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Zernike phase contrast cryo-electron tomography of whole bacterial cells

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Abstract

Cryo-electron tomography (cryo-ET) provides three-dimensional (3D) structural information of bacteria preserved in a native, frozen-hydrated state. The typical low contrast of tilt-series images, a result of both the need for a low electron dose and the use of conventional defocus phase-contrast imaging, is a challenge for high-quality tomograms. We show that Zernike phase-contrast imaging allows the electron dose to be reduced. This limits movement of gold fiducials during the tilt series, which leads to better alignment and a higher-resolution reconstruction. Contrast is also enhanced, improving visibility of weak features. The reduced electron dose also means that more images at more tilt angles could be recorded, further increasing resolution.

Keywords

Defocus phase contrast (DPC); Zernike phase contrast (ZPC); cryo-electron tomography (cryo-ET); bacteria; *Caulobacter crescentus*; *Vibrio vulnificus*

1. Introduction

Cryo-ET is currently the only method available through which small, unique objects such as bacterial cells can be imaged to produce three-dimensional (3D) structures at ~2 to 10 nm resolution (Milne and Subramaniam, 2009). The rapid freezing of specimens onto EM grids preserves the native arrangement of macromolecules, and eliminates the need for stains, chemical fixation, plastic embedding, and sectioning, which introduce artifacts (Lucic et al., 2008; Murphy and Jensen, 2007). However, several challenges are associated with cryo-ET imaging of bacterial specimens. First, cryo-ET data has an inherently low signal to noise ratio (SNR). Images are acquired under low electron dose conditions in order to preserve specimen integrity. Typically, total electron doses of ~60 to ~200 e⁻/Å² are fractionated over an entire tilt series of ~65 to ~130 images, which corresponds to ~1 to ~2 e⁻/Å² per image. Many researchers have implemented approaches to improve the SNR of the individual images of the tilt series by using high sensitivity/high resolution CCD cameras

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(Booth et al., 2006; Faruqi, 1998; Milazzo et al., 2011; Zhang et al., 2003), energy filters (Grimm et al., 1997), and post-data collection image processing procedures (Frangakis and Hegerl, 2001; Narasimha et al., 2008). These efforts enable investigators to both record greater numbers of images and improve alignment of the tilt series images, which subsequently result in 3D reconstructions of higher final quality. In addition, due to the low contrast of cryopreserved, unstained biological specimens, micrographs are taken several μm under focus (i.e. a defocus of 4 – 16 μm under focus) in order to enhance the contrast of low-resolution features, including cellular membranes. Unfortunately, the enhanced contrast gained from defocus phase contrast (DPC) is achieved at the expense of reducing information from high-resolution features, such as viral capsid proteins.

To overcome these limitations, Zernike phase contrast transmission electron microscopy (ZPC-TEM) was advanced so that researchers could generate higher contrast images of ice-embedded specimens examined near-to-focus and eliminate the compromise between contrast and resolution. The development and improvement of ZPC cryo-EM tools for 2D image acquisition and single particle analysis of biological specimens has only been recently realized (Danev et al., 2009; Danev and Nagayama, 2001, 2008; Rochat et al., 2011; Shigematsu et al., 2010). The first application of ZPC-TEM for cryo-ET was demonstrated with T4 bacteriophage (Danev et al., 2010). Further studies have shown the technology's promise when used to examine other small macromolecular complexes such as epsilon15 bacteriophage (Murata et al., 2010), the isolated flagellar hook-basal bodies from *Vibrio alginolyticus* (Hosogi et al., 2011), as well as the thinnest edges of eukaryotic cells (Fukuda and Nagayama, 2011). All the data presented in our study of bacterial cells was collected with a JEOL JEM-2200FS 200 kV FEG-TEM (JEOL Ltd., Japan) equipped with the Zernike phase plate airlock system located in the back-focal plane of the objective lens, an in-column energy filter (slit width 20 eV), a cryo-transfer specimen holder (Model 914; Gatan, Pleasanton, CA), and a $4\text{k} \times 4\text{k}$ Gatan Ultrascan CCD camera. Here we present definitive results illustrating the success of ZPC cryo-ET technologies when applied to the generation of tomographic reconstructions of bacteria. Specifically, we have used ZPC cryo-ET to study intact, frozen-hydrated *Caulobacter crescentus* and *Vibrio vulnificus* cells.

2. Results and Discussion

The total electron dose employed for cryo-ET data collection depends on the 'dose sensitivity' of the biological target. Many of the bacterial species that have been studied using cryo-ET are able to withstand electron doses up to $\sim 200\text{ e}^-/\text{\AA}^2$ with no apparent damage to the cell. However, there are species that are more radiation labile and this may limit studies of their ultrastructure. To establish the dose sensitivity of the two bacterial species in this study, cumulative electron dose series were collected (Figs. 1a and 1b). Each dose series image corresponded to an electron dose of $\sim 5\text{ e}^-/\text{\AA}^2$ with a defocus of 4 μm under focus applied. We determined that the *C. crescentus* cells could withstand electron doses between 120 and $200\text{ e}^-/\text{\AA}^2$ without observed damage to the cell cytoplasm, cell periphery or flagellum, which is consistent with previous reports (Fig. 1a) (Briegleb et al., 2006). However, upon inspection of the images of *V. vulnificus* cells, while the integrity of the cell membranes and the cytoplasm was stable to a total dose of $\sim 120\text{ e}^-/\text{\AA}^2$, the sheathed bacterial flagellum incurred visible radiation damage (Fig. 1b). Strikingly, radiation damage

was first observed in the confines of the sheathed flagellum after a cumulative dose of only $\sim 60 \text{ e}^-/\text{\AA}^2$ (Fig. 1b). The restriction of dose sensitivity to only the flagellum of *V. vulnificus* is unusual and has not been observed with flagella or appendages of other bacterial species examined. Most likely, the reduced dose tolerance is due to the macromolecular crowding within the membranous confines of the flagellar sheath, which limits the diffusion of the beam-induced radiolytic fragments and byproducts. In order to avoid visible radiation damage, the total electron dose for tilt series acquisition of *V. vulnificus* must be restricted to much less than $\sim 60 \text{ e}^-/\text{\AA}^2$. For DPC cryo-ET, the reduced dose would dampen the SNR, hamper the alignment of the images, and diminish quality of the final 3D reconstruction, especially in bacterial cells such as *V. vulnificus*.

In order to assess the application of ZPC for tilt series image acquisition, we acquired sequential images of multiple individual bacterial cells. One image was collected with standard DPC conditions and the other was acquired using ZPC settings. The ZPC data was collected near-to-focus using a Zernike-style phase plate with a thin amorphous carbon film applied to a 5×5 array phase plate aperture disc. The carbon film thickness of the phase plate was $\sim 27 \text{ nm}$, which corresponded to a $\pi/2$ phase shift (Danev et al., 2009), and a central hole dimension of $\sim 0.75 \text{ }\mu\text{m}$ (cut on periodicity of $\sim 35 \text{ nm}$). Each recorded image was limited to an electron dose of ~ 1.5 or less $\text{e}^-/\text{\AA}^2$, which matches the dose of a single tilt series image. After examining the DPC and ZPC images (Fig. 2 and Supplementary Fig. 1), we concluded that the ZPC images had improved SNR that allowed us to resolve features that were either barely discernable or undetectable in the equivalent DPC image. Some of the complexes unambiguously identified in the ZPC images include: individual pili, the core filament of the sheathed flagellum, macromolecular complexes throughout the cytoplasm, and outer membrane vesicles and their cargo. In addition, we quantified membrane intensity peaks from linear density profiles derived from measurements taken across the membranes of *V. vulnificus* cells (Figs. 3a, 3b and 3c). From the measurements, we concluded that the improvement in image contrast of the ZPC data was beneficial for achieving greater clarity of the measured spacing between the inner and outer membranes of the bacterial cell wall. In addition, the peak associated with the peptidoglycan layer (* in Figs. 3b, 3c) was also resolved in the ZPC data, whereas it was imperceptible in the DPC data.

To test the utility and potential of ZPC cryo-ET for the structural characterization of bacteria, we collected and analyzed multiple data sets of *C. crescentus* and *V. vulnificus* cells collected under both DPC and ZPC conditions at total electron doses of between $\sim 30 \text{ e}^-/\text{\AA}^2$ and $\sim 120 \text{ e}^-/\text{\AA}^2$. We determined several important points. First, using ZPC approaches, we reduced the total electron dose used to acquire a tilt series by 75%, in other words, from $\sim 120 \text{ e}^-/\text{\AA}^2$ to $\sim 30 \text{ e}^-/\text{\AA}^2$ and generated high contrast, high SNR 3D reconstructions of bacterial cells. A decrease in electron dose combined with ZPC will enable cryo-ET to be applied to radiation sensitive samples, such as the sheathed flagellum of *V. vulnificus*. In addition, reduction of the electron dose for tomography is important because the ice and the specimens embedded within the ice move due to repeated exposure to the beam (Brilot et al., 2012; Wright et al., 2006). The motion of objects in the ice can substantially limit the generation of higher resolution 3D maps and volumes. For single particle analysis, groups have improved data collection strategies in order to minimize movement and utilized

electron detection devices that allow for the sub-selection and alignment of image frames to reduce blurring (Brilot et al., 2012; Li et al., 2013). In our study, we sought to define the impact of ice mobility on the 10 nm gold particles commonly used to align the tilt series images used for construction of the 3D tomogram. We observed that gold particles in a higher dose ($\sim 120 \text{ e}^-/\text{\AA}^2$) tilt series were displaced by up to 3 nm in the x-y plane (Fig. 4a, 4c), whereas the gold particles in lower dose ($\sim 30 \text{ e}^-/\text{\AA}^2$) tilt series were displaced by no more than 1.25 nm in the x-y plane (Fig. 4b, 4c). This illustrates that fiducial movement within the ice layer can be reduced by $\sim 50\%$ when a lower electron dose of $\sim 30 \text{ e}^-/\text{\AA}^2$ is used for data collection, which equates to an overall better alignment of the images for the generation of higher-quality tomographic reconstructions.

To characterize the improvement in tomogram SNR and the definition of cellular structures, DPC and ZPC cryo-ET data was analyzed from the same *C. crescentus* cell. Tomographic reconstructions were generated using IMOD (Kremer et al., 1996). As shown in Fig. 5, each 2.2 nm slice through the ZPC cryo-ET 3D volume had significantly higher SNR and clarity of the cell membranes (white arrows), s-layer periodicity (white arrowheads), and macromolecules within the cytoplasm when compared to individual 2.2 nm or averaged 11 nm slices from the DPC data. In our current and previous investigations (Guerrero-Ferreira et al., 2011) as well as in Fig. 5 and Supplementary Movies 1, 2, and 3, we have analyzed features such as the cell membranes, flagellum, flagellar basal body, bacteriophage capsid, and bacteriophage head filament, which typically require enhancement through the application of a defocus. All of these structures were more evident in the ZPC data because they were represented as uniform projections of the object mass density.

To further assess the resolution of low electron dose ($\sim 35 \text{ e}^-/\text{\AA}^2$) tomography data of bacterial cells collected using either DPC or ZPC methods, we utilized the noise-compensated leave-one-out in two dimensions (NLOO-2D) method (Cardone et al., 2005) as implemented in Bsoft (Heymann et al., 2008) to determine the in-plane resolution of the full tomograms. The in-plane resolution of the DPC tomograms was estimated as $\sim 25 \text{ nm}$ (0.3 threshold) and the in-plane resolution of the ZPC tomograms was estimated as $\sim 12 \text{ nm}$ (0.3 threshold). The percent improvement in resolution of the ZPC tomograms as compared to the DPC tomograms was $\sim 50\%$. However, it should be noted that due to sample geometry and thickness; the angular increment or step-size used for data collection; and the number of images acquired, the quantification of tomogram resolution is extremely variable and might be misleading. Nevertheless, resolution calculations are valuable first appraisals of tomogram quality prior to additional processing and analysis. Further increases in data resolution could be realized as sub-tomogram averaging methods are optimized and microscope hardware is upgraded with direct-electron detectors that will permit the collection of data with higher SNR.

During the analysis of the DPC and ZPC tomography data, we studied the impact of the defringing algorithm (Danev et al., 2010) on the quality of final reconstructions. We used the algorithm as described before but varied the power coefficient in the exponent of the filter function to between 5 and 10. When we examined slices from the DPC, ZPC, and tomograms generated with defringed ZPC tilt series (Supplementary Fig. 2), we observed that defringing reduced some of the fringe artifact associated with the cut on frequency of

the phase plate. However, its use also reduced and flattened signal associated with bacterial membrane components and macromolecular complexes within the cytoplasm in individual 2D images. Upon examining the effect of defringing on the FFT of individual images and the radial average of the FFT (Supplementary Fig. 3), we observed the reduction of the fringe background in the FFT as well as the reduced peak height of the cut on frequency in the radial average. In the 2D FFTs and the radial averages we noted a marked decrease in the intensity of the frequency domains surrounding the peak of the cut on frequency that was reduced by the defringing program. However, reconstructions of ZPC tilt series processed for fringe reduction generated good quality tomograms with reduced fringe artifacts, no loss of resolution, and maintained improved ZPC contrast, as previously reported (Danev et al., 2010).

3. Conclusions

Here we demonstrated that the thin carbon film ZPC technology allows one to reduce the electron dose applied to the specimen and maintain high contrast; improve image alignment due to the greater SNR of each image and the reduction of fiducial displacement; and achieve higher contrast and resolution in each tilt series image and in the final 3D reconstruction. One challenge associated with ZPC TEM is the charging and ageing of the phase plate over time that necessitates phase plate exchange (Danev et al., 2009). We are working to determine the utility of other materials for the thin film of the phase plate when used for cryo-ET applications. We continue to improve and streamline the cryo-ET data collection scheme in order to extend the usable lifetime of the thin carbon film phase plate. In the current study, we have successfully collected up to eight individual tilt series using one phase plate aperture by employing SerialEM (Mastronarde, 2005). SerialEM was used to automate data acquisition, reduce the time taken to obtain a tilt series from the 90 minutes reported by Danev et al. (Danev et al., 2010) to 45 to 60 minutes, and lower the total electron dose applied to the phase plate during the collection of each data set to less than $240 \text{ e}^-/\text{\AA}^2$. Several teams are engineering phase plate airlock systems that can be retrofitted onto existing microscopes in order to offer the ZPC technology to a larger group of investigators. Due to the significant improvements that employing thin carbon film ZPC affords to cryo-ET data, the technology should be advantageous to structural studies of a wide-range of biological specimens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Booth CR, Jakana J, Chiu W. Assessing the capabilities of a 4k×4k CCD camera for electron cryo-microscopy at 300kV. *J Struct Biol.* 2006; 156:556–563. [PubMed: 17067819]
- Briegel A, Dias DP, Li Z, Jensen RB, Frangakis AS, et al. Multiple large filament bundles observed in *Caulobacter crescentus* by electron cryotomography. *Mol Microbiol.* 2006; 62:5–14. [PubMed: 16987173]
- Brilot AF, Chen JZ, Cheng A, Pan J, Harrison SC, et al. Beam-induced motion of vitrified specimen on holey carbon film. *J Struct Biol.* 2012; 177:630–637. [PubMed: 22366277]
- Cardone G, Grunewald K, Steven AC. A resolution criterion for electron tomography based on cross-validation. *J Struct Biol.* 2005; 151:117–129. [PubMed: 15964766]
- Danev R, Glaeser RM, Nagayama K. Practical factors affecting the performance of a thin-film phase plate for transmission electron microscopy. *Ultramicroscopy.* 2009; 109:312–325. [PubMed: 19157711]
- Danev R, Kanamaru S, Marko M, Nagayama K. Zernike phase contrast cryo-electron tomography. *J Struct Biol.* 2010; 171:174–181. [PubMed: 20350600]
- Danev R, Nagayama K. Transmission electron microscopy with Zernike phase plate. *Ultramicroscopy.* 2001; 88:243–252. [PubMed: 11545320]
- Danev R, Nagayama K. Single particle analysis based on Zernike phase contrast transmission electron microscopy. *J Struct Biol.* 2008; 161:211–218. [PubMed: 18082423]
- Faruqi AR. Design principles and applications of a cooled CCD camera for electron microscopy. *Adv Exper Med Biol.* 1998; 453:63–72. [PubMed: 9889815]
- Fontana J, Cardone G, Heymann JB, Winkler DC, Steven AC. Structural changes in Influenza virus at low pH characterized by cryo-electron tomography. *J Vir.* 2012; 86:2919–2929.
- Fontana J, Steven AC. At low pH, influenza virus matrix protein M1 undergoes a conformational change prior to dissociating from the membrane. *J Vir.* 2013; 87:5621–5628.
- Frangakis AS, Hegerl R. Noise reduction in electron tomographic reconstructions using nonlinear anisotropic diffusion. *J Struct Biol.* 2001; 135:239–250. [PubMed: 11722164]
- Fukuda Y, Nagayama K. Zernike phase contrast cryo-electron tomography of whole mounted frozen cells. *J Struct Biol.* 2011; 177:484–489. [PubMed: 22119892]
- Grimm R, Barmann M, Hackl W, Typke D, Sackmann E, et al. Energy filtered electron tomography of ice-embedded actin and vesicles. *Biophys J.* 1997; 72:482–489. [PubMed: 8994635]
- Guerrero-Ferreira RC, Viollier PH, Ely B, Poindexter JS, Georgieva M, Jensen GJ, Wright ER. Alternative mechanism for bacteriophage adsorption to the motile bacterium *Caulobacter crescentus*. *Proceedings of the National Academy of Sciences of the United States of America.* 2011; 108:9963–9968. [PubMed: 21613567]
- Heymann JB, Cardone G, Winkler DC, Steven AC. Computational resources for cryo-electron tomography in Bsoft. *J Struct Biol.* 2008; 161:232–242. [PubMed: 17869539]
- Hosogi N, Shigematsu H, Terashima H, Homma M, Nagayama K. Zernike phase contrast cryo-electron tomography of sodium-driven flagellar hook-basal bodies from *Vibrio alginolyticus*. *J Struct Biol.* 2011; 173:67–76. [PubMed: 20705140]
- Keller PW, Adamson CS, Heymann JB, Freed EO, Steven AC. HIV-1 maturation inhibitor bevirimat stabilizes the immature Gag lattice. *J Vir.* 2011; 85:1420–1428.
- Kremer JR, Mastronarde DN, McIntosh JR. Computer visualization of three-dimensional image data using IMOD. *J Struct Biol.* 1996; 116:71–76. [PubMed: 8742726]
- Li X, Mooney P, Zheng S, Booth CR, Braunfeld MB, et al. Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. *Nat Methods.* 2013; 10:584–590. [PubMed: 23644547]
- Lucic V, Leis A, Baumeister W. Cryo-electron tomography of cells: connecting structure and function. *Histochem Cell Biol.* 2008; 130:185–196. [PubMed: 18566823]
- Mastronarde DN. Automated electron microscope tomography using robust prediction of specimen movements. *J Struct Biol.* 2005; 152:36–51. [PubMed: 16182563]

- Milazzo AC, Cheng A, Moeller A, Lyumkis D, Jacovetty E, et al. Initial evaluation of a direct detection device detector for single particle cryo-electron microscopy. *J Struct Biol.* 2011; 176:404–408. [PubMed: 21933715]
- Milne JL, Subramaniam S. Cryo-electron tomography of bacteria: progress, challenges and future prospects. *Nat Rev Microbiol.* 2009; 7:666–675. [PubMed: 19668224]
- Murata K, Liu X, Danev R, Jakana J, Schmid MF, et al. Zernike phase contrast cryo-electron microscopy and tomography for structure determination at nanometer and subnanometer resolutions. *Structure.* 2010; 18:903–912. [PubMed: 20696391]
- Murphy GE, Jensen GJ. Electron cryotomography. *Biotechniques.* 2007; 43:413–421. [PubMed: 18019332]
- Narasimha R, Aganj I, Bennett AE, Borgnia MJ, Zabransky D, et al. Evaluation of denoising algorithms for biological electron tomography. *J Struct Biol.* 2008; 164:7–17. [PubMed: 18585059]
- Rochat RH, Liu X, Murata K, Nagayama K, Rixon FJ, et al. Seeing the portal in herpes simplex virus type 1 B capsids. *J Vir.* 2011; 85:1871–1874.
- Shigematsu H, Sokabe T, Danev R, Tominaga M, Nagayama K. A 3.5-nm structure of rat TRPV4 cation channel revealed by Zernike phase-contrast cryoelectron microscopy. *J Biol Chem.* 2010; 285:11210–11218. [PubMed: 20044482]
- Wright ER, Iancu CV, Tivol WF, Jensen GJ. Observations on the behavior of vitreous ice at ~82 and ~12 K. *J Struct Biol.* 2006; 153:241–252. [PubMed: 16434212]
- Zhang P, Borgnia MJ, Mooney P, Shi D, Pan M, et al. Automated image acquisition and processing using a new generation of 4K × 4K CCD cameras for cryo electron microscopic studies of macromolecular assemblies. *J Struct Biol.* 2003; 143:135–144. [PubMed: 12972350]

Figure 1.

DPC cryo-EM images of frozen-hydrated bacteria. **(a)** Electron dose series images of *C. crescentus*. No beam-induced destruction of the cell or its appendages was observed up to doses of $\sim 120 \text{ e}^-/\text{\AA}^2$. Magnified views are of the boxed region at $2 \text{ e}^-/\text{\AA}^2$ and $120 \text{ e}^-/\text{\AA}^2$. **(b)** Electron dose series images of *V. vulnificus*. Indications of beam-induced damage to the flagellum noted after a dose of $\sim 60 \text{ e}^-/\text{\AA}^2$. Magnified views of the boxed region illustrate the unharmed flagellum structure at a dose of $\sim 30 \text{ e}^-/\text{\AA}^2$ and the increase in damage to the flagellum at doses of $60 \text{ e}^-/\text{\AA}^2$, $90 \text{ e}^-/\text{\AA}^2$, and $120 \text{ e}^-/\text{\AA}^2$, arrowheads point to 'bubbles'. Scale bars, 200 nm.

Figure 2.

Enlarged views of DPC and ZPC cryo-EM images of *V. vulnificus* cells (whole cell images, Supplementary Figure 1). Single low dose ($\sim 1.5 \text{ e}^-/\text{\AA}^2$) DPC (a, b, and c) and ZPC (d, e, and f) images of portions of individual *V. vulnificus* cells. Each image set highlights the improvement in image contrast and visibility of the flagellum, outer membrane vesicles, and pilus that was obtained with ZPC (d, e, and f) versus DPC (a, b, and c). Scale bars, 200 nm.

Figure 3.

DPC and ZPC cryo-EM images and linear profiles through the cell wall envelope of a *V. vulnificus* cell. **(a)** Enlarged views from single low dose ($\sim 1.5 \text{ e}^-/\text{\AA}^2$) DPC (left) and ZPC (right) images of the same *V. vulnificus* cell (OM: outer membrane; IM: inner membrane). **(b)** Linear profiles through the *V. vulnificus* envelope from regions highlighted with dashed boxes in **a**; OM: outer membrane peak; IM: inner membrane peak; the star (*) represents the peak in the ZPC profile corresponding to the discernible peptidoglycan layer. **(c)** Overlaid linear profiles through the *V. vulnificus* envelope from regions highlighted in **a**. OM: outer membrane peak; IM: inner membrane peak; the star (*) represents the peak in the ZPC profile corresponding to the peptidoglycan layer. Scale bars, 200 nm.

Figure 4.

Gold fiducial positions overlaid on pre- and post-cryo-ET tilt series data acquisition images of frozen-hydrated bacteria. **(a)** Overlaid fiducial positions on an image of a *C. crescentus* cell acquired after a tilt series collected at high total dose ($\sim 120 \text{ e}^-/\text{\AA}^2$). **(b)** Overlaid fiducial positions on an image of a *V. vulnificus* cell acquired after a tilt series collected at low total dose ($\sim 30 \text{ e}^-/\text{\AA}^2$). Fiducial positions before (yellow open circles) and after (blue open circles) tilt series collection are presented. **(c)** Displacement in the x-y plane of 10 nm gold particles after exposure to high ($\sim 120 \text{ e}^-/\text{\AA}^2$, filled triangle) and low ($\sim 30 \text{ e}^-/\text{\AA}^2$, filled circle) electron doses used for cryo-ET data collection. Scale bars, 200 nm.

Figure 5.

Cryo-electron tomograms of the same *C. crescentus* cells. Tomographic slices through the same *C. crescentus* cell. Single 2.2 nm DPC slice (**a**), averaged 11 nm DPC slice (**b**), single 2.2 nm ZPC slice (**c**). Insets are magnified views of the boxed region (bacterial envelope) highlighting the outer and inner membranes (white arrows) and s-layer periodicity (white arrowhead). Other complexes are noted including, a macromolecular complex (dashed-boxed region), storage granule (black arrow), and ribosomes (black arrowheads). Scale bars, 200 nm.