Preface

Blood–brain barrier (BBB) breakdown leading to cerebral edema occurs in many brain diseases—such as trauma, stroke, inflammation, infection, and tumors—and is an important factor in the mortality arising from these conditions. Despite the importance of the BBB in the pathogenesis of these diseases, the molecular mechanisms occurring at the BBB are not completely understood. In the last decade a number of molecules have been identified not only in endothelial cells, but also in astrocytes, pericytes, and the perivascular cells that interact with endothelium to maintain cerebral homeostasis. However, the precise cellular interactions at a molecular level in steady states and diseases have still to be determined. The introduction of new research techniques during the last decade or so provide an opportunity to study the molecular mechanisms occurring at the BBB in diseases.

The Blood–Brain Barrier: Biology and Research Protocols provides the reader with details of selected morphologic, permeability, transport, in vitro, and molecular techniques for BBB studies, all written by experts in the field. Each part is preceded by a review that emphasizes the advantages and pitfalls of particular techniques, as well as offering much relevant current information. The techniques provided will be helpful to both beginners in BBB research and those more experienced investigators who wish to add a specific technique to those already available in their laboratories. Although a number of in vitro techniques are included, it is suggested that they be complemented with data derived from in vivo studies to gain a truer picture of the biological process.

It is hoped that the methods described in *The Blood–Brain Barrier: Biology and Research Protocols* will aid researchers in the isolation of molecules not yet described, and increase our understanding of how they interact at the BBB to maintain cerebral homeostasis as well as of the mechanisms that result in BBB breakdown in diseases. Advances in technology will necessitate collaboration among researchers having expertise in many of these techniques to solve biological questions.

A greater understanding of the molecular mechanisms occurring at the BBB in diseases is also necessary in order to identify substances/molecules that can be targeted for pharmacological manipulation and/or gene therapy and to determine when therapeutic intervention can attenuate the disease process.

I would like to acknowledge Prof. John M. Walker for this opportunity and for his help and all the authors who have contributed their protocols.

This book is dedicated to Mohit Kumar and Labonya Nag.

Sukriti Nag

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Studies of Cerebral Vessels by Transmission Electron Microscopy and Morphometry

Sukriti Nag

1. Introduction

Transmission electron microscopy (TEM), a once popular research tool, is used less frequently now. However, the isolation of novel proteins in the past decade has led to renewed use of electron microscopy for the subcellular localization of these proteins. This chapter will describe the standard method for preparation of brain tissue for TEM studies of cerebral vessels.

Optimum tissue fixation is essential to obtain good electron micrographs and in this chapter, primary fixation with an aldehyde mixture containing both glutaraldehyde and paraformaldehyde that crosslink proteins ***(1)*** will be described. The advantage of an aldehyde mixture is that formaldehyde penetrates cells faster than glutaraldehyde and temporarily stabilizes structures, which are subsequently more permanently stabilized by glutaraldehyde. Structural preservation is superior when the combined aldehyde fixative is used rather than either fixative alone. Paraformaldehyde reacts with proteins, lipids, and nucleic acids. Glutaraldehyde results in the formation of intermolecular and intramolecular links between amino acids, yielding rigid heteropolymers of proteins thus stabilizing cell structures and preventing distortion during processing. It also increases tissue permeability to embedding media. Glutaraldehyde does not stabilize lipids; hence, cell membranes are not visible unless tissues are post-fixed in osmium tetroxide.

Secondary fixation is done using osmium tetroxide, which reacts with unsaturated lipids, proteins, and lipoproteins. It is electron-dense and stains phospholipids in the cell membrane resulting in deposition of lower oxides of osmium, although a small degree of density may be contributed by organically bound but unreduced osmium. Osmium does not react with ribonucleic acid or deoxyribonucleic acid. The next optional step is tertiary fixation of blocks using aqueous uranyl acetate. This increases the overall contrast and further stabilizes membranous and nucleic acid containing structures. However, glycogen is extracted from the tissue. Tissues are dehydrated through ascending concentrations of ethanol, then propylene oxide before embedding in a resin mixture whose major constituent is Epon 812. The latter is the most widely used embedding medium for electron microscopy because it can be easily sectioned and sections can be stained without difficulty. In addition, ultrathin sections of Epon can tolerate the intense

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heat and strong vacuum in the electron microscope and sections show greater contrast in the electron microscope than do comparable Araldite sections. Since Epon was discontinued in the 1970s, substitutes for Epon 812 became available.

Semithin and especially ultrathin sectioning require patience and practice. The basics of sectioning are included in this chapter as are staining of semithin and ultrathin sections. However, detailed problem solving of sectioning difficulties is beyond the scope of this chapter and the reader is referred to more comprehensive texts for further information ***(2,3)***.

2. Materials

Chemicals used should be of high purity and of analytic grade (*see* **Note 1**).

2.1. Primary Fixation

1. Buffer: 0.2 *M* sodium cacodylate buffer containing 8.7% sucrose, pH 7.4. Sodium cacodylate has an osmolality of 400 mOsM while sucrose provides an osmolality of 250 mOsM (*see* **Note 2**). This buffer is diluted in a ratio of 1 : 1 for use so the final osmolality is approx 300 mOsM.
2. Fixatives: Paraformaldehyde powder and 25% or 70% glutaraldehyde stock solution.
3. 1% Calcium chloride solution.
4. A perfusion apparatus, which consists of a 1L bottle having a rubber stopper through which a glass connector tube and a plastic Y-shaped connector are inserted (**Fig. 1**). The latter is attached to a) tubing that leads to a pressure pump, and b) a segment of tubing at the end of which a Hoffman clamp is placed so that the pressure in the system can be adjusted (*see* **Fig. 1**). The inner end of the glass connector tube in the stopper is attached to tubing, which should be long enough to reach the bottom of the bottle so that the last few drops of fixative can flow into the system. The outer end of the glass connector tube is connected to tubing, which is connected to a plastic Y-shaped connector, which connects to segments of tubing that are attached to a) a manometer, and b) a metal stopcock. The beveled end of a 16-gage needle is filed so the tip has a straight edge. This needle is fitted on the stopcock.

2.2 Secondary Fixation

1. 2% Osmium tetroxide in 0.1 *M* cacodylate buffer is prepared as follows:
   a. Wash the vial containing the osmium tetroxide crystals and score the marked ring on the neck of the vial with a file.
   b. Break off the tip of the vial and drop the vial into an amber colored bottle containing the required amount of distilled water to prepare a 4% solution and replace the lid of the bottle but do not secure tightly.
   c. Stir solution in a fume hood for a few hours until it is clear.
   d. Add an equal volume of 0.2 *M* cacodylate buffer to get a 2% solution of osmium.
   e. Tighten the lid and store at 4°C.
   f. Ensure that the solution is clear before use because this solution precipitates if kept too long.
   g. Osmium tetroxide is a hazardous chemical (*see* **Note 3**).
2. Pasteur pipets, glass tubes 10×75 mm or shell vials 12×35 mm (Kimble) with cork stoppers, parafilm.
3. Nalgene wash bottles.

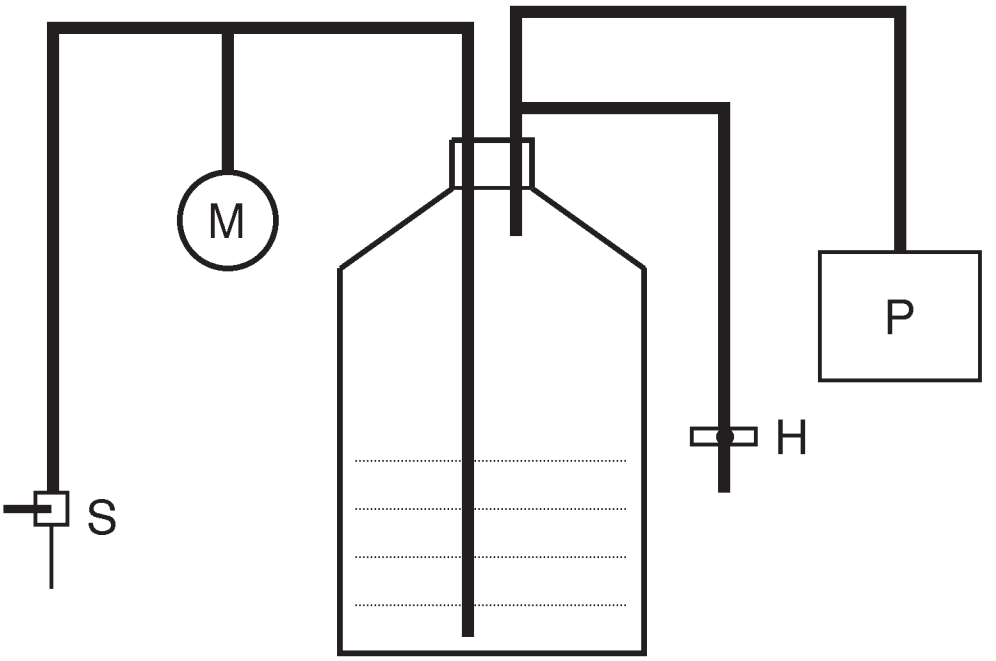

Fig. 1. The diagram shows the apparatus used for vascular perfusion of mice and rats that is described in **Subheading 2.1., step 4**. Abb. S = metal stopcock at the end of which a 16-gage needle is fitted; M = Manometer; H = Hoffman clamp, which can be adjusted to attain the correct perfusion pressure; P = the pressure perfusion pump.

2.3. Tertiary Fixation

1. 0.05 *M* Sodium hydrogen maleate buffer, pH 5.15 for washes and pH 6.0 for the preparation of uranyl acetate stain, is prepared fresh before use as follows:
   a. Prepare a 0.1 *M* sodium hydrogen maleate stock solution containing 1.16% maleic acid and 0.4% NaOH in distilled water.
   b. Prepare a 0.2 *M* NaOH solution
   c. Add the 0.2 *M* NaOH to 50 mL of the sodium hydrogen maleate stock solution until the required pH is reached and bring the total volume to 100 mL with distilled water.
4. 3% Uranyl acetate in 0.05 *M* sodium hydrogen maleate buffer, pH 6.0. Filter using no. 50 Whatman paper (*see* **Note 4**).

2.4. Dehydration and Embedding

1. Ethanol, propylene oxide.
2. Epon mixtures A and B are prepared depending on the weight per epoxide equivalent (WPE) of Epon 812, which is obtained from a table supplied by the manufacturer. If the WPE of Epon 812 is 145, then Mixture A contains 200 g of Jembed 812 resin (J.B. EM Services, Pointe Claire, Quebec) and 254 g of dodecenyl succinic anhydride, and Epon mixture B contains 250 g of Jembed 812 resin and 212 g of nadic methyl anhydride. The Epon mixtures are prepared as follows:
   a. The required amounts of the constituents are added by weight to an amber-colored 1 L bottle placed on a top-loading balance.

   b. Stir using a glass rod.
   c. Tighten lids of bottles and store at 4°C. These stocks are good for several months.
3. The hardness of the final block depends upon the ratio of mixture A and B and an increase in the proportion of mixture B will harden the block. In our laboratory the final resin mixture contains 1 part of Epon mixture A and 4 parts of Epon mixture B to which 1.8% of 2,4,6-tri (dimethylaminomethyl) phenol (DMP-30), an accelerator for epoxy resin is added. This mixture is prepared as follows:
   a. Leave the stock solutions at room temperature for at least 2 h before use.
   b. Pour the required amount of the stock solutions into graduated disposable tri-pour polypropylene beakers.
   c. Place the beaker in a 60°C oven for 10 min and then stir contents for 5 min using a glass rod.
   d. Add the required amount of DMP-30 in a fume hood and stir for at least 10–15 min.
   e. Cover the beaker with a paper lid and let it sit at least 45 min before use so that the air bubbles, which form during stirring, break up.
4. Round mold or flat molds, small paper labels (5 × 7 mm) with case numbers written in pencil.

2.5. Sectioning

1. A knife maker, strips of plate glass (6 mm thick) to make glass knives, masking tape for making a boat at the cutting edge of the glass knife.
2. Dissecting microscope, a diamond knife, an ultramicrotome, tissue sectioner.
3. Small and large plastic petri dishes, gelatin capsules.
4. Segments of hair taped to an applicator stick, platinum wire loop.
5. Copper grids having a 3.05-mm outside diameter and a 300 hexagonal or square mesh. Grids obtained from the manufacturer are cleaned as follows:
   a. Place grids in a glass vial containing 10% HCl and swirl for 2–3 min. 10% HCl is made every week.
   b. Pour out the HCl solution and rinse in several changes of filtered distilled water and then filtered ethanol.
   c. Decant most of the ethanol and pour grids on a filter paper. Separate with forceps and allow them to dry.
   d. The filter paper is placed in a plastic petri dish, which is covered to keep out the dust.
   e. Freshly cleaned grids are required each day ultrathin sectioning is done.
   f. Grids can be cleaned only once otherwise they flake.

2.6. Staining

1. Stainless steel Dumont tweezers, 30-mL capacity amber-colored bottle, 50-mL glass flask with a glass lid.
2. Sodium hydroxide pellets, 5 *N* sodium hydroxide.
3. Silicone rubber plates with numbered squares on the surface.
4. Stain for semithin sections: Add 1 g of toluidine blue and 1 g of sodium borate to 100 mL of distilled water. Stir for 30 min to dissolve. This stain keeps for long periods when stored at 4°C. It is filtered each time before use using a Whatman no. 1 filter paper or it can be dispensed in a 5-mL syringe fitted with a 0.45-µm filter.
5. Stain for ultrathin sections:
   a. Saturated solution of uranyl acetate in water: Add uranyl acetate to a 30-mL amber-colored bottle containing filtered distilled water and stir for a few hours. A residue remains at the bottom indicating that it is a saturated solution. Store at room temperature in the

staining area. When staining, pipette a few drops from just below the surface of the solution taking care not to disturb the solution. This stain is only good for 2 wk (*see* **Note 5**).

b. Lead citrate stain: Boil 100 mL of distilled water in an Erlenmeyer flask to remove carbonates from the water and allow it to cool. Rinse an acid-cleaned 50 mL volumetric flask having a glass stopper with the boiled distilled water and dry. Add 160 mg of lead citrate to 45 mL of the boiled distilled water. Do not use a metal spoon while weighing the lead because it reacts with the metal. Add the glass stopper and shake vigorously for 3–4 min. Add 5 *N* NaOH drop by drop over 15 min until the solution clears. Clean the sidewalls of the flask by pipetting solution down the sidewalls and rinse the glass stopper with carbonate-free water. Allow the solution to settle. It can be used after 2–3 h. This solution is made every week because of the tendency for lead carbonate precipitate to form on being kept. This solution is kept at room temperature (*see* **Note 5**).

3. Methods

The methods described below outline 1) primary fixation, 2) secondary fixation, 3) tertiary fixation, 4) dehydration and embedding, 5) sectioning, 6) staining, and 7) morphometry.

3.1. Primary Fixation

A mixture of paraformaldehyde and glutaraldehyde in cacodylate buffer is used at a pH of 7.4, which is the pH of most animal tissues. Cacodylate buffer is widely used for preparation of tissues for electron microscopy. One of its advantages is that calcium can be added to the fixative solution without the formation of precipitate. It is also resistant to bacterial contamination during specimen storage. Addition of calcium chloride to the fixative solution has many beneficial effects, including 1) decrease in the swelling of cell components, 2) maintenance of cell shape, 3) reduction in the extraction of cellular materials, and 4) membrane and cytoskeletal stabilization.

3.1.1. Paraformaldehyde-Glutaraldehyde Mixture

This fixative contains 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2 containing 4.35% sucrose and 0.05 % calcium chloride. Preparation of 100 mL of this fixative is done as follows:

1. Heat 35 mL of distilled water to about 60–70°C in an Erlenmeyer flask in a fume hood. Add 2 g of paraformaldehyde and stir for a few minutes using a magnetic stirrer.
2. Add 1–2 drops of 5 *N* NaOH and stir for few minutes until the solution clears.
3. Cool the solution and filter using a Whatman no.1 filter.
4. Add 10 mL of 25% glutaraldehyde, 50 mL of 0.2 *M* cacodylate buffer, and 5 mL of a 1% $CaCl_2$ solution.
5. This solution is used undiluted for immersion fixation. It is diluted in a 1:1 ratio with 0.1 *M* cacodylate buffer for vascular perfusion of experimental animals.
6. The fixative is cooled to 4°C before vascular perfusion. For long-term storage (*see* **Note 6**).

3.1.2. Alternate Fixative

If both electron microscopy and immunohistochemistry have to be performed using the same tissue, then it is best to use a fixative that does not contain glutaraldehyde,

which inhibits demonstration of some proteins. In our laboratory, we use 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 for vascular perfusion. Phosphate buffer is one of the most physiologic buffers, because it is found in living systems in the form of inorganic phosphates and phosphate esters. Microorganisms grow readily in this buffer therefore it is advisable to sterilize this solution and store at 4°C. Another disadvantage is that calcium cannot be added to this fixative because a precipitate of calcium phosphate may form which can be visualized by electron microscopy:

1. Prepare 50 mL of 6% paraformaldehyde as described previously (*see* **Subheading 3.1.1**).
2. Add 25 mL of distilled water and 25 mL of 0.4 *M* phosphate buffer, pH 7.2 which is prepared by adding 5 *N* NaOH to a 0.4 *M* solution of KH_2PO_4 until the pH is 7.2.
3. Filter before perfusion using a Whatman no.1 qualitative filter.

3.1.3. Vascular Perfusion of Fixative

This is the best method for obtaining good preservation of animal tissues for electron microscopy. Animals should be perfused in a fume hood to avoid inhaling paraformaldehyde or glutaraldehyde vapours which are irritant to the eyes as well. The method used for perfusing rats in our laboratory is:

1. Load fixative cooled to 4°C into the 1-L bottle of the perfusion apparatus (*see* **Fig. 1**). Switch the pump on and allow pressure to build in the system. Tighten the Hoffman clamp until the manometer reads 110 mm Hg. Open the stopcock above the needle and allow fluid to flow until all air bubbles are expelled. Close the stopcock.
2. Rats are anesthetized using metofane or halothane inhalation. In accordance with animal experimentation regulations, animals must be kept anesthetized throughout the surgery. They do not move when their paws are held tightly and the corneal reflex is absent.
3. The abdominal wall just below the ribcage on the left side is lifted up using toothed forceps and a hole is made. Scissors are then inserted into the hole and a linear cut is made upwards through the diaphragm and the costal cartilages left of the sternum up to the level of the clavicle. A transverse cut is made in the diaphragm.
4. The heart is then grasped using the index and thumb of the left hand or a pair of forceps with blunt ends and the 16-gage needle is inserted through the apex of the heart into the left ventricle. Slide the needle along the interventricular septum of the left ventricle until it enters the aorta.
5. If the heart is pulled down gently the tip of the needle will be seen in the ascending aorta.
6. Clamp the needle in place by placing a pair of curved hemostatic forceps on the heart below the atria.
7. Open the stopcock above the needle and allow fixative to flow into the aorta (*see* **Note 7**).
8. A slit is made in the right atrium for efflux of the perfusate, which initially is bloody and later becomes clear.
9. Perfusion is continued for 10 min or until 500 mL of fixative is perfused (*see* **Note 8**).
10. Perfusion is usually not satisfactory if the lag between death and entry of fixative in the animal exceeds 60–90 s. An adequately fixed brain is firm and yellow in color when an aldehyde mixture is used and white if paraformaldehyde is used alone. A poorly perfused brain showing reddish areas of discoloration on the surface should be discarded since electron microscopy invariably shows poorly preserved tissue.
11. Brains are removed and placed in the same fixative solution for 2 h at room temperature.
12. Our usual practice is to obtain coronal slices of 50–60 µm thickness, from each hemisphere using a tissue sectioner (Sorvall TC-2 Sectioner or a Vibratome).

13. Brain slices should show only few red blood cells in the vasculature on light microscopy if fixation is optimum.
14. Slices are viewed under the stereoscope and rectangles of cortex measuring 2.5 × 4 mm and having central arterioles are cut using a sharp razor blade, which is cleaned with acetone. These blocks are fixed overnight and processed for resin embedding next morning (*see* **Note 9**).

3.2. Secondary Fixation

The minimum processing time for 50–60 µm thick blocks is given. For thicker blocks the time in the different solutions have to be increased. Processing is done in glass tubes or shell vials with cork stoppers and solutions are added or removed using Pasteur pipettes.

1. Rinse blocks in 3 changes of 0.1 *M* cacodylate buffer for 5 min each.
2. Postfix in 2% osmium tetroxide in 0.1 *M* cacodylate buffer for 30 min in a fume hood on ice. The tissue becomes uniformly black at the end of osmication.
3. Rinse blocks in 2 changes of 0.1 *M* cacodylate buffer for 5 min each and then in 3 changes of maleate buffer for 5 min each.

3.3. Tertiary Fixation

1. Perform *en bloc* staining by placing tissues in 3% uranyl acetate in 0.05 *M* maleate buffer for 1 hour in an incubator at 37°C (*see* **Note 10**).
2. Wash in 3 changes of maleate buffer for 5 min each.

3.4. Dehydration and Embedding

These steps remove water from the tissue and replace it with a medium that will withstand the stress of cutting. These steps are also done using a fume hood to avoid inhalation of solvents.

1. The solutions listed below are filled in Nalgene wash bottles for quick dispensing.
   a. 2 changes of 70% ethanol for 7 min each.
   b. 2 changes of 85% ethanol for 7 min each (*see* **Note 11**).
   c. 2 changes of 95% ethanol for 7 min each.
   d. 3 changes of 100% ethanol for 7 min each.
   e. 3 changes of propylene oxide for 5 min each.
2. Infiltration
   Blocks are then placed in the final resin mixture and propylene oxide in a 1 : 1 ratio for at least 2 h and in a 2 : 1 ratio for 36 h. Place cork stoppers on the tubes containing the blocks and wrap parafilm at the junction of the cork and glass tube to prevent evaporation, otherwise the resin mixture may become too viscous (*see* **Note 12**).
3. Embedding
   Fill freshly prepared resin mixture in a 5-mL plastic syringe and fill blank molds. Cubes of brain are embedded in polyethylene BEEM embedding capsules while 50–100 µm thick blocks or vessels are embedded in flat silicone rubber molds.
   a. Pour the contents of the glass tube with the sections onto card paper. Use bamboo splints to transfer the sections into the molds.
   b. Use a stereoscope to position the tissue such that the required surface is at the cutting edge (**Fig. 2A**).

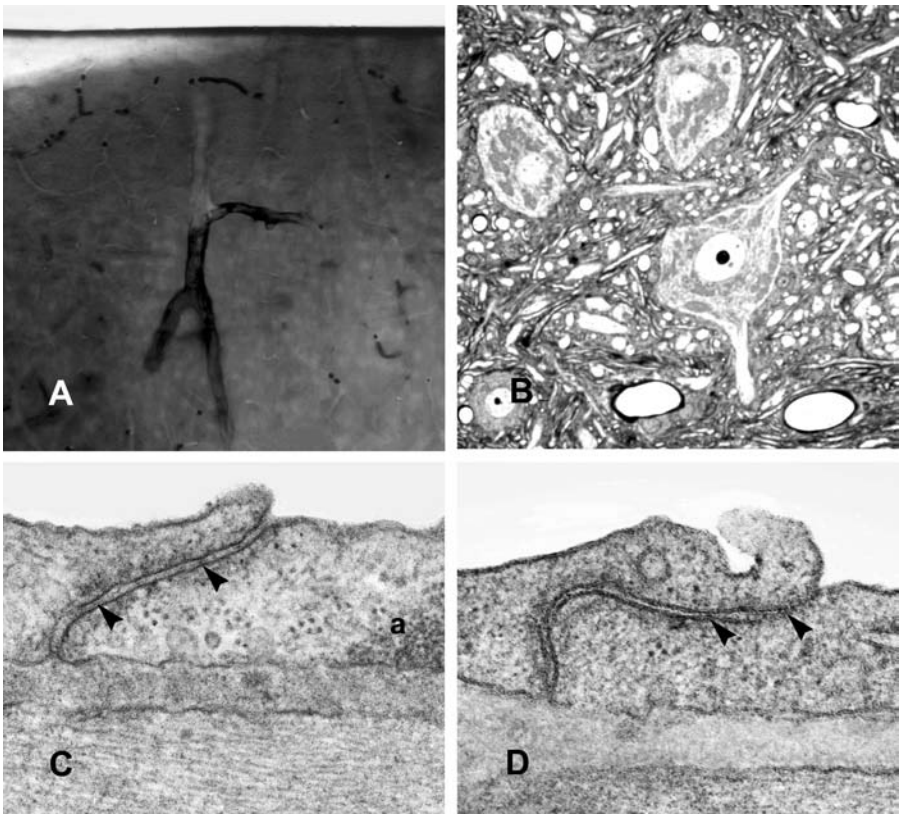

Fig. 2. **(A)** The cutting edge of an epon block prepared using a flat silicone mold, shows a 50-µm thick section of cerebral cortex embedded within the block. The block viewed using a 3.5X objective shows an arteriole permeable to HRP. The vessel details allow the researcher to determine the precise level at which to section this vessel. **(B)** A semithin section stained with toluidine blue provides good visualization of neurons, nerve processes, and blood vessels present in the block. Such a preparation can be used for morphometry to determine the number of neurons or the density of vessels in a particular area. Segments of cerebral endothelium of vessels permeable to horseradish peroxidase processed without **(C)** and with **(D)** *en bloc* stain are shown. Note that unit membranes are better preserved in **(D)** than in **(C)**. The fusion of the outer leaflets of the plasma membranes at tight junctions (**arrowheads**) are clearly seen in **(D)**. Cross sections of actin filaments **(a)** are indicated. **A**, X150; **B**, X185; **C**, X70,000; **D**, X75,000.

c. Puncture any air bubbles in the resin mixture with a needle and leave molds at room temperature for a few hours. If tissues have moved from the cutting edge reposition them using the bamboo splints or needles.
d. Place molds in a 60°C oven overnight.
e. Remove resin blocks from the molds and place in a glass beaker. If blocks are sticky put them back in the oven for a further 2–4 hr (*see* **Note 13**).
f. Next place blocks in a 90°C oven for 2–3 d to cure.
g. Residual resin mixture is polymerized in the beaker by placing in the 90°C oven until it hardens. It can then be discarded.
h. Any Epon spills can be cleaned with ethanol.

3.5. Sectioning

Both semithin and ultrathin sections are cut using an ultramicrotome. Before cutting, wipe the ultramicrotome with a damp Kimwipe or soft cloth to remove dust or debris from previous sectioning. This also reduces static electricity.

3.5.1. Semithin Sectioning

Resin-embedded sections are superior to paraffin sections because tissue shrinkage is less and cellular components are better preserved (**Fig. 2B**). Semithin sections have many uses such as 1) they can be used to determine whether fixation, dehydration, and infiltration are carried out properly; 2) they are suitable for assessment of morphological findings and for morphometric assessments such as number of neurons/mm^2, number of vessels/mm^2, and the diameter of vessels; 3) they are helpful for selection of areas of interest for ultrathin sectioning. Semithin sections are cut using glass knives, which are made using a knife maker as per instructions supplied with the machine. An old diamond knife may also be used to cut these sections. Semithin sections are stained with an alkaline toluidine blue solution (*see* **Fig. 2B**).

1. Trimming of blocks
   a. Place the block in a microtome chuck, which is then mounted on a special base and viewed by transmitted illumination using a dissecting microscope.
   b. Use a fresh single-edge razor blade to trim away the resin around the tissue to form a four-sided pyramid, with the tissue at the top of the pyramid.
   c. The block face is usually rectangular with the top and bottom edges parallel to one another to obtain a ribbon during sectioning. Cut off one corner. This cut end can be seen in sections by light microscopy and helps to orient the block when the size of the block face is cut down further in preparation for ultrathin sectioning.
2. Sectioning
   a. Reset the advance mechanism of the microtome after each block or each day depending on the number of blocks being cut.
   b. Retract both the coarse and fine stage advances.
   c. Mount the chuck containing the block into its holder in the machine and tighten the screw holding it in place. The block is positioned so that its long axis is at right angles to the cutting edge.
   d. Mount the knife and screw tightly into place. Adjust the angle between 2° and 5°.
   e. Cycle the microtome manually and stop when the face of the block is slightly above the height of the cutting edge.
   f. Position the binocular microscope and adjust the angle of illumination system so that some light is reflected on the block face.
   g. Bring the mounted knife forward slowly until the cutting edge and the block face can be viewed through the microscope.
   h. A syringe or micropipette is used to fill filtered distilled water in the trough adjacent to the diamond knife. Sufficient water is added so that the surface of the water is convex in order to wet the knife-edge. Water is withdrawn from the trough using a syringe until the surface is horizontal (*see* **Note 14**).
   i. Adjust the knife holder so that the best portion of the knife is used for sectioning.
   j. Slowly advance the knife toward the block face, by using the coarse adjustment and then the fine adjustment.

k. Cutting Speed: This is the rate at which the specimen block passes the knife during the cutting phase and is expressed in mm/s. Automatic ultramicrotomes maintain a constant cutting speed over a specified distance. As a general rule, sectioning should be performed at a relatively slow speed of 2–3 mm/s. However, the appropriate speed has to be established by trial and error for the tissue being cut (*see* **Note 15**).
l. The ultramicrotome has a control to adjust section thickness. In addition, thickess of sections in a water bath are also judged by the interference colors that are produced when the light reflected from both the upper surfaces of the sections and from the section-water interface move out of phase. A color scale is available, which gives the thickness of sections having a particular color and this is applicable to all embedding materials having a refractive index close to 1.5, including epoxy resins ***(4)***. Semithin sections are dark gold and have a thickness of approx 900 nm.
m. When floating on the trough liquid, the sections should appear uniformly colored both within each section and from section to section in the ribbon.
n. The surface of sections should be smooth without wrinkles, folds, or knife marks.
o. Make sure that the ribbon is straight.
p. Detach the ribbon from the cutting edge of the knife using a clean hair, which is mounted on wooden handles or cluster sections.
q. Sections are picked up from the water bath and placed on a glass slide using the wire loop.
r. Slides are placed on a hot plate set at 80°C for 10 min to flatten the section, evaporate the water and ensure adhesion of sections to the slides.

3. Staining semithin sections
   a. Place a drop of toluidine blue stain on sections and place on the hot plate for a minute or until a dried ring is seen at the edge of the stain.
   b. Wash slide with distilled water and dip in acetone to differentiate.
   c. Dehydrate in 2 changes of 100% ethanol and then in 2 changes of xylene for 2 min each.
   d. Place a drop of Permount on the section and then a cover slip.

3.5.2. Ultrathin Sectioning

Ultrathin sections are cut from a preselected area of the block, usually based on information obtained by viewing the semithin section. Most sections within the silver to pale gold range (60–90 nm) are suitable for normal work, although thinner sections may be required when high resolution is needed. Pale gold sections having a thickness of about 90 nm give well-contrasted, good-quality electron micrographs while micrographs obtained from silver sections usually lack contrast. Ultrathin sections are cut using a diamond knife whose cutting angle is specified by the manufacturer. These sections can also be cut with freshly prepared glass knives. Follow the sectioning method outlined in **Subheading 3.5.1., step 2.**

1. A plexiglass draft protector, which is supplied with the ultramicrotome should be used when ultrathin sections are cut to prevent the sections being blown around in the trough.
2. Collection of Sections
   a. Detach the ribbon from the cutting edge of the knife using the clean hair taped to an applicator stick.
   b. One edge of a clean copper grid is bent using a pair of tweezers to produce a 90° angle with the rest of the surface. The grid is held at this end and lowered into the trough and positioned under the ribbon such that the rim of the grid is under the edge of the first section. Lift the grid vertically out of the water. Sections are picked up on the dull side of the grid (*see* **Note 16**).

c. Touch the edge of the grid on the surface of filter paper and place on a filter paper in a petri dish with the section side up and dry grids at least 20–30 min before staining.
3. Usually two to three grids of sections are cut from one block.
4. After sectioning, clean the diamond knife with distilled water contained in a spray bottle and dry it using a container of compressed air. Periodic cleaning in an ultrasonic cleaner is recommended to remove any adherent resin.

3.6. Staining

May be done using an automatic stainer. Manual staining is done in a designated clean area of the laboratory preferably using a bench top hood. The stain solutions are left undisturbed in this hood. The method for manual staining of ultrathin sections is as follows:

1. If *en bloc* staining with uranyl acetate is done proceed to lead citrate staining (**step 4**).
2. Place a drop of uranyl acetate on a silicone rubber plate contained in a petri dish. Float the grid on this drop with the section side down for 20 min. Because the surface of this rubber plate is divided into numbered squares, four to five sections can be stained at a time.
3. Rinse the grid by 10–15 quick dips in a 250-mL beaker containing filtered water. Use a dry forceps to hold the opposite side of the grid and dip again 10–15 times in another beaker containing filtered water (*see* **Note 17**).
4. Touch the edge of the grid to filter paper to remove excess water and place the grid on a drop of lead citrate stain for 1–1.5 min. This stain is placed on the silicone rubber plate, which is surrounded by NaOH pellets in a large petri dish. Keep petri dish covered while staining.
5. Rinse sections as in **step 3**.
6. Place the grid with the section side up on a filter paper and allow it to dry for few minutes and then place the grid in a gelatin capsule, which can be labeled with the case number (*see* **Note 18**).

3.7. Morphometry

Measurements of various parameters of pial and intracerebral cortical vessels such as diameter, cross sectional area of the media and intima and density of cerebral microvessels are available in the literature ***(5–7)***. In addition, measurements are also available of lengths of tight junctions, density of fenestrations and organelles in cerebral endothelium such as mitochondria, and endothelial vesicles ***(8–10)***. Details on how to perform these measurements are beyond the scope of this chapter because it depends to a certain extent on the type of image analyser being used. An example of a morphometric technique follows.

3.7.1. Determination of the Ratio of the Wall-Lumen Area of Cerebral Cortical Arterioles

1. Areas of the different layers of the vessel wall can only be measured when vessels are fixed after achieving maximal dilatation. This is achieved by perfusing the rat initially with Kreb's solution for 15 min followed by perfusion of a fixative solution.
2. Flat embedded blocks are placed on a glass slide and examined using a 3.5X objective of a light microscope to determine whether the block has an arteriole (*see* **Fig. 2A**). Arterioles having external diameters ranging from 15–25 μm and a single layer of smooth muscle are selected.

3. Sectioning arterioles at a particular depth from the cortical surface is a laborious process. To section arterioles at a depth of 300 μm from the cortical surface:
   a. Place a calibrated micrometer scale in the eyepiece of a light microscope and measure the length of the block using a 3.5X objective. If 1 division of the eyepiece scale is equal to 34.5 μm, then 8.5 divisions of the top of the block have to be cut away to reach a depth of 300 μm from the cortical surface.
   b. The block face is trimmed and sectioned. Once the cortical surface is reached the ultramicrotome is set at a thickness setting of 10,000Å and 300 sections are discarded to reach a depth of 300 μm.
   c. The block can then be re-measured to determine whether the correct depth has been reached.
   d. The block face may have to be trimmed further before thin sections are cut.
4. Overlapping electron micrographs are taken along the circumference of arterioles at a screen magnification of 2700. Only vessels sectioned perpendicular to their long axis are used for photography. These vessels show unit membranes along their entire circumference and the cell wall has a fairly uniform thickness excluding the areas having endothelial nuclei.
5. The magnification of the electron microscope is checked using a carbon diffraction replica.
6. Electron micrographs printed at a constant magnification are taped together to reconstruct the entire vessel. This is placed on an illuminated copy stand (Kaiser). A high resolution CCD Camera is used to transmit the image to an image analyzer.
7. Image analysers such as the Microcomputer Imaging Device system (Imaging Research, St Catherines, On) can receive images from negative of electron micrographs placed on a light box via a CCD camera. The image obtained on the screen can be inverted to obtain a positive image.
8. The image analyser is calibrated for linear measurements in micrometers or area measurements in square-micrometers. The cross-sectional area occupied by the vessel lumen and the total cross sectional area of the vessel (lumen + media and intima) of vessels in the different experimental groups is measured using the image analyzer. Cross sectional area of the arterial wall is calculated by subtracting the lumen area from the total vessel area. The ratio of the wall-lumen area is then calculated.
9. Vessel wall dimensions for the different experimental groups are compared using the unpaired *t* test.
10. Interpretation: These measurements were used to establish that the ratio of the wall-lumen area of cerebral arterioles is significantly higher ($p > 0.001$) in rats with chronic renal hypertension as compared to arterioles of normotensive rats ***(7)***.

Quantitation of endothelial organelles such as plasmalemmal vesicles is given in Chapter 8, Subheading 3.1.2.1.

4. Notes

1. Most chemicals used for electron microscopy such as solvents, resins, and buffers are hazardous and gloves should be worn for all procedures listed in this chapter and is optional only when sectioning.
2. Sodium cacodylate contains approx 30% arsenic by weight and is a health hazard. It should be weighed and dissolved in a fume hood.
3. Osmium tetroxide is hazardous and should be used in a fume hood. It is disposed in a container containing vegetable oil and kitty litter. The Biohazard Department of the Institution has to be contacted to dispose this waste.
4. Uranyl acetate and lead salts used for staining grids are toxic and have to be handled with care. Uranyl acetate is a radiochemical as well. Both these substances are disposed in

different labeled containers, which are disposed by the biohazard department of the institution.

5. Distilled water filtered using a no. 50 Whatman filter is used for the preparation of stains for ultrathin sections and to fill the trough adjacent to knives.
6. Undiluted fixative can be frozen in 250-mL amounts in Nalgene flasks. It is defrosted before use and diluted with an equal amount of 0.1 *M* cacodylate buffer containing sucrose for perfusion.
7. Some researchers perfuse a buffer or Ringer's solution containing heparin for few minutes to clear the blood followed by the perfusion of the fixative solution. In our studies preservation of endothelium is not as good when buffer followed by fixative is used as compared with fixative alone. Heparin is known to increase cerebral endothelial permeability to protein tracers ***(11)*** and therefore should not be used when permeability studies are undertaken. Perfusion with Ringers solution is done if vessel morphometry is required to achieve maximal dilatation of vessels before fixation.
8. A perfusion time of 10 min is selected because the results show good preservation of tissue. Shorter periods result in poor preservation and fragile slices.
9. If brain blocks or slices are immersion fixed for longer than 48 h there is extraction of tissue constituents and organelles and nerve processes appear swollen.
10. The advantage of *en bloc* staining is that preservation of membranes is superior (*see* **Figs. 2C** and **D**), and it eliminates having to stain grids with uranyl acetate prior to viewing. The latter technique is more likely to produce precipitates on sections making them unsuitable for photography.
11. Ethanols, which are 85% and higher, are collected in a safety container for disposal by the biohazard department of the institution.
12. Rotary shakers are available for agitating the contents of the tubes to promote infiltration of resin into blocks. These shakers are not suitable for tissue blocks, which are 50 µm thick as the tissue gets caught between the cork and glass tube and disintegrates.
13. If semithin sections are required quickly blocks can be cut after being kept at 60°C overnight. These blocks do have to be placed in the 90°C oven to cure the Epon before ultrathin sections can be cut.
14. A convex meniscus generally tends to overwet the cutting edge resulting in wetting of the block face of the specimen and sometimes the back of the knife face also picks up some trough fluid. Sections cannot be obtained under these conditions. A concave meniscus may not wet the entire knife face, which results in the sections sticking to the knife and crumpling.
15. If the cutting speed is too high, variations in section thickness result. Excessive compression, wrinkles and fine chatter parallel to the cutting edge of the section can also occur. A very slow cutting speed may drag the trough fluid over the back of the knife and changes in temperature and draught during a cycle will cause thermal drift.
16. An alternate method for collecting sections is to place the grid flat on the upper surface of the sections.
17. Static may be a problem during staining especially during the winter months when the air is very dry. This is overcome by running a humidifier near the staining area.
18. An inexpensive way to store grids is to place them in a gelatin capsule, which is numbered with the grid number. Capsules containing grids from one case are placed in a cardboard pill box that is labeled with the case number.

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