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A Cryogenic Fluorescence Microscope Retrofittable in Coincidence with a FIB/SEM

Daan B. Boltje[1,8*], Jacob P. Hoogenboom[1], Arjen J. Jakobi[2], Grant J. Jensen[3], Caspar T.H. Jonker[8], Abraham J. Koster[4], Mart G.F. Last[8], Jürgen M. Plitzko[5], Stefan Raunser[6], Sebastian Tacke[6], Roger Wepf[7] and Sander den Hoedt[8]

[1.] Department of Imaging Physics, Delft University of Technology, Lorentzweg 1, 2628 CJ Delft, The Netherlands.
[2.] Kavli Institute of Nanoscience, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands.
[3.] California Institute of Technology, Pasadena, CA 91125, United States.
[4.] Department of Cell and Chemical Biology, Leiden University Medical Center, Einthovenweg 20, 2333 ZC Leiden, The Netherlands.
[5.] Department of Molecular Structural Biology, Max Planck Institute of Biochemistry, 82152 Planegg-Martinsried, Germany.
[6.] Department of Structural Biochemistry, Max Planck Institute of Molecular Physiology, Otto-Hahn-Str. 11, 44227 Dortmund, Germany.
[7.] Centre for Microscopy and Microanalysis, The University of Queensland, St. Lucia Queensland 4072, Brisbane, Australia.
[8.] Delmic B.V., Kanaalweg 4, 2326 EB Delft, The Netherlands.
* Corresponding author: boltje@delmic.com

Cryogenic Electron Tomography (Cryo-ET) opens up the window to life's complex machineries at the atomic scale [1,2]. In Cryo-ET, a sample is flash frozen, thinned to the appropriate thickness and a tomogram is acquired using a cryo Transmission Electron Microscope (TEM). A prerequisite for high resolution Cryo-ET is that the sample is thinner than the inelastic mean-free-path of electrons in vitreous ice; practically, this means a sample thickness of approximately 100 to 200 nm [3]. Focused Ion Beam (FIB) fabrication is essential to prepare a frozen hydrated lamellae for Cryo-ET containing a native protein environment from the cell interior. In the standard workflow, FIB fabrication is combined with Scanning Electron Microscopy (SEM), which leads to a blind procedure without prior knowledge of the precise location of the structure or protein of interest. Cryogenic Fluorescence Microscopy (FM) can be used to identify a fluorescence-expressing Region of Interest (ROI) and registration markers or fiducials can assist in locating this region of interest in the FIB-SEM [4-6]. Transfer between stand-alone Cryo-FM and Cryo-FIB/SEM is, however, susceptible to contamination. Furthermore, the registration accuracy may be limited, especially in 3D, hampering targeted milling of a 100-200 nm thin lamella containing the fluorescence ROI from a frozen hydrated cell. Integrated solutions combining FM with SEM and FIB milling in one instrument, may overcome these limitations.

Add-on FM solutions to Cryo-FIB/SEM systems have recently been presented [7, 8]. While greatly reducing contamination risks, registration between the FM and FIB/SEM may still limit accurate 3D registration and precise targeting of a ROI. As the sample cannot be imaged with the SEM, FIB, and FM without repositioning of the sample, these methods do not allow for FM-guided lamella milling. To maximize accuracy of ROI targeting in Cryo-FIB milling, an in-situ coincident geometry is required [9], which can also be adopted to allow for super-resolution FM localization and improved targeting accuracy.

We present a coincident 3-beam cryogenic Correlative Light and Electron Microscopy (CLEM) solution by combining a compact cryogenic microcooler with a custom positioning stage and an inverted widefield FM, thus allowing for in-situ FM-guided fabrication of frozen hydrated lamella. The system is easily retrofittable to existing FIB/SEM platforms as the hardware resides on a high-vacuum door which replaces the original door of the microscope (Figure 1). The central feature is a customized Joule–Thomson cryogenic microcooler [10, 11] (Demcon-kryoz) optimized for its low vibrations, drift and small footprint. The microcooler is mounted on a tailor-made piezo positioning system (SmarAct GmbH). The inverted widefield FM and corresponding objective lens (0.85 NA, 1 mm WD) is placed directly under the sample with a pre-tilt of 28 degrees to allow for live imaging whilst FIB milling occurs. Repositioning of the stage is therefore not needed and registration errors are hence avoided. The sample is mounted into a custom shuttle allowing for coincident FM/SEM/FIB imaging whilst shielding it from contamination, and is transferred in via a high-vacuum load lock system.

The technical description of the system along with the thermal and mechanical performance of the Joule-Thomson microcooler is presented. We discuss the design of the 5 degrees of freedom (*X, Y, Z, Rx, Rz*) sample stage and its (re)positioning accuracy. We also show fabrication of a frozen hydrated lamella based on FM targeting and in-situ FM monitoring during milling (Figure 2, top). Inspection after milling is done to check whether the structure of interest is still present and this data is also used to overlay on the TEM overview image. Based on this overlay, tomograms are acquired and reconstructed, showing no signs of devitrification (Figure 2, bottom). We further discuss and show implementation of astigmatic PSF shaping by means of a cross-cylinder lens set for improved repositioning accuracy in the *Z* direction [12].

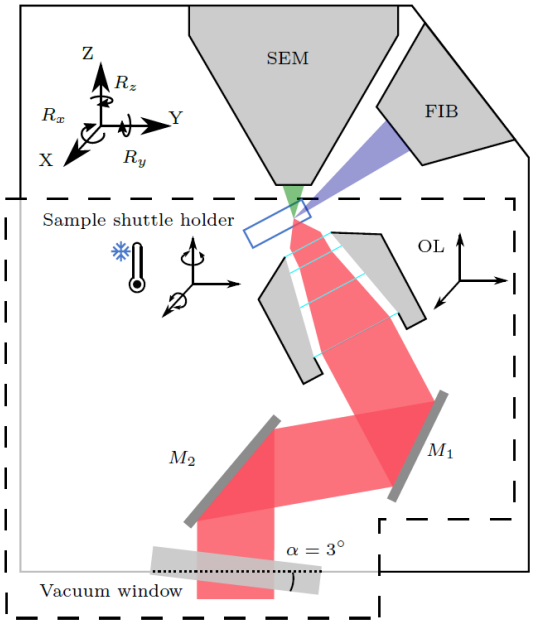

Figure 1. Schematic illustration showing the retrofitted hardware (dashed box) separated from the FIB/SEM hardware in respectively the lower and upper half-spaces. The Degrees of Freedom (DOF) of the objective lens (OL) and sample shuttle holder are denoted by arrows. The OL is mounted with a pre tilt of R_x = -28º.

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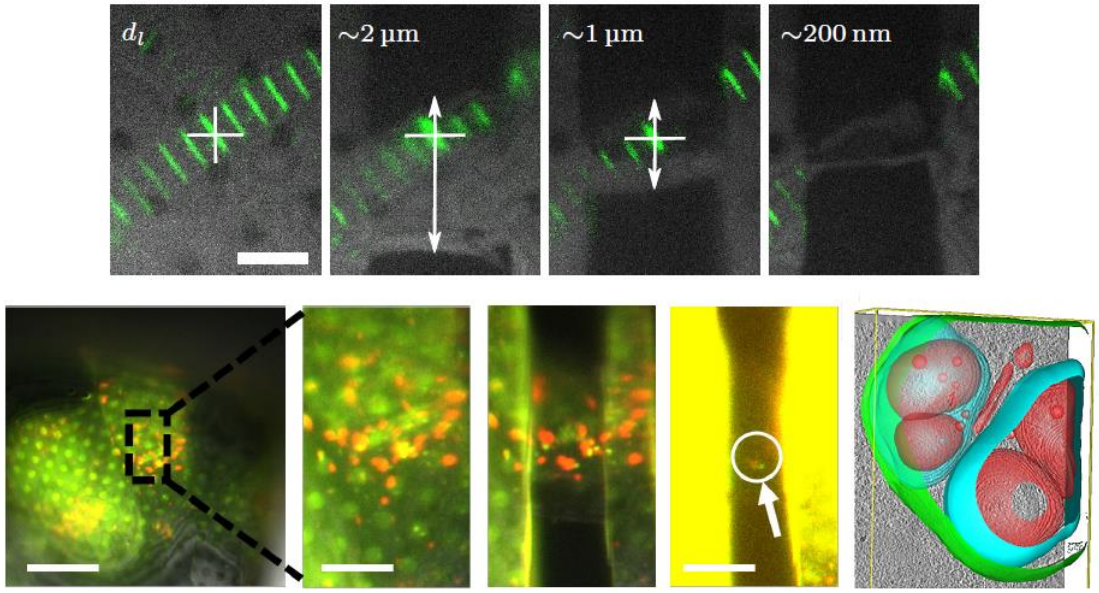

Figure 2. Top: adjusting the fabrication procedure when making a lamella based on predefined milling patterns of i.e. 2, 0.6 and 0.2 µm. After each step RLM and FM images are acquired. The feature of interest marked by the white cross, the vertical arrow show the misalignment and d_l denotes the estimated lamella thickness. Scalebar 5 µm. Bottom: light targeted FIB milling of a frozen hydrated lamella. HeLa cells expressing LC3-RFP-GFP were nutrient starved to induce the autophagy pathway. Green & yellow: autophagosomes and red: autophagolysosomes. Going from left to right; the ROI is selected by centering and focusing the FM field of view, milled and a high contrast FM image is acquired for inspection (white circle). FM overlay is used to acquire a tomogram at this site (segmentation, last image). Scalebars 40 µm (first) and 10 µm (other FM) images.

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