2017 Nobel Prize in chemistry – for CryoEM

The 2017 Nobel Prize in Chemistry has been awarded to Jacques Dubochet, Joachim Frank and Richard Henderson for developing electron cryomicroscopy (cryoEM) for high-resolution determination of biological macromolecules in solution. It has long been realized that electron microscopy has the potential to reveal atomic details of macromolecules and this year's award is reflective on the various developments that have been made on specimen preparation, theoretical and computational approaches and detectors over the past four decades. In describing the involvement of the three scientists in development of cryoEM, a brief history of electron microscope (EM) in life sciences is presented together with contributions from other scientists who helped the growth of cryoEM.

From the time Ernst Ruska built the EM and developed electron optics (for which he was awarded the Nobel in 1986), life scientists have been using it from the beginning initially with air-dried macromolecules (like viruses). As biological molecules are made of light atoms, they scatter electrons weakly, resulting in low contrast and to visualize them the method of negative staining was invented. As the solution of heavy metal covers the surface of the specimen and provides the contrast, only low resolution information (typically the shape) is preserved and internal features of the macromolecules cannot be visualized. Freeze drying, freeze substitution and freeze fracture are also other techniques commonly used in life sciences and developed in early days, where the first steps involve freezing but the imaging is generally done with dried specimen and often at room temperature. During the early years with negative stained images, Aaron Klug along with David Derosier developed optical methods, and the theory behind the image processing and reconstruction (for which Aaron was awarded the Nobel in 1982). Subsequently, the method was extended to large viruses using the common lines approach resulting in maps that were informative at that time[1]. It was realized that staining with heavy metal had its limitations and other ways to improve specimen preparation and imaging were sought. Thus the story of this year's Nobel Prize to cryoEM starts with imaging of unstained crystalline specimen, followed by development of computational methods to analyse single particles, the development of plunge freezing and the introduction of new detectors.

Electron crystallography

Richard Henderson was born in Scotland and obtained his physics degree in Edinburgh. After graduation, Richard contemplated whether to pursue research on nuclear fusion or molecular biology, a field that was very new. He decided wisely on the latter and joined David Blow at the Laboratory of Molecular Biology to work on the structure of chymotrypsin. At that time only a couple of protein structures were known and Richard was a key member of David's team in the structure determination of chymotrypsin. As a Helen-Hey Whitney fellow, Richard went to Yale to study ion-channels and soon realized that they are difficult and it was not the right time to pursue them. In a conference, Richard listened to a talk by Walter Stoeckenius, who described a light-driven pump from *Halobacterium salinarum* and found it very interesting as the protein was densely packed and already crystalline in the membrane. When he came back to the Laboratory of Molecular Biology (LMB), Cambridge he started working on the purple membrane initially by X-ray scattering. In the annual talks at LMB, Nigel Unwin's description of imaging biological macromolecules by EM turned Richard into an electron microscopist. In 1971, Bob Glaeser quantified the amount of radiation damage on organic and biological molecules and described the need for averaging a large number of molecules and recommendation for low-dose[2]. Following upon this, Richard and Nigel used a solution of glucose to replace water and imaged the unstained 2D crystals of BR (as well as catalase crystals) at room temperature using low dose and produced projection maps[3]. Subsequently, collecting tilted images and combining the data they were able to determine the first lower resolution map of bacteriorhodopsin at 7 Å revealing for the first time an architecture of a membrane protein (Figure 1)[4]. This structure was a milestone both in membrane protein structural biology and electron microscopy. Ken Taylor and Bob Glaeser meanwhile were able to obtain diffraction patterns of frozen catalase crystals (without any cryoprotectant) to a very high resolution. However, at that time it was only possible to maintain the temperature at –120°C (refs 5, 6). After the landmark publication in 1975, it took Richard close to 15 years to obtain an atomic model of BR using cryoEM, which required several technical improvements in electron microscopy (described in detail below).

Photos of Jacques Dubochet, University of Lausanne, Lausanne; Richard Henderson, MRC Laboratory of Molecular Biology, Cambridge (photo: Nobel Foundation); Joachim Frank, Columbia University, New York (photo from the profile in PNAS by Kasper Mossman).

Single particle EM

While the structures of highly ordered specimens such as 2D crystals and helical arrays were feasible by using low-dose method, whether this was applicable to macromolecules as single particles, in particular those without any inherent symmetry, was unclear. When observing macromolecules as single particles, one

of the important questions was to know how much dose is necessary for a particle to be visible and sufficient signal remained for further alignment and averaging. The development of computational approaches for image analysis of asymmetric molecules can be attributed to Joachim Frank and his colleagues. After his Diploma in Physics, Joachim Frank joined the lab of Walter Hoppe at Max-Planck Institute for Biochemistry in Martinsried and his thesis was on cross-correlation to align carbon films. Joachim obtained the Harkness fellowship and joined the Jet Propulsion Laboratory, Pasadena to learn image processing. Then he joined Bob Glaeser at UC Berkeley, where he was primed to elaborate methods for averaging lot of molecules so that 3D structures can be obtained. After a short stint in Cambridge, Joachim joined the Wadsworth Centre in Albany and started working on the problem of obtaining structures by averaging single particles. Together with Marin van Heel, he developed the multivariate statistical analysis for classification of particles in 2D, and with Michael Radermacher developed the random conical tilt method to find the relationship of the 2D projections in 3D[7,8]. Joachim and his co-workers thus developed a tool for processing of images called SPIDER – 'System for Processing of Image Data in Electron Microscopy and Related fields' and along with IMAGIC (developed by Marin van Heel) popularized single particle electron microscopy[9,10]. The whole process of image alignment in 2D, how they are related in 3D and thereby determining five parameters for each particle, forms the basic behind single particle reconstruction and used in many of the softwares that are currently available. One such example is shown in Figure 2, where one can relate the various views seen of a membrane protein complex in the micrographs to 2D classes and the resultant final 3D map[11].

Specimen preparation for cryoEM

John Kendrew, the then Director of European Molecular Biology Laboratory (EMBL) recruited Jacques Dubochet to develop a process for freezing suitable for EM. Jacques was born in Switzerland and studied in Lausanne and got a certificate to use electron microscopy. He was interested in studying DNA. At EMBL, he and his group studied the process of freezing of thin film of water by investigating in an electron microscope including the rate of freezing and various morphology. Water is an interesting molecule, which when frozen can exist in different states. For visualization in EM, it was necessary that water was frozen very rapidly without allowing to form crystals. Dubochet and his colleagues characterized different forms of ice and came up with a method that was very simple. Few microlitres of protein of interest is applied to a carbon coated or holey EM grids, blotting the excess liquid and plunge freezing in liquid ethane (maintained at about –185°C with liquid nitrogen)[12,13]. They made these grids with home-made plunger (similar to the one shown in Figure 3). The inspection of such grids required high vacuum chamber in the microscope and this was developed by Philips at that time. Dubochet and his colleagues also developed a cold shield that was used to protect the grid after it was inserted into the microscope. Excellent images of several macromolecules were obtained by plunge freezing and slowly, the cryoEM started to gain momentum. An extensive review on vitrification was written by Dubochet and his colleagues, which remains one of most read articles in electron microscopy[14]. The cold stages where the cryo grids were mounted and maintained at liquid nitrogen temperature and inserted into the microscope were developed in different labs at that time. Some of the initial ones were very simple made of

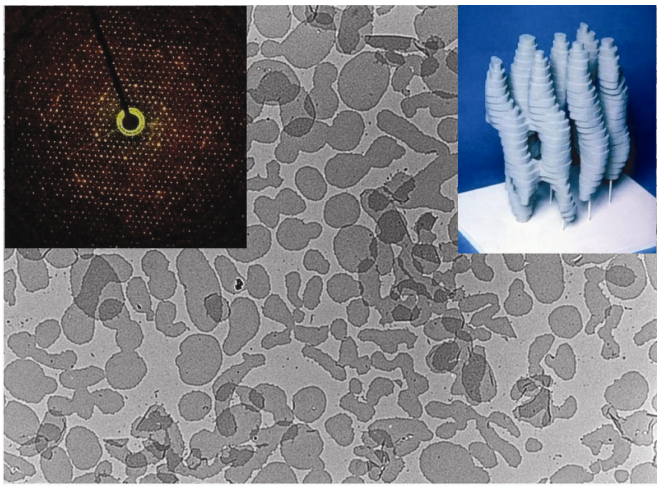

Figure 1. Unstained 2D crystals of bacteriorhodopsin and insets show an electron diffraction pattern and the 3D model obtained by Henderson and Unwin in 1975. The figure was provided by Richard Henderson.

Figure 2. The steps involved in generation of 3D maps of macromolecules by single particle cryoEM is explained with bovine mitochondrial complex I. Protein molecules of interest in solution (complex I in detergent micelles) are rapidly frozen in ethane and imaged with an electron microscope and a direct detector. The micrographs in ***a*** show projections of the molecule adapting different orientations on the support film (scale bar is 500 Å). The information content of these images is limited by radiation damage and signal to noise. Many of these projections are then averaged to obtain high resolution information as shown in the 2D class averages (***b***). The box size is 481 Å. The 2D classes of complex I show the TM helices in the membrane domain and densities for accessory subunits. Subsequently, the orientation parameters of individual particles (the three Euler angles and *X* and *Y* translations) are determined and a 3D map is reconstructed (***c***). The represented map is the raw data, which is then used to interpret and build model. Figures based on ref. 11.

foam to hold nitrogen but over the years cryo holders were perfected to have low drift and more stability. The current specimen loading mechanism is completely automated with the grid mounted on a small cartridge and this is loaded into the column and the specimen chamber is often isolated from environment thus increasing stability. A parallel development in EM included the introduction of the field emission guns (FEG), which provided a more coherent source and brighter beam and the vacuum within the microscope column was dramatically improved allowing imaging of the specimen for longer period with reduced contamination rate. All these advances together allowed Richard Henderson to obtain an atomic model of bacteriorhodopsin in 1990 (ref. 15).

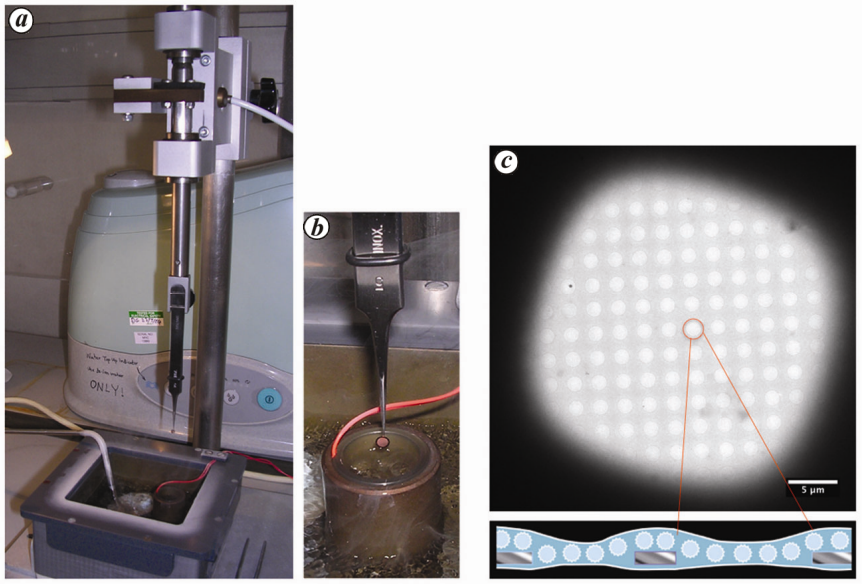

Figure 3. Specimen preparation for cryoEM. A typical manual plunger is shown in (***a***). It consists of a rod that hosts a forceps and an EM grid is attached to its tip. At the bottom, the box consists of a chamber where ethane is liquified and outer chamber is cooled with liquid nitrogen. ***b***, The grid in the forceps after blotting for excess liquid is plunged into ethane by gravity. The whole plunger mechanism can be placed in a controlled environment with a set humidity. ***c***, A low magnification image of a holey carbon grid (quantifoil) is shown. There are regular arrays of holes and after plunge freezing uniform thickness of ice is formed. Each of these holes is imaged and it is expected that these will have single particles of the protein of interest as shown in the magnified view. The photograph of manual plunger was taken by Shaoxia Chen at the LMB, Cambridge.

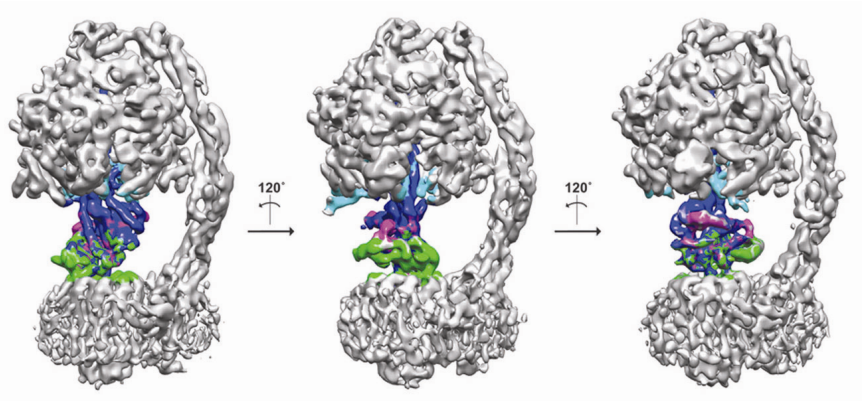

Figure 4. Sorting of conformational heterogeneity by cryoEM. As the molecules in solution are rapidly frozen, different functional states of macromolecules that might exist can be trapped and computationally classified to obtain many different structures and thus the dynamics of macromolecules can be studied by cryoEM. The mitochondrial F_1F_0–ATP synthase from *Pichia angusta* in three different states is shown as an example here. ATP synthases are dynamic enzymes that use the proton gradient to synthesize ATP and can exist in multiple different states. Using a protein inhibitor found in mitochondria, the enzyme has been trapped in different states and computationally these states can be separated from a mixture of population. The central stalk that consists of three proteins is coloured (blue, magenta and green) and the inhibitor protein in cyan. The three states are related roughly by 120° rotation. Figure reproduced from Vinothkumar *et al.*[20].

CryoEM – new era

In 1995, making some simple assumptions Richard asked if perfect images from an EM were obtained limited only by radiation damage what would be the smallest single particle that can be oriented[16]. This analysis revealed that macromolecules as small as 40 kDa can be oriented and a 3 Å structure can be determined with as little as 12,000 asymmetric units (if a particle had no symmetry then this corresponded to 12,000 particles and for particles with symmetry this would be less). When this was proposed there was general scepticism if this was possible, as the structures obtained till then were low resolution and often revealing just the shape (called as blobs). The number of particles required was subsequently estimated to be 600 or 1400 using different criterion but in practical terms the resolution of the maps obtained remained low and the number of particles remained high[17,18]. Beam-induced motion and charging, radiation damage and the signal-to-noise ratio (aka detection) were thought to be the key factors limiting the quality of EM images and improvement in all three might allow one to achieve resolutions and particle numbers as determined from theory. Traditionally, recording of EM images was performed on photographic films, which were developed, rinsed, dried and digitized prior to processing. The whole process of exposure, developing and scanning involved a lot of work and often the number of films that can be exposed at any given time was limited to about 50 and the quality of the grid, ice, specimen was unknown to the users until at a later stage. This limitation led to development of charged-couple device (CCD) cameras, where there was no need to develop and scan; images can be seen immediately and evaluated. However, at the voltage (300 kV) where the beam penetration and

charging are minimal the detective quantum efficiency (DQE) was lower than films. Thus, much of 2000's was focused on development of new detectors that had higher DQE[19]. At the end of 2012, three detectors were introduced co-developed by academic labs and commercial vendors. These detectors were based on the complementary metal-oxide sensors (CMOS) technology specifically designed to withstand the bombardment of electrons. In addition to increased DQE than films or CCD, these detectors were running in rolling shutter mode allowing to capture movies instead of just still images. These movies have revolutionized the way EM images are processed and allowed the analysis of the effect of electrons on the specimen (for example to correct for drift). A parallel development has been faster computers and better algorithms that have brought the time required for processing EM data from months to days and currently hours. Thus the introduction of detectors and sophisticated computing programs has resulted in determination of many biologically important molecules in the last four years and has revolutionized the way structural biology is currently perceived. One of the great beauties of cryoEM is that the freezing process can result in preserving distinct conformations of a macromolecule that existed in solution. The different functional states can then be separated computationally and from a single dataset it is thus possible to obtain multiple structures. An example of such sorting of different states of the highly dynamic ATP synthase is shown in Figure 4 (ref. 20).

Future of cryoEM

It is our desire to know the structure of all proteins in cells and understand how they function and interact with other proteins. Structures of macromolecules previously thought to be difficult are now routinely imaged with cryoEM. However, it is clear that there is still significant gain to be made, in particular with the development of next generation detectors with increased DQE and faster frame rate and methodological improvement in specimen preparation. Beyond visualizing purified proteins as single particles, imaging these proteins within the cells by electron cryotomography and obtaining high-resolution structures will be the next big step. With the new detectors and the introduction of phase plate, already sub-tomogram averaging is starting to produce sub-nanometre resolution structures of larger macromolecules *in situ*. Thus, cryoEM will be a technique that will be used by structural biologists and by cell and neurobiologists. The purchase, maintenance and running cost of an EM is expensive and the limited expertise has led many governments to pool resources and initiate centres for cryoEM similar to synchrotrons for X-ray crystallography such that any scientist with an interesting biological question will be able to get access to high-end cryoEM. One such facility placed at Instem-NCBS campus (Bangalore) will soon be available for users and we hope that India will have many more of these in the near future and allow Indian scientists to realize the full potential of electron cryomicroscopy.

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