

Suggested Standard Operating Procedures (SOPs) for the Preparation of Electron Microscopy Samples for Toxicology/Pathology Studies in a GLP Environment

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ABSTRACT

We provide a set of Standard Operating Procedures (SOPs) for preparing samples for electron microscopic evaluation that allow storage of samples in the primary fixative for at least 17 years without noticeable degradation, do not compromise the ability to prepare the same samples for standard light microscopic evaluation, and provide tips for orientation of samples that may be necessary for evaluation. Guidelines for proper sample size, buffer composition, and fluid concentrations during processing are given. The impact of these procedures on specimen quality, ability to produce truly comparable samples for drug development studies, and ways to minimize time spent by technicians preparing these samples during necropsies is evaluated. Although many laboratories routinely employ most of these techniques, this compilation will facilitate the simultaneous light and electron microscopic preparation by the pathologist of comparable specimens that can be stored long-term at 4°C in McDowell's and Trump's 4F:1G fixative (4F:1G).

Keywords. Sample preparation; TEM; SEM; SOPs.

INTRODUCTION

Transmission electron microscopy (TEM) is a valuable tool for characterizing a variety of subcellular changes in tissues exposed to potential therapeutic pharmaceuticals under development. Peroxisomal proliferation or increased quantities of smooth endoplasmic reticulum in liver in response to exposure to various compounds is readily confirmed by TEM. Corneal and retinal tissues are often examined for evidence of potential compound-related changes. In studies where effects on pulmonary function are observed, mineralization, phospholipidosis, and other morphologic alterations in pulmonary cellular populations can be demonstrated. Changes in reproductive tissues such as improperly developed spermatozoa can be documented with TEM. Studies on antiviral drugs often include examination of cellular populations for the presence of viral particles. Development of antiviral nucleoside analogs typically requires analysis of mitochondrial populations in heart, skeletal muscle, and liver for changes similar to those reported with exposure to fialuridine (10) and zidovudine (9).

Scanning electron microscopy (SEM) is used less often in pharmaceutical development studies but has proved valuable with implanted medical devices. A number of SEM studies have been conducted examining host responses to intraocular devices, indwelling catheters, artificial vascular grafts, and novel suture materials. Bacteria associated with these materials have also been examined with SEM.

Frequently, companies outsource the relatively small number of electron microscopy (EM) studies associated with drug development to contract toxicology and pathology laboratories specializing in EM. Some of these companies conduct their animal studies in-house and send the tissues collected at necropsy to contract pathology laboratories for further preparation, yet other sponsors have their animal studies conducted entirely by a contract laboratory. To achieve the most meaningful results from ultrastructural evaluation of tissues, initial sample collection and fixation procedures must be optimally performed. Because the laboratory performing the in-life study may lack staff with expertise in the area of EM, sample collection and preparation may be less than optimal. The purpose of this paper is to present some guidelines for sample collection and preparation to ensure successful ultrastructural examination of tissues and cells in a GLP-compliant environment.

A set of Standard Operating Procedures (SOPs) for EM ideally will produce excellent EM samples while not

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compromising the capacity to examine portions of the same samples by standard light microscopy. Unfortunately, some laboratories incorrectly think that light and electron microscopy samples require different fixation procedures. The SOPs should specify a suitable primary fixative, the optimal size of samples, the timeliness for collection at necropsy, the orientation landmarks needed for some tissues, and the number of pieces needed for standard EM studies. The SOPs should specify the tissue volume/fixative volume ratio for optimal fixation, the temperature of fixation, the proper storage of samples before processing, and the proper tonicity, pH, and chemical composition of buffering solutions to be used. They should provide guidelines for storage of tissues in the primary fixative and provide a schedule for complete processing of samples to resin embedment and polymerization, resulting in specimens suitable for producing semithin and ultrathin sections for examination by light microscopy and EM, respectively. A separate, slightly modified SOP for SEM samples is required.

MATERIALS AND METHODS

General SOP Guidelines for Electron Microscopy Samples

1. Primary Fixation of Samples

- a. McDowell's and Trump's 4F:1G (11) is recommended as a primary fixative, both for light and electron microscopic samples. This fixative was originally developed for kidney perfusions, so it is a good choice for organ and whole-body perfusions (eg, for peripheral nerves, reproductive tissues, CNS, kidneys, and lungs). Hayat (7) and Robards and Wilson (12) discuss specific perfusion techniques.
- b. Tissues should be removed from the animal as quickly as possible postmortem and immersed in the primary fixative during dissection into EM-sized pieces.
- c. The tissue samples for EM should be no more than 1-mm thick in at least 1 dimension but need not be minced into 1-mm³ pieces.
- d. The fixative fluid (and any other processing fluids) should be approximately 5–10× the volume of the sample.
- e. EM samples should not be taken from large pieces of previously fixed tissues farther than 1 mm from the surface.
- f. Sample surfaces should never be allowed to dry.

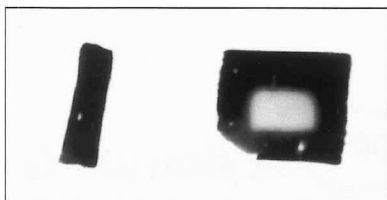


FIGURE 1.—Rat liver fixed in 4F:1G in 1997 and stored at 4°C until further processing in 2002. The narrow piece was trimmed to approximately 0.75-mm thick in 1 dimension before being osmicated, while the larger piece was trimmed to approximately 2 mm³ before being placed in osmium tetroxide. Note the light area in the center of the larger piece, indicating the area without significant osmium tetroxide penetration during the 1 hr osmication step at room temperature. ×8.5.

2. Trimming and Orientation of TEM Samples

Materials Needed:

- Paraffin-filled Petri dish
- New single-edge razor blades
- Applicator sticks with 1 end shaved down to a flat surface
- 4-dram specimen vials
- 4F:1G fixative
- Pasteur pipets and bulbs

Procedure:

Necropsy: During necropsy, quickly cut off a small piece of fresh tissue several mm in thickness and place it into a large droplet of fixative on the surface of the paraffin in a Petri dish. Slice the tissue sample with a razor blade into pieces 1 mm or less in thickness. Examine the list following and determine if a long dimension needs to be obvious for sample orientation. Prepare 4 pieces per tissue sample. Pick up the 1-mm-thin slices of tissue by touching them with the flattened end of an applicator stick, and then place them into 4-dram vials containing 1–2 ml of 4F:1G fixative.

- a. **Skeletal Muscle.** Cut long, thin superficial slices from muscles and hold them in clamps or carefully tie them to applicator sticks prior to primary fixation (3). Failure to have them held in this fashion will lead to



FIGURE 2.—Skeletal muscle from a found-dead woodchuck, formalin-fixed, osmicated, and embedded in Spurr's resin. Note the prominent swollen mitochondria (M) with minimal matrix content. ×12,600.

contraction during fixation. If sarcomere length is not a critical issue for the study, clamping prior to fixation is not necessary. Each sample should be flat-embedded, with 2 blocks oriented for longitudinal sections and 2 blocks oriented for transverse sections. If there is limited sample quantity, make the longitudinal blocks first, because they are generally the only ones examined.

- b. Heart Muscle. The wall of a heart can be oriented as described for skeletal muscle. Attaching the sample to pieces of applicator stick prior to primary fixation will prevent contraction. If a longitudinal strip of ventricle is processed and embedded so that longitudinal sections can be cut, the image is very similar to that of a longitudinal section of skeletal muscle. Orient 2 of the 4 pieces of tissue so that they can be cut for transverse sections.
- c. Neural Tissue.
 1. Peripheral Nerves. Orient long sections of tissue for transverse sections.
 2. Central Nervous System. Random orientation is usually adequate.
- d. Eyes. For best fixation of eye tissue, the eye is incised on 1 side, top to bottom, midway between the cornea

and optic nerve. The entire eye should then be quickly placed into a container with 10× more fixative than the eye volume. With large eyes (eg, dog and primate eyes), a slit can be made in the side of the globe, the entire eye submerged for approximately 5 minutes in fixative, and then the slit can be further opened with scissors and the vitreous gently removed to hasten fixation of the other ocular tissues. Specific areas of interest should be dissected out after primary fixation, but before further processing, since tissue blackening after osmication makes identification of specific areas in the eye difficult.

Embedding

- a. Samples Where Surfaces are Important (eg, skin, gastrointestinal tract, in situ cell culture): Flat-embed the samples with the surface either up or down in the mold. When trimming and sectioning, leave some empty embedding resin beyond the surface of the tissue so that sections will be less likely to fold over when exposed to the electron beam.
- b. Samples That are Generally Homogeneous (eg, liver, kidney cortex): These samples can be embedded either in BEEM capsules or flat molds, because orientation is not critical.

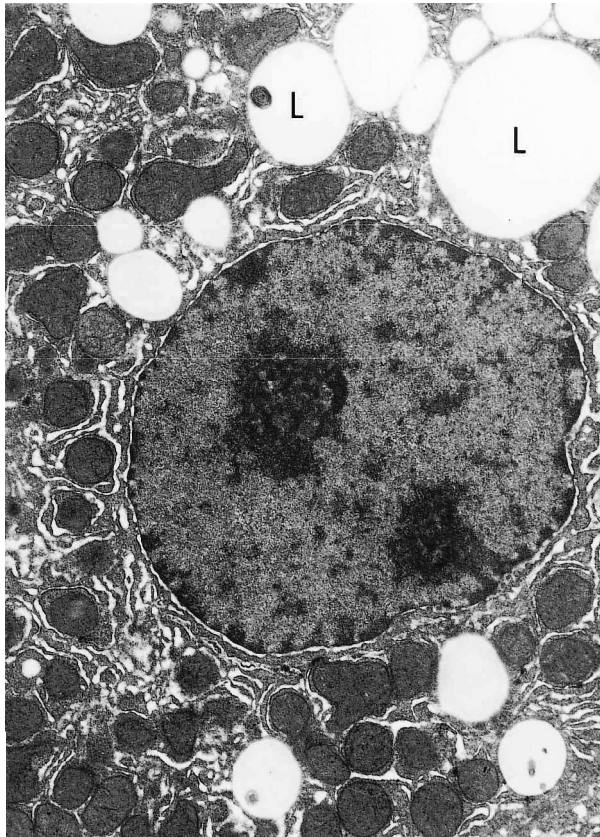


FIGURE 3.—Rat liver fixed in 4F:1G, osmicated, and embedded in Spurr's resin, illustrating slightly suboptimal fixation quality. Note the highly extracted lipids (L), seen as large vacuolar spaces, and the lacy appearance of the cytoplasm due to the swollen ER as well as the uneven spacing between the inner and outer nuclear envelope membranes. $\times 8,000$.

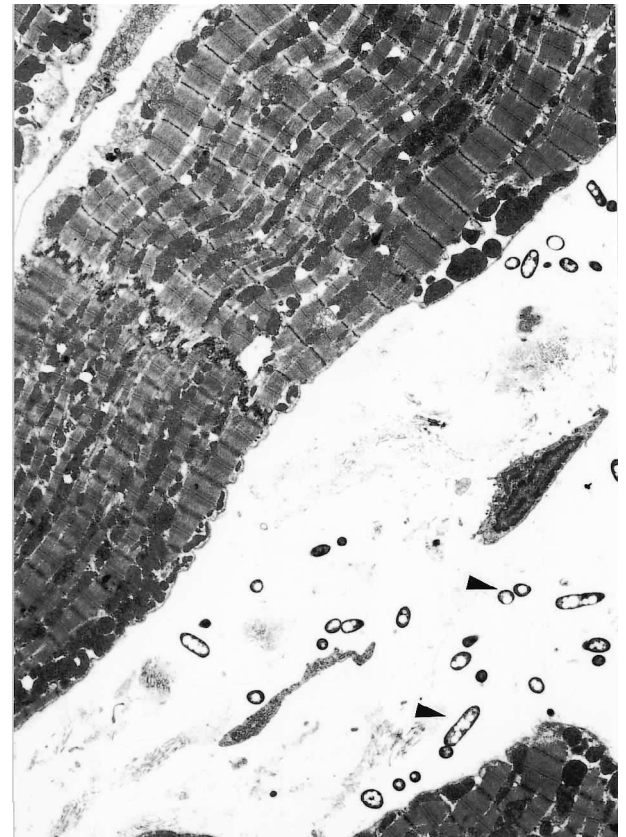


FIGURE 4.—Cynomolgus monkey cardiac muscle stored in buffer at 4°C for several weeks following primary fixation. Note the bacterial cells (arrowheads) that grew in the buffer storage solution and the lack of host response, indicating a postmortem invasion. $\times 3,136$.

Calcified Tissues

Samples That May Have Calcified Areas or Other Hard Substances (eg, growth plates, fish scales, marrow, platelets, samples collected from nature that may have dirt associated with them): Always cut semithin sections of these with a glass knife before attempting to cut ultrathin sections. If there is any evidence of hard material in the sample that could damage a knife edge, either trim that area away before cutting ultrathin sections with a diamond knife or cut sections only with a glass knife.

3. Buffers: For most samples, a 0.1 M phosphate buffer (or cacodylate buffer for cytochemical work employing charged molecules, or when working with marine samples) at the physiological pH for the tissue/cells is appropriate. If the exact physiological pH is not known, a pH of 7.2–7.4 is recommended.
4. Dehydration: A standard ethanolic dehydration series followed by passage through 100% acetone as outlined next is recommended for samples to be embedded in epoxide resins such as Spurr resin (14) or any of the Epon substitutes currently available. Propylene oxide is neither needed nor recommended, due to its toxicity (2). For acrylic resins, omit the acetone steps to avoid poor polymerization.

5. Processing Particulate Samples for TEM: If the sample consists of suspended cells (yeasts, bacteria, protozoans, etc) or small metazoans (eg, shrimp embryos), agar-embed them (3) after washing out the primary fixative with buffer. Do not centrifuge osmicated samples in molten agar, as they may be fractured by the shear forces produced.
6. Processing Particulate Samples for SEM: Put a suspension of the particulates onto a poly-L-lysine-coated coverslip (3) before standard SEM processing.
7. Sample Storage: If you must store samples after primary fixation, but before processing, store them in the primary fixative at approximately 4°C.
8. Embedding Molds: Use either BEEM-type capsules or silicone flat-embedding molds for epoxide embedment and BEEM-type capsules or gelatin capsules for acrylic resins. For epoxide resins, never cap the BEEM capsules. For acrylic resins, make sure the BEEM-type capsules or gelatin capsules are capped.
9. Choice of Embedding Media: Spurr epoxide resin is recommended for standard structural studies, while acrylic resins (eg, LR White, LR Gold, or the Lowicryl resins) are recommended for immunocytochemistry and general cytochemistry.

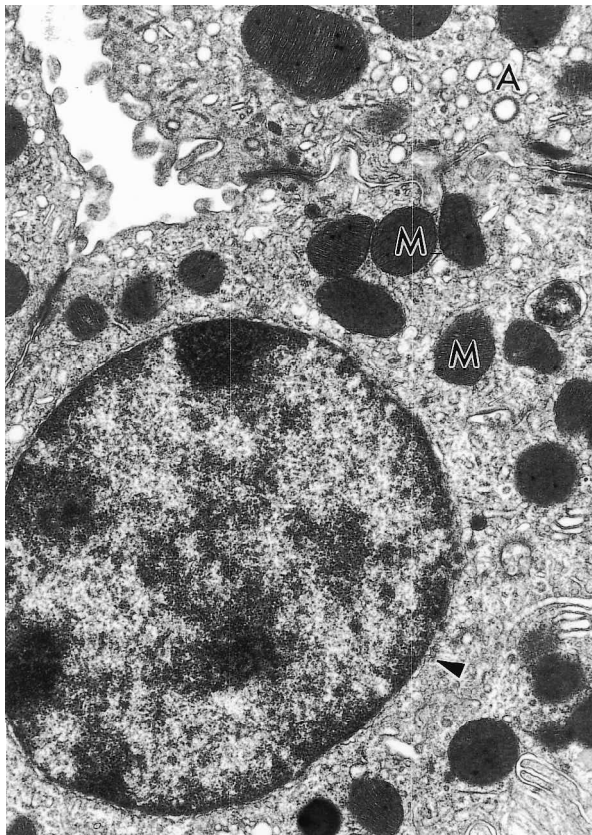


FIGURE 5.—Rat kidney distal convoluted tubule. Tissue fixed in 4F:1G and processed to block in Spurr's resin on the same day in 1985. Note the dense mitochondria (M), tight nuclear envelope (arrowhead), intact apical vesicles (A), and dense nucleoplasmic and cytoplasmic ground substance. $\times 15,000$.

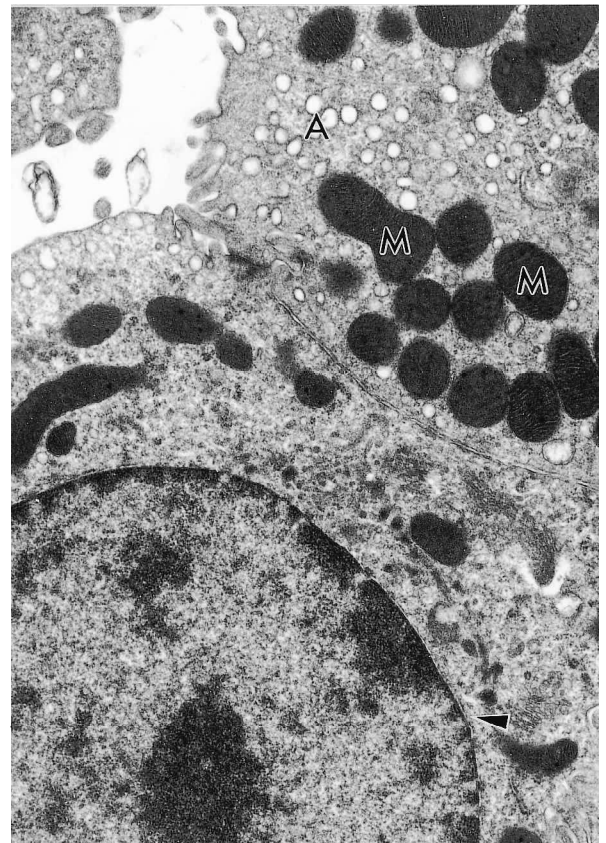


FIGURE 6.—Rat kidney distal convoluted tubule. The same tissue sample fixed in 1985 shown in Figure 4, except that the sample was stored in the fixative at 4°C until final processing in 2002. Note the dense mitochondria (M), tight nuclear envelope (arrowhead), intact apical vesicles (A), and dense nucleoplasmic and cytoplasmic ground substance. $\times 15,000$.

10. Labeling Blocks: Block labels can be prepared with a laser-jet printer. They should be predried in a polymerizing oven before use to avoid water contamination of the resin. All labels should be placed within the resins in the molds, so that the information cannot be separated from the sample.

RESULTS

Samples that are more than 1-mm thick or those taken further than 0.5–1 mm from the surface of samples stored in the primary fixative will not be well-penetrated by either aldehydes nor osmium tetroxide. The larger piece of tissue in Figure 1 was approximately 2 mm³ when originally exposed to 1% osmium tetroxide in Sorenson's phosphate buffer for 1 hour at room temperature. After dehydration to 75% ethanol, the block was cut down the middle with a razor blade, revealing the unosmicated (light) central region of the sample. The unosmicated area would show suboptimal fixation in the form of extracted nucleoplasmic and cytoplasmic ground substance (proteins), leading to high-contrast images and an apparent lack of nuclear and cytoplasmic content. Mitochondria may be swollen with decreased matrical content. Cytoplasmic membranes may be discontinuous, and the normal even spacing between adjacent membranes of the endoplas-

mic reticulum (ER) and nuclear envelopes may be replaced by uneven spacing and evidence of artifactual swelling. Animals found dead and fixed for evaluation often exhibit these artifacts (Figure 2). Suboptimal fixation, often due to slightly oversized samples (thicker than 1 mm), can produce the subtle changes seen in Figure 3, which shows large vacuolar spaces where lipid has been extracted. Perinuclear ER has somewhat wavy edges due to the fact that the normally parallel membranes bounding the ER lumen are irregularly swollen. In addition, the nuclear envelope membranes are also expanded in most places, rather than being closely appressed to each other as is found in well-fixed nuclear envelopes.

Samples stored in buffer solutions even at 4°C will frequently be invaded by fungi or bacteria, so storage in fixative is preferred. Postmortem invasion of a tissue sample is suggested when no host response to the microorganism is seen, as with the bacteria in Figure 4.

Long-term storage in McDowell's and Trump's 4F:1G (4F:1G) does not produce marked changes in tissue ultrastructure. A rat kidney fixed and embedded on the same day in 1985 shows dense nucleoplasmic and cytoplasmic ground substance, mitochondria with dense matrical contents, and no evidence of swelling of the ER or nuclear envelope membranes (Figure 5). The same tissue sample

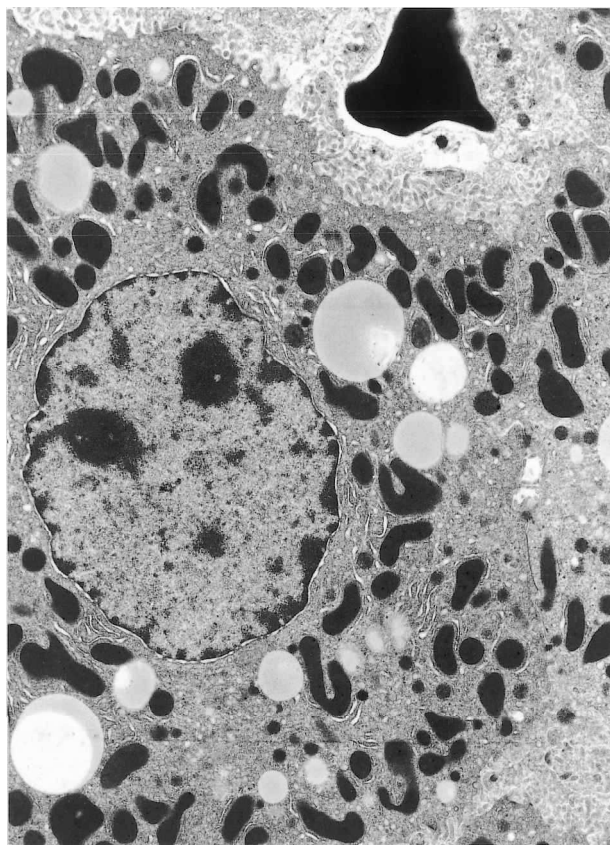


FIGURE 7.—Mouse liver fixed in 4F:1G in 1989 and stored in the fixative at 4°C until final processing in 2002. The hepatocyte has dense mitochondria, the lipid bodies are well-preserved, and the nucleoplasmic and cytoplasmic ground substance is well-preserved. Sinusoidal microvilli are evident near the single darkly-stained erythrocyte. $\times 5,760$.

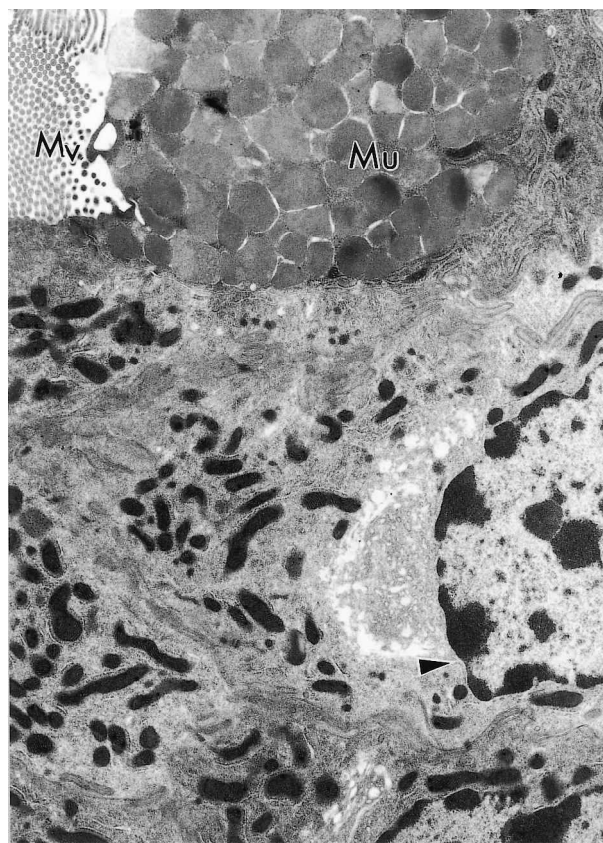


FIGURE 8.—Mouse small intestine fixed in 4F:1G in 1989 and stored in the fixative at 4°C until final processing in 2002. The mitochondria are dense, the mucous droplets (Mu) and luminal microvilli (Mv) are well-preserved, and the nuclear membrane is tight (arrowhead). Nucleoplasmic and cytoplasmic ground substance is well-preserved. $\times 7,200$.

stored in the original 4F:1G fixative at approximately 4°C until further processing in 2002 (Figure 6) is virtually indistinguishable from the sample fixed and processed in 1985 (Figure 5).

Mouse liver fixed in 4F:1G in 1989 and then stored in the fixative until further processing in 2002 is shown in Figure 7. The nuclear envelope and ER membranes show less evidence of the swelling seen in Figure 3, and the mitochondria show neither swelling nor diminished matrical content.

Mouse small intestine fixed in 1989 and stored at approximately 4°C until final processing in 2002 showed good preservation of mucous droplets and microvilli and no evident swelling of the ER and nuclear envelope. In addition, there was good nucleoplasmic and cytoplasmic ground substance (Figure 8). The quality of preservation of cytoplasmic structures, including mucous droplets, is sufficient to provide a sample suitable for evaluation of potential treatment-related changes.

Figure 9 shows a typical randomly oriented piece of ventricle muscle from a ground squirrel fixed with 4F:1G. The somewhat random orientation of sarcomeres would cause problems comparing samples from control and treated groups, while the longitudinally oriented rat ventricle tissue shown in Figure 10 would allow easier selection of comparable areas between samples for evaluation.

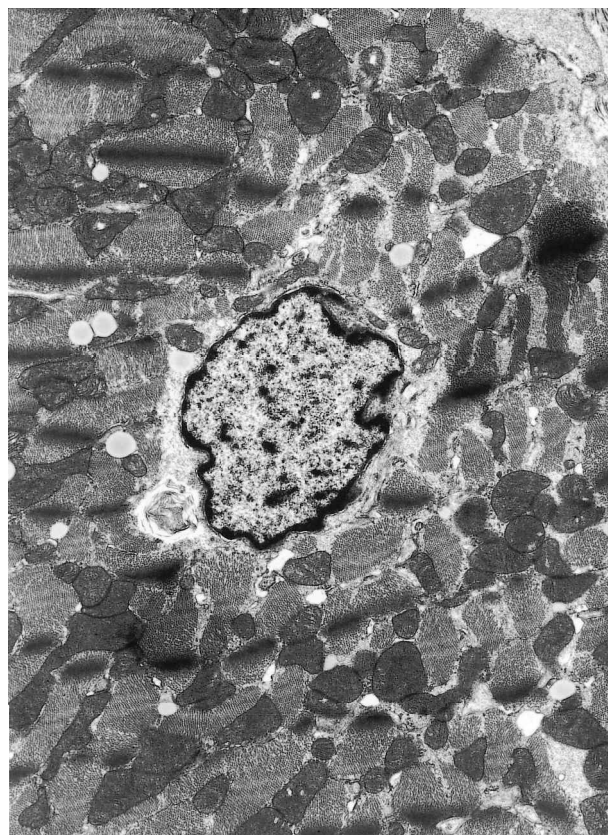


FIGURE 9.—Ground squirrel cardiac muscle fixed in 4F:1G and processed into Spurr's resin. The samples were diced into mm³ pieces that made orientation in the resin blocks random. Note that some of the sarcomeres are in near-longitudinal section, while others are nearly transverse. $\times 7,500$.

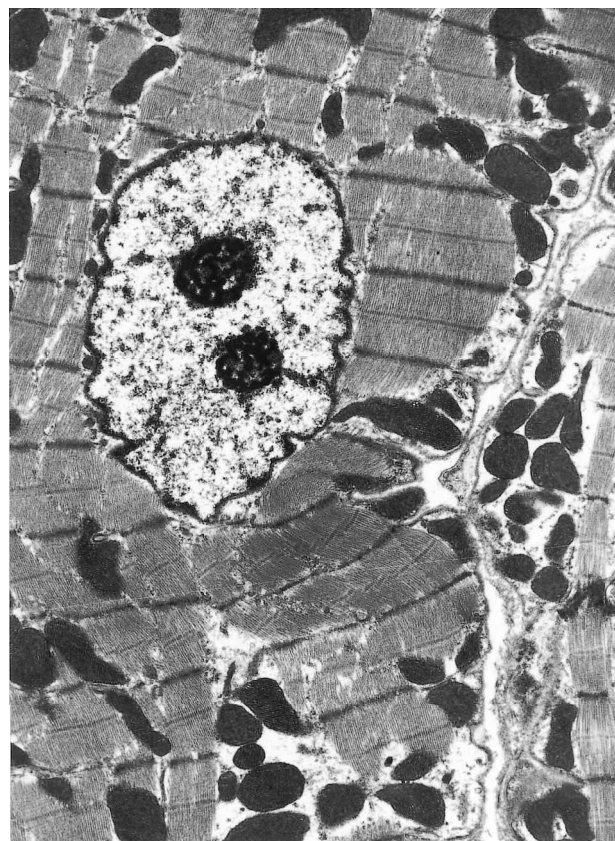


FIGURE 10.—Rat cardiac muscle cut into strips prior to primary fixation in 4F:1G so that longitudinal sections could be cut from the samples after embedding in Spurr's resin. Note that the sarcomeres are clearly defined in longitudinal section. $\times 7,500$.

Samples fixed with concentrations of glutaraldehyde that are 2% or higher exhibit nonspecific periodic acid-Schiff's reagent (PAS) reactivity and will appear uniformly pink following the staining procedure. In addition, these samples are often brittle when paraffin-embedded and produce suboptimal histological preparations. The blocked tissues typically exhibit chatter after sectioning and are generally difficult to section. Utilizing 4F:1G as the fixative of choice for samples that must be split between light and electron microscopic preparation is recommended, because the samples show good ultrastructural preservation, as illustrated in the examples already shown, and produce histological material that sections and stains well with typical histological stains such as H & E (Figure 11) and PAS (Figure 12). The brush borders of the proximal convoluted tubules are stained deeply by the PAS procedure compared to the other tubular details (Figure 12).

DISCUSSION

The suggestions for sample preparation for electron microscopy provided in this paper are meant to provide easier, more consistent sample collection and quality electron micrographs for the assessment of potential ultrastructural changes in drug development studies. Conditions surrounding primary fixation are the most critical for producing good

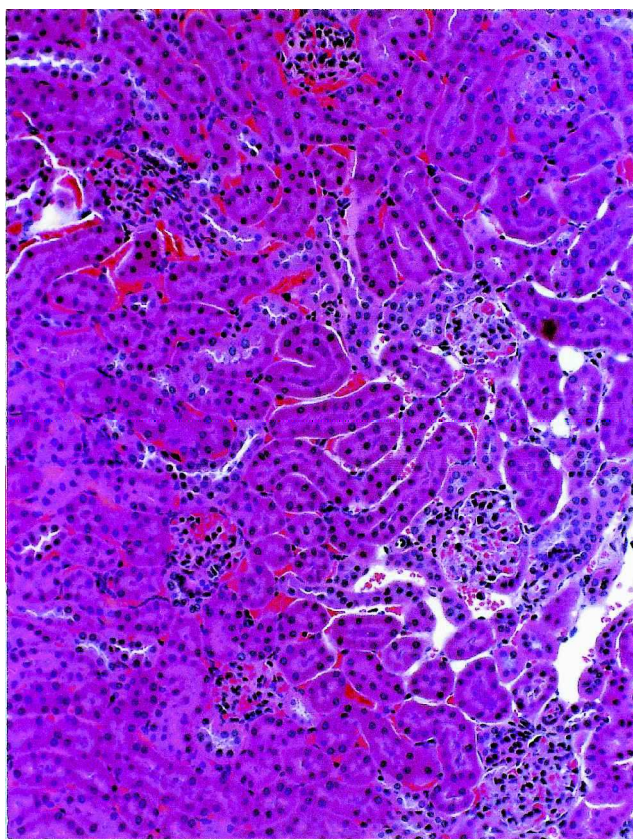


FIGURE 11.—Mouse kidney cortex fixed in 4F:1G and embedded in paraffin. Stained with H & E. $\times 190$.

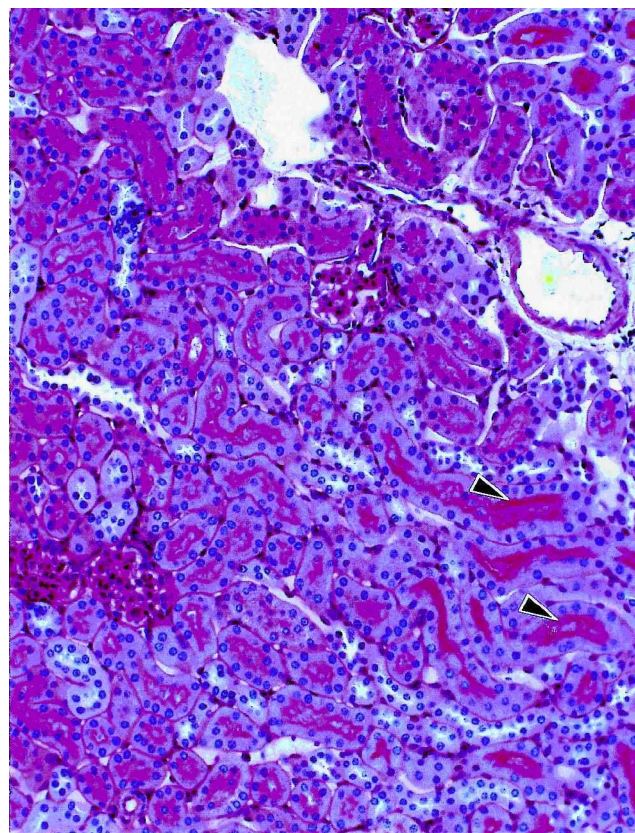


FIGURE 12.—The same paraffin-embedded block of mouse kidney cortex shown in Figure 10, stained with PAS. Note the prominent staining of the proximal convoluted tubule brush borders (arrowheads). $\times 190$.

samples. Rapid fixation of specimens in a 2-aldehyde fixative such as 4F:1G at room temperature produces excellent samples suitable for both histological techniques and electron microscopy, without compromising either approach. Most primary fixatives and the osmium tetroxide used as a secondary or postfixative rarely penetrate deeper into a tissue than 0.5 mm in 1 hour at room temperature. Autolysis of many tissues will be well underway within 1 hour, which reinforces the concept that samples should be no more than 1-mm thick in at least 1 dimension (2, 3, 4). At the same time, routinely cutting 1-mm³ pieces of minced tissue is often not advantageous because of the loss of orientation capability as described under the earlier section on necropsy procedures. Another critical issue is to assure that fixative volume and the volume of other fluids used during processing are 5–10 times the volume of the sample being prepared. Osmicated tissues are typically so dark that landmarks visible in unfixed tissue are no longer recognizable, so samples need to be taken in such a way that specific sites of interest can still be recognized after processing. This “landmarking” is often done by cutting wedges of tissue that are wider on the capsular surface of an organ and narrow toward the center. Preparation of samples that can be oriented for sectioning from comparable areas allows valid comparisons between tissues from control and treated animals. If samples are to be taken from previously fixed blocks of tissue, sampling from only the outermost 0.5–1 mm of the larger tissue piece is imperative so that less optimally fixed areas are avoided.

Rather than mincing tissue into 50 or 60 pieces that are approximately 1 mm³, we recommend that prosectors provide only 4 pieces of tissue for electron microscopy, because more than 2 or 3 blocks are rarely examined during the analytical stage of a microscopy study. Thus, technical staff time at necropsy devoted to preparing EM samples can be minimized.

Histopathologic evaluation of tissues prior to electron microscopic examination is often the most cost-effective procedure. A number of laboratories attempt to store fixed tissues in buffer solutions with or without sucrose, the sucrose intended to minimize osmotic changes. After aldehyde fixation, membranes are molecularly no longer osmotically responsive, so the sucrose is not needed. In addition, the addition of sugars can foster fungal growth, which can permeate the tissue and compromise the samples. As Figure 4 illustrates, storing the samples in buffer for extended periods of time at 4°C can also lead to bacterial growth and should be avoided.

Most general texts do not address the issue of prolonged storage of tissues in any medium (1, 4, 13). Hayat (8) says “The most desirable practice in the preparation of specimens for electron microscopy is to fix and embed them immediately after their collection.” He does not state what may happen to the specimens if this is not done, though he does say that storage of aldehyde-fixed samples in buffer “. . . is not recommended.” The potential morphological changes are not specified. In one of Hayat’s earliest texts (6), he discusses good results obtained by investigators who had stored tissues in

various aldehydes for several months or buffered formalin for up to 1 year. Chapter 5 in *Procedures in Electron Microscopy* (12) discusses tissue storage and repeats the conventional wisdom that "... best preservation of biological specimens is achieved when processing immediately follows primary fixation." They go on to point out that prolonged storage of perfusion-fixed liver, kidney, brain, and heart samples from rats fixed and stored at either 4° or 21°C for up to 12 months produced rare myelin-like whorls of membrane in all of the tissues, but most prominently in the brain tissue at 6 months of storage. By 12 months, the whorls were associated with "areas of lucency," suggesting lipid leaching. Myelin-like whorls were also seen in skin samples stored for up to 4 weeks in phosphate-buffered 2.5% glutaraldehyde. Gastrin cells stored in 4% glutaraldehyde for 24 hours resulted in a marked decrease in dense-cored granules in the cells.

Our results have demonstrated that mammalian tissues can be stored in 4F:1G for at least 17 years at 4°C without noticeable changes in ultrastructural integrity. Storage in fixative at 4°C minimizes the biological activity of lysosomal enzymes, thus decreasing eventual cytoplasmic and nucleoplasmic extraction during tissue processing. The added feature that this fixative does not make paraffin-embedded samples excessively brittle for histology technicians, as glutaraldehyde concentrations 2% or above tend to do, is a bonus. Finally, 4F:1G fixative does not compromise PAS staining of histological sections or cause problems with any of the other standard histological stains.

We recommend the use of Spurr's resin for all standard structural samples because it has low viscosity, allowing superior sample penetration, so that even tissues that are hard to embed such as skin or eyes will be assured a reasonable chance of producing workable specimens for the microtome.

If these protocols are followed, technician time spent at necropsy preparing these samples is minimized, well-fixed samples are collected, and critical orientation to allow analysis of truly comparable samples is assured.

REFERENCES

- Bozzola JJ, Russell LD (1999). *Electron Microscopy*. 2nd ed, Jones and Bartlett Publishers, Boston.
- Dykstra MJ (1992). *Biological Electron Microscopy*. Plenum Press, New York.
- Dykstra MJ (1993). *A Manual of Applied Techniques for Biological Electron Microscopy*. Plenum Press, New York.
- Flegler SL, Heckman JW Jr, Klomparens KL (1993). *Scanning and Transmission Electron Microscopy*. W.H. Freeman and Co, New York.
- Glauert AM (1975). *Fixation, Dehydration and Embedding of Biological Specimens*. North-Holland Publishing Company, New York.
- Hayat MA (1970). *Principles and Techniques of Electron Microscopy: Biological Applications*, Vol 1. Van Nostrand Reinhold Company, New York.
- Hayat MA (1981). *Fixation for Electron Microscopy*. Academic Press, New York.
- Hayat MA (2000). *Electron Microscopy: Biological Applications*. 4th ed, Cambridge University Press, New York.
- Lewis W, Papoian T, Gonzalez B, Louie H, Kelly DP, Payne RM, Grody WW (1991). Mitochondrial ultrastructural and molecular changes induced by Zidovudine in rat hearts. *Lab Invest* 65: 228-236.
- Lewis W, Griniuvienė B, Tankersley KO, Levine ES, Montione R, Engleman L, de Courten-Meyers G, Ascenzi MA, Hornbuckle WE, Gerin JL, Tennant BC (1997). Depletion of mitochondrial DNA, destruction

tion of mitochondria, and accumulation of lipid droplets result from Filaridine treatment in Woodchucks (*Marmota monax*). *Lab Invest* 76: 77-87.

- McDowell EM, Trump BF (1976). Histologic fixatives suitable for diagnostic light and electron microscopy. *Arch Pathol Lab Med* 100: 405-414.
- Robards AW, Wilson AJ (eds). (1993). *Procedures in Electron Microscopy*. Wiley, New York.
- Robinson DG, Ehlers U, Herken R, Herrmann B, Mayer F, Schürmann F-W (1987). *Methods of Preparation for Electron Microscopy*. Springer-Verlag, Berlin.
- Spurr AR (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* 26: 31-43.
- Wischnitzer S (1981). *Introduction to Electron Microscopy*. Pergamon Press Inc, New York.

APPENDIX

Specific Procedural Instructions for Processing Transmission Electron Microscopy (TEM) Samples to Block

1. Materials Needed

McDowell's and Trump's 4F:1G fixative (4F:1G) (11)

The following items are added sequentially, with stirring

86 ml distilled water
10 ml Fisher F-79 (37-40% formaldehyde), or equivalent
4 ml 25% biological-grade glutaraldehyde
1.16 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$
approximately 0.27 g NaOH (check pH after mixing and adjust to pH 7.2-7.4 with 1 N NaOH)

0.2 M and 0.1 M Sorenson's sodium phosphate buffer, pH 7.2-7.4

Mix 23 ml of stock solution A (27.6 g/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and 77 ml of stock solution B (28.4 g/L anhydrous Na_2HPO_4) to make 100 ml of 0.2 M buffer. To make 0.1 M buffer, mix equal volumes of the 0.2 M buffer and distilled water. Check the pH after formulation of the 0.2 M buffer and adjust with 1 N NaOH or 1 N HCl as needed.

2% aqueous osmium tetroxide
100% ethanol (to make the dilution series)
100% acetone (transitional solvent)
Spurr resin (6.3 g DER recipe)

Recipe: Add the following components, in order, to a clean 100 ml screw-cap Nalgene bottle. Cap the bottle and swirl the contents vigorously for approximately 30 seconds after all the components have been added. The resin may be used immediately, or Parafilm can be wrapped tightly around the cap and the resin stored for up to 2 months at approximately -20°C before use.

10 g Vinylcyclohexene dioxide (ERL 4206)
6.3 g DER 736
26 g Nonenyl succinic anhydride (NSA)
0.4 g Dimethylaminoethanol (DMAE)

2. Processing Schedule For TEM Samples (All of the times are approximate and represent minimum recommended times. The temperatures listed are also approximate.)

- a. Fix samples for 1–2 hours at room temperature. Samples may then be stored in the fixative at approximately 4°C for at least several years.
- b. Rinse tissue 2 times (15 minutes each) in 0.1 M Sorenson's sodium phosphate buffer at pH 7.2–7.4.
- c. Postfix sample in 1% osmium tetroxide/0.1 M phosphate buffer for 1 hour at room temperature (made by mixing equal volumes of 2% aqueous osmium tetroxide and 0.2 M Sorenson's sodium phosphate buffer, pH 7.2–7.4).
- d. Rinse tissue in distilled water 2 times (5 minutes each).
- e. Dehydrate sample by passing it through the following alcohol series (always using fluid volumes at least 5–10× greater than the sample volume):

50% ethanol	15 minutes
75% ethanol	15 minutes (If necessary, samples can be left overnight at 4°C)
95% ethanol	15 minutes, 2 times
100% ethanol	30 minutes, 2 times
100% acetone	10 minutes, 2 times

- f. Infiltrate with Spurr resin 6.3 recipe (2).

Spurr:100% acetone (1:1)	30 minutes
100% Spurr resin	60 minutes
100% Spurr resin	60 minutes
Fresh 100% Spurr resin; put in appropriate molds with labels	

- g. Polymerize in 68–70°C oven overnight to 3 days.

Specific Procedural Instructions for Processing Scanning Electron Microscopy (SEM) Samples

1. Materials Needed

McDowell's and Trump's 4F:1G fixative (11)
0.2 M and 0.1 M Sorenson's sodium phosphate buffer, pH 7.2–7.4 (see previous recipe)

100% ethanol for preparation of the dehydration series
2% aqueous osmium tetroxide solution (optional)

2. Processing Schedule for SEM Samples (Times and temperatures are all approximate, and the times listed represent the minimum recommended.)

- a. Prepare samples that can easily fit on the type of SEM stubs for the particular microscope that will be used to examine them. The samples must also be small enough to fit easily into the types of containers used with the critical point drying apparatus available in the laboratory. Samples should be less than 2–3-mm thick whenever practical to lessen chances of bulk charging. Fix samples in 4F:1G fixative for 1–2 hours at room temperature. As with TEM samples, specimens may be stored in 4F:1G at approximately 4°C for at least several years. For difficult samples expected to charge (generally, large samples or those with a lot of topography as with intestinal villi), postfixing for 1 hour at room temperature in 1% osmium tetroxide in phosphate buffer at pH 7.2–7.4 after primary fixation may be useful (see the previous TEM schedule).

- b. Rinse sample in distilled H₂O 2 times (5 minutes each).
- c. Dehydrate sample:

50% ethanol	15 minutes
75% ethanol	15 minutes
95% ethanol	15 minutes
100% ethanol	15 minutes (2 times)

- d. Critical point dry the sample, freeze-dry, or use the alternative drying process employing dimethylsilazane (3).

- e. Mount samples on SEM stubs and sputter-coat according to the sputter-coating unit instructions. A coating of approximately 20 nm of gold-palladium is sufficient for most conventional SEM studies, though high-resolution, field-emission, gun-equipped instruments require chromium coating to achieve proper resolution.