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Supplemental Information

Site-Specific Cryo-focused Ion Beam Sample Preparation Guided by 3D Correlative Microscopy

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Supporting Material

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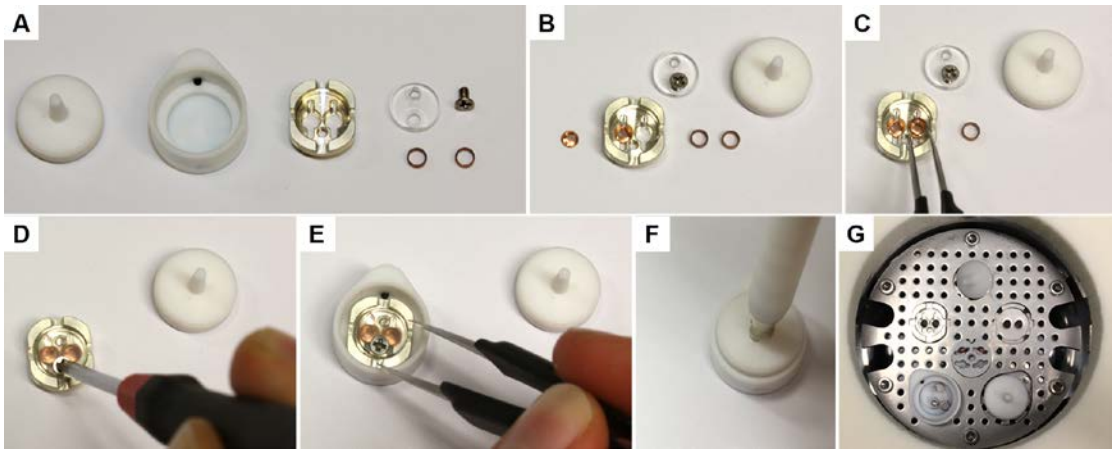
Supporting Material

Preparation of vitrified biological Sample
apply 1 μm Dynabeads (life Technologies, dilution 1:10) and 200 nm Tetraspeck (life Technologies, dilution 1:100)
prepare cellular sample: cell cycle synchronization and addition of vital fluorescent dye
plunge freezing
fix grids into autogrids customized for FIB-milling
mount specimens into *FEI Corrsight* shuttle (as described in Figure S1)
Cryo-FLM
overnight purging of the cryo-stage with 60°C dry N_2 and of the microscope chamber with dry N_2
load shuttle into cryo-stage at cryogenic temperatures
acquire grid overviews with 5x or 20x air objective and widefield microscopy using *MAPS*
acquire spinning disk confocal stack with 40x air objective at ROI using *MAPS*
select grids with appropriate ROIs and sufficient number of fiducials for further processing
Cyo-FIB milling
remove grids from shuttle and mount into dual-beam microscope at cryogenic temperatures
acquire low magnification montage of the grid with SEM using *MAPS* and align in 2D to FLM data
acquire high magnification images with SEM and FIB at ROI at the appropriate angle for milling
select markers that are visible in FLM, SEM and FIB images using Fiji
compute correlation using coordinate transformation
identify coordinates of feature of interest in FIB image
prepare thin lamella and acquire final images of lamella in SEM and FIB
Cyo-TEM
Transfer grids to the TEM at cryogenic temperatures
identify locations of lamellas on the microscope Fluorescence screen
acquire montage of lamella at intermediate magnifications using *SerialEM*
use features on lamella edges for 2D correlation to SEM image after lamella preparation
use transformation to overlay original confocal FLM data onto the TEM montage

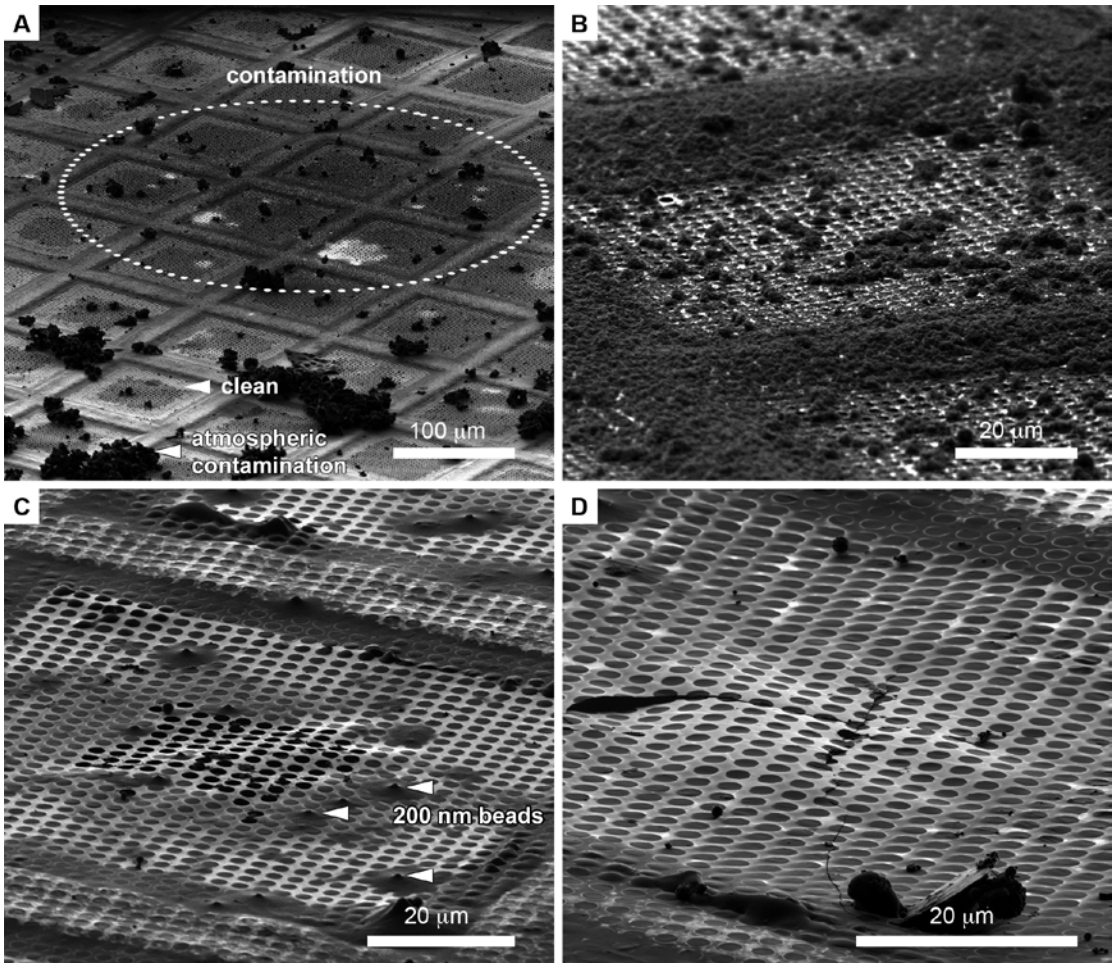
Supplementary Table 1. Protocol for 3D cryo-correlative workflow.

Supplementary Figure 1.



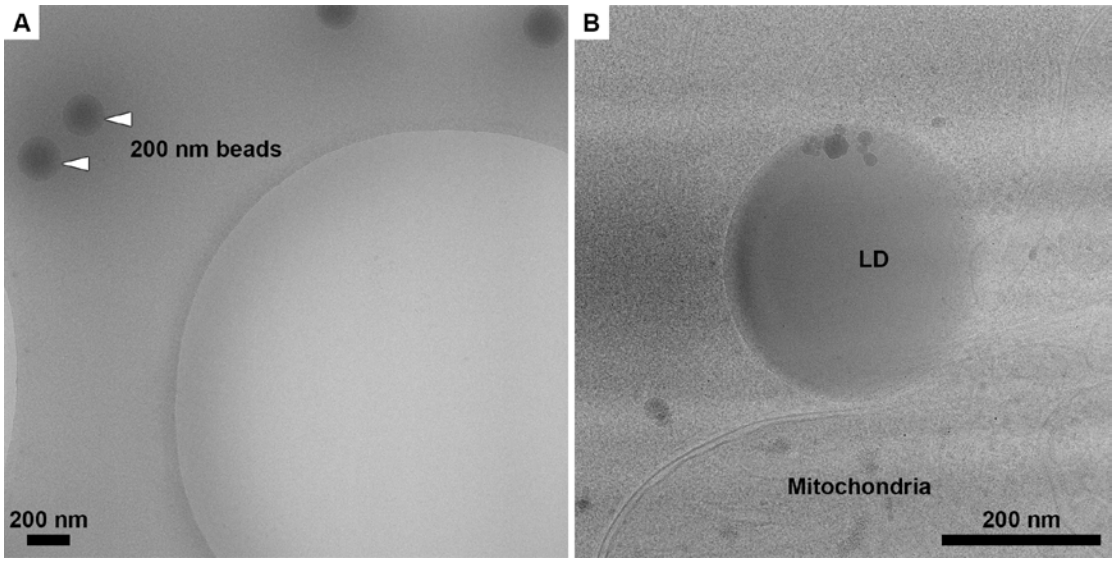
Supplementary Figure 1. A. Components of the shuttle system used for transfer and mounting of autogrid-clipped samples into the cryo-stage. **B.** Grids are inserted into the shuttle and fixed with cylinders (C). **D.** A translucent lid is screwed into place to secure the cylinders and seal the grids from the top. **E.** For safe transfer, the shuttle is stored in a box closed by a lid (F). **G.** All steps are performed in LN_2 in a customized loading station.

Supplementary Figure 2.



Supplementary Figure 2. A. Grids showed severe contamination after imaging in the *FEI CorrSight*. The occurrence of contamination was random, but appeared in confined locations on the grid. **B.** Severe contamination is in the form of small ice crystals (<1 µm). This type of contaminations renders further FIB processing of the sample impossible and hinders correlation, as markers cannot be distinguished on the surface. The appearance of the contamination and its localization had no relation to whether the grid was imaged or not. Contamination accumulated only when the shuttle with the grids was mounted into the imaging position. **C.** Magnification of a clean grid after imaging in the *FEI CorrSight* obtained after purging with heated N_2 and sealing of the cryo-stage. **D.** Comparison with a sample, which was not previously examined by cryo-FLM.

Supplementary Figure 3.



Supplementary Figure 3. Samples after cryo-FLM imaging in the cryo-stage show no signs of de-vitrification. A. Thin samples of 200 nm fluorescence beads in PBS. **B.** HeLa cell after correlation-guided cryo-FIB preparation (corresponding to Fig. 4). LD: lipid droplet.