Introduction to Phase Plates and the FEI® Phase Plate Solution

The use of a phase plate in electron microscopy has shown renewed interest, triggered by a publication on this topic in 2001 by Danev and Nagayama [1]. This interest can be understood when the fact that many samples that are studied in TEM, are weak phase objects (especially in life sciences) is taken into account. The use of a phase plate is the obvious method of choice to convert otherwise invisible phase modulations into visible amplitude modulations in the detected intensity profile. A phase plate can provide in-focus phase contrast, unlike the conventional method where a strong defocus is needed to generate contrast at low spatial resolutions, with the added consequence of introducing contrast reversals as a function of frequency.

Although the basic principle of a phase plate is well understood and phase contrast light microscopy is widespread, it has turned out to be technologically challenging to perfect this technique in electron microscopy. The main hurdles in making a device that performs well, is the sensitivity of the electron beam for any disturbances in or near the back focal plane of the microscope, the plane where the phase plate is inserted, and the strong interaction of electrons with phase plate materials. Disturbances are caused by charging, contamination, and deterioration or aging of the phase plate materials due to the electrons that impinge on the device. The result is a distorted wave front of the electron beam, instead of a well-controlled phase shift between the unscattered and scattered electrons.

Many types of phase plates have been proposed, and for a long time the most successful was the original thin-film Zernike phase plate. This type of phase plate has shown promising performance, especially in life science applications. The most widely tested film is amorphous carbon, but this type of film suffers from aging problems, making frequent exchanges of the phase plate necessary. Alternatives to amorphous carbon have been investigated and silicon-based films show promise in terms of longevity [2].

At FEI we have investigated new methods of coming to a workable phase plate solution, addressing the problems that are still associated with phase plate usage. The work was done in collaboration with the Max Planck Institute of Biochemistry, with emphasis on practical aspects of phase plate usage in the prevailing workflow in cryo-electron microscopy.

In the course of this collaboration program, we have developed a new type of phase plate with properties that make it very suitable for implementing it as a user friendly device in our TEMs. Foremost, it produces high-contrast images, providing excellent contrast transfer in the low resolution range which is particularly relevant for cryo-electron tomography. No fringing effects around high-contrast features are observed, and CTF oscillations can be avoided up to high spatial resolution. Also, transmission losses by the phase plate are very modest. Moreover, the phase plate shows uncompromised performance for at least half a year of usage. Finally, the phase plate allows for automated use in real-life applications such as tomography and single particle acquisition.

To facilitate routine phase plate usage, we have added extra alignments and control panels to the microscope software. Step-by-step guided alignments have been added to set up proper illumination conditions for the phase plate. Also, highly accurate adjustments of beam deflection pivot points is taken care of to ensure a stable beam position at the plane of the phase plate. Furthermore, software was developed to easily navigate the phase plate in the back focal plane. All together, these alignments ensure seamless integration of the phase plate in the (automated) applications.

The FEI phase plate has been tested in various applications. Especially in low-dose cryo-electron tomography, our phase plate has proven its added value, but also in Single Particle Analysis, good results have been obtained.

The use of a phase plate, especially when combined with a highly sensitive direct electron detector, will give a tremendous increase in the image contrast of weak phase objects, such as unstained cryo specimens.
Figure 1 displays Tecnai™ F20 results from cryo-electron tomography on doxorubicin using conventional TEM at 4 µm defocus (left) and phase plate TEM (right) at 0.5 µm defocus. Experimental conditions: total dose of 85 e-/Å2, tilt range ±60°. Courtesy of Radostin Danev.

Physical Selection and Movement of the Phase Plate

NOTE The phase plate should always be retracted when changing samples, as this is the most likely source of particulate contamination of the phase plate film.

The phase plate is located on a heated phase plate holder (Phase Plate holder, see Figure 2) which in most cases contains two objective apertures and one phase plate. The phase plate consists of up to 6 rectangular windows with rounded corners (6 slots, see Figure 3), and the surface of the phase plate can be observed at high defocus in low mag mode (low mag, see Figure 4). The principle of the Volta Phase Plate (VPP) is that since the intense undiffracted beam self creates the phase plate, no physical alignment of a feature on the phase plate is required: one can put the beam at any position on the carbon film and create a phase plate. However, one also does not want a previously used Volta potential too close on the phase plate that it disturbs the current experiment. To avoid this, the aperture is moved in steps of 20 µm to new positions (aperture movements, see Figure 5). This movement is controlled by an extension to the normal aperture control panel (apertures, see Figure 6).
After insertion into the column, the phase plate needs to be left for approximately 5 minutes to allow the holder to reach mechanical and thermal equilibrium. When moving from one phase plate position to another using Next, only a few seconds are required.

**Phase Plate Holder**: Photograph of the VPP Heating Holder, with image of phase plate inset.
6 Slots: Within each Phase Plate slot are lines of predefined preset positions. Click **Next** to move to the next preset position of the selected Phase Plate slot. After the last preset position, the first one is selected again. The index number of the selected Phase Plate preset position is displayed in the Control Panel.
Low mag: Low magnification image of a grid in the sample holder, a phase plate inserted. The surface of the phase plate can be inspected by defocusing ~100 mm in low mag mode where Volta potentials appear as white spots.
Aperture movements: When Adjust is pressed, the Multifunction knobs will control the position of the Phase Plate. This functionality can be used for temporarily working on another part of the Phase Plate. When the Next button is used, the Phase Plate will be positioned on the next preset position again.
Phase Plate User Manual  ■  Physical Selection and Movement of the Phase Plate

Apertures: In the Phase Plates tab of the Apertures Control Panel, you can select a Phase Plate slot and move within the usable area of the Phase Plate. The Apertures strip contains one Phase Plate Grid which accommodates six Phase Plate slots. Up to six Phase Plate slots can be selected in the drop-down list (in a similar way as Apertures).
Phase Plate Alignments (Alignments Tab)

The Phase Plate alignment (Alignments) sets two things:

- A diffraction lens value that focuses on the phase plate (saved as eucentric diffraction focus on the highest camera length).

  The idea of the first step alignment is to make it easier than simply observing the structure of the phase plate via a Ronchigram method, described by Danev et al PNAS 2014 (though you can also use this method).

- Accurate beam shift pivot points.

Alignments: Alignment tab for phase plates - right hand panel

The Phase Plate Alignment procedure does not adjust the on-plane condition for the imaging mode that you are going to use. It is a calibration step that uses reference conditions that enables finding the on-plane condition for any condenser setting later on.
There are different alignment procedures for Titan™ (3 condenser lens) and Tecnai/Talos™ (2 condenser lens) microscopes. This is mostly due to the conditions that result in a parallel beam, which is the same condition in which the beam is focused at the back (and front) focal plane and therefore the phase plate. For a Titan, a C2-C3 zoom mechanism works together to change the illuminated area size, while maintaining parallel illumination. Because of the C2-C3 zoom, either microprobe or nanoprobe modes can be used, and therefore both modes are aligned. For a Tecnai/Talos, there are only discrete beam sizes, one for each C2 aperture, that are parallel, and only in nanoprobe the illuminated area matches well with the size of the detector. Therefore, there is only a nanoprobe alignment. These alignments only apply to systems with fast cameras. Alternate instructions will be provided for those with only a Flu-screen.

The phase plate alignments should be done with the same spot size that is used with the imaging beam. This needs to be changed manually from the automatically set spot size in the first alignment step.

**Titan Alignments**

Below is the extracted Help text for the Phase Plate alignments combined with the actual steps in the alignment procedure.

**Phase Plate Alignment Microprobe**

**Purpose:** Basic alignments for phase plate usage in HM-TEM mode (microprobe).

- Fine tune diffraction lens at highest camera length, to focus accurately on phase plate plane.
- Fine tune beam shift pivot points, such that pivot points accurately coincide with phase plate plane.

**Method:** Starting position is in low magnification SA mode (microprobe) with the objective lens at eucentric focus.

**Preparation step:** (Alignment tab)

1. Remove specimen.
2. Remove SA aperture.
3. Select 50 um C2 aperture.
4. Center beam [NX, NY].
5. Spread beam [Intensely] to fill the 40 mm circle.
6. If necessary make beam round with
Corresponding help text:

- Remove the specimen from the beam and remove the SA aperture.
  ***if it is inserted***
- Center C2 aperture:
  ***note that proper C2 alignment on a 3 condenser lens system requires that C3 is turned off first in free lens control***
  - Focus spot and center it on the screen.
  - Turn the Intensity knob to overfocus (clockwise).
  - Center the aperture until the illuminated area is symmetrical around the screen center.
- Remove astigmatism by tuning the condenser stigmator to make illuminating beam round.
- Insert the phase plate.

**Set on-plane condition:**

Change intensity [Intensity] to find on-plane condition [Focusing method].

If necessary recenter beam [MF-X,Y].

If necessary, toggle to condenser stigmator [C2] (see online help).

The on-plane condition is realized if the beam is exactly focused at the phase plate.
Focusing is done with the condenser system.
The on-plane condition can be recognized with the help of a phase plate Ronchigram (in-line hologram). Such a Ronchigram becomes visible in imaging mode, using optimized contrast settings of the Flu-Cam. This can be done by adjusting the contrast range selection in the intensity histogram of the Flu-Cam image (and changing the exposure settings to maximize the dynamic range of the Flu-Cam). A fast CCD camera such as an FEI Ceta camera, US1000, or K2 can be used in place of a Flu-Cam. If lines are apparent in the Ronchigram, the condenser astigmatism needs to be adjusted so that the beam expands on either side of the blow-up point concentrically.

Figure 7    Histogram of the Intensity Distribution on the Flu-Cam Image

The on-plane condition is achieved at the blow-up point, where the magnification of phase plate detail is infinitely high. Examples of off-plane and on-plane Ronchigrams are shown below.

Figure 8    Example of an Off-Plane Ronchigram
Setting the diffraction lens correction value (stored as the eucentric diffraction lens focus value):

Once the on-plane condition is found, a correction value can be determined for the diffraction lens (at the largest camera length). To this aim, the diffraction lens is focused so that the smallest beam spot is observed. This spot coincides with the phase plate plane. The on-plane condition is later adjusted in the phase plate panel with the adjustment of the condenser system (MF-Y adjustment).

Setting the beam shift pivot points:

The beam shift pivot points are the points where a beam shift does not result in a movement of the beam in the back focal plane and therefore on the phase plate. It is a minimization of the beam tilt induced by beam shift.

A first, coarse alignment of the beam shift pivot points is done in diffraction mode, at the largest camera length. A second, more accurate beam shift pivot point alignment is done in imaging mode, by minimizing the effect of a beam shift on the phase plate Ronchigram (the alignment text is not included here for the Ronchigram beam shift pivot point).
Phase Plate Alignment Nanoprobe

**Purpose:** Fine tune beam shift pivot points for nanoprobe such that pivot points accurately coincide with phase plate plane.

**Method:** Starting position is in low magnification SA mode (nanoprobe) with the objective lens at eucentric focus.

**Preparation step:**

1. Remove specimen from the beam and remove the SA aperture.
2. Center C2 aperture:
   - Focus spot and center it on the screen.
   - Turn the **Intensity** knob to overfocus (clockwise).
   - Center aperture until the illuminated area is symmetrical around the screen center.
3. Remove astigmatism by tuning the condenser stigmator to make the illuminating beam round.
4. Insert the phase plate.

**Set on-plane condition:**

- Center spot [MF, XY]
- Accurately focus spot [INTENSITY]
- If necessary, toggle to diffraction stigmator [F2] (see online help).

It is assumed that the focus setting of the imaging system has already been aligned in the phase plate alignment microprobe. Therefore, it is sufficient to focus the beam to a spot with the condenser system.
Set beam shift pivot points:

Minimize spot movement [MF-X,Y]
If necessary, toggle to Diffraction Shift [F12]

The beam shift pivot points are aligned with respect to the phase plate plane, first in diffraction mode (coarse alignment), and then in imaging mode (precise alignment).

EFTEM (only if filter present)

If an image filter is present, there is an additional step to align the diffraction lens focus in EFTEM mode.

Tecnai and Talos Arctica (also any 2 condenser lens with a fast CCD or direct detector)

The beam is only parallel (and therefore on-plane) under discrete conditions for each C2/spot size setting, with the C2 lens having the main contribution, and spot size a smaller contribution. However the strategy is similar to on the Titan in that a diffraction lens value is determined by the alignment procedure, so that when on-plane, the diffraction spot is a minimum size. Then, for any setting, the Intensity (C2 excitation) is adjusted so that the diffraction spot is again a minimum size.

Phase Plate Alignment Nanoprobe (Microprobe cannot be used)

Purpose: Basic alignments for phase plate usage in HM-TEM mode (Nanoprobe).

- Fine tune diffraction lens at highest camera length to focus accurately on phase plate plane.
- Fine tune beam shift pivot points, such that pivot points accurately coincide with phase plate plane.

Method: Starting position is in low magnification SA mode (nanoprobe) with the objective lens at eucentric focus.

Preparation step: (Alignment tab)

1. Remove specimen.
2. Remove SA aperture.
3. Select and center 50µm C2 aperture (see online help).
4. Center beam [MF-X,Y]
5. Spread beam [Intensity] to fill the 40 mm circle.
6. If necessary make beam round with the condenser alignment (toggle with F12)
7. Tilt phase plate.
Corresponding help text:

- Remove the specimen from the beam and remove the SA aperture.
  ***if it is inserted***

- Center C2 aperture:
  - Focus spot and center it on the screen.
  - Turn the Intensity knob to overfocus (clockwise).
  - Center the aperture until the illuminated area is symmetrical around the screen center.

- Remove astigmatism by tuning the condenser stigmator to make the illuminating beam round.

- Insert the phase plate.

**Set on-plane condition:**

The on-plane condition is realized if the beam is exactly focused at the phase plate.

Focusing is done with the condenser system.

The on-plane condition can be recognized with the help of a phase plate Ronchigram (in-line hologram). Such a Ronchigram becomes visible in imaging mode, using optimized contrast settings of the Flu-Cam. This can be done by adjusting the contrast range selection in the intensity histogram of the Flu-Cam image (and changing the exposure settings to maximize the dynamic range of the Flu-Cam). A fast CCD camera such as an FEI Ceta camera, US1000, or K2 can be used in place of a Flu-Cam. If lines are apparent in the Ronchigram, the condenser astigmatism needs to be adjusted so that the beam expands either side of the blow-up point concentrically.

*Figure 10  Histogram of the Intensity Distribution on the Flu-Cam Image*
The on-plane condition is achieved at the blow-up point, where the magnification of phase plate detail is infinitely high. Examples of off-plane and on-plane Ronchigrams are shown below.

*Figure 11  Example of an Off-Plane Ronchigram*

*Figure 12  Example of an On-Plane Ronchigram*
Setting the diffraction lens correction value (stored as the eucentric diffraction lens focus value)

Once the on-plane condition is found, a correction value can be determined for the diffraction lens (at the largest camera length). To this aim, the diffraction lens is focused such that the smallest beam spot is observed. This spot coincides with the phase plate plane. The on-plane condition is later adjusted in the settings panel with the adjustment of the condenser system (Intensity-C2 excitation).

Setting the beam shift pivot points:

The beam shift pivot points are the points where a beam shift does not result in a movement of the beam in the back focal plane, and therefore on the phase plate. It is a minimization of the beam tilt induced by beam shift.

The beam shift pivot points are aligned with respect to the phase plate plane, first in diffraction mode (coarse alignment), and then in imaging mode (precise alignment).
Phase Plate Operation (saving different modes and setting the on-plane condenser settings)

Introduction

In order for the phase plate to work properly, the cross-over in (or near) the back focal plane of the objective lens must be set as accurately as can be achieved at the phase plate. The Phase Plate control panel aids operation with the phase plate by allowing storage of three sets of optical settings that can be recalled as needed, as well as saved and re-loaded at a later time. These stored settings are not often used in normal phase plate operation as only one on-plane condition is allowed. They can be useful for troubleshooting, during which one wants to look at the volta potentials created (an easy way of doing this is creating off-plane modes and saving them). When the Phase Plate panel is active, an additional correction of focus-dependent beam tilt correction will be active when present.

The settings stored and recalled are:

- Optical mode
- Spot size
- Illuminated area
- Fine focus back focal plane (or C2 excitation for 2 condenser lens system)
**Active**

Before the phase plate settings controls can be used, the phase plate control must be turned on by pressing the **Active** button (which will turn yellow).

**Settings 1, 2, 3**

The three sets of optical settings are accessed through the buttons **Settings (1, 2, 3)**. When a button is pressed, the particular setting is selected. There are two possible consequences:

- The button turns red with white text. The particular setting has been selected, but there are as yet no optical settings defined.
- The button turns yellow. In this case the pre-defined optical settings are put into the TEM optics.

**Define**

To define the electron-optical settings associated with one of the three settings:

1. Select the particular setting (press the button so it becomes red or yellow).
2. Press the **Define** button (it becomes yellow).
3. Manually set the optical settings on the microscope.
4. Press the **Define** button again (it becomes grey again).

If the **Settings** button was red before, it will now turn yellow. This is only possible in an electron-optical mode that is suitable for phase plate operation.

**MF-Y Focus to Back Focal Plane (not present on 2 condenser lens systems, Tecnai and Talos)**

To tune the cross-over at the phase plate, the **MF-Y** knob can be connected to the optical setting that allows fine control to do so. Note that the beam must be in the parallel range (not condensing or spreading).
Using the Phase Plate

Activation
The phase shift develops as a function of dose, which can be measured using the program AutoCTF, or other CTF determination software with a phase shift measurement included (CTFFIND4). The normal operation of the phase plate is to pre-activate the phase plate with a dose that brings the phase shift to ~0.5 pi, or to the part of the curve that is slowly increasing. This is done by moving to a new phase plate area (next), and simply exposing the phase plate with a given dose (calculated from the beam current and time).

Imaging
Once the phase plate is activated, imaging can be performed normally. However, the condenser system cannot be changed, as this will change the on-plane condition and result in a movement of the beam, away from the Volta potential that was created by activation.

Using the Phase Plate with the Low Dose System
Familiarity with the FEI Low Dose system is assumed.
It is also assumed that the Phase Plate Alignments have been performed.

| NOTE | Beam tilt pivot points should NOT be adjusted in the Direct alignments. The perpendicular component is shared with the beam shift pivot point alignments and will cause errors in the beam shift pivot points. |

On a Tecnai or Talos, Nanoprobe mode must be used for Focus and Exposure (on-plane modes), and preferably also used for Search.

On a Titan, either Microprobe or Nanoprobe can be used for Focus and Exposure, and Nanoprobe is preferable for Search, which also must be set outside of the parallel range (~15 µm illuminated area).

Some example settings are shown below for a Tecnai F20 TWIN:

- **Search**: x4,400, spot 7, C2 53%, defocus ~ -100 µm
- **Focus**: x25,500, spot 7, C2 38.360%, Obj: ~ 88.50%, focus offset ~ 4 µm
- **Exposure**: x25,500, spot 7, C2 38.360%, Obj: ~ 88.50%

These are just example settings but:

1. All normalizations should be on.
2. Adjusting the on-plane condition:
— On a Tecnai/Talos, the Exposure mode on-plane condition should be found first with the C2 as follows:
  • Turn on the Active tab in the Phase Plate control panel
  • Switch to diffraction mode and set the Camera length to the longest possible
  • Make the beam as small as possible with C2 (Intensity)
  • Make the beam round with the condenser stigmators
  • Switch out of diffraction

— On a Titan, the Exposure mode on-plane condition should be found first as follows:
  • Turn on the Active tab in the Phase Plate control panel
  • Make sure the beam settings selected are within the parallel range
  • Switch to diffraction mode and set the Camera length to the longest possible
  • Check the MF-Y Fine Focus Back Focal Plane box
  • Use MF-Y to minimize the size of the beam
  • Use condenser stigmators to make the beam round
  • Switch out of diffraction and uncheck the MF-Y Fine Focus Back Focal Plane box

3. Copy the settings from Exposure to Focus. They must be made exactly the same and should not be changed at all (intensity or spot size).

4. Click Next in the phase plate tab in the Apertures menu to move to a new phase plate position (with the beam blanked by lifting the flu-screen). Wait a few seconds for phase plate drift to stabilize (this needs to be at least 5 minutes after inserting the phase plate from being fully retracted).

5. Activate the phase plate by direct exposure to the electron beam and deliver a pre-determined electron dose. This can be done by either lowering the flu-screen or taking images and thereby illuminating the phase plate).

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<td>There is variation in the electron dose that is needed to achieve a phase shift of 0.5 pi. Values may range from 20 nC to 200 nC, the number is phase plate and system dependent. Also, you may decide to work at smaller values of the phase shift (e.g., 0.25 pi), and allow for some residual phase shift built up during the experiment. Delivering a dose of 50 nC, working with 1 nA beam current, will take 50s. Dose versus phase shift curves need to be created for each phase plate, and checked every ~1 month.</td>
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The phase shift can be measured using the AutoCTF tool provided.

The phase plate often creates a small amount of objective astigmatism compared to no phase plate, but it does not change very much at different phase plate positions. This can be corrected manually, or with the AutoCTF tool.
— If you have problems with the image contrast/phase shift changing upon switching from the off-plane search mode, try the following:

- Manually normalize all lenses before putting the screen down to activate the phase plate.
- After switching to search mode to identify and move to the target area, switch back to Focus mode (using the camera and not the screen), manually normalize all lenses again.
- Additional normalize all lenses may be required if the focus changes significantly before the exposure is taken, though this has not been fully explored.

**Other Considerations**

The Phase Plate heating should be turned off while the column is not under vacuum, whether a planned or unplanned venting of the column.