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Processing Apoferritin with the Appion Pipeline

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Abstract

The 3DEM map challenge provided an opportunity to test different algorithms and workflows for processing single particle cryo-EM data. We were interested in testing whether we could use the standard Appion workflow with minimal manual intervention to achieve similar or better resolution than other challengers. Another question we were interested in testing was what the influence of particle sorting and elimination would be on the resolution and quality of 3D reconstructions. Since apoferritin is historically a challenging particle for single particle reconstruction and the authors of the original map challenge data used only a fraction of the particles present in the dataset, we focused on the apoferritin dataset for our entry. We submitted a 3.7 Å map from 25,844 particles and a 3.6 Å map from 53,334 particles and after assessment were among the best of the apoferritin maps that were submitted. Here we present the details of our reconstruction strategy and compare our strategy to that of another high-scoring apoferritin map. Altogether, our results suggest that for a relatively conformationally homogeneous particle like apoferritin, including as many particles as possible after elimination of junk leads to the highest resolution, and the choice of parameters for custom mask creation can lead to subtle but significant changes in the resolution of 3D reconstructions.

Keywords

Cryo-EM; map challenge; best practices; single particle reconstruction

Introduction

Apoferritin is historically a challenging particle for structure determination by cryogenic electron microscopy (cryo-EM) (Henderson and McMullan, 2013; Russo and Passmore, 2014). In projection images, it appears as a relatively featureless hollow sphere with no

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obvious features that distinguish different views. The asymmetric unit is a four α -helix bundle that is arranged with octahedral symmetry to generate a spherically circumscribable ball. In order to determine the relative Euler angles of cryo-EM images of apoferritin, the particles must be imaged with high-enough resolution to align on the α -helices instead of the gross morphology of the molecule. As such, it is an excellent particle for assessing the quality of sample preparation, imaging, and processing for single particle cryo-EM.

Russo and Passmore (Russo and Passmore, 2014) showed that by using holey EM grids made entirely out of gold, they could dramatically improve the image quality of apoferritin images. Compared to carbon, the gold substrate dramatically limits beam-induced motion by increasing the conductivity and by reducing the effect of differential contraction (Russo and Passmore, 2016). The authors showed that by collecting cryo-EM data of apoferritin prepared on gold foil grids, they increased the resulting image quality enough to be able to determine the structure to 4.7 Å. In that study, the authors showed that the beam-induced motion of apoferritin decreased substantially on gold grids. They initially processed ~6000 particles and found that the highest resolution was found by limiting their data to the 478 best particles. We have previously found that the resolution of reconstructions is strongly influenced by the number of particles that contribute to the map (Stagg et al., 2014), and the Map Challenge apoferritin data contained over 70,000 particles. We hypothesized that we could achieve higher resolution by using a substantially larger number of particles than the original authors. Furthermore, for the map challenge, we were interested to find out if we could use the standard Appion pipeline for data processing (Lander et al., 2009) to achieve a single particle reconstruction with similar or better quality than Russo and Passmore and to see if we could achieve similar quality to the other map challengers.

Methods

Details of the Apoferritin dataset

The map challenge apoferritin dataset consisted of 178 images collected on a FEI (now ThermoFisher) Polara operated at 300 keV. Images were recorded on a FEI Falcon II at a sampling rate of 1.346 Å/pixel. The total dose was 16 e-/Å² which was spread out over 16 movie frames. Underfocus values ranged between 1.3–3.6 µm.

Frames

We chose to use the movie frames provided from the map challenge for our data input. The first step was to make summed images for CTF estimation and particle picking. We used tools from the Leginon/Appion package and numpy to read in movies and make summed images. We noticed that there was a large border at the edge of the frames that was unusable, so we masked the border after summing the images. The summed images were uploaded to the Appion database for particle picking and CTF estimation. After picking, the particles were adjusted for per-particle beam-induced motion using the DE frame alignment script that is available from directelectron.com and is wrapped in Appion (Spear et al., 2015). The DE frame aligner makes rolling averages for sets of frames, aligning them to the sum of all frames, and iterating the alignment for two 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. For these data, we used 5 frames for the rolling average and used the dose compensation scheme worked out in Grant and Grigorieff (Grant and Grigorieff, 2015).

Particle picking

We picked particles using a combination of reference-free difference-of-Gaussians (DOG) picking (Voss et al., 2009) and template-based picking (Roseman, 2004). Our first step was to use the Appion DOG picker to pick particles in a reference-free manner so as to not bias the reconstruction later. DOG picker works best for particles that are not hollow and does not center particles particularly well, so it had many false negatives missing most of the particles in the apoferritin dataset (Fig. 1A). Of the particles picked, however, there were few false-positives. The DOG picks were used to create an unaligned average template that represents the 2D rotational average of the particles (Fig. 1B). This was used as a template in template-based picking using FindEM in Appion (Roseman, 2004). The template-based picks were much better centered, had fewer false positives, and many fewer false negatives (Fig. 1C). The new picks were again used to make an unaligned average that was used as a template for another round of template based picking (Fig. 1D, E). The final picks were additionally limited by their distance to the border in order to exclude the border mask at the edges of the images (Fig. 1E).

CTF estimation

The CTF was estimated using multiple runs of ACE (Mallick et al., 2005) and CTFFIND3 (Mindell and Grigorieff, 2003). ACE does not estimate astigmatism but does a good job getting an accurate, but not necessarily precise defocus estimate. CTFFIND3 does a good job estimating astigmatism and defocus more precisely than ACE, but it is more dependent on the starting value for defocus. Our strategy was to estimate the defocus first with ACE, then refine the defocus estimate using CTFFIND3 using the ACE value as a starting point. CTFFIND3 was then run a third time using the previous value as a starting point (Fig. 2). Appion was then used to rank the CTF estimates using a reliability metric that scores CTF estimates based on how well they match the data (Sheth et al., 2015). Surprisingly, 29.3% of the micrographs had better estimates in ACE than they did in CTFFIND3. We speculate that this is due to the fact that CTFFIND3 almost always finds some astigmatism. If images had no or little astigmatism, then ACE could have a higher resolution estimate.

Stack making

After particles were picked and CTF estimated, stacks were made using makeStack in Appion. This resulted in a stack of 79,467 particles. Bad picks and poorly aligning particles were eliminated by performing 2D classification using the topology alignment tool within Appion (Ogura et al., 2003). This resulted in a final stack of 65,495 particles. Per-particle frame alignment as described in *Frames* above was then performed on individual particles to generate a frame-aligned dose-compensated stack of apoferritin particles.

Refinement

Refinements were performed using both FREALIGN (Grigorieff, 2007) and RELION1.4 (Scheres, 2012). We initially performed alignments with FREALIGN. An initial model was generated by assigning particles with random Eulers and reconstructing. A global Euler search was then performed in FREALIGN using mode 4 in FREALIGN. Iterations of global searches and reconstructions with octahedral symmetry applied were performed until the structure converged on a reconstruction resembling apoferritin. Then a custom particle-shaped mask was created, and the refinement was switched to mode 1 for local Euler refinement. The refinement converged on a 6 Å map, and we were unable to further increase the resolution with FREALIGN. We then moved to refining in RELION. We were particularly interested in the influence of the number and quality of particles for overall map resolution and quality, so we compared eliminating particles by 2D classification to eliminating them by 3D classification before using auto-refinement to generate the final reconstruction. Our approach with the 2D classification strategy was to run all the particles through 2D alignment and classification, and select only particles that contributed to class averages that showed clear secondary structure for auto-refinement (Fig. 3A). The averages with neighboring particles apparent in the average were not excluded unless they were poorly defined. This resulted in a set of 53,334 particles that refined to 3.6 Å by the FSC_{0.143} criterion (Fig. 3B, Supplemental Fig. 1A). The reconstruction showed clear side-chain densities that were consistent with the reported resolution. The second approach was to use 3D classification to sort particles into two classes. Only one of the classes showed well-resolved secondary structure (Fig. 3C), and the 25,844 particles from this class were then used in an auto-refinement and resulted in a 3.7 Å reconstruction by the FSC_{0.143} criterion (Fig. 3D, Supplemental Fig. 1A). The reconstruction was of similar quality to the 3.6 Å reconstruction and had similar details though some side chains appeared slightly less resolved, though overall, it was hard to distinguish the two by eye. Many other particle sorting strategies were attempted in order to find the “best” particles such as using only particles with 2D class averages with no neighboring particles and splitting the particles into more 3D classes, however none of these approaches resulted in higher resolution than from using all 53,334 particles.

A final try at improving the resolution with FREALIGN was attempted. We took the Euler angles and shifts from RELION and imported them into FREALIGN. With no further refinement, the particles reconstructed identically to the RELION reconstruction, however, when we attempted to further refine the Euler angles, the resolution dropped substantially, and we could never get better than 4.5 Å. After the map challenge results were released, we noticed that the highest resolution challenger used FREALIGN for all of the processing (emcd121), which indicates that there is a strong user dependence to refinement in FREALIGN. After the map challenge was complete, a new version of the FREALIGN refinement was released in a new package called cisTEM (Grant et al., 2018). cisTEM heuristically determines many of the refinement decisions that the user would have to determine with FREALIGN, and this has the potential of removing some of the user-to-user differences with that software. Thus, as a final test, we ran the original 65,495 particle stack through the cisTEM workflow using the default parameters. 2D classification was performed in cisTEM, from which 61,636 particles were selected for auto-refinement. The auto-refinement

converged on a 3.4 Å structure that had noticeably better side chain densities than the maps we submitted (Fig. 4D). This suggests that the refinement algorithm in cisTEM helps eliminate much of the user-to-user variation we observed in the map challenge for Frealign refinements of apoferritin.

Results and Discussion

As described in the Methods, we used standard Appion tools for particle picking, CTF estimation, frame alignment, and particle sorting to generate stacks that were refined with RELION, Frealign, or cisTEM. We ultimately submitted two apoferritin maps to the map challenge: a 3.6 Å map from 53,334 particles and a 3.7 Å map from 25,844 particles (Fig. 3,4). The maps were very similar in quality and details, and it was hard to distinguish them visually. Both maps ranked very highly in the map assessment and out of the nine entries were either the top ranked or just behind the top-ranked maps among multiple assessment metrics. In our own assessment, which ranked maps according to how well the automatic modeling algorithms in Rosetta (DiMaio et al., 2015) were able to create all-atom models (Mendez and Stagg, current issue), the maps were the ranked third for the 3.6 Å map and fourth for the 3.7 Å map. We isolated single apoferritin asymmetric units from each of four maps: our two maps emcd118 and emcd166, emcd112, which was the top ranked apoferritin map from our assessment, and our new map from the cisTEM refinement. A horse spleen apoferritin crystal structure, PDB ID 2W0O (de Val et al., 2012), was rigid-body fit into each map, and the maps were contoured such that they enclosed an equal volume. When they were visualized in the same orientation, overall, they all looked similar, but some slight differences could be observed particularly in the quality of certain side chains (Fig. 4). For instance, at the same relative contour level, lysine 97 (* in Fig. 4) was only resolved with the higher-quality, higher-resolution maps. It should be noted, however, that lysine 97 could be observed in all four maps, but only at lower contour levels for the lower-resolution maps.

Our submitted maps and emcd112 were processed in similar ways, which suggests the question, why was emcd112 higher resolution than our maps? The most likely reasons are differences in the frame alignment and dose compensation schemes and the custom mask used for refinement. We used per-particle frame alignment and a fixed per-frame dose compensation scheme whereas in emcd112, they used the “particle polishing” algorithm in RELION in which the dose compensation is not fixed but estimated per-particle based on alignment quality. Comparing the custom masks, our mask was more conservative with smoother contours and lower resolution features (Fig. 5). Since we were able to get to 3.4 Å resolution with cisTEM (which uses a dynamic masking algorithm) with the same dose-weighted particles that we used in RELION, we believe that the difference in resolution of the refinements was due to the masking. A more thorough analysis on the influence of the masking in Euler angle determination and estimation of resolution would be necessary to determine that definitively.

Based on our analyses we suggest a few conclusions. First, pre-processing using standard Appion tools and minimal user intervention and decision making produces high quality results. Second, once bad picks and junk are eliminated from a stack of particles, including as many particles as possible produces the highest-quality highest-resolution reconstructions

for apoferritin. There is likely a strong specimen dependence for this observation as apoferritin is relatively homogeneous in conformation and composition. Nonetheless, given that resolution is ultimately dependent on the number of particles contributing to a map (LeBarron et al., 2008; Rosenthal and Henderson, 2003; Spear et al., 2015; Stagg et al., 2014) our results suggest that care should be taken when throwing out particles. Determining what constitutes bad particles is often subjective and can vary from person to person. Particles that might not align well with reference-free 2D classification may well align properly with 3D projection matching. Third, there can be substantial variations between the results of refinements between different users in the different refinement packages. For instance, we are very experienced with single-particle reconstructions and have used FREALIGN to achieve better than 3 Å resolution reconstructions in the past (Spear et al., 2015; Xie et al., 2017), but we were unable to achieve a high-resolution reconstruction using FREALIGN alone in for apoferritin. However, as the software packages mature, best practices are emerging. For instance, in the new implementation of FREALIGN in cisTEM, we achieved a similar resolution and quality as the best FREALIGN entry for apoferritin in the map challenge. Thus, as best-practices emerge, some of the user-to-user variability is being eliminated. We look forward to more best practices emerging in future map challenges.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Particle picking. A) Particles picked first using DOG picker. B) An unaligned rotational average of DOG picks. C) Template-based picks using (B). D) An unaligned rotational average of template-based picks. E) Template-based picks using (D).

Fig. 2.

CTF estimation. In each panel, a different Appion CTF assessment is shown. In the Appion CTF assessment panel, the top left shows the raw 1D power spectrum of an image with the noise fit in red. The top right shows the noise-subtracted spectrum with the envelope fit. The middle panel shows the noise and envelope-corrected power spectrum together with the fitted CTF from a given CTF estimation run. The bottom panel scores the CTF fit across different resolution bands with green being the best agreement between the data and the model and red being worst. A) CTF estimation using ACE. B) CTF estimate using CTFFIND3 with the ACE defocus value as a starting point. C) CTF estimate using CTFFIND3 with (B) as a starting point.

Fig. 3. Map challenge refinements. A) 2D classification. Particles that contributed to averages that showed secondary structure were selected for (B). B) Refinement from 53,334 particles. C) 3D classification. D) Particles that contributed to the structure on the left were selected for (D). D) Refinement from 25,844 particles.

Fig. 4.

Comparison of our submitted maps with higher-resolution maps. All maps are shown with the 2W0O crystal structure fitted in the density map in the same orientation for all panels. The maps were contoured such that they enclosed the same total volume. A) Reconstruction from particles sorted by 3D classification. B) Reconstruction from particles sorted by 2D classification. C) Map challenger emcd112 that was processed similarly to our submissions. D) Re-refinement of our particles in cisTEM. The density for lysine 97 (*) was only seen at the given contour level in the higher-resolution maps.

Fig. 5. Comparison of surface contours (top panels) and central slice (bottom panels) for emcd112 (A) and our maps, emcd118 and emcd166 (B).