Improved specimen reconstruction by Hilbert phase contrast tomography

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A R T I C L E   I N F O

Article history:
Received 12 December 2007
Received in revised form 29 July 2008
Accepted 30 July 2008
Available online 7 August 2008

Keywords:
Transmission electron microscopy
Electron tomography
Phase contrast
Phase plate
Differential interference contrast

A B S T R A C T

The low signal-to-noise ratio (SNR) in images of unstained specimens recorded with conventional defocus phase contrast makes it difficult to interpret 3D volumes obtained by electron tomography (ET). The high defocus applied for conventional tilt series generates some phase contrast but leads to an incomplete transfer of object information. For tomography of biological weak-phase objects, optimal image contrast and subsequently an optimized SNR are essential for the reconstruction of details such as macromolecular assemblies at molecular resolution. The problem of low contrast can be partially solved by applying a Hilbert phase plate positioned in the back focal plane (BFP) of the objective lens while recording images in Gaussian focus. Images recorded with the Hilbert phase plate provide optimized positive phase contrast at low spatial frequencies, and the contrast transfer in principle extends to the information limit of the microscope. The antisymmetric Hilbert phase contrast (HPC) can be numerically converted into isotropic contrast, which is equivalent to the contrast obtained by a Zernike phase plate. Thus, in-focus HPC provides optimal structure factor information without limiting effects of the transfer function. In this article, we present the first electron tomograms of biological specimens reconstructed from Hilbert phase plate image series. We outline the technical implementation of the phase plate and demonstrate that the technique is routinely applicable for tomography. A comparison between conventional defocus tomograms and in-focus HPC volumes shows an enhanced SNR and an improved specimen visibility for in-focus Hilbert tomography.

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1. Introduction

Cryo electron tomography (CET) is a unique technique for gaining insight into the three-dimensional structure of biological specimens, such as whole cells or cryo sections of tissues, with a typical structural resolution of ~4–5 nm at present (Beck et al., 2004, 2007; Jensen and Briegel, 2007). An important goal of CET is to extend the resolution to a level where individual molecules can be recognized and distinguished by their shape (Medalia et al., 2002; Nickell et al., 2007). It is generally expected that a resolution of 2–3 nm will be necessary for such shape recognition (Robinson et al., 2007). At this level of resolution, the effect of the microscope point spread function (PSF) can hamper the reconstruction. This is well known from single particle averaging work, where the correction of the contrast transfer function (CTF, as the Fourier transform of the PSF) is imperative for reaching high resolution from strongly defocused images. Strong defocusing is often necessary for reaching a level of object contrast sufficient for current image processing algorithms. Therefore, it is advantageous to improve image contrast by the use of a physical phase plate. It provides strong in-focus phase contrast and a microscope PSF which corresponds to the ideal contrast transfer (Gamm et al., 2008).

Most image artifacts that arise from the convolution of the projection image with the PSF can be analyzed and corrected by the appropriate correction of the structure factors of the object in Fourier space. As is commonly known, the CTF produced with the conventional defocus method is oscillating and approaching zero for low spatial frequencies. Only certain bands of spatial frequencies with alternating positive (black atomic density on white background) and negative (white on black) are represented in the image. Between these bands, close to the CTF zeros, the signal transfer is particularly low and object structure factors are lost. This is especially the case for the lowest spatial frequencies, corresponding to extended features or the overall shape of the imaged object. For CET, these low-resolution features are essential, e.g. to obtain continuous membrane structures within the reconstructed volume. Therefore, large defocus values have to be applied, often in the order of 10–15 μm. As a consequence, the structural resolution is limited to a narrow spatial frequency band.

To improve the contrast of weak-phase objects in CET, we are currently evaluating a variety of different phase plates. Images recorded in focus with a phase plate contain strong contrast, reaching from the lowest (currently ~1/50–1/100 nm −1) to the highest spatial frequencies in Fourier space. The corresponding phase plate PSF is particularly narrow, resulting in a well-de-
fined object point representation. As no defocus artifacts arise, in-focus phase contrast images represent a true projection of the object mass density. Consequently, in-focus tomograms yield a more faithful representation of the structure in question and can be interpreted more safely. For imaging with phase plate-mediated contrast, there are no inherent resolution limits imposed by oscillations and transfer gaps of the CTF.

In this article, we describe the optical principle of Hilbert phase contrast and its first application in electron tomography. A theory of TEM image formation for thick objects is outlined. It is experimentally shown that high resolution object information is almost entirely transferred via phase contrast. The contrast of a typical native frozen-hydrated specimen can be enhanced by a Hilbert phase plate while recording images in focus. We present a number of examples for the numerical correction of the antisymmetric Hilbert PSF, which is essential for the 3D reconstruction by any kind of back projection algorithm. A quantitative comparison of tomograms from conventional defocus phase contrast and Hilbert phase contrast shows the improvements obtained by in-focus phase contrast. Of particular importance is the ease of use of the Hilbert phase plate in a conventional electron microscope. Current experience with different kinds of phase plates in practical work (Majorovits et al., 2007; Barton, 2008) favors the Hilbert phase plate for ET for conventional electron optics, as alignment artifacts and obstructing effects of the diffraction pattern are minimal.

2. Theory of defocus and in-focus contrast generation for biological electron tomography

2.1. Image formation for thick biological objects

Objects investigated in CET, typically consisting of organic material embedded in vitrified ice, may have a thickness up to 500 nm. The attenuation of the beam by such specimens is considerable, predominantly resulting from inelastic scattering and subsequent zero-loss energy filtering. It is also not clear whether the phase distortion of the specimen exit wave is still small, as in the case of a thin weak-phase object. The exit wave of a thick object may be written in the general form

$$\psi(r) = A(r) \exp\{-i\eta(r)\},$$

where \(r = (x,y)\) are coordinates within the specimen plane. Here, \(A(r)\) and \(\eta(r)\) denote the modulation of amplitude and phase of the incident plane wave.

Simulations show that the phase shifting potentials (or electron refractive indices) \(\eta' = d\eta/dz\) of organic material (e.g. protein) and the embedding vitrified ice differ only slightly. Majorovits (2002) finds a value of 2.18°/nm for protein and 1.96°/nm for vitreous ice, so that \(\Delta\eta' \approx 0.22°/\text{nm}\). The total phase distortion of the incident plane wave after passage through the specimen is thus

$$\eta(x,y) = \int_{-\frac{1}{2}}^{\frac{1}{2}} \Delta\eta'(x,y,z)\,dz.$$  

For a specimen of thickness \(t = 500\) nm, the phase shift could theoretically be as large as \(110°\), but only in the very unrealistic case that the organic material is arranged in “columns” along the beam axis \(z\). Cellular systems studied in CET consist of lipid membranes, protein particles and vesicles that can be distributed almost homogeneously. It was shown that even for an object of 500 nm thickness which is embedded in vitreous ice, the projection \(\eta(r)\) will have values in the range of 4–8° (Barton, 2008). In any case, the exit wave front can be considered as the superposition of a uniformly phase shifted plane wave and a slightly distorted wave, \(\eta(r) \rightarrow H + \eta(r)\). Without loss of generality, the “global” phase \(H\) can then always be set to zero, as it applies to both the scattered and the reference wave. Therefore, the approximation \(\eta \ll 2\pi\) is justified even for a thick frozen-hydrated specimen, and the exit wave phase can be written as

$$\exp\{-i\eta(r)\} \approx 1 - i\eta(r) - \frac{\eta^2(r)}{2}.$$  

This holds also e.g. for unstained, resin-embedded biological samples.

On the other hand, the wave amplitude will decrease exponentially with the specimen thickness. The factor \(A\) in Eq. (1) generally quantifies the wave amplitude reduction (“electron loss”). This loss may originate from removing inelastically scattered electrons by the use of an energy filter or from intersection of high angle scattering by an objective aperture. Although thick specimens often reduce the amplitude of the incident wave by \(>50\%\) (Koster et al., 1997), the variation of the lateral amplitude distribution \(A(r)\) is small (<1) for a typical biological object (Barton, 2008). With this assumption, the exit wave amplitude can be rewritten as the sum of a constant, global amplitude reduction \(A\) and the small variation \(a(r)\):

$$A(r) \rightarrow A + a(r)$$  

The variation \(a(r)\) represents the object amplitude signal. The amplitude attenuation is proportional to the logarithm of the object’s mass density, while the phase shift is directly proportional. Therefore, \(a(r)\) can be assumed to be small compared to the phase variation \(\eta(r)\) for thick specimens. Finally, the exit wave of a thick biological specimen can be rewritten as

$$\psi(r) \approx [A - a(r)]\left[1 - i\eta(r) - \frac{\eta^2(r)}{2}\right]$$

$$\approx A\left[1 - a(r) - \frac{\eta^2(r)}{2} - i\eta(r)\right].$$  

Here, terms of the order \(a\eta\) and \(a\eta^2\) are neglected. Apart from the constant amplitude factor \(A\), Eq. (5) is equivalent to the weak-phase-weak-amplitude approximation for thin objects. It is possible that Eq. (4) does not hold for the entire field of view, i.e. if \(A(r)\) contains large “steps”. An example is a dense cell located next a “void” region with nothing but ice. For such a case, the field of view can always be split up in sub-regions where Eq. (4) can be applied. The entire image is then formed from superposition of the sub-images, using different \(A\), and thereby retrieving the original amplitude distribution.

2.2. Contrast transfer and SNR

The imaging theory for a weak-phase-weak-amplitude object is described in general by Danev and Nagayama (2004). The image intensity for a phase object is

$$I(r) = A^2 - 2AF^{-1}\mathcal{S}(k).$$  

Here, \(F^{-1}[\mathcal{S}(k)]\) is the inverse 2D Fourier transform of the image spectrum \(\mathcal{S}(k)\). The latter can be written as

$$\mathcal{S}(k) = F[a(r)/A + \eta(r)/2]a\text{CTF}(k) + F[\eta(r)]p\text{CTF}(k).$$  

In other words, the transfer of the specimen’s amplitude and phase information is modulated by the amplitude and phase contrast transfer functions aCTF and pCTF, respectively. The CTFs are instrument-specific functions. It is worth noting that with Eq. (6) the SNR of an image directly depends on the CTFs:

$$\text{SNR} = \frac{\sigma\text{(signal)}}{\sigma\text{(noise)}} \approx \frac{2A}{\sigma\{N(r)\}} \frac{1}{n} \sum_{|k|} \left|F^{-1}[\mathcal{S}(k)]\right|.$$
The index \( \mathbf{r} \), denotes the summation over \( n \) image pixels \((\sigma, 2D \text{ standard deviation}; N(\mathbf{r}), \text{arbitrary image noise distribution}) \). A direct proportionality of contrast and SNR is also given by Reimer (1984, p. 443).

For conventional TEM (cTEM), the contrast transfer functions are

\[
\begin{align*}
\text{aCTF}(k) &= \cos W(k) \\
\text{pCTF}(k) &= \sin W(k)
\end{align*}
\]

(9)

with the spatial frequency \( k \) and the wave aberration

\[
W(k) = -Zj k^2 + j C_0 k^4
\]

(10)

\((Z, \text{defocus}; \lambda, \text{electron wavelength}; C_0, \text{spherical aberration coefficient})\). As a result of the sine-dependence of the conventional pCTF, a weak-phase object generates negligible contrast when recorded close to Gaussian focus \((Z \rightarrow 0)\).

2.3. In-focus phase contrast transfer by a Hilbert phase plate

To overcome the problem of low phase contrast and incomplete signal transfer, the Nomarski or differential interference contrast (DIC) technique has been routinely applied for decades in light microscopy. For TEM, its equivalent was first described and tested by Nagayama and Danev, who refer to it as the Hilbert phase contrast (HPC), as it involves an optical Hilbert transform (Nagayama, 2002). The principle of the HPC consists in adding a constant phase shift, the \( \text{pCTF} \) Eq. (8) becomes

\[
\text{pCTF}_H(k) = \begin{cases} 
\sin W(k) & |k| \leq g \\
-\text{sign}(k_i) \cos W(k) & |k| > g.
\end{cases}
\]

(11)

The cut-on frequency \( g \) takes into account that the edge of the half-plane will be positioned at some finite distance \( d = \lambda f g \) from the center of the diffraction pattern, in order to leave the central unscattered beam unaffected \((f, \text{focal length of objective lens})\). Within a “gap region” where \( k_i \leq g \), the object signal is transferred as the sine of the wave aberration, as in cTEM. Outside the gap, the pCTF equals to a cosine-dependent transfer, with an additional multiplication of structure factors at \( \pm k \) by \( \pm i \) (see Fig. 2A). Due to the sine-to-cosine transformation of the pCTF, the Hilbert phase plate produces particularly strong contrast especially for low spatial frequencies, as \( \cos(0) = 1 \). Caused by the \( \text{sign}(k_i) \) modulation, the resulting image PSF is antisymmetric. As shown in the experimental Hilbert images, this leads to a topographic representation of density gradients within the object. The image appears similar to a landscape that is illuminated from one side, the illumination direction determined by the direction of the phase plate (see Fig. 2B).

Besides the desired phase shift, incoherent inelastic and multiple elastic scattering of electrons by the carbon film lead to a reduction of coherent signal amplitude (Majorovits, 2002; Danev and Nagayama, 2008). This “loss” \( 1 - a(t) \) occurs only in one half-plane of the BFP. Therefore, it can be accounted for by introducing a single-sideband term into Eq. (11). Thus, a more realistic description of the HPC signal transfer outside the gap is given by the sum of an antisymmetric cosine and a single-sideband term:

\[
\text{pCTF}_H(k) = -\text{sign}(k_i) \left[ a_i \cos W(k) + \frac{1 - a_i}{2} \exp(i \text{sign}(k_i) W(k)) \right]
\]

(12)

(Danev and Nagayama, 2004). It is worth noting that the amplitude reduction by the factor \( a_i \) gives rise to single-sideband signal transfer rather than causing complete information loss (Majorovits et al., 2007). If an image is acquired in Gaussian focus and the gap is made infinitesimally small \((W \rightarrow 0 \text{ and } g \rightarrow 0)\) the Hilbert pCTF becomes

\[
\text{pCTF}_H(k) = -\frac{1 + a(t)}{2} \text{sign}(k_i).
\]

(13)

This holds for a resolution range where the spherical aberration term in Eq. (10) can be neglected. The antisymmetric \( \text{sign}(k_i) \) artifact can be eliminated numerically by multiplying the image Fourier Transform with \( i \text{sign}(k_i) \) (cf. Fig. 2). If a HPC image is recorded in focus and numerically corrected, it will represent a nearly perfect projection of the object’s phase shifting potential. Even if spherical aberration is taken into account, the corrected Hilbert pCTF will have a value greater than 50% for spatial frequencies \( k \approx 0.9 (C_0^2 \lambda^2)^{-1/4} \). For a 300 kV microscope with \( C_0 \) of 2.0 mm, this means high contrast for all sample features at resolutions up to 0.39 nm.

3. Material and methods

3.1. Implementation of Hilbert phase plates

The technical implementation of HPC chosen for this work is based on the phase plate design by Nagayama and Danev (Nagayama, 2002; Danev and Nagayama, 2004). A layer of amorphous carbon film with a sharp, straight edge is inserted in the back focal plane of the objective lens, its surface perpendicular to the optical axis. The edge is positioned laterally as close as possible to the optical axis without obstructing the high intensity of the central beam. In practice, this can be as close as 200 nm, as is shown in Fig. 2. When passing through the carbon layer of thickness \( t \), the electrons are exposed to a positive mean electrostatic potential \( U(t) \) generated by the atomic nuclei. The total energy is changed during the passage, and the phase of the electron wave is thereby shifted by an amount

\[
\phi(t) = \pi \frac{U(t)}{U_0} t.
\]

(14)

\( \chi(U(t)) = 2(\text{mc}^2 + eU)/(2 \text{mc}^2 + eU(t)) \) is a relativistic constant depending on the acceleration voltage \( U_0 \), \text{m} \), and \( e \) are the rest mass and charge of the electron, respectively. Different values for the mean potential \( U(t) \) can be found in the literature. While Reimer gives
a value of +7.8 V (Reimer, 1984), we used a more recent value for the potential of $U_r = +10.7$ V (Harscher and Lichte, 1998; Wanner et al., 2006). For an acceleration voltage of $U_a = 200$ kV, this results in a carbon film thickness of 40.4 nm to obtain a phase shift of ~180°. For a 300 kV microscope, a thickness of 45.0 nm is necessary.

Novel designs of implementing Zernike- or Hilbert-type phase plates have been recently described that generate phase shifts by spatially confined electrostatic (Schultheiss et al., 2006; Majorovits et al., 2007; Cambie et al., 2007; Schröder et al., 2007) or magnetic (Yasuta et al., 2006) potentials in the BFP. All these are in the process of development or require major modifications of the electron optics, whereas the simple Hilbert phase plate can be used for biological imaging with minimal modifications to the electron microscope.

### 3.2. Specimen preparation

As test specimens, we chose 1st—a frozen-hydrated preparation of lipid vesicles mixed with ferritin molecules and 2nd—slightly contrasted sections of resin-embedded skeletal muscle from mice and unstained, uncontrasted sections of resin-embedded *Pichia pastoris* (yeast) cells. The cryo samples were prepared on standard specimen grids coated with holey carbon film (Quantifoil, Jena, Germany) by plunge-freezing in liquid ethane held at liquid nitrogen (N$_2$) temperature. The samples were kept at N$_2$ temperature during imaging in the TEM. The mouse muscle tissue was fixed with 2.5% glutaraldehyde (GA) and 0.1 M Na-cacodylat buffer, embedded in low viscosity resin (Agar), microtome-sectioned (~100 nm thick) and subsequently exposed to osmic acid vapor. The yeast cells were fixed with 4% paraformaldehyde, 1% GA and 0.1 M Na-cacodylat buffer and then embedded in LR White Resin (London Resin Company). The cells were microtome-sectioned without any additional staining. The sections were mounted on a standard electron microscopy grid coated with a thin carbon support film. Unstained yeast cell sections were chosen to test the new technique for a sample that generates an even weaker phase contrast than it is obtained from typical frozen-hydrated preparations. This is caused by the almost identical mass densities of the organic material and the embedding medium (resin).

### 3.3. Image and tilt series acquisition

Cryo images of ferritin-decorated lipid vesicles were recorded using a 300 kV TEM (Tecnai G2 “Polara”, FEI Company, Hillsboro, USA) on a 4 × 4 k pixel CCD camera with 14 μm pixel size (Gatan, Pleasanton, USA), binned to 2 × 2 k. Zero-loss energy filtering was applied. The images were recorded using a primary magnification of 22,100× resulting in an image pixel size of 1.27 nm. The applied electron dose of ~9 Å$^{-2}$ per image was kept as low as possible to allow for comparison of two images (cTEM and HPC) of the same specimen area without major radiation damage effects. For HPC images, a carbon half-plane (thickness 45 ± 5 nm) was positioned in the BFP. A rough mechanical alignment perpendicular to the beam axis using a standard objective aperture drive was followed by an electron-optical fine alignment by slightly varying the image tilt, so that the zero beam passes in ~200 nm distance to the carbon edge of the phase plate. A distance in this range was kept to avoid exposure of the carbon foil to the high intensity of the focal spot, as this leads to accumulation of insulating contamination by surface diffusion (Danev, personal communication). As for the Polara instrument the illumination is nonparallel (conical) by default, the objective lens crossover plane can be adjusted to the phase plate plane by varying the strength of the 2nd condenser lens (“illumination”). Specimen and phase plate were surrounded by an anticontaminator held at liquid nitrogen temperature. This allowed for the recording of >50 images without observable contamination effects. After recording an image series, contamination of the phase plate could be either observed or was assumed, and the phase plate was mechanically shifted along the edge by ~10 μm to obtain a fresh area of carbon foil. While using this method, no additional heating of the phase plate was necessary.

To test the experimental applicability and performance of Hilbert phase contrast for electron tomography, we recorded tilt series images at high defocus without a phase plate (conventional technique) as well as in focus with a Hilbert phase plate and compared the results. The tomogram tilt series were recorded using a 200 kV FEG TEM with a corrected in-column 90° energy filter (“SE-SAM II”, Carl Zeiss NTS, Oberkochen, Germany). Images were zero-loss energy-filtered and recorded with a 1 × 1 k pixel slow scan CCD camera (Gatan, Pleasanton, USA) with a pixel size of 24 μm. The primary magnifications were 8,500 and 16,500×. The conventional tilt series were recorded with a defocus of 15–20 μm. For acquisition of the HPC in-focus tomograms, a Hilbert phase plate as described in Section 2.2 was inserted into the back focal plane of the objective lens, while tilt images were recorded in focus. The necessary positioning of the phase plate with an accuracy of <10 nm was achieved by a piezo-driven nanomotor (Kleindiek, personal communication).
Reutlingen, Germany). The SESAM provides parallel ("Köhler") illumination. By varying the excitation of the 3rd condenser lens, we introduced a slightly conical illumination to match the crossover plane perfectly to the phase plate plane. To minimize contamination and charging, an anticontaminator surrounding the phase plate was kept at liquid nitrogen temperature. With the objective chamber at room temperature, the recording of a tomogram tilt series was not possible, owing to a rapid build-up of image artifacts caused by charging of the carbon foil. Image focus and lateral position of the carbon edge were checked by inspection of the power spectrum and adjusted if necessary after each set of ~10 images. This proved to be sufficient to maintain close-to-optimum Hilbert contrast for the entire tilt series. Otherwise, the phase plate contrast was not affected by specimen tilting or electron beam exposure. Exchanging the phase plate was not necessary during acquisition of individual tilt series. Linear tilt schemes, i.e. constant tilt angle increments, were used in all the experiments. For the mouse muscle sections, an increment of 1.5° with a maximum tilt angle of ±45° was used. The total electron dose applied was ~1500 Å² for both HPC and cTEM. For the yeast cell sections we tilted ±60°; with an increment of 2°. Here, a dose of ~400 Å² was used. About 30 min. were necessary to record the tilt series (61 images), and the acquisition time was not substantially increased for the HPC series. Tomograms were recorded without applying low-dose conditions because of the low radiation sensitivity of resin-embedded samples. Gold markers were added to the sections for subsequent tilt series alignment.

3.4. Image processing and tomogram generation

Images from the conventional defocus phase contrast tilt series were determined from periodograms, and the phases of Fourier spectra flipped accordingly (Frank and Penczek, 1995; Fernandez et al., 2006). Considering the sample area and the achievable resolution, it was sufficient to measure one average defocus value for each tilt image. The Hilbert PSF (cf. Section 2.3) was numerically symmetrized (see Fig. 2) by multiplication of

$$p_{CTF_n}(k) = 2i \left[1 + \exp \left(\frac{k - g}{T}\right)\right]^{-1} - \frac{1}{2}$$  \hspace{1cm} (15)

in Fourier space. The temperature factor $T = g/\ln(1/0.8 - 1)$ was chosen so that the Fermi function has a value of ±80% at the borders of the Hilbert gap. A Fermi edge was applied rather than a sharp edge to account for a blurring resulting from a slight misalignment of the phase plate in beam direction. The rotation of the Hilbert edge was determined once for each image series using the zero tilt image and the Hilbert gap width was measured automatically for each individual image to account for lateral drift of the phase plate during acquisition. For all image processing steps, dedicated algorithms compiled under the program package MatLab (The MathWorks, Natick, USA) were used. After Hilbert PSF correction of each individual image, tomograms were generated using the IMOD package (Boulder Laboratories, Boulder, USA) using marker points for image alignment. The resulting volumes were slightly denoised using nonlinear anisotropic diffusion (Frangakis and Hegerl, 2001), while the same parameters were used for conventional and Hilbert contrast volumes.

4. Results

4.1. Quantification of signal loss and pCTF phase shift

Fig. 3 shows experimental power spectra calculated from images of amorphous carbon film, recorded with a 300 kV TEM (Tecnai G² “Polara”, FEI company, Hillsboro, USA). Conventional and HPC image data recorded with strong underfocus (~20 µm) are compared. Fig. 3A contains the typical pattern of concentric Thon rings. In the HPC spectrum (Fig. 3B), the sine-to-cosine transition of the pCTF can be directly observed: the Thon rings inside the gap (vertical stripe) follow a sine functionality, whereas outside the gap the Thon ring pattern is inverted. This indicates a 90° phase shift of the pCTF and thus a cosine-shaped phase contrast transfer for spatial frequencies affected by the phase plate.

The radially averaged intensities of the power spectra are compared in Fig. 3C. The amplitude of the HPC contrast transfer maxima is reduced by ~30% compared to the data without phase plate. This is a consequence of the expected loss of coherent signal caused by interactions of the scattered electrons with the carbon film. This is a good transfer of low spatial frequencies, because the dominating intensity contribution comes from scattering angles within the HPC gap and is therefore not affected by the carbon film (cf. Fig. 1). Both images are zero-loss energy-filtered, so that inelastic scattering by the phase plate does not contribute to the background noise of the data. According to Eq. (12), the amplitude reduction of the Hilbert pCTF peaks is equal to the carbon film transmissivity $a_c$, which was thereby measured to be ~70%. This value roughly agrees with the published results of other groups (Kaneko et al., 2005).

4.2. Hilbert phase contrast imaging of frozen-hydrated specimens

Fig. 4 shows a comparison of conventional defocus and in-focus HPC images of a typical CET specimen. As expected from theory, the conventional near-focus image (Fig. 4A) of frozen-hydrated lipid vesicles decorated with ferritin (an iron-containing protein molecule) show particularly weak contrast. The outlines of the vesicles and individual ferritin molecules become visible by strong defocusing of ~25 nm (Fig. 4B). A substantial enhancement compared to the defocus phase contrast is achieved when the same object is imaged in focus with a Hilbert phase plate inserted in the BFP (Fig. 4C). The membranes of the rather extended vesicles, being 100–500 nm in diameter, appear with strong topographic contrast. This indicates a good transfer of low spatial frequencies, which is a characteristic feature of the phase plate imaging technique. In comparison, the lowest spatial frequencies are underpronounced in the defocus images (Fig. 4B); a consequence of the sine-shaped contrast transfer. The difference in object representation between the defocus and the in-focus HPC technique also becomes apparent in Fig. 4D–G, where close-ups from Fig. 4B and C are shown. The images prove that beside the strong low-resolution contrast, the phase plate images also contain significant contrast for structural details (individual ferritin particles). The ferritin molecules are clearly visible in both the conventional and the HPC image. The advantage of HPC is demonstrated by a sharper and more localized representation of membranes and ferritin molecules. This reflects the fact that the conventional PSF, corresponding to the rapidly oscillating defocus CTF, is significantly broader than the Hilbert PSF. Producing sharp images and strong contrast at the same time is the main advantage of the novel HPC technique over conventional defocus CET.

4.3. Phase and amplitude contrast of thick objects

Han et al. (1995) showed that the problem of weak phase contrast persists for thick specimens (0.3–1 µm), such as ice-embedded cells or organelles as they are analyzed in CET. Objects with a thickness above 0.5 µm cause about 60% inelastic scattering which can be partially converted into amplitude contrast by energy filtering (Angert et al., 2000). However, this component contributes only at resolutions well below 25 nm. The exit wave reconstruction
Fig. 3. (A) Power spectrum of a conventional 300 kV bright-field image of amorphous carbon film recorded with ~20 μm underfocus (41 kx primary magnification). The bright concentric Thon rings correspond to the contrast transfer maxima of the conventional pCTF = sin W(κ). Dark rings indicate spatial frequency bands with vanishing signal. (B) Power spectrum from the same specimen area imaged with a Hilbert phase plate (~45 nm thick) in the BFP of the objective lens. The Thon ring patterns in the "gap" region and the HPC region of the spectrum are inverted, indicating the sine-to-cosine transition of the pCTF for the spatial frequencies influenced by the phase plate. (C) Radial intensities of the power spectra A and B. The contrast transfer amplitude of the HPC signal (height of the oscillation peaks above the background, cf. Eq. (12)) is reduced by 29% with respect to the transfer of the corresponding bright-field image (cf. Section 2.3). The defoci of the images are slightly different, so that the pCTF phases cannot be directly compared.

Fig. 4. Images of native, frozen-hydrated lipid vesicles mixed with ferritin molecules (image pixel size 1.27 nm). The weak-phase contrast of the close-to-focus image (A) is substantially enhanced by either strong underfocusing of ~25 μm (B) or the insertion of a Hilbert carbon film phase plate while imaging close to Gaussian focus (C). The pseudo-topographic contrast of the HPC image yields a clear outline of the vesicular structures with dimensions of 100–500 nm (white arrow in C). In comparison, the contrast for these low-resolution features of the sample is less pronounced in the conventional defocus image (B). This effect originates in the better transfer of low spatial frequencies by the in-focus HPC technique, corresponding to the mass density projection of extended objects. Both the defocus cTEM and the in-focus HPC image show significant contrast for the individual ferritin complexes with a size of ~5 nm (arrowheads in C), representing high resolutions. (D–G) Close-ups from B and C. In the defocused cTEM micrographs (D and F) the individual layers within membrane stacks ('M') cannot be clearly distinguished. The HPC images (E and G) show a better resolution of the layers (arrows). Furthermore, due to the narrower PSF, the blurring of the ferritin molecules ('F') is reduced in the HPC images. This yields a more precise representation of the actual size of the molecules. The same electron dose of ~9 Å⁻² was applied for the HPC image (recorded 1st), the defocus image (2nd) and the close-to-focus image (3rd).
for thick specimens showed significant phase contrast, and this contribution dominates for resolutions better than 10 nm (Han et al., 1995). These findings are confirmed by the data shown in Fig. 5. Images of medium thick (~150 nm) sections of unstained, resin-embedded yeast cells are high-pass filtered in Fourier space. The bright-field image recorded in Gaussian focus (Fig. 5A) is generated predominantly from amplitude contrast, as the phase contrast is \( \sin W(k) \approx 0 \) for \( Z = 0 \). In the corresponding high-pass filtered image, where the lowest object frequencies \( (<1/100 \text{ nm}^{-1}) \) are removed, almost no object structures remain visible (Fig. 5E).

If, on the other hand, a phase contrast image is high-pass filtered, cellular features such as membranes and protein complexes can be recognized (Fig. 5F, defocus phase contrast; Fig. 5G, HPC). High-resolution information for thick biological specimens is almost entirely transferred via phase contrast. Consequently, apart from a low-resolution amplitude contribution, such objects have to be treated as phase objects for high resolution imaging and tomography.

4.4. Simulation of images, constrains on the experimental implementations

The two alignment parameters for the Hilbert carbon film phase plate are the orientation of the carbon edge and the distance of the edge from the unscattered, central beam in the BFP (Fig. 2). To understand better the influence of these parameters, HPC images of ribosomes were simulated as shown in Fig. 6. Model ribosomes (EMD entry 1055, Valle et al., 2003) were imaged with a simulated vitrified ice-embedding corresponding to 40% Gaussian noise. Conventional defocus images show the expected weak phase contrast, so that the particles can hardly be distinguished from the background noise. Numerical CTF correction does not improve the situation (Fig. 6B,C).

In comparison, the in-focus HPC simulations show a substantially enhanced SNR, and thereby an excellent visibility of the individual ribosome particles (Fig. 6D–K). It is evident that the edge position, i.e. the cut-on frequency \( g \) for the cosine-shaped contrast transfer, has the strongest effect on the final PSF-corrected HPC images (cf. Fig. 6F,G and J,K). To retrieve spatial frequencies as low as possible and thereby obtain the highest possible SNR, the carbon edge has to be positioned as close as possible to the focal spot of the BFP. However, a few 100 nm of edge distance is required in typical TEMs to prevent damage of the carbon foil by the intense zero beam. In practice, a distance as close as 200 nm can be realized without apparent damage of the phase plate (cf. Fig. 2). In comparison to the influence of the gap width, a small deviation from the ideal thickness of the Hilbert phase plate has a negligible effect. The ribosome images simulated for 190° and 170° show no apparent difference in phase contrast (Fig. 6D,E and H,I), and this does not change upon Hilbert PSF correction (Fig. 6F,G and J,K). Likewise, the effect of the orientation of the carbon edge appears to be marginal (data not shown).

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Fig. 5. Images of unstained, uncontrasted thin sections of resin-embedded P. pastoris cell sections (section thickness about 150 nm, magnification 13.3 kx, image pixel size 1.80 nm) with and without a Hilbert phase plate. Unlike in frozen-hydrated samples, the mass densities of the organic material and the embedding medium (London resin) are nearly the same, which further decreases phase contrast. (A) Image without phase plate close-to-focus. The low-resolution elastic and inelastic (zero-loss energy filter) amplitude contrast reveals few structural details. (B) Image recorded with high defocus (~15 nm). The contrast is increased, but the image shows point spread artifacts such as an over-pronounced granularity in the cytoplasm, most likely originating from the carbon support film. The structural resolution is compromised by the CTF oscillation, as indicated by Thon rings in the power spectrum inset. (C) Focused image recorded with a Hilbert phase plate in the BFP. (D) Same image as (C) after numerical PSF correction. (E–H) Close-up of the framed area in A, after removing all spatial frequencies below 1/300 nm\(^{-1}\) in images (A–D). The enclosed mitochondrion is invisible in the focused image (E), indicating that the image contains no high resolution phase contrast. The defocus image (F) has faint contrast. In the HPC image (G), the mitochondrion and its cristae (arrow) are clearly visible. After Hilbert PSF correction (H), some objects in the cytoplasm can be distinguished (inset).
defocus of about 15–20
number of projections of randomly oriented ribosomes from the EM database (EMD, Tagari et al., 2002) were generated in vacuum. (B–K) Simulated images for a 200 kV TEM with f = 3.0 nm. Gaussian noise was added to simulate ice-embedding. The mean projected densities of protein and ice were adjusted to be 1.36 (Kühlbrandt, 1981), and the standard deviation (SD) of the gaussian noise intensity to be 40% of the SD of the ribosome projections. Phase contrast images were then simulated according to the theoretical description in Section 2. (B) Bright-field image with an underfocus of 1 μm. (C) Same image as B, corrected for the CTF by numerical phase-flipping. (D–G) In-focus HPC images with an edge distance from the beam axis of 500 nm, corresponding to a cut-on frequency g = 1/(15 nm) (see text). For phase shifts of 170° (D) and 190° (E), i.e. ±6° deviation from the ideal value of 180°, no significant difference can be observed. (F and G) Images (D and E) after numerical correction for the antisymmetric Hilbert PSF using Eq. (15). (H–K) HPC simulations for an edge distance of 200 nm, resulting in a cut-on at 1/(37.6 nm). The contrast is significantly stronger than in (D–G), due to the increased transfer of lower spatial frequencies. (H and I) Again, a small deviation from the ideal 180° has a negligible impact on imaging. (J and K) After Hilbert PSF correction, both images yield a near-perfect representation of the projected object density A.

Theory predicts zero contrast in the image direction corresponding to the carbon edge. However, for the general visibility of the molecule outline, whose edges do not coincide with the orthogonal direction of the carbon edge in the diffraction plane, such vanishing contrast in a singular direction is not relevant. In the PSF-corrected HPC images (Fig. 6F,G and J,K), the particular orientation of the phase plate can hardly be recognized.

4.5. Unstained yeast cell sections as a test for cryo Hilbert tomography

To investigate the improvement of the SNR by the Hilbert phase plate for specimens with a very low intrinsic contrast, we recorded tilt series of thin unstained sections of yeast cells embedded in resin. As a consequence of the low difference in densities of the embedding polymer and the biological components, the cells remain almost invisible when imaged in Gaussian focus (Fig. 5A,E). Features such as cell organelles or the cell wall can be visualized only with strong defocus or by use of a Hilbert phase plate (Fig. 5B,C).

During acquisition of the conventional tilt series, we applied a defocus of about 15–20 μm in order to generate sufficient contrast for the most prominent object features (Fig. 7A,C). On the other hand, when imaging with the Hilbert phase plate the cells and their internal features were clearly visible in focus (Fig. 7B,D). In Fig. 8 we show sections through the reconstructed volumes, comparing the resulting object visibility of both techniques. In the conventional reconstruction, the mitochondrial membranes (Fig. 8A,C) appear with faint contrast. In comparison, the Hilbert phase contrast reconstruction yields a much clearer outline of the cellular features (Fig. 8B,D). Densities inside the mitochondrial membranes appear with strong contrast in the HPC volume (Fig. 8G), while they can hardly be recognized in the conventional reconstruction (Fig. 8E). Despite the low magnification of 8,500×, intracellular protein complexes close to the mitochondrial surface, most likely ribosomes, can be distinguished in the HPC slice (arrowheads in Fig. 8F).

4.6. Tomography of mouse muscle sections

The enhanced contrast of individual myosin filaments in the HPC tomogram of a mouse muscle section can be clearly seen in the reconstructed volume shown in Fig. 9. In the reconstruction calculated from defocus images, the filaments have a granular appearance, an artifact of the convolution of the object density with the extended, ring-shaped defocus PSF (Fig. 9A–C). The filaments break apart into artificial fragments, as can be seen in the close-ups from the surface-rendered isosurface representations (Fig. 9C). Individual filaments can hardly be tracked throughout the volume or counted (Fig. 9B). On the other hand, the Hilbert reconstruction contains continuous filaments that are well separated from each other (Fig. 9E). This continuous appearance underlines the presence of structure factors for low spatial frequencies...
that are lost in defocus images. At the same time, the overemphasized granularity owing to CTF oscillations at higher spatial frequencies is strongly reduced. When comparing the close-up of the 3D isosurface visualization in Figs. 9C and F, it is obvious that the HPC reconstruction is a better and less noisy representation of the myosin filaments.

5. Discussion

The first experimental HPC tomograms of thick biological objects demonstrate the favorable imaging conditions of in-focus phase contrast. The overall improvement in contrast and signal for weak-phase objects has been demonstrated before for Zernike-type carbon phase plates in general (Danev and Nagayama, 2001), and more recently also in a quantitative study (Danev and Nagayama, 2008). Hilbert-type phase contrast imaging has so far only been tested in a qualitative way (Kaneko et al., 2005, 2006), whereas our study shows for the first time a quantitative application. It is shown that after numerical PSF correction, in-focus HPC images can be used for tomographic reconstructions.

An important result of the experiments is the surprising ease of use of the carbon Hilbert phase plate even for the acquisition of long image series, such as extended tilt series. Zernike-type carbon phase plates are hampered by difficult alignment and contamination (Marko et al., 2005; Majorovits, 2002). In contrast, the technically simple Hilbert phase plate requires alignment in only one direction. The intense zero beam irradiates the carbon foil less often, which substantially reduces the accumulation of contamination on the phase plate surface during tilt series acquisition, compared to a Zernike phase plate with round geometry. A large number of physical phase plates can be stored inside the microscope column at the same time by using a simple setup as described in Fig. 1. When image artifacts caused by contamination occur, the carbon edge can easily be replaced simply by moving along the edge. The effective contamination rate of the phase plate may be further reduced by heating of the phase plates (Danev and Nagayama, 2001). The simulations and the experimental results demonstrate that the low contrast transfer in one direction (corresponding to the carbon edge in the BFP) does not impede the visibility of biological objects or their quantitative reconstruction in a tomogram after correction for the specific Hilbert PSF.

Our experiments and the previously published results show that in-focus phase plate imaging improves object contrast and SNR also for thick samples (Setou et al., 2006). This is equally true for frozen-hydrated preparations (Fig. 4) and for the mixed amplitude and phase contrast observed for resin-embedded sections (Fig. 5). Although we are well aware of the more complex nature of the image formation for thick objects, it is interesting to note that the weak-phase approximation is obviously applicable. We present here a theoretical approach to explain this phenomenon.

The two biological samples used in this study to test the application of in-focus Hilbert phase contrast in tomography demonstrate a major visual improvement of SNR in the resulting volumes. However, a quantitative assessment of the level of improvement is difficult. In a recent study of single particles (Danev and Nagayama, 2008), the answer to the question of contrast enhancement is not very clear: resolution did not improve, but the number of particles needed to obtain a given resolution decreased as result of the improved visibility. The situation in our case seems to be similar: the processing of the myosin thick filament array did not show any additional features in the periodicities of the filaments. However, a thresholding of the volume as a first step to segmentation shows a clearer representation of the outline and organization of the filaments within the muscle fiber (Fig. 9). Similarly, internal structures in whole cells (Fig. 8) are visible in the HPC tomograms, whereas even after CTF correction they are not recognizable in defocus phase contrast tomograms.

We have shown that in-focus Hilbert phase contrast can be experimentally applied to tomography and leads to better visibility and generally to a more faithful representation of the object density. In the future it needs to be shown that HPC can also be used for CET under low-dose conditions. There were some difficulties encountered for CET under low-dose conditions preventing the comparison of HPC tomography of frozen-hydrated samples with the conventional technique within the timeframe of this study. One of the difficulties was that the automated tilt series routine featured in the utilized commercial TEM did not provide the required axial beam alignment stability. This made positioning of the phase plate during tomogram acquisition difficult. However,
finding an appropriate optical alignment with two defined points (rotation center of image and focal spot in the BFP) and making the necessary minor modifications to the software should be possible in the future. From the current experience, we expect that only when using vitrified samples and state-of-the-art equipment it will indeed be possible to achieve an improved resolution by Hilbert phase contrast electron tomography.

6. Conclusion

Our trial tomograms show that the application of a Hilbert phase plate in electron tomography can enhance the signal-to-noise ratio of the 3D reconstruction significantly, while the applied electron dose and all other acquisition parameters are unchanged. As a result of the contrast enhancing phase shift by a physical phase plate, the microscope can be operated in focus while recording high contrast images of weak-phase objects. Charging or contamination of the carbon film was not a problem, nor was the positioning of the phase plate in the BFP very critical for tilt series acquisition. After a straightforward, automated procedure correcting for the antisymmetric PSF, Hilbert phase contrast yields tomograms with high signal for features such as the outline of large structures or macromolecular complexes.

In Hilbert ET, the object information encoded in the object exit wave is almost ideally converted into image contrast. Contrast generation by a phase plate does not compromise resolution as in the case of conventional, highly defocused images. Given the limitation of electron dose with radiation-sensitive specimens, the Hilbert phase plate has the potential to enhance the SNR of tomograms, which allows an interpretation with higher accuracy. It is worth noticing that the integration of a Hilbert phase plate does not compromise the use of other
electron-optical components, such as energy filters or aberration correctors and does not require additional expensive hardware.

Acknowledgments

The authors would like to thank Radostin Danev and Kuniaki Nagayama for initial discussions on the use of carbon films as Zernike and Hilbert phase plates. They also thank Götz Hofhaus for cryo specimen preparation, and Werner Kühlbrandt for his continuous support. The work was funded by the Deutsche Forschungsgemeinschaft filed under Schr 424/11-1.

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