Rahman, S.H., Cornu, T.I., et al. (2008) Unexpected failure rates for modular assembly of engineered zinc fingers. *Nat. Methods* **5**, 374–375.
17. Cai, C.Q., Doyon, Y., Ainley, W.M., Miller, J.C., Dekelver, R.C., Moehle, E.A., et al. (2009) Targeted transgene integration in plant cells using designed zinc finger nucleases. *Plant Mol. Biol.* **69**, 699–709.
18. Streisinger, G., Coale, F., Taggart, C., Walker, C., and Grunwald, D.J. (1989) Clonal origins of cells in the pigmented retina of the zebrafish eye. *Dev. Biol.* **131**, 60–69.
19. Lamason, R.L., Mohideen, M.A., Mest, J.R., Wong, A.C., Norton, H.L., Aros, M.C., et al. (2005) SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. *Science* **310**, 1782–1786.
20. Halpern, M.E., Ho, R.K., Walker, C., and Kimmel, C.B. (1993) Induction of muscle pioneers and floor plate is distinguished by the zebrafish *no tail* mutation. *Cell* **75**, 99–111.
21. Schulte-Merker, S., van Eeden, F.J.M., Halpern, M.E., Kimmel, C.B., and Nüsslein-Volhard, C. (1994) *no tail (ntl)* is the zebrafish homologue of the mouse T (Brachyury) gene. *Development* **120**, 1009–1015.
22. Beumer, K.J., Trautman, J.K., Bozas, A., Liu, J.L., Rutter, J., Gall, J.G., and Carroll, D. (2008) Efficient gene targeting in Drosophila by direct embryo injection with zinc-finger nucleases. *Proc. Natl. Acad. Sci. USA* **105**, 19821–19826.
23. Miller, J.C., Holmes, M.C., Wang, J., Guschin, D.Y., Lee, Y.L., Rupniewski, I., et al. (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat Biotechnol.* **25**, 778–785.
24. Szczepek, M., Brondani, V., Büchel, J., Serrano, L., Segal, D.J., and Cathomen, T. (2007) Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat Biotechnol.* **25**, 786–793.

Chapter 21

Derivation of Mouse Embryonic Stem Cell Lines from Blastocysts Produced by Fertilization and Somatic Cell Nuclear Transfer

Zhongde Wang

Abstract

This chapter describes a detailed protocol for deriving mouse embryonic stem (ES) cell lines from blastocysts that are produced either by fertilization or by somatic cell nuclear transfer (NT or cloning). Rather than function as a scientific communication with the experts in this field, this protocol is written with researchers who are new to mouse ES cell line derivation in mind. Therefore, researchers who want to establish such methods in laboratories where mouse ES cell line derivation has never been done before should find this chapter helpful.

Key words: Embryonic stem cells, ES, ntES, blastocysts, mouse, derivation, feeder, pluripotency, chimeric, tetraploid complementation.

1. Introduction

The establishment of mouse embryonic stem (ES) cells from preimplantation stage embryos, first achieved in 1981 (1, 2), has contributed to modern biology in a way that few other biological inventions in the last 30 years have. Gene targeting in ES cells through homologous DNA recombination has revolutionized mammalian genetics. Mice generated from such genetically modified ES cells allow scientists to elucidate a gene's function in live animals for embryonic development, adult physiology, and disease (3, 4). In vitro studies of mouse ES cells have also contributed greatly to our current understanding of the epigenetic mechanisms that regulate cellular pluripotency and differentiation (5).

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_21,

As the "prototype" of mammalian ES cells, mouse ES cells also have been used as the experimental model for developing protocols to derive ES cells from other mammalian species, such as rat, monkey, and human (6–8). The recent breakthroughs in deriving induced pluripotent stem (iPS) cells from somatic cells with pluripotent transcription factors would not have been possible without the knowledge gained from the epigenetic studies with mouse ES cells (9). Following the success of mouse cloning by NT, ES cell lines have also been derived from NT blastocysts (ntES) (10). This type of ES cells has served as a unique experimental model for both basic research and medical applications. For example, ntES cells derived from blastocysts cloned from cancer cells have been used to investigate the contribution of epigenetic alterations to cancer development (11). Undoubtedly, many other novel applications will be explored by scientists using cloning and ntES cells (12).

Different protocols have successfully been used for deriving mouse ES cell lines. For improving the efficiency of ES cell line derivation, especially from the mouse strains refractory to the standard ES cell establishment protocols, several strategies have been employed and shown to be very beneficial. These strategies include selective ablation of differentiated cells (13), the use of conditioned media (2, 14), and the application of pharmacological inhibitors and cytokines (15, 16). Different developmental stages of mouse preimplantation embryos have also been used for deriving ES cell lines (17–19). When deriving ES cell lines from blastocysts, both intact blastocysts (20) and inner cell mass (ICM) cells isolated from blastocysts through an immunosurgery procedure are used (2, 16).

This chapter presents a protocol for deriving mouse ES cell lines from intact blastocysts that are produced by either fertilization or cloning. Mouse ES cell lines from commonly used mouse strains, such as 129, B6ABL/C, and B6D2F1, have been efficiently established with this protocol. Cognizant of the needs of researchers who are new to this field, this protocol avoids using any complicated procedures, even though such procedures may increase the efficiency of mouse ES cell line derivation.

2. Materials

2.1. Equipment

A standard laboratory setup for mammalian cell culture is generally sufficient for deriving and culturing mouse ES cell lines. The major equipments needed for such laboratory operations are listed in this Section.

1. Class II biosafety cabinet: All the procedures that require a sterile condition (e.g., preparing media, changing culture medium, and passaging and harvesting cells) should be performed in a class II biosafety cabinet (which provides personnel, product, and environmental protections). Turn on the cabinet about 20–30 min before it is being used to clean the air in the cabinet. Disinfect the cabinet by wiping down all surfaces with 70% ethanol or isopropanol before and after each use. For a cabinet that is equipped with a short-wave UV light (recommended), UV light can be turned on for several minutes to sterilize the surfaces before and after each use of the cabinet. Class II biosafety cabinets manufactured by Labconco or other manufacturers can be used.
2. Clean bench: A clean bench is used for providing a particulate-free environment to host a stereomicroscope for transferring blastocysts onto feeder cells and picking up ES colonies. The purifier horizontal clean bench from Labconco (providing product protection only) can be used when working with nonhazardous materials. When working with hazardous materials (such as ES cells expressing live viruses), the recently designed and manufactured class II biological safety cabinets with the scope-ready option from Labconco (Type A2 Purifier Cell Logic Biological Safety Cabinet) allowing a user-supplied microscope to be integrated into the cabinet should be used.
3. CO_2 incubator: An atmosphere of 5% CO_2 in a CO_2 incubator is required for maintaining the pH of the medium for culturing mouse embryos, ES cells, and fibroblast cells. Whenever a CO_2 incubator is mentioned in this protocol, it is set at 37°C and 5% CO_2 (95% air). Make sure that a pan of water is kept filled at all times to keep the humidity in the incubator. CO_2 incubators can be purchased from Thermo Scientific or other vendors. For researchers interested in growing ES cells in low oxygen conditions, CO_2 incubators with oxygen control should be used.
4. Microscopes: A stereomicroscope (e.g., Nikon, Tokyo), normally hosted in a mouse procedural room where surgical procedures are performed, is needed for the steps of flushing blastocysts out of mouse uterine horns and blastocyst collection. Another stereomicroscope, normally hosted in a cell culture room, is needed for transferring blastocysts onto feeder cells and picking up ES cell colonies. This stereomicroscope should be placed on the clean bench or in the scope-ready biological safety cabinet mentioned above to keep its environment particulate free.
   An inverted phase contrast microscope is used for visualizing ES cells (colonies), mouse embryonic fibroblast (MEF)

cells, and blastocyst outgrowths. An inverted microscope with differential interference contrast (DIC) optics and objectives (e.g., Nikon, Tokyo) can be used. Such a microscope is also used for injecting mouse ES cells into mouse embryos if forming chimeras is used for testing the pluripotency of the derived ES cell lines; in this case, a piezo-driven system (Prime Tech, Ibaraki, Japan) attached to a micromanipulator (e.g., Narishige) is also needed.

5. Electroporator: Electroporator such as BioRad Gene Pulser Xcell Electroporator or BTX ECM-600 can be used for electroporating DNA constructs into mouse ES cells.
6. Cryopreservation equipment: Liquid nitrogen tanks (e.g., MVE Cryosystem 6,000; Select Genetics) can be used for long-term storage of mouse ES cells and MEF cells. For better temperature control and for storing a large number of cell vials, such as in the case of establishing a large ES cell bank, the Cryoplus series storage systems (Thermo Scientific) can be used.
   For cryopreserving ES or MEF cells, a –80°C freezer is needed for slowly freezing cells from room temperature to –80°C overnight before freezing them in liquid nitrogen. Any of the common brands of freezers with good temperature control works well for such purpose. Isopropanol filled high heat capacity freezing containers (Nalgene Labware) are needed to hold the cryovials of ES or MEF cells for freezing them in the –80°C freezer.
7. Irradiator: radiation source such as a Gammacell 40 Exactor (gamma source) from MDS Nordion or RS 2000 (X-ray source) from Rad Source Technologies, Inc. is needed to mitotically arrest MEF cells for making MEF feeder cells.
8. Hemocytometer (Patterson Scientific). It is used to count MEF and ES cells.
9. Cell culture plates: 4-well plates (Nunc), 6-well plates (Corning Incorporated), 12-well plates (Corning Incorporated), 24-well plates (Corning Incorporated), V-bottom 96-well plates (Corning Incorporated).
10. Cell culture flasks: T25 cm^2 flasks (Corning Incorporated), T75 cm^2 (Corning Incorporated); cell culture dishes: 10 cm^2 dishes (Corning Incorporated).

2.2. Culture Media and Other Reagents and Materials

1. MEF medium: HEPES-buffered Dulbecco's modified Eagle's Medium (DMEM, high glucose, Gibco) supplemented with heat-inactivated fetal calf serum (HyClone, final concentration 15%), non-essential amino acids (100× stock from Gibco, final concentration of total amino acid is 0.1 mM), beta-mercaptoethanol (Sigma, final

concentration of 0.1 mM), and penicillin/streptomycin (final concentrations 100 units/mL for penicillin and 100 μg/mL for streptomycin). This medium is also used for culturing mitotically arrested MEF feeder cells.

2. Mouse ES cell medium: MEF medium supplemented with leukemia inhibiting factor (LIF, Chemicon, ESGRO) to a final concentration of 1,000 units/mL (*see* **Note 1**). At the step of mouse ES cell line derivation, PD98059 MEK1 inhibitor (Cell Signaling Technology) is also added to the ES medium (final concentration 5×10^{-5} M). This inhibitor is optional for culturing established ES cells.
3. Cryopreservation medium for mouse ES, primary MEF, and MEF feeder cells: Working medium is MEF medium containing 10% DMSO. A 2× stock medium can be made with MEF medium containing 20% DMSO. Use the 2× stock medium within 2 weeks from its preparation.
4. Medium used for isolation mouse blastocysts: M2 medium (Sigma) supplemented with penicillin/streptomycin (final concentrations 100 units/mL for penicillin and 100 μg/mL for streptomycin).
5. Mitotically arrested MEF feeder cells: Mitomycin treated or gamma- or X-ray-irradiated MEF feeder cells can be purchased from several vendors, such as from The Jackson Laboratory. For the researchers interested in preparing MEF feeder cells from primary MEF cells in house, *see* **Section 3.2** for detailed procedures for making MEF feeder cells. For culturing ES cells under drug selection, MEF feeder cells prepared from the DR-4 mouse strain displaying resistance to all of the four drugs commonly used in mammalian cell culture (G418, 6-thioguanine, puromycin, and hygromycin) should be used. DR-4 MEF (or mice if DR-4 primary MEF cell lines are to be established in house) can be purchased from The Jackson Laboratory.
6. HEPES buffer: 20 mM HEPES buffer (diluted from Invitrogen's 1 M HEPES buffer solution, with water) is used for rinsing away culture medium from cultured fibroblasts and ES cells before trypsinization.
7. Ca/Mg-free PBS (Dulbecco's phosphate-buffered saline (D-PBS); Gibco) can also be used (as the equivalent of HEPES buffer) for rinsing away culture medium from cultured fibroblasts and ES cells before trypsinization.
8. Trypsin/EDTA solution: 0.05% trypsin with 0.02% EDTA 4Na (Invitrogen) is used to trypsinize cultured mouse fibroblast and ES cells.
9. Acid Tyrode's solution (Sigma): used for dissolving zona pellucida of mouse blastocysts.

10. Gelatin solution: 0.2% gelatin solution is used to treat cell culture wares used for ES cell culture. This solution is made by dissolving 1 g gelatin (Type A from porcine skin from Sigma) in 500 mL water. Autoclave the solution for sterilization. Store the gelatin solution at 4°C or at room temperature (up to 2–3 months).
11. Mitomycin C (Sigma): *see* **Section 3.2.2** for the procedures of preparing mitomycin C solutions.

3. Methods

3.1. Culturing Primary MEF Cells

3.1.1. Reviving Primary MEF Cells from Frozen Stocks

1. Retrieve frozen MEF cells in a cryovial from the liquid nitrogen tank where the cells are stored (or from dry ice if cells are just received from a vendor in dry ice).
2. Thaw the cells rapidly in a 37°C water bath (proceed to step 3 as soon as the medium in the vial is melted; leaving cells in water bath too long will compromise cell viability).
3. Transfer the content from the cryovial to a 15 mL sterile conical tube containing 10 mL pre-warmed (at room temperature) MEF medium.
4. Gently mix the cells with the medium by pipetting the cells a few times.
5. Centrifuge the tube at 1,200 rpm for 5 min in a bench-top centrifuge (at 4°C or room temperature) to pellet the cells.
6. Aspirate away supernatant, resuspend the cells in 5 mL MEF medium by pipetting, and transfer the content to a T25 (for 1–2 $\times$ 10^6 cells) tissue culture flask (*see* **Note 2**).
7. Place the flask into a CO_2 incubator with a setting of 5% CO_2 and 37°C to allow MEF cells attach to the flask and grow.
8. On the next day, change medium by aspirating away the medium and refilling the flask with 5 mL pre-warmed fresh MEF medium.
9. Change medium every 2–3 days until the cells reach 95–100% confluency.

3.1.2. Passaging Primary MEF Cells

1. Aspirate away the medium from the tissue culture flask where cells have reached 95–100% confluency.
2. Rinse the adherent MEF cells with 5 mL of HEPES buffer or Ca/Mg-free PBS buffer (for a T25 flask; if cells are cultured in different sizes of cell culture wares, the volumes of washing buffer should be adjusted accordingly—use 0.2–0.5 mL

of buffer per 1 cm^2 of the bottom surface area of the cell culture ware) to remove any dead cells and traces of serum.

3. Add 2 mL of 0.05% trypsin/0.02% EDTA to the flask and tilt the flask until the solution completely covers the cell layer.
4. Return the flask to the CO_2 incubator for about 5 min. Monitor cells being lifted off from the flask under an inverted microscope every 1–2 min.
5. Once most cells are loosened from the flask, add 5 mL MEF medium to the flask and immediately rinse the cells off from the flask. Collect the cells into a 15 mL conical tube and pipette up and down a few times to create a single-cell suspension.
6. Centrifuge the tube at 1,200 rpm for 5 min in a bench-top centrifuge to pellet the cells. Remove the supernatant and resuspend the cell pellet in 10 mL pre-warmed MEF medium.
7. To further expand the cells, split the cells into new cell culture wares at a 1:3 ratio (e.g., divide equally into three T25 or transfer to a T75 flask). Add proper amount of MEF medium according to the size of the cell culture ware used (use 0.2 mL medium for each square centimeter of the bottom surface of the culture ware. e.g., 5 mL for a T25 flask and 15 mL for a T75 flask), and culture the cells under 5% CO_2 at 37°C in the CO_2 incubator until they reach 95–100% confluency (medium is changed every 2–3 days if extended culture is needed).
8. Repeat steps 1–7 until enough MEF cells can be harvested (*see* **Note 3**).

3.1.3. Freezing Primary MEF Cells

1. At step 6 in **Section 3.1.2** above, count the cells with a hemocytometer and record the cell number.
2. Adjust the cell suspension with MEF medium to make a cell concentration of 1–5 × 10^6 cells/mL (if cells are too diluted, pellet the cells again by centrifugation, remove the supernatant, and resuspend the cells with MEF medium at the desired concentration, *see* **Note 4**).
3. Add equal volume of 2× freezing medium to the cell suspension and aliquot cells into cryovials (1 mL per vial).
4. Fill the high heat capacity freezing containers in full with 200 mL room temperature isopropanol and place the cryovials containing the cells in the container.
5. Place the container into a –80°C freezer to slowly freeze the cells (at about 1°C per min; it takes about 4–6 h for the container to reach –80°C).

6. After 4–6 h or overnight, move the cryovials from the container into standard square boxes (perform this step on dry ice) and immediately place the boxes into a liquid nitrogen storage tank for long-term storage.

3.2. Making MEF Feeder Cells for ES Cell Line Derivation and ES Cell Culture

Primary MEF can be purchased from several vendors, such as from The Jackson Laboratory (www.jax.org). If researchers want to establish primary MEF from mouse embryos, follow the protocol described in Chapter 22, this volume. Once MEF cells are available, there are two ways to make MEF feeder cells: one is with irradiation treatment and another with mitomycin treatment.

3.2.1. Making MEF Feeder Cells with Gamma- or X-Ray-Irradiation Treatment

To mitotically inactivate MEF cells, 3,000 rads (30 Gy) of irradiation are required. When gamma irradiation is used, calculate the time required for irradiating the cells based on the dose rate of the radiation source in the researcher's facility (dose rate may vary from radiation source to radiation source); when X-ray irradiation is used, follow the manufacturer's instructions to calculate the radiation time required to expose cells to 3,000 rads. If this is the first time that the researcher uses the irradiator, training is required for its proper handling.

1. Harvest MEF cells from a culture that has reached 95–100% confluency by trypsinization as described above.
2. Transfer MEF cells into a 15 or 50 mL conical tube, pellet cells by centrifuging the tube at 1,200 rpm for 5 min in a bench-top centrifuge.
3. Remove the supernatant and resuspend the cell pellet into MEF medium (cell concentration can be in the range of $1–5 \times 10^7$ cells/mL).
4. Close the conical tube with a cap and place it on ice in a small container (such as a plastic beaker) that can fit into the irradiator. Carry the cells on ice in the small container to the room where the irradiator is located.
5. Expose cells to 3,000 rads of gamma or X-ray radiation.
6. Return the cells to the cell culture hood and adjust the volume of cell suspension to a concentration of 6×10^6 cells/mL with MEF medium.
7. Add equal volumes of 2× cryopreservation medium to the cell suspension (final DMSO concentration is 10%) and gently mix the solution. The final cell concentration should be 3×10^6 cells/mL (*see* **Note 5**).
8. Aliquot the mitotically inactivate MEF feeder cells into cryovials (1 mL per vial) and freeze them in the same way as described when freezing primary MEF cells for long-term storage.

3.2.2. Making MEF Feeder Cells with Mitomycin C Treatment

1. Prepare a 1 mg/mL mitomycin C stock solution by dissolving 2 mg mitomycin C in the manufacturer's vial with 2 mL sterile PBS (since mitomycin C is toxic, perform this procedure in a fume hood or in a well-ventilated place). Wrap the vial with aluminum foil to prevent it from light and store the stock at 4°C (for up to 2 weeks). Prior to use on the same day, dilute the stock into MEF medium to a final concentration of 10 μg/mL.
2. Aspirate away the medium from a 95–100% confluent MEF cell culture and replace it with the same volume of MEF medium containing 10 μg/mL mitomycin C. Incubate cells for 2–3 h in the CO_2 incubator.
3. Aspirate away the medium and wash the cell layer with HEPES buffer or Ca/Mg-free PBS three times to thoroughly remove mitomycin C.
4. Trypsinize the mitotically inactivated MEF cells as described in **Section 3.1.2** and resuspend the cells in MEF medium at a concentration of 6×10^6 cells/mL.
5. Follow steps 7–8 in **Section 3.2.1** to freeze down the mitomycin C-treated MEF feeder cells.

3.3. Preparing MEF Feeder Cell Culture Wares for ES Cell Line Derivation and ES Cell Culture

All of the cell culture wares used for deriving ES cell lines and culturing ES cells need to be gelatinized with 0.2% gelatin solution.

3.3.1. Gelatinizing Cell Culture Wares

1. Add enough volume of 0.2% gelatin solution to cell culture wares to cover the whole surface area.
2. Incubate the cell culture wares with gelatin solution for at least 5 min at room temperature (*see* **Note 6**).
3. Completely remove the gelatin by aspiration and the cell culture ware is ready to use.
4. For the gelatin-treated cell culture wares that are not used immediately following treatment, keep them sealed at room temperature in the plastic bags in which they were packaged by the manufacturer. They can be used within a month.

3.3.2. Seeding MEF Feeder Cells into Culture Wares

1. Follow the same steps as detailed in **Section 3.1.1** to revive frozen MEF feeder cells.
2. Seed MEF feeder cells at a density of about 5×10^4 per cm^2 with 0.5 mL MEF medium into gelatin-coated cell culture wares.
3. Culture the MEF feeder cells under 5% CO_2 at 37°C in the CO_2 incubator for several hours or overnight before use (*see* **Note 7**).

3.4. Deriving Mouse ES Cell Lines From Blastocysts

This section describes the procedures for obtaining mouse blastocysts produced from natural mating. For researchers interested in deriving mouse ES cell lines from cloned mouse blastocysts, follow the mouse cloning procedures described in Chapter 22, this volume, to clone blastocysts.

3.4.1. Flushing 3.5 Days Post-coitum Blastocysts from Females

1. At 3.5 days post-coitum (dpc), sacrifice the female mice that were successfully mated by males (follow the approved IACUC protocols in your institution for proper handling of mice).
2. Cut the uterine horns from the mice and collect them into a 35 mm petri dish containing 2 mL M2 medium (prewarmed at room temperature).
3. Hold the upper end of a uterine horn with a pair of forceps and flush the blastocysts out of it with M2 medium by using a 32-gauge hypodermic needle (blunted by cutting off the tip with a metal cutter) connected to a 5 mL syringe.
4. Repeat step 3 until all of the uterine horns have been flushed.

3.4.2. Culturing Mouse Blastocysts on MEF Feeder Cells to Produce ICM Outgrowths

1. One day before blastocyst isolation, prepare 4-well MEF feeder plates as described in **Section 3.3.2** (*see* **Note 8**).
2. In the morning before sacrificing female mice for blastocysts isolation, replace the MEF medium with ES medium containing PD98059 inhibitor in the 4-well MEF feeder plates. Return the 4-well plates back to the CO_2 incubator until use.
3. Under a stereomicroscope hosted in a clean bench, transfer the isolated blastocysts (**Section 3.4.1**) into a 35 mm dish containing 0.5 mL acid Tyrode's solution to dissolve the zona pellucida. It takes about 1–2 min to dissolve the zona pellucida (*see* **Note 9**).
4. Immediately collect the zona pellucida-free blastocysts and wash them in 2 mL ES medium in a 35 mm dish.
5. Transfer the zona-free blastocysts individually into each well of the 4-well MEF feeder cell plates prepared at step 1 above.
6. Return the plates to a CO_2 incubator. Don't disturb the plates for a minimum of 24 h to let the blastocysts attach to the feeder layer (*see* **Note 10**).
7. Once the blastocysts have attached to the feeder layer, monitor ICM outgrowth daily under an inverted microscope. Prepare more 4-well MEF feeder plates (**Section 3.3.2**) one or a few days before performing step 8 below.
8. When the ICM outgrowths are about three to four times the size of a blastocyst (*see* **Note 11**), aspirate away the ES

medium and wash the ICM outgrowths once with Ca/Mg-free PBS or HEPES buffer.

9. Add 2–3 drops of 0.05% trypsin/0.02% EDTA solution to cover the ICM outgrowth in each well. Return the plates back into the CO_2 incubator for about 2 min.
10. Add 500 μL of ES medium plus PD98059 inhibitor to each well and immediately pipette the cells up and down about 10 times with a P200 pipette (*see* **Note 12**).
11. Transfer the entire cell suspension from each well into a well of a new 4-well MEF feeder plate (prepared ahead of time at step 7) in which the MEF medium from each well has been replaced by ES medium plus PD98059 inhibitor a few hours or 1 day earlier. The final volume of ES medium in each well should be about 1 mL.
12. After culturing the cells overnight, replace medium with pre-warmed fresh ES medium plus PD98059 inhibitor. ES colonies will appear 2–3 days later (*see* **Note 13**).
13. Replace medium with pre-warmed fresh ES medium plus PD98059 inhibitor once more and culture the ES colonies for another day for them to be large enough (an appropriate colony diameter is about 5–10 times the diameter of a P200 pipette tip at its narrow end) to be passaged into 6-well MEF feeder plates for further expansion. Prepare 6-well MEF feeder plates as described in **Section 3.3.2** on this day (they will be needed at step 16 below).
14. Aspirate away culture medium and wash once the wells with HEPES buffer or Ca/Mg-free PBS; trypsinize the colonies along with the feeder cells in the same way as trypsinizing MEF cells (**Section 3.1.2**) and transfer the cells from each well into a 15 mL conical tube (one well per tube).
15. Pellet ES cells by centrifuging them at 1,200 rpm for 5 min in a bench-top centrifuge.
16. Resuspend the ES cell pellet in 3 mL ES medium (with or without PD 98059 inhibitor, *see* **Note 14**) and transfer the cells from each tube into a well of 6-well plate containing MEF feeder cells.
17. Add 3 mL ES medium and return the plates back to the CO_2 incubator. ES colonies (the number of ES colonies could range from 0 to a few dozen) should emerge 24 h later.
18. Culture the ES colonies for 1–2 more days until they reach about 70% confluency (change medium if its color becomes yellowish). ES colonies are ready to be trypsinized for further passaging (**Section 3.5**), to be frozen down for long-term storage (**Section 3.6**), or for other applications. If for

any reason the researcher wants to pick up ES colonies individually, follow **Section 3.7.2**. The ES cell lines at this step are defined as passage 0 (P0, *see* **Note 15**).

3.5. Culturing and Passaging Mouse ES Cells

1. Prepare feeder cell culture wares for ES cell culture by following the procedures described in **Section 3.3.2**.
2. Revive a vial of ES cell stock in the same way as reviving MEF cells (**Section 3.1.1**) and plate them onto a T25 flask containing feeder cells (*see* **Note 16**).
3. Change ES medium every day. ES cells will be ready for passaging within 2–3 days (at a confluency of about 70%).
4. Aspirate away ES medium and wash the ES cell colonies with HEPES buffer or Ca/Mg-free PBS once. At this step, also prepare more feeder cell culture wares, such as T25 flasks, to be used in step 9 below.
5. Add 1 mL 0.05% trypsin/0.02% EDTA, tilt the flask to allow the trypsin solution to cover the colonies, and incubate the flask at 37°C in the CO_2 incubator for 5 min.
6. Add 5 mL ES medium and pipette the cells up and down several times with a P1000 pipette to disaggregate the ES colonies.
7. Transfer ES cells into a 15 mL conical tube and pipette ES cells up and down a few more times to make sure cells are in single-cell suspension (*see* **Note 17**). Count the ES cells if needed (*see* **Note 18**).
8. Pellet ES cells by centrifugation at 1,200 rpm for 5 min in a bench-top centrifuge.
9. Resuspend ES cells into single-cell suspension in 5 mL ES medium by gentle pipetting. ES cells are then split into three to five T25 flasks (prepared with MEF feeder cells at step 4 of this Section) for further expansion (*see* **Note 19**).
10. Repeat steps 3–9 above if more rounds of cell passaging are needed; otherwise, proceed to **Section 3.7** to freeze ES cells for long-term storage or use the ES cells for any application.

3.6. Freezing ES Cells

1. Aspirate away ES culture medium from a 70% confluent ES cell culture and wash once the ES colonies with HEPES buffer or Ca/Mg-free PBS.
2. Trypsinize the ES colonies along with the feeder as described in **Section 3.5**, steps 5 and 6 above. Count ES cells and adjust the cell concentration to 2×10^6/mL (or any other concentration the researcher prefers, *see* **Note 20** in step 3 below) with ES medium.

3. Add an equal volume of 2× ES freezing medium to the ES cells and gently but thoroughly mix the content using a Pipetman. Dispense cells as 1 mL aliquots into cryovials (1×10^6 ES cells per vial, *see* **Note 20**) and freeze them in the same way as freezing MEF cells for long-term storage (**Section 3.1.3**).

3.7. Electroporation of ES Cells with DNA Constructs and Establishment of Transgenic ES Cell Lines

Since one of the most common applications of mouse ES cells is to perform genetic modifications by introducing foreign DNAs into ES cells, for the convenience of the readers detailed procedures for introducing foreign DNA constructs into ES cells through electroporation and for establishing transgenic ES cell lines are described in this Section (*see* **Note 21**).

3.7.1. Electroporation of ES Cells with DNA Constructs

1. Grow ES cells on MEF feeder cells in a T25 flask until they reach about 70% confluency (*see* **Note 22**).
2. Prepare DR4 MEF feeder cell culture wares as described in **Section 3.3.2**. Use 100 mm petri dishes or 6-well plates to prepare the MEF feeder cells (*see* **Note 23**).
3. Trypsinize and collect ES cells from the culture flask into a 15 mL conical tube as described in **Section 3.5**, steps 4–8.
4. Wash the ES cells in the tube twice with 10 mL Ca/Mg-free PBS.
5. Pellet again ES cells by centrifugation and resuspend the cell pellet gently but thoroughly (to achieve single-cell suspension) in a volume of Ca/Mg-free ice-cold PBS to achieve a final concentration of ES cells in the range of 2×10^6 to 2×10^7 per mL (a 70% confluent ES cell culture in a T25 flask normally yields 5×10^6 to 2×10^7 ES cells). Keep the cells on ice.
6. For each electroporation, mix 20–50 μg (about 1 μg/μL) plasmid DNA (for the sizes of plasmid in the range of 3–10 kb) with 0.8 mL of the ES cell suspension in an electroporation cuvette (e.g., 0.4 cm electrode gap cuvette from BioRad).
7. Select the parameters for electroporation. If BioRad Gene Pulser Xcell Electroporator is used, a setting of 400 V, 25 μF, and 0.2 ms can be used. Place the cuvette into the electroporation chamber and zap the cuvette.
8. Let cells sit on ice for 20 min in the cuvette and then transfer them to 5 mL ES medium in a 15 mL conical tube. Fully mix the ES cells with the medium.
9. Split the electroporated ES cell suspension into fresh MEF feeder cell dishes/plates for further cultivation (*see* **Note 24**).

10. Based on the size of culture wares used, add proper amount of ES medium (0.2 mL/cm^2 of the bottom surface of the cell culture ware) and culture the ES cells in the CO_2 incubator at 37°C.
11. Twenty-four hours later, aspirate away the culture medium from the culture and replace it with selection ES medium (ES medium containing the proper selection drugs) (*see* **Note 25**).
12. Change medium (with selection ES medium) every 1 or 2 days until drug-resistant ES colonies reach a diameter that is about 5–10 times the diameter of a P200 pipette tip at its narrow end. ES colonies are ready to be picked up.

3.7.2. Picking up ES Colonies and Establishing Transgenic ES Cell Lines (Subclones)

1. Prepare 12- or 24-well plates with DR4 MEF feeder cells as described in **Section 3.3.2** (*see* **Note 26**).
2. Prepare a V-bottom shape 96-well plate for collecting ES colonies by filling each of its wells with 30 μL HEPES buffer with a multichannel pipettor.
3. Wash the dishes/plates containing the ES colonies that are to be picked up with HEPES buffer once.
4. Add a thin layer of HEPES buffer after the wash to just barely cover the ES colonies.
5. Under a stereomicroscope on a clean bench, use a P200 μL pipette tip connected to a P200 Pipetman to gently scrape around a colony and make a cut on the MEF feeder cell layer surrounding the colony.
6. With the same pipette tip, immediately pick up the colony by pipetting it up along with some HEPES buffer (the P200 Pipetman can be set at 50–100 μL). Transfer the content into a well of the V-bottom shape 96-well plate prepared at step 2 above (*see* **Note 27**).
7. After the desired number of ES colonies is picked up, add 30 μL of 0.05% trypsin/0.02% EDTA solution with a multichannel pipettor to each of the wells containing ES colonies in the 96-well plate.
8. Incubate the 96-well plate in a CO_2 incubator at 37°C for about 5 min.
9. Add 100 μL ES medium to each well with a multichannel pipettor and pipette up and down the colonies a few times to disaggregate colonies into a single-cell suspension.
10. Transfer 75 μL of the content from each well of the 96-well plate into a well of the 12- or 24-well plates containing DR4 MEF feeder cells prepared at step 1 above. The ES cells left in the 96-well plate can be directly used for genomic DNA isolation for genotyping or frozen at –80°C

(in this latter case, cover the plate tightly with a piece of parafilm) to be genotyped later.

11. Culture the ES cells in the 12- or 24-well plate until they reach about 70% confluency (*see* **Note 28**).
12. Trypsinize ES cells as described in **Section 3.5** (steps 3–9) and further expand them by culturing them in larger MEF feeder cell culture wares such as in T25 flasks.
13. When ES cells reach about 70% confluency, cryopreserve ES cells from each flask as described in **Section 3.6** (*see* **Note 29**). Alternatively, ES cells can be immediately used for other applications or further expanded by splitting onto new MEF feeder cell culture wares.

3.8. Characterizing Mouse ES Cells

Established ES cell lines should be fully characterized before being used in any application. Although there are many ways to characterize ES cells both in vitro and in vivo, this author prefers using only two methods: karyotyping (for euploidy) and making mouse chimeras (for pluripotency). Once an ES cell line is established, karyotyping should be performed to make sure it is euploid. Such assays should also be performed periodically after several rounds of passaging have been performed on an ES cell line. As the procedures involved in ES cell karyotyping are simple and quick, researchers working with ES cells should establish such an assay in their laboratory. The protocols described by Nagy et al. (21) are commonly used for karyotyping ES cells.

The true test for pluripotency (epigenetic integrity) of mouse ES cells is by demonstrating their ability to contribute to the germline of chimeric mice after being injected or aggregated with mouse preimplantation stage embryos or, alternatively, by producing ES mice through ES-tetraploid embryo complementation (22). The procedures involved in such assays are beyond the scope of this chapter. Readers interested in such procedures can find an excellent reference in Nagy et al. (21) or at the Website of the Nagy lab (http://www.mshri.on.ca/nagy/default.htm). Alternatively, such tests can be conducted through commercial services provided by many contract research organizations.

4. Notes

1. Other types of DMEM media with high glucose after supplementation with LIF are also suitable for culturing mouse ES cells. HEPES-buffered media maintains the pH better when cell cultures are being handled outside of CO_2 incubator, such as when picking up ES colonies.

2. Choose the proper size of culture flask to seed MEF cells according to the number of MEF cells stored in the cryovial. MEF cells generally grow well in culture when seeded at a density of approximately 5–10 $\times$ 10^4 cells/cm^2.
3. When such MEF cells are used for making MEF feeder cells, a passage number of not more than five is recommended. Cellular activity from highly passaged cells may be compromised.
4. Other concentrations of cells can also be made depending on the future applications of the MEF cells by the researcher. As MEF cells are normally seeded at a density of around 5–10 $\times$ 10^4 cells/cm^2 (*see* **Note 2**), 1–5 $\times$ 10^6 MEF cells from a cryovial can be conveniently cultured in T25 or T75 flasks with minimal adjustment of cell numbers.
5. Similar to when freezing primary MEF cells, other concentrations of MEF feeder cells can also be made for cryopreservation. 3 $\times$ 10^6 cells/mL MEF feeder cells in 1 mL/cryovial aliquots provide a convenient number of MEF feeder cells for their applications. For example, since 5 $\times$ 10^4 cells/cm^2 of MEF feeder cells should be used for ES cell line establishment and ES cell culture, 3 $\times$ 10^6 cells from one cryovial are good for one 6-well plate (the surface area for each well of the 6-well plate is 9.6 cm^2), for 10 4-well plates (the surface area for each well of the 4-well plate is 1.5 cm^2), or for two to three T25 flasks (surface area is 25 cm^2); these cell culture wares are the most commonly used ones for culturing mouse ES cells.
6. Some researchers prefer to treat the cell culture ware with gelatin solution for minimum an hour or more before use, but this author does not find this necessary.
7. MEF feeder cells prepared in the cell culture wares can be used within a week if kept in a CO_2 incubator at 37°C.
8. For the steps of deriving mouse ES cell lines, 4-well plates such as the Nunc brand ones (Cat# 176740) are used to culture single mouse blastocysts. The volume of ES medium (0.5–1 mL) in a well of the 4-well plates provides a suitable cell culture condition (the suitable strength of the autocrine and paracrine signals from the blastocysts and/or feeder cells) for ICM outgrowth when a single mouse blastocyst is plated in it.
9. This process should be closely monitored, since overexposing blastocysts to acid Tyrode's solution may reduce cell viability. Do not wait for the zona pellucida to be completely dissolved by digestion with acid Tyrode's solution. Instead, when the zona pellucida becomes a thin layer

during digestion, use a mouth-controlled pipette to aspirate in and out the blastocysts a few times to break the blastocysts free from the zona pellucida. Since zona pellucida-free blastocysts tend to stick to transfer pipettes, rinse the transfer pipette with ES medium a few times before manipulating the blastocysts.

10. It normally takes about 24–48 h for fertilization-derived blastocysts to attach to the feeder layer, but this may take longer (up to 3–4 days) for cloned blastocysts.
11. For fertilization-derived blastocysts, it normally takes 2–3 days after the blastocysts attach to the feeder layer for the ICM outgrowths to reach the adequate size. A cloned blastocyst tends to take longer time (1–2 more days) to give rise to the adequate size of ICM outgrowth, possibly due to inefficient epigenetic reprogramming or the relatively smaller number of ICM cells in a cloned blastocyst. Therefore, an extra day(s) should be given to cloned blastocysts before disassociation for ES cell line derivation. Add more ES medium to the wells if medium loss occurs from evaporation. The percentage of blastocysts that eventually form ICM outgrowths may vary depending on the mouse strain used and whether the embryos are fertilization derived or cloned (normally 20–100% for fertilization-derived blastocysts and 0–10% for cloned blastocysts).
12. The ICM outgrowth should be disassociated to such extent that while most cells become single-cell suspension, other cells still exist as small cell clumps. Excessive or inadequate disassociation of the ICM outgrowths reduces the chance of deriving ES cell lines from the outgrowths.
13. The efficiency of ICM outgrowths giving rise to ES colonies may vary. The major factors affecting the success of deriving ES colonies at this step include the strains of mouse used, how the blastocysts are produced (fertilization or cloning), and the experience of the researcher. For fertilization-produced mouse blastocysts in the 129 strain background (one of the most efficient mouse strains for deriving ES cells), an efficiency of 80–100% can be expected for an experienced researcher.
14. The MEK1 inhibitor PD98059 is only needed for the steps of culturing mouse blastocysts to produce ICM outgrowths and for producing ES colonies from the outgrowths. Once ES colonies are established, PD98059 is optional for the subsequent culture of ES cells.
15. Upon reaching this step, stable ES cell lines have been successfully derived from blastocysts. ES cells at this stage are ready to be used for different downstream applications,

such as being electroporated with DNA for gene targeting or injected into mouse blastocysts for making chimeric mice. However, in order to make a large stock of ES cell lines, ES cells from each line at this stage are normally further expanded by being passaged one to three more times. Freeze a few vials of ES cells at each passage so that an ES cell stock from each of these early passages will be available for future applications. As extensively passaged ES cells may become aneuploid or gain epigenetic errors (such as loss of genomic imprinting), ES stocks from early passages provide a researcher with a supply of high-quality ES cells.

16. Normally, established mouse ES cells are cultured in T25 flasks, 100 mm dishes or 6-well plates, as 70–90% confluent ES cells from one of such size cell culture wares provides sufficient ES cells for most of the common downstream applications. However, any other size of cell culture wares can also be used depending on the number of ES cells needed for the application. It is recommended to seed ES cells at a density of about 5×10^4 cells/cm^2, i.e., the same density as seeding MEF feeder cells; although ES cells can be seeded at several fold different densities from this density, one should avoid seeding ES cells too dense or too diluted as this can cause differentiation. For different ES cell lines, tests need to be conducted for finding the optimal density of ES cells to be seeded.
17. To ensure that each ES colony to be formed is derived from a single ES cell, single-cell suspension needs to be achieved with this step.
18. As ES cells tend to overwhelmingly outnumber the feeder cells in a 70% confluent ES cell culture, no distinction needs to be made between ES cells and feeder cells when counting ES cells.
19. When passaging mouse ES cell lines, single-cell suspension of ES cells harvested from a roughly 70% confluent ES cell culture should be reseeded onto new cell culture wares containing MEF feeder cells at a three- to fivefold dilution, i.e., ES cells harvested from 1 cm^2 bottom surface area of a cell culture ware should be reseeded onto 3–5 cm^2 bottom surface area of a new cell culture ware. If not all of the ES cells harvested from a culture are needed to be further passaged, reseed only 1/3 or 1/5 of the ES cells into a new culture ware with the same bottom surface area as the one from which the ES cells were grown. The rest of the ES cells can be discarded, frozen down, or used for other applications.
20. Cryopreservation at 1×10^6 cells per vial (1×10^6 cells/mL) provides a convenient cell number for

subsequent ES cell culture. As explained above (*see* **Note 16**), ES cells from one of such cryovial can be directly cultured in a T25 flask or in 2 wells of a 6-well plate without adjusting cell numbers.

21. While electroporation is one of the most commonly used methods to introduce foreign DNA constructs into ES cells, other methods such as lipofection can also be used. Plasmid DNA constructs used for electroporation need to be linearized (if permanent integration of the DNA into the ES genome is intended) and purified with phenol extraction and ethanol precipitation (the routine protocols used in standard molecular laboratories for DNA digestion and purification work well for this purpose).
22. Depending on the numbers of ES cells to be used for downstream applications, different sizes and numbers of cell culture wares can be used.
23. These MEF feeder cell culture wares will be used for culturing ES cells after electroporation. Since ES colonies from transfected ES cells will be picked up with a pipette, transfected ES cells need to be cultured in petri dishes or cell culture plates (where the cover of cell culture wares can be lifted for picking up colonies) but not in flasks (where colonies will be unreachable by a pipette). 100 mm petri dishes or 6-well plates are commonly used for such purposes. If drug selection is used for selecting transfected ES cell colonies, DR4 MEF feeder cells should be used. Such MEF feeder cell culture wares can be prepared on the same day or a few days earlier (not more than a week) before electroporating ES cells.
24. ES cells should be seeded at a density at which the to-be-formed ES colonies are well-separated so that they can be individually picked up. As a rule of thumb, from a 70% confluent ES cell culture used for electroporation split it at 1:6–1:8 ratio after electroporation for future cultivation. Depending on the efficiency of permanent integration of the DNA constructs into the ES cell genome, the number of drug-resistant ES cell colonies formed can vary. It will be helpful if the electroporated ES cells are seeded at three to five different densities so that at least some of the cultures will form well-separated ES colonies.
25. For the proper concentration of the selection drugs, consult the product specification sheets from the manufacturers. Drug-resistant genes under the control of different promoters may require different concentrations of drugs. If necessary, trials should be conducted to find the optimal concentration of drugs to be used.

26. 12- or 24-well plates are normally used to culture ES cells derived from single colonies. This is because when an ES cell colony is ready to be picked up it contains roughly 0.1×10^6 ES cells and the surface area of a well from either 12- or 24-well plates works best for growing this number of ES cells.
27. Since not all of the ES colonies are correctly targeted or targeted in the same way by the transfected DNA construct, pipette tips should be change after each colony pick up to avoid ES cells from one colony to be carried over to another. For the same reason, avoid picking ES colonies that grow very close to each other. In the case that, due to the breakdown of some ES colonies during the picking up process, some ES cells are floating in the HEPES buffer, gently wash such loose ES cells away by adding 5 mL of HEPES buffer to the dish/plate and subsequently aspirating it away. Add a new thin layer of HEPES buffer to cover the colonies and resume the colony pick up process.
28. It is normal if ES cells from some of the ES colonies do not survive after transferring to 12- or 24-well plate, possibly due to spontaneous differentiation or cellular damage incurred during colony pick up and disaggregation.
29. ES cells derived from individual ES colonies are normally called subclones of the original ES cell line that is used for gene targeting. An ID should be given to each of the subclones corresponding to the wells of the 96-well plate where a portion of each of the ES colonies is kept, so that genotyping results from the ES cells in the 96-well plate can be correctly assigned to each of the ES subclones. The subclones at this stage are considered as passage 0 (P0) post-gene targeting. Researchers should design easy interpreting systems to label their ES cell lines. For example, if an ES cell line with a name of Beatles1 at P6 is used for gene targeting, a subclone #5 derived from this line can be called Beatles1P6#5(P0). The P0 in parenthesis refers to the passage number of the subclone to distinguish the passage number of the parental ES cell line. If the subclone is further passaged, use P1, P2, etc., in the parenthesis to record the passaging numbers.

Acknowledgments

This work is supported by Hematech, Inc. The author thanks Angie Edwards for proofreading the manuscript.

References

1. Evans, M. J. and Kaufman, M. H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.
2. Martin, G. R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638.
3. Mak, T. W. (2007) Gene targeting in embryonic stem cells scores a knockout in Stockholm. *Cell* **131**, 1027–1031.
4. Smithies, O. (1993) Animal models of human genetic diseases. *Trends Genet* **9**, 112–116.
5. Niwa, H. (2007) How is pluripotency determined and maintained?. *Development* **134**, 635–646.
6. Li, P., Tong, C., Mehrian-Shai, R., Jia, L., Wu, N., Yan, Y., Maxson, R. E., Schulze, E. N., Song, H., Hsieh, C. L., Pera, M. F., and Ying, Q. L. (2008) Germline competent embryonic stem cells derived from rat blastocysts. *Cell* **135**, 1299–1310.
7. Mitalipov, S., Kuo, H. C., Byrne, J., Clepper, L., Meisner, L., Johnson, J., Zeier, R., and Wolf, D. (2006) Isolation and characterization of novel rhesus monkey embryonic stem cell lines. *Stem Cells* **24**, 2177–2186.
8. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147.
9. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872.
10. Wakayama, T., Tabar, V., Rodriguez, I., Perry, A. C., Studer, L., and Mombaerts, P. (2001) Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. *Science* **292**, 740–743.
11. Hochedlinger, K., Blelloch, R., Brennan, C., Yamada, Y., Kim, M., Chin, L., and Jaenisch, R. (2004) Reprogramming of a melanoma genome by nuclear transplantation. *Genes Dev.* **18**, 1875–1885.
12. Wang, Z., Meissner, A., and Jaenisch, R. (2004) Nuclear cloning and epigenetic reprogramming. In *Handbook of Stem Cells*, Lanza, R. et al., Eds. Elsevier Academic Press, London, vol. 1, pp. 119–127.
13. McWhir, J., Schnieke, A. E., Ansell, R., Wallace, H., Colman, A., Scott, A. R., and Kind, A. J. (1996) Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a nonpermissive genetic background. *Nat. Genet.* **14**, 223–226.
14. Schoonjans, L., Kreemers, V., Danloy, S., Moreadith, R. W., Laroche, Y., and Collen, D. (2003) Improved generation of germline-competent embryonic stem cell lines from inbred mouse strains. *Stem Cells* **21**, 90–97.
15. Ying, Q. L., Nichols, J., Chambers, I., and Smith, A. (2003) BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* **115**, 281–292.
16. Nichols, J., Silva, J., Roode, M., and Smith, A. (2009) Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development* **136**, 3215–3222.
17. Tesar, P. J. (2005) Derivation of germ-line-competent embryonic stem cell lines from preblastocyst mouse embryos. *Proc. Natl. Acad. Sci. USA* **102**, 8239–8244.
18. Wakayama, S., Hikichi, T., Suetsugu, R., Sakaide, Y., Bui, H. T., Mizutani, E., and Wakayama, T. (2007) Efficient establishment of mouse embryonic stem cell lines from single blastomeres and polar bodies. *Stem Cells* **25**, 986–993.
19. Chung, Y., Klimanskaya, I., Becker, S., Marh, J., Lu, S. J., Johnson, J., Meisner, L., and Lanza, R. (2006) Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. *Nature* **439**, 216–219.
20. Axelrod, H. R. (1984) Embryonic stem cell lines derived from blastocysts by a simplified technique. *Dev. Biol.* **101**, 225–228.
21. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003) *Manipulation of Mouse Embryo: A Laboratory Manual*, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
22. Wang, Z. and Jaenisch, R. (2004) At most three ES cells contribute to the somatic lineages of chimeric mice and of mice produced by ES-tetraploid complementation. *Dev. Biol.* **275**, 192–201.

Chapter 22

Cloning Mice and ES Cells by Nuclear Transfer from Somatic Stem Cells and Fully Differentiated Cells

Zhongde Wang

Abstract

Cloning animals by nuclear transfer (NT) has been successful in several mammalian species. In addition to cloning live animals (reproductive cloning), this technique has also been used in several species to establish cloned embryonic stem (ntES) cell lines from somatic cells. It is the latter application of this technique that has been heralded as being the potential means to produce isogenic embryonic stem cells from patients for cell therapy (therapeutic cloning). These two types of cloning differ only in the steps after cloned embryos are produced: for reproductive cloning the cloned embryos are transferred to surrogate mothers to allow them to develop to full term and for therapeutic cloning the cloned embryos are used to derive ntES cells. In this chapter, a detailed NT protocol in mouse by using somatic stem cells (neuron and skin stem cells) and fully differentiated somatic cells (cumulus cells and fibroblast cells) as nuclear donors is described.

Key words: Nuclear transfer, animal cloning, epigenetic reprogramming, ES cell, ntES cell, somatic stem cell, oocyte, embryo.

1. Introduction

Since the first success of mouse cloning through NT achieved by Wakayama and his colleagues in 1998 (1), numerous studies have used this technique both for addressing some of the fundamental epigenetic questions and for developing novel strategies for cell therapy. Particularly, mouse cloning has contributed greatly to the understanding of how the epigenetic flexibility (reprogrammability) of a cell is controlled by the epigenetic modifications in the genome (2); it also has served as a powerful tool for exploring the possible use of cloning to generate isogenic stem cells for cell therapy (3).

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_22,

Despite the great power of mouse cloning for basic research and biomedical applications, and despite the fact that the mouse is one of the most popular experimental models, the numbers of laboratories that can perform such procedures are very limited at present. One of the reasons for the limited success in promoting such technology is that the procedures involved in mouse cloning are very technically challenging, requiring extensive training and diligent practice for a researcher to master the skills. Additionally, the current lack of procedural standardization among laboratories is another contributing factor for the technical challenges; it is the author's view that frequent comparisons and sharing of cloning protocols among the laboratories is essential for quickly improving and promoting this technology. Another challenge facing the mouse cloners is that, in comparison to most other vertebrate species where NT has been successful, mouse cloning is very inefficient. In the past several years, however, substantial efforts have been spent on improving this technology and some great improvements have been made (4). In this chapter, a detailed protocol used by the author to clone mouse embryos from different donor cell types, ranging from somatic stem cells to fully differentiated somatic cells, is described.

Since the NT procedures for cloning mouse embryos for producing mice and for establishing ntES cell line are the same, the NT protocol described here can be used for both of these applications. For the readers interested in reproductive cloning, information on how to perform embryo transfer to surrogate mothers and how to foster the cloned pups is included in this chapter; for the readers interested in deriving ntES cell lines, the ES cell line establishment protocol described in **Chapter 21**, this volume can be used.

2. Materials

2.1. Equipment

1. Stereomicroscope (e.g., Nikon, Tokyo), for dissecting oviducts, collecting oocytes, and transferring oocytes (before and after enucleation) and reconstituted NT embryos between medium drops in a culture dish.
2. Inverted microscope, with differential interference contrast (DIC) optics and objectives (e.g., Nikon, Tokyo), for the enucleation and nuclear injection steps.
3. A piezo-driven system (e.g., Primetech, Ibaraki, Japan) attached to a micromanipulator (e.g., Narishige or Leica), for enucleation and nuclear injection.

4. Flaming/Brown micropipette puller (model P-97, Sutter Instruments Co.), for pulling pipettes.
5. Microforge (model MF-900, Narishige), for breaking the tips of pipettes and for polishing the holding pipettes.
6. Micropipettes: For enucleation and nuclear injection, pipettes can be directly purchased (Humagen). Alternatively, such pipettes can be made in house from capillary tubes (*see* 2.1.7 below). The inner diameter (ID) of the enucleation pipettes is 7–8 μm, and the ID of the nuclear injection pipettes is 5 μm when neuron and skin stem cells are used as nuclear donors and 6 μm when fibroblast cells are used as nuclear donors (*see* **Note 1**).
7. For making pipettes in house, Friedrich & Dimmock Coagulation capillary tubes (Fisher) with an outside diameter (OD) of 1.2–1.4 mm (wall thickness is 0.2 mm) are used. Pipettes are made by pulling coagulation capillary tubes with a Flaming/Brown micropipette puller. Pipette tips are broken with a Microforge at a position of the pipettes with the desired ID. The ID of pipettes at their tips for collecting donor cells, oocytes, and embryos is about 120–180 μm. The ID and OD of a holding pipette at its tip after being polished with the Microforge is 10–20 and 40–60 μm, respectively.
8. Threaded Plunger Syringes (Hamilton), for controlling holding, enucleation, and nuclear injection pipettes on the micromanipulator. They are linked to the pipettes with Tygon R-3603 laboratory tubing (with an ID of 1/32″ and an OD of 3/32″).
9. Transfer pipette connected to a roughly 2-foot long latex tubing (with an ID of 1/8″, VWR) through a micropipette holder, for collecting donor cells, oocytes, and embryos.
10. CO_2 cell culture incubator, for culturing donor cells, cloned embryos, and ntES cells.
11. Culture dishes and centrifuge tubes and other plastic and glass wares (e.g., Fisher).

2.2. Animals

1. Superovulated C57BL/6 X DBA/2 F1 (B6D2F1) female mice are used as oocyte donors for NT. At the time of oocytes collection, mice should be between 8 and 10 weeks old. Mice can be purchased from Charles River.
2. Krt1-15-EGF transgenic mice generated in Dr. George Cotsarelis' lab (5) are used for isolating hair follicle stem cells.
3. B6D2F1 adult females are used as cumulus cell donors.
4. B6D2F1 adult females and males are used for deriving adult fibroblast donor cells. Fetuses produced by mating

C57BL/6 females with DBA/2 males are used for deriving fetal fibroblast donor cells. Mice can be purchased from Charles River.

5. For full-term development, pseudopregnant Swiss females are used as surrogate mothers for transferring the cloned embryos. These mice should weigh no more than 35 g at the time of use (overweight mice will dramatically reduce pregnancy rate). Fertile female mice can be purchased from Charles River. Pseudopregnancy is induced by mating these females with vasectomized Swiss males and confirmed by having a copulation plug in the vagina of the recipient females in the first morning after mating.
6. Lactating BALB/c albino females are used to foster the cloned pups. Young and fertile mice can be purchased from Charles River and naturally mated in-house for producing lactating mothers. These BALB/c albino females should be those who have naturally given birth 0–2 days prior to the time when cloned pups are retrieved from the Swiss surrogate mothers with C-sections.

2.3. Donor Cells

1. When cumulus cells are used as nuclear donors, they are isolated from B6D2F1 cumulus–oocyte complex (COC) at oocyte collection.
2. When fetal fibroblast cells are used as donors, they are derived from the fetuses produced by mating C57BL/6 females with DBA/2 males.
3. When tail tip fibroblast cells are used as nuclear donors, cells are derived by culturing tail tip biopsies from B6D2F1 adult mice.
4. Neuronal stem cells used as donors in our laboratory were gifts from Austin Smith's lab (6).
5. When hair follicle stem cells are used as donors, they are isolated from skin biopsies from Krt1-15 transgenic mice (5).

2.4. Media

1. Medium for culturing mouse embryonic fibroblast (MEF) and adult fibroblast cells: HEPES-buffered DMEM (high glucose, Gibco) is supplemented with 15% heat-inactivated fetal calf serum (HyClone), 0.1 mM non-essential amino acids (Gibco), 0.1 mM beta-mercaptoethanol (Sigma, add 4 μL to 500 mL medium), and penicillin/streptomycin (100 units/mL for penicillin and 100 μg/mL for streptomycin). This medium is called MEF medium in this protocol.
2. Medium for culturing mouse neuron stem cells: NS-A medium (Euroclone) plus modified N2 supplement (Gibco) and 10 ng/mL for both fibroblast growth factor-2 (FGF-2)

and epidermal growth factor (EGF) (R&D Systems Inc.). For details, *see* Conti et al. (6).

3. Medium for isolating mouse oocytes: HCZB (or M2) medium containing about 100 units/mL of hyaluronidase to release cumulus cells from the COC, and HCZB (or M2) for washing the oocytes and cumulus cells (if cumulus cells are used as donor cells for the experiment). For HCZB medium formulation (7), *see* **Tables 22.1**, **22.2**, and **22.3**. Hyaluronidase solution is made by dissolving the enzyme at 0.2 mg/mL (about 100 units/mL; depending on the specific activity of the enzyme from different vendors, dissolve the enzyme in appropriate volume) concentration in HCZB (or M2) containing 0.01% BSA. As hyaluronidase solution is not stable for long-term storage, aliquots should be made and stored at –20°C. Use a freshly thawed tube each time. The frozen stock normally can maintain its hyaluronidase activity for about 1–2 months at –20°C.

Table 22.1
Formulations for 10× master salt for H/MCZB media

Chemicals	Weight or volume
MilliQ water	990 mL
NaCl	47,600 mg
KCl	3,600 mg
$MgSO4{\cdot}7H_2O$	2,900 mg
EDTA·2Na	400 mg
KH_2PO_4	1,600 mg

Adjust the solution with MilliQ water to a final volume of 1000 mL.

Table 22.2
Formulations for H/MCZB medium stock

Chemicals	Weight or volume
MilliQ water	400 mL
10× master salt for H/MCZB	50 mL
D-Glucose	500 mg
Na-lactate	2.65 mL
Pen/strap	5 mL

Adjust the solution with MilliQ water to a final volume of 500 mL. Filter the stock for sterilization with 0.22 μm filter.

Table 22.3
Formulations for HCZB medium

Chemicals	Weight or volume
H/MCZB stock	90 mL
Polyvinyl alcohol (PVA)	10 mg
HEPES-Na	520 mg
$NaHCO_3$	42 mg
$CaCl_2 \cdot 2H_2O$ 100× stock (170 mM)	1 mL

Use 1 N HCl to adjust the pH to 7.5 and adjust the final volume to 100 mL with MilliQ water. Filter the medium for sterilization with 0.22 μm filter.

4. Medium for enucleation: HCZB medium containing 5 μg/mL cytochalasin B. Cytochalasin B is made as a 1,000× stock (5 mg/mL) by dissolving cytochalasin B powder in DMSO. Aliquot cytochalasin B stock with a volume of 5–10 μL into eppendorf tubes and store them at –20°C. Use freshly thawed cytochalasin B stock for each experiment and discard the leftover.
5. Medium for nuclei isolation and nuclear injection: HCZB containing 3% polyvinylpyrrolidone (PVP) is used for nuclei isolation, and HCZB containing 10% PVP is used to wash injection pipettes. PVP solutions are made by adding PVP powder onto HCZB medium in a 50 mL sterilized bottle. Let PVP powder dissolve into the HCZB medium slowly without stirring. This can be achieved by leaving the bottle at 4°C overnight. Gently mix the solution without causing any air bubbles on the next day to make sure a homogeneous solution is formed. Slowly filter the solution with a 0.45 μm filter and aliquot the solution. Store the aliquots at 4°C until use.
   Pure HCZB medium (without PVP) is used for nuclear injection.
6. Medium for culturing oocytes and cloned embryos: KSOM (Specialty Media) or MCZB medium made in house. *See* **Table 22.4** for MCZB formulations. Whenever KSOM is used, MCZB can also be used in this protocol.
7. Medium for egg activation: Calcium-free MCZB medium plus cytochalasin B and $SrCl_2$ made in house. *See* **Table 22.4** for Ca-free MCZB formulations. $SrCl_2$ is made as a 1000× stock (10 M) by dissolving it in MilliQ water. Aliquot the stock into eppendorf tubes and keep them at room temperature. *See* step 4 for making cytochalasin B solutions.
8. Medium for establishing and culturing ntES cell lines: HEPES-buffered DMEM (high glucose, Gibco) is supplemented with 15% heat-inactivated fetal calf serum

Table 22.4
Formulation for MCZB and Ca-free MCZB

Chemicals	Weight or volume
H/MCZB stock	90 mL
$NaHCO_3$	211 mg
Na-pyruvate	3 mg
L-Glutamine	15 mg
BSA	500 mg
$CaCl_2 \cdot 2H_2O$ 100 × stock (170 mM)	1 mL for MCZB; no $CaCl_2$ is added for Ca-free MCZB

Adjust the solution with MilliQ water to a final volume of 100 mL.

(HyClone), 0.1 mM non-essential amino acids (100× stock from Gibco), 0.1 mM beta-mercaptoethanol (add 8 μL to 1 L medium), 1,000 units/mL of leukemia inhibiting factor (LIF, Sigma), 5 × 10^{-5} M PD98059 MEK1 inhibitor (Cell Signaling Technology), and penicillin/streptomycin (final concentrations: 100 units/mL for penicillin and 100 μg/mL for streptomycin).

3. Methods

3.1. Superovulate Oocyte Donor Female Mice with Hormones

B6D2F1 female mice are superovulated with 5 U of gonadotropin from pregnant mare serum (PMSG) through intraperitoneal injection followed by 5 U of human chorionic gonadotropin (hCG) 46–48 h later. PMSG and hCG injections are normally done in the afternoon around 4–6 pm, so that oocyte collection can be performed at 7–9 am in the morning of the day of cloning (15 ± 1 h post-hCG injection, **Section 3.3**).

3.2. Prepare the Dishes

1. The oocyte collection dish:
   Prior to sacrificing the superovulated females in the morning of the day of cloning, an oocyte collection dish is prepared by placing a large drop of M2 medium (about 500 μL; HCZB medium can also be used) containing hyaluronidase (100 units/mL) and 4–6 smaller drops of M2 medium (about 200 μL; HCZB medium can also be used) onto a 100 mm × 15 mm cell culture Petri dish as depicted in **Fig. 22.1a**. Cover the medium drops with a thin layer of mineral oil by gently pouring the mineral oil directly from manufacturer's container.

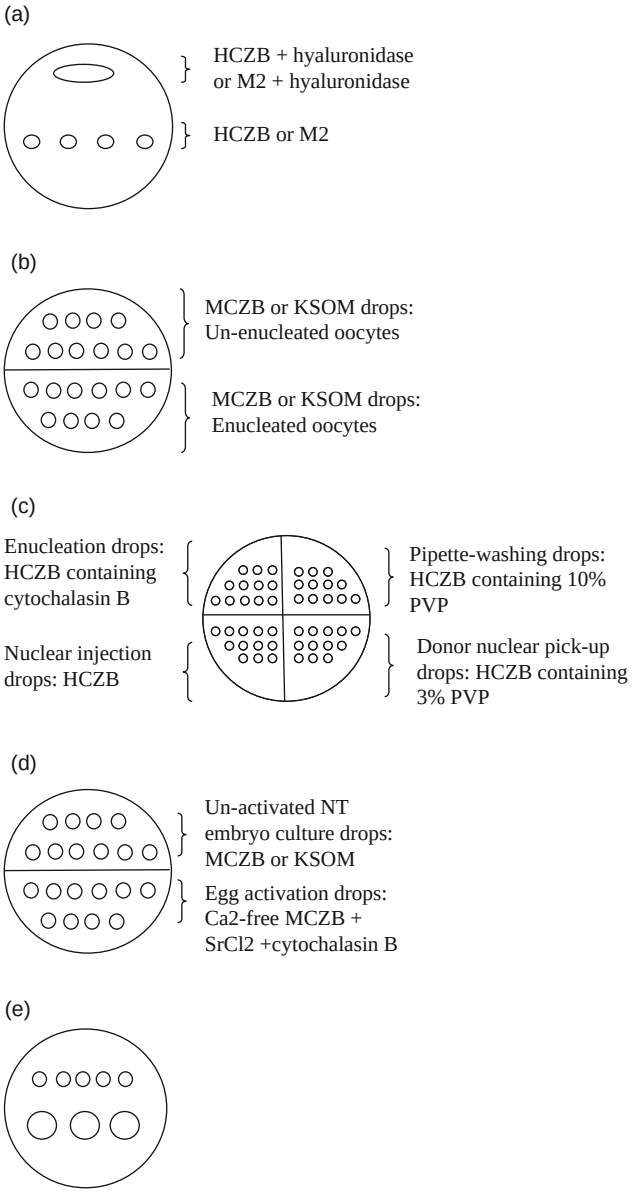

Fig. 22.1. Diagrams of dish set ups (*see* **Section 3.2** in the text for details). (**a**) Oocyte collection dish setup. (**b**) Oocyte culture dish setup. (**c**) Micromanipulation dish setup. (**d**) Egg activation dish setup. (**e**) Embryo culture dish setup.

2. The oocyte culture dish:
   Draw a line on the back of a 100 mm × 15 mm cell culture Petri dish and flip it over for making KSOM (MCZB can also be used) drops into it (about 50–100 μL for each drops). The drops above the line are used for culturing the oocytes prior to enucleation (un-enucleated oocytes) and the ones below the line are used for culturing enucleated oocytes

(**Fig. 22.1b**). Cover the medium drops with mineral oil in the same way as described when preparing the oocyte collection dish. Keep the prepared dish in a CO_2 incubator (37°C, 5% CO_2) for at least 20 min before use.

3. The micromanipulation dish:
   A cover lid of a 100 mm × 15 mm cell culture Petri dish is used for holding medium drops in which enucleation and nuclear injection are performed. A cross is drawn on the back of the Petri dish lid to separate the dish into four sections. Flip the lid over and quickly place ~30 μL drops of media as depicted in the diagram of **Fig. 22.1c**. Drops from four types of media are made in this dish, one is HCZB containing 5 μg/mL cytochalasin B for enucleation, one is HCZB for nuclear injection, one is HCZB containing 3% PVP for nuclei isolation, and one is HCZB containing 10% PVP for washing enucleation and nuclear injection pipettes. Cover the medium drops with mineral oil in the same way as described for preparing the oocyte collection dish. The arrangement of different types of medium drops among the four sections of the divided dish lid can be made according to the operator's preference, but the one depicted in **Fig. 22.1c** generally works well for a right-handed operator.
4. Egg activation dish and cloned embryo culture dish:
   Thaw a vial of 1000× $SrCl_2$ solution at room temperature or by hand and add 5 μL $SrCl_2$ and 5 μL cytochalasin B (*see* **Note 2**) stocks to 490 μL pre-warmed calcium-free MCZB and mix the medium well. This is to be used as egg activation medium. Similar to preparing the oocyte culture dish, a line is drawn on the back of a 100 mm × 15 mm cell culture Petri dish. Flip the dish over and two types of medium drops are made into this dish: KSOM (MCZB can also be used) medium drops are made above the line for culturing un-activated cloned embryos, and Ca-free MCZB plus $SrCl_2$ and cytochalasin B medium drops are made below the line for egg activation (**Fig. 22.1d**). The drop sizes are in the range of 50–100 μL. Cover the medium drops with mineral oil in the same way as described in preparing other types of dishes above.

For culturing activated cloned embryos, KSOM or MCZB medium are used for making drops. Use five to six drops (50–100 μL) in the upper row to wash the embryos for removing the activation medium and use three to four larger drops (100–150 μL) in the lower row for culturing the embryos (**Fig. 22.1e**).

3.3. Collection of Oocytes

Superovulated B6D2 F1 females are sacrificed 15 ± 1 h post-hCG injection in order to retrieve oocytes. Oviducts are cut off

between the ovaries and the uterus horns and dropped onto the oil in the oocyte collecting dish. Once all of the oviducts are collected onto the dish (for 5–6 mice, this process takes about 2 min), use a pair of forceps to hold a oviduct while using another pair of forceps to tear the swollen upper part of the oviduct (the ampulla) to release the cumulus–oocyte complex (COC) onto the oil (multiple COCs immersed in the oviduct fluid will exist as water drops in the oil). Once all of the oviducts are torn to release COCs, use a pair of forceps to drag all of the water drops containing the COCs into the hyaluronidase medium drop (a process that will take about 20 s). Leave the dish at room temperature to let the cumulus cells to be released from COCs in the hyaluronidase medium drop (it takes about 5 min). The release of cumulus cells from COCs is monitored under the stereomicroscope. Once the COCs collapsed, sequentially wash the oocytes into the four smaller M2 medium drops to thoroughly remove hyaluronidase. Then wash the cumulus cell-free oocytes into KSOM medium drops placed in the oocyte culture dish that has been equilibrated in the CO_2 incubator for at least 20 min. Return the oocyte culture dish containing the oocytes to the incubator (37°C with 5% CO_2) until use. When cumulus cells are used as nuclear donors, wash them in the same way as washing oocytes with M2 medium but keep them in the M2 (or HCZB) medium drop at 4°C (either by keeping the oocyte collection dish on wet ice or in a 4°C fridge) until use.

3.4. Preparation of Donor Cells

1. When cumulus cells are used as donors, the cumulus cells released from the COC from oocyte collection are used. *See* **Section 3.3** on how to collect the cumulus cells.
2. When fibroblast cells are used as donors, they are derived from fetuses or adult mice (*see* **Note 3** for protocols of establishing mouse fetal fibroblast and postnatal fibroblast cells).
3. When neuronal stem cells are used as donors, NS5, NSV6.5, and Cor1-5 cell lines originally established in the Austin Smith's lab (7) are used. Cells are cultured as described by Conti et al. (7) and are harvested and resuspended in the culture medium right before nuclear injection. Keep the cells on wet ice until use. When skin stem cells are used as donors, they are freshly isolated from the k-15 transgenic mice generated in George Cotsarelis' lab (5). The isolation of skin stem cells is detailed by Morris et al. (5)

3.5. Set Up the Micromanipulator

1. Attach the piezo-driven micromanipulator system to the inverted Nikon microscope
   For right-handed operator, it is generally more convenient if the holding pipette holder is attached to the microscope on the left side of the operator (when the operator is at the

operating position) and the enucleation/injection pipette on the right side of the operator so that the operator uses his/her left hand to control holding pipette and right hand for enucleation/injection pipette.

2. Set up the holding pipette:
   Fill the syringe used for controlling the holding pipette with distilled H_2O and use the syringe to push the water all the way through the connection tubing to the metal pipette holder. Attach the holding pipette firmly (water tight) to the pipette holder and adjust the pipette to allow its beveled tip portion to be parallel to the bottom of the dish. Push water from the syringe to fill the holding pipette to its tip. Make an oil–medium interface at the pipette tip by sucking in first a small amount of oil from the covering oil and subsequently medium from the medium drop from the micromanipulation dish (the interface in the holding pipette during micromanipulation can be used to visualize the sucking and expelling motions exerted from the control syringe).
3. Set up the enucleation pipette:
   Fill 4–5 μL mercury to the enucleation pipette with a 2 cc syringe from the back of the pipette and push the mercury to the tip of the pipette. Fill the syringe used for controlling the enucleation pipette with distilled water as described in setting up the holding pipette above. Attach the enucleation pipette to its holder firmly (water tight) and align the beveled portion of the pipette parallel to the bottom of the dish. Wash the enucleation pipette in the PVP medium drop by sucking in and expelling out the PVP medium several times.
4. Set up the nuclear injection pipette:
   After enucleation, the enucleation pipette is replaced by the nuclear injection pipette. The nuclear injection pipette is set up in the same way as with the enucleation pipette. Wash the enucleation pipette in the PVP medium drop by sucking in and expelling out the PVP medium several times (*see* **Note 4**). This will make the injection pipette less sticky to the cell debris that will be generated during nuclear injection (sticky injection pipette will lyse the oocyte).

3.6. Enucleation of Oocytes

1. Load about 25 oocytes into an HCZB + cytochalasin B drop in the micromanipulation dish. Depending on your speed in enucleation, the number of oocytes should not be more than what can be enucleated within 15 min. Wait about 8–10 min before beginning enucleation (*see* **Note 5**).
2. Locate the metaphase II spindle (as a shaded spot) by turning an oocyte around with the enucleation pipette. Once

the metaphase II spindle is located, push the oocyte with the enucleation pipette against the holding pipette while exerting a gentle suction with the holding pipette to firmly hold the oocyte. The oocyte should be held with its metaphase II spindle positioned toward the enucleation pipette side (1–5 o'clock, if enucleating from the right-hand side).

3. Gently push the enucleation pipette against the zona pellucida adjacent to the metaphase II spindle and apply one or two piezopulses to cut through the zona pellucid. Care must be taken to avoid breaking the oocyte membrane at this step (*see* **Note 6**).
4. Insert the enucleation pipette through the cut into the space between the oocyte and the zona pellucida. Push the enucleation pipette against the metaphase II spindle and exert a gentle suction by the enucleation pipette to hold the metaphase II spindle. Once the metaphase II spindle is firmly held by the enucleation pipette, remove the metaphase II spindle from the oocyte by dragging away the enucleation pipette from the oocyte with the micromanipulator in a slow motion so that the oocyte membrane wrapping around the metaphase II spindle will pinch off from the oocyte. Repeat this process until all of the oocytes are enucleated.
5. Transfer the enucleated oocytes with a mouth-controlled transfer pipette into a pre-equilibrated KSOM medium drop prepared in the oocyte culturing dish that has been kept in the CO_2 incubator (37°C, 5% CO_2). Wash the enucleated oocytes through three to four KSOM medium drops to completely remove the enucleation medium. Culture the enucleated oocytes in KSOM medium in the CO_2 incubator until all of the oocytes are enucleated (which takes about 1–2 h, depending on the speed of the operator). Culture the last batch of enucleated oocytes in KSOM medium in the CO_2 incubator for about 30 min before using them for nuclear injection.

3.7. Nuclear Injection

1. Load enucleated oocytes onto the micromanipulation dish: Use an oocyte collection pipette to pick up about 25 enucleated oocytes from the oocyte culture dish and transfer them into an HCZB + cytochalasin B drop in the micromanipulation dish. Carry as little volume of the KSOM medium as possible into the HCZB + cytochalasin B drop where the nuclear injection is performed. This can be ensured by quickly washing the enucleated oocytes in another HCZB + cytochalasin B drop before transferring them into the enucleation drop.

2. Load donor cells onto the micromanipulation dish:
   Use the donor cell collection pipette to pick up a small volume of donor cells (about 2 μL) from the donor cell suspension and load the cells into the PVP drop in the micromanipulator dish. Gently mix the cells with the collecting pipette by stirring the drop with slow motions until the cells are completely mixed with the PVP medium.
3. Isolate donor nuclei:
   Use the nuclear injection pipette to pick up a nice looking donor cell (round shaped with relatively smooth-looking cell membrane). Aspirate the donor cell in and out a few times to fully break the oocyte membrane (part of the cell membrane and cytoplasm will be removed during this process). Aspirate the isolated nucleus about three to five cell diameters away from the injection pipette tip to make room for next nucleus isolation. Repeat this step until five to six nuclei are picked up.
4. Move both the holding pipette and injection pipette to the medium drop where enucleated oocytes were loaded. Hold an enucleated oocyte firmly by the holding pipette, cut the zona pellucida using piezopulses (the power of the piezo should be set at a level at which two to three pulses are required to cut the zona pellucida). Reduce the piezopower level and push the last picked up nucleus forward in the injection pipette until it reaches the tip of the pipette.
5. Push the injection pipette through the cut on zona pellucida and press it against the oocyte membrane along the oocyte diameter to make a deep depression into the oocyte without breaking the oocyte membrane. When the injection pipette reaches a position about 1/4–1/8 of the oocyte's diameter away from the oocyte membrane on the opposite side of the injection pipette, apply one or two piezopulses to puncture the oocyte membrane (the lowest possible piezopower should be used to avoid lysing the oocyte). Immediately inject the donor nucleus into the enucleated oocyte cytoplasm with as little volume as possible of the PVP medium. Withdraw the injection pipette from the oocyte with the micromanipulator in a smooth and gentle motion to avoid lysing the oocyte (*see* **Note 7**).
6. Repeat steps 3–5 until all of the isolated nuclei are injected into the enucleated oocytes.
7. Move the injection pipette into a 10% PVP drop to wash away any cell debris from the injection pipette by sucking in and expelling some PVP medium several times and by expelling a few drops of mercury while applying a few piezopulses.

8. Repeat steps 2–7 until all of the enucleated oocytes loaded on the dish are injected.
9. Keep the injected oocytes in the injection drop for about 10 min and then wash them into KSOM (or MCZB) medium drops in the embryo culture dish.
10. Repeat steps 2–9 until all of the enucleated oocytes are injected. Culture the reconstructed NT embryos in the CO_2 incubator (37°C, 5% CO_2) for 1–3 h before activation.

3.8. Egg Activation

1. Transfer the injected oocytes into activation medium by washing them through three drops of activation medium. Since some of the injected oocytes will lyse during activation and the released cellular debris will precipitate with $SrCl_2$, divide the injected oocytes into a few drops of the activation medium (each drop holds about 20–30 injected oocytes). Activate the injected oocytes for 4–6 h in a 5% CO_2 incubator at 37°C.
2. Check activation rate under the micromanipulation microscope (by detecting the formation of pseudopronuclei) after activation (*see* **Note 8**).
3. Transfer the activated oocytes into KSOM medium by washing them through three drops of KSOM medium to completely remove the activation medium.
4. Continue to culture the cloned embryos for 3 days until blastocysts are formed.

3.9. Establish ntES Lines or Embryo Transfer for Producing Cloned Mice

3.9.1. ntES Cell Line Establishment from Cloned Blastocysts

When the cloned blastocysts are used for ntES cell line establishment, follow the protocols detailed in **Chapter 21**, this volume.

3.9.2. Producing Cloned Mice from Cloned Blastocysts

When the cloned blastocysts are used for producing cloned mice, the following procedures are used.

1. On the same day of cloning, mate estrous Swiss females with vasectomized Swiss males by putting two to three females with one male in the same cage (matting is normally set up around 4–6 pm). Pseudopregnancy is confirmed by having a copulation plug in the vagina of the recipient females in the next morning after mating. These pseudopregnant mice are considered as 0.5 day of postcoitum (pdc) in the

morning when plugs are checked. Separate the pseudopregnant females from unmated females and transfer them into separated cages until embryo transfer.

2. On the fourth day of cloning when cloned embryos have developed to the morulae/blastocysts stage, they are transferred to the uterus horn of the 2.5 pdc Swiss females under general anesthesia induced with Avertin. Generally, five to ten cloned morulae/blastocysts are transferred into each uterus horn of a recipient female. For detailed procedures on performing embryo transfer, *see* Nagy et al. (8). In the case that too few cloned embryos are available (e.g., fewer than 5), to improve pregnancy rate, a few fertilization-produced mouse embryos should be mixed with the cloned embryos and transferred them together to the uterus horns of a recipient female. Such fertilization-produced mouse embryos should be with distinct genetic background from the cloned embryos (different coat colors are recommended) so that clones and fertilization-produced mice can be identified after birth.
3. Cesarean section (C-section) is performed at 19.5 dpc to retrieve the cloned pups (*see* **Note 9**). For detailed procedures on performing C-sections, *see* Nagy et al. (8).
4. After cutting the uterus open, dissect the cloned pups off their placentae. Put the cloned pups into a Petri dish or directly onto the bedding in a mouse cage and move them under a heating light to maintain their body temperature (depending on the power of the heating light, the heating light should be kept about 2 ft away from the pups so that the surface temperature of the Petri dish or the bedding is about 37°C; care must be taken to avoid over-heating the pups).
5. Under the heating light, clean the amniotic fluid from the pups, especially in the nose and mouth areas, with a cotton swab. Stimulate the pups to breathe by gently pocking them with the cotton swab. It may take anywhere from a few seconds to about 10 min for a cloned pup to start vigorous breathing. High percentage of cloned pups may never initiate vigorous breathing and will die from the so-called respiratory stress.
6. Once the cloned pups start vigorous breathing, mix them with pups borne naturally within 0–2 days by BALB/c albino females and leave them with the lactating mothers to be fostered (*see* **Note 10**).

4. Notes

1. The ID of nuclear injection pipette is determined by the sizes of donor cells. Normally, a nuclear injection pipette with an ID of about half the diameter of the donor cells works well. For certain donor cell types with either too big or small nuclei, trials need to be performed to determine the right size of nuclear injection pipette. The pipette ID should be small enough to squeeze and break the cytoplasmic membrane of the donor cell but not too small to feel the donor nucleus being "stuck" at the pipette tip when a donor cell is being picked up. The smaller the cut made to the oocyte membrane, the less the chance that the oocyte will lyse after injection. Therefore, whenever possible a small injection pipette diameter is preferred.
2. Cytochalasin B is needed when cells at the G1 stage of the cell cycle (diploid, with 2C DNA content) are used as donors. In this case, cytochalasin B suppresses secondary polar body extrusion from the cloned embryos through its action of inhibiting microfilament formation, preventing the cloned embryos from becoming haploid. When metaphase cells (after DNA synthesis, therefore with 4C DNA content) are used as donors, cytochalasin B is omitted in the activation medium allowing second polar body extrusion from the cloned embryos to result in a normal diploid (2C) genome.
3. Establishing mouse fetal and adult fibroblast cell lines:
   For establishing mouse embryonic fibroblast cells, day 13.5 mouse embryos are retrieved via C-section. The following procedures should be performed in a sterile hood. Embryos are first rinsed with 10 mL HEPES buffer or with fibroblast culturing medium and then dissected individually in a 100 mm Petri dish to remove the head and internal organs. The carcasses are rinsed to remove any tissue debris and blood. Add 0.5 mL 0.05% trypsin/0.02% EDTA to a carcass and mince it into fine pieces with a scalpel. Add another 1.5 mL trypsin to the minced tissues and further mince the tissues by passing the tissues several times through an 18 gauge syringe needle attached to a 10 mL syringe until the tissues have a sludgy consistency. Place the dish containing the minced tissues into a cell culture incubator (37°C, 5% CO_2) for 30 min. Add 10 mL MEF culture medium to the dish and transfer the content into a 50 mL conical tube. Wash the dish one more time with another 10 mL MEF culture medium and combine it with the content in the conical tube. Transfer the content from the conical tube

into a 175 cm^2 culture flask. Culture the cells in a cell culture incubator (37°C, 5% CO_2) for 24–36 h or until cells reach confluence. Cells are trypsinized and resuspended in single-cell suspension with fibroblast culture medium and directly used for cloning or to be frozen for future usages.

For establishing postnatal mouse fibroblast cells, both ear biopsies and tail tip biopsies are used. In either case, place the biopsies in a Petri dish in a sterile hood and remove the skin from the biopsies by peeling it off with forceps. Add a few drops of trypsin to the tail tip biopsies and mince them with a scalpel into fine pieces. Spread the tissue pieces across the dish and leave the tissues in the dish with the lid open in the hood for about 10 min (biopsies will stick to the bottom of the culture dish better after some of the trypsin solution has evaporated). Gently add 10 mL mouse fibroblast culture medium without disturbing the biopsies off the bottom of the dish and culture the tissues in a cell culture incubator (37°C, 5% CO_2). It normally takes about 24–48 h for fibroblast cells to grow out from the biopsies. Change medium as needed and passage the fibroblast cells by trypsinization when most of the area in the culture dish is covered with fibroblast cells.

Once the fibroblast cell line is established, the cell line can be further expanded by passaging and culturing. Single-cell suspension of freshly trypsinized fibroblast cells from a confluent cell culture is used as nuclear donors.

4. Prewashing the injection pipette with fluoric acid:
It is often the case that the nuclear injection pipette tends to stick to the oocyte membrane during nuclear injection, and a sticky injection pipette will lyse the oocyte. To prevent this, a prewash of the injection pipette with fluoric acid is recommended. Attach a nuclear injection pipette to a 0.2 cc syringe through thin plastic tubing. Place a small drop of fluoric acid in a Petri dish and wash the injection pipette by sucking a small amount (about 1 cm into the injection pipette) fluoric acid into the pipette from the drop and immediately release it out back to the drop. Rinse the pipette in the same manner in a distilled H_2O drop and then in a 95% ethanol drop.
5. To facilitate enucleation of the oocytes, the f-actin-based cytoskeleton is disrupted with cytochalasin B. This is a reversible process, as the cytoskeleton will reform upon the removal of the drug.
6. Since the intensity of piezopulses needed to break the zona pellucida is too strong for oocyte membrane (the oocyte membrane will lyse immediately if the impact from a piezopulse at this intensity is exerted onto the oocyte

membrane), an obvious space (about the thickness of the zona pellucida, or larger) between the zona pellucida and the oocyte membrane should exist at the time of applying piezopulses, so that the impact of the piezopulses will not affect the oocyte membrane. If such space does not exist at the position where the zona pellucida is to be cut, use the enucleation pipette to press the zona pellucida a few times where the cut is to be made, in order to push the oocyte away from zona pellucida in this region. Once an adequate space is generated between the oocyte and the zona pellucida, gently press the enucleation pipette against the zona pellucida (generate a barely noticeable bending of the zona pellucida by the pipette but without significantly reducing the space between the zona pellucida and the oocyte membrane) and then apply one or two piezopulses to penetrate the zona pellucida.

7. Successful penetration of the injecting pipette through the oocyte membrane is reflected by a sudden relaxation of the indented oocyte membrane from the tip of the injection pipette. The injection of the nucleus into the oocyte cytoplasm should be performed almost simultaneously as the oocyte membrane is being penetrated. The injection pipette, after injecting the nucleus into the oocyte cytoplasm, should be pulled away from the oocyte in a smooth and gentle motion by the micromanipulator before the oocyte membrane is fully relaxed from the indentation. If the oocyte membrane is fully relaxed from the indentation, the cut on it made by the injection pipette will not reseal and the oocyte will lyse.
8. Upon treatment with $SrCl_2$, the donor chromosomes in a reconstructed NT embryo will form sub-cellular structures resembling the pronuclei that are formed following fertilization. Wakayama et al. (1) referred such structures as pseudopronuclei. Only the oocytes with distinct pseudopronuclei are considered to be activated.
9. For yet unidentified reasons (possibly due to the delay of development and/or abnormal physiology of the cloned placentae), pregnancies carrying cloned embryos tend to fail in inducing natural labor. Also, cloned mouse embryos at term tend to be larger than those produced from natural reproduction (the so-called large offspring syndrome in cloning). Due to these reasons, C-section is necessary to deliver the cloned pups.
10. If many cloned pups are to be fostered by a lactating mother, remove some of the naturally born pups from the litter (fewer than 10 pups in total per foster mother

is recommended). To increase the chance of the foster mother accepting the cloned pups, mix the cloned pups first with bedding from the mouse cage in which the foster mother has been kept (the bedding contains some waste generated by the foster mother, and its scent will help her accept the cloned pups) before mixing them with the naturally born pups.

Acknowledgments

The work is supported by Hematech, Inc. The author thanks Emma Wang from Harvard College for proofreading the manuscript.

References

1. Wakayama, T., Perry, A. C. F., Zuccotti, M., Johnson, K. R., and Yanagimachi, R. (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**, 369–374.
2. Blelloch, R., Wang, Z., Meissner, A., Pollard, S., Smith, A., and Jaenisch, R. (2006) Reprogramming efficiency following Somatic Cell Nuclear Transfer is influenced by the differentiation and methylation state of the donor nucleus. *Stem Cells* **24**, 2007–2013.
3. Rideout, W. M., 3rd, Hochedlinger, K., Kyba, M., Daley, G. Q., and Jaenisch, R. (2002) Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* **109**, 17–27.
4. Kishigami, S., Mizutani, E., Ohta, H., Hikichi, T., Thuan, N. V., Wakayama, S., Bui, H. T., and Wakayama, T. (2006) Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. *Biochem. Biophys. Res. Commun.* **340**, 183–189.
5. Morris, R. J., Liu, Y., Marles, L., Yang, Z., Trempus, C., Li, S., Lin, J. S., Sawicki, J. A., and Cotsarelis, G. (2004) Capturing and profiling adult hair follicle stem cells. *Nat. Biotechnol.* **22**, 411–417.
6. Conti, L., Pollard, S. M., Gorba, T., Reitano, E., Toselli, M., Biella, G., Sun, Y., Sanzone, S., Ying, Q. L., Cattaneo, E., and Smith, A. (2005) Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol.* **3**, e283.
7. Chatot, C. L., Ziomek, C. A., Bavister, B. D., Lewis, J. L., and Torres, I. (1989) An improved culture medium supports development of random-bred one-cell mouse embryos in vitro. *J. Reprod. Fertil.* **86**, 679–688.
8. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003) *Manipulation of mouse embryo: A laboratory manual*, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Chapter 23

Keeping Two Animal Systems in One Lab – A Frog Plus Fish Case Study

Hazel Sive

Abstract

For two decades, my lab has been studying development using two vertebrate animals, the frog *Xenopus* and the zebrafish, *Danio*. This has been both productive and challenging. The initial rationale for the choice was to compare the same process in two species, as a means to find commonalities that may carry through all vertebrates. As time progressed, however, each species has become exploited for its specific attributes, more than for comparative studies. Maintaining two species simultaneously has been challenging, as has the division of research between the two and making sure that lab members know both systems well enough to communicate productively. Other significant issues concern funding for disparate research, figuring out how to make contributions to both fish and frog communities, and being accepted as a member of two communities. I discuss whether this dual allegiance has been a good idea.

Key words: Zebrafish, *Danio*, frog, *Xenopus*, two models in one lab.

1. Introduction: Why Fish Plus Frog?

A long time ago, in 1991, as I was setting up my own lab, I decided to include an additional animal model in our research. Since an undergraduate, I had used the frog *Xenopus laevis* as a tool to study developmental questions. Frog embryos are large enough to manipulate by transplant and explant assays, and until the last decade, essentially everything known about vertebrate development came from studies in amphibians, primarily *Xenopus*. However, certain assays were frustrating in *Xenopus*. There were no embryonic mutants, and loss of function assays had to be performed by expression of dominant negative constructs or by antibody injection. There was the promise of antisense (1), but

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_23,

nothing usable. Promoter analysis was very difficult, as transient transgenics made by DNA injection expressed only in a highly mosaic fashion, and as *X. laevis* has a generation time of 2 years, stable transgenics were difficult to make and seemed no better. Nonetheless, almost everything that was known about early vertebrate development had come from amphibian embryos (for example, (2–6), reviewed in (7)), due to the ease of explanting and transplanting embryonic tissue, and the ability to obtain large numbers of embryos for biochemical or molecular assays. These attributes made *Xenopus* very attractive.

In 1991, the zebrafish, *Danio rerio,* had yielded some information about early mesodermal and neural development (for example, (8–10)), but the attraction of the system was its promise. Pre-eminent among the vertebrates, the zebrafish could readily be used for forward genetic screens, yielding mutants and identifying genes required for vertebrate development. Interesting mutants already existed (for example, (11, 12)), and massive zygotic mutant screens were underway. Preparation of transgenic lines was not established, but was being worked on (13). All this promised a system that was more tractable than the frog at identifying genes required for development, and at assaying true loss-of-function effects. The drawback to the fish is that the embryo is small, and transparent, which is great for imaging, but tough for microdissection-based assays, as one cannot readily distinguish specific regions, and once these are removed, they can vanish easily in the Petri dish. Thus, explant assays, so valuable in the frog, had not been developed for the fish. In addition, the zebrafish fate map is not as stereotypical as that of the frog embryo (14, 15), further complicating embryological assays. Nonetheless, it was clear that the zebrafish was becoming a very important vertebrate system.

One thing that bothered me about both frogs and fish was their evolutionary distance from mammals, and whether what we learned in frogs would extend to mammals. Amphibians and teleosts diverged more than 200my ago, frogs and mammals, about 150my ago. As the distance between fish and frogs is very great, and it seemed therefore, that if one identified a process conserved in both *Xenopus* and zebrafish, it was more likely to be conserved throughout the vertebrates than one identified in frogs or fish alone.

These considerations made the power of frogs and fish a compelling dual system in which to perform both embryological and genetic assays, and so we set up both systems to address the molecular basis of nervous system determination and patterning. *The overarching rationale was that asking the same questions in two animal systems, was a powerful way to compare and define conserved principles of vertebrate neural development.*

2. The Progression of Our Research Using Two Models

Our initial analyses using zebrafish were entirely comparative with those in *Xenopus*. We had isolated a set of genes expressed very early during *Xenopus* neural patterning, and compared expression patterns and time of specification of forebrain-expressed genes in fish and frog. This yielded several good papers, although each paper used either the frog (16, 17) or fish (18, 19), not both. If we did the studies today, they would compare species in a single paper.

Then, we moved on to the hindbrain, and here, something interesting happened. The question of how the hindbrain is set aside and patterned had been started in *Xenopus* (20). However, we had isolated, by subtractive cloning, a set of genes expressed specifically in the zebrafish hindbrain (21), and most of these had not yet been isolated in frog. Fish hindbrain mutants had already given very interesting information (reviewed in (22)). So the zebrafish hindbrain project moved rapidly, with analyses of new hindbrain gene function (23), as well as analysis using a *vhnf1* mutant and Fgf signaling (24). The fish studies moved ahead of the frog, and a catch-up game with frog did not seem useful, or a fair project for a student or postdoc, who would get less novel publications than the authors of the fish studies.

During the fish hindbrain study, which involved looking at the brain a lot, we started thinking about brain morphology. This led us to the fascinating question of why the vertebrate nervous system is tubular, what the cavities (brain ventricles) are for, and later, why the tube bends (reviewed in (25)). From the outset, it was clear that the fish was a much better system than was frog with which to address questions of brain morphogenesis and brain ventricle formation – there were mutants already, and now the transparency of the fish was very useful. We could make amazing live movies of the brain cells changing shape, and look in the mutants to see what had gone wrong (26–28). It was clear that this was going to be a productive approach, and that it did not include the frog, at least in our lab.

At the same time, we had, for a long time, studied the extreme anterior of the embryo in *Xenopus*. We had productively studied the cement gland, an amphibian-specific anterior organ (many years of work reviewed in (29)), but then moved dorsally, to the primary mouth, which is highly conserved. In the frog, we were able to determine which cells contribute to the primary mouth and which tissue interactions are necessary, and began to study which factors were required, identifying Wnt antagonists as pivotal (30, 31). There was no way that this study could have been done in fish – the primary mouth (stomodeal) region is difficult

to image as the eyes are in the way, the germ layers in the fish are not distinct without lineage-specific gene markers, which are not available, and face transplant assays (31), which have been crucial in figuring out whether specific gene function is required locally in *Xenopus*, are not possible in fish.

The distinct attributes of each species were reinforced by an early project we performed, to ask whether frog explant techniques could be applied to the zebrafish. This was very challenging because the fish embryo is small and transparent, but two talented postdocs succeeded in isolating and culturing embryonic explants and performing ectoderm/mesendoderm induction assays, to show that neural induction occurs in the fish (18, 19). A later paper compared frog and fish neural specification, emphasizing the usefulness of having parallel techniques available, and of comparative studies (32). Developing these techniques was a tour de force, but the assays are difficult, and still, *Xenopus* is a much simpler system for this approach.

Overall, these experiences added up to a move away from direct comparative studies, rather using the attributes of each species to address specific questions. *Thus, the initial rationale of fish/frog comparison led to useful insight; however, more recent studies have used each species for its greatest attributes.*

3. Advantages and Challenges of Fish + Frog

Overall, having two animal species as experimental tools has been good, but not really for the reasons I thought. As discussed above, the notion of comparative studies turned out to be more cumbersome than the original rationale suggested, although this approach remains very important. The greatest advantage, I think, is that we have been able to study a much broader array of questions in two systems, than we would with one. This outcome arose due to technical considerations. Both fish and frog methods have improved enormously in the last two decades: including efficient methods to make stable or transient transgenics (for example, (33–35)), facilitating promoter analysis and tissue-specific or inducible gene expression; and antisense morpholino-modified oligonucleotides which have been extremely useful in both systems (reviewed in (36)). However, the genetic power of the fish remains supreme, even with the promise of mutants from *Xenopus tropicalis*. Thus, *X. laevis* and *D. rerio* remain species with distinct attributes, which can be combined to address a single question, or applied to different questions that make use of the distinct attributes.

An unexpected advantage is that our group members become familiar with two models, and with a little effort can become facile with both. Several former lab members have switched systems, more easily than would be possible without the two animal exposure. Further, use of a technique in one species in our lab often inspires researchers working on the other system to rapidly try out the technique.

On the challenging side is the issue of maintaining healthy colonies of two species. Both *Danio* and *Xenopus* may be aquatic, but they have their own water quality and temperature requirements, food needs, and techniques of embryo collection. We successfully raise some *X. laevis* to adulthood, but raising enough zebrafish to keep the group stocked is a continual and huge task. Separate technicians for each animal have been necessary, and separate animal rooms are essential. Where one aquatic system goes wrong frequently, two do so even more frequently! As anyone who works with aquatic species knows, a disaster of temperature change or lack of proper feeding can lead to no or poor embryos for protracted periods, and this is amplified when two species are used. On the flip side, exchange between the animal managers of each species is synergistic and beneficial.

Another challenge is the need for a large enough group to have a critical mass of investigators using each system, both to get the research done and to share techniques and responsibilities for the animals. Associated with that is the challenge of securing funding for separate lines of research in separate animal systems. This increases the need for proven technical expertise in the particular system. For example, although expertise in making transgenic frogs bodes well for success in the fish, it is nowhere near as valuable as having actually prepared transgenic fish. An ongoing challenge, has been participating extensively in two communities, and mostly, ensuring that the group is viewed as committed to the fish or the frog community. In the case of frog, multiple *Xenopus* investigators moved entirely to zebrafish, and we are one of the very few groups who added fish, but also stayed with the frog. We have tried to emphasize our commitment to both communities, but the perception of not fully participating has been frustrating at times.

In sum, having two animals in the lab has clear advantages: the ability to ask diverse questions, exposure of lab members to the practicalities and techniques of more than one system. The challenges include extensive husbandry required, the need to be facile with techniques in both species, and the challenge of participating in two different animal communities.

4. I Would Do It Again. Circle YES/NO

Here is the question. Would I have pursued both fish and frog models, had I known the challenges involved? I love the gentle frogs, large enough to hold and to encourage to lay their eggs. I love frog embryos, which are really beautiful, where their lack of transparency makes the changing parts of the embryo readily visible. I can't imagine not working with these embryos. The extreme anterior projects we have worked on are fascinating, and if anything, I would devote more time to these if I did it again. On the other hand, fish have grown on me. The adults are small and don't have the personality of frogs. One has to look very hard at the embryos to see their features. But when the cells are GFP labeled, and the imaging is done right, cells moving, changing shape or dividing are easy to see, deep within the living brain, and the embryos are very wonderful. We could not have performed the primary mouth study in fish. Period. Conversely, we could not have performed the brain studies in frogs. Certainly, we could have focused on just one question, but that is not my style – the pull of so much interesting biology waiting to be explored is too strong. So, circle YES for me, please.

5. Last thoughts

Finally, if you are thinking of starting another animal system in your lab, here are some questions that may help you explore whether this is really the path you want to take.

1. Why do you want a second animal system in your lab?
2. Could you collaborate with another group working on the second system, rather than maintain both systems yourself?
3. Which two animals juxtapose effectively in terms of the questions you are addressing? Should they both be vertebrates, or would an invertebrate be useful?
4. Which two animal species juxtapose effectively in terms of the husbandry involved? Would expertise be shared between the two?
5. Can one animal caretaker maintain both systems?
6. How do you plan to split research between the two systems?
7. How would you ensure that a small group of researchers working on one animal system connect with others working on the same system?

8. Would research questions in the two systems be overlapping, or distinct?
9. If distinct, do you have sufficient funding and personnel to make a scientific contribution to each project?
10. What strategies would you employ to optimize the contribution of your group to the communities of each animal model?

And, if you have the energy to go for two systems, best of luck!

Acknowledgments

Our research has been supported by the NIH (including DE021109 to H.S.), NSF and multiple foundations including Searle, MOD, Whitehall, the Stanley Medical Research Institute, via their grant to the Stanley Center for Psychiatric Research at the Broad Institute and the Simons Foundation (SFARI 95091 to H.S.). My warmest acknowledgment to the students and postdocs who contributed to our studies, and I apologize that I could not cite all of your work. Another place, I will. Apologies to other groups whose material was not cited – this was often for arbitrary reasons, as I used good examples, but did not attempt a comprehensive listing.

References

1. Izant, J. G. and Weintraub, H. (1984) Inhibition of thymidine kinase gene expression by anti-sense RNA: a molecular approach to genetic analysis. *Cell* **36**, 1007–1015.
2. Sokol, S., Christian, J. L., Moon, R. T., and Melton, D. A. (1991) Injected Wnt RNA induces a complete body axis in *Xenopus* embryos. *Cell* **67**, 741–752.
3. Cho, K. W., Morita, E. A., Wright, C. V., and De Robertis, E. M. (1991) Overexpression of a homeodomain protein confers axis-forming activity to uncommitted *Xenopus* embryonic cells. *Cell* **65**, 55–64.
4. Smith, J. C., Price, B. M., Green, J. B., Weigel, D., and Herrmann, B. G. (1991) Expression of a *Xenopus* homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* **67**, 79–87.
5. Sive, H. L. and Cheng, P. F. (1991) Retinoic acid perturbs the expression of Xhox.lab genes and alters mesodermal determination in *Xenopus laevis*. *Genes Dev.* **5**, 1321–1332.
6. Slack, J. M. (1991) The nature of the mesoderm-inducing signal in *Xenopus*: a transfilter induction study. *Development* **113**, 661–669.
7. Sive, H. L. (1993) The frog prince-ss: a molecular formula for dorsoventral patterning in *Xenopus*. *Genes Dev.* **7**, 1–12.
8. Holder, N. and Hill, J. (1991) Retinoic acid modifies development of the midbrain–hindbrain border and affects cranial ganglion formation in zebrafish embryos. *Development* **113**, 1159–1170.
9. Krauss, S., Johansen, T., Korzh, V., and Fjose, A. (1991) Expression pattern of zebrafish pax genes suggests a role in early brain regionalization. *Nature* **353**, 267–270.
10. Chitnis, A. B. and Kuwada, J. Y. (1991) Elimination of a brain tract increases errors

in pathfinding by follower growth cones in the zebrafish embryo. *Neuron* **7**, 277–285.
11. Hatta, K., Kimmel, C. B., Ho, R. K., and Walker, C. (1991) The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature* **350**, 339–341.
12. Eisen, J. S. and Pike, S. H. (1991) The spt-1 mutation alters segmental arrangement and axonal development of identified neurons in the spinal cord of the embryonic zebrafish. *Neuron* **6**, 767–776.
13. Culp, P., Nüsslein-Volhard, C., and Hopkins, N. (1991) High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. *Proc. Natl. Acad. Sci. USA* **88**, 7953–7957.
14. Kimmel, C. B., Warga, R. M., and Schilling, T. F. (1990) Origin and organization of the zebrafish fate map. *Development* **108**, 581–594.
15. Woo, K. and Fraser, S. E. (1995) Order and coherence in the fate map of the zebrafish nervous system. *Development* **121**, 2595–2609.
16. Kuo, J. S., Patel, M., Gamse, J., Merzdorf, C., Liu, X., Apekin, V., and Sive, H. (1998) Opl: a zinc finger protein that regulates neural determination and patterning in *Xenopus*. *Development* **125**, 2867–2882.
17. Gamse, J. T. and Sive, H. (2001) Early anteroposterior division of the presumptive neurectoderm in *Xenopus*. *Mech. Dev.* **104**, 21–36.
18. Sagerström, C. G., Grinblat, Y., and Sive, H. (1996) Anteroposterior patterning in the zebrafish, *Danio rerio*: an explant assay reveals inductive and suppressive cell interactions. *Development* **122**, 1873–1883.
19. Grinblat, Y., Gamse, J., Patel, M., and Sive, H. (1998) Determination of the zebrafish forebrain: induction and patterning. *Development* **125**, 4403–4416.
20. Kolm, P. J., Apekin, V., and Sive, H. (1997) *Xenopus* hindbrain patterning requires retinoid signaling. *Dev. Biol.* **192**, 1–16.
21. Sagerström, C. G., Kao, B. A., Lane, M. E., and Sive, H. (2001) Isolation and characterization of posteriorly restricted genes in the zebrafish gastrula. *Dev. Dyn.* **220**, 402–408.
22. Moens, C. B. and Prince, V. E. (2002) Constructing the hindbrain: insights from the zebrafish. *Dev. Dyn.* **224**, 1–17.
23. Hoyle, J., Tang, Y. P., Wiellette, E. L., Wardle, F. C., and Sive, H. (2004) nlz gene family is required for hindbrain patterning in the zebrafish. *Dev. Dyn.* **229**, 835–846.
24. Wiellette, E. L. and Sive, H. (2003) vhnf1 and Fgf signals synergize to specify rhombomere identity in the zebrafish hindbrain. *Development* **130**, 3821–3829.
25. Lowery, L. A. and Sive, H. (2009) Totally tubular: the mystery behind function and origin of the brain ventricular system. *Bioessays* **31**, 446–458.
26. Lowery, L. A. and Sive, H. (2005) Initial formation of zebrafish brain ventricles occurs independently of circulation and requires the nagie oko and snakehead/atp1a1a.1 gene products. *Development* **132**, 2057–2067.
27. Gutzman, J. H., Graeden, E. G., Lowery, L. A., Holley, H. S., and Sive, H. (2008) Formation of the zebrafish midbrain–hindbrain boundary constriction requires laminin-dependent basal constriction. *Mech. Dev.* **125**, 974–983.
28. Gutzman, J. H. and Sive, H. (2010) Epithelial relaxation mediated by the myosin phosphatase regulator Mypt1 is required for brain ventricle lumen expansion and hindbrain morphogenesis. *Development* **137**, 795–804.
29. Wardle, F. C. and Sive, H. L. (2003) What's your position? The *Xenopus* cement gland as a paradigm of regional specification. *Bioessays* **25**, 717–726.
30. Dickinson, A. J. and Sive, H. (2006) Development of the primary mouth in *Xenopus laevis*. *Dev. Biol.* **295**, 700–713.
31. Dickinson, A. J. and Sive, H. L. (2009) The Wnt antagonists Frzb-1 and Crescent locally regulate basement membrane dissolution in the developing primary mouth. *Development* **136**, 1071–1081.
32. Sagerström, C. G., Gammill, L. S., Veale, R., and Sive, H. (2005) Specification of the enveloping layer and lack of autoneuralization in zebrafish embryonic explants. *Dev. Dyn.* **232**, 85–97.
33. Amaya, E. and Kroll, K. L. (1999) A method for generating transgenic frog embryos. *Methods Mol. Biol.* **97**, 393–414.
34. Suster, M. L., Sumiyama, K., and Kawakami, K. (2009) Transposon-mediated BAC transgenesis in zebrafish and mice. *BMC Genomics* **10**, 477.
35. Soroldoni, D., Hogan, B. M., and Oates, A. C. (2009) Simple and efficient transgenesis with meganuclease constructs in zebrafish. *Methods Mol. Biol.* **546**, 117–130.
36. Heasman, J. (2002) Morpholino oligos: making sense of antisense? *Dev. Biol.* **243**, 209–214.

Chapter 24

Laboratory Guidelines for Animal Care

Marcelo Couto

Abstract

Animal research is a controversial subject because of the ethical and moral implications of using unwilling research subjects in potentially painful or distressful procedures usually ending in euthanasia. As such, it must be conducted in a compassionate and responsible manner geared toward maximizing the animals' quality of life prior to and during experimentation. Because of its contentious nature, the conduct of animal research is highly regulated at the federal, state, city, and institutional levels. It is essential that researchers acquire a working knowledge of the procedures and regulations in order to protect themselves and their staff from occupational hazards as well as protect their labs from criticism or attack from animal rights organizations. Perhaps the best way to protect from the latter is to avoid inadvertent instances of noncompliance with their research protocol or applicable regulations. Regulatory noncompliance can also have serious negative consequences on investigators' research ranging from temporary suspension of their protocols to loss of funding or principal investigator status. To minimize such events, it is advised that researchers build positive and collaborative relationships, trust and rapport with key institutional players, such as the veterinary staff, the Institutional Animal Care and Use Committee (IACUC), and top administrators. Guidance is provided regarding the appropriate handling of regulatory noncompliances.

Key words: Animal research, ethics, animal facilities, organizational relationships, animal welfare regulations, regulatory compliance.

1. Introduction

This chapter is intended to provide useful background information and practical advice to investigators using animals in research, teaching, training, and testing, herein described as "research," with regard to the organizational infrastructure and key stakeholders in an animal program, applicable regulations, different types of animal facilities, occupational risks, and the proper handling of regulatory noncompliance.

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6_24, © Springer Science+Business Media, LLC 2011

The use of animals in research is a privilege and must be conducted responsibly. As opposed to human clinical trials, where patients volunteer for a clinical study and sign an informed consent, laboratory animals obviously do not voluntarily participate in research activities and therefore require the intervention of advocates, such as the researchers, the veterinary staff, and the Institutional Animal Care and Use Committee (IACUC) (1).

Positive and collegial interactions and collaboration among researchers, the veterinary staff, the IACUC, and the institutional administration are essential for the safe, effective, and responsible conduct of animal research.

It is vital that the well-being of the animals be ensured to the maximum extent possible during the conduct of research. This requires balancing research goals with the compassionate use of the animals. By and large, current animal research is conducted responsibly. This was not always so, however. Before and during the 1960s, a relatively small number of experimenters were found to be involved in mistreatment of animals as well as questionable practices for acquiring their animal research subjects. When this information became public, people were justifiably enraged. Stories of animal abuse and stolen pets flooded the evening news and the press (2). In response to public outrage and mounting pressure from animal advocacy groups, Congress passed in 1966 the Federal Animal Welfare Act (3). This legislation effectively sanctioned the humane care and use of animals and established tight oversight mechanisms.

Although a detailed knowledge of animal research regulations and guidelines is not strictly required, scientists new to animal research in addition to those established researchers who have recently joined a new institution would do well to become acquainted with, and have a working knowledge of, applicable animal welfare regulations as well as institutional policies and procedures.

It is recommended that primary responsible research personnel (e.g., principal investigator [PI] and lab associates) contact the institutional attending veterinarian in the early planning stages of research not only to learn about the logistics of the particular animal facilities (e.g., security access requirements, availability of housing and procedural space, hours of operation, lighting schedules, and other restrictions) but also to seek assistance and advice with experimental design of studies, writing of grants and animal protocols and with getting the lab ready for mandatory inspections and site visits by the *IACUC*; the *U.S. Department of Agriculture (USDA)* and the *Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International* (see below) (4).

2. Primer on Regulations, Standards, and Guidelines Regarding Laboratory Animals

Animal research is tightly regulated via numerous laws and agencies (5). Rather than dictate how research should be conducted, the intent of the regulations is twofold: (i) minimize the use of animals in research and (ii) enhance the quality of life of research animals.

2.1. The "3Rs"

The spirit of the regulations is embodied by the principles of animal experimentation known as the "3Rs": Replacement, Reduction, and Refinement (6).

Replacement refers to the use of non-animal alternatives in research, such as in vitro methods and mathematical or computer (*in silico*) modeling. Its interpretation may be extended to the replacement of higher animal species with phylogenetically lower species. The latter is based on the assumption that animals with less developed central nervous systems are less likely to feel pain, distress, and other negative sensations.

Reduction means that the number of animals used in research should be reduced to the lowest necessary that will yield biologically and statistically significant data. This principle applies to both minimizing animal numbers in individual studies and avoiding *unnecessary* duplication of published results.

Refinement refers to the efforts toward minimizing animal pain, distress, discomfort, fear, boredom, and other negative experiences during the conduct of research. Refinement is achieved through the use of proper animal husbandry, environmental enrichment, handling, restraint, and the appropriate use of drugs, such as anesthetics, analgesics, and tranquillizers.

2.2. Key Points Regarding Regulations

Regulations and standards can be confusing, as their applicability varies with the species of animal under consideration, the source of the funding, and the institutional accreditation status. Although there is considerable regulatory overlap, the vast majority of responsible institutions follow the stricter standards. The regulatory maze of animal research is easier to navigate if we keep in mind a few key points:

2.2.1. Some "Rules" Have the Force of Law

The *Animal Welfare Act* (AWA) and associated *Animal Welfare Regulations* (AWR) (7) are administered and enforced by the USDA. The *Health Research Extension Act of 1985* (8) and associated *Public Health Service (PHS) Policy* (9), and *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* (10) are administered and enforced by the Office of Laboratory Animal Welfare (OLAW) at the National Institutes of Health (NIH) (11). In addition, some

states require the filing of special permits. For example, California's Department of Fish and Game mandates that *Restricted or Detrimental Species* permits be filed for the housing or breeding of *Xenopus* frogs, transgenic zebrafish, and other exotic or genetically modified species (12).

2.2.2. Some Non-legislated Standards Have Become De Facto Law

The Institute of Laboratory Animal Research (ILAR), a branch of the National Academy of Sciences, publishes the *Guide for the Care and Use of Laboratory Animals* (13) with support from the NIH, the USDA, and the Department of Veterans Affairs. Though the latest version of this document was published in 1996, a revised edition is expected in mid-2010. As of this writing, the 2010 edition has not yet been adopted. The *Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International*, a private, nonprofit organization, that promotes the humane treatment of animals in science through voluntary accreditation and assessment of animal programs, uses *The Guide* and other animal research regulations as its reference documents and publishes useful position statements on best practices with regard to animal care, staff training, and occupational health.

2.2.3. Different Regulations may Apply to Different Species

Whereas the PHS policy and the guide apply to all live vertebrate species, the AWA and AWR apply only to what is known as "USDA-covered species." The AWR define "animals" as "any live or dead dog, cat, nonhuman primate, guinea pig, hamster, rabbit, or any other warm-blooded animal, which is being used, or is intended for use, for research, testing, experimentation, or exhibition purposes, or as a pet". The AWR explicitly excludes "birds, rats of the genus *Rattus*, and mice of the genus *Mus*, bred for use in research, horses not used for research purposes, and other farm animals, such as, but not limited to livestock or poultry, used or intended for use as food or fiber, or livestock or poultry used or intended for use for improving animal nutrition, breeding, management, or production efficiency, or for improving the quality of food or fiber". It is worth noting that "*rats of the genus* Rattus, *and mice of the genus* Mus*, bred for use in research*" comprise the vast majority of laboratory animal species currently used in the USA.

3. Establishing and Managing Key Relationships

As indicated previously, the importance of establishing a positive, cordial, and professional relationship with the veterinary service, the IACUC, and the Administration cannot be overemphasized. Take time to get to know them in person during the planning of studies and ask for input with grant and protocol writing. Waiting

until a problem arises may start the relationship on a negative note and set the wrong tone going forward. The benefits derived from developing trust and rapport will more than pay off, likely resulting in greater cooperation and assistance with experimental design, protocol reviews, and grant transfers. No doubt, this will save time, money, and aggravation down the road as well as minimize research delays and non-compliance incidents. It is also important to spend some time learning about services offered by the veterinarian or the animal technical staff (e.g., breeding, injections, drug administration, surgery, pathology, laboratory tests). Knowing that experimental animals are in the competent hands of husbandry and veterinary staff members will give researchers peace of mind and allow them to focus their time and energy on their specific research.

The veterinary staff will welcome your help with identifying and reporting animal health or husbandry problems that may have been found by you or your staff during the course of your studies. Problems occasionally arise that may not have been obvious during routine health rounds by the veterinary staff or may have developed quite quickly (such as a flooded mouse cage). Some of these problems, if undetected or unreported, could endanger the animals and your research. There should be no hesitance in contacting the veterinary staff for assistance or to notify them early of health issues or logistical problems.

When establishing and managing relationships, do not forget to include the individuals in top administrative posts that support your research in less direct—though no less important—ways. Such influential members may include the dean of your school, the Provost, the Vice Chancellor, or the Vice President for Research. More than likely, one of them is the appointed Institutional Official (IO). The IO oversees the IACUC activities and evaluates the performance of the attending veterinarian. The IO is ultimately (and legally) responsible for ensuring animal research at his or her institution is conducted humanely and in compliance with all applicable regulations. It is also the IO's job to ensure sufficient resources (mainly money and space) are allocated to the animal program to maintain or improve its quality and effectiveness. Enlisting the IO's cooperation can be invaluable in the likely event that you might need financial or logistical assistance, especially with startup costs, subsidization of certain research activities with high institutional priority, specialized equipment purchases, or to cover unforeseen expenses.

Other valuable relationships include the office of Contracts and Grants, Physical Plant or Facilities Management, Capital Programs, and the organization's Security or Police departments. The latter is particularly important to protect researchers and their research from becoming the target of animal rights activists. Animal rights organizations are known to have infiltrated research

laboratories or animal resources departments and post online recorded videos of lab animal-related activities. More recently, non-violent tactics have yielded to violent terrorist acts, such as fire bombings of researchers' residences and vehicles. Because of this, it is advisable to conduct thorough background checks on potential lab employees—and conduct your research in strict accordance with your approved animal protocol.

4. Animal Facilities and Equipment

4.1. Housing and Procedural Space

4.1.1. Centralized Versus Decentralized Facilities

Vivarium facilities may be centralized, decentralized or combinations of both.

Centralized facilities have the advantage that all activities involving animals (housing, breeding, and experimental procedures) occur within a single, secure, and environmentally controlled area. This is particularly important when working with rodents, as their health or infectious status may preclude transporting animals from one facility to another for breeding or experiments. Another advantage of a centralized vivarium is economies of scale as a result of availability of shared resources (personnel, equipment, and services). The greatest disadvantage of centralized facilities is their relative vulnerability to catastrophic losses due to power failures, natural disasters, infectious outbreaks, and animal rights attacks, which may potentially decimate large populations of valuable experimental animals.

Decentralized facilities consist of multiple vivaria, typically under the management of the animal resources department. Advantages of decentralized facilities include greater protection against catastrophic losses and, in general, added convenience for researchers, as the animal facility may be located near the investigator's laboratory. Disadvantages of decentralized facilities include less efficient use of personnel, equipment, and services as well as duplication of resources and need for additional security.

Regardless of the type of housing, it is imperative that researchers receive appropriate instruction on the humane care and use of animals, in addition to proper facility orientation and regulatory instruction and training from the animal resources staff. Specifically, researchers may be required to obtain security clearances, keys, or electronic card access to the animal facilities and must learn about applicable standard operating procedures (SOPs).

The choice of location for a vivarium or study area is also critical. In particular, the structural integrity of facilities for the housing of aquatic species must be carefully assessed, as the weight of water-filled tanks can exceed the maximum weight tolerance

of the floor. For this reason (and to mitigate potential floods), most aquatic facilities are set up in the bottom floor of a building, immediately above the structure's slab.

4.1.2. Assignment of Vivarium Housing Space

This critical and delicate issue is typically delegated to the attending veterinarian or to the facility manager. In most instances there is good rationale for the fair assignment of space, such as degree of grant support or departmental or institutional priorities. In other cases, space is assigned on a first-come-first-serve basis. Nevertheless, this process is almost always highly political and inherently flawed. It is advisable to contact your animal resource department well in advance of planned experiments or if you anticipate an imminent or significant increase in the need for animal space, e.g., when a grant is funded or has received a high priority score. Furthermore, the attending veterinarian may be in a position to lobby on your behalf to help you secure needed support from the administration regarding justification for additional space.

4.1.3. Study Areas or Satellite Facilities

Another form of animal housing is what is variously known as "study areas" or "satellite facilities." The Animal Welfare Regulations define "study area" as any building room, area, enclosure, or other containment outside of a core facility or centrally designated or managed area in which animals are housed for more than 12 h. The PHS Policy defines a "satellite facility" as any containment outside of a core facility or centrally designated or managed area in which animals are housed for more than 24 h. Study areas or satellite facilities are essentially "pockets" of vivarium space scattered throughout a campus or a particular building. Generally, these rooms are an extension of an individual researcher's laboratory whose own personnel look after the husbandry or breeding of the animals.

Investigator-maintained study areas are particularly prevalent among research laboratories housing aquatic species, such as zebrafish and *Xenopus* frogs, although they can also be part of specialized ("core") applications, such as surgery, imaging, or electrophysiology centers (see below).

Satellite facilities must conform to all federally mandated environmental standards for housing of laboratory animals, such as appropriate lighting cycles; control of temperature and humidity within acceptable ranges for the species; adequate ventilation, sanitization, and disinfection methods; and animal health check schedules. Researchers who maintain study areas are required to file with the IACUC a detailed SOP for the facility; maintain and periodically submit records of staff training and daily room activities, such as health checks, husbandry practices, and room sanitization; and undergo IACUC inspections at least semiannually.

In practice, IACUCs and institutional administrators, as well as regulatory agencies and accrediting bodies, typically discourage

the establishment or maintenance of study areas for multiple reasons, such as occupational health concerns, relatively lax security, and, frequently, inadequate veterinary oversight (see under *Disadvantages*). Study areas must be inspected, qualified, and approved by the IACUC prior to their use for animal housing or procedures. Though at times it might be tempting to house animals or do experimental procedures in unapproved areas, such as your own laboratory, you must be aware that such practice is universally condemned by IACUCs and is a serious noncompliance reportable to both the NIH/OLAW and the USDA.

Advantages of maintaining a study area include:

(1) Added researcher freedom, convenience, and flexibility of access and schedules;

(2) Protection of unique, delicate, or valuable equipment;

(3) Avoidance of per diem charges normally assessed in regular vivaria.

Disadvantages of maintaining a study area include:

(1) Decreased personal health and safety. The main occupational health risk is exposure to laboratory animal allergens (LAA). Personnel working directly with the animals (primary exposure) as well as those involved in nearby non-animal activities (incidental or "second-hand" exposure) are more likely to become exposed to LAA in a study area setting. This is due to (a) lack of specialized protective equipment, such as change stations, laminar flow hoods, and bedding disposal units, which are typically available in regular centrally managed vivaria, and (b) lack of or inappropriate use of personal protective equipment (PPE) or apparel that would otherwise be mandatory in regular vivaria. Additionally, personnel experiencing incidental exposure are less likely to be enrolled in institutional occupational health and safety programs or receive awareness training in this area;

(2) Compromised animal health. Infrequent or less rigorous veterinary health checks of animals housed in study areas may lead to the delayed detection of sick or injured animals;

(3) Compromised animal biosecurity. Insufficient or improper room sanitization or health surveillance practices can result in higher incidence of infections among laboratory animals. In addition, the usual absence of health surveillance programs in study areas can lead to delayed detection of outbreaks of animal infection or disease. Cross-traffic of research personnel between study areas and vivaria can potentially endanger the health status of the latter by sparking infectious outbreaks;

(4) Lower physical security. Reduced security can potentially lead to equipment theft or cause research staff to become exposed to harassment or attacks by animal rights activists and other criminal activities;

(5) Absence of economies of scale. Added equipment expenses and duplication of resources (personnel, equipment, supplies), as the provision of caging equipment (racks, cages, tanks) necessary for the housing of animals in study areas is typically the financial responsibility of the PI, whereas in regular vivaria these expenses are covered by grant funds or are built into the per diem rates;

(6) Inefficient and costly utilization of lab space. Valuable laboratory space is typically sacrificed or lost to the housing or breeding of animals. In addition, necessary physical renovations, upgrades to the ventilation system, and general maintenance of the study areas are the financial responsibility of the investigator. In most instances, a realistic cost–benefit analysis tends to favor housing animals in centrally managed vivaria;

(7) Compliance costs. Researchers maintaining animals in study areas may incur potential additional expenses for IACUC inspections and veterinary health checks. Since these inspections are mandated by law, reduced cost-effectiveness associated with decentralization may lead to the assessment of compliance charges, which are normally covered by institutional indirect cost recovery funds are not typically built into the per diem rates.

4.1.4. Satellite Surgical Facilities

Institutions that lack a central surgical resource have little choice but to approve the establishment of researcher-maintained satellite surgery facilities, typically within the confines of a researcher's laboratory. Even when a central surgery area may be available, investigators may successfully justify the creation of their own satellite surgery areas on the basis of their need for specialized surgical or data collection equipment that may not be available, practical or safe to place in a centrally managed shared resource. By and large, the pros and cons listed above for study areas apply also to satellite surgery facilities. One important additional aggravating factor with satellite surgeries is inability of the institutional veterinary staff to adequately monitor (if at all) the surgical manipulations, post-operative recovery, and pain management of animals undergoing surgery in remote locations. The latter may lead to institutional criticism by external regulatory or accrediting bodies.

4.1.5. Research Core Facilities

These are specialized forms of satellite study or surgical areas. Such dedicated core facilities may be devoted to maintaining ani-

mal breeding colonies (e.g., mice, zebrafish); providing assisted reproductive technologies services (creation of transgenic or other genetically manipulated animals, rederivation, embryo or gamete cryopreservation, or in vitro fertilization); imaging technologies (e.g., MRI, PET, CT scanning, optical imaging, ultrasonography); behavioral testing of rodents, fish, or nonhuman primates; surgery; metabolic and nutritional studies; irradiation; biocontainment, etc. Besides being subject to the same minimum standards as study or satellite surgery areas with regard to IACUC approval and oversight, some of these facilities may be subject to even higher standards and additional requirements. For example, because of their potential for nefarious misuse or bioterrorism, users of irradiation facilities based on radioactive sources (e.g., cesium or cobalt) and the use of microbial "select agents" are subject to background checks by the Department of Homeland Security and the FBI, biometric identification and required to maintain extraordinary security measures, such as sophisticated alarm systems connected to a central security office or a campus police department.

5. Animals, Equipment, and Supplies

5.1. Sources of Laboratory Animals and Tissues

The quality and reliability of research data obtained from animals is closely correlated with the quality of the animals, particularly their genetic purity (i.e., inbred strain versus outbred stocks), microbiological status and incidence of injury or disease, as well as the integrity and consistency of the environmental conditions. Concurrent infections that may not show external signs of disease (subclinical) can still affect research results in profound ways (14, 15). Reputable animal vendors have stringent health surveillance programs in place and generally their animals are free of specified pathogens. This is particularly true for the main rodents and rabbit suppliers. In the rare cases when an infectious outbreak is detected in their commercial breeding facilities, they immediately contact the institutional purchasing agents, who in turn notify the attending veterinarian and researchers who may have purchased animals from a contaminated source. Because health status of aquatic species, particularly *Xenopus* frogs and zebrafish, is less well defined, the choice of suppliers becomes all the more critical. Annually published laboratory animal buyers guides are good resources for information regarding animal suppliers (16, 17).

It is important to procure animals from institutionally approved sources, using proper purchasing or animal transfer channels and abiding by any Materials Transfer Agreement (MTA) restrictions. In addition, the creation or importation of

genetically modified animals, usually transgenic or knockout mice, may require registration with the Institutional Biosafety Committee (IBC) in accordance with the NIH Guidelines for Research Involving Recombinant DNA (18). Prior to importing new animal genetic lines, it is recommended that you consult with the IBC to find out whether the desired strain of animal may already be available in your organization. This extra step may help you minimize research delays and save a significant amount of grant or departmental money.

Responsible institutions have strict animal quarantine or rederivation requirements for the importation of animals from non-traditional sources. The goal of these mechanisms is to protect institutional animal colonies against the introduction of infectious agents. It is critical that you consult with the institutional veterinarian prior to the importation of animals from non-routine suppliers.

Tissues and biologic products of animal origin (mainly cell lines and serum) may have become contaminated with pathogens, which could trigger outbreaks of disease at your institution upon their inoculation into animals housed in a "clean" colony. Prior to introduction, these products must be assessed by polymerase chain reaction (PCR) for the presence of pathogens.

Tissues and biologic products of human origin may also carry pathogens that could potentially expose animals and animal workers to infection or disease. Additionally, use of primary tissues or cells from human patients may require approval from the Institutional Review Board (IRB). If you plan to work with human embryonic stem cells in animal research, be aware that the National Academies called in 2005 for the establishment of institutional Embryonic Stem Cell Research Oversight (ESCRO) committees and provided guidelines for the management of ethical and legal concerns in human embryonic stem cell research (19).

5.2. Equipment

The conduct of animal research requires that appropriate housing (racks, cages, tanks) be provided for the well-being and comfort of the animals in order to maximize reliability and reproducibility of research data. As indicated above, provision of physical space, maintenance of proper environmental conditions, and supply of personal protective equipment (PPE) are typically the responsibility of the animal resources department in centrally managed vivaria whereas they become the researchers' responsibility in investigator-maintained satellite facilities. Researchers must be aware that the goal of wearing appropriate PPE is to protect the health of the animals (biosecurity) as well as that of the researchers and animal care personnel (occupational medicine).

5.3. Feed, Bedding, and Water

The quality and reliability of data obtained from research animals are also closely linked to the quality of the feed, bedding, and water used. Reputable suppliers of feed and bedding offer consistent, high quality, products. The goal is to maintain consistent environmental conditions for research animals to avoid the introduction of uncontrolled variables. The Buyers' Guides mentioned above also list feed and bedding suppliers. Keep in mind that even high-quality feed and bedding may cause unwanted research interference. For example, seasonal variation in the phytoestrogen content in standard rodent diets can affect reproductive function in mice. Similarly, corncob bedding may be inappropriate for certain metabolic or nutritional studies in rodents where strict fasting may be necessary.

The quality of the drinking water is critical for immunocompromised animals, as even ubiquitous or opportunistic bacteria and fungi can infect the animals causing disastrous clinical or subclinical problems.

The quality of the tank water is absolutely vital for amphibians and fish. Commercial test kits are available to measure critical parameters, such as alkalinity, hardness, pH, salinity/conductivity, chlorine/chloramines, ammonia, nitrite, and nitrates.

6. The Animal Research Environment

6.1. Protecting Researchers and Their Staff from Research Risks

Researchers must be aware of the risks involved in working with animals and enrol appropriate staff in the institutional Occupational Health and Safety program (OHSP). Generally speaking, research institutions have broad OHSP guidelines as well as requirements that are specific to particular research or species of animal used. Requirements may range from the filing of a medical history questionnaire, to specific vaccinations, TB testing, Hepatitis B, or other disease serum titers, to respiratory function tests and serum banking. Some infectious risks are inherent to working with certain animal species (Herpes B virus in macaque monkeys, Q fever in sheep, Chlamydia in birds) whereas physical injury due to bites, scratches, and splashes may happen with just about any species.

Perhaps the most important non-infectious occupational risk of working with animals is the development of laboratory animal allergies (LAA). Allergy symptoms can range from mild skin, eye, or nose irritation to fatal asthma attacks. Exposure to animal dander, mouse urine proteins, or skin of aquatic species may sensitize workers to LAA. To learn more about occupational hazards you may wish to consult the book, *Occupational Health and Safety in the Care and Use of Research Animals* (20).

When planning to do animal work with specific infectious microorganisms, biologic toxins or recombinant DNA, the IBC should be contacted for guidance with completing the protocol, assignment of appropriate biocontainment and protection levels and room signage. CDC's Biosafety in Microbiological and Biomedical Laboratories (21) is an excellent resource on appropriate biocontainment levels and engineering protective controls. Recombinant DNA guidelines can be found on NIH's Office of Biotechnology Activities (OBA) web site (22).

When toxic chemicals (e.g., carcinogens or mutagens, such as BrdU, tamoxifen, paraformaldehyde) or radioactive agents are used with animals, the institution's environment health and safety office or radiation safety departments should be contacted for guidance on appropriate measures to protect your staff as well as animal care personnel from inadvertent exposure to hazardous reagents. Whenever possible, consider the use of non-infectious, non-radioactive, or less toxic alternatives to hazardous research reagents.

6.2. Non-surgical Procedures on Laboratory Animals

The most common non-surgical techniques are injections, administration of oral substances (gavage), and collection of blood and other tissues or fluids. Although most non-surgical procedures are minimally invasive and cause only slight or momentary pain, it is sometimes necessary to provide anesthesia to the animals for the purpose of immobilization. It must be recognized that certain nonsurgical procedures have the potential to cause distress and are closely scrutinized by IACUCs and animal advocacy groups. Examples of these procedures are prolonged physical restraint of conscious animals, food or water restriction, or deprivation and use of death as an endpoint (typically in infectious or tumor-related studies).

6.3. Surgical Procedures on Laboratory Animals

The conduct of surgery on laboratory animals is subject to federal and institutional requirements that vary with the species under consideration and with the nature of the surgery. Regardless of the type of surgery, all animals must be provided adequate anesthesia at sufficient levels that will abolish consciousness and pain perception during the procedure. Animals intended to recover from the procedure (i.e., survival surgery) must be given adequate pain relief (analgesia) for the expected duration of the painful phase of the post-operative recovery. Though the length of the potentially painful period will depend on the invasiveness of the procedure, as a general guideline, post-operative analgesia should be given for a *minimum* of 48 h after major surgery or 24 h after minor surgery. However, the animal should continue to be assessed for signs of pain following this period and analgesia administered when necessary.

Knowledge of the following definitions from the AWA and key points from the regulations will help to determine how to adequately categorize your animal protocol:

(a) *Major surgery* (Major operative procedure): This is defined in the AWR as "any surgical intervention that penetrates and exposes a body cavity or any procedure which produces permanent impairment of physical or physiological functions." Major operative procedures on USDA-covered species must be conducted only in facilities intended for that purpose (i.e., dedicated operating room) operated and maintained under aseptic conditions. Rodent, bird, fish, and amphibian surgery may be done in clean and temporarily dedicated areas of the lab. The veterinarian or IACUC representative should be consulted for specific guidance regarding appropriateness of the surgery areas.

(b) *Survival surgery*: Animals are intended to recover from anesthesia (i.e., regain consciousness) after the procedure. All survival surgical procedures must be conducted using aseptic technique to minimize contamination of the wound or the development of systemic infection after the procedure. The indication for aseptic preparation of the skin in *Xenopus* frogs and other aquatic species is controversial, however. Chemical antiseptic agents may disrupt the normal skin flora of these species whereas surgical drapes can easily damage their delicate skin. NIH veterinarians report that the incidence of clinical complications following surgical oocyte harvesting without strict aseptic preparation is rare. On the other hand, post-surgical pain relief must always be provided unless scientifically justified by the PI and approved by the IACUC.

(c) *Non-survival surgery*: Animals are euthanized at the conclusion of the procedure *before* they regain consciousness.

(d) *Multiple major survival surgery*: Multiple surgeries *on the same animal* may be done only as an integral part of a protocol, i.e., if the two or more surgeries are related components of a single study. Multiple major survival surgeries must be scientifically justified by the PI and approved in advance by the IACUC. Consult USDA Policy #14 for details on this topic (23).

The veterinarian should be consulted if surgical training is needed. Alternatively, arrangements may be made for qualified members of the veterinary staff to perform the experimental surgeries for the research lab.

7. Pain and Distress in Laboratory Animals

The recognition and alleviation of pain and distress in laboratory animals is a difficult subject to address because their expression varies greatly among the different species used in research settings. Mild to moderate pain in lower species, such as fish, frogs, rodents, and chickens, may be nearly impossible to recognize. For details on this subject, the reader is referred to the texts, *Recognition and Alleviation of Pain in Laboratory Animals* (24) and *Recognition and Alleviation of Distress in Laboratory Animals* (25).

Because of these limitations in the proper recognition of signs of pain and distress, animals must be given the benefit of the doubt regarding the need for pain medication. When in doubt, the best approach is to administer painkillers to the animals unless withholding such medications has been scientifically justified by the PI and approved by the IACUC. The U.S. Government Principles IV, V, and VI address the issue of pain and distress thus (26),

> IV. Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals.
>
> V. Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia, or anesthesia. Surgical or other painful procedures should not be performed on unanesthetized animals paralyzed by chemical agents.

VI. Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed at the end of the procedure or, if appropriate, during the procedure. The three main drugs used to relieve pain, distress, and anxiety in laboratory animals are anesthetics, analgesics, and tranquilizers.

Anesthetics can have local, regional, or general effects. Local anesthetics typically abolish pain perception in specific areas of the body where they are injected or applied. Regional anesthetics block specific nerves that supply larger areas of the body, which become desensitized by the injection of a local anesthetic agent. Neither local nor regional anesthetics depress the central nervous system (CNS) and therefore the animal remains conscious. General anesthetics depress the CNS leading to loss of consciousness, chemical immobilization, and lack of pain perception after injection, inhalation, or topical exposure (such as for fish and amphibian anesthesia).

Local anesthetics, such as lidocaine (Xylocaine®) and bupivacaine (Marcaine®), are injected into the skin or immediately

underneath (subcutaneously) in order to numb the area and block pain sensation that might otherwise result from a surgical incision or the insertion of a large-bore needle (trocar). Local anesthetics may be applied also topically to skin or mucous membranes or injected, much in the same way dentists do on human patients. General anesthetics include agents such as isoflurane inhalant gas, injectable agents, such as ketamine/xylazine combination and pentobarbital (Nembutal®), and topical tricaine methanesulfonate (MS-222). The latter is commonly used for general anesthesia of aquatic species and is applied by immersion and exposure of the animals in medicated water solution.

Analgesics (painkillers) are drugs that abolish or minimize pain perception after systemic administration, usually orally or by injection. Common analgesics include morphine and related compounds (buprenorphine, oxymorphone) as well as drugs that have both analgesic and anti-inflammatory properties, such as ibuprofen (Advil®), carprofen (Rimadyl®), and meloxicam (Metacam®). Even though animals under general anesthesia do not feel pain, it is important to administer an analgesic also in order to control post-operative pain upon recovery from anesthesia. It is best if the analgesic is given pre-emptively, i.e., before the surgical incision is made, as this will likely reduce the postoperative need for analgesia.

Tranquilizers are seldom used in laboratory animals except in large animals, mainly dogs, cats, rabbits, nonhuman primates, and farm animals. The drugs most commonly used are diazepam (Valium®), midazolam (Versed®), and acepromazine.

On occasion, the administration of analgesics or tranquilizers may interfere with the goal or outcome of the study. In these cases, researchers must present to the IACUC detailed scientific justification for withholding pain medication subsequently to a potentially painful or distressful procedure. These studies are classified as "Pain category E"—based on the USDA annual report form 7023—and undergo extensive IACUC review.

8. Neuromuscular Blockers (NMB; Paralyzing Drugs)

The use in animal research of paralyzing drugs, such as vecuronium and curare, is a delicate matter and one that receives close scrutiny from IACUCs and veterinarians. The concern is that a paralyzed animal is unable to display signs of pain—not unlike the unfortunate situation where an insufficiently anesthetized human patient wakes up during surgery but, because of the effects of the NMB, is unable to signal perception of pain.

Therefore, use of NMBs must be accompanied by the administration of general anesthetics and assurance of proper respiratory function (ventilation). In mammals, ventilation of a paralyzed animal is accomplished by endotracheal intubation and the use of respirators (ventilators) or manually assisted ventilation. Use of NMBs in the absence of general anesthesia requires extraordinary scientific justification by the PI and IACUC approval.

9. Euthanasia of Laboratory Animals

Euthanasia means painless and humane killing and, with few exceptions, it is administered to laboratory animals at the conclusion of a study as soon as possible after the experimental endpoint described in the research protocol has been reached. Euthanasia is also administered for humane reasons if the animals experience unanticipated pain and distress, even if the experimental endpoint has not yet been reached.

Although the choice of euthanasia method and agent largely depends on the animal species and the scientific goals, whenever possible, animals should be administered an overdose of anesthetic, such as inhalant isoflurane, injectable pentobarbital, or topical MS-222. This is preferable to physical methods, such as exposure to ice water, decapitation, cervical dislocation, pithing, or exposure to high concentrations of carbon dioxide. The document, *Guidelines on Euthanasia* from the American Veterinary Medical Association (AVMA) (27) is the most comprehensive resource on this subject and forms the basis for what regulatory agencies in the USA consider acceptable. The most recent version of this document was published in 2007 and periodic revisions appear every 5–7 years. NIH also has developed guidelines on euthanasia of embryos, fetuses, and adult animals of various species and published other relevant references (28, 29).

When making specific recommendations on euthanasia methodology, guidelines generally distinguish between adult and fetal or larval stages based on their degree of development of the neural mechanisms necessary for detecting noxious stimuli and perceive pain (28). For example, there is no evidence to indicate that zebrafish are capable of perceiving pain or distress during the first week of development. Consequently, zebrafish embryos ≤ 7 days post-fertilization (dpf) may be euthanized by exposure to a bleach solution. For ≥ 8 dpf zebrafish, on the other hand, the preferable method of euthanasia is an overdose of tricaine (MS222) by immersion for ≥ 10 min after opercular movement ends. Other acceptable methods (e.g., when the previous method interferes with data collection) include (i) immobilization by

submersion in ice water for at least 10–20 min following cessation of opercular movement, (ii) anesthesia with MS222 followed by rapid freezing in liquid nitrogen, or (iii) decapitation with a sharp blade (28). The latter physical method requires scientific justification and IACUC approval.

While adult rodents are typically euthanized by an overdose of anesthetic of exposure to CO_2, in embryos and fetuses up to day 14 gestational age, neural development is minimal and pain perception is unlikely. For this reason, euthanasia of the mother (using methods appropriate for adult rodents) or removal of the embryos or fetuses from the uterus is considered acceptable as this would ensure their rapid death due to interruption of oxygen supply and lack of independent viability at this developmental stage. Fetuses at 15 days of gestation or older may have developed neural pain pathways and the possibility of pain perception. Because of their lack of sensitivity to inhalant anesthetics and CO_2, however, unconsciousness must be induced by chilling on a wet ice slurry and euthanasia administered by decapitation with sharp surgical scissors or rapid freezing in liquid nitrogen (29).

10. Following Protocol, Policies, and Procedures

Principal investigators and staff members must be thoroughly familiar with approved protocols. Adherence to the research protocol as approved by the IACUC and all applicable policies, rules, and regulations is paramount.

The PHS Policy establishes that the following instances must be reported to NIH/OLAW (30):

(1) Any serious or continuing noncompliance with this Policy,

(2) Any serious deviation from the provisions of the *Guide*, and

(3) Any suspension of an activity by IACUC

10.1. Common Causes of Noncompliance

Common causes of noncompliance include the following:

a. *Failure to respond promptly to veterinary directives.* Institutions and regulatory agencies fully expect that researchers will respond promptly to communications from the veterinary service relative to the health and well-being of research animals under their protocols, especially when following its directives and recommendations with regard to treatment or euthanasia of sick or injured animals. At least one veterinarian serves on the IACUC and he or she has delegated authority to suspend research activities if the health, welfare, or safety of the animals is at risk.

b. *Failure to follow the approved protocol.* Be aware that even seemingly minor departures, such as failure to list staff members who work with the animals (including temporary students) or slight changes in the experimental design, can have severe repercussions and trigger lengthy and painful investigations of noncompliance during which a protocol is likely to be suspended. Not only will the suspension delay the study but can also result in exposure of the institution (and the researcher personally) to criticisms or attacks from animal rights and advocacy groups. It is essential that the veterinarian be consulted regarding any planned protocol changes and IACUC approval secured before the implementation of any protocol related changes. Some changes may not require *immediate* IACUC approval, however. These typically involve refinements of your study as a result of a veterinary recommendation (with verbal or written concurrence from the IACUC), such as substituting anesthetics or analgesics with the goal of improving the welfare of the animals. Nevertheless, changes that are intended to become permanent must be formalized in an amendment to the original research protocol.

c. *Conducting research without IACUC approval* (absence of an approved protocol; use of an expired protocol). These violations are automatically reported to NIH or USDA, as appropriate, and usually result in lengthy suspension of animal research privileges or placement of a principal investigator on probationary status.

It is worth noting that, in addition to the research delays, there may be financial repercussions from NIH for conducting research in a manner that violates PHS Policy, such as during protocol suspension or after protocol expiration. According to a recent guidance document from NIH-OLAW, institutions must certify that PHS funds were not used during continued care and maintenance of animals during periods of protocol suspension (due to noncompliance) or IACUC expiration of protocols (31).

10.2. Responding to Noncompliance Reports

Frequent and effective communication with the attending veterinarian and the IACUC will avert many problems related to animal health and noncompliance issues. Many IACUCs and veterinary services publish newsletters or publish-regulatory and policy updates on their web sites. Be sure to check these regularly.

Nevertheless, if you are presented with a report or an allegation of noncompliance, the best course of action is to fully cooperate with the IACUC's investigation by providing prompt and candid responses. IACUCs recognize that honest mistakes do occasionally happen and they generally take steps to handle the noncompliance in a manner acceptable to the regulatory agencies while attempting to restore the protocol to active status as soon

as possible. This may not be the case if the IACUC perceives that the PI is not forthcoming with the required information, attempts to hide or distort facts or stonewalls the investigation.

11. Conclusion

Responsible conduct of animal research is expensive, onerous, and subject to myriad restrictions and strict regulatory oversight. Nevertheless, in many instances animal research is necessarily the only vehicle available to answer particular scientific questions. Whenever possible, however, researchers should make every effort to pursue or consider non-animal alternatives (*Replacement*). When these are unavailable, attention should be directed at minimizing the number of animals used (*Reduction*) as well as reducing the invasiveness of the interventions in an attempt to minimize pain and distress (*Refinement*). Collaborations with the veterinary staff and other key members of the animal program will facilitate navigation of the regulatory maze, expedite fulfilment of training and administrative protocol requirements, and streamline the overall conduct of your studies. When things do not go as planned and regulatory noncompliances occur, it is best to admit errors, cooperate fully with any investigation, be forthcoming with the requested information, and learn from the experience in order to avoid making similar mistakes in the future. Bear in mind that the regulations are designed to protect animal welfare as much as they are for the protection of personnel from occupational risks and protection of the institution from regulatory sanctions and criticism or attack from animal rights activists, including violent terrorist acts.

References

1. IACUC Guidebook, 2nd edition, 2002, http://grants.nih.gov/grants/olaw/GuideBook.pdf
2. Adams, B. and Larson, J. (2009). Legislative History of the Animal Welfare Act. USDA. National Agricultural Library. Animal Welfare Information Center, http://www.nal.usda.gov/awic/pubs/AWA2007/intro.shtml
3. Federal Animal Welfare Act. USDA National Agricultural Library http://awic.nal.usda.gov/nal_display/index.php?info_center=3&tax_level=4&tax_subject=182&topic_id=1118&level3_id=6735&level4_id=11092&level5_id=0&placement_default=0
4. Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International. http://www.aaalac.org/
5. NIH Office of Laboratory Animal Welfare http://128.231.74.189/grants/olaw/olaw.htm
6. Russell, WMS., Burch, RL (1959). The principles of humane experimental technique. London: Methuen; http://ehpnet1.niehs.nih.gov/members/1996/104-8/zurlo.html
7. USDA-APHIS website http://www.aphis.usda.gov/animal_welfare/downloads/awr/awr.pdf
8. NIH website http://grants.nih.gov/grants/olaw/references/hrea1985.htm

9. DHHS-NIH website http://grants.nih.gov/grants/olaw/references/phspol.htm
10. NIH website http://fmp-8.cit.nih.gov/oacu/guidepi/references/2govprinciple.pdf
11. DHHS-NIH website http://128.231.74.189/grants/olaw/olaw.htm
12. California Department of Fish and Game website http://www.dfg.ca.gov/licensing/pdffiles/fg1518.pdf
13. The National Academies Press. *The Guide* (1996); http://www.nap.edu/catalog.php?record_id=5140. The 8th edition (2010) of the Guide is published at: http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf
14. Baker, D. G. (1998). Natural Pathogens of Laboratory Mice, Rats, and Rabbits and Their Effects on Research. http://cmr.asm.org/cgi/reprint/11/2/231.
15. Baker, D. G. (2003). Natural Pathogens of Laboratory Animals: Their Effects on Research. ISBN-13: 978-1555812669. http://www.amazon.com/Natural-Pathogens-Laboratory-Animals-Research/dp/155581266X
16. Lab Animal Buyers' Guide; Nature Publishing Group: http://guide.labanimal.com/guide/index.html
17. ALN Buyers' Guide: http://www.alnmag.com/alnbg/
18. NIH Guidelines for Research Involving Recombinant DNA: http://oba.od.nih.gov/rdna/nih_guidelines_oba.html
19. The National Academies Press (2005). Guidelines For Human Embryonic Stem Cell Research: http://www.nap.edu/openbook.php?isbn=0309096537
20. Occupational Health and Safety in the Care and Use of Research Animals, 1997, http://www.nap.edu/catalog.php?record_id=4988.
21. Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th edition, http://www.cdc.gov/biosafety/publications/bmbl5/index.htm
22. NIH's Office of Biotechnology Activities (OBA) http://oba.od.nih.gov/rdna/nih_guidelines_oba.html
23. USDA Policy #14: http://www.aphis.usda.gov/animal_welfare/downloads/policy/policy14.pdf
24. Recognition and Alleviation of Pain in Laboratory Animals, 2009, http://books.nap.edu/catalog/12526.html
25. Recognition and Alleviation of Distress in Laboratory Animals, 2008, http://www.nap.edu/catalog.php?record_id=11931
26. U.S. Government Principles. http://grants.nih.gov/grants/olaw/references/phspol.htm#USGovPrinciples
27. American Veterinary Medical Association (AVMA). 2007. Guidelines on Euthanasia http://www.avma.org/resources/euthanasia.pdf
28. NIH Guidelines for Use of Zebrafish in the NIH Intramural Research Program. http://oacu.od.nih.gov/ARAC/documents/Zebrafish.pdf
29. National Cancer Institute. Guidelines for Euthanasia of Rodents. http://web.ncifcrf.gov/rtp/LASP/intra/acuc/fred/guidelines/ACUC18EuthanasiaGuidelines.doc
30. Wolff, A. 2009. Reporting noncompliant events to OLAW. Seminar. http://grants.nih.gov/grants/olaw/090305_seminar_transcript.pdf
31. DHHS-NIH-OLAW Guidance on Confirming Appropriate Charges to NIH Awards during Periods of Noncompliance for Activities Involving Animals. NOT-OD-10-081; Release Date: April 15, 2010. http://grants.nih.gov/grants/guide/notice-files/NOT-OD-10-081.html

INDEX

F.J. Pelegri (ed.), *Vertebrate Embryogenesis*, Methods in Molecular Biology 770,
DOI 10.1007/978-1-61779-210-6, © Springer Science+Business Media, LLC 2011

E

F

G

H

I

K

L

M

N

O

P

R

S

T

U

V

W

X

Z