**Editorial comments:**  
  
1) 1.5 What is done, stepwise, for cryocycling?

Answer: Cryocycle a function of the microscope, which warms up the microscope and cools it down to cryogenic temperature again. To perform, one clicks the “cryocyle” button the microscope user interface.  
  
2) 1.6 What steps are taken to align the microscope?

Answer: We have a detailed protocol for microscope alignment and our staff performs this protocol ahead of each session. Such alignment is routine in any EM facility. Due to the limited time available for the video-taping, it is not possible to perform these steps in the video and, accordingly, we did not list these steps.  
  
3) Figure 2 should have some indicator that both left hand images are Figure 2a, both center images are 2b, ect.. or the lower images in the panel should be labeled and described as being a different magnification/view type.

Answer: Changed as suggested.  
  
4) As a note, this can be removed: "Video link - The video component of this article can be found at http:// xxxxxxxxxxx". Text integration will include this automatically once the article is put online.

Answer: Removed in text.  
  
5) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.   
Answer: We have taken time to edit the text carefully. Thank you for reminding us.  
  
**Reviewers' comments:**  
  
**Reviewer #1:**   
General Comments  
Dr. Zhou and colleagues are leading expert in pushing the single particle reconstructions at near atomic resolution. Their experience will be useful for other investigators particularly for the steps that are not commonly practiced in the routine cryoEM structure studies at lower resolution. This paper should serve this purpose to describe procedures including grid preparation, microscope alignment, data processing and model building. There are major weaknesses in the present format of this manuscript. One is the need of improving the readability of the text. A thorough edit with spelling and grammar check is needed. Another weakness is the failure of providing details on possibly key steps that are presumably needed to generate high resolution images and maps. The followings are some points that the authors need to address in the revision.  
  
1. Baking grids is a step rarely used in the cryoEM practice. More details should be included in terms of dose rate and total dose to irradiate the grids effectively; the length of time that the baked grids are still useable after the baking; and the storage of method of the baked grids.

Answer: The time and dose rate for baking the grids are empirical. We use the same beam settings and variable time and got the same results. We typically bake them under the strongest possible 100kV beam (LaB6 filament) that we have at the imaging plane for 1-3 days. The grids are still usable after baking for 3 days. The total dosage is on the order of a few to a few hundred e/A2, depending on the condition of the microscope. The baked grids can be stored in air in a petri dish.  
  
2. How are the microscope alignment done for coma and astigmatism? Is this done manually or based on the software supplied by the microscope company ? How often this alignment was done (e.g. daily or hourly?)

Answer: We noted in the revised manuscript that this is done manually. We also noted that we check the alignment every couple of days.  
  
3. How do they cope with charging or beam induced movement? If there is none in their data, it should be mentioned. What is the percentage of their data are good, acceptable and bad as exemplified in one of their figures?

Answer: The baking of the grids greatly reduces the extent of charging. Beam induced movement is inevitable and cannot be easily quantified. The percentage of acceptable and unusable data depends on the imaging condition. The percentage of acceptable data ranges from 70% to 95%. However, we decide not to talk about the statistics because of the variability. Simply, discard the unusable data and keep the useful data.

4. In the image processing steps, most parameters used here seem to be default in EMAN. Critical steps should be emphasized for obtaining 3-4 Å resolution but not for lower resolution studies.

Answer: We omitted the details about how to use EMAN package and left it to the original authors of EMAN to discuss it. In summary, the protocols suggested by the original authors are sufficient to get to atomic resolution. The only difference that we introduced is the phase flipping with astigmatic CTF, which we noted in the revised manuscript. We believe that it is the data quality that has made the difference, not the adequacy of using image processing software.  
  
5. How is the map validated and what is the resolution criterion ?

Answer: We verify the resolution in a few ways and try to get the consensus of them. First, we look at the features of the maps. When a map improve from 4.0 to 3.3 angstroms, the side chains of small hydrophobic residues start to show differences; the aromatic residues begin to show thinning on the direction perpendicular to the ring. Second, we use FSC to determine the resolution. Third, we refine the atomic model against the maps, and estimate the resolutions based on the R factors.  
  
6. How does one know that the model is refined optimally and not over-refined?

Answer: We presume that by “model” the review refers to our atomic models. The quality of the refinement of the models can be judged by the R factors and the geometric validation (namely, RAMA plot, bond lengths and angles, etc).  
  
Specific Comments: The followings are specific suggestions throughout the text for consideration in the revision.   
  
Line 63 - Distinguishing your "technical aspects" from that of "others" really is not done in this paper. A possible explanation of why others don't routinely obtain this resolution would be very helpful.

Answer: There are many reasons for failure. The purpose of this video is to show how we performed the published work so that others can repeat or improve on it.

Line 78 - The term initially makes it seem like this never happens, however this strategy is probably more common.

Answer: Removed initially in text and modified sentence.  
  
Line 93 - References should be added.

Answer: References added.  
  
Line 93-95 - Poorly worded sentence.

Answer: Corrected.

Section 1.1 - If this is an overview, an explanation of why these methods are being done would be helpful. If no explanation, citing a review on sample prep would help.

Answer: This is not an overview but rather an operational demo.   
  
Line 118 - "fin" should be fine.

Answer: corrected.  
  
Section 1.3 - It really should be emphasized that this protocol may, and most likely will be different for various instruments.

Answer: A note was added at the beginning of section 1 to address this within the text.

Line 143 - Why use these parameters? Or reference a paper that has used them in the past.

Answer: Again, this is an operational demo for work published previously. Readers can refer to cited papers.

Line 147 - An explanation of "good data and bad data" should be given, not just an image. Why is the data good? Why is it bad? What could be done to improve the data?

Answer: See figure legend.  
  
This leads back to the workflow (Figure 1.1) where it shows that the only thing that can improve resolution is the image processing. Is this really true? Do we need the very expensive high end instrument as used by the authors?

Answer: Figure 1 is not intended to suggest such a conclusion. Taking high-resolution images is the first step of that figure. We do not intend to suggest that one has to use an expensive instrument for high-resolution imaging.  
  
There are review papers that described high resolution image processing. They should be referenced.

Answer: References added (last line in introduction).

Section 2.4 How can one tell this? Should the reader know all this? If the reader does know all this why state these steps?

Answer: We clarified the section by changing “CTF” to “2D power spectra”. We will show examples of these cases in the video.  
  
Section 3 - No mention of using "Gold-standard" practices. One really needs to start validation at the image processing level to reduce bias and ensure that the resulting models are an accurate representation of the biological sample.

Answer: At the time of preparing the paper, we did not incorporate “Gold-standard” FSC criteria. We understand that the FSC could be easily affected by many parameters such as masking. However, we typically judge our maps by the features. We also judge the resolution by the R-factors given by pseudo-crystallographic refinement.  
  
Section 3.1.1 - Again, using the term "good" is very subjective. What makes them "good"? How many should one keep?

Answer: “good” changed to “selected” to reflect the terminology used by the program.  
  
Section 3.1.3 - To possibly improve this paper, a list of exact commands would be helpful. This has been brought up at various meetings (Keystone Conferences for instance) that published data would be accompanied with exact commands.

Answer: The commands used are provided in parentheses in Fig. 3 and are also *italicized* in the text.   
  
Line 298 - Capitalize Phenix.

Answer: Edited in text.  
  
Section 4.1.1 - An example of this would be helpful.

Answer: we provided such demonstrations in Fig. 7.  
  
Section 4.1.2 - You are essentially doing "baton-building" a reference to a crystal structure that has done this would be helpful.

Answer: We now indicate that this is baton-building. We deleted the reference to X-ray structures.

Section 4.1.3 - Again, "Good enough" is very subjective. What should one be looking for?

Answer: We removed the sentence.

Section 4.1.4 - What tool are you using to do this?

Answer: Clarified.

Section 4.1.5 - How is this "cutting" (segmentation) done?

Answer: Clarified.

Section 4.1.6 - If you have a segmented density and you remove 5-10% this is really hurting your test set. To avoid this real space refinement should be done.

Answer: We did what was described in order to take advantage of existing X-ray crystallography refinement package, CNS. We recognize that this could be a potential issue and we are considering real space refinement. However, we feel this discussion is outside the scope of the current video illustration.

Section 4.2.1 - Where is this "vector residue" parameter used? in CNS?

Answer: Yes, it refers to CNS. Since that line is a sub-section of 4.2, we omitted the reference to CNS.  
  
Line 336 - Could you explain the constant? Just providing a number is not helpful.

Answer: This is totally empirical and gives acceptable results.  
  
Figure 6c - If you could point out regions that are misfit, it would really help.

Answer: We now indicate the misfit regions in this panel.  
  
Section 4.2.3 - Do you see density for all residues equally? What do you do for poorly resolved regions?

Answer: We build model anyway for the poorly resolved regions. They may not be accurate. However, this is common practice in crystallography as well.  
  
Section 4.2.9 - Using NCS symmetry may reduce high resolution information due to map differences. How do you deal with this loss of data? The side chains become less constrained, correct?

Answer: The maps are reconstructed with the NCS symmetry imposed. Therefore, using the constraint or restraint does not harm the resolution. We simply use NCS symmetry in order to refine molecular contact.  
  
Section 4.2.11 - Ramachandran (check your spelling) may harm models at resolutions >3Å. Essentially, it overweights geometry and reduces the fit to map. Also, why would you consider this optional?

Answer: We would like to let it “harm” the fit to the map and thus get to a worse R factor. We do this intentionally so that the resolution is not over-estimated.  
  
Representative Results section - Some of this detail should be above when describing your protocol.  
  
Line 397 - Why "pseudo"-crystallographic R-factors? If they are pseudo, can they really be trusted?

Answer: We say “pseudo” because it is not crystallographic data; but we followed crystallographic practices. This is widely accepted term.  
  
Line 398 - 2.7Å? is this simulated?

Answer: By “reconstructed” we suggested that we include data to that (2.7 Angstroms) resolution. The map may not reach that resolution. We simply want to give the audience an impression that including too much data to the high resolution side will introduce noise. We clarified this in the revised manuscript by pointing out “(resolution limit) to”.  
  
Line 401 - The Figure should have modeled side chains showing. Only showing the density and a backbone does not help.

Answer: Side chains are shown in other figures when appropriate. We experimented with displaying side chains in Fig. 7 and found that to defeat the purpose of the figure. For examples of side chains, viewers can refer to Figure 5.  
  
Line 403-404 - What about β-sheets? Loop regions?  
Answer: We added the following: “At 3.6Å, loops are resolved, strands in β sheets are well separated” and “At 4.5Å resolution, strands become hard to resolve”.

Line 418 - Refining the atomic model multiple times is not shown in the workflow image (Figure 1). How does one know when he has completed the model? Line 419 - This is restating the previous protocol. What about results? How are the models validated?  
  
Answer: This section is to illustrate the results. The protocol is detailed above.

Figure 1 - Colors are practically meaningless. One could obtain the same information if it were black and white. Also, this workflow makes it seem like atomic-resolution is only based on image processing and reconstruction methods. Sample preparation and imaging conditions may be altered if ideal resolution is not obtained.

Answer: colors refer to stages of distinctive steps that are performed at either the same instrument or program and are grouped together. As we have answered this above, high resolution imaging is also part of our procedure.  
  
Figure 3 - When does one proceed to segmentation?  
Answer: Prior to atomic modeling.

Figure 5 and corresponding text - The CPV manuscript explains the model building procedure much better than this review. One should just reference that paper and not try to re-describe the methods.

Answer: Indeed, the current work is a video illustration of our previously published protocol.   
  
Line 473 - More detail on this would be really helpful, or at least a reference.

Answer: References are now provided.   
  
Line 488-490 - This statement should be avoided for a review. Discuss current published data and not unpublished data.

Answer: This is not a review but a video demonstration of practices.   
  
Line 498 - A reference to this is required? What other groups? Would this research help labs that didn't have the same technology?

Answer: We have indicated that this is by personal communication with Wim Hagen.

Line 505 - The term "kicks in" is not a proper term. It should be something like introduced and should include a reference.

Answer: Edited in text.  
  
Line 542 - This manuscript ends very abruptly. What impact does solving these high resolution structures have?

Answer: Again, this is not a review where significance is summarized. The last section happens to be Discussion. Our discussion begins with a summary of the significance of the protocol presented. This section was expanded to include various issues that remain to be addressed in future efforts.

Also, what validation measures are taken to ensure no model bias and that the final molecular model is correct?

Answer: Our protocol does not include the use of reference models in refinement. The atomic structures resulting from this protocol are validated by consistence with amino acid sequence. The models are automatically validated by the refinement programs. This situation is different from others where no atomic structures are obtained.  
  
Lots of crystallographers are not satisfied with data worse than 3-3.5Å. What makes these models more credible? Do you refine a larger complex? How do you refine interfaces? Do you use symmetry when refining models?

Answer: It is beyond the scope of this video demonstration to argue either way. The last three question are answered by cited papers.  
  
Table of Reagents / Materials Used - Film is becoming quickly outdated. I realize this is what you used for your experiments but more discussion should be given on new sensor technology and the potential improvement it can provide to ones reconstruction.

Answer: We believe that the recent development of direct electron detector is beyond the scope of this paper and warrants another paper. Nevertheless, we added a reference to the direct electron detector in the first paragraph of Discussion.  
  
  
**Reviewer #2:**   
The manuscript illustrated the procedure to obtain near atomic reconstructions of macromolecular complexes by cryo-EM and build atomic models de novo based on the EM density maps. The authors used tobacco mosaic virus (TMV) and cytoplasmic polyhedrosis virus (CPV) as examples of helical and icosahedral reconstructions and demonstrated the approach to derive atomic models de novo with cryo-EM maps reconstructed to 3-4 Å resolution.  
  
Comments:  
1. In Step 3. Image processing, the authors used extensive space to illustrate the helical reconstruction procedure of TMV. If the authors include the atomic model of TMV built de novo with the cryo-EM map and compare that to the structure solve by crystallography (Step 4), the demonstration and continuity of the paper will be further improved.

Answer: We had discussed about the atomic model of TMV and its difference to the X-ray model elsewhere. Therefore, we are not to include this in the current paper.  
  
2. In step 4.1.2, could the authors comment on potential issues of tracing the c-alpha backbone in regions with poor density or regions where the connectivity of secondary structural elements is ambiguous?

Answer: We suggest refraining from modeling in such areas. Our experience is that there is typically no connection ambiguity for maps at resolutions better than 4Å.   
  
3. In step 4.1.4, could the authors comment on how to discern multiple possible assignments of amino acid sequence to the backbone model?

Answer: At the resolution of <4Å, amino acid sequence can typically be unambiguously assigned.  
  
Minor comments:  
1. Step 3.2.1, 3.2.2 and 3.2.3 are the sub-steps of 3.2. The font should not be in bold just for the clarity and consistency with the style of sub-steps of 3.1.

Answer: Removed bolding from these areas in the revised text.  
  
2. In line 198, "The box size should be 3-4 times larger than the particle for thin helixes and ..." Does " the particle" refer to the width or length of the particle?

Answer: Specified “diameter of the particle” in the revised text.

3. Placing Fig7, progressing of TMV refinement, subsequently to Fig4, Helical reconstruction, will create better flow of the paper.

Answer: We believe that Fig. 7 serves a different purpose than illustrating a typical result of helical reconstruction. The actual purpose is to illustrate the features of the map at different resolutions. Therefore, we would like it to stay as Fig. 7.  
  
4. Fig 7, what are the densities colored in yellow vs. cyan?

Answer: We color the two different views in yellow and cyan, respectively.  
  
**Reviewer #3:**   
The manuscript "Deriving de novo atomic models by cryo electron microscopy" by Ge et al. in Hong Zhou's laboratory very nicely illustrates the value of JoVE in visualizing and outlining critical methods to both experts within the field and outside of the field. The manuscript provides an excellent overview on how Dr. Zhou's laboratory achieves their ground-breaking results. Minor adjustments, as outlined below, are requested:  
  
p. 2, l. 65: "important" used twice, could be changed to "critical"

Answer: Changed in revised text  
  
p. 2, l. 67: "(CPV" second bracket missing

Answer: Added “)” in revised text  
  
p.3, l. 109: "his/her sample" change to "the sample studied"

Answer: Changed in revised text  
  
p.3, l. 118: "fin" change to "thin"

Answer: Changed in revised text  
  
P. 4, l. 133: Mention which specific cryo-EM is appropriate as it would otherwise imply that any TEM can be used

Answer: Per the editor’s requirement, we removed trademark names from our manuscript.  
  
p. 6, l. 212: "flip the phase" -use technical terms

Answer: Changed to “Do phase-flipping ctf correction”.  
  
p.7, l. 266: "bfactor (Nikolaus Grigorieff group) program" -provide reference

Answer: This is just a utility program which doesn’t have a citation.  
  
p.7, l. 267: format "1/A2" properly

Answer: Changed in revised text  
  
p. 7, l. 268: use reference or provide further details for "Bfactor can be adjusted based on the microscope's performance."

Answer: Changed in revised text  
  
p. 8, l. 304: format "1/A2" properly

Answer: Changed in revised text

p. 8, l. 308: "4.5Å maps" change to "4.5Å resolution maps"

Answer: Changed in revised text  
  
p.10, l. 390: Why are the authors discussing "CCD frame" when they mentioned the use of film or direct electron detectors before?

Answer: We now clarify it so that the preceding line reads “we test our microscope alignment by taking test images with a CCD camera in two steps”.  
  
p.10, l. 391: "thong" should read "Thon"

Answer: Changed in revised text  
  
p. 11, l. 404: "At 8Å" change to "At 8Å resolution"

Answer: Changed in revised text  
  
p. 11, l. 431: "upscale microscope" - be precise what type of cryo-EM is meant such as 300kV FEG, etc.

Answer: Attempted to be more specific in the revised text, however the editors have asked us to avoid trade names.  
  
p. 11, l. 435: "thong" should read "Thon"

Answer: Changed in revised text  
  
p. 12, l. 470: "structural biology method" change to "a structural biology method"

Answer: Changed in revised text  
  
p. 12, l. 487-488: The sentence "It is the size of protein that is under particle alignment during refinement of the structure." is not clear and needs to be rephrased.

Answer: We deleted the sentence.  
  
p. 13, l. 490: The recent channel structure by Yifan Cheng's lab needs to be discussed and cited in the context of particle size.

Answer: We believe that the recent development of direct electron detector is beyond the scope of this paper and warrants another paper.  
  
p. 13, l. 505: "kicks in" change to "shows its effect"

Answer: Changed in revised text  
  
p. 13, l. 506-8: It is generally known that the phases have a strong effect. It is fine though, however, to keep this statement in the discussion.

Answer: Agreed.  
  
p. 13, l. 514: "everything" - too casual  
Answer: Changed in revised text  
  
**Reviewer #4:**   
See external attachment

Answer: The suggested changes marked in the PDF file have been incorporated in the revised manuscript.   
  
**Reviewer #5:**   
In this manuscript Ge et al. present their approach to solve viral protein structures by cryo-EM. The manuscript is nicely written and will give the reader/viewer a good guide through the different steps in structure determination by cryo-EM. However, it reads a bit as if it has been written 1-2 years ago. In the introduction, the structure determination of the mitochondrial ribosome by Ramakrishnan and colleagues and of the TrpV channel by Cheng and colleagues is not mentioned at all. Besides in a single sentence on p. 4 (1.7) the application of direct detectors is also not mentioned. The authors should therefore modify the introduction and the discussion accordingly. Sometimes it would be good to know why the authors perform the described experiments/steps. For example, why do they soak the grids in EDC. Why do they apply an additional carbon layer on the grid….I think it would help the reader/viewer a lot to have this information. It would be also important to point out the differences (and limits) between working with film and direct detectors.

Answer: What we present here is simply how we do things. We follow many early practices as to eliminate as many possible resolution barriers as possible.

For the direct electron detector, we believe that the recent development of it warranties another paper.  
  
On p. 10 (representative results) Fig. 1d and Fig 1e are missing, but are important for the reader to understand the text. It is not "thong rings" but "Thon rings", named after the German physicist Thon.

Answer: We initially wanted to include these figure panels. However, we decided that the words are enough to illustrate the points and decided not to include these panels. We failed to have edited and removed these references. We have now removed them.  
  
Fig. 6: Would be better to always show the same region of the protein, otherwise it is confusing.

Answer: In Fig. 6, we are trying to show the change of the protein during the refinement process. No single region would be able to illustrate all effects of the refinement. Therefore, we chose to show different regions.  
  
Fig.7 Figures should be arranged such that the lowest resolution is on top and the highest on the bottom. At higher resolutions the differences are difficult to see. I would recommend to concentrate on two helices in one and on two ß-strands in the other panel, but to zoom in on them..

Answer: We would like to retain our sequence of panels. The yellow panels are to show the detailed features and the cyan panels are to show the overall visual impressions of the protein.  
  
It is not a "fin layer of carbon" but a "thin layer of carbon".

Answer: Changed in revised text  
  
But besides these few suggestions, I recommend the paper for publication in JoVE and look forward to seeing the video.