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CRYO-ELECTRON MICROSCOPY: RECENT DEVELOPMENTS FOR MEMBRANE PROTEIN STRUCTURE DETERMINATION

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ABSTRACT

Electron Microscopy (EM) is a powerful structure determination tool for biological specimens as it allows imaging of biological molecules with dimensions spanning several orders of magnitude [1]. Virology and cell biology have benefitted greatly from developments in EM. Molecular structural biology using electron microscopy was traditionally limited to structure determination for biological mega-molecules such as ribosome complexes and fitting of previously determined atomic structures into EM density blobs. Recent developments in cryo-EM are allowing atomic structure determination for small as well as asymmetric molecules. This certainly is a giant leap from 'blobology' [2]. The advent of direct electron detectors and maximum likelihood based image classification have been widely accepted as the roots of these new developments. Membrane protein research has benefitted the most from these developments. Previous difficulties in structural determination resulting from low sample concentration, non-homogeneous conformation and composition and tedious sample preparation are being bypassed. In this essay, an attempt has been made to delineate the important recent developments in EM, and their (possible) impact on membrane protein research.

CONTENTS

INTRODUCTION

Membrane proteins account for nearly a quarter of the human proteome and are involved in several important biological functions, such as selective or non-selective transportation of molecules across membranes, signalling between inside and outside of the cell, identification of other cells and relevant surfaces, interaction with other cell and surfaces and catalysis of biochemical reactions. In humans, the origins of many diseases such as depression, schizophrenia, cystic fibrosis, pernicious anaemia etc. can be traced to membrane proteins. Because of their active role in the signalling pathway(s), membrane proteins are targets for nearly half of all drugs [3], including Herceptin (breast cancer drug, targets HER2 receptor), Salbutamol (asthma drug, targets β_2 Adrenergic Receptor) and Escitalopram (antidepressant, targets serotonin transporter) etc. Knowledge about prokaryotic membrane proteins such as vitamin and drug transporters and pore forming toxins is enabling development of new generation of antibiotics. In addition to these biomedical applications, membrane proteins have also found their importance in development of novel nano-technological devices such as biosensors, molecular motors, energy generators and structural scaffolds etc. [4]. These important applications of membrane proteins underscore the significance of understanding the mechanisms of their action. The biological functions of proteins are the resultant of their spatial conformation and atomic interactions. This emphasizes the need for their three-dimensional structure determination, preferably at atomic resolutions.

With exponential advancements in technology, many techniques such as molecular dynamics, fluorescence spectrometry etc. have been contributing towards the discovery of the structure-function relationship of proteins. Nearly all of them are heavily dependent on the outcome of three structure-determination techniques namely X-Ray Diffraction (XRD) Crystallography, Nuclear Magnetic Resonance (NMR) Spectroscopy and Electron Microscopy (EM). X-Ray Crystallography is a powerful technique for protein structure determination and so far it as dominated the atomic-resolution structure-determination of proteins. The general work-flow of structure determination by X-Ray Crystallography involves protein production and ultra-purification, crystal growth, diffraction by crystals and solving the electron density maps. While this strategy is straightforward for most of the soluble proteins, problems faced at each of these steps make X-Ray Crystallography very complicated for membrane proteins. Hydrophobic membrane proteins often need a very particular environment to retain their native folding and sometimes, even a slight deviation from the 'optimum condition' might lead to a total loss of structure and activity. It is for this reason that molecular biologists have to invest a lot of time in optimizing production and purification of membrane proteins. Unlike soluble proteins, membrane proteins have to be stored in detergents and/or liposomes. At best, one can only guess about the native environment of the given membrane protein while selecting these detergents and lipids, so they might not mimic the biological environment. Membrane proteins often form huge complexes, and are generally very difficult to crystallize. To overcome this problem, crystallographers generally go for crystallization in parts, where they crystallize different domains separately and merge them together after individual crystal structure determination. Advent of methods such as Lipidic Cubic Phase (LCP) crystallization

has brought some relief to the crystallographers, but they still face problems because the transmembrane domains of membrane proteins make only a few and weak contacts, which leads to growth of small and less ordered crystals [3]. Procuring a good crystal is still necessary for higher resolution diffraction. It is not uncommon in membrane protein crystallography that even well scored crystals fail to diffract because of high internal disorder. We simply do not know enough about the crystallization of proteins. The involved phase diagrams are still mysterious for us. When this is combined with insufficient knowledge about the local environments of membrane proteins, the problems we face during X-Ray Crystallography of membrane proteins are increased manifold.

Apart from being used for protein structure determination, NMR is also used for studying the dynamic processes in proteins and effects of ligand binding. The major advantage over XRD Crystallography is that there is no need for crystallization, which allows NMR to determine the structure of internally disordered proteins. But for NMR studies, proteins have to be labelled with isotopes, and sometimes expression of such proteins could be problematic [3]. Due to slow relaxation time of larger protein molecules, currently the upper limit on protein size for NMR spectroscopy is below 40-50 kDa [5]. This restricts the use of NMR to study membrane proteins, where the functional units are generally present as large macromolecular complexes. NMR also requires relatively large amount of pure protein, which could be problematic due to low expression of membrane proteins.

Electron crystallography has been used for atomic structure determination of membrane proteins using Electron Microscopes. For employing this technique, membrane proteins are reconstituted in a lipid bilayer, and conditions are optimized for formation of two dimensional (2D) crystals, which is essentially a monolayer of periodic array of membrane proteins in a lipid bilayer. Electron crystallography not only can reveal protein atomic structure, but also provides knowledge about lipid-protein interaction. But the membrane protein crystallization is problematic even in lipid environment as seen in the case of LCP crystallization. Additionally handling of atomically flat 2D crystal specimens extremely challenging.

Electron microscopy has played a significant role for structure determination of biological ‘mega-structures’ such ribosome complex or virus assemblies. These structures used to be solved with nanometer resolutions, which sometimes could unravel the working mechanism in large multi-domain complexes. Absence of high resolution information in electron micrographs has traditionally precluded the application of Electron Microscopy for studying smaller protein molecules. However, in recent few years, new technological developments in the field of Cryo-Electron Microscopy have provided it a major boost to compete with aforementioned structure determination techniques. This phase of accelerated development has been referred to as “the Resolution Revolution” by some scientists [6]. In this essay, an attempt has been made to evaluate the potentials of Cryo-Electron Microscopy in the post resolution-revolution scenario, particularly for studying membrane proteins and related biological complexes.

ELECTRON MICROSCOPY

A microscope is an optical instrument used for viewing small objects. In a microscope, electromagnetic waves are either converged or diverged using lenses, to form a highly magnified image of the sample. The phenomenon of diffraction of waves puts a limit on the best achievable resolution, which is proportional to the wavelength of the wave used for sample illumination. In other words, using a wave of smaller wavelength allows us to clearly distinguish between two points closer to each other. In Electron Microscopy, a beam of high velocity electrons is used as the source of illumination while exploiting the 'wave-particle duality' of electrons. The De Broglie equation relates the wavelength of a 'matter-wave' to its

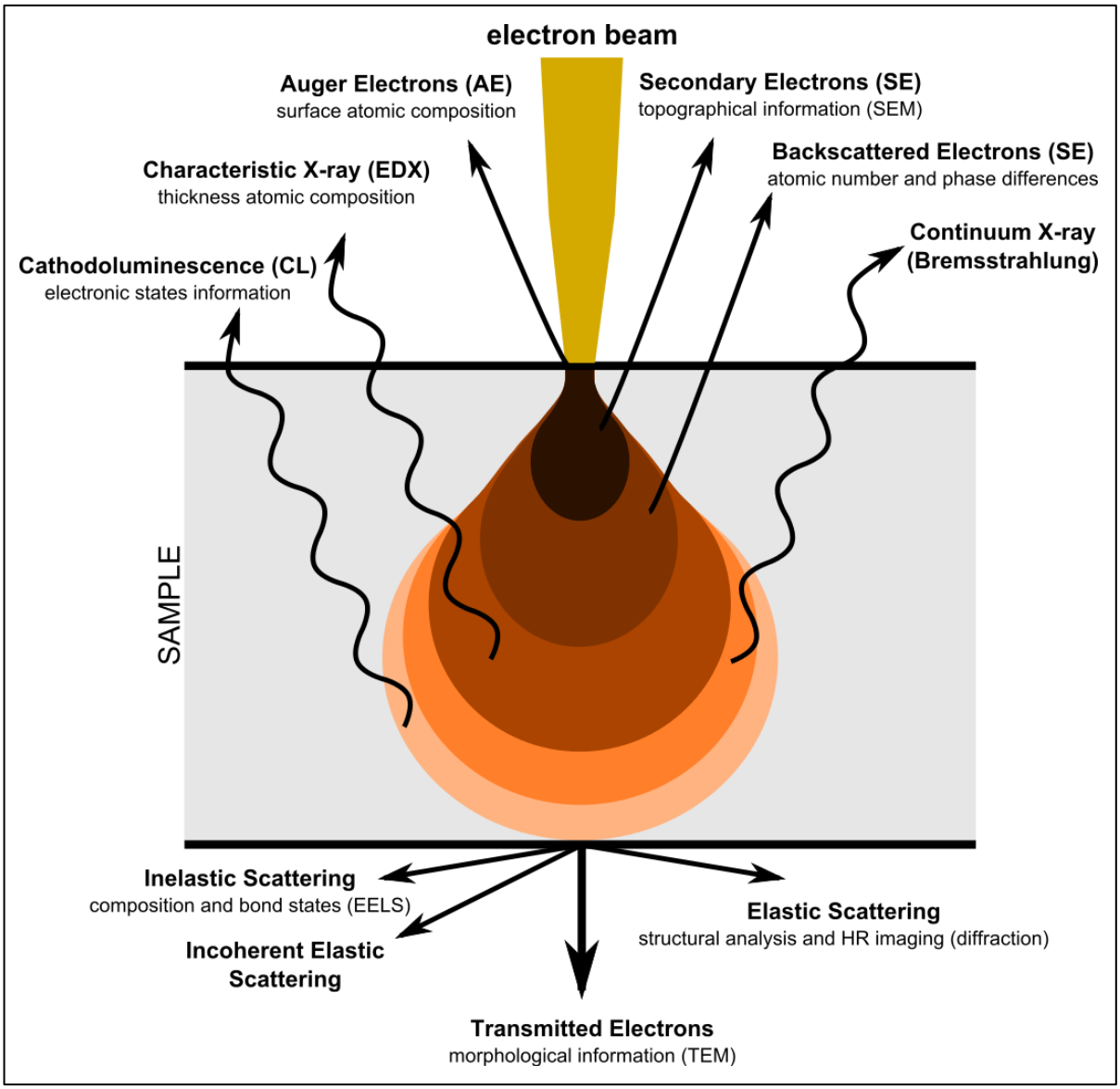

Figure 1 Interactions of electrons with matter and resultant modules of Electron Microscopy [7].

Straight lines represent backscattered or transmitted electrons and curved lines represent electromagnetic waves that are generated after interaction of high energy electrons with matter. Secondary electrons are used for Scanning Electron Microscopy (SEM), while transmitted and elastically scattered electrons are used for Transmission Electron Microscopy (TEM).

momentum as $\lambda = h/p$ (where λ is the wavelength, h is the Plank's constant and p is the momentum of the particle). High velocity electrons have a wavelength which is about 100,000 times smaller than the wavelength of light, which allows us to achieve resolving power of over 1 Å at a magnification of over 1,000,000 x.

Electron microscopes are mostly of two kinds: Scanning Electron Microscopes (SEMs), using secondary electrons for surface reconstruction and Transmission Electron Microscopes (TEMs), using transmitted electrons for 2D projection formation (Figure 1) [7]. Some electron microscopes (such as STEM) provide a facility for simultaneous acquisition of SEM and TEM micrographs, for a holistic understanding of the sample.

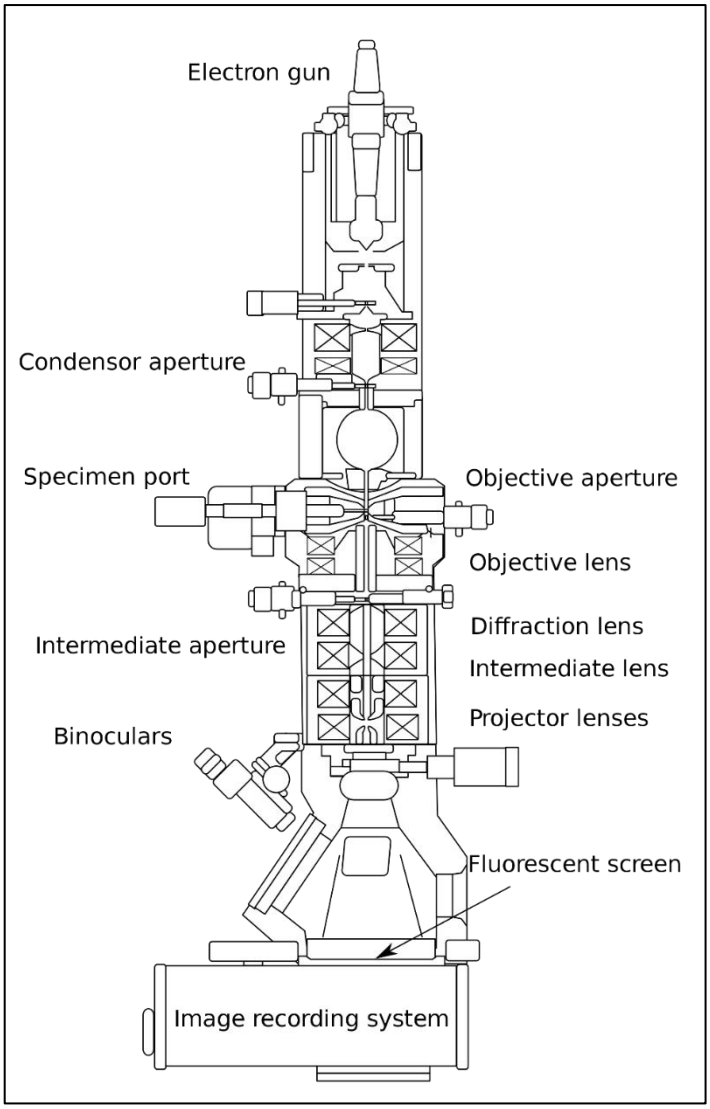

Figure 2 Functionally Essential Components of TEM [7].

Both SEM and TEM have their advantages and limitations. SEM image relies on electrons' interaction at the surface, rather than their transmission, so it is able to image bulk samples and has a good depth of view. SEM images provide us three dimensional and topological information about the sample surface [8] Sample preparation for SEM is much easier when compared with TEM.

TEM (Figure 2) has a resolution which is about an order of magnitude better than SEM [8] TEM can also be used for three dimensional (3D) tomography, which involves collecting TEM images successively by tilting the sample and later using these images to reconstruct a three-dimensional image of the sample. Additionally, TEM can be used with 'image mode' and 'diffraction mode', which allows more flexibility for data collection. In contrast to these benefits, the sample needed for TEM has to be very thin and its preparation is often very laborious. For non-biological materials, atomic resolution is easily achievable, but since biological samples are easily damaged by radiation, the resolution is limited.

In general, some of the drawbacks of electron microscopy include its high cost of installation, operation and maintenance, requirement of special building facilities (for isolation of

vibrations and external magnetic fields), specialized training for its operation and introduction of artefacts during sample preparation and/or data acquisition.

DEVELOPMENTS IN ELECTRON MICROSCOPY FOR BIOLOGICAL STUDIES

Application of Electron Microscopy for studying non-biological materials in their solid state environment, has always been easy when compared to a suspension of soft biological macromolecule in water [9]. This is because biological samples are very susceptible for radiation damage when exposed to high energy electron beam. When studies have to be performed on tissues and sub-cellular structures, there is a risk of total structural collapse due to water evaporation in high vacuum conditions of electron microscope interior. Low contrast of biological molecules leads to very low signal to noise ratio and makes it difficult to distinguish the sample from surrounding. Early developments of Electron Microscopy addressed these issues, to make it 'biofriendly' and to increase its efficacy. Over the years, there were developments such as positive and negative staining of samples to improve image contrast, chemical and cryo fixation of samples for maintaining the sub-cellular structure, metal-shadowing, freeze fracture and freeze etching for studying membrane structures and cell surfaces, ultramicrotomy for producing extremely thin sections of specimens and cryo-techniques. While these developments were relevant mostly for study of tissues and sub-cellular structures, they certainly have matured EM for studying biological macromolecules as well.

SINGLE PARTICLE CRYO-EM

Biological suspensions of macromolecules in vitrified ice provide a low contrast and a low signal to noise ratio (SNR). While it is possible to visualize a single molecule of a protein in an electron micrograph, due to low SNR, it is difficult to extract significant structural information form a single micrograph of a molecule of protein. On the other hand, if similar micrographs of the protein would be averaged, the random noise would reduce significantly with respect to the signal. By averaging a large number of micrographs, the SNR can be greatly enhanced, making it possible to extract structural information from the averaged micrograph. This approach is referred to as single particle analysis, as it involves image processing of micrographs of individual protein molecules. Single particle analysis for electron microscopy has been around since 1980s and has contributed significantly towards pushing the resolution of biological samples into the sub-nanometer range. It would be very appropriate to say that this approach lies at the heart of high resolution structure determination for biological samples using EM.

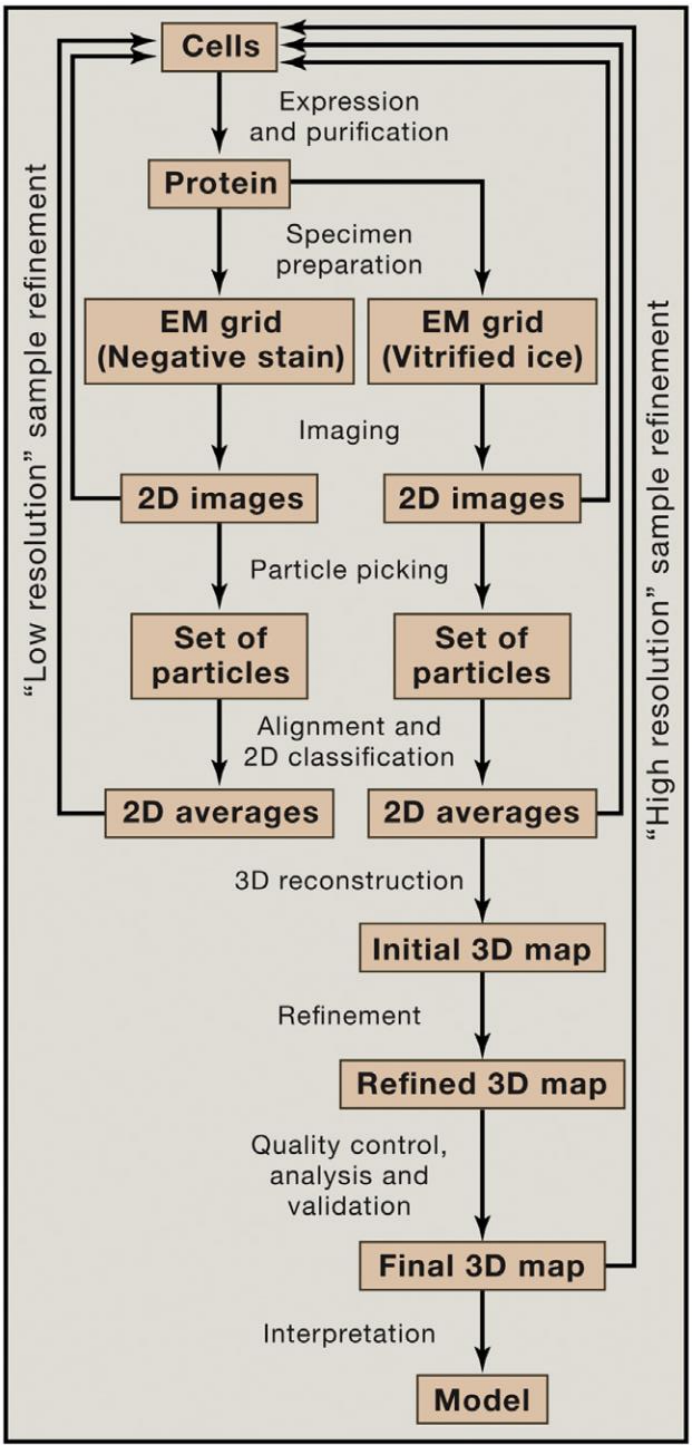

Figure 3 Structure Determination by Single Particle Analysis [10].

Generally, high resolution structure determination by cryo-EM single particle analysis involves nine essential steps (Figure 3) [3,10]. After the sample is prepared by vitrification, it is imaged and a micrograph is collected. This micrograph would contain multiple copies of the protein, possibly in several different orientations and spatial conformations. Individual particles are picked and windowed and they are corrected according to the Contrast Transfer Function (CTF).

After this, they are grouped according to their orientations (2D classification) and an initial screened potential map (or simply density map) is calculated. Now the images are classified according to the projections of initial density map (3D classification) which allows further refinement of the density maps. Finally the density map is validated and a 3D model of protein is built.

Sample and EM-Grid Preparation: For single particle analysis, the sample is a buffered solution of biological macromolecules. Sample preparation begins with purification of proteins. While in most situations, an overexpression system is used, in some cases when it is not possible, native purification can be used. On one hand, sample yield is high in rDNA assisted protein overexpression and it is well stablished and straightforward, on the other hand, native purification ensures that all post translational modifications are present and the sample is more relevant biologically. Integral membrane proteins have huge hydrophobic domains that are natively buried inside a lipid bilayer. Hence, solubilisation of membrane proteins generally requires use of detergents in concentrations above their critical micelle concentration.

Sample homogeneity is an important aspect for modern day single particle cryo-EM. To ensure this, the biomolecular sample is often subjugated to ultra-purification techniques such as HPLC. Even with such precautions, conformational and compositional heterogeneity might be present in the sample.

After protein purification in buffering solution, the sample is subjugated to grid preparation, which can proceed either by vitrification or by negative staining. Negative staining is done for low resolution reconstruction, and since it provides a high negative contrast, it is generally used for optimizing the biochemical preparation of the sample. Heavy metal salts such as uranyl acetate, uranyl formate, ammonium molybdate and phosphotungstic acid can be used for negative staining. The sample is imaged at room temperature in dehydrated conditions, which presents high risk of molecular flattening.

Vitrification of biological molecules is the only reliable technique for high-resolution biomolecular structure-determination using single-particle analysis. It simultaneously allows fixation, radiation protection and prevents water evaporation inside the high vacuum chamber of electron microscope. Vitrification is achieved by flash freezing the sample in liquid ethane, which in-turn is cooled in a bath of liquid nitrogen. Due to rapid cooling, the water molecules in the sample do not have enough time to rearrange themselves as crystals, so transparent amorphous ice or vitreous ice is formed, as well as, the native structural information of the protein is also preserved. Ideally, the thickness of vitrified water layer should be only slightly more than the size of the concerned biomolecule (10 – 100 nm) [11].

While theoretically it seems very straightforward, sample and grid preparation for EM still needs a lot of optimizations for each kind of molecules. Since the contrast in vitrified samples comes from the density difference between protein ($\sim$1.36 g/cm^3) and vitrified buffer ($\sim$0.93 g/cm^3), high concentration of salts is avoided, as it would reduce the contrast [1]. Presence of

glycerol and ethyl glycol causes bubble formation in electron beams, so these cryo-protectants must be avoided as well [1].

Image Acquisition: Data acquisition for high resolution of final reconstruction requires high quality of micrographs. While acquiring data, proper sample illumination must be there, image contrast must be uniform throughout the sample volume and sample drift should be reduced to minimum. Precautions observed during sample preparations can improve signal to noise ratio. It must also be remembered that the sample gets damaged over time due to continuous electron bombardment, so image must be acquired with a limited total electron dose. Overexposure may cause high resolution data loss due to beam induced motion and radiation damage.

2D Alignment and Sorting: Micrographs obtained from EM might contain hundreds of thousands of images of the concerned molecule and they can be expected to be in all possible relative orientations. After image acquisition, a supervised windowing process is followed by image sorting based on relative orientations while rejecting false positives. Images within one sorted group are aligned, superimposed and averaged.

3D Density Map Reconstruction: After the 2D sorting and averaging is done, each image represents the 2D projection of the three dimensional Screened Nuclear Potential (or simply 3D density) of the molecule. The spatial orientation and position of the molecule are defined by six geometric parameters - three Euler angles (which define molecular rotation), two in-plane positional parameters (x and y) and the defocus (which defines the z position along the direction of the electron beam). Defocus is often assumed to be constant for all particles in a micrograph. Class averages of different orientations are combined to give a three dimensional density map. The resolution of the reconstruction is improved iteratively by refining the geometric parameters (except the defocus, since it is constant) for each particle to high accuracy [2].

Model Generation: After 3D density maps are obtained, model generation follows approximately the same course as X-Ray crystallography. The resolution of the 3D density map puts a restriction on the final resolution. If 3D structures of homologues are available, they could be used for direct initialization of high resolution refinement, but this method is prone to risk of introducing model bias [12]. The method of common lines, which depends on interdependence of 2D and 3D Fourier transforms of given data-sets, makes *de novo* structure determination possible for electron microscopy, but it is too sensitive to noise An optimized algorithm is a compromise between preserving computational efficiency with increasing number of data-sets and considering all possible models for given data-sets. This search is still in progress. Meanwhile, single particle cryo-EM borrows a lot of algorithms and computer applications from X-Ray Crystallography, for model building.

Validation: Structure validation is a very important part of any structure determination technique, be it X-Ray Crystallography or Electron Microscopy. This is a final check, an assurance that correct information has been interpreted out of the diffraction patterns or the images. One of the ways in which a EM model can be validated is by comparing it with a complete or incomplete model generated from an independent source, such as atomic cross linking coupled with mass spectrometry, NMR or crystallography, although it must be noted that the secondary structures are prone to changes with change in the molecular environment. Of course 'independent source' lays emphasis on the fact that the model which is used for comparison should not have been used for initialization in 3D reconstruction. When other techniques cannot be involved, a tilt-pair test is used for validation of the low resolution shape of the molecule (Figure 4), where 3D models are generated independently using an 'untilted' and a 'tilted' set of data and later compared for the 'tilt-angle' between them. Model bias or noise bias goes undetected in tilt-pair test. Single particle structure validation is a field that still needs a lot of effort before its maturity.

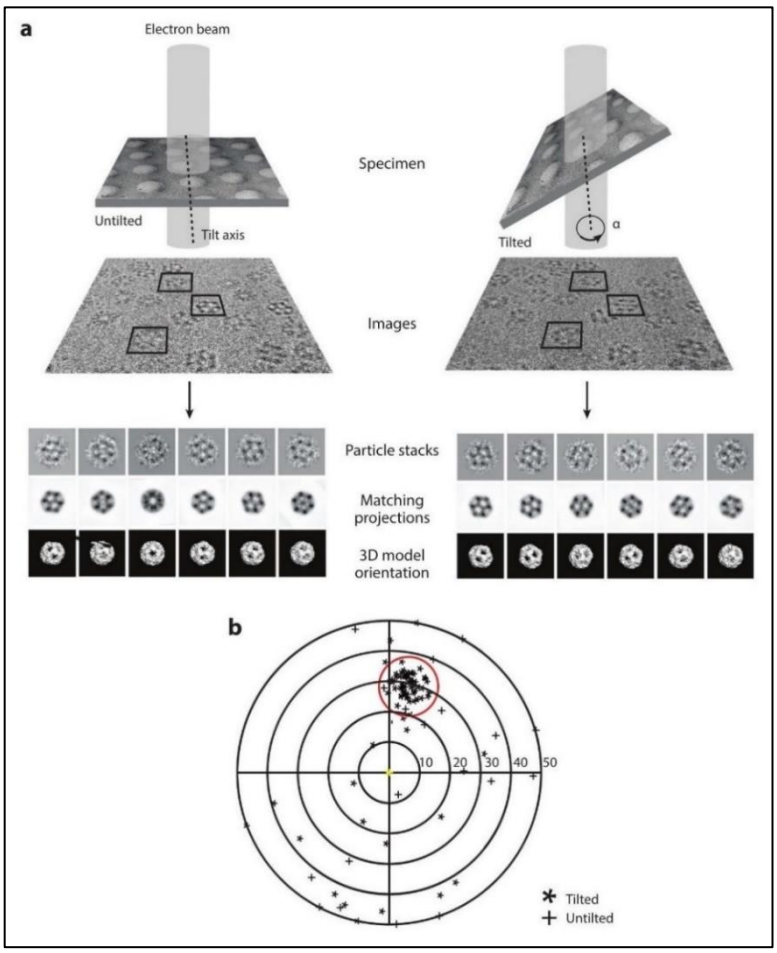

Figure 4 Schematics of Tilt-Pair Test [12].

a. Data is acquired and model is created in two sets - 'untilted' and 'tilted' sample.
b. Geometric relationship between untilted and tilted model is compared with the tilt angle.

THE RESOLUTION REVOLUTION

While recent advances have improved the applicability of cryo-EM for biomolecular structure determination, cryo-EM in its current form is a result of years of continuous developments. It seems appropriate to acknowledge these slow and steady developments before discussing about the quantum leap. Over the years, the accelerating potential of electron microscopes have improved from 200 kV to nearly 400 kV nowadays, leading to production of high energy electrons [13]. While reducing electron wavelength, this development is very elementary for looking into vitreous ice [14]. Use of field emission guns has caused a nearly 500 fold increase in brightness and spatial coherence, when compared to the previously used tungsten filament thermionic emission electron gun [13]. A significant drop in the temperature of cathode has decreased the spread in electron energy and hence has increased the temporal coherence [13]. Stable cold stages have reduced the sample motion induced by temperature fluctuation. Energy filtering technology has allowed imaging with zero-loss energy filtration to reduce sample noise. High vacuum technology has prevented ice contamination build-up around the sample [13]. And finally, automation has played a significant role in obtaining good quality and high quantity of data, something which is of utmost importance for single particle analysis.

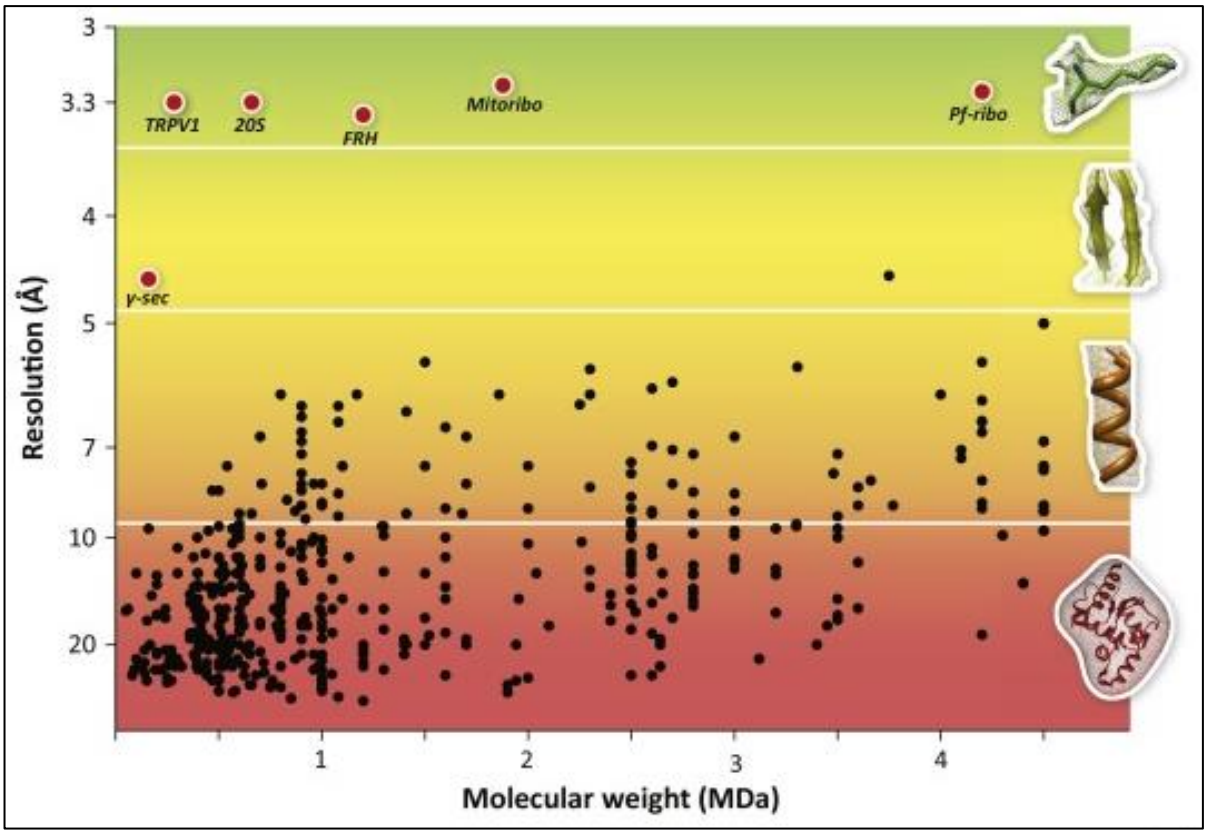

Figure 5 Impact of resolution revolution [5].

The black dots represent structure determination form traditional EM. Red dots represent structure determination by using direct electron detectors and improved classification algorithms.

"Resolution revolution" was a term coined by Werner Kühlbrandt [6], while referring to recent developments in EM image acquisition and image processing technologies. Together, these two developments have pushed the resolution limits achieved by cryo-EM, not only for larger molecules, but for smaller molecules as well (Figure 5). In this section, these significant developments, have been summarized.

DIRECT ELECTRON DETECTORS

Traditionally, photographic films were used for EM data collection, which were later digitized. They provided higher sensitivity for electrons with 80 - 120 keV energy, but low signal to noise ratio (SNR) due to a low value of the total dose (20 - 30 electrons/$Å^2$) [14,15]. Recording on these films required imaging at a higher defocus to ensure sufficient image contrast. This would cause loss of high resolution information. For electronic detectors, the electron image is focussed on a thin layer of phosphor scintillator, which is optically coupled with a camera

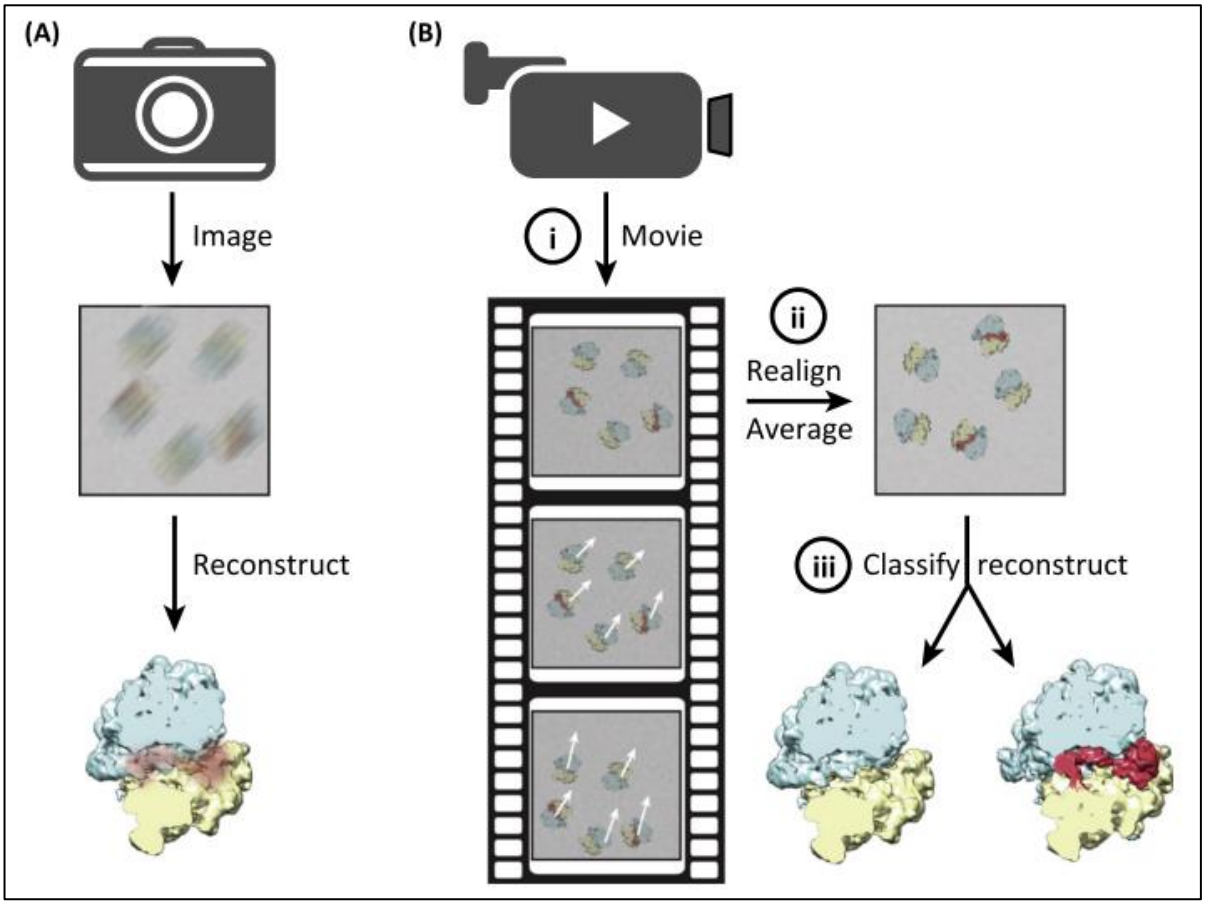

Figure 6 Classical Detectors vs Direct Electron Detectors [5].

(A) The biological sample drifts due to accumulation of charge. Classical detectors are not quick enough, so the drift is captured as blurring. This results to poor final resolution.

(B) Direct Electron Detectors capture the image very rapidly, so rather than recording a single image of a sample, a movie can be recorded, which means that same exposure time would be divided over multiple snapshots. This reduced the effects of the sample drift in the image frame. Image classification becomes easier and different conformations can be easily identified.

sensor such as charged couple device (CCD) or complementary metal oxide semiconductor (CMOS) chip. Although these detectors worked well in 80 - 120 keV range, they had poorer high-frequency detective quantum efficiency (DQE) and smaller area, when compared to photographic plates, making them suboptimal for low electron dose [15]. But despite these shortcomings, electronic detectors had a significant advantage over photographic plates - they could be automated very easily.

Both these detectors were unable to cope with the development of higher energy electron microscopes. Higher energy electrons deposit lower and variable amount of energy at their initial point of contact with the detector surface [14]. They also penetrate deeper, causing increased formation of backscattered electrons, hence causing a general loss of resolution. In general they were not suited for high-resolution electron microscopy.

In recent few years, direct electron detectors have been developed. They use back-thinned Monolithic Active Pixel Sensor (MAPS) technology [5]. This technology is most commonly used in the camera chips of modern smartphones [6]. They are directly fabricated on silicon, with most of the support matrix removed. This back-thinning significantly reduces noise and resolution loss due to backscattered electrons [14]. Since these detectors are sensitive to individual electrons, so they get easily saturated even at low electron doses. When this property is combined with their fast frame read-out rate, it allows them to spread the same exposure time of biological molecules over multiple frames (Figure 6). In other words, they can be used in rolling shutter mode or movie mode, where one movie is composed of hundreds of frames with a much shorter exposure time. This also allows use of a higher total dose, which can enhance image contrast. It also allows detection of sample movement and reversing it using a computer. The movie mode of data acquisition has allowed us to visualize and correct for the beam induced motion of the sample, eventually, to improve the signal to noise ratio. This is done by realignment of individual frames of the movie acquired from direct electron detectors. Also, by using the Bayesian framework, it is also possible to track the motion of individual particles and reverse it during image processing, hence making the realignment technique automated and high-throughput.

Direct electron detectors can acquire data via two modes - either the number of electron events could be counted (electron-counting mode) or charges generated from striking of electron to detector surface could be integrated (linear charge-integration mode). Electron-counting detectors are relatively ideal for gathering high resolution information since they reduce Landau noise and readout noise [2]. The electron counting mode improves DQE at low resolution, which allows imaging at lower defocus, which in practice means that high resolution information is better preserved, while providing sufficient contrast for particle picking [3]. Another major advantage of direct electron detectors is the super resolution mode, which allows the possibility of inferring the initial point of incidence to sub pixel resolution and hence to obtain information beyond the traditional Nyquist frequency limit [14]. Due to all these factors, electron counting detectors have proved to be very relevant for high resolution structure determination.

MAXIMUM LIKELIHOOD BASED CLASSIFICATION

After data acquisition, hundreds of thousands of images of individual molecules are obtained. These images have the necessary information for reconstruction of 3D density maps, in a truly homogeneous sample, this information would comprise different orientations of the macromolecule. However, a certain degree of conformational and compositional heterogeneity is always present. Ignoring these differences during 3D density reconstruction would mean that the density map would represent 'average' features from all possible conformations, rather than the correct structure. This underscores the importance of sorting images into different classes, which can be based on the orientation, conformation or type of imaged biomolecule. Automation of the sorting process is necessary for practical reasons (since hundreds of thousands of images have to be analysed) and for keeping subjective errors at bay.

Automated image sorting is achieved using 'unsupervised 2D and 3D-classification' algorithms, which are mostly based on maximum-likelihood but are also refined by using other statistical algorithms such as multivariate statistical analysis, 3D variance calculation by bootstrapping or nonstatistical multiparticle refinement [5,16]. In the maximum likelihood approach, direct assignment of an orientation is avoided, but a fuzzy assignment of orientation is done based on calculated probabilities (maximum likelihood) [17]. For 3D reconstruction, the probabilities of orientations are used. There are two major advantages of the maximum likelihood approach: firstly, during 3D reconstructions, more reliable orientations have higher weight; and it is easy to automate particle sorting based on conformational heterogeneity. Over the years, these algorithms have been integrated into major reconstruction software [2]. Many high-resolution structures from Cryo-EM are resultant of the maximum likelihood method. Since small protein molecules are not able to provide sufficient contrast, atomic structure determination for protein complexes below 100 kDa in size is difficult, if not impossible. In practice, small antibody fragment of 50-kDa, known as Fab fragment, is attached to the protein molecule, which increases the effective molecular size. It also acts as a fiducial marker for assisting in image alignment [3.] Fab fragments may also promote sample homogeneity by stabilizing a particular protein conformation. Care must be taken however in designing and selecting these molecular appendages so that they bind to specific rigid sites on the proteins and with high affinity, otherwise they would defy their purpose of improving single particle analysis [3.] Using Fab fragments, cryo-EM structure analysis for proteins as small as 65 kDa was demonstrated [3,23,24.] presents an example for the structure determination of ribosomes [18].

SAMPLE PREPARATION

Membrane protein structure determination has benefitted a lot from improved sample preparation. Detergents ideal for cryo-EM, such as maltose-neopentyl glycol-3 (MNG-3), are being developed [3]. These detergents feature low critical micelle concentration (CMC), which enable them to be easily removed from the protein solution. Eventually, it is possible to form thinner vitreous ice layers [3]. Amphipols are amphipathic polymers that interact with trans-

membrane domains in semi-irreversible manner. So after treatment with amphipols, membrane proteins behave like soluble proteins in solution. Cryo-EM resolution was improved for certain membrane proteins due to use of amphipols [19–22]. Inspired by the β-barrel proteins of gram negative bacteria, special β-sheet peptides have been designed and optimized to stabilize membrane proteins [3]. To ensure that the membrane proteins remain in their native lipid environment, some proteins have been studied in liposomes. This approach has caused more problems than solutions, because of difficulties in determining protein orientation parameters. This issue has been addressed to some extent by 'random spherically constrained single particle reconstruction method' [3]. Developments in nanodisc-technology

for reconstituting single membrane protein complexes is also expected to have positive influence on high resolution cryo-EM of membrane proteins.

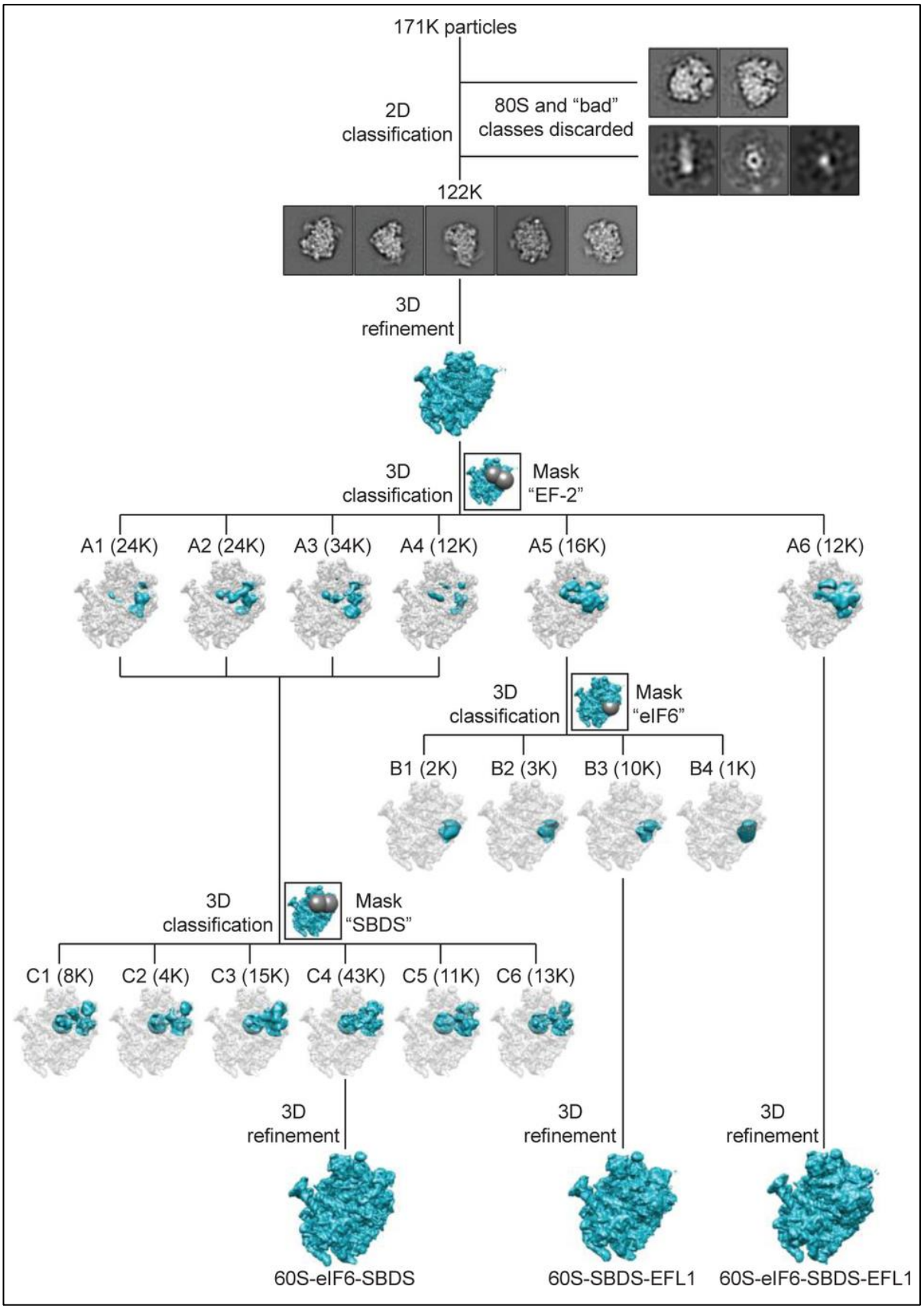

Figure 7 Example for high resolution image processing work-flow for cryo-EM data [18].

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On top of protein purification, optimization of biochemical conditions can tremendously improve the achievable resolution [11]. The ProteoPlex technique is a high-throughput transition-temperature-monitoring technique, which has been shown to assist in this [11]. It relies on the properties of an environmentally sensitive fluorescent dye, where quantum yield of the dye significantly increases during protein unfolding [11]. Generally, if the transition temperature is higher, the condition is more suitable for cryo-EM. Mild chemical fixation techniques coupled with density gradient centrifugation have been shown to preserve tertiary and quaternary structure of proteins, hence promoting conformational and compositional homogeneity [11]. Gradient Detergent Removal (GraDeR) has been shown to enhance contrast for membrane proteins. Essentially, this technique involves solubilisation of membrane proteins in lauryl maltose-neopentyl glycol (LMNG), followed by density gradient removal of excess LMNG [11].

POSSIBILITIES AFTER THE RESOLUTION REVOLUTION

The developments in image processing have efficiently complemented the developments in image acquisition to improve the achievable resolution of EM. On one hand the confidence in individual images has increased due to high signal to noise ratio, and on the other hand improved individual images have contributed to better classification and reconstruction. Although there are a few theoretical limitations to what can be accomplished, the 'resolution revolution' has already achieved a few 'supposedly impossible' feats, for example 2.2 Å cryo-EM structure of a 465 kDa enzyme β-galactosidase [11]. It is now possible to solve the atomic models of protein molecules using Electron Microscopy, without relying on prior structures [19,20,25]. At ~3 Å resolution, even small molecules bound to macromolecular complexes can be identified [26]. At any given resolution, *de novo* model reconstruction yields better results for EM as compared to X-Ray crystallography, probably because unlike X-Ray crystallography, the phase information is preserved in EM micrographs [5]. In recent years, atomic structures of relatively small proteins have been determined by EM [19,22]. When symmetry is present in the complex, protein structure determination becomes easier. Although it must be noted that in many cases EM is still not able to reach to a resolution where atomic models can be unambiguously determined.

New developments in EM have shown most promising results for the cases where sample has a high degree of heterogeneity, purification is inefficient to get rid of contaminants or where expression altogether is very difficult rendering low concentration of sample. These problems render NMR as well as X-Ray crystallography useless, but EM is able to produce good results even with these conditions [27]. One of the major implications of these developments is the fact that now it is possible to catch protein molecules while 'in action' [20,25]. It must be noted, however, that even with single particle analysis, sample heterogeneity complicates structure determination, hence only so much can be done if a very high degree of sample heterogeneity is there.

MEMBRANE PROTEIN STRUCTURE DETERMINATION USING CRYO-EM

Membrane protein research is expected to greatly benefit from recent developments in cryo-EM. Already several atomic structures of membrane proteins have been published. These results are significant because they are not only solving age-old problems of structural biology, but are also successfully challenging some of the established norms of membrane protein research, forcing us to look at membrane proteins from a fresh perspective. These developments are discussed using some notable examples involving application cryo-EM for studying membrane protein complexes.

STRUCTURE AND MECHANISM OF TRPV1 ION CHANNEL [19,20]

TRPV1 stands for *member 1 of subfamily 5 of the Transient Receptor Potential (TRP) non-selective cation channels*. TRPs are known to be involved in calcium adsorption, sensory transduction, as well as in diseases such as Olmsted syndrome, dysplasia syndrome. TRPV1 is a receptor for capsaicin as well as it is thermosensitive, which means it can sense changes in ambient temperatures [19]. It has a molecular weight of around 300 kDa. TRPV1 is activated by noxious heat and modulated by inflammatory agents, such as extracellular protons and bioactive lipids, which contribute to pain hypersensitivity [19]. Due to its involvement in pain sensation, TRPV1 and other somatosensory TRPs are considered to be an important target for analgesic drugs. It is believed that structure of TRPs is similar to Voltage Gated Ion Channels

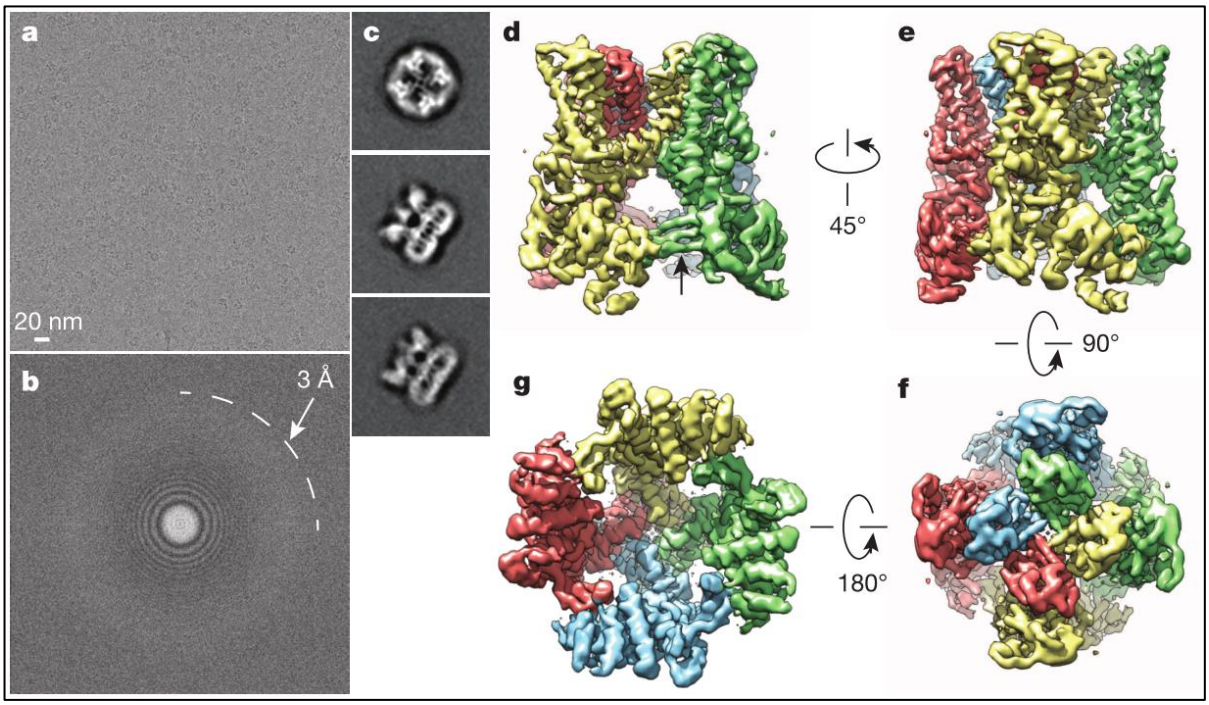

Figure 8 3D Reconstruction of TRPV1 by Cryo-EM Single Particle Analysis [19].

a. Micrograph acquired by using direct electron detector containing many images of TRPV1.
b. Fourier transform of the micrograph shown in a with Thon rings extending till nearly 3 Å.
c. 2D class averages of different relative orientations.
d to g. Electron density maps in different views at 3.4 Å resolution.

(VGICs) but they are extremely difficult to crystallize so their atomic structure was not previously known. *Liao M. et.al.* [19] have not only reported the atomic structure of TRPV1, but this was the first reported atomic structure of a membrane protein complex using cryo-EM.

Micrographs were acquired using frozen hydrated amphipol-solubilized monodisperse TRPV1 at 300 kV using a direct detection camera in movie mode to allow dose fractionation and correct for sample motion. After motion correction and 2D classification, final *de-novo* 3D reconstruction of TRPV1 had an overall resolution of 3.4 Å, using gold-standard Fourier shell correlation (FSC) = 0.143 criteria.

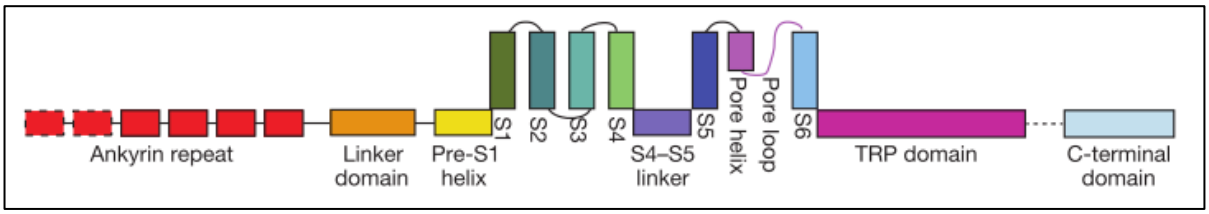

Figure 9 Arrangement of TRPV1 Complex [19].

The TRPV1 protein complex begins with Ankyrin repeats in the N-Terminal, sequentially followed by a transmembrane domain, TRP domain and a C-Terminal domain (Figure 9). Two linkers are present, one between Ankyrin repeats and trans-membrane helix S1 and another between S4 and S5. In the final reconstruction, side-chain densities of all transmembrane helices and TRP domain were well resolved. Densities for the first two Ankyrin were absent, but anti-parallel beta-sheets were visible for rest of them. The structure of Ankyrin repeats (residues L111 - E359) was docked on to the *de-novo* created atomic model of the rest of the structure (residues L360 - A719).

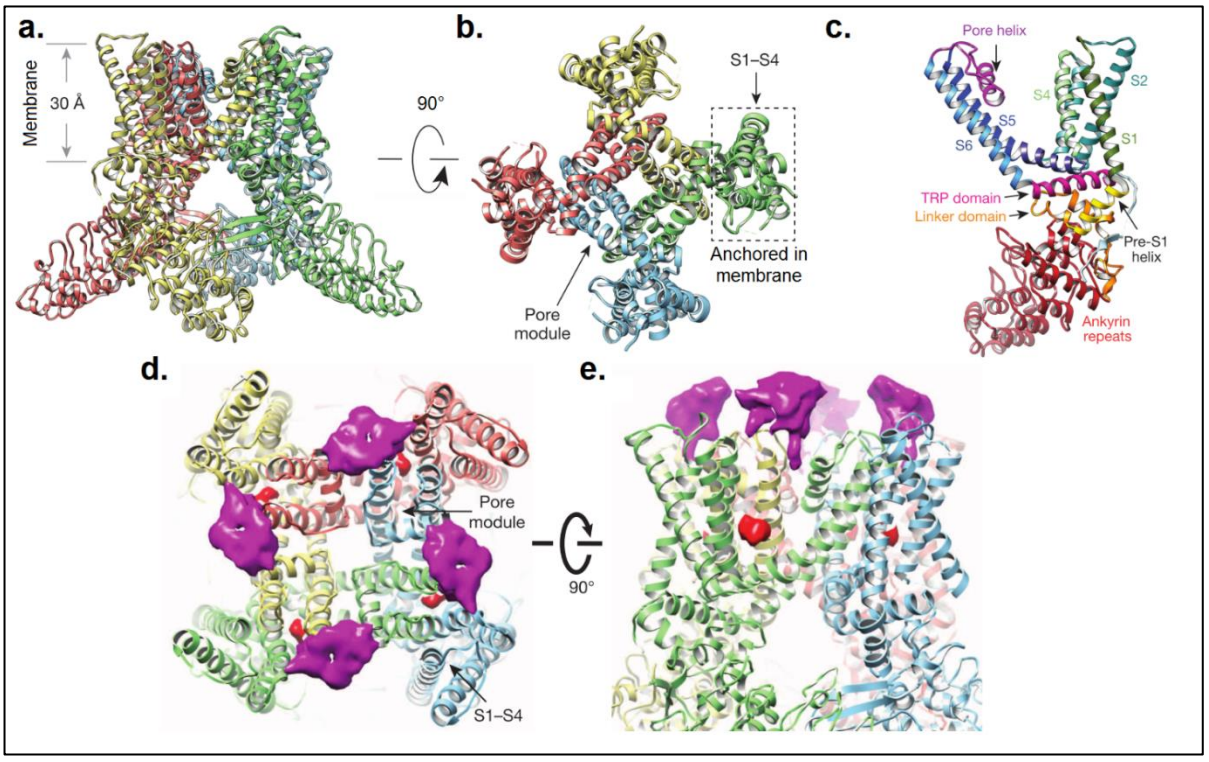

Figure 10 Structure of TRPV1 and binding sites [19,20].

TRPV1 has a tetrameric architecture. Each subunit has six transmembrane alpha helices that span the bilayer (Figure 10). The transmembrane domain has two distinct substructures: helices S5, SP6 and Pore-Helix constitutes the pore, while helices S1 - S4 are rigidly anchored in the membrane and provide external surface for binding of lipophilic ligands. The helical TRP domain is separated from the S6 transmembrane helix by a sharp bend, and is believed to be responsible for allosteric modulation of channel gating, by facilitating coupling between channel domains. The TRPV1 pore has a funnel like structure, and midway inside the pore a selectivity filter is present with side chains pointing towards the cavity. Lack of hydrogen-bonds between pore helices makes TRPV1 very flexible in nature and susceptible to pore dilation. The Ankyrin repeats are mostly responsible for packaging of the cytosolic part of TRPV1. While doing so, it can modulate TRPV1 gating, depending on its interaction with various intracellular factors.

TRP channels respond to diverse physical and chemical stimuli, and hence are believed to be very dynamic in nature, which in turn could be the reason why they are so difficult to crystallize. Single particle cryo-EM has allowed to circumvent this problem to determine a high resolution structure of TRPV1 and has improved our understanding of the mechanism of its action.

THREE DIMENSIONAL STRUCTURE OF HUMAN γ-SECRETASE [21,22]

Human γ-Secretase is a membrane embedded aspartyl protease that cleaves a large number of transmembrane substrate proteins. The cleavage product serves as signalling molecule in a process called regulated intramembrane proteolysis (RIP). Human γ-Secretase (HγS) is responsible for cleavage of amyloid precursor protein (APP) and the Notch receptor, so its structure determination is pertinent to understanding the manifestation of Alzheimer's disease as well as several forms of cancer. The Human γ-Secretase complex comprises four subunits, namely *PS1*, *PEN-2*, *APH-1* and *Nicastrin*, which have a combined molecular weight of 170 kDa, along with an additional 30 - 70 kDa of glycosylation in nicastrin extra cellular domain. Researchers have faced many challenges in structural studies of Human γ-Secretase because of difficulties faced in its production and intact purification.

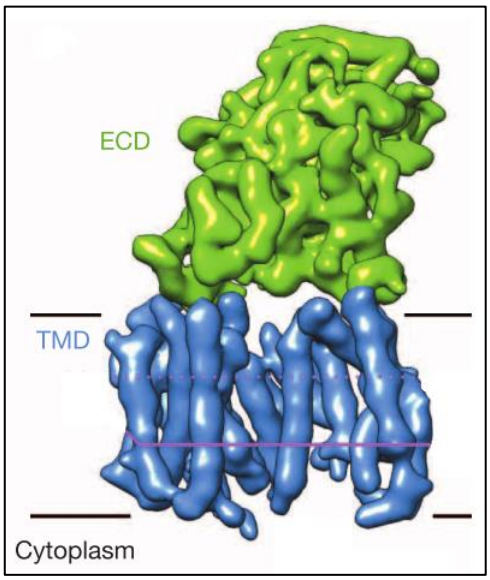

Figure 11 HγS structure at 4.5 Å [21]

In 2014, *Lu et.al* [21] reported a three dimensional structure of intact Human γ-Secretase, where HγS was solubilized in amphipol A8-35 and data was collected using direct detector camera in electron counting mode to achieve high signal to noise ratio at lower spatial frequencies. After movie processing and statistical image classification, an overall resolution of 4.5 Å was achieved [21]. Later in 2015, *Bai et.al.* [22] from the same group changed their data acquisition and image analysis approach to report the first atomic structure of Human γ-Secretase at a significantly better 3.4 Å resolution. To achieve this, a larger data set was collected, using zero-loss energy filtered imaging, a higher magnification and a lower dose rate on a single electron counting detector, and a 3D classification approach focussed on relatively rigid trans-membrane domain was employed.

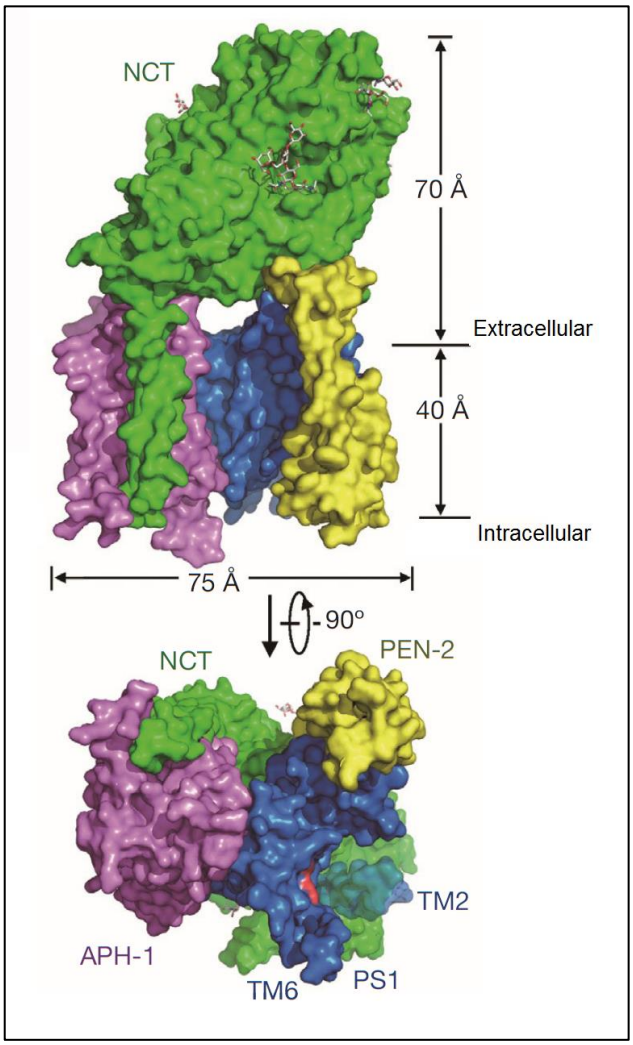

Figure 12 Human γ-Secretase structure at 3.4 Å [22].

These two studies performed by *Bai et.al.* and *Lu et.al* are significant because they demonstrate the potentials of cryo-EM for studying small monomeric and asymmetric complexes. While intrinsically flexible and dynamic regions still pose a serious challenge for determining structures of entire complexes, cryo-EM has good potential to provide significant structural and mechanistic information of protein complexes. These studies also demonstrate that for the same sample, experimenting with different approaches for structure determination can sometimes lead to significantly better results.

GATING MECHANISM OF CorA MAGNESIUM CHANNEL [25]

CorA is a nearly 200 kDa Mg^{2+} dependent ion channel responsible for the uptake of magnesium by prokaryotes. Homeostasis through the CorA takes place by a negative feedback loop, where excess of Mg^{2+} in cells leads to closure of the ion channel, and low concentrations promote the conductive state. So for CorA ion channel Mg^{2+} acts as both gating ligand as well as the substrate for transport. The negative feedback mechanism of CorA is in contrast with the mechanism of most ligand-gated ion channels, where energy released during the binding of ligands to proteins is responsible for opening of the channel cavity. Studies have shown contradicting results for activation/deactivation mechanisms of CorA channels. On one hand X-Ray crystallography has suggested no significant difference between the Mg^{2+} bound and the unbound state of CorA, on the other hand, Electron Paramagnetic Resonance (EPR) studies have suggested huge deviation of the unbound quaternary structure from the bound one [25]. *Matthies et.al.* [25] have reported two very different conformations of Mg^{2+} bound and unbound CorA, while explaining its gating mechanism as well. Differing from the commonly accepted norms, the unbound state has been reported as an asymmetric structure.

CorA was either solubilized in DDM or reconstituted in nanodiscs and plunge frozen in liquid-ethane was used for micrograph acquisition. EM Data for DDM solubilized CorA was collected with FEI Titan Krios microscope, operated at 300 kV and aligned for parallel illumination. GIF Quantum energy filter, operated in zero-energy-loss mode, was used before image detector. K2 Summit camera was used in super-resolution mode for acquiring 38 frames per image and at an accumulated dose rate of $\sim 40\ e^{-}/Å^2$. For nanodisc data-collection a second microscope with Falcon II direct electron detector (1.4 Å pixel size) was used. During image processing, movie alignment, CTF determination, particle picking and 2D class averaging were performed before generating a low resolution model with 5 fold symmetry (for Mg^{2+} bound state only). This low resolution model was used for 3D classification, refinement and post processing to generate a final map of 3.8 Å resolution. Symmetry was not imposed for unbound CorA. While *de-novo* atomic model determination was possible for Mg^{2+} bound CorA, for determination of asymmetric unbound CorA, the Mg^{2+} bound atomic model was fragmented into seven overlapping pieces per subunit and fitted into a 7.1 Å filtered density map. A 7.1 Å filtered model was also

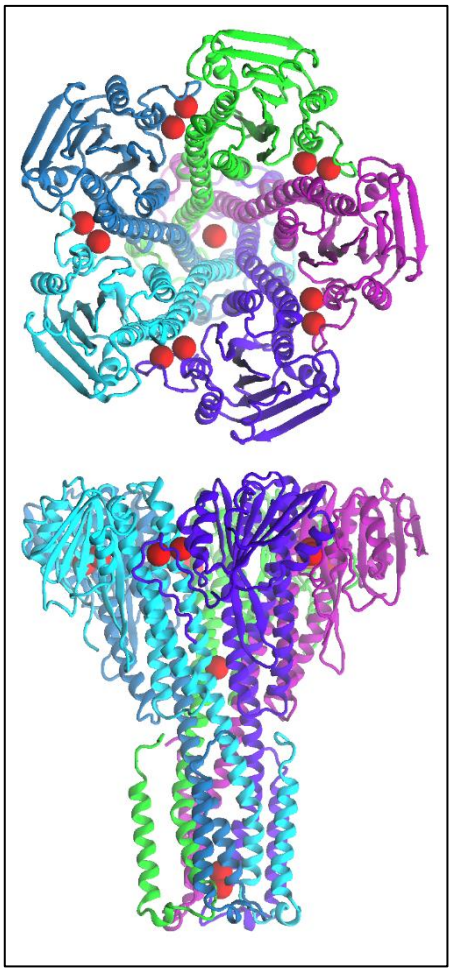

Figure 13 CorA with Mg^{2+} bound (3JCF.pdb).

generated for Mg^{2+} bound CorA by using a similar approach. Even in these low resolution models, by locating well separated α-helices and β-sheets, it was possible to unambiguously trace the polypeptide chains. Mg^{2+} densities could be detected at subunit interfaces.

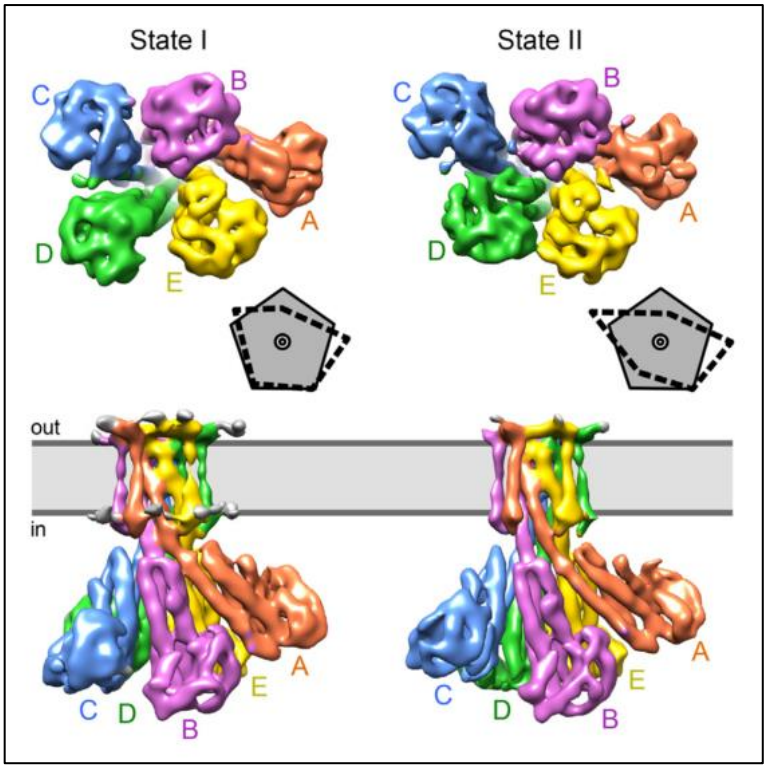

Figure 14 Loss of fivefold symmetry in unbound CorA [25].

It was discovered that the closed (Mg^{2+} bound) state was mostly similar to that reported by XRD crystallography, with some differences in the dynamic regions of the channel. Overall, CorA was present as a homopentamer, with fivefold symmetry (Figure 13). Mg^{2+} density could be seen at the monomer interface, as well as inside the CorA channel cavity. In case of ligand free CorA, the fivefold symmetry of the cytoplasmic domain was lost due to subunit movement (Figure 14) but the trans-membrane domain retains approximate fivefold symmetry. The physiological relevance of the loss of fivefold symmetry of the cytoplasmic domain was supported by several other experiments such as cryo-EM of CorA reconstituted in lipid nanodiscs, subunit crosslinking experiments and molecular dynamics simulations with dummy spin labels [25].

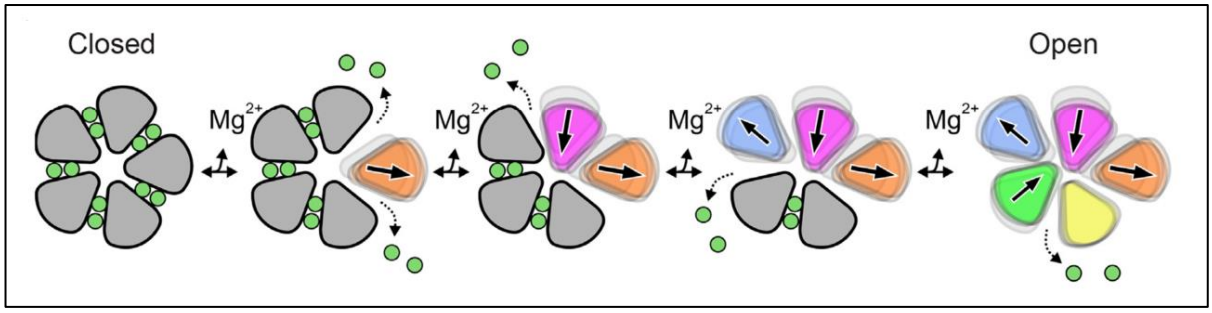

Figure 15 Gating mechanism of CorA [25].

Based on these observations, a gating mechanism was proposed, where in presence of Mg^{2+}, inter-subunit forces stabilize the quaternary structure, but as intracellular Mg^{2+} concentration falls, Mg^{2+} is released from the interface (Figure 15). This destabilizes the quaternary structure, making the cytoplasmic domains more dynamic and flexible. The loss of cytoplasmic domain rigidity is translated to the transmembrane domain, which causes random and transient opening of the ion channel.

In this study, classification of images allowed identification of several conformations from same sample set, which in turn elucidated the mechanism of action. Compared to this, XRD crystallography presents a 'static snapshot' representing the average of all conformations present in a crystal, with a higher B-factor for the more flexible domains. So, this study shows that even a lower resolution cryo-EM structure (3.8 Å or 4.4 Å in this study) can reveal more significant physiologically relevant information about the underlying biomolecular mechanisms as compared to a higher resolution XRD crystal structure (3.2 Å) [28].

DISCUSSION

Cryo-EM structure determination had a quantum leap few years ago, with improved image acquisition technology and image classification technique. Rather than providing a density map (blob) for fitting of previously known structures, single particle cryo-EM is nowadays is able to *de-novo* produce high resolution and atomic structures, and not just for large molecular complexes such as ribosomes or viruses [29], bur for small asymmetric macromolecular complexes as well. As years pass by, the single molecule Cryo-EM technique has produced several high impact researches related to membrane protein complexes, which earlier eluded structural-biologists due to numerous complications in good quality sample preparation and high resolution data acquisition. Apart from providing high resolution structures of protein molecules, cryo-EM has also played a pivot role in understanding of the underlying mechanisms by providing snapshots of structural intermediates [19,20,25] from the same biological sample. In this aspect, cryo-EM has significant advantage over XRD Crystallography because rather than producing a binary result (success or failure) for protein structure determination, this technique almost always provides some structural insight [2]. This is because even at lower cryo-EM resolutions, the prominent features such as alpha helices and beta sheets are visible, which can elucidate the mechanism of action [23]. Despite these successes, single particle cryo-EM for atomic structure determination is relatively young and needs numerous improvements.

First and perhaps the most important of all, the DQE of direct detectors needs improvements as it is well below what is physically possible (~0.5 as compared to ~0.8) [5]. Higher DQE would lead to better signal to noise ratio, leading to improved confidence in unsupervised automated image processing.

Even with rolling shutter mode or movie mode of direct detector cameras, beam induced sample movement is a problem. It is known that even with one tenth of regular electron dose, nearly 99% of 3 Å is destroyed during initial exposure [5]. And yet these initial phases of data collection face most serious sample movement, and data from these frames has to be weighed down or discarded altogether. Stopping sample movement would be a new revolution in electron microscopy. Some success has been achieved for this by use of graphene and gold EM-grids [11,30]. Better understanding of causes of beam induced motion and change of approach for sample preparation and data collection can bring some success in this direction [5,13].

Some efforts are also needed to standardize and perfect biological sample preparation for electron microscopy. While it is true that most biological samples, membrane proteins in particular, need their own 'special optimization', the underlying principles of sample preparation remain more or less the same. New innovations are required to ensure stability of fragile molecular complexes and to provide higher contrast for smaller macromolecules without compromising their structural integrity.

Cryo-EM can also potentially benefit from commercial availability of phase plates, which is a device that produces phase contrast by introducing a phase shift between scattered and unscattered waves [31]. By introducing additional contrast in imaging of particles, phase plates have shown their applicability for low resolution and near atomic resolution single particle structure determination [31,32]. Use of such devices has to be standardized and further incorporated in routine electron microscopy.

While most single particle cryo-EM structures are now available above 3 Å resolution, pushing this number to below 3 Å or 2.5 Å would greatly reduce the enormous amount of time spent in *de-novo* model building and structure refinement [2]. Also, since at this resolution ions and bound ligands would be visible as well, this development would have positive influence on drug discovery and related biomedical researches.

For better data sorting and image processing, related algorithms have to be improved. As a matter of fact, each post-imaging step of single particle analysis can benefit from improved algorithms. Some of our current limitations in this area include inability to handle high heterogeneity in the sample, inefficient computational power to handle newer and/or better algorithms and lack of tools and methodologies for efficient model building, refinement and structure validation [2]. Significant time must be spent to resolve these shortcomings. Only then can we be more confident of the reported cryo-EM structures. It is worthwhile to mention here that *de novo* development of any technology may not always be the best answer to the existing problems and same is true for EM as well. Cryo-EM 3D density maps are homologous to electron density maps obtained from X-Ray crystallography. Hence many existing X-Ray crystallography tools developed for the building and stereo-chemical refinement of molecular structures have been readily adopted for cryo-EM [5], and in the future, it can be expected that single-particle cryo-EM and X-Ray crystallography would greatly benefit from each other, not only in terms of better algorithm development for model building and validation, but also for sample preparation [11].

Automation of different steps would also benefit single particle cryo-EM by introducing a standard practice and hence reducing human biases and errors during sample preparation and data analysis. In theory, a sample can be imaged in a cryo electron microscope for days. Automation of data collection would help in increasing the quantity and possibly the quality of data. During data analysis, an efficient algorithm for unsupervised image alignment and 2D and 3D classification would significantly reduce the time spent in processing hundreds of thousands of molecular images. Of-course any such be rigorously tested before introduction in mainstream cryo-EM.

Atomic resolution biomolecular structure determination with EM was considered impossible few years ago. This was limited mostly by technological advancements. Recent success of cryo-EM can make us hopeful about several other developments such as time resolved cryo-EM and continuum structure determination [33]. Time resolved EM could allow imaging of short lived (10-1000 ms) intermediates of proteins [33]. It would mostly involve developments in sample preparation methods, so that chronologically separated molecular events could be

temporally separated on the EM grid. Continuum structure determination, as the name suggests, would allow recovering of an entire continuum of coexisting species [33]. Successful continuum structure determination would produce cryo-EM movies, rather than cryo-EM images. While this sounds tempting, this would require imaging of millions of particles and would involve some serious developments in current image classification algorithms and computational power. For both these developments, sound scientific principles exist, but technological development is awaited [33].

Finally, given the immense potential of biomedical research, for propagation of cryo-EM, academia, industry and governments have to work together. Modern electron microscopes are very expensive, so only a select few institutions can afford them. This thwarts the reaping of full potentials of EM. It would be beneficial to invest in synchrotron-like cryo-EM facilities [2], which could provide latest hardware and computation infrastructure to researchers at nominal costs. Additionally, since applications of cryo-EM are expected to proliferate, it would help the field to invest time and resources on training of cryo-EM experts.

REFERENCES

1. Rubinstein, J. L. Structural analysis of membrane protein complexes by single particle electron microscopy. *Methods* **41,** 409–416 (2007).

2. Cheng, Y. Single-Particle Cryo-EM at Crystallographic Resolution. *Cell* **161,** 450–457 (2015).

3. Zorzi, R. De, Mi, W., Liao, M. & Walz, T. Single-particle electron microscopy in the study of membrane protein structure. *Microscopy* **65,** 81–96 (2016).

4. Curnow, P. Membrane proteins in nanotechnology. *Biochemical Society transactions* **37,** 643–652 (2009).

5. Bai, X., McMullan, G. & Scheres, S. H. . How cryo-EM is revolutionizing structural biology. *Trends in Biochemical Sciences* **40,** 49–57 (2015).

6. Kühlbrandt, W. The Resolution Revolution. *Science* **343,** 1443–1444 (2014).

7. Wikimedia Commons Open Source Information. Wikimedia Commons. *Wikipedia* at <http://www.wikipedia.org/>

8. John Innes Centre. at <https://www.jic.ac.uk/>

9. Schröder, R. R. Advances in electron microscopy: A qualitative view of instrumentation development for macromolecular imaging and tomography. *Archives of biochemistry and biophysics* **581,** 25–38 (2015).

10. Cheng, Y., Grigorieff, N., Penczek, P. A. & Walz, T. A Primer to Single-Particle Cryo-Electron Microscopy. *Cell* **161,** 438–449 (2015).

11. Stark, H. & Chari, A. Sample preparation of biological macromolecular assemblies for the determination of high-resolution structures by cryo-electron microscopy. *Microscopy (Oxford, England)* dfv367 (2015). doi:10.1093/jmicro/dfv367

12. Elmlund, D. & Elmlund, H. Cryogenic Electron Microscopy and Single-Particle Analysis. *Annual Review of Biochemistry* **84,** 499–517 (2015).

13. Henderson, R. Overview and future of single particle electron cryomicroscopy. *Archives of biochemistry and biophysics* **581,** 1–6 (2015).

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

15. Ruskin, R. S., Yu, Z. & Grigorieff, N. Quantitative characterization of electron detectors for transmission electron microscopy. *Journal of Structural Biology* **184,** 385–393 (2013).

16. Elmlund, D. & Elmlund, H. Cryogenic electron microscopy and single-particle analysis. *Annual review of biochemistry* **84,** 499–517 (2015).

17. Sigworth, F. J. Principles of cryo-EM single-particle image processing. *Microscopy* **65,** 57–67 (2016).

18. Weis, F. *et al.* Mechanism of eIF6 release from the nascent 60S ribosomal subunit. *Nature structural & molecular biology* **22,** 914–919 (2015).

19. Liao, M., Cao, E., Julius, D. & Cheng, Y. Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature* **504,** 107–12 (2013).

20. Cao, E., Liao, M., Cheng, Y. & Julius, D. TRPV1 structures in distinct conformations reveal activation mechanisms. *Nature* **504,** 113–8 (2013).

21. Lu, P. *et al.* Three-dimensional structure of human γ-secretase. *Nature* **512,** 166–170 (2014).

22. Bai, X. *et al.* An atomic structure of human γ-secretase. *Nature Article* **525,** 212–217 (2015).

23. Kim, J. *et al.* Subnanometre-resolution electron cryomicroscopy structure of a heterodimeric ABC exporter. *Nature* **517,** 396–400 (2015).

24. Wu, S. *et al.* Fabs enable single particle cryoEM studies of small proteins. *Structure* **20,** 582–592 (2012).

25. Matthies, D. *et al.* Cryo-EM Structures of the Magnesium Channel CorA Reveal Symmetry Break upon Gating. *Cell* **164,** 747–756 (2016).

26. Wong, W. *et al.* Cryo-EM structure of the Plasmodium falciparum 80S ribosome bound to the anti-protozoan drug emetine. *eLife* **2014,** 1–20 (2014).

27. Yamamoto, H. *et al.* Structure of the mammalian 80S initiation complex with initiation factor 5B on HCV-IRES RNA. *Nature Structural & Molecular Biology* **21,** 721–7 (2014).

28. Guskov, A. *et al.* Structural insights into the mechanisms of Mg2+ uptake, transport, and gating by CorA. *Proceedings of the National Academy of Sciences* **109,** 18459–18464 (2012).

29. Sirohi, D. *et al.* The 3.8 Å resolution cryo-EM structure of Zika virus. *Science* **5316,** 1–7 (2016).

30. Russo, C. J. & Passmore, L. A. Ultrastable gold substrates for electron cryomicroscopy. Supplementary. *Science (New York, N.Y.)* **346,** 1377–80 (2014).

31. Danev, R. & Baumeister, W. Cryo-EM single particle analysis with the Volta phase plate. *eLife* **5,** e13046 (2016).

32. Sharp, T. H., Koster, A. J. & Gros, P. Heterogeneous MAC Initiator and Pore Structures in a Lipid Bilayer by Phase-Plate Cryo-electron Tomography. *Cell Reports* **15,** 1–8 (2016).

33. Chen, B. & Frank, J. Two promising future developments of cryo-EM: capturing short-lived states and mapping a continuum of states of a macromolecule. *Microscopy* dfv344 (2015). doi:10.1093/jmicro/dfv344