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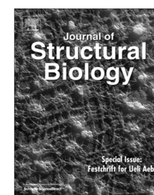

In-situ integrity control of frozen-hydrated, vitreous lamellas prepared by the cryo-focused ion beam-scanning electron microscope

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ABSTRACT

Recently a number of new approaches have been presented with the intention to produce electron beam transparent cryo-sections (lamellas in FIB-SEM terminology) from hydrated vitreously frozen cryo samples with a Focused Ion Beam (FIB) system, suitable for cryo-Transmission Electron Microscopy (cryo-TEM). As the workflow is still challenging and time consuming, it is important to be able to determine the integrity and suitability (cells *vs.* no cells; vitreous *vs.* crystalline) of the lamellas. Here we present an in situ method that tests both conditions by using the cryo-Scanning Electron Microscope (cryo-SEM) in transmission mode (TSEM; Transmission Scanning Electron Microscope) once the FIB-made lamella is ready. Cryo-TSEM imaging of unstained cells yields strong contrast, enabling direct imaging of material present in the lamellas. In addition, orientation contrast is shown to be suitable for distinguishing crystalline lamellas from vitreous lamellas. Tilting the stage a few degrees results in changes of contrast between ice grains as a function of the tilt angle, whereas the contrast of areas with vitreous ice remains unchanged as a function of the tilt angle. This orientation contrast has subsequently been validated by cryo-Electron BackScattered Diffraction (EBSD) in transmission mode. Integration of the presented method is discussed and the role it can play in future developments for a new and innovative all-in-one cryo-FIB–SEM life sciences instrument.

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1. Introduction

Cryo electron microscopy, both cryo-Transmission Electron Microscopy (cryo-TEM) and cryo-Focused Ion Beam – Scanning Electron Microscopy (cryo-FIB–SEM), is a steadily developing field because of its ability and potential to study biological specimens in their native state. Optimized freezing protocols (Müller and Moor, 1984; Medalia et al., 2002) prevent water from crystallization inside and outside the cells, thereby preventing damage to the ultrastructure of the cell during ice crystal formation. The state of samples, when correctly frozen by these freezing protocols, is called vitreous.

For cryo-TEM observations, the area of interest must be thinned down to electron transparency while maintaining its vitreous status. Both cryo-ultra microtomy (Al-Amoudi et al., 2004) and cryo-FIB milling (Marko et al., 2006, 2007; Edwards et al., 2009; Rigort et al., 2010, 2012a; Hayles et al., 2010; Wang et al., 2012; Strunk et al., 2012) are techniques that can achieve this.

Although the viewing area of the FIB-made lamella is much smaller compared to that of a cryo-ultra microtome, major advantages are the absence of artifacts caused by cryo-sectioning (no crevasses, compression (Al-Amoudi et al., 2005)), the possibility to vary the thickness and achieve greater final thicknesses and the potential of site specificity (Jiménez et al., 2010). Especially for cryo-electron tomography, thicker cryo lamellas allow larger volumes/structures to be investigated.

The workflow for producing FIB-made lamellas while maintaining the vitreous status is at the moment time consuming and labor intensive. Therefore it is very useful and important to be able to conduct integrity controls throughout the process; firstly confirming the presence of the cells of interest in the lamella and secondly the vitreous status of the ice. In cases where the presence of ice crystals or absence of cells is evident then the process can be stopped and restarted with a new sample, saving important time of the operator and use of the instrument.

In the workflow described by Hayles et al. (2010) the first control takes place during trimming of the sample with the cryo-ultra

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microtome. Experienced operators can visually check the vitreous status of the sample. Exposed vitreous ice appears black inside the cryo-ultra microtome chamber under standard room TL-tube illumination. Crystalline samples appear whitish. In addition trimming is hampered by the presence of crystals in the sample, resulting in the breaking out of small parts of the sample's block face instead of cutting sections.

The method described in the present paper examines the integrity of the FIB-made lamella in the cryo-FIB–SEM microscope after the milling has finished and the sample still under vacuum. Once the lamella is made, both the presence of cells and the confirmation of vitreous status of the ice are done with cryo-SEM imaging in transmission mode, called cryo-TSEM. The name of a SEM in transmission mode varies in the literature. It is simply referred to as STEM (Scanning Transmission Electron Microscopy), but to distinguish it from the same scanning mode available in the TEM, it is also called STEM-in-SEM or TSEM (Transmission Scanning Electron Microscopy) (Klein et al., 2012). Throughout this paper we will use the latter term, TSEM.

Due to the relatively low acceleration voltage of the SEM (30 kV) in comparison with a TEM (80–300 kV), the contrast between the vitreous ice and the cells is very strong, enabling imaging of cells in absence of any chemical staining. With the presence of ice crystals, different crystallographic orientations of these crystals will create contrast, also known as channeling contrast. The standard method to determine the vitreous state is recording diffraction patterns in a cryo-TEM. In order to validate orientation contrast within the cryo-SEM, a recently introduced technique called t-EBSD (Transmission-Electron BackScatter Diffraction) (Keller and Geiss, 2011) is extended to cryo conditions and is applied to confirm the relation between orientation contrast and the presence of ice crystals by diffraction patterns.

Therefore both the presence of cells and the status of the ice can be verified in-situ in the cryo-FIB–SEM, which together will help decide whether to continue to the cryo-TEM for further examinations or to start a new experiment. The presented work is the first application of cryo-TSEM with t-EBSD on samples from life sciences.

2. Materials and method

All the cryo-TSEM experiments are performed in a Nova Nanolab 600 Dualbeam (FEI Company, Eindhoven, The Netherlands). The microscope is equipped with a PP2000T cryo transfer system with a CHE2000 12 l Dewar and an Advanced Transfer Unit (ATU) (Quorum Technologies Ltd., Ringmer, UK). The preparation chamber facilitates a Pt sputter coating unit for metal coating of the sample prior to the cryo-FIB–SEM work and a cooled through vacuum probe to manually manipulate the sample. A cold trap is present in the cryo-FIB–SEM chamber and kept at −175 °C to collect any water vapor that comes within the vicinity of the sample, so protecting the sample from contamination.

Also installed is a Nordlys II Electron BackScatter Diffraction (EBSD) detector (Oxford Instruments HKL technology, Abingdon, UK). A Gas Injection System (GIS) with a Pt precursor is used to in-situ planerize the sample (Hayles et al., 2007).

2.1. Samples

Fresh bakers yeast (*Saccharomyces cerevisiae*) was used as a sample. Yeast in an amount of 5 g was suspended in 100 ml miliQ. The yeast cells were rehydrated for 2 h at room temperature under gentile agitation, using a top-down Almicon stirrer. After rehydration, yeast cells were gently spun down in 1.5 ml aquilots at 105 RCF. Supernatant was removed and the pellet was re-suspended resulting in a 30 μl cell-suspension.

2.2. Preparation of the sample

The samples have been frozen in a High Pressure Freezer (HPF) (Leica EMHPF, Leica Microsystems, Vienna, Austria). Sample loading is done in 100 μm deep membrane carriers (Leica Microsystems). In order to freeze the membrane carriers in the HPF system, sandwiches are made which fit the 1 mm deep cavity of the standard specimen holder (Fig. 1). The sandwich consists of a 400 μm thick spacer ring on which the membrane carrier is positioned. After filling the sandwich with 0.6 μl concentrated yeast suspension the sample is covered with a 100 mesh copper grid with a carbon coated formvar film (film towards the sample) followed by a type B platelet (300 μm recess, Wohlwend engineering office, Sennwald, Switzerland), flat side down. Special care is taken to prevent the presence of air bubbles in the sandwich. The type B platelets were coated with 2% lecitin in 100% ethanol to facilitate removal of the platelet after freezing. The grid in the sandwich prevents the lecitin from dissolving into the sample. After the freezing the platelet and spacer rings are removed from the carriers under LN2 (liquid nitrogen). Subsequently the carrier is trimmed using a cryo-ultra microtome (UCT/FC, Leica Microsystems, Vienna, Austria). The membrane carrier is orientated perpendicular to the knife and trimmed in this orientation until the edge of the membrane carrier is removed. Next the knife is rotated about 45 degrees, trimming the membrane carrier into a wedge shape, taking away the metal sides surrounding the sample. The trimming procedure for the wedge is indicated in Fig. 1 as dashed lines across the membrane carrier. The result is similar to the one shown in Hayles et al., 2010 (Fig. 7a therein). The trimming conditions were: knife speed 50 mm/s and a feed of 170 nm, while in the cryo-ultra microtome the chamber, the knife and the stage were kept at a temperature lower than −160 °C.

2.3. The cryo-TSEM stage

The cryo-TSEM stage is a special designed stage (Quorum Technologies Ltd., Ringmer, UK) consisting of a redesigned top block with an additional hole projecting downwards in the center. At the bottom a thermally and electrically insulated diode is mounted to collect the TSEM signal. The cryo-TSEM stage accepts a standard cryo-sledge. A standard cryo-sledge was modified with a hole to allow a signal to pass through to the diode below. On top of the cryo-sledge a clamping mechanism is mounted which holds the sample (the membrane carrier) above the hole and the diode. The clamping mechanism is made from the tip of a standard TEM grid holder which is mounted such that it can be tilted from a horizontal position (TSEM position) to 38 degrees (milling position) where it clicks in place. The moveable part is called the flipper. Fig. 2 depicts both the cryo-sledge and the cryo-TSEM stage.

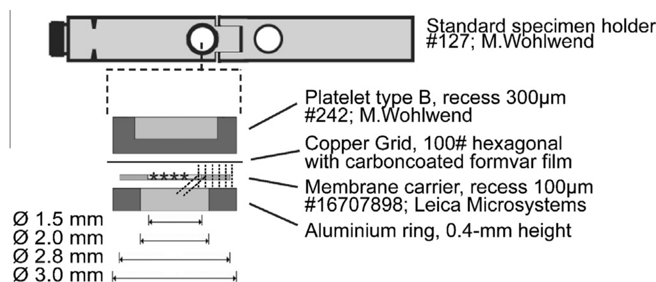

Fig.1. The specimen sandwich used to high pressure freeze the sample with a High Pressure Freezer. The * indicates the position of the sample and the dashed lines across the membrane carrier indicates the cutting of the cryo-ultra microtome.

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