

Cryo-FIB Preparation for Cryo-TEM Tomography

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We have previously shown that a focused ion beam (FIB) can be used to thin vitreously frozen specimens. First we demonstrated that ice can be FIB-milled without devitrification [1]. Then we showed that vitrified small cells (bacteria) can be FIB-milled to a thickness suitable for cryo-TEM, without detectable artifacts [2].

The impetus for our cryo-FIB development is to carry out high-resolution cryo-TEM tomography on vitreous biological specimens. Our ultimate goal is to identify and reconstruct macromolecular complexes *in-situ*, and to compare these 3-D structures to those obtained from high-resolution single-particle reconstruction of the isolated complexes.

Although we pioneered the use of cryo-ultramicrotomy to prepare thin specimens of high-pressure frozen material for cryo-TEM tomography [3], we found that the process of cryo-ultramicrotomy is prone to artifacts. With care, we found that many of the artifacts can be avoided [4], with the exception of compression of the section, which amounts to about 30% in the cutting direction. Compression is not uniform; some structures resist it better than others, so the distortion due to compression cannot be simply computationally corrected.

In developing cryo-FIB as a TEM preparative method, we recognize three types of specimens: (1) plunge-frozen suspensions of small cells or organelles that are thin enough for vitreous freezing, but too thick for cryo-tomography; (2) high-pressure-frozen cells that are seeded directly on TEM grids; (3) high-pressure-frozen tissue samples. In our previous work we provided proof of concept for the first of these. Currently, we are concentrating on FIB-milling high-pressure-frozen cell pellets and tissue.

Vitreously frozen material is little different from any other material with respect to the actual FIB-milling--the key to cryo-FIB TEM preparation is specimen handling. The vitreously frozen specimens to be used for high-resolution TEM must be maintained below about -140°C at all times after the initial freezing step. Following FIB-milling, the specimens must be kept free of frost during transfer out of the FIB instrument, into storage, and into the cryo-TEM. Thus, our major focus now is developing (1) new tools and fixtures to mount the specimen in the high-pressure freezer, and then conveniently handle it after freezing and during pre-processing for transfer into the FIB instrument, (2) a reliable method to transfer the specimen into and out of the FIB instrument without frost accumulation, and (3) accessories and tools for convenient transfer of the specimen into the TEM cryo-transfer holder.

We have explored a few ideas for accomplishing frost-free transfer into and out of the FIB instrument, and have eventually settled on a method that is not greatly different from that used for TEM cryo-transfer. We are still “tuning up” the details of our cryo-FIB system as a whole.

Meanwhile, with respect to the actual FIB-milling process itself, we have been able to overcome the common FIB-milling artifact known as “curtaining”, which appears as streaks or grooves on the milled surface parallel to the ion beam. The streaks are due to roughness of the surface

perpendicular to the ion beam, and are commonly attenuated by platinum deposition. A variant of this is possible for cryo specimens as well [5], but we found it not to be necessary in our case. The key to a smooth milled surface is that the sample surface perpendicular to the ion beam be smooth. We use the common “H-bar” milling geometry. For subsequent cryo-TEM tomography, it is important that the specimen region surrounding the thinned H-bar be sufficiently thin so as not to occlude the area of interest at high tilt in the TEM. By pre-trimming the specimen in a cryo-ultramicrotome, the thickness of the specimen is reduced to about 20 μm , and the surface perpendicular to the ion beam is made sufficiently smooth to avoid curtaining (Fig. 1). Our long-term roadmap includes development of a simpler, lower-cost alternative to a cryo-ultramicrotome for this step.

References

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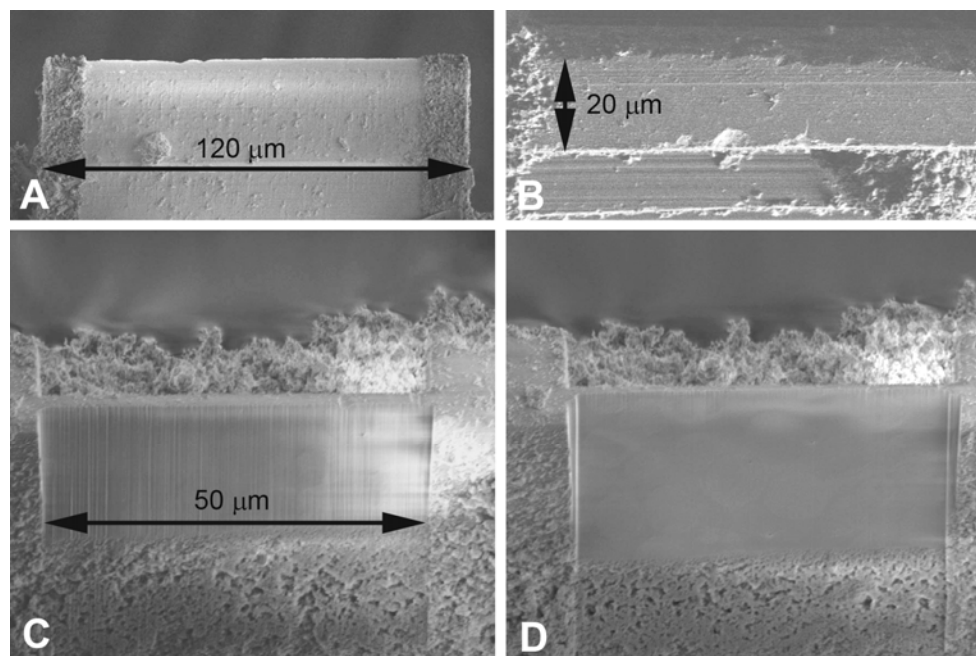


FIG. 1. (A) Side view of a trimmed cell pellet, supported on the left and right by remains of an aluminum high-pressure-freezing specimen carrier. (B) End view of the edge of the pellet after smoothing with a cryo-knife. (C) Oblique view of a 1- μm thick H-bar milled from this specimen. (D) Same H-bar after thinning to 500 nm by a final smoothing cut.