# Experimental Neuroanatomy

## A Practical Approach

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# 1

# Preparation of central nervous system tissue for light and electron microscopy

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## 1. Introduction

Techniques of experimental neuroanatomy are those that enable one to characterize the position of neurones in the microcircuits of the nervous system on the basis of their morphology, chemistry, and connections. The essence of the techniques are that the morphology, chemistry, and output region of neurones are identified first at the light microscopic level and then the chemistry, origin, and pattern of afferent synaptic input are identified at the electron microscopic level. Information of this nature is a pre-requisite to the characterization of the microcircuitry or neuronal networks of a region and provides a framework within which functional data can be interpreted. For the analysis of individual neuronal elements or populations of neurones in the microcircuitry of an area it is necessary to examine the tissue at the light microscopic level. For the analysis of *synaptic* interactions of individual neurones or populations of neurones, it is necessary to examine the tissue at the ultrastructural level with an electron microscope. The object of this chapter is to introduce to the reader the basic techniques used in the preparation of tissue from neuroanatomical experiments for light and electron microscopy. Since a high proportion of modern experimental neuroanatomical procedures can be applied to ultrastructural analysis and it is by electron microscopy alone that synaptic connections between neurones can be identified, the main emphasis of this chapter will be on the preparation of the material for *electron microscopy*. However, in the use of modern experimental neuroanatomical techniques, material is generally prepared for light microscopy first, and then the same material is examined at the electron microscopic level. The techniques for the preparation of material for light and electron microscopy will not be dealt with in an exhaustive manner but will be confined to those that have proved successful in the hands of the author and his close colleagues for the analysis of many areas of the central nervous system.

## 1.1 Sequence of procedures in experimental neuroanatomy

The sequence of events or procedures for the preparation of material for light and/or electron microscopy in neuroanatomical experiments are virtually the same no matter what the system or experimental manipulation that has been performed.

(a) Carry out experimental manipulation on animal, for example injection of retrograde tracers (Chapter 2) or injection of anterograde tracers (Chapter 3).

(b) After the appropriate survival time fix the CNS, preferably by perfusion (*Protocol 3*).

(c) Remove the fixed brain from the animal, dissect out areas of interest, and cut sections preferably on a vibrating microtome (*Protocol 4*).

(d) Carry out histochemical or immunohistochemical procedure to reveal injected or endogenous substances (see individual chapters).

(e) Mount the sections prepared for light microscopy on coated slides, dehydrate, clear, and apply coverslips (*Protocols 5* and *6*).

(f) Post-fix the sections prepared for electron microscopy in osmium tetroxide (*Protocol 7*).

(g) Carry out single section Golgi-impregnation if required (*Protocol 12*).

(h) Dehydrate sections and flat-embed in an electron microscopic resin on microscope slides (*Protocol 8*).

(i) Examine the sections in the light microscope, select, record, and re-embed neurones and regions of interest in blocks of resin suitable for ultrathin sectioning (*Protocol 9*).

(j) Cut ultrathin sections at 60–90 nm on an ultramicrotome and collect on coated single-slot grids (*Protocol 10*).

(k) Carry out post-embedding immunocytochemistry (Chapter 6).

(l) Stain the ultrathin sections (*Protocol 11*) and examine in the electron microscope.

# 2. Fixation

The first step in the preparation of tissue from neuroanatomical experiments for light and electron microscopy is fixation. The process of fixation is in effect the preservation of tissue so that it will survive in its natural state or near-natural state during subsequent processing, during storage, and during examination in the light and/or electron microscopes. The functions of fixation are thus to stabilize the cellular organization such that:

- the tissue will no longer undergo degradation in response to endogenously released enzymes or as a response to the action of micro-organisms
- subsequent processing steps do not extract tissue components
- the tissue is hardened or strengthened to aid sectioning

Furthermore, an ideal fixative is one that penetrates into the tissue rapidly, acts rapidly, and is essentially irreversible.

Although many different substances may be used for fixation of biological tissue for light and electron microscopic analyses (1, 2) the most commonly used system in electron microscopic experimental neuroanatomy is a double fixation, initially with a buffered aldehyde (specifically paraformaldehyde and glutaraldehyde) solution followed by post-fixation in osmium tetroxide (3). For light microscopy only the primary fixation in aldehydes is necessary. The aldehydes react primarily with proteins, stabilizing the tissue by cross-linkage whereas the osmium tetroxide, exposed to the tissue at a later time, reacts with various tissue components but especially unsaturated lipids. The osmium tetroxide has the added advantage that it also stains the tissue providing extra contrast at the electron microscopic level. Aldehyde fixatives are generally prepared and used in a buffered solution at physiological or slightly higher pH. The most commonly used buffers for the preparation of aldehyde fixatives and the most commonly used in histochemical or immunohistochemical techniques are phosphate buffers, the preparation of which is described in *Protocol 1*.

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**Protocol 1.** Preparation of phosphate buffer (PB) and phosphate-buffered saline (PBS)

1. Prepare stock solution of 0.2 M $Na_2HPO_4 \cdot 2H_2O$, (35.6 g/litre): solution A.[a]
2. Prepare stock solution of 0.2 M $NaH_2PO_4 \cdot 2H_2O$, (31.2 g/litre): solution B.[a]
3. For a stock solution of 0.2 M phosphate buffer at pH 7.4 mix solutions A and B in a ratio of 4:1. Check pH, adjust with solutions A or B as necessary. Stock may be kept up to one week at 4°C. Different pH values are prepared by mixing solutions in different ratios.
4. For a working solution of 0.1 M dilute stock one in two with distilled water. For 0.05 M dilute the stock 1 in 24 in distilled water.
5. Phosphate-buffered saline is prepared by mixing:
   - 0.2 M PB 50 ml
   - NaCl 8.76 g
   - KCl 0.2 g
   - distilled water up to 1 litre

[a] The stock solutions may be stored for long periods at 4°C, but should be warmed to ensure that all crystals are dissolved before use.

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The preparation of buffered paraformaldehyde/glutaraldehyde fixatives are described in *Protocol 2*. The concentration of aldehydes in the fixative depends upon the procedures that are to be applied to the tissue and the reader is referred to individual chapters. In general terms the higher the concentrations of aldehydes, the better preserved is the tissue and in particular the higher the concentration of glutaraldehyde the better the ultrastructural preservation. For light microscopy it is often only necessary to use paraformaldehyde (~ 4%) alone. It should be remembered however, that the process of fixation may also reduce the activity of endogenous or exogenous enzymes that are subjects of histochemical reactions and may obscure or denature antigenic sites. Strong fixation with high concentrations of glutaraldehyde may also hinder penetration of reagents into the tissue. It is therefore necessary to attain a balance between the degree of preservation of the tissue and the maintenance of antigenic sites or of an enzyme activity. Thus in immunocytochemistry it is generally necessary to use only low concentrations of glutaraldehyde (although there are notable exceptions see Chapters 5 and 6). Some enzymes, both exogenous and endogenous tolerate high concentrations of glutaraldehyde and their histochemical detection can be carried out in well fixed tissue. For the specific requirements of fixation for different histochemical procedures see individual chapters.

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**Protocol 2.** Preparation of paraformaldehyde–glutaraldehyde fixative in phosphate buffer[a]

*Materials*

- paraformaldehyde (Aldrich or TAAB)
- glutaraldehyde (supplied as a 25% aqueous solution from all EM suppliers, stored at 4°C)
- 0.5 M NaOH
- 0.2 M phosphate buffer (*Protocol 1*)

*Method*

All steps should be carried out in a fume hood.

1. Heat distilled water (just under half of the final volume of fixative required) to 65–70°C.
2. Weigh out the paraformaldehyde powder and add to the water in a fume hood. Stir continuously with a magnetic stirrer.
3. Add 0.5 M NaOH dropwise, until the solution clears. There is a slight delay in this so do not add too much sodium hydroxide.
4. Filter the paraformaldehyde solution.

5. Add 0.2 M phosphate buffer (half final volume). The fixative can be prepared to this step the evening before fixation (stored at 4°C) if being used immediately, cool to appropriate temperature.
6. Add appropriate volume of the 25% glutaraldehyde to give the desired final concentration (see *Table 1*).
7. Make up to final volume with distilled water.

*Examples*

For 500 ml of 4% paraformaldehyde/0.5% glutaraldehyde: dissolve 20 g paraformaldehyde in 200 ml of water, add 250 ml 0.2 M phosphate buffer, add 10 ml of 25% glutaraldehyde solution, and make up to 500 ml with distilled water.

For 250 ml of 3% paraformaldehyde/0.1% glutaraldehyde: dissolve 7.5 g paraformaldehyde in 100 ml of water, add 125 ml 0.2 M phosphate buffer, add 1.0 ml of 25% glutaraldehyde solution, and make up to 250 ml with distilled water.

See *Table 1* for volumes of 25% glutaraldehyde solution to give selected final concentration.

[a] **Safety note 1.** Paraformaldehyde and glutaraldehyde are both extremely toxic by ingestion, inhalation, and contact with the skin. Wear appropriate protective clothing and avoid contact with the skin. *All* manipulations with the aldehydes, including perfuse–fixation, should be carried out in a fume cupboard. Waste should be washed away with large volumes of water. Aldehydes should be stored away from 'fixable' reagents, e.g. antisera.

**Table 1.** Volume of 25% glutaraldehyde solution required to prepare fixatives

| | Volume of fixative (ml) required | | | | | | |
|---|---|---|---|---|---|---|---|
| **Conc. of glutaraldehyde required** | **100** | **200** | **300** | **400** | **500** | **750** | **1000** |
| 0.01% | 0.04 | 0.08 | 0.12 | 0.16 | 0.2 | 0.3 | 0.4 |
| 0.05% | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | 1.5 | 2.0 |
| 0.1% | 0.4 | 0.8 | 1.2 | 1.6 | 2.0 | 3.0 | 4.0 |
| 0.2% | 0.8 | 1.6 | 2.4 | 3.2 | 4.0 | 6.0 | 8.0 |
| 0.5% | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | 15.0 | 20.0 |
| 1.0% | 4.0 | 8.0 | 12.0 | 16.0 | 20.0 | 30.0 | 40.0 |
| 2.0% | 8.0 | 16.0 | 24.0 | 32.0 | 40.0 | 60.0 | 80.0 |
| 2.5% | 10.0 | 20.0 | 30.0 | 40.0 | 50.0 | 75.0 | 100.0 |
| 5.0% | 20.0 | 40.0 | 60.0 | 80.0 | 100.0 | 150.0 | 200.0 |

## 2.1 Perfusion–fixation

Fixatives are exposed to the tissue by either vascular perfusion (*Protocol 3*) or by immersion. The former, perfusion–fixation, is by far the superior since it is possible to remove blood from the vasculature, fixation is more rapid and is more even, and excess fixative can be removed by perfusion with buffer. If it is necessary to perform immersion fixation, say for human post-mortem or biopsy tissue, then the tissue should be cut as thinly as possible to aid penetration of the fixative, and at least the initial fixative solution should be cold to slow down any degradation of the tissue. For most purposes, especially when experimental manipulations have been performed on the animal, perfusion–fixation will be the method of choice. The technique of perfusion–fixation is essentially the same for different experimental procedures and species, the exact details for the individual procedures are given in the appropriate chapters. The essence of perfusion–fixation is that the fixative is administered to a deeply anaesthetized animal directly into the vasculature by means of a cannula inserted through the heart into the aorta. The fixative is pumped around the circulation by means of a peristaltic pump or a gravity feed mechanism, and a mixing chamber is included to give a gradual change from the vascular rinse to the fixative.

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**Protocol 3.** Perfusion–fixation (see also Chapter 10, *Protocol 2*)

The following are required:

- anaesthetic
- peristaltic pump or gravity feed system
- mixing chamber
- dissection instruments (large and small scissors, clamps, arterial clip)
- wide bore (approximately 2 mm internal diameter) blunt-ended needle
- vascular rinse solution, (e.g. Tyrode's solution (see Appendix, this chapter) or 0.9% NaCl solution)
- fixative
- dissection board

*Method*

See *Safety note 1* in *Protocol 1*.
The following method is for a 200–300 g rat.

1. Deeply anaesthetize the animal. We use 350 mg/kg chloral hydrate (intraperitoneally) as a 3.5% solution in 0.9% NaCl solution, but any suitable anaesthetic will be adequate.
2. Pin out the deeply anaesthetized rat on a dissection board either in a large

tray in a fume cupboard or over a ventilated down-draught sink. Set perfusion apparatus flowing with vascular rinse at a rate of approximately 20 ml/min.

3. Open abdominal cavity, take hold of xyphoid cartilage with clamp, and lift to expose the diaphragm. Cut away connections between the liver and the diaphragm.
4. With blunt-ended scissors cut the diaphragm away from the rib cage and cut along sides of the rib cage, lift the cut rib cage to expose the heart, and cut away the pericardium if necessary. Once the thoracic cavity is opened then speed is of the utmost importance as the brain is no longer oxygenated.
5. Grasp the heart between thumb and forefinger and insert the cannula into the base of the left ventricle, push gently into the aorta, and clamp (with arterial clip) in position above the heart.
6. Immediately cut the right atrium with scissors. Blood should flow freely. After 60–90 sec change to the fixative.[a] Move lungs to one side and clamp the descending aorta.[b]
7. Continue the perfusion at the rate and for the time recommended in individual procedures. An average rate and time for a rat is 10 ml/min over a period of 20–30 min.[c]
8. Post-perfuse with buffer if indicated in the individual procedures.

[a] If the lungs turn white and become bloated, then the cannula is in the wrong position, i.e. it has been pushed into the left atrium and not the aorta.

[b] Should the hind legs stiffen it implies that the clamping of the descending aorta is ineffective.

[c] A good perfusion is characterized by fasciculations of muscles when first exposed to the fixative, followed by a rapid hardening.

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On completion of the perfusion the head of the animal is removed, the skin and muscle are cut away from the top of the skull, and with the aid of bone snippers, the brain is carefully removed. The brain should be firm to the touch and creamy-white in colour when a low concentration of glutaraldehyde is used, and yellow when a high concentration is used. Any pink coloration indicates the presence of blood and a poor perfusion and hence poorly fixed tissue. Slightly soft brains can be hardened by post-fixation in paraformaldehyde solutions at 4°C and indeed it is recommended in some histochemical procedures to do so. Once the brain is removed, the areas of interest are cut into blocks or slices of about 5 mm thickness. This can be carried out freehand with a single or double-edged razor blade, or using a perspex guide into which the brain is placed and cut along guide lines. Alternatively, when a specific plane of section is required, for instance in relation to an atlas, a stereotaxic frame can be used to make guide cuts. To do this the top of the brain is exposed and the head is placed in a stereotaxic frame, a razor blade held in a

micromanipulator is then used to produce guide cuts in the required plane and thickness.

## 3. Sectioning of the brain

Sections suitable for histochemical reactions and histological analysis by light microscopy can be prepared using frozen tissue cut on a cryostat or freezing microtome or alternatively, non-frozen tissue cut on a vibrating microtome (*Protocol 4*). In the electron microscope, however, frozen sections have very poor ultrastructure and they are generally not used for preparation of sections for electron microscopy. The most commonly used system to prepare sections for electron microscopy is the vibrating microtome (*Protocol 4*). Several models of vibrating microtomes are available on the market (Vibratome; Vibroslice). The essential features are that the tissue (which can be of almost any hardness from fresh to well-fixed), is glued to the stage, a razor blade then advances over the tissue whilst vibrating in the plane of section but at 90° to the direction of advance (i.e. a sawing motion), and the thickness of the sections adjusted by raising the stage. The vibration of the blade enables it to cut through even quite soft tissue. In general terms the higher the frequency or amplitude of vibration and the slower the speed of advance, the better the sections are.

For light microscopy, whole brain sections or hemisections are cut. The same may also be applied to electron microscopic sections, however it may be necessary to trim the blocks down to include only the area of interest, as large sections often become damaged during subsequent processing and it is often difficult to keep them flat.

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**Protocol 4.** Vibrating microtome sections

*Materials*

- vibrating microtome
- cyanoacrylate adhesive
- phosphate buffer or phosphate-buffered saline (see *Protocol 1*)
- labelled glass vials (or some other receptacle for collecting the sections)
- artist's paint brush
- agar (5% in distilled water)

*Method*

1. Gently dry the block or slice of fixed brain by dabbing on absorbent paper and glue to the stage of the microtome with cyanoacrylate glue.
2. If tissue requires support, surround it with agar that has been melted in a water bath and allow it to set.

3. Mount the stage in the microtome and fill the bath with cold PBS or PB.
4. Cut sections at 20–100 μm (usually 50–70 μm) using maximum amplitude of vibration and slow speed of advance.
5. Pick up sections with a paint brush and place in a vial containing PB or PBS.
6. Wash sections several times in PB or PBS by pipetting the solutions from the vials with a disposable Pasteur pipette.
7. Carry out the histochemical procedure or store at −23°C after equilibration in a cryoprotectant solution consisting of: 160 ml 0.05 M phosphate buffer (pH 7.4), 120 ml ethylene glycol, and 120 ml glycerol.

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Failure to cut sections or the cutting of irregular sections can be due to several reasons:

- blunt or damaged blade
- tissue not adequately stuck to the microtome stage
- tissue block is too high which results in its movement before the advancing blade
- insufficient support for tissue

## 4. Processing of sections

It is at this stage that histochemical or immunohistochemical procedures are carried out and the reader is referred to individual chapters. The reactions are generally carried out on free-floating sections in the scintillation vials (or similar) of about 15 ml capacity and subjected to constant, gentle, shaking although sections for light microscopy can be incubated by the various histochemical procedures after mounting on slides (see below). The most effective shakers are those that move in a rotary fashion, (e.g. IKA-Vibrax VKR). For individual shakers and vials, the optimum volume of solution should be determined that results in gentle 'wafting' of the sections when put on the shaker. For 15 ml glass vials and a IKA-Vibrax VKR shaker, the optimum volume is 1–3 ml. It is at this stage that the paths divide for sections prepared for light microscopy and those prepared for electron microscopy since the conditions of incubation are often different and the subsequent procedures are different.

## 5. Mounting of sections for light microscopic analysis

Those sections that are prepared from tissue that is to be used for light microscopy alone, and those sections in electron microscopic studies that will

only be used for light microscopic analysis, i.e. injection sites, are then mounted on to microscope slides. The sections can of course be mounted on to slides first and then reacted on the slides, however it is generally more economical in reagents and convenient to carry out the reactions on free-floating sections, and in general, the quality of staining is better. The microscope slides are coated or subbed with a substance, (e.g. gelatin, egg albumin) that aids the adherence of the sections to the glass (*Protocol 5*, see also Chapter 8, *Protocol 1* and Chapter 6, *Protocol 1*). The sections are mounted by gently rolling them around a soft artist's paint brush and then 'unrolling' them on to the slide. Alternatively, the sections can be placed in a trough of buffer, the microscope slide dipped into the trough at an angle of 20–45°, the sections gently moved into position above the slide with an artist's paint brush, and the slide gently lifted out while keeping the section relatively still. The section can be moved around and correctly positioned with the paint brush whilst they are still wet. Several sections are generally placed on each slide and they are then left to air-dry at room temperature for about 12–18 h. This time may be reduced by the use of a cold air blower.

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**Protocol 5.** Coating glass microscope slides

*Materials*

- acid alcohol (a few drops of glacial acetic acid added to absolute ethanol)
- microscope slides
- chrome alum (chromic potassium sulphate)
- gelatin

*Method*

1. Soak slides in acid alcohol for several hours.
2. Remove slides from acid alcohol using forceps and place in slide carriers.
3. Wash under running water (preferably hot) for several minutes and then wash in several changes of distilled water.
4. Dry in an oven away from dust.
5. Prepare slide coating medium by mixing:
   - chrome alum 0.1 g
   - gelatin 1.0 g
   - distilled water 200 ml

   Heat gently to dissolve. Cool before use. This solution may be stored up to several weeks at 4°C.
6. *Either*: take slides one at a time, hold by edges in thumb and forefinger,

and dip into the cold coating solution. Allow to dry in the upright position in a dust-free environment.
*Or*: immerse slide carrier containing slides in staining dish containing the gelatin solution. Drain on absorbent paper and dry in a dust-free environment.

7. Store coated slides in a closed container to avoid contamination with dust.

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In order to examine sections in the light microscope it is necessary to infiltrate them with a medium that 'fills' all spaces in the tissue, in effect, it evens out the changes in refractive index of the tissue (clearing). Most mounting media are not miscible with water, so in order to completely infiltrate the tissue it is necessary to remove water or dehydrate the tissue prior to exposure to the medium. The tissue is dehydrated in graded dilutions of ethanol before soaking in a link reagent (a substance in which both the alcohol and mounting medium dissolve), e.g. xylene, and infiltrating with the mounting medium. Coverslips are then applied to the section, the remaining link reagent and the solvent of the mounting medium evaporate, and the medium sets hard (*Protocol 6*). In this form the sections can be stored indefinitely and can be examined in the light microscope.

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**Protocol 6.** Dehydration, clearing, and mounting light microscopic sections

*Materials*

- graded series of volume for volume dilutions of ethanol in water (50%, 70%, 90%, 100%)
- dry absolute ethanol (ethanol stored over anhydrous cupric sulphate)
- xylene
- mounting medium such as XAM or DPX (BDH)
- coverslips
- staining dishes and slide carriers (alternatively Coplin jars may be used)

*Method*

1. Place the slides with dried-on sections in the slide carrier. Place the slide carrier in the dishes containing the increasing concentrations of ethanol, removing excess ethanol solutions from the slide carrier between each dilution by dabbing on absorbent paper.
2. 50% ethanol for 10–15 min.
3. 70% ethanol for 10–15 min.
4. 90% ethanol for 10–15 min.

**Protocol 6.** *Continued*

5. 100% ethanol for 10–15 min.
6. Dry absolute ethanol for 10–15 min.
7. Two changes of xylene for 10–15 min each.[a,b]
8. Apply a 'line' or 'blob' of mounting medium, (e.g. XAM or DPX) on a coverslip using a glass rod.
9. Remove one slide from the xylene and place it, sections down, on the coverslip. Turn the slide with adhering coverslip over and apply gentle pressure to the coverslip to spread mounting medium completely over the sections. Do not allow the sections to dry out before they are exposed to the mounting medium.
10. Leave the slides to set dry.[c]

[a] **Safety note 2.** Xylene and xylene-based mounting media are toxic and inflammable, all manipulations should therefore be carried out in a fume cupboard.

[b] Do not use solvent-based markers to label slides as these wash off in the xylene. Use a diamond marker or pencil if slides have frosted ends.

[c] Coverslips can be removed, even after the mounting medium is set, by soaking for several hours (up to days) in xylene. This may be necessary if the sections partially dry out during mounting or insufficient medium was put on the coverslip.

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## 5.1 Counterstaining of light microscopic sections

It is sometimes desirable to counterstain sections that are prepared for light microscopy to enable injection sites, sites of transport of axonal tracers, and immunocytochemically stained structures to be correctly identified.

### 5.1.1 Nissl staining

A most effective and reliable stain for light microscopic sections is the Nissl stain using cresyl violet. The sections are mounted on to coated slides and allowed to dry, as described above. Prior to staining they are 'defatted' by passing through graded dilutions of ethanol for about three minutes each in a manner similar to that described in *Protocol 6*, after which they are rehydrated by passing back through decreasing concentrations of ethanol, and finally back into water. Place the sections in cresyl violet solution for 10 to 30 minutes consisting of:

- 1 M sodium acetate 15 ml
- 0.2 M formic acid 75 ml
- 0.5% aqueous cresyl violet 150 ml

When the sections have an overall, even blue appearance dehydrate, infiltrate with a mounting medium and apply a coverslip as described in *Protocol 6*. The ethanol solutions act to differentiate the stain, causing myelin and other components to lose colour whereas perikarya retain the colour. Differ-

entiation can be accelerated by slightly acidifying the 70% and 90% ethanol with acetic acid.

# 6. Preparation of sections for electron microscopic analysis

## 6.1 Post-fixation in osmium tetroxide

After processing for the histochemical and immunohistochemical procedures described in the individual chapters, sections that have been selected for electron microscopy are post-fixed in a buffered osmium tetroxide solution (*Protocol 7*). During treatment with osmium the sections will turn black, will harden considerably, and will become brittle. Any unevenness or folds in the sections will be difficult to remove after osmium treatment and even more so after subsequent treatments, it is therefore important to ensure that the sections are completely flat before treatment.

**Safety note 3**. Osmium tetroxide is a powerful oxidizing agent, it is extremely toxic, and is volatile. *All* manipulations should be carried out in a fume hood and wearing protective gloves and clothing. Stock osmium solutions should be stored in ground glass-stoppered bottles, preferably *double* ground glass-stoppered, at 4°C in a fridge dedicated to toxic substances. Waste osmium solutions can be rendered less hazardous by placing in a strongly alkaline solution. All waste and washings are therefore placed in a bottle containing sodium hydroxide pellets from which the metal can be reclaimed.

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**Protocol 7.** Post-fixation with osmium tetroxide

A. *Preparation of stock and working solutions of osmium tetroxide*

*Materials*

- osmium tetroxide (usually supplied as 1 g solid in sealed ampoules; OXKEM Ltd. or Electron Microscope Suppliers)
- 0.2 M phosphate buffer (*Protocol 1*)
- ground glass-stoppered bottles (preferably double stoppered)
- laboratory film, e.g. Parafilm (made by American Can Company)

*Method*

1. To prepare a 4% stock solution, open 1 g ampoule of osmium tetroxide and dissolve in 25 ml of distilled water in ground glass-stoppered bottle. This may take up to 24 h without stirring but the time is considerably shortened with stirring. The stock solution is stored at 4°C for several months, but should be in a fridge dedicated to toxic substances since even with ground glass stoppers and several layers of Parafilm, the osmium escapes.

**Protocol 7.** *Continued*

2. To prepare a working solution of 1% in 0.1 M phosphate buffer (pH 7.4) mix 4% stock solution, 0.2 M phosphate buffer, and distilled water in the ratios 1:2:1. Thus for 20 ml mix the following:
   - stock 4% osmium tetroxide 5 ml
   - 0.2 M phosphate buffer (pH 7.4) 10 ml
   - distilled water 5 ml

B. *Post-fixation with osmium tetroxide*

*Materials*

- 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4
- 0.1 M phosphate buffer, pH 7.4 (*Protocol 1*)
- Petri dishes (if the number of sections is large)
- artist's paint brush

*Method*

1. Equilibrate sections with 0.1 M phosphate buffer (pH 7.4).
2. Pipette off the phosphate buffer and ensure that the sections are flat using a paint brush. If there are many sections, transfer to a Petri dish.
3. Carefully pipette 1–2 ml of the 1% osmium tetroxide solution using a glass Pasteur pipette and cover. (Sections in Petri dishes will require a greater volume.) Ensure that the sections do not float on the solution. The sections will rapidly turn black and become very brittle, any sections that are folded will remain so. Caps of the vials will also become black and should therefore be saved only for osmium treatment or discarded after use.
4. The incubation time depends on the thickness of the sections:[a]
   - for sections of 50 μm incubate for approximately 20–25 min
   - for sections of 70 μm sections incubate for 30 min
   - for sections of 100 μm incubate for 40 min
   - for 1 mm cubes of tissue incubate for 1 h
   - for slabs of tissue (approximately 5 × 5 × 1 mm) incubate for up to 4 h
5. Pipette off the osmium solution and place in waste osmium bottle.
6. Wash sections at least three times for 15 min each in phosphate buffer. If the sections are not to be subjected to further processing, e.g. Golgi-impregnation, it is preferable to dehydrate and embed in an electron microscopic resin immediately (*Protocol 8*).

[a] If the osmium solution changes from straw colour to a deep purple it should be replaced with fresh solution.

## 6.2 Dehydration and embedding in resin

Following post-fixation with osmium tetroxide the sections may be subjected to Golgi-impregnation (see *Protocol 12*), but if this is not required they should be embedded in an electron microscopic resin. The main reason for embedding tissue for electron microscopy in resin is to provide support during the preparation of ultrathin sections. However, one of the advantages is that the procedures, as with light microscopic preparations, clears the tissue and results in a preparation that is suitable for high resolution light microscopic analysis. Also, as is the case with light microscopic preparations, most resins are not miscible with water; in order to obtain complete infiltration of the tissue it is first necessary to dehydrate the tissue with ethanol to remove all water. Once all the water is removed and the tissue equilibrated in an appropriate link reagent, (i.e. a substance that is miscible with both ethanol and the resin) it is infiltrated with the resin in a liquid form, this is then allowed to polymerize. The ideal resin is one that is soluble in the dehydrating agent, does not change volume on polymerization and polymerizes evenly, is easy to cut with an ultramicrotome, has good optical qualities, and is stable in the electron beam of the electron microscope. The most commonly used resins in the preparation of neural tissue for electron microscopy are epoxy resins but several other types are available including those that are miscible with water prior to polymerization (1, 2).

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**Protocol 8.** Dehydration and embedding in resin

*Materials*

- graded series of volume for volume dilutions of ethanol in water (50%, 70%, 90%, 100%)
- dry absolute ethanol (ethanol stored over anhydrous cupric sulphate)
- propylene oxide[a]
- electron microscopic resin (e.g. Durcupan ACM, Fluka)
- aluminium foil for making boats

*Method*

Osmium-treated and washed sections are treated sequentially in glass vials with the following.

1. 50% ethanol for 15 min. Use a large volume (~ 10 ml) to ensure that all the phosphate is removed since any remaining phosphate may react with the uranyl acetate in the following step to produce an electron dense precipitate in the tissue.

**Protocol 8.** *Continued*

2. 0.5–1.0% uranyl acetate[b] in 70% ethanol for 20–30 min. Prepare the uranyl acetate solution before it is required (~ 30 min) since it takes time to dissolve. Filter before use.
3. 95% ethanol for 15 min.
4. 100% ethanol for 10–15 min.
5. Dry absolute ethanol for 10–15 min.
6. Two changes of propylene oxide for 10–15 min each.[a]
7. During dehydration prepare aluminium boats using vials as a mould, attach these to an appropriate container (we use photographic paper boxes) with double sided adhesive tape.
8. Prepare resin[c] according to manufacturer's instructions. For Durcupan, mix the components A:B:C:D in the ratios 10:10:0.3:0.2 by weight, in a disposable plastic beaker. Mix thoroughly with a disposable glass pipette or wooden spatula and add 1–2 ml to each aluminium boat. The resin components should be weighed out in the fume hood.
9. Using a paint brush (dedicated) transfer the sections from the last propylene oxide to resin in the aluminium boats and leave overnight in the fume cupboard. Ensure that the sections do not dry out at this stage and that they are completely submerged in the resin. The label from the scintillation vial can be transferred to the aluminium boat, care should be taken to avoid the propylene oxide washing the writing off the labels.
10. Gently warm the boats containing the sections and the resin on a hot plate and transfer the sections, one at a time, with forceps to uncleaned microscope slides.[c] Allow them to settle for a few minutes.
11. Briefly examine the slides under a dissection stereo-microscope (protected from contamination with resin by covering in laboratory or kitchen film) to ensure that sections or fragments of sections are not overlying each other. The sections or fragments can be manoeuvred about the slide with cocktail sticks or a paint brush.
12. Place a coverslip on top of sections.[d] Allow to settle again and then press down gently to remove all air bubbles and any excess resin. The amount of resin should be sufficient to cover the sections and spread to the edge of the coverslip by capillary action but not to emerge from the sides when the coverslip is pressed. Excess resin can be removed by absorbing it on to filter paper and extra resin can be added by placing drops along the edge of the coverslip.
13. Place the slides in containers (photographic boxes lined with aluminium foil) and polymerize the resin by heating in oven at 60°C for 48 h. All materials contaminated with resin are heated under the same conditions

and then disposed of. The sections are now in a form suitable for light microscopic analysis prior to electron microscopy and can be stored indefinitely.

[a] **Safety note 4**. Propylene oxide is potentially extremely toxic by ingestion, inhalation or absorption through the skin, and is highly volatile and flammable, it should be handled *only* in a fume cupboard. Do not wear gloves as it will dissolve in them and concentrate next to the skin. Place waste propylene oxide in a 500 ml loosely stoppered bottle containing 200 ml ethanol and 200 ml of ammonia solution (35%). When full leave for approximately two days and then wash down the sink with large volumes of water. All materials contaminated with the propylene oxide should be left in the fume hood for at least one day to allow complete evaporation.

[b] **Safety note 5**. Uranyl acetate is toxic both because it is a heavy metal and is radioactive, protective clothing and gloves should be worn and it should only be handled in a fume hood.

[c] **Safety note 6**. Some components of epoxy resins are likely to be toxic. Wear protective clothing and gloves, only handle in a fume hood, and polymerize all contaminated materials.

[d] The slides and coverslips should not be cleaned and may in fact be passed gently through the fingers to coat them with grease or they may be silicon-coated. This aids the removal of the coverslip during the re-embedding of the sections at a later date (*Protocol 9*). Alternatively the sections may be embedded between acetate sheets (see Chapter 10) or plastic coverslips may be used.

## 6.3 Light microscopic analysis prior to electron microscopy

Once the resin has polymerized the sections are in a form in which they may be stored indefinitely. They are suitable for detailed light microscopic analysis and in fact the osmium treatment and embedding in an epoxy resin produces material of high quality and allows more detail to be resolved than material prepared for light microscopy alone. The main advantage however, of tissue flat-embedded on microscope slides is that the *same* sections that are examined in the light microscope are examined in the electron microscope, and indeed the *same neuronal structures* that are identified in the light microscope can be examined in the electron microscope (correlated light and electron microscopy). The sections are examined for structures that are stained by the histochemical or immunocytochemical protocols. Neurones, neuronal structures, or regions of interest are noted, sometimes drawn (with the aid of a drawing tube) and photographed. The region of interest, containing the neurones or neuronal structures that are to be examined in the electron microscope or that are to be subjected to post-embedding immunocytochemistry (Chapter 6), is then re-embedded in blank blocks of resin, or glued on to blank blocks of resin that are suitable for further sectioning for electron microscopy (*Protocol 9*).

### **Protocol 9.** Re-embedding for electron microscopy

*Materials*

- dissection microscope
- single edged razor blades

**Protocol 9.** *Continued*

- scalpel with pointed blades
- blank tubes of resin or truncated electron microscopy embedding capsules
- cyanoacrylate glue or electron microscopy resin
- hot plate

*Method*

1. Ensure that all excess resin around the edge of the coverslip is removed.
2. Completely remove coverslip from the slide or remove just that part of the coverslip which overlies the neurones or regions of interest by gently inserting a razor blade between the coverslip and the resin and pushing until the coverslip is free. Once the coverslip is removed the slide can still be examined in the light microscope even using oil immersion lens, although care should be taken not to damage the tissue since it is no longer protected by the coverslip.[a]
3. Identify neurones or regions to be re-embedded in the light microscope and make guide cuts in the resin with a razor blade or pointed scalpel blade.
4. Warm slide gently on a hot plate to soften the resin,[b] cut around neurones or areas of interest with a scalpel blade under the dissecting microscope, and remove piece of tissue with forceps.
5. Flatten the small piece of resin-embedded tissue by placing it on a warm razor blade or slide and even sandwiching between two of them if necessary.
6. *Either*: glue to the end of a blank tube of resin[c] with cyanoacrylate glue (Superglue) or araldite.
   *Or*: place face down on a fresh microscope slide, place truncated embedding capsule over it, fill with resin, place label in, and seal with a coverslip. Polymerize the resin at 60°C for 48 h. Once polymerized, remove the coverslip and capsule with the aid of a razor blade. The block should come away from the slide easily.

[a] If a section is damaged after the coverslip has been removed it can be repaired by applying a small drop of resin and a new coverslip, and curing for 48 h at 60°C. Similarly, after cutting out the region of interest, the slide can be restored by the same method. Following removal of the coverslip, good optical qualities of the slide can be restored temporarily, by applying a drop of immersion oil and a new coverslip.

[b] The resin is brittle at room temperature: should it be cut without warming there is the possibility of splitting or cracking areas of interest. Furthermore once a piece of tissue is cut away from the slide it should only be further trimmed when warmed as trimmed pieces or, just as likely, the area of interest may 'flick' away.

[c] The blank tubes of resin are made either using a mould or by cutting off the pointed end of an embedding capsule, placing it on a slide, over-filling with resin, placing a coverslip over the end, and curing for 48 h at 60°C. The resultant blocks should have two optically good surfaces that allow the examination of the re-embedded tissue in the light microscope. The blank blocks measure 8 mm in diameter and about 10 mm in height.

## 6.4 Re-sectioning for electron microscopy

The sections prepared for examination in the light microscope prior to the electron microscope are in the region of 50–100 μm in thickness. For electron microscopy, however, it is necessary to have sections of a thickness in the region of 60–90 nm. In order to examine this material in the electron microscope or indeed material prepared for direct electron microscopic analysis, it is necessary to re-section at a thickness suitable for electron microscopy. Sectioning at this thickness can only be carried out on commercially available microtomes, referred to as ultramicrotomes (Leica). Tissue that has been re-embedded in blocks of resin in the manner described above are suitable for sectioning on an ultramicrotome. A precise description of sectioning on an ultamicrotome is beyond the scope of this volume, it is a difficult process that requires instruction in relation to a particular ultramicrotome. Only the basic principles will be briefly described here.

### 6.4.1 Principles of sectioning for electron microscopy

The essential features of all ultramicrotomes are:

- chuck to hold specimen
- knife holding block
- advance mechanism; either thermal feed or mechanical
- stereo-microscope
- system of illumination
- glass or diamond knife

A block with the re-embedded tissue is examined in the light microscope by placing on a glass microscope slide and treating as a normal light microscopic preparation; the neurones, neuronal structures, or regions of interest are located. The block is then placed in the ultramicrotome chuck which is put in a position for direct viewing of the block surface. The surplus resin and tissue are trimmed away with a razor blade under stereo-microscopic guidance. With frequent comparisons with the image seen in the light microscope, the block is trimmed to produce a face suitable for ultrathin sectioning (in the region of 1 $mm^2$ or less, and the top and bottom edges parallel). The chuck with trimmed block is placed in the cutting position. The chuck is adjusted to make the block surface parallel to the knife edge. The knife holding block, which is stationary during the operation of the microtome, is advanced to the block surface by a coarse and fine manual feed. Fine adjustments to make the block surface parallel to the knife edge are made; the chuck and the knife holder between them can be adjusted in all three planes. The close adjustment is aided by the special illumination system that gives a reflection of the knife edge on the block surface. Once 'lined-up' the boat, which is an integral part of a diamond knife but must be attached to a glass knife, is filled with

water. The machine is turned on and the specimen passes the knife edge and is then advanced by either a mechanical or thermal feed. Sections float on the water; the thickness is monitored by their interference colours and adjusted accordingly. A well trimmed specimen with clean, parallel edges will result in a ribbon of sections of consistent thickness. Series of up to 100 sections are cut in a single session. If analysis of serial sections is to be carried out, which is usually the case in the analysis of synaptic connections, the sections are collected on single-slot coated grids (*Protocol 10*) rather than on mesh grids, as a significant area of the sections would be obscured by the bars of a mesh grid. The grids are dried and stored in a commercially available grid box (Agar Scientific Ltd. or Cambridge Instruments).

### 6.4.2 Some useful hints for ultrathin sectioning

(a) The smaller the block face the easier it is to cut long series of sections, and the easier it is to locate structures in correlated light and electron microscopic analyses.

(b) An asymmetric shape to the block surface makes orientation in the electron microscope easier.

(c) Dirt is easily picked up during sectioning so always clean forceps, syringe needles, eyelashes etc. with ethanol prior to any manipulation of sections or electron microscope grids.

(d) Always store sections in the grid boxes in the same way, i.e. sections either to the right or to the left.

### 6.4.3 Semi-thin sectioning on an ultramicrotome (see also Chapter 6, *Protocol 1*)

The ultramicrotome can be used to cut sections at 1–5 μm from resin embedded tissue (semi-thin sections). The same material that is prepared for electron microscopy can be cut, and indeed one of the main reasons for cutting semi-thin sections is that they aid in orientation of sections in the electron microscope and the localization of structures first identified in the light microscopic section. The sections are only cut with glass knives since sections of this thickness damage the cutting edge of a diamond knife. As with ultrathin sections the semi-thin sections are floated on water, picked up with a drop of water with the point of a hypodermic needle or a specially designed loop, and placed on a drop of water on coated microscope slides (*Protocol 5*). The sections are dried on to the slide with gentle heat on a hotplate. The sections can then immediately have a coverslip applied with a mounting medium (XAM or DPX) or they can be stained with a mixture consisting of:

- 50 ml 1% aqueous Azur II
- 50 ml 1% toluidine containing 0.5 g sodium tetraborate
- 6 g sucrose

Filter the stain before use, place a drop on the section, heat gently on a hotplate, and wash in running water. Alternatively, they can be subjected to post-embedding immunocytochemistry (see Chapter 6).

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**Protocol 10.** Coating of single-slot electron microscope grids

*Materials*

- single-slot electron microscope grids (copper, nickel, or gold; from any EM supplier)
- chloroform
- grid-coating resin (Pioloform, Butvar or Formvar, Fisons Polaron)
- distilled water
- staining trough or dish
- grid-coating apparatus (from Agar Scientific Ltd.) or small vessel with tap at bottom
- laboratory film, e.g. Parafilm (made by American Can Company)
- Petri dishes

*Method*

1. Clean grids in chloroform, dry on filter paper, and store in Petri dish.
2. Prepare a 1% solution of grid-coating resin in chloroform, filter, and store at 4°C.
3. Fill coating apparatus with the resin solution. Put cleaned microscope slide in the solution in the upright position. The slide is then coated with the Pioloform by either opening the tap at the bottom of the vessel and allowing the solution to flow out, or by slowly lifting the slide vertically out of the vessel. The thickness of the film is dependent on the rate of removal of the slide from the solution or the rate of flowing out of the solution (the slower the rate the thinner the film).
4. Once all the chloroform has evaporated, remove the film from the slide by scoring the bottom edge of the slide with a razor blade and lowering it into the water at an angle of about 45°. The film should separate from the slide, and by continuing to lower the slide into the water, the film will float off completely. The correct thickness of the film appears silver in colour when illuminated from a lamp above the surface of the water.[a]
5. Place grids on to the film, one at a time, with the matter side down. Cover the whole film with grids but take care that they do not overlap.
6. Remove the film with grids adhering from the water by placing a piece of laboratory film (Parafilm) on top of them and gently lifting. Place the Parafilm with coated grids in a clean Petri dish.

**Protocol 10.** *Continued*

7. Before using a batch of grids, examine a few from each film in the electron microscope to check for strength, evenness, and cleanliness.

[a] Cover water surface with foil when not in use to prevent dust falling on to it.

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### 6.4.4 Manipulations of ultrathin sections

Ultrathin sections collected on coated *gold* or *nickel* grids are in a form that is suitable for immunocytochemistry by the immunogold method (post-embedding immunocytochemistry: see Chapter 7). For most applications *copper* grids are used. The sections can be immediately examined in the electron microscope but it is usually necessary to stain the sections with heavy metals to improve contrast. The two most commonly used stains are uranyl acetate and lead citrate (*Protocol 11*). It is usual to expose the tissue to uranyl acetate during the dehydration procedure (see *Protocol 8*), but this is not necessarily the case, and it can be performed on sections prior to the lead staining.

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**Protocol 11.** Staining of ultrathin sections with Reynolds' lead citrate[a] (4)

*Materials*

- lead nitrate
- tri-sodium citrate
- 4 M sodium hydroxide
- sodium hydroxide pellets
- Petri dish
- laboratory film, (e.g. Parafilm)
- filter paper
- wash bottle containing distilled water
- hair dryer
- Pasteur pipettes

A. *Preparation of the lead citrate*

1. Weigh out 0.27 g of lead nitrate (we use glass vials).
2. Weigh out 0.35 g of tri-sodium citrate in a separate vial.
3. Add 9.6 ml of distilled water to the lead nitrate. Put cap on vial and shake to dissolve.

4. Add the tri-sodium citrate to the lead nitrate solution. Shake to dissolve. At this stage the solution is milky in appearance.
5. Add 0.4 ml of 4 M NaOH. Replace cap quickly[b] and shake.

B. *Staining of sections*[c]

1. Prepare a Petri dish containing a piece of laboratory film and sodium hydroxide pellets to absorb $CO_2$.
2. Take up some of the lead citrate solution in a Pasteur pipette, discard first drop, and place separate drops on to the Parafilm, only removing the cover of the Petri dish as far as is necessary.
3. Place grids, sections down on the drops of stain (one grid per drop) and leave for 1.5–5.0 min.
4. Pick grids up one at a time, wash in a stream of distilled water, dry excess water off with filter paper, particularly between the points of the forceps, and completely dry with warm air from a hair dryer.
5. Replace grid in grid box.

[a] **Safety note 7.** Lead and lead salts are toxic, appropriate precautions should be taken to prevent contact with the skin.

[b] This solution can be stored up to two weeks at 4°C. Prevent contact as far as possible between lead citrate and the air as atmospheric carbon dioxide reacts to form an insoluble, electron dense precipitate.

[c] The same method can be applied to staining of sections with uranyl acetate (1% aqueous solution that is filtered before use), except that the precautions to avoid contact with $CO_2$ are not necessary, and the staining time is 20–60 min (see *Safety note 4, Protocol 8*).

## 7. Examination of sections in the electron microscope

If the tissue has not been subjected to any histochemical or immunocytochemical staining procedure then the sections are examined in the electron microscope by standard procedures. Similarly, if the material contains a high density of labelled structures, for instance anterogradely labelled terminals, then analysis can be by standard procedures. It must be remembered however, that in many histological or immunocytochemical procedures the penetration of the reagents into the tissue may be very limited, it is thus important to examine those ultrathin sections that are closest to the surface. In some cases it may even be necessary to angle the tissue during ultrathin sectioning so that the very surface of the tissue is included in the sections.

If the structures under investigation are rare, are rarely stained, or the ultrastructural analysis needs to be related to the three-dimensional or light microscopic morphology of the neuronal structure under investigation, then it is necessary to identify and examine the *same* structures in the electron

microscope that were identified in the light microscope. The same is also often the case in multiple labelling studies (see Chapter 11) where a particular event, for instance the close apposition of an anterogradely labelled terminal and a retrogradely labelled cell, is rare for technical reasons. Thus a neurone that is characterized first at the light microscopic level by its morphology (by for instance, Golgi-impregnation see *Protocol 12*, or the intracellular injection of HRP see Chapters 9 and 10), by its projection area (see Chapters 2 and 4), or its chemical characteristics (see Chapters 5 and 7) can then be examined in the electron microscope to characterize its ultrastructure, and its afferent and efferent synaptic connections.

Furthermore, by examination of light microscopically identified neurones in the electron microscope the topography or pattern of afferent synaptic terminals to a particular neurone can be assessed, and *quantitative* aspects of both the input and output can be established. In this way the position of individual neurones and eventually classes of neurones, in the neuronal microcircuitry or networks of a particular area of the brain can be established. The technique by which a structure that is first identified in the light microscope is then examined in the electron microscope, termed 'correlated light and electron microscopy', was pioneered by Blackstad (5, 6) and Smogyi (7, 8) and is described in detail elsewhere (9).

## 7.1 Correlated light and electron microscopy

Probably the most important step in correlated light and electron microscopy is the detailed records of the structure under investigation at the light microscopic level. In addition to aiding the analysis of the structure in the electron microscope, it is important to make as detailed recordings as possible because once they are sectioned for electron microscopy they are lost. Detailed high magnification photomicrographs and drawings are necessary. It is also valuable to note and record the positions of easily identifiable structures, (e.g. capillaries, neuronal perikarya, etc.) that may be used as 'landmarks' to help locate the neuronal structure under study in the electron microscope. Having decided and recorded those structures that are to be examined in the electron microscope, the coverslip is removed and the tissue re-embedded for ultrathin sectioning (*Protocol 9*).

Once re-embedded the tissue within the block can again be examined in detail in the light microscope and if necessary photographs may be taken, (e.g. see Figure 2A of Bolam *et al.* (10), which was taken from the surface of the block). The block is trimmed to include the area of interest or structures of interest. Rather than using the classical symmetrical trapezium shape for the trimmed block surface, an asymmetrical shape is used to simplify the orientation of the sections in the electron microscope. The distance from the surface of the block to the tissue can be measured by focusing on the surface, and then on a structure in the tissue, and reading off the distance on the microscope vernier. The tissue (or structure of interest) is now approached on

an ultramicrotome under manual control, cutting one or two micron sections with regular monitoring in the light microscope. At each examination in the light microscope the size of the block is assessed and trimmed further if necessary to reach optimal size for serial sectioning; the distance of the structure of interest from the surface is also measured. When less than 1 μm from the structure of interest, the ultramicrotome is turned on to automatic, and serial ultrathin sections (silver/grey, approximately 70 nm) are cut. Short series of 50–100 sections are generally cut in one session and are collected on coated single-slot grids (*Protocol 10*). When the sections have been collected, the block is removed from the ultramicrotome chuck and examined again in the light microscope. The location within the block, of the parts of the neuronal structure that have been completely or partly sectioned are noted, and their positions marked on both the drawings and the high magnification micrographs.

After staining the ultrathin sections with lead citrate (*Protocol 11*) they are examined in the electron microscope. The identified structures, (i.e. those examined in the light microscope) are located within the section and examined at high magnification. To localize, or find, the identified structures in the electron microscope, constant comparisons are made between the positions of structures seen in the block in the light microscope (it is useful to have a light microscope adjacent to the electron microscope), the image in the electron microscope, and the high magnification light micrographs. The positions of the identified structures can then be confirmed by their relationships to the 'landmarks' (for instance, unstained neuronal perikarya or blood vessels) in the block and in the light micrographs. Once located, structures are easily examined in serial sections and with careful sectioning on the ultramicrotome, from series to series.

An additional aid to the location of identified structures in the electron microscope are 1 μm sections taken at the beginning or at the end of the series of ultrathin sections (see Section 7.4.3). These sections, suitably counter-stained, are more easily compared than the block surface with the image in the electron microscope. The major problem with 1 μm sections is that structures can no longer be recovered for electron microscopy or can only be done so with great difficulty, thus should only be taken at times when important structures will not be lost.

Records of the observations of the structures examined in the electron microscope should include low and high power light and electron micrographs that include 'landmarks' to demonstrate or 'prove' that the structure examined in the electron microscope is indeed the same structure that had been examined in the light microscope.

## 8. Single section Golgi-impregnation (11)

Many of the histological procedures designed to stain neurones that are used in experimental neuroanatomy, particularly when preparing for electron

microscopy, result in staining of only cell bodies and proximal dendrites. This is a major disadvantage if it is difficult to morphologically characterize a neurone on the basis of the morphology of the cell body and proximal dendrites. It is also a major problem when examining the synaptic input to the neurone since without the distal dendritic tree, both qualitative and quantitative data will be severely limited. One approach to overcome this problem is to Golgi-impregnate the neurones that are stained by the histochemical or immunocytochemical process (11–14). Golgi-impregnated neurones are often stained in their entirety and, following gold-toning, can be examined in the electron microscope (5–8). Although the impregnation of neurones appears to be a random process, staining of sufficient sections eventually results in the impregnation of histochemically stained neurones. A simple and rapid method of impregnation of tissue sections is described that is a modification of the 'single-section-Golgi' procedure of Freund and Somogyi (13).

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**Protocol 12.** Single section Golgi-impregnation (11)

*Materials*

- 3.5% aqueous potassium dichromate
- 1–2.5% aqueous silver nitrate
- glycerol
- microscope slides and coverslips
- artist's paint brush
- filter paper
- tape (electrician's insulation tape or masking tape)

*Method*

1. After histological processing and treatment with osmium tetroxide (*Protocol 7*) place the sections in 3.5% aqueous potassium dichromate for 1–3 h, or overnight if convenient, at room temperature.
2. Remove sections, one at a time, and place at one end of a microscope slide. Trim with a razor blade to remove large masses of myelinated axons and ventricles which may hamper diffusion of silver nitrate solution through the section.
3. Remove excess potassium dichromate solution with filter paper and place another slide directly on top of the first. The two slides are held together by gently taping at the opposite end to the sections with the electrician's insulation tape. If the sections are small, then two may be placed side-by-side on a single slide.
4. Place the slide upright in a small beaker that is approximately one third

full of silver nitrate solution. The space between the slides immediately fills with the silver nitrate solution by capillary action.[a] Several slides can be placed in one beaker of silver solution.

5. Monitor progress of the impregnation by examination with a microscope taking care to protect the microscope and lenses from the silver nitrate solution.
6. On completion of impregnation, usually 6–24 h, remove the sections by cutting the edge of the tape that holds the slides together, separating the slides with a razor blade. Lift off the section with an artist's paint brush dipped in glycerol. Place each section between coverslips.

[a] If the silver solution flows over the surface of the section crystals of silver chromate will be formed that will hinder impregnation and obscure any impregnated cells. Remove the section from the slide, briefly rinse in water, and put back into the potassium dichromate solution.

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For electron microscopic analysis of Golgi-impregnated neurones it is necessary to carry out a de-impregnation of the neurones to remove the electron dense silver chromate precipitate that fills the cytoplasm of impregnated structures. De-impregnation is carried out by reacting with gold chloride (gold-toning) which results in the deposition of metallic gold within the impregnated neurones. The gold deposit is less dense and allows the visualization of histochemical markers within the neurones at the light level, and allows more detailed ultrastructural analysis of the identified cells and their synaptic inputs (15–17).

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**Protocol 13.** Gold-toning of Golgi-impregnated structures

*Materials*

- fibre optic lamp (15 V 150 W bulb supplied by Schott)
- cool air blower
- 0.07% $NaHAuCl_4 \cdot 2H_2O$ containing 0.25% glycerol
- 0.2% oxalic acid
- 1% sodium thiosulphate

*Method*

1. Illuminate sections whilst still sandwiched between two coverslips in glycerol for 30 min using a fibre optic illuminating device containing a 15 V, 150 W bulb focused into an 8 mm circle at full strength. Throughout the illumination the sections are cooled by cold air blowing from a hair dryer.
2. Store sections at 4°C until gold-toning is to be carried out. Remove

**Protocol 13.** *Continued*

sections from the coverslip by gently separating them with a razor blade, picking them up with a fine paint brush soaked in glycerol, and place in glass vials.

3. Incubate for 15–30 min in 0.07% aqueous solution of $NaHAuCl_4 \cdot 2H_2O$ containing 0.25% glycerol at 0°C with occasional shaking.[a]
4. Wash sections for 3 × 2 min in distilled water, 2 min in 0.2% oxalic acid, and then 3 × 2 min in distilled water.
5. Wash for 3 × 15 min in 1% sodium thiosulphate at room temperature.
6. Rinse briefly in distilled water, dehydrate, and embed in electron microscopy resin according to *Protocol 9*.

[a] The longer the time in the gold solution the heavier is the gold-toning. Select optimal time on each run by incubating test sections.

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## Appendix: Tyrode's solution

1. Add to 950 ml distilled water in the following order:
   - 8.0 g NaCl
   - 0.2 g KCl
   - 1 ml of 26.5% $CaCl_2 \cdot 2H_2O$ (the calcium may be omitted)
   - 1 ml of 5% $NaH_2PO_4 \cdot 2H_2O$
   - 1 g $NaHCO_3$
   - 1 g glucose (if solution is made up in advance this should be added on the day of use)
2. make up to 1000 ml with distilled water
3. gas the solution with a mixture consisting of 95% $O_2$ and 5% $CO_2$ prior to use.