Structure determines function.

That, at least, is what structural biologists will tell you. And these days they have a powerful new tool—or rather, a vastly improved old one—for determining the structure of biological molecules, and thereby ascertaining what they do and how they do it.

The tool is cryo-electron microscopy (cryo-EM), a suite of methods that allows researchers to construct three-dimensional images of microscopic objects using focused beams of electrons and super-cold temperatures. Until recently, the technique could only produce fuzzy, blob-like images of biological macromolecules with nothing like the fine detail available through methods like x-ray crystallography and nuclear magnetic resonance (NMR). “A few years ago, we were considered the ‘blob-ologists,’” says Melanie Ohi, PhD, associate professor of cell and developmental biology and a member of the Center for Structural Biology at Vanderbilt University.

But over the past several years, cryo-EM has begun to produce images with the kind of near-atomic resolution that was once limited to its rivals—but without their drawbacks.

“The field is in a revolution,” says Klaus Schulten, PhD, a computational biophysicist at the University of Illinois. Cryo-EM is now producing high-resolution structures of large biomolecules and molecular machines such as chromatin, supercoiled DNA, intracellular vesicles, membrane pores, ion channels, and individual virus particles. At the same time, it is being used to hone in on the atomic-level structures of smaller and smaller individual molecules at an extraordinary level of detail. And on top of that, researchers are figuring out how to extract different conformations of the same molecules from...
cryo-EM data—allowing them to create simple animations that illustrate how a molecule’s structure changes as it does its job in the cell.

**BEETTER THAN CRYSTALLOGRAPHY**

Before the cryo-EM revolution started, the gold standard for determining molecular structure at atomic resolution was x-ray crystallography. This method requires that proteins be crystallized before they can be scanned, but crystallization is not always possible. Nor is it necessarily desirable. As Schulten points out, crystallizing proteins forces them to line up “like Prussian soldiers in crystal,” denying researchers the opportunity to capture the various shapes, or conformations, that the biological molecules assume as they go about their business—conformations that provide the key to a molecule’s function. Potential insights into the workings of the largest molecular machines—which tend to be extremely flexible and dynamic systems with many moving parts—have thus remained obscured.

NMR offers resolution on par with x-ray crystallography without the need for crystallization. But it has a different limitation: It can only determine the structure of relatively small objects—certainly nothing as large as a molecular machine such as a ribosome or an ion channel, much less the cell that contains it.

Cryo-EM, by comparison, can handle a wide range of scales; though the method can’t yet resolve the smallest objects available to NMR, it’s getting there, and it can already be used to image much larger ones with ease. In addition, the freezing process used in cryo-EM allows proteins to remain in something resembling their native state, offering the possibility of gleaning more information about function.

**THE FREEZE**

In cryo-EM, researchers plunge thin films of sample solution into baths of ethane that have been cooled to the temperature of liquid nitrogen (−180°C). The biological material in the samples is flash-frozen in a delicate layer of glass-like ice, which can then be bombarded with electrons.

Cryo-EM comes in two principal flavors: single-particle analysis and cryo-electron tomography (CET). In single-particle analysis, researchers capture two-dimensional projections of hundreds of thousands, even millions, of the same kinds of biological objects—membrane proteins, cellular complexes, and viral capsids—that have been randomly distributed throughout the ice in different orientations. Image-processing algorithms then sort and average those 2-D–projections to construct a three-dimensional structure.

Cryo-electron tomography, on the other hand, involves taking multiple images of a single object. By tilting the object at various angles relative to the electron beam, researchers can again build up a 3-D structure. The resolution offered by CET is currently lower than that of single-particle analysis, but it can be used to image larger one-of-a-kind objects like organelles or even entire cells. In the most cutting-edge applications of cryo-EM, the two methods are used in combination.

**THE REVOLUTION: LEAVINGBlob-ology BEHIND**

For many years, cryo-EM could not achieve the kind of near-atomic resolution available to NMR and x-ray crystallography, nor could it handle the smallest molecular structures—in part because researchers had to limit the power of their electron beams in order to avoid destroying the samples they were trying to study.

Recent technological breakthroughs now allow researchers to collect higher resolution images using fewer of the tiny charged particles, however, reducing the amount of damage done to the samples while improving the quality of the resulting structures. Schulten himself helped revolutionize the field by developing computational methods for fitting high-resolution structures generated by x-ray crystallography into the relatively blobby, low-resolution structures derived from cryo-EM. This so-called hybrid approach uses computational modeling to develop realistic and highly detailed structures that conform to what scientists have learned about the dynamic behavior of biological molecules through decades of computer simulations. Schulten and his colleagues recently used precisely such methods to determine the atomic structure of the Rous sarcoma virus, a cancer-causing retrovirus that is used in cancer and HIV research.

Recently, however, cryo-EM has begun to achieve resolutions similar to x-ray crystallography all by its lonesome. This past year, for example, Sriman Subramaniam, PhD, a senior investigator at the National Cancer Institute’s Center for Cancer Research, imaged a small metabolic enzyme called beta-galactosidase at a resolution of 2.2 Å, or .22 billionths of a meter, using nothing but single–particle analysis. At that level of detail, one can see individual water molecules bound to the protein—something that would have been unimaginable with cryo-EM just a few years ago, and that could eventually assist in drug design.

Much of this progress is due to the development of new, highly sensitive cameras called direct electron detectors, and to the advent of powerful computing clusters that can be used to process the enormous volumes of data they generate. (“We’re generating many terabytes every day,” Subramaniam says.) But it is also due to the development of new and improved image-processing algorithms—algorithms that play a vital role in virtually every stage of cryo-EM.

In single-particle analysis, for example, image-processing algorithms must determine the relative orientations of enormous numbers of 2-D projections, then align and average them in order to reconstruct a 3-D image. Researchers like John Briggs, PhD, at the European Molecular Biology Laboratory in Heidelberg, have been applying similar techniques to improve the resolution of cryo–electron tomography, essentially taking multiple images of repeating structures within a given molecular machine and averaging them, much as multiple images of individual objects are averaged in single–particle analysis. Briggs and his colleagues have used this approach, which is known as subtomographic averaging, to resolve the structures of protein complexes that allow vesicles to travel from one cell compartment to another, and that enable the HIV-1 virus to self-assemble.
Discerning how coated vesicles form. Trafficking vesicles bud from one cellular compartment and fuse with another to transport material within cells. To form such vesicles, membrane coats localize cargo and polymerize into cages to bend the membrane. Although extensive structural information is available for components of these coats, the heterogeneity of trafficking vesicles has prevented an understanding of how complete membrane coats assemble on the membrane. Using a combination of cryo-electron tomography, subtomogram averaging, and cross-linking mass spectrometry, researchers derived this complete model of the highly interconnected coat protein complex I (COPI), a coat involved in vesicle traffic between the Golgi and the endoplasmic reticulum. At left is a ‘triad’, the building block of the COPI coat. At right, the complete COPI–coated vesicle made of an assembly of triads. The development of this model provided novel insights into how coated vesicles form. From SO Dodonova et al., A structure of the COPI coat and the role of coat proteins in membrane vesicle assembly, Science 349, 195 (2015). Image: Svetlana Dodonova, European Molecular Biology Laboratory (EMBL).
Finding the path through the nuclear pore complex.
The nuclear pore complex (NPC), one of the largest protein complexes in the cell, is responsible for mediating or blocking the exchange of materials between the nucleus and cytoplasm. Cryo-EM was recently used to determine the structure of the NPC as shown above. The NPC is comprised of three layered rings: the cytoplasmic ring (gold), the spoke ring which forms the pore (blue) and the nucleoplasmic ring (green) as shown in (a) and (b) below (inverted views of the same complex). In cross-section (c), extended linker structures protrude from the nucleoplasmic ring (C and D), as well as from the cytoplasmic ring (E). Likely nuclear transport routes pass through the nuclear pore complex barrier, as illustrated by solid and dashed curves. The axes show the dimensions of the NPC in the $x$- and $y$-direction. From M Eibauer, M Pellanda, Y Turgay, A Dubrovsky, A Wild, and O Medalia: Structure and Gating of the Nuclear Pore Complex. *Nature Communications*. June 26, 2015. doi: 10.1038/ncomms8532.
Propellers with a soft touch. Mechanosensitive cation channels serve key roles in converting mechanical stimuli into various biological activities, such as touch, hearing and blood pressure regulation, through a process termed mechanotransduction. The Piezo family of cation channels, in humans and other mammals, plays broad roles in multiple physiological processes, including body proprioception, sensing shear stress of blood flow for proper blood vessel development, regulating red blood cell function and controlling cell migration and differentiation. Until now, researchers have not known the overall structural architecture and gating mechanisms of Piezo channels.

In recent work published in *Nature*, researchers determined the cryo-electron microscopy structure of the full-length (2,547 amino acids) mouse Piezo1 cation channel at a resolution of 4.8 Å. Here, four representative cryo-EM Piezo1 structures are shown (a). They consist of a trimeric propeller-like structure with the extracellular domains resembling three distal blades and a central cap. The rather flexible extracellular blade domains are connected to the central intracellular domain by three long beam-like structures. The red dashed lines, which represent observed positions of the propeller blades, reveal that the blades are not always positioned 120 degrees apart (black solid lines). By overlaying the third and fourth structures (b) in orange and cyan, one can see the centripetal movement of the blades (top) and the tilted movement of the beams relative to the plasma membrane plane (bottom). It’s possible that Piezo1 uses its peripheral regions as force sensors to gate the central ion-conducting pore, as diagrammed in (c), where the blue and orange models represent the closed and open state channels, respectively, and red arrows indicate force-induced motion. Red dashed lines indicate the possible ion-conduction pathways. Reprinted by permission from Macmillan Publishers Ltd: J Ge, W Li, Q Zhao, et al., Architecture of the mammalian mechanosensitive Piezo1 channel, *Nature* (2015) doi:10.1038/nature15247.
A clearer picture of chromatin structure.

DNA packs itself into the small space inside the cell nucleus by wrapping around histone proteins to form nucleosomes. These basic elements repeat as beads-on-a-string, interconnected by sections of linker DNA. In addition, a linker histone called H1 coils the beads-on-a-string structure into a 30 nm chromatin fiber whose structure has been a matter of debate. Cryo-EM structures recently described in *Science* are now offering a clearer picture. Here we see a cryo-EM map of the 30 nm chromatin fiber (A). This structure was used to build the model of a longer fiber as shown in (C). Reprinted with permission from F Song, P Chen, D Sun et al., Cryo-EM Study of the Chromatin Fiber Reveals a Double Helix Twisted by Tetranucleosomal Units, *Science* 344 (6182), 376-380 (2014).
**The flexibility of supercoiled DNA revealed.**

DNA supercoiling regulates access to the genetic code, which strongly affects DNA metabolism. Researchers recently used cryo-electron tomography together with biochemical analyses and computer simulations to investigate the various shapes taken by individual purified DNA minicircle topoisomers with defined degrees of supercoiling. They found that each topoisomer adopts a unique and surprisingly wide distribution of three-dimensional conformations including circles, handcuffs, “racquets” and figure eights. Molecular dynamics simulations independently confirmed this conformational heterogeneity and provide atomistic insight into the flexibility of supercoiled DNA. These images show the structure of the DNA calculated with the supercomputer simulations (in color); and in the images to the right, superimposed upon the cryo-electron tomography data (in white or yellow). From RN Irobalieva, JM Fogg, DJ Catanese Jr, T Sutthibutpong et al., The Structural Diversity of Supercoiled DNA, *Nature Communications* 6 (2015). Image credit: Thana Sutthibutpong.
**Helical measles.** Viruses rely on their capsid proteins to package and protect their genome. For the measles virus and other viruses in the same family, multiple capsid proteins together form a helical shell around the viral RNA and are collectively called the nucleocapsid. In recent work, researchers determined the high-resolution cryo-EM structure of the measles virus nucleocapsid at near-atomic resolution. The nucleocapsid consists of a series of connected N-nucleoproteins (D) wrapped around the viral RNA (green). This figure shows the nucleocapsid structure in front view (B) and cutaway view (C), with colors denoting the N-nucleoprotein’s two domains [N-terminal domain (blue) and C-terminal domain (pink)], as well as its N-terminal (dark blue) and C-terminal (yellow) arms that hold nucleoproteins together contributing to the stability of the whole architecture. Close-ups of three consecutive N-nucleoproteins from the exterior (E) and interior (F) of the helix reveal the nucleoproteins stacked into a helical shape. The structure reveals how the nucleocapsid assembles and how the nucleo-protein and viral RNA interact, both of which may inform drug design. From I Gutsche, A Desfosses, G Effantin, et al., Near-atomic cryo-EM structure of the helical measles virus nucleocapsid, *Science* 348:6235:704-707 (2015). Reprinted with permission from AAAS.
Understanding how ribosomes spot stop codons.

As a ribosome chugs along a strand of messenger RNA, it adds amino acids to a peptide chain according to the prescription of trios of mRNA nucleotides known as codons. In coordination with molecules called release factors, the ribosome stops adding more amino acids when it comes across one of three universally conserved stop codons: UAA, UAG or UGA. Eukaryotes rely on an omnipotent release factor (eRF1) that recognizes all three stop codons, but avoids each of the 61 sense codons for amino acid addition. To better understand how eRF1 discriminates between stop codons and sense codons, researchers trapped some ribosomal complexes at this crucial recognition step and examined the complexes using cryo-EM. Using a Bayesian classifier, they isolated five distinct classes of ribosomal complexes as shown here. One of these classes was further sub-classified (bottom line) to identify only the eRF1-containing particles, permitting a high-resolution view of the recognition event. By comparing the structures for each of the three stop codons, the researchers could see how eRF1 remodels mRNA in a way that permits their sequence to be queried. The results provide a molecular framework for understanding eukaryotic stop codon recognition. Reprinted by permission from Macmillan Publishers Ltd: A Brown, S Shao, J Murray, RS Hegde, V Ramakrishnan, Structural basis for stop codon recognition in eukaryotes, Nature 524:7566 (2015).

Seeing the spokes of a molecular motor.

The main skeleton of cilia and flagella is a microtubule doublet (MTD). The structure of tubulin, the main component proteins of MTD, has been previously solved at atomic resolution. In recent work, researchers analyzed the three-dimensional structure of the entire MTD from *Tetrahymena* cilia at ~19 Å resolution by single particle cryo-electron microscopy to reveal how various proteins such as tubulin isoforms, dyneins (motor proteins), radial spokes and microtubule inner proteins (MIPs) bind to the MTD to generate or regulate force. The image above shows various MIPs (in color) bound to the inside of the MTD as well as an external view of the MTD showing its tubulin subunits. Reprinted with permission from A Maheshwari, JM Obbineni, et al., α- and β-Tubulin Lattice of the Axonemal Microtubule Doublet and Binding Proteins Revealed by Single Particle Cryo-Electron Microscopy and Tomography, *Structure* 23:9:1584-95 (2015).