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Electron Cryo-Microscopy: The Frozen Frontier of Structural Biology

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When the electron microscope was conceived in the early 1950s, it was originally to be a tool mainly for atomic physicists who were interested in obtaining more detailed analyses of materials such as metals at an atomic resolution. Using a beam of electrons rather than a beam of light, it would be possible to gain the sufficient resolution needed to visualize the comparatively much larger nuclei of atoms. It was not until biologists seized upon the opportunities afforded by this new technology that the truly revolutionary application, with respect to cellular biology, was made for the electron microscope. Detailed structures within the cell, never before seen through the crude lens of the light microscope, were now laid out before the awe-filled eyes of the nascent ultrastructuralists. Heretofore unknown subcellular organelles were now seen, and some of the initial observations on cellular membrane traffic — the dynamics of cellular compartmentalization that are responsible for most of what makes eukaryotic life possible — were made, ultimately to yield a Nobel Prize in Biology and Medicine in 1974 for George Palade, founder of the world-renowned Department of Cell Biology at Yale University School of Medicine.

However, in the tradition of scientists, more is always better, and in this case size matters. Microscopists have been pushing the envelope to achieve greater and greater levels of resolution using the electron microscope. A collaborative effort from laboratories in Berkeley, California, United States; Cambridge, England; and Berlin, Germany brought to fore the use of the electron microscope for high-resolution imaging. An example is the work of Nigel Unwin in England on the nicotinic acetylcholine receptor over the past two decades [1-3]. Using the electron microscope, Unwin has shown the rough three-dimensional structure of the ion channel visualized through high-magnification imaging and electron density mapping. It has become clear that the

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[b] Abbreviations: Å, Ångstrom; cryoEM, electron cryo-microscopy; GTP, guanosine triphosphate; GED, GTPase effector; PH, pleckstrin-homology.

channel is an actual porous complex of five subunits through which ions may pass or be blocked depending on the bound state of the acetylcholine molecule [1-3]. The technique used to visualize the nicotinic acetylcholine receptor was electron cryomicroscopy, and that technology has now been brought to the Yale University School of Medicine. With the recent acquisition of three new high-power electron microscopes, Yale is intending to remain on the forefront of the development of this technology. Yale has created a facility dedicated toward the ultrastructural determination of biological specimens, a pursuit important to biochemists, biophysicists, physiologists, and cell biologists alike. Current projects already underway include the structural elucidation of ion channels, intrinsic membrane proteins, inter-cellular adhesion proteins, and other macromolecular complexes.

BACKGROUND

The limit of resolution for any microscope is a function of the wavelength of the incident beam and the numerical aperture of the objective lens. Given the wavelength of visible light, even with the best objective lenses resolution is limited to no better than 200 nm. Using electrons for imaging, however, yields a much higher degree of resolution due to the relatively smaller deBroglie wavelength of electrons [4]. The limit of resolution for an electron microscope, it turns out, is determined more by the low numerical apertures of the objective lenses used, due to problems with spherical aberrations at higher numerical apertures. Even so, it is possible to obtain images of 1.6 Ångstrom (Å)[b] resolution, the typical distance between two atomic nuclei in biological specimens. To date, however, atomic resolution has never been achieved for a biological specimen using an electron microscope. This is mostly due to a question of imaging — the

purpose of microscopy. In order for our eyes to visualize a specimen, there must be sufficient contrast within the specimen to distinguish it from non-specimen materials. Contrast for an electron microscope is basically due to scattering of the incident beam of electrons, which then generates mostly a phase-contrast image. Stains, comprising large, electron-dense atoms such as uranium or lead, are often used to enhance electron scattering and therefore specimen contrast. These heavy metal stains are acceptable when imaging at the low cellular and subcellular levels, when the goal is visualization of gross cellular anatomy and membranous organelles, because the size of the heavy-metal atoms is much smaller than that of the cellular structures desired to be observed. For the deconstructionist paradigm pervading science, which craves molecular detail, however, these methods are vastly inadequate, since the atoms used in the stains are some of the largest atoms on the periodic table of elements — completely overshadowing their smaller, biologically more relevant cousins, and thereby rendering any possibility of observing molecular detail moot.

Over the past few decades, however, use of the electron microscope has moved beyond the cell and down to the protein macromolecules, which are the infrastructure and machinery by which the cell operates. This achievement necessarily had to come with a new strategy for obtaining specimen contrast. Heavy metal staining will still yield some structural detail for the larger protein macromolecular complexes, but there will never be the level of detail obtained from an atomic resolution image. The heavy metals are thrown out and in their stead is used the nectar of life, pure water. A major advantage of using water is that one maintains the aqueous environment in which the specimen is naturally found and, thereby, may study the specimen in a more "functional" state than what one may find after using crude artificial

fixatives which may be prone to producing artifacts [4]. In order for water to be used, it needs to provide a solid embedding support for the specimen, and its structure needs to remain amorphous, as it is in its liquid state. Liquid water cannot be used in the vacuum system of the electron microscope, and while ice is compatible with this vacuum, the crystals will ruin the ultrastructural detail of the specimen [4]. In order to resolve this dilemma, the specimen is plunged into liquid ethane kept at the extremely low temperatures of liquid nitrogen (<180°C), inducing a rapid freeze which occurs quickly enough that the water does not have a chance to order itself into crystals (a cooling of roughly 10^5°C per second). Instead water becomes vitrified, in a state similar to glass, and the specimen is in what is called a frozen-hydrated state [4]. In this way, the still disordered water molecules provide a solid base around the biological specimen, yet they do not interfere with imaging of high resolution detail within the specimen.

Unfortunately, the major drawback from using water instead of heavy metal atoms is that omission of heavy metal stains renders the specimen sensitive to damage from electron radiation. It is well known that specimen interactions with electrons produces non-reversible changes that damage and eventually destroy the specimen [4]. However, by using extremely low temperatures while working with vitrified water, the degree of specimen degradation by the electron beam is reduced. In turn, this reduction in radiation sensitivity allows higher doses of electrons to be used for the imaging process than would be possible at higher temperatures. Consequently, imaging of a frozen hydrated specimen results in better signal-to-noise ratios at higher resolution. This highlights yet another advantage of cryo-microscopy toward achieving as close as possible to the true structure of the specimen.

Achieving truly high resolution images still requires the specimen to be in a regularly ordered array similar to what is typically needed for X-ray crystallography, the standard tool for structural biology. For electron cryo-microscopy, one needs what is called a "2-D crystal," which is a two-dimensional array of molecules organized in a plane [4]. The main advantage of such 2D-crystals is that the imaging information of the specimen is constrained to discrete diffraction spots rather than being continuous in reciprocal space, the domain in which structural data are handled. Hence, the signal can more easily be distinguished from the background noise. Furthermore, a whole arsenal of digital image processing methods have been developed to analyze data from such specimens and even to computationally improve crystal quality by removing certain types of disorder from the crystal lattice.

LOOKING AHEAD

So why all this fuss over a new technique which has finnicky quirks to boot? There are advantages to using electron cryo-microscopy compared with other techniques of structural biology. For one thing, macromolecules can be imaged in their natural state as opposed to in a potentially unknown conformation that happens to form a crystal, which is a concern with X-ray crystallography. Observing the structure in a near native state is also a benefit of nucleic magnetic resonance (NMR) structural studies, which can solve the molecular structure of a macromolecule in solution. However, one major drawback to NMR is that relatively small macromolecules (<200 amino acids, for example) are routinely amenable to study by this method . In addition, a large advantage of electron microscopy over other structural methods is that there are no limitations on what specimens can be ana-

lyzed. Furthermore, using cryo-EM, one can visualize complexes that otherwise would not form the standard crystals necessary for X-ray crystallography. This is particularly important for proteins that polymerize, such as cytoskeletal proteins, since the polymerized state of the protein will not necessarily be the state under which three-dimensional crystals will form. So if one is interested in the polymeric state of proteins, electron cryo-microscopy will potentially be a better option than the standard X-ray crystallography. Moreover, because three-dimensional crystals are not needed, one can, in theory, resolve the three dimensional structure of a protein or complex under a variety of different states (structural conformations) triggered under various experimental conditions. This will allow researchers to determine, for example, how an interaction between two proteins or a protein and an auxiliary compound will be transduced structurally, thus providing crucial information about the mechanisms of action for these proteins and complexes. This information could then be used for rational mutagenesis of critical amino acid residues to test for their function, or for the application and development of various pharmaceutical targets. Finally, the National Institutes of Health is planning a large-scale high-throughput crystallization effort, whereby they hope to have the crystal structures of well over 10,000 proteins solved by an automated method within the next decade or so. This will shift the focus of academic structural biology more toward the techniques of NMR and electron cryo-microscopy, which allow for more fluid and sophisticated structural experimentation compared with the static crystallographic information.

The most exciting application of these methods toward biology will be a synergy with all three major techniques of structural biology. X-ray crystallography remains currently the method consistently providing the highest resolution structures. But as mentioned above, the electron cryo-microscopic data provide priceless information on orientation and dynamic components of macromolecules. Therefore, a blend of both methods, where one would use the image gathered by cryo-EM in order to correctly orient the more detailed structure obtained by X-ray crystallography, could produce very promising results to support the various hypotheses surrounding the functional domains of proteins.

An example of this is the recently published three-dimensional structure of dynamin, a large GTP hydrolyzing molecule important in endocytosis [5] (Figure 1). Endocytosis, a critical biological process necessary for the internalization of cellular membranes, receptors, and extracellular substances, is important in many functions, including metabolism, the immune system, the nervous system, and cancer. Therefore, elucidation of the molecular mechanisms of endocytosis will be important toward figuring how this process works, ultimately to yield a better understanding of the related pathology and potential therepeutics of diseases involving endocytosis. Understanding how the dynamin protein works will be a big step toward this goal.

The dynamin molecule has several domains which are each predicted to confer certain abilities and attributes to this protein. One domain (the pleckstrin-homology [PH] domain) was thought to be involved in the interactions of this protein with the membrane [5]. Another domain (the GTPase effector [GED] domain) was hypothesized to be important in the oligomerization of the dynamin protein [5]. Through the combined use of X-ray crystallography and cryo-EM, both of these hypotheses have been substantiated [5]. The authors of this study used the high resolution three-dimensional crystal structures of a similar PH domain and a similar
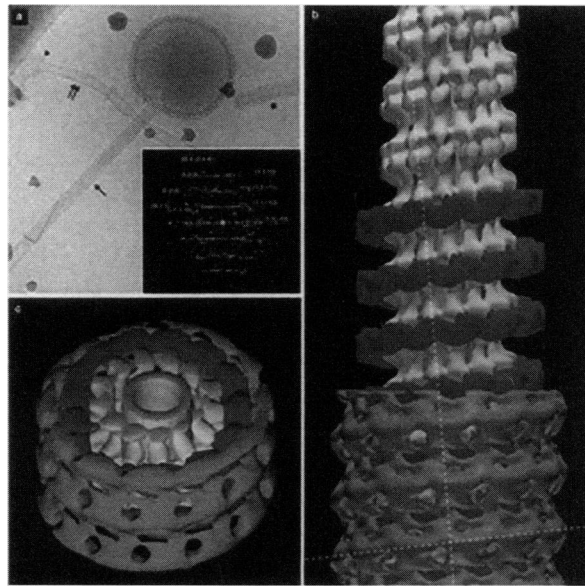
Figure 1. Three-dimensional reconstruction of a dynamin protein complex forming a helical array by electron cryo-microscopy. a: Micrograph of a lipid bilayer being deformed into a tube by dynamin, as visualized by electron cryomicroscopy. Double arrows distinguish an undecorated lipid tube from a dynamin decorated tube (single arrow). The inset shows a computed Fourier transform of the diffraction pattern generated. **b** and **c:** Three-dimensional reconstruction map viewed from two different angles showing an image at 20 Å resolution. Reprinted by permission from Zhang, P. and Hinshaw, J.E. *Nature Cell Biology*, Vol. 3, No. 10, pp. 923. Copyright © 2001, Macmillan Magazines, Ltd.

GTP hydrolyzing domain (both from different proteins), in order to orient them in space according to the relatively lower resolution images obtained by cryo-EM. Through this computation, it became clear that the PH domain was clearly in a position to interact with the membrane, whereas the GED domain was in a position appropriate for it to interact with other dynamin molecules [5]. This type of analysis is made more robust by the fact that several proteins have domains with similar three-dimensional structures, which can then be used to visualize a comparable domain in a protein with unknown structural detail imaged by cryo-EM, as these authors have done [5]. As it becomes more evident that many protein domains share a common three-dimensional structure despite very little homology at the

amino acid level, one can envisage using a cryo-EM structure to screen the three-dimensional databases for potential domains which would fit into the structure solved by cryo-EM, thereby more quickly localizing certain functional domains which would give a clue to the mechanism of action for the protein of interest. This information would be very useful in designing further experiments and drug targets.

Thus, the emerging technology of electron cryo-microscopy is in a position to help make great advancements in biology and medicine. Researchers interested in various aspects of protein function could benefit from pursuing cryo-EM structural studies in parallel with other techniques, increasing the creative repertoire of experimentation. As techniques and equipment

become better and better, scientists in the field believe that the ultimate goal of delivering on the promise of atomic resolution will be attained. Together with X-ray crystallography and NMR, cryo-EM will expand the arsenal of tools available for the structural determination of biological molecules. Through the development of the new microscope facility, Yale University will continue its strong tradition of staying at the leading edge of development in structural biology.

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