Electron Cryomicroscopy

Secondary article

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Electron cryomicroscopy makes it possible to observe hydrated material, especially biological samples, in a close to native state, preserving the structural integrity down to atomic resolution. It is best combined with image processing for three-dimensional reconstruction, taking advantage of all possible symmetries in the object.

Article Contents

Introduction

Biological matter makes life difficult for an electron microscopist. On one hand, liquid water, which is by far the most abundant constituent of biological material, must be completely eliminated before the sample can be introduced into the vacuum of the electron microscope. On the other hand, biological matter is damaged by the electron beam: the very electrons that are necessary for imaging a nanometre-sized biological structure destroy it before it can be seen. Elegant solutions have been found for both of these difficulties.

Vitrification

The water problem can be solved when the liquid is cooled at such a low temperature that it does not evaporate significantly. This idea was proven correct in 1975 when Taylor and Glaeser (1976) showed that various biological particles could be directly observed with an adequate contrast in the frozen state. The trouble, however, is that water crystallizes upon freezing, and, when ice forms, it causes severe damage to most biological samples. Ideally, the liquid should be cooled so rapidly that it has no time to crystallize before the molecules are immobilized into a vitreous state. Vitrification was thought to be fundamentally impossible because of some fundamental understandings of thermodynamics – which had to be revisited when vitrification of liquid water became an experimental fact. It should be noted, though, that the vitrification paradox is not yet solved. It points towards new concepts about the structure of water. Vitrification, however, was not the whole story. Other questions, such as how to form a thin uniform film of solution, how to manipulate and transfer a submicrometre-thick vitreous layer and how to obtain sufficient contrast, found answers that made electron cryomicroscopy of vitrified thin films of biological suspensions a simple and practical method. We call it the Adrian method because Marc Adrian was instrumental in solving many of these problems (Adrian *et al.*, 1984).

Image analysis

To provide a high-resolution image, the specimen must be irradiated with an enormous dose of electrons, which practically incinerates the specimen. Glaeser, again, was the person who rationalized the optimal trade-off between using sufficient electrons to obtain a sharp image but not so many as to destroy the specimen. His conclusion was that any biological structure smaller than a few nanometres cannot be visualized with an electron microscope (Glaeser, 1971). In 1975, however, Unwin and Henderson (1975) reported the projected structure of bacterial rhodopsin at a resolution of 0.7 nm. What happened? They used a simple trick: instead of irradiating one particle with a high dose, they irradiated many particles, regularly arranged in a crystal, and incorporated the information gathered from all the particles into one high-resolution image. The method can be extended to less regular objects, and even to single particles. The only requirement is that a large number of identical particles be observed in a perfectly preserved state. Electron cryomicroscopy is the method of choice for preparing such specimens, together with optimal image processing.

Preparation of Biological Suspensions: The Adrian Method

The preparation commences with an electron microscopy grid, held in tweezers, mounted on a special plunger. It is convenient that the grid is covered with a perforated carbon film. A drop of suspension is put on the grid, and most of the drop is removed with blotting paper. It is remarkable that, whereas most of the drop soaks into the blotting paper in less than 1 s, the last layer of only a fraction of a micrometre thickness is relatively stable and remains for at least another second. This thin layer of liquid, spanning the holes of the perforated carbon supporting film, is rapidly plunged into a good cryogen, in which it vitrifies. As the layer is only about 50 nm thick,

the cooling speed is very high, perhaps $10^{8\circ}C\,s^{-1}$, leading to immobilization in not more than 1 μs. Once the specimen is vitrified, it is transferred into the electron microscope equipped with a good cold stage, and observed at a temperature that should be colder than $-160^{\circ}C$.

When a specimen is prepared by the Adrian method, the thin film containing the particles to be observed stands free for a good fraction of a second before being vitrified. The particles in the layer are still mobile and they tend to rearrange in a two-dimensional order. The interactions at the air–liquid interface also help to position the particles. Consequently, it frequently happens that elegantly ordered patterns are formed in the thin layer. **Figure 1** is a micrograph obtained with the Adrian method, modified by the incorporation of heavy salt in the solution in order to create an effect similar to that of negative staining (Adrian *et al*., 1998).

It might be thought that the lack of contrast would place a severe limit on the possibility of observing unstained specimens immersed in water. This is not so because it is not the contrast that is important but the signal to noise ratio; the latter is good for vitrified specimens because there is no noise as a result of the stain, the supporting film or distortion of the specimen. The excellent visibility of even the finest specimen is illustrated in **Figure 2**, which depicts a 1868 base pairs, closed, supercoiled deoxyribonucleic acid (DNA) molecule floating in a low salt buffer. The stereoscopic visualization shows that the molecule is really floating in the liquid layer. A three-dimensional model of the molecule is shown in the middle.

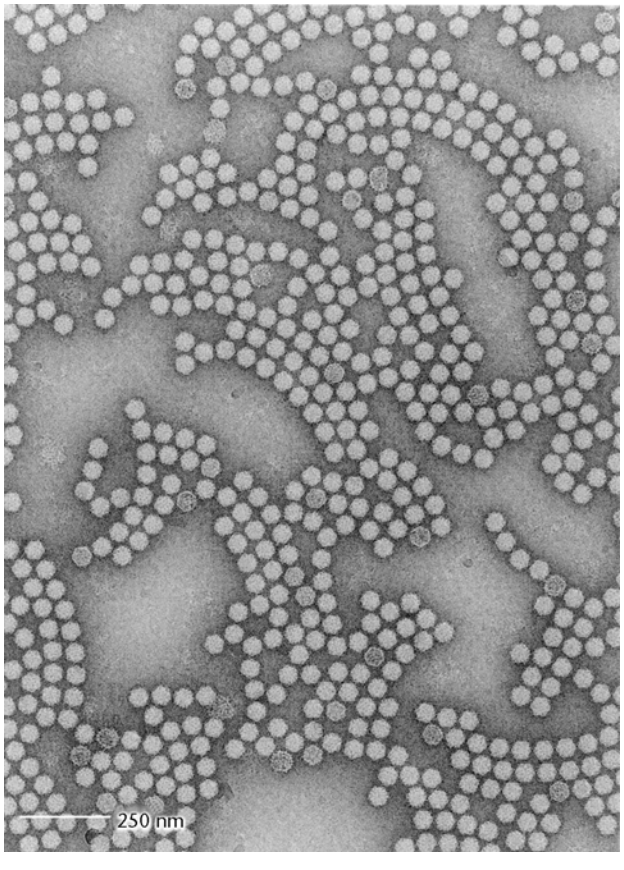

Figure 1 Tomato Bushy Stunt Virus solution prepared by cryonegative staining. Bar, 250 nm. Virus, courtesy J. Witz; micrograph, M. Adrian.

Three-dimensional Image Reconstruction

Electron cryomicroscopy can produce images of biological samples in their native environment without the addition of a contrast-enhancing stain. This allows the perfect preservation of the unaffected structure of the sample, but, especially in the case of small biological molecules, the images are very noisy. This noise can be so strong that the samples are almost invisible with the naked eye. Nevertheless the samples are there, and the images do contain detailed structural information – it is just hidden in the fog. Computational image treatment is necessary to retrieve the structural data from these images. Averaging of several images is done to eliminate the noise, and views from identical particles from different orientations are combined to obtain the three-dimensional structure.

Three-dimensional reconstruction from single particles

The structure of the hepatitis B virus was determined by this technique by Böttcher *et al*. (1997), and is reproduced in **Figure 3a**. Some 6400 images of differently oriented virus particles were aligned. For each image the orientation of the virus was determined and this three-dimensional structure was reconstructed by a method called 'back-projection'. Such image reconstructions require a program

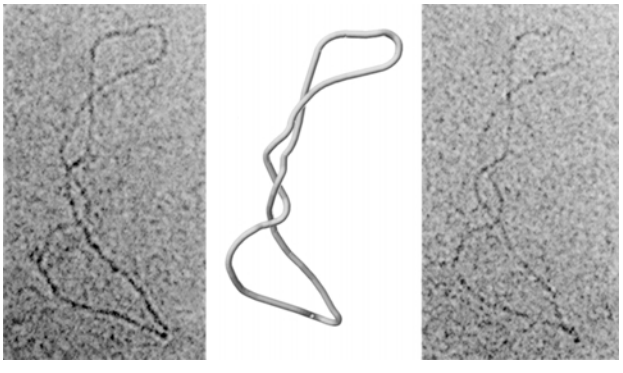

Figure 2 Closed, circular, 1868 base pairs, supercoiled deoxyribonucleic acid (DNA) molecule (pUC 18 fragment). Left and right images form a stereo pair from which a three-dimensional model has been determined (middle). Micrograph, Eric Larquet; model, Catherine Zucker.

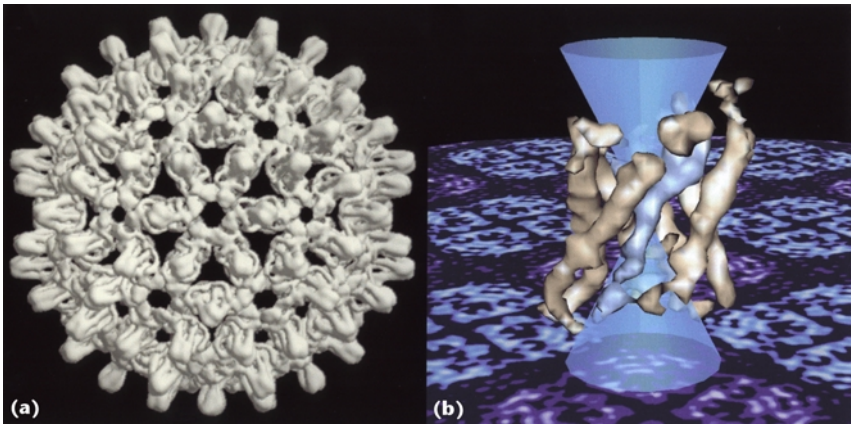

Figure 3 Three-dimensional structures obtained by electron cryomicroscopy. (a) The hepatitis B virus core protein shell contains 240 subunits arranged as 120 dimers. This reconstruction has 0.74 nm resolution, the virus diameter is 37.0 nm. Reproduced with kind permission from Böttcher *et al.* (1997). A similar structure was obtained by Conway *et al.* (1997). (b) Aquaporin 1 from red blood cells is constructed from six rod-like α helices that surround a central water pore, indicated by the cone (Walz *et al.*, 1997). This three-dimensional map has a resolution of 0.6 nm; the diameter of the protein in the horizontal membrane plane is 3.2 nm. The 'floor' in this image is an example of a two-dimensional image from electron cryomicroscopy after image treatment. Figure kindly provided by B. Heymann, University of Basle, Switzerland.

that allows the alignment and manipulation of large numbers of images. Images of single particles can be processed with the program packages SPIDER (Frank *et al.*, 1996) or IMAGIC (van Heel *et al.*, 1996).

Three-dimensional reconstruction from two-dimensional crystals

Another major application area of electron cryomicroscopy is the structure determination of membrane proteins. These proteins usually resist all efforts to crystallize them into a three-dimensional crystal, which would be the prerequisite for structure determination by X-ray diffraction. But, frequently, these proteins can be purified and reconstituted in the presence of lipids into well-ordered two-dimensional crystals. A noisy electron cryomicroscopical image of such membranous crystals contains many thousands of projections of identical proteins in identical orientations. The computational image treatment can rely on the well-ordered crystalline packing and therefore the positions of the membrane proteins can easily be determined. An electron microscopic image of the untilted sample usually shows the 'top views' of all proteins. The electron cryomicroscopist can now tilt the sample in the microscope to obtain tilted side views up to an angle of, usually maximally, 60°. Such a tilted image comprises the same tilted view of many thousands of molecules in the two-dimensional crystal. Each differently tilted photo gives after the averaging process a noise-reduced view of the protein, seen from a different angle. The three-dimensional structure of the protein can then be reconstructed by the backprojection method, combining all these differently oriented views.

Three-dimensional structures of membrane proteins reconstructed by this technique usually have a very good resolution in the membrane plane. However, resolution in the direction perpendicular to the membrane is less good. This is due to the fact that it is not possible to tilt the sample in the microscope to large angles close to 90°, because such images would just show a thin line and could not be analysed. The real side views of the proteins are missing in the data set. We call this the problem of the 'missing cone' in the three-dimensional data set. Further, two-dimensional crystals of membrane proteins nicely stabilize the membrane-spanning parts of the molecules in precisely periodic positions, but the extramembranous loops of these proteins are not necessarily fixed in a crystalline conformation. Variable loop conformations are invisible in an average image.

Figure 3b shows an example of a three-dimensional structure of the membrane protein Aquaporin 1 from *Escherichia coli*, as determined by electron cryomicroscopy of two-dimensional crystals (Walz *et al.*, 1997). The structure reveals a ring of six rod-like α helices surrounding a central water-permeable pore, as indicated by the blue cone. The above-mentioned difficulties are the reason why the α-helical transmembrane segments are well resolved but the helix-connecting loops are missing in the structure. Intensive work with highly tilted samples in the microscope is still necessary to increase the resolution perpendicular to the membrane plane such that a convincing model can be fitted into the three-dimensional structure, even without knowing the exact position of the helix-connecting loops.

The structure of the membrane proteins bacteriorhodopsin from *Halobacterium salinarum* (Henderson *et al.*, 1990) and the light-harvesting I complex from plants (Kühlbrandt and Wang, 1991), as well as tubulin (Nogales

et al., 1998), have been solved to atomic resolution by this technique of electron cryomicroscopy of two-dimensional crystals. Images of two-dimensional crystals are best processed with a collection of programs written by Richard Henderson, Medical Research Council, Cambridge, UK (Henderson *et al*., 1990).

Cryoultramicrotomy

Specimens that can be prepared in a thin liquid layer by the Adrian method or similar procedure are more the exception than the rule. In general, cells and tissues must be sectioned into slices thinner than 100 nm in order to be observed. The ultimate method – the one that electron cryomicroscopists dream of – would be like this. A piece of tissue, large enough for excellent preservation, is extracted in its native state and immediately vitrified by rapid cooling. The sample is sectioned in a cryoultramicrotome under conditions that preserve the vitreous state. The section is then observed, still at low temperature, depicting all the fine structures perfectly preserved in a state of suspended time.

It came as a surprise, therefore, in 1982, when it was found that the dream could come true in some cases. Sections of vitreous cells and tissues showing, distinctly, all the features known from conventional electron microscopy – although with some noticeable differences – have been obtained at many occasions (for a review see Dubochet *et al*., 1988). However, three major difficulties still limit the practical use of the method.

The first problem is rather down to earth. To obtain a good image of a good cryosection, a dozen preparation steps in a row must be executed successfully. None of the steps is very difficult, but all have their problems. If the probability of success is 50% for each step, the method is hopeless. The difference between hopeful and hopeless is determined by the skill, experience and patience of the scientist. This limitation is slowly fading away with the constant improvement of the procedures.

Vitrification is the second difficulty. In many cases even a single cell is too large to be fully vitrified, and only some tissues are adequately cryoresistant. High-pressure freezing improves the vitrification depth by about one order of magnitude, which is sufficient for most practical cases. However, the technology for high-pressure freezing involves more artillery than a delicate specimen preparation method. The new generation of high-pressure freezing apparatus should change the situation.

Finally, sectioning water – even if vitrified – is not easy. Water flows, deforms and cracks, sometimes very badly, sometimes astonishingly little.

How can obtaining the best vitreous sections become the rule rather than the exception? The first answer is: more work! By chance, from time to time, observations are made that surpass by far all that has been obtained by conventional methods. Such results show avenues for further improving the method and refresh the motivation for 'more work'. One of these remarkable observations is shown in **Figure 4**.

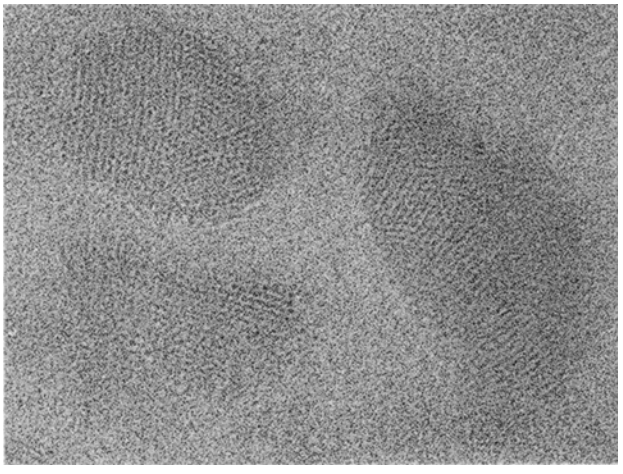

Figure 4 Thin vitreous section of partially decondensed stallion spermatozoa. Deoxyribonucleic acid (DNA)–protamine filaments are arranged in hexagonally packed bundles. The distance between filaments is 2.7 nm. Preparation and micrograph by Nathalie Sartori Blanc.

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Further Reading

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