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In-focus electron microscopy of frozen-hydrated biological samples with a Boersch phase plate

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ABSTRACT

We report the implementation of an electrostatic Einzel lens (Boersch) phase plate in a prototype transmission electron microscope dedicated to aberration-corrected cryo-EM. The combination of phase plate, C_s corrector and Diffraction Magnification Unit (DMU) as a new electron-optical element ensures minimal information loss due to obstruction by the phase plate and enables in-focus phase contrast imaging of large macromolecular assemblies. As no defocussing is necessary and the spherical aberration is corrected, maximal, non-oscillating phase contrast transfer can be achieved up to the information limit of the instrument. A microchip produced by a scalable micro-fabrication process has 10 phase plates, which are positioned in a conjugate, magnified diffraction plane generated by the DMU. Phase plates remained fully functional for weeks or months. The large distance between phase plate and the cryo sample permits the use of an effective anti-contaminator, resulting in ice contamination rates of <0.6 nm/h at the specimen. Maximal in-focus phase contrast was obtained by applying voltages between 80 and 700 mV to the phase plate electrode. The phase plate allows for in-focus imaging of biological objects with a signal-to-noise of 5–10 at a resolution of 2–3 nm, as demonstrated for frozen-hydrated virus particles and purple membrane at liquid-nitrogen temperature.

1. Introduction

In transmission electron microscopy (TEM), organic materials composed of light elements such as H, C, N and O can mostly be treated as weak-phase objects. The weak-phase approximation is applied to practically all biological specimens investigated by electron cryo-microscopy (cryo-EM), as well as to many polymers and other soft materials studied in materials science. The entire information on the projected Coulomb potential distribution of a weak-phase object is contained in a small distortion of the object exit wave plane, which for ice-embedded protein corresponds to a phase ripple of only a few degrees [1]. These small phase shifts have then to be converted into phase contrast to create an interpretable image. Amplitude contrast for such objects, which is created by scattering outside the objective lens aperture, only accounts for ~7% of the image contrast [2]. For the faithful reconstruction of a weak-phase object, phase contrast has to be maximized, while keeping the electron dose at a minimum. At electron energies of 100–300 keV typically used in TEM, inelastic scattering outweighs elastic scattering by roughly a factor of 4 for light elements. This causes dose-dependent energy deposition, and the associated radiation damage limits the electron dose that can be applied to biological samples to 500 to 5000 e^-/nm^2, depending on the object temperature and desired resolution. To make matters worse, the detection quantum efficiency (DQE) of available electron detectors is only in the range of 10–50% and images are subject to substantial noise. Thus there is a great need for methods that maximize the information content per scattered electron, and especially to enhance the inherently weak signal-to-noise ratio (SNR) in cryo-EM.

Even for a weak-phase object, the phase of the object exit wave is not represented directly by the image intensity, but convolved with a linear, instrument-dependent point-spread function (PSF). The contrast transfer function (CTF), as the Fourier transform of the PSF, modulates object structure factors in frequency space. Obtaining a SNR sufficient for reconstructing weak-phase objects thus depends critically on the CTF of the electron microscope. The phase contrast transfer function (pCTF) for a given spatial

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frequency k in conventional TEM is given by the sine of the wave aberration w, damped by an envelope function E_{stv}:

$$pCTF(k) = \sin[w(k)] \cdot E_{stv}(k). \quad (1)$$

E_{stv} is the product of damping envelopes originating from limited spatial and temporal coherence, as well as mechanical vibrations. For a perfect, aberration-free image, phase contrast close to focus is zero. This is particularly unfortunate as the advent of aberration correctors now produces sub-atomic resolution images of objects that are less radiation-sensitive, such as metals or semiconductors [3]. For atomic resolution imaging, contrast can be generated for single atom columns by introducing small residual aberrations [4], and the exit wave can be calculated from focal series [5]. However, in biological TEM and many soft materials applications, atoms cannot be resolved, because the required radiation dose would destroy the object features of interest.

In conventional TEM, contrast for weak-phase objects is generated by underfocussing the image. This results in an oscillating pCTF with undesirable, alternating bands of positive and negative contrast, with regions of negligible contrast between these bands (Fig. 1a). Except at a high defocus of several μm, the contrast for features of a few nm in size is always weak. Even perfect C_S correction will not improve this situation. On the one hand, it will not increase phase contrast at low and intermediate (>1 nm) resolution for imaging close to focus, as shown in Fig. 1. On the other hand, at an underfocus of 100 nm to several μm, which is often used in biological TEM and electron tomography (ET) to produce intermediate resolution contrast, C_S correction will not change the unfavorable pCTF characteristics described above. Although aberration correction will extend the information limit of the instrument, this is usually not what limits the resolution achieved with dose-sensitive objects. Without a phase plate, a C_S corrector therefore would seem to offer no advantage for imaging soft materials in a conventional TEM. However, in combination with a phase plate that modulates the contrast transfer of the microscope, aberration correction will generate optimal phase contrast [6] in the entire range from low ($\sim$50 nm) to high resolution ($\sim$0.2 nm). The resulting contrast transfer (Fig. 1b) is almost ideal for cryo-EM.

Here we present the first implementation of an electrostatic Boersch phase plate (BPP) in an aberration-corrected TEM dedicated to low-dose cryo-EM of frozen-hydrated biological specimens, referred to as the PACEM (Phase contrast Aberration-Corrected EM). The prototype is based on a Zeiss Libra 200 electron microscope (Carl Zeiss, Oberkochen, Germany). The PACEM images weak-phase objects in Gaussian focus using C_S correction and thus maximizes phase contrast generated by the phase plate. We demonstrate in-focus cryo imaging with a SNR of $\sim$5 for object features in the 2–3 nm range in frozen-hydrated tobacco mosaic virus (TMV). The phase contrast signal for 2D crystals of bacteriorhodopsin is increased by a factor of two by the phase plate in images recorded within 50 nm of Gaussian focus.

We also address the problem of the limited lifetime of the Einzel lens devices [7,8]. We compare devices fabricated by a new, scalable process that makes use of UV lithography and a cleanroom environment, and compare them to the electrostatic phase plates fabricated by the process described by Schultheiß et al. [9]. Given their limited lifetime, a steady supply of fresh phase plates is critical for future routine application of BPPs in biological electron microscopy.

2. Theoretical background: In-focus TEM with a physical phase plate

Theory predicts that in-line holography with an electrostatic Boersch phase plate (BPP), mounted in an aberration-corrected TEM, should give near-optimal image SNR for weak-phase objects [6]. This optimal condition results from a phase plate that converts the sine pCTF to a cosine pCTF, in combination with a C_S corrector that minimizes lens aberrations. An ideal phase plate adds a constant $\pm 90^\circ$ phase shift to the unscattered wave relative to the scattered wave in the diffraction plane of the TEM. In theory, this results in near-perfect contrast transfer for aberration-corrected images of weak-phase objects (Fig. 1b), with

$$pCTF_{BPP}(k) = \sin(\pm 90^\circ) \cdot E_{stv}(k) = \pm E_{stv}(k). \quad (2)$$

This holds if the wave aberration is sufficiently well corrected within the observed range of spatial frequencies ($w=0$). Even in an uncorrected TEM, in-focus imaging with a phase plate would produce a substantial increase in phase contrast transfer, compared to conventional defocus contrast (Fig. 1b; [10]).

Due to the optimized phase contrast transfer, phase plate imaging directly retrieves the phase of the object exit wave from a single image of a weak-phase object. Although this approach has long been advocated [11], technical problems have precluded the

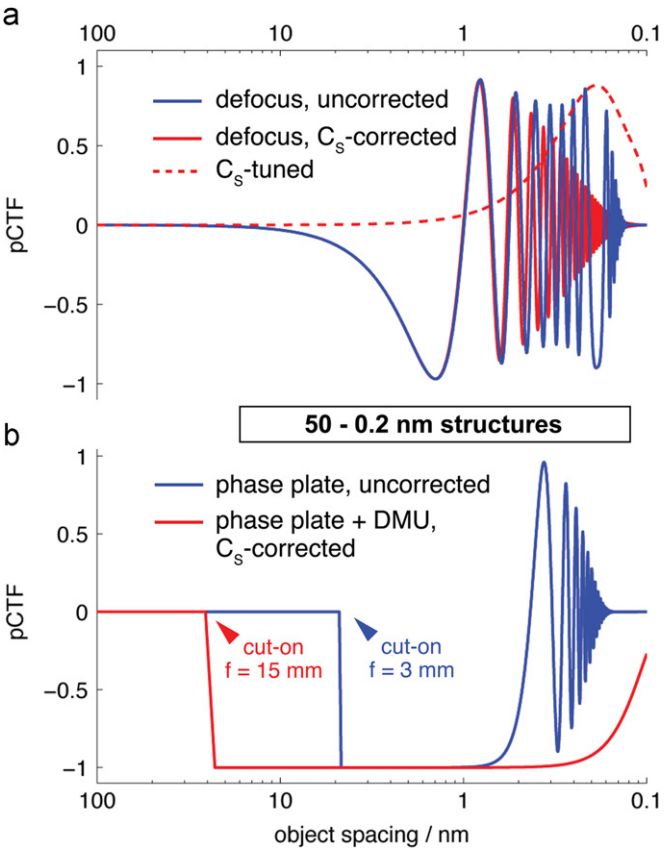

Fig. 1. C_S correction combined with a phase plate results in optimal phase contrast for cryo-EM. (a) Applying an underfocus of 400 nm in a conventional, uncorrected TEM generates an unfavorable pCTF with zeros and contrast inversions for periodicities <1 nm, while signal transfer at low resolution is poor. C_S correction does not improve this situation, instead theory predicts an even steeper envelope for $C_S=0$ [25]. Optimal tuning of defocus and spherical aberration ($C_S=-21$ μm, 8.0 nm overfocus; [5]) yields good transfer for large spatial frequencies, but contrast transfer is even worse than defocus contrast at low to intermediate resolution. (b) In-focus pCTFs obtained using a BPP with 3.2 μm outer electrode diameter and -90° pCTF phase shift. In an uncorrected TEM with $f=3$ mm, good signal transfer is generated within a resolution band reaching from the cut-on frequency to the first oscillation caused by the wave aberration. A near-ideal transfer for phase objects is achieved by combining a C_S corrector with a phase plate in a magnified diffraction plane ($f=15$ mm), as realized in the PACEM. Signal transfer now covers almost the entire range of structure factors of interest for structural biology. All calculations assume 200 keV electron energy, a convergence semi-angle of 0.2 mrad, a chromatic defocus spread of 2.9 nm and $C_S=2.2$ mm for the uncorrected TEM.