

Low-dose cryo-electron ptychography of proteins at sub-nanometer resolution



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is a very interesting paper using a fast hybrid pixel detector to push the boundaries for achievable resolution using cryogenic-electron ptychography of proteins. Noteworthy results include the demonstration of sub-nanometer reconstructions from 3 different protein complexes using the described ptychographic method, as well as a 1.1 angstrom map of apoferritin solved by conventional single particle approaches as a benchmark comparison. Although the demonstrated examples are limited to large protein complexes, this work is viewed as a significant step in the field and lays a foundation for future work pursuing resolution optimization and extension of the method to other complexes. The work is original, and the manuscript flows well. Overall, the methodology is sound and does meet expected standards in the field, but the presentation of the results is a bit incomplete in a few areas as described below. That said, I believe the authors can address these concerns without any need for further experimentation and simply through inclusion of additional text and one supplemental figure.

Concerns:

1) As written, the ResLog estimate for ultimate resolution is presented as though the same analysis method for understanding particle number versus resolution for conventional cryo-EM directly applies to the ptychographic data described here. However, based on the data provided it is not clear if the ResLog estimate is directly applicable or just assumed to be applicable. The structural comparisons with conventional cryo-EM datasets are not done at a same particle number level but rather 35x different and no other papers have set precedent showing ResLog holds true for ptychographic datasets. Furthermore, no ResLog plots were provided, nor were any description of the empirically determined values for the "constant" parameter for each of the datasets meaning there is no way for the reviewer to easily evaluate the ResLog based claims without trying to calculate the value from other partially provided parameters. The authors did include ResLog B-factor and ultimate resolution values in Supplemental Table 1 along with a section (lines 557 -578) describing background about ResLog, but the assertions in the paper aren't fully supported by the provided data and there is mixed messaging. For example, Line 119 ("sub-nm resolution can be achieved from less particles than usually imaged in cryo-EM") seems to imply fewer particles are needed to get sub-nm resolution compared to conventional cryo-EM. Additionally, Line 240 ("ptychography resulted in 3D reconstructions at sub-nm resolutions, obtained from very few imaged particles") echoes a similar message, but conventional cryo-EM data of the highly symmetric samples can also generate sub-nanometer resolution from such few particles. Those statements also appear in conflict with Line 240 ("Nevertheless, this resolution would still be worse than what is achievable by conventional cryo-EM") so better clarification of the main takeaways would be helpful.

2) It would also be helpful for the authors to provide a supplemental figure showing the calculated ResLog plots for each of the samples imaged to visualize the hopefully linear trend/slope and the ultimate resolution achieved at 1,000,000 particles as mentioned in the paper. This could be added as a supplemental figure and should include both the conventional cryo-EM dataset and the ptychographic datasets on the plots. Without this visualizing or supporting data, it is difficult for the reviewer to assess the rigor of the estimate for achievable resolution. Case in point, how many particles from the conventional single particle dataset would be needed to match the 5.8 angstrom resolution of the ptychographic dataset? Based on "Figure 3(a) ResLog plots for apoferritin collected in counting" in Mendez et al. 2019 IUCrJ 6:1007-1013, DOI: 10.1107/S2052252519012661), only 150 particles would be needed to reach the same 5.8 angstrom resolution for the conventional cryo-EM case. That is roughly 75 times fewer particles than the ptychography approach.

3) In terms of whether enough detail is provided in the methods for the work to be reproduced, there

is very little context for how the CSA was chosen for each dataset. A background section on lines 98-106 briefly highlights 4D-STEM and first references the CSA, but all 3 of the proteins were imaged with different CSA values and very little discussion was provided as to why. Since the CSA appears to have a critical impact on the results, it would be helpful for the authors to expand the methods and discussion sections to better detail how a future researcher could match CSA to sample when replicating the method. On line 241, the authors state: "However, here we applied 4D-STEM with a CSA of only 4 mrad to increase specimen height variation tolerance and data collection efficiency; a higher CSA would increase resolution." Was the CSA empirically determined by trial and error for each sample, or was it calculated somehow based on particle size or overall ice thickness? If it was calculated, what equation was used, or what software? Also, the statement about a "higher CSA would increase resolution" is mentioned but is not further elaborated and it has limited supporting context. If a higher CSA could improve resolution, why wasn't the higher value chosen, and what are the tradeoffs in the selection of CSA for imaging of proteins with cryo-electron ptychography? A few additional sentences could be added to the results or discussion sections to briefly address these questions.

4) The abstract reads like the motivation for the paper (and its impact) is to enable structural analysis of small proteins. For example, Line 29 states: "However, for particles smaller than 50kDa, single particle cryo-EM often fails due to the limited imaging contrast of individual micrographs." and Line 42 states: "Ptychography is a promising tool to study the structure of smaller frozen hydrated protein particles, and, once combined with electron tomography tilt series, bears the potential to provide unique insight into the ultrastructure of vitrified biological tissue." The latter sentiment is then reiterated in the closing paragraph on Line 256: "For cellular samples, where the uniqueness of the sample does not allow the averaging of signal from multiple particles, and where thicker slices of the sample are often of interest, the cryo-electron ptychography is especially promising to gain insight into the structural arrangement of smaller structures in cells and tissue."

While the authors appear to be "selling" the potential impact as aiding structure determination of small proteins, none of the samples imaged fall into the small protein category - which the authors self-identify as less than 50kDa. The smallest sample imaged in the current manuscript is roughly 10x larger at 485kDa. So there seems to be a disconnect as the data only depicts results for large and highly symmetric protein complexes. Either the authors should include a few sentences on caveats and limitations to their new method and then mention that future work may enable breakthroughs with structural analysis of small proteins, or the authors could simply rework the 3 sentences above to remove the link to small proteins and just focus the impact on providing the first sub-nanometer reconstructions of proteins with a hybrid pixel detector and cryo-electron ptychography - which is a publication worthy result on its own.

5) The authors used an aberration corrected microscope for this work but intentionally used high defocus. The main benefits of aberration correction are usually realized at low defocus so this raises the question of whether this work absolutely needs an aberration corrected microscope or if it could be duplicated on non-corrected microscopes. A few sentences in the discussion section could easily clarify the importance or benefits of using aberration correction. If the method can be applicable to instruments without aberration correction then it increases the potential impact and immediate adaptation of the technique by a broader cohort of microscopists.

Reviewer #1 (Remarks on code availability):

The code on Github does not appear to be publicly accessible yet and is likely to be officially released upon manuscript publication. So I was not able to evaluate the code at this stage.

Reviewer #2 (Remarks to the Author):

Review of the manuscript 'Low-dose cryo-electron ptychography of proteins at sub-nanometer resolution' by Berk Küçükoğlu et al., Manuscript # NCOMMS-24-14736

This manuscript details an impressive study of three biological samples made possible by key advances in the technique of electron ptychography, including image processing and acquisition details developed in the authors' laboratory. A comparison is made to state-of-the-art cryo-EM single-particle analysis for one of these proteins, murine apoferritin.

Whilst the applications demonstrated here are remarkable, the true potential is yet to be realised. In this sense, the authors show key advances to the application of ptychography to proteins and viruses but fall well short of the optimistic forecasts in the Abstract pertaining to sub-50 kDa proteins.

I recommend that this study be published with only very minor revisions. Rephrasing certain sentences will not detract from my assessment, viz. that this study is well worth publishing on its own merits. Unusually, my main issue with this manuscript is the somewhat misleading wording of the Abstract, which gives rise to unfair expectations (and potentially unwarranted criticisms) of what is a very useful contribution to the literature.

- What are the noteworthy results?

The authors show new and promising developments in the technique of cryo- electron ptychography, which enabled them to solve the structures of four protein structures: the benchmark cryo-EM single-particle standard, apoferritin, the tail as well as the core of the Phi92 bacteriophage, and a classical biological EM (helical) standard, tobacco mosaic virus (TMV). They implement a refined version of their ptychography image processing pipeline to demonstrate the feasibility of acquiring data within a reasonable timeframe. Although this can currently approach the speed of their own conventional single-particle processing workflow only to within 1-2 orders of magnitude, it should be noted that the conventional workflow has undergone concerted development in recent years, for which the authors' acquisition rate matches the highest speed possible at the present time (more than 900 movies per hour).

Taken in context, these results are indeed significant. The authors solve the structure of apoferritin to 5.8 Å, the Phi92 capsid and sheath to 12.1 Å and 8.4 Å, respectively, and the TMV filament to 6.4 Å. The authors also hint at the fact that these results should improve with better statistics, which are understandably very modest at this stage of development. It is remarkable that these resolutions have been achieved with so few 'particles'.

- Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.
The work builds upon existing literature but is novel. One could argue that the cryo-EM structure of apoferritin at high resolution is not novel but this appears to be an exercise aimed solely at comparing what can be expected of the two methods at the current time. As such, I find this to be useful.

- Does the work support the conclusions and claims, or is additional evidence needed?
The claims and conclusions are very straightforward and represent an honest appraisal of the experimental results. This is not always the case with studies claiming to have led to improved resolution. In other words, the conclusions are not overstated.

- Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?
In my opinion, there are no discernible flaws in the data analysis, interpretation or execution of the study. As suggested in my comments, I feel that a minor data processing exercise will lead to a more intuitive means of assessing the conclusions concerning apoferritin structure. This is a simple 'extra'

and does not require a reformulation of the existing text.

- Is the methodology sound? Does the work meet the expected standards in your field?

The methodology is sound and should withstand scrutiny. My only request is that the comparison of ptychography data with conventional cryo-EM single-particle analysis be given more systematic support by confirming that the sample grids were indeed comparable in terms of thickness, i.e. please give a little more information on how the ice thickness was assessed to be 50-100 nm and what measures were taken to use comparable preparations.

- Is there enough detail provided in the methods for the work to be reproduced?

The detail provided in this manuscript to enable reproduction of the experiments is exemplary. This includes all aspects of the workflow including the provision of original scripts where appropriate, sample preparation details, and all data processing parameters.

Detailed comments

Abstract

The Abstract is well written. My main criticism is that it mentions the tantalising possibility of solving sub-50 kDa proteins but then goes on to talk mostly about an almost 500 kDa protein, apoferritin. It also fails to mention the other proteins solved, which I feel are noteworthy. I understand that imaging small proteins is one of the main motivations for developing a CTEM-alternative. The simplest way to address this is to mention that reconstruction of < 50 kDa proteins requires some kind of advance in CTEM image processing or alternatively, the development of a plausible alternative to CTEM (ptychography). The Abstract is phrased as though the authors are about to demonstrate solving a < 50 kDa protein. They go on to talk about subnanometer resolution for single protein particles (impressive, yes), only for the reader to find that these particles are not anywhere near the sub-50 kDa size regime.

Second, there are two mentions of 'contrast'. Pixel contrast can be improved with any image processing software, and is thus an arbitrary concept. What matters is differential contrast, i.e. signal-to-noise ratio, and this **is** clear from the main text.

Third, the mention of applications to tomography (c.f. single-particle analysis) is interesting but it is not immediately clear what advantage is conferred by ptychography over conventional cryo- electron tomography involving CTF correction or the use of e.g. phase plates, which also provide contrast close to Scherzer focus.

Fourth: the Abstract alludes to high-throughput ("high speed", "large datasets"), which is relative. I agree that the authors have made real progress in terms of what was previously possible with the acquisition rate for ptychography but the throughput compared to conventional cryo-EM SPA is still very low, and this apparent difference is exacerbated by the 900-plus movies per hour the authors managed using CTEM.

The rest of the text is exemplary. As such, given a re-write of the Abstract, I feel that this manuscript should be published with only the addition of a small extra exercise: CTEM analysis of a random subset of the existing data to facilitate comparison of CTEM with ptychography, in terms of a similar particle number. I believe that this can be accomplished within a couple of hours.

Some minor comments:

Main text

Lines 50-52: "...if the proteins are available in sufficiently large quantities and homogeneity, and if the protein particles have a molecular weight larger than 50 kDa".

I would argue that this misleads the non-practitioner into thinking that these are the only criteria that must be fulfilled, leading to unrealistic expectations. While these criteria are of primary importance, different proteins behave differently with respect to the air-water interface, for example. It is not always trivial to solve structures of larger proteins that exhibit flexibility, dissociation etc.. The authors are clearly trying to compare two techniques with a well-behaved sample, which does have merit. The comparison is of two optical modes, which is independent of the sample and the identical sample preparation.

The literature reviewed in the introduction is well-summarized and, to the best of my knowledge, accurate.

Lines 114-116: this sentence cites two articles with reference to "...application of 4D-STEM ptychography to frozen-hydrated viruses". Reference 29 does not mention viruses or any other biological samples, for that matter.

Figure 2. It is not clear to me that the colors in this Figure contribute anything to an understanding of the workflow. It works well as a Powerpoint slide but should be revised for publication. In any case, the colors are not referenced in the figure caption.

Section beginning with Line 160 "Conventional Cryo-EM imaging of Apoferritin": The authors discuss a conventional bright-field cryo-TEM reconstruction of their apoferritin. I agree that the (ResLog) B-factor of 28 Å² as well as the final resolution of 1.09 Å attest to the quality of their cryo-EM sample preparation expertise and microscopy. What is not clear to me, however, is how the samples imaged by ptychography and cryo-EM single-particle analysis are related. What measures were taken to ensure that the imaged regions were comparable in terms of ice thickness and particle number per field of view? In this context, Line 515 states "These locations had an ice thickness between 50 and 100 nm."; however, I was unable to determine how this was calculated or estimated.

Also, the SPA treatment represents state of the art in aberration-free acquisition, speed, and processing, and thus contained 40-fold refined particles compared to the largest dataset obtained by ptychography. This is impressive as-is but the question remains: "What if the ptychography data was more comprehensive?" I do not dispute that there has been a substantial effort made to accelerate ptychography data acquisition, and it would therefore seem reasonable to me to simply compare this with similar particle numbers from SPA. To this end, I recommend that the authors take a random subset of their initial SPA dataset corresponding to the number of particles assessed by ptychography. Showing this information with a slightly reduced resolution would serve as a fair comparison. It is perfectly acceptable to keep the full SPA analysis to show what can be realised with a large dataset, and without question, this should be included. The only other way I could reconcile such a comparison is if the two apoferritin acquisitions were comparable in terms of acquisition time. This seems less relevant because ptychography will undoubtedly catch up to some extent.

Lines 157-159: "To test the quality of the AF protein and its suitability for high-resolution data collection, apoferritin was first imaged by conventional TEM (CTEM)..." – This reads as though the utility of apoferritin must be established when apoferritin (and indeed the murine construct from the Kikkawa lab) is used routinely for such purposes. Please rephrase to show that your work is capable of reproducing what is done elsewhere because the results show that it is a formality for this team.

Line 162: typo, 'kDa' should read 'kDa'.

Lines 162-164: "It shows a highly robust structural integrity when prepared for cryo-EM imaging, making it the ideal test specimen for high-resolution assays". This is true but to avoid inevitable criticism from those who frown upon anything 'apoferritin', I suggest mentioning that certain properties of apoferritin (octahedral symmetry, spherical shape) make it unusually unrepresentative of proteins but that these properties can be exploited for benchmarking purposes.

Lines 166-168: and subsequently, e.g. Supplementary Table 1: the particle numbers contain apostrophes rather than commas. Perhaps this is a result of conversion to pdf format from Latex or similar. Also, I suggest avoiding the non-standard abbreviation 'AF' for 'apoferritin'.

Lines 241-243: "...here we applied 4D-STEM with a CSA of only 4 mrad to increase specimen height variation tolerance and data collection efficiency; a higher CSA would increase resolution." – It is not immediately clear why a higher CSA was not used in the first place. Different CSA values are apparent in the data collection statistics. Could the authors please comment on these values and how they came to be chosen? Was this based on learnings from apoferritin, i.e. what was the order of data collection, and how did this influence subsequent data collection parameters?

Lines 252-256: "The strong contrast of cryo-electron ptychography evident in the reconstructed images, the low numbers of particles needed to obtain sub-nm reconstructions, and the trustworthiness of the projections obtained from the purely positive CTF suggests that 4D-STEM combined with cryo-electron ptychography tomography bears great potential for 3D reconstructions of biological tissue samples." I agree with this sentence but suggest that it should read: "The strong differential contrast...". It would be even better if the authors could talk about SNR but I understand that measurement of signal versus noise is not trivial for the data shown.

Line 383 "3 μ L aliquots of the apoferritin at 80 mg/ml..." – Could the authors please confirm that the protein concentration was, in fact, 80 mg/mL? I do not suggest it is wrong but it seems like a very high concentration for a 3s blot when a monolayer coverage of apoferritin can be achieved with < [5 mg/mL].

Data availability

Presumably, the several mentions of placeholder 'xxx' will be made available after acceptance.

References

The references are mostly complete and use the accepted journal abbreviations. Reference 6 appears to be missing page information (e13046). For the article titles, there is a mix of all words with initial capitals and first-word initial capital only. These should be reviewed for consistency.

I commend the authors on an excellent and well-written study.

End of comments.

Reviewer #2 (Remarks on code availability):

I was unable to see any README file or instruction for the Jupyter Notebook provided. This code refers only to the Phi92 dataset but I assume that this is intentional at this stage.

Reviewer #3 (Remarks to the Author):

The manuscript describes ptychography experiments that have allowed the 3D reconstruction of two different proteins and a virus. The authors correctly point out that ptychography offers a route to phase contrast at low doses without the contrast reversals that can be seen with defocused conventional transmission electron microscopy.

The paper is very nicely and clearly written, the results are impressive and the methodology sound. My main concern is that, having read the paper, I am not clear what new science has been learnt. Cryo-ptychography and 3D reconstruction of a viruses has been previously demonstrated, for example [Pei et al, Nat. Comm. 14 (2023) 3027] using a defocused probe, and [Lazic et al. Nat. Mat. 19 (2022)

1126] for in-focused probe cryo 4D STEM imaging of a protein and a virus. Note that Lazic et al used a quadrant detector, but the in-focus conditions allow for reduced detector plane sampling so this should still be regarded as a 4D-STEM phase retrieval approach. As yet, all these 4D-STEM approaches, including the current work, examine test systems, and these approaches have not yet demonstrated a capability that cannot be met by the well-established cryo-TEM methods. It is not clear from the present manuscript whether the authors feel they have demonstrated an improvement over cryo-TEM in the current work. This is very nice imaging work, but until there is a clear and quantitative assessment of what the advances are that are offered by the work in this manuscript, I cannot recommend publication.

There is also no discussion of how the imaging parameters were selected. I accept the authors assertion that the contrast transfer function is not a well-defined quantity for these phase retrieval methods, but there is developing a reasonable understanding of what the resolution limit is for a particular convergence angle, beam energy etc.

A final technical question I have is whether the ResLog B-factor relies on Poission noise statistics. Cryo-TEM does follow Poisson statistics, but ptychography does not, and I wonder if that would impact on this calculation.

Response to reviewers

Low-dose cryo-electron ptychography of proteins at sub-nanometer resolution

Reviewer #1 (Remarks to the Author):

This is a very interesting paper using a fast hybrid pixel detector to push the boundaries for achievable resolution using cryogenic-electron ptychography of proteins. Noteworthy results include the demonstration of sub-nanometer reconstructions from 3 different protein complexes using the described ptychographic method, as well as a 1.1 angstrom map of apoferritin solved by conventional single particle approaches as a benchmark comparison. Although the demonstrated examples are limited to large protein complexes, this work is viewed as a significant step in the field and lays a foundation for future work pursuing resolution optimization and extension of the method to other complexes. The work is original, and the manuscript flows well. Overall, the methodology is sound and does meet expected standards in the field, but the presentation of the results is a bit incomplete in a few areas as described below. That said, I believe the authors can address these concerns without any need for further experimentation and simply through inclusion of additional text and one supplemental figure.

We thank the reviewer for the kind words. Below, we address the reviewer's concern, highlighting the changes to the manuscript.

Concerns:

1) As written, the ResLog estimate for ultimate resolution is presented as though the same analysis method for understanding particle number versus resolution for conventional cryo-EM directly applies to the ptychographic data described here. However, based on the data provided it is not clear if the ResLog estimate is directly applicable or just assumed to be applicable. The structural comparisons with conventional cryo-EM datasets are not done at a same particle number level but rather 35x different and no other papers have set precedent showing ResLog holds true for ptychographic datasets. Furthermore, no ResLog plots were provided, nor were any description of the empirically determined values for the "constant" parameter for each of the datasets meaning there is no way for the reviewer to easily evaluate the ResLog based claims without trying to calculate the value from other partially provided parameters. The authors did include ResLog B-factor and ultimate resolution values in Supplemental Table 1 along with a section (lines 557 -578) describing background about ResLog, but the assertions in the paper aren't fully supported by the provided data and there is mixed messaging. For example, Line 119 ("sub-nm resolution can be achieved from less particles than usually imaged in cryo-EM") seems to imply fewer particles are needed to get sub-nm resolution compared to conventional cryo-EM. Additionally, Line 240 ("ptychography resulted in 3D reconstructions at sub-nm resolutions, obtained from very few imaged particles") echoes a similar message, but conventional cryo-EM data of the highly symmetric samples can also generate sub-nanometer resolution from such few particles. Those statements also appear in conflict with Line 240 ("Nevertheless, this resolution would still be worse than what is achievable by conventional cryo-EM") so better clarification of the main takeaways would be helpful.

We agree with this reviewer. We now have removed the ResLog B-factor calculation for the 4D STEM datasets. While the recorded diffraction patterns have Poisson-distributed electron counts, this does not apply to the reconstructed ptychography images. We have kept the ResLog B-factor calculation for the real-space cryo-EM map, and for this we added the ResLog plot in Extended Data Fig. 8.

2) It would also be helpful for the authors to provide a supplemental figure showing the calculated ResLog plots for each of the samples imaged to visualize the hopefully linear trend/slope and the ultimate resolution achieved at 1,000,000 particles as mentioned in the paper. This could be added as a supplemental figure and should include both the conventional cryo-EM dataset and the ptychographic datasets on the plots. Without this visualizing or supporting data, it is difficult for the reviewer to assess

the rigor of the estimate for achievable resolution. Case in point, how many particles from the conventional single particle dataset would be needed to match the 5.8 angstrom resolution of the ptychographic dataset? Based on “Figure 3(a) ResLog plots for apoferritin collected in counting” in Mendez et al. 2019 IUCrJ 6:1007-1013, DOI: 10.1107/S2052252519012661), only 150 particles would be needed to reach the same 5.8 angstrom resolution for the conventional cryo-EM case. That is roughly 75 times fewer particles than the ptychography approach.

As stated above, the ResLog plot for the CTEM ApoF structure is shown in Extended Data Fig. 8. This is for the 1.09Å structure from 411,780 particles.

To compare the signal-to-noise ratio of CTEM images with 4D-STEM images, we also collected another ApoF dataset from the very same Titan Krios instrument that was also used for the 4D-STEM work. This is a Titan Krios that is equipped with a Probe Cs aberration corrector. Here, we collected 38,000 particle images, so that in CTEM and in 4D-STEM we are dealing with the same numbers of particles. This CTEM work from 38,000 particles allowed reaching a 3D reconstruction at 1.49Å resolution, which is FAR better than 4D-STEM currently was able to achieve, as this reviewer correctly predicted. Sigh...

But the path to higher SNR and resolution for 4D STEM is becoming clear: Dose-fractionation, sparse sampling, combination of separately collected scans into ptychography reconstructions that take particle movement and increasing beam damage into account through particle motion correction and beam-dependent frequency weighting as routinely done for real-space cryo-EM images, multi-slice reconstructions to take multiple scattering in thicker samples into account, in combination with tomography tilt series, etc.. We are convinced that the field will eventually be able to fully employ the potential of 4D STEM also in life sciences, once all these technologies have been developed for 4D STEM.

3) In terms of whether enough detail is provided in the methods for the work to be reproduced, there is very little context for how the CSA was chosen for each dataset. A background section on lines 98-106 briefly highlights 4D-STEM and first references the CSA, but all 3 of the proteins were imaged with different CSA values and very little discussion was provided as to why. Since the CSA appears to have a critical impact on the results, it would be helpful for the authors to expand the methods and discussion sections to better detail how a future researcher could match CSA to sample when replicating the method. On line 241, the authors state: “However, here we applied 4D-STEM with a CSA of only 4 mrad to increase specimen height variation tolerance and data collection efficiency; a higher CSA would increase resolution.” Was the CSA empirically determined by trial and error for each sample, or was it calculated somehow based on particle size or overall ice thickness? If it was calculated, what equation was used, or what software? Also, the statement about a “higher CSA would increase resolution” is mentioned but is not further elaborated and it has limited supporting context. If a higher CSA could improve resolution, why wasn't the higher value chosen, and what are the tradeoffs in the selection of CSA for imaging of proteins with cryo-electron ptychography? A few additional sentences could be added to the results or discussion sections to briefly address these questions.

Recording 4D STEM data with a higher CSA is experimentally difficult. At higher CSA, the height tolerance for correct focus during the scan over the sample is reduced, and at higher CSA, the image SNR is reduced. We have included a discussion of this in the manuscript in the Discussion section.

The information limit of most STEM imaging modalities, including ADF, DPC, and parallax, is set by diffractive optics given by twice the CSA. While ptychography can in-theory exceed this information limit, enabling “super-resolution”, this requires meaningful scattering intensities outside the BF disk which are in-practice difficult to achieve for dose-limited samples. Assuming that the information limit of ptychography in the dose-limited datasets presented here is similarly given by twice the CSA, we can use the following formula to calculate the resolution limit:

$$\text{information limit } [\text{\AA}] = 1/(2 * \text{CSA } [\text{mrad}]) * 1000 * \lambda(\epsilon) [\text{\AA}]$$

where $\lambda(\epsilon)$ is the relativistic electron wavelength in Angstroms. For a CSA=4mrad at 300kV, this corresponds to an information limit of ~2.5Å.

Moving away from ultimate information limits, it's important to realize that the contrast transfer function of ptychography – while not known analytically – varies with spatial frequencies relative to the CSA. As such, increasing CSA affects the image contrast, and experimentally we often observe reduced SNR. Moreover, recording with a higher CSA results in reduced height tolerance for the correct defocus of the scan over

the sample. We have included a discussion of this in the manuscript.

4) The abstract reads like the motivation for the paper (and its impact) is to enable structural analysis of small proteins. For example, Line 29 states: “However, for particles smaller than 50kDa, single particle cryo-EM often fails due to the limited imaging contrast of individual micrographs.” and Line 42 states: “Ptychography is a promising tool to study the structure of smaller frozen hydrated protein particles, and, once combined with electron tomography tilt series, bears the potential to provide unique insight into the ultrastructure of vitrified biological tissue.” The latter sentiment is then reiterated in the closing paragraph on Line 256: “For cellular samples, where the uniqueness of the sample does not allow the averaging of signal from multiple particles, and where thicker slices of the sample are often of interest, the cryo-electron ptychography is especially promising to gain insight into the structural arrangement of smaller structures in cells and tissue.”

While the authors appear to be “selling” the potential impact as aiding structure determination of small proteins, none of the samples imaged fall into the small protein category - which the authors self-identify as less than 50kDa. The smallest sample imaged in the current manuscript is roughly 10x larger at 485kDa. So there seems to be a disconnect as the data only depicts results for large and highly symmetric protein complexes. Either the authors should include a few sentences on caveats and limitations to their new method and then mention that future work may enable breakthroughs with structural analysis of small proteins, or the authors could simply rework the 3 sentences above to remove the link to small proteins and just focus the impact on providing the first sub-nanometer reconstructions of proteins with a hybrid pixel detector and cryo-electron ptychography - which is a publication worthy result on its own.

We agree. For clarity (and as pointed out by another reviewer below), we refer now to SNR instead of contrast. The SNR of ptychography is an open-question and under intensive discussions. What is the SNR per beam damage that can be obtained from a specific imaging technique? For materials-science samples at high electron fluences, electron ptychography has been shown to be a very efficient data collection method. However, for dose-limited biological samples, we are not yet able to quantify the technique’s dose efficiency and SNR. We have therefore removed all statements from the manuscript, where we previously claimed a superior SNR.

Instead, in order to contribute to the discussion about SNR, we have added another dataset of Bacteriorhodopsin (BR) 2D crystals to this manuscript. BR 2D crystals allow during image processing a quantitative evaluation of the signal-above-background and the background intensities of reflection spots in the computed Fourier transforms. The spots each correspond to a specific spatial frequency, and for each we can evaluate the SNR from the signal-above-background divided by the background. This is possible to obtain from a single such BR 2D crystal image. This is added as Extended Data Fig. 9. We applied this technique to images reconstructed using Parallax and Ptychographic methods. The differences in SNR between the two techniques appear to be in qualitative agreement with a recently-proposed numerical model on white-noise objects by some of us [arXiv:2309.05250]. Further work (in another manuscript) will be required to systematically evaluate the SNR of each 4D STEM method in biological samples and how it benefits from certain settings such as dose, probe position overlap, CSA, defocus, or sparse sampling. Nevertheless, for this manuscript, we believe that Extended Data Fig. 9 is already an interesting contribution to the ongoing discussion about the SNR of this method.

5) The authors used an aberration corrected microscope for this work but intentionally used high defocus. The main benefits of aberration correction are usually realized at low defocus so this raises the question of whether this work absolutely needs an aberration corrected microscope or if it could be duplicated on non-corrected microscopes. A few sentences in the discussion section could easily clarify the importance or benefits of using aberration correction. If the method can be applicable to instruments without aberration correction then it increases the potential impact and immediate adaptation of the technique by a broader cohort of microscopists.

We agree and have included in line 140 a statement that the aberration correction is not necessary for the work presented here.

Reviewer #1 (Remarks on code availability):

The code on Github does not appear to be publicly accessible yet and is likely to be officially released

upon manuscript publication. So I was not able to evaluate the code at this stage.

The code is now available on github, at <https://github.com/LBEM-CH/4D-BioSTEM>.

In addition, all data available on the EMDB and PDB. Raw cryo-EM images are already on EMPIAR, and we are in the process of uploading the raw 4D STEM data also to EMPIAR. Access numbers are given in the manuscript for the datasets, and we will add the 4D STEM EMPIAR access numbers before publication.

Reviewer #2 (Remarks to the Author):

Review of the manuscript 'Low-dose cryo-electron ptychography of proteins at sub-nanometer resolution' by Berk Küçükoğlu et al., Manuscript # NCOMMS-24-14736

This manuscript details an impressive study of three biological samples made possible by key advances in the technique of electron ptychography, including image processing and acquisition details developed in the authors' laboratory. A comparison is made to state-of-the-art cryo-EM single-particle analysis for one of these proteins, murine apoferritin.

We thank this reviewer for the kind words.

Whilst the applications demonstrated here are remarkable, the true potential is yet to be realised. In this sense, the authors show key advances to the application of ptychography to proteins and viruses but fall well short of the optimistic forecasts in the Abstract pertaining to sub-50 kDa proteins.

As discussed above, we agree also with this reviewer and have removed our overly advertising claim of superior SNR and <50kDa capabilities for 4D STEM.

I recommend that this study be published with only very minor revisions. Rephrasing certain sentences will not detract from my assessment, viz. that this study is well worth publishing on its own merits. Unusually, my main issue with this manuscript is the somewhat misleading wording of the Abstract, which gives rise to unfair expectations (and potentially unwarranted criticisms) of what is a very useful contribution to the literature.

We thank the reviewer for their positive recommendation of our manuscript. We address the reviewer's concerns below, highlighting the changes to the manuscript.

• What are the noteworthy results?

The authors show new and promising developments in the technique of cryo- electron ptychography, which enabled them to solve the structures of four protein structures: the benchmark cryo-EM single-particle standard, apoferritin, the tail as well as the core of the Phi92 bacteriophage, and a classical biological EM (helical) standard, tobacco mosaic virus (TMV). They implement a refined version of their ptychography image processing pipeline to demonstrate the feasibility of acquiring data within a reasonable timeframe. Although this can currently approach the speed of their own conventional single-particle processing workflow only to within 1-2 orders of magnitude, it should be noted that the conventional workflow has undergone concerted development in recent years, for which the authors' acquisition rate matches the highest speed possible at the present time (more than 900 movies per hour).

Taken in context, these results are indeed significant. The authors solve the structure of apoferritin to 5.8 Å, the Phi92 capsid and sheath to 12.1 Å and 8.4 Å, respectively, and the TMV filament to 6.4 Å. The authors also hint at the fact that these results should improve with better statistics, which are understandably very modest at this stage of development. It is remarkable that these resolutions have been achieved with so few 'particles'.

We thank this reviewer for the positive summary.

- Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.

The work builds upon existing literature but is novel. One could argue that the cryo-EM structure of apoferritin at high resolution is not novel but this appears to be an exercise aimed solely at comparing what can be expected of the two methods at the current time. As such, I find this to be useful.

We thank this reviewer for the positive summary.

- Does the work support the conclusions and claims, or is additional evidence needed?

The claims and conclusions are very straightforward and represent an honest appraisal of the experimental results. This is not always the case with studies claiming to have led to improved resolution. In other words, the conclusions are not overstated.

We thank this reviewer for the positive summary.

- Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?

In my opinion, there are no discernible flaws in the data analysis, interpretation or execution of the study. As suggested in my comments, I feel that a minor data processing exercise will lead to a more intuitive means of assessing the conclusions concerning apoferritin structure. This is a simple 'extra' and does not require a reformulation of the existing text.

We agree, as discussed below.

- Is the methodology sound? Does the work meet the expected standards in your field?

The methodology is sound and should withstand scrutiny. My only request is that the comparison of ptychography data with conventional cryo-EM single-particle analysis be given more systematic support by confirming that the sample grids were indeed comparable in terms of thickness, i.e. please give a little more information on how the ice thickness was assessed to be 50-100 nm and what measures were taken to use comparable preparations.

We agree with the reviewer. We have now added another (smaller) dataset that we had collected from the very same ApoF grid on the very same Cs-probe-corrected Titan Krios that had also been used for collecting the 4D STEM datasets. The ApoF images never show multiple layers of ApoF molecules. This implies that the ice layer is thin enough (we chose the thinnest regions) such that only one single layer of ApoF fits into the ice layer. Since the diameter of ApoF is around 12 nm, and since we assume an additional water layer on top and bottom of that, we believe that the ice layer must have been around 50 nm thick. We have corrected the manuscript to this now.

- Is there enough detail provided in the methods for the work to be reproduced?

The detail provided in this manuscript to enable reproduction of the experiments is exemplary. This includes all aspects of the workflow including the provision of original scripts where appropriate, sample preparation details, and all data processing parameters.

We thank this reviewer for the positive summary.

Detailed comments

Abstract

The Abstract is well written. My main criticism is that it mentions the tantalizing possibility of solving sub-50 kDa proteins but then goes on to talk mostly about an almost 500 kDa protein, apoferritin. It also fails to mention the other proteins solved, which I feel are noteworthy. I understand that imaging small proteins is one of the main motivations for developing a CTEM-alternative. The simplest way to address this is to mention that reconstruction of < 50 kDa proteins requires some kind of advance in CTEM image processing or alternatively, the development of a plausible alternative to CTEM (ptychography). The

Abstract is phrased as though the authors are about to demonstrate solving a < 50 kDa protein. They go on to talk about subnanometer resolution for single protein particles (impressive, yes), only for the reader to find that these particles are not anywhere near the sub-50 kDa size regime.

We thank the reviewer for the positive comment. The same criticism concerning the overly bold claim of SNR (formerly “contrast”) was also given by Reviewer #1. As described above, we have now removed the claim of higher SNR and removed the claim of <50kDa protein capability. Instead, we have added an analysis of the SNR, using 2D crystals of bacteriorhodopsin, as described above.

Second, there are two mentions of ‘contrast’. Pixel contrast can be improved with any image processing software, and is thus an arbitrary concept. What matters is differential contrast, i.e. signal-to-noise ratio, and this is clear from the main text.

We agree, and now termed this SNR.

Third, the mention of applications to tomography (c.f. single-particle analysis) is interesting but it is not immediately clear what advantage is conferred by ptychography over conventional cryo- electron tomography involving CTF correction or the use of e.g. phase plates, which also provide contrast close to Scherzer focus.

4D STEM, depending on the method of data analysis, can produce image reconstructions with a high SNR and these reconstructions have a CTF that is always positive without zero crossings or gaps [arXiv:2309.05250]. This is also now shown and explained in detail in Extended Data Figure 10. This feature, together with the fact that multislice ptychography analysis of 4D STEM datasets has modest depth resolution, has a very promising potential for cryo-electron ptychography tomography. It opens the door to tomography with a significantly reduced missing wedge, and with a significantly improved resolution in the Crowther Criterion measure, even when using larger tilt angle increments. We have added a discussion of this into the manuscript now.

The promise of ptychography over conventional cryo electron tomography is three-fold:

- i) The CTF of ptychography, as shown in Extended Data Figure 10 and in a recent study by some of us [arXiv:2309.05250], shows no contrast-reversals or zero-crossings - suggesting a more faithful model for the scattering physics.
- ii) Multi-slice ptychography can include non-linearities in the scattering physics arising due to multiple-scattering and Fresnel propagation. Further, multi-slice ptychography results in a volumetric reconstruction with modest depth resolution. Combined with tilting, the out-of-plane information in multi-slice ptychography results in an improved Crowther Criterion measure, scaling with the number of “super-slices” used.
- iii) Finally, the 4D-data as a function of tilt-series can be inverted to give a 3D volume directly, without resorting to 2D projections first - in a technique called “joint ptychographic tomography” [arXiv:2309.05250]. This results in higher dose efficiency and a significantly reduced missing-wedge artifact.

We note, however, that demonstrating these advantages will still require significant experimental and algorithmic advances.

Fourth: the Abstract alludes to high-throughput (“high speed”, “large datasets”), which is relative. I agree that the authors have made real progress in terms of what was previously possibly with the acquisition rate for ptychography but the throughput compared to conventional cryo-EM SPA is still very low, and this apparent difference is exacerbated by the 900-plus movies per hour the authors managed using CTEM.

We thank this reviewer for the positive comments, and we also agree that our 4D STEM speed and dataset size are relative. We have toned down the manuscript in this regard now.

The rest of the text is exemplary. As such, given a re-write of the Abstract, I feel that this manuscript should be published with only the addition of a small extra exercise: CTEM analysis of a random subset of the existing data to facilitate comparison of CTEM with ptychography, in terms of a similar particle number. I believe that this can be accomplished within a couple of hours.

We agree with the reviewer that such an analysis would be beneficial. Instead of re-analyzing a subset of the CTEM data that led to the 1.09 Å ApoF structure, we now include data from another CTEM dataset that had been collected on the very same instrument that we also used for the 4D STEM analysis, and on the very same ApoF grid. This new CTEM dataset is of the same size as the 4D STEM dataset, which is around 38k particles. The CTEM image processing in cryoSPARC even from this tiny dataset, still reached 1.49Å, which is significantly better than our 4D STEM ptychography analysis, which only reached 5.8Å. So, we have a long way to go. But the path is more and more becoming clear: Dose-fractionation, sparse sampling, combination of separately collected scans into ptychography reconstructions that take particle movement and increasing beam damage into account through particle motion correction and beam-dependent frequency weighting as routinely done for real-space cryo-EM images, multi-slice reconstructions to take multiple scattering in thicker samples into account, in combination with tomography tilt series, etc.. We are convinced that we will eventually be able to fully employ the potential of 4D STEM also in life sciences.

We have kept both CTEM datasets in the manuscript, the one at 1.09 Å from many particles (and collected on another Titan Krios), and the one at 1.49Å from few particles. This is because the first one shows the quality of the ApoF sample, while the second shows the quality of the grid used for 4D STEM analysis.

Some minor comments:

Main text

Lines 50-52: "...if the proteins are available in sufficiently large quantities and homogeneity, and if the protein particles have a molecular weight larger than 50 kDa".

I would argue that this misleads the non-practitioner into thinking that these are the only criteria that must be fulfilled, leading to unrealistic expectations. While these criteria are of primary importance, different proteins behave differently with respect to the air-water interface, for example. It is not always trivial to solve structures of larger proteins that exhibit flexibility, dissociation etc.. The authors are clearly trying to compare two techniques with a well-behaved sample, which does have merit. The comparison is of two optical modes, which is independent of the sample and the identical sample preparation.

We agree and have rephrased those sentences at the beginning of the main text.

The literature reviewed in the introduction is well-summarized and, to the best of my knowledge, accurate.

Lines 114-116: this sentence cites two articles with reference to "...application of 4D-STEM ptychography to frozen-hydrated viruses". Reference 29 does not mention viruses or any other biological samples, for that matter.

We thank this reviewer for catching this error. The reference has now been corrected.

Figure 2. It is not clear to me that the colors in this Figure contribute anything to an understanding of the workflow. It works well as a Powerpoint slide but should be revised for publication. In any case, the colors are not referenced in the figure caption.

We thank this reviewer for the comment. The figure has now been improved.

Section beginning with Line 160 "Conventional Cryo-EM imaging of Apoferritin": The authors discuss a conventional bright-field cryo-TEM reconstruction of their apoferritin. I agree that the (ResLog) B-factor of 28 Å² as well as the final resolution of 1.09 Å attest to the quality of their cryo-EM sample preparation expertise and microscopy. What is not clear to me, however, is how the samples imaged by ptychography and cryo-EM single-particle analysis are related. What measures were taken to ensure that the imaged regions were comparable in terms of ice thickness and particle number per field of view? In this context, Line 515 states "These locations had an ice thickness between 50 and 100 nm."; however, I was unable to determine how this was calculated or estimated.

We thank this reviewer for the positive comments. We have now included another, smaller CTEM dataset from the same instrument and same grid, as used for the 4D STEM work.

Also, the SPA treatment represents state of the art in aberration-free acquisition, speed, and processing, and thus contained 40-fold refined particles compared to the largest dataset obtained by ptychography.

This is impressive as-is but the question remains: “What if the ptychography data was more comprehensive?” I do not dispute that there has been a substantial effort made to accelerate ptychography data acquisition, and it would therefore seem reasonable to me to simply compare this with similar particle numbers from SPA. To this end, I recommend that the authors take a random subset of their initial SPA dataset corresponding to the number of particles assessed by ptychography. Showing this information with a slightly reduced resolution would serve as a fair comparison. It is perfectly acceptable to keep the full SPA analysis to show what can be realised with a large dataset, and without question, this should be included. The only other way I could reconcile such a comparison is if the two apoferritin acquisitions were comparable in terms of acquisition time. This seems less relevant because ptychography will undoubtedly catch up to some extent.

We agree, and have included a smaller CTEM dataset, as suggested.

Lines 157-159: “To test the quality of the AF protein and its suitability for high-resolution data collection, apoferritin was first imaged by conventional TEM (CTEM)...” – This reads as though the utility of apoferritin must be established when apoferritin (and indeed the murine construct from the Kikkawa lab) is used routinely for such purposes. Please rephrase to show that your work is capable of reproducing what is done elsewhere because the results show that it is a formality for this team.

We have rephrased this now accordingly.

Line 162: typo, ‘kDA’ should read ‘kDa’.

We thank the reviewer for catching this. We have now corrected this..

Lines 162-164: “It shows a highly robust structural integrity when prepared for cryo-EM imaging, making it the ideal test specimen for high-resolution assays”. This is true but to avoid inevitable criticism from those who frown upon anything ‘apoferritin’, I suggest mentioning that certain properties of apoferritin (octahedral symmetry, spherical shape) make it unusually unrepresentative of proteins but that these properties can be exploited for benchmarking purposes.

We agree and have rephrased this now accordingly.

Lines 166-168: and subsequently, e.g. Supplementary Table 1: the particle numbers contain apostrophes rather than commas. Perhaps this is a result of conversion to pdf format from Latex or similar. Also, I suggest avoiding the non-standard abbreviation ‘AF’ for ‘apoferritin’.

We have replaced the apostrophes with commas now, and refer to apoferritin as “ApoF”.

Lines 241-243: “...here we applied 4D-STEM with a CSA of only 4 mrad to increase specimen height variation tolerance and data collection efficiency; a higher CSA would increase resolution.” – It is not immediately clear why a higher CSA was not used in the first place. Different CSA values are apparent in the data collection statistics. Could the authors please comment on these values and how they came to be chosen? Was this based on learnings from apoferritin, i.e. what was the order of data collection, and how did this influence subsequent data collection parameters?

As also described above, work at higher CSA becomes technically more challenging. A higher CSA reduces focal depth tolerance, reduces height tolerance for the grid’s planarity, and reduces SNR in the reconstructed images. We have added text to explain this.

Lines 252-256: “The strong contrast of cryo-electron ptychography evident in the reconstructed images, the low numbers of particles needed to obtain sub-nm reconstructions, and the trustworthiness of the projections obtained from the purely positive CTF suggests that 4D-STEM combined with cryo-electron ptychography tomography bears great potential for 3D reconstructions of biological tissue samples.” I agree with this sentence but suggest that it should read: “The strong differential contrast...”. It would be even better if the authors could talk about SNR but I understand that measurement of signal versus noise is not trivial for the data shown.

We agree and have now replaced “contrast” with SNR.

Line 383 “3 μ L aliquots of the apoferritin at 80 mg/ml...” – Could the authors please confirm that the protein concentration was, in fact, 80 mg/mL? I do not suggest it is wrong but it seems like a very high

concentration for a 3s blot when a monolayer coverage of apoferritin can be achieved with < [5 mg/mL].

We thank the reviewer for their astute observation in catching this typo. The concentration was 8 mg/mL, and has now been corrected..

Data availability

Presumably, the several mentions of placeholder 'xxx' will be made available after acceptance.

Data are now all publicly available. Accession numbers (EMPIAR, EMDDB, PDB, GitHub) are all in the manuscript, with the exception of the 4D STEM datasets to EMPIAR, which we are still in the process of uploading. These accession numbers will also be available before publication.

References

The references are mostly complete and use the accepted journal abbreviations. Reference 6 appears to be missing page information (e13046). For the article titles, there is a mix of all words with initial capitals and first-word initial capital only. These should be reviewed for consistency.

We thank this reviewer for catching that. We have corrected the references now.

I commend the authors on an excellent and well-written study.

We once again thank the reviewer for their in-depth review of our manuscript..

End of comments.

Reviewer #2 (Remarks on code availability):

I was unable to see any README file or instruction for the Jupyter Notebook provided. This code refers only to the Phi92 dataset but I assume that this is intentional at this stage.

A README file is now available for the Jupyter Notebook.

Reviewer #3 (Remarks to the Author):

The manuscript describes ptychography experiments that have allowed the 3D reconstruction of two different proteins and a virus. The authors correctly point out that ptychography offers a route to phase contrast at low doses without the contrast reversals that can be seen with defocused conventional transmission electron microscopy.

We thank this reviewer for the positive comments.

The paper is very nicely and clearly written, the results are impressive and the methodology sound. My main concern is that, having read the paper, I am not clear what new science has been learnt. Cryo-ptychography and 3D reconstruction of a viruses has been previously demonstrated, for example [Pei et al, Nat. Comm. 14 (2023) 3027] using a defocused probe, and [Lazic et al. Nat. Mat. 19 (2022) 1126] for in-focused probe cryo 4D STEM imaging of a protein and a virus. Note that Lazic et al used a quadrant detector, but the in-focus conditions allow for reduced detector plane sampling so this should still be regarded as a 4D-STEM phase retrieval approach. As yet, all these 4D-STEM approaches, including the current work, examine test systems, and these approaches have not yet demonstrated a capability that cannot be met by the well-established cryo-TEM methods. It is not clear from the present manuscript whether the authors feel they have demonstrated an improvement over cryo-TEM in the current work. This is very nice imaging work, but until there is a clear and quantitative assessment of what the advances are that are offered by the work in this manuscript, I cannot recommend publication.

Yes, our work is incremental. We continue on the path of others (Elbaum, Kirkland, Wang, Kourkoutis, Muller, and many more.). But we still think that our work is worth publishing, since we significantly improved the achieved resolution over so far published work. We describe how this was done, and we discuss the strength and pitfalls of the method, the challenges still to overcome, and provide insight into

signal formation in ptychography (Extended data figure 10).

There is also no discussion of how the imaging parameters were selected. I accept the authors assertion that the contrast transfer function is not a well-defined quantity for these phase retrieval methods, but there is developing a reasonable understanding of what the resolution limit is for a particular convergence angle, beam energy etc.

We thank this reviewer for alerting us to this. We have now included some explanations how the imaging parameters were chosen, especially the CSA, and we added an analysis of SNR from 2D crystals and from different amplitude and phase reconstruction methods in ptychography.

A final technical question I have is whether the ResLog B-factor relies on Poisson noise statistics. Cryo-TEM does follow Poisson statistics, but ptychography does not, and I wonder if that would impact on this calculation.

We agree and have removed the ResLog B-factor calculation for the 4D STEM datasets.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

With the current revision, the authors have adequately addressed all my prior concerns and they have expanded the clarity and impact of the manuscript. Several key updates to the narrative provide extra details about the data collection and reconstruction methods as well as better link the discussion of the ResLog analysis to the data presented. The inclusion of new conventional cryo-EM datasets for apoferritin with a total particle count similar to the 4D-STEM experiments was helpful to more directly compare the approaches, and the addition of the 2d crystal bacteriorhodopsin dataset was useful for showing a quantitative assessment of resolution through the intensity of the diffraction spots above background. The results presented by the authors represent a significant advancement in the field and the manuscript is ready to be accepted for publication with only minor edits to the narrative. In my opinion, the requested minor edits do not need further peer review but would be handled sufficiently at the editor or copy editor level.

All of the minor text edits below relate to the new manuscript version containing long sentences that disrupted the flow of the narrative and required re-reading in order to interpret the author's intent. I have provided my best interpretation for the various sentences as reference.

- On page 3, lines 47-52: "Cryo-EM has been shown to be able to determine in a few hours at near-atomic resolution the structure of proteins that have been frozen as single particles in a thin aqueous layer, provided the proteins are available in sufficient concentration as homogeneous populations, sufficiently stable conformations, and embedded in random orientations in a thin ice layer without excessive adsorption to the air-water interface." I believe the authors meant something like "Cryo-EM has been shown to only require a few hours to determine the near-atomic resolution structure of proteins that have been frozen as single particles in a thin aqueous layer. The method generally requires that the proteins are available in sufficient concentration as homogeneous populations, adopt sufficiently stable conformations, and are embedded in random orientations in a thin ice layer without excessive adsorption to the air-water interface."

- On page 11, lines 266-270: "For ptychography reconstructions, computed Fourier transforms of the 2D potential maps obtained by computing ptychography phase-only reconstructions, while fixing the amplitudes to constant "1", show for 4D datasets recorded with a defocus of 2 μm the presence of ring like amplitude oscillations (Fig. 3), and the width of the rings is wider (with more narrow dark gaps) when data closer to focus are recorded (Extended Data Fig. 9)." I believe the authors meant something like "For ptychography reconstructions, computed Fourier transforms of the 2D potential maps were obtained by computing ptychography phase-only reconstructions. While fixing the amplitudes to constant "1" shows the presence of ring like amplitude oscillations (Fig. 3) for 4D datasets recorded with a defocus of 2 μm , the width of the rings is wider (with more narrow dark gaps) when data closer to focus are recorded (Extended Data Fig. 9)."

- On page 10, lines 240 - 244: "Crystallographic processing of these two reconstructions with the FOCUS software allowed the reconstruction of the projection maps at around 6 Å resolution from each, which required for the parallax reconstruction a correction of the oscillating CTF by Wiener filtration, while the ptychography map allowed reconstruction of the correct projection map without any CTF correction." I believe the authors meant something like "Crystallographic processing of these two reconstructions with the FOCUS software allowed the reconstruction of the projection maps at around 6 Å resolution from each dataset. The parallax reconstruction required a correction of the oscillating CTF by Wiener filtration, while the ptychography map allowed reconstruction of the correct projection map without any CTF correction."

- On page 6, lines 138-141: "However, as ptychography reconstructs both, the complex-valued sample exit wave and the complex-valued electron probe function including its aberrations, aberration

correction is not a requirement for the work presented here, since CSAs of only 4 to 6 mrad and larger defocus values around 2 μm were used." I believe the authors meant something like "However, ptychography reconstructs both the complex-valued sample exit wave and the complex-valued electron probe function including its aberrations. As such, aberration correction is not strictly a requirement for the work presented here since CSAs of only 4 to 6 mrad and larger defocus values around 2 μm were used."

Reviewer #1 (Remarks on code availability):

Scripts for Py4DStem are available on GitHub

Reviewer #2 (Remarks to the Author):

This is an addendum to my original report. I have studied the authors' responses as well as the comprehensive and thoughtful replies to the comments of all reviewers. The authors have addressed the concerns to my satisfaction, and as such, I recommend that this manuscript be published in the revised format. In particular, the authors have supplied missing links to code as well as supplementary data to address the major criticisms.

Reviewer #2 (Remarks on code availability):

I have not tested the code but I have examined it, and find it to be relatively straightforward.

Reviewer #3 (Remarks to the Author):

The revisions made by the authors have not really addressed my main concerns regarding this paper. I am still struggling to discern what is actually learnt from the work scientifically. In their response to my concern, the authors state that they have improved the resolution over previously published work, yet the very closely related quadrant detector work which they cite (reference 7) has reached 3.5 angstrom resolution whereas their work claims 5.8 angstrom. There does not appear to have been any rational quantitative basis for their choice of parameters, in particular the convergence semi angle, apart from a vague comment that it is a compromise between resolution and depth selectivity. I would have liked a more detailed and quantitative comparison with previous work that would then help steer developments in this area. As it stands, the paper is really just a discussion of some successful imaging experiments reported somewhat in isolation from the understanding developing in this field.

Having said that, it is a very nicely presented piece of work and the experimental data is very nice. I can that the other reviewers are more positive about the work than I am, and given that I have no specific technical concerns about the work I do not want to stand in the way of its publication. It may will be a useful addition to the literature as it stands.

My only comment is that py4DSTEM is, as I understand it, not a single piece of software but a collection of methods which have in many cases been developed elsewhere. I assume an iterative reconstruction method was used, and I would like to know the type of algorithm used as there are several different types. I would like the authors to give some further details about the algorithm, how convergence was test etc.

Response to reviewers

Low-dose cryo-electron ptychography of proteins at sub-nanometer resolution

Reviewer #1 (Remarks to the Author):

With the current revision, the authors have adequately addressed all my prior concerns and they have expanded the clarity and impact of the manuscript. Several key updates to the narrative provide extra details about the data collection and reconstruction methods as well as better link the discussion of the ResLog analysis to the data presented. The inclusion of new conventional cryo-EM datasets for apoferritin with a total particle count similar to the 4D-STEM experiments was helpful to more directly compare the approaches, and the addition of the 2d crystal bacteriorhodopsin dataset was useful for showing a quantitative assessment of resolution through the intensity of the diffraction spots above background. The results presented by the authors represent a significant advancement in the field and the manuscript is ready to be accepted for publication with only minor edits to the narrative. In my opinion, the requested minor edits do not need further peer review but would be handled sufficiently at the editor or copy editor level.

We thank the reviewer for the kind words. Below, we address the reviewer's concern, highlighting the changes to the manuscript.

All of the minor text edits below relate to the new manuscript version containing long sentences that disrupted the flow of the narrative and required re-reading in order to interpret the author's intent. I have provided my best interpretation for the various sentences as reference.

- On page 3, lines 47-52: "Cryo-EM has been shown to be able to determine in a few hours at near-atomic resolution the structure of proteins that have been frozen as single particles in a thin aqueous layer, provided the proteins are available in sufficient concentration as homogeneous populations, sufficiently stable conformations, and embedded in random orientations in a thin ice layer without excessive adsorption to the air-water interface." I believe the authors meant something like "Cryo-EM has been shown to only require a few hours to determine the near-atomic resolution structure of proteins that have been frozen as single particles in a thin aqueous layer. The method generally requires that the proteins are available in sufficient concentration as homogeneous populations, adopt sufficiently stable conformations, and are embedded in random orientations in a thin ice layer without excessive adsorption to the air-water interface."

Thank you, that is excellently phrased. We replaced our long sentence with your suggestion in lines 48-52.

- On page 11, lines 266-270: "For ptychography reconstructions, computed Fourier transforms of the 2D potential maps obtained by computing ptychography phase-only reconstructions, while fixing the amplitudes to constant "1", show for 4D datasets recorded with a defocus of 2 μm the presence of ring like amplitude oscillations (Fig. 3), and the width of the rings is wider (with more narrow dark gaps) when data closer to focus are recorded (Extended Data Fig. 9)." I believe the authors meant something like "For ptychography reconstructions, computed Fourier transforms of the 2D potential maps were obtained by computing ptychography phase-only reconstructions. While fixing the amplitudes to constant "1" shows the presence of ring like amplitude oscillations (Fig. 3) for 4D datasets recorded with a defocus of 2 μm , the width of the rings is wider (with more narrow dark gaps) when data closer to focus are recorded (Extended Data Fig. 9)."

We thank this reviewer for catching the unclear phrasing in that paragraph. We have now modified that paragraph in lines 239-245 to:

For ptychography reconstructions, we reconstructed 2D potential maps for the sample. This was done with the iterative ptychography algorithm implemented in py4dstem by setting the amplitudes in each iteration to "1", so that all specimen signal was directed towards the phase map. The computed Fourier transformations of such "potential maps" show for 4D-STEM datasets recorded with a defocus of 2 μm

the presence of ring like amplitude oscillations (Fig. 3). The width of the rings is wider (with more narrow dark gaps) when data closer to focus are recorded (Extended Data Fig. 9).

- On page 10, lines 240 - 244: "Crystallographic processing of these two reconstructions with the FOCUS software allowed the reconstruction of the projection maps at around 6 Å resolution from each, which required for the parallax reconstruction a correction of the oscillating CTF by Wiener filtration, while the ptychography map allowed reconstruction of the correct projection map without any CTF correction." I believe the authors meant something like "Crystallographic processing of these two reconstructions with the FOCUS software allowed the reconstruction of the projection maps at around 6 Å resolution from each dataset. The parallax reconstruction required a correction of the oscillating CTF by Wiener filtration, while the ptychography map allowed reconstruction of the correct projection map without any CTF correction."

We thank this reviewer for catching the unclear phrasing in that paragraph. We have now modified that paragraph in lines 213-217 to:

Crystallographic processing with the FOCUS software allowed the reconstruction of the projection maps at around 6 Å resolution from the parallax and the ptychography reconstructions. The parallax map required a correction of the oscillating CTF by Wiener filtration, while the ptychography map allowed reconstruction of the correct projection map without any CTF correction.

- On page 6, lines 138-141: "However, as ptychography reconstructs both, the complex-valued sample exit wave and the complex-valued electron probe function including its aberrations, aberration correction is not a requirement for the work presented here, since CSAs of only 4 to 6 mrad and larger defocus values around 2 µm were used." I believe the authors meant something like "However, ptychography reconstructs both the complex-valued sample exit wave and the complex-valued electron probe function including its aberrations. As such, aberration correction is not strictly a requirement for the work presented here since CSAs of only 4 to 6 mrad and larger defocus values around 2 µm were used."

Thank you, that is excellently phrased. We replaced our long sentence with your suggestion in lines 130-133.

Reviewer #1 (Remarks on code availability):

Scripts for Py4DStem are available on GitHub

We thank the reviewer for the very helpful work.

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Reviewer #2 (Remarks to the Author):

This is an addendum to my original report. I have studied the authors' responses as well as the comprehensive and thoughtful replies to the comments of all reviewers. The authors have addressed the concerns to my satisfaction, and as such, I recommend that this manuscript be published in the revised format. In particular, the authors have supplied missing links to code as well as supplementary data to address the major criticisms.

Reviewer #2 (Remarks on code availability):

I have not tested the code but I have examined it, and find it to be relatively straightforward.

[We thank the reviewer for the kind comments.](#)

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Reviewer #3 (Remarks to the Author):

The revisions made by the authors have not really addressed my main concerns regarding this paper. I am still struggling to discern what is actually learnt from the work scientifically. In their response to my concern, the authors state that they have improved the resolution over previously published work, yet the very closely related quadrant detector work which they cite (reference 7) has reached 3.5 angstrom resolution whereas their work claims 5.8 angstrom. There does not appear to have been any rational quantitative basis for their choice of parameters, in particular the convergence semi angle, apart from a vague comment that it is a compromise between resolution and depth selectivity. I would have liked a more detailed and quantitative comparison with previous work that would then help steer developments in this area. As it stands, the paper is really just a discussion of some successful imaging experiments reported somewhat in isolation from the understanding developing in this field.

We thank the reviewer for the comments. The iDPC work with the quadrant detector (reference 7) is from some of us. Indeed, the resolution achieved there of 3.5 Å was higher than what we show here for ptychography. Also, our here included work with real-space CTEM, reaching 1.09 Å is at much higher resolution than what we show here for ptychography. Nevertheless, we believe that our work here has a merit on its own. Each approach, real-space CTEM, iDPC and Ptychography, has its advantages and challenges, which we discuss in our work.

Having said that, it is a very nicely presented piece of work and the experimental data is very nice. I can that the other reviewers are more positive about the work than I am, and given that I have no specific technical concerns about the work I do not want to stand in the way of its publication. It may will be a useful addition to the literature as it stands.

We thank the reviewer for the kind comments.

My only comment is that py4DSTEM is, as I understand it, not a single piece of software but a collection of methods which have in many cases been developed elsewhere. I assume an iterative reconstruction method was used, and I would like to know the type of algorithm used as there are several different types. I would like the authors to give some further details about the algorithm, how convergence was test etc.

We thank the reviewer for the request. We have now added a more detailed description of the algorithms employed to the manuscript in lines 558-604, which reads:

Data analysis: Parallax and ptychographic reconstructions

Each of the acquired 4D-STEM datasets were analyzed using the py4DSTEM software³⁴ using the following steps:

Preprocessing

- Data loading and flat-field correction: Electron diffraction patterns from one scan in h5 format were loaded, flat-field normalized, and hot/dead pixel corrected by replacing values of identified non-functional pixels with median values computed from their neighbors. Scan metadata such as scan step size were stored separately.*
- Extraction of key parameters: Following normalization, the mean diffraction pattern was computed to identify the pixels inside the brightfield disk. The diameter of this disk was subsequently used for reciprocal space pixel size calibration and in the parallax reconstruction algorithm.*

Parallax reconstruction:

- Cross-correlation of virtual brightfield images: Each pixel inside the brightfield disk was used to form a virtual brightfield (VBF) image corresponding to a tilted planewave illumination. The VBF images were iteratively aligned (starting with a large bin value to boost signal) using cross-correlation to obtain a vector field.*
- Aberration-fitting using aligned vector field: The obtained cross-correlation vector field – which is proportional to the gradient of the aberration surface – was used to fit low-order aberrations across the field of view, notably defocus and the relative rotation between real and reciprocal-space coordinate systems.*

- *Phase-flipping CTF correction:* The estimated low-order aberrations were then used to correct the aligned brightfield image by phase-flipping negative portions of the CTF. Note that the steps listed up to here for a parallax reconstruction²⁴ are computationally inexpensive. The CTF-corrected aligned brightfield image was used to monitor the acquisition in “real-time”.

Ptychographic reconstruction:

- *Object and probe preprocessing:* The scattering potential was initially assumed to be empty, and the probe was initialized using an aperture obtained by the mean of a 4D-STEM measurement over vacuum and the estimated aberration surface from the parallax reconstruction.
- *Single-slice reconstruction:* The object and probe were jointly reconstructed using a batched stochastic gradient descent algorithm. The error was monitored using the L2 norm of the difference between the measured diffraction intensities and those modeled using the “single-slice approximation” model. Convergence was typically achieved within 30 iterations, i.e., 30 passes through the batched diffraction patterns, the order of which was randomized between passes to accelerate convergence.
- *Object and probe constraints:* The scattering potential was assumed to be a pure-phase object, i.e., its amplitude was fixed to unity during the iteration. The probe aperture (i.e., its Fourier amplitude) was replaced by the vacuum measurement mean at each iteration, while the aberration surface was fitted to a low-order Zernike-like expansion up to radial and angular order 3, to ensure smoothness and remove residuals²⁴.
- *Potential map postprocessing:* The reconstructed micrograph was cropped if necessary and saved for subsequent analysis. See Extended Data Figure 10 to compare different ptychography data models.

The workload was distributed across four GPUs, each processing a single dataset. This parallel approach allowed the processing of 70 datasets per hour with a 4-GPU computer.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I have previously reviewed two versions of this manuscript. The edits made by the authors in the current revision have addressed all my concerns, and the work is certainly of a quality suited for Nature Communications. I therefore support the publication of this manuscript as-is.

Reviewer #1 (Remarks on code availability):

The code was previously reviewed and is available on GitHub.

Reviewer #3 (Remarks to the Author):

I appreciate the authors' responses to my comments, and in particular the additional details they have provided about the reconstruction methods. I have no further points to raise and I am content that the paper be published.