

AN IMPROVED OsO_4 MACERATION METHOD FOR THE VISUALIZATION OF INTERNAL STRUCTURES AND SURFACES IN HUMAN BIOPTIC SPECIMENS BY HIGH RESOLUTION SCANNING ELECTRON MICROSCOPY

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Abstract

We have further modified the aldehyde/ OsO_4 /dimethylsulfoxide (DMSO)/freeze cracking/DMSO/ OsO_4 /DMSO maceration method, in order to render it more suitable to the analytical study of normal and pathological human bioptic samples. The new technique, which has been made easier and quicker, allows the visualization not only of cytoplasmic organelles but also of internal surfaces. The changes consist of the use of 1% OsO_4 -1.25% $\text{K}_4\text{Fe}(\text{CN})_6$ as a secondary fixative and in the sectioning of tissues at room temperature by a chopper. The OsO_4 -ferrocyanide mixture, by binding osmium to the tissue, allows the omission of further treatment with OsO_4 and tannic acid. It also allows the storage of specimens in phosphate buffered saline at 4°C for several days before the start of the maceration. Since internal structures are exposed by cutting and not by freeze fracturing, the treatment with DMSO becomes unnecessary. Furthermore, 44 hours at 25°C is enough to macerate specimens with 0.1% OsO_4 thereby reducing the total time for preparation to 48 hours. By shaking the specimens during or after maceration, we were able to remove organelles and to expose the inner surface of the plasmalemma and its special structures (e.g., folds, intercellular canaliculi, and cellular junctions). A further result is the visualization, in hepatic needle biopsies, of pathological features of possible diagnostic value.

Key Words: High resolution scanning electron microscopy, maceration, intracellular structures, human biopsies, liver.

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Introduction

Owing to its unique ability of providing, on the same specimen, both panoramic and highly magnified views, the scanning electron microscope (SEM) appears the instrument of choice for complementing, even at the 3-dimensional (3D) level, information obtained with the light microscope (LM) and the transmission electron microscope (TEM). However, despite the availability of the new field emission (FE) microscopes and the reintroduction (Tanaka, 1980; Tanaka and Naguro, 1981; Tanaka and Mitsushima, 1984) of the old OsO_4 maceration method (Bolles Lee and Henneguy, 1887) for the visualization of intracellular structures, the use of SEM in the study of human tissues and particularly of biopsies is still rather limited. Apart from our studies on salivary glands (Riva *et al.*, 1995, 1996, 1998; Segawa and Riva, 1996; Segawa *et al.*, 1998) and liver (Faa *et al.*, 1994, 1997) there are in fact only the findings reported by Lea *et al.* (1994) on a single human hepatic needle biopsy. In our experience this is mainly due to the fact that the most popular osmium maceration method (Tanaka and Mitsushima, 1984) requires the fracture of tissues frozen with liquid nitrogen, a procedure unsuitable for the analytical studies of small samples since it does not allow the cutting of the entire specimen into sections of controlled thickness (Table 1). Moreover, even though we (Riva *et al.*, 1993) succeeded in removing this obstacle by cutting the bioptic samples at room temperature (RT) by a tissue chopper, the protocol is still too long and rigid. A further disadvantage of the technique, that renders it inconvenient in the study of human specimens, of which the arrival is unpredictable, is the fact that, once started, the protocol can not be stopped at any stage.

Materials and Methods

Tissues studied consisted of specimens obtained from 60 patients undergoing surgery for the removal of tumors and of 60 hepatic needle biopsies (measuring about $1 \times 1 \times 15 \text{ mm}^3$) for diagnostic use. Informed consent was obtained from each patient to participate in this study. Normality of tissues of surgical origin was assessed by parallel LM stud-

Table 1. Preparation of tissue for SEM according to Tanaka and Mitsushima (1984).

1. *Fixation*: strips of tissue of 1x1x3 mm³ in 0.5% glutaraldehyde + 0.5% formaldehyde in 0.1 M cacodylate buffer (CB) 1x15 min, pH 7.4 at RT
2. *Rinsing*: PBS 3x10 min
3. *DMSO treatment*: 25% and 50% in water for 30 min each
4. *Freeze cracking*: specimens frozen on a metal plate chilled with liquid nitrogen are cracked in two by a razor blade and a hammer.
5. *Thawing*: the cracked pieces are placed in a 50% aqueous DMSO solution at RT
6. *Rinsing*: PBS until DMSO is completely removed
7. *Postfixation*: 1% OsO₄ in 1/15 (0.067) M phosphate buffer (PB) 1 x 60-120 min
8. *Maceration*: 0.1% OsO₄ in 1/15 (0.067) M PB 72 hours at 293 K (20°C)
9. *Rinsing*: PBS 3x10 min
10. *Second postfixation*: 1% OsO₄ in 1/15 (0.067) M PB for 1 hr
11. *Rinsing*: PBS 3x10 min
12. *Conductive staining*: 2% aqueous tannic acid solution overnight; rinsing in PBS 3x10 min; 1% OsO₄ in 1/15 (0.067) M PB for 1 hour
13. *Rinsing*: PBS 3x10 min
14. *Dehydration* with ethanol, *critical point drying* from CO₂, *coating* with platinum (about 3 nm) with an ion coater
15. Observation by a FE SEM (Hitachi HFST)

ies. Prior to fixation some specimens from salivary glands were stimulated *in vitro* (Segawa *et al.*, 1998) with the secretagogue 5-hydroxydopamine (Abe *et al.*, 1996). The protocol used here was the following:

1. *Fixation*: Strips of tissue of 1x1x5 mm were fixed with 0.5% glutaraldehyde + 0.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2), 1 x 15 min at RT.
2. *Rinsing*: phosphate buffered saline (PBS) 3 x 10 min at RT.
3. *Postfixation*: 1% OsO₄-1.25% K₄Fe(CN)₆ in distilled H₂O, 2 hours in the dark at RT.
4. *Rinsing*: PBS 3x10 min at RT; specimens can be stored in this solution at 4°C for a maximum of 7 days.
5. *Sectioning*: specimens are embedded in 1% agarose in distilled H₂O and cut into 150 µm thick sections by a Sorvall (New Town, CT) TC2 tissue sectioner (this company no longer exists, but an equivalent instrument is the McIlwain Tissue Chopper, Mickle Laboratory Engineering Co., Guildford, Surrey, UK).
6. *Rinsing*: PBS 3x10 min at RT.
7. *Second postfixation*: 1% OsO₄ -1.25% K₄Fe(CN)₆

(Figures 1-4 on facing pages)

Figure 1. Survey view of a portion of a submandibular gland serous acinus. C: intercellular canaliculus; N: nucleus; secretory granules: arrows.

Figure 2. Cells of a parotid gland salivary duct filled with mitochondria (m). The plasmalemma exhibits the typical basal folds (arrows). N: nucleus; AP: apical projection; L: lumen.

Figure 3. Cells of a kidney proximal convoluted tubule bearing long microvilli (mv). Their cytoplasm contains many mitochondria (m). N: nucleus.

Figure 4. Portion of a secretory tubule of a ceruminous gland. A well developed smooth endoplasmic reticulum (E) occupies most of the cells. Some mitochondria (m) and lipofuscin granules (arrow) are present. N: nucleus.

in distilled H₂O, 1 hr in the dark at RT.

8. *Rinsing*: PBS 3x10 min at RT.

9. *Maceration*: 0.1% OsO₄ in PBS, 44-48 hrs at 25°C.

10. *Rinsing*: PBS 3x10 min at RT.

11. *Dehydration* through a graded acetone series, critical point drying from CO₂, coating with platinum (2 nm) by an Emitech (Ashford, UK) 575 turbo sputtering apparatus.

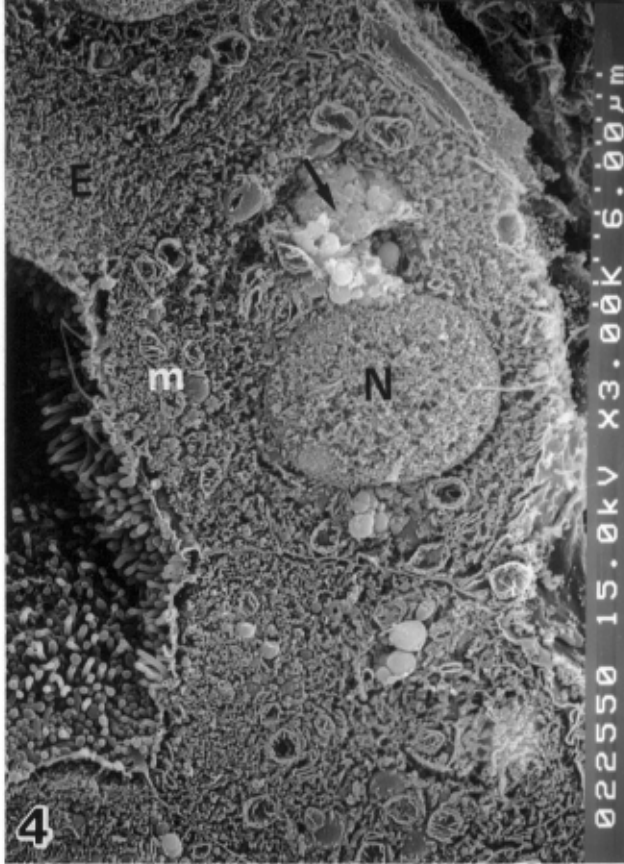
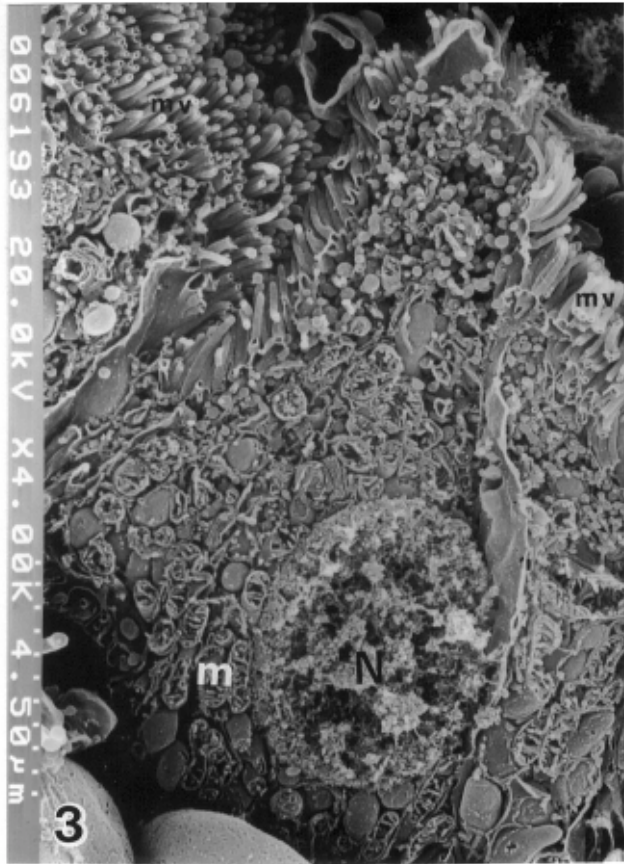
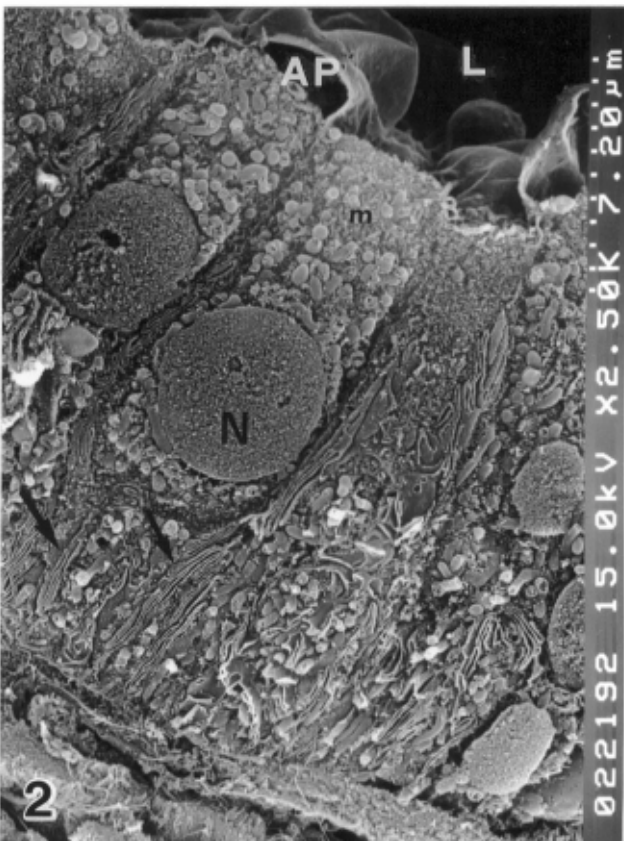
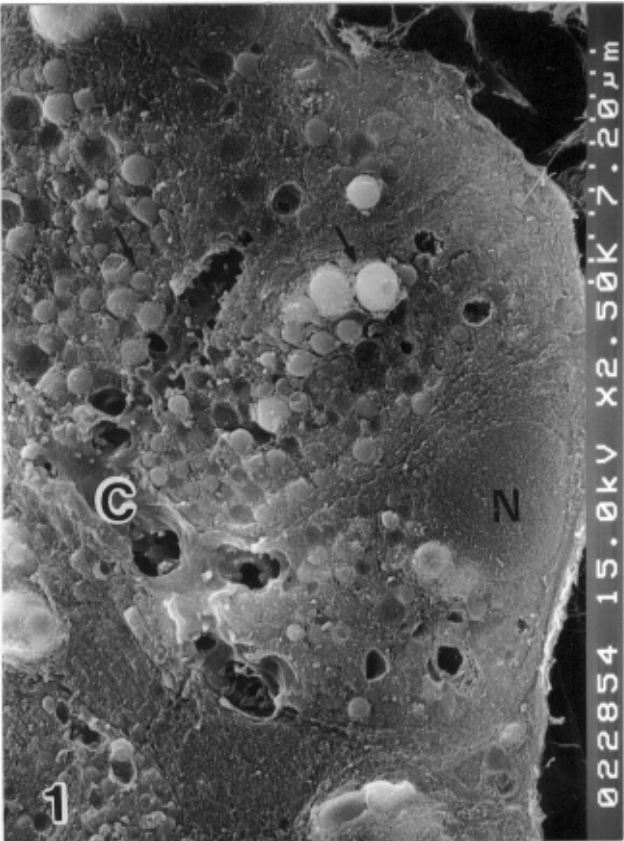
12. *Observation* in a field emission Hitachi (Tokyo, Japan) S4000 SEM operated at 15-20 kV.

Note: During rinsing the use of a rotating agitator is advisable; specimens can be shaken also during maceration in order to remove cytoplasmic organelles.

Results and Discussion

The method

Fixation and postfixation. A major improvement in our protocol is the use of the osmium tetroxide-ferrocyanide mixture as a secondary fixative in the postfixation step. The mixture, introduced in TEM by Karnovsky (1971) because of its capability of enhancing membrane contrast, is more stable and easy to handle than the original OsO₄ buffered fixative and can be employed even in the absence of a fume hood, a very useful characteristic when human biopsies are to be fixed in the surgical ward. The mixture (Hayat, 1989, 1993) contains intermediate osmium compounds that are chelated by donor atoms in the tissue macromolecules resulting in a greater deposition of osmium. This abolishes the need for further conductive staining with tannic acid introduced by Tanaka and Mitsushima (1984) in order to bind more osmium to tissues. Following postfixation with the osmium-ferrocyanide mixture, tissues can be stored for several days in PBS at 4°C without



impairing the results of the maceration process. This possibility to interrupt the procedure in an early stage of the preparation is particularly useful in the case of human biopsies, not only because it allows the processing of specimens that come in early or late hours of the day or at times when technicians are not available, but also because tissues stored in PBS can be mailed from a peripheral hospital to a laboratory endowed with a FE SEM.

At variance with fixation with buffered OsO₄ that blackens the tissues, the osmium-ferrocyanide mixture gives them a brownish color which tends to darken during storage in PBS.

Sectioning. As described previously (Riva *et al.*, 1993) tissues stored in PBS are cut at RT into sections of approximately 150 µm by a Sorvall TC2 tissue sectioner. To fix the specimen to the stage of the tissue sectioner, we use a drop of 1% aqueous solution of agarose warmed to 37°C. We have now ascertained that the treatment with dimethyl sulfoxide (DMSO), introduced by Tanaka and Naguro (1981) and formerly recommended also by us (Riva *et al.*, 1993), can be omitted.

Second postfixation. A second treatment with the osmium-ferrocyanide mixture improves the following maceration process. However, it can be omitted in tissues that have been stored in PBS for more than 24 hrs.

Rinsing. As specified in Materials and Methods, sections must be carefully rinsed in PBS at every step to avoid the presence of precipitates. The use of a rotating agitator is strongly advised.

Maceration. Following several trials we found that, on average, best results are obtained with a maceration time of 44-48 hours at 25°C. Of course, however, the maceration time can be shortened or lengthened according to the tissue studied and to the peculiar needs of the experiment. Temperature is very important and a thermostatic stove should be used. In general, higher temperatures speed up the process while lower temperatures slow it down. At 45°C (Riva *et al.*, 1993) 3 hours are enough to macerate most specimens. Such a treatment, however, though useful because it reduces the whole preparation time to 10 hours, cannot be used routinely because parts of the specimen are always damaged, which makes it difficult to study the specimen in a more than purely qualitative way. During the procedure it is mandatory to check, from time to time, the macerating solution and replace it when it is no more limpid and colorless. In our experience, we found that this should be done at least after the first 3 hours of maceration and in the morning following an overnight treatment.

Maceration can be performed also on specimens that are shaken with a rotating agitator during the whole process. In this case many cells lose their complement of organelles thereby allowing the direct visualization of the plasmalemma and of its specializations (e.g., folds or inter-

(Figures 5-8 on facing pages)

Figure 5. Golgi apparatus of a submandibular gland serous cell. The *cis* face presents numerous fenestrations (arrow). RE: rough endoplasmic reticulum.

Figure 6. Detail of the cytoplasm of a parotid secretory cell. Note (arrow) some small fenestrations in the cisterns of the rough surfaced endoplasmic reticulum. G: secretory granule.

Figure 7. Mitochondrion from a cell of a kidney convoluted tubule.

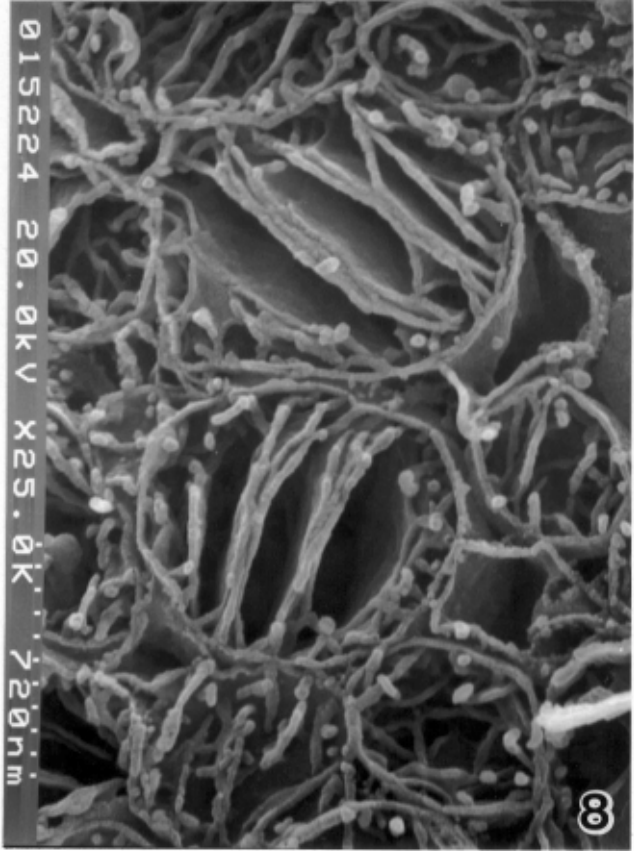
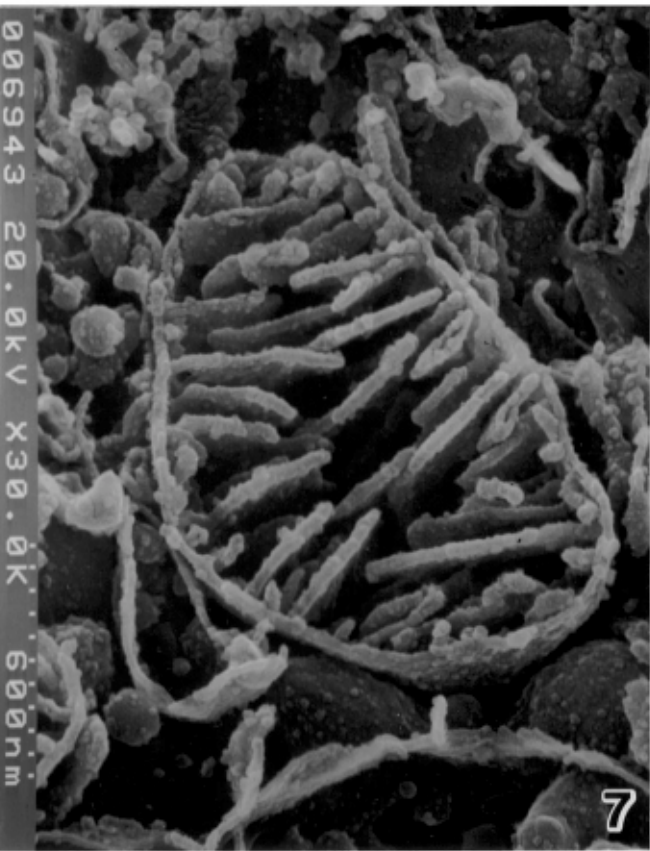
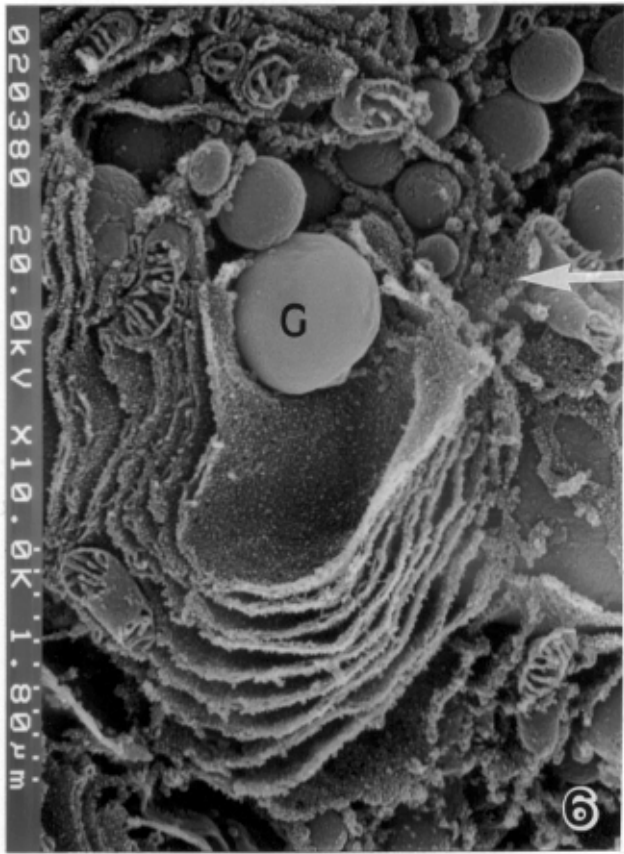
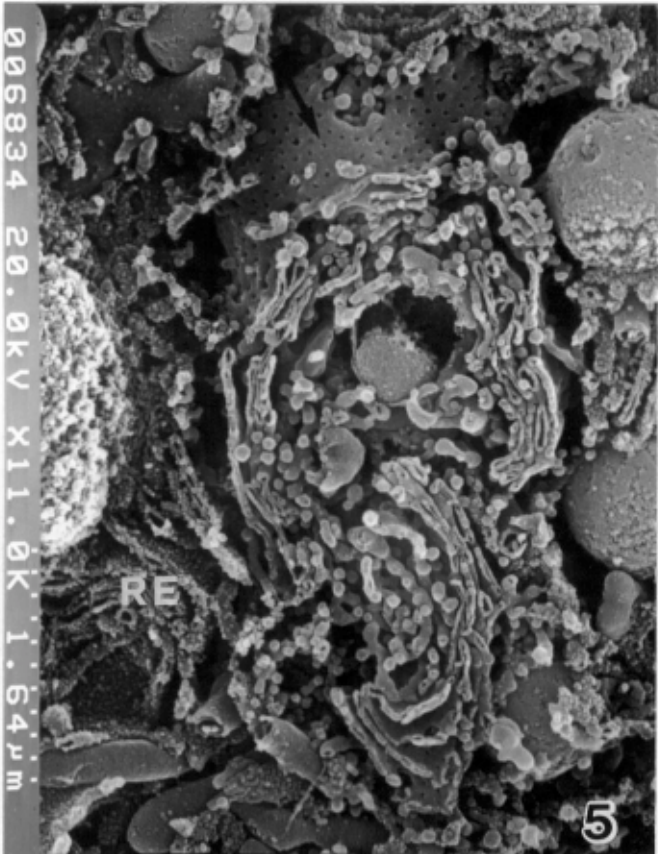
Figure 8. Deep posterior lingual gland. Mitochondria from an oncocyte. The two mitochondria in the center exhibit closely packed lamellar cristae.

cellular canaliculi).

Morphological findings

As shown in Figures 1-4, our technique allows low magnification views of various kinds of cells. Figures 1-2 are panoramic views of acinar and duct cells of major salivary glands that give interesting information on their cytoarchitecture. In Figure 1, besides the granules that occupy the apical cytoplasm, a few intercellular (secretory) canaliculi are observed. The cells of salivary gland ducts (Fig. 2) exhibit the typical basal folds while their cytoplasm is filled with mitochondria; on their luminal surfaces the so called apical projections (Riva, 1992) are seen. Numerous mitochondria and long microvilli are observed (Fig. 3) in a cell from a kidney proximal convolute tubule, while an arrangement of cytoplasmic organelles similar to that described in TEM (Testa Riva and Puxeddu, 1980) is seen (Fig. 4) in a sectioned tubule of a ceruminous gland.

At higher magnification our method gives details of cytoplasmic organelles, fully comparable with those obtained in animal tissues with the original freeze cracking technique (e.g., Tanaka, 1980; Tanaka and Naguro, 1981; Tanaka and Mitsushima, 1984; Hanaki *et al.*, 1985; Tanaka *et al.*, 1986; Tanaka, 1987; Lea and Hollemberg, 1989; Lea *et al.*, 1994; Naguro and Iwashita, 1992). Figure 5 represents a single Golgi complex from a secretory cell of a submandibular gland, clearly showing its relationship with the rough surfaced endoplasmic reticulum (RER) and the fenestrations on its *cis* cistern. As shown in Figure 6, small fenestrations are appreciable even in the cisterns of the RER of a serous secretory cell. Figures 7 and 8 illustrate sectioned mitochondria from a cell of a kidney convoluted tubule and from an oncocyte of a lingual gland. The latter shows closely packed cristae that encompass the entire breadth of the organelle with an arrangement matching that seen by TEM (Tandler, 1966; Riva *et al.*,



1988). By shaking the specimens with a rotating agitator during maceration, cytoplasmic organelles may be partially or totally removed, leaving the cytoplasmic side of the plasmalemma available for inspection. This applies also to nuclei where the chromatin can be removed, allowing the visualization of the inner side of the nuclear envelope and of its complement of nuclear pores. In some cases (Fig. 9), when the nuclear envelope is broken and turned upside, its outer sheet studded with ribosomes is visualized as well. In a secretory cell of a major sublingual gland we also observed some annulate lamellae endowed with pores (Fig. 10) which look more numerous and regularly arranged than those observed in the nuclear envelope.

In cells where organelles are completely removed, the inner side of the plasmalemma can be seen (Figs. 11 and 12). Moreover, in serous cells such as those of salivary glands, the cytoplasmic aspect of intercellular canaliculi becomes evident (Figs. 11-14). Seen from this side, these canaliculi look similar to the biliary canaliculi (Riva *et al.*, 1995) and to intracellular canaliculi of parietal cells of rat stomach (Ogata and Yamasaki, 1993). They appear as tubular structures fenestrated by holes (Figs. 13 and 14) corresponding to the bases of microvilli deprived of their cytoskeleton. They also show occasional bulges and indentations possibly related to the process of exocytosis. In previous studies (Segawa and Riva, 1996; Segawa *et al.*, 1998) we have shown that canaliculi of human salivary glands, stimulated *in vitro* with isoproterenol or carbachol, exhibit morphological alterations peculiar to the relevant secretagogue. This has been demonstrated in the present study as well (Figs. 13 and 14) where the false transmitter 5-hydroxydopamine (a stimulant of both α and β sympathetic receptors according to Abe *et al.*, 1996) has been used. The canaliculi show, in fact, protrusions of the size of a secretory granule possibly due to the induced exocytosis. All along the plasmalemma, bordering the unstimulated and stimulated canaliculi, there are often clusters of particles (Figs. 13 and 14) of about 40 nm which have been related (Riva *et al.*, 1998) to the presence of desmosomes.

As stated above, intercellular canaliculi, seen from the cytoplasmic side, look similar to biliary canaliculi. In the vast majority of human liver specimens so far examined (Fig. 15) and in rodent liver (Riva, unpublished results), the intracellular canaliculi do not, however, show the bulges and the indentations seen in intercellular canaliculi of serous glands. Similar to what has been shown for these serous glands (Riva *et al.*, 1996), opened biliary canaliculi can be observed on the lateral plasmalemma of hepatocytes following the detachment of adjacent cells (Fig. 16). In this case, they look like grooves bordered by numerous short micro-villi.

(Figures 9-12 on facing pages)

Figure 9. Parotid gland. Portion of a nucleus of a salivary duct cell seen after removal of the chromatin. A breakage in the envelope allows the visualization of its outer aspect studded with ribosomes (R). Pores are evident on the internal (arrows) and on the external (double arrows) side of the nuclear envelope.

Figure 10. Annulate lamellae in a serous cell of a major sublingual gland. Note that they are continuous with the elements of the rough reticulum studded with ribosomes (arrow).

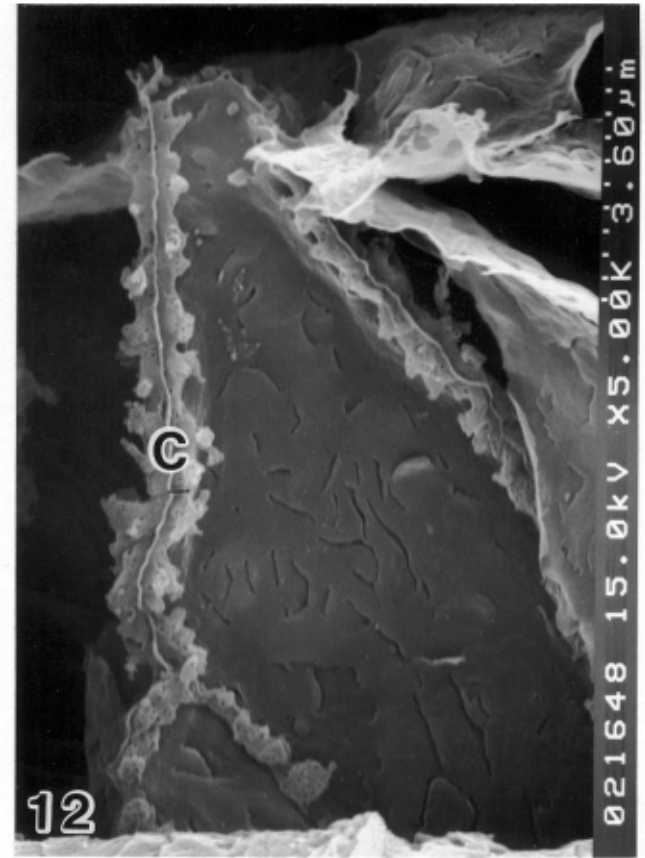
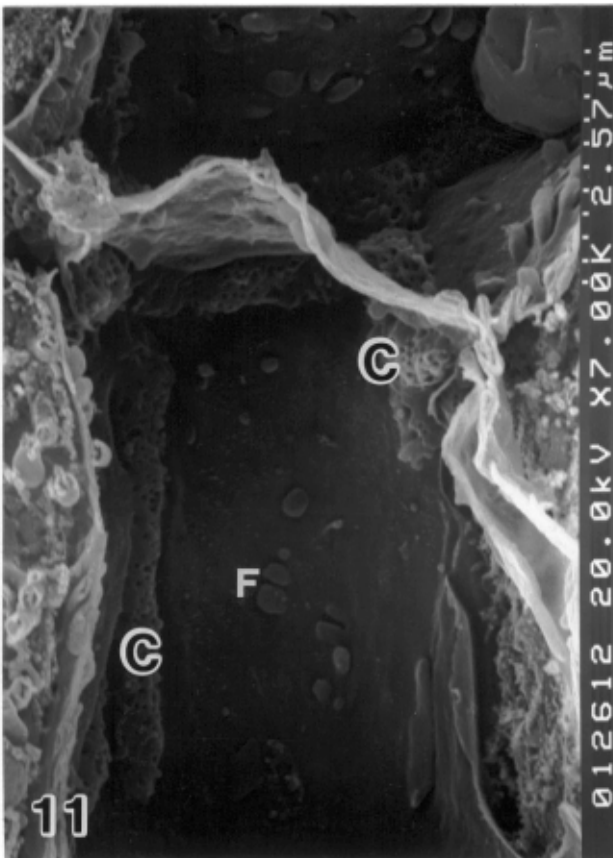
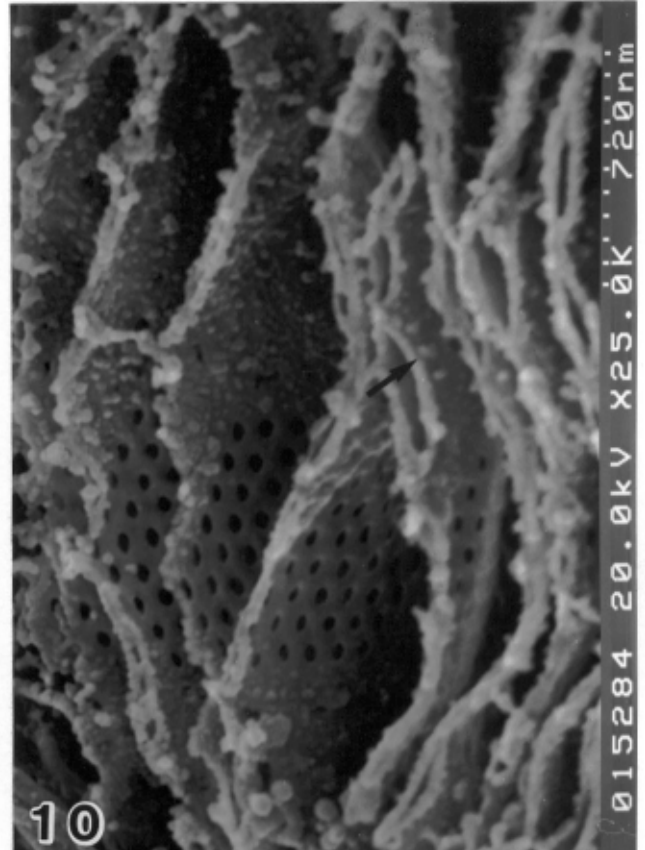
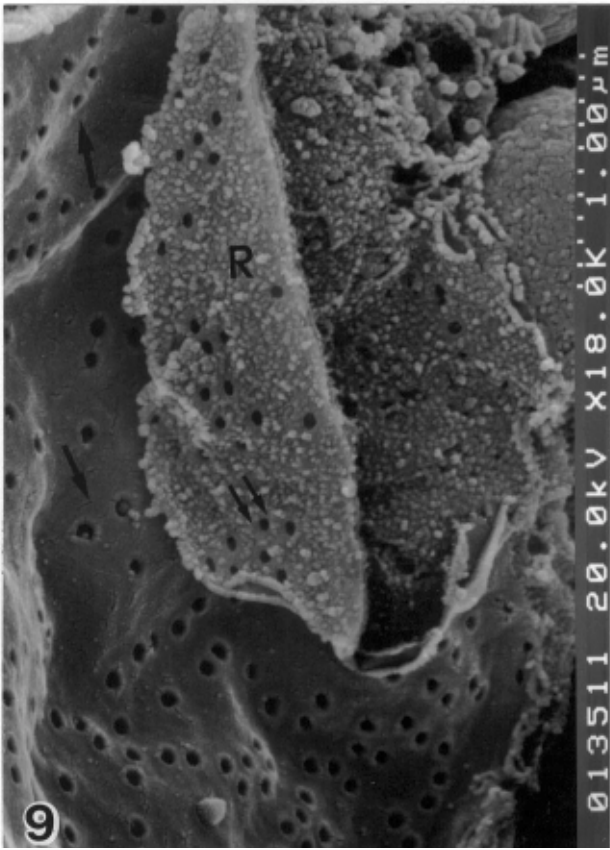
Figure 11. Interior of submandibular gland serous cells seen following removal of cytoplasmic organelles. C: intercellular canaliculi. F: folds of the plasmalemma seen from their cytoplasmic side.

Figure 12. Cytoplasmic side of the plasmalemma of a parotid secretory cell showing fissures and reliefs corresponding to its folds. C: intercellular canaliculus.

Pathology

Although we have published a few papers (Faa *et al.*, 1994, 1997) reporting findings obtained in some liver diseases by our previous method, results obtained in diagnostic histopathology are still preliminary and incomplete, not only because of the drawbacks of the technique, but also because of the lack of data on normal tissue. This applies especially to liver because, despite several attempts, we did not as yet succeed in getting needle biopsies that looked truly normal neither by LM nor TEM. There are, however, some results which seem to demonstrate the possible diagnostic value of our technique. Figure 17 shows a biliary canaliculus with an irregular profile and small bulges, possibly related to the peculiar pathology involved (vanishing bile duct disease). At present, we are now studying all the liver material we have in order to ascertain whether or not it is possible to correlate the 3D morphology of biliary canaliculi to the liver disease involved. Another example of the potential application of our method is its ability to detect very early stages of fibrosis not seen by LM and difficult to detect by TEM, because of its narrow field of view. This can be seen in Figure 18 which shows intralobular pericellular collagen fibril deposition in a case of β -thalassemia major.

The technique we have presented here is relatively simple and requires time for preparation comparable to that needed for standard histology or for TEM. The possibility of interrupting the procedure and also that of mail-



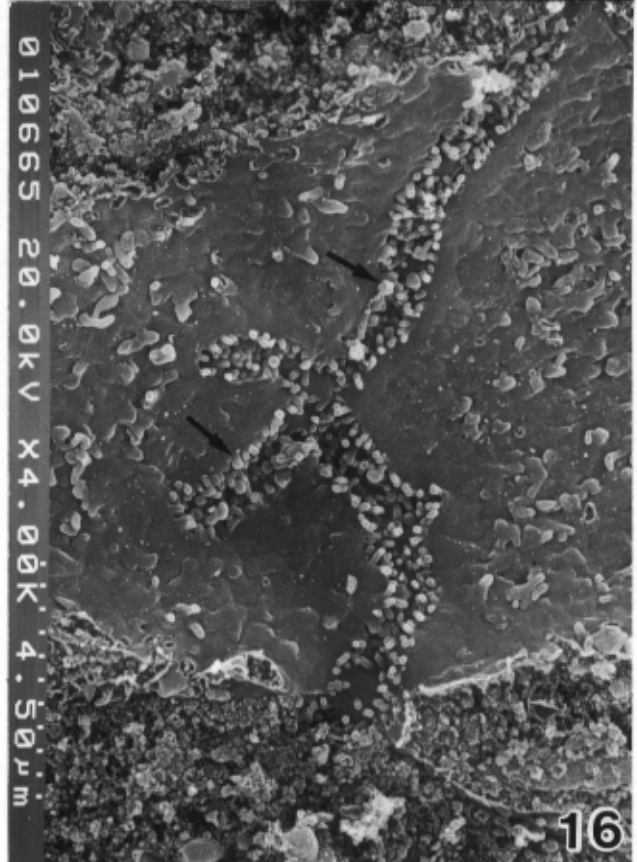
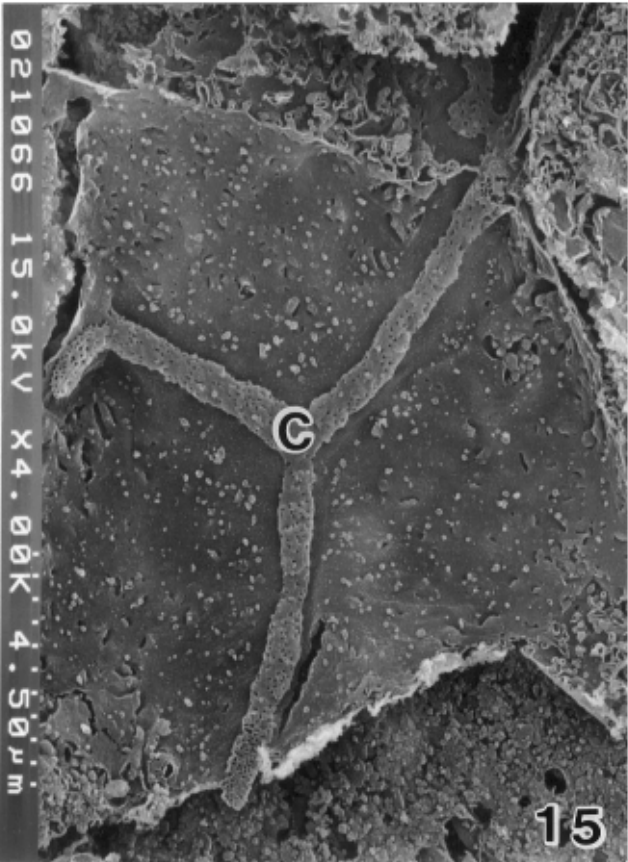
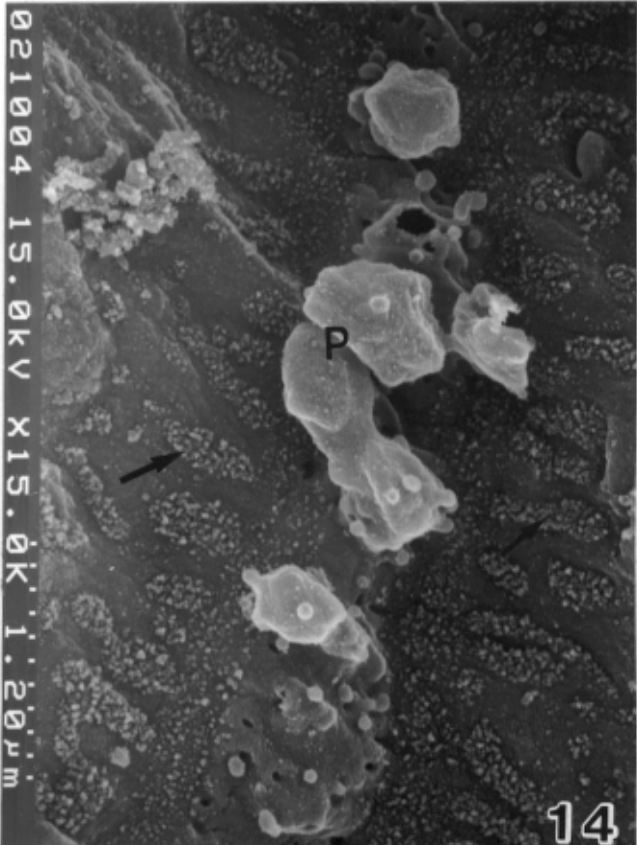
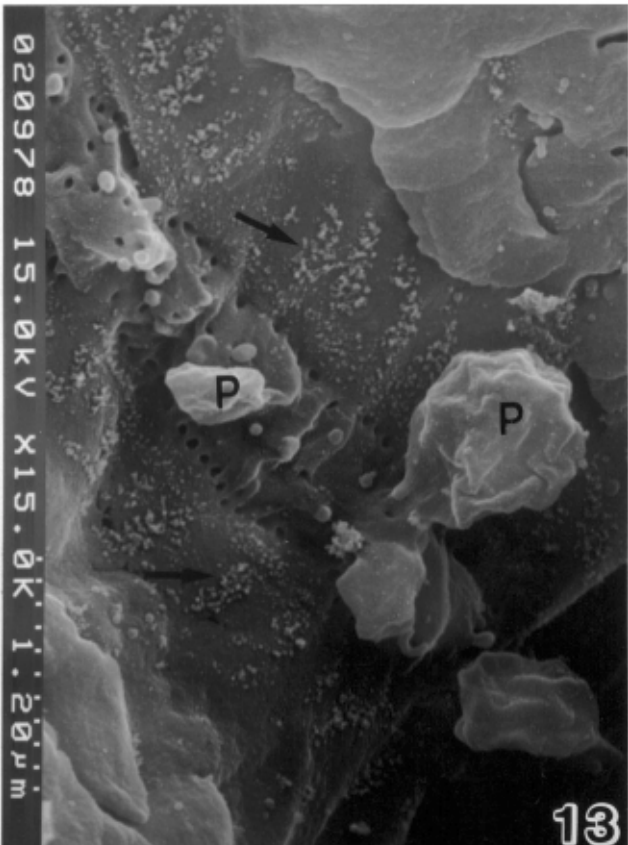
ing the specimens from a peripheral hospital to the SEM laboratory, further enhances its usefulness in diagnostic histopathology. We hope that a widespread use of the method may fully demonstrate its potential as a complement, at the 3D level, to information gained by LM and TEM.

Acknowledgements

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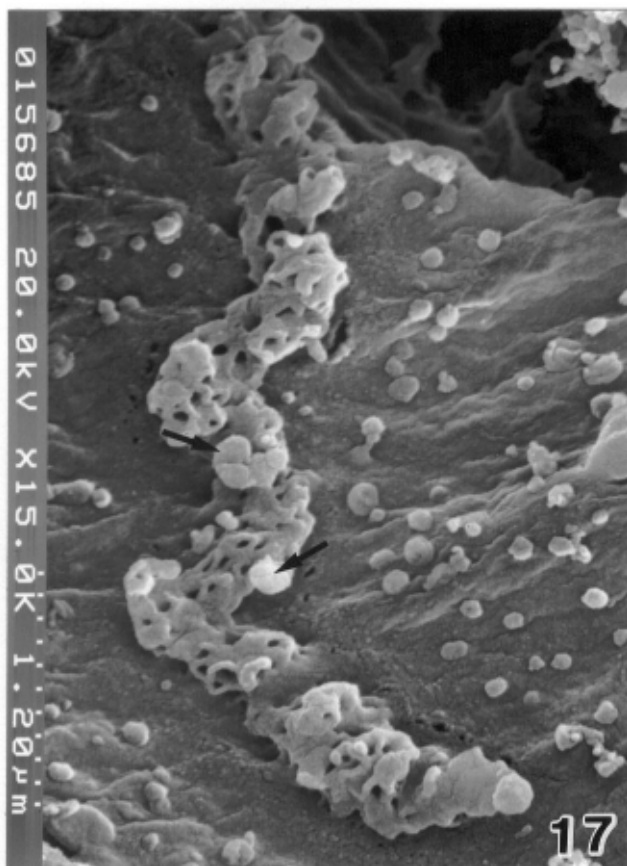


Figure 17. Liver. Altered canalculus in a case of vanishing bile duct disease. The canalculus, which exhibits a tortuous course, shows small protuberances (arrows) possibly related to the pathological process.

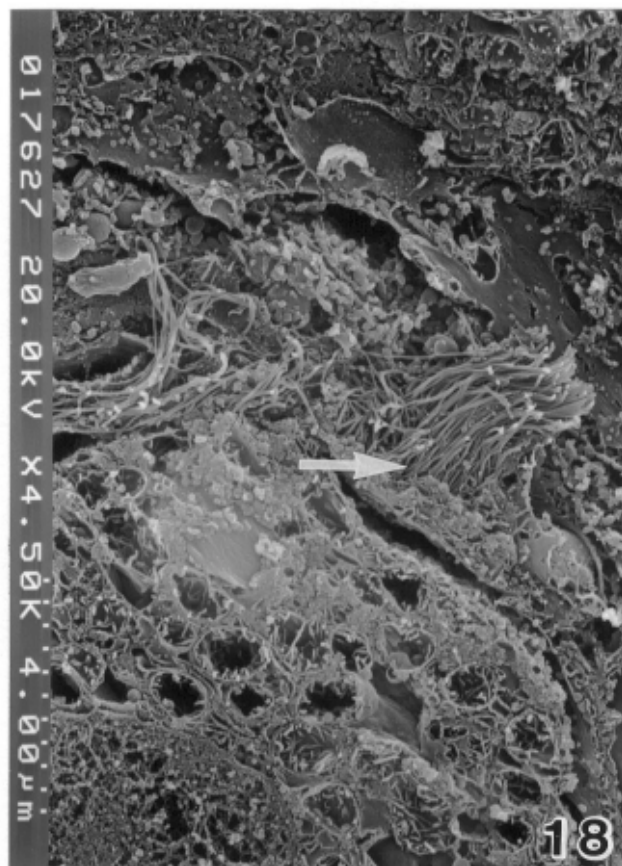


Figure 18. Liver. Intralobular fibrosis in a case of b-thalassemia major. The arrow indicates the bundles of collagen fibrils.

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Discussion with Reviewers

I. ap Gwynn: This method clearly preserves the membranous structures of the cytoplasm, but not the cytoskeleton. Have you attempted to modify the treatment in order to preserve at least some of the cytoskeleton?

Authors: No, it is something we are planning to do in the near future.

R. Wróblewski: Have you tried to use a backscattered electron (BSE) detector, especially when working at low magnification where your images look somewhat pale?

Authors: We have just ordered a high resolution BSE detector and we will try it as soon as it is available.

R. Wróblewski: Could you please explain why the PBS rinse between each preparation step is so important?

Authors: To avoid precipitates produced by chemical interaction or by materials extracted during maceration.

W. Jongebloed: The work shows a good example of what information can be gained when tissue has been subjected to a maceration procedure, although no comparison is given with images of similar non macerated tissue or tissue which has been macerated according to the method of Tanaka and Mitsushima (1984) to show the differences. Particularly the differences with the “Tanaka method” should be shown with a few examples.

Authors: We have not employed here the “Tanaka method” because, for the reasons given in the text, it is unsuitable for the analytical study of human biopsies. If one compares published results obtained by the latter method with those presented here it emerges that they are qualitatively similar. Our technique, however, besides producing a far greater number of surfaces available to inspection, is considerably shorter and simpler.

W. Jongebloed: It is somewhat questionable whether this technique can be used to study *pathological* tissue, because even the authors themselves are not fully sure in certain cases (liver?), whether the observed changes are the result of the maceration process or the result of a pathological process.

Authors: We present a protocol technically suitable for the SEM study of human biopsies. As also was the case in the application of TEM to pathology, the distinction between artifacts and alterations is a matter of controls and experience. This can be assessed only following a prolonged use of our method on different tissues.

W. Jongebloed: The main reason behind the fact that there is not much literature about human biopsies is more likely caused by the fact that human material is not easily available, rather than that a maceration procedure is not at hand.

Authors: The situation may vary among different countries. On the other hand, pathologists will send biopsies to SEM laboratories only when this will be useful to their diagnostic purposes.

W. Jongebloed: Why have the authors used a tissue sectioning apparatus together with agar, while the production of vibratome sections of approximately 150 nm is

very easy without possible contamination?

Authors: A vibratome does not allow the cutting of the entire specimen since part of it must be glued to the stage. Moreover, needle biopsies must be held vertically in order to produce sections in the plane perpendicular to their long axis.

W. Jongebloed: The authors mention the omission of tannic acid, because it should not be necessary for conductivity enhancement. Tannic acid as a ligand in non coating procedures improves internal and external conductivity of a sample, so no external conductive layer is required. The samples studied by the authors all have been coated with Pt. Could you comment on this point?

Authors: To improve internal conductivity we have used the osmium-ferrocyanide mixture instead of tannic acid which requires a much longer time. Our treatment seems to be effective since Tanaka and Mitsushima (1984) used, following the tannic acid treatment, an “external” coating with platinum of 3 nm, while a 2 nm coating was enough for our specimens.

W. Jongebloed: Why have you used 20 kV in almost all cases, while your technique allows more specific surface information to be visualized which is almost impossible with these high accelerating voltages? Moreover the use of low kV (in the case of FE SEM even 1-3 kV) is more attractive when tannic acid is used. Could you comment on this?

Authors: Although Tanaka and Mitsushima (1984) do not report the acceleration voltage employed, 25 kV is the one mentioned in a subsequent article by the Tanaka group (Hanaki *et al.*, 1985), while Lea and Hollemberg (1989) used a voltage of 20 kV. We used a voltage of 15-20 kV in order to be able to compare our findings with those reported by authors who have applied the method of Tanaka and Mitsushima (1984).

W. Jongebloed: You state that a tissue, after the light (0.5% GA + 0.5% paraformaldehyde for 15 minutes) prefixation and the first postfixation with osmium/ferrocyanide, can be stored without problems. How can you be sure that post mortem changes do not occur, particularly with the proteinic contents of the tissue? Why did you store the tissue in PBS, whereas you performed the prefixation in cacodylate buffer?

Authors: We compared morphological results obtained with tissues stored in PBS up to 7 days with those immediately processed after postfixation with osmium/ferrocyanide and found no differences. Storage of specimens with cacodylate buffer was less satisfactory, because tissues showed sign of corrosion not present in controls. Cacodylate buffer that, actually, was the same buffer used

by Tanaka and Mitsushima (1984), was preferred for its ability to prevent the formation of contaminating molds and bacteria in the diluted aldehyde solution used as primary fixative.

W. Jongebloed: The original “Tanaka maceration” was carried out at 20°C, your maceration is carried out at 25°C; is the process too slow at 20 C°? Would you comment on that in the light of your discussion of the temperature?

Authors: We tried different temperatures starting from 20°C and a maceration time of 72 hours and found that

25°C gave morphological results comparable to those obtained at 20°C with a shorter maceration time (44 hours). Higher temperatures provoked corrosion.

W. Jongebloed: You mention the brownish color of the osmium/ferrocyanide treatment, changing to dark in due time in PBS while the osmium method stains rather dark from the beginning. Does it mean that more osmium is reduced and bound to the tissue when they are in PBS?

Authors: This is possible, but we do not have any evidence to support this hypothesis.