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Feasibility of Focused Ion Beam Milling for Preparation of TEM Specimens of Biological Material Embedded in Vitreous Ice

M. Marko*, C.-E. Hsieh*, W.J. MoberlyChan**, C.A. Mannella*, and J. Frank***

* Resource for Visualization of Biological Complexity, ***Howard Hughes Medical Institute, Wadsworth Center, Empire State Plaza, Albany, NY 12201-0509
**Center for Imaging and Mesoscale Structure, Harvard University, 17 Oxford St., Cambridge, MA 02138

The Albany Resource has recently developed the technique of electron tomography of frozen-hydrated tissue sections [1-4]. The sections are obtained from plunge-frozen or high-pressure frozen material by cryo-ultramicrotomy at -160°C. Specimens prepared in this way can be studied in a "native" state, since no chemical fixatives, stains, or dehydration are involved. Collection of the "dry" frozen-hydrated sections on EM grids after microtomy is difficult, especially when the sections have to be mounted firmly, and placed in suitable positions on the grid to permit electron tomography. It has recently been shown that a cryo-equipped dual-beam focused ion beam (FIB) instrument may be used to mill frozen biological tissue to expose interior surfaces for SEM imaging [5]. We show here that FIB may be used to prepare frozen-hydrated material for TEM, as an alternative to cryo-ultramicrotomy. Previously, we have used tomography to demonstrate that some artifacts of sectioning frozen-hydrated material are confined to the surface of the sections, while others occur throughout the sections [1]. Of the latter, the most problematic may be compression, a shortening of the section in the cutting direction, which is accompanied by an increase in thickness. To date, improvements in cryo-ultramicrotomy have been largely unsuccessful in solving the compression problem [6]. By using FIB milling to prepare frozen-hydrated specimens for electron tomography, we may be able to obtain the first undistorted 3-D views of the ultrastructure of "native" biological tissue.

To avoid the damaging effects of ice crystals, the water in frozen-hydrated biological specimens must remain in the "vitreous" solid phase. Irreversible devitrification of amorphous ice occurs between -133 and -138°C. Devitrification is readily apparent in the TEM (Figs. 1A-D) by changes in both the image (loss of smoothness) and the diffraction pattern (occurrence of discrete crystalline reflections). FIB milling at -160°C does not cause a temperature rise in the bulk ice layer such that devitrification occurs. A droplet of water on a TEM grid, vitrified by plunge-freezing in liquid ethane, was thinned from about 1 μm to about 100 nm in the FIB (Fig 1F). TEM images and diffraction patterns clearly indicate that the remaining water layer remains vitreous (Figs 1G-I). An FEI DB235 dual-beam FIB with a Gatan Alto 2500 cryo system was used for these experiments. A special specimen holder (Fig. 1E) that prevents frost formation and warming during cryotransfer was constructed for the Alto system. The specimen is covered during cryo-transfer in the shuttle, and the cover can be opened and closed in the Alto using the knife tool. [7,8]

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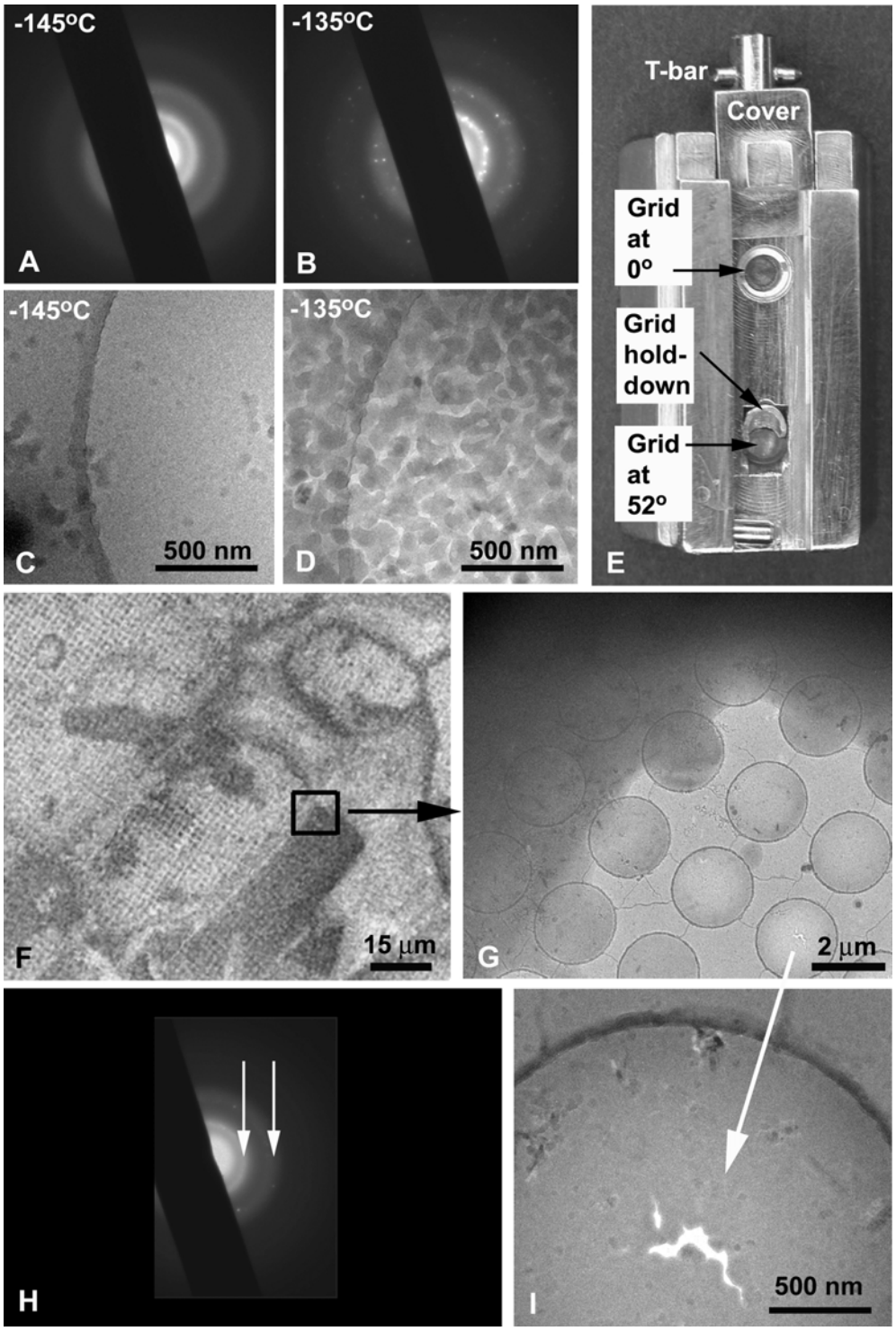

FIG. 1. (A-D) TEM of ice layer: A, C vitreous; B,D crystalline. (E) FIB cryotransfer holder with cover. (F) SEM image of slot FIB-milled at 15° to surface of water droplet on TEM grid. (G) TEM of area boxed on F. (H) Diffraction pattern from area in I showing typical diffuse rings of vitreous ice. (I) TEM of circular perforation (from G) in carbon Quantifoil grid, covered with thin ice layer. Note smooth appearance of vitreous ice. TEM images at 400keV with zero-loss filtering using JEOL JEM4000FX and Gatan GIF2002.

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