Journal of Visualized Experiments

Deriving de novo atomic models by cryo electron microscopy

--Manuscript Draft--

Manuscript Number:	JoVE51436R2
Full Title:	Deriving de novo atomic models by cryo electron microscopy
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Electron microscopy; cryo electron microscopy; atomic modeling; TMV; CPV; eman; iterative helical real space reconstruction; single particle reconstruction
Manuscript Classifications:	5.5.595: Microscopy; 5.5.595.402: Microscopy, Electron; 5.5.595.402.150: Cryoelectron Microscopy
Corresponding Author:	Hong Zhou, Ph.D. UCLA Los Angeles, CA UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	Hong.Zhou@UCLA.edu
Corresponding Author's Institution:	UCLA
Corresponding Author's Secondary Institution:	
First Author:	Peng Ge, PhD
First Author Secondary Information:	
Other Authors:	Peng Ge, PhD
	Nicole Poweleit
	Robert P Gunsalus, PhD
Order of Authors Secondary Information:	
Abstract:	Cryo electron microscopy is a structural biology technique for determining three-dimensional structures of supramolecular complexes in solution. In cryoEM, samples in their physiological, non-crystalline state are embedded in vitreous ice during low dose imaging. A three-dimensional density map of the sample is obtained by combining tens of thousands of cryoEM images. In recent years, the resolution of cryoEM has improved steadily, reaching a resolution sufficient for building atomic models from the cryoEM density map alone. In this video article, we illustrate how such atomic models can be derived by cryoEM. Others have described the detailed procedures for preparing frozen hydrated samples, image acquisition and the basic steps of data processing of cryoEM 1,2. We will focus on several important technical aspects important for reaching 3-4 Å resolution and for building reliable atomic models. We use the tobacco mosaic virus (TMV) and cytoplasmic polyhedrosis virus (CPV) as representative complexes with helical and icosahedral objects, respectively. De novo atomic models of these objects have previously been determined by cryoEM and published, and their biological relevance is described in previous publications3-6.
Author Comments:	This is an invited contribution.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	

Cover Letter

UNIVERSITY OF CALIFORNIA, LOS ANGELES **UCLA**

BERKELEY • DAVIS • IRVINE • LOS ANGELES • MERCED • RIVERSIDE • SAN DIEGO • SAN FRANCISCO SANTA BARBARA • SANTA CRUZ

Z. Hong Zhou, PHD
PROFESSOR & DIRECTOR
Electron Imaging Center for Nanomachines
California NanoSystems Institute (CNSI)
Los Angeles, CA 90095-7151
http://EICN.ucla.edu and http://www.mimg.ucla.edu/

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY & MOLECULAR GENETICS
DAVID GEFFEN SCHOOL OF MEDICINE AT UCLA
Box 951594
Los Angeles, CA 90095-1594
Courier delivery: BSRB 250B, 615 Charles E Young Drive S, Los Angeles, CA 90024
EMAIL: Hong.Zhou@UCLA.edu; PHONE: 310-206-0033

August 27, 2014

Re: JoVE manuscript JoVE51436R1 by Ge et al.

Jaydev Upponi, Ph.D.
Science Editor
JoVE

Dear Dr. Upponi,

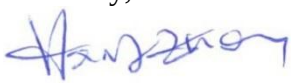
We would like to extend our appreciation again for inviting us to submit a manuscript to JoVE. We are pleased to submit now our revised manuscript.

We apologize for the delay in our revision of our manuscript. However, we felt that the reviewers were asking for information outside the scope of this current project, and that some questions are really tedious to answer, specifically, issues raised by reviewers 1 & 4. Nevertheless, we took painstaking efforts and extended time to respond to these questions as detailed in our Responses to Reviewers.

We tried to avoid adding excessive text so that the length of the manuscript would be maintained.

Thank you again for consideration.

Sincerely,



Z. Hong Zhou, PhD
Professor & Director Electron Imaging Center for Nanomachines, CNSI/MIMG, UCLA

Title:
Deriving *de novo* atomic models by cryo electron microscopy

Authors:
Peng Ge[1], Nicole Poweleit[1,2], Robert P. Gunsalus[2,3], Z. Hong Zhou[1,2,*]

Author Affiliation:
Ge, Peng [1],
[1]Electron Imaging Center for Nanomachines (EICN),
California NanoSystems Institute,
University of California,
Los Angeles
gepeng1983@gmail.com

Poweleit, Nicole [1,2],
[1]Electron Imaging Center for Nanomachines (EICN),
California NanoSystems Institute,
University of California,
Los Angeles

[2]Department of Microbiology,
Immunology and Molecular Genetics,
University of California,
Los Angeles
poweleit@ucla.edu

Gunsalus, Robert P. [2,3],
[2]Department of Microbiology,
Immunology and Molecular Genetics,
University of California,
Los Angeles

[3]UCLA Institute of Genomics and Proteomics,
Los Angeles, CA
robg@microbio.ucla.edu

Zhou, Z. Hong [1,2,*]
[1]Electron Imaging Center for Nanomachines (EICN),
California NanoSystems Institute,
University of California,
Los Angeles

[2]Department of Microbiology,
Immunology and Molecular Genetics,
University of California,
Los Angeles
Hong.Zhou@UCLA.edu

Keywords:
Electron microscopy, cryo electron microscopy, atomic modeling, TMV, CPV, eman, iterative helical real space reconstruction, single particle reconstruction

***Corresponding Author:**
Z. Hong Zhou,
Hong.Zhou@UCLA.edu

Short Abstract:
Cryo electron microscopy (cryoEM) can be employed to derive *de novo* atomic models of macromolecular complexes in solution. The steps involved in high resolution cryoEM of biological molecules, from image recording, to data processing, to atomic modeling based on the resulting cryoEM density map, are illustrated.

Long Abstract:
Cryo electron microscopy is a structural biology technique for determining three-dimensional structures of supramolecular complexes in solution. In cryoEM, samples in their physiological, non-crystalline state are embedded in vitreous ice during low dose imaging. A three-dimensional density map of the sample is obtained by combining tens of thousands of cryoEM images. In recent years, the resolution of cryoEM has improved steadily, reaching a resolution sufficient for building atomic models from the cryoEM density map alone.

In this video article, we illustrate how such atomic models can be derived by cryoEM. Others have described the detailed procedures for preparing frozen hydrated samples, image acquisition and the basic steps of data processing of cryoEM [1,2]. We will focus on several criticle technical aspects important for reaching 3-4 Å resolution and for building reliable atomic models. We use the tobacco mosaic virus (TMV) and cytoplasmic polyhedrosis virus (CPV) as representative complexes with helical and icosahedral objects, respectively. *De novo* atomic models of these objects have previously been determined by cryoEM and published, and their biological relevance is described in previous publications[3-6].

Introduction:
Beginning in the early 1970s, electron microscopy has been used in structural biology to investigate macromolecular assemblies at a “biologically significant” resolution [7]. Atomic resolution protein structures determined by x-ray crystallography or NMR were docked into the lower resolution (30-8 Å) electron microscopy density maps (for example [8,9]). In this way, interactions between subunits of a large complex could be assessed to give hints at the overall function of a macromolecular assembly. While this approach is still frequently used for complexes with low or no symmetry, advances over the last twenty years in sample preparation, microscope design, data collection, and data processing have made *de novo* atomic model building, based on the EM density map alone, possible for certain types of samples [3,6].

Three broad types of sample are suitable for *de nove* atomic model building by cryoEM: 2D crystalline arrays, such as bacteriorhodopsin [10] and tubulin [11], helical assemblies, such as tobacco mosaic virus [3] and the bacterial flagellum [12], and complexes with high symmetry, such as icosahedral viruses [4-6,13] and GroEL [14]. Our laboratory has been successful in deriving several atomic models for helical assemblies and icosahedral viruses [3,5,6]. Based on past experiences we present the current article, which is intended to highlight some essential steps which allow for the electron density map to be refined to 3-4 Å resolution.

Below, we provide a working protocol with detailed steps for determining icosahedral and helical structures to about 3-Å resolution and for deriving atomic models. We also provide representative results and a discussion focusing on crucial steps in sample preparation and image processing for achieving atomic resolution structures. For in-depth understanding of the underlying theory and recent progress, viewers are advised to read recent reviews on this topic [15-17].

Protocol:
A general workflow of atomic model determination by cryoEM is given in Fig. 1. In the following protocol, items 1, 2 and 4 are shared in both single particle- and helical-structural determination. Item 3.1 is for single particle structures while item 3.2 is for helical structures. Therefore, one either will follow 1, 2, 3.1, 4 or 1, 2, 3.2, 4 depending on the nature of the sample being studied.

1) Grid preparation, sample preparation, and image collection

1.1) Place commercial cryoEM grids in a petri dish lined with several pieces of filter paper, and add 1,2-dichloro-ethane (ethylenedichloride, EDC) so that the liquid level is about one half to one centimeter. Gently push any floating grids into the liquid. Close the petri dish with a glass cover and seal with plastic film. Leave the sealed dish in a fume hood for a week, then remove from EDC and dry.

NOTE: Specific microscope settings will vary based on the type of microscope being used and the control software associated with the microscope. Results may also vary based on the microscope being used.

1.2) Coat dried grids with a fine layer of carbon by placing the grids on a flat open surface in a vacuum evaporator and establishing vacuum. Adjust a piece of graphite so it is held in the clamps above the grids and contact sparks will create fine carbon particles which coat the grids.

1.3) Layer carbon-coated grids on a closed style film image plate. Put this plate, with its projection chamber open, into the imaging plane of a 100kV electron microscope. Remove all apertures and irradiate with the smallest size beam which is sufficient to cover all the grids. Leave the grids in the microscope to “bake” for 10-50 hours. This approach removes all contaminants on the grids like oil from the oil pump of the vacuum evaporator and reduces charging of the grid during imaging.

1.4) Plunge freeze samples using an automated or manual freezing apparatus. Perform sample perpetration as demonstrated in other JoVE articles [1,2].

1.5) Prepare the electron microscope for imaging by cryo-cycling at least overnight, and load the grid(s) into the electron microscope. To do this click the “cryocyle” button the microscope user interface.

NOTE: Cryocycle a function of the microscope, which warms up the microscope and cools it down to cryogenic temperature again.

1.6) Carefully perform direct alignment of the microscope, including coma free alignment (manually, not with the software). A well aligned microscope is essential for obtaining high resolution data. Two things are especially important for a better cryoEM image: minimal coma and higher coherence of the beam. We typically check the alignment every one to two days.

1.7) Take images on films or using a direct electron detector either manually (with low dose) or with leginon automation software. Use 1.5 µm defocus for larger particles and up to 3 µm defocus for particles which are difficult to see [3,5,6].

1.8) Develop and scan the films to obtain an electronic copy of the data if appropriate or transfer electronic files to another location for data processing (See Fig. 2 for examples of good and bad data).

2. Preprocessing of images

Many of the steps in this and the following procedures require a large commitment of computer processing: transfer the files to a computer cluster if available.

2.1) If the data files are in a format other than .mrc, convert the data files to .mrc.

2.2) Determine the contrast transfer function (CTF) of each image using ctfffind3.exe [18].

2.3) Create a filtered version of the data where the particles are clearly visible. There are many types of filters available through the proc2d command in EMAN [19], use a Wiener filter (according to the ctf), a bilateral filter and a low pass filter at first, but use other filters or different filters if necessary to see the particles. Filtered images are ONLY used during pre-processing.

2.4) Examine the 2D power spectra of each image. Images that are well outside the defocus target value set while imaging (e.g., autofocus failed), that have any serious charging or drifting problem, or that are very astigmatic, can be ignored henceforth.

2.5) Examine the real space image. Any image with apparent ice contamination can be ignored henceforth.

3. Image Processing

3.1) Image Processing for Icosahedral Single Particles (Windows or Linux).

This protocol utilizes highly optimized programs in the IMIRS package[20] and consists of three main processing steps -- orientation determination, orientation refinement and 3D Fourier inversion -- executed in an iterative fashion (Fig. 3).

3.1.1) For all particles picked, run *AutoOrtElim* to generate a list of selected particles with initial orientation and center parameters using common lines.

3.1.2) Run *hrefine* program to refine the orientation and center parameters for all particles with an initial orientation estimate. The program is based on minimization of phase residuals between the particle image and a set of projection images

3.1.3) Run *batch_reconstruct_ISAF* or a GPU-accelerated 3D reconstruction program, *g3d* (Ref. [21]), to merge the Fourier transforms of all "good" particles according their refined orientation and center parameters and generate a 3D density map. Steps 3.1.1 and 3.1.2 are iterated until no further improvement in the 3D density map is obtained.

3.2 Image Processing for Helical Structures (Linux)

This protocol assumes that the reader has an in-depth understanding of EMAN software package (further than using graphic interface and default parameters). The work flow is outlined in Fig. 4.

3.2.1) Preparing segmented particles and 2D analysis

3.2.1.1) Box the particles using the helixboxer command of EMAN. The box size should be 3-4 times larger than the diameter of the particle for thin helixes and 60% larger for very thick helixes. The box size should be factorable to simple primes (e.g. 192, 256, 432, etc.)

3.2.1.2) Do not box particles or portions of particles which: are on the carbon, are on the rings at the edge of the micrograph, are off the micrograph, are obscured by contaminants, are overlapped by other particles, or have bad ice.

3.2.1.3) Create a directory to house the particles which will be boxed out and run the helixbatchboxer command.

3.2.1.4) Each particle should overlap the previous particle by one helixal turn. If the size of the helical turn is not known or too large, start with a 90% overlap.

3.2.1.5) Do phase-flipping ctf correction for each particle based on the astigmatic CTF parameters given by 2.2. Create a directory for the phase flipped particles and copy to the particles the ctf parameters which were determined in 2.2.

3.2.1.6) Create a refine2d directory, and copy the refine2d.py script to this location. Create or copy other accessory scripts which will be needed to run the refine2d.py script such as a script file (R2D.sh) for submission into the scheduler in the refine2d directory. Similarly, use script to group refinement commands too.

3.2.1.7) Create a start.hed file using lstcat.py.

3.2.1.8) Create class averages for the boxed particles. Use the start.hed file to run refine2d.py, this will result in reference free classification of all the particles. Set up the R2D.sh script to run the averaging process several times (about 20 iterations). For helices, a specially modified version of refine2d.py can be used to align the filament vertically.

3.2.1.9) Create an averaged Fourier transform of the final iteration of class averaging. Based on the resulting pattern, determine the helicity of the sample. This is traditional helical cryoEM work [22].

3.2.1.10) To determine helicity, create a grid on the FFT using the perimerdian line and periequitorial line as the defining pattern. From this grid determine n,l, and m numbers for the equation l = nt + um. Use one line in the perimerdian and one in the periequitorial to set up a system of equations and solve for the unknowns, t and u; where t is the number of turns and u is the number of subunits (i.e. u/t = subunits/turn).

3.2.2) Generate the first structure

3.2.2.1) Use the *make3d* command from *EMAN* to create a preliminary 3D density map using the classaverages with random angular assignment.

3.2.2.2) Create a symdoc.dat file based on an initial estimation of the helical parameter. This file is in *Spider* document format.

3.2.2.3) Use *himpose* of *IHRSR* package [23] to apply the helicity; then convert it back to mrc for further use in *EMAN*.

NOTE: If the helicity of the sample is not clear, several models will have to be built using different symmetry parameters and used as starting models for trial runs. The trial runs may or may not converge to the same helicity. The most plausible result will be used henceforth (plausible as judged empirically).

3.2.3) Refine the starting structure

3.2.3.1) Follow the guidelines given by the EMAN authors for refining a cryoEM structure. Between every two iterations, IHRSR programs are used to refine the helicity and the newly refined helicity is applied to the refinement result. maxshift=<pixel> (set to (100% - overlap) * boxsize / 2) is appended to the refine command-line to prevent moving the new data (100% - overlap) of a segment out of the refinement box (volume).

3.2.3.2) Use EMAN style CTF correction (option ctfc=<resolution> in EMAN refine program). To simplify the CTF determination, EMAN style ctf parameter is built directly from ctffind3.exe results. Set amplitude to 1, bfactor to 0, noise baseline to a constant 0.

3.2.3.3) Use B-factor (Nikolaus Grigorieff group) program to boost high resolution factors of the structure. A typical refinement uses a 0 B-factor in initial refinement, a ~160 1/Å^2 B-factor for further atomic resolution refinement. B-factor can be adjusted based on the microscope's performance.

3.2.3.4) Reduce hsearch range by angular increments as the structure refines.

3.2.3.5) Set resolution cutoff and gradually increase the resolution as the structure refines.

3.2.3.6) Gradually decrease angular interval for projection as prescribed by EMAN authors. Projections can be limited to between 90 and 70-80 degrees altitude to save computational load, since helical objects have very limited out-of-plane tilt in the ice. (The filament aligns to the Z direction in the volume. When projected with 90 degrees altitude, the projection image should see a perfect side view of the filament.) A Vernier based angular sampling scheme can be used to reduce the number of necessary projections [3].

NOTE: A typical refinement with 64,000 segments of 640x640 box can take 1000 cpu*days for one iteration in the last few iterations. Reduce the overlap between adjacent segments to save computational load if necessary.

3.2.3.7) When the helicity refinement converges, use the final helicity (may be average from last few iterations) to refine the structure for several more iterations with Fourier space helicizing followed by real space symmetrizing (by himpose) [3]. This approach tremendously reduces the number of projections if the helical asymmetric unit is small enough (in terms of angular span) and enables refinement with even smaller angular intervals.

4) Building the Atomic Model

First build the atomic models for cryoEM structures with Coot and then refine the model structure with CNS [24] and Phenix [25]. An example (CPV) of atomic modeling is illustrated in Fig. 5.

4.1) Start building the atomic level model before the refined structureis final. Use the preliminary atomic model to generate a theoretical radially averaged (one dimensional) structure factor for the purpose of scaling Fourier amplitudes [26]. A B-factor of 40-100 1/Å^2 can be used to sharpen the structure after scaling. Then continue to build and refine the atomic models to achieve the completed model.

4.1.1) Start the atomic model when the reconstruction maps achieve 4Å resolution: 4.5Å resolution maps can also be modeled but are more challenging (See Representative Results).

4.1.2) Trace C-alpha backbone using "baton-building" in Coot. When tracing the C-alpha, it is advisable to pay attention to the sequence as well and trying to have matching residue registration between the map and the sequence particularly at this step. This will simplify the next several steps.

NOTE: At a region of poor density, try to register the sequence before and after it. Then one will be able to more accurately trace the residues corresponding to this region.

4.1.3) Generate backbone model (poly A) with the appropriate program, typically Coot [27] or REMO [28].

4.1.4) Mutate the backbone model into full atomic model with the protein sequence using "Mutate Residue Range" in Coot.

4.1.5) Convert the cryoEM map into "hkl" pseudo-crystallographic reflections (CNS: em_map_to_hkl.inp). Before conversion, the map is typically tailored (the map is cut into a smaller volume with "Tools -> Volume Data -> Volume Viewer -> Sub-region selection" in Chimera to save memory and segmented to include the subunits of interest only).

4.1.6) Mark out the testing set (5-10%) with CNS (make_cv.inp, resulting in a .cv file).

4.2) Refine the automatically generated full atomic model with CNS against the reflections (.cv file). This time the refinement is limited to a single copy of a single protein at a time (segmentation needed). The refinement process is illustrated in Fig. 6. It starts with a crude model (Fig. 6a, green model) that is resulted from 4.1.4 above.

4.2.1) Use "vector residue" target instead of amplitude-only targets if there is reliable phase information. The refinement is more powerful when phases are also included.

4.2.2) Use simulated annealing to boil down the structure and make an initial good fit (CNS: anneal.inp). The key of not losing the fit is to lock down the C-alpha atoms with harmonic restraints. Use 20 as the constant (result in Fig. 6b, green model).

4.2.3) In Coot, manually fit any misfit residue (result in Fig. 6c, green models). (Typically, only <5% of all the residues are misfit.)

4.2.4) Refine group B-factor (CNS: bgroup.inp).

4.2.5) Minimize the structure (CNS: minimize.inp) with reduced (10) harmonic constant. Restrain both C-alpha and C-beta atoms this time.

4.2.6) Refine group B-factor again (CNS: bgroup.inp).

4.2.7) Minimize the structure again with more reduced (5) harmonic constant.

4.2.8) Refine group B-factor again (result in Fig. 6d, green model).

4.2.9) Put all refined structures of single proteins together. Use non-crystallographic symmetry (NCS) to deal with symmetry. Use either NCS constraint or restraint. When using restraint, make multiple copies of the proteins and dock them into symmetry-related copies. With the next iteration, release all harmonic restraints.

4.2.10) Manually adjust the structure if serious clashes appear after applying symmetry. These clashes typically result from long residues fitted to densities that belong to other copies. Use openmp, parallel version of CNS to reduce calculation time since refinement against an entire virus can be very time-consuming. Typical refinement of a virus takes a few days on 8 cpu cores.

4.2.11) Optionally, improve the structure by refining it in Phenix with Ramachandran restraints.

Representative Results:

The steps of cryoEM structure determination include sample purification and vitrification, low-dose imaging, orientation determination and refinement, 3D reconstruction, and atomic model building. First, samples particularly suitable for high-resolution cryoEM analysis are those with adequate size (>1MDa molecular weight, < 150 nm in thickness) such that sufficient contrast for visualization is provided, and with structural uniformity and integrity such that all particles are structurally identical for averaging. We show here an example of a good image of a suitable sample (Fig. 2c). (Notice that we intentionally selected a good image with some scanning defect – the Newton rings at the top-left corner of the image.) Second, a good cryoEM image should be without any ice contamination or drifting / charging. Figure 2a and 2b show an examples of ice-contaminated sample and an example of an image with drifting / charging, respectively. In an electron microscope, without applying any correction, one should see crystal lattices of crystal ice in the sample (Fig. 2a, top, inset), which give rise to the reflections in corresponding Fourier transforms (Fig. 2a, bottom, inset).

Although modern cryoEM instruments are capable of recording images at ~1Å resolution, when high-resolution cryoEM is concerned, the coherence of the beam plays a critical role in the resulting resolution. Therefore, we test our microscope alignment by taking test images with a CCD camera in two steps: first, the optical alignment is tested by imaging carbon film at 250,000x magnification with eight times low-dose dosage, under these conditions, the Thon rings should reach the point resolution of the microscope; second, the coherence of the beam is tested by imaging carbon film at 120,000x magnification with the working dose for cryoEM low-dose imaging, under these conditions, a single 4kx4k CCD frame should show Thon rings reaching 3Å resolution. Since the dosage is low, the Thon rings would have been hidden by noise if the signal over resolution drops off rapidly: an indication of poor beam coherence.

When reconstructed to different resolution, a cryoEM structure shows different features. Sometimes, the resolution of a structure can be judged empirically by its features, such judgments match resolution given by pseudo-crystallographic R-factors in the majority of cases. Figure 7 shows the TMV structure reconstructed (with resolution limit) to different (2.7Å, 3.3Å, 3.6Å, 4Å, 4.5Å, 5Å, 6Å, 7Å, 8Å, 10Å) resolutions. Notice that at 3.3Å resolution, side-chain features are very visible, sometimes showing difference between Val, Leu and Ile. At 3.6Å, loops are resolved, strands in β sheets are well separated and small side-chains start to blur. At 4.5Å resolution, strands become hard to resolve and only big (i.e., aromatic, arginine) side-chains can be seen. 5.5Å is the first resolution in which decent alpha-helical grooves are visible. At 6-7Å resolution, alpha-helices show up as sausages, although the size and length of them match well with that of the atomic model. At 8Å resolution, alpha-helices start to blur, and they are marginally distinguishable at 10Å resolution.

When building the model, the phase information in a cryoEM structure helps tremendously to automatically build the model, as compared to the amplitude-only data of X-ray crystallography. At 3.5Å resolution, atomic models can be built with ease. First, a c-alpha trace is built from the density map; be very sure that the amino acid registration is correct at this stage. When building loops that are not easily traced, be sure to consult structure elements before and after the loops so that there are no extra or missing residues in the loop (Fig. 6a, red). Then simply convert the tracing into a poly-alanine main-chain model and mutate the main-chain model to match the known sequence (Fig. 6a, green). Then run a simulated annealing with c-alpha atoms restrained by harmonic potentials (result: Fig. 6b, green). Fix any largely misfit residues (Fig. 6c, red to green). Then refine the atomic structure several times, gradually reducing the harmonic restraint potential until zero (result: Fig. 6d, green).

Figure Legends:

Figure 1 Workflow of *de novo* atomic model generation by cryoEM

This flowchart briefly lists the steps of atomic structure determination by cryoEM from sample preparation to final product. Light blue boxes represent sample preparation, red boxes represent imaging, yellow boxes represent micrograph processing, green boxes represent initial model building, dark blue boxes represent model refinement, and the purple box represents applications for the model.

Figure 2 Examples of problematic micrographs

(a) An image of an ice-contaminated sample. This kind of images contains diffraction contrasts. In a 300kV, high-end microscope, the lattices of the crystalline-ice areas are visible at higher magnification (inset). (b) A drifted image. The corresponding Fourier transform shows its characteristic envelope. (c) An acceptable image. Notice that we intentionally included some area with digitizing artifact for illustrative purpose (top left corner). The Fourier transform of this image has Thon rings reaching 3.5 Å resolution.

Figure 3 Single-particle reconstruction

Flow chart showing the basic steps of single-particle reconstruction implemented in the IMIRS package [17] (Protocol section 3.1 or the yellow boxes from Figure 1)

Figure 4 Helical reconstruction
Flow chart showing the basic steps of helical reconstruction using the Iterative Helical Real Space Reconstruction protocol (Protocol section 3.2 or the yellow boxes from figure 1.) Cyan boxes are typical of the IHRSR protocol. Yellow boxes mark improved steps for high resolution structure determination.

Figure 5 Atomic modeling of a cryoEM reconstruction
(a) CryoEM reconstruction of CPV. (b) Atomic models built for individual subunits. (c) Density maps with atomic models superimposed, showing good match between the features of the densities and their corresponding models. Most of the side-chain densities in this panel match well with their models.

Figure 6 Refinement of atomic models against cryoEM density maps
In each of the panels, red models stand for the result of the last step; green models stand for the result of this step; density maps are colored navy blue. (a) From Cα tracing to full atomic model. At this step, the atomic models don't fully match the densities. (b) Simulated annealing, protocol 4.2.2. (c) Manually adjust misfit models. Some models are too far away from their corresponding densities to be effectively refined into these densities. Protocol 4.2.3. (d) Final refinements. Constraints are gradually released and models gradually refine into densities. Protocol 4.2.4-8.

Figure 7 Progression of resolution during cryoEM structure refinement
CryoEM structure of TMV reconstructed to different resolutions as marked on the panels. Notice how α helices become visible around 8 Å resolution, and large individual amino acids become identifiable around 4 Å resolution.

Discussion:
With advances in imaging hardware and software, cryoEM has come of age as a structural biology method enabling *de novo* atomic models based on density maps alone. However, some major limitations persist for this technique, in particular the limitations in sample types and preparations which are suitable for use in cryoEM. Thus when considering cryoEM for structural determination, the most important question to ask is still: is this the right technique for my sample [15-17]? While X-ray crystallography and NMR remain the best choice for small, individual proteins; cryoEM has developed into an excellent tool for obtaining structural data of macromolecular complexes, both at the level of individual proteins and protein interactions within complexes. Continued advances in physics and material science, computer science and computer processing, sample preparation procedures, and the very recent direct electron detection or counting technology, make cryoEM a growing field of interdisciplinary research which will continue to push limitations and provide unique structural insights.

To get an idea of how big the complex would be or could be, the size of our CPV and TMV structures may be referred to. CPV has an alignable protein shell of nearly 30 MDa in size, although smaller viruses like Hepatitis B Virus (HBV) capsid can also be determined to atomic resolution. On the other hand, the helical structure of TMV contains a 6.5 MDa segment of the much longer helical virus. Other unpublished

structures demonstrate that one can reach the resolution necessary for a *de novo* atomic model with an alignable size as small as 4.5 MDa.

Several factors are important for reaching atomic resolution. First, the stability of the microscope plays a vital role, especially the stability of magnification. With a three condenser lens system, modern microscopes can control the parallelism of the beam very well. It was estimated that without the three condenser system, parallelism can be aligned to within 1% error; with the three condenser system, it can be aligned as well as 0.1% error. This helps to stabilize the magnification of the microscope tremendously (Wim Hagen, personal communications). However, several groups have previously achieved ~ 4Å resolution with earlier generation microscopes. Second, coma has a big negative effect on final resolution. Uncorrected residue beam tilt after coma free alignment generates considerable phase shift in images at high resolution and was considered to be the major barrier beyond 3Å resolution [29]. However, a poorly aligned beam tilt (including pivot point, a misalignment of which generates beam tilt when the beam is shifted) can be even worse. Third, the use of astigmatic CTF correction allowed us to have better defined phases in the images. This factor impacts models at about 4Å resolution. It is also interesting that it is the phases (via phase flipping) of the images that play a more critical role than the amplitudes. Proper astigmatic phase flipping with circular amplitude correction allows for a resolution sufficient for atomic modeling, whereas further application of astigmatic amplitude correction doesn't obviously improve the resolution.

There are still more issues that are not possible to enumerate in a limited space. "Full-screen anti-aliasing" of the phase flipping process gives better features on the structure. The unceasing discussion of whether to integrate all data processing steps into a well-designed software package with optimized user-interface (as IMIRS package in protocol 3) or to leave all modules of a package accessible to expert users so that individuals can tweak parameters or even software code at the cost of poor user-interface (as combined IHRSR / EMAN 1 workflow in protocol 4) has yet to be concluded. (We consider the most recent package, EMAN 2 [30], to be an acceptable balance between the two extremes, bearing a windowed user-interface with usable default parameters and a command-line interface for advanced users to customize the process.) In any case, specialized handling of sample and data requires far more than words to describe and working with a group or individual with considerable experience in *de novo* atomic model building may be essential.

Acknowledgements:
Xuekui Yu provided CPV data for analysis. We acknowledge funding from NIH (GM071940 and AI094386) and NSF (DBI-1338135), PG and NP receive funding from American Heart Association Western Affiliate (13POST17340020) and the NIH Biotechnology Training Program (T32GM067555), respectively.

Disclosures:
The authors declare no conflict of interests.

References:

1 Meng, X., Zhao, G. & Zhang, P. Structure of HIV-1 capsid assemblies by cryo-electron microscopy and iterative helical real-space reconstruction. *J Vis Exp*, doi:10.3791/3041 (2011).

2 Meyerson, J. R. *et al.* Determination of molecular structures of HIV envelope glycoproteins using cryo-electron tomography and automated sub-tomogram averaging. *J Vis Exp*, doi:10.3791/2770 (2011).

3 Ge, P. & Zhou, Z. H. Hydrogen-bonding networks and RNA bases revealed by cryo electron microscopy suggest a triggering mechanism for calcium switches. *Proc Natl Acad Sci U S A* **108**, 9637-9642, doi:10.1073/pnas.1018104108 (2011).

4 Yu, X., Ge, P., Jiang, J., Atanasov, I. & Zhou, Z. H. Atomic model of CPV reveals the mechanism used by this single-shelled virus to economically carry out functions conserved in multishelled reoviruses. *Structure* **19**, 652-661, doi:10.1016/j.str.2011.03.003 (2011).

5 Liu, H. *et al.* Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* **329**, 1038-1043 (2010).

6 Zhang, X., Jin, L., Fang, Q., Hui, W. H. & Zhou, Z. H. 3.3 A cryo-EM structure of a nonenveloped virus reveals a priming mechanism for cell entry. *Cell* **141**, 472-482 (2010).

7 Beer, M., Frank, J., Hanszen, K. J., Kellenberger, E. & Williams, R. C. The possibilities and prospects of obtaining high-resolution information (below 30 A) on biological material using the electron microscope. Some comments and reports inspired by an EMBO workshop held at Gais, Switzerland, October 1973. *Q Rev Biophys* **7**, 211-238 (1974).

8 Bharat, T. A. M. *et al.* Structure of the immature retroviral capsid at 8[thinsp]A resolution by cryo-electron microscopy. *Nature* **487**, 385-389, doi:10.1038/nature11169 (2012).

9 Li, S., Hill, C. P., Sundquist, W. I. & Finch, J. T. Image reconstructions of helical assemblies of the HIV-1 CA protein. *Nature* **407**, 409-413, doi:10.1038/35030177 (2000).

10 Kimura, Y. *et al.* Surface of bacteriorhodopsin revealed by high-resolution electron crystallography. *Nature* **389**, 206-211, doi:10.1038/38323 (1997).

11 Nogales, E., Wolf, S. G. & Downing, K. H. Structure of the alpha beta tubulin dimer by electron crystallography. *Nature* **391**, 199-203, doi:10.1038/34465 (1998).

12 Yonekura, K., Maki-Yonekura, S. & Namba, K. Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy. *Nature* **424**, 643-650, doi:10.1038/nature01830 (2003).

13 Zhang, X. *et al.* Structure of Sputnik, a virophage, at 3.5-A resolution. *Proc Natl Acad Sci U S A* **109**, 18431-18436, doi:10.1073/pnas.1211702109 (2012).

14 Ludtke, S. J. *et al.* De novo backbone trace of GroEL from single particle electron cryomicroscopy. *Structure* **16**, 441-448, doi:10.1016/j.str.2008.02.007 (2008).

15 Grigorieff, N. & Harrison, S. C. Near-atomic resolution reconstructions of icosahedral viruses from electron cryo-microscopy. *Curr Opin Struct Biol* **21**, 265-273, doi:10.1016/j.sbi.2011.01.008 (2011).

16 Zhou, Z. H. Atomic resolution cryo electron microscopy of macromolecular complexes. *Adv Protein Chem Struct Biol* **82**, 1-35, doi:10.1016/B978-0-12-386507-6.00001-4 (2011).

17 Zhou, Z. H. Structures of viral membrane proteins by high-resolution cryoEM. *Curr Opin Virol* **5**, 111-119, doi:10.1016/j.coviro.2014.04.001 (2014).

18 Mindell, J. A. & Grigorieff, N. Accurate determination of local defocus and specimen tilt in electron microscopy. *J Struct Biol* **142**, 334-347 (2003).

19 Ludtke, S. J., Baldwin, P. R. & Chiu, W. EMAN: semiautomated software for high-resolution single-particle reconstructions. *J Struct Biol* **128**, 82-97, doi:10.1006/jsbi.1999.4174 (1999).
20 Liang, Y., Ke, E. Y. & Zhou, Z. H. IMIRS: a high-resolution 3D reconstruction package integrated with a relational image database. *J Struct Biol* **137**, 292-304 (2002).
21 Zhang, X. & Zhou, Z. H. Low cost, high performance GPU computing solution for atomic resolution cryoEM single-particle reconstruction. *J Struct Biol* **172**, 400-406, doi:10.1016/j.jsb.2010.05.006 (2010).
22 De Rosier, D. J. & Klug, A. Reconstruction of Three Dimensional Structures from Electron Micrographs. *Nature* **217**, 130-134 (1968).
23 Egelman, E. H. The iterative helical real space reconstruction method: surmounting the problems posed by real polymers. *J Struct Biol* **157**, 83-94, doi:S1047-8477(06)00168-7 [pii]10.1016/j.jsb.2006.05.015 (2007).
24 Brunger, A. T. Version 1.2 of the Crystallography and NMR system. *Nat. Protocols* **2**, 2728-2733, doi:10.1038/nprot.2007.406 (2007).
25 Adams, P. D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-221, doi:10.1107/S0907444909052925 (2010).
26 Zhang, J. *et al.* Mechanism of folding chamber closure in a group II chaperonin. *Nature* **463**, 379-383, doi:10.1038/nature08701 (2010).
27 Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallographica Section D* **66**, 486-501, doi:10.1107/S0907444910007493 (2010).
28 Li, Y. & Zhang, Y. REMO: A new protocol to refine full atomic protein models from C-alpha traces by optimizing hydrogen-bonding networks. *Proteins: Structure, Function, and Bioinformatics* **76**, 665-676, doi:10.1002/prot.22380 (2009).
29 Zhang, X. & Zhou, Z. H. Limiting factors in atomic resolution cryo electron microscopy: no simple tricks. *J Struct Biol* **175**, 253-263, doi:10.1016/j.jsb.2011.05.004 (2011).
30 Tang, G. *et al.* EMAN2: an extensible image processing suite for electron microscopy. *J Struct Biol* **157**, 38-46, doi:10.1016/j.jsb.2006.05.009 (2007).

Figure 1
Click here to download Figure: JoVE FinalRev figure 1.pdf

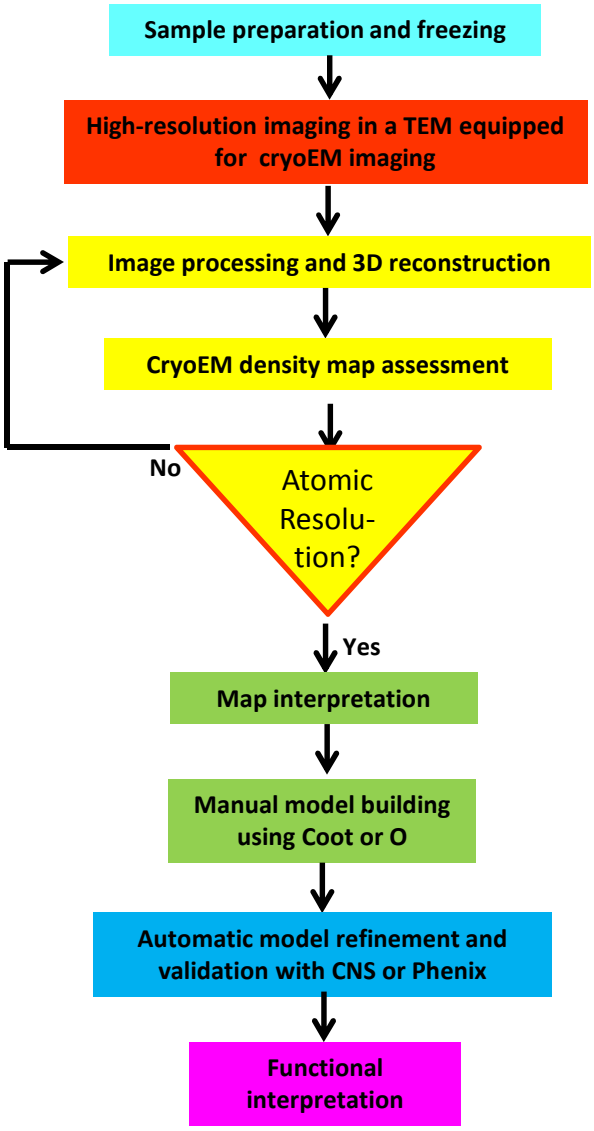


Figure 2
Click here to download Figure: JoVE FinalRev figure 2.pdf

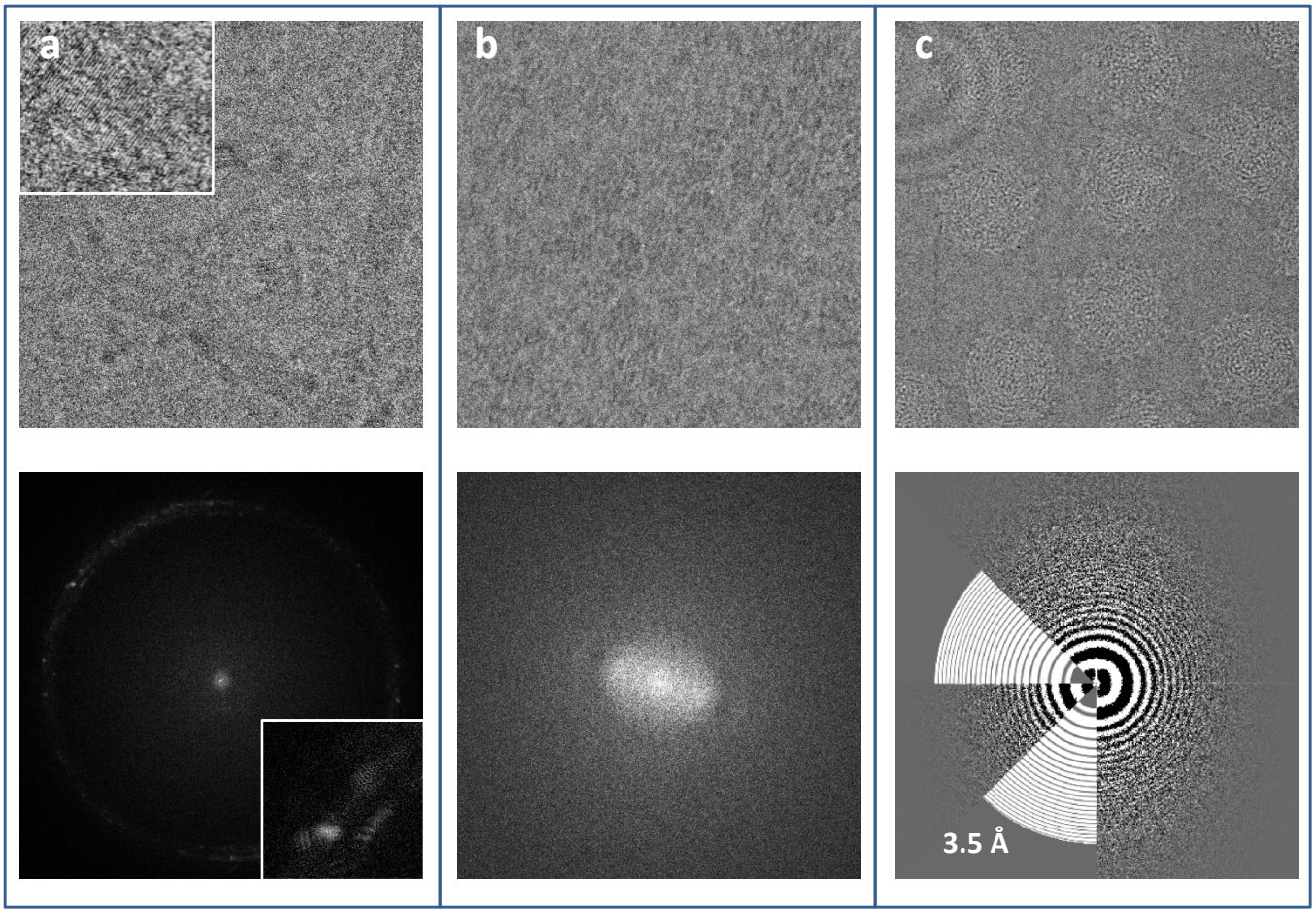


Figure 3
Click here to download Figure: JoVE figure 3 rev.pdf

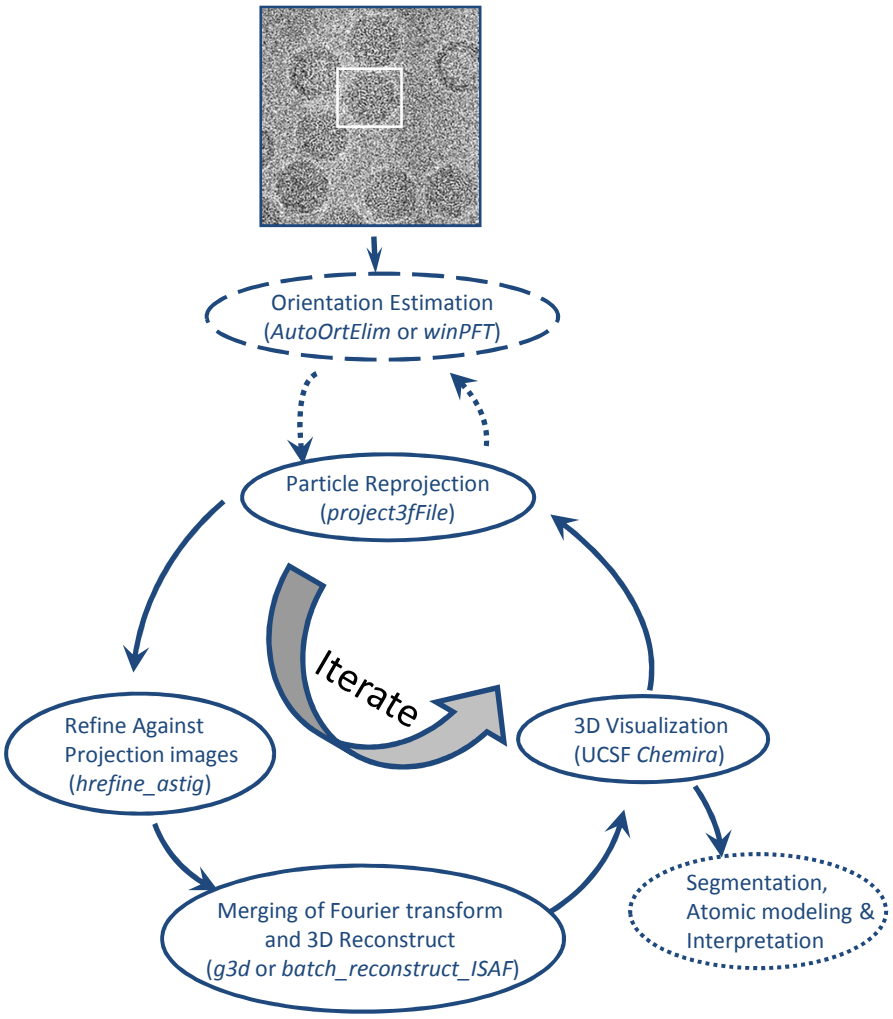


Figure 4
Click here to download Figure: JoVE figure 4 rev.pdf

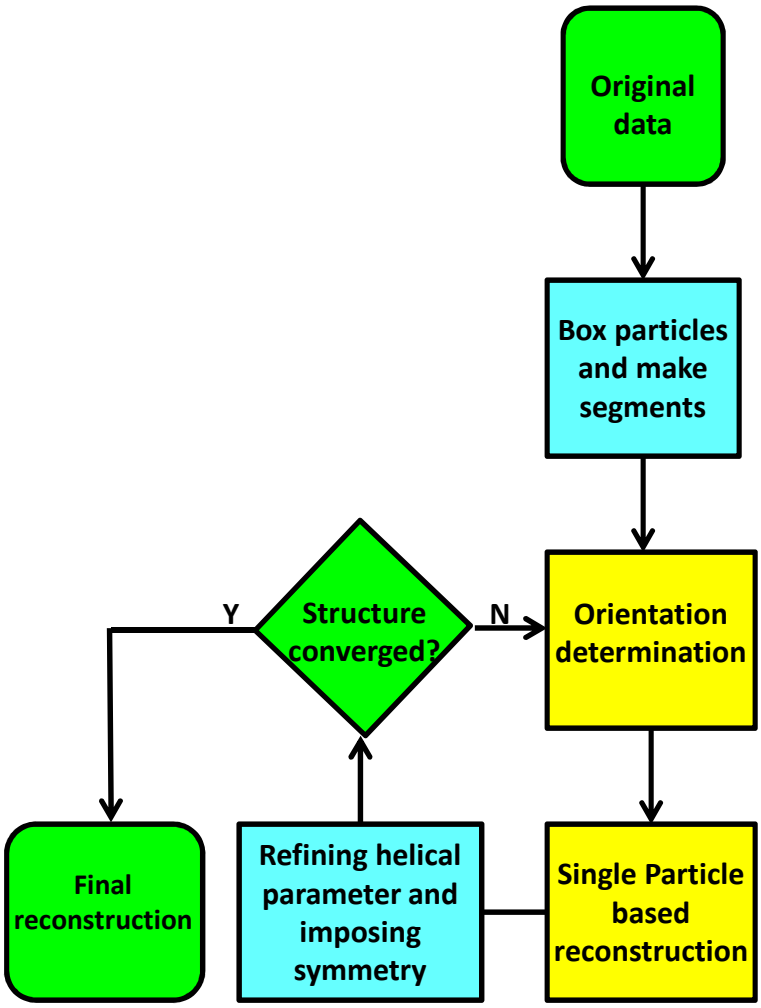


Figure 5
Click here to download Figure: JoVE figure 5 rev.pdf

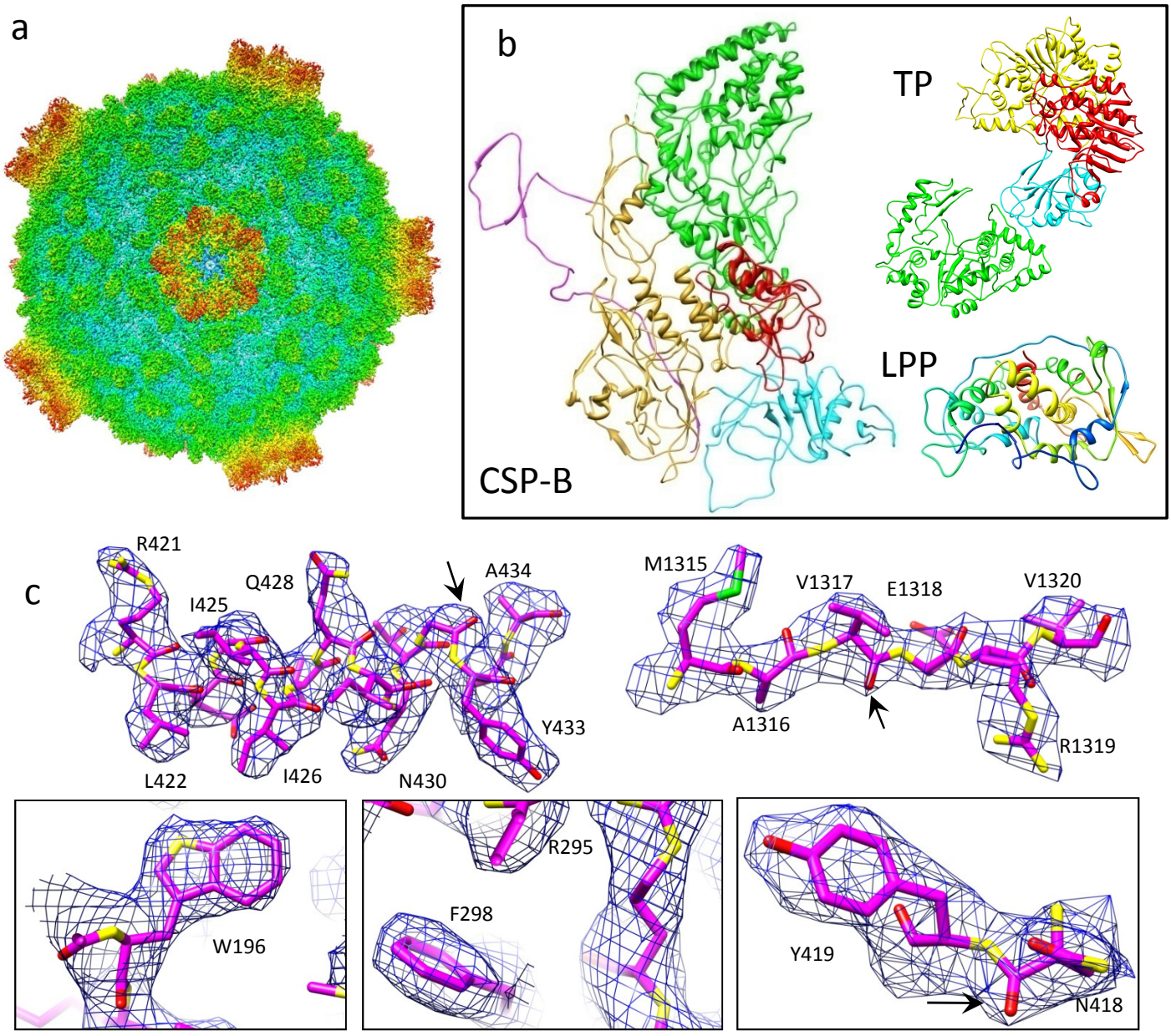


Figure 6

Click here to download Figure: JoVE FinalRev figure 6.pdf

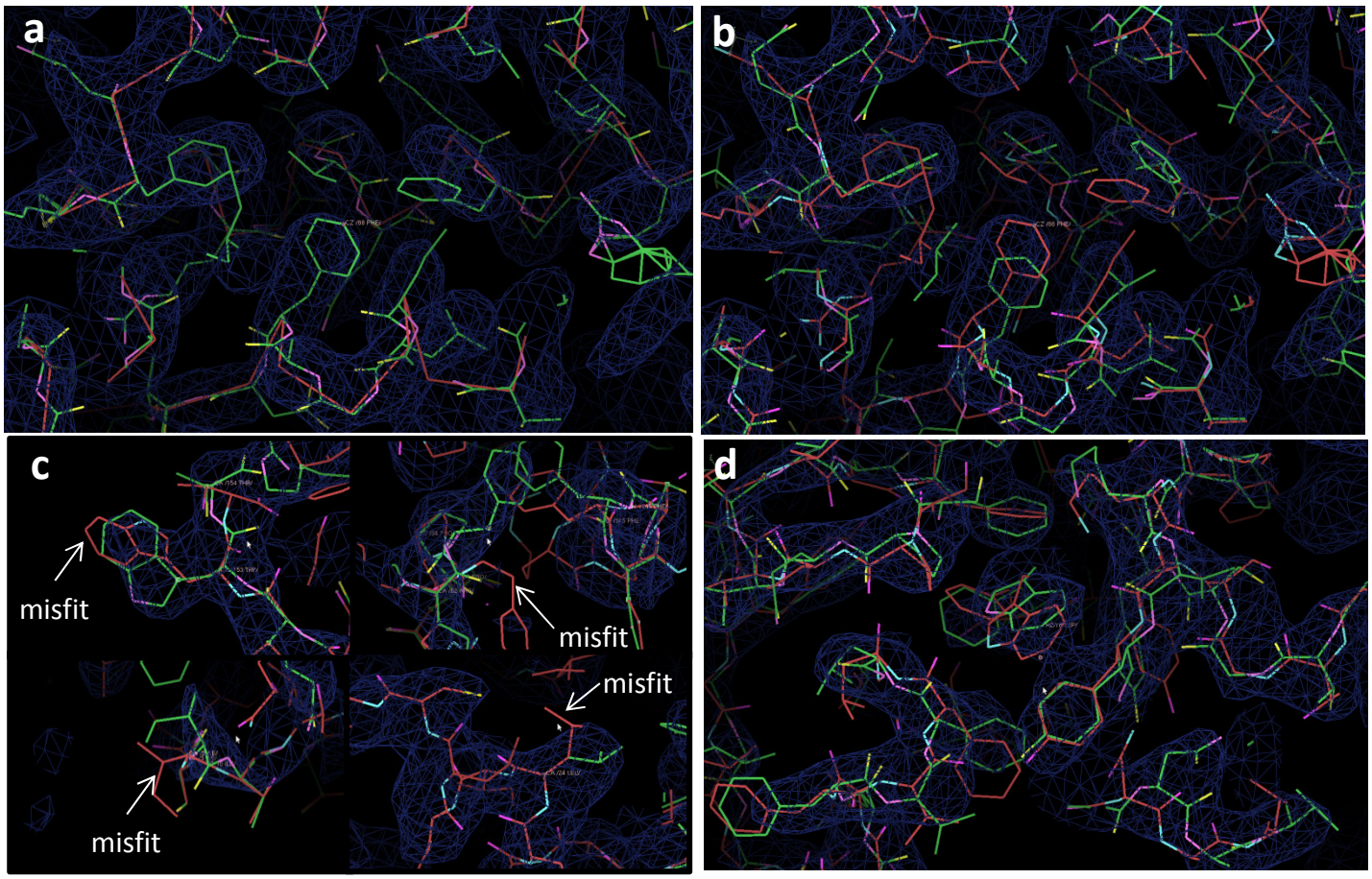
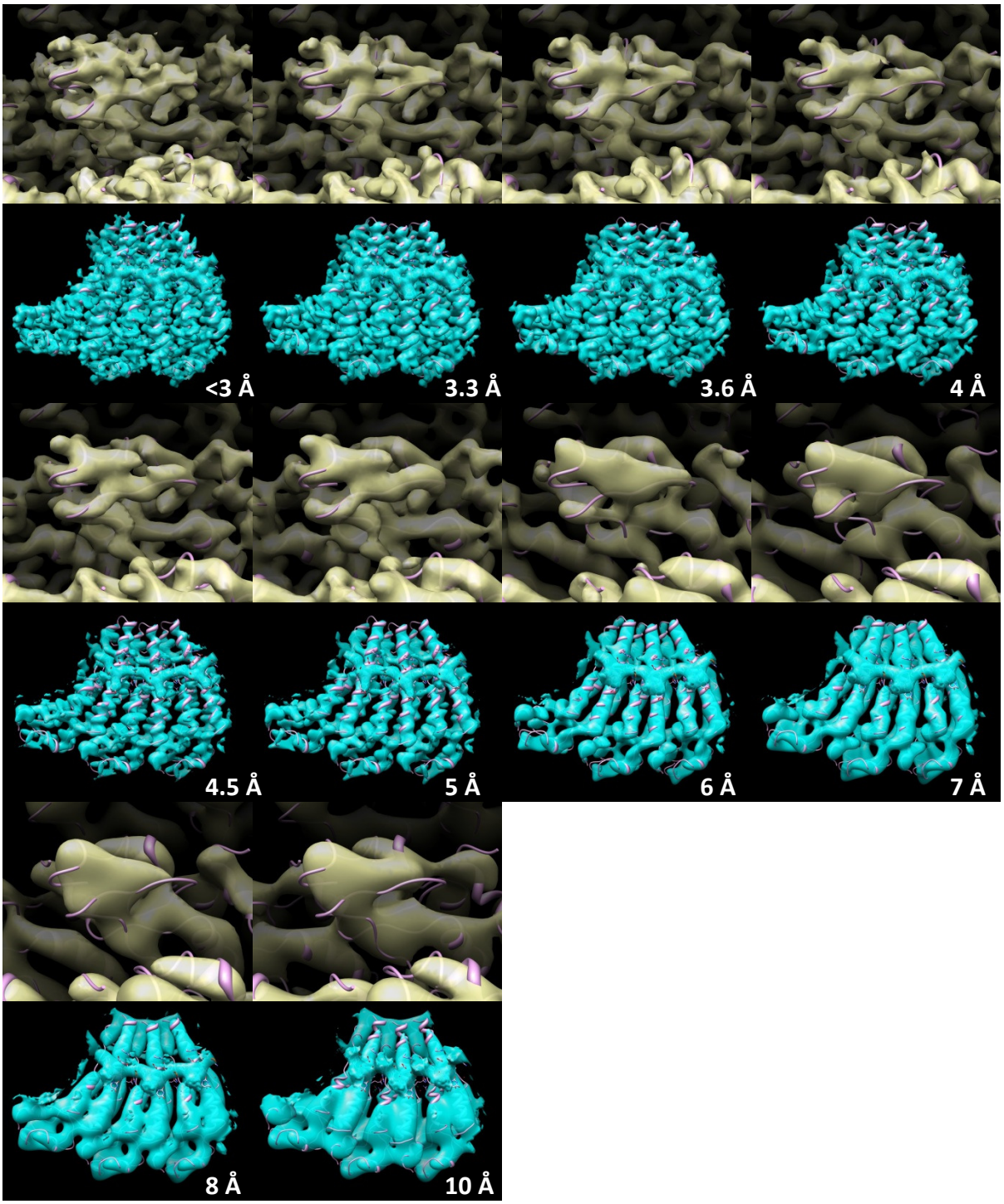


Figure 7
Click here to download Figure: JoVE figure 7 rev.pdf



Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Equipment and Reagents JOVE 8-28-14.xlsx

Name of Equipment	Company	Catalog Number	Comments
Vitrobot	FEI		
Titan Krios	FEI		
Quantifoil cryoEM grids	Quantifoil microtools GmbH		http://www.quantifoil.com/qfstr_en.php4
1,2-dichloro-ethane	Sigma-Aldrich	107-06-2	
SO163 Films and developer/fixer	Kodak		
Super CoolScan 9000 ED	Nikon		
EMAN 1,2	Baylor College of Medicine		http://blake.bcm.edu/emanwiki/EMAN/
Linux cluster with scientific linux (redhat enterprise linux)	Redhat / Fermilab		
Chimera	UCSF		http://www.cgl.ucsf.edu/chimera/
Coot			http://lmb.bioch.ox.ac.uk/coot/
CNS			http://cns-online.org/v1.3/
Phenix			http://www.phenix-online.org/

*Article & Video License Agreement
Click here to download high resolution image

jove
17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name: Hong Zhou

Department: MIMG

Institution: UCLA

Article Title: Deriving de novo atomic models by Cryo Electron Microscopy

Signature: [signature] Date: 2/19/2013

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy as a PDF to the *JoVE* submission site upon manuscript submission (preferred);
2) Fax the document to +1.866.381.2236; or
3) Mail the document to *JoVE* / Attn: *JoVE* Editorial / 17 Sellers St / Cambridge, MA 02139

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use):

*Article & Video License Agreement
Click here to download high resolution image

17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Deriving de novo atomic models by cryo electron microsc

Author(s): Peng Ge, Nicole Poweleit, Hong Zhou

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

*Article & Video License Agreement
Click here to download high resolution image

ARTICLE AND VIDEO LICENSE AGREEMENT

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

Response to Early Revisions

Click here to download Rebuttal Comments: Resonse to revisions request.docx

1. “We have recently updated our manuscript guidelines and now require between 6 and 12 key words per manuscript. Please add at least 1 more keyword.”

We added three key words, eman, iterative helical real space reconstruction, and single particle reconstruction

2. “Personal pronouns (we, you, our, etc.) should be used sparingly in most sections and not at all in the protocol section. Please reduce the use of personal pronouns throughout the manuscript. Please remove all instances of personal pronouns from the protocol.”

We removed all personal pronouns from the protocol and from the introduction and discussion sections.

3. “Please adjust the numbering of your protocol section to follow JoVE instructions for authors, 1. should be followed by 1.1) and then 1.1.1) and so on, and all steps should be lined up at the left margin with no indentations. Please use only numbers, not letters. Introductory text (such as the text you have at the very beginning of the protocol, before section I) does not need to be numbered.”

The steps of the procedure were renumbered in accordance with this request.

4. “Please add a space between each numbered step.”

Done

5. “Please update instances within the text where you refer to steps by number according to the updated numbering scheme.”

Such instances were changed to reflect the new numbering scheme.

6. “Please ensure that all steps in the protocol are written in the imperative tense (do this, mix that, etc.). For instance, "Sample preparation is performed as demonstrated in other JoVE articles." should be "Perform sample preparation as demonstrated?.."”

Changed any obvious instances of protocol steps which were not in the imperative tense.

7. “Introductory text segments and explanatory notes do not need to be written in the imperative tense. However this type of text should be used sparingly and the majority of the protocol section should contain action steps in the order they should be completed.”

Reduced explanatory notes.

8. “Your protocol currently exceeds the JoVE 3 page guideline and will be even longer when you add a space between each step. After revising your protocol as described above, please highlight (with yellow text background) less than 2.75 pages of text (including spaces between steps) to identify which portions of the protocol are most important to film; i.e. which steps should be visualized to best supplement the written section of the protocol. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader. Notes or comments that will not be filmed can be excluded from the highlighted portion. You may combine short steps that are related to shorten the protocol section. Ideally, each numbered step will contain 1-3 related actions. As a guideline, individual steps should not be longer than step 3 in section 1.”

The steps we intend to be filmed are now highlighted and correspond with screenwriter special request form.

9. “As you highlight please consider which steps will benefit most from being visualized and translate most appropriately to a video format. While a large portion of your protocol is computational, we ask that you include both experimental and computational methods in the video.”

See screenwriter special request form.

10. “For all steps that you will film, it is important that enough details are included so that a user can replicate the protocol and our script writer can plan the video properly. For instance "Dried grids are coated with carbon." How? Using what equipment / parameters? If this step is highlighted for filming please re-write it as a sequence of actions to be performed. Please ensure that all steps chosen to be filmed include enough detail for a user to properly replicate them.”

Modified some steps, including some of those we intend to film.

11. “This applies to both experimental and computational steps. Computational steps should be re-written as a sequence of distinct actions such as: open this program, select this window, click this option etc. For instance for than "Run hrefine program..." how is this done?”

See above.

12. “As you are considering the detail level of the protocol, please think about how you will demonstrate each step when you film your video, and the actions that are involved, and use these to guide the protocol text. Steps should be written with specific details for the example you will demonstrate rather than in vague or generic terms.”

Streamlined protocol to be more compliant with this request.

13. “After you have highlighted 2.75 pages of steps to film, please see the special request below. Please note that this request only applies to the steps you have highlighted. Please ensure that all desired revisions are made to the protocol section prior to completing this request.”

Done

14. “Please move the figure titles and captions from the individual figure files into the main text file. Please insert these at the end of the results section. Each figure should have a title and a brief description, and these should stand alone from the results text itself. There should not be any text in the figure files other than within the figures themselves. Please ensure that the protocol steps referred to in the figure captions use the correct numbers.”

Figure legends were removed from figures and placed in the appropriate location.

15. “If any of your figures are being reprinted from a previous work please obtain and provide the required permission. This is very important since you have requested expedited processing to be in press and not providing permission for reprints will delay processing your manuscript later on.”

No permissions need to be requested. Figures are original.

16. “JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript and use more generic terms to refer to materials and equipment. While information such as brand and company names is helpful to the user, these details should not be included in the manuscript text but should be included in the table of materials and equipment. Examples of commercial language in your manuscript include: Titan Krios microscope; software brand names (unless open source); etc.”

Removed trademarked names and described instruments in more general terms.

And 17. “In instances where the use of a specific type of material or equipment will directly affect the protocol outcome or parameters you may use the specific name when you introduce the equipment and then refer to it by more generic terms thereafter. No trademarks symbols can be published anywhere in the manuscript.”

Edited out use of trade names and applied more generic names to instruments.

Response to Reviewers
Click here to download Rebuttal Comments: Responses to Reviewers-JoVE-edits-08-28-14-NP.docx

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:// xxxxxxxxxx". 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 (LaB_6 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/A^2, 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.

Scriptwriter file
Click here to download Supplemental File (as requested by JoVE): 51436_SW.docx