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Ion Beam Preparation and Transmission-SEM Imaging of Frozen-Hydrated, Vitreous Lamellas Prepared by the Cryo-FIB-SEM: An All-In-One Instrument

D.A.M. de Winter[1], Rob J. Mesman[1,2], M.F.Hayles[3], C.T.W.M. Schneijdenberg[1], C. Mathisen[4], J.A.Post[1]

[1.] Biomolecular Imaging, Faculty of Science, Utrecht University, Utrecht, The Netherlands.
[2.] Present address: Department of Microbiology, Faculty of Science, Radboud University Nijmegen, Nijmegen, The Netherlands
[3.] Independent expert for Cryo-FIB-SEM technology, Eindhoven, The Netherlands
[4.] FEI Company, Hillsboro, Oregon, United States.

Cryo-electron microscopy in life sciences has been a steadily developing field as it allows studying biological specimen in their native state at unprecedented resolving powers. Ultrafast freezing protocols enable vitrification of the specimen, preserving the ultra structure. Subsequently the specimen can be sectioned by a cryo-ultra microtome for further cryo-Transmission Electron Microscopy (cryo-TEM) investigation.

Recently a number of new strategies have been proposed, producing cryo-TEM samples (lamellas) with a FIB (Focused Ion Beam) under cryo conditions [1, 2, 3]. These techniques yield high quality, artifact free lamellas, but the procedures are very labor intensive. To improve the success rate, the lamellas need to be checked for (a) the presence of structures of interest (in this case cell structures) and (b) the vitreous status, prior to the transfer to the cryo-TEM. Here we present a method to check both conditions within the cryo-FIB-SEM instrument, directly after the preparation of the lamella by the FIB. Details on the lamella preparation can be found in reference 3.

The key to checks (a) and (b) is the combination of a transmission mode option in the SEM and the low acceleration voltage of the SEM compared to the TEM. In standard cryo-TEM's, the contrast between the cell structures and the surrounding ice is generally weak. At low acceleration voltages (typically 30 kV) the electron beam interaction becomes much stronger, resulting in stronger contrast. Therefore, the images are recorded using a Transmission Scanning Electron Microscopy (TSEM) detector. In figure 1 cryo-TSEM images are shown, demonstrating the strong contrast and that the presence of cells can be verified.

To determine the vitreous versus crystalline status, cryo-TSEM imaging can be used. Ice crystals will create diffraction (or channeling) contrast and different crystallographic orientations will interact differently with the electron beam (figure 2). Absence of ice crystals will be reflected by the absence of diffraction contrast.

In between the cells shown in figure 1, structures are visible, but a distinction must be made between extracellular material and a crystalline phase. Diffraction contrast depends on the orientation of the lamella relative to the electron beam; hence tilting the lamella should result in different relative contrast of the structures in case of crystallinity, e.g. dark becomes bright and bright becomes dark.

Figure 1 shows a tilt series (-2°; 0° and +2°) and it is clear that the structures do not change contrast. Therefore it is concluded that the lamella is vitreous. A crystalline sample in figure 2 does show orientation contrast when tilting the lamella, proving its crystalline nature.

Further validation of the method of tilting the lamella to identify crystalline phases comes from EBSD (Electron BackScatter Diffraction). We've succeeded in recording EBSD patterns in transmission mode under cryo conditions, which could be indexed using the structure files of ice. Cryo-transmission-EBSD provides a direct link between the orientation contrast and crystalline nature of the lamella.

The current work expands the applicability of cryo-FIB-SEM instruments in life science research and hints towards an all-in-one tool for both sample preparation and imaging.

References:

[1] M. Marko *et al*, Nature Methods 4(**3**) (2007), p. 215 – 217.
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[3] M.F. Hayles *et al*, Journal of Structural Biology 172 (**2**) (2010), p. 180 – 190.
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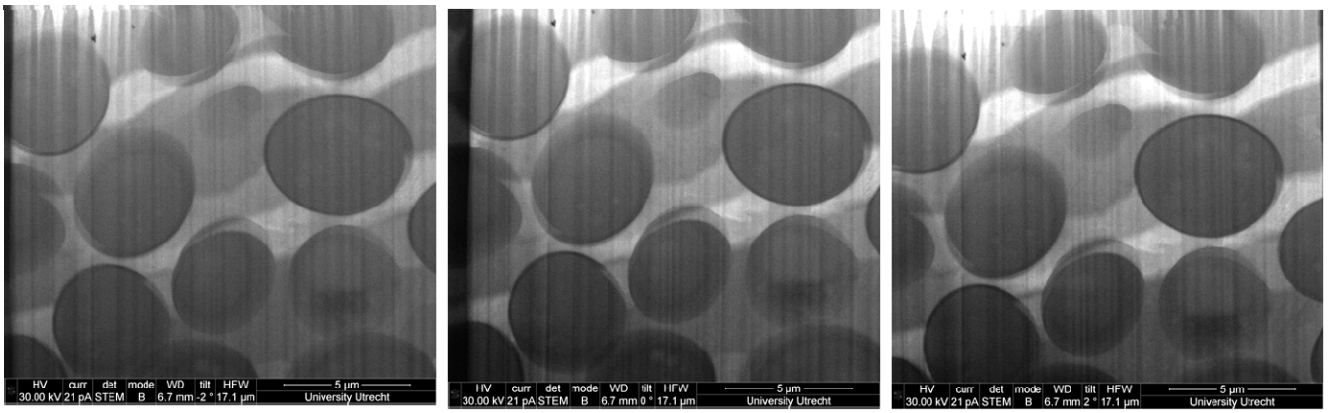

Figure 1. Cryo-TSEM images at different tilt angles demonstrating both the strong contrast of cellular materials and the absence of diffraction contrast; proof for vitreous status.

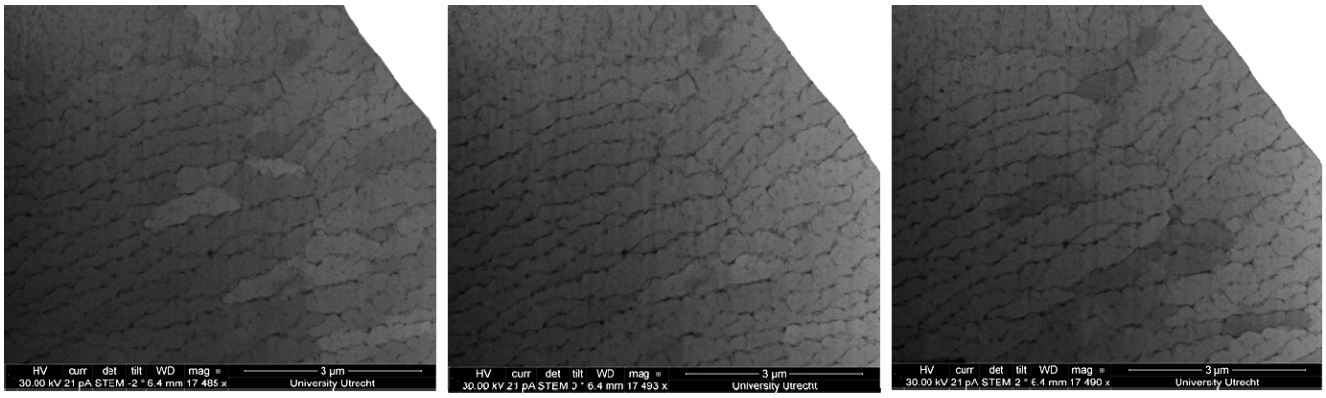

Figure 2. Cryo-TSEM images at different tilt angles demonstrating both the strong contrast of different ice crystals and the changes in diffraction contrast as a result of tilting; proof for crystalline status.

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