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Opening windows into the cell: focused-ion-beam milling for cryo-electron tomography

Elizabeth Villa[1], Miroslava Schaffer[1], Jürgen M Plitzko[1,2] and Wolfgang Baumeister[1]

Addresses
[1] Department of Molecular Structural Biology, Max Planck Institute of Biochemistry, Martinsried 82152, Germany
[2] Bijvoet Center for Biomolecular Research, Utrecht University, 3584 CH Utrecht, The Netherlands

Corresponding authors: Plitzko, Jürgen M (plitzko@biochem.mpg.de) and Baumeister, Wolfgang (baumeist@biochem.mpg.de)

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Introduction

The trend in Structural Biology is towards the study macromolecular complexes and supramolecular assemblies of ever-increasing size and complexity. The ultimate goal is to study them in the context of the intricate network and crowded environment in which they operate, that is, the cell. Over fifty years ago, electron microscopy was instrumental in launching the field of Modern Cell Biology [1] and until today it continues to be an important tool for the study of cellular ultrastructure [2]. In the past decade, super-resolution light microscopy (SRLM) has advanced towards revealing information at the molecular level by determining the position of specific molecules with a precision of 50–100 nm [3]; however, it does not provide information on their shape and structural context. At the same time, cryo-electron microscopy (cryo-EM) has established itself as a key technique for studying the structure of large and flexible macromolecular complexes at subnanometer resolution. The challenge today is to bring the molecular resolution enabled by cryo-EM to cellular studies, bridging the divide between molecular and cellular structural studies.

In single-particle cryo-EM, macromolecules are isolated, deposited on a grid, and rapidly cooled to liquid nitrogen temperatures. The high speed of cryo-fixation prevents ice-crystal formation; instead, the water is fixed in an amorphous or vitreous state which preserves the structures under scrutiny in a close-to-life state. The samples are subsequently imaged with a transmission electron microscope (TEM) and a three-dimensional structure is derived from a large data set of identical particles present in different orientations [4]. For cells and other pleomorphic structures, a different imaging modality, cryo-electron tomography (CET), is used. In this case, the sample is tilted inside the TEM (typically from -60° to 60°) to acquire enough information for computing a three-dimensional reconstruction of the sample [5]. TEM relies on detecting the electrons that pass through the sample with no or minor alteration to their direction and energy. For biological samples at intermediate voltages, that is, 300 keV, the mean free path or average distance travelled by an electron at which they undergo a single scattering event is ~280 nm. With thicker samples, electrons undergo multiple inelastic scattering, that is, energy-loss events that result in image blurring. Practically, cellular samples up to 500 nm can be imaged with TEM, but the signal-to-noise ratio of the images drops considerable already at 300 nm. This limitation renders most cells too thick for CET, and thus most CET studies describe isolated or reconstituted systems [6,7], small prokaryotic cells [8], and peripheral regions or appendages of eukaryotic cells [9,10].

Since vitrification arrests cells in a close-to-native state, tomograms under such conditions capture snapshots of molecular landscapes of the cells at the time of freezing. Thus, extending this methodology to be applicable to any region of a cell is highly desirable and will enable the study of cell biology problems in which not only structural information is needed, but also quantitative analysis of the data. Examples of such analysis include molecular census of populations according to their absolute and relative locations, and organization of cytoskeletal elements such as filament networks. Furthermore, some supramolecular assemblies involved in key cellular processes cannot be studied in isolation; since they are so deeply entangled in their environments that it is impossible to isolate them without disrupting their structural integrity and cellular context. Thus, CET can bridge the gap between Molecular and Cellular Structural Biology, yield high resolution while studying the structures under scrutiny in their molecular context, and circumvent the adverse effects of fixation, staining, and labelling.

To overcome the sample thickness limitation of CET, cryo-ultramicrotomy has been developed as a technique to obtain thin samples from vitrified material. A diamond knife is used to cut thin sections (60–80 nm) from samples of cells or tissue that have been high-pressure frozen inside capillaries [11,12]. These sections are deposited

on EM grids and imaged by CET. Unfortunately, the technique requires very experienced users, typically has a low throughput, and the samples suffer from disturbing artefacts, including curved sections, crevasses introduced by the knife, and compression of up to 30–50% in the direction of the cut. Because of the irreproducibility of these artefacts, quantifying the results or combining results from different data sets becomes challenging, and thus limits cryo-ultramicrotomy to descriptive results. Furthermore, structures of interest often occupy a volume that cannot be accommodated within a single thin section.

Here, we review a new technique, cryo-focused-ion-bean milling of cellular samples, as a preparation tool for CET that is applicable to a large variety of cell types. The ability to do tomography directly in cells unaltered by sample preparation opens the possibility of quantitative molecular cell biology, in which cells can be perturbed to represent different physiological scenarios and examined in molecular detail.

Opening windows into the cell: the workflow

Focused ion beam milling is a technique used in material sciences for preparing thin samples for TEM studies [13]. A focused ion beam system operates similarly to a scanning electron microscope (SEM), except that instead of electrons, it uses a focused beam of ions, typically gallium. The ion beam can be used at low beam currents for imaging. At high beam currents, it can be used in an ablation mode to mill samples by sputtering atoms from the surface of the material. Typically, a FIB system is combined with a SEM system in the same chamber. Such a dual-beam system can be used to navigate and mill samples with the FIB under visual control of the SEM; the latter provides the advantage of reducing the radiation damage on the sample caused by FIB imaging. Adapted to cryo-temperatures, this instrument can be used for micro-machining biological material in a controlled and systematic manner.

The proof-of-principle that FIB milling could be used for preparing biological materials for TEM imaging was published by Marko and colleagues [14••]. We developed the necessary instrumentation to realize routine FIB milling of cells that are grown or deposited on an EM grid [15••,16••], and designed a workflow for structural studies of cellular samples, including sample vitrification, localization of regions of interest through cryo-fluorescence microscopy, sample preparation using FIB milling, and CET [17]. Our cryo-FIB set up has been reproduced with minor modifications [18•]. Other efforts have been focused on FIB milling of cells that have been subject to high-pressure freezing [19,20••,21].

To adapt FIB milling for biological specimens, the dual beam has to be equipped with a cryo-system that consists of a cryo-stage, a preparation system to mount the grids, and a transfer system for insertion and extraction of the samples into the chamber kept at high vacuum and cryo-temperatures. We modified a commercial system from Quorum Technologies to fit the needs for FIB milling of cells, as described in [16••]. Notably, we modified the commercial rigid specimen support *Autogrid*™ (FEI Company, Eindhoven, the Netherlands), which provides stability to the malleable gold EM grids that need to be used for cell culture to avoid cytotoxicity. The Autogrid was modified to make the sample accessible to the beam at shallow incident angles. Furthermore, a gas-cooled cryo-stage that attaches to the microscope stage was designed to enable 360° in-plane rotation of the shuttle, and precise targeting of height and tilt, allowing for the sample to be properly oriented for milling with respect to the ion beam.

To prepare cellular samples for CET, cells are grown or deposited on an EM grid and plunge frozen in a cryogen, typically liquid ethane (Figure 1a). EM grids are clipped in Autogrids and loaded into a shuttle that is inserted into a cooled stage in the chamber of the dual beam for milling. The grids are oriented such that the angle of incidence of the ion beam is almost parallel to the surface of the grid. This shallow angle of incidence results in milled samples that are correspondingly almost parallel to the grid, allowing for conventional tilting schemes during CET. Furthermore, at low angles of incidence, the ions and the atoms they dissociate from the sample are projected away from the sample, so that deposition of milled material or ions on the sample is minimized (Figure 1b). The preparation is done by selecting patterns to be milled. The ion beam rasters through these patterns, sputtering away cellular material, yielding thin sections supported by the surrounding unmilled cellular volume. The process is done in multiple steps, where the bulk of the material is removed at high current (100–300 pA), and the last step is done at low current (30–50 pA) to minimize radiation damage at the surface of the sample. Typically, a milled area results in a surface of tens to a few hundreds of square microns, and one can target a thickness adequate for CET, that is, between 60 and 350 nm, effectively opening an electron-transparent window into the cell. The aim is to have a window thin enough for CET that still contains the structure of interest. Depending on the shape and size of the sample, different milling geometries are used, as described below. To ensure a smooth surface of the milled area, a gas injection system can be used to release a precursor gas that is adsorbed by the sample, resulting in a organometallic platinum layer covering the grid that protects the sample from irregular sputtering of the sample during milling (Figure 3a,b).

After milling, the grids are removed from the dual beam chamber, and stored for later CET in a cryo-TEM (Figure 1c). In a single session of four to eight hours,