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Cryo-electron Tomography: An Overview

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Introduction

Viruses are macromolecular machineries which hijack cellular metabolism for replication. Viruses that are enveloped, comprise a large variety of DNA and RNA virus, many of which are notorious human or animal pathogen. Despite their importance, presence of lipid bilayers in their assembly had made most enveloped viruses too pleomorphic to be reconstructed as whole, by traditional structural biological methods. Structural biology of the virus lifecycle was hindered by sample thickness. Here is the report of the recent advances in the applications of cryo-electron tomography (cryo-ET) on enveloped viral structures and intracellular virus activities.

Electron cryotomography (CryoET) is an imaging technique which is used to produce high resolution (~1 – 4 nm) 3D views of samples, often biological macromolecules and cells. CryoET is a specialized application that is transmission electron cryomicroscopy (CryoTEM) in which, samples are imaged as they are tilted which resulting in a series of 2D images which can be combined to produce 3D reconstruction, similar to that of a CT scan of the human body. In contrast to other electron tomography techniques, samples are imaged under cryogenic conditions (<−150 °C). For cellular material, the structure is immobilized in non-crystalline, vitreous ice, allowing to be imaged without chemical fixation or dehydration, which would otherwise distort or disrupt biological structures.

Description of the technique

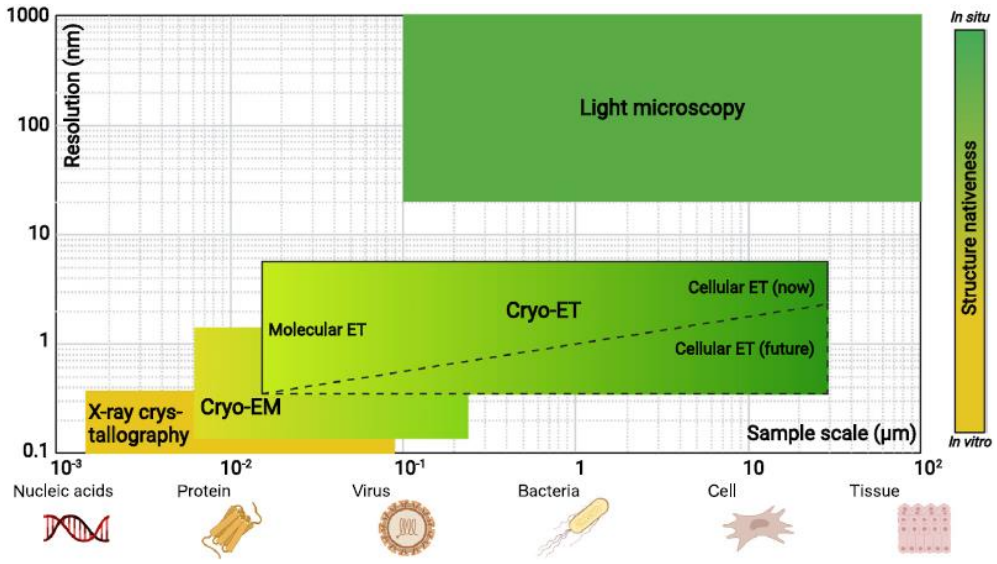
In electron microscopy (EM), samples are imaged in a high vacuum, such vacuum is incompatible with biological samples like, cells; water would boil and pressure would explode the cell. In room temperature, EM techniques, samples are therefore prepared by fixation and dehydration. Another approach to stabilize biological samples, is to freeze them (electron cryo-microscopy). As in other electron cryo-microscopy techniques, samples for CryoET (typically Bacteria, Archaea, or viruses) are prepared

in aqueous media and applied to an EM grid. The grid is then plunged into a cryogen (liquid ethane) so that water molecules do not have time to rearrange into a crystalline ground. The resulting water state is called "vitreous ice" that preserves native cellular structure, like, lipid membranes. Samples are imaged in transmission electron microscope (TEM). As in other electron tomography techniques, sample is tilted to different angle relative to electron beam (typically at every 1 or 2 degrees from about − 60° to + 60°) and an image is acquired at each of the angle. This tilt series of images then can be computationally reconstructed into a 3D view of the object. This is called “tomogram” or “tomographic reconstruction”.

Applications

In transmission electron microscopy (TEM), because of electrons, interact strongly with matter, resolution is limited by thickness of sample. Also, thickness of sample increases, as the sample is tilted and thicker samples can completely block electron beam, making image darker or completely black. Therefore, in CryoET, samples should be less than ~ 500 nm thick to achieve "macromolecular" resolution (~ 4 nm). For this particular reason, most ECT studies have focused on purified macromolecular complexes, viruses, or small cells such as of many species of Bacteria and Archaea. Cryo-tomography was used to understand encapsulation of 12nm size protein cage nanoparticles inside 60nm sized virus like nanoparticles.

Larger cells and even most of the tissues, can be prepared for CryoET by thinning, either by cryosectioning or by focused ion beam (FIB) milling. In cryosectioning, frozen blocks of cells or tissue are being sectioned into thin samples with a cryo-microtome. In FIB milling, plunge frozen samples are exposed to focused beam



of ions, typically Gallium, that precisely whittle away the material from top and bottom of sample, leaving thin

lamella suitable for ECT imaging. The strong interaction of electrons with the matter also results in an anisotropic resolution effect. As sample is tilted during imaging, electron beam interacts with relatively greater cross sectional area at higher tilt angle. In practice, tilt angle greater than approximately 60–70° do not yield much information and therefore not used much. This results in "missing wedge" of information infinal tomogram which decreases resolution parallel to electron beam. For structures which are present in multiple copies in one or more tomograms, higher resolution (even ≤ 1 nm) can be obtained by sub-tomogram averaging. Similar to single particle analysis, sub-tomogram averaging computationally combines image of identical objects to increase the signal - to - noise ratio.

Comparison of structural biology and microscopy methods

A major obstacle in CryoET is identifying structures of interest within the complicated cellular environments. One solution is to apply correlated cryo fluorescence light microscopy and even super resolution light microscopy (Cryo-PALM) and CryoET. In this technique, sample containing fluorescently tagged protein of interest is plunge frozen and first imaged in light microscope equipped with special stage to allow sample to be kept at sub crystallization temperature (< − 150 °C). The location of fluorescent signal is identified and the sample is then transferred to CryoTEM, where same location is then imaged at high resolution by CryoET.

Synergy between cryo-ET and in situ structural virology

By recording 3D volume data, Cryo-ET is less restricted by sample heterogeneity or purity and requires only relatively larger structures, such as glycoproteins (GPs), or locally ordered lattices/grounds, such as matrix proteins (Ms), for high resolution structure determination. This advantage has made Cryo-ET particularly well-suited for studies on the *in-situ* structures of enveloped virus and viral activities in infected cells, which remain challenging for traditional structure biology method due to structural heterogeneity and crowded background, respectively.

Over the past two decades, structural virology and cryo-ET method developments had built synergy in conducting mutual progress, providing structural insights into the assembly of the most threatening viruses, including Lassa (LASV), Ebola (EBOV), Marburg (MARV), Rift Valley fever virus (RVFV) and HIV. Cryo-ET has also enabled quick response to Coronavirus disease-2019 (COVID-19) pandemic, illustrating intact severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virions, the flexibility of its spike protein (S), its intracellular viral activities and antigen conformation and distribution

for vaccine candidates. The realistic illustration of the virus revealed by cryo-ET have also provided scientifically accurate visual for publicity and education. In return, curiosities into virus structure and replication mechanisms have driven cryo-ET method developments into higher resolution, advanced labelling using biomaterial and multiscale imaging. A classic example is HIV capsids studies, which have driven cryo-ET into sub-nanometer and near the atomic resolution, yielding a series of method that impact the overall *in situ* field of structural biology.

Cryo-ET of isolated viruses

Unlike nonenveloped viruses, enveloped viruses are assembled by the oligomerization of one or more structural proteins (SPs), including GPs (Glycoproteins), Ms, capsid proteins (Cs) and nucleoproteins (Ns) that together with the lipid envelope. Loose protein – protein interactions and presence of lipid bilayer in their assembly have contributed to the vast diversity of enveloped viral morphology. These challenges in heterogenous structure have been efficiently tackled by cryo-ET, as by the recent advancement of the *in-situ* structures of GPs and Ms.

Cryo-ET unveiled the secret life of viruses from infected cells

Although direct imaging of the cell periphery by cryo-ET uncovered viral attachment and entry mechanism, most key step of a virus lifecycle, including endocytosis, uncoating, genome replication, protein translation, assembly and egressing, happen intracellularly and the structural details remained enigmatic. Observation of such activities by this technoque is hindered by thickness of the cell. Traditionally, cells freeze substituted, resin embedded, thinned using diamond knife and contrasted with heavy metal ions before imaging. The method provides extraordinary contrast at the cost of sample native-ness and resolution. Over the past 15 years, advances in phase plate, cryo focused ion beam milling (cryo-FIB), cryo-ET, correlative light electron microscopy (CLEM) and STA techniques provided possibilities in visualizing intracellular viral activities at nano-meter resolutions. Here, there is recent discoveries into two parts depending on the type of functioning of the viral proteins:

(i) Uncoating, assembly and egressing, which are mediated by viral SP; and
(ii) replication, which is mediated by non-structural proteins (NSPs).

Concluding remarks

Technical development built on the previous cryo-ET studies on the isolated enveloped viruses had been quickly applied on intact SARS-CoV-2, providing the vital structural and statistical references for vaccine candidates. Similarly, characterizations of other Coronaviruses will expand our knowledge on their viral assembly and evolution. Experiences on cryo-ET of VLM on IAV and RVFV are expected to be applied to the study of Coronavirus liposome fusion, which will explain how orchestration of trypsin,

angiotensin converting enzyme 2 (ACE2), and acidic pH triggers S-mediated membrane fusion. Since enveloped virus is much understudied, expanding these experiments to other pathogenic enveloped virus is likely to uncover their unnoticed weakness, novel neutralizing mechanism, or inspire the drug or antibody developments that will inhibit fusion and genome release. Increasing the throughput of these techniques is desirable to accumulate significant data for solving high resolution structures. Furthermore, accurately locating virus related events requires the improvement of super resolution (SR)-CLEM to achieve 50 - 100 nm lateral precision. The success of these technical developments will be crucial for visualization of intracellular viral activities at sub-nanometer resolution.

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