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ORIGINAL ARTICLE

Focused ion beam (FIB)/scanning electron microscopy (SEM) in tissue structural research

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Abstract The focused ion beam (FIB) and scanning electron microscope (SEM) are commonly used in material sciences for imaging and analysis of materials. Over the last decade, the combined FIB/SEM system has proven to be also applicable in the life sciences. We have examined the potential of the focused ion beam/scanning electron microscope system for the investigation of biological tissues of the model organism *Porcellio scaber* (Crustacea: Isopoda). Tissue from digestive glands was prepared as for conventional SEM or as for transmission electron microscopy (TEM). The samples were transferred into FIB/SEM for FIB milling and an imaging operation. FIB-milled regions were secondary electron imaged, back-scattered electron imaged, or energy dispersive X-ray (EDX) analyzed. Our results demonstrated that FIB/SEM enables simultaneous investigation of sample gross morphology, cell surface characteristics, and subsurface structures. The same FIB-exposed regions were analyzed by EDX to provide basic compositional data. When samples were prepared as for TEM, the information obtained with FIB/SEM is comparable, though at limited magnification, to that obtained from TEM. A combination of imaging, micromanipulation, and compositional analysis appears of particular interest in the investigation of epithelial tissues, which are subjected to various endogenous and exogenous conditions affecting their structure and function. The FIB/SEM is a promising tool for an overall examination of epithelial tissue under normal, stressed, or pathological conditions.

Keywords Histopathology · Hepatopancreas · Terrestrial isopods · Crustacea · EDX analyses

Abbreviations

FIB	Focused ion beam
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
EDX	Energy dispersive X-ray
BSE	Back-scattered electrons

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Introduction

The scanning electron microscope (SEM) is an instrument which allows visualization of a sample surface. A beam of high energy electrons used in SEM can yield information on topography, morphology, chemical composition, and crystallographic information (Bozzola and Russell 1998).

Recently, novel approaches in which SEM can be adapted to investigate the three-dimensional architecture of cells have been reported. Samples under study, dried or embedded in plastic, were sectioned outside (Denk and Horstmann 2004) or inside (Heymann et al. 2006; Drobne

et al. 2008; Knott et al. 2008; De Winter et al. 2009; Hekking et al. 2009; Heymann et al. 2009; Lešer et al. 2009) the chamber of the microscope. When samples were sectioned inside the microscope chamber, a focused ion beam/scanning electron microscope (FIB/SEM) system was used. In this system, in addition to an electron beam column, an ion beam column with gallium as ion source is also installed (Young and Moore 2005).

Focused ion beams have been used since the 1960s to investigate the chemical and isotopic composition of materials (Mackenzie and Smith 1990; Perrey et al. 2004). Because of its sputtering capability, the FIB is used also as a micro-machining tool to modify or machine materials at the micro- or nano-scales (Mackenzie and Smith 1990; Young 1993).

Today's FIB capabilities have proved to be applicable to a range of disciplines ranging from materials to biological sciences. FIB/SEM is popular for advanced circuit editing, for revealing subsurface defects in advanced materials and devices, and for site-specific two-dimensional sectioning and imaging materials or biological micro-structures (Young et al. 1993; Phaneuf 1999; Inkson et al. 2001; Chaiwan et al. 2002; Haswell et al. 2003; Sivel et al. 2004). FIB/SEM investigation of biological samples can be conducted on bulk specimens, prepared for conventional SEM (Drobne et al. 2008; Lešer et al. 2009), frozen specimens (Hayles et al. 2007), or bulk plastic-embedded specimens prepared for conventional TEM. The latter is at present most widespread in investigation of biological samples (Heymann et al. 2006; Knott et al. 2008; De Winter et al. 2009; Heymann et al. 2009; Hekking et al. 2009).

Here we present examples of the efficient incorporation of FIB/SEM into morphological and compositional research dealing with biological samples.

Morphological research

Secondary electron (SE) imaging is used to generate an image based on topographic contrast. The major strength of FIB/SEM systems in morphological investigations of biological samples is their ability to manipulate a specimen in situ and site-specifically employing a wide range of magnifications (Drobne et al. 2004). The introduction of FIB for in situ exposure of subsurface structures offers an attractive possibility to expand sample surface investigations by subsurface structural research at any location of interest.

Compositional research

Back-scattered electrons (BSE) are elastically scattered high energy electrons. They may escape from considerable depths in the specimen and exit at some distance from the point of electron beam entry. In BSE imaging, micrographs are produced which identify areas occupied by domains of different density. Z-contrast is based on differences in atomic number; if the atomic number increases, the yield of both secondary and back-scattered electrons increases because heavier elements have more orbital electrons available, with a weaker binding energy, to interact with migrating electrons (Bozzola and Russell 1998).

Techniques based on BSE are not commonly used for compositional research of biological samples prepared for conventional SEM. The potential contribution of FIB/SEM in BSE imaging is to generate planar surfaces.

Beam-sample interaction is a well-known problem in the X-ray microanalysis of biological specimens. It is difficult to generate baseline X-ray mapping conditions that can provide reasonable results for bulk biological specimens (Bozzola and Russell 1998), but these problems can be avoided by using resin-embedded sections of biological samples to generate X-ray maps.

For the purposes of EDX analysis, as in BSE imaging, the contribution of FIB/SEM in providing flat surfaces at any selected location is anticipated.

Linking FIB/SEM to conventional TEM

Traditional SEM is not intended to be commonly used for subcellular investigation. With plastic-embedded biological samples, FIB/SEM proved to be reliable for a direct comparison with TEM, supporting a link between the two techniques (Heymann et al. 2006; Knott et al. 2008; De Winter et al. 2009; Heymann et al. 2009; Hekking et al. 2009).

The aim of this work was to employ and assess an FIB/SEM system for structural and compositional research of the digestive gland epithelium of a model invertebrate species (*Porcellio scaber*, Isopoda, Crustacea). Digestive glands of *P. scaber* have been investigated extensively and their structure is well known (Wägele 1992; Hames and Hopkin 1991; Žnidaršič et al. 2003). The epithelium consists of a single layer composed of large dome-shaped big cells (B cells) and interspersed between them, wedge-shaped small cells (S cells). The most prominent feature of the large B cells is lipid droplets and of the small S cells is metal granules (Hopkin and Martin 1982; Hames and Hopkin 1991; Morgan et al. 1995). We investigated the gross morphology and most characteristics of the subsurface structures of epithelial cells by SE and BSE and in addition, have investigated a FIB-exposed cell surface by EDX. The benefits of the use of FIB/SEM in structural and compositional studies of biological samples are discussed.

Materials and methods

Sample preparation

In this work, digestive glands of a terrestrial isopod, *P. scaber* Latreille, 1804 (Crustacea: Isopoda), were selected as a model tissue. Animals were collected under concrete blocks and pieces of decaying wood (Ljubljana, Slovenia). The animals were decapitated and put in physiological saline (Hagedorn and Ziegler 2002). The digestive gland tubes were isolated and fixed in 1.0% glutaraldehyde and 0.4% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.2, osmolarity 289 mOsm/kg) for 2.5 h at room temperature.

a) Morphological research

The chemically fixed samples were followed either by OsO_4/thiocarbohydrazide/OsO_4/thiocarbohydrazide/OsO_4 (OTOTO) conductively staining and dehydration (Lešer et al. 2009) or by direct dehydration. After dehydration in a graded series of ethanol, the samples were dried at the critical point (Balzers Critical Point Dryer 030, Liechtenstein) and gold coated (Sputter coater SCD 050, BAL-TEC, Germany).

b) Compositional research

When samples were prepared for BSE imaging or EDX, no metals were introduced during the sample preparation. In these cases, the samples were only fixed with glutaraldehyde and paraformaldehyde, dehydrated and critical point dried, and secondary fixation and conductive staining were omitted. After drying, the samples were coated with carbon (Sputter coater SCD 050, BAL-TEC, Germany).

c) Linkage of FIB/SEM to conventional TEM

Samples were postfixed in OsO_4, en bloc stained in uranyl acetate, dehydrated and embedded in plastic (Agar 100 resin kit, Agar scientific, United Kingdom) as previously described (Heymann et al. 2006) in a procedure modified for our samples (Lešer et al. 2009). The sample was then cut with diamond knife on an ultramicrotome (Reichert Ultracut S, Liechtenstein). Ultrathin sections were inspected with a Philips CM100 transmission electron microscope (Department of Biology, University of Ljubljana, Ljubljana, Slovenia) and images documented with a Bioscan 792 camera (Gatan, United Kingdom). Remaining sample of the same material (bulk plastic embedded sample) was examined by FIB/SEM.

FIB/SEM operation

The samples were fixed on brass holders with silver paint (High purity silver paint, SPI), mounted on the sample holder in the specimen chamber of a dual beam system for FIB/SEM operation (FEI Strata DB 235 M, CNR-INFM, University Modena, Modena, Italy). Rough milling conditions to open a trench employed ion currents of 5 to 7 nA, at 30 kV. Lower beam currents of 100 to 300 pA were used to polish the cross section. The spot size in the case of rough milling was approximately 100–150 nm in diameter and for polishing it ranged from 20 to 35 nm in diameter. Secondary electron detectors were: Everhardt Thornely Detector, Continuous Dynode Electron Multiplier, and Back-Scattered Electron Detector. The dwell time for milling was 1 μs and the overlap was 50%. The SEM imaging was performed with the FEG electron column available in the same system with a resolution of 1 nm at 30 kV. The spot size in the case of SEM was in the nanometer range. In some samples, a protective platinum strip, 1–2 μm in thickness, was deposited on the sample prior to milling (Drobne et al. 2007).

Plastic embedded samples that had been previously edge cut with a diamond knife were fixed on brass holders with silver paint (see above) and examined by Quanta 3D FEG (FEI Company, Eindhoven, the Netherlands). Prior to FIB milling, a platinum protective strip about 1 μm in thickness was deposited. To mill the primary trench, a current of 50 nA was used, and subsequent steps for cleaning across the section were carried out with currents in the range of 1–15 nA. A beam current of 300 pA was used for the final polishing.

EDX analyses

The EDX analyses were performed by Nova NanoSEM (FEI Company, Eindhoven, the Netherlands) with an integrated EDX detector and FIB. Analysis in spot mode was carried out at 15 kV, beam current used was 650 pA, and acquisition time was 150 live seconds.

Results

Digestive glands of the isopod *P. scaber* were prepared either as for conventional SEM or for TEM. Both types of samples were milled by FIB to expose the cell interiors. Some samples prepared for SEM were also analyzed by EDX.

Morphological research

For observation of digestive glands, morphology samples were prepared by OTOTO procedure and dried (Fig. 1a). Selected cells were FIB milled (Fig. 1b, c) to expose the cell interiors. Intracellular structures that could be recognized in B cells were lipid droplets of different sizes (Fig. 1c, d), lamellar bodies, different vesicles (Fig. 1d),

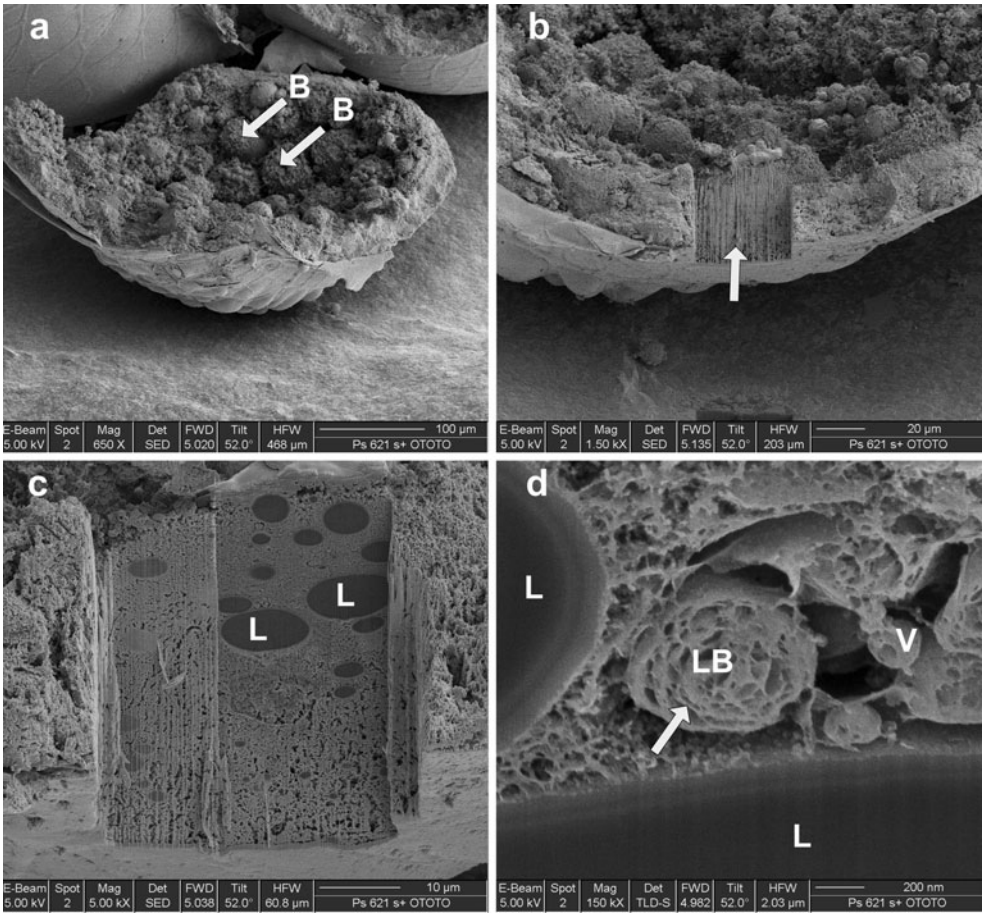

Fig. 1 Secondary electron micrographs of digestive gland epithelium investigated by FIB/SEM. **a** Manually broken digestive gland tube with clearly observable B cells (*arrow*). **b** A FIB-milled region at lower and **c** higher magnification. **d** High magnification image of a FIB-milled area. Lipid droplets and different vesicles with lamellar (*arrow*) or non-lamellar structure can be recognized. The cytoplasmic ground substance is netlike. *B* B cell, *L* lipid droplet, *LB* lamellar body, *V* vesicle

and nuclei. Cytoplasmic ground substance had a netlike appearance. No other organelles could be unequivocally identified. In S cells recognizable organelles were nuclei, small lipid droplets, and different vesicles. Some of vesicles were probably metal granules, but could not be distinguished from other vesicles. The ground cytoplasmic substance was netlike.

Compositional research

To study the chemical composition of digestive gland cells, samples were only aldehyde fixed while postfixing with osmium tetroxide or conductive staining (OTOTO) was omitted in order to avoid introduction into the sample of elements with high atomic number. Samples were first imaged by back-scattered electrons and regions of interest were further analyzed by EDX.

a) BSE imaging

The gross morphological features of the samples were similar to those of samples prepared by the OTOTO method. The intracellular features of B cells were empty holes, where lipid droplets had been originally present, and a porous or netlike cytoplasmic ground substance. With the exception of the nucleus, no other organelles could be recognized in the B cells. In small S cells the cytoplasmic ground substance was also porous or netlike. Organelles that were recognizable were nuclei and different vesicles.

Selected regions of FIB-milled area were imaged by back-scattered electrons (BSE; Figs. 2, 3). BSE micrographs clearly distinguish the regions on the cell surfaces exposed by FIB that are composed of elements with atomic number higher than the surroundings (Fig. 2b). The same regions can also be seen in a SE micrograph, where they are only slightly brighter than their surroundings (Fig. 2a). In BSE images the same regions are seen as bright spots on a darker background. An elemental analysis of some selected regions was performed by EDX (see below).

b) EDX analysis

Spot mode EDX analysis was used to check the chemical composition of the exposed surface of S cells (Fig. 3). When the two spectra are compared, the difference in metal composition can be seen clearly. As expected in the spectrum major amounts of copper and zinc were detected, since a metal granule of S cells was analyzed. Significant contributions to the spectra originate from

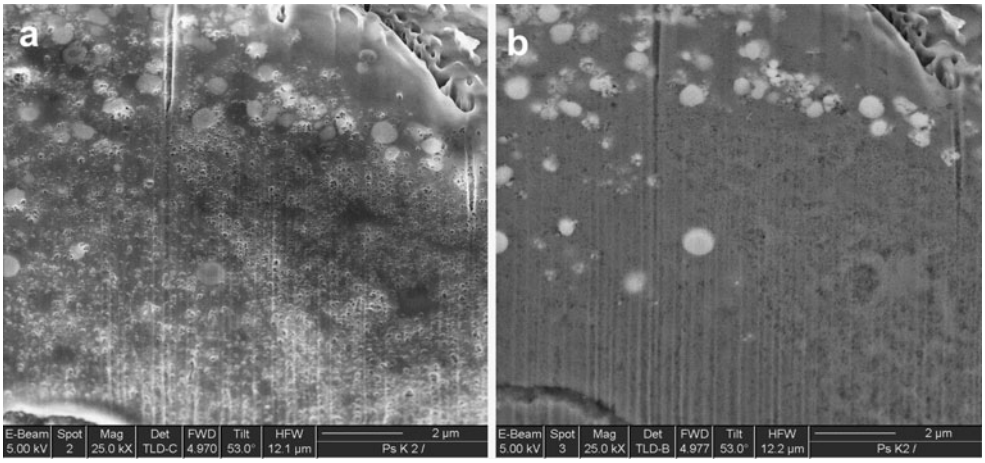

Fig. 2 **a** Secondary electron micrograph of an S cell and **b** back-scattered electron micrograph of the same region in the S cell. *Bright regions* on back-scattered electron micrograph indicate a significant presence of elements with atomic number higher than the ones typical of basic elements of organic materials. The sample was prepared without conductive staining

phosphorous, sulfur, carbon, oxygen, and calcium, chemical elements commonly found in living organisms. Gallium and silver were introduced to the specimen during the preparation procedure.

Linkage of FIB/SEM to conventional TEM

To compare the information given by FIB/SEM with that from TEM, the digestive gland of the same animal was embedded in plastic and prepared as for TEM. After TEM investigation the bulk sample was transferred into an FIB/SEM, FIB milled (Fig. 4b) then examined with a back-scattered electron detector. Back-scattered electron micrographs were inverted (reverse-contrast) to allow straightforward comparison with TEM images (Fig. 4c, d). Back-scattered electron micrographs and transmission electron micrographs, up to a certain magnification, provided very similar information on the ultrastructure of digestive gland cells (Figs. 4c, d, and 5).

Discussion

The FIB/SEM application could significantly contribute to the quality of structural investigation of biological specimens by simultaneous investigation of specimen gross morphology, surface and subsurface structures, and EDX analyses. Our study also provides evidence that FIB/SEM subsurface investigation, up to a certain magnification, produces results comparable to those from TEM.

a) Simultaneous investigation of specimen gross morphology and of some selected intracellular structures with characteristic shape and composition

We have demonstrated that FIB/SEM is suitable for simultaneous investigation of sample surfaces and subsurface structures. In SEM, the contrast is based primarily on specimen topography and in TEM, on the number of electrons transmitted through the specimen. Consequently, the cell ultrastructure, as imaged by SEM or TEM has a different appearance (Drobne et al. 2008).

Published data provide evidence that the aldehyde fixed and OTOTO-processed samples ensure the best ratio between the extracted and preserved material resulting in relief sufficient to allow distinction between different intracellular structures (Lešer et al. 2009). Accordingly, we selected this procedure for the FIB/SEM investigation of gross morphology and subcellular structures. The dried FIB/SEM samples prepared for conventional scanning electron microscopy are suitable for characterization of those intracellular morphological features, which have membranous/lamellar appearance and structures with composition of different density from the rest of the cell.

Compositional investigation by BSE imaging

The FIB/SEM system appeared to be a suitable tool with which to produce planar surfaces on biological samples for BSE imaging and also for other so-called analytical electron microscopes.

In this work, we provide evidence that the FIB/SEM can be used to make a planar surface for either SE or BSE imaging. The cells investigated in our study store metals as granules (Hopkin and Martin 1982; Morgan et al. 1995), which can easily be identified by BSE. BSE imaging of FIB-prepared biological samples can be used in investigations of those biological samples which contain not only low atomic numbered elements, but samples such as metal storing tissues or skeletons in which high atomic numbered elements are also expected.

Our results confirm that the higher atomic numbered elements appear slightly more intensely than lower atomic numbered elements in a secondary electron detector and significantly more intensely in a back-scattered electron detector (Bozzola and Russell 1998).

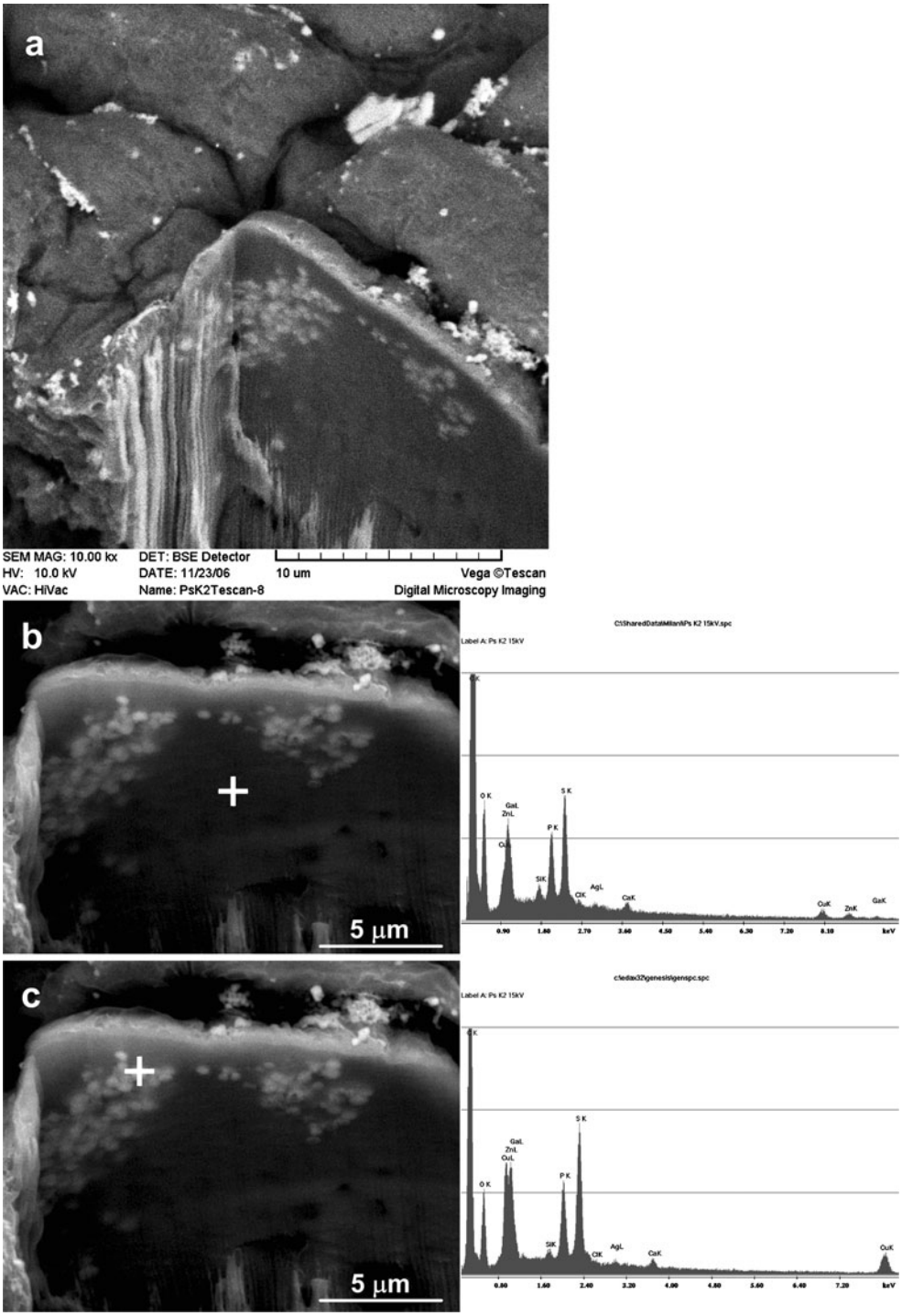

Fig. 3 Back-scattered electron micrograph of FIB-milled region. **a** FIB-milling operation was performed from the outer towards the inner part of a digestive gland tube. **b**, **c** The FIB-exposed region was EDX analyzed. The back-scattered electron micrographs with corresponding EDX spot spectra at two different locations on the FIB-exposed S cell surface of digestive gland tube are shown. The *crosses* on micrographs indicate the site of measurement **b** region of S cell without metal granules, **c** region of the S cell with metal granules (metal granules are seen as *bright spots*)

In theory, BSE imaging has lower resolution than SE imaging because of the larger region from which SE signals originate (Bozzola and Russell 1998). In practice, at the tissue level, the information obtained by BSE imaging is of satisfactory resolution compared to that of SE imaging and constitutes complementary information. Since samples were prepared without metal postfixing or conductive staining, charging effects due to their low conductivity could be expected. Our results, however, indicate that charging was not a problem.

Compositional investigation using EDX analyses

The incidence of irregular surfaces is one of the problems related to X-ray analyses of biological samples. This problem can be ameliorated by FIB milling but still, the

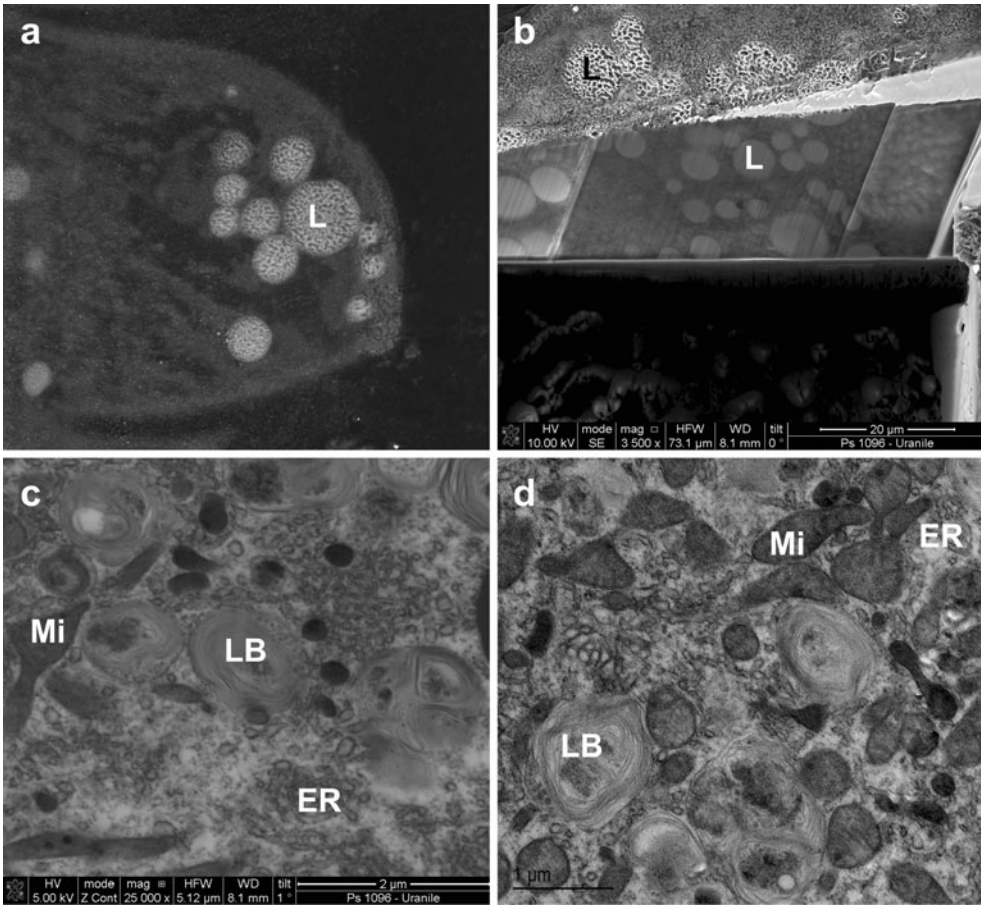

Fig. 4 Secondary electron micrographs of digestive gland tube embedded in plastic and cut with diamond knife **a** before and **b** after FIB milling. **c** Reverse-contrast back-scattered electron micrograph of FIB-milled area. All organelles can be recognized as in the corresponding transmission electron micrograph **d** made of the same digestive gland. *L* lipid droplet, *LB* lamellar body, *Mi* mitochondrium, *ER* endoplasmic reticulum

bulk biological sample of heterogeneous composition is not the best choice for X-ray analyses. It is, however, sufficient for screening of the predominant elements and their distribution at the tissue level. If elemental analysis is a primary concern, EDX analysis is a significant contribution to the proper selection of future approaches.

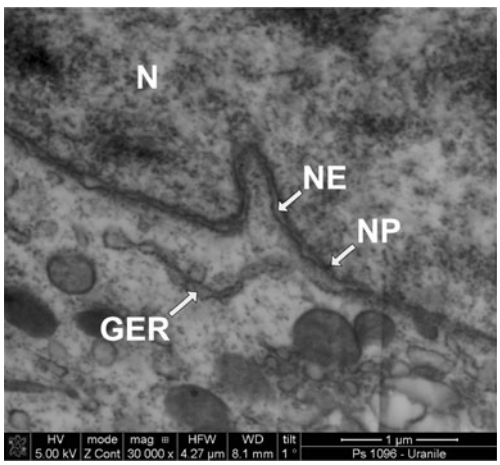

Fig. 5 Reverse-contrast back-scattered electron micrograph of FIB milled area at higher magnification. Note the detailed structure of the nuclear membrane. *GER* granular endoplasmic reticulum, *N* nucleus, *NE* nuclear envelope, *NP* nuclear pore

Linking FIB/SEM to conventional TEM

In the work presented here we provide evidence that when samples are embedded in plastic as for TEM, FIB/SEM enables both detailed recognition of cell ultrastructure and investigation of sample surface morphology. When samples, on the other hand are dried as for conventional SEM, subcellular composition can be analyzed. The FIB/SEM of dried samples does not allow unambiguous recognition of cellular organelles, but these can be recognized by FIB/SEM when samples are fixed, postfixed, and embedded in plastic as for conventional TEM then imaged by back-scattered electrons (Heymann et al. 2009; Lešer et al. 2009). This is explained by the fact that in plastic embedded samples a compositional contrast is predominant and recorded, while in dried samples a topographical contrast predominates and is more suitable for a different kind of characterization. In both cases, by using BSE imaging in either SEM or TEM, the compositional contrast can be recorded and this is why the images can be compared directly. However, in the case of FIB/SEM at higher magnifications, the yield of back-

scattered electrons is low and fails to provide satisfactory resolution. As a consequence, the FIB/SEM could be applied only up to a certain magnification, depending also on the biological features of the sample and preparation protocol used. For our samples the critical magnification is around 50,000. Above that magnification TEM is the logical choice for cell ultrastructural research.

We conclude that FIB/SEM has numerous advantages in structural research of biological samples. It enables simultaneous investigation of sample gross morphology, cell surface characteristics, and subsurface structures. It also allows in situ manipulation of a specimen to expose subsurface structures for SE, BSE imaging, or EDX analyses. When samples are prepared as for TEM, a straightforward comparison between FIB/SEM images and TEM images of major cellular structures, such as mitochondria, Golgi vesicles, or nuclei, is possible. The possibility of an overall structural and compositional and dynamic examination of a biological sample subject to normal, stress, or pathologic conditions is particularly interesting. Such information could provide new knowledge on the mode and extent of action of different stressors. FIB/SEM could be seen as a preceding step in morphological and structural research prior to a more detailed ultrastructural investigation by TEM or compositional analysis by EDX or any similar methods.

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Conflict of interest The authors declare that they have no conflict of interest.

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