Applied Microscopy
Korean Society of Microscopy

pISSN 2287-5123 · eISSN 2287-4445
http://dx.doi.org/10.9729/AM.2016.46.1.1

Review Article

Advanced Cryo-Electron Microscopy Technology: High Resolution Structure of Macromolecules

Jeong Min Chung, Hyun Suk Jung*

Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chuncheon 24341, Korea

*Correspondence to:
Jung HS,
Tel: +82-33-250-8513
Fax: +82-33-259-5664
E-mail: hsjung@kangwon.ac.kr

Received March 7, 2016
Revised March 18, 2016
Accepted March 18, 2016

Recent cryo-electron microscopy (EM) studies reported the structure of various types of proteins at high resolution which is sufficient to visualize the intermolecular interaction at near atomic level. There are two main factors that cause the advances in cryo-EM; the development of image processing techniques, such as single particle analysis, and the improved electron detection devices. Although the atomic structures of small and asymmetric proteins are not yet to be determined by cryo-EM, this striking improvement implies the bright prospect of the application in biomedical studies. This study reviews the recently published studies reported high resolution structures using improved imaging analysis techniques and electron detectors. Furthermore, we will discuss about the future aspects of cryo-EM application.

Key Words: Cryo transmission electron microscopy, Single particle analysis, Electron detectors, Proten structure

INTRODUCTION

The main aim of structural biology is to understand how macromolecular complexes play their roles in the living cell through determination of the three-dimensional (3D) arrangement of their atoms. There are several techniques that can be used to determine such structures including X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and cryo-electron microscopy (Cryo-EM). So far, X-ray crystallography and NMR are highly used in the determination of atomic resolution structure. X-ray crystallography may provide high-resolution structure without size limitation of the sample unless irregular and unstable crystal of the interest complex is formed. However, this technique requires large amounts of pure sample and there are many proteins that cannot be properly crystallized. NMR may provide unique structural information on dynamics and interactions, but its resolution is restricted to the size of complexes (small size complexes with approximately 40 to 50 kDa).

Cryo-EM can visualize the molecular interactions accompanying their functional alterations in cell. Since 2013, several cryo-EM 3D structures have been reported at atomic resolution. Although only few cryo-EM structures of small and asymmetric complexes have reported at a resolution about 4 to 7 Å, it is getting close to reach an atomic resolution. There are two crucial points that improve the resolution of cryo-EM 3D structures; the advances in electron detector hardware, including direct detection system, and the improved image processing techniques such as single particle analysis. Even though, a structural determination of native protein as small as 100 kDa waits to be realized, those technical and equipmental developments will open the door for countless proteins especially small protein complexes. In this paper, we review the latest structural biology studies using advanced cryo-EM technology. Furthermore, we discuss the potential and scope of this technique in future.

This study was supported by Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT & Future Planning (NRF-2015R1C1A1A01053611 to H. S Jung) and Next-Generation BioGreen Program (SSAC, PJ011891 to H. S Jung).

TRANSMISSION ELECTRON MICROSCOPY

Since its invention in 1930s by Ruska brothers, transmission electron microscopy (TEM) has become a powerful tool for studying the structural details of biological samples ranging in size from atomic level imaging of single protein molecules to the whole cell. The basic operating principle of TEM is same as the light microscope but uses electrons instead of light. TEM uses beam of electrons as a "light source" emitted from the top of the microscope which travels through vacuum in the column of the microscope. In the TEM, electron magnetic lenses that are analogous to glass lenses in light microscope focus the electrons into a thin beam. The electrons then travel through the specimen resulting in the formation of image which is magnified and focused onto an imaging devise including fluorescent screen or a charge-coupled device (CCD) camera (Culling, 1974; Ellis & Herbert, 2001; Kourkoutis et al., 2012). Since electrons must be kept in a high vacuum to prevent unwanted scattering in their path, biological samples in EM should therefore be stabilized or preserved. There are two common sample preparation methods that depend on the nature of the sample; negative staining and the frozen hydrated (cryo) preparation technique. Negative staining uses uranyl acetate stain as the heavy atoms in the stain efficiently scatter electrons, inducing a high degree of negative contrast for visualizing the specimen and minimizing electron beam damage. This method, which is a quick and simple, became popular during the 1960s and remains so. However, it causes the sample deformation during staining and drying which can hinder visualization of protein structure. Recently, the frozen hydrated sample preparation technique has been used to overcome these challenges. Followed by early attempts by Henderson and Unwin (1975), the cryo preparation technique allows snapshots of fragile assemblies preserved in a fully hydrated state. In cryo-EM work, the sample is immobilized using vitrification by plunge-freezing in slurry of coolant such as liquid ethane at about –188°C. The frozen hydrated sample preparation technique prevents the specimen distortion on drying due to flattening and non-uniform stain distribution between top and bottom of the specimen. It also gives higher resolution than negative staining due to the native contrast between protein and solution.

DEVELOPMENT OF IMAGE PROCESSING

Image processing refers to any technique that extracts and displays the accurate structural information of specimen images. The aim of image processing is to obtain the detailed features of sample (signal) from non-regular details (noise). As the images obtained by cryo-EM having low contrast and noise, image processing is necessary to improve the signal-to-noise ratio (SNR). Since the late 1960s several image processing techniques have been developed that are now used in structural studies of biological samples. Previously, DeRosier and Klug (1968) demonstrated the reconstruction of 3D model from two-dimensional (2D) projections of negatively stained bacteriophage images in different directions. This process is called electron crystallography which records electron micrographs of periodic arrangement of molecules (2D crystals) to reconstructs 3D model. It allows determination of high-resolution structural details as a single micrograph of 2D crystals of large numbers of identical subunit arranged in same orientation. The combination of this technique and cryo-sampling method showed its potential to obtain sufficient structural details identifying the position of individual atoms in molecule. Electron crystallography requires extensive symmetry in crystals to facilitate proper 3D reconstruction process. In the absence of such order (noncrystalline specimens), 3D reconstruction can be directly calculated from projections of individual particles, called single-particle analysis. It averages the images of individual protein molecules to improve SNR. The method requires identical particles that are randomly oriented to obtain projection images of particles from almost all directions. The single particle technique is normally used for large macromolecules or complexes and it can be applied for the molecules that are not symmetric provided if they are in the same conformation. For single particle reconstruction, particle images are selected from digitized raw micrographs or CCD frames. These selected images are always noisy due to the use of low dose of electrons to minimize radiation damage and loss of structural details of specimens. Image processing for single particle analysis is largely divided into two steps: first, similar 2D projections are averaged to minimize noise in the selected particle images to be used for 3D reconstruction; second, contrast transfer function (CTF) correction is applied by using estimated parameters such as defocus and astigmatism. Once the particles are selected, they are first aligned and then grouped into several classes representing views in various orientations. Particle alignment is the process that repositions the selected particles in the same orientation by in-plane rotation and translation resulting in generation of averaged images with improved SNR (Thuman-Commike, 2001). The alignment process is affected by challenges such as the low contrast and inherent noise in electron micrographs. Several approaches have been applied to overcome such obstacles and most effective one has been the reference-based alignment method that compares each selected particle image against a reference image (Frank et al., 1981). However, this approach produces severe bias towards the reference image. The bias can be reduced by multiple comparisons with different (multi) references (multi-reference alignment method) (Van Heel & Stoffler-Meilicke, 1985)

or in the absence of reference (reference-free alignment method) (Penczek et al., 1992). To improve the accuracy of classification, accurate alignment should be performed prior to the classification. Alignment, however, requires appropriate references that are generated from previous rounds. As the first round of alignment and classification may not reach the optimal level an iterative refinement process may be needed to improve the result (Thuman-Commike, 2001). The single particle image processing method is not only applied to the globular proteins, but also it can be used to reconstruct 3D model of helically ordered objects using the iteratice helical real-space reconstruction (IHRSR) method (Meng et al., 2011). Using the algorithm, 3D reconstruction of flexible and disordered helical objects, including actin filaments tobacco mosaic viruses and microtubules, is now attainable. Although the improvement of the algorithm in single particle analysis, there is still room for the further advances in the method. The complexes that are small (less than 100 kDa), or that contain flexible domains are remained to be solved with improved 3D classification algorithms.

IMPROVED DIRECT-ELECTRON DETECTOR

Performance of electron detector is another crucial point in the study of electron sensitive samples such as in cryo-EM, where the SNR is poor due to the use of low dose of electrons to avoid the radiation damage (McMullan et al., 2014). The detector performance is defined by detective quantum efficiency (DQE) which represents the amount of additional noise added during the detection process. When detector does not add any additional noise to a recorded image, the DQE value will be 1. However, in practice, all detectors degrade SNR and so have values less than 1. Until recently, photographic film was used to record a high resolution cryo-EM images. This traditional image detector can generate a large field-of-view and it has relatively high DQE value of ~0.3 (Krivanek & Mooney, 1993; Spence & Zuo, 1988). Alternative detectors, such as CCD cameras, perform well at low electron energies, but DQE value of these device reduced to ~0.1 at the higher electron energies which is preferable for high-resolution cryo-EM (Bai et al., 2015). Although the significant weakness of the detectors, they are highly used in current research field due to the convenience of not having to develop and scan photographic film (Booth et al., 2004; Suloway et al., 2005).
Recently, a convenient high-DQE detector so called "direct electron detector" has been introduced and it has showed great promise in current research field. The direct detectors use backthinned monolithic active pixel sensors (MAPS) which is fabricated on a very thin epitaxial layer of semiconductor such as silicon in which the incident electron energies are deposited (Milazzo et al., 2005). In recent studies assessing the performance and characteristics of the backthinned direct electron detectors, such as the FEI Falcon II (FEI, USA) and the Gatan K2 Summit (Gatan, USA), these electron detectors showed relatively high-DQE value than that of traditional photographic film. Moreover, they are electronic detector which is designed to compensate for the inconvenient process of photographic film including development and scanning film (Ruskin et al., 2013). Another advantage of the MAPS detectors is that they have high readout speeds, reducing the effects of electron beam damage of sample, which is crucial point for the highly sensitive biological sample for cryo-EM. The high sensitive and fast readout speed detectors are able to produce improved images in low dose electron field by electron counting mode which detects individual incident electrons and this allows to reconstruct the final image from the information contained in processed sub-images of individual incident electrons (McMullan et al., 2009). As a result, the detectors are possible to provide movie-mode functionally in low-dose exposures and this can be used in the liquid EM which enables direct in situ imaging of processes in solutions (Evans et al., 2012). Therefore, the improvement of detectors allows us to take one step forward for visualizing high resolution biological process and dynamics in real-time.

ADVANCES IN BIOLOGICAL STUDIES

In early 1980s, the image processing for symmetric and asymmetric complexes were demonstrated by negatively stained specimens (Radermacher et al., 1987; Verschoor et al., 1984). Followed by the development of cryo-technique, the achievement of several near-atomic resolution of 3D models shows the optimistic aspect of theoretical considerations made about the potential of the method 20 years ago (Henderson, 1995). During the 1990s, 3D structures of ribosome in ice have reached a resolution near 10 Å (Frank et al., 1991; Gabashvili et al., 2000). Since 2008, several cryo-EM 3D structures have been reported at a resolution of 3 to 4 Å allowing for the determination of the protein backbone as well as some amino-acid side chain: these include ribosomes from human pathogen (Wong et al., 2014) or ion channels (Liao et al., 2013) or some of key enzymes involved in biogenesis of methane (Allegretti et al., 2014). The recent advances described here are caused by the improvement of both imaging process techniques and electron detectors (Table 1) (Amunts et al., 2014; Bartesaghi et al., 2014, 2015; Campbell et al., 2015; Grant & Grigorieff, 2015; Hussain et al., 2014; Jiang et al., 2015; Li et al., 2013; Liao et al., 2013; Lu et al., 2014). Nevertheless, there are only few cryo-EM structures of small and asymmetric complexes reported at a near atomic resolutions (about 4~7 Å) (Lu et al., 2014). Therefore there are still limitations that should be solved in the coming years, including the advances in electron detectors with a DQE value of 1, reducing the electron beam-induced motion that leads to

Table 1. Recently reported reference lists for high-resolution cryo-EM structure of proteins and image processing software used

Protein (molecular weight)	Resolution (Å)	TEM	Electron detector	Image processing software used	Reference
Proteasome (700 kDa)	3.30	TF30 Polara (FEI)	Gatan K2 Summit	SPIDER and FREALIGN	Li et al., 2013 (*Nat. Methods*)
TRPV1 (475 kDa)	3.40	TF30 Polara (FEI)	Gatan K2 Summit	SPIDER and FREALIGN	Liao et al., 2013 (*Nature*)
Ribosome (>1 MDa)	4.00	Tecnai G2 (FEI)	FEI Falcon II	EMAN2 and RELION	Hussain et al., 2014 (*Cell*)
Ribosome (>1 MDa)	3.20	Titan Krios (FEI)	FEI Falcon II	EMAN2 and RELION	Amunts et al., 2014 (*Science*)
γ-secretase (170 kDa)	4.50	Titan Krios (FEI)	Gatan K2 Summit	RELION	Lu et al., 2014 (*Nature*)
β-Gal (465 kDa)	3.20	Titan Krios (FEI)	Gatan K2 Summit	EMAN2, FREALIGN, COOT	Bartesaghi et al., 2014 (*PNAS*)
Proteasome (700 kDa)	2.80	Titan Krios (FEI)	Gatan K2 Summit	APPION and RELION	Campbell et al., 2015 (*Elife*)
PA pore (440 kDa)	2.90	Titan Krios (FEI)	Gatan K2 Summit	EMAN2 and RELION	Jiang et al., 2015 (*Nature*)
Rotavirus VP6 (540 kDa)	2.60	Titan Krios (FEI)	Gatan K2 Summit	TIGRIS, EMAN2, FREALIGN, RELION	Grant & Grigorieff, 2015 (*Elife*)
β-Gal–PETG (465 kDa)	2.20	Titan Krios (FEI)	Gatan K2 Summit	EMAN2, FREALIGN, COOT, PHENIX	Bartesaghi et al., 2015 (*Science*)

Full name for the abbreviation of each image processing software and their websites: SPIDER, System for Processing Image Data from Electron microscopy and Related fields, http://spider.wadsworth.org/spider_doc/spider/docs; FREALIGN, Fourier Reconstruction and Alignment, http://grigoriefflab.janelia.org/frealign; EMAN2, Electron Micrograph Analysis, http://blake.bcm.edu/emanwiki/EMAN2; RELION, Regularised Likelihood Optimisation, http://www2.mrc-lmb.cam.ac.uk/relion; COOT, Crystallographic Object-Oriented Toolkit, http://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot; APPION, Automated Pipeline for the Processing of Images, http://appion.org; TIGRIS, Tile and General Research Imaging System, https://sourceforge.net/projects/tigris; PHENIX, Python-based Hierarchical ENvironment for Integrated Xtallography, https://www.phenix-online.org.
TEM, transmission electron microscope.

blurring of the images and improvement in the classification methods for flexible specimens displaying the large amount of structural heterogeneity. To obtain the further improvements in the coming years, both academic and industrial research effort must be applied in structural biological field.

CONCLUSIONS

Recently, several cryo-EM studies reported the structure of relatively small sized complexes (<500 kDa) at high resolution (Bartesaghi et al., 2015; Grant & Grigorieff, 2015; Zhao et al., 2015). This is due to the improvement of image processing technology and development of hardware such as electron detectors. High resolution structural details are required to reveal the intermolecular interactions between proteins and their associative proteins. The information is crucial for understanding of cellular process which can be applied to human disease model. Cryo-EM has specific ability to provide snapshots of single protein structure with their native cellular status. This powerful application will play a critical role in the identification of novel therapeutic target of drug.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

REFERENCES

Allegretti M, Mills D J, McMullan G, Kühlbrandt W, and Vonck J (2014) Atomic model of the F420-reducing [NiFe] hydrogenase by electron cryo-microscopy using a direct electron detector. *Elife* **3**, e01963.

Amunts A, Brown A, Bai X C, Llácer J L, Hussain T, Emsley P, Long F, Murshudov G, Scheres S H W, and Ramakrishnan V (2014) Structure of the yeast mitochondrial large ribosomal subunit. *Science* **343**, 1485-1489.

Bai X C, McMullan G, and Scheres S H (2015) How cryo-EM is revolutionizing structural biology. *Trends Biochem. Sci.* **40**, 49-57.

Bartesaghi A, Matthies D, Banerjee S, Merk A, and Subramaniam S (2014) Structure of β-galactosidase at 3.2-Å resolution obtained by cryo-electron microscopy. *Proc. Natl. Acad. Sci. USA* **111**, 11709-11714.

Bartesaghi A, Merk A, Banerjee S, Matthies D, Wu X, Milne J L, and Subramaniam S (2015) Electron microscopy. 2.2 Å resolution cryo-EM structure of β-galactosidase in complex with a cell-permeant inhibitor. *Science* **348**, 1147-1151.

Booth C R, Jiang W, Baker M L, Zhou Z H, Ludtke S J, and Chiu W (2004) A 9 angstroms single particle reconstruction from CCD captured images on a 200 kV electron cryomicroscope. *J. Struct. Biol.* **147**, 116-127.

Campbell M G, Veesler D, Cheng A, Potter C S, and Carragher B (2015) 2.8 Å resolution reconstruction of the Thermoplasma acidophilum 20S proteasome using cryo-electron microscopy. *ELife* **4**, e06380.

Culling C F A (1974) *Modern Microscopy: Elementary Theory and Practice*

(Butterworth and Company Ltd., London).

DeRosier D J and Klug A (1968) Reconstruction of three dimensional structures from electron micrographs. *Nature* **217**, 130-134.

Ellis M J and Herbert H (2001) Structure analysis of soluble proteins using electron crystallography. *Micron* **32**, 541-550.

Evans J E, Jungjohann K L, Wong P C, Chiu P L, Dutrow G H, Arslan I, and Browning N D (2012) Visualizing macromolecular complexes with in situ liquid scanning transmission electron microscopy. *Micron* **43**, 1085-1090.

Frank J, Penczek P, Grassucci R, and Srivastava S (1991) Three-dimensional reconstruction of the 70S Escherichia coli ribosome in ice: the distribution of ribosomal RNA. *J. Cell Biol.* **115**, 597-605.

Frank J, Verschoor A, and Boublik M (1981) Computer averaging of electron micrographs of 40S ribosomal subunits. *Science* **214**, 1353-1355.

Gabashvili I S, Agrawal R K, Spahn C M, Grassucci R A, Svergun D I, Frank J, and Penczek P (2000) Solution structure of the E. coli 70S ribosome at 11.5 Å resolution. *Cell* **100**, 537-549.

Grant T and Grigorieff N (2015) Measuring the optimal exposure for single particle cryo-EM using a 2.6 Å reconstruction of rotavirus VP6. *Elife* **4**, e06980.

Henderson R (1995) The potential and limitations of neutrons, electrons and X-rays for atomic resolution microscopy of unstained biological molecules. *Q. Rev. Biophys.* **28**, 171-193.

Henderson R and Unwin P N (1975) Three-dimensional model of purple membrane obtained by electron microscopy. *Nature* **257**, 28-32.

Hussain T, Llácer J L, Fernández I S, Munoz A, Martin-Marcos P, Savva C G, Lorsch J R, Hinnebusch A G, and Ramakrishnan V (2014) Structural changes enable start codon recognition by the eukaryotic translation initiation complex. *Cell* **159**, 597-607.

Jiang J, Pentelute B L, Collier R J, and Zhou Z H (2015) Atomic structure of anthrax protective antigen pore elucidates toxin translocation. *Nature* **521**, 545-549.

Kourkoutis L F, Plitzko J M, and Baumeister W (2012) Electron microscopy of biological materials at the nanometer scale. *Annu. Rev. Mater. Res.* **42**, 33-58.

Krivanek O L and Mooney P E (1993) Applications of slow-scan CCD cameras in transmission electron microscopy. *Ultramicroscopy* **49**, 95-108.

Li X, Mooney P, Zheng S, Booth C R, Braunfeld M B, Gubbens S, Agard D A, and Cheng Y (2013) Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. *Nat. Methods* **10**, 584-590.

Liao M, Cao E, Julius D, and Cheng Y (2013) Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature* **504**, 107-112.

Lu P, Bai X C, Ma D, Xie T, Yan C, Sun L, Yang G, Zhao Y, Zhou R, Scheres S H W, and Shi Y (2014) Three-dimensional structure of human γ-secretase. *Nature* **512**, 166-170.

McMullan G, Clark A, Turchetta R, and Faruqi A (2009) Enhanced imaging in low dose electron microscopy using electron counting. *Ultramicroscopy* **109**, 1411-1416.

McMullan G, Faruqi A R, Clare D, and Henderson R (2014) Comparison of optimal performance at 300 keV of three direct electron detectors for use in low dose electron microscopy. *Ultramicroscopy* **147**, 156-163.

Meng X, Zhao G, and Zhang P (2011) Structure of HIV-1 capsid assemblies by cryo-electron microscopy and iterative helical real-space reconstruction. *J. Vis. Exp.* **9**, 3041.

Milazzo A C, Leblanc P, Duttweiler F, Jin L, Bouwer J C, Peltier S, Ellisman M, Bieser F, Matis H S, Wieman H, Denes P, Kleinfelder S, and Xuong N H (2005) Active pixel sensor array as a detector for electron microscopy. *Ultramicroscopy* **104**, 152-159.

Penczek P, Radermacher M, and Frank J (1992) Three-dimensional reconstruction of single particle s embedded in ice. *Ultramicroscopy* **40**, 33-53.

Radermacher M, Wagenknecht T, Verschoor A, and Frank J (1987) Three-dimensional structure of the large ribosomal subunit from Escherichia coli. *EMBO J.* **6**, 1107-1114.

Ruskin R S, Yu Z, and Grigorieff N (2013) Quantitative characterization of electron detectors for transmission electron microscopy. *J. Struct. Biol.* **184**, 385-393.

Spence J C H and Zuo J M (1988) Large dynamic range, parallel detection system for electron difrraction and imaging. *Rev. Sci. Instrum.* **59**, 2102-2105.

Suloway C, Pulokas J, Fellmann D, Cheng A, Guerra F, Quispe J, Stagg S, Potter C S, and Carragher B (2005) Automated molecular microscopy: the new leginon system. *J. Struct. Biol.* **151**, 41-60.

Thuman-Commike P A (2001) Single particle macromolecular structure determination via electron microscopy. *FEBS Lett.* **505**, 199-205.

Van Heel M and Stoffler-Meilicke M (1985) Characteristic views of E. Coli and B. Stearothermophilus 30S ribosomal subunits in the electron microscope. *EMBO J.* **4**, 2389-2395.

Verschoor A, Frank J, Radermacher M, Wagenknecht T, and Boublik M (1984) Three-dimensional reconstruction of the 30 S ribosomal subunit from randomly oriented particles. *J. Mol. Biol.* **178**, 677-695.

Wong W, Bai X C, Brown A, Fernandez I S, Hanssen E, Condron M, Tan Y H, Baum J, and Scheres S H (2014) Cryo-EM structure of the Plasmodium falciparum 80S ribosome bound to the anti-protozoan drug emetine. *Elife* **3**, e03080.

Zhao M, Wu S, Zhou Q, Vivona S, Cipriano D J, Cheng Y, and Brunger A T (2015) Mechanistic insights into the recycling machine of the SNARE complex. *Nature* **518**, 61-67.