doi:10.1017/S1431927613006375
Microsc. Microanal. 19 (Suppl 2), 2013

Reproducibility in Focused Ion Beam sample preparation – a Key Requirement for Cryo-Electron Tomography of Eukaryotic Cells

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Specimen thickness is a major limitation for investigation of eukaryotic cells using cryo-electron tomography (CET). This can be circumvented using cryo-FIB for thinning of frozen-hydrated specimens. By sputtering with gallium ions at a shallow angle, multiple electron transparent lamellas of <500nm thickness are created directly on a TEM grid containing intact cells embedded in vitreous ice. The lamellae are not subject to sample compression and associated structural distortions encountered in conventional cryo-ultramicrotomy [1,2]. Homogeneously thick areas spanning 150μm^2 or more can be prepared in a controlled and targeted manner, suitable for subsequent tomographic studies. Such a large field-of-view is often essential for locating specific features of interest within the cell. The power of cryo-FIB milling and its potential for structural biology has been demonstrated and discussed in recent work [3].

Biological specimens are often only laboriously obtained in small quantities, e.g., primary cell culture of neurons, and CET observations require repetition for statistical significance. High preparation reproducibility and yield are therefore key aspects of cryo-FIB technique development. In this presentation we will show recent advancements towards this goal. Besides general improvements of instrumental stability, two issues needed particular consideration:

(i) protection from lamella surface contamination during all of the necessary transfer and preparation steps, and
(ii) reproducibility of lamella quality with respect to overall thickness and thickness homogeneity.

Lamella surface contamination is most often caused either by re-deposition of sample material during milling, or by other contaminants, e.g., water vapor or hydrocarbons, infiltrating the preparation/transfer system. While mild frost contamination can be considered unavoidable and does not generally affect data acquisition, continuous contamination layers that render the sample thicker by tens or hundreds of nanometers prevent successful CET investigations. A major leap in increased preparation yield has been reached by using appropriate milling strategies and systematically isolating and eliminating possible sources of contaminants.

Inhomogeneity in specimen surface topology, either inherent to the varied composition of the biological system or caused by ice-contamination created during plunge-freezing and transfer, is a major cause of FIB milling artifacts known as ‘curtaining effects’ which give strongly inhomogeneous lamella thickness. These effects can be heavily reduced by applying smooth and homogeneous protection coating, e.g., by cold CVD deposition of organometallic platinum [4], prior to the lamella preparation. Due to cryo-conditions, such a layer can be directly deposited with an in-situ gas-injection-system (GIS) without electron or ion beam radiation. Protection layers in combination with scan parameter optimization result in substantially improved sample quality. We will discuss measures to reduce the

amount of contaminations and to improve the resulting lamellae quality. Example applications of different biological systems will demonstrate the versatility and power of this new technique.

References:

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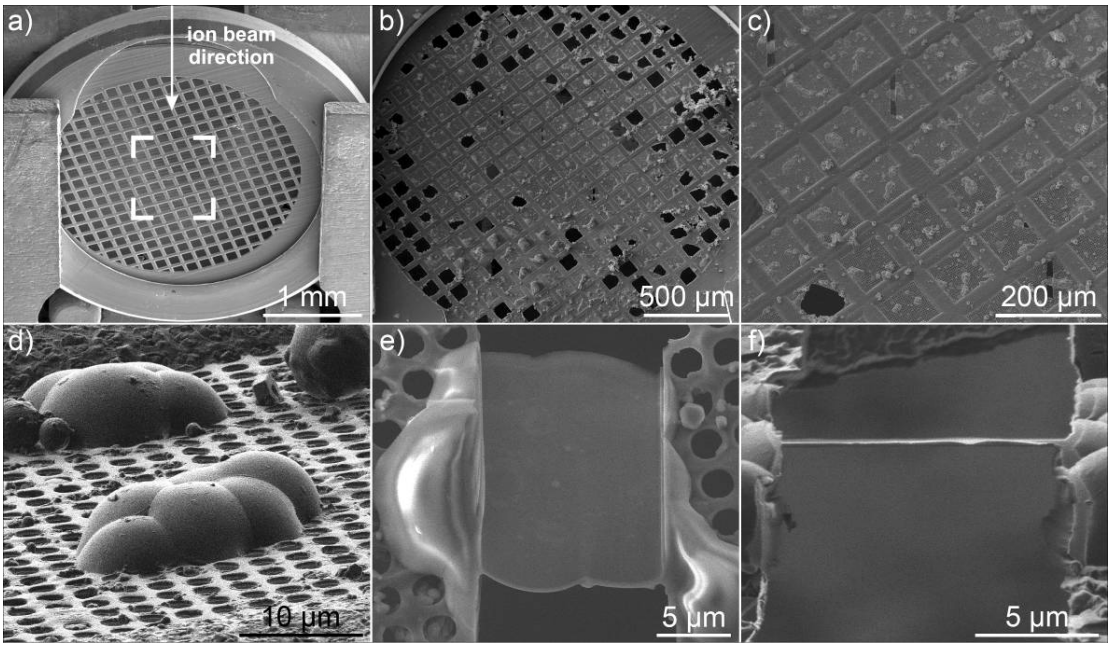

Figure 1. Cryo-FIB preparation of *Chlamydomonas reinhardtii* cells. TEM grid mounted in modified autogrid cryo-holder (a), SEM images of cell material on TEM grid (b-d), SEM image of final lamella (e), FIB SE image of final lamella edge-on (f).

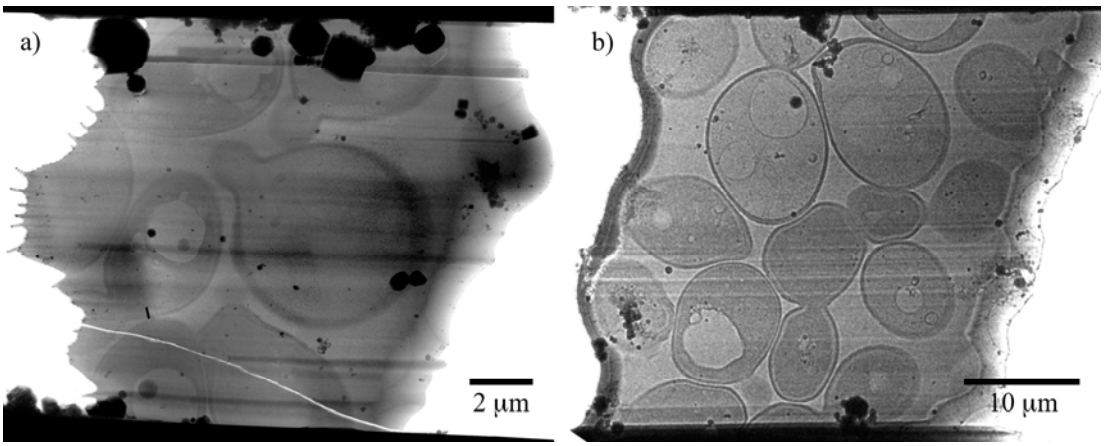

Figure 2. Comparison of yeast-cell lamella cryo-TEM images: Preparation without (a) and with (b) protective platinum layer deposited prior FIB milling.

https://doi.org/10.1017/S1431927613006375 Published online by Cambridge University Press