Fate maps and cell lineage analysis

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1.1. Introduction

Knowledge about the fates of cells during development is of fundamental importance to our understanding of developmental mechanisms (1). In organisms like the nematode Caenorhabditis elegans, cell number is sufficiently reduced and the embryo is small and transparent enough to make lineage analysis by direct observation of cell divisions relatively straightforward. However, in systems where cell number is greater, special techniques have to be introduced to follow cell fates.

Lineage analysis requires the introduction of markers that allow the descendants of specific cells to be identified. The marker itself must not affect developmental fate, it must be easily identified, it should be cell autonomous (not transferred between lineally unrelated cells) and, ideally, it should not become diluted by cell division. Lysinated fluorescent dextrans (2, 3) or horseradish peroxidase can be injected into single cells and the labelled progeny followed for up to about 10 cell divisions. Groups of cells can also be labelled using lipophilic carbocyanine dyes (e.g. Dil, DiO) (4); these are intensely fluorescent and therefore can be used to follow cell fate over very long periods. Markers that do not become diluted include the construction of inter-specific (e.g. quail-chick, Xenopus laevis/Xenopus borealis) or inter-strain (e.g. between different strains of mice) chimaeras, the induction of mosaic by genetic recombination (e.g. the yellow or minute loci in Drosophila), and the introduction of marker genes into single cells (e.g. lacZ; see also Chapter 20).

Here, we review some of the most widely used of these methods. The advantages and disadvantages of each are dependent on the system being studied, and their suitability for the question being addressed should be decided by the investigator, but the account given here should help in deciding an appropriate technique.
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2. Preparation of embryos of different species for fate mapping or cell lineage analysis

In principle, embryos of any animal species can be used for cell lineage analysis with the methods described here, provided that the cells of interest are accessible at the relevant developmental stage and that embryos with labelled cells can develop to the stage of interest either in whole embryo culture or in vivo. Methods for achieving these aims are described for most groups in Chapters 1–7.

3. Fate mapping with lipophilic carbocyanine dyes

A simple way to construct fate maps makes use of the recently introduced carbocyanine dyes (4), DiI (red), DiO (green), and related compounds (marketed by Molecular Probes, Inc). These are applied extracellularly and become incorporated into cell membranes because of their lipophilic nature, and they are not generally transferred between neighbouring cells. They are also intensely fluorescent. These properties make them very suitable as general markers for fate mapping. Usually the dye is made up in some organic solvent and applied through a microelectrode by air pressure so that a small group of cells (between two and 100 cells, depending on the species, stage, location, and injection conditions) becomes labelled. In our experience, descendants of the labelled cells can still be found in the chick embryo up to one week after labelling. The availability of dyes of different colours (contact Molecular Probes for the latest news on available dyes) makes it possible not only to label two or more groups of cells in the same embryo but also to control in each case for the possible transfer of dye between groups: two adjacent groups of cells are labelled, one with each of two dyes, and embryos are scored after the desired incubation period for doubly-labelled cells. We will describe the simplest method for constructing fate maps which is suitable for most species of animals.

Protocol 1. Constructing a fate map with DiI and DiO

1. Make up a stock of dye. Three methods are particularly successful:

(a) Make up DiI or DiO at 0.3% in dimethylformamide\textsuperscript{a}.
(b) Make up DiI at 0.5% and DiO at 0.25% in absolute ethanol at room temperature. Store at \(-20^{\circ}\text{C}\).\textsuperscript{b}
(c) Make up DiI at 0.2–0.3% in vegetable cooking oil (Wesson vegetable oil in the USA, Mazola in the UK).\textsuperscript{c}

2. Make electrodes. A simple way is to use thick-walled 50 μl capillary glass tubing (e.g. Sigma P1049), pulled with any standard electrode puller so that they have a very long, gradually tapering tip (very low heat and, in a vertical puller, using gravity only).

3. Break the extreme tip of the electrode (done most easily by touching the tip with a hard surface such as the back of a pair of forceps). It should still look very fine under a dissecting microscope.

4. Prepare embryos of the species and stage of choice as described in the chapter for the relevant species (see Chapters 1–7). Most species may be either in whole embryo culture or in vivo, but any system that allows easy access to the embryo and region of interest can be used. Avian embryos may be labelled either in New culture or in ovo. Mammalian embryos may be labelled in culture or in utero (the latter only for relatively late postimplantation stages when the embryo can be seen through the wall of the uterus or when the uterine wall may be opened without leading to abortion).

5. For most purposes, injection does not require special equipment. Although a pressure injection apparatus may be used (e.g. PicoSpritzer, General Valve Corp.), and the amount of dye expelled controlled very accurately in this way, this is not necessary and injection can be done through a mouth tube (e.g. Sigma A5177). If using the latter, aspirate some of the dye of choice to fill the tip of the electrode. Use separate electrodes for each dye used, if more than one, to avoid contamination.

6. Carefully expel a very small amount of dye to the region of choice in the embryo, looking under the dissecting microscope. There should be a faintly coloured, very small pool of dye at the site of injection. A very small spot will usually be sufficient. Always check under fluorescence optics to assess how many cells were labelled and their position.

7. Incubate the embryo to the desired stage and fix it in 4% formaldehyde in PBS containing 0.25% glutaraldehyde. Store them in the fixative at 4 °C in the dark but analyse labelled progeny as soon as possible. DiO, in particular seems to leach out over time, even in fixative.

8. Examine labelled progeny with conventional epifluorescence optics or using intensifying electronics (see below).

* Although this has been used successfully in some systems, we have found that this solution is toxic to cells in early avian embryos.

a It is also possible to dilute ethanol-based stocks of dye in aqueous solution, just before use. To do this, first heat up 45 μl of 0.3 M sucrose made up in crude distilled water (not deionized water) and the ethanol stock of the dye, in separate Eppendorf tubes, to 45 °C. Take up 5 μl of the warm solution of dye and quickly pipette it directly into the warm sucrose solution (not the sides of the tube). Immediately vortex for a few seconds. It should look clear and a pellet will not be seen if briefly centrifuged. This solution must be used the same day as it is made as it will come out of solution within several hours.
Protocol 1. Continued

This requires repeated heating of the dye/oil to about 45 °C for 30 min each time, alternating with vortexing for one whole minute each time. Continue this until dye has dissolved completely, which may take up to 4 h. The stock can be kept in the dark at room temperature for up to about 3 weeks without visible deterioration.

Because these dyes are lipophilic and water insoluble, embryos containing labelled cells cannot be sectioned easily without the dye spreading. Some techniques to do this have been reported in the literature, in which frozen sections of gelatin-embedded labelled embryos are cut and mounted in aqueous medium as soon as possible after cutting. However, there is a better technique which stabilizes the dye by photoconverting the fluorescence to an insoluble precipitate of dianinobenzidine, allowing conventional wax sections to be cut (Protocol 2).

Protocol 2. Photooxidation of carbocyanine-dye-labelled cells

1. Wash embryos containing labelled cells (previously fixed in 4% formaldehyde in PBS containing 0.25% glutaraldehyde) three times (10 min each or longer if large embryos) in 0.1 M Tris pH 7.4. Subsequent processing has to be done one embryo at a time.

2. Incubate the embryos in a large volume (e.g. 10 ml) of a freshly made, 500 µg/ml solution of dianinobenzidine (DAB)\(^a\) in 0.1 M Tris pH 7.4, in the dark at room temperature for 1–3 h, depending on their thickness, so as to infiltrate the tissue very well.

3. Place one embryo in a fairly deep (≈3–5 mm) glass cavity slide immersed in fresh DAB solution and place a coverslip over it. Carefully remove any excess with paper tissues.\(^a\)

4. Place slide with embryo on the stage of an epifluorescence microscope. Using a 10× or 20× objective, illuminate the regions containing labelled cells, focusing every 10 min or as required, until all fluorescence disappears completely in the field being illuminated and for a few minutes thereafter. This may take up to 1 h for each field, higher power objectives (with higher numerical aperture) being more efficient and producing less background.

5. Move the slide to illuminate all regions containing labelled cells as described in step 4 until no fluorescence remains visible in the embryo.

6. If steps 4 and 5 take more than about 2 h, replace the solution in the cavity slide with fresh DAB solution and repeat this every 2 h or so, as the solution becomes exhausted.

7. Remove embryo carefully\(^a\) from the cavity slide and transfer to a small bottle containing tap water (which helps to intensify the DAB precipitate
and to blacken it). Wash embryo three times (30 min each) in tap water, then once in distilled water (10 min).

8. Dehydrate through a series of alcohols (70%, 95%, absolute twice; 20 min each). Clear in xylene (Histoclear gives less good results) and either view/photograph with transmitted light or proceed for conventional wax embedding and sectioning.

DAB is a suspected carcinogen. Exercise appropriate care. Anything that has come into contact with DAB solution or powder must be oxidized in a solution of household bleach before discarding it.

4. Single cell lineage analysis by injection of fluorescent dextrans, horseradish peroxidase, or RNA

4.1 Intracellular pressure-injection of large cells (e.g. amphibian embryos)

4.1.1 Introduction

Amphibians have very large cells during the early stages of their development, which allows intracellular injection using air or water pressure. Fate maps and lineage analysis have been performed in the frog by injection of stable enzymes, such as horseradish peroxidase (5, 6), or mRNAs encoding these, such as lacZ transcripts encoding β-galactosidase (7), or inert tracers such as LRD or LFD (2, 3, 8). It is possible, however, that cells may take up small amounts of inert, stable, intracellular lineage tracers from dying neighbours and this possibility needs to be controlled for in any new type of experiment.

Protocol 3. General method for intracellular injection into Xenopus embryos

1. Place oocytes, eggs, or embryos in 3% Ficoll, 1× MMRa (IS injection solution) for at least 5 min before injection. IS helps the embryo to cleave in a stereotyped manner and the hypertonicity of IS will prevent the outward flow of cytoplasmic material from the injection site. Embryos should be treated with cysteine, to remove the jelly coat, prior to injection (see Chapter 5).

2. Place embryos in IS drops on siliconized and autoclaved microscope glass slides, 1–15 embryos per drop.

3. Make injection micropipette. Simple injection pipettes can be made with a basic puller and borosilicate glass micropipettesb. For injections into relatively young embryos, when cells are large, the tip must not be too fine. Beveling the needlesc will facilitate penetration and will diminish injection injury.
Protocol 3. Continued

4. Fill the micropipette. This can be done manually by introducing the material to inject through the back end of the pipette with a Pasteur pipette pulled to a fine tip. It is easier to use a pressure-driven injector (e.g., Narishige) and apply reverse pressure to fill the needle. If you have excess material to inject, fill the needle with a maximum of 3 μl. It is always better to keep materials to inject in closed tubes at low temperature rather than inside the needle at room temperature for a long period.

5. Calibrate the injection pipette. The method of calibration will depend on the injection machine used but generally division of 1 μl in the needle by injection time or needle length will do. Use sterile or DEPC-treated water to calibrate needles.

6. Place slides with embryos in IS drops on a dissecting microscope stage. Focus on the embryos and carefully bring the needle close to the embryos to inject. Depending on the pressure, some material may leak out, so equalize the pressure and keep the needle out of the IS buffer when not injecting.

7. Inject embryos by puncturing the vitelline and cellular membranes with the needle at an angle of about 90° to the vertical. Different angles of penetration and exit of the needle can cause serious damage so keep the same angle of injection on the way in and on the way out. The embryos will display a small resistance to the needle. You can provide some support by holding a pair of tweezers in the back of the embryo being injected (Figure 1). Apply pressure to release materials to inject. Do not move the needle while materials are being delivered. Dead embryos will appear very soft. If the needle becomes clogged, try shaking it gently in the IS buffer. If this does not clear it, break back the tip and recalibrate, or replace the needle.

8. After injection, collect the embryos in a Petri dish containing 10–15 ml of IS. Embryos can be transferred with a wide-bored pipette.

9. Progressively exchange the IS buffer for 0.1 × MMR over a period of about 2–3 h. The first buffer change must never be made before 3–4 cleavages occur. The hypertonicity of IS prevents the outward flow of cytoplasmic material from the injected embryo or blastomere while it heals. However, IS can cause gastrulation defects if it is left for too long. A small protrusion of tissue may be observed at the site of injection. This will not affect the rest of the embryo and can sometimes be cut out. If injecting oocytes these can be brought back to 1 × MBSH in a similar manner.

10. Normally, 10–20% of injected embryos die. Keep track of the injected embryos and discard dead ones as soon as these are recognized (large,
whitish, soft). Dead and decomposing embryos can affect the development of their neighbours. Make sure that embryos are kept in clean, sterile, and well aerated buffers.

- 10 X MMR medium. Mix together 58.4 g NaCl and 12 g Hepes. Add water to 700 ml and adjust pH to 7.6. Add 1.5 g KCl, 2.4 g MgSO₄·7H₂O and 0.4 g EDTA. Make up to 1 litre.
- For RNA injections, treat micropipettes with DEPC and autoclave prior to pulling them.
- Bevellers are commercially available but can also be made from the motor of an old record player with a 4-5 cm diameter Perspex (Plexiglass) disc on the turntable, covered with very fine abrasive paper.
- DEPC-treated water. Add diethylpyrocarbonate to water to 0.1%. Shake. Allow to stand overnight at room temperature. Autoclave 20 min. Note: DEPC is highly toxic.

4.1.2 Notes on injection in frogs

Injection of large numbers of developing *Xenopus* embryos requires a fast injection technique. A simple microinjector, such as a Singer macro-manipulator, allows, with some practice, for the injection of several hundred embryos of the same batch (*Figure 2*). The development of embryos to be injected can also be slowed down by decreasing the temperature of the buffers, thus allowing for a longer period over which to inject. A pedal to activate the pressure machinery for injection is recommended since this leaves the hands free to hold a pair of tweezers and the needle holder.

Injection volumes should be of 20–30 nl maximum per embryo. If injecting single blastomeres, the volume will have to be reduced accordingly (but you can increase the concentration of whatever you are injecting!). The time of injection is not critical but very short bursts are not recommended. Injection into one-cell embryos is best if done just after the first cleavage furrow is visible. One can inject equatorially, animally, or vegetally. Alternatively, one

Figure 2. Injection/dissection set-up.

can inject into one or two blastomeres at the two-cell stage, a time when the embryo appears to take injection trauma better. Practice and the appropriate concentration of injected materials will increase the percentage of injected embryos that develop normally. Toxicity of different materials should be carefully considered and monitored. Dextran tracers can be injected routinely at 10–50 mg/ml, plasmid DNAs and synthetic mRNAs at 50–200 µg/ml, and purified antibodies (e.g. IgG; crude serum is very toxic) at 100–500 µg/ml. However, a variety of dilutions should be tried to find the optimal concentration. Synthetic mRNAs should be purified by running a small Sephadex G-50 column followed by ethanol precipitations. High concentrations of unincorporated nucleotides are toxic. The stability of injected RNAs (9) can be generally increased by cloning the desired cDNA into pSP64T (Promega), which provides 5’ and 3’ untranslated sequences and a polyA.polyC tail (10). The localization of injected and endogenous RNAs can be determined by whole mount in situ hybridization (see Chapter 26). In general, plasmids neither integrate into the host genome nor replicate to high levels. These are maintained extrachromosomally and display mosaic distribution patterns in the embryo. Antibodies are very stable in the cytoplasm of injected embryos and display quite homogeneous distribution similar to that observed for dextran tracers (11, 12).
4.2 Intracellular microinjection into single small cells
(e.g. avian, mammalian embryos)

Successful methods for injection of tracers or nucleic acids into single cells in avian and mammalian embryos are essentially similar to those described above for other species, but the cells of these higher vertebrate embryos tend to be smaller than equivalent cells in frogs. It is therefore impossible to use air pressure to drive the dye or nucleic acid into individual cells, as the resulting increase in volume would kill the injected cell. For this reason, the material to be injected is usually expelled by iontophoresis: pulses of electrical current are used to drive the charged electrolyte into the cell, with no appreciable change in cell volume. For mammalian embryos, methods for cell lineage analysis by intracellular injection have been reviewed recently (13) and the following sections are intended to complement it.

4.2.1 Iontophoretic injection of tracers into single avian/mammalian cells in vivo

The equipment required for injection into single avian and mammalian cells, in addition to those for handling the embryos themselves, is also similar to that described above. The best type of glass for making microelectrodes for injection by iontophoresis is alumino-silicate capillary (1.2 mm outside diameter, 0.9 mm inside diameter, 10 cm long, with inside filament) manufactured by A-M Systems Inc. (catalogue No. 5820). Alumino-silicate glass requires more heat than borosilicate for pulling, and although any conventional electrode puller can be used, some (e.g. Narishige) do not seem to produce enough heat to pull this kind of glass. Any basic electrophysiological system can be used. Requirements are: a high input impedance headstage and preamplifier with current injection and resistance monitoring facilities (e.g. Digitimer Neurolog system with NL102G), modules for controlling frequency and duration of pulses to trigger the current injection module (e.g. Digitimer Period Generator NL304 and Digital Width NL401), and a suitable output device. The latter may be any digital storage oscilloscope but a less expensive alternative is to purchase a conventional (non-storage) oscilloscope and to use a digital storage adaptor (e.g. Thurlby instruments DSA511).

In addition to a manipulator to move the electrode, it is almost essential for both penetration and withdrawal of the electrode from cells to use a stepper motor controlled by a microcomputer. A cheap and convenient setup for this (SignificAt; catalogue No. SCAT-01e) is marketed by Digitimer Ltd and includes the computer (Epson HX20), interface, software, stepper motor, and even a three-axis manipulator. The manipulator and/or stepper motor drive should be mounted on a conventional (upright) microscope equipped with epifluorescence optics even for injecting non-fluorescent compounds (e.g. RNA, DNA, peroxidase), because it is always useful to include some
rhodamine-dextran to visualize the cell being injected. Most important, the
microscope should be fitted with both a low power objective (e.g. 2.5× or
4×) to locate the embryo and with a suitable higher power objective with
ultra-long working distance. For most applications, the best type of high
power objective is a 20× with a working distance of 1–3 cm (e.g. Olympus
20× ULWD). It is also important that the microscope used should focus by
movement of the nosepiece of the microscope rather than the stage. If the
stage moves, then the micromanipulator must be mounted on it rather than
on the bench. Finally, it is important for injection into small cells to control
vibration, which can cause problems. Any vibration isolator can be used. The
cheapest way of doing this, however, is to use an inflated inner tube from a
car tyre on which is placed a smooth slab of stone or metal (e.g. a heavy slab of
marble or granite from your local undertaker or churchyard). If metal, this
must be connected to earth (e.g. a water pipe) independently of the electrical
set-up to avoid an earth loop, which is dangerous.

Protocol 4. Iontophoretic injection into single avian or mammalian
cells

1. For avian embryos, prepare embryos in New culture (Protocols 3 or 4,
Chapter 6) or as if for in ovo operations (Protocol 2, Chapter 12). For
rodent embryos, set up the embryo as if for culture (Protocol 2, Chapter
7) and place it in culture medium on the lid of a 3 cm or 5 cm Petri dish.

2. Pull electrodes from aluminosilicate glass as indicated in the notes
above. Back-fill the tip with the dye or solution to be injected a by
inserting a Pasteur pipette that has been pulled to a very fine tip into the
back of the pulled electrode and expel enough liquid to fill the end of the
electrode up to the start of the shoulder (a small fraction of a
microlitre). Using a small piece of plasticine or Bluetak secured to the
wall or other vertical object, stand the electrode upright, tip down, for a
few minutes to allow any bubbles to clear. If more than 5–10 min, place
electrodes upright in an air-tight container (e.g. jam jar) humidified
with a pool of distilled water, but do not allow the electrode tips to come
into contact with the water.

3. Use 1.2 M LiCl to fill the electrode holder (e.g. holder for 1.2 mm glass,
with Ag/AgCl electrode, Clarke Electromedical) with a hypodermic
syringe and the rest of the shank of the electrode itself with a pulled
Pasteur pipette. Place the electrode in the holder, making sure to avoid
any bubbles.

4. Place the embryo under the microscope. Illuminate it with a fibre optic
source. For mammalian embryos, use a second micromanipulator,
controlling a holding pipette with gentle suction applied to hold the
embryo steady and to move it around the dish.
5. Connect the bath of saline containing the electrode to the ground connection on the headstage, using a thin, flexible wire or agar/silver wire bridge immersed in the bath.

6. Close down the illuminating aperture of the epifluorescence source to the minimum and use the small light spot to locate the embryo in the dish and to focus the microscope roughly (no need to look into the microscope). Now position the electrode tip roughly using the manipulator to be close to the illuminated spot.

7. Both the desired region of the embryo (which will only be visible if there is enough light from the fibre optic source) and the electrode tip (visible mainly from the fluorescence once the aperture is opened) should be in the field of view of the 20x objective, but the electrode tip will be at a different focal plane from the embryo. If either electrode or embryo are not in the field, adjust as necessary.

8. Check electrode resistance (e.g. by passing 1 nA pulses of current and reading the voltage on the oscilloscope; instructions for this should be found in the manual accompanying the current injection module of the preamplifier; on the Digitimer NL102G, simply turn on the switch labelled: RESIST.CHECK). Electrode resistance is calculated by Ohm’s law: \( R (\Omega) = V (V)/I (A) \). It should be between 50 and 110 M\( \Omega \).

9. Impale a cell in the region of interest using the manipulator and/or stepper motor. The oscilloscope may reveal a noisy signal when the outside of a cell is touched. If this is seen, use negative capacitance or a large current pulse to penetrate the cell (on the Neurolog NL102G, press the button labelled NEG.CAP for about 1 sec). The trace will now be much less noisy and there should be a shift in the baseline. The difference in level will reflect the resting potential of the cell, provided the embryo has been kept warm.

10. Apply a single current pulse of about 2 nA (on the Neurolog NL304, push the button labelled SINGLE). With the fibre optic illumination turned off and the fluorescence on, you should see a cell faintly filling up. If not, increase the current and apply another pulse. If maximum current is applied and the cell does not fill up, you are probably not in a cell, or the electrode may be blocked. If the latter, the trace on the oscilloscope will be noisy. Clear with pulses of current and bursts of negative capacitance.

11. Repeat steps 9 and 10 until a cell is impaled successfully.

12. Now apply 2–6 nA pulses of current, each 500 msec duration every 500 msec (1 pulse per second) for about 1–2 min or until the cell looks brightly fluorescent. Too much dye, however, can lead to compartmentation in the cell resulting in a spotty appearance of the fluorescence when the clone is examined later.
Protocol 4. Continued

13. Turn off pulses, wait 2 sec, and then withdraw the electrode as fast and as smoothly as possible. If using the stepper motor with the SCAT01e, press '0' on the Epson keyboard to do this. Look at the screen of the oscilloscope to monitor the resting potential again, which will probably be a little less than on first impalement.

14. Quickly check that the cell is still fluorescent and that only one cell has been injected. Discard any embryos in which more than one cell was labelled.

15. Incubate the embryo for the desired time (for fluorescent dyes or peroxidase, the maximum is generally 1.5–2 days, depending on the cell division rate of the cells in question; clones of up to 1000 cells can be seen with suitable image intensification) as suitable for the species being used.

16. Prior to analysis, fix the embryo as suitable for the marker being used. For peroxidase use 4% formaldehyde in PBS for 30 min at room temperature or absolute methanol overnight at −20 °C. For fluorescent dextran-amines (LRD, LFD) use 4% formaldehyde containing 0.25% glutaraldehyde in PBS for 1 h.

a Use high quality deionized water rather than a saline to dissolve the compound to be injected. If using fluorescent dextrans (e.g. rhodamine-dextran, neutral, molecular weight 10 000, lysine fixable, Molecular Probes Inc.), these are made up in deionized water at 10 mg/ml and 10 μl aliquots stored frozen until the day of use. Do not refreeze once thawed. Enzymes may be made up and stored in the same way, at a suitable concentration for each enzyme (usually around 1 mg/ml). RNA and DNA should also be made in water, which in the case of RNA should have been treated with DEPC. If injecting RNA, pretreat the electrode glass with DEPC for 1 h and autoclave it before pulling it. When injecting non-fluorescent compounds, include a small amount of any fluorescent tracer (e.g. rhodamine-dextran) in the stock solution so that injection can be monitored.

b Agar/silver bridges. Melt 3% agar in 0.9% NaCl by boiling. Fill a 15 cm length of flexible plastic tubing (≈0.35 cm diameter) with the molten agar/saline. Before it sets, insert a 5–7 cm piece of chlorinated silver wire half-way into it so that about 2 cm protrude. Allow to set at room temperature. Seal the joint with Epoxy resin and cover with heat shrink tubing. Connect the protruding end of the silver wire to the reference plug of the headstage using thin electrical cable fitted with a suitable plug for the equipment used. Silver wire can be chlorinated electrolytically in a saturated solution of KCl or dipped in molten AgCl (dipped wires will last longer than plated wires).

4.2.2 Analysis of clones in avian or mammalian embryos

The methods used will depend on the tracer selected. For horseradish peroxidase or alkaline phosphatase, the methods described for peroxidase- or alkaline-phosphatase-coupled antibodies in whole mounts or sections (Chapter 21) can be followed. Embryos can be sectioned either before or after development of the colour reaction for the enzymes.

For fluorescent tracers, mount the embryo or sections in Gelvatol (see
Chapter 21) to reduce quenching. In most cases some form of image intensification will be required. This can be a silicon intensifier target (SIT or ISIT) camera, a multi-channel plate (video intensifier) or a charge couple device (CCD) camera with Peltier cooling (which reduces the dark current) and the ability to integrate. For clones of up to about 500 cells, however, it is possible to visualize them and even to photograph them on conventional high-sensitivity colour slide film (e.g. Fuji 1600P). It is also possible to use a confocal laser scanning microscope for imaging.

4.3 Injection of fluorescent dextran into single cells in zebrafish embryos

Because zebrafish embryos are optically transparent, a single cell can be labelled with fluorescent dye and its clonal progeny mapped throughout development (14, 15). When screened in the one-day embryo, when many tissues have begun the process of differentiation, labelled cells are identified on the basis of their morphology within a tissue (14, 15). If a cell has not been identified at this time, it can be observed over the following several days until its tissue identity can be finally determined. Cells can be followed with this technique in the live embryo for up to a week.

4.3.1 General method for injection into single fish cells

The basic techniques used for dye injection into single zebrafish cells are similar to those given above for amphibians and amniotes. Electrophysiology equipment is required to penetrate the cells and to monitor the impalement, as in amniotes. Up to the blastula stage, air pressure is used to expel the dye from the electrode once impalement has been achieved, as for amphibian cells.

Protocol 5. Labelling single fish early blastula cells

Electrodes are usually made on the day before the experiment. The basic set-up for labelling requires basic electrophysiology equipment (pulse generator, preamplifier, and oscilloscope; see Section 4.2.1) and a source of air pressure.

1. Pull thin wall borosilicate glass tubing with internal filament using any suitable electrode puller, so that they have a resistance of 150–200 MΩ when filled with 0.2 M KCl.

2. Fill the tips of electrodes like these with a fresh, filtered solution of neutral tetramethylrhodamine dextran (RD), molecular weight 10 000 (Molecular Probes), at a concentration of 5 mg/ml in 0.2 M KCl.

3. Store RD-filled electrodes overnight in a moist sealed container at 4 °C. This removes air bubbles from the electrode tips.
Protocol 5. Continued

4. Mount embryos on glass depression slides in a ‘bath’ of embryo medium (see Chapter 4), and view them with Nomarski DIC optics.

5. Fill the shanks of RD-filled electrodes and the electrode holder with 0.2 M KCl.

6. Connect the bath to ground with a silver chloride ground wire embedded in agar (see footnote b, Protocol 4).

7. Break the tip of the electrode to a resistance of 30 to 100 MΩ (see Protocol 4) and apply a brief pulse of depolarizing potential (4–14 V) or of negative capacitance (see Protocol 4) to penetrate the cell membrane. Monitoring voltage with an oscilloscope helps to determine when the electrode is within a cell and whether the cell remains undamaged: a healthy resting potential is around −30 mV in fish cells.

8. Air pressure will gently expel dye into the cell. The cell should look faintly pink when viewed under Nomarski DIC optics, but at high concentrations the dye is toxic to cells.

9. Place the embryo containing the labelled cell in an agar-coated dish full of embryo medium (Chapter 4).

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* A ‘simple’ set-up consists of: stimulator (model SD9, Grass Instruments), intracellular amplifier (model 5A, Getting Instruments Inc. or Intra 767, World Precision Instruments Inc.), oscilloscope (model 2245A, Tektronix), air pressure (PV820 Pneumatic Picopump, World Precision Instruments Inc.). The equipment described in Section 4.2.1 can also be used.

* In fish, lysinated dyes are toxic. If a fixable tracer is required, make a 3 mg/ml solution of biotin-dextran (10 000 molecular weight, Molecular Probes Inc.) in 0.2 M KCl and add to the RD (5 mg/ml) solution. After injection with this mixture and subsequent development, embryos can be fixed in 4% paraformaldehyde in PBS. Biotin is then detected using the ABC kit/Vectastain from Vector Laboratories and the DAB reaction (see Chapter 21). After staining, embryos can be dehydrated in alcohol, cleared in methyl salicylate, and mounted in Permount.

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Cells in embryos older than the early blastula stage are small and do not tolerate pressure injection. Here, iontophoretic injection should be used, similar to the protocols given above for amniotes. We will point out some differences between the two.

Electrodes pulled from thick-walled borosilicate glass with internal fibre should have a resistance of about 100 MΩ when filled with 0.2 M KCl. To penetrate the cell membrane, negative capacitance or a depolarizing potential may be used. Apply pulses of current as described for amniotes or use the capacitance compensator (CAP COMP) button on the preamplifier, gently turning it up or down. The dye should be RD or RD mixed with biotin-dextran as described in Protocol 5. The cell should be visible but not brightly fluorescent.

After injection, place the embryo in an agar coated dish full of embryo medium to develop.
4.3.2 Screening for labelled clones in fish embryos

Orientate gastrula stage embryos in 3% methylcellulose in embryo medium (see Chapter 4, Protocol 2) on glass depression slides. For older embryos use either bridged coverslip chambers* or embed in 1.2% agar in embryo medium on microslides. Labelled cells can be viewed with a low light level video camera mounted on a compound microscope (see Section 4.2.2). For fish experiments, we prefer the red sensitive intensifier from Videoscope used with their Newvicon high resolution camera. Output from the video display monitor may also be relayed to a storage device, such as a high resolution video tape recorder (e.g. Panasonic AG6720), a computer (e.g. Macintosh II or Quadra, with a Quick Capture Board from Digital Design), or an optical disc recorder (Panasonic or Sony). Once an embryo has been screened, it should be removed to a dish full of embryo medium to continue development as required.

* Microcoverglass chambers: two 22 mm × 22 mm No. 1 glass coverslips are glued to each end of a 24 mm × 60 mm No. 1 coverslip. After placing an embryo in the centre in an excess of medium, a second 24 mm × 60 mm No. 1 coverslip is placed on top. The medium will hold the coverslips together by capillary action. Using this chamber, embryos can be observed from either side.

References