Chapter 5
Epoxy Resins for Light and Transmission Electron Microscopy

Michael John Sumner

5.1 Introduction

Epoxy resins were first introduced as an embedding medium for electron microscopy in 1956 by Glauert et al. [1], and by Richardson et al. [2] in 1960 for light microscopy. The liquid resin mixture contains three main ingredients: (A) an epoxy resin (an aliphatic or aromatic epoxide), (B) a hardening agent which when heated forms a solid cross-linked polymer that is rigid enough to produce ultrathin sections (between 50 and 100 nm) and is stable under the transmitted electron beam and (C) an accelerator. The three most commonly used resins are Araldite, Epon and ERL. Using the recipes of Luft, [3], Epon 812 has been the most widely used epoxy embedding medium for electron microscopy [4]. Epon 812 was discontinued by the manufacture Shell in 1984. There are a number of Epon substitutes [4] now available from a variety of EM supply companies. These resin mixtures section easily, provide good specimen contrast when stained with heavy metals, and have a relatively low viscosity as compared to Araldite. This allows the Epon resin mixture to penetrate the tissue faster than Araldite, which has a much higher viscosity. According to Ellis [5], the various Epon substitutes on the market today are not identical to the original Epon 812 produced by Shell, and users should include in publications the precise mixture and chemistry of the Epon substitute used. In 1969, Spurr [6] introduced a new epoxy resin, vinylcyclohexene dioxide (VCD) (ERL 4206 of Union Carbide—now Dow Chemical), having a much lower viscosity than Epon 812 and its substitutes, that facilitates rapid infiltration into tissues. ERL 4206 and its hardener, nonenyl succinic anhydride (NSA) alone produce a very hard block. Spurr countered this problem by adding a reactive flexibilizer, diglycidyl ether of polypropylene glycol (DER 736), to the embedding medium. The hardness of the resin block is controlled by varying the amount of DER 736.

M. J. Sumner (✉)
Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada
e-mail: Michael.Sumner@umanitoba.ca

E. C. T. Yeung et al. (eds.), *Plant Microtechniques and Protocols,*
DOI 10.1007/978-3-319-19944-3_5

ERL 4206 is no longer available and has been substituted with ERL 4221. ERL 4221 is somewhat more viscous than ERL 4206; however, the properties of the new Spurr's resin medium, using ERL 4221, is very similar to the original formulation. Glauert and Lewis [7] recommend using benzyldimethylamine (BDMA) as the accelerator in the Spurr's epoxy medium in place of the original Spurr's accelerator, dimethylaminoethanol (DMAE). DMAE is widely used as the accelerator in the Spurr's embedding medium and continues to produce satisfactory results in our lab. The low viscosity of the Spurr's embedding medium makes it ideal for the infiltration of plant tissue which, unlike animal tissue, has the complexity of hard extracellular cell walls, starch grains, calcium oxalate crystals and a waxy cuticle that are difficult to infiltrate using traditional Epon and Araldite embedding media. A new epoxy resin has been recently introduced by Electron Microscopy Sciences (Ultra Bed Low Viscosity Epoxy catalogue # 14310) and Polysciences (Embed-it catalogue # 64-86-8). These new very low viscosity resin kits are reported to be similar to their Spurr ERL 4221 low viscosity embedding kits and are less hazardous *(see Note 3)*. This chapter focuses on the standard protocols used in the preparation of Spurr's epoxy embedded plant tissue for transmission electron microscopy as well as the protocols used to section and stain epoxy embedded tissue for light microscopy.

5.1.1 Tissue Preparation for Transmission Electron Microscopy [7–11]

The high-resolution transmission electron microscope (TEM) is but one of the many tools available to biologists in the quest to understand the structure and function of the living cell. Start your anatomical investigation with a thorough examination of the anatomical literature specific to the subject matter, followed by an in-depth examination of the relevant tissues of the living organism using techniques presented in Chaps. 1–4 of this book. Only after a thorough understanding of the three-dimensional structure and organization of the relevant tissues is achieved should one proceed to the use of the TEM in a research project; of course, its use is dependent on the research question being asked.

In general, the preparation of tissue for TEM involves six fundamental steps: fixation, dehydration, infiltration, embedding, sectioning and staining. The process starts with the careful dissection of small (less than 1 mm) segments of hydrated living tissue with a sharp razor blade and ends with nonliving dehydrated tissue that has been infiltrated and embedded in an epoxy resin matrix. Thin (1–2 μm) survey sections are affixed to coated glass slides, stained and viewed with a light microscope. Ultrathin (60–80 nm) sections are subsequently cut from the same block, picked up on copper or nickel grids, stained and viewed with a TEM to localize the subcellular structures that we hope are representative of the once living tissue. However careful and precise we are during the preparation process, artifacts are an unavoidable consequence at all steps of the process [12].

5.2 Materials

5.2.1 Major Equipment

1. TEM
2. Compound light microscope
3. Ultramicrotome
4. Glass knife maker
5. Diamond knife for ultrathin sectioning *(see Note 1)*.

5.2.2 General Laboratory Equipment and Supplies

1. Glass vials (10–15 mL), vial holder and small identification labels inserted into the vial
2. Double-sided razor blades and high quality dissecting kit
3. Top loading balance for measuring Spurr's resin components
4. Disposable plastic beakers and pipettes
5. Rotary mixer to hold the glass vials during fixation, dehydration and infiltration
6. Aluminum embedding (weighing) dishes (44 mm)
7. Fine-bladed piercing saw for cutting out 2–3 mm pieces of embedded tissue
8. Plastic Beem® capsules containing blank polymerized epoxy resin stubs
9. Fine carpenter's file for precise flattening (90° to the long axis) and removal of the pyramidal end of the blank epoxy resin stub
10. Five minute epoxy glue to glue the pieces of embedded tissue to the end of the epoxy resin stub (termed the tissue block)
11. Rotary tools (e.g. Dremel) can be used for careful removal of excess resin from around tissue block (optional)
12. Silver tape and dental wax to make glass knife boat, clean boated triangle glass knives for thin survey sectioning
13. Ten cc syringes fitted with 0.45 µm filters for distilled water and stains
14. Eyelash attached to wood stick by dental wax for manipulating ultrathin sections floating on the surface of the diamond knife boat
15. Standard and locking fine forceps (# 5)
16. Drawn glass microprobe, loop or shaved wooden spatula stick to pick up thin sections from the glass knife boat
17. Copper and nickel TEM grids *(see Note 2)*
18. Petri dishes with filter paper to store each type of cleaned TEM grid
19. Petri dish with a numbered rubber pad for keeping track of stained grid sections
20. Stained grid storage containers
21. Microscope slides, number 1.5 coverslips
22. Slide warmer
23. Glass strips for making knives.

5.2.3 Embedding Kits, Chemicals and Stains

1. Spurr low viscosity embedding kit (EMS catalogue # 14300) (Ted Pella catalogue # 18108) (Polysciences catalogue # 01916-1) *(see Note 3)*
2. 2–4 % glutaraldehyde primary fixative in buffer *(see Note 4)*
3. 1–2 % osmium tetroxide (OsO_4) secondary fixative in buffer
4. Ethanol or acetone for dehydration, propylene oxide for infiltration (optional when using Spurr's resin)
5. Filtered distilled water
6. Light microscope stains and related reagents: crystal violet, toluidine blue O (TBO), borax to stain for thin survey sections,aniline blue black, calcofluor, aniline blue stains, periodic acid, Schiff's reagent, sodium metabisulphite, DNPH (2, 4-dinitrophenol-hydrazine), gelatin, glycerol, chromium potassium sulphate for coating frosted microscope slides, non-permanent mounting media (70 % sucrose)
7. Chloroform for stretching thin and ultrathin sections
8. TEM stains and related reagents: uranyl acetate and lead citrate stains for ultrathin sections, thiocarbohydrazide, silver proteinate, acetic acid, potassium ferricyanide, calcium chloride and cacodylate buffer.

5.3 Methods

5.3.1 Chemical Fixation for Conventional TEM

A comprehensive discussion of fixation is found in Chap. 2 of this book. A standard protocol is to fix tissue in 3 % glutaraldehyde in 0.05 M phosphate or cacodylate buffer (pH 6.8–7.2) for 2 h at room temperature, followed by 24 h at 4 °C in the same fixative, then post-fixed for 4 h in 2 % OsO_4 in 0.05 M phosphate or cacodylate buffer (pH 6.8–7.2, *see Note 5*).

5.3.2 Dehydration (see Note 6)

Tissues are dehydrated in a graded ethanol series 30, 50, 70, (20–30 min each), 95 (two changes 20–30 min each), 100 % (three changes 30 min each) followed by successive changes of ethanol:propylene oxide (3:1; 1:1; 1:3; for 30 min each), and three changes of 100 % propylene oxide (30 min each). The tissues are constantly rotated during all stages of dehydration. Be careful while decanting solutions during dehydration; tissue samples *must not be allowed to dry out*.

5.3.3 Infiltration

1. Spurr's resin medium [6, 8, 10] *(see Note 3)*

	EMS (mL)	Polysciences (g)
ERL—4221	18	4.10
D.E.R.736	14	1.43
NSA	48	5.90
DMAE	0.6	0.1

2. In our lab, tissues are typically infiltrated with a mixture of propylene oxide and Spurr's low viscosity epoxy resin 3:1; 1:1; for 2 h each; 1:3 for 48 h, followed by daily changes of 100 % fresh Spurr's resin for 3–7 days depending on the type of plant tissue being infiltrated.
3. Tissues containing abundant starch and particularly thick cell walls should be infiltrated for a week. Infiltration is a key step in the process and the precise time needed for complete infiltration can only be determined by trial and error. The tissues are constantly rotated during all stages of infiltration.

5.3.4 Embedding

1. Following infiltration, the tissues are embedded in 100 % Spurr's resin in shallow 44 mm diameter aluminum dishes, separated from each other using the shaved tip of a wooden stick and polymerized at 70 °C under partial vacuum for 8–12 h.
2. Observe the embedded tissue (black due to OsO_4 fixation) within the disk of epoxy resin using a stereomicroscope and a transmitted light source. Draw a rectangular outline around the tissue using an extra fine black felt pen and note the orientation of the tissue within the resin.
3. Secure the resin disk in a vice and slowly cut the outlined cube of embedded tissue from the resin disc using a fine-bladed piercing saw. Using fast curing epoxy adhesive such as the 5-minute epoxy glue, glue the cube to a cylindrical Beem resin stub with the long axis of the tissue precisely parallel to the flat stub face for cutting longitudinal sections or with the long axis of the tissue precisely perpendicular to the flat stub face for cutting transverse sections *(see Note 7)*.

5.3.5 Sectioning

5.3.5.1 Thin Sectioning for Light Microscopy

1. Start by reading the microtome instruction manual to gain a thorough understanding of how the machine operates.

2. The block of epoxy embedded tissue being initially used for light microscopy is typically trimmed to a rhomboid shaped surface of 1 mm^2 or less. Because of the small size of the block face, microtomes used for thin sectioning should be fitted with a binocular magnification system similar to that found on an ultramicrotome.
3. Reset the microtome advance mechanism and retract the course and fine stage manipulators.
4. Insert and firmly secure the block into the chuck. Be sure the lower block face is parallel to the knife-edge.
5. Clamp a "wet" triangular glass knife onto the stage and initially set the knife angle to 3°. Adjust the water level in the knife boat to wet the knife-edge.
6. Carefully advance the stage knife toward the block using the course and fine adjustments. When the knife-edge is about 0.5 mm from the block face, carefully advance the stage toward the block using the fine adjustment while simultaneously advancing the block toward the knife at a rate of 2 μm per cycle until a small portion of the block face is initially cut. Reset the microtome to 1 μm and continue to cycle the block until the entire block face is being cut with each cycle.
7. If the face of the block has been previously rough trimmed to remove damaged wound tissue at the initial cut line, you can begin to collect sections with a drawn glass microprobe (or a wire loop or a wooden spatula) and transfer them to drops of filtered distilled water on clean Haupt's coated microscope slides *(see Note 8)*. Allow the slide to dry on a slide warmer at 85 °C. Stain the 1–2 μm survey sections with crystal violet [13] or TBO [10].
8. Once the target zone is reached, stop, retract the microtome stage and carefully remove the block from the microtome to be retrimmed for ultamicrotomy using a diamond knife.

5.3.5.2 Ultrathin Sectioning for Transmission Electron Microscopy

1. Start by reading the ultramicrotome instruction manual to gain a thorough understanding of how the machine operates.
2. Novice users should practice and become proficient at ultrathin sectioning with a "wet" triangular glass knife prior to sectioning valuable research material with a clean diamond knife.
3. Examine the most recent stained light microscope survey sections to locate the centre and extent of the target zone.
4. Using a new clean fine razor blade, carefully retrim the block from Sect. 5.3.5.1 to a size that is as small as possible (maximum 0.5 mm) and still includes the target zone. Be sure the top and bottom faces of the block face are trimmed precisely parallel to each other to allow for a straight ribbon of ultrathin sections to be cut using the ultramicrotome. Trim the sides to maintain the rhomboid shape of the block face. Clean all trim debris from the block.

5. Insert the trimmed block into the ultramicrotome chuck, making sure that no more than 5 mm of the block extends beyond the chuck to minimize vibrations that produce ultrathin sections with chatter [12].
6. Insert a clean diamond knife *(see Note 1)* into the ultramicrotome stage holder and set the knife angle according to the manufacturer's instructions. Fill the boat with water from a clean filtered (0.45 μm) syringe. Slowly raise the water level in the boat (slightly convex) to wet the knife-edge. Withdraw a small amount of water until a slightly flat to concave water level is achieved. With proper adjustment of the illumination from the ultramicrotome stereomicroscope system, the knife-edge, the surface of the water and the face of the block should become visible. From this point onward, all observations will be made through the microscope.
7. Carefully advance the stage knife toward the block face using the coarse and fine adjustments of the stage. Manually cycle the block until it is level with the knife-edge. Use the stage fine adjustment to advance the knife toward the block until the shadow of the knife appears on the illuminated block face.
8. Manually cycle the ultramicrotome to advance the block face toward the knife-edge at 1 μm intervals until the block face shadow of the knife-edge is very thin. Using the chuck fine adjustment, rotate the block until the lower edge of the block face is perfectly parallel with the knife-edge.
9. Switch the ultramicrotome to automatic with a cutting speed of about 2–3 mm/s and a section thickness of 200 nm (purple interference colour). Continue cycling until the block face first contacts the knife-edge. Stop the ultramicrotome.
10. Set the section thickness to between 80 nm (pale gold) to 60 nm (silver) and begin to collect ribbons of sections. Dip the end of an applicator stick in chloroform or xylene and pass the stick over the sections for 5 s. The vapours of the solvent will stretch the sections. Periodically add water to the boat to maintain the surface illumination and section interference colours. Gold coloured sections are fine for low magnification electron microscopy while grey to silver are better for higher magnification work. It is advisable to cut and collect both for future use.
11. Using the tip of the eyelash applicator stick, move the ribbons toward the centre of the boat and arrange in clusters of 2–3 ribbons per cluster.
12. Pick up the edge of a dry clean grid *(see Note 2)* with a pair of fine (# 5) locking forceps. Bend the edge of the grid so that the locking forceps are at a 45° angle relative to the grid surface. With a steady hand lower the grid (dull side down) over a grid cluster. The cluster will be attracted to the grid surface along with a drop of boat water.
13. Place the wet grid (section side up) on a filter paper in a glass petri dish. Cover and let dry. Repeat the process for each section cluster.

5.3.6 Staining

5.3.6.1 Staining Epoxy Sections for Light Microscopy *(see Note 9)*

Unlike glycol methacrylate (GMA), a water permeable plastic, the epoxy resins are relatively water impermeable. Traditional staining techniques developed for fresh, paraffin-embedded and GMA-embedded tissue rely heavily on dyes dissolved in water or aqueous buffers. The majority of these staining techniques have proven to be ineffectual when applied to epoxy-embedded plant tissue. Limited success has been achieved using cationic dyes (e.g. TBO, methylene blue, azure II) at high pH (10).

Crystal Violet [13] *(see Note 10)*

1. Superior contrast is obtained by staining epoxy sections, on a slide warmer at 85 °C, with 2 % ethanolic crystal violet in 0.05 M ammonium oxalate buffer at pH 6.7 for 0.5–1.0 min. Use a filtered (0.45 μm) syringe and apply drops of stain directly on the sections on the slide.
2. Rinse in running distilled water for 5 min. Use Coplin jars.
3. Decolourize in 95 % EtOH if necessary
4. Mount in 70 % sucrose in distilled water using size 1.5 coverslips *(see Note 11)*

TBO [10] *(see Note 12)*

1. Stain tissue with 1 % TBO in 1 % aqueous borax at 50–85 °C on a slide warmer for 1–5 min. Use a filtered (0.45 μm) syringe and apply drops of stain directly on the sections on the slide.
2. Rinse in running distilled water for 5 min. Use Coplin jars.
3. Mount in 70 % sucrose in distilled water using size 1.5 coverslips *(see Note 11)*.

Aniline Blue Black [14] *(see Note 13)*

1. Sections are stained in 1 % aniline blue black in 7 % acetic acid for 15 min at 60 °C. Use Coplin jars in a water bath.
2. Rinse in 7 % acetic acid to remove excess stain. Use Coplin jars.
3. Mount in glycerol containing 5 % acetic acid using size 1.5 coverslips.

Periodic Acid—Schiff Reaction (PAS) [10] *(see Note 14)*

1. Block aldehydes using a saturated solution of DNPH (2,4-dinitrophenol-hydrazine) in 15 % acetic acid. Use Coplin jars.
2. Wash in running distilled H_2O for 10 min.
3. 1 % aqueous periodic acid for 30 min.
4. Wash in running distilled H_2O for 5 min.
5. Schiff's reagent 35–45 min.
6. 0.5 % sodium metabisulphite in 1 % dilution of concentrated HCl (optional) 2 × 2 min.
7. Wash in running distilled H_2O 10 min.
8. Counter stain (optional) with TBO, crystal violet or aniline blue black.
9. Mount crystal violet and TBO stained sections in 70 % sucrose in distilled water using size 1.5 coverslips *(see Note 11)*.
10. Mount aniline blue-black stained sections in glycerol containing 5 % acetic acid using size 1.5 coverslips.

5.3.6.2 Staining for Fluorescence Microscopy

1. Sections from material fixed in glutaraldehyde -OsO_4 are immersed in 1 % periodic acid or 10 % hydrogen peroxide for 30 min to bleach osmium from the tissues.
2. Using a diamond point pencil on the bottom of the glass slide, outline the clusters of epoxy sections adhering to the Haupt's adhesive on the upper surface of the slide. Prior to staining epoxy-embedded material with fluorochromes (e.g. calcofluor, aniline blue) the resin must be removed from the sections *(see Note 15)*

Calcofluor [15] *(see Note 16)*

Epoxy sections are stained in 0.01 % aqueous calcofluor white M2R for 5 min, rinsed in running water, mounted in 70 % sucrose and viewed with ultraviolet (UV) light using a UV excitation filter (330–380 nm), dichroic mirror DM400 and barrier filter transmitting above 420 nm.

Aniline Blue [10] *(see Note 17)*

1. Epoxy sections are stained for 5 min in 0.05 % aniline blue in 0.01 M phosphate buffer at pH 8.5, mounted in the buffered stain and illuminated with UV light using a UV excitation filter (330–380 nm), dichroic mirror DM400 and barrier filter transmitting above 420 nm.

5.3.6.3 Staining for Transmission Electron Microscopy *(see Note 18)*

Conventional Staining of Aldehyde/OsO_4 Fixed Tissues with Uranyl Acetate Followed by Lead Citrate [7–11]

A. Uranyl Acetate *(see Note 19)*

1. Add 3.75 g uranyl acetate to 50 mL of 50% methanol.
2. Stain grids with a filtered(0.45 µm) syringe of a saturated solution of uranyl acetate in 50% methanol at room temperature for 5–25 min.
3. Cover sections during staining to block light induced precipitates.
4. Rinse 2x in 50% methanol; 2x filtered degassed H_2O.
5. An optional procedure is en bloc staining with uranyl acetate prior to dehydration [10, 11].

B. Lead citrate [16] (alternative methods *see* [17, 18])

1. Add 0.02 g lead citrate to 10 mL of degassed distilled water in centrifuge tube.
2. Add 0.1 mL of 10 N NaOH, seal and shake to dissolve.
3. Stain grids with a solution of lead citrate for 5–10 min. Centrifuge before use.
4. Staining must be done in a CO_2 free environment to prevent the formation of lead carbonate precipitates *(see Note 20)*.
5. Place drops of stain on squares of Parafilm, dental wax or plastic petri dishes.
6. Rinse in degassed filtered water.
7. Dry.
8. Excellent contrast with sections that have been fixed with OsO_4.

Periodic Acid—Thiocarbohydrazide—Silver Proteinate (PA-TCH-SP) [19] *(see Note 21)*

1. Collect ultrathin sections of tissue, fixed in glutaraldehyde—OsO_4 (*see* Sect. 5.3.1), on clean dry nickel grids *(see Note 2)*.
2. Float grids section-face down on 1% aqueous periodic acid for 30 min in a high humidity chamber, followed by three changes of 10 min each in distilled water.
3. Grids are transferred to 0.2% thiocarbohydrazide (TCH) in 20% aqueous acetic acid for 5 h followed by successive rinses of 10, 5 and 1% aqueous acetic acid (20 min each) and three rinses of distilled water (30 min each).
4. The grids are then immersed in 1% aqueous silver proteinate for 30 min in the dark followed by three successive rinses of 1 h each in distilled water.

OsO_4 and Potassium Ferricyanide (OsFeCN) [20] *(see Note 22)*

1. Tissue is fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 6.8) with 5 mM calcium chloride for 2 h at room temperature followed by 24 h at 4 °C in the same fixative.

2. Following a wash in 0.05 M caycodylate buffer (pH 6.8) with 5 mM calcium chloride, the tissue is post-fixed in a mixture of 2 % OsO_4 and 0.8 % potassium ferricyanide in 0.05 M cacodylate buffer with 5 mM calcium chloride for 4 h at room temperature.
3. The methods of dehydration, infiltration, embedding, orientation, sectioning and staining are the conventional TEM methods described previously.
4. The OsFeCN method enhances overall membrane contrast and in particular the ER and nuclear envelope.

5.4 Notes

1. We highly recommend investing in a diamond knife for TEM studies. "Wet" triangular glass knives will cut ultrathin sections; however, the longevity of the glass knife-edge is short and unpredictable. Weeks of valuable time in the preparation of tissue for TEM are potentially wasted if the sharp edge of the glass knife becomes dull as one enters the target zone. Retracting the knife stage, inserting a new glass knife in the knife holder and precisely realigning the knife to the block face is very difficult. Resuming sectioning with the new glass knife generally results in the loss of some if not all of the target zone tissue.
2. TEM grids (analogous to the glass slide in light microscopy) are round (3.05 mm) typically copper or nickel mesh supports, which hold the stained ultrathin sections of tissue. Grids come in a wide variety of mesh shapes and sizes [8]. In our experience, hexagonal and rectangular (75 × 300) mesh grids allow for adequate support of the epoxy sections while maximizing the area of tissue that can be exposed to the electron beam and thus be visible in the TEM. Prior to use, grids should be cleaned first in acetone then in ethanol in a small beaker using a periodic swirling motion. The ethanol is decanted, the beaker (containing the ethanol wet grids) is inverted over a filter paper and placed in a 60 °C oven to dry. The clean dry grids are stored in a clean dust free petri dish.
3. All epoxy resin components are skin irritants and potential carcinogens. ERL 4206 and ERL 4221 in particular are toxic and hazardous. Wear gloves and work in a fume hood over paper towels to absorb any spilled epoxy resin medium liquids. Collect all contaminated vials, paper towels, plastic beakers, etc. within the fume hood in a covered container. Polymerize prior to disposal.
4. Use only electron microscopy-grade glutaraldehyde (available in sealed 25–70 % ampoules).
5. Depending on the research question being asked, there are numerous protocols in the literature for fixing tissues for electron microscopy [4, 7–11, 21].
6. ERL 4221 resin is miscible in ethanol or acetone and does not require a transitional solvent such as propylene oxide following dehydration [4]. Many microscopists still use propylene oxide following ethanol dehydration as a transition solvent with ERL 4221 infiltration and to further facilitate complete dehydration.

7. This step is critical for obtaining precise longitudinal and transverse sections. The orientation of the block of embedded tissue in the microtome/ultramicrotome chuck as well as the glass or diamond knife can be changed by altering the adjustment mechanisms for the microtome chuck and knife holder. However, we have found it preferable to obtain the desired orientation of the tissue during the post-embedding process that then only requires minimal adjustments to centre the trimmed tissue block relative to the knife-edge.
8. Clean microscope slides in a 70% ethyl alcohol solution containing 0.5% of 1 N HCl, followed by thorough washing with distilled water. The cleaned slides are then taken directly (without drying) from distilled water, dipped in Haupt's adhesive solution and placed vertically in a dust-free area to dry. Haupt's adhesive [22]: Dissolve 1 g of gelatin in 100 mL of distilled water at 90 °C. Cool to 30 °C add 15 mL of glycerine, stir and filter. Finally the adhesive is stirred and filtered and stored at 4 °C. As a preservative, 2 g of phenol may be added. Another excellent adhesive [23] may be prepared by dissolving 5 g of gelatin in 1000 mL of warm distilled water, and then adding 0.5 g of chromium potassium sulphate (*see* Chap. 4).
9. Prior to staining material fixed in glutaraldehyde—OsO_4 the sections should be immersed in 1% periodic acid (as part of the PAS reaction) or 10% hydrogen peroxide for 30 min to bleach osmium from the tissues.
10. The use of crystal violet (a cationic dye) to stain plant tissues embedded in epoxy resins has not been previously reported, though it is commonly used in bacteriology as part of the Gram staining procedure, and in animal histology to metachromatically stain amyloid. We routinely use crystal violet to stain epoxy survey sections in our lab and find the results superior to other cationic stains (e.g. TBO, methylene blue, azure II at high pH).

    Crystal violet stain recipe:
    Solution A: 2 g crystal violet (certified) in 20 mL 95% EtOH
    Solution B: 0.8 g ammonium oxalate in 80 mL distilled H_2O
    Mix solutions A and B, store for 24 h, filter and store at room temperature in a dark bottle.

11. After viewing with the compound light microscope, wash the slides in Coplin jars using running distilled water to remove the sucrose and coverslip. Store dry.
12. TBO is a cationic metachromatic dye which will stain certain chemical components of tissues a colour different from that of the dye in solution. TBO generally binds to macromolecules containing large numbers of polyanionic binding sites (e.g. carboxyl, sulphate and phosphate groups). The colour shift is from blue to red with the strongest metachromatic reaction (i.e. reddest) being for polymers containing sulphated groups; polymers containing carboxyl groups exhibit a slightly weaker reaction (i.e. pink); and polymers containing phosphate groups exhibit the weakest metachromatic reaction (purple). Sulphated polysaccharides (e.g. fucoidan) and carboxylated polysaccharides (e.g. pectic acids, alginic acids) will stain red to dark pink, RNA will stain purple and DNA will stain blue. By what is termed negative metachromasia, lignin and some

polyphenols stain green [24]. The high pH TBO used to stain epoxy sections shows a much weaker metachromatic reaction when compared to using the stain on fresh sections (*see* Chap. 1) or GMA sections (*see* Chap. 4). Crystal violet also exhibits weak metachromatic reactions similar to high pH TBO.

13. Aniline blue black, an anionic dye, is used to localize protein. The cytosol of healthy cells stain light blue while that of degenerating cells, presumably rich in hydrolytic proteins, stain dark blue. Mitochondria, plastids and regions of nuclei stain light to dark blue. It provides excellent contrast when used as a counter stain following the PAS reaction.
14. The PAS reaction is used to localize insoluble carbohydrates. The mechanism of this two-step histochemical procedure is well documented [25]. Periodic acid (PA) oxidizes vicinal 1,2 glycol groups (hydroxyl groups at the 2nd and 3rd carbon) in carbohydrates to aldehydes, which react with the Schiff's reagent to produce a magenta colour at the reaction site. Glucans with 1–4 linkages, such as pectic compounds, hemicellulose, starch and the oligosaccharide side chains of glycoproteins, are PAS positive. Cellulose, as it occurs in cell walls, is reported to be PAS negative, due perhaps to steric hindrance of available vicinal glycol groups [10]. Compounds will be PAS negative where the hydroxyl groups are not attached to vicinal carbon atoms or where one of the vicinal carbons is involved in linkages (Beta 1–3 glucans, e.g. callose). Certain compounds, other than carbohydrates, will also exhibit a PAS positive reaction. These include the amino acids serine, threonine and hydroxylysine, which have vicinal hydroxy-amino groups that can be oxidized to dialdehydes. Certain lipids will also react positively [26]. Lignin will combine with Schiff's reagent and give a positive reaction without prior periodic acid oxidation [10].
15. Prior to staining Spurr's epoxy-embedded material with fluorochromes, the resin must be removed from the sections using a modification of the procedure of Lane and Europa [27]. Sections mounted on Haupt's gelatin-coated slides are immersed in a saturated solution of potassium hydroxide in 95% ethanol for 2 min, rinsed in three changes of 95% ethanol, followed by 5 min in running water. The resin is removed to facilitate access of the fluorochrome to the tissue and to eliminate the problem of background autofluorescence caused by the resin.
16. Calcofluor has been used as a general stain for plant cell walls [28] and in the study of the site of cellulose synthesis and microfibril assembly [29]. Calcofluor has some specificity for mixed linkage β-glucans [30] and it has been shown to bind to cellulose and chitin fibrils [31].
17. The fluorochrome in aniline blue is thought to be specific for the β1–3 glucan, callose [22], though Smith and McCully [32] question the absolute specificity, suggesting it may also bind to other cell wall polysaccharides. They have shown that aniline blue specificity for callose is enhanced by prior treatment of the tissues with TBO (to block carboxylated polysaccharides) and the PAS procedure (to block polysaccharides with vicinal glycol groups).
18. The most common way to stain TEM sections is when they are mounted on grids [10]. The grid is floated section-down on a drop of stain in a petri dish

on a sheet of dental wax or Parafilm for the appropriate period of time. The stained grid is picked up with locking forceps and thoroughly washed by running drops of filtered distilled water over the sections for 30–60 s. Alternatively, the stained grid is dipped in filtered distilled water in a beaker (10–15 times in each of three fresh changes of distilled water). Air-dry and store in grid boxes. TEM stains contain heavy metals and as such are poisonous. Dispose of using appropriate safety procedures.

19. Uranyl acetate solution is photolabile (store foil wrapped bottles), radioactive and poisonous. It provides good contrast for structures rich in nucleic acids; some proteins, some cell walls.
20. A simple CO_2-free staining chamber is a petri dish containing a reservoir of NaOH solution [recommended by 10] or NaOH pellets that traps CO_2. Place 3–4 drops of lead citrate on the strips of fresh dental wax or Parafilm and quickly float a grid section-down on each drop of stain. Immediately place the cover on the chamber. Alternatively, we routinely use a glass chamber with an outlet and an inlet connected by a hose to a tank of nitrogen gas.
21. The PA-TCH-SP procedure, used to localize insoluble carbohydrates, is a modification of the PAS procedure. PA is used to selectively oxidize vicinal glycol or glycol-amino groups to aldehydes. The aldehydes are then condensed with thiocarbohydrazide (TCH) to produce thiocarbohydrazones, which are strong reducing agents [26]. Subsequent staining with silver proteinate (SP) results in the deposition of reduced silver over the reaction site. Compounds that are PA-TCH-SP positive or negative correspond to those described previously for the PAS reaction. The control most commonly used in this study was the substitution of water for periodic acid. Additional controls were the blockage of aldehydes with aqueous 0.1 % sodium borohydride and the omission of TCH or SP from the staining procedure [26].
22. The OsFeCN post-fixation procedure is used to stain the endoplasmic reticulum (ER) and nuclear envelope. The initial use of this procedure in plant ultrastructure was by Hepler [20] in a study of the distribution of ER in dividing cells of barley. The chemical basis of the staining reaction is unknown; however, White et al. [33] suggested that metal-containing proteins on membranes chelate the cyano-bridged osmium-iron complexes resulting in the deposition of these electron-opaque complexes on the membrane sites. The removal of calcium chloride from the fixatives or the addition of phosphate buffer, which would precipitate the calcium, abolishes the selective staining of the ER and nuclear envelope [20]. We have found that the OsFeCN procedure does not ubiquitously stain all ER. Frequently, in contiguous cells, with equal access to the stain, the ER cisternae of one cell were stained positively, while the ER cisternae of the adjacent cell connected via a common plasmodesma were unstained. There must be a difference in the chemical content of the stained and unstained ER at the time of fixation; however, until the mechanism of staining is understood, the value of the OsFeCN method will be to discern the distribution of ER in the cell and to enhance overall membrane contrast (Figs. 5.1, 5.2, 5.3).

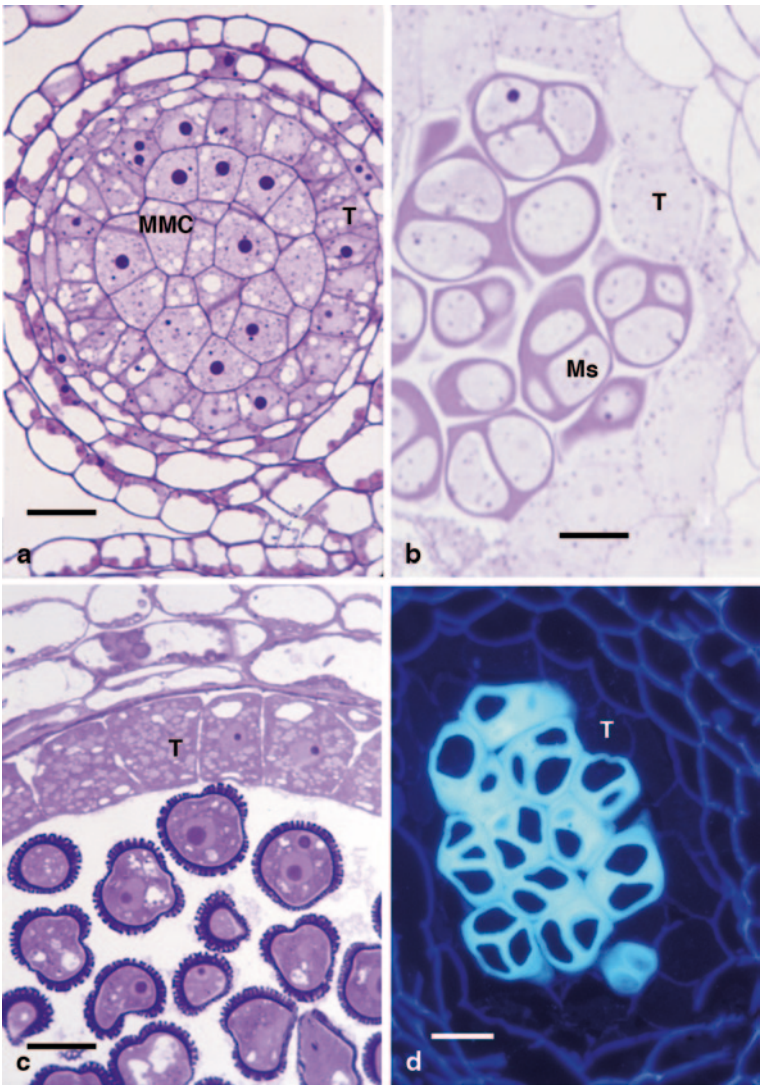

Fig. 5.1 Light and fluorescence micrographs of various stages of pollen development in Saskatoon. **a** Premeiotic anther showing microspore mother cells *(MMC)*, tapetum *(T)* and four outer anther wall layers. Stained with crystal violet. Scale bar = 14.5 µm. **b** and **d** Mature tetrads containing microspores *(Ms)* surrounded by tapetum *(T)*. **b** Stained with PAS for insoluble carbohydrates. Scale bar = 9.0 µm. **d** stained with aniline *blue* for callose and viewed with UV light. Scale bar = 4.5 µm. **c** Anther loculus containing two-celled pollen grains. Tapetum *(T)*. Stained with crystal violet. Scale bar = 15 µm

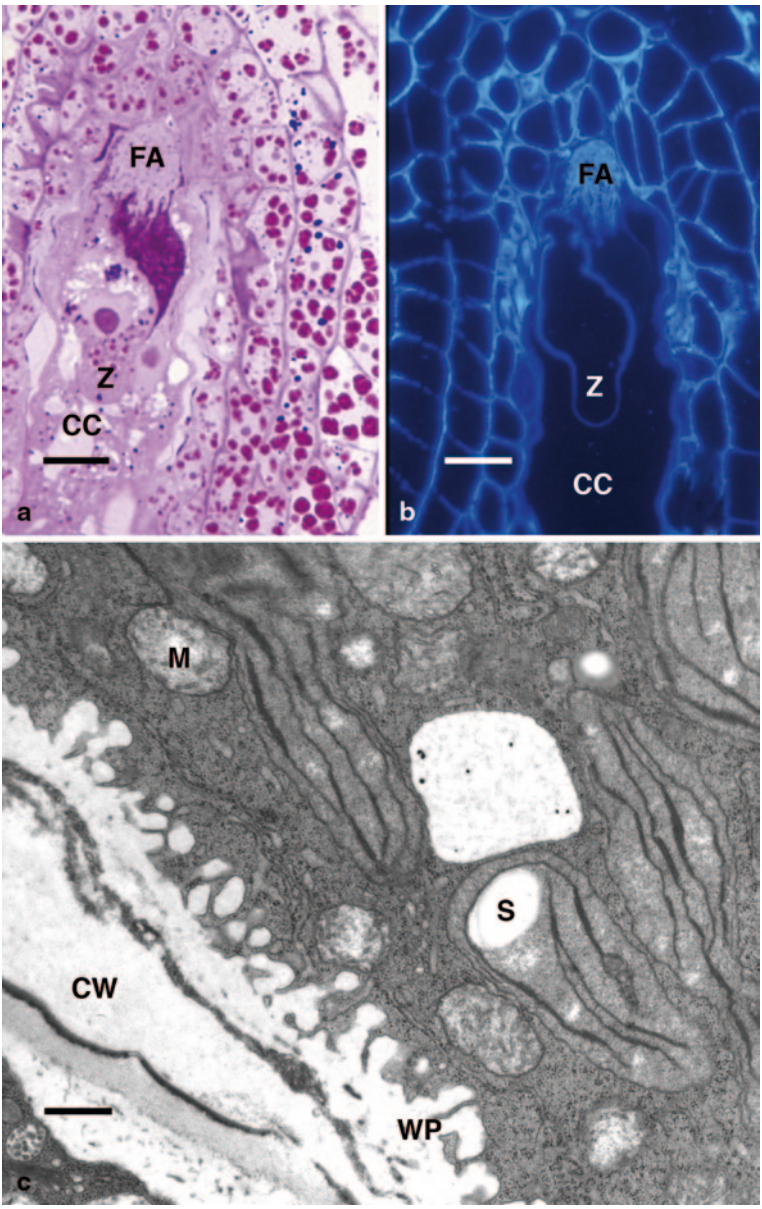

Fig. 5.2 Light, fluorescence, and TEM micrographs of a young seed of Canola. **a** The zygote *(Z)* is contiguous with the embryo sac central cell *(CC)* and a degenerate synergid, containing a PAS positive filiform apparatus *(FA)*. The surrounding integument tissue is rich in PAS positive starch. Stained with PAS and crystal violet. Scale bar = 10.5 μm. **b** Fluorescence micrograph of a serial section to **a**. The section was stained with Calcofluor and viewed with UV light. Beta 1,4 glucan cell wall material of the zygote *(Z)*, the degenerate synergid filaform apparatus *(FA)* and the integument tissue fluoresce a *pale blue*. Scale bar = 10.5 μm. **c** The lateral cell walls *(CW)* of the central cell adjacent to the egg apparatus, zygote and young embryo contain numerous electron-transparent cell wall projections *(WP)* and central cell chloroplast starch *(S)* and mitochondria *(M)*. Stained with uranyl acetate and lead citrate. Scale bar = 0.45 μm

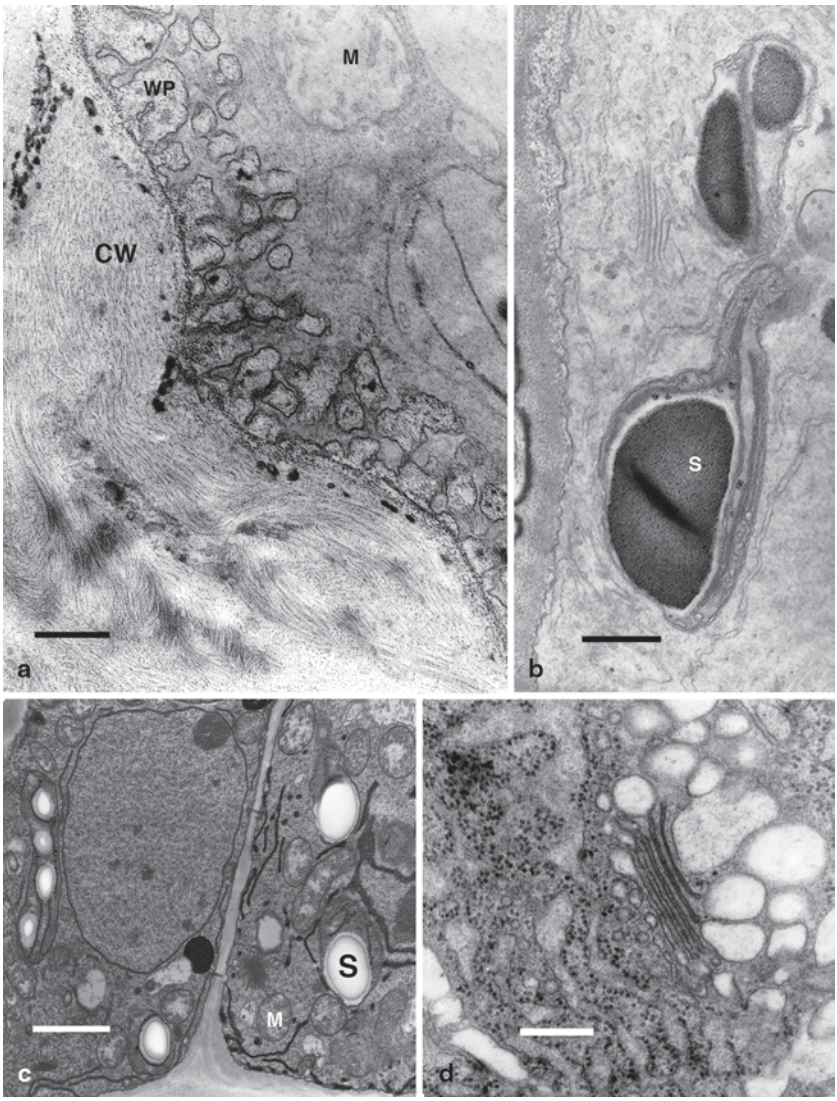

Fig. 5.3 TEM micrographs of a young seed of Canola. **a** There are deposits of electron-opaque silver over the central cell wall *(CW)* and wall projections *(WP)*. Mitochondrion *(M)*. Stained with PA-TCH-SP for insoluble carbohydrates. Scale bar = 0.4 µm. **b** There are deposits of electron-opaque silver over integument cell starch *(S)* and cell wall. Stained with PA-TCH-SP. Scale bar = 0.65 µm. **c** Two integument cells stained by OsFeCN resulting in electron-opaque deposits within the ER cisternae and enhanced staining of the nuclear envelope. Starch *(S)*, Mitochondrion *(M)*. Scale bar = 2.2 µm. **d** Median section through a synergid cell Golgi apparatus. Stained with uranyl acetate and lead citrate. Scale bar = 0.27 µm

5.5 Concluding Remarks

One does not become accomplished at the use of anatomical techniques overnight, and in particular, the many techniques related to light and electron microscopy. This chapter is a brief overview and a starting point for learning the basic techniques of light and electron microscopy. The techniques are challenging but doable if one follows the protocols carefully and accurately. There are no short cuts. A final comment from O'Brien and McCully [10] "There are three general working attitudes necessary to become a good microtomist: patience; organization; intolerance of dirt and grease. Knives, water, slides, grids, forceps and working surfaces are potential sources of contamination. Fingers are a source of grease and the atmosphere is a source of dust". The traditional use of TEM in plant anatomy has been to provide a high-resolution method of describing the cellular contents of cells. Improvements in fixation techniques, the use of immunocytochemistry and advances in all aspects of genetics, molecular and cell biology, biochemistry and physiology has provided a renaissance in the use of TEM to elucidate the structure and function of the living cell.

References

1. Glauert AM, Rogers GE, Glauert RH (1956) A new embedding medium for electron microscopy. Nature 178:803
2. Richardson KC, Jarett L, Finke EH (1960) Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol 35:313–323
3. Luft JH (1961) Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol 9:409–414
4. Hayat MA (2000) Principles and techniques of electron microscopy: biological applications, 4th edn. Cambridge University Press, Cambridge
5. Ellis AE (2014) No more Epon 812: this product does not exist today. Microscopy Today 22:50–53
6. Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43
7. Glauert AM, Lewis PR (1998) Biological specimen preparation for transmission electron microscopy. Princeton University Press, Princeton
8. Bozzola JJ, Russell LD (1999) Electron microscopy, 2nd edn. Jones and Bartlett, Sudbury
9. Russell SD, Preston L, Strout G (2014) Principles and techniques of transmission electron microscopy. Samuel Roberts Noble Microscopy Laboratory, University of Oklahoma online resource. http://www.ou.edu/research/electron/bmz5364/. Accessed 13 July 2015
10. O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Termarcarphi, Melbourne
11. Hayat MA (1986) Basic techniques for transmission electron microscopy. Academic, London
12. Chang RFE, Klomparens KL (eds) (1988) Artifacts in biological electron microscopy. Plenum, New York
13. Gephart P, Murray RGE, Costilow RN, Wood WA et al (1981) Manual of methods for general bacteriology (Amer Soc Microbiol). ASM Press, Washington, D.C.
14. Fisher DB (1968) Protein staining of ribboned epon sections for light microscopy. Histochemie 16:92–96

15. Fulcher RG, Wong SI (1980) Inside cereals—a fluorescence microchemical view. In: Inglett GE, Munch L (eds) Cereals for food and beverages—recent progress in cereal chemistry. Academic, New York, pp 1–26
16. Venable JH, Coggeshall R (1965) A simplified lead citrate stain for use in electron microscopy. J Cell Biol 25:407–408
17. Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212
18. Hanaichi T, Sato T, Hoshin M, Mizuno N (1986) A stable lead stain by modification of Sato's method. Proc XIth Int Cong Electron Microscopy. Kyoto, pp 2181–2182 (English corrected)
19. Thiery JP (1967) Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. J Microsc 6:987–1017
20. Hepler PK (1981) The structure of the endoplasmic reticulum revealed by osmium tetroxide-potassium ferricyanide staining. Eur J Cell Biol 26:102–110
21. Hayat MA (1981) Fixation for electron microscopy. Academic, New York
22. Jensen WA (1962) Botanical histochemistry: principles and practice. Freeman, San Francisco
23. Pappas PW (1971) The use of a chrome alum-gelatin (subbing) solution as a general adhesive for paraffin sections. Stain Technol 46:121–124
24. O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373
25. Hale AJ (1957) The histochemistry of polysaccharides. Int Rev Cytol 6:193–263
26. Roland JC (1978) General preparation and staining of thin sections. In: Hall JL (ed) Electron microscopy and cytochemistry of plant cells. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 1–62
27. Lane BP, Europa DL (1965) Differential staining of ultrathin sections of epon-embedded tissues for light microscopy. J Histochem Cytochem 13:579–582
28. Hughes J, McCully ME (1975) The use of an optical brightener in the study of plant structure. Stain Technol 50:319–329
29. Schnepf E, Deichgraber G, Herth W (1982) Development of cell wall appendages in *Acanthosphaera zachariasi* (Chlorococcales): kinetics, site of cellulose synthesis and microfibril assembly, and barb formation. Protoplasma 110:203–214
30. Wood PJ, Fulcher RG (1978) Interactions of some dyes with cereal beta glucans. Cereal Chem 55:952–966
31. Herth W, Schnepf E (1980) The fluorochrome, calcofluor white, binds oriented structural polysaccharide fibrils. Protoplasma 105:129–133
32. Smith MM, McCully ME (1978) A critical evaluation of the specificity of aniline blue induced fluorescence. Protoplasma 95:229–254
33. White DL, Mazurkeiwicz JE, Barrnett RJ (1979) A chemical mechanism for tissue staining by osmium tetroxide-ferrocyanide mixtures. J Histochem Cytochem 27:1084–1091