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Comparison of different preparation methods of biological samples for FIB milling and SEM investigation

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Summary

When a new approach in microscopy is introduced, broad interest is attracted only when the sample preparation procedure is elaborated and the results compared with the outcome of the existing methods. In the work presented here we tested different preparation procedures for focused ion beam (FIB) milling and scanning electron microscopy (SEM) of biological samples. The digestive gland epithelium of a terrestrial crustacean was prepared in a parallel for FIB/SEM and transmission electron microscope (TEM). All samples were aldehyde-fixed but followed by different further preparation steps. The results demonstrate that the FIB/SEM samples prepared for conventional scanning electron microscopy (dried) is suited for characterization of those intracellular morphological features, which have membranous/lamellar appearance and structures with composition of different density as the rest of the cell. The FIB/SEM of dried samples did not allow unambiguous recognition of cellular organelles. However, cellular organelles can be recognized by FIB/SEM when samples are embedded in plastic as for TEM and imaged by backscattered electrons. The best results in terms of topographical contrast on FIB milled dried samples were obtained when samples were aldehyde-fixed and conductively stained with the OTOTO method (osmium tetroxide/thiocarbohydrazide/osmium tetroxide/thiocarbohydrazide/osmium tetroxide). In the work presented here we provide evidence that FIB/SEM enables both, detailed recognition of cell ultrastructure, when samples are plastic embedded as for TEM or investigation of sample surface morphology and subcellular composition, when samples are dried as for conventional SEM.

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Introduction

First evidence of a successful application of the focused ion beam (FIB) / scanning electron microscope (SEM) for biological samples was published in 1993 (Young *et al.*, 1993). Within a decade, a bunch of papers appeared reporting the applicability of FIB/SEM in exposing and investigating the subcellular structures of a variety of biological samples (see Milani *et al.*, 2006; Hayles *et al.,* 2007). Despite the potential advantages of FIB/SEM, it is still not accepted as a method of choice in biological structure research at submicrometre scale. A great deal of this restraint against the application of FIB/SEM in biology arises from the fact that the sample preparation methods and FIB manipulation artefacts are not well documented. Besides, specific morphological criteria for identifying cell elements in FIB-milled cells are lacking (Drobne *et al.*, 2007). The aim of the paper presented here is to allow a critical, evidence-based assessment of applicability of FIB/SEM in structural research of biological samples at micrometre and submicrometre scales.

The FIB/SEM is a scanning microscope with an electron column and an ion column embedded in the same specimen chamber. Both beams are aiming the same point on the specimen surface. The major strength of application of FIB/SEM system in investigations of biological samples is the *in situ* site-specific manipulation of a specimen and a wide range of magnifications (Drobne *et al.*, 2004).

In biological electron microscopy many preparatory methods exist. For each separate step in the sequence of a preparation there are many alternative recipes.

For electron microscopy, chemical fixation is the most widely used method for preserving biological samples. The same criteria for fixative selection apply to transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Hayat, 2000).

In SEM the outermost structures are examined, which are first in contact with the fixative and are fixed immediately. For

this reason, some preparation artefacts in the underlying tissue can be tolerated. Also, in conventional SEM the specimen size is of a lesser concern than in TEM. When FIB/SEM is used as an extension of SEM for subcellular research, the question appears whether the usual SEM sample preparation is appropriate.

Chemical fixation continues to dominate the field of biological electron microscopy despite the advantages in cryopreservation methods. In TEM and SEM, the most frequently used fixative is glutaraldehyde or a combination of glutaraldehyde and formaldehyde. Glutaraldehyde is efficient in cross-linking proteins and maintaining cell ultrastructure, but it penetrates the tissue rather slowly. Formaldehyde is the smallest and the simplest aldehyde and penetrates tissues rapidly, apparently due to its low molecular weight. For postfixation, osmium tetroxide appeared most popular. It acts not only as a fixative but also as an electron stain. It preserves many lipids and it is able to stabilize some proteins by transforming them into clear gels, without destroying many of the structural features. The biphasic effect of osmium tetroxide on tissue constituents is well known; first it gelates and then extracts certain cellular components. The prefixation with glutaraldehyde does not prevent leaching of some proteins when specimens are postfixed with osmium tetroxide (Hayat, 2000).

Uranyl acetate can be used for staining thin sections as well as for *en bloc* staining before dehydration. It also acts as both, an electron stain and as a fixative. The use of uranyl acetate, following double fixation with glutaraldehyde and osmium tetroxide and before dehydration, is used for increasing the overall contrast and further stabilization of membranous and nucleic-acid-containing structures. Uranyl acetate also reacts with proteins (Hayat, 2000).

In the standard protocols for SEM, specimens are dried, mounted onto the specimen stub and coated with a conductive layer of a few to 20 nm, or, alternatively, non-coating techniques, like conductive staining are used (Davies & Forge, 1987; Dunnebier *et al.*, 1995). The non-coating techniques involve the use of the ligands in combination with osmium tetroxide in a given sequence for various periods. In the OTOTO (osmium tetroxide/thiocarbohydrazide/osmium tetroxide/thiocarbohydrazide/osmium tetroxide) non-coating technique, thiocarbohydrazide is used as a ligand smium tetroxide/thiocarbohydrazide/osmium tetroxide/ thiocarbohydrazide/osmium tetroxide). Thiocarbohydrazide acts as a bivalent mordant of binding the osmium tetroxide to itself. In this manner osmium's natural affinity for unsaturated lipids is enhanced or amplified. It is well known that the electrical conductivity imparted to the tissue by this increased deposition of osmium reduces the deleterious effects of specimen charging (Friedman & Ellisman, 1981).

The contrast in SEM images is proportional to the intensity of electrons ejected from the sample. The emitted signal is primarily related to surface topography and to the atomic number of elements on the surface. For traditional SEM of biological specimens, samples are air dried to allow observation of surface morphology. However, the FIB/SEM allows investigation also of plastic embedded samples (Knott *et al.*, 2008).

In the work presented here we tested different sample preparation methods in order to select the most appropriate one for FIB milling and SEM imaging of biological samples. Samples were either plastic embed as for conventional TEM and investigated by FIB/SEM or dried as for conventional SEM and investigated by FIB/SEM. In a parallel to FIB/SEM, same samples were investigated by TEM to allow assessment of the outcome of different sample preparation procedures. We discuss the role of sample preparation when FIB/SEM is used for investigating biological samples.

Materials and methods

Terrestrial isopods, *Porcellio scaber* Latreille, 1809 (Crustacea: Isopoda), were collected under concrete blocks and pieces of decaying wood. They were kept in glass tanks containing the soil and leaf litter from their natural environment until the dissection. The animals were decapitated and put in physiological saline (Hagedorn & Ziegler, 2002). The digestive glands (hepatopancreas) were isolated with tweezers and immediately transferred to the primary fixative. Digestive gland tubes were prepared either for conventional SEM or TEM (Fig. 1). Each of four digestive gland tubes was processed separately, following one of four different fixation procedures: (a) aldehyde primary fixation – procedure I, (b) aldehyde primary fixation and osmium postfixation – procedure II, (c) aldehyde primary fixation, osmium postfixation and *en bloc* staining with uranyl acetate – procedure III and (d) aldehyde primary fixation, osmium postfixation and conductive staining TOTO (thiocarbohydrazide/osmium tetroxide/thiocarbohydrazide/osmium tetroxide) – procedure IV.

The samples were examined with Strata DB235 (Modena, Italy), Quanta 3D FEG (Eindhoven, Netherlands) or Helios Nanolab (Eindhoven, Netherlands) FIB/SEM systems.

(a) For FIB operations with Strata DB235 (milling and polishing) gallium ion source was used (Drobne *et al.*, 2005, 2007). The ion currents were for milling in the range of 5 to 7 nA, and for cleaning mill in the range of 0.3 to 1.0 nA. Beam energy was 30 keV Ga^+. In some samples the protective platinum strip (1–2-μm thick) was deposited on the sample prior milling (Drobne *et al.*, 2007).

(b) For FIB operations with Quanta 3D FEG (milling and polishing) similar procedure as described above (a) was used. Ion beam current values were different due to instrument features. Also in this case a Pt protection strip of 1–2-μm thick was deposited as first step of the process. To mill the primary trench a current of 50 nA was used, the next steps of cleaning cross section were

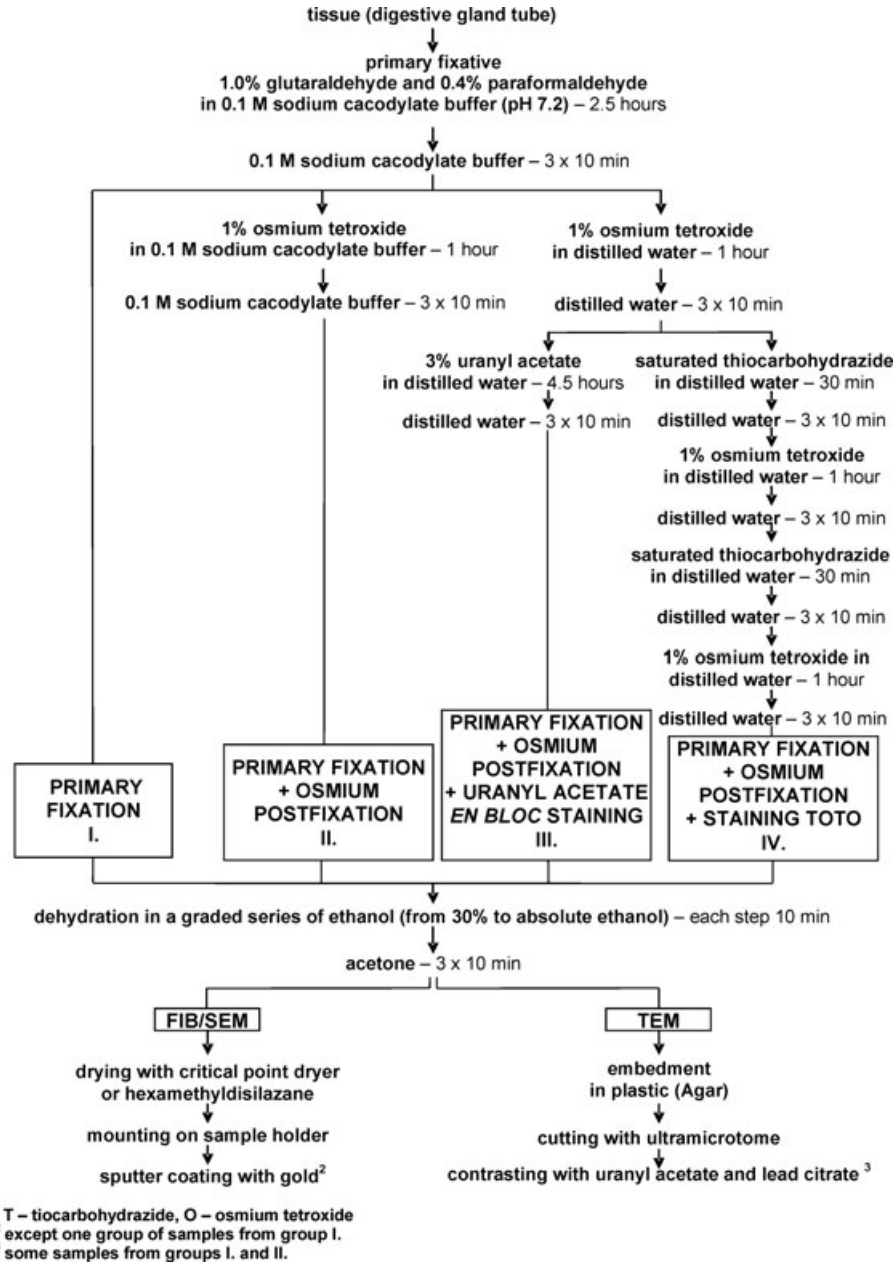

Fig. 1. Scheme of digestive glands preparation methods for observation with focused ion beam / scanning electron microscopy and transmission electron microscopy. All preparation steps were done at room temperature.

made with a range of current from 15 to 1 nA. For final polish a beam current of 300 pA was used. The cross sections made with this system have a peculiarity. The signal collected to build such images comes from the back scattered electrons (BSE) by mean of a solid state detector placed below the final lens pole piece. A typical cross section is obtained by a sample surface oriented perpendicular to the ion beam (sample surface tilted at 52 degrees); in this condition the take off angle for the BSE collection from a cross section (90 degrees to the sample surface – 38 degrees w.r.t the E-beam) is not optimal. To increase the collection yield the c/s was milled with the sample surface at 0 degrees this allows a better orientation of the sidewall (now the c/s surface is at 52 degrees w.r.t. the E-beam) towards the solid state back scattered electron detector (SSBSED) (see Fig. 10b).

(c) For FIB operations with Helios Nanolab (milling and polishing). The primary trench has been milled at 30 kV Ion Beam accelerating voltage and at 21 nA beam current. Following steps for cleaning the cross

Table 1. Summary of preparation methods of digestive glands of isopod *P. scaber* (SEM: scanning electron microscopy; TEM: transmission electron microscopy; +: yes; –: no)

Procedure/fixation and staining	I	II	III	IV
Aldehyde primary fixation	+	+	+	+
Osmium tetroxide postfixation	–	+	+	+
Staining with uranyl acetate	–	–	+	–
Conductive staining TOTO	–	–	–	+
Figures				
SEM dried sample	Fig. 2a, b	Fig. 4a, b	Fig. 6a, b	Fig. 8a–c Fig. 10c
SEM plastic embedded				Fig. 10b
TEM	Fig. 3a, b	Fig. 5a, b	Fig. 7	Fig. 9a, b Fig. 10a

section were done with beam current range from 6.5 nA to 0.3 nA. In order to improve the quality of the cross section and minimize the artefact due to the ion beam, a final step of polish at low accelerating voltage has been done. For this step an Ion Beam Acceleration of 5 kV and a current of 150 pA were used.

The numbers of animals examined were: 14 for procedure I, 6 for procedure II, 3 for procedure III and 21 for procedure IV. In most animals up to seven cells were examined. Ultrathin sections were examined with the Philips CM100 TEM (Ljubljana, Solvenia). The ultrastructure of *P. scaber* digestive glands is well known (Köhler *et al.*, 1996; Žnidaršič *et al.*, 2003), but anyway we examined the digestive glands of up to 10 animals under TEM.

Results

The preparation methods which were evaluated in our work are listed in Table 1 (for details see Fig. 1). No differences were found regarding gross morphology of cells and the ultrastructure between the critical point dried samples and the samples dried with hexamethyldisilazane (data not shown).

Procedure I: primary aldehyde fixation

The main ultrastructural characteristic of aldehyde-fixed and dried cells (procedure I) examined with FIB/SEM is ground substance with holes of different size and shape (Fig. 2a) and spherical nuclei with rough network (Fig. 2b). Larger holes are areas where lipid droplets were originally deposited. The ground substance was either entirely homogeneous (Fig. 2a) or netlike (Fig. 2b).

On TEM micrographs of the aldehyde-fixed samples microvilli (Fig. 3a) and nucleus (Fig. 3b) were recognized. Other cellular structures could not be defined due to the lack of contrast. Lipid droplets were rinsed during the preparation procedure and seen as electron lucent oval areas (Fig. 3a and b).

Procedure II: primary aldehyde fixation and osmium tetroxide postfixation

The cell components recognized in dried FIB/SEM investigated samples fixed with aldehydes and postfixed with osmium tetroxide were microvilli (Fig. 4a) and lipid droplets (Fig. 4b). Lipid droplets were homogeneous and bright. The cytoplasmic ground substance was either netlike (Fig. 4a) or homogenous (Fig. 4b).

On TEM images of the samples fixed with aldehydes and postfixed with osmium tetroxide we observed spherical nuclei with clearly seen inner and outer nuclear membranes, euchromatin, heterochromatin and nucleolus; oval or elongated mitochondria; lipid droplets with a lucent halo (halo was not present on all images); granular endoplasmic reticulum organized as short cisternae or as long, parallel cisternae; Golgi apparatus composed of cisternae; and different vesicles (Fig. 5a and b). At the apical part of the cells, finger-like microvilli (Fig. 5a). The cytoplasmic ground substance was electron lucent.

Procedure III: primary aldehyde fixation, osmium tetroxide postfixation and uranyle acetate en bloc staining

In the aldehyde-fixed, osmium tetroxide postfixed and uranyl acetate *en bloc* prepared samples examined with FIB/SEM, the following cell structures were recognized: finger-like mikrovilli and homogeneous lipid droplets (Fig. 6a). In the cytoplasmic ground substance a variety of structures with different shape and size were observed; however, due to the lack of specific characteristics, none of them could be unequivocally identified (Fig. 6a and b). We used also backscattered imaging to obtain compositional contrast, but we did not improve the recognition of cellular organelles (Fig. 6b). The cytoplasmic ground substance appeared porous or netlike. As expected, in the samples investigated by TEM (Fig. 7a and b), the

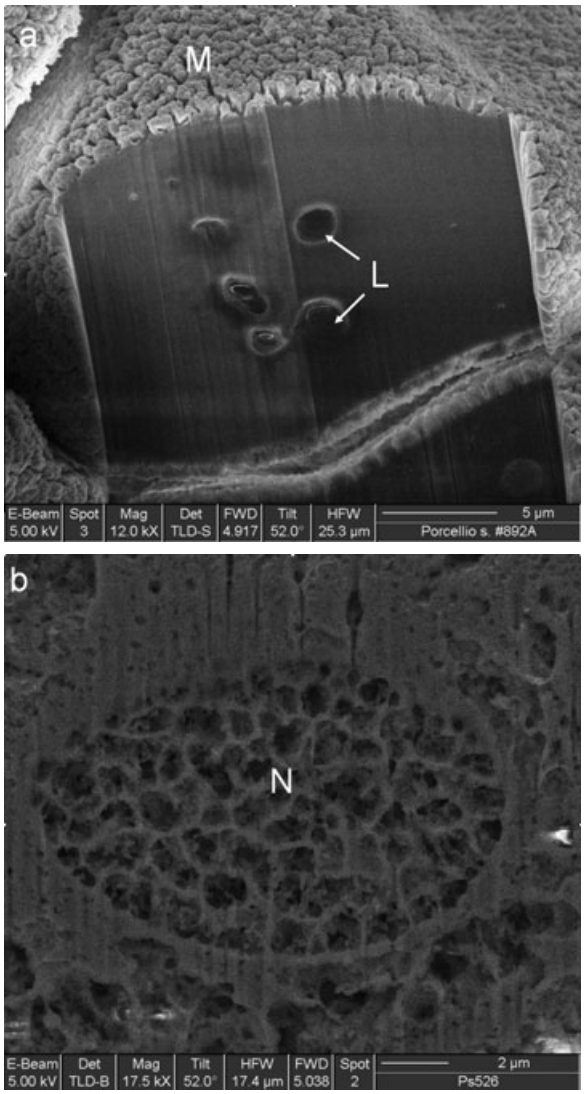

Fig. 2. Electron micrographs of focused ion beam / scanning electron microscopy investigated of aldehyde fixed samples (FEI Strata DB 235 M). (a) Cell cut at the apical part. Homogeneous ground substance with extracted lipid droplets. (b) In the milled median part of a cell, nucleus is recognized because of its different composition as the rest of the cell. (L – lipid droplet; M – microvilli; N – nucleus).

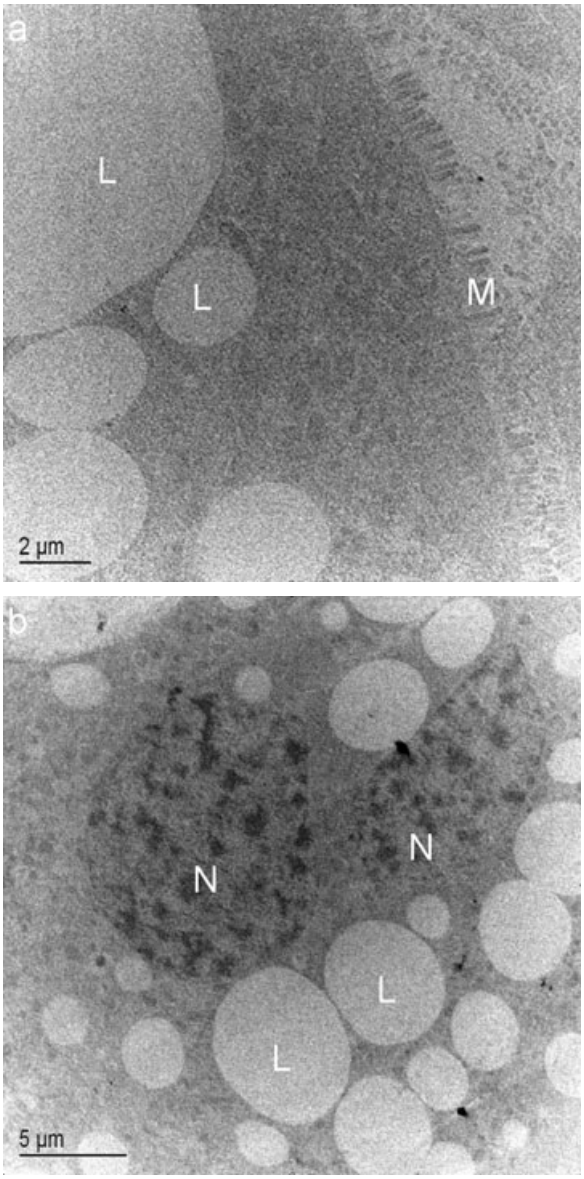

Fig. 3. Transmission electron microscopy investigation of aldehyde fixed sample (Philips CM100). Sections were either contrasted (b) or not (a). (a) Apical part of the cell with microvilli. Organelles are hard to define. (b) Basal part of the cell with nuclei and electron lucent spaces, where lipid droplets were deposited. (L – lipid droplet; M – microvilli; N – nucleus).

same structural characteristics were observed as in the aldehyde-fixed and osmium tetroxide postfixed samples (procedure II; Fig. 5a and b).

Procedure IV: primary aldehyde fixation, osmium tetroxide postfixation and TOTO

In the aldehyde-fixed and OTOTO processed samples the following cell structures were recognized when investigated by FIB/SEM (Fig. 8a–d): finger-like mikrovilli; homogeneous lipid droplets with multilayered halo; different lamellar structures with well seen lamella. The cytoplasmic ground substance was always netlike.

The major differences between the TEM images of the samples prepared according to procedures II (Fig. 5a and b) and III (Fig. 7a and b) and the sample prepared following procedure IV (Fig. 9a and b) were: in the latter, the cytoplasm was very electron dense and the lipid droplets had an electron-dense multilayered halo.

Straightforward comparison between two preparation principles for FIB/SEM (critical point drying d, e or plastic

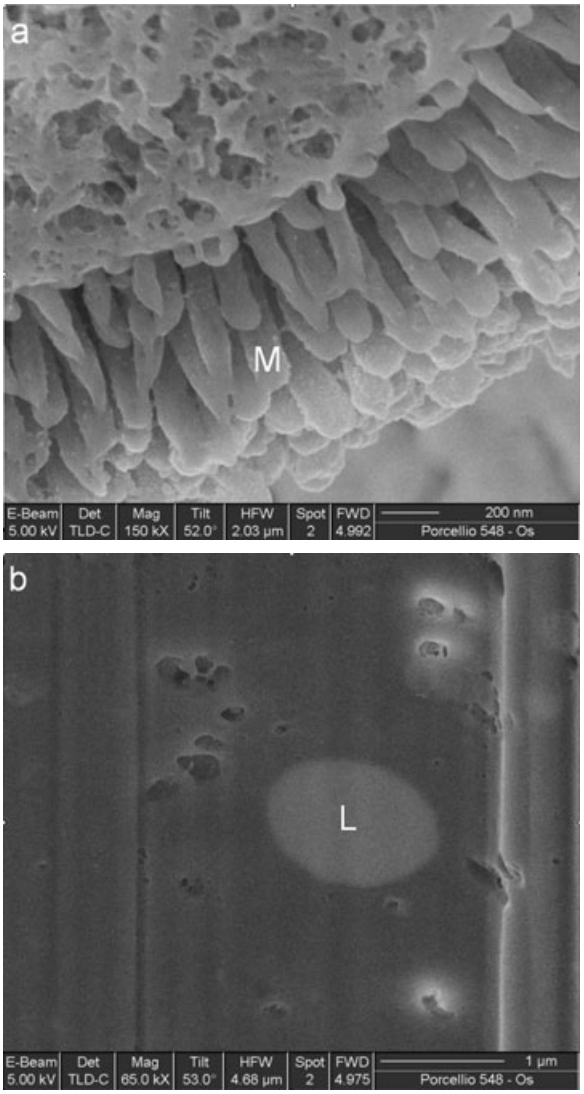

Fig. 4. Electron micrographs of focused ion beam / scanning electron microscopy investigated aldehyde fixed and osmium postfixed sample (FEI Strata DB 235 M). The same digestive gland cut through two different cells. (a) Structured and (b) homogeneous cytoplasmic ground substance. (L – lipid droplet; M – microvilli).

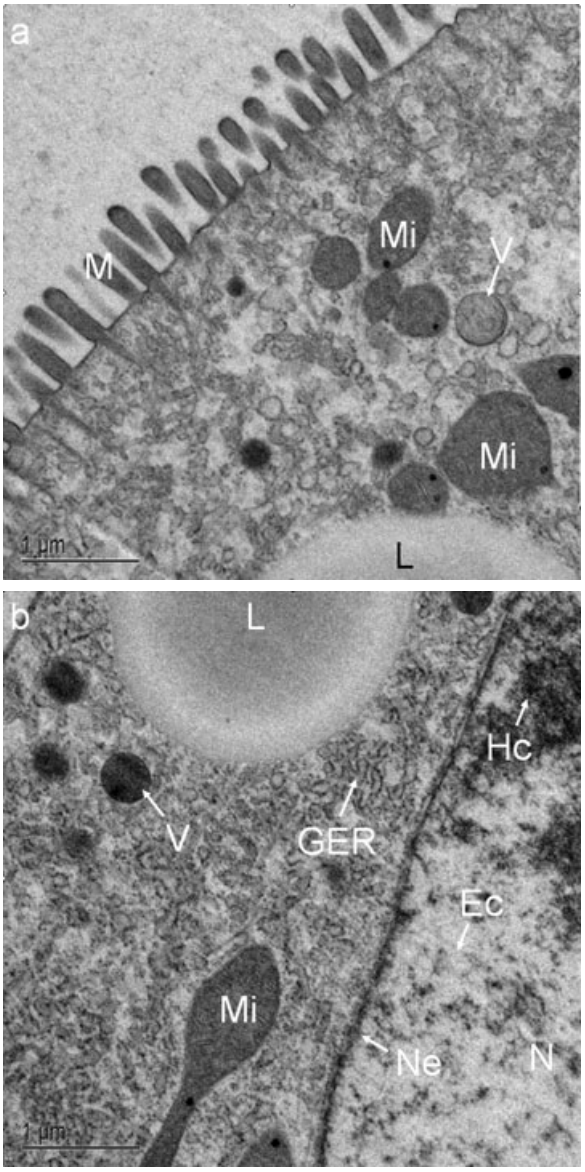

Fig. 5. Transmission electron microscopy investigation of aldehyde fixed and osmium postfixed samples (Philips CM100). Sections were contrasted. All organelles and membrane structures are well defined (a, b). (GER – granular endoplasmic reticulum; Ec – euchromatin; Hc – heterochromatin; L – lipid droplet; M – microvilli; Mi – mitochondrion; N – nucleus; Ne – nuclear envelope; V – vesicle).

embedding, a–c) and conventional TEM (a) is provided on the same sample (Fig. 10a–c). Digestive glands of the same animal were aldehyde fixed, osmium postfixed and uranyl acetate *en bloc* stained (procedure III). Part of a sample was plastic embedded and investigated by TEM (Fig. 10a) or FIB/SEM (Fig. 10b and c). The other part of a sample was processed as for SEM (dried and sputter coated) and investigated by FIB/SEM (Fig. 10d and e). TEM and FIB/SEM of plastic embedded samples provide actually the same ultrastructural information (Fig. 10a and c). FIB/SEM of dried sample provides another type of information which is complementary to the previous two.

Discussion

We proved that FIB/SEM can be used for investigating sample external and subsurface morphological characteristics as well as cell ultrastructure. FIB milling and conventional SEM imaging of dried samples provide structural information on tissue and cell external morphology and reveals morphological characteristics of cell interior. However, here recognition of cellular organelles and straightforward comparison with TEM images is not possible. FIB milling and SEM imaging

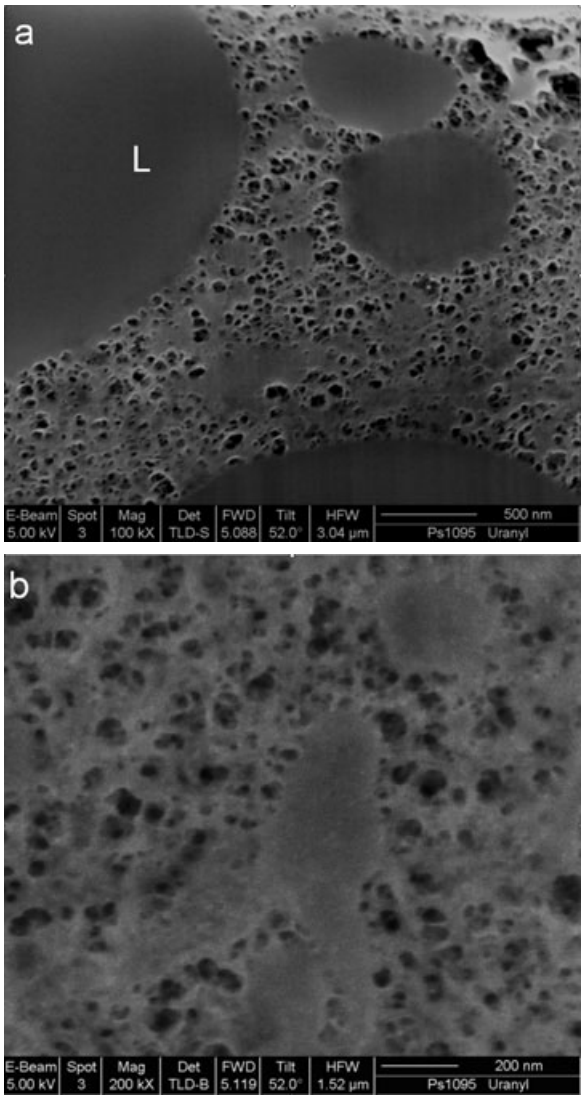

Fig. 6. Electron micrographs of focused ion beam / scanning electron microscopy investigated aldehyde fixed, osmium posfixed and uranyl acetate stained sample (FEI Strata DB 235 M). a) secondary electron image. b) backscattered image. Cell organelles cannot be recognized. The cytoplasmic ground substance is porous. Lipid droplets, are seen as dense structures. (L – lipid droplet).

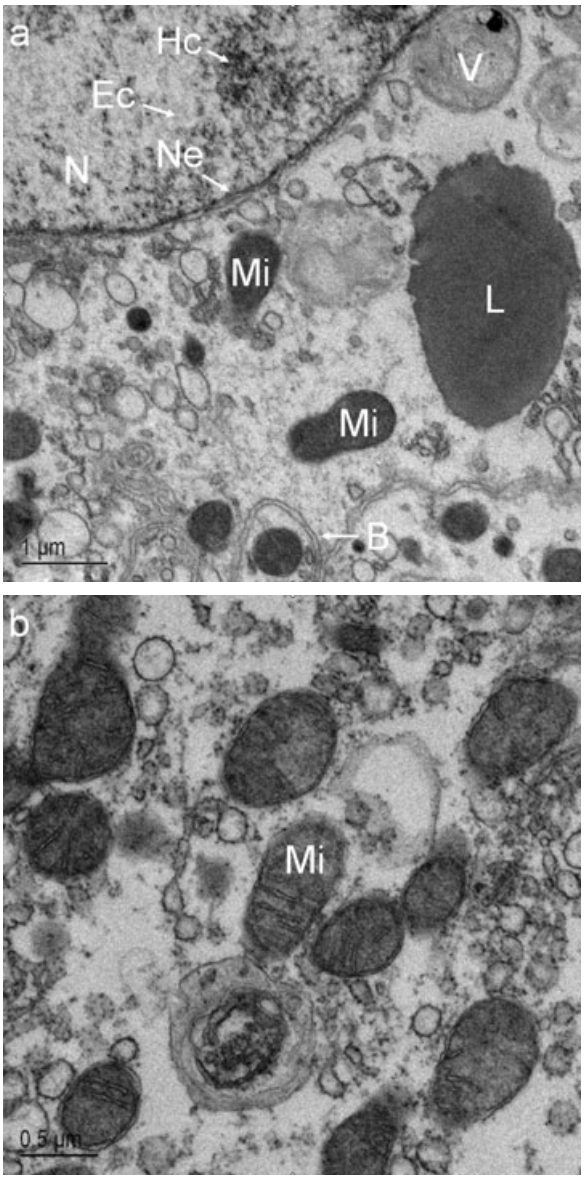

Fig. 7. Transmission electron microscopy investigation of aldehyde fixed, osmium postfixed and uranyl acetate *en bloc* stained sample (Philips CM100). Sections were not contrasted. All organelles and membrane structures are well defined (a, b). (B – basal labyrinth; Ec – euchromatin; GA – Golgi apparatus; GER – granular endoplasmic reticulum; Hc – heterochromatin; L – lipid droplet; Mi – mitochondrion; N – nucleus; Ne – nuclear envelope; V – vesicle) .

of plastic embedded samples enables recognition of cellular organelles and straightforward comparison with TEM images. Also, the 3D architecture of the cell interior is generated on plastic embedded samples by sequential FIBing and imaging as described by Heyman *et al.* (2006) and Knott *et al.* (2008).

At the moment, FIB/SEM is much more popular and more frequently applied on plastic embedded biological samples as on dried samples (Heymann *et al.*, 2006; Drobne *et al.*, 2008). This can be explained by the fact that due to the long history of TEM in life sciences, the specific morphological criteria for identifying cell elements are well known. The traditional SEM is not intended to be commonly used for subcellular investigation; therefore virtually nothing is known how the cellular elements should look like when the cells are opened by FIB milling and imaged by SEM. One of the core advantages of FIB/SEM of plastic embedment biological samples is that the image acquisition process can be completely automated and the ultramicrotoming can be avoided. Additional benefits of FIB/SEM are that combination of preparation methods, i.e. plastic embedment and drying allows both TEM and SEM

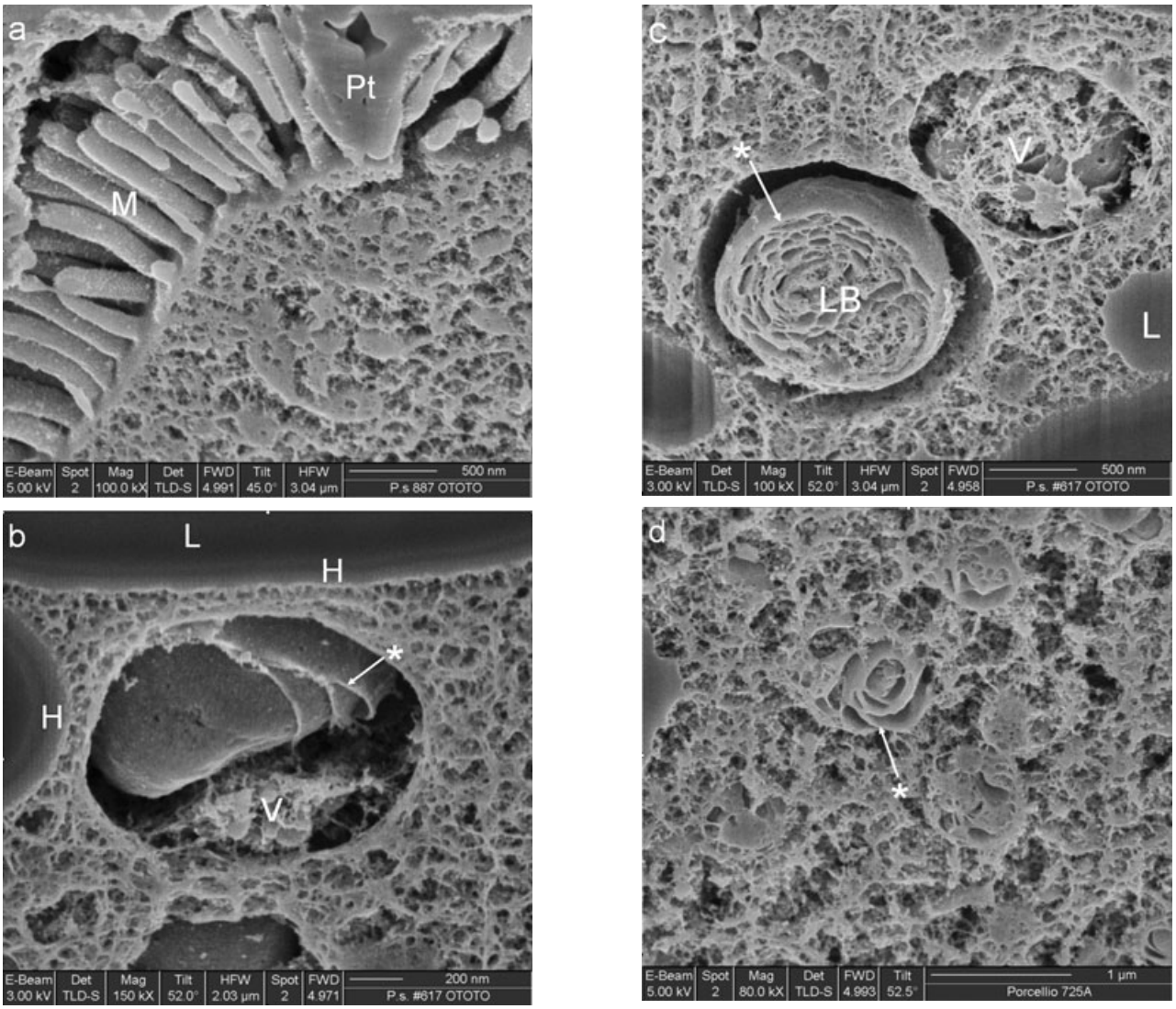

Fig. 8. Electron micrographs of focused ion beam / scanning electron microscopy investigated aldehyde fixed, osmium postfixed and TOTO conductively stained samples (FEI Strata DB 235 M). In all samples cytoplasmic ground substance is netlike (a–d). Organelles cannot be determined except lipid droplets and lamellar bodies (b, c, d). (H – halo; LB – laminar body; L – lipid droplet; M – microvilli; Pt – protective platinum strip; V – vesicle; * – lamella).

Fig. 8. Continued.

imaging mode and therefore a direct comparison with existing electron microscopy data of biological samples.

A lot of knowledge exists on sample preparation for TEM of biological samples, but data on sample preparation for subcellular research with FIB/SEM are very rare. For FIB/SEM of dried samples best results in terms of topographical contrast were obtained with aldehyde-fixed and OTOTO-processed samples. The compositional contrast did not contribute significantly to the image formation. In SEM, the criteria for good tissue preservation are not clearly determined. In our work, we assessed the quality of tissue fixation for SEM by TEM. The TEM images show that aldehyde fixation followed by osmium tetroxide postfixation (procedure II), satisfactorily preserved the tissue. However, the samples that were not postfixed provided too low contrast for satisfactory high magnification imaging (procedure I). In cases where osmium tetroxide postfixation was followed by uranyl *en bloc* staining (procedure III) no differences in terms of structural characteristics were observed when compared with the aldehyde-fixed and osmium tetroxide postfixed samples (procedure II). In samples where osmium tetroxide postfixation was followed by the TOTO conductive staining (procedure IV) also no differences in preservation of cell structures were observed; however, here the cellular ground substance as well as other cellular structures appeared very electron dense. Here also, some features appeared more pronounced than those in the samples prepared following

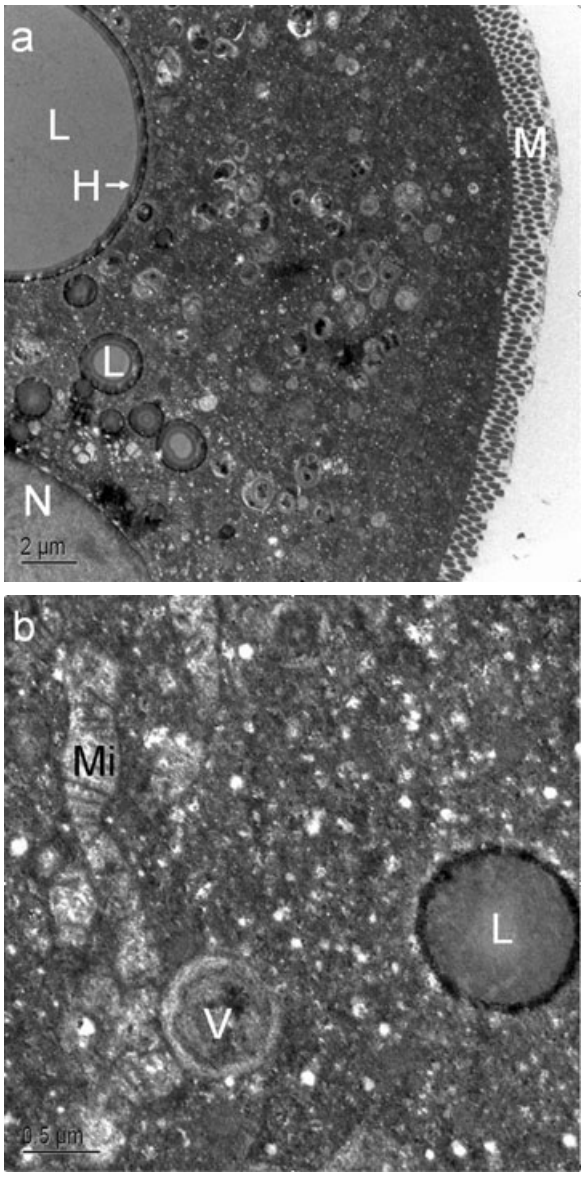

Fig. 9. Transmission electron microscopy investigation of aldehyde fixed, osmium postfixed and TOTO conductively stained sample (Philips CM100). Sections were not contrasted (a, b). All organelles and membrane structures are well defined. Cells are electron very dens due to introduction of large quantities of osmium tetroxide into the sample during the preparation procedure. Lipid droplets have well seen halo. (H – halo; L – lipid droplet; M – microvilli; Mi – mitochondrion; N – nucleus; V – vesicle).

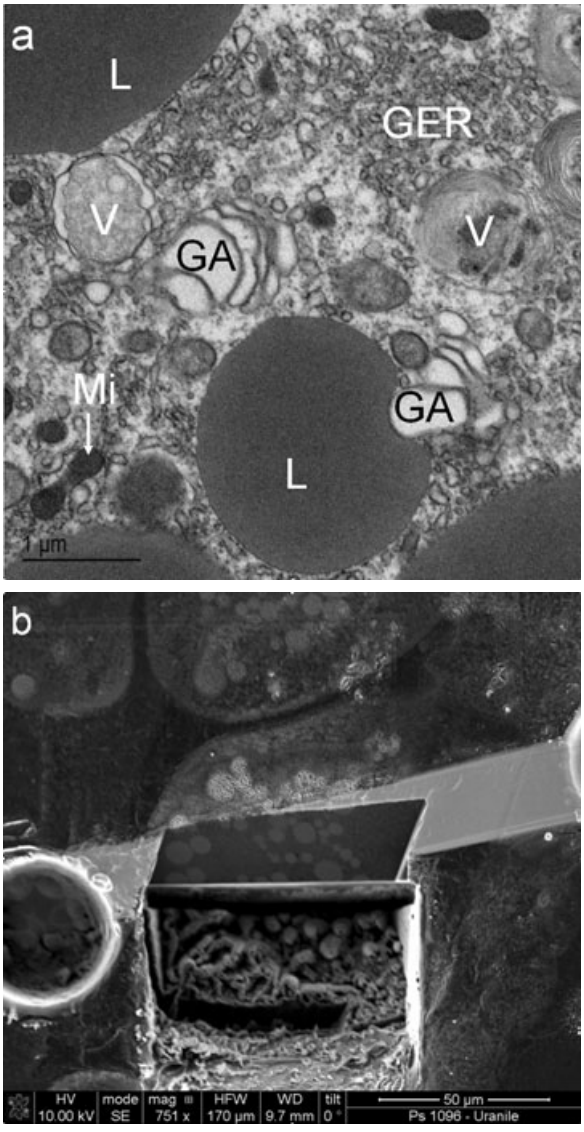

Fig. 10. Isolated digestive glands of the same animal were aldehyde fixed, osmium postfixed and uranyl acetate *en bloc* stained (procedure III). Part of a sample was then plastic embedded and investigated by transmission electron microscopy (TEM) (a, Philips CM100) or focused ion beam / scanning electron microscopy (FIB/SEM) (b, c, Quanta 3D FEG). FIB manipulated region is shown (b) The other part of a sample was dried and sputter coated and investigated by FIB/SEM (d, e, Helios). On TEM image (a) and on FIB/SEM backscattered image of plastic embedded sample (c) all expected cellular organelles are recognized. On secondary electron FIB/SEM image of dried sample (e) one can see diverse intracellular structures but they are not recognizable. Exceptions are lipid droplets due to their distinctive homogenous composition. (GA – Golgi apparatus; GER – granular endoplasmic reticulum; L – lipid droplet; M – microvilli; Mi – mitochondrion; N – nucleus; V – vesicle).

other procedures. The multilayered halo of lipid droplets is evident only in the OTOTO-processed samples. However, due to the higher electron density of a sample, some details are not evident on TEM images, like lamellae of lamellar structures in some vesicles; however, mitochondrial cristae are clearly visible and sometimes also lamellae in lamellar structures.

The aldehyde-fixed and OTOTO-processed samples (procedure IV) provided the best ratio between the extracted and preserved material which resulted in enough relief to allow the distinction among different intracellular structures

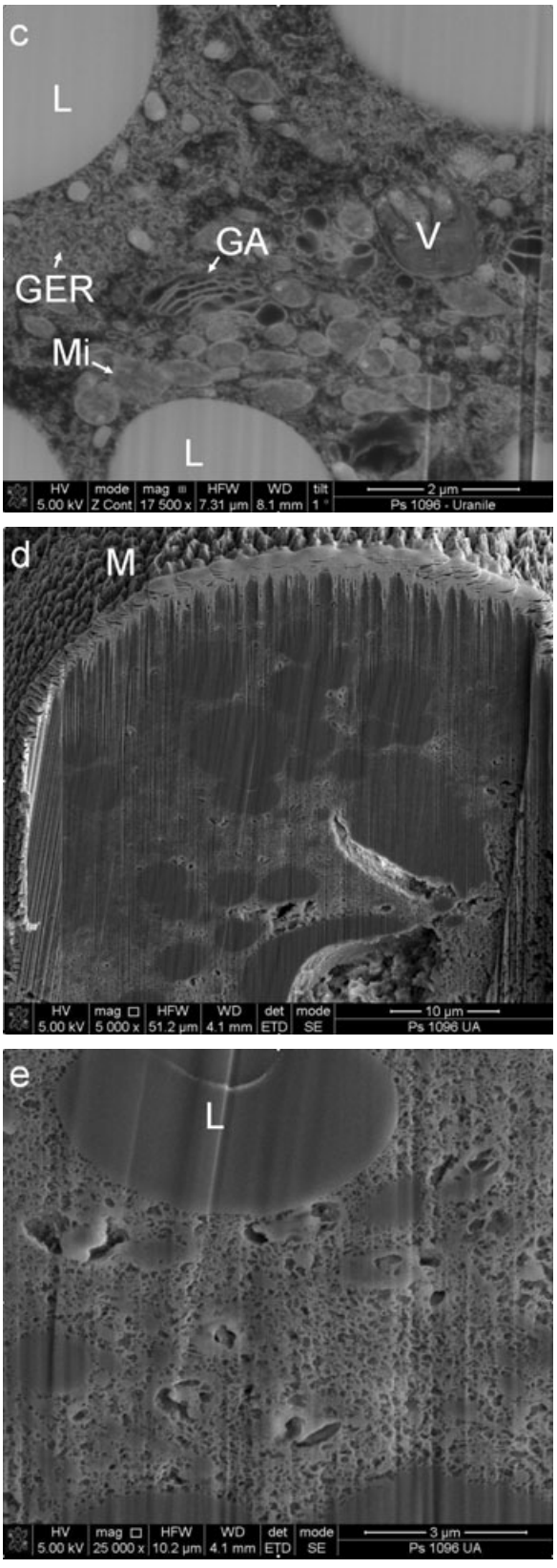

Fig. 10. Continued.

Here, the separation of the liquid phase from the solid phase of the protoplasm is most suitable.

Apart from the preservation of material, fixatives cause the loss of a substantial amount of carbohydrates, proteins, lipoproteins, nucleic acids and lipids during and after fixation. The longer duration of fixation tends to cause diffusion of enzymes, extraction of cellular material and shrinkage or swelling of the tissue (Hayat, 2000). Also, dehydration and embedding may affect the final amount of the fixed material in tissue. The degree of extraction is primarily dependent on the duration of fixation and dehydration, type of buffer used and the type of proteins present in the sample (Hayat, 2000). We explain the net-like appearance of the cytoplasmic ground substance by the loss of material during the TOTO (5-h) procedure. Our results show that more relief in the cell interior is not related to the smaller amount of material inside a cell, but rather to the fixation pattern. Namely, in samples that were only aldehyde-fixed, less material was preserved, but the cell interior appeared in general as a bulk with holes of different size and not net-like or spongiform as the one in the OTOTO-processed samples.

The aldehyde-fixation, uranyl acetate staining samples (procedure III) and plastic embedment proved to be the best preparation for FIB/SEM investigation of cell ultrastrucutre. Here, the backscattering contrast was used to visualize the heavy-metal staining of tissue prepared using techniques that are routine for transmission electron microscopy (Denk & Horstmann, 2004). Aldehyde-fixed and OTOTO-processed samples provide best results for FIB/SEM investigation of dried samples. We explain the differences in sample preparation method by differences in imaging mode. In plastic embedded samples a compositional contrast is recorded, whereas in dried samples a topographical contrast is of interest.

To conclude, the FIB/SEM has a unique possibility to couple simultaneous investigation of sample gross morphology, cell surface and subsurface characteristics and cell ultrastrucutre investigation with *in situ* sample manipulation. However, for investing external structures of investigated sample, it has to be prepared as for conventional SEM (dried). For investigating cell ultrastrucutre, samples have to be plastic embedded. With recent developments of FIB/SEM systems, their resolution in plastic embedded samples can compete with that of TEM and thus satisfy the need for ultrastrucutral information (Heymann *et al.*, 2006).

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