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Atomic Cryo-EM Structures of Viruses

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Abstract

During the development of single particle cryo-EM in past five decades, icosahedral viruses have led the resolution progress owing to their large mass and high symmetry. Many technical advances in cryo-EM were first established with viruses. Since reaching ~4 Å resolution in 2008, it has become a relatively routine task to solve the atomic structure of isolated viruses. The future of structural virology will be increasingly focused on remaining challenges including solving structures of jumbo viruses, intermediate functional states during assembly, maturation, and infection, and *in situ* structures. Recent demonstrations of near-atomic resolution structure with electron tomography and sub-tomogram averaging opens a new direction for high resolution studies of pleomorphic viruses and the pleomorphic states of icosahedral viruses that have defied past efforts using the single particle cryo-EM approach.

Introduction

In recent years, single particle cryo-electron microscopy (cryo-EM) has matured into a revolutionary structural biology method that can determine near-atomic resolution (2–4 Å) structures of a wide range of macromolecular assemblies from ~64 kDa hemoglobin [1] to ~150 MDa adenovirus (Figure 1E) [2]. In the five decades since late 1960s, viruses, with their advantageous large mass and high symmetry, have played critical roles as model systems for the development of new techniques essential for high resolution imaging and 3-

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D reconstruction. With a single image containing sufficient 3-D information due to the helical symmetry, the T4 phage tail was the first 3-D structure determined from 2-D images in 1968 [3]. By computationally determining the relative views of multiple particle images of tomato bushy stunt virus in 1970 [4], the concept of single particle TEM imaging and 3-D reconstruction was demonstrated. In 1984, the high contrast image of Adenovirus embedded in vitreous ice by plunge freezing [5] signaled the arrival of cryo-EM. In 1997, cryo-EM progressed to 7.4 Å [6] at which the helices of the capsid protein of Hepatitis B virus core particles could be resolved. With multiple viruses solved at ~4 Å resolution in 2008 [7,8,9], single particle cryo-EM finally broke the barrier for near-atomic resolution. The 3.3 Å aquareovirus structure solved in 2010 (Figure 1F) [10] represents a major advance that allowed *de novo* full-atomic modeling utilizing the well resolved sidechain densities. With the arrival of direct electron detectors, single particle cryo-EM could also obtain sufficient signal to solve the structure of smaller, lower symmetry macromolecular complexes to near-atomic resolution [11,12]. However, viruses continued to play important roles in further technical developments in cryo-EM. With movie images of viruses, beam induced sample motion [13] was observed and optimal weighting of movie frames [14*] was developed to compensate radiation damages to later frames. Elliptic distortion of the TEM imaging lenses was characterized and computationally corrected [15*,16*], and affinity grid method was shown capable of reaching 2.6 Å resolution (Figure 1B) [17**].

Cryo-EM is the method of choice for structural virology

In the 1970s and early 1980s, X-ray crystallography became the first method capable of determining 3-D structures of icosahedral viruses at atomic resolution [18,19]. Since then, X-ray crystallography has been used to solve atomic structures of over 300 icosahedral viruses (<http://viperdb.scripps.edu>). However, it remains difficult to achieve atomic resolution for large viruses. Virus-like particles (VLP) assembled from heterologously expressed capsid proteins or truncated/mutated versions were often used to facilitate assembly and crystallization. In contrast, cryo-EM can be utilized to image authentic virions and solve near atomic resolution structures within a considerably shorter time span, making cryo-EM the method of choice for structural studies of viruses. For example, an altered adenovirus with short fiber was necessary to obtain crystals diffracting to 3.5 Å resolution [20] while the cryo-EM structure of similar resolution was determined in much shorter time using a wild type virion (Figure 1E) [2]. A recent example is the structure of Zika virus solved by cryo-EM within months of outbreak (Figure 1C) [21**,22*]. Crystals have not yet been reported for any of the large number of tailed dsDNA phages. It took significant effort over a long period of time [23] to achieve 3.44 Å resolution crystal structure of the ~650 Å diameter bacteriophage HK97 VLP [24,25]. In contrast, a single cryo-EM session was used recently to reach a 2.9 Å resolution structure of the icosahedral capsid of bacteriophage Sf6 virion of similar size (Figure 1D) [26**]. This, together with other near atomic resolution cryo-EM structures of large bacterial viruses [27,28,29], has led to new and unbiased understanding enabled by more reliable atomic models.

It has essentially become a routine task to solve symmetric virus structures to near-atomic resolutions using cryo-EM. There has been an explosion of 4 Å and higher resolutions virus structures (Figure 2) across a wide range of sizes (Figure 3).

Virus structures beyond icosahedral symmetry

The difficulty in crystalizing authentic phage virions is mainly due to tail structure that does not follow the symmetry of the icosahedral capsid [30,31]. Such asymmetric structural features have fallen beyond the reach of methods relying on icosahedral averaging. One ubiquitous asymmetric structural feature in viruses is the viral genome. X-ray crystallographic studies showed that fractions of viral genome were ordered as a result of interactions with capsid proteins, but most of the viral genome was not resolved due to non-crystallographic symmetry averaging and averaging during crystallization. In last two years, cryo-EM has dramatically contributed to our understanding of genome organization in dsRNA [32**,33**] and ssRNA viruses [34,35**]. In the recent 3.6 Å resolution cryo-EM asymmetric reconstruction of (+)RNA bacteriophage, MS2, ~80% of the 3,569 nucleotides in the viral genome could be traced [35**].

Moreover, cryo-EM has been successfully applied in high resolution structural studies of filamentous viruses [36] and HIV capsid structures [37**], which are refractory to crystallization. In the latter cases, the cone-shaped HIV capsid has variable sizes, preventing direct averaging of entire particles by single particle cryo-EM. Instead, cryo-electron tomography (cryo-ET) and subtomogram averaging were utilized to achieve 3.9 Å resolution (Figure 1G) [37**].

Future perspective of structural virology using cryo-EM

As summarized above, it has now essentially become routine to solve the 3-D structure of many small to intermediate sized (<100 nm diameter) icosahedral viruses to near-atomic resolutions using single particle cryo-EM. Nevertheless, many remaining challenges in structural virology remain. We will briefly discuss these challenges and comment on potential solutions.

Atomic structures of jumbo viruses (>100nm)

Currently, the largest virus solved to near-atomic resolution is adenovirus (~90 nm in diameter, 3.6 Å) [2]. Although there is no fundamental barrier to extending similar achievements to jumbo viruses, for example, Herpes (~125 nm) [38], PBCV (~190 nm) [39], and mimivirus (~500 nm) [40], large particle size does pose several practical challenges.

Geometric distortion of the TEM imaging lenses

Several Titan Krios microscopes were found to have 2–3% elliptic distortion which limits the structures of viruses of 70–80 nm in diameter to 7–8 Å resolutions [15*, 16*]. Computational estimation and correction of the distortion were essential to improve these virus structures to 2–4 Å resolutions. The jumbo viruses will be more sensitive to distortion and require more accurate correction. Small variations of magnification and distortion might require per-micrograph or per-particle level correction [16*,41*]. Correction of additional distortions such as spiral, barrel, and pincushion [42] might become essential.

Focus gradient within a single virus particle

This is equivalent to the Ewald sphere problem. Current CTF correction methods, which assume a single constant defocus for the entire particle, might be insufficient for large, high resolution structures. Although several algorithms have been proposed [43,44,45,46], successful application to experimental data remains elusive. New algorithms will be needed to overcome this challenge. A practical, brutal force approach for addressing this issue is to use a larger number of particles and taking advantage of the icosahedral symmetry. Regardless of the orientation, the asymmetric units near the equator plane of a virus particle will provide accurate structural information consistent with the mean defocus. In contrast, the asymmetric units at the top or bottom of the particle will be out of focus. The effective symmetry of the 60-fold icosahedral symmetry is thus reduced to 30 or fewer, which can be compensated by a larger number of particles. While inelegant, this practical approach is expected to determine atomic structures of jumbo viruses of 100–200 nm in diameter in the near future.

Data collection

Collection of the same number of particles will take significantly more time for jumbo viruses than for smaller viruses. Larger area detectors, for example, the new 8kx8k DE-64 camera, which is about 4x of the size of other direct electron detectors, will help significantly increase data collection efficiency. As observed in the 2.9 Å resolution structure of PCV2 (Figure 1A) solved using a close-packed particle strategy [41*], grids with high particle densities can also be utilized to further improve data collection efficiency.

Non-crystallographic symmetry (NCS) averaging

Larger virus particles also have larger T-numbers, i.e. more subunits of the same protein localized at quasi-equivalent environments in an asymmetric unit [47]. Although the conformations of these subunits can vary significantly, for example, the subunits in the “T=2” inner shell of dsRNA viruses [10,48] and the T=7 dsDNA phages [9,25,26**,27,28], the conformations of these subunits in many viruses, for example, rotavirus VP6 trimers [7,14*], are indistinguishable even at near-atomic resolutions. NCS averaging of these subunits can effectively increase the total symmetry of the particles up to 60T and significantly reduce the number of particles required to reach high resolutions. The reduction of particle number requirement will be proportional to the T-number, potentially reducing the number of particles by several orders for viruses such as: T=16 for Herpes [38], T=169 for PBCV [39], and 972 T 1200 for mimivirus [40]. By averaging all or a fraction of the subunits, it is possible to keep the data collection effort largely at similar levels for viruses of different sizes, which would allow the jumbo virus structures to be solved more easily.

Computational resources

Large image sizes needed for jumbo virus data analysis pose serious computational challenges. For example, the ~190 nm PBCV would require ~32GB memory for each 3D map of ~2000³ voxels, which is beyond the memory of current GPUs used by Relion [49] and CryoSPARC [50] software and the per-core memory of almost all Linux cluster nodes used by jspr [51] and EMAN [52] software. Image processing programs will need to

explicitly consider the symmetry and only store a fraction of the structure to reduce the memory requirement. Without algorithmic improvements, a period of 5+ years would be required for future computers to have sufficient resources for image processing of jumbo virus cryo-EM data assuming Moore's Law.

Structural complexity and heterogeneity

It is a common misunderstanding that viruses are simple icosahedrons. Almost all viruses are more complex for which the complete structural solution is beyond an icosahedral reconstruction.

Symmetry mismatches

All viruses have structural components that have different symmetries than the icosahedral shell. Well known examples include the genome encapsulated inside the capsid (discussed above), the tail attached to the portal vertex of dsDNA phages [30,31], the fibers attached to Adenovirus vertices [53], the RNA polymerases attached to the inner surface of dsRNA viruses [32**,33**], and the nucleocapsid of Flaviviruses [21**,22*,54], *etc.* When there are three or more components with mismatched symmetries, such as the corestack in the T7 phage capsid I with 5, 12, 8, and 4-fold symmetries in tandem at one of the icosahedral vertices, the combinatorial assembly of these components can create a large number, for example, $5 \times 12 \times 8 \times 4 = 1920$, of states that are imaged as a mixture [55]. A focused refinement strategy is needed to zoom in on individual components to resolve them separately [55,56]. The nucleocapsid of Flaviviruses, such as Zika and Dengue, remains elusive to structural studies [21**,22*,54,57]. It is unclear if the core has icosahedral symmetry but with a fixed or random orientation offset to the surrounding icosahedral glycoprotein layer, a different symmetry, or even without a defined structure. We hope the recently increased awareness of Zika virus will help bring sufficient attention to solve this puzzle.

Intermediate states

Many viruses undergo dramatic conformational changes in their assembly and maturation process. Most structural studies have focused on one or a small number of stable states that can be purified in large quantity [28,58,59]. These structures provide a few key frames for the dynamic process of virus assembly and maturation. It is time to extend structural studies to additional sub-states to address questions that cannot be answered by sparse key frames. For instance, how do the trimers in Flavivirus immature particles morph into a raft of dimers in mature particles before being released from host cells and then again into trimers triggered by low pH after infecting new host cells [58,60]? Structural studies of these intermediate states are challenged by the high level of structural heterogeneity of the particles [61,62]. It is conceivable that the paths between the major states represents one or even multiple trajectories and each of the particles can assume any points in the paths. Such a continuously varying conformational heterogeneity is unsolvable by current methods which are limited to a small number of discrete states. Development of new algorithms, such as manifold learning [63**], will be needed to solve this class of problems.

Pleomorphic state of icosahedral virus and inherently pleomorphic viruses

When an icosahedral virus is morphing between two major states, will the intermediate states maintain icosahedral symmetry? Current studies by cryo-EM [61,62] and molecular dynamics simulations typically assume preservation of icosahedral symmetry [64]. The symmetry was even explicitly embedded to accelerate computations [65]. However, more evidence has suggested that symmetry is not necessarily preserved through the maturation process as shown by the half immature and half mature Dengue virus particles [66]. We argue that deviations from symmetry are more likely as the triggers of conformational changes, such as receptor binding, are not likely to occur synchronously for all the 60T subunits and the propagation of the changes cannot be instantaneous. When the 60T subunits undergo independent conformational changes, the virus particles can assume an astronomic number of conformations, for example, 2^{60} or 10^{18} states for the simplest system of 2 states per subunit for T=1 viruses. The symmetry has become a curse instead of benefit for structural studies and the overall structure is now better regarded as pleomorphic just as those inherently pleomorphic viruses such as influenza and HIV. New reconstruction methods have been proposed to solve this class of problem by only assuming symmetry for the ensemble average instead of individual particles [67]. It remains to be seen if such method can reach high resolution. The recently demonstrated HIV capsid protein at 3.9 Å resolution using sub-tomogram averaging [37**] suggests that tomography has now become a viable approach for high resolution studies of pleomorphic structures by local region classification and averaging.

Structural virology beyond purified virus particles

For viruses to propagate they need to successfully bind the receptor on the host cell surface, enter the cell, replicate the genome, assemble of progeny virions, and release the progeny virions for subsequent rounds of infection. High resolution cryo-EM studies will be needed to provide structural insights to these critical steps of viral infection.

Native state structures

Most structural studies have used highly purified virus particles. The short-lived states or low abundance states could easily be lost by the lengthy purification process. It is also a concern as to whether the virus particles, especially for those unstable enveloped viruses, such as Dengue virus, still maintain their native, functional states and if the cryo-EM structures do genuinely represent the functional states. This concern is corroborated by the discrepancy between the virus concentrations based on plaque assays (i.e. PFU) and particle counts. For example, the particle to PFU ratio for human herpes simplex virus 1 is ~10:1 [68] but ~40,000:1 for Varicella-zoster virus, another human herpes virus [69]. Affinity cryo-EM methods [70,71,72] capture target particles directly from cell lysate without pre-purification. The virus particles are more likely to resemble the native states. In one of the first applications of this approach, multiple capsid states of T7 phage captured from cell lysate were solved to 7–8 Å resolutions [73]. Using Tulane virus as a model system, the antibody affinity cryo-EM approach was shown capable of reaching 2.6 Å resolution [17**], the highest resolution published virus structure. An increase in use of the affinity cryo-EM strategy is anticipated to cross-validate the functional relevance of structures solved using

purified samples, and more importantly, to discover novel functional states lost to purification. The affinity cryo-EM method will help reduce the health risk associated with structural studies of highly contagious or dangerous viruses by using smaller quantity samples. It will also help expedite structural solutions to new virus outbreaks by directly utilizing patient samples and forgoing the need of establishing a cell culture system which can take decades for some viruses, such as human noroviruses [74].

***In situ* structures**

A truly native structure is ideally obtained from virus particles in the act of infecting a host cell or being assembled inside a host cell. Such structures are currently available at low resolutions by electron tomography of cell sections [75,76] or small bacterial cells [77,78]. As shown by the success with HIV [37**], sub-tomogram averaging of virus particles, either at the whole particle level or using local regions of the particles, is a promising method for extending tomographic studies to near-atomic resolutions. An alternative approach, which has not been reported yet, is to simply treat the virus particles in the cell sections as standard single particle cryo-EM projects. The crowded cytoplasmic background will be noisier than chemical buffers, however, the section can be set to a thickness just slightly larger than the particle diameter to reduce this effect. A partially cut virus particle should still have sufficient signals to allow reliable determination of its orientation owing to the high redundancy of the icosahedral symmetry. Once the technique barriers for high resolution *in situ* structures have been overcome, the entire dynamic process of receptor binding, cell entry, uncoating, assembly, and release processes can then be systematically investigated at atomic model resolutions.

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Highlights

- Large, symmetric viruses played critical roles in developing cryo-EM techniques
- Cryo-EM can routinely solve icosahedral virus structures to 2–4 Å resolution
- Cryo-EM has become the method of choice for structural virology
- Cryo-EM is poised to solve structures of jumbo viruses and pleomorphic viruses
- Structural virology is moving towards solving atomic structures of intermediate functional states and viruses *in situ*

Figure 1.

Cryo-EM maps of representative viruses (surface rainbow-colored radially) and atomic models of their major capsid proteins (N→C rainbow-colored ribbon diagrams). Cryo-EM maps in (A-F) are in the same scale, with the bar in (E) corresponding to 200 Å. The bar in (G) is 200 Å. The capsid protein ribbon diagrams are in the same scale.

Figure 2.
Cryo-EM structures of viruses at better than 4 Å resolution plotted against years.

Figure 3.
Cryo-EM structures of viruses at better than 4 Å resolution plotted against assembly mass.