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The influence of frame alignment with dose compensation on the quality of single particle reconstructions

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ABSTRACT

As direct electron detection devices in cryo-electron microscopy become ubiquitous, the field is now ripe for new developments in image analysis techniques that take advantage of their increased SNR coupled with their high-throughput frame collection abilities. In approaching atomic resolution of native-like biomolecules, the accurate extraction of structural locations and orientations of side-chains from frames depends not only on the electron dose that a sample receives but also on the ability to accurately estimate the CTF. Here we use a new 2.8 Å resolution structure of a recombinant gene therapy virus, AAV-DJ with Arixtra, imaged on an FEI Titan Krios with a DE-20 direct electron detector to probe new metrics including relative side-chain density and ResLog analysis for optimizing the compensation of electron beam damage and to characterize the factors that are limiting the resolution of the reconstruction. The influence of dose compensation on the accuracy of CTF estimation and particle classifiability are also presented. We show that rigorous dose compensation allows for better particle classifiability and greater recovery of structural information from negatively charged, electron-sensitive side-chains, resulting in a more accurate macromolecular model.

1. Introduction

The introduction of direct electron detection devices (DDDs) has precipitated a revolution in 3DEM. The combination of their high detective quantum efficiency (DQE) with their high frame rates has allowed for the determination of several single particle reconstructions to better than 3 Å resolution (Bartesaghi et al., 2015; Fischer et al., 2015; Grant and Grigorieff, 2015; Jiang et al., 2015; Campbell et al., 2015), and many reconstructions with high enough resolution to model atomic structures with high confidence (DiMaio et al., 2015). The advantage of the high DQE of DDDs is that they outperform film and CCD cameras in terms of contrast and signal-to-noise ratios at all spatial frequencies (McMullan et al., 2014), and this in-turn allows for better alignment and classification. The advantages of the high frame rate are that it allows for the compensation of beam induced motion and for compensation of specimen damage due to the ionizing radiation of the electron beam (Campbell et al., 2012; Li et al., 2013; Scheres, 2014; Wang et al., 2014).

In spite of the ability to compensate for radiation damage, a common observation among the high-resolution single particle reconstructions that have been determined thus far is that the density values for acidic residues are low relative to the other residues (Bartesaghi et al., 2014; Fromm et al., 2015; Jiang et al., 2015). It has been suggested that acidic residues are more sensitive to radiation damage than other residues (Bartesaghi et al., 2014). Another possibility is that their electron scattering factors are lower than the other residues (Mitsuoka et al., 1999). Several methods have been used to mitigate the effects of beam damage. One approach is to align and classify particles on the lower-noise high-dose images then reconstruct based on the early frames that have suffered less beam damage (Liao et al., 2013). Another approach is fitting relative B-factors to reconstructions from individual frames before combining them into a map containing all frames (Scheres, 2014). Yet another is to combine the frames into one image after applying a low-pass filter to each that is proportional to the cumulative electron dose the specimen has experienced, then summing all the frames (Grant and Grigorieff, 2015; Wang

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et al., 2014). In the current work, the filtering resolution is set by calibration to the dose-dependent attenuation of catalase electron diffraction (Wang et al., 2014), while it is also possible to similarly calibrate against the resolutions of prior single particle reconstructions (Grant and Grigorieff, 2015). Here, we test the effects of the advances in frame alignment with dose compensation on other aspects of single particle reconstruction, including CTF estimation, particle classification (Euler angle assignment), and examine the impact on resolution and side-chain densities.

2. Materials and methods

2.1. Expression and purification

Virus-like particles of AAV-DJ were expressed in insect cells from a baculovirus construct as previously described (Lerch et al., 2012). Empty capsids were purified as before using three rounds of CsCl density gradient ultracentrifugation, followed by heparin affinity chromatography, eluting with a NaCl gradient. Capsids were then diluted in 50 mM Hepes, 25 mM MgCl2, 25 mM NaCl, pH = 7.4.

2.2. Cryo-EM sample preparation

3 μl of 0.6 mg/ml AAV-DJ was applied to Quantifoil® (Jena, Germany) R2/2 200 mesh copper grids that were rendered hydrophilic by glow discharge in 75/25% Ar/O. The grid was hand blotted and further incubated with 3 μl of Arixtra (pharmaceutical preparation) for 15 s at a concentration of 5.7 mM. Arixtra was dissolved in ultra pure water. After addition of Arixtra, the grids were vitrified in liquid nitrogen-cooled ethane using an FEI Vitrobot (FEI, Hillsboro OR) with the following parameters: blot force = 1, blot time = 3 s, total blots = 1, humidity = 100%, temperature = 4 °C. Vitrified samples were stored in liquid nitrogen until further use.

Kvβ2 was expressed and purified as previously published with no modifications (Weng et al., 2006). For cryo-EM sample preparation, the final sample was dialyzed overnight at 4 °C against glycerol-free buffer (20 mM Tris pH 8.0, 300 mM KCl, 1 mM beta-mercaptoethanol) in a 5000 molecular weight cut off (MWCO) dialysis cassette (Thermo, USA). 3 μl of Kvβ2 at 0.9 mg/ml was applied to Quantifoil® R2/2 200 mesh grids that were pre-treated in the same fashion as mentioned above. Grids were vitrified in liquid nitrogen-cooled ethane using an FEI Vitrobot (FEI, Hillsboro OR) with the following parameters: blot force = 2, blot time = 2 s, total blots = 1, humidity = 100%, temperature = 4 °C. Vitrified samples were stored in liquid nitrogen until further use.

2.3. Image acquisition

Two AAV-DJ Arixtra datasets were obtained from the same grid with differing defocus ranges. A final exposure magnification of 29,000 was maintained for all images. All images experienced a similar total dose (66 $e^-/Å^2$) and were acquired using an FEI Titan Krios (FEI, Hillsboro OR) using Leginon (Suloway et al., 2005) for image collection. The images were recorded on a DE-20 direct electron detector (Direct Electron, San Diego, CA) with the dose fractionated across 45 frames leading to a dose rate of 1.5 $e^-/Å^2$ per frame. The first dataset was collected at a defocus range between 1.5 and 3.0 μm and the second set at 0.75 – 1.75 μm. Defocus estimates were continually made throughout the image acquisitions to ensure that the defocus range did not drift outside of the defined parameters using the automated contrast transfer function (CTF) estimator ACE (Mallick et al., 2005) and CTFFIND3 (Mindell and Grigorieff, 2003). The two datasets yielded a total of 1428 images that were acquired over a total of 40 h of data collection. Of those images, 377 were discarded due to contamination or mistargeting of the exposure.

2.4. Dose compensation and frame alignment

Frame alignment and dose compensation was performed using the DE_process_frames-2.5.1 software that comes with direct electron cameras. Briefly, the algorithm makes rolling averages for sets of frames, aligning them to the sum of all frames, and iterating the alignment a user-defined number of iterations. Dose compensation is achieved by applying a low-pass filter to individual frames where the cutoff frequency for a given frame is dependent on the cumulative dose for that frame. The specific cutoff frequencies were calibrated from the fading of spots of images of catalase crystals (Direct Electron, personal communication) determined similarly to that described in Baker et al. (2010). A scaling factor can optionally be applied to make the filtering more or less aggressive, and this was used to generate stacks with four different dose compensation schemes. The individual per frame low-pass filtering schemes for each stack are given in Fig. S1. The DE_process_frames software was wrapped into the Appion pipeline so that if a user has access to the DE_process_frames software, frame data can be processed in a high-throughput manner. For the datasets here, full frames were first aligned and dose compensated, then after particles were picked, individual particles were realigned and dose compensated.

2.5. Image processing

Of the two AAV-DJ Arixtra datasets, the first image set yielded 503 full-frame image exposures, producing 65,978 particles. The second image set totaled 548 full-frame image exposures and produced 54,188 particles. Particle picking was completed semi-automatically using the template picker FindEM (Roseman, 2003) within Appion (Lander et al., 2009). The template used for picking was a rotational average of the unaligned average of a stack of manually picked particles. Particles over carbon were manually deselected. Initial Eulers were generated using EMAN1 (Ludtke et al., 1999) by refining for 2 iterations with an angular increment of 1° starting from an initial model of AAV that had been low-pass filtered to 20 Å. The Euler angles were then further refined with 9 iterations of FREALIGN (Grigorieff, 2007) refinement. An inverse B factor was applied to the final FREALIGN maps using EMBFactor (Fernández et al., 2008) after refinement was complete.

2.6. Atomic modeling and fitting

The effective EM magnification, an envelope correction, and the resolution of a low-pass filter were least-squares refined using RSRef (Chapman et al., 2013) by optimizing the agreement between the density of the EM reconstruction and the atomic model. The starting model was derived from a prior AAV-DJ structure at 4.5 Å resolution (Lerch et al., 2012). It was manually adjusted using Coot (Emsley and Cowtan, 2004) and then refined by simulated annealing torsion angle optimization against the AAV-DJ-Arixtra complex map. This was done using a real-space objective function calculated by RSRef embedded in CNS v1.3 (Brünger et al., 1998).

Arixtra was refined against a difference map between the Arixtra complex and native. Prior to difference map calculation, the reconstructions were scaled in reciprocal space using EMAN1 to limit the impact of differences in power spectra. They were also scaled in real-space, putting each on an absolute scale with reference to the atomic model using RSRef (Chapman et al., 2013). The model for the Arixtra ligand was refined against this difference