

# [1] Light and Electron Microscopy of Vertebrate Photoreceptors Including a Technique for Electron Microscopic Autoradiography

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## Introduction

Light and electron microscopy continue to play fundamental roles in the development of our understanding of photoreceptor cell function. The purpose of this chapter is to acquaint the reader with the tools necessary to produce high-quality tissue preservation and tissue contrast so that photoreceptors may be studied by these techniques. The chapter provides an introduction to photoreceptor cell structure and step-by-step procedures for the preservation of retinal tissue by the standard technique of primary fixation in aldehydes, secondary fixation in osmium tetroxide, and embedment in resin along with the subsequent staining of tissue sections for transmitted light and electron microscopy. A method is also provided for producing autoradiograms that can be observed by electron microscopy, thus providing a high-resolution technique for the subcellular localization of radiolabeled molecules. Illustrations, derived from mammalian retinas prepared by the procedures described here, serve to familiarize readers with the appearance of each anatomical compartment of the photoreceptor cells in tissue generally considered to be “well preserved” for ultrastructural study.

## Overview of Photoreceptor Cell Structure

Fundamental anatomical differences between rod and cone photoreceptors as observed by light microscopy are demonstrated in Fig. 1, which shows an example from the parafoveal region of a rhesus monkey retina. Cone outer segments, although somewhat tapered, are not really conical in mammalian retinas; rather, it is the large size of the cone inner segment relative to the outer segment that gives the cones an apparent conical shape. Figure 2

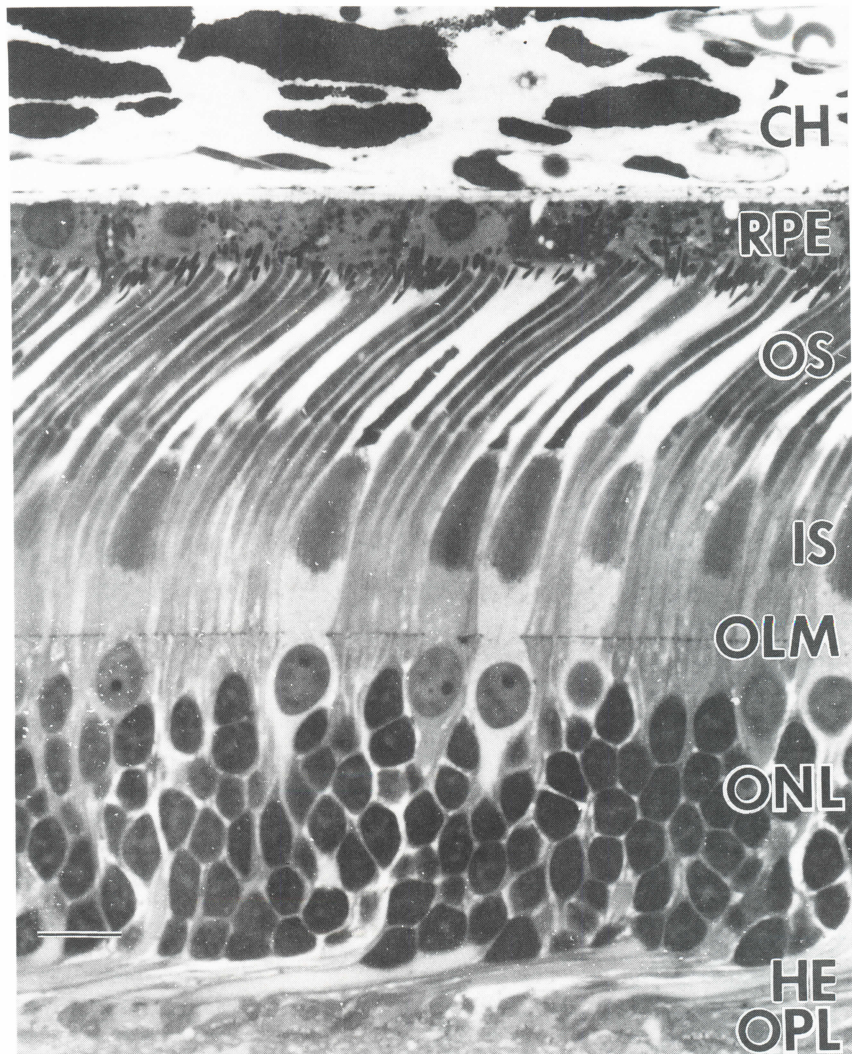


FIG. 1 Light micrograph from the parafoveal region of a rhesus monkey retina. The uppermost cellular layer is the choroid or choriocapillaris (CH). Nutrients and metabolic products must be exchanged between the choroidal capillaries and the layers of photoreceptor cells (OS through OPL) by way of the second cellular layer, the retinal pigment epithelium (RPE). The long, thin rod outer segments (OS) interdigitate directly with the apical border of the RPE, whereas the much shorter cone outer segments are, in primates, contacted by thin villuslike processes from the RPE [F. S. Sjöstrand, *Rev. Mod. Phys.* **31**, 301 (1959)]. The layer of inner segments (IS) can be divided into the densely stained ellipsoid and lightly stained myoid regions.



presents, in diagrammatic form, the basic structural features of vertebrate photoreceptors, and Figs. 3 and 4 are electron micrographs showing these general features.

In most species, rod outer segments are longer than cone outer segments, although this difference can be minimal, for example, in various species of diurnal squirrels or in the fovea (1–3). The highly ordered structure of the outer segment was clearly defined by early electron microscopic observations of rod outer segments (4–6), which demonstrated that they are composed of a stack of bimembranous disks separate from a surrounding plasma membrane. Although structural connections between the disks and the plasma membrane were shown later (7), the fundamental observation in rods remains that the molecular events in visual transduction associated with the disk membranes are physically isolated from the ion channels located on the plasma membrane.

In cone outer segments, however, the disks (although more properly referred to as “lamellae,” disks is used here for convenience) are in continuity with the plasma membrane (5, 8–10). In nonmammalian species this fact is easily demonstrated by electron microscopy of thin sections, where patency of the disks occurs along the length of the cone outer segment in any given section. In mammalian cones, outer segment disks often appear patent only at the basal one-third of the outer segment; above this region the outer segment appears rodlike, with disks separate from the plasma membrane (10, 11; Fig. 5). This ultrastructural appearance probably results from the fact that as cone outer segments develop the “disks” become only partially enveloped by a rim separate from the plasma membrane, resulting in a narrow region of disk–plasma membrane continuity that may not be apparent in a single 90-nm-thick section (10). In fact, the issue of how many, if any, cone disks occur isolated from the plasma membrane remains unresolved. In rod outer segments, only the basalmost, newly forming disks are open to the extracellular space, and this compartment may be infiltrated with extracellular tracer molecules (8). Indeed, this phenomenon has been used, as an alternative, higher resolution, less time-consuming method to autoradiography for studying outer segment membrane renewal (12).

Outer segment disks in both rods and cones develop from evaginations of the membrane of the cilium that connects the inner and outer segments (13, 14). These evaginations can be seen as outgrowths of the connecting cilium

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The outer limiting membrane (OLM) divides the layer of inner segments from the layer of photoreceptor cell bodies (ONL, outer nuclear layer). In the micrograph, the cell bodies connect to the layer of synaptic terminals that form the outer border of the outer plexiform layer (OPL) by way of relatively long “axons” called fibers of Henle (HE). Bar, 10  $\mu\text{m}$ .

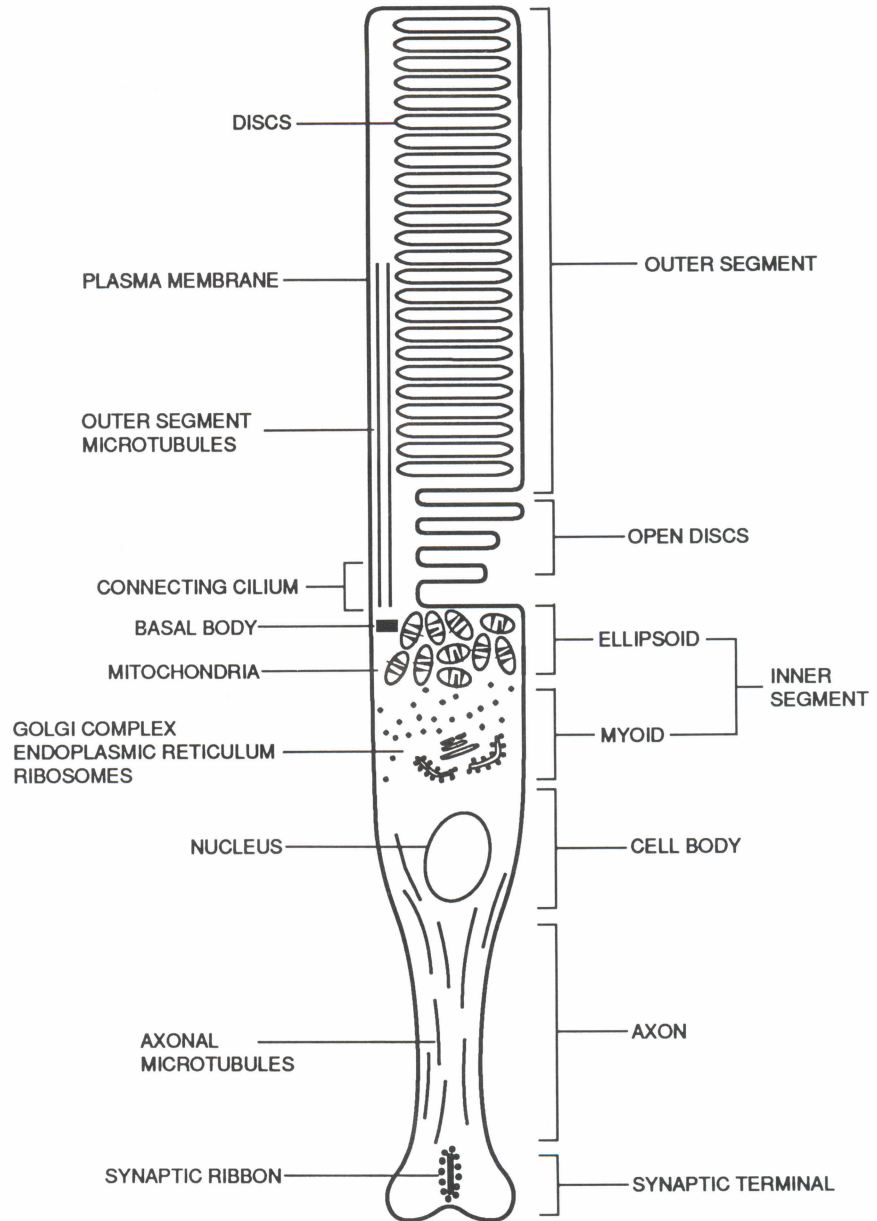


FIG. 2 Diagrammatic representation of a vertebrate photoreceptor emphasizing the high degree of polarity and compartmentalization in these cells. A rod photoreceptor is represented; in cones, many (perhaps all, see text) of the outer segment disks

that grow toward the margin of the outer segment (Fig. 5). Each surface of an evagination will form the uppermost and lowermost surfaces of two adjacent disks. As the disks mature, a secondary event occurs: the rim of the disk and new outer segment plasma membrane form by growth of the ciliary membrane in a "zippering-like" process between adjacent disk-surface evaginations (14, 15). When this process is completed in rods, the disk becomes isolated from the plasma membrane; in cones, it is incomplete, such that the disks remain patent to varying degrees depending on species.

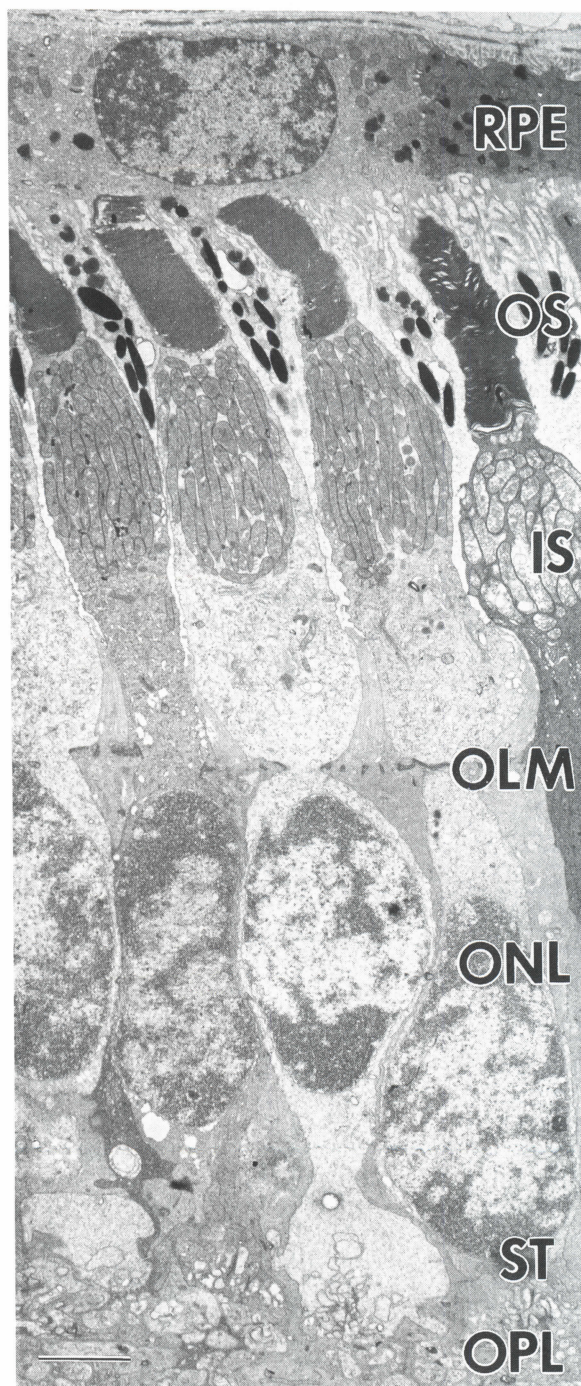
The process of outer segment disk formation occurs throughout the life of an organism, providing a mechanism for continual renewal of outer segment components. This process was first described in an elegant series of studies using light and electron microscopic autoradiography after labeling of the organism with tritiated amino acids (16; see Ref. 17 for a review). The autoradiographic studies also demonstrated a fundamental difference in the labeling pattern of rods and cones (16, 17). Rod outer segments always show the formation of an isolated "band" of radioactive protein at their base. This band of newly formed membrane then migrates toward the outer segment apex, where it is shed and phagocytosed by the adjacent retinal pigment epithelium (18). Cones, on the other hand, show a diffuse pattern of labeling in which silver grains are scattered over the length of the outer segment. The labeling pattern in rods can be explained as the literal "entrapment" of new molecular components in the disk membranes as the disks mature and become isolated from the plasma membrane. The diffuse labeling pattern in cones is generally interpreted as reflecting the fact that the "disks" of cone outer segments are in continuity with the plasma membrane, allowing the free diffusion of newly inserted protein molecules throughout the outer segment length (18, 19). Consistent with this hypothesis is the demonstration that with short labeling times a gradient of label does occur from the base to the tip of cone outer segments (20).

The connecting cilium emerges from the inner segment early in photoreceptor development and provides the only intracellular pathway for the movement of molecular components between the inner and outer segments. There have been rare observations of "cytoplasmic bridges" between the inner and outer segment (21), and Besharse and Horst (22) have presented a model in which vesicles budding from the apex of the inner segment may deliver molecular components to the outer segment. Examples of these nonmotile

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would be in continuity with the plasma membrane. The region of open disks represents the new disk surface evaginations as they grow to their full width. In rods, these will become isolated disks by the growth of the disk rim and plasma membrane.





cilia are shown in Figs. 5 and 6. For a discussion of their specialized properties, readers are referred to Besharse and Horst (22).

The compartment of the photoreceptor inner segment from which the cilium arises is known as the ellipsoid and contains structures associated with the cilium (basal body, striated rootlet), vesicles of various sizes, cytoskeletal elements such as microtubules and actin filaments (22–24), and a large, densely packed population of mitochondria. Intermediate (10 nm) filaments and “wavy” filaments occur in this region of photoreceptors in at least some species, although the composition of the filaments has not yet been determined (Figs. 5 and 6).

Vesicles are often seen congregated around the basal body of the connecting cilium (13, 22) in a region known as the periciliary ridge complex (25). At least some of these vesicles, as well as those found elsewhere in the inner segment, are presumed to be transport vesicles for delivery of molecular components (e.g., opsin) destined for the outer segment (26). Others, however, are known to be endocytic vesicles (27).

Just proximal to the ellipsoid is the myoid region of the inner segment (Fig. 7). This compartment contains most of the subcellular machinery for protein synthesis, posttranslational modification of proteins, and their packaging and transport (ribosomes, rough endoplasmic reticulum, Golgi apparatus, etc.). It is also rich in cytoskeletal elements, especially microtubules. It is from this region that newly synthesized proteins must be transported to other locations in the cell (17).

The myoid region connects to the cell body of the photoreceptor cell. At the transition between the two compartments, photoreceptors are joined to each other and to the apical processes of Müller cells by adhering junctions that form a line along the outer border of the layer of photoreceptor nuclei (Figs. 1, 3, and 4; see Ref. 28 for immunocytochemical studies of these junctions). This is a landmark easily recognizable by light microscopy and is termed the outer limiting membrane, a misnomer left over from early light microscopic studies. This array of junctions may function as more than simply points of cell–cell adhesion. It may act as a molecular sieve because molecules with a Stokes radius of greater than 36 Å (about 44,000 molecular

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FIG. 3 Low-power electron micrograph showing the cone photoreceptors of a California ground squirrel fixed by perfusion of mixed aldehydes. The very dark-staining photoreceptor on the right is found in regular arrays in the retina of several species and may represent a subclass of cones that are sensitive to short-wavelength light [i.e., blue-sensitive cones; P. K. Anhalt, H. Kolb, and R. Pflug, *J. Comp. Neurol.* **255**, 18 (1987)]. ST, Synaptic terminal. Bar, 2  $\mu\text{m}$ .



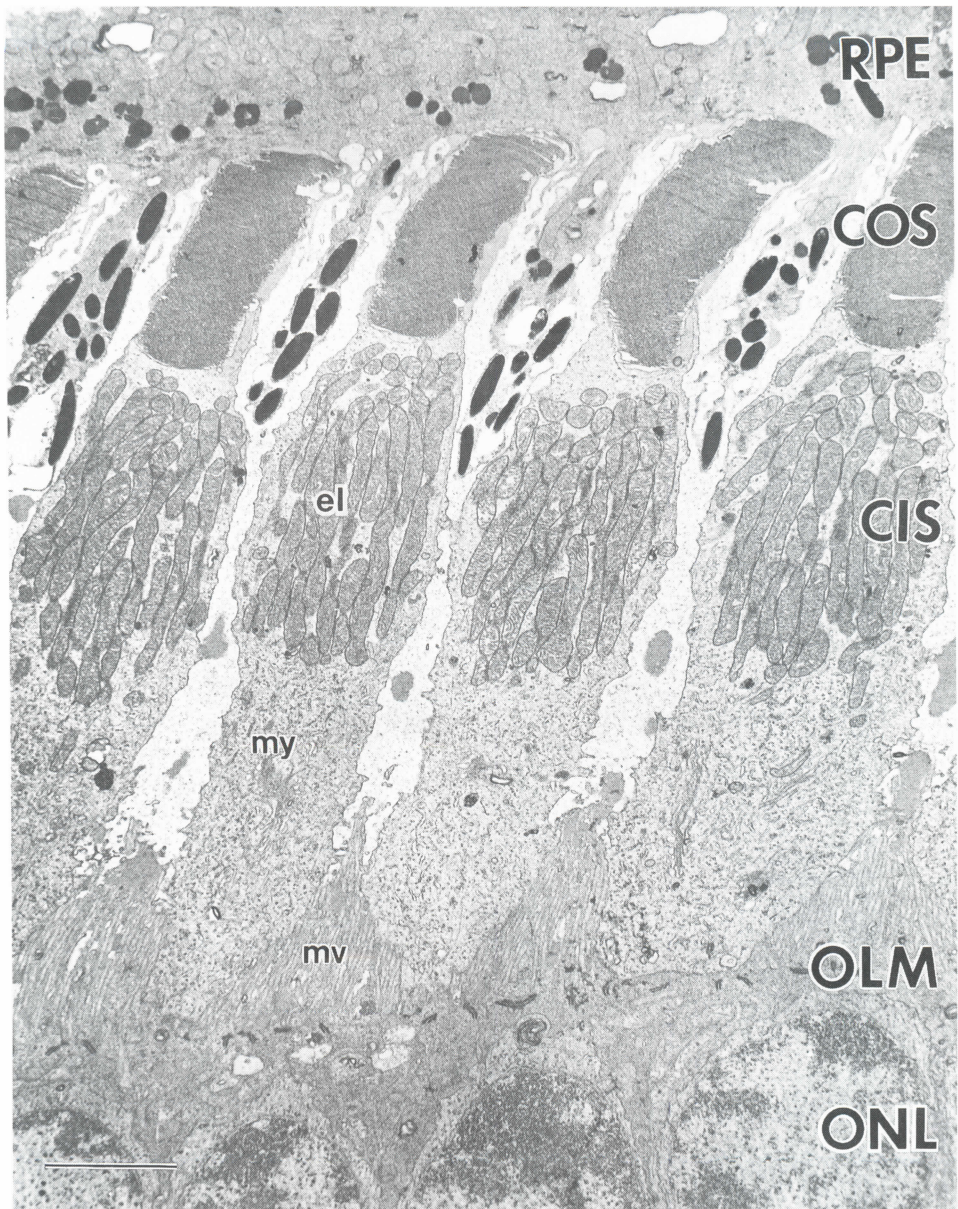


FIG. 4 Low-power electron micrograph of an array of cone photoreceptors (COS, cone outer segments; CIS, cone inner segments) in the retina of a ground squirrel fixed by mixed-aldehyde perfusion. el, Ellipsoid region of inner segment; my, myoid region of inner segment; mv, Müller cell microvilli. Bar, 2  $\mu$ m.



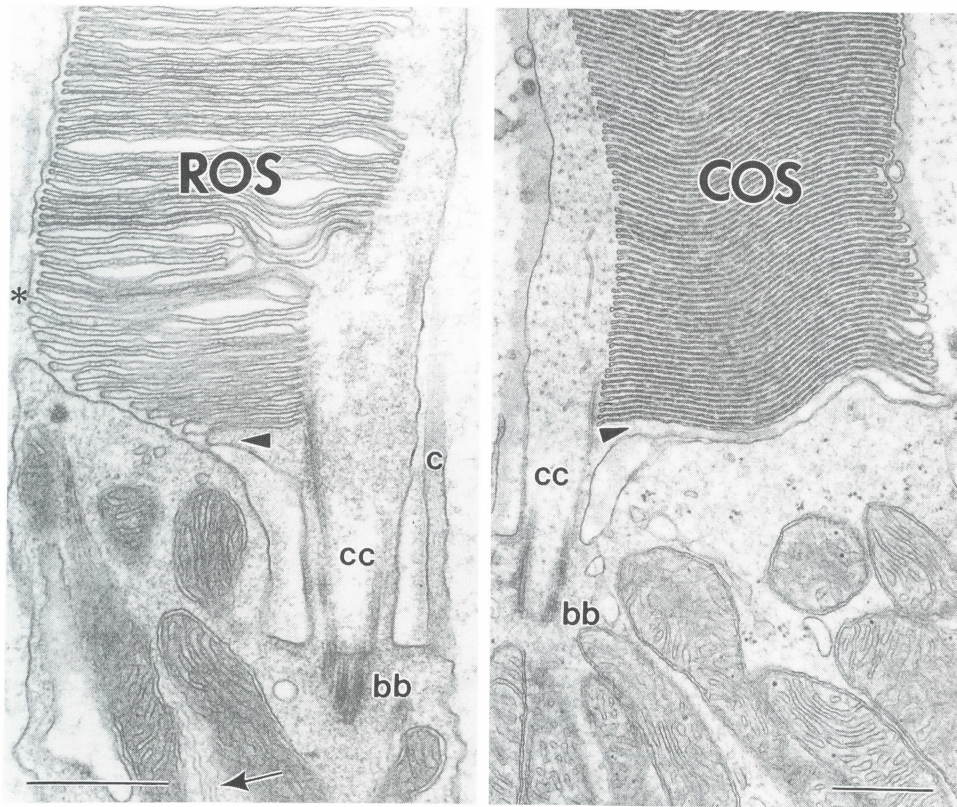
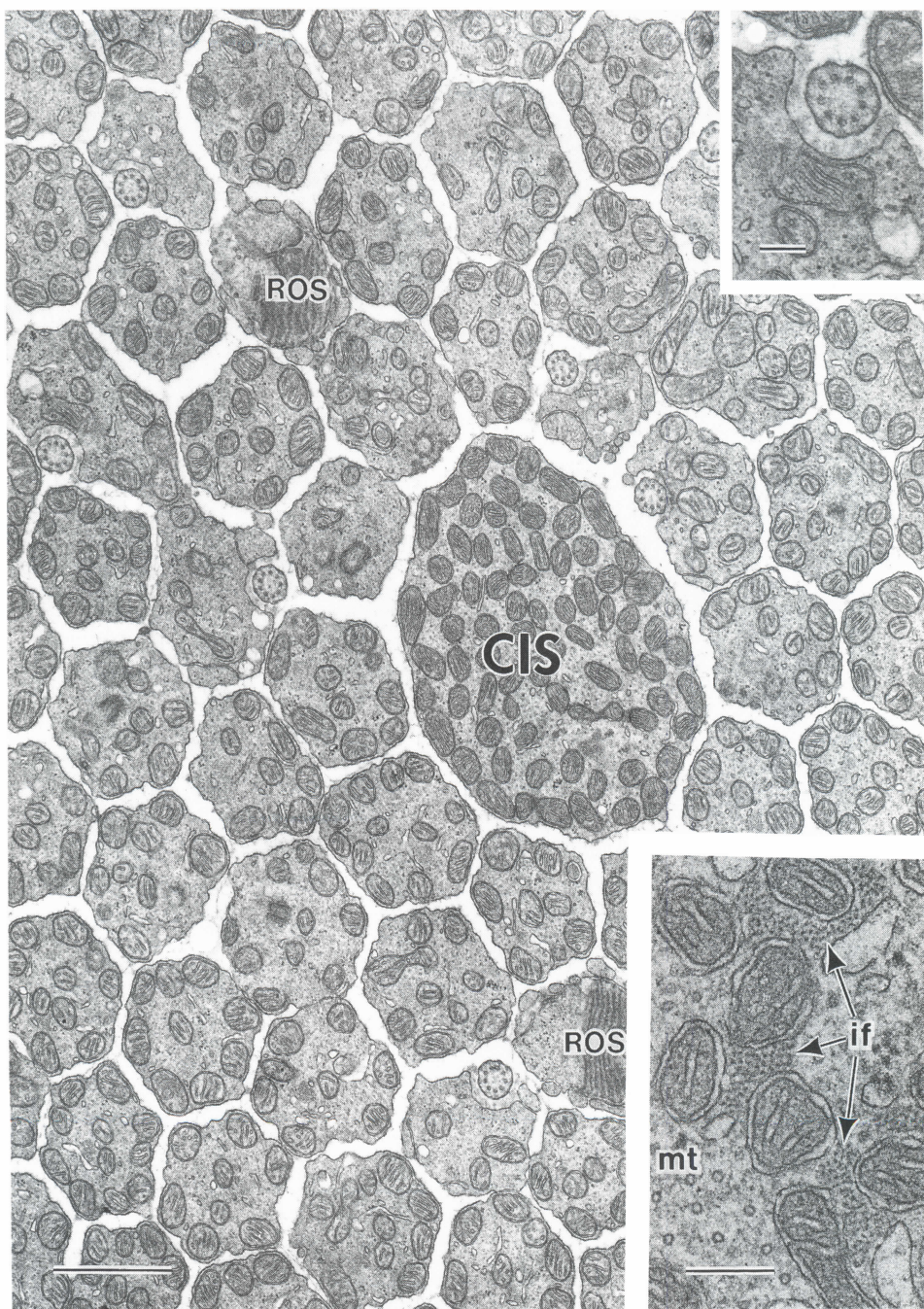


FIG. 5 Basal regions of rod (ROS) and cone outer segments (COS) from rhesus monkey and ground squirrel retina, respectively. Arrowheads indicate the cytoplasm of the evaginations that give rise to new disk surfaces. The asterisk indicates the beginning of the plasma membrane that encloses the rod disks; below that point the disks are open. Calycal processes (c) are slender, cytoplasmic, actin-filled processes that arise from the inner segment and extend along the outer segment. They occur in different numbers and lengths in different species. Note the accumulation of mitochondria in the apical inner segment, the membranous vesicles near the basal body (bb), and the small cluster of 10-nm-diameter wavy filaments (arrow). cc, Connecting cilium. Bar, 0.5  $\mu\text{m}$ .

weight) cannot diffuse through the junctions when applied to the photoreceptor side of an isolated retina (29).

The photoreceptor nucleus resides in the cell body, and a so-called axon arises from the distal end of the cell body (Fig. 8). The axon can be essentially absent, with the synaptic terminal being directly adjacent to the cell body





(2), or it can be quite long, as in the fibers of Henle that occur in the macula (see Fig. 1, for example). When present, these fibers have the ultrastructural appearance of axons elsewhere in the nervous system, that is, they contain few organelles other than microtubules and membranous vesicles presumably associated with the transport of proteins to and from the synaptic terminal (Fig. 8) (17, 30). Photoreceptors are often interconnected by gap junctions; in some species these occur between cytoplasmic "fins" that radiate from the inner segment of the cell (31), and in other species they occur between processes that extend from the synaptic terminals (Fig. 9; 32, 33).

The complex nature of information processing that must occur at the first-order synapse in the visual system was revealed by the pioneering ultrastructural studies of Sjöstrand and Missotten (34, 35) in which it was demonstrated that photoreceptors make numerous and complex contacts with their postsynaptic cells, the bipolars and horizontal cells (Fig. 10). In general, cones terminate as large pedicle-shaped endings with multiple synaptic invaginations and basal contacts, whereas rods terminate in spherule-shaped endings with a single synaptic invagination (35). These distinctions, however, are less clear in some species like the diurnal squirrels (2).

These synapses have received considerable attention in ultrastructural studies, especially those designed to disentangle their elaborate connectivity with secondary neurons (33, 36–38). It should be noted that the morphology of these synapses may also change depending on the adaptive state of the eye or time of day (39, 40). The reader is also referred to Chapter 9 of *Histology of the Human Eye* by Hogan *et al.* (41) for exquisite micrographs of photoreceptors from the human eye, and to books by Rodieck (42) and Dowling (43) for invaluable introductions to photoreceptor physiology, structure, and synaptic architecture.

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FIG. 6 Electron micrograph of a section cut transversely across the ellipsoid region of a cat retina fixed by immersion in mixed aldehydes. A large cone inner segment (CIS) is surrounded by smaller rod inner segments. The plane of section is at a level that cuts through the base of two rod outer segments (ROS). Many connecting cilia occur in the field. The upper right inset demonstrates that the pattern of microtubules in the cilia is mirrored in the extracellular matrix trapped between the ciliary stalk and the inner segment. The lower right inset shows three clusters of intermediate (10 nm diameter) filaments (if) in a rod inner segment from a rhesus monkey retina. Also note the numerous microtubules (mt) that occur in the cytoplasm. Bar, 1  $\mu\text{m}$ ; inset bars, 0.2  $\mu\text{m}$ .



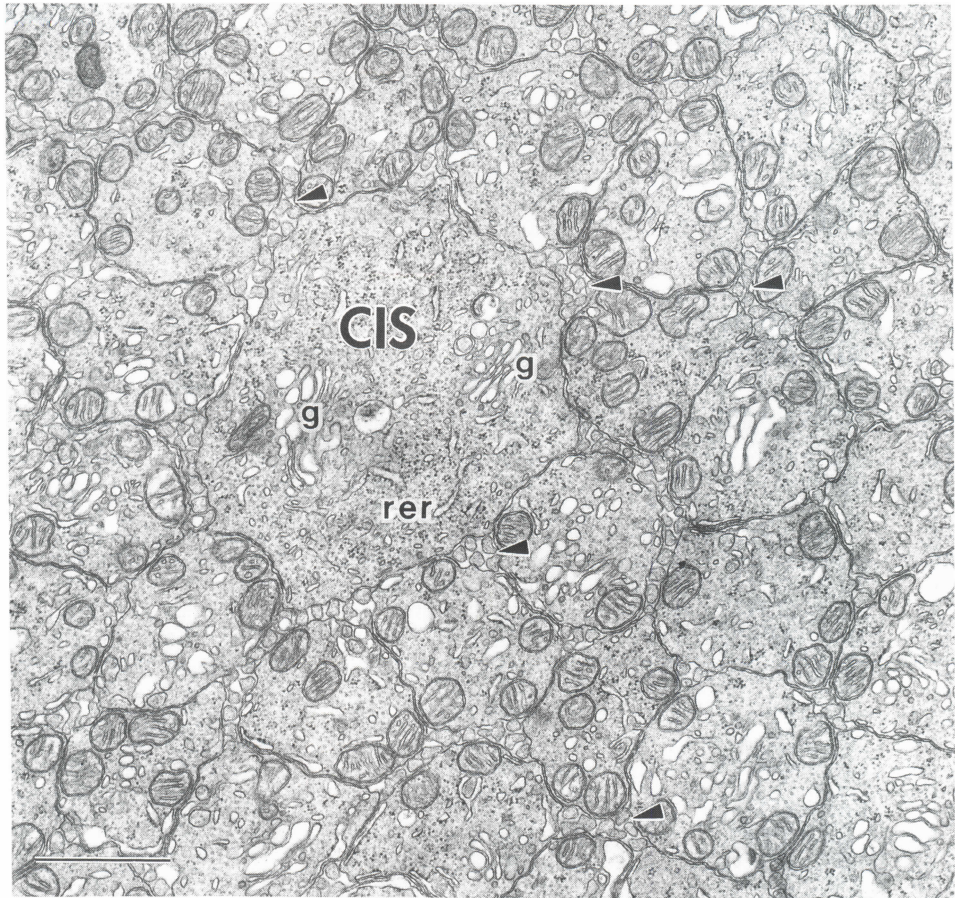


FIG. 7 Electron micrograph of a section cut transversely across the myoid region of a cat retina. A large cone myoid (CIS) is surrounded by smaller rod inner segments. Compare the tight packing that occurs in this region to that in the ellipsoid region (Fig. 6). The myoid area contains the majority of the metabolic machinery of the cell. Note the numerous cisternae that lie adjacent to the plasmalemma of the inner segments. Müller cell microvilli interdigitating between the inner segments are indicated with arrowheads. g, Golgi apparatus; rer, rough endoplasmic reticulum. Bar,  $1 \mu\text{m}$ .

## Methods

Fixation of retinal tissue can be accomplished either by immersion of an eye whose anterior and posterior segments have been separated or by intracardiac



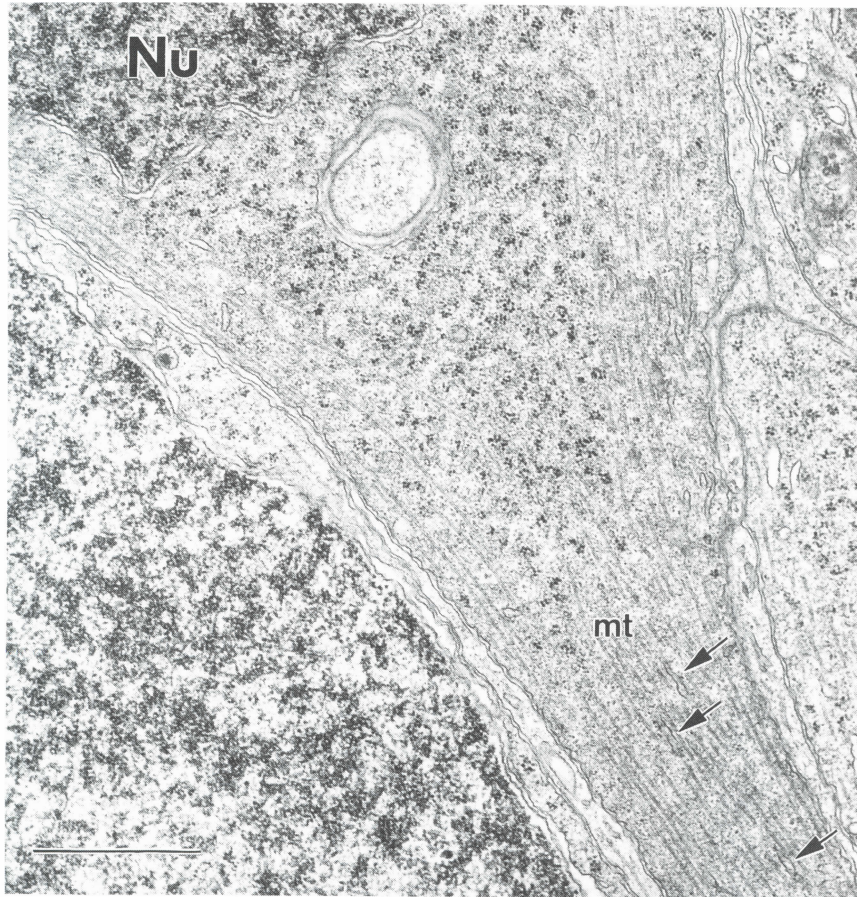


FIG. 8 Electron micrograph showing the emergence of an axon from a cone cell body in a rhesus monkey retina. Note the funneling of microtubules (mt) into the axon and the paucity of other organelles in that region. The small vesicles indicated by arrows may be associated with the transport of material through the axon. Nu, Nucleus. Bar, 1  $\mu\text{m}$ .

perfusion of fixative. The choice of method will depend somewhat on the species (e.g., squirrels must be fixed by perfusion to obtain acceptable ultrastructure) and on the needs of the particular experiment. In our experience the best ultrastructure is obtained by fixation in mixed aldehydes and postfixation in osmium tetroxide. However, glutaraldehyde is often used alone, and osmium tetroxide can be used itself as a fixative. Again the choice of (plastic)

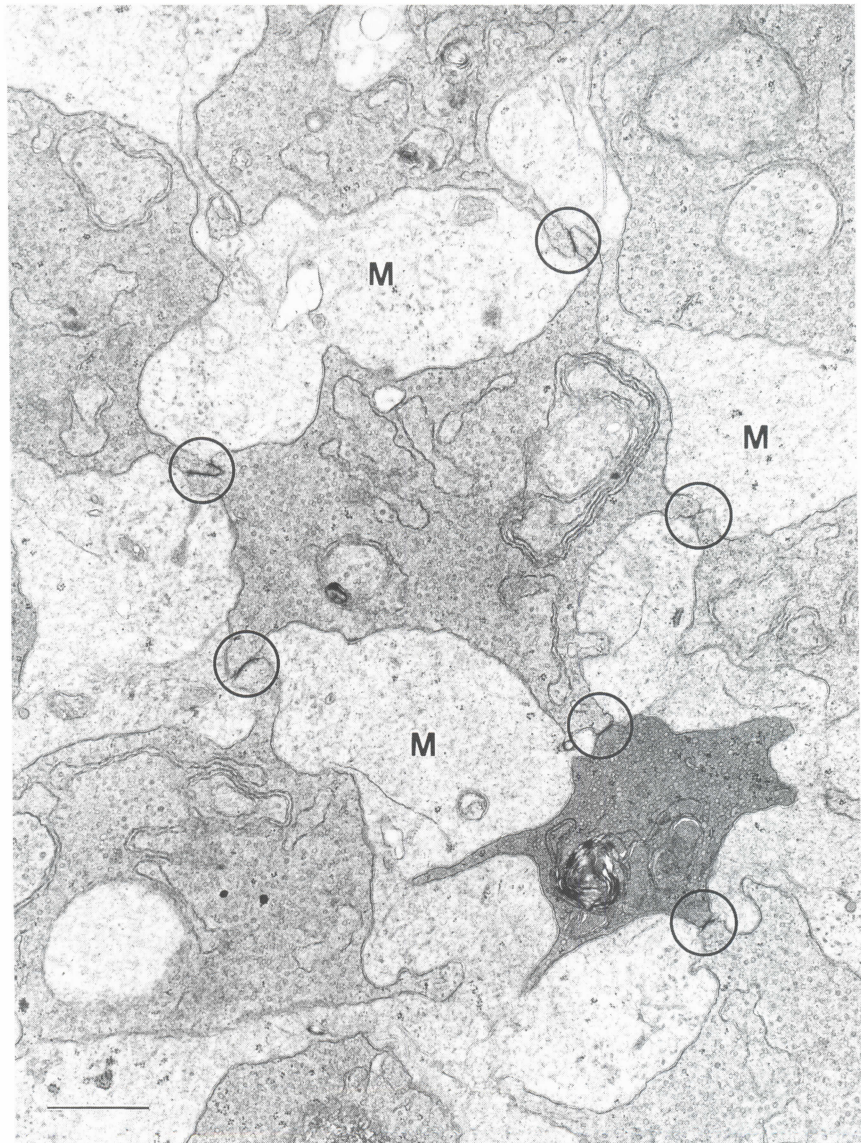


FIG. 9 Electron micrograph of a transverse section taken just above the layer of photoreceptor terminals in a ground squirrel retina. The photoreceptors are electrically linked at this point by gap junctions that occur between projections of the terminals (circles). The terminals are surrounded by Müller cell cytoplasm (M). Bar, 1  $\mu\text{m}$ .



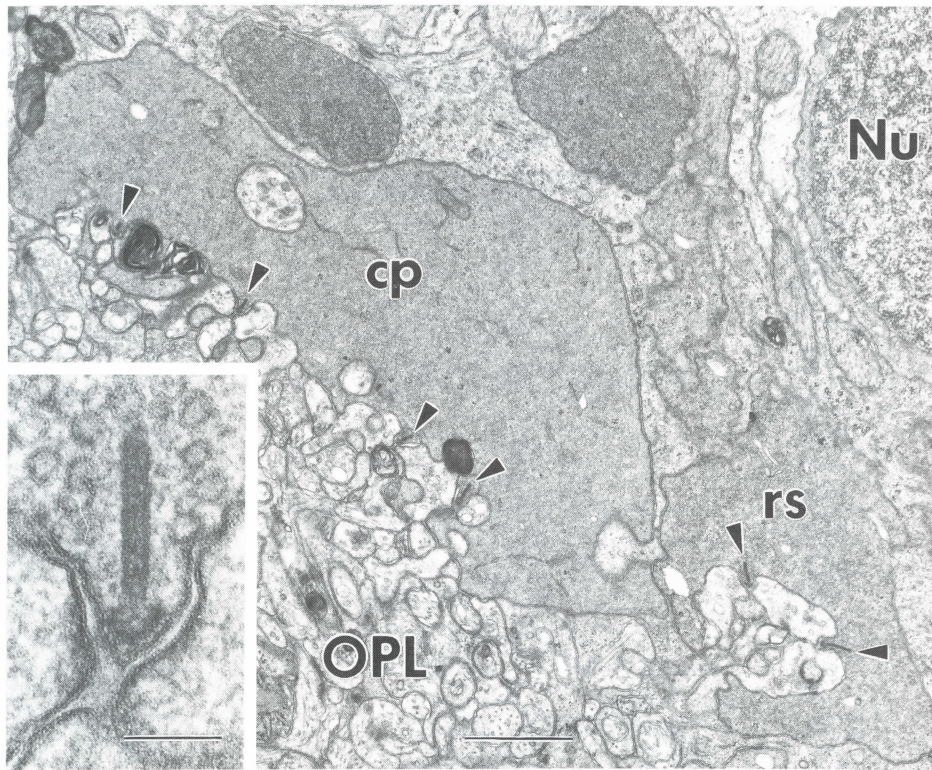


FIG. 10 Electron micrograph through the synaptic terminal layer of a human retina fixed by immersion in glutaraldehyde and postfixed in osmium tetroxide. Note the difference in shape between the rod spherule (rs) and cone pedicle (cp). Arrowheads indicate synaptic ribbons that demark the synaptic complexes of these terminals. Not apparent at this magnification are the basal junctions that occur between “flat” bipolar dendrites and the cone terminals. The inset shows a synaptic ribbon in a human rod spherule. The ribbon is surrounded by synaptic vesicles, and an electron-dense structure, known as the “arciform density,” occurs between the ribbon and the photoreceptor membrane. Bar, 1  $\mu\text{m}$ ; inset bar, 0.1  $\mu\text{m}$ .

resin for tissue embedment will be dictated by the specifics of the experiment as well as the preference of the individual. Hydrophobic epoxy resins such as Spurr’s (Polysciences, Washington, PA) and Araldite (Polysciences) have superior sectioning qualities and increased stability in the electron beam compared to hydrophilic methacrylate plastics like London Resin White (LR White; Ted Pella, Redding, CA).

## *Preparation of Stock Solutions*

### *Sodium Phosphate Stock Buffer*

The basic buffer solution is 0.172 *M* sodium phosphate adjusted to a pH of 7.2. For each 100 ml of buffer stock, use 0.68 g of monobasic sodium phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ) and 1.73 g of dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ). Add sequentially to double-distilled water while stirring vigorously. Begin with less than the target volume. After dissolving the phosphate salts, dilute to the final volume and then adjust the pH with 1 *N* HCl or 1 *N* NaOH. Isotonicity approximately equal to that of mammalian blood will be achieved later by dilution with double-distilled water or, in the case of the fixative, with 2% paraformaldehyde dissolved in double-distilled water.

### *2% Paraformaldehyde*

Into a volume of double-distilled water that is one-half of the volume of fixative needed, add fresh paraformaldehyde crystals to create a 2% (w/v) solution (i.e., 2 g per 100 ml). The water needs to be heated to approximately 80°C to help dissolve the paraformaldehyde. Do not allow the temperature to rise much beyond 80°C or potential degradation of the formaldehyde may occur. The best way to accomplish this without overheating, which causes turbidity, is to immerse the paraformaldehyde solution in a boiling water bath and heat it, with frequent swirling, until it dissolves (about 20–30 min). Remember to keep the flask loosely covered (a 2-inch square of aluminum foil is good), so that loss by evaporation is minimized. Beware of vapors since they are highly toxic. An alternative method is to heat the water to 80°C with a Bunsen burner, transfer to a hot plate with a magnetic stirrer, and stir while dissolving the paraformaldehyde. A few drops of 1 *N* NaOH will assist in dissolving the paraformaldehyde (which is clear when dissolved). It is important to make the solution well in advance so it can cool to room temperature before use. The 2% paraformaldehyde solution can be stored at 4°C but should be brought to room temperature before use.

### *2% Glutaraldehyde in Stock Buffer*

Into a volume of stock 0.172 *M* phosphate buffer that is one-half that of the final volume of fixative desired, dilute enough 70% (v/v) electron microscopy (EM) grade glutaraldehyde to yield a concentration of 2% (v/v) (use 2.9 ml of 70% glutaraldehyde for each 100 ml buffer). The solution is very toxic so gloves and a fume hood are recommended.



### *1:1 Aldehyde Fixative*

To prepare the final fixative, mix equal volumes of the cooled 2% paraformaldehyde and 2% glutaraldehyde solutions to yield a mixture containing 1% paraformaldehyde and 1% glutaraldehyde in 0.086 M buffer.

### *2% Osmium Tetroxide*

Osmium tetroxide ( $\text{OsO}_4$ ) is very toxic so always handle it under a fume hood. *Always wear latex gloves* since  $\text{OsO}_4$  can penetrate vinyl gloves.

1. Dissolve 2.0 g of  $\text{OsO}_4$  crystals in 50 ml double-distilled water (in a hood with a magnetic stirrer or sonicator).
2. To 50 ml of 0.172 M sodium phosphate buffer add the 50 ml of 4%  $\text{OsO}_4$ . This yields 2% (w/v)  $\text{OsO}_4$  in 0.086 M sodium phosphate, pH 7.2.

Alternately, prepared  $\text{OsO}_4$  solutions that are ready to use may be purchased; they are, however, more expensive. The 2%  $\text{OsO}_4$  fixative can be stored for several months at 4°C if the storage bottle is free of contaminants and well sealed (double seal over the cap with Parafilm). Osmium tetroxide vapors are difficult to contain, so it is recommended that the fixative solution not be kept in the same refrigerator with sensitive materials (antibodies, cells, etc.).

### *Wash Buffer (0.137 M)*

Isotonic wash buffer (~315 mOsmol) used in the tissue processing steps is prepared by diluting 4 parts of 0.172 M sodium phosphate stock buffer with 1 part of double-distilled water.

## *Immersion Tissue Fixation*

1. Following enucleation, wash the globe in 0.137 M wash buffer, then make a small slit just behind the iris to allow fixative access to the inside.
2. Immerse the globe in fixative at room temperature for 5 to 10 min.
3. Remove the globe from the fixative and carefully cut around the perimeter of the globe by enlarging the original slit, until the anterior segment structures are removed. Although not as critical in smaller eyes, we routinely remove the vitreous body from larger eyes, such as those of the cat, to allow faster penetration of the fixative.
4. Reimmerse the eye cup in the fixative for 12 to 24 hr at 4°C.

An alternative method is to leave the globe in the fixative while enlarging the original slit and removing the anterior segment structures and vitreous body. This technique is very gentle on the globe, but it requires some practice to cut the globe while it is immersed in fixative. An additional benefit to this procedure is that the globe can be bisected right away after immersion in fixative, thus allowing more rapid penetration of the fixative to the retina.

### *Fixation by Cardiac Perfusion*

Figure 11 illustrates a simple apparatus that can be used for perfusion fixation.

1. The animal is euthanized by an overdose of appropriate anesthetic and then placed on its back in a sink or other receptacle which can serve to catch the blood and excess fixative. After opening the chest cavity, the descending aorta is clamped with a hemostat.

2. Air is infused into a flask above the level of primary fixative. The flask also has a second line extending to the bottom of the flask and leading to an 18-gauge blunted/beveled needle. The needle is placed in the left ventricle of the heart and secured with a small hemostat if necessary. A manometer is connected to the air line before it enters the flask in order to monitor the air pressure. Perfusion pressure should never exceed normal blood pressure

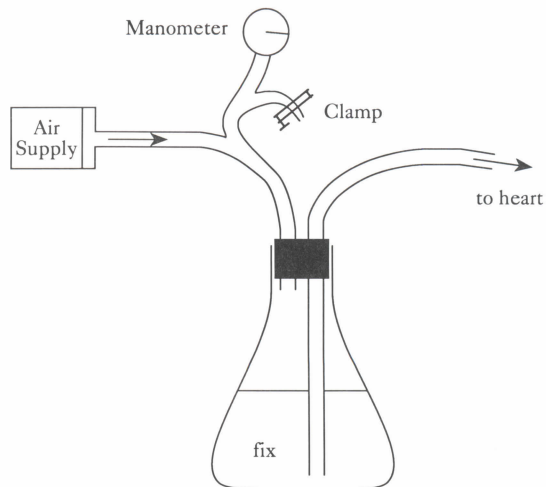


FIG. 11 Diagram of apparatus for use in perfusion fixation.



for the species being perfused or artifactual vacuolization can occur, particularly in the basal half of the retinal pigment epithelium.

3. Air pressure is adjusted by means of a clamp installed in a small side line between the air source and the fixative flask. Opening or closing the clamp will regulate the pressure until it reaches the desired level. A small slit is made in the right atrium to allow for drainage. Continue perfusing until there is only a small amount of blood in the fixative draining from the heart, then turn off the air and enucleate the eye. Remove the anterior structures as for immersion tissue fixation (see above) and immerse in the same fixative at 4°C for 12 to 24 hr.

### *Dissection of Retinal Tissue after Fixation*

For optimal dehydration and embedment, eyes larger than 1.5 cm in diameter should be dissected into at least four pieces. We regularly cut the posterior pole into superior and inferior hemispheres, then into temporal and nasal quadrants. Tissue pieces of that size or smaller will dehydrate and embed properly.

It is a good idea to keep track of retinal quadrants and their orientation. Most tissues become completely black after  $\text{OsO}_4$  fixation, and tissue landmarks may be difficult to find. A notch may be cut in the periphery of each quadrant or in the eyecup at an identifying location to allow orientation after  $\text{OsO}_4$  fixation. After dissection, transfer the tissue to small vials; glass scintillation vials work well.

### *Postfixation in 2% Osmium Tetroxide*

Postfixation in 2% buffered  $\text{OsO}_4$  is virtually required for routine study of tissue by electron microscopy to optimize membrane stability as well as to increase membrane density in the electron beam. (*Note:* There are procedures, such as postembedding immunocytochemistry, in which this step must be omitted; see Refs. 45 and 46.)

1. After overnight fixation, rinse the tissue using three changes of 0.137 *M* sodium phosphate buffer for 15 min each rinse.

2. Immerse the tissue pieces for 1 to 2 hr in a volume of 2%  $\text{OsO}_4$  solution sufficient to cover. The tissue will usually darken or turn black. Fixation with  $\text{OsO}_4$  hardens the tissue, preventing osmotic damage during subsequent exposure to solutions that are far from isotonic.

### *Dehydration*

1. Make the following percentage ethanol solutions using 100% ethanol and double-distilled water as the diluent: 30, 50, 70, 85, and 95% (v/v).
2. Wash the osmicated tissue in double-distilled water three times for 15 min each. To be safe, treat the wastewater as a dilute  $\text{OsO}_4$  solution and exercise appropriate precautions and disposal procedures.
3. Dehydrate the tissue sequentially through the 30, 50, 70, 85, and 95% ethanol solutions, each time leaving the tissue for 10 min in a volume of ethanol sufficient to cover the tissue twice over. (*Note:* During dehydration there is the option of staining the tissue *en bloc* with 2% uranyl acetate dissolved in 70% ethanol to increase tissue contrast. This is particularly valuable when embedding in Spurr's resin or LR White since they impart low contrast in the electron beam. Simply add one change in the uranyl acetate solution for 1 hr after the 50% ethanol solution and then continue the series with the 70% ethanol step. See Ref. 45 for details.)
4. Complete the dehydration process with three changes of 100% ethanol, for 20 min each. For the final two changes, the vials should be capped so that the alcohol will not absorb water from the air.
5. Process the tissue through two 10-min changes of propylene oxide, a transitional solvent between alcohol and resin.

### *Embedment*

Embedding resins are usually bought as kits containing the different components. The following are slight modifications of the standard instructions.

#### *Embedment in Araldite Resin*

Araldite 6005 is usually the most brittle of the epoxy resins; however, for thin sectioning many find its stability and hydrophobicity an advantage. One batch of resin (made according to the following recipes) is sufficient to infiltrate or to embed tissue from one cat eye. Resins are toxic and potential carcinogens, so use gloves and a fume hood.

1. Into a 100-ml disposable beaker weigh out 25 g of Araldite 6005 resin.
2. Add 22.25 g of DDSA (dodecyl succinic anhydride). A convenient way to make this mixture is to weigh out the Araldite and then slowly add the DDSA from a large syringe (12–20 ml) to a total weight of 47.25 g. Use a disposable pipette to add 1.09 ml of BDMA (benzyl-diethylamine).



3. Mix the components thoroughly using a glass rod. The resulting mixture will be very viscous.
4. Add an equal volume of propylene oxide to the resin mixture and stir until well incorporated.
5. Cover the tissue with enough of the 1:1 (v/v) propylene oxide–resin mixture to cover the tissue twice over. For good infiltration place the vials, uncapped, overnight on a rotating platform at about a 40° angle.
6. For embedment make up another batch of resin mixture and stir until well mixed. Try not to incorporate too much air as it can prevent proper infiltration. Air bubbles can be reduced by placing the mixture in a 45°C oven for 15 min. Remove the tissue from the resin–propylene oxide mixture (by now much of the solvent should have evaporated). Place the tissue pieces on Whatman (Clifton, NJ) No. 4 filter paper and place into a 45°C oven for 10–15 min to remove residual solvent–resin mixture. Place the tissue pieces in small aluminum weighing pans and cover with fresh resin. Place the pans in a 45°C oven for 12 to 24 hr and then transfer to a 60°C oven for an additional 12 hr.

#### *Embedment in Spurr's Resin*

Spurr's resin is a low-viscosity resin, suitable for embedding large specimens (47). However, this plastic has a number of idiosyncrasies which can make it difficult to work with. First, it is toxic and may be a potent carcinogen. Consequently, all work should be done with gloves, under a fume hood. Second, the plastic tends to be harder than either Araldite or Epon, and it is usually best to work with either the "soft" or even the "softer" version of the plastic formulation (Table I). Third, thin sections of this plastic are difficult to stain, and it is recommended that the specimens be stained *en bloc* with uranyl acetate. Finally, when mixing the components, add the

TABLE I Formulas for Spurr's Resin

Component <sup>a</sup>	Amount (g)			
	Hard	Firm	Soft	Softer
ERL 4206	10.0	10.0	10.0	10.0
DER 736	4.0	6.0	8.0	9.0
NSA	26.0	26.0	26.0	26.0
DMAE	0.4	0.4	0.4	0.4

<sup>a</sup> ERL, vinylcyclohexane dioxide; DER, diglycidyl ether of polypropylene glycol; NSA, nonenyl succinic anhydride; DMAE, dimethylaminoethanol.

accelerator DMAE (dimethylaminoethanol) last and only after the previous components have been thoroughly mixed.

1. Infiltrate the tissue in 2 parts propylene oxide and 1 part Spurr's resin for 30 min with rotation at room temperature. Special platforms for rotating samples during infiltration can be purchased from most EM supply companies (e.g., Ted Pella). See Table I for formulas for various Spurr's mixtures. Note that the hard, firm, and soft formulations are from the manufacturer's recommendations, while the softer formulation was provided by Dr. Brian Matsumoto (personal communication, 1991).

2. Infiltrate in 1 part propylene oxide and 2 parts Spurr's resin for 30 min with rotation at room temperature.

3. Remove the infiltration mixture and replace with fresh Spurr's resin. Rotate for 2.5–24 hr.

4. Transfer specimens to fresh vials or pans containing the resin. Polymerize between 60 and 80°C at least overnight.

#### *Embedment in London Resin White*

LR White can be used for conventional light and electron microscopy. In addition, with some tissue processing modifications LR White is suitable for postembedding immunocytochemistry owing to its slightly hydrophilic nature. This is a single component resin that does not require propylene oxide as a transitional solvent. For complete details on tissue processing and embedment, see Erickson *et al.* (46).

### *Microtomy*

All of the resins may be cut either as 0.5- to 1- $\mu$ m-thick sections for light microscopy or 60- to 90-nm-thick sections for transmission electron microscopy (for complete details, see Ref. 48).

### *Staining Sections*

#### *Staining for Light Microscopy*

The following stains are suitable for resin-embedded sections. The stains may be used either alone or in combination. The staining effect can be tailored to the particular needs of an experimenter. Basic fuchsin, which primarily stains extracellular matrix, connective tissues, and nuclei, is useful when visualizing silver grains in autoradiograms. The blue stains allow for



easier visualization of nuclei, although they may obscure labeling on autoradiograms or sections used for immunocytochemistry. Lipophilic stains such as *para*-phenylenediamine (PPDA) stain photoreceptor outer segments nicely and can be used along with either of the above stains.

#### Azure II–Methylene Blue–Toluidine Blue

1. To make 400 ml of stain, start with 350 ml of double-distilled water in a 16-ounce (~480 ml), screw-capped bottle.
2. While stirring add 1.0 g sodium borate (0.25%) and stir until dissolved.
3. Add 1.0 g azure II (0.25%), 1.0 g methylene blue (0.25%), and 1.0 g toluidine blue (0.25%).
4. Add 50 ml double-distilled water, cap the bottle tightly, and shake. Remove the cap and stir until dissolved.

#### Basic Fuchsin

1. To make 400 ml of stain, start with 380 ml of double-distilled water in a 16-ounce (~480 ml), screw-capped bottle. Add 20 ml of 50% ethanol and stir.
2. Add 2.0 g basic fuchsin (0.5%), cap the bottle tightly, shake, remove the cap, and stir until dissolved.

#### *para*-Phenylenediamine

*para*-Phenylenediamine is used as a saturated solution; we usually make 200 ml at a time. Crystalline PPDA should be added to double-distilled water until it no longer goes into solution. Care should be used when handling PPDA since it is carcinogenic.

#### Staining Procedure

The following procedure is applicable for staining using any of the reagents mentioned above.

1. Pipette a small drop of stain onto the sections (we usually stain on a hot plate at 100–125°C to speed up the process). Staining times will vary with temperature, thickness, tissue type, and resin. Do not let the stain dry, or precipitates will form.
2. Wash the sections thoroughly in double-distilled water and let dry.
3. Coverslip with a mounting medium (e.g., Permount from Fisher Scientific, Pittsburgh, PA).

*Note:* Various combinations of stains can be used in sequence to achieve different effects. In addition, if precipitates form, filter the stains through filter paper.

#### *Staining for Transmission Electron Microscopy*

The most common problems encountered in the staining of thin sections are dirt and salt precipitates. To avoid the former, always store grids covered in a petri dish or grid box. To avoid the latter, which is caused primarily by exposure to  $\text{CO}_2$ , we highly recommend Grid Sticks (Ted Pella). In this system, grids are attached to a grid holder and inserted into a glass pipette. Stains are then drawn into the pipette, minimizing exposure to air. Alternatively, the grids can be floated on drops of stain placed on a wax substrate or immersed in small pools of stain in depression slides.

The most common stains used for transmission electron microscopy (TEM) are Reynolds lead citrate (49) and uranyl acetate (50). Caution is advised in handling either of these heavy metal salts as both are dangerous if inhaled.

#### Lead Citrate

1. To make 100 ml of lead citrate, start with 60 ml of double-distilled water and, with stirring, add 2.66 g of lead nitrate,  $\text{Pb}(\text{NO}_3)_2$ , and stir until completely dissolved.
2. Add 3.52 g of trisodium citrate,  $\text{Na}_3(\text{C}_6\text{H}_5\text{O}_7) \cdot 2\text{H}_2\text{O}$ , and shake for 1 min.
3. Let stand for 30 min with intermittent shaking. The solution is milky white during this stage.
4. Add 16 ml of 1 N NaOH to clear the solution.
5. Bring the final volume to 100 ml with double-distilled water. Store tightly capped at  $4^\circ\text{C}$ .

#### 1% Uranyl Acetate

1. To make 100 ml of uranyl acetate, add 1 g of uranyl acetate,  $\text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$ , to 85 ml of double-distilled water and stir for about 5 min.
2. Bring the final volume to 100 ml with double-distilled water, store tightly capped in a brown or foil-wrapped bottle at room temperature.

#### Use of Grid Sticks

1. Attach grids to the holder and insert into a glass pipette.
2. Slowly draw uranyl acetate into the pipette; leave for the recommended time (see Table II).



TABLE II Staining Times for Various Embedding Media

Stain	Staining time (min)		
	Araldite	Spurr's	LR White
Uranyl acetate	10	15	5
Lead citrate	15	20	7

3. Rinse several times with double-distilled water and then repeat the process using lead citrate.

*Note:* Staining times will vary depending on the type of resin and tissue. We have found that the times listed in Table II work well for mammalian photoreceptors. Carbon coating of the grids in a vacuum evaporator may be necessary to stabilize and electrically ground the sections for viewing (for full details, see Ref. 48).

### *Electron Microscopic Autoradiography*

Autoradiography, the process of detecting radioactive isotopes in a section of tissue by the use of an overlying photographic-type emulsion, is a powerful technique that can be used at either the light or electron microscopic level. Although time consuming, this procedure can provide high-resolution localization of labeled molecules and results that can be quantified. It has proved useful in a variety of studies of photoreceptor metabolism (see Refs. 17 and 20). Bok (51) provides a complete technique for light microscopic autoradiography especially tailored to studies on photoreceptors. The method presented below is a modification of that described by Young and Droz (52). This modified technique should result in the recovery of over 90% of the sections as usable autoradiograms. Typical results that can be expected from this technique are illustrated in Fig. 12.

#### *Slide Preparation*

1. Prepare a 2% (w/v) solution of Parlodion (purified pyroxylin, Ted Pella) in isoamyl acetate. Stirring overnight often is required for the resin to dissolve completely.
2. Mark frosted glass slides with a diamond scribe as shown in Fig. 13. Scribe two + marks in the location indicated on the back, that is, the non-frosted side of the slide. These will be used as guides in the placement of

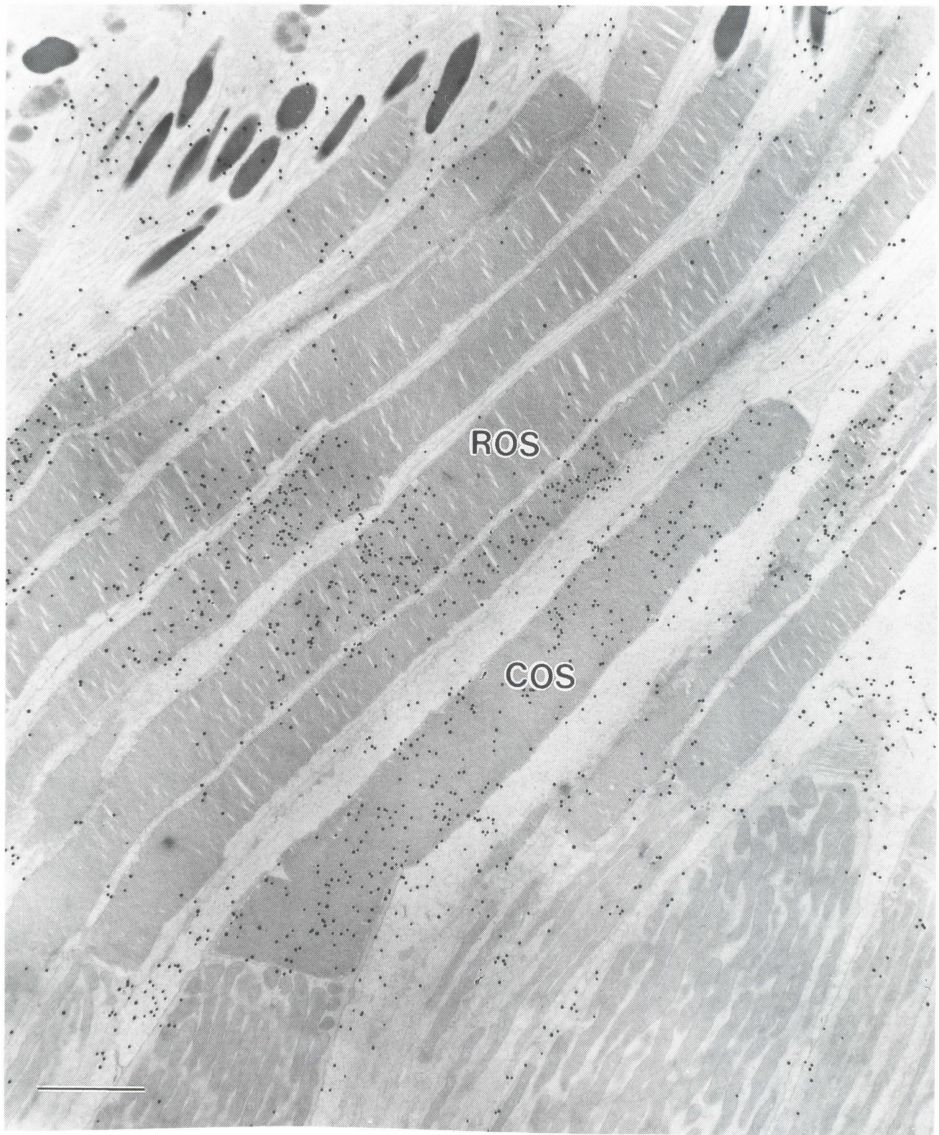


FIG. 12 Electron microscopic autoradiograph of photoreceptors from a rhesus monkey retina. The animal was labeled with  $[^3\text{H}]$ fucose 96 hr before perfusion fixation by the method described in the text. The label is accumulated in restricted "bands" in the rod outer segments (ROS) but occurs diffusely scattered over the cone outer segment (COS). Bar, 2  $\mu\text{m}$ .



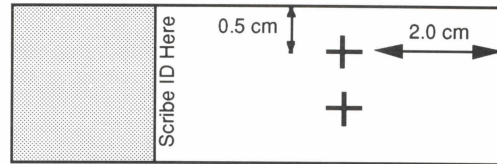


FIG. 13 Appropriate marking of glass microscope slides for the location of the sections to be used in EM autoradiography.

the sections later on. Also scribe an identification of some sort on the front of the slide just below the frosted part. You can also write on the frosted glass to further identify the specimen.

3. Clean the slides as follows:
  - a. Wash each slide in Liquinox or 7X detergent (Ladd Research Industries, Burlington VT) for a few minutes by rubbing the slides between your fingers (its a good idea to wear rubber gloves during this procedure). Overcleaning can result in films that stick so well they will never float off. It is strongly suggested that you go all the way through Step 5 and then try to get some films off. If you have trouble, try again.
  - b. Rinse each slide several times in double-distilled water.
  - c. Dip each slide in 95% ethanol and wipe dry with a lint-free cloth or Kimwipe.
  - d. Store in a dust-free environment until use.
4. Conduct the first Parlodion dip as follows:
  - a. Dilute the 2% parlodion 1 : 1 with isoamyl acetate to produce a 1% (v/v) solution.
  - b. Dip the nonfrosted end of the slide into the Parlodion to a point just below the identification marking.
  - c. Dry the slides in a clean place at about a 30° angle. This layer of Parlodion will provide a substrate on which to place the thin sections.
5. Conduct the second Parlodion dip as follows: Using the 2% Parlodion, redip only the bottom 1 cm of each slide and dry as before. This provides an “anchor” that will assist in getting the Parlodion to come off the slide at the end of the procedure.

#### *Transferring Sections to Slides*

1. Make a plastic or wire loop that is the same size as the diameter of an EM grid (e.g., about 3 mm inside diameter). It will also be best if the loop is quite thin. (*Note:* Several EM supply houses, such as Ted Pella, sell section transfer loops. They work well but have inside diameters of 2 mm

or less, thus restricting the size of the sections that can be used. For this reason we make our own out of very thin but flexible plastic. Plastic rulers are good for this if they are not more than 0.5 mm thick. If you have made a loop it will be necessary to attach it to a handle, such as a pair of forceps, using some method; premade loops will come with a handle.)

2. Take very thin sections of the tissue (silver to pale gold at the thickest), then group a bunch of sections, enough to fill the loop, away from the other sections in the knife boat.

3. Gently lower the loop over the sections until you touch the surface of the water surrounding the sections.

4. *Very* gently withdraw the loop, carrying the sections in the film of water remaining in the center of the loop.

5. Place the loop over one of the scribed + marks (Parlodion-coated side up and scribed + on the underside) and slowly lower the loop until the water touches the Parlodion-coated glass surface. Be careful here that you do not touch the loop to the Parlodion coating as this may rupture it, causing the film to lift off during later stages in the procedure.

6. While holding the loop steady, wick away the water from beneath its edge using a wedge of filter paper. Be careful not to touch the sections or draw the water away too quickly, or the sections may end up on the filter paper.

7. Repeat the process so that there is one group of sections over each scribed + mark on the slide. (*Note:* Once the sections have dried onto the coated slide they will be nearly invisible!)

8. Store in a clean dust-free place.

### *Staining with Heavy Metals*

1. Prepare a 5% aqueous solution of uranyl acetate.

2. Filter the solution and dilute with an equal volume of absolute ethanol.

3. Using a Pasteur pipette, place drops of this solution, about twice the diameter of the loop (e.g., 6 mm) over each + mark on the slides. Let sit for about 20 min. As with any heavy metal staining do not breathe onto the drops if possible as this may cause precipitates (more important with the lead citrate).

4. Wash the slides (gently), under running deionized water for 10 or 15 sec each.

5. Dry slides thoroughly and protect from dust and other contaminants.

6. Prepare a standard solution of Reynolds lead citrate as described above.

7. Place a drop of lead citrate, the same size as before, over each group of sections. Stain for 5–7 min.

8. Wash and dry as before.



### *Carbon Coating*

Coat slides with a thin layer of carbon using a vacuum evaporator. Exact procedures are described by Hyatt (48). Too thick a layer will make the film brittle and difficult to remove. Carbon is necessary to provide subsequent stability in the electron beam and to prevent an undesirable interaction between any  $\text{OsO}_4$  in the tissue and the nuclear track emulsion applied later.

### *Coating with Emulsion*

The following is done in a darkroom under dim red (No. 4) or sodium vapor (recommended) illumination. These instructions are for  $^3\text{H}$ - and  $^{14}\text{C}$ -labeled specimens specifically; check the suitability of the emulsion before using these methods for other isotopes.

1. Coat about 50 clean slides with a single layer of 1% Parlodion. These will be used for removing bubbles from the emulsion and checking for proper thickness of the emulsion layer.
2. Stabilize a water bath at 40°C.
3. In a container (a 100-ml Tri-Pour beaker is good for this) that will remain stable in the water bath (e.g., not float around), mark lines with a permanent marker at 60 and 75 ml. This will be used to obtain a 1 : 4 dilution of emulsion in water.
4. Fill the container up to the lower (60 ml) line with deionized water, cover with Parafilm, and place in the water bath to stabilize at 40°C. This will take about 30 min.
5. Assemble the following materials: aluminum foil, black electrician's tape, porcelain spoon, slide boxes lined with filter paper, and small packages (about 4 cm  $\times$  2 cm  $\times$  2 cm) of desiccant wrapped in a couple layers of cheesecloth.
6. Under safe illumination, open a bottle of Ilford L4 nuclear track emulsion (Polysciences) and spoon chunks of it into the beaker of warmed deionized water until the level reaches the 75 ml mark. Return the beaker to water bath to melt the emulsion. Use fresh emulsion only, or high background can result.
7. After 15 min, stir the emulsion gently with the porcelain spoon. Repeat after another 15 min, and if there are still lumps repeat once more.
8. After the final stir, dip some of the blank, Parlodion-coated slides into the emulsion until no bubbles can be seen on the surface of the slide after dipping. Then dip several more slides to just below the scribed identification area and let dry at a fixed angle (about 30°).
9. Wait until the test slides dry (about 10 min in normal humidity) and then take a couple of slides out into the light to examine. You should have

an even surface, free of bubbles, and the coating over the area where the + marks are located should have a purple-green interference color when held at about a 45° angle to a light source. This will assure a proper thickness for producing a monolayer of silver grains. If the color is too dark (e.g., blue) then the emulsion needs to be diluted. If the color is too light (e.g., greenish-gold), then the emulsion is too thin and more should be added. When the thickness is correct go to Step 10.

10. Dip each slide containing the tissue into the emulsion to just below the scribed area, then blot the end onto filter paper to remove excess emulsion from the slide. Allow to dry for 1 or 2 hr at the same angle as the test slides.

11. When dry, place the slides into slide boxes, making sure you have several small groups of slides to use as test boxes for determining the proper exposure. Each test box should contain at least one slide from each experiment. There should be enough test boxes so that the proper exposure time can be safely determined. For example, if the estimated exposure time is 10 weeks, then there should be enough test slides to develop one at 8 weeks, one at 9 weeks, etc. Into each slide box place a couple packages of desiccant, using a blank slide to separate them from the dipped slides. Close the slide boxes and tape shut with black electrical tape, then place them into light-tight plastic bags (e.g., the kind Ilford photographic paper comes in) and wrap them with aluminum foil. Store at 4°C.

*Note:* A good rule of thumb is that the electron microscopic autoradiographic exposure time will be approximately equal to 10 times the exposure time for light microscopic autoradiograms cut from the same tissue sample (e.g., light microscopy, 7 days; electron microscopy, 10 weeks).

### *Development*

The following development procedure uses phenidone (Lauder Chemicals, San Mateo, CA), which produces small round grains. If it is desired to visualize the entire track (worms), then elon development is suggested (53).

1. Prepare an ice bath that is large enough to hold the containers of fixer and developer.
2. Prepare fresh fixer (recipe makes 600 ml). Dissolve 180 g of crystalline sodium thiosulfate in 500 ml of double-distilled water and bring up to 600 ml. This fixer does not harden the gelatin or remove the stain.
3. Prepare phenidone developer as follows:
  - a. In 300 ml deionized water dissolve in sequence 6.0 g ascorbic acid and 1.0 g 1-phenyl-3-pyrazolidone (phenidone).
  - b. Vacuum filter (will take time), then add 2.4 g potassium bromide,

- 5.2 g sodium carbonate, and 80 g sodium sulfite (will saturate the solution and make it cloudy).
- c. Vacuum filter the solution (will take time), then add 24 g potassium thiocyanate (Mallinckrodt, St. Louis, MO, recommended).
  - d. Bring the volume to 400 ml with double-distilled water, stir, vacuum filter, and use at once.
4. Bring a beaker of developer and two beakers of fix to 15°C. (*Note:* The temperature is critical to control of grain size; warmer developer means larger grains.)
  5. Remove a slide or slides from the box and immerse into the developer to a level above the sections but below the edge of the Parlodion coating. This is to give the film an anchor so that it will not float off of the slide during processing. Plastic holders (Peel-A-Way, Polysciences, Warrington, PA) that can accommodate five slides are convenient for this and are available from most EM supply companies.
  6. Develop for 1 min.
  7. Rinse in the first beaker of fixer for 5 sec and then transfer into another beaker of fix for 5 min.
  8. Wash in three sequential double-distilled water baths (at room temperature), for 1 min each.
  9. Let the slides sit in double-distilled water for 5 to 10 min. Watch for signs of the film coming loose at the top of the slides, and if this occurs take them out at once.

### *Removing Films*

1. Let slides dry for 1 to 2 min, then remove the bottom 3–4 mm of coating from the slide with a fresh razor blade, using a fresh blade for each slide. Score around the sides and top of the film with a razor blade.
2. Holding the frosted end of the slide, slowly immerse it into a large dish of deionized water at about a 45° angle. In the best of all possible situations the film should be loosened and float onto the surface of the water. They may need some encouragement: use a paper clip bent into an L shape to support the loose end of the film and gently pull the slide down and back while holding the film steady. (This is one of these steps you have to practice. It is not easy.)
3. Carefully place an EM grid over the sections on the floating Parlodion film. You should be able to see them if the film is dry and the light is just right.
4. Gently lay a strip of Parafilm onto the surface of the film, then slowly lift it up steadily from one end while rolling it over (thus inverting the film)



at the same time. With luck, the film and grids will come with the Parafilm. Place it, inverted (wet side of the Parlodion film up), onto a flat surface to dry thoroughly.

5. Using a pair of fine forceps punch all around the edges of the grid and then carefully lift the grid away from the surrounding Parlodion film and off of the supporting Parafilm.

6. View in the electron microscope.

#### *Miscellaneous Notes*

Electron microscopic autoradiography is not a trivial procedure. If you attempt it you can expect a trying time, but with some luck and caution you should be able to get through it the first time. Many of the steps require much time. For example, plan on 1–2 hr just to make the developer. The isoamyl acetate used for the Parlodion should be used in a fume hood. Peeling the films is an acquired skill. The first time you try, expect to lose up to 85% of the sections. As you become proficient you can expect up to 95% recovery. The thickness of the sandwich (e.g., Parlodion–section–emulsion) is very important, but some allowances can be made for the capacity of the microscope to deal with thicker (e.g., >90 nm) sections. In general, do not use sections thicker than necessary, in order to maximize resolution (i.e., start with silver sections).

A good reference for the theory and techniques of autoradiography is *Techniques of Autoradiography* (53).

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