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Accessing Subcellular Structural Information with Advanced Targeting and Sectioning Techniques

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Our understanding of cell biology would benefit greatly from three-dimensional mapping of the macromolecular assemblies that carry out most cellular functions. The prerequisites for a comprehensive spatio-temporal analysis are accurate preservation of macromolecular architecture, an ability to capture sufficiently detailed images at critical points in time (for example: key events in the cell cycle or a stage of infection with a virus), and an ability to acquire information from pre-selected sites without compromising structural integrity. Improvements in cryogenic light microscopy and non-destructive specimen thinning are priorities for realising the full potential of cellular cryo- electron tomography.

1) Correlative cryo-microscopy. We and others have designed and implemented liquid nitrogen –cooled stages for the light microscope that fulfil a "search and relocate" function compatible with the acquisition of structural data by low-dose electron tomography [1,2]. We have now developed a second-generation cryogenic stage for an inverted light microscope (Figure 1). This stage allows imaging of multiple EM grids using a 63×, 0.75 numerical aperture objective lens. Continuous cooling with liquid nitrogen provides capabilities for long-term studies of e.g. photobleaching. The system utilises only one glass layer between objective lens and specimen to reduce gross distortions. As in the previous version, phase contrast imaging with Köhler illumination as well as epifluorescence and confocal imaging are possible. Using monochromatic light with a wavelength of 500-550 nm, the theoretical (Rayleigh) resolution for such a configuration is 330-370 nm, respectively, although systematic tests are necessary to assess the practically attainable resolution of ice-embedded specimens with a defined thickness as well as the detection efficiency for biologically relevant fluorophores such as chlorophyll or green fluorescent protein and its spectral derivatives.

2) Cryogenic focussed ion beam milling. A Quanta 3D DualBeam instrument (FEI Company, Eindhoven, The Netherlands) equipped with a cryo transfer stage (Quorum) has been installed as an alternative to vitreous sectioning using an ultramicrotome. The principle advantage compared to mechanical sectioning is that ion beam –milled specimens do not suffer from compression; however, serial sectioning transmission electron microscopy/tomography is not possible due to the loss of material resulting from the sputtering process, emphasizing the need for selective milling of target regions. We are currently working on software-controlled combination of three techniques to enable the selective elimination of material that surrounds target structures destined for tomography that have previously been identified by cryo-correlative methods. New developments, e.g. in time-resolved vitrification will allow us to exploit these strategies most efficiently in the future, and we will report on our recent findings regarding the ideal targeting and sectioning techniques for 3 dimensional cellular studies at the best achievable resolution.

References

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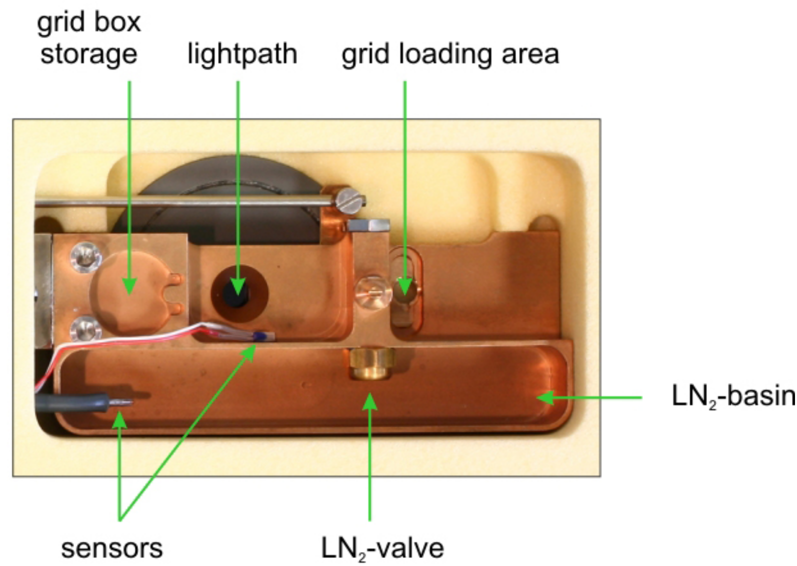

Figure 1: Second-generation stage for correlative cryo- light microscopy. The stage comprises a liquid nitrogen reservoir that is continuously refilled via level-sensor control, and a slider for moving the desired grid into the optical path.

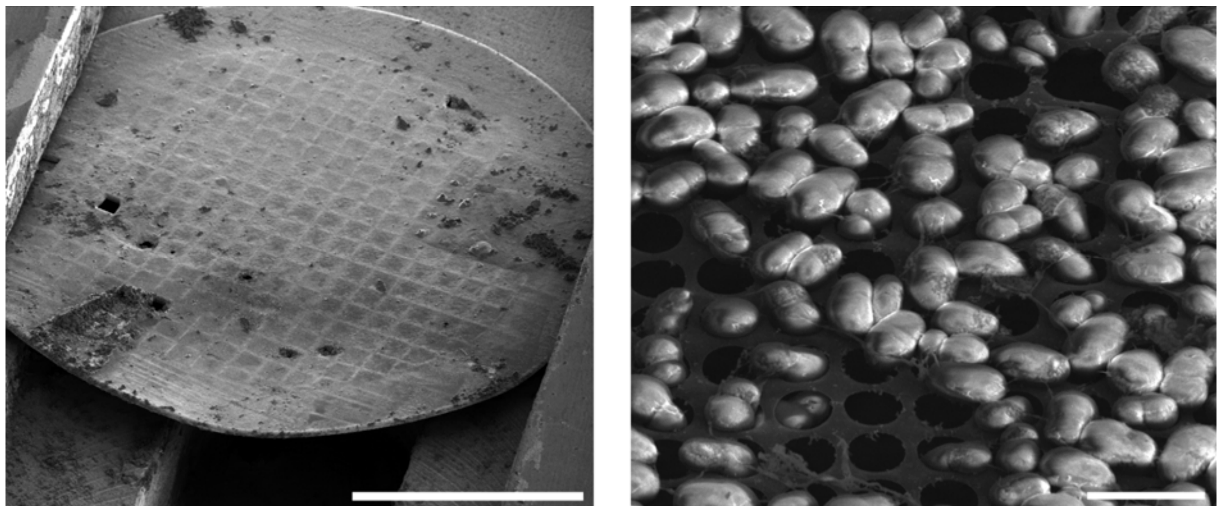

Figure 2: Cryo- scanning electron micrographs of unicellular algae on EM grids mounted in a DualBeam (SEM / focussed ion beam) instrument. **(A)** Sub-monolayer coverage of cells in a layer of vitreous ice after thin-film vitrification ('plunge' freezing in liquid ethane). A very homogeneous film thickness is obtained due to the regular size and distribution of the cells. The impression left by the forceps is visible on the lower left of the grid. Some contamination with frost has occurred during storage and transfer. Scale bar: 1 mm. **(B)** Exposed cells resting on the carbon film of the grid after rapid sublimation of the interstitial ice. Scale bar: 5 µm.

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