doi:10.1017/S1431927610054048
Microsc. Microanal. 16 (Suppl 2), 2010

Requirements for Phase-Plate Cryo-Electron Tomography

M. Marko*,**, C. Hsieh*, A. Leith*, and C. Mannella*

*Wadsworth Center, Empire State Plaza, Albany, NY 12201
** College of Nanoscale Science and Engineering of the University at Albany, 251 Fuller Rd., Albany, NY 12203

Phase-plate imaging is particularly beneficial for cryo-electron tomography. The resolution of tomographic reconstructions of vitreously frozen biological specimens is limited by the total electron dose required to collect a tilt series, thus individual images are noisy due to low electron dose. Phase-plate imaging improves image contrast, by as much as five times at certain spatial frequencies [1]. Thus, the electron dose per image can be reduced, allowing the recording of more images and consequently better resolution in the reconstruction. Alternatively, if the electron dose per image is not reduced with phase-plate imaging, lower-contrast features become more visible. An additional advantage is that images can be recorded close to focus when using a phase plate, avoiding the high-underfocus typically used for cryo-tomography,. As a result, the transfer of information is excellent over a wide range of spatial frequency, greatly improving the fidelity of representation of the 3-D mass density in the sample.

For the near future, the Zernike phase plate (ZPP) is the most easily implemented type; its use is detailed in Danev et al. [1]. The ZPP, placed in the objective aperture (objective lens back focal plane), has a small central hole to pass electrons not scattered by the specimen, while most of the scattered electrons pass through the film and are phase-shifted, usually by $\pi/2$.

The "cut-on frequency" of a phase plate (d) is the spatial frequency below which information is not transferred by phase contrast. It is determined by $d=f(\lambda/r)$, where f is the focal length of the objective lens, λ is the electron wavelength (determined by the accelerating voltage), and r is the radius of the central hole in the phase plate. It is recommended that the cut-on frequency correspond to twice the size of the largest particle or feature to be imaged [1]. The minimum diameter for the central hole in a ZPP is about 0.3 μm for an FEG TEM, and about 0.7 μm for a LaB_6 TEM. Thus, one can calculate the appropriateness of an existing TEM for ZPP work with a particular specimen. In the case of thin vitreous sections of cells, a cut-on frequency corresponding to 15 nm is adequate for imaging membrane boundaries with high contrast. This spatial frequency is possible with many existing cryo-TEMs.

Current carbon-film ZPPs are subject to contamination in most TEMs, unless they are heated to 150-200°C. In addition, the ZPP has to be accurately centered on the unscattered beam. Thus, a modified objective aperture holder with heating and extra-fine centering controls is desirable. The phase plates themselves are made by applying a carbon film to an aperture and then milling a small central hole using a focused ion beam. For a phase shift of $\pi/2$, the carbon-film thickness should be about 15-30 nm, depending on the accelerating voltage of the TEM (100-400 kV)[1].

The quality of the ZPP should be checked in the TEM before use. In most cases, the carbon film charges to some extent, even when freshly made. In mild cases, the effect of charging is simply to shift the CTF slightly, which can be compensated by a focus adjustment. The easiest way to determine the proper focus offset is take a focal series of images of a carbon-film specimen over a range of about +/- 500 nm, and pick the defocus at which the Fourier power spectrum drops off only

gradually from the cut-on frequency to the edge of the image. If a CTF dip is observed over this entire range, charging is probably excessive and the ZPP should not be used.

Alignment of the TEM is more critical for ZPP work than it is for routine microscopy. The objective aperture should be at the height in column corresponding to the back focal plane when the objective lens is operated at optimal current. In order that the unscattered beam-spot be as small as possible at the ZPP, the illumination on the specimen should be as parallel as possible (parallel illumination, and use of a small "spot size" setting of the condenser, improve the envelope function of the EM, so are recommended in any case). With some TEMs, a compromise will be necessary, and the illumination may not be very close to parallel when the beam-spot is minimized at the ZPP. The unscattered beam must be precisely centered on the ZPP central hole, primarily by mechanically centering the aperture. A small amount of beam tilt can be used for "fine tuning", but excessive beam tilt will result in coma and degrade imaging at high resolution.

During low-dose imaging and tilt-series collection, the beam is blanked and unblanked many times. In the typical CCD camera installation, the beam-blank function applies a signal to a gun deflector via an optocoupler (solid-state relay). When using a ZPP, the beam must return to within about 10 nm of the center of the ZPP, but the default method of beam blanking may not be reproducible enough. In that case, it may be necessary to use the TEMs internal computer control for beam blanking.

With modern TEMs that have goniometers with good eucentricity (not more than a few micrometers focus change or image shift over the full tilt range), academic tilt-series collection systems such as SerialEM [2] and Leginon Tomography [3] can be adapted to include automated phase-plate centering. The position of the ZPP central hole can be observed by temporarily defocusing the final condenser lens, and image cross-correlation can be used to find the direction and amount of shift to be applied, by piezo control and/or beam tilt.

If the goniometer eucentricity is poor, a large amount of electronic image shift is needed to track the specimen during the tilt series. This results in an inconsistent correspondence in ZPP centering between the off-axis centering condition (e.g. "Focus") and the on-axis imaging condition (e.g. "Exposure"). In this case, or if fully automated software is not installed, a robust semi-automated tilt-series collection method can be used. In this method, focus, specimen-tracking and ZPP-centering operations are carried out manually and on-axis; however, on-axis imaging is carried out at 1/10th the "Exposure" magnification (i.e., 1/100th of the electron dose rate). A simple automation script can be written to cycle the TEM through the different settings of the lenses and deflectors, to tilt the specimen stage and to capture serial images.

References

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[4] Supported by NIH / NCRR Biomedical Research Technology Program Grant RR01219 (P.I . C. Mannella).