A beginner's guide to cryogenic electron microscopy

Christos Savva
(Midlands Regional Cryo-EM Facility, Leicester Institute of Structural and Chemical Biology, University of Leicester, UK)

The 'Beginner's Guides' are a new ongoing series of feature articles in the magazine, each one covering a key technique and offering the scientifically literate, but not necessarily expert audience, a background briefing on the underlying science of a technique that is (or will be) widely used in molecular bioscience. The series will cover a mixture of techniques, including some that are well established amongst a subset of our readership but not necessarily familiar to those in different specialisms. Our first 'Beginner's Guides' covers cryogenic electron microscopy.

Introduction

In 2017, the Nobel Prize in Chemistry was awarded to Joachim Frank, Jacques Dubochet and Richard Henderson for their contributions to the development of cryogenic electron microscopy (cryo-EM). In 2015, cryo-EM was hailed as 'Method of the Year' by Nature publishing group. One only has to look at the growth in the field since 2013 to appreciate why this technique has become so popular and important to structural biology. For example, from 2002, when depositions began, up until 2013, there were approximately 2,110 EM structures deposited in the EM Database (EMDB). From 2013 up to 2018, there were an additional 5,204 structures deposited, which corresponds to over 1,000 new EM structures a year and growing. Even though X-ray crystallography still leads in quantity with almost 10 times the number of deposited structures every year, cryo-EM is catching up and its current output is the equivalent of X-ray crystallography back in the mid-90s.

What is cryo-EM?

Although we mainly associate the term cryo-EM with macromolecular EM, the 3D study of macromolecules, viruses, organelles and cells using a transmission electron microscope (TEM), cryo-EM is a general term for the observation of low-temperature specimens using an electron microscope. This term can also be used for the observation of frozen material by a scanning electron microscope (SEM), which produces vastly different images from those of a TEM.

Electron microscopes are imaging devices that use electrons instead of light and electromagnetic lenses instead of glass lenses to produce magnified images of an object being studied. Electrons can either pass through the sample, and in doing so get slightly scattered (as in TEM), or 'bounce off' the sample (as in some forms of SEM). The interaction of these electrons with the sample gives rise to various types of images. Macromolecules imaged by a TEM in ice are referred to as projections and can be thought of as a sum of the density through the macromolecule from a particular orientation, similar to looking through one's hand in an X-ray picture taken at a hospital. Due to the scattering nature of electrons, a very high vacuum is required inside the microscope which means that biological specimens have to be stabilized accordingly.

Alongside X-ray crystallography (which can determine the structures of macromolecules using X-rays scattered by well-ordered 3D crystals) and nuclear magnetic resonance spectroscopy (NMR, which is a solution-state technique especially suited for smaller proteins and yields dynamic as well as structural information), cryo-EM is a very versatile structural determination technique that in itself, comprises several branches. The study of isolated macromolecules embedded in random orientations in a thin layer of ice is the branch of cryo-EM known as single particle analysis (SPA). Here, thousands of molecular images of the sample, from different orientations, are aligned and combined to produce a high-resolution structure. SPA can be used to obtain structures of macromolecular complexes with or without symmetry, viruses and large fibre-like structures. If the macromolecules arrange in a well-ordered 2D array, or 2D crystal, the branch of cryo-EM referred to as electron crystallography can be

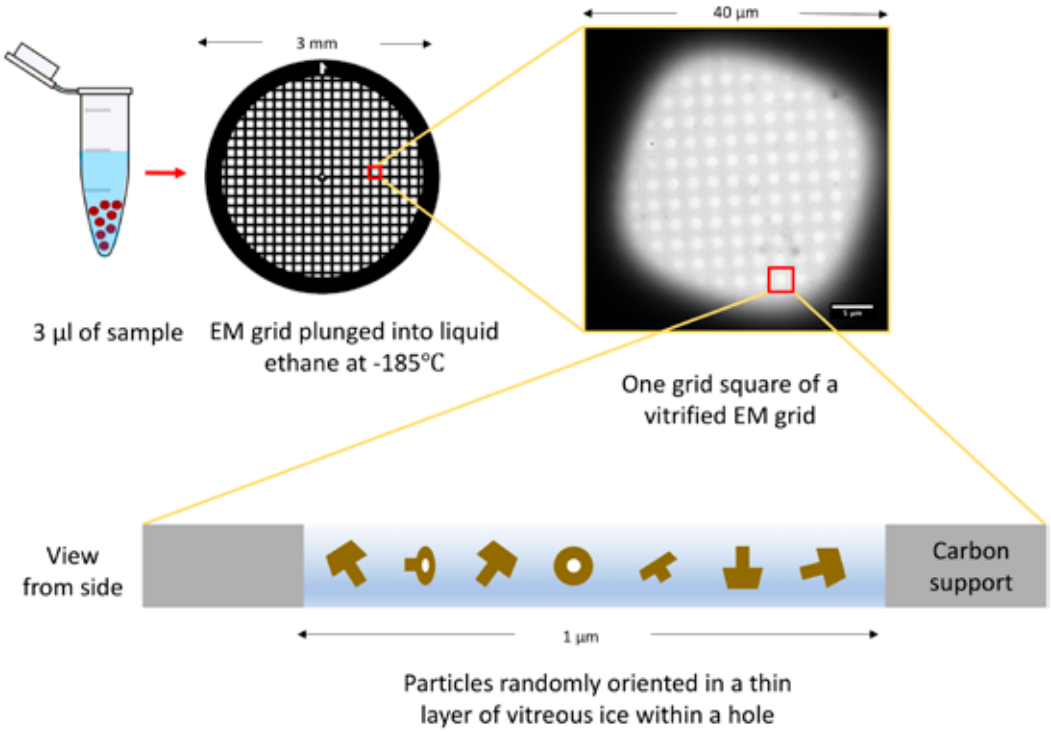

Figure 1. Overview of the cryo-EM sample preparation procedure.

used to solve the repeating unit's structure. In addition, if the biological object of interest has no repeating or regular structure, or is pleomorphic, cryo-electron tomography (cryo-ET) can be employed, whereby the object is tilted in respect to the electron beam and a 3D volume is reconstructed containing structural information of this object.

How did we get here?

To better explain how we got to this point let us consider some of the contributions of our Nobel laureates. Joachim Frank was amongst the first to observe that one could take noisy images of asymmetric macromolecules, using a TEM, and by aligning and averaging these molecular images, the signal in the images could be increased to the point where details in the 2D averages allowed interpretation of the molecular structure. He is one of the key figures responsible for starting the branch of macromolecular EM called SPA. The images are noisy as the specimen preservation technique, the radiation damage caused by the interacting electrons, the imperfect microscope lenses and the image-forming devices (e.g., film, CCD cameras) all introduce noise to our images. It is worth noting that in the early days of SPA, this type of image analysis could only be performed on samples embedded in a heavy-metal stain which replaced the buffer, and not in cryogenic conditions.

Amongst their many other contributions, Richard Henderson and his colleague Nigel Unwin, at the MRC Laboratory of Molecular Biology in Cambridge, discovered how to use TEM images to solve the structures of macromolecules arranged in a repeating, 2D array or 2D crystal. This branch, now called electron crystallography, allowed Richard and Nigel to solve the very first structure of a membrane protein in its lipid environment. But again, their early efforts studied 2D arrays of bacteriorhodopsin embedded in a sugar and not cryogenically preserved samples. Early adopters of macromolecular EM soon realized that macromolecules, which were surrounded by stains or other compounds in the microscope's vacuum, would not necessarily represent the true structure of the object being imaged and would suffer distortion effects. Enter our third Nobel laureate: Jacques Dubochet and his colleagues at EMBL discovered a way to achieve the best structural preservation for macromolecular EM. The answer was to keep the macromolecules in their native aqueous environment, or buffer, and to rapidly freeze them in a cryogen, a very low-temperature liquid. By doing so, water molecules did not have time to form the regular arrays found in crystalline ice such as that in your freezer but remained in a state reminiscent of water at room temperature. Imaging of molecules in this vitreous ice opened up a new direction for macromolecular EM. Over the next couple of decades cryo-EM, though still a very specialized technique and restricted to a limited number of groups around the world, was slowly showing us what could be achieved, and complemented the high-resolution X-ray and NMR techniques. Most cryo-EM reconstructions were limited to resolutions in the 1–2

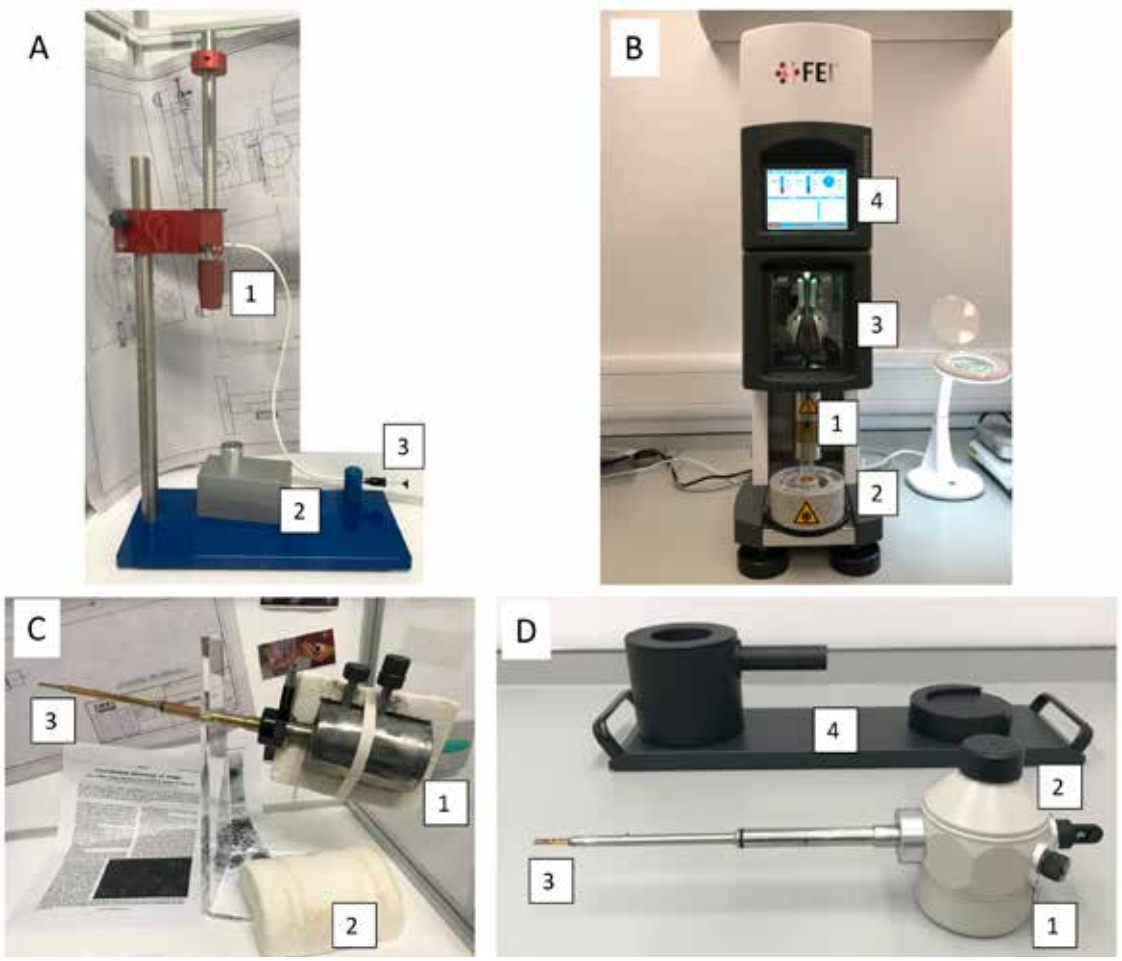

Figure 2. New and old: equipment used for cryo-EM sample preparation and imaging.

A) One of the original gravity-fed plungers on display at EMBL. The rod attached to the tweezers (1) dropped into a metal cup (2) which would contain a cryogen. The release was facilitated by a camera aperture release mechanism (3) and blotting was performed manually.

B) The Vitrobot is the modern equivalent. The tweezers are attached to (1) and will plunge into liquid ethane in the container (2). The sample is blotted from 2 sides in a temperature- and humidity-controlled chamber (3). The vitrification parameters including humidity and blotting time are adjusted on the computer (4).

C) One of Dubochet's original cryo-specimen holders also on display at EMBL. A container filled with LN_2 (1) and insulated by polystyrene (2) cooled the vitrified EM grid located at the tip (3).

D) A modern side-entry cryo-specimen holder with a double-wall vacuum-insulated container (1) which could be topped up with LN_2 from the top (2) and a retractable cryo-shield (3) to protect the specimen during transfer to the microscope. Loading of EM grids occurs in a dedicated transfer station (4).

nanometre range, with the exception of highly ordered 2D crystals and other very high-symmetry macromolecules such as icosahedral viruses and helical assemblies. During this time improvements in microscope hardware, such as more coherent electron sources and better vacuum systems, were crucial for what was to come. During the mid-90s, Richard Henderson wrote a now pivotal paper on the potential of macromolecular EM and he predicted that given ideal conditions for images taken with an electron microscope, it should be possible to obtain high-resolution (better than 0.4 nm) structures from a few tens of thousands of molecular images. Richard Henderson and his colleagues, as well as other groups, also made strides to develop better detectors in order to realize the full potential of cryo-EM. By 2013, the first high-resolution structures of symmetric (and asymmetric) macromolecules solved using this new generation of direct electron detectors began to emerge and were the humble beginnings of what would be an avalanche of cryo-EM research output. In conjunction with the arrival of these detectors which created the clearest images yet of macromolecules embedded in vitreous ice, new image-processing algorithms made it possible to extract the maximum information possible from these images.

The steps involved in solving a cryo-EM structure

1. Sample preparation

Ask any cryo-electron microscopist what the most crucial step is in the cryo-EM pipeline and they will all tell you (or *should* tell you) it all comes down to the sample (Figure 1). A well-behaved sample not only makes data collection more straightforward but can also make the computational structure determination procedure much easier. It all begins with a well-characterized, freshly purified sample; and sometimes less is more, meaning fewer purification steps may be an advantage. Unlike X-ray crystallography where a small contaminant may cause crystallization issues, cryo-EM samples can tolerate some contaminants as long as their size is not similar to the sample being studied. Speeding the process of sample purification may even help fragile complexes avoid falling apart. A frequent mistake when one is starting cryo-EM is to use a sample that has been in the freezer or even the fridge for some time, thinking that this sample is very stable and behaves well on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel or size-exclusion chromatography. While some complexes can be very stable, most are not, and using

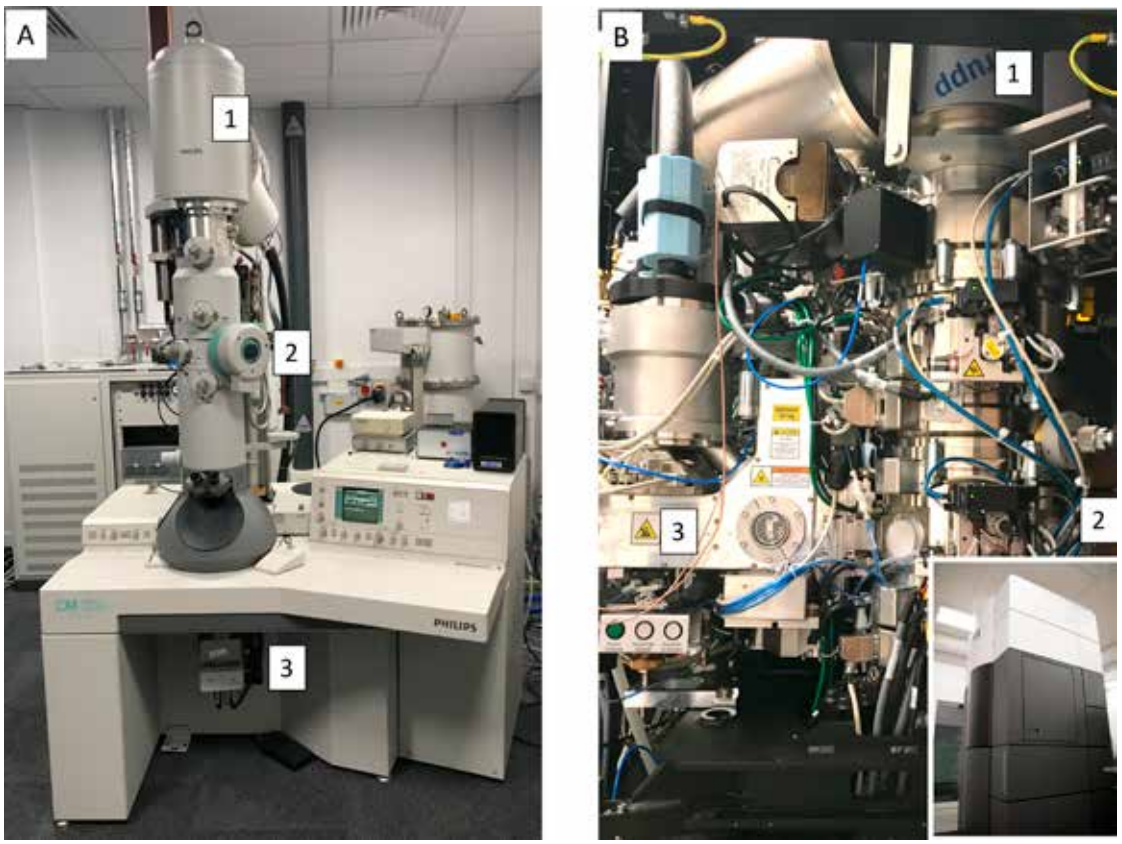

Figure 3. Cryo-TEMs. **A**) Circa 1996 Philips Electron Optics CM200 FEG cryo-capable TEM. This microscope was cutting-edge at the time with a coherent field emission gun (1) and a computer-controlled specimen stage (2). A Charged Couple Device camera is placed in the projection chamber allowing digital capture of images (3). **B**) Inside the Thermo Fisher Titan Krios G3 circa 2018. This Krios (shown in the inset), based at the University of Leicester, also boasts a high brightness FEG and a very stable microscope stage (2). The Krios also has an automated loading system which can change any of the 12 samples at the click of a button. These modern microscopes negate the need for a side-entry specimen holder shown in Figure 2D.

the freshest sample will involve a lot less troubleshooting down the line and will actually save the researcher time. Once a good sample is obtained, it has to be vitrified on a support medium. EM grids, as they are called, are very thin 3 mm diameter disks made out of metal such as copper (Cu) or gold (Au) that usually have metal bars running in perpendicular directions, similar to a round BBQ grate. An additional thin foil, usually perforated carbon, is layered on top of a grid and made hydrophilic, or water-loving, by plasma treatment. The freezing process then takes place on a piece of equipment called a plunge freezer (Figure 2A,B). These are either gravity-fed or spring-loaded devices that will plunge a pair of tweezers grasping an EM grid into the cryogen, usually liquid ethane, at –185°C. Back to the freezing process. A small amount, 3–5 microlitres, of the purified sample is then added to this hydrophilic grid surface and excess liquid is blotted away with paper before plunging the grid into the ethane. It is estimated that the sample freezes at a rate of 10^6 °C per second. The ideal result is to create a thin layer of amorphous (non-crystalline) ice that is slightly thicker than the maximum dimension of your macromolecule. The grids are then stored in special containers in liquid nitrogen until observation using the TEM.

2. Screening and data collection

Transmission electron microscopes come in lots of different configurations and prices (Figure 3). The higher-end data collection microscopes equipped with the latest generation of direct electron detectors and other 'toys' cost £3–5 million and microscope time is so precious that a new sample must be initially observed on a lower-end instrument to make sure it is suitable for data collection. Because of their high purchase and maintenance costs, not every university or institute can afford to have their own. In the UK, there are approximately 20 of these data collection microscopes scattered around the country including four located at the electron Bio-Imaging Centre (eBIC) in Oxfordshire. Some serve the actual facilities where they are based. Others are used by a consortium or partnership of institutes, while still allowing external paid access. The microscopes at eBIC are accessible for free by anyone around the world through an application process. Other microscopes around Europe are also freely available to researchers through such initiatives as Instruct and iNEXT.

Screening, or checking the suitability of a sample for data collection, takes place on smaller, less automated TEMs, often equipped with older CCD or CMOS scintillator-based cameras, where an electron signal is converted to

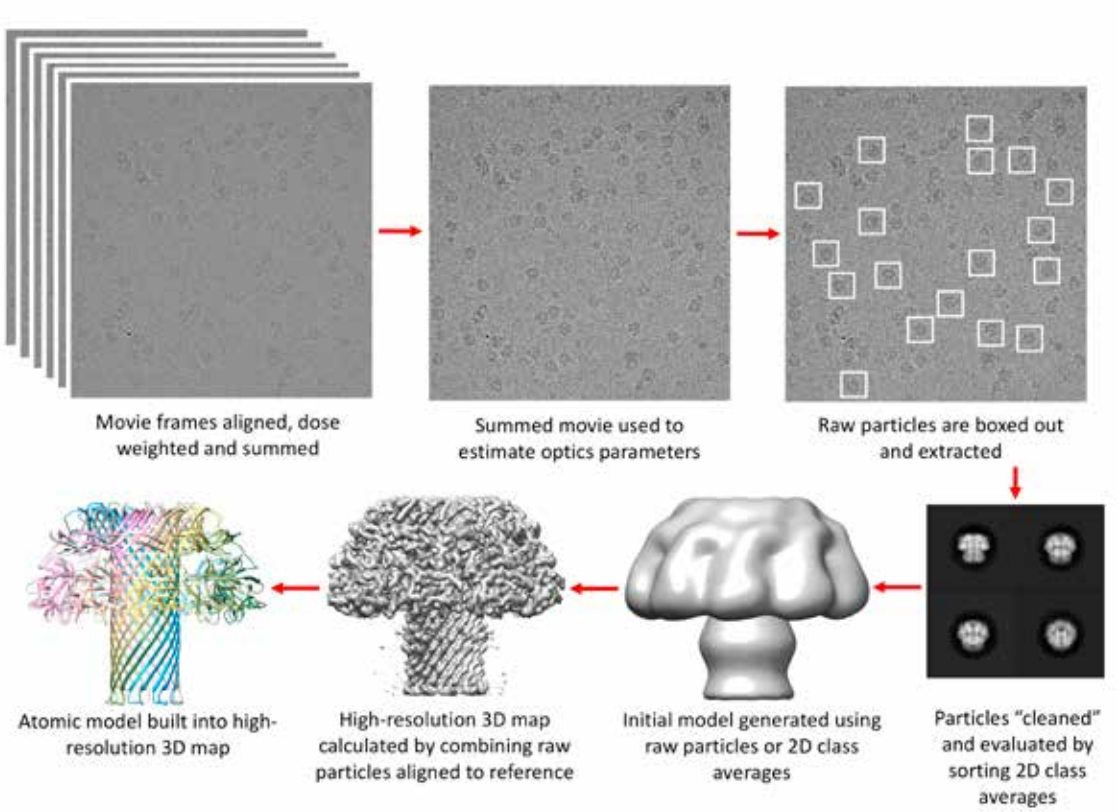

Figure 4. Image processing outline illustrated with data from the small pore-forming toxin lysenin.

photons for detection. These instruments are adequate to judge whether the sample is nicely dispersed in a vitreous ice layer and if the sample is homogeneous and of an adequate concentration. On newer screening microscopes it may also be possible to collect small datasets that, when processed, can provide further information on the sample's suitability for high-end data collection. In general, screening instruments often exist in many universities and institutes, usually serving non-cryo projects, but can sometimes be made cryo-capable with the acquisition of a cryo-specimen holder.

In most cases, a new sample will require several rounds of optimization and screening before an adequate sample is obtained. This may involve modifying the purification procedure or the sample composition and in others it may require using different support substrates (EM grids, support foils, etc.) or the use of an additive. Frustratingly, each sample is unique and optimal conditions have to be found on a per sample basis. However, as mentioned before, this process is the most crucial step and should be performed diligently and methodically.

Optimized specimens are then loaded onto a high-end microscope and, with the use of dedicated software that controls the electromagnetic lenses, electron beam and microscope stage (the part that holds and moves the specimen around), the user sets up data collection on the best EM grid, usually judged by eye. Ideally, a few thousand images or electron micrographs are then collected over a period of a few days in order to yield enough images for a 3D reconstruction to high resolution. Once data collection is set-up, the user can leave the microscope unattended and monitor data collection remotely or even start processing the incoming data whilst its being collected.

3. Data processing

The thousands of micrographs collected are then processed using sophisticated image-processing software. Depending on the type of cryo-EM data collected (single particles, tomograms, 2D crystals etc.) software and methodology varies. Let's look at the most popular of these techniques: SPA (Figure 4).

The first step is to take our micrographs and to correct for the instability of the microscope stage. The new generation of detectors are not only very good at acquiring high signal-to-noise images, but also very fast, recording images at 100s of frames per second. This means we can record a movie with many sub-frames instead of a single image and then align these frames to account for the stage or sample movement during the exposure. When a microscope stage is quite unstable, images are reminiscent

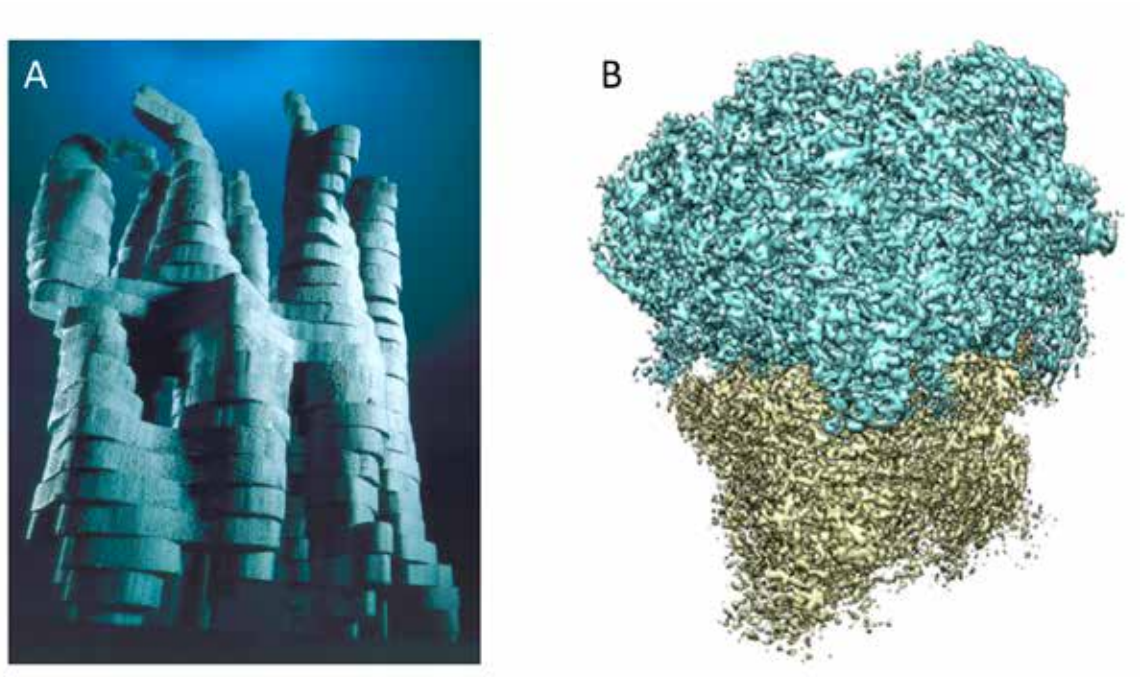

Figure 5. Key structures solved by macromolecular EM. **A**) A model of bacteriorhodopsin at 7 Å resolution, the very first membrane protein structure to be solved by Richard Henderson and Nigel Unwin in 1975. Image courtesy of MRC LMB Image archive. **B**) One of the first cryo-EM structures to be solved using the new generation of detectors and software, the 80S ribosome from Saccharomyces cerevisiae at 4.5 Å resolution. [Image reproduced with permission from (EMD-2275) Bai, X.C., Fernandez, I.S., McMullan, G., Scheres, S.H. (201 3) Ribosome structures to near-atomic resolution from thirty thousand cryo-EM particles. *ELIFE* **2** e00461-e00461]

of taking a photograph from a speeding car with a long exposure time. Everything is blurred and in a single shot the objects in the images cannot be distinguished. A further advantage of recording movies is that we can also adjust the effect of radiation damage in each frame. Earlier frames will have less damage with more high-resolution information whilst later frames will have less high-resolution information which can be downweighed.

The corrected images are then analysed to determine certain parameters related to the imperfect microscope optics and then the user will semi-automatically or in a completely automated way select sub-regions of the micrograph, or boxes, around each macromolecule. Each micrograph will ideally have tens or hundreds of these 'particles' leading to tens, or even hundreds, of thousands of molecular images.

The next step varies depending on the software used, but usually these molecular images are classified into groups in which the particles are in the same orientation. These 'similar' projections are then averaged to produce 2D images with a higher SNR. In the past, these 2D class averages could also be used to calculate a 3D structure or map. Their relative orientations were calculated, and they were then combined to create a 3D map. In more recent software, the 2D classification process serves to 'clean up' a dataset from junk particles (ice contamination, carbon edges etc.) and to interpret the sample's homogeneity (or heterogeneity) since domain structure and secondary structure elements (e.g., alpha helices) can clearly be seen in good 2D classes. The next step is to calculate a 3D map from these 'clean' particles. An initial low-resolution model is required to do this and can be obtained by the particles of the same dataset or from other homologous structures. This initial model is used to create artificial, high-signal, molecular images from different views simulating the experimental projections in ice. Each raw particle is then compared with, and aligned in respect to, its best matching high-signal reference. Once each particle is aligned these projections are combined in 3D space to create a 3D map of the original sample. This process of re-projecting the 3D map and re-aligning each particle is iterated and with each iteration, an improved 3D map is obtained. Eventually, no further improvement is made and one obtains the final high-resolution 3D map.

In the final step of calculating an atomic structure, the high-resolution map is used in a very similar way to an X-ray map to model the polypeptide chain (or chains) inside the map density. With the aid of 3D modelling software and the known protein sequence, each amino

acid is placed manually to complete the polypeptide chain. The 3D model is finally corrected for basic geometry errors and the researcher can begin interpreting the model to understand the molecular basis of function (Figure 5).

Conclusions

The structural biology field of cryo-EM has undergone a revolution in recent years thanks to hardware and software developments. One of the advantages of this explosion is also the fact that many more groups are now practising cryo-EM and thinking about how to improve the technique, which according to the experts, has not yet reached its full potential. Further improvements in detectors, microscopes and software are expected and, in parallel, improvements in the way we prepare samples are being developed. Before this revolution, cryo-EM was described as a technique complementary to X-ray crystallography due to the lower resolution one could achieve. Although this is no longer the case, with resolutions better than 1.8 Å being reached, the greatest strength of cryo-EM lies in the ability to solve structures that are not amenable to other techniques. For example, structural determination of very large macromolecular complexes such as the ribosome and spliceosome have suddenly become feasible and are providing a wealth of information on translation and RNA splicing. Even more remarkable is the fact that we can also study highly heterogeneous samples derived from patients, such as amyloid fibres, giving us insight into diseases including Alzheimer's and Parkinson's. Finally, the field of cryo-ET allows us to study biological structures in the context of their native environment. ■

Christos Savva is the facility manager of the Midlands Regional Cryo-EM Facility located at the University of Leicester. He entered the field of macromolecular EM in 1999 as an undergraduate at the University of Leeds working with Andreas Holzenburg. After obtaining his PhD from Texas A&M University he did a postdoc at Birkbeck College in London studying the structure of pore-forming proteins. He then joined the Cryo-EM Facility at the MRC LMB in Cambridge in 2013 before moving to the University of Leicester in 2018.
Email: gwcs1@leicester.ac.uk.

Further reading

- Bai, X.C., McMullan, G. and Scheres, S.H. (2015) How cryo-EM is revolutionizing structural biology. Trends Biochem. Sci. **40**, 49–57. doi: 10.1016/j.tibs.2014.10.005. Epub 2014 Nov 7. Review. PubMed PMID: 25544475
- MRC LMB Cryo-EM course: https://www2.mrc-lmb.cam.ac.uk/research/scientific-training/electron-microscopy/
- Comprehensive Cryo-EM video tutorials: https://em-learning.com/
- Ceska, T., Chung, C.W., Cooke, R., Phillips, C. and Williams, P.A. (2019) Cryo-EM in drug discovery Biochem. Soc. Trans. doi: 10.1042/BST20180267 http://www.biochemsoctrans.org/content/early/2019/01/14/BST20180267
- Kim, D.N. and Sanbonmatsu, K.Y. (2017) Tools for the cryo-EM gold rush: going from the cryo-EM map to the atomistic model. Biosci. Rep. **37**. doi: 10.1042/BSR20170072 http://www.bioscirep.org/content/37/6/BSR20170072

