JOURNAL OF ELECTRON MICROSCOPY TECHNIQUE 6:35–41 (1987)

Steedman's Polyester Wax Embedment and De-Embedment for Combined Light and Scanning Electron Microscopy

JON L. NORENBURG AND J. MICHAEL BARRETT
Mount Desert Island Biological Laboratory, Salsbury Cove, Maine 04672 (J.L.N.) and Department of Anatomy, Medical College of Georgia, Augusta, Georgia 30912 (J.M.B.)

KEY WORDS Technique, SEM, Histology, Polyester wax

ABSTRACT Steedman's polyester wax mixture is a good, general-purpose histological embedding medium that is suitable and convenient to use when it is desirable to combine light microscopy with scanning electron microscopy (SEM). A range of properties recommend this wax: it has a low melting temperature (37°C), is readily soluble in most dehydrating agents, results in negligible tissue shrinkage, preserves tissue antigenicity, and may even be used as a solvent for fixative agents. We prepare and embed tissues in polyester for light microscopy much as they would be for paraffin wax. For SEM, the block surfaces is micro- or ultraplaned, utilizing, respectively, a standard rotary microtome with razor blade knives or an ultramicrotome with glass knives. The block is de-waxed in absolute alcohol and then taken to critical point drying. Similarly, sections mounted on coverslips or glass slides may be brought to the SEM after removing the wax. This enables one to bring to the SEM relatively large block faces or sections with good control over orientation. We find the results to be superior to similar procedures employing paraffin. We believe it to be more versatile and equivalent or superior to a variety of other techniques designed to gain access to the interior of tissues with SEM.

INTRODUCTION

Access for visualization of the internal structure of biological tissues with scanning electron microscopy (SEM) can be obtained through a variety of methods. Principally, they are variations on the themes of 1) fracturing (e.g., tearing, cutting, cryofractography, ultraplaning), 2) dissociation (e.g., enzymatic digestion, sonication), or 3) combinations of these (see reviews by Jones, 1982; Kuzirian and Leighton, 1983). Microtomy of embedded tissues allows one to survey tissue with light microscopy (LM) and/or transmission electron microscopy (TEM) prior to SEM. Hence, it is possible to view with SEM a surface that is planed and whose histotopographical orientation and position are evident, provided one can remove the embedding medium. The efficacy of de-embedding procedures is determined by the criteria of preservation of structure, sectioning characteristics, reliability of the de-embedment protocol, and general convenience. Various de-embedment procedures have been proposed to optimize one or more of these criteria, depending largely on the desired priority, e.g., orientation or correlative microscopy.

One can view with LM, TEM, and SEM those surfaces obtained within one micrometer of each other by utilizing epoxide resin that can be dissolved. Procedures for dissolving epoxide resins from biological samples were described by Mayor et al. (1961), Kaissling (1973), Ng et al. (1982), and others. Although we have obtained excellent results with sodium methoxide de-epoxification, we have not found the method sufficiently reliable to use routinely, as was noted previously by Geissinger (1976). Others (Humphreys and Henk, 1979; Humphreys et al., 1979; Kuzirian and Leighton, 1983) utilize oxygen plasma etching to good effect in removing epoxy at the surface of a specimen. Some workers, attempting to retain the desirable sectioning attributes of hard epoxides, employ other embedments, such as styrene (Arakawa et al., 1977), diethylene glycol distearate (DGD) (Capco et al., 1984), and polyethylene glycol (PEG) (Wolosewick, 1980, 1984; Kondo, 1984). Clearly, de-embedding

Received May 28, 1986; accepted October 17, 1986.

Address reprint requests to Dr. Jon L. Norenburg, Bay View Drive, Salsbury Cove, ME 04672.

from DGD or PEG has useful applications in TEM (Capco et al., 1984; Wolosewick, 1984; Kondo and Ushiki, 1985), but we have not found them to be convenient when extensive LM is desired prior to SEM. This is because of the poor sectioning quality of these embedments using routine histological (rotary) microtomes. Polyester wax brings the desirable characteristics of being soluble in general lab solvents, has a low melting point, and is readily sectioned for light microscopy using the standard rotary microtome.

Several investigators have tried the "obvious" approach of using paraffin wax to obtain specimen orientation or to combine LM and SEM (McDonald et al., 1967; Myklebust et al., 1975; Pattee et al., 1984). This report details a similar approach, using polyester wax (Steedman, 1957, 1960; Sidman et al., 1961), which we find to be quite suitable for de-embedding and whose properties we believe make it more desirable than paraffin for routine histology. We report the use of polyester wax in routine histological and histochemical studies with LM, which then may be combined with de-embedment for SEM at the investigator's convenience.

MATERIALS AND METHODS

We have examined with SEM various tissues fixed by a variety of protocols using polyester wax. The tissues described here were prepared in the following manner. Kidneys of Munich-Wistar and Wistar rats were fixed by retrograde abdominal aortic perfusion with 2.5% glutaraldehyde in 0.16 M sodium cacodylate buffer, pH 7.4 (Barrett et al., 1975). Each kidney was trimmed to the central core of tissue (Schmidt-Nielsen et al., 1985). The core was cut into three pieces: 1) an outer, cortex block, 2) a middle piece bracketing the outer medulla, and 3) the remainder, most of the inner medulla, including the papilla. These pieces were rinsed multiple times in buffer, postfixed 1 hr in 2% osmium tetroxide in buffer, dehydrated in a graded series of ethanols, followed by infiltration and embedding in Steedman's polyester wax (see below).

The pedal aperture glands (PAG) of *Mya arenaria* L., the soft-shelled clam, were fixed in situ by cutting the adductor muscles, separating the valves, and immersing them in Hollande's cupri-picri-formal-acetic fixative or Helly's mercuric fixative (Humason, 1972). After a few minutes, the PAG of each valve was excised along with some surrounding tissue and placed in fresh fixative. The tissues were post-treated in osmium tetroxide, dehydrated through a graded series of ethanols, infiltrated, and embedded in polyester wax.

It is appropriate to describe in detail the general use of Steedman's polyester wax, as it is not widely known. Stock is prepared in lots of 200 or more grams, depending on projected need. We and others (Åkesson, 1966) have found that premixed lots formerly available from suppliers often were unreliable. The stock is made by melting 90 parts polyethylene glycol 400 distearate (not PEG 400!) at 60°C and then adding 10 parts 1-hexadecanol (cetyl alcohol), with occasional stirring until a uniform fluid is obtained (Polysciences, Warrington, PA and Aldrich Chemical Company, Milwaukee, WI stock both components). This is set aside to cool and solidify at room temperature, or some may be placed in an oven at 37–40°C for immediate use. From the stock mixture, the amount needed for that working day is removed and melted at 37–40°C. Working stock should not be used after more than 24 hr in the oven and should not be melted more than once after it first solidifies; repeated melting appears to adversely affect sectioning properties.

In general, tissue is dehydrated to 95% ethanol (Table 1). The final dehydrating fluid is mixed 1:1 with wax. Infiltration is, as with paraffin, dependent on the size and type of tissue. Åkesson (1966) offers some valuable tips for dealing with orientation of small structures and recommends staining such specimens before infiltration. A few drops of a saturated solution of acid fuchsin in the final dehydrating fluid is convenient. The final wax for embedding should be freshly melted and, if necessary, filtered (in the oven). The low melting temperature allows ample time for orientation of specimens. The block is allowed to solidify at room temperature. Blocks should harden for several hours before handling. We mount the blocks on wooden stubs and then do the final trimming. We also embed small tissue pieces in Beem capsules. The cap and tapered tip of the capsule are cut off, the tissue is placed in the cap with a small amount of wax, and the remaining portion of the capsule is inserted into the cap and filled with wax. This enables one to utilize standard holders for this shape of block, which may then be clamped in the vice of a standard rotary microtome or an

TABLE I. Sample schedule for embedding and de-embedding with polyester wax

Dehydrate	Ethanol: 30%, 50%, 70%, 85%, ½ to 1 hr each; 95% or 100%, 3 × 15 min
Infiltrate	1:1 Mixture of final solvent and wax, 30 min with occasional agitation; pure wax, 3 × ½ to 1 hr; pure wax, 1–12 hr
Embed	Pure wax, freshly melted and filtered. Allow blocks to cool at room temperature 24 hr
Section	3–4 μm
De-embed	100% ethanol, 3 × 5 min for sections; 3 × 15 min @ 37°C for blocks
CP Dry	CO_2

ultramicrotome. The first enables us to obtain sections 2–20-μm thick and a microplaned block face with a steel blade or razor blades. The block face subsequently may be ultraplaned on an ultramicrotome with glass knives, but sections cannot be obtained this way (presumably the heat of friction is not conducted rapidly enough). Sections may be cut in a few hours, but cutting properties improve significantly after 2 or 3 days (Steedman, 1960; Åkesson, 1966).

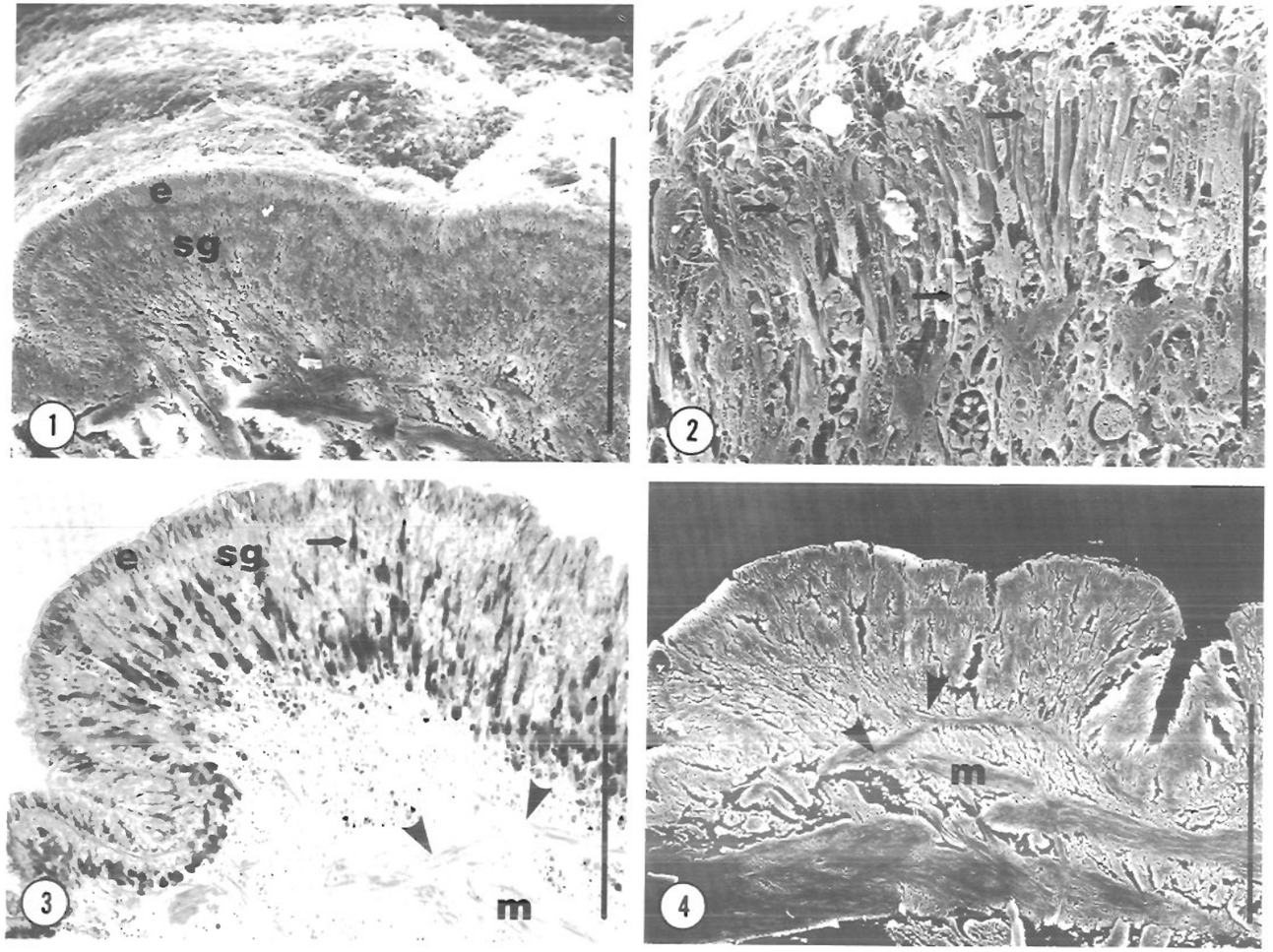
Ribbons of sections are placed on slides coated with albumen. We use 5 to 10% formalin for floating sections. The slide is tipped on end on a paper towel, and the formalin is dripped so that it runs underneath the ribbons, briefly lifting them. This results in the sections expanding slightly and flattening smoothly on the albumen. To obtain proper bonding to the albumen, slides are air-dried for about 24 hr (a desiccator may be necessary in high humidity). No heat, including that of direct sunlight, should be applied to the sections. Sections for viewing with SEM may be placed on coverslips and processed similarly. Sections, or the remaining block of tissue, are de-waxed in 3 or 4 changes of absolute alcohol and critical-point dried in CO_2. We then mount the coverslip or tissue block on stubs with silver paint and sputter-coat them lightly with silver. We examined them with an AMR 1000A SEM operated at 20 kV. If one wishes to process alternate sets of 2 or more sections for LM and SEM, a closer correspondence between sets may be obtained by placing every other set upside-down on its coverslip. If correlation between last section and block face is not critical, SEM resolution of cytological detail of the block face may be improved by ultraplaning the embedded block face with a glass knife.

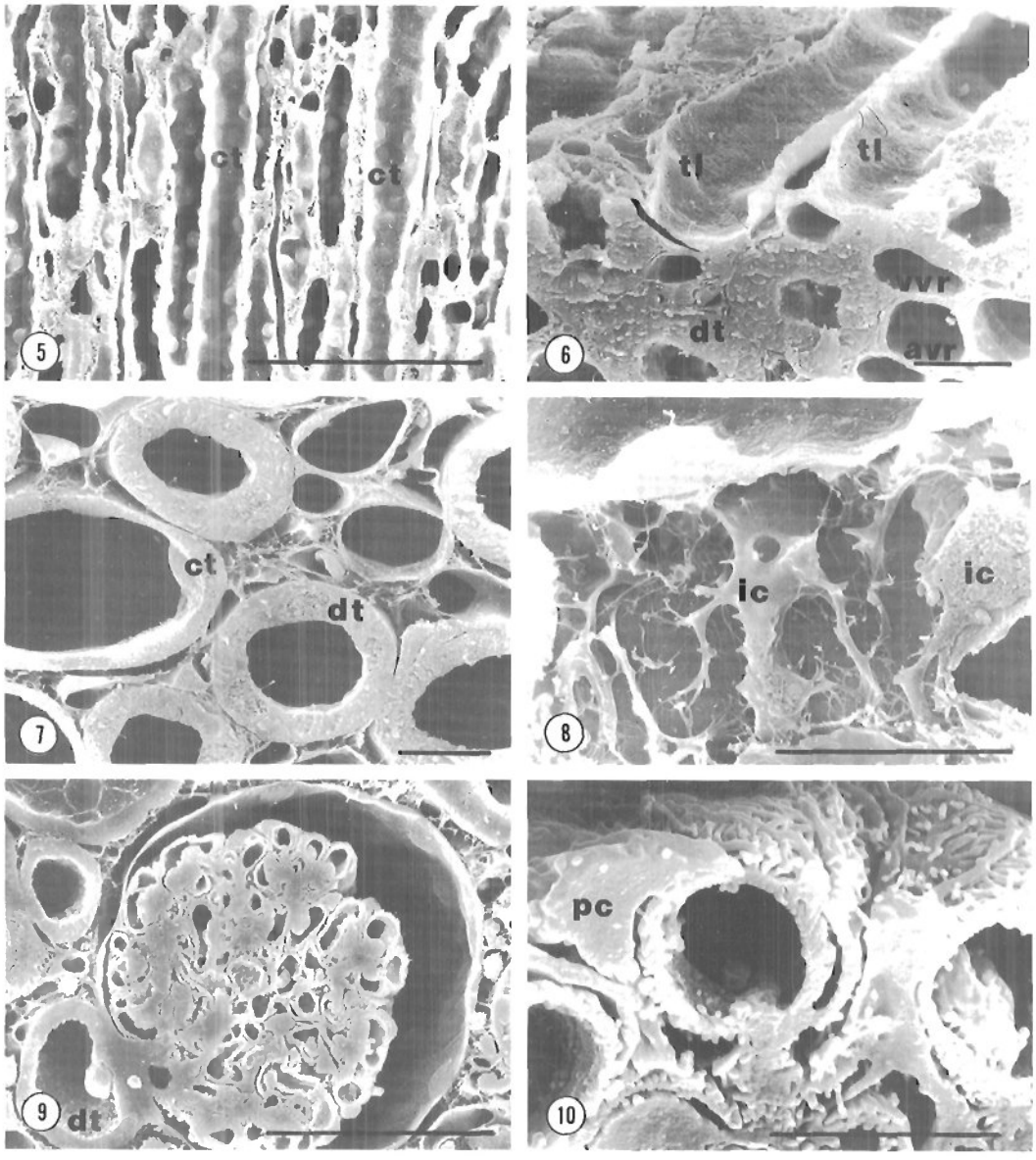
Some of the kidney tissue was de-embedded in absolute ethanol, followed by two 15-minute changes of propylene oxide, and then infiltrated and embedded in Poly/Bed 812 (Polysciences). Silver to gold sections of this tissue were cut on a diamond knife with a Sorvall MT-5000 ultramicrotome. The sections were collected on copper mesh grids, stained with uranium and bismuth (Barrett et al., 1975), and examined with a Philips 400 TEM.

RESULTS AND DISCUSSION

Polyester wax has a melting temperature of 37°C, is miscible with ethanol, methanol, acetone, 2,2-dimethoxypropane (and other common laboratory solvents), and can tolerate up to 5% water in these without noticeable effects. It sections readily in the same range as paraffin, i.e., 4–12 μm. Sections less than 4-μm thick require cooling and special effort. Absolute alcohol need not be anhydrous to remove the wax, and tissue may be stained with any of the protocols used in paraffin work.

In its simplest form we use the de-embedding protocol to examine the micro- or ultraplaned surface of the tissue block remaining after sectioning for LM (Figs. 1, 2, 9, 10). It also is possible to examine sections with SEM that have already been examined with LM (Figs. 3, 4, 7). In complex structures, such as the renal medulla, we use this method to obtain optimal orientation of a relatively large block surface (Norenburg et al., 1985), thus maximizing the availability of luminal surfaces of the nephron segments (Fig. 5). In this way we are able to obtain long views of these segments and know precisely our histotopographic position relative to the medullary zones (Barrett et al., in preparation). It


e
sg
1
2
e
sg
m
3
m
4



Figs. 1–4. Pedal aperture gland of *Mya arenaria*. 1. SEM of de-embedded block with microplaned surface shown in transverse section. Epidermis (e), subepidermal gland zone (sg). Bar = 500 μm. 2. Enlargement of epidermal zone in Figure 1, showing ciliated cells and necks of subepidermal glandular cells containing secretory packets (arrows). Bar = 50 μm. 3 and 4. Section (3-μm thick) from block shown in Figure 1. Section mounted on a coverslip, stained with iron hematoxylin, then mounted on slide with Permount, viewed and photographed with LM (Fig. 3). The coverslip was then released in toluene, processed for and observed with SEM (Fig. 4). Arrowheads mark points of identity in muscles (m). Epidermis (e), subepidermal gland cells (sg and arrows). Bar = 500 μm.

Figs. 5–10. SEM of Munich-Wistar rat kidney. 5. Upper portion of inner medulla cut along its long axis. Collecting tubule (ct). Bar = 100 μm. 6. Outer strip of outer medulla sectioned along long axis of tubules and then sectioned transversely. Note part of vascular bundle at lower right. Descending long thin limbs (tl), distal tubule (dt), arterial vasa recta (avr), venous vasa recta (vvr). Bar = 10 μm. 7. Transverse section, 7-μm thick, of tubules of outer stripe. Collecting tubule (ct), distal tubule (dt). Bar = 10 μm. 8. Portions of stellate interstitial cells in the inner medulla with most of extracellular matrix extracted during storage in fixative. Interstitial cell (ic). Bar = 10 μm. 9. De-embedded block of cortex with surface ultraplaned with glass knives, showing glomerulus. Distal tubule (dt). Bar = 50 μm. 10. Enlargement from glomerulus shown in Figure 9. Podocyte (pc). Bar = 5 μm.

is possible to determine position in relation to vascular bundles or other elements by also sectioning the block transversely (Fig. 6).

The SEM results obtained with polyester-embedded tissues are comparable to or better than those that we obtained with de-epoxified tissues, or that we have seen in the literature for tissues that have been cryofractured, or hand- or vibratome-cut tissues (Fig. 10). These results are clearly superior to those demonstrated with paraffin de-embedding. They are comparable to the results reported for styrene, PEG, or DGD procedures, but the ease with which the tissues are de-embedded make the polyester wax more convenient to use. Each of the other embedment media has value in specific instances, but polyester wax has been more versatile in correlative LM/SEM applications. Johannessen (1977) and others (van den Bergh Weerman and Dingemans, 1984) de-embedded paraffin tissues and re-embedded them in epoxide resin for subsequent TEM of thin sections suitable for pathological study. We examined thin sections with TEM of kidney which had been stored embedded in polyester wax for eight months and then re-embedded in epoxide resin. General histo- and cytotopography appear to be adequately preserved when compared with tissue embedded directly in epoxide resin. However, at high magnifications, membranes and fibrous components were indistinct and granular in appearance, as if proteins had fragmented. Although it may yet be possible to improve the ultrastructural appearance of polyester wax-embedded tissues for TEM, we consider the wax to be more than adequate for topographic work with SEM. Fixation probably is the greatest variable in tissue appearance, even with SEM (Russell and Daghlian, 1985). For instance, there was significant extraction of the abundant extracellular matrix in the renal medulla (Fig. 8) during prolonged storage in primary fixative; this can be desirable when attempting to study interstitial tissue components.

Mammalian kidney, because of the difficulty in preparing it for histological and cytological study, also was selected as a test organ by Baker (1958) and Steedman (1960) for evaluating fixatives and embedding media. We have used this wax for 15 years in routine histology of soft-bodied invertebrates, which present similar difficulties. We concur with Åkesson (1966) that "a good polyester wax mixture prepared according to Steedman's formula is superior to paraffin wax for all objects where shrinkage is a major problem." Furthermore, Kusakabe et al. (1984) found that tissue antigenicity in polyester wax is preserved for at least six months. Bancroft and Stevens (1977) describe two methods whereby fixation and infiltration can be achieved simultaneously by taking advantage of the solvent properties of this wax. Hence, there are compelling arguments for using polyester wax in routine and specialized applications.

ACKNOWLEDGMENTS

We are grateful to Professor Nathan W. Riser of Northeastern University for introducing us to polyester wax and its use in histology. We thank Mr. Hal Church for photographic assistance. This work was supported in part by NIH grant 2R01 AM15972-13 to Dr. Bodil Schmidt-Nielsen at Mount Desert Island Biological Laboratory and was Contribution no. 147 from the Marine Science and Maritime Studies Center, Northeastern University, Nahant, MA.

REFERENCES

Åkesson, B. (1966) Orientation and embedding of small objects in Steedman's polyester wax for sectioning in definite planes. Arkiv Zoologi 13:247–249.

Arakawa, M., Edanaga, M., and Tokunaga, J. (1977) The application of scanning electron microscopy to the histological diagnosis of renal biopsy specimens. Scanning Electron Microsc. II:171–177.

Baker, J. (1958) Principles of Biological Microtechnique. London: Methuen & Co. Ltd., London.

Bancroft, J., and Stevens, A. (1977) Theory and Practice of Histological Techniques. New York: Churchill Livingstone.

Barrett, J., Heidger, P., and Kennedy, S. (1975) The use of bismuth subnitrate as a stain for electron microscopy. J. Histochem. Cytochem. 23:780–783.

Barrett, J., Norenburg, J., Ator, A., and Schmidt-Nielsen, B. (198) Morphometry and fine-structure of the descending thin limb of long loops of Henle relative to reduced protein in the diet of Munich-Wistar rats, in preparation.

Capco, D., Krochmalnic, G., and Penman, S. (1984) A new method of preparing embedment-free sections for transmission electron microscopy: Applications to the cytoskeletal framework and other three-dimensional networks. J. Cell Biol. 98:1878-1885.

Geissinger, H. (1976) Intermicroscopic (LM, SEM, TEM) correlation. In M.A. Hayat (ed): Principles and Techniques of Scanning Electron Microscopy, Vol. 5. New York: Van Nostrand Reinhold, pp. 94–121.

Humason, G. (1972) Animal Tissue Techniques, 3rd ed. San Francisco: W.H. Freeman.

Humphreys, W., and Henk, W. (1979) Ultrastructure of cell organelles by scanning electron microscopy of thick sections surface-etched by an oxygen plasma. J. Microsc. 116:255–264.

Humphreys, W., Henk, W., and Chandler, D. (1979) Scanning electron microscopy of biological specimens sur-

face-etched by an oxygen plasma. Scanning Electron Microsc. II:345–354.
Johannessen, J. (1977) Use of paraffin material for electron microscopy. Pathol. Annu. 12:189–224.
Jones, D. (1982) Scanning electron microscopy of isolated dog renal tubules. Scanning Electron Microsc. II:805–813.
Kaissling, B. (1973) Histologische und histochemische Untersuchungen an semidünnen Schnitten. Gegenbaurs. morph. Jahrb. Leipzig 119:1–13.
Kondo, H. (1984) Polyethylene glycol (PEG) embedding and subsequent de-embedding as a method for the structural and immunocytochemical examination of biological specimens by electron microscopy. J. Electron Microsc. Tech. 1:227–241.
Kondo, H., and Ushiki, T. (1985) Polyethylene glycol (PEG) embedding and subsequent de-embedding as a method for the correlation of light microscopy, scanning electron microscopy, and transmission electron microscopy. J. Electror Microsc. Tech. 2:457–462.
Kusakabe, M., Sakakura, T., Nishizuka, Y., Sano, M., and Matsukage, A. (1984) Polyester wax embedding and sectioning technique for immunohistochemistry. Stain Technology 59:127–132.
Kuzirian, A., and Leighton, S. (1983) Oxygen plasma etching of entire block faces improves the resolution and usefulness of serial scanning electron microscopic images. Scanning Electron Microsc. IV:1877–1885.
Mayor, H., Hampton, J., and Rosario, B. (1961) A simple method for removing the resin from epoxy-embedded tissue. J. Biophys. Biochem. Cytol. 9:909–910.
McDonald, L., Pease, R., and Hayes, T. (1967) Scanning electron microscopy of sectioned tissue. Lab. Invest. 16:532–538.
Myklebust, R., Dalen, H., and Saetersdal, R. (1975) A comparative study in the transmission electron microscope of intracellular structures in sheep heart muscle cells. J. Microsc. 105:57–65.
Ng, W.-L., Ma, L., and So, K. (1982) Preparation of Epon-embedded renal tissue for scanning electron microscopy. J. Clin. Pathol. 35:1384–1387.
Norenburg, J., Barrett, J., and Schmidt-Nielsen, B. (1985) Steedman's polyester wax as a tool in correlated LM and SEM of mammalian kidney. Proc. Southeastern Electron Microsc. Soc. 8:11.
Pattee, H., Mohapatra, S., and Agnello, E. (1984) A technique for comparative light and scanning electron microscopy of the same deparaffinized microtomed section. J. Microsc. 137:111–114.
Russell, S., and Daghlian, C. (1985) Scanning electron microscopic observations on deembedded biological tissue sections: Comparison of different fixatives and embedding materials. J. Electron Microsc. Tech. 2:489–495.
Schmidt-Nielsen, B., Barrett, J., Graves, B., and Crossley, B. (1985) Physiological and morphological responses of the rat kidney to reduced dietary protein. Am. J. Physiol. 248:F31–F42.
Sidman, R.L., Mottla, P.A., and Feder, N. (1961) Improved polyester wax embedding for histology. Stain Tech. 36:279–284.
Steedman, H. (1957) Polyester wax. A new ribboning embedding medium for histology. Nature 179:1345..
Steedman, H. (1960) Section Cutting in Microscopy. Springfield, Illinois: Charles C. Thomas.
van den Bergh Weerman, M., and Dingemans, K. (1984) Rapid deparaffinization for electron microscopy. Ultrastruct. Pathol. 7:55–57.
Wolosewick, J. (1980) The application of polyethylene glycol (PEG) to electron microscopy. J. Cell Biol. 86:675–681.
Wolosewick, J. (1984) The "rediscovery" of polyethylene glycol, and its use as an embedding matrix. Proceedings of the 42nd Annual Meeting of the Electr. Microsc. Soc. Amer. 1984:28–31.