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# Cryo-EM for small molecules

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## Abstract

In recent years, protein structure analysis using cryo electron microscopy(cryo-EM) has expanded and improved. In this review, we discuss many recent improvements to the field, the problems those improvements hope to solve, and some of the still unanswered questions. Most notably, this review will discuss improvements in resolving small or fragmented protein structures, as well as methods to improve the signal to noise ratio of the data by increasing the contrast of the image using carbon-based systems. We will also describe how, in the last 5 years, methods improvements have allowed for better 3D image resolution by capturing a continuum of 3D images. We will provide examples of these methods in practice and discuss how these improved methods may be used in small molecule drug discovery and development.

### Keywords

cryo electron microscopy; cryo-EM; Protein biochemistry; Structural variability analysis

## Introduction

The so-called "EM revolution" has been very active in recent years. New and old tricks to enhance structural information include improved sample prep, imaging, and new data processing/analysis programs. While keeping more particles shows small sacrifices in resolution, it also helps decipher additional details that show up in single particle analysis. However, these details may be absent in the final reconstruction. New particle variance analysis enables the determination of drug activity on macromolecular structure without seeing the drug in the final reconstruction. Such new analyses have paved the way for small molecule drug investigation via cryo-EM. Macromolecular structures, including membrane proteins, previously determined by x-ray crystallography(McNulty, Ulmschneider, Luecke, & Ulmschneider, 2013; Strugatsky et al., 2013) are incorporating new information found in cryo-EM reconstructions(Cui et al., 2019). Herein, we discuss aids to resolve proteins with higher resolution while concurrently characterizing heterogeneity in cryo-EM. We explore some well-known procedures such as, increasing protein size, increasing contrast, focused classifications, and methods of negative stain. We also explore recent developments

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in the past 5 years which exploit deviations in protein subunits that enable quantification of variability in 3D structure. After discussion of specific recent and traditional methods to analyze EM data, we provide a procedure and example for cryo-EM processing of mouse apoferritin images. We begin with summarizing traditional and recent tricks as follows.

## Cryo-EM Overview

Macromolecule imaging in cryo-EM requires very homogeneous samples. Upon purification, samples are typically first screened in negative stain to assess homogeneity and sample concentration. Typically, cryo-EM concentrations are screened around ten times the concentration for which it appears well distributed in negative stain. This is because the blotting process used in cryo-EM sample prep removes most of the protein. Purified protein is applied to a glow-discharged grid to create a hydrophobic environment such that protein will be attracted to the surface. Upon blotting the grid from both sides with filter paper to remove excess buffer, the grid is plunged into liquid ethane. This causes the protein suspended in buffer to freeze much faster than water hexagonal ice crystals can form, resulting in the desired vitreous ice. Grids are stored in liquid nitrogen dewars until ready for image acquisition.

Images are typically acquired several frames per second using a direct detector camera, which allow per pixel quantifications of electron dose. Since the electron beam causes particles to move during image acquisition, the frames may be aligned for the entire micrograph and/or per particle to enhance resolution. Data processing on the aligned micrographs may be done using a single program which include CryoSPARC(Punjani, Rubinstein, Fleet, & Brubaker, 2017), RELION(Scheres, 2012), FREALIGN(Lyumkis, Brilot, Theobald, & Grigorieff, 2013), EMAN2(Tang et al., 2007) or a suite of programs such as those included in APPION(Lander et al., 2009). Aligned micrographs are corrected for contrast transfer function (CTF). Particles may be picked in an automated fashion using a difference of gaussian picker(Voss, Yoshioka, Radermacher, Potter, & Carragher, 2009) which can distinguish the signal of noise (vitreous ice) to the macromolecule. If the automated picker is unsuccessful, particles should be picked manually. Boxed particles are then aligned land classified into several 2D -class averages. This involves rotating 2D particles and grouping those with similar features and orientations. In Fourier space, these 2D images will have a common plane if they are from the same object. Programs will search for these common planes or lines and arrange them in 3D. The final reconstruction is viewed in real space.

Although a 3D reconstruction can be generated from negative stain data, the analysis will not always work well because of the low-resolution limits of negative stain. For example, if a low-pass filter of 25 angstroms is applied to apoferritin, the 24-mer oligomer (around 500 kDa) will look like a featureless ball. Thus, crisp 2D class averages or 3D reconstructions would not be expected from apoferritin negative stain data. For such a protein, the solution is cryo-EM. Cryo-EM is also used when a resolution sub-nanometer or better is needed to answer the research question.



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## Adding monoclonal antibodies to proteins to increase size while elucidating structure

Using cryo-EM to identify epitopes and their structures provides information for pharmaceutical drug development. Many virus epitopes, such as those in flaviviruses, are relatively conserved in their structure, therefore increasing the significance of cryo-EM imaging (Tyagi, Ahmed, Shi, & Bhushan, 2020). In addition to understanding the mechanism of broadly neutralizing antibody action, the molecular determinants of antibody recognition can be used to design better therapeutic neutralizers and help facilitate 3D structure determination. Alignment algorithms used in data processing perform rotation of 2D images to align particles in similar projections based on the overall shape. Proteins that are small and have limited number of features have poor alignment scores, and therefore produce very low resolution structures. The use of antibodies to aid in structure determination is not new and has been used with great success (Lyumkis, Julien, et al., 2013; Sauer et al., 2021). However, because of the way the alignment programs work, a monoclonal antibody must be used since it binds to a single region of a protein (unlike a polyclonal antibody which can bind to several regions). Moreover, since antibodies have a flexible region that will vary in space from particle to particle, only the Fab portion of the antibody should be used for cryo-EM structure determination. These Fab's will bulk up the protein and aid in alignment of particles, because they will add around 50 kDa to the protein (Kim et al., 2019). In addition to locating protein domains and understanding protein-antibody interactions, this strategy can give smaller proteins a better chance at having their structures determined. It is even more powerful if the protein is capable of dimerizing or forming other higher symmetry structures which will provide increased redundancy and enhance resolution.

## Adding large imaging scaffolds to small proteins

One challenge has been resolving structures of proteins under 50 kDa. A motivation for this work stems from the fact that many proteins are less than 50 kDa, with bacterial proteins averaging 30 kDa, and eukaryotic proteins averaging 45 kDa. A large point of failure in resolving lower atomic weight proteins using cryo-EM pertains to low signal to noise of precluding images, making the later 3D images difficult to establish. Recent work has been done to try and improve imaging of these smaller proteins (Yeates, Agdanowski, & Liu, 2020). Some methods have to do with altering the protein itself optimize size, purity, abundance, and homogeneity. One example involves complexing antibodies to a specific part of an ambiguous protein structure to help improve resolution and orientation. However, the methodology itself could be improved, instead of the target. The specific method of interest here pertains to engineering smaller molecules to become part of a large imagining scaffold for improved visualization using cryo-EM. A method for improving the resolution may be attaching such "cargo" (smaller proteins) to a "scaffold" (larger well classified imaging target). Creating a macromolecule scaffold also allows for constructed symmetry of the sample, allowing for higher image resolution with fewer particles, and the avoidance of preferred particle orientation of the EM grid.

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## Increasing contrast with thin carbon

Graphene and graphene oxide have been used to enhance the contrast of biological molecules in cryo-EM, especially for small proteins. Graphene and its derivative provide a single layer crystalline lattice that minimizes noise from electron scattering upon data collection, unlike that of amorphous carbon supports which is often detrimental to visualization of small proteins. This support also has much higher conductivity than that of amorphous carbon, decreasing movement and charge interference (Pantelic, Meyer, Kaiser, Baumeister, & Plitzko, 2010). Cryo-EM freezes the protein in vitreous ice within the holes of the grid, minimizing contact with the air-water interface. This is often a challenge with small proteins that adhere to the carbon support grid, resulting in radiation damage and their denaturation. Graphene carbon can be emitted from a rod at high temperature to coat thin layers of carbon on mica support. The mica-containing graphene is cut into small pieces and placed into deionized water to float the thin layer of carbon off the mica support. Then a grid containing holes is used to scoop up the floating carbon. These grids are airdried and subsequently glow-discharged and are ready for sample application for cryo-EM imaging. This technique requires a lower concentration of sample protein than that of traditional cryo-EM imaging methods.(Palovcak et al., 2018) Use of graphene and its lower oxygen species indicates that the thickness of the vitreous ice increases significantly while maintaining an even layer of the sample. This decreases radiation damage of the protein when in contact with the air-water interface. The ability to manipulate the hydrophilicity of the grids is also a crucial factor needed for the dispersion of various proteins(Wu, Wu, Zhu, & Wu, 2021).

## Negative stain before cryo-EM

Since the signal to noise (S/N) ratio is often low when imaging vitrified protein, it can be useful to add a negative stain to visualize the overall architecture of the protein complex(McNulty et al., 2015). Negative stain solutions are heavy metal salts that form a shell around the protein. This makes the protein appear white in a background of dark stain. Acquiring sample images will show if enough particles are concentrated in the field of view at the magnification required for imaging, how homogeneous the sample is, and if buffer components need to be optimized before attempting structure determination(Gewering, Januliene, Ries, & Moeller, 2018). It is generally better to optimize these components at the bench instead of troubleshooting particle image alignment and classification. Transmission electron microscopy imaging in the presence of negative stain limits the overall resolution of visible information to around 20–30 angstroms. Because of the heavy metal coverage, the protein is often flattened and shows no high-resolution data. Nonetheless, this resolution is sufficient to generate initial models via Random Conical Tilt (RCT). This density is often low-pass filtered in cryo-EM structure determination. Negative stain reconstructions can also be useful for investigating domain interactions, arrangement of protomers, and macromolecular complex stoichiometry, and dynamics.

## Focused classifications

Since alignment programs use features of the entire particle to produce class averages, information can be lost or blurred when domains are moving relative to the rest of the complex. Use of class averages that are more variable lower the overall resolution of the

desired protein. To visualize the dynamics of specific protein complex regions, software can be used to create a soft mask around the region of interest. This area is then cropped out of the particles and analyzed via 2D or 3D classification(C. Zhang et al., 2019). Particles in a similar dynamic state are classified together. Analysis of the different masked class averages allows movies to be made, showing the dynamics of protein domains. Moreover, if a subset of the sample has a bound and unbound states, this percentage can be identified by the masking procedure.

## From 3D classification to variability – multi-body refinement

It is becoming increasingly popular to produce a continuum of cryo-EM reconstructions instead of solely one 3D structure. Previously, portions of macromolecular structures with intrinsically disordered regions, or simply unstructured regions, would either have poor resolution or be completely absent in the reconstruction. These unmapped regions of heterogeneous samples are important to the structure and interactions of the protein. Lacking this information limits development in targeting these variable regions. However, in the last 5 years strategies exist to analyze the entire 3D structure, which includes stable high-resolution regions along with dynamic and poor resolution components. Multi-body refinement is a combination of focused classification and signal subtraction,

The human spliceosome is a perfect example of this phenomenon. Reported resolutions vary significantly throughout the structure of the whole spliceosome. The spliceosome catalytic core consisting of U2/U6 RNP is relatively stable. However, the periphery components are highly dynamic in nature and typically produce lower-resolution reconstructions. The spliceosome maturation process is such that components need to add to the complex followed by disassociation and combination of new components. Macromolecular structures with such a dynamic assembly mechanism are possibly required to ensure productive complex formation and functionality. In the case of the spliceosome, an approach has been developed that combines iterative 3D classification with a newly developed procedure that uses principal component analysis (PCA)(Haselbach et al., 2018). The procedure involves collecting a large data set consisting of 2.2 million particle images to produce over 100 spliceosome structures, which become ordered according to conformational similarities post applying PCA. This allows identity of dynamic patterns of flexible components that cover a conformational continuum with major and minor states. The particle numbers for each observed state allows the conformational landscape to be converted to a Boltzmann energy landscape, which quantitatively describes the energy barriers between conformational states. Thus, the 3D classification strategy can be used to produce several reconstructions that have multiple components, assembled, disassembled, or the reconstructions may vary in relative position of subunits. Therefore, regardless of the reason, all the reconstructions can have their energy landscape mapped to the structure via 3D PCA. If ATP/GTP is not included in the sample, then the energy barriers for each conformation change can be overcome with available thermal energy. Such inherent thermal energy can produce spliceosome rotations up to 40 degrees and translations up to several hundred angstroms mapped over 8 reconstructions at 3.4 Å resolution.



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## Analyzing virus dynamics with cryo-EM

Cryo-EM has been used to capture viruses in never-before-seen states that include ejecting genome from viral heads(McNulty et al., 2018). Another recent method using cryo-EM to look at macromolecular dynamics has been done with NωV virus. The virus undergoes an autocleavage event which is required to convert from a procapsid to a mature capsid. The procapsid is stable at neutral pH 7.6, but exposure to pH 5 causes cleavage of the gamma peptide (residues 571–644) and virus compacts yielding the mature icosahedral virion with the gamma peptide non-covalently associated with the mature capsid. The virus has a single protein to make up the coat but has T=4 quasi symmetry. The A-subunits form the 5-fold while the B, C, and D subunits form pleated hexagons. Thus, there are different interactions for coat monomers that make up the 5-fold vertices vs. hexagonal subunits(Doerschuk, Gong, Xu, Domitrovic, & Johnson, 2016). An analysis of the particle variance pixel by pixel that is contributing the reconstruction allows the dynamic properties of each subunit to be quantified. Thus, the time course of the cleavage event can be mapped to the 3D structure by looking at the 2D pixel variance. In this example, A and D subunits stabilize quickly while B and C subunits take longer to get into position as evidenced by their higher standard deviation at earlier timepoints. Even if a dynamic event is so fast that it is on average invisible, the event can be captured and described by creating a variance map of the electron density.

## Elucidating dynamic protein structures

Cryo-EM has been used to image heterodimeric ABC transporters in human cell lines. These transporters have two nucleotide binding domains that participate in ATP hydrolysis and ATP binding, which are necessary for movement across a membrane. These complexes are either in a nucleotide bound (IF) or nucleotide free ($OF^{open}$) state. Prior to cryo-EM, this dynamic protein had images for only its bound state and lacked structure determination of its inactive unbound form. TmrAB was reconstituted into lipid nanodisks and imaged in both states. IF is either in a narrow-bound state or a wide state, depending on the location of transmembrane helix 6 (TM6). Cryo-EM imaging indicates that these states are in dynamic equilibrium and are hard to isolate, due to the free exchange of ATP and ADP. Nuclear binding domains are dimerized upon ATP binding, opening the extracellular gate, permitting the release of substrate out of the cell. Upon release of the substrate, TmrAB will change from $OF^{open}$ and $OF^{occluded}$ conformation until inorganic phosphate is released from the catalytic site(Hofmann et al., 2019). Structural determination of the bound and unbound states is crucial to understanding how to target either conformation in drug design or further research.

## Mapping variable protein functional states by pooling particle datasets

Protein degradation by the proteosome is at the same time highly conserved and poorly understood on many levels. A large regulator of this process is ubiquitin-specific protease 14 (USP14), which allows the proteosome to interact and act on substrates. However, the structural activity and mechanism of interaction of this protease with the proteosome remains unknown. That is until a recent study(S. Zhang et al., 2022) was able to characterize the different interactions and intermediate confirmations of a USP14-proteosome complex

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by using a time resolved confirmational continuum. Nowadays it is almost standard for every complex to be submitted with several maps of the varying functional states.

## Small molecule drug development and cryo-EM

Cryo-EM can also be an effective tool when elucidating protein structural changes caused by small molecule drug candidates. In a recent study (Raisch et al., 2021), they discuss how insect specific binding pockets (and thereby activation) of a slowpoke potassium channels (Slo) may display variability in this otherwise conserved domain. The Slo channels are activated by high concentrations of intracellular $Ca^{2+}$, and in specific cases such as in neuronal or muscle tissue, must be coupled to $K^{+}$ ion concentration. In this study, four different cryo-EM structures of this channel are reported, depending on if they are $Ca^{2+}$ bound or not, and if they were in complex with specific ligands (verruculogen and emodepside). $Ca^{2+}$ free samples were simply prepared by incubating the Slo protein in EDTA on ice for 15 minutes before grid preparation. Calcium-bound samples were prepared by incubating Slo with Aflatrem on ice for 3 hours. For each of the ligand bound complexes, Slo was incubated with each ligand at 30 uM for 3 hours on ice. Importantly, cryo-EM analysis has not only characterized insect specific binding pockets, but also specific and different activation of this channel depending on the ligand and binding pocket. Using heterogeneously expressed Slo protein, the $Ca^{2+}$ bound and the $Ca^{2+}$ free confirmations could be studied. Potential binding pockets were founding using the BiteNet software, which uses a neural network to analyze protein structure. Such analysis may allow for the identification of drug binding pockets. Both reconstructions were achieved at over 2.4 Å, allowing for visualization down to the side chain level. One specific binding domain, called the RCK2 pocket, is structurally dependent of $Ca^{2+}$ causing confirmational changes of the gating ring. Analysis of the Slo ligands verruculogen and emodepside was also performed. The test substances used were tremorgenic mycotoxin verruculogen and the anthelmintic drug emodepside. Verruculogen is known to strongly inhibit Slo, and emodepside desensitizes Slo to $Ca^{2+}$ regulation, specifically in motor neurons, causing non-regulated $K^{+}$ ion conductive behavior. Both cryo-EM structures bound to a ligand and $Ca^{2+}$ were resolved at over 2.5 Å. By understanding more about how small molecules contribute the protein restructuring may allow for, in this case, better species-specific insecticide compounds, and, more broadly, novel small molecule drug development.

## Protein fragment analysis and fragment screening

High resolution mining for small molecule drug discovery may be performed using fragment-based drug discovery (FBDD) or fragment screening in combination with cryo-EM. Upfront preparation still needs to be optimized for fragment screening to work with cryo-EM, but recent research shows the ability of cryo-EM to produce high resolution structures of protein complexes and screening across cryo-EM specific fragment libraries. Examples of cryo-EM resolving particles/ligands at the fragment level are displayed in the study(Saur et al., 2020) Three fragment sized ligands for *Escherichia coli* Bgal (L-ribose, 1-deoxy-galactonojirimycin (DGN), and phenylethyl β-D-thiogalactopyranoside (PETG)) are known to bind at different binding sites and cause different confirmational changes of the loop. Upon determination with cryo-EM, all three of these ligands in complex with Bgal were able to resolve at a resolution of ~2.3 Å and, when compared against previous X-ray

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structures, appeared correct. The confirmational changes caused by the complexation of these ligands to Bgal could also be observed, specifically the rotation of side chain Phe601 caused by L-ribose and PETG, and and a larger confirmational change across almost 20 loop residues. Upon comparison to previous X-ray structures, these observations appear to be correct. Using the analysis software webcryo greatly improves sample processing and the utility of cryo-EM for this type of fragment analysis and fragment screening. Analysis of the oncological target PKM2 was used to qualify the use of cryo-EM in fragment screening. The M2 isoform of this protein kinase, which is upregulated in many cancer cells, causes the final step of glycolysis. 68 fragments were included in this initial fragment screen with a goal of having data processed in 2–3 days. One example, L-threonine complexed with PKM2, a small nonplanar complex, was able to resolve at over 2.5 Å using cryo-EM, where Cmp5 co-complexed with PKM2 at 3.2 Å, and was still able to be used to determine the binding of the ligand, even though resolution was worse than 3 Å. In both examples, key interactions between residues and these ligands were also discovered. Overall, this study highlights the possibility for cryo-EM to structurally evaluate proteins at the fragment level, and the possibility to use cryo-EM as a screening tool for fragment sized small molecule drug discovery.

### Exploring protein variability using cryoSPARC

Dynamic flexibility and variability can also be found in proteins thought to be very stable. For example, the T20S proteosome structure, which contains 14-fold D7 symmetry is large and considered to be a rock that makes a good benchmark protein for cryo-EM studies(Campbell, Veesler, Cheng, Potter, & Carragher, 2015). This data was reanalyzed from 84,605 particle images processed in *cryoSPARC*. As these particles were duplicated around the 14-fold access, 1,184,470 particles were used for 3D variability analysis. The 3DVA process in *cyroSPARC* allows for direct deciphering molecular movements, flexibility, and heterogeneity. Although the T20S protein is relatively stable, *cryoSPARC* was able to detect five types of continuous bending and flexibility within the molecule. This included a stretching-compression movement for the top and bottom regions of the proteosome barrel, a rotational twist of the top and bottom barrel regions in opposite directions, and non-symmetrical (asymmetric) bending of the whole barrel in two different directions. The authors also reported similar striking new finds in the 80S ribosome, $Na_v1.7$ sodium channel, and the spliceosome.

## Concluding Remarks

In summary, there are numerous tools to enhance the amount of information one can distill from single particle EM images. Low resolution reconstructions caused by flexibility or dynamic equilibrium were previously undesirable and the sample was typically optimized to yield a homogenous sample. However, many are starting to realize that intrinsic flexibility and heterogeneity can be biologically relevant. So, it is equally important and more informing to describe the ensemble of structures when this is the true physiological form of a macromolecular complex than it is to focus on producing a single reconstruction that is an average of multiple structures.

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## Literature Cited

Campbell MG, Veesler D, Cheng A, Potter CS, & Carragher B (2015). 2.8 A resolution reconstruction of the Thermoplasma acidophilum 20S proteasome using cryo-electron microscopy. Elife, 4. doi:10.7554/eLife.06380

Cui Y, Zhou K, Strugatsky D, Wen Y, Sachs G, Zhou ZH, & Munson K (2019). pH-dependent gating mechanism of the Helicobacter pylori urea channel revealed by cryo-EM. Sci Adv, 5(3), eaav8423. doi:10.1126/sciadv.aav8423 [PubMed: 30906870]

Doerschuk PC, Gong Y, Xu N, Domitrovic T, & Johnson JE (2016). Virus particle dynamics derived from CryoEM studies. Current opinion in virology, 18, 57–63. doi:10.1016/j.coviro.2016.02.011 [PubMed: 27085980]

Gewering T, Januliene D, Ries AB, & Moeller A (2018). Know your detergents: A case study on detergent background in negative stain electron microscopy. Journal of Structural Biology, 203(3), 242–246. doi:10.1016/j.jsb.2018.05.008 [PubMed: 29852220]

Haselbach D, Komarov I, Agafonov DE, Hartmuth K, Graf B, Dybkov O, … Stark H (2018). Structure and Conformational Dynamics of the Human Spliceosomal Bact Complex. Cell, 172(3), 454–464.e411. doi:10.1016/j.cell.2018.01.010 [PubMed: 29361316]

Hofmann S, Januliene D, Mehdipour AR, Thomas C, Stefan E, Brüchert S, … Moeller A (2019). Conformation space of a heterodimeric ABC exporter under turnover conditions. Nature (London), 571(7766), 580–583. doi:10.1038/s41586-019-1391-0 [PubMed: 31316210]

Kim JW, Kim S, Lee H, Cho G, Kim SC, Lee H, … Lee J-O (2019). Application of antihelix antibodies in protein structure determination. Proceedings of the National Academy of Sciences - PNAS, 116(36), 17786–17791. doi:10.1073/pnas.1910080116

Lander GC, Stagg SM, Voss NR, Cheng A, Fellmann D, Pulokas J, … Carragher B (2009). Appion: an integrated, database-driven pipeline to facilitate EM image processing. J Struct Biol, 166(1), 95–102. doi:10.1016/j.jsb.2009.01.002 [PubMed: 19263523]

Lyumkis D, Brilot AF, Theobald DL, & Grigorieff N (2013). Likelihood-based classification of cryo-EM images using FREALIGN. J Struct Biol, 183(3), 377–388. doi:10.1016/j.jsb.2013.07.005 [PubMed: 23872434]

Lyumkis D, Julien JP, de Val N, Cupo A, Potter CS, Klasse PJ, … Ward AB (2013). Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. Science, 342(6165), 1484–1490. doi:10.1126/science.1245627 [PubMed: 24179160]

McNulty R, Cardone G, Gilcrease EB, Baker TS, Casjens SR, & Johnson JE (2018). Cryo-EM Elucidation of the Structure of Bacteriophage P22 Virions after Genome Release. Biophys J, 114(6), 1295–1301. doi:10.1016/j.bpj.2018.01.026 [PubMed: 29590587]

McNulty R, Lokareddy RK, Roy A, Yang Y, Lander GC, Heck AJR, … Cingolani G (2015). Architecture of the Complex Formed by Large and Small Terminase Subunits from Bacteriophage P22. J Mol Biol, 427(20), 3285–3299. doi:10.1016/j.jmb.2015.08.013 [PubMed: 26301600]

McNulty R, Ulmschneider JP, Luecke H, & Ulmschneider MB (2013). Mechanisms of molecular transport through the urea channel of Helicobacter pylori. Nat Commun, 4, 2900. doi:10.1038/ncomms3900 [PubMed: 24305683]

Palovcak E, Wang F, Zheng SQ, Yu Z, Li S, Betegon M, … Cheng Y (2018). A simple and robust procedure for preparing graphene-oxide cryo-EM grids. Journal of Structural Biology, 204(1), 80–84. doi:10.1016/j.jsb.2018.07.007 [PubMed: 30017701]

Pantelic RS, Meyer JC, Kaiser U, Baumeister W, & Plitzko JM (2010). Graphene oxide: A substrate for optimizing preparations of frozen-hydrated samples. Journal of Structural Biology, 170(1), 152–156. doi:10.1016/j.jsb.2009.12.020 [PubMed: 20035878]

Punjani A, Rubinstein JL, Fleet DJ, & Brubaker MA (2017). cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. Nat Methods, 14(3), 290–296. doi:10.1038/nmeth.4169 [PubMed: 28165473]

Raisch T, Brockmann A, Ebbinghaus-Kintscher U, Freigang J, Gutbrod O, Kubicek J, … Raunser S (2021). Small molecule modulation of the Drosophila Slo channel elucidated by cryo-EM. Nat Commun, 12(1), 7164. doi:10.1038/s41467-021-27435-w [PubMed: 34887422]

Sauer MM, Tortorici MA, Park YJ, Walls AC, Homad L, Acton OJ, … Veesler D (2021). Structural basis for broad coronavirus neutralization. Nat Struct Mol Biol, 28(6), 478–486. doi:10.1038/s41594-021-00596-4 [PubMed: 33981021]

Saur M, Hartshorn MJ, Dong J, Reeks J, Bunkoczi G, Jhoti H, & Williams PA (2020). Fragment-based drug discovery using cryo-EM. Drug Discov Today, 25(3), 485–490. doi:10.1016/j.drudis.2019.12.006 [PubMed: 31877353]

Scheres SH (2012). RELION: implementation of a Bayesian approach to cryo-EM structure determination. J Struct Biol, 180(3), 519–530. doi:10.1016/j.jsb.2012.09.006 [PubMed: 23000701]

Strugatsky D, McNulty R, Munson K, Chen CK, Soltis SM, Sachs G, & Luecke H (2013). Structure of the proton-gated urea channel from the gastric pathogen Helicobacter pylori. Nature, 493(7431), 255–258. doi:10.1038/nature11684 [PubMed: 23222544]

Tang G, Peng L, Baldwin PR, Mann DS, Jiang W, Rees I, & Ludtke SJ (2007). EMAN2: an extensible image processing suite for electron microscopy. J Struct Biol, 157(1), 38–46. doi:10.1016/j.jsb.2006.05.009 [PubMed: 16859925]

Tyagi A, Ahmed T, Shi J, & Bhushan S (2020). A complex between the Zika virion and the Fab of a broadly cross-reactive neutralizing monoclonal antibody revealed by cryo-EM and single particle analysis at 4.1 Å resolution. Journal of Structural Biology: X, 4, 100028–100028. doi:10.1016/j.yjsbx.2020.100028 [PubMed: 32647830]

Voss NR, Yoshioka CK, Radermacher M, Potter CS, & Carragher B (2009). DoG Picker and TiltPicker: software tools to facilitate particle selection in single particle electron microscopy. J Struct Biol, 166(2), 205–213. doi:10.1016/j.jsb.2009.01.004 [PubMed: 19374019]

Wu K, Wu D, Zhu L, & Wu Y (2021). Application of Monolayer Graphene and Its Derivative in Cryo-EM Sample Preparation. International journal of molecular sciences, 22(16), 8940. doi:10.3390/ijms22168940 [PubMed: 34445650]

Yeates TO, Agdanowski MP, & Liu Y (2020). Development of imaging scaffolds for cryo-electron microscopy. Curr Opin Struct Biol, 60, 142–149. doi:10.1016/j.sbi.2020.01.012 [PubMed: 32066085]

Zhang C, Cantara W, Jeon Y, Musier-Forsyth K, Grigorieff N, & Lyumkis D (2019). Analysis of discrete local variability and structural covariance in macromolecular assemblies using Cryo-EM and focused classification. Ultramicroscopy, 203, 170–180. doi:10.1016/j.ultramic.2018.11.016 [PubMed: 30528101]

Zhang S, Zou S, Yin D, Zhao L, Finley D, Wu Z, & Mao Y (2022). USP14-regulated allostery of the human proteasome by time-resolved cryo-EM. Nature, 605(7910), 567–574. doi:10.1038/s41586-022-04671-8 [PubMed: 35477760]



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**Table 1:**

An overview of the following described method and developments, noting core principles discussed, and key citations for each topic.

| Topic | Core Principles | Key Citations |
|---|---|---|
| **Adding large imaging scaffolds or monoclonal antibodies to proteins to increase size and structural clarity** | Antibody fabs or imaging scaffolds can be used to aid structural determination of smaller proteins. | Tyagi, Ahmed, Shi, & Bhushan, 2020; Lyumkis et al., 2013; Sauer et al., 2021; Kim et al., 2019; Yeates, Agdanowski, & Liu, 2020 |
| **Increasing image contrast with thin carbon** | Graphene carbon coating on EM grids improves image contrast, allowing for lower sample concentration requirements, and better visualization of small proteins. | Pantelic, Meyer, Kaiser, Baumeister, & Plitzko, 2010; Palovcak et al., 2018; K. Wu, Wu, Zhu, & Wu, 2021 |
| **Negative stain before cryo-EM** | Lower resolution TEM with negative stain may be used as a screening and troubleshooting tool prior to cryo-EM analysis to avoid wasting thetime and resources of cryo-EM. | McNulty et al., 2015; Gerwering, Januliene, Ries, & Moeller, 2018 |
| **Focused Classification** | Softwares can create masks around specific domains of interest during reconstruction to help visualize moving domains and/or domains in different conformational states. | C. Zhang et al., 2019 |
| **Multi-Body alignment** | A continuum of cryo-EM reconstructions may be used to better resolve disordered, unstructured, or otherwise heterogeneous samples. The human spliceosome offers a prime example of this technique. | Haselbach et al., 2018 |
| **Analyzing virus and protein dynamics and mechanisms through cryo-EM** | Cryo-EM can be used to analyze dynamic structures of proteins and viruses to help classify function. For example, the cleavage event of NωV virus, the unbound state of heterodimeric ABC transporters, and interaction between the USP14 protease and the proteosome have been 3D imaged | McNulty et al., 2018; Doerschuk, Gong, Xu, Domitrovic, & Johnson, 2016 |
| **Small molecule drug development and cryo-EM** | Cryo-EM shows changes in protein structure caused by interactions with small molecules drug candidates, as with the identification of insect specific binding pockets and ligand-specific channel activation in the case of the Slo channels in insects. | Raisch et al., 2021 |
| **Protein fragmentation analysis and fragment screening** | Fragment based drug discovery and fragment screens may be done in tandem with cryo-EM to structurally analyze proteins at the fragment level and identify interactions between residues. | Saur et al., 2020 |
| **Exploring protein variability using cryoSPARC** | Imaging software like cryoSPARC is a valuable tool to further quantify protein structure, especially small variabilities in small proteins. Specifically, the 3DVA process allows for direct molecular movement deciphering of flexibility, and heterogeneity. | Campbell, Veesler, Cheng, Potter, & Carragher, 2015 |