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ENZEL - A cryogenic, retrofittable, coincident fluorescence, electron, and ion beam solution for the cryo-electron tomography workflow.

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Cryogenic Electron Tomography (Cryo-ET) opens up the window to life's complex machineries at the atomic scale [1,2]. Focused Ion Beam (FIB) fabrication is essential to prepare a frozen hydrated lamella for Cryo-ET containing a native protein environment from the cell interior. In the standard procedure, 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 retrieving this region of interest in the FIB-SEM [3-5]. 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, to accurately mill a 100 nm thin lamella from the interior of a frozen hydrated cell. Integrated solutions combining FM with SEM and FIB milling in one instrument, are needed to overcome these limitations.

Add-on FM solutions to cryo-FIB/SEM systems have recently been presented [6, 7] but these still require an in-vacuum transfer between sample positions for FM and FIB/SEM. While greatly reducing contamination risks, coordinate system alignment between the FM and FIB/SEM may still limit accurate 3D registration and precise targeting of a ROI. A cryogenic FM/FIB/SEM system that allows for coincident FM, EM, and FIB would enable highly precise coordinate registration and could allow in-situ monitoring of the ROI via FM. Standard solutions to keep the sample at cryogenic temperature are however incompatible with the limited space available in such a 3-beam coincident system.

Here, we present a coincident 3-beam cryogenic 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 systems as the hardware resides on a high-vacuum door which replaces the original door of the microscope. The central feature is a customized Joule–Thomson cryogenic microcooler [8, 9] (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) are placed directly under the sample and are aligned with the EM optical axis. All retrofittable hardware is separated from the EM hardware in respectively the lower

and upper hemispheres of the FIB/SEM. The sample is mounted into a custom shuttle allowing for coincident FM/SEM/FIB imaging whilst shielding it from contamination as much as possible and is transferred in via a high-vacuum load lock system

We present and discuss the technical description of the system along with the thermal and mechanical performance of the Joule-Thomson microcooler. We discuss the design of the 5 degrees of freedom (X, Y, Z, Rx, Rz) sample stage and its (re)positioning accuracy. The FM objective is mounted on a 3 degrees of freedom piezo positioning stage (X, Y, Z) to allow for accurate alignment with the EM optical axis. Finally, we present some initial results on an improved lamella-fabrication process for the cryo-ET workflow which is faster, without transfer steps and significantly reduces ice contamination. Being able to check the lamellae between milling steps and after the milling process resulted in a higher success rate in lamellae fabrication and therefore in a reduced imaging time in the cryo-ET spent on sub-optimal samples.

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