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A cylindrical specimen holder for electron cryo-tomography

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ABSTRACT

The use of slab-like flat specimens for electron cryo-tomography restricts the range of viewing angles that can be used. This leads to the "missing wedge" problem, which causes artefacts and anisotropic resolution in reconstructed tomograms. Cylindrical specimens provide a way to eliminate the problem, since they allow imaging from a full range of viewing angles around the tilt axis. Such specimens have been used before for tomography of radiation-insensitive samples at room temperature, but never for frozen-hydrated specimens. Here, we demonstrate the use of thin-walled carbon tubes as specimen holders, allowing the preparation of cylindrical frozen-hydrated samples of ribosomes, liposomes and whole bacterial cells. Images acquired from these cylinders have equal quality at all viewing angles, and the accessible tilt range is restricted only by the physical limits of the microscope. Tomographic reconstructions of these specimens demonstrate that the effects of the missing wedge are substantially reduced, and could be completely eliminated if a full tilt range was used. The overall quality of these tomograms is still lower than that obtained by existing methods, but improvements are likely in future.

1. Introduction and theory

Electron cryo-tomography (cryo-ET) is a three-dimensional imaging technique capable of visualising unstained biological material in a near-native state at a resolution of a few nanometres. Cryo-ET has rapidly increased in popularity in recent years, and many reviews are now available [1–5]. The technique has produced some highly significant results [6–9], but two major limitations currently restrict the quality of reconstructions generated by cryo-ET.

First, unstained biological specimens are easily damaged by the electron beam, and have low inherent contrast in the TEM, producing images with a low signal-to-noise ratio (S/N). Higher electron doses improve the S/N, but also damage the specimen, resulting in a compromise between the electron dose and the attainable resolution [10,11]. The S/N is reduced further by the use of thick specimens, so samples must be kept as thin as possible (well under 1 μm) for best results. Various technological improvements are currently being developed in an attempt to improve the situation, including direct electron detectors to improve the S/N of individual images [12,13], phase plates to increase image contrast [14], and cryo-sectioning and focussed ion beam milling to reduce specimen thickness [15–18].

The second major limitation that affects cryo-ET is the limited range of angular sampling imposed by the use of extended flat specimens, prepared on standard TEM grids. When a slab-like flat specimen is tilted for tomography, the effective specimen thickness in the electron beam increases, decreasing the S/N in the images. At high tilt angles, this effect becomes severe and images provide no useful signal above the background noise. After a tomogram is reconstructed, a wedge-shaped region in Fourier space is left containing no information, and this is named the "missing wedge" problem.

The missing wedge causes several deleterious effects in tomograms. These are demonstrated in the simulation presented in Fig. 1 and can also be seen in the real example shown in Fig. 2. Most strikingly, horizontally-oriented features disappear almost entirely as the size of the missing wedge increases. In Fig. 1, this effect can be seen most clearly on the horizontal lines and the large circle, but also on the horizontal part of the central star shape, showing that horizontally-oriented parts of more complex structures are also lost. In Fig. 2, the cell membranes are obviously incomplete, and the ice layer that contains the cell is entirely missing from the reconstruction, since its surfaces are horizontal and therefore aligned with the missing wedge. Compact objects – such as the small circle in Fig. 1 and the ribosomes in Fig. 2 – are not lost completely, but do suffer from elongation in the direction of the missing wedge, reducing the resolution in this direction. This elongation has been expressed as a multiplication factor by

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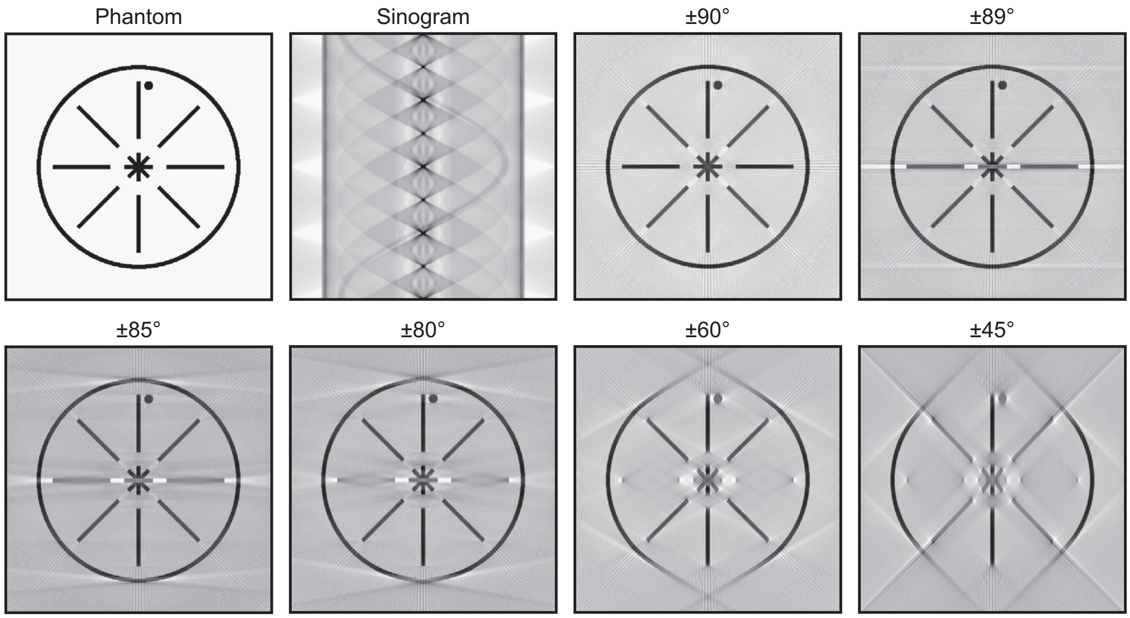

Fig. 1. Simulation of the effects of the missing wedge. A phantom image was projected at 1° intervals to produce the sinogram shown. A reconstruction from a full 180° range of angles (±90°) shows some loss of contrast and minor artefacts due to the tilt increment, but is otherwise an accurate copy of the original phantom. As the range of angles used for reconstruction is reduced, more serious artefacts appear and horizontally-oriented structures are almost completely lost from the images.

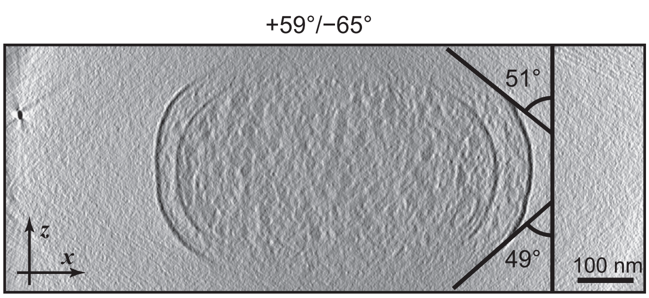

Fig. 2. A real example of the effects of the missing wedge. The image shows a cross-section from a reconstruction of a frozen *Escherichia coli* cell, with a gold fiducial marker visible at the extreme left. The cell membranes are only resolved at the sides of the cell, and white streaking artefacts are visible around the gold particle. The black lines show tangents to the outer cell membrane and their angles from the *z* axis. The angular range of the tilt series used for this tomogram is shown above the image.

Radermacher [19], describing the lengthening of an ellipsoidal point spread function (PSF) along the *z* axis. However, this treatment does not explain the complete loss of extended horizontal structures from the reconstruction, and it seems the full effect of the missing wedge cannot simply be explained by convolution of an ellipsoidal PSF with the points composing the object.

Another effect of the missing wedge is the presence of white halos and streaking artefacts that appear at a tangent from objects with high contrast (most features in Fig. 1, and the gold fiducial marker on the left of Fig. 2). It is notable that curved linear structures, such as the large circle in Fig. 1, are visible only between these tangents, and their angles match the size of the missing wedge. This is instructive when observing real tomography data: the tangent angles where the cell membranes fade away can give an indication of the angular range where useful information is obtained from the images. These angles are often measured to be lower than the full range that was used for image collection. In the example shown in Fig. 2, tangents to the last clearly-visible parts of the cell membrane have angles of +49° and −51°, even though images were collected over a much larger range of +59° to −65°. This suggests that the images from higher tilt angles contribute little or no information to the reconstructed tomogram (due to the increased thickness at high tilt and the resulting low signal-to-noise ratio in the images) and so the missing wedge is larger than expected. This could have some relevance in situations where the size of the missing wedge is used in subsequent calculations, such as the estimation of resolution or the masking of objects for sub-tomogram averaging.

An important point to notice from Fig. 1 is the size at which the missing wedge becomes a problem. Some artefacts are visible even when a ±89° tilt range is used, but above approximately ±80° the effects are not too severe. With a larger missing wedge, the quality of the reconstructions deteriorates quite seriously as structures with certain orientations are no longer resolved. Typical cryo-tomograms are obtained using a ±60° range, but as discussed above, often the effective size of the missing wedge will be larger than this. The situation in these cases is likely to lie somewhere between the ±60° and ±45° panels in Fig. 1, which show that a large amount of information about the specimen will be lost. Increasing the tilt range to ±80° would clearly help this situation, while reaching ±90° would be ideal, providing a full 180° of tilt.

Several approaches have been taken to minimise or eliminate the problems of the missing wedge. Conceptually, the simplest option is to use a cylindrical specimen, which does not increase in thickness at high tilt angles and so allows image collection over a complete angular range. It is important to note that since the thickness does not change, images of a cylinder from all tilt angles have an equal S/N. Therefore even if the angular range is restricted (by mechanical limitations, for example), the effects of the missing wedge will be minimised, because images at high tilt angles will contribute useful information to the reconstruction. This stands in contrast to the situation in flat extended specimens, in which high-tilt images contribute very little to the tomogram.

Cylindrical specimens have been used in materials science, where cryogenic temperatures are not required and specimens are less sensitive to radiation. Several groups have succeeded in manufacturing cylindrical specimens for electron tomography [20–24], and modified specimen stages have been developed allowing full rotation of the specimen [24–28]. In biology, only one experiment has been reported where cylindrical specimens