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Perspectives on electron cryo-tomography of vitreous cryo-sections

Jason Pierson^{1,2}, Matthijn Vos^{1,2}, J. Richard McIntosh³ and Peter J. Peters^{1,4,*}

¹Division of Cell Biology II, The Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands, ²FEI Company, Achtseweg Noord 5, PO Box 80066, 5600 KA Eindhoven, The Netherlands, ³Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309, USA and ⁴Kavli Institute of Nanoscience, Delft University of Technology, Delft, The Netherlands

*To whom correspondence should be addressed. E-mail: p.peters@nki.nl

Abstract A major objective of modern structural biology is to appreciate the cellular organization by elucidating the spatial arrangement of macromolecular complexes within a cell. Cryogenic sample preparation, combined with cryo-ultramicrotomy, enables large cells and pieces of biological tissues to be thinned for electron cryo-tomography, which provides a three-dimensional view of the biological sample. There are, however, limitations associated with the technique that must be realized, addressed and overcome for the procedure to become mainstream. Here, we provide perspectives on the continued advancements in cryogenic sample preparation for vitreous cryo-sectioning, image collection and post-image processing that have expanded the attainable information limit within the three-dimensional reconstructions of cells and pieces of biological tissues.

Keywords electron cryo-tomography, vitreous cryo-sectioning

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Introduction

Cryogenic biological sample preparation for electron cryo-microscopy and tomography is at the forefront of modern biological structural analysis. Ultra-rapid freezing immobilizes biological samples and ensures that they are in a close-to-native state during imaging [1]. Compared with conventional electron microscopy (EM), which involves chemical fixation, dehydration and staining, cryogenic sample preparation methods have greatly improved our understanding of the biological ultra-structure and macromolecular organization within a cellular context [2]. The technique has its own limitations, however, which must be recognized and addressed.

One of the major difficulties in imaging frozen-hydrated biological samples is their sensitivity to the electron beam; therefore, a limited dose must

be used during image acquisition to reduce the potential of ultra-structural damage to the sample. It has been well documented that beam damage, in the form of vitreous section ‘flow’, during image acquisition [3,4] significantly limits how one should distribute electron dose during single-projection imaging and also during tilt series acquisition. The result is a low-dose imaging method with an increased amount of statistical noise due to inadequate sampling that limits both the observable cellular detail and the subsequent procedures for the alignment of multiple examples of the same biological structure.

Confident recognition of macromolecular complexes within the three-dimensional (3D) snapshots of cells imaged with low electron doses remains a challenge, partially due to the low signal-to-noise

ratio caused by low-dose imaging. Non-isotropic sampling and the absence of an *in vivo* labeling technique are additional important problems. Only large macromolecular complexes and cellular components [5–10] can be recognized with confidence. Here, we briefly review the current state of progress in addressing these problems and also present data from a few alternative approaches that may hold promise for future applications.

Methods for cryo-immobilization

The biological sample and its extracellular medium must be treated in order to prevent ice crystal formation. To successfully freeze large cells or pieces of tissue, the sample must be high-pressure frozen [11]. This ensures that no crystallization of intracellular water will occur. Preventing crystallization, which is often called ‘vitrification’, is important because growing ice crystals may damage the ultrastructure of the cell [12]. To achieve vitrification, the pressure is raised more than 1000 times, which could potentially alter chemical equilibria in cells. However, the speeds with which pressure is applied and freezing is accomplished are likely to provide reliable cryo-immobilization.

Large mammalian cells and pieces of tissue often do not vitrify well, however, resulting in partial immobilization within non-vitreous water. To help

solve this problem, some form of cryo-protectant is often added prior to freezing to ensure proper vitrification. Often high-molecular-weight compounds (low osmolarity), such as Dextran [13], provide cryo-protection without a significant alteration in the osmolarity of the extracellular medium. Such compounds may still, however, affect the chemistry of the extracellular medium, so we sought an alternative approach to freeze large mammalian cells.

Yeast cells and some bacteria are relatively easy to vitrify by high pressure freezing (HPF), even without extracellular cryo-protection. Dried yeasts suspended in water have been used for years as a paste to mix with cells of interest, minimizing the amount of extracellular fluid that must be frozen as heat is withdrawn from the sample [14]. We therefore explored mixtures of mammalian cells [e.g. normal rat kidney (NRK)] with isotonic sucrose and healthy yeast cells, grown to mid-log phase and harvested by filtration to form a paste. The small, easily frozen cells provide a benign, but effective cryo-protection (Fig. 1a). Tomographic reconstruction (Fig. 1b) shows a representative area of the NRK cell at higher magnification. Many distinct cellular features can be recognized, such as a mitochondrion (M), the nucleus (N) and various large cellular macromolecular complexes. The representative area from (b) appears well vitrified, which is confirmed by the accompanying select-area diffraction pattern (b').

Fig. 1. (a) An image from a medium magnification montage EM grid map [using a Tecnai F20 (FEI, Eindhoven) EM at 140× nominal magnification] showing a representative area of *Saccharomyces cerevisiae* (denoted by the Y) and NRK (outlined with black dotted line) cells. The cells were mixed together, high-pressure frozen and vitreous cryo-sectioned. (b) A 10-nm slice from a reconstructed tomogram of an NRK cell. The letter M represents a mitochondrion and N the nucleus. (b') A selected area diffraction pattern from (b) indicating that the sample is vitreous [3]. Scale bars = 100 nm.

Methods for cryo-ultramicrotomy

Specimens smaller than 1 μm can be prepared for electron cryo-microscopy/tomography by plunge-freezing techniques [15], however, most mammalian cells and pieces of tissue are too thick to be viewed by EM *in toto*, due to frequent plural electron scattering; these samples must therefore be thinned, either using cryo-ultramicrotomy [16] or focused-ion beam milling technology [17].

Recently, there have been great improvements in the equipment and procedures for generating quality vitreous cryo-sections. Among them are micromanipulation [18], electrostatic charging for section attachment to a support film and an anti-contamination glove box [19,20]; all these contribute to producing ribbons of vitreous cryo-sections more quickly and in a more reproducible manner. Figure 2 displays the results of using such an equipment to produce long

Fig. 2. Electrostatic charging technique for attaching a ribbon of vitreous sections to an EM support film. (a) An EM grid is placed at the diamond knife-edge using tweezers with a bent tip. (b) A ribbon of vitreous sections (white arrowheads) is guided over the EM grid using an eyelash (white arrow) attached to a wooden dowel. (c) Once the ribbon of vitreous sections is of suitable length (white arrowheads), approximately the diameter of the EM grid, the generator is switched from the discharge mode to the charge mode, causing the ribbon to get attached to the EM grid. (d) A low magnification image showing the ribbon of vitreous sections after electrostatic charging. Scale bar = 50 μm . (e) A medium magnification micrograph showing a vitreous section from within the ribbon. Note that the section is smooth and relatively flat with no apparent mechanically induced damage as is often seen with stamping/pressing techniques. *Saccharomyces cerevisiae* cells can be seen scattered throughout the vitreous section (black arrows). Holes within the C-Flat EM grid can be seen and one such is denoted with a white asterisk. Scale bar = 2 μm . (f) A selected area diffraction pattern confirming vitreous ice. (g) A 5-nm-thick tomographic slice from a vitreous section, (V) vacuole, (M) mitochondrion, (ER) endoplasmic reticulum and black arrows indicate putative 80S ribosomes. Scale bar = 100 nm. (h) A flipped view (about the x -axis) from the tomographic reconstruction in (g). The vitreous section (black arrowheads) is well attached to the support film (white arrowheads). A fiducial gold marker is seen on the surface of the carbon support film (white arrow). Scale bar = 100 nm. (i) A surface rendered visualization of the tomographic volume in (g). Mitochondrion, purple; vacuole, blue; endoplasmic reticulum, red; cell membrane, white; unidentified vesicle structures, green; unidentified macromolecular complexes including putative ribosomes, yellow.

ribbons of vitreous cryo-sections (Fig. 2a–d) that can be attached to the support film of an EM grid.

Artifacts induced by the process of sectioning (knife marks, crevasses and compression) have been described in detail and must be taken into consideration [21]. It is possible to minimize crevasses by using thinner sections [22]. Indeed, we have observed that the depth of crevassing is a constant fraction of the thickness of the section. This means that in thicker vitreous cryo-sections crevassing is more obvious, but in thinner sections crevasses are probably still present until the electron beam irons them out.

Compression has been regarded as the most problematic and complex artifact of vitreous cryo-sections. However, images of ribosomes [19,20] (Fig. 3), microtubules [23] and other cellular components appear to resist compression at the given resolution, which may imply that compression is inhomogeneous. Whole cells are affected, but their constituents may not be. This is, however, a topic that deserves much more work.

Electron cryo-imaging strategies

Electron cryo-tomography of thinned vitreous cryo-samples is the only currently available technique for 3D imaging of large cells and pieces of tissue in a close-to-native state (Figs 1b, 2g–i and 3). As mentioned in Introduction, the electron dose for imaging frozen-hydrated samples must be low, especially when it is distributed over an entire tilt series. Therefore, reducing the ‘overhead’ dose used to pinpoint an area of interest before acquisition would be very helpful. This can be done using low-magnification montage or grid mapping.

Grid mapping allows the operator to quickly explore a grid and find regions that appear promising for further imaging. It reduces the amount of time the user must invest to find an adequate area for imaging within an EM grid. In addition, it limits the amount of electron dose used for scanning EM grids for areas of interest. For example, in the case of serial EM [24], a low-magnification map of the entire EM grid is initially created at a nominal magnification of 140 $\times$. Areas of interest from the initial low-magnification map are selected, and

intermediate maps (nominal magnification of 3100 $\times$) are subsequently made. Cells and cellular outlines should be observable in these medium magnification maps. From such data, one can choose regions for a more detailed study at magnifications that will reveal interesting cellular architecture. Alternatively, these portions can be stored and used in a future batch tomography processing.

Small macromolecular complexes can be visually recognized based on their size and distribution. However, the true identity of these protein complexes remains subjective until a suitable labeling, and correlative approach or template matching procedure is developed for electron cryo-tomography. One major limitation is the lack of a ‘green fluorescent protein-like’ label for EM. Therefore, localizing individual protein complexes in cells remains a challenge. There have been recent advancements, though, that correlate cryo-light microscopy images with cryo-EM images to mimic protein labeling, or correlative light-electron microscopy [25–27]. This technique is rapidly developing and may replace the need for a ‘green fluorescent protein-like’ EM marker in the future.

The next step in the imaging is to collect either single-projection images of the area of interest or to acquire a tilt series. Cells are inherently three-dimensional. EM provides 2D images, so densities are superimposed along the projection direction and the resulting image lacks basic 3D information. Electron tomography addresses this problem by tilting the stage of the microscope, which in turn tilts the sample, and by recording images from multiple angles. These can be combined to generate a 3D volume, albeit the reconstruction being imperfect, due to the limited range of angles that can usually be employed [28,29]. In the best cases, using the current methods, molecular resolution can be obtained, and multi-resolution maps of macromolecular complexes that enhance the structural interpretation of such protein complexes can often be constructed [2]. Figure 4 shows one such multi-resolution map from an 80S yeast ribosome constructed from semi-automated particle selection and volumetric averaging from electron cryo-tomograms of vitreous cryo-sections [19,20].

Fig. 3. Vitreous cryo-section-induced compression explored at the macromolecular level using 80S ribosomes. (a) A 5-nm-thick slice through a reconstructed tilt series from a ~65-nm-thick vitreous cryo-section of an *S. cerevisiae* cell: CW, cell wall; V, vacuole; M, mitochondrion; ER, lamellae of endoplasmic reticulum; R, cytoplasmic 80S ribosomes. Scale bar = 100 nm. (a') The surface of the reconstructed volume (a) that shows the diamond knife marks used to approximate the cutting direction (black arrow). (b) A simple volume-conserving transformation (Section 2.4.1) was applied to the original reconstructed volume (a) to compensate for vitreous cryo-section-induced compression. (c) A single cross-section of an 80S ribosome sub-volume that was used to measure (d) the dimensions in relation to the cutting direction (denoted in a'). Scale bar = 10 nm. (e) A sum of 400 isolated 80S ribosome volumes from the reconstructed volume (a). The measurements are presented in the graph (i), which displays the relationship between dimensions measured parallel (blue) and perpendicular (red) to the cutting direction. The graph shows that the average height of the 80S cellular ribosomes was measured to be $21 \text{ nm} \pm 2.4$, and the width $22 \text{ nm} \pm 2.5$. The same measurements were done for the transformed volume (b) in (f–h) and presented in the corresponding graph (j). For the 80S ribosomes in the transformed volume, the average height was measured to be $27 \text{ nm} \pm 3.5$ and the width to be $21 \text{ nm} \pm 2.5$. Comparison of plunge frozen and HPF, vitreous cryo-sectioned isolated 80S ribosomes. (k) A 5-nm-thick slice from a reconstructed volume of isolated, plunge frozen 80S yeast ribosomes. Scale bar = 100 nm. (k') A summed volume of 300 particles from the reconstruction in (k). Scale bar = 10 nm. (l) A 5-nm cross-section from a reconstructed volume of isolated 80S yeast ribosomes that were HPF, vitreous cryo-sectioned. The black arrow denotes the cutting direction on the surface of the section. The asterisk represents a carbon edge of the C-Flat EM grid used to support the vitreous cryo-section during imaging. (l') A summed volume of 300 particles from the reconstruction in (l).

Fig. 4. Multi-resolution mapping of an 80S *S. cerevisiae* ribosome density map obtained from tomographic reconstructions of vitreous cryo-sections. (a) 80S ribosome density map obtained from tomography of vitreous cryo-sections. The small (40S) and large (60S) subunits are denoted along with representative ribosomal landmarks including the head region (h), beak (bk), shoulder (Sh), base (b), stalk (St), and stalk base (SB). (a') 180° rotated view from (a) that shows the L1 protuberance (L1). (b) A high resolution single-particle 80S *S. cerevisiae* ribosome obtained from single-particle cryo-EM analysis [35] is fit into the density map in (a and a'). (c) The theoretical X-ray crystallographic map fit into the density map from (a). Green color represents the 40S small subunit and blue color is the 60S large subunit.

Future prospects

In the future, it will be necessary to cut thinner vitreous cryo-sections to maximize the information obtained. Alternatively, other methods/novel equipment must be integrated in order to successfully sample thicker vitreous cryo-sections. Although the characterization of 'thin' is relative, here we mean that the vitreous cryo-section must be electron-transparent, i.e. transmit a sufficient fraction of the forward scattered electrons to the detector, so an interpretable image is produced. If the vitreous cryo-section is thinner than the mean-free path for inelastic interaction, then less energy is transferred to the specimen and the result is less damage, due to heating effects. In modern electron microscopes, spherical and chromatic aberrations of the objective lens can be corrected, but if the sample is too thick, then specimen-induced aberrations (both spherical and chromatic) dominate the information limit. These problems can only be corrected by reducing the inherent thickness of the sample.

In materials research, scanning transmission electron microscopy (STEM) in combination with a high-angle annular dark-field (HAADF) detector is widely used for structural and chemical analyses of various materials at high spatial resolution [30]. The principle of STEM tomography is to use a fine

electron probe to scan the sample. The beam must scan parallel to the optical axis at all times, so it mimics the parallel beam in a transmission electron microscopy (TEM) (even though it is scanning). The image quality of STEM depends on the electron probe size, which is expressed as the convolution between the object function of the specimen and the shape of the probe used to analyze it [31].

A potential advantage for STEM is that lenses do not form the image (the probe does), therefore the defects in the imaging lenses do not apparently affect the final image resolution limits, which is limited simply by beam dimensions. For instance, for thick vitreous cryo-sections, chromatic aberrations can seriously limit TEM image resolution. In the STEM imaging mode, chromatic aberrations are mostly absent [32] and therefore for thick vitreous cryo-specimen this would be a rather significant advantage. In addition, STEM imaging is also useful for beam-sensitive specimen because a scanning beam enables a precise control of the irradiated region of the sample, and therefore it can be compared with an improved low-dose TEM imaging method.

In 2002, Ziese *et al.* [33] showed that HAADF-STEM revealed a higher contrast and better signal-to-noise ratio (SNR) in biological material when compared with normal bright-field TEM. The contrast is greater

in HAADF-STEM because the camera length can be adjusted to maximize the ratio of the number of scattered electrons that hit the detector to the number of electrons passing through the hole in the middle of the detector. Dark-field TEM images also showed poorer contrast and noisier images than HAADF-STEM, but they displayed a higher resolution. Unfortunately, STEM tomography has not become a mainstream technique in the life science field yet, but we suspect that it may in the future.

Although there have been many technological advances on the imaging side, significant data collection from vitreous sections is still very much limited by sample preparation and the time it takes to produce and locate suitable vitreous cryosections and regions of interest. The route to produce large amounts of reliable data is often long and requires screening and imaging many samples. In this respect, the introduction of multiple samples with automatic loaders, for instance, in the FEI Titan Krios™, is useful to quickly screen 12 grids. In addition, it reduced the amount of possible EM grid transfer damage and contamination that build up with frequent sample changes. The sample can remain usable in the column for at least 3 days without visible contamination, maximizing the imaging time on an optimized area.

In summary, the combination of easy sample screening, longer specimen lifetime in the microscope, new imaging modalities (e.g. microprobe STEM) and more sensitive detectors (complementary metal-oxide semiconductor- or CMOS-based direct electron detectors) [34] should provide a fertile ground in which vitreous sections can become an analysis technique of great importance for imaging cells in their close-to-native state at molecular resolution.

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