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Limits of Specimen Thickness for Energy-Filtered Tomography of Stained Plastic Sections at 120 kV Beam Voltage

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Energy-filtered transmission electron microscopy (EFTEM) offers advantages for electron tomography of sectioned cells when the specimen thickness is greater than the inelastic mean free path [1]. By zero-loss filtering it is possible to eliminate completely the inelastically scattered electrons, which reduce image contrast due to the blurring effect of the microscope's chromatic aberration. Furthermore, it is generally necessary to tilt the specimen to at least 60°, effectively doubling the specimen thickness in the beam axis. If tilt angles as high as 80° are used, the specimen thickness effectively increases by a factor of nearly six. To date electron tomography is performed on specimens that are prepared by (i) fixation then plastic embedding, sectioning, and staining or by (ii) rapid freezing and cryo-electron microscopy. Previous studies have shown that energy-filtering is important in optimizing contrast in frozen-hydrated, unstained specimens, where the low mean atomic number gives a high inelastic to elastic scattering ratio [1]. However, most tomography to date has been applied to plastic sections, which provide a wealth of 3D structural information, particularly when combined with high-pressure freezing and freeze-substitution [2]. Contrast in stained specimens is mainly due to high-angle scattering outside the objective aperture and the image series are typically recorded close to focus.

Here we address two questions. First, whether energy-filtering is useful for tomography of stained plastic sections at a beam voltage of 120 kV. Second, how thick the specimen can be for tomography at 120 kV. We have recorded tilt series from (i) BSC-1 cells that had been infected with vaccinia virus, and (ii) cultured rat hippocampal neurons. Sections were cut to a nominal thickness of 100, 200 and 300 nm. The specimens were coated with approximately 3 nm of carbon on both sides to stabilize against beam-induced shrinkage. Colloidal gold particles of diameter ~10 nm were deposited on the top of the sections to facilitate alignment. The specimen thickness t was determined from the low loss spectrum, in terms of the inelastic mean free path λ_{i},, by the equation: $t = \lambda_i \log_e(I_{tot}/I_o)$, where I_{tot} is the total intensity in the energy loss spectrum and I_o is the zero-loss intensity. Tilt series were acquired with an FEI CM120 electron microscope equipped with a Gatan GIF100 imaging filter, and were processed with the IMOD software package developed by the University of Colorado to provide tomographic reconstructions [3].

Figure 1 shows energy loss spectra from a plastic section cut to a nominal thickness of 300 nm at tilt angles θ = 0°, 10°, 20°, 30°, 40°, 50° and 60°. As expected, the measured values of t/λ_i for these tilts are proportional to $1/\cos(\theta)$ with the projected thickness doubling when the specimen is tilted to 60°. These values are consistent with a mean free path of 150 nm for the embedded cell, although this ignores some collapse of the specimen in the z-direction. Figure 2 compares filtered and unfiltered images recorded at a tilt of 60°, from which the improved contrast is evident after filtering. A comparison of the slices through the reconstruction obtained from the unfiltered and the filtered series is shown in Figure 3. There is a significant improvement in resolution in the energy-filtered reconstruction, but the unfiltered reconstruction gives a surprisingly good result considering that the sample thickness is four inelastic mean free paths or 0.6 µm thick at the 60° tilt. One problem with energy-filtering is that 98% of the electrons are inelastically scattered for a 300 nm thick specimen that is tilted to 60°, which increases the required dose leading to more damage.

References

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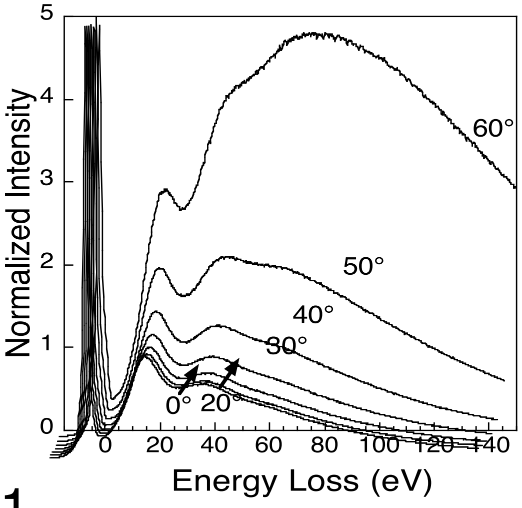
Table 1. Measured relative thickness (t/□) as a function of tilt angle obtained from the spectra in Figure 1.

Tilt Angle (□°)	1/cos(□)	Nominal Thickness in Beam Direction (nm)	Measured t/□
0	1.000	300	1.921
10	1.015	305	1.933
20	1.064	319	2.018
30	1.155	345	2.167
40	1.305	392	2.471
50	1.556	467	2.974
60	2.000	600	3.975



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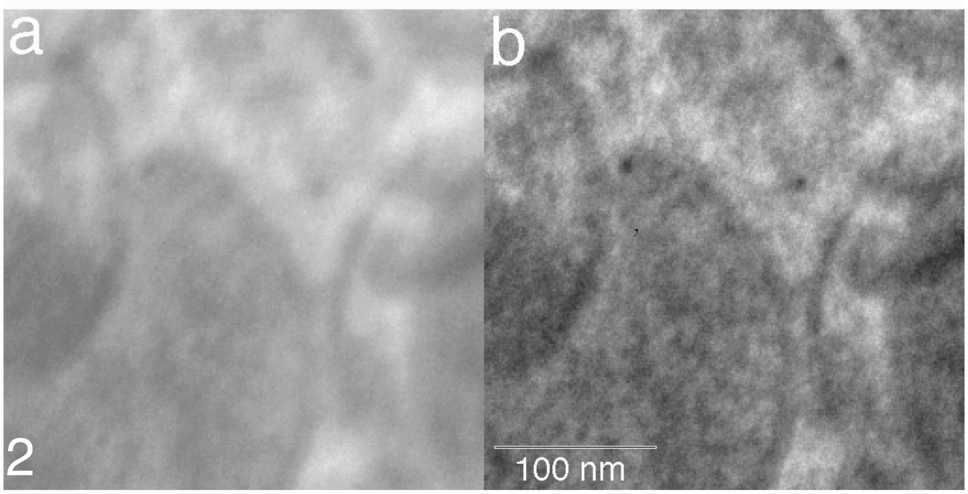

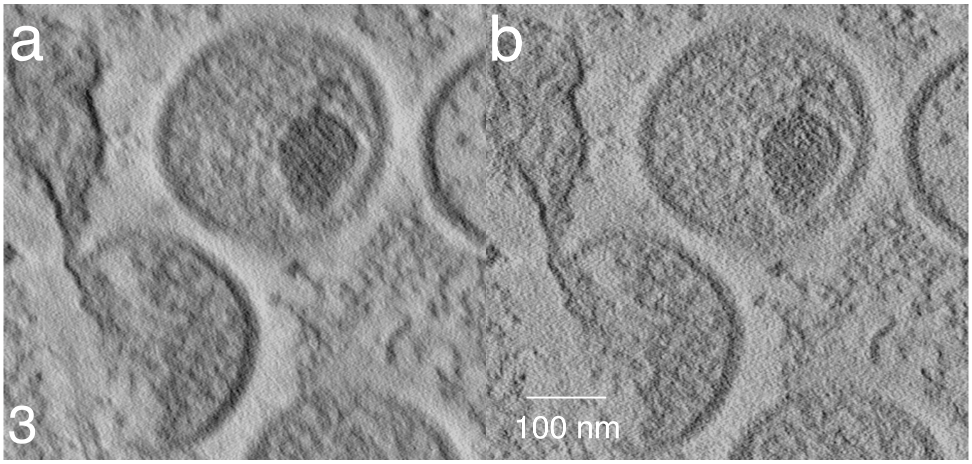

FIG. 1. Energy loss spectra recorded at 120 kV beam voltage from 300 nm thick plastic section of BSC-1 cells at tilts from 0° to 60°.
FIG. 2. Unfiltered and filtered micrographs of stained plastic section tilted to 60°.
FIG. 3. Slice of thickness 1.3 nm through reconstruction of vaccinia viruses in infected BSC-1 cells from energy-filtered tilt-series of ~300 nm thick section.