Microsc. Microanal. 24 (Suppl 1), 2018
doi:10.1017/S1431927618004592

Cryo-FIB Lamella Milling: A Comprehensive Technique to Prepare Samples of Both Plunge- and High-pressure Frozen-hydrated Specimens for in situ Studies.

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In recent years, in situ cryo-electron tomography (cryo-ET) of macromolecules inside cells at sub-nanometre resolution has become possible through ground breaking developments in sample preparation. Using a cryo-focused ion beam (cryo-FIB) microscope, vitrified specimens are locally thinned to electron transparency, offering cross-sectional views of the sample without introducing preparation artefacts. Such cryo-FIB lamellas can be reproducibly prepared with suitable quality for Volta phase plate contrast-enhanced imaging, enabling in situ studies of membrane-bound macromolecules [1-4].

However, the established cryo-FIB method is only suitable for specimens that can be vitrified in toto by plunge-freezing and are sufficiently small to allow complete removal of material on both sides of the area of interest by ion milling [5]. An interesting challenge is the extension of cryo-FIB sample preparation to high-pressure-frozen (HPF) bulk samples, which would enable studies of tissue or any large macroscopic specimen that can be fully vitrified. Several preparation schemes from materials science, including lamella lift-out with a micro-manipulator needle and in-place bulk-sample H-bar milling, seem promising for adaptation to cryo-preparation of biological samples. However, successful application for cryo-ET at molecular resolution has so far not been shown, possibly due to the stringent sample quality requirements and geometric constraints of the technique.

In this work, we describe a novel cryo-FIB lift-out sample preparation scheme as an integral part of a complete cryo-ET workflow. Utilizing a cryo-adapted micromanipulator ‘gripping’ tool, which avoids issues of localized material deposition, we selectively extracted fluorescently-labelled volumes of interest from large HPF bulk samples and transferred them onto a customized TEM half-grid for final thinning (Fig. 1). The sample size, thickness and overall quality of the final TEM samples were comparable to the standard lamella-milling approach, enabling high-resolution in situ cryo-ET studies on HPF biological specimens for the first time.

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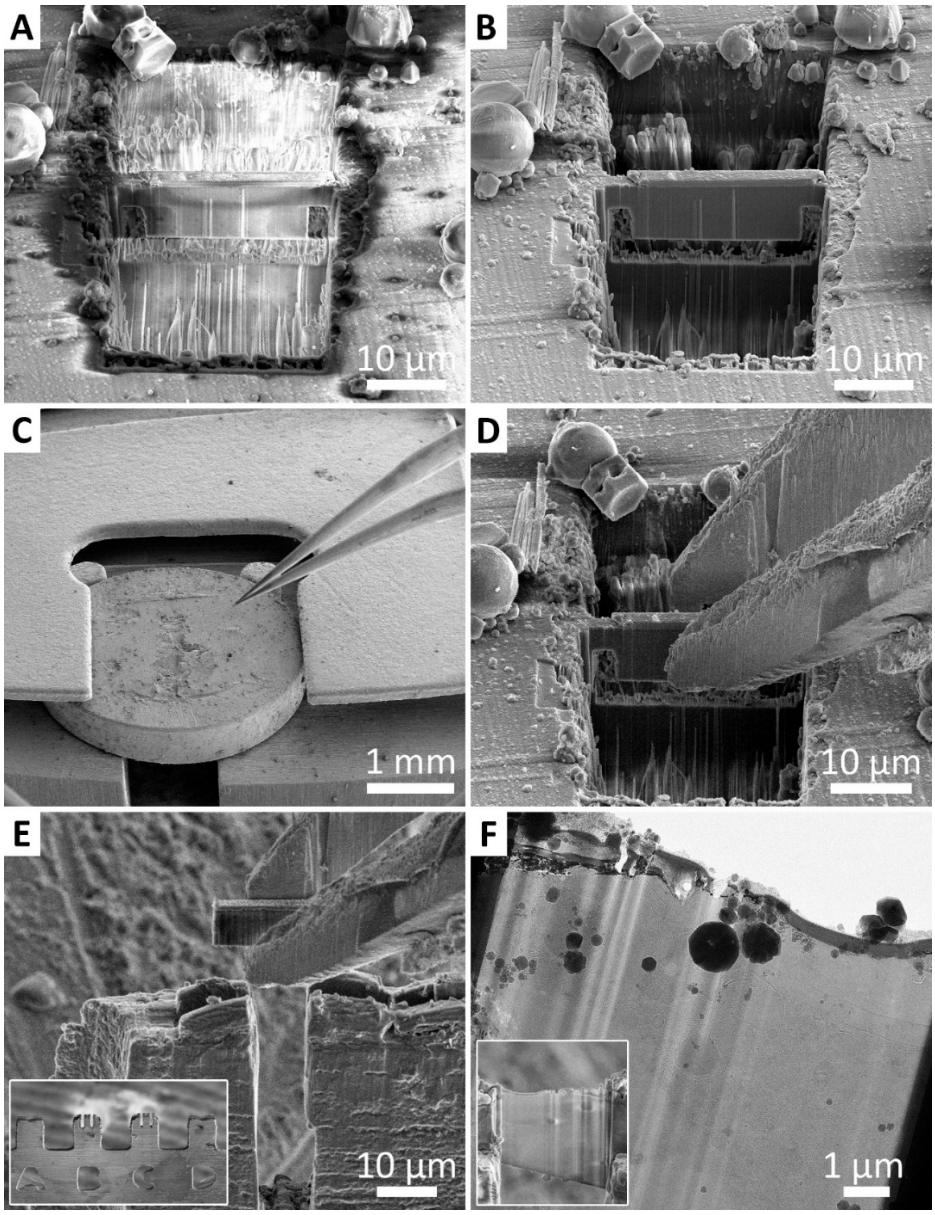

Fig. 1: Cryo-FIB sample preparation utilizing a Kleindiek cryo-gripper micromanipulator. A volume of interest is prepared in an HPF bulk sample by milling two trenches (A) and subsequently under-cutting the volume using an angle such that only two connecting bridges remain (B). The cryo-gripper micromanipulator is inserted (C) and, using a tweezer-like motion, grabs the volume of interest (D), which is then cut loose by the ion-beam milling. The volume is transferred into custom-prepared slits on a TEM half-grid (E) before final thinning to electron transparency. The TEM overview image of the final lamella (F) demonstrates the overall quality of the sample from which cryo-ET data was subsequently acquired and analysed.

https://doi.org/10.1017/S1431927618004592 Published online by Cambridge University Press