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A Universal Method of *In Situ FIB* Lift-Out for Cryogenic Samples

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Cryogenic electron-imaging (EM) of biological materials enables the observation of cells which are hydrated and not tainted by sample preparation. Cryogenic EM is not limited to biological materials as a range of beam sensitive materials (i.e. polymers and III-V semiconductors) and vacuum incompatible (solid-liquid interfaces, i.e. in hydro gels and batteries) also benefit. However, the use of cryogenic conditions restricts the ability to produce specimens for transmission electron microscopy/tomography.

There are two established methods of producing electron transparent cryogenic samples, cryo-ultramicrotome [1] and on grid cryo-FIB thinning [2, 3]. Both methods are tailored for biological applications and have limitations. Cryo-ultramicrotomy introduces significant deformation and has a limited capability to prepare site specific samples. On grid, cryo-FIB thinning offers a significant improvement in sample preparation as the specimen is not deformed and the region thinned can be selected. On grid thinning requires that the feature of interest to be positioned in the central region of a TEM grid before freezing, which is not trivial. This method also requires the full thickness of the sample and grid to be milled, which is restrictive.

A common approach to site specific TEM lamella preparation of non-cryo samples is the *in situ* FIB lift-out. This method uses a FIB, nanomanipulator and gas injection system (GIS) to extract a sample from bulk and re-position to a TEM grid. The method is not suitable for cryo due to manipulator temperature and lack of a suitable attachment method. This process can be adapted by modifications to the OmniProbe nanomanipulator to work on cryogenic specimens, both biological and non-biological.

An OmniProbe nanomanipulator was modified with a custom manipulator shaft, an oxygen free copper cooling piece to cool the probe tip was attached to the shaft. This was cooled by a flexible connection to the stages anti contamination device (ACD), so the probe tip was below the water vitrification temperature. A thermocouple mounted on a port feedthrough monitored the probe temperature. The probe temperature was directly linked to ACD and temperatures below -170 °C were achieved. A Cryo-lamella containing yeast cells was FIB milled using the conventional room temperature approach, Figure 1. Pt deposition was used as the stress buffer; the deposition of Pt on cryogenic samples produces a thick blanket layer. The Pt layer can be electron beam annealed to form a smooth surface [4], this deposition is not however suitable for a lift-out attachment.

We demonstrate that low pressure water injection via a standard GIS acts to form a thin vitreous ice layer on the sample, attaching the probe to the lamella (figure 1a, b) in ~10 seconds. The same method is used to attach the lamella to a TEM grid. The ice attachment thickness was controlled by manual opening/closing of the GIS control valve. The speed of the ice attachment significantly exceeds that of conventional beam assisted deposition attachments, but is not site-specific (figure 1e), requiring additional milling (figure 1f). Marko et al [2] show that aggressive (fast) 30 kV Ga^+ milling of vitreous ice (1.2 μm milled to 150 nm) does not induce sufficient heating to cause devitrification, thus extra milling does not impact the result and time saved in the attachment offsets the additional milling.

Use of a FIB with near standard attachments (modified OmniProbe, cryo-stage and GIS's with reduced heating) we demonstrate a powerful method to extract a site specific cryogenic TEM specimen. The technique is suitable for both biological and not biological samples. Use of a standard port-mounted accessories and conventional lamella lift-out technique means the requirements on sample geometry/thickness are significantly relaxed.

References

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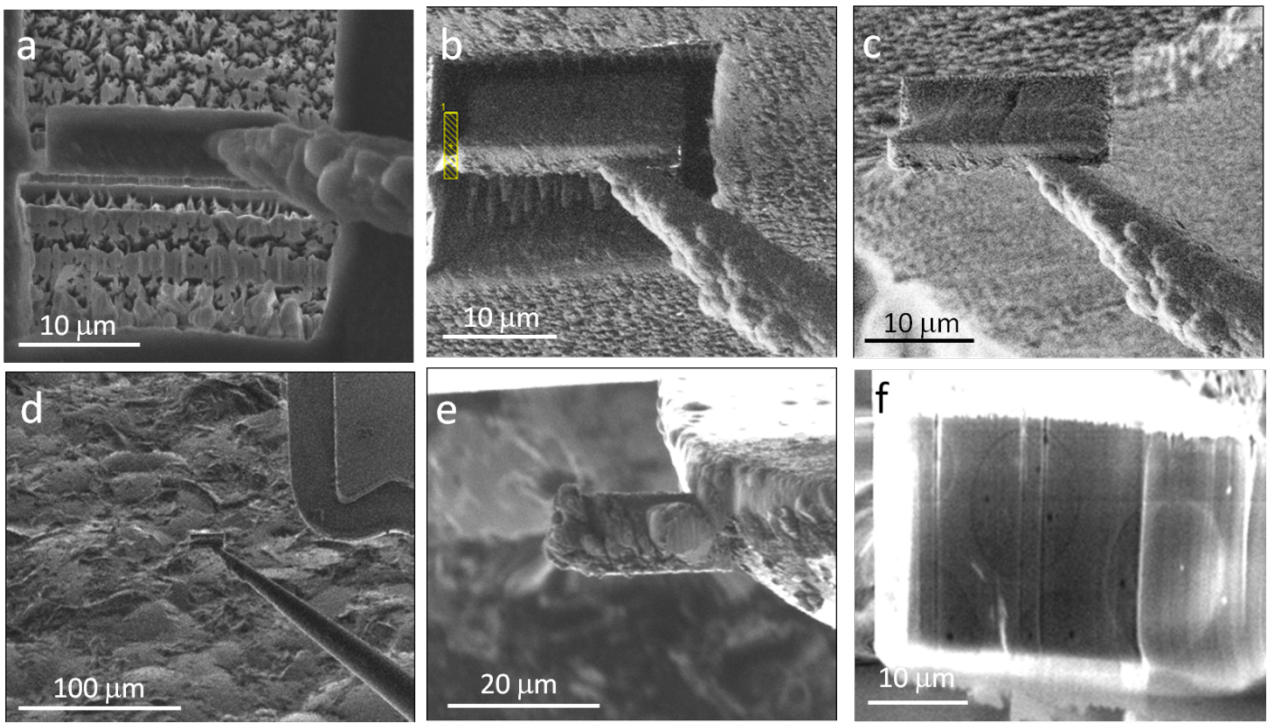

Figure 1. Cryogenic lift out of yeast cells. The sample was prepared by the modified total release methodology and attached to the Cryoprobe with vitreous ice (a, b electron/ion image of attachment respectively). The lamella was extracted (c) and repositioned to a TEM grid (d). The sample was attached to the grid using vitreous ice deposition (e). Excess ice was aggressively removed (f) to prepare the sample for conventional FIB thinning.

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