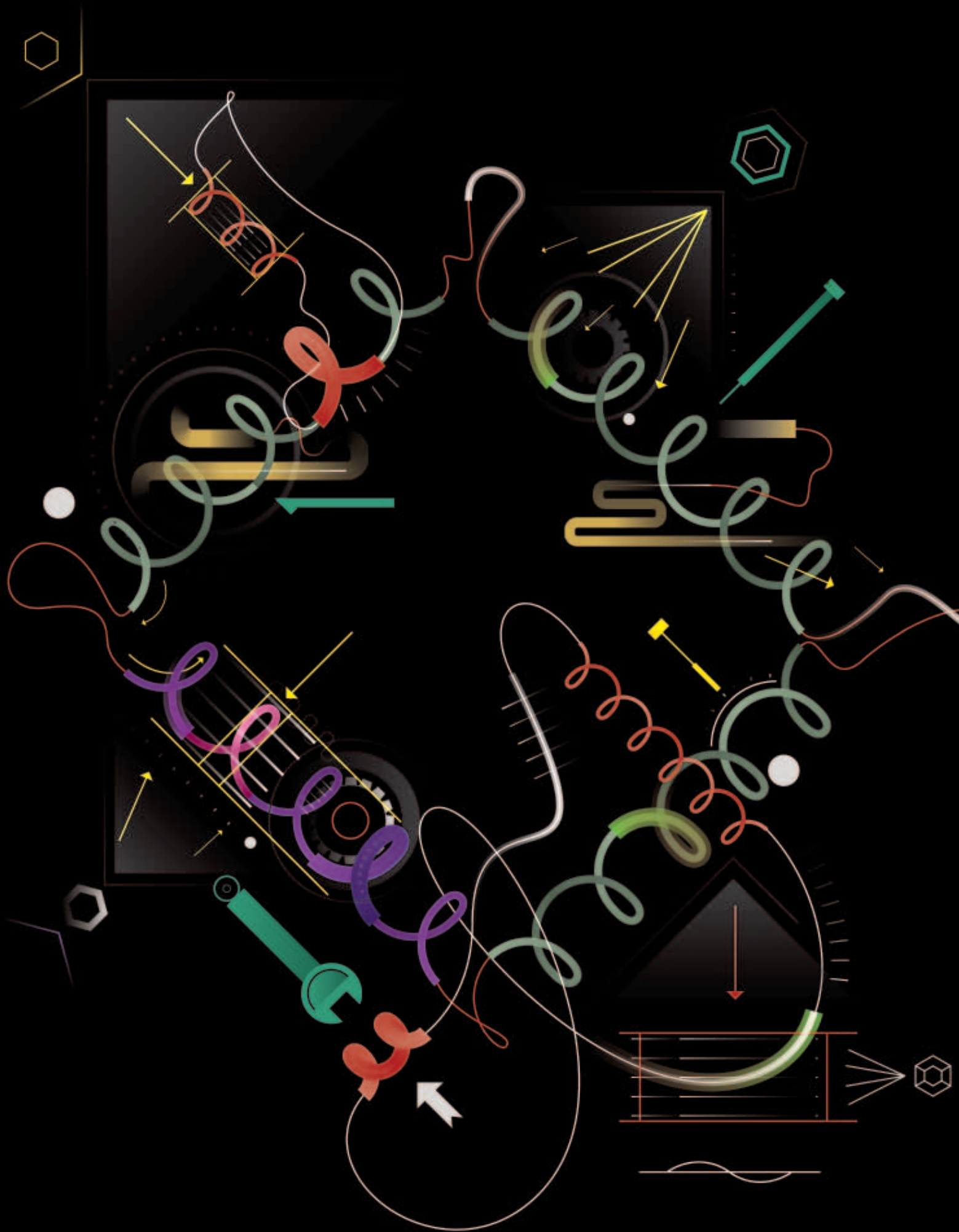
A STRUCTURAL TOOLBOX

No longer content with static snapshots, biologists are using a medley of techniques to get a global view of molecules at work.

By Nicole Kresge
Illustration by Leandro Castelao



Part 2 of 2: This is the second in a series of articles that commemorates the 25th anniversary of HHMI's structural biology program. Part 1 explored the program's genesis and its first investigators.

Natalie Strynadka wants to design a better antibiotic. Her strategy: learn about the molecules bacteria use to invade cells. Her tool: structural biology.

Easier said than done. Some structural biology techniques can't handle the big, membrane-embedded complexes bacteria use to overtake their hosts. Others don't give the level of detail Strynadka wants. And still others can't show her exactly how individual molecules interact.

So, she's combining multiple techniques—electron microscopy (EM), nuclear magnetic resonance (NMR), and x-ray crystallography—to visualize her molecular subjects. "When you're tackling these larger assemblies you really need to have a multidisciplinary approach," says Strynadka, an HHMI senior international research scholar at the University of British Columbia. "You aren't going to capture the whole picture in just one image. You have to piece together the information."

Strynadka is one of a handful of HHMI scientists who have been molding the tools of structural biology to fit their interests (see box on page 23 for a description of these tools). Twenty-five years ago when the Institute started its structural biology program, the goal was basic: to discover how molecules looked. Researchers spent years using a single method to solve a single structure.

"In the early days, we tried to choose interesting proteins for which the structures could be determined, but they also had to be proteins that were sufficiently well behaved and were easy to purify," recalls Brian Matthews, a biophysicist and HHMI investigator alumnus at the University of Oregon. "Now the progress is really driven by the biology."

Today, researchers want more than a simple structure. They want to watch molecules in action. They want to learn how proteins work together in molecular assemblies. They want to understand why cellular membrane pores let some things inside but deter others.

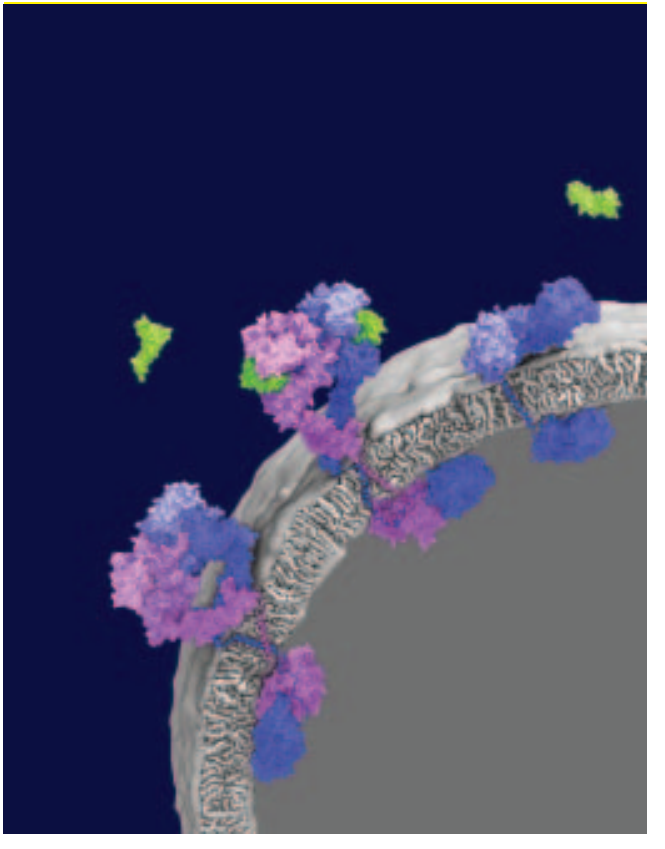
To do so, they routinely tweak and merge structural techniques or combine them with approaches from other fields—cell biology, genetics, and biochemistry—to create tools that can make their wishes come true. They are assembling multidisciplinary teams in their labs, mixing experts in x-ray crystallography, EM, and NMR with biologists, physicists, and computational biologists. They are going across the halls and across the country to initiate collaborations to address a dizzying number of questions—from watching enzymes remodel themselves to matching proteins with their undiscovered binding partners.

A COLLABORATIVE APPROACH

In her lab at Brandeis University, HHMI investigator Dorothee Kern studies the dynamics of enzymes as they catalyze, or speed, chemical reactions along. She then uses the data to create real-time movies of enzymes in action. The perfect technique for following these tiny movements is NMR, which allows molecules to perform their biological functions while they're being visualized at atomic resolution.

One of her more unexpected findings came from human cyclophilin A—the cellular target for the immunosuppressive drug cyclosporin A and the enzyme that is hijacked by HIV. After characterizing cyclophilin's motion during catalysis, Kern discovered that the enzyme has a "dynamic personality." Even when it's not bound to its substrate, cyclophilin A goes through the entire range of motions it uses during catalysis.

To learn more, Kern turned to x-ray crystallography—a tool usually called on to look at static structures—and modified it to also get a sense of structural change. Kern had a hunch that x-ray crystallography data contained overlooked information about a molecule's motions. When a protein forms a crystal, each molecule in the crystal is frozen in a slightly different conformation.



John Kuriyan and his collaborators used a combination of molecular biology, nuclear magnetic resonance, x-ray crystallography, and computational analysis to explore the membrane-bound epidermal growth factor receptor (EGFR). They learned that two molecules of EGFR (purple and pink) come together to form an active receptor.

D. E. Shaw Research

"NOWADAYS, NO ONE WHO DOES STRUCTURAL BIOLOGY STUDIES JUST THE STRUCTURE. IT IS VERY IMPORTANT THAT WE PUT OUR STRUCTURES IN BIOLOGICAL CONTEXT."
→ EVA NOGALES

A scientist's map of the protein's atoms is the average of all the different arrangements. Kern believed one could tease out these minute movements from the data.

She called on a close friend—x-ray crystallographer Tom Alber at the University of California, Berkeley (UC Berkeley)—who developed an algorithm to look more closely at the data. When they used the new program on cyclophilin A, Alber and Kern saw several of the molecule's alternative structures in the electron density data, previously written off as noise. The molecular movements were the same ones she'd seen in solution with NMR, but x-ray crystallography provided high-resolution images. NMR delivered information on the speed of these conformational changes.

Alber and Kern published their results in *Nature* in 2009 and the algorithm has caught on. "I think the whole field has really changed in the last few years," says Kern. "Now we're all talking about conformational ensembles. We can ask how proteins work rather than just look at a molecule's shape."

HHMI investigator Eva Nogales, whose specialty is EM, agrees. "Nowadays no one who does structural biology studies just the structure," she says. "It is very important that we put our structures in biological context."

Much like Alber and Kern, Nogales joined forces with another scientist to learn more about a molecular assembly. Shortly after she arrived at UC Berkeley in 1998, Robert Tjian, then an HHMI investigator at UC Berkeley and now president of HHMI, approached Nogales about collaborating. He had been trying to get a look at the transcription factor II D (TFIID) complex—a sizable assembly of 13 or 14 proteins that jumpstarts gene expression by binding to regions on DNA known as promoters. The complex was proving to be a difficult target for x-ray crystallography. Because TFIID had to be isolated from human cells, it was hard to get enough protein to grow crystals. And the flexible nature of the complex, so essential to its function, was making it difficult to crystallize.

Tjian was hoping that EM would succeed where x-ray crystallography had failed. It did.

Within a year, Nogales and Tjian had published in *Science* the structure of human TFIID in complex with two other transcription factors, TFIIA and TFIIB. Next, Nogales decided to try cryo-EM.

The cryo-EM data, published in *Cell* in January 2013, revealed that the hulking TFIID can reorganize itself. This property is likely essential for its regulation by activators and cofactors. One-third of the multiprotein complex shifts across a large central channel, creating a very different looking structure. With Jim Kadonaga at the University of California, San Diego, Nogales's team combined the cryo-EM data with biochemical analysis to reveal that one conformation of TFIID binds DNA much more tightly than the other.

"Cryo-EM was absolutely essential to visualize this conformational flexibility," says Nogales. "My collaboration with [Tjian] made me realize that my set of experimental tools is ideally suited to study this system."

In March 2013, Nogales published a paper in *Nature* that took the work even further. She created the EM equivalent of a stop-motion movie (see Web Extra animation). The short film shows several transcription factors assembling into what's known as the preinitiation complex—a massive structure that helps position RNA polymerase II on DNA to start gene transcription. Nogales did this by adding the transcription factors sequentially, taking pictures of the growing complex after each addition.

IN-HOUSE EXPERTISE

Instead of collaborating with other lab groups, some HHMI scientists have gathered the structural techniques they need right under their own roof. An x-ray crystallographer by training, Strynadka has added EM, cryo-EM, NMR, and customized mass spectrometry approaches to her own lab. She needs them all to study the membrane protein assemblies she is targeting for antibacterial and vaccine development.

Strynadka is building a detailed picture of the type III secretion apparatus, an impressive needle-like complex bacteria use to inject host cells with a dose of proteins so they can infect the cells. The entire complex consists of more than two dozen different proteins and spans three membranes—two bacterial membranes and the host membrane. She uses cryo-EM to get an outline of the complex's general features. X-ray crystallography and NMR give her the structures of the individual proteins that make up the assembly. Mass spectrometry allows her to see how the structures interact. And computational approaches, such as those developed by HHMI investigator David Baker at the University of Washington, are ultimately used to fit the structures and supporting data into EM-generated silhouettes—like solving a big jigsaw puzzle.

So far, her group has characterized several of the major structural components of the type III secretion system, including a series of rings that act as a molecular lock to support the extracellular needle components—details published in *PLoS Pathogens* in April 2013. She's using her collection of techniques to look at a cluster of a half-dozen membrane-spanning proteins known as the export apparatus, which binds, sorts, and secretes type III specific virulence toxins.

Many of the molecules Strynadka studies are dynamic, multidomain protein complexes that span multiple membranes. These

"I THINK THE WHOLE FIELD HAS REALLY CHANGED IN THE LAST FEW YEARS. WE CAN ASK HOW PROTEINS WORK RATHER THAN JUST LOOK AT A MOLECULE'S SHAPE."
→ DOROTHEE KERN

unwieldy research subjects typically lack stability when out of the membrane, are very flexible, and have water-averse hydrophobic surfaces—all characteristics that make crystallization challenging.

Strynadka tries to circumvent this problem by extracting the proteins in a solution of detergents and lipids that mimic the native membrane environment. "It comes down to trying to understand, by a variety of analyses, what mixture of conditions, including potential binding partners, may make a protein amenable to crystallization."

That mixture can be elusive, however. Especially when it comes to certain membrane-bound receptor tyrosine kinases—enzymes that add phosphate atoms to proteins. Attempts to crystallize these molecules by surrounding them with detergents have met with little success.

HHMI investigator John Kuriyan is a structural biologist who has managed to tackle the problem. In his lab at UC Berkeley, he uses molecular biology to slice proteins into specific regions, or "domains"—an extracellular domain, an intracellular domain, and a transmembrane domain. Then he determines the shapes of the extra- and intracellular domains with x-ray crystallography and visualizes the transmembrane domains with NMR, with the help of his Berkeley colleague David Wemmer.

"Then the problem becomes stitching them together and seeing how they work," says Kuriyan. For this, he collaborates with HHMI investigator Jay Groves, also at Berkeley. By creating mutant versions of the kinases and measuring the effects of these changes on receptor activity in mammalian cells, Kuriyan and Groves have been able to get a grasp of the protein's function.

Kuriyan has used the same approach to learn about a kinase called the epidermal growth factor receptor (EGFR), an enzyme important to cell division that is overactive in many cancers. By analyzing crystal structures of EGFR and carrying out experiments using artificial membranes, he discovered that the receptor is activated by coupling with another EGFR molecule. Recently, he teamed up with computational biochemist David E. Shaw to do computer simulations of EGFR in the membrane. In two papers published January 2013 in *Cell*, they detailed how one EGRF molecule interacts with another to turn it on.

METHOD MASH-UPS

In his lab at Janelia Farm Research Campus, Tamir Gonen applies a powerful but little-used method called electron crystallography to get at the structures of membrane proteins. His results have helped account for observations that other structural biology techniques couldn't make sense of.

Gonen coaxes membrane-embedded proteins to form two-dimensional crystals, which he then exposes to intense electron beams produced by powerful microscopes at Janelia. The result: a protein structure in an actual membrane rather than in detergent.

Using electron crystallography, Gonen produced an exquisitely detailed portrait of aquaporin-0 while working as a postdoc in the lab of HHMI investigator Thomas Walz at Harvard Medical School. The channel protein allows water molecules to pass, one at a time, through the cell membrane. Since then, Gonen, in collaboration with HHMI investigator Stephen Harrison, has visualized the entire membrane around aquaporin-0 and learned about the interactions that occur between lipids and proteins.

Membrane biologists had known that channel proteins

Natalie Strynadka is studying bacterial structure to design better antibiotics and Tamir Gonen is making sense of membrane proteins that allow water or sugar molecules into cells.

Strynadka: Kent Kallberg Gonen: Paul Fetters

interact with their environments in certain ways, such as pairing positively and negatively charged atoms, but they had never seen this process in action. "Once we had the structure of a complete bilayer around a protein, we actually saw these things for the first time," says Gonen. As his next step, he's combining biochemistry, x-ray crystallography, EM, NMR, and computer simulations to learn how the channel is regulated.

Gonen is also using electron crystallography to explore a bacterial membrane protein, galactose permease, that transports sugar into cells. Malfunctions in a human version of the protein have been linked to diabetes, heart disease, and hyper- and hypoglycemia. Gonen and his team have managed to grow two-dimensional crystals of galactose permease embedded in membranes. Next, they aim to produce a detailed structure, which they hope will shed light on the mechanism of how sugar enters a cell.

Christopher Garcia, an HHMI investigator at Stanford University School of Medicine, is also interested in things people haven't seen before. He is combining x-ray crystallography and proteomics—the study of an organism's entire set of proteins—to uncover hidden interactions between receptors and their ligands. He calls the effort his "deorphanization project."

"The vast majority of receptors in any genome are orphans," explains Garcia. "We don't know what their ligands are. And the same is true for secreted proteins. We don't know what their receptors are. So I thought, 'what can I do as a biochemist and a structural biologist to address this problem in a way that others can't?'"

Garcia is taking a pairwise approach to the problem. He and his team are using proteomics to find orphan receptors and orphan-secreted proteins in the fruit fly *Drosophila* and then matching them up to find naturally occurring interactions to explore with x-ray crystallography. So far, they've discovered more than 63 pairs.

Garcia hopes to embark on a genome-wide receptor deorphanization program. While his project is one of the more ambitious structure-based HHMI efforts, the trend is undeniable. Today, very few scientists are pure structural biologists. Instead, they are researchers who use all the tools and expertise at their disposal to find out about biology.

"About 25 years ago when HHMI started the program, the emphasis in all of our labs was determining structures," says Kuriyan. "Now, increasingly the structure determination endeavor is carried out within a larger biological framework. If I had my way, I'd just remove the label 'structural biology.' I would call myself a biologist or a molecular biologist—I prefer to use the broadest label." ■

WEB EXTRA: To read about how HHMI is ensuring its investigators have access to a very powerful x-ray crystallography tool, go to www.hhmi.org/bulletin/spring2013.

COMING THIS SUMMER: Look for a special Web-only article on the future of structural biology in July.

TOOLS OF THE TRADE

X-ray crystallography
In x-ray crystallography, molecules are coaxed to form crystals, which are bombarded with intense beams of x-rays. Each atom in the crystal scatters the x-rays, producing what's called a diffraction pattern. Scientists gather diffraction data from many angles by rotating the crystal in the beam. With help from a high-powered computer, the data are combined with a known protein sequence to produce a three-dimensional map of the molecule.

Electron microscopy
Electron microscopes illuminate samples with electrons, producing high-magnification images. The technology requires only small amounts of sample and is well suited to visualizing larger molecular assemblies. Because the molecules aren't locked into a crystal, it's possible to capture the dynamics of very flexible proteins.

Electron crystallography
In this mash-up of electron microscopy and x-ray crystallography, two-dimensional protein crystals are exposed to an intense beam of electrons from an electron microscope. The resulting diffraction patterns are similar to those produced in x-ray crystallography. The technique is ideal for membrane proteins that cannot easily form large three-dimensional crystals.

Nuclear magnetic resonance
This method exposes molecules to a giant magnet that causes their nuclei to absorb and re-emit electromagnetic radiation. This emitted energy gives clues about each atom's orientation and location in the protein. Because the molecules are free to move about, the technique is useful for watching molecules move and interact.

Cryoelectron microscopy
In this method, samples are plunged into liquid nitrogen and flash frozen before going into the microscope. The freezing preserves a layer of water around the protein, capturing it in a more natural environment than that created by the harsh stains required in regular electron microscopy.

Mass spectrometry
In mass spectrometry, a molecule is sliced into pieces that are then ionized—electrons are added or removed to create charged particles. The fragments are sorted by their mass and charge, and then the fragmentation pattern is compared with predicted patterns for the protein to get an idea of which parts of a molecule are near each other. The technique can also be used to learn how two molecules interact with each other by cross-linking the proteins—permanently binding the molecules together—and studying the resulting fragmentation pattern.