In vivo veritas: electron cryotomography of cells

Jürgen M. Plitzko, Achilleas S. Frangakis, Stephan Nickell, Friedrich Förster, Ariane Gross and Wolfgang Baumeister

Automated electron cryotomography enables the study of organelles or whole cells embedded in vitrified ice in a close-to-life state and with a resolution of a few nanometers. This technology will provide new vistas of the supramolecular architecture inside cells that orchestrates higher cellular functions.

Electron tomography: principles and problems

Given the large depth of focus, electron microscopic images are essentially 2D projections of the object in the direction of the electron beam. Features from different levels within the object overlap and cannot be separated. Tomographic techniques acquire projections of the object as viewed from different directions and then merge them computationally to obtain a 3D reconstruction of the object volume. In electron tomography (ET) the specimen holder is tilted incrementally around an axis perpendicular to the electron beam and images are recorded at each position. Before the 3D density map can be calculated – most commonly by a ‘weighted backprojection’ algorithm [3] – the projections must be mutually aligned to establish a common system of coordinates (Fig. 1).

The relationship between resolution d of a tomographic reconstruction of a cylindrical object of diameter D, from N equally spaced projections covering the full angular range of 180°, is given by the Crowther relationship d ~ π D/N [4]. For the reconstruction of an object of 200 nm diameter with a resolution of 2 nm one would therefore need 320 projections and tilt increments of 0.6°. For an optimal sampling of objects of constant thickness, non-equidistant tilt angles are advantageous [5]. Owing to physical constraints (e.g. limitations of specimen holders and increase of specimen thickness at higher tilt angles) the full angular range cannot be accessed; in practice, it is restricted to about ±70°. Considering the projection theorem [6], the limited tilt range implies that data are missing in a wedge-shaped region of the Fourier space resulting in distortions of the reconstruction, most notably a loss of resolution in the z-direction (discussed later).

A key problem in ET, which for more than two decades stood in the way of establishing it as a practicable technique, is reconciling two requirements that are in conflict with one another. To obtain “high” (<5 nm) resolution tomograms with minimal distortions, one has to collect data over as wide a tilt range as possible and with increments as small as possible. At the same time, the cumulative electron dose must be kept within tolerable limits to prevent radiation damage erasing the finer details of the structure or, in the worst case, rendering reconstructions meaningless. Samples embedded in amorphous ice are extremely sensitive to radiation damage and should not be exposed to >2000–5000 e nm⁻² (e = electrons) for visualizing molecular structures.

The successful realization of electron cryotomography is based on dose fractionation. Early theoretical considerations [7] had suggested that, in principle, the electron dose, which is required to visualize structural features...
close to the resolution limit, is the same for 2D and 3D images containing equivalent information. Thus, the separation of features in z-direction would be a bonus one has not to pay for! By combining the information of all projections of a tilt series, the signal-to-noise ratio improves as it does by adding up statistically noisy 2D images. More recent computer simulations have confirmed that the dose fractionation theorem holds, even in the case of (not too strongly) absorbing specimens [8]. Therefore, one could, in principle, fractionate the dose over as many projections as an optimized tilt geometry might require. There is, however, a practical limitation: the signal-to-noise ratio must still enable alignment of the projections accurately enough to establish a common system of coordinates. This is done by cross-correlation, and facilitated by adding fiducial (gold) markers to the specimen.

The alignment problem is aggravated by the limited mechanical accuracy of specimen holders. Deviations from an ideally eucentric tilt geometry, instability and drift of the cryoholders cause movements in the x-, y- and z-direction that translate into lateral shifts and changes of focus. Therefore, following each change of tilt angle, the specimen (or the image) has to be realigned and refocused. This can be done manually, but at the expense of additional exposure of the specimen to the electron beam. In fact, the dose ‘overhead’ spent on these alignments can far exceed the dose spent usefully on recording data.

**Instrumentation and automation**

With the advent of computer-controlled transmission electron microscopes equipped with eucentric goniometers, and the availability of large-area charge-coupled device (CCD) cameras, it became possible to develop and implement complex image-acquisition procedures running in a fully automated fashion [9,10]. This made possible the recording of tomographic datasets with the specimen remaining centered and at a uniform level of focus, while the cumulative electron dose is kept within tolerable limits [11]. At lower magnifications suitable for imaging large structures at the resolution of ~5 nm, datasets comprising up to 180 projections can be recorded with a total dose as low as 5000 e nm⁻². At higher magnifications with resolution targets in the 2 nm range, one aims not to exceed a dose of 3000 e nm⁻². The fraction of the dose that is spent on overhead [search, recentering, (auto)focusing] can be kept as low as 3% of the total; in other words, almost all electrons are used for retrieving information. This has changed the perspectives of electron cryotomography quite dramatically. It is now possible to study specimens embedded in vitreous ice as demonstrated with phantom cells (lipid vesicles encapsulating macromolecules) [12,13], organelles [14,15] or whole prokaryotic cells [16,17].

Vitrification by rapid freezing ensures close-to-life preservation of the structures to be studied and enables the capture of dynamic events [18]. It avoids the risk of artifacts traditionally associated with chemical fixation and staining or with the dehydration of cellular structures. Equally important, tomograms of frozen-hydrated structures represent their natural density distribution while staining reactions tend to produce intricate mixtures of positive and negative staining, and therefore compromise interpretation in molecular terms.

A major limitation in electron tomography is specimen thickness and here one has to take into account that for simple geometric reasons a specimen with a thickness of 200 nm (at 0° tilt) will increase in thickness to 540 nm at 70° tilt. A thickness larger than the mean free path of electrons brings us into a multiple scattering regime. In particular, the contribution from inelastically scattered electrons (the inelastic mean free-path is ~200 nm at an acceleration voltage of 120 kV; for 300 kV it increases to 350 nm) [19] can degrade the quality of the images and tomograms quite significantly. Here, energy filters offer some remedy: when operated in a zero-loss mode, the adverse contribution (‘blurring’) of the inelastically scattered electrons can be removed and contrast is improved. Samples much thicker than 0.5–1.0 μm cannot be studied in this way and, therefore, must be cryosectioned before tomographic analysis.

**Exploiting the information contained in cryotomograms**

Using automated procedures, cryotomograms can be obtained more or less routinely within a few hours. Recording a tilt series and calculating a tomogram is indeed less cumbersome and time consuming than going through the conventional procedures of plastic embedding and sectioning the material. Once the tomogram is produced, it can be sectioned in silico in any desired direction and segmented for visualization (Fig. 2). With smaller structures (e.g. bacteriophages docked onto liposomes), a resolution...
Figure 2. An example of a tomographic reconstruction

Electron tomographic reconstruction of the Archaeon Methanospirillum hungatei embedded in vitrified ice. The tomogram shown is reconstructed from 60 projections; the tilt increments were 1.5° and the effective tilt range was ±45°. The cumulative dose for recording the whole dataset was ~2000 e−/nm². Once a tomogram is obtained, it can be ‘sectioned’ in silico in any direction desired. (a) Two orthogonal slices are displayed, one parallel to the long axis of the cell showing the multilayered cell wall and one perpendicular to it at the level of a ‘plug’. (b) The plug structure and the flagella traversing it are also shown in an isosurface representation. The tomogram has been ‘de-noised’ by nonlinear anisotropic diffusion and manually contoured to highlight the cell wall and the flagella [21].

Beyond easily recognizable features, such as the multilayered cell wall structure in Fig. 2, a cryotomogram of a cell contains an imposing amount of information. It is, in fact, a 3D image of the entire proteome of the cell and it depicts the whole network of macromolecular interactions that provide the basis for higher cellular functions. However, new strategies and innovative image analysis techniques are needed for ‘mining’ this information. Exploitation is confronted with two major problems: cryotomograms are contaminated by substantial residual noise and distorted by missing data – in spite of optimized image acquisition systems. ‘De-noising’ techniques, while improving the signal-to-noise ratio, modify the signal in a way that precludes quantitative post-processing. Moreover, the cytoplasm is densely populated (‘crowded’) with molecules literally touching each other [20]. It is therefore virtually impossible to perform a segmentation and feature extraction based on a visual inspection of the tomograms. In principle, one could find ways to introduce electron-dense labels marking the positions of the molecules under scrutiny and facilitating their detection. Such an approach, however, would no longer be non-invasive and it would be difficult, if not impossible, to achieve quantitative detection.

Therefore, we prefer a different strategy, namely a detection and identification based on structural signatures [21,22]. Provided that a high- or medium-resolution structure of the macromolecule of interest is available, this can be used as a template to perform a systematic search of the reconstructed volumes for matching structures (Fig. 3). Such an approach is computationally demanding because not only are the positions of the molecules unknown, but also are their orientations. Image simulation [13] and experimental studies using phantom cells (A.S. Frangakis et al., unpublished results) have shown that template matching is indeed a feasible approach and can achieve a satisfactory level of fidelity. The search should be performed in an objective and reproducible manner and, therefore, should be entirely machine-based, not requiring manual intervention. Ideally, the search is exhaustive, detecting all copies of the target molecule, and it should be fast to enable the analysis of large datasets. The perfection of such pattern recognition tools will be crucial for taking full advantage of the power of electron cryotomography [21].

Ongoing instrumental developments and prospects

With the current (non-isotropic) resolution of 4–5 nm, we can address only larger (>400 kDa) complexes in a cellular context. To widen the scope of cellular tomography we need to overcome some of the present limitations.

As illustrated in Fig. 4 for a lipid vesicle, distortions and artifacts resulting from the ‘missing wedge’ can be reduced quite significantly by dual-axis tilting combining two single-axis tilt series with a discrete or continuous
90° rotation around the z-axis [23,24]. We have recently designed and built a dual-axis tilt cryoholder and tested it successfully (S. Nickell et al., unpublished results).

It is expected that keeping the specimen at (near) liquid helium temperature (~10K and below) instead of liquid nitrogen temperature (~90–100K) will increase its radiation resistance. Although measurements performed in the past came to quite divergent results regarding the protection factors, there is a broad consensus that the cryoprotection factor will be (at least) in the range of 2–3 [25]. While this might appear to be only a modest improvement, it would help considerably in tomography; it would in fact enable us to double the number of projections, and the finer sampling in 3D should bring us closer to the 2 nm resolution target. This, in turn, would enable the detection of smaller particles in cellular volumes and improve the identification fidelity.

With the new generation of TEMs, which can be operated at both liquid nitrogen and liquid helium temperature, a more accurate assessment of the protection factor will become possible.

Improvements can be expected to come from optimizing detection systems for intermediate voltage (300–400 kV) microscopes [26,27]. With the slow-scan CCD cameras, performance is affected by the discrete pixel size, electron (back) scattering, photon propagation and the (non-optimal) coupling between the scintillator and the CCD chip of the camera. Currently, novel designs are being explored that exclude the adverse contribution of back-scattered electrons, intrinsic to fiber coupling. Such lens-coupled cameras might improve the detection efficiency twofold.

Taken together, these developments should help to push the limits of electron cryotomography and brighten the prospects to explore the uncharted territory of the molecular architecture of the cytoplasm [28,29].
References