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A NEW APPROACH FOR STUDYING SEMITHIN SECTIONS OF HUMAN PATHOLOGICAL MATERIAL: INTERMICROSCOPIC CORRELATION BETWEEN LIGHT MICROSCOPY AND SCANNING ELECTRON MICROSCOPY

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Abstract

In order to obtain useful and complete information on the study of pathological material, we observed by scanning electron microscopy (SEM) the same semithin sections observed by light microscopy (LM). For this purpose, the specimen must have, at the same time, chromatic and electron dense characteristics.

We thus developed different specimen preparation methods, subjecting the semithin sections to specific polychromatic staining with high atomic number (Z) elements, to monochromatic staining followed by routine contrasting with uranyl acetate and lead citrate, and to specific cytochemical and immunocytochemical procedures.

The specimens were examined in sequence by LM, by SEM equipped with secondary electron, backscattered electron, transmitted electron detectors and by scanning transmission electron microscopy (S(T)EM).

KEY WORDS: Comparative Observation, Light Microscopy, Semithin Sections, Secondary Electron, Backscattered Electron, Transmitted Electron, X-Ray, Material Preparation, Staining Pathological Material

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Introduction

Examination of biological embedded material, by transmission electron microscopy (TEM), is usually preceded by preliminary examination by light microscopy (LM) of semithin sections obtained from the same specimen. This permits localization, in a rather large area, of limited zones of high diagnostic value to be examined by TEM (4). Examination by LM of such sections properly stained provides particularly interesting information (4). The difficulty to survey by TEM the same entire area of a large specimen is, in pathology, a great limitation (3, 4, 8).

With the introduction of new processing methods for biological embedded and sectioned material, there is, now, the possibility of viewing, in sequence, the same field of observation both by LM and by scanning electron microscopy (SEM).

The main problem to be solved is to obtain a specimen with chromatic and electron dense characteristics at the same time. It would therefore be possible, after LM examination, to transfer the same specimen into the scanning electron microscope. In this way, diagnostic surgical material may be studied by means of secondary (SE), transmitted (TE), backscattered (BSE) and X-ray detectors.

Materials and Methods

The specimen to be observed by LM and by SEM may be processed in two different ways. It is possible to observe sections mounted on grids,by using a scanning electron microscope fitted with an accessory for transmitted electron (TE) detector. On the other hand these sections may be mounted on glass slides and observed by simply utilizing an SE detector (11).

Sections collected on grids. Sections (0.5-1 μm), obtained from an embedded biological specimen, are transferred by a small wooden

spatula from the knife of the ultramicrotome to a drop of distilled water on a clean glass slide covered with a thin film (30 nm) of formvar. After drying on a 150° C hot plate, the sections can be stained directly (for instance with toluidine blue or other dyes), observed and photographed by LM. The sections, at this point, may be removed from their support by immersing the glass slide into a Petri dish full of chloroform. This operation should be carried out under a fume-cupboard. Floated sections may be transferred by a Pasteur pipette into a dish with distilled water in which the sections float as a consequence of surface tension and may therefore be collected on grids and observed directly (one step staining) or after conventional staining with uranyl acetate and lead citrate (two step staining) in a scanning electron microscope fitted with TE, SE and BSE detectors. The sections, before observation, must be covered by a thin layer of evaporated carbon (20 nm).

Sections collected on glass. The semithin sections, to be observed by LM, are processed in the above-mentioned way without covering the glass support with formvar and without removing the sections in chloroform. In this case the sections do not have to be removed from the glass. For SEM observation the glass slides must be reduced to the same size (1x1 cm) as the observation stubs. The small pieces of glass are then glued, by means of silver conducting paint, to the aluminium stubs. The specimen is rendered more conductive by placing a silver paint bridge between the stub and the cut glass slide. After drying at 60°C in a oven, the specimens, covered under vacuum with a thin layer of pure evaporated carbon (20 nm), are observed in a scanning electron microscope equipped with SE and BSE detectors.

Staining procedures. Two different staining methods, applicable to semithin sections mounted either on grids or on glass, were utilized in order to obtain a specimen with both chromatic and electron dense characteristics.

One step staining. The use of histological dyes containing high atomic number (Z) elements gives the specimen, in a single solution, good chromatic and electron dense characteristics. Among the dyes of this type, we used mainly the toluidine blue-silver methenamine-safranin method, with some modifications in order to obtain, at LM level, differential polychromatic staining of tissue components and good morphological and compositional contrasting at SEM level.

Two step staining. This staining procedure permits any type of histological staining both for detection (monochromatic staining) and for diagnostic (polychromatic staining), independently of its specific electron density. The sections, in fact, after LM examination, are stained with conventional electron dense dyes (uranyl acetate and/or lead citrate) and then observed by SEM.

The following methods (reviewed in refs. 1, 4, 5, 6, 12) are of easy application and are helpful in the study of normal and pathological material:

Toluidine blue

Dye solution:
0.5 per cent sodium carbonate in distilled water plus toluidine blue (1 g/100 ml). It is better to use a week old toluidine blue solution because a fresh one may remove the sections from the glass slide.

Staining procedure:
1. Stain with a few drops of this solution by heating on 80°C hot plate for 20 sec.
2. Drain excess stain and rapidly rinse in distilled water.
3. Dry by heating on 150°C hot plate, very quickly.

Malachite green and safranin

Dye solutions:
1. Two per cent aqueous malachite green solution.
2. Two per cent aqueous safranin solution.

Staining procedure:
1. Stain with malachite green solution by heating on 80°C hot plate for 2 min.
2. Drain excess stain, rapidly rinse in distilled water and dry by heating on 150°C hot plate for 5 min.
3. Stain with safranin solution by heating on 80°C hot plate for 30 sec.
4. Drain excess stain, rapidly rinse in distilled water and dry by heating on 150°C hot plate, very quickly.

Toluidine blue-silver methenamine-safranin

Dye solutions:
1. 0.5 per cent sodium carbonate in distilled water + toluidine blue (1 g/100 ml). It is better to use a week old toluidine blue solution because a fresh one may remove the sections from the glass slide.

2. Silver solution (3 per cent aqueous hexamethylenetetramine solution, 50 ml.; 5 per cent aqueous silver nitrate solution, 5 ml.; 3 per cent aqueous sodium tetraborate solution,6 ml.
3. Two per cent aqueous safranin solution.
4. One per cent aqueous periodic acid solution.

Staining procedure:

1. Postpolymerization on 150°C hot plate for 5 min.
2. Remove osmium tetroxide employing the periodic acid solution in 60°C oven for 15-20 min. If better nuclear contrast is required, less osmium tetroxide should be removed by using a periodic acid solution for 10 min. at room temperature.
3. Wash with two rapid changes of distilled water.
4. Stain with an old toluidine blue solution in a 60°C oven for 20 min.
5. Wash with two rapid changes of distilled water.
6. Postpolymerization on 150°C hot plate for 10 min.
7. Stain with silver methenamine solution in a 60°C oven for 40 min.
8. Wash in distilled water.
9. Repeat twice the silver methenamine staining and washing, controlling each time the staining (sections appear brown).
10. Wash with 5% sodium thiosulfate for 10 min. at room temperature. Then wash with two changes of distilled water.
11. Postpolymerization on 150°C hot plate for 15 min.
12. Stain with safranin solution on 60°C oven for 5-10 min. (this step is very critical, staining must be controlled from time to time).
13. Rinse in distilled water and dry by heating on 150°C hot plate very quickly.

Anti-actin immunocytochemical reaction

Tissue samples were fixed in periodate-lysine-paraformaldehyde (7), dehydrated and embedded in Araldite. The protein A- gold immunocytochemical technique (10) was applied on semithin sections for the detection of actin. Labelling was enhanced by a silver precipitation reaction (1).

Electron dense staining

Uranyl acetate

Solution:

1. Add 3 g of uranyl acetate to 100 ml of 50 per cent ethanol; shake until uranyl acetate is dissolved.

Staining procedure:

1. Stain for 15-30 min. at room temperature in the dark.
2. Wash with distilled water.

Lead citrate

Solution:

1. Add 0.125 g of lead citrate to 49.5 ml of distilled water in a clean graduated glass cylinder.
2. Add 0.5 ml of 10 N sodium hydroxide in the cylinder. Shake vigorously until the lead citrate is dissolved.

Staining procedure:

1. Stain for 5-10 min. at room temperature in the dark.
2. Wash in distilled water.

Double staining with uranyl acetate followed by lead citrate produces more effective contrast than either stain on its own. We employed first a 10 min.staining in saturated uranyl acetate in 50% ethanol at room temperature and another 10 min. staining in lead citrate at the same temperature.

Instruments and operating procedures

All the specimens were observed by LM and successively examined in a scanning electron microscope Philips 505 and 515 equipped with TE, SE, BSE detectors, under different conditions:

- primary electron (PE) energies : ranging from 3 to 30 kV;
- beam spot size (S): ranging from 20 to 200 nm;
- specimen stage tilt (Tilt): ranging from 0° to 30°.

Images obtained: transmitted electron image (TEI), secondary electron image (SEI), back-scattered electron image (BEI).
We used the negative polarity mode (reversed signal) in SE and BSE mode in order to obtain the best correlation between TEI, SEI and BEI. The negative (-) image is a contrast reversal from the positive (+) or normal image (2).

At the same time , some specimens were observed by TEM Philips 400 T fitted with a S(T)EM unit. The operating procedures were:

- PE: ranging from 60 to 100 kV;
- S: ranging from 10 to 100 nm;
- Tilt: ranging from 0° to 30°.

In addition, energy dispersive X-ray analysis was performed by using an EDAX detecting unit.

Results

Some significant examples have been selected from all the biological material taken into consideration in our study.

One step staining

Fig. 1 refers to a semithin section of the human stomach, mounted on a glass slide, stained with toluidine blue-silver methenamine-safranin and viewed by LM. The same field observed in SE(-) mode by SEM gives an image with a contrast similar to that of the LM image (LMI) (Fig. 2). The same section can be viewed by using both SE(-) and BSE(-) modes (Figs. 3a, 3b). By comparing Figs. 1, 2 and 3, it can be seen that cellular details (e.g., mucous granules) are more distinct at SEM.

Fig. 4 is an LMI of a semithin small intestine section of a patient with acquired immunodeficiency syndrome (AIDS). The section was processed and stained as described above. In Fig. 5a, the section as showed by SE(-) mode. The lamina propria of a shortened villous presents some distended vessels and numerous macrophages containing argentaffin granules which are better seen at higher magnification in Fig. 5b.

In Fig. 6, from a semithin small intestine section, stained as above, of a patient affected by Whipple's disease, it is possible to observe by LM the characteristic histiocytes localized at lamina propria level. Well resolved correlated SEI(-) (Fig. 7a) and BEI(-) (Fig. 7b), may be obtained when the same section is examined by SEM. Fig. 8 shows, in detail, the argentaffin particles contained within the histiocytes.

Fig. 9 refers to a semithin section of the human stomach of a patient treated with Aspirin for six months. The section was collected on a grid, stained as above and observed by LM. Note the pseudo-polypoid aspect of the gastric mucosa. The same section was observed in TE mode (Fig. 10). TEI may be well compared to those obtained in SE(-) and BSE(-) mode (Fig. 11).

Two step staining

Fig. 12 is an LMI of a semithin section of normal human stomach, collected on glass and stained with toluidine blue. After post-staining with uranyl acetate and lead citrate, the same field may be viewed in SE(-) mode (Fig. 13). Contrast image and resolution are improved .

In Fig. 14 an actin specific immunocytochemical reaction, visible by LM at brush border level of the absorptive epithelial cells, was applied to a semithin section of the small intestine. After contrasting with uranyl acetate, SEI(-) shows actin localization and morphological details. (Fig. 15a). Fig. 15b is a BEI(-) at high magnification.

Fig. 16 is an LM view of a semithin section of the human bone marrow of a patient with sideroachrestic anaemia. The section was mounted on glass and stained with malachite green and safranin. Erythroblastic islands are showed. After LM examination, the section was collected on grid, stained with uranyl acetate and viewed by S(T)EM. TE (bright field) image reveals the presence of sideroblasts containing pathological inclusions and sidero-mitochondria (Fig. 17a). The X-ray microanalytical examination performed on the mitochondria evidenced the iron accumulation at this level (Fig. 17b).

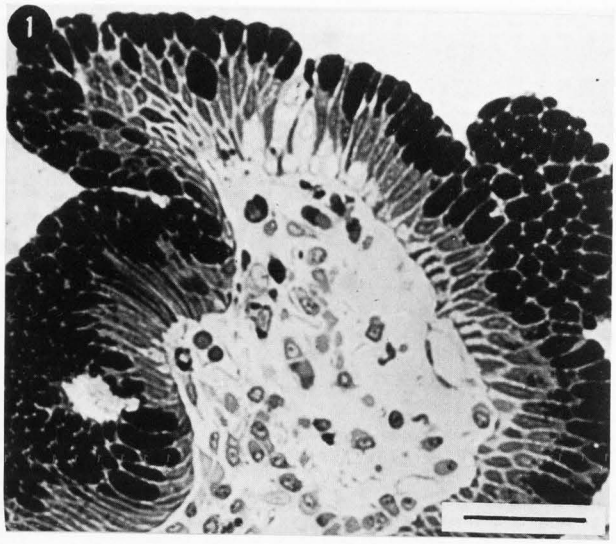
Fig. 18 shows an LMI of a semithin kidney section of a patient with chronic lead poisoning, collected on glass, stained with toluidine blue, silver methenamine and safranin. After post-staining with uranyl acetate and lead citrate it was not possible to evidence, by SEM, the specific intranuclear inclusions at proximal tubule level (Fig. 19a).
However, X-ray examination by S(T)EM on an adjacent unstained section showed the presence of lead (Fig. 19b), probably localized as molecular aggregates not visible at morphological level.

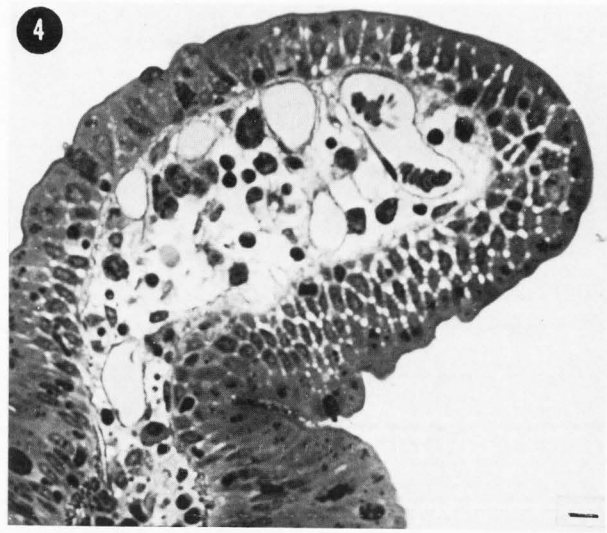
Conclusions

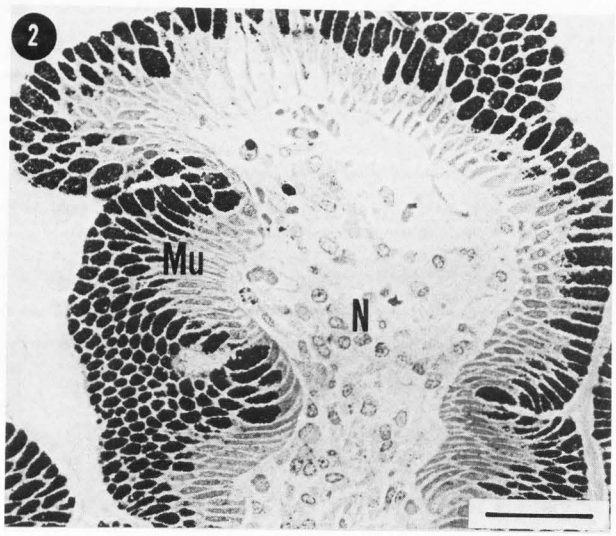
Semithin sections obtained from embedded pathological material and studied by LM, provide some important information to the pathologist (4). Because of low LM resolution, further observation of biological sections are required by electron microscopes (2, 3, 8). TEM does not permit to observe the same field examined by LM, and micrographs obtained are often difficult to correlate with LMI in morphological studies (2). SEM allows examination of semithin sections with a resolution in the range between the light microscope and the transmission electron microscope (2).

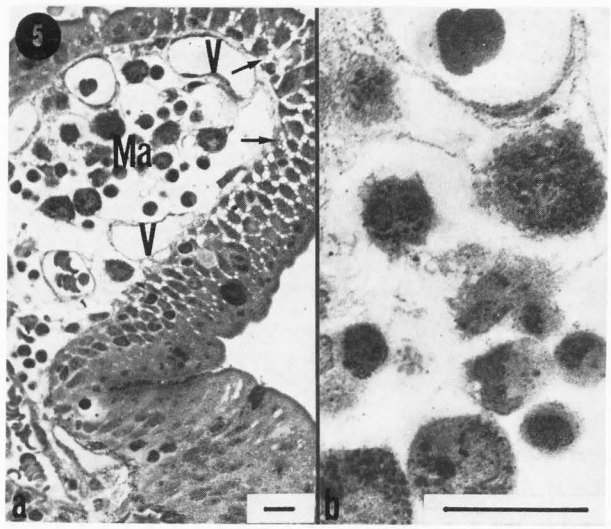
In order to examine the same semithin section, first by LM and then by SEM, both chromatic and electron dense characteristics should be present at the same time. This may be achieved in two different ways: either by using histological dyes with high atomic number (Z) elements (e.g. silver, phosphotungstic and phosphomolybdic acids) or by staining the section, first with a traditional histological dye (e.g. toluidine blue, malachite green, safranin) and secondly by post-staining, after observation by LM, with a conventional electron dense dye (uranyl acetate, lead citrate). In this case better defined electron microscopic images are obtained.

Moreover, two different specimen processing methods are utilized, in relation to the type of









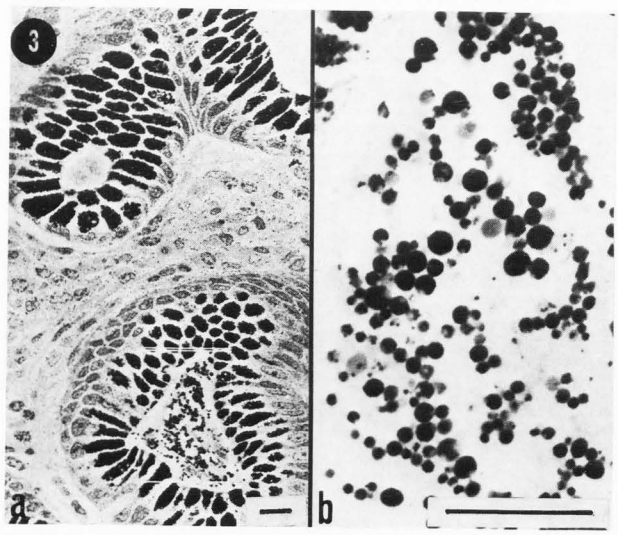


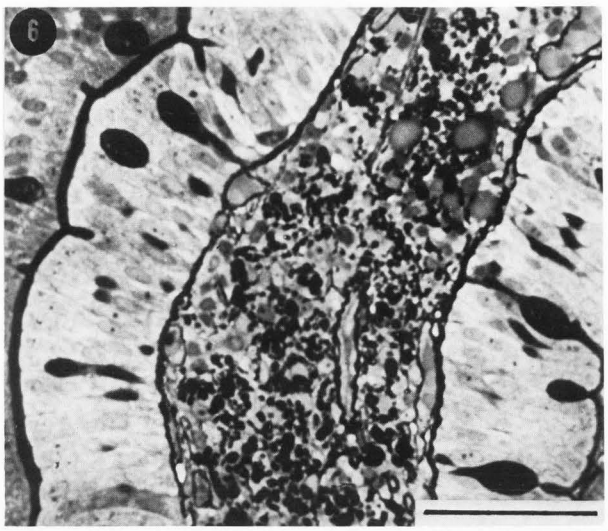
Fig. 1. One step staining. LM of human stomach (1 µm section, mounted on glass and stained with toluidine blue, silver methenamine, safranin). Bar = 50 µm.

Fig. 2. SEI(-) of the same field as seen in Fig.1 Mucous (Mu) and nuclei (N) are well visible. SEI resolution is better than LM resolution. PE 15 kV, S 100 nm, Tilt 0°. Bar = 50 µm.

Fig. 3. a) SEI(-) and b) BEI(-) of another field from human stomach. BEI shows mucous granules in detail. PE 30 kV, S 100 nm, Tilt 0°. Bar = 10 µm.

Fig. 4. One step staining. LM of pathological (AIDS) human small intestine (1 µm section, mounted on glass and stained with toluidine blue, silver methenamine, safranin). Bar = 10 µm.

Fig. 5. SEI(-) of the section as seen in Fig. 4. a) SEI(-) shows a shortened and thickened villous. The covering epithelium presents some increase in the intercellular spaces (arrows). The lamina propria presents some distended vessels (V) and numerous macrophages (Ma) containing argentaffin granules. b) SEI(-) shows these granules in detail. PE 30 kV, S 100 nm, Tilt 15°. Bar = 10 µm.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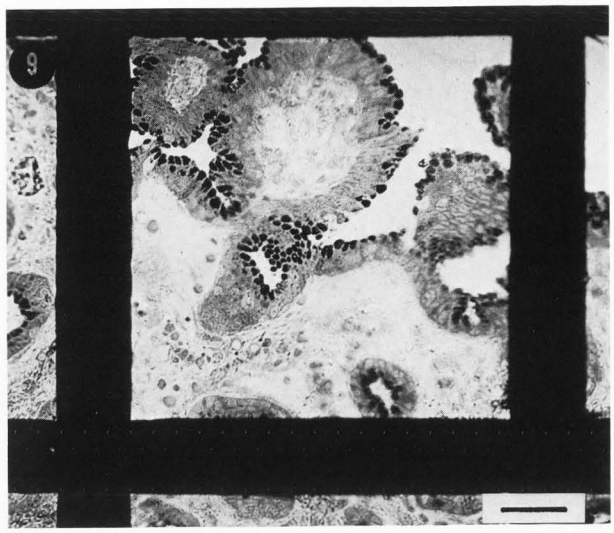
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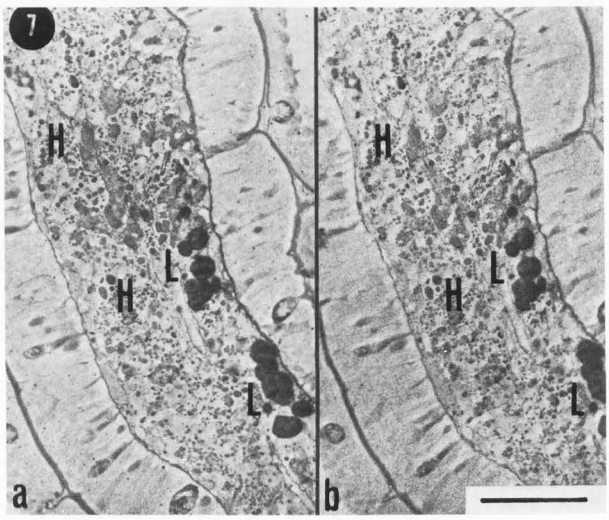
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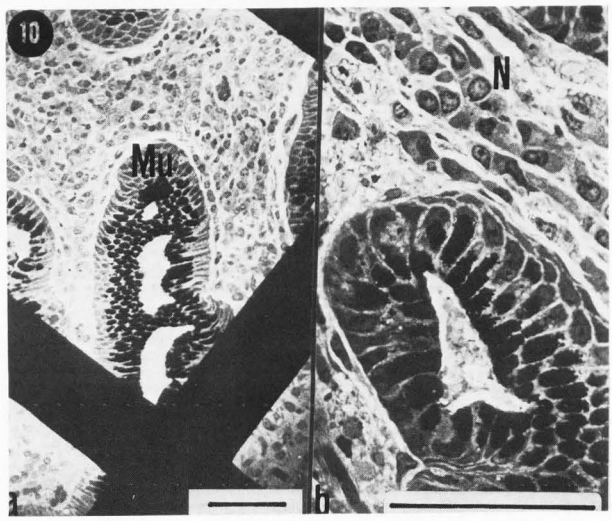
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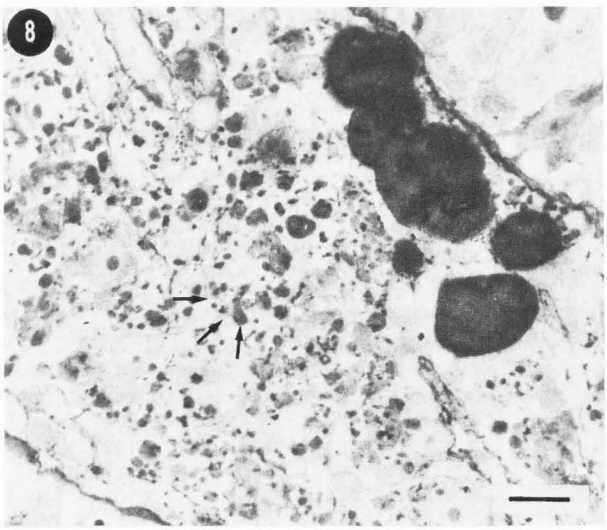
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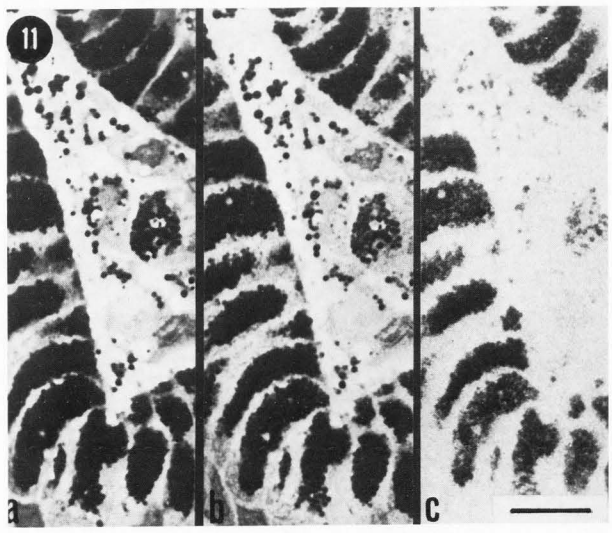
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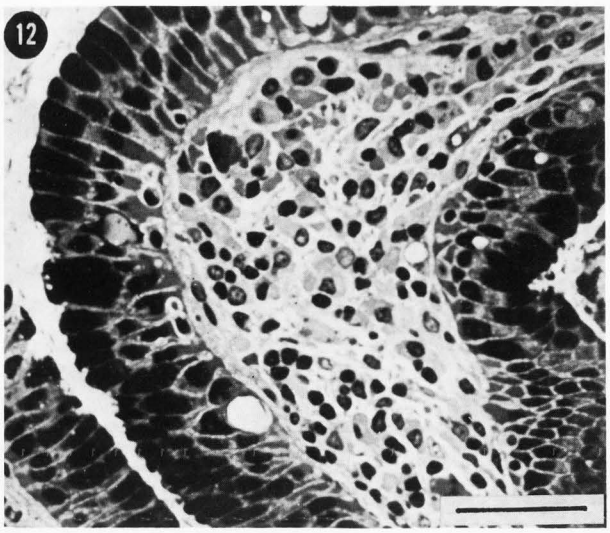
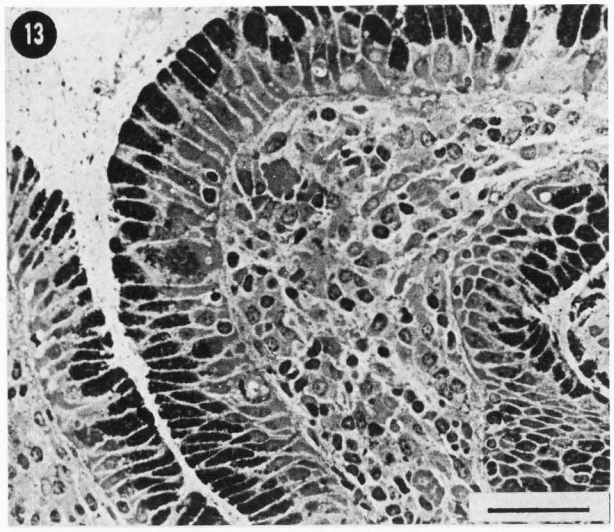
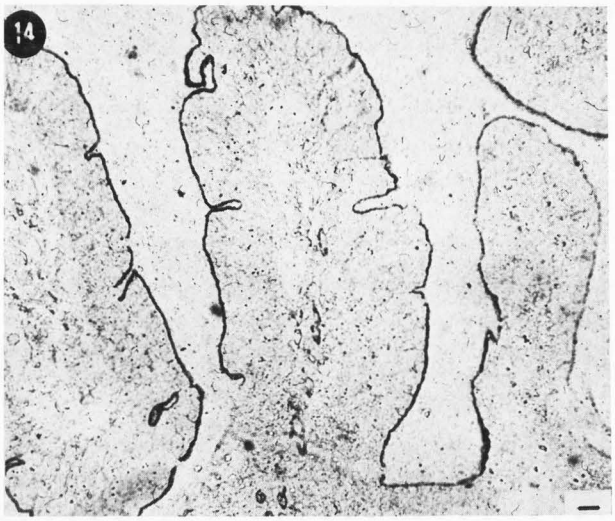






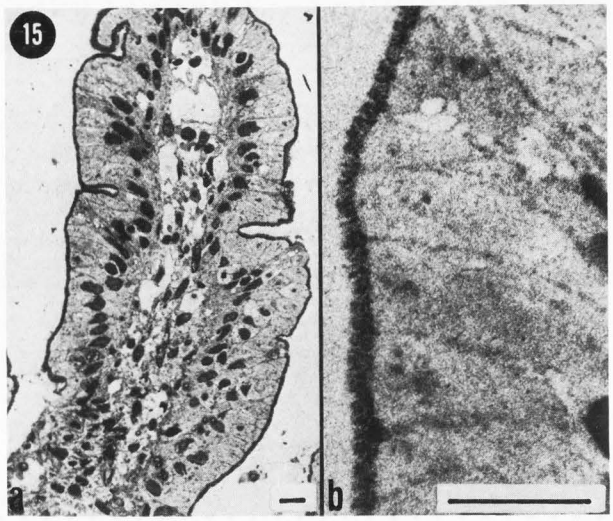


Fig. 6. One step staining. LM of pathological (Whipple's disease) human small intestine (1 µm section, mounted on glass and stained with toluidine blue, silver methenamine, safranin). Bar = 10 µm.

Fig. 7. a) SEI(-) and b) BEI(-) of the same field as seen in Fig. 6. The covering epithelium does not appear to be affected. The lamina propria is bloated by masses of histiocytes (H). Lipid drops are also visible (L). PE 30 kV, S 100 nm, Tilt 15°. Bar = 50 µm.

Fig. 8. SEI(-) of Fig. 7 at higher magnification A detail of argentaffin particles contained within the histiocytes (arrows). PE 30 kV, S 100 nm, Tilt 15°. Bar = 10 µm.

Fig. 9. One step staining. LM of pathological (Aspirin gastritis) human stomach (1 µm section, mounted on grid , stained with toluidine blue, silver methenamine, safranin). Note the pseudopolypoid aspect of gastric mucosa. Bar = 50 µm.

Fig. 10. TEI of the section as seen in Fig. 9. Mucous (Mu) and nuclei (N) are very visible. PE 30 kV, S 100 nm , Tilt 0°. Bar = 50 µm.

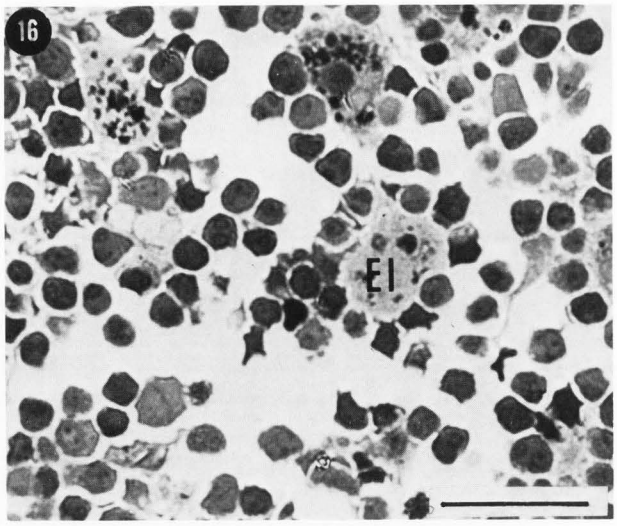
Fig. 11. A correlating a) TEI, b) SEI(-) and c) BEI(-) of the same field viewed in Fig. 10. PE 30 kV, S 100 nm, Tilt 15°. Bar = 10 µm.

Fig. 12. Two step staining. LM of human stomach (1 um section, mounted on glass, stained with toluidine blue). Bar = 50 µm.

Fig. 13. SEI(-) of the same field as seen in Fig. 12; uranyl acetate and lead citrate counterstain. PE 15 kV, S 100 nm, Tilt 0°. Bar = 50 µm.

Fig. 14. Two step staining. LM of human small intestine (1 µm section, mounted on glass, and stained to visualize actin through a protein A-gold immunocytochemical technique of which the intensity has been enhanced by silver precipitation). Bar = 10 µm.

Fig. 15. a) SEI(-) and b) BEI(-) of the same field as seen in Fig. 14. Uranyl acetate counterstain. SEI shows nuclei and actin localization. BEI is a high magnification at brush border level. PE 20 kV, S 100 nm, Tilt 0°. Bar = 10 µm.



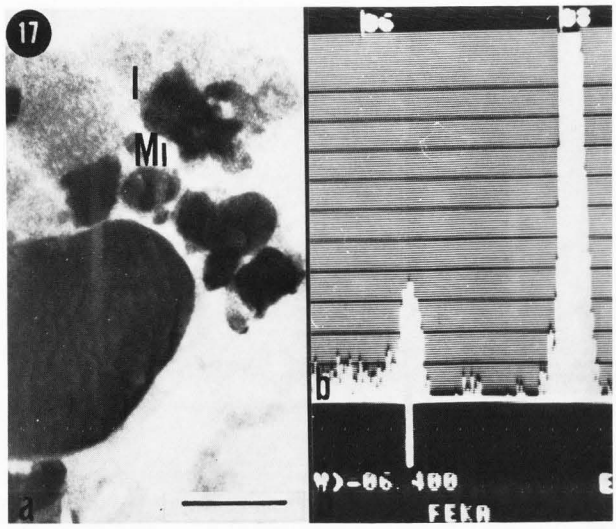


Fig. 16. Two step staining. LM of human pathological (sideroachrestic anaemia) bone marrow (1 µm section, mounted on glass and stained with malachite green and safranin). Note the presence of three erythroblastic islands (EI). Bar = 50 µm.

Fig. 17. a) S(T)EM image of sideroachrestic anaemia (1 µm section, mounted on grid and stained with uranyl acetate). A sideroblast containing pathological inclusions (I) and sidero-mitochondria (Mi). PE 80 kV, S 20 nm, Tilt 0°. Bar = 10 µm. b) EDXA spectrum performed on mitochondria revealed the presence of iron.

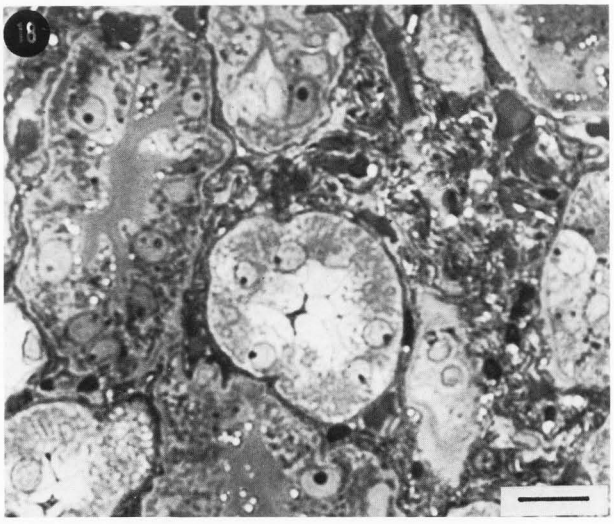


Fig. 18. Two step staining. LM of human pathological (lead poisoning) kidney (1 µm section, mounted on glass and stained with toluidine blue, silver methenamine, safranin). Proximal tubules are evident. Bar = 10 µm.

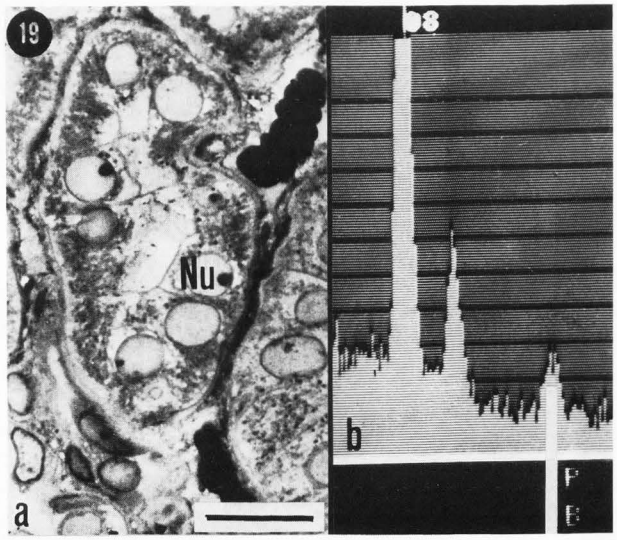


Fig. 19. a) SEI(-) of the section viewed in Fig. 18, after post-staining with uranyl acetate and lead citrate. The specific intranuclear lead inclusions are not visible. Some nucleoli are present (Nu). PE 30 kV, S 200 nm, Tilt 15°. Bar = 10 µm. b) EDXA spectrum performed on a similar unstained section in S(T)EM revealed the presence of lead.

support required by the electron observation procedures (grid or glass) and depending on the type of detectors used for examination.

Various studies carried out on these specimens have demonstrated the possibility of applying SEM in the examination of embedded and sectioned biological material. The simplest method to obtain suitable, reproducible specimen was determined. The most convenient procedures is, unless TEI or clear X-ray spectra are required (without the background due to the glass support), to cut the embedded material, to collect the sections directly on a glass slide and to histologically stain them; after observation and photographing by LM, they are stained with electron dense dyes, covered with a protective carbon evaporation layer and observed by SEM.

These procedures for biological sections are simple and give a good ultrastructural preservation. SEM examination allows the survey of the same entire area of a large specimen previously observed by LM and correlated information may be obtained if SE, BSE, TE and X-ray detectors are used. The following may be obtained from a specimen prepared as above:

. SE images equivalent to BSE images.

. Well resolved SE images with a total compositional signal collected (due to the absence of the specimen's surface morphological contribution (8, 9) and a contrast comparable to that of LMI.

. X-ray spectra showing better spatial resolution than that obtained from traditional bulky specimens.

In conclusion, SEM seems to be effective to survey biological embedded and sectioned material. When ultrastructural investigation is necessary at high magnification, preliminary examination by SEM may be of great help in localizing the areas to be observed by TEM.

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Discussion with Reviewers

Reviewer I: How much additional time is required to process the semithin sections for SEM before examination in the TEM ?

Authors: Not much additional time. Semithin sections collected on glass require only the time to reduce the glass to the same size as the SEM stubs. For semithin sections mounted on grid, it is necessary the time which depends on the operator, to transfer the sections from the glass to the grid. Concerning the staining time, utilization of two step staining time method adds 10-20 minutes more. Obviously, the one step staining does not require more consuming time.