Towards the Visual Proteomics of *C. reinhardtii* Using High-throughput Collaborative *in situ* Cryo-ET

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Cryo-electron tomography (cryo-ET) has become a vital tool in structural biology for determining the 3D structure of macromolecular complexes in their native context inside cells or tissues. Cryo-ET and subsequent subtomogram averaging have produced structures at resolutions sufficient to visualize sidechains (3-5Å), with the specimens being cryo-fixed in vitrified ice 200nm or thinner. While thicker vitrified single cells and tissues from multicellular organisms have been milled using Gallium cryo-focused ion beam (Ga+-cryo-FIB), subtomogram averages of macromolecular complexes within these cryo-FIB milled lamellae have often been limited to resolutions lower than 10 Å due to poor lamella quality, redeposition, and beam-induced motion during tilt-series data acquisition. A next-generation cryo-FIB that uses plasma instead of Gallium (cryo-plasmaFIB) from Thermo Fisher Scientific reduces redeposition and ion beam damage while improving the throughput with automated sample transfers, making it possible to collect large in situ cryo-ET datasets on lamellae of cells and tissues to determine high-resolution molecular atlases or visual proteomics.

In this study, we benchmarked the performance of cryo-plasmaFIB using *S. cerevisiae* as an established model system, which resulted in a 6.5 Å subtomogram average of 80S ribosomes from 7 tomograms acquired on a single lamella, cryo-FIB milled using Xenon plasma. The analysis of Rosenthal-Henderson B-factors of 80S ribosomes from lamellae with varying thicknesses demonstrates an inverse relationship between specimen thickness and signal-to-noise ratio (SNR). As a proof of principle of visual proteomics approaches, we generated a large in situ cryo-ET dataset containing thousands of tomograms from *C. reinhardtii*. We show that we were able to obtain sub-nanometre subtomogram averages of biomolecular complexes ranging in size from megadaltons to several hundred kilodaltons, and hence feasibility to determine the Visual Proteome of *C. reinhardtii*.

However, the increasing number of known and unknown macromolecular complexes in such large datasets poses a challenge for individual efforts to identify, average, classify, and curate macromolecular complexes, calling for a collaborative effort. The availability of large curated molecular atlases will prove instrumental in the development of new computational tools for in situ cryo-ET. Overall, the study highlights the potential of cryo-plasmaFIB for high-throughput and high-resolution cellular cryo-ET and visual proteomics approaches to gain insights into the molecular functioning of fundamental cellular processes.