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Reproducible lamella preparation for electron cryo-tomography by in-situ thickness estimation during fluorescence-guided FIB milling

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Recent advances in electron cryo-tomography (Cryo-ET) have made it possible to image protein complexes in their native cellular environment. With subtomogram averaging, even interacting macromolecules and supramolecular complexes within a frozen-hydrated cell can be structurally resolved at <1 nm resolution, crucial for understanding molecular mechanisms of cellular processes underlying disease. The macromolecular complexes of interest need to be contained in a 100-200 nm thin lamella that is prepared from a >1,000 μm^3 cell through FIB milling, while the exact location of the complex itself is unknown [1]. The achievable resolution from tomography is directly related to sample thickness, which currently can only be estimated from side views of the lamella for which the milling process needs to be interrupted.

Here, we present a calibration-free method to accurately estimate local lamella thickness in-situ. Our method is based on an integrated cryo-fluorescence-FIB-SEM instrument that allows, unlike commercially available fluorescence microscopes (FM), coincident imaging using FM, FIB or SEM beams [2]. To estimate lamella thickness, we utilise the optical unit in our integrated setup as a high-resolution 2D-STEM camera (Fig. 1a) by replacing the original ITO covered glass beam stopper with an optically transparent scintillator CRY18 (Crytur) [3]. This setup allows recording the light-converted scattering pattern of the sample on a CCD or CMOS camera while preserving fluorescence imaging capabilities. Switching between FM and 4D-STEM is achieved by moving the objective focal plane.

The scattering pattern from amorphous samples contains information about local properties such as sample thickness. If the center of the scattering pattern can be accurately located, the signal of the virtual STEM segments (Fig. 1b-d; in our case dark field (DF)/bright field (BF) ratio) or the most common scattering angle can be compared to Monte Carlo simulations to estimate local sample thickness [4]. To demonstrate the accuracy of the method in the thickness range relevant for lamella preparation, we analyzed a sample of latex nano spheres ranging from 95 and 303 diameter and found excellent agreement between geometrical size and experimental thickness estimates (Fig. 2b).

Our method allows monitoring cryo-lamella thickness during FIB milling and paves the way to a robust workflow for reproducible fluorescence-guided lamella preparation for Cryo-ET imaging.

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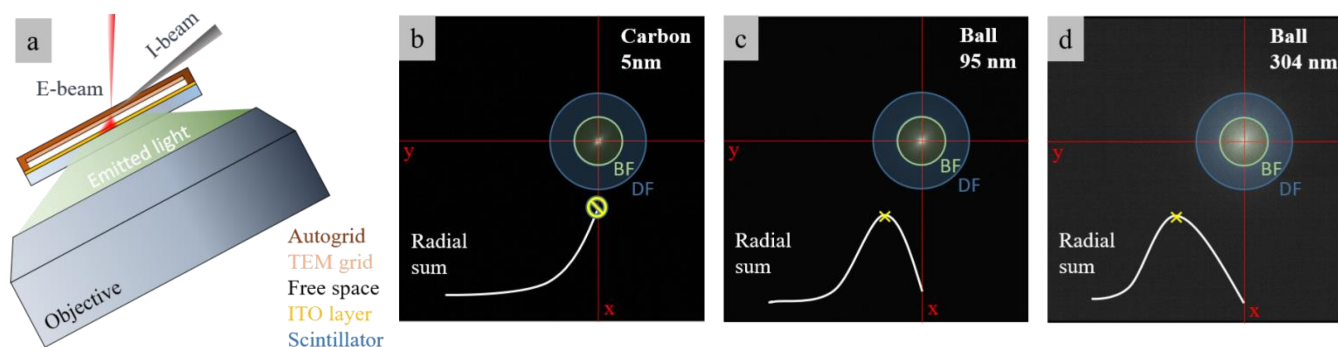

Figure 1. (a) Transformation of a fluorescence microscope into a 2D-STEM detector. (b-d) Transmitted scattering patterns of various beam positions. The center of the scattering patterns is highlighted by cross hair (red) and virtual STEM segment positions (in green and blue). Radially averaged profile plots are also shown. Yellow crosses show peaks and crossed circle its absence.

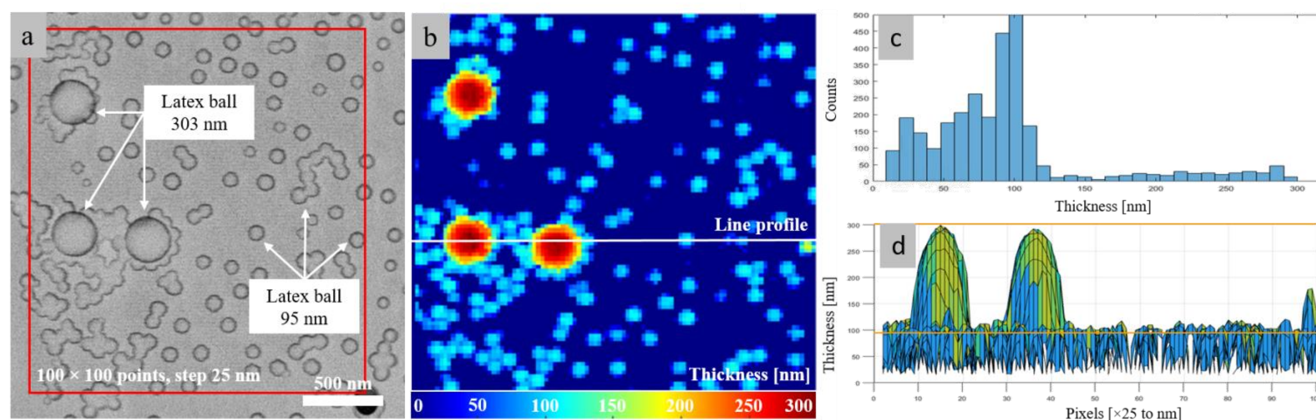

Figure 2. (a) Example dataset of latex beads (95 and 303 nm) consisting of 1K images in acquired in a 100 × 100 matrix (red rectangle). (b) Local thickness map of rectangular area in (a). (c) Histogram of size populations shows broad distributions around 100 and 300 nm, corresponding to the latex bead diameters. (d) Line profile (indicated in (b)) of measured thickness vs. expected bead diameter (yellow lines).

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