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Accurate modelling of single-particle cryo-EM images quantifies the benefits expected from using Zernike phase contrast

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Abstract

The use of a Zernike-type phase plate in biological cryo-electron microscopy allows the imaging, without using defocus, of what are predominantly phase objects. It is thought that such phase-plate implementations might result in higher quality images, free from the problems of CTF correction that occur when images must be recorded at extremely high values of defocus. In single-particle cryo-electron microscopy it is hoped that these improvements in image quality will facilitate work on structures that have proved difficult to study, either because of their relatively small size or because the structures are not completely homogeneous. There is still a need, however, to quantify how much improvement can be gained by using a phase plate for single-particle cryo-electron microscopy. We present a method for quantitatively modelling the images recorded with 200 keV electrons, for single particles embedded in vitreous ice. We then investigate what difference the use of a phase-plate device could have on the processing of single-particle data. We confirm that using a phase plate results in single-particle datasets in which smaller molecules can be detected, particles can be more accurately aligned and problems of heterogeneity can be more easily addressed.

Keywords

Zernike phase contrast; cryo-electron microscopy; multislice; image simulation; heterogeneity

Introduction

In single-particle cryo-electron microscopy (cryo-EM) a field of single molecules frozen in a thin layer of vitreous ice is imaged using a transmission electron microscope (TEM). Biomolecules, made up predominantly of protein or nucleic acid, are similar in density to the surrounding water, and they are normally modeled as weak phase objects, with little amplitude contrast. In standard single-particle cryo-EM, image contrast is generated by defocusing the microscope, which results in a contrast transfer function (CTF) with low values at low resolution and values that oscillate between +1 and -1 at high resolution (Frank, 2006). Recent developments in instrumentation have focussed on practical methods

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for imaging biomolecules without using defocus, instead generating contrast using a Zernike phase plate (Boersch, 1947). Methods have been developed using both a thin carbon-film phase plate (Danev et al., 2009) and electrostatic fields (Cambie et al., 2007; Majorovits et al., 2007; Schultheiss et al., 2006).

It is thought that using such a phase plate will improve the quality of the raw images taken in single-particle cryo-EM, allowing smaller molecules to be imaged and also allowing for the detection of smaller differences in structurally heterogeneous samples. It is important to quantify just how great the benefits of Zernike phase contrast can be, however, both to motivate the development of phase-plate technology and to provide a benchmark that can be used to judge how closely the experimental work approaches what should be physically possible to achieve. Simulating single-particle cryo-EM imaging allows us to investigate the effect of different parameters in phase-plate designs as well as to compare images with those taken with conventional defocus. The simulated imaging system can be used to generate raw image datasets, which in the current work were for a fixed electron energy of 200 keV, that can then be processed in the same way that is used for experimental data, giving insight into the practical advantages of phase contrast microscopy. While Chang et al. have recently investigated this question in detail (Chang et al., 2010), we show here that their investigation underestimates the improvement in contrast and signal-to-noise ratio that is achievable at low spatial frequencies, due to using a rather high value for the cut-on frequencies in their simulations

For the purposes of this investigation, much of the electron microscope can be ignored, paying attention only to the propagation of an incident plane wave through the sample followed by imaging of the transmitted wave (exit wave) by the objective lens. The simplest model for propagation of a wave through the sample is to assume that its transmission function can be calculated from the projected two-dimensional (2-D) potential of the whole sample (Kirkland, 1998). While this “projection approximation”, and the frequently used “weak phase-object” model that is derived from it, are sufficient for very thin specimens, as we demonstrate here, biological macromolecules as thin as Tobacco Mosaic Virus (TMV) clearly cannot be considered to be weak phase objects. For even thicker specimens, the projection approximation can also fail due to curvature of the Ewald sphere (Glaeser et al., 2007). As a result, it can be an over simplification to use a thin-specimen model for cryo-EM (Kirkland, 1998).

To overcome the theoretical limitations mentioned above, a multislice approximation is commonly used (Goodman and Moodie, 1974), in which the specimen is computationally split into a series of thin slices. The transmission of the incident wave through the entire specimen is then calculated by successive transmission through each slice followed by Fresnel propagation to the position of the next slice. Imaging of the exit wave is then calculated as in the (single) thin-slice method. Employing the multislice formulation to simulate images of biological macromolecules may appear to be both more complicated and computationally more expensive than using the weak phase-object approximation. In practice, however, the complication is transparent to the user when employing an already existing computer program, and the increased length of time is trivial with modern computers. Thus, there is hardly any excuse to not do the simulations with the more exact approximation to begin with, whether it is really necessary or not to do so.

In order for an imaging simulation to be useful we must be able to accurately model (describe computationally) the specimen being imaged. For single-particle cryo-EM we are generally interested in protein structures. It is therefore convenient to use the atomic coordinates from a PDB structure (Bernstein et al., 1977) as a starting point. In an actual specimen, however, a protein is embedded in a thin layer of vitreous ice, which has a

molecular arrangement similar to that of liquid water. It is therefore possible to accurately model the entire specimen by placing the PDB coordinates of a protein into a simulated box of liquid water.

While single-particle cryo-EM has been successfully used in the investigation of many large macromolecules, the practical results for smaller macromolecules are still restricted by the quality of the raw images that can be achieved by defocus-based phase contrast. Currently most investigations are limited, for example, to a particle size of >250kDa. One of the major factors affecting the processing of images of small macromolecules is that the increased defocus used in order to be able to detect the particle results in a CTF which is more difficult to correct due to the rapid oscillation at high resolution. As we now demonstrate in a quantitative way, the use of Zernike phase contrast can improve the processing of images of small macromolecules. We demonstrate that for a 100kDa particle Zernike phase contrast both improves the ability to identify small particles in simulated images and greatly reduces alignment errors for such particles.

Even when particles are fairly large, the relatively low contrast provided by defocus-based phase-contrast images has a detrimental effect on the ability to process cryo-EM data. One of the limits of cryo-EM, currently at the forefront of investigation, is particle heterogeneity (Leschziner and Nogales, 2007). Many methods have been developed that address the problem (Fu et al., 2007; Hall et al., 2007; Penczek et al., 2006; Scheres et al., 2007; Shatsky et al., 2010; White et al., 2004), but all are limited by the imperfect quality of raw images. As we demonstrate here, the use of Zernike phase contrast can improve the sorting of heterogeneous particles. In the example that we investigated here, involving particles of RNA polymerase II with three possible compositions whose masses differed by less than 10%, the homogeneity of sorted data sets is improved from ~55% for images simulated with defocus-based phase contrast to ~75% for images simulated with Zernike phase contrast.

Methods

Our simulation and evaluation of a phase-plate type device is split into four distinct parts. First a model of the system being imaged is generated, resulting in a structure of a protein embedded within a box of water, in which both components are represented by atomic coordinates. Then, using a multislice approximation, the transmission of an electron wave through the model is simulated, resulting in an estimation of the exit wave. The imaging of this exit wave is then simulated, using either a Zernike phase plate or the phase shift due to defocus. The images generated from the simulation are then evaluated using standard single-particle algorithms.

Model building

The target proteins were modelled using coordinates for non-hydrogen atoms taken from the protein data bank (PDB) (Bernstein et al., 1977). Using the molecular simulation package Gromacs (Hess et al., 2008), hydrogen atoms were then added to the amino acid residues in the protein structure using the OPLS – AA force field definitions (Jorgensen et al., 1996).

Separately, again using Gromacs, a $375\text{\AA} \times 375\text{\AA} \times 375\text{\AA}$ box of water was generated with a periodic boundary using the SPC 216 model of water (Teleman et al., 1987). As the SPC 216 water model only contains 216 molecules of water (within a box $\sim 12\text{\AA}$ on edge) our larger box of water was generated by tiling the SPC 216 water model, resulting initially in a periodic, or “super-lattice” array of water molecules within the larger box. This periodically continued model of water is unsuitable for imaging simulations due to the intense diffraction spots that are generated in a computed Fourier transform. To overcome the artificial, crystal-type arrangement of the 216 water molecules, a short (20 picoseconds) molecular dynamics

(MD) simulation was run on the large box of water, effectively randomizing the positions of water molecules while maintaining a liquid-water like structure. While the resulting model is not expected to be accurate enough for studies on the physical chemistry of liquid water, we believe that it can provide an accurate simulation of contrast matching by vitreous ice in cryo-EM images.

Using Gromacs (Hess et al., 2008) the protein structure was added to the box of water, and water molecules whose Van der Waals surface overlapped with the calculated Van der Waals surface of the protein structure were deleted. Note while it is possible to add the protein structure to the water before running the MD simulation, possibly resulting in a more accurate model of how the protein is solvated, we do not believe that the questions being addressed in our simulations of image contrast would benefit from adding that additional complexity to our calculations. Examples of the water model and solvated protein can be found at <http://cryoem.berkeley.edu/multislice>.

Multislice simulation

The multislice calculations reported here were performed with code adapted from that published in (Kirkland, 1998). The model of the atomic coordinates being imaged is split into slices 10Å deep (operational parameter), and the projected atomic potential along the z axis, $v_z(\mathbf{x})$, is calculated for each slice. For simplicity the linear superposition of the potentials of each neutral atom, j , is used in this calculation

$$v_z(\mathbf{x}) = \sum_{j=1}^N v_{zj}(\mathbf{x} - \mathbf{x}_j), \quad (1)$$

not taking into account the rearrangement of electrons due to bonding. Calculation of a more accurate potential taking into account bonding is not yet computationally feasible for such a large model. The Coulomb potential for individual atoms was taken from tabulated parameters (Kirkland, 1998). While the effects of chemical bonding can be as large as 15% for peptide bonds at small scattering angles (Kirkland, 1998), and even more than that for ion pairs, this is still a relatively small effect compared to the differences in contrast computed for defocus-based phase contrast versus Zernike phase contrast, and in any case the associated error is systematically the same in the two cases.

The transmission function for each slice n , is calculated

$$t_n(\mathbf{x}) = e^{[i\sigma v_{zn}(\mathbf{x})]}, \quad (2)$$

and bandwidth limited to prevent aliasing artefacts. The parameter σ is the interaction constant:

$$\sigma = \frac{2\pi m e \lambda}{h^2} \quad (3)$$

where m is the relativistic mass of an electron, e the elementary charge, λ the wavelength and h Planck's constant. All simulations reported in this paper were done for an electron energy of 200 keV.

The exit wave for the n th slice, $\psi_0(x, y)$, is propagated through the next slice of the model:

$$\psi_{n+1}(x, y) = p_n(x, y, \Delta z_n) \otimes [t_n(x, y) \psi_n(x, y)], \quad (4)$$

where p_n is a spherical wave representing Fresnel propagation through a slab of thickness Δz_n . On exiting the final slice, the transmitted wave is Fourier transformed,

$$\Psi_n(k_x, k_y) = FT[\psi_n(x, y)], \quad (5)$$

where k_x and k_y are the Cartesian components of the two-dimensional spatial frequency, k .

Simulation of imaging of the exit wave

To simulate imaging within the microscope, the Fourier transform of the exit wave is multiplied by the coherent transfer function of the imaging system. We use the phrase “coherent transfer function” to describe the linear relationship between the exit wave function and the image wave function that exists in a coherent optical system (Goodman, 2005). We continue, of course, to use the phrase “(phase) contrast transfer function”, and the abbreviation CTF, to describe the linear relationship that exists between the exit wave function for a weak (phase) object and the image intensity. In the absence of astigmatism and additional aberrations that are normally not considered, the coherent transfer function is given by

$$\begin{aligned} H_0(\mathbf{k}) &= e^{[-i\gamma(\mathbf{k})]} A(\mathbf{k}) \\ \gamma(k) &= \pi \lambda k^2 (0.5 C_s \lambda^2 k^2 - \Delta f) \end{aligned} \quad (6)$$

where C_s is the spherical aberration coefficient, and Δf is the amount of defocus. $A(\mathbf{k})$ is the aperture function, which under normal defocus imaging conditions is represented as:

$$\begin{aligned} A(\mathbf{k}) &= 1; \lambda k = \alpha < \alpha_{\max} \\ &= 0; \text{otherwise} \end{aligned} \quad (7)$$

where $\alpha_{\max}$ is the maximum semiangle of the objective aperture. When simulating a phase plate, the aperture function $A(\mathbf{k})$, can be replaced by a phase-plate function,

$$\begin{aligned} P(\mathbf{k}) &= 1; \quad \lambda k = \alpha < \alpha_{\text{cut-on}} \\ &= e^{i(\frac{\pi}{2})}; \quad \lambda k = \alpha_{\text{cut-on}} < \alpha < \alpha_{\max} \\ &= 0; \quad \lambda k = \alpha > \alpha_{\max} \end{aligned} \quad (8)$$

Multiplication of the coherent transfer function by the Fourier transform of the exit wave produces the modified scattered wave function in the back focal plane,

$$\Psi_i(\mathbf{k}) = H_0(\mathbf{k}) \Psi_n(\mathbf{k}). \quad (9)$$

To calculate the simulated image intensity we compute the square modulus of the inverse Fourier transform of the modified scattered wave function

$$I(\mathbf{x}) = |FT^{-1}[\Psi_i(\mathbf{k})]|^2. \quad (10)$$

To simulate the electron shot noise associated with typical, low electron dose imaging, a Poisson distribution is used to randomly generate the number of electrons in each pixel. The probability of n electrons within a square angstrom is given by

$$f(n; \mu) = \frac{\mu^n e^{-\mu}}{n!}, \quad (11)$$

where μ is equal to the average exposure times $I(x)$, i.e. the expected number of electrons per square angstrom, given the theoretical variation in the image intensity.

Model structures

We used tobacco mosaic virus (TMV) (PDB-2TMV)(Namba et al., 1989) as the modeled sample to investigate the expected increase in image contrast resulting from the use of a Zernike phase plate. TMV has been widely used as a test sample in electron microscopy (Henderson, 1992; Sachse et al., 2007; Sander et al., 2005), and it has been used to test early physical phase-plate implementations (Danev et al., 2009).

In order to test the hypothesis that a phase plate will make it possible to process much smaller structures, a test structure of a 100kDa protein (Arsenite Oxidase, PDB 1G8J) was generated. Nine models of the structure were simulated in a single orientation, each model in a different block of random water.

To test the effect of using Zernike phase-contrast images during the refinement of heterogeneous single-particle data, we used the structure of the yeast RNA polymerase II elongation complex (PDB-1Y1W) (Kettenberger et al., 2004). This complex contains multiple protein subunits, DNA and RNA, allowing us to synthetically generate multiple biochemically heterogeneous structures. Note, however, that the artificial structures we used for this purpose are not biologically relevant. For our analysis we used three different assemblies: the complete PDB structure (the ternary elongation complex); the structure minus the DNA and RNA (RNAPII alone), involving a loss of mass of ~12.5 kDa; and the structure minus DNA, RNA and 2 protein subunits (RNAPII- LK), involving a loss of mass of ~33 kDa. For each assembly we generated 50 models of the structure in random orientations, with a different randomised block of water for each orientation. The small number of test images is due to the time taken to produce a fully random block of water (~32 cpu hours).

Parameters that were varied during comparisons

To analyse the improvement in contrast when using a phase plate, images of TMV were simulated using a range of defocus values (1.2 μ m, 2.4 μ m, 3.6 μ m, 4.8 μ m, 6.0 μ m), and a

range of phase-plate cut-on frequencies ($k_{cut-on} = \frac{1}{25\text{\AA}}, \frac{1}{75\text{\AA}}, \frac{1}{125\text{\AA}}, \frac{1}{250\text{\AA}}, \frac{1}{500\text{\AA}}$), with an exposure of 20e/ $\text{\AA}^2$ and a pixel size of 0.375 $\text{\AA}$ /pixel. To test if using a phase plate will allow the processing of smaller structures, the models of the 100kDa protein were used to simulate images with the same range of parameters as the TMV simulation. Images were also generated of random water, without protein. To test how well the structures could be detected above noise and how well they could subsequently be processed, the simulated images were aligned against reference images using a standard cross correlation function (CCF).

Analysis of the heterogeneous RNAPII structures was carried out by generating images with the same defocus and phase-plate cut-on frequencies used in the other experiments. The simulated defocus images were corrected for oscillations of the CTF by phase flipping,

using the known CTF parameters, and all images were coarsened to a final pixel size of 1.5 Å/pixel. The simulated images were aligned against multiple models in order to test if the different assemblies could be distinguished by multiple-model template matching, a common tool that is used to process heterogeneous data (Gao et al., 2003). The three reference models, which accurately represented the differences in the dataset, were filtered to 20 Å before reprojecting at an angular step of 15°, resulting in 212 templates for each model. Alignment and multi-model template matching was carried out using the Spider image processing package according to the protocols described in (Shaikh et al., 2008). Many methods exist to sort heterogeneous data, the Spider multimodel reference based procedure was chosen because of its simplicity and robustness when given accurate starting models (Gao et al., 2003). A raw image was designated as being correctly assigned if, after alignment to all reprojections of all models, the best matching template is of the same conformational state.

Results

Contrast of TMV

Tobacco mosaic virus (TMV) is a rod-shaped particle with a diameter of ~18 nm. Images of a short section of TMV, simulated for an electron energy of 200 keV, are shown in figure 1(a), where the left-hand column contains images for different amounts of defocus (in μm) and the right-hand column contains images for different values of the phase-contrast cut-on frequency (in Å⁻¹). For reference, figure 1(b) shows the one-dimensional profile of the phase shift in the exit wave, averaged along the length of the TMV particle.

The series of images that is presented in figure 1(a) provides a visual way to appreciate that the phase plate produces higher quality results than does defocusing the image, provided that the cut-on frequency of the phase plate is low enough. The defocus phase-contrast images show the well known increase in contrast with the amount of underfocus, which is better represented by the quantitative intensity profiles plotted in figure 1(c). As is also well known, however, the increase in contrast that is achieved by increasing the amount of defocus is accompanied by detrimental blurring of the image. The phase-plate images, on the other hand, are in focus in all cases, and thus the high-resolution features are not degraded by the effects of increasing amounts of defocus. Instead, the only difference, shown quantitatively in figure 1(d), is an increase in low-frequency information, i.e. an increase in the contrast of low-resolution features, as the value of the cut-on frequency decreases.

To more accurately assess the effects of changing defocus on the one hand and changing cut-on frequency on the other, 1-D intensity profiles across the TMV images were calculated, figure 1(b), by projecting the image intensities shown in figure 1(a) onto the horizontal axis. The profiles show increasing contrast for both increased defocus and reduced cut-on frequency, although the increasing contrast is much more pronounced for the

phase plate. The maximum contrast for $k_{cut-on} = \frac{1}{500\text{Å}}$ is ~50% (average intensity of the protein / average intensity of the background), while for a defocus of 6.0 μm it is only ~10%. The 1-D profiles also show, more clearly than the gray-scale images, that the Zernike phase-plate images have a pronounced halo (fringe), while a weaker halo is also present in the defocused images.

Detection of a 100kDa protein

The results from the simulation (at an electron energy of 200 keV) of the detection of a 100kDa protein (Arsenite Oxidase) are summarized in figure 2. This enzyme has a globular

structure with a long dimension of ~7.5 nm and a short dimension of ~5 nm. Figure 2(a) shows the distribution, represented by box plots, of values for the maximum peak in the CCF (Cross-Correlation Function; see pp 94 and 95 of (Frank, 2006)) for various types of simulated images and a fixed template. As is indicated in the figure legend, images were simulated at defocus values ranging from 1.2 μm to 6.0 μm , or for values of the cut-on frequency ranging from $1/(25\text{\AA})$ to $1/(500\text{\AA})$, using 9 models that included protein and 9 models of water alone. All 18 models are independent, i.e. different randomized water models and shot noise simulations. From this plot it can be seen that increasing the defocus or decreasing the phase-plate cut-on frequency results in progressively higher CCF peaks. Only at defocus values of 2.4 μm or more are the peaks above those for noise, whereas all

phase-plate simulations other than for $k_{\text{cut-on}} = \frac{1}{25\text{\AA}}$ have a distribution of CCF peaks above noise. The results presented in Figure 2(a) also show that the peak heights obtained in the simulation of the phase-plate images using the lowest cut-on frequency are about 3 times greater than the peak heights obtained in the most highly defocused images.

Figure 2(b) shows how the relative heights of the CCF peaks, reported in Figure 2(a) for different values of defocus or cuton frequency, respectively, affect alignment error. For all

phase plate tests other than when $k_{\text{cut-on}} = \frac{1}{25\text{\AA}}$, the center of the particle is found with an error of less than one pixel as opposed to ~10 pixels for images simulated without a phase

plate. Setting $k_{\text{cut-on}} = \frac{1}{25\text{\AA}}$, however, results in images of the 100kDa protein that are not distinguishable from water, using a standard CCF, due to the lack of low-frequency

information. From figure 2(c) it can also be seen that at $k_{\text{cut-on}} = \frac{1}{25\text{\AA}}$ the particle is almost indistinguishable from the background noise.

Representative examples of images of this 100 kDa particle are shown in Figure 2(c) in order to provide a visual impression of how well such a particle might be recognized for different imaging conditions, when using an exposure of 20 electrons/ $\text{\AA}^2$ and an electron energy of 200 keV. Examples of images simulated for different values of defocus (indicated in μm) are shown in the upper row, while examples of Zernike phase-contrast images simulated for different cuton frequencies (indicated in $\text{\AA}^{-1}$) are shown in the lower row.

Detection of particle heterogeneity

Using different RNA polymerase II (RNAP II) subunit assemblies is one way to test the effectiveness of using a phase plate to detect particle heterogeneity. RNAP II is roughly ellipsoidal in shape, with a long dimension of ~26 nm and a short dimension of ~14 nm. Figure 3(a) shows an example of the raw images, simulated at an electron energy of 200 keV, for different values of defocus between 1.2 and 6.0 μm (conventional images, upper row) and phase-plate cuton frequencies between $1/25$ and $1/500 \text{\AA}^{-1}$ (Zernike phase-contrast images, lower row), respectively. Figure 3(b) shows the accuracy of the assignment of structural (compositional) state for the three simulated datasets of particles with slightly different structures that are described in Methods. A total of 135 images were simulated for each dataset, 45 of each conformation. The accuracy of assignment for the phase-plate datasets (indicated by dark gray bars) is consistently better than for the defocus data

(indicated by light gray bars) for all simulations other than $k_{\text{cut-on}} = \frac{1}{25\text{\AA}}$. Comparing the best cases for simulations of both underfocused images and images produced with a phase plate, the fraction of particles that were assigned to their correct group was increased from ~55% for defocused images to ~75% for images produced with a phase plate.

Discussion

It is clear from all results obtained in these simulations, for images recorded at an electron energy of 200 keV, that we can expect an improvement in single-particle EM image quality when using Zernike phase-plate technology. It is also clear that this increase in image quality will especially enhance the ability of single-particle methods to process smaller macromolecules, whereas the improvement in dealing with particle heterogeneity will be more modest.

The results from the TMV simulation show that when the phase-plate cut-on frequency is reduced below $\sim 1/(\text{the particle size})$, the simulated in-focus phase-plate images have progressively more contrast than do images for which contrast is produced only by defocus. The maximum contrast is ultimately achieved when the cut-on frequency of the phase plate approaches $1/(\text{three times the particle size})$. At this point the contrast is calculated to be ~ 5 times that which is achieved without a phase plate, using a defocus as large as 5 or 6 micrometers. Of course, the increased contrast to which we refer here corresponds mainly to the lower-resolution features of the structure, where the CTF for even a highly defocused image remains very small.

The amount of phase contrast that is shown in our simulations is significantly greater than that reported by Chang et al. (Chang et al., 2010), who also modelled Zernike phasecontrast image formation for macromolecules embedded in vitreous ice. We believe that the discrepancy in results is due mainly to the fact that Chang et al. did not apply a sufficiently low cut-on frequency to produce the full effect that can be achieved with a phase plate. The amount of contrast predicted by our simulations of TMV is also approximately 4-5 times greater than that reported for experimental images obtained with a thin carbon-film phase plate (Danev et al., 2009). We believe that this discrepancy is due to the fact that the performance of the particular phase plate used in recording that image had already degraded due to the “aging” phenomenon described in the same paper.

Finally, the fact that our simulations indicate that the full amount of phase contrast for ice-embedded TMV should be about 40% demonstrates that even such a thin biological particle cannot be accurately described as being a weak phase object. The difference of 0.2 radians in the integrated phase shift between TMV and ice, shown in figure 1(b) would generate $\sim 40\%$ contrast according to the weak phase-object approximation compared to 37% contrast when deviations from the weak-phase approximation are accounted for. Further information about how the actual image contrast deviates from that expected according to the weak-phase approximation can be found in figure 7a of (Danev et al., 2009). As is shown in that figure, increasing amounts of phase shift result in progressively smaller increases in contrast, and beyond a certain point the contrast actually decreases and ultimately reverses. As a result, it will not be appropriate to use a quarter-wave phase plate to image objects that are much thicker than TMV. For example, the weak phase-object approximation (WPOA) overestimated the contrast by 30% if the phase shift is 0.4 (twice that of TMV), and deviation from the WPOA rises steeply thereafter. The use of a weaker phase plate can, however, still be an effective way to image such “strong phase objects” (Danev et al., 2009).

Our simulations of phase-contrast images of TMV demonstrate that the images have a substantial, unwanted halo (fringe). Highly defocused images have a similar fringe, but it is generally less noticeable, simply because the contrast is much less than that achieved with a phase plate. In addition, although not shown in these simulations, it is known experimentally that the unwanted fringe produced by a phase plate is sensitive to very small amounts of misalignment, including that produced by the local variation of the beam tilt that can arise from local charging in a specimen. When the phase plate is not properly centered, the fringe

becomes asymmetric around the particle because low-frequency Friedel mates in the scattered electron wave are no longer affected by the phase plate in the same way.

In our simulations of images of a 100kDa protein, we have shown that the increase in contrast and image quality provided by Zernike phase contrast can lead to both more accurate identification of single particles above background and more accurate alignment when aligning against a perfect model. The results of this simulation are consistent with the experimental demonstration of greatly improved visibility for both the 100 kDa domains of a 200 kDa dimer (dissimulatory sulfite reductase) and the 88 kDa subunits of structurally heterogeneous assemblies of VacA toxin (Danev et al., 2009). The improved ability to work with smaller macromolecules that is demonstrated experimentally as well as in these simulations should greatly extend the usefulness of single-particle cryo-EM. Recent experimental results have also demonstrated significant improvement in data quality for larger particles. Murata et al. demonstrated that the number of particles of epsilon15 bacteriophage could be reduced by a factor of ~3 (Murata et al., 2010), for example, and Rochat et al. were finally able to visualize the small portal complex within herpes simplex virus type 1 capsids (Rochat et al., 2011)

The tests that we performed with different models of structural heterogeneity show that the improvement in image quality provided by a phase plate will result in data for which it is easier to sort particles into structurally more homogeneous subsets. While the examples of structural heterogeneity that we used to investigate this point in no way explore all of the different types of compositional and conformational heterogeneity that will exist for real specimens, this one example does show that using images obtained with a Zernike phase plate will make it easier to sort particles into more homogeneous groups. Currently, heterogeneity is a major problem in single-particle cryo-EM, stalling the investigation of many systems for which it is impossible to biochemically isolate homogeneous states. The improvement in image quality gained from using a phase plate, together with improvements in the computational algorithms for clustering heterogeneous data, should thus result in a significant increase in the usefulness of single-particle cryo-EM for investigating the dynamic nature of macromolecular assemblies.

While we believe that the way in which we have embedded macromolecular particles in amorphous water accounts reasonably well for the reduction in contrast that is expected relative to simulations in which the same particle would be in vacuum, there are at least two aspects of our model of vitreous ice where further improvements could be considered. First of all, a full MD simulation of water plus protein, like that done by Wang et al. for streptavidin monolayer crystals (Wang et al., 2008), might improve the high-resolution features of image simulations by more realistically modeling the hydration shell of the protein. Since the number of atoms in the hydration shell is a small fraction of the number of atoms in the protein, however, it seems unlikely that a proper simulation of the hydration shell would have a significant effect on the results. In fact, we believe that the continuum model of water used by Chang et al. (Chang et al., 2010), which merely represents the vitreous ice by some value of the inner potential, serves equally well to account for the partial “contrast matching” between protein and its embedment. The main purpose of using an atomistic model of vitreous ice was to facilitate our use of existing “multislice” software to calculate the exit wave. A second, more important improvement, we believe, would be to use MD simulations of water that were performed at the same density of water molecules that is present in vitreous ice. Since the density of amorphous ice is about 5% to 10% less than that of liquid water, our current results necessarily underestimate the contrast achievable, in principle, for cryo-EM. On the other hand, we believe that the relative improvement reported here for Zernike phase contrast images versus conventional, defocused images would not be changed substantially by using an improved model of

vitreous ice. This is because a similar (i.e. proportionate) increase in the contrast of conventional and Zernike phasecontrast images would occur if a slightly lower density of water molecules were used in both cases.

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Figure 1.

(a) Simulated images, for 200 keV electrons, of ice-embedded TMV (left column) at various amounts of underfocus (given in μm) and (right column) with progressively decreasing values of the phase-plate cut-on frequency (given in $\text{\AA}$). All images are shown with a simulated exposure of $20 \text{ e}/\text{\AA}^2$. (b) One-dimensional profile of the phase of the exit wave, averaged along the length of the TMV particle. Shot noise is not added in this case. (c) Averaged intensity profiles across the width of images of TMV particles that were simulated with various amounts of underfocus. (d) Averaged intensity profiles across the width of images of TMV particles that were simulated with decreasing values of the phase-plate cut-on frequency.

Figure 2.

The effect of various imaging conditions on the ability to detect a 100kDa protein and to align it to a noise-free projection. Box plots indicate the minimum, lower quantile, median, upper quantile and maximum for the distribution of values obtained for multiple simulated images (9 in total), all performed for an electron energy of 200 keV. In panels (b) and (c), results obtained when contrast is produced by defocusing the image are indicated by “Def” with the defocus indicated in Å, while results obtained for Zernike phase contrast are indicated by “Phase” with the resolution at which phase contrast first begins indicated in Å. (a) Plot showing the distribution of CCF peak heights when the simulated images are cross correlated with a perfect noise free image. + indicates that the protein is present in the image, - that the simulated image contains only water. (b) Plot showing the alignment error, i.e. the distance of the CCF peak from the position corresponding to correct alignment. (c) Example images for simulations of a 100kDa protein (top row) at various amounts of underfocus (given in μm) and (bottom row) with different values of the phase-plate cut-on frequency (given in Å⁻¹).

Figure 3.

The effect of various imaging conditions on the ability to sort out images of structurally heterogeneous particles into homogeneous groups. (a) Some representative images of RNAPII simulated at a fixed electron energy of 200 keV with (top row) various amounts of underfocus (given in μm) and (bottom row) different values of the phase-plate cut-on frequency (given in $\text{\AA}^{-1}$). (b) Plots of the percentage of particles that were assigned to their respective, correct group, using multi-model projection matching. For each experiment a total of 135 images were used, 45 from each conformation. Results obtained when contrast is produced by defocusing the image are indicated by “Def” with the defocus indicated in $\text{\AA}$, while results obtained for Zernike phase contrast are indicated by “Phase” with the resolution at which phase contrast first begins indicated in $\text{\AA}$.