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Cryo-electron microscopy for structural biology: current status and future perspectives

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Recently, significant technical breakthroughs in both hardware equipment and software algorithms have enabled cryo-electron microscopy (cryo-EM) to become one of the most important techniques in biological structural analysis. The technical aspects of cryo-EM define its unique advantages and the direction of development. As a rapidly emerging field, cryo-EM has benefitted from highly interdisciplinary research efforts. Here we review the current status of cryo-EM in the context of structural biology and discuss the technical challenges. It may eventually merge structural and cell biology at multiple scales.

cryo-electron microscopy, structural biology, cell biology, three-dimensional reconstruction

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Since the 1980s, structural biology has become a booming area in life sciences. By determining the three-dimensional (3-D) structures of proteins, nucleic acids, and their complexes, structural biology has given us accurate insights into concerning the shapes of complicated bio-macromolecules, the arrangements of atoms and molecules, and physical properties such as electro-potential distributions and hydrophobicity. These structures provide a crucial understanding of how bio-macromolecules execute specific biological functions. As techniques of structural biology become more mature in the 21st century, they assume more significant roles in various sub-disciplines of life sciences. Of the more than 10^5 protein structures that have been deposited in the Worldwide Protein Data Bank, most were solved by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy (Figure 1A).

Recent technical progress has stimulated the use of new structural biology tools. In December 2013, two landmark *Nature* articles revealed the atomic-resolution structure of the TRPV1 ion channel in membranes that was imaged by cryo-EM [1,2]. With cryo-EM, Yifan Cheng and David Julius et al. [1,2] at the University of California, San Francisco, imaged for the first time the 3-D structure of the membrane channel protein TRVP1 with a resolution of 3.4 Å, and constructed an atomic model. In addition, several nearly atomic models of viruses, proteasomes, and ribosomes have been resolved by cryo-EM in the past few years [3−6]. The significance of the work was to obtain high-resolution structural information from a very small amount of sample, without requiring protein crystallization, and to simultaneously obtain the structures of the protein complex in different states over a relatively short time. In only one year, cryo-EM has received extensive attention as a means to directly obtain atomic structures of bio-macromolecules.

Electron microscopy has been used in structural biology for many years. Since the 1950s, it has revealed cellular, sub-cellular, and bio-macromolecular structures. Based on completely different principles from X-ray crystallography and NMR spectroscopy in solving the structures of bio-macromolecules, mature image acquisition techniques and

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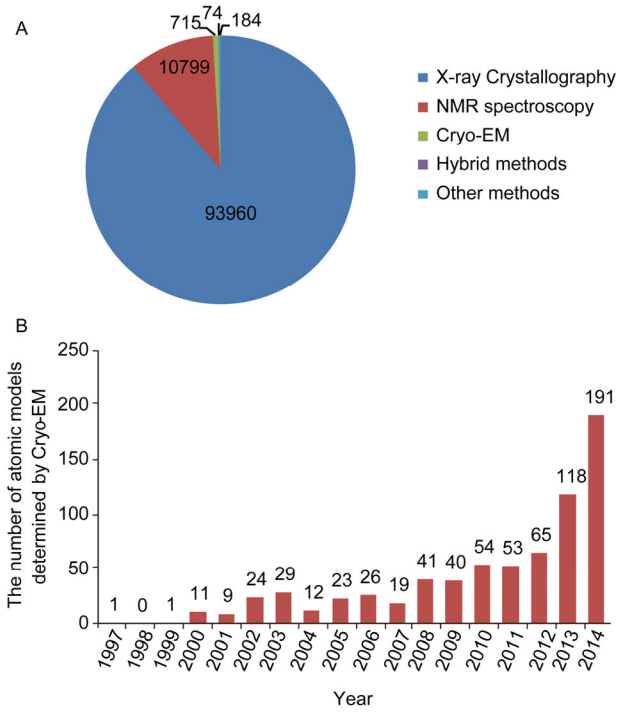

Figure 1 Statistics for atomic models in the Worldwide Protein Structure Data Bank. A, Breakdown of methods used for structures in the Worldwide Protein Structure Data Bank by January 20, 2015. B, Number of atomic models determined by cryo-EM from 1997 to 2014.

analysis algorithms of cryo-EM have been gradually established. In the past 10 years, the sophisticated technologies and the increasing number of cryo-EM research teams have resulted in revolutionary breakthroughs in imaging and structure analysis. Since 2008, the number of atomic structural models for various bio-macromolecular complexes based on cryo-EM data has risen rapidly (Figure 1B).

1 Basic principles of cryo-EM

Cryo-EM is based on transmission electron microscopy (TEM), including the basic steps of sample preparation, imaging, image processing, and structure analysis (Figure 2). In TEM, electrons generated by an electron gun are accelerated to very high energies in the high vacuum of the microscope column. Because high-speed electrons are deflected by magnetic fields, a series of electromagnetic lenses condense the electrons and then focus them on the specimen. The transmitted electrons are detected and form recorded images that are magnified by factors of 10^3–10^5. Computer programs then solve the detailed structure of the sample from the magnified images.

Biological samples present several technical challenges for TEM [7]: (i) the high vacuum is not compatible with hydrated samples; (ii) biological samples are mainly composed of light elements that are vulnerable to damage from high-energy electrons; and (iii) light elements interact with electrons weakly, lowering image contrast. In 1974, Robert M. Glaeser et al. [8] discovered that freezing biological samples at liquid nitrogen temperatures dramatically reduced damage from the electrons. In 1984, Jacques Dubochet et al. [9] improved the liquid nitrogen freezing technique and observed, for the first time, virus particles in vitreous ice. The preparation of rapidly frozen hydrated

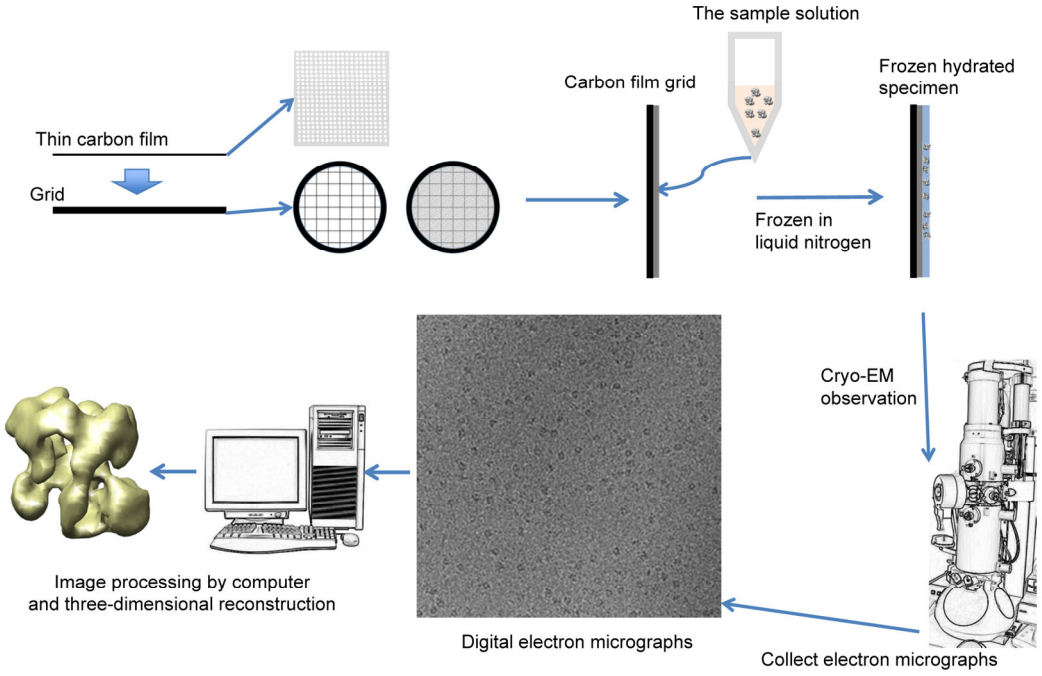

Figure 2 Workflow of cryo-EM structure determination.

biological samples at liquid nitrogen temperatures solves the first problem because the saturated vapor pressure of vitreous ice at that temperature is much lower than the vacuum pressure inside the TEM, and it also solves the second problem because the electron-induced damage is much lower.

During TEM imaging, electron beams penetrate the specimen and project its 3-D electric potential distribution function onto a two-dimensional (2-D) plane that is perpendicular to the direction of the electron beam. In 1968, Aron Klug formulated the "central section" theorem (Figure 3), and proposed that one may use 2-D projections of a 3-D object from different angles to perform a computer 3-D reconstruction of the object [10]. Thus, TEM can be used to obtain magnified projection images of a biological sample from multiple angles, and reconstruct the 3-D structure by computer.

The Fourier transformation of a 2-D projection of a 3-D real-space object along a certain angle is equal to the central section, which is perpendicular to the projection vector of the Fourier transformation of the 3-D object. According to the central section theorem, the 3-D structure can be reconstructed from the 2-D projections of a 3-D object from different angles.

In cryo-EM structural determinations, various imaging and 3-D reconstruction methods are used depending on the nature and geometries of the biological samples. Currently, the most widely used methods to solve bio-macromolecule and sub-cellular structures are electron crystallography, single-particle reconstruction, and electron tomography reconstruction.

1.1 Electron crystallography

Electron crystallography can determine bio-macromolecule structures by using TEM to acquire images or diffraction patterns of highly ordered repeating structures formed in one, two or three dimensions. This method is appropriate for 10–500 kD samples, and the highest reported resolution is about 1.9 Å. Both X-ray crystallography and electron crystallography require highly homogenous, periodic arrangements of bio-macromolecules. However, to determine a structure, electron crystallography can use images of a crystal or its diffraction patterns, whereas X-ray crystallography can only use the latter.

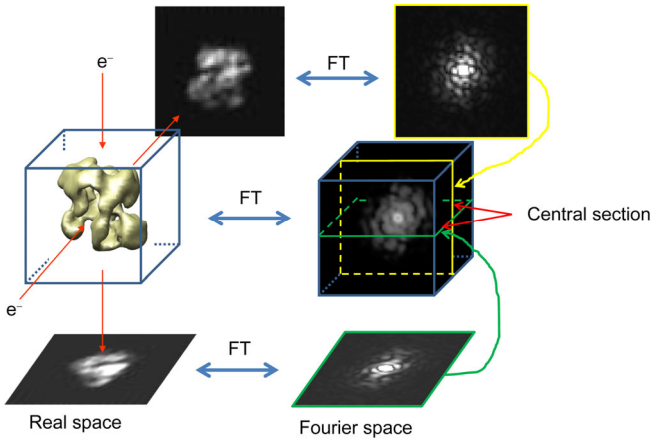

Figure 3 Central section theorem.

1.2 Single-particle reconstruction

In the single-particle reconstruction method, large numbers of sparsely distributed bio-macromolecules are imaged by TEM. The 2-D images are computer-analyzed statistically to obtain enhanced signal-to-noise ratios (SNRs) by alignment, classification, and averaging, based on the assumption that they represent different views of the homogenous 3-D structure. The spatial projection relationships among the various 2-D images are determined to reconstruct the 3-D structure (Figure 4). The method is appropriate for 80 kD–50 MD biomolecules; with the highest reported resolution of about 3 Å. Single particle reconstruction techniques include the common lines, random conical tilt, and random initial model iterative convergence refinement methods. The principal aim of these methods is to determine the correct spatial relationships among the various 2-D images.

1.3 Electron tomography reconstruction

Electron tomography reconstruction uses 2-D TEM images of a specimen that is rotated at multiple angles and subsequently reconstructs a 3-D structure based on the rotational relationships (Figure 5). This method is mainly used for cellular, sub-cellular, and bio-macromolecule complexes without rigid structures (~800 kD). The highest reported resolution is about 20 Å.

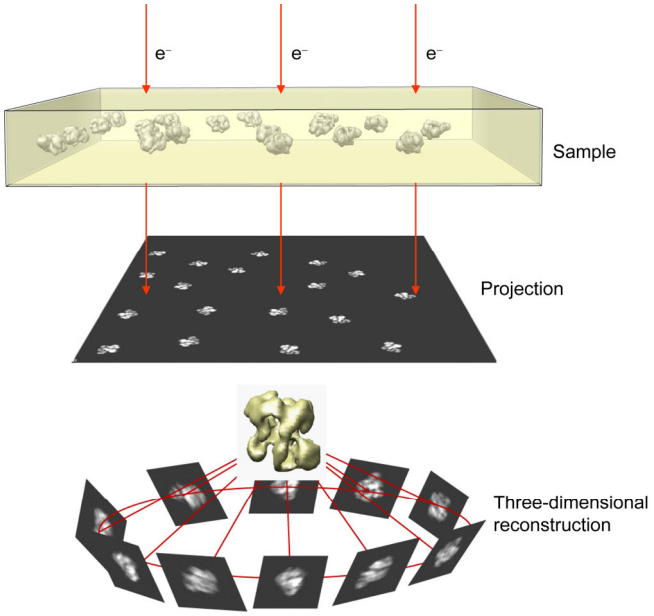

Figure 4 Principle of single-particle reconstruction methods.

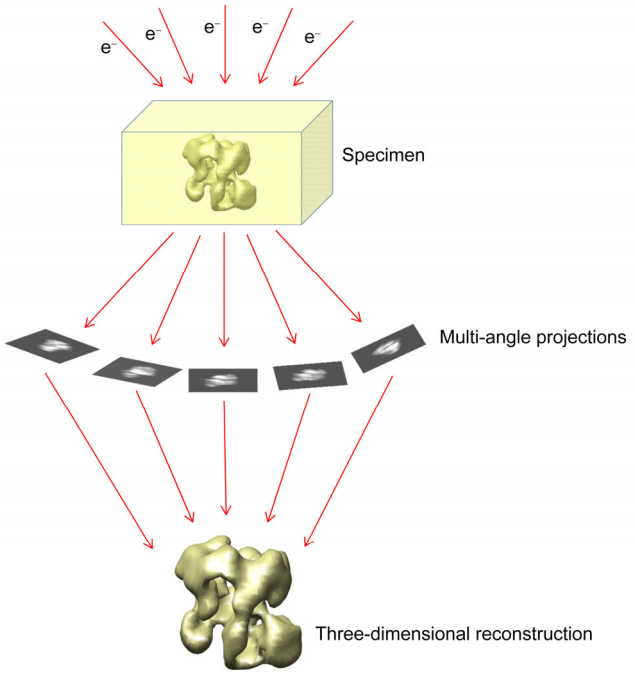

Figure 5 Principle of electron tomography.

2 Advantages of cryo-electron microscopy

2.1 Small samples

Relative to other structural biology techniques, cryo-EM requires very small samples for structure determinations. For example, single-particle reconstruction requires 10^5 particles to reconstruct a near-atomic-resolution structure, as long as the samples have good biochemical properties and high conformational homogeneity [1]. For bio-macromolecule complexes with high symmetry, such as viruses, only 10^4 particles may be needed [11]. A cryo-EM specimen requires 3–5 μL of protein solution at a concentration of 0.1–1 μmol L^{-1}. This is in marked contrast to the much larger samples needed for X-ray crystallography and NMR spectroscopy.

2.2 Samples are closer to the physiological state

For cryo-EM, samples are frozen very rapidly (10^5°C s^{-1}) to fix them in vitreous ice to preserve their hydrated state and to prevent scattering from ordered ice. Therefore, the structural information basically reflects the instant state of the sample before freezing, which is much closer to the natural physiological state than crystallization.

2.3 Wide range of samples

Cryo-EM can be used to characterize samples over a wide range of scales such as cells, organelles, or macromolecule complexes over 500 kD. Recently, with the great improvement in hardware, especially image capture devices, we are able to determine high-resolution structures of 200 kD proteins. The record for even smaller molecular weights is continuously being broken.

2.4 Inhomogeneous samples

A unique advantage of cryo-EM is the direct acquisition of images at high magnification that are statistically analyzed to reconstruct structural information. The statistical analysis can classify different possible molecular structures from the same sample to separate molecules with different conformations or compositions. The algorithm for classifying different structures from 2-D and 3-D information is relatively mature and has enabled us to obtain high-resolution structures from inhomogeneous samples [12]. More importantly, the classification of a large number of single molecular structures also provides a statistical distribution of different states. Thus different temperatures, solution conditions, and biochemical reaction time-points will yield thermodynamics and kinetics information for bio-macromolecule complexes correlated with structural information. Overall, these data lead to a deeper insight into molecular mechanisms [13,14].

3 The current status of cryo-EM in structural biology and cell biology

Initially, cryo-EM was used to image highly ordered 2-D crystals, helical structures, and viruses with icosahedral symmetry. Recently, given the rapid development of computer technology, we have witnessed the continuing emergence of new algorithms for structural determinations as well as high-throughput, high-resolution data collection. We have also seen hardware improvements, better ways of identifying and purifying macromolecule samples from advances in proteomics, molecular biology, and biochemistry, and improved cellular and sub-cellular sample preparation. Overall, there have been many avenues of progress in cryo-EM.

3.1 Diversified samples

It is now possible to acquire molecular-level cryo-EM images of asymmetric bio-macromolecules with low molecular weights and high flexibility. With the improved data quality, we can now reconstruct high-resolution structures of 300 kD asymmetrical proteins or nucleic acids in vitrified samples. Because the collection of 10^4 images of single particles has become routine, the conformational heterogeneity analysis of flexible molecules is more accessible by statistical classification methods. Cryo-EM single-particle reconstruction is now able to solve structures of almost all bio-macromolecules above 300 kD. Recently, it has been shown that certain protein structures under 200 kD can be solved

by cryo-EM single-particle method [15,16]. For example, Yigong Shi et al. from Tsinghua University, working with Sjors Scheres et al. [16] from the MRC Laboratory of Molecular Biology, Cambridge Biomedical Campus, solved the structure of the γ-secretase complex at a resolution of 4.5 Å. This involved determining the 170 kD protein portion of the complex and setting a new record.

3.2 Resolution improvements

A significant technical breakthrough in cryo-EM has been the advent of high-resolution image capturing devices. Direct electron-detection devices, based on complementary metal-oxide-semiconductor transistors, have considerably improved the SNR relative to films or charge coupled devices [17]. The latter had to first convert the electronic signal to an optical signal, and then covert the optical signal back to an electronic signal. Direct electron detection enhances the imaging quality because it eliminates electron-photon-electron data transfer and, more importantly, it can perform continuous imaging at high speeds. The video-like image capture, in combination with computer image processing, is able to eliminate most image blurring caused by instabilities in the sample or stage [5,6]. Last year, this significant technical breakthrough contributed to a dozen solved protein structures (and their atomic models), with resolutions beyond 4 Å. Yifan Cheng et al. benefitted tremendously from this breakthrough in the TRVP1 structure determination (Figure 6). This technical progress has completely changed the approach to characterize certain macromolecule complexes. For example, cryo-EM structures of viruses and ribosomes have exceeded 3.5 Å resolution and are improving. Hence, cryo-EM has replaced X-ray crystallography as the major tool in structural studies of viruses and ribosomes.

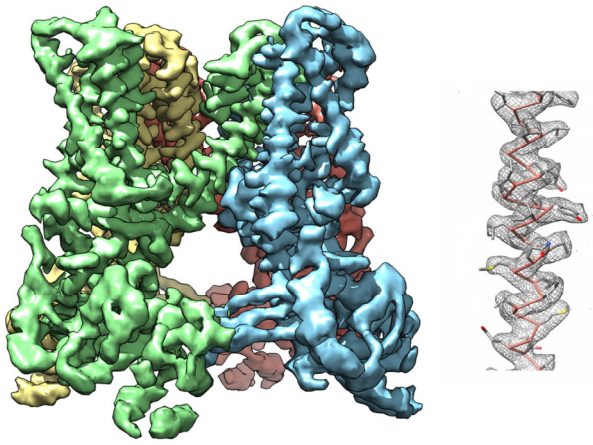

Figure 6 High-resolution (3.4 Å) 3-D reconstruction of TRPV1 by Yifan Cheng et al. [1]. The partial model in the right panel illustrates that high-resolution structures can reveal side chains of an α-helix and thus can be used to build *de novo* atomic models.

3.3 New methods and techniques

Various software programs for high-throughput, automatic data collection, image processing, and structure analysis are being developed (http://en.wikibooks.org/wiki/Software_Tools_For_Molecular_Microscopy). The software makes it easier to enter the cryo-EM field and enables its popularization in structural biology, biochemistry, and cell biology. Increased use of cryo-EM encourages new approaches and integration of various techniques. One cryo-EM technique that will widely extend the range of samples for protein crystallography is micro-crystal electron diffraction, where 100 nm crystals of macromolecules are analyzed via electron diffraction patterns [18].

Cryo-EM tomography is developing rapidly because of the increased stability of the microscope and the high-throughput data collection that divides, aligns, and classifies hundreds of 3-D tomographic reconstructions for sub-tomogram volume averaging. It can be used to analyze macromolecule complexes inside a cell and obtain their high-resolution structure *in vivo* [19]. New frozen, hydrated sample preparation methods such as vitreous sections [20] and focused ion beam milling [21] enable better images of cellular structures. In addition, the emerging technique of serial block-face scanning electron microscopy [22] provides important spatial information on intercellular processes in neurobiology and immunology.

Correlative light and electron microscopy (CLEM) is becoming an important way to obtain high-resolution structural information for cellular features that have been labeled with fluorescent probes. This method could also be used to correlate the molecular dynamics of a cellular process with high-resolution structures *in vivo* [23].

4 Technological challenges and future perspectives

As with other methods in structural biology and biophysics, major breakthroughs in cryo-EM hardware have solved many critical technical difficulties and enabled widespread applications. However, other technical difficulties have emerged for structure determinations and become new bottlenecks that require research and investments.

4.1 Sample preparation

Sample preparation is always the key and limiting step in cryo-EM. For the structural study of bio-macromolecules, a proper density of single particles distributed evenly in optimally thick vitreous ice is necessary for high-quality data. However, the distribution of bio-molecules in a sample will depend on interactions and solution behavior in the supporting films. Similarly, for cellular structure analysis, thick cellular samples must be thinned for cryo-EM imaging.

Frozen sectioning methods keep improving, but still require long-term training and practical experience to be mastered. Focused ion beam milling is another approach for cellular cryo-sample structure analysis. Major breakthroughs in sample preparation are still necessary for cryo-EM to become the method of choice in structural biology.

4.2 High-resolution structural determination and model building

The number of near-atomic-resolution structures (<4 Å) solved by cryo-EM in the last two years almost exceeds the total number solved by cryo-EM in the last few decades. Even more structures were solved at a resolution of 4–8 Å within this short time frame. In contrast with X-ray crystallography, the resolution of cryo-EM single-particle reconstruction cannot be determined by the strength of diffraction signals coming from a crystal lattice. The objective resolution of a 3-D reconstruction is an important problem in cryo-EM. Moreover, atomic models must be built from reconstructions at different resolution levels to understand molecular functions at the atomic level. For 3-D reconstructions at resolutions beyond 4 Å, X-ray crystallography model building methods can be applied directly. For reconstructions at resolutions below 4 Å, however, there are no well-accepted methods for building highly reliable models. Attempts to build atomic models at this resolution level are being made with homologous modeling and molecular dynamics simulations.

4.3 Analysis of bio-macromolecule conformational heterogeneity

A major advantage of cryo-EM single-particle reconstruction over X-ray crystallography is the ability to obtain structures of bio-macromolecules in solution instead of requiring crystals. However, flexible conformations of bio-macromolecules are no longer fixed as they are in crystals, and can preclude determination of high-resolution structures. Therefore, separating molecules into different conformations is an important step to improve resolution. Furthermore, as mentioned above, different conformations may reflect different functional states that provide insights into the molecular mechanism. Heterogeneity analysis is thus a major technical challenge for cryo-EM structural determination. Several algorithms use classification analysis and maximum probability analysis to reveal important aspects of bio-macromolecular mechanisms, but additional approaches are needed.

4.4 New electron optics

Super-resolution imaging in material sciences has been improved significantly based on the development of new electron optics and new imaging methods. These include spherical aberration correction, chromatic aberration correction, and scanning transmission electron microscopy. The most advanced systems are able to achieve 0.5 Å resolution. Thus there is an opportunity and challenge to utilize these ultra-high resolution systems for cryo-EM determination of biological structures. Other new techniques include phase-plates that facilitate single-particle reconstruction of small molecules and tomography reconstruction of cellular structures [24].

4.5 *In vivo* structures

Structural biologists have primarily used purified bio-macromolecules *in vitro* to solve over 10^5 structures and have thus greatly enhanced our understanding on the molecular mechanisms in biological processes. *In vivo* atomic-resolution structures are still not feasible without further development of cryo-EM techniques, especially tomography. With more stable EM systems, more efficient data collection, and more powerful computer processing tools, 3-D tomography may potentially help us to reconstruct and statistically analyze specific cellular structures. Labeling specific molecules inside cells with high efficiency and specificity is still a major technical challenge for cryo-EM. Once the problem is solved, cryo-EM could fill the gaps between structural and cell biology, thus allowing us to achieve a more comprehensive understanding of processes at different temporal and spatial scales.

5 Summary

Cryo-EM started in the 1950s, experienced a period of development in the 1980s and 1990s, matured in the early 21st century, and has experienced revolutionary technical breakthroughs and growth in the past two years. The major goal in the next few years for structural biologists and cell biologists is to take advantage of these new developments for investigating biological processes at a much deeper level. Researchers in mathematics, physics, computer sciences, material sciences, and chemistry will contribute significantly to improved techniques in collaboration with those in the cryo-EM field. The latter need to keep an open mind and actively welcome challenges to stay on the cutting edge and push the frontier of the field into more diverse areas.

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