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TOPICAL REVIEW — Electron microscopy methods for the emergent materials and life sciences

Cryo-ET bridges the gap between cell biology and structural biophysics*

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Cryo-electron tomography (cryo-ET) is a cutting-edge technology providing three-dimensional *in situ* ultra-structural information of macromolecular machineries, organelles, and eukaryotic cells in their native environment at an unprecedented level of detail. Cryo-ET enables the direct observation of dynamic macromolecular architectures of bio-samples in their naturally occurring physiological state, without any harmful artifacts introduced by heavy metal staining, dehydration, and chemical fixation, which occur in traditional transmission electron microscopy. Over decades, cryo-ET has been providing insights into numerous aspects of cellular biology by revealing the pristinely preserved ultra-structures of different cellular components comprising the crowded and complex environment of the cell, thus, bridging the gap between cellular biology and structural biophysics. In this paper, we review the fundamentals of this technique, its recent advances in optics, detection devices, and computational algorithms. The enhancement of our understanding of structural cellular biology by combining these improvements, when integrated with other methods, such as cryo-focused ion beam milling, correlative light and electron microscopy, is discussed via a few examples from research groups worldwide. We also believe that cryo-ET applications in cell biology continue to provide fundamental insights into the field, revolutionizing structural biology itself.

Keywords: sub-tomogram averaging, segmentation, direct detection, phase plate

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1. Introduction

Following the discovery of the wave–particle duality of electrons, transmission electron microscopy (TEM) was invented in the 1930 s to study the structure of both materials and biological specimens.[1] Owing to the short wavelength (pm range, 10^{-12} m) of electrons under a high accelerating voltage (generally between 120 kV–300 kV for bio-TEM), TEM has a much better resolving power than light microscopy.[2] Theoretically, TEM can obtain a resolution better than 1 Å. However, achieving atomic resolution is difficult,[3] especially for biological specimens, which are fragile when exposed to a high-energy sample-damaging electron beam.[4] Some of the electrons, interacting with biological materials in the microscope column, deposit energy into the sample, resulting in a series of detrimental events including chemical bonds rupture, free radical formation, and structural rearrangements,[5] thus destroying the structural integrity of the specimen. This process, namely the radiation damage,[6] limits the amount of the electron dose used in the image acquisition session resulting in a low single-to-noise ratio (SNR) of the image formed, fundamentally limiting the achievable resolution in TEM research. In addition to radiation damage, another challenge for TEM research is dehydration. Water molecules evaporate in the vacuum of the microscope, resulting in the collapse of the biological specimen. Both challenges need to be addressed.

To reduce radiation damage and dehydration, biological specimens are cooled to cryogenic temperatures in rapidly vitrified water. The low temperatures protect the samples against radiation damage, enabling a better tolerance for higher electron doses; thus, improving the SNR and the final resolution.[5] The vitreous water, namely the rapidly solidified water in its liquid form, around the biological material retains the samples in their natural shape even in vacuum.[7] Following the acquisition of the images of these frozen-hydrated bio-samples, a computational reconstruction procedure[8] is required to transform these fuzzy pictures, which represent two-dimensional (2D) projections of the three-dimensional (3D) bio-samples of interest, into sharp information-rich 3D structures.[9] This technique, which allows scientists to visualize bio-molecules in their natural physiological forms, is known as cryo-electron microscopy (Cryo-EM).[10] Three scientists, Jacques Dubochet, Joachim Frank, and Richard Henderson were awarded the 2017 Nobel Prize in Chemistry for

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developing this technique. Although the prize was awarded for cryo-EM, it is still a relatively new technique, under active development.[11] The prize was primarily given for single-particle analysis (SPA) of cryo-EM, a technique mainly used to study protein complexes known as "single particles".[12] The other direction of development of cryo-EM is cryo-electron tomography (cryo-ET), which has been increasingly used to study bio-molecules, organelles and cells *in situ*,[13] and holds a vast potential for revolutionizing structural biophysics again in the near future,[14] and it is the subject of this review.

2. Fundamentals of cryo-ET

2.1. Cryo-EM and cryo-ET

In structural cellular biology applications, cryo-EM has two main focuses. One is SPA, a method routinely used to determine the 3D structures of macromolecular complexes with sub-nanometer resolution without the need of crystals or intrinsic symmetries.[12] SPA is an averaging technique, which requires a large number of structurally identical protein complexes of interest, called "single particles", to be frozen, imaged, aligned, and averaged. Recent advances in detector devices[15–17] and software algorithms[16,18–23] enable researchers to study structures of these architectures at atomic resolution.[24–28] The compositional and conformational heterogeneity of the protein complexes of interest is a challenging problem, which needs to be addressed in order to achieve a sharp electron density map, upon which an atomic model can be automatically built.[29]

Although SPA is a powerful technique, it has limitations. For example, structurally unique or pleiomorphic objects such as organelles, intact cells, and multi-cell systems are not accessible by SPA. Extracting high-fidelity structural information of certain supramolecular assemblies in their native, physiological, unperturbed, crowding cellular, or organellar environment is also challenging to SPA. Furthermore, as certain crucial cellular processes are carried out by molecular actors, which are only fully functional in transiently formed assemblies in the presence of their crowded cellular environment, a genuine understanding of their structural mechanism requires the acquisition of their molecular architecture *in situ*. Furthermore, several of these functional multi-component machineries are either challenging to purify[30] or difficult to reconstitute *in vitro*; therefore, other methods are needed.

The other focus of cryo-EM is cryo-electron tomography (cryo-ET),[31–33] which is a method with the potential to tackle these intriguing scientific problems. Cryo-ET enables dissecting faithfully preserved molecular mechanisms directly in frozen-hydrated cells. It allows direct visualization of unperturbed 3D structures of protein assemblies, organelles, cellular fragments, and multi-cellular systems with varying molecular detail *in situ*.[14,34] Tomographic reconstruction, often followed by sub-tomogram averaging, has the capacity to achieve sub-nanometer resolution;[35,36] the pipelines of the promising technique are reviewed in the following sections. Besides SPA and cryo-ET, there are other commonly used TEM approaches, such as electron crystallography,[37,38] helical reconstruction,[39,40] and icosahedral symmetry reconstruction.[41] The different approaches of TEM are outlined in Table 1.

Table 1. Typical Approaches of TEM.

	Single particle reconstruction	Electron crystallography	Helical reconstruction	Icosahedral symmetry	Electron tomography
Symmetry	any symmetry	2D crystals	helixes	icosahedral	any symmetry
Representative	Ribosome (2.9 Å, 2015)	Bacteriorho-dopsin (3 Å, 1999)	TMV (4.5 Å, 2007) microtubule (3.5 Å, 2015)	Capsid of bacteriophage Sf6 virion (2.9 Å, 2017)	Immature HIV-1 CA-SP1 (3.9 Å, 2016)
	Ryanodine receptor (3.8 Å, 2015)	aquaporin-1 (3.8 Å, 2000)	actin (8 Å, 2013)	Aquareovirus (3.3 A, 2010)	immature HIV-1 CA-SP1 with novaCTF (3.4 Å, 2017)
	GroEL (3.5 Å, 2017)	MAPEG proteins (2005)	acetylcholine receptor (4 Å, 2003) flagella (4 Å, 2003)	tulane virus (2.6 Å, 2016)	salmonella type III secretion machine (17 Å, 2017)

2.2. Pipeline of cryo-ET and sub-tomogram averaging

Molecules in cells rarely act alone; they interact with each other, stably or transiently, to form large functional modules.[42,43] A thorough study of the spatial relationships of these intricate ensembles and complicated macromolecular machines would expand our knowledge of the mechanisms underlying their specialized cellular tasks.[44] To achieve this essential *in situ* structural information, instead of 2D, a reliable 3D structural signature of these functional cellular content is absolutely necessary.[45] Cryo-ET, developed by combining tomography with cryo-EM, provides faithful 3D structural blueprints for these cellular components (Fig. 1).[46]

2.2.1. Sample preparation for cryo-ET

For decades, TEM has become the technique of choice for researchers worldwide to observe biological specimens and cellular morphologies.[47–50] In terms of techniques, sample preparation methods, such as heavy metal staining, chemical fixation, resin-embedding, and thin-sectioning, have been ac-

tively developed to promote this scientific visual inspection procedure.[51–55] Nowadays, a significant part of knowledge of the organizations of the cell comes from this method, which widens our scope of view in structural biology in cells.[56–60] TEM has been used for more than 50 years in cellular imaging and laid the foundation for modern *in situ* structural biology. However, biological samples, especially membranes, when going through these harsh preparation methods, suffer from disruption and distortion, occasionally causing artifacts, which may interfere with or even compromise the accurate interpretation of the visualized data.[13,61–63] Therefore, an improved sample preparation method is needed, which preserve the biological material better.

To avoid these potential artifacts due to the traditional TEM sample preparation procedures, another sample processing scheme was devised in the 1980s.[7] Researchers prepared frozen-hydrated biological specimens in a thin layer of vitreous ice by rapidly plunge-freezing the biological sample to liquid nitrogen temperatures using FEI Vitrobot,[64] Gatan CP3, or Leica EM GP. Colloidal gold particles, serving as fiducial markers to facilitate the alignment procedure in the subsequent 3D reconstruction step, can be either added to the EM grids before applying the sample, or simply mixed with the sample solution.[65] Then, 2 μL–3 μL of the solution is applied to the EM grid, blotted by a pair of filter papers, and then plunged into cryogens like liquid ethane or propane with rapid cooling rates. Thus, the formation of crystalline ice is circumvented, and the biological material under scrutiny is exquisitely preserved in a thin layer of amorphous ice in its native, physiological, functional state ready for further inspections in the electron microscope.[66] Practically, the frozen grid can be kept for months or even years, in the liquid nitrogen before use. This specimen preparation procedure opens up new perspectives to *in vivo* explore the complexity of structural biology.[67]

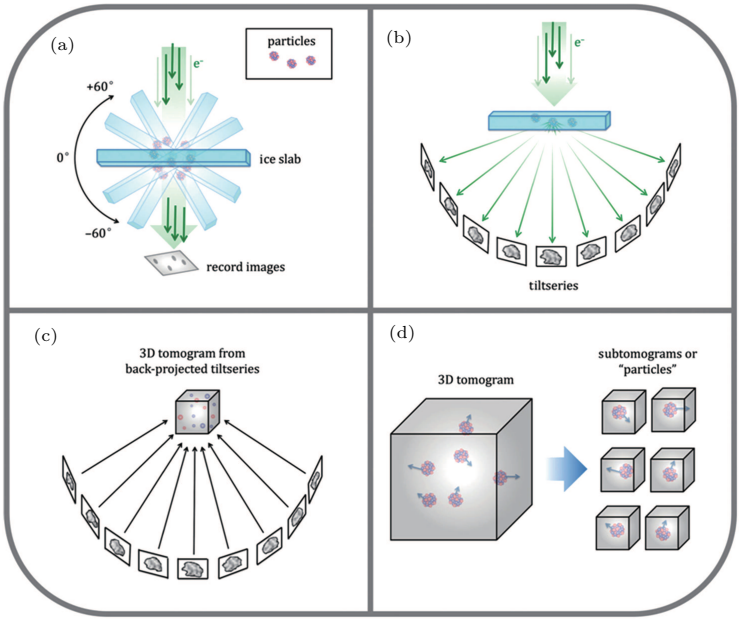


Fig. 1. (color online) Basic pipelines of cryo-ET. Basic pipeline for cryo-ET. (a) The sample stages are usually tilted from −60° to +60°, (b) allowing projection images at varying angles to be recorded. (c) These 2D projection images are then reconstructed computationally to generate a 3D volumetric structure known as a "tomogram". Structural features of interest in the tomogram are segmented either manually or automatically by a specialized visualization software, to create 3D annotations, which can then be used for both visualization and statistical analysis. (d) Repetitive structures in a tomogram can be extracted as "sub-tomograms" for furthering averaging. Modified with permission from Galaz-Montoya *et al.*, 2017.

2.2.2. Image acquisition and reconstruction of cryo-ET

After being frozen into cryogens at liquid nitrogen temperatures, the biological material under observation is transferred onto the surface of a glow-discharged[68] or plasma-cleaned holey carbon grid, which is placed onto the tip of a cryo-holder, which is then inserted into the column of a typical transmission electron microscope for further imaging and data acquisition by specialized software packages.[69] Depending on the type of specimen (protein complexes, organelles, cells, etc.), different types and sizes of grids need to be chosen for the study. Furthermore, depending on the cell line, polylysine, collagen, or fibronectin can be used for cellular samples to facilitate the adhesion and spreading of cells on the grid surface.

Following the grid transfer, microscopes are accurately aligned by well-trained users and image stacks comprising of 2D projection pictures of vitrified specimens at a series of different orientations are collected by physically rotating the sample stage through a range of tilt angles (−64° to +64°, typically) at constant intervals (2°, for instance)[70] with specialized software packages such as Serial-EM.[71] Electron doses are fractionated into each tilting projection in a tomographic tilt series, while several data acquisition schemes are available. Among these, the dose-symmetrical scheme is generally

beneficial for retrieving and maximizing the high-frequency information better, which is important for the subsequent sub-tomogram averaging procedure.[72] The tilt series of projection images is then computationally aligned, with or without the fiducial tracers, and reconstructed with software packages such as IMOD,[73] dynamo,[74] Autom, etc.[75] into a 3D volumetric image known as a tomogram, which represents the block of amorphous ice where the specimen resides. Several computational algorithms, such as the weighted-back projection (WBP), simultaneous iterative reconstruction technique (SIRT),[76] and iterative compressed-sensing optimized non-uniform fast fourier transform reconstruction (ICON),[77] have been developed for the reconstruction step.

2.2.3. Sub-tomogram averaging and segmentation

The tomogram can be further analyzed in several ways. Macromolecular assemblies in multiple copies in a tomogram, such as phages at a certain stage in a bacterium, can be identified visually and then extracted into smaller volumes known as sub-tomograms.[78] Sub-tomograms can be aligned and averaged to produce 3D structures with medium-to-high resolution (Fig. 2). This methodology is called sub-tomogram averaging[79] and has increasingly been adopted for *in situ* observation of molecular architectures of macromolecular assemblies with multiple copies in the cell, such as macromolecular complexes, phages, ribosomes.[80] Florian K M Schur *et al.* have determined the structure of HIV-1 capsid-SP1 to 3.9 Å using this method.[81] Sub-tomogram averaging is widely used to solve structures of protein machines *in vivo*, often as a complementary method for the cryo-EM SPA technology. It is particularly useful when the studied complex is superimposed in projection by confounding densities and rendering the 2D classification in SPA is very difficult.

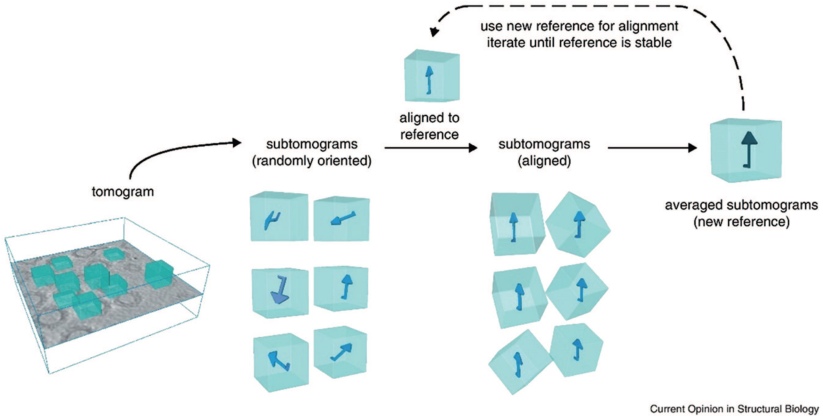


Fig. 2. (color online) Overview of sub-tomogram averaging. Sub-tomograms are extracted from the tomogram. Each sub-tomogram contains a "repeating particle" of interest. Sub-tomograms are rotationally and translationally aligned against a reference. Then, the aligned sub-tomograms are averaged to generate a new reference. Then, the new reference is used for alignment of the sub-tomograms again. This procedure is repeated until the reference stabilizes. Modified with permission from Briggs *et al.*, 2013.

For further non-uniform cellular features of interest, the segmentation method can be applied. Segmentation is performed either manually with specialized visualization packages such as Amira/Avizo[82] or can be achieved automatically with algorithms such as the template-based matching developed by Wolfgang Baumeister's group[83] or convolutional neural-network (CNN) based tools implemented in EMAN 2.2,[84] to produce a multicolor volume for purposes of better visualization. Informative movies clearly illustrating the spatial relationship of each features of interest can be created with segmented data, clarifying the observed morphology and therefore facilitating the derivation of the hypothesis.[85] In certain cases, quantitative statistical analysis can be applied on the segmented tomograms to understand the biological/clinical problems in a more specialized way.[86]

2.3. Integration of cryo-ET with other techniques

When integrated with other techniques, cryo-ET has the potential of mechanistically understanding cellular structural biology across wide size and time scales, sometimes in a more dynamic way.[14] Although cryo-ET can provide structural information on the actual mechanisms of molecular assemblies *in situ* at molecular resolution, atomic details are often missing. This information gap can sometimes be partially complemented by atomic details of those mechanisms *in vitro*, from scientific tools like x-ray crystallography, nuclear magnetic resonance (NMR), and cryo-EM SPA. The integration of cryo-ET with these high-resolution *in vitro* techniques can generate structural information in a more native cellular context, closer to the empirical evidence.[14] Cryo-ET also suffers from shortcomings, such as its smaller field of view, which can be complemented by fluorescent microscopy.[87] Correlative light and electron microscopy (CLEM)[88] is often used to pinpoint areas of interest in a cell on the EM grid before loading the sample for cryo-ET visualization, which gives the researcher structural details at a much higher resolution compared to that of light microscopy. Another challenge for cryo-ET is the thickness of the large eukaryotes. This problem can be solved by an essential integrative technique for cryo-ET: the cryo-focused ion beam (cryo-FIB) milling.[89–91] Cryo-FIB has been shown

to reproducibly yield homogenously thin, distortion-free, vitreous sections which are suitable for cryo-ET study.

Therefore, when combined with integrative approaches, cryo-ET has the potential to investigate structural biophysics in the cellular context. Consequently, cryo-ET is capable of bridging the gap between structural biophysics and cell biology.

2.4. Some examples of cryo-ET

Followed by sub-tomogram averaging, Cryo-ET has widely been adopted to study structural details of macromolecular assemblies.[80] For example, it has been used to obtain the first 3D structure of the flagellar motor of leptospira, with novel features in the flagellar C ring, export apparatus, and stator.[92] Cryo-ET has also been applied to understand the architecture of the flagellar motor, which is responsible for bacterial motility, revealing its functional mechanism from a structural point of view.[93] It has been adopted to reveal the organization of microtubule and actin filaments[94] to substantial detail in both eukaryotic and prokaryotic cells. It is crucial in understanding the nuclear pore complex (NPC) (Fig. 3) and the underlying nuclear lamina.[95]

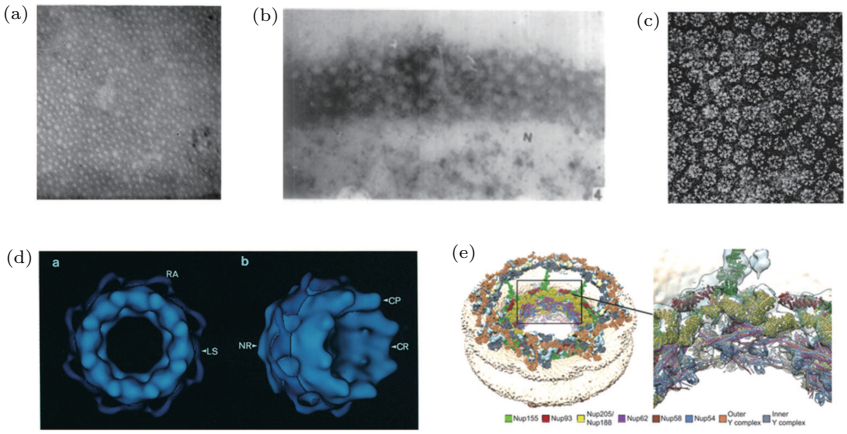


Fig. 3. (color online) TEM Study of nuclear pore complex (NPC). (a) Callan and Tomlin isolated the nuclear membrane of the oocyte of Xenopus laevis, and reported orderly array pores in the outer layer by TEM for the first time in 1950. (b) Using the ultra-thin sectioning technique, Bahr and Beermann reported on the nuclear membrane of salivary glands. (c) Faberge isolated the nuclei of Oocytes of the new Taricha granulosa, and directly demonstrated the eight-fold symmetry of the nuclear pores. (d) Akey and Radermacher isolated the Oocytes of Xenopus, and solved the 3D structure of NPC by Cryo-EM in 1993, with the resolution of 25 Å. (e) Composite structure of the human NPC acquired by docking all available single or subcomplex crystal structures into the cryo-ET map of the intact human NPC (EMD-3103).

Another example should be mentioned is ribosome. Although its purified structure had been extensively studied by cryo-EM SPA,[96–99] little was known about its dynamics and *in situ* structure. Robert Englmeier *et al.* discovered that mitoribosomes are organized in clusters in the inner membrane of the mitochondria, and they also identified the region, which mediates the contact of ribosome with the inner membrane by using sub-tomogram averaging.[100] In the future, more and more attempts are expected to be made to decipher the detailed mechanisms and functions underlying the *in situ* structural architecture of the ribosome using cryo-ET and sub-tomogram averaging.

In certain cases cryo-ET is capable of discovering new phenomena, indicated by structures, which were directly visualized for the first time by it. Two examples are the architecture of the ring formed by tubulin homologue FtsZ in bacterial cell division[101] and bacterial compartmentalization structures created by protein diffusion barriers.[102]

In addition to high-resolution structures obtained from averaging, pleomorphic structures are also extensively studied by cryo-ET, often followed by other integrative methods such as CLEM or cryo-FIB. For example, research on human platelet structural variation with cryo-ET (Fig. 4) indicates the possibility of using cryo-ET as a clinical diagnostic tool.[86] Furthermore, an *in situ* structure of Chlamydomonas chloroplast is visualized using cryo-ET, in combination with cryo-FIB,[103] and cryo-ET, together with CLEM, are used to discover a novel member of the bacterial cytoskeleton.[104]

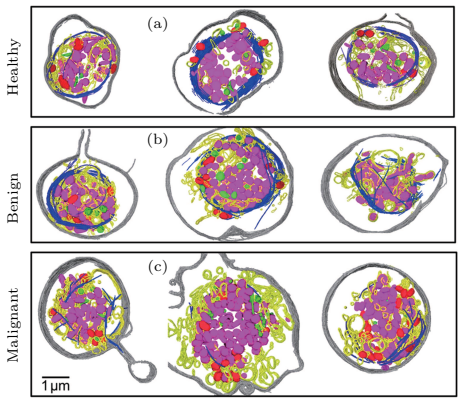


Fig. 4. (color online) Cryo-ET has potential as a clinical diagnostic technique on a single cell level. Platelets from control subjects and patients with cancer, annotated tomogram. (a) Three randomly selected annotated platelets from healthy donors. All of them have an intact marginal band of microtubules (blue) enclosing most of their granules (α pink, dense green) and mitochondria (red) inside. The plasma membrane is gray and the low-contrast vacuole-like (LCV) feature is yellow. (b) Three randomly selected annotated platelets from patients with benign masses. Their structures are similar to those shown in panel (a). (c) Three randomly selected annotated platelets from patients with invasive ovarian cancer. Their morphologies appear to be more heterogeneous compared with the six platelets in the other two panels. They seem to have more LCV, fewer and shorter microtubule filaments, and more mitochondria. Modified with permission from Wang *et al.*, 2015.

3. Technical advances of cryo-ET

Cryo-EM associated technologies have been actively developed in the last few years. In this chapter, we briefly discuss them with an emphasis on their applications in the cryo-ET field.

3.1. Direct detection device

Previously, charge-coupled detectors (CCDs) were used in cameras for image acquisition for cryo-EM and cryo-ET.[105] Electrons are not directly detected in a CCD camera, but they are converted to photons when passing through a scintillator. Following this, a fiber-optic bundle transmits the photons and converts them to charges before detection on the sensor. Although faster than the film as a recording device, the conversions not only degrade the high-frequency information, but also inevitably append noise to the image, reducing the chance of retrieving high-resolution signal significantly.[11] The introduction of direct detector device (DDD) cameras[106] revolutionized both cryo-EM and cryo-ET.

As its name suggests, DDD cameras have a complementary metal-oxide semiconductor (CMOS) chip enabling direct detection of electrons.[107] These cameras exhibit a significant improvement in detective quantum efficiency (DQE) compared to CCDs.[108] DDDs also have a high frame rate, enabling fine-tuning of the acquired data if motion correction is performed. Motion correction is crucial for achieving atomic resolution in cryo-EM studies as it significantly reduces the beam-induced movement of the sample during exposure.[16] Among the commercially available DDD cameras, the Gatan K2 Summit is specifically designed for counting individual electrons. Counting further improves DQE, eliminates noise, and reduces the artifacts due to the point spread function (PSF) effect from each incident electron hitting the camera.[109] With the application of DDDs, both cryo-EM and cryo-ET have experienced a so-called "resolution revolution".

3.2. Phase plate

Although the "resolution revolution"[110] significantly enhanced the performance of both cryo-EM and cryo-ET, both techniques still suffer from the fundamental dose limit problem. This is further exacerbated by the low contrast of the cryo-ET images of frozen hydrated biological samples predominantly comprising of light elements. As phase contrast, not amplitude contrast is the dominant form of contrast in the cryo-ET pictures, enhancing the phase contrast of the cryo-ET images is crucial for retrieving structural information from biological specimens. Traditionally, the phase contrast in a typical cryo-ET imaging session is generated by defocusing the objective lens.[111] However, images produced using this method, especially those taken under high under-focus conditions, suffer from a dramatic degradation of high-frequency signal; therefore, a novel methodology is required.

The idea of phase plate was theoretically proposed during the early application of cryo-EM, and phase contrast can be generated over a wide range of spatial frequencies without the need of defocusing.[112] Phase plate has been adopted in cryo-ET studies and it enables the visualization of unprecedented ultrastructural details in a cellular environment. The application of phase plate to cryo-ET begin with the use of the thin film Zernike phase plate (ZPP),[113] which comprises a thin carbon film with a tiny hole at the center. Dai *et al.* successfully used ZPP to investigate the maturation process of cyanophage Syn5 inside Synechococcus host cells.[110,111] Although high quality results can be achieved, ZPP has practical limitations. The first problem with ZPP is its short lifespan: the performance of ZPP degrades quickly and therefore it needs to be replaced frequently. A further problem is that ZPP cannot be used in automated data acquisition sessions, as the hole of the ZPP on the beam path needs to be centered manually. These problems lead to low efficiency in ZPP tomographic data collection; therefore, restricting its range of application.

Another type of phase plate, the Volta phase plate (VPP), which solved most of the practical issues with ZPP, was proposed by Radostin Danev.[114] The design of the VPP is very similar to that of the ZPP, but without the central hole. When being irradiated by an electron beam, the heated carbon film displays a negative "Volta potential" which, when used constructively, generates a phase shift with the same sign and magnitude to that of a ZPP. Experimental evidence suggests that beam-induced surface modifications are essential in the process resulting in the "Volta potential". Contrary to the ZPP, the VPP has a relatively longer usable life. Furthermore, images taken with the VPP do not show fringing artifacts commonly seen in images taken with the ZPP. Moreover, as the phase shift is self-created by the electron beam, a VPP can be easily implemented in the automated tomographic image acquisition schemes. Owing to these improvements over the ZPP, the VPP has been widely used in cryo-ET studies in several systems following its recent introduction. Asano *et al.* studied the spatial distribution, state of assembly, and conformational states of 26S proteasome in neurons using VPP cryo-ET, proving the potential of cryo-ET to reveal structural information *in situ*.[115] Mahamid *et al.* observed the meshwork of nuclear lamina *in situ* for the first time with this approach.[116] Englmeier R *et al.* studied ribosomes with VPP cryo-ET. In their work, human ribosomes tethering to the inner membrane of mitochondria could not be identified clearly in the tomograms for later classification and for sub-tomogram averaging procedures without the application of VPP.[100] The performance of the VPP was also tested in combination with other techniques. For instance, Fan X *et al.* demonstrated the feasibility of combining the VPP with spherical aberration (Cs) correction for high-resolution imaging at both over and under-focus conditions.[117] VPP cryo-ET is powerful, but still suffers from practical issues, such as the electrostatic charging of the specimen[118] and challenges for maintaining accurate focus throughout the tilt series.[118,119]

3.3. Cryo-focused ion beam

A further important limitation of the cryo-ET is the accessible thickness of the specimen; thus, the cryo-ET approach is not suitable for most of the eukaryotic cells with thickness over 1 μm. Therefore, the thickness problem needs to be addressed.

Several solutions have been suggested to address this problem. One approach is to mechanically cut the biological specimens to sufficiently thin sections with a diamond knife.[120] This approach is known as cryo-electron microscopy of vitreous section, or CEMOVIS, for short.[121,122] So far, different types of specimens, such as mitochondria, human skin, microtubules, bacteria, and bacterial spores, etc., have been studied with this method.[123–127] However, this technique does have limitations. The most severe one is the compression along the cutting direction, which almost inevitably distorts the observed structure to a certain degree,[128] significantly complicating the accurate scientific interpretation of the data. Because of this problem, another approach, the cryo-focused ion beam (cryo-FIB) milling has emerged as an alternative.[129–132]

In cryo-FIB milling, the regions of interest within the sample are abraded layer-by-layer by a beam of accelerated ions from both above and below, and consequently, lamellas with thickness of approximately 200 nm, suitable for cryo-ET analysis, are obtained.[133] Cryo-FIB milling significantly expands the boundaries of cryo-ET, rendering almost any interior regions of interest in any types of cells suitable for cryo-ET study.[89] As a result, cryo-FIB milling, in combination with cryo-ET, has been increasingly applied in dissecting interesting features, reactions, and processes in cells or simple organisms. In the following, a few examples are provided. Jasnin *et al.* studied the effect of reorganization of actin filaments on the ability of Dictyostelium discoideum to adapt to the environment.[94] Hagen and colleagues discovered the mechanism underlying the herpesvirus capsid budding from the nuclear envelope.[134] A research conducted by Wolfgang Baumeister's group showed the native architecture of Chlamydomonas chloroplast *in situ*.[103] These significant achievements by cryo-FIB milling demonstrate the feasibility of this technique used together with cryo-ET to prepare and study thin, distortion-free lamellae of frozen hydrated biological materials *in situ*.[135]

3.4. Correlative light and electron microscopy

Correlative light and electron microscopy (CLEM) has become widely used in structural studies of cells and tissues. Cryo-CLEM combines spatiotemporal information from dynamic fluorescent microscopy with high-resolution *in situ* structural architecture from cryo-ET.[88,90,136] As previously shown, cryo-ET alone has several limitations. Sample thickness is a major issue, which can be solved by combing cryo-ET with cryo-FIB.[137] Its inability to unambiguously locate a particular reaction or process within the crowded cellular environment, partly due to the low contrast, low signal-to-noise ratio (SNR), and low copy number of target molecules of cryo-ET, is another limitation which restricts its applicability. Moreover, cryo-ET typically has a relatively smaller field of view compared to light microscopy, which further limits its potential of locating specific areas of interest in large cells. CLEM, which undergoes many technical improvements recently,[88] can be adopted as a complementary approach to cryo-ET, because it can accurately label and target specific complexes or events using fluorescent dye. This integrative method has been applied to study cell division in vitrified Streptomyces bacteria.[138] It was also applied to study virus infected or transfected mammalian cells.[139] Therefore, CLEM together with cryo-ET, has the potential of bridging the gap between cellular biology and structural biophysics.

3.5. Accurate contrast transfer function correction

Observing macromolecular complexes *in situ* is a significant accomplishment, but currently achieving near-atomic resolution routinely is very challenging for sub-tomogram averaging. One major problem is the difficulty to accurately perform contrast transfer function (CTF) correction for the tomographic data. As CTF is an oscillating sine function depending on the defocus parameter, determining the accurate defocus is essential. It should be noted that there is a defocus gradient across the tilting angle within each tilt series collected. Therefore, each tilting projection needs to be CTF-corrected multiple times. This is commonly performed by a strip-based correction algorithm. Moreover, thick specimens, such as cells, have multiple features of interests reside in different *Z*-heights within the sample. A recent publication by Turonova *et al.* introduced a NovaCTF-correction algorithm, where both the CTF gradient across the tilting angles and the CTF gradient due to the sample thickness are considered.[36] They achieved a result with 3.4-Å resolution with sub-tomogram averaging using this correction method. Galaz-Montoya *et al.* also demonstrated their CTF-correction method, which can routinely yield sub-tomogram averages close to 4/5 Nyquist frequency of the detector under their experimental conditions.[140] Performing accurate CTF correction for tomographic data has been gradually becoming a routine in the cryo-ET field.

4. Concluding remarks

Although the Nobel Prize in Chemistry 2017 was awarded for the cryo-EM, it continues to advance in various aspects, such as detection device, specimen thinning, image acquisition, computational algorithms, etc. These advancements, combined with integrative approaches such as CLEM and cryo-FIB, significantly enhance the applicability and performance of cryo-ET and turning it into a widely chosen technique in the near future.

Due to sample thickness, one of the major limitations of cryo-ET, only small cellular fragments, such as platelets as

shown in Fig. 4, or certain bacterial cells, can be directly observed with cryo-ET. Recently, FIB milling is increasingly becoming a complementary approach to cryo-ET. Cryo-FIB uses a beam of gallium close to the horizontal axis of the sample in a cryo-SEM to ablate material, generating thin sections (typically 100 nm–200 nm) suitable for cryo-ET study.

Another challenge of performing structural biology *in situ* with cryo-ET is to accurately locate the molecules of interest within large cells or tissues. This can be addressed by combining cryo-ET with CLEM. It should be noted that the relatively low resolution of light microscopy is one major limitation for this technique. Recently, the application of super-resolution fluorescence microscopy to cryogenic conditions is under development. This integrative approach, which exploits the advantages of both CLEM and cryo-ET, bridges the gap between cellular biology and structural biophysics.

Advances in detection devices, optics, and computational algorithms are also important. The imaging with 300-kV Titan Krios, using a parallel electron beam, energy filter, VPP, and a K2 Summit camera (or an even better K3 camera) in both super-resolution and electron counting mode with the motion correction applied, can significantly enhance the quality of the information-rich raw tilt images before reconstructing them into 3D tomograms. Furthermore, with the development of computational algorithms like ICON, the missing-wedge problem due to incomplete angular sampling during the image acquisition might be alleviated. Automated segmentation tools like CNN noticeably reduces the time needed to perform laborious manual tomogram segmentation, raising the possibility of achieving high-throughput tomography. The accurate three-dimensional CTF correction algorithm in sub-tomogram averaging enables the era of visual proteomics at atomic resolution.

Theoretically, with integrative approaches, such as CLEM and cryo-FIB, cryo-ET, together with the advancements in optics, detection devices, and computational algorithms, have the potential to study any macromolecular complexes, cellular events, metabolic processes, in any types of cell, to high-resolution detail (shown in Fig. 5). To reach the ultimate aim of structural cellular biology, there is still a long way to go, but we believe that the bright future of cryo-ET is getting closer.

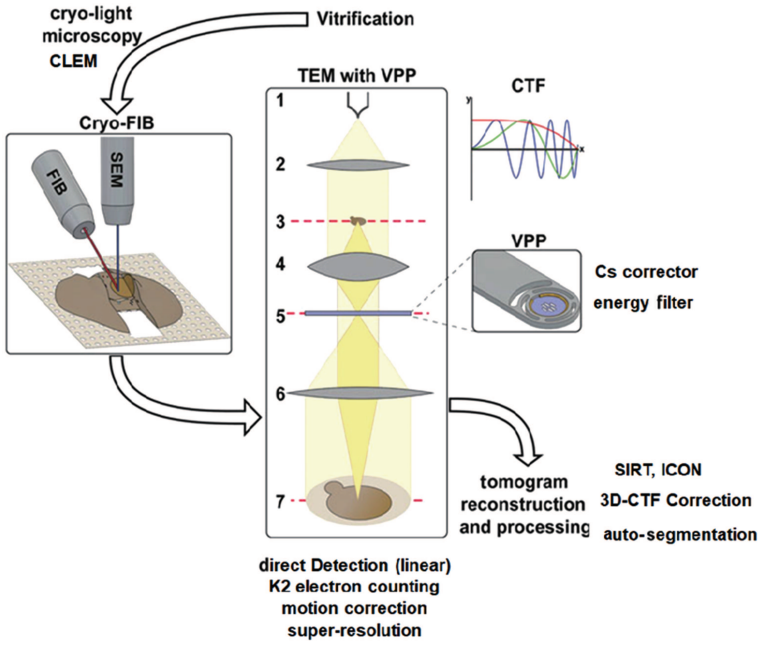


Fig. 5. Future of cryo-ET. Cryo-ET alone has a variety of limitations. By integration with other approaches, such as CLEM, cryo-FIB milling, it can target any interior structures within any types of cell. The advancements in optics, such as the VPP, spherical aberration corrector and energy filter, the improvements in detection devices, such as direct detection, K2 summit electron counting, and also better computational algorithms, such as SIRT, ICON, 3D-CTF correction, and automatic segmentation, enable high-resolution imaging of any macromolecular complexes, cellular events within any type of cell, in the future. Modified with permission from Wagner *et al.*, 2017.

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