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Cryo-FIB specimen preparation for use in a cartridge-type cryo-TEM

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Abstract

Cryo-electron tomography (cryo-ET) is a well-established technique for studying 3D structural details of subcellular macromolecular complexes and organelles in their nearly native context in the cell. A primary limitation of the application of cryo-ET is the accessible specimen thickness, which is less than the diameters of almost all eukaryotic cells. It has been shown that focused ion beam (FIB) milling can be used to prepare thin, distortion-free lamellae of frozen biological material for high-resolution cryo-ET. Commercial cryosystems are available for cryo-FIB specimen preparation, however re-engineering and additional fixtures are often essential for reliable results with a particular cryo-FIB and cryo-transmission electron microscope (cryo-TEM). Here, we describe our optimized protocol and modified instrumentation for cryo-FIB milling to produce thin lamellae and subsequent damage-free cryotransfer of the lamellae into our cartridge-type cryo-TEM.

Keywords

Cryo-ET; Cryo-FIB; TEM cartridges; Yeast; Organelles; Alcohol-oxidase

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1.0 Introduction

Cryo-electron tomography is a powerful method for studying three-dimensional (3D) structures of macromolecular complexes or sub-cellular organelles in vitreously frozen cells and tissues, which are preserved in near-native state (Harapin et al., 2013; Lucic et al., 2013). However, application of cryo-ET is typically restricted to regions of specimens that are thinner than 500 nm. The inelastic mean-free path of 300 keV electrons in biological specimens embedded in vitreous ice is hardly more than 300 nm, yet almost all eukaryotic cells and tissue samples are thicker than 500 nm. Thus, micrograph quality and image resolution decrease as the specimen thickness increases, due to chromatic aberration of the TEM and due to the weaker signal from increased total scattering. Vitreously frozen eukaryotic cells and tissue can be prepared for cryo-ET by cryo-ultramicrotomy (Hsieh et al., 2006; Vanhecke et al., 2007), however, forces during mechanical sectioning cause distortion, and the “cryosections” adhere poorly to the specimen support, making cryo-ET unreliable (Hsieh et al., 2006; Marko et al., 2007). To address these problems, focused-ion-beam (FIB) milling, a mature technique from materials science, was adapted for preparing thin, vitreously frozen biological specimens for cryo-ET (Marko et al., 2006; Marko et al., 2007). This technique was further developed for cultured cells on TEM grids (Rigort et al., 2010), cell suspensions (Hayles et al., 2010) and tissue (de Winter et al., 2013; Hsieh et al., 2014). Recently, successful application of cryo-FIB for vitreously frozen muscle tissue using specially designed fixtures compatible with a side-entry cryo-TEM stage was described (Wagenknecht et al., 2015).

Although it has been a decade since the first proof-of-principle application, routine use of cryo-FIB for cryo-TEM preparation has only been reported from a small number of laboratories, due to significant technical challenges. Commercially available systems have needed modification or the development of ancillary fixtures, often specific to a particular TEM, such as the FEI Polara (Rigort et al., 2012; Wang et al., 2012) and FEI Titan Krios (Schaffer et al., 2017; Zhang et al., 2016). Here, we report our recently re-engineered Leica cryo-transfer holder that facilitates efficient cryo-FIB preparation for cryo-ET in our cartridge-type JEM-3200FSC cryo-TEM (JEOL Ltd., Tokyo, Japan).

A typical cryo-FIB preparation protocol includes four steps: (1) Vitreously freezing the sample; (2) transferring the sample onto the coldstage in the FIB-SEM instrument; (3) FIB-milling the sample; (4) cryotransferring the sample out of the FIB-SEM and into the TEM. While it is essential to maintain the sample at -140°C or below at all times, the final step is the most critical. The very fragile TEM lamella needs to be transferred from FIB-SEM to TEM without damage or frosting from exposure to room air. Our solution for cartridge-type TEMs is to accommodate the cartridge itself in the FIB-SEM, thus avoiding direct handling of the sample and allowing the FIB-prepared sample to be manipulated in the same way as a routine cryo-EM specimen. This involves in a relatively simple re-engineering of the Leica cryo-transfer holder to accept a JEOL cartridge, and milling a slot in the JEOL EM specimen cartridge to allow access by the ion beam.

2.0 Methods

2.1 Freezing yeast cells

Yeast cells from the strain *Pichia pastoris* were cultured in BMMY media with 1–2% methanol added, following the protocol reported previously (Wang et al., 2016). Cells were cultured for 4 days at 30°C, then harvested by 1000×g centrifugation and re-suspended in water immediately before plunge-freezing. For plunge-freezing, 3 µl of the cell suspension was placed on 200-mesh Quantifoil grids (R 2/1) and plunge-frozen in liquid ethane using an FEI Vitrobot IV (FEI, Hillsboro, OR).

2.2 Handling the sample in the cryo-TEM cartridge

Our strategy for protecting the sample after FIB-milling is to mount the cryo-TEM grid directly in a modified TEM cartridge before introducing it into the FIB-SEM coldstage. This required modification of the TEM cartridge to allow FIB-beam access to the sample, and also modification of the pre-tilted cryo-transfer holder to hold the TEM cartridge during cryotransfer into and out of the FIB-SEM.

We use a Leica VCT-100 cryo-system (Leica Microsystems, Vienna, Austria) with a Zeiss Neon EsB-40 FIB-SEM (Carl Zeiss, Oberkochen, Germany). The VCT-100 system includes a cryotransfer shuttle system as well as the coldstage and anticontaminator in the FIB-SEM chamber. Leica provides a pre-tilted cryo-transfer holder (Leica product no. 16770266) for holding TEM grids, which fits both the shuttle and the coldstage. We have described the use of this system in detail, together with modifications and special fixtures for cryo-transfer of FIB-milled samples into a side-entry cryo-TEM (Hsieh et al., 2014).

With the original Leica cryotransfer holder, the TEM grid is mounted directly in the holder for cryo-FIB milling (Fig. 1a). After cryotransfer from the FIB-SEM, the FIB-milled TEM grid, with its fragile lamellae, is removed from the holder and inserted into the cryo-TEM cartridge. However, in our experience, handling the FIB-milled TEM grid usually causes breakage of almost all the FIB-thinned lamellae. We found that mounting the FIB-milled TEM grid into the JEOL specimen cartridge (Fig. 1e and f, which involves careful manipulation of a thin washer and a tiny screw-down ring of 0.32 mm thickness to be done under liquid, resulted in broken lamellae. Therefore, we designed and re-engineered a system that facilitates mounting the TEM grid in the cryo-TEM cartridge before FIB-milling, so that the fragile sample does not need to be directly handled after FIB-milling.

In our new workflow, we mount the TEM grid in a modified cartridge (Fig. 1g) before FIB-milling. The cartridge is then mounted in a modified Leica cryo-transfer holder (Fig. 1c, d, h and i). The holder was modified to accommodate the cartridge, while still allowing automatic opening of the protective shutter upon insertion into the FIB-SEM coldstage. The shutter is closed during cryotransfer and not opened until the holder is in the FIB-SEM stage. The cartridge is pre-tilted in the holder (Fig. 1h and i) so that little additional tilt is needed to reach the proper FIB-milling angle.

2.2.1 Details of cartridge and transfer holder modifications—The original transfer holder is shown in Figs. 1a,b. TEM grids (arrows in Fig. 1a) are held in a clamping

mechanism and are accessible for loading and for FIB milling when the shutter is open. The shutter is manually opened for grid loading (Fig. 1a) and closed (in Fig. 1b) during cryotransfer in the shuttle. After cryotransfer, the shutter opens automatically when the holder is inserted into the FIB-SEM coldstage.

In order to accommodate the TEM cartridge in the holder, we machined three small parts and installed them with the screws into the holder as shown in Figs. 1c,d. First of all, we removed the grid-clamping mechanism as shown in Fig. 1b to make space for the modification. The empty space in the open side (the right side in Fig. 1c and d) was filled with a semi-cylindrical copper block, marked as part C in Fig. 1d, to prevent entrance of contaminants when the shutter is closed. Then, we built a holding mechanism (consisting of two parts marked A and B in Fig. 1c) for the TEM cartridge in the left side of the holder (Fig. 1c), which normally would be exposed when the shutter is open. A semi-circular block, part B in Fig. 1c, holds the cartridge at the correct angle for FIB-milling, and the cartridge is fixed in place with a leaf spring, part A in Fig. 1c.

After our modification, the shutter assembly had to be reoriented by 180° compared to the original version, so that it opens automatically upon insertion of the holder into the coldstage. The shutter is opened and closed by a rod with a semicircular cross-section, which is inserted into a D-shaped hole in the modified holder (blue arrows in Fig. 1a, b, h and i) to handle the holder in the shuttle and in the FIB-SEM coldstage. The rod is inserted with the shutter closed. After transfer to the FIB-SEM coldstage, rotation of the rod activates the four grippers (black arrows in Fig. 1a) that fix the holder in the slot in the FIB-SEM coldstage, and at the same time the shutter is rotated to the open position, making the sample accessible to the ion beam. In addition, material marked by the circle in Fig. 1b had to be removed to allow the shutter to fully close.

The above modifications allow the cryo-transfer holder to be handled in the normal way, and permits FIB-milling from the original ion-beam direction. For a detailed assembly of the device, please refer to the carton movies supplied in the Supplementary Materials.

To prepare satisfactory thin lamellae from cells on TEM grids, the ion beam must be inclined at about 10° with respect to the grid. Although the cartridge is pre-tilted in the transfer holder to present an appropriate angle, the edge of an unmodified TEM cartridge would occlude the ion beam over most of the area of the TEM grid. Therefore, a slot having a width of 1.5 mm, and with a depth of 0.36 mm (sufficient to reach the surface of the TEM grid), was milled in the back side of the cartridge (Fig. 1g). This allows full access in both the FIB and the TEM to the same area of a TEM grid that is originally available for tilt-series collection over a full tilt range of 120–140 degrees in an unmodified cartridge.

2.3 Cryo-FIB milling

The FIB-SEM has a field-emission electron source and a gallium ion source. The electron beam is used for monitoring milling progress with minimal irradiation damage. Typically, the electron beam energy is 1.3 keV, and secondary electrons are captured by the in-lens detector. The ion beam, always operated at 30 keV, can be used at low current (e.g. 10 pA) for imaging, and ion-excited secondary electrons are collected by an Everhart-Thornley

detector. As seen in Fig. 2a, the ion beam images in the direction of cutting, enabling set-up of the milling pattern, while the electron beam images a tilted plan view of the upper surface of the sample.

FIB milling is carried out by rapidly rastering rectangular patterns, as set up in the “ion-beam view”. A pattern about 5 μm in height and 10 μm in length is milled at 500 pA on both sides of the sample, creating a lamella about 1.5 μm thick (Fig. 2b and c). The final lamella is thinned in steps with rectangles of decreasing size and decreasing current, from 50 to 20 pA, yielding a final lamellae 200–300 nm in thickness. Creation of a single lamella takes about 15 minutes. The quality of the lamellae is monitored by electron-beam imaging, by which faint outlines of the cells can be seen (Fig. 2d).

2.4 Cryo-transfer to the TEM

After FIB-milling, the cryo-transfer holder is placed under liquid nitrogen in our TEM cryo-transfer workstation (Fig. 2e), the shutter is opened, and the cartridge is moved to the standard JEOL JEM-3200FSC three-sample cryo-transfer block (Fig. 2f). The block is transferred to the TEM and the cartridge is inserted into the TEM cryostage in the normal manner.

2.5 Electron tomography

All electron tomographic tilt series data sets were collected using SerialEM (Mastronarde, 2005) with a cosine tilt increment starting with 2 or 3 degrees. The JEM-3200FSC was operated at 300 keV with zero-loss energy filtering, slit width 24eV. The pixel size on the specimen was 0.36 nm and the total electron dose was about 70 $\text{e}^-/\text{Å}^2$. Images were recorded at a target underfocus between 4 and 8 μm on a K2 Summit direct-electron-detection camera (Gatan, Pleasanton, CA) with 5 to 8 frames per second during an exposure time of 1 to 1.5 sec. Frame alignment was carried out with the “Unblur & Summovie” package (Brilot et al., 2012; Campbell et al., 2012; Grant and Grigorieff, 2015). Marker-free alignment and tomographic reconstruction were carried out using Etomo/IMOD (Kremer et al., 1996; Mastronarde and Held, 2017)

3.0 Results

Our modified JEOL cryo-TEM cartridge and Leica cryo-transfer holder successfully eliminated the problem of damage to the lamellae during post-FIB transferring, which significantly increased the success rate of the cryo-FIB preparation workflow. We have used this method to visualize macromolecular complexes within virtuously frozen yeast cells, which were cultured with the addition of 1 or 2% methanol. Although no metal coating was used at any stage of the workflow, we did not have charging problems that impacted the FIB milling, SEM imaging, or TEM imaging in a way that prevented us from getting the desired results.

Typically, multiple yeast cells can be found in one lamella (Fig. 3a). The addition of methanol induced alcohol-oxidase crystals (Veenhuis et al., 1980; Veenhuis et al., 1981; Vonck et al., 2016), which were found within most peroxisomes seen in our cryo-tomograms (Fig. 3c,d and f). The crystalline lattice is poorly appreciated in un-tilted projection

micrographs as shown in Fig. 3b, but it is clearly visible in tilted slices from the tomograms. Fig. 3c displays a highly tilted 7.2-nm-thick slice from the tomogram, in which the crystal lattice is clearly recognizable. This demonstrates the effectiveness of the marker-free alignment algorithm in IMOD. Thus, the step of adding colloidal gold particles to the thin FIB lamellae as fiducial markers (Gruska et al., 2008; Masich et al., 2006), can be avoided -- along with concomitant risk of damage to the lamellae -- unless structural features are too faint for adequate alignment of the tilt-series images or if the best possible alignment accuracy is required. In addition to the alcohol-oxidase crystals, cytoplasmic ribosomes are also clearly seen in the 3.6-Å-thick tomographic z-slices, as indicated by the white arrows in Fig. 3e, as well as rows of putative ATP synthase within an adjacent mitochondrion, as indicated by the black arrows (Davies et al., 2011; Strauss et al., 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Specimen-cartridge and cryotransfer-holder modifications. The unmodified cryotransfer holder is shown in (a), with the shutter open and with the four grippers indicated at the black arrows. Two TEM grids (each cut in half) are shown at the red arrows; the blue arrows indicate the “D”-shaped hole for the operating rod, with the arrow parallel to the flat edge of the hole. The original cryotransfer holder can easily be disassembled by removing two screws as shown in (b). The grid-clamping block was removed, and the mechanism for holding the TEM cartridge was built in its place, using our newly made parts A, B and C,

and the 5 screws shown in the assembly figures **(c)** and **(d)**. The leaf spring (part A) and the semi-circle (part B) are fixed onto the holder by the screws indicated with the red arrows in **(c)** and **(d)**. Together, the two parts hold the modified TEM cartridge in place at a pre-tilted angle as shown in **(h)** and **(i)**. The semi-cylindrical copper block (part C in **(d)**), fills the empty space that was created by removal of the original TEM-grid clamping block, and prevents entrance of contaminants when the shutter is closed. Note that in the modified holder, the shutter assembly is oriented 180° from its position on the original holder, as seen in **(a)** and **(b)**. The blue arrow indicates the D-shaped hole that accepts the operating rod, which also controls the shutter. The red circle in **(b)** indicates some material that had to be removed so that the reoriented shutter could be fully closed. In order to maintain the original milling direction with the modified holder, the holder had to be inserted in the FIB-SEM coldstage re-oriented end-for-end. Thus, with our modification, the shutter can be operated in the normal way to protect the specimen. The original JEM-3200FSC specimen cartridge is shown in top view **(e)** and underside view **(f)**. Insertion of the thin washer and the small hold-down ring, seen in **(e)**, is done under liquid nitrogen, which often results in damage to the FIB-milled lamella due to direct handling of the grid. The cartridge was modified by milling a 0.36-mm-deep slot in its underside, as indicated by the arrow in **(g)**. This allows access to the ion beam at the desired 10° inclination to the surface of the TEM grid. In **(h)** and **(i)**, the modified cartridge is shown mounted in the modified Leica cryotransfer holder.

Fig. 2.

Cryo-FIB specimen preparation. (a) The modified cryotransfer holder, holding a modified cartridge, mounted on the FIB-SEM coldstage, showing the relative orientations of the electron and ion beams. (b) An “electron-beam view” of two FIB lamellae (in dotted squares). (c) An “ion-beam view” (end-on) of a FIB lamella. (d) An electron-beam view at higher magnification of a FIB lamella, showing faint outlines of the yeast cells. (e) The TEM cryotransfer loading station. Under liquid nitrogen, the shutter of the modified FIB-SEM

cryotransfer holder is opened and the cartridge is inserted into the TEM cryotransfer block, shown in **(f)**. **(f)** A TEM cartridge inserted in the TEM cryo-transfer block.

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Fig. 3. Electron tomography of vitreously frozen *Pichia pastoris* cells prepared by cryo-FIB. Multiple yeast cells can be found in a FIB prepared lamellae, as shown in (a). An alcohol-oxidase crystal lattice inside a peroxisome is hardly visible in an un-tilted projection image (b). However in (c), the lattice is clearly seen in a highly tilted 7.2-nm-thick tomographic slice, attesting to good markerless alignment of the tilt-series images. In a z-slice from another cryo-tomogram (d), large protein complexes are seen. In the white circle, magnified in (e), cytoplasmic ribosomes (white arrows) are seen. Some mitochondrial complexes,

which are likely rows of ATP synthase, are also clearly resolved (black arrows). Within the black circle in **(d)**, shown magnified in **(f)**, is a crystalline array of alcohol oxidase.

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