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Practical Experience with Hole-Free Phase Plates for Cryo Electron Microscopy

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Abstract

Phase plate (PP) imaging has proven to be valuable in transmission cryo electron microscopy of unstained, native-state biological specimens. Many PP types have been described, however until the recent implementation of the “hole-free” phase plate (HFPP), imaging has been challenging. We found the HFPP to be simple to construct and to set up in the transmission electron microscopy, but care in implementing automated data collection is needed. Performance may be variable, both initially and over time, thus it is important to monitor and evaluate image quality by observing the power spectrum. We found that while some HFPPs gave transfer to high resolution without CTF oscillation, most reached high resolution when operated with modest defocus.

Keywords

phase plate; contrast-transfer function; cryo electron tomography (cryo-ET); cryo electron microscopy (cryo-EM); SerialEM

Introduction

In the past 10–15 years there have been numerous reports on the success of transmission electron microscopy (TEM) phase plate (PP) imaging, which is well-suited to low-dose cryo-TEM applications. Two of the most recent are Danev & Baumeister (2016) and Mahamid et al. (2016). Several types of PP that are potentially suitable for such applications have been described, as reviewed by Glaeser (2013). Until recently, the most practical PP design was the Zernike phase plate (ZPP; Danev et al., 2009), which typically consists of a

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Supplementary Movies 1 and 2

Supplementary Movies 1 and 2 can be found online. Please visit journals.cambridge.org/jid_MAM

thin carbon film placed in the objective-lens back focal plane. The ZPP has a small central hole for passage of the unscattered beam, whereas the inner potential of the film applies a $\pi/2$ phase shift to electrons scattered by the sample.

More recently, it has been found that a similar (and thinner) film, but without the central hole, can also be used as a “self-forming” PP (Malac et al., 2012; Danev et al., 2014). The hole-free phase plate (HFPP), also known as the Volta PP, has proved to be a more-practical alternative to the ZPP.

With the HFPP, a small patch forms on a thin (typically carbon) film as a result of electron irradiation. This patch shifts the phase of the unscattered beam relative to the electrons scattered by the specimen, again by the desired $\pi/2$ amount, and the phase shift remains stable over time (Malac et al., 2012; Danev et al., 2012). The size of the patch corresponds to the illumination crossover in the objective-lens back focal plane, typically only 20–100 nm in diameter. The edges of the patch are not abrupt; thus, as predicted in Danev et al. (2009), “ringing” fringes are not observed at the edges of extended objects. Once the patch is formed, it is only necessary that neither the beam nor the PP move during the imaging session. Thus, the need to initially center the unscattered beam on a small hole in the film is eliminated.

Materials and Methods

Any cryo electron microscopy (cryo-EM) lab should have the capability to make a suitable carbon film, so use of an HFPP only requires the installation of a heated objective aperture holder. Although both FEI and JEOL can now provide complete HFPP systems for their current-model TEMs, a PP retrofit could be adapted for any suitable TEM, as we have done for our JEOL JEM-4000FX (Marko et al., 2011), with the help of a competent machinist/engineer.

PP Construction

We evaporated amorphous carbon (from rods originally supplied by Fullam or Bal-Tec, and now supplied by Ladd Research Industries, Burlington, VT, USA), and used either a Denton Desktop Turbo vacuum evaporator (Denton Vacuum, Moorestown, NJ, USA) or a Bal-Tec (Leica) MED 020 evaporator, both with oil-free pumping systems. In the Denton, we used resistance heating, and estimated the film thickness by color. In the Bal-Tec, we used electron beam evaporation and measured the thickness by a crystal-thickness monitor. The film thickness was about 12 nm. The films, deposited on freshly cleaved mica, were floated off on distilled water and picked up on 2-mm-diameter discs that had a 5×5 array of 100- μm -diameter apertures (a gift from Kazuyoshi Murata, Okazaki, Japan, but available from Daiwa Techno Systems, Tokyo, Japan). We call these 25 holes the “PP apertures,” and during an imaging session, we think of each aperture as a different HFPP.

TEM

All the data were collected on a JEOL JEM-3200FSC/PP field-emission cryo-TEM (JEOL Ltd., Tokyo, Japan). The accelerating voltage was 300 kV and the beam current was 1.3 nA.

This TEM is equipped with two heated PP holders with computer-controlled micro-positioning, and each holder is capable of heating the PP to 500°C. For this work, the PP temperature was set to ~260°C, and the PPs were heated continuously. One PP holder operates in the objective-lens back focal plane and the other in a conjugate focal plane formed by a modified objective minilens that acts as a transfer lens. The objective-lens focal lengths are 5 and 15 mm, respectively. The cryo specimen stage was normally operated at ~100 K. The stage has a tilt range of 140° and has a long-range piezo control along the tilt axis, which shifts the specimen for the off-axis focus and tracking needed for low-dose cryo-TEM. There are separate anticontaminators (“cryo boxes”) around each PP and around the specimen. The anticontaminators were kept 10–20 K colder than the sample. Although this TEM is specially configured for use with several different types of PP, not all features are needed when used with the HFPP. Images were recorded on a Gatan K2 Summit direct-electron detector and, in a few cases, a TVIPS F-416 CMOS camera (TVIPS, Gauting, Germany).

Samples

HFPP performance tests were based on imaging (nominally) amorphous carbon films in the normal specimen position. We used the thick (~12 nm) carbon film that surrounds the holes in commercial specimen supports (Quantifoil Microtools, Jena, Germany).

Tomographic tilt series were recorded from plunge-frozen bacteriophage T4 samples (a gift of F. Maley, Wadsworth Center), over film-free holes in Quantifoil R2/2 or R2/4 grids. Before plunge-freezing, the T4 samples were mixed with a suspension of 10-nm colloidal gold particles, to serve as alignment fiducials.

Images were also recorded from samples of plunge-frozen tobacco mosaic virus particles (a gift of R. Diaz-Avalos, HHMI Janelia Farm).

Data Collection

PP performance for use in low-dose, high-resolution imaging was tested by imaging the Quantifoil film and observing the power spectra of the images. Quantifoil grids, as received, have a minor amount of plastic surface contamination. This contamination is not visible without use of a PP, thus it serves as a quick indication of a functioning PP. The carbon film for the PP was 12 nm thick and heated to 260°C. For these tests, the pixel size on the specimen was 0.74 Å, and the dose rate on the K2 camera (Gatan Inc.) was 6.5 electrons/pixel/s. A 3 s exposure with 0.1-s frames was used, for a specimen dose of ~35 e⁻/Å². Frame alignment and summing was done in Gatan Digital Micrograph (although there was insignificant specimen drift). Some PPs were also used for imaging at 6.8 Å pixel size to specifically assess the low-frequency transfer for tomography applications.

Comparison of using an HFPP in the normal objective-lens back focal plane versus the magnified conjugate plane was done under the same conditions. The separate anticontaminators around each PP, as well as that around the specimen, were all used.

For tomographic tilt-series collection, a specially modified version of SerialEM (Mastronarde, 2005) was used, as described below. Tomographic tilt series were recorded

close to focus at 2° intervals over a 120° tilt range. Images were recorded at 300 keV with zero-loss energy filtering, slit width 20 eV. The pixel size (after 2 × binning) was 6.8 Å and the total electron dose was ~80 e⁻/Å². The exposure per “movie” frame was 0.2 s, and five to seven such frames were recorded at each tilt. The frames were aligned and summed within Gatan Digital Micrograph (SerialEM “Align in DM”). In any case, drift was minimal. The ice thickness was 150 nm (as measured by electron energy-loss t/λ and confirmed by the tomographic reconstruction). For testing the effect of PP heating, images were recorded as above, but without specimen tilting or PP heating.

Results and Discussion

Here, we do not attempt to add to understanding of the underlying mechanism of the HFPP, but only to provide an example of how we use the HFPP for cryo-TEM. The benefits of imaging with the HFPP are illustrated in Figure 1.

Formation and Characterization of the Phase-Shifting Patch

The phase-shifting patch should be made as small and round as possible. This is accomplished by imaging a particle of debris on the carbon film of a PP aperture while the TEM is in the normal imaging mode, but with the condenser lens at an off-plane setting. As the condenser is adjusted to approach the on-plane condition (i.e., crossover in the objective-lens back focal plane), the particle appears to become larger, and its shape may change if the condenser astigmatism is not corrected. The on-plane condition is reached when the particle size increases such that it is no longer visible. This adjustment is rather critical. If the HFPP is truly in the objective-lens back focal plane, this procedure yields perfectly parallel illumination on the specimen, which is ideal for high-resolution imaging.

After performing HFPP set-up as the above, the result can be checked by imaging the PP film directly, using the low-magnification function of the TEM (objective-lens off, focusing with the objective minilens). When the film is imaged using several millimeters of underfocus, the patch is darker than the surrounding film; it is not visible when in focus, and when overfocused it is lighter than the surrounding film (Fig. 2a–2c). This behavior demonstrates that the patch is visible due to phase contrast and not to amplitude contrast, as would be the case if the patch were due to contamination (Danev et al., 2009; Marko et al., 2011). It is best to record results from this test using a camera that can tolerate high-intensity images (we used our TVIPS F-416 camera for this).

Our “on-plane” patch size is ~100 nm in diameter, according to JEOL’s specification of the minimum size of the illumination crossover in the objective-lens back focal plane with our particular TEM. The expected patch sizes, both for the normal mode and for “transfer mode” (see below) was roughly confirmed by comparing with a known-sized, focused ion beam (FIB)-drilled hole in the film (Fig. 2f, 2g).

The small on-plane patch forms in 15–30 s at a beam current of ~1 nA, typical for low-dose cryo-EM. Contrast quickly builds up as the HFPP patch forms (Supplementary Movie 1), and the Thon rings in the power spectrum slightly contract, if the TEM is underfocused (Supplementary Movie 2). The image appears to reach maximum contrast after about 15 s.

The Thon rings contract in the first few seconds, and then the central disc of low-frequency transfer becomes more uniform during an additional 15–30 s. The PP aperture drifts for a few seconds after being selected, so a 15-s wait is observed when selecting a new PP aperture position.

Once the patch forms, phase-contrast imaging typically remains stable for a few hours of use. As imaged with strong underfocus in the low-magnification mode, the patch is observed to persist without additional electron irradiation. However, patches fade sufficiently after a few days (Fig. 2d, 2e), so that the same PP aperture can be re-used. The fading is the same whether or not the PP anticontaminator is in continuous use. “Old” patches that happen to be within a few micrometers of a new patch often appear as small dark regions in the low-frequency region of the power spectrum (as seen in Fig 6a, to be described below). These have no significant effect on the image, and the artifacts slowly disappear with continued irradiation of the new patch.

Effect of an Enlarged (Conjugate) Objective-Lens Back Focal Plane

For microfabricated PPs, which have a central structure such as that of the ZPP, the “cut-on” frequency (below which the PP does not function) is determined by the spatial frequency corresponding to the size of the central structure. In this case, magnification of the back focal plane will improve low-frequency transfer. Our TEM has a transfer lens and a lower PP holder for just this purpose, providing a conjugate back focal plane with a magnification of three times (focal length 15 mm instead of 3 mm). However, there is no benefit in the case of an HFPP because the patch becomes larger in the same proportion as the back focal plane is magnified (Figs. 2f–2g).

Effect of Heating the HFPP

In the first few seconds, a HFPP may perform the same whether it is heated or not. However, without heating severe artifacts may develop almost as fast as the patch is formed. Although good contrast could still be obtained, contrast reversal was often observed (Fig. 3), as was distortion of the expected power spectrum (Marko et al., 2013). Although contamination was suspected (which could lead to dark-field imaging or excessive phase shift of the unscattered beam), the cause was not determined. The speed of deterioration was independent of use of the PP anticontaminator. We do not recommend attempting to use an HFPP without heating to above 200°C.

Comparative Performance Tests of HFPP Based on Power Spectra

In agreement with Danev et al. (2014, 2016), we observed that the optimal focus setting with the HPFF is quite critical, if contrast transfer function (CTF) oscillations are to be avoided. If the highest spatial frequency without oscillations is to be obtained, the focus tolerance is only about 20 nm. Therefore, it is essential to set the focus by observation of the power spectrum, often using a longer exposure at an off-axis position on the specimen to obtain a clear power spectrum. In the case of possible local tilt of the sample along the tilt axis, the required compensation for a more-accurate focus setting may be accomplished by the use of equal and opposite off-axis displacements along the tilt axis.

We followed each HFPP film in a 25-hole PP aperture disc over time, and we tested several such discs. For each test, we imaged an amorphous carbon film at 1-week intervals for up to 6 weeks (Fig. 4). Typically, power spectra compared well with the calculated CTF. We allowed 1 week between each test to ensure that previous patches in a given aperture disc had substantially faded. During an imaging session, there often was a time-dependent tendency for reduction of the maximum spatial frequency in the low-frequency area that could be transferred (Figs. 5 and 6), as well as a gradual reduction of artifacts in the power spectra that are sometimes seen with a given HFPP (Fig. 6). The objective-lens (focus) setting that yielded the most-uniform transfer to the highest spatial frequency varied among PP apertures, as did the required correction for objective astigmatism. Our reference for focus and astigmatism was a 100- μm objective aperture without the carbon film. We reset the EM defocus readout to zero with such a non-HFPP aperture by observing Gaussian focus in the power spectrum. This enabled us to measure the defocus introduced by the PP aperture. A PP aperture typically introduced 200–300 nm of underfocus.

A summary of the test results from 41 HFPP apertures is given in Table 1. As mentioned below, while most HFPPs were suitable for tomography, only a few were suitable for high-resolution single-particle imaging without CTF oscillations. To assess the maximum “resolution” obtained without oscillation, we visually measured the length in pixels of a radius ending at the midpoint of the first minimum in the power spectrum, and converted it from spatial frequency to periodicity. We are investigating the physical characteristics of HFPP films with the aim to increase near-focus performance and stability. Modifications of the carbon film, or use of different materials, may lead to improvements. Meanwhile, high-resolution work usually requires CTF correction at higher spatial frequencies.

Effect of Accumulated Electron Dose on the HFPP Film

Repeated imaging using the same HFPP patch gives stable results after the first several seconds of patch formation (Fig. 5, Supplementary Movies 1 and 2). By imaging with moderate underfocus so as to generate oscillations, it was found that the positions of the power-spectrum minima do not change on repeated imaging over a period of 1–2 h. There is a slight shrinkage of the strong low-spatial-frequency transfer range, as also seen in Figure 6.

Use of the HFPP at Underfocus

We observed that when operated in an underfocus condition, the full resolution of the TEM can be obtained with nearly every HFPP, although the resulting CTF oscillations at higher spatial frequencies would require correction. We found that a HFPP that did not provide transfer to high resolution without oscillation could nevertheless provide high-resolution transfer when used slightly underfocus (e.g., 1,000 nm underfocus). The performance of a typical HFPP at underfocus is shown in Figure 6. As discussed in Danev & Baumeister (2016), the critical attention to focus is relaxed if CTF oscillations are accepted. The result is faster data collection and little change to “standard” image processing, while retaining the advantage of greatly increased contrast at low spatial frequency. In the case of imaging for electron tomography (ET), where resolution beyond 1 nm is not normally expected, nearly all of our HFPPs provided adequate non-oscillating transfer.

High-Resolution Performance

Although most tests were done with a pixel size of 0.74 Å and an electron dose of 35 e⁻/Å² on the specimen, we confirmed that an HFPP can easily pass the full resolution of the TEM when used at higher magnification and higher dose, i.e., the presence of an HFPP in the back focal plane does not dampen the high-resolution performance of the TEM, as can be seen in Figure 7.

When we recorded images with a pixel size of 0.3 Å and an electron dose at the specimen of 100 e⁻/Å², the 2.3 Å lattice reflection in gold particles was as apparent with a HFPP as when close to Scherzer focus in the absence of a PP. However, it is clear that the lower spatial frequencies are transferred much better with the HFPP. It is notable that with the HFPP the actual structure of the carbon film is apparent, rather than the characteristic “granularity” resulting from defocus phase-contrast imaging. The shape of the PP curve in Figure 7 is due to a small amount of relative underfocus (~50 nm) needed to achieve about the same resolution with the HFPP as that obtained near Scherzer focus with no PP.

Implementation of Automated Tilt-Series Collection in SerialEM

We found that JEOL’s original graphical user interface (GUI) for storing, recalling, and annotating the positions of an array of 25 PP apertures (Fig. 8a) was very convenient, and we modified it to include both normal objective apertures and PPs in the selected area aperture (SA) position (“transfer mode”).

Although accurate centering of the PP aperture is not needed before forming the HFPP patch, it is essential that the unscattered beam remains accurately centered on the patch throughout subsequent imaging. The most certain way to ensure this is to avoid any changes to the TEM’s illumination system after the initial set-up. This is possible in our case because we can accurately shift the specimen between the off-axis Focus and Trial (tracking) positions and the on-axis Record position simply by controlling a piezo driver to move the specimen stage along the tilt axis (Fig. 8b). However, with most TEMs the off-axis position is obtained by electronic image shift and compensatory beam shift. In both cases, a few iterations of the data collection cycle are needed before start of data collection, to avoid hysteresis effects.

If the specimen stage (goniometer) is eucentric enough to limit specimen shift during tilting to within about 1 μm, and the illuminated area on the specimen is large enough when the HFPP is in the “on-plane” condition, then electronic image shift for tracking, with compensatory beam shift, is not needed, and this function can be turned off in SerialEM (Mastronarde, 2005).

If tracking by electronic image shift with compensatory beam shift is required, then the “shift purity” must be accurate, so that there is no beam tilt resulting from beam shift. Although compensatory beam shift keeps the illumination centered on the camera, any beam tilt that might be introduced moves the unscattered beam off the HFPP patch.

The standard TEM alignment protocol includes an adjustment for eliminating beam tilt when shifting the beam, and this needs to be carefully carried out. In the case of many JEOL

TEMs, this procedure needs to be done at the condenser-lens setting that puts the HFPP “on-plane,” and we have provided a GUI for this alignment (Fig. 8c). Once “shift purity” is established, it is not necessary to turn off beam shift in SerialEM, and any electronic tracking excursions that may be needed can safely be compensated by beam shift.

For our TEM, SerialEM was modified to allow beam shift to be disabled during a tilt series. In addition, a connection was established to use our piezo specimen shift instead of electronic image shift for off-axis trial (tracking) and focus; this includes a 20-s wait time for specimen-stage drift settling. These additions are part of the current SerialEM distribution (<http://bio3d.colorado.edu/SerialEM/>).

As mentioned above, an accurate focus setting is needed for widest transfer of spatial frequency information without oscillations. The required focus setting is determined by observing the power spectrum of an off-axis image, with the HFPP patch fully formed. As this setting will not be the same as Gaussian focus (as determined in SerialEM by beam tilt), the required focus offset (i.e., “target”) must be saved to SerialEM during setup. Because beam tilt is used for auto-focus, additional patches will be formed on the HFPP. To avoid distortion of the power spectrum by nearby patches, the amount of beam tilt for autofocus should be set to place subsidiary patches at least 5 or 6 μm from the main path. The subsidiary patches create small spot-like dark artifacts in the power spectra (as seen in Fig. 6a), but have no significant effect on image quality.

Example Tomograms of T4 Bacteriophage

Figure 9 shows x - y plane images (z -slices) from electron tomograms of plunge-frozen bacteriophage T4, recorded at 300 keV with zero-loss energy filtering and a total electron dose on the specimen of about $80 \text{ e}^-/\text{\AA}^2$. The sample slice shown in Figure 9a is from a tomogram with imaging at 4 μm underfocus without a PP. This underfocus places a CTF maximum at a periodicity of 4 nm (that of the phage tail), thus this periodicity is also visible in slices from tomograms recorded without a PP. Note that the slices from HFPP tomograms have much greater contrast. Figure 9a–9f are 1-nm-thick slices, whereas Figure 9g–9i are 10-nm-thick slices. Most of the panels show full capsids (“heads”), although an empty capsid is shown in Figure 9i, and condensed DNA is seen in one of the capsids in Figure 9e. The central tube is clearly seen in Figure 9f. A long fiber is seen just above the tail in Figure 9g. The spiral arrangement of tail-sheath subunits is seen in Figure 9d and 9h. In Figure 9i, the tail-sheath spiral is superimposed on the tail core. The structures seen in these slices can be favorably compared with those seen in slices from conventional tomograms of T4 (Hu et al., 2015). Some features (e.g., tail-sheath subunits) which can be seen in individual slices from HFPP tomograms are only seen in sub-tomogram averages from conventional tomograms.

Protocol

For convenience, we list a protocol for setting up automated tilt-series acquisition with HFPP imaging, using SerialEM. The protocol is independent of the imaging camera (in our case a Gatan K2 Summit or a TVIPS F-416), and in principle also independent of the TEM model:

1. Confirm the setting and re-setting of the positions of several suitable PP apertures in the 25-hole disc. This is needed so that a new PP aperture can be quickly selected when needed during the tilt series. This pre-centering of the apertures is best done in diffraction mode.
2. If no long-range stage piezo is available, carefully adjust the TEM alignment so that there is no beam tilt associated with beam shift. This is best done after first setting the condenser-lens “on-plane” condition.
3. Start SerialEM with Tasks > Special Features “Skip beam shift during tilt series” = On, if the goniometer is precise enough that beam shift is not needed, and also select “Use piezo for low-dose” (if the TEM has that function). 4. If setting up the tilt series for the first time, the position of the unscattered beam on the HFPP film when the beam is tilted for SerialEM’s autofocus, needs to be confirmed. To do this, first set the condenser lens to the approximate on-plane condition, perform autofocus, and record the change in the TEM’s beam-tilt deflector. Then manually apply the same change in beam-tilt deflector while observing the beam in diffraction mode. This should be done with the diffraction focus adjusted for a sharp edge of the objective aperture, using a small (e.g., 10 or 30 μm) objective aperture to aid in judging distances. The beam should move not less than 5 or 6 μm , so that the subsidiary HFPP patches created during autofocus do not interfere unduly with the main patch used for Record. If necessary, the amount of beam tilt for autofocus can be adjusted in SerialEM.
4. Select the desired magnification and accurately set the final condenser lens to the on-plane condition by observing the expansion of a small piece of debris on a PP film; if necessary, adjust the condenser astigmatism to maintain the feature’s shape as it increases in size. It is usually adequate to do this only once, at one location on the PP aperture disc, after which all PP apertures on the disc will be “on plane.” Record the lens current or digital code for the on-plane condition, in case it needs to be subsequently re-set.
5. Set up Trial and Focus with the same settings as Record, the only difference being the position on the specimen. Set up View, ideally avoiding use of a lower magnification range than used for Record (i.e., it is best that the objective-lens has the same excitation in View and Record). In any case, avoid the TEM’s “low magnification” mode, which

can cause hysteresis and thermal drift from cycling the objective-lens current.

6. Locate a “sacrificial” area on the specimen and set up concentricity of Record and View. Go through the cycle of positions a few times to eliminate any hysteresis.
7. Insert a PP aperture and image in Trial for a total of 30–60 s to form a patch. Check that returning to Record after cycling through Trial, View, and then again Trial does not compromise HFPP imaging with the present patch.
8. Adjust the focus in Record, while observing the power spectrum (FFT: fast Fourier transform), such that the contrast transfer is strong and without oscillation up to the desired spatial frequency. If not possible, select a different PP aperture. Use SerialEM to measure the defocus, and use that defocus target for the tilt series.
9. Set up the tilt series with your chosen parameters (including energy filtering if available), and start data collection on a fresh location on the specimen. 11. If the Record image becomes defective with respect to HFPP contrast, stop the tilt series and select a new PP aperture (or a new position on the same HFPP film but at least 5 μ m away from the previous position). Before proceeding, form a new patch by imaging at the Trial position, then observe the power spectrum and re-set the focus target if necessary.

Conclusions

HFPPs are easy to use, inexpensive to implement, adaptable to automated data collection, and they offer excellent performance with respect to the several advantages of PPs. It is very likely that most biological applications of PPs will employ the HFPP for the present time.

1. For applications in cryo-ET, HFPPs provide a dramatic increase in contrast (this facilitates higher resolution without increasing the electron dose, which risks specimen damage).
2. For applications in single-particle cryo-EM, HFPPs can provide excellent transfer to high resolution without CTF oscillation. Nevertheless, the greatly improved low-frequency contrast (which aids in particle-picking, alignment, and sorting heterogeneous particles) is achieved even if oscillations in the CTF occur at higher frequencies.
3. The TEM can be operated in such a way that the beam does not move during automated data collection; thus once the

- HFPP is formed, it need not be re-centered during an imaging session.
4. HFPPs have a very favorable low spatial frequency cut-on because the “patch” is formed by—and is only slightly larger than—the unscattered electron beam in the objective-lens back focal plane. This patch is much smaller than the size of a typical microfabricated PP element (e.g., the central hole of a ZPP).
 5. HFPPs have no “ringing” fringe artifacts because the cut-on is gradual, not sharp as in the case of the central hole of a ZPP.
 6. As the HFPP patch is formed in the back focal plane by the illumination crossover, there is no benefit from using an HFPP with long-focal-length optics.
 7. HFPPs vary in quality from one to another, but the user can easily make selections before an imaging session.
 8. Heating the HPFF greatly reduces apparent charging and contamination and provides long-term stable performance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Hole-free phase plate (HFPP) comparison images. **a–c:** High-dose ($40 \text{ e}^-/\text{\AA}^2$) images of T4 bacteriophage recorded at 300 keV with zero-loss energy filtering, using the Gatan K2 Summit “dose fractionation” mode (4 s exposure with 0.2 s/frame); near-focus HFPP imaging (**a,b**), compared with defocus phase-contrast imaging (**c**). **a:** An ideal overview HFPP image; the periodicity on the T4 tail is 4 nm. **b,c:** Comparison between an HFPP image (**b**) and an image (**c**) taken 5 μm underfocus without a phase plate (PP), but with the same objective-aperture diameter (100 μm). **d–g:** Images of tobacco mosaic virus recorded at 300 keV with zero-loss energy filtering (K2 dose fractionation, 2 s exposure, 0.2 s/frame, $\sim 12 \text{ e}^-/\text{\AA}^2$). The 2.3-nm periodicity is seen in the images and the power spectra. The PP image (**d–e**) demonstrates higher low-frequency contrast, whereas the 2.3-nm periodicity is also seen in the defocus-contrast image (**f–g**), which was recorded at 1,000 nm underfocus (1,000 nm underfocus positions a CTF maximum at $\sim 2 \text{ nm}$).

Figure 2.

Beam-induced patches on an hole-free phase plate (HFPP) carbon film. **a–c:** The patch on the HFPP film can be studied in the low-magnification mode of the TEM. When imaged with underfocus (**a**), the patch (arrow) appears dark. When imaged in-focus (**b**), the patch is not visible, indicating that the contrast is not due to contamination (which would be visible with amplitude contrast). When imaged with overfocus (**c**), the patch is bright. Initially, the patch is clearly visible with strong underfocus (**d**), but it fades after 1 week (**e**). The patch at the objective-lens back focal plane [(**f**), arrow] is about 100 nm in diameter, seen next to a 700-nm-diameter FIB-drilled hole seen adjacent to the patch. When the patch is formed on an HFPP film located in the conjugate back focal plane, it appears to be at least three times larger [(**g**), arrow], due to the three-times enlargement of the conjugate back focal plane (fringes make it difficult to accurately measure the patch size).

Figure 3.

Variation in imaging with unheated hole-free phase plate (HFPPs). Three images of T4 phage recorded with non-heated HFPPs. Note the great differences in contrast, presumably due to differences in charging and contamination. **a:** Image recorded while the HFPP patch is just forming. **b:** Image recorded when the patch has completely formed. **c:** Image recorded after several additional minutes of irradiation.

Figure 4.

hole-free phase plate (HFPP) imaging of amorphous carbon films. **a–c:** Power spectra of images at a magnification typical for tomographic tilt-series collection. The Nyquist frequency (i.e., the right edge of the image frame) corresponds to a periodicity of about 1.1 nm. **a:** Near-focus image recorded without phase plate (PP); almost no transfer below a periodicity of about 4 nm. **b:** HFPP image recorded on the same day the carbon film was made; note the strong low-frequency transfer where there was almost none in (a). **c:** Image from a new HFPP patch formed on the same PP aperture after the film was kept in the TEM with continuous heating at 260°C for 3 weeks. **d–e:** Power spectra of high-magnification images. The first zero (near the center of the image frame) corresponds to a periodicity of about 3.2 Å. **d:** Scherzer focus with no PP. **e:** Image recorded with a HFPP patch on a freshly made carbon film. **f:** Image recorded 3 weeks later, with a new patch on the same carbon film as in (e). **g:** Calculated CTF showing transfer corresponding to no PP [as in (d)] and with HFPP [as in (e) and (f)]. **h:** Plot of the maximum HFPP resolution obtainable without power-spectrum oscillation, as the carbon films were kept in the transmission electron microscopy with continuous heating at 260°C. A new HFPP patch was formed each week on the carbon film of each of eight different PP apertures; each data point is an average of the results from the eight PP apertures.

Figure 5.

Effect of irradiation of the hole-free phase plate (HFPP) patch. Images and power spectra of “as received” Quantfoil carbon films recorded under low-dose conditions at 1,000 nm underfocus. The structure comes from plastic contaminants on the surface, which is not apparent in-focus without a PP. The beam current was 1.3 nA, and the exposure time was 3 s/exposure, thus the electron dose on the patch was 3.9 nC/image. Use of underfocus facilitates phase-shift determination by CTF fitting. **a–c:** Irradiation of “PP12,” as shown in (g). This HFPP started with a phase shift of 0.3π , which increased to almost 0.6π , but the image maintained constant quality. **d–f:** Irradiation of “PP8,” as shown in (g). This HFPP started at 0.5π phase shift and increased to over 0.8π . In this case, the image quality (dominated by low-frequency information) worsened due to decreased low-frequency transfer, evidenced by a slight “shrinkage” of the first minimum of the power spectrum. (a) and (d) are the third images in the series, (b) and (e) are the 30th images, and (c) and (f) are the 55th images in the series.

Figure 6.

Dose series of an hole-free phase plate (HFPP) operated underfocus; effect of a nearby residual patch. **a:** Power spectrum of the fifth image in a series, recorded with an additional 3.9 nC of electron irradiation on the HPFF film for each subsequent image. Arrowheads indicate artifacts due to a nearby patch formed 1 week previously. **b:** Power spectrum of the 30th image in the series; the white circle corresponds to a periodicity of 3 Å and the arrowhead corresponds to a periodicity of 1 nm. Note the fading of the artifacts seen in (a). With such underfocus imaging, low spatial frequencies are still strongly transferred without oscillation, and the power spectrum extends to high resolution, although with oscillations. **c:** Power spectrum of the 60th image; the range of good low-frequency transfer contracts slightly with accumulating electron dose. The apparent astigmatism (i.e., distortion) at the lowest spatial frequencies is likely caused by local, non-rotationally symmetric charging of the film. All images were recorded with 1,000 nm under-focus and a pixel size of 0.74 Å.

Figure 7.

High-resolution imaging with a hole-free phase plate (HFPP). Graphitized-carbon/gold-island test specimen imaged near Scherzer focus without a phase plate (PP) (**a**) and near focus with an HFPP (**b**). In the images, variability in the prominence of graphite lattice lines in certain directions, and the appearance of spots in the power spectrum that do not exactly correspond to the known lattice spacing, is due to the heterogeneous and non-planar nature of the sample. The power spectra and the calculated CTF plot (insets) show improved low-frequency transfer. Note that the granularity of the carbon film is an artifact of conventional (defocus) phase contrast, whereas the PP reveals the true structure. Transfer to 2.3 Å appears to be as strong with the HFPP as without it. Imaged at 300 kV with zero-loss energy filtering.

Figure 8.

Controls for phase plate (PP) operation implemented on our TEM. **a:** The positions of PP apertures and normal objective apertures are selected by this GUI. The 25 PP apertures on the PP disc are sorted for quality and are color coded. Once the positions are stored, any PP aperture can be selected, with no need for further alignment. The positions can be stored separately for both upper (objective lens (OL) mode) and lower (“SA” = transfer-lens mode) and for both PP and normal apertures. Normally, the upper (OL) 400- μm objective aperture is used in combination with a lower PP, so both OL and SA apertures can be selected at the same time. This GUI can be accessed by an external computer, although we have not found it necessary. **b:** The GUI for piezo control of the specimen stage and PP. This GUI controls piezo centering of either PP, as well as translation of the specimen stage. “Z” is not used for PP, but for the specimen stage, “Z” is the long-range piezo (4.5- μm travel along the tilt axis). During tilt-series acquisition, this application is controlled remotely by SerialEM. **c:** The GUI for tuning the illumination system. This GUI is used to perform coma-free alignment and to achieve precise beam shift/tilt compensation.

Figure 9.

Example slices from electron tomograms of bacteriophage T4. **b–i:** Z-slices of a tomogram recorded with an hole-free phase plate (HFPP), compared with **(a)**, the same but without a PP. **(a–f)** are 1-nm-thick slices and **(g–i)** are 10-nm-thick slices. **a:** No PP, but with 4 μm underfocus (CTF first-maximum periodicity = 4 nm). Note that the 4-nm periodicity in the tail is visualized in both (as expected), but the overall contrast is much higher when the HFPP is used. **d:** Detail of a compressed tube sheath with subunits visible (white arrows). **e:** Condensed DNA is shown in one of the heads. **f:** Detail of central tube. **g:** One of the particles from **(b)**, but in a 10-nm-thick slice. **h:** Five subunits of the spiral tail-sheath are seen between the two white arrowheads. **i:** The projected 4-nm tail periodicity seen below the spiral structure. For scaling, the width of the T4 head is ~ 90 nm.

Table 1

Overall Quality and Longevity of Carbon Films for Hole-Free Phase Plates (HFPPs).

Maximum Resolution (Å) Without Power-Spectrum Oscillations	Number of PP Apertures	Average % Resolution Decrease After 1 Week
Overall	41	8.5
3.0–3.4	3	7.0
3.5–3.9	10	13.5
4.0–4.4	4	8.4
4.5–4.9	9	7.1
5.0–5.4	4	6.9
5.5–5.9	0	N/A
6.0–6.4	2	7.2
6.5–6.9	4	2.6
7.0–7.4	1	14.9
7.5–7.9	1	–5.3
8.0–8.4	1	5.1
8.5–8.9	2	12.7

In all cases, the objective-lens focus was adjusted to yield a power spectrum (from the amorphous carbon film specimen) with the highest-possible spatial frequency without visible oscillations in the power spectra. Although the carbon films of relatively few phase plate apertures demonstrated the HFPP performance shown in Figures 4e–4g, nearly all HPFFs transferred high resolution—even after the film aged for a few weeks—if underfocus was applied, although CTF oscillations then occurred at higher spatial frequencies (Fig. 6). Furthermore, nearly all HFPPs provided oscillation-free transfer up to a periodicity of 1.5 nm, as would be suitable for tomography.