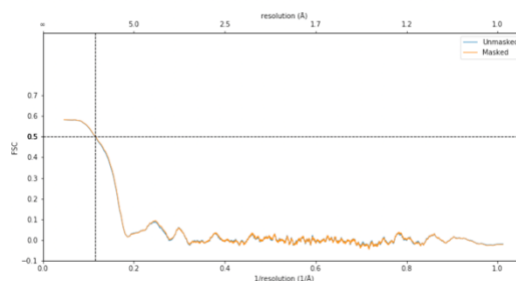


I initially feared that reading through and understanding the massive amount of material sent to me for evaluation would be a huge undertaking. Pleasantly, I found that the Reader has actually done almost all of the work needed to understand the serious concerns with the paper by Casasanta et al. The Reader should be commended for all of the effort that he/she has invested in properly analyzing this paper. In contrast, the more material supplied by the Authors to rebut the Reader, the more questions arose about both what was published and what has been done since. Rather than recapitulating all of the points raised by the Reader, let me just summarize what appears to me to be the salient points:

- 1) There are five different pixel values cited in the paper and the depositions, with no explanations. As the Reader noted, one cannot simply “rescale” the pixel values by a large amount without redoing the entire processing, which never appears to have been done. The only significant difference between the original EMDB deposition (EMD-22982) and the “improved” one (EMD-24147) appears to be the pixel size.
- 2) It was stated that the images were collected with “<5 electrons per Å²”. This is impossible to believe, as the shot noise from such ultra-low doses (described by Poisson statistics) would preclude the alignment of images.
- 3) After multiple requests from the Reader for the half-maps that were used to calculate the FSC, the Authors supplied such maps in ChimeraX sessions. As the Reader notes, these cannot possibly be the half maps of either EMD-22982 or EMD-24147. The simplest glance at these would show anyone that there is significantly more high frequency noise in the full maps than in the half maps, while the opposite should be the case. It seems the Authors do not understand that one of the consequences of averaging more particles together is that noise is reduced. They would have us believe that more noise appears in the full data set than in half data sets.
- 4) The Authors initially refused to provide the PDB of the model used in their paper. They argued (correctly) that it was not appropriate for PDB deposition. But such models should be provided in the Supplementary Material, particularly when this model appears in a number of figures, as well as in Supplementary Movie 1 showing the fit of the model to the density map. They have now made a model available, but the Reader makes an extremely strong argument why this is not the model shown in the paper nor is it the output of Phyre2 without “refinements”.
- 5) It has been argued in numerous papers that the FSC is not really a measure of resolution, but a measure of reproducibility. This becomes very clear when symmetry is involved, and imposing the wrong symmetry can lead to two independent half-maps that are well correlated, but both are nonsense. Thus, the true “gold standard” is not the FSC curve, but the visual appearance of the map. This has also been true in x-ray crystallography, where the interpretability of the map is far more important than any simple metric (such as FOM). Taking the N-model_Phyre2.pdb file, part of the ChimeraX session meta-data-overlay.cxs, I did a map:model FSC with the “final” map, EMD-24147 (right). Using the rather standard



threshold of 0.5 for such map:model comparisons, the resolution is judged to be 8.6 Å. This makes sense, and is consistent with the visual appearance of the map. Keeping in mind that this model appears to be altered from the Phyre2 output model used in the paper in order to better fit the map (such as by the loss of secondary structure, etc.), it is therefore reasonable to expect that the resolution would be judged even worse using the original model. Phenix, which was used for this FSC curve, returns a map:model correlation of 0.44. This is quite consistent with the overall correlation between map and model that the Authors cite of 0.472, but shows that such an overall correlation tells one nothing about the resolution. The FSC, by definition, is showing the correlation in Fourier space as a function of resolution. Thus, the resolution of 4.3 Å stated by the Authors is judged to be absurd.