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Precise 3D-correlative FIB-milling of biological samples using METEOR, an integrated cryo-CLEM imaging system

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Cryo-electron tomography (Cryo-ET) is currently the principal method for high resolution visualization of subcellular structures and macromolecular complexes in their native environments (Beck and Baumeister 2016). However, in order to study the architecture of cells by transmission electron microscopy (TEM), most of them need to be thinned to lamellas through cryo-focused ion beam (FIB) milling (Marko et al 2006, Rigort et al 2012).

In recent years, cryo-correlative light and electron microscopy (CLEM), which consists of taking cryo-fluorescence images prior to lamella preparation, has proven to be a powerful tool for guiding the FIB-milling toward specific macromolecular assemblies (Rigort et al 2010, Arnold et al 2016). The power of the cryo-CLEM approach has been exemplified by the localization of neurotoxic aggregates in the range of 1-3 µm (Baeuerlein et al 2017, Guo et al 2018), and more recently, by the precise localization of cellular structures as small as 400 nm, such as the novel Ede1-dependent endocytic protein deposit (END), a phase-separated aggregate which is degraded by autophagy (Wilfling et al 2020).

Despite its success, this two-step sample preparation workflow is laborious, time-consuming, and prone to sample contamination and damage due to multiple transfer steps. To overcome this limitation, fluorescence imaging systems which are directly integrated in the FIB/SEM chamber have been developed in recent years (Gorelick et al 2019).

Here, we present METEOR, a commercially available, FIB/SEM integrated, top-down widefield fluorescence microscope and the associated workflows, which enable one-step 3D-correlated cryo-FIB-milling of biological samples. We show the preparation of lamellas on a variety of samples, including eGFP-Ede1-labelled END condensates, located at the *Saccharomyces cerevisiae* plasma membrane.

Our “all-in-one” FIB/CLEM workflow provides numerous advantages. Besides avoiding unnecessary transfer steps, it allows the user to verify the presence of the targeted fluorescent signal in lamellas during and after the milling process (Figure 1). This is achieved by acquiring z stacks at positions of interest and correlating the deconvolved fluorescence signal to the FIB view using fiducial beads and the publicly available 3D-

Correlation Toolbox (3DCT; Arnold et al 2016). By imaging with METEOR during different lamella preparation stages, we can furthermore monitor the milling progress using the change of fluorescence signal in planes just above and below the targeted puncta, and re-adjust the milling patterns if necessary.

Finally, a fluorescence stack of the finished lamella is used to verify the presence of the target structure and for accurate correlation with the TEM overviews – without the risk of contaminating the sample through additional transfers. In this regard, METEOR should prove especially useful in correlative cryo-ET projects targeting novel or hard to identify structures.

To further enhance the quality and resolution of the fluorescence signal, we designed a new FIB/SEM shuttle compatible with higher numerical aperture (NA) and lower working distance objectives (Figure 2). Such high-NA objectives enable a more precise localization of small subcellular structures and hence increase the success rate of the 3D-correlation procedure.

In conclusion, we established a workflow using METEOR which increases the yield and quality of sample preparation by correlative FIB milling. Thereby, METEOR enables novel "on the fly" imaging strategies, which can be used to further optimize and streamline the 3D cryo-CLEM workflow.

Author contributions: O. H. Schiøtz, C. Capitanio and A. Bieber contributed equally to this work. Corresponding authors are P. S. Erdmann and J. M. Plitzko.

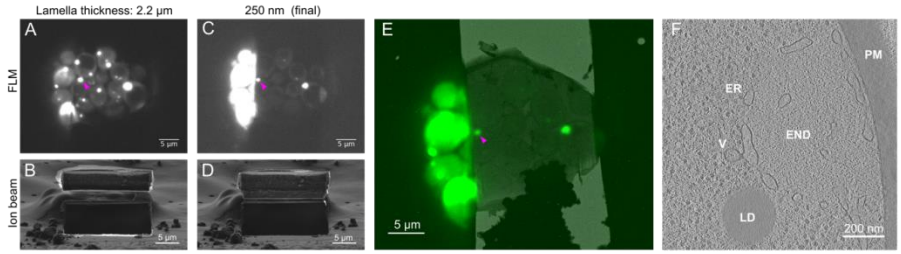

Figure 1. Correlative cryo-FIB milling and cryo-ET workflow using METEOR, demonstrated on a sample of *S. cerevisiae* expressing eGFP-Ede1. A-D. Maximum intensity projections (MIPs) of fluorescence stacks (A,C) and ion beam views (B,D) of a lamella during rough milling (A,B) and after fine milling (C,D). Fluorescence stacks were acquired with excitation at 484 nm, emission filter set to 525/30 nm and a z step size of 400 nm. E. Overlay of the final lamella MIP (green) on the TEM lamella overview. Magenta arrowheads in A, C & E indicate the targeted fluorescent punctum where the tomogram in (F) was taken. F. 2D slice of a tomogram after denoising, acquired on the indicated position on the lamella. The target structure, a phase separated END compartment at the plasma membrane (PM), surrounded by fenestrated ER, can be clearly distinguished from the surrounding cytosol containing ribosomes. LD: lipid droplet, V: coated vesicle.

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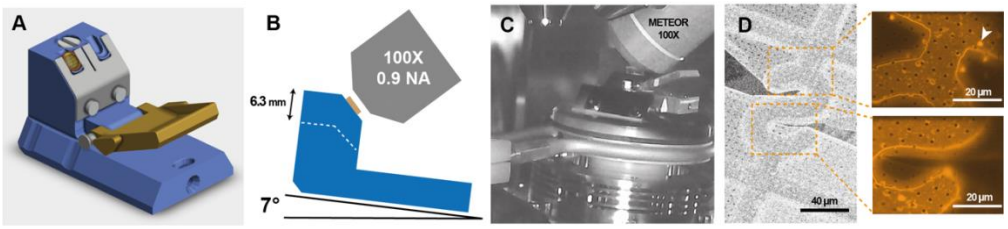

Figure 2. A new FIB/SEM shuttle design allows using high NA objectives with low working distance. A. 3D model of the new shuttle design. B. Scheme of the shuttle at METEOR imaging position. This system is 6.3 mm higher than the standard FIB/SEM shuttle for the Scios/Aquilos (white dashed line) and compatible with 1 mm working distance objectives. C. Infrared camera view of the shuttle inside of the FIB/SEM chamber. The shuttle is located at LM imaging position, at 7° tilt, with a 100X/0.9 NA objective. D. SEM field of view of a gold Finder grid. On the right, two fluorescence views acquired in that area with METEOR, at 100X magnification, using the new shuttle design. In the top figure, a white arrow indicates an autofluorescent 1 µm fiducial bead.

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