Cryo-FIB-SEM serial milling and block face imaging: large volume structural analysis of biological tissues preserved close to their native state

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Abstract

Many important biological questions can be addressed by studying in 3D large volumes of intact, cryo fixed hydrated tissues ($\geq 10,000 \mu\text{m}^3$) at high resolution (5-20 nm). This can be achieved using serial FIB milling and block face surface imaging under cryo conditions. Here we demonstrate the unique potential of the cryo-FIB-SEM approach using two extensively studied model systems; sea urchin embryos and the tail fin of zebrafish larvae. We focus in particular on the environment of mineral deposition sites. The cellular organelles, including mitochondria, Golgi, ER, nuclei and nuclear pores are made visible by the image contrast created by differences in surface potential of different biochemical components. Auto segmentation and/or volume rendering of the image stacks and 3D reconstruction of the skeleton and the cellular environment, provides a detailed view of the relative distribution in space of the tissue/cellular components, and thus of their interactions. Simultaneous acquisition of secondary and back-scattered electron images adds additional information. For example, a serial view of the zebrafish tail reveals the presence of electron dense mineral particles inside mitochondrial networks extending more than 20 μm in depth in the block. Large volume imaging using cryo FIB SEM, as demonstrated here, can contribute significantly to the understanding of the structures and functions of diverse biological tissues.

Introduction

Biological structures are complex, hierarchical and often graded, and most importantly are hydrated. Deciphering these structures at high resolution in their in vivo or as close as possible to the in vivo conditions, is challenging to say the least. Biological structures have been probed for more than 300 years, and a diverse array of instruments and methods are currently available including optical, electron and X-ray imaging techniques. Here we describe one of the newest methods for biological structural analysis that integrates the enormous benefits of cryo-imaging and analyzing large volumes, in 3 dimensions. Cryo-FIB-SEM (Focused Ion Beam-SEM) can produce 3D image stacks of a volume of thousands of micrometers cubed at an image pixel size down to 5 nm, while still embedded in a hydrated state in vitreous ice. This method thus fills a valuable niche in biological cryo-imaging by closing the gap between light microscopy and TEM tomography.

Cryo-FIB-SEM merges two technologies. The FIB-SEM instrument was developed in the 1990's mainly for applications in the semiconductor industry (Krueger, 1999). In 2006 Subramaniam and his colleagues demonstrated that this instrument could be adapted for producing 3D volumes of biological tissues by using the heavy ion beam to remove thin (20 nm) slices off the surface of a traditionally embedded block of a fixed and stained biological tissue (Heymann et al., 2006). The electron beam is then used to image each newly exposed block face. This process of serial FIB milling and SEM imaging is iterated hundreds of times and all the images are aligned to produce a 3D stack (Heymann et al., 2006; Stokes et al., 2006). This technology has been applied to diverse biological tissues, either traditionally embedded, or after high pressure freezing and freeze substitution. The examined tissues include yeast cells, tumor tissue (Heymann et al., 2006) and cancer cells (Revach et al., 2015), bone tissue (Reznikov et al., 2013; Schneider et al., 2010; Schneider et al., 2011), malaria parasite (Weiner et al., 2011), bacterial invasion of cells (Weiner et al., 2016) and brain tissue (Knott et al., 2008;

Merchán-Pérez et al., 2009). The technique provides 3D imaging of large volumes of tissue, suffering however from artifacts deriving from fixation, staining and drying.

Dubochet revolutionized EM biological imaging by developing a method for rapid freezing such that the water does not crystallize, but remains amorphous or vitreous, thus preserving the biological structures in almost their native state (Adrian et al., 1984; Dubochet et al., 1983). Such rapid freezing was limited to very thin samples. Moor, Muller and colleagues resolved this problem by developing the high pressure approach to freezing and thus enabled relatively large volumes of biological tissue with a thickness of up to 200 μm to be vitrified (Moor, 1987; Studer et al., 1988). This opened the way to merging the FIB-SEM “slice and view” approach with the huge benefits of cryo-fixation. It also required new developments in detector technology so as to enable contrast to be obtained on unstained biological materials embedded in vitreous ice. The recently developed cryo-FIB-SEM technique (Schertel et al., 2013) allows three-dimensional imaging of high pressure frozen biological samples under conditions that are very close to their native state, without any chemical treatment (Bennet et al., 2015; Sviben et al., 2016; Vidavsky et al., 2015). Contrast is achieved by detection of low energy secondary electrons that are influenced by differences in the surface potential of chemically distinct species (Schertel et al., 2013). The voxel size of the 3D data can be modified according to the imaging requirements of the region of interest even within the same specimen. Using in-lens secondary electron detection, the cellular ultrastructure can be directly visualized in the freshly exposed serial FIB cross-sections. By simultaneous detection of the InLens SE and the InLens backscattered electron (EsB) signals complementary information on the tissue ultrastructure and material contrast/localization of heavier elements can be acquired slice by slice (Bennet et al., 2015; Sviben et al., 2016). The cryo-FIB-SEM workflow is extremely fast, requiring less than an hour from organism sacrifice to the first results.

Here we demonstrate this methodology using tissues which we are actively investigating, namely the developing sea urchin embryo and zebrafish larvae – both

important models for developmental biology (Driever et al., 1994; Giudice, 1973; Kimmel et al., 1995; Lawson and Weinstein, 2002).

Our aim is to understand how cells produce mineralized tissues. Both the sea urchin embryonic skeleton and the larval zebrafish bone form through highly unstable transient precursor mineral phases. These precursor phases are first formed in vesicles inside cells (Akiva et al., 2015; Beniash et al., 1999; Kerschnitzki et al., 2016; Mahamid et al., 2011; Mahamid et al., 2010). The precursor phases can easily dissolve or change during conventional sample preparation, but are well preserved by vitrification. The cells and the associated mineralized tissues occupy volumes of thousands of cubic micrometers. Our scientific objectives include the characterization of contacts between cells, between a cell and the extracellular environment, and the spatial relations between the forming mineralized element and the surrounding membranes. Cryo-FIB-SEM is thus probably the only technology that can currently provide this information, under conditions that still preserve the intracellular and extracellular mineral deposits.

Materials and Methods:

Sea urchin embryonic/larval culture. Mature cultured adult sea urchins *Paracentrotus lividus* were supplied by the Israel Oceanographic and Limnological Research Institute. Spawning was induced by injecting 1 mL of 1M KCl solution into the coelomic cavity. The egg suspension was collected, washed, and kept in sea water containing 30 mg/L Penicillin G sodium salt (Sigma–Aldrich, 69578) and 15 mg/L Streptomycin sulfate salt (Sigma-Aldrich, 3810740) at 18 °C. Sperm was kept at 4 °C undiluted. The fertilization was carried out by sperm dilution in sea water and rapid mixing with the egg suspension inside sea water containing 30 mg/L Penicillin G sodium salt (Sigma–Aldrich, 69578) and 15 mg/L Streptomycin sulfate salt (Sigma-Aldrich, 3810740). The culture was kept at 18 °C with gentle shaking (100 rpm). The developmental stage of the embryo was determined from the spicule and embryo characteristic morphology, using a light microscope with normal and polarized light. When the prism developmental stage was

reached (Giudice, 1973), the samples were high pressure frozen without any processing, as described below. The research involving sea urchins is approved by the Israel Oceanographic and Limnological Research, National Center for Mariculture.

Zebrafish husbandry. Adult and larval zebrafish were raised and maintained as described (Avraham-Davidi et al., 2012) and were handled according to the guidelines of the Weizmann Institute Animal Care and Use Committee. Embryos were generated by natural spawning and raised in water in an incubator at 28 ± 0.5 °C as described (Westerfield, 2000).

Sample preparation for cryo-imaging:

Sea urchin embryos: 10 μ L of the embryo suspension in sea water mixed with 10% dextran (40,000 Da) (Sigma, 31389), were sandwiched between two metal discs (3-mm diameter, 0.1 mm and 0.05 mm cavities) and cryo-immobilized in a high pressure freezing device (HPM10; Bal-Tec).

Zebrafish larvae: Tails were dissected from 25-30 days post fertilization (dpf) zebrafish larvae and were immersed immediately in 10% dextran (Sigma, 31389), sandwiched between two metal discs (3 mm diameter, 0.1 or 0.05-mm cavities with flat cover above). The samples were cryoimmobilized in a high-pressure freezing device (HPF, HPM10; Bal-Tec). After HPF, the flat top was removed.

Cryo FIB:

The samples were transferred into the preparation box of the VCT shuttle system (Leica Microsystems, Vienna, Austria) in a liquid nitrogen bath. The sample carriers containing the high-pressure frozen samples were mounted on a cryo-sample holder for 3mm HPF sample carriers (Leica Microsystems, Vienna, Austria). Under cryo-conditions the cryo-holder was transferred into the cryo-sputter coater (Leica Microsystems, Vienna, Austria) using the Leica VCT sample shuttle.

During imaging the angle between the cross-section normal and the SEM column was 36 deg. The raw images were recorded using tilt correction for 36 deg. For tilt correction the separation between horizontal scan lines is reduced to obtain the same distance

between pixels in both directions in the plane of the cross-section. Using tilt correction the images have square pixels and appear like recorded without any tilt.

Sea urchin embryos imaging: The sample was sputter-coated with 8 nm platinum using the Leica SCD500 cryo sputter coater (Leica Microsystems, Vienna, Austria) and was transferred in the Zeiss Auriga60 FIB-SEM (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) using the VCT100 shuttle (Leica Microsystems, Vienna, Austria). Throughout the imaging the samples were kept below -145°C and the system vacuum pressure was 2.2×10^{-6} mbar. A coarse trench about 50 µm wide was milled for SEM observation using the 30kV@16nA FIB probe current. No cold deposition was done and prior to serial sectioning and imaging no further processing besides the initial sputter-coating was done. For serial FIB milling and SEM imaging the slice width was 45 µm and for FIB milling the 30kV@600pA probe current was used. After milling of each slice InLens and EsB SEM images were simultaneously collected at 1.8 kV acceleration potential using the 15 µm aperture in normal mode. The EsB grid was set to 870V. The image size was 2048 x 1536 pixels. For noise reduction line average with a line average count N=90 at scan speed 2 was used. The final data cube after alignment and cropping is composed of 526 serial sections comprising a volume of X= 20µm, Y= 12µm Z=11 µm, with a voxel size of 10x10x20 nm.

Zebrafish tail imaging: An 8nm platinum layer was sputter-coated at -110 °C on the sample or freeze-fractured surface using the Leica ACE600 cryo sputter coater (Leica Microsystems GmbH, Vienna, Austria), in an attempt to reduce curtaining. The sputter conditions were 35mA current, 2.5×10^{-2} mbar argon pressure and 0.07 nm/sec sputter rate. After sputter coating the sample holder was transferred into a Zeiss Auriga60 FIB-SEM using the VCT500 prototype shuttle (Leica Microsystems GmbH, Vienna, Austria). The Leica cryo-stage temperature was set to -120°C and the system vacuum pressure was 3×10^{-7} mbar.

Notwithstanding the relatively high temperature used, if devitrification occurred during surface sublimation before platinum-coating, the ice crystals are below the resolution

limit and do not cause detectable changes in morphology. During imaging, ice sublimation due to the sample temperature alone is not expected to result in significant surface topography since after every image a new cross-section is cut. This is supported by the fact that if a second image of the same area is taken, the image is similar and no significant changes in surface topography are observed (besides beam damage being obvious after prolonged scanning).

As a viewing channel for SEM observation, an initial 100 μm wide FIB trench was coarse milled using a FIB probe current of 16 nA. In order to minimize the curtaining or water fall effect a Pt precursor protection layer was deposited by manually opening and immediately closing the gas valve for several times. For cold deposition, the platinum precursor reservoir was not heated and temperature was 25°C. For serial FIB milling and SEM imaging a 77 μm wide cross-section was milled using the 30kV@2nA FIB probe current. After removal of each FIB slice InLens SE and EsB backscattered electron images of the freshly exposed cross-section were taken simultaneously at 2.3 kV acceleration potential using the 30 μm aperture in normal mode. The EsB grid was set to -1500 V. The image size was 204x1536 pixel and for noise reduction line average with a line average count N=171 at scan speed 1 was used.

A data cube of 609 serial sections comprising a volume of X= 40 μm , Y= 23.5 μm , Z=24.5 μm , with a voxel size of 20x20x40 nm was obtained after alignment and cropping. A second data set was obtained with a voxel size of 15.1x15.1x100 nm. Note that the Z direction referred to in the voxel size refers to the thickness of the milled material removed from the surface. For each slice InLens and EsB images were obtained simultaneously. For this reason, two corresponding set of images were acquired with different detectors displaying diverse information (ultrastructure versus material contrast) which can be overlaid.

Image analysis. The obtained images were aligned using the ImageJ (US National Institutes of Health, Bethesda, Maryland, USA) and Avizo 9 software (FEI). After alignment, the stacks were cropped to achieve a final stack with fully overlapping

images. Noise filtering, including elimination of curtaining, was carried out by using the ImageJ software through FFT-based corrections. As an example, one zebrafish tail raw image and the corresponding processed image are reported in Figure S1. Contrast enhancement was obtained with the “enhance contrast” process of ImageJ. 3D reconstruction was done with Avizo on the InLens image stack. Automatic segmentation and volume rendering was carried out after bandpass filtering. After smoothing, iso-surfaces were generated from the segmented data. Brightness, contrast and gray levels of single images were adjusted by Adobe Photoshop CS 4 Extended (Adobe Systems, San Jose, CA).

Results

Here we demonstrate the use of cryo-FIB SEM to visualize in 3D two well studied model systems in developmental biology and biomineralization: the sea urchin embryo and the zebrafish larva. In the zebrafish larvae, we concentrate on the forming tail fins.

Cryo-FIB SEM imaging strategies

The Zeiss Auriga60 FIB-SEM has two beams: a focused gallium ion beam for sample serial milling and an electron beam for imaging. The milling and imaging are applied to a high pressure frozen sample, and are carried out under cryogenic conditions. The voxel size is determined by the lateral resolution in XY and by the distance between slice surfaces (i.e. slice thickness) in depth (Z), and can be set according to the question being addressed. For example, the imaging of a large volume (e.g., 10 μm in depth) at an image resolution of 5nm will require 2000 slices, and hence a long acquisition time and a very large data set, especially when both in-lens secondary electron and the EsB detector micrographs are acquired. We use the in-lens secondary electron detector for imaging of the ultrastructure and the EsB detector for detection of minerals, as materials with higher Z atomic number such as calcium appear brighter in the EsB detector. The combination of these detectors provides complementary information (ultrastructure versus material contrast/localization of bio minerals) and enables better interpretation of the gray level of the different imaged features. Acquisition and

exposure time can be reduced, as it is possible to acquire both secondary and backscattered electron images simultaneously.

Unlike conventional FIB-SEM imaging, in cryo-FIB-SEM imaging the samples are not artificially stained with heavy metals. The mechanism by which the image contrast is obtained in the unstained cryo-fixed samples is not well established. It was suggested that the contrast results from local differences in surface potential: local negative charge yields repulsion of electrons and hence a brighter signal, and local positive charge yields darker signals because less electrons leave the surface (Schertel et al., 2013).

The Inlens detector collects the low energy secondary electrons generated by the direct interaction of the primary beam with the sample at the focusing point. These low energy electrons are easily influenced by the presence of different local potentials at the surface.

Sea urchin embryo

In the sea urchin embryo, the cellular ultrastructure is clearly observed in each of the micrographs of the acquired stack. The cellular organelles are recognized based on their distinct morphologies: the plasma membrane, nuclei, nuclear membrane, nuclear pores, polymembranous bodies and filopodial extensions, endoplasmic reticulum, mitochondria, Golgi apparatus and vesicles are all clearly identifiable (Fig. 1 A-C).

The observed organelles have various morphologies and appear with different gray levels, which presumably reflect their chemical compositions and/or morphologies. The aqueous cytosol being rich in water appears bright, while the membranes and mitochondria that are composed of lipids appear dark. Dark particles that are surrounded with a brighter cross shape are observed. These dark particles are lipid or proteinaceous bodies (Fig. 1 C), showing local charging by the electron beam. The cross-shaped artifact is enhanced by image processing. The micrometer size vesicles containing dark particles are identified as the vesicles in which the initial amorphous

calcium carbonate precursor phase is deposited (Beniash et al., 1999). This identification is based on their size and abundance (Vidavsky et al., 2014). In some cases, there are discontinuities in the plasma membranes, which could be due to imaging artifacts caused by local charging associated with inhomogeneous charge dissipation. The local charging may overwhelm the contrast provided by chemically distinct species (Fig. 1 C).

Analyzing this huge amount of data can be carried out in various ways. The most straightforward way is to prepare a video of all the images in sequence, i.e. the entire data base. Video 1 shows an example of a whole acquisition stack with a large field of view (hence lower than maximal resolution) showing many of the features observed in figure 1. The video is periodically paused, and the figure legend points out interesting features that can be observed in each pause.

Fig. 1

Cryo-FIB-SEM micrographs of high pressure frozen sea urchin embryos showing various cellular compartments. (A) A cell containing several different organelles and vesicles, with distinct morphologies. Intracellular polymembranous bodies can be seen (green arrow). A filopodial extension is indicated (red arrow). Mitochondria (blue arrows) are distinguished according to their size, morphology and internal structure. Inset: magnification of mitochondrion. Inset dimensions: 800 nm x 1000 nm. (B) Intracellular compartment adjacent to the nucleus, which is most likely part of the endoplasmic reticulum (green arrow). (C) Intracellular dark organelles that may be lipid or proteinaceous bodies (red arrow). The dark bodies have a characteristic bright cross shape around them, due to local charging enhanced by image processing. The endoplasmic reticulum or Golgi apparatus (green arrow) is surrounded by small dark particles, which are most likely secretion products. Some discontinuities in the membranes are observed (red asterisk). N- Nucleus.

In addition to studying the image sequences, both from the imaging direction and from the reconstructed directions, a complementary way of approaching the large data set is by 3D segmentation and/or volume rendering. Volume rendering of the sea urchin embryo enables the characterization of the cellular compartments in 3D, and the elucidation of their functions according to their spatial locations and contacts with their environment. Figure 2 shows a large volume of the sea urchin embryo: the stack is composed of more than 500 slices with a lateral field of view of $20 \times 12 \mu\text{m}$. the pixel size of each acquired image is $10 \times 10 \text{ nm}$. The images were taken 20 nm apart from each other in a serial manner, which results in a total volume of $20 \times 12 \times 10.5 \mu\text{m}$ (Fig 2 A). The cross section of the embryonic endoskeleton, the spicule, is gray, as are the cells, which are easily differentiated from the spicule (Fig. 2 A). A detailed automated volume rendering (Fig. 2 B) of the whole stack reveals complex spatial arrangements of the cellular compartments. This is challenging to study due to the crowded details. However by focusing in on specific details, interesting observations can be made. For example, fine cellular filopodia that are barely recognized in 2D can be seen at high resolution in such an isolated volume, located on the outer surface of a cell (Fig. 2 C). An important advantage of 3D data is the ability to rotate the data and collect information from different viewing angles. For example, the spicule surroundings are packed with vesicles, which most likely take part in the spicule formation process. In order to understand the spatial interaction between the spicule and the vesicles, and whether there are contact sites between them, 3D examination of the data is required. Some vesicles that are in close proximity to the spicule may appear to be in contact with it, but are actually at a micrometer distance from it (Fig. 2 D-E). Alternatively the 3D model of the acquired and processed data cube can be re-sliced in any direction/orientation providing electronically reconstructed cross-sectional images for further specific analysis.

Fig. 2

Cryo FIB-SEM micrographs of a high pressure frozen sea urchin embryo: (A) A spicule cross section (S) and the surrounding organelles. Scale bar: 1 μm . Inset: Global view of a stack of 526 serial micrographs after 3D reconstruction. The stack has a volume of $X=20\mu\text{m}$, $Y=12\mu\text{m}$ $Z=11\mu\text{m}$. (B) 3D automatic reconstruction of the stack presented in A. The volume is packed with details, making it challenging to isolate each compartment. (C) Magnification of the area marked with a rectangle in the segmented data shown in B, showing thin filopodial extensions of the cell (arrow). The observed filopodia are not detectable in the 2D images, and are clearly visible as a result of the volume rendering. (D) Enlargement of the spicule environment in the segmented data shown in B, in a longitudinal view of the spicule. The spicule surroundings are packed with intra- and extracellular vesicles. The 3D view enables a better understanding of the vesicle spatial orientation. The vesicles marked with arrows are both in close proximity to the spicule, and rotation is needed in order to verify their exact spatial location relative to the spicule. (E) Enlargement of the vesicles marked in D after 3D rotation. In this orientation the vesicle marked with a green arrow is clearly contacting the spicule, whereas the vesicle marked with a red arrow is approximately 1 μm away from the spicule. S= spicule.

Zebrafish larval tail

Figure 3 shows individual micrographs and reconstructed images from a particularly large volume of the zebrafish larval tail at a higher pixel size imaged in cross section. This stack comprises 609 slices that were imaged with a lateral pixel size of 20 nm (Fig. 3 A-C). The milling step was 40 nm, covering an overall depth of 24.5 μm , composing a total imaged volume of 40x23.5x24.5 μm . The imaged volume provides a detailed view of the fish tail anatomy and of the interactions between the bone, cells, blood vessel and other extracellular compartments (Fig. 3, 4). The entire larval tail tissue thickness is 15-25 μm (Fig. 3A-C). The mineralized bone and the outer epithelial cell layer are clearly distinguished by their characteristic morphologies and textures. While the nuclei and cell cytosol have similar gray levels as those observed in the sea urchin, other features in the tissue are much darker. A thin dark gray layer (80-100 nm) separates the epithelial cell layer from the inner cellular content of the tissue (Fig. 3A, blue arrow). This thin layer is most likely the basement membrane that consists of different types of collagenous proteins and glycoproteins and connects the epithelial layer to the bone-cell layer (Huang et al., 2009; Smith et al., 2008). Figures 3B and C show the YZ plane of the acquired stack after reconstruction. Despite the fact that the resolution in this direction is significantly lower than the pixel size in the XY plane, additional features are detected in the YZ plane. The bone structure along the growth direction of the tail comprises a continuous thin layer of single cells separating the bone from the basement membrane and the epithelial layer (Fig. 3B, marked by *). The blood plasma texture shows particles with gray levels that can be distinguished from the characteristic appearance of the blood cell nuclei. Interestingly, the borders of the blood vessels are not well defined in the XY plane, but can be easily detected in the YZ plane as a long and continuous tubular structure (Fig. 3 C).

By combining different magnifications and pixel sizes, large volume structures can be better understood. A higher resolution stack with a lateral pixel size of 5.2 nm of the bone growth region was thus acquired. The inset in Figure 3D shows small black

particles inside the blood vessel (artery). A wavy structure separates the blood plasma from the thin conjugated endothelial cells. Different mineralization stages can be observed in the non-mineralized collagen region, based on the different gray levels. The mineralized region has a porous structure, which is most likely due to its early maturation stage. The cell-collagen interface is also shown. Some of the cells show undefined membrane borders, which are most likely in the process of exocytosis.

Fig. 3

Cryo-FIB-SEM micrographs of a high pressure frozen zebrafish larval tail acquired in a serial manner from 2 large volumes of the specimens. (A-C)The micrographs are part of a stack of 609 serial micrographs comprising a volume of $X=40\mu\text{m}$, $Y=23.5\mu\text{m}$, $Z=24.5\mu\text{m}$, with a voxel size of

20x20x40 nm. (A) A micrograph from the XY plane showing a cross section of the fish tail. The tissue thickness is 15-25 μm (tissue borders are marked with white arrows). The outer epithelial cell layer (red dashed line) is confined by a basement membrane (darker gray layer, blue arrow). Two hemi-cylindrical bone segments of one bony ray (Bo) appear dark gray on the right side of the image. The bones are surrounded by cells (cell nuclei – N). The vein is located next to the cells, between different rays (cyan dashed line). (B-C) Slices from the YZ plane of the cryo-FIB-SEM stack after reconstruction. (B) Two hemi-cylindrical bone segments of one bony ray (Bo) in longitudinal view and the inner part of the ray (green dashed line). A thin cell layer separates the bone from the basement membrane (*). (C) The blood vessel is observed in a longitudinal view as a continuous tubular entity (cyan dashed line), confined by a basement membrane. Inset: Small gray particles are distributed along the blood vessel. Two cells (*) are also observed inside the blood vessel. (D) Higher magnification of the growth region of the bone, obtained from a different stack with a pixel size of 5.2x5.2 nm. BM-basement membrane, NMC-non-mineralized collagen, N-nucleus, Ar-artery, * - endothelial cell, ** - a wavy structure delimiting the blood vessel, #- a hole caused by a beam damage while focusing in this area. Inset: particles located inside the artery. Scale bar: A-C 2 μm , D-500 nm.

By combining different detectors, various aspects of the tissue are better understood. Figures 4 A and B show two different mineral-associated compartments of the forming zebrafish larval tail using the InLens (Fig. 4A) and the EsB (Fig. 4B) detectors. The two mineral-associated compartments are mineralized bone and mineral particles inside the mitochondrial network. Both compartments contain calcium and phosphate minerals (see also (Martin and Matthews, 1970; Sayegh et al., 1974; Sutfin et al., 1971)), but their gray levels using the InLens detector are different (Figure 4 A). The small CaP particles are detected in the InLens cryo-FIB SEM images as bright white particles, whereas the bones are much darker. The difference in the gray levels between the bone and the mitochondrial particles is attributed to the fact that CaP mineral only comprises about half the volume in bone, whereas the mitochondrial particles are composed almost entirely of mineral. This difference in CaP density will result in higher backscattered electron yield for the mineral particles and lower BSE yield in case of the bone due to its protein collagen content. This can explain the contrast difference in the InLens SE image since backscattered electrons generate low energy secondary electrons (SE2) while exiting the sample. Confirmation that the small white particles are indeed calcium-containing material is shown in the EsB image (Fig. 4B). While bone is easily detected both by InLens and EsB detectors, the small mitochondrial particles are harder to detect

by the EsB, possibly due to their small size (~ 50 nm) relative to image pixel size and due to the low signal to noise ratio of the EsB compared to the InLens image. Figure 4C shows a 3D reconstruction and segmentation of the bones and the mitochondrial network with their mineralized particles. The presented stack is of $24.5\ \mu\text{m}$ in depth, allowing a view of the organelles and their relation to the forming bone in large volume. The interactions between the different mitochondrial networks are visible in videos 2 and 3, where the same image stacks are presented in two different planes (XY for video 2 and XZ for video 3). Some of the mitochondrial networks show the presence of white mineral particles whereas other mitochondrial networks are detected without these particles. Video 4 shows a 3D reconstruction and segmentation of one mitochondrial network with the mineralized particles, taken from the same image stack as in videos 2 and 3.

Fig. 4

Cryo FIB-SEM micrographs of a high pressure frozen zebrafish larval tail. InLens (A) and BsE (B) micrographs were acquired simultaneously. (A-B) The micrographs are a part of a stack of 154 serial micrographs comprising a volume of $X = 31\mu\text{m}$, $Y = 23\mu\text{m}$, $Z = 15.4\mu\text{m}$, with a voxel size of $15.1 \times 15.1 \times 100\text{ nm}$. (A) InLens micrograph showing the ultrastructure of the biological tissue and mineralized structural features at various gray levels. Bone appears in dark gray while brighter particles are observed inside the mitochondria (purple arrow). Inset: digital magnification showing the mitochondria containing the bright particles. (B) BSE signal of (A) providing material contrast. Bones that contain high concentration of calcium are light grey. Small white particles located inside the mitochondria (arrow) show high back-scattered electron yield. Note that the non-mineralized region of the joint (*) is black in the BsE image indicating the absence of high density concentrations of minerals. Scale bar: $2\mu\text{m}$. (C) 3D reconstruction and segmentation of the bones (yellow) and the mitochondrial network (purple), which show back-scattered electron signal yield in the BsE image and bright white signal in the InLens detector. The segmentation was derived from the stack presented in figure 3, showing a volume of $40 \times 23.5 \times 24.5\mu\text{m}$. The segmentation is presented in video 4.

Discussion

We show here how cryo-FIB-SEM serial block face imaging and reconstruction can be effectively used to study large volumes of biological samples in 3D. We image high pressure frozen samples that did not undergo any chemical processing or staining, thus minimizing potential damage and morphological changes in the sample. Intracellular organelles and compartments were imaged and characterized in 3D by using the InLens secondary electron detector, while minerals were localized in 3D by using the backscattered electron (EsB) detector. The maximum imaging resolution, around 5 nm isotropic voxel size, is high, when considering the large volume imaged. Combining the advantages of relatively high resolution with the large volume imaging and the Z-contrast characterization by the EsB detector, sheds new light on the nature of various cellular compartments and components and their interactions with other compartments.

The sizes and morphology of easily identified cellular organelles, such as nuclei and mitochondria, imaged with cryo-FIB-SEM are similar to what was previously reported (Ingersoll et al., 2003). So even though conventional preparations involve fixing and dehydration, we do not observe major size differences.

Cryo-FIB SEM does not visualize the actin network that comprises the cytoskeleton of the cell. This is most probably due to the actin filament size (<7 nm) that is below or comparable to the FIB-SEM imaging pixel size used (5 to 20 nm). Actin filaments are observed in cryo EM tomography methods that have a higher resolution (Elad et al., 2013; Medalia et al., 2007; Patla et al., 2010).

In contrast to samples that were stained with heavy metals, in cryo-FIB-SEM of intact specimens the image contrast and quality are more sensitive to charging because low energy electrons are detected instead of backscattered electrons for imaging the ultrastructure.

In the sea urchin embryo we repeatedly noticed the existence of imaging artifacts around dark spherical bodies and around the cross section of the spicule. In both cases, the imaging artifacts most likely result from charging caused by differences in charge dissipation. The mineralized spicule, which is denser than the cellular environment around it, is most likely milled differently by the Ga beam of the FIB. The dark spherical bodies are probably lipid bodies that charge positively because carbon and hydrogen rich molecules more easily lose electrons compared to oxygen rich molecules. Both spicules and lipid bodies often produce charging artifacts.

We show that the calcium carbonate and calcium phosphate biominerals observed here have different contrasts when imaged by inLens secondary electrons, and it is yet unclear what causes these differences. The spicule, which is composed almost entirely of calcium carbonate ($\sim 95\%$), appears light gray, whereas the small mitochondrial calcium phosphate particles in the zebrafish tail appear bright white. The gray levels obtained cannot be compared directly because the images were acquired using different EHT voltages and the image contrast depends on the beam energy. However, gray level differences between calcium carbonate and calcium phosphate are likely to be detected even where the imaging conditions are identical. We suggest that the differences can be explained at least in part by the different electronic structures that affect the amount of low energy secondary electrons emitted. A material that can be

easily ionized will produce more signal, especially in the low voltage regime where the interaction volume is small and close to the escape depth of secondary electrons. Joy et al. (Joy and Joy, 1996) suggested that the InLens detector can respond to changes in the sample chemistry, thus providing chemical SE contrast mechanism. The yield of the low energy secondary electrons, both SE1 and SE2, strongly depends on the surface potential of the sample, and is extremely relevant for poorly conductive biological objects. By setting the landing voltage close to the critical energy (E_2), where a charge balance between incoming and outgoing electrons is satisfied and the integrated yield from a given area approaches unity, the local charging can be minimized to enhance the native contrast. Since the E_2 values of CaP and CaCO_3 should be different and are probably lower than 3kV, the differences in contrast between the two compounds at these low voltages can be extreme (Goldstein et al., 2003). Monte Carlo simulations (Drouin et al., 1997; Hovington et al., 1997; Li et al., 2011) show that calcium phosphate produces much more back-scattering signal than calcium carbonate at 1.5 kV acceleration potential (Fig. S2). Since backscattered electrons generate low energy secondary electrons (SE2) while exiting the sample, it is conceivable that the back scattered and InLens electrons have the same relative behavior.

Another parameter that may influence the obtained gray levels is the environment. A particle dispersed in an aqueous vitrified solution may yield a different gray level than the same material in bulk, because of different charge dissipation. This could be the reason for charging. Finally, because the zebrafish sample was imaged at a higher temperature (-120°C), imaging beam induced sublimation may contribute to the observed local surface potentials because charged particles are leaving the surface.

Cryo-FIB-SEM imaging of large volumes yields large data sets that contain hundreds of images, making image processing and analysis crucial. Manual segmentation of the reconstructed data is beneficial when the 3D structures of specific features need to be emphasized, but is a highly time consuming process. Automatic volume rendering of the InLens stacks yields a volume that is packed with details of many cellular and

extracellular compartments. When imaging large volumes with low pixel size it becomes challenging to interpret the richness of the obtained segmented data. In some cases, splitting the data into sub-regions of interest may be helpful.

Cryo FIB SEM can be compared to other cryo 3D imaging techniques. Cryo electron tomography (Dierksen et al., 1993; Dubrovsky et al., 2015; Lučić et al., 2013; Medalia et al., 2002a; Medalia et al., 2002b; Nicastro et al., 2000), including cryo- scanning transmission electron microscopy (STEM) and cryo- scanning transmission electron tomography (STET) (Wolf et al., 2014; Wolf et al., 2015) provide 3 dimensional visualization under cryogenic conditions and at resolutions higher than cryo-FIB SEM serial block face imaging. Cryo EM tomography methods can be used to solve the 3D structures of proteins and other cellular components at a few Å resolution. However, the imaging volumes using cryo EM tomography are limited, as the sample thickness is only hundreds of nanometers. Cryo soft X-ray tomography allows the imaging of higher volumes, with sample thickness of up to a few micrometers, but with lower resolution (around 50 nm) (Hanssen et al., 2011; Le Gros et al., 2005; McDermott et al., 2009). Note too that cryo- FIB milling has been used to prepare samples for cryo EM tomography (Harapin et al., 2013; Harapin et al., 2015; Lučić et al., 2013; Mahamid et al., 2016).

The novel cryo-FIB-SEM technique offers extensive possibilities for correlative workflows between light, electron and X-ray microscopy. Regions of interest could be fluorescently labeled and imaged before or after sample freezing, followed by correlation with cryo-FIB-SEM imaging. Also, during serial milling and imaging, cryo EDS may be applied to the exposed surface of the sample, although this could result in damage of the exposed surface. Another powerful tool is the combination of cryo-FIB-SEM imaging with a lift-out of a thin slice of the sample, milled by the FIB, and cryo-transferred into the TEM where complimentary high resolution microscopy and tomography may be applied.

Concluding Comment

Cryo FIB SEM fills a very important niche in the cryo imaging repertoire. Sample volumes large enough to include many cells and other tissue constituents can be imaged at relatively high resolution under conditions that are close to those in vivo. These capabilities combined with the possibilities of directly correlating molecular targets labelled and imaged in vivo in the light microscope with the volumes obtained at high resolution in the FIB SEM, offer many opportunities for solving structural and mechanistic biological problems.

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