Recent developments in FEI's *in situ* cryo-electron tomography workflow

Studying the molecular machinery of cells from atomic details to the cellular context and beyond is a great challenge for cell biology. It requires the integration of dynamic and structural information obtained using different ranges of spatial resolution. In order for this to be accomplished, a comprehensive workflow is needed covering vitreous freezing, cryo-fluorescence microscopy, sample thinning by focused ion beam microscopy, and high-resolution cryo-electron tomography. This Application Note provides an overview of FEI's integrated product suite, providing a complete cryo-transmission electron microscopy (cryo-TEM) tomography workflow.

Structural biologists have made great progress in discovering the structures of individual proteins and protein complexes. Much of the recent progress has resulted from advances in cryo-TEM, especially single-particle analysis, which has made the elucidation of near-atomic-scale structures for large proteins and protein complexes almost routine. However, single-particle analysis requires large numbers of identical particles to be purified and separated from their original context. The ultimate goal of structural and cell biologists is to derive the structures of the molecular machines that constitute all living systems in their natural, functional contexts, where their interactions with other components can be observed. Cryo preparations for TEM can preserve samples in a near-natural, fully hydrated state, frozen in non-crystalline vitreous ice. Now advances in *in situ* cryo-TEM tomography, which combines cryo-focused ion beam (cryo-FIB)-based sample preparation and cryo-electron tomography, are enabling researchers to prepare site-specific thin sections (called cryo-lamellas) containing targeted structures and acquire nanometer-scale 3D models of those structures *in situ* in the native cellular context[1,2]. Here we describe the suite of instruments that enable FEI's cryo-electron tomography workflow.

As **Figure 1** illustrates, FEI's cryo-TEM tomography workflow comprises five steps: vitrification, cryo-correlative microscopy, cryo-FIB sample thinning, cryo-TEM tomography, and analysis/visualization.

(I) Vitrification: Cells prepared by routine culture methods are surrounded by physiological solution and are grown on carbon-coated gold electron microscopy (EM) grids. In an FEI Vitrobot™ device, excess solution is blotted away and the sample grid is plunge-frozen in a cryogenically cooled fluid, typically liquid ethane. The water in the sample vitrifies, freezing so rapidly that it does not crystallize, thus avoiding the molecular-scale disruption that would occur with a normal freezing process.

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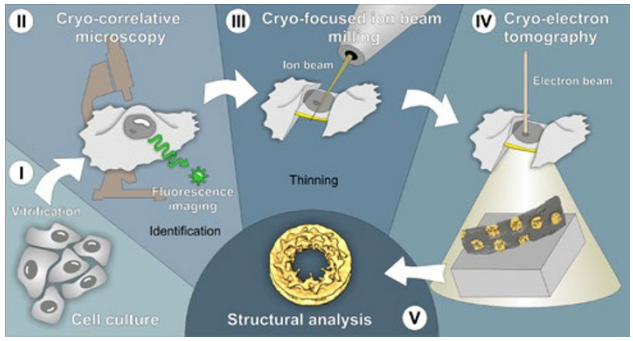

Figure 1 | The five essential steps of the cryo-TEM tomography workflow. Each step is supported by/within FEI's product portfolio/Workflow. Vitrobot (I), CorrSight (II), Scios (III), Titan Krios (IV), Amira (V).

(II) Identification: The sample is transferred to an inverted cryo-confocal microscope, where structures of interest are identified by genetically encoded fluorescent tags (such as GFP). The inverted design of FEI's CorrSight™ light microscope provides ample room for the cryo-cooling module required to keep the sample in its vitrified state during cryo-fluorescence imaging.

(III) Thinning: The sample is transferred using an FEI cryo-transfer system to the Scios™ DualBeam microscope, which combines an FIB and a scanning electron microscope (SEM) with a cryo-stage for the sample. The FIB uses a finely focused beam of Ga^+ ions to remove material from the sample through the process of sputtering atoms. The milling process can be controlled at the nanometer scale. A gas injection system is used to deposit a micrometer-thick layer of metal (usually platinum) onto the frozen sample. The coating protects the sample during the preparation of the thin, TEM-transparent cryo-lamella. The SEM can be used to monitor the progress of the FIB milling process. To assist in locating and navigating to the target structures identified in the previous step, the DualBeam system overlays the SEM image with

the fluorescent image exported electronically from the previous step using MAPS™ software. Perhaps the key development that has enabled *in situ* cryo-TEM tomography is a specialized FIB milling procedure[3] that removes material above and below a plane through the sample, leaving the target structure within a thin, uniform lamella ideal for TEM imaging and analysis (**Fig. 2**).

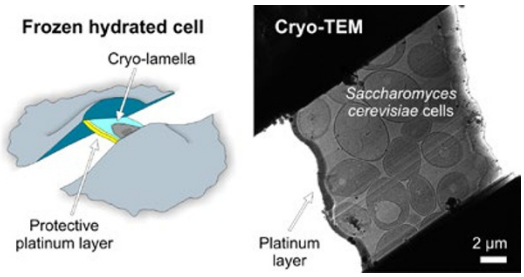

Figure 2 | A key development enabling *in situ* cryo-TEM tomography is the specialized FIB milling procedure enabled by the Scios DualBeam that removes sample material above and below the desired thin lamella. Adapted with permission from ref. 1.

(IV) Tomography: The thin lamella sample on the EM grid is transferred to an automated cryo-TEM (Talos Arctica™ or Titan Krios™), which acquires a series of 2D images from different perspectives as the sample is tilted in precisely determined increments (cryo-electron tomography). A computer then reconstructs the images into a 3D tomographic data set of the imaged volume.

(V) Structural analysis: Small subsets containing the structures of interest can be computationally extracted from the 3D tomogram and subjected to image processing methods. In cases where multiple identical structures are present in the sample (e.g., pores in the cell's nuclear membrane), the reconstruction and analysis software can combine data from individual structures, averaging out noise and increasing contrast to create a composite model with substantially higher resolution (**Fig. 3**). Using FEI's Amira™ visualization framework, the operator can segment, color, and cross-section the model in a variety of ways to enhance its display and presentation.

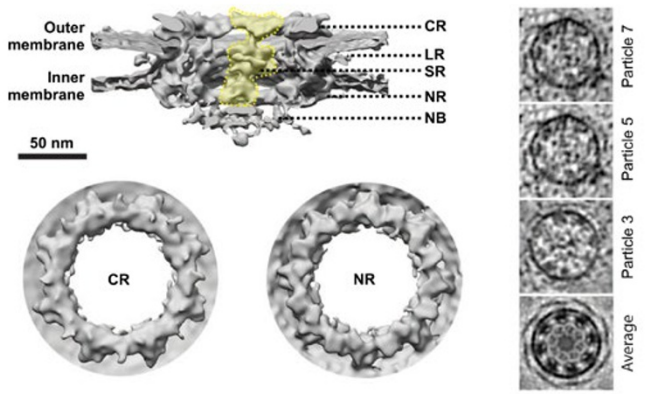

Figure 3 | Averaged structure of a HeLa cell nuclear pore complex (NPC) obtained from a cryo-electron tomogram acquired using FEI's Volta phase plate technology. Average images of NPCs perpendicular to and along the nucleo-cytoplasmic axis. CR, cytoplasmic ring; LR, luminal ring; SR, spoke ring; NR, nuclear ring; NB, nuclear basket. A protomer is highlighted in yellow. (right) Filtered 2D central slices of the average and representative particles. Image courtesy of J. Mahamid (Department of Molecular Structural Biology, Max Planck Institute for Biochemistry, Martinsried, Germany). Adapted with permission from ref. 4.

Conclusion

In situ cryo-TEM tomography can provide nanometer-scale 3D models of biological structures in their native cellular context. The FEI cryo-electron workflow yields repeatable, high-quality results. The ability to see these structures in their native context allows researchers to observe their functional relationships and interactions with other components in the cellular environment and promises to become an important tool in the investigative toolbox as scientists seek a better understanding of living systems spanning the molecular and cellular levels.

For more information regarding systems and instruments designed to support this workflow, please visit https://www.fei.com/life-sciences/structural-biology.

1. Rigort, A. & Plitzko, J.M. *Arch. Biochem. Biophys.* **581**, 122–130 (2015).
2. Schaffer, *M. et al. Bio Protoc.* **5**, e1575 (2015).
3. Rigort, A. *et al. Proc. Natl. Acad. Sci. USA* **109**, 4449–4454 (2012).
4. Mahamid, J. *et al. Science* **351**, 969–972 (2016).