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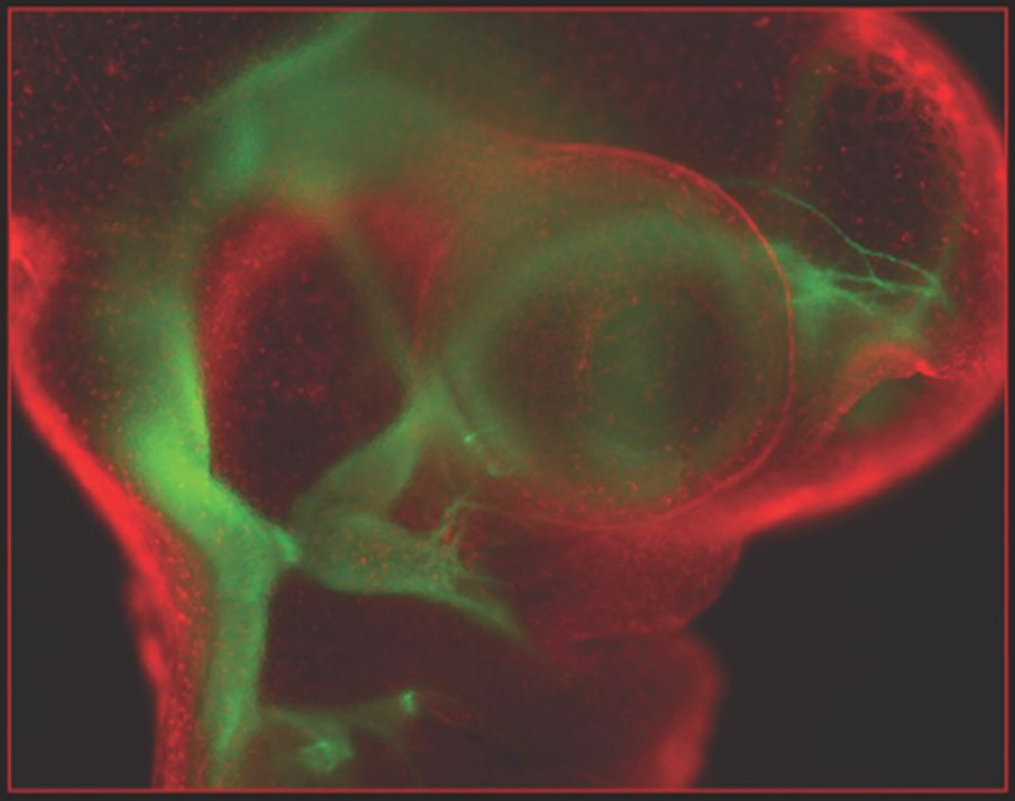
Avian Embryology

2nd edition



Edited by

Marianne Bronner-Fraser

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Methods in Cell Biology

VOLUME 87

Avian Embryology, 2nd Edition

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Methods in Cell Biology

VOLUME 87

Avian Embryology, 2nd Edition

Edited by

Dr. Marianne Bronner-Fraser

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Front Cover Photo Credit: Three day old quail embryo immunostained with anti-neuronal class III β-tubulin (green) to show the developing central and peripheral nervous systems, and with QH-1 (red) to show vascular development. *(Photograph by Peter Lwigale)*

Back Cover Photo Credit: Section through an quail-chick neural crest chimera in a three day old embryo immunostained with QCPN antibody (red) to show quail neural crest-derived contributions to the head. Section is counterstained with DAPI to show all nuclei. *(Photograph by Peter Lwigale)*

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PREFACE

For centuries, embryologists have been intrigued with the central question in developmental biology: How does a complex organism arise from a single cell? This question remains at the forefront of modern developmental and cell biology. Recent advances in molecular techniques, transgenesis, and genome sequencing have greatly added to the tool kit of approaches available to tackle this fascinating problem.

The developmental questions being asked in various systems and tissues are wide-ranging and include how cells proliferate, migrate, differentiate, and communicate. Different model organisms have proved particularly amenable to addressing these topics. For vertebrates, the mouse, zebrafish, frog, and chicken embryos are the most commonly used systems, the former two because of the ease of genetics and in the case of zebrafish, their transparency that allows imaging. The latter two organisms are favored for their ease of transplantation and amenability to experimental perturbation. Furthermore, avian embryos are particularly similar in their early development to human embryos. This coupled with new techniques in electroporation and other types of transgenesis as well as the sequencing of the chick genome have placed the avian embryo in a unique and prominent position for studies of vertebrate development.

This volume represents a compilation of diverse techniques for application to a number of important developmental questions that can be approached in the avian embryo. The methodologies range from classical embryological approaches to modern methods in molecular biology, image analysis, and genomic analysis. Chapters in this volume include descriptions of embryonic transplantations, cell culture, organ culture, *in situ* hybridization, dye labeling, electroporation and other methods of gene transfer, overexpression and loss-of-function analyses, and bioinformatics approaches to interrogate the chick genome. The unique feature of this volume is that it is specifically devoted to providing detailed approaches with adaptations that work optimally in avian embryos.

The authors have attempted to include detailed methodologies that describe both the potentials and pitfalls of various techniques. The goal was to allow direct implementation of these techniques in the reader's laboratory.

I thank all the authors for their thoughtful and thorough contributions and particularly for their attention to detail. I also take this opportunity to thank the excellent staff at Elsevier for helping bring this volume to fruition.

Marianne Bronner-Fraser

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PART I

Embryological Microsurgery and Tissue Culture Methods

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CHAPTER 1

Operations on Primitive Streak Stage Avian Embryos

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I. Introduction

The body plan of vertebrate embryos begins to be laid down just before and during the process of gastrulation, when the embryo first generates its three definitive germ layers: ectoderm, mesoderm, and endoderm from a single initial embryonic layer, the epiblast. In amphibians and fish, the two new cell layers arise by ingression through a blastopore (amphibians) or marginal zone and shield (teleost fish). In amniotes (reptiles, birds, and mammals), gastrulation occurs through a

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midline structure, the primitive streak. At the tip of the primitive streak lies the amniote organizer, Hensen's node. It is around the time of gastrulation that the different axes of polarity (head–tail, axial–lateral, and left–right) become fixed, that many cells are set aside for different lineages, and that the embryonic disc starts to form a bilateral axis. The acquisition of different fates is largely reliant on inductive processes, by which some cells emit signals that influence the fate of their neighbors. The mesoderm, endoderm, and neural plate are all thought to arise from the epiblast as a result of such inductive interactions (for review see Stern, 2004a,b).

To study these events, it is essential to be able to manipulate cells, either to map their normal fates in the embryo, or to challenge their developmental potential by placing cells in a new environment where they may be exposed to different signals, or to test the inducing properties of groups of cells by placing them adjacent to other putative responding tissues. This requires the embryo to be amenable to transplantation and culture to stages after these processes have taken place. The avian embryo lends itself perfectly to these manipulations: at the time of laying (equivalent to the blastula stage), it is a large (3 mm diameter), flat, and translucent disc that can be cultured easily to early organogenesis stages (at least the 15- to 20-somite stage) outside the egg. In this chapter, we present the basic techniques necessary for transplantation of cells between regions of the primitive streak stage embryo. We describe transplantation of Hensen's node between quail and chick embryos as a prototype example, and present variations of this technique for obtaining other tissues for grafting and guidelines for homotopic or heterotopic grafting.

II. Staging of Embryos

One advantage of avian embryos is that very precise staging systems have been described. The most widely used, covering from the appearance of the primitive streak until hatching, is that of Hamburger and Hamilton (1951) for chick embryos. A complete staging system also exists for quail embryos (Zacchei, 1961); however, this is rarely used and the Hamburger and Hamilton (HH) system is equally useful for quails. Partial staging systems have also been described for other birds, including turkey and duck embryos (e.g. Bakst *et al.*, 1997).

Some time ago it was realized that the intermediate stages between those suggested by Hamburger and Hamilton are required for additional precision. Two attempts to subdivide primitive streak stages have been made. Vakaet (1970) suggested that the stages HH1–4 should be redefined as seven separate stages. However, this turned out to be very confusing because stage HH5 would follow Vakaet's stage 7. An alternative scheme (Schoenwolf *et al.*, 1992) subdivides stage HH3 into four substages: 3a, b, c, and d, where stage 3d corresponds to stage HH4. One problem with this staging system is that the time lapse films

reveal that stage 3c (a short streak with a groove) is only observed in a minority of embryos. Another problem is that the stage HH4 is often misdiagnosed: while Hamburger and Hamilton's textual description refers to a primitive streak with a defined Hensen's node but no head process, their illustration shows an embryo with a distinct head process primordium in front of the node (Schoenwolf stage 4). To avoid confusion while still benefiting from finer subdivisions, Selleck and Stern (1991) suggested that the original proposal (Hamburger and Hamilton, 1951) of using + and − should be used to indicate intermediate stages. Using this system, the following stages can be distinguished (Fig. 1):

- Stage HH2: short, triangular streak.
- Stage HH2^{+}: a streak that is almost parallel-sided except at its base, which is slightly concave.
- Stage HH3 (=Vakaet stage 3, Schoenwolf stage 3a): perfectly parallel-sided streak; the epiblast surface is smooth with no groove visible. The middle layer has not yet left the primitive streak, which appears as a solid rod. No obvious middle layer is outside the streak itself.
- Stage HH3^{+} (=Vakaet stage 4, Schoenwolf stage 3b/c): the streak is narrowed and slightly elongated; a distinct longitudinal groove is visible in the epiblast. Node not yet distinct, the groove ends suddenly without a pit. Middle layer (mesendoderm) starts to emerge lateral to the streak.

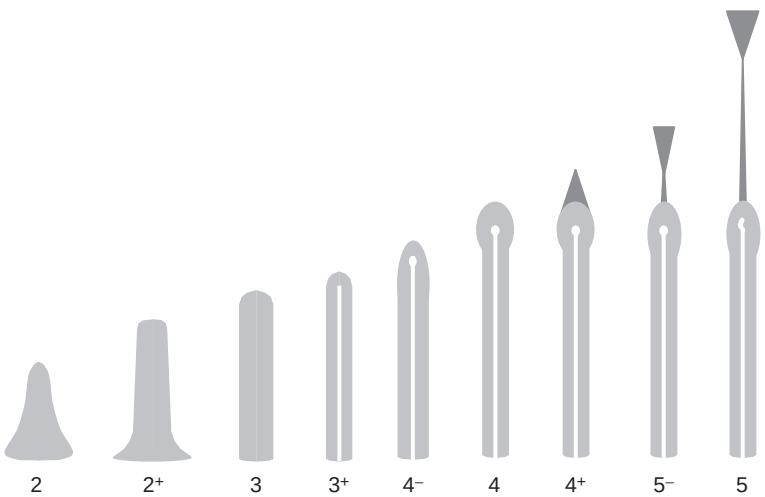

Fig. 1 Staging of primitive streak stage chick embryos. Morphology and appearance of the primitive streak are the decisive features for staging early chick embryos (Hamburger and Hamilton, 1951). Finer subdivision of the stages is indicated by the use of + and −. The diagrams illustrate the appearance of the streak (gray) and the emerging axial mesoderm (dark gray) at different stages (numbers). See text for detailed description.

- Stage HH4^{-} (=Vakaet stage 5, Schoenwolf stage 3d): the primitive groove terminates anteriorly in a clear pit, but the edges of the node are not yet distinct;
- Stage HH4 (=Vakaet stage 6, Schoenwolf stage 3d): Hensen's node is clearly visible at the anterior tip of the streak, the primitive pit lies at its center. There is no indication of mesendoderm anterior to the edge of the node.
- Stage HH4^{+} (=Vakaet stage 7, Schoenwolf stage 4): a small triangular mesendodermal extension protrudes from the front of the node. The broad side of this triangle abuts the node itself and its tip points forward.
- Stage HH5^{-}: the triangular structure in front of Hensen's node is now inverted, with its tip pointing posteriorly (or touching the node) and its base facing anteriorly. There may be a short intervening length of head process (notochord) between the node and this triangle.
- Stage HH5: a distinct long head process extends in front of the node, but no head fold is visible.

Staging the embryos precisely is important for many types of manipulations. For example, in neural induction assays, the competence of the area opaca epiblast (see below) disappears suddenly between stages HH4 and HH4^{+}, and the ability of the node to induce starts to decline immediately after stage HH4 (Dias and Schoenwolf, 1990; Gallera, 1970, 1971a,b; Gallera and Ivanov, 1964; Kintner and Dodd, 1991; Storey *et al.*, 1992, 1995). In addition, while these stages are defined by the overall appearance of the embryo, it is important to realize that the cells making up the various structures may be constantly changing because of the extensive cell movements taking place at these stages (Joubin and Stern, 1999).

III. Anatomy of the Primitive Streak Stage Embryo

Although the primitive streak stage embryo has relatively few structures, it is important to understand the precise relationships between them. Under transmitted light, the embryo contains a translucent central area (area pellucida) and a peripheral darker area (area opaca). Until stage 3, the embryo comprises two cell layers, except within the primitive streak itself and in the peripheral area opaca (Fig. 2). The upper layer, or epiblast, is a one-cell-thick epithelium, continuous throughout the embryonic disc. Within the central area pellucida, the cells are columnar; in the peripheral area opaca, they are cuboidal, the last two cells at the periphery of the area opaca epiblast having a squamous morphology. Beneath this, the area pellucida contains a single-cell thick, loose tissue of large yolky cells, derived from early islands of cells (hypoblast) and ingrowing cells from the posterior margin (endoblast), both of which will contribute to the extraembryonic yolk sac stalk (Stern, 1990, 2004a). The deep layers of the area opaca are multicellular and made up of very large, yolky cells (germ wall). The hypoblast/endoblast is

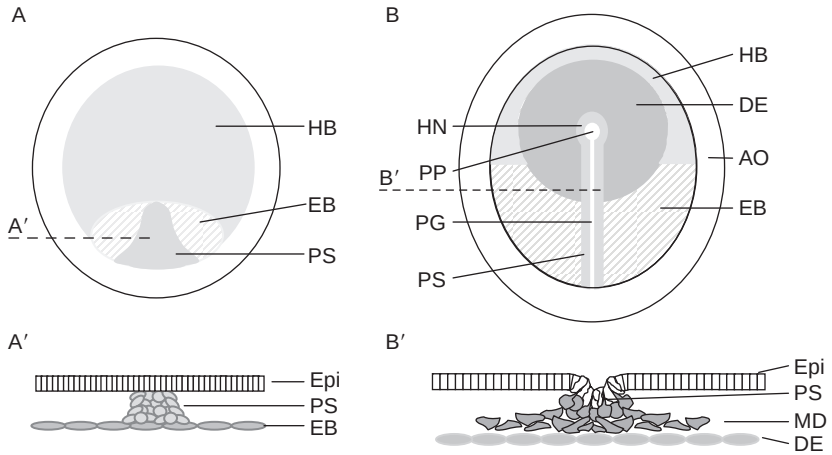

Fig. 2 Anatomy of the chick embryo at HH2 and HH4. (A) At stage 2, the primitive streak (PS) is triangular; the ingrowing endoblast (EB; striped) has begun to displace the hypoblast (HB; gray) from the posterior margin toward the anterior of the embryo. Cells forming the primitive streak accumulate underneath the epiblast (Epi; see section A′); no groove is visible. (A′) Diagram of a section through the primitive streak at the level indicated by the line in panel A. (B). At stage 4, Hensen's node (HN) has formed at the tip of the primitive streak (PS) with the primitive pit (PP) in its center. The primitive groove (PG) is clearly visible. The endoblast (EB; striped) has expanded and the hypoblast (HB; gray) has been displaced anteriorly. The definitive endoderm (DE) has ingressed through the streak and displaced endoblast and hypoblast layers. AO: area opaca. (B′) Diagram of a section through the primitive streak at the level of the line in panel B. In the primitive streak (PS), bottle-shaped epiblast (Epi) cells ingress to give rise to mesoderm (MD).

loosely attached to the deep layer of the area opaca at the germ wall margin, a region where the germ wall is not attached to the epiblast but forms a protruding flap.

At stage 3^{+}, a middle layer starts to emerge from the primitive streak Fig. 2, as cells migrate out at right angles to its long axis. This layer contains both endoderm and mesoderm precursors. The endoderm inserts into the lower layer close to the anterior tip of the primitive streak, gradually replacing the hypoblast/endoblast to form the definitive (gut) endoderm. In the middle layer, the cells migrating laterally out of the primitive streak will give rise to the lateral plate, intermediate and paraxial mesoderm. At stage 4^{+}, cells that start to form the head process just in front of the node occupy the anterior midline. This is the cephalic portion of the notochord, which will later (stage 6 onward) extend caudally. At the tip of the head process, the mesendodermal cells fan out as an inverted triangle, first visible at stage 5^{-}: this is the prechordal mesendoderm. Immediately anterior to the prechordal mesendoderm, the definitive endoderm is thickened and contacts the epiblast directly, without any intervening mesoderm: this is the prechordal plate proper (Seifert *et al.*, 1993).

IV. New Culture

In the avian embryo, operations at primitive streak stages (10–20 h incubation, HH3–5) are most easily performed in whole embryo culture, as described by New (1955; see also Stern and Bachvarova, 1997). Even though slightly more involved than "EC-culture" (Chapman *et al.*, 2001), embryos develop better in New culture: growth is less stunted and head abnormalities are less common.

The following protocol for preparing cultures is based on the method of New but with some modifications as previously described (New, 1955; Stern and Bachvarova, 1997; Stern and Ireland, 1981). The main difference between this method and that originally described by New is the use of rings cut from glass tubing, rather than bent from a glass rod. The advantage of these rings, with rectangular profile, is that they grip the vitelline membrane tightly and therefore allow transfer of the assembly to a flat plastic dish. We recommend rings of 27 mm outer diameter because it is easier to wrap the membrane around these for a novice. However, if larger (29–30 mm diameter) rings are used, the embryos can develop up to 6–9 h longer. The longevity of the cultured embryo appears to depend both on the amount of thin albumen under the ring and on the length of time for which it can be cultured before the edges of the *area opaca* reach the ring (Stern and Bachvarova, 1997).

A. Protocol

Collect the following materials (very clean but not necessarily sterile):

- Hens' eggs incubated 12–18 h (depending on stage needed)
- Dissecting microscope with transmitted light base
- Pannett–Compton saline:

solution A: 121 g NaCl, 15.5 g KCl, 10.42 g $CaCl_2 \cdot 2H_2O$, 12.7 g $MgCl_2 \cdot 6H_2O$, H_2O to 1 liter;

solution B: 2.365 g $Na_2HPO_4 \cdot 2H_2O$, 0.188 g $NaH_2PO_4 \cdot 2H_2O$, H_2O to 1 liter;

(both of these solutions may be kept at 4 °C if autoclaved)

before use, mix (in order): 120 ml A, 2700 ml H_2O, and 180 ml B.

- Two pairs of watchmakers' forceps, number 4 or 5
- One pair of coarse forceps, about 15 cm long
- One pair of small, fine scissors, with straight blades about 2 cm long
- Container for egg waste
- One small beaker (50–100 ml)
- Pasteur pipette (short form), end lightly flamed to remove sharp edges; rubber teat
- Pyrex baking dish about 5 cm deep, 2 liter capacity

- Watch glasses, about 5–7 cm diameter
- Rings cut from glass tubing, approximately 27 mm outer diameter, 24 mm inner diameter, and 3–4 mm deep
- 35 mm plastic dishes with lids (bacteriological grade)
- Plastic box with lid for incubating culture dishes
- Incubator at 38 °C

Proceed as follows to set up the cultures:

1. Fill the large Pyrex dish about 3/4 full with saline (about 1.5 liters).

2. Open an incubated hen's egg by tapping the blunt egg with coarse forceps, and carefully remove pieces of shell. Discard the thicker albumen, assisted with the coarse forceps, and collect thin albumen separately in a small beaker. Try to remove as much albumen as possible, which will simplify the later steps.

3. When the yolk is clean and free from adhering albumen, carefully tip it into the saline container, taking care not to damage the vitelline membrane on the edges of the broken shell. The blastoderm should face upward. If not, carefully turn the yolk with the side of the coarse forceps. Now place a watch glass and a glass ring into the container.

4. Using small scissors, make a cut into the vitelline membrane enveloping the yolk just below the equator. Continue to cut all the way around the circumference of the yolk.

5. With two pairs of fine forceps (one to pull the edge of the membrane and the other to hold the yolk down as it becomes exposed), slowly but very steadily 'peel' the North Pole of the vitelline membrane, all the way off the yolk. It is best to pull slightly upward (about 25–30^{o} angle from the yolk surface) rather than tangentially along the yolk because the latter tends to detach the embryo from the membrane. Do not stop during the process. The embryo should come off with the membrane. Let the membrane rest on the bottom of the dish, inner surface (containing the embryo) facing upward.

6. Slide the vitelline membrane, preserving its orientation, onto the watch glass, and arrange the ring over it so that membrane protrudes around the ring. Pull out the assembly from the saline.

7. With fine forceps, work carefully to fold the cut edges of the vitelline membrane over the edge of the ring, all the way around its circumference. Do not pull too tightly but ensure that the bottom of the membrane is smooth and free from any large wrinkles as you work around the circumference.

8. Place the watch glass over a black surface. Suck off as much fluid as possible from the outside of the ring with the flamed Pasteur pipette. If there is much yolk remaining over and/or around the embryo, wash it carefully with clean saline. Discard any embryo in which the vitelline membrane inside the ring has been damaged. If there is too much vitelline membrane collecting inside the ring, trim the excess by lifting the edges with fine forceps as you trim with small scissors.

9. Working under the microscope, carefully remove any remaining saline, both inside and outside the ring. It is important that the embryo and the inside of the ring remain completely dry during incubation.

10. Now pour some thin albumen (about 2- to 3-mm-thick layer) on the bottom of a 35 mm plastic dish. Slide the ring with vitelline membrane off the watch glass and transfer it to the dish, lowering it (insert it slightly obliquely to make sure that no air is trapped underneath) over the pool of egg albumen. Press lightly on the ring with two forceps to allow it to adhere to the dish.

11. If the level of albumen comes close to the edge of the ring, remove the excess. Also aspirate any remaining fluid from inside the ring. It is best if the vitelline membrane bulges upward, above a good pool of albumen. This will also help to drain off further fluid accumulated during culture to the edges of the ring.

12. Wet the inside of the lid of the plastic dish with albumen and spread it all over. Discard the excess. Now check once more that there is not too much albumen in the dish (i.e., the ring should not be floating)—otherwise remove some and finally use the wetted lid to seal the dish.

13. Place the dish in a plastic box containing a piece of tissue paper or cotton wool wetted in distilled water, seal the box, and place it in an incubator at 38 °C.

Using this culture method, embryos should survive for 24–36 h (about stage 14).

V. Hensen's Node Grafts and Other Induction Assays

Induction is defined as an interaction between two tissues, whereby signals from one tissue change the direction of differentiation (fate) of the other, responding tissue (Gurdon, 1987; Slack, 1991). To test this it is therefore important to use, as responding cells, a region of the embryo that does not contain cells fated to contribute to the tissue assessed as a result of the induction. Such a contribution may be exacerbated by abnormal cell movements caused by the graft, whereby cells of the endogenous domain become attracted to the graft site. Moreover, self-differentiation of the graft itself should be taken into account: it is possible that the grafted tissue contains some cells whose fate is the same as that being assessed.

For example, an obvious region to assess neural induction would be the embryonic, nonneural ectoderm (prospective epidermis). However, this is a very narrow region in the anterior lateral area pellucida, and grafts of the organizer (Hensen's node) into it always lead to a bifurcated nervous system showing continuity with the host, where recruitment of host neural plate cannot be discounted. One way to overcome this is to place the grafts into a peripheral ring of the avian blastoderm, the inner third of the area opaca. During normal development, this region only contributes to extraembryonic tissues, but is nevertheless able to respond to a graft of Hensen's node by generating a complete embryonic axis, where the host epiblast changes its fate from extraembryonic ectoderm to neural tissue (Dias and Schoenwolf, 1990; Gallera, 1971a,b; Storey *et al.*, 1992, 1995). The competence

of this region to respond to such a graft declines rapidly, such that by stage HH4$^+$, it is no longer able to respond to grafts of nodes derived from donors of any stage (Dias and Schoenwolf, 1990; Gallera, 1971a,b; Gallera and Ivanov, 1964; Storey *et al.*, 1992, 1995; Streit *et al.*, 1997).

To distinguish donor from host cells, interspecies chimeras can be used, for example, quail donors and chick hosts (Le Douarin, 1973), whose cells can be distinguished by either the Feulgen–Rossenbeck technique or using antiquail cell antibodies (e.g., QCPN) or species-specific riboprobes in *in situ* hybridization analysis (Izpisúa-Belmonte *et al.*, 1993). Another way to trace the fate of the grafted cells is to label the transplanted tissue with a cell-autonomous vital dye, such as the carbocyanine dye DiI (Selleck and Stern, 1991, 1992; Storey *et al.*, 1995).

We will now describe Hensen's node grafts as a prototype for induction assays and then discuss the isolation of other tissues for similar experiments.

A. Hensen's Node Grafts for Neural Induction

Collect the materials listed under Section IV.A above and the following (clean but not necessarily sterile):

- Fertile quails' eggs incubated to HH3$^+$–4$^-$
- 500 ml Tyrode's saline
- 10–15 cm Petri dish
- Small spoon
- Very fine needles (e.g., entomological size A1 or D1) or sharpened tungsten wire mounted by melting the fine end of a Pasteur pipette (to act as a handle) or into a metal needle holder, or two 30-gauge syringe needles mounted on 1 ml syringes as a handle
- Pasteur pipette with the end cut-off at the shoulder, the stump flamed to remove sharp edges, and a rubber teat

Incubate chick host embryos to stage 3$^+$/4$^-$ and set them up as described for New culture earlier (Section IV.A) up to step 8. Add Pannett–Compton saline to the inside of the rings to ensure that the embryos are completely submerged; add saline to the outside of the ring to avoid drying out and sticking to the watch glass. The embryos can be kept like this on the bench for several hours while preparing the donors.

Collect the donor embryos as follows:

1. Remove quail eggs from incubator. With the scissors, gently tap near the blunt end of an egg so as to penetrate the shell. Use the tip of the scissors to cut off a small cap of shell near this end, carefully so as to avoid damaging the yolk.

2. Allow egg white to pour into waste bucket, assisted by the scissors, taking care to avoid damage to the yolk. You may need occasionally to cut through the thick albumen using the scissors.

3. Once most of the albumen has been poured off, make sure the embryo is uppermost; if not, turn the yolk by stroking it very gently with the sides of the scissors.

4. Use the scissors to make four cuts into the vitelline membrane around the embryo. If the embryo does not lie exactly in the center of the egg, make the first cut on the side of the embryo nearest the shell, and proceed in this way until all four cuts have been made. Make sure that all the cuts meet.

5. Pick up the square of embryo/membrane with the spoon, trying to collect only a minimal amount of yolk.

6. Transfer the yolk/embryo/membrane with the spoon into the large Petri dish with Tyrode's saline under a dissecting microscope. With fine forceps, turn the square of yolk/membrane/embryo so that the embryo is uppermost.

7. After the desired number of donor embryos have been placed into the Petri dish, use two pairs of forceps to separate the embryo from adhering yolk. Working at low magnification, pick up a corner of the square of vitelline membrane with one pair of forceps and slowly but steadily fold it back, steadying the yolk with the other pair of forceps. During the whole procedure, the membrane and embryo should remain totally submerged in the saline. The embryo should be attached to the membrane; if not, peel the membrane completely and then use forceps gently to remove the embryo from the underlying yolk.

8. Pick up the embryo, with or without adhering membrane, with the wide-mouth Pasteur pipette and transfer it to a 10 cm dish with clean saline for final cleaning and dissection. The edges of the extraembryonic membranes will be perfectly circular, provided that the embryo has not been damaged during the explantation procedure.

Having prepared both donor quail and host chick embryos, you are ready for the operation. Follow the steps below:

9. Place the watch glass with the host chick embryo under the microscope. Using a fine needle, carefully lift up a portion of the flap of yolky cells (germ wall margin) that covers the inner margin of the area opaca, working outward from the area pellucida and taking care not to penetrate the ectoderm underneath, which is only one cell thick. This will produce a pocket into which the graft can be inserted. Alternatively, just remove a small region of yolky endoderm at the future site of grafting, using the needle.

10. Remove the watch glass with the host and bring the dish with the donor quail embryo under the microscope, arranging it so that its ventral (endoderm) surface is uppermost. Using a fine needle, carefully cut out the very tip of the primitive streak, cutting through the whole thickness of the embryo. It is best to cut the two sides first, working as close as possible to the primitive streak edges, then free the anterior tip and finally detach the node (which is quite small—about as long as it is wide) from the rest of the streak.

11. Lower the magnification of the microscope, keeping track of the excised node, and pick this up with a Gilson P20 fitted with a yellow tip, set to 1–2 µl.

12. Move the dish with donor embryos away and bring back the watch glass with the host into the field. While looking down the microscope, insert the tip of the Gilson under the saline covering the host embryo and gently expel the quail node onto its surface, keeping track of it at all times.

13. Use fine needles to manipulate the donor node to close to the desired grafting site and slide it into the pocket, pushing it as deep as possible so that when the flap of germ wall margin is replaced, it will cover the graft completely. Alternatively, if you have only cleaned some yolky endoderm, manipulate the node to the denuded site.

14. Now finish the culture following steps 9–11 of the protocol in Section IV.A. Take care while removing excess saline from the operated embryo so that the grafted node remains in place. As fluid is removed, the graft should become well attached to the host.

After overnight incubation, the induction should be clearly visible as a small ectopic axis.

B. Other Induction Assays

In the earlier example, the transplanted tissue (Hensen's node) comprises all three layers of the blastoderm. For some induction assays, it is desirable to test the inducing ability of a single germ layer. At early neurula stages (HH5–7), separation of the germ layers is difficult without the aid of enzymes. Here we describe the isolation of stage 5–6 mesoderm as an example of this procedure.

Collect the materials described in Sections IV.A and V.A, but incubate donor embryos to HH6. In addition, you will also need:

- 35 mm Sylgard-coated dish; to make these, mix the elastomer and accelerator of clear Sylgard 184 (Dow Corning) following the manufacturer's instructions and pour 2–3 ml into one 35 mm Petri dish. Leave at room temperature until any bubbles have disappeared and then cure overnight at 38–50 °C in a dry incubator or oven.
- Entomological A1 pins.
- Dispase (Roche); keep aliquots of 5 mg/ml dispase in Tyrode's saline at −80 °C and dilute to working concentration (25 µg/ml) just before using. Keep on ice.
- Aspirator tube (Sigma A5177).
- 50 µl Borosilicate glass capillaries pulled to a long tip with a vertical electrode puller, tip broken off.
- 35 mm Petri dish.
- 2- to 3-ml 2.5% serum (any species) in Tyrode's saline.

Set up primitive streak stage host embryos as described in Section IV.A, steps 1–8; keep the embryos submerged in saline until the donor tissue is dissected and ready to graft. Prepare donor embryos from stage 5/6 as described in Section V.A, steps 1–7. To isolate head mesoderm, use the following procedure:

1. Using a wide mouth Pasteur pipette, transfer an embryo to a Sylgard-coated dish filled with Tyrode's saline.
2. Using fine forceps, turn the embryo ventral side up; secure the embryo onto the Sylgard by piercing the area opaca with four entomological pins so that the embryo is as flat as possible.
3. Use 30 gauge hypodermic needles to score the endo/mesoderm along the edges of the intended graft.
4. Use the capillary to inject a small amount of dispase along the cuts and wait one minute.
5. Use the back of the needle tip to peel the endoderm off the mesoderm and discard it.
6. Loosen the mesoderm from the underlying ectoderm using the same technique; work from the anterior toward the posterior edge of the graft. The mesoderm will retract a little as you loosen it.
7. Release the mesoderm from the ectoderm and using a 5 μl Gilson pipette, transfer it to a Petri dish containing Tyrode's saline with 2.5% serum. Keep on ice.
8. Collect all donor tissue and keep on ice until ready for transplantation.
9. Pick up one graft at a time from the dish using a 5 μl Gilson Pipette; rinse in Tyrode's saline to remove serum.
10. Place a watch glass with a primitive streak stage chick host prepared as described in Section V.A, step 9 under the microscope and under low magnification release the graft onto the embryo, keeping track of it all the time.
11. Use fine needles to manipulate the donor tissue close to the desired grafting site and slide it into the pocket, pushing it as deep as possible so that when the flap of germ wall margin is replaced, it will cover the graft completely. Alternatively, if you have only cleaned some yolky endoderm, manipulate the node to the denuded site.
12. Now finish the culture following steps 9–11 of the protocol in Section IV.A.

VI. Other Types of Graft

On occasion it may be necessary to place a graft into an equivalent site from which it was taken (homotopic graft). This approach is useful for fate mapping using chick–quail chimeras or when electroporated or transgenic donors or hosts

are needed. This can be done into a host embryo either of the same stage (isochronic) or of a different stage (heterochronic). Finally, it may also be desirable to challenge cells by placing them into a site different from their origin (heterotopic) to assess their developmental potential.

For operations involving only mesoderm or endoderm, the same techniques as described earlier in Section V.B are used, except that it is also necessary to separate the layers of the host embryo as well as those of the graft. For this, use a drop of dispase to loosen and remove the appropriate tissue from the host before grafting the donor tissue.

Operations involving the ectoderm present an additional difficulty: these grafts do not heal well. To overcome this, a few parameters are important (Stern and Bachvarova, 1997):

- It is critical that the dorsoventral orientation of the graft is the same as that of the host. It is not necessary to mark the graft, because upon cutting ectoderm tends to roll up with its basal surface inward.
- It is essential that immediately after placing the graft, the embryo is dried completely, in particular the junction between graft and host tissue. This can be achieved using the capillary action of a very fine capillary (as described above for dispase); apply the capillary without suction to all the edges of the graft, to knit donor and host tissue together.
- Healing is greatly enhanced if the graft is completely covered by host mesendoderm. To achieve this, do not remove these layers when exposing the graft site but rather generate a flap that can later be used to cover the graft.
- It helps to leave the finished grafted New culture to heal at room temperature for 1–3 h before incubating.
- As the embryo expands, it creates considerable tension that may open the wound. This tension is generated by the peripheral cells of the area opaca as they attach and crawl on the vitelline membrane (New, 1959). Removal of the outermost edge of the area opaca delays this process as the embryo regenerates these cells and thus allows the graft to heal. Take care during this procedure not to puncture the vitelline membrane.

VII. Fixing Operated Embryos for Analysis

For analyzing grafted embryos either as whole mounts or in sections, it is important that they should remain flat during processing. This can be achieved in either of two ways: they can be pinned out through the area opaca on a Sylgard dish before fixation or flattened out on the lid of a plastic Petri dish. The latter procedure is particularly suitable for embryos younger than stage 8: place embryos in individual drops of saline onto the lid, remove saline with a Pasteur pipette while the embryo spreads out on the plastic surface, and then add fixative directly onto the embryo.

In either case, the embryo needs to be removed from the vitelline membrane before fixation. For this, use a Pasteur pipette to fill the ring containing the embryo with phosphate-buffered saline (PBS) quickly, to avoid the embryo floating away. Using the end of a closed fine forceps gently loosen the edge of the area opaca all around the embryo and then pick it up with a wide mouth Pasteur pipette.

For whole mount *in situ* hybridization, either alone or in combination with immunohistochemistry, fix embryos in freshly made 4% paraformaldehyde in PBS containing 1 mM EGTA for 4 h at room temperature or overnight at 4 °C (Streit and Stern, 2001). For cryosectioning and immunostaining, fix for 20–30 min in 4% formaldehyde in PBS. For *in situ* hybridization on wax-embedded sections, use Carnoy's fixative overnight.

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CHAPTER 2

Quail–Chick Transplantations

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I. Introduction

Because it is easily accessible to experimentation, the avian embryo has long been a popular subject of study for embryologists. The combination of cells or rudiments from two related avian species (quail, *Coturnix coturnix japonica*, and chick, *Gallus gallus*), adapted as a means to identify cells that migrate during embryogenesis (Le Douarin, 1973a), has served as a useful tool for the study of many developmental biology problems. The use of quail–chick grafts was motivated by the need to selectively label defined groups of cells in order to follow their pathways of migration and identify interactions during prolonged period encompassing morphogenesis and organogenesis.

The method is based on the observation (Le Douarin, 1969) that all embryonic and adult cells of the quail possess condensed heterochromatin in one (sometimes two or three) large mass(es) in the center of the nucleus, associated with the nucleolus. As a consequence, this organelle was strongly stained with the reaction by Feulgen and Rossenbeck (1924). When combined with chick cells, quail cells can readily be recognized by the structure of their nucleus, thus providing a permanent genetic marker (Fig. 1).

The main purpose in constructing quail–chick chimeras was to follow the fate of defined embryonic territories not only to their ultimate destinations and fates in the mature bird but also at intermediate time points during embryonic development. The investigations carried out on the neural crest provide a good example of the utility of employing the quail–chick chimera system, as they have uncovered the nature of the tissues and organs derived from this structure as well as showing the migratory pathways taken by neural crest cells (NCCs) en route to their destination (Le Douarin, 1982; Le Douarin and Kalcheim, 1999)—another clear example stems from the mapping of the neural primordium (Le Douarin, 1993). The transformation from the early neural ectoderm to the mature brain involves an

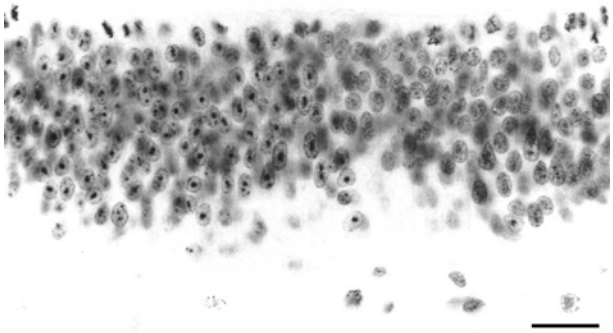

Fig. 1 Chimeric neuroepithelium at 4 days of incubation. A quail hemimesencephalon was grafted orthotopically, 2 days earlier, in a 2-day-old chick embryo. Quail cell nuclei (to the left) show the typical quail nucleolus with a large heterochromatin clump, whereas chick cells (to the right) display dispersed heterochromatin. The section is 5 μm thick; Feulgen–Rossenbeck staining with counterstain. Scale bar: 20 μm.

enormous complexity that comes about via differential growth of various regions of the neuroepithelium, extensive cell migrations, and assembly of the very complicated wiring taking place between the neurons of the central nervous system (CNS). As will be shown in this chapter, quail–chick chimeras provide a means to unveil some of the mechanisms underlying these complex processes.

This type of study assumes that the developmental processes occur in the chimeras as they do in the normal embryo. To achieve this, transplantations of quail tissues into chick embryo (or vice versa) do not consist of adding a graft to an otherwise normal embryo but rather in removing a given territory in the recipient and replacing it as precisely as possible by the equivalent region of the donor of the same developmental stage.

Quail and chick are closely related in taxonomy, although they differ by their size at birth (the quail weight is about 10 g and that of the chick is 30 g) and by the duration of their incubation period (17 days for the quail and 21 days for the chick). However, during the first week of incubation, when most of the important events take place in embryogenesis, the size of the embryos and the chronology of their development differ only slightly. When the dynamics of development of a given organ are to be studied by the quail–chick substitution method, the exact chronology of development of this organ in each species must first be established. This is a prerequisite to choose the exact stage of donor and host embryos at operation time as well as for later interpretation of the results. An example of this requirement can be found in the study that was performed on the origin of the calcitonin-producing cells that develop in the ultimobranchial body and of the enteric nervous system (see Le Douarin, 1982, and references therein).

In order to rule out possible differences in developmental mechanisms between the two species, it is beneficial not only to carry out the grafts from quail to chick

(the most often performed because it is easier to recognize one isolated quail cell within chick tissues than the other way around) but also to perform reciprocal control experiments from chick to quail.

In addition to isochronic–isotopic substitutions, grafts can be placed into normal embryos without previous extirpation of the corresponding territory to study certain developmental processes. For example, this was instrumental in demonstrating the colonization of the primary lymphoid organ rudiments (thymus and bursa of Fabricius) by hemopoietic cells and in showing that this process occurs according to a cyclic periodicity (Le Douarin *et al.*, 1984, and references therein).

Quail–chick chimeras generated by isotopic–isochronic grafts of embryonic territories appear to develop normally. To further verify this, the chimeras were examined after hatching and postnatal survival. This was tested in a variety of experimental designs: neural chimeras in which parts of the CNS (including the brain) or the peripheral nervous system of chick were replaced by their quail counterpart (Kinutani *et al.*, 1986; Le Douarin, 1993, and references therein) or by immunological chimeras in which the thymus rudiment of the chick was replaced by that of the quail (Ohki *et al.*, 1987). Neural chimeras are able to hatch and exhibit an apparently normal sensory motor behavior even when their brain is chimeric. Because quail and chick exhibit species-specific behavioral characteristics, these chimeras can be analyzed to determine whether a particular trait is linked to a specific area of the neuroepithelium, as demonstrated for certain songs of quail and chick (Balaban *et al.*, 1988).

However, the analysis of quail–chick chimeras after birth is time-limited. Although there is no immune rejection during embryogenesis, when the immune system is immature, the transplant is rejected at various times after birth. For neural grafts, a long delay is observed between the onset of immune maturity and rejection due to the relative isolation of the CNS from circulating lymphocytes by the blood–brain barrier as well as the low immunogenicity of the neural cells. This delay, which may be more than a month, allows behavioral studies to be carried out in early postnatal life.

The immune rejection of the implant raised a series of interesting problems concerning the mechanisms of self–nonself discrimination. This demonstrated an unexpected role of the epithelial component of the thymus in tolerance to self (Belo *et al.*, 1989; Martin, 1990; Ohki *et al.*, 1987, 1988). In allogeneic (chick–chick) grafts, it was found that embryonic neural grafts between major histocompatibility complex (MHC)-mismatched chick species trigger little or no immune response from the host. This led to identification of brain areas responsible for an autosomic form of genetic epilepsy (Fadlallah *et al.*, 1995; Guy *et al.*, 1992, 1993; Teillet *et al.*, 1991).

For many years, analysis of chimeras relied on the differential staining of the nucleus by either the Feulgen–Rossenbeck reaction or other DNA staining methods such as acridine orange or bizbenzimide (Hoechst 33258, Serva, Heidelberg) which could be combined with immunocytochemistry (see, for example, Fontaine-Perus *et al.*, 1985; Nataf *et al.*, 1993). Subsequently, species-specific antibodies that recognize either quail or chick cells have been developed. In addition,

cell type-specific reagents are available either as monoclonal antibodies (MAbs) or as nuclear probes that distinguish, at the single resolution, whether a cell produces a particular product and if it belongs to the host or the donor.

Section II reviews the technical requirements and experimental procedures for production and analysis of quail–chick chimeras.

II. Differential Diagnosis of Quail and Chick Cells

A. Nucleolar Marker

The interphase nucleus of quail cells has an immediately apparent feature even when stained with a common nuclear dye like hematoxylin. The nucleus contains a very large, deeply stained inclusion, the so-called "quail nucleolus," even in cells where the nucleolar ribonucleoproteins are not abundant. DNA-specific techniques like the Feulgen–Rossenbeck reaction or the use of acridine-orange or bizbenzimide and electronic microscopy have revealed that this inclusion is essentially composed of heterochromatin associated with the nucleolus (Le Douarin, 1973b; also see Fig. 2). This contrasts with the nucleoli of most species that contain only small amounts of chromatin, usually below the limit of detectability in the light microscope. The quail-like nucleolus has been found in several other bird species, as well (Le Douarin, 1971).

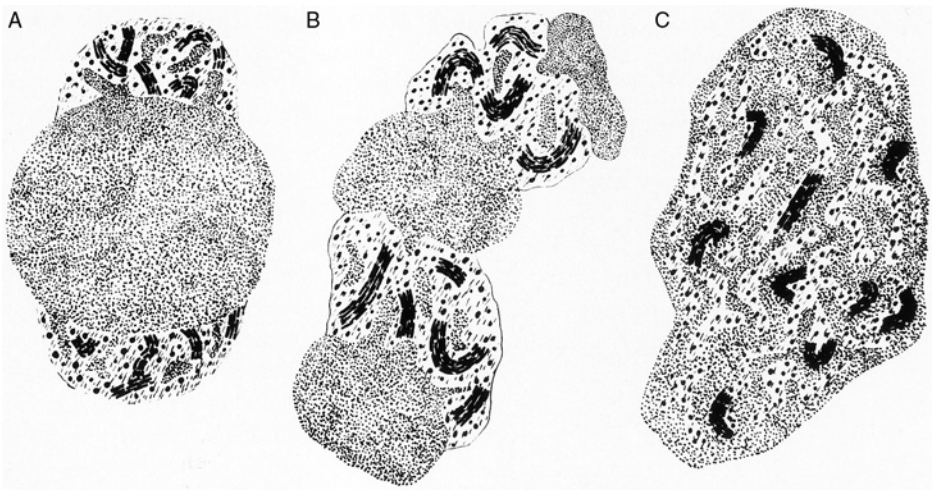

Fig. 2 The quail nucleolus: schematic drawings of the ultrastructural organization of quail nucleoli. (A) Type 1 nucleolus of quail cells; a large chromatin condensation is flanked by lateral clumps of nucleolar material (granular and fibrillar containing structures and amorphous matrix) inside which chromatid strands are located. (B) Type 2 nucleolus of quail cells observed in hepatocytes; several DNA condensations are linked by nucleolar RNA. (C) Type 3 nucleolus of quail cells; RNA granules and fibrils are localized inside the large centronuclear DNA condensation. Reprinted with permission from Le Douarin (1973b).

Some variations in morphology of the quail nucleolar DNA are observed depending on the cell type (Le Douarin, 1973b; Fig. 3). In early embryonic cells, such as young blastoderm cells or early hematopoietic precursors, the centronuclear chromatin mass is very large, stains lightly, composed of irregular outlines and a reticulated structure. In most later embryonic cells and differentiated cells, the nucleus contains one or two centronuclear chromatin clumps, which are compact, brightly stained, and precisely outlined, for example, in kidney, lung, thyroid, suprarenal gland, neural tube, and neural derivatives. In hepatocytes, there may be two to four heterochromatin masses, some attached to the nuclear membrane. In lymphocytes, many chromocenters are dispersed in the nucleus and smaller ones are located juxtaposing the nuclear membrane. In muscular fibers, three to five masses line up along the axis of the elongated nuclei.

In the chick (see Fig. 3), the chromatin network is made up of inconspicuous chromocenters, homogeneously distributed in the nucleoplasm, with only small variations in this pattern between cell types. In hepatocytes where the nucleolus is large, the nucleolus-associated DNA appears as a thin ring around the nucleolar RNA. In thymocytes, numerous tiny chromocenters are scattered in the whole nucleus.

As a rule, the differences between nuclei of corresponding cell types from the two species are obvious and can be used as markers to distinguish the origin of cells. Before analysis, it is important to compare the relevant cells or tissues of each species and establish appropriate criteria taking into account possible cell type variations.

B. Species-Specific Antibodies

Antibodies have been prepared that recognize virtually all cell types of the quail but not the chick. Examples are the chick antiquail serum raised by Lance-Jones and Lagenaur (1987) and the MAb Quail/Chicken PeriNuclear (QCPN) prepared by Carlson and Carlson, available through the Developmental Studies hybridoma bank (Department of Biology, University of Iowa, Iowa City, IA). The QCPN MAb is particularly useful for studying derivatives of quail grafts in young embryos and can be easily combined with other antibodies (Fig. 4). Other MAbs are both species- and cell type-specific such as the MB1 and QH1 MAbs (Fig. 4) (Pardanaud *et al.*, 1987; Péault *et al.*, 1983) that recognize a glycosylated epitope carried by surface proteins expressed in quail leucocytes and endothelial cells but not present in chick (Péault and Labastie, 1990). These antibodies are very useful for studying development of the vascular and hemopoietic systems in quail and chimeric embryos (Caprioli *et al.*, 1998; Dieterlen-Lièvre *et al.,* 2001; Pardanaud and Dieterlen-Lièvre, 1993, 1995; Pardanaud *et al.*, 1989, 1996).

A series of reagents is available to study development of the immune system. These include MHC molecules such as TAC1 and TAP1 that identify a common determinant of quail and chick class II MHC, respectively (Le Douarin *et al.*, 1983)

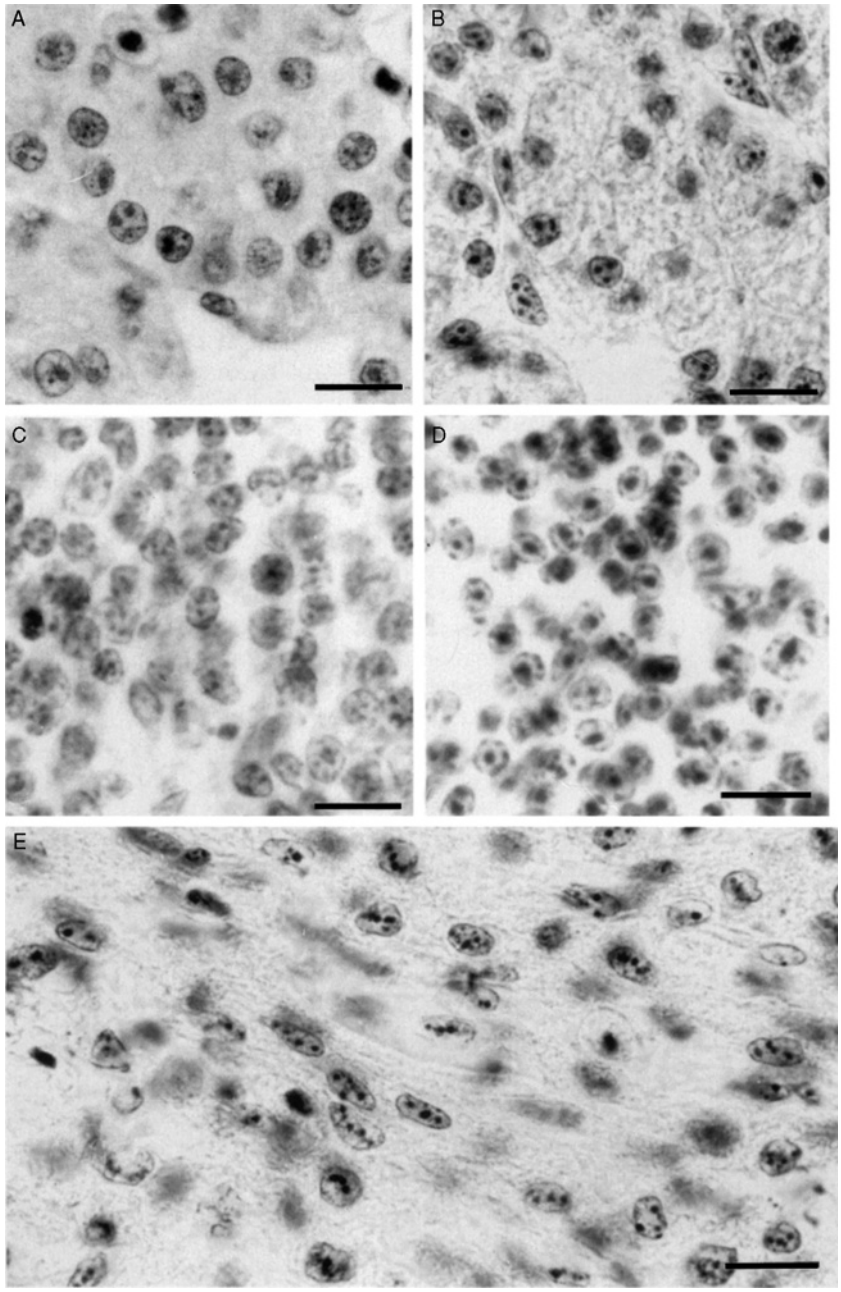

Fig. 3 Appearance of nuclei in different types of chick and quail cells. (A) Hepatocytes in a 15-day-old chick embryo. Very fine heterochromatin dots and an occasional pale staining nucleolus are shown. (B) Hepatocytes of a 15-day-old quail embryo. Chromatin condensations are larger and more

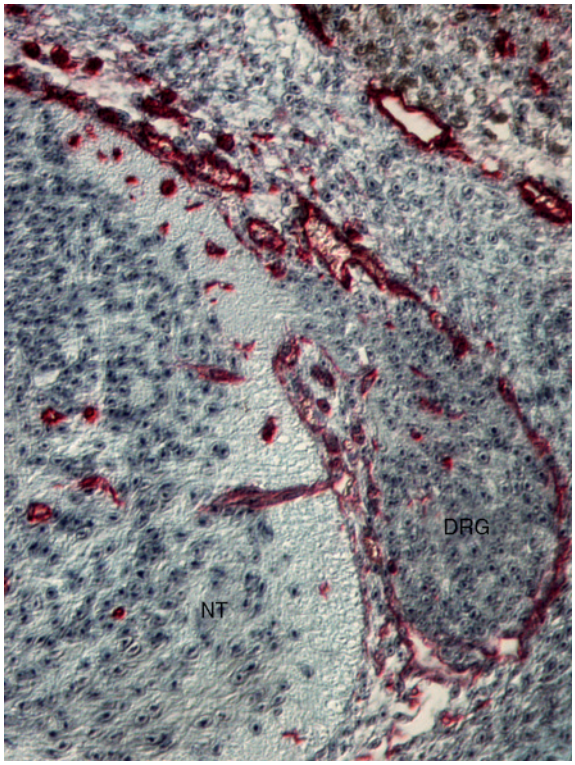

Fig. 4 QCPN/QH1 double immunostaining of a 5-day quail embryo section at the trunk level. All cell nuclei appear in dark blue (QCPN affinity revealed through alkaline-phosphatase, NBT/BCIP). Quail vascular endothelial cells display dark blue nuclei (QCPN+) and red cytoplasm (QH1 affinity revealed through alkaline-phosphatase, fast red). In a quail/chick chimera, only endothelial quail cells would be labelled with both QH1 and QCPN antibodies. Other grafted quail cells would be labeled only by QCPN and chick cells would not be labeled. NT, neural tube; DRG, dorsal root ganglion. Courtesy of C. Vincent. (See Plate no. 1 in the Color Plate Section.)

and a number of chick-specific T-cell markers (see Table I) that have been widely used in the study of the maturation of the immune function in quail–chick chimeras (Bucy *et al.*, 1989; Coltey *et al.*, 1989).

Finally, the analysis of neural chimeras is greatly facilitated by the availability of MAbs that recognize either neuronal cell bodies or neurites of one or the other species (Tanaka *et al.*, 1990; also see Table I).

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deeply stained. (C) Chick thymic cell nuclei display several fine, dispersed chromocenters. (D) Quail thymic cells present one large heterochromatin mass and several smaller ones against the nuclear membrane. (E) Myocardal cells of a quail, 10 days after birth. As in skeletal muscle quail cell nuclei, several chromatin condensations are present. Feulgen–Rossenbeck staining. Scale bars: 10 μm.

Table I
Specifities of various monoclonal antibodies that recognize either quail or chicken antigens

Cell type	Quail	Chicken
All	Chick antiquail serum (Lance-Jones and Lagenaur, 1987) QCPN (hybridoma bank)	
Neurones	QN (neurites) (Tanaka *et al.*, 1990)	37F5 (neuronal cell bodies), 39B11 (neurites) (Takagi *et al.*, 1989), CN (neurites) (Tanaka *et al.*, 1990)
Hemangioblastic lineage	MB1/QH1 (Péault *et al.*, 1983; Pardanaud *et al.*, 1987)	
MHC	TAC1 (Cl II) (Le Douarin *et al.*, 1983)	TAP1 (Cl II) (Le Douarin *et al.*, 1983)
T-cell markers		αTCR1 ($\gamma\delta$) (Chen *et al.*, 1988) αTCR2 ($\alpha\beta$) (Cihak *et al.*, 1988) αCT3 (Chen *et al.*, 1986) αCT4 (Chan *et al.*, 1988) αCT8 (Chan *et al.*, 1988)

C. Species-Specific Nucleic Acid Probes

In addition to antibodies, species-specific cDNA probes have been used to analyze quail–chick chimeras; for example, there is a chick probe for the homeobox gene *goosecoid* that has been used to demonstrate induction of this gene in a chick host by grafting quail goosecoid-producing tissues (Izpisùa-Belmonte *et al.*, 1993). The quail-specific Schwann cell myelin protein probe (Dulac *et al.*, 1992) is currently used in quail–chick neural chimeras to distinguish quail and chick oligodendrocytes (Cameron-Curry and Le Douarin, 1996). Furthermore, chick *Wnt1* and quail *Wnt1* probes have been combined to demonstrate ectopic *Wnt* expression in heterotopic quail–chick chimeras (Bally-Cuif and Wassef, 1994).

III. Material and Equipment

A. High-Quality Fertilized Eggs

It is important to select freshly laid eggs from vigorous strains of chick and quail. The eggs should be stored no more than 1 week at 15 °C. The choice of a rapidly growing strain of chickens is judicious because early stages of development will proceed at the same speed in donor and recipient embryos. Such is the case for the JA57 strain (I. S. A. Lyon, France), which is particularly resistant and normally shows a high rate of hatching. Other features may guide the choice of the host strain, such as feather pigmentation, that can serve as an additional marker

when grafts of NCCs are involved. Usually the chick host is obtained from a nonpigmented strain as the quail wild-type phenotype is heavily pigmented.

B. Incubators

Incubators must be equipped with temperature (38 ± 1 °C) and humidity regulators [45% up to embryonic day 17 (E17) for the chick, 75% thereafter, up to hatching time]. Automatic rocking is required in certain cases. A timed programmer is also useful in order to ensure precise stages, especially for operations performed at the early phases of development. The developmental tables of Hamburger and Hamilton (1951), Eyal-Giladi and Kochav (1976), and Zacchei (Z) (1961) are used to stage chick and quail embryos.

IV. Egg Holders

There are several kinds of egg holders that serve different purposes. Multiple wire tongs are used to hold series of chick eggs in a horizontal position and to manipulate them prior to operations. Wooden circles of appropriate sizes serve to hold eggs during operations. After the operation, eggs are stacked horizontally in the incubator on hollowed out wooden slats.

A. Optical Equipment

Microsurgery is performed under a stereomicroscope allowing a continuously progressive magnification (zoom), for example, from 6× to 50×. The possibility of adding photographic or video equipment without losing stereoscopic vision is beneficial. Illumination is best obtained from optic fibers, which have limited heat load.

B. Microsurgical Instruments

Microscalpels adapted to each type of operation are required for surgery. For excising fragments of the neural tube or brain vesicles, microscalpels generated by stropping and honing steel needles on an Arkansas oil stone are the most convenient because they can be both extremely thin and resistant (Fig. 5A). Prepared with a smooth tip, they are used for dissociating tissues after enzymatic treatment. Tungsten microscalpels (Conrad *et al.*, 1993) or microscalpels made from entomology needles are quicker to prepare but are more fragile. They are useful for precisely dissecting very small pieces. Other instruments (Fig. 5B) include curved and straight small scissors, iridectomy scissors (Pascheff-Wolff, Moria-Instruments, Paris), thin forceps, a transplantation spoon, microscalpel holders, and black glass needles.

C. Other Equipment

Glass micropipettes are hand drawn from Pasteur pipettes, curved, and calibrated according to use, injections of liquid, or transfer of pieces of tissues (see Fig. 5B). Calibration of the micropipette according to the size of the rudiment to be transplanted (for instance, neural tube versus brain) is an important consideration.

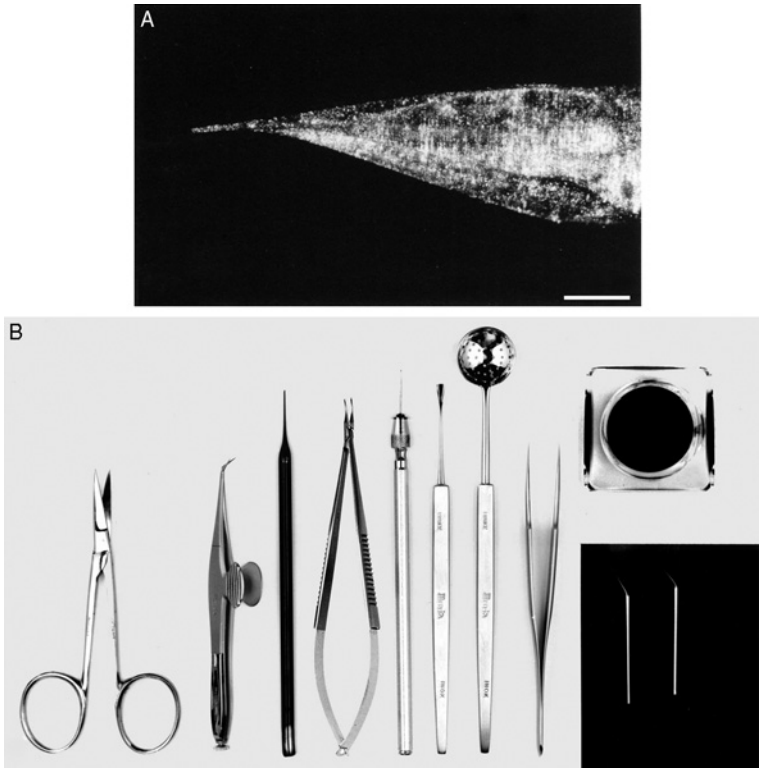

Fig. 5 (A) Microscalpel made from a steel needle. Only the extreme tip is used for microsurgery. Scale bar: 200 μm. (B) Microsurgery instruments: from left to right, curved scissors; Pascheff-Wolff iridectomy scissors; black glass needle; microscalpel in a holder; transplantation spoon and skimmer; and No. 5 Dumont forceps. Top right: glass dish with black rhodorsil base and entomology needles. Bottom: micropipettes.

The pipettes are attached to plastic tubes for mouth use. Indian ink diluted with a physiological solution containing antibiotics can be injected under the embryo to create a dark background against which young embryos become easily visualized (Fig. 6). Phosphate-buffered saline (PBS) or Tyrode solution devised for avian cells may be used for dissections and treatments of the embryos. These are typically supplemented with antibiotics, penicillin, and streptomycin. Enzymes for tissue dissociation, trypsin, pancreatin, or collagenase are used to dissociate the epithelia from the mesenchyme. The concentration of the enzyme, normally diluted in Ca^{2+}, Mg^{2+}-free Tyrode solution, and the application time have to be empirically determined for each rudiment because the purpose is to partly degrade the basement membrane without interfering with cell-to-cell adhesion in order to cleanly separate coherent epithelial sheet from the mesenchyme. A dish with a black resilient base and entomology needles are needed to immobilize the donor embryo for dissection. A rhodorsil base (Rhône-Poulenc) is preferred to paraffin. Carbon

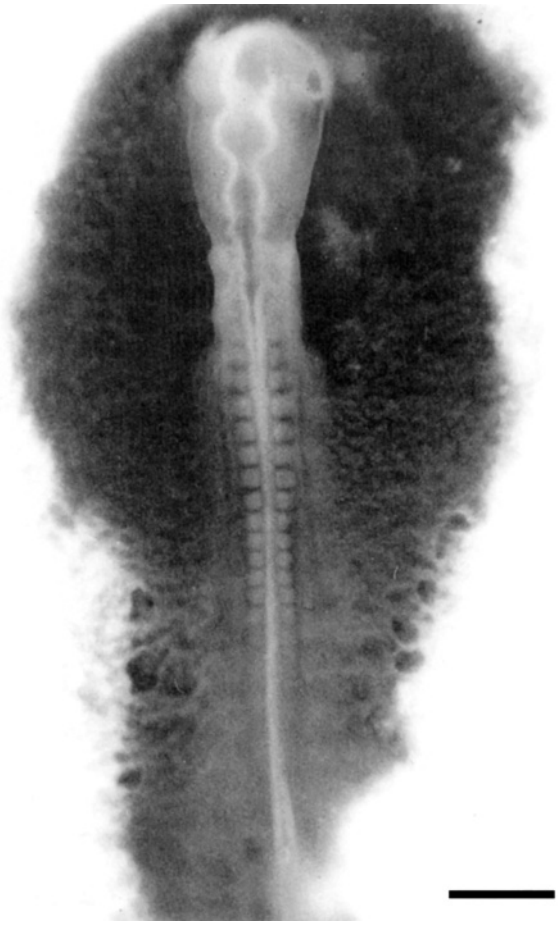

Fig. 6 Chick embryo with 15 pairs of somites prepared for *in ovo* surgery against a black background. A few drops of diluted Indian ink (1 v/v PBS) have been injected beneath the blastoderm using a curved glass micropipette inserted through the extraembryonic area. Scale bar: 200 μm.

black is added to the commercial transparent preparation in order to obtain a black suspension that is poured in a dish of adequate size and shape and polymerized by UV illumination (according to provided directions). Disposable syringes (1 or 2 ml) and needles (0.8 mm) are used to remove albumin from host eggs. Transparent scotch tape (5 cm in width) serves to seal the shell of operated eggs.

D. Feulgen–Rossenbeck Staining

Zenker's or Carnoy's fluids are used to fix the tissues for the reaction by Feulgen and Rossenbeck (1924). Carnoy's fluid is particularly appropriate because it allows the application of both the Feulgen–Rossenbeck technique and various antibodies

or nuclear probes on alternate paraffin sections. Feulgen–Rossenbeck staining is carried out according to the directions described by Gabe (1968). This staining is performed on 5-μm paraffin sections.

V. Preparation and Sealing of Eggs

Eggs are incubated with their long axis horizontal for operations before E4 and their long axis vertical (air chamber up) for operations from E4 onward. The blastoderm normally develops on the upper surface of the yolk and is located against the shell membrane. If the egg was incubated horizontally, the blastoderm would be injured when a window is cut in the shell; thus, usually, a small quantity of albumin (about 1–3 ml) is removed before the window is opened, using a 1- or 2-ml syringe equipped with a 0.8-mm needle inserted at the pointed pole of the egg. The hole is then closed with a drop of paraffin or a small piece of tape. A more practical way to open the shell without injuring the E2 or E3 embryos is to perforate the air chamber and turn the egg upside down. The blastoderm comes back to the top immediately and lies away from the shell. If the eggs are incubated air chamber up, from E4 onward a window can be cut through the upper part of the shell (i.e., in contact with the air chamber) without other premanipulation.

After Indian ink injection, if necessary, the vitelline membrane is torn open with a microscalpel at the site chosen for the graft. When the grafting operation has been performed, the window is sealed with a piece of tape and the egg is reincubated in the same orientation. Daily gentle manual rocking of the operated eggs enhances embryo survival.

VI. Neural Tissue Transplantations

Different types of neural tissue transplantations have been classified according to the purpose of the experiments.

A. Neural Tube Transplantations

1. Orthotopic Grafting

This operation has allowed the detection of NCC migration pathways and the construction of a neural crest fate map (Fig. 7; Le Douarin, 1982; Le Douarin and Teillet, 1973; Le Lièvre and Le Douarin, 1975).

NCCs leave the dorsal aspect of the neural tube progressively from rostral (5- to 6-somite stage at the dimesencephalic level; Le Lièvre and Le Douarin, 1975) to caudal levels (E4.5 and E5 in quail and chick embryos, respectively) (Afonso and Catala, 2005; Catala *et al.*, 2000). An interspecific graft is performed at a level where the NCCs are still inside the apex of the neural anlage, that is, in the neural

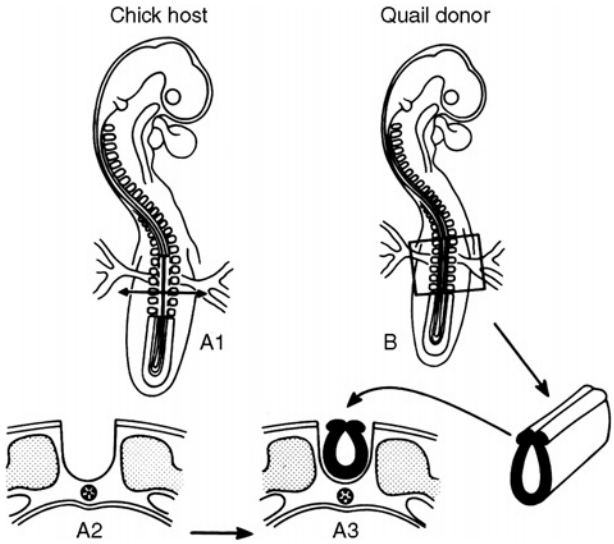

Fig. 7 Scheme of neural tube orthotopic transplantation. A fragment of the neural tube is microsurgically removed *in ovo* from the chick host at the level of the last segmented somites (A1 and A2). The corresponding level of a quail embryo at the same stage is submitted to enzymatic digestion (B) and is dissociated. The neural anlage free from surrounding tissues is inserted in the chick host (A3).

folds (NFs) (see next paragraph) of the cephalic area and at the level of the last formed somites for the cervical and dorsal regions. Quail and chick embryos are stage matched (the most precise way to determine the stage at E2 is to rely on the number of somites formed); the stage is selected according to the rostrocaudal level of the neural tube that is to be transplanted since neural crest migration starts at the level of a given somite only a few hours after it is formed.

a. Excision of Host Neural Tube

The selected neural tube fragment is excised from the host embryo by microsurgery *in ovo* (Fig. 8A). A longitudinal slit through the ectoderm and between the tube and the adjacent paraxial mesoderm is made bilaterally along the chosen part of the neural tube. The latter is then gently separated from the neighboring somites and is cut transversally, rostrally, and caudally. Using a microscalpel, it is then progressively separated from the underlying notochord and is finally sucked out with a glass micropipette.

b. Preparation of Graft

The transverse region of the donor embryo comprising the equivalent fragment of the neural tube plus surrounding tissues (ectoderm, endoderm, notochord, and somites) is retrieved with iridectomy scissors, and subjected *in vitro* to enzymatic

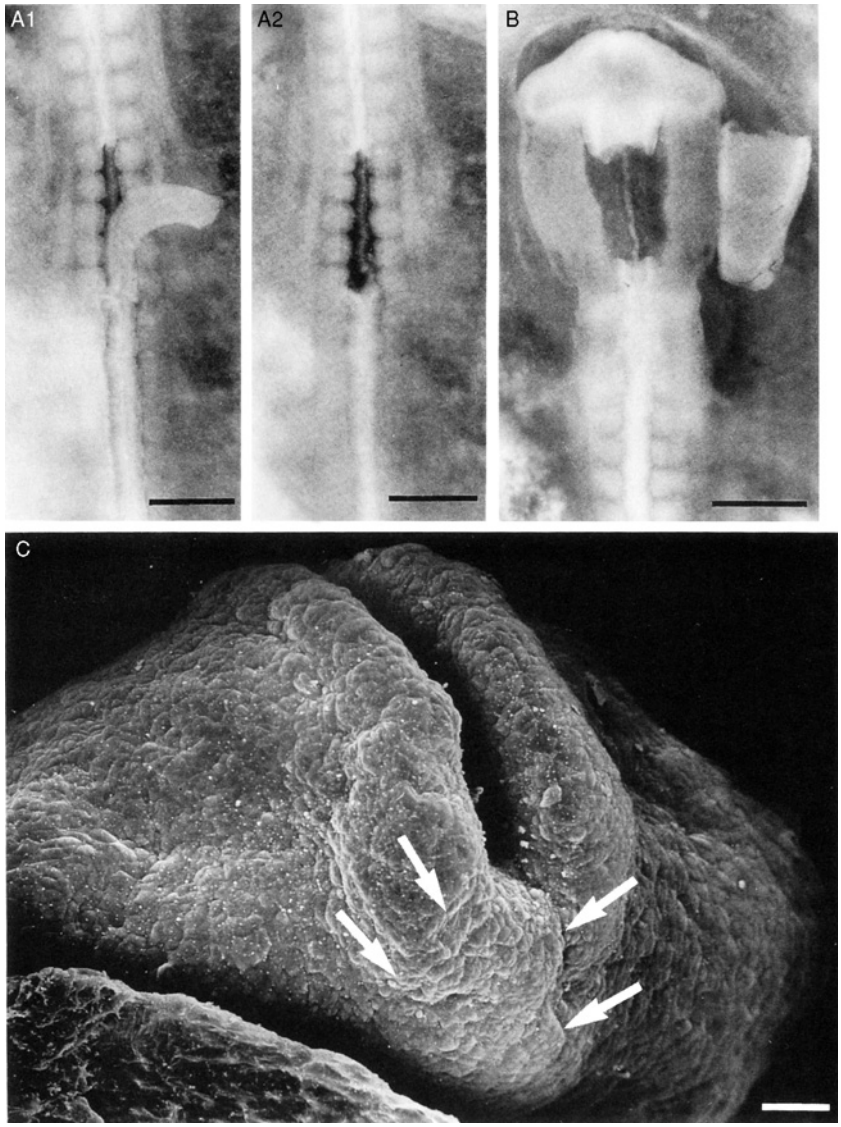

Fig. 8 Neural tissue microsurgery photographed at different steps of the procedure. (A) Ablation of a fragment of the neural tube at the level of the last segmented somites in an 18-somite stage embryo *in ovo*. Scale bars: 100 μm. (A1) The neural tube is partially removed. (A2) The notochord is visible at the level from which the neural tube has been completely removed. (B) The mesencephalic and met encephalic vesicles have been microsurgically excised from a 12-somite stage chick embryo *in ovo*. The equivalent quail vesicles that are to be grafted in the free space are positioned next to the host encephalon. Scale bar: 100 μm. (C) Scanning electron micrograph of a rostral neural fold graft (arrows) in a 5-somite stage chick embryo, 2 h after the operation. The neural fold has been replaced at the 3-somite stage by an equivalent fragment excised from a quail embryo. This region of the neural fold gives rise to the adenohypophysis (Couly and Le Douarin, 1985). Note the perfect incorporation of the quail-grafted tissue (courtesy of G. Couly and P. Coltey). Scale bar: 25 μm.

digestion (pancreatin 1:3 in PBS or Tyrode) for 5 to 10 min on ice or at room temperature according to the age of the embryo, and is then rinsed with PBS or Tyrode. The addition of calf serum inactivates the proteolytic enzymes.

c. Grafting Procedure

The donor neural tube is transplanted to the host embryo using a calibrated micropipette and is placed in the groove produced by the excision in the normal rostrocaudal and dorsoventral orientations.

Labeling of NCCs by orthotopic grafting of a fragment of quail neural anlage into a chick embryo (or vice versa) theoretically can be accomplished at any rostrocaudal level. However, the lumbosacral and caudal neural tube arising from the tail bud develops late (at E3–E4), when this type of microsurgery becomes virtually impossible because of the curvature of the tail. For this reason, operations bearing on the region caudal to somite 25 have to be performed on presumptive territories at E2 and checked for accuracy on the following days using the newly segmented somites as landmarks (Catala *et al.*, 1995).

2. Heterotopic Grafting

This type of grafting of fragments of the neural tube to different locations is instrumental in studying whether the fate of NCCs is specified when the operation is carried out. The graft is taken from the donor at a more rostral or more caudal level than the acceptor level (see Le Douarin and Teillet, 1974). Depending on the latter, the donor embryo will be older or younger than the recipient (see Le Douarin, 1982, and references therein).

3. Grafting of a Neural Tube Compartment

A unilateral compartment of the neural tube can be selectively exchanged in order to reveal differences between contralateral NCC migrations.

B. Hensen's Node Transplantations

Hensen's node (HN), the avian equivalent of Spemann's amphibian organizer, the shield in fish and the node in mammals, is a pit located at the rostral end of the primitive streak that persists and moves progressively caudally during elongation of the embryo. At the caudal end of the neural axis, it is called the chordoneural hinge (CNH). Fate mapping and clonal lineage analysis of HN had been made at early stages [4 of Hamburger and Hamilton (HH)] (Selleck and Stern, 1991). We have performed fate mapping of the CNH and HN regions, respectively at 25ss (caudal bud stage) and 5–6ss (midgastrula stage), through transplantations from quail to chick embryos (Catala *et al.*, 1995, 1996). At the 5–6ss, the HN lies as a "button" under a depression of the neural plate, the median pit, in the middle of the caudal neural plate in the "sinus rhomboidalis." It is situated between the growing

notochord and the remnant of the primitive streak. In order to determine the fate of the mass of tissue situated under the median pit, we replaced this structure of the chick *in ovo* by its quail counterpart (see Fig. 2 in Charrier *et al.*, 2005).

After removing the vitelline membrane, the tissue to be excised from the chick embryo is demarcated by four incisions using a fine microscalpel. The delimited tissue comprises the button itself, plus narrow strips of the neural plate bordering it. This tissue is removed, leaving in place a small part of the underlying endoderm to support the graft when it is inserted. The equivalent button (endoderm included) is removed from the ventral side of a quail embryo of the same stage. It is grafted in place into the chick embryo, on the remaining endodermal support.

One day after the graft (25ss), notochord, floor plate, and dorsal endoderm, as seen in sagittal and parasagittal sections, are composed of quail cells from the level of the graft down to the CNH (see Fig. 2 in Charrier *et al.*, 2005). Quail-labeled lateroventral neural tissue indicates the level of the graft.

Recently, three zones (a, b, and c) have been distinguished in HN according to morphological and molecular criteria (Charrier *et al.*, 1999, 2005). In zone a, situated above the median pit, notochord and floor plate are separated by a basement membrane and express *HNF3β* and *Shh*. Zone b lies at the level of the median pit and expresses *HNF3β* throughout but *Shh* only in its ventral portion (future notochord), which is not physically separated from its dorsal portion (future floor plate). A mesenchymal zone c expressing *HNF3β* but not *Shh* is in close contact with the primitive streak.

Surgical excision of zone c (or a barrier introduced between zone c and primitive streak) stops the rostral-caudal progression of HN and the deposition of the floor plate territory and notochord at the level of the caudal neural tube (Charrier *et al.*, 2001).

These experiments clearly show that the floor plate of the neural tube has a different origin from the rest of the neural tube. This results from the rostral-caudal migration of HN, leaving in its wake the median floor plate territory and the notochord (Charrier *et al.*, 2002 and also see Charrier *et al.*, 2005, and references therein).

C. Transplantations of Anterior NFs and Neural Plate at Early Neurula Stage

Fate maps of the early rostral neural primordium have been established. These involved substitution of neuroepithelial territories at the neurula stage in embryos having 0–5 somites (Couly and Le Douarin, 1985, 1987, 1988; Couly *et al.*, 1993).

Very thin, sharp microscalpels (made from tungsten fibers or entomology needles) are used to excise precise fragments of the NFs. The grafts are not subjected to enzymatic treatment because no mesoderm is present in the NFs at that stage. An ocular or objective micrometer is used to measure the pieces of tissue that are to be removed and grafted. Pieces of NFs are grafted orthotopically (Fig. 8C) for studying normal development of the cells comprising this structure or

heterotopically to discover the level of autonomy of the grafted territory (Grapin-Botton *et al.*, 1995).

D. Transplantations of Brain Vesicles

This operation has been devised to label defined regions of the brain and to study cell migration within the neuroepithelium itself (Alvarado-Mallart and Sotelo, 1984; Hallonet *et al.*, 1990; Martinez and Alvarado-Mallart, 1989; Tan and Le Douarin, 1991) or to transfer a defined brain region thought to encode a genetic behavioral or functional trait from host to recipient in either xenogeneic or isogeneic combinations (Balaban *et al.*, 1988; Le Douarin, 1993; Teillet *et al.*, 1991, and references therein). For these different purposes, either the entire encephalon or fragments of encephalic vesicles can be exchanged between chick and quail or between normal and mutant chick embryos.

Donor and recipient embryos are chosen around somite stage 12, which is particularly appropriate for the following reasons: (1) brain vesicles are clearly demarcated by constrictions but extensive brain curvature or covering of the brain by the amnion have not yet occurred, (2) the neural tissue does not adhere strongly to the notochord, and (3) the neuroepithelium is not yet vascularized. At younger stages, landmarks are lacking and precise measures have to be taken to know exactly what part of the brain is dissected and grafted (see Couly and Le Douarin, 1985). Moreover, separation of the neural tissue from the underlying notochord is much more difficult (see Grapin-Botton *et al.*, 1995).

Equivalent brain vesicles or parts of them are excised microsurgically in the stage-matched donor and recipient (Fig. 8B). The dorsal ectoderm is slit precisely at the limit between the neural tissue and the cephalic mesenchyme on each side of the selected brain region. The neural epithelium is then separated from the cephalic mesenchyme, cut out transversally (and longitudinally) at the chosen levels, and finally separated from the notochord if necessary. Heterotopic grafts can be made to study specific problems (Grapin-Botton *et al.*, 1995; Le Douarin, 1993; Martinez and Alvarado-Mallart, 1990; Martinez *et al.*, 1991; Nakamura, 1990, and references therein).

In order to study the extent of the territory that yields the cerebellar cortex, very refined experiments have been performed (Hallonet and Le Douarin, 1993). Fragments of the alar plate extending from 20° to 120° from the sagittal plane were exchanged between chick and quail embryos at the level of the mesencephalon and metencephalon (Fig. 9). This provided insights into the pathways of cell migrations taking place within the neural epithelium, and precise the nature and extent of the morphogenetic movements affecting this region of the brain.

Interestingly, when the graft is successfully incorporated into the host encephalon, the chimeric brains develop with a gross anatomy very close to normal (Fig. 10). Brain chimeras can hatch and show apparently normal behavior (Fig. 11). Histologically, one can see that the grafted cells are perfectly integrated into the host tissues. Cell migrations are so abundant in the brain that small- and

medium-sized grafts become entirely chimeric because of the penetration of host cells. Similarly, the host neural structures are invaded by donor cells. It is remarkable that the pattern of these migrations is highly reproducible for each type of graft. Therefore, it can be assumed that these migrations reflect normal cell

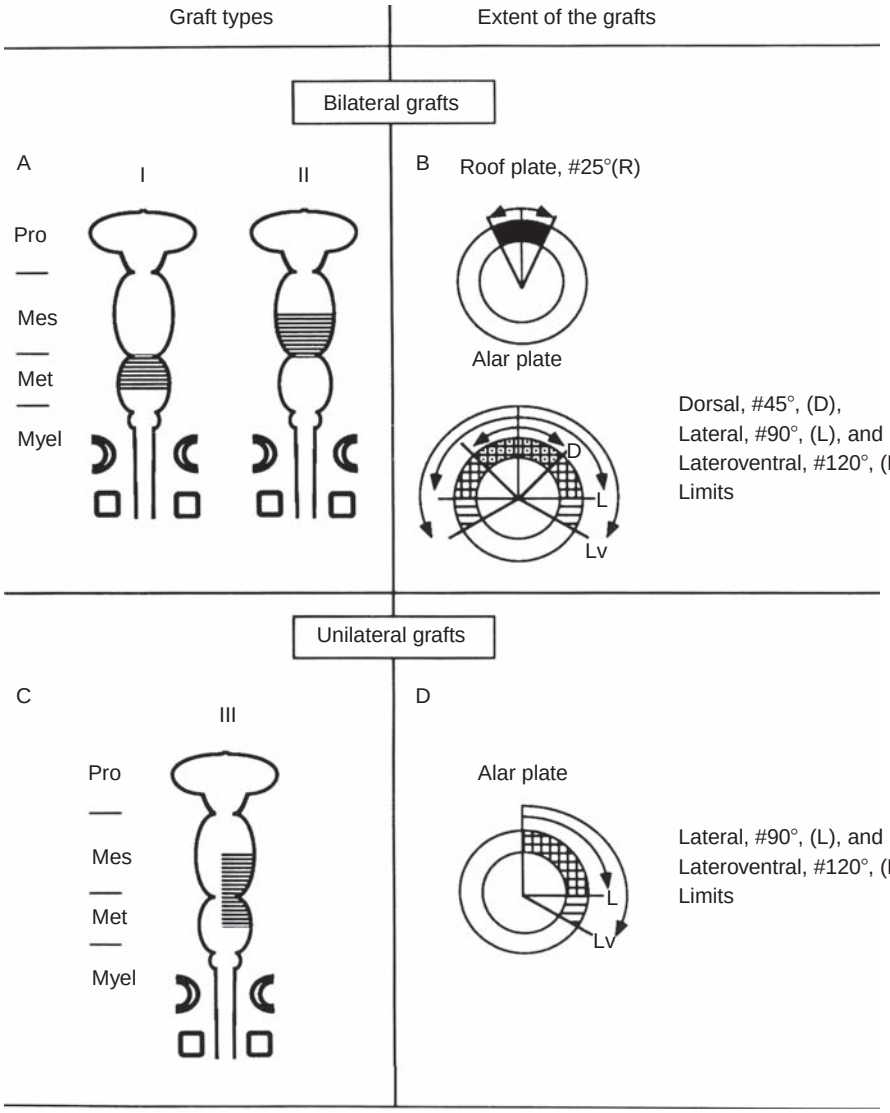

Fig. 9 Different types of isotopic and isochronic reciprocal changes made between chick and quail embryos in order to study cerebellum origin and development. These grafts are bilateral (A and B) or unilateral (C and D) and cover different dorsoventral sectors of the alar plate (B and D). Reprinted with permission from Hallonet and Le Douarin (1993).

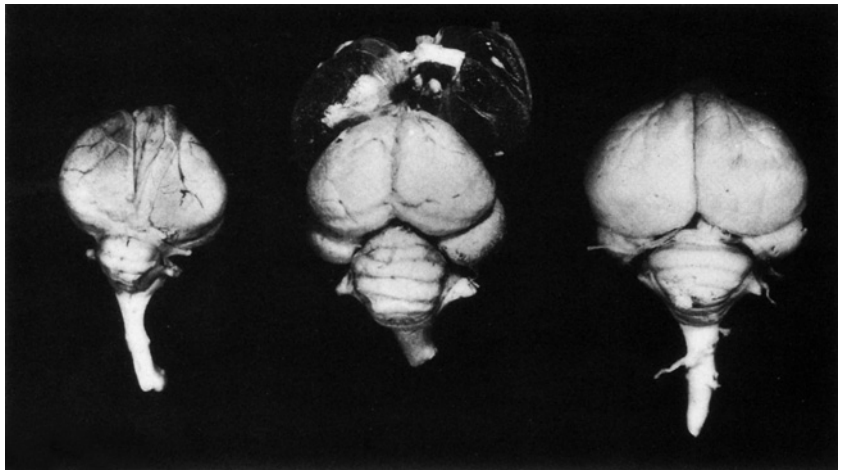

Fig. 10 The brain of a chimera (center) 7 days after hatching between the brains of normal quail (left) and chick (right) of the same age. The chimera was constructed by replacing the prosencephalon of a chick embryo with that of a quail. The grafted quail hemispheres are narrower than the optic tecta of chick origin. The eyes have been left in place in the chimera.

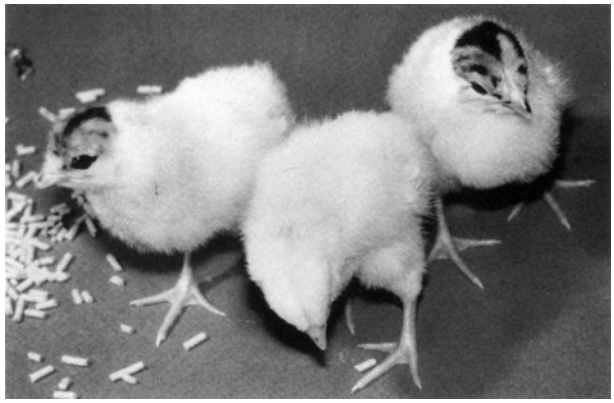

Fig. 11 Two quail–chick brain chimeras and a control chick 4 days after hatching. In the chimeras, a quail dorsal prosencephalon was grafted *in ovo* at the 12-somite stage. Quail melanocytes of neural crest origin decorate the head feathers of the chimeras at the level of the graft.

movements in the neuroepithelium. Notably, the cells of the ependymal epithelium do not mix, which means that the quail–chick limit at this level indicates, in fact, the initial limits of the graft. This allows evaluation of the extent of migration of the host cells into the graft and vice versa (Fig. 12).

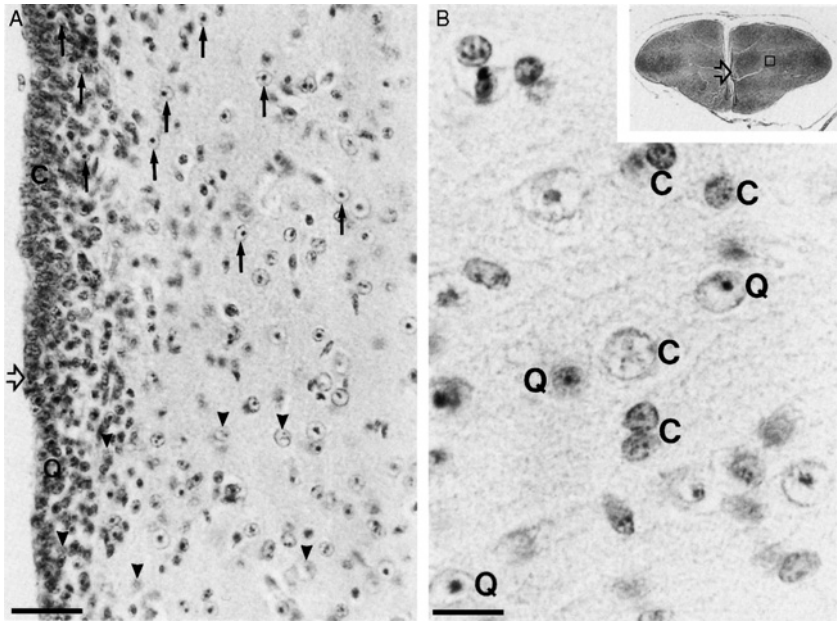

Fig. 12 A Feulgen-stained section through the cerebral hemispheres of a brain chimera in which the dorsal part of a quail prosencephalon has been orthotopically grafted at the 12 somite stage. (A) At the level of the ventricular epithelium, quail (Q) and chick (C) cells are clearly segregated (open arrow). A mixing of quail (arrows) and chick (arrowheads) cells in the subventricular zone indicates tangential cell movements during development; this produces chimeric brain regions, one of which framed in the insert is enlarged in B, where quail and chick neuronal and glial cells are mixed. The large open arrow indicates the ventricular boundary of the graft. Scale bars: (A) 30 μm; (B) 10 μm. Reprinted with permission from Balaban *et al.*, (1988).

VII. Early Transplantations in Blastodiscs

A. Blastoderm Chimeras

These chimeras can be made for investigating immunological tolerance or with the aim of producing transgenic birds (Watanabe *et al.*, 1992). The area pellucida of stages XI–XIII (Eyal-Giladi and Kochav, 1976) quail blastoderms is dissected out and cleaned free of yolk in Tyrode's solution, then cells are dissociated with or without enzymatic treatment. Seven hundred to 2000 cells suspended in 1.3 ml of Tyrode are injected into the subgerminal cavity of Stage XI to 2 (HH) chick embryos with a 70- to 100-μm tip diameter siliconized glass pipette using a micromanipulator. Injections are made in different locations of the blastodisc (central or posterior) according to the stage of development of the chick host. Chick–chick chimeras have been constructed according to the same pattern and were able to hatch (Petitte *et al.*, 1990).

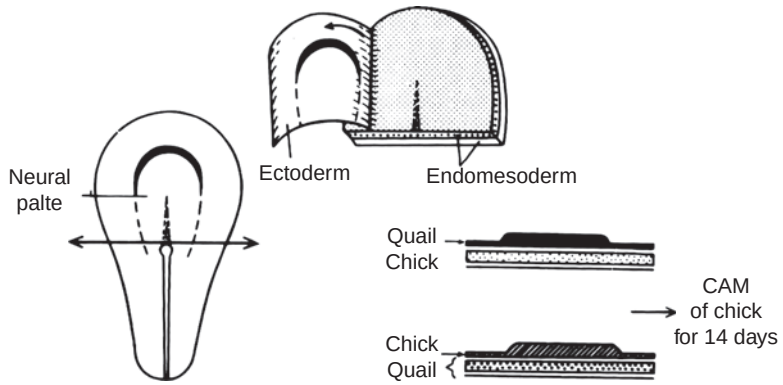

Fig. 13 Germ layer recombination.

B. Germ Layer Combinations

Germ layer chimeras have been constructed and cultured *in vitro* and have been used to study gastrulation (Fontaine and Le Douarin, 1977; Vakaet, 1974). Blastoderms incubated for 5–8 h are dissociated mechanically into a hypoblast and an epiblast. The layers are then exchanged between quail and chick, and the recombinants are cultured, epiblast side down, for 24–50 h according to a culture technique by New (1955). At slightly later stages [head process to head fold (stages 5–6 of HH)], pieces of the area pellucida are dissociated by trypsin treatment (0.1% in Tyrode solution minus calcium and magnesium) into ectoderm on the one hand and endomesoderm on the other hand. Recombined layers are cultivated for 12 h on a semisolid medium to ensure their association and are then grafted onto the chorioallantoic membrane (CAM) of chick hosts (Fig. 13).

VIII. Transplantation of Epiblast or Primitive Streak Fragments

These transplantations, bearing on small segments of the selected structures, are used to map territories in the young blastodisc and to reveal the cell movements that occur during gastrulation and early neurulation (Schoenwolf *et al.*, 1989, 1992). Isotopic and isochronic grafts of a plug of epiblast or of short segments of the primitive streak are made *in vitro* from quail to chick or vice versa. Host blastoderms are cultured ventral side up according to New's technique for an additional 24 h. Similar experiments can also be performed *in ovo* (Catala *et al.*, 1996).

IX. Hemopoietic Organ Rudiment Transplantations

Similar to the cells of the neural primordium, migrations of cells are an inbuilt feature of hemopoietic cells. However, these migrations are more extensive in the hemopoietic system than in the nervous system since they continue past beyond

development, they may affect cells at different times of their maturation process, they can be resumed after a period of arrest, they do not need to follow defined pathways, and they respond to physiopathological cues. Cell labeling has revolutionized the long-held classical view of the ontogeny of the hemopoietic system by revealing one of its essential features: that stromal cells of hemopoietic organs do not yield hemopoietic cells. This was first hypothesized from chicken combinations where the sex chromosomes were used as markers (Moore and Owen, 1965, 1967) and could be definitively and precisely established by means of quail/chick transplantations (for a review see Le Douarin *et al.*, 1984).

A. Grafts on Chorioallantoic Membrane (CAM) and Injections into Chorioallantoic Vessels

The CAM of the chick embryo is an excellent culture environment for many viruses, microorganisms, and normal or tumorous tissues. When avian hemopoietic organ rudiments are grafted onto it, they are colonized by blood-borne extrinsic stem cells and thus become chimeric. This technique has the advantages of ease and rapidity. Indeed, it is sufficient to deposit the tissue or rudiment in an area devoid of large vessels on the CAM of 6- to 10-day embryos and the tissues become vascularized. If the frame of a stromal rudiment is grafted, it becomes colonized by hemopoietic cells from diverse hosts including between various avian species (e.g., quail, chick) or between reptiles and birds (e.g., quail and turtle; Vasse and Beaupain, 1981) but not between birds and mammals. Thus, a mouse thymic rudiment grafted onto the quail or chick CAM remains uncolonized (Moore and Owen, 1967). Blood vessels from the CAM invade the grafted rudiment and/or connect with those in the rudiment.

Alternatively, it is possible to inject hematopoietic cells into a vein of the CAM at 13 days of incubation. To this end, the egg is candled and branching vessels are identified and marked on the shell. Using a circular saw, a triangle is cut from the shell around the branching vessels, making sure that the shell membrane remains undamaged. A drop of paraffin oil is applied to the window, making it transparent, so that the vessels become visible. The vessels remain adherent to the shell membrane, and the needle may be inserted tangentially in the direction of the branching. This procedure is mainly used to study the influence of adult lymphoid cells on the avian embryo and to analyze the process known as the graft versus host reaction (reviewed in Simonsen, 1985).

B. Grafts in Somatopleure

When the host has reached approximately 30-somite stage (around 52 h of incubation), the vitelline membrane is torn apart using watchmaker's forceps, and the amnion is split away. The graft, marked with a few particles of carbon black, is deposited near the grafting site. The somatopleura, that is, the body wall constituted by ectoderm and mesoderm, is split and the graft is inserted into the cleft such that it remains wedged into the opening. The size of the cleft must be

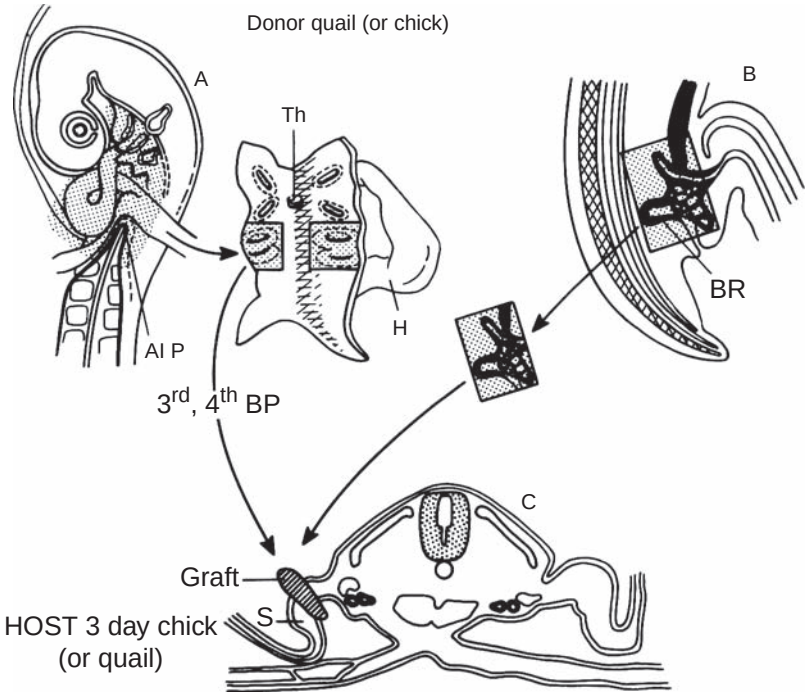

Fig. 14 Somatopleural grafting in the case of the thymus or bursa of Fabricius. AIP, anterior intestinal portal; BP, branchial pouch; H, heart; S, somatopleura; Th, thyroid. (A) A 3-day donor for the floor of the pharynx, (B) a 6-day donor for the bursa rudiment (BR), and (C) a transverse section in the trunk of a 2-day recipient showing the position of the graft.

adapted to that of the graft. Somatopleural grafting (Fig. 14) has been extensively used by Le Douarin's group to investigate the colonization schedule of the thymus and bursa of Fabricius (Le Douarin and Jotereau, 1975; Le Douarin *et al.*, 1975, 1984).

C. Coelomic Grafts

These are performed around 3 days of incubation and consist of introducing the graft into the coelom through a cut into the abdominal body wall. Applied to the quail chick model, this classical mode of grafting has been useful for showing that the allantoic bud has a hematogenous potential (Caprioli *et al.*, 1998, 2001).

D. Grafts in Dorsal Mesentery

Purely mesenchymal tissues, introduced through a deep cleft made along the ventral aspect of the aorta, embed within the dorsal mesentery of the host, which provides a hemopoietic microenvironment (Fig. 15). The wall of the dorsal aorta, grafted in this location, gives rise to hemopoietic foci (Dieterlen-Lièvre, 1984).

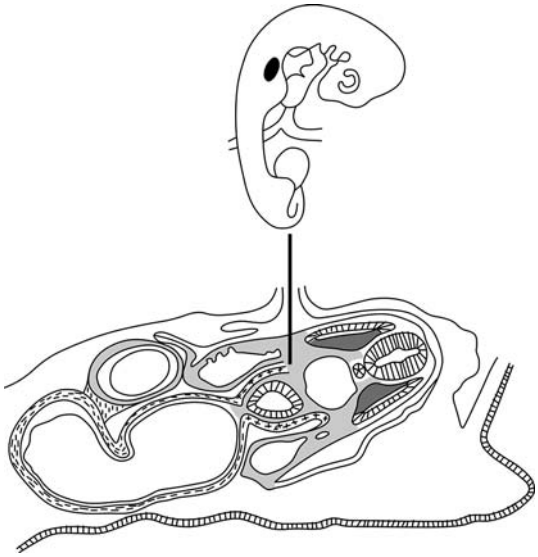

Fig. 15 Grafting into the dorsal mesentery. The graft is inserted through a cleft in the body wall at the wing level just ventral to the aorta (black dot in the schematized embryo). The arrow indicates the location of the graft, ventral to the aorta in a section.

E. Parabiosis

Parabiosis between two chicken embryos is a classical technique (see Metcalf and Moore, 1971). A simple method can be used to achieve parabiosis between two quail embryos (Le Douarin *et al.*, 1984). The contents of two quail eggs are poured into an emptied chick shell that serves as a culture dish. Vascular anastomoses form when the two embryos develop. The transfer of the two eggs to the empty shell can be done until day 2 of incubation. This system has been used to demonstrate the physiological nature of the cyclic periodicity of thymus colonization by hemopoietic precursors in intact embryos (Le Douarin *et al.*, 1984).

F. Orthotopic Transplantations of Thymus and Bursa of Fabricius

The heterotopic transplantation of the thymus and bursal rudiments into the somatopleure of an E3 host has been instrumental in demonstrating the hemopoietic origin of the T and B lymphocytes, the colonization schedule of the primary lymphoid organs, and the emergence of T-cell subpopulations within the thymus (see Dieterlen-Lièvre and Le Douarin, 1993, and references therein). More refined techniques have been devised (Belo *et al.*, 1985; Martin, 1983) *in ovo* to substitute the thymic and bursal epitheliomesenchymal rudiments of the chick by their quail counterpart prior to their colonization.

1. Microsurgery of Thymus

a. Extirpation of Thymus

Extirpation is carried out when the chick embryo is at stage 25–26 of HH (E5). The shell is opened above the air chamber, and the shell membrane is removed. Using forceps, a window is carefully opened in the amnion and the chorion is pushed aside. The tegument of the neck is then cut with a microscalpel above the vagus nerve, which can be seen transparently through the skin. The two thymic primordia that derive from the third and the fourth branchial pouches appear as white masses between aortic arches III–IV and IV–V, respectively. They are dissected and sucked out with a micropipette that has an internal tip diameter of 0.1 mm. The proximity of the aortic arches and jugular veins makes the operation quite delicate. If these vessels are injured, the embryos usually die within 24 h. The glossopharyngeal nerves are present near the posterior thymic primordia and make their complete ablation difficult. For bilateral thymectomy, the embryo that usually lies right side upward is first operated on the right side and is then rotated inside the amniotic cavity.

b. Grafting

The quail thymuses that are to be grafted are taken either from 5-day-old embryos, still uncolonized by lymphoid precursor cells, or from 6- to 8-day-old embryos, which have already been seeded. Each thymic rudiment, which includes a minimum of surrounding mesenchyme, is inserted into the space made by the extirpation of the host thymic primordium.

2. Microsurgery of Bursa of Fabricius

The bursa develops much better when it is grafted in an orthotopic position instead of a heterotopic site. This experimental model has been used to study the immunological status of birds in which B-cell progenitors develop in a foreign microenvironment (Belo *et al.*, 1985; Corbel *et al.*, 1987). As in the case of the thymus, surgery involves two steps: bursectomy followed by *in situ* implantation of a foreign bursal rudiment (Fig. 16). The method, described next, which permits precise replacement of the recipient bursa by a foreign rudiment, is well tolerated so that animals hatch and survive until adulthood. This method has replaced a former operation in which the rump of the embryos was cut out at 72 h of incubation (Fitzsimmons *et al.*, 1973). This resulted not only in the absence of the bursa but also in the absence of the caudal gut and was not compatible with prolonged postnatal survival.

a. Bursectomy

Bursectomy is performed in the E5 chick embryo. After opening the shell and the shell membrane, a window is made in the chorion and the amnion, above the posterior part of the embryo. The right hind limb of the embryo is deflected by

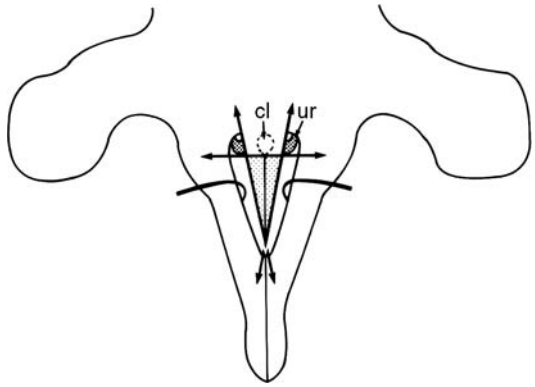

Fig. 16 The posterior part of a chick embryo at 5 days of incubation after a superficial section over the cloacal area. Arrows indicate the transverse and lateral cuts that will liberate the bursal region (represented as a dotted area), which will be removed. Ur, arrival of the uretera in the cloaca; cl, view of the cloacal cavity across the superficial ectoderm.

means of a humidified cotton thread, the ends of which are applied to the egg shell where they adhere. The tail is extended with curved forceps, thus providing access to the cloacal region. The bursal rudiment is isolated by a transverse cut in the anal plate just behind the ureters and is then removed by two lateral cuts.

b. Isotopic and Isochronic Transplantation of Quail Bursal Rudiment

The bursal primordium to be grafted is taken from quail embryos at E5, and the graft is inserted at the exact site of chick bursa removal. The superficial sheets of mesenchymal cells are peeled off from the quail bursal rudiment in order to avoid contaminating the graft by peribursal blood vessels and donor blood cells. The quail bursa, slightly smaller than its chick counterpart, is deposited in the space made by the extirpation of the host bursal rudiment; care should be taken to position the graft with the proper anteroposterior and dorsoventral orientation. The leg and the tail of the recipient embryo are then placed back in their normal positions. The amnion is sealed by joining and cutting together the edges of the opening. The shell membrane is pushed back into its initial position, covering the embryo, and the shell is sealed with adhesive tape.

G. Yolk Sac Chimeras

This microsurgical technique (Martin, 1972) consists of suturing the quail embryonic body onto the chick extraembryonic area (Fig. 17). The operation is performed during the second day of incubation on embryos ranging from 8 to 22 pairs of somites. The blastodiscs providing the two components of the association are matched for stage. White Leghorn chick eggs are incubated 6 h earlier than the quail to achieve synchrony. The quail blastoderms, donors of the embryonic area,

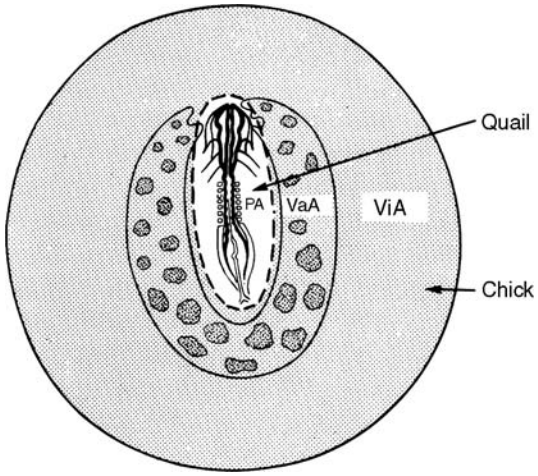

Fig. 17 Construction of a yolk sac chimera. The stippled line indicates the suture between the two components. PA, pellucid area; VaA, vascular area; ViA, Vitelline area.

are taken out in Tyrode's solution and the vitelline membrane is removed. The central area of the blastoderm is trimmed, but a small margin is left around the head and somites. This margin will be resected as the central area is seamed onto the extraembryonic area.

The recipient chick blastodisc can be made more visible by depositing a few particles of neutral red on the vitelline membrane. As neutral red diffuses, it stains the blastodisc evenly. The trimmed donor embryo is transplanted by means of a wide-mouthed pipette onto the recipient blastoderm and is positioned correctly, with respect to the germ layers as well as with respect to the cephalocaudal axis, side by side with the original embryo. Only then is the vitelline membrane of the host blastoderm torn apart and the embryo excised. The transplanted embryo is moved above the cavity, and the edges of the two partner blastodiscs are seamed together by resecting their margins simultaneously with Pascheff-Wolff scissors. The success of the operation depends on the localization of the blastodisc, which, ideally, should be at the center of the egg opening. If the blastodisc is in an oblique position, the suture, submitted to shear stress, may crack open. After the operation, the egg is sealed with tape and reincubated. New blood vessels grow across the seam and the chimeras develop according to a pattern very similar to that of normal quail embryos. The weight of the grafted quail embryo is statistically increased at all ages of incubation, and the retraction of the yolk sac, which is under thyroid control, proceeds more rapidly than in a control chick embryo. Around 10% of the operated embryos continue development and reach day 13 or 14 of incubation. In these chimeras, definitive hemopoietic organs are exclusively colonized by hemopoietic stem cells from the embryo proper, not from the yolk sac (Dieterlen-Lièvre, 1975; Martin *et al.*, 1978), while circulating red

blood cells are from the chick species until E6 and become progressively intermixed with erythrocytes from quail thereafter (Beaupain *et al.*, 1979).

This type of chimera has also been made between two chick partners from inbred lines differing by their gender (Lassila *et al.*, 1978), by presumptive immunoglobin allotypes (Martin *et al.*, 1979), or by their major histocompatability antigens (Lassila *et al.*, 1982). Such chick–chick chimeras were able to hatch and grow to adulthood.

Finally, chick embryos have also been grafted onto quail extraembryonic regions, that is, according to the reverse combination (Cuadros *et al.*, 1992). The principle of the operation is basically the same. It should be pointed out, however, that suturing the transplanted embryonic area onto the recipient blastodisc is very difficult in this configuration because of the small size and the more pronounced curvature of the quail vitelline globe. These chimeras have been raised only until day 5 of incubation.

A variant of the yolk sac chimera consists of replacing part of the embryonic body of one species with the homologous part of the other species. These so-called "complementary chimeras," made either *in vitro* (Didier and Fargeix, 1976) or *in vivo* (Hajji *et al.*, 1988; Martin *et al.*, 1980), have proven useful for studying the development of the kidney and the gonad.

X. Genetic Manipulation by Electroporation in the Quail–Chick System

Electroporation aims at transferring nucleic acid sequences into living cells. The principle is based upon a transient permeabilization of the cell membrane triggered by an electrical impulse, which enables an exogenous nucleic acid sequence to enter the targeted cells, and become trapped in the cytoplasm as the cell membrane recovers its integrity. First devised *in vitro*, this technique has been successfully adapted to *in vivo* transfection in the mid nineties. It turns a unique pulse of high voltage—with an exponential decay—into a series of square low-voltage iterative pulses applied to the two electrodes, anode and cathode. When placed in a polarized electrical field, nucleic acids (globally negative molecules) are displaced toward the cathode. According to the above principle, the interposition of an epithelium between the nucleic acid solution and the cathode results in the directional transfer of the foreign nucleic sequence into the epithelial cells.

First introduced by Harakazu Nakamura (Tohoku University, Japan), this technique that enables an instantaneous and highly efficient penetration of the vector into the cells (Muramatsu *et al.*, 1997; Sakamoto, *et al.*, 1998) has rapidly conquered the field of the avian embryology, in which no powerful means of transgenesis had been achieved before. This technique has allowed the study of gene function in avian developmental processes with the added feature that, in this class of vertebrates, classical embryonic tissue manipulations can be combined with molecular approaches. Moreover, due to the accessibility of the embryo, in

the egg, at a range of developmental stages, transgenesis can be performed in stage- and space-specific manner.

Two kinds of constructs, plasmids or retroviral vectors, can be used to achieve either ectopic gene expression or *in situ* overexpression. The main drawback of plasmids resides in the nonpermanent integration in the transected-derived cells, thus leading to progressive loss of the vector and finally turning off the foreign gene expression (whose kinetics tightly depend on the proliferate rate of the recipient tissue). In contrast, the use of retroviruses that randomly integrate into the host genome by means of their long terminal repeat sequences ensures a stable transfection of the exogenous DNA. Exploiting the species-restricted infectious ability of viruses into either a permissive or repellent species environment provides an elegant approach to limit the spread of retroviral contamination in quail–chick chimeras.

An example of this procedure is the electroporation of the NF of a quail embryo prior to NCC emigration, using a quail-specific retroviral construct that is unable to infect chick cells. In a second step, the transfected NF (recognized by Green Fluorescent Protein simultaneous transfection with a GFP construct) is isotopically and isochronically grafted into a naive chick embryo (Creuzet *et al.*, 2002).

The problem raised by the electroporation of the NCCs is that the cells emanating from the strands of NF merge along the dorsal midline before migrating bilaterally. As a consequence, the ''control'' and ''experimental'' sides receive a substantial contribution of transfected and untransfected cells, respectively, that can strongly bias the interpretation of resulting phenotypes. To solve this problem, a third electrode (e.g., one anode, rostral, and two cathodes, lateral) makes it possible to generate a triangular electric field leading to bilateral dispersion of the nucleic acid sequences.

An alternative method for tackling this problem consists in the unilateral electroporation of the NF followed by the bilateral transplantation of the transected tissues. For this purpose, two donor embryos are used; one for the right and another for the left NF (see Creuzet *et al.*, 2002 for technical description). When performed in interspecific environment (i.e., quail–chick chimeras), this procedure offers the unique opportunity to precisely correlate the forced gene expression to the fate of the transfected cells (by using the QCPN antibody to detect the quail cells). This combined strategy enables one to discriminate the fate of the targeted graft-derived cells from the untransfected host-derived environment (Creuzet *et al.*, 2002). This technique is therefore particularly useful to decipher the molecular basis of cell interactions that regulate morphogenetic events.

The electroporation method has been recently enriched to perform not only gain- but also loss-of-function experiments on the targeted gene. Loss-of-function can be achieved either transcriptionally through the use of morpholinos or post-transcriptionally using RNA interference (RNAi).

Morpholinos are sophisticated hexameric base antisense oligomeres typically designed against the sequences upstream to the initiation site of a cognate gene. When bound to the targeted sequence, morpholinos inhibit protein translation (for a review, see Summerton and Weller, 1997). Using this approach, Tucker and

coworkers switched off the expression of integrin subunits in the neural primordium and, as a consequence, observed perturbations in NCC epitheliomesenchymal transition and emigration (Kos *et al.*, 2003; Tucker, 2004).

The most popular way to produce loss-of-function is via the use of RNAi. While the mechanisms behind RNAi are not fully understood, its empirical application to experimental embryology has rapidly gained favor with avian developmental biologists. A pioneer in this field, Ester Stoeckli (University of Zurich, Switzerland) designed a potent and practical procedure to synthesize and prepare double-stranded RNA (dsRNA) by using the full length cDNA as a template (Pekarik *et al.*, 2003). The introduction of dsRNA in the targeted cells triggers a prompt cleavage by Dicer and generates multiple small RNAi that optimize the efficiency of RNA degradation for gene silencing. This system has paved the way for functional genomics in chick (Pekarik *et al.*, 2003; Stoeckli, 2005).

In our model, the electroporation of dsRNA against Fgf8 morphogen production in the presumptive BA1 ectoderm in early neurula hampers NCC migration and colonization of the underlying territories. The strong deficit of NC-derived mesenchymal cells on the transected side was interpreted as an effect of FGF8 on both survival/proliferation and migration of NCCs during branchial arch formation (Creuzet *et al.*, 2004).

XI. Results, Discussion, and Perspectives

The quail–chick transplantation technique has many useful applications. In our laboratory, it has been used to follow the migrations of cells of the neural, hemopoietic, somitic, and angiogenic lineages. For the first time, the wide dispersion of cells emerging from the neural crest could be visualized in the embryo itself from the moment they depart from the neural primordium up to when they have homed to their definitive location and fully differentiated.

The possibility of selectively labeling small populations of NCCs, for example, the vagal region that subsequently invades the whole gut, gives a striking view of the remarkably invasive capabilities of these cells (see Le Douarin, 1982 and Le Douarin and Kalcheim, 1999). The analysis of the fate of the cephalic NF performed in the early neurula (Couly *et al.*, 1993) and at later stages of development (see Le Douarin, 1982 and Le Douarin and Kalcheim, 1999, references therein) disclosed the paramount role of this structure in the morphogenesis of the vertebrate head.

The plasticity of the NCC populations arising from each level of the neural axis was revealed by heterotopic transplantations of fragments of quail neural primordium into chick embryos (Le Douarin and Teillet, 1974). This shows that the environment in which the NCCs migrate is critical in determining their fate.

Experiments involving construction of brain chimeras are particularly well suited in following the migrations of cells moving within the plane of the neuroepithelium (Le Douarin, 1993). As the ependymal layer does not mix, it demarcates

the initial limits of the quail and chick territories. Thus, either chick or quail cells that cross these limits can be easily recognized even if they have migrated far from their point of origin. It was not possible to detect such migrations, tangential in relationship to the surface of the neuroepithelium, using conventional radioisotopic method based on pulses of tritiated thymidine; however, the latter revealed radial cell migrations very efficiently.

The quail–chick method has clearly shown the tangential migrations of cells of the rhombic lip in the myelencephalon (Tan and Le Douarin, 1991) and has been used to study the origin of oligodendrocytes in the spinal cord (Cameron-Curry and Le Douarin, 1996).

Quail–chick chimeras have revealed morphogenetic movements affecting large areas of the brain vesicles during neurogenesis. This was shown for the cerebellum (Hallonet *et al.*, 1990) and analyzed in detail by transplantation restricted to defined strips of neuroepithelium more or less distant from the sagittal plan (Hallonet and Le Douarin, 1993). This technique nicely showed that mesencephalic areas could induce diencephalic neuroepithelial territories to express the engrailed gene (En2) and to acquire the tectal structure and properties (Itasaki and Nakamura, 1992; Itasaki *et al.*, 1991; Martinez *et al.*, 1991 and see Le Douarin, 1993, for more references).

Those are only a few examples of the results obtained by means of the quail–chick neural chimeras. Clearly this simple method, which can be combined with immunological reagents and molecular probes, still has a large array of future uses for deciphering the complexity of neurogenesis.

Another area where the quail–chick system has brought important and indisputable data concerns the development of the primary lymphoid organs. Prior to this, there were long controversy concerning the embryonic origin of the lymphocytes in the thymus and, in birds, in the bursa of Fabricius. The hematogenic hypothesis proposed by Moore and Owen (1965, 1967) suggested that lymphocytes and blood cells that develop in the hemopoietic organs have an extrinsic origin; this has been fully confirmed through the use of the quail–chick chimeras. Moreover, thanks to this technique, it was shown that the hemopoietic organs, with the exception of the yolk sac, are totally devoid of blood-forming capacity if they are not colonized by hematopietic precursor cells. It was also demonstrated that, at least in birds, the hemopoietic precursors that function during adult life originate from the embryo itself and not from the yolk sac as proposed by Moore and Owen (see Metcalf and Moore, 1971). Increasing evidence has accumulated suggesting that in mammals as in birds the yolk sac does not provide the adult with hemopoietic stem cells (Dieterlen-Lièvre, 1975; Dieterlen-Lièvre and Le Douarin, 1993). The mesoderm in the neighborhood of the aorta (Fig. 18; Dieterlen-Lièvre, 1994; Dieterlen-Lièvre and Martin, 1981) and the allantois were shown to produce new populations of stem cells (Caprioli *et al.*, 1998, 2001).

The quail–chick marker system has shown that the avian embryonic thymus is seeded by successive waves of incoming hemopoietic cells. Figure 18 summarizes the major events in the development of the hemopoietic system, all of which were

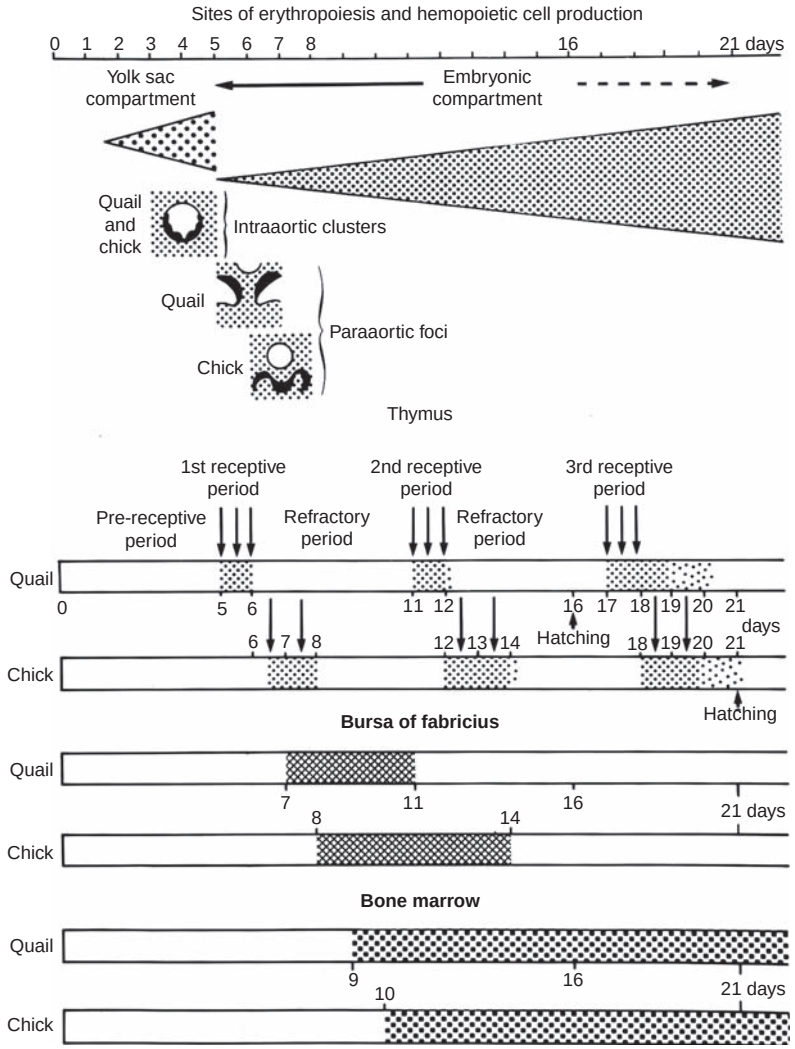

Fig. 18 Timetable of the major events in the ontogeny of the hemopoietic system in quail and chick embryos. During the first phase (symbolized with thick dots), the yolk sac functions with its own stem cells. During the second phase (finer dots), it receives additional stem cells from the embryo, as demonstrated in the chick yolk sac/quail embryo chimeras (Beaupain *et al.*, 1979). The intraaortic and paraaortic hemopoietic processes and the colonization periods of organ rudiments are schematized. This dynamic view of the ontogeny of the hemopoietic system was acquired by means of different quail/chick transplantation schemes; the quail nucleolar marker and the MB1/QH1 monoolonal antibodies were used to identify the species of origin of the component cells.

revealed by interspecific transplantations. The use of species-specific markers of T-cell subpopulations made it possible to precisely determine the type of receptor these T cells produce at different developmental times (Coltey *et al.*, 1989).

Moreover, the knowledge thus acquired on thymus ontogeny led to the demonstration that the thymic epithelium plays a major role in tolerance to self (Ohki *et al.*, 1987, 1988). Angiogenesis, the ontogeny of blood vessels, and the differentiation of their constitutive endothelial cells represent a crucial domain of developmental biology that is necessary for understanding several important biological processes such as morphogenesis and growth, as well as for tumor biology (review in Folkman and Shing, 1992). The avian embryo, in which blood vessel development can be easily visualized and followed, has served as an experimental system for a number of pioneering studies in this field (Noden, 1989; Pardanaud and Dieterlen-Lièvre, 1993, 1999; Pardanaud *et al.*, 1987, 1989, 1996; Péault *et al.*, 1983; Poole and Coffin, 1989). In particular, it has been shown that two different mechanisms are responsible for the emergence of the endothelial tree in the embryo: the body wall is colonized by extrinsic precursors, while the mesenchyme of internal organs gives rise to endothelial cells *in situ* (Pardanaud and Dieterlen-Lièvre, 1993).

The recent development of the transgenic technique by electroporation applied to the avian embryo has been an important asset for the Heuristic value of this model in developmental biology. The fact that gene loss or gain of function experiments can be combined with the chimeric technique brings about even more precision in the analysis of the developmental events under scrutiny as shown in recent works performed in our laboratory.

To conclude the quail/chick transplantation system has yielded new views about the development of a number of developmental systems including limbs, kidney, and gonads that are applicable to amniotes. The new tools such as electroporation reinforce powerfully the usefulness of the quail/chick model.

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CHAPTER 3

Other Chimeras: Quail–Duck and Mouse–Chick

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I. Introduction

A well-established and immensely effective experimental approach for studying the developmental histories of anatomical structures in avian embryos has been the quail–chick chimeric system (Baker *et al.*, 1997, 1999; Borue and Noden, 2004; Cobos *et al.*, 2001; Couly and Le Douarin, 1990; Couly *et al.*, 1992, 1993; Köntges and Lumsden, 1996; Le Douarin, 1973; Le Lièvre and Le Douarin, 1975; Lwigale, 2001; Marcucio *et al.*, 2005; Noden and Schneider, 2006; Noden, 1978, 1983, 1986; Olivera-Martinez *et al.*, 2000; Schienda *et al.*, 2006, 2001; Schneider, 1999; Selleck and Bronner-Fraser, 1995). Because quail and chick are closely related birds with comparable rates of growth and morphology, transplants between them have enabled the behaviors, functions, and fates of cells to be followed throughout embryogenesis (Le Douarin *et al.*, 1996). However, because of the similarities between quail and chick, subtle morphological changes that may have been induced by donor cells have, for the most part, gone unnoticed. In contrast, other avian chimeric systems have long included domestic duck as a means to study those patterning mechanisms that make embryos morphologically distinct (Dhouailly, 1967, 1970; Hampe, 1957; Pautou, 1968; Sohal, 1976; Sohal *et al.*, 1985, 1990; Waddington, 1930, 1932; Yamashita and Sohal, 1986, 1987; Zwilling, 1959). For example, recently published results using quail–duck chimeras have demonstrated the ability of neural crest mesenchyme to generate species-specific facial morphology (Schneider and Helms, 2003; Tucker and Lumsden, 2004) as well as feather pattern (Eames and Schneider, 2005) through the regulation of host gene expression.

The quail–duck chimeric system affords several benefits for discerning the effects of the donor on the host, or vice versa (Fig. 1). First, quail and duck embryos are morphologically distinct especially in terms of overall size and shape, which depending on the behavior of donor cells in the host environment, offers a direct way to explore donor- or host-specific mechanisms of pattern formation. Second, quail and duck embryos have considerably different rates of maturation (17 days vs 28 days), which contingent upon donor cells maintaining their internal clock following transplantation provides a means to determine the extent to which the donor can regulate the timing of gene expression, tissue interactions, histogenesis, and other events during development. That donor cells maintain their intrinsic rates of maturation has been confirmed by molecular and histological analyses of facial and cranial feather development (Schneider, 2005; Merrill *et al.*, 2008). Finally, as with the quail–chick chimeric system, the readily available and ubiquitous anti-quail nuclear antibody (Q¢PN) does not recognize duck cells and thus, allows donor and host contributions to be distinguished from one another (i.e., Q¢PN-positive vs Q¢PN-negative). By design, the quail–duck chimeric system allows the role of different cell populations during embryogenesis to be better understood and provides a potent experimental model for identifying time-dependent signaling interactions.

Although the avian embryo is easily accessible for experimental manipulations and highly suitable for embryological and molecular procedures such as microsurgical grafting of tissues and cells (Lwigale *et al.*, 2004, 2005), use of

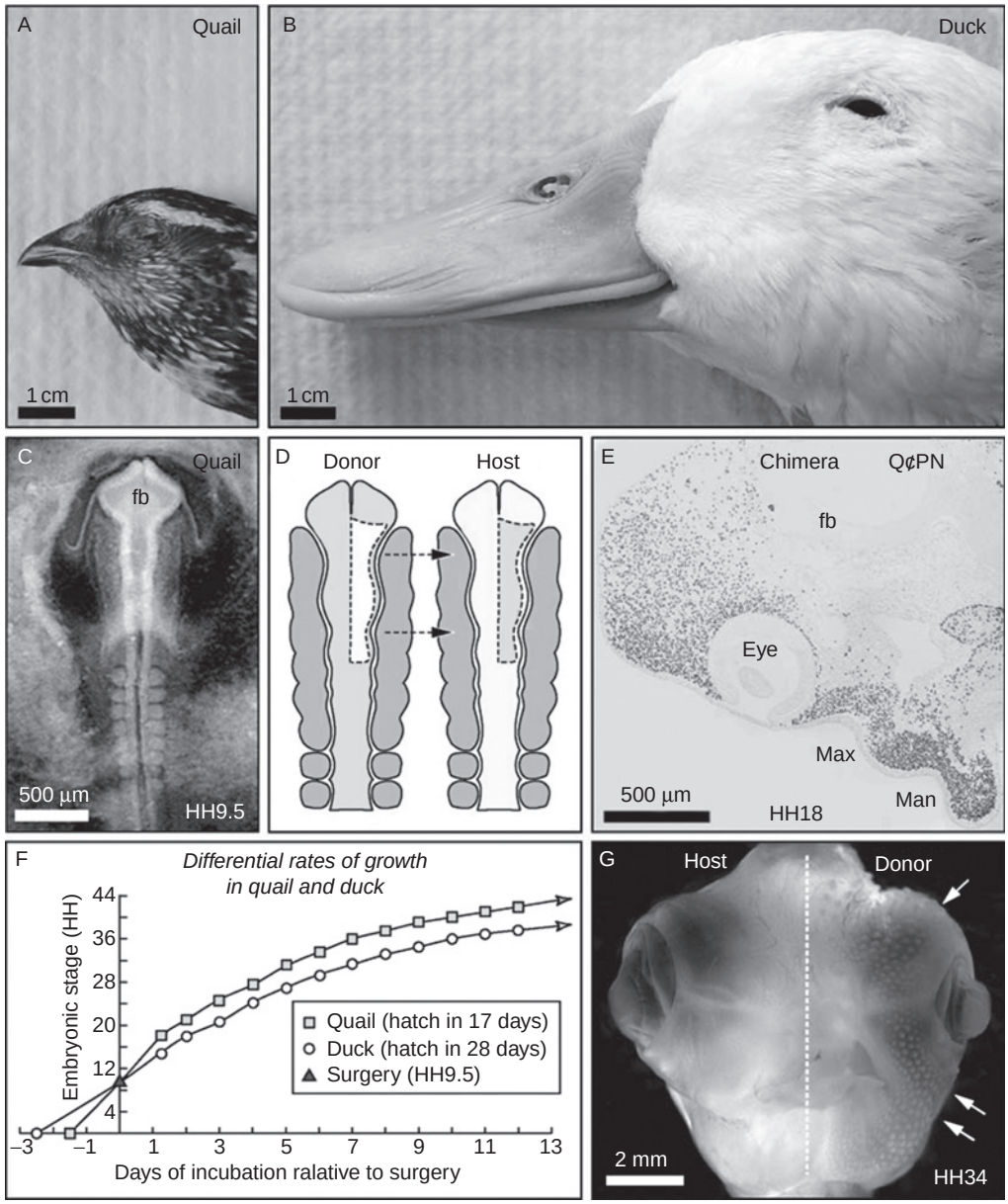

Fig. 1 (A) Japanese quail and (B) White Pekin duck display considerable morphological differences, and thus, are ideally suited for a chimeric system (Eames and Schneider, 2005). (C) Embryos can be prepared at a variety of stages (HH) for transplant as shown here in dorsal view up to the level of the forebrain (fb). (D) Neural crest cells can be cut unilaterally and transplanted from quail donors into duck hosts stage-matched at HH9.5. (E) Quail donor cells (black) can be followed in chimeras using an anti-quail antibody (Q¢PN) as shown in sagittal section through the maxillary (max) and mandibular

markers to track cell lineages (Cerny *et al.*, 2004; Serbedzija *et al.*, 1989), and perturbation of gene function by electroporation or transfection with genetic material (Krull, 2004), there are limitations to the system as a genetic model. In contrast, the mouse embryo has been well studied as a mammalian genetic model using transgenesis and gene targeting, but is not easily amenable to embryological manipulations. Moreover, the fact that some genes are embryonic lethal precludes the use of many mouse mutants for studying gene function at later stages of development.

Limitations that hinder the use of the avian and mouse models separately have been successfully circumvented by performing mouse–chick tissue recombination experiments (Lemus, 1995; Wang *et al.*, 1998) and by generating mouse–chick (Fontaine-Perus and Cheraud, 2005; Fontaine-Perus *et al.*, 1996, 1997; Fontaine-Perus, 2000a,b; Mitsiadis *et al.*, 2003, 2006; Serbedzija and McMahon, 1997) and/or mouse–quail chimeras (Pudliszewski and Pardanaud, 2005). The mouse–chick chimera technique has several advantages. First, grafted mouse tissue readily incorporates into the chick and contributes to the development of the chimera. Second, grafted mouse cells can be easily identified in the chick. Third, the behavior of genetically altered mouse cells can be analyzed after grafting into a normal chick host. Fourth, genetic effects on cell behavior can be studied at stages beyond that normally allowed by some embryonic lethal mutations.

In the following sections, we describe major techniques and methods that we generally employ to generate either quail–duck or mouse–chick chimeras. We also give a few examples of types of experiments that have already proven successful and yielded valuable information. Overall, we believe that using disparate taxa in chimeric systems offers unique advantages for addressing many outstanding questions in developmental and evolutionary biology, especially given that many of the genetic and molecular tools currently applied to conventional model systems (i.e., chick and mouse) work equally well in quail–duck and mouse–chick chimeras.

II. Preparation of Quail, Duck, Chick, and Mouse Embryos

A. Obtaining Avian Eggs and Conditions for Incubation

Fertilized eggs for a variety of avian species are readily available from breeders, farms, and agricultural schools. Often such eggs can be shipped overnight via commercial courier services directly to the laboratory. Because Japanese quail

(man) primordia, which respectively give rise to the upper and lower portions of the beak. (F) Due to their distinct maturation rates, quail and duck embryos that are stage-matched for surgery grow apart that provides a potent assay to identify donor-mediated events. (G) Quck chimeras have a side derived from the duck host as well as a side containing donor quail cells, which in this case leads to premature feather differentiation (arrows; B. Eames, unpublished data).

(*Coturnix coturnix japonica*), White Pekin duck (*Anas platyrhynchos*), and domestic chick (*Gallus gallus domesticus*) are typically bred in large numbers for the food industry, their fertilized are eggs relatively inexpensive and generally of high quality. When eggs arrive in the lab they are immediately unpacked, wiped down with 70% ethanol, placed on plastic egg trays, and stored for up to 1 week in a refrigerator set at 14–16 °C. Such cold storage can keep embryos arrested at very early stages of development, but to grow embryos further in a manner that minimizes shock, eggs should be acclimated to room temperature for several hours prior to being set in a heated incubator. Quail, duck, and domestic chick can all develop successfully at 37.5 °C in a chamber with 85–87% humidity. Eggs should be placed horizontally and the highest point marked with pencil (the change in humidity causes the embryos to rotate to the top of the yolk, which ideally will be directly below the pencil mark). The importance of adequate humidity cannot be overemphasized; humidity should be monitored and trays containing distilled water must be filled regularly. In general, there are many different small and large incubators manufactured from various materials (e.g., wood, Styrofoam, metal, Lucite) that provide appropriate environmental conditions for avian development. Regardless of the size or type, all incubators should be cleaned occasionally with 10% bleach, 70% ethanol, and then with distilled water in order to minimize contamination from bacteria and yeast.

B. Windowing Avian Eggs and Visualization of Embryos

Eggs can be incubated for any desired amount of time depending on the species of bird and the type of surgery to be performed. From fertilization, Japanese quail hatch in 17 days, White Pekin duck in 28, and domestic chick in 21. Thus, to perform surgery at comparable stages between different species, total incubation time needs to be adjusted appropriately. To access embryos during development, a small piece of outer shell from the top of the egg (i.e., at the pencil mark) can be removed with forceps, taking care not to puncture the inner shell membrane. Then a syringe with an 18 gauge by 1 inch needle can be used to poke a hole at the narrowest tip of the egg and to withdraw approximately 1–2 ml of albumen. This allows the embryo to drop down and also creates a working distance under the shell. The hole can be sealed with a small piece of transparent tape or a dab of melted paraffin, and then a window can be cut along the top surface of the egg using curved scissors (transparent tape may also be applied before cutting the egg shell to prevent pieces from falling on the embryo). To enhance visualization, either a small amount of Neutral Red (0.02 g/ml sterile saline) can be brushed lightly with a blunt glass rod over the embryo, or an ink solution (one part Pelikan Fount India ink mixed with nine parts sterile saline) can be injected underneath the blastoderm using a 1 ml syringe with a curved 25 gauge by 5/8 inch needle until the white translucent embryo is contrasted against the black background.

C. Staging Avian Embryos and Preparation for Surgery

Although development is a continuum, avian embryos can be matched at equivalent stages based on external morphological characters that are relatively independent of body size and incubation time (Hamilton, 1965; Ricklefs and Starck, 1998). The Hamburger and Hamilton (HH) staging system for chick is well-established (Hamburger and Hamilton, 1951) and can also be used for classifying quail (Le Douarin *et al.*, 1996). Separate staging systems are available for duck (Koecke, 1958) and quail (Padgett and Ivey, 1960) but these can be adapted to the scheme used for chick (Schneider and Helms, 2003; Yamashita and Sohal, 1987). Criteria utilized to determine a particular stage change over time depending on which structures become prominent. For early ontogeny, neurulation and somitogenesis (i.e., number of somites) are principally employed as identifiers of embryonic stage, whereas for later, features such as the limbs, pharyngeal arches, eyes, and feathers become more apropos. Typically, for surgeries involving cranial neural tube grafts or neural crest cell injections, embryos between HH9 (7 somites) and HH10 (10 somites) are used because at this stage neural crest cell migration is just under way in the midbrain region (Le Lièvre and Le Douarin, 1975; Tosney, 1982), and host embryos are very receptive to grafted neural crest cells. For trunk grafts, embryos at HH11 (13 somites) or older can be used. To stage-match avian embryos, eggs from desired avian species simply need to be placed in the incubator for different intervals prior to surgery. For example, to perform surgery at HH9.5, duck embryos are incubated for approximately 2.5 days, chick embryos for almost 1.5 days, and quail embryos for a little more than 1 day (Fig. 1).

Once avian embryos have reached the desired stage and are ready for surgery, flame-sharpened tungsten needles can be used to cut and pull back the vitelline membrane over the embryo, and a drop of sterile saline can be spread around and over the embryo to prevent dehydration (Schneider, 1999). To manufacture surgical needles, tungsten rods (A-M Systems, Everett, WA) are cut in half and threaded through the tapered end of a borosilicate glass Pasteur pipette. Leaving approximately 3/4 inch of wire sticking out of the glass, the wire is adhered to the glass with a small bead of dental wax or hot glue (refills for a hot glue gun can be melted with an alcohol flame). Using a pair of forceps, the tip of the tungsten wire (about 1/4 inch from the top end) is bent until a 45° angle is achieved. Holding the Pasteur pipette, about 1/8 inch of the tip of the tungsten wire is placed into the flame of a propane torch. Preferably, a propane fuel cylinder with a screw-on brass ''pencil flame'' torch is used because the type of torch controls the size of the flame. The tip of the wire should be held steady and perpendicular to the flame. The concept is that the very tip of the needle will get hotter relative to the rest of the wire and eventually the heat differential will cause only the very tip of the wire to burn off leaving a very sharp end. If this is done correctly, a tiny orange speck of wire flies off of the needle and is carried up the flame. The moment this speck is observed the needle should be pulled out of the flame. Tungsten needles can be flame sterilized repeatedly in an alcohol flame and will stay sharp until they are inadvertently bent.

Embryos that are ready for surgery can be covered with small pieces of parafilm or transparent tape and kept at room temperature until needed.

D. Preparation and Staging of Mouse Embryos

Mouse embryos are generated by setting up overnight timed matings. Typically, one male is paired with one female per cage, and males can be reused 2 days after each successful plug. Females from each pair are examined in the morning and those with signs of copulation plugs are separated from the males. Mice with plugs are considered to be pregnant and the day of conception is designated 0.5 day postcoitus or embryonic day (E0.5).

After the desired period of pregnancy, mice are euthanized using CO_2 and/or by cervical dislocation. The uterus is immediately removed through an incision in the abdomen and placed into sterile Ringer's solution. Using fine tipped No. 5 forceps, each embryo is dissected from the implantation sac and placed in cold Dulbecco's modified Eagle's medium (DMEM, Invitrogen/GIBCO) on ice (embryos are best if used within 2 h after dissection). After all embryos have been collected, the developmental stage of each embryo is determined based on published criteria (Rugh, 1968; Theiler, 1989). Embryos are transferred one at a time into a dish containing fresh cold DMEM where neural tube grafts can be extirpated using pulled glass or flame-sharpened tungsten needles.

Unlike avian embryos (chick, quail, and duck), which are roughly similar in size and morphology during early stages of development, mouse embryos are slightly smaller and neural crest migration is initiated earlier (i.e., prior to cranial neural fold closure). Therefore, matching mice to avian embryos based on published staging criteria is complicated. Instead, one approach is to match as closely as possible the mouse donor tissue itself to the stage of the tissue to be extirpated in the chick host. For cranial neural tube grafts, E8–9 (5–7 somites) mouse embryos are used to match HH9 chick with a similar amount of neural crest migration (Fig. 2A–C; Table I), and for trunk neural tube grafts, E9–9.5 (15–21 somites) mouse embryos are used to match HH14 chick embryos (Fig. 2B–D; Table I).

III. Generation and Analysis of Quail–Duck Chimeras

A. Operations on Donor and Host Embryos

Numerous types of surgical operations can be performed in avian embryos and by extension, in the quail–duck chimeric system. For example, either unilateral (which provides an internal control) or bilateral populations of neural crest cells can be grafted orthotopically at HH9.5 from quail to duck to generate chimeric "quck" or from duck to quail to produce chimeric "duail" (Fig. 1). Flame-sharpened tungsten needles and Spemann pipettes can be used for surgical operations (Schneider, 1999). Donor graft tissue is positioned and inserted into a host that has a comparable region of tissue removed. For controls, orthotopic grafts and sham operations can

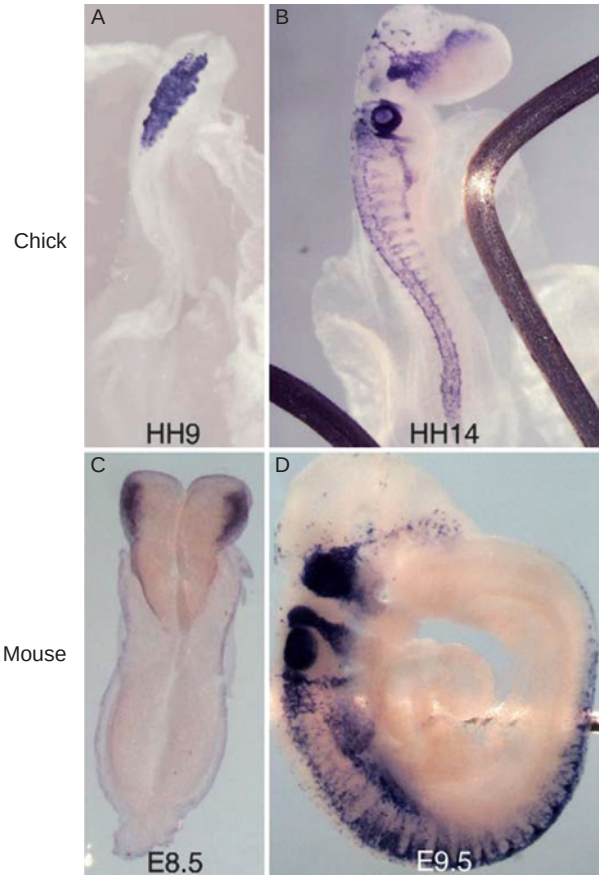

Fig. 2 *In situ* hybridization with Sox10 probe to mark neural crest cells at different stages of development. Sox10 is expressed by migrating neural crest in the cranial region of HH9 chick (A) and E8.5 mouse (C) in a similar pattern. Later in development, Sox10 is expressed by the cranial neural crest as they form ganglia and by the migratory trunk neural crest in an identical pattern in HH14 chick (B) and E9.5 mouse embryos (D). (See Plate no. 2 in the Color Plate Section.)

be made within each species (Noden, 1983; Schneider and Helms, 2003; Schneider *et al.*, 2001; Schneider, 1999). Controls should be incubated alongside chimeras in order to ensure that the stages of grafted cells in the donor, host, and chimeras are accurately assessed. Again, the host side of a unilateral transplant offers an excellent internal control for the effects of surgical exposure, incubation, and differential growth (Eames and Schneider, 2005; Tucker and Lumsden, 2004). After surgery, eggs can be sealed with transparent tape and incubated until reaching stages appropriate for analysis. When staging chimeric embryos, a combination of morphological characters can be used, but emphasis should be placed on embryonic structures that will not be significantly affected by the surgery.

Table I
Comparison of Mouse and Chick Developmental Stages at which Neural Tube Grafts are Performed

	Mouse			Chick		
	Theiler stage[a]	Embryonic age	Number of somites	Hamburger and Hamilton stage[b]	Embryonic age	Number of somites
Cranial grafts	13	8.5	5–7	9	1.5	7–10
Trunk grafts	14	9	15–20	13–14	2–2.5	19–22

[a]Embryonic stages are based on Theiler (1972).
[b]Embryonic stages are based on Hamburger and Hamilton (1951).

B. Visualization of Quail Cells and Detection of Donor-Induced Changes

Control and chimeric embryos can be collected at any necessary stage of development. For the best detection of quail cells, embryos can be fixed in cold Serra's fixative (100% ethanol:formalin:glacial acetic acid, 6:3:1) overnight at 4 °C, dehydrated, paraffin embedded, cut into 7 μm sections, and mounted on microscope slides. To detect quail cells, representative sections can be immunostained with the quail nuclei-specific Q¢PN antibody (Developmental Studies Hybridoma Bank, Iowa) following a previously published protocol (Schneider, 1999). This technique provides a permanent labeling of quail cells by using a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) that is reacted with Diaminobenzidine (Sigma St. Louis Missouri). Quail cells can be clearly observed using differential interference contrast (i.e., Nomarski) microscopy without any counterstain (Fig. 1). For subsequent analyses, paraffin sections can be selected for analysis from quck cases with large populations of labeled quail donor-derived cells (i.e., Q¢PN-positive) and from duail cases with large populations of unlabeled duck donor-derived cells (i.e., Q¢PN-negative), and these can then be used for histological, immunocytological, and/or gene expression studies.

IV. Generation and Analysis of Mouse–Chick Chimeras

A. Operations on Donor and Host Embryos

To prepare the chick host, the vitelline membrane is removed from the region directly above the neural tube and a drop of sterile saline can be added to prevent dehydration of the embryo. Slits are made on either side and then at the desired anterior and posterior levels of the graft to separate the neural tube from the adjacent ectoderm. The neural tube is then removed using either a Spemann pipette or micropipette, taking care not to damage the underlying notochord. For cranial grafts, the neural tube is excised from the midbrain to the forebrain region (Fig 3A). For trunk grafts, the neural tube is taken out from a region of interest (usually encompassing at least four somites) (Fig. 3B).

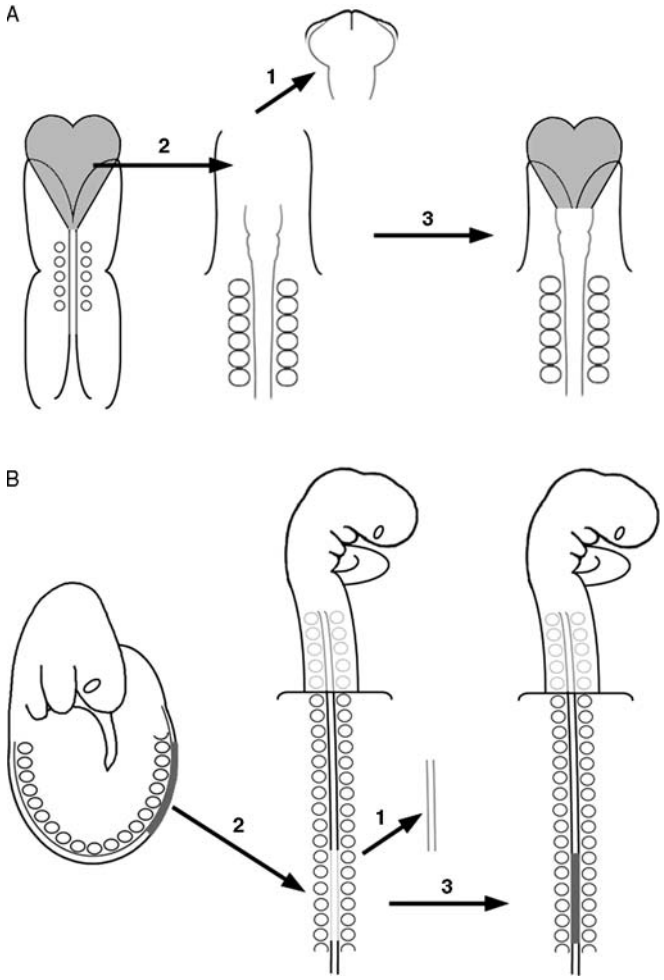

Fig. 3 Schematic representation of (A) cranial neural tube and (B) trunk neural tube grafts from mouse donors into chick hosts. Desired region of the neural tube is microsurgically removed from the chick host *in ovo* (1). Similar region is dissected from donor mouse (2) and grafted in the same orientation into the extirpated chick host (3). Resulting chimeras are incubated for the desired duration to analyze neural crest migration or differentiation.

The level of cranial or trunk neural folds to be grafted is determined using an established fate map of mouse cranial and trunk neural primordium (Osumi-Yamashita *et al.*, 1994; Serbedzija *et al.*, 1990; Trainor and Tam, 1995). Mouse and chick embryos are stage-matched according to levels of neural crest migration from the dorsal neural tube. Neural tubes are dissected from mouse donor embryos and transferred to the chick host embryo using a micropipette. The graft tissue is carefully fitted into the chick embryo maintaining the proper anteroposterior and

dorsolateral orientations. Excess Ringer's solution covering the embryo is removed and the egg is sealed with transparent tape, labeled, and transferred to the incubator for the desired period of time.

B. Visualization of Mouse Cells and Detection of Donor-Induced Changes

There are several reliable techniques for identifying and tracing grafted mouse neural crest cells in the chick host embryo. Many general published methods can be used to study neural crest migration and development. Migratory neural crest cells can be tracked in the chimeras after 1–2 days incubation. Chimeras incubated for more than 2 days can be prepared for histological analysis of neural crest differentiation. Mouse cells can be tracked in chick host embryos using any of the methods listed in Table II. Such methods can be used either individually or in combination to track grafted mouse cells, which will likely allow for much progress in studying mammalian development and disease by way of avian embryos. Particularly, mouse neural crest cells have been shown to respond well to cues in the chick environment making them an excellent cell type for studying genetic defects intrinsically associated to their migration and differentiation.

Mouse neural crest cells can be labeled with the cell marker DiI (1,1-dioctadecyl-3,3,3′,3′-tetramethlindocarbocyanine perchlorate; Molecular Probes), which is normally useful for labeling migratory neural crest cells without grafting but can be adapted to mouse–chick chimeras by grafting DiI-labeled mouse neural tubes into unlabeled chick host embryos then tracking DiI-labeled mouse cells in whole-mount or sections. An advantage of using this method is that the migratory behavior of fluorescently labeled mouse neural crest cells may be visualized

Table II
Markers and Mouse Lines Used to Identify Migratory and Differentiated Neural Crest

Marker/transgenic line	Cells labeled	References
DiI	Migratory neural crest in cultured mice embryos	Serbedzija *et al.*, 1989, 1990, 1992
		Trainor and Tam, 1995
		Osumi-Yamashita *et al.*, 1994
Bisbenzimide (Hoechst)	Distinguishes grafted mouse	Fontain-Perus *et al.*, 1995, 1996, 1997
DAPI	Cells from chick host cells based on DNA repartition in the nucleus	Mitsiadis *et al.*, 2003, 2006
Feulgen–Rossenbeck		
Acradine orange		
β-Galactosidase staining	Grafted mouse cells are traced using lacZ staining	Fontain-Perus *et al.*, 1996, 1997
		Fontain-Perus and Cheraud, 2005
Rosa26-lacZ	All cells are labeled	Soriano, 1999
Wnt1-Cre/R26R	Neural crest	Jiang *et al.*, 2000, 2002
Wnt1/lacZ		Echelard *et al.*, 1994

in ovo and followed using time-lapse video microscopy. Limitations of this method are that the DiI becomes diluted as the cells divide making it difficult to track later during development, and DiI may also leak into tissues that are not targeted for tracing and result in false positive labeling.

Mouse cells can also be tracked based on DNA repartition in the nucleus. In the mouse nucleus, chromatin is condensed into several masses whereas in chick, chromatin is evenly distributed. Based on these differences, nuclear staining methods such as Feulgen–Rossenbeck, Acradine orange, Bisbenzimide (Hoechst), and DAPI can be used to identify grafted mouse cells in the chick host (Fig. 4A and B). These methods are very helpful when following large numbers of grafted cells but become less reliable when very few cells are involved. For example, mouse neural crest cells migrate from the grafted neural tubes and incorporate with the chick cells in regions where chick neural crest normally migrate.

Additionally, mouse cells can be identified using mouse-specific antibodies and probes for *in situ* hybridization. For example, the anti-P75 antibody (Rao and Anderson, 1997) does not cross-react with chick and can therefore be used to follow migratory grafted mouse neural crest cells in the chick host. Similarly, grafted mouse cells can be labeled using mouse-specific RNA probes that are expressed by migratory neural crest cells and/or their derivatives. For example, msox10 can be used to track grafted migratory neural crest in chick whereas mMsx1, mMK, mPax9, and mBarx1 have been used to study differentiation of grafted mouse neural crest (Mitsiadis *et al.*, 2003). Finally, mouse cells can be tracked by exploiting transgenic reporter constructs. Stable transgenic mouse lines have been generated to express lacZ reporter in all cells, as in the case of the Rosa26-lacZ line (Soriano, 1999). Better yet, stable transgenic lines with the lacZ reporter expressing under the control of the Wnt1 enhancer have been generated (Echelard *et al.*, 1994; Jiang *et al.*, 2000, 2002) in which migratory and

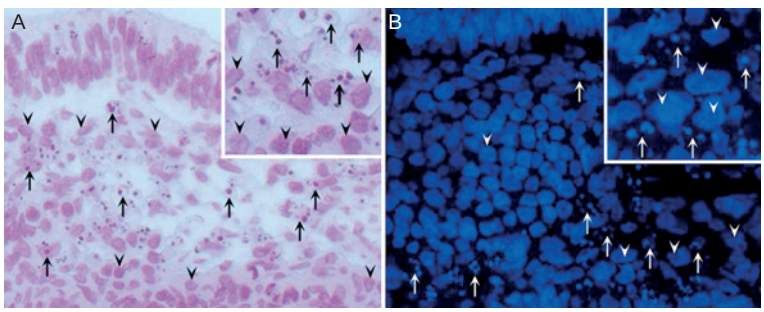

Fig. 4 Visualization of mouse-derived neural crest cells based on DNA repartition in the nuclei in mouse–chick chimera (A) after Feulgen–Rossenbeck staining and (B) after Hoechst staining. In both staining methods, mouse neural crest cells can be distinguished from chick cells by the appearance of condensed heterochromatin in their nuclei (arrows), whereas the heterochromatin in chick nuclei appears evenly distributed (arrowheads). (See Plate no. 3 in the Color Plate Section.)

differentiated neural crest continue to express LacZ. Grafted mouse neural crest cells from these lines can be visualized in the chick after β-galactosidase staining.

V. Conclusion

Although in classical Greek mythology observing a chimera (made of lion, snake, and goat) was considered a sign of impending doom, we take a more optimistic view, and think the future after chimerism holds much promise for developmental biology. By emphasizing what makes animals different, heterogeneous chimeras composed of donor and host parts can help identify embryonic events that regulate the spatial and temporal patterning of anatomical elements, reveal the hierarchical levels of organization that enable precise structural and functional integration, elucidate signaling interactions that drive histogenic differentiation, and point to molecular and cellular mechanisms that underlie species-specific evolution. The potential for novel applications of quail–duck and mouse–chick chimeras seems great, especially in conjunction with the rapid advancement of modern techniques in genomics, proteomics, stem cells, and regenerative medicine.

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CHAPTER 4

Manipulations of Neural Crest Cells or Their Migratory Pathways

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I. Introduction

The formation of the embryo involves intricate cell movements, cell proliferation, and differentiation. The neural crest has long served as a model for the study of these processes because neural crest cells undergo extensive migrations and give rise to many diverse derivatives. Neural crest cells arise from the dorsal portion of the neural tube. Several unique properties of these cells make the neural crest an ideal system for studying cell migration and differentiation. First, these cells migrate extensively along characteristic pathways. Second, they give rise to diverse and numerous derivatives, ranging from pigment cells and cranial cartilage to adrenal chromaffin cells and the ganglia of the peripheral nervous system. Third, the characteristic position of premigratory neural crest cells within the dorsal portion of the neural tube makes them accessible to surgical and molecular manipulations during initial stages in their development.

This chapter summarizes techniques for the isolation, induction and identification of neural crest cells in tissue culture as well as various manipulations of neural crest cells and some of the tissues with which they interact in the embryo.

II. Preparation of Avian Neural Crest Cultures

Because neural crest cells migrate away from the neural tube, it is possible to isolate them from surrounding tissues by explanting the neural tube or neural folds into culture. The remaining neural tube tissue can then be scraped away, leaving a relatively pure population of neural crest cells. When grown in a rich medium containing embryo extract, these cells differentiate into a number of normal neural crest derivatives, including pigment cells, adrenergic cells, and cholinergic cells. During the first day in culture, this technique makes it possible to examine the migration of neural crest cells on two-dimensional substrates. In addition, the effects of different culture conditions and growth factors on the differentiation of neural crest cells in longer term cultures can be monitored.

A. Preparation of Medium

Standard medium for neural crest cultures:

- 75% Eagle's minimal essential medium (MEM)
- 10% horse serum
- 15% embryo extract

The levels of embryo extract can be varied. In addition, some authors have developed more defined culture conditions for growing neural crest cells

(Sieber-Blum, 1991). Although it is possible to purchase powdered embryo extract, it is best to prepare embryo extracts from 10- to 11-day-old chick embryos for optimal neural crest cell differentiation.

The following protocol, adapted from Cohen and Konigsberg (1975), works well as a complete medium for neural crest cells. It is best to collect all of the materials the day before embarking on this procedure as it takes the better part of a day. Note that this procedure is done as cleanly as possible, but that the glassware need not be sterilized since the embryo extract is filtered through a 0.22 μm filter prior to addition to the medium.

Materials

- 1–2 liter of MEM
- 100 ml of horse serum
- Sterile millipore filters (0.22, 0.45, 0.8, and 1.2 μm)
- Sterile bottles
- 2 × 250 ml beakers
- One large beaker with gauze over the top
- Two to three graduated cylinders
- Scissors/forceps
- Large tubes for ultracentrifugation

Embryo extract

1. Have ready 10 dozen chicken eggs that have been incubated for 10–11 days. "Candle" eggs by holding them up to a light source so that only viable eggs are visualized and opened. Wipe the eggs with 70% ethanol.
2. With curved sterile scissors, cut a circular opening in the blunt end of the egg. Remove the embryo by sliding one arm of the forceps under the neck of the embryo. The membranes, for the most part, will be left behind. Place the embryos in a large petri dish filled with cold MEM.
3. Using the scissors, remove the eyes and beaks from the embryos and make a few slits in the belly. Then place the embryos into another dish containing cold MEM.
4. To drain excess fluid, place the embryos onto a beaker covered with a double layer of gauze. Rinse with cold MEM to remove excess blood.
5. After draining the embryos, transfer them to a sterile beaker and mince with large scissors.
6. Transfer the minced embryos to a 50 ml plastic syringe and expel into a 500 ml sterile preweighed bottle containing a sterilized stir bar.
7. Weigh the minced embryos and add an equal amount of MEM (1 g = 1 ml). Stir for 1 h at 4 °C.
8. Add hyaluronidase (Worthington Biochemical) for the last 15 min of step 7. The grams of hyaluronidase added equals the number of milliliters of embryo extract times 4×10^{-5}. Chill and filter sterilize the hyaluronidase prior to use.

9. Ultracentrifuge for 30 min at 20,000 rpm at 4 °C.
10. Separate supernatant from pellet and ultracentrifuge supernatant at 35,000 rpm at 4 °C for 60 min.
11. Collect the supernatant and filter through progressively smaller pore size filters (1.2 μm followed by 0.8 μm followed by 0.45 μm followed by 0.22 μm). After filtration, the embryo extract should have an orange-reddish color and appear slightly cloudy.
12. Combine horse serum (10%), embryo extract (15%), and MEM (75%) to make complete medium and stir for 10 min.
13. Filter the complete medium through sterile 0.22 μm filters into 30 ml sterilized bottles. A bottle holds approximately 20 ml of complete medium and can be kept frozen at –80 °C for up to 6 months.

B. Preparation of Two-Dimensional Substrates

Typically, cultures are plated onto substrates coated with extracellular matrix molecules. The protocol for coating dishes with most matrix molecules is similar. Therefore, fibronectin will be used as a typical example.

1. Prepare Howard Ringer's solution (or utilize a saline of your choice). Ringer's solution consists of 7.20 g of NaCl, 0.17 g of $CaCl_2$, 0.37 g of KCl, and 1000 ml of H_2O (distilled).
2. Incubate plastic tissue culture dishes (35 mm diameter or a 15-mm four-well multidish; Nunclon) with 25 μg/ml fibronectin (New York City Blood Bank) in Ringer's solution at 38 °C for 1 h.
3. Remove the excess fibronectin solution and incubate the dish with complete culture medium at 38 °C for another hour.

Similar protocols can be used for other matrix components including collagen, laminin, or fragments of these molecules. In addition, the coating concentrations and nature of the salts can be varied to provide distinct types of substrates.

C. Preparation of Three-Dimensional Substrates

For some experiments, it is advantageous to grow neural crest cells or embryonic tissues in three-dimensional substrates. The collagen gels described next are typically used.

Materials

- Rat tail collagen (Collaborative Research)
- Dulbecco's minimum essential media (DMEM) powder high glucose, no bicarbonate (GIBCO-BRL)
- 7.5% bicarbonate
- Horse serum
- F12 nutrient medium (GIBCO); N2 supplement (GIBCO)

- L-Glutamine
- Penicillin/streptomycin
- Four-well multidishes (Nunc)

1. Add 90 μl of rat tail collagen (3.13 mg/ml) to 10 μl of a 10× DMEM solution (pH 4). Vortex to mix. Avoid introducing bubbles. Bring to neutral pH with 7.5% sodium bicarbonate; add approximately 2–4 μl to the mixture. The collagen mixture will turn from a bright yellow to a faint orange color.

2. Place a thin layer of collagen (~10 μl) onto the bottom of a four-well Nunc multidish. Spread the collagen around with the tip of a pipette to make a mound about one-third, the diameter of the well. This procedure must be done quickly before the collagen polymerizes. Allow it to set at room temperature for 15–30 min. Avoid letting the collagen dry.

3. Using a pipetteman or other means of transfer, place the explants onto the gelled collagen. Take care to remove the medium in which the explant was transferred to avoid problems with gelation of the collagen. Overlay it with another layer of collagen. The amount of collagen will vary with the size of the tissue. Make sure that the tissue is covered. Because the explant will often float to the top, use a blunt tunsgsten needle to gently poke the tissue into the liquid collagen and to position the explant in the desired orientation.

4. Allow the gel to set for 15–40 min at room temperature followed by 5 min at 37 °C.

5. Gently pipette approximately 300 μl of complete media, F12 plus N2 or media of choice into the culture dish and incubate at 37 °C in a CO_2 incubator.

6. Gels can be fixed and processed for immunostaining or *in situ* hybridization. Peel the gel of the tissue culture dish using watchmaker's forceps.

Another three-dimensional substrate that has been used successfully is Matrigel (Maxwell and Forbes, 1990) which can be made according to the manufacturer's directions.

D. Primary Neural Crest Cultures from the Trunk Region

These methods are essentially those described by Cohen and Konigsberg (1975). For most cultures, quail embryos are used because the neural crest cells from this species differentiate well *in vitro*. However, similar procedures can be followed using other avian embryos and have also been adapted for mammalian embryos. Figure 1 illustrates a typical neural crest explant immediately after plating and after 1 day in culture.

Materials

- 60 mm glass petri dish filled with black dental wax mixed 1:1 with paraffin; sterilize by flaming the surface with a Bunsen burner and cover immediately with a sterilized top;

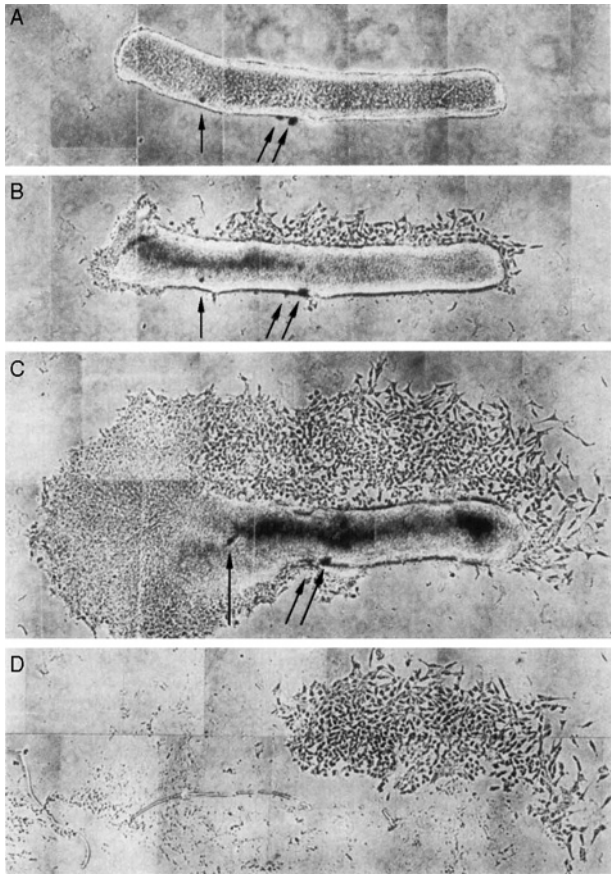

Fig. 1 Composite phase-contrast photomicrographs of a single living neural tube after explantation *in vitro*. (A) During the first 2 h in culture, no neural crest cells emigrate. Arrows indicate carbon particles used to mark the ventral surface of the neural tube. (B) By 8 h after explantation, some mesenchymal neural crest cells have migrated away from the neural tube. (C) Within 20 h, hundreds of cells have left the dorsal neural tube. In addition, flattened mesenchymal cells from the neural tube form an epithelial sheet. (D) The non-neural crest cells have been scraped away with a tungsten needle, leaving the neural crest population. From Cohen and Konigsberg (1975).

- Sterilized fine scissors and forceps;
- Sterile Pasteur pipettes with a bent end (made by holding over a Bunsen burner until the glass bends); the large end of the pipette should be plugged with cotton;
- Electrolytically sharpened tungsten needles;
- Dispase (Worthington, 2 mg/ml in 20 mM HEPES in buffered DMEM);
- Sterile three-well dishes;
- Complete culture medium (as described earlier);
- 2 × 250 ml sterile beakers;

- Sterile Ringer's solution;
- Sterile fibronectin-coated dishes; and
- Quail eggs (*Coturnix coturnix japonica*) incubated at 38 °C until they reach stages 13–14 by the criteria of Hamburger and Hamilton (1951); approximately 47 h of incubation.

Procedure

1. In a horizontal laminar flow hood, wash the quail eggs with 70% ethanol. Open the eggs gently with scissors and remove a small piece of the shell, allowing access to the embryo. Grasp the extraembryonic membranes of the embryo, cut it away from the yolk, and place in the black wax dishes filled with Ringer's solution. Embryos can also be put in sterilized dishes without black wax, if desired. Rinse the embryos and replace the Ringer's solution several times until the excess yolk is removed.

2. Use sharp tungsten needles to excise the region of the trunk consisting of the six to nine most posterior somites as well as the unsegmented mesenchyme from each embryo. Dissect by making two longitudinal cuts lateral to the somites and two transverse cuts through the neural tube and adjacent tissue.

3. Remove explants and place them into well dishes containing Ringer's solution for washing. Replace the Ringer's solution with dispase and incubate for 15 min on ice followed by 10 min at 37 °C (the exact length of time is empirical and can vary somewhat with age and specific activity of dispase).

4. While in dispase, aspirate and expel the explants through a bent sterile Pasteur pipette. This generates shear forces that separate the neural tube from the ectoderm, endoderm, somites, and notochord, although the latter sometimes remains attached.

5. Segregate neural tubes from other tissue and place in a well filled with complete medium, which contains endogenous enzymatic inhibitors that stop the reaction. Rinse with fresh medium.

6. Place isolated neural tubes in a few drops of medium onto prepared tissue culture dishes coated with fibronectin or the substrate of choice (Fig. 1). Incubate for 1 h at 37 °C and fill the culture dish with 1.5 ml of complete medium.

7. After 8–24 h, scrape away the neural tube and any groups of cells with epithelial morphology using the blunt end of a tungsten needle (Fig. 1). Remove the media and debris. Replace with fresh culture medium. Feed the cultures fresh media every other day.

E. Primary Neural Crest Cultures from the Cranial Region

Materials

- Same as described earlier.
- Incubate quail eggs until they reach stage 8 (four to six somites), typically takes about 24 h.

Procedure

1. In a laminar flow hood, wash the eggs with 70% ethanol. Open the eggs as described earlier. Place the embryos in Ringer's solution.

2. Excise midbrain region with sharp tungsten needles. Make two lateral cuts between the neural tube and the ectoderm. Make a longitudinal cut underneath the notochord and two transverse cuts, one between the midbrain and forebrain and a second between the midbrain and hindbrain. Gently tease off the neural folds using a sharp point of the tungsten needle.

3. Wash the neural folds with Ringer's solution and transfer in a few drops of medium onto substrate-coated tissue culture dishes. Incubate at 37 °C for 30–60 min and then feed with 1.5 ml of complete medium.

F. Secondary or Clonal Cultures

After neural crest cells have migrated away from the explanted neural tube and the neuroepithelial tissue is scraped away, secondary cultures can be prepared by dissociating the neural crest cells and replating them at sparse density. Single cells can be isolated using this procedure, thus making it possible to perform a clonal analysis (Baroffio *et al.*, 1988, 1991; Cohen and Konigsberg, 1975; Sieber-Blum and Cohen, 1980). A simple procedure for preparing secondary cultures is described next. Despite the fact that this procedure is in principle quite straightforward, neural crest cells do not grow well at low density. Therefore, a number of investigators have used more exacting procedures including a more complicated medium (Sieber-Blum, 1991) or have grown clonal neural crest cultures on feeder layers of 3T3 cells (Baroffio *et al.*, 1988).

1. Rinse the primary cultures with Ringer's solution and add a crude preparation of 0.25% collagenase (Worthington CLS) for approximately 20 min at 37 °C. Other enzymes such as dispase or trypsin can be substituted, although the length of treatment must be altered. In addition, cells can be isolated from the substrate by prolonged exposure to calcium-free buffers. Pipette the solution intermittently to dislodge the cells.

2. When the cells are loosened from the substrate, add an equal volume of cold complete medium. Endogenous enzyme inhibitors in the serum will stop the enzyme reaction.

3. Centrifuge the cell suspension at 800 rpm for 5 min. Remove the supernatant by aspiration and resuspend the pellet in approximately 0.4 ml of complete medium.

4. A Petroff–Heuser bacterial cell counter is used to count the number of cells in the suspension, although other types of hemocytometers can be used. Secondary cultures are typically inoculated with 200 cells per 60 mm Falcon petri dish coated. Various substrates can be used, including fibronectin, polylysine, or a feeder layer of 3T3 cells.

5. For clonal cultures, it is important to verify that single cells have been obtained. This can be accomplished by plating the resuspended cells into multiwell plates and verifying the presence of a single cell per well using an inverted phase-contrast microscope.

6. Cells are fed fresh complete medium every 2–3 days.

G. Whole Trunk Explants

An alternative approach for studying neural crest migration is an explant preparation that allows direct visualization of migrating cells in normal living tissue (Krull *et al.*, 1995). The whole trunk region of the chicken embryo, excised and placed in explant culture, appears to continue normal development for up to 2 days. Neural crest cells migrate in their typical segmental fashion, and the morphological and molecular properties of the somites are comparable to those in intact embryos.

Materials

- Similar to those used earlier;
- Millicell inserts;
- Six-well Falcon culture plates; and
- Chicken eggs incubated to stage 11.

Procedures

1. Cut embryos from egg as described previously and place in Ringer's solution. Using tungsten needles, carefully dissect a region of the trunk, stretching from the fifth to the eleventh most recently formed somites (somites V–XI; Ordahl, 1993). For the dissection, transverse incisions are made just caudal to somite V and just rostral to somite XI. To free the tissue, incisions are made perpendicular to the first, extending longitudinally and lateral to somites V–XI. The resulting explant contains the neural tube, including presumptive neural crest cells, multiple pairs of discrete somites, and other associated structures including the ectoderm and endoderm.

2. Place the ventral surface of the explant onto the Millicell polycarbonate membrane, leaving the dorsal surface of each explant exposed to the atmosphere. Then, underlie the Millicell insert with medium. A defined culture medium composed of neurobasal medium (GIBCO), supplemented with B27 (GIBCO) and 0.5 mM L-glutamine (Sigma), is used.

These cultures have the advantage of maintaining relatively normal *in vivo* development for 2 days while being readily accessible to both visualization and addition of perturbing reagents.

III. Induction and Specification Assays for Neural Crest Cells

Because their precursors are surrounded by prospective neural, non-neural ectoderm tissues, and underlain by mesoderm, it has been suggested that a combination of these tissues is responsible for neural crest induction. The original idea of inducers, and specifically for tissues involved in neural crest induction can be traced back to the 1940s. Induction in developmental biology refers to the capacity of a cell to signal a responsive second cell yielding a third yet different type of cell. Neural crest induction can be recapitulated *in vitro* by combining two different tissues and monitoring the appearance of neural crest markers. The pioneering work of Raven and Kloss, from the 1940s in amphibians, inspired researchers to assess the role of different tissues in several other organisms. Below, we describe assays to monitor induction of avian neural crest cells, either by different tissues or by specific molecules (Basch *et al.*, 2006; Dickinson *et al.*, 1995). Then, we provide a section examining the early specification of neural crest cells by testing the capacity of isolated tissues to generate neural crest cells. These experiments with chick can easily be adapted to use quail or a combination of both, chick and quail embryos.

A. Naive Intermediate Neural Plate and Non-Neural Ectoderm (Prospective Epidermis) from Stage 10 Embryos

1. In a horizontal laminar flow hood, wash chicken eggs incubated for 36 h with 70% ethanol. Crack-open the eggs into a large pyrex plate. Making sure not to overflow, the yolks should rise above the albumin. Using fine scissors carefully cut within the area opaca a rectangle around the embryo, fish it out from the yolk, and place it in a sterile petri dish. Rinse the embryos and replace the Ringer's solution several times until the excess yolk is removed.

2. Place the embryos into well dishes containing Ringer's solution for washing. Replace the Ringer's solution with dispase and incubate for 15 min on ice followed by 10 min at 37 °C (the exact length of time is empirical and can vary somewhat with age and specific activity of dispase).

3. Rinse the dispase solution three times using Ringer's solution, and transfer the embryos to a petri dish with Ringer's solution. Stretch the caudal region of the embryo using two to four pins (if silgard plates are used) or with the aid of a horseshoe shaped wire to provide some weight.

4. Using sharp needles (tungsten or glass), carefully separate the caudal open neural plate from the adjacent epidermis. Usually, we introduce the needle in between both tissues at the caudal portion of the open neural plate, and slide the needle toward the anterior region of the embryo, separating them precisely at the border. Next we introduce the needle between the presomitic mesoderm and the neural plate near the last or second to last-formed somite, and slide it toward the caudal end of the neural tube. The caudal portion of the open

neural tube is detached from the rest of the embryo by making two transverse cuts, one at the somitic level, and the second just caudal to the center of the open neural plate.

5. The portion of the open neural plate can now be removed and placed in a clean region on the petri dish, where the final dissection can be performed. The notochord is still attached to the central-most region of the open neural plate (future ventral) and is a useful landmark. Using the two needles, the open neural plate is further dissected to separate the lateral one-sixth territories containing the border of the prospective epidermis and the neural plate (containing prospective neural crest) on both sides, and then the central one-third containing the notochord is also removed. These dissections render two slabs (one-sixth each) of tissue corresponding to the intermediate neural plate, a naïve tissue competent to respond to neural crest induction signals. Each slab can be further dissected into two to three pieces for independent analysis.

6. Using a thin needle attached to a mouth pipette tube, transfer the isolated intermediate neural plates to a Terazaki plate well with 10 μl of serum-free PB1 medium and keep on ice.

Unused regions of the embryos described earlier could be used as a good source for prospective epidermis. If these tissues are not appropriate, repeat steps 1–3 and stretch embryos as in step 4.

7. Using the fine needles cut a rectangular segment of the non-neural ectoderm of the caudal region of the embryo lateral to the open neural plate, adjacent to the area opaca–area pellucida border. Verify that the non-neural ectoderm is free from underlying tissues, if this is not the case, carefully remove the lower tissue layers. The non-neural ectoderm tissue can be cut into 100–150 μm^2 sections.

8. Transfer the non-neural ectoderm tissue to a Terazaki plate using a mouth pipette fitted with a fine needle as above, and keep on ice.

9. Prepare collagen gel just before setting, the tissue cultures, essentially as described on p. 78.

In an eppendorf, add 90 ml collagen (type I rat tail; Collaborative Research), 10 ml 10× DMEM and vortex briefly.

Then add 4.5–5.0 ml 7.5% sodium bicarbonate (store at 4 °C), and vortex immediately for 20 sec. Store on ice. The solution should be straw yellow with a pink meniscus. If the solution is pink, the pH is low and the collagen will set quickly. Small variations in the pH generated by the sodium bicarbonate will alter the precise time required for the collagen to set which in turn will limit the available time to place the tissues in the desired arrangements. Therefore, the precise amount of sodium bicarbonate must be titrated in advance.

10. Mouth pipette the tissues to be cultured and place them at the edge of the thin needle, so that they can be transferred in a short time to the collagen gel, and in a very small volume (avoiding the transfer of excess liquid).

11. Pipette 10 ml of the freshly made collagen gel onto the bottom of a tissue culture well (four-well dish) spreading the bottom of the drop slightly to generate a domed looking droplet.

12. Quickly mouth pipette the tissues placed at the edge of the needle into the collagen droplet avoiding the transfer of excess liquid. Gently mix the tissues with the collagen to ensure a homogeneous collagen solution, and make sure that the tissues remain fully surrounded by collagen. It is critical to prevent the tissues from ending on the bottom of the plate or on the surface of the collagen. If two different tissues are to be juxtaposed, bring them together until they touch each other.

13. Allow to set for 5 min at 38 °C, 5% CO_2 (this helps prevent dehydration). Verify that the tissues are actually placed as expected. If needed, additional fresh collagen (1–3 μl) can be added to correct placement (repeat 5 min incubation). Once the tissues are set correctly, allow the gel to set for 15 min at room temperature and verify that the collagen has set properly (a change in color is obvious).

14. Cover gel with 350 μl of Ham's F12 with N2 supplement (GibcoBRL) or other desired medium. BMP, WNT, or any other molecules to be tested for induction should be premixed with the medium before adding to the gels. These molecules could be added to the naïve intermediate neural plate alone, which alone cannot generate neural crest cells. Incubate at 38 °C with 5% CO_2 overnight or as long as desired.

15. Fix in 4% paraformaldehyde for 10 min to 1 h at room temperature. Wash two to three times, 5 min each, in PBT [phosphate-buffered saline (PBS) with 0.1% bovine serum albumin, 0.1% Triton X-100] or PTw (PBS with 0.1% Tween-20). The explants can be now monitored for neural crest markers using any of the many available antibodies (Pax3, Pax7, Snail2, SoxE, cMyc, HNK-1, etc).

B. Early Epiblast Tissues from Stages 3 and 4 Chick Embryos

1. Cut 1 cm squares of Whatman filter paper and remove the center using a hole punch. The paper provides support, and tension for the vitelline membrane and the embryo.

2. Incubate chicken eggs for 12–16 h to obtain stages 3 and 4 embryos. Clean the egg's shells with 70% ethanol in a horizontal laminar flow hood.

3. Using blunt forceps, crack a small portion of the shell's concave end, and remove the debris. Holding the egg in one hand, tilt it and start to pull thick albumin off the egg. Monitor the appearance of the early embryo blastodisc, and discard undeveloped ones.

4. Continue to remove the thick albumin, tilting the egg to drain thin albumin while gently cutting or removing more and more of the egg's shell with the forceps. Avoid piercing the yolk, and aim to leave it in a small portion of the shell. Remove as much albumin as possible, and make sure that no thick albumin remains on top of the embryo (look for altered light reflection on the yolk surface to monitor the transparent thick albumin).

5. Center the filter paper round the embryo, making sure it attaches firmly on all its surface to the surrounding vitelline membrane. Using fine scissors, cut the vitelline membrane around the perimeter of the filter paper. Hold the filter paper with fine forceps and gently transfer the embryos into a petri dish containing Ringer's solution for washing.

6. Turn the embryos upside down so that the lower layers of the embryo are placed uppermost. Under the microscope, orient the embryo guided by the primitive streak and push gently, with fine forceps, the lower layers from the edge of the area opaca–area pellucida border toward the center of the embryo, exposing the epiblast. Use a motion that replicates the rolling of a carpet. The separation of the layers can be facilitated through a short incubation in dispase (30–60 sec).

7. Cut a 100 μm wide stripe of epiblast perpendicular to the primitive streak posterior to Hensen's node, from one side of the area opaca, through the primitive streak and into the area opaca in the opposite side. Remove the stripe of tissue into a clean location of the dish.

8. Cut the stripe by the middle, precisely at the primitive streak, which being thicker than the rest of the tissue provides an excellent landmark.

9. Using sharp needles (tungsten or glass), carefully perform five consecutive, equidistant, cuts perpendicular to the long axes, toward the primitive streak in one of the two halves. These generates six pieces of approximately 80 μm, with the thicker primitive streak being number 6, and the most lateral segment being number 1. Make sure to separate and arrange each piece according to its original position in the stripe and transfer each one to a different well (filled with 10 μl of PB1) of a Terazaki plate using a mouth pipette.

Repeat steps 10–15 as above.

10. Mouth pipette the tissues to be cultured and place them at the edge of the thin needle, so that they can be transferred in a short time to the collagen gel, and in a very small volume (avoiding the transfer of excess liquid).

11. Pipette 10 ml of the freshly made collagen gel onto the bottom of a tissue culture well (four-well dish) spreading the bottom of the drop slightly to generate a domed looking droplet.

12. Quickly mouth pipette the tissues placed at the edge of the needle into the collagen droplet avoiding the transfer of excess liquid. Gently, mix the tissues with the collagen to ensure a homogeneous collagen solution, and that the tissues remain fully surrounded by collagen. It is critical to prevent the tissues to end on the bottom of the plate or on the surface of the collagen.

13. Allow the collagen to set for 5 min at 38 °C, 5% CO_2 (this helps prevent dehydration). Verify that the tissues are actually placed as expected. If needed, additional fresh collagen (1–3 μl) can be added to correct placement (repeat 5 min incubation). Once the tissues are set correctly allow the gel to set for 15 min at room temperature and verify that the collagen has set properly (a change in color is obvious).

14. Cover gel with 350 µl of Ham's F12 with N2 supplement (GibcoBRL) or other desired medium. BMP, WNT, or any other molecules to be tested for induction should be premixed with the medium before adding to the gels. Incubate at 38 °C with 5% CO_2 overnight or as long as desired.

15. Fix in 4% paraformaldehyde for 10 min to 1 h at room temperature. Wash two to three times, 5 min each, in PBT (PBS with 0.1% bovine serum albumin, 0.1% Triton X-100) or PTw (PBS with 0.1% Tween-20). The explants can be now monitored for neural crest markers using any of the many available antibodies for neural crest development (Pax3, Pax7, Snail2, SoxE, cMyc, HNK-1, etc).

IV. Microinjection of Cells and Antibodies into Embryos

By microinjecting function-blocking antibodies that recognize cell surface, extracellular matrix, or cell adhesion molecules, it is possible to perform *in vivo* perturbation experiments. This approach makes it possible to characterize the nature of cell–cell or cell–matrix interactions required for normal cell migration in living embryos. Antibody perturbation can be done by injecting purified antibodies at selected concentrations or by introducing hybridoma cells that secrete antibody. If introducing hybridoma cells, it is useful to label the cells with a vital dye to allow visualization of the source of antibody.

An additional advantage of this microinjection approach is that any cell type can be injected into selected regions of the embryo. This approach makes it possible to use the embryo as an *in vivo* culture system to examine the differentiation of the microinjected cells. Alternatively, the migratory patterns of these cells can be examined within the embryo.

Similar approaches are used for cell labeling and microinjecting cells/antibodies into chick embryos.

A. Labeling Cells Prior to Microinjection

Cells for microinjection into embryos can be labeled with a variety of vital dyes. Two labeling procedures are described next.

1. *Labeling cells with DiI*: A stock solution of 0.5% 1,1-dioctadecyl-3,3,3′,3′-tetramethlindocarbocyanine perchlorate (DiI; Molecular Probes, Junction City, OR) in 100% ethanol (w/v) is prepared and can be stored for up to 2 weeks. For cell labeling, the stock is diluted 1:100 in 0.3 M sucrose and is centrifuged to remove any crystals that might have precipitated.

2. *Labeling cells with CFSE*: An alternative method whereby cells remain labeled for a short time (~24 h) is the vital dye, 6-carboxyfluorescein diacetate succinimyl ester (CFSE). A stock solution of 10 mM CFSE is prepared in dimethyl sulfoxide which is stored at 4 °C. For labeling cells, the stock solution is diluted 1:300 in PBS, pH 7.4.

3. Cells can either be labeled in suspension or on the culture dish. Rinse the cells with Ringer's solution. For cell suspensions, centrifuge the cell suspension at low speed and add 1.5 ml of the DiI or CFSE solution. For cultures, add the vital dye directly to the culture dish. Incubate for 60–90 min at 37 °C. Rinse again with Ringer's solution. For cell cultures, remove the cells from the dish by incubating with 0.25% trypsin (GIBCO) for approximately 10 min. The enzyme activity is stopped with the addition of fresh culture medium.

4. Place cells into siliconized centrifuge tubes using siliconized pipettes. Wash and centrifuge three times in Ringer's solution, with the final wash being performed in a microfuge tube to reduce the volume of liquid containing the labeled cells.

B. Microinjection of Cells or Antibodies into Embryos

Essentially identical procedures are used for injecting cells or antibodies into embryos. Only the size of the injection pipette varies since cells need a larger opening than antibody solutions.

Procedures

1. Eggs are windowed as described in Chapter 1 by Streit and Stern, this volume. Chicken embryos are incubated at 38 °C until they reach the desired stage of development (typically 36 h for injection onto cranial neural crest migratory pathways and 60 h for injections onto trunk pathways). A window is cut in the shell over the embryo. India ink (Pelikan Fount) diluted 1:10 in a saline solution is injected under the blastoderm to aid in visualization of the embryo. The vitelline membrane is removed using an electrolytically sharpened tungsten needle.

2. Approximately 5 µl of antibodies and/or labeled cells is backfilled into a pulled micropipette. The micropipette is held in an adapter that is connected to a micromanipulator. The pipette tip is broken off to have an opening of 10–30 µm and the tip is then lowered into the desired region of the embryo (Fig. 2). For cranial injections, the antibodies/cells are typically expelled into the mesenchyme adjacent to the neural tube, whereas for trunk injections they are inserted into one or more somites at the wing level.

3. The antibodies/labeled cells are expelled with a pulse of pressure. This is accomplished by connecting the pipette to a pressure source such as a picospritzer or a house air line.

4. Following the injections, the eggs are sealed with cellophane tape (Scotch Magic 3 M) and returned to the incubator until the time of fixation. Embryos injected with DiI-labeled cells are fixed in 4% paraformaldehyde or 4% paraformaldehyde/0.25% glutaraldehyde and prepared for cryostat sectioning. Embryos with CFSE-labeled cells are fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned.

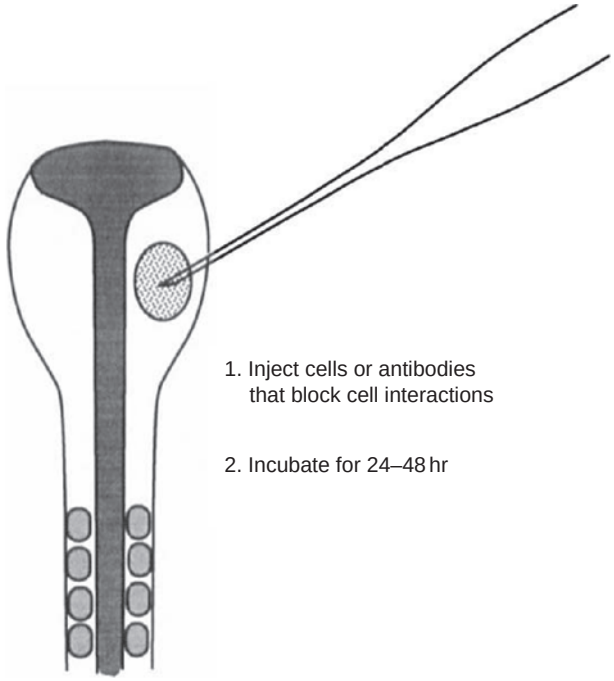

Fig. 2 The technique used for microinjecting cells or antibodies into the cranial mesenchyme of a 1.5-day-old chick embryo. Cells are labeled prior to injection. Cells and/or antibodies are backfilled into a micropipette having an opening of approximately 20 μm. The embryo is incubated for an additional 1–2 days and is then fixed and stained.

V. Labeling of Neural Crest Cells *In Vivo* with Vital Dyes

A number of useful cell-marking techniques are used to examine the pathways of neural crest migration. Classically, neural tube transplantations have provided a wealth of information about migratory pathways and, in particular, neural crest derivatives in avian embryos (see Chapter 2 by Le Douarin *et al.*, this volume). Neural tubes or neural folds from quail embryos have been transplanted to the same or different axial levels of chick hosts (Le Douarin, 1982). An alternative approach is to label neural crest cells in fixed embryos with antibodies that recognize neural crest cells (HNK-1 and NC-1; Tucker *et al.*, 1984). Although this provides a nonsurgical alternative to grafting paradigms, most antibodies are not entirely specific. They recognize numerous cell adhesion molecules associated with many non-neural crest cells (Kruse *et al.*, 1984) and do not recognize all neural crest populations.

Another cell-marking technique for labeling the neural crest is to inject the lipophilic dye DiI (Sechrist *et al.*, 1993; Serbedzija *et al.*, 1989) into the lumen of the neural tube (denoted as whole neural tube injections) or directly into the neural

folds (denoted as focal neural fold injections). Because the dye is hydrophobic and lipophilic, it intercalates into all cell membranes that it contacts. Injection into the neural tube marks all neural tube cells including presumptive neural crest cells within its dorsal aspect. Because the time and location of injection can be controlled, this technology provides a direct approach for following migratory pathways. In addition, the dye can be used to follow neural crest pathways in a number of species, including chick, mouse, and frog. Despite the differences in the nature of the techniques involved, DiI labeling, quail/chick chimeras, and antibody staining provide similar pictures of neural crest migratory pathways.

Two techniques for DiI injections to label neural crest cells are provided. For a more detailed discussion of vital dye labeling of other populations of cells in avian embryos, including methods for labeling individual precursor cells, see Chapter 8 by Ahlgren, this volume.

A. Whole Neural Tube DiI Injections

By injecting the DiI into the lumen of the neural tube, the dye intercalates into all neural tube cells, including premigratory neural crest cells. Because DiI is lipophilic and hydrophobic, it is necessary to make up a stock solution of DiI in a nonaqueous solvent. Ethanol (100%) is typically used, although DMSO and other solvents can be substituted. Because ethanol is toxic to cells, the dye must be diluted prior to putting large amounts of ethanol into the embryo. By diluting the stock solution in isotonic sucrose, the dye remains in solution and damage to the embryo is minimal or nonexistent.

1. A 0.05% solution (w/v) of DiI (Molecular Probes) is made by diluting the stock solution (0.5% in 100% ethanol) 1:10 in 0.3 M sucrose.

2. The injection micropipette is backfilled with the DiI solution which is then attached to a forced air pressure source (either a picospritzer or a house air line). The tip of the pipette is broken with fine forceps to have an opening of about 10–20 μm. The micropipette is inserted into the lumen of the neural tube using a micromanipulator. Enough dye is expelled to fill most of the neural tube.

3. After injection, the eggs are sealed with cellophane tape and returned to the incubator until the indicated times of fixation. DiI-labeled embryos are fixed in 4% paraformaldehyde or 4% paraformaldehyde/0.25% glutaraldehyde and prepared for cryostat sectioning.

B. Focal Injections of DiI into Neural Folds

Premigratory neural crest cells arise from the dorsal neural folds shortly after tube closure in the chick embryo. By placing small, focal injections of DiI directly into the neural folds, one can label a subpopulation of neural crest cells and examine their subsequent migration over time. For this method, it is necessary to use undiluted DiI to produce intense and localized labeling. Because only a small amount of DiI in ethanol is expelled, ethanol damage to the embryo is minimal.

1. An undiluted stock solution of DiI (0.5% in 100% ethanol) is backfilled into the micropipette as described previously.
2. A small amount of DiI is expelled into the regions of the neural fold. At the time of the injection, the injection site is visible as a small red spot of dye in the tissue through the epifluorescence microscope (Sechrist *et al.*, 1993).
3. DiI can be visualized in living embryos by epifluorescence or after fixation in 4% paraformaldehyde. Embryos are prepared for cryostat sectioning.

VI. Grafting Techniques

A number of embryonic manipulations can be used to alter the rostrocaudal or dorsoventral position of neural crest cells. In addition, the prospective neural crest can be removed or the position of the tissue encountered by the neural crest can be altered, such as the notochord. A few representative manipulations are described next. For an extensive discussion of other types of embryonic manipulations, see Chapter 2 by Le Douarin *et al.*, this volume.

A. Neural Tube Rotations

1. Prepare glass knifes by pulling thin glass rods to a sharp tip using an electrode puller. Any electrode puller can be used for the preparation of glass needles. A needle with a long, slow taper is preferred.

2. Operations can be performed on embryos between 1.5 and 2 days of development. For neural tube rotations, a lateral cut is made between the neural tube and the adjacent mesenchyme as well as under the notochord. After separating the neural tube/notochord from the adjacent tissue, two transverse cuts are made rostrally and caudally. The whole neural tube, which is now loose in the embryo, can be rotated rostrocaudally, dorsoventrally, or both. An example of dorsoventral neural tube rotation is illustrated in Fig. 3.

3. Eggs are resealed with cellophane tape and returned to the incubator until the time of fixation.

B. Neural Fold Ablations

1. Segments comprising about one-third to one-half of the dorsal neural tube are removed bilaterally with glass needles. Incisions are made perpendicular to the long axis of the neural tube at the rostral and caudal edges of the site to be ablated. Longitudinal cuts are then made at both the boundary between the epidermis and the neural folds and the desired level within the neural tube (Fig. 4).

2. To avoid any possible contribution from the ablated tissue, it is removed from the egg by capillary action through a micropipette.

3. Eggs are resealed with cellophane tape and returned to the incubator until the time of fixation.

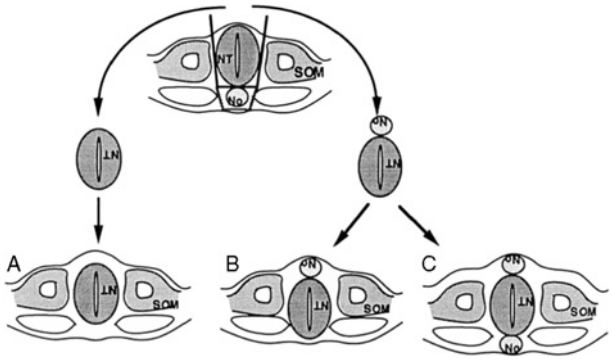

Fig. 3 The neural tube can be rotated in place either rostrocaudally or dorsoventrally. This schematic diagram illustrates dorsoventral rotation either with or without the notochord attached. With a fine glass needle, slits are made between the neural tube (NT) and somites (SOM) and either between the neural tube and notochord (No) or underneath the notochord. The polarity of the tissue is then inverted and it is replaced into the same embryo or into another host prepared in the same way. This makes it possible to produce an embryo in which (A) the neural tube is inverted dorsoventrally in the absence of a notochord, (B) the neural tube is inverted dorsoventrally in the presence of a notochord dorsally, or (C) the neural tube is inverted dorsoventrally in the presence of notochord dorsally and ventrally. For experimental results, see Stern *et al.* (1991).

C. Notochord Implants

1. Notochords are isolated using the procedure for isolating neural tubes as described earlier for trunk neural crest cultures. Briefly, a rectangular block of tissue, including the notochord, is dissected out of the embryo at the desired axial level using an electrolytically sharpened tungsten needle. The notochords are isolated from surrounding tissues with dispase treatment. The notochords then are allowed to recover in complete medium for 1 h prior to implantation.

2. A pulled glass knife is used to make an incision between the neural tube and the adjacent somite.

3. A donor notochord is transferred to the embryo in 2 μl of medium. The notochord is oriented parallel to the incision and is inserted laterally to the neural tube by pushing in with a glass needle.

4. Eggs are resealed with cellophane tape and returned to the incubator until the time of fixation.

D. Notochord Ablations

1. Using a pulled glass needle, an incision is made along both sides of the neural tube of stages 9–10 embryos. A third incision is made perpendicular and posterior to the first incisions, and the neural plate is carefully lifted and folded back (Fig. 5A).

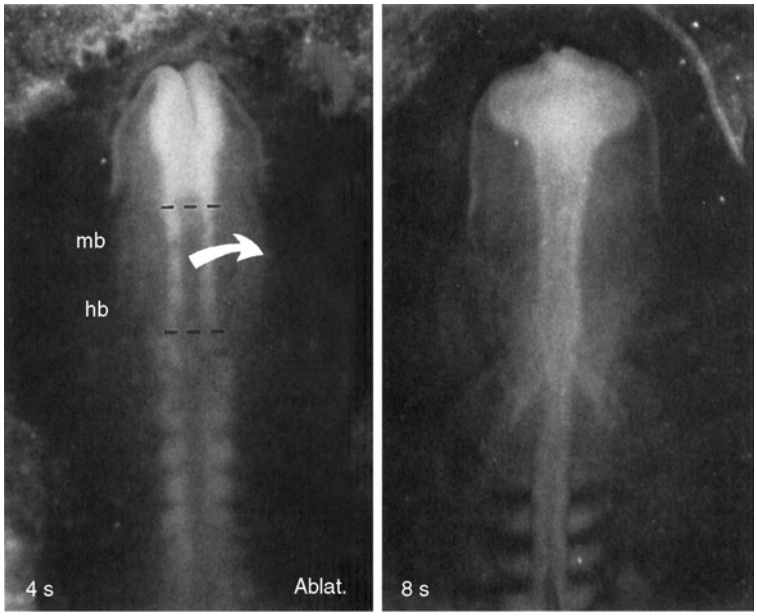

Fig. 4 Bright-field micrographs of an embryo after ablation of the dorsal neural folds in the midbrain and hindbrain region of an embryo operated at the four-somite stage (left). Using a glass knife, slits are made lateral to the neural tube and the top half to third of the dorsal neural tube is removed. The same embryo several hours later (right) at the eight-somite stage. The neural tube has closed and the embryo appears relatively normal morphologically.

2. The notochord is scraped off the underside of the neural plate, which then is returned to its original position (Fig. 5B). Trypsin (0.15%) (GIBCO) can be added to help separate the notochord from the underlying endoderm. A few drops of complete medium are added to dilute the enzyme and to stop the reaction when the operation is complete. Approximately 100–700 μm of notochord tissue is removed at the time of surgery.

VII. Conclusions

The methods described in this chapter provide a number of techniques that can be applied to the study of neural crest specification, migration and differentiation. Simple adaptations of these techniques make them applicable to other regions of the embryo as well. Because neural crest cells interact with neighboring tissues, it is often useful to combine studies of neural crest development with the development of adjacent structures, including the neural tube, somites, and notochord, as described in other chapters of this volume.

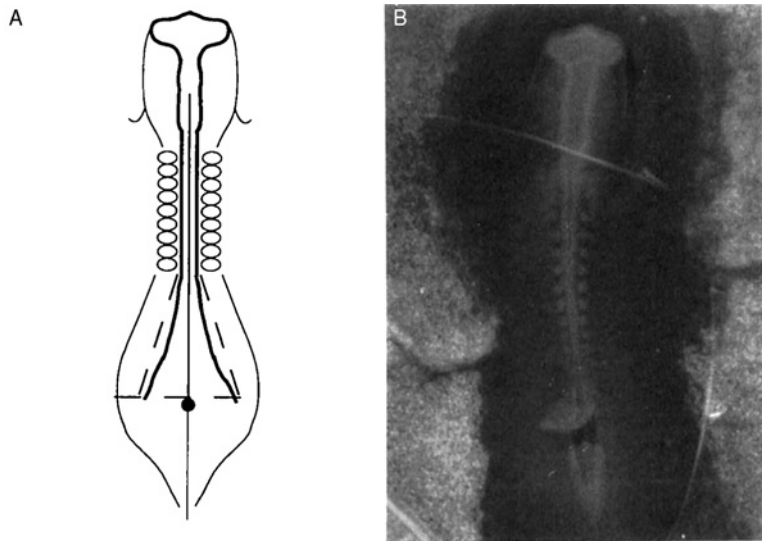

Fig. 5 (A) The microsurgical operation used to ablate the notochord. Incisions were made along the dotted lines as indicated in stages 9 and 10 chick embryos (7- to 10-somite stage). The neural plate was deflected and the notochord was subsequently removed. The neural plate was then returned to its original position and the embryo was allowed to develop for 1–4 days. (B) Photomicrograph of a stage 9 embryo in which the neural plate has been deflected and the notochord removed. Data from Artinger and Bronner-Fraser (1993).

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CHAPTER 5

Embryo Slices and Strips: Guidance and Adhesion Assays in the Avian Embryo

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I. Introduction

Despite the fact that reductionist methods such as cell culture have excelled at revealing developmental mechanisms at molecular levels, an actual embryo poses a much more complex environment. To fully understand mechanisms, developmental biologists must ultimately refer their investigations back to the complexities of the embryo. To facilitate such analysis, this chapter describes two preparations developed in our laboratories (see Hotary and Tosney, 1996; Hotary *et al.*, 1996; Krull and Kulesa, 1998; Tosney *et al.*, 1996) that in large part replicate the complexity of the embryonic milieu and the numerous factors that affect cell interactions and guidance, and that nonetheless offer the pliability and visibility of tissue culture. These preparations use the avian embryo, which has been particularly valuable in elucidating elements of motoneuron and neural crest cell guidance (e.g., Eberhart *et al.*, 2002, 2004; reviews: Creuzet *et al.*, 2005; Krull and Koblar, 2000; Landmesser, 2001). While slice preparations have been used in chick for other purposes (e.g., Arai *et al.*, 2007; Brunet *et al.*, 2007; Kasemeier-Kulesa *et al.*, 2006), the preparations described here are particularly useful for dissecting guidance interactions by visualizing movements and interactions of cells and growth cones either *in situ* or seeded onto complex embryonic environments. These preparations are also amenable to experimental intervention.

II. Preparing and Culturing Embryo Slices

This section describes a chick embryo slice preparation devised to study axon guidance through peripheral tissues. These slices, like the better studied slices of the central nervous system used for neurophysiological studies (see Dingledine *et al.*, 1980), maintain many characteristics of the intact embryo and allow developmental and physiological events to be directly observed and manipulated in a more normal environment. In addition, embryo slices can be manipulated relatively easily, both physically and molecularly. Thus, this preparation provides many advantages of whole embryo manipulations and cell cultures while also overcoming many limitations of these approaches. It is a simplified *in vivo* system, with *in vitro* accessibility and visibility.

The following protocols describe how to prepare transverse slices of the caudal half of chick embryos, although slices can be made in any orientation from any region of the embryo, with minor modifications depending on the embryonic stage and the specific hardware used to cut slices (see Krull and Kulesa, 1998). Regardless of whether embryos are intact or have been altered *in ovo* (e.g., by surgery, or electroporation), embryos are first harvested, rinsed free of yolk granules, eviscerated, and trimmed. The region to be sliced is then isolated and embedded in agarose, which provides the necessary stiffness for sectioning on a vibratome. Slices are cultured either adhered to a substratum or free floating, depending on the experimental intent (Fig. 1).

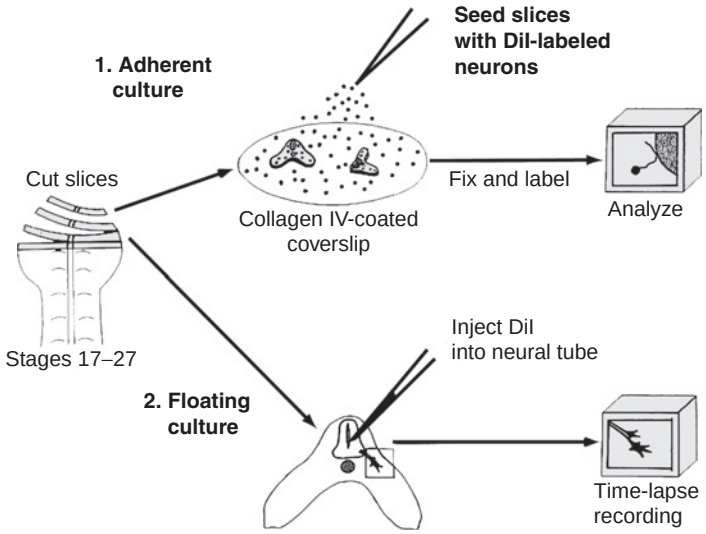

Fig. 1 Alternative applications for preparations. (1). Labeled neurons or cells may be dissociated and seeded onto an adherent slice (or onto the strip preparation) and assessed after fixation. (2). Neurons or cells may be labeled either in the embryo (not shown) or in the slice and assessed with time lapse.

A. Preparing the Embryo

1. Candle eggs (hold a light pipe directly against the shell) and mark the position of the embryo. Lower the embryo by withdrawing 5–10 ml of albumen through an 18 gauge needle attached to a 10 ml syringe. The lower level of the embryo minimizes the risk of damage when removing the overlying shell. Remove the shell over the embryo using blunt forceps or small scissors.

2. Cut through the yolk sac around the outside of the embryo with Vanna's scissors (Fine Science Tools, Foster City, CA) or with fine dissecting scissors and lift the embryo out of the shell using curved blunt forceps as a ladle. Place the embryo in a petri dish filled with a sterile physiological saline (e.g., Hanks' balanced salts or Tyrodes solution) containing penicillin–streptomycin (50–100 U/ml; Invitrogen).

3. Rinse the embryo in the sterile saline to remove adherent yolk granules by gently rocking the petri dish. Remove extraembryonic membranes using jeweler's forceps and Vanna's scissors.

4. Transfer the embryo through three successive washes in sterile saline containing penicillin–streptomycin (50–100 U/ml). After the last wash, transfer the embryo to a glass petri dish coated with 5–7 mm layer of silicone elastomer (Sylgard; Dow Corning) and filled with sterile saline/penicillin–streptomycin (50–100 U/ml).

5. Decapitate the embryo by firmly grasping the neck just rostral to the heart with a pair of jeweler's forceps and squeezing.

6. Pin the embryo ventral side up using insect pins 5–10 mm long. Insert one pin through the neck and another through the tail, pulling the embryo taut, but not so taut as to tear it.

7. Remove the heart and make a slit in the ventral body wall from the neck to the tail using Vanna's scissors.

8. Pin the loose flaps of skin laterally to expose the body cavity and internal organs. Again, pull the embryo taut, without tearing it. The tension will ease evisceration in the succeeding step. The most secure attachment is usually achieved by placing pins through the loose ectoderm and the limb buds; however, if the limb buds are to be included in the culture, pin only the loose ectoderm.

9. Eviscerate the embryo. With jeweler's forceps, grasp the foregut rostrally and pull toward the tail (a pin through the neck is usually sturdier than a pin at the tail, so pulling the viscera caudally minimizes the risk of tearing the embryo). In older embryos (stage 20+), it is usually possible to remove the gut and most other organs in a single piece. The mesonephros can then be removed in one strip from each side of the embryo. In younger embryos, the gut and mesonephros usually must be removed in several small pieces.

10. Remove the dorsal aorta with jeweler's forceps and clean off any remaining bits of loose tissue. Trim off loose ectoderm, as fragments will later stick in the embedding agarose, making it difficult to free slices from the embedding medium.

B. Cutting

1. Embed the embryo in 2–5% low melting-point agarose (GIBCO ultrapure LMP agarose) made in sterile physiological saline in a plastic boat of the type commonly used for paraffin embedding (available from VWR Scientific). Place the embryo in the boat once the agarose has sufficiently cooled but has not yet started to set. Use forceps or a birch-handled probe to move the embryo into the desired slicing orientation.

2. Once set, cut out a block of agarose containing the embryo and mount on a vibratome specimen platform with cyanoacrylate adhesive (Krazy Glue, Borden, Inc., Columbus, OH).

3. Mount the platform on the vibratome and cut slices. Clean and semisterilize the chamber in which the slices are cut by washing in 70% ethanol followed by several rinses with sterile distilled water and then several rinses in sterile saline. It is advantageous to allocate one vibratome for slicing live material only, as residual fixative can cause problems from tissue sticking or even lethality. To keep the agarose stiff during the slicing, keep the bath solution on ice until use and keep the slicing chamber cold. We cut slices 150–220 μm thick on an EMCorp (Chestut Hill, MA) Model H1200 vibratome or on a vibratome 1000 plus sectioning system (Product No. 064018, Technical Products International). To avoid contamination, we recommend that fixed and live tissues be sectioned with different microtomes.

4. Remove slices to a petri dish containing ice-cold sterile saline and remove any adhering bits of agarose or remaining loose pieces of tissue.

5. Transfer cleaned slices to a petri dish containing culture medium (see media and solutions). Slices can be easily transferred with minimal damage using a wide-bore plastic pipette that has been prewetted with culture medium (to prevent the slices from sticking to the pipette walls). Slices can be kept in their dishes for several hours at 37 °C in 5% CO_2 until transferred to their final culture dishes.

C. Culturing

Slices may be cultured either free-floating or attached to a substratum. Floating cultures make it easy to reorient a slice (e.g., when making time lapse recordings of endogenous cell movements). Attached cultures are advantageous when slices will be examined after fixing and staining, as the slices stay in place during procedures. Each method can, however, affect slice architecture. In floating cultures, tissue growth is not confined to two dimensions. For instance, growing limb buds may curl upward. Conversely, in attached cultures, the substrate must be carefully chosen to prevent cells from spreading away from the slice.

For either method, slices are transferred to culture chambers prepared as below from the holding dish (step 5 above) using a prewetted wide-bore pipette. Move them into the desired position with forceps. How many slices each well can accommodate varies with the size of the slice. For stage 17–26 hindlimb slices, a maximum of 6 are commonly cultured per culture.

1. *For floating cultures*, we place slices in specially constructed chambers made by first drilling a 12-mm-diameter hole in the bottom of a 35-mm plastic petri dish. The hole is then covered from the outside with a glass coverslip (Clay Adams Gold Seal) attached with silicon cement. This chamber supports clear visualization through an inverted microscope. If environmental conditions are unstable, slices can drift, but drifting can be prevented using a sliver of coverslip that is stuck to the coverslip with a small bead of silicone grease. The grease acts as a hinge, allowing the coverslip segment to be lowered gently onto the slice. The glass holds the slice gently but firmly, and can be lifted to reorient the slice if needed.

Other chamber types can be used for floating cultures, as long as they do not allow slides to settle in one place for a long period. Slices will eventually attach to nearly any substrate, including the plastic surface of a petri dish. Long-term floating cultures may require periodic rocking to hinder attachment.

2. *For attached cultures*, the culture substrate chosen is crucial both to keep the slices stationary and to maintain tissue integrity. Type IV collagen (200 μg/ml in carbonate buffer; Sigma) coating acid-washed coverslips (Clay Adams Gold Seal) provides good slice attachment without promoting excessive spreading onto the coverslip. Other substrates tested (laminin, polylysine, and several commercially prepared substrata) either were insufficiently adhesive so that part or all of the slice floated free or were overly adhesive so that cells preferred the substrate over their neighbors and migrated from the slice, with resultant loss of tissue structure. Attached slices may be cultured in the chambers described

earlier, or on 12-mm-diameter coverslips placed in 15-mm-diameter four-well dishes (Nunc).

D. Fixing and Mounting

The following protocol is written specifically for fixing attached cultures in their culture wells, and subsequently mounting the coverslips on slides for observation with an upright microscope. The same solutions and procedures are used for floating cultures, except that the slices are transferred in a wide-bore pipette from one solution to the next. To successfully transfer slices without damage and with minimal contamination by the previous fluid, hold the pipette upright above the next fluid until the slices settle at fluid meniscus. The meniscus will protrude slightly but the slices will be held by surface tension. Without pressing fluid out, simply touch the meniscus to the fluid in the next dish; without the barrier of the surfaced tension, the slices will gently transfer to the new fluid.

1. Rinse slices three times for 10 min each in Kreb's buffer/sucrose (solutions are described below). At each rinse, withdraw one-half of the volume of fluid in the well and replace with fresh buffer. Avoid draining the wells completely in this and all subsequent steps, as any air–water interface can dislodge the slices.
2. After the final rinse, replace one-half of the fluid in the well with 4% paraformaldehyde (in Kreb's/sucrose) and let stand for 10 min.
3. Replace one-half of the fluid in the well with fresh 4% paraformaldehyde solution and fix for 30 min to overnight.
4. Rinse the fixed slices three to five times (10 min each) in phosphate-buffered saline (PBS). The slices are now ready for further specific processing, such as antibody or lectin labeling or mounting.
5. To mount adherent slices for observations on an upright microscope, make a ring of silicon grease of about 20 mm diameter on a microscope slide. Make the ring higher than the thickness of the slices. With jeweler's forceps, gently lift the coverslip with its adherent slices out of the culture well and place them slice side up in the middle of the grease ring. Flood this well with PBS or some other mounting solution and cover with a clean coverslip. Push the second coverslip down gently until it is just above the slice surface: avoid squashing the slice!
6. Mount floating slices between two coverslips. First, ring the periphery of the coverslip with silicone grease and transfer the loose slices to the rings well with a wide-bore pipette. Cover the coverslip with a second like-sized coverslip and press together to hold the slices stationary. This slice sandwich can be turned to view from either side with an upright microscope. Support the assembly on a microscope slide.

E. Solutions

1. Culture Medium for slices

The basic medium used for both adherent and floating cultures lacks serum because serum promotes rapid overgrowth of ectoderm that then covers the cut surfaces. It is based on F-12 with the following supplements, all of which are available from Sigma or GIBCO:

- 33 mM glucose
- 22 mM glutamine
- 5 μg/ml insulin
- 6 ng/ml progesterone
- 1.6 μg/ml putrescine
- 8 ng/ml sodium selenite
- 5 μg/ml transferring
- 100 U/ml penicillin–streptomycin.

When sensory neurons are of major interest, or are seeded on the slice, the culture medium is further supplemented with 100 ng/ml 7S nerve growth factor. F-12 medium is formulated to equilibrate in an atmosphere containing 5% CO_2. In cases when the slices will be out of a CO_2 atmosphere for prolonged periods (e.g., during time lapse recording), 10–20 mM HEPES can be added to the medium to buffer pH changes. Alternatively, Leibovitz's L-26 medium, which buffers pH in an ambient atmosphere, could be substituted.

2. Kreb's Buffer and Sucrose

The following final concentrations of Kreb's buffer and sucrose are used during slice fixation. In practice, separate 4× solutions of both buffer and sucrose are made and then the two are mixed with distilled water in a 1:1:2 (Kreb's:sucrose: water) ratio for use in the initial rinses. The fix solution (4% paraformaldehyde) is made in the same ratio, replacing the water with 8% paraformaldehyde.

- 145 mM NaCl
- 5 mM KCl
- 1.2 mM $CaCl_2$
- 1.3 mM $MgCl_2$
- 1.2 mM NaH_2PO_4
- 10 mM glucose
- 20 mM HEPES
- 400 mM sucrose

III. Preparing and Culturing Somite Strips

Somite strips are a hybrid *in vivo–in vitro* assay that exposes anterior and posterior sclerotomes to view and yet retains in culture both the segmental architecture and the typical molecular characteristics such as differential binding to peanut agglutinin lectin (PNA; see Oakley and Tosney, 1991). Labeled neurons or cells sprinkled directly on the strip are visible and have direct access to guidance cues. Natural elements such as neurite lengths provide quantitative measure of relative permissiveness that can be used to monitor effects of treatments designed to block or alter the activity of guidance molecules. Orientations and trajectories distinguish positive from inhibitory interactions, and long-distance from contact-mediate interactions. The assay supports a variety of investigations into inhibitory and permissive properties.

As an overview, the embryo is filleted by removing the viscera, notochord, neural tube, and limbs, leaving only the somites and covering ectoderm. This strip of tissue is placed ectoderm side down on a laminin substratum. The ectoderm spreads on the laminin, but the somite does not spread on the ectodermal undersurface, and therefore retains its integrity. The cells to be assessed are labeled with a nontoxic fluorescent dye, dissociate, sprinkled over the somite strip, and allowed to extend axons overnight. They can be visualized in living cultures with confocal microscopy, or fixed for viewing.

A. Preparing Coverslips

Prepare coverslips the night before and incubate them overnight. The next morning, wash, dry, and place the coverslips in four-well plates. Because only one side of the coverslip is laminin-coated, keep the orientation of the coverslip in mind throughout the manipulations.

1. Use acid-washed 12-mm-round coverslips (Clay Adams, Gold Seal). With forceps, dip each coverslip in 95% ethanol, dab off excess fluid against the lip of the beaker, and ignite the ethanol in a flame. Do not hold the coverslip in the flame longer than it takes to ignite it, to prevent cracking. After a brief pause for cooling, place the coverslip in a sterile 35-mm petri dish. Add 25 μg of laminin (100 μg/ml in 50 mM carbonate buffer, pH 9.6) to each coverslip. Place a second flamed coverslip on each drop of laminin to make a laminin–coverslip sandwich. Cover the dish and place it in a CO_2 incubator overnight.

2. Wash coverslips the next day, at lease 30 min before adding somite strips. With sterile forceps, pick up a coverslip, dip it several times in a beaker of sterile distilled water, change forceps, and dip again. The forceps must be changed because the first will take up solution by capillary action and, when opened, deposit it again on the coverslip. Place the coverslip laminin side up on filter paper to dry.

B. Dissecting

Embryos are removed from the egg, washed extensively to limit contamination and eviscerated. The notochord, spinal cord, and mesonephros are then removed to expose the somites, and the strips are cleaned and trimmed to size. All manipulations are in a laminar flow hood.

1. *Select the correct stage*. Strips are best prepared from lower thoracic and upper lumbar levels of stage 17–18 (3-day) embryos. Somites at these levels have developed sufficiently to have distinct boundaries between sclerotome populations. More mature somites will have contaminating cells from mesonephros and blood cells, and the anterior sclerotome will have begun to acquire inhibitory properties (Oakley and Tosney, 1991)

2. *Prepare dishes*. Fill three 35-mm petri dishes and one 60-mm Sylgard-bottomed dish with Hanks balanced saline (HBS: Gibco). Sylgard (Dow Corning) is an optically clear, autoclavable polymer. A layer 1 cm deep is polymerized in the bottom of a glass dish according to manufacturer directions. Pins can be stuck in it. Add 750 units of pen-strep (Gibco) to each 35 mm dish and 2500 units of pen-strep to the Sylgard dish.

3. *Remove embryo from the egg*. To see the embryo *in ovo*, candle the egg: hold an egg adjacent to a light source to determine where the embryo lies. Trace a circle around the embryo. Swab the egg with 70% ethanol. Use an 18-guage needle attached to a 10-ml syringe to remove 5–10 ml of albumin from the egg: insert the needle through the shell at the fat end of the egg, angling the needle down and toward the side to avoid piercing the yolk. With the albumin removed, the embryo falls away from the shell and can be removed with less chance of damage or contamination. Using curved forceps like scissors, cut out the penciled circle of egg shell. Using dissecting scissors, cut a circle around the embryo through the blastodisc and yolk sac. Lift the embryo out by scoping it with curved forceps; do not try to grasp the embryo or it will be crushed. Transfer the embryos to a prepared 350-mm dish and gently swirl to wash away as much yolk as possible.

4. *Remove embryonic membranes*. Place the petri dish on a dissecting scope stage and observe at low magnification for all further steps. Grasp membranes on either side of the midline at the leg level using two sterile forceps. Gently pull laterally to open a hole, and then continue opening the membranes toward the head. Pull the membranes from both sides to the ventral midline, where they attach to the body wall. Sever the attachment. Decapitate the embryo using Vanna's scissors (Fine Science Tools, Foster City, CA) and discard the head. Cut off and discard the heart.

5. *Wash embryos*. Using curved forceps, transfer embryos to the second and then the third prepared petri dishes. Each time, gently swirl the dish for a minute to rinse the embryos. After the final wash, transfer the embryos to the Sylgard-coated dish.

6. *Pin the embryos*. Gently hold an embryo on its back using sterile forceps. Insert a minutien pin (2 mm, Fine Science Tools, Foster City, CA) through the notochord and push is securely into the Sylgard. Now push a pin through the tail

and *gently stretch* the embryo in the anterior to posterior direction and push the pin into the Sylgard. If the embryo is not stretched, it will flop around while the ventral tissues are removed, making the task difficult. Use four more pins to pin back the body wall and expose the ventral tissues. Because the body wall is thin and fragile, pin it through the limb buds on each side, applying gentle tension to stretch the body cavity. If more than one embryo is being dissected, pin them down like spokes in a wheel, with their necks toward the hub.

7. *Remove the gut*. The midgut may be attached by a thing, membrane-like extension to the ventral body wall. Free the midgut from the body wall using forceps. Remove the entire gut by grasping it in the neck region with the forceps, and pulling toward the tail. Expert force only along the anterior–posterior axis; pulling laterally often tears the embryo in two.

8. *Remove the notochord*. Use forceps to sever the notochord at the upper forelimb level. Grasp the notochord just posterior to the break and carefully peel it toward the tail. If the notochord is difficult to remove, use two forceps: work the tips of one pair between the notochord and neural tube, and use the second pair to pull toward the posterior. Pull the notochord free just past the leg buds, and then cut it off. Trying to pull it away from the tail often pulls off the entire tail, and sometimes the limb buds as well.

9. *Remove the spinal cord*. Using forceps, pick up a minutien pin. Use the sharp end of the pin to split the neural tube at the midline. As the neural tube is opened, each half will fold laterally to cover the medial aspect of the somites. Now, remove one-half of the spinal cord by grasping it at the forelimb level with forceps and pealing it posteriorly. Repeat for the other side. Be gentle to avoid damaging the somites and the underlying ectoderm.

10. *Remove the mesonephros*. Unless the strip is going to be labeled later with a marker for posterior (e.g., PNA; see Oakley and Tosney, 1991), apply a marker to distinguish anterior from posterior. Carbon particles can be used, or DiI (Molecular Probes) can be injected into a posterior segment at one end of a strip. To prepare DiI stock, dissolve 2.5 mg DiI in 1 ml 100% ethyl alcohol. This stock solution can be kept refrigerated for up to a week. Just before use, dilute 200 μl of stock in 800 μl of 10% sucrose in distilled water, and filter with a 0.5 μm syringe-type filter. Pull micropipettes from electrode glass to form a long tapered end. Break off the tip at a diameter of $< 1/10$ the diameter of a somite, as determined by eye. For injection, use an assembly with a 1-cc syringe inserted into a 0.22 μm filter with an 18.5 gauge need at its tip. Attach propylene tubing between the needle and the micropipette. Suck DiI into the pipette and inject a very small amount into a posterior half-somite.

11. *Trim strip*. Remove pins except for those securing the neck and tail. Use Vanna's scissors to remove limb buds and cut the strip into a clean rectangle. Do not separate the bilateral pairs of somites: single rows of somites curl up. In double rows, the ectoderm spreads on the substrate, securing and displaying the sclerotomes.

C. Culturing

The strips are placed ectoderm side down on the laminin-coated coverslips and held in place with sterile tungsten wire. A variety of culture media are appropriate, but F12 culture medium supplemented as for slices works well. Avoid serum that promotes overgrowth by contaminating cells.

1. *Transfer strips to culture plates*. Add 0.9 ml of culture medium to each 15-mm-diameter well of a four-well plate (Nunc). Culture each strip in a separate well. Place one laminin-coated coverslip in each well, laminin side up, making sure it settles to the bottom without trapping bubbles. Prewet a sterile Pasteur pipette with medium to prevent the tissue from sticking to the glass. Carefully suck up a somite strip by placing the pipette tip at one end of the strip and slightly releasing the bulb. When done correctly, the strip will enter the pipette readily (it tends to fold along the midline) and will lie close to the tip of the pipette. Transfer to the well with a minimum of fluid transfer.

2. *Secure strips*. Use a dissecting scope to examine the strips in wells. If necessary, use forceps to slip the somite strip over to have the ectodermal side facing the laminin. Use forceps to dip small pieces (0.5 cm) of tungsten wire (0.005 mm in diameter, R.D. Mathis, Long Beach, CA) in 95% ethanol and flame briefly. Hold wire for a minute to cool. Place a wire across the strip at the posterior end between anterior and posterior halves of the last somite pair. Once this wire is positioned correctly, push it into the tissue to secure the posterior end. Be gentle to avoid severing the tissue. Repeat for the anterior end, stretching the strip as flat as possible.

3. *Incubate*. Carefully move the plate into a 5% CO_2 incubator at 37 °C. Incubate for 2–4 h before adding dissociated neurons. This period allows the ectoderm to attach and begin to spread on the laminin.

D. Labeling Neurons

Neurons of your choice may be labeled, dissociated, and sprinkled on the adherent strips, and cultured overnight. This section describes preparing sensory neurons (DRG) and labeling after dissociation. A method for labeling by injecting nerves in intact preparations is described in the original publication on DiI by Honig and Hume (1986).

1. *Dissect*. Remove a 5–6 day (stage 24–26) embryo from the egg, rinse, decapitate, and remove membranes as described above. Transfer to a Sylgard-bottom dish with 10 ml of HBS and 750 units of pen-strep. Pin the embryo on its back, slit open the body wall, and pin them down. Eviscerate and clean the body cavity of loose tissue. In the anterior, use Vanna's scissors to cut transversely through the vertebrae, and then the cut the vertebral processes to each side. With forceps, pull the flap of vertebral tissue to the posterior. It may be necessary to cut vertebral processes as you pull. When the tail is reached, cut off the vertebral strip.

Remove the neural tube to expose the DRG. Pluck out 20–30 DRG using forceps and clean off debris. Collect the ganglia in one area of the dish. Prewet the tip of a pipette with HBS and use it to transfer the DRG into a 35 mm petri dish containing 5 ml of HBS.

2. *Dissociation label.* Mince each DRG and transfer the piece into a 10 ml plastic tube with 4.5 ml modified Puck's glucose and 0.5 ml of 10× trypsin (Gibso). Incubate 20 min in a water bath at 37 °C. Add 5 ml of medium containing 5% horse serum to quench the trypsin reaction, mix, and then centrifuge to pellet the cells. Use medium with 5% serum for all following steps, except the final resuspension. Triturate the pellet in 1 ml of medium. Resuspend in 9 ml of medium and centrifuge. Decant fluid. Make, sonicate and filter a DiI stock solution composed of 2.5 mg DiI in 100 µl DMS and 900 µl 100% ethyl alcohol. Resuspend pellet in 1900 µl of medium and 100 µl of DiI stock solution. Triturate and incubate for 40 min at 37 °C in a 5% CO_2 tissue culture incubator. Add 9 ml of medium and centrifuge. Repeat trituration and rinsing steps for more times and then resuspend in 1 ml of medium without serum. Add 150–200 µl to each culture well, adjusting cell density so only a few neurons adhere to each strip.

E. Fixation

Wash cultures gently with medium at 37 °C to remove floating cells. Fix overnight in 4% paraformaldehyde (5 ml of 8% paraformaldehyde, 2.5 ml 4× Kreb's buffer, and 2.5 ml 1.6 M sucrose in distilled water). Rinse in PBS.

IV. Perspectives

A. Characteristics

For these preparations to be useful, they must remain viable in culture; retain normal tissue morphologies, relationships, and molecular composition; and support normal developmental processes. Our evaluation shows these criteria are met to a realistic degree (Hotary and Tosney, 1996; Hotary *et al.*, 1996; Krull and Kulesa, 1998; Tosney *et al.*, 1996).

Slices and strips retain embryonic morphology and tissue relationship for 1–2 days in culture (Figs. 2 and 3). Tissues and borders between tissues are easily visualized using phase or Nomarski optics. Tissues do not become necrotic. Cell death, other than in the normal sites of apoptosis, is not detected with acridine orange, calcein-AM/ethidium homodimer-1 (live/dead cell assay; Molecular Probes, Eugene OR). Dead cells and debris are absent from cut surfaces. Expression patterns should be checked for the proteins of particular interest, but clearly a marker for inhibitory tissues, PNA labels appropriately. In slices, tissue relationships are retained. In strips, the borders are clearly visible and are retained in culture. After a couple of days, slice structure can be disrupted by differential tissue

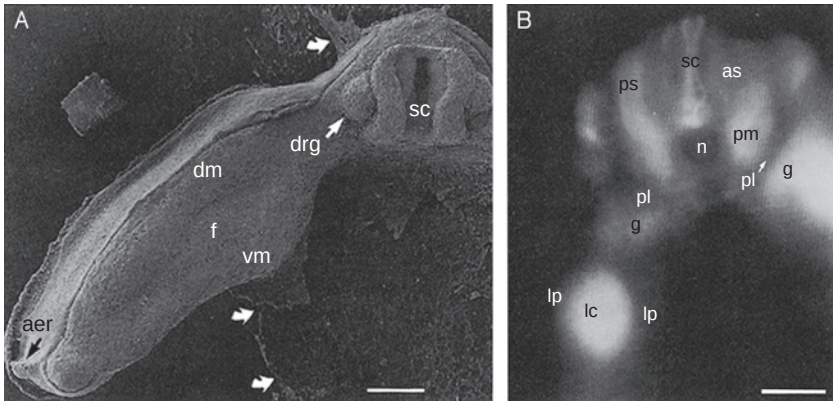

Fig. 2 (A) Scanning electron micrograph of a stage 22 chicken embryo slice after 20 h in culture. The slice surface is free of debris, leaving internal tissues exposed (ventral tissues have been removed in this slice). Embryonic structures and tissue relationships are normal, and the dorsal (dm) and ventral (vm) muscle masses as well as the femur (f) are beginning to emerge in the limb. Note that some cells in contact with the substrate have migrated out, and help anchor the slice. er, ectodermal ridge; drg, dorsal root ganglion; sc spinal cord. (B) Epifluorescent micrograph of the peanut agglutinin lectin-labeling pattern in a stage 26 slice after 20 h in culture. The section is oblique, so both posterior (ps, labeled) and anterior (as, unlabeled) sclerotomes are visible. Other tissues inhibitory to axon advance (pm, perinotochordal mesenchyme; pg, pelvic girdle; lc, limb core) are labeled whereas other permissive tissues (pl, plexuws; lp, limb path) are not. n, notochord. Scale bars, 150 μm.

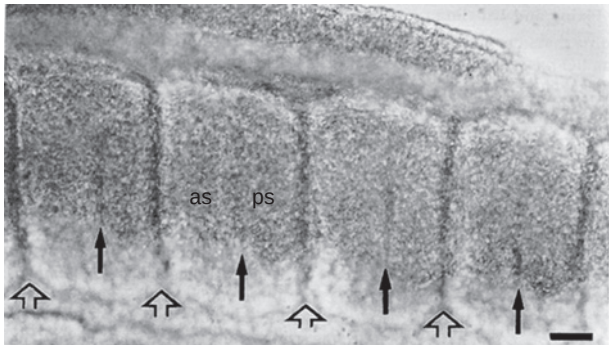

Fig. 3 Half of a bilateral somite strip after 18 h of culture. Open arrows mark the borders between somites whereas dark arrows mark the border between somite halves. The ectodermal edge is visible at top right. as, anterior sclerotome; ps posterior sclerotome. Scale bar: 50 μm.

growth or obscured by ectodermal growth over the cut surface. In strips, the utility diminishes with development because the anterior sclerotome begins to assume inhibitory characteristics, as it also does in the embryo.

For somite strips, the retention of morphology is critically dependent on the ectoderm. In this preparation, the ectoderm maintains the segmental structure of

the somites. To maintain tissue relationships, the ectoderm must be intact and well-spread on the laminin substrate. If sclerotome tissues touch the laminin substratum directly, they will prefer it, spread upon it as a monolayer, and the segmental tissue will be dispersed. In addition, if the ectoderm looses contact with the substratum so the slice is held only by the wire, the slice will assume an hourglass shape, or simply curl up. Good ectoderm contact with the substrate is thus crucial. The ectoderm is evidently attached at its cut edges, and is easily dislodged, so cultures must be handled with minimal mechanical disturbance. Strips of six to eight somites are generally more stable and tend to curl up less, than longer strips.

Because somite strips are designed to provide a natural permissive/inhibitory environments in repeated array, and because these environments change their characteristics naturally during development, the stage and axial level of somites used are crucial. Somites change in two ways during development that complicate the simple dichotomy between anterior and posterior functional domains. Unless you are alert to these developmental features, you could, for instance, be confounded by apparently inhibitory qualities in anterior sclerotome. First, anterior sclerotome normally becomes less permissive as somites mature (Oakley and Tosney, 1991). Second, the ventral portion of both anterior and posterior sclerotome becomes inhibitory on interacting with the notochord (Tosney and Oakley, 1990). Removing the notochord early prevents this interaction and allows ventral–anterior sclerotome to retain its permissive qualities. If strips are prepared from more mature somites, the interaction would already have commenced. This process can, of course, be studied in strips, but an inhibitory marker would be essential, because an obvious border between dorsal

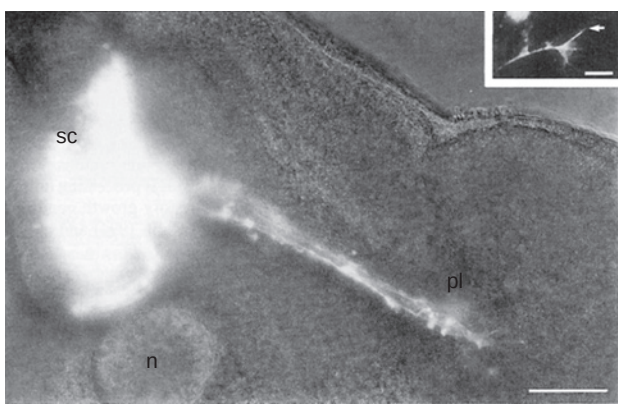

Fig. 4 DiI-labeled motor neurons within slices. A bolus of DiI was pressure injected into the spinal cord (SC) of a stage 22 slice. After about 3 h, a group of motor neurons, some of which extend as far as the plexus at the limb base (pl), are brightly labeled. n, notochord. Scale bar: 50 μm. The inset shows a higher magnification view of a motor axon growth cone from a different slice labeled in the same way. The DiI label allows clear visualization of individual filopodia (arrow) in growth cones. Scale bar: 20 μm. Note that these micrographs are standard epifluorescence; confocal will of course give even sharper images.

permissive and ventral inhibitory tissue is absent. When prepared as described, the preparation supports analyzing cell and growth cone interactions with natural substrates differing in permissiveness.

B. Applications

These two assays offer access to more complex embryonic environments in a dish, but differ in geometry and application. The strip offers a geometrically simple set of rectangular tissues that comprise a representative set of permissive and inhibitory tissues for axon and cell guidance. While the slice preparation is obviously better suited to examine labeled neurons and cells as they move *in situ* (Figs. 4 and 5), cells and neurons of different sources and stages can be seeded either on strips or on slices (Fig. 6), to assess, for instance, temporal differences in

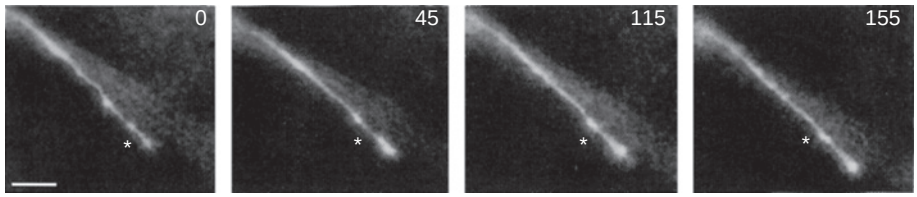

Fig. 5 Time lapse series of DiI-labeled motor axon advancing within a stage 23 slice. Over a total recording period of 4.5 h, this axons advanced steadily at an average rate of about 12 μm/h. Numbers indicate elapsed time in minutes. Asterisks mark a fixed reference point in the slice. Scale bar: 25 μm.

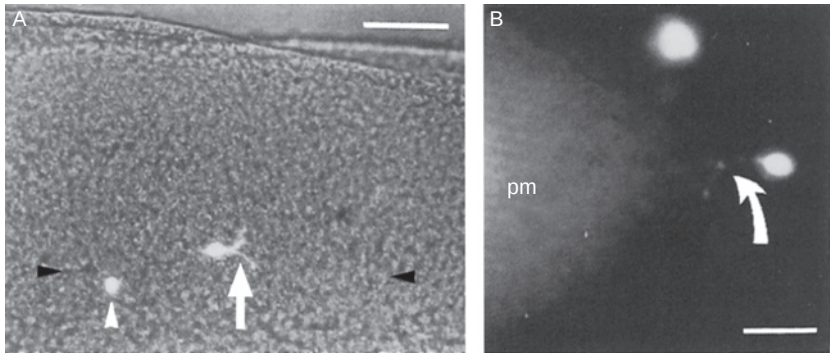

Fig. 6 DiI-labeled neurons deposited on slices and cultured for 18 h. (A) Two DiI-labeled motor neurons on the limb exhibit differential outgrowth according to which tissues contact. One neuron (arrowhead) landed on precartilaginous femur tissue (limb core) and sprouted only a very short neurite. A second neuron (arrow) on the nearby limb path has extended two longer neuritis. The black arrowheads indicate the boundary between path and core. (B) A neuritis (curved arrow) from a sensory neuron in the plexus contacted the border of the PNS-labeled perinotochordal mesenchyme (pm) and turned. Scale bars: 25 μm.

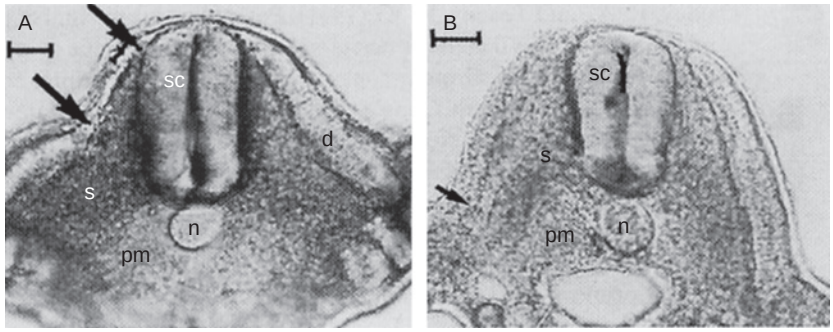

Fig. 7 Slices in which dermamyotome has been removed. (A) The dermamyotome (d) has just been removed from the region between the two arrows. The overlying ectoderm and the sclerotome (s) remain intact. After a few hours in culture, the wound made by removing the dorsal dermamyotome is no longer visible following a similar operation in a different slide. (B) The arrow indicates the remaining dermatome and myotome on the operated side. Other labels as in Fig. 2. Scale bars: 50 μm.

cell sensitivities to guidance cues or the degree to which different neurons detect the same cues. Tissues may be removed from slices (Fig. 7) or added to slices. Either preparation can be subject to earlier manipulation, for instance, by labeling cells *in ovo*, embryonic surgery, or electroporation of reagents (e.g., Chesnutt and Niswander, 2004; Itasaki *et al.*, 1999; Krull, 2004, Muramatsu *et al.*, 1997; Pekarik *et al.*, 2003; Swartz *et al.*, 2001). The assays share a significant similarity: both use portions of embryos that are big enough to simulate the real internal environment of the embryo, but both are placed in a culture setting that is highly accessible to intervention, to exogenous chemicals, to visualization, and to analysis.

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CHAPTER 6

Neural Crest, Sensory Neuron, and Muscle Cultures

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I. Introduction

In vitro culture is now a routine and effective technique for studying the behavior of cells and/or tissues when isolated from the environment of the embryo. Although *in vitro* conditions may not completely recapitulate *in vivo* development, the ability to control isolated cells and experimental conditions can be very powerful. To study cells and tissues freshly dissociated from the embryo requires a number of skills that involve their isolation, purification, and knowledge of their behavior in the embryonic environment, in combination with cell and tissue culture techniques. Optimization of culture conditions is also required for cell maintenance or cell differentiation.

New techniques such as DNA electroporation and RNAi, which have been proven to work in the embryo, mean that transfected cells can be isolated and the effect of a particular gene on cell behavior can be studied in culture under controlled conditions and then used to further understand its function in the embryo. In contrast, cells or tissues can be isolated from the embryo, transfected with a gene of interest *in vitro*, and then returned into the embryo.

In this chapter, we review culture techniques that are commonly used and we introduce methods that extrapolate them to include recent techniques involving isolation of pure cells, transgenesis, and immunocytochemistry. Here, we outline cultures for three different cell types from embryonic avian embryos—neural crest, sensory neurons from the trigeminal ganglia, and muscle cells. Some techniques are specific to the cell type under study but most can be modified and applied to other cultures. For instance, one of the methods we employed for enrichment of neural crest cells is immunoselection, which is used routinely by immunologists and now increasingly popular among developmental biologists.

Sensory neurons from the trigeminal ganglia originate from two distinct embryonic cell populations: the neural crest and ectodermal placodes. In the trigeminal ganglion,

neural crest gives rise to neurons and supports cells whereas placode cells give rise to only neurons. We describe how neural crest-derived versus placodal-derived neurons can be isolated and enriched from the trigeminal ganglia by a combination of dissection based on their locations as well as their differential needs for neurotrophins. Furthermore, we discuss methods by which neural crest- and placode-derived neurons can be distinguished using molecular markers. In this way, one is able to separate and study distinct populations of sensory neurons, which cannot be achieved with dorsal root ganglion sensory neurons as they are all neural crest-derived. In this chapter, we focus on the trigeminal ganglion, the largest of all cranial ganglia, but the methods described can be extrapolated to other cranial ganglia of dual origin.

Finally, we provide a protocol for generating striated muscle cultures. Because of the relative ease of these cultures, they are often used in neuron-muscle cocultures; thereby, providing opportunities to study relationship between neurons and their interacting tissues such as synapse formation and neuronal activities.

II. Materials

A. Eggs

Fertilized chick or quail eggs are obtained from commercial sources. To ensure good survival, eggs should be stored between 15 and 20 °C and incubated within 5 days after they are laid. Fertilized eggs are incubated at 38 °C with humidity until the desired stages.

B. Materials for Microsurgery

Dissecting microscope with fiber optic illumination
Egg holder for stabilizing the egg during dissection
Small scissors
Iridectomy scissors
Curved iridectomy knife
Blunt-ended curved forceps for cracking eggs and removing embryos
Dumont No. 5 and No. 55 forceps
Wipes (Kim wipes)
Sterile petri dishes (Fisher or VWR)
70% Ethanol in a squirt bottle for sterilizing egg surface
70% Ethanol in a beaker lined with cotton gauze for sterilizing tools
Ice bucket

C. Materials for Cell and Tissue Culture

All the following material should be kept sterile:

15 and 50 ml screw cap conical tubes (various sources)

Sterile plastic petri dishes (100, 60, and 35 mm, Fisher)

4-well or 8-well chamber slides (Nunc or Falcon)

24-well tissue culture plates (various sources)

Cell strainers (40 μm, Falcon)

Cotton-plugged pasture pipettes (Fisher or VWR)

5- and 10-ml serological pipettes (Fisher or VWR)

Refrigerated bench-top centrifuge (various manufacturers)

D. Solutions and Media

Ringer's solution for dissecting tissues from embryos

Ringer's solution +0.1% bovine serum albumen (BSA, Sigma, St. Louis, MO, USA) for storing dissected tissues

Dulbecco's modified Eagle's medium (DMEM, Invitrogen/GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Invitrogen (Grand Island, NY, USA) or Atlanta Biologicals, Irvine, CA, USA) + 1% glutamine + 1% penicillin–streptomycin (Invitrogen/GIBCO, Grand Island, NY, USA)

Dispase (1 mg/ml in DMEM, Roche, Indianapolis, IN, USA)

0.05% Trypsin (Cellgro, Manassas, VA, USA)

Type I rat tail collagen (BD Biosciences, San Jose, CA, USA)

Collagen matrix gel [type I rat tail collagen (BD Biosciences, San Jose, CA, USA) + 10× DMEM, adjust to pH 7.2 with 7.5% Na_2HCO_3]

Poly-D-lysine (Sigma)

Fibronectin (1 mg/ml; BD Biosciences or Sigma)

Laminin (BD Biosciences)

Rat anti-mouse IgM beads and magnetic stand (Dynal Biotech, Oslo, Norway)

Neurotrophins: nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), both from Alomone Laboratory (Jerusalem, Israel) or other commercial sources

E. Reagents for Immunocytochemistry

Phosphate-buffered saline (PBS)

PBST (PBS + 0.1% Triton X-100)

4% Paraformaldehyde in PBS, pH 7.4

Donkey or goat serum

Bovine serum albumin (Sigma)
Primary antibodies (various sources, Table II)
Secondary antibodies (various sources)

III. Methods

A. Incubation of Eggs and Staging Embryos

Eggs are incubated for different periods until the desired stages are reached (Table I) at 38 °C in a humidified forced-draft incubator. Prior to removal of the embryo, eggs are sterilized with 70% ethanol. Each culture type may require a different technique for removal of the embryo; these techniques are described separately in relation to the type of culture. Embryos are staged according to the development tables of Hamburger and Hamilton (1951).

B. Fixing and Immunocytochemistry

For most immunostaining, media are first removed from cultured cells or explants. Cultures are briefly rinsed with PBS to remove residual media, then fixed in 4% paraformaldehyde for 20 min to 1 h at room temperature followed by three 10-min washes in PBS. Immunostaining is usually performed on freshly prepared samples, otherwise they can be stored for a short period of time at 4 °C. For most antibody stainings, we typically incubate cultures with a blocking solution containing PBS with 5–10% serum (donkey or goat) and 0.1% BSA for 1 h followed by incubation in primary antibodies, diluted in blocking solution. A list of antibodies that we routinely use to identify different cell types both *in vivo* and *in vitro* is compiled in Table II. The choice of secondary antibodies can be fluorescence (Alexa 488- or Alexa 594-conjugated) or enzymatic (horseradish peroxidase- or alkaline phosphatase-conjugated).

Table I
Optimal Age for Collecting Tissues for Culture

Culture type	Optimal HH stage	Somites	Embryonic day
Late migrating trunk neural crest cells	19	37–40	3
Pectoral muscles	37–39	N/A[a]	11–13
Trigeminal ganglion	32–35	N/A[a]	8–9

[a]All somites have formed at these stages; therefore, other morphological features are used for identifying their age.

Table II
Cell Type-Specific Markers

Cell type	Marker	Source	References
Neural crest	HNK-1	ATCC[a]	Tucker *et al.*, 1984, 1988
Neurons	TuJ1	Covance	Lee *et al.*, 1990; Lwigale and Bronner-Fraser, 2007; Moody *et al.*, 1989
	Neurofilament M	Invitrogen	Lee *et al.*, 1987; Sechrist and Bronner-Fraser, 1991
	HuC/D	Invitrogen	Marusich *et al.*, 1994
	Trk A, B, C	Dr. L. F. Reichardt (University of California, San Francisco)	Buchman and Davies, 1993; Clary *et al.*, 1994; Genc *et al.*, 2005; Huang *et al.*, 1999; von Bartheld *et al.*, 1996
Trigeminal Placode	Pax3	DHSB	Baker *et al.*, 1999
Quail-derived cells	QCPN	DHSB	Lwigale *et al.*, 2005
Quail-derived neurons	QN	Dr. Hideaki Tanaka	Lwigale, 2001; Tanaka *et al.*, 1990
Cartilage	Collagen II	Chemicon, DSHB[b]	Lwigale *et al.*, 2004
Smooth muscle	Smooth muscle-specific actin	Sigma	Skalli *et al.*, 1986
Striated muscle	MF20	DSHB	Bader *et al.*, 1982
Dividing cells	Phosphohistone H3 (M phase)	Chemicon/Millipore	Hendzel *et al.*, 1997
	BrDU (S phase)[c]	DSHB, Roche	Sechrist and Marcelle, 1996

[a]ATCC = American Type Culture Collection.
[b]DSHB = Developmental Studies Hybridoma Bank.
[c]Require application of BrDU prior to fixation.

IV. Neural Crest Culture

A. Obtaining and Identifying Neural Crest Cells

Neural crest can easily be obtained *in vitro* by explanting the neural tube prior to neural crest emigration. After one day in culture, neural crest cells have migrated and can be seen as a halo around the neural tube. Details of methods for neural tube explant culture can be found in Chapter 4 by Bronner-Fraser and Garcia-Castro, this volume (and the last edition, Bronner-Fraser, 1996). This is a very

simple and convenient way to obtain neural crest cells especially for emigration or early migration assays; however, long-term neural crest culture can be challenging because different culture conditions may be required for survival and differentiation of these cells. This is particularly problematic if one would like to study neural crest cells during later stages of migration—they cannot be isolated from neural tube because they have migrated out, and neural crest cells generated from neural tube explants tend to differentiate into neurons quickly; thus, neural crest explants that have been cultured for more than 3 days may not faithfully represent their counterpart *in vivo*.

In avian embryos, migrating neural crest cells can be visualized by a monoclonal antibody called HNK-1 (Tucker *et al.*, 1984, 1988). HNK-1 stains neural crest cells as soon as they have exited the neural tube and its expression persists during migration and in neural crest-derived ganglia. In order to isolate migrating neural crest at different stages of development, we have optimized a protocol where we take advantage of HNK-1 as a cell surface marker and use immunoselection techniques to enrich for neural crest cells from dissected tissues. We have outlined a general protocol for isolating and purifying neural crest cells from stage 19 chick embryos. Specific dilutions of antibodies and choice of magnetic separation techniques should be determined by individual end users depending on their experiment endpoints and cell type of interest. A similar method was used to purify neural crest cells from the gut in older chick embryos (Pomeranz *et al.*, 1993); thus, this type of protocol can be modified for specific stage of development and cell type of choice. We have outlined here the technique to isolate neural crest cells from stage 19 embryos but it can also be applied to younger embryos with optimizations.

B. Isolation and Purification of Neural Crest Cells

Collect stage 19 embryos (37- to 40-somite stage) and rinse several times in Ringer's solution. Dissect the trunk region of stage 19 embryos between somite levels 11 and 22; remove limb bud if possible. Tissues are then incubated with dispase at 37 °C for 30 min, followed by three rinses in Ringer's solution +0.1% BSA. Using Dumont No. 5 forceps, remove ectoderm and notochord (notochord expresses HNK-1 at this stage). Collect all explants without notochord into a 50 ml conical tube and dissociate into a single-cell suspension with a fire-polished Pasteur pipette in about 3 ml of PBS + 0.1% BSA by passing the tissue up and down the pipette until no clumps can be seen. Pass the cell suspension through a 40 μm cell strainer to remove cell clumps. Incubate cells with the mouse HNK-1 antibody for 15–30 min at 4 °C with gentle shaking, followed by three washes in PBS + 0.1% BSA. Incubate with rat anti-mouse IgM conjugated with beads for 15–30 min at 4 °C with gentle shaking (make sure you mix the rat anti-mouse IgM beads thoroughly before use). Rinse cells three times in 1 ml of PBS + 0.1% BSA by placing the tube containing cell sample in a magnetic stand and let stand for 2 min. The positively selected cell suspension (HNK-1 + cells) + rat anti-mouse IgM

beads will appear reddish brown and adhere toward the side of the magnetic stand. Discard supernatant, which contains HNK-1 negative cells, by removing it gently with a pipette. Remove tube from magnetic stand and add fresh PBS + 0.1% BSA to the selected cells and mix gently. Repeat this washing step two more times. After the final wash, remove PBS + 0.1% BSA and resuspend the selected cells gently in DMEM + 10% FCS. Purified cells can now be plated on fibronectin-coated tissue dishes for culture or other downstream purposes. Remove an aliquot of cells after each experiment to estimate yield and purity of resulting cells by counting HNK-1 stained cells under the fluorescence microscope.

C. Cell Culture

Purified neural crest cells can be plated on fibronectin- or laminin-coated slides or tissue culture-treated dishes. The choice of media will depend on the purpose of the experiment. A typical basic medium is DMEM + 10% FCS + 2% chick embryo extract. Purified neural crest cells can be subjected to migration or other cell behavior analyses such as the Boyden chamber assay or live imaging, which is typically short-term (5–10 h). Alternatively, they can be used to study cell differentiation by culturing for longer periods. For instance, neuronal differentiation can be observed usually 24 h after plating as judged by the presence of long neurites in addition to HuC/D and neurofilament immunoreactivity. Tyrosine hydroxylase, a marker for sympathetic neurons, can be observed after 2–3 days *in vitro*.

V. Sensory Neuron Culture

The following methods for isolating and culturing trigeminal ganglion neurons are modified from those described by Nishi (1996) for autonomic and sensory neurons.

A. Dissection of Trigeminal Ganglia

A well-condensed trigeminal ganglion (composed of both neural crest and placodal cells) is formed by embryonic day 4–5 (E4–5; Covell and Noden, 1989). At this time, the majority of differentiated neurons are of placode origin (D'Amico-Martel and Noden, 1980, 1983); neural crest cells have just begun to exit the cell cycle and differentiate into neurons and glia. The best time to dissect trigeminal ganglia for *in vitro* culture is between E8 and E10 (Lwigale and Bronner-Fraser, 2007), before the cranial bones harden. Also at these stages of embryonic development, the ganglia are fairly large and the number of both neural crest and placodal neurons is high (Covell and Noden, 1989). Older ganglia may also be

dissected if necessary. This would require careful removal of the scalp and underlaying cranial bones, followed by the entire brain. Trigeminal ganglia and their connections to the central nervous system remain in skull and are located in the middle cranial fossa resting on the petrous bone in Meckel's cave. The ganglia are covered by membranes that have to be carefully removed using forceps. Once the ganglia are visible, the two major branches are cut at the point where they enter the skull, then each ganglion is teased apart from underlying membranes.

To dissect trigeminal ganglia, curved blunt-ended forceps are used to pinch off the heads from the embryos. The heads are transferred into a 100 mm petri dish or 50 ml tube containing cold Ringer's solution, rinsed twice, then stored on ice. The head to be operated is transferred into a 60 mm petri dish and placed on the stage of a dissecting microscope. Using forceps to hold down the head, one ganglion is dissected from each side of the embryo. Remove the ectoderm between the ear and the eye and locate the trigeminal ganglion within the slightly ossified cranial tissue. Looking through the microscope at the lowest magnification, the ganglion will appear as a dense white bilobed mass of cells surrounded by cranial cartilage and relatively loose cells. Once the ganglion has been identified, carefully remove the surrounding tissue using fine forceps following the outline of the ophthalmic and maxillomandibular branches. With iridectomy scissors, first cut the connection of the ganglion to the central nervous system, then the distal parts of the ophthalmic and maxillomandibular nerve branches. Using a Pasteur pipette, transfer the ganglion into a 35 mm petri dish containing Ringer's solution with 0.1% BSA on ice. Unless specified, for the remaining steps, Ringer's solution with 0.1% BSA is used. Remove any debris from the ganglion with forceps (if this can be carefully done without damaging the ganglion, there is no need to use dispase at this point). Repeat this process on the contralateral side. Store the dissected ganglia on ice until the desired number is obtained.

B. Identifying Neural Crest- and Placode-Derived Neurons

At early stages of gangliogenesis, neural crest-derived neurons, which are usually small, can be distinguished from placode-derived neurons that are relatively large (Covell and Noden, 1989). However, at later stages of development (such as those used for *in vitro* culture), neural crest and placode neurons can no longer be distinguished morphologically (D'Amico-Martel and Noden, 1980). Nonetheless, they can be identified by: (1) electroporating either cell precursors with a green fluorescent protein plasmid (personal observation) prior to ganglion assembly, (2) grafting either cell precursors from quail into chick then tracing the neurons with QCPN or QN antibodies (Lwigale, 2001; Lwigale *et al*., 2004), or (3) *in vitro* culture with neurotrophic factors that support development of either trkA-positive neural crest (NGF) or trkB-positive placode-derived neurons (BDNF; Barde *et al*., 1980; Davies, 1997; Enokido *et al*., 1999; Lindsay and Rohrer, 1985; Lindsay *et al*., 1985).

C. Preparation of Ganglion Explants

After all trigeminal ganglia have been collected, transfer one ganglion at a time into another petri dish containing ice-cold Ringer's solution on the microscope stage. Lay the ganglion down then maneuvering with a pair of forceps, use a tungsten needle to trim off the central connection to the brain, and then cut off the nerves from the ophthalmic and maxillomandibular branches close to the ganglion. Cut the ganglion approximately half way across (Fig. 1C). The region of the ganglion proximal to the brain should contain mostly neural crest-derived

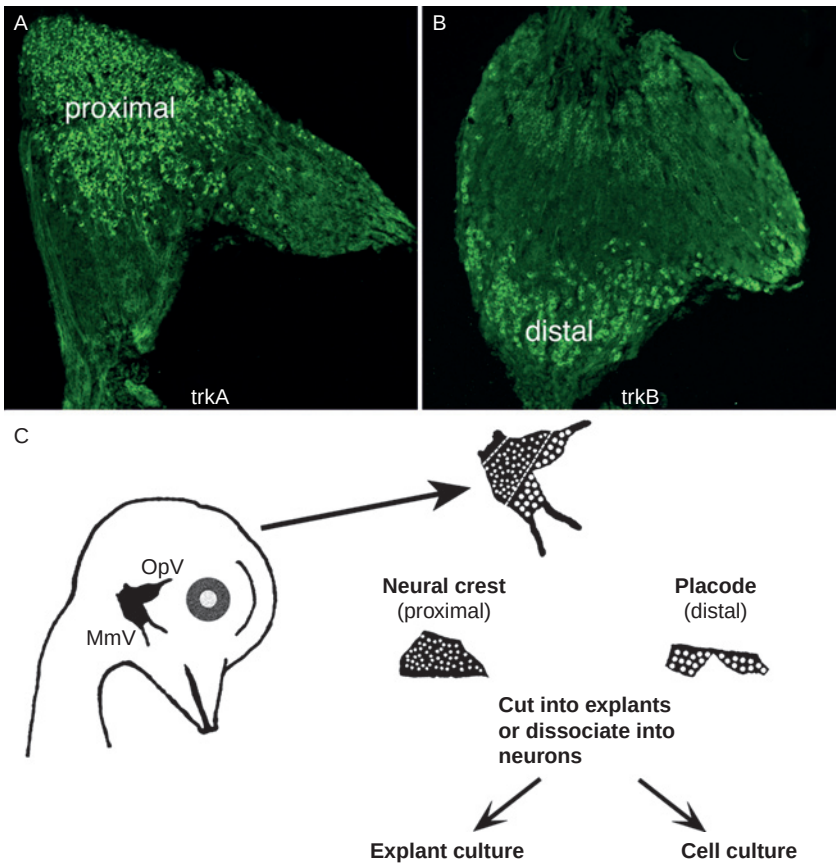

Fig. 1 (A) Neural crest-derived trigeminal neurons are located in the proximal region of the ganglion and stain positive for trkA, whereas (B) placode-derived trigeminal neurons are located in the distal region of the ganglion and stain positive for trkB. (C) Location and procedure for dissecting the trigeminal ganglion. Neural crest- and placode-derived neurons can be initially separated by physically dissecting the ganglion into proximal and distal portions, which may either be cut into neuron explants or dissociated into cells. (See Plate no. 4 in the Color Plate Section.)

neurons that are trkA-positive (Fig. 1A) and the distal part mostly placode-derived neurons that are trkB-positive (Fig. 1B; Davies, 1997; Lwigale *et al.*, 2004; Rifkin *et al.*, 2000). The proximal and distal portions of the ganglion are separated and cut into smaller explants of desired size. Neuronal explants are transferred into cold DMEM and kept on ice until ready for culture experiments.

D. Dissociation of Trigeminal Ganglia

Trigeminal neurons can be isolated and studied separately as neural crest- or placode-derived neurons, or cultured together then later identified using cell markers as explained earlier. Ganglion tissue is cut into smaller pieces (to speed up the enzyme digestion process) then transferred into a 15 ml screw cap tube containing 5 ml of 0.05% trypsin. Minced ganglion pieces are digested in a 37 °C water bath for 10–20 min and are then centrifuged briefly to pellet the cells. The pellet is gently triturated with a flamed Pasteur pipette (also a 200 μl pipette tip can do if cells are resuspended in media containing serum) in 1 ml DMEM, and is then washed twice by centrifugation at 2000 rpm for 5 min, each time removing the supernatant and adding 1 ml of fresh media. When dealing with a large amount of tissue, the triturated cell mixture is diluted tenfold and washed in 10 ml of media to ensure complete removal of the enzyme. After the residual enzyme has been removed, the pellet is resuspended at desired concentration in media supplemented with 2 ng/ml of NGF (neural crest-derived neurons), BDNF (placode-derived neurons), or NGF + BDNF for a mixed population of neurons.

E. *In Vitro* Culture of Ganglion Explants and Dissociated Neurons

1. Ganglion Explants

Neuronal explants are transferred into collagen solution on ice and then plated in tissue culture dish or chamber slide. Media supplemented with neurotrophin(s) is added until the collagen gels containing the explants are completely submerged. Neural crest-derived neurons grow best when the media is supplemented with NGF, whereas placode-derived neurons grow best in media supplemented with BDNF (Barde *et al.*, 1980; Davies, 1997; Enokido *et al.*, 1999; Lindsay and Rohrer, 1985; Lindsay *et al.*, 1985). Explants are cultured in a CO_2 tissue culture incubator at 37 °C. Trigeminal neuron explants can be cocultured with other tissues, cells, or media containing test additives with desired biological activity (Lwigale and Bronner-Fraser, 2007; Rochlin and Farbman, 1998).

Axon outgrowth from the explants may be visible as early as 6 h of culture, but maximum outgrowth is usually seen after 48 h. Controls should be added to each experiment to be sure of the normal extent of axon outgrowth.

2. Dissociated Neurons

To promote cell adhesion, culture dishes/slides should be pretreated with poly-D-lysine and then coated with fibronectin or laminin (Nishi, 1996). Neurons are resuspended in DMEM supplemented with neurotrophins, then cultured at desired concentration in a CO_2 tissue culture incubator at 37 °C. Similar to neuronal explants, dissociated neurons may also be cocultured with other tissues, cells, and media containing test additives.

VI. Pectoral Muscle Culture

A. Dissection and Dissociation of Pectoral Muscle Tissue

Pectoral muscle cultures are fairly easy to obtain owing to the ease of dissection and the availability of large pieces of tissues. Muscle cultures can be used to study the differentiation of striated muscles from myoblasts as well as used in muscle/neuron cocultures to study nerve-muscle development such as neuromuscular junctions and synapse formation. Here, we provide a general and simple protocol for generating muscle cultures from avian embryos modified from that described by Link and Nishi (1997).

E11 pectoral muscles are dissected in Ringer's solution and muscle stroma removed from cartilage. Remaining muscle tissues are then cut in small pieces and dissociated into single cell suspension by triturating with a fire-polished Pasteur pipette. Cell suspension is then passed through a 40 μm cell strainer (Falcon) to remove cell clumps and any large chunks of tissues that may be remaining. Pellet cells by centrifuging at 1000 × *g* for 15 min and resuspend in DMEM + 10% FCS.

B. Cell Culture

Plate cells in a 100 mm tissue dish and incubate at 37 °C for 30 min; fibroblasts will attach to uncoated dishes readily while muscle precursors preferred a substrate such as collagen. Collect nonadherent cells from the uncoated dish and seed them onto tissue cultures dishes treated with rat tail collagen I. Myoblasts should be exposed briefly to serum to boost cell proliferation, either DMEM + 10% FCS or 10% horse serum is a suitable medium. Myotubes will form in serum-containing medium after several days *in vitro*; however, fibroblasts have usually overgrown by then. Therefore, muscle cultures are typically seeded in DMEM + 10% FCS or 10% horse serum for a day and then switched to a serum-free, defined medium containing insulin to promote differentiation while minimizing fibroblast proliferation (Link and Nishi, 1997).

VII. Results and Discussions

A. Neural Crest Culture

Neural crest cells can be enriched to 95–99% purity using the immunoselection and magnetic separation method outlined above. Cells remain healthy and viable after the purification (Fig. 2) and can be used for further *in vitro* or *in vivo* studies. Similarly, this protocol can be adapted to select or enrich other cell types if a cell surface marker is available. In addition, fluorescent activated cell sorting (FACS) can be used to select HNK-1+ or other cells labeled by fluorescent markers. In case of HNK-1, an anti-mouse IgM secondary antibody conjugated to a fluorescent dye (e.g., Alexa 488-conjugated or Alexa 594-conjugated IgM) will be used in place of the IgM beads. Alternatively, the HNK-1 antibody can be directly conjugated to fluorophores such as Alexa dyes instead of using a two antibodies, indirect method. Either way, HNK-1+ cells that are bound to the fluorescent IgM antibody can be interrogated in a cell sorter and positively selected. A major advantage of FACS over magnetic separation is that live cells can be isolated by one or multiple fluorescent marker(s), regardless of their cellular localization, that is, one is no longer restricted to the availability of cell surface markers. An increasingly common and powerful use of FACS is to isolate a specific cell type when an enhancer driving green fluorescent protein (GFP) or red fluorescent protein (RFP) is used to visualize labeled cells of interest. In mouse embryos, neural crest stem cells and Schwann cells were isolated by using mice expressing GFP under the control of the proteolipid protein promoter, and then GFP+ cells were purified by FACS (Buchstaller *et al.*, 2004). In avian embryos, several studies have demonstrated

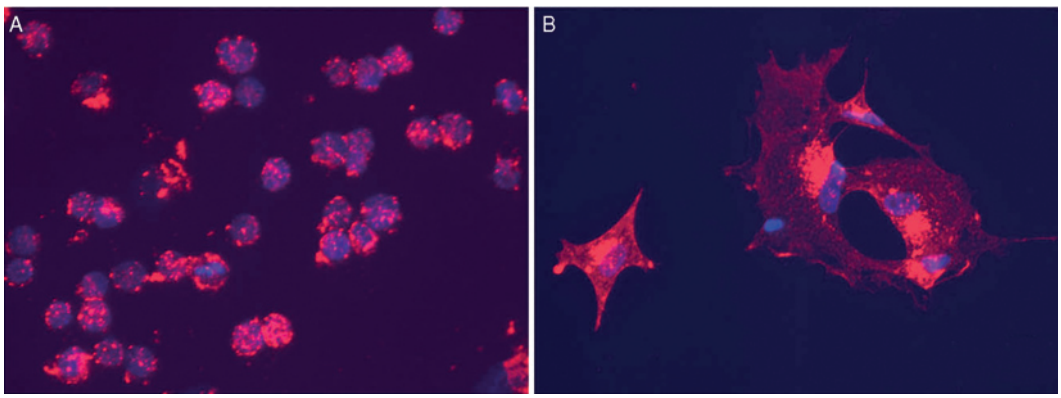

Fig. 2 Neural crest purified using the HNK-1 antibody. (A) Freshly purified neural crest cells (red), nuclei are counterstained with DAPI (blue), virtually all the cells are HNK-1+ in this view. (B) Purified neural crest after 9 h in culture. Neural crest cells were plated onto fibronectin-coated slides after purification and allowed to attach. They appeared healthy and have cell morphology typical of neural crest cells in culture. (See Plate no. 5 in the Color Plate Section.)

the identification and visualization of enhancer using GFP and RFP constructs and electroporations (Uchikawa *et al*., 2003, 2004; and Chapter 17 by Kondoh and Uchikawa, this volume). In addition, overexpression of transgenes by bicistronic vectors containing GFP and RFP and gene knockdown using fluorescein-tagged morpholino or RNAi vectors containing GFP or RFP have become common techniques in avian embryology (Basch *et al*., 2006; Chen *et al*., 2004a,b; Das *et al*., 2006; Kos *et al*., 2001, 2003; Krull, 2004; Megason and McMahon, 2002; Swartz *et al*., 2001; and Chapter 5 by Krull and Tosney and Chapter 12 by Sauka-Spengler and Barembaum, this volume). These can be combined with FACS to separate FITC/GFP/RFP+ cells in experimental versus control embryos. In these types of experiments, GFP+ or RFP+ cells can be isolated for further analyses such as gene expression profiling, examining the effects of gain- and loss-of-functions of different genes, and testing differentiation potential in specific cell populations.

B. Trigeminal Explant and Neuron Culture

Trigeminal sensory neurons are derived from neural crest and placodal cells (D'Amico-Martel and Noden, 1980; Lwigale, 2001; Noden, 1975). These two distinct cell types are distinguished early during gangliogenesis by the expression of markers such as HNK-1 and Sox10 (neural crest) or Pax3 and Islet1 (placodes). Other markers of trigeminal neurons and references of how they were used are listed in Table II. As the ganglion matures, neural crest-derived neurons aggregate in the region proximal to the central nervous system, whereas placode-derived neurons aggregate in the distal region. Despite their locations in the ganglion, the neurons cannot be distinguished morphologically in the mature ganglion. Fortunately, these neurons can now be distinguished using markers such as trkA, which labels only neural crest-derived neurons in the proximal region, and trkB which labels placode-derived neurons in the distal region (Fig. 1A and B). Knowledge of the location of each neuronal cell type in the ganglion combined with neuron-type specific markers can therefore be utilized to isolate and culture (Fig. 1C) trigeminal neurons. Using the techniques highlighted in this part of the chapter, the behavior of neural crest- or placode-derived neurons can be studied beginning with relatively pure cell populations.

After isolation from the trigeminal ganglion, neuron explants or dissociated neurons may be cultured separately, with other cells (Fig. 3) or tissues (Lwigale and Bronner-Fraser, 2007; Rochlin and Farbman, 1998). Trigeminal neuron explants (from either the proximal or distal parts of the ganglion) show normal radial outgrowth of axons when cocultured with GFP-transfected HEK293T cells (Fig. 3A), but are repelled by cells transfected with Semaphorin3A (Fig. 3B). Dissociated neurons are cultured at various concentrations depending on the type of experiment. If one wishes to study the behavior of numerous neurons, they can be plated at 1000 cells per 35 mm dish and they would appear as

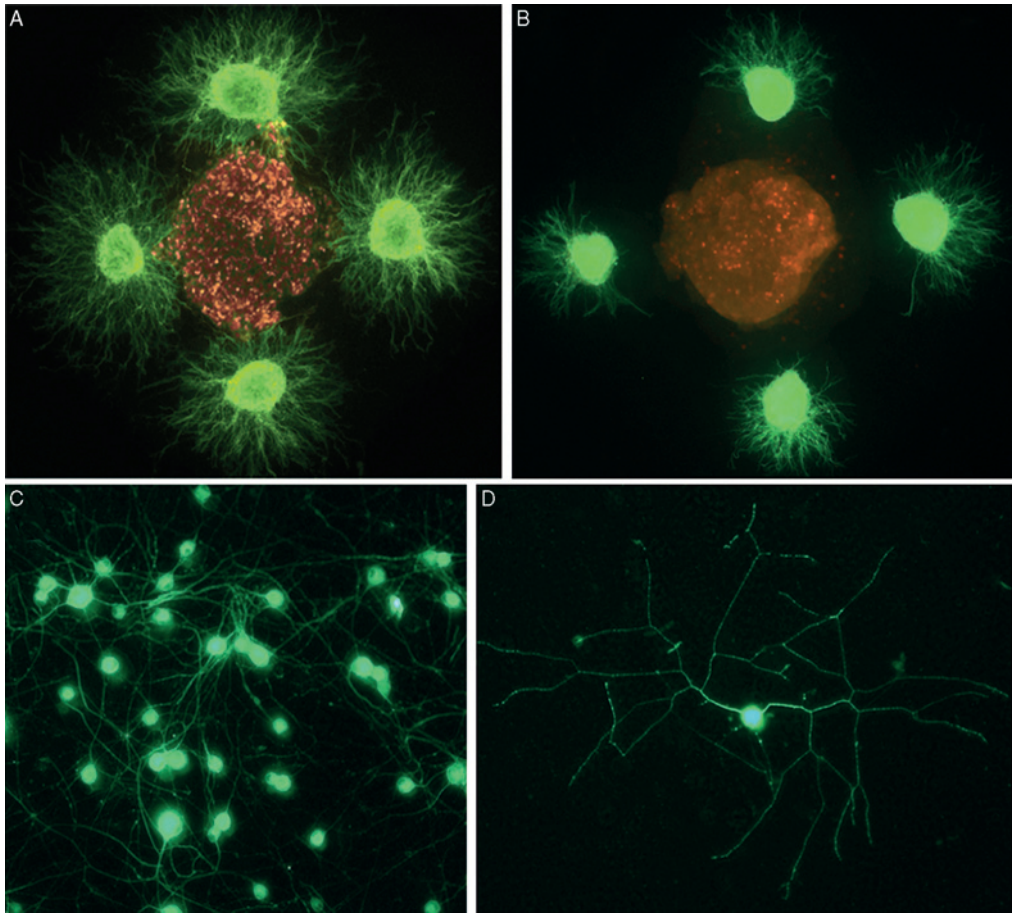

Fig. 3 Coculture of trigeminal neuron explants (A) with GFP-transfected HEK cells showing radial outgrowth of axons from all quadrants of the explants and (B) with Hsema3A transfected HEK cells showing repulsion of axons in the proximal but not in the distal quadrants of the explants. Dissociated trigeminal neurons cultured at (C) high concentration and (D) low concentration after immunostaining with Tuj1 antibody. (See Plate no. 6 in the Color Plate Section.)

numerous cell bodies with an array of axons after immunostaining with a neuron antibody such as TuJ1 (Fig. 3C). If the experiment requires studying individual neurons, cells are further diluted 10- to 20-fold to obtain 50–100 cells per 35 mm dish, which normally yields isolated neurons (Fig. 3D) whose morphology can be easily visualized and analyzed.

In addition to using cell markers (Table II), the quail–chick chimera technique described in Chapter 2 by Le Douarin *et al.*, in this volume can be used to identify and track the developmental origin of trigeminal neurons. Neural crest- and

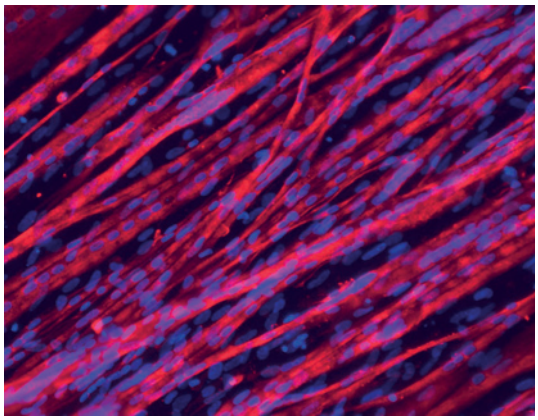

Fig. 4 E11 pectoral muscle cells in culture. Pectoral muscle cultures were maintained for 3 days in culture and immunostained with an antibody to the myosin heavy chain (red, MF20). Multinuclei (blue, DAPI) myotubes could be seen throughout the culture. (See Plate no. 7 in the Color Plate Section.)

placode-derived trigeminal neurons can be tracked with the QCPN antibody after grafting quail midbrain dorsal neural tube or adjacent presumptive placode ectoderm, respectively, into a stage-matched chick host (D'Amico-Martel and Noden, 1983; Lwigale, 2001). Therefore, trigeminal ganglia from quail–chick chimeras can be adapted to the culture methods described here to complement the cell markers by providing additional information about the identity of the neurons.

C. Pectoral Muscle Culture

Muscle cells and myotubes can be observed as early as 2 days after cultures but they tend to be small and do not contain many nuclei. After 3–4 days in culture, numerous long, multinucleated myotubes can be obtained. These myotubes express muscle markers such as myosin heavy chain revealed by MF20 immunostaining (Fig. 4). Pectoral cultures can be seeded either on plastic or on glass slides; however, they are much healthier and robust on plastic—the myotubes formed are larger on plastic compared with those on glass. In addition, if the presence of fibroblasts is not a concern, then preplating and collagen coating are not strictly necessary. We found that myoblasts attached, survived, and formed myotubes (although they tend to be smaller) when cultured in uncoated plastic tissue culture dishes.

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CHAPTER 7

Methods in Avian Embryology Experimental and Molecular Manipulation of the Embryonic Chick Limb

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I. Introduction

The developing limb appendage, from a vertebrate or an invertebrate animal, is a beautiful paradigm to study many questions in developmental biology such as pattern formation, skeletal development, cell fate, morphogenesis, signaling, gene regulation, and cell and molecular interactions. Limb development can be perturbed without affecting the development of other tissues or the animal itself and hence provides a simple system to test the function of genes that may be required more generally during embryonic development. Much of our understanding of vertebrate limb development has come from studies utilizing the chick embryo due to the accessibility of the developing limb, its relative large size, and the ease of experimental and molecular manipulation.

Focusing on the developing chick limb, classical experimental embryology studies performed over the past 60 years have revealed the critical organizing centers that govern the three axes of the limb: proximal–distal (Pr–D; shoulder to tips of digits), anterior–posterior (A–P; thumb to little finger), and dorsal–ventral (D–V; knuckle to palm). The experiments that lead to these exciting discoveries are outlined below as they still are fundamental to the experiments that are performed today to understand the molecular basis of limb development.

Many of the experimental techniques and theoretical models that have driven an exploration of limb development and placed the chick limb as a unique experimental system can be attributed to a long line of creative experimental embryologists including John Saunders, Lewis Wolpert, Cheryll Tickle, Madeline Kieny, and John Fallon. Their work and the work of many scientists since the explosion of the molecular era have produced a level of understanding of limb development that is on one ''hand'' tremendously complete for such a complex structure, and on the other hand still lacking in mechanistic insight. For instance, although we now know the patterning signals that instruct the three axes of the limb, we do not know how these patterning mechanisms are translated into the formation of skeletal elements in the right place with the right shape and size. Even less is known about the formation of other limb mesenchyme-derived tissues (i.e., tendon, connective tissue) or ectoderm structures like the nails and foot pads.

It is hoped that the experimental strategies outlined below will help foster continued understanding and trigger innovative experimental methods that will open new avenues of exploration. Moreover, our understanding is deep enough that it is now instructive and exciting to combine the advantages of different animal systems: the chick with its ease of experimental manipulation and ability to continue *in ovo* development after manipulation and the mouse with its tremendous genetic and molecular genetic resources and to bring this knowledge to bear to other non-model organisms that have dramatically diversified the limb structures such as the bat or marsupial or those animals that display a great potential for regeneration of the limb structures like the axolotl.

The later sections represent an interweaving of questions surrounding the cellular and molecular determinants that direct patterning of the limb along its three axes with the methodological methods that have been used to experimentally address these questions.

II. Visualization of the Limb Bud and Analysis of the Experimental Outcome

A. Visualization of the Limb Bud and Continued Development of the Embryo

The egg is "windowed" to allow access to the embryo and the extraembryonic membranes over the limb pulled away. The vitelline membrane is difficult to see but the Nile Blue stain used below will help to visualize it for removal with a forceps. The forelimb is usually chosen for manipulation as the hindlimb lies close to the allantois, which should not be punctured. The forelimb bud initiates at Hamburger and Hamilton stage 16 (Hamburger and Hamilton, 1992) and morphological form is fully apparent by approximately stage 27. To better visualize the limb bud during the early stages of its development and for the experimental manipulations below, Nile Blue sulfate dye (0.5% in water) is applied using either a glass needle whose balled tip has been coated with agar and impregnated with the dye or by using pipteman with a few microliters of dye to spread the dye around the limb bud. Nile Blue staining is also a good method for revealing the distribution of apoptotic cells in the limb.

In all cases following experimental manipulation, the goal is to continue embryonic development until the time to analyze the experimental outcome. As the limb is not vital for embryonic survival, essentially all aspects of limb development can be manipulated, the "window" in the eggshell sealed with tape or parafilm (for the latter, heat a spatula to melt the parafilm to the shell) and the egg placed back in the incubator (without rocking) to continue development. The chick heals rapidly, even after relatively drastic manipulation of the limb, such that within a few hours the cut surfaces will have healed. Chick embryos are remarkably resistant to infection but the egg and instruments should be wiped with 70% ethanol and a few drops of penicillin–streptomycin solution (similar to that used for cell culture) added to the embryo after operation.

B. Analysis of the Experimental Outcome

Analyses are unlimited but the major tools consist of assessing gene/protein expression patterns or analysis of the skeletal elements.

1. RNA *In Situ* Hybridization and Immunohistochemistry

RNA *in situ* hybridization and immunohistochemistry in tissue sections or in whole mount can be performed using standard techniques not described here. For RNA *in situ* hybridization, the most important consideration occurs when the gene

of interest is expressed in the ectoderm and/or AER. This single-cell layer can be damaged by strong proteinase K treatment: a good rule of thumb is to treat for 5 min at room temperature with 1–5 μg/ml proteinase K or to perform a detergent-based permeabilization (for instance RIPA buffer).

2. Cartilage and Bone Staining

Analysis of the skeletal pattern is an easy way to begin to understand the effect of loss or manipulation of key genes that regulate limb development. For example, effects of microsurgery on the generation of the skeletal pattern of the limb can be demonstrated by fixing embryos 8–10 days after the start of egg incubation and staining for cartilage and bone using Alcian Blue and Alizarin Red dyes. One can begin to visualize the cartilage at 5 days of incubation with Alcian Blue but the staining will be weak and not observed distally. The embryonic limb skeleton can be visualized at early stages using molecular markers such as Sox9 or Collagen type IIa by whole-mount RNA *in situ* hybridization.

Cartilage staining

(a) Acid alcohol: 75% ethanol and 25% glacial acetic acid by volume.

(b) Alcian Blue or Alcian Green: dissolve 75 mg dye in 100 ml acid alcohol.

- Fix embryo in 4% paraformaldehyde, wash two times in water, then stain overnight in Alcian Blue dye.
- Rinse one time in acid alcohol for a few minutes and then place in fresh acid alcohol for 1–2 h. Pour off acid alcohol and place in 100% ethanol for two changes of 1 h each. Clear tissue by changing to a graded series of methyl salicylate (2:1 ethanol:methyl salicylate, 1:2 ethanol:methyl salicylate, 100% methyl salicylate).

Cartilage and Bone staining

Cartilage (Alcian Blue) and bone (Alizarin Red) staining of older chick embryos (>day 8)

- Fix embryo in 4% paraformaldehyde, wash two times in water, then stain in Alcian Blue for 24 h at room temperature (75 mg/100 ml acid alcohol).
- Wash two times in acid alcohol, then wash overnight in 100% ethanol.
- Place embryo in 0.5% KOH until it sinks. Change the solution and destain overnight in 0.5% KOH.
- Stain for 24 h in Alizarin Red (2 ml of 0.1% Alizarin Red solution in 8 ml 0.5% KOH).
- Destain in 0.5% KOH, changing several times.
- Clear embryo in glycerol (50% plus a drop of H_2O_2 to aid clearing, then 75% glycerol for 24 h each). Store in 80% glycerol.

III. Proximal–Distal Limb Development

The organizing center that is necessary to allow Pr–D outgrowth of the limb is called the apical ectodermal ridge (AER). In the 1940s, John Saunders demonstrated the importance of the AER in allowing the Pr–D pattern to be realized by removing the AER from the chick limb bud and studying the resulting skeletal pattern (Saunders, 1948). Removal at progressive stages of development results in progressively more distal limb truncation such that removal of the AER at an early stage results in a severely truncated limb; removal at later stages results in loss of only the most distal elements (Saunders, 1948; Summerbell, 1974).

A. Apical Ectodermal Ridge Removal

The AER rims the distal edge of the limb running from anterior to posterior and forming a border between the dorsal and ventral ectoderm. The AER is better visualized by staining the limb bud with Nile Blue. The following experiment describes AER removal but this technique will also be referred to for creating an AER "loop" to test ZPA activity (Section IV.A).

Starting from the anterior side, a tungsten or glass needle or sharp iridectomy blade (i.e., Moria microblades from Fine Science Tools) is used to cut through the dorsal ectoderm surface near the AER by pulling the needle up (dorsally) and making continued small upward cuts until the posterior side of the dorsal ectoderm is reached. Starting again at the anterior side, the needle is used to separate the AER from the mesenchyme, upward cuts again from anterior to posterior may help to separate the layers. When the ectoderm on the ventral surface is reached, the ectoderm on the anterior side is pierced and cut through with upward cuts until the AER is completely free, forming a large free loop. The anterior and posterior sides of the AER are clipped and the AER removed from the egg. It is fine if the AER tears during this process or is removed in pieces but care must be taken to ensure that all fragments are removed from the tip of the limb bud.

B. Understanding the Molecular Signals, Part One: Bead Preparation and Implantation

The AER removal experiments provided the perfect assay system to then test the relevance of genes that encode signaling molecules that are normally expressed in the AER. This lead to the realization that fibroblast growth factors are the key molecular signals that mediate the activities of the AER (Fallon *et al.*, 1994; Niswander *et al.*, 1993). These studies were done by removal of the AER and application of recombinant FGF protein supplied by a bead implanted onto the limb as outlined below. Mouse genetic studies have borne out the validity of the conclusion that FGF signaling is important in limb development as loss of two *Fgf*

genes, *Fgf8* and *Fgf4*, results in the formation of only a tiny limb bud that is lacking skeletal elements (Boulet *et al*., 2004; Sun *et al*., 2002).

The bead technique has now been used to apply secreted proteins or drugs to cells within many tissues of the embryo. Bead size should be selected based on the tissue of interest. The properties of the protein/drug and the bead should be considered. The heparin acrylic and Affi-Gel Blue beads have been shown to be suitable for supplying FGF, BMP, Noggin, and SHH. It is likely that other proteins will work equally well. The AG-1X2 beads are suitable for retinoic acid (RA) experiments and have been used for pharmacological inhibition of signaling pathways. It is important to realize that bead experiments are appropriate for secreted proteins but are not suitable for proteins that act within the cell or on its cell surface. In the latter cases, other approaches such as retroviral transfection or *in ovo* electroporation are required (see Section IV.B).

Heparin Acrylic beads: Sigma H-5263 (large range in bead size).

Affi-Gel Blue beads: Bio-Rad 153–7301 (75–100 μm diameter); Bio-Rad 153–7302 (100–200 μm diameter).

- Select appropriate size beads using forceps to pick up and collect in a 100 μl drop of PBS in 35 mm petri dish.
- Prepare another 35 mm petri dish with many small (~15 eight microliter) drops of PBS around the dish, excluding the center (this is for humidification). In the center, place a 1–3 μl drop of protein diluted as directed by manufacturer.
- Pick up bead and transfer to the protein drop. It is important to not dilute the protein. To remove PBS carried along with the bead in the forceps, work back and forth between a dry area on plate and a humidifying drop until you can pick up the bead dry. For example, drop the bead onto the dry portion of plate, hold forceps together close to bead and use capillary action to pull PBS up into forceps, discard PBS, continue until bead is dry, pick up bead on one tip of forcep, and place in protein drop.
- Greater than twenty 100 μm beads can be placed in a 1 μl drop of protein. Soak the beads in protein for 1 h at room temp or overnight at 4 °C. Rinse beads in PBS just prior to implantation, etc.
- Bead implantation depends on the experiment desired. Following AER removal, the bead can be "stapled" at the edge of the exposed mesenchyme using very fine (0.025 mm) platinum wire (from Goodfellow Scientific) bent to form a staple. A bead can be placed into the mesenchyme by cutting a slit in the tissue. However, when the tissue heals it tends to force the bead out so a staple may help to keep it in place. Affigel Blue beads tend to be stickier than heparin beads so they stick in tissue better. To help prevent the heparin beads from slipping out, chip off a small piece at the edge of the bead and then soak in protein. The chipped region tends to hold well in the tissue. The studies which showed that RA can repolarize the A–P axis of the limb used a loop of the AER to hold the bead in place between the AER and mesenchyme (see Section IV.A).

C. RA or FGF/MEK Inhibitor Soaked Beads

Ion exchange beads (for RA or SU5402 or Mek inhibitor) Bio-Rad AG1-X2: 140–1231 (50–100 mesh). These beads come in chloride form and to use them for RA they must be formate derivatized.

RA is dissolved in DMSO. RA is light sensitive and it will diffuse out of the beads so prepare and use when needed.

Place a 100 μl drop of diluted RA on a piece of parafilm.

Pick up formate derivatized AG1-X2 bead with forceps and place at the bottom of the RA drop (the beads will swell to approximately two times the dry size so select smaller beads than will be required).

Soak in RA for 20 min.

Prepare a strip of parafilm with two rows of 100 μl drops of media containing phenol red (equal number of drops relative to number of beads). Serum is not needed and any type of tissue culture medium is fine.

Place each bead in first row of drops (the beads will take up the phenol red dye making them easier to visualize).

Move each bead to the second row of drops.

Beads are now ready for use.

To formate derivatize the AG1-X2 ion exchange beads:

- Place beads in an eppendorf tube and add 0.5–1 M formic acid.
- Pipet off the formic acid and wash beads with dH_2O until pH $\sim$5 (test the withdrawn water with pH paper).
- Pipet beads into a 100 mm petri dish.
- Dry at room temperature or in 37 °C incubator overnight with lid slightly ajar.
- Transfer to an eppendorf tube and seal with parafilm. Store at room temperature.

D. Recombinant Limb Bud

The studies above demonstrated that FGF is the signal from the AER that is needed for limb outgrowth and for the Pr–D pattern to be revealed. Another question that was first addressed by experimental manipulation is whether the AER acts in an instructive manner to dictate the pattern of the Pr–D elements that derive from the mesenchyme or whether the AER acts as a permissive signal. The experiment to test this idea used a recombinant limb bud technique to place the ectoderm (and AER) from a developmentally older limb onto a young limb bud mesenchyme or, conversely, a young ectoderm on an old mesenchyme bud. The outcome of this experiment showed that the AER provides a permissive, not an instructive, signal as the limb skeleton conformed to the age of the mesenchyme, not the AER (Rubin and Saunders, 1972).

The experiments outlined in Sections (III.D.1) and (III.D.2) require some practice to perfect but the results have been very informative. For instance, experiments

in Section III.D.1 have addressed the influence of the age of the ectoderm on Pr–D patterning and the influence of the ectoderm orientation relative to the mesenchyme with respect to D–V patterning (MacCabe *et al.*, 1974; Rubin and Saunders, 1972). Another possibility is use chick experimental embryology to test the functions of tissues from other animals, for instance using an ectodermal jacket from a mouse limb mutant (Kuhlman and Niswander, 1997) or testing ZPA potential (see Section IV.A) from tissues of other creatures (Cameron and Fallon, 1977). Experiments in Section III.D.2 have tested the ability of cells from different positions of the limb to form specific skeletal elements or to recognize one another as described under Pr–D and A–P patterning (Dudley *et al.*, 2002; Omi *et al.*, 2002).

1. Testing the Interactions Between the Limb Ectoderm and the Mesenchyme

Enzymatic separation of limb tissues and replacement of the ectodermal cap onto a bud of denuded limb mesenchyme.

In this experiment, an intact ectodermal jacket from one limb bud is used to cover a denuded limb mesenchyme bud. It is best to match the stage/size of the limbs used for the ectodermal jacket and mesenchymal core: stage 21/22 is optimal to practice with. The limbs can be cut from embryos taken out of the egg or the limb can be removed *in ovo*, if a comparison with the original donor is desired.

1. Cut off the limb bud and rinse in two or more changes of calcium- and magnesium-free (CMF) PBS.
2. To prepare a mesodermal core.

The mesodermal core remaining after preparation of the ectodermal jacket below can be used; however, the trypsin treatment leaves the mesoderm a bit soft. To prepare a firm mesodermal core to receive a covering of ectoderm, incubate an isolated, CMF-treated limb bud in 1% EDTA (ethylene diamine tetracetate) at 37 °C for about 30 min. The ectoderm can then be removed, often in patches, by means of fine needles. The mesoderm core is then placed in a Sylgard (Dow Corning) coated dish in 10% fetal bovine serum (FBS) in Ringer's (or PBS) and kept on ice or on a cold stage (an inexpensive cold stage can be created from a metal block placed at −20 °C until use; a few blocks left at −20 °C are useful so that the stage can be exchanged if it starts to warm up).

(c) To prepare an ectodermal jacket, use one of the two trypsin treatments:

(i) Place the limb bud in a solution of 2–2.5% trypsin at 4 °C for about 30 min to several hours depending on the age of the limb. When the ectoderm begins to curl up near the cut edges, this indicates that the ectodermal layer is loosening from the mesoderm. Test that the ectoderm is pulling away from the mesoderm. Transfer the limb to the same dish as the isolated mesodermal core in Ringer's:FBS. By means of fine forceps, gently slide the ectoderm from its mesodermal core. Note the A–P and D–V orientation of the

ectoderm if important for the experiment. This is fairly obvious from the limb shape. Keep the media cold because if it is allowed to warm, the ectoderm will shrink into a configuration useless for further manipulation.

(ii) After treatment with CMF, incubate the isolated limb at 37 °C in a solution of 2% trypsin plus 1% pancreatin for less than 30 min. This should suffice to loosen the ectoderm. Transfer the limb to the dish with the mesodermal core and remove the ectoderm. Keep the ectoderm cap cold.

(d) Using a cold stage to keep the tissues cold, slide/coax the ectoderm over the mesoderm core using tunsten needles and/or forceps. Place 1 or 2 pins (insect pins [Fine Science Tools] or 0.025 mm platinum wire [Goodfellow Scientific]) through the ectoderm/mesoderm and into Sylgard dish to hold the ectoderm on the bud. Carefully transfer the dish to the bench at room temperature for approximately 20 min. Then place the dish in a 37 °C incubator. As the limb warms, the ectoderm shrinks tightly around the mesoderm core. Now the recombinant limb is ready for grafting to a host embryo (~stage 22; see Section III.E) for continued development. Ensure that the mesenchymal base (the proximal surface) is not covered by ectoderm as the ectoderm will prevent revascularization. If necessary, cut the base to expose the mesenchyme before grafting.

With the recombinant limb bud technique, one can also consider experiments that combine the advantages of chick experimental embryology and mouse genetics. The ectoderm from a mouse limb can be used in place of the chick ectoderm (Kuhlman and Niswander, 1997). The rest of the protocol remains the same. However, in the mouse/chick experiments, the following considerations must be kept in mind. First, the limb bud of the mouse is smaller than the chick and therefore it may be necessary to remove part of the chick limb mesenchyme: the anterior portion can be removed and this will still allow for normal wing formation. Another possibility is to use a quail limb bud mesenchyme (graft transplanted to either chick or quail host). This experiment also highlights the fact that the mesenchyme holds the intrinsic information as to limb type [forelimb vs hindlimb (Zwilling, 1955); chick wing vs mouse arm] and Pr–D pattern. Second, unfortunately, the converse experiment (mouse mesenchyme with chick ectoderm) does not work effectively. If the recombinant limb is grafted to the chick host, the mouse tissue does not become vascularized and hence becomes necrotic.

2. Testing the Behavior of Dissociated Limb Mesenchyme, Part One

a. Dissociation of the Limb Bud Mesenchyme and Placement into an Ectodermal Cap

This experiment, in which the mesenchymal cells are dissociated and reaggregated randomly before placing back into an ectodermal jacket, has been used to ask questions related to Pr–D and A–P patterning. For instance, recent studies related to the Early Specification model of Pr–D patterning outlined later, suggested that cells from the distal tip of an early limb bud form the most distal

skeletal elements (Dudley *et al.*, 2002). A–P differences have been demonstrated by marking the limb mesenchyme with colored dyes at different A–P positions, dissociating the mesenchyme into single cells, packing them back together, and inserting into an ectodermal cap. Following further development, this showed a differential sorting of anterior cells with anterior cells and posterior cells with posterior cells (Omi *et al.*, 2002).

Mesodermal cores isolated by either of the trypsin methods above can be processed into single-cell suspensions. After removal of the ectoderm cap, continue to incubate the mesoderm in the trypsin solution for another approximately 20 min. Transfer the mesoderm to Tyrode's:FBS solution and make a single-cell suspension by pipeting the mesoderm up and down repeatedly through small-bore pipettes. Such suspensions can be pelleted centrifugally and stuffed into ectodermal jackets, and the recombinant limb grafted to a host embryo (MacCabe *et al.*, 1973).

E. Graft of the Donor Tissue to a Host to Allow Continued Development

Further development of a recombinant limb bud or a part of the limb bud requires that the tissue becomes revascularized. This can be done by grafting the bud to a host chick. The graft site can be many places: over the somites, removal of a limb and graft of tissue to the limb stump, into the coelom (Kardon, 1998), or onto the chorioallantoic membrane. The following describes grafting to the somites.

1. Graft to the Somites

One of the easiest places to graft a limb bud (or other tissue) is on the dorsal surface of the host embryo. Host embryos of stages 22–24 are optimal. The host membranes are removed over the forelimb and adjacent somites. Tissue will be removed over the somites (approximately four somites long) and extending over the forelimb if the graft tissue is large. A tungsten needle is used to "fillet" and remove this area and a small amount of underlying mesenchyme to create the wound bed in which there is some bleeding from the ruptured capillaries.

The donor limb bud is then transferred to the host and its mesodermal surface is apposed to the surface of the wound bed. Surface tension of the amniotic fluid is usually sufficient to hold the graft in place. However, a platinum wire pin (0.025 mm from Goodfellow Scientific) can be inserted through the graft into the host. Once the graft is in place, some antibiotic is added, the egg sealed and placed in the incubator.

F. Pr–D Information Within the Mesenchyme

The AER-derived FGFs are clearly sufficient and necessary for continued Pr–D development; however, it is less clear how they act in this process. There is little evidence that the AER plays a role in regulating cell proliferation. However, the

AER does promote mesenchyme cell survival and also appears to regulate the number of mesenchymal cells that initially contribute to the limb bud as well as ensuring that there are sufficient number of mesenchymal cells to undergo condensation to initiate cartilage formation (Barrow *et al*., 2003; Dudley *et al*., 2002; Sun *et al*., 2002).

Switching from considerations of the AER signal, the next question relates to when the mesenchyme becomes specified as to its Pr–D pattern. Although it is clear that the intrinsic information for Pr–D pattern resides in the mesenchyme, the nature of the Pr–D information still remains an enigma. Moreover, there is still considerable controversy as to when Pr–D information is specified and when the mesenchymal cells are committed.

There are two distinct models that have been proposed as to when Pr–D specification occurs—the Progress Zone model and the Early Specification model. The Progress Zone model posits that Pr–D fates are specified progressively as the limb bud grows (Summerbell *et al*., 1973). The Progress Zone is defined as the undifferentiated mesenchyme cells near to and under the influence of AER. As the limb grows and cells are pushed out of the Progress Zone, they become fixed as to the positional information they acquired while under the influence of the AER or, in other words, progressively specified as to their Pr–D fate. Cells that leave the Progress Zone early form more proximal structures, those that leave later form more distal structures. Although the original experiments and their interpretation can now be reevaluated on the basis of approximately 35 years worth of intervening work and molecular markers, the Progress Zone model still influences thinking to this day.

The Early Specification model of Pr–D limb specification was recently proposed. This model postulates that Pr–D fates are specified early and cells with different fates are localized in Pr–D ''layers'' in the early limb bud (Dudley *et al*., 2002). In this model, the AER serves to expand these populations that have already been specified as to their Pr–D fate. The experiments that lead to this idea in part consist of fate mapping experiments in which fluorescent dye was applied to cells at different distances from the AER along the Pr–D axis, and the limbs then analyzed later when the cartilage had formed. From this rather broad labeling, it was found that cells near the AER contributed to the autopod (hand), cells near the base of the limb to the stylopod (humerus) and cells in between to the zeugopod (radius/ulna). This fate map has been recently refined (Sato *et al*., 2007), indicating that the borders between the Pr–D elements is not discrete but that there is some overlap. However, in both cases, these fate maps inform us as to what the cells in particular positions can contribute to but does not tell us when they have acquired Pr–D information.

More recently, it has been argued that neither model fits the known molecular gene expression patterns and that perhaps neither model is correct (Tabin and Wolpert, 2007). However, in light of the recent studies of the behaviors of cells from different Pr–D levels of the very early limb bud (using the low density micromass assay described in Section III.H), it appears clear that proximal and

distal cells display differential properties and can distinguish amongst themselves (Barna and Niswander, 2007). Hence, this suggests that Pr–D fates are specified at the onset of limb development, although yet there are no discrete molecular markers of this specification.

G. Fate Mapping

Fate mapping experiments have played an important part in revealing the potential of cells to contribute to specific embryonic tissues. This has been useful in many organisms and tissues, the chick limb included. Although it is a powerful technique, it must be remembered that this provides information as to what structures the cells can contribute to but it cannot be used to infer when those cells are specified as to their fate nor their commitment. In general, the chick limb fate mapping experiments have been done using lipophilic fluorescent dyes [for instance, DiI (1,1-dioctadecyl-3,3,3,3′-tetramethyl indocarbocyanine perchlorate) and DiO; Molecular Probes] which label the membranes of a group of cells surrounding the injection site. Other approaches include transplantation of cells from quail or GFP labeled (transgenic or electroporated tissue) embryos to a host chick embryo. Quail cells are recognized by the QCPN monoclonal antibody (Developmental Studies Hybridoma Bank).

H. Testing the Behavior of Disassociated Limb Mesenchyme, Part Two

1. Low Density Micromass Cultures

Micromass experiments have been performed for more than 30 years as a model system for cartilage formation. In the traditional micromass cultures, the limb bud mesenchyme is dissociated into single cells and then plated at high density and cultured over a period of days (Daniels *et al*., 1996). Within 3 days, cartilage nodules are formed which express early cartilage markers. These nodules are surrounded by fibroblastic cells many of which have differentiated into muscle.

Recently, the micromass assay has been modified to use a low-density culture which allows the analysis of individual cells (Barna and Niswander, 2007). This has revealed a wide variety of cell behaviors that mimic those in the developing limb. Moreover, it provides a simple means to manipulate gene function to begin to determine the cell biological processes regulated by these genes, which have been difficult to define during *in ovo* limb development.

The modified micromass technique can be used to address a number of questions relating to limb development. This has revealed the sorting of cells from different Pr–D positions at earliest stages indicating that proximal and distal cells have intrinsic differences at the onset of limb development (Barna and Niswander, 2007) in support of the Early Specification model. Also the molecular steps involved in early cartilage formation can be studied to provide a better understanding of the cell behaviors regulated by, for instance, genes implicated in achondrodysplasias as

demonstrated by manipulation of Bmp signaling or Sox9 function (Barna and Niswander, 2007). It may also prove useful in understanding how patterning information is ultimately translated into the cell behaviors that regulate skeletal formation, for instance by using limbs from embryos with defects in A–P patterning (polydactyly or syndactyly) or Pr–D patterning, as well as to consider experiments relating to D–V patterning.

Chick (or mouse) limb buds are removed from embryos and transferred to calcium–magnesium free Hank's balanced salt solution containing 0.2% trypsin–0.1% collagenase and incubated at 37 °C for 30 min. A Pasteur pipette is used to disperse cell aggregates into approximately single-cell suspension. To ensure a single-cell suspension, the cells can be filtered through a 40 mm nylon cell strainer (BD Falcon). Cells are placed in D-MEM/F-12 media with 10% FBS, pelleted, washed, and resuspended in approximately 100 µl DMEM + FBS. Cells are plated at a concentration of 2.5×10^5 cells in a 96-well glass flat-bottomed plate (EM Sciences). Cells are allowed to attach to the plate for at least 30 min. The cells can then be imaged in time-lapse to follow individual cell behaviors.

IV. Anterior–Posterior Patterning

Turning our attention to the A–P or thumb-little finger axis, classical experimental embryology studies revealed the organizer of A–P patterning of the limb. The history behind the original experiments is of interest. It had been noted by John Saunders that there is a population of cells in the posterior of the limb bud that undergoes programmed cell death (there is also extensive apoptosis in the AER as well as in the "opaque zone" which is thought to correspond to the region that will form the space between the zeugopod elements) (Saunders *et al.*, 1962). To determine whether the posterior mesenchyme cells could be prevented from dying, Saunders transplanted them to other areas of the embryo. When the tissue was transplanted to the anterior side of the limb bud, the result was surprising and it opened a completely different field of study. It was found that the posterior mesenchyme could change the polarity of the limb, creating skeletal duplications in a mirror-image symmetry to the normal elements (Saunders and Gasseling, 1968). When posterior mesenchyme cells were implanted under the AER on the anterior side of the limb, this generated the digit pattern 4*, 3*, 2*, 2, 3, 4 from A–P: with the asterisk (*) indicating ectopic digits and 2 and 4 representing the normally anterior- and posterior-most digits, respectively [the chick wing digits have traditionally been classified as digits 2–4 but see Vargas and Fallon (2005) for arguments for classification as digits 1–3]. The posterior tissue that has this repolarizing activity has been named the zone of polarizing activity or ZPA.

It is now realized from the work of Cliff Tabin's lab that the activity of the ZPA resides in the expression of the *Sonic Hedgehog* gene (Riddle *et al.*, 1993). They showed this by transfecting cells with the *Shh* gene (the cells used for transfection do not appear to be critical [chick or mouse embryonic fibroblasts, Cos, etc.])

to produce SHH protein and then grafting a pellet of cells under the AER as done for the ZPA transplants. Alternative methods are to implant a bead soaked in recombinant SHH protein or infection of the limb mesenchyme with a retroviral vector expressing *Shh*.

A. Test of ZPA Activity

The mesenchymal tissue defined as having ZPA activity is located in the posterior and distal part of the limb bud (approximately stages 17–28) (MacCabe *et al*., 1973). The donor embryo can be removed from the egg to facilitate graft removal. The posterior mesenchyme is cut using a tungsten needle. The ectoderm can be left in place or removed physically or with light trypsin treatment.

ZPA activity is best tested by placing grafts or beads soaked in (or cells expressing) the protein of interest under a loosened loop of AER, as interactions between the limb mesenchyme and AER are important. The technique used to make an AER loop is very similar to that described in the section on AER removal. Here, the AER from an approximately stage 20 embryo is separated from the mesenchyme from the anterior side to a bit past midway to the posterior side and it is not clipped off (if the AER breaks, discard the embryo and start again). This results in a free loop into which the graft/bead is gently inserted by means of a fine needle or forceps. The loop will shrink back and serve to hold the graft. It is best if there is very little mesoderm attached to the AER to intervene between the graft and AER. Antibiotic is added, the egg sealed and placed in incubator.

B. Understanding the Molecular Signals, Part Two

1. Cell Pellets

A gene of interest can be transfected into cells (Cos, embryonic fibroblasts, etc.) to provide a source of a secreted signalling molecule. Start with one 100 mm approximately confluent plate of transfected cells. Trypsinize the cells as usual, resuspend in 10 ml of complete media, spin down the cells (1000 RPM for 10 min), then aspirate the media leaving approximately 250 μl over the cells. Resuspend the cells by vigorously tapping the tube. Pipet a 35–50 μl drop onto a 35 mm petri (not tissue culture) dish. Flip the plate in one smooth movement to invert. Culture for at least 1 h in a 37 °C CO_2 incubator. Flip the plate back over and, using a tungsten blade, cut the cell pellet into small pieces for grafting under the AER loop.

2. Retroviral Vectors

Retroviral vectors can be used to misexpress wildtype, constitutive active or dominant-negative constructs. Replication-competent avian-specific retrovirus (RCAS) (Hughes *et al*., 1987) has been used successfully to express high levels of protein from genes encoding transcription factors, signaling molecules, receptors,

intracellular components, and histological markers in the chick embryo (Fekete and Cepko, 1993; Morgan and Fekete, 1996; Morgan *et al*., 1992; Riddle *et al*., 1993). The RCAS vectors are particularly advantageous for generating proteins that need to be produced in the cell of interest. *In ovo* electroporation (described in Section IV.B.3) is also useful for this purpose and has largely surplanted the use of the retrovirus in chick. However, it is difficult to generate fully transfected limb tissue by electroporation, whereas, the RCAS virus continues to replicate and infect the surrounding cells. The preparation of the retrovirus and methods to introduce it into the limb are described in the references earlier.

3. *In Ovo* Electroporation

In ovo electroporation uses short electrical pulses to transfect plasmid DNA into the tissue of interest. It can be used to misexpress a gene or genes or to generate loss of function phenotypes using dominant-negative DNA or short hairpin RNA constructs. To compare electroporation to retroviral infection: (1) it is easy to misexpress more than one gene by electroporation (the different plasmid DNAs will in general be co-introduced into the same cell), whereas superinfection of virally transfected cells is blocked unless a different subtype of virus is introduced; (2) the size of the DNA to be introduced is relatively unlimited by electroporation, whereas RCAS vectors can only accept approximately 2 kb of exogenous DNA; (3) strong expression is observed within 24 h postelectroporation and GFP can be detected 4–6 h postelectroporation, whereas the RCAS vectors usually show weak expression at 24 h which becomes stronger with time; (4) the electroporation construct remains episomal, which means that as cells divide its effect becomes diminished (this is usually not a problem unless high levels of gene expression are required many days after transfection), RCAS will continue to express the gene of interest; and (5) electroporation can produce a discreet region of transfected cells whereas, depending of the RCAS vector and chick host used, the viral infection will continue to spread. It must be borne in mind that electroporation will result in mosaic limbs as the transfection rate is not 100% of cells.

The limb ectoderm is relatively easy to fairly uniformly transfect by electroporation. This should be done prior to limb bud formation (approximately stages 14–17) by placing an electrode above and below the lateral plate at the forelimb level, applying the electroporation DNA construct over the ectoderm of the lateral plate, and providing three 50 msec square pulses, typically at 25 V to drive the DNA into the ectoderm. In general, mesenchyme is more difficult to electroporate. It is best to electroporate early (approximately stages 16–19) when the pool of limb mesenchyme cells is relatively limited to get the best tranfection efficiency. The DNA is microinjected into the mesoderm at a few places within the forelimb field. Fast green (2.5%) can be added to the DNA for visualization purposes. The DNA is usually 0.5–5 μg/μl but the concentration of DNA should be empirically tested to determine the most efficient transfection. In some cases, it may be desired to target a limited region of the limb and judicious placement of the electrodes and DNA

injection within the limb mesenchyme allows good control of the region of transfection. See Krull (2004) for preparation of DNA and basic electroporation technique.

V. Dorsal–Ventral (D–V) Patterning

The third axis of the limb is the dorsal–ventral axis. Classical embryological studies demonstrated the importance of the ectoderm in conferring D–V polarity to the mesenchyme. The original experiment essentially followed the methodology above on creating recombinant limbs with an ectodermal cap. However, in this case, the ectoderm was removed from the limb mesenchyme, rotated 180°, and then replaced onto the mesenchyme such that ventral ectoderm touches dorsal mesenchyme and vice versa. This results in a reversal in the polarity of the mesodermally derived structures indicating that D–V patterning information resides in ectoderm which then confers D–V polarity on the mesenchyme (Geduspan and MacCabe, 1987; MacCabe *et al*., 1974).

Now, it is clear what the key ectodermal signals are: *Wnt7a* in the dorsal ectoderm and *Engrailed-1* and *Bmp* in the ventral ectoderm. Within the mesenchyme, *Wnt7a* signaling regulates the expression of the transcription factor *Lmx1b* in the dorsal mesenchyme (Ahn *et al*., 2001; Chen and Johnson, 1999; Pizette *et al*., 2001). These molecular interactions have been shown by misexpression of these genes by retroviral transfection of the chick ectoderm and by mouse genetics. In terms of evaluating changes in D–V polarity, these genes themselves provide very good molecular markers of ectoderm and dorsal mesenchyme polarity. Ectodermally derived structures are easy to assess (hair, nails, foot pads, sweat glands) but D–V polarity of resulting mesenchymal structures is more subtle and is most reliably assessed by analysis of the tendons and sesmoid bones.

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CHAPTER 8

Cell Division, Differentiation, and Death in Avian Embryos

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I. Introduction

In this chapter, we will describe methods for studying cell death, proliferation, and differentiation in chicken embryos. The development of any organism is controlled precisely by a coordinated set of patterning, proliferation, survival, and differentiation signals. The study of these events has progressed from careful cytological studies to more sophisticated use of antibodies and enzymatic markers. Each technique has built on previous observations and improves specificity and sensitivity of analysis. The development of multiple antibodies that are specific for cell proliferation and cell death detection, combined with markers for progenitor

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and mature cell types, now allow for a great deal of information to be gleaned from developing chick embryos, both as whole mount and in sections.

II. Studies in Cell Death

Cell death is an important part of normal development, as demonstrated by abnormal phenotypes seen when cell death is misregulated (Ahlgren *et al.*, 2003; Garcia-Domingo *et al.*, 1999). Among other places, developmental cell death is needed for the normal development of the brain, for sculpting tissues such as the digits, as well as pruning the number of sensory neurons to match the field of innervation. Hamburger and Levi-Montalcini (1949) were the first to appreciate that cell death occurs during normal development when studying the chick dorsal root ganglia. Subsequent studies have demonstrated cell death as a component of normal development in every tissue.

The presence of normal cell death during development went unappreciated initially due to the rapid clearance of dying cells, as well as balancing proliferation that helps maintains tissue volume. The study of cell death in all contexts, but specifically during development, was greatly enhanced by the findings from the study of cell number in *Caenorhabditis elegans*, which demonstrate exquisite control of both proliferation and survival. Mutant in *C. elegans* that demonstrated excess and reduced cell survival were key in understanding the mechanism of programmed cell death. Vertebrate homologues of these genes have been identified and contribute to our understanding of cell survival and cell death mechanisms. While initial counts of total neurons were used to demonstrate that cell death occurred, this method is labor intensive and prone to errors in population definition and counting. Other studies have sought to mark dying cells in tissue sections to confirm that the loss of cell number was because of cell death (Hamburger and Levi-Montalcini, 1949; Oppenheim *et al.*, 1978). An understanding of the mechanism of programmed cell death has lead to better and better techniques to mark dying cells.

The term apoptosis first appeared in 1972, to delineate a distinctive pathway of cell death (Kerr *et al.*, 1972). The morphological features are cell shrinkage, cell surface blebbing, and cell disintegration. The nucleus undergoes condensation and breakdown. Changes in the cell surface molecules allow neighboring cells to recognize and phagocytose apoptotic cells. This carefully orchestrated progress allows for the death and removal of cells without releasing potentially immunogenic contents. Necrosis, on the other hand, is a pathological process that is marked by cell swelling and ultimately lysis. The condensation of the nuclear chromatin is preserved. Necrosis is a passive cell death process, brought on by noxious treatments. During necrosis, the cell membrane is disrupted, followed by influx of calcium ions and water, causing the cell to swell and ultimately to lyze. Although necrosis is not thought to play an important role during normal development, it may contribute to loss of cells when development is manipulated. A third mechanism termed necroptosis has been identified, (Degterev *et al.*, 2005;

Teng *et al.*, 2005) which resembles apoptosis but is resistant to classic antiapoptotic drugs. Currently, there are no specific embryological studies to look at the role of necroptosis during development.

A number of techniques are available to study cell death during development. TdT-mediated dUTP-biotin nick-end labeling (TUNEL) is often considered the most accurate indicator of apoptosis (Smith and Cartwright, 1997). While this is a very useful technique, it is important to recognize that there have been some reports suggesting that TUNEL-staining is not apoptosis-specific (Grasl-Kraupp *et al.*, 1995; Hughes, 2003). Another technique that is useful for whole-mount visualization of cell death is live staining with acridine orange or related dyes (Ahlgren and Bronner-Fraser, 1999). There are a number of companies making antibodies to activated caspase-3 that is highly useful in conjunction with other antibodies when examining sections. A number of dyes, including Propidium Iodide, 4′,6-diamidino-2-phenylindole (DAPI), Hoechst, and Sytox Green dye, all of which stain DNA, are useful to visualize pyknotic nuclei, especially in combination with antibody staining (Ahlgren and Bronner-Fraser, 1999).

III. Studies in Cell Proliferation and Differentiation

The total number of cells in a tissue or culture system is a product of the combination of cell survival and proliferation. Cell proliferation is the measurement of the number of cells that are proceeding through the cell cycle. The study of cell proliferation/cell division started with the histological observation of cells in cycle. The cell cycle progresses from mitotic phase (M), which is easily visualized by the condensed chromosomes, followed by a growth or gap phase termed G1. Replication of DNA takes place (synthesis, or S-phase) followed by another gap phase termed G2. While mitosis easy to visualize with standard histological stains, the rest of the cell cycle is not. During early development, the cell cycle is rapid and fairly uniform. As cell types differentiate, cell cycle length begins to vary between tissues and cell types. Using particular labels during development allows researchers to determine the fate of cells that became postmitotic at the time of labeling, using the concept of birth-dating cells.

One place where birth-dating of cells has provided interesting results is the process of neural differentiation. The pseudostratified columnar epithelium of the neural plate, neural folds, and early neural tube consists of a stem cell population undergoing rapid expansion without differentiation. As individual neuroblasts leave the cell cycle, they migrate to specific layers of the outer neural wall. A similar pattern is seen in cortical and retinal development, with the position and fate of the newly developed neuron dictated by the timing of the last division. To determine whether a cell is a proliferating progenitor or a postmitotic young neuron, researchers commonly combine S-phase-labeled nucleotide incorporation with markers of committed cells, using antibodies to factors such as neurofilament and the neural transcription factor Hu. In the chicken, the first postmitotic neurons are found in the hindbrain as part of the reticular complex (Sechrist and Bronner-Fraser, 1991).

The ability to incorporate of tagged nucleotides during S-phase has expanded the ability to study this aspect of cell division. Initially radioactive thymidine was added to living tissue to detect cells in S-phase (Boswald *et al*., 1990). Radioactive detection methods have mostly been replaced by the use of bromodeoxyuridine (BrdU) and antibodies that recognize this molecule. BrdU is incorporated into DNA, replacing thymidine during the S-phase of the cell cycle. One caveat with the use of BrdU is the observation that it should not be used for long-term studies, as it is a teratogen (Bannigan *et al*., 1981; Novotna *et al*., 1994; Wilt and Anderson, 1972).

An alternative to labeling cells in S-phase is to examine the mitotic index. The mitotic index is the ratio between the number of cells in mitosis and the total number of cells. The cells in mitosis can be easily visualized by the use of the antibody, antiphospho histone H3 Millipore (Massachusetts), which can be used in conjunction with antibodies of a different isotype. Another useful antibody is one which detects Proliferating Cell Nuclear Antigen (PCNA) which is an intranuclear polypeptide found in cells currently in the cell cycle (Uni *et al*., 1998).

The studies of cell proliferation and survival/cell death are commonly combined with studies of differentiation. Markers of differentiation can include cytological, such as the acquisition of perineurial sheath (Du Plessis *et al*., 1996), or development of lens structures (Bassnett and Winzenburger, 2003). Further information as to the differentiation status of a tissue can be obtained using specific antibodies. For instance, in neural tissue, both antineurofilment and anti-Hu antibodies stain neurons and not progenitor cells (Fischer *et al*., 2002). Muscle development is marked by a transition from progenitor markers such as Pax7 to more mature markers including myoD, accompanied by development of morphologically mature muscle fibers (Amthor *et al*., 2006). Other tissues have similar maturation patterns that can be marked by antibody or morphological changes during development. The combination of antibodies for cell death (anti-activated caspase-3), cell proliferation (anti-phospho histone H3) with markers of early, and/or late differentiation allows for powerful analysis of the sequence of events that lead to tissue development and the control of tissue size.

IV. Selected Protocols

A. Apoptosis/Cell Survival

1. Electron Microscopy

Widely used during early studies of cell death, electron microscopy was thought to be the definitive method for recognizing apoptosis and necrosis. It is still used in chicken embryos occasionally (Mao *et al*., 2006). Using electron microscopy, apoptotic cells can be seen to contain condensed chromatin followed by disintegration of the nucleus in to discrete fragments (Wyllie, 1987). Necrotic cells, however, feature a mild clumping of nuclear chromatin, dilation of endoplasmic reticulum, and swelling of mitochondria (Ho and Duffield, 2000). For a detailed description of the preparation of tissue for transmission electron microscopy, see Kerr *et al*. (1995).

2. Histological Stains

Quantitative analysis of degenerating cells in the tissue of question coupled with a loss in the total number of healthy cells of the same type or in the same tissue is a valuable means for linking the cell death to the reduction in healthy cells. In neural tissue, staining for Nissl staining does double duty identifying cytoskeletal ribosomes and staining for nuclear structures (Hamburger *et al*., 1981). Anatomical recognition of dying cells by light microscopy takes advantage of the morphological changes in the nucleus that accompany apoptosis. A pyknotic cell is described as one large particle, spherical in shape, deeply stained, or a group of small deeply stained particles clustered together (Hamburger and Levi-Montalcini, 1949; Hamburger *et al*., 1981).

Any number of nuclear stains can be used to assess the relative levels of cell death. In order to determine the population of cells to be counted in a section, it is important to define a specified area to be counted. One common stain used in tissues is hematoxylin/eosin that provides excellent staining of tissue morphology (see below). Hematoxylin colors the nuclei blue, and eosin stains the cytoplasm pink. Because of the chemicals used in this form of staining, it is not appropriate to use with most antibody stains.

3. TUNEL Procedure

The TUNEL method has been developed that detect the DNA degradation that accompanies apoptosis (Gavrieli *et al*., 1992; Wijsman *et al*., 1993). It relies on the observation that DNA breaks are one of the first events that occur during programmed cell death. It relies on the action of the enzyme terminal deoxynucleotydyl transferase (TdT) on exposed ends of DNA to add labeled nucleotide molecules. The signal can then be amplified by precipitate reaction (alkaline phosphotase or HRP), if needed. While alternative approaches using DNA polymerase 1 were also developed (Wijsman *et al*., 1993), the TdT reaction is now generally used. A number of kits have been developed (Roche, *in situ* cell death detection kit, R&D systems TdT *in situ*, Chemicon ApopTag, and Upstate TUNEL apoptosis detection kit, just to name a few). While the kits are not designed for use with whole-mount embryos, Smith and Cartwright (1997) have published a detailed description of the method adapted for chicken embryos. One of the reasons TUNEL is useful, especially when combined with a nuclear tag, is that TUNEL labels cells at a very early stage in apoptosis, and these cells may not appear to be pyknotic by other methods.

4. Activated Caspase-3 staining

The process of apoptosis involves the progressive cleavage of the intracellular components of a cell. A key set of enzymes, the caspase protease family was initially identified in studies in *C. elegans*, where it was demonstrated that worms lacking *Ced3* did not have the normal number amount of cell death compared to wild-type

worms (Horvitz *et al.*, 1994). Caspases have an obligatory cysteine in their active site and cleave adjacent to aspartate residues. Vertebrate caspases have been demonstrated to constitute an autocatalytic cascade. Caspases appear to be present in most cells in inactive proenzyme form. Some caspase-activating signals have been identified, while others are still being sought (Zou *et al.*, 1997). Caspase-3 (also known as CPP32) is thought to be the last step in this cascade. Therefore, activated caspase-3 has been a very useful tool to study caspase-dependent cell death.

5. Fluorescent-activated cell sorting (FACS) Sorting

When dealing with large amounts of cell death in an embryo, or when dealing with primary cells in culture, the detection of cell death can be accomplished by florescent sorting of cells based on DNA content, where dying cells demonstrate DNA contents $<1\times$. This is an especially valuable technique when considering the balance between cell death and proliferation because one readout (DNA) content is useful for both assays. The DNA content is determined by using any variety of DNA-binding dyes, including propidium iodide, Hoeschst dye, and DAPI. However, for chicken embryology, this approach is less useful because the starting material for cell sorting should be somewhat uniform. This uniformity can be accomplished by cosorting with a cell surface marker.

6. Methods for Studying Cell Viability

A cell viability measurement is often used to determine the health of cells after treatment in culture or FACS sorting. These measurements determine what percentage of the cells in a solution or in a culture are alive. When cells are not actively dividing or dying, they still are viable and there are times when the measurement of viability is useful.

Permeability: Trypan blue is used to determine the fraction of healthy cells in a suspension, after sorting or dissociation. It is combined with the use of a hemocytometer, which provides an account of the total number of cells in the solution. Trypan blue is excluded from cells with intact healthy membranes, and those cells appear ''phase bright'' and not blue. Cells with compromised membranes integrate trypan blue and appear ''phase dark'' and blue.

Metabolic activity: When cells have been placed in culture and exposed to a variety of treatments, there are a number of methods to determine if the cells have compromised membranes, but there are also useful measures of cell metabolic activity. Tetrazaolium salt is cleaved into a colored precipitate by metabolic activity, and can provide the most accurate readout of healthy cells in a culture.

Dimethylthiazolyl tetrazole (MTT) is widely used to quantitate cell viability in 96 well plate assays. MTT is one of several tetrazolium salts that are reduced by the succinate–tetrazolium reductase system, to produce a foramazan colored reaction product. This reductase system belongs to the mictochondrial respiratory chain, which is active only in viable cells. The readout can be done either by manual visualization or by ELISA reader.

B. Cell Proliferation

1. BrdU Immunohistochemistry

The introduction of monoclonal antibodies to BrdU has lead to the expanded use of BrdU in cell proliferation studies. When BrdU is available, it replaces thymidine during S-phase of the cell cycle. The advantages of BrdU compared with ([^{3}H]-TdR) is the ease of application, fast processing times, and the avoidance of radioactivity.

a. BrdU Application

5-Bromo-2′-deoxyuridine can be purchased from either Sigma (Missouri) or Roche (Switzerland). A stock solution is made in water, with further dilutions for application dissolved in either phosphate-buffered saline (PBS) or Ringer's solution. For embryos up to Hamburger and Hamilton (HH) stage 6, BrdU (50 µl of a 10^{-2} M solution) is applied directly onto the vitalline membrane. At later stages, the vitalline membrane can be broken by using a sharpened glass or tungsten needle, and the BrdU placed adjacent to the embryo. The typical incubation time is 1–2 h at 38 °C. Embryos are then sacrificed and fixed in 4% paraformaldehyde (PFA) made in PBS. Do not overfix the embryos (recommended no longer than overnight at 4 °C), as the BrdU epitope is somewhat sensitive to fixation.

b. Antibody Detection

Whole-mount embryos: For whole-mount staining (which works best for surface features or localizing changes in proliferation relative to grafts or bead implants), fixed embryos are removed from fixative and washed in PBS/0.1% Tween (PBT), followed by a series of methanol steps to dehydrate the embryo. Embryos can also be stored in methanol at −20 °C, followed by rehydration in PBT. To inactivate endogenous peroxidases, embryos are then incubated for 30 min in PBT/H_2O_2 (0.3%). All antibody and blocking preparations are done in PBS/2 mg/ml BSA/ 0.1% Triton. Embryos are blocked in 20% goat serum (or serum matching the species of the chosen secondary antibody) for 2 h at room temperature; primary antibody incubation (1:10–1:30 dilution) is done overnight at 4 °C, followed by vigorous washing in PBS at room temperature for 6–8 h. Secondary antibody, tagged with horseradish peroxidase (HRP), is then added at 1:1000 dilution, again overnight. Alternatively, a biotin-tagged secondary could be used followed by a HRP-tagged avidin. 3,3′-Diaminobenzidine tetrahydrochloride (0.05 mg/ml final concentration in water) is used as the substrate for the HRP enzymatic reaction.

Sectioned embryos: When embryos have been sectioned, a more accurate analysis of cell populations can be obtained. In this case, sections are first treated with 2 N HCl for 10 min at room temperature, to expose the DNA. Then move the slides to 0.1 M sodium borate ($Na_2B_4O_7$; pH 8.5) to neutralize the HCl for 5–10 min. Wash in PBS, then block with 20% heat-inactivated goat serum (or serum matching secondary antibody). The anti-BrdU antibody can be used at 1:30–1:50 dilution in PBS/2 mg/ml BSA/0.1% Triton for 1 h at room temperature. After washing in PBS

three times, slides are incubated with secondary antibody, using the brightest labeled appropriate secondary for at least 1 h at room temperature. This protocol can be combined with other antibody staining as long as the primary antibodies are detected by different secondary antibodies.

C. General Protocols

1. Preparation of Chick Embryos for Experimental Manipulation

1. Incubate at 37–39 °C until desired stage. Staging is performed using the criteria of Hamburger and Hamilton (1951). Incubators can be set on timers to allow precise incubation times. Eggs left at room temperature have variable viability, depending on time from laying to delivery. Incubated eggs can be removed from the incubator and stored at room temperature for a period not to exceed 24 h without incurring extensive increase in abnormalities. When eggs are to be manipulated *in ovo*, it is most helpful to place the eggs horizontally on egg flats for incubation.

2. It is often useful to first wash eggs with 70% EtOH prior to opening. Removal of 1–2.5 ml of albumin, using a 3–5 ml syringe and an 18 gauge needle, drops the embryo down from the eggshell, which is useful to visualize and manipulate the embryo. After removing the albumin and before cutting the egg shell, it is useful to place Scotch magic tape on the middle of the egg, to reduce egg cracking. Using sharp surgical scissors, cut a hole in the top of the egg in the tape-covered area, about the size of a quarter.

3. Prepare an inking syringe by attaching a 1/2″ 25 gauge embryo to a 1 ml syringe, bending the needle between 45 ° and 90 °. Fill the syringe with diluted India ink (Kohl-Noor fountain pen ink) or diluted vegetable dye (typically 8–10 drops in 5 ml Ringer's solution). Carefully slip the needle below the embryo as visualized by the white spot on top of the egg yolk. Gently release a small amount of the dye as a contrast agent below the embryo. Keep the embryo moist using Ringer's solution.

4. After embryo manipulation, the hole in the egg shell is covered with either Scotch tape or Tear-by-Hand packing tape, making sure the hole is well sealed to prevent drying. Embryos are placed back in a humidified 37 °C incubator.

2. Embryo Collection

1. Carefully cut the tape and egg shell to make a hole large enough to remove the embryo. Using either straight or bent surgical scissors, or iris scissors, cut a large area around the embryo while holding one edge of the embryo with forceps. Gently remove the embryo to a petri dish filled with Ringer's solution, saline, or PBS solution. In the petri dish, gently separate the embryo from the vitallene membrane, and trim the embryo to desired size using fine scissors. Younger embryos can be picked up by placing a small piece of filter paper, which will stick to the embryo, and

cutting around it before transferring to the petri dish. Larger embryos may require the use of a slotted spoon to transfer from the egg to wash solutions.

2. The choice of fixative and fixation time largely depends on the nature of the experiment, what antibodies or stains are to be used, and the age and size of the embryo or piece of the embryo to be used. For many experiments, it is typical to fix in a 4% PFA/PBS solution overnight at 4 °C. Following fixation, rinse the embryos in PBT several times. Embryos can be stored at 4 °C in PBT for a short period (1–2 weeks) or moved through a methanol /PBT series into 100% methanol for longer storage. Not all antibody epitopes will work with the above fixation protocol, so some troubleshooting may be required.

3. General Fixatives and Histological Stains

a. 4% PFA in 0.1 M PBS solution, pH 7.0–7.5

Make PFA in the hood and be sure to wear gloves—it is carcinogenic and toxic. Add 400 ml of distilled water and 2 ml of 1 M NaOH in a 1 liter beaker. Heat on a stirring hot plate in the fume hood. Add 32 g of PFA powder. When dissolved, add 324 ml of 0.2 M Na_2HPO_4 slowly (prepare 400 ml by dissolving 11.4 g in 400 ml distilled water). Then add 76 ml of 0.2 M NaH_2PO_4 slowly (prepare 200 ml by dissolving 4.8 g in 200 ml of distilled water). Cool and check the pH using pH paper (never the pH meter). Adjust as needed for differences in initial water pH.

b. Carnoy's Fix

This is good for DNA and cartilage staining: glacial acetic acid (10 ml), absolute ethyl alcohol (60 ml), and chloroform (30 ml). Fix for 3–6 h. Rinse in ethanol.

c. Howard's Ringer's Solution

This solution consists of 7.20 g NaCl, 0.17 g $CaCl_2(2H_2O)$, 0.37 g KCl, and bring up to 1000 ml with H_2O. Filter sterilize (do not autoclave). Aliquot into 80–100 ml bottles, use fresh each time.

d. Gelatin for Embedding

Heat 100-ml 1-M PBS solution in a beaker with a stir bar. Add 7.5% gelatin and 15% sucrose. Stir until dissolved, but do not cook. This solution can be kept at 4 °C for up to 3 weeks. To use, scoop small amounts into conical tubes and melt briefly (10–20 sec) in the microwave as needed.

4. Embedding Procedures

a. Paraffin Embedding

a. After fixation, wash embryos in PBS (1 h for embryos up to E3, longer for older embryos).
b. Dehydrate embryos in ascending ethanol series: 60%, 75%, 90%, and 100% (2×) for 15–20 min each for embryos up to E3, longer for older embryos (1–2 h at E9).

c. Place embryos in toluene, xylene, or Histosol twice for the same time as step b. These solvents will clear the embryo.
d. Infiltrate the specimen in melted paraffin (Paraplast). Paraffin should not be heated above 60 °C, and a vacuum oven is useful to maintain melted paraffin. To ensure that all solvent from previous steps is removed, three changes of paraffin should be used.
e. Embed the embryo by using warm forceps to position the embryo in a plastic mold. Once embedded, blocks can be stored at room temperature.
f. Blocks are trimmed with a razor blade to expose the embryo with only a small rim of wax surrounding it. Sectioning is performed on a microtome using disposable blades. Ribbons of 5- to 10-μm sections are floated on water dropped on a subbed slide placed on a heated block (45 °C). The water will help the ribbon flatten out as it evaporates.

b. Embedding in Gelatin for Frozen Sections

a. After fixation, embryos are washed in PBS for 1 h or more.
b. Place the embryos in 5% sucrose (in PBS) for 4–8 h at 4 °C. Embryos should sink, although some embryos fail to sink even when equilibrated in sucrose.
c. Place the embryos in 15% sucrose (in PBS) for 4–8 h at 4 °C.
d. Equilibrated the embryos in 15% sucrose/7.5% gelatin in PBS (see above) for 2–4 h at 37 °C.
e. Place embryos in embedding molds and orient. Check that the orientation is maintained as the gelatin sets. Embryos can be kept for a short time (1 week) in a humidified chamber at 4 °C.
f. Rapidly freeze the embryo in the mold in liquid nitrogen. Alternatively, a dry ice methanol bath can be used. The frozen block can be cut out using a razor blade, but often will drop out if a sharp rap is given to the back of the block. The gelatin block is attached to the cryostat chuck with OCT, and then allowed to equilabrate to the temperature of the cryostat.
g. Section at −30 °C in a cryostat at 10–12 μm.

Additional embedding protocols are available in Sechrist and Marcelle (1996).

5. Staining Procedures

a. Hematoxylin and Eosin Stain

This classic stain provides the best visualization of tissue architecture, but is not recommended in conjunction with florescent antibody labeling. The basic nature of this stain is to use two contrasting dyes with different binding characteristics. Hematoxylin is a dark purple dye that stains nuclear material. Eosin is a pink-red dye that stains cytoplasmic material. Thus, the cell is seen as a pink tissue with a purple nucleus, and cell membranes typically stain darker pink-red than the cytoplasm. There are various protocols adapted to perform hematoxylin and

eosin (H&E) staining, depending on the manner of sectioning employed, and the desired intensity of staining. The following protocol is designed for sections fixed in PFA and cut on the cryostat. For sections exposed to organics, the samples need to be moved from organic to water by a series of hydration steps.

a. Immerse sections in the Harris hematoxylin for 1–3 min.
b. Wash three times in tap water, with agitation.
c. Immerse sections in eosin stain for 1–5 min.
d. Wash three times in tap water, with agitation, until water is clear.

Mount coverslips in aqueous mounting media (Gelmount, Biomedia, California), or dehydrate with ascending alcohol solutions followed by xylene and mount coverslips with Permount (Biomedia).

6. Antibody Labeling on Sectioned Materials

Using conventional compound microscopes, it is possible to combine antibodies in two or three combinations to best utilize the information from each section. More information can be obtained by staining alternate sections with H&E. The combinations that can be used are limited by the nature of the primary antibodies available. While the following protocol uses directly tagged secondary antibodies, further amplification can be obtained by using an avidin-biotin amplification step.

1. Fix embryos in desired fixative and section on cryostat or microtome. If sectioned in paraffin wax, deparaffinize in two changes of histol, followed by rehydration in 1000%, 100%, 95%, 70%, and 30% ethanol for 1 min each. Transfer slides to 0.1 M PBS solution. If sectioned on a cryostat using gelatin, place slides in PBS warmed to 42 °C for 5 min, followed by one wash in room temperature PBS.

2. Block the slides with heat-inactivated serum that is appropriate for the chosen species of secondary antibody.

3. Incubate with the first primary antibody for 2 h at room temperature or 4 °C overnight. Dilution of the primary antibody needs to be determined independently in advance. Antibodies are best diluted in a 0.1% BSA/PBS solution, with or without 0.1% Triton, which should be determined for each antibody. Lay slides flat in a plastic container (an empty slide box with moist towels at the bottom is a useful tool). A coverslip can be used to spread small amounts (no less than 100 μl) of antibody on the slide. Often in the case of hybridoma supernatants, no further dilution is required.

4. Rinse the slides 3 × 5 min in 1× PBS.

5. Incubate with FITC or TRITC or similar conjugated secondary tagged antibodies for 1–2 h at room temperature. Secondary antibodies should be specific for the chosen primary and not general enough to label a second antibody, if one is used.

6. Rinse the slides 3 × 5 min in 1× PBS (can proceed to coverslipping at this time point).

7. Incubate with the second primary antibody, 2 h room temperature or overnight at 4 °C.

8. Rinse the slides 3 × 5 min in 1× PBS.

9. Incubate with FITC or TRITC or similar conjugated secondary tagged antibodies (not the same flourophore as used for previous antibody) for 1–2 h at room temperature. Secondary antibodies should be specific for the chosen primary and not general enough to label a second antibody, if one is used.

10. Counterstain, if desired, in Hoescht or DAPI. Hoechst 33258 is a DNA-specific counterstain. Use a stock solution of 10 mg/ml to make a working solution of 1 mg/ml in PBS, stain for 15 min, and wash in PBS, followed by coverslipping. DAPI can be made as a 1 mg/ml stock solution in water, diluting to a 0.1–0.2 mg/ml working solution (in water), incubate 10 min followed by PBS washes and coverslipping.

11. After antibody staining, the coverslip is affixed using Gel Mount, an aqueous mounting media designed to preserve fluorescence. Keep slide flat overnight, then seal with clear nail polish, if desired.

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PART II

Labeling and Transgenesis Approaches

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CHAPTER 9

In Situ Hybridization Analysis of Chick Embryos in Whole-Mount and Tissue Sections

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I. Introduction

Detection of the temporal and spatial regulation of gene expression in embryos is essential for elucidating the developmental functions of genes and for elucidating the cell interactions that regulate tissue patterning and differentiation. Patterns of gene expression can be visualized by detecting the encoded protein product by immunocytochemistry or mRNA using *in situ* hybridization (Wilkinson 1992; Wilkinson and Nieto, 1993). The detection of protein has the advantage of being a more accurate guide to sites of gene action, because, due to translational regulation, RNA and protein expression do not always correlate. Furthermore, immunocytochemistry reveals the subcellular location of protein that can be an important clue to how it functions. However, the production of specific antibodies can be difficult and time-consuming. In contrast, specific probes for *in situ* hybridization to mRNA can easily be produced.

In situ hybridization to RNA involves a series of procedures:

1. synthesis of a labeled nucleic acid probe complementary to the target mRNA.
2. fixation and permeabilization of tissue (sectioned or whole embryo).
3. hybridization of probe to the tissue and washing to remove unhybridized probe.
4. detection of the probe.

Many types of probe and methods of labeling and visualization have been used for *in situ* hybridization to embryos. Hapten-labeled single-stranded RNA probes are most commonly used, as they enable high sensitivity, a single cell resolution of signal, the ability to visualize gene expression in whole embryos, and detection of multiple RNAs can be carried out (Lopez-Sanchez, 2004; Nieto *et al.*, 1996; Stern, 1998). Following hybridization and washing, the location of probe is detected with a hapten-binding protein conjugated to an enzyme. This latter enzyme catalyzes the conversion of a substrate to an insoluble, colored, or fluorescent product, and thus a signal is produced at the sites of the target mRNA.

A number of haptens and enzyme conjugates of hapten-binding proteins are available: haptens include digoxigenin (DIG), fluorescein, and dinitrophenol

(DNP) that can be detected with commercially available antibodies, and enzymes include alkaline phosphatase (AP) and horse radish peroxidase (HRP). For each of these enzymes, a variety of substrates can be used that yield different colored or fluorescent products. For detection of a single RNA, the reagents that have found widespread favor because of their high sensitivity and low backgrounds are DIG-labeled probes detected with an AP-conjugated anti-DIG antibody and the chromogenic substrate mixture of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) plus 4-nitro blue tetrazolium chloride (NBT). For detection of two RNAs, a mixture of RNA probes labeled with DIG and fluorescein can be used followed by sequential detection with AP-conjugated antibodies to generate different colored products (Protocols 1–6). Alternatively, fluorescent products can be generated by using HRP-conjugated antibodies (Protocols 8–10).

For many purposes, the *in situ* hybridization of whole embryos is the method of choice as it is easier and provides a broader picture of the gene expression pattern than the hybridization of sections. If sections are required, it is less work to section embryos after whole-mount hybridization and signal detection than it is to prepare sections and then hybridize. However, the extent of penetration of reagents into tissues limits the size of embryos that can be used for whole-mount *in situ* hybridization. Although we have obtained low backgrounds with chick embryos up to stage HH25 (Hamburger and Hamilton, 1951), much stronger signals are obtained when the tissue is at the surface than when it is internal, and thus some sites of expression could be missed. This limitation can be alleviated to some extent by longer hybridization and washing steps. In addition, access of the reagents can be increased by bisecting embryos or dissecting out the tissue of interest prior to hybridization.

II. Solutions

TE buffer: 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0.

5 × transcription buffer: 200 mM Tris-HCl, pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl.

Nucleotide mix: 10 mM GTP, 10 mM ATP, 10 mM CTP, 6.5 mM UTP, 3.5 mM DIG-UTP or fluorescein-UTP, pH 7.5 (Roche).

Phosphate-buffered saline (PBS): prepared using Dulbecco "A" tablets (Oxoid) and treated with DEPC.

PBT: PBS, 0.1% Triton X-100 (Tx).

Proteinase K: 10 mg/ml stock in sterile H_2O.

Paraformaldehyde fixative: 4% paraformaldehyde in PBS. Heat at 65 °C with occasional agitation until dissolved, cool, and then filter. Use on the day of preparation. Note: take precautions with paraformaldehyde fumes that are toxic.

Hybridization solution: 50% formamide, 5 × SSC, 2% Roche blocking powder, 0.1% Tx, 50 mg/ml heparin, 1 mg/ml Torula yeast RNA, 1 mM EDTA, 0.1% CHAPS, DEPC-treated dH_2O.

20 × SSC stock solution: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.

KTBT: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, 0.3% or 0.1% Tx.

NTMT: 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tx, 1 mM levamisole.

NBT stock solution: 75 mg/ml NBT (Roche) in 70% dimethylformamide.

BCIP stock solution: 50 mg/ml BCIP (Roche) in dimethylformamide.

III. Single and Multiple Detection of RNA in Floating Sections or Whole-Mount Embryos

It is important that solutions used for processing the embryos prior to hybridization are ribonuclease-free to avoid the degradation of the cellular RNAs. We find it sufficient to autoclave the PBS used for making fixative and pretreatment solutions and to use disposable plastic tubes. In order to obtain low backgrounds, it is important that the washes are thorough, but do not damage the embryo. We use a variable speed rocking platform adjusted such that the embryos are gently agitated during prehybridization, hybridization, and washing steps; this is easier to achieve if the container is not completely full. For the high-temperature incubations, we place microtubes in a heater block turned on its side on a rocking platform. Alternatively, an incubator containing a rocking platform can be used. When changing solutions, allow the embryos to settle to the bottom of the container and leave some liquid above them otherwise surface tension can flatten them. A variety of different containers can be used, partly depending upon the equipment available. We use 7 ml flat-bottomed tubes for the fixation and pretreatment of embryos and 2 ml microtubes for hybridization, washing, and immunodetection.

The following protocol has been used extensively to detect RNA transcripts by *in situ* hybridization of embryos from different species such as chick (Fig. 1A–E), mouse, zebrafish, lizard, turtle, and amphioxus, as well as in adult tissues including human samples. This multiple detection of RNA can also be combined with immunohistochemical protocols (Fig. 1F and G) to detect protein expression.

A. Protocol 1: Preparation of Labeled RNA Probes

To detect one or two different RNAs, probes are synthesized labeled with one of two different haptens: DIG or fluorescein. The protocol used to synthesis each probe is to carry out *in vitro* transcription of the DNA template in the presence of ribonucleotides, to one of which DIG or fluorescein is conjugated (usually UTP).

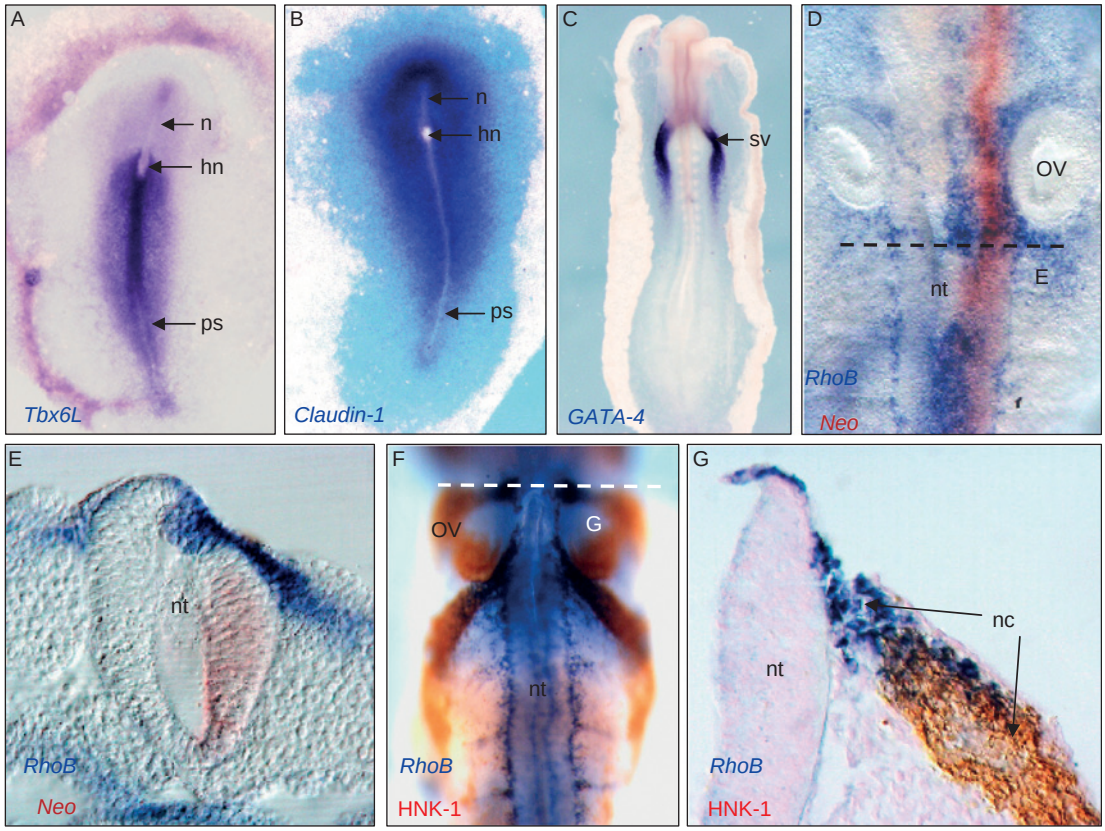

Fig. 1 Multiple detection of gene transcripts and proteins in chick embryos using nonfluorescent *in situ* hybridization and immunohistochemistry. (A) *Tbx6L* expression in the mesoderm of an HH6 chick embryo (B) *Claudin-1*-expression in the epiblast of an HH5 embryo. (C) *GATA-4* expression in the sinus venosus and the cardiogenic mesoderm of an HH10 embryo (D) Simultaneous detection of *RhoB* (blue) and *Neomycin* expression (red) shown in a dorsal view of the hindbrain at HH13. The *Neomycin* coding region was included in a plasmid vector electroporated in the right-hand side of the neural tube. (E) Vibratome section obtained from the embryo shown in (D) at the level of the dotted line. (F) Dorsal view of the posterior hindbrain region of an HH13 embryo hybridized with *RhoB* (blue) and immunostained for the HNK-1 carbohydrate (brown). (G) Section taken from the embryo shown in (F) at the level indicated by the dotted line (adapted from Del Barrio and Nieto, 2004) allows the visualization of different neural crest populations. Abbreviations: n, notochord; hn, hensen's node; nc, neural crest; nt, neural tube; ov, otic vesicle; ps, primitive streak; sv, sinus venosus. (See Plate no. 8 in the Color Plate Section.)

The labeled RNA synthesized should be complementary or antisense to the target mRNA. When possible, long probes are recommended (around 0.5–2 kb) to increase specificity and signal strength. Before probe synthesis, the DNA template should be linearized at a restriction site located at the 5′ end of the cDNA (inside the cDNA or in the plasmid's multiple cloning site) using an enzyme that produces a blunt or 5′ overhanging end. After checking that linearization is complete,

the DNA is purified using either columns or phenol/chloroform extraction and ethanol precipitation. We generally redissolve the DNA at a concentration of approximately 1 μg/μl in TE buffer. Usually probes are synthesized using SP6, T7, or T3 RNA polymerases, the promoters for which are generally present in most commercial vectors. The transcription buffer is generally supplied with the enzymes.

1. Mix these reagents in the following order at room temperature:

   8.5 μl sterile distilled water
   4 μl 5 × transcription buffer
   2 μl 0.1 M dithiothreitol
   2 μl nucleotide mix
   1 μl linearized plasmid (1 μg/μl)
   0.5 μl RNasin (100 U/μl) (Promega)
   1 μl SP6, T7, or T3 RNA polymerase (10 U/μl)
2. Incubate at 37 °C for 2 h.
3. Add 2 μl of 3 M ammonium acetate and 50 μl ethanol, mix, and incubate at −20 °C for 2 h.
4. Spin for 10 min in a microfuge at 4 °C, wash the pellet with 70% ethanol, and air dry.
5. Redissolve the pellet in 20 μl ice-cold DEPC-treated dH_2O and 20 μl formamide.
6. Remove 2 μl aliquot and run on 1% agarose/TAE gel to check the amount of RNA synthesized.
7. Store the synthesized probe at −70 °C.

B. Protocol 2: Preparation of Embryos and Tissue Sections

A crucial step in this procedure is the way in which the embryos are obtained because the dissection has to be performed quickly in ice-cold PBS in order to minimize any degradation of RNA. With mouse (from E10.5) and chicken embryos that are at least 2 days of age, it is necessary to make a small hole in the cavities (like the dorsal hindbrain) to prevent probe or antibody trapping that can give rise to nonspecific signals. Paraformaldehyde fixation preserves the RNA transcripts, the embryonic tissues, and the general morphology of the embryos. Normally for chick embryos (up to stage HH17), a fixation time of 2 h is sufficient but frequently we use overnight fixation. During all these steps, the embryos or sections are kept on ice.

1. Dissect out the embryos in ice-cold DEPC-treated PBS.

2. Fix the embryos overnight at 4 °C in 4% paraformaldehyde prepared in DEPC-treated PBS. When working with vibratome sections, cut the sections at a thickness of 50–100 μm and refix the tissue sections in 4% paraformaldehyde.

3. Wash the embryos or sections with ice-cold PBT, twice for 5 min.

4. Wash the embryos with ice-cold 25%, 50%, 75% methanol in PBT for 5 min in each solution and then twice with 100% methanol. When dealing with bigger embryos extend the length of the washes to up to 15 min. The embryos or sections can be stored at –20 °C in 100% methanol, although it is better to store them after the prehybridization step.

C. Protocol 3: Prehybridization Treatments and Hybridization

The permeabilization steps that constitute the prehybridization process are important to improve the penetration of the probes and antibodies into the embryos and tissue sections. Proteinase K treatment partially digests cellular proteins and facilitates probe penetration into embryonic tissues. However, excessive digestion with proteinase K will alter embryonic morphology and it is not recommended for early embryos (up to stage 8 in the chick and E8 mouse embryos). A treatment with H_2O_2 is necessary to inactivate endogenous peroxidase activity. This step can be omitted if peroxidase will not be used later in the detection procedure. In the following protocol, the embryos are kept on ice, except for steps 4 and 6.

1. Rehydrate the sections or embryos through 75%, 50%, 25% methanol in PBT and then wash twice with PBT.

2. If required, incubate the sections or embryos in 1% hydrogen peroxide in PBT for 20 min. For embryos from stage HH12 or for whole organs, incubation can be extended up to 1 h.

3. Wash the sections or embryos with PBT three times for 5 min.

4. Treat the sections or embryos with 10 μg/ml proteinase K in PBT for 15 min at room temperature. The appropriate treatment depends on the age of the embryos. Proteinase K should not be used for embryos up to stage HH8 (particularly if they have been cultured *in vitro*), while 5–7 min treatment is appropriate for embryos up to stage HH12. For vibratome sections, we recommend a 3 min digestion. Under no circumstances should the digestion be extended for longer than 20 min.

5. Wash the sections or embryos for 5 min each with freshly prepared 2 mg/ml glycine in PBT and twice with PBT. This step is optional.

6. Refix the sections or embryos with fresh 4% paraformaldehyde in PBT for 20 min.

7. Wash the sections or embryos twice for 5 min with PBT.

8. Add 1 ml of the hybridization solution and transfer the embryos to a 2 ml screw-capped tube. Incubate at 60 °C for 5 min to equilibrate.

9. Replace the hybridization solution with fresh solution and incubate the material overnight at 60 °C with gentle agitation. The sections or embryos can be stored indefinitely in this solution at −20 °C.

10. Denature the anti-DIG and/or antifluorescein probes by heating them at 65–70 °C for 3 min.

11. Remove the hybridization mix and add fresh solution containing around 2 µl of each RNA probe per milliliter of hybridization mix (equivalent to 1–5 µg of the purified probe). The volume used should be sufficient to cover the embryos or sections.

12. Incubate overnight at 60 °C with gentle agitation. For higher stringency, hybridization can be carried out at higher temperatures, provided the probe is longer than 400 bp.

D. Protocol 4: Posthybridization Washes and Signal Detection

Following hybridization, embryos and sections should be washed at moderate stringency to remove the unhybridized probe. The bound probe is then detected with an AP-conjugated antihapten antibody. The washes after antibody binding serve to remove the unbound antibodies. Overnight washing is not always necessary but it helps to reduce background in older embryos or organs. The embryos are then incubated with a chromogenic substrate for AP. This produces a colored precipitate at the site where the probe RNA and antibody have bound to the target RNA. The time required for the color reaction to develop normally varies from 30 min to 48 h and depends on several factors such as the quantity of target RNA, probe quality, and reagent penetration. With some probes, the reaction may even take longer than 48 h to visualize the reaction product in whole-mount embryos or tissue sections, but care must be taken so that the background levels of staining do not prevail or reach unacceptable levels. After visualization of the staining of the material, embryos or sections can be stored for a long time in PFA 4% in PBS or glycerol 50% + sodium azide 0.02% in PBS at 4 °C.

1. Wash twice for 5 min with 2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation.

2. Wash three times for 30 min each with 2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation. For older embryos, increase the length and number of washes.

3. Wash three times for 30 min each with 0.2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation.

4. Wash three times for 5 min each with KTBT (0.1% Tx) at room temperature.

5. Block the sections or embryos by incubating with 15% sheep serum, 0.7% Roche blocking powder in KTBT (0.1% Tx), for 2–3 h at 4 °C.

6. Incubate with 1/1000 dilution of AP-conjugated anti-DIG antibody (Roche) in blocking solution. The antibody can be preabsorbed for 1 h with embryo powder (prepared as in Protocol 5), but this step is optional. To preabsorb the antibody with embryo powder, incubate 3 mg of embryo powder in KTBT

(0.1% Tx) for 30 min at 70 °C. Allow the mixture to reach room temperature and preabsorb the desired amount of anti-DIG antibody for 1 h at 4 °C. Spin in a microfuge at 12,000 rpm for 1 min at 4 °C and dilute the supernatant in the appropriate amount of blocking solution.

7. Incubate the sections or embryos with antibody overnight at 4 °C.

8. Wash the sections or embryos in KTBT (0.3% Tx) eight times for 1 h each wash at room temperature.

9. Leave washing overnight in KTBT (0.3% Tx) at room temperature.

10. Wash the sections or embryos three times with NTMT, 15 min each wash at room temperature.

11. Incubate with AP substrate (3 μl/ml NBT + 2.3 μl/ml BCIP in NTMT) at room temperature until a blue precipitate is readily apparent.

12. Wash in KTBT (0.3% Tx) twice, 5–10 min each wash at room temperature. If you need to develop the reaction longer, the sections or embryos can be left overnight in KTBT and the staining resumed at step 10 on the following day.

E. Protocol 5: Preparation of Embryo Powder

1. Homogenize embryos (12.5–14.5 days mouse embryos or 4–5 days chick embryos) in a minimum volume of ice-cold PBS.
2. Add four volumes of ice-cold acetone to the homogenate, mix and incubate on ice for 30 min.
3. Centrifuge at 10,000 × *g* for 10 min and remove supernatant. Wash the pellet with ice-cold acetone and repeat the centrifugation.
4. Spread the pellet out and grind it into a fine powder on a sheet of filter paper. Air-dry the powder and store it at 4 °C.

F. Protocol 6: Detection of the Second RNA

To detect a second RNA, one of the two probes is labeled with fluorescein and detected with AP-conjugated antifluorescein and a substrate that yields a distinct color from that generated for the DIG-labeled probe detected above (Fig. 1D and E). It is essential to inactivate the AP activity of the anti-DIG antibody prior to the detection of the fluorescein-labeled probe. This inactivation is carried out by acid treatment. Note that the INT/BCIP precipitate generated in the second color reaction is not very stable and can disappear if extensively washed.

1. Incubate the sections or embryos in 0.1 M glycine in PBS, pH 2.2, two times for 10 min each at room temperature.

2. Wash the sections or embryos in KTBT (0.1% Tx) three times for 5 min each at room temperature.
3. Block the sections or embryos by incubating with 15% sheep serum, 0.7% Roche blocking powder in KTBT (0.1% Tx), for 2–3 h at 4 °C.
4. Incubate overnight at 4 °C with antifluorescein antibody (Roche) at 1/3000 dilution.
5. Incubate the sections or embryos with antibody overnight at 4 °C.
6. Wash the sections or embryos in KTBT (0.3% Tx) eight times for 1 h each wash at room temperature.
7. Leave washing overnight in KTBT (0.3% Tx) at room temperature.
8. Wash the sections or embryos three times with NTMT, 15 min each wash at room temperature.
9. Incubate with INT/BCIP 75 µl /10 ml NTMT until a red-brown precipitate appears.
10. Wash in KTBT (0.3% Tx) twice, 5–10 min each wash at room temperature.
11. Wash in PBS and store embryos or sections in 4% paraformaldehyde or 50% glycerol at 4 °C.

G. Protocol 7: Immunodetection of Protein

To detect protein expression after *in situ* hybridization follow protocol 6 up to step 10 and then continue as indicated below (Fig. 1F and G).

1. Block again the embryos by incubating with 10% goat serum in KTBT for 2–3 h.
2. Incubate embryos overnight at 4 °C with an antibody against the protein you wish to detect at an appropriate dilution in 10% goat serum in KTBT. By inclusion of 0.01% azide at this step, incubation can go up to three days.
3. Wash the embryos three times for 5 min with KTBT.
4. Wash the embryos 12 times for 20 min with KTBT.
5. Incubate with a biotinilated, anti-IgG secondary antibody specific for the species of the primary antibody overnight at 4 °C in blocking solution.
6. Wash the embryos three times for 5 min with KTBT.
7. Wash the embryos 10 times for 20 min with KTBT.
8. Incubate with ABC kit Vectastain (Vector Laboratories) for 3 h and wash overnight.
9. Wash in 0.5 mg/ml diaminobenzidine in KTBT for 30 min.
10. Develop in the same solution containing 0.03% H_2O_2 at room temperature. This reaction is usually extremely fast and is completed in less than 2 min.

IV. Photography and Sectioning

Low-power photographs of the embryos can be taken on a dissecting microscope with overhead illumination (Fig. 1A–C). We usually place the embryo on a 3% agarose/PBS gel (in a 35 mm petri dish) that gives us a nice light blue background. Alternatively, embryos in 50% glycerol can be mounted under a coverslip in order to take photographs at higher magnification (Fig. 1D). Place two drops of petroleum jelly or silicon grease about 1 cm apart on a microscope slide, pipette the embryo in the middle, and orientate as desired. Lower a coverslip on top, gently pushing it down as required. Depending on the site of expression, it can be very useful to partially dissect embryo tissues to improve the observation and help focusing the structures of interest.

Embryos can then be embedded in gelatin/albumin or paraffin wax to be sectioned on a vibratome (Fig. 1E) or microtome (Fig. 1G), respectively. The dehydration steps used to embed in wax can produce a loss of signal, especially of INT/BCIP precipitates. Vibratome sectioning is the preferred method to analyze embryos subjected to double detection of mRNAs including INT/BCIP precipitates and paraffin sectioning when signals are resistant to treatments for embedding.

V. Whole-Mount Fluorescent *In Situ* Hybridization

The use of fluorescent *in situ* hybridization has two major advantages. One is that very good cellular resolution can be obtained, enabling the detection of coexpression or complementary expression of genes in adjacent territories. Second, it is possible to simultaneously detect up to three different messenger RNAs in whole-mount embryos (Fig. 2A) or sections. In addition, this multiple detection can be combined with fluorescent immunodetection of proteins (Fig. 2B and C). The use of a confocal microscope capable of analyzing many different excitation wavelengths is indispensable to obtain sufficient cellular resolution, although it is also possible to obtain very good images using a conventional fluorescence microscope.

The fluorescent whole-mount *in situ* hybridization protocol is very similar to the general protocol described above but it contains a few modifications that mainly affect the type of antibodies and reagents used to visualize the hybridized RNA probe. In the following protocol, we shall describe the triple localization of RNAs and their immunodetection in whole-mount embryos. The order in which the different probes are detected may be important when strong and weak signals are expected. Normally the stronger signal is detected with the fluorescein-conjugated probe and revealed with FITC-tyramide, while the weakest signal should be visualized with the DIG-conjugated probe and Cy3-tyramide. The protocol presented here is modified from Denkers *et al.* (2004) with an additional step to detect proteins by immunochemistry. It is important to keep embryos protected from light throughout the entire *in situ* hybridization process, especially during and after developing.

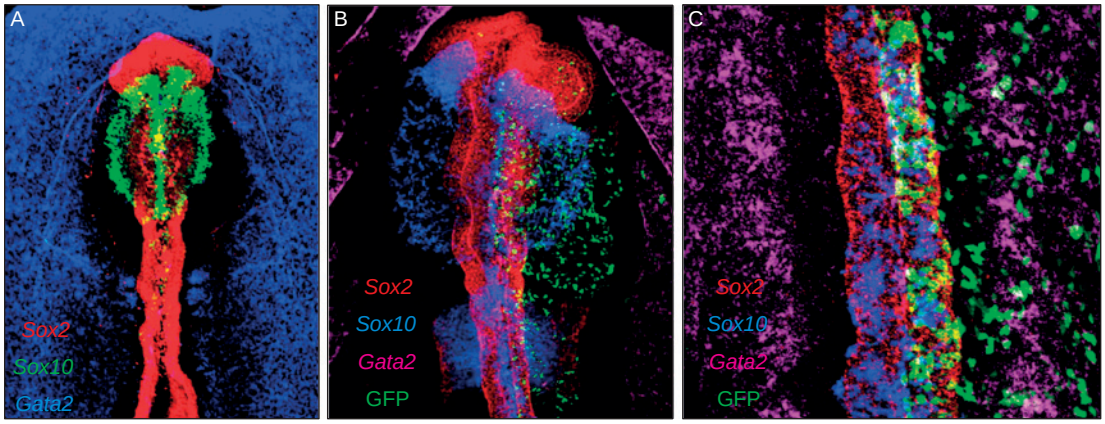

Fig. 2 Multiple detection of gene transcripts and proteins in chick embryos using fluorescent *in situ* hybridization and immunohistochemistry. (A) Three-color *in situ* hybridization in a HH9 chick embryo for the epidermal marker *Gata2* (blue, artificial color), the neural marker *Sox2* (red), and the neural crest marker *Sox10* (green). (B) Three color *in situ* hybridization in the head of an HH9 embryo for *Gata2* (magenta, artificial color), *Sox2* (red), and *Sox10* (blue). This embryo was electroporated with a plasmid encoding the green fluorescent protein (GFP). Cells expressing the GFP protein were detected by immunohistochemistry (green). (C) A higher power image of a similar embryo showing the trunk region of an HH9 embryo. Images were acquired using a Leica DM IRE2 confocal inverted microscope. (See Plate no. 9 in the Color Plate Section.)

To detect three different RNAs, three probes are synthesized with different epitopes: DIG-UTP, fluorescein-UTP, and DNP-UTP. The protocol for probe synthesis is as described in Protocol 1, using a nucleotide mix of 10 mM GTP, 10 mM ATP, 10 mM CTP, 6.5 mM UTP, 3.5 mM DIG-UTP or fluorescein-UTP (Roche), or DNP-UTP (Perkin Elmer). The preparation of embryos, prehybridization, and hybridization are all carried out as described in Protocols 2 and 3, with the use of three probes in the hybridization solution.

A. Protocol 8: Posthybridization Washes and Signal Detection

1. Wash twice for 5 min with 2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation.
2. Wash three times for 30 min each with 2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation. For older embryos, increase the length and number of washes.
3. Wash three times for 30 min each with 0.2 × SSC, 0.1% CHAPS at 60 °C with gentle agitation.
4. Wash three times for 5 min each with KTBT (0.1% Tx) at room temperature.
5. Block the sections or embryos by incubating with 15% sheep serum, 0.7% Roche blocking powder in KTBT (0.1% Tx), for 2–3 h at 4 °C.

6. Incubate the embryos with HRP-coupled antifluorescein antibody (1/500, Perkin Elmer) in blocking solution overnight at 4 °C.
7. Wash the embryos in KTBT (0.3% Tx) three times, 5 min each at room temperature.
8. Wash the embryos in KTBT (0.3% Tx) 10 times, 20 min each at room temperature.
9. Wash the embryos for 1 min with the amplification buffer supplied by the manufacturer in the TSA kit.
10. Incubate the embryos with amplification buffer including freshly added Cy3-labeled tyramide (1:100, Perkin Elmer) for up to 1 h at room temperature.
11. Wash the embryos in KTBT (0.3% Tx) three times for 5 min each at room temperature.
12. Incubate the embryos for 45 min with 1% H_2O_2 in KTBT (0.3% Tx) to inactivate the HRP activity.
13. Wash the embryos in KTBT (0.3% Tx) three times for 5 min each at room temperature.

B. Protocol 9: Detection of the Second RNA

1. Block the embryos by incubating with 15% sheep serum, 0.7% Roche blocking powder in KTBT (0.1% Tx) for 2–3 h at 4 °C.
2. Incubate overnight at 4 °C with HRP-coupled anti-DIG antibody (1/1000, Roche).
3. Wash in KTBT (0.3% Tx) three times, 5 min each at room temperature.
4. Wash the embryos in KTBT (0.3% Tx) 10 times, 20 min each at room temperature.
5. Wash the embryos for 1 min with the amplification buffer supplied by the manufacturer in the TSA kit.
6. Incubate the embryos with amplification buffer including freshly added FITC- labeled tyramide (1:100, Perkin Elmer) for up to 1 h at room temperature.
7. Wash in KTBT (0.3% Tx) twice for 5–10 min each at room temperature.

C. Protocol 10: Detection of the Third RNA

1. Block the embryos by incubating with 15% sheep serum, 0.7% Roche blocking powder in KTBT (0.1% Tx) for 2–3 h at 4 °C.
2. Incubate overnight at 4 °C with HRP-coupled anti-DNP antibody (1/500, Perkin Elmer).
3. Wash in KTBT (0.3% Tx) three times, 5 min each at room temperature.
4. Wash the embryos in KTBT (0.3% Tx) 10 times, 20 min each at room temperature.

5. Wash the embryos for 1 min with the amplification buffer supplied by the manufacturer in the TSA kit.
6. Incubate the embryos with amplification buffer including freshly added Cy5-labeled tyramide (1:100, Perkin Elmer) for up to 1 h at room temperature.
7. Wash in KTBT (0.3% Tx) twice for 5–10 min each at room temperature.

D. Protocol 11: Immunodetection of Protein

1. Block the embryos by incubating with 10% goat serum in KTBT for 2–3 h.
2. Incubate embryos overnight at 4 °C with an antibody against the protein you wish to detect. To avoid possible cross-reaction of secondary antibody with the HRP-coupled mouse antibodies used previously, do not allow antibodies to be produced in mouse.
3. Wash the embryos three times for 5 min with KTBT.
4. Wash the embryos 12 times for 20 min with KTBT.
5. Incubate the embryos for 3 h with an Alexa 405-conjugated anti-IgG specific for the species of the primary antibody (1/500, Molecular Probe) in blocking solution.
6. Wash the embryos three times for 5 min with KTBT.
7. Wash the embryos 10 times for 20 min with KTBT.
8. Wash the embryos with PBS and store in the dark at 4 °C in 4% paraformaldehyde or in 50% glycerol.

VI. Photography and Sectioning

For image acquisition, embryos are maintained in 50% glycerol. Low-power photographs of the embryos can be taken on a dissecting microscope with epifluorescence illumination. In addition, embryos can be mounted as previously described to take photographs at higher magnification with a fluorescence microscope. For confocal image acquisition, a microscope with laser emission at 405 nm (UV), 488 nm (FITC), 541 or 561 nm (Cy3), and 633 nm (Cy5) is necessary to get four color images (Fig. 2B and C). Early embryos in glycerol (50%) should be placed without a coverslip in a 35 mm petri dish, the plastic base of which has been substituted by a glass microscope cover. After photographing, the embryos can then be embedded in gelatin/albumin and sectioned using a vibratome. Although both gelatin and albumin autofluoresce, this does not interfere with confocal image acquisition.

VII. *In Situ* Hybridization to Tissue Sections

The method for *in situ* hybridization to tissue sections involves steps identical (probe preparation, embryo fixation) or with simple adaptations (pretreatments, hybridization, washing, and the immunocytochemical detection of probe) to those

used in whole-mount hybridization. As described for whole mounts, precautions should be taken to avoid ribonucleases degrading cellular RNA prior to hybridization. In addition to using autoclaved PBS, we avoid using any slide holders that have been exposed to ribonucleases.

A. Protocol 12: Preparation of Tissue Sections and Subbed Slides

In the method described below, tissue sections are prepared by embedding fixed embryos in paraffin wax, cutting sections, and drying them onto slides that have been subbed with TESPA. An alternative is to cut cryostat sections. To maximize signal, it may be advantageous to cut thick sections.

1. Dissect the embryos and fix them in 4% paraformaldehyde in PBS, overnight at 4 °C.
2. Wash the embryos with PBS, twice for 10 min.
3. Dehydrate by taking embryos through methanol series in PBT (25% methanol, 50% methanol, then 75% methanol) then twice in 100% methanol, for 10 min each. Later stage embryos should be washed for longer to ensure complete dehydration.
4. Equilibrate embryos with toluene, three times for 20 min, then with molten paraffin wax at 60 °C, three times for 20 min, occasionally agitating the vial. Take precautions to avoid breathing toluene fumes.
5. Transfer the embryos to glass embryo dishes (preheated to 60 °C), orientate them with a warmed needle under a dissection microscope and allow the wax to set. Paraffin wax blocks can be stored indefinitely at 4 °C until required for use.
6. On a microtome, cut 6 μm sections as ribbons that are then floated on a bath of distilled water at 50 °C until the creases disappear and collected on TESPA-subbed slides.
7. Dry the sections onto the slides at 37 °C overnight. They can be stored desiccated at 4 °C.

TESPA-subbed slides are prepared as follows:

1. Dip the slides in 10% HCl/70% ethanol, followed by distilled water and 95% ethanol, for 1 min each, and then air-dry.
2. Dip the slides in 2% TESPA (3-aminopropyltriethoxysilane) in acetone for 10 sec.
3. Wash twice with acetone, and then with distilled water.
4. Dry at 37 °C.

B. Protocol 13: Prehybridization Treatments

Prior to hybridization, the sections are dewaxed, permeabilized by proteinase K treatment followed by refixation, and dehydrated. The probe is then spread over the sections under a coverslip. This protocol does not include a prehybridization

blocking step. The same general considerations apply as for whole-mount hybridizations, with the additional factor that overdigestion with proteinase can lead to the sections falling off the slides. Except where otherwise stated, we place the slides in holders suitable for 250 ml slide dishes and use 200–250 ml of the solutions.

1. Dewax the slides in Histoclear, twice for 10 min, and then place them in 100% methanol for 2 min to remove most of the Histoclear.
2. Transfer the slides through 100% methanol (twice), 75%, 50%, and 25% methanol/PBT for 1–2 min in each solution, then wash twice in PBS for 5 min.
3. Immerse the slides in fresh 4% paraformaldehyde in PBS for 20 min.
4. Wash the slides with PBS, three times for 5 min.
5. Drain the slides and place horizontally on the bench. Overlay the sections with 10 μg/ml proteinase K (freshly diluted in PBS from a 10 mg/ml stock in dH_2O) and leave for 5 min.
6. Shake off excess liquid and wash the slides with PBS for 5 min.
7. Repeat the fixation of step 3; the same solution can be used.
8. Wash the slides twice with PBS for 5 min. Dehydrate by passing through 25%, 50%, 75% methanol/PBT, then twice in 100% methanol, for 1–2 min in each solution. Allow to air-dry.
9. Apply the hybridization mix to the slide adjacent to the sections ($\sim$5 μl/cm^2 of coverslip is sufficient) and gently lower a clean coverslip so that the mix is spread over the sections. Hybridization mix and probe are made exactly as described for whole mounts.
10. Place the slides horizontally in a box containing tissue paper soaked in 50% formamide, 5 × SSC, seal the box, and incubate overnight at 55–65 °C.

C. Protocol 14: Posthybridization Washing and Immunocytochemical Detection

The slides are washed and immunocytochemistry carried out under identical conditions as described for whole-mount hybridization. It may be possible to reduce the times given for these steps without affecting background.

1. Place the slides in a slide rack and immerse in prewarmed 2 × SSC, 0.1% CHAPS at 55–65 °C until the coverslips fall off. Gentle encouragement with forceps may be necessary.
2. Wash with 2 × SSC, 0.1% CHAPS, twice for 30 min at 55–65 °C.
3. Wash with 0.2 × SSC, 0.1% CHAPS, twice for 30 min at 55–65 °C.
4. Wash with KTBT, twice for 10 min at room temp.
5. Quickly drain each slide and place horizontally in a sandwich box containing moist tissue paper. Take care that the sections do not become dry, and quickly overlay them with 20% sheep serum in KTBT. Seal the box and incubate for 2–3 h.

6. If desired, the antibody can be preabsorbed as described in Protocol 4.
7. Remove the 20% serum from the embryos, replace with the diluted antibody and incubate in a moist box at 4 °C overnight.
8. Wash with KTBT for 5 min, three times, and then for 30 min, three times.
9. Wash with NTM, three times for 5 min.
10. Incubate in the dark with NTM containing 4.5 μl NBT, 3.5 μl BCIP per milliliter.
11. Occasionally monitor, and when sufficient signal has developed, stop the color reaction by washing with PBT.
12. Fix the signal by immersing the slides in 4% paraformaldehyde in PBS for 2 h, dehydrate quickly through a graded methanol series followed by Histo-clear, then mount under a coverslip using Permount mounting agent.

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CHAPTER 10

Vital Labeling of Embryonic Cells Using Fluorescent Dyes and Proteins

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I. Introduction

Developmental biology strives to understand the establishment of biological form. In almost every example, embryonic development involves groups of cells that once appeared indistinguishable from one another undergoing morphogenetic movements and phenotypic differentiation, thereby becoming different. Thus, this generation of diversity is in many ways the central task of developmental biology, whether studied at the tissue, cell, or molecular level. Progress at any of these levels requires reliable knowledge of the fate map of the embryo and of the cell lineage of single precursor cells. These are closely related experimental questions. Fate maps are depictions of what cells in various regions of an embryo will become during normal development. Cell lineages identify the range of phenotypes that arise from single cells. As an example of the importance of this class of data, consider the development of diverse cell types within a single tissue from an apparently homogenous group of precursors. One extreme possibility is that there is an inherent diversity underlying the apparent homogeneity of the cells; the population of precursors is a heterogeneous mixture of unipotent cells, each fated to become a predetermined cell type. Another extreme possibility is that there is a homogeneous set of precursors, able to give rise to many of the different cell types (multipotent or pluripotent) or to all of the cell types (totipotent) in the tissue. In these two scenarios, the environment of the cells would be proposed to play much different roles: in the case of unipotent precursors, it might play no role or it might play a role only in selecting which cell types survive or differentiate; in the case of multipotent or totipotent precursors, it must play a more instructive role. Thus, an important first step in understanding the cell interactions and molecular mechanisms that guide cell phenotype selection must be to test the potency of the precursor cells.

The experimental requirements for cell lineage and fate map studies are very similar. Both require a means of labeling a cell (or distinct group of cells) in a defined region of the embryo, of identifying the progeny of the labeled cell(s) over time, and of scoring the final phenotypes and positions of the progeny. Of course, the ideal label should be indelible so that all descendants of the labeled cell(s) are identified; similarly, it should be unable to pass to neighboring cells. Failure of either of these qualities would result in false negative cases, where true descendants are missed, or false positive cases, where not all of the labeled cells are true descendants. At present, there is no truly ideal approach, but traditionally two powerful cell marking techniques have been utilized that permit a cell and its progeny to be followed as they move and differentiate: (1) marking the cells with an injectable lineage tracer or (2) marking the cells by infection with a recombinant retrovirus. Intracellular microinjection of the individual cells with fluorescent dextran (Bronner-Fraser and Fraser, 1988; Wetts and Fraser, 1988) or with horseradish peroxidase (HRP) (Holt *et al*., 1988) can be used to trace lineages because both compounds are large and membrane impermeant. Such tracers are passed

from the injected cell solely to its progeny at cell division; thus, all labeled cells must be derived from the injected precursor. In the second approach, precursors are infected with a recombinant retrovirus containing the *lac Z* gene but lacking sequences needed for the infected cell to shed the virus. Therefore, only the descendants of the infected cell will carry the integrated *lac Z* gene (reviews: Cepko, 1988; Sanes, 1989). The two techniques have complementary advantages (for a more detailed treatment, see Fraser, 1992). The site and timing of the marking are both under experimental control in the tracer injection experiments, but the finite amount of injected compound can be diluted by continued mitotic activity. The retrovirus approach avoids the potential pitfall of dilution, but the identification of any given cell as a member of a clone might rely primarily on statistical arguments. In recent years, a third approach has come to light (no pun intended), which capitalizes on the ongoing development of "optical highlighters" or photoactivatable fluorescent proteins (PAFPs) and is rapidly gaining prominence as a method of choice for noninvasively labeling superficial and deep embryonic cells (for reviews see Chapman *et al*., 2005; Lukyanov *et al*., 2005; Shaner *et al*., 2007). While lower phototoxicity and good cellular resolution allow live imaging of the labeled cells, photostability of the photoactivated fluorophore imposes limitations on the length of time for which the labeled cell can be followed.

This chapter presents techniques used for labeling single cells or small groups of cells with fluorescent dyes and proteins. Fluorescent dextran and PAFP labeling of cells offer a direct means to label single cells or small groups of cells, making it appropriate for either fate mapping or cell lineage studies. Lipid-soluble carbocyanine dyes (e.g., DiI and DiA; Honig and Hume, 1986) offer a simpler means to label groups of cells for fate mapping studies; the drawback of the lipid dyes is that it is difficult or impossible to be certain that only a single cell and its progeny were labeled. Although none of the approaches meets all of the criteria of an ideal cell tracer, each can generate useful and valid data if the potential shortcomings are kept in mind.

II. Iontophoretic Microinjection of Lineage Tracers

A straightforward means to trace cell lineage is to microinject a precursor cell with a macromolecule that is trapped within the cytoplasm; the progenies of the injected cell are recognized by the presence of the tracer within their cytoplasm of the marker that they inherit at mitosis. Such an approach, using the enzyme HRP, was developed for tracing early lineages in the leech embryo (Weisblat *et al*., 1978) and was first applied in vertebrate embryos to determine the descendants from the blastomeres of early cleavage stage embryos (Hirose and Jacobson, 1979). Because HRP is an enzyme not found in normal animal tissues, sensitive histochemical stains permit the descendants of the injected cell to be identified after considerable development of the embryo. To permit injected cells to be visualized without the need to fix and process the tissue, fluorescent macromolecules, such as fluorescent

peptides and fluorescent dextrans (Gimlich and Braun, 1985), were developed for use in cell lineage studies. The dextrans have the added advantage that they are not degraded by the labeled cells, permitting lineages to be assayed after long developmental times. Simple refinements in the injection techniques have permitted the intracellular microinjection of fluorescent dextran (Bronner-Fraser and Fraser, 1988; Wetts and Fraser, 1988) or HRP (Holt *et al.*, 1988) to be applied to the much smaller precursor cells of the vertebrate nervous system. In most settings, the sensitivity of the detection of HRP is outweighed by the ability of fluorescent dextran to be observed in living cells. Because of this advantage and because equivalent results have been obtained with both tracers (Holt *et al.*, 1988; Wetts and Fraser, 1988), only microinjection of fluorescent dextrans will be discussed here.

The microinjection approach can satisfy only some of the criteria for an ideal lineage tracer. Because the microinjection technique is under the control of the experimenter, the position and timing of the labeled cell can be dictated. Of course some cells can be extremely challenging to microinject because of their size or their position. If fluorescent dextran is used, an epifluorescence microscope can be used to validate the presence of only a single labeled cell at the desired location. The dye is not taken up from neighboring cells even when the labeled cell is intentionally killed; instead, it is passed from the injected cell solely to its progeny at cell division. Thus, all labeled cells must be derived from the injected precursor. Fluorescent dextran is not degraded by the labeled cells, making the labeling long-lived; however, it is not indelible. Only a fixed amount of the tracer is injected into the precursor, and subsequent growth and division must lead to the dilution of the dye. This dilution can be sufficient to render the dye invisible after several mitoses, although clones as large as 100–1000 can be recovered routinely (Bronner-Fraser and Fraser, 1988; Stern *et al.*, 1988). Because the dextran can be fixed in place for histological processing, the fluorescently labeled descendants can be double labeled by a number of immunocytochemical and *in situ* hybridization techniques.

A. Apparatus for Iontophoretic Dextran Injection

The equipment needed for intracellular dye injection is a straightforward application of the technique long used to record intracellularly from cells with sharp micropipettes. The goal is to introduce the tip of the micropipette through the cell membrane, to iontophoretically inject the dye into the cell, and to withdraw the pipette, leaving a labeled, living cell behind in the embryo. The components of the system needed and their role in achieving this goal are outlined below:

1. Pipette

A pipette is a pulled piece of thin-walled glass tubing that must be sharp enough to enter the cell membrane without severely damaging the cell, yet have a large enough lumen to permit dye to quickly flow into the cell. Of course any pipette is a compromise between these two goals, as discussed in the later section on pipette design.

2. Pipette Holder

The holder plays both a mechanical and an electrical role. It mounts the pipette to the micromanipulator and serves as a "half-cell" that connects the liquid inside of the pipette to the input of the current-passing amplifier. The typical holder has a small reservoir that holds a concentrated salt solution and a silver–silver chloride electrode that connects to the amplifier input.

3. Micromanipulator

The manipulator is responsible for moving the pipette into position and holding it stable during the dye microinjection. Among different users, preferences for types of manipulators can approach a religious fervor. Experience shows them all to be correct. Any manipulator that is convenient for the user and that drifts in position less than 1 μm in a few minutes will suffice.

4. Intracellular Amplifier

A current-passing amplifier built for intracellular electrophysiology is needed to record the membrane potential of the impaled cell and to pass current through the pipette tip. The amplifier measures the potential between the inside of the pipette (the inside of the cell when the pipette tip is in a cell) and an indifferent or reference electrode (a silver–silver chloride wire) placed into the egg white through a small hole in the shell. As discussed later, the membrane potential is a useful method for monitoring the successful impalement of the cell and the health of the injected cell, as well as a diagnostic for the pipette slipping into a second cell. A current-passing capability of 10 nA should suffice for most applications.

5. Oscilloscope

An oscilloscope is used for monitoring the potential recorded by the amplifier. A simple oscilloscope will suffice as the signals are not overly fast ($<$1 kHz) or small ($>$10 mV). The most convenient models to use are digital storage oscilloscopes with the ability to perform a "roll" display. Using such an oscilloscope, new data are added to the right side of the screen, pushing old data off of the left side so that the most recent 20–30 sec of data can be observed at a glance.

6. Specimen Holder

A specimen holder is a mount for eggs (or culture dishes) that stabilizes the specimen on the stage of the microscope. It should cover the condenser lens to protect it from the inevitable spills of saline or egg albumen. In the simplest case, the specimen holder can be a large microscope slide held with the conventional slide-holding device of the microscope stage. The egg is stabilized with a ring of modeling clay or a 1-cm segment of foam pipe insulation.

7. Microscope

A fluorescence microscope permits the pipette to be positioned most accurately and allows the quality of an injection to be measured as soon as the injection is completed. The epiillumination light source should be shuttered and neutral density filters employed to minimize the light exposure to the preparation. This minimizes the bleaching of the dye, hence maximizing the dye signal in the cell and avoiding cell death from the by-products of the dyes being bleached. While some cell types are very resistant to such damage, others can be killed in a few seconds of illumination. Electronic shutters (Uniblitz; Vincent and associates) between the light sources and microscope are best as they permit the light paths to be controlled without directly touching, and hence wiggling, the microscope. To document the dye injection with a minimum of light exposure, a light-intensifying video camera (a SIT or an intensified CCD) can be used to capture images with about tenfold less light exposure than required for film.

B. Tools and Tricks

1. Pipette Design

The pipette must accomplish two opposed goals: it should have a very small tip, with an outer diameter so small that it can be inserted through the membrane of the targeted cell with little or no damage, and it should have a very large inner diameter so that dye can move quickly into the cell cytoplasm from the pipette lumen, allowing the pipette to be removed from the cell before movement of the embryo or the pipette causes damage to the cell. Finding the appropriate compromise between these two can be a major challenge of the approach and may be different for each cell type, and even each stage of development. The availability of thin-walled aluminosilicate glass tubing has made this task somewhat easier. The glass is somewhat harder than conventional borosilicate glass, requiring a higher heat setting on the pipette puller. When pulled properly, the glass yields micropipettes with a very sharp tip and a large inner diameter. An added benefit is that most cell types seem to seal to the pipette tip more quickly, minimizing the deleterious effects of the impalement. The only drawback to the aluminosilicate glass is that it can be more brittle than conventional glass, requiring some care in tip design. Figure 1 presents the double-pull pipette tips that have been proven the most reliable. The goal is to create a rapidly tapering tip that is sufficiently long to reach conveniently into tissue, yet tapers rapidly enough to have sufficient physical strength and low electrical resistance. The first pull of the double-pull design shown in Fig. 1 creates a small-diameter shaft immediately behind the pipette tip, making it easier not only for the pipette to pass through tissue but also to view the preparation as the optical path is less severely distorted by the smaller diameter of the pipette immediately behind the tip. For most preparations, double-pull micropipettes with a resistance of 35–80 MΩ have proved to be the most serviceable.

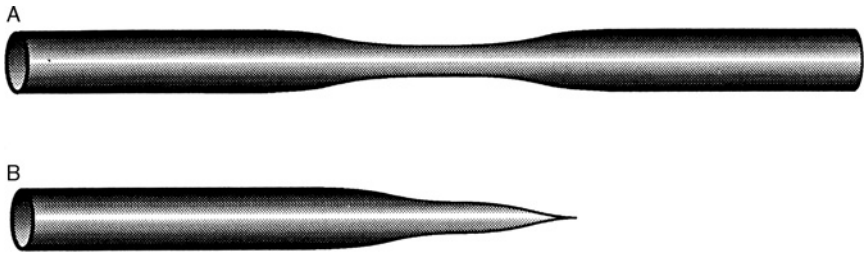

Fig. 1 Pipette tip design. (A) Using the programming feature of the pipette puller to apply a cooling jet of air creates an hourglass shape in the capillary. (B) A second step, in which less cooling and a much greater pull strength is employed, creates a rapid taper tip on the micropipette.

2. Pipette Filling Solutions

For cell labeling, small fluorescent dextran dyes perform very well. The 3- and 10-kDa dyes are too large to pass from cell to cell through gap junctions, yet they are small enough to rapidly pass from the pipette into the impaled cell. Larger dyes are considerably slower to eject from the pipette tip. The dextran dyes are available commercially (Molecular Probes has the largest variety) or can be synthesized using conventional techniques for labeling antibodies or other proteins.

The dextrans are well tolerated by most cells, although some cell types seem more sensitive; some batches of the dye are toxic for such sensitive cell types. Different lots of the same dye, made by the same recipe or purchased under the same catalog number, can vary wildly in their toxicity. The toxicity of any of the dyes can be minimized by ''cleaning'' them before use with Micro-Centricon ultrafiltration tubes (Amicon). Make a 100-mg/ml solution of the dye and place it into the Micro-Centricon tubes. The tubes, when spun in a microcentrifuge, let solutes smaller than their size cutoff (3 kDa) pass through the membrane to the lower chamber. Repeated resuspending of the retained dye in distilled water followed by spinning effectively removes the small by-products that are toxic. After the final spin, resuspend the dye in distilled water to a final volume identical to the starting dye solution. After cleaning, aliquot the dye into 100-µl volumes in sealed tubes and freeze at $-20\ ^{\circ}C$. Rinsing all lots of dye is recommended as rinsed dyes can be injected to much higher concentrations in the cells.

In contrast to the injection solutions most typically used for pressure-injecting substances into cells, which can contain salts and buffers, the best iontophoretic injections are obtained from mixing the dyes in distilled water alone. This is because iontophoresis uses the flow of current to move the dye out of the pipette and into the cell. If the solution contains a significant concentration of salt or buffer, these small, more mobile ions will serve as the major charge carriers, proportionately reducing the expulsion of the dye. With some micropipettes, particularly those with higher tip resistances, the electrical performance of the pipette

may be unstable if there are no small salts in the solution. In those cases, add the minimum amount of salt that will stabilize the electrical performance of the micropipettes.

3. Pipette Filling

The dye solutions or other reagents to be iontophoretically injected can be rare or expensive; therefore, a filling technique that minimizes the volume of filling solution is required. A small amount of the dye solution, about 0.25–0.5 μl, is drawn into a filling pipette and is deposited inside the aluminosilicate pipette at the taper, as close to the tip as possible. Allow a minute for capillary action to carry the dye into the tip and for any air bubbles to be displaced. The micropipettes with the dye in the tip can be stored in a humidified storage jar for a day. Immediately before the pipette is to be mounted on the manipulator, a second filling pipette is used to back fill the shank of the pipette with electrolyte solution. Dilution of the dye and bubbles will be minimized if the tip of the filling pipette is brought close to, but not into, the dye solution (0.5 mm from the meniscus of the dye). A gentle expulsion of the electrolyte from the filling pipette will "layer" the electrolyte solution on top of the dye solution; 1.2 M LiCl is typically employed as the electrolyte because it is less likely to crystallize in the tip of the filling micropipettes between uses.

For handling and depositing small volumes, a clear filling pipette is best because it permits the solution to be observed and deposited accurately. A homemade filling pipette made from 1-cc disposable plastic tuberculin syringes can be used. Remove the plunger from the syringe body and set it aside. Heat the tip of the syringe body immediately next to the flame (not over) of a microburner until the plastic becomes transparent (not flaming); the tip is then pulled slowly away from the syringe body under visual control. The faster the pull, the smaller the diameter of the tip. Becasue the molten plastic provides little resistance, it cannot be pulled by "feel" in the manner typically used for glass tubing. After allowing the plastic to solidify (become translucent), a razor blade is used to cut the tip to length (typically 5 cm). Replace the plunger and press; if air cannot move out of the tip with medium to gentle force on the plunger, the tip is too small in diameter.

4. Iontophoretic Injection

The success of the injections will be maximized by remembering two facts of electrical currents. First, electrical currents only flow in loops. As a result, any break in the pathway from the amplifier, to the electrode holder, through the pipette and preparation, into the indifferent (ground) electrode, and back to the amplifier will be sufficient to prevent the iontophoretic injection. The two most common examples of this problem are air bubbles in the injection pipette or failure to position the indifferent electrode properly. Second, electrical current will take the path of least resistance. Saline or backfilling solution smeared on the outer surface of the injection pipette, on the pipette holder, or near the plug where the

pipette holder joins with the amplifier can provide a pathway of much lower resistance than that through the very small opening at the pipette tip. This can short circuit the current flow into the cell, resulting in failed dye injections even though current appears to be flowing.

C. Intracellular Injection Protocol

1. Pull and fill an injection pipette as outlined earlier and mount it into the pipette holder. Make certain that there are no air bubbles in the injection pipette or in the electrode holder. The electrolyte solution must cover the back opening of the injection pipette to provide a pathway for the current from the amplifier to the injection pipette.

2. Mount the specimen on the stage of the microscope. Insert the indifferent (ground) electrode into the preparation. For *in ovo* injections, the indifferent electrode can be inserted through a needle hole in the end of the chicken egg. Align the specimen so that the targeted region is in the field of view.

3. Turn on the fluorescence epiillumination and coarsely position the injection pipette so that it is above the preparation, in the beam of exciting light. Under visual control, lower the pipette to the preparation. Contact with the saline can be determined by the motion of the meniscus and by the oscilloscope trace. A stable electrical recording is impossible before the electrical continuity provided by the pipette entering the solution. Adjust the oscilloscope trace position with the voltage offset controls to position it in the upper half of the oscilloscope screen.

4. Position the pipette with the micromanipulator directly above the cell to be injected and turn off the epiillumination source. Lower the electrode slowly, watching the preparation in bright-field and/or the oscilloscope screen. Contact of the pipette tip with the surface of the cell typically causes a slight deflection of the oscilloscope trace and an increase in the width (noise) of the trace.

5. ''Ring'' the pipette tip with the negative capacitance control on the injection amplifier as briefly as possible. This causes a brief electrical oscillation in the amplifier and is thought to facilitate impaling the cell by slightly wiggling the pipette tip and/or destabilizing the membrane structure. Once inside the cell, these currents can kill the cell so make the ring as brief as possible. Some amplifiers have a special control to provide an instantaneous ring. Those using amplifiers without such a circuit develop very quick wrist action on the negative capacitance knob. Some workers prefer to ''tap'' into a cell. This requires finding a spot on the vibration table, microscope, or manipulator where a small tap causes a small wiggle of the pipette. For injecting chicken eggs, this is hard to control as even small taps can slosh the contents of the opened egg.

6. In an ideal cell penetration (Fig. 2), the potential recorded through the pipette drops precipitously after the ''ring'' to a new stable value reflecting the cell membrane potential. A sharp transition shows that the cell membranes have quickly sealed to the pipette tip. The value recorded varies with the cell type,

stage of the embryo, temperature of the egg, and pipette design, but is typically between 10 and 100 mV. The recorded value is not an accurate measure of the cell membrane potential because the dextran solutions are not ideal electrolytes. However, the cell membrane potential reported can be used to monitor the health of the cell, as well as warn of the pipette moving to another cell. Figure 3 presents the variety of real-world traces that might be obtained on the oscilloscope.

7. The dye is iontophoresed into the cell with pulses of current, typically positive currents of 2–10 nA at frequencies of 0.5–2 Hz. The periods during the current flow eject the dye, and the periods between pulses can be used to monitor the cell membrane potential. Although some amplifiers are equipped with bridge circuits to permit the potential to be recorded during the pulses, the dextran-filled micropipettes do not perform well in this mode because the dextran is not a well-behaved electrolyte. As a result, the resistance of the pipette tip varies with the flow of current, making it difficult, if not impossible, to properly set the bridge. If better performance is required, some smaller ions must be added to the solution, but this will decrease the iontophoretic injection of the dextran.

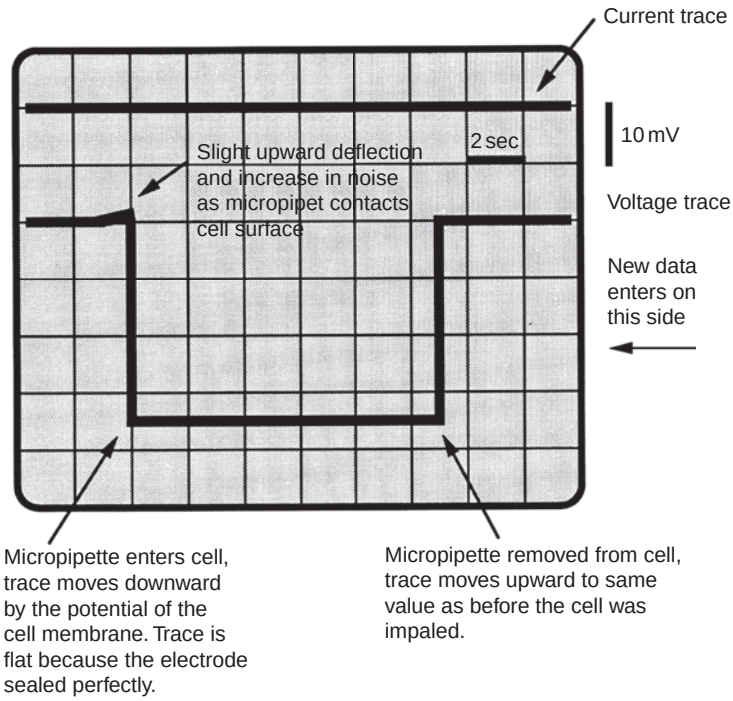

Fig. 2 Ideal oscilloscope trace. The sketch shows the appearance of the oscilloscope screen if a digital storage oscilloscope with a roll display is employed. Data continuously enter on the right, pushing previous data toward the left. In an actual injection experiment, pulses of current would interrupt the current and voltage traces; these have been omitted here for clarity.

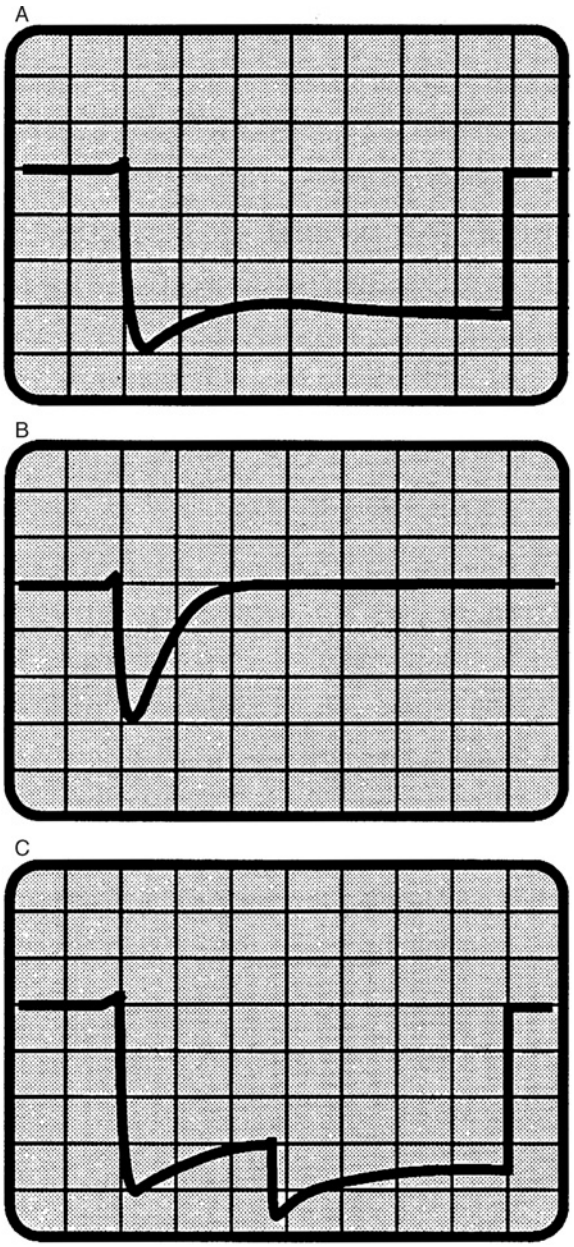

Fig. 3 More realistic examples of oscilloscope traces. (A) This trace shows a nearly ideal injection sequence. The micropipette sealed in rapidly, resulting in only a slight loss of the membrane potential of the cell, followed by a later downward slope of the trace. (B) A stable membrane potential is never recorded in cases where the cell dies or the pipette falls out of the cell before sealing in place. (C) A failed lineage injection is indicated by the sudden change in the potential recorded by the micropipette. When the micropipette drifts between cells in this fashion, at least two labeled cells can be expected.

The shape of the voltage trace can be used as a method to monitor the electrode tip. If the voltage trace does not return to a somewhat stable value within the first 0.1 sec after a current pulse, the pipette is probably plugging; if it returns immediately, the tip may have broken off.

Some robust cells can be injected quickly with current from continuously "ringing" the pipette with negative capacitance. This should be used with care on small embryonic cells as it kills most of them. Some cell types are too fragile to inject with pulses of current. These are best injected by slowly ramping up the current to 2–4 nA, although it is difficult to measure membrane potential during such injections.

8. The duration of dye iontophoresis varies with a number of factors, including the size of the dextran (smaller = faster), the size of the injection current (larger = faster), and the size of the cells (smaller = faster). For most embryonic cells, 20–30 sec at 4 nA should suffice. Turn off the current and wait for the electrode potential to stabilize.

9. Remove the pipette quickly by turning the knobs of the micromanipulator to a predetermined amount. A distance of two- to three-cell diameters or more, directly along the axis of the pipette, works best. This snaps the pipette free from the cell membrane, allowing it to reseal quickly. Moving the pipette away more carefully and slowly increases the chance of tearing the cell open or of plucking the cell from the tissue by it remaining adhered to the pipette tip. Note the size of the potential change recorded by the pipette on exiting the cell; ideally, it should be close to the transition recorded on entering the cell (see Figs. 2 and 3).

10. Turn on the fluorescence epiillumination light source and focus on the labeled cell(s) quickly. Score the presence of a single cell and its position. Those embryos with more than one cell cannot be used for cell lineage studies but can be used for fate mapping or cell migration studies.

III. Iontophoretic Application of DiI

Despite the many advantages of iontophoretic injection of fluorescent dextrans, there are several factors that limit its use, ranging from the difficulty of impaling fragile embryonic cells to the considerable expense of the equipment required. Iontophoretic application of the fluorescent lipids DiI and DiO offers a much simpler and less expensive alternative for those experiments in which single-cell lineage data are not required. Iontophoretic application is a refinement of the DiI microinjection techniques covered in other chapters. Although in some settings, it has been used to label single cells reliably (Myers and Bastiani, 1993), in the avian embryo, iontophoretic application of DiI is most reliable in labeling from 2 to 30 cells. DiI labels cells brightly and appears to be less phototoxic than fluorescent dextran, making it well suited for time-lapse studies and *in vivo* microscopy. A red-shifted carbocyanine dye, DiI(5) (excited with red light; emits in the infrared) performs very

well in this regard. The spectra of DiI(5) are ideally suited to the red excitation line of an Ar–Kr laser on a confocal microscope, and background fluorescence is low at these wavelengths. The dye bleaches very slowly; even prolonged viewing does not seem to cause cell death. The availability of DiI-CM, a fixable derivative of DiI, solves one of the long-standing limitations of DiI for use with immunocytochemistry or *in situ* hybridization studies.

A. Apparatus for DiI Iontophoresis

The apparatus for DiI iontophoresis can be as complicated as that used for the dextran injections, but excellent results can be obtained with very simple equipment. The injection pipette is not placed intracellularly so there is no need for an oscilloscope or recording amplifier. A simple current source of a 9-V alkaline battery in series with a 100-MΩ resistor suffices; a push button switch or a foot switch can be used to turn the current on and off. Because the dye is being applied to the surface of the cell(s), the pipette can even be held by hand rather than by a micromanipulator.

B. Iontophoretic Application Protocol

1. Pull injection micropipettes in the same fashion as for dextran injections. The ideal tip should be slightly less fine than used for dextran injections (10 MΩ if filled with the dextran solution). Do not break off the tip as typically done for pressure injections.

2. Backfill the tip and about 5 mm of the electrode shank with a 0.5% solution (w/v) of DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine, perchlorate; Molecular Probes, in absolute ethanol). Mount the pipette into a pipette holder with a silver wire that runs down the pipette shank and touches the DiI solution. Unlike for dextran injections, the silver wire does not need to be chloridized.

3. The indifferent (ground) electrode should be inserted into the egg as for dextran injections. The rules of current apply here as well, so apply the same cautions about alternate current paths.

4. Lower the pipette tip into the preparation and close the switch, allowing current to flow for 2–30 sec. Examine the preparation under epiillumination. Some workers find it convenient to monitor the labeling process continuously with the epifluorescence microscope. In such cases, it is important to monitor the preparation for possible phototoxic effects such as cell blebbing or beading.

5. If no labeled cells result, check for a plugged tip by examining the injection pipette with an epifluorescence microscope. A plugged tip usually has a small crystal of dye fluorescing a different color and brightness. Although it may seem counterintuitive, making the pipette tip sharper and smaller reduces plugging of the micropipettes. A larger tip permits the alcohol to leak out of the tip and water to leak in, precipitating the dye.

IV. Relative Advantages of Dextran and DiI

The two dye-labeling approaches share some of the same limitations. For example, both can be diluted to the point that they are no longer detectable by prolonged mitotic activity. The advantages of the dextran technique are that it can reliably label single cells and that the dextran dyes do not transfer from cell to cell. Injecting large amounts of dextran into the extracellular space or intentionally killing a brightly labeled cell does not result in labeled neighbors. The advantages of DiI or related carbocyanine dyes are the ease with which they can be applied and the reduced phototoxicity they exhibit. Perhaps the most reliable way to exploit both sets of advantages is to employ both approaches and compare the results.

V. Photoactivation of Fluorescent Proteins in Single Cells

Since the demonstration of heterologous expression of the jellyfish *Aequorea victoria*, green fluorescent protein (GFP) in the bacteria *Escherichia coli* and in the nematode worm, *Caenorhabditis elegans* more than a decade ago (Chalfie *et al*., 1994; Inouye and Tsuji, 1994), its biological applications have proven manifold and pervasive (for reviews, see Haseloff and Siemering, 2006; Hastings and Morin, 2006; Hazelrigg and Mansfield, 2006; Hitchcock *et al*., 2006; Phillips, 2006; Shimomura, 2006; Tsien, 1998; Valdivia *et al*., 2006; Ward, 2006; Ward and Lippincott-Schwartz, 2006; Zacharias and Tsien, 2006). Detailed mutational and crystallographic analysis of its primary, secondary, and tertiary protein structure and corresponding spectroscopic output has enabled its potential to be exploited at many levels (Cubitt *et al*., 1999; Phillips, 2006; Prendergast, 1999). In its unaltered state, it can function as a reporter of gene expression or provide information on the subcellular localization of a protein it is fused to (Misteli and Spector, 1997). Its deliberately modified forms have a plethora of functions including their use as fine-tuned pH and calcium sensors, indicators of protease activity and detectors of closely associated proteins in the cell via fluorescence resonance energy transfer (March *et al*., 2003; Nehrke, 2006; Tsien, 1998). In its latest avatar, created by mutating selected residues in the chromophore or its proximity, the fluorescence of GFP can be specifically activated by a particular wavelength and intensity of light (Patterson and Lippincott-Schwartz, 2002; Sawin and Nurse, 1997; Yokoe and Meyer, 1996).

This landmark discovery of "photoactivatable" GFP (PAGFP) has since led to a burgeoning class of fluorescent proteins known as PAFPs whose spectroscopic properties such as absorption and emission spectra can be altered at will (see Table I; Chapman *et al*., 2005; Lippincott-Schwartz and Patterson, 2008; Lukyanov *et al*., 2005; Shaner *et al*., 2007). Next generation fluorescent proteins viz. yellow and red-shifted GFP homologues found in coral reef organisms (class Anthozoa) have also been tapped for their ability to be photoactivated. Based on their mechanism of activation, PAFPs can be classified in three groups

(Henderson and Remington, 2006; Lukyanov *et al*., 2005). The first group includes PAGFP (Patterson and Lippincott-Schwartz, 2002) and photoswitchable cyan fluorescent protein (PSCFP) (Chudakov *et al*., 2004) that when activated by ultraviolet (UV) light results in a large increase in green fluorescence (~510–520 nm) when excited with blue light (~460–480 nm). Photoactivatable monomeric red fluorescent protein (PAmRFP) on the other hand undergoes a modest increase in red fluorescence when activated by UV-light (Campbell *et al*., 2002). A second group of PAFPs including Kaede (Ando *et al*., 2002), EosFP (Wiedenmann *et al*., 2004), and KikGR (Tsutsui *et al*., 2005) undergoes an irreversible green to red conversion on illumination with UV to violet light. Dendra, which is similarly dual-color, does not belong to this group of proteins as it is not activated by UV-violet light, but rather by the commonly used blue 488-nm laser line (Gurskaya *et al*., 2006). Finally, the PAFPs, kindling fluorescent protein-1 (KFP-1) (Chudakov *et al*., 2003), and Dronpa (Ando *et al*., 2004) are unique in their ability to undergo reversible photoactivation. KFP-1 does not fluoresce of its own accord; instead when illuminated with low-intensity green light, it ''kindles'' and temporarily emits red fluorescence but in the absence of this light, it spontaneously reverts back to a dark state (Chudakov *et al*., 2003; Henderson and Remington, 2006). Interestingly, when illuminated with high-intensity green light for a sustained period of time, it undergoes irreversible conversion to a red fluorescent protein; in fact, this fluorescence can last for as long as a year if kept in the dark (Chudakov *et al*., 2003). Unlike KFP-1, Dronpa (''dron'' comes from a ninja term for vanishing and ''pa'' stands for photoactivatable) cloned from a coral Pectinidiiae is only capable of reversible photoswitching (Ando *et al*., 2004). In its native conformation, it fluoresces green (emission at 518 nm). When the fluorescence of the protein is visualized by strong excitation at around 490 nm, the protein bleaches rapidly. However, the extinguished protein is able to reacquire its bright fluorescence when excited using 405-nm light. It will once again revert to its dark state if imaged at this time. This reversible photoswitching, which occurs on a millisecond timescale, can be carried out greater than a 100 times without any appreciable loss in fluorescence intensity. It has been used to demonstrate fast protein dynamics such as nucleocytoplasmic shuttling of ERK1-Dronpa fusion protein (Ando *et al*., 2004). More recently, it has also been used to reconstruct neuronal connectivity in the zebrafish embryo (Aramaki and Hatta, 2006). The mechanism of photoactivation or photoconversion remains incompletely understood and varies depending on the protein in question. In some cases, it is thought to involve a *cis–trans* isomerization of the chromophore buried within the protein (Henderson and Remington, 2006) while in the case of PAGFP, for example, a single point mutation is sufficient to alter its state to a predominantly anionic form which on activation with UV-light shows increased absorbance at a lower wavelength (Patterson and Lippincott-Schwartz, 2002).

Using PAFPs to label single cells has distinct advantages over existing techniques, including the ability to mark a cell deep within an embryo and sustain individual cell health with minimal invasiveness. Additionally, a PAFP such as

Table I
A Comparison of the Spectral Qualities of Selected Photoactivatable Fluorescent Proteins for Their Use in Chick Embryos (Adapted from Lukyanov *et al.*, 2005)

PAFP characteristics	PAGFP	Kaede	PSCFP2	KFP1	EosFP	KikGR	Dendra
Source of fluorescent protein	*Aequorea Victoria* (hydrozoa)	*Trachyphyllia geoffroyi* (anthozoa)	*Aequorea coerulescens* (hydrozoa)	*Anemonia sulcata* (anthozoa)	*Lobophyllia hemprichii* (anthozoa)	*Favia favus* (anthozoa)	*Dendronephthya* (anthozoa)
Oligomeric state	Monomer[¶]	Tetramer[§]	Monomer[¶]	Tetramer[§]	Tetramer/dimer[§d]	Tetramer[§]	Monomer[¶]
Activating wavelength	UV-violet (405 nm)[§]	UV-violet (405 nm)[§]	UV-violet (405 nm)[§]	Green (532 nm)[¶]	near UV-light (390 nm)[§]	UV-violet (405 nm)[§]	Blue (488-nm laser line)[¶]
Change in absorbance spectrum (nm)	400–504	508–572	400–490	Increase at 590	506–571	507–583	486–558
Change in emission spectrum (nm)	Increase at 517 (faint to bright green)	518–580 (green to red)	470–511 (cyan to green)	Increase at 600 (none to bright red)	516–581 (green to red)	517–593 (green to red)	505–575 (green to red)
Reversibility of photoactivation	Irreversible	Irreversible	Irreversible	Reversible and irreversible	Irreversible	Irreversible	Irreversible
Time to reach maximum photoconversion (sec)	837.25[a]	107.02[a]	4008[a]	N/A	60[b]	37.7[a]	<60[c]
High brightness	√√	√√√	√√	√	√√√	√	√√√
High contrast	200-fold	2000-fold	>2000-fold	N/A	ND	2000-fold	1400-fold
Dual color fluorescent form	No	Yes	Yes	No	Yes	Yes	Yes
Utilized in chick embryos[e]	Yes	Yes	Yes	No	No	Yes	No
Reference	Patterson and Lippincott-Schwartz, 2002	Ando *et al.*, 2002	Chudakov *et al.*, 2003	Chudakov *et al.*, 2003	Wiedenmann *et al.*, 2004	Tsutsui *et al.*, 2005	Gurskaya *et al.*, 2006)

Advantages ([¶]) and disadvantages ([§]) of photoactivatable fluorescent proteins. N/A, not applicable; ND, not determined.

[a]Data obtained from Stark and Kulesa (2007); recorded with 2% 405-nm laser power.

[b]Data obtained from Wacker *et al.* (2007).

[c]Data obtained from Gurskaya *et al.* (2006).

[d]EosFP has also been engineered to be monomeric (mEosFP); however, this protein can fold and function only below 30 °C, making it impracticable to use in the chick embryo.

[e]See Stark and Kulesa (2007).

PAGFP can be re-excited with 405-nm light to extend the duration of the increased fluorescent signal in a cell. The DNA constructs for the photoactivatable proteins are relatively easy to obtain either from private or commercially available sources and the method of introducing them into cells via electroporation is also relatively straightforward; the challenge is the process of photoactivation and subsequent imaging which requires highly specialized equipment viz. scanning confocal microscopes or two-photon microscopes with the appropriate laser lines for photoactivation and photoconversion. Another significant drawback in contrast to the use of DiI and dextrans is the lack of immediacy. The DNA construct for the PAFP has to be transcribed, translated, and the protein has to accumulate to high enough levels before photoactivation or photoconversion can begin. Thus, there is a significant time lag between when the PAFP construct is first introduced into cells and when cells with photoactivated proteins can be followed.

A. Considerations in Choosing the Optimal PAFP

The number of PAFPs available to the scientific community has mushroomed since the generation of PAGFP in 2002. Table I summarizes key information about some of the photoactivatable/photoswitchable proteins that can be utilized for cell fate and cell lineage analysis in the chick embryo. This table does not include Dronpa and PAmRFP1 as both are better suited for following protein dynamics within a cell. The parameters to keep in mind when choosing a suitable PAFP for your application include:

1. Maturation time of the protein:

This would encompass the time it takes for the protein to be produced at high enough levels, to fold, and to reach peak fluorescence. For most PAFPs listed in Table I, this can take between 5 to 10 h.

2. Photoefficiency of photoactivation or photoconversion:

When tested in the chick embryo, both Kaede and KikGR are particularly fast at reaching maximal photoconversion while PSCFP2 can take over an hour to undergo cyan to green photoconversion (Table I), at the same low level (2%) of 405-nm laser power. These time-frames can sometimes be species-specific and equipment dependant.

3. Photostability:

Another critical parameter in choosing an appropriate PAFP is its photostability once activated. In this regard, both PAGFP and PSCFP2 have been shown to be the most stable when imaged repetitively. In addition, PSCFP2 also allowed photoactivated cells to be distinguished from nonphotoactivated cells for the longest period (48 h). PAGFP was distinguishable in photoactivated cells for 24 h without reactivation while the fluorescence intensity ratio of Kaede dropped sharply and cells in which it was activated could be distinguished only up to 14 h (Stark and Kulesa, 2007).

B. Apparatus for Photoactivation of Fluorescent Proteins

1. PAFP construct:

PAGFP and EosFP, are not commercially available. These constructs can be obtained from the laboratories in which they were generated (Table I). PSCFP2, Dendra and KFP1 are both available from Evrogen while MBL International sells Kaede, KikGR, and Dronpa.

2. Equipment required for electroporation of chick embryos is detailed in Chapter 5 by Krull and Tosney and Chapter 12 by Sauka-Spengler and Barembaum, this volume, while the material required for time-lapse imaging *in ovo* and in culture is detailed in Chapter 11 by Ezin and Fraser, this volume.

3. Fluorescence stereomicroscope with halogen light source and appropriate filters (LP-DAPI, TRITC; Leica).

4. Confocal inverted or upright laser scanning microscope with 488- and 405-nm laser lines (Zeiss LSM5 PASCAL or Zeiss LSM 510) and cell tracking software (optional).

C. Photoactivation Protocol

To date, four of nine PAFPs, discussed above, have been utilized to label single cells or groups of cells in chick embryos (Table I, Stark and Kulesa, 2005, 2007). These include PAGFP, PSCFP2, KikGR, and Kaede (Stark and Kulesa, 2007). Because PAGFP is the best-studied and most widely used PAFP at present, we provide a protocol to photoactivate PAGFP in individual cells (Fig. 4). However, parameters for photoactivating PSCFP2, KiKGR, and Kaede will be briefly mentioned (for details refer to Stark and Kulesa, 2007).

1. Plasmid DNA containing the coding sequence of the photoactivatable protein of choice is electroporated at a concentration of 5 μg/μl into the chick embryo at an appropriate stage and location (refer to Chapter 5 by Krull and Tosney and Chapter 12 by Sauka-Spengler and Barembaum, this volume for details regarding the most effective strategy to target different cell populations by electroporation either *ex vivo* or *in vivo*). If PAGFP (only dimly fluorescent before activation) is being utilized, then electroporate in a 1:1 ratio with a tracer construct such as H2B-mRFP, that is, a fusion protein construct with a different emission spectrum. Coelectroporation is highly recommended in this instance as it can control for electroporation conditions as well as highlight the region where the PAGFP-expressing cells are likely to be. Most cells will receive both constructs; however, a few cells will receive only one or the other of the constructs. This control is not necessary for dual-color PAFPs such as PSCFP2, KikGR, and Kaede.

2. Cells in older embryos can be photoactivated either in an explant culture system or *in ovo* (see Fig. 4; refer to Chapter 11 by Ezin and Fraser, this volume for protocols for imaging *in vivo* or *ex vivo*). Note that in New culture or in the explant

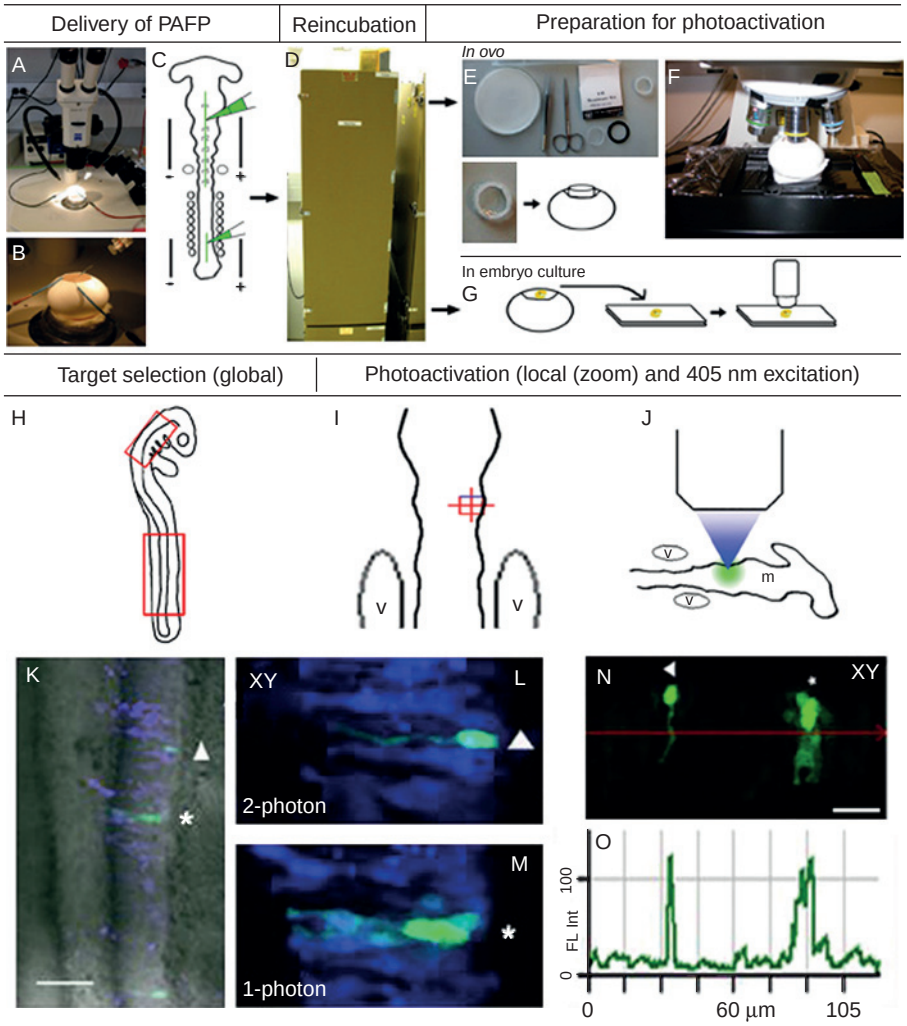

Fig. 4 Photoactivation of fluorescent proteins in the chick embryo. (A–C) Delivery of photoactivatable fluorescent proteins (PAFP). (A) *In ovo* injection and electroporation of PAFP constructs using a streomicropscope and platinum electrodes. (B) Higher magnification of panel (A). (C) Schematic representation of injection and electroporation of PAFPs into the neural tube of a HH stage 8 chick embryo to label premigratory neural crest cells. Electrodes are placed on either side of the embryo to target the construct to one half of the neural tube. PAFPs can either be injected into the cranial and/or trunk region of the embryo. (D) After *in ovo* delivery of PAFPs, the eggs are resealed and reincubated in a humidified 37 °C incubator until desired stage for photoactivation. (E–G) Preparation for photoactivation. (E) Supplies needed to prepare Teflon membrane to place over the embryo for *in ovo* photoactivation. From left to right: beeswax, forceps, scissors, Teflon membrane sheets, plastic ring, rubber *o*-ring. Lower panel shows prepared Teflon membrane sealed around the plastic ring using beeswax and a schematic showing placement over the embryo *in ovo*. (F) Preparation of microscope stage for *in ovo* photoactivation. Plastic wrap is placed underneath the microscope stage to catch any

culture system, the embryo is dorsal side down: therefore, activation and imaging requires an inverted confocal microscope. Photoactivation and imaging in an *in ovo* situation necessitates the use of an upright confocal microscope such as the LSM 5 PASCAL or the LSM 510. If using an *ex vivo* setup, place the culture dish onto the microscope stage and bring the sample into focus using a low-magnification objective, for example, using a 10× or 20× objective lens. Use a mercury lamp source and an appropriate filter cube to locate the population of cells expressing the control construct since expression of PAGFP is very dim 8–10 h after electroporation and is only slightly above normal autofluorescence levels. A long-pass DAPI filter can be used to view it, as long as prolonged exposure to UV light is avoided. In fact, for PAGFP, Kaede, and KikGR, cells expressing fluorescence can be located using 488-nm laser excitation at low laser power (1–10% for PAGFP, 1–2% with Kaede and KikGR) with a 10×/0.3 Zeiss objective at 512 × 512 pixels. This avoids any inadvertent photobleaching and/or photoconversion. However, PSCFP2-expressing cells (cyan fluorescence prior to photoactivation) should be located using 405-nm laser excitation at 1–3% laser power.

3. If there is an option for multitrack excitation scanning available with the confocal imaging software, choose a multitrack configuration to allow emission pre- and postphotoactivation to be captured as well as in the case of PAGFP to include GFP and any excitation that is required for highlighting the host cell population. The multitrack configuration will create an image without any bleed-through from the two constructs.

4. To create an image of PAGFP-expressing cells, turn up the detector gain, pinhole diameter, and apply four to eight averages to eliminate noise. If the expression is still too low to visualize, then increase the laser power past 10% on an argon laser.

5. To target an individual cell for photoactivation, continue to zoom in on a particular region. To focus on an individual cell, crop the region of excitation and scan around the area of the cell. The PAGFP construct is located in the cytoplasm and will diffuse rapidly during and postphotoactivation. The selected

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media or albumin that may leak. Shown here, an upright Zeiss LSM5 Pascal. Egg is placed on the microscope stage with Teflon membrane in place over the embryo, and a 10× objective is slowly focused down on embryos. (G). Alternatively, the embryo can be removed from the egg, transferred to a Mat-tek dish with a glass bottom, and covered from above by a Teflon membrane that rests on spacers of silicon grease to reach a height above the embryo, then photoactivated. The embryo can then be cultured in an incubator or on a heated microscope stage for up to 6 h. For longer culture periods (up to 24 h), the photoactivation can take place while the embryo rests on a culture insert inside a sealed six-well plate with culture media and sterile water. (H) Global target selection of the embryo. (I) Local target selection of cranial neural tube cells, shown by red box and cross hairs. (J) 405 nm excitation of region selected in cranial neural tube. Objective positioned above embryo and green circle depicts area of photoactivation by 405 nm excitation (blue). (K) Single-cell photoactivation of PSCFP2 in the chick neural tube using (L) two-photon and (M) single photon and (N and O) comparison to show photoactivation due to out-of-plane confocal laser light. Scale bars = 50 μm (K) and 20 μm (N), r, rhombomere; v, otic vesicle; m, midbrain. (See Plate no.10 in the Color Plate Section.)

cell does not have to be completely located inside the cropped box before photoactivation can occur.

6. To photoactivate PAGFP within a cell, use a 405-nm laser with a power setting between 2% and 5%. Then, scan a single image to photoactivate the PAGFP within the cell. A single scan may not be enough to optimize the photoactivation.

7. Evaluate the increase in mean GFP fluorescence in the cell by following the 405-nm excitation scan with a 488-nm excitation scan. A second or third scan at 405 nm may be necessary. Maximum photoconversion of PAGFP within a cell can be reached by monitoring the line intensity profile after each 405-nm excitation scan. Repeat as needed for other cells in the embryo. As the image is zoomed in, the power from the laser increases by:

$$\text{laser dosage} = \frac{\text{laser intensity} \times \text{scan time}}{\text{scan area}}$$

The higher the zoom, the lower laser power required. It is recommended to have 2–3% (5–15% for PSCFP2) laser power if greater than or equal to 35× zoom and 3–5% (15–30% for PSCFP2) laser power if less than 34× zoom. If photobleaching occurs immediately, the 405-nm power is too high and must be lowered accordingly for the subsequent cell(s). The current cell is no longer suitable for photoactivation. Often, photoactivated cells become so bright that they are overexposed. At this point, the detector gain and pinhole can be reduced to create a more acceptable confocal image. If laser power levels were increased, then begin by reducing the power level.

8. Once photoactivation is complete in a single or a number of cells in an embryo, begin collecting time-lapsed images or reincubate the embryo for cell lineage studies. For explanted embryos (as per protocol in Chapter 11 by Ezin and Fraser, this volume), add 0.5 ml culture medium to the well. If the culture insert begins to float, remove some medium so that the culture insert sits on the bottom of the dish. Wrap the dish in parafilm and place in a 37 °C incubator for the designated amount of time. Alternatively, to start imaging directly postphotoactivation, place the six-well culture dish or the egg on the confocal microscope with a specially designed heater box around it.

Configure the confocal imaging software to collect images at a desired time interval. A minimum of a 2-minute interval setting between time points is recommended.

D. Troubleshooting

In general, once photoactivated, PAGFP proves to be fairly stable when scanned consistently using 488-nm light. This makes it a good candidate for four-dimensional imaging. However, photoactivated cells may be virtually indistinguishable within a short time period (less than 24 h) for a number of reasons. Among these are cell death, migration deep into the embryo, suboptimal photoactivation, and photobleaching as a consequence of persistent photoactivation at

high magnification or zoom using the 405-nm laser. In order to ensure optimal photoactivation, electroporated cells should be given sufficient time to allow the PAFP to be expressed at high levels and to reach peak fluorescence prior to photoactivation. To prevent premature photobleaching, it is a good idea to start the experiment with low-power laser or use an objective with low magnification such as a 10×0.3 NA Plan Neofluar objective. Unfortunately, once cells start to photobleach, they are no longer suitable for photoactivation. However, if photoactivated cells have not started photobleaching, they can be reactivated. While this may not lead to another 100-fold intensity increase, a strong discrimination will still be observed in comparison to nonphotoactivated cells.

VI. Conclusions and Emerging Technologies

In conclusion, there are a number of methods available to label single cells as well as small populations of cells in the chick embryo. While intracellular injection of rhodamine dextran is a very reliable technique for labeling individual cells, it is also a highly invasive technique. Consequently, cell death occurs in more than half the injected cells. DiI on the other hand is less invasive instead of noninvasive, but is best used for labeling more than one cell. Finally, PAFPs provide both a noninvasive and reliable way to label single cells (particularly when used in conjunction with two-photon excitation) or populations of cells; however, the equipment required is highly specialized and expensive. Both DiI and photoactivation of fluorescent proteins have the added advantage that they can be combined with live cell imaging to enable dynamic lineage analysis. An up and coming trend in fluorescently labeling cells and vital imaging is the use of small semiconductor nanocrystals known as quantum dots. So far, there has been only one report of its biocompatibility with chick embryos where it was used to visualize the chorioallantoic membrane vasculature (Smith *et al.*, 2007), but it is only a matter of time before it becomes a more widely used technology. Combining the spectral range of quantum dots—which is virtually endless as their size governs their emission properties and their size can be very finely engineered—with a microscope that can detect colors separated by a few nanometers in wavelength such as the Zeiss LSM Meta could enable us to label hundreds of cells with different colors and watch their interactions in real time: a dream come true for developmental biologists.

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CHAPTER 11

Time-Lapse Imaging of the Early Avian Embryo

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I. Introduction

Morphogenesis transforms the embryo from a simple geometrical shape (a disk in the case of the avian embryo) into a complex, functioning organism. This transformation requires a range of dynamic events from the molecular level to the tissue level, resulting in specific cell numbers, locations, shapes, and fates. As they execute their behavior, cells exert mechanical forces that reshape tissues and ultimately change the appearance of the developing embryo as a whole (Farge, 2003; Wallingford *et al.*, 2002). Similarly, cell lineages can be analyzed directly by following the descendents of labeled progenitors over time (Stern and Fraser, 2001). In each of these brief examples, time-lapse imaging offers the most powerful tool for the collection of data. Time-lapse analysis records a series of still images of a developing embryo over time. When aligned and assembled into an animated sequence, time-lapse imaging reveals surprisingly dynamic events, which could be captured with no other methods. Filming the development of the early chick embryo has a long history (Bortier *et al.*, 1996), starting with the filming of gastrulation *in ovo* (Gräper, 1929). A great many other studies have been performed such as the movements of precardiac cells (De Haan, 1963), the regression of the node (Vakaet, 1967), and the spatiotemporal occurrence of cell division (Stern, 1979).

By combining live imaging with molecular experiments and microdissection, it has been possible to experimentally address the role(s) of given molecules or cell types in morphogenesis. Thus, time-lapse imaging revealed the origin of the primitive streak and endoderm (Stern, 1990), as well as the movements preceding and associated with the formation of the streak during blastula stages (Cui *et al.*, 2005; Chuai *et al.*, 2006; Voiculescu *et al.*, 2007). During gastrula stages, the ingression of primitive streak is regulated by chemotactic signals from FGFs (Fibroblast Growth Factor; Yang *et al.*, 2002), while somite formation occurs through ingression of epiblastic tail bud and regressing streak cells accompanied by movements of the extracellular matrix (Filla *et al.*, 2004; Iimura *et al.*, 2007). During somite boundary formation, cells can move across the forming boundary into regions of different gene expression (Kulesa and Fraser, 2002). Neural tube closure in chick occurs from anterior to posterior, involves interaction with the lateral epidermis, and variable mode of head and neural fold formation at the

anterior neuropore (Brouns *et al.*, 2005; Moury and Schoenwolf, 1995). Segmentation of the closed tube into eight rhombomeres occurs through a combination of growth and constriction (Kulesa and Fraser, 1998a), while neural crest cells interact with one another during migration away from the neural tube toward branchial arches and to form ganglia (Krull *et al.*, 1995; Kulesa and Fraser, 1998b, 2000; Kulesa *et al.*, 2000, 2005; Kasemeier-Kulesa *et al.*, 2006; Rupp and Kulesa, 2007). In older embryos, organogenesis of the heart shows abnormal looping in embryos with extirpated cardiac neural crest (Yelbuz *et al.*, 2002). Thus, time-lapse imaging of the early embryo is versatile and adaptable to many early embryonic stages and questions.

A. Advantages of the Chick Embryo

The early avian embryo (blastula and gastrula) is well suited for time-lapse imaging because it is relatively transparent, flat as it develops quickly outside the mother. Its transparency is sufficient to permit the visualization of cells beneath the surface, especially if those cells are fluorescently labeled. The flatness of the embryo facilitates maintaining the embryonic tissues in focus during time-lapse imaging. In addition, because the embryo is planar, imaging either ventral or dorsal surface is relatively simple, requiring only that the embryo be oriented with the surface of interest facing the microscope objective. Versatile culture methods, compatible with time-lapse imaging, allow its development *in vitro*. Finally, chick embryos develop quickly, completing blastulation, gastrulation, and the early steps of neurulation in approximately 36 h. These factors make the avian embryo ideal for time-lapse imaging.

B. Constraints of the Chick Embryo

Disadvantages of the avian embryo are associated with its culture. Earlier than Hamburger and Hamilton (HH) stages 7–8, explanted chick embryos must be cultured dorsal side down (Chapman *et al.*, 2001). This introduces some logistical issues, requiring researchers to determine the culture methods to be used and type of microscope needed for their investigative needs. The culture conditions must provide nutrition to the embryo placed *in vitro* (Chapman *et al.*, 2001). Moreover, because chick embryos *in vitro* develop abnormally past HH 18–21, time-lapse imaging of older embryo must take place *in ovo* or *ex ovo*; preparation for *in ovo* imaging is time-consuming. Finally, imaging the developing avian embryo requires engineering a warm (37 °C) and humid environment to promote growth and avoid dehydration (reviewed in Stern and Bachvarova, 1997). The need for an incubator on the microscope can be one of the most challenging and time-consuming aspects of setting up for time-lapse imaging of avian embryos.

C. The Quail Embryo as an Avian Model

Quail embryos lend themselves to time-lapse imaging, as they look very similar to chick embryos at all stages, although they develop slightly faster than chick embryos (Zacchei, 1961). Some researchers feel that quail resist better the hardships

of explantation, manipulation, and time-lapse imaging. Quail embryos offer the promise of easier labeling because transgenic lines with tissue- or cell-specific fluorescent protein expression are becoming available (Scott and Lois, 2005; also see Chapter 15 by Poynter and Lansford, this volume). All of the labeling approaches that work in chick work well in quail, including infection with virus particles modified to drive the expression of various fluorescent markers (LaRue *et al.*, 2003), lipophilic dye labeling, and electroporation (Chapter 12 by Sauka Spengler and Barembaum, this volume; Cui *et al.*, 2005). With its small size (2 cm), the quail egg fits conveniently into the apparatus for Magnetic Resonance Imaging microscopy, making possible the generation of an atlas of quail development starting at day 5. Through this noninvasive magnet-based technology, researchers image the 3-dimensional aspect of the embryo, compiling an annotated, digitally accessible atlas of quail development (Ruffins *et al.*, 2007). The methods presented in this chapter are described for the chick egg and embryo, but can be applied to quail, with only minor modifications.

II. The Technology of Time-Lapse Imaging

Capturing the development of the live embryo has benefited from key technical advances in three domains: image-recording hardware and software, culturing techniques, and tissue labeling methods. The current technology in those three domains has made time-lapse imaging of the avian embryo more accessible and effective.

A. Hardware and Software

1. Advances in Hardware and Software for Time-Lapse Imaging

The technology for recording morphogenesis has become progressively user-friendlier. Early imaging of chick development used film and was then replaced by tape (Bortier *et al.*, 1996). As the value of recording morphogenesis became clear, the technology improved. Imaging development is now carried out digitally, with images captured at specific time intervals through specialized image-capture software installed on a computer. In combination with image processing software, digital time-lapse imaging makes processing, editing, annotating, and viewing the developmental sequence forward or backward veritably easy. In addition, the use of the Internet avails time-lapse sequences to a larger audience, allowing movies to be downloaded, shared, viewed, critiqued, discussed, displayed, and analyzed with other researchers.

2. Equipment Needed for Time-Lapse Imaging of the Avian Embryo

To generate a time-lapse movie of a developing embryo, one needs a microscope with a camera and imaging software. A widely used type of camera is the cooled charge-coupled device camera. These are versatile cameras that perform well with

low-light (fluorescence) imaging and high-light imaging (bright field) without damage to the camera. To obtain the best possible images, the camera must be correctly connected to the microscope, including correctly installed adapters (Oshiro *et al*., 2005). Microscopes used for time-lapse imaging can vary widely. Stereoscopes, confocal microscopes, and compound scopes are all viable options. In addition, imaging software that can be programmed to open and close the camera shutter and activate the capture mechanism as specified by the user adds convenience to the endeavor of time-lapse imaging.

The chick embryo requires a 37 °C for development. To create such an environment during time-lapse imaging, a box is built around the microscope stage. Hot air from a heater circulates into that box and a proper temperature controller maintains the environment around 37 °C. This entire apparatus can be made inhouse at low cost, with a cutter, a ruler, cardboard, reflectrix insulation (from a local hardware store), flexible 4-in. dryer duct (from a local hardware store), and duct-tape. Cutout shapes, carved from cardboard and insulated with reflectrix insulation wrap, are assembled like pieces of a puzzle around the microscope each time the box is needed. Homemade boxes can last through years of repeated use (Kulesa and Kasemeier-Kulesa, 2007). To generate warm air into the box, one uses heaters for egg incubators or a variety of commercial heaters, connected to the box with the flexible 4-in. dryer duct and set the heating unit outside the box. To monitor the temperature around the microscope stage, one plugs the heater unit to a temperature controller (Fisher scientific 11-463-47A) and mounts the temperature probe of the temperature controller onto the stage of the microscope.

Alternatively, commercial incubation chambers, adapted to specific microscopes, can be purchased from companies such as Solent Scientific or Oko Labs. These commercial chambers offer an option for a CO_2 inlet and control of humidity. The temperature control in commercial chamber can be more stable than it is in their homemade counterparts. For chick embryos, provision of CO_2 is not absolutely necessary, though it does not seem to harm the developing embryo.

B. Culture Techniques

1. Advances of *In Vitro* Culture

The *in vitro* technique devised by New (1955),revolutionized the experimental biology of the chick embryo in general and its developmental imaging in particular. The New technique consists of collecting the chick embryo with as much of its vitelline membrane as can be laid out flat. The embryo is then stretched onto a glass ring and cultured ventral side up in a dish atop egg white. Normal development until stage 16 ensues, availing both sides of the embryo for imaging and allowing experimental manipulations. This technique has been many times modified (reviewed in Stern and Bachvarova, 1997), and a widely used, recent modification collects the embryo on a filter paper ring, a method known as EC method (Chapman *et al.*, 2001).

2. Culture Techniques for Time-Lapse Imaging

a. In Vitro Culture Systems: Thin Egg White, Millicell Insert, and Semisolid Substrate

In vitro culture is the method most widely used in combination with time-lapse imaging. A key point applicable to all *in vitro* methods for avian embryos bears mentioning emphatically: embryos younger than stage 7–8 will not develop normally if cultured ventral side down onto a substrate, including glass or plastic (Chapman *et al.*, 2001). Therefore, the surface to be imaged, the stage of the embryo, and the type of microscope must be carefully coordinated to allow 4-dimentional imaging of the desired morphogenetic events. *In vitro* cultures are usually carried out in plastic six-well dishes, modified to image through a glass surface by replacing the bottom of a well, or the top of the dish cover with a glass coverslip. Both whole embryos and embryonic explants can be cultured *in vitro* (Fig. 1).

In vitro methods provide the embryo with nutrition to promote long-term culture, with moisture to avoid dehydration, and allow access to either dorsal or ventral embryonic surfaces. The six-well dishes, in which embryos for time-lapse imaging are cultured *in vitro*, offer two advantages: they fit onto a microscope stage and the unused wells are filled with sterile water to generate moisture. There are three common approaches:

1. Avian embryos prepared for *in vitro* culture are collected on a filter paper ring (Chapman *et al.*, 2001) and cultured in a pool of thin egg white (Chapman *et al.*, 2001), suspended over tissue culture media on a millicell membrane insert (Kulesa and Fraser, 1998b; Rupp *et al.*, 2003) or laid on a semisolid bed of bacto agar and egg white (Chapman *et al.*, 2001). Culture of the embryo in thin egg white has the advantage of speedy preparation and optical transparency during time-lapse imaging. The correct amount of egg white must be added, in order to avoid dehydration of the embryo (too little albumen) or its detachment from the filter paper (too much albumen).

2. Avian embryos are explanted with or without Whatman filter paper, placed on a millicell membrane insert, and suspended above sterile tissue culture medium. The millicell insert becomes moist from the medium below, keeping the embryo from dehydration. An adaptation of the millicell insert, engineered to further optimize optical transparency, consists of removing the membrane from the millicell insert, and replacing it with surgical sutures, spaced 2-mm apart (Rupp *et al.*, 2003).

3. In the semisolid agar method, the thickness of the agar must be controlled to remain compatible with optical transparency requirements of time-lapse imaging. The thick semisolid substrate makes this culture technique maladapted to imaging from the bottom, through that substrate, on an inverted microscope; therefore, the best results are obtained with an upright microscope.

Each of the *in vitro* culture methods has implications for the accompanying microscopy modality. The semisolid agar/egg white substrate is most compatible with imaging from the top, using an upright microscope. Given that early embryos must be cultured ventral side up, these agar cultures work best imaging on the ventral side of the embryos. To explant the embryo dorsal side up, the embryo

must be older than stage 8. The egg white and the millicell insert method work well and are compatible with imaging from above or below the embryo (upright and inverted microscope, respectively), allowing flexibility in the embryonic surface to be imaged (Figs. 2 and 3).

b. In Ovo Culture and Time-Lapse Microscopy

When the desired length of imaging extends beyond 24–30 h, methods that minimize disruption of the embryo are appropriate. In the *in ovo* method, the embryo remains within the egg and is imaged through a window opened in the eggshell (Kulesa and Fraser, 2002; Kulesa *et al.*, 2000; McLennan and Kulesa, 2007; Stark and Kulesa, 2005). The *ex ovo* method consists of dispensing the content of the egg in a dish, eliminating the need to handle the large, fragile, and ovoid egg. For early embryos, the *in ovo* method has been used in conjunction with time-lapse imaging. The main disadvantage of *in ovo* imaging is the lengthy preparation time. The configuration of the egg restricts the researcher to imaging from above the embryo with an upright microscope. However, compared to the *in vitro* culture systems, the *in ovo* method offers the benefits of imaging beyond 1 day, while maintaining excellent morphology. The best results for *in ovo* imaging are obtained when embryonic tissues are fluorescently labeled.

C. Labeling Techniques

1. Advances in Tissue Labeling Techniques

The technology for labeling cells in a way that makes them visible during the development of the embryo has become progressively better. Labeling parts of the embryo helps to track the movements of specific cell populations. Early methods for labeling cells in chick embryos include the application of carbon or iron oxide particles and vital stain. These techniques presented drawbacks: whether carbon particles remained attached to the cells originally labeled or moved to a different cell population was uncertain, while vital stains become less crisp as the embryo develops (Argüello *et al.*, 1975; Campbell, 1973; Selleck and Stern, 1991; Spratt, 1955; 1957). Newer methods for labeling chick embryos for time-lapse imaging include the injection of fluorescent lipophilic dyes onto living cells, injection of fluorescent hydrophilic polysaccharides (dextrans) into living cells, and the introduction of genetically encoded fluorescent proteins.

2. Current Labeling Techniques for Time-Lapse Imaging of the Avian Embryo

There are several labeling options when the research goal is to capture the movements and behaviors of subpopulations of cells, instead of the overall morphogenesis of the embryo. Tight groups of 30 or more cells can be injected with lipophilic dyes such as 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI), DiO, and DiI (Molecular Probes), which permeate the cell membrane and are passed to daughter cells upon division (Cui *et al.*, 2005; Gavalas *et al.*, 2001; Honig and Hume, 1989; Serbedzija *et al.*, 1989; Stern, 1990).

It is also relatively easy to inject several spots, of different colors. Time-lapse sequences of embryos labeled in this way help to understand the movement of cell populations with respect to one another (Bhattacharyya *et al.*, 2004; Cui *et al.*, 2005; Kulesa and Fraser, 2000). The disadvantage of lipophilic dye labeling is that the dye becomes diluted with cell division, and the fluorescent signal looks fainter after 2 days of incubation.

Scattered populations of individual cells can be made to express fluorescent proteins by electroporation of genetically encoded fluorescent molecules. In this method which has been widely combined with live imaging, the fluorescent proteins, encoded on plasmids, are introduced into the cells through the application of brief pulses of current (Chapter 12 by Sauka-Spengler and Barembaum, this volume; Teddy *et al.*, 2005; Yamamoto *et al.*, 2004). The fluorescent molecules can be targeted to subcellular components such as cell nuclei, cell cytoplasm, and cell membrane (Teddy *et al.*, 2005; Yamamoto *et al.*, 2004). The signal in electroporated cells does not fade with cell division, unlike lipophilic dyes. The disadvantage of electroporation compared with fluorescent lipophilic dye injection is that the fluorescent signal in electroporated cells takes 3–6 h before it is visible.

Additional fluorescence labeling methods are available. For example, iontophoretic labeling of single cells with fluorescent dextrans can be used to delineate cell lineage and to assess the potency of progenitor cells (see Chapter 10 by Bhattacharyya *et al.*, this volume; Birgbauer and Fraser, 1994; Bronner-Fraser and Fraser, 1988; Bronner-Fraser *et al.*, 1991; Clarke and Lumsden, 1993, Wong *et al.*, 1993). Avian embryos can also be labeled using viral particles modified to drive the expression of various fluorescent proteins (LaRue *et al.*, 2003; Okada *et al.*, 1999). The new technology of quantum dots, which are fluorescent nanocrystals, can label the embryo with virtually no background (see Chapter 10 by Bhattacharyya *et al.*, this volume).

III. Considerations Before Time-Lapse Imaging

There are several choices the experimenter should decide upon before beginning time-lapse imaging:

- Use of chick or quail
- Labeling the embryo
- Preparation time
- Ways to incubate the embryo, length of time-lapse sequence, and appropriate type of microscope
- Ways to culture the embryo
- Ways to visualize the embryo
- How often to capture events
- Possible drawbacks: phototoxicity, dehydration, and abnormal development
- Trial runs

A. Use of Chick or Quail

This depends on the user's preferences. Advantages of chick include a greater availability of markers, including antibodies and RNA probes. If the cell movements seen in time lapse must be integrated with molecular manipulations, chick may be a better option. However, many markers work in quail as well as they do in chick; the user can easily confirm their expression pattern. The quail embryo has the advantage that it is sturdier when manipulated than the chick embryo. With the emergence of lines of transgenic quails that express fluorescence in tissue-specific patterns become widely accessible, the transgenic stocks may sway the balance in favor of using quail (see Chapter 15 by Poynter and Lansford, this volume).

B. Labeling the Avian Embryo

How to label the embryo depends on one's question. For interactions between polyclones (groups of cells), vital dyes of different colors are an excellent tool. To investigate cell–cell or –tissue interactions, the most effective labeling choice is electroporation. When using electroporation, one must expect as long as 3–6 h of development before fluorescent signal becomes visible.

C. Preparation Time

One should estimate approximately 2 h to prepare for time-lapse imaging, excluding time needed for labeling the embryonic tissues with fluorescent dyes or proteins. This includes installing the correct objectives on the microscope, assembling the warm box around the microscope, waiting as the temperature rises to 37 °C, cleaning and sealing the imaging dish for *in vitro* imaging, collecting thin egg white or making semisolid agar, cutting paper rings, or setting the embryo in culture. In addition, if embryos were previously labeled with lipophilic dyes or electroporated, time must be planned for the researcher to screen the embryos to decide on the best candidate for imaging.

D. Ways to Incubate the Embryo, Length of Time-Lapse Sequence, and Appropriate Type of Microscope

Several questions must be answered to identify a suitable culture method:

1. *At what stage will imaging begin?*

The chick embryo must be cultured ventral side up earlier than stage HH7–8 *in vitro*. This necessitates the use of an inverted microscope.

2. *At what stage will imaging stop?*

For imaging that will go beyond turning stages (stage HH13–14), one should consider *in ovo* imaging if imaging the head because it can develop poorly *in vitro* (Chapman *et al.*, 2001). If the head is not of interest, *in vitro* works well up to early stage 20s (Cui *et al.*, 2006). The ideal solution for imaging embryos older than stage HH14 is to combine both *in vivo* and *in vitro* methods if possible.

3. *Is the tissue of interest on the dorsal or the ventral side of the embryo?*

Chick embryos younger than stage 7–8 must be cultured ventral side up if they are placed *in vitro* (Chapman *et al.*, 2001). Therefore, if the ventral side is your interest, and the embryo is younger than stage HH7–8 at the start of the time lapse, an upright microscope will be needed, and an imaging dish containing a hole in the cover will have to be made (cf. Rupp *et al.*, 2003). If dorsal events are of interest, a dish with hole at the bottom covered with a coverslip is needed (Kulesa and Fraser, 2005).

4. *Is the tissue of interest found deeper in the embryo than can be seen from the surface of the embryo?*

Performing a few tests will answer this question. If the answer is "yes," an explant system will need to be devised and tested. To test whether the tissue is too deep to image from the surface of the embryo requires labeling the tissue of interest such that the tissue is labeled at the *end stage* of the ideal time-lapse sequence. One then images the fixed embryo. The ideal imaging method is a confocal or multiphoton microscope used to optically section the embryo. Based on the quality of the images in the resulting Z-stack, one decides whether imaging the intact embryo will afford adequate access to the tissue of interest. If the images are not of high quality, it will be necessary to devise an explant system that allows the tissue of interest both to be visible and be to behave and differentiate relatively normally in culture.

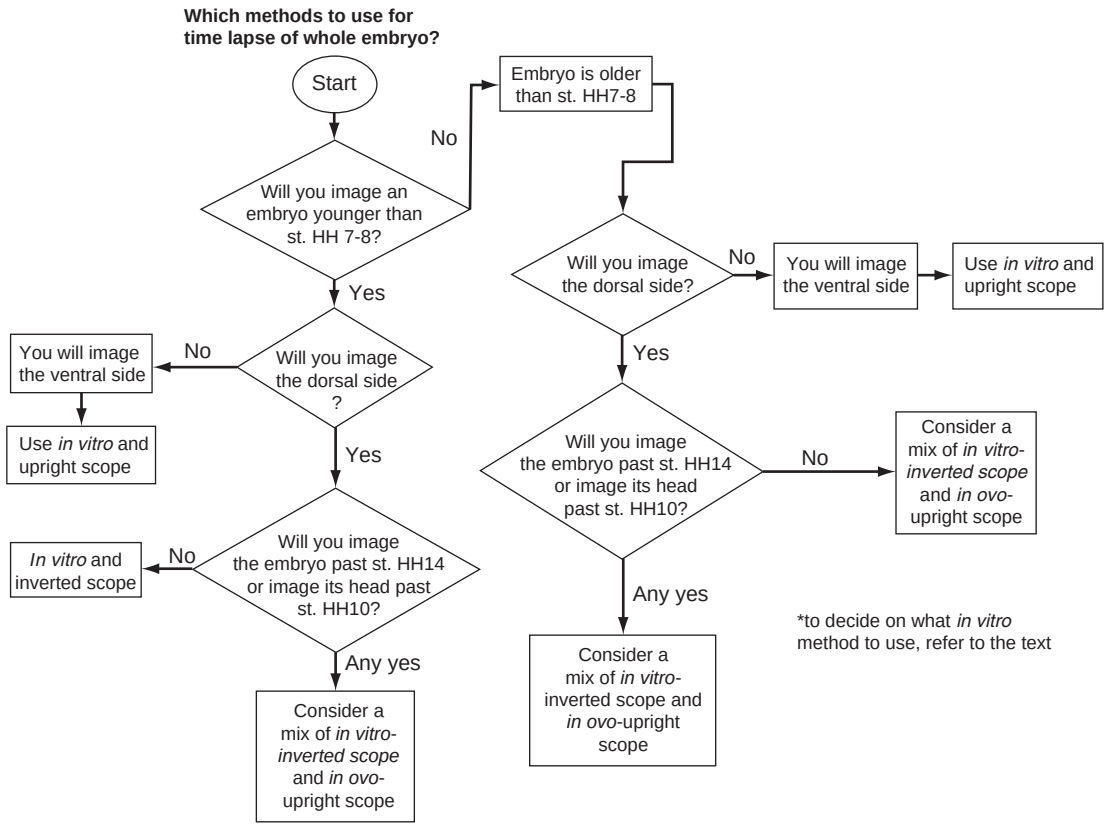
The following flowchart suggests what method may work well for imaging (Questions 1–3 above).

E. Ways to Culture the Embryo

Embryos can be cultured *in vitro* or *in ovo*. The flowchart above helps determine which modality to use. If using *in vitro* and imaging the ventral side of the embryo, all three *in vitro* options (thin egg white, millicell insert, and semisolid substrate) are viable options in combination with an upright microscope, imaging from above the embryo. If imaging the dorsal aspect of the embryo, then the millicell insert and the thin egg white methods are appropriate, in combination with an inverted microscope, imaging from beneath the embryo. The choice between equivalent options is user-dependent.

F. Ways to Visualize the Embryo

Time-lapse imaging of the chick embryo can be carried out on a stereoscope, compound microscope, or confocal microscopes. To visualize overall morphogenesis in the embryo, a combination of bright field and low-magnification objectives (generally 2.5, 5, and 10×) are necessary. To view cell–cell interaction, cell behaviors, and cell division through fluorescently labeled cells, fluorescence microscopes are required. If the labeled cells of interest are sharply visible from the surface of the embryo, both epifluorescence microscopes and confocal microscope work well. If labeled cells are several layers deep in the embryo, confocal



microscopy is necessary. The objectives appropriate for visualization of cell behaviors generally include 10×, 20× (or higher). When using these magnifications, long working distance objectives (1–2 mm) are helpful to compensate for the movements of the developing embryo, which moves out of the plane of focus or out of contact with the imaging surface (the coverslip).

G. How Often to Capture Images

To study morphogenesis in bright field, or to study the trajectory of fluorescently labeled groups of cells, intervals of 3–5 min are a good starting point. Intervals can be as long as 15 min (Rupp *et al.*, 2003). If the goal is to investigate the behavior of individual cells, or cell–cell interactions, intervals of 60–120 sec are appropriate (Kulesa and Fraser, 2000).

H. Possible Drawbacks: Phototoxicity, Dehydration, and Abnormal Development

Chick embryos are sensitive to phototoxicity, therefore, when using confocal microscope to image fluorescence, the laser power should remain below 15% of

total power. Dehydration of the embryo during imaging is prevented by adding water to the wells of the six-well dish in which the embryo is cultured and sealing the dish with parafilm. The combination of explantation, *in vitro* culture, phototoxicity can lead to abnormal development during time-lapse imaging (Fig. 4).

I. Trial Runs

The first steps before time-lapse imaging consists of optimizing live samples for static imaging. This includes identifying a suitable culture method, determining the types of dishes compatible with the sample and with time-lapse imaging and familiarizing oneself with the imaging software. Next, it is important to test a sample on the microscope stage, with the heated box installed, to troubleshoot the humidity and temperature of the environment. The first time-lapse imaging trials will help determine how often to capture images, how to avoid phototoxicity, and how fast the embryo develops on the microscope stage.

IV. Methods

A. Technique to Explant and Spread a Whole Chick Embryo for Imaging

This method spreads the embryo dorsal side down on a fibronectin-coated surface. It has been adapted to embryos from stage 7 to 12 or so (Bhattacharyya *et al.*, 2004; Krull *et al.*, 1995; Kulesa and Fraser, 1998, Kulesa *et al.*, 2005). The advantages are that the embryo remains relatively flat. This method also affords good resolution for imaging the embryo in both bright field and fluorescence channels. Disadvantages include abnormal morphogenesis over time, as the embryo can become very flat after 8–12 h. The head of the embryo can often develop abnormally, though the remainder of the axis is less susceptible to poor development.

The nutritive requirements for the embryo are supplied by culture media. The imaging chamber (a six-well dish with one or more hole cut in the bottom of some of the wells) in which the embryo is imaged can be washed with water, sprayed with 70% ethanol, dried, protected in paper towels, and reused many times.

Preparation (before opening eggs):

Falcon 3046 six-well dish with dime-sized hole cut at the bottom of one well (Beckton Dickinson, NJ)

In the tissue culture hood:

Spray w/70% EtOH (keep flame off)
Put dish in tissue culture hood with UV approximately few hours or O/N
Dot small amount silicon grease uniformly around top of hole in well 1
Flame a 25 mm coverslip
Center and seal the coverslip onto the well

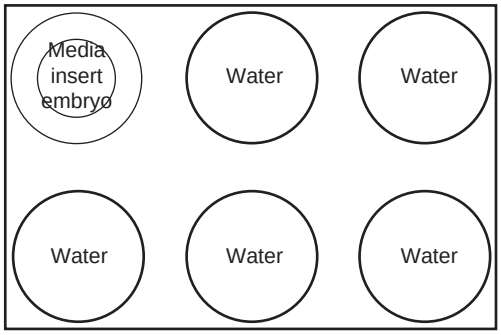


Fig. 1 Six-well dish showing the position of water for humidity and the embryo to be imaged. Courtesy Paul Kulesa and Carole Lu.

Remove excess silicon grease (razor blade if bottom)

Fill five other wells with about 9 ml sterile water each

Warm in 37 °C incubator

Keep at 37 °C between remaining steps:

Put tissue culture insert into well 1 (Millipore Millicell-CM PICM 03050, Bedford, MA)

Add 200 µl of fibronectin (Gibco, 33016-015, Grand Island, NY) at 20 µg/ml to insert

Swirl to spread evenly. Soak for 30 min to 1 h

Remove fibronectin (FN) solution

Add 1.5 ml of neural basal media (Gibco, 21103-049, Grand Island, NY) supplemented with B27 (Gibco, 17504-044, Grand Island, NY)

Return to 37 °C incubator

Checklist before disturbing eggs:

- "Sterile" six-well dish with water and neural basal media-coated coverslip
- Dishful of filter paper rings (these are $\sim 1 \times 1$ cm^2) cut out in Whatman filter paper, with a hole punched in the center. These rings are used to remove embryo from the egg (Chapman *et al.*, 2001)
- FN-treated culture insert (1–2 h)
- Sterile Ringer's solution
- Forceps, scissors, and transfer pipette
- Three dishes of sterile Ringer's solution warmed to 37 °C
- Warm heater box on microscope
- Correct objectives on the microscope, including long-distance objective for imaging at higher magnifications (higher than 10×)

Explant procedure:

- Remove eggs from incubator
- Open egg (reopen if previously injected)
- Put filter paper ring around the embryos and cut around the ring
- Transfer embryo (adhering to the ring) to warm Ringer's solution with plastic pipette
- Clean embryo and wash off yolk platelets
- Transfer to culture insert, ventral side up
- Center embryo, line up along AP axis
- Remove excess Ringer's solution to help spread out embryo
- Put culture insert in well 1
- Put lid on six-well dish
- Return to incubator for 1 h (good for humidity, etc)
- Seal the dish with parafilm while holding securely
- Tape down the corners of the parafilm (see Fig. 4)
- Take to confocal microscope and image

Supplemented neural basal media:

Gibco BRL neural basal media (Gibco BRL, Grand Island, NY)
2% B-27 Supplement
1% L-Glutamine
1% Pen-Strep

B. Technique to Explant a Whole Chick Embryo with EC Culture and to Image Its Dorsal Side

This method, a modification of the New culture, uses Whatman filter paper to collect the embryo (Chapman *et al.*, 2001; New, 1955). The protocol detailed below is specifically adapted to the use of an inverted microscope. This is important when

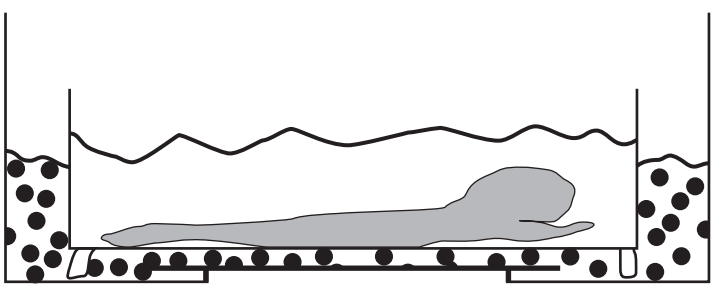


Fig. 2 The relative positions of the explanted embryo, the millicell insert, neural basal media, and the well and coverslip are shown. Dots = supplemented neural basal media. Embryo is gray, covered in Ringer's solution in the culture insert, surrounded by neural basal media in the well. Courtesy Paul Kulesa and Carole Lu.

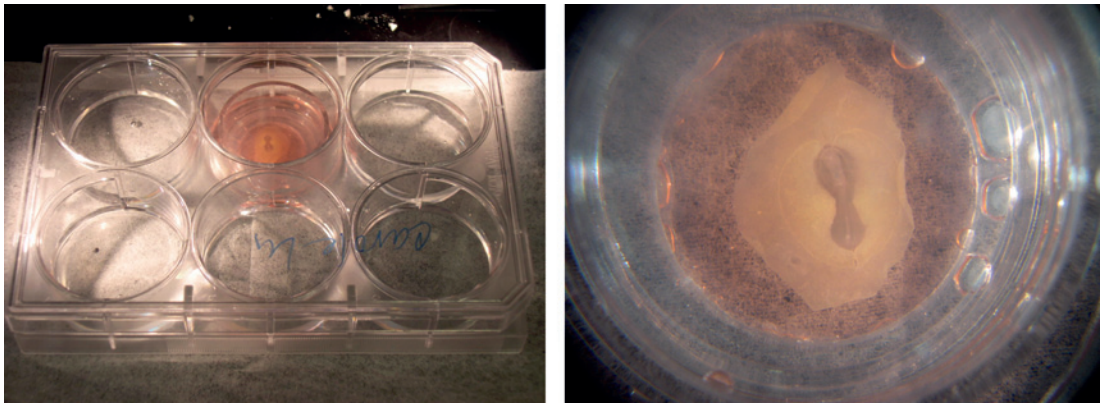


Fig. 3 Explanted chick embryos in millicell insert placed in a six-well dish. Courtesy Carole Lu. (See Plate no. 11 in the Color Plate Section.)

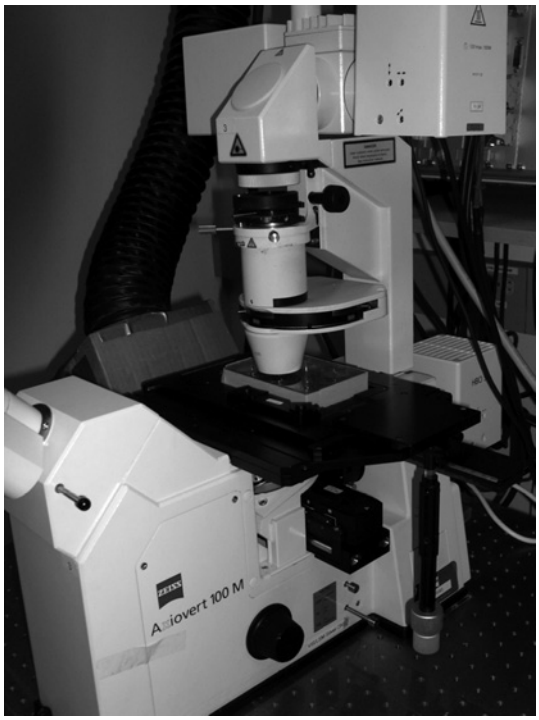


Fig. 4 Microscope set up with sealed six-well dish and embryo. The heated box was removed to expose the dish. Courtesy Carole Lu, 2007.

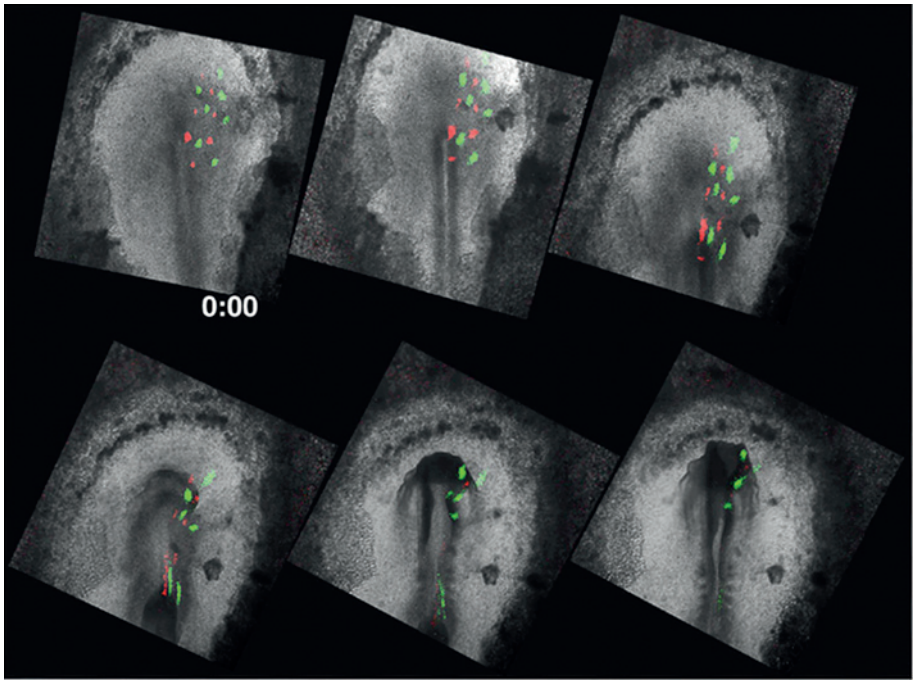


Fig. 5 Frames from a time lapse using embryos explanted with the EC method and cultured in egg white. (See Plate no. 12 in the Color Plate Section.)

imaging the dorsal side of embryos that must be cultured dorsal side down (Chapman *et al.*, 2001). The culture medium is thin egg white in the protocol detailed below. Egg white culture is well adapted for imaging embryos as old as stage 18 and is compatible with both inverted microscope and upright microscopes (Fig. 5). The disadvantages are that the embryo can detach from the filter paper in egg white and develop abnormally. Culturing the embryo on a millicell membrane insert in tissue culture media can deliver the same results as the thin egg white method (See Section IV.C; Rupp *et al.*, 2003).

Preparation (before opening eggs):

Falcon 3046 six-well dish with 1- to 2.5-cm-diameter hole cut in 1, 2, or 3 of the wells

In the tissue culture hood:

- Spray w/70% EtOH (keep flame off)
- Put dish in tissue culture hood with UV approximately few hours or O/N
- Dot a small amount silicon grease uniformly around top of hole in well 1 and others if applicable.
- Flame 25 mm coverslips for 1 cm hole, or 45 × 50-1 coverslips (Fisher Scientific, 12-545-H, 45 × 50-1, Pittsburgh, PA) for 2.5 cm holes

- Center and seal the coverslip onto the well(s)
- Remove excess silicon grease (use razor blade if coverslip is on bottom of dish)
- Fill other wells with about 9 ml sterile water each
- Warm in 37 °C incubator

Checklist before disturbing eggs:

- "Sterile" six-well dish with water
- Thin egg white from incubated eggs (12–24 hs) collected in sterile container, and kept at 37 °C.
- Filter paper ring to remove embryo
- Sterile Ringer's solution
- Forceps, scissors, transfer pipette, and trashbag for eggs
- Three dishes of sterile Ringer's solution warmed to 37 °C
- Warm heater box on microscope
- Correct objectives on the microscope, including long-distance objective for imaging at higher magnifications (higher than 10×)

Explant procedure:

- Remove eggs from incubator
- Open eggs (reopen if previously injected)
- Put Whatman filter paper circle around the embryo and cut around the paper
- Transfer embryo to warm Ringer's solution with plastic pipette
- Clean embryo, wash off yolk platelets
- Identify the best, second, and third best embryos for imaging
- Add 1.5 ml of warm thin egg white to each of the coverslipped wells.
- Transfer the embryos to the wells ventral side up: the embryo will bathe in a pool of egg white, even sinking a little into the thin egg white.
- Center embryo, line up along AP axis
- Adjust amount of egg white so that a layer thinner than 1 mm covers the embryo to allow gas exchange, help avoid the drying up or detaching of the embryo
- Smear a thin layer of egg white inside the lid of the dish to avoid condensation
- Put lid on six-well dish
- Return to incubator for 1 h (good for humidity, etc) if possible
- Seal the dish with parafilm while holding securely
- Tape down the corners of the parafilm (Fig. 4).
- Take to microscope and start time-lapse imaging

C. Technique to Explant a Whole Chick Embryo with EC Culture and to Image Its Ventral Side

This method is similar to the one detailed above for dorsal imaging. We will focus on the differences (Rupp *et al.*, 2003).

1. Use of an Upright Microscope

Young embryos must necessarily be cultured ventral side up, and older embryos develop well ventral side up as well. To image the ventral side of embryos cultured in this way, one uses an upright microscope.

2. The Imaging Chamber

A six-well dish is used, but holes are not cut at the bottom of any of the wells. Instead, the holes are made in the cover of the dish. They are sealed with coverslips through which the embryos are imaged.

3. Embryo Culture

The wells in which embryos are cultured are filled with semisolid agarose or thin egg white (Chapman *et al.*, 2001) or culture media (Rupp *et al.*, 2003), all of which work well.

To make semisolid agarose:

Heat a water bath to 49–55 °C

Boil saline (7.19 g of NaCl/ 1 liter of distilled water)

Add 0.72 g bacto agar and stir using a stir bar to dissolve it

In the meantime, collect thin egg white and set aside

Place the flask of dissolved agar in the 49–55 °C water bath to equilibrate

Add the thin egg white to the dissolved agar and swirl until evenly mixed

Add 5 U/ml of penicillin–streptomycin

Aliquot no more than 2.5 ml of semisolid agar in wells that will be used to image the embryo, avoiding bubbles

Aliquot the remaining agar in 35 mm dishes and store at 4 °C for up to a week.

To make culture media:

Leibovitz-L15 medium supplemented with 2 mM L-glutamine

10% chicken serum and 1% penicillin–streptomycin

4. The Use of a Long-Distance Objective

A long-distance objective is critical as the embryo is at a distance from the imaging surface. The use of an objective with a working distance up to 20 mm (Rupp *et al.*, 2003) is recommended.

D. *In Ovo* Time-Lapse Imaging

Imaging the chick embryo *in ovo* presents invaluable advantages as detailed above. The main disadvantage of time-lapse imaging *in ovo* is that it can be a rather fastidious process to complete. Of course, *in ovo* imaging must take place on an upright microscope and the use of a long-distance objective is recommended for magnifications above 10× (Kulesa and Fraser, 2000). Even though it might seem that the embryo can be imaged indefinitely *in ovo*, several changes make it difficult, and would have to be resolved for prolonged observation. Specifically, embryos older than day 4 become heavier and the yolk becomes thinner as embryo uses its nutrients. The result is that the embryo sinks away from the microscope objective and away from adequate oxygen supply critical for proper growth (Kulesa and Fraser, 2005). In addition, the faster heart beat and movements of older embryo make the time-lapse imaging difficult, unless a fast frame acquisition method is available.

Chick embryos can develop when the egg content is dispensed into a dish. This method can be adapted to time-lapse imaging, although sometimes, embryos in the dish develop cardiovascular abnormalities (Tutarel *et al.*, 2005).

Important note: Incubate your eggs horizontally

Preparation (before opening eggs):

- If needed, remove the condenser from the microscope
- Determine what type of support would safely lift the egg close up enough to the objectives of the microscope
- Add the objectives on the microscope. Include long-distance objectives if necessary
- Melt the beeswax on a heater set to 37 °C

In the tissue culture hood:

Not required

Checklist before disturbing eggs:

- Sterile Ringer's solution warmed to 37 °C
- Egg white collected with sterile techniques
- Forceps, transfer pipette, scissors to open or reopen the egg, trashbag for eggs
- Warm heater box on microscope
- High-sensitivity, oxygen-permeable Teflon membrane (Fisher #13-298-83; 3.8 cm × 7.5 cm × 15 μm, Pittsburgh, PA)
- Cylindrical acrylic ring (2.2 cm inner diameter × 2.6 cm outer diameter × 0.5 cm height)
- Melt white beeswax (Eastman Kodak Co. #1126762, Rochester, NY)
- Rubber *o*-ring from a local hardware store (2.4 cm outside diameter 2.1 cm inside diameter)
- Wooden probe or spatula

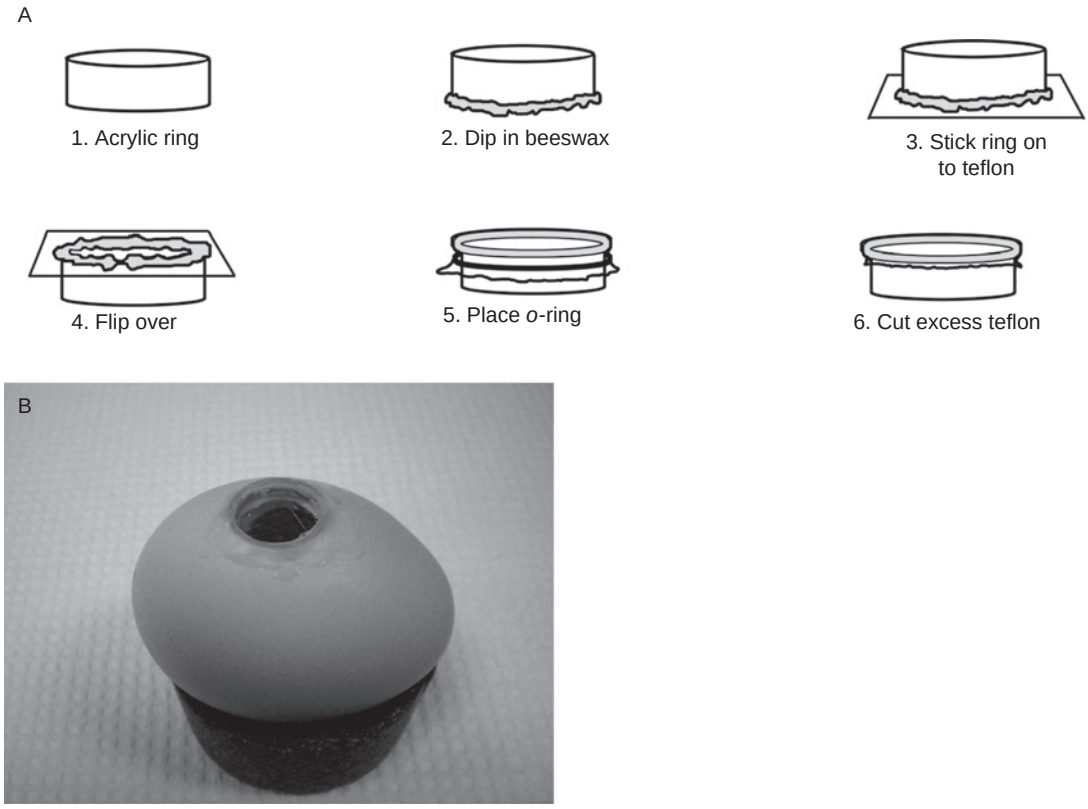


Fig. 6 Panel A (steps 1–6) show the procedure for securing the *o*-ring to the Teflon membrane and making sure the Teflon membrane is taught. Panel B shows a windowed egg with Teflon membrane insert, sealed with melted beeswax.

Explant Procedure:
None needed
In Ovo Procedure:

- Set the Teflon membrane on a clean surface near the warm wax
- Using forceps, dip the acrylic ring into the warm white beeswax for approximately 10 sec
- Lay the ring, beeswax side down onto the center of the Teflon
- Carefully flip over the ring with Teflon loosely attached
- Immediately, before the beeswax hardens, roll the rubber *o*-ring down snuggly over the Teflon and around the circumference of the ring: this will pull the Teflon taut around the acrylic ring
- Do not disturb for 5 min

- Remove the rubber *o*-ring and cut away excess Teflon
- Remove up to 3 ml of egg white from the blunt end of the egg with a needle (if not done before)
- Trace the acrylic ring with a sharpie onto the top of the egg (if not done before)
- Open or reopen the egg by cutting around the trace of the acrylic ring
- Place the ring—Teflon membrane side down—onto the embryo
- Add thin egg white to raise the embryo up close to the top of the egg
- Dip the metal end of the wooden probe into the melted beeswax
- Use to bind the ring to the eggshell repeatedly until the egg is sealed all around the ring
- Take the egg to the microscope, remove the condenser if need be and start imaging (Fig. 6)

E. Gaining Access to Tissues Deep Within the Embryo by Organ Explant and Imaging Method

When the cells of interest are too deep within the embryo, it may be possible to resort to explanting the tissue and exposing those cells populations to the microscope. The main advantage is, of course, access to those cells.

The disadvantages of the organ explant include difficulty separating the artifacts of the culture method from the normal behavior of the cells. Choosing the appropriate culture medium and limiting wound healing are extremely important and will require some experimentation. Finally, the length of culture is limited by the health of the organ in culture.

Here, we show the example of neural crest migrating to the dorsal root ganglia and the sympathetic ganglia (Kasemeier-Kulesa *et al.*, 2005). They migrate between the somite and the neural tube, and are therefore not easily viewed from the (accessible) dorsal side of the embryo. Cutting out a section of trunk of stage 17–22 embryos, and cutting the neural tube sagittally allows access the labeled neural crest (Hamburger and Hamilton, 1951).

Preparation (before opening eggs):

Falcon 3046 six-well dish with dime-sized hole soldered in 1, 2, or 3 of the wells

In the tissue culture hood:

- Spray dish w/70% EtOH (keep flame off)
- Put dish in tissue culture hood with UV approximately few hours or O/N
- Dot a small amount silicon grease uniformly around top of hole in well 1 and others if applicable
- Flame 25-mm-diameter coverslips
- Center and seal the coverslip onto the well(s)
- Remove excess silicon grease (use razor blade if coverslip is on bottom of dish)
- Fill other wells with about 9 ml sterile water each

- Warm in 37 °C incubator
- Put tissue culture insert into well 1 (Millipore Millicell-CM PICM 03050, Bedford, MA)
- Add 200 μl of fibronectin at 20 μg/ml to insert
- Swirl to spread evenly. Soak for 30 min to 1 h
- Remove fibronectin solution
- Add 1.5 ml of supplemented neural basal media with B27 supplement at 1:50 to well 1 and others if applicable
- Place the tissue culture insert into well 1
- Adjust the level of supplemented neural basal media so that the level of media touches the tissue culture insert without the insert floating up, away from the bottom of the dishes
- Return to 37 °C incubator

Checklist before disturbing eggs:

- "Sterile" six-well dish with water, tissue culture insert, and neural basal media at 37 °C
- Filter paper ring to remove embryo
- Sterile Ringer's solution
- Forceps, scissors, tungsten needle (A-M Systems, 717000, Sequim, WA), transfer pipette, trashbag for eggs
- Three dishes of sterile Ringer's solution warmed to 37 °C
- Objectives on the microscope, including long-distance objective for imaging at higher magnifications (higher than 10×)
- Heater box on microscope, set to 37 °C and stably holding temperature

Explant procedure:

- Remove eggs from incubator
- Open eggs (reopen if previously injected)
- Put Whatman filter paper circle around the embryo and cut around the paper, through any blood vessels
- Transfer embryo to warm Ringer's solution
- Clean embryo, wash off yolk platelets
- Identify the best, second, and third best embryos for imaging
- Detach the embryo from the filter paper using forceps
- Transfer embryo to a new dish of warm Ringer's solution
- Cut the section of trunk of interest with tungsten needle
- Position the section of trunk dorsal side up
- Hold on with forceps and use a razor blade to slide sagittally, along the midline, through the thickness of the entire neural tube

- Add a few drops of supplemented neural basal media to the tissue culture insert
- Transfer the explants to the tissue culture inserts and use forceps to position medial or lateral side down, as you choose
- Center explant, line up along AP axis
- Smear a thin layer of egg white inside the lid of the dish to avoid condensation
- Put lid on six-well dish
- Return to incubator for 1 h (good for humidity, etc) if possible
- Seal the dish with parafilm while holding securely
- Tape down the corners of the parafilm (Fig. 4)
- Take to microscope and start time-lapse imaging

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CHAPTER 12

Gain- and Loss-of-Function Approaches in the Chick Embryo

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Abstract
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Abstract

The chicken embryo has been used as a classical embryological model for studying developmental events because of its ready availability, similarity to the human embryos, and amenability to embryological and surgical manipulations. With the arrival of the molecular era, however, avian embryos presented distinct experimental limitations, largely because of the difficulty of performing targeted mutagenesis or transgenic studies. However, in the last decade and a half, a number of new methods for transient transgenesis have been developed that

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allow efficient alteration of gene function during early embryonic development. These techniques have made it possible to study the effects of gene inactivation or overexpression on downstream transcriptional regulation as well as on embryonic derivatives. This, together with sequencing of the chicken genome, has allowed the chicken embryo to enter the genomic era. While attempts to establish germ line transgenesis are ongoing, methods for rapid, transient spatiotemporally targeted gene alterations have thus again re-established the chick embryo as an important experimental niche by making it possible to apply genetics in concert with classical embryological techniques. This provides a unique tool to explore the role of developmentally important genes (Ishii and Mikawa, 2005; Itasaki *et al*., 1999; Krull, 2004; Ogura, 2002; Swartz *et al*., 2001).

Transient transfection methods have allowed for efficient mis- and overexpression of transgenes. For long-term analyses, retrovirally mediated gene transfer has particular advantage. For short-term experiments, electroporation and adenoviral-mediated gene transfer methods provide transient expression, largely because of the short persistence time of the transgene within the cell. More recently, Tol2 transposon-mediated constructs have been employed, allowing for integration into the genome and prolonged expression of the transgene (Sato *et al*., 2007), see Chapter 14 by Takahashi *et al*., this volume). These methods today are routinely used for gain-of-function analysis, to overexpress or ectopically express genes of interest (Arber *et al*., 1999; Barembaum and Bronner-Fraser, 2007; Bel-Vialar *et al*., 2002). Loss-of-function experiments are also possible using electroporation of dominant-negative constructs that act as competitive inhibitors (Bel-Vialar *et al*., 2002; Renzi *et al*., 2000; Suzuki-Hirano *et al*., 2005), morpholino antisense oligos (Basch *et al*., 2006; Kos *et al*., 2001; Sheng *et al*., 2003) that block translation or splicing, or constructs expressing small interfering or small hairpin RNAs (siRNAs or shRNAs) (Chesnutt and Niswander, 2004; Das *et al*., 2006; Katahira and Nakamura, 2003).

Electroporation as the most popular method of the transient transfection into the chick embryos

Electroporation of chicken embryos involves application of an electric field to the exposed tissue that transiently disrupts the stability of the cell plasma membrane, creating reversible pores through which nucleic acids or their analogues can be readily transported into the cytosol. The use of this method for transfection into the vertebrate embryos has been facilitated by adapting the voltage parameters and the type and the duration of the electric pulse. By applying several successive square pulses at a very low voltage, with long rest periods in between, one can successfully deliver a DNA construct or another small charged particle into the cytoplasm, with minimal cell death, high efficiency of the uptake and good embryonic survival rate. The size limit of the DNA molecule that can be transfected in such a way is not yet known, though it is more likely that the size limitation in this procedure (if any) lies within the practical problems of cloning large fragments into the plasmid. We routinely overexpress constructs containing 3–4 kb inserts and coharboring a

GFP or RFP reporter whose translation is initiated from an internal ribosomal entry site (IRES), thus allowing easy detection of the electroporated cells.

I. Gain-of-Function by Electroporation

The coding regions of genes of interest need to first be cloned into the expression construct. There are a number of different vectors available for this purpose, but those containing the β-actin promoter with the CMV-IE enhancer, such as pCIG (Megason and McMahon, 2002) and pMES (Swartz *et al.*, 2001), give excellent results. Vectors containing CMV promoter and enhancer (such as pIRES2-EGFP, Clontech) generally produce lower levels of expression and are not ubiquitous; this characteristic, on the other hand, might be desirable if the protein is suspected of having toxic effects at very high doses. Finally, tissue-specific enhancer-driven constructs hold promise for the future and should allow us to express the genes of interest in a very precise spatiotemporal manner (Uchikawa *et al.*, 2004).

For each type of construct, one typically performs parallel electroporations with an empty vector to control for nonspecific effects of the procedure. Many vectors of choice contain IRES driving GFP expression as a marker for the cells that have received the construct. The expression of GFP can first be detected about 4 h after electroporation because of its long posttranslation folding time, but the target protein is likely to be present earlier.

When designing the construct, it is important to ensure that a consensus Kozak sequence for efficient translation initiation (Kozak, 1987) is included with the open reading frame. This element can be added by including 6–10 bp. of the endogenous sequence, directly upstream of the start codon or the consensus Kozak sequence, into the oligonucleotide used for PCR amplification of the fragment.

DNA used for electroporation is prepared using a Qiagen endotoxin-free plasmid kit and eluted at high concentration, of at least 5 μg/μl. Occasionally DNA prepared using other methods has been found to be toxic to the embryos. Endotoxin-free plasmid preparations typically do not have this problem. Generally, GFP expression can be detected for as long as 72 h after electroporation. However, a very significant decline in the level of GFP after 96 h has been observed. Plasmids expressing RFP also work well in chick embryos. Recently, newer vectors have been introduced, taking advantage of the transposon-mediated gene transfer technique (see Chapter 14 by Takahashi *et al.*, this volume) that allow stable integration of the gene construct into chick genomic DNA and its prolonged expression (Sato *et al.*, 2007).

A number of different chick embryonic tissues have been transfected using electroporation (see Table I). We have used the protocols below to transfect ectoderm and placode-derived structures in the head, or neural crest cells at the cephalic or trunk levels of the neural tube (Fig. 1A–C).

The electrodes for electroporation are made of two pieces of platinum wire (19 gauge) approximately 4 cm long and soldered to speaker wires. The platinum

Table I
Electroporation Targeting Different Tissues in Chicken Embryos

Target	Reference
Limb bud	Krull, 2004; Maas and Fallon, 2004; Oberg *et al*., 2002
Somites	Delfini *et al*., 2005; Krull, 2004; Scaal *et al*., 2004
Cerebellum (Purkinje cells)	Luo and Redies, 2004
Gut endoderm	Pedersen and Heller, 2005
Optic vesicle	Ishii and Mikawa, 2005; Momose *et a l*., 1999
Older embryo, *ex ovo*	Luo and Redies, 2005
Lens	Chen *et al*., 2004
Lung	Sakiyama *et al*., 2003

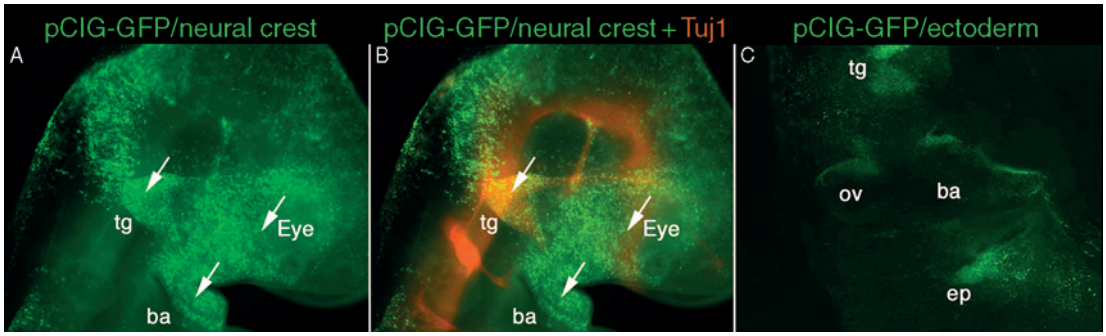

Fig. 1 Electroporation into the cephalic neural crest and ectoderm of the stage HH8 embryo. pCIG-GFP expression construct was electroporated into the neural tube (A, B) or ectoderm (C) at the cephalic level and analyzed for GFP expression approximately 48 h later, at stage HH18. (A, B) White arrows indicate the expression in the neural crest cells settled within the trigeminal ganglion (tg), branchial arches (ba), and periocular region (eye). Antibody staining against early neuronal marker class III β-tubulin (TuJ1) labels newly differentiating neurons in red. (C) After electroporation into the cephalic ectoderm at HH8, GFP-expressing cells are found concentrated in the cephalic ganglia (tg, trigeminal ganglion; ep, epibranchial ganglia) as well as in the forming otic vesicle (ov). (See Plate no. 13 in the Color Plate Section.)

wires are set 4 mm apart and fixed onto a handle, usually the outer cover of a ballpoint pen, with Epoxy resin and bent at 45 °C from the horizontal axis, about 5 mm from the end. Commercial electroporators are available (Krull, 2004) but one can also use electroporators that are simple and manufactured in house. The conditions described below should be adjusted and optimized for the specific equipment used, and are meant as a starting point or basic settings. Too high a voltage can lead to embryonic death and, if this occurs, the voltage should be decreased. Conversely, if the survival is good but efficiency of GFP/RFP expression is low, the voltage should be increased. Once an appropriate voltage is found

that combines both good survival and efficient transfection, the number of pulses and their duration can be varied to further optimize the electroporation efficiency. If one encounters problems with bacterial contamination, it is possible to add penicillin/streptomycin to the Ringer's solution to help prevent infection.

The extent of electroporation can be monitored on a dissecting microscope with UV fluorescence and a FITC or GFP filter. Removing the embryo from the egg allows detection of fainter fluorescence signal that is sometimes hardly visible *in ovo*. After fixation with 4% formaldehyde, electroporation-specific fluorescent signal decreases and there is a concomitant increase in the background fluorescence. We recommend not fixing in methanol because GFP fluorescence is lost during this procedure and the anti-GFP antibody does not work well on methanol-fixed tissue. After sectioning of the embryos fixed in 4% paraformaldehyde, much of the original GFP signal will no longer be visible. However, applying an antibody against GFP onto sections allows ready detection of electroporated cells. After *in situ* hybridization procedure, the GFP signal may be difficult to detect, even with use of anti-GFP antibody. In this instance, increasing the concentration of the antibody and the incubation time can improve detection.

A. Protocol for *In Ovo* Ectoderm Electroporations

This method can be used to obtain transfection of ectodermal placodes (e.g., trigeminal, epibranchial, and otic placodes; Barembaum and Bronner-Fraser, 2007). In addition, it can be used to transfect presumptive epidermis.

1. Eggs are incubated on their side without rocking to stages 8 and 9 (four to eight somites). Electroporations on older embryos, up to stage 13, can also be done using this approach.

2. About 3 ml of albumin is removed using a syringe with an 18 gauge needle and carefully piercing the shell at the small end. The eggs are windowed and the embryo is visualized by injecting a small amount of India ink (1:50 dilution with Ringer's solution) immediately below the embryo. The eggs are tape-closed until needed.

Ringer's Solution

7.2 g NaCl
0.17 g $CaCl_2$
0.37 g KCl
0.115 g Na_2HPO_4
0.02 g KH_2PO_4
Adjust to pH 7.4
Bring volume to 1 liter and filter sterilize

3. Just prior to the electroporation, dilute DNA to 2.5 μg/μl with Ringer's solution. Use 1 μl of vegetable dye (2% FD&C Blue in Ringer's solution) in 20 μl of DNA, prepared on the same day. Fast green has also been used to visualize the DNA solution but has sometimes been found to be toxic.

4. Back fill a glass needle (made from a 0.8- to 1.1-mm-diameter glass capillary tube) with the DNA solution. Break off the tip to create a narrow opening.

5. Insert the tip of the glass needle through the vitelline membrane near the caudal end of the embryo and slide the tip parallel to the embryo to the site that will be electroporated. Fill the space between the embryo and the vitelline membrane with the DNA solution to cover the region to be electroporated (as visualized with the vegetable dye in the solution). Avoid using a strong stream as it may damage the embryo. Keeping the vitelline membrane intact helps prevent the DNA from leaking out. This approach will give bilateral electroporation. By adding DNA to only one side of the embryo, unilateral electroporations can be achieved.

6. Make a hole with a 25 gauge needle in the blastoderm about the midpoint between the embryo and the edge of the blastoderm. Larger holes reduce survival rate.

7. Insert the positive electrode through the hole in the blastoderm and line it up under the embryo. Place the negative electrode over the region to be electroporated. The negative electrode should be in contact with the vitelline membrane but not pressed onto the embryo. Use 3 pulses of 8–10 V of 30 msec duration with an interval of 100 msec in between. Carefully remove the electrode and add Ringer's solution to the embryo to prevent it from drying and seal the egg with tape.

8. Incubate at 38 °C for the desired length of time. The survival of the electroporated embryos depends on the quality of the eggs, and can range from 70% to 10% after 48 h incubation. Optimal expression is observed between 12 and 48 h postelectroporation. GFP can be detected as early as 4 h after electroporation and positive cells have been detected after 96 h, though much reduced in numbers. Longer incubations have not been attempted.

B. Protocol for *In Ovo* Neural Tube/Neural Crest Electroporations

This method is based on the method described by Itasaki *et al.* (1999). It works well in neural crest populations at all axial levels (Barembaum and Bronner-Fraser, 2004), with appropriate variation for age and position of the embryo.

1. Eggs are incubated on their side without rocking until they reach stages 7–12. The most anterior neural crest population around the eye will only be transfected when using three-somite embryos or younger. To electroporate neural tube and neural crest at hindbrain or spinal cord levels, older embryos are preferable since these populations onset migration later and it is easier to inject DNA solution into the lumen of the closed neural tube.

2. About 3 ml of albumin is removed using a syringe with an 18 gauge needle, by carefully piercing the shell at the small end. The eggs are windowed and the embryo is visualized by injecting a small amount India ink (1:50 dilution with Ringer's solution) immediately below the embryo. The eggs are tape-closed until needed.

3. Just prior to the electroporation, dilute DNA to 2.5 μg/μl with Ringer's solution. Use 1 μl of vegetable dye (2% FD&C Blue in Ringer's solution) in 20 μl of DNA, prepared on the same day. Fast green has also been used to visualize the DNA solution but has sometimes been found to be toxic.

4. Back fill a glass needle (made from a 0.8- to 1.1-mm-diameter glass capillary tube) with the DNA solution. Break off the tip to create a narrow opening.

5. Insert the tip of the glass needle through the vitelline membrane near the caudal end of the embryo and slide the tip parallel to the embryo to the site that will be electroporated. When electroporating the open neural plate, fill in the region between the neural folds. To inject the closed neural tube, slide the needle through one side of the tube and inject the DNA slowly, making sure that the tube is filled (as visualized by the expansion of the tube). Avoid using a strong stream as it may damage the embryo.

6. Place the electrodes on either side of the embryo, straddling the target site and 4 mm apart. Make sure the electrodes are immersed in the Ringer's solution and touching the vitelline membrane. By placing the positive electrode on the right side of the embryo, the neural crest on the right side will be labeled, thus allowing the embryo to be examined for GFP or RFP fluorescence *in ovo*. Use between 2 and 5 pulses of 18–25 V of 30–50 msec duration with a 100 msec intervening time. We get excellent survival using 5 30 msec pulses of 18 V. If more extensive electroporation is desired, we use up to 2 30 msec pulses of 25 V, though the survival in this instance is reduced. The parameters can thus be adjusted within these ranges to give optimal results. Take care not to tear the embryo when lifting out the electrode.

7. Seal the egg and incubate at 38 °C for the desired time. The survival of the electroporated embryos depends greatly on the quality of the eggs, ranging from 90% to 10% after 48 h incubation. Optimal expression of the transgene usually occurs between 12 and 48 h postelectroporation. GFP can be detected as early as 4 h after electroporation and positive cells have been detected after 96 h, though much reduced. Longer incubations have not been attempted. In general, most of the neural tube neural crest cells are labeled on only one side, though it is common to see a few labeled neural crest cells that have migrated to the contralateral side. It is also possible to label portions of the ectoderm in addition to the neural tube, especially if there has been substantial leakage of DNA onto the ectoderm, but the labeled cells are typically found contralateral to the labeled neural tube and crest cells.

II. Loss-of-Function by Electroporation

The development of new knockdown methods, involving strong binding nucleic acid analogues or deployment of small interfering RNAs, can be used in combination with electroporation to achieve transient loss-of-function in the embryos at

various times and locations. Three different methods yield important and reproducible results: electroporation of morpholino antisense oligos, dominant-negative constructs, and small interfering RNAs.

A. Dominant-Negative Constructs

Most dominant-negative constructs express truncated or variant forms of proteins that act as competitive inhibitors of the corresponding wild-type forms, disrupting the function of the endogenous proteins when coexpressed in the same cells. They can be introduced in the same manner as wild-type proteins in overexpression/ectopic expression assays described above (Barembaum and Bronner-Fraser, 2007; Eberhart *et al*., 2004; Ethell *et al*., 2001; Lee and Pfaff, 2003).

The inhibitory action of dominant-negative forms occurs because they retain some but not all activities of the wild-type protein, allowing them to compete for the target or substrate of the wild-type protein, but not to carry out its essential functions. For example, dominant-negative mutants of the transcription factors often have intact DNA-binding domains, allowing them to compete with the wild-type forms for the binding sites within the chromatin, but have mutations or truncations in their transactivation domains and therefore cannot carry out their transcriptional functions. Similarly, dominant-negative receptors are often characterized by the absence of the predicted cytoplasmic domain, while their extracellular domains remain intact, thus allowing them to compete with the wild-type receptors for the ligand, but not to transmit the signal intracellularly. The design of the dominant-negative constructs should therefore be adapted to the type of the protein whose function is to be inhibited and ideally tested in an independent *in vivo* or *in vitro* assay.

B. Morpholinos

Although originally utilized in *Xenopus* and zebrafish, morpholinos, modified antisense oligomers, have recently been adapted to the chicken. These are nucleic acid analogues in which deoxyribose sugar moieties are replaced by morpholine rings (GeneTools, LLC; http://www.gene-tools.com). They provide a powerful tool to knock down protein function in many vertebrate models. If the morpholino is designed against the proximal region of the translation initiation site, they bind to a specific mRNA transcript and sterically inhibit the initiation complex, thus halting the translation of a given protein. Alternatively, when designed to encompass the intron/exon boundaries within the pre-mRNA, they can interfere with the spliceosome, creating the alternative splicing variants or eliminating exons essential for the function of a given protein. The use of morpholinos, delivered into the chicken embryos by electroporation has provided a reproducible and solid means of gene inactivation (Basch *et al*., 2006; Kos *et al*., 2001; Sheng *et al*., 2003).

Electroporation serves to enhance uptake of morpholinos into chick embryonic cells [there is evidence that morpholinos alone can penetrate the plasma membrane and enter the cytosol, (Kos *et al*., 2003)] and to spatially target their application to a

desired tissue. As standard nonlabeled morpholinos have no charge, researchers should either use Gene Tools charged morpholinos, consisting of nonionic morpholino/DNA heteroduplexes electrostatically bound to ethoxylated polyethylenimine or fluorescently tagged morpholinos (fluorescein- or lissamine-tagged). In addition to facilitating visualization of morpholino uptake, tagging also fortuitously introduces a weak charge by virtue of the tag (negative or positive, respectively). Lightly charged morpholinos have been shown to be more amenable to electroporation, even though some success has been reported with the electroporation of the standard noncharged morpholino into trunk neuroepithelial cells (Kos *et al.*, 2001, 2003). Although the exact mechanism is unknown, it is thought that the application of electric pulses to the cells temporarily disrupts the stability of the plasma cell membrane creating the transient pores through which the nucleic acid molecules (DNA or RNA) to be transected are transported into the cytosol. The directed electric field orients and enhances movement of charged molecules into the cell. Although the role of simple diffusion of small charged molecules has never been assessed, it is possible that it could add the additional drive and mediate the transport through pores in the destabilized plasma membrane.

Administration of morpholinos can be achieved with precise spatiotemporal precision. In order to obtain protein depletion, care must be taken to apply the morpholino prior to accumulation of the gene transcript. It is possible to achieve targeted knockdown by electroporating the morpholino into selective regions of the embryo, such as the ectoderm, neural tube, sensory placodes, limb mesenchyme, paraxial mesoderm, and many other embryonic sites. The electroporation procedure can be performed *in ovo*, as described above, or *ex ovo* using embryo culture systems, described in other chapters of this volume (see Chapter 1 by Streit and Stern, this volume). The procedure for electroporation of morpholinos into embryonic ectoderm or neural tube is very similar to the one described for DNA constructs above. The tissue targeting, choice of electrodes, and fine-tuning of the electric pulse conditions (number of pulses, voltage level, and ON/OFF pulse durations) should be adapted to each application separately.

Here, we focus on electroporation of morpholinos into the early chicken embryo. Chick gastrulae and early neurulae adapt well to this perturbation, with survival rates as high as 90% for 1 day postelectroporation. Epiblast cells demonstrate very efficient uptake of the morpholino, with relatively even distribution across the targeted cell population. This is likely because of the loose association of undifferentiated cells at these stages. Electroporation into early embryos not only allows targeted knockdown by taking advantage of the various fate maps of early gastrulae and neurulae but also provides the possibility of administering the knockdown agent before the onset of transcription of the gene of interest. It is possible to deliver the morpholino unilaterally, with the nonelectroporated side serving as an internal control.

As in other vertebrates, one problem with morpholino-mediated knockdown in chicken embryos is the penetrance of the morphant phenotype to later derivatives. Due to a highly regulative nature of vertebrate embryos, redundancy and

compensation by cell cycle control mechanisms lead to partial or complete correction of late phenotypes. Thus, the primary utility of morpholinos is for short-term experiments.

1. The Use of Appropriate Controls

When planning a morpholino electroporation experiment, the experiment must be accompanied by a proper set of controls, both for possible toxicity of the knockdown agent as well as for its off-target, nonspecific effects. Standard control morpholinos serve to rule out the possibility that the observed phenotype is due to a general toxicity of the knockdown agent or alterations in the embryo produced by application of the electrical charge. While five-mismatch morpholinos are recommended as a specificity control for off-target effects, caution must be used because five-base pair mismatched morpholinos also can have nonspecific effects. If the match is too close, they also can partially block the original target. Ideally, prior to the electroporation into the embryos, each ordered morpholino should be tested for its knockdown efficiency. This can be done by: (1) using *in vitro* translation systems to demonstrate that a morpholino specifically impairs protein translation of a given transcript or (2) *in vivo*, by electroporating/transfecting the morpholino together with its target mRNA. For this purpose, we routinely use mRNA/morpholino coinjections into a single cell *Xenopus* embryos to assess effects on production of exogenously introduced protein. In short, capped C-terminally tagged mRNA coding for the targeted gene of interest is injected with and without corresponding morpholino and the presence of protein in the embryo lysate assessed using SDS-PAGE and Western blot with an antibody against the C-terminal tag. An additional method to address specificity is to obtain the same phenotype using two different preferably nonoverlapping morpholinos.

Theoretically, the best method to demonstrate that the morphant phenotype is due to specific binding of the morpholino to its predicted target is to perform a rescue experiment. This approach consists of coelectroporating the morpholino with the expression construct containing the coding sequence of the targeted gene in question. So that it is unaffected by the morpholino, the sequence of the rescue expression construct is either altered or obtained from a different species such that the morpholino does not affect its translation. Successful rescue suggests that the observed morphant phenotype was due to the abolition of the targeted protein. Unfortunately this experiment is not as straightforward as it appears at first blush. First of all, when the DNA expression plasmid is coelectroporated with negatively charged morpholino (either fluorescein- or special delivery lissamine-tagged), there is the discrepancy in the size of the morpholino-positive domain versus that of the plasmid, probably due to differences in size. The former is slightly larger, though this difference diminishes as the transcripts accumulate. Furthermore, the levels of the electroporated DNA construct have to be titered and the concentration response curve established, to avoid the overexpression phenotype due to excess DNA-introduced. To make this experiment reproducible and interpretable, the number of experimental embryos needs to be

sufficiently high to be statistically relevant. Performing a concentration response curve is also recommended to establish the gradation and penetrance of the phenotype.

The most reliable confirmation of the results of a transient knockdown experiment is the employment of two independent methods (e.g., morpholino with shRNA or dominant-negative competitive construct) to obtain the same phenotype.

2. Analysis of the Morphant Phenotype After the Morpholino Electroporation

When embryos have reached the desired stage, the extent of integration of the knockdown agent should be checked using a fluorescence dissecting microscope. Embryos are fixed and the distribution of morpholino recorded (photographed). Embryos with the poor transfection efficiency/extent are discarded.

Experimental and control embryos are analyzed for alteration in expression of markers genes of interest by whole-mount and/or section *in situ* hybridization as well as antibody staining. If antibody against the targeted gene is available, immunocytochemistry on the morphant and control embryos should confirm the depletion of the targeted protein. If the antibody does not work in the immunocytochemistry procedure, immuno blot analysis can be performed as an alternative. Alterations in the cell numbers should be assessed by performing proliferation and cell death assays to eliminate the possibility of nonspecific toxic effects of the electroporations, as well as to test for the involvement of the investigated target in cell cycle regulation.

Finally, one can dissect and isolate regions of interest from injected and contralateral sides of both morphant and control embryos and perform microextractions of total RNA and cDNA syntheses independently. This procedure allows monitoring changes in expression level of a wide range of potential downstream factors by quantitative RT PCR.

Here, we present an example of the protocol for morpholino electroporation into the stage Hamburger and Hamilton (HH) 4–7 embryos. We routinely use a modified New culture system to explant and culture the embryos following the procedure (Chapman *et al.*, 2001). This method simplifies the approach introduced by New (1955, 1966) for chick whole-embryo culture *ex ovo*, by using filter paper carriers to support the early blastoderm/vitelline membrane under sufficient tension to allow development on a layer of thin albumen or agar–albumen substrate.

C. Protocol for *Ex Ovo* Morpholino Electroporations into the Early Chick Embryos

1. Eggs are incubated in the upright position for the desired length of time (this time may vary depending on the quality of the eggs and the season, as well as on the expected embryonic stage, for stage HH4 incubation usually takes 18–24 h).

After incubation, eggs are allowed to cool for 15–20 min. Shells are sprayed with 70% ethanol for sterility.

2. The methods for extracting the embryo vary. We routinely create a small opening in the uppermost extremity of the shell and carefully pour and pinch off the excess thick albumin, while preserving the thin albumin in a falcon tube for the later culturing step. The shell containing the intact yolk is reduced to minimum, such that thick albumin covering the blastoderm can be carefully removed by gentle horizontal scraping movements with a pair of large blunt forceps. The thick albumen can also be removed using a piece of folded Kimwipe. Alternatively, the egg can be carefully cracked and the contents delivered into a glass petri dish, and the thick albumen subsequently removed (for more details, see Chapman *et al.*, 2001).

3. Position the square piece of the filter paper that has an opening in the middle over the cleared area of the vitelline membrane, such that the opening is centered on the blastoderm. To prepare the filter paper, cut 1.5–2.0 cm squares and make an opening in the middle using a hole puncher. The improperly cleaned vitelline membrane surface will interfere with the adherence to the filter paper, resulting in poor or arrested development. Using sharp scissors cut the entire perimeter around or through the filter paper and gently pull it away from the yolk. Immerse the filter paper/blastoderm, ventral side up into a petri dish containing Ringer's solution and swirl gently to remove excess yolk (care should be taken not to detach the embryo from the vitelline membrane) and move it to the clean Ringer's dish. We never keep the explanted embryos for prolonged period of times in the Ringer's solution, as this can result in detachment of the blastoderm from the vitelline membrane.

4. Prior to electroporation, thaw an aliquot of the morpholino and heat it to 65 °C for 5 min to dissolve the precipitates. On receiving the morpholino, reconstitute the desiccated agent to 1 mM stock solution in nuclease-free water. As the optimal concentrations vary from morpholino to morpholino, we routinely test a range between 250 μM and 1 mM and use the highest concentration that does not have toxic effects on the developing embryos, because the penetrance of phenotype is higher under these conditions. When morpholinos are fluorescently tagged, they are also visible upon injection, so the use of solution dyes such as vegetable dye (2% FD&C Blue in Ringer's solution) or Fast Green is unnecessary. Moreover the use of Fast Green in morpholino solutions has been reported to inhibit the uptake of the morpholinos into the cells (Kos *et al.*, 2003). A recent recommendation by GeneTools specialist is to reconstitute the morpholino in nuclease free-water to a desired stock concentration of 1-2 mM, but to keep both stock solution and individual aliquots in a dry and obscure place at room temperature.

5. Back fill a glass needle (made from a 0.8- to 1.1-mm-diameter glass capillary tube) with the morpholino solution. Break off the tip to create a narrow opening.

6. Position the embryo on the filter paper ventral side up into the electroporation chamber. The embryo should be submerged into the thin layer of Ringer's solution. Our electroporation chamber is petri dish-shaped, with the 4-mm-thick solid

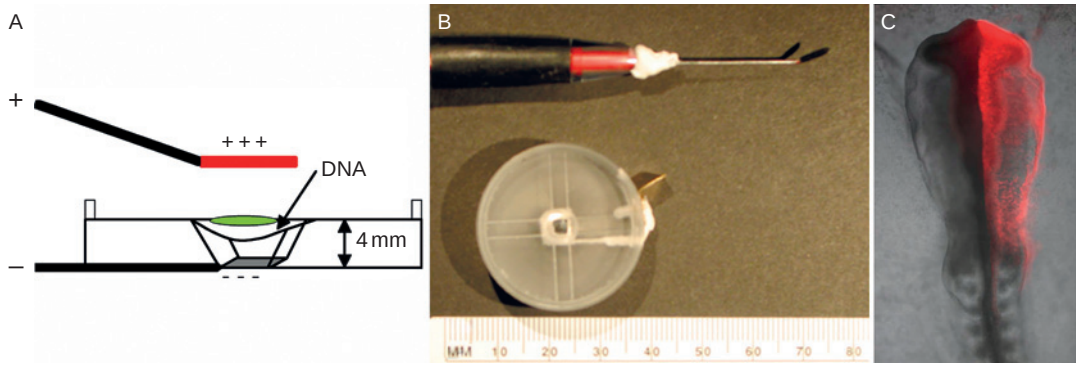

Fig. 2 Morpholino electroporations into the early chicken embryo at stages HH4–7. (A) Schematic of the apparatus used for *ex ovo* early embryo electroporations. (B) Early embryo electroporation apparatus consists of the single upper platinum electrode in the form of the thin sheet and a petri dish-shaped electroporation chamber, with a 4-mm-thick bottom and an inverted blunt-cone shaped opening in the center, covered with thin platinum layer. (C) Unilateral incorporation of the lissamine-tagged morpholino, electroporated at stage HH4, into the neural crest and neural tube progenitors at the cephalic level and visualized at stage HH10. Image courtesy of J. Khudyakov. (See Plate no. 14 in the Color Plate Section.)

isolation base that has an inverted blunt-cone shaped opening in the center, and whose bottom is covered with thin platinum sheet (Fig. 2A and B). The depth of this depression (and therefore the distance of the platinum sheet from the embryo positioned above it in the chamber) is 4 mm. The blastoderm on the culture paper is positioned ventral side up in the chamber and the morpholino injected underneath it. The platinum sheet on the bottom of the electroporation chamber is therefore under the embryo and will serve as a negatively charged electrode (cathode) in the case of FITC-tagged, slightly negatively charged morpholinos, or as anode (positively charged electrode) if the morpholino is lissamine-tagged (carries a slight positive charge).

7. Inject the morpholino solution into the desired position in the periembryonic space in between the blastoderm and the vitelline membrane. This is achieved by inserting the tip of the glass needle through the blastoderm at the presumptive position of the targeted progenitors, sliding it parallel to the plane of the disk and lifting the tissue slightly, without tearing it, to assure that the vitelline membrane is not pierced.

8. Immediately prior to application of the upper platinum electrode, shaped like a thin sheet, make sure that the blastoderm is positioned directly above the lower electrode portion of the chamber and not over the isolator base. Position the upper electrode precisely above the embryo and apply the electric pulses. Make sure that the electrode is in full contact with the Ringer's layer overlying the embryo, but not touching the embryo, as this action could result in burning

the cell layer that contacts it. We usually apply between 3 and 5 square 7 V pulses with duration of 50 msec and a 100 msec intervening rest time. These conditions can be adjusted as a function of the survival rate and the electroporation efficiency.

9. After electroporation, the embryo is left to rest for about a minute and then transferred to the 3 cm culture dish, coated with approximately 1 ml of thin albumen. The dish is sealed using small volume of thin albumen deposited inside the rim of the lid. The explanted electroporated embryo is then incubated for the desired amount of time at 38 °C in the CO_2 tissue culture incubator. The survival of the electroporated embryos depends not only on the quality of the eggs but also on the morpholino used and can range from 70–80% after 20 h of incubation to only 10% after 48 h. The length of the incubation time varies and depends primarily not only on the stage of the embryo ultimately desired for the phenotypic analysis but also on the quality of the particular egg batch and should be assessed on the case per case basis.

D. RNAi

The use of double-stranded RNAs (dsRNAs), originally adapted to animal models in *Caenorhabditis elegans* and *Drosophila melanogaster*, provides a powerful knockdown approach. Termed RNA-mediated interference, it triggers silencing of genes homologous to the introduced RNA sequence. In higher vertebrates, injection of long dsRNAs (>30 nucleotides) appears to induce a cytotoxic reaction that leads to the nonspecific RNA degradation and the overall shutdown of protein synthesis within the host cell. However, this toxicity can be circumvented by the use of small interfering RNAs (dsRNA molecules of 21–22 nucleotides) that can be chemically synthesized or shRNAs that are processed by the cellular RNAi machinery into small dsRNAs. These can be introduced by several types of DNA vectors that drive shRNA expression. Commonly used constructs developed for use in other developmental systems exploit the characteristics of the RNA polymerase III to terminate transcription upon incorporating a sequence of 3–6 uridines, thus satisfying the requirement that siRNAs possess defined 3′ termini that hybridize to a mRNA target. RNA PolIII promoters (usually mammalian U6 or H1 promoters) chosen to drive expression of sh/siRNAs are relatively simple and all elements necessary for their activity lie directly upstream of the sequence transcribed, thus eliminating any need to include promoter sequence within the siRNA. In chick, electroporation can be employed to achieve gene silencing by either synthetically produced siRNAs (Hu *et al*., 2002) or vector-based shRNA (Katahira and Nakamura, 2003; for review see Nakamura *et al*., 2004). These approaches are not yet widely used in the chick system due to difficulties in producing robust phenotypes, possibly due to the rapid degradation of siRNAs or inefficient transcription from a PolIII promoter (Hernandez and Bueno, 2005). More recently, the application of shRNAs present within the naturally occurring microRNA (miRNA) context has been successfully utilized (Silva *et al*., 2005; Zeng and Cullen, 2005; Zeng *et al*., 2002). Pre-miRNA transcripts transcribed from this improved vector-based RNAi system are

processed within their natural cellular context, first by Drosha in the nucleus, subsequently protected and exported in association with exportin-5 and finally excised by Dicer to produce siRNAs (Kim, 2005).

Recently, Das *et al.* (2006) have introduced retroviral RNAi vectors optimized for gene silencing in chicken embryos. Pre-miRNAs are transcribed from a chicken U6 promoter and express shRNAs embedded within the context of microRNA mir-30, to ensure natural processing. The authors present very promising results, suggesting that the increased knockdown efficiency (90% silencing of target genes modeled) is due to use of both the chicken U6 promoter and the microRNA operon expression context. All of the RNAi plasmids as well as precloned mir-shRNAs used therein can be obtained from ARK-Genomics.org. One pitfall with the use of vectors producing shRNAs flanked with microRNA arms is the relatively frequent occurrence of nonspecific effects, possibly due to the overload of the system causing the delay in the transcriptional machinery. Although RNAi holds future promise, further improvements to the technique may increase efficiency and decrease off-target effects.

III. Retrovirus-Mediated Protein Expression

The use of replication-competent and replication-defective retrovirus vectors allows the expression of a protein of interest in the infected cells (Morgan and Fekete, 1996). Replication-competent retroviral systems, such as RCASBP (Replication-Competent, Avian leukemia virus long terminal repeat, Splice acceptor, Bryan high-titer Polymerase), contain all genes necessary for completion of the viral replication cycle and can therefore spread from the initially infected cells into their environment, constantly enlarging the domain of transgene expression. RCASBP virion binds to a cell through interaction with a receptor and the replication cycle of the retrovirus starts, leading to the release of the viral core into the cytoplasm of the host cell. The core is subsequently uncoated and the single-stranded RNA genome is reverse transcribed into double-stranded DNA. During M phase, the viral DNA can be integrated into the host genome and the mRNA of the transgene, introduced by the construct, can be transcribed. Thus, the timing of the expression of the gene of interest is dependent on the proliferative state of the target tissue and one should take this into account when considering overexpression or misexpression in postmitotic cells. In the neural tube injected at stage 9, proteins expressed from RCASBP have been detected as early as 9 h after infection, with most of the cells expressing protein after 18 h (Homburger and Fekete, 1996). Another important consideration is the envelope subgroup that determines which cellular receptor the virion will bind to. Subgroup B has been widely used in our laboratory and will infect neural tube and neural crest cells efficiently (Homburger and Fekete, 1996).

The replication-defective retrovirus lacks most of the viral genes, but retains *cis*-regulatory elements necessary for packaging, reverse transcription, integration,

and transcription, allowing the integration and expression of the desired protein only in the cells that were initially infected. Because the absence of the essential viral genes does not permit further cycles of the viral infection, these vectors have been very useful in cell lineage studies. However, in order to produce infectious viral particles, introduction of this vector into packaging cell lines is required.

For the most part, retroviruses have been superceded by plasmid electroporations. The DNA expression vectors are more flexible and can contain GFP or other reporter proteins that allow the transfection efficiency to be rapidly evaluated. The replication-competent retroviruses also have a limitation in the size of the transgene they can contain, as inserts longer than 2.5 kb interfere with the assembly of the virions, resulting in a very low titer. Furthermore, while EGFP expressed from a electroporated DNA construct can be first detected several hours after transfection, retroviral proteins can only be detected after 9 h or more, depending on the replication state of the target cells. Plasmid electroporations also do not require the production of high titer virus. Finally, expression of retroviral proteins may affect the host cell itself. However, the replication-competent retrovirus vectors can be useful in their ability to spread to other cells, which may increase the number of cells receiving the transgene. This can be particularly helpful in cases when target tissue is difficult to electroporate/transfect, such as mesenchymal cell populations. This approach also ensures the prolonged expression of the transgene because of its integration into the host genome. It is worth noting that recently described Tol2 transposon-mediated gene transfer using electroporation into chick tissue helps achieve the same goal (Sato *et al*., 2007).

The RCASBP retroviruses are constructed using an adapter plasmid that contains all the retrovirus sequence in a plasmid that can be grown in bacteria. Once transfected into a chicken cell, the viral messages are transcribed and infectious virus produced. The DNA coding the protein of interest is cloned into the appropriate site in this plasmid so that the protein will be expressed from the integrated form of the retrovirus (provirus), under the control of the viral enhancer elements. RCASBP adapter plasmid SLAX13 can be used to clone the desired DNA fragment into RCASBP(B) vector. The SLAX13 plasmid can be grown and purified using standard techniques. The QIAGEN Maxi preps have been adequate for the purpose of obtaining DNA of sufficient purity for transfection.

To produce the virus, it is necessary to transfect chicken fibroblasts with the adapter plasmid. The cells are then passaged several times to allow the retrovirus particles to be produced and to infect the rest of the cells in the cultures. The infected cells continue to produce virus and secrete them into the culture media during incubation. The media is collected and the viruses are concentrated by centrifugation. The concentrated viruses can then be used for infecting chicken embryos. The virus-infected cells can be detected using an antibody to an epitope of the viral *gag*-encoded Matrix protein (such as the antibody AMV-3C2 from the Developmental Studies Hybridoma Bank).

The following protocols are based on those provided by Dr. Donna Fekete.

A. Protocol for Primary Cultures of Chick Fibroblasts

1. Incubate 1 dozen line 0 eggs [for RCASBP(B)] for 10 days. Collect four to six embryos in a petri dish. Cut off the limbs and heads. Remove the viscera.
2. Mince well with a razor blade.
3. Transfer tissue into Ehrlenmeyer flask with 10 ml trypsin solution at 37 °C. Add stir bar and mix at low speed for 15 min.
4. Allow the big chunks to settle for 5 min and transfer supernatant to a sterile 50 ml tube.
5. Add an equal volume of 100% fetal calf serum (FCS). Mix by inverting.
6. Spin at 1000 rpm for 5 min. Save the pellet.
7. Resuspend pellet in 100% FCS and spin again.
8. Resuspend pellet in chick culture media (10% FCS, 2% chicken serum, in DMEM).
9. Plate cells into multiple 10 cm tissue culture plates at three different concentrations: 10^7, 10^6, or 3×10^5 cells per plate. Incubate at 37 °C.
10. Once the cells have begun to grow, choose the fastest growing plates. When they are 80–90% confluent, collect primaries by freezing at a ratio of three vials per dish (1 ml per dish) in 10% FCS, 12% DMSO in DMEM. Secondaries are passaged once at 1:4 using trypsin digestion and frozen 1–2 days later when they are 80% confluent. Use secondaries for virus work.
11. Thaw 1 ml aliquots into three 6-cm plates. Passage every other day at 1:4 and 1:8 dilutions.

B. Protocol for Lipofectamine Transfection

1. Prepare tube A (6 μg DNA, in 840 μl Optimem) and tube B (56 μl Lipofectamine, 840 μl Optimem). Mix tubes A and B and incubate at room temperature for 30 min.
2. Use cells in a 10 cm plate at 80% confluency. Wash cells in Optimem.
3. Add 6.4 ml of Optimem to mix of tubes A and B. Remove Optimem from cells and add Lipofectamine mix to cells and incubate at 37 °C for 5 h.
4. Replace media with chick culture media. Grow at 37 °C.
5. Passage several times to ensure that all the cells have been infected.

C. Protocol for Adjusting Virus Concentration

1. When the plates are just confluent, replace media with low serum media (2% FCS, 0.2% chick serum) and grow for 24 h.
2. Collect media from confluent plates in 50 ml tubes. Keep on ice from this time on. Spin at 3000 rpm for 30 min to remove cellular debris.

3. Remove supernatant and pool.
4. Add to sterile polyallomer tubes and spin in a Beckman SW-28 rotor at 20,000 rpm for 2 h and 15 min.

Pour off as much of the supernatant as possible. Keep tubes on ice. Resuspend the pellet in the remaining liquid. This can be done by triturating slowly with a 200 μl pipette. Once the pellet is dissolved split the virus solution into 20 μl aliquots and store at −80 °C.

D. Protocol for Retroviral Infection of Neural Tubes

1. Thaw a tube of high titer RCASBP(B) virus on ice. Add polybrene to 8 μg/ml.
2. Back fill a glass needle (made from a 0.8- to 1.1-mm-diameter glass capillary tube) with the virus solution. Break off the tip to create a narrow opening.
3. Open and window chicken eggs of stage 8–9. About 3 ml of albumin is removed using a syringe with an 18 gauge needle and carefully piercing the shell at the small end. The eggs are windowed and the embryo visualized by injecting a small amount India ink (1:50 dilution with Ringer's solution) immediately below the embryo. The eggs are tape-closed until needed. Add Ringer's solution to cover the embryo.
4. Remove some of the vitelline membrane above the egg. Inject the neural tube at the desired level with the virus solution. Be certain that the tube is filled (usually the tube expands a bit when it is filled). Adjust the air pressure so that only as much virus as needed is added.
5. Incubate at 37 °C for appropriate amount of time.

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CHAPTER 13

Manipulation and Electroporation of the Avian Segmental Plate and Somites *In Vitro*

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Abstract

The chick embryo has been a leading model in embryological studies, including somitogenesis, because of its easy accessibility for manipulation during most of its development. The recent development of gain- and loss-of-function strategies of specific genes by electroporation has made the chick embryo an even more attractive model for embryological studies. *In vitro* electroporation, combined with whole chick embryo culture techniques, provides a wide range of possible approaches to study early stages of chick embryogenesis. We will describe *in vitro* electroporation techniques that are useful to study the development of somites (and any other derivative of the primitive streak and epiblast), and discuss potential applications of these methods.

I. Introduction

Somites are one of the key structures in the embryonic vertebrate body, and they appear as a series of paired epithelial blocks of paraxial mesoderm cells lying on both sides of the neural tube and notochord. The somitic series begins immediately after the otic vesicles and extends posteriorly to the caudal tip of the embryo. Pairs of somites are sequentially and rhythmically formed from the rostral extremity of the mesenchymal presomitic mesoderm (PSM; also called the segmental plate). In parallel, new cells are sequentially added to the posterior PSM as a result of paraxial mesoderm formation from the regressing primitive streak and then from the tail bud during embryonic body axis elongation. This patterning strategy establishes the metameric organization of the vertebrate body as is evident by the periodic distribution of vertebrae, associated muscles, and peripheral nerves. The avian embryo has been a leading model in somitogenesis studies since the nineteenth century, and much of our current knowledge of the vertebrate segmentation process derives from studies performed in chick embryos (Pourquie, 2004). The recent introduction of electroporation techniques has now made it possible to express nucleic acid constructs in the developing embryo to interfere with the role of genes during development (Nakamura and Funahashi, 2001). Such techniques have led to novel molecular insights into the patterning of the vertebrate paraxial mesoderm (Dale *et al*., 2003; Dubrulle *et al*., 2001; Iimura and Pourquie, 2006; Iimura *et al*., 2007; Nakaya *et al*., 2004). In this chapter, we will describe the *in vitro* electroporation procedure and a grafting method of the electroporated

tissue that contains somite precursors to achieve focal targeting of gene transfer by electroporation. Further potential applications of these methods will also be also discussed.

II. Rationale

The basic principle of electroporation is that application of an electric field to cells generates the reversible opening of pores in the cellular membrane, which then allows charged macromolecules, such as DNA, RNA, and proteins, to penetrate the cells. The difficulty in applying this technique in living tissue had been that the electric pulses often damage the tissue, resulting in significant cell death. This issue was overcome through the important discovery that using a repetitive series of low-voltage, square-wave pulses, instead of average- or bell-shaped exponential pulses, successfully reduced the lethal damage to the tissue. Muramatsu *et al.* (1997) first reported the use of such electroporation techniques *in ovo* to overexpress genes in the chick embryo. Subsequently, the conditions for routine use of *in ovo* electroporation in various tissues have been established, and a wide range of potential applications has been developed (Inoue and Krumlauf, 2001; Itasaki *et al.*, 1999; Nakamura and Funahashi, 2001). Electroporation of embryonic tissues is particularly well-suited for tissues in which cells are electrically coupled by gap junctions like epithelia. Thus, the technique has been widely used to overexpress various constructs in the neuro-epithelium or epiblast. In contrast, overexpression of mesenchymal tissues, such as the PSM, is very inefficient. We developed a method that targets the somitic precursors *in ovo* at the stage when they are still epithelial, in the epiblast (Dubrulle *et al.*, 2001). To achieve misexpression of genes of interest in somites and in the PSM, we targeted somite precursors in the anterior epiblast (Hatada and Stern, 1994; Iimura and Pourquie, 2006; Iimura *et al.*, 2007; Schoenwolf *et al.*, 1992) and in the anterior primitive streak at stages 3+ to 7 Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992; Iimura and Pourquie, 2006; Iimura *et al.*, 2007; Nicolet, 1971; Psychoyos and Stern, 1996; Rosenquist, 1966).

It had been difficult to establish electroporation methods for the epiblast in early gastrulating embryos because of their small size and fragility. The use of flat electrodes, combined with an *in vitro* culture system, has made it possible to overexpress genes in embryos at the primitive streak stage (Yang *et al.*, 2002; Yasuda *et al.*, 2000). Commercially available electrodes (Kobayashi *et al.*, 2002) and an improved *in vitro* culture method for early stage chick embryos using paper rings (Chapman *et al.*, 2001) have made it possible to reliably carry out *in vitro* electroporation during gastrulation stages of the chick embryo (around stages 3–7 HH). For this purpose, a square platinum cathode is embedded in the bottom of a silicon rubber dish that is glued in the center of a petri dish (Fig. 1A). A flat platinum anode of the same size is placed within a given distance of a gap above the cathode (Fig. 1B), with the embryo placed on the paper ring in between (Fig. 1C). The use of this *in vitro* method permits the precise staging of chick embryos, which

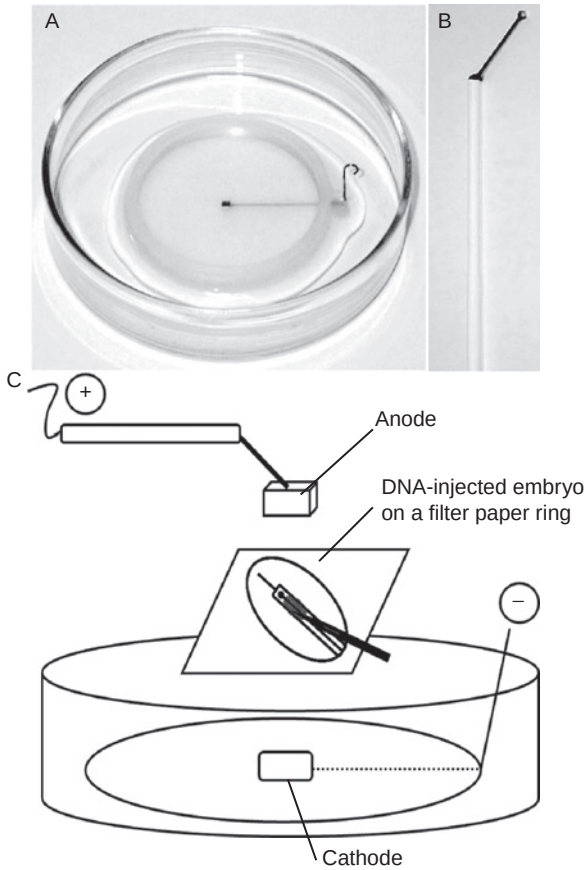

Fig. 1 *In vitro* electroporation. (A and B) Petri dish-type electrodes (CUY701P2L), cathode (A) and anode (B). (C) Schematic drawing for the basic procedure of *in vitro* electroporation. An embryo is supported by a filter paper ring (Chapman *et al.*, 2001), injected with DNA solution, and then placed between the cathode and the anode for electroporation.

is critical for targeting the desired somitic level along the anteroposterior (AP) axis. This issue will be discussed later in this chapter.

III. Methods

A. General Procedure for *In Vitro* Electroporation

1. Preparation of Embryo

Fertilized chick eggs are obtained from commercial sources. Eggs are stored at 15 °C for up to 1 week and are then incubated at 38 °C in a humidified (60–80%) incubator. After incubating eggs for the desired length of time, embryos are

prepared for EC culture (Chapman *et al*., 2001) and staged according to Hamburger and Hamilton (1992) and by counting the somites when possible. The embryo on the paper ring is then placed ventral side up into the inner silicone ring of the petri dish-type electrodes (cathode) (CUY701P2L, NEPA Gene) (Kobayashi *et al*., 2002) in which the inner ring is fully filled with phosphate-buffered saline (PBS) or Hank's solution (Fig. 1).

2. *In Vitro* Electroporation

Using a fine glass needle, approximately 0.2 μl of plasmid DNA solution is injected into a slit between the vitelline membrane and the epiblast, targeting the anterior primitive streak groove (Fig. 2). Promptly after the DNA injection, the position of the embryo is gently adjusted so that the tissue area injected with the plasmid DNA lies above the cathode. Subsequently, the anode is placed onto the surface of the filled solution in the inner ring, ensuring that the electrode covers the targeted area of the embryo (Fig. 1). In this way, the distance between the two electrodes is always maintained at approximately 4 mm. Square-wave pulses are applied (e.g., 6–8 V, 50 msec, 5 pulses with 150 msec intervals) by an electroporator (Electro-Square Porator CUY21, NEPA Gene) using a foot pedal or a push button. The electroporated embryo is then placed in an agar–albumin culture dish and reincubated at 38 °C; GFP fluorescence of the electroporated reporter is usually first detected after 3–4 h of reincubation.

B. Targeting the Electroporated Area by Tissue Grafting

The petri dish-type electrode (CUY701P2L, 2 × 2 mm) largely covers the whole embryonic tissue (area pellucida) at stages 3–5 HH. Because of the pressure between the vitelline membrane and the epiblast, the injected DNA diffuses quite fast, thus resulting in a large electroporated domain when using the petri dish electrodes. One possible way to limit the electroporated area is through focal DNA injection. Alternatively, microsurgical tissue grafting, a well-established technique in avian embryology, can be used to restrict the electroporated area to a defined zone (Fig. 3).

1. Excision of the Donor Fragment from the Epiblast/Primitive Streak

An electroporated embryo, prepared as above, is placed ventral side up in an agar–albumin plate. Figure 3A illustrates the sequence of steps necessary to obtain a donor fragment from the primitive streak. Using a tungsten needle, the first vertical incision is made along the outer margin of the primitive streak. Then, the second incision is made along the other outer margin of the primitive streak. If a smaller area (half the width of the streak) is grafted, the second incision is made along the primitive streak groove. Finally, two horizontal incisions are made to excise the donor tissue fragment. The isolated fragment of the primitive streak is then transferred onto a host embryo using a glass capillary (see next section).

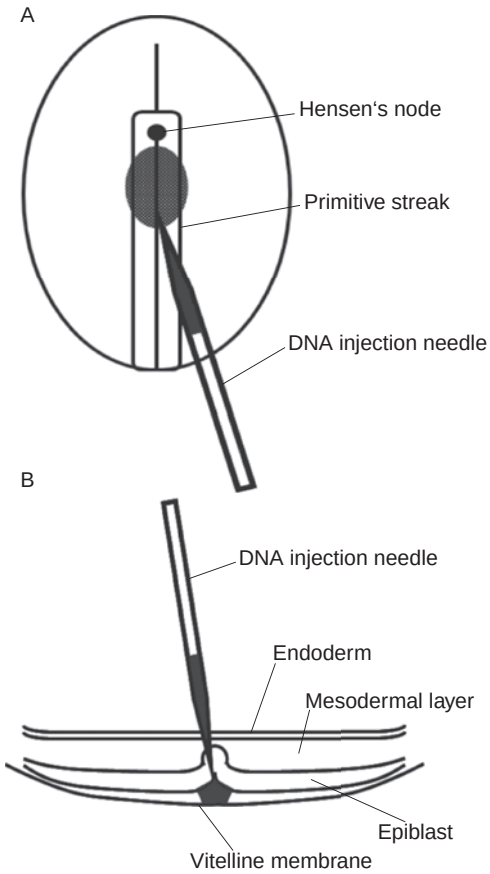

Fig. 2 Schematic drawing for DNA injection. (A) Targeting somitic precursors in the anterior primitive streak and neighboring epiblast. DNA solution (blue) is injected through a capillary needle by mouth-pipetting, targeting the anterior one-third of the primitive streak level. The DNA solution flows along the primitive streak groove anteriorly and spreads laterally. (B) Targeting the space between the epiblast and vitelline membrane, the transverse section of a gastrulation-stage embryo, ventral side up. The injection needle penetrates the three germ layers from the endoderm side and stops when the tip of the needle touches the vitelline membrane so that the DNA solution (blue) is injected into the space between the vitelline membrane and the epiblast. If the vitelline membrane is broken accidentally, a flow of blue DNA solution beneath the embryo will be visible.

2. Preparation of the Host Embryo

A host embryo of the same stage as the donor embryo is prepared for EC culture and placed ventral side up on an agar–albumin plate. A fragment of equivalent size to that of the donor fragment is removed as described above at exactly the same position as in the donor, thus leaving a hole in the primitive streak/epiblast. Keeping the donor fragment adjacent to the operation site in the donor is a good way to estimate the size of the tissue to remove.

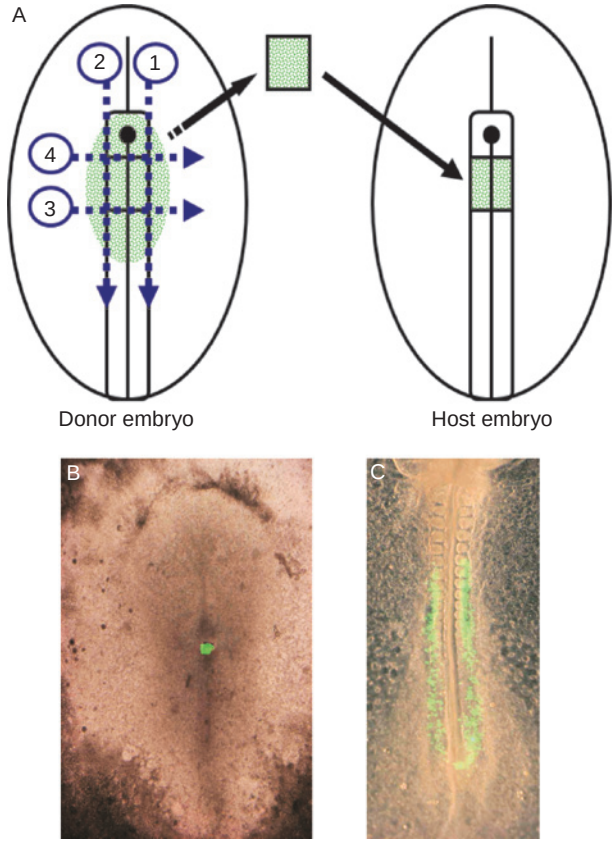

Fig. 3 Primitive streak graft. (A) Schematic drawing of a primitive streak graft. A tissue fragment of the anterior primitive streak with the underlying tissue layers is removed from an electroporated donor embryo. To dissect the tissue fragment from the donor embryo, incisions are made in the order shown from 1 to 4 using a sharp tungsten needle. An equivalent fragment is removed from the host embryo and the electroporated tissue fragment from the donor is grafted onto the same position into the host embryo. (B) Stage 5 HH embryo just after the graft. The fluorescent grafted tissue fragment is located at the level of the anterior primitive streak. (C) The same embryo shown in (B) 24 h after reincubation at 38 °C. EGFP-positive descendent cells (green) largely contribute to somites and the presomitic mesoderm. Ventral views, anterior to the top. (See Plate no.15 in the Color Plate Section.)

3. Implantation of the Donor Fragment of the Primitive Streak

After confirming that the tissue fragment is thoroughly removed from the host embryo, the donor fragment is gently transferred next to the tissue hole using a tungsten needle and then positioned so as to replace the excised fragment, ensuring that it is properly oriented along the dorsoventral and AP axis. For this purpose, a part of the donor fragment may be marked by a vital dye (such as DiI) beforehand, especially because the AP orientation is not visible morphologically.

The transplanted host embryo is then left at room temperature for 10–30 min to allow the grafted embryo to heal. The operated embryo is then reincubated at 38 °C for the desired time period and then processed for subsequent analysis (Fig. 3C). Repeating this procedure at different primitive streak positions, allows two-color grafts to be generated (Iimura and Pourquie, 2006; Iimura *et al.*, 2007).

C. Focal Electroporation Using a Rod-Type Electrode

Another way to limit the area of the electroporated tissue is by using a rod-type electrode (e.g., CUY195P0.3, NEPA Gene). By combining this rod-type anode and a petri dish-type cathode (e.g., CUY701P2L), the electroporation area is limited to 0.5–0.3 mm in diameter (Iimura and Pourquie, 2006; Fig. 4A). Three successive square-wave pulses of 5–7 V for 25 msec with 150 msec intervals were applied in this experiment.

D. Coelectroporation

It is possible to coexpress two distinct constructs in the same cells, much as in an *in vitro* transfection experiment. The two expression constructs (1.0 μg/μl each) harboring different fluorescent protein reporters (e.g., pCAGGS-EGFP and pCAGGS-DsRed Express) (Iimura and Pourquie, 2006) are mixed, and the solution is then used to electroporate the anterior primitive streak/epiblast. This electroporation results in the two distinct constructs being expressed in essentially the same cells (see the same distribution patterns of EGFP-positive cells and DsRed-positive cells in Fig. 4B). In our experience, if two reporters driven by distinct promoters are coelectroporated, a slightly different distribution pattern between the two populations of reporter-positive cells is often observed, probably due to some degree of tissue or cell preference even with "ubiquitous" promoters (McGrew *et al.*, 2004).

E. Successive Electroporation

One way to achieve overexpression of two genes in different sets of cells of the same tissue is by performing serial tissue grafts of electroporated primitive streak/epiblast in the same host embryo as described above. Another less challenging method is to perform successive electroporations using the rod-type electrode to obtain expression of the two genes in distinct populations of cells (Iimura and Pourquie, 2006). The low voltage of square-wave pulses using the rod-type electrode allows the survival of the embryos. A first focal electroporation is performed with the first DNA construct, and subsequently, the second construct is electroporated in the same territory. This procedure, in contrast to the coelectroporation described above, results in the two different constructs being expressed in largely distinct populations of cells (Fig. 4C and D). Nevertheless, some cells coexpress the two reporter constructs (see yellow cells in Fig. 4D). If better separation of the two distinct reporter populations is required, a slight shift of the DNA injection point

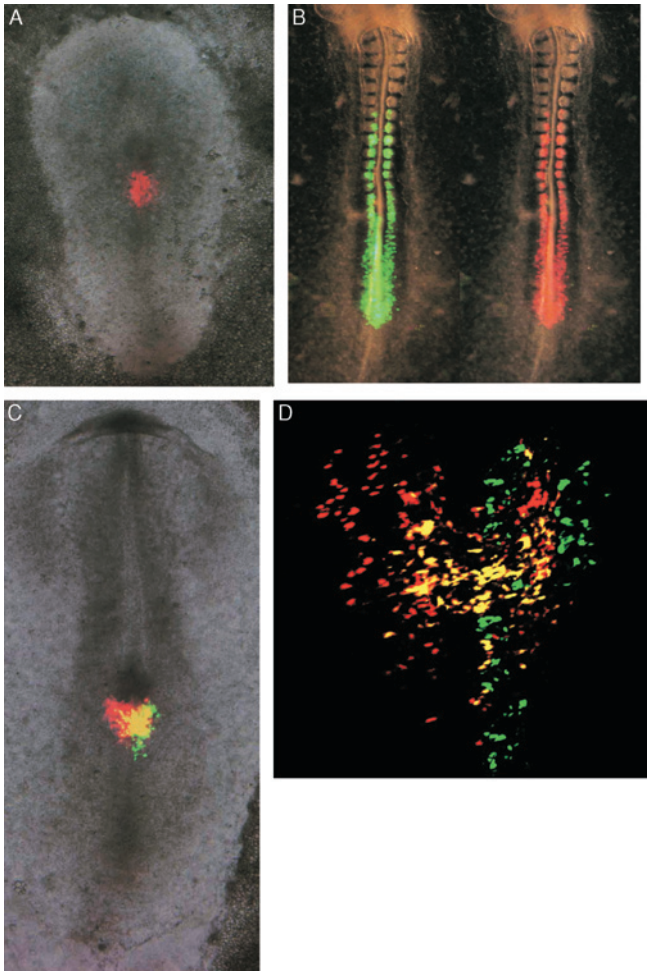

Fig. 4 *In vitro* electroporation using a rod-type anode. (A) Stage 5 HH embryo 4 h after electroporation. A stage 4 HH embryo was electroporated with a pCAGGS-DsRed vector at the anterior primitive streak level using a rod-type anode (CUY195P0.3, NEPA Gene, Japan). Four hours after reincubation at 38 °C, DsRed-positive cells (red) are observed in the anterior primitive streak region. (B) Coelectroporation of two distinct constructs. pCAGGS-EGFP and pCAGGS-DsRed were mixed and electroporated in the anterior primitive streak level of a stage 5 HH embryo using the same method as described in panel A. Twenty-four hours after reincubation, cells marked by each fluorescent reporter exhibit the same tissue distribution, indicating that most cells coexpress both reporter constructs (EGFP-positive cells in green and DsRed-positive cells in red in the left and the right panels, respectively). (C and D) Stage 7 HH embryo successively electroporated by pCAGGS-EGFP and pCAGGS-DsRed at the anterior primitive streak level (EGFP-positive cells in green and DsRed-positive cells in red). (C) pCAGGS-EGFP and pCAGGS-DsRed were successively electroporated at the anterior primitive streak level in a stage 5 HH embryo. Seven hours after reincubation at 38 °C, EGFP and DsRed single-positive cells are observed in the anterior primitive streak region and the newly formed mesoderm. (D) Confocal image of the embryo shown in panel C. EGFP (green) and DsRed (red) label largely distinct populations of cells. A number of cells coexpressing both reporters is, however, observed in these conditions (yellow). Ventral views, anterior to the top. (See Plate no.16 in the Color Plate Section.)

will provide a lower amount of double-positive cells. For example, the first DNA injection is performed on one side of the anterior primitive streak groove, and then the other side, at the same level of the streak, is targeted for the second DNA injection.

IV. Materials

A. Electroporator and Electrodes

Electroporator (Electro-Square Porator CUY21) and petri dish-type electrodes (CUY701P2L) are available from NEPA Gene (Tokyo, Japan).

B. Purified DNA Solutions of Expression Vectors

Vectors in which the expression of constructs is driven by the CAGGS promoter (containing the CMV enhancer and a chicken β-actin promoter; Niwa *et al*., 1991) are frequently used and drive a high level of expression compared with SV40 or TK promoters (Iimura and Pourquie, 2006). Vectors containing a Rous sarcoma virus enhancer and the chicken β-actin promoter are also used routinely (Suemori *et al*., 1990; Wakamatsu *et al*., 1997). Commercially available expression vectors for mammalian cells can be used as well (e.g., ones with CMV or SV40 promoter). Plasmid DNA is purified using an endotoxin-free purification system (e.g., Endo-Free Plasmid Maxi Kit, QIAGEN No. 12362). DNA is dissolved in 1 mM $MgCl_2$ in PBS and adjusted to a final concentration of 1–10 μg/μl, depending on the purpose and the DNA constructs. Fast Green (1%; Sigma F7250) is added to the DNA solution to visualize the DNA injection process (final concentration: 0.1–0.05%). To monitor the tissues that have been electroporated with the DNA construct, a GFP-expressing vector is often coelectroporated with the vector containing the construct to be overexpressed. Alternatively, we have constructed the pCIZ and pCIRX vectors in which a CAGGS promoter drives the gene of interest and a fluorescent reporter protein (ZsGreen and DsRed-Express, respectively) cloned after an internal ribosomal entry sequence (Iimura and Pourquie, 2006). These expression vectors facilitate the monitoring of electroporated cells at a single-cell resolution level. Recent innovations in novel vector technologies such as applications of a tetracycline (Tet)-dependent promoter and transposon-mediated gene integration, and their potential application for *in vitro* electroporation will be discussed later in this chapter (Sato *et al*., 2007; Watanabe *et al*., 2007).

C. Injection Needles

To make a fine open tip, injection needles are pulled from glass capillaries (e.g., outer diameter = 1 mm; inner diameter = 0.58 mm; length = 100 mm; Sutter Instrument, Novato, California).

D. Solutions

PBS
Hank's solution
EC culture
EC cultures are essentially performed as described by Chapman *et al.* (2001)

V. Discussion

A. Staging Embryos and Targeting Different Somitic Levels Along the AP Axis

Because the paraxial mesoderm is formed sequentially by the anterior primitive streak and the neighboring epiblast during body axis elongation, cells produced earlier occupy a more anterior position in the somitic column, while cells produced later are located at a more posterior position. The embryonic stage of an embryo at the time of electroporation thus defines the level of the electroporated somites along the AP body axis. Electroporations of the anterior primitive streak/epiblast at stages 3+/4 HH, 4 HH, 5–7 HH, and 8 HH will drive expression in somitic columns, showing an anterior-most limit of expression at the levels of the head mesoderm, the occipital, the cervical, and the thoracic region, respectively.

B. Controlling Expression of Electroporated Genes

It is possible to vary the expression level of electroporated genes by using different promoters. CAGGS promoter (CMV enhancer + chicken β-actin promoter) shows a five to six times higher expression level than SV40 in the chick electroporation system (Stamataki *et al.*, 2005). In addition to these ubiquitous promoters, tissue-specific promoters can be used to control temporal and spatial control of gene expression by electroporation. Analysis of *cis*-regulatory elements in a promoter sequence can be performed using this method (Inoue and Krumlauf, 2001; Itasaki *et al.*, 1999; Uchikawa *et al.*, 2003).

C. Loss-of-Function Experiments

Gain-of-function experiments have demonstrated that electroporation is a viable method to misexpress exogenous genes. Unless a dominant-negative form of a given molecule is known, loss-of-function experiments are more difficult to achieve. However, *in ovo* electroporation can be used to achieve gene silencing by overexpressing siRNA expression vectors (Das *et al.*, 2006; Katahira and Nakamura, 2003) or morpholinos targeting the gene of interest (Kos *et al.*, 2003). In addition to these methods, dsRNAs of 21 nucleotides have been electroporated to target endogenous mRNAs to downregulate their functions (Dasen *et al.*, 2005).

D. Targeting Other Tissues

Because the epiblast contains precursors for lineages of the three germ layers, simply changing the targeted area of the epiblast and the embryonic stage makes it possible to misexpress DNA in different tissues and location, such as the neural plate, head mesoderm, prechordal mesoderm, lateral plate mesoderm, heart mesoderm, and endoderm.

E. Time-Lapse Imaging

Misexpression of the fluorescent reporter protein(s) by electroporation and *in vitro* culture can also be used to fluorescently label cells for time-lapse live imaging (Iimura *et al*., 2007; Rupp *et al*., 2003; Yang *et al*., 2002). The flat shape of the early chick embryo and the reproducibility of the *in vitro* culture assay make this approach easier to implement and of higher resolution when compared with mouse embryo imaging.

F. Combination of *In Vitro* Electroporation and *In Ovo* Culture

The limited culture period of the *in vitro* culture system can be overcome by a combination of *in vitro* electroporation and *in ovo* culture. Grafting a targeted tissue by electroporation *in vitro* into a host embryo *in ovo* enables the fate and effect on the tissue to be tracked for a much longer time.

G. Tet-on-Inducible System

One of the major concerns with electroporation using a ubiquitous promoter is the lack of temporal control over the overexpressed construct. For example, electroporated precursors of the paraxial mesoderm first have to undergo ingression during gastrulation before the PSM is formed. If an electroporated construct causes a deleterious effect at an earlier developmental step (such as mesoderm ingression), then analysis of its role in later stages will be challenged. To overcome this issue, Tet-controlled gene expression methods can be combined with *in ovo* electroporation (Watanabe *et al*., 2007). With this system, expression of an electroporated gene can be controlled in a timely manner simply by adding the antibiotic doxycycline, so that stage-specific roles of a gene of interest can be more directly investigated. This approach will be described in more detail in the Chapter 14 by Takahashi *et al*., this volume.

H. Transposon-Mediated Gene Transfer

The expression of genes of interest by electroporation is retained only for a couple of days because the electroporated plasmid becomes degraded and diluted by cell division. Application of a transposon Tol 2 system to *in ovo* electroporation

can successfully facilitate integration of a transgene into the genome, resulting in persistent expression of the transgene (Sato *et al.*, 2007). This system can be combined with the Tet-on system to provide various tools to investigate developmental regulations from very early to very late stages.

VI. Summary

In vitro electroporation allows precise tissue targeting in early avian embryos. Combining this method with other manipulations discussed in this chapter provides a variety of modern tools, allowing sophisticated temporal and spatial conditional expression of transgenes for embryonic studies.

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CHAPTER 14

Transposon-Mediated Stable Integration and Tetracycline-Inducible Expression of Electroporated Transgenes in Chicken Embryos

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I. Introduction

Nakamura and colleagues' invention of the *in ovo* electroporation technique (Funahashi *et al.*, 1999) has enabled genetic manipulation of cells in early chicken embryos. This has led to considerable progress in understanding the molecular mechanisms by which morphogenesis and cell differentiation are regulated in developing embryos. Most of these experiments use the ubiquitously active promoter CAGGS that contains both the CMV and the β-actin promoters (Niwa *et al.*, 1991), which efficiently drive gene expression soon after the cells are electroporated (Momose *et al.*, 1999).

Despite these advantages, there are several technical limitations. For example, expression of a transgene is transient, lasting only a few days after electroporation due to the inability to integrate. Thus, the amount of gene product is diluted with cell proliferation. Another limitation is that CAGGS-promoter cannot be conditionally manipulated. This can be critical because if the effect of an electroporated gene is fatal for the cells at the time of electroporation, one is unable to investigate the effects (this could also be "roles") of the gene at later stages of development.This is particularly important because the same gene sets tend to be used reiteratively during development and may be used repeatedly in different developmental contexts. This is exemplified by the role of Notch signals during somitogenesis, in which Notch signals are repeatedly used in multiple steps ranging from the segmentation clock operating in the presomitic mesoderm (PSM) to the morphological segmentation, the event following the clock (Giudicelli and Lewis, 2004; Sato *et al.*, 2002; Takahashi, 2005).

Recently, techniques have been developed to circumvent these shortcomings (Sato *et al.*, 2007; Watanabe *et al.*, 2007). This chapter first describes the tetracycline (Tet)-dependent inducible expression system, optimized for manipulations in chickens. Then, we describe a novel technology that exploits transposon-mediated gene transposition to allow stable integration of transgenes into the genome. The combination of these two methods allows a stably integrated transgene to be conditionally expressed in cells in a temporally controlled manner at relatively late stages in development, including during organogenesis.

II. Tet-Inducible Expression of Electroporated Transgenes

A. How the Tet-Inducible System Works

The method of Tet-dependent inducible expression has been used for diverse studies, ranging from in vitro cell cultures to mouse embryos (Furth *et al.*, 1994; Gossen and Bujard, 1992). Both transcriptional activators [the reverse

Tet-controlled transcriptional activator (rtTA) and the Tet-controlled transcriptional activator (tTA)] act on the *cis*-element promoter, tetracycline-responsive element (TRE) (Figs. 1A, and 2A). rtTA binds TRE only in the presence of doxycycline (an analogue of Tet; Dox), and activates transcription of the TRE-driven gene (''Tet-on'') (Fig. 1A). In contrast, tTA binds to the TRE constitutively and activates the TRE-driven gene in the absence of Dox. When added to a cell with Dox, tTA is released from TRE, leading to the inactivation of the TRE-driven gene ('Tet-off'') (Fig. 2A). Thus, it is possible to control the onset or termination of the expression of a transgene by altering the time of Dox administration.

B. Plasmids

rtTA2^{s}-M2 (commercially available at Clontech, Japan (Tet-On Advanced (#631069), created by the Hillen's group, is a modified and more efficient version of the conventional rtTA (Urlinger *et al*., 2000). Tet-Off Advanced (#631070) and pTRE-Tight-BI-AcGFP1 (#631066) have replaced previous versions tTA and pBI-EGFP, respectively, which were used in Watanabe *et al*. (2007). DsRed2 and short half-life EGFP (d2EGFP) are also purchased from Clontech.

A fragment of rtTA2^{s}-M2, tTA, or DsRed2 was subcloned into a pCAGGS expression vector (Momose *et al*., 1999; Niwa *et al*., 1991). The pBI plasmid contains a cassette in which two different genes can be bidirectionally transcribed under the control of a single TRE. This plasmid was designated as pTRE− in Watanabe *et al*. (2007), but here it is identified as pBI−.

C. Dox-Dependent Induction of Electroporated EGFP by Tet-on System

A particularly useful applicaiton of the Tet-on method has been to early somitogenesis. For delivering a transgene into the PSM, one must electroporate the ingressing cells at the anterior end of primitive streak of stage 8 chicken embryos (3–4 somites) (Sato *et al*., 2002). If constitutive overexpression of the electroporated gene is lethal to these ingressing cells, this precludes studies of the role of this gene at later stages of somitogenesis, as is true for *cMeso1*, *Pax 2*, and *RhoA* (Watanabe *et al*., 2007). The Tet-on method makes it possible to circumvent this early lethality.

For this purpose, three different plasmids, pBI-EGFP, pCAGGS-rtTA2^{s}-M2, and pCAGGS-DsRed2, are coelectroporated into the embryo. For electroporation, the ratio of the amount of the plasmids pCAGGS-rtTA2^{s}-M2 and pBI-EGFP is 1:2 in a DNA solution. While pCAGGS-DsRed2 is not necessary for the Tet-induction, it is useful to select embryos with successful transgenesis because TRE-regulated EGFP is not visible before injection with a Dox solution. To observe expression of the transgene, a Dox solution of 250 ng (0.5 ml of 0.5 ng/μl in PBS) is injected into the egg between the embryo and the yolk. As seen in Fig. 1, which shows an example of a Dox-injected embryos that has developed to stage 12 (∼15 somites), the induced EGFP signal can be seen as early as 3 h after the Dox

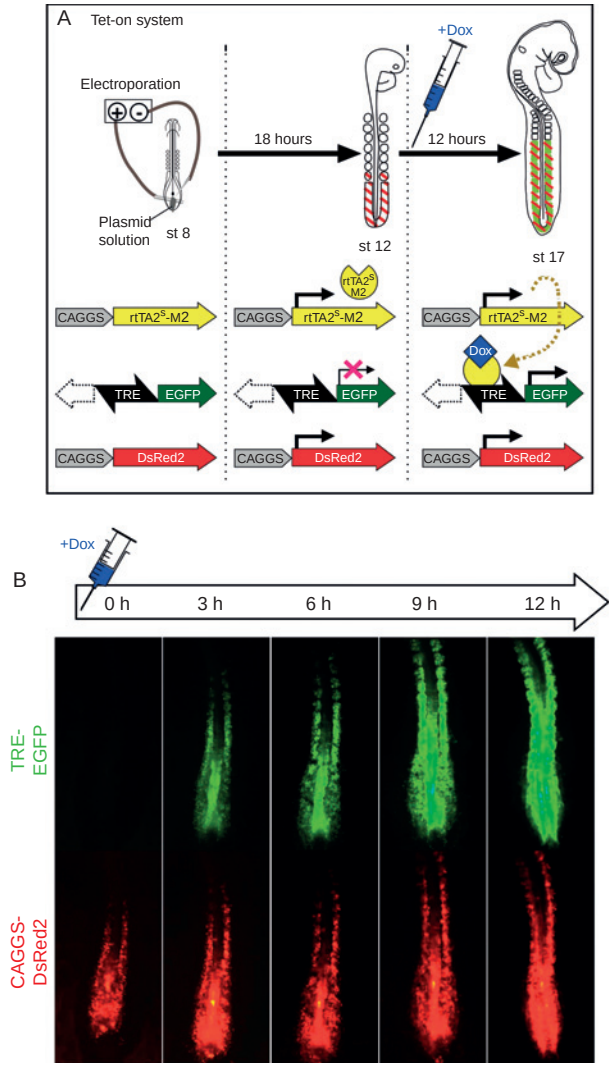

Fig. 1 Inducible expression of electroporated DNAs in developing somites by the Tet-on method. (A) A diagram showing the principle of the Tet-on system. *In ovo* electroporation of the PSM is carried out at stage 8 of development as previously described (Sato *et al.*, 2002). Soon after the electroporation, rtTA2^{s}-M2 protein (yellow) is produced by the CAGGS-driven cDNA. The rtTA2^{s}-M2 protein can bind to TRE (*cis*-element shown in black) when Dox (blue) is provided into an egg. CAGGS-driven DsRed2 (red) is constitutively produced. A DNA cassette containing TRE is designed to transcribe two genes bidirectionally (Clontech). (B) The Dox-dependent induction of electroporated EGFP in early chicken embryos. The three plasmids shown in A were coelectroporated. Three hours after the Dox administration, EGFP signal started to be observed. Modified from Watanabe *et al.* (2007). (See Plate no. 17 in the Color Plate Section.)

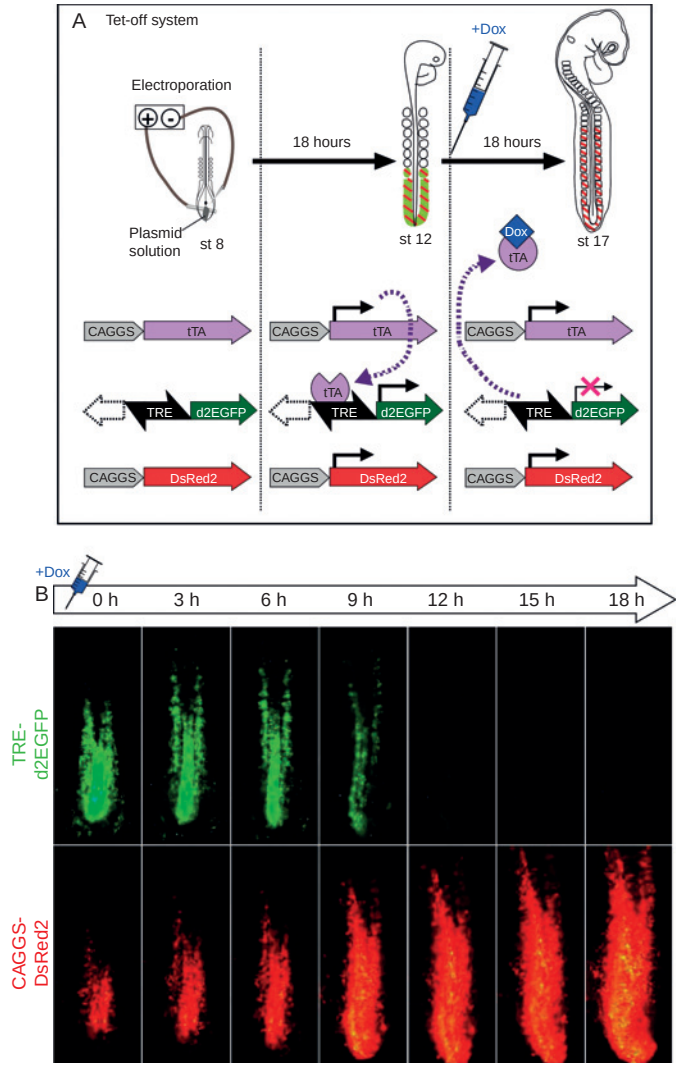

Fig. 2 Controlled termination of the expression of electroporated DNA by the Tet-off system. (A) The experimental design for this method is similar to that shown in Fig. 1. Instead of the rtTA2^{S}-M2 and EGFP used in Fig. 1, tTA and d2EGFP are used. tTA, a constitutive transcription activator bound to TRE, is released from TRE in the presence of Dox. (B) Dox-dependent termination of the expression of electroporated DNA in developing chicken embryos. The three plasmids shown in panel A were coelectroporated into the embryo. By 9 h after the administration of Dox, d2EGFP signals had started to diminish, and by 15 h, the signal could no longer be detected. Modified from Watanabe *et al.* (2007). (See Plate no. 18 in the Color Plate Section.)

injection, and signal intensity increases with time. No toxicity was found for doses of Dox up to 50 μg. In somites, a single administration of Dox solution is sufficient for maintenance of induced expression for 48 h. One can alter the timing of the Dox injection depending upon specific experimental context.

D. Dox-Dependent Termination of Electroporated EGFP by Tet-off System

The conditional shut-off of TRE-EGFP expression using tTA can be seen in Fig. 2. This is achieved by electroporating embryos with three plasmids: pBI-d2EGFP, pCAGGS-tTA, and pCAGGS-DsRed2. Thus, it differs from the Tet-on system in its use of tTA instead of rtTA2^{s}-M2, and d2EGFP instead of EGFP. Soon after the electroporation, which is done in the absence of Dox, TRE-driven d2EGFP is turned on by the transcriptional activity of tTA, resulting in a pattern similar to that of CAGGS-DsRed2. After Dox administration, however, the intensity of the d2EGFP signals diminishes from 9 h onward, and the signals disappear completely by 15 h (Fig. 2).

III. Stable Integration of Electroporated Transgenes

A. Tol2-Mediated Transposition

Transposons are genetic elements that move from one locus in the genome to another. As a result, they represent powerful tools for experiments in both animals and plants. Recently, the *Tol2* transposable element, which was originally found in medaka (Koga *et al.*, 1996), has been reported to undergo efficient transposition in a wide variety of vertebrate species including zebrafish, frogs, and mice (Kawakami, 2005; Kawakami *et al.*, 2004). When a DNA plasmid, containing the transposon construct that carries a gene expression cassette, is introduced into vertebrate cells with a transposase activity, the transposon construct is excised from the plasmid. Subsequently, the cassette is integrated into the host genome (Fig. 3A) (Kawakami, 2005; Sato *et al.*, 2007)

B. Plasmids

A DNA cassette that is to be integrated into the chromosome is placed into the pT2K vector, which includes a pair of Tol2-sequences required for the transposition. Transient expression by pCAGGS-T2TP appears to be sufficient for supplying transposase activity for the transposition of an electroporated gene. Therefore, pT2K-CAGGS-EGFP and pCAGGS-T2TP must be coelectroporated into the embryo (Fig. 3B).

C. Persistent Expression of Tol2-Mediated Transgene

As shown in Fig. 3B, embryos coelectroporated with pT2K-CAGGS-EGFP and pCAGGS-T2TP along with pCAGGS-DsRed2 into PSM show almost identical patterns of expression of EGFP and DsRed2 for the first couple of

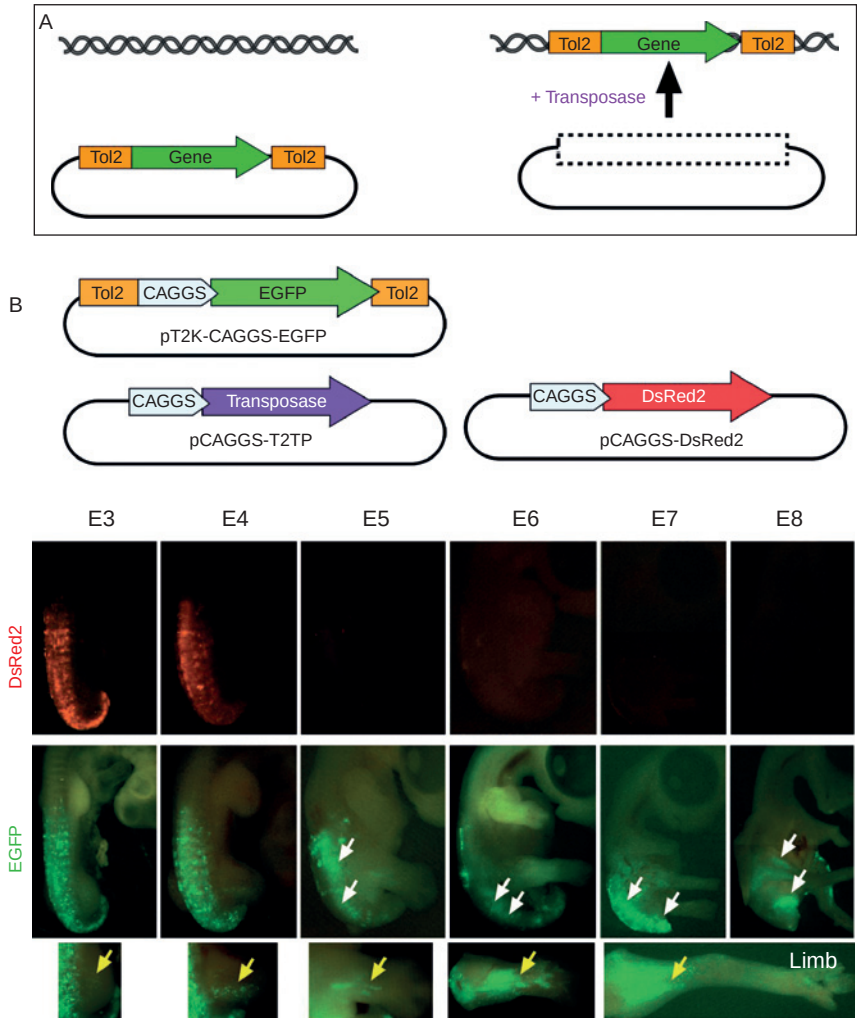

Fig. 3 The method of Tol2-mediated DNA transposition that allows a transgene to be stably expressed in the chicken embryo. (A) A DNA cassette cloned in the *Tol2* plasmid construct is excised from the plasmid, transposed, and integrated into a host genome in the presence of transposase activity. (B) Persistent expression of EGFP in developing chicken embryos electroporated with Tol2 constructs. The three kinds of plasmids shown at the top of the figure were coelectroporated into the PSM. The upper and lower pictures are of the same specimen at each embryonic stage analyzed. Until E4, the signals of both DsRed2 (not carried by the *Tol2* construct) and EGFP (carried by the *Tol2* construct) were detected in a similar pattern. However, the EGFP signal persisted until E8 (white arrows), the latest stage analyzed, whereas the DsRed2 signal disappeared by E5. EGFP signals were also observed in muscle precursor cells in developing limb buds (yellow arrows), which are somitic derivatives. Modified from Sato *et al*. (2007). (See Plate no. 19 in the Color Plate Section.)

days. In contrast, from E5 (3 days postelectroporation) onward, DsRed2 signals provided by nonintegrated plasmid diminish, and EGFP signals are retained in a substantial number of cells and tissues. Persistent EGFP expression can be attributed to a stable integration of the transgene into the chromosomes, as revealed by genomic Southern blot analyses (Sato *et al.*, 2007). The Tol2-mediated transgene is retained in a variety of somite-derived tissues including axial bones, dorsal muscles, and muscles in limb buds (Fig. 3B). This method can also be applied to the developing retina, where persistent expression lasts until at least E12 (Sato *et al.*, 2007).

IV. Stage-Specific Manipulation of Stably Integrated Transgenes

A stably integrated transgene by the Tol2-mediated transposition can also be experimentally controlled for onset of its expression in combination with the Tet-on method. To accomplish this, the three kinds of plasmids, pT2K-CAGGS-rtTA2^{s}-M2,[1] pT2K-BI-TRE-EGFP, and pCAGGS-T2TP, are required for coelectroporation (Fig. 4A).Because the TRE element of pT2K-BI-TRE-EGFP acts bidirectionally, one additional gene can be placed under the control of TRE in the opposite direction to EGFP. For *in ovo* electroporation, coelectroporate with a forth plasmid, pCAGGS-DsRed2, is recommended because no cells are EGFP positive prior to injection of Dox (see below). Without the signal driven by pCAGGS-DsRed2, it would be difficult to select embryos with successful transgenesis.

Figure 4B shows an example of embryos electroporated with the four above-mentioned plasmids. In this series of experiments, Dox was administered at the end of E5. DsRed2 signals derived from nonintegrated transgene vanished by E5, after which the expression of chromosomally integrated cDNA of EGFP could be induced by the administration of Dox. For somite-derived tissues, a single injection of Dox is sufficient to retain the induced expression for at least 3 days (from E5 to E8). However, the perdurance of expression varies from tissue to tissue: in retinas, for example, it is necessary to reintroduce Dox at least once a day (Tanabe *et al.*, 2006).

V. Summary

The Tol2-mediated transposition approaches are novel techniques for the molecular manipulation of chicken embryos by which an exogenous gene can be integrated into the host genome. The integrated gene(s) can be stably expressed when driven by a ubiquitous promoter. This method can also be used for conditional expression when combined with the Tet-on system to achieve temporal control. The Tol2-transposition method allows for expression at stages even later than E5, which otherwise could not be analyzed with the conventional

[1] This plasmid was originally designated as pT2K-CAGGS-rtTA-M2 in Sato *et al.* (2007).

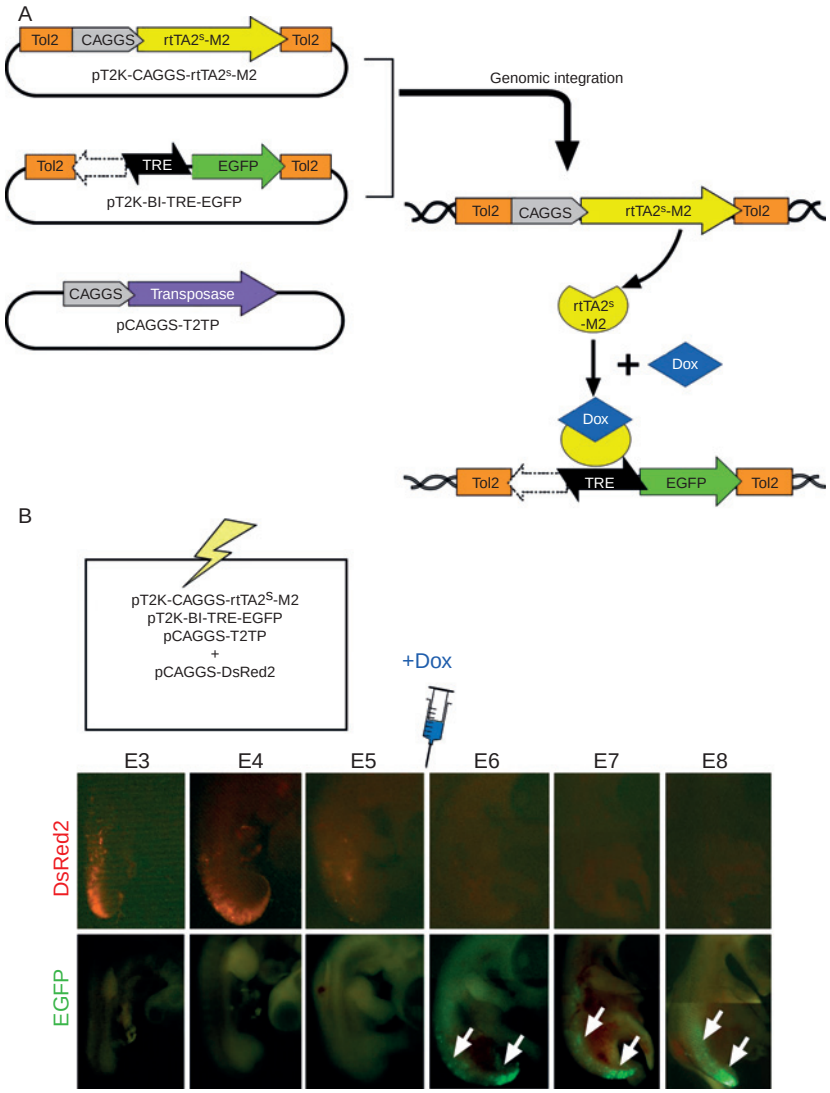

Fig. 4 The combination of the method for Tol2-mediated stable integration and the Tet-on method of inducing the expression of electroporated genes. (A) The three plasmids: pT2K-CAGGS-rtTA2^{S}-M2, pT2K-BI-TRE-EGFP, and pCAGGS-T2TP are used. (B) The embryos were coelectroporated with the three plasmids shown in panel A along with pCAGGS-DsRed2. The upper and lower photos at each time point of analysis show the same specimen for DeRed2 and EGFP, respectively. Until E5 when Dox solution was injected, TRE-driven EGFP signals were not detected whereas the signals of CAGGS-driven DsRed2 (not carried by the *Tol2* construct) were seen, revealing that both electroporation and transgenesis were successful. Following the administration of Dox, the TRE-regulated expression of EGFP (carried by the *Tol2* construct) started to be observed, and these signals were persistently found until at least E8, the latest stage analyzed. Modified from Sato *et al*. (2007). (See Plate no. 20 in the Color Plate Section.)

electroporation technique. These developmental stages are critical for organogenesis, where numerous tissues interact to generate the complex structures of functional organs. Thus, the techniques presented here are extremely useful for understanding how cells contribute to organogenesis at the molecular level.

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CHAPTER 15

Generating Transgenic Quail using Lentiviruses

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I. Introduction

The present mismatch between the best species for molecular and imaging experiments motivates us to develop a tool for transgenesis in the avian embryo. The avian embryo permits unsurpassed accessibility to detailed study, and has long been a favorite model system of classical embryologists. Our understanding of myogenesis, vasculogenesis, angiogenesis, skeletogenesis, wound healing, immunology, developmental biology, neurobiology, virology, and teratology have progressed significantly as a result of studies on avian embryos (Mizutani, 2002). The methodology needed to manipulate avian embryo is now well known.

Access to the avian embryo simply requires the researcher to cut a small opening in the eggshell. After experimental manipulation, the opening is sealed with transparent adhesive tape and the egg is incubated to the desired development stage. Dynamic imaging of the developing embryo can be accomplished through a similar aperture in the shell and sealed with O_2 permeable Teflon to prevent evaporation. The windowed embryos can be videorecorded continuously for up to 5 days without deleteriously affecting normal embryogenesis. The creation of GFP-expressing transgenic avians now permits us to molecularly manipulate the developing embryo and to dynamically image cellular events from the blastoderm stage throughout development in normal and mutated embryos.

It has been possible to make transgenic chickens through the use of viral vectors (Harvey *et al*., 2002; McGrew *et al*., 2004; Mozdziak *et al*., 2003; Salter *et al*., 1987; Scott and Lois, 2005) and transplantion of embryonic blastodermal cells (Pain *et al*., 1996; Petitte *et al*., 1990; van de Lavoir *et al*., 2006; Zhu *et al*., 2005) for some time. However, transgenic avians are not readily available primarily because of the low efficiency of germline integration of the transgene and the subsequent poor expression of integrated transgenes.

A. Avian Transgenesis

Numerous techniques have been developed for isolation, culture, and reintroduction of blastoderm cells to generate chimeric animals (Pain *et al*., 1996). ES cells can be collected from the area pellucidae of stage X embryos (Petitte *et al*., 1990) or PGCs from the gonadal anlage of E3 embryos (Kuwana, 1993). These cells can be either infected with viruses or transfected with desired expression vectors prior to their return into the subgerminal cavity of the host embryo (Brazolot *et al*., 1991; Etches *et al*., 1996; Pain *et al*., 1996). To increase the efficacy of obtaining transgenic avian animals, other researchers have used busulphan or irradiation to compromise host cells (Aigegil and Simkiss, 1991; Bresler *et al*., 1994; Petitte *et al*., 1990; Reynaud, 1977; Vick *et al*., 1993). Approximately 200 manipulated cells introduced into compromised stage X (Eyal-Giladi and Kochav, 1976) chick embryos appear sufficient to generate chimeric founder birds that can produce germline progeny (Petitte *et al*., 1990).

Lentiviruses have been used to successfully generate transgenic chickens (Chapman *et al*., 2005; McGrew *et al*., 2004) and quail (Scott and Lois, 2005). Sang and colleagues successfully maintained germline transmission and expression of a GFP transgene introduced using the equine infectious anemia lentivirus vectors (McGrew *et al*., 2004). The injected chicken embryos were cultured *ex ovo* to hatch (Perry and Sang, 1993). HIV-based viral vectors injected into the area pellucidae of stage X embryos *in ovo* and then incubated to hatch, also maintained germline transmission and expression of their GFP transgene (Chapman *et al*., 2005; Scott and Lois, 2005).

B. Quail as a Model System

The following list highlights several reasons why we think quail are the best choice for studying embryogenesis:

- Quail are small, permitting a colony of 60 breeding pairs in less space than needed to house one rack of mouse cages.
- The quail life cycle is surprisingly fast, becoming sexually mature 5–6 weeks after hatching. Thus, their life cycle is significantly shorter than that of mice, chickens, or zebrafish.
- Quail embryos are very hardy, surviving manipulation and culture better than do chicken embryos
- Their small size permits them to be imaged more conveniently than chicken embryos by both light and MRI microscopes.
- Quail embryo development is virtually identical to chicken embryo development, allowing the large database of chicken data to be applied to the quail.
- The genome of the quail and chickens are 99% (Hillier *et al*., 2004; Wallis *et al*., 2004) homologous, permitting information from sequencing the chick genome to be directly applied to quail experiments
- Avid hobbyists, commercial growers, and scientists have defined several strains of quail that have been identified for feather coloration, increased growth rate and size, and some serve as models for human disease.

Thus, the quail combines the needed background data, developmental rate, and accessibility to make it ideal for both molecular and imaging studies. The approach for generating transgenic quail that we describe can also be directly applied for the generation of transgenic chickens.

C. Using Viruses to Fluorescently Label Cells

Imaging cell and tissue movements throughout embryogenesis is crucial if we are to better understand body patterning in developing organisms. Cell tracking studies that rely upon microinjection or microapplication of vital dyes find that

their dyes can bleach from prolonged imaging or be diluted out as the labeled cells divide. Using vectors that express fluorescent proteins provide both a renewable source of the fluorescent label via continued gene expression, as well as tissue-specific expression to study subsets of cells. Introducing such vectors into avian embryos is typically done by either electroporation or viral vectors. The problem with current electroporation technologies is that the DNA vector does not integrate within the cell genome, and therefore is likely to be lost during subsequent cell divisions. Viruses that are able to stably integrate within the host genome are reliably passed on to their daughter cells, providing a useful tool for cell tracing and cell fate studies.

The infected cells appear to follow their standard migratory routes and undergo normal differentiation (Carleton *et al.*, 2003; Okada *et al.*, 1999) and express sufficient levels of GFP to allow *in vivo* time-lapse video-microscopy, laser scanning confocal microscopy, and two-photon microscopy. A researcher can make viruses that are targeted to various subcellular locations (e.g., H2B-GFP targets to the cell nucleus) and that express various GFP color variants (e.g., CyanFP, GreenFP, YellowFP, mRedFP, CherryFP, to name a few of the possible color options) (Shaner *et al.*, 2005). The ability to localize different GFP variants to specific cellular organelles allows these structures to be specifically imaged, cell divisions to be followed, and morphological changes to be dynamically observed.

II. Experimental Procedures

A. Lentiviruses

We prefer using lentiviral vectors to introduce foreign genes into both quail and chicken because we can recapitulate and maintain endogenous gene expression in differentiating somatic cells and in germ cells (Chapman *et al.*, 2005; McGrew *et al.*, 2004; Scott and Lois, 2005). We and others have found that MoLV-based vectors are able to adequately infect avian blastoderm and stem cells and make transgenics, but they do not consistently maintain their expression profiles through germline transmission.

We have generated a large family of replication-defective, GFP-expressing retroviruses and lentiviruses that infect avian embryonic cells. The HIV- and Moloney-based retroviruses have been pseudotyped with VSV-G (Zavada, 1982) to both increase their host range and permit their concentration by ultracentrifugation, with minimal loss of biological activity (Burns *et al.*, 1993). By uniting and modifying techniques and reagents previously reported (Dull *et al.*, 1998; Kinsella and Nolan, 1996; Ory *et al.*, 1996), we typically obtain GFP-expressing viruses with titers greater than 1×10^9 infectious virions/ml. We typically do not attempt to make transgenics using viral titers below 1×10^8 virions/ml, much preferring to use viral titers above 5×10^8 virions/ml.

B. Transfection into 293 FT Packaging Cells

We recommend including a negative control (no DNA, no Lipofectamine™ 2000) in your experiment to help you evaluate your results. You could also include a positive control—a GFP-expressing retroviral construct that has already been prepared, such as pRRLsin.PPT.hPGK:GFP. The plate should be ~50% confluent to maximize the amount of lentivirus generated.

Harvest cells with trypsin–EDTA and count using a hemacytometer. Plate 5×10^6 cells per 10 cm Falcon TC plate. You can also coat the surface of the dish with 0.1% gelatin prior to plating, which helps the 293FT cells stay attached to the plate during the transfection. Incubate 293FT cells at 37 °C/5% CO_2 overnight.

- For each transfection sample, prepare DNA–Lipofectamine™ 2000 (Invitrogen) complexes as follows, which closely accords the manufacturers recommendations:
  - In a sterile 5-ml tube, dilute 9 μg (μl) of the Invitrogen ViraPower™ Packaging Mix and 3 μg of your pLenti expression construct DNA in 1.5 ml of Opti-MEM® I Medium without serum. Mix gently.
  - In a separate sterile 5-ml tube, add Lipofectamine™ 2000 gently before use, then dilute 36 μl in 1.5 ml of Opti-MEM® I Medium without serum. Mix gently and incubate for 5 min at room temperature.
  - After the 5-min incubation, combine the diluted DNA with the diluted Lipofectamine™ 2000. Mix gently.

- Incubate for 20 min at room temperature to allow the DNA:Lipofectamine 2000 complexes to form. The solution may appear cloudy, but this will not impede the transfection.
- While DNA–lipid complexes are forming, prepare 5 ml of Opti-MEM® I Medium with 10% FBS. At the end of the 20-min incubation, aspirate the growth medium off the plate containing 293FT cells and replace with the 5 ml of DMEM + FBS that was just prepared.
- Add the DNA–Lipofectamine™ 2000 complexes to the 10-cm tissue culture plate containing 5 ml of DMEM, 10% FCS, and adherent 293FT cells. *Note:* Do not include antibiotics in the medium.
- Mix gently and then incubate cells at 37 °C in a CO_2 incubator for 4–6 h.
- Aspirate the medium containing the DNA–Lipofectamine™ 2000 complexes and replace with DMEM + 10% FBS or complete media.
- By using a fluorescent microscope, you should be able to see GFP expression within 12–24 h post-transfection.
- Collect virus every 12 h, beginning 24 h post-transfection. Centrifuge supernatant at 400 $\times g$ for 10 min at +4 °C to pellet cell debris and filter through a 0.8-μm filter to remove debris. Supernatants can be stored at −80 °C for several months to a year. (*Caution*: Remember that you are working with infectious virus at this stage. Follow the recommended guidelines for working with BL-2 organisms).

At this point, your virus titer should be at $\sim 1 \times 10^5$ to 1×10^7 infectious units/ml (IU/ml). If a higher titer is desired (i.e., *in vivo* work), the following protocol can be followed to increase the titers to $\sim 1 \times 10^9$ IU/ml. Set aside 0.5 ml in order to check starting virus titer.

- Add your filtered supernatants to a Centricon Plus-20 or 70 (20 or 70 ml capacity), and spin at the manufactures recommended conditions in a swinging bucket rotor at +4 °C (typically 10–30 min). Invert the filter cartridge and collect retentate with a quick spin. The supernatant should be at a volume of <700 µl at this point.
- Transfer the viral supernatant to a presterilized ultracentrifuge tube (100% EtOH). Spin at 50,000*g* for 2 h at +4 °C.
- Remove the supernatant, avoiding the pellet at the bottom of the tube. Remove excess supernatant by inverting the tube onto a clean paper towel. Add 50–100 µl of HBSS or DMEM to the pellet and allow the pellet to dissolve several hours to overnight at +4 °C. To resuspend, hold tube at an angle and pipet fluid over the pellet 20 times.
- Use the virus immediately or dispense virus in 5–10 µl aliquots, flash-freeze in liquid nitrogen, and store at −80 °C. Freezing should not deleteriously affect virus titer, but avoid multiple freeze-thaws, which lower virus titer.

C. Titering Your Lentiviral Stock

Plate cells to be infected by the virus to 2×10^4 cells/cm^2 in 12-well dishes the day prior to infection. Your test cells should be well spaced and healthy. We use 293FT or NIH 3T3 cells for vectors with ubiquitous promoters, YSE murine endothelial cells for vascular promoters such as TIE-1 and TIE-2 (De Palma *et al*., 2003), and C6 rat glioma or C1300 CLONE NA neuroblatoma for neural-specific promoters such as synapsin I and α-CaMKII (Dittgen *et al*., 2004). QEF and CEF cells are good for titering your virus in avian cells if this is desired.

Determining titer with fluorescent microscope:

- Prepare sequential 5× dilutions, starting with a 1000-fold dilution of your stock virus. For example, dilute 1 µl of virus stock into 1 ml of culture media as your starter. Next, make 5× dilutions after that into culture tubes so you will have 5000 dilution, then 25,000 dilution, then 125,000 dilution, and so on. Be sure to mix the virus containing media by pipetting up and down before transferring to the next dilution set.
- Replace the culture medium in your desired cells in each well with 1 ml of the diluted virus-containing medium. Make sure that you leave two wells blank so as to include negative controls; you mock infect these wells with DMEM + media, lacking virus. Replace media with fresh DMEM+ media after 12 h.
- After 48 h post-infection, trypsinize cells, inactivate trypsin with DMEM+, pellet the cells at 1000 rpm for 5 min, and resuspend in cold PBS. Count the percentage of GFP+ cells using a Neubauer cell counting chamber and a

fluorescent microscope or with a FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, CA, USA). Use a well that has between 0.1% and 10% of cells expressing GFP to determine the viral titer.

- GFP-positive cells can be identified 2 days after infection. To calculate the viral titer use the following formula: viral titer $= (F \times Co/V) \times D$. F is the frequency of GFP-positive cells determined by Neubauer cell counting chamber or FACScan; Co is the total number of target cells infected; V is the volume of the inoculum per millileter; D is the virus dilution factor. GFP titers should be calculated at several lentivector dilutions (0, 10^{-1}, 10^{-2}, 10^{-3}).

You should strive for $>5 \times 10^8$ GFP+ virions/ml (transducing units/ml; TU/ml) for successful embryo injections. Virion concentration of 10^9 TU/ml is readily obtained with this protocol. Thus, to titer your viral stock, assume you will need to dilute your virion sample 10^3–10^5.

D. Virus Injection Station

Our injection station (Fig. 1) consists of the following: a secluded table and chair, an ergonomically unfriendly Zeiss dissecting scope, a steel platform, a magnetic stand, a micromanipulator, Harvard Apparatus PLI-90 microinjector, an egg holder, a heating plate set to maintain melted paraffin wax, a good music source to maintain a good state of mind, and a nearby, baggy lined trash can. A small Humidare incubator, located on the floor beneath the injection station, is set to 38 °C to hold eggs prior to and after injection.

Consideration should be given to the design and location of your injection station. Bear in mind that you are using an injection apparatus ended with a sharp needle that contains highly concentrated virus. Position your viral injection station away from high traffic areas to lessen the chance that one of your lab mates will inadvertently bump the back of your chair.

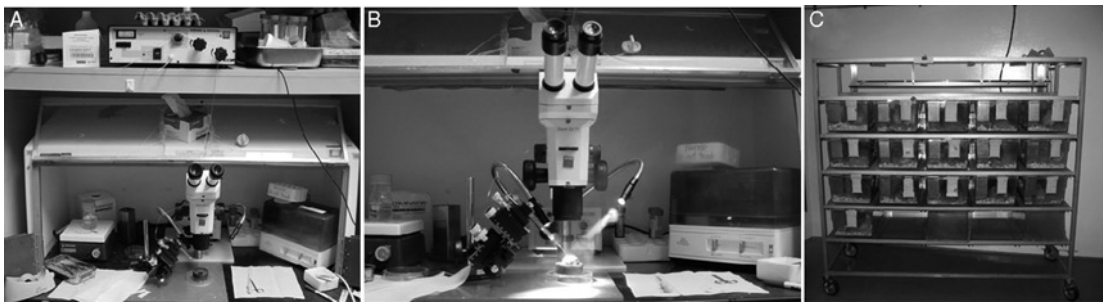

Fig. 1 Egg injection and breeding quail facility. (A) Egg injection area. (B) Close up of injection area showing dissecting microscope, microinjector, ultrasonic humidifier, and quail egg on mount. (C) Quail breeding rack.

We typically attempt to inject 30 eggs per day. An injection session typically lasts 2–5 h. Consider adjusting your seat and dissecting scope to be as comfortable as possible. A stereomicroscope equipped with an adjustable eye-piece is ideal. Your back will appreciate such forethought; stretching and pull-ups seem to help too.

E. Injection Procedure

- We obtain our eggs from either the quail facility at Caltech or from commercial vendors such as AA LabEggs (Westminster, CA). Care should be taken to use eggs soon after laying, if possible. Eggs can be stored 3–7 days at 16–17 °C with minimal consequences; longer storage tends to decrease hatchability.
- Spray eggs with 70% ethanol in water (v/v) to disinfect and then place eggs on their side at RT for 1 h (this hopefully permits the embryo to rotate to the top side of the horizontal egg).
- Directionally maintain the egg when placing it onto an egg holder (cut and carved plumber insulation works well). Turn on the ultrasonic humidifier; be sure the humid effusion is oriented toward your dissection area under the dissecting scope. Wipe the egg with Kimwipe wetted with 75% ethanol in water (v/v) to sterilize the egg surface. Use dissecting scissors to create a 3–5-mm hole in the top of the egg (hopefully right above the blastoderm now at the summit). *Immediately apply a drop of HBSS gently above the blastoderm*. Remove air bubbles from the egg using a short glass Pasteur pipette. We also add back a few drops of the extracted thin albumen to float the embryo higher in the egg to facilitate air bubble removal and the virus injections into the embryo.
- Angle the injection needle into place against the vitelline membrane, just above the blastoderm (a well-trained eye will note a change in the light diffraction pattern above the vitelline membrane once it has been displaced by the injection needle). Gently puncture the vitelline membrane so that the virus-filled needle is inserted into the area pellucida of the blastoderm. The area pellucida is about 3 mm long by wide and is encircled by the area opaca. (We do not use dye to visualize the blastoderm. If you do choose to use a dye, we suggest you avoid the overused India ink due to undefined contaminants that may deleteriously affect hatching. We use food coloring dyes instead; a mixture of yellow and blue seems to work well). Gently inject the virus.
- Virus and DNA injections are made using heat pulled quartz needles, and a microinjector at 0.1–0.5 psi. Injecting at higher flow rates often causes the loosely associated embryonic cells to be disbursed in a deleterious manner. Very gently inject the viral payload into the area pellucida. We do not use the 'inject' knob, rather we turn the positive pressure knob from 0.1 to 0.5 psi for several seconds (actual injection time varies somewhat due to variability in the size of the quartz needle opening). Withdraw the needle from the embryo.
- Wipe the perimeter of the egg window with a Kimwipe wetted with 95% ethanol in water (v/v) to remove any residual water, HBSS, or albumen from the egg surface to permit a better seal with the SteriStrip. It is best to seal within 10 min of opening.

Use blunt end forceps to place a precut SteriStrip over the aperture. Use your finger to be sure the egg is well sealed. Place the egg on its side in the small nonrotating Humidare incubator. Using the small Humidare conveniently permits eggs to recover from the injection at their preferred incubation temperature of 38 °C and lessens the number of times the large Petersime incubator needs to be opened. Occasionally, our sealed eggs become dehydrated, which prevents hatching. We have had good success at preventing dehydration by additionally placing a thin layer of molten paraffin wax over the SteriStrip seal of the egg opening.

- At the end of the injection session, place the egg holder containing the virus-injected eggs onto the egg tray of the large rotating Petersime incubator. Be sure to include some unmanipulated eggs to control for proper incubation conditions. Place the eggs or 'blunt end up' to ensure proper orientation of the embryo within the egg for hatching. Incubate the eggs at 38 °C (~60% RH) with gentle rocking for the next 14–15 days. Eggs should be rocked at least three times a day to avoid detrimental effects on the hatchability, growth of embryos, development of chorioallantois, and subembryonic fluid formation to name a few (Tazawa and Whittow, 2000).

F. Hatching Quail Embryos

Hatching manipulated quail is not trivial. There are numerous obstacles that must be appreciated and then overcome to successfully hatch manipulated avian embryos at high frequencies. Great care must be given to maintain stable temperature (38 °C) and relative humidity (60%) conditions throughout the incubation period. In addition, particular attention must be given to the manner the eggs are opened, to the conditions maintained during manipulation of the embryo, and to securing the embryo back within the shell. We are able to hatch 10–25% of embryos manipulated at E0 and 75% of embryos manipulated at E3. The quail eggs are incubated in a Petersime Model 4 rotary incubator for 15 days; the egg racks are gently rotated a total of 15 ° from the horizontal axis to prevent spillage from the bandaged opening.

On day 14–15, the quail eggs are moved to the hatching incubator. The hatcher is kept at 38 °C and 70% RH; addition of several water trays helps to stabilize the relative humidity inside the hatcher. The eggs are placed on their sides in an enclosed rack lined with deodorizing drop pan paper (the paper is rough enough to prevent splay leg, Hayes, 1995, p. 105) in the hatchlings and also absorbs excrement fairly well). The incubator should be thoroughly cleaned after each hatching.

The newly hatch Coturnix likely needs water within 24 h of hatching, but not necessarily food. The Coturnix embryo draws the yolk sac into their body cavity by day 15. At hatching, ~30% of the original lipid rich yolk is still available as an nutrient reserve for the newly hatched chicks (Etches, 1996).

G. Rearing Manipulated Quail Embryos

A day after hatching, the quail chicks are moved to the quail room in the Caltech animal facility and placed in a brooder for 4 weeks. The room temperature is typically 20 °C, so we heat the cages using a 9 × 9-heating lamp. The hatchlings

should be kept at 35 °C for the first week, 32 °C for the second week, and 29 °C for the third week (Hayes, 1995). A blender is used to break down Matzuri Exotic Gamebird Starter feed for our hatchlings. Water is carefully provided in a Plastic Water Fount for quail. Jewelry beads are placed in the water trough to prevent the hatchlings from drowning.

After 4 weeks in the brooder, two females and one male are placed together within a cage. The bird's diet is switched to Matzuri Exotic Gamebird Breeder, which provides more calcium for our egg layers. Japanese quail begin to lay fertile eggs around weeks 6–7, typically providing 10–12 eggs/hen/2 weeks. They typically provide ~90% fertile eggs until they are about 1 year old; at this point the egg fertility begins to decline. Quail are rather aggressive toward one another during mating (Hayes, 1995), so pay close attention for open wounds or overly aggressive birds. Immediately separate injured birds from one another to permit healing and recovery.

H. Verifying Transgenic Integration

Stable integration of the retrovirus constructs is typically verified using Southern blot analysis of genomic DNA according to standard protocols (Sambrook and Russell, 2001).

I. Tools and Reagents

- Clean lab bench
- Petersime Model 4 rotary incubator (Gettysburg, OH)
- Humidare incubator (Model 20, New Madison, OH)
- SteriStrip (#R1547; 3M Healthcare, St. Paul, MN)
- Deodorizing drop pan paper (#1629, G.Q.F. MFG. Co., Savannah, GA)
- Specimen forceps (4.5″) (#25719–044; VWR, West Chester, PA)
- Dissecting scissors (4.5″) (#25870–002; VWR, West Chester, PA)
- Ultrasonic humidifier (#HM455; Holmes Product Corp. Milford, MA)
- Molten paraffin wax (Parowax; Service Assets Corp. Newport Beach, CA)
- Sterile needles (#305122; Becton Dickinson and Company, Franklin Lakes, NJ)
- 3 cm^3 syringe (#309585; Becton Dickinson and Company, Franklin Lakes, NJ)
- Microloader Pipet Tips (#930001007; Eppendorf, Westbury, NY)
- Sterile water
- Sterile Hanks Balanced Salt Solution (#14065–056; Invitrogen Corporation, Carlsbad, CA)
- Lyon Electric Hatcher (PRF1-H; Chula Vista, CA)
- Dissecting scope (Zeiss Stemi SV11, Oberkochen, Germany)
- Harvard Apparatus microinjector (PLI-90; Holliston, MA)
- Micromanipulator (#AH-60–0570; Harvard Apparatus, Holliston, MA)

- Needle Puller (Sutter Instrument Company, Novato, CA)
- Silica needles (#Q100–70–10; Sutter Instrument Company, Novato, CA)
- Temperature and Humidity Recorder (#CT-485B-110V-G-AL; Omega, Stamford, CT)
- Quail egg trays (# 0205; G.Q.F. MFG. Co., Savannah, GA)
- Ultrasonic dissecting tool cleaner
- Matzuri Exotic Gamebird Starter (#5637; Purina, St. Louis, MO)
- Matzuri Exotic Gamebird Breeder (#5639; Purina, St. Louis, MO)
- 9 × 9 heating lamp (#910–04; Lyon Electric Co. Inc., Chula Vista, CA).

DMEM + (293 FT) media:

- DMEM (Irvine, #9031)
- FCS 10%
- P/S (Irvine, #9366) 1×
- L-Glu (Irvine, #9317) 2 mM
- HEPES (Irvine, #9319) 10 mM
- MEM–NEAA (Gibco-BRL, #1140–050) 0.1 mM
- Sodium pyruvate (Mediatech, Inc. #25–000-CI) 1 mM

III. Concluding Remarks

This chapter has presented several methods used in our laboratory for the purpose of generating transgenic avians. Avian embryos are ideal for imaging because they are accessible to study at all stages of development outside of their vertebrate mother, unlike mammals. The ability to combine transgenic avian technology with the use of dynamic imaging can be used to dynamically follow cell movements in living embryos.

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PART III

Functional Genomics

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CHAPTER 16

Gene Discovery: Macroarrays and Microarrays

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I. Introduction

Gene expression profiling is a powerful approach to characterize global changes in gene expression and to identify novel genes. Traditional methods that evaluate gene expression, such as Northern blotting and RT-PCR, monitor the levels of individual genes. In contrast, gene arrays permit high throughput, genome-level transcript profiling, where the expression levels of thousands of genes are measured at the same time. Since their introduction, the various array technologies have undergone extensive evolution. Here, we will discuss DNA hybridization arrays and highlight the advantages of using chick embryos for expression analysis studies.

II. Chicken as a Model System: Integrating Embryology and Genomics

Although there are currently more resources available for the human, mouse, and rat genomes, chicken embryos offer many attractive features for genomic studies, especially for the developmental biologist. Chicken embryos are easy and inexpensive to obtain. While maintaining a mouse colony can cost $0.60–1.00 per cage a day, fertilized eggs can usually be purchased for less than $6 per dozen and require no animal husbandry or per diem fees. Because chick embryos develop externally *in ovo*, one can readily obtain, manipulate, and culture tissues at almost any developmental stage without having to sacrifice the mother or use special culture conditions. Furthermore, because chick is a classical embryological system, it is possible to apply genomic approaches to characterize changes in gene expression as a consequence of developmental events, such as the response to an inductive interaction or signal (Stern, 2005). The embryology of the chicken (and quail) are very well described and their embryos are large relative to those of frog, zebrafish, and mouse; it is thus less difficult to collect sufficient starting materials for constructing cDNA libraries or making probes. The chick is also an amniote and its developmental processes and morphology closely resemble that of human embryos. The chicken genome is one-third the size of mammalian genomes because it contains less repetitive sequence, pseudogenes, and duplications (Consortium, 2004). This is a real advantage when assessing functional elements and identifying coding sequences, and makes the chick genome extremely useful for comparative genomics (see Chapter 18 by Brown and Chapter 12 by Sauka-Spengler, this volume). As a food source, interest in and resources for the chick genome are further motivated by the poultry sciences (Cogburn *et al.*, 2003). In fact, avian macroarray and microarray experiments have assessed the fat content in different strains of chickens (Bourneuf *et al.*, 2006; Wang *et al.*, 2006, 2007) and identified immune-related genes in order to understand the molecular basis of disease pathogenesis and responses to infections (Bliss *et al.*, 2005; Dar *et al.*, 2005; Sarson *et al.*, 2007; Smith *et al.*, 2006).

III. The Array: Choosing a Platform

Prior to the genome era, researchers who were interested in gene expression analysis used a variety of techniques. Differential colony hybridization utilized a replica-plated traditional library, screened with two different probes, and compared signal intensity (Cochran *et al.*, 1987). This approach is cumbersome and identifies only abundant clones. Differential display and subtracted cDNA libraries are more sensitive approaches (Wan *et al.*, 1996), but yield only a limited view of gene expression differences.

Array technology has overcome these past limitations. On a DNA array, each clone (either cDNA or oligonucleotide) is situated in a fixed location, which greatly lessens the burden of identifying corresponding clones post-hybridization. With current technology, all known genes in a genome can be placed on a single array, or the genes arrayed can be tailored to the interest of the researcher. Importantly, multiple copies of any array can be produced, allowing gene expression to be evaluated at different time points and under varying conditions. Sensitivity issues are overcome by using minimal hybridization volumes, or subtracted probes (see below).

Array formats can be broadly divided into macroarrays and microarrays. We summarize the pros and cons of these arrays in this section and in Table I, and provide information for researchers that are interested using these technologies in following sections.

A. Macroarrays

Macroarrays usually consist of bacterial libraries (cDNA or genomic) from which individual clones are arrayed into 384-well plates and spotted in duplicate onto 20 cm $\times$ 20 cm nylon filters (Clark *et al.*, 1999). Many filters of the same library can be manufactured, allowing researchers to screen the library under multiple conditions and share libraries among themselves. In contrast to randomly plated libraries, each clone on a macroarray is located in a fixed coordinate, so it is straightforward to identify the correct clones from hybridization spots on the filters. Once the target spot location "address" has been identified, one simply has go back to the 384-well plates and pick the clone from the glycerol stocks.

A major advantage of a macroarray is that it is an "open system." Although they are more often used, microarrays include a constrained selection of known sequences (often from EST projects) spotted onto slides, thereby confining the pool of genes to be analyzed. To create a macroarray, no preexisting EST or genome project is required, thus maximizing the chance for new gene discovery in an unbiased manner. The researcher can make their library at a particular time point during development, or from a particular structure, to increase the likelihood of identifying interesting genes. In addition, it is possible to array full-length cDNA libraries, which facilitates subsequent experiments such as *in situ* hybridization or overexpression studies.

Table I
An overview of the Major Differences Between Macroarrays and Microarrays

	Advantages	Disadvantages
Macroarray	Results in full-length clones	Have to array own filters (labor intensive)
	Convenient to obtain clones in expression plasmids	Have to sequence each clone
	Filters can be reused	Finite life of filters
	Versatile screening protocols	Need expensive PhosphoImager screens
	Can make customized libraries to suit your needs	May not have complete coverage of rare transcripts
	Do not require prior sequence information	Usually only screen once with each probe
	Unbiased coverage of genes on the array	Screening labor-intensive
		Quality of libraries/filters can vary
		The amount of DNA at each spot location can vary from filter to filter
		Sequential hybridization to compare two populations
Microarray	Clone identity known right away	Need to obtain full-length clones for further study
	Commercial arrays available for purchase	Arrays can only be used once
	Representation of rare genes can be more complete	Need fluidic station and reader to perform hybridization
	Simple screening protocol, also many companies offer screening and data analysis services	General arrays more common, custom arrays can be expensive
	Quality of arrays (especially commercial ones) is more consistent	Gene coverage varies depending on the company and EST database used for design
	Can start with total or messenger RNA to make probes	Sequence information needed to create the array
	Single hybridization to compare two populations	Quality of "home-spotted" arrays varies

Macroarrays are relatively simple to manufacture (although they do require use of a Genetix Q-bot robot), and they are amenable to different screening strategies. The amount of DNA spotted can vary from filter to filter, but by double-spotting each clone, including hybridization controls, and carefully managing comparisons, reliable changes in gene expression can be observed. Macroarrays remain the best option for large-scale screening in organisms that do not have genome projects or well-annotated genomes.

Macroarrays do have several drawbacks. Perhaps the biggest advantage of a macroarray is also one of its limitations: each clone picked must be sequenced to determine its identity. This can be costly and annotations of potential novel genes can prove inadequate. Another shortcoming is that low abundance transcripts can be omitted from the array. However, if the library is not amplified before spotting, and sufficient clones are spotted, cDNA or genomic libraries with adequate coverage can be obtained (Rast *et al.*, 2000). An additional complication is that macroarray filters must be hybridized in large volumes (10–20 ml) compared to the small volume used (<100 μl) for microarrays. Because chick and quail embryos are inexpensive to

obtain and they are relative large, it is feasible to collect sufficient tissues to prepare probes for hybridizations. Alternatively, rather than obtaining greater quantities of starting tissue, cDNA populations can be linearly amplified with T7 polymerase, and the hybridization signal of rare transcripts boosted by subtraction (see Section VI. Protocol; Rast *et al.*, 2000). Whereas microarrays compare two cDNA populations with a single hybridization, macroarrays must be sequentially hybridized. However, careful analysis of the hybridization kinetics suggests this is a minor issue, especially if the same filters are used for both hybridizations (Rast *et al.*, 2000).

B. Microarrays

The alternative to macroarrays is microarrays. Microarrays come in two forms: cDNA arrays printed onto glass slides, and oligonucleotide arrays synthesized at high density on a solid support. In both cases, the small size of a microarray allows minimal hybridization volume, and increased signal from rare genes as a result. Expression microarrays include coding regions (cDNA inserts or oligonucleotides complimentary to short segments of a gene) and are designed using existing sequence information, so the identity of each spot on an expression microarray is known—that means one can find out immediately which genes are up- or down-regulated without having to sequence each clone, as with macroarrays. Whole chick genome tiling arrays are also commercially available from Affymetrix and Nimblegen; however, tiling arrays include both coding and intergenic regions, and so are not well-suited for gene expression analysis. In addition, as a result of the ever-expanding amount of sequence information available, there are many options for creating custom arrays, as well as companies offering full-service screening options.

A major disadvantage of microarrays is that full-length clones must be obtained to further study a gene of interest. The coverage on an expression microarray can also vary, depending on the EST database used to select clones for inclusion on the array. In addition, purchasing and screening microarrays can involve significant expense and specialized equipment. Oligo arrays have some advantages (uniform DNA spots, probes to different regions of a gene, consistent melting temperatures across the array), but the consensus is that good data can be generated with either.

Microarrays can also be synthesized by printing PCR-amplified inserts from a library of interest, and some labs have used this approach to great success (Bailey *et al.*, 2003; Schultheiss and Afrakhte, 2004). To some extent this combines the advantages of micro- and macroarrays, however, the coverage of these microarrays ($\sim$10,000 clones) is much lower than that of a macroarray ($>$100,000 clones). Microarrays can be constructed from subtracted libraries in order to increase representation on the array, but this added manipulation risks the loss of interesting transcripts. There is also considerable expense involved in amplifying and sequencing every insert that will be spotted.

With the completion of the chick genome project, a variety of commercially available chick microarrays are now available. The following sources that we know of sell chick expression microarrays:

1. cDNA arrays

   ARK-Genomics

   http://www.ark-genomics.org/resources/chickens.php

   Fred Hutchinson Cancer Research Center

   http://www.fhcrc.org/science/shared_resources/genomics/dna_array/spotted_arrays/chicken_array/

   University of Arizona Genomics Research Lab

   http://www.grl.steelecenter.arizona.edu

   The Royal Institute of Technology in Sweden

   http://www.biotech.kth.se/molbio/microarray/Array%20Types/projects/KTH%20UniChicken%2014k%20cDNA%20Microarray/KTH%20UniChicken%2014k%20cDNA%20Microarray.htm

2. Oligo arrays

   Affymetrix

   http://www.affymetrix.com/products/arrays/specific/chicken.affx

   Agilent Technologies

   http://www.chem.agilent.com/cag/bsp/gene_lists.asp?arrayType=gene

   Nimblegen (currently no stock chick expression arrays are available. However, custom expression arrays can be produced, and chick genome tiling arrays are stock items).

   http://www.nimblegen.com

IV. Strategy: Devising the Best Screen Possible

Regardless of the choice of DNA array, a critical consideration in any expression analysis is the careful design of the conditions to be used for the experiment. The resulting data will only be as focused as the comparison that is made. Care should be taken to hold constant as many factors as possible, so that only the intended features are compared. Spatial, temporal, and environmental variations in the experience of the tissue will create differences in gene expression that will impact the data you obtain from the array. The complexity of the tissue samples is also a factor. A greater number of cell types leads to greater diversity of gene expression, and more background to be subtracted out (literally or figuratively) so that the differences of interest are apparent. The most successful screens compare two populations that are as similar and as simple as possible, with the difference

temporally and spatially restricted to the defined developmental window of interest. In this respect chick embryological assays hold a real advantage over comparisons of wildtype versus mutant mouse tissue; unless a conditional knockout is used, development proceeds for many days in the absence of a gene, and it is not possible to know at what point the differences in gene expression developed. In designing your assay, however, it is important to keep your conditions as close to the *in vivo* situation as possible. These principles are illustrated by a screen in which neural crest induction was recreated *in vitro*. Identical pieces of neural plate and nonneural ectoderm were cultured either in apposition or as isolates for equivalent lengths of time and compared (Gammill and Bronner-Fraser, 2002). In this way, gene expression resulting from the neural crest inductive interaction (explants touching) could be separated from gene expression in the neural plate and nonneural ectoderm.

Another common and critical consideration of successful gene expression profiling experiments is the quality of the starting material. When samples are pooled to obtain sufficient quantities of RNA, it is important that the tissue is generated as consistently as possible, keeping in mind the rule that any variations will be detected as differences in gene expression. If feasible, it is worthwhile to check for expression of known differentially expressed genes by RT-PCR or Q-PCR before pooling to verify the intended consequences. In addition, the resulting RNA needs to be of the highest possible quality, and the integrity of total RNA should be verified either by gel electrophoresis or other methods such as the Agilent RNA 6000 Nano or Pico kits or the 2100 Bioanalyzer.

V. Analysis: Sorting through Several Thousand Data Points

Generating long lists of differentially expressed genes is relatively easy; the difficulty comes in determining which differences are real and interesting. To some extent, this depends upon whether the goal of the experiment is to characterize regulatory relationships or to identify new genes involved in a process. To define pathways, coregulated genes are identified using clustering algorithms. Many programs are available to aid in statistical and clustering analyses of microarray data, such as dChip, GeneSifter, GeneSprings, etc. For gene discovery, upregulated genes are usually clustered functionally to better understand the process studied. For this aspect, array annotation is key. Ideally it should be as complete and accurate as possible; however, because the annotation of the chick genome is still in progress, this is rarely the case. The good news is that most companies provide the latest annotations as information becomes available. However, if a macroarray is used, one starts with no annotation whatsoever, and each gene must be sequenced and annotated individually. Clustering genes by function is greatly facilitated by the gene ontology (GO) database (Consortium, 2000), which provides a description of the function, localization, and biological processes attributed to a gene product

based on sequence and homology. GO terms can easily be obtained for the products of a macroarray screen, and Affymetrix, for example, includes GO terms for each gene on the newest release of the chick whole genome array.

To verify genes of interest, differential expression must be validated by independent means. To support upregulation of a gene in the biological assay used for screening, real-time quantitative PCR is the method of choice (Provenzano and Mocellin, 2007). This assay is especially useful when determining gene regulatory relationships, but necessitates collection of tissue samples equivalent to those used in the subtraction, which can be a laborious task depending on the assay. When gene discovery is the goal, we find *in situ* hybridization to be the most informative, as it provides spatial and temporal information about the relative expression level of a gene in the tissue of interest and neighboring cells. This information is not only useful in confirming differential gene expression, it is extremely helpful in postulating function and designing future experiments.

VI. Protocol: Macroarray Screening

The main issue for macroarrays is their dimensions: filters must be hybridized in a large volume. For differential gene expression analysis using complex probes, this means that it is difficult for rare transcripts in a large volume to hybridize to the extent that a hybridization signal is detectable (in other words, they do not reach a sufficient C_0t, which is the product of the DNA concentration and the time of incubation; it is a measure of hybridization kinetics). To overcome this obstacle, subtracted probes must be used when screening macroarrays with complex probes (Rast *et al.*, 2000). Subtraction removes common genes from two populations being compared, effectively decreasing the complexity of the cDNA pool of interest and increasing the representation of differentially expressed transcripts. This enriches the population for those genes, so that even rare transcripts can hybridize to a C_0t with a detectable hybridization signal in a reasonable length of time (higher concentration, less time).

The following protocol for generating subtracted probes for macroarray screening is largely that of Rast *et al.* (2000) with our notes added. See this reference for a variety of controls for this method. Standard molecular biology protocols (ethanol precipitation, formaldehyde gels, electroelution, butanol extraction, etc) are described in Sambrook and Russell (2001). Materials are indicated within each section.

A. Preparation of PolyA RNA for cDNA Synthesis

Prepare polyA RNA from pooled tissue using the method or kit of your choice. We prefer to prepare total RNA first so that the integrity can be checked on a gel before proceeding.

B. Preparation of Tracer and Driver for Subtraction

1. First and Second Strand cDNA Synthesis

Many kits are available to synthesize first and second strand cDNA. We used the SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen). First strand cDNA is random-primed with the following biotinylated oligo:

LT7RND-BT:	5′-[biotin]-CGGAGGTAATACGACTCACTATAGGGAGNNNNNN-3′

After first and second strand incubation, purify the cDNA using Qiaquick columns (Qiagen), including an extra 500 µl 35% guanidine hydrochloride (GuHCl) wash after binding and before the PE wash to completely get rid of the long oligos. Elute in 50 µl EB, then ethanol precipitate with 0.5 volumes 7.5 M ammonium acetate and 2 volumes ethanol using 5 µl tRNA as a carrier. Resuspend in 6 µl DEPC H_2O.

2. Linker Ligation

On the reverse strand, the following linkers contain a 3′ dideoxy residue to prevent filling in of overhang, and a 5′ phosphate for blunt ended ligation to the cDNA. Anneal linkers at 1 µg/µl using standard protocols:

S-linker:	5′-GGGTGCTGTATTGTGTACTTGAACGGGCGGCCGCA-3′
	3′-ddCCCGCCGGCGT-P-5′
D-linker:	5′-GCCAACGTATGTAAGGTTGAGTTCCGGGCAGGT-3′
	3′-ddCCCGTCCA-P-5′

In a 10 µl reaction, Ligate linkers to the cDNA O/N at 4 °C, using annealed S-linker for tracer, D-linker for driver.

Qiaquick purify, including extra GuHCl wash to remove unligated linkers. Elute in 50 µl EB.

3. PCR Amplification and Size Selection

Set up three pilot PCR reactions using 5% of the cDNA (2.5 µl) in each reaction to determine the optimal cycle number needed to amplify 300 ng each of driver and tracer populations. For the tracer population, the goal is to obtain sufficient product for size selection on an agarose gel. For the driver population, the goal is to prepare template for driver transcription.

LT7	5′-CGGAGGTAATACGACTCACTATAGG-3′
SLINK	5′-GGGTGCTGTATTGTGTACTTGAACG-3′
DLINK	5′-GCCAACGTATGTAAGGTTGAGTTCC-3′

Amplify one tube each for 5, 15, or 25 cycles.

PCR parameters: 95 °C 2 min (initial denaturation) – 95 °C 1 min/60 °C 1 min/72 °C 3 min (PCR cycles) – 72 °C 8 min (final extension)

Qiaquick purify, elute in 50 µl.

Run half of each reaction on a gel, use the other half to determine the concentration with a spectrophotometer (OD).

Using the yield from the pilot reactions as a guide, set up new PCR reactions using half (25 µl) of the cDNA, adjusting the cycle numbers accordingly to obtain 300 ng product.

Example: The 15 cycle pilot is close to 300 ng. Since 10-fold more template will be used, you will need 10-fold less amplification. PCR does not give perfect doubling, so $(1.9)^3 = 6.9$ while $(1.9)^4 = 13$, so can drop to 11 or 12 cycles to get 300 ng product.

4. Preparation of Single-Stranded Tracer

Run the PCR-amplified Tracer cDNA on a 1.5% agarose prep gel. Excise the 300-500 bp region of the smear and electroelute. Resuspend precipitated DNA in 50 µl total Qiaquick buffer EB.

Perform pilot PCR reactions amplified 5, 15, or 25 cycles using 5% of the size-selected cDNA and a biotinylated LT7 primer (BT-LT7). Your goal is to make about 4 µg of tracer cDNA from 4 or 5 pooled 100 µl reactions, with each reaction containing about 5–10% of the template cDNA and yielding about 700 ng product. It is important not to overamplify or the products will start multimerizing (up to about 0.7–1 µg of product, amplification proceeds normally).

Qiaquick purify the scaled up reactions, binding all of them to one Qiaquick column by sequential addition/centrifugation. Elute in 50 µl EB.

Run 1 µl on a gel to check for multimerization and determine concentration by absorbance.

Remove and set aside 1 µg of double-stranded tracer BT-cDNA for preparation of unsubtracted probe. This should leave 3 µg for single-stranded tracer preparation.

The sense strand of tracer DNA is prepared by capturing the antisense strand with Streptavidin magnetic beads (Dynal) using the manufacturers protocol for strand-specific probe template and elution by alkali treatment.

Ethanol precipitate the eluate with 0.1 volume 3 M sodium acetate and 2.5 volumes ethanol incubating at −20 °C overnight.

Note: Treat the cDNA as if it were RNA, as it will be incubated with RNA. Use clean or DEPC-treated solutions.

5. Driver Transcription

Qiaquick purify the Driver cDNA amplified in step B3, eluting in 50 µl EB.

Ethanol precipitate the eluate with sodium acetate and resuspend in 16 µl H_2O

Antisense Driver RNA is transcribed using the Megascript kit (Ambion). The resulting RNA is treated with DNase and recovered by phenol:chloroform extraction and isopropanol precipitation according to the manufacturers instructions.

C. Subtractive Hybridization

Note: This protocol utlizies hydroxylapatite chromatography to separate double- and single-stranded nucleic acid. Please see Rast *et al.* (2000) for a discussion of why this is preferable to other techniques.

1. Analyze Tracer and Driver

Spin down the driver and tracer (each currently being precipitated), wash with 75% EtOH/DEPC H_2O, and resuspend each in 8 µl DEPC H_2O.

OD the driver and tracer to determine concentration, and run a small amount on a formaldehyde (denaturing) gel to check size, integrity, etc.

2. Subtraction

Prepare 1 M Phosphate Buffer (PB) pH 6.8 by mixing equal amounts of 1 M NaH_2PO_4 and 1 M Na_2HPO_4. Filter each before mixing and store separately.

Mix	200 ng tracer ssDNA	3 µl
	10 µg driver RNA	6 µl
	* 10 pg control ssDNA	1 µl
		10 µl

Denature 95 °C for 5 min, quick chill on ice

Warm sample to 65 °C, add 10 µl 2× HAP subtraction buffer (0.68 M PB + 0.02% SDS) prewarmed to 65 °C

Place in a 50-ml tube with a wet Kimwipe (already equilibrated to 65 °C) and hybridize for 48 h at 65 °C

**Note:* For the control ssDNA we subcloned a 300 bp fragment of lambda DNA then PCR amplified with a BT primer and isolated the sense strand using Dynabeads. Any fragment with known sequence (for making primers) that is not present in the cDNA population will do.

3. Prepare Hydroxyapatite (HAP) Suspension

Weigh out 2.5 g of hydroxyapatite (HAP; BioRad DNA grade, catalog # 130–0520). Place in a 50-ml tube and equilibrate in 5 volumes 0.12 M PB for 30 min at RT, mixing occasionally by inversion

Boil 15 min. Resuspend occasionally while boiling.

Resuspend HAP and let settle for 20 min (no shorter and no longer!). Discard liquid and add 5 volumes 0.12 M PB, repeat 3× total. Store HAP suspension at 4 °C.

4. Hydroxylapatite Chromatography

Prepare fresh 0.12 M PB + 0.05% SDS and 0.5 M PB + 0.05% SDS, equilibrate at 60 °C

A 0.2 ml HAP column bed is prepared in a 2 ml water-jacketed column maintained at 60 °C using a circulating water bath, running water from bottom to top of the column. Before pouring the column, first flush the frit with water to dislodge any particles. Run 0.05% SDS through the column, then close stopcock, fill again with 0.05% SDS let sit about 1 h. Let flow through or remove with a transfer pipette.

With the stopcock closed, add 800 µl of resuspended HAP suspension. Gently resuspend several times using a transfer pipette as the suspension warms to 60 °C. Let the HAP pack for about 15 min, then drain.

Wash column with 3× 2 ml of 0.12 M PB + 0.05% SDS

Note: Never let column dry out and always use it straight away! Never use more than once.

To prepare the subtraction for HAP chromatography, bring the 20 µl 0.34 M PB subtractive hybridization reaction to 200 µl in 0.12 M PB by adding (maintaining the subtraction and all solutions at 60 °C on a heat block):

36.67 µl 0.05% SDS

143.33 µl 0.12 M PB + 0.05%

Remove 10 µl of the subtraction and set aside for "before" quantitation by Q-PCR

Add the sample to be analyzed to the HAP column with the stopcock closed, incubate 2 min

Open the stopcock and collect the sample, then add 800 µl 0.12 M PB + 0.05% SDS at 60 °C and collect in the same tube

Wash 2× with 1 ml 0.12 M PB + 0.05% SDS, collecting each wash in a separate, labeled tube

Wash 3× with 1 ml 0.5 M PB + 0.05% SDS, collecting each wash in a separate, labeled tube

5. Concentrate and Desalt the HAP Eluates, Test Efficiency of Subtration

Concentrate 0.12 M PB fractions 1 and 2 (single-stranded fractions) by butanol extraction.

Desalt using NICK spin columns (Pharmacia) or similar G50 resin spin columns.

Remove 1/20 (e.g., 5 µl of 100 µl) and set aside for "after" quantitation by Q-PCR

Ethanol precipitate with ammonium acetate using 1 µl glycogen as a carrier. Store at −20 °C until needed.

To determine the efficiency of subtraction, quantitate levels of ubiquitously expressed genes, the control ssDNA (added in C2) and any known differentially expressed genes in cDNA before and after subtraction by real-time quantitative PCR (according to manufacturers' instructions).

6. Fill in the Subtracted cDNA

Spin down subtracted DNA. Wash with 70% EtOH and resuspend in 20 µl DEPC H_2O or EB.

Set up a PCR reaction with:

10 µl subtracted cDNA
5 µl Advantage taq buffer
2 µl LT7 primer
2 µl S-link primer
1 µl dNTPs
29 µl H_2O
+ 1 µl Hi-fidelity taq
50 µl

Run 5–8 cycles of PCR with the usual settings. Number of cycles depends on your recovery; can do a pilot reaction (as in step B3) to check. Run out 2 µl of the reaction on a gel to check quality (and absence of multimers).

Qiaquick purify the remainder, elute in 35 µl EB, determine concentration of 1 µl using specrophotometer.

D. Library Screening with Complex Probes

One probe synthesis is needed for four filters in one bottle. Probe the same filters sequentially, first with probe transcribed from subtracted and then unsubtracted cDNA. Never use different filter copies for comparison as variations in spotting can give false results.

1. Probe Labeling

Probe synthesis is performed using the Maxiscript kit (Ambion) and 800 Ci/mM ^{32}P-UTP according to the manufacturers instructions. Unincorporated nucleotides should be removed by G-50 column purification. The reaction typically yields 2–2.5 mg of probe.

2. Filter Hybridization

Roll a stack of four filters separated by mesh spacers in 5× SSPE, avoiding bubbles. Insert into a clean hybridization bottle, add some 5× SSPE and slowly unroll the filters. Drain SSPE.

1× SSPE = 0.15 M NaCl, 10 mM phosphate, 1 mM EDTA, pH 7.4

Prehybridize in 80 ml of hybridization solution for 2 h at 65 °C.

Hybridization solution	in 100	ml
20× SSPE	25	ml
10% Sodium pyrophosphate (NaPPi)	1	ml
20% SDS	25	ml
10 μg/μl tRNA	200	μl
H_2O	48.8	ml

Heat the RNA probe to 95 °C for 2 min and mix with 15–20 ml of 65 °C hyb. Pour off prehyb and add the hyb/probe mix to the wetting volume.

Hybridize for 48 h at 65 °C.

Wash filters in 500 ml:

a. 2× 20 min at RT in 2×SSPE; 0.1% SDS; 0.05% NaPPi
b. 2× 20 min at 65 °C in 1 × SSPE; 0.1% SDS; 0.05% NaPPi
c. 1× 15 min at 65 °C in 0.1 × SSPE; 0.1% SDS; 0.05% NaPPi

Filters are sandwiched between two layers of plastic wrap and exposed to a phosphoimager plate for 24 h. Record exact time of exposure. Calibrate longer exposure so that the darkest spots nearly saturate the phosphor screen.

3. Filter Stripping

Note: Filters must either be allowed to decay or be stripped. For stripping to work efficiently, the filters must never dry out. Store them at −80 °C until you are ready to strip them to keep them wet. Never store filters in plastic wrap at room temperature or they will become moldy.

a. 20 min at 45 °C in 0.2M NaOH
b. 30 min at 65 °C in 0.2M Tris pH7.4; 0.1x SSC; 0.1% SDS
c. soak briefly in 0.2M Tris pH7.4; 0.1x SSC; 0.1% SDS; 10mM EDTA
d. dry on filter paper and store between sheets of filter paper in a cool, dry place.

4. Analysis

Spot intensities are quantitated using BioArray software, described in Brown *et al.* (2002). Upregulated genes are identified by comparing and sorting subtracted and unsubtracted values in Excel (Microsoft). Genes of interest are then picked and sequenced.

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CHAPTER 17

Dissection of Chick Genomic Regulatory Regions

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I. Introduction

An old view of vertebrate gene regulation in embryonic development held that a gene is associated with a few ''tissue-specific'' regulatory elements, which are usually located in the 5′ proximal region of the gene. Recent advances in functional genomics, boosted by the sequence determination of the entire genome of model animals, completely revised the old view, unveiling the reality. Namely, a single gene directly involved in the regulation of embryonic development is usually regulated by many regulatory elements, which are widely scattered on both sides of the genes and have distinct spatiotemporal specificities. In other words, a continuous expression domain of a gene in a developing tissue may be regulated by the combined effects of specific subdomain- or stage-confined regulatory elements (Matsumata *et al*., 2005; Uchikawa *et al*., 2003).

This apparent complexity of gene regulation may reflect the fact that regulation of a developmentally important gene needs to satisfy the following two requirements: (1) The gene must be continuously expressed in order to sustain the tissue identity and to complete a developmental process, whereas (2) the extracellular signals and cell–cell interactions that activate/repress the gene change dynamically depending on the stage and location of a developing embryo.

One aim of this article is to familiarize readers with genetic tools to untangle the problems of complex regulatory elements, tools that fully take advantage of the features of chicken embryos.

The availability of the whole genome sequences of various animal species also opened up new horizons of functional genomics. Alignment of syntenic regions of different animal species clearly indicates that many discrete non-protein-coding regions are well conserved in DNA sequences. The stretches of such conserved sequences vary from a few hundred base pairs to a few kilobases (e.g., Woolfe *et al*., 2005).

The extent of sequence conservation of these regions tends to be longer and higher when two phylogenetically related species are compared, but some regions are conspicuously and consistently conserved even from mammals to fishes. It is a generally accepted notion that these regions are conserved because of regulatory importance, and available evidence strongly supports this view. However, there have been only a few limited cases of systematic examination of how the sequence conservation is functionally significant. We have developed methods to systematically analyze regulatory sequences, taking advantage of chicken embryo electroporation, and applying them for genes encoding the transcription factor SOX2 (Uchikawa *et al*., 2003) and the cell adhesion molecule N-cadherin (Matsumata *et al*., 2005), which provide the basis of this chapter.

Another advantage of the chicken embryo is its unique phylogenetic position and genome size. Chickens and other birds are only distantly related to mammals, yet together with mammals belong to the amniotes, placing them at a unique phylogenetic distance from mammals, and this facilitates the evaluation of

conserved sequences. Additionally, the size of the chicken genome is two-thirds that of mammalian genomes; this is also an advantage in the functional analysis of the conserved regions.

In this chapter, we describe fundamental principles and basic strategies for utilizing chicken embryos, and discuss various practical issues. The genes involved in developmental processes are under dynamic regulation, which relies on a battery of gene-associated regulatory regions of distinct specificities. The majority of the regulatory regions of a gene are widely distributed in a genomic span that extends as much as several hundred kilobases. The chicken genome and chicken embryos have advantages for the characterization of these regulatory regions, owing to the availability of the powerful technique of embryo electroporation, to the unique phylogenetic distance from the mammals, and also to other features. Vectors to be used in the electroporation-based identification and analysis of the regulatory regions (in particular enhancers), a high-throughput system to analyze a genomic region of a BAC clone size and strategies to analyze the regulatory regions with resolution of a few base pairs, which allow identification of interacting transcription factors and upstream signals, are described and discussed here in great detail, together with the underlying principles. The phylogenetically conserved, noncoding sequences are compared with the regulatory sequences determined by unbiased functional assays, and advantages and pitfalls of phylogenetic comparison-based predictions of the regulatory regions are discussed.

II. Rationale

A. Where to Search for the Regulatory Regions in the Genome

How far away a regulatory region is located from the gene of interest is not predictable. However, our previous survey of potential regulatory regions for *Sox2* expression suggests that major regulatory sequences are located within a 100-kb region encompassing the gene, if the gene itself is not gigantic. As the *Sox2* gene is located in a region of the genome sparse in protein-coding genes (sometimes referred to as a ''gene desert''), the analysis of regulatory regions was straightforward without interference by those of neighboring genes. It is also known that transgenesis of various model animals using BAC (bacterial artificial chromosome)-based transgenes of the size of 200–300 kb usually results in recapitulation of the endogenous expression pattern of the gene (e.g., Heintz, 2000), in support of the view that a region of a few hundred kilobases covers the majority of regulatory sequences. Of course, there may be some regulatory regions further away, as indicated by documented cases of regulatory regions Mb remote from the gene (e.g., Kleinjan and van Heyningen, 2005; Sagai *et al*., 2004).

Given that various BAC-based genomic libraries are available for many animal species, a BAC clone may be good material with which to start the analysis of the regulatory regions associated with a gene.

B. Significance of Phylogenetically Conserved Sequence Blocks

The extent to which the noncoding sequences are conserved depends on the genetic locus. However, mere comparison of genomic sequences of a few mammalian sequences is not sufficient, as important regulatory sequences tend to be masked by the bulk of less important sequences that are also conserved to significant degrees. However, as the phylogenetic distance between the two species increases, the extent of sequence conservation in the less important regions drops steeply, and the phylogenetic distance between the chicken and mammals (e.g., humans) turns out to be just appropriate for predicting the regulatory sequences. If a region of conserved noncoding sequence is identified in the chicken and human genomes, it is a strong candidate for a regulatory region (Uchikawa *et al*., 2003, 2004).

This conservation of the potential regulatory sequences to a large extent guarantees that the mechanism of gene regulation *per se* is also conserved. Therefore, regulatory functions of the conserved sequences of various animal species, from mammals to fish, can be studied in developing chicken embryos, using the methods described in this chapter.

It is to be noted that not all regulatory sequences are identifiable as conserved sequences. Therefore, phylogenetically conserved regulatory sequences may be the first choice in the hunt for regulatory sequences, but it should be borne in mind that this approach alone has a caveat of overlooking some important elements. Another reservation is that some of the conserved sequences may correspond to exons of protein-noncoding transcripts, although they tend to diversify more extensively than do the regulatory regions (Gustincich *et al*., 2006; Mattick and Makunin, 2006).

C. Vectors of Choice

For either investigating many potential regulatory sequences or studying a single regulatory sequence in detail, it is desirable to have a basic expression vector of versatile utility. An important requirement for such a vector, especially when the vector is used to study enhancers (activating elements), is that the vector is inactive (or nearly so) without insertion of a regulatory sequence, but is strongly activated in such a way as to faithfully reflect the regional and temporal specificities of the enhancer.

After trials with various constructs, we chose ptk-EGFP (green fluorescence) and ptk-mRFP1 (red fluorescence) for our routine use. They have virtually no background of fluorescent protein expression without insertion of an enhancer, but express these fluorescent proteins with clear tissue/stage specificities of the enhancer inserted, in electroporated chicken embryos and in transfected cultured cells. This allows live recording of the enhancer activity, and enables tracing a temporal change of enhancer activity even using a single embryo.

The fluorescence emitted from tissues or cells expressing tk-EGFP or tk-mRFP1 genes is usually strong enough for quantitative assessment. Using afluorescent microscope equipped with software for photon counting of a defined area, or

using 96-well fluorescence readers, relative enhancer activities in electroporated embryos and transfected cells can be scored.

The performance of these vectors is excellent when they are introduced into embryos and cells as closed circular DNAs and used for "transient expression assays," but they are not recommended for assays based on chromosomally integrated genes, for example, transgenic mice, as the expression levels of these genes are generally very low under the latter condition.

D. Embryos and Embryonic Cells for Functional Assays

The great advantage of the chicken embryonic system is the applicability of embryo electroporation, which provides an efficient and inexpensive means to assess enhancer activity of even many DNA sequences, which is difficult to accomplish employing other means.

Electroporation of an embryo can be done *in ovo*, but when it is carried out using embryos that are developing in culture, it shows further advantages, especially concerning early developmental stages. For instance, time-lapse fluorescent image recording enables detailed analysis of the spatiotemporal change of the enhancer activity, and in combination with cell labeling technique, makes it possible to follow the fate of cells that once had the enhancer activity. Especially, when electroporation is performed in early (e.g., stage 4) chicken embryos, the cells of a wide embryonic area, including the entire area pellucia, can receive DNA, either in the epiblastic or hypoblastic layer.

There are of course a few limitations to this approach: for later stage embryos undergoing organogenesis and having complex tissue organization, electroporation can be performed only locally, for example, in the spinal cord or a somite, and for much older embryos electroporation is not practical any more.

One way to circumvent this limitation of the developmental stages to which the electroporation technique can be successfully applied is the use of isolated cells of various embryonic tissues of different embryonic stages placed in primary cultures. The same vector DNAs can be used to transfect these cells in primary culture. This provides a robust way of monitoring dynamic switches of active enhancers that take place during development (e.g., Matsumata *et al.*, 2005).

E. Breaking a Regulatory Region into Modules

1. Determination of the Minimal Essential Core Element

A major purpose of identifying regulatory regions of a gene is to identify distinct molecular mechanisms of gene regulation that operate in different regions or developmental stages of an embryo. The following features shared by many "tissue-specific" enhancers should be borne in mind while dissecting an enhancer. (1) A tissue-specific enhancer, which may be several hundred base pairs long,

is usually composed of a minimal essential core element responsible for generating the specificity, and several less-specific subsidiary elements that primarily contribute to generating a strong enhancer effect according to the specificity of the core element. (2) Removal of the core element from the full-length enhancer usually results in total inactivation of the enhancer, while removal of a subsidiary element generally leads to reduction of the enhancer strength. (3) A monomeric core element in isolation is generally very weak as an enhancer, but multimerization of the core element is usually sufficient to elicit a strong enhancer effect with specificity that largely recapitulates the specificity of the original enhancer. Good examples of this have been reported for the lens-specific δ-*crystallin* enhancer (Goto *et al*., 1990) and enhancer N-1 and enhancer N-3 of the *Sox2* gene (Takemoto *et al*., 2006; Inoue *et al*., 2007).

The core sequences are functionally definable by introduction of series of deletions in an enhancer sequence, and are usually in the size range of 30–100 bp, comprising several transcription factor binding sites. The core element sequence tends to be the most strongly conserved across the phyla in the entire enhancer sequence. Thus, when an enhancer region of several hundred base pairs is functionally defined, and the entire region highly conserved between the chicken and mammals, it is worth looking for a subregion conserved in lower vertebrates, such as fish. Such a subregion, if found, would possibly include the core element.

Some enhancers may be more complex regarding the organization of the elements, and the core element may not be clearly definable. Even in such cases, it is valuable to determine the minimal essential region of the enhancer in order to analyze interacting nuclear factors and upstream signal cascades that determine the enhancer activity.

2. Dissecting the Core Region

An important aim of defining and dissecting the regulatory regions of a gene of developmental interest is to clarify in molecular terms how the gene is regulated. The core elements of enhancers associated with a gene is, as a first approximation, the major sites of regulation of the gene. Therefore, a detailed analysis of the core elements is promising for uncovering the central regulatory processes.

Software is available to predict possible transcription factor binding sites in a given sequence. Such predictions are no doubt informative, but are not accurate at all, as (1) the binding consensus sequences, determined based on various criteria, are not necessarily well-defined, (2) functional transcription factor binding sequences very often deviate from the ''consensus,'' and (3) interactions with unlisted important transcription factors are often overlooked.

A straightforward approach to identifying transcription factor binding sites is to introduce a series of mutations into the core sequence and assess the effects of the mutations on the enhancer activity, in order to define functional modules. If the DNA sequence of the functional module matches a known binding sequence of a transcription factor, the factor is a strong candidate for the functional regulator. If a module

does not match known sequences, biochemical identification of the binding proteins followed by their functional assessment will be required. Recent advances of new proteomics-based techniques will be a powerful aid to this approach.

Once a core element is defined, effects of various extracellular signals on the core-dependent enhancer activity can also be investigated using chicken embryo electroporation or transfection of cells. One should be alert to the fact that such effects of signaling molecules could be indirectly, rather than directly, affecting the activity of transcription factors that bind to the core sequence.

III. Methods

A. Basic Vectors for Analyses of Enhancers in Electroporated Chicken Embryos

For assessing an enhancer activity associated with a DNA fragment in live developing embryos and cells, fluorescent protein genes are the first choice. It is preferable to have similar vectors with different fluorescent colors for simultaneous introduction into the same embryo/cells, one serving as a control and the other for testing. For this purpose we have found the combination of EGFP (green fluorescence) and mRFP1 (red fluorescence) satisfactory: similar brightness to our eyes, similar activation time for fluorescence, and also similar response to enhancers inserted in a vector under the regulation of a neutral promoter for transcription.

The most important factor is the choice of the promoter, which must satisfy the following conditions: (1) the absence of expression of the gene in the vector without insertion of an enhancer sequence, and (2) strong gene activation precisely reflecting the specificity of the enhancer.

Strong promoters tend to give a high background level of gene expression, whereas weak promoters respond to enhancers too poorly. We found that the 260-bp promoter of the Herpes simplex virus thymidine kinase (tk) gene in combination with EGFP/mRFP1 coding sequences is satisfactory.

Figure 1A shows the DNA map of the current version (v3) of ptk-EGFP and ptk-mRFP1 vectors having multiple unique restriction sites upstream and downstream of the transcription unit. These sites may be used to insert and multimerize a genomic sequence to be tested for enhancer activity (Fig. 1B).

It should be noted that the vectors we developed were to be used as circular plasmids in electroporation or transfection and to be transcribed in an extrachromosomal state in a nucleus.

B. Inserting DNA Fragments Derived from a BAC-Size Genomic Sequence in a Vector for Assessing an Enhancer Activity

As discussed in the Rationale section, major regulatory sequences of a gene may be scattered in a genomic region of over tens to hundreds of kilobases. If BAC or analogous genomic clones are available, it is convenient to start hunting for enhancers with such clones.

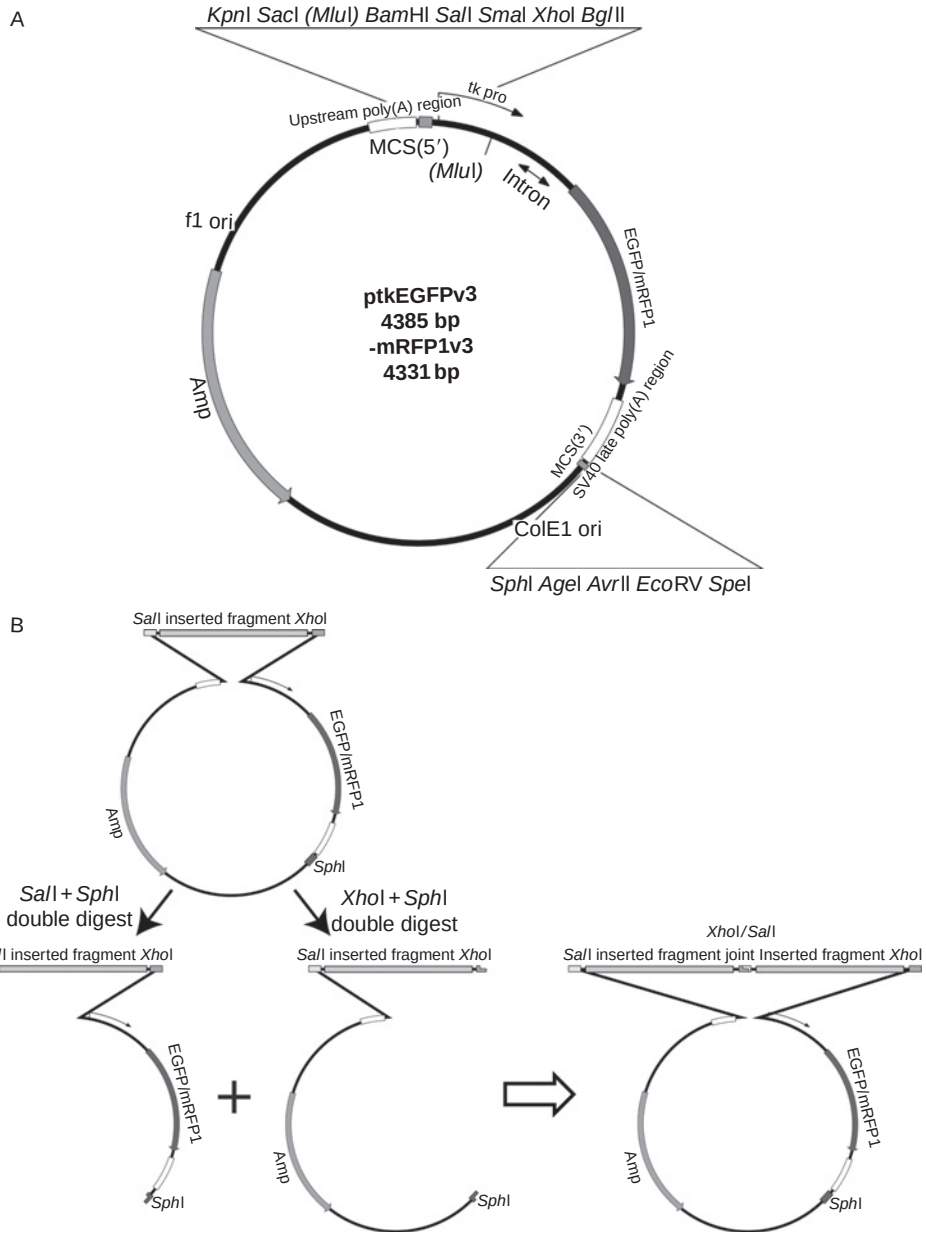

Fig. 1 (A) Basic structure of ptk-EGFP and ptk-mRFP1 (version 3). Nucleotide sequences are available as supplementary data. EGFP and mRFP1 coding sequences are preceded by the multiple cloning sites (MCS) for *Kpn*I, *Sac*I, *Bam*HI, *Sal*I, *Sma*I, *Xho*I, *Bgl*II, 254 bp Herpes simplex virus tk promoter and an intron sequence, and followed by SV40 poly(A) addition region, and cloning sites for *Spe*I, *Eco*RV *Avr*II, *Age*II, and *Sph*I. (*Mlu*I sites in the parentheses occur twice in the plasmid.) The upstream poly(A) region

1. Inserting Defined DNA Fragments

If the base sequence of the genomic region is fully determined, and if the sequences of interest [e.g., phylogenetically conserved sequences (Woolfe *et al*., 2005), predicted transcription binding sites (Hallikas *et al*., 2006), or targets of chromatin immunoprecipitation (Wei *et al*., 2006)] have already been determined, these sequences may be inserted in a site of ptk-EGFP/tk-mRFP1 vectors after amplification (from BAC or genomic sequences) by PCR, or by using the Red recombinase-dependent gap-repair reaction (Lee *et al*., 2001; Liu *et al*., 2003).

2. Inserting Random DNA Fragments for a Shotgun Approach

However, the most unbiased assessment of enhancer sequences is a shotgun approach starting from random fragments (of a BAC insert), if a high throughput system to systematically analyze many DNA fragments is available, as will be described later. Random fragments of a BAC clone DNA can be generated either by shearing DNA molecules by passing the solution through a narrow opening, or by sonication. To obtain a DNA fragment population of the size of a few kilobases, the former technique appears to be easier to regulate the DNA-breaking reactions. As the electroporation efficiency drops with DNA inserts larger than 6 kb in ptk-EGFP/ptk-mRFP1, DNA fragments shorter than 5 kb are used in practice. The mechanically generated DNA fragments are flush ended by sequential treatment with T4 DNA polymerase (efficient removal of 3′ protrusion, filling-in of the 5′ extrusion) and Klenow fragment of DNA polymerase I (a lower 3′ exonuclease activity guaranteeing generation of flush ends in the double-stranded DNAs), followed by T4 polynucleotide kinase reaction to generate 5′ phosphorylated ends, rendering the DNAs substrates of T4 DNA ligase. They are then inserted in linearized ptk-EGFP/ptk-mRFP1 plasmids with blunt ends (after cleavage with *Sma*I, *Eco*RV, etc.) by the reaction of the DNA ligase. The following are the conditions employed in the identification of enhancers associated with the *N-cadherin* gene of the chicken (Matsumata *et al*., 2005) (Fig. 2A).

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terminates transcription from further upstream caused by cryptic promoter activities, and reduces the background level of EGFP/mRFP1 expression in the absence of inserted enhancer. (B) Production of the multimers of an inserted sequence using the multiple cloning sites. A monomeric DNA sequence to be multimerized is inserted in a site (*Sma*I site in the case of ptk-EGFP/mRFP1) flanked by a pair of different 6-base restriction sites generating compatible cohesive ends, such as *Sal*I [G*TCGA*C] and *Xho*I [C*TCGA*G] (or *Bam*HI [G*GATC*C] and *Bgl*II [A*GATC*T]; *Spe*I [A*CTAG*T] and *Avr*II [C*CTAG*G]). Then, the plasmid is digested by *Sal*I plus *Sph*I, and by *Xho*I plus *Sph*I, and the insert-containing fragments of the two different digestions are combined and ligated. The clones derived from this ligation should have the dimerized insert in the form *Sal*I site-insert-GTCGAG joint-insert-*Xho*I site at the multiple cloning sites. As the GTCGAG joint is cleaved by neither of *Sal*I or *Xho*I, repeating the same steps yields tetrameric inserts separated by the GTCGAG joint sequence. Arbitrary multimers can also be produced, for example, by combination of the dimer digest and monomer digest yielding a trimer.

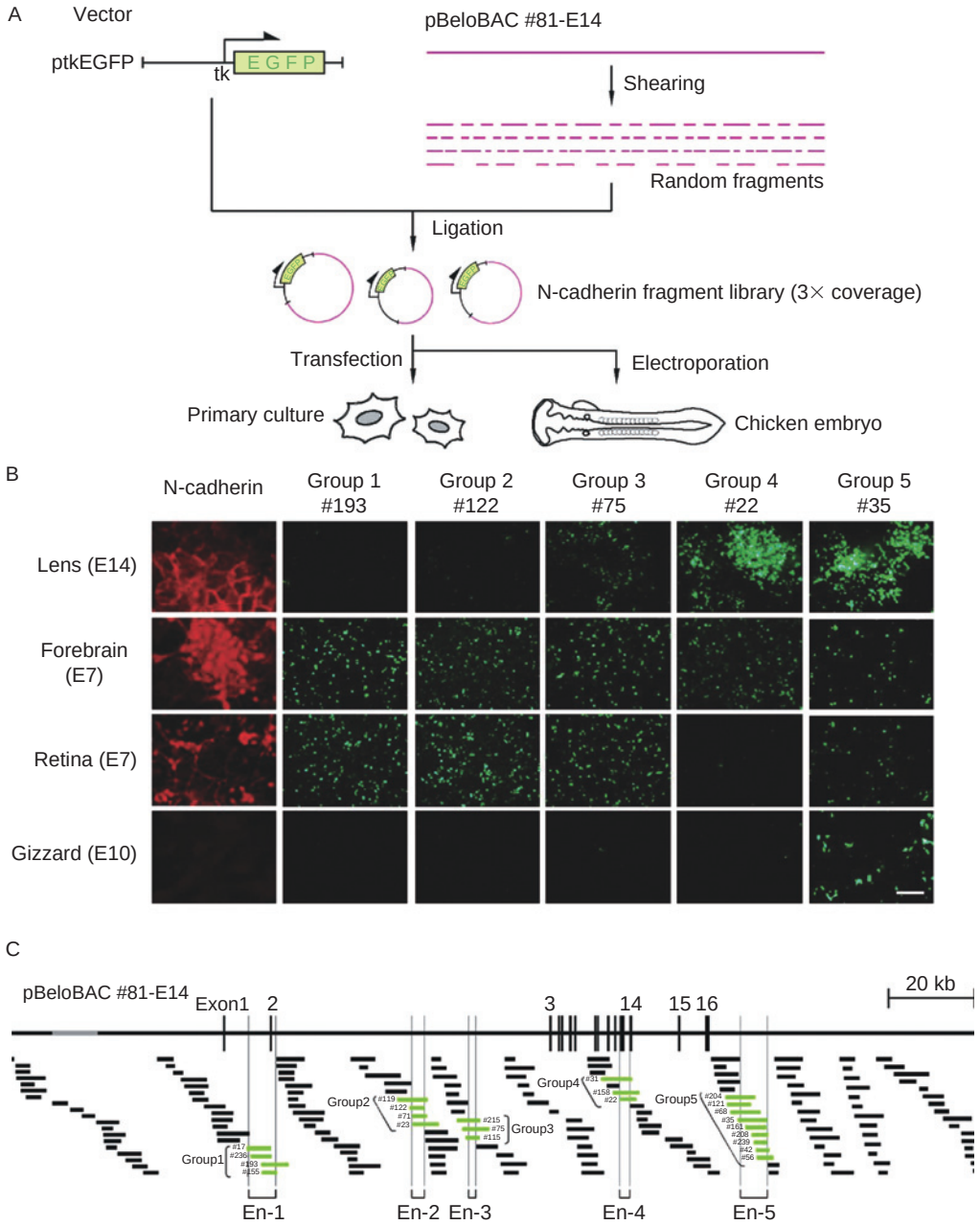

Fig. 2 Systematic screening of enhancers located in a BAC clone, taking the example of the *N-cadherin* enhancers active in the sensory and neural tissues. (A) A schematic illustration of screening of various *N-cadherin* gene subfragments for enhancer activity using transfection. Random subfragments of the genomic *N-cadherin* locus DNA were inserted into the tk-EGFP reporter vector to

3. Protocol for Construction of a Random Fragment Library of a BAC DNA Using ptk-EGFP

1. Suspend the BAC clone DNA at 0.2 μg/μl in 100 μl of 10 mM Tris–HCl, 1 mM EDTA.

2. Fragment the DNA by mechanical shearing caused by pushing and sucking of the solution through a 27-gauge needle of 19 mm length attached to a hypodermic syringe 350 times at the rate of 2 s per cycle, until an average fragment length of 4.5 kb is attained.

3. Remove the fragments larger than 6 kb and shorter than 3 kb by agarose gel electrophoresis and purify the DNA fragments of interest using, for example, a QIAquick Gel Extraction Kit (Qiagen).

4. After flushing the sheared DNA ends using sequential treatment with T4 DNA polymerase and Klenow's fragment in the presence of dNTPs and 5′ phosphorylation with T4 polynucleotide kinase, insert the fragments by T4 DNA ligase treatment into blunt-ended and alkaline phosphatase-treated ptk-EGFP vector at the insert/vector molar ratio of 1.

5. Transform *Escherichia coli* recipients, and randomly pick a sufficient number of clones for covering three times the length of the BAC DNA by the inserts.

6. Expand each clone for DNA preparation, and purify the plasmid DNA, for example, by using aQIA Plasmid Midi Kit (Qiagen).

7. The purified DNA is used for DNA sequence determination at both ends of the insert (which determines the position of the insert DNA in the BAC sequence), and for transfection of cultured cells and electroporation of embryos.

C. Introduction of the Vectors into Chicken Embryos and Cells

Once a library of DNA fragments derived from a BAC (or analogous) clone is generated in an expression vector, with multiple coverages of the original DNA by the inserts, the library is ready for biological assays (Fig. 2A).

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construct a subfragment library. (B) Representative transfection data in combination with the map positions, which allowed classification of the enhancer-bearing fragments into five groups. Panels in the "N-cadherin" column show anti-N-cadherin immunostaining of the cultures used for transfection. Other panels show EGFP fluorescence, reflecting the enhancer activity of the inserted subfragments. Inserts of groups 1 and 2 show enhancer activity in the forebrain and retinal cultures; group 3 inserts in the lens, forebrain, and retinal cultures; and group 4 inserts in the lens and forebrain cultures. Group 5 inserts showed enhancer activity in all cultures, including lung and dermal fibroblasts (not shown here). The scale bar indicates 100 μm. (C) Alignment of the *N-cadherin* locus subfragments indicated by bars on the genomic sequence. Green bars indicate subfragments with enhancer activity of assigned groups, while black bars indicate those without enhancer activity in cultures used for the assay. Reprinted from Matsumata, M., Uchikawa, M., Kamachi, Y., and Kondoh, H. (2005). Multiple N-cadherin enhancers identified by systematic functional screening indicate its Group B1 SOX-dependent regulation in neural and placodal development. Developmental Biology 286, 601–607. (See Plate no. 21 in the Color Plate Section.)

1. Transfection of a Panel of Embryonic Cells in Primary Culture

A high-throughput assay system that have we developed consists of a panel of embryonic tissues in primary culture to be used for transfection with individual clones. Taking embryonic brain tissues as an example, various brain regions (e.g., forebrain, midbrain, etc.) can be isolated separately from embryos of various developmental stages, and then plated for transfection. Such a panel provides data of enhancer activities with sufficiently high spatiotemporal resolution to determine the specificity.

Transfection of these panels of cells with the library of genomic fragments in the fluorescent protein expression vectors results in the generation of fluorescence in a specific set of cultures that depends on the particular genomic fragment inserted in the vector, allowing easy scoring of the enhancers by digital image recording or even by visual inspection. An example is shown in Fig. 2B.

The relative expression levels of the fluorescent proteins reflecting the enhancer strength can easily be quantified by preparing cell extracts from the transfected cultures and placing the extracts in a 96-well plate and measuring fluorescence per well using a fluorescence reader. The procedures for transfection and fluorescence measurement are provided in the next section.

By locating the enhancer activity-positive clones on the map of the BAC insert, the enhancer sequences can be defined from the overlap of such clones (Fig. 2C).

2. Protocol for Transfection of Primary Cultures

1. The method for isolating embryonic tissues and dissociating them into cells for plating in culture dishes varies depending on the tissue and developmental stage. Tissues of chicken embryos older than E5 are usually dissociated by treatment with 0.25% trypsin in 1 mM EDTA and cultured in Dulbecco's modified MEM (Gibco).

2. Dishes of 3.5-cm diameter or wells in a 6-well plate are convenient for transfection, digital image recording, and measurement of fluorescence strength. Inoculate neural cells (brain and retina) at 2×10^6 cells per well, and nonneural and fibroblastic cells at 2.5×10^5 cells per well.

3. After 2 days in culture, transfect the cells with 2.2 μg of DNA per well, using Fugene 6 reagent (Roche) or other reagents. Forty-eight hours after transfection, photorecord the EGFP fluorescence in transfected cells using a CCD camera-equipped epifluorescence dissecting microscope.

4. Scrape, suspend, and lyse the cells in 300 μl of 100 mM potassium phosphate (pH 7.8), 1 mM DTT containing 1% NP40, dispense 100-μl aliquots into a 96-well plate and measure EGFP fluorescence using a 96-well fluorescence reader. The fluorescence intensity of EGFP can be normalized using cotransfected reference mRFP1 fluorescence.

3. Electroporation and Culturing of Chicken Embryos

With the information of the data of primary culture transfection or even without it, one may proceed to electroporation of developing embryos. The technique of electroporation varies depending on the tissues and developmental stages of interest, and standard techniques are described in other chapters of this volume. We shall confine our description of the electroporation to that used for epiblastic cells of stage 4 embryos placed in a modified new culture. This technique allows electroporation of the entire region of the epiblast and facilitates time-lapse recording of the developmental alterations of the enhancer activity in the same developing embryo (Fig. 4B) up to stage 15.

The following is the standard protocol used for this purpose.

1. Preparation of the agar support medium

   a. Pour the yolk and albumen of a cracked egg into a 10-cm diameter dish, and collect 10–15 ml of thin albumen in a sterile bottle. After addition of glucose using 10% sterile stock solution to 0.3% (final), stir the albumen for 15 min at room temperature, and then equilibrate at 49 °C.

   b. Equilibrate melted 0.6% Agar Noble (Difco) in 123 mM NaCl at 49 °C, add the melted agar to an equal volume of the albumen (final 0.3% agar), and mix the bottle's contents by gentle swirling for 30–60 s.

   c. Dispense 2-ml aliquots of the agar/albumen mixture in Petri dishes (1008, Falcon) using pipettes.

   d. Harden the agar by placing dishes at 4 °C for more than 1 h, and store them at 4 °C for use within 1 week.

2. Preparation of 5% yolk supernatant

   a. From the remainder of the egg, collect 0.5 ml of the yolk in a 1.5-ml Eppendorf-type tube and mix with 0.5 ml of Hank's solution by brief vortexing, and centrifuge at 13,000 rpm at room temperature for 10 min.

   b. Mix 0.5 ml of the supernatant with 4.5 ml of Hank's solution, which thus yields 5% yolk supernatant (Li *et al.*, 1994). The yolk supernatant can be stored at 4 °C for up to 1 week.

3. Preparation of the ring support for embryo culture

   a. Cut a ring of 6-mm internal diameter and 18-mm external diameter out of 3 MM filter paper (Whatmann).

   b. Sterilize the rings under UV light illumination for 30 min in a clean bench.

4. Isolation and culture of stage 4 chicken embryos *in vitro*

   a. Incubate fertilized eggs at 38 °C in a humidified chamber to stage 4 (usually for 18–19 h), and briefly sterilize them by swabbing the egg shell with a cotton ball containing 70% ethanol, followed by air-drying.

   b. Crack the shell and pour the egg into a 10-cm-diameter dish.

c. Position the blastodermal embryo on the upper yolk surface by rolling the yolk with a spoon, and remove the thick albumen adhering to the embryo area using scissors or forceps.

d. Place the filter paper ring so that it surrounds the embryo area, and cut the vitelline membrane at the periphery of the ring.

e. Remove the embryo from the yolk and place it in warm Hank's solution by using the paper ring to hold it. Rinse away the adhering yolk so that the embryo can be used for electroporation or culturing.

5. Electroporation of chicken embryos

a. Clean electrodes in a stream of Hank's solution or using an interdental brush.

b. Place an embryo upside down (the vitelline membrane on the bottom) above the cathode made of a $2 \times 2\text{-mm}^2$ platinum plate located in a 2-mm concavity made in the silicone platform.

c. Inject 1–2 μl of DNA solution of containing the vector DNAs at 2–4 μg/μl between the blastoderm and the vitelline membrane using a glass capillary.

d. Quickly place an anodal electrode on the hypoblast side of the embryo with an interelectrode distance of 4 mm, and perform electroporation using an electroporating apparatus (e.g., CUY21 electroporator, BEX Co., Ltd; T820, BTX) with 5 pulses of 10 V for a duration of 50 ms at intervals of 100 ms–1 s (Fig. 3).

6. Culture of electroporated embryos.

a. After electroporation, rinse the embryo with warm Hank's solution and incubate it with the hypoblast side up on the warmed agar culture medium prepared as described earlier.

b. Overlay the embryo with 5% yolk supernatant in Hank's solution and incubate it at 38 °C at 100% humidity overnight.

D. Analyses of Identified Enhancers

When an enhancer associated with a gene is defined with its regional and developmental stage specificities, the enhancer can be subjected to the analysis of transcription factors and upstream signals directly involved in the regulation of the enhancer.

1. Defining the Minimal Essential (Core) Element of the Enhancer

As discussed in the Rationale section, many enhancers include minimal essential "core" element, which also plays a major role in the determination of the specificity of the enhancer. Other less-specific and nonessential elements are primarily involved in the augmentation of the enhancer activity. The definition of the core region, therefore, facilitates analysis of the regulation of the enhancer. The simplest approach to define the core element is to create sequential deletions

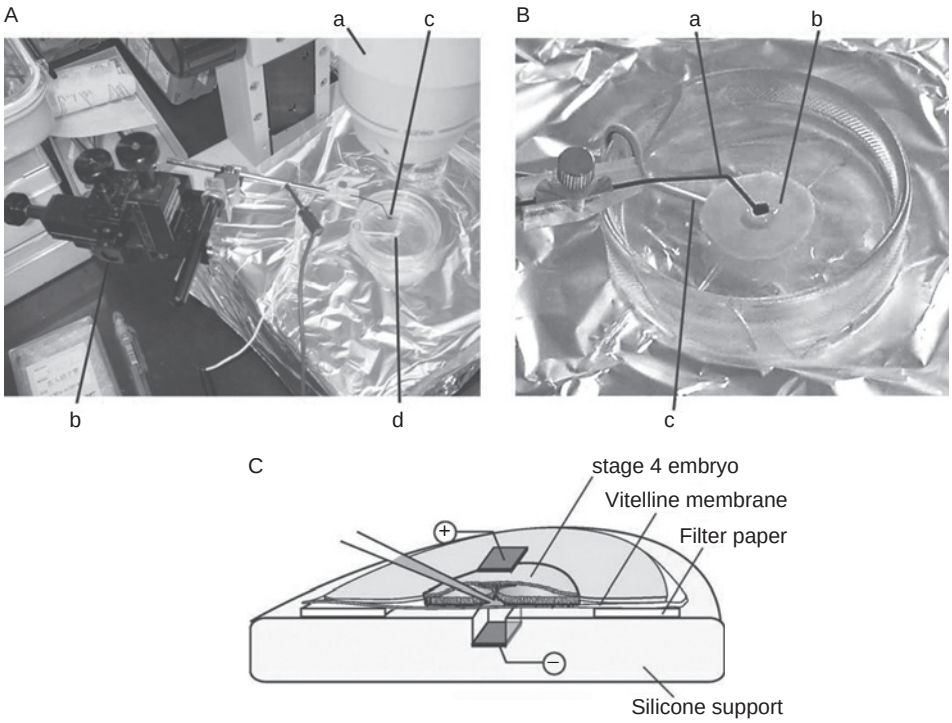

Fig. 3 A setup for chicken embryo electroporation. (A) An overall view of the setup for embryo excision and electroporation. (a) Dissecting microscope, (b) micromanipulator to hold the anodal electrode, (c) anodal electrode tip, and (d) cathodal electrode embedded in a silicone platform. (B) An embryo placed in an electroporation chamber underneath the anode hanged over. (a) Anodal lead, (b) an embryo on a filter ring support, and (c) cathodal lead. (C) A diagram of the spatial arrangement of the electrodes and the embryo. Reprinted from Uchikawa, M., Takemoto, T., Kamachi, Y., and Kondoh, H. (2004). Efficient identification of regulatory sequences in the chicken genome by a powerful combination of embryo electroporation and genome comparison. Mechanisms of Development 121, 1145–1158.

from either side of the enhancer sequence, and the boundaries of deletions causing total loss of the enhancers provide landmarks for the core sequence. During the analysis of partly deleted enhancer sequences, augmentation of the enhancer activity is sometimes observed in a way that is inconsistent with other deletion data. In our experience, such effects reflect creation of a new binding site of a transcription factor by the fusion of sequences flanking the deleted sequence, rather than removal of a repressive element.

It is also common that the core or other elements represented by subfragments of the enhancer are very low in enhancer activity by themselves, but multiplication (2–8-mers) of the same subfragments is usually sufficient for demonstrating their potential activities and specificities as enhancers (Goto *et al*., 1990; Kamachi and Kondoh, 1993; Takemoto *et al*., 2006). The combinations of *Sal*I/*Xho*I, *Bam*HI/

*Bgl*II, or *Spe*I/*Avr*II sites, with shared cohesive end sequences, are conveniently utilized for multimerization of an insert sequence in a vector, as illustrated in Fig. 1B.

2. Mutational Analysis of the Enhancer

An unbiased way of analyzing a core or analogous element of an enhancer, usually shorter than 100 bp, is to introduce base alterations in consecutive blocks of 2–10 bp that span the entire sequence. Of course, specific base alterations targeted to potential transcription factor binding sites are also informative. In any case, the base alterations should be designed primarily based on transversion mutations G ↔ T and A ↔ C, but two important factors should also be considered. (1) Simple base substitutions of the transversion types often create a new transcription factor binding site. Therefore, once base substitutions are introduced in a base sequence *in silico*, the sequence must be checked for the possible creation of a new transcription factor binding site, for instance using software for transcription binding site predictions. If that happens, transition-type base substitutions (A ↔ G, T ↔ C, A ↔ T, G ↔ C) will be mixed with those of transversion, so that the sequence motif of the mutated version substantially deviates from the original, and avoids creation of a new transcription factor binding site.

Even after a careful design of the mutated sequence, a new cryptic binding site sometimes occurs in the mutated sequence. Such cases are suggested if an unexpectedly high enhancer activity is observed using the mutated sequence, or if the effects of mutations in the neighboring blocks are too discordant with each other. When such a case is suspected, a second mutated sequence should be prepared to see if the same mutational effect is obtained using unrelated base substitutions of the same sequence block. (2) If the base alterations are to be introduced in a stretch with a very high A/T or G/C content, naive transversions will alter the physical characters of that DNA stretch, namely, the form of double helices, stability of the base pairs, bendability of DNA after transcription factor binding, etc. Therefore, care must be taken to minimize the alteration of the local physical properties of DNA while the base sequences are altered substantially to affect possible transcription factor binding. Considerations relevant to the possible creation of new transcription factor binding sites apply to these cases as well.

The base alterations are usually introduced by use of synthetic oligonucleotides. When this is done in the monomeric (e.g., enhancer core) sequence, it is then unidirectionally multimerized step-wise in a plasmid, using the strategy described in the previous section (Fig. 1B) (Kamachi and Kondoh, 1993; Takemoto *et al.*, 2006; Inoue *et al.*, 2007). Attempts to create multimers directly from synthetic oligonucleotides by multimeric ligations followed by plasmid-based cloning often end in a problematic situation, for the following two major causes: (1) The error rate of synthetic oligonucleotides is too high, and it is not easy to obtain a DNA sequence of, for example, four repeats of 50 bp without a synthetic error. The presence of a monomeric segment with a wrong nucleotide insertion in a tetrameric sequence is

deleterious to the enhancer activity. (2) Perfect multimeric DNA sequences tend to be refractory to being cloned in a plasmid vector. For this reason, cloning of multimeric ligation products of synthetic DNAs directly in a plasmid vector results in enrichment of aberrant sequences, often short deletions. We therefore strongly recommend the step-wise multimerization strategy described earlier (Fig. 1B).

When multimers of a defined sequence (e.g., with base substitutions compared to the natural (wild-type) sequence) are made in a plasmid, then the multimers may be transferred to an expression vector through ordinary recloning steps. However, the expression vectors ptk-EGFP and ptk-mRFP1 shown in Fig. 1A are designed so that the multimerization steps can be done on these expression vectors themselves, which facilitates the enhancer analysis described in this section.

Once a series of multimeric enhancer elements having various base substitutions is prepared, enhancer activities of the multimeric elements are evaluated in transfected embryonic cells or in electroporated embryos. For this purpose, cotransfection/electroporation of the tester multimers in an expression vector (e.g., ptk-EGFP) together with an expression vector giving different fluorescence (e.g., ptk-mRFP1) and carrying the wild-type enhancer element will allow precise evaluation of the effects of base substitutions (Fig. 4A).

IV. Discussion

A. Advantages of the Combined Use of Multiple Fluorescence Colors for the Vectors

In the Methods section, the advantage of the use of two vectors expressing different fluorescent proteins was already discussed in a simple situation, such as one (e.g., red fluorescence) serving as the control, and the other (green fluorescence) reflecting variables, for example, mutational effects. Such an example is shown in Fig. 4A. Of course, tricolored analysis may be carried out when appropriate, if a relevant optical system is available.

Two-color analysis can be utilized in various ways. Figure 4B shows an example with temporal changes of the spatial relationship of the activities of two different enhancers (N-1 and N-2 of *Sox2)* in an embryo developing in a culture. Figure 4C represents a case where the spacial boundaries of the enhancer activity (enhancer N-4 of *Sox2)* are determined using a rhombomere-specific marker vector.

So far, only activating regulatory elements have been discussed as the subject of analysis. However, repressive elements (silencers) can also be studied, under conditions in which the unrepressed expression level of an enhancer-activated reporter gene is appropriately controlled. For this purpose, ptk-mRFP1 activated by an enhancer may serve as a reference, and ptk-EGFP activated by the same enhancer plus insertion of an additional genomic fragment may be tested for the possible silencer effect of the fragment. It should be borne in mind that the effect of a repressive element may depend on the nature of the enhancer. Therefore, one should be alert to the choice of the enhancer employed in the assay of repressive elements.

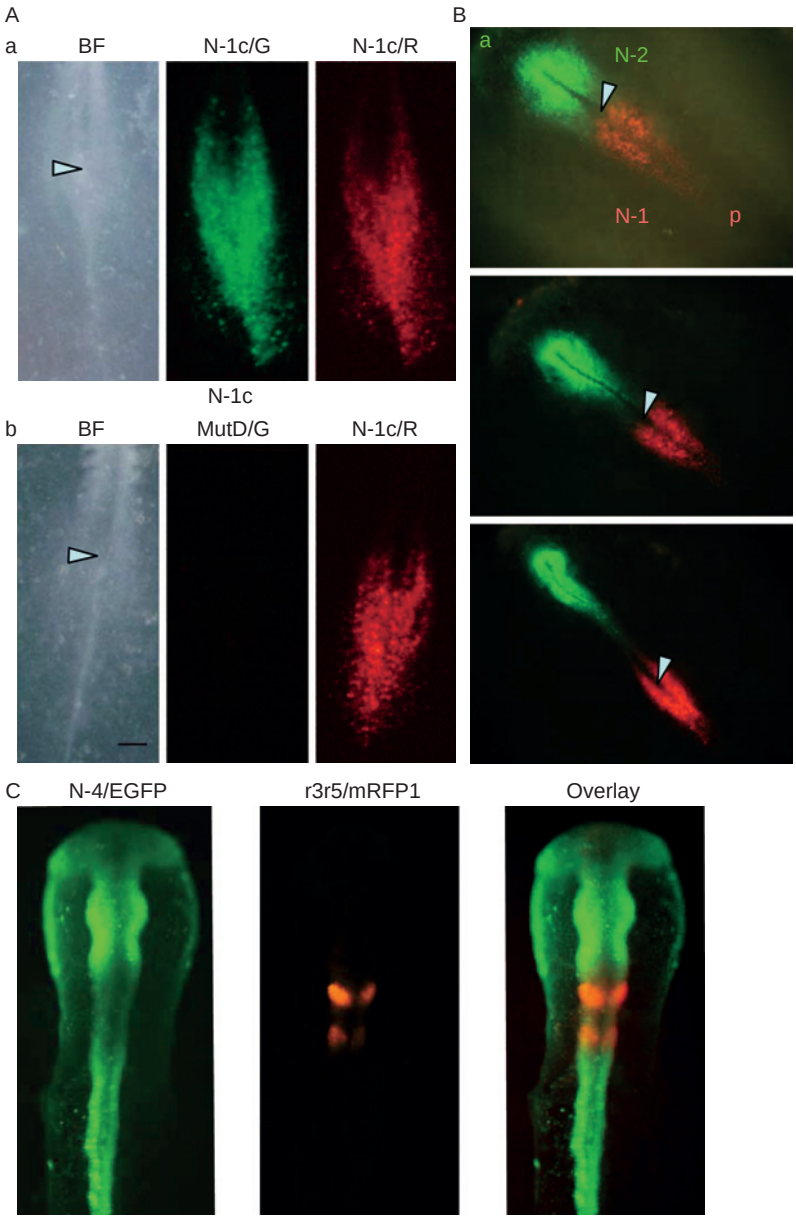

Fig. 4 Various applications of the use of coelectroporated ptk-EGFP and ptk-mRFP1. (A) Assessment of the effect of a mutation introduced in the enhancer sequence. Both embryos are electroporated with ptk-mRFP1 activated by the wild-type enhancer N-1 core trimer (N-1c/R), serving as an internal control, and ptk-EGFP activated by the same wild-type N-1 core trimer (N-1c/G) (a) and by a mutation in Block D of the N-1 core sequence (N-1cMutD/G), demonstrating a deleterious effect of the mutation on the enhancer activity (Takemoto *et al.*, 2006). The tissue areas surrounding the node [arrowheads in

It is often observed that deletion of a DNA sequence from the genomic fragment under investigation causes some increase of the enhancer activity. This does not necessarily indicate that the deleted region contains a repressive element, especially when the increase of the enhancer activity is modest. Altered spacing between activating elements, creation of a transcription factor binding site as a consequence of deletion of a sequence, or other causes may alter the enhancer strength. Without functional assessment as described earlier, a repressive regulatory region cannot be defined.

B. Resolving Complex Regulations into Elements

The transient assay systems employing embryo electroporation and embryonic cell transfection described in this chapter are limited by the following two conditions: (1) The effects of the regulatory regions are assessed while the reporter vectors stay as extrachromosomal copies in a nucleus. Therefore, only classical enhancers and silencers will be detected, without much reflecting the regulation that is highly dependent on the chromatin structure. (2) Efficient electroporation of a DNA into embryonic tissues has a size limit around 10 kb, with an allowance of a maximum of 6 kb to be inserted in ptk-EGFP/ptk-mRFP1. The assay system is thus very efficient for the analysis of individual enhancers/silencers, but not necessarily suitable for the analysis of integrated regulations of a genomic locus.

Perhaps the best way of totally clarifying the developmental regulation of a gene is the combination of the transient assays using chicken embryos as described in this chapter, and the BAC-based transgenic mouse embryos (Heintz, 2000), the former facilitating detailed analyses of individual enhancers/silencers, and the latter allowing assessment of the contribution of each element in the overall regulation in the locus.

Suppose there is a locus with clustered genes whose regulatory regions are intermingled in such a way that an enhancer of one gene is located in a position separated from that gene by a few different genes. The enhancer/silencer activity of individual regulatory regions can be evaluated using the high-throughput assay, using chicken embryos, regardless of which gene is dependent on the particular regulatory region. This analysis may be followed by the study of BAC-based transgenic mouse embryos in which one of the regulatory regions is altered. This

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the bright field (BF) panel] are indicated with a scale bar of 200 μm. (B) The temporal change of the spatial relationships of the activities of enhancers N-1 and N-2 in the same developing embryos labeled by the expression of tk-mRFP1 and tk-EGFP, respectively. The panels indicate 4, 6, and 9 hours (from top) after electroporation. The activity of enhancer N-1 occurs posterior to Hensen's node (arrowhead) which also moves posteriorly (p), while that of enhancer N-2 stays at the anterior end (a) of the neural plate. (C) Defining the territory of the enhancer activity. Activity of the enhancer N-4 in stage 11 embryo is marked by tk-EGFP expression, while rhombomeres 3 and 5 are labeled by tk-mRFP1 activated by a rhombomere *EphA4* enhancer (M. Inoue, unpublished data), indicating that the activity of enhancer N-4 is low in the region of the neural tube corresponding to rhombomeres 2–5. (See Plate no. 22 in the Color Plate Section.)

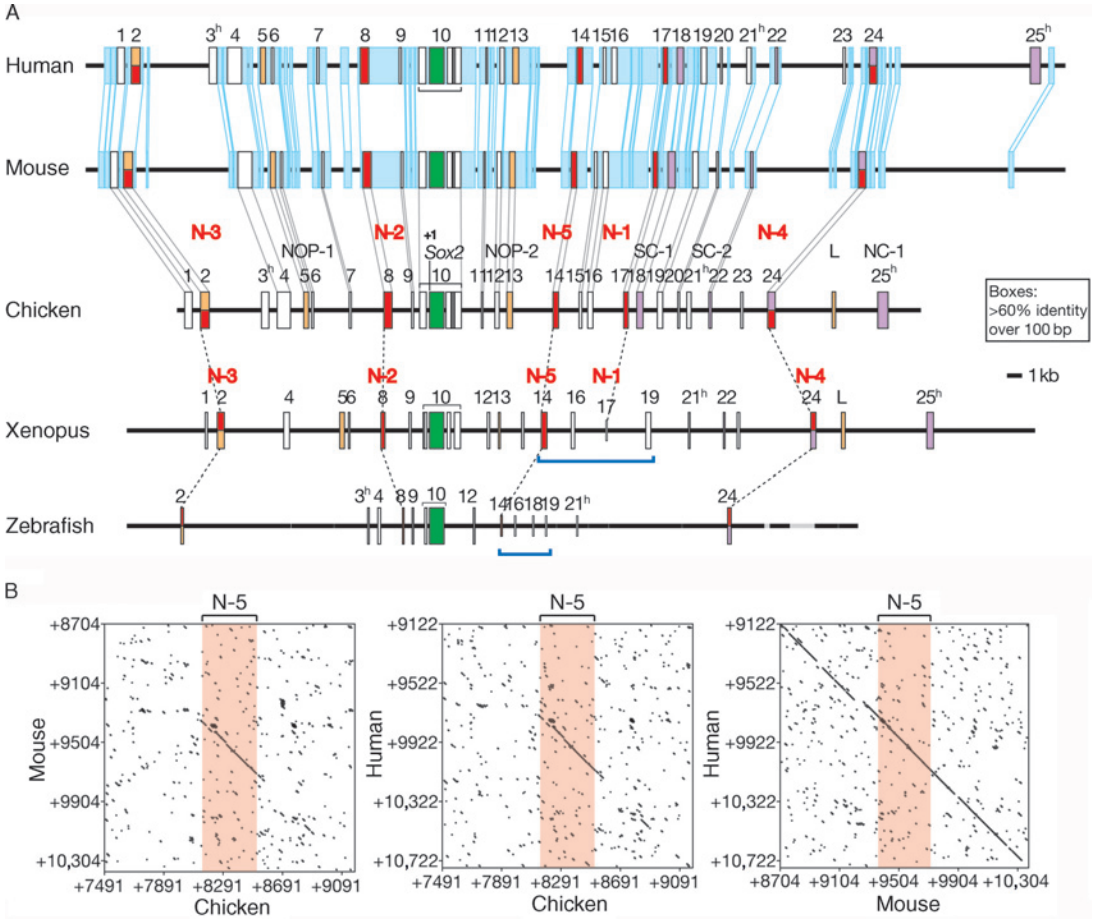

Fig. 5 Correspondence of the functionally defined enhancers with the conserved sequence blocks shared by the chicken, mouse, and human *Sox2* locus sequences. (A) A summary of conserved sequence blocks nos. 1–25 of the *Sox2* locus, which are indicated by the boxes. On the top, the enhancers corresponding to these blocks are indicated. The box numbers with the superfix 'h' indicate those sequence blocks that conserved between chicken and human, but are missing in the mouse sequence. Reprinted from Uchikawa, M., Takemoto, T., Kamachi, Y., and Kondoh, H. (2004). Efficient identification of regulatory sequences in the chicken genome by a powerful combination of embryo electroporation and genome comparison. Mechanisms of Development 121, 1145–1158. (B) Dot matrix analysis comparing the DNA sequences of the three animal species of the region encompassing conserved sequence block no. 14, corresponding to enhancer N-5. A dot indicates a 10-bp sequence with 60% matching. Between the chicken and mammalian sequences, only the enhancer sequence is significantly conserved, whereas between human and mouse, the enhancer sequence is buried in a wider region of high sequence conservation. Reprinted from Uchikawa, M., Ishida, Y., Takemoto, T., Kamachi, Y., and Kondoh, H. (2003). Functional analysis of chicken Sox2 enhancers highlights an array of diversity regulatory elements that are conserved in mammals. Developmental Cell 4, 509–519. (See Plate no. 23 in the Color Plate Section.)

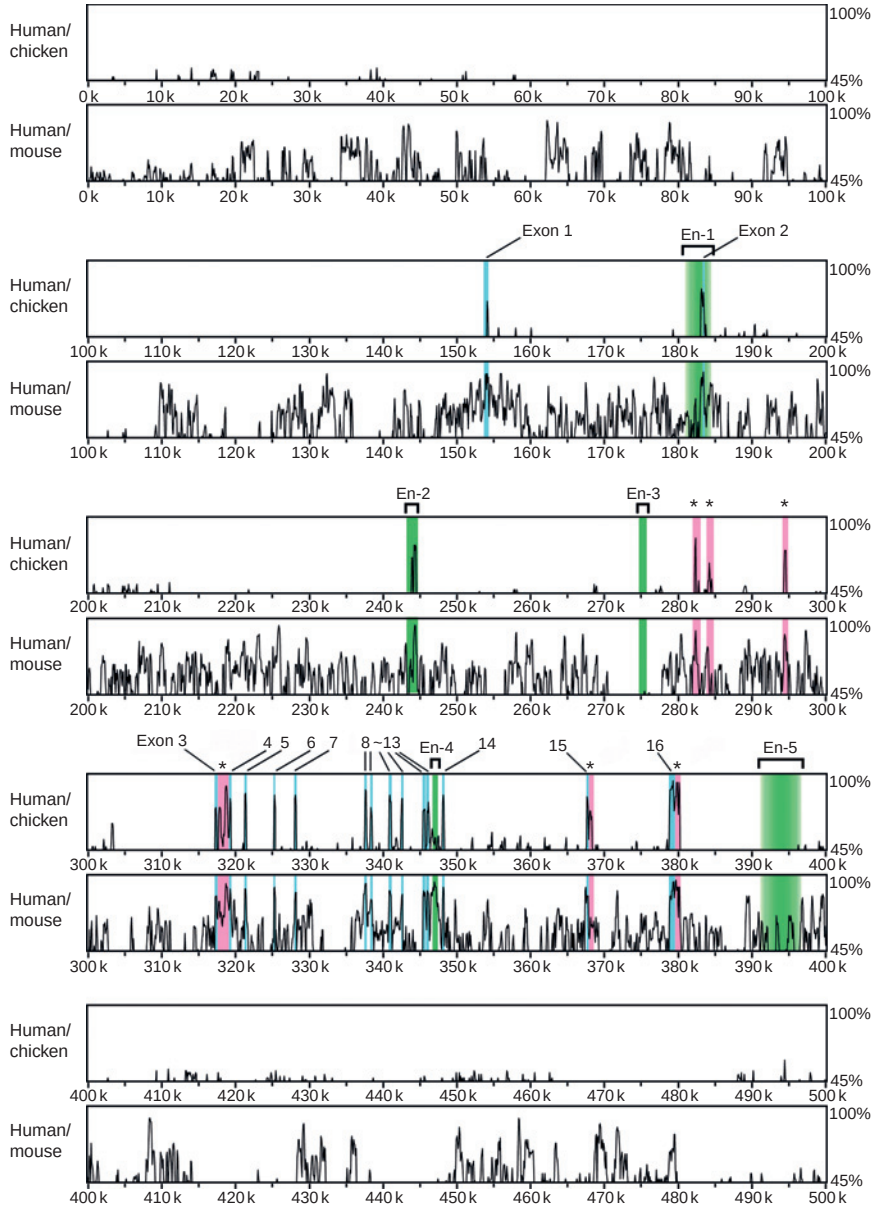

Fig. 6 Analysis of sequences conserved between chicken and mammalian *N-cadherin* genes using the VISTA program. Upper rows: Comparison of chicken versus human *N-cadherin* sequence, evaluating every 50-bp alignment and plotting regions with greater than 45% sequence identity. The abscissa represents human sequence coordinates. Exons are numbered and indicated by blue stripes, five enhancers identified in this study are indicated by ''En'' and green stripes, and other highly conserved regions found between the human and chicken sequences by asterisks and pink stripes. Lower rows,

will identify the gene under direct control of the regulatory region, and indicate the significance of the regulatory region in the overall regulation of the genes. These combined approaches will establish the functional relationships between the regulatory regions and the genes clustered in a genomic locus. Also, if regulatory regions are found that are effective in transgenic mouse embryos but not in electroporated chicken embryos, these regions would be strong candidates for those involved in regulation dependent on the chromatin structure. Of course, the highly conserved regulatory mechanisms between the chicken and mouse embryos underlie this strategy.

C. Considerations Regarding Phylogenetically Conserved Sequences

One widely employed strategy is first to find phylogenetically conserved noncoding sequences and then to investigate regulatory functions associated with the sequences (Woolfe *et al.*, 2005). Indeed, a substantial fraction of the conserved sequence blocks have demonstrable enhancer activities (Fig. 5). However, not all the regulatory regions are identified as those conserved as sequence blocks across many phyla. In addition, the degree of the conservation of the regulatory regions varies considerably depending on the genes of interest. Inspecting the published data of various cases, we find that the regulatory regions tend to be more highly conserved for genes with regulatory functions (e.g., transcription factor genes) than for those with structural functions (cytoskeletal, cell adhesion, etc.) (Figs. 5 and 6).

We can consider a few different mechanisms that might account for the regulatory regions not being conserved among species. (1) The genomes of higher vertebrates have gone through multiple rounds of duplications. Functional allotment among the genes derived from the same ancestral gene may vary depending on the branch of phylogenetic diversification. For instance, enhancer N-1, a major enhancer regulating the *Sox2* gene in the chicken and mouse (Fig. 4), is only partially conserved in the *Xenopus Sox2* locus, and absent from the zebrafish genomes, despite strong conservation of other enhancers. This is because the enhancer N-1-dependent function of *Sox2* in the chicken and mouse embryos is overlapped by the function of *SoxD* in *Xenopus*, and replaced by the function of *Sox19* in zebrafish (Okuda *et al.*, 2006). (2) Details of the gene expression patterns

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comparison of mouse and human *N-cadherin* genomic sequences based on the same criteria. Conservation of the Enhancer 4 sequence between human and chicken is less pronounced compared with that of Enhancers 1, 2, or 5, but the significance of the conservation is indicated by the remarkably high score of this region between human and mouse. Enhancers 3 and 5 sequences determined in the chicken genome do not have corresponding sequences in mammalian genomes and are positioned roughly in a way that reflects their coordinates in the chicken sequence. Note three asterisk-marked (pink-shaded) prominent peaks in intron 2, which may correspond to mesodermal enhancers or sensory/neural enhancers active in later development. Reprinted from Matsumata, M., Uchikawa, M., Kamachi, Y., and Kondoh, H. (2005). Multiple N-cadherin enhancers identified by systematic functional screening indicate its Group B1 SOX-dependent regulation in neural and placodal development. Developmental Biology 286, 601–607. (See Plate no. 24 in the Color Plate Section.)

may vary even between related animal species. For instance, regulation of a gene may not be identical between the species to its details, resulting in a difference in the regulatory regions responsible for the gene expression in these domains. In chicken lens development, the *Sox2* gene is expressed in both lens epithelium and lens fibers, being regulated by the enhancers N-3 and L, respectively (Uchikawa *et al*., 2003). However, in the mouse embryonic lenses, *Sox2* is expressed only in the lens epithelium (Kamachi *et al*., 1998), reflecting the lack of the enhancer L sequence in the mouse *Sox2* locus. (3) We further speculate that there are cases in which regulatory regions are conserved not as a long sequence but rather as a shuffled version of the regulatory motifs to attain very similar regulations. This situation is indeed observed frequently between the genomes of ascidians *Ciona intestinalis* and *Halocynthia roretzi* (Oda-Ishii *et al*., 2005).

Thus, the information about the conserved noncoding sequences should be utilized with some reservations. For a thorough understanding of the regulation of a gene during embryonic development, unbiased approaches must be combined with those relying on predictions based on various criteria.

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CHAPTER 18

Computational Approaches to Finding and Analyzing *cis*-Regulatory Elements

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Abstract
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II. Effective *cis*-Regulatory Sequence Analysis
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Abstract

The transcription of almost all developmental genes is driven by tissue- and time-specific regulatory elements. These transcriptional regulatory elements lie in the genomic DNA proximal to the gene, and hence are *cis*-regulatory (as opposed

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to *trans*-regulatory elements like transcription factor genes). Over the past three decades, a number of techniques have been applied to the problem of finding and characterizing these regulatory elements. In this chapter, I discuss some computational approaches that have been particularly useful in identifying developmental *cis*-regulatory regions, and provide a tutorial on how to apply these approaches to the study of chick development.

I. Structure and Function of *cis*-Regulatory Elements

Over the last two decades, a consistent view of the structure of *cis*-regulatory elements has emerged from work in *Drosophila*, sea urchins, and mouse (Davidson, 2006). *cis*-Regulatory apparatus can be spread over hundreds of kilobases of genomic DNA, and even several megabases; this apparatus largely consists of a number of relatively small (300 bp–3 kb) modules of regulatory DNA. Each module contributes to the tissue-specific expression of at least one gene, regulating it either positively or negatively. Modules are often capable of acting independently: that is, an individual module can direct or repress expression of a reporter construct independently of the presence of other regulatory modules. Thus the most common method of testing the *cis*-regulatory function of a region of DNA is to link it to a reporter gene encoding either β-galactosidase (LacZ) or a fluorescent protein, make a transgenic animal or line containing the reporter construct, and then look for reporter activity *in vivo*.

While relatively few regulatory modules have been analyzed in exhaustive detail, those that have been analyzed yield a consistent picture of their internal structure (Davidson, 2006). Each regulatory module contains several – from two to dozens – binding sites for transcription factors. Some of these binding sites bind tissue-specific positive or negative regulators that respond, in turn, to their own regulatory apparatus. Others bind ubiquitous transcription factors that modify DNA structure or link regulatory elements to the basal transcription apparatus.

The ultimate goal of *cis*-regulatory analysis is to understand precisely how the transcription of a particular gene is controlled by upstream factors, and to place this understanding in the larger context of development and disease. There are two subsidiary questions. First, what are the *cis*-regulatory modules controlling the expression pattern of a particular gene? And second, what transcription factors bind within those *cis*-regulatory modules? Once answers are known to these questions, there are many ways to study how the different transcription factors combine to control gene expression.

These two questions are, however, difficult to answer experimentally. Unlike protein-coding genes and small RNAs, there is no systematic whole-genome method for identifying *cis*-regulatory elements, and genome-scale binding site identification is difficult to do *in vivo*. Because vertebrate genomes are so large, with well over 100 kb of genomic DNA surrounding many genes, it is not easy to identify even proximal regulatory elements for a particular gene. Moreover, once located, analyzing

regulatory elements for binding sites is not straightforward: biochemical analysis cannot usually be done *in vivo*, and extracting proteins from small, localized domains of tissue is challenging. Thus the opportunity for computational aid is significant.

Computationally, *cis*-regulatory elements are also difficult to identify. Unlike protein-coding genes, which are transcribed and must make a valid protein sequence, *cis*-regulatory elements have no obvious properties that let us identify them easily in single genomes. And, while we know a number of binding sites, transcription factors bind to quite variable sequences, leading to an immensely high false positive rate that has been estimated at 99.9% when current search techniques are applied to whole-genome assemblies (Wasserman and Sandelin, 2004). Even when we can reliably identify individual binding sites, we may not be able to identify the entire *cis*-regulatory element necessary for proper expression.

The advent of multiple whole-genome sequences across a range of evolutionary distances has led to a revolution in the identification of *cis*-regulatory elements. This is because *cis*-regulatory sequences, like protein-coding genes and other functional elements in the genome, are evolutionarily constrained and can be identified by comparative sequence analysis. At the same time, large-scale techniques including chromatin immunoprecipitation followed by either an array assay ("ChIP-chip") or massively parallel sequencing ("ChIP-seq") have begun to provide genome-wide sets of binding sites, empowering binding site search (Hudson and Snyder, 2006; Johnson *et al.*, 2007).

II. Effective *cis*-Regulatory Sequence Analysis

I discuss computational approaches to solving three different types of *cis*-regulatory problems below: first, the problem of finding putative *cis*-regulatory elements within genomic DNA by using comparative sequence analysis to look for evolutionarily conserved noncoding DNA; second, the problem of identifying binding sites for known transcription factors within regulatory elements; and third, the problem of locating novel binding sites in coregulated DNA. Computational sequence analysis techniques have been applied to all of these questions, and the effectiveness of the solutions is in the order given above: comparative sequence analysis has been effective at finding regulatory elements, and when known transcription factor binding sites are sought within relatively short sequences, this approach too has been valuable. Computational discovery of binding sites within several coregulated sequences has been least effective, both because the computational problem is quite difficult and because the biological information is rarely sufficient to narrow down the search sequence or provide enough coregulated sequence elements.

The approach that I recommend is as follows: locate candidate elements with comparative sequence analysis; test the functional relevance of these candidate elements *in vivo*; scan functional sequences with a database of transcription factor

binding sites; and finally, test these sites for function by site-directed mutagenesis and perturbation analysis of upstream genes. This approach uses computation to restrict the number of experiments, while providing quick feedback as to functional significance of sequence elements; it has been successfully used in several laboratories to study many regulatory elements.

A. Selecting Candidate Genomic Regions in Chick

Many regulatory elements lie within the genomic neighborhood of the gene they regulate, although there are notable exceptions. While there are no rules that will work for every gene, generally the place to start looking for regulatory elements is within the region bounded by neighboring genes (including both upstream and downstream sequence, as well as intronic sequence). Here, I show how to extract the chicken *Sox2* genomic region from the UCSC genome browser and determine the appropriate region for investigation.

1. Finding the *Sox2* Genomic Region in Chick

First, go to the UCSC genome website at http://genome.ucsc.edu/.

Select "Genomes" on the upper left to get to the Genome Browser Gateway.

In the search form at the top of the page, choose "Chicken" in the "Genome" pull-down menu. Enter "Sox2" into the box labeled "position or search term". Click submit (see Fig. 1).

You will now see a list of matches to "Sox2" in the chicken genome annotations. The next task is to choose one of these matches. In the case of *Sox2*, this is easy: *Sox2* is in a well-assembled region of the chicken genome and there is a RefSeq gene match to a location on a specific chromosome (chromosome 9, positions 17990091–17991429). RefSeq matches (and UCSC Gene matches) are the highest quality annotation and should be used when present.

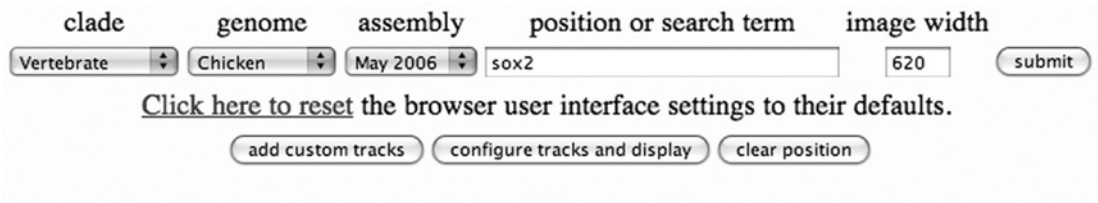


Fig. 1 Searching for chick *Sox2* using the UCSC Genome Browser Gateway.

There are two other common types of search results. The first is a match to an unmapped portion of the genome; for example, if you search for "Dlx3," a member of the *distal-less* gene family, you will find that it is currently (as of the May 2006 assembly) mapped to "chrUn random," which is shorthand for "unmapped region." If your gene of interest is in an unmapped region, you may find that the assembly is imperfect or incomplete; such regions should be treated with caution. (One good tactic for resolving such issues is to compare the region to another, better-assembled genome, such as the mouse genome; if you find discrepancies in exon organization or the position of neighboring genes, then you may be looking at a misassembly.)

Another kind of search result is one in which you simply fail to find a chick RefSeq gene match. For example, a search for "Dlx2" in the May 2006 assembly finds four "Non-Chicken RefSeq genes," corresponding to *Dlx2* TBLASTN matches on chromosome 2. If you examine these matches, you will find that they match to Xenopus *Dlx2*, two zebrafish *Dlx2* paralogs, and a Medaka *Dlx2*. More importantly, the chick gene they match is actually *Dlx5*, not *Dlx2* – *Dlx2* is not present in the May 2006 chick genome assembly! In this case, you are stuck: there is no general way to determine the chick genomic sequence for your gene, although you might be able to use other genomic sequences like mouse or human for comparison or motif search.

Now, select the *Sox2* RefSeq gene match on chromosome 9.

This next Web page shows the *Sox2* RefSeq gene mapped onto the genome; the initial view shows only the *Sox2* RefSeq sequence. In this case, *Sox2* is mapped on the bottom strand (see the "<<" arrows) and only has one exon (see Fig. 2).

Next, zoom out until you can see all of the genomic sequence surrounding *Sox2* up to the neighboring genes; use the 'move' buttons to shift the view so that you include as few neighboring genes as possible. For *Sox2*, this view should include about 600 kb of genomic DNA (see Fig. 3). (For most genes, this should be less than 250 kb.)

This is the region that you want to use for conservation and motif analyses. The figures below use the region **chr9:17,600,308–18,251,061**.

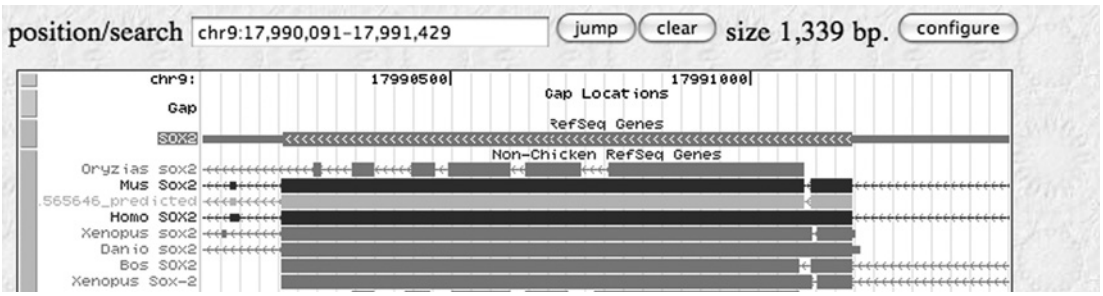


Fig. 2 Chick *Sox2* default view.

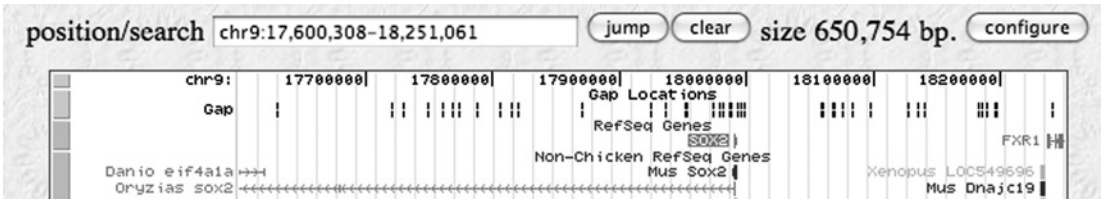


Fig. 3 Chick *Sox2* view, including nearby genes.

2. Saving *Sox2* Genomic DNA into a FASTA File

Most programs do not interact directly with the UCSC browser, and require that you provide DNA sequence in the FASTA format. To extract the genomic region into a FASTA file, go to the UCSC genome view that includes precisely the region you want to analyze, and select "DNA" on the top toolbar. There are several sequence formatting options. The two most important are "Mask repeats," which should be selected (be sure to also select "to N"), and "reverse complement," which (because *Sox2* is on the bottom strand) should be selected in this case. Now select "get DNA."

The next Web page will be a very large text-only screen containing a header (starting with "> galGal3") and all of the genomic sequence you requested.

Use "Save As..." to save this into a text file, or copy and paste it into a word processor such Notepad, TextEdit, or Microsoft Word. Be sure to save the DNA file as a text file (with a ".txt" ending, in plain, text-only format); the extra formatting that is usually added to word processor files will confuse most bioinformatics programs.

At this point, you are ready to import this DNA into a variety of analysis systems (several of which are described below).

III. Approaches and Tools for Finding Conserved Sequence Elements

Conserved noncoding regions are excellent candidates for *cis*-regulatory elements. The most important consideration in looking for conserved noncoding regions seems to be the choice of companion species: there are now over a dozen vertebrate genomes available in draft form, ranging from teleosts to primates. A number of studies have shown that regulatory elements can be conserved between species as distant as primates and teleosts, but there are some indications that the mammalian genomes are the optimal partners for comparison with the

chicken genome. For example, a systematic experimental analysis of the *Sox2* regulatory regions in chick showed that 10 distinct chick regulatory regions were conserved between human, mouse, and chick (Uchikawa *et al.*, 2004). A study of the *SCL* gene region found that five of eight known mouse enhancers were present in the chick *SCL* region, while only two were found in *Fugu rupribes*, a teleost, and none were found in zebrafish (Gottgens *et al.*, 2002). Systematic analysis of *Gdf6* regulation in mouse demonstrated that many of the known regulatory regions were also conserved between mouse and chick, but fewer were present in zebrafish (Portnoy *et al.*, 2005). Thus it seems that while comparisons with zebrafish sequence can find some regulatory regions in amniotes, there are amniote-specific regulatory elements that can only be found by comparisons between chick and mammals.

A. Comparing Sequences

There are three kinds of computational approaches used in comparative sequence analysis: global alignments, local alignments, and dotplots. Global alignments (like those computed by AVID and LAGAN) seek to generate an alignment of two entire sequences, and either do not detect or else minimize rearrangements in the two sequences. With a global alignment, each base on one sequence is aligned to a base or a gap in the other sequence.

Local alignment programs (like NCBI BLAST and blastz) compute all stretches of local similarity between two sequences. Each position in one sequence may be aligned to zero, one, or many other positions in the other sequence. Local alignments can detect evolutionary duplications, shuffled pieces of DNA, or regions that have been inverted in orientation. Local alignment programs usually use simple heuristics to "seed" alignments around points of high local similarity, following which the alignment is expanded subject to a significance threshold; this results in marked speedups, which is why BLAST-style algorithms can be used for whole-genome comparisons.

The third kind of comparison is the dotplot comparison, which uses a very simple algorithm to compare all fixed-width (ungapped) windows of DNA on one sequence with all fixed-width windows of DNA on the other sequence. Because dotplots do an exhaustive comparison, they tend to be much slower but potentially more sensitive than the other kinds of comparisons.

Later, I will show how to establish and visually compare all three types of comparisons. Although there has been no systematic analysis of the effectiveness of each kind of comparison, where they vary in utility may be at the extremes of evolutionary distance: global alignments are unlikely to work well at large evolutionary distances, where sequences may be very dissimilar. At small evolutionary distances, regions are unlikely to have been shuffled or inverted, so a global alignment may more specifically outline conserved sequences. Regardless, all three types

of comparisons have been successfully used to find conserved noncoding regions, and in general it seems that all three types of comparisons identify the same regions of DNA.

B. Tools for Finding Conserved Regions in Genomic DNA

There are a number of tools that can be used for finding conserved regions in genomic DNA, and essentially all of them work well on vertebrate sequence. Two of the most popular are the VISTA browser (Frazer *et al.*, 2004) and PipMaker (Schwartz *et al.*, 2000). One that we have used successfully to study chick elements is the Cartwheel/FamilyRelationsII system, which lets users run several different kinds of analyses and display them simultaneously (Brown *et al.*, 2005).

Later, I will show how to use the UCSC genome browser to survey conservation, and then give a detailed guide to establishing your own comparative analysis in Cartwheel and then viewing it in FamilyRelationsII.

The *Sox2* region has been well studied experimentally, so we will use it for our examples (Inoue *et al.*, 2007; Takemoto *et al.*, 2006; Uchikawa *et al.*, 2003). Later I will show you how to extract homologous regions to *Sox2* from the mouse genome for use in a pairwise comparison.

1. Using the UCSC Genome Browser to Survey Conservation near to *Sox2*

The UCSC Genome Browser is an excellent place to start looking at conservation. By default, the genome browser shows conservation scores calculated using the MULTIZ program (Blanchette *et al.*, 2004). See Fig. 4 for a view of conservation between the chick *Sox2* locus and several other vertebrates; the blue peaks represent the overall strength of conservation, while the individual lines below represent matches in the human, mouse, rat, opossum, frog, and zebrafish genomes.

The UCSC Genome Browser conservation track is useful for an overall view, and it will show you whether or not there is any conservation at all in the area. However, it is not possible to do your own searches or change conservation thresholds within the UCSC Genome browser. Later I will show you how to use the Cartwheel server and FamilyRelationsII software to display several different conservation and similarity calculations, adjust parameters, and extract DNA for further work.

2. Finding the *Sox2* Genomic Region in Mouse and Extracting DNA

To extract the *Sox2* genomic region from mouse, repeat the process for finding the *Sox2* genomic region in chick but select the mouse genome instead. (Be sure to reverse complement the mouse genomic sequence as well, since the *Sox2* gene is on the bottom strand in the mouse genome view.) Save the mouse genomic region into a different FASTA file.

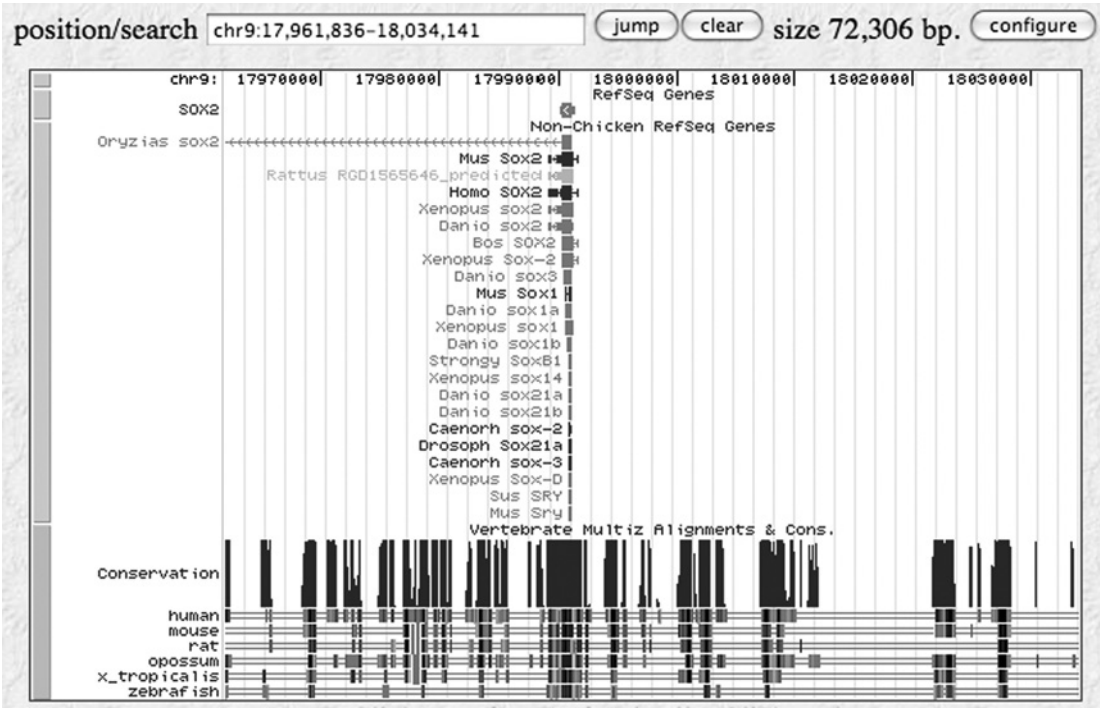


Fig. 4 Sequence conservation near chick *Sox2* in the UCSC Genome Browser.

3. Comparing Chick *Sox2* to Mouse *Sox2* with Cartwheel

The Cartwheel server is a website that lets users upload sequences and build custom annotations and comparisons of the uploaded sequences (Brown *et al.*, 2005). Unlike the UCSC Genome Browser, Cartwheel is targeted at custom analyses of relatively small sequences; it was originally developed to compare BAC-sized genomic sequences from two sea urchins (Brown *et al.*, 2002). An introductory tutorial is available at http://family.caltech.edu/tutorial/.

To make use of Cartwheel, first go to the Cartwheel website, http://woodward.caltech.edu/canal/. Create an account (using "add account") and choose a lab space (the "Public Space for Testing" lab is a good default, if your lab does not yet have its own space).

Now log into your account, enter a folder name describing your project, and click on the "create a new subfolder" button at the bottom of the page. The folder type to create should be left at the default, "analysis folder". Now click on the new folder name.

You will now be in your new folder, which is your personal work space. Next, we need to upload the sequences. Click on "manage sequences," and then select

"upload a FASTA file." Do this for both the mouse and chick sequences you saved from above.

Once you are back at the sequence list, you should rename the sequences to something shorter than the default names, e.g. "chick sox2 genomic" and "mouse sox2 genomic."

Next, we will create a custom view of these sequences containing some comparisons. To do this, return to the folder view, and click on "create an analysis group." Name it "chick/mouse sox2," select "Pairwise analysis of two sequences," and then select "create analysis group," On the next screen, set the top sequence to "chick sox2 genomic" and the bottom sequence to "mouse sox2 genomic." Select "set sequences."

Next, create several pairwise comparisons. We will add two to start with: first, a global alignment with a VISTA-like comparison, and second, a local alignment with a BLAST algorithm.

To do this, select "add pairwise comparison." The next page offers a menu of choices; let us start by running a VISTA comparison with LAGAN. Select this menu option, and then select "create analysis." Set the analysis name to "LAGAN analysis" and select "continue." The next screen offers some options: the window-size specifies how big a window to use for the comparison score (see "Global alignments", above), and the "minimum percent" sets a threshold for the similarity, below which features will not be shown. The default window size (100) and percent similarity (50) are both good places to start. The third option, orientation, specifies whether the second sequence should be compared in forward orientation or reverse complement; this depends on whether or not the *Sox2* genes in the sequences extracted from UCSC are on the same strand in both sequences. (If you followed the directions above, both genes should be on the top strand.) By default, Cartwheel assumes that the sequences should be compared in the same orientation.

Now repeat the process, but add a "PipMaker-style blastz comparison" instead. There are no parameters set for this kind of comparison.

These comparisons are now entered into a processing queue, and we need to wait for them to run. In general, neither type of comparison is very time-consuming, so this should take only a moment or two; reload the browser window a few times until "Job Status" changes to "completed."

The next step is to view the analyses in the FamilyRelationsII viewer.

4. Viewing the Comparisons in FamilyRelationsII

To view the comparisons, you need to download the FamilyRelationsII ("FRII") desktop viewer. This (freely available) viewer loads analyses from Cartwheel and supports zooming, threshold changing, and copy-and-pasting of DNA sequences.

To download the FRII viewer, go to http://family.caltech.edu/ and select the "download" link. There are versions available for both Windows and Mac OS X. Unpack it and start the FRII application.

Now enter your Cartwheel username and password, and go find the analysis folder you created above. Double click on the name of the analysis.

After a moment while the analysis loads, you will see a dotplot-style view of the two sequences, with a series of blue dots marking points of strong similarity between the two sequences (Fig. 5A). Where the two sequences absolutely identical, there would be a blue line of dots running diagonally from the upper left corner to the lower right corner; however, because conservation is patchy between the chick and mouse *Sox2* loci, you will see regions of high similarity interspersed with regions of no similarity. By clicking on "Pair View," you can also switch to an alignment-style view where lines are drawn between similar points on the two sequences (Fig. 5B).

At this point there are a number of things to try.

First, uncheck the "show comparison" box on the right side, in the (blue) "blastz" parameter window. This turns off display of the blastz comparison, and shows only the LAGAN-VISTA matches (in red). These two comparisons overlap almost entirely, demonstrating that (in the case of *Sox2*) the global and local alignments show nearly identical points of similarity.

One can also adjust parameters specific to each analysis. In the case of both LAGAN-VISTA and blastz comparisons, there is only one parameter: threshold. The LAGAN-VISTA threshold is the number of base in a 100 base window that must be aligned to identical bases in order to be displayed.

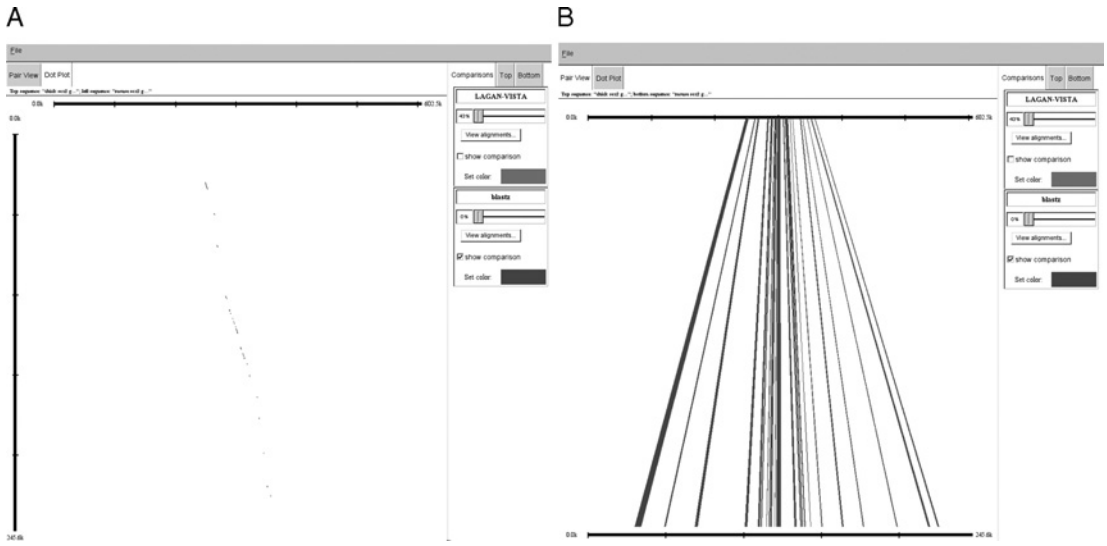


Fig. 5 A FamilyRelationsII view of blastz and LAGAN-VISTA comparisons between the chick (top) and mouse (left/bottom) *Sox2* sequences. The left figure is a dotplot-style view where similarities are plotted at the intersection of positions in the chick sequence (top) and the mouse sequence (left); the right figure is an alignment-style view where lines are drawn between similar points on the chick sequence (top) and the mouse sequence (bottom).

Next, select some small portion of the comparison and zoom. You can do this in two ways: either drag-select on the sequences, by pushing down the left mouse button and moving the mouse along the sequence, or drag-select a rectangle on the dotplot view. Now click in the blue boxes to zoom in a bit. Holding down the SHIFT key and clicking on the sequence will zoom the view back out.

You can zoom in to view the actual sequence. For blastz, select a region of interest, and click on ''view alignments.'' This will bring up a window showing a close-up view of the alignments, together with a window showing the actual nucleotide matches in black (exact match) and red (mismatch) (Fig. 6A).

You can also do a motif search; to bring up the motif search window, right-click or CTRL-click on the sequence of interest, and select motif search. A new window will open up with forms to search for up to five motifs (Fig. 6B). The motif entry form takes IUPAC-style fuzzy motifs like ''WGATAR,'' discussed in detail below. There is also a mismatch slider for each motif, with which you can select how many mismatches to allow for each motif match.

In order to extract sequences, simply select a region of DNA and click on it with the right mouse button (or CTRL-click on a computer with a single mouse button). A menu will pop up; select ''copy sequence,'' and the selected DNA sequence will be put in the system paste buffer, where it can be pasted into another program.

The purpose of the FamilyRelationsII view is to let users establish some comparisons, adjust parameters, and explore the resulting analyses in order to

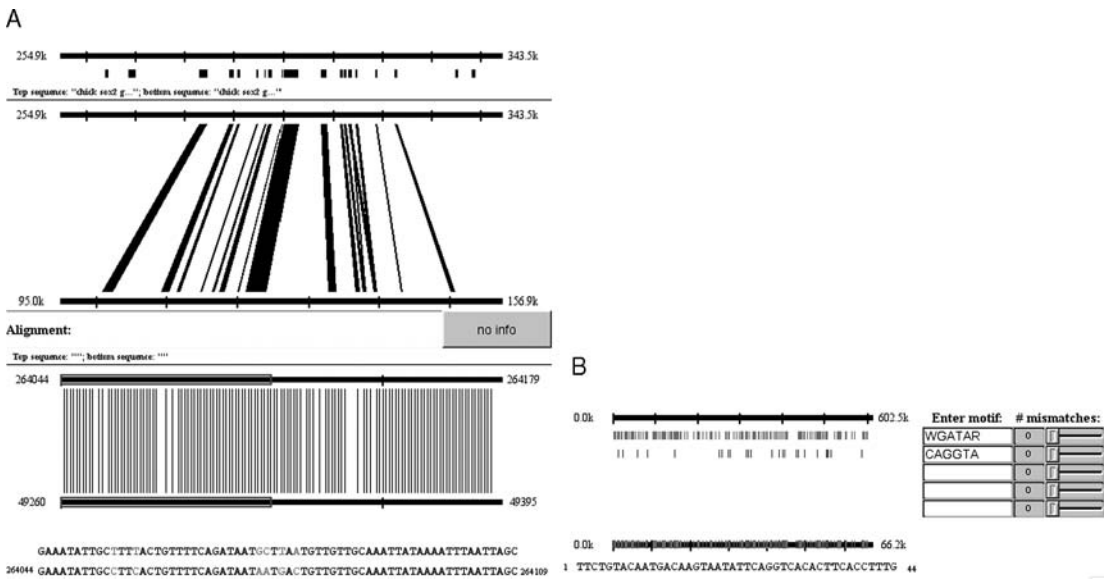


Fig. 6 An alignment close-up view (A) and a motif search window (B) in FamilyRelationsII.

determine which regions of genomic sequence are most likely to be interesting for an experimental test. LAGAN-VISTA and blastz both find similar regions of DNA, and they have effectively helped find regulatory elements in vertebrate comparisons; another program, paircomp, may be slightly more sensitive to trace similarities but can only be used on smaller pieces of DNA.

5. Doing more Sensitive Local Comparisons with Paircomp

The paircomp program does a very straightforward all-by-all dotplot comparison of two regions of DNA, and can be used on regions where each sequence is less than 250 kb of DNA (Brown *et al.*, 2005). Unlike LAGAN-VISTA and blastz, which first build gapped alignments, paircomp compares all fixed-width windows on one sequence to all fixed-width windows on the other sequence and reports those with similarity above a particular threshold. Paircomp was originally developed for sea urchin BAC comparisons, where we found that it worked well on draft unannotated sequence. While no systematic comparison has been done between paircomp, blastz, and LAGAN-VISTA, the paircomp program allows users to specify more stringent or more sensitive comparisons by changing the window size and threshold used. Thus it has been useful in mouse/human comparisons, with a high level of background similarity, as well as in comparisons of fast-evolving sea urchin DNA, with only some trace similarities (Adachi and Rothenberg, 2005; Ransick and Davidson, 2006).

To establish a paircomp comparison, go back to the pairwise analysis in the Cartwheel web server and select ''add pairwise comparison.'' Now select ''add paircomp analysis'' and set a name. The next page contains several parameters: window size and threshold, which control the size of the fixed-width window and the number of matching bases required for a match to be displayed, and also start/stop coordinates for the analysis on both sequences. To select the region proximal to the *Sox2* gene, set the start coordinate to '200,000' and the stop to '400,000' for the chick sequence. On the mouse sequence, choose 200kb centered on the *Sox2* coding region. The choice of window size and threshold can be set to individual taste; for smaller regions (50 kb by 50 kb), a 20 bp window with an 80% threshold is a good place to start.[1] Now submit the analysis to the job queue. Note that paircomp analyses may take a few minutes to run.

Once the analysis is finished, load the pairwise comparison group into Family RelationsII again. The paircomp analysis will be displayed in a third color (green, by default), and the threshold parameter can be adjusted upwards to make the analysis more stringent.

Figure 7 shows a paircomp analysis of the *Sox2* locus in chicken and mouse. Both the coding region (marked in red) and surrounding noncoding sequence show many matches at a threshold of 80% (40 of 50 bases match in each window);

[1] For more information on window size/threshold combinations, see the FamilyRelationsII tutorial at http://family.caltech.edu/tutorial/.

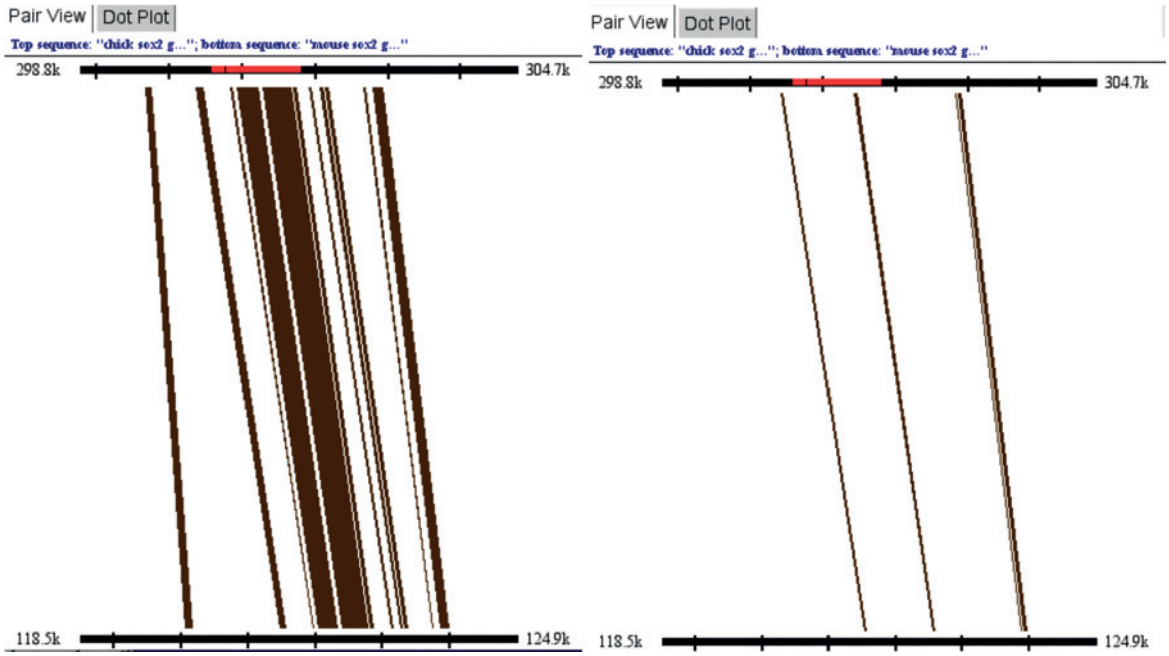


Fig. 7 A view of a 50 bp paircomp analysis comparing chicken (top) and mouse (bottom) DNA surrounding the *Sox2* gene (marked in red on the chicken sequence). (A) 80% threshold and (B) 90% threshold. (See Plate no. 25 in the Color Plate Section.)

by increasing the threshold to 90% (45 of 50 bases match), only three distinct conserved patches remain.

6. Choosing Sequences to Test

Whether you are using LAGAN-VISTA, blastz, or paircomp to do comparisons, the ultimate goal is to test regions for regulatory functionality. How should you choose the regions to test?

In general, regulatory elements seem to be co-linearly conserved that is, regulatory elements are rarely inverted or shuffled in order. This is not just because some programs are biased in favor of detecting co-linear features: while LAGAN-VISTA performs a global alignment first, thus restricting its similarity analysis to co-linear features, both blastz and paircomp are capable of finding points of similarity that have been inverted or shuffled. This conservation of order and orientation in regulatory elements seems to be a general feature of regulatory elements in deuterostomes, and is probably due to the nature of change in genomic

DNA (Cameron *et al.*, 2005). I recommend adjusting comparison thresholds until the only features remaining are co-linear, and then extracting and testing those features. (All of the features in the *Sox2* comparison in Fig. 5 are co-linear: none of the lines in the pairwise view cross each other.)

C. Beyond Pairwise Comparisons

This discussion has focused entirely on pairwise comparisons. Where there are more avian genomic sequences, multiway sequence comparisons might be useful for finding avian-specific fast-evolving sequences, as has been done within the mammals (Margulies *et al.*, 2003). Until the zebra finch genome is completed, pairwise comparisons between chick and a mammal may be the best way to find chick regulatory elements. This is because the most distant genomic sequence has a disproportionate impact on conservation scores (Pollard *et al.*, 2006).

D. "Lies, Damned Lies, and Statistics"

Before continuing, let us return to the question of what kind of comparison algorithm to use. Each kind of comparison – global alignments (LAGAN-VISTA), local alignments (blastz), and dotplot comparisons (paircomp) – implicitly contains assumptions about how sequence evolves, and uses these assumptions to calculate a significance or recommend a threshold. These assumptions are often hidden in the algorithm, and parameters are chosen based on how well a particular choice of parameters performs for specific alignment problems; whether or not the default parameters work well for your problem is unknown.

In this sense, the biggest advantage of dotplot algorithms is that they assume very little about the nature of sequence evolution, and they do not base significance calculations on the length of the match. This is one reason a dotplot comparison like paircomp can be more sensitive (although increased sensitivity can also lead to more false positives).

Thus our advice is to use LAGAN-VISTA and blastz to examine conservation on a large scale, and then do more sensitive, targeted analyses of sequence elements with dotplots.

E. Distal Regulatory Elements and Synteny

If you are interested in a gene that maintains long distance syntenic relationships, then you may need to look further afield for regulatory elements. For example, an enhancer for the *Shh* gene is located one megabase away from the *Shh* gene itself, and this may be the driving force behind the extremely conserved gene order across the 7q36 human locus containing *Shh* (Goode *et al.*, 2005). Long-range gene regulation may also be one cause of the highly conserved *Hox* gene complex (Lee *et al.*, 2006). Kikuta *et al.* (2007) speculate that maintenance of long-range relationships between regulatory elements and genes may be a general constraint on genomic rearrangements leading to synteny (Kikuta *et al.*, 2007).

F. When does Comparative Sequence Analysis not Work?

While comparative sequence analysis has been an effective way to find candidate regulatory elements, there are two scenarios in which it simply cannot work. The first is the obvious case in which the gene in the species of interest has a different expression pattern than the gene in the species being used for comparison. In this case, either the regulatory elements themselves or the spatiotemporal activity of the upstream regulators has diverged, and the regulatory elements may not be present in both species. This case can often be detected by looking at the expression pattern of the gene in the available species.

The other scenario in which comparative sequence analysis fails is when regulatory sequence for a particular gene has diverged even though the expression pattern driven by the two different sequences is similar. This is a form of neutral evolution, and it can be difficult to detect without exhaustive experimental *cis*-regulatory analysis or detailed knowledge of the important binding sites. While there are a number of examples of divergent regulatory sequence with similar regulatory function outside the vertebrates, we know of only one clear example in the vertebrates: the *SCL* gene locus in Fugu contains a regulatory element that shares no detectable sequence identity with the known regulatory elements in mouse, yet is capable of driving correct expression in *Fugu* (Barton *et al.*, 2001). There is no effective way of finding such elements computationally. Note that this may be one of reasons why McGaughey *et al.* (2008) found that comparative tools identified regulatory elements with relatively poor sensitivity across great evolutionary distances.

IV. Identifying Transcription Factor Binding Sites Computationally

Precisely locating individual transcription factor binding sites is distinct from the problem of locating functional *cis*-regulatory modules. While *cis*-regulatory modules are often capable of driving or repressing gene expression in reporter assays independently of any other regulatory element, binding sites rarely act alone; *cis*-regulatory modules often contain combinations of binding sites for several different transcription factors (Davidson, 2006). Thus *cis*-regulatory modules are larger than individual binding sites and are often amenable to detection by conservation analysis, perhaps because the strength, spacing, and orientation of the binding sites is constrained (Cameron *et al.*, 2005).

Individual transcription factor binding sites, however, are much harder to find and characterize. Even fairly sequence-specific transcription factors may recognize and bind to a variety of sites; these sites may be as long as 15–20 bases, but the invariant core sequence can be as few as 4 bases, e.g., for a GATA factor. This degeneracy in binding sites contributes to many false positives and complicates the *de novo* discovery of binding sites based solely on patterns in sequence.

Experimental techniques for finding binding sites do not always work well: electrophoretic mobility shift assay (EMSAs), or "gel shifts," require large amounts of starting protein, as do DNAse footprints; chromatin immunoprecipitation requires many input cells as well as a good antibody; and large-scale assay techniques are expensive and lack well-defined positive controls.

The degeneracy of known binding sites and the paucity of detailed biochemical models of specific transcription factor binding make large-scale sequence search for known binding sites a tricky business. Nonetheless, there are a variety of techniques that can be used to find potential binding sites for a specific transcription factor family, given some knowledge of existing binding sites. Discovering new binding sites from sequence data alone is much less reliable, and seems to be very dependent on the input data, search tools, and parameters used for the search.

Here I discuss useful techniques for performing a computational search with known binding sites, and also mention the problem of predicting new binding sites from sequence data.

A. Searching for Known Binding Sites

Suppose that we are interested in searching for binding sites for a particular transcription factor within a given sequence, and some known binding sites for that transcription factor have already been garnered from *in vitro* or *in vivo* assays. Were one to search for only the known binding sites, a large number of slightly divergent sites would be missed. Therefore the approach of choice in this case is to *generalize* from the existing set of binding sites.

There are two commonly used techniques for generalizing from a set of known binding sites. The first technique is a search with a fuzzy motif, which can be written in a variety of ways. For example, if one wanted to match either an A or a T, then G-A-T-A, and then either an A or a G, the search motif would be written as [AT]GATA[AG], or alternatively as WGATAR using IUPAC notation. The second technique is to use a matrix scoring system, variously known as a position-frequency matrix (PFM), position-weight matrix (PWM) or a position-specific scoring matrix (PSSM) (Wasserman and Sandelin, 2004). A matrix-based search allows for the definition of preferred or accepted nucleotides in each position. For example, this matrix finds [AT]GATA[AG] binding sites with equal weighting on an A or a T at the beginning, and an A or a G at the end:

	1	2	3	4	5	6
A	0.5	-	1.0	-	1.0	0.5
C	-	-	-	-	-	-
G	-	1.0	-	-	-	0.5
T	0.5	-	-	1.0	-	-

One can read this matrix from left to right, as allowing either an A or a T, then a G, A, T, A, and then either an A or a G. (For clarity, the "-" marks represent zeroes in the matrix.) If, however, you know that an A is preferred over a T in the first position, you can change the matrix accordingly:

	1	2	3	4	5	6
A	**0.8**	-	1.0	-	1.0	0.5
C	-	-	-	-	-	-
G	-	1.0	-	-	-	0.5
T	**0.2**	-	-	1.0	-	-

For the first position, this matrix weights an A higher than a T, which in turn is weighted higher than a C or a G. Note that the consensus binding site is read off simply by finding the nucleotide with the highest score in each column – AGATA [AG], for this matrix.

In order to construct a motif, we start with a set of known binding sites, gathered either from a high-throughput experiment like SELEX or from individually verified sites (Ellington and Szostak, 1990). These sites are subsequently aligned without gaps, using (for example) CLUSTALW with a high gap-opening penalty. Then, for each position in the alignment, which of the four nucleotides show up in that position is recorded. For example, given an alignment of three short motifs,

AGATAA
TGATAA
AGATAG
—****—

one can see that the core four bases are always "GATA," with either an A or a T in the first position and an A or a G in the last position. This yields a motif of [AT]GATA[AG].

Much the same procedure is used to construct a search matrix, except that frequency of each nucleotide is taken into account. For example, the alignment above would yield the following PFM:

	1	2	3	4	5	6
A	$\frac{2}{3}$	-	$\frac{3}{3}$	-	$\frac{3}{3}$	$\frac{2}{3}$
C	-	-	-	-	-	-
G	-	$\frac{3}{3}$	-	-	-	$\frac{1}{3}$
T	$\frac{1}{3}$	-	-	$\frac{3}{3}$	-	-

This weight matrix can then be used to score *any* subsequence of six bases as a potential match: for each position in the putative site, one uses the matrix to look up the score for the nucleotide at that position, and then sums the scores to obtain a total. This total is then used to rank the sites. For example, using this matrix to score the three sites used to construct the matrix, we get:

AGATAA	2/3 + 4 + 2/3	= 16/3
TGATAA	1/3 + 4 + 2/3	= 15/3
AGATAG	2/3 + 4 + 1/3	= 15/3

As would be expected from looking at the matrix, "AGATAA" is the highest-scoring site of the three. Also note that this matrix gives site TGATAG a score of 14/3, ranking it only one step below the three known sites but above all other sites – suggesting that TGATAG may also be a valid binding site for this transcription factor. This ability to generalize from a small set of known binding sites to a larger set of sites is the reason why people use both motifs and matrix-based approaches, as I mentioned earlier.

There are two more interesting features of matrices. The first is that the highest-scoring binding site calculated from matrices may not actually be in the list of sites used to calculate the matrix. For example, suppose we construct a PFM from the following four sites:

AGATAG
AGATAT
TGATAA
GGATAA
—****—

The highest-scoring motif under the resulting matrix will be "AGATAA," which is not in the list of input sites! This is because the matrix construction process counts the frequency of nucleotides in each position independently, so all that matters is that there are two 'A's in both the first and last columns. The other interesting feature of matrices is that the scores calculated from specific types of matrices – PWMs, which are calculated by taking the logarithm of each entry in a PFM – are, in theory, related to the actual biochemical binding strength of the site. Thus not only are high-scoring sites predicted to be more likely actual binding sites, but these high-scoring sites may be more strongly bound by the actual transcription factor. While this relies on a number of limiting assumptions – the primary being that each position in a site must contribute independently to the strength of binding – there are some indications that it holds true for some transcription factors (Stormo, 2000).

Matrices are a flexible way to search for binding sites, and they are at the foundation of most binding site search programs. Unfortunately, there are

essentially no tools that let biologists create matrix-based motifs from lists of known sites, although websites like CONSITE (discussed later) let you search with predefined matrices and there are several programs that support motif discovery (also discussed later).

So, which of the two search techniques – motifs or matrices – is better? The unfortunate answer is that the easier-to-use technique supported by many tools, searching with motifs, is considerably worse. This is because as the number of known binding sites increases, the resulting motif must become less specific in order to include all of the permutations of the individual bases, while position-weight matrices usually become more specific given more known motifs. Moreover, matrices rank candidate binding sites by putative strength or similarity to the known binding sites, unlike motifs which simply report matches. Motifs are therefore generally poor choices for transcription factor binding site search, because they lead to less specific searches and do not rank sites. Conversely, matrices are problematic because they require choosing a threshold below which you no longer consider a site to be relevant, and there are no generally agreed-upon techniques for choosing that threshold computationally. (Note that motifs are technically a special case of matrices: every motif search can be implemented as a matrix search, although not every matrix can be represented as a motif.)

Both matrices and motifs have several drawbacks as models of transcription factor binding. First, neither matrices nor motifs can represent synergistic interactions between positions in binding sites; were the above WGATAR binding site a strong binding site only when an A was present in both the first and last positions at the same time, neither search technique would be able to accurately rank the site. Second, neither matrices nor motifs can find sites that have insertions or deletions (''indels'') in them. And, of course, both matrices and motifs score *individual* sites, and not collections of sites.

With these drawbacks, it is worth asking why we should use either motifs or matrices! While there are more complicated binding site representations that can take into account synergistic interactions between positions in a binding site, gaps, or indels, and neighboring binding sites, we rarely have enough information about real binding sites to make use of these other representations. For example, to accurately calculate pairwise interactions between each pair of positions from a set of known sites, one needs several *hundred* known sites. Moreover, by moving to a more complicated binding site representation, one also must move to a more complicated statistical calculation that is likely to admit more false positives into the search results. Thus it seems that matrices, as imperfect as they are, represent a good starting point for motif searches.

B. Practical Motif Searching

The primary practical problem with searching for binding sites in vertebrate sequence is that there is so much sequence to search! On average, each gene in the human genome has over 70 kb of noncoding sequence surrounding it, all of which

may contain functional binding sites. While there are some relatively simple ways to narrow the search – for example, looking at sequence conservation and synteny relationships, as described earlier – you may still need to search several kilobases of DNA. Given the short length of most known sites and the low specificity of matrices, this can lead to many more predicted sites than are likely to be functional.

While there is no general way to limit this false positive rate, there are several control "experiments" that can give you an intuitive feeling for the likely false positive rate for a particular search. If searching only within conserved regions, one can also search nearby nonconserved sequence to get an estimate of how many sites are found in DNA that is unlikely to be functional. Alternatively, one can do the same motif search in sequence taken from somewhere else in the genome, near to genes that are unlikely to be regulated by the transcription factor in question. While it is always possible that real binding sites are situated in nonconserved sequence or randomly chosen genomic sequence, it is unlikely enough to serve as a reasonable control. Be sure to avoid comparing searches of your candidate DNA with searches on repetitive sequence or searches on sequence with a very different GC content; both repeat elements and GC- or AT-rich sequences will bias your results.

Another source of high false positive rates is a poor choice of threshold for a matrix search. As described earlier, matrices rank binding sites with a numerical score, and the lower the score the less likely it is that that site is a real binding site. Where should the threshold be set? Again, there is no general technique to determine a good threshold, but the scores of the sites that were used to construct the matrix should give an idea of what scores to consider: a threshold that includes many but excludes a few of the known sites is likely to be safe. For example, using the GATA-site PFM above, we score the three input sites as follows:

AGATAA	2/3 + 4 + 2/3	= 16/3
TGATAA	1/3 + 4 + 2/3	= 15/3
AGATAG	2/3 + 4 + 1/3	= 15/3

In this case, these three sites are the three highest ranked sites possible with this matrix. The next best site is "TGATAG," with a score of 14/3, and there are four sites (GGATAT, GGATAC, CGATAT, CGATAC) that have a score of 12/3. All other six-base sites have scores of 11/3 or below; because of the combinatorics of DNA, there are dozens of sites that have a score of 10/3. By ranking the input sites, we can guess that a choice of 15/3 for a threshold is good, and anything lower than 12/3 will probably admit many false positives.

This illustrates a general problem with matrices: as the cutoff threshold decreases, the number of matching sites increases dramatically. For example, as you decrease the threshold for the JASPAR HFH-2 PWM from its maximum of 58 to 40, the number of sites found by the PWM rises from 1 to over 420,000 (Fig. 8).

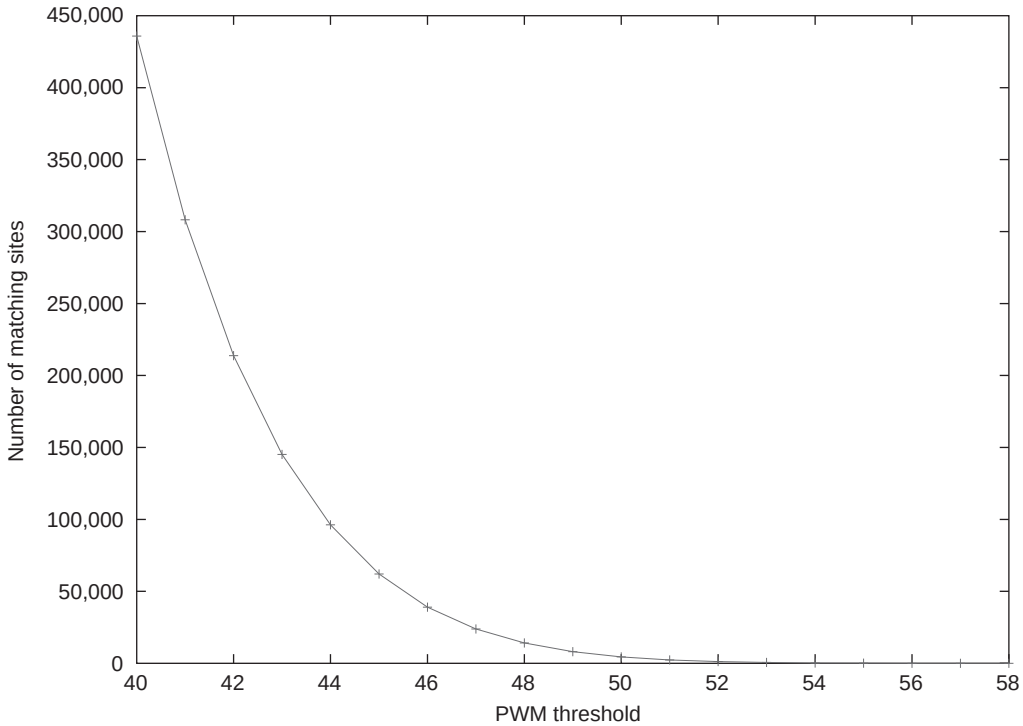


Fig. 8 How the number of sites matching a PWM increases as the threshold decreases. Calculations were done using the HFH-2 Forkhead matrix taken from the JASPAR binding site collection, against a genomic background with no A/T bias. The Y axis is the number of possible 12 base sites that match the PWM at or above the given threshold. Calculations were done with the motility toolkit, unpublished software available at http://cartwheel.caltech.edu/motility/.

This corresponds to a PWM/threshold combination that discovers two sites in every 1 kb fragment!

This result demonstrates the mathematical tendency of binding site searches towards false positives that underlies the so-called "Futility Theorem" (Wasserman and Sandelin, 2004). The Futility Theorem states that 99.9% of binding site predictions in undistinguished genomic DNA are probably false positives, or, equivalently, that a given binding site match is 99.9% likely to be a false positive.

C. Using JASPAR and CONSITE to Scan DNA with a Binding Site Library

There are several sites that can search a sequence against a library of binding site matrices. The JASPAR collection of sites is a high-quality library of multicellular transcription factor binding sites based primarily on SELEX data (Vlieghe *et al.*, 2006). The CONSITE service uses JASPAR as a source of binding site

data and lets users search any piece of DNA against the library (Sandelin *et al.*, 2004).

To demonstrate this service, let us take the *Sox2* N-1c regulatory element and use CONSITE to scan it for known binding sites.

First, retrieve the 55 bp N-1c sequence from UCSC by searching for the region **chr9:17,980,31817,980,373** in the *Gallus gallus* genome. Export this DNA as described above; note that to get it in the same orientation as in Takemoto *et al.* (2006) you need to export the reverse complement.

Now go to the CONSITE website, http://www.phylofoot.org/consite/, and select "Analyze single sequence." Paste the sequence into the top sequence box, and set the sequence name to "Sox2 N-1c." Select "Proceed to transcription factor selection."

On this next screen, you can select a search set of JASPAR transcription factors from the list, or simply ask CONSITE to search for all of them. For this search, we will ask for a global search, which is the default.

The other important setting on this page is the specificity setting, which is set to "10 bits" by default. This is analogous to the threshold for matrix searches described above, and this parameter can be varied to get different results: lowered, for less stringency, or raised, for more. The mathematical meaning of a bit in this case is that each precisely specified individual base is worth two bits, so a 10 bit threshold would find an exact match to a 5-base site. (The meaning is not actually so straightforward because matrices perform fuzzy matching, but the math works out to 2 bits per exact base match.)

Leave the setting to 10 bits and select "Analyze the sequence(s) with all TFs." This will run a search against the entire JASPAR library. You will see an image like that in Fig. 9A, where the name of the matrix match is provided along with a pointer to the location of the match. To see the match position, matrix itself, the transcription factor name, and the organism in which the data for the matrix was recorded, click on the matrix name (Fig. 9C).

Rerun the analysis with a lower threshold of 8 bits (Fig. 9B).

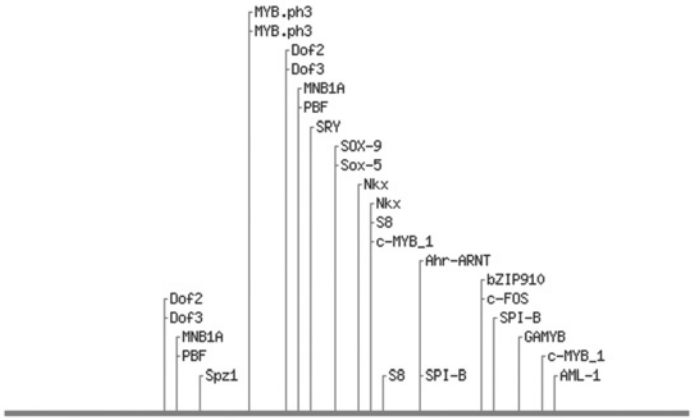
As expected, lowering the threshold only a little bit increases the number of matches significantly; this is again because of the fuzzy matching "Futility Theorem" problem described earlier. Moreover, none of the binding sites found match the known binding sites in this region, because the actual binding sites are too different from the matrices in the library; one would have to admit many more false positives in order to find the two Tcf/Lef binding sites in this region, for example. Nonetheless, JASPAR and CONSITE are good tools to have in the toolbox, and as we have shown it is quite easy to try them out.

D. Searching for Combinations of Known Motifs

Very few transcription factors act alone, and many seem to work in combination with the same partners to coregulate different genes. Therefore, searching for closely grouped combinations of known motifs can be an effective way to reduce

A **Putative transcription factor binding sites found along *sox2_n-1c***

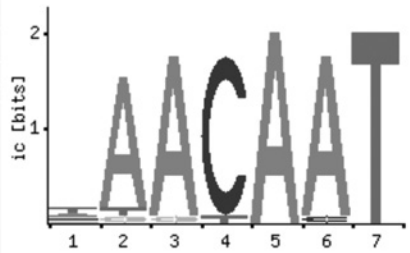
B **Putative transcription factor binding sites found along *sox2_n-1c***



C

Score	Position	Sequence
7.562	28	sox2_n-1c

ID	MA0087
Name	Sox-5
Class	HMG
Species	Mus musculus
Sequence	
Lit ref	
Medline	




```
A  [ 8 21 22  0 23 22  0 ]
C  [ 4  0  0 22  0  1  0 ]
G  [ 3  1  1  0  0  0  0 ]
T  [ 8  1  0  1  0  0 23 ]
```

Fig. 9 Using CONSITE to search for JASPAR matrix matches in the *Sox2* N-1c enhancer. (A) Results with a more stringent threshold of 10 bits; (B) results with a less stringent threshold of 8 bits and (C) information on the Sox-5 match from (A). The matrix is given in both numbers of each nucleotide at each position and in the ''sequence logo'' format, where the height of each nucleotide indicates the importance of that nucleotide to the match score.

the false positive rate inherent in the single-site search techniques discussed above. However, the sparseness of our current biological knowledge of sites and associated factors limits the effectiveness of combinatorial search, because it is more than likely that we know neither what partners are involved nor what their binding sites are.

One of the easiest to use combinatorial search tools is the ClusterBuster program developed by Zhiping Weng's lab at Boston University (Frith *et al.*, 2003). Let us first try a simple search recapitulating the discovery of two TCF/Lef binding sites in the *Sox2* N-1c enhancer (Takemoto *et al.*, 2006).

For this search, you will need to retrieve the genomic sequence surrounding the N-1c enhancer. The genomic coordinates for the 1 kb including this region in the May 2006 chicken genome assembly are **chr9:17,980,000–17,981,000**; go to the UCSC genome browser site and enter this position into the search box (Fig. 10). Now extract the DNA so that you see it in your browser window (click on "DNA," followed by "Submit") and copy it into your paste buffer.

Now go to the ClusterBuster website search form, http://zlab.bu.edu/cluster-buster/cbust.

Paste the DNA into the search box at the top of the form, and enter the following matrix (representing a TCF/Lef binding site) into the motif box, under "Select a bunch of motifs."

```
>tcf-lef
0100
0110
0001
0001
0001
0010
1000
```

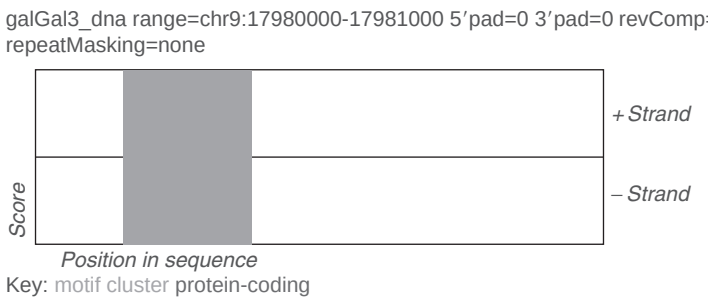


Fig. 10 Using ClusterBuster to find a pair of TCF/Lef binding sites in the *Sox2* N-1c enhancer.

Also change the gap parameter to 5, to allow the binding sites to be close together. Now submit the search with "Find my clusters."

The result will show you a single cluster, in green, located 150 bases into the 1 kb fragment; this is the cluster identified and tested experimentally by Takemoto *et al.* (2006).

ClusterBuster can also be used to search larger sequence regions. Taneyhill *et al.* (2007) identified a *Cadherin6b* regulatory element by searching for pairs of *Snail2* binding sites within the 8 kb of genomic sequence surrounding the start codon of *Cadherin6B*. If you recapitulate their search with ClusterBuster, using the DNA from chick **chr2:70,535,000–70,545,000**, a cluster threshold score of 2, and the following snail matrix representing "CAGGTA,"

```
>snail
0100
1000
0010
0010
0001
1000
```

you will see multiple clusters (Fig. 11), the second, fourth, and fifth of which correspond to those tested in Taneyhill *et al.* (2007).

Although both of these examples show searches for homotypic clusters, or clusters of the same binding site, it is possible to add multiple matrix entries into the search box. In this case, combinations of different matrices will be used in the search.

The drawback of this kind of search is that one needs to know what binding sites are likely to be found in combination. It is also possible, although much more difficult, to discover unknown binding sites given several coregulated regulatory regions.

E. Predicting Unknown Binding Sites Computationally

There are a number of methods for predicting unknown binding sites computationally, but they do not work well until at least four or five sequences containing similar motifs are available. Even then, results are poor, especially on metazoan sequences; an initial survey of computational tools found that sensitivity and

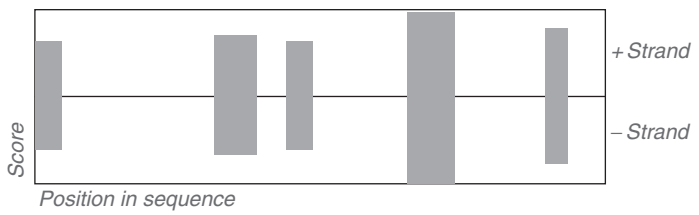


Fig. 11 Using ClusterBuster to find a pair of snail binding sites near the *Cadherin6b* translation start.

predictive value of most tools were under 15% (Tompa *et al.*, 2005). While such assessments are difficult to do properly because of our general lack of known-good binding sites, these results do not bode well for biologists attempting to use such programs on their own sequences. One encouraging result of this study was that combining results from multiple binding site search programs is valuable. In one case, combining the predictions from two programs doubled the correlation between nucleotides predicted to be part of a site and those actually part of a site. To quote,

> Biologists would be well advised to use a few complementary tools in combination rather than relying on a single one and to pursue the top few predicted motifs of each rather than the single most significant motif.

The review contains links to a number of programs for identifying "common" binding sites in a selection of sequences (Tompa *et al.*, 2005). The WebMOTIFs site at http://fraenkel.mit.edu/webmotifs/ is one site that allows combined searches with several motif discovery programs.

F. Integrating Conservation and Motif-Search Evidence

Above, I suggest starting with experimental analysis of conserved regions to identify functional *cis*-regulatory modules, and then proceeding to binding site analysis to identify individual binding sites. Many discussions of binding site analysis focus on using conservation as independent evidence for functionality. Unfortunately, if conservation is used to narrow down the amount of sequence to be searched with a motif, conservation cannot then be used as independent evidence of binding site functionality. Given the generally poor quality of binding site search and the high false positive rate of bioinformatic methods in general, I have found that the relatively straightforward procedure of experimentally testing conserved elements is the most effective way to find functional DNA. Once functional regulatory elements are known, they become amenable to the more sensitive but less specific bioinformatics techniques such as motif searching, and they can also be probed with a variety of experimental techniques such as deletion, mutation, footprinting, and ChIP analysis.

If conservation analysis does not find the regulatory element of interest, I recommend trying the "shotgun" approach used by Uchikawa *et al.* (2003).

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CHAPTER 19

Investigating Regulatory Factors and Their DNA Binding Affinities Through Real Time Quantitative PCR (RT-QPCR) and Chromatin Immunoprecipitation (ChIP) Assays

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References

I. Introduction

In the past, assessing gene expression in avian embryos has relied solely upon non- or semiquantitative techniques, such as *in situ* hybridization and traditional PCR. Although *in situ* hybridization provides invaluable spatiotemporal information with respect to gene expression, the *quantity* of a given transcript in a particular tissue cannot be measured. Similarly, traditional PCR provides information with respect to the presence or absence of a transcript, but it is, at best, semiquantitative. With advances in molecular biology and appropriate protocol adaptations, it is now possible to precisely measure transcript levels in the embryo through the use of real time quantitative PCR (RT-QPCR). Furthermore, recent advances made in genomics afford the opportunity to apply robust biochemical assays to the embryo in order to study the association of regulatory factors with their binding sites. In conjunction with RT-QPCR, chromatin immunoprecipitation of regulatory factors allows for the dissection of the spatiotemporal interactions of these proteins with their DNA recognition sites in putative targets. Taken

together, these assays will help elucidate regulatory networks involved in all aspects of chick embryonic development.

In this chapter, we examine the utility of RT-QPCR for use in analyzing gene expression in chick embryos, and discuss the various types of assays currently available. We also describe the technique of chromatin immunoprecipitation (ChIP) and how ChIP, in combination with RT-QPCR, provides a powerful system to quantitatively measure the affinity of various regulatory factors to their targets *in vivo*. The information in the following sections was compiled from various sources, including literature available from companies such as Applied Biosystems and BIO-RAD, as well as from primary research literature (indicated in the References section).

A. RT-QPCR vs. Traditional PCR

Traditional PCR, or end-point PCR, relies upon the determination of the amount of amplified product at the end of the PCR by agarose gel electrophoresis. This end-point corresponds to the plateau phase of a PCR in which the reaction has slowed down and/or has stopped. In replicate samples processed by traditional PCR (which start out identical and in which the amount of amplified product should not change), the plateau phase for each sample does inevitably vary, reflecting different amounts of amplified product. Thus, it is more accurate to make measurements for amplification in the beginning of the PCR (during the exponential phase), where products are doubling at every cycle (assuming 100% reaction efficiency), and it is upon this principle that RT-QPCR is based (for review see Bustin, 2005 and Kubista *et al.*, 2006, as well as literature available from Applied Biosystems and BIO-RAD).

RT-QPCR eliminates the need to process samples by gel electrophoresis because PCR data are acquired in ''real time'' by software associated with the PCR machine, thus reducing experimental time (for comparison of traditional and RT-QPCR, see Fig. 1). A tungsten lamp is used to excite the fluorescent dyes, and emission is measured between 500 and 660 nm during the extension phase of the PCR. Lenses, various filters, and a dichroic mirror are then used to focus the emission into a charge-coupled device (CCD) camera. The system software collects the data from the camera and uses mathematical data algorithms to analyze it, presenting it in a user-friendly format for further examination by the researcher. Because of its real time nature, the RT-QPCR method does not have any of the limitations of traditional/end-point PCR, which include poor precision, low sensitivity, low resolution, size-based discrimination only, and lack of quantitation. RT-QPCR is designed to collect data in the exponential phase of the PCR as the reaction is proceeding, and allows for the measurement of the amount of PCR product at any given cycle number. Thus, RT-QPCR provides a fast and sensitive means by which to measure both quantitative changes in gene expression in a given sample, as well as qualitative differences among samples.

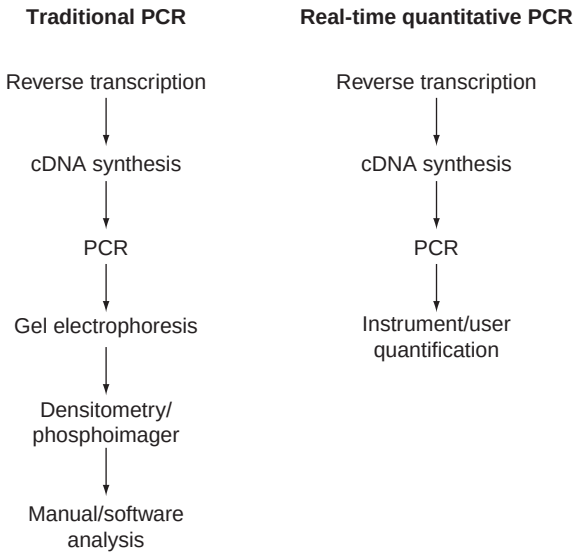

Fig. 1 Flow-chart of traditional and RT-QPCR. An overview of methods required for both traditional and RT-QPCR is presented. Note the simplification achieved by RT-QPCR, particularly with respect to data analysis. Adapted from Bustin (2000, p. 15).

B. RT-QPCR Chemistries

The two types of chemistries for the detection of RT-QPCR products include DNA binding dyes (SYBR Green I) and dye-labeled, sequence-specific oligonucleotide primers (Amplifluor, Scorpions, LUX, and BD Qzyme) or probes (molecular beacons, TaqMan, hybridization, and Eclipse). The most common chemistries chosen for assays, however, are SYBR Green I and TaqMan. The following outline of RT-QPCR chemistries is a synopsis of available information from BIO-RAD and Applied Biosystems. For a more in depth review, see further information from these companies, as well as Bustin (2005) and Kubista *et al.* (2006).

1. DNA Binding Dyes

SYBR Green I is a fluorescent dye that binds indiscriminately to double-stranded DNA, resulting in up to a 1000-fold increase in fluorescence. The main advantages of using SYBR Green I is that these assays are easy to design (only 2 primers) and less expensive (no need for probes), and one can perform a dissociation curve to check the specificity of the QPCR. Conversely, the main disadvantages of SYBR Green I-based assays include their lack of specificity (a dissociation curve is essential to ensure that all captured fluorescence is due to the amplification of the gene of interest and not to some nonspecific amplification) and their inability to be used in multiplex QPCR, as the dye binds to all double-stranded DNA present and will not distinguish separate PCR products.

2. Fluorescent Primers/Probes

Although they use different mechanisms to allow for target gene detection, fluorescent primers and probes (end-labeled with a 5′ reporter dye and a 3′ quencher dye) commonly rely upon the principle of fluorescent resonance energy transfer (FRET). Due to the proximity of the quencher dye to the reporter dye, the reporter dye does not fluoresce upon excitation by a light source. Only in the presence of amplified product will emission of probe fluorescence occur because of the nature of its reaction chemistry. The main advantages of using fluorescent primers/probes is that this method allows for built-in specificity, due to the binding of an additional oligonucleotide rather than a nonspecific DNA binding dye, and the ability to perform multiplex RT-QPCR. The following chemistries are currently available, although the most commonly used ones for RT-QPCR are TaqMan probes and molecular beacons.

a. TaqMan Probes (5′ Nuclease Assay)

The chemistry for this particular RT-QPCR assay relies upon a sequence-specific, labeled oligonucleotide probe that anneals between the primers, and the use of Taq DNA polymerase that possesses 5′ exonuclease activity to remove DNA downstream of the amplicon as it is being produced. As the polymerase approaches the probe during the PCR, its 5′ exonuclease activity displaces and cleaves the probe, such that the probe no longer sits on the DNA. Upon cleavage of the probe, the quencher dye can no longer suppress reporter dye fluorescence because the distance between the reporter and quencher dyes has increased. The FRET principle no longer applies, resulting in the emission of fluorescence by the reporter dye after excitation. This reporter dye fluorescence recorded by the RT-QPCR machine is directly proportional to the amount of amplicons being produced in the PCR. Commonly used reporter–quencher pairs are fluorescein (FAM)-TAMRA and FAM-Black Hole Quencher 1. Advantages of using TaqMan probes include high specificity, high signal-to-noise ratio, and ability to perform multiplex reactions. The main disadvantage, however, is the high cost and potentially difficult assay design.

b. Molecular Beacons

RT-QPCR assays using molecular beacons are very similar to the TaqMan assay described above. Much like their TaqMan probe counterparts, molecular beacons are end-labeled with reporter and quencher dyes, and bind to a specific sequence between two primers. The difference, however, lies in the addition of sequence to the end of the molecular beacon that allows it to form a hairpin in the absence of any PCR product, and the lack of 5′ nuclease activity inherent to the polymerase. As the gene of interest is amplified, the molecular beacon anneals to its target sequence, and the reporter dye fluoresces because it is no longer in close proximity to the quencher dye (no hairpin). Advantages of molecular beacons include their

high specificity and use in multiplex PCR, while the main disadvantage is that the hairpin design of such beacons is not trivial, leading potentially to unintended fluorescence (weak hairpin) or lack of fluorescence (strong hairpin).

c. Hybridization Probes

Assays conducted using hybridization probes consist of two sequence-specific primers and two sequence-specific probes. The probes anneal to sequence between the primers and adjacent to one another. The first probe is labeled with a donor dye on its 3′ end, and the second probe is labeled with an acceptor dye on its 5′ end. The emission spectrum of the donor dye overlaps with the excitation spectrum of the acceptor dye, and the PCR is monitored at the wavelength corresponding to the acceptor dye. During the PCR, the probes anneal and, using the FRET principle, excitation of the donor dye allows for fluorescence of the acceptor dye.

d. Eclipse Probes

Like TaqMan probe assays, Eclipse probes utilize two primers in the presence of a sequence-specific probe that is labeled with a 5′ reporter dye and 3′ quencher dye (nonfluorescent in the absence of the amplicon) and contains a DNA minor groove binder. The minor groove binder facilitates hybridization of the probe to the amplicon, subsequently allowing for the separation of the reporter and quencher dyes and fluorescence indicative of product.

e. Amplifluor Chemistry

Amplifluor chemistry utilizes two gene-specific primers and a universal primer that forms a hairpin with reporter and quencher dyes at each end (UniPrimer). One gene-specific primer contains a 5′ extension sequence (Z sequence) that is also found at the 3′ end of the UniPrimer. Annealing of the Z primer to the gene of interest and extension occur initially, followed by annealing of the UniPrimer to its complementary sequence in the Z primer, forming a product that serves as a template for amplification by the second gene-specific primer. Extension in the presence of this second primer causes the hairpin to unfold, and the FRET principle allows for fluorescence of the reporter dye as the reporter and quencher dyes become separated.

f. Scorpions Primer

Scorpions primer-based assays rely upon the ability of the Scorpions primer to also act as a probe that forms a hairpin structure (a loop with reporter and quencher dyes labeling the ends). During annealing, the primer containing the hairpin structure binds and is extended, and during the next denaturation step, the reporter and quencher separate as the loop binds to its complementary sequence in the target.

g. Lux

LUX primers also form a hairpin and are labeled with a 5′ reporter dye that is quenched by the secondary structure intrinsic to the hairpin. During denaturation, the hairpin structure is lost as the primer anneals and is extended, resulting in reporter fluorescence.

h. BD QZyme

BD QZyme primer-based assays consist of a zymogene primer that encodes a catalytic DNA, a target-specific reverse primer, and an oligonucleotide end-labeled with a 5′ reporter dye and 3′ quencher dye. The zymogene primer anneals to the target sequence, and subsequent extension by the reverse primer results in the production of the catalytic DNA. In the next annealing step, the catalytic DNA hybridizes to the probe and cleaves the probe, permitting reporter dye fluorescence.

II. Materials for RT-QPCR

A. Templates

Templates for RT-QPCR usually consist of tissue from which RNA, cDNA, or DNA has been prepared, containing the gene of interest for amplification. The use of cDNA templates is common, with RNA being isolated from tissue through the use of commercially available kits (Ambion, QIAGEN) or through standard reagents and methods (Trizol or guanidine isothiocyanate), and converted to cDNA through the use of random hexamers and/or oligo(dT) primers (Kubista *et al.*, 2006). As in any PCR, the quality and quantity of the template are important to yield maximal results. Quality control of templates is advisable; this can be done by performing a standard PCR for a housekeeping gene such as *GAPDH* to ensure the presence of starting material. Measurement of cDNA or DNA concentration can also be done using standard techniques (spectrophotometry). One of the main advantages of RT-QPCR, however, is that very little template is required due to the increased sensitivity of RT-QPCR. The concentration of template used in the PCR will be dependent on the relative level of the gene of interest present in the sample. Preliminary experiments should be conducted to assess the quantity of template required to give a measurable fluorescent signal in the PCR.

B. Primer Design

RT-QPCR amplicons are typically on the order of 50–150 bp in length. On the basis of this criterion, primers should be chosen within the sequence of interest. These primers should be specific for the gene of interest and not amplify other sequences nonspecifically. In addition, primers may be designed across the intron–exon boundaries of a gene (if genomic sequence is available) to increase specificity. Several software programs are available to facilitate primer design, either online

(e.g., Primer3) or from companies such as Applied Biosystems (Primer Express). The following parameters are recommended by Applied Biosystems for primer design:

G-C content: 20–80%;

Melting temperature: 58–60 °C;

Avoid runs of identical nucleotides, particularly guanines (4 or more);

The 5 nucleotides at the 3′ end of each primer should have no more than two guanines and/or cytosines; and

Primers should be as close as possible to the probe without having any overlap with the probe sequence.

Typical RT-QPCR primer concentrations range from 75 to 900 nM. As in any PCR reaction, the concentration of primers is critical for proper amplification. It is best to perform initial experiments to determine the correct concentration of primer that will yield the best amplification for the chosen sample. It is important to note that primer concentrations may vary depending upon the gene whose expression is being studied.

C. Probe Design

Probes prepared for the 5′ exonuclease activity may also be chosen using the software described above for primer design. The following parameters are recommended by Applied Biosystems for the design of probes containing 5′ reporter and 3′ quencher dyes for use in single-probe assays:

G-C content: 20–80%;

Melting temperature: 68–70 °C (10 °C higher than primer melting temperature);

Avoid runs of identical nucleotides, particularly guanines (4 or more);

Avoid putting a guanine on the 5′ end of the probe; and

Select the strand that gives the probe more cytosines than guanines.

RT-QPCR probe concentrations will also vary, depending upon the gene being studied, and initial experiments should be carried out to determine the amount of probe required for a 5′ nuclease assay. A typical concentration for a TaqMan probe is 450 nM.

D. RT-QPCR Instruments

Many RT-QPCR platforms are commercially available, from companies such as Applied Biosystems, BIO-RAD, and Roche. In general, these instruments use the same experimental set-up protocols and data processing software, and differ primarily in the number of fluorophores they can read and the types of plates or

tubes they can handle (Kubista *et al.*, 2006). The type of instrument chosen by the researcher is determined mainly by the type of assays to be performed.

III. General Principles and Definitions

This section discusses some of the critical principles and definitions associated with RT-QPCR. It is a summary of information available from Bustin (2005) and Kubista *et al.* (2006), and from websites for companies that specialize in RT-QPCR (Applied Biosystems and BIO-RAD; www.appliedbiosystems.com and www.biorad.com, respectively).

A. Baseline

Fluctuations in fluorescence levels, particularly in the initial phases of the PCR, may be evident due to changes in the reaction medium. This background level of signal dictates the baseline fluorescence for the entire RT-QPCR. The baseline value refers to a defined range of PCR cycles (for example, the default for Applied Biosystems machines is 3–15) over which this level of background fluorescence is calculated. This background is then used to calculate a subtracted amplification plot ($\triangle R_n$; see below) that is then analyzed by the researcher. The baseline can be manually adjusted by the researcher, particularly if the gene being amplified is abundant, such that signal is measured after the range of baseline values, usually one to two cycles before the earliest amplification.

B. Threshold and Threshold cycle (C_T)

A threshold line, corresponding to the level of detection of the amplicon (or fluorescence) above a background level, is set during the exponential phase of the RT-QPCR and is a statistically significant point above the baseline. The cycle number at which the fluorescence generated by a sample meets this threshold line is known as the threshold cycle (C_T), and this value is critical in determining the amount of the amplicon produced in the RT-QPCR.

C. Normalized Reporter Signal (R_n) and $\triangle R_n$

The normalized reporter signal is the reported fluorescence in a well (reaction) that is calculated by the division of the fluorescent reporter signal by the signal of a passive reference dye (such as ROX; explained later). $\triangle R_n$ refers to the adjusted value of the normalized reporter after the elimination of background fluorescence through the baseline calculation.

D. Passive Reference Dye

The passive reference dye is a component of the RT-QPCR master mix that is present at a constant concentration in each of the samples. Its presence is used to normalize fluorescence from the reporter in order to account for subtle variations

in reporter fluorescence that are not due to PCR (such as pipetting errors), and to allow for well-to-well comparison of reporter signal. A common passive reference dye used is called ROX.

E. Standard

This term refers to a sample (usually DNA) that is of known concentration that is used to construct a standard curve.

F. Normalizer Gene (or Active Reference)

The normalizer gene is a chosen target that is used to standardize experimental results. Normalizers can be endogenous (present and amplified in the sample by the PCR reaction) or exogenous (added into the sample at a known concentration). Normalizers are convenient to adjust for differences in starting material concentrations, thus allowing for quantitative comparisons among multiple samples. In addition, expression of the chosen normalizer gene should not be affected by experimental treatments. Commonly chosen normalizer genes include *GAPDH*, *ubiquitin*, *β-actin*, and *ribosomal 18S*. RT-QPCR is performed for the normalizer gene in an analogous fashion to that for the gene of interest.

G. Calibrator (Control)

The calibrator sample is one that is used as a means of comparison relative to other experimentally treated samples.

IV. Types of Assays

In RT-QPCR, one can either measure the absolute number of transcripts of interest in a given amount of tissue, known as ''absolute quantification,'' or the fold change of transcript in equivalent amounts of tissue, known as ''relative quantification.'' For all methods of RT-QPCR, results from experiments must be normalized in order to make data meaningful. In the case of absolute quantification, this means that the number of particles or transcripts is expressed as compared to the amount of initial sample (such as ''per ml of sample'' or ''per 1000 cells''). Relative quantification, on the other hand, allows the calculation of expression as a fold change between equivalent amounts of the sample being evaluated and the control sample.

A. Absolute Quantification

Absolute quantification is an assay where the desired output is the quantity of the target transcript. A standard curve is created by dilutions of a template with known concentration. Unknowns are then assayed with these standards

(on a single plate) and these are then used to determine the initial amount of transcript present in the tissue through interpolation. While absolute quantification gives a number that is more appreciably concrete than the fold change of relative quantification (i.e., that there are 200,000 ± 15,000 transcripts in a given sample), researchers must be aware that several steps can affect this result. Messenger RNA is not trivial to isolate, and it is very unstable – any loss of transcripts at this point may dramatically affect the outcome of the RT-QPCR. Reverse transcription reactions are more predictable, but no two are going to have the same exact efficiency. It is for these reasons that reactions must be repeated a number of times from the original starting materials in order to ensure that results are repeatable and meaningful.

B. Relative Quantification

Relative quantification involves the comparison of the amounts of the transcript in question between two different samples (e.g., experimentally-treated samples and control-treated samples) while normalizing to ensure that they are compared between equivalent amounts of tissue. In this case, the samples can be normalized by unit mass or as compared to a "housekeeping" gene. In the case of normalization by unit mass, the starting material is measured either in cell number or microgram of nucleic acid. Total RNA is prepared from an equal amount of tissue. The problem with this method is that one must be sure to carefully measure the starting amount of material. In addition, the separate reactions might give different extraction and reverse transcription efficiencies. To determine equivalent amounts of sample necessitates either the extraction of equal amounts of tissue and the hope that these separate extractions have the same efficiency, or the use of a "normalizing" or "housekeeping" gene which does not change between the treated and the control sample in order to evaluate an equivalent amount of DNA. When normalizing relative quantification to a reference gene, the necessary assumption is that the reference gene levels do not change between the control and the unknown samples. Genes such as *18S*, *GAPDH*, *ubiquitin*, and *β-actin* are commonly used as normalizing genes. There can, of course, be individual variation with these normalizing genes, but the use of multiple reference genes can solve this problem. This is especially useful in multiplex reactions, which would not increase the number of needed reaction tubes. Researchers must keep in mind the same caveats here that exist with absolute quantification – namely that these reactions must be statistically significant and repeatable in order to have any meaning despite inherent differences in the steps leading up to the assay.

C. Plus/Minus Assays

Plus/Minus assays are similar to the use of regular reverse transcription-based PCR in order to identify the presence or absence of transcript in a sample as compared to a control. This can be more sensitive than regular reverse transcription-based

PCR and can therefore be more useful with small tissue samples or with a low copy number transcript. It is not acceptable to compare the "plus" and "minus" numbers in terms of fold change, however, because the absence of transcript in one sample makes it impossible to calculate a fold change in gene expression.

D. Multiplex Assays

Multiplexing is the amplification of more than one target in a single reaction tube. This is only possible using fluorescent primers or probes that specifically recognize each separate amplification target and do not spectrally overlap. Despite the initial higher cost, these multiplexing reactions can be beneficial in the long run for many reasons including a dearth of starting template, whether it is physically hard to obtain or economically challenging, because more genes can be tested using the same amount of material. Additionally, more samples can be run on an individual plate, allowing multiple samples to be tested at once. Multiplex reactions also provide the opportunity to test each individual sample for a control target, which can facilitate the elimination of unrecognized individual well contamination. Multiplex assays have not been published in an avian system as yet, but because of the benefits of this assay platform, we predict that this will soon become an important tool in avian RT-QPCR.

V. Methods of Analysis

A. Standard Curve

RT-QPCR is based on the initial amount of a target, and the C_T value measured through amplification. In order to determine the initial amount of target in an unknown sample, a standard curve can be constructed by measuring the C_T value of a range of standards of known quantity. These C_T values are then plotted along the *y*-axis while the corresponding logarithm of the initial copy number of the standards forms the *x*-axis. The plot of the line is therefore [$C_T = m$(log quantity of initial copy number) + b] in [$y = (mx + b)$] form. The R^2 value represents the fit of the experimental data to this line, providing a measure of assay replicate variability and amplification efficiency. The R^2 value must always be greater than 0.98 for any experiment and if it falls below this value the data should be considered not meaningful and discarded.

Once the standard curve has been made, the C_T values of the unknown are plotted on the line and the quantity of the gene of interest is interpolated. It is important to make sure that standards are always run on the same plate as the unknown samples in order to ensure the validity of the C_T measurements.

In the case of absolute quantification, standards curve measurements are best made of a plasmid containing the specific gene of interest. Relative quantification assays have also been successfully performed using whole tissue cDNA dilutions as standards. This is possible because the dilution of whole embryo cDNA should include target message at a copy number that decreases with the dilution factor.

Amplification efficiency can be calculated from the slope of the standard curve in the following manner: If the equation of the line is expressed in [$y = (mx + b)$] form, the amplification efficiency E can be calculated as [$E = 10^{-1/\text{slope}}$]. An ideal reaction is one in which the amount of product would double with each cycle of amplification, with an efficiency of 2, giving -3.32 as the ideal slope of the line. Percentage efficiencies are calculated through the following formula:

$$\text{Percent efficiency} = (E - 1) \times 100\%$$

The percentage efficiency of any given reaction should be between 90 and 100%. If the percentage efficiency falls outside of this range, primers and probes should be redesigned following the specified guidelines.

B. Livak Method or $2^{(-\triangle\triangle CT)}$ Method

The first step in this method is to verify that both target and reference genes are being amplified at near 100% efficiency, and no more than 5% apart from each other. Relative differences in expression level between different samples can then be calculated as follows.

First, the C_T of the target gene must be normalized to that of the reference gene for all samples:

$$\Delta C_{T(\text{unknown})} = C_{T(\text{target, unknown})} - C_{T(\text{reference, unknown})}$$

$$\Delta C_{T(\text{control})} = C_{T(\text{target, control})} - C_{T(\text{reference, control})}$$

Then the $\triangle C_T$ of the unknown is normalized against the $\triangle C_T$ of the reference gene:

$$\Delta\Delta C_{T()} = \Delta C_{T(\text{unknown})} - \Delta C_{T(\text{control})}$$

Finally, the normalized expression ratio can be stated as:

$$2^{(-\Delta\Delta C_T)} = \text{Normalized expression ratio}$$

which is the fold change of the target sequence in the unknown sample relative to the control sample, normalized to a reference gene.

C. The $\triangle C_T$ Method Using a Reference Gene

The $\triangle C_T$ Method using a reference gene is a simplified form of the Livak method that uses the difference between the reference and target C_T values in a given sample.

$$\text{Ratio}\left(\frac{\text{Reference}}{\text{Target}}\right) = 2^{[C_{T(\text{reference})} - C_{T(\text{target})}]}$$

If you then divide this result by the expression value of the control sample, the results will be precisely what they were by using the Livak method.

D. The Pfaffl Method

If there is a situation in which the amplification efficiencies of the target and reference genes are not similar, the Pfaffl method is used. Generally speaking, this is a more efficient formula than the Livak method, because whether your amplification efficiencies are precisely the same, or quite a bit different (though within specified guidelines), the ratio can still be accurately calculated. In this case, one must first determine the efficiencies of both the target and the reference genes, here called E_{target} and $E_{\text{reference}}$. The change in C_T between the unknown and the control samples for the target and reference genes must also be calculated and are shown as $\triangle C_T$, target (control − unknown) and $\triangle C_T$, reference (control − unknown).

$$\text{Ratio} = \frac{[(E_{\text{target}})^{\Delta C_T,\ \text{target(control}-\text{unknown)}}]}{[(E_{\text{reference}})^{\Delta C_T,\ \text{reference(control}-\text{unknown)}}]}$$

E. Setting the Baseline and the Threshold

Once a run is complete, you can begin your analysis. First, perform the baseline correction to set each curve at the origin. Using the specific software controls, set the graph such that the *y*-axis is linear. Then reset the baseline so that it represents these initial cycles showing no amplification. Make sure that the chosen portion of the baseline is the narrowest possible and that the end cycle is approximately 2–5 cycles before any amplification is visible.

The C_T or threshold cycle is where the threshold line intersects the amplification trace. In order to set the threshold, select all the wells that were active in the run to see where noise exists and where the linear portion of the curve is, viewing the data in the logarithmic format. Move the threshold setting to a point above the noise in the narrowest portion of the linear phase of the amplification curve. New $\triangle R_n$ values are then calculated for all of the samples. Make sure to reanalyze your data according to your software instructions after resetting these values.

VI. Assay Setup

The exact methods surrounding assay set-up will depend upon the type of RT-QPCR instrument used and corresponding software, as well as the RT-QPCR chemistry chosen (5′ nuclease assay versus SYBR Green I). For experiments in which gene expression is being assessed under different conditions, and for ChIP assays, the ''absolute quantification'' plate setting is recommended. The following describes the experimental set-up for an Applied Biosystems 7000 machine.

Each RT-QPCR can be done in a final volume of 25 or 50 µl. Amounts reported here are for 50 µl reactions. RT-QPCR reactions for each sample tested (a particular standard DNA concentration, unknown, and no template control) should be done in at least triplicate replicates, and the final value reported will reflect an average of these replicates.

Set up RT-QPCR to contain the following:

5′ nuclease assay

Universal master mix (with appropriate buffers, dNTPs and passive reference dye)

Forward primer (final concentration of 75–900 nM)

Reverse primer (final concentration of 75–900 nM)

Probe (final concentration of 150–900 nM)

DNA template

Nuclease-free water to 25 or 50 µl.

SYBR Green Assay

Universal master mix (with appropriate buffers, dNTPs and passive reference dye)

Forward primer (final concentration of 75–900 nM)

Reverse primer (final concentration of 75–900 nM)

DNA template

Nuclease-free water to 25 or 50 µl.

DNA template for target genes should be present at a range of between 150 and 700 ng, but this number is best determined by running different concentrations of template in order to determine the rarity of the transcript and the efficiency of the primers. Housekeeping or normalizing genes may need to be run at more dilute levels (proportionally) because of the high level of transcript present (unless it is a multiplex reaction).

Run RT-QPCR according to the default settings on the RT-QPCR machine.

Perform data analysis (see Section V).

VII. ChIP and RT-QPCR

Chromatin immunoprecipitation (ChIP) is a valuable technique for the study of the spatiotemporal nature of protein:DNA interactions in an *in vivo* context. This method was initially used to explore the interaction of RNA polymerase and topoisomerase with genes in the fly (Gilmour and Lis, 1985, Gilmour *et al.*, 1986), and was later used to describe histone interactions with DNA in systems such as the fly and yeast (Braunstein *et al.*, 1993; Solomon *et al.*, 1988). ChIP has been more recently employed to identify the *in vivo* association of transcription factors to their DNA binding sites in frog, mouse and chick (Sachs and Shi, 2000;

Skowronska-Krawczyk *et al.*, 2004; Zhou *et al.*, 2005; Taneyhill *et al.*, 2007). For a more thorough review of the historical development and refinement of the ChIP assay, the reader is referred to (Kuo and Allis, 1999; Orlando, 2000; Orlando *et al.*, 1997). In brief, ChIP involves the preparation of protein:DNA complexes by formaldehyde crosslinking of tissue, and these complexes are subsequently sheared by sonication to generate smaller, uniform fragments (see Fig. 2 for protocol outline). An antibody to the protein of interest (regulatory factor) is then employed to immunoprecipitate potential protein:DNA complexes, which are captured using protein A or G beads. After crosslink reversal and protein degradation via proteinase K, the immunoprecipitated DNA is purified and then analyzed for the presence of the sequence of interest, usually by PCR or hybridization techniques. The information below summarizes the ChIP technique (Cuthbert and Bannister, 2006, www.abcam.com and Allis and Wu, 2004); further information may be found in these references.

A. Crosslinking of Proteins to DNA Via Fixation

Crosslinking of proteins to DNA, as well as proteins to one another, occurs by fixation of tissue through the use of 1% formaldehyde. Crosslinking is necessary when looking at the association of regulatory factors with DNA because of their

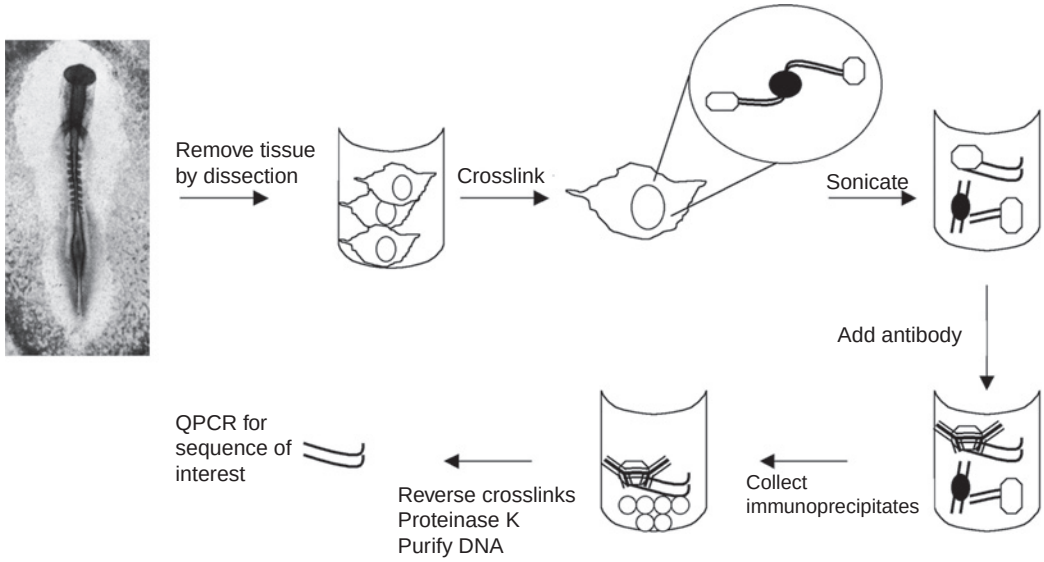

Fig. 2 Diagram of chromatin immunoprecipitation (ChIP) procedures. See text for additional details. Fixed tissue is subjected to sonication in order to achieve fragments of appropriate size for immunoprecipitation by the antibody of interest. Antibody:protein:DNA complexes are immunoprecipitated and captured using protein A or G beads, and immunoprecipitated DNA is subjected to RT-QPCR using primers (and probes) to the sequence of interest.

general weaker affinity for DNA (as opposed to more abundant proteins such as histones). However, times of fixation will vary, depending upon the regulatory factor being examined. Increased times of fixation may preclude antibody recognition of the regulatory factor because of possible alterations in accessible epitopes by the fixation procedure, as well as the loss of access to these epitopes due to the presence of other bound proteins. Conversely, decreased fixation times may not crosslink all of the regulatory factor to the DNA, leaving little protein available for immunoprecipitation. Thus, it is necessary to titrate fixation times to examine the ability of the antibody to immunoprecipitate the regulatory factor after fixation.

B. Sonication

ChIP requires careful optimization of sonication conditions. Fragmentation of chromatin into pieces of less than 1000 bp is suggested, as this assists in the recognition of the antibody for the regulatory factor. Recommended sonication procedures are delineated below, but it is essential to assess chromatin size by agarose gel electrophoresis prior to carrying out the rest of the immunoprecipitation procedure.

C. Use of Antibodies in ChIP

The ChIP method requires the use of a specific antibody that efficiently immunoprecipitates the protein of interest under conditions of formaldehyde fixation. Many antibodies work well in standard immunoprecipitation reactions, but these antibodies may not work well in ChIP, as the epitopes to which they bind may be masked by chromatin architecture and/or altered beyond antibody recognition after fixation. Thus, it is necessary to use an antibody that will recognize the regulatory factor *after* formaldehyde fixation. Performing preliminary experiments designed to test the efficiency of immunoprecipitation of the regulatory factor by the chosen antibody after fixation can help address this question. In addition, polyclonal antibodies are preferable, as they recognize multiple epitopes and increase the chance that the antibody will bind to the regulatory factor, even if some epitopes are masked by the presence of other DNA binding proteins. Finally, ChIP experiments work best when the regulatory factor of interest crosslinks efficiently to the chromatin.

D. Use of Relevant Controls

The use of relevant controls (for both immunoprecipitation and PCR) is requisite in order to definitively prove the association of a regulatory factor to a particular sequence. A common positive control antibody for ChIP experiments performed on actively transcribed genes is one that recognizes trimethylated lysine 4 of histone H3. Recommended negative control antibodies for ChIP include a nonchromatin epitope (such as GFP, HA) and/or IgG antisera, as well as a ChIP

reaction performed in the absence of added antibody. For the RT-QPCR, controls include the use of several PCR primer pairs (and possibly probes) over the region of interest, as well as RT-QPCR performed on an "input" sample that has not undergone immunoprecipitation by any antibodies. An excellent negative control is the use of primers (and possibly probes) designed to recognize a region of sequence to which the regulatory factor should not bind, such as a region of 3′ coding sequence in the gene of interest.

E. ChIP and RT-QPCR

In the past, ChIP experiments were analyzed by hybridization techniques or agarose gel electrophoresis, followed by imaging and the use of appropriate software, to assess relatively semiquantitative interactions of a regulatory protein with its DNA binding site (see methods in Kuo and Allis, 1999). The advent of RT-QPCR, however, has allowed for more direct quantification of this association, eliminating the need for electrophoresis and subsequent imaging analysis. Immunoprecipitated samples are subjected to RT-QPCR using a primer set (and possibly probe(s)) that are specifically designed to amplify a particular DNA sequence. The specificity of immunoprecipitation, coupled with the sensitivity of RT-QPCR and availability of data analysis software programs associated with the RT-QPCR machine, provide a more direct means by which to quantitatively analyze association of regulatory proteins with target sequences.

The following protocol is an adaptation of that available from Upstate, and was developed and refined for tissue from chick embryos to assess the interaction of regulatory factors with chromatin. Thus, this protocol may require adaptation for materials obtained from other organisms and/or tissues. Alternative protocols are offered from other companies that also provide ChIP kits and reagents for purchase.

1. Chromatin Immunoprecipitation (ChIP) Protocol

1. Dissect out tissue of interest and fix tissue in 1% formaldehyde in PBS for 10 min at room temperature with agitation. Stop reaction by adding glycine to final concentration of 0.1M.
2. Quick spin to pellet embryos. Remove liquid to waste.
3. Wash 1× with PBS, quick spin to pellet, and remove liquid to waste.
4. Incubate in 240 μl SDS Lysis Buffer (+25 × protease inhibitors (PI) = 240 μl lysis buffer + 10 μl PI), vortex and pipet up and down to break up embryos/tissue, and incubate on ice for 10 min.
5. Sonicate at 50% output, using microtip, turning up the setting from 1 to 2 on sonicator (Branson 450 Sonifier). Use 20–30 s bursts with 1 min cooling on ice in between sonication. Sonicate to see a ladder of chromatin products,

ranging from 200 to 1000 bp. Sonication time and amount must be adjusted accordingly to see this ladder.

6. Remove 20 µl for checking for the amount of shearing and as the "input" fraction (see below).
7. Spin maximum speed, 10 min, 4 °C. Remove supernatant and transfer to 2 ml tube. Add 80 µl PI inhibitors + ChIP Dilution Buffer (and beads—see below) to 2 ml.

2. Preclearing

1. Prepare protein A (or protein G depending upon antibody isotype) Sepharose beads as follows: Weight out 0.1 g beads and add 500 µl RNAse/DNAse free-water (GIBCO). Pellet by quickly spinning at room temperature, maximum speed, 30 s. Remove water and wash 3× with water. Prepare 50% slurry as indicated in "Buffers" section.
2. Add 75 µl protein A or G to each chromatin prep and incubate at 4 °C for 30 min to 1 h to eliminate nonspecific binding.
3. Quick spin at room temperature to pellet beads. Remove supernatant of sheared chromatin and use in IP reaction.

Chromatin	Divided equally amongst all tubes (~500 µl)
Antibody	Volume range depends on antibody concentration (must be determined experimentally to find an antibody concentration that will immunoprecipitate efficiently; also use no antibody and non-specific antibodies, such as GFP or HA, as negative controls)

IP reaction

1. Set up IP reaction as follows:
2. Incubate overnight with rotation at 4 °C.
3. Add 60 µl protein A or G bead slurry and incubate at 4 °C for at least 1 h with rotation.

Washes

1. Pellet beads by spinning at 1000 rpm, 4 °C, 1 min. Remove supernatant.
2. Wash by adding 1 ml appropriate wash buffer (indicated below), incubating with rotation for 3–5 min, then spinning as in 1):
   a. Low salt wash buffer—1×
   b. High salt wash buffer—1×
   c. LiCl wash buffer—1×
   d. 1× TE, pH 8—2×

3. Elution

1. Prepare fresh elution buffer (1% SDS, 0.1 M $NaHCO_3$).
2. Add 250 µl elution buffer to tubes, vortex and incubate with rotation for 15 min.
3. Pellet beads by spinning at room temperature, 1000× rpm, 1 min, and carefully remove eluate. Transfer to new tube.
4. Repeat 2–3.

4. Crosslink reversal/Proteinase K treatment for immunoprecipitates and input sample

1. Add 20 µl 5 M NaCl (1.0 µl for the 20 µl sample from sonication—"input") and incubate at 65 °C, 4 h.
2. Add 10 µl 0.5 M EDTA, 20 µl 1 M Tris, pH 6.5, and 2 µl proteinase K and incubate at 45 °C for 1 h.

5. Phenol/Chloroform/Isoamyl + Ethanol precipitation

1. Add 500 µl phenol/chloroform/isoamyl to each sample. Shake 20 s, let sit at room temperature for 2 min.
2. Spin 2 min, maximum speed, room temperature. Take aqueous phase.
3. Precipitate by adding 1/10 volume 3 M sodium acetate + 1 µl glycogen + 2.5× volumes 100% ethanol.
4. Incubate at −80 °C, at least 30 min, or at −20 °C 1 h to overnight.
5. Spin 4 °C, maximum speed, 20 min. Decant.
6. Wash w/1 ml 75% ethanol, and spin as in (5) for 5 min. Decant.
7. Air dry pellet for 5–10 min.
8. Resuspend pellet in appropriate volume RNase/DNase-free water for use in QPCR (25–40 µl).
9. Perform RT-QPCR on immunoprecipitated DNA using TaqMan primers and probes as described previously and the standard curve method or the ΔΔ Ct method, doing at least triplicate replicates for each set of primers and probes used. To generate standard curves, use input sample as template and appropriate QPCR primers and probes. As a negative control for the ChIP reaction, perform QPCR using primers and probes designed to a region of your gene of interest to which your regulatory factor DOES NOT bind (i.e., downstream genomic sequence).
10. Analyze data as described previously, calculating the amount of sequence immunoprecipitated in each ChIP reaction. For data analysis, subtract background value (calculated for the averaged result obtained for the no antibody and nonspecific antibody controls) from the value obtained for the regulatory factor. The resulting number is the amount of sequence immunoprecipitated by your regulatory factor.

6. Buffers

1× PBS: phosphate-buffered saline

Protease inhibitors: complete protease inhibitors (Roche)

Proteinase K: Roche

Beads: 50% slurry of protein A/G Sepharose or Agarose (Roche), 600 μg salmon sperm DNA, 1.5 mg BSA in 1× TE, pH 8.0

SDS lysis buffer: 1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0

ChIP dilution buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, 167 mM NaCl

Low salt wash buffer: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, 150 mM NaCl

High salt wash buffer: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, 500 mM NaCl

LiCl wash buffer: 0.25 M LiCl, 1% NP-40, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris–HCl, pH 8.0

1× TE buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0

Elution buffer: 1% SDS, 0.1 M $NaHCO_3$

VIII. Conclusions and Perspectives

A. Caveats

Care must be taken, as with all disciplines and techniques, to perform adequate controls for RT-QPCR. Samples must be tested a minimum of three times, and at least three different experimental and control samples must be examined for each condition to make sure that changes seen are consistent and statistically significant.

B. Multiplex Considerations

Multiplexing seems simple in theory, and at first glance it might appear that one can just combine all the necessary primers and probes in a single reaction tube. This is not true, however, because amplifications in a single tube can affect each other. For example, amplification of less efficient or abundant targets can be inhibited by amplification of more efficient or abundant targets because reaction components become limited in later cycles. This is especially important when considering target transcripts with a low copy number such as transcription factors and transcripts with a high copy number like those used as reference genes (*18S* or *GAPDH*). In order to combat this problem, reaction amounts of DNA polymerase, dNTPs, and $MgCl_2$ can be altered until the reaction is optimized. Another important consideration is that of probes and primers. Primers should all have about the same T_m = 55–60 °C and probes should be about 5–10 °C higher.

Primers and probes should all be examined for complementarity as they will all be present in the same reaction. Additionally, individual reactions should be optimized to 90–100% efficiency before attempting a multiplex reaction. Reactions should then be run both individually and in multiplex on the same plate. Comparison of C_T values for target genes should not show significant differences. If they do, reaction components should be titrated until the efficiencies indicate optimization.

Reporters are another consideration in multiplex reactions. Emission spectra must be examined to make sure that they overlap as little as possible. Check with your individual machine and reaction supplier for recommended fluorophores for multiplex reactions.

Reaction components for Multiplex Assay from BIO-RAD:

1/10 of an RT reaction that used 100 ng of total RNA

400 nM of each forward and reverse primer

200 nM each probe

200 nM each dNTP

5 mM $MgCl_2$

3.75 units of iTaq DNA polymerase

Following standard multiplex reaction conditions.

C. Method Relevance

The use of RT-QPCR is highly relevant in many systems because it allows researchers to examine real time changes in gene expression based on chosen perturbations. These changes are not simply qualitative in nature, and can many times indicate a level of transcript change much smaller than previously considered relevant. Along with the proper controls and repeats, these changes have been found to be quantifiable and statistically significant – changing the way that researchers look at their data. One of the best examples of this change is in fields that examine transcription factors. Very small changes in transcription factor level can cause massive up- or down-regulation of downstream factors, but prior to the use of RT-QPCR, the detection of the changes in transcription factor levels was difficult or impossible.

In conjunction with RT-QPCR, ChIP assays are leading the way in the adaptation of biochemical assays to chick. We now have the appropriate tools with which to better describe biologically relevant interactions of a regulatory factor with its cognate target site *in vivo*, in a highly quantitative manner.

D. Future Uses

RT-QPCR will continue to become more sensitive and accurate over time, so that a smaller amount of sample can be used per reaction and more accurate measurements will be achieved. Additionally, we assume that multiplex assays will soon be published using an avian system. As more of the chick genome

becomes sequenced and assembled, it will be possible to use ChIP and RT-QPCR to identify putative regulatory factor binding sites for many genes, making the elucidation of molecular networks a true possibility in this model system.

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