Cryo-electron microscopy: taking back the knight

Stephen Fuller

Why cryo-EM?

An instructor in an introduction to an X-ray crystallography course some years ago explained the difference between electron microscopy and X-ray diffraction as structural techniques. Imagine, he said, setting out to determine the structure of a man. One approach would be to cover him in a suit of armour, place him in a vacuum until all the water is gone and broil him under a high energy beam until the armour began to melt and flatten, and finally take a picture of the remnants and call it the structure. That, he said, is the electron microscopist's approach. The X-ray crystallographer, in contrast, maintains his sample in an aqueous environment and determines the structure of the entire sample in three dimensions rather than only that of a shell of a metal shadow or a heavy metal stain. From such a perspective, the choice between the two techniques was an obvious one to any serious student of structure.

Today the situation has changed dramatically and few structural biologists would omit electron microscopy as part of their characterization of a biological system. The advent of cryo-electron microscopy (cryo-EM) has allowed the observation of biological samples in a layer of vitrified water. This avoids the drying associated with classical approaches. The use of phase contrast to image the specimen allows one to perform unstained microscopy so that the entire density of the specimen contributes to the image. Image processing techniques have developed so that the contrast of these images can be accentuated and the three-dimensional structure determined by combining the projected densities in the individual micrographs. Finally, measures of the reliability of the resultant structures have been developed and validated by comparing them with structures derived from X-ray diffraction of the same complexes. This has led to the use of a divide and conquer approach to many systems. First, the atomic resolution structures of subcomponents of a macromolecular complex are determined by either X-ray crystallography or NMR. Then, cryo-EM is used to provide a context for these structures by showing their placement in the complex and indicating where the structure must be altered during assembly.

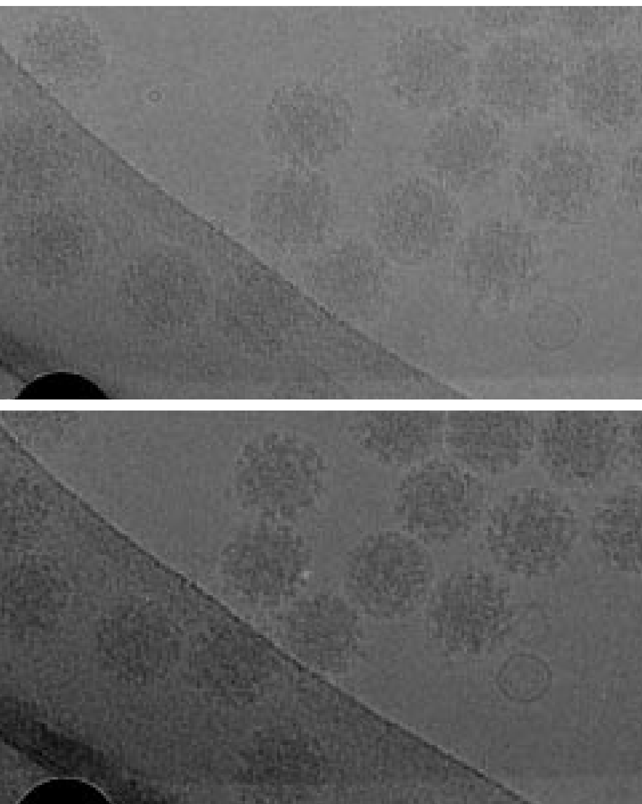

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Fig. 1. Cryo-electron micrographs of SFV taken on a Philips CM200 equipped with a field emission gun at 2 µm (upper) and 4 µm (lower) underfocus. The 700 Å diameter virions sit in a layer of vitrified water suspended over the holes of a carbon film. The phase contrast nature of the image causes different resolution ranges to be accentuated at different defocuses.
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Vitrified samples

The preparation of a sample for cryo-EM takes advantage of the properties of water. When water is cooled slowly (a few degrees per second) it forms hexagonal crystals of ice, the form found in ice cubes and snow flakes. Very rapid cooling (thousands of degrees per second) traps the water in a vitrified state in which the structure has not been allowed to rearrange into a crystalline form. Practically, this is usually accomplished by a method developed 20 years ago by Jacques Dubochet and collaborators at the European Molecular Biology Laboratory (EMBL). A small aliquot of the sample in suspension is placed on a holey carbon film blotted to generate a layer of ~1000 Å thickness and plunged into a bath of ethane slush held in a container of liquid nitrogen. Ethane is a very efficient cryogen since, unlike nitrogen, it does not boil at the temperatures used for vitrification. Nitrogen is not used directly since the formation of gas around the specimen when it is introduced into liquid nitrogen slows the cooling and leads to the formation of ice.

The vitrified state is not an equilibrium one but rather a metastable one. It can be maintained at liquid nitrogen temperatures for long periods. Our group has examined specimens stored in liquid nitrogen for more than 10 years without loss of the vitrified state. Raising the temperature above about −140 °C causes devitrification and the formation of cubic ice. Paradoxically, vitrified water freezes upon warming. Cooling the sample again does not restore the vitrified state, it simply generates cooler ice. The vitrified sample is transferred to the microscope by passing it rapidly between baths of liquid nitrogen and then mounting it in a liquid-nitrogen-cooled specimen holder which is inserted into the microscope. Other schemes have been used for generating vitrified specimens. The requirements are sufficiently rapid cooling to avoid the formation of ice and maintenance of the vitrified state by keeping it at low temperature at all times, usually by storage in liquid nitrogen.

The requirement for very rapid cooling is a limitation of the technique. The method works best with specimens, such as suspensions of viruses or protein complexes, which can be made very thin so that cooling is rapid. Thicker objects such as cells must be handled differently and represent a challenge to the field. Suspension samples also present problems if they contain solutes which interfere with vitrification. Samples containing salt or sucrose show a phase partitioning after vitrification. Crystalline specimens are usually handled in a somewhat different way. These samples are often placed on a continuous carbon film in glucose or tannic acid and cooled directly in liquid nitrogen or in the holder rather than using ethane as a cryogen. This is possible because very high concentrations of these sugars act as a cryoprotectant and inhibit ice formation.

The vitrified sample is typically maintained at near −170 °C during microscopy. The specimen is extremely sensitive to radiation damage from the electron beam of

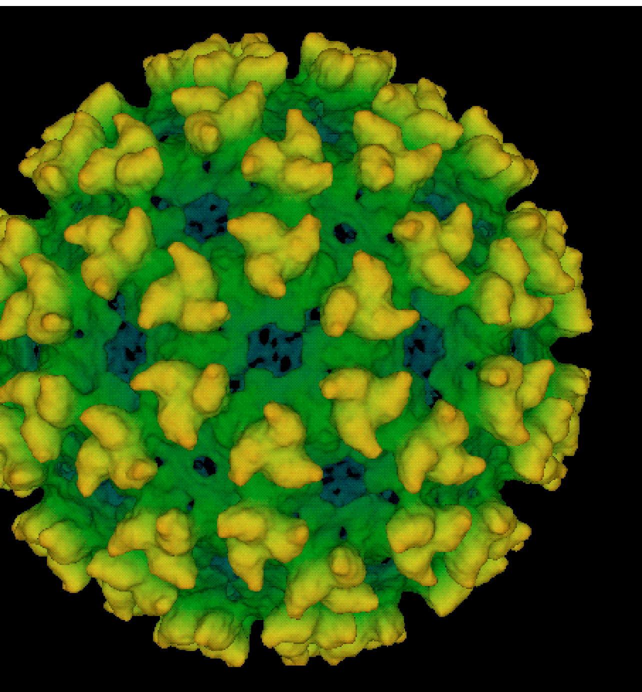

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Fig. 2. Three-dimensional reconstruction of SFV generated from 10,000 particle images such as those seen in Fig. 1. The triangular profiles of the spikes are seen as well as the thin protein sheets which overlie the membrane of the virus.
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the microscope. An image of an undamaged specimen is obtained by using low dose techniques. The specimen is first imaged at low magnification so that areas of interest can be identified with minimal irradiation (< 0·1 electrons $Å^{-2}$). The image is then captured at higher magnification with the first electrons to hit the specimen. Typically, the image is formed with a dose of 5–10 electrons $Å^{-2}$. Work with crystalline specimens at –170 °C indicates that this dose does not damage the structure at resolutions coarser than 10 Å.

The images obtained by cryo-EM show details of the entire specimen. In contrast to negative staining or metal shadowing which show the surface of the sample, as the armour models the features of the knight, the image is a superimposition of all of the density in the sample. Interpreting such a projected image requires either restricting oneself to the edges of the object or accounting for the projected nature of the image. Three-dimensional reconstruction techniques take advantage of the fact that the image is a projection. The most powerful approach is that of electron crystallography. However this can only be applied when the specimen is co-operative enough or the investigator inventive enough to produce the sample in an ordered two-dimensional crystal. The simplest of the alternative approaches takes advantage of the homogeneity of the specimen. A field of particles such as the one of Semliki Forest virus (SFV) shown in Fig. 1 can be viewed either as images of separate particles or as images of the same structure from a variety of orientations. The latter is key to determining the structure. The process of three-dimensional reconstruction involves identifying the orientations of each of the views represented by the particle images and then combining these images to determine the structure (Fig. 2). Obviously, the reliability of the assignment of the orientation and the resolution of the information contained in the image limit the final resolution of the structure. A process of refinement allows one to determine the orientations to higher reliability; however this process is only effective if the resolution of the data allows determination of the orientations with high precision. The homogeneity of the sample is critical to this process. The reconstruction method assumes that the differences between separate particle images reflects the change in orientation rather than sample variation.

Three-dimensional reconstructions of particles with higher symmetry, such as icosahedral viruses, are particularly well developed. Building on algorithms formulated by R.A. Crowther (MRC Laboratory of Molecular Biology, Cambridge), a number of groups have developed methods for treating the case of icosahedral particles. More than 150 such structures have already been published and the number continues to grow. The use of a highly symmetric particle allows the symmetry to serve as a guide in determining the orientation and makes the process of computing the structure itself much more efficient. Tests for symmetry in the particle images allows identification of those particles in the population which have been distorted and hence would blur the reconstruction if they were included in the average. Reconstruction methods have also been developed for non-symmetric particles particularly by the groups of M. van Heel (London) and J. Frank (Albany, USA). The power of these methods has recently been demonstrated by high resolution reconstructions of the ribosome.

Cryo-EM comes of age

Cryo-EM and three-dimensional reconstruction are undergoing a renaissance. A few years ago, the best icosahedral reconstructions achieved resolutions of slightly better than 30 Å. Higher resolution was the domain of electron crystallographers and others working with ordered specimens such as helices. Two developments have been key in bringing a new excitement to the field. The first is the establishment of methods for processing large numbers of images and combining them to produce a three-dimensional structure. Combining thousands of particles enhances the relatively low signal to noise ratio in the unstained, low dose image of a single particle. The second development is instrumental. The use of high voltage and field emission gun sources has increased the strength of the high resolution information transferred from the specimen to the film. This is a consequence of the fact that the image of a thin unstained specimen is dominated by phase contrast. A more coherent electron source produces a better phase image. The effect is particularly important for images of single particles. Accurate determination of orientations requires refinement against high resolution information which is, in turn, necessary for enhancing the signal to noise ratio at high resolution.

The determination of the fold of the hepatitis B capsid protein by the groups of R.A. Crowther (MRC, Cambridge) and A. Steven (NIH, Bethesda, USA) from the icosahedral reconstruction of hepatitis B cores signalled a coming of age in the field. The methods used by the two groups exemplified the two developments described above. The Cambridge group used thousands of images taken on a microscope equipped with a field emission gun source to produce a 7·6 Å resolution reconstruction which allowed visualization of the α-helices of the structure. Since this

marvellous demonstration of the potential of the methods, a number of groups have applied this approach successfully to larger systems. Work by W. Chiu's group (Baylor, Houston, USA) on herpesvirus and our group on SFV (Fig. 2) have produced structures at better than 10 Å resolution in which secondary structure can be identified. This progress is not limited to icosahedrally symmetric structures. The work on the ribosome mentioned above has now reached resolutions of 13 Å and is serving to map out the changes in conformation with the functional state of the system.

The increased resolution and demonstrable reliability of the information provided by the combination of cryo-EM and image reconstruction have altered the gentle rivalry which once existed between X-ray crystallographers and electron microscopists into a respectful working relationship. Three-dimensional structures derived from cryo-EM not only provide context for the higher resolution structures of components but are also increasingly used to investigate large macromolecular complexes by crystallography. The determination of the structure of the bluetongue virus core by D. Stuart's group (Oxford) (see p. 59) utilized a three-dimensional reconstruction from B. Prasad (Baylor, Houston, USA) to generate the initial model used for phasing. The progress on the crystallography of the ribosome has been aided by the use of reconstructions to serve as an initial phasing model and a control for heavy atom phasing at higher resolution.

The future: promises and challenges

Cryo-EM also provides resolution in time. The vitrification method can be adapted to capture intermediates. The approach depends both on the timescale and the system but a large range of conditions is accessible. We employed a simple spray technique in which the blotted grid was exposed to a mist of low pH while it was plunging towards the ethane bath to examine the structures formed by the SFV spike during the first 50 ms after exposure to low pH. At this early stage, the virion remains icosahedrally symmetric and hence a three-dimensional reconstruction could be performed by standard methods. Simple mixing experiments have allowed us to examine the slower interaction of the pH-activated virion with the target vesicle. Naturally, the structure does not remain symmetrical upon contact and hence other approaches must be used to determine three-dimensional structure.

Despite the recent progress, challenges still remain. A typical example is shown in the image of immature HIV (Fig. 3). HIV is an ideal case for 'divide and conquer' since the structures of parts of all the structural proteins are now known. This image contains a wealth of information. It shows the layer of spikes on the surface, features within the membrane itself and the radial arrangement of the independently folded domains of Gag. It shows two other key features which represent challenges for the determination of the three-dimensional structure. First, the particles are not icosahedral. Rather they seem to be formed of independent hemispherical sectors with closely packed, radially arranged Gag protein under the membrane with defects in the packing between the sectors. Second, the particles do not all have the same structure and hence averaging of images cannot make use of this basic property. Approaching such a system requires a continual interaction between biochemists and structural biologists to prepare homogeneous subassemblies such as the helical tubes formed by segments of the Gag protein. It also will require methodological developments such as the improvement of techniques for cryo-EM tomography to determine the unique structures of individual particles.

The future of the technique remains full of promise. Although methods are still under development, current techniques have reached such a level of sophistication that they can be applied in investigating a very broad range of biological problems. No longer are we restricted to working on the best behaved systems. Cryo-EM has passed from being a tool of the specialist to being an essential tool of the microbiologist.

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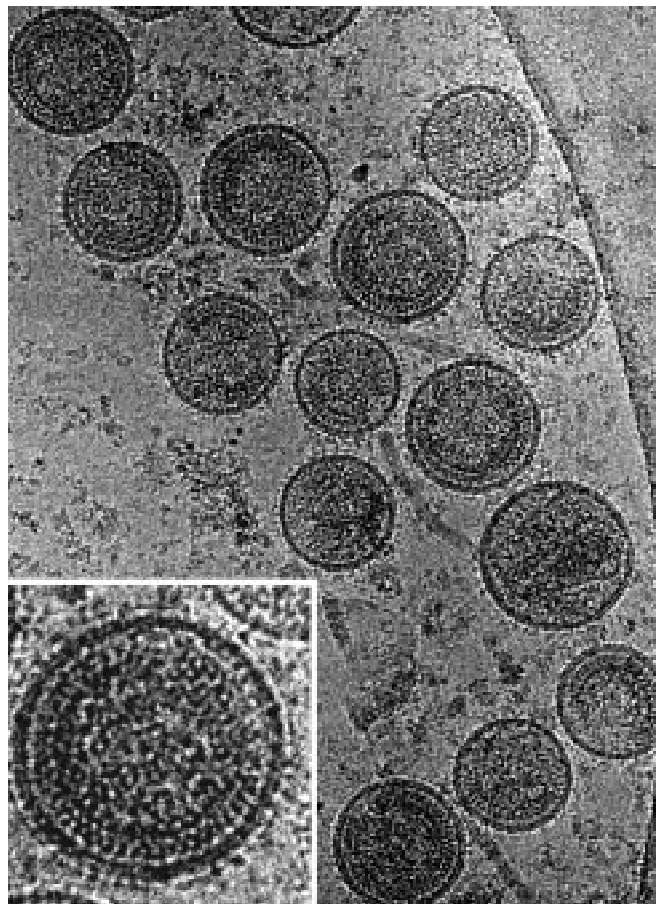

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Fig. 3. Cryo-electron micrographs of immature HIV produced by infected cells in the presence of protease inhibitors. The envelope proteins, features of the lipid bilayer, radial disposition of the Gag protein and heterogeneity in particle size and shape are easily seen.
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