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Methods for testing Zernike phase plates and a report on silicon-based phase plates with reduced charging and improved ageing characteristics

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Abstract

Imaging with Zernike phase plates is increasingly being used in cryo-TEM tomography and cryo-EM single-particle applications. However, rapid ageing of the phase plates, together with the cost and effort in producing them, present serious obstacles to widespread adoption. We are experimenting with phase plates based on silicon chips that have thin windows; such phase plates could be mass-produced and made available at moderate cost. The windows are coated with conductive layers to reduce charging, and this considerably extends the useful life of the phase plates compared to traditional pure-carbon phase plates. However, a compromise must be reached between robustness and transmission through the phase-plate film. Details are given on testing phase-plate performance by means of imaging an amorphous thin film and evaluating the power spectra of the images.

Keywords

Phase plate; Zernike; CTF; image contrast; cryo-EM; cryo-tomography

1.0 Introduction

In the TEM, a phase plate is used to introduce an additional phase shift between unscattered electrons and the electrons that are elastically scattered by the specimen. Therefore, the TEM does not need to be defocused in order to create strong phase contrast, and the oscillations in the contrast-transfer function (CTF) caused by defocus are avoided. As a result, excellent, nearly-uniform contrast transfer is obtained over a very wide spatial-frequency range. The contrast increase at low spatial frequencies is especially important for

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cryo-electron tomography, and for alignment of small or heterogeneous molecules in single-particle cryo-EM.

The usefulness of in-focus TEM phase-plate imaging has long been appreciated, and has recently been affirmed (Malac et al, 2008; Chang et al., 2010; Hall et al., 2011), but practical problems with its implementation for “real-world” use have hindered widespread adoption (see reviews by Nagayama and Danev, 2009; Danev et al., 2009; Danev and Nagayama, 2010; Nagayama 2011). Nevertheless, the current state of development has enabled early applications to biological problems, validating the technical advantages of phase-plate imaging (Danev and Nagayama, 2008; Fukuda and Nagayama, 2012; Rochat et al., 2011; Hosogi et al, 2011; Danev et al., 2010; Murata et al., 2010; Shigematsu et al., 2010; Yui et al., 2008; Yamaguchi et al, 2008).

Many types of phase plates have been proposed, and most of them have already yielded good results on test samples (reviews cited above, also Malac et al., 2010; Buijsse et al., 2011; Barton et al., 2011; Hettler et al., 2012; Edgecombe et al., 2012; Kuo et al., 2013). However, only the thin-film type of phase plate is at a sufficiently mature state of development to be used for practical biological applications. Of these, the “Zernike” configuration, placement of a thin film with a central hole in the TEM back focal plane (the objective aperture position), is most often used.

The most serious obstacle in Zernike phase-plate imaging is “ageing” of the phase plate (Danev et al., 2009; Marko et al., 2011). Ageing is thought to reflect charging of the phase-plate film, causing the phase plate to act as a lens that changes the CTF, resulting in an incorrect phase shift or a seriously compromised CTF. It is generally believed that charging is caused by non-conductive contaminants (probably involving oxygen) on the phase-plate film, which are either present from the start, or which build up during use in the TEM. Heating the phase plate can help drive off contaminants (or reduce their build-up), but does not completely solve the problem.

Phase plates that lack a thin film, such as electrostatic types (Cambie et al., 2007; Majorovits et al., 2007; Hettler et al., 2012) or a partially blocking type (Buijsse et al., 2011) may not avoid ageing, since they can also become contaminated. Phase plates with no material intruding into the diffraction pattern in the objective-lens back focal plane (or in a conjugate plane), such as the anamorphic (Schröder et al., 2007) or the ponderomotive-force (Müller et al., 2010) types may solve the problem, but such devices have not yet been realized in a practical TEM. Therefore, as an interim solution, Zernike phase plates that are less subject to ageing are critically needed.

Current carbon-film Zernike phase plates are expensive if purchased, or time-consuming to make, and they usually have a short lifetime. Our silicon-based phase plates are easier to make and more reproducible, longer lived, and could be mass-produced to be available at a moderate cost.

2.0 Methods

2.1 Silicon-base characteristics

Phase plates were created by using a focused-ion-beam instrument to mill a small central hole in a thin amorphous silicon film supported in a thick mono-crystalline silicon frame. As manufactured, the films are completely free of oxygen; however over time with exposure to room air, they likely acquire a layer of SiO₂ about 1 nm thick on both surfaces. The phase-plate silicon-frame “blank” is very similar to the silicon TEM grids available from TEMWindows (TEMWindows.com). For the present work, we designed chip sets

containing 42 discs, each 2 mm in diameter and 0.1 mm thick (Fig. 1). Each 2-mm disc had 16, 50×50-μm square, thin “windows” in which the phase plates are made. These discs are suitable for the holders supplied with our JEOL (Akishima, Japan) TEMs. However, disks could be made of any diameter, or a rectangular strip could be made, to fit any objective aperture holder or phase-plate holder.

We experimented with silicon films of 5 and 15 nm thickness. Our task was to find over-coatings that gave the correct $\pi/2$ phase shift for a given TEM accelerating voltage, while maximizing operational life and electron transmission through the film.

2.2 Focused-ion-beam milling

Silicon chips were held in a custom sub-stage (Fig. 1A) in a Zeiss (Oberkochen, Germany) Neon 40 EsB FIB-SEM. Central holes, 700 nm in diameter, were made in the individual phase-plate films, using a 30 keV gallium ion beam with a current of 1 pA and a milling time of 20 sec. (independent of the silicon-film thickness). In future, the making of the central hole will be transferred to a photolithographic process, substantially reducing the fabrication cost. Handling will also be reduced, which is expected to improve the performance consistency across all phase plates on the disc.

2.3 Phase-plate preparation and coating

Immediately before coating, the silicon chips were cleaned by plasma sputtering for 90 seconds on each side in Fischione (Export, PA) Model 1020 plasma cleaner. Pure argon gas was used in an effort to avoid oxygen. The sample was placed deep in the chamber, within the region of the RF coil, and the plasma was tuned for maximum using a Bird (Solon, OH) Model 43 wattmeter. With this method, the cleaning action was sputtering rather than conventional plasma-cleaning. We expected this treatment to remove most of the SiO₂ layer, although we have not yet analyzed the films to confirm it. We also tried pure-argon plasma-cleaning using a compact plasma cleaner (PDC-32G, Harrick Plasma, Ithaca, NY), which we carefully cleaned and then pumped only with an oil-free turbo pumping station. Phase plates that had no initial charge could not be produced with the compact 18-watt plasma cleaner (even after cleaning for 10 min), and required use of the 40-watt Fischione plasma cleaner, even though both plasma cleaners used RF coils operating at about the same frequency.

Coating was performed in a South Bay (San Clemente, CA) ISBe dual-argon-ion-beam sputter coater, using target materials from the same supplier. The system was flushed with pure argon before use. Sputter-coating was done individually on each side, at 8 keV and 6 mA at a pressure of 6×10^{-6} Torr. The stage was rotated continuously while tilted to 40° during coating, so that the sides of the “wells” in the silicon frame would also be coated for good conductivity. The coating time varied between 5 and 20 minutes, depending on the material and the thickness desired. The thickness was measured by a crystal thickness monitor, individually calibrated for the material to be used. When multiple-material layers (e.g. aluminum, then carbon) were coated, the sample remained at high vacuum between coatings.

We did not do any elemental analysis of the phase-plate layers, either individually or as the completed phase plate. We did not characterize the state of the amorphous carbon-film overcoat, but we did observe that phase plates were initially charged in the TEM when they had been exposed overnight or longer to high room humidity. This may be due to the porosity of the carbon over-coat. The initial charging could be reduced -- but not eliminated -- by heating in the JEM-3200FSC/PP TEM vacuum for several hours at 300°C. The carbon films that performed best were light in color, sputtered at 10^{-6} Torr, rather than dark in

color, sputtered at 10^{-5} Torr, in spite of the likelihood that the former were diamond-like and thus might be expected to be less conductive. Detailed analysis of the phase-plate layers will be the subject of a future paper, which will also include statistics on lifetime and shelf-life in our laboratory and in other laboratories.

As shown in Figure 2, the phase-plate film is uniform. Fig. 2A was recorded with very high defocus phase-contrast (“off-plane condition”) such that “Berriman footprints” would be visible, as shown in Marko et al., 2011. Since contrast of these footprints is due to charging, not build-up of contamination, such images show that there are no islands of charging. Fig. 2B shows an in-focus image of the phase-plate film revealing no contaminants that give strong amplitude (scattering) contrast. A further indication of the uniformity of the phase-plate film is that, even in the case of phase plates that did show initial charging, the power spectra showed no distortions of the type that would come from islands of charge.

2.4 Testing for phase shift and charging

Immediately after coating, individual phase-plate discs were separated from the silicon chip and inserted into the objective aperture holder of a JEOL JEM-4000FX TEM equipped with a post-column energy filter and a LaB_6 cathode. Some phase plates that performed well were also used for imaging tests at 300 keV in a JEOL JEM-3200FSC/PP TEM.

The two important electron-optical parameters of a Zernike phase plate are the cut-on frequency and the phase shift (Danev et al., 2009; Marko et al., 2011). The cut-on frequency is determined by the size of the central hole in the phase plate, the electron-beam energy, and the objective-lens focal length. The phase shift is primarily determined by the effective thickness of the phase-plate film (its total inner potential) and the electron-beam energy. The effect of objective-lens aberrations, varied over the range of 1 to 10 mm found in any TEM in common use, is not of practical significance in determining the phase shift. Thus, by calculating the CTF (Fig. 3A) obtained when using a phase plate with a given total inner potential (as expressed by the equivalent thickness of a carbon film), we were able to evaluate phase plates for a correct phase shift when used at 200, 300 or 400 keV, by examining the power spectra of images recorded with our 400 keV TEM. In fact, as seen in Figure 3B, the CTF plots hardly differ between 400 and 300 keV, and are only slightly shifted at 200 keV. This reflects the wide tolerance for phase-plate thickness mentioned in Danev et al. (2009)

The defocus readout of the JEM-4000FX was calibrated by comparing the calculated CTF (Fig 3A “Conventional”) to the power spectrum of an image of an amorphous carbon-film specimen (imaged without a phase plate). A nominal underfocus setting of 1000 nm corresponded to an actual underfocus of 1250 nm. The long-term stability of the TEM with respect to focus was found to be excellent, with no detectable change in the minima of the power spectrum after two hours of periodic 3-sec irradiation of the carbon film specimen at $1600 \text{ e}^-/\text{nm}^2/\text{sec}$, simulating a phase-plate electron-dose-series test (see below). The carbon film used was that found between the holes in a Quantifoil (Jena, Germany) TEM grid, which is nominally 15 μm thick (however, Quantifoil GmbH notes that a thin residual layer of plastic overlays the carbon). Colloidal gold particles 25 nm in diameter were applied to the film as an aid both in focusing (using the image wobbler) and in phase-plate centering (by observing the fringe symmetry around the particle).

In both of our JEOL TEMs, the objective aperture holder is located at the true back focal plane of the objective lens. Thus, when the phase plate is in the “on-plane” condition, the illumination crossover is in the plane of the phase plate, and the illumination at the specimen is parallel. The JEM-4000FX was set for parallel illumination, using a 50 μm condenser aperture, with the spot size (first condenser lens) and gun bias adjusted to give a mean CCD

count of about 2100 (equal to 300 primary electrons) for a 3 sec exposure at 0.25 nm pixel size with respect to the specimen. Images were recorded with two-times binning on the Gatan (Warrington, PA) MegaScan 794IF/20 CCD camera of a GIF 2002 post-column energy filter, operated with the slit retracted. Power spectra were compared to a calculated CTF for a $\lambda/2$ phase shift, using a convenient Excel spreadsheet courtesy of Radostin Danev. The positions of the first five power-spectrum minima were recorded.

Any significant charging of the phase plate, as well as the effect of a focus change induced by the phase plate, is very apparent by examination of the power spectra. The effect of charging of the phase plate might be slightly less at 400 keV than it would be 200 or 300 keV, although the thinner films used at those lower energies might have less tendency to charge.

2.5 Phase-plate dose-toleration testing

Each image of the carbon-film specimen, as described above, represented an electron dose on the specimen of about $4800 \text{ e}^-/\text{nm}^2$. Imaging was repeated 30 times at approximately one-minute intervals. The spatial-frequency positions of the first five power-spectrum minima were plotted against the image number, representing the accumulating electron dose.

2.5 Phase-plate film transmission testing

The most accurate means of measuring phase-plate transmission requires placing the phase plate at the TEM specimen position (Danev et al, 2009). However this is inconvenient when many different phase-plate films need to be evaluated. Thus, we have adopted a simpler method, which yields a transmissivity value as a “figure of merit” that indirectly compares the relative electron scattering within the various films under test (see discussion).

With no sample in the specimen stage, the phase plate (while in place at the objective lens back focal plane) was brought into focus by using the objective minilens instead of the objective lens. Focus was judged by using the image wobbler and by observing the fringes of the phase-plate central hole. An image was recorded with a 5 eV energy-selecting slit inserted, and transmission through the phase-plate film was measured by taking the ratio of mean CCD counts over the film to that within the central hole (Danev et al., 2009).

While the transmission of a given phase-plate film will be less at 300 or 200 keV compared with 400 keV, the film needs to be thinner at the lower energies in order to provide the $\lambda/2$ phase shift. Thus the transmissivity values we determine at 400 keV reasonably reflect what would be obtained at the lower energies, with the material layers of the phase-plate film being proportionally thinner.

3.0 Results and discussion

Table 1 shows the compositions tested and summarizes the results.

3.1 Rationale for material selection

We decided to start our search for a good metal coating with the noble metals, known for their good electrical conductivity and poor reactivity. Fresh surfaces of these metals immediately form a monolayer of oxygen (not an oxide), and then remain unreactive unless heated to high temperature (www.encyclopedia.com). Of these, platinum has long been used to sputter-coat samples for high-resolution SEM, so we expected a fine-grained, uniform coat from these elements. We first tried iridium, the most corrosion-resistant metal known, and then rhodium because it has a lower atomic number (resulting less electron scattering). We also tried chromium, because of an earlier report of its use (Irsen et al., 2012). In

addition, we tried an experimentally applied proprietary carbon-based coating, as well as sputter-coated carbon. We also tried aluminum which, if not for the immediate formation of a thick oxide coat in air, would have been an excellent choice for a Zernike phase plate (Danev and Nagayama, 2001). We prevented oxide formation by over-coating with carbon without exposing the aluminum to air. A final amorphous carbon coat may also have an advantage since it is not expected to form an oxygen layer thicker than a monolayer on its surface.

3.2 Phase shift

For thin biological specimens (which do not have a strong intrinsic phase shift), a phase-plate phase shift of $\pi/2$ (i.e. 90 degrees) is optimum. With a Zernike phase plate, the inner potential of the thin film shifts the phase of the electrons that are scattered by the specimen. The plot in Figure 3A shows our standard for testing phase plates: Nominal 1000 nm underfocus (actual 1250 nm); 400 keV; effective phase plate thickness as for 32 nm of pure carbon. A CTF plot under the same conditions but without a phase plate is shown for comparison. The spatial frequencies of the first five CTF zeros are used as the standards of comparison for the phase plates under test.

The inner potential of the phase-plate film varies with the thickness and the elemental composition. For a carbon-film phase plate the thickness should be 32 nm for 400 keV, 31 nm for 300 keV, or 27 nm for 200 keV (Danev et al., 2009). Calculated CTF plots for images recorded with phase plates of these three thicknesses, if imaged at 400 kV in our JEM-4000FX, are shown in Figure 3B.

Figure 4 shows power spectra of images of amorphous carbon films recorded at a nominal 1000 nm underfocus, representing phase plates with slightly excessive, correct, and slightly insufficient phase shifts for a $\pi/2$ phase plate. For comparison, a power spectrum from an image recorded with no phase plate is shown, demonstrating the 90-degree phase shift and the increased transfer at low spatial frequencies. Slight phase-shift variation is typical among the 16 phase-plate “windows” in a given 2-mm disc. In the case of our current best composition (Table 1, Type 22), 8 to 10 of the 16 windows typically had the correct phase shift, but most were useable.

3.3 Charging

Charging is evaluated by observing the power spectra from images of an amorphous carbon film. Charging is considered present if (1) the positions of the power-spectrum minima at a standard underfocus (nominally 1000 nm) do not fall where they should based on the defocus setting and the electron-optical parameters of the TEM; (2) the objective lens setting for the in-focus condition is not the same with or without the phase plate in the back focal plane; or (3) there are artifacts at low spatial frequency, near the cut-on frequency.

After finding that an iridium coating provided good resistance to charging, we tried rhodium, also a noble metal, since is a lower-z material and would contribute less to unwanted scattering of electrons in the phase plate. Rhodium was found to be as effective as iridium in reducing charging. About half of the films coated with iridium or rhodium showed no charging, while the others showed slight charging evidenced by low-frequency artifacts (as in Fig. 4D) or subtle changes in the expected positions of the power-spectra minima.

Gold sputter-coating, an attempt to deposit an oxidation-resistant protective second coat over rhodium, was very uneven, so the phase plates could not be properly evaluated. The traditional carbon phase plates preformed properly at first, but quickly deteriorated during use, as observed by most other users. Silicon films coated with chromium, or an

(undisclosed) carbon material, or uncoated films of silicon with n-type doping all showed severe charging, as shown in the power spectra in Figure 5A,B,C.

In general, the effects of charging that we observed did not change over time (weeks) in the TEM or during prolonged electron irradiation. Sometimes the central artifacts in the power spectrum (Fig. 4D) were reduced or eliminated during irradiation.

3.4 Electron Irradiation of the phase-plate film

It is of interest to estimate the number of electrons incident on the phase-plate film when imaging the amorphous-carbon specimen during an electron-dose test.

When in the “on-plane” (parallel illumination) condition, the illumination on the specimen was 1.6 μm in diameter, however the size of the unscattered beam on the phase plate was about 500 nm in diameter, as determined by the “Berriman effect” as shown in (Marko et al., 2011). This is crossover-spot size of the JEM-4000FX as operated in the on-plane condition for this test.

The total beam current below the specimen was 9.6 nA (measured by a Faraday cage). Thus for the 3-sec exposure, not more than 6×10^{10} electrons could fall on the phase plate film. If all these electrons were in the unscattered beam, with 500-nm diameter, the electron dose would be $9 \times 10^5 \text{ e}^-/\text{nm}^2$. However, during imaging, most of the electrons pass through the central hole of the phase plate, and the phase-plate film is only briefly irradiated the small, intense beam during alignment.

The electrons which the specimen forward-scatters away from the central beam fall on the phase-plate film, with a generally decreasing radial density from the center. The ratio of all the electrons in the post-specimen beam to those which could fall on the phase-plate film (when the phase plate is properly aligned on the central beam) was estimated by recording an electron diffraction pattern of the amorphous carbon-film specimen, with a camera length (30 cm) sufficient to cover the angular range of scattering over which a signal could be detected. We found that the ratio of the electrons that could fall on the phase-plate film at the back focal plane, to the total electrons exiting the specimen, was about 0.28.

3.5 Electron-dose tolerance and ageing

In routine use of carbon-film Zernike phase plates, in-focus images with a good phase plate appear sharp, and as the phase plate ages, the images become blurry. Although we did not observe such a change with our phase plates, we could compare in-focus images of the same region of an amorphous film, using phase plates of different quality (Fig. 6). The quality of images from our successful phase plates does not change during prolonged electron irradiation, as seen in Figure 7. If a phase plate initially performs somewhat poorly (as in Fig. 6 C,D), the same (low) quality persists throughout the electron irradiation test.

Figure 7A shows representative electron-dose tolerance results with our “best” phase-plate composition, a 15-nm silicon core coated on both sides with 2 nm of aluminum and then 6 nm of carbon. Such behavior was also observed with 5 nm of iridium, 2 nm of rhodium, or 1 nm of rhodium on both sides of a 5-nm silicon base. Note that at each of the 30 steps of the electron dose series, the specimen had an *additional* electron dose of $4800 \text{ e}^-/\text{nm}^2$, each step considerably greater than that used in a single single-particle cryo-EM image (1000 to 3000 e^-/nm^2), and far greater than that used for an individual member of a cryo-electron tomography tilt series (about $100 \text{ e}^-/\text{nm}^2$ per image).

In some cases, the minima in the power spectrum shifted slightly (by a final shift of about 0.1 nm^{-1}) to lower spatial frequencies during an exposure series. If the series was repeated,

but with the TEM's anticontaminator cooled, the shift did not occur; the lower anticontaminator surface was about 0.2 mm from the upper surface of the phase plate. Such a shift in the power-spectra minima would require build-up of a layer of ice or hydrocarbons about 10 nm thick, which is not likely; a much thinner, but charge-prone, layer may have built up. Nevertheless, deterioration in the image and power spectrum, as represented (although not observed) in Figure 6 did not occur.

The characteristics of phase plates with sufficiently thick coatings of iridium or rhodium, or of aluminum with a carbon coating, remained the same for at least three weeks of equivalent use in the TEM for routine single-particle or tomographic data collection, even without heating. Shelf life was at least three weeks. Shelf-stored phase plates were always argon plasma-cleaned immediately before introducing into the TEM, otherwise phase plates were stored in a vacuum desiccator that had been evacuated with an oil-free pumping station.

3.6 Transmissivity

The drawback of coating with a relatively high-*z* metal is that scattering in the phase plate is increased, thus decreasing the contrast improvement of in-focus phase-plate imaging. In this regard, pure-carbon Zernike phase plates performed best, as shown in Table 1. Our measurement of transmissivity, while unconventional, gave a result for the carbon film consistent with that reported by Danev et al. (2009). We believe that for many applications, in particular for electron tomography, the advantages of our best-performing phase plates can outweigh the decrease in transmissivity. Our best example had a transmissivity of 70%, compared to 80% for a pure-carbon phase-plate film.

With respect to electron scattering within the phase-plate film, the electrons that we are most concerned with are those which are elastically scattered by the phase plate (whether also scattered by the specimen or not) and which therefore result in an increased noise level in the image. All electrons that are scattered inelastically (whether from the specimen or the phase plate) can be removed by the energy filter.

Nevertheless, our transmissivity measurement (intensity ratio of film to empty hole) reflects primarily inelastic scattering. When a 20- μ m aperture at the standard "selected-area" position was introduced, the apparent transmission typically decreased by about 14%, indicating cut-off of electrons scattered at higher angles. However, when a 5-eV-wide energy-selecting slit was introduced instead, transmission typically decreased by about 30%, and inserting the selected-area aperture had no effect. Therefore, we did all our measurements with the 5-eV slit alone.

By comparing unfiltered and energy-filtered electron diffraction patterns, we estimated the ratio of elastic to inelastic scattering outside the central beam to be about 0.39. This is consistent with estimations from data shown in Danev and Nagayama, (2001), yielding an approximate value of 0.30 for C, Si, and Al, rising to about 0.35 for Ir. Thus, although we do not directly measure the amount of elastic scattering in the phase plate film, our transmissivity measurement is a reasonable "figure of merit" for the present purpose.

3.7 Comparison images

Figure 8 shows the practical performance of our current most-favored silicon-based phase plate type (Table 2, type 22). Actual measurement of the performance of the new phase plates, with specimens of various types, and in both LaB₆ and FEG-equipped TEMs at 200, 300 and 400 keV, will be presented in a subsequent report.

4.0 Conclusions

We found that phase plates made on a silicon base can perform well, with reduced charging characteristics and improved performance under prolonged electron irradiation compared to traditional pure-carbon phase plates. Note that these tests were done in a conventional TEM, without a heated phase-plate holder. It might be expected that our phase plates would last even longer in a TEM specially equipped for phase-plate use.

Silicon films provided with a layer of noble metal (e.g. Ir and Rh) at least 2 nm thick performed well with respect to charging and longevity, but when the films were thick enough to provide the correct $\pi/2$ phase shift, the transmissivity (due to scattering in the phase-plate film) was considerably reduced compared to pure-carbon phase-plate films.

We found that use of a lower-z conductor (aluminum), when protected from excessive oxidation by carbon-coating, provided the same level of longevity and freedom from charging as the noble-metal-coated films, but had improved transmissivity. We believe that the 10% reduction in transmissivity compared to pure carbon is an acceptable trade-off for more robust performance.

It is also of considerable importance that silicon-based phase plates can be mass-produced at low cost, since current Zernike phase plates require costly equipment and careful work to make, or are expensive to purchase.

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Figure 1.

Phase-plate construction. A. Sub-stage for mounting a silicon chip in the FIB-SEM. Each chip can contain 42 2-mm-diameter phase-plate discs. B. SEM of two phase-plate discs. Each of these discs has 25 “windows” (we normally use 16) containing a thin silicon film, in which a central hole is FIB-milled. C. High-magnification SEM showing the texture of a final carbon coat near the edge of a phase-plate central hole. This represents the surface of our current best candidate for a Zernike phase plate; the silicon film is coated with 2 nm of aluminum and then 6 nm of carbon.

Figure 2.

Uniformity of the phase-plate film. A. Image of the central-hole region of a typical Si-Al-C phase plate, taken at the “off-plane” setting of the final condenser lens. This image has very strong defocus phase contrast, such that subtle “islands” of charge would be seen, if they existed (Marko et al., 2011). B. In-focus image of the central-hole region of the same phase plate. This image reveals only amplitude (scattering) contrast. The phase-plate film is slightly wrinkled, as can be seen from the wide, diagonal slightly darker streaks, but no islands of contamination are evident.

Figure 3.

Reference CTF plots used for testing phase plates. A. CTF plots representing imaging with a $\lambda/2$ phase-plate and with conventional imaging, using our JEM-4000FX at 400 keV and a nominal underfocus setting of 1 μm . The phase shift of phase plates under test is evaluated by noting the positions of the power-spectra minima from images of amorphous films, and comparing those to the calculated CTF zeros (shown at dots). B. Amorphous-film imaging at 400 keV can be used to confirm the correct phase shift for thinner phase plates to be used at 300 and 200 keV. The CTF plot shifts very little with thinner phase plates.

Figure 4.

Phase-shift determination from power spectra of images of amorphous carbon films at 400 keV, with a nominal underfocus of 1 μm . Individual phase-plate windows on a single 2-mm disc may vary in their phase shift to the extent shown here. A. Slightly too much phase shift -- the first minimum occurs too close to the center of the pattern. B. Correct phase shift -- the spatial frequencies of the minima match the calculated CTF. C. Slightly insufficient phase shift -- the first minimum occurs too far from the center of the pattern. Nevertheless, all three phase plates (A-C) would be useable, and would require only a slight adjustment in defocus. D. Power spectrum without phase plate -- the vertical lines demonstrate the 90° phase shift relative to the example of a true $\pi/2$ phase plate. The low intensity at the center

of the pattern (arrow) corresponds to the loss of low-spatial-frequency information, as indicated in Fig. 3A “Conventional”.

Figure 5.

Examples of excessively charged phase plates. Power spectra of images of amorphous carbon films at 400 keV, with a nominal underfocus of $1\mu\text{m}$. Note that with severe charging the spacing of the minima increases rather than decreases. A. Si core of 5 nm, with 7 nm Cr on both sides. B. Si core of 5 nm with 10 nm of experimental carbon material on both sides. C. Si film, 5 nm thick, with n-type doping. D. Si core of 15 nm with 5 nm Rh and then 5 nm C on both sides. In this pattern, the power-spectrum minima are in approximately the correct locations, but there is a region of poor transfer near the center of the pattern (arrow). This effect is sometimes observed from individual phase-plate windows in a disc containing otherwise good phase plates.

Figure 6.

Effect of charging with in-focus phase-plate imaging. A. A sharp image of an amorphous carbon film (with a 25-nm-diameter Au particle), as obtained with a good phase plate. B. The corresponding power spectrum shows good intensity at low spatial frequencies and a gradual, uniform radial decrease in intensity. C. The same amorphous film imaged with a suboptimal phase plate yields a slightly "blurry" in-focus image. D. The power spectrum has good low-frequency intensity but higher-resolution information is attenuated.

Figure 7.

Tolerance of silicon-based phase plates to prolonged electron irradiation. A. Plots of the minima of power spectra from images of an amorphous carbon film specimen. At each image number, the specimen received an additional $4800 \text{ e}^-/\text{nm}^2$. The shift in spatial frequencies of the first five minima is shown. B, D. Image and power spectrum of image 10. C, E. Image and power spectrum of image 30. Note that the fine texture of the carbon-film specimen remains the same (e.g. as in Fig. 6a) and that there is no significant change in the power spectra.

Figure 8.

Phase-plate image comparison, using phase plate Type 22 from Table 1, recorded at 300 keV in the JEM-3200FSC/PP, with a 20-eV energy-selecting slit, and a phase-plate cut-on periodicity of 28 nm. The electron dose was $140 \text{ e}^-/\text{nm}^2$ for each image; the total dose for (C) was $420 \text{ e}^-/\text{nm}^2$. Spirochete bacteria courtesy of Justin Radolf. (A) In-focus without phase plate, (B) Underfocused $15 \mu\text{m}$ without phase plate, (C) Near focus with phase plate. In (C), note better visibility of the peptidoglycan layer (short arrows) and much clearer representation of apparent fine filaments (upper long arrow) and a flagellum (lower long arrow).

Summary of testing results on coating of silicon-based phase-plates. Comparisons can be made to each other and also to a traditional pure-carbon-film phase plate (Type 1). At least two samples of each type were made. For these tests, 2-mm silicon discs having 16 50- μ m-square windows were used. The characteristics of the phase plates in individual windows always varied somewhat. Types with appropriate phase shift are in bold; while all these types also have good dose tolerance, many have poor transmission compared to pure-carbon films. The best compromise is Type 22.

Table 1

Type	Core	First Layer, per side	Second Layer, per side	Transmission	Phase Shift	Charging	Dose Tolerance
1	32 nm C	---	---	80%	Most correct	Moderate	Poor
2	15 nm Si	---	---	86%	Undetermined	Moderate	Undetermined
3	5 nm Si	10 nm C *	---	Undetermined	Undetermined	Severe	Undetermined
4	15nm Si	7 nm C	---	80%		Moderate	Undetermined
5	5 nm Si	7 nm Cr	---	65%		Severe	Undetermined
6	5 nm Si	(Si had n-type doping)		Undetermined		Severe	Undetermined
7	5 nm Si	3 nm Ir	---	55%	All too little	Slight	Undetermined
8	5 nm Si	5 nm Ir	---	38%	Most correct	Slight	Excellent
9	5 nm Si	2 nm Ir	3 nm Au	Gold coating excessively rough and uneven			
10	5 nm Si	1 nm Rh	---	75%	All too little	Slight	Poor
11	5 nm Si	2 nm Rh	---	69%	All too little	Slight	Undetermined
12	5 nm Si	3 nm Rh	---	60%	Most too little	Slight	Undetermined
13	5 nm Si	5 nm Rh	---	Undetermined	Some correct	Slight	Undetermined
14	5 nm Si	6 nm Rh	---	46%	Most correct	Slight	Very Good
15	5 nm Si	7 nm Rh	---	41%	Most too much	Slight	Undetermined
16	15 nm Si	3 nm Rh	5 nm C	42%	Most too much	Slight	Fair
17	15 nm Si	5 nm Rh	5 nm C	46%	All too much	Slight	Fair
18	15 nm Si	5 nm C	1 nm Rh	70%	All too little	Moderate	Undetermined
19	15 nm Si	2 nm Rh	4 nm C	64%	Most correct	Slight	Excellent
20	15 nm Si	2 nm Rh	5 nm C	62%	Most correct	Slight	Very good
21	15 nm Si	4 nm Al	5 nm C	61%	Most correct	Slight	Very good
22	15 nm Si	2 nm Al	6 nm C	70%	Most correct	Slight	Excellent

(*proprietary carbon coating)