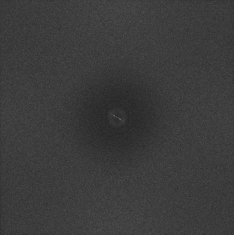
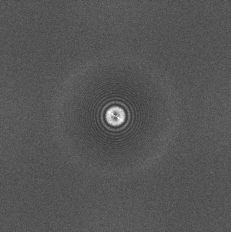
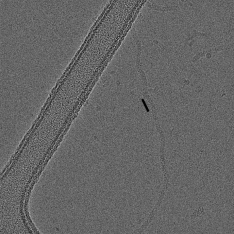
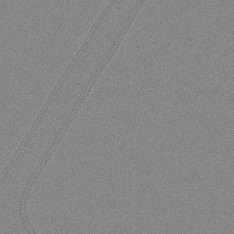
The 180 page PDF “Technical Response” sent by the senior author is a perfect example of a document dump, as described by Wikipedia (hDps://en.wikipedia.org/wiki/Document\_dump): A **document dump** is the act of responding to an adversary's request for information by presenting the adversary with a large quantity of data that is transferred in a manner that indicates unfriendliness, hostility, or a legal conflict between the transmitter and the receiver of the information. The shipment of *dumped* documents is unsorted, or contains a large quantity of information that is extraneous to the issue under inquiry, or is presented in an untimely manner, or some combination of these three characteristics. The phrase is often used by lawyers, but is in increasing use in the blogosphere. It is often seen as part of the characteristic behavior of an entity that is engaging in an ongoing pattern of activities intended to cover up unethical or criminal conduct.

The original map associated with the Nanoscale paper is EMD-22982, which has a scale of 0.93 Å/px but described in the paper as 1.05 Å/px. After concerns were voiced about the original map, an “improved” map was deposited, EMD-24147, based upon the same micrographs. The scale in this new map is mysteriously 0.83 Å/px. The authors have responded to previous concerns about the lack of half-maps by sending such maps (Map1\_50.mrc, Map2\_50.mrc, half-map1.mrc, half map2.mrc). These appear to have been resampled at either 1.85 or 2.0 Å/px. EMD-40656 and EMD 40657 have 1.4 Å/px. The authors appear to be so confused by these different values that they state in the Technical Response: “A newer version of the N protein map (EMD-29002) was deposited in 2022, also with a pixel value of ~0.93 Å / pixel.” In fact, this deposited map has a scale of 0.8 Å/px. So from the same original micrographs the authors have pixel values of 0.8, 0.83, 0.93, 1.05, 1.4, 1.85 and 2.0 Å. EMD-29002 is linked to their 2023 M&M paper (Casasanta et al., 2023), and that paper states that the pixel size is 1.05 Å. So in 2022, when they deposited EMD-29002, they knew that the correct pixel size was either 0.8 or 0.93, but in 2023 they wrote that it was 1.05 Å? None of this can be justified, and the arguments that the author makes about the thickness of the grid changing the pixel size are absurd. They are asking us to believe that the 30% change in scale (from the published 1.05 Å/px to the latest 0.8 Å/px) might be due to a shift of the Z-position of the sample due to the increased thickness of the grids.

The absurdities are apparent in Figure 1 of the Technical Response, where they display both EMD-29002 and EMD-24147 with 0.93 Å/px, when the actual deposited maps have values of 0.8 and 0.83 Å/px, respectively. So if one looks at the progression of these maps, from the original deposition EMD-22982 linked to the Nanoscale paper (0.93 Å/px), to EMD-24147, an “improved” map linked again to the same Nanoscale paper (0.83 Å/px), to the latest map EMD-29002 (0.8 Å/px) linked to the 2023 M&M paper (Casasanta et al., 2023), the reconstruction is shrinking in volume over time to provide a better fit to their model.

The author insists that the EMs used were recorded with a total dose of 5 electrons/Å2, as it was stated in the Nanoscale paper that the dose was < 5 e/Å2, and provides Fig. 2 in the Technical Response as an example of such an image. But in the Methods of the Nanoscale paper it is actually stated that “Images were recorded under low-dose conditions (<5 electrons per Å2 per s) at 200 kV. Sixty movies were acquired at 0.25 second exposures”. This would mean a total dose of ~ 1.25 e/Å2 since exposures were for only a quarter of a second. This is even more absurd than the claim of 5 e/Å2. Dr. Kelly seems unaware of basic math and physics, as such an image with 5 e/Å2 (ignoring the more absurd 1.25 e/Å2) would be dominated by the electron shot noise due to the Poisson statistics and would have almost no contrast for small objects.

I show an image recorded on a 200 kV microscope with 5 e/Å2 (left), compared with a “normal” dose of 50 e/Å2 (right):



The power spectrum for each of these images is shown below the image. The black bar (50 e/Å2 image on the right) is 200 Å long, and the fairly massive filament (practically invisible on the left) has a mass per unit length of ~ 2.4kDa/Å. So the mass in the length of the black bar would correspond to 10 N protein monomers. Thus, the statement that these images in the Nanoscale paper were recorded with 5 e/Å2 cannot possibly be true. Not only would one be unable to see the particles at such a dose, but one would be unable to observe the oscillations of the CTF in the power spectra needed to accurately determine the defocus. Dr. Kelly *now* states in the Response that the total dose was actually 5-60 e/Å2 for all images collected, not either < 5 e/Å2 or 1.25 e/Å2 as they published in Nanoscale, and “The best images used in our structural analysis contained the least amount of radiation damage and were obtained at the lower end of the dose range.” If we look at the subsequent “updated” depositions by the authors described in the Technical Response, EMD-40632,

EMD-40633, and EMD-40647 state that the total dose was 60 e/Å2, while EMD-40653 and EMD 40656 state 50 e/Å2. This raises an obvious question. If 5 e/Å2 was able to achieve the beautiful contrast shown in Fig. 2 of the Technical Response, and the best images contained the lowest dose, why did the authors use 10-12 times this total dose in the “updated” and improved maps? So rather than simply say that the 5 e/Å2 and 1.25 e/Å2 were a mistake and they meant 50-60 e/Å2, Dr. Kelly has chosen to defend the impossible.

Dr. Kelly never really addresses the egregious artifact in most FSC plots shown by them, which is that these curves almost never fall to 0.0. She states that this was due to the binning procedure, but this is nonsense. For two independent maps, there should be no correlation at the highest spatial frequencies. In other words, beyond the true resolution of the maps, almost all modulation is noise, which should be uncorrelated between two independent maps. Yet if one looks at the FSC plots in this “document dump”, the FSC never falls below 0.2 in 40632, 40633, and 40647. In 40656 and 40657 (based upon resampling to 1.4 Å/px) the author-supplied FSC curves show such a steep drop that serious questions are raised as to what was actually done, as Fourier theory dictates that the rate of change in a Fourier Transform cannot be greater than 1/D, where D is the dimension of the compact support. The steep drop in the FSC plots should be compared to the ideal behavior of the curves published in Fig. 2C of the Nanoscale paper, purportedly showing both map:map and map:model FSCs. Unfortunately, the authors have never provided the data used to generate that plot despite multiple requests.

Related to the problems of the FSC plots, Dr. Kelly states that in Phenix “the auto-box process yielded a rectangular box around the center of the map, rendering it no longer cuboidal”. This does not appear to be the case. Rather, when supplied with a model (as the authors provided), the mtriage utility in Phenix that was most likely used for these FSC calculations has an option, box\_map\_before\_analysis, which defaults to False. From the Phenix documentation:

You can box the map (cut out box a little bigger than supplied model) before any analysis if you want. This will make the analysis faster, **but it can introduce masking artifacts** [my emphasis].

It appears that this must have been set to True, generating these huge artifacts. A reasonable scientist would see these artifacts in the FSC plots and be concerned. Such a reasonable person would actually look at the map and see that the calculated resolution (and the published resolution in the Nanoscale paper) could not possibly be true, based upon prior knowledge of what proteins look like at different resolutions. But Dr. Kelly has argued instead that everything supports the original statements of resolution in the Nanoscale paper. It is suggested in the Technical Response that I may have inadvertently compressed the map as my estimate of resolution using a map:model FSC differed by a factor of 2 from their values. I did not compress the map, and my estimate of 8.6 Å was actually very consistent with the visual appearance of the map. Their estimates of < 5 Å are entirely inconsistent with such a visual inspection, as would be immediately obvious to anyone with experience looking at cryo-EM volumes.

Lastly, the authors declared no conflicts of interest for their Nanoscale paper. However, the penultimate author of the paper, Dukes, lists her affiliation as Applications Science, Protochips, Inc, Morrisville, NC 27560, USA. The paper is clearly promoting the use of particular commercial microchips, produced by Protochips: “To prepare cryo-EM specimens, we employed microwell integrated microchips (Protochips, Inc., EPB-42A1-10) with imaging windows having dimensions of 10 μm × 10 μm in the *x*- and *y*-dimension and ∼20 nm thick.” In fact, the central role of these microchips even appears in the Title (“**Microchip-based** structure determination of low-molecular weight proteins using cryo-electron microscopy”) and the Abstract: “Here we contribute a **microchip based toolkit** to perform complementary structural and biochemical analysis on low-molecular weight proteins…To complement our structural findings, we engineered **microchip-based** immunoprecipitation assays that led to the discovery of the first antibody binding site on the N

protein [my emphasis].” The failure to declare this blatant conflict of interest appears to be a further and serious basis for retraction.

Casasanta, M.A., Jonaid, G.M., Kaylor, L., Luqiu, W.Y., DiCecco, L.A., Solares, M.J., Berry, S., Dearnaley, W.J., and Kelly, D.F. (2023). Structural Insights of the SARS-CoV-2 Nucleocapsid Protein: Implica\ons for the Inner-workings of Rapid An\gen Tests. Microsc Microanal *29*, 649- 657.