

May 24, 2012

Title: "Movies of ice-embedded particles enhance resolution in electron cryo-microscopy"

Editor's Remarks to Author:

Both reviewers come up with a fairly long list of questions, mainly about experiments that have not been reported and may not yet have been done. One reviewer is more positive than the other, but the bottom line is that they both think that not quite enough new work has been done to merit immediate acceptance for PNAS. The authors should be offered the opportunity to revise the manuscript particularly by adding more data or more analysis to address the criticisms of the referees. The most serious issues are (a) the apparent absence of rotational particle motions induced by the beam, reported in previous papers using the same specimen: one referee has a list of questions about this, while the other doubts the interpretation (How can the authors be sure?), and (b) whether the experiments reported might have too many changed parameters since the 2008 data set to make reliable deductions. Both of these criticisms could potentially be answered by either more data or more analysis or both.

Reviewer Comments:

Reviewer #1:

Subject Quality? Yes
Sufficient General Interest? Yes
Conclusions Justified? Yes
Clearly Written? Yes
Procedures Described? Yes

Comments:

This manuscript reports a significant advance in cryo-EM of biological specimens. It will be of interest to hundreds of scientists in the field, and I expect that it will result in follow-up experiments performed by others over a period of several years.

The manuscript could be further improved if the authors would make the following, minor revisions.

1. I suggest that more quantitative information be provided for the following:
 - a. The authors state that the observed reduction of the B-factor that resulted from alignment of particles from one frame of a "movie" to the next should increase the signal by 50%. What I find missing, however, is a statement of the factor by which the number of particles is expected to be reduced solely because of the improved B-factor. In other words, how much of the 10-fold reduction in number of particles is due to the reduced B-factor and how much must be due to the other factors that the authors discuss?
 - b. The authors state that the fraction of electrons that are inelastically scattered at 200 keV is much larger than it is at 300 keV. Since the energy dependence of scattering cross sections is well known, the authors should say explicitly what the fraction is for a typical specimen thickness.
2. It would be very helpful to the rest of the field if the authors provided much more detail about the currently observed beam-induced movement. I found it important that they did not observe rotation of the virus particles in these experiments, for example, and that they offered a possible explanation for this difference from earlier work. Other details that might be covered include

- a. How did the authors distinguish between stage drift and beam-induced translation?
 - b. How much of the motion (quantified in Figure 2) was due to stage drift?
 - c. Was there any correlation between the direction in which a particle moved and the direction of the edge of the hole in the carbon film?
 - d. Was there any correlation between the amount by which a particle moved and its location relative to the edge of the hole in the carbon film?
 - e. In the current experiments, did neighboring particles move by similar amounts and in a similar direction, as they did in the previous experiments?
3. Finally, I recommend two changes in the way that the authors appear to present themselves:
- a. The final phrase in the abstract is likely to be interpreted as being provocative, although I do not think that this was the intent of the authors. I suggest that they replace the statement "and is expected to reduce the need for the highest-end microscopes to achieve the best possible resolution." by something similar to what they wrote in the Discussion: "The ability to record movies therefore represents a way to improve the performance of mid-range electron microscopes that may have limited mechanical stability and beam coherence."
 - b. I find it curious that the authors still include helium cooling in the list of improvements that have been made recently in electron microscopes. I am not sure whether a helium stage is still offered by any of the major manufacturers - in any case, the community as a whole no longer is interested in such an option. I thus recommend that they delete that item from their list.

Reviewer #2:

Subject Quality? No
Sufficient General Interest? No
Conclusions Justified? No
Clearly Written? Yes
Procedures Described? Not Applicable

Comments:

In this manuscript, Grigorieff and colleagues report the use of successive images collected with a direct electron detector to reduce the extent of beam-induced motion in cryo-electron microscopy. The authors show that by using a subset of the images collected during continuous electron exposure, fewer particles are necessary to get to near-atomic resolution for the 70 MDa rotavirus DLP particles.

While it is nice to see that direct electron detectors can be used in this mode, the manuscript is superficial in many respects. As the authors point out, the idea of aligning movie frames to reduce blurring was already introduced in ref. 24. In the present manuscript, the authors use this technique in order to obtain a single-particle reconstruction of the rotavirus DLPs at 4.4 Å; and compare it to a previously published structure at 4.1 Å resolution (ref. 27) obtained using photographic film.

The benefit of motion compensation in exposure series is clear and somewhat predictable based on the general premise of single-particle reconstruction. The more interesting comparison is the one the authors make with the '2008 data set'. However, the interpretation of the results is not as clear-cut as the authors imply, and practitioners of single particle EM will want to see a lot more before arriving convincingly at the preferred conclusions the authors wish to draw. The authors present the result of one comparison, but there are numerous possible differences that are still uncalibrated. Differences between the data collection and imaging

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strategies utilized in the two cases include different voltages (300 vs 200kV), film-vs-direct-detector, different pixel size (1.2 vs. 1.42), and also substrates and microscope illumination conditions. All of these elements can contribute to the differences observed by the authors between the maps, and it is difficult to determine the contribution of each component separately.

The absence of measurable rotations within movie frames presented in this manuscript compared to ref. 24 is attributed to the differences in imaging setup (exposing entire holes as opposed to only exposing one side of a hole). How can the authors be sure about this? What happens when rotations are present? This was touched upon in ref. 24 but it is not discussed here.

July 24, 2012

Title: "Movies of ice-embedded particles enhance resolution in electron cryo-microscopy"

Authors: Campbell et al.

Dear Dr. Grigorieff,

I regret to inform you that the PNAS Editorial Board has rejected your revised manuscript. The expert who served as the editor obtained 2 re-reviews, which are included below. After careful consideration, the editor decided that we cannot accept your manuscript. Our policy is that a single negative review, with which the editor agrees, is sufficient to recommend rejection.

Once a paper has been rejected, it may not be resubmitted through an Academy member. Note that the PNAS License to Publish conveyed at initial submission is terminated.

Thank you for submitting your manuscript to PNAS. I am sorry we cannot be more encouraging this time, and I hope you will consider submitting future work to PNAS.

Sincerely yours,

Editor-in-Chief

Editor's Remarks to Author:

Although the work reported is clearly important, there are enough concerns about the origins of the reported improvements in image quality and resolution that I think PNAS should reject the paper in its current form. The authors will then have to decide whether (a) to publish elsewhere in a more technically oriented journal in which the reasons for the improvements are less important than the technology for achieving them, or (b) do more work to eliminate or quantify the component of the improvement due to stage drift compensation, which is indeed not so interesting for PNAS, and then submit a different paper.

Reviewer Comments:

Reviewer #1:

Suitable Quality? Yes
Sufficient General Interest? Yes
Conclusions Justified? Yes
Clearly Written? Yes
Procedures Described? Yes

Comments:

All issues mentioned in my review of the initial manuscript have been fully addressed. I believe that this paper will be much cited, and that the quality of work accomplished by others will benefit considerably from this paper.

Reviewer #2:

Suitable Quality? No
Sufficient General Interest? No

Conclusions Justified? No
Clearly Written? Yes
Procedures Described? Yes

Comments:

The line of experiments the authors are pursuing is interesting and important, but I remain concerned about the quality of work presented in this manuscript. The authors draw broad conclusions about the origin of resolution in cryo-EM from a relatively small amount of data on images recorded from frozen-hydrated specimens of viruses. They report experiments where they attempt to fractionate the dose delivered to the specimen, and compare the final reconstructions using earlier and later images, and with or without aligning sub-exposures to each other. They find that alignment improves the resolution. The origins of this improvement could be either because they are compensating for "beam-induced movement" or mechanical stage-drift. However, here is the problem. If the improvements are due to the former, then the result is interesting, although it is still rather preliminary because the relevance of extending this to other samples, substrates and different data collection conditions remains to be established. However, if it is primarily from stage drift, the result is less interesting and is largely an advertisement for the detector that has the capability of recording such movies.

To discriminate between these two parameters, the authors use a script that monitors stage drift and record images when it is less than 7 $\mu\text{m}/\text{sec}$ (although they say that the actual drift was only 1.6 $\mu\text{m}/\text{sec}$). It is no surprise that stage drift is a concern for recording useful images. However, there is a flaw in the design of the experiment (page 7):

"Smaller translations were performed (less than 2 μm) to record movies on different holes in the same area. Although no further monitoring of the stage drift was performed after these smaller translations it is reasonable to assume that the average drift rate did not change significantly."

There is no basis to assume that the drift is any smaller with small translations of the stage. While this may be true of certain specialized piezo stages, the mechanically driven stages the authors use can in fact lead to significant drift rates, especially for small translations. So the authors have no way of knowing the actual stage drift for most of the data they have collected, which undermines their essential conclusion that the measured shifts (Figure 2D) arise from beam-induced motion. This is a key point that needs to be addressed experimentally. Without careful quantitation of the relative contribution of stage movement vs. beam induced movement, the results can be misleading.

There is also an internal inconsistency within the manuscript. At the end of the abstract and the final paragraph, the authors note that using movies should improve the performance of microscopes with poor stage stability or beam coherence. Beam-induced motion is going to be much the same in a higher-end or lower-end microscope, so it is inconsistent to make the argument that this is generally applicable to lower end microscopes with poorer mechanical stability, given that they argue that it is beam-induced motion and not stage drift that is the reason for the degradation they observe in the absence of fractionating the dose.

In summary, I think this manuscript is not yet ready for publication. The question being addressed is of broad general interest to the cryo-EM community, but the experiments and their analysis is not yet sufficiently rigorous.