

## Secondary Sublimation Removes Ice Contamination for Improved Visualization of Structures by Cryo-SEM

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Cryo-preparative techniques allows preservation of biological samples closer to their native state, thus maintaining components and structures that are routinely lost or adversely affected using conventional room temperature fixation. These techniques are only useful for cryo-scanning electron microscopy (cryo-SEM) if ice contamination resulting from specimen transfer between instruments can be minimized. Although some transfer devices exist to keep specimens under vacuum in cryo-conditions after fracturing, a similar device is not available for transfer into an in-lens SEM. The goal of this study was to develop methods to reduce the accumulation of problematic ice contamination for improved visualization of frozen biological specimens with a Hitachi S-5200 in-lens SEM.

Poliovirus (PV) infected HeLa cells were fixed with 2% paraformaldehyde (PFA) to render the virus biologically inactive and then osmicated allowing fractures through lipid bilayers[1]. 1.5 ul droplets of cells suspended in 10% BSA/Hanks buffered saline solution were pipetted into Leica freeze fracture hats and covered with fracture rings. The assembly was high pressure frozen with the Leica EMPACT2. Freeze fracture hats were then quickly transferred into a liquid nitrogen bath and mounted on a pre-cooled stage for insertion into a BAF 060 (Leica) freeze etching device, vacuum  $1 \times 10^{-6}$  mbar and stage temperature at  $-145^{\circ}\text{C}$  for fracturing. After fracturing, the BAF 060 was heated to  $-90^{\circ}\text{C}$  for 15 – 20 minutes to remove ice through sublimation [2]. The BAF 060 was then cooled to  $-145^{\circ}\text{C}$ , and samples were coated by electron beam evaporation with 1.8 – 3.5 nm of platinum at a fixed angle of  $45^{\circ}$  for shadowing followed by an additional 14-19 nm of carbon, rotary shadowed at  $90^{\circ}$  to apply a uniform conductive surface without obscuring small structural details [3].

After coating, frozen samples were quickly transferred into a liquid nitrogen bath, and when ready to examine, placed in the liquid nitrogen filled Gatan CT-3500 workstation for mounting into the specimen holder (Fig.1) which was pre-cooled to  $-150^{\circ}\text{C}$ . After inserting the specimen into the cryo-holder it is shielded and inserted into the Hitachi S-5200 in-lens microscope. Even with great care, ice contamination proved to be problematic, rendering visualization of the specimen nearly impossible due to ice obscuring the structures of interest and charge artifacts making imaging difficult (Fig. 2 a & b). Secondary sublimation was performed within the microscope by heating the cryo-stage  $-105^{\circ}\text{C}$  to  $-110^{\circ}\text{C}$  for 15-20 minutes with the shield protecting the specimen. The stage was then cooled to  $-150^{\circ}\text{C}$ , and the specimen viewed at 3

kV. Secondary sublimation greatly reduced ice contamination allowing visualization of the regions of interest using both backscatter and secondary modes. (Figs. 3 a-c)

#### References:

[1] BT Hansen et al, *Microsc. Microanal.* 12 (suppl.2)(2006) 1134-35.

[2] A Cavalier, et al, *Handbook of Cryo-Preparation Methods for Electron Microscopy* (2009) 395-410.

[3] P Walther, et al, *J. of Microscopy* 179(1995)229-37.

[4] The authors would like to acknowledge the contribution of PV infected HeLa cells by Drs. George Belov and Ellie Ehrenfeld.

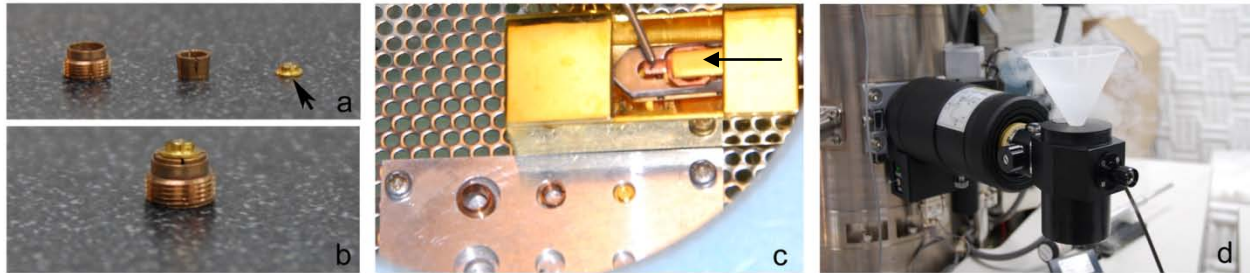


Figure 1: (a) The freeze fracture hat (arrow) is placed into a collar which is secured into a threaded ring (b). The assembly is then screwed into the stage and a shield (arrow) covers the specimen (c) for transfer into the in-lens SEM (d).

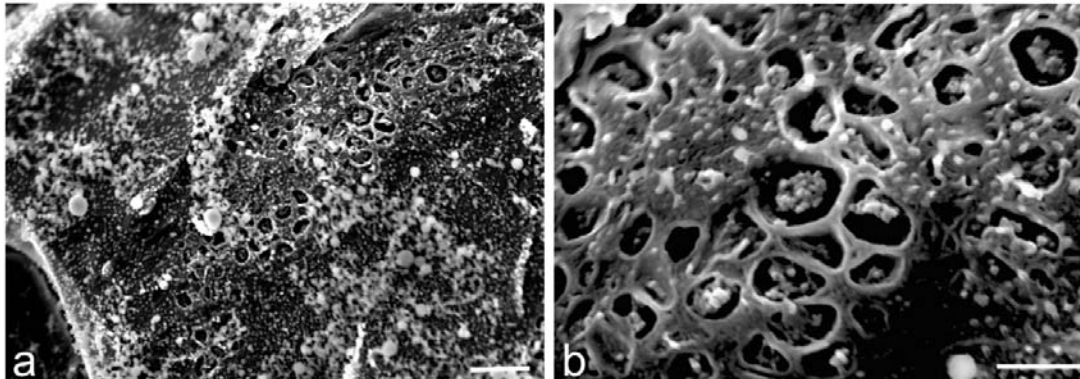


Figure 2. (a) Ice contamination obscures many structures of interest, and even in areas where structures are visible makes imaging difficult due to charge artifacts (b). Scale bar in (a) = 0.5  $\mu\text{m}$ , and in (b)=100 nm

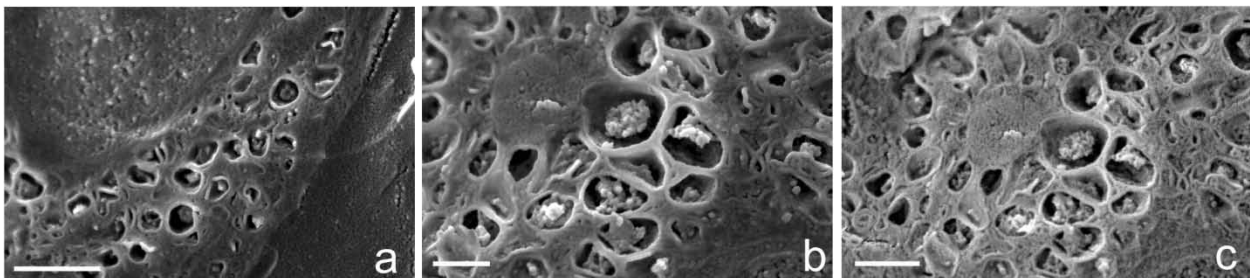


Figure 3. Secondary sublimation within the microscope removes much of the contamination (a) and allows better viewing of specimen in both secondary (a & b) and backscatter modes (c). Scale bar in (a) = 0.5  $\mu\text{m}$ , and in (b & c)=100 nm