Correlative Light and Electron Microscopy of the Same Sections Embedded in HPMA, Quetol 523 and MMA

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—Received for Publication, July 31, 1992—

Key Words: Correlative microscopy, Semithin section, Water-miscible methacrylates, Quetol 523, Fine structure

Summary: Semithin sections, cut from tissues stained with acid and basic dyes after embedding in 2-hydroxypropyl methacrylate, Quetol 523 and methyl methacrylate, showed cytoplasmic components at a high resolution by light microscopy. These same sections could then be viewed, after osmium tetroxide, uranyl and lead staining, by the electron microscope. These sections had a number of inherent advantages: they could be observed with a light microscope; they facilitated analysis of cellular structures in the identical sites, and they were frequently the optimum thickness to provide three-dimensional information. We clearly established the structural detail of this same-section correlative light-electron microscopy approach by showing that the coloured materials observed in such sections of cells followed the distribution of fine structures within the same sections as determined by electron microscopy.

In some instances the fidelity of the correlation between the distribution of the coloured area and cytoplasmic components in identical cells of the same section revealed significant details which could not be visualized in thin sections. This technique, therefore, provided a simple and useful solution to many problems that require the localization of cellular components in identical cells selected previously by light microscopy.

Correlative light and electron microscopy is a tool used in histology and histochemistry to study the structural details of identical cells and for the visualization of specific reaction sites which contain particular substances like glycosaminoglycans, proteins or enzymes. The electron microscope can be used to investigate the morphological details of cellular components and to obtain three-dimensional information on images provided by light microscopy of the same sites.

The ability to study biological material in semithin sections by light microscopy, prior to examining the same section by electron microscopy, provides the following advantages. First, biological material within sections thinner than 2.0 μm can be visualized with excellent resolution of detail by light microscopy (Bennett et al., 1976, Kushida et al., 1981, Rieder and Bowser, 1983). Therefore, electron microscopic findings can be obtained in the identical site previously observed by light microscopy. Examination of a matched set of serial thick and thin sections is unnecessary. Second, precise correlation between the results obtained by light microscopy and those obtained by electron microscopy is readily achieved. Using this approach, the distribution of coloured deposits can be correlated with the ultrastructure in histochemical (Nagato et al., 1980; 1984, Nagato and Kushida, 1985) or immunohistochemical (Rieder and Bowser, 1985) staining techniques. Third, structural relationships between cellular components which are not visible in traditional thin sections may be revealed in semithin sections. The thicker sections facilitate studies requiring an analysis of serial sections since fewer sections are needed to identify or reconstruct the structure of interest.

Semithin sections embedded in water-miscible methacrylates can be examined when the same sections are within 0.2–0.3 μm in thickness by both light and electron microscopy operating at 100 kV (Kushida, 1977; 1978, Kushida and Kushida, 1978; 1981). Thus structural relationships can be revealed after staining with various acid and basic dyes (Kushida et al., 1977; 1981). Such sections are also used in histochemical staining procedures to identify reaction deposits by electron microscopy (Nagato et al., 1980; 1984). It is, however, difficult to determine the composition of cellular components at an electron microscopic level.

Recent developments in technique have provided reliable methods for the correlation of cellular details. The development of the intermediate voltage (200–500 kV) electron microscope has enabled investigators to examine sections which are appreciably thicker than the conventional thin sections
The ability of these instruments to produce high resolution images of biological components in semithin sections has restored an appreciation of the third dimension of biological ultrastructure by eliminating many of the problems encountered in reconstructing biological ultrastructure from serial thin sections (Glauert, 1974). Furthermore, cellular structures may be clearly identified in images of specimens, since the higher acceleration potential is available to obtain high resolution.

With this approach, fixed materials are embedded prior to sectioning in a mixture of 2-hydroxypropyl methacrylate (HPMA), Quetol 523 and methyl methacrylate (MMA), a low viscosity water-miscible methacrylate, which is used to prepare specimens for observation under an electron microscope operating at 200 kV (Kushida et al., 1985). This method was applied to reveal the significant structural differences in ectopic neurons of the cerebellum in neurological mutant mice (Nagato et al., 1991).

In the present study, the results of correlative light and electron microscopy for animal tissues embedded in the above methacrylate resins are described and comments are made on the fixation and staining procedures. In addition, a comparison is made with the results of previous methods.

Materials and Methods

Fixation and embedding

Small pieces of animal tissues were immersed in a mixture of 4% formaldehyde and 1–2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, as described previously (Nagato et al., 1981). Postfixation with osmium tetroxide should be avoided. Fixed preparations were rinsed with buffer solution and then dehydrated using a graded series of ethanols. According to the method described in detail by Kushida et al. (1985), they were then infiltrated and embedded by the procedure using a mixture of 2-hydroxypropyl methacrylate (HPMA), Quetol 523 and methyl methacrylate (MMA) (Nissin EM Co. Ltd., Tokyo, Japan and others). After dehydration, the specimens were transferred to a 1:1 (v/v) mixture of absolute ethanol:resin mixture, and then further infiltrated with two changes of fresh resin mixture. Tissues were rotated on a rotary shaker at room temperature. Tissue samples were transferred to gelatin capsules (size 0 or 00) filled with fresh resin mixture. They were incubated overnight at 60°C in an oven for curing. The final hardness of the block was adjusted by altering the ratio of HPMA and Quetol 523 to suit the particular specimen.

Sectioning

Sections 0.3–0.5 μm thick and approximately 2 × 2 mm in size were cut by glass knives on an ultramicrotome with a lowered water level in the boat to avoid wetting the block face. Ribbons of sections display a green or red interference colour after expansion on the water surface. Each section was separated from the ribbons and placed on collodion-coated grids for staining by the same techniques used in thin sectioning.

Light microscopy

The grids containing sections were inserted into the small slits of polyethylene tubing for staining as described previously (Kushida et al., 1979). They were immersed in a staining solution of hematoxylin-eosin or toluidine blue. The sections require longer staining times than semithin sections used for light microscopy (Nagato et al., 1989, Iijima et al., 1992). After staining, the grids were carefully removed from the polyethylene tubing and placed on the slide glass for examination and photography.

Staining for electron microscopy

The same sections that were viewed by light microscopy were then stained with heavy metals using the polyethylene tubing as a grid-holder (described above). In general, they can be adequately stained with such heavy metals by the following procedures: (1) 1% aqueous osmium tetroxide for 5–10 min; (2) uranyl acetate for 3 min; (3) Reynolds’ lead citrate for 1 min.

Electron microscopy

Selected sections, which contained the area or structures of interest as determined by comparing photomicrographs, were examined and photographed at low magnification in an electron microscope operated at 200 kV.

Results

Semithin sections of stained tissues, 0.3–0.5 μm in thickness, showed clear high resolution patterns under a light microscope, and this range of section thickness could be examined, after osmium tetroxide, uranyl acetate and lead citrate staining, under an electron microscope operated at 200 kV. Using this approach, it is therefore possible to correlate the light microscopic image from a semithin section with the corresponding ultrastructure in the same section. However, the sections must be examined and photographed under the light microscope prior to staining for electron microscopy, since heavy metals quench all colour.
A comparison of light and electron micrographs from the same sections revealed an accurate correlation between the distribution of cytoplasmic organella as seen in the photo and electron micrographs (Figs. 1–8). Figures 1A and B are an example of a correlation of granular convoluted tubule in the submandibular gland from a mouse. Supranuclear secretary granules and basal endoplasmic reticulum, stained with toluidine blue, were located with excellent detail and contrast under an electron microscope (Figs. 1A, B and C). In small intestine from a mouse, the long filamentous supranuclear and granular infranuclear structures detectable in the light microscopic image correlated, at the electron microscopic level, with single tubular mitochondria, although individual cristae were less distinguishable (Figs. 2A, B and C). Staining with toluidine blue enabled goblet cell granules with various stainability to be observed. Examination by electron microscopy showed various contrast the granules of goblet cells. Correlation of the identical area in the same section revealed their contrast accurately associated the stainability at the light microscopic level (Figs. 2A, B and C).

The fidelity in the correlation between light and electron microscopic images in sections of cells examined for ultrastructure prompted us to explore whether this same approach could be used to determine the ultrastructure in the developmental stage of germ cells. A pair of photo and electron micrographs was obtained in the course of investigating the developmental changes in germ cells of the seminiferous tubules from a mouse testis (Figs. 3A and B). Light microscopy of semithin sections from the early stage of testis, stained with hematoxylin and eosin, showed distinct colouring of cytoplasmic structure (Fig. 3A). A comparison of the light microscopic image from sections of these cells with the ultrastructure in the same section clearly revealed that the structural differences between germ cells were situated toward the lumen and Sertoli cells localized in the peripheral region (Figs. 3A, B and C). Under high magnification, mitochondria and vesicular endoplasmic reticulum could be seen (Fig. 3C). An example of correlative light and electron microscopy after staining with hematoxylin–eosin is also illustrated in Fig. 4. It shows a section of renal tubule from a mouse kidney. The basal half of epithelial cells showed long rod-like mitochondria; their orientation could be identified clearly by electron microscopy with excellent detail and contrast (Figs. 4A, B and C). It can be seen that filamentous microvilli overlapped this area of the section.

This approach revealed the composition and structure of cellular components that are not visible in thin sections because of the thickness of the section (Figs. 5–8). Figure 5 shows secretory granules in a granular convoluted tubule of the submandibular gland. A higher magnification demonstrated the various sizes of the granules, whose location was clearly distinguishable within the section, since the overlapping of some granules occurred. Tissue sections also allowed the distribution of the membrane to be examined. Figures 6 and 7 are electron microscopic images of the distribution of membrane; this information could not be obtained from thin sections. Figure 6 shows the overlapping of microvilli with an irregular cell membrane and terminal bar from intestinal epithelial cells. Figure 7 illustrates the glomerulus of the cortex from kidney. Staining with heavy metals clearly revealed the basement membrane, although such structures are not stained by eosin. An example of this approach is also presented in Fig. 8. It shows a meiotic spindle in the germ cells of a seminiferous tubule from a mouse testis. The section was processed for electron microscopy after toluidine blue staining. The electron micrograph revealed the distribution of spindle microtubules.

**Discussion**

Seminifer sections have been used in a high voltage microscope when specific ultrastructural information is required that can not be obtained by thin sections embedded in epoxy resins (Rieder *et al.*., 1985). In recent years, the introduction of water-miscible methacrylates as an embedding medium has enabled the examination of tissue structure using semithin sections in an electron microscope operated at 100 to 200 kV, instead of at a high voltage (500–1,000 kV). The use of the HPMA-Quetol 523-MMA mixture for embedding is an example of the adaptation of a light microscopic procedure to electron microscopy (Kushida *et al.*., 1985). This method has been used with an electron microscope operating at 200 kV instead of the previous methods that used embedding in GMA-Quetol 523 or GMA-Quetol 523-MMA (Kushida 1977; 1978, Kushida and Kushida 1978; 1981), since satisfactory results have not been obtained when sections of these resins were applied for the observation of cellular structures under an electron microscope operating at 100 kV (Kushida *et al.*., 1977; 1981).

The present study revealed that seminifer sections embedded in HPMA-Quetol 523-MMA can be viewed and photographed with good resolution and contrast in an electron microscope operating at 200 kV. Tissues can also be visualized with excellent resolution of detail by light microscope. The...
procedure is useful for studies requiring the ultra-
structural examination of a selected area or structures
that are large enough to be visualized by a light
microscope. The present study suggests that the
light microscopic images of biological materials in
sections prepared for electron microscopy depend
on the section thickness, the type of fixation, and
staining. Sections thicker than 0.5 μm were unsatis-
factory for electron microscopy and coloured sections
thinner than 0.3 μm were too weak to observe the
tissue image. Therefore, satisfactory results were
obtained when sections 0.3–0.5 μm in thickness
were used for the examination. Materials fixed in
a mixture of formaldehyde and glutaraldehyde
showed better contrast than those fixed only with
glutaraldehyde. The contrast of material in stained
sections could be further enhanced by staining with
heavy metals. The best contrast in semithin sections
was obtained after osmium tetroxide, uranyl acetate
and lead citrate staining. Under these conditions,
the image increased in contrast and the higher mag-
nification revealed the fine structure.

Examination by electron microscopy demon-
strated the secretory granules in a selected area of
tissue sections stained by toluidine blue. Staining
with hematoxylin and eosin was also useful for the
determination of cell type and cellular components
by electron microscopy. Our same-section cor-
relative approach has allowed us to characterize
cellular structures in the developing seminiferous
epithelium at the ultrastructural level. These results
could not have been obtained by light or electron
microscopy alone.

An additional advantage of using semithin in-
stead of thin sections is that structures which are not
visible, or go unnoticed, in thin section studies may
be clearly visible in semithin sections of the same
material. In some instances examination revealed the
distribution of the discrete granules in granular
convoluted tubules of the submandibular glands,
the long filamentous structures of mitochondria
which appear in epithelial cells, and meiotic spindle
in germ cells that have been shown to consist of
microtubules.

The fidelity in the correlation between light and
electron microscopic images of the same section
indicates that this technique can provide an alterna-
tive approach to histochemical methods as long as
the reaction products in question appear localized
by light microscopy, since such correlation suc-
cceeded in demonstrating the localization of alkali-
ne phosphate activity, glycosaminoglycans and
glycoproteins after histochemical staining without
the enhancement of contrast (Nagato et al., 1980;
1984). This method might be useful, for example,
to determine the ultrastructural localization of the
antigen of a specific region of the cell visible by light
microscope.

In conclusion, correlative light and electron
microscopy has been introduced for the observation
of cytoplasmic details from sections embedded in
HPMA-Quetol 523-MMA. The results indicated
here correlated coloured areas with cellular struc-
tures which could not be observed in thin sections.
Through this method, electron microscopy provides
an opportunity to observe the distribution of specific
antigens using immunohistochemical techniques.

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Explanation of Figures

Plate I

Fig. 1A, B and C. Correlative photo (A) and electron (B and C) micrographs of granular tubules in the submandibular glands from a mouse. A 0.5 μm thick section was stained by toluidine blue (A), followed by staining with heavy metals (B and C). Panel C is an electron micrograph of secretory cells enlarged from panel B. The various sizes of granules (G) and endoplasmic reticulum (ER) are clearly shown.

Fig. 2A, B and C. Photo (A) and electron (B and C) micrographs of the same 0.5 μm thick section from a mouse small intestine. Panel A is stained by toluidine blue; Panels B and C are stained by heavy metals after toluidine blue staining. A comparison of these micrographs indicates that the coloured image correlates with the distribution of cytoplasmic structures.
Plate II

Fig. 3A, B and C. A pair of photo (A) and electron (B) micrographs of developmental seminiferous tubules from a mouse testis. Panel C is enlarged from panel B. A 0.5 μm section was stained by hematoxylin-eosin for light microscopy, and was then treated by heavy metals for electron microscopy. A comparison of these micrographs reveals cytoplasmic structures, such as mitochondria, microvilli, basement membrane and nucleus, of the identical site.

Fig. 4A, B and C. Photo (A) and electron (B and C) micrographs of a 0.5 μm thick section from the cortex of a mouse kidney. Hematoxylin and eosin staining (A), followed by osmium tetroxide, uranyl and lead treatment (B and C) was carried out on the section.
Plate III

Fig. 5. Electron micrograph of the 0.5 μm section from a granular convoluted epithelial cell of submandibular gland stained by hematoxylin-eosin and then incubated with heavy metals.

Fig. 6. Electron micrograph of epithelial cells from a mouse small intestine. The 0.5 μm thick section was stained by hematoxylin-eosin, followed by heavy metals. It clearly illustrates overlapping of the cell membrane and microvilli.

Fig. 7. Electron micrograph of the cortex of a mouse kidney showing glomerulus and renal tubules. The 0.5 μm section was processed for staining with hematoxylin-eosin and heavy metals. It indicates the distribution of the basement membrane and cytoplasmic organelles.

Fig. 8. Electron micrograph of meiotic division in a germ cell of a mouse testis. The 0.5 μm section was treated for staining with toluidine blue and heavy metals. It reveals the distribution of the meiotic spindle microtubules.