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HEAVY METAL STAINING OF PARAFFIN, EPOXY AND GLYCOL METHACRYLATE EMBEDDED BIOLOGICAL TISSUE FOR SCANNING ELECTRON MICROSCOPE HISTOLOGY

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Abstract

This paper describes three techniques for preparing biological tissue sections for correlation of light microscopy (LM) and scanning (SEM) and transmission (TEM) electron microscopy. Tissues were embedded in paraffin, epoxy (Epon-Araldite), and butoxyethanol-glycol methacrylate (BGMA). Paraffin sections were stained with silver. Epoxy embedded tissue was postfixed in OsO_4, mounted on carbon-coated nylon grids and stained with uranyl acetate and lead citrate. BGMA sections were stained with uranyl acetate, lead citrate, silver proteinate and phosphotungstic acid. Epoxy and BGMA sections on polished carbon planchets were stained by flooding the surface of the planchet. Paraffin and BGMA sections were histologically stained for LM. Sections were viewed in the SEM using transmitted (TSEM), backscattered (BSE) and secondary (SE) electron imaging. Summation of BSE and SE signals was also used.

SE imaging of deparaffinized sections shows tissue surface, while BSE imaging reveals deeper internal structure. Osmicated tissue shows good intracellular detail with SEM. BSE and SE images of BGMA and epoxy sections resemble LM and TEM images of alternate sections. TSEM images of epoxy sections provide good definition of thin extra-cellular fibrils. Large BGMA or many small epoxy sections can be stained and examined on a single planchet.

The following application is recommended for each technique. Paraffin embedding - for the best correlation of surface topography and internal structure. Epoxy embedding - for the best intracellular details and tissue patterns, without interference from grid bars. BGMA embedding - for the best correlation of LM, SEM and TEM.

KEY WORDS: Backscattered Electron Imaging, Paraffin, Epon, Araldite, Glycol Methacrylate, Biological Tissue, Heavy Metal Staining, Histology, Pathology, Scanning Electron Microscopy.

Introduction

Conventional light microscopy (LM) and transmission electron microscopy (TEM) are used extensively in biological research and clinical diagnosis. The images provided by these techniques are often difficult to correlate in morphological studies. The LM image (LMI) obtained from a thick (2,000 - 10,000 nm) section does not correlate well with a TEM image from a thin (60 - 100 nm) section. Examination of thick and semi-thick (500 - 2,000 nm) sections with TEM is possible using the high voltage transmission electron microscope (HVEM), but this does not in anyway facilitate correlation with LM. In fact, the HVEM image of thick and semi-thick sections presents additional problems of interpretation since the depth of field in TEM is always greater than the section thickness and effective resolution is decreased because of overlapping details. Scanning transmission electron microscopy (STEM) routinely uses semi-thick and thick sections, like HVEM, but resulting images, as in conventional TEM, are high magnification profiles of cellular and extracellular tissue constituents that lack histological perspective.

A potentially productive alternative to HVEM and STEM observation of sectioned material thicker than 100 nm is scanning electron microscopy (SEM) using backscattered electron imaging (BSI) and heavy metal staining. Resolution is less than HVEM and STEM, but the magnification and resolution obtained with backscattered electrons (BSE) are intermediate between LM and TEM allowing better tissue correlation with cellular structure. Previous investigations[1-3] with BSI and correlation with secondary electron imaging (SEI) show that paraffin-embedded tissues are useful for comparing surface structure of tissues with subsurface relationships. Here the BSI resembles an LMI. Recently, DeNee and Frederickson[4] have shown that epoxy-embedded tissues appear to add a further dimension to BSI, because better correlation can be made with TEM. Here the most useful section thickness is in the semi-thin (100 - 500 nm) range.

The purpose of this report is to introduce a new SEM technique for the study of biological

tissue in the range between the LM and TEM. This technique involves heavy metal staining for the SEM and chromatic staining for LM of 500 - 2,000 nm sections of butoxyethanol-glycol methacrylate (BGMA) embedded tissue. The technique appears to be one which will best correlate with LM, SEM and TEM. It overcomes some of the limitations fundamental to both LM and TEM. These include: (1) low resolution in LM, (2) a limited sample size and restricted view with the TEM, (3) the impracticality of attempting serial sections in TEM, (4) long preparation times for both LM and TEM, (5) distortion of the tissue resulting from heating during paraffin processing, (6) questionable ultrastructure of cells resulting from conventional fixatives for paraffin embedding, and (7) the difficulty in using histochemical staining for TEM.

Methods and Materials

Tissue Processing

In this study three different embedding media were used to prepare the tissue for sectioning, staining and ultimate observation with LM, SEM and TEM. These media were paraffin, epoxy and butoxyethanol-glycol methacrylate (BGMA). Heavy metal stains were used to obtain SEM and TEM contrast. They contained Pb, U, Ag, W and Os. Conventional chromatic stains were used for LM.

Paraffin embedding followed conventional procedures. Mouse lung was fixed in 10% formalin buffered to a pH of 7.0 with 0.2M phosphate buffer. The tissue was dehydrated routinely and embedded in paraffin. The technique for preparing paraffin sections for SEM has been described previously[1]. Sections were cut at about 5µm using a metal knife and were placed on glass slides, deparaffinized, rehydrated and stained with ammoniacal silver by Willard's modification of Wilder's reticulum stain[1]. After staining, the sections were dehydrated and critical point dried with CO_2. The sections were coated with approximately 20 nm of carbon before viewing in the SEM. Additional sections were chromatically stained for LM[5].

The tissue embedded in epoxy (Epon-Araldite)*[6] was chick embryo fixed in a combination of 1% glutaraldehyde and 1.25% formaldehyde[7], buffered to a pH of 7.4 - 7.6 with 0.2M sodium cacodylate. Postfixation was with 2% osmium tetroxide (OsO_4), also buffered with 0.2M sodium cacodylate. Following conventional embedding procedures sections were cut at thicknesses of 60, 100, 200, 400, 600 and 800 nm using either a Sorvall MT-2B or JB-4A microtome. They were then mounted on either 300 mesh carbon-coated nylon grids or polished carbon planchets[8] and were double stained with lead citrate[9] (20 min) and uranyl acetate[10] (60 min). The grids were stained by flotation and washed for 1 min by continuous flow with distilled water after each staining. The sections on carbon planchets were heated on a hot plate for 5 - 10 minutes to improve adhesion and then stained by flooding the upper surface of the planchet with stain solution. The solutions, times and washes were the same as for the grids. The sections were observed in the SEM without coating. A few of the 800 nm sections were placed on glass slides and were stained for LM, with toluidine blue.

Tissue embedded in BGMA (JB4 Embedding Kit)[11] was fixed in the same buffered aldehyde solution used with the epoxy technique. None of this tissue was postfixed in OsO_4. Tissues sampled were lung, kidney, gall bladder, ovary, placenta, heart and liver. The embedding follows established procedures (Polysciences)[11]: Sections were cut on a JB-4A microtome in the range of 500 - 2,000 nm (semi-thick), and alternate serial sections were placed on glass microscope slides and carbon planchets. The tissues on glass slides were stained with hematoxylin and eosin (H & E)[12] for LM and the tissues on carbon planchets for SEM were double stained following the procedure described for epoxies. Some sections were singly stained with uranyl acetate (60 min). Two special TEM stains used to demonstrate glycoprotein were also used on the BGMA sections. These stains contained the heavy metal compounds silver proteinate[13] and phosphotungstic acid[14].

Modes of Study

Both paraffin and BGMA sections stained with H & E were observed with the LM. BGMA and epoxy sections on carbon planchets were observed in the SEM using -BSE, -SE and +SE, [note that the negative (-) image is a contrast reversal from the positive (+) or normal image]. In addition, these tissues were observed by electrically summing the BSE and SE signals, Σ(-BSE, -SE). The paraffin sections were observed in +SE and -BSE. Epoxy sections on nylon grids were observed in -BSE, -SE, +SE, transmission scanning electron microscopy (TSEM), Σ(-BSE, -SE), and HVEM. (All sections were viewed at normal incidence unless noted.)

The sections were observed on an ETEC Autoscan SEM equipped with a special solid state BSE detector and a standard PMT TSEM detector. The special BSE detector is mounted just below the final lens and has 6 chips located in an annular fashion surrounding the beam. At a working distance of 10 mm the BSE electrons which have been scattered through an angle of 130° to 152° (145° to 160° for a 16 mm working distance) are detected. At a working distance of 10 mm the TSEM detector collects all transmitted electrons that are scattered through an angle of up to 11.3° (11.7° for 16 mm working distance). Normal working voltage for the SEM was 20 kV and

*Mention of specific brand names is for information only and does not imply endorsement by the National Institute for Occupational Safety and Health.

beam current ranged from 0.1 to 1.0 na. The HVEM used to observe the grids was a JEOL 1000B.

Results and Discussion

Paraffin embedding

The technique of paraffin embedding and heavy metal staining has been described previously.[1-3] SE imaging of thick (2-10μm), deparaffinized sections shows excellent surface topographic details as shown in Fig. 1a, while -BSE imaging reveals the heavy metal-(silver)-stained internal structures of cells such as nuclei and nucleoli, as shown in Fig. 1b. By comparing the two images the physical relationships of the internal and external structures can be seen. In addition, when alternate serial sections are histologically or histochemically stained for LM, structure and function can be correlated. To date, this method has only succeeded with glass as the substrate. It was attempted with a carbon planchet as a substrate, but portions of the sections came loose, apparently due to the porosity of the planchet. Work is continuing on this problem.

Certain other characteristics of paraffin embedding are worth noting. Differential heating of the tissue during the paraffin embedding process leads to some distortion. Since paraffin is the softest of the three embedding materials, the tissue must also be relatively "softer" than tissue embedded in plastic in order to be sectioned. Therefore, the tissue cannot be fixed with glutaraldehyde, a good fixative for ultrastructural preservation, because this fixative made the tissue too hard. Because of paraffin softness, trauma during sectioning creates distortion of the tissue. Also, the softness of the paraffin limits thinness of the sections and the relatively thick sections show cluttered, overlapping details, leading to a loss of resolution.

Epoxy Embedding

In this method the tissue was fixed in glutaraldehyde, which is compatible with fine structural studies, and embedded in a relatively hard material (epoxy). Heating occurs only for polymerization after the tissue is very firm, and sectioning is performed with an extremely sharp glass or diamond knife. Therefore, much of the distortion which occurs in paraffin sections is eliminated.

An important limitation of TSEM imaging is dramatically illustrated in Fig. 2, which is a low power comparison of the same section as viewed with TSEM and BSE imaging. In Fig. 2a, only about 25% of the tissue section can be seen in the TSEM image because of grid bars. However, the complete tissue section can be seen in the BSE image (Fib. 2b). (The latter allows unrestricted observation of total tissue continuity.)

Figs. 3a and 3b show higher magnification images of a similar section of tissue. It can be seen by comparing Figs. 3a and 3b, that cellular detail is more distinct in the BSI (Fig. 3b). Under the conditions of the experiment it was determined that TSEM was marginally adequate for sections of 60 to 100 nm and optimal at 200 nm. Above 200 nm, contrast and resolution deteriorated markedly (Fig. 3a). Conversely, the BSI lacks contrast for sections of 60 to 100 nm, but was sufficient for thicknesses of 200 nm and greater. Therefore, the optimum section thickness for using both TSEM and BSE imaging of epoxy sections was found to be 200 nm for the particular staining times used.

The epoxy sections can be viewed in both the

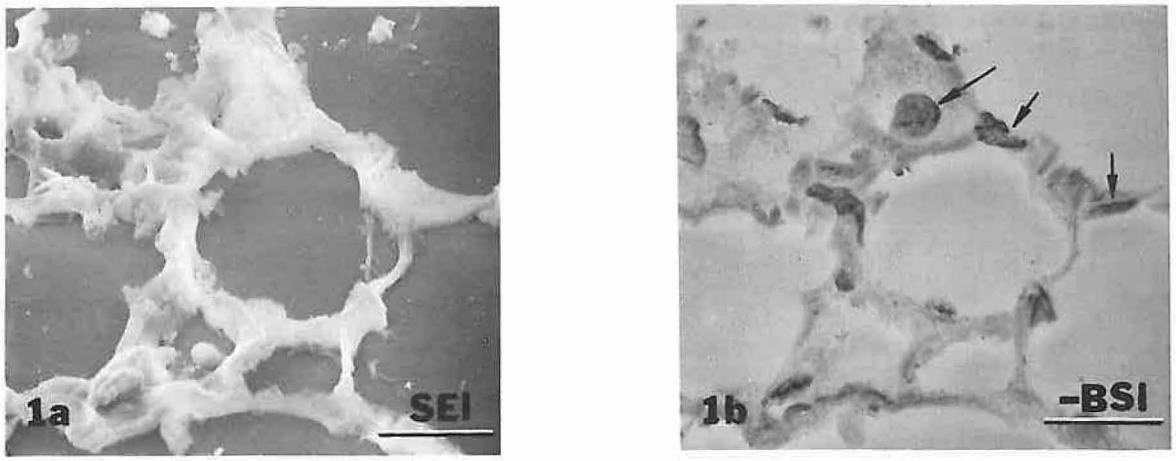


Fig. 1 3μm paraffin section of mouse lung showing alveolar septa; stained with ammoniacal silver. a) SEI showing cut surface of epithelial cells; no cellular staining visible. b) -BSI showing silver stained nuclei (arrows) not visible in SE image. [Note that the (-) negative image indicates contrast reversal] Marker = 10μm. Specimen tilt = 45°

SEM and high voltage TEM by using the same grids for both instruments without any separate preparation. Tissue lying over the grid bars and not visualized with the HVEM can be viewed readily with the SEM using BSI (Fig. 2). Resolution, of course, is significantly greater with the HVEM, but total tissue perspective is easily "overlooked." Fig. 4 illustrates a portion of chick embryo notochord visible in one grid square, while numerous similar regions are obstructed by grid bars.

The epoxy sections mounted on a carbon planchet can also be viewed in the SEM by BSE or SE imaging. Fig. 5a is a-SEI and Fig. 5b a -BSI from an epoxy section of chick embryo similar to the ones seen in Figs. 2, 3 and 4. The BSE resolution is the same for a section on a planchet as it is for one on a grid. The advantages of using carbon planchets are: Ease in handling, many serial sections can be viewed on the same specimen holder, and the specimens are supported on a flat substrate thus eliminating sagging.

Glycol Methacrylate Embedding

This is the first time that BGMA has been applied to correlative studies using all of the forms of microscopic imaging available in both LM and SEM. The BGMA used in this report differs from the glycol methacrylate (GMA) which has been used previously for TEM thin sections. BGMA is softer and less brittle than GMA, thus it is more suitable for thicker (0.5 - 4 μm) sections.

BGMA sections mounted on polished carbon planchets provide a method of scanning large histological regions at low magnification and selected areas at high magnification (and high resolution). Correlation with LM of an adjacent section of the same thickness can be done simultaneously. Figure 6a shows a -BSI of a 0.5μm section of kidney stained with uranyl acetate and lead citrate. Good resolution of both nuclear and cytoplasmic features of kidney tubule cells is seen. Contrast is high. Figure 6b is a light micrograph of an adjacent 0.5μm section. Because of the thinness of the section, staining with H & E requires extended times and triple strength hematoxylin[12] to get adequate staining intensity. Even then, the staining is relatively light. However, because of this thinness, the amount of detail (resolution) in the tissue is maximum for a light micrograph.

Because of section thinness it is possible to see sections of the same cells in both LM and SEM (BSE) micrographs. Thus, we can now correlate the two images. Enzyme and other histochemistry with chromatic LM stains can be correlated with the structure of cells and tissues revealed both by SEM and TEM. In fact, heavy metal-containing histochemical stains usually applied to TEM can be performed just as readily on these sections for SEM and in an extremely simple fashion.

Several other advantages are apparent when BGMA sections are used on carbon planchets. Large sections of tissue can be cut, stained, and studied in the SEM without the interference of grid bars which is so annoying in the TEM. parallel ribbons of serial sections can be studied in the SEM on the same substrate (a polished 25 mm dia. carbon planchet). This is also an advantage over TEM where the size of the grid (3 mm) is a limiting factor. One carbon planchet holds as many sections as about 70 TEM grids. All of the sections on a single planchet can be stained simultaneously, thus promoting uniformity from section to section. In addition, since the sections can be embedded and the material polymerized at or below room temperature, thermally induced artifacts are prevented.

Image Formation

The SEI results from both backscattered electrons and from surface roughness. Thus, deparaffinized sections stained with heavy metals yield an SEI which includes both the surface topography of the tissue and contribution from the heavy metal staining. Careful subtraction of the BSE signal from the SE signal (i.e., the summation of the SE signal and the negative BSE signal) yields a "true" surface topography. The BSE signal from the same tissue yields only the heavy metal stained tissue components such as reticulin, collagen, nucleus, etc.

Since the surfaces of epoxy and BGMA sections are extremely smooth when properly cut and mounted on polished carbon planchets, the main contribution to the SEI from these sections is secondary electrons resulting from backscattered electrons. Thus, the -SEI and -BSI are nearly identical for epoxy and BGMA sections on carbon planchets as long as (a) the surfaces of the sections are smooth and free of contamination and tears, (b) there is no specimen charging, and (c) the substrate is smooth and of uniform density. The -SEI and -BSI were found to be not identical for epoxy sections on grids when observed in the SEM. Using carbon planchets the major difference between the SE and BSE signals is one of brightness, since there are assumed to be more secondary electrons generated than backscattered electrons, and the SE and BSE detectors are in different locations and have different collection efficiencies. Thus, for SEI one can operate at a lower beam current (smaller spot size), lower accelerating voltage and/or lower electronic gain than for BSI.

Fig. 5a and 5b are a -SEI and a -BSI of an epoxy section of chick embryo on a carbon planchet which was stained with lead citrate and uranyl acetate. The two images are nearly identical except for small tears (arrow). In the SEI (Fig. 5a) these tears show up as dark areas which appear to be heavily stained. In the BSI (Fig. 5b) these areas are less discernible and do not appear heavily stained.

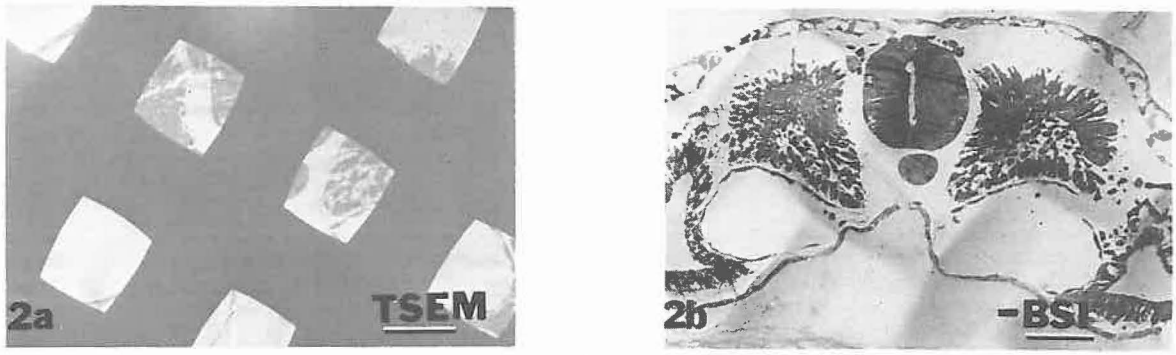


Fig. 2 Low magnification of 200 nm thick section of epoxy-embedded chick embryo on a carbon-coated nylon grid stained with uranyl acetate and lead citrate. Postfixed with OsO_4. a) TSEM image. b) -BSI. Note that grid bars do not interfere with the -BSI. Marker = 50µm.

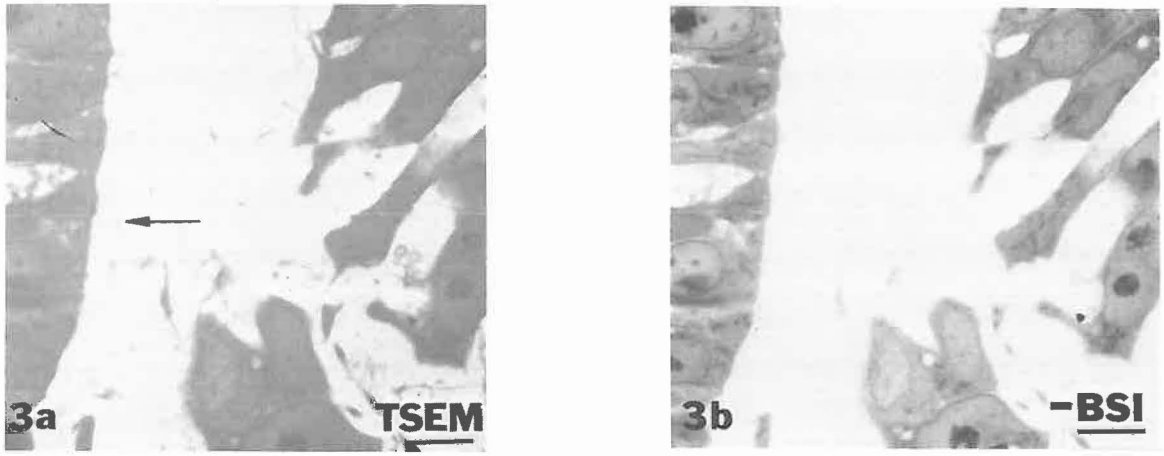


Fig. 3 Higher magnification of 400 nm thick section of epoxy-embedded chick embryo on a carbon-coated nylon grid, stained with uranyl acetate and lead citrate. Postfixed with OsO_4. a) TSEM image. Note poor cellular detail but good definition of extracellular material (arrow). b) -BSI. Note good cellular detail with mitochondria visible in cytoplasm, but extracellular material is not evident. Marker = 5µm.

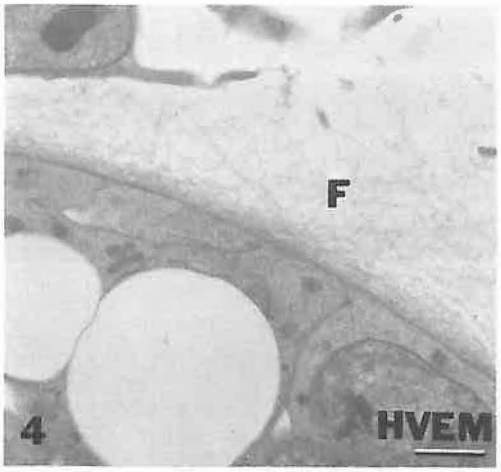


Fig. 4 HVEM micrograph of 500 nm thick section of epoxy-embedded chick embryo stained with uranyl acetate (60 min) and lead citrate (60 min). Postfixed with OsO_4. Representative view of notochord and extracellular fibrils (F). Marker = 3 µm.

One advantage of having nearly identical -BSE and -SE signals available is the ability to electronically sum the two signals thus allowing operation at lower beam currents (smaller spot size) and/or lower electronic gains (for both the BSE and SE signals). This is illustrated in Fig. 7. The section was viewed with a beam current which was too low to give either a good SE or BSE signal. The summation allows one to improve the signal/noise ratio and/or resolution obtainable from heavy metal stained sections.

An interesting observation is that the embedding of tissue in a medium causes loss of cell surface topography which is normally visible in deparaffinized sections. An analogous situation is observing a glass chip in air and in water. It is visible in air but disappears in water.

Comparison of TSEM and BSE Images

The explanation for the difference in appearance between the extracellular and intracellular

materials, as observed by TSEM and BSE imaging of epoxy sections, involves the physical principles of electron scattering. For the current staining procedure of 20 min for lead citrate and 60 min for uranyl acetate, it appears that the effective stain penetrates about 200 nm into the epoxy sections. Thus, only the thinner sections are completely stained.

TSEM imaging involves single low angle scattering events, i.e., scattering $> 11^{o}$ while BSI is involved with single and multiple high angle scattering events, i.e., scattering $> 130^{o}$. According to Wells,[15] for a short path length 10 nm, electron deflections occur as a result of single event scattering except for low angle scattering from high Z material which involves both single and multiple scattering events. For a long path length $\sim$200 nm, electron deflections result from both single and multiple scattering events. Thin sections, therefore, have fewer high angle backscattered events than the thicker ones where there are multiple scattering events, thus the contrast in cellular material is improved in the BSI as the effective stain depth increases. This also explains why the thin extracellular material, which is 10 to 20 nm in diameter, gives poor contrast.

For TSEM, the multiple low angle scattering events from the extracellular material gives rise to good contrast with the surrounding epoxy, while the contrast of cellular material decreases as section thickness increases due to multiple scattering events.

Morphological Features of Plastic Embedded Tissues

The -BSI and -SEI of plastic tissue sections provide contrast that is similar to black and white micrographs obtained with LM or TEM. This enables more direct correlation between those forms of microscopy. Resolution is considerably better than with LM, and both histological and cytological features can be seen; also, extracellular materials can be readily distinguished from intracellular materials. However, resolution is not as good as TEM and cytoplasmic fine structure is not well defined. This is compensated for by better observation of larger histological expanses of tissue in these SEM images and, thus, tissue continuity is more easily examined. Since these sections can be as thick as 2 μm or more, stereophotography is possible, and much information can be obtained from the added three-dimensional perspective. Optimum stereo viewing is determined by the degree of penetration by the heavy metal stains. In addition, analytical procedures can be performed more quickly over a greater amount of tissue and at good resolution of semi-thick sections, but they are again limited by sample size and grid bar obstruction. The methods described in the present study can be used with a significantly less expensive instrument, are simple and quick to produce results, and specimens, prepared in a common manner, can be examined by LM, SEM and TEM in a correlative study.

Tissues which were fixed in osmium tetroxide in addition to the buffered aldehydes displayed better contrast and resolution of cellular constituents than those receiving only the aldehyde fixation (epoxy sections). This is possibly a result of the increased deposition of heavy metal in the tissue, because osmium has a great general affinity for numerous cellular constituents. This "primary" staining is then enhanced with uranium and lead during the final staining step. Also, double staining with uranyl acetate and lead citrate is superior to single staining with only one of these compounds. Preliminary results using several differential staining procedures involving heavy metals, such as silver and tungsten, show localization that is consistent with the reported specificity of the stains.[13,14] These procedures seem to be a promising area for further investigation.

Examples of cytological features that have been observed consistently in the present study include: mitochondria in a variety of cells; myofibrillae in cardiac muscle; secretion granules in respiratory epithelium; cilia in a variety of cells; and lipid droplets in hepatocytes. Cell boundaries (position of plasma membranes) are readily identifiable and extracellular materials, such as collagen fibers are distinct. SEM imaging of these sections provides, in addition to good correlation with LM and TEM, an alternative to the slow and tedious survey procedures some times attempted with TEM on thin sections. A significantly greater amount of tissue can be screened at this lower magnification and, especially with epoxy sections, a considerable resolution of cytoplasmic components is still achieved. See for example Figure 3b.

Conclusions

In conclusion, we do not suggest that any one of the three methods of tissue embedding is best. Each method gives a different perspective to the study of biological tissue. The following is a list of the advantages and disadvantages of each technique along with our recommendations for the best application of each.

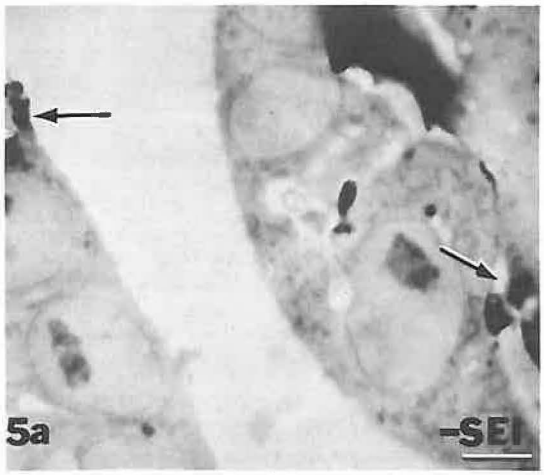
Paraffin Embedding

Advantages:

a) Surface topography of cells can be seen along with their internal structure (no need for replication) and the two images compared.

b) Embedding medium is one which is in common use, inexpensive, easy to section and easy to remove.

c) Alternate serial sections can be stained for LM histology and histochemistry and correlations made between structure and function.



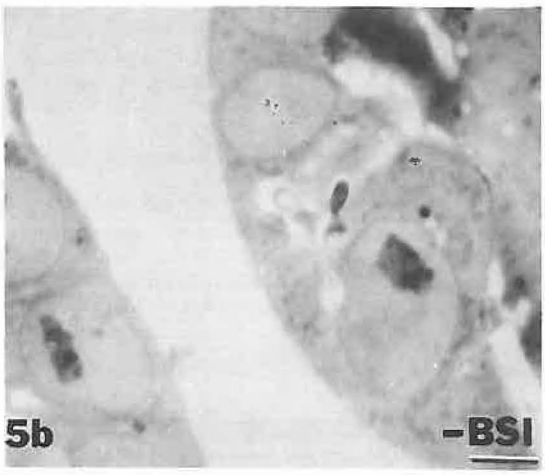
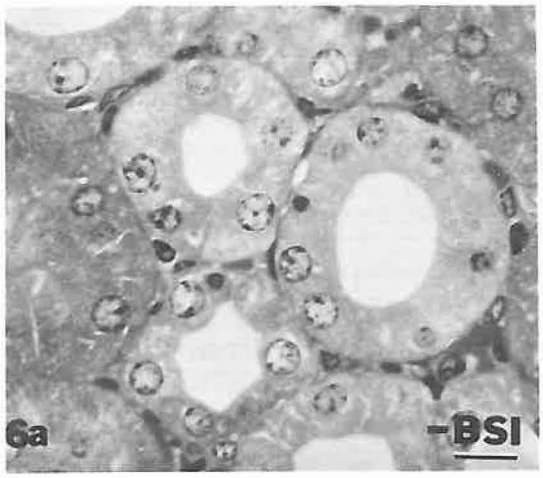


Fig. 5 View of 200 nm thick section of epoxy-embedded chick embryo on a carbon planchet stained with uranyl acetate and lead citrate. Postfixed with OsO_4. a) -SEI. Note tears (arrows) giving appearance of intense staining. b) -BSI. Note absence of dark areas which confirms lack of heavy staining. Marker = 3 µm.



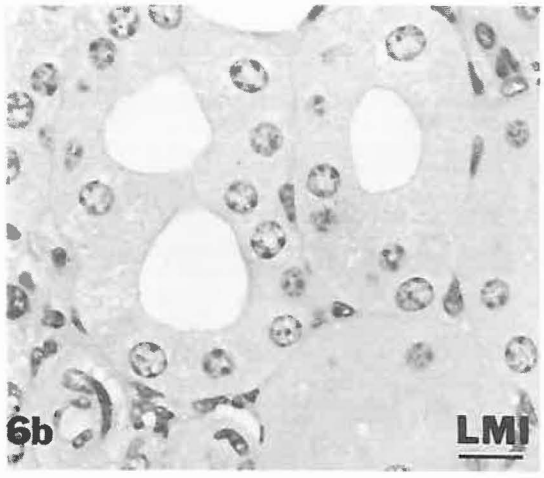
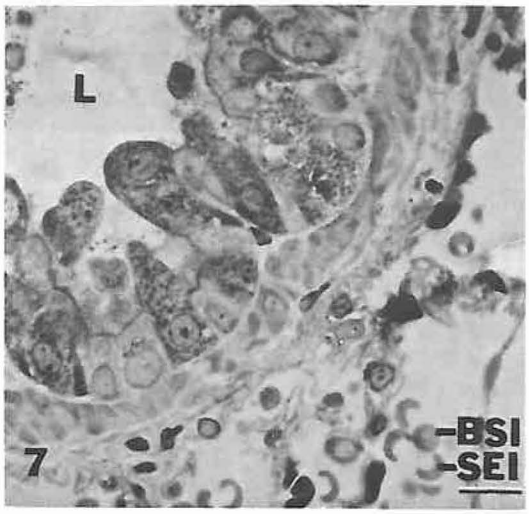


Fig. 6 500nm thick adjacent section of BGMA-embedded guinea pig kidney. a) -BSI of section on carbon planchet stained with uranyl acetate and lead citrate. b) LMI of adjacent section on glass slide, stained with H&E. Less contrast than with Fig. 6a. Marker = 10 µm.

Fig. 7 500 nm thick section of BGMA-embedded guinea pig lung on a carbon planchet, stained with uranyl acetate and lead citrate. Electrical summation of -SE and -BSE signals. Note good contrast and variety of identifiable cell types forming bronchiole. Lumen (L). Marker = 10 µm.



Disadvantages:
a) Some distortion of the tissue because of heating during paraffin processing and trauma during sectioning.
b) Poor correlation with TEM.
c) Difficult to observe same cell in serial sections.
d) Visualization of internal structure not as good as other two methods because of section thickness.
e) Carbon coating required for SEM observation because sections placed on glass substrate.

Epoxy Embedding

Advantages:
a) Ease of comparison of serial sections TEM (thin), with SEM (semi-thin).
b) Can view same sections (semi-thin) on grids in SEM (TSEM, BSE & SE imaging) and high voltage TEM.
c) Can view single cells in serial sections (SEM, TEM and LM).
d) OsO_4 fixation provides better visualization of internal cellular structure.
e) Equivalence of SEI and BSI from smooth sections on carbon planchets.
f) Can use grids or carbon planchets as substrate; no coating necessary.

Disadvantages:
a) Limited to small sample size.
b) Lack of 3d appearance.
c) Difficult to stain for LM, few LM (chromatic) stains available.
e) Can't observe surface topography.
f) Can't use BSI on TEM (thin) sections.
g) Must be sectioned using a glass or diamond knife.
h) Longest preparation time of the three methods.

Glycol Methacrylate Embedding

Advantages:
a) Fastest preparation time.
b) Ability to look at large samples limited only by size of knife, hardness of material and skill of technician.
c) More LM (chromatic) stains available than for epoxy.
d) Compatible with enzyme histochemistry because of water permeability of sections and ability to polymerize block without heating (polymerized with UV light at or below room temp.).
e) Elimination of thermal distortion by low-temperature (UV) polymerization.
f) Able to view same cells with high voltage TEM, LM, and SEM (BSI and SEI).
g) Most compatible with all three forms of microscopy (LM, SEM and TEM) for correlative studies.
h) Equivalence of SEI and BSI.

Disadvantages:
a) Difficult to postfix butoxyethanol glycol methacrylate (BGMA) with OsO_4 for better cellular detail (inhibits polymerization).
b) Can not cut thin sections well with BGMA.
c) Difficult to remove embedding medium.

The authors wish to make the following recommendations for the most effective use of the three methods.

Paraffin Embedding should be used when the best three dimensional observation of the tissue is desired and when it is important to correlate surface topography with internal structure. It is also recommended when the best correlation with chromatic LM stains is desired.

Epoxy Embedding should be used when the best intracellular detail (but less than TEM) is desired and tissue patterns are to be observed without interference from grid bars. It can be used when no correlation with LM chromatic stains is desired.

Glycol Methacrylate Embedding should be used when the best correlation among LM, SEM and TEM is desired and when less intracellular detail is needed (BGMA). It should be used when large areas of tissue are to be examined.

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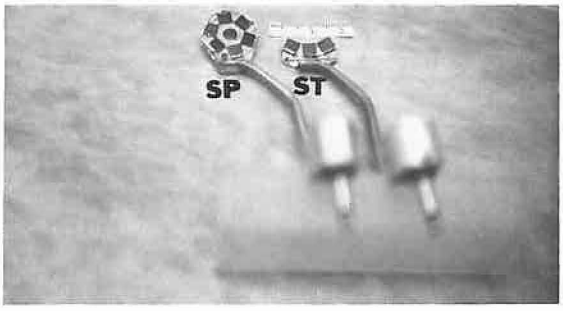
DISCUSSION WITH REVIEWERS

Reviewer II: What is the solid angle of your BSE detector at various working distances? Please include a photograph of your special BSE detector.
Authors: The special BSE detector has 6 chips and the standard BSE detector has 3 chips. Each chip has an area of approximately 10 mm^2. Therefore, at a working distance of 10 mm the solid angle for the special detector is approximately 0.6 steradians, which is twice the solid angle for the standard detector.



The photograph shows the special BSE detector (SP) and the standard BSE detector (ST) removed from the SEM. There is a photograph of the standard detector in place in the SEM on page 255 of Reference No. 2.

Reviewer III: Your six chip solid state BSE detector would appear to detect only a small fraction of the total BSE signal. A different explanation of the greater brightness of the SE signal over the BSE signal is improved collection efficiency of the SE detector for SEs, when compared to the BSE detector for BSEs. Have you any figures to support your statement that there are more SEs than BSES?
Authors: No.

Reviewer II: What is the capacitance of your special BSE detector compared to the standard detector?
Authors: The capacitance for the 6 chip (special) detector should be twice that for a 3 chip (standard) detector.

Reviewer II: Do you have to work at unusually slow scan rates with your BSE detector?
Authors: Yes.

Reviewer V: What do the authors mean by "correlation of structure and function" with respect to the use of alternate sections for LM and SEM?
Authors: Tissue and cell structure can be revealed well by SEM and alternate sections can be stained for LM using histochemical procedures which reveal "function". Our use of the term "structure" designates the physical appearance of both macromolecular constituents of cells and the composition of cells within tissues including such qualities as presence or absence and precise distribution of morphological features. With respect to this type of microscopic structure, "function" is used to describe certain properties of cell specialization, both individually (internally) and as aggregations in the form of tissues. Differential cytochemistry and histochemistry provide information, such as the localization of specific enzyme systems, the presence or absence of specific glycoproteins and the distribution of certain characteristic lipids. All of these procedures reveal "function" based on cell specialization.

Reviewer IV: Why do you contend that heating of a fixed section in paraffin distorts it, and heating of a fixed section in plastic does not ?
Authors: The shock heating of this tissue (paraffin) occurs during infiltration of the paraffin whereas the heating of the tissue (plastic) occurs slowly during polymerization.

Reviewer I: At the magnifications employed in Figs. 3b, 5b, 6 and 7, it is not clear that the BSE imaging mode offers improved resolution over the LM.
Authors: The improvement in resolution comes with higher magnification.

Reviewer V: What is the advantage of glycol methacrylate for enzyme histochemistry?
Authors: Glycol methacrylate is one of the standard embedding media used for electron microscopic cytochemistry. There are several reasons

for this: 1) Glycol methacrylate (GMA) can be used as a dehydrating agent and tissue specimens can be embedded without coming in contact with organic solvents; 2) Polymerization of (GMA) occurs at room temperature or lower (with UV light), thus making an excellent embedding media for enzyme histochemistry; 3) It is felt that water-miscible embedding media allow better penetration of sections by water-soluble histochemical reagents (e.g., enzymes), especially if a certain amount of water is retained within the polymerized medium.

Reviewer II: In your section on Image Formation, you say that SE(-) and BSE(-) images are nearly identical for plastic sections on carbon planchets but not for plastic sections on grids. Please explain further why this is so.

Authors: The substrate affects the SEI but not the BSI. There is a higher SE yield from the epoxy areas in the grid opening than from the epoxy over the grid bars. This is due to both SEs escaping from the bottom surface and from SEs generated from transmitted primary electrons striking the metal specimen holder and stage.

Reviewer I: Assuming that the stain penetration is uniform, how does the resolution of the BSE image vary with section thickness?
Authors: The resolution of the BSI appears to remain constant for sections 200 nm and thicker.

Reviewer I: For optimal resolution of the surface, should not you use a less energetic beam than 20 kV? Have you determined the accelerating voltage for which you obtain optimal image contrast and resolution for most of your stained sections?
Authors: For our equipment the accelerating voltage which gives the best contrast and resolution in BSI is 20 kV. The contrast is poor much below 20 kV while the resolution is poor above 20 kV. Although the optimum resolution of the surface may be obtained at voltages below 20 kV, we wish to compare SEI and BSI, thus we work at the same voltage.

Reviewer III: Your results show an equivalent of the BSE and SE images for smooth sections free from contamination, charging, and tears, when placed on a smooth substrate of uniform density. This would appear to indicate that the SE yield is as heavily dependent upon electron penetration and scattering as the BSE yield, in this situation. As such, observations of the distribution of histochemical stains in smooth sections can be performed in any SEM fitted with a SE detector, and does not need a special BSE detector. Do you agree?
Authors: If all criteria for the sections are met, SEI is all that is necessary, but BSI must be used to verify that these criteria are met.

Reviewer II: In figure 7, what beam current was used? Please show the SE(-) and BSE (-) images individually formed at the exact gain settings for the summed picture.
Authors: The beam current was approximately 0.05 na. Under normal working conditions, this would have given an adequate SEI but a poor BSI. Shielding was used to simulate the worse possible case. Under these modified conditions, both -SEI and -BSI lacked sufficient contrast to photograph but the summation (Fig. 7) provided an adequate image.

Reviewer IV: What organelles are better visualized by your methods than currently available methods of LM?
Authors: LM demonstrates the presence of certain organelles such as cilia, mitochondria and nuclei by means of specific stains but does not reveal morphologic details. Our method reveals these details.

Reviewer III: The techniques you have described for the production of thin sections are well known in LM and TEM. The epoxy and BGMA embedding media cannot be easily removed to enable viewing of surface topography, which is, after all, a big advantage of a SEM over a TEM. Why not use cryosectioning for the preparation of thin sections? It would appear to have many advantages over the techniques you have described.
Authors: We have limited our paper to three standard techniques for tissue embedding and processing. There are many other techniques such as cryosectioning, freeze fracturing, ion etching, etc., which would be useful.