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Focused Ion Beam/Scanning Electron Microscopy Studies of *Porcellio scaber* (Isopoda, Crustacea) Digestive Gland Epithelium Cells

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Summary: The focused ion beam (FIB) was used to prepare cross sections of precisely selected regions of the digestive gland epithelium of a terrestrial isopod *P. scaber* (Isopoda, Crustacea) for scanning electron microscopy (SEM). The FIB / SEM system allows ad libitum selection of a region for gross morphologic to ultrastructural investigation, as the repetition of FIB/SEM operations is unrestricted. The milling parameters used in our work proved to be satisfactory to produce serial two-dimensional (2-D) cuts and/or three-dimensional (3-D) shapes on a submicrometer scale. A final, cleaning mill at lower ion currents was employed to minimize the milling artifacts. After cleaning, the milled surface was free of filament- and ridge-like milling artifacts. No other effects of the cleaning mill were observed.

Key words: focused ion beam, scanning electron microscopy, ultramicroscopy, sample preparation, digestive glands, terrestrial isopod *Porcellio scaber*

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Introduction

The focused ion beam (FIB) system is a tool used in the characterization and modification of a wide variety of materials in the semiconductor and microcircuit fields. The FIB has also been used successfully as both an analytical instrument and a means for preparing specimens for subsequent analysis by scanning electron microscopy (SEM), transmission electron microscopy (TEM), secondary ion mass spectrometry (SIMS), x-ray photoelectron spectroscopy (XPS), and Auger electron spectroscopy (Dobrzhinetskaya *et al.* 2003, Haswell *et al.* 2003, Mackenzie and Smith 1990, Vasile *et al.* 1999). The FIB has also been proven to pattern materials with nanometer dimensions (Pickard *et al.* 2003, Rubanov and Munroe 2004, Xie *et al.* 2004). In the second half of the 1990s, applications combining in situ FIB sectioning with FIB imaging have attracted significant attention in the materials science community (Phaneuf 1999, Rubanov and Munroe 2004). In life sciences, the FIB sample preparation and subsequent FIB or SEM imaging is in an early stage of application (Drobne *et al.* 2004).

The FIB milling involves scanning a beam of ions across a sample. The beam is aligned to the exposed face either parallel to make a cross cut or perpendicular to make a trench. The momentum of the ions as they strike the surface of the sample is so high that the materials, atoms and ions, are sputtered away. In this way it is possible to prepare site-specific sections. The major strength of the FIB technique, as a specimen preparation technique, is the precision by which material and structures can be removed or sectioned on a submicrometer scale (Van Meerbeek *et al.* 1995). In addition, FIB cross sectioning exposes clean, uncontaminated surfaces. However, drawbacks with this technique involve artifacts, such as filament- and ridge-like artifacts, as a consequence of the nonuniform milling, melting effect, redeposition of the milled material onto the exposed face and implantation of gallium ions (Ga^+).

In life sciences, FIB has been used for the removal of surface layers from biological material to reveal underlying anatomical features (Young *et al.* 1993) and cellular structures (Ballerini *et al*. 1997, Drobne *et al*. 2004, Milani *et al.* 2004, Moulders 2003).

In the work presented here, digestive gland cells (hepatopancreas) of *Porcellio scaber* (Isopoda, Crustacea) were selected as a test system for performing the FIB / SEM operations. The terrestrial isopod *P. scaber* is one of the most studied organisms in ecophysiology and ecotoxicology (Drobne 1997). The digestive system of *P. scaber* has been thoroughly studied by means of light microscopy,

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SEM and TEM (Drobne and Štrus 1996, Žnidaršič *et a1*. 2003).

The aim of our work was: (1) to perform in situ FIB milling and SEM imaging of a single cell, (2) to perform in situ FIB milling and SEM imaging of a larger part of the digestive gland tube, and (3) to make step-wise in situ FIB milling and SEM imaging of the resulting three-dimensional (3-D) cut. The FIB milling parameters and the precision of a 3-D cut are discussed.

Materials and Methods

Terrestrial isopods, *Porcellio scaber* (Latreille 1809) (Isopoda, Crustacea), were collected under concrete blocks and pieces of decaying wood (Ljubljana, Slovenia). Digestive gland tubes were isolated and fixed in 1.0% glutaraldehyde and 0.4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2.5 h at room temperature. After dehydration in a graded series of ethanols, the digestive gland tubes were dried at the critical point (Balzers Critical Point Dryer 030, Bal-Tec, Balzers, Germany) and gold sputtered (sputter coater SCD 050).

The samples were mounted on brass holders with silver paint (high purity silver paint, SPI), fixed on a silicon wafer holder (5 cm diameter, 6-axis eucentric stage), and placed into a dual beam system for FIB/SEM operation (FEI Strata DB 235 M dual beam, Modena University, Italy). The ion Ga^+ currents for milling were in a range of 5 to 7 nA, beam energy was 30 keV Ga^+. Ions were field emitted from a liquid metal ion source. Lower beam currents in the range of 0.3 to 1.0 nA were used for the cleaning mill. The spot size in the case of milling was 200 to 300 nm in diameter, and in cleaning 50 to 100 nm in diameter. Dwell time for the milling was 1 μs/spot and the overlap was 50%. The SEM imaging was performed by means of the field emission gun (FEG) electron column available in the same system, with a SEM beam voltage of 5 kV. The spot size in the case of SEM was 5 nm in diameter. The system operated with column pressures in the 10^{-5} Pa range, with the work chamber between 10^{-4}–10^{-3} Pa.

Results

In situ FIB milling and SEM imaging were performed on a mechanically broken median region of a digestive gland tube (Fig. 1). The milling of a single digestive gland cell was in the direction from the apical part of the cell toward its basal part (Fig. 2a, perpendicular view to the ion beam). The apical part of the cell appeared homogeneous with numerous empty round-shaped holes of different sizes (Fig. 2b). These can be interpreted as areas where lipid droplets were originally deposited. The lipids were washed out by alcohol during the preparation because the sample was not postfixed with osmium. On the basal part of the milled cell, ridge-like structures can be observed, which are artifacts of the high current milling (Fig. 2a, c). After milling with high currents, milling with lower ion currents was used to clean the exposed surface (Fig. 2a, b). Due to the low current, the cleaning ions were stopped before reaching the basal part of the cell, and the difference between the cleaned and noncleaned surface is clearly seen (Fig. 2a).

Next, a region of 60×35 μm of the same gland tube was milled gradually. The location and dimensions of the milled area on the gland tube is shown in Figure 3 (from side view) and Figure 4a, b, and c (vertical view parallel to the ion beam). The structural integrity of the milled cells at different depths of the cut is shown in Figure 5a, b, and c (perpendicular view to the ion beam), and in Figure 6a and b (perpendicular view to the ion beam). The milled cells were not cleaned with a lower current beam. The arrows in Figure 5a, b, and c indicate the border of the same cell nucleus, which was cut at three different depths. The difference in the depth between the exposed surfaces in Figure 5a and b is approx. 1.5 μm, and between the exposed surfaces in Figure 5b and c the difference is approximately 1.0 μm. The time needed for milling a 0.5 μm deep cut was 4 min. A further step-wise milling operation, 35 μm deeper into the cells (Figs. 4b, c), revealed median parts of cells with nuclei and large empty holes corresponding to the location of lipid droplets (Fig. 6a, b). The edge of the step is shown magnified in Figure 6b.

Discussion

The FIB makes it possible to produce both two-dimensional (2-D) cuts at nm scale, and 3-D shapes of determined dimensions (Haswell *et al*. 2003, Inkson *et al*. 2001, Picard *et al*. 2003, Vasile *et al*. 1999, Young *et al*. 1993). The FIB/SEM system allows ad libitum selection of a region for

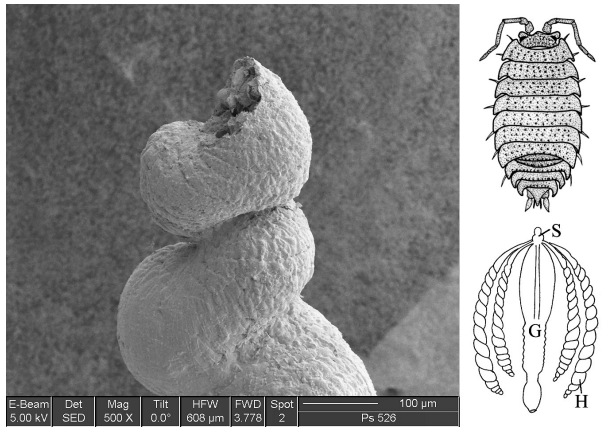

FIG. 1 The *Porcellio scaber* digestive system. Upper right corner: drawing of *P. scaber*. Lower right corner: scheme of *P. scaber* digestive system (S = stomach, G = gut, H = hepatopancreas). Left: scanning electron micrograph of a mechanically broken digestive gland tube.

the gross morphologic to ultrastructural investigation. The repetition of FIB/SEM operations is unrestricted. The milling parameters used in our work proved to be satisfactory for producing serial 2-D cuts and/or 3-D shapes on a submicrometer scale.

The artifacts associated with the FIB operations are not well understood, especially those in non-semiconductor materials (Cairney *et al.* 2000, Cairney and Munroe 2003). In our work, the final cleaning mill at an aperture corresponding to 0.3 to 1.0 nA was employed to minimize the milling artifacts. Filament- and ridge-like artifacts are eas-

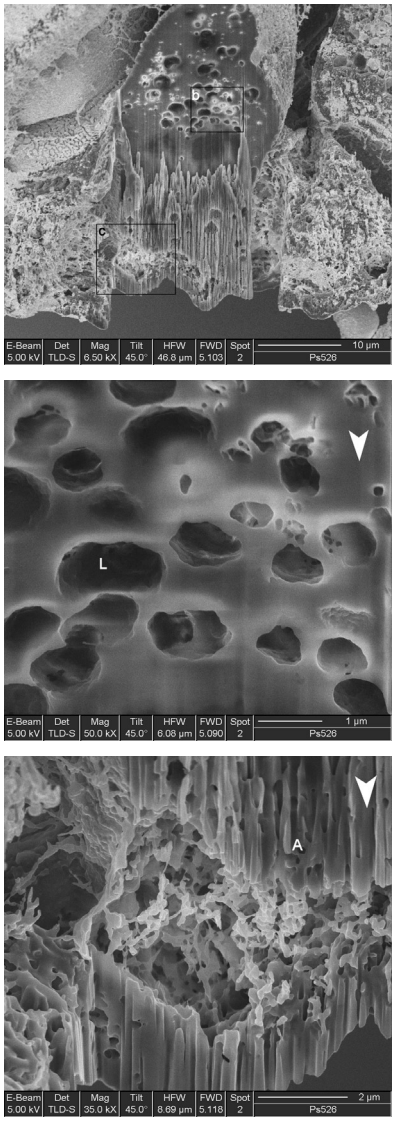

FIG. 2 (a) Scanning electron micrograph of a cut of a single cell. Rectangles indicate regions which were magnified and shown in b and c. (b) Scanning electron micrograph of the middle part of the cleaned cell. L = empty holes where lipids were deposited, arrowhead = direction of the ion beam. (c) Scanning electron micrograph of the basal part of the cell with pronounced vertical ridge-like milling artifacts (A) Arrowhead = direction of the ion beam.

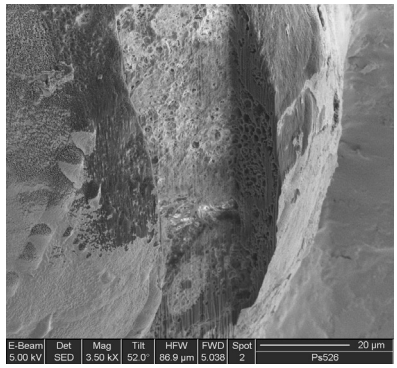

FIG. 3 Scanning electron micrograph of the external part of the gland tube. The shape and dimensions of the cut are shown from the side view.

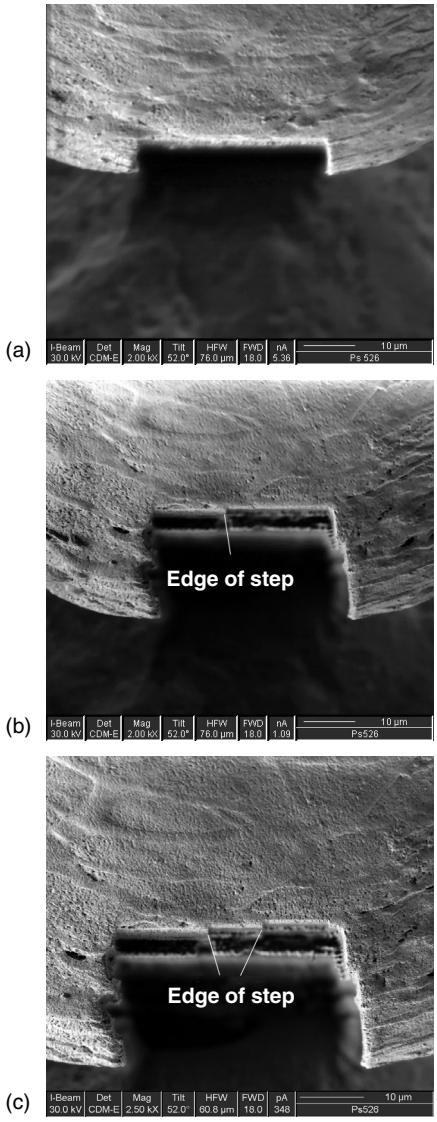

FIG. 4 (a) Ion micrograph shows the same region of the digestive tube as in Figure 3, but from a different perspective. (b) Shape of the deeper double step cut. (c) Shape of the triple step cut.

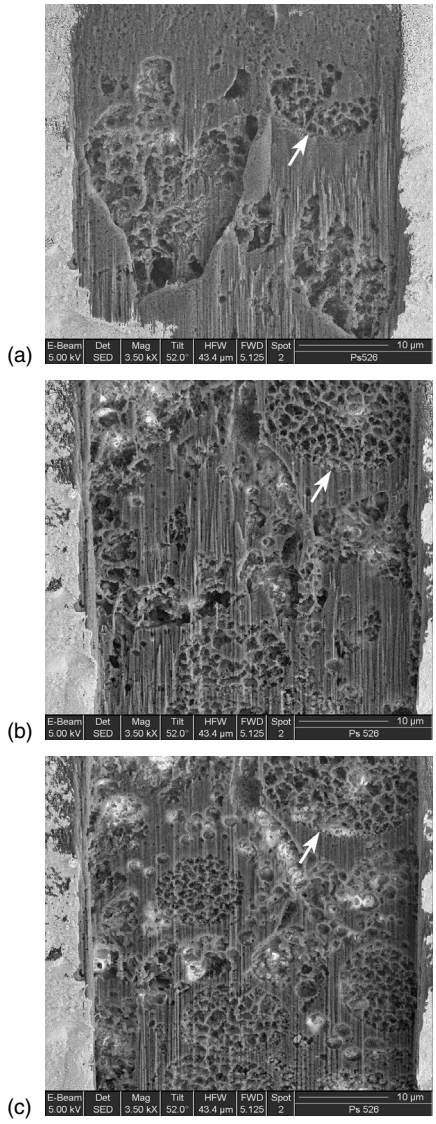

FIG. 5 (a–c) Scanning electron micrographs of the basal part of cells which were cut at different depths. The arrow indicates the border of the same nucleus which was cut at different levels.

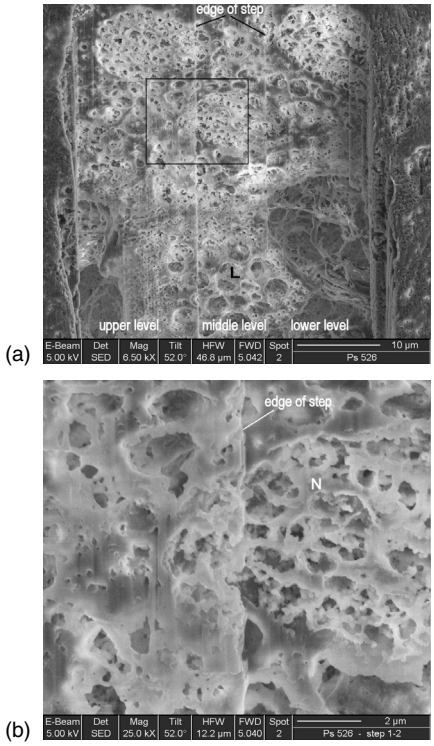

FIG. 6 (a) Scanning electron micrograph of the basal part of cells where the triple step cut was performed. (b) Magnified edge of the cut. N = nucleus, L = empty holes where lipids were deposited.

ily recognized; they have the same direction as the impinging ion beam responsible for the cut. After the final cleaning mill, the milled surface was smooth. Similar milling irregularities appearing in the form of striations were also observed by Young *et al.* (1993) when milling the cuticular structures of arthropods. Low-energy ions are also used to remove the presence of thin layers on the exposed surface as a result of melting, implantation of Ga^+ ions, or redeposition of milled material (Haswell *et al.* 2003). We observed no difference in the structural composition between the cleaned and noncleaned surfaces. In spite of that, some milling artifacts cannot be excluded. To reduce and control the artifacts caused by the FIB on biological samples, a great deal of fundamental research for a detailed understanding of the beam-specimen interaction remains to be performed.

The microscopic anatomy of a digestive gland epithelium of a terrestrial isopod *P. scaber* has already been thoroughly studied by light microscopy, SEM, and TEM (Drobne and Štrus 1996, Žnidaršič *et a1.* 2003). The application of FIB / SEM revealed new characteristics of the structural integrity of cells, which were not observed before by other microscopies (Drobne *et al.* 2004). A novelty of the FIB / SEM approach is in the simultaneous investigation of gross morphology of the organ and ultrastructure of cells in different regions of the organ. The potential of FIB / SEM lies in the microscopy coupled to in situ sample preparation for further quantitative imaging of the chemical composition at nanometer resolution (Chandra 2001).

The FIB/SEM system was virtually unknown in the life sciences community half a decade ago. Conference presentations and journal articles that mention the use of FIB/SEM in life sciences are rare. Initially driven by semiconductor industry, commercial manufacturers are now turning their attention to materials scientists and life scientists to elicit the feedback regarding future developments of this technology. In our opinion, the future of FIB/SEM in life sciences is bright, and the present holds many opportunities for both research and application-driven use of this technology.

References

Ballerini M, Milani M, Costato M, Squadrini F, Turcu ICE: Life science applications of focused ion beams (FIB). *Eur J Histochem* 41, 89–90 (1997)

Cairney JM, Munroe PR: Redeposition effects in transmission electron microscope specimens of FeAl–WC composites prepared using a focused ion beam. *Micron* 34, 97–107 (2003)

Cairney JM, Munroe PR, Schneibel JH: Examination of fracture surfaces using focused ion beam milling. S*cripta Material* 42, 473–478 (2000)

Chandra S: Studies of cell division (mitosis and cytokinesis) by dynamic secondary ion mass spectrometry ion microscopy: LLC-PK1 epithelial cells as a model for subcellular isotopic imaging. *J Microsc* 204, 150–165 (2001)

Dobrzhinetskaya LF, Green HW, Weschler M, Darus M, Wang YC, Massonne HJ, Stockhert B: Focused ion beam technique and transmission electron microscope studies of microdiamonds from the Saxonian Erzgebirge, Germany. *Earth Planetary Sci Lett* 210, 399–410 (2003)

Drobne D: Terrestrial isopods—a good choice for toxicity testing of pollutants in the terrestrial environment. *Environ Toxicol Chem* 16, 1159–1164 (1997)

Drobne D, Štrus J: The effect of Zn on the digestive gland epithelium of *Porcellio scaber* (Isopoda, Crustacea). *Pflügers Archive* 431, 2, R247–R248 (1996)

Drobne D, Milani M, Ballerini A, Zrimec A, Berden Zrimec M, Tatti F, Drašlar K: Focused ion beam (FIB) for microscopy and for in situ sample preparation: application on a crustacean digestive system (*Porcellio scaber*, Isopoda, Crustacea). *J Biomed Opt* (2004) 1238–1243

Haswell R, McComb DW, Smith W: Preparation of site-specific cross-sections of heterogeneous catalysts prepared by focused ion beam milling. *J Microsc* 211, 161–166 (2003)

Inkson BJ, Mulvihill M, Möbus G: 3-D determination of grain shape in a FeAl-based nanocomposite by 3-D FIB tomography. *Scripta Material* 45, 753–758 (2001)

Mackenzie RAD, Smith GDW: Focused ion beam technology: A bibliography. *Nanotechnology* 1, 163–201 (1990)

Milani M, Batani D, Ballerini M, Squadrini F, Cotelli F, Lora Lamia-Donin C, Poletti G, Pozzi A, Eidmann K, Lucchini G: High-resolution microscopy techniques for the analysis of biological samples: a comparison. *Eur J Phys: Appl Phys* 26, 123–131 (2004)

Moulders H: The use of a SEM/FIB dual beam applied to biological samples. *G.I.T. Imaging Microsc* 2, 8–10 (2003)

Phaneuf MW: Applications of focused ion beam microscopy to materials science specimens. *Micron* 30, 277–288 (1999)

Picard YN, Adams DP, Vasile MJ, Ritchey MB: Focused ion beam-shaped microtools for ultra-precision machining of cylindrical components. *Prec Eng* 27, 59–69 (2003)

Rubanov S, Munroe PR: The application of FIB milling for specimen preparation from crystalline germanium. *Micron* 35, 549–556 (2004)

Van Meerbeek B, Conn Jr. LJ, Duke ES, Schraub D, Ghafghaichi F: Demonstration of a focused ion-beam cross-sectioning technique for ultrastructural examination of resin-dentin interfaces. *Dental Mater* 11, 87–92 (1995)

Vasile MJ, Nassar R, Xie J, Guo H: Microfabrication techniques using focused ion beams and emergent applications. *Micron* 30, 235–244 (1999)

Xie DZ, Ngoi BKA, Fu YQ, Ong AS, Lim BH: Etching characteristics of TiNi thin film by focused ion beam. *Appl Surf Sci* 225, 54–58 (2004)

Young J, Dingle T, Robinson K, Pugh PJA: An application of scanned focused ion beam milling to studies on the internal morphology of small arthropods. *J Microsc* 172, 81–88 (1993)

Žnidaršič N, Štrus J, Drobne D: Ultrastructural alterations of the hepatopancreas in *Porcellio scaber* under stress. *Environ Toxicol Pharmacol* 13, 161–174 (2003)