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Practical workflow for cryo focused-ion-beam milling of tissues and cells for cryo-TEM tomography

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ABSTRACT

Vitreous freezing offers a way to study cells and tissue in a near-native state by cryo-transmission electron microscopy (cryo-TEM), which is important when structural information at the macromolecular level is required. Many cells – especially those in tissue – are too thick to study intact in the cryo-TEM. Cryo focused-ion-beam (cryo-FIB) milling is being used in a few laboratories to thin vitreously frozen specimens, thus avoiding the artifacts and difficulties of cryo-ultramicrotomy. However, the technique is challenging because of the need to avoid devitrification and frost accumulation during the entire process, from the initial step of freezing to the final step of loading the specimen into the cryo-TEM. We present a robust workflow that makes use of custom fixtures and devices that can be used for high-pressure-frozen bulk tissue samples as well as for samples frozen on TEM grids.

1. Introduction

Currently, the most faithful electron tomographic reconstructions of eukaryotic cells and tissue are usually carried out on material that has been high-pressure frozen, freeze-substituted, plastic-embedded, sectioned, and imaged at room temperature in the TEM. Since the technique is reliable, not difficult, and does not require specialized equipment (beyond the freezing instrument), it is widely used.

However, for almost two decades, cryo-electron tomography of vitrified specimens has been the premier method for the study of cellular ultrastructure in a near-native state, but technical challenges have prevented it from being adopted more widely for eukaryotic cells and tissue. More recently, sub-tomogram averaging has emerged as a way to increase resolution in determining macromolecular structure *in-situ* (e.g. Zhu et al., 2006; Frank, 2006; Förster et al., 2008; Wu et al., 2009; Winkler et al., 2009; Amat et al., 2010; Yu and Frangakis, 2011; Heumann et al., 2011; Kuybeda et al., 2013). This facilitates study of macromolecular interaction with neighboring cellular components, while yielding sufficient resolution to compare their *in-situ* structures with examples of high-resolution structural maps obtained from isolated macromolecules using the single-particle method or other techniques, most commonly X-ray crystallography.

Tomographic resolution decreases with increasing sample thickness, unless the number of tilt images can be increased. However, increasing the number of tilt images normally increases the electron dose, thus potentially limiting resolution because of specimen damage. In addition, electron-optical image quality decreases with thicker samples, especially when the specimen is tilted to high angles and the electron path length doubles or triples. Thus, samples generally need to be in the thickness range of 100–300 nm for good results in sub-tomogram averaging. While samples just thicker than the macromolecule of interest give the best resolution, the 3-D relationships within the cell may be lost if the sample is too thin.

Although small cells such as bacteria, or the periphery of larger cells, fall into the suitable thickness range for cryo-TEM tomography, most cells, and certainly bulk tissue, need to be thinned. Cryo-ultramicrotomy of vitreously frozen specimens is an effective method of thinning the sample, and the technology has been steadily improving (e.g. Al-Amoudi et al., 2004, 2005; Pierson et al., 2010). The sample area on a grid that is suitable for tilt-series acquisition can be wide, allowing a good choice of targets for reconstruction. Nevertheless, artifacts due to the mechanical

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cutting action have not yet been completely overcome, and the wrinkled topology of the sections, along with their poor attachment to the TEM grid, can make them difficult to use for cryo-tomography (Hsieh et al., 2006; Marko et al. 2006a).

To avoid the drawbacks of vitreous cryo-sectioning, we proposed (Marko et al., 2005, 2006b), and then demonstrated (Marko et al., 2007) that cells could be thinned for cryo-TEM by means of focused-ion-beam (FIB) milling. We confirmed that samples remained vitreously frozen when observed in the TEM, and that they lacked the artifacts seen after cryo-ultramicrotomy. Since then, development of the technique has been taken up intensively at the Baumeister laboratory (Plitzko et al., 2009; Rigort et al., 2010, 2012a,b), and also in the Zhang laboratory (Strunk et al., 2012; Wang et al., 2012), a group in The Netherlands (Hayles et al, 2010; de Winter et al., 2013), and others (Edwards et al., 2009; Rubino et al., 2012).

However, cryo-FIB preparation remains a challenging technique, often with very low "throughput". The challenges are (1) handling a small, fragile specimen, (2) keeping the specimen below the devitrification temperature (about −140 °C) at all times and (3) avoiding frost accumulation. Each lab that has worked with the cryo-FIB method has approached these problems by specially designed fixtures and devices, and here we show our versions. Unlike any of the other protocols so far published, we emphasize the use of bulk tissue prepared by high-pressure-freezing (HPF), while also ensuring that our workflow can accommodate cells plunge-frozen on TEM grids.

2. Methods and results

2.1. High-pressure freezing

We use an HPF carrier (also known as a platelet, planchet or hat) that has a narrow slot (0.3 mm wide and 2 mm long) rather than a large circular depression. This is desirable so that when the tissue is exposed in the trimming step (Section 2.3 below), the walls of the slot will support the tissue and provide a reasonably short path to ground for charge reduction during FIB-milling. Although the "sectioning quality" of FIB milling is less sensitive to freezing quality than is cryo-ultramicrotomy (Hsieh et al., 2006; Marko et al., 2006a,b), it is best to heed the advice of Studer et al. (1989, 1995) and optimize heat-transfer conditions by using a platelet with the smallest possible volume of tissue and the thinnest possible freezing windows.

For the Bal-Tec HPM 010 (now produced by ABRA Fluid AG, Widnau, Switzerland), the Leica HPM 100 (Leica Microsystems, Vienna, Austria) or the Wohlwend HPF Compact 02 (TechnoTrade International, Manchester, NH), the best platelet choice is the Wohlwend slot type, part number 446. For the Leica EM PACT or EM PACT2 instruments, the best choice is the "Biopsy Carrier", part number 16706896.

2.2. Intermediate sample holder

The trimmed-down HPF samples are small and difficult to handle under liquid nitrogen, and the FIB-milled TEM lamellae are especially fragile. In order to limit the need to handle the specimen (HPF carrier or TEM grid) directly, we mount the specimen in an intermediate specimen holder (ISH), as shown in Fig. 1. Working

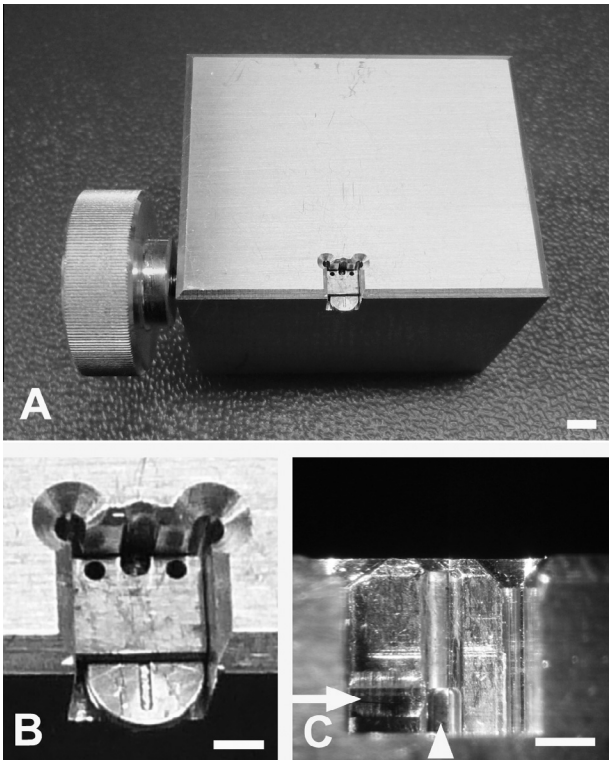

Fig.2. ISH loading block. A. The knob on the left spreads the jaws of the ISH by inserting the pin, shown by the arrow in (C). B. View of an ISH in the loading block. The countersunk holes in the loading block are for re-positioning the ISH, if necessary. C. Side view of the empty loading block showing the locating pin (arrowhead) and the spreading pin (arrow). Bars: A = 3 mm; B,C = 1 mm.

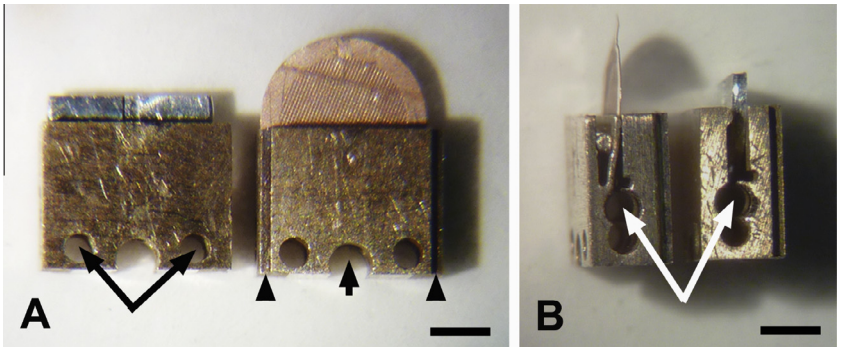

Fig.1. Intermediate Specimen Holder (ISH). Two types are shown. On the left in (A) is an ISH designed for holding a standard HPF carrier, which is cut down (nearly in half in this case, see Fig. 3) to expose the tissue. On the right in (A) is an ISH designed for a TEM grid. A. Top and bottom views: channels on the bottom (arrowheads) fit into the TEM cryo-transfer holder (Fig. 8). The central hole (short arrow) accepts the locating pins in both the loading block (Fig. 2) and in the TEM cryo-transfer holder (Fig. 8). The side holes (long arrows) allow the ISH to be easily manipulated by forceps. B. Side views of both types of ISH (grid-type on the left and HPF-carrier type on the right): the long arrows show the holes where the loading-box retractable pin inserts to spread the jaws. The lower hole forms the hinge. Bars = 1 mm.