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In Situ Tomography of Membrane Proteins Enabled by Advanced Cryo-FIB Sample Preparation and Phase Plate Imaging

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The development of cryo-focused ion beam (cryo-FIB) microscopy into a highly reliable sample preparation technique for frozen-hydrated specimens has recently enabled cryo-electron tomography (CET) of eukaryotic cells with unprecedented resolution and image quality [1,2,3]. The ability to prepare distortion-free lamellas of homogenous, user-defined thickness has allowed *in situ* studies of cellular structures in their native state, revealing new insights into biological processes [4]. Additionally, cryo-fluorescence microscopy has been combined with cryo-FIB for the *in situ* targeting of fluorescently-labelled cellular structures [5].

While cryo-FIB milling has increased the accessibility of cellular volumes, thereby widening the biological applications of CET, independent developments in low-dose TEM imaging have pushed the boundaries of CET to new detection limits. The latest generation of direct detection cameras greatly reduces noise and thereby improves sensitivity. Additionally, a new type of TEM phase plate, the Volta phase plate (VPP), can strongly increase in-focus phase contrast of thin biological specimens with relatively little additional experimental effort [6]. However, sample charging in lamellas prepared by cryo-FIB has proven to be highly detrimental to VPP imaging.

In this work, we show that this obstacle can be overcome by adding a step to the cryo-FIB workflow. Coating the surfaces of a freshly prepared lamella with a thin, sputtered layer of platinum (~1 nm) sufficiently reduces charging during CET without strongly impeding image quality. However, in cases where the small contrast reduction due to this Pt coating of the lamella must be avoided, an alternative method can be employed. Prior to FIB milling, a sandwich-type coating layer is deposited by applying a conventional sputtered Pt layer, followed by a thick organometallic protective layer deposited by the FIB gas injection system, and then a final sputtered Pt layer (~20nm). Charging on the subsequently-milled lamella in close proximity to this thick protective layer is sufficiently reduced to allow CET in combination with VPP imaging.

Here, we describe the modified cryo-FIB sample preparation workflow. We further show that the combined application of this workflow with CET, using both direct electron detection and VPP imaging, has enabled the first *in situ* visualization of integral membrane proteins in eukaryotic cells.

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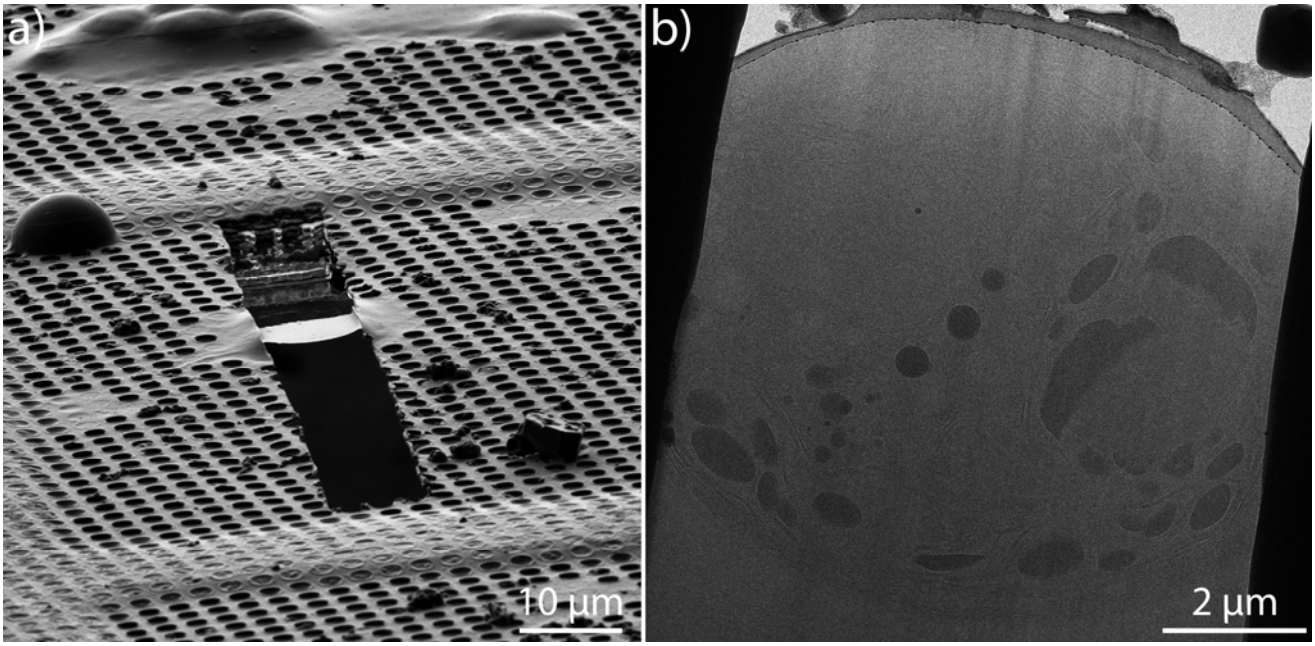

Figure 1. Cryo-FIB sample preparation of *Chlamydomonas reinhardtii* cells. SEM image (a) and TEM image (b) of a finished lamella.

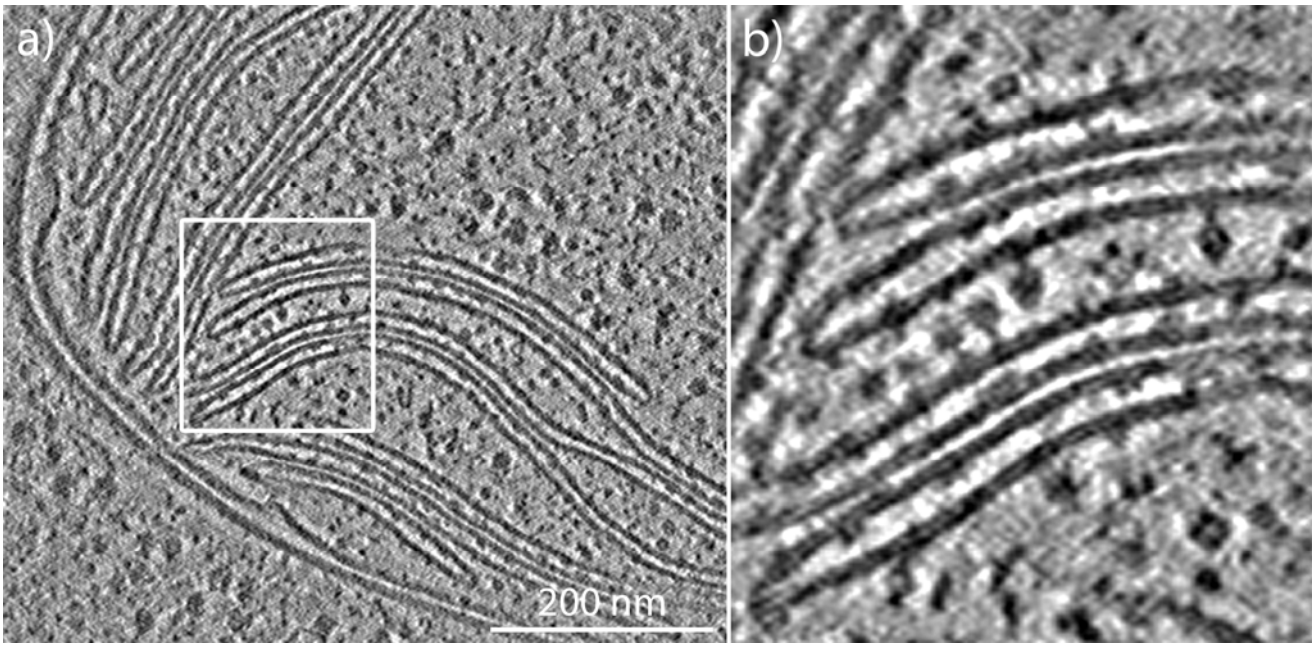

Figure 2. Slice from a reconstructed tomogram revealing the chloroplast of a *Chlamydomonas reinhardtii* cell. Overview (a) and detail (b) images of thylakoid membranes, clearly showing ATP synthases bound to the exterior thylakoid surfaces and photosystem II complexes projecting into the thylakoid interiors.

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