Received: 19 November 2019 | Revised: 12 December 2019 | Accepted: 16 December 2019

DOI: 10.1002/pro.3805

REVIEW

THE PROTEIN SOCIETY WILEY

# Developments, applications, and prospects of cryo-electron microscopy

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**Funding information**
Starting fund for talent introduction of Fenyang College of Shanxi Medical University, Grant/Award Number: 2020A01

**Abstract**

Cryo-electron microscopy (cryo-EM) is a structural biological method that is used to determine the 3D structures of biomacromolecules. After years of development, cryo-EM has made great achievements, which has led to a revolution in structural biology. In this article, the principle, characteristics, history, current situation, workflow, and common problems of cryo-EM are systematically reviewed. In addition, the new development direction of cryo-EM—cryo-electron tomography (cryo-ET), is discussed in detail. Also, cryo-EM is prospected from the following aspects: the structural analysis of small proteins, the improvement of resolution and efficiency, and the relationship between cryo-EM and drug development. This review is dedicated to giving readers a comprehensive understanding of the development and application of cryo-EM, and to bringing them new insights.

**KEYWORDS**

3D reconstruction, 3D structure, cryo-electron microscopy, structural biology, X-ray crystallography

## 1 | OVERVIEW OF CRYOELECTRON MICROSCOPY

For decades, electron microscopy has been used to study biomacromolecules and related complexes. With the accumulation of experience and the deepening of understanding, electron microscopy has been developed to study the structure of biomacromolecules. In recent years, the continuous development of cryogenics and rapid freezing technology has led to the birth of cryo-electron microscopy (cryo-EM), that is, the micro technology of using transmission electron microscope to observe samples at low temperature.

The basic principle of cryo-EM is to image biological macromolecules frozen and fixed in glassy ice, thereby obtaining the projection of protein molecules in all directions. A computer is then used to process and calculate a large number of 2D (two-dimensional) images, and reconstruct the 3D (three-dimensional) structure of the biomacromolecule.[1] 3D reconstruction is used to deduce 3D structure from 2D images. Its theoretical basis is the central section theorem proposed by Aaron Klug in 1968[2]: That is, the Fourier transform of a function projected along a certain direction is equal to the cross-section function of the function whose Fourier transform passes through the origin and is perpendicular to the projection direction.

In recent years, cryo-EM has made great achievements in the determination of macromolecular structure, especially the structures of supramolecular systems. The breakthrough of this technology has led to a revolution in structural biology. In 2017, the Nobel Prize in chemistry was awarded to three biophysicists, Jacques Dubochet, Joachim Frank, and Richard Henderson (Figure 1), for their pioneering contributions to the development of cryo-EM.

Xu Benjin and Liu Ling contributed equally to this work

  


**FIGURE 1** Three Nobel Prize winners in chemistry in 2017 (https://www.nobelprize.org/prizes/chemistry/2017)

## 2 | THE AGE OF TRIPARTITE CONFRONTATION

Structural biology was born in the middle of the last century. It is a discipline that elucidates life phenomena by studying the structure and function of biological macromolecules. At present, there are three kinds of commonly used structural biology research methods, namely, X-ray crystallography, nuclear magnetic resonance (NMR), and cryo-EM, which together constitute the basis of high-resolution structural biology research. In the past half century, X-ray crystallography has been dominant in structural biology. As of October 17, 2019, the crystal structures in the PDB database account for about 89% of the total, the NMR structures account for 8.2%, and the EM structures account for 2.5% (Figure 2). X-ray crystallography is mainly applicable to proteins with molecular weight of 10~150 kDa. After decades of development and maturity, more than 140,000 protein structures have been resolved, which has become the main means to analyze the structure of biological macromolecules. NMR is often used to study proteins with molecular weight less than 80 kDa. The advantage of this method is that it can determine the structure of protein molecules in solution, and study the dynamic process and the corresponding energy change of biological samples. However, the application of NMR is limited due to the requirement of high sample concentration (usually at the mM level) and high stability. NMR structures are also at relatively low resolution.

With the rapid development of cryo-EM, the minimum molecular weight limit of sample particles has been

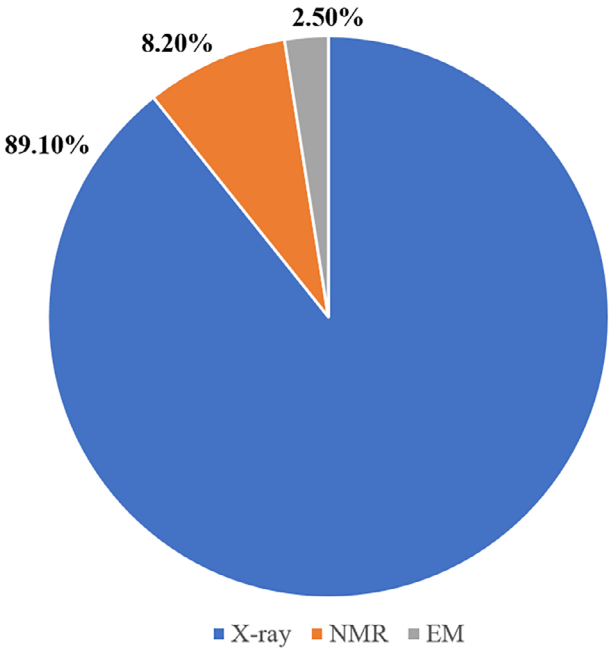


**FIGURE 2** Structure proportion of the three structural biology methods. Blue corresponds to structures determined by X-ray crystallography, dark orange corresponds to structures determined by nuclear magnetic resonance, and gray corresponds to structures determined by electron microscopy

pushed to 52 kDa,[3] the resolution has also been continuously improved, and the freezing method has been more advanced. This has made cryo-EM faster and more efficient, so that it can compete or even replace X-ray crystallography in many aspects.[4] Compared with traditional structural biology methods such as X-ray crystallography

and NMR, cryo-EM has the following advantages: (a) it does not need crystals; (b) it is suitable for proteins and their complexes of large molecular weight; (c) it reduces radiation damage and maintains the native activity and functional state of samples, including posttranslational modifications; (d) multiple different conformational states can be captured in one experiment; (e) it is suitable for the structural analysis of membrane proteins such as GPCR and their complexes; (f) when encountering some structures that cannot be resolved by conventional X-ray crystallography, cryo-EM is still the mainstream. Of course, cryo-EM also has its own shortcomings or defects: (a) cryo-EM can only be used for a large protein complex, which generally requires a minimum of 150 kDa or even more than 300 kDa, otherwise, the protein is too small to find under the electron microscope; (b) the requirement of sample homogeneity is very high, it will be difficult to get good results if the protein is flexible; (c) the current resolution of cryo-EM is about 3 Å, and is below 3 Å only in a few cases, which is still a certain gap from the demand of pharmaceutical research and development (2 Å).

## 3 | HISTORY AND CURRENT SITUATION OF CRYO-EM

In 1931, Max Knoll, a German physicist, and his student Ernst Ruska, invented the first transmission electron microscope, and in 1933, they broke through the limit of the optical microscope for the first time, making it possible to see even smaller particles. Electron microscopy imaging needs to be performed in a high vacuum, and the radiation damage of electrons to biological samples is very large. Therefore, in the following decades, scientists could only image biological samples through heavy metal salt staining.[5] In 1968, De Rosier and Klug proposed and established the general concept and method of 3D reconstruction, and reported the 3D structure of the T4 phage tail,[2] the first electron microscope structure. In order to reduce the radiation damage caused by high-energy electrons, Taylor and Glaeser in 1974 proposed the use of cryo-EM.[6] The wheel of historical development is rolling forward! In 1981, Jacques Dubochet and his colleague Alasdair McDowall made a breakthrough in electron microscopy technology. They introduced the method of rapid freezing,[7,8] which quickly froze molecules in a single layer of vitreous state water. This solved the above two problems of high vacuum and radiation damage. The process of vitrification can not only keep the sample in its natural state, but also protects them from dehydration.[5]

However, there are still many challenges to achieve atomic resolution. For example, the contrast and signal-to-noise ratio in electron microscope images is low. Also one needs to transform 2D images into 3D structure. Joachim Frank and his colleague, Marin van Heel, took the lead in using computational image processing techniques[9–11] to calculate and average multiple image copies of biomacromolecules to improve the signal-to-noise ratio. After obtaining 2D images with different angles, 3D reconstruction is carried out by computer software to analyze the 3D structure of biomolecules. By 1999, the resolution of 50S large subunit of *Escherichia coli* ribosome had been improved to 7.5 Å.[12] After about 2000, the number of electron microscope structures began to increase year by year, but the resolution was not high. However, after 2013, the number of structures analyzed by electron microscopy began to improve rapidly and the resolution also increased to near atomic level. The structure of β-galactosidase reported in 2015 had a resolution of 2.2 Å.[13] Also, the resolution of the 20S proteasome[14] and the 70S-EF-Tu complex[15] were 2.8 Å and 2.9 Å, respectively (Figure 3).

During the past few years, cryo-EM has made a lot of progress in computing image processing, such as the development of user-friendly software,[16–18] and the use of direct electronic detectors. In 2016, the 3D structure of glutamate dehydrogenase (334 kDa) was reported, and the resolution even reached 1.8 Å.[19] It is the development of these technologies that has made the application of structural analysis based on cryo-EM more and more extensive (see timeline in Figure 3). By October 17, 2019, the total number of electron microscope structures in the PDB database had reached 3,874, and it is believed that the growth momentum will be more rapid in the future.

## 4 | PROCEDURE AND COMMON PROBLEMS OF STRUCTURE ANALYSIS BY CRYO-EM

The general process of structure analysis by cryo-EM is as follows: (a) protein expression and purification: samples with high purity, homogeneity, and integrity need to be obtained, and the molecular sieve needs to show a single peak and symmetrical distribution; (b) negative staining: the sample molecules are embedded in a layer of heavy metal salt (commonly uranium acetate) solution, so that the heavy metal salt surrounds the molecules. With the yeast small subunit processome, for example, 4 μl of SSU processome at an absorbance of 0.6 mAU at 260 nm can make a sample[20]; (c) data collection of negative staining sample (sometimes omitting this step according to the situation); (d) freezing sample: This is very important and usually includes two steps, first load the sample on the grid to form a thin water film, and then quickly freeze. In most cases, water can be made glassy by immersing the

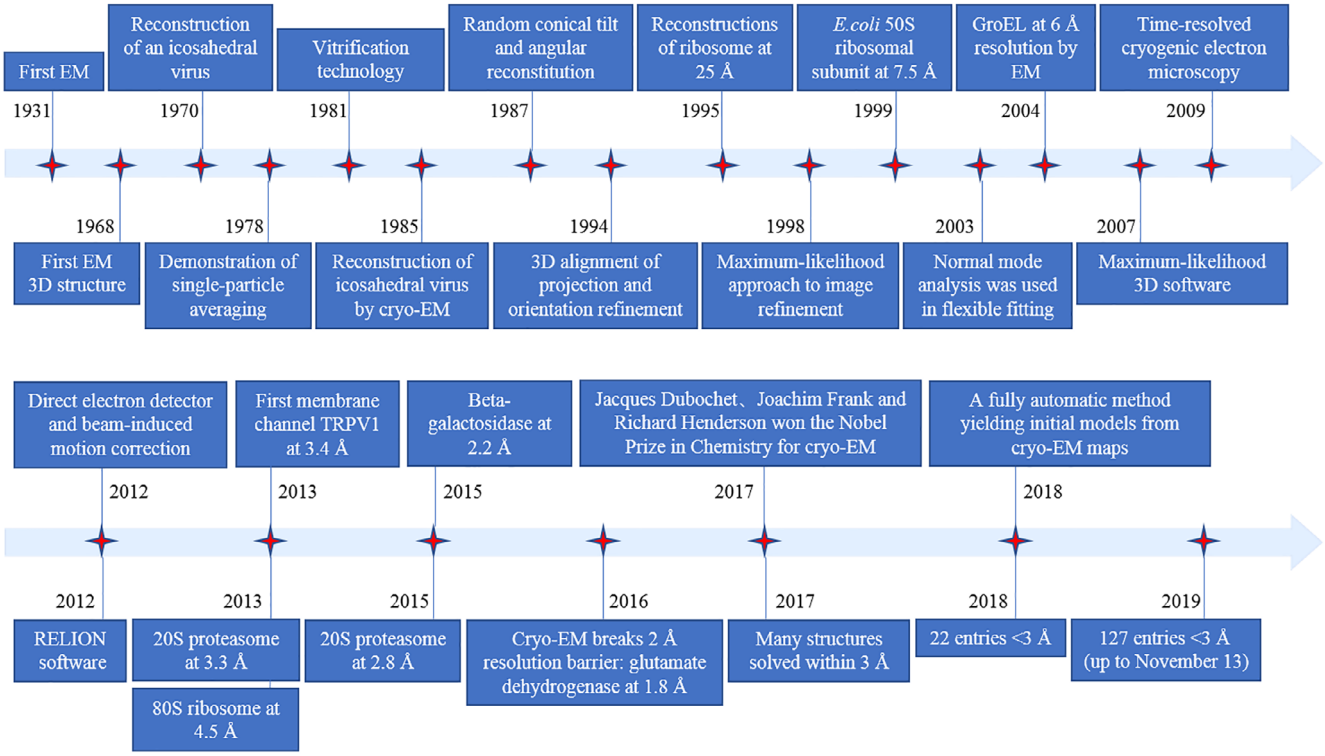


**FIGURE 3** Timeline of key events in the development of cryo-EM

grid into liquid ethane quickly by manual operation, which has the advantage that the sample can be close to the "natural" state. Because biochemical reactions, especially some enzymatic reactions, take place rapidly, another method named fast mixing/spraying microfluidic chips[21–23] has been developed in order to obtain the structural information of the intermediate state of the reaction. That is, mixing the two molecular systems in milliseconds, and then freezing them quickly so that one can capture the intermediate steps in the biochemical reactions. This method can achieve time resolution of tens of milliseconds, which is very suitable for studying short-term biological events, such as ribosome recycling, translation initiation, and other processes.[23,24] Generally speaking, the sample concentration of soluble protein is about 1 mg/ml and membrane protein is about 5 mg/ml. Take eukaryotic ribosome for example, 3 μl of ~80 nM can make a sample[25]; (e) load the sample in the cryo-EM; (f) screen sample: it is necessary to screen the sample before data collection to check whether the water in the sample is in a glassy state and whether the thickness of ice layer is appropriate. If there is a problem, the sample needs to be prepared again; (g) data collection: select good particles to take photos, and the minimum exposure technology must be used in the photography because the high-energy electrons have radiation damage to the sample; (h) three-dimensional reconstruction.

Some problems may be encountered in the process of using cryo-EM, mainly in the following aspects: (a) the sample is unstable, degraded, or aggregated; (b) some ligands with small molecular weight, may not be seen in the density map; (c) there may be organic substances such as sugar, DMSO, or glycerin in the buffer, resulting in the decrease of sample contrast and resolution; (d) the purity of the sample may be good or even very good, but the homogeneity is poor, which greatly reduces the resolution; (e) the target area may have greater flexibility, after 2D or 3D averaging, the resolution of the target area becomes very poor; (f) samples may be destroyed during freezing; (g) in addition to the samples, there are many parameters to be optimized, such as sample concentration, block time, temperature, grid specifications, and so forth. Therefore, cryo-EM needs rich experience and sufficient machine time, and good experimental results really need the right time and the right place.

## 5 | BREAKTHROUGHS IN CRYO-EM

During the last few years, a series of important breakthroughs have been made in the field of structural biology by the combination of cryo-EM and 3D

reconstruction. The breakthrough of cryo-EM mainly comes from the development of hardware and software. The hardware includes the electron microscope and the image recording device; the software includes 3D reconstruction algorithms and image data processing.[1]

### 5.1 | Electron microscope

The Titan Krios is the most advanced cryo-EM at present, which has made great improvements in the following six aspects: (a) 300 kV field emission low temperature transmission electron microscope, equipped with field emission electron gun and three-stage condenser system, which can realize parallel illumination within a certain illumination range; (b) an automatic sample injection system that can load and store 12 frozen samples at one time, improving the sample efficiency; (c) an automatic liquid nitrogen filling system, which can automatically maintain the low temperature of the sample chamber and the lens cone for extended operation; (d) a maximum tilt angle of the sample table of 70°, and a horizontal rotation of 90°; (e) a constant power mode magnetic lens system that ensures the high stability of imaging; (f) a point resolution that can reach 0.25 nm, and on information resolution limit which can reach 0.14 nm.

### 5.2 | Direct electron detector

The development of new detector hardware led to the resolution revolution of cryo-EM. A series of new and attractive near atomic resolution structures have been obtained, which fully proves this point. For example, the complex structure of pathogen ribosome and drug molecules,[26] the large subunit structure of mitochondrial ribosome,[27] the structure of mammalian ribosome complex,[28] and the structure of TRPV1 ion channel.[29] The new direct electronic detector is more sensitive and faster than the traditional medium (CCD camera or photographic film), and can record image data at the rate of multiple frames per second instead of single long-time exposure. The direct electronic detection camera not only has a significant improvement in the detection quantum efficiency, but also has a high frame rate, enables images to be acquired as "movies" made of stacked frames.[30] In the future, desire for higher resolution will certainly promote the development of electron microscopy in many aspects: thinner detector chip and faster readout rate, so as to greatly improve the signal-to-noise ratio of resolution; improve the sample preparation method and use the holder that is not easy to move under electron bombardment; develop new image recording programs to reduce sample movement caused by light beams.[31]

### 5.3 | Image processing and algorithm

Low signal-to-noise ratio or poor image contrast is the main challenge of cryo-EM, which is difficult to avoid as biological samples are sensitive to radiation. It can only be improved by reducing the electron dose, which in turn results in a very low signal-to-noise ratio. The development and application of new direct electronic detectors have greatly promoted the development of image processing and algorithms.[32,33] In order to extract valuable structural information from noisy images, researchers have developed an assembly based on the maximum possible and prior information of undetermined structure.[34] This new software can work without user intervention to a large extent, and provides objective resolution standards, which quickly become the "gold standard" of cryo-EM.[31] The new standardized verification procedure makes the results (including particle orientation, absolute chirality, and contrast loss) more reliable,[35] and can avoid over fitting noise to generate clearer images.[36] The powerful classification method can separate different molecules or different structural states of the same molecule in the same field of vision. Therefore, the development and progress of the new algorithm make people have a new understanding of sample processing: Samples are purified in computers, rather than through arduous, time-consuming, and often destructive biochemical processes.[31]

## 6 | APPLICATIONS OF CRYO-EM

### 6.1 | Gene expression and regulation related complexes

Cryo-EM is mainly used to determine the structure of biological macromolecules and macromolecular super complexes. The most typical examples are spliceosomes and chromosomes. The splicing of RNA is performed by the spliceosome, a super protein enzyme complex, and abnormal splicing leads to many genetic diseases. Yigong Shi, a member of Tsinghua University, reported the high-resolution cryo-EM structures of yeast and human spliceosomes in different catalytic states,[37–41] revealing the mechanism of spliceosome catalyzed mRNA processing, providing an important basis for understanding the RNA splicing process of higher organisms. In 2014, Song et al.[42] successfully established a platform for in vitro reconstruction and structural analysis of

chromatin, and took the lead in analyzing the high-definition 3D structure of 30 nm chromatin by using cryo-EM, making an important breakthrough in the research of higher-order structure of 30 nm chromatin, the carrier of "life information," which provides a structural basis for understanding the mechanism of epigenetic regulation of organisms. In 2019, Li et al.[43] analyzed the cryo-EM structure of the Snf2-nucleosome complex in different nucleotide states, answered the basic principle of DNA translocation in the process of chromatin remodeling, and revealed the mechanism of chromatin remodeling.

## 6.2 | Protein synthesis and degradation related complexes

The most typical examples are the ribosome and the proteasome. The ribosome is a molecular machine for protein synthesis, the determination of its 3D structure is the basis for correct understanding of ribosome function. The high-resolution structures of prokaryotic and eukaryotic cytoplasmic ribosomes have been resolved by X-ray crystallography and cryo-EM, but the high-resolution structures of eukaryotic mitochondrial ribosomes have not been resolved for a long time. The breakthrough of cryo-EM and the development of data classification algorithm provide a powerful means for the determination of mitochondrial ribosome structure and for the investigation of internal rearrangements of the translational apparatus. V. Ramakrishnan of MRC molecular biology laboratory analyzed a series of high-resolution structures of mitochondrial ribosomes by means of cryo-EM. In 2014, they reported the structures of human[44] and yeast[27] mitochondrial ribosome large subunits with resolutions of 3.4 Å and 3.2 Å respectively; they also resolved the structures of human mitochondrial ribosome[45] and yeast mitochondrial ribosome,[46] with resolutions of 3.5 Å and 3.3 Å, respectively. In addition, Ban et al. analyzed the structure of mammalian mitochondrial ribosomal large subunits in 2014, with resolutions of 4.9 Å[47] and 3.4 Å,[48] respectively. These results provide an important structural basis for understanding the molecular mechanism of protein synthesis in mitochondria. The proteasome is a giant protein complex that is common in eukaryotes and archaea, and also exists in some prokaryotes. In eukaryotes, proteasome is widely distributed in the cytoplasm and nucleus. Its core function is to regulate the concentration of specific proteins and to remove misfolded proteins in cells. It is one of the indispensable large-scale all enzyme supramolecular complex machines in the cell, and also the largest protein degradation machine found so far. In 2012, the low resolution cryo-EM structures of human[49] and yeast[50] 26S proteasome were reported. In 2016, Huang et al.[51] and Schweitzer et al.[52] reported the atomic structure of human 26S proteasome with resolution of 3.5 Å and 3.9 Å, respectively. In 2018, Dong et al.[53] determined the high-resolution (2.8~3.6 Å) atomic structures of seven intermediate conformations of human 26S proteasome during the degradation of substrate by combined use of cryo-EM and machine learning technology. This work provides an important structural basis for the activation mechanism of the proteasome core particle gated switch and the kinetic mechanism of substrate transport channels of regulatory particle subcomplex.

## 6.3 | Membrane proteins

These include various ion channels, transporters, and supermembrane protein complexes. They are very important in organisms that participate in many biological processes such as cell proliferation and differentiation, energy transfer, signal transduction, and material transport. It is estimated that about 60% of the drug targets are membrane proteins. The membrane proteins are difficult to purify and crystallize, and it is difficult to resolve their 3D structure by X-ray crystallography. Therefore, the development and breakthrough of cryo-EM bring new hope for the structural analysis of membrane proteins and their complexes. In 2016, Gong et al.[54] first reported the 4.4 Å cryo-EM structure of human cholesterol transporter NPC1; Wu et al.[55] reported the first high-resolution 3D structure of the eukaryotic voltage-gated $Ca^{2+}$ channel, which laid a foundation for understanding the working mechanisms of voltage-gated $Ca^{2+}$ and $Na^{+}$ channels with important physiological and pathological functions. In addition, Zhao et al.[56] in 2019 reported the cryo-EM structure of the mammalian voltage-gated $Ca^{2+}$ (Cav) channel and ligand complex. This clarified the molecular basis for the recognition and regulation of Cav channel by three ligands at the atomic level, and laid the foundation for the future drug research and development of Cav channel disease. In the field of membrane complexes, Wei et al.[57] reported the 3.2 Å cryo-EM structure of spinach photosystem II-light-harvesting complex II (PSII-LHCII) supercomplex in 2016. The results are of great significance to further understand the time dynamics and light protection mechanism of energy transfer in PSII-LHCII supercomplex at the molecular level. In 2017, Su et al.[58] reported the structure of pea PSII-LHCII supercomplex, revealing the supramolecular basis of efficient light harvesting in plants under low light conditions. In 2019, Pi et al.[59] reported the cryo-EM structure of PSII-FCPII supercomplex of *Chaetoceros gracilis*, which provided an important basis for elucidating the unique

mechanism of light energy capture, transfer and transformation and efficient light protection in PSII-FCPII supercomplex of diatom. In addition, Gu et al.[60] first reported the cryo-EM structure of the respiratory chain super complex, respirasome, which has the highest resolution so far in 2016. The results of this study provide an important structural basis for further understanding of the assembly form, molecular mechanism of mammalian respiratory chain complexes and the treatment of cellular respiratory related diseases.

## 6.4 | Viruses and related protein complexes

Viruses are pathogens that cause various diseases of human or animals. The determination of their 3D structures is not only helpful to elucidate the mechanism of virus self-assembly and infection, but also has guiding significance for drug design based on structure. The size of virus particles is larger than that of proteins, so it is more suitable to analyze their 3D structure with cryo-EM. In 2018, Yuan et al.[61] first reported the atomic resolution structure of the nucleocapsid of herpes simplex virus type 2 (HSV-2) of the herpesvirus α family. They clarified the complex interaction mode and fine structural information of the nucleocapsid protein, and proposed the assembly mechanism of the nucleocapsid of the herpesvirus. In the same year, Song et al.[62] revealed the cryo-EM structure of the spike glycoprotein of SARS coronavirus and its receptor ACE2 complex. Yuan et al.[63] revealed the structure and function of MERS-CoV and SARS-CoV spike proteins, providing a key 3D structure map for the follow-up design of broad-spectrum antibody and vaccine. In 2019, Liu et al.[64] first reported the key mechanism of human herpesvirus genome packaging and the genome structure of the virus, which is helpful to prevent and control a variety of diseases caused by herpesvirus, and is expected to transform herpesvirus for targeted treatment. Wang et al.[65] determined the cryo-EM structure of African swine fever (ASF) virus capsid, with a resolution of 4.1 Å, which revealed the basis of capsid stability and assembly, and opened a new way for the development of ASF vaccine.

## 6.5 | Neurodegenerative disease-related proteins

Neurodegenerative disease results in dysfunction due to the gradual loss or even death of neuron structure or function, including amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and Huntington's disease, among others. At present, the cause of this kind of disease is not clear and cannot be cured, which seriously threatens human health and daily life. In 2019, Yang et al.[66] reported the cryo-EM structure of human γ-secretase in complex with a notch fragment, which revealed the structural basis of notch recognition, and is of great significance for γ-secretase to recruit amyloid precursor protein. Then, Zhou et al.[67] reported the cryo-EM structure of human γ-secretase in complex with transmembrane app fragment, which provided important support for the design of substrate specific inhibitors and the understanding of the function of γ-secretase and the pathogenesis of AD. In the same year, Gu et al.[68] resolved the cryo-EM structure of mammalian ATPase tetramer. This explained the structural composition pattern and molecular mechanism of mammalian ATPase, the synergistic relationship between respiratory chain complexes and the influence on the shape of mitochondrial cristae, which provided an important structural basis for the treatment of energy metabolism diseases and neurodegenerative diseases.

## 6.6 | The innate immune system related proteins

The innate immune system forms an evolutionarily ancient defense line, which can resist invading pathogens and endogenous danger signals. In some innate immune cells, inflammasome can sense a series of stimuli, which can produce inflammatory response. The imbalance of inflammatory signals leads to a wide range of immune disorders leading to diseases such as gout, Crohn's disease and sepsis.[69] In 2015, Hu et al.[70] reported the cryo-EM structure of PrgJ-NAIP2-NLRC4ΔCARD complex (inflammasome) in mice, revealing the molecular mechanism of NLRC4 protein induced self-activation in NAIP-NLRC4. Zhang et al.[71] reported the cryo-EM structure of the activated NAIP2-NLRC4 inflammasome and revealed its nucleated polymerization mode. In 2019, Sharif et al.[72] analyzed the cryo-EM structure of human NLRP3-NEK7 complex, and revealed the molecular mechanism of NEK7 mediated activation of NLRP3 inflammasome through the multi-interface interaction with NLRP3 subunit. Wang et al.[73,74] reported the assembly, structure, and function analysis of plant NLR protein complex. This work revealed the key molecular mechanism of NLR function, promoted people's understanding of plant immune mechanism, and was a milestone event in plant immune research. Dong et al.[75] reported the cryo-EM structure of human TCR α/β in complex with CD3 hexamer complex. It revealed the structural basis of TCR-CD3 complex assembly, provided clues for TCR triggering, and laid a foundation for the rational design of immunotherapy for the complex.

## 7 | A NEW DIRECTION OF CRYO-EM—CRYO-ELECTRON TOMOGRAPHY

At present, most of the biomacromolecule complexes analyzed by cryo-EM are assembled in vitro. Only a few have been studied in vivo, as will be necessary to understand the essence of life phenomenon. The next development goal of structural biology will be "in situ structural biology", that is, to study biological macromolecules in physiological and pathological states directly from the cell in situ, and to unify structure and function in the cell.[1,76]

Cryo-electron tomography (cryo-ET) can obtain the 2D projection of the sample in multiple directions by tilting the sample in the microscope, and then reconstructing the 3D structure by calculation. It combines the 3D imaging technology with the sample preparation method to maintain the complete cell structure, so as to achieve the purpose of studying the 3D structure of macromolecules in a more real state.[77] It is very suitable to study the 3D structure of viruses, proteins, and organelles with heterogeneous structure on the nanometer scale.[78] Cryo-ET can not only map the position and interaction of macromolecules in the whole cell environment, but also reveal their in situ high-resolution structures.[79] The 3D reconstruction of cryo-ET generally includes four steps: (a) data collection of 2D electronic projection images with different angles; (b) registration of a series of oblique projection images; (c) 3D reconstruction of tomograms by reverse projection; (d) denoising and distinguishing of reconstruction results.

Cryo-ET is widely used. It can not only analyze the 3D structure of proteins and their complexes at the molecular level, but also study the organelles at the subcellular level and even the tissue structure at the cell level. In recent years, much new knowledge has been gained in the field of cell biology by means of cryo-ET, including the ultrastructure of mitochondria, ribosomes, the endoplasmic reticulum and the Golgi complex, the structure of spindles involved in cell division, the tissue structure of insect flying muscles, the 3D structure of the nuclear pore complex, and the structure of asymmetric and polymorphic viruses.[80]

Compared with single particle technology, the sample resolution obtained by the cryo-ET is not as high, but it is suitable to analyze the structure of some viruses and their complexes that are asymmetric, amorphous, and important in function.[78] Cryo-ET can effectively make up for the shortcomings of other structural analysis techniques, and will be widely used in more laboratories. As predicted by Timothy Baker, a microscientist at Purdue University, in the next 10 years or so, cryo-ET will show explosive development, and it will undoubtedly play a leading role in the field of cell biology.[81]

## 8 | PROSPECTS FOR CRYO-EM

### 8.1 | Structural analysis of small proteins

For a long time, the poor contrast transfer function (CTF) made it difficult for cryo-EM to analyze the structure of small molecular complexes. Therefore, cryo-EM has been considered to be only useful for the structure determination of macromolecular complexes with molecular weight greater than 500 kDa.[82] However, many drug targets are of low molecular weight. In recent years, cryo-EM has developed rapidly, more and more high-resolution structures less than 150 kDa have been resolved. In 2017, Khoshouei et al.[83] introduced the voltage phase plate (VPP) into cryo-EM. By doing so, they analyzed the structure of 64 kDa human hemoglobin, and pushed the lowest molecular weight limit of the electron microscope sample to a new height. In 2019, Hongwei Wang's group from Tsinghua University reported the high-resolution structure of 52 kDa streptavidin with a resolution of 3.2 Å, which is the smallest protein resolved by cryo-EM.[3] Although the phase plate technology needs to be further improved in imaging stability, it provides the possibility for cryo-EM to break through the limit of small molecular scale.

### 8.2 | Resolution improvement

Cryo-EM has made a lot of progress in the past 40 years, but there is still a lot of room for improvement. One of the keys is to further improve the resolution to about 2 Å. The homogeneity of the sample and the flexibility of the particle interior are two limiting factors of the resolution. A nonhomogeneous sample will lead to difficulty in data processing, and may also damage the integrity of the structural information and reduce the resolution. At present, many cryo-EM studies still use intuition to get structure through continuous multiple rounds of classification. We expect focused 3D alignment and classification methods to overcome this problem in the future. In addition, the resolution can also be improved by optimizing the grid preparation process, data acquisition, and data processing. Moreover, the further improvement of direct electronic detectors and phase plates, as well as the comprehensive upgrades of software and hardware, including more advanced optical lens systems and better detectors and algorithms, will enable us to enter the golden age of cryo-EM, by then, the resolution will reach a new height.

## 8.3 | Efficiency improvement

Some in industry point out, it may take another 5–10 years for cryo-EM to become a conventional tool for new drug development. But for many pharmaceutical and biotech companies, now may be the best time to integrate this tool into the R&D system. In X-ray crystallography, data collection only takes a few minutes, and structure analysis is very fast, making it the main means of structure-based research.[5] Cryo-EM samples, however, need to be optimized many times before data collection, and data collection takes hours, if not days. Therefore, the throughput is much less than for crystallography. Therefore, the automation and optimization of sample preparation is one of the most urgent needs of cryo-EM development in the future.[84]

## 8.4 | Cryo-EM and drug development

At present, the rapid, perfect, automatic, and easy to quantify crystal structure screening system is mature. It is hard to imagine what cryo-EM will become in the next few decades. Therefore, it is not impossible for cryo-EM to replace crystallography for conventional drug discovery. However, the more likely outcome is that the two technologies will continue to be the mainstream means of drug discovery, complementing each other and developing together for a long time.

With the continuous development of cryo-EM, we firmly believe that our understanding of many molecular mechanisms, such as those that drive translation or transcription, DNA repair, RNA splicing, cytoskeletal transport, and channel gating mechanisms, will be more profound.

## CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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**How to cite this article:** Benjin X, Ling L. Developments, applications, and prospects of cryo-electron microscopy. *Protein Science*. 2020;29: 872–882. https://doi.org/10.1002/pro.3805