Chapter 14

Techniques for dye injection and cell labelling

PETER MOBBS, DAVID BECKER, RODDY WILLIAMSON, MICHAEL BATE and ANNE WARNER

1. Introduction

The introduction of compounds into cells via iontophoresis or pressure injection from micropipettes is a powerful technique of wide application in modern biology. The many uses to which this technique can be put include:

(i) Cell identification following electrophysiological recording.

(ii) Delineation of cellular architecture in anatomical studies.

(iii) Tracing neuronal pathways.

(iv) Identification of cell progeny in lineage studies.

(v) Investigations of the transfer of molecules from one cell to another via gap junctions or other routes.

(vi) The introduction of genetic material that affect protein synthesis or gene expression.

(vii) The measurement of intracellular ion concentrations, for example pH or calcium ion.

This chapter describes the techniques used to inject cells and focuses upon the design of experiments for some common applications of these methods. In the final sections, we offer sample protocols and advice on the necessary equipment.

The basic methods for cell injection are similar whatever the compound to be used. This chapter concentrates on techniques that involve iontophoresis or pressure injection using intracellular micropipettes while section 9 describes some other routes by which compounds can introduced into cells. For each application described below, we concentrate upon the factors that influence the

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PETER MOBBS, Department of Physiology, University College London, Gower St., London WC1E 6BT, UK
DAVID BECKER AND ANNE WARNER, Department of Anatomy and Developmental Biology, University College London, Gower St., London WC1E 6BT, UK
RODDY WILLIAMSON, The Laboratory, Citadel Hill, Plymouth, PL1 2PB, UK
MICHAEL BATE, Department of Zoology, University of Cambridge, Downing St., Cambridge CB2 3EJ, UK

choice of the compound to inject, since this is usually the factor most crucial to success.

2. Microinjection methods

Manufacturing micropipettes

Pipettes for intracellular microinjection can be produced on any standard microelectrode puller. The best pipettes generally have the following characteristics: (a) a relatively short shank (b) a relatively large tip diameter. The latter is frequently a limitation because, for successful penetration of small cells without damage, the tip diameter also must be small. When the diameter of the tip is small then both the iontophoresis and pressure injection of compounds is impeded, the former by the charge on the glass and the electrical resistance of the tip and the latter by the tip's resistance to bulk flow of solution. Several different types of glass are available for the production of micropipettes. A number of manufacturers (see appendix B) provide suitable capillaries with a variety of outside diameters, with thick or thin walls, with and without internal filaments, made from soda or borosilicate glass. Pipettes made from thick-wall borosilicate glass are usually the most robust and useful for penetrating tough tissue. However, thin-wall glass has the advantage that the channel through the tip is usually larger, and thus the resistance is lower, for any given tip size. The characteristics of micropipettes for use in microinjection experiments can sometimes be improved by bevelling (see Chapter 11). Soda-glass is somewhat less fragile than borosilicate glass but is difficult to pull to fine tips, it has been dropped from some supplier's lists. No matter what the theoretical expectations, the best electrodes to use are those that work!

Pipette filling

Modern micropipette glass incorporates an internal 'filament' (actually a second narrow capillary). The filament increases the capillarity of pipettes so that fluid is drawn into the tip. This characteristic can be exploited to enable very small volumes of fluid to be loaded into the pipette tip, which is useful where the compound to be injected is expensive. Solutions can be introduced into the back of the pipette either by immersion or by bringing into contact with a drop of fluid. The volume drawn into the tip depends upon its diameter. Pipettes with tips of 1 μm will draw up about 100 nl and those of 5 μm will fill with about 1 μl of fluid. Coarse pipettes can be filled by sucking fluid directly through the tip. Electrical connections to pipettes in which only the tip is filled can usually be effected simply by sticking a wire into the pipette lumen. The presence of a thin trail of electrolyte along the outside of the internal filament provides the necessary path for current flow. It is advisable to centrifuge all solutions before use to remove material that may block the tip.

Iontophoresis

Iontophoresis involves the ejection of a substance from a pipette by the application of

current. The polarity of the ejection current employed depends on the net charge on the substance to be injected (negative pulses are used to eject negatively charged molecules). Most modern microelectrode amplifiers are equipped with a current pump that can be used to provide an iontophoretic current that is, within limits, independent of the electrode resistance (see Chapters 1 and 16). If only a simple amplifier is available, or the current pump is unable to provide sufficient voltage to drive the required current through the electrode tip, then it is possible to use a battery and a current limiting resistor as a current source. If a battery is employed then the headstage of the amplifier should be switched out of the circuit when the battery is connected. Obviously the current provided by this crude arrangement will be governed by Ohm's Law. The current applied to a cell should be as small as is consistent with the introduction of sufficient of the compound into the cell. In all events the voltage produced by the passage of the iontophoretic current must be limited (to say +100 to −100 mV) to avoid damage to the cell membrane.

Continuous application of current should be avoided since it often causes the electrode tip to block. This block can sometimes be relieved by reversing the polarity of the current for a short time. However, once an electrode shows signs of block the trend is usually irreversible and the pipette should be discarded. Often the best strategy is to employ short duration current pulses of alternating polarity. Whatever the form of the pulse, small currents for long periods are usually more successful than high currents for shorter times. To recognise electrode block and standardise procedures, it is essential to monitor the *current* flow through the electrode. It is not sufficient simply to monitor the voltage applied to the electrode! If the amplifier employed does not have a current monitor then a simple one can be improvised by measuring the voltage drop across a resistor in the earth return circuit. The membrane potential of the cell should be measured during electrode insertion, before switching to current injection. It is sensible to check the condition of the cell by measuring its resting potential at intervals during iontophoresis. Such measurements are simplified by using a bridge amplifier (see Chapters 1 and 16) that enables the membrane voltage to be monitored continuously during current passing experiments. For a detailed discussion of the circuits for current injection and current monitoring see Purves (1981).

A useful technique for achieving bulk flow from the electrode tip is to cause high frequency oscillations of the voltage across the electrode resistance. This is achieved by pressing the 'buzz' or 'zap' buttons present on some amplifiers. The effect of these can be imitated by turning up the capacity compensation control, found on nearly all microelectrode amplifiers, to the point at which the electrode voltage oscillates (termed 'ringing').

In theory the amount of a substance ejected from the pipette during an iontophoretic pulse can be estimated from a consideration of its transport number (Purves, 1981). In practice, these estimates are highly unreliable and the transport number is often unknown for the compound employed.

Pressure injection

Pressure ejection is the method of choice for the injection of neutral molecules and

those of low iontophoretic mobility. Commercial pressure injection devices are available (see list of suppliers) that enable the application of calibrated pressure pulses to the back end of the injection pipette. Essentially a pressure injection system consists of a gas cylinder connected, via a timing circuit, a solenoid-operated valve and a pressure regulator, to a side-arm pipette holder. Commercial equipment is expensive, but a home-made rig can be simply made from the components listed above. The timing circuit can be replaced by a manually operated switch. Take care to ensure that the connections and tubing are safe at the pressures employed and that the pipette is firmly held within the holder. The pressure and timing of the pulse can be roughly established by measuring the diameter of a drop expelled from the pipette tip into a bath of liquid paraffin. However, this method frequently over-estimates the back-pressure from the cytoplasm and quantification of pressure injection is often as uncertain as in iontophoresis.

Patch-pipettes

Many substances can be introduced into cells from patch-pipettes while recording in the whole-cell mode. The concentration that a compound reaches within the cell during whole-cell recording is equal to that within the patch-pipette solution. Thus for most dyes and labels the concentrations to employ are a fraction of those used in iontophoresis or pressure injection experiments. For example, Lucifer Yellow CH incorporated into the patch-pipette solution at 1 mg ml^{-1} will produce intense fluorescence of the cell (40 mg ml^{-1} is used in sharp electrodes for iontophoresis; Fig. 1B).

3. Techniques for visualizing cells

Visualizing cells prior to injection

In order to inject a cell you must be able to guide your micropipette toward it. There are three techniques available to aid in the steering of electrodes:

(a) Stereotaxic movements combined with continuous electrical recording (mainly used for penetration of cells in brain nuclei).

(b) Visual guidance using white light and interference contrast optics to visualize the cell and identify targets.

(c) Visual guidance using cells prelabelled with fluorescent dyes as the target.

In solid tissue, whatever technique is chosen to guide the electrode, the target must lie along initial trajectory of the electrode. Manipulation out of this axis will break the electrode.

1. *Stereotaxis*. This method requires that you know precisely where your target cells lie even though you can not see them. Such information is sometimes available from stereotactic atlases. Micromanipulators can be roughly calibrated to give depth measurements but errors always arise as a result of tissue distortion during electrode penetration. The identification of the target cells can sometimes be achieved through knowledge of their electrical properties or synaptic connections,

for example by the response to current injection or stimulation of a peripheral nerve.

2. *Interference contrast optics.* Phase contrast and differential interference contrast techniques (Nomarski) are good for visualising living cells. Phase contrast is useful for cells in tissue culture but does not work well for tissue slices. Nomarski optics provide high resolution and can be used to provide effective optical sections of transparent tissue. The more recently introduced Hoffman optics are cheaper than Nomarski optics and are useful for viewing tissue slices because they provide a greater depth of field.

3. *Prelabelling with a fluorophore.* There are two approaches to the prelabelling of cells to identify them as targets for subsequent microinjection experiments. Cells can either be bathed in a dye that becomes internalized (Fig. 1E), or labelled by retrograde transport of a marker from their axons (Fig. 1A). Whilst some dyes are either actively taken into cells or simply diffuse across the membrane others only enter if the membrane is disrupted by osmotic shock or through exposure to dimethyl sulphoxide. Whatever the method of prelabelling, the choice of the label is crucial to success. Ideally the label should be visible under the same filter set as the dye used in subsequent injection experiments and the intensity of the prelabel's fluorescence should not mask that of the injected fluorophore. Since the prelabel may remain inside the cell for an extended period, it is important that it is non-toxic.

Retrograde labelling of neurons via their axonal projections is an extremely useful means of identifying populations of cells that project to particular targets. Fast blue and diamidino yellow are amongst the most popular of the labels available for this purpose. Fast blue labels the cell cytoplasm and diamidino yellow stains the nucleus (Fig. 1E). Both pass rapidly across the cell membrane and can be used to label cells from their axon terminals or from cut axons. General labelling of all the cells in a tissue can be achieved by bathing in a dilute solution of the dyes. Both of these dyes work well on formaldehyde-fixed tissue. Target cells identified with these prelabel dyes can subsequently be injected with Lucifer Yellow, carboxyfluorescein or Cascade Blue which are visible with the same filter set (Fig. 1E).

Some fluorophores with useful properties are neither taken up nor transported by cells. However, they can be made into useful labels through conjugation to lectins, dextrans or plastic microspheres. Lectins bind to sugar moieties on the cell membrane, are brought into the cell through endocytosis and transported. Dextrans can also be conjugated to most fluorophores. Plastic microspheres can be coupled to fluorescent molecules. They are available in a variety of materials and sizes. Applied to damaged axons they are taken up by and retrogradely transported. Microspheres are visible in the electron microscope.

Visualizing labelled cells

The object of many microinjection experiments is to render the cell under study visible by introduction of a label. The majority of such labels are either fluorescent or can be processed to produce a coloured reaction product. Below we describe the techniques for visualizing and recording the results of cell labelling experiments.

Often labelled cells can be visualised without any histological processing and some labels can be used to follow changes in cell morphology that occur over extended periods of time (Purves *et al.* 1986). Methods for the fixation of tissue and the histological processing of tissue containing labels are given later.

Fluorescent labels are excited by light at one wavelength and emit light at another longer wavelength. The user must choose the excitation and emission filters most suitable to their application (see appendix A). It is convenient to have the microscope used for positioning the electrode equipped with a light source and filters capable of exciting the label. This allows the user to determine the endpoint of the injection experiment by observation. Many of the labels in common use are excited by far blue or UV light. The tungsten or quartz halogen bulbs found in most microscope illuminators do not provide much light at these wavelengths and an additional mercury or xenon light source is required. Most manufacturers provide some convenient means for switching between the white and UV light sources. If this switching mechanism is to be used during the course of a labelling experiment, it is imperative that it operates without vibration if the microelectrode is to remain in the cell under study.

The factors involved in the choice of the optics and light sources for fluorescence microscopy are complex. The short discussion below is offered as an introduction that may be supplemented by consulting some of excellent free literature provided by major manufacturers (see for example the booklets offered by Zeiss, Lieca and Nikon). Mercury lamps are cheaper than xenon lamps. However, the emission spectrum of a xenon lamp is relatively continuous throughout the UV and visible spectrum while that of mercury lamps consists of a series of sharp peaks (emission lines). With mercury lamps, it is important to ensure that a line exists at a wavelength appropriate to the dye in use. Most modern fluorescence microscopes employ epi-illumination, a system in which the light used to excite the dye is focused on the specimen through the same objective used to view the light emitted by the dye.

The choice of objective is critical in fluorescence microscopy. Quartz objectives pass much more short wavelength light than those made from glass. However, quartz objectives are expensive and unnecessary for use with dyes excited by light in the visible and near UV regions of the spectrum. It is crucial that the objective has a high numerical aperture (NA) since both the intensity of the light focused on the specimen and the light gathering power of the lens increase with the square of the aperture. An objective with an NA of 1.0 will yield 16 times as much light as a 0.5 NA lens. High NA objectives have shorter working distance and need an immersion medium - water, oil or glycerol (for UV). For injection of cells in thick preparations on an upright microscope water immersion lenses are preferable to those that work in air because they have a greater NA and there is no optical distortion due to meniscus effects of the micropipette on the bath surface. On the other hand, very long working distance air electrodes can be covenient, if optically inferior. Two particularly useful lenses are Zeiss ×40 0.75 NA W water immersion and the Nikon ×40 ELWD air (NA 0.5) with correction collar. Intensity of fluorescent light also depends upon the magnification. It

decreases as the square of the magnification: a ×10 eyepiece produce an image of 25% the intensity of an image formed by a ×5 eyepiece. Low magnification eyepieces are therefore preferable for visual observation.

Fluorescent images can be recorded on film or by analog or digital video techniques. There are many black and white, colour print and transparency films suitable for recording fluorescence images. Generally a film of high speed and acceptable grain should be chosen. Colour films of speed greater than 400 ASA tend to be too grainy, however, black and white films such as Kodak's TMAX give excellent results even at 2400 ASA (must be developed in TMAX developer). In normal photography, the reciprocity law applies and the total amount of exposure is given by the product of the luminance and the exposure time. Thus an exposure of 1/60th of a second at f8 is the same as for 1/30th at f11. With dim objects the reciprocity law fails to predict the exposure and the exposure time has to be increased. Most film manufacturers provide a guide to the performance of their films at low light intensities. In practice it is often better simply to take several exposures of increasing duration starting with the exposure time indicated by the meter on the camera.

The advent of cheaper video cameras that operate at low light intensities has opened up the possibility of recording fluorescent images either on video-tape or in digital form on a computer. Digital image recording has the advantage of allowing complex analysis of an image.

Labels that result in a coloured or opaque reaction product are much simpler to photograph than those labelled with fluorescent compounds. No special equipment is required.

4. Labelling cells for subsequent identification and for determination of overall cell architecture

Dyes injected for these purposes should have the following properties: (a) they should be visible, either immediately or after chemical reaction; (b) they should remain in the injected cell, either because they are too large to move across the cell membrane and through gap junctions or because they are strongly bound by the cytoplasm; (c) they should not be toxic, although this requirement can be relaxed if the tissue is to be processed immediately after the cell has been injected; (d) they should be stable and not break down to give products with different properties; (e) they should withstand histological processing. In practice, property (e) is the most difficult to achieve.

Six classes of compound are used for this purpose:

1. *Inherently fluorescent molecules and those tagged with a fluorescent probe.* Lucifer Yellow (MW 457) and carboxyfluorescein (MW 376) are the most popular fluorescent compounds for determining overall cellular architecture. However, they are far from ideal for this purpose. Both pass through gap junctions (see below) and carboxyfluorescein cannot be fixed. Lucifer Yellow withstands fixation well but as

with all other dyes some fluorescence intensity is lost. Passage through gap junctions can be prevented by conjugation of the fluorophore to dextrans. Dextrans (MWs 3000-70000) can be coupled to fluorescein, rhodamine isothiocyanate or Texas Red. They can be prepared in the laboratory (see Gimlich & Braun, 1985) or purchased commercially (Molecular Probes, 48-49 Pitchford Avenue, Eugene, Oregon, OR97402-9144 USA). Cascade Blue and sulphrhodamine 101 are also useful for determining cellular architecture and extend the range of colours available for double marking experiments. For examples of multiple labelling see Fig. 1D,F.

Advantages:

Can be pressure injected or iontophoresed.

Can be seen in living cells with appropriate fluorescent illumination.

Are not toxic provided the amount injected is kept fairly low.

Do not break down.

Will withstand routine fixation and embedding techniques, provided the fixative or mountant does not generate auto-fluorescence. Glutaraldehyde fixation, for example, must be avoided. Many commercial mountants, such as DPX, are unsuitable for this reason. Mountants that are designed to reduce fading can now be obtained (e.g. Citifluor, City University, London).

Disadvantages:

Limit of detection determined by threshold of fluorescence. Detection levels can be improved by electronic image intensification.

Fluorescence fades under continuous illumination. This can be reduced by using anti-fade mountants.

Fluorescein fades particularly fast, but is more fluorescent than rhodamine or Texas Red.

Sometimes become incorporated into cellular organelles with time, making fluorescence particulate.

Margin between visible not toxic, and visible but toxic is narrow.

2. *The carbocyanine dyes.* Octadecyl(C_{18})-indocarbocyanine (DiI) and oxycarbocyanine (DiO) (MWs 934 and 882) are highly fluorescent lipophilic compounds. They dissolve in, and diffuse throughout, the lipids of the plasma membrane. They are not toxic and they have been reported to remain in the cell membrane for up to one year (Kuffler, 1990). They will also diffuse along membranes in lightly fixed tissue. In the absence of any sites of membrane fusion the carbocyanines label single cells. The diffusion rate for these compounds is slow (about 6 mm/day, slower in fixed tissue), however, carbocyanines with unsaturated alkyl chain segments (FAST-DiI and FAST-DiO) exhibit accelerated diffusion rates. The polyunsaturated "DiASP" compounds (N-4(4-dilinoleylaminostyryl)-N-methylpyridinium iodide and related molecules) (MW~800) are also reported to diffuse more rapidly. Because the carbocyanines are insoluble in water they must either be pressure injected into cells in solution in

DMSO or alcohol or applied to the cell membrane in which they rapidly dissolve. DiI and DiO can be visualized by fluorescence microscopy. DiI has similar excitation properties to rhodamine, excited by green it fluoresces red. DiO is similar to fluorescein in that it is excited by blue light and produces green fluorescence. DiAsp has a broad excitation spectrum and fluoresces orange. These dyes can be converted into a permanent reaction product via the Maranto reaction (Maranto, 1982) in which the singlet oxygen released by illumination is used to oxidise diamino-benzidine (DAB).

Advantages:

They are not toxic and can remain in the cell membrane without harm over several years.

Disadvantages:

Not water soluble.
They tend to fade quickly particularly in laser scanning confocal microscopy.
Long diffusion times.
Can only be pressure injected.

3. *Enzymes such as horse radish peroxidase.* Horse radish peroxidase (HRP) is reacted with diamino-benzidine or other chromogens to generate a product visible in the light or electron microscope. There are many protocols for developing HRP (see Mesulam, 1982 and Heimer & Robards, 1981 for a selection). Widely used in studies in the central nervous system. The injection of enzymes can also be used to kill individual cells (e.g. pronase). This is potentially useful in lineage and regeneration studies.

Advantages:

Can be pressure injected or iontophoresed.
Not toxic.
Remains within the injected cell, provided the preparation is free from micro-peroxidases. Will cross synapses, which can be useful when tracing pathways.
Does not break down.
Good visibility.
Reaction product visible in the electron microscope.

Disadvantages:

Can only be seen after reaction product produced. However, by using a fluorescent peroxidase conjugate, such as RITC-peroxidase (Sigma P5031), an indication of the staining can be obtained during the fill period (see Fig. 1A-C).
Can get reaction product from endogenous peroxidases, so method has to be modified if this is likely to be a problem.
The penetration of chromogen into tissue is rather poor (about 100 μm), so that whole mounts or slices have to be below this thickness.

Much of the enzyme activity is lost on fixation. If possible the material is best fixed after reaction.

4. *Biocytin*. A recently introduced intracellular marker (Horikawa & Armstrong, 1988) comprising a highly soluble conjugate of biotin and lysine (MW 372.48) that has a high binding affinity for avidin. The injected biocytin is visualised by attaching a label to avidin, e.g. a fluorescent label such as FITC or rhodamine, or a chromogenic enzyme such as HRP. Suitable avidin conjugates are widely available (e.g. Sigma, Vector Labs.). A small molecular weight biotin compound, biotinamide (MW 286), is also available (Neurobiotin, Vector Labs, 16 Wulfric Square, Bretton, Peterborough PE3 8RF, UK) and may be easier to inject (Kita & Armstrong, 1991).

Advantages:

Highly soluble in aqueous solutions.
Can be pressure injected or iontophoresed.
Low toxicity.
Does not break down.
Good fluorescent, visible light, or electron microscopic visibility after avidin reaction.

Disadvantages:

Can only be seen after avidin reaction.
Reaction penetration limited to about 100 μm even with detergents or surfactants so tissue may have to be sectioned.
Some ultrastructural degradation from penetration agents.
Can pass between coupled cells.
Occurs naturally in trace amounts.

5. *Heavy metals such as cobalt and nickel.* The metal is precipitated with ammonium sulphide or hydrogen sulphide. The sensitivity can be improved by intensification with silver (Pearse, 1968; Bacon & Altman, 1977). Double labelling can be achieved by using different metals in the same preparation followed by precipitation with rubeanic acid (Quicke & Brace, 1979); this results in precipitates of different colours depending on the metal, e.g. cobalt = yellow, nickel = blue, copper = olive.

Heavy metal complexes, such as lead EDTA (Turin, 1977) can be suitable in cells that are not linked to their neighbours by gap junctions (see later section). In principle, it is possible to prepare a range of heavy metal complexes of different sizes so long as the complex is firmly held, so that there is no free metal or anion which might be toxic, and the metal has a much higher affinity for sulphide than for the anion used to make the complex. This is essential to ensure precipitation of the metal out of the complex. The advantage of a heavy metal complex is that the complex can be much less toxic than the heavy metal itself and may be much easier to eject from the pipette. However, some metal sulphides will re-dissolve if the precipitant (usually ammonium sulphide) contains polysulphides. Freshly prepared solutions saturated with H_2S do not suffer from polysulphide formation.

(i) Cobalt and nickel

Advantages:

Can be iontophoresed or pressure injected.
Strongly bound to cytoplasm, therefore retained in the cell despite small size.
Good visibility after reaction, very good after intensification.
Very good in whole mount, because the sulphide precipitation step permeabilizes the cells. Electron opaque, so product visible in the electron microscope.
Will withstand fixation.
Multiple labelling possible with rubeanic acid precipitation.

Disadvantages:

Electrodes liable to block and require a high current for a long period to eject sufficient cobalt. Nickel filled electrodes suffer less from this.
Treatment with sulphide compounds interferes with cytological appearance.
Toxic, therefore only suitable when precipitated immediately after injection.

(ii) Lead EDTA (as an example of a heavy metal complex).

Advantages:

Very easy to inject iontophoretically or by pressure.
Not toxic; very well tolerated by cells.
Electron opaque.

Disadvantages:

Moves easily through gap junctions therefore not suitable if the cell is linked to others by electrical synapses or gap junctions.
Requires intensification to improve sensitivity.
Sulphide treatment spoils cytology.

6. *Compounds tagged with radioactive label and then visualized with autoradiography.* In principle any suitable molecule can be labelled. Large proteins can be tagged with ^{125}I, but a tritium or carbon label is preferable if autoradiography is to be used. Proline has been useful in studies of the central nervous system; like Horse Radish Peroxidase, proline is transported trans-synaptically and can therefore trace extensive, interlinked neuronal pathways.

Advantages:

Not toxic provided total radioactivity kept low.
Compound normally present in the cell can be used.
Permanent preparation, no fading.
If appropriate compound (usually one that is not normally found within cells, e.g. deoxyglucose) is chosen, no breakdown.
Very good for tissue cultured cells, because no sectioning required.

Disadvantages:

Label only withstands fixation if compound is bound to cell contents.

If a naturally occurring molecule is used, it may be broken down by cell metabolism.
Can only be used on tissue sections.
Potential delay in obtaining results introduced by autoradiography.

5. Identifying the progeny of the labelled cell (lineage tracing)

This technique is used extensively in developmental biology, as a way of analysing the prospective fate of a cell and its progeny at different stages of development. The technique also can reveal the extent to which the progeny of the injected cell remain as coherent clones and so provide valuable information on the degree to which cells mix during development. The most important factor when selecting a suitable compound as a lineage label is the degree to which the label is diluted during cell division and growth. Cell labelling by injection is, therefore, often only suitable at certain stages of development, when cell division and growth are relatively slow. This method of determining lineage has been most successful in the amphibian and leech embryos, because early development in these animals involves reduction in the size of each cell without extensive growth, so that the cytoplasm of the egg is gradually partitioned into smaller and smaller units. In species such as the mouse, where the embryo arises from a very small number of cells formed during the early cleavages (most of which contribute to extraembryonic structures), there is extensive growth and cell division causing dilution of the label. In this situation, other methods that rely on cell autonomous labels, such as differences in enzymes, have been more successful (see Gardner, 1985). The incorporation of self-replication defective retro-viruses into the genome of a host, as yet undifferentiated, cell is proving useful as a way of providing a cell autonomous label.

1. *Fluorescently labelled compounds such as labelled dextrans.* The overall properties of these compounds are dealt with above. The specific advantage for cell lineage studies is that fluorescent compounds can be observed in living cells, provided the level of illumination is kept low. This means that the way in which the fluorescent cells are distributed in the embryo can be followed sequentially in living specimens. In order to avoid damage from illumination, it is sensible to use an image intensifying system. Dilution is not a serious problem in the amphibian and leech embryos and these compounds remain at an analyzable level for two or three days of development. They have been applied also to studies of the zebra fish embryo, an increasingly popular model system for the study of developmental processes.

2. *Horse Radish Peroxidase (HRP).* This has been used extensively in the amphibian embryo (e.g. Jacobson & Hirose, 1978) and more recently in *Drosophila* (Technau & Campos Ortega, 1985). Its advantages and disadvantages are as above. In amphibia the degree of dilution is much the same as for lysinated Dextrans. Some caution should be exercised since there have been reports (see Serras & Biggelaar,

1987) showing that HRP can induce an exocytotic/endocytotic cycle, which causes artefactual transfer of HRP and any other compound injected with it.

3. *Radioactively labelled molecules that are incorporated in DNA and/or RNA.*

Advantages:

Label is permanent.
Will withstand fixation.
As long as the precursor is available to be incorporated into DNA or RNA the label will not be diluted out.

Disadvantages:

Precursors to DNA and RNA, such as small nucleotides, may not be restricted to the injected cell.
Labelled breakdown products may not be restricted to the injected cell.
Once all available label is incorporated, dilution occurs at each division.
Levels of radioactivity, and therefore concentration of precursor, have to be kept low to reduce radiation damage. This exacerbates the dilution problem.
Only usable on sections or very thin whole mounts.
May be long interval between experiment and obtaining results.

4. *Labelled proteins, which are usually tagged radioactively.*

Advantages:

Foreign protein can be used, so reducing likelihood of breakdown.

Disadvantages:

May require special fixative to ensure the protein will withstand histological processing.
Foreign protein may be toxic, or be handled by cell metabolism in an unpredictable way.
Naturally occurring proteins may be broken down into small metabolites which could leave the cell.

5. *Incorporation of viral or foreign DNA and recognition of the products of expression of the foreign genes.* This technique is expanding rapidly, and has been successfully used in tracing the lineage of some cells in the vertebrate nervous system. Lineage studies have depended on deficient retro-viruses, modified so that they can no longer spontaneously replicate and can therefore transfect only one cell. Since single copies only are incorporated into the host cell, the virus may insert only into one copy of cellular DNA and so replicate within 50% rather than 100% of the progeny of the transfected cell. This complication often is inadequately recognized. Transfection of single cells frequently is achieved by the injection of virus into the extracellular fluid (such as the cerebro-spinal fluid) at very low concentration. This technique relies on dilution by the CSF to reduce infection level and thus requires careful controls to ensure that single clones are chosen for analysis.

The generation of chimeric embryos also has given information on lineage. In this

case either a single cell, or group of cells, may be injected into the blastocoel cavity of the mammalian embryo, become incorporated into the embryonic and extra-embryonic lineages, and are then recognized at specified intervals after injection. Alternatively aggregation chimaeras of whole embryos can be made. There are a number of strategies for recognizing the foreign cell(s) and the progeny. These include: (i) incorporation of genetic material that puts expression of, for example, the enzyme alkaline phosphatase under the control of the promoter for the foreign gene and (ii) molecular recognition (by *in situ* hybridization) of DNA specific to the injected cells.

Advantages:

Can be injected by iontophoresis (because of overall charge on DNA and RNA) or by pressure injection.

Label is autonomous and is amplified at each cell division. This is undoubtedly the major advantage of the approach because it eliminates the problems associated with dilution at each cell division.

If the appropriate gene is selected the product will be retained within the cell.

Disadvantages:

In order to make the product of gene expression visible some reaction step is likely to be required.

The label is unlikely to withstand fixation so that frozen sections, or permeabilization of the labelled cells may be necessary before reaction.

Expression of the foreign or viral gene may not be uniform throughout all the progeny of the injected cell because of difficulties with transcription or translation, or because expression of the foreign gene is subject to controls on gene expression exerted during development, which may be tissue or product specific. The site at which the foreign DNA is incorporated into the host genome cannot be controlled; this may lead to aberrant expression patterns and/or differentiation (see below).

6. Studying cell-cell communication

One of the commonest uses of dye injection is to determine the ability of cells to communicate with each other. The experiments may require simple determination of the presence or absence of cell-cell communication, or may be directed towards determining the size range of molecules that can be exchanged. This section considers the problems involved in determining direct cell-cell communication; that is, specifically, the exchange of small molecules from one cell to the next without recourse to the extracellular space, through the morphologically identified structure, the gap junction. The properties of gap junctions are discussed extensively in the literature; a useful start may be obtained by examining recent reviews (e.g. Seminars in Cell Biology Ed: Gilula, 1992). Transfer from cell to cell also can occur through the extracellular space, as with molecules like HRP, which can cross synapses,

probably because they are successfully exocytosed and then endocytosed by adjacent cells.

The requirements of suitable molecules for tracing pathways of gap junctional communication are necessarily very different from those associated with lineage studies or labelling for subsequent identification. The *size* and *charge* of the injected molecules is of importance, because this will determine whether the molecule moves from one cell to the next. The most sensitive way of recognizing communication through gap junctions is to examine the spread from one cell to the next of injected current, where the voltage change induced in neighbours of the injected cell by injection of a current pulse reflects the ability of small ions to move through gap junctions. Because gap junctions allow the transfer of a range of small molecules (MWs generally less than 1000) in addition to small ions, the injection of dyes allows the upper limits of gap junction permeability to be explored. When working near the cut-off limit, dye transfer is the most useful technique, because it can reveal relatively small differences in permeability. It is important to recognize that the lower limit of available methods for detection of the selected compound can determine whether transfer from one cell to the next is recognized. Dye transfer is, therefore, inherently less sensitive than electrophysiological methods and failure to observe transfer may be a reflection of the detection method, rather than the permeability of gap junctions.

The major requirements when selecting compounds to examine the permeability of gap junctions are: (a) the compound should be visible at the time of injection; (b) it should be freely diffusible in the cytoplasm, so that transfer from cell to cell is not limited by binding; (c) preferably the compound should withstand fixation, so that the distribution can be examined in greater detail at the end of the experiment, possibly in sections; (d) it should not be toxic; (e) it should not influence intracellular pH, intracellular free calcium or intracellular cyclic AMP because the permeability of gap junctions is sensitive to pH, Ca^{2+} and cyclic AMP; (f) it should not influence the properties of the junction itself; (g) ideally the size and charge of the molecule should be known. In practice the molecular weight is often used as an indicator of size**,** because the degree of hydration and shape of the injected molecule are not available; (h) the injected compound should not be able to cross the surface membrane of the cell, so that entry into cells cannot take place if dye leaks into the extracellular space from the pipette or from damaged cells.

A large number of compounds have been used to trace the degree and pattern of cell-cell communication through gap junctions. Few of these compounds possess all the desirable characteristics. A useful discussion of the approach to synthesizing compounds with the appropriate properties can be found in Stewart (1978) and Stewart & Feder (1985). However, good chemists with an interest in generating suitable compounds are in short supply and most workers have proceeded on a trial and error basis. The reagents currently in most common use are:

1. *Fluorescein and 6-carboxy fluorescein.* Low molecular weight (fluorescein: 332) highly fluorescent compounds.

Advantages:

Easily injected iontophoretically or by pressure.

High quantum yield on excitation, so that low levels of dye can be detected easily.

Not toxic.

Not bound to cellular components.

Disadvantages:

Will not withstand fixation, so can only be used in live preparations.

Can cross cell surface membranes, although 6-carboxy-fluorescein is better in this respect.

2. *Lucifer Yellow* (MW 457). Two versions of this dye were originally available: CH and VS. Most published papers use the CH form; when no indication is given it is likely that the CH form has been used. A detailed description of the properties of these dyes is given in the two Stewart references (see above), which also indicate the variety of purposes for which Lucifer Yellow may be used. Lucifer Yellow, introduced in 1978, remains probably the most popular dye currently in use. It has proved a useful dye for developmental studies because the transfer of LY seems to be particularly sensitive to regional differences in gap junction properties so that its transfer can be restricted even when electrical coupling and the transfer of other molecules is not (e.g. Warner & Lawrence, 1982; there are now many examples in the literature). Several new forms of Lucifer Yellow are now available (see the Molecular Probes catalogue for details).

Advantages:

Easily injected by iontophoresis or pressure.

Highly fluorescent.

Diffuses through the cell rapidly, although it does become bound to cell contents and particularly nuclei with time.

Not toxic.

Withstands fixation, provided formalin or formaldehyde fixative used.

Permanent preparations can be made with an antibody to Lucifer Yellow (Taghert et al. *1982).*

Will react with diamino-benzidine in the presence of irradiating light to give an electron dense product (Maranto, 1982).

It can be injected into cells after weak formaldehyde fixation. The prefixation technique can be useful for examining the structure of small cells that are liable to excessive damage by penetration of the electrode when alive.

Disadvantages:

Forms an insoluble precipitate with potassium so that electrodes must be backfilled with lithium chloride when iontophoresing. This is not a problem for short term experiments, but can lead to problems when injecting early embryos because lithium is extremely teratogenic at low intracellular

concentrations. The potassium salt of Lucifer Yellow can be obtained from Molecular Probes, but it is much less resistant to fixation than the lithium salt.
Electrodes tend to block during iontophoresis, probably because Lucifer in the electrode tip is precipitated by potassium ion from the cytoplasm. The block can be temporarily relieved by applying depolarizing pulses.
Binding to cell components means that Lucifer is only available for transfer to adjacent cells for short periods of time, so that its distribution at longer times is not simply a reflection of the presence of gap junctions.
Some fading on irradiation.
Some loss of dye on fixation.
Illumination for long times leads to damage from singlet oxygen (but see below).

Other uses for Lucifer Yellow: The damage induced by over-irradiation provides a useful way of precisely killing a single cell.

3. *Tetramethyl Rhodamine Isothiocyanate (TRITC)/sulphrhodamine.* Both dyes are excited by green and emit red light. TRITC (MW 444) is poorly fluorescent, toxic and strongly bound, and therefore not a dye of choice, but can be useful if another label is to be used simultaneously. Sulphrhodamine 101 (MW 607) is a better choice, it has a high quantum yield and fades only slowly. It does not pass through gap junctions as fast as Lucifer Yellow or Cascade Blue but can be useful in multiple-label studies.

4. *Cascade Blue.* A relatively new dye available from Molecular Probes which shares many of the properties of Lucifer Yellow. Versions are available with MWs between ~600 and 700. These dyes are excited by near-UV light and fluoresce blue with a high quantum yield. They can be usefully combined with Lucifer Yellow in multiple-labelling experiments. Remains visible within fixed tissue.

5. *Biocytin/Neurobiotin* (see earlier for properties). These small molecules (MW 372/286) have proved useful as tracers of gap junctions because they are sufficiently small to give a diffusion pattern much closer to that predicted by electrical coupling studies than observed with Lucifer Yellow. This has proved particularly advantageous in the central nervous system, where both biocytin and Neurobiotin have revealed extensive networks of coupled cells, many times larger than seen with Lucifer Yellow (Vaney, 1991, Peinado et al., 1993).

6. *Heavy metal complexes.* For example lead EDTA, potassium argentocyanate (Turin, 1977). It must be possible to precipitate the metal out of the complex with hydrogen sulphide and ammonium sulphide. It is also important to ensure that traces of free metal or chelating anion are not present in the preparation. By choosing appropriate metal complexes, molecules of a wide range of molecular weight and dimensions can be generated. These compounds have not yet been widely used.

Advantages:
Easily injected iontophoretically or with pressure.
Not toxic, if the compound has been chosen with care.
Good sensitivity after intensification.
Permanent preparation, which does not fade.

Disadvantages:

Can only be visualized after chemical reaction.

Artefact can result from the presence of polysulphides in ammonium sulphide, which can redissolve the precipitated metal sulphide. This can lead to an over-estimate of the distribution of, and a false pattern for, the injected compound.

6. *Sugars and small peptides (and other small molecules) coupled to a fluorescent label such as fluorescein or rhodamine.* This approach allows the range of molecules that can be tested for the ability to pass through gap junctions to be greatly extended (see Simpson *et al.* 1977). However, considerable care must be taken to ensure that the label is not split off by metabolism and also that the test molecule is not broken down to smaller metabolites.

7. Achieving functional 'knock-out', ectopic expression and the generation of transgenics

The techniques of intracellular microinjection form the basis of a number of new and important approaches and methods for the analysis of cellular function. A full description is inappropriate here but they are introduced in order to demonstrate that skills obtained when learning intracellular injection can be translated immediately to exploit a variety of new technologies.

One way of determining the functional contribution of a particular molecule or mechanism is to neutralize its function by injection of an antibody or anti-sense RNA/oligonucleotides. This is a potentially powerful approach since, in principle, it allows direct demonstration of a specific function. Antibodies can be injected with pressure; however, "ringing" the electrode is also a rapid and efficient means of introduction. Careful selection of pipettes (for glass, tip size and shape) can improve the success rate. It can be helpful to include a low concentration of fluorescent dye to confirm that the injection has been successful. To be convincing, such experiments require a set of adequate controls (pre-immune serum, other antibodies, IgGs and, preferably, Fab fragments), which can make them time consuming and labour intensive. Nevertheless their considerable power makes them extremely informative.

The intracellular injection of RNA and/or oligonucleotides (both sense and antisense) to achieve over-expression, ectopic expression or functional knockout is now used widely as a method of exploring the functional role of genes and molecules. The *Xenopus* oocyte has proved to be a useful expression system, whether for expression of crude RNA extracts (e.g. the early experiments on the properties of neurotransmitters) or for expression of pure, *in vitro* synthesized RNA transcripts (e.g. Parke *et al.* 1993: a recent example from a very large literature). The large size of the oocyte (1 mm in diameter) allows substantial volumes to be ejected under pressure from relatively large tipped pipettes (up to 10 μm). Relatively

unsophisticated (and therefore inexpensive) equipment is adequate; a simple manipulator and a dissecting microscope will suffice. The difficulties relate to the need to culture the oocytes to allow expression levels to build up, which requires sterile injections and good quality oocytes. Failure to obtain expression may reflect oocyte quality rather than difficulties with injected RNA. There are concerns about certain aspects of such experiments (e.g. whether the injection of foreign RNA induces inappropriate expression of endogenous genes and whether down-stream signalling cascades activated by, for example, exogenously expressed 5-HT, reflect the properties of the oocyte or that of the signalling mechanism in the system from which the RNA is drawn). Both concerns are known to be valid in some circumstances, but not in others.

For developmental studies, ectopic expression, achieved by injection of sense RNA into a cell where the protein product is not found normally, also has proved illuminating.

Knock out, by injection of antisense RNA or oligonucleotides, also can be achieved by intracellular injection. Again, the problems relate to the injected material and the way it is handled by the injected cell (and its progeny) rather than the injection itself.

A high quality microinjection set-up, based on a compound microscope and good manipulators, also can be used for the generation of transgenic mice, where the gene of choice is injected into the nucleus. The tricks associated with nuclear injection relate primarily to the preparation and it will generally be necessary to ensure sterility, since injected embryos must be returned to foster mothers to continue development. Nevertheless, the skills associated with microinjection of dyes and molecules into small cells (such as using electronic oscillation to eject molecules of DNA) are not often the province of mammalian developmental biologists; any one competent in microinjection should be capable of acquiring the basic technology relatively quickly.

8. The injection of indicators and buffers for ions

Indicators

A wide range of molecules are available that can act as fluorescent indicators of the concentrations of ions inside cells. Molecules are available to measure the concentration of most ions of biological interest (see the Molecular Probes catalogue). The mostly widely employed compounds are those used to measure free calcium levels (aequorin, Quin-2, Fura-2: Blinks *et al.* 1982; see Chapter on fluorescent indicators) and pH (e.g. BCECF, Rink *et al.* 1982) . The use of these dyes in imaging and microspectrofluorimetry is the subject of Chapter 12 in this volume and will not be covered here.

Indicator reagents can be introduced into single cells by injection through micropipettes and the underlying principles are the same as for other compounds. The difficulties are generated by methods of detection and measurement, which are beyond the scope of this chapter.

Buffers

Similar comments apply to the injection of buffers such as BAPTA and EGTA. Buffers allow the experimenter to set the intracellular level of the ion of interest and can be important when exploring the role of particular ions in, for example, controlling the permeability of gap junctions or controlling current flow through ion channels (e.g. calcium currents in invertebrate neurones).

Note: BAPTA is far superior to EGTA as a calcium buffer because its ability to complex calcium is not pH sensitive making calculation of the free calcium concentration much easier (see Chapter 11), and it has faster binding kinetics.

9. Other methods for introducing compounds into cells

For the sake of completeness we finish with a brief summary of methods other than pressure injection and iontophoresis that can be used to load cells with reagents. All these methods are directed towards loading cells in large numbers, rather than singly.

(i) A membrane soluble derivative of the chosen compound, which is converted by metabolism into an insoluble form is used, so that the compound can enter, but not leave, the cells. Usually an ester of the chosen compound is used. Esters cross the cell membrane rapidly and are then acted on by intracellular esterases. This method has been used to load cells with fluorescein (fluorescein diacetate) and Quin-2 and Fura-2 (acetoxymethyl esters; see Chapter 12). The hydrolysis of the ester inside the cell also generates hydrogen ions, so that a small fall in intracellular pH is inevitable. It is important to ensure that other products of the hydrolysis are not toxic.

Fluorescein diacetate can be used as a vital dye because fluorescein liberated inside the cell will only cross membranes of damaged cells, rendering intact cells fluorescent at FITC wavelengths, and can be combined with ethidium bromide staining of nuclei of the dead cells (fluorescent at rhodamine wavelengths).

(ii) The compound is dissolved in a reagent such as DMSO which permeabilizes the cell so that quite large molecules can gain entry. On return to normal solution the compound is trapped inside the cell. Used in prelabelling techniques (see above).

(iii) The cells are permeabilized transiently by osmotic shock. The exact sequence of changes in osmotic pressure that is most effective (i.e. from high to low or vice versa) depends on the cells being used. Low permeability is restored on return to normal osmotic strength.

(iv) Artificial endocytosis. Lipid vesicles are loaded with the substance to be incorporated into the cell. These vesicles then fuse with the cell membrane, releasing their contents to the cell interior (see Spandidos & Wilkie, 1984).

(v) Electroporation (e.g. Potter *et al*. 1984). Brief, high voltage shocks allow molecules to enter through holes made in the membrane by the electric field. This method is used routinely to incorporate DNA into cells (as when transforming cell lines). Voltages of about 4000 V cm^{-1} are required. Because of the high voltages

used, this method is potentially hazardous and should not be attempted without advice from someone who is already experienced in its use.

(vi) Retrograde and anterograde labelling of neurones. These methods are widely used for tracing anatomical pathways and are covered, for example, in Helmer & Robards (1981). Some methods rely on the uptake of the tracer by damaged neurones others upon spontaneous endocytosis (see below). In the periphery the chosen axon is cut and sucked up into a pipette containing the label. The compound (HRP, Wheat germ agglutinin either fluorescently tagged or complexed with HRP, cobalt chloride and radioactively labelled proline have all been widely used) then enters the neurone and is transported back to the cell body. The transport of the label can be enhanced by applying a standing voltage to the cut end. In the central nervous system, the marker is injected fairly crudely into the brain or spinal cord and is taken up by damaged cells close to the injection site. The degree to which the pathway is traced depends on the time allowed for axonal transport.

(vii) Spontaneous endocytosis. Some compounds (particularly lectins) are avidly taken up by cells via an endocytotic pathway. Dye can be applied in an Agar pellet or in a small piece of gelatine sponge. The compound enters the lysozomes and provides a relatively permanent label so long as it is not broken down. Substances that are relatively toxic when directly injected are well tolerated by cells if allowed to enter by this natural pathway (e.g. tetra-methyl rhodamine, Texas Red). Cells labelled with TRITC have been used to examine the commitment of embryonic cells in *Xenopus laevis* (Heasman *et al.* 1985). Texas Red has been used to follow the outgrowth of neurones from retinal ganglion cells during the establishment of retino-tectal connections in *Xenopus* (O'Rourke & Fraser, 1986).

(viii) Scrape labelling. Cells are damaged by scraping! Compounds present in the external solution enter the cell during the period before the cell membrane reseals (El-fouly *et al*. 1987). Not a method of choice when studying gap junctions although many authors do so.

10. Sample protocols

Intracellular injection of HRP

(i) Fill microelectrode with solution of HRP. There are a number of different recipes in the literature. We have used 4% HRP in 0.2 M KCl.

An alternative is to use 4% HRP in 0.2 M Tris and 0.2 M KCl at pH 7.4. Some authors use slightly less HRP, slightly more KCl and may or may not add Tris. The type of HRP used also varies. Type II can be used, but many people prefer Type VI. Type VI is a single isozyme of HRP, type II a mixture. If you wish the preparation to survive for long periods of time after injection then type VI is probably the best.

(ii) Inject cell with positive going pulses of about 10 nA and 0.5 sec duration and a frequency of about 1 Hz for 15-20 min. You may see the cell swell as it fills.

(iii) When filling cells with fine processes it often helps to leave the preparation for 20 to 40 min after the end of injection to allow the HRP to diffuse. If the preparation will stand it, put it in the fridge.

(iv) Fix the preparation for about half an hour. 4% glutaraldehyde in saline or 0.1 M phosphate buffer for 15 to 20 min gives acceptable results.

There are various opinions in the literature concerning the concentration of fixative to employ. Some authors suggest fix as low as 0.8% glutaraldehyde. It may be important to use a low level of fixative to prevent inactivation of the peroxidase. In general the right amount and time of fixation are determined for each preparation by trial and error.

(v) Wash the preparation thoroughly in buffer.

(vi) Transfer preparation to 0.5 mg ml^{-1} diaminobenzidine (DAB) in 0.1 M phosphate buffer for 10 min. This allows the DAB to penetrate.

(vii) Add one or two drops of 1% hydrogen peroxide ml^{-1} of DAB solution, to the solution bathing the preparation. Watch the reaction. When the brown colour is fully developed wash in buffer.

(vii) Dehydrate in alcohol and clear for permanent preparation.

(ix) There are many alternative methods for HRP in the literature. Most of them work so it probably doesn't matter which you use. The main variant lies in the reagent used to visualise the HRP.

Many of the chromagens are potentially carcinogenic.

Some protocols recommend including 0.02% cobalt chloride and 0.02% nickel ammonium sulphide in the incubation medium (Adams, 1981). This can help to intensify the reaction product.

Biocytin injection technique

(i) Fill electrode with a 2-4% solution of biocytin or Neurobiotin (Vector Labs) in 2 M potassium acetate.

(ii) Inject with 1 nA depolarising pulses for up to 10 minutes.

(iii) Fix in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for at least 2 hours, depending on tissue thickness.

(iv) Rinse in buffer and, where necessary, slice tissue into sections (<100 μm).

(v) Wash well in buffer containing 0.4% Triton X-100 (or Tween 20) for at least 2 hours.

(vi) Incubate in 'ABC' (Streptavidin-biotinylated-HRP) complex (Vector Labs.) in Triton/ buffer for at least 2 hours; some protocols recommend 24 hours. (A variety of alternative avidin complexes, including a number of fluorescent conjugates are also available.)

(vii) Rinse well with 3 changes of Triton / buffer.

(viii) React with freshly mixed 0.05% diaminobenzidine (Caution, DAB is a potential carcinogen) and 0.003% hydrogen peroxide in Triton / buffer.

(ix) Wash well in buffer, if appropriate, mount sections, dehydrate in an alcohol series and clear in Histoclear or methyl salicylate

Cobalt injection technique

(i) Fill electrode with either 6% cobalt hexamine or 100 mM cobaltous chloride. Cobalt hexamine is best.

(ii) Inject with positive going pulses, 1-10 nA for about 10-15 min. For very small cells a shorter time will probably be enough.

(iii) Rinse preparation with bathing medium.

(iv) Either add a few drops of ammonium sulphide to the bath and observe appearance of dark brown precipitate, or saturate the bathing medium with hydrogen sulphide gas and then add to preparation; a dark brown precipitate of cobalt sulphide will appear.

Although both work, hydrogen sulphide is preferable if available. It produces a finer grain precipitate and does not contain polysulphides. Polysulphides can cause the sulphide-metal complex to re-dissolve.

(v) Fixation: The intensification procedure that follows works well with a glacial acetic acid/ethanol mix made up as:

1 part glacial acetic acid

4 parts 70% ethanol

The method will also work satisfactorily with glutaraldehyde fixation, provided the preparation is adequately washed. If a formaldehyde- or formalin-based fixative is essential, then the preparation should be washed extensively with chloral hydrate before proceeding to intensification.

For small preparations half an hour should be sufficient.

Method for intensification of a metal precipitate

There are two main physical developer methods of intensification, one based on Timm's solution, using gum Arabic as a protective colloid (e.g. Bacon & Altman, 1977), and a second using tungsto-silicic acid as the protective colloid (e.g. Szekely & Gallyas, 1975). Both methods give good results but the former is carried out in the dark at 60ºC, whereas the latter, illustrated below, can be done at room temperature in the light.

We are indebted to Barry Roberts for introducing us to this intensification method.

(i) Wash preparation in distilled water for 15 min.

(ii) Incubate in 2% sodium tungstate - 10 min for sections, half an hour for whole mounts.

(iii) Place in intensification solution in Petri dish. Intensification solution should be freshly prepared.

(iv) Observe under microscope until tissue begins to discolour (2-10 min).

(v) Rinse with 3 changes of distilled water.

(vi) For permanent preparation, dehydrate through graded alcohols and then clear in xylene or methyl salicylate.

If the tissue is over-intensified, it can be worth attempting a partial de-intensification using Farmer's photographic reducer method (Pitman, 1979).

Intensification solution (for step iii)

A	distilled water	355 ml
	1% Triton X-100	15 ml
	sodium acetate $3H_2O$	1.5 gm
	glacial acetic acid	30 ml
	silver nitrate	0.5 gm

This solution can be kept in the fridge until a silver precipitate begins to appear.

B 5% sodium tungstate

C 0.25% ascorbic acid in distilled water.

Make immediately before use.

Mix A, B, C in proportions 8A:1B:1C, freshly prepared. For 40 ml of solution take 32 ml A:4 ml B:4 ml C.

APPENDIX A

Table of absorption and emission maxima for some common fluorophores

Compound	MW	ABS (nm)	EM (nm)	Zeiss filter set
bis-Benzimide (Hoechst 33258)	534	365	480	01, 02
bis-Benzimide (Hoechst 33342)	562	355	465	01, 02
Carboxyfluorescein	376	492	516	09, 10, 16, 17, 23
Cascade Blue	607	375/400	410	02, 05, 18, 21, 30
DAPI	457	347	458	01, 02, 18
DiASP	787	491	613	09, 10, 16, 17
DiI/Fast DiI	934	550	565	14, 15, 23
DiO/Fast DiO	882	484	501	09, 10, 16, 17, 23
Diamidino Yellow	NA	~365	~480	05, 18
Ethidium bromide	394	526	605	14, 15
Fast Blue	NA	~365	~480	05, 18
Fluorescein (FITC)	389	495	519	09, 10, 16, 17, 23
Lucifer Yellow	453	428	535	05, 06, 18
Propidium iodide	668	536	617	14, 15
Rhodamine (TRITC)	444	544	570	14, 15, 23
Sulphrhodamine 101	607	~586	607	00, 14, 15, 23
Texas Red	625	589	615	00, 14, 15, 23

It is worth experimenting with different filter combinations to obtain the best result for any particular application.

ABS, absorbance max; EM, emission max; NA, not available.

APPENDIX B

Equipment

1. Electrode glass. From many suppliers, including:

Clarke Electromedical Ltd, P.O. Box 8, Pangbourne, Reading, RG8 7HU, UK. Clarke also supply pollers, electrophysiological equipment and act as agents for a number of manufacturers. A helpful firm.

Glass Company of America, Bargaintown, New Jersey, USA.

2. Electrophysiological equipment. No special requirements related to injection. Simple amplifiers can be constructed at low cost (see circuits in Purves, 1981). High quality amplifiers are available from:

Axon Instruments Inc., 1101 Chess Drive, Foster City, CA 94404 USA

Digitimer Ltd., 37 Hydeway, Welwyn Garden City, AL7 3BE, UK

World Precision Instruments, Astonbury Farm Business Centre, Unit J, Aston, Stevenage, Herts SG2 7EG UK (Obtainable from Clarke).

3. Pressure Injection. Ready made devices are available from:

General Valve Corporation, East Hanover, New Jersey, USA. (Picospritzer 11., cheap).

Eppendorf Geratebau, P.O. Box 630324, 2000 Hamburg 63, F.D.R. (not cheap).

Alternatively devices can be made using Agla (micrometer driven) syringe, plastic tubing and liquid paraffin which is cheap and messy, or using a gas cylinder and a solenoid operated tap; available from General Valve Corp., (above) and from:

RS Components Ltd, P.O. Box 99, Corby, NN17 9RS.

4. Optical equipment. A fluorescence microscope (preferably epifluorescence) is an absolute essential for many injection experiments and potentially expensive.

A dissecting microscope may be used for injection, but a compound microscope with a fixed stage and head focussing, or an inverted microscope (for cultured or dissociated cells), extends the range of injectable cells downwards to about 5 μm. Ideally the fluorescence head should be fitted to the microscope used for injection. Zeiss, Nikon and Leitz microscopes are available to order with a fixed stage.

Advice and a wide range of microscopes are available from:

Micro Instruments (Oxford) Ltd, 18 Nanborough Park, Long Hanborough, Oxford OX7 2LH, UK. They manufacture a fixed stage micromanipulation microscope.

Leica UK Ltd (Leitz), Davy Avenue, Knowlhill, Milton Keynes, MK5 8LB, UK

Nikon UK Ltd, Instrument Division, Haybrook, Halesfield, Telsford, TF7 4EW, UK

Carl Zeiss (Oberkochen) Ltd, PO Box 78, Woodfield Road, Welwyn Garden City, AL7 1LU, UK

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N.B. Additional references, which are not quoted in the text, are included in this list for information.

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