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Expanding the boundaries of cryo-EM with phase plates

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Phase plates have long been considered as a means for improving the performance of cryo-electron microscopy (cryo-EM). But practical limitations, such as a short lifespan or cumbersome usage have prevented their widespread adoption. The recently developed Volta phase plate overcomes most of the practical issues and it is now commercially available. Results from both, electron cryo-tomography (cryo-ET) and single particle analysis (SPA), have demonstrated the benefits of using a phase plate. In CET phase plates have helped to visualize cellular ultrastructure in unprecedented detail. In SPA phase plates allowed to determine the structures of small proteins at near-atomic resolutions. Further improvements in phase plate technology are possible and new designs are already under development.

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For a complete overview see the Issue and the Editorial

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Introduction

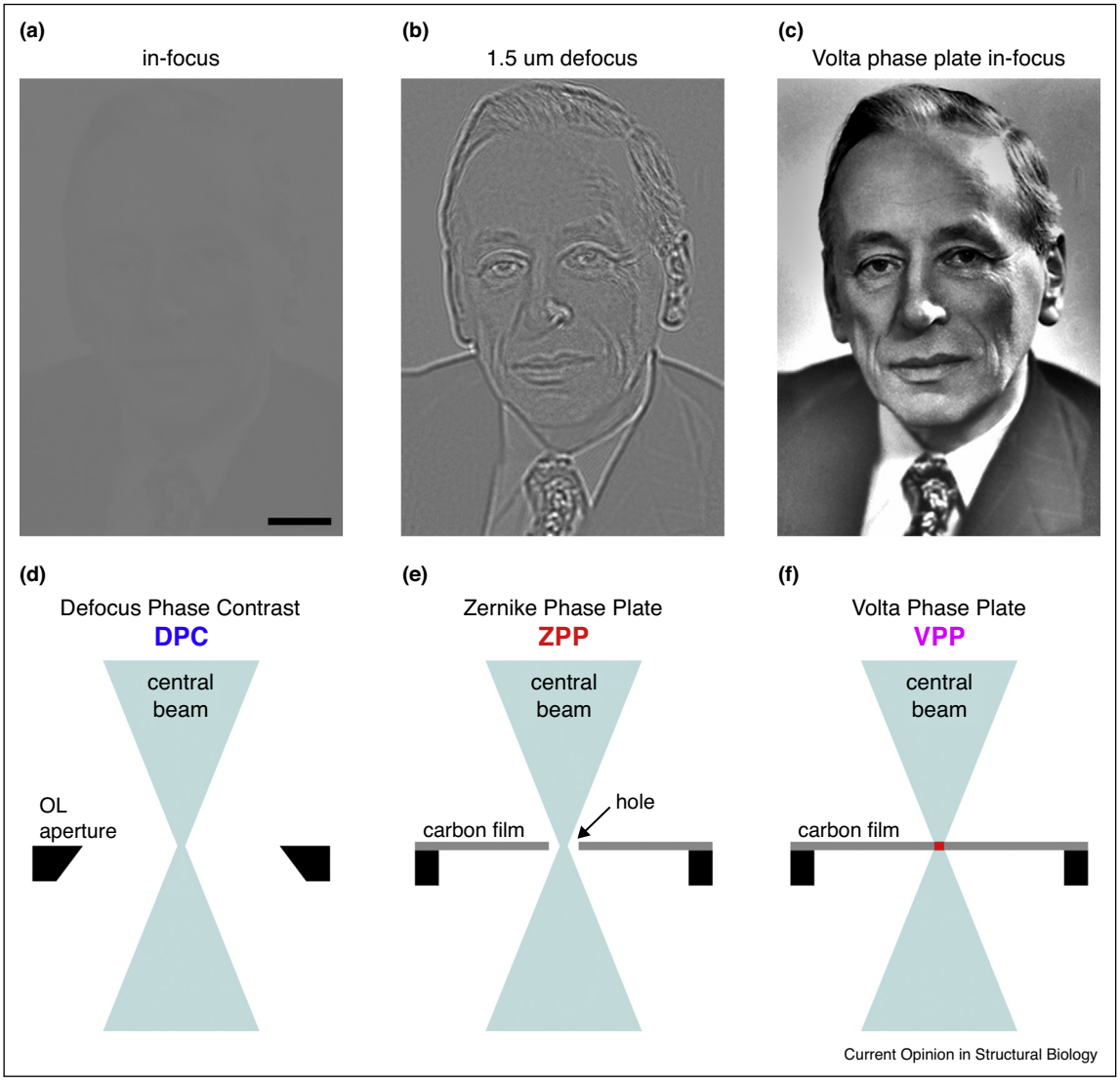
In recent years, in the wake of technological and methodological improvements, cryo-electron microscopy (cryo-EM) has reached a new level of performance; some refer to this as the 'resolution revolution' [1]. And yet, the performance is still fundamentally limited by the radiation sensitivity of biological materials and the need to minimize exposure to the electron beam. As a consequence, signal-to-noise ratio of the images is relatively poor and necessitates the application of averaging to retrieve structural information. The dose limit problem is exacerbated by the low contrast of frozen-hydrated biological samples which consist predominantly of light elements. Electron-optically, they behave like weak-phase objects, that is, they modify weakly the phase of the electron wave but not its amplitude (Figure 1a) [2]. Therefore, phase contrast is essential for extracting a maximum of information from biological samples with a tolerable electron dose.

Phase contrast in cryo-EM is traditionally produced by defocusing the objective lens, generating defocus phase contrast (DPC) [2] (Figure 1d). This method provides good information transfer for intermediate to high spatial frequencies but performs poorly at low spatial frequencies. Consequently, DPC images have a high-pass-filter-like appearance and overall low contrast (Figure 1b) which makes them difficult to interpret directly and necessitates various image restoration techniques.

Phase plates are devices which generate phase contrast without the need of defocusing and over a wide range of spatial frequencies (Figure 1c) [3]. They were proposed and theoretically considered already in the early days of transmission electron microscopy (TEM) [4]. Various types of phase plates were tested over time but for several decades no usable device could be built [5]. In the early 2000s a new wave of phase plate experiments started and a promising candidate was developed – the thin film Zernike phase plate (ZPP) (Figure 1e) [6]. It consists of a thin amorphous carbon film with a small hole at the center. For over 10 years the ZPP was tested and used in proof-of-principle applications with some success [7]. Good quality data could be collected, but the ZPP suffered from a number of practical problems [8]. The first issue was the short lifetime of the ZPP. After a few days in the electron microscope the performance degraded and the phase plate had to be exchanged. The second problem was the requirement to precisely center the hole of the ZPP on the beam path. This could not be automated and data collection had to be carried out manually.

A few years ago, a new type of phase plate was proposed – the Volta phase plate (VPP) (Figure 1f) [9]. It is very similar in design to the ZPP but without a central hole. The VPP takes advantage of a phenomenon, which as of today is not yet fully understood; the phase shift is created by the interaction of the electron beam with the continuous carbon film. The current hypothesis is that the beam causes physicochemical changes on the surface of the film which lead to a local change of the work function and in turn to a local surface potential difference. Those changes are temporary and after a few days the surface of the film recovers to its original state by reactions with the residual gasses inside the electron microscope. After their recovery, positions on the VPP can be reused in the following experimental sessions. Consequently, the VPP has a very long practical lifespan and if not damaged mechanically can be used for years. The fact that the phase shift is self-created by the electron beam means that the VPP does not require precise centering and thus can be easily implemented in automated data acquisition schemes.

Figure 1



(a–c) Simulation of a weak-phase object imaging in an electron microscope. The simulation was performed using a portrait of Frits Zernike (1888–1966), the inventor of phase contrast microscopy, as a model object with a maximum phase shift of 0.2π. Scale bar: 20 nm. **(a)** An in-focus image of a weak-phase object shows almost no contrast. **(b)** A defocus phase contrast (DPC) image of a weak phase object has a 'ghost-like' appearance due to the high-pass-filter effect of DPC. **(c)** A Volta phase plate image of a weak-phase object is a close-to-original representation of the model object. (d–f) Phase contrast methods in electron microscopy with their configuration at the back-focal-plane of the objective lens (OL). **(d)** Defocus phase contrast uses an aperture at the back-focal plane. **(e)** Zernike phase plate consists of a thin amorphous carbon film with a small hole centered on the central diffraction beam. **(f)** The Volta phase plate comprises a continuous amorphous carbon film. The phase shift is generated by the modification of the surface properties of the film by the central diffraction beam.

However, the VPP has some limitations. Because it uses a carbon film in the beam path some of the electrons are scattered which leads to ~18% (at 200 kV) signal loss [9]. Additionally, different positions on the VPP could introduce varying amounts of astigmatism due to local variations of the film quality or wrinkling of the film. An ideal phase plate device should not introduce any materials in the beam path to avoid electrostatic charging or scattering effects. Such a device is already under development; in one such design the phase shift is created by a high-intensity focused laser beam [10,11]. If it can be successfully implemented, the laser phase plate could become a permanent phase contrast solution for TEM.

Cryo-tomography with phase plates

Electron cryo-tomography (cryo-ET) is severely limited by the radiation sensitivity of the biological material and the low contrast of ice-embedded biological samples [12].

Cellular structures are subject to stochastic variations and therefore they are unique structures, limiting the application of image averaging techniques and posing challenges for image segmentation. When tomograms contain repetitive structures, subtomogram averaging can be used to improve the signal-to-noise ratio. Subtomogram analysis permits amplification of the signal by averaging of multiple instances of the same object. In such cases the signal-to-noise ratio of a tomogram determines the accuracy of the alignment of the subtomograms as well as their classification.

Phase plate applications in cryo-ET began with the ZPP. The initial results were promising, showing greatly improved contrast [13], visibility of fine features [14,15] and improved subtomogram averaging performance [16]. A striking example of ZPP cryo-ET is a work by Dai *et al.* [17] investigating the maturation process of cyanophage Syn5 inside *Synechococcus* host cells (Figure 2a). To that end, they manually acquired tens of tomograms with the ZPP. On average it took them ~12 hours on the microscope to acquire 3–4 tomographic tilt series [18]. In comparison, an automated tilt series acquisition nowadays takes ~45 min.

The VPP solved most practical issues of the ZPP and is being used with the standard automated tilt series acquisition routines. In the two years since its introduction, the VPP demonstrated its usefulness for cryo-ET in the structural studies of several systems. Fukuda *et al.* [19•] analyzed the practical cryo-ET performance of the VPP alone and in combination with an energy filter. They measured a ~16% improvement in contrast for cellular membranes by zero-loss energy filtering and ~61% by the VPP. Asano *et al.* [20••] used VPP cryo-ET to investigate the spatial distribution of 26S proteasomes in intact neurons and were able to determine their state of assembly and conformational states (Figure 2b). This was a pioneering demonstration of the potential of cryo-ET for studying of protein complexes *in situ*. Sharp *et al.* [21] analyzed the membrane attack complex (MAC) pore structure in lipid bilayers. Chlanda *et al.* [22] studied the hemifusion structure formed between influenza virus-like particles and liposomes. Mahamid *et al.* [23•] demonstrated the usefulness of phase plates for visualizing in unprecedented detail the molecular sociology of the periphery of a HeLa cell nucleus (Figure 2c). In their work, the delicate meshwork of the nuclear lamina was visualized for the first time *in situ* and it provided a glimpse of the dynamics of nuclear pores. Khoshouei *et al.* [24] compared the subtomogram analysis performance of the VPP with that of DPC using ribosomes as a test sample. There was no significant difference in resolution but the Fourier shell correlation curve (FSC) of the phase plate data was smoother and did not show the information gaps characteristic for DPC (Figure 4 in [24]).

The main practical problem in using phase plates for cryo-ET is electrostatic charging of the specimen [5,25]. Non-conductive samples become charged by the electron beam and this could move or deform the beam on the phase plate. The only practical solution is to have a conductive support film or to coat the samples with conductive material [25]. Another practical issue with phase plates is accurate focusing. Most phase plate tomograms are acquired in-focus and the resolution is determined by the ability to focus precisely [24]. Non-flat samples, such as cryo-FIB lamellas, pose challenges for maintaining precise focus throughout the tilt series [25].

Single particle analysis with phase plates

Single particle analysis (SPA) recently entered the atomic resolution age with the adoption of direct electron detectors [1]. The significant boost in performance provided by these cameras enabled high-resolution studies of smaller protein complexes than previously possible but there are still limitations to the technique. Proteins smaller than ~200 kDa are challenging for DPC single particle analysis [26] and so far there are only a few examples of reaching near-atomic resolutions.

Phase plates are expected to expand the capabilities of cryo-EM by simplifying the study of small (<200 kDa) proteins [3]. Such samples are challenging for DPC because of the poor signal-to-noise in the images which affects the ability to accurately align them to a reference and determine their orientation. Phase plates provide additional low spatial frequency signal which improves the performance of such operations. In a theoretical study, Henderson [27] estimated that the smallest particle size for which the orientations can be determined is approximately $38/C^2$ kDa, where $0 < C \leq 1$ is the contrast relative to that of a perfect phase contrast image. Thus with an ideal phase contrast electron microscope ($C = 1$) one should be able to solve the structures of $\geq$38 kDa molecules to atomic resolutions. The above relation between molecule size and contrast indicates that improving the contrast has a strong influence on the minimum size which allows a high resolution structure determination, and to that effect phase plates are an obvious solution. In a later work, Glaeser [28] estimated that even smaller molecules (~20 kDa) should be accessible by single particle analysis. Using computer image simulations, Chang *et al.* [29] investigated the performance of ZPP for several molecules of different size. According to their results, for a small (100 kDa) molecule the phase plate significantly outperforms DPC in terms of number of particles required to reach near-atomic resolution. In another simulation study, Hall et al. [30] concluded that the improved contrast provided by a phase plate will lead to more accurate alignment of 100 kDa protein images against a reference and make it easier to classify particles and to disentangle coexisting states.

Figure 2

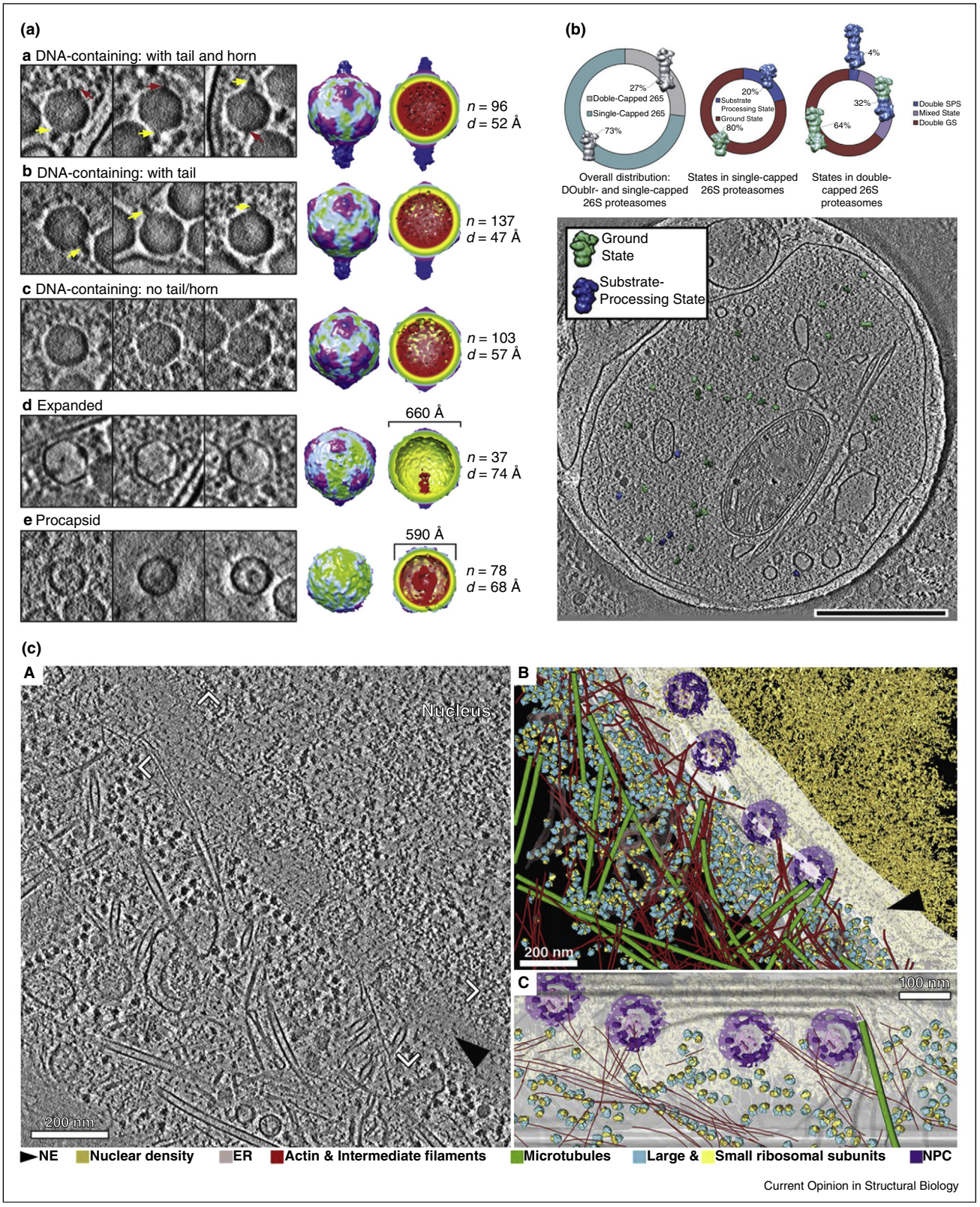

(a)
a DNA-containing: with tail and horn
n = 96
d = 52 Å
b DNA-containing: with tail
n = 137
d = 47 Å
c DNA-containing: no tail/horn
n = 103
d = 57 Å
d Expanded
660 Å
n = 37
d = 74 Å
e Procapsid
590 Å
n = 78
d = 68 Å
(b)
27%
Doble-Capped 265
Single-Capped 265
73%
20%
Substrate Processing State
Ground State
80%
4%
32%
64%
Double SPS
Mixed State
Double GS
Overall distribution: DOublr- and single-capped 26S proteasomes
States in single-capped 26S proteasomes
States in double-capped 26S proteasomes
Ground State
Substrate-Processing State
(c)
A
Nucleus
200 nm
B
200 nm
C
100 nm
NE
Nuclear density
ER
Actin & Intermediate filaments
Microtubules
Large &
Small ribosomal subunits
NPC
Current Opinion in Structural Biology

Table 1

Summary of Volta phase plate single particle analysis results

	20S proteasome [33]	20S proteasome [34••]	Peroxiredoxin-3 [35]	Nucleosome [36]	Hemoglobin [37•]
Molecular weight [kDa]	700	700	257	200	64
Defocus [nm]	In-focus	500 nm	In-focus	In-focus	500 nm
Pixel size [Å]	1.35	1.06	1.74	1.38	0.525
Electron dose [e^{-}/A^{2}]	30	39	20	31	40
Number of particles	13 469	93 596	8580	26 060	175 374
Symmetry	D7	D7	D6	C2	C2
Number of asymmetric units	188 566	1 310 344	102 960	52 120	350 748
B-factor [$Å^{2}$]	123	74	137	79	200
Resolution [Å]	3.2	2.4	4.4	3.9	3.2

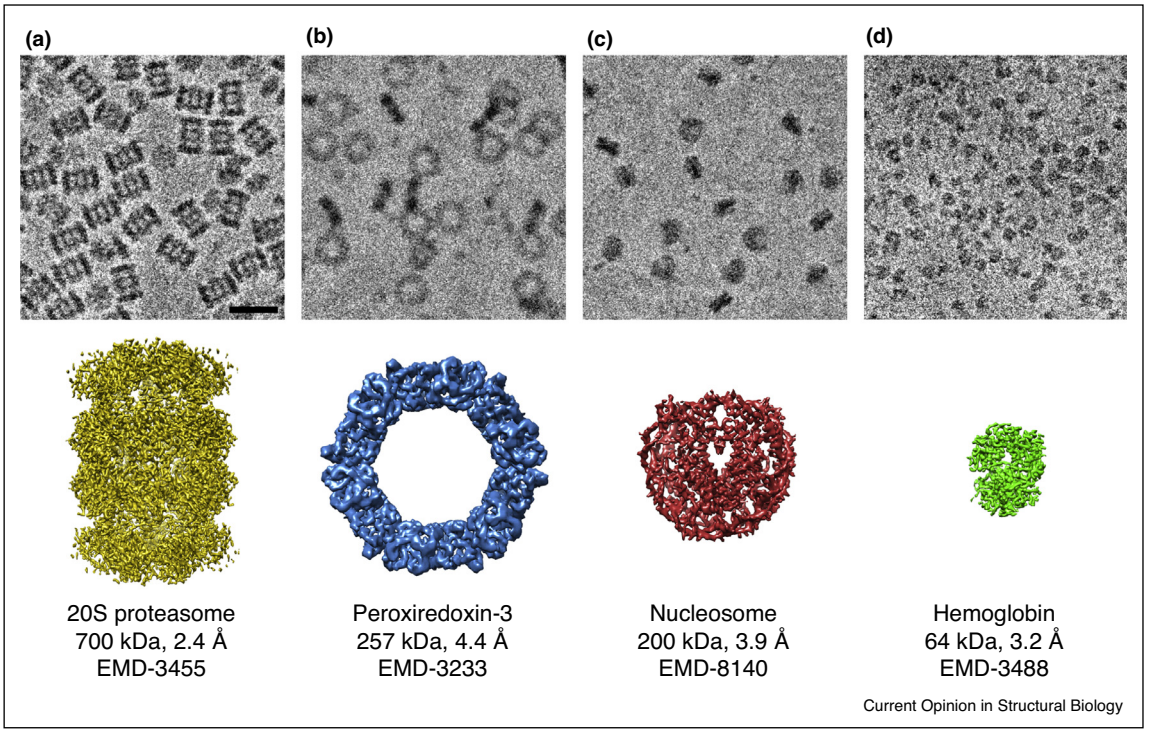
Initial experimental trials with phase plates in SPA, like in cryo-ET, started with the ZPP [16,31]. In those studies the data had to be collected manually which limited the size of the datasets. Focusing was performed manually and this affected focusing accuracy. Therefore, the resolution in all ZPP SPA studies was limited to ~1 nm. Nevertheless, the advantage of using phase plates for SPA was successfully demonstrated by Rochat *et al.* [32] by visualizing the portal complex of herpes simplex virus type 1 capsids. Previous studies based on DPC were unable to positively identify the location of the portal because of its small size. Despite the modest resolution, which was partially due to the use of CCD cameras in those early works, there were already good indications of the ability of phase plates to visualize small proteins. Danev *et al.* [8] presented high-contrast in-focus ZPP images of 200 kDa (dissimilatory sulfite reductase) and 88 kDa (VacA toxin) molecules. Glaeser [3] later demonstrated the ability to detect single 55 kDa streptavidin molecules with a special single-sideband 'tulip' aperture.

The VPP offering the possibility for automation and its use in conjunction with direct electron detectors enabled for the first time near-atomic resolution structure determination with phase plates. The first such demonstration with the 20S proteasome, a complex of ~700 kDa, reached a resolution of 3.2 Å [33] (Table 1) and used an in-focus data acquisition approach. From a theoretical point of view the in-focus approach is optimal because it provides uniform information transfer in Fourier space, without the characteristic contrast transfer function (CTF) oscillations. However in practice, the in-focus method is quite challenging because it requires very precise focusing and stigmation (in the order of nanometers) to reach high resolutions [33]. The iterative focusing steps slow down the data acquisition and thus reduce the overall efficiency. In addition, the resolution of in-focus data reconstructions is limited to ~3 Å by the spherical aberration of the objective lens [33]. A follow-up work [34••] combined the VPP with a small amount of defocus (500 nm) and improved the resolution to 2.4 Å (Figure 3a and Table 1). In this case, defocusing served a different purpose. In DPC the defocus is the main means of generating contrast whereas with the VPP it is used to enable CTF fitting and correction. The combination of VPP and defocus has several practical advantages. It mitigates the requirement for precise focusing and thus improves the data acquisition speed. The possibility to measure and correct the CTF removes the resolution limitation stemming from spherical aberration. And last but not least, the visibility of CTF rings allows for a quantitative evaluation of the quality of each micrograph. Both works described above were technical evaluations of the performance of the VPP and used a relatively big (~700 kDa) and highly symmetric (14-fold) test specimen, the 20S proteasome. Such samples are easy to solve without a phase plate and do not pose a challenge for DPC.

Actual biological applications of the VPP began less than two years ago and with its help already several challenging structures could be solved to high resolution. Khoshouei *et al.* [35] determined the structure of the human peroxiredoxin-3, a 257 kDa dodecamer, at 4.4 Å resolution (Figure 3b and Table 1). The challenge with this sample, apart from its size, was that the researchers had to collect data in relatively thick ice areas because of a preferred orientation issue in thin ice. Thicker ice reduces the contrast and the signal-to-noise ratio but with the help of the VPP they were able to achieve near-atomic resolution. In a recent paper, Chua *et al.* [36] presented the first high-resolution cryo-EM structure of the nucleosome core particle, at 3.9 Å resolution (Figure 3c and Table 1). The DPC challenge with this sample was its small size of ~200 kDa and the disk shape of the complex making certain orientations difficult to detect in DPC micrographs. The most recent work by Khoshouei *et al.* [37•] has demonstrated the ability to solve the structure of

(Figure 2 Legend) Phase plate electron cryo-tomography (cryo-ET) examples. **(a)** Maturation steps of cyanophage Syn5 inside Synechococcus host cells observed with Zernike phase plate cryo-ET [17]. **(b)** *In situ* census of 26S proteasomes in intact neurons investigated by Volta phase plate (VPP) cryo-ET [20••]. Scale bar: 500 nm. **(c)** Molecular sociology of the HeLa cell nuclear envelope visualized by VPP cryo-ET [23•].

Figure 3



Single particle analysis with the Volta phase plate (VPP) at near-atomic resolution. **(a)** 20S proteasome at 2.4 Å resolution [34••]. **(b)** Human peroxiredoxin-3 at 4.4 Å resolution [35]. **(c)** Nucleosome at 3.9 Å resolution [36]. **(d)** Human hemoglobin at 3.2 Å resolution [37•]. Scale bar: 20 nm.

small molecules. With the help of the VPP they were able to solve the structure of human hemoglobin to 3.2 Å resolution (Figure 3d and Table 1). At the time of this writing, this is the smallest single protein structure determined by cryo-EM SPA at near-atomic resolution.

The main challenges in using phase plates for single particle analysis include focusing, CTF determination and phase shift management. The original theoretical goal of using phase plates in-focus requires very precise focusing and stigmation, which complicates and slows down the data acquisition [33]. On the other hand, combining the VPP with a small amount of defocus simplifies the experiment but requires CTF fitting and correction [34••]. The fits must include an additional parameter for the VPP phase shift, which makes them less robust than DPC CTF fits. To ensure reliable results, the output of the CTF fitting has to be monitored for consistency and if necessary the fitting parameter ranges must be adjusted. Usually, a narrower defocus range and a higher low resolution limit (~20 A) improve the VPP CTF fit accuracy [34••]. The VPP phase shift is not constant and increases with the accumulated dose on the phase plate. During data collection the phase plate has to be moved to a new position after every ~50 images. The exact number depends on the beam settings [34••].

Summary and outlook

Phase plates are an emerging technology in cryo-EM which can improve performance beyond what is already provided by direct electron detectors. Results are already routinely obtained with the current generation of phase plates, namely the VPP. In cryo-electron tomography, phase plates allow the study of cellular structures *in situ* and with unprecedented resolution. For subtomogram analysis phase plates offer advantage when small (<200 kDa) protein complexes are studied *in situ*. In single particle analysis, the VPP has clear advantage for structure determination of small particles. Samples of sufficient size (>200 kDa) and homogeneity can be solved using DPC and usually do not require the use of a phase plate. Small samples (<200 kDa), although sometimes possible to solve by DPC, are easier to solve with a phase plate. Apart from providing the ability to determine the structures of small molecules, phase plates, with their improved contrast and lack of CTF artifacts, make it much easier to judge the sample quality and homogeneity by simply looking at the first few micrographs obtained with a new sample. The current generation of VPPs occasionally suffers from performance inconsistencies but future generations will hopefully solve the manufacturing issues.

Graphene has to be explored for the manufacturing of VPPs, instead of the currently used amorphous carbon

films. Graphene would probably allow to use thinner layers and thus reduce the information loss by scattering caused by the phase plate. Higher acceleration voltage microscopes could also provide improvement in phase plate performance by reducing the phase plate scattering loss, in addition to an increased depth of focus and better sample penetration. New phase plate devices, such as a focused high-intensity laser, could provide a stable, lossless phase plate with a real-time phase shift control. Ultimately, a more robust, optically stable microscope platform with intelligent software control could make the phase plate as simple to use as DPC. With such a system there would be no need to revert to DPC, even for 'easy' samples, and all data could ultimately be collected with the phase plate.

Conflict of interest

Radostin Danev is a co-inventor with no financial benefits in US patent US9129774 B2 'Method of using a phase plate in a transmission electron microscope'. Wolfgang Baumeister is on the Scientific Advisory Board of FEI Company.

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