Cryo-EM and Single Particles

Cryoelectronmicroscopy is a method for the imaging of macromolecules in the electron microscope. It was originally developed to determine membrane protein structures from two-dimensional crystals, but more recently “single-particle” techniques have become powerful and popular. Three-dimensional reconstructions are obtained from sets of single-particle images by extensive computer processing; the methods are being applied to many macromolecular assemblies.

Cryoelectronmicroscopy (cryo-EM), also called electron cryomicroscopy, is a method for obtaining images and 3-D reconstructions of macromolecules. In cryo-EM the specimen—typically an unstained protein preparation embedded in vitreous ice—is held at cryogenic temperatures while images are formed in the electron microscope.

Cryo-EM is actually one of a family of methods of specimen preparation for 3-D electron microscopy. The classical preparation methods for cell biology, involving fixation, sectioning, plastic embedding, and staining, are now also used for observing membranes and organelles in 3-D by tomographic reconstructions (54) but do not provide the resolution for visualizing nanometer-sized structures such as single macromolecules and macromolecular complexes. For these smaller structures, replicas by heavy-metal shadowing (5, 31) or casts by negative staining provide a view of surface topography. Negative staining (6) is a particularly simple and rapid way to obtain a surface view of macromolecular complexes and can even provide millisecond time resolution (70). In this technique the macromolecules of interest are embedded in a dried film of heavy-metal salt such as uranyl acetate or phosphotungstic acid. Reconstructions in three dimensions of protein complexes such as myosin–actin systems, ion channels, and DNA complexes continue to be made from negative-stain images, but there can be considerable geometric distortion due to the flattening of the stain film as it dries. The resolution of negative-stain images is reputed to be limited by the grain size to ~20 Å, but much better resolution has been reported in very low-dose images (30).

A recent development is the cryonegative-stain technique (1). Instead of drying down the thin film of stain and macromolecules, the film is rapidly frozen. The resolution and geometric fidelity of this method is much better than with traditional negative stain. However, because it is a stain technique, it provides only a representation of the surface of a macromolecule; it does not provide information about the interior structure.

Why not image a protein molecule directly, without stain, in the electron microscope? We can, but there are substantial technical problems to be overcome. Most seriously, the protein is rapidly blown to bits while any image is acquired. This is because the high-energy electrons break the chemical bonds in the protein, forming volatile fragments that quickly vanish into the microscope’s vacuum system. The problem can be solved by immobilizing the protein at a very low temperature, embedded in ice. This way many chemical bonds can be broken while the atomic nuclei remain approximately in place, and much larger electron doses (~10 electrons per square angstrom of specimen area) can be applied before destroying the specimen. If the aqueous film containing the protein specimen is frozen very rapidly and maintained below ~160°C, the water remains glassy and no ice crystals form.

Cryo-EM methods were proposed more than 40 years ago (15) and developed in the 1970s and 1980s (12, 57). A modern cryo-EM specimen consists of an aqueous film that spans holes in a thin carbon film (FIGURE 1). The film is blotted to ~1,000-Å thickness, and the grid is rapidly plunged into liquid ethane. Cooled by liquid nitrogen, the ethane is maintained well below its boiling point and therefore serves as an excellent cryogen.

The cryo-EM image of a protein molecule has much lower contrast than a negative-stain image because the protein’s light elements produce only a weak phase-shift signal. The situation is not helped by the fact that phase-contrast imaging in the electron microscope is primitive. Zernike phase-contrast optics for the electron microscope are only in the experimental stage (11), and at present the only practical technique for phase-contrast imaging is to throw the electron microscope out of focus. This produces an image with a complicated contrast-transfer function and with quality that depends strongly on the coherence of the electron beam. For high-resolution work, a microscope with a highly coherent “field-emission” electron source is required.

2-D crystals

The EM image of a single protein molecule has not only low contrast but also much shot noise from the fact that a limited number of electrons is used in forming the image. The result is a very low signal-to-noise ratio (SNR), which must be overcome by some sort of signal averaging to yield a useful image. The SNR decreases with spatial frequency, and therefore much more averaging (10⁶ particle images or so) is required to achieve near-atomic resolution of 3–4 Å than...
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is required for lower resolution (a few thousand images for 20-Å resolution). Averaging is simplest in the case of 2-D crystals. A 2-D crystal of membrane protein consists of a lipid bilayer incorporating a high density of the protein, so high that the protein molecules form a regular array. A smaller sort of regular array is provided by the icosahedral symmetry of virus capsids, where signal averaging can also be performed over 60 unit cells.

Perhaps the most famous cryo-EM project has been Nigel Unwin’s determination of the structure of the nicotinic acetylcholine receptor (AChR). In the 1980s it was discovered that postsynaptic membrane vesicles from the electric organ of Torpedo marmorata can be coaxed into forming membrane “tubes” of ~800 Å in diameter. In these the AChRs are arranged in a 2-D crystal that is rolled up into a cylinder, forming a helical crystalline array (7). From this start, 20 years of technical innovations followed; as one example, consider the problem of catching AChRs in the open state, which due to desensitization has a very brief lifetime. Berriman and Unwin (4) constructed an apparatus that sprays droplets of an acetylcholine solution onto a grid just before it plunges into liquid ethane. The result is a fast application of ligand with a time resolution of ~1 ms. In the end, with images acquired with Yoshi Fujiyoshi’s incomparable microscope (18), Unwin has obtained a refined, 4-Å structure of the closed receptor channel (58). This effort and a study of the aquaporin channel (44) have yielded the highest-resolution structures of proteins frozen in ice.

Single particles

Why can’t one just average the images of individual protein molecules without having a crystalline array of them? Within limits one can, and this is what single-particle reconstruction is all about (16). There are various detailed methods for processing the images, but they share a general strategy. First, because the individual molecules (particles) are randomly orientated in the ice (or stain) layer, the particle images must be classified into groups representing the various views of the original 3-D particle. Second, the particle images in a group must be aligned to each other so that they can be averaged together appropriately. Third, once the averaged views are on hand, a “map” of the density throughout the volume of the particle can be calculated by using the same Fourier-transform trick—the projection theorem (10)—that is the basic idea behind the reconstruction process also in tomography and in X-ray crystallography.

FIGURE 2 demonstrates these steps in a recent cryo-EM study by Ludkte et al. (35) of the calcium-release channel of skeletal muscle, ryanodine receptor 1 (RyR1). First, 28,000 particle images are selected from hundreds of micrographs. Meanwhile, from an initial 3-D model, a few hundred reference images are computed as projections through the model at a complete set of angles. Each particle image is then aligned against each reference and scored for similarity; by this process each particle image is assigned to a reference’s class. Class averages are computed and inserted into a new 3-D model at the reference’s projection angle. This process is iterated maybe 10 times until it converges to a stable structure.

All of these computations, especially the classification and alignment steps, are quite intensive. A single-particle reconstruction project may require hundreds to tens of thousands of CPU-hours, depending on the size of the data set and the final resolution achieved. Clusters of networked computers are employed for high-resolution projects.

Determining the resolution of a single-particle reconstruction is not a straightforward issue. Whereas in crystallography the quality of a crystal (and the eventual density map) is apparent in the pattern of diffraction spots, how is one to determine the quality of a reconstruction made from individual, noisy particle images? The most widely used approach is to split the data set in half, make two “independent” reconstructions, and compare their similarity as a function of spatial frequency. The similarity comparison is usually done by computing...
the Fourier shell correlation (FSC), but there remains controversy about what threshold correlation value to use, and there is a danger of artifacts (20, 60). In the end the important question is what structural features are visible in a map. For example, at 10-Å resolution long α-helices can be seen as cylinders of density. Beyond 5 Å, individual strands of β-sheet may be resolved.

The RyR1 structure obtained by Ludtke et al. (35) has a resolution, by an FSC measure, of 9.6 Å; another RyR1 structure by Samso et al. (48) is 13.6 Å by the same conservative measure. Both show tilted rods that appear to be the α-helices of the ion-conducting pore, which would be expected to be closed in the EGTA buffer used in each of these studies. The transmembrane region of the Ludtke et al. structure is shown in Figure 3, where the authors have identified rods corresponding to the pore helices and inner helices of potassium channels. The surprise here is that the kink in the inner helices matches well the “open” MthK potassium channel structure (28).

**Uses of single particles**

In principle a suspension of any purified, monodisperse protein can be applied to an EM grid, frozen, and imaged, and a reconstruction can be performed. Indeed, considerable efforts are underway to automate the image collection (55) and processing (37) to make this a straightforward process. The requirement that individual particle images be properly classified and aligned means that there must be strong-enough features in each image to be recognized in the presence of the large noise. In practice this places a lower bound of ~500 kDa on the size of a particle that can be analyzed in this way. Many macromolecular structures have been studied by single-particle techniques; we provide only a sampling here.

The first particles imaged in vitreous ice were adenovirus particles (2); the size, symmetry, and rigidity of virus capsids have allowed structures with resolutions better than 10 Å to be obtained in many cases, allowing the identification of protein folds and elucidation of infection mechanisms (23, 42). On the other hand, the most-analyzed “low-symmetry” single particles have been ribosomes, which served for many years as the test specimen for the development of single-particle techniques. Although recently high-resolution structures of the two ribosomal subunits became available from X-ray crystallography (36), the structural analysis of the ribosome’s elongation cycle has continued to be pursued through single-particle cryo-EM studies (53). A new structure at 13- to 15-Å resolution of a ribosome, complexed with particular cofactors and substrates, can be obtained in a few weeks. Two recent examples are the observation of the reconstituted 70S initiation complex, trapped through the use of a nonhydrolyzable GTP analog (28), and the structures of ribosomes stalled following the translation of three different proteins (3, 19). Similarly, the reaction cycle of the GroEL protein-folding machine has been studied extensively with cryo-EM (47), and recently a structure of GroEL with a bound protein substrate has been obtained (14).

**Ion channels are excellent candidates for single-particle work, where their various conformational states may be investigated.** For cryo-EM the RyRs have been well suited, having a very large cytoplasmic domain that allows good particle alignment (Figure 2). The other intracellular calcium-release channels, the inositol trisphosphate receptors, are also being investigated by negative stain and cryo-EM (56). Ion channels in the six-transmembrane-segment family, including voltage-gated channels, have been studied mainly by negative stain. This is because their size (300–600 kDa) and compact shape yield cryo-EM particle images that are at the limit for reliable image alignment. Recent negative-stain studies of Kv1 channels (39, 51) and Kv4 channels (29) located the positions of accessory subunits and demonstrated the architecture that is now visible in greater detail in the X-ray crystal structure of Kv1.2 (34). The bacterial voltage-gated channel KvAP, bound to Fab antibody fragments that augmented its size, was imaged using cryonegative stain (25); this has yielded the best EM resolution for a protein of this family: 10.5 Å. Voltage-gated calcium channels have

![FIGURE 2. An example of single-particle structure determination](http://physiologyonline.physiology.org/ by 10.220.32.247 on June 6, 2017)
tein preparation is not pure, or if there is too much conformational flexibility, a high-quality crystal cannot be grown. With cryo-EM, images can be taken of heterogeneous mixtures of particles; the challenge, however, is to deduce the correct structures from them. The fundamental assumption of the single-particle method is that each imaged particle is identical. If there is a mixture of particle types, one could apply the image-classification methods not only to separate the various particle views but also to separate out the various particle types. An elegant example of this is the work of Heymann et al. (22) on the maturation of the herpes simplex virus capsid. From micrographs taken at four time points, they sorted out a spectrum of 17 density maps, showing the structures of the procapsid, the mature capsid, and various intermediate states. This impressive result was made possible by the large size of the virus particles, so that differences among particle images were relatively easy to detect. Other general methods have been proposed for distinguishing heterogeneous particles in various views, for example, Ref. 66. But what about particles that are small, so that in some views of the particles the differences are not detectable?

A powerful approach to this problem, which shows promise for studies of many small particles, is a directed random conical tilt method recently applied by Rodal et al. (45) to the Arp2/3 actin nucleation complex. They made use of a classical strategy of acquiring pairs of images of the same field of particles, one with the specimen tilted and one with the specimen in its untilted position. Particles were selected from the untilted image and classified, and one class that clearly showed a view of a particular particle conformation was identified. For just this set of particles, the corresponding tilted views were selected, and from these a 3-D reconstruction was made by using the random conical tilt method (17). In the end Rodal et al. made 3-D reconstructions of three different conformations, showing an open, intermediate, or closed cleft between the Arp2 and Arp3 subunits. The open and closed conformations correspond to inactive and activated states of the complex.

A second challenge has to do with membrane proteins. Most 2-D crystals have been grown from membrane proteins present at high density in a

been imaged as well (50, 65, 68) and a cryo-EM structure of sodium channels has been obtained at the technical limits of single-particle reconstruction (49).

Myosins have also been studied extensively by EM techniques. Due to the small size of the head region (~80 kDa) and the extreme flexibility of the neck and tail regions, no single-particle cryo-EM has been carried out for single myosin molecules. Reconstruction of helical filaments has been exploited in cryo-EM studies of various myosins bound to actin (43, 61, 67). Recent work shows myosin II filaments in the relaxed state (69) by exploiting an application of single-particle computational methods to helical structures (13). Negative-stain techniques have been remarkably useful in studying individual myosin molecules, allowing them to be visualized (62, 63), in determining their oligomeric states (32, 33, 52), and in observing transient states during the ATPase cycle (8, 64). The use of negative-stain and single-particle image processing to explore myosin dynamics has been reviewed recently (9).

**Challenges for the future**

Crystallographers know that if a protein preparation is not pure, or if there is too much conformational flexibility, a high-quality crystal cannot be grown. With cryo-EM, images can be taken of heterogeneous mixtures of particles; the challenge, however, is to deduce the correct structures from them. The fundamental assumption of the single-particle method is that each imaged particle is identical. If there is a mixture of particle types, one could apply the image-classification methods not only to separate the various particle views but also to separate out the various particle types. An elegant example of this is the work of Heymann et al. (22) on the maturation of the herpes simplex virus capsid. From micrographs taken at four time points, they sorted out a spectrum of 17 density maps, showing the structures of the procapsid, the mature capsid, and various intermediate states. This impressive result was made possible by the large size of the virus particles, so that differences among particle images were relatively easy to detect. Other general methods have been proposed for distinguishing heterogeneous particles in various views, for example, Ref. 66. But what about particles that are small, so that in some views of the particles the
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References


