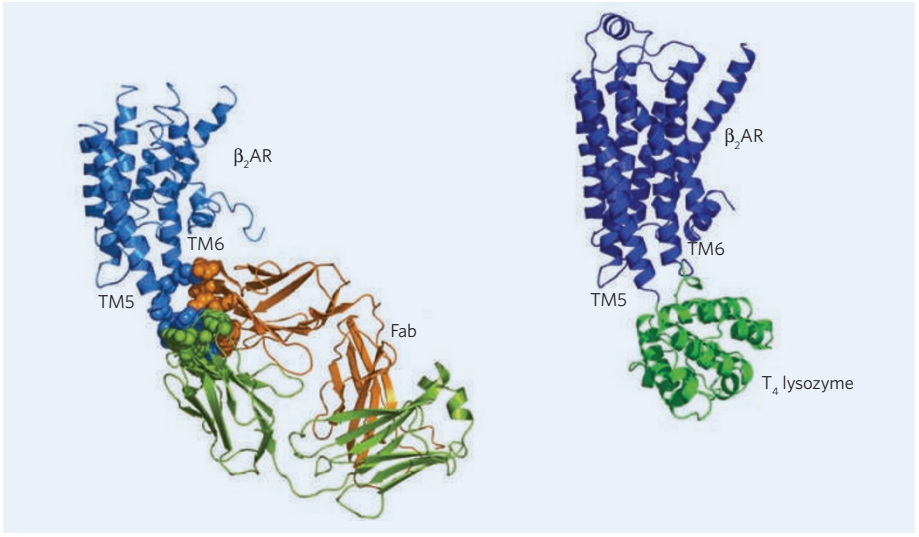
The gatekeepers revealed

Proteins in cell membranes are notoriously hard to crystallize, but new techniques give scientists the means to map them. Monya Baker scouts out the tools for cracking the structure of membrane proteins.

If a cell is a house and the cell membrane its walls, then proteins serve as the doors, windows and electricity and telephone lines. Membrane-bound proteins, anchored within the cell's lipid bilayer, regulate the influx and efflux of molecules and information. How and when these membrane proteins change shape determines essential processes, including whether a drug slows a racing heart, an eye detects light or a virus invades a cell.

Yet scientists studying these proteins often know only the rough outlines of their shapes. Structures have been solved for fewer than 250 membrane proteins, and almost all of these are from microbes such as bacteria and yeast. Of the 7,000 human membrane proteins, researchers have found high-resolution structures for fewer than 12, and each structure captures only one of the protein's many possible forms.

Functional protein assays are important, but are better suited to determining whether a protein performs a certain task than to explaining how or why it does so. Mapped-out structures can give researchers ideas about why a mutation changes a protein's behaviour or help researchers to design drugs. But without a structure, researchers can only speculate about the reasons for drug or mutation effects, says Brian Kobilka, a biochemist at Stanford University in Palo Alto, California. "When you have a structure," he says, "you can begin to understand."



B. KOBILKA

The floppy helices of a G-protein-coupled receptor can be stabilized with an antibody fragment (left, orange) or by the insertion of another protein (right, green).

B. KOBILKA LAB.

Brian Kobilka solves high-resolution crystal structures.

Researchers hoping to solve membrane-protein structures face a cruel paradox: to work on a protein's shape, they must remove it from the cell membrane, destabilizing it and disrupting the conformation. "When you solubilize the protein, you are taking away the belt that holds it together," says Raymond Stevens, a biochemist at the Scripps Research Institute in La Jolla, California. Researchers have tended to avoid this added hassle by focusing on proteins that float free in water.

According to So Iwata, head of the Human Receptor Crystallography Project at the Japan Science and Technology Agency in Kyoto, membrane structural biology is lagging 20–30 years behind the study of soluble proteins. But the field is catching up fast as researchers learn better ways to make, purify and crystallize membrane proteins. The first atomic-resolution crystal structure of a membrane protein, the reaction centre of a photosynthetic bacterium, was published in 1985 (ref. 1). It was important not just for the structure, but as proof that membrane proteins could be crystallized. Still, it was 13 years before crystal structures had been solved for 20 membrane proteins. Techniques have improved: in 2006 alone, 21 proteins were reported, mostly from *Escherichia coli* and other bacteria; this year, that number was surpassed by mid-May (see 'Protein progress').

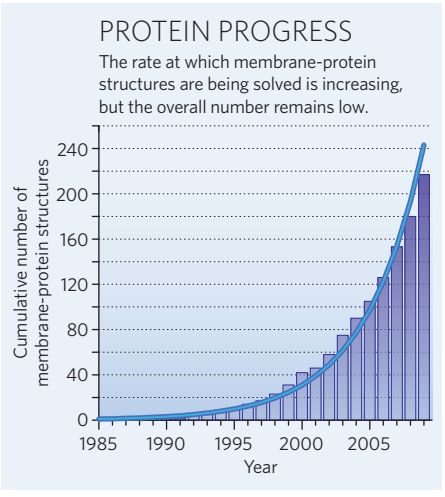
But there's a long way to go. James Bowie, a structural biologist at the University of California, Los Angeles, estimates that representing 90% of structural families would need structures from around 1,700 membrane proteins[2]. His work indicates that most disease-causing mutations are likely to perturb the structure[3], so the number of clinically relevant structures could be much greater.

One sign that crystal structures are easier to solve is that pharmaceutical companies are looking for the structures of membrane proteins to target with drugs. "Before, they wouldn't spend money on it because it was too risky," says Iwata.

Added stability

The largest family of membrane proteins is among the most challenging for biologists. The G-protein-coupled receptors (GPCRs) consist of seven helices that twist and turn through the membrane. Flexible loops extending beyond the lipid bilayer interact with water and the inner helices are normally surrounded by lipids. Ligands that bind these receptors on the outside of the cell membrane cause conformational shifts on the inside that can trigger the cell to respond.

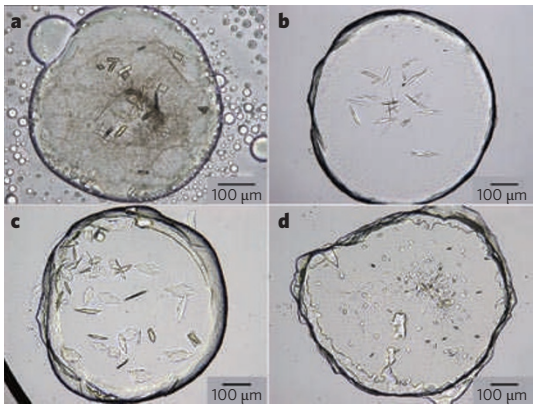
The roughly 800 different GPCRs control pretty much everything that happens in the body — smell, sight, even response to neurotransmitters and immune signals. Many frequently prescribed drugs, from antihistamines to β-blockers, target this class of receptor, and researchers think that crystal structures can help to find more and better drugs more quickly.



SOURCE: S. WHITE, UNIV. CALIFORNIA, IRVINE

The first known GPCR structure, published in low resolution in 1993 (ref. 4) and in high resolution in 2000 (ref. 5), was of the bovine version of rhodopsin, the photoreceptor that enables vision in low light conditions. Rhodopsin, however, is an unusual GPCR because it is particularly stable and is expressed in high enough concentrations to be collected from natural sources — two characteristics not usually associated with this protein family. As such, "rhodopsin didn't tell us how to get structures for other GPCRs", says Kobilka. It wasn't until 2007 that researchers solved the structure of a second GPCR[6] — the human β_2-adrenergic receptor, which is involved in cardiovascular and pulmonary function. Kobilka led a team that designed an antibody to bind two of the protein's helices, stabilizing the receptor and providing a polar surface that helped crystals to form[7]. In other work, Kobilka and the Scripps Research Institute's Stevens solved a high-resolution structure from another crystal, in which the protein was stabilized by replacing an intracellular loop with another protein[6,8].

Researchers are also hunting for compounds that can boost proteins' stability without adding another protein, a strategy that has allowed Stevens to obtain a portrait of another important GPCR, the A_{2A} adenosine receptor[9], which is involved in many physiological processes and is blocked by caffeine. Receptos, a drug development company co-founded by Stevens and based in San Diego, California, used the same method to solve the structure for sphingosine-1-phosphate receptor subtype 1, a drug target for multiple sclerosis. This year, the company announced a clinical drug candidate designed with reference to this structure.



a, adrenoreceptor β_2AR; b, adenosine receptor A_{2A}AR; c, chemokine receptor CXCR4; d, dopamine receptor D_3.

R. STEVENS LAB.

Another tactic is to stabilize GPCRs through targeted mutagenesis rather than through third-party agents such as antibodies and extra proteins. Researchers led by Chris Tate and Gebhard Schertler at the Medical Research Council's Laboratory of Molecular Biology in Cambridge, UK, for example, identified a handful of mutations in GPCRs that boost stability with no apparent effect on function[10]. In 2007, Tate co-founded Heptares Therapeutics in Welwyn Garden City, UK, which uses the stabilized GPCRs, known as StaRs, to inform drug design and has solved several crystal structures of GPCRs with bound ligands.

Sticking with a membrane

Techniques for purifying membrane proteins without denaturing them go beyond tool compounds and engineering. Anatrace in Maumee, Ohio, part of Affymetrix, based in Santa Clara, California, sells detergents and lipids used for solubilizing and stabilizing proteins, including Chobimalt, a water-soluble cholesterol derivative; A8-35, a polymer that wraps itself around the membrane protein; and tripod amphipiles, which limit protein mobility and interactions. In 2007, the company launched three or four new products for membrane proteins; last year, it rolled out twenty. The growing number of publications and tools has brought in scientists who previously restricted themselves to soluble proteins, says Ben Travis, the company's research and development manager. "They're finding the membrane-protein field more accessible," he says.

Perhaps the biggest shift in the field is the ability to accommodate membrane proteins' structural need for fat. "Before, people tried to purify membrane proteins so they didn't have lipids associated with them, but now we know that it probably isn't a good idea," says Stephen White, a biophysicist at the University of California, Irvine.

To address this, many structural biologists have turned to a technique called lipidic cubic phase (LCP) crystallization, also known as *in meso* crystallization. In this technique, proteins are dissolved in lipids to form membrane-like bilayers around water-filled cavities. This mixture feeds lipids and proteins into crystals as they grow. The idea that one could obtain crystals from a protein embedded in a bilayer was "pretty radical", says Bowie, who has developed a variant of the technique using bicelles of lipid and detergent. "A number of protein structures would remain unsolved without the LCP method."

The LCP mixture, however, is incredibly difficult to work with. "You end up with something that looks and feels like very sticky toothpaste," says Martin Caffrey, a biochemist from Trinity

CRYSTAL-CLEAR IMAGES

Getting proteins to form crystals is only one step for the structural biologist. The next step to sleuthing out a protein's structure involves placing the crystals in an intense beam of X-rays. This radiation bears little resemblance to the broad, diffuse X-rays used in medicine: the powerful X-rays that work best for protein crystallography are produced at giant facilities called synchrotrons, of which only a few dozen exist.

At the Advanced Photon Source synchrotron at Argonne National Laboratory, Illinois, for example, electrons race around a 1.1-kilometre track at close to the speed of light. Radiation generated from the electrons is collected into a 70-metre beamline, which focuses X-rays into a 25-square-micrometre area where crystals can be positioned for analysis.

Proteins in the crystal scatter the X-rays as they pass through, and researchers can decipher a protein's structure from the resulting diffraction pattern. To generate a complete pattern, the crystal must be rotated within the beam so that X-rays pass through in different orientations. The process requires precision: researchers have to collect enough data to solve a structure, while limiting radiation damage to the crystal.

Technologies for manipulating crystals and keeping them at temperatures below 0 °C to decrease radiation damage have got better, but the most dramatic improvement is that experiments can now be done using very small crystals or crystals with many poorly diffracting regions, says So Iwata, who heads the Human Receptor Crystallography Project at the Japan Science and Technology Agency in Kyoto. "Crystals that would have been turned away ten years ago are welcome now," he says.

Robert Fischetti matches crystals with X-rays.

ARGONNE NATL LAB.

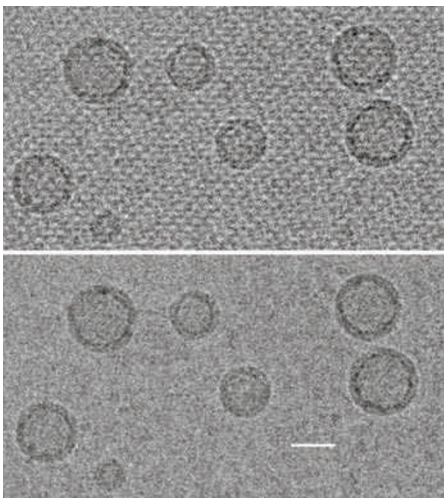
Still, a crystal must be as wide as or wider than the beam passing through it to generate a reliable diffraction pattern. That's a problem, because crystals of membrane proteins tend to be small, says Robert Fischetti, a senior scientist at Argonne National Laboratory, which has produced data for crystal structures of several membrane proteins, including the β_2-adrenergic receptor (S. G. Rasmussen *et al. Nature* 450, 383–387; 2007). Technologies such as lipidic cubic phase crystallization have helped researchers to grow crystals, he says, but these are often only 5–10 micrometres across, a tenth the size of most crystals submitted for analysis and much smaller

College Dublin, Ireland, who is widely credited with popularizing the technique by inventing a way for researchers to homogenize the solutions. His method involves two syringes, one filled with protein, detergent and water, and the other with lipid. The syringes are coupled together so that each injects into the other; researchers mix the contents by pushing the plungers back and forth. Finally, the mixture is placed onto a crystallization plate along with a solution that promotes precipitation. "And then," he says, "you pray for crystals."

Although Caffrey says that researchers with access to a good machine shop should be able to build syringe-coupling devices themselves, Emerald Biosystems, a protein reagents and services firm in Bainbridge Island, Washington, makes a kit consisting of plates, a variety of precipitant solutions formulated for cubic phase and a syringe device prefilled with lipids. Formulatrix in Waltham, Massachusetts, sells crystal-imaging and other technologies that work with the LCP technique.

Late last year, QIAGEN in Hilden, Germany, began offering a product that allows researchers to grow crystals using the LCP method while following many of the protocols for soluble proteins, such as vapour diffusion. The hardest part, says Frank Schäfer, associate director of protein sciences at QIAGEN, was outfitting fluid-handling robots so that they could deal with such viscous material and dispense it into standard crystallization plates. Their success meant that researchers who buy the product don't have to work with the lipids at all. "This has the potential to be used by everybody. There is no special equipment," he says.

Stevens, who switched to using LCP technology about five years ago, after Vadim Cherezov, who had previously worked with Caffrey, joined his lab, welcomes the development of commercial products for the technique, but believes that they need further refinement. "Those kits have not advanced to the stage where it's routine," he says. Researchers familiar with LCP methods don't use the kits, and less-experienced labs have trouble using them. Time and communication should solve that problem, though. The National Institutes of Health Roadmap meeting on membrane-protein technologies this November includes a workshop, organized by Stevens and Cherezov, on LCP crystallization technologies.



F. SIGWORTH

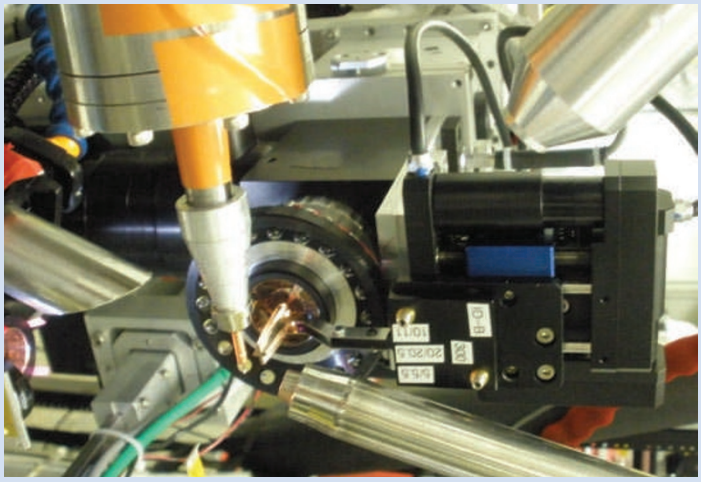
Large membrane complexes can be embedded in liposomes, and then imaged with cryo-electron microscopy to yield a structure. Scale bar, 25 nm.

Computational boosts

Laboratory techniques will continue to improve, but some of the most important advances will happen *in silico*. Computer programs are getting better at filling in gaps from incomplete or ambiguous data sets. In April, for example, Axel Brunger, professor of molecular and cellular physiology at Stanford University, described a technique for improving the accuracy of low-resolution structures[11]. The added precision provided by his software means that researchers can pinpoint specific amino acids, which should help with protein and drug engineering. So far, Brunger has reported using the algorithm only with soluble protein structures, but it should work for membrane proteins too, he says.

But what if you don't have a structure to start with, even a crude one at poor resolution? David Baker at the University of Washington in Seattle is pursuing an approach that builds models of proteins using data that are too sparse to solve any structure at all. His computer program, Rosetta, evaluates possible protein conformations to find the most stable — and hence most likely — shape. Although the number of possibilities makes such calculations impractical for all but the smallest proteins, even sparse structural data can be used to refine the search by excluding improbable conformations. Baker compares his software to a vast team of explorers scouting Earth for the lowest possible point. If data showed that it was not in North America, say, then the explorers could search more effectively by focusing their efforts only on other continents, he says.

Another program, MODELLER, by Andrej Sali at the University of California, San Francisco, is also very popular among structural biologists and modellers. Sali's software uses sequence homology to create three-dimensional best guesses for proteins of unknown structure. These and other computer programs will improve as more protein structures are



ARGONNE NATL LAB.

A collimator produces a 5-micrometre minibeam that allows the study of a tiny crystal, mounted on a copper post.

than the X-ray beam used in crystallography studies.

Researchers led by Fischetti have developed a new version of a collimator, a device that blocks most of the X-rays to produce a 'minibeam' of 5 micrometres or less. Collimators, essentially engineered strips of platinum, are placed about 3 centimetres from the sample and are much more than simple pinhole apertures, says Fischetti. "We started out with a single collimator with three parts — the beam-defining pinhole aperture, the capsule around the pinhole and a forward scatter guard tube."

The first versions of the device caused X-rays to scatter in a way that interfered with the diffraction pattern, but his team has since engineered features, such as a layer of molybdenum, to overcome these problems. They also created double and triple collimators to let users pick the beam size. After one user damaged a collimator by spilling liquid nitrogen on it, they modified the design, eventually making a more robust version with four aperture settings but fewer parts.

What has made the collimator most practical, says Fischetti, is automating the process of selecting the aperture to match the crystal. In the past, technicians had to refocus the beam manually to shrink its size, which took hours. Now, he says, "we can do it in seconds with just clicking".

Besides allowing the study of smaller crystals, smaller beams let researchers identify the parts of the crystal that diffract better. "In the past, if you had a large crystal that was not homogeneous, you'd look at the crystal and say it was bad," says Fischetti. "Now, people can find the region that is the best to look at." M.B.

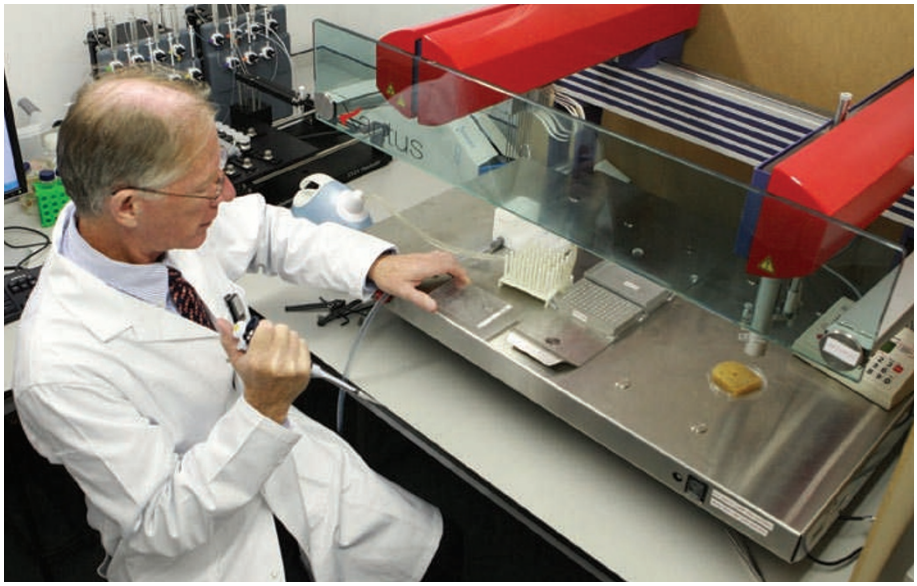
solved — and will in turn allow more structures to be found. They could pave the way for researchers to get high-resolution structures using assorted data sources, including X-ray diffraction (see 'Crystal-clear images'), cryo-electron microscopy (cryoEM) and nuclear magnetic resonance (NMR).

Structure without crystals

The prospect of refining coarse structures through computer modelling entices scientists such as Fred Sigworth at Yale University, New Haven, Connecticut, who studies ion channels using cryoEM, a type of electron microscopy performed at very low temperatures. CryoEM can't match the resolution of classic methods, but it addresses a nagging question: is this how the protein looks in the membrane or does the lack of the lipid bilayer distort it? Instead of extracting proteins and putting them through a crystallization matrix, cryo-EM involves embedding membrane proteins in artificial liposomes, which are then frozen and can yield thousands of pictures. In recent years, robots from companies including FEI in Hillsboro, Oregon, which sells the Vitrobot, and Gatan in Pleasanton, California, which sells the Cryoplunge, have helped to automate liposome preparation and freezing, preserving researchers' time and samples. With the cryoEM photo library, researchers can apply a technique called single-particle reconstruction to sort through all the two-dimensional images and calculate what kind of three-dimensional proteins could have generated them.

Besides falling short of the resolution of most crystal structures, the technique has other drawbacks. For one, it works best on very large, rigid structures, such as ribosomes. Last year, Sigworth used the single-particle technique to solve the structure of a membrane protein — in this case, the human large-conductance calcium- and voltage-activated potassium channel — which, with a molecular mass of 0.5 megadaltons, is small for cryoEM[12].

Other important groups of proteins, such as the GPCRs, are flexible and just a fraction of that size, and thus are not amenable to cryo-EM studies. That hasn't dimmed Sigworth's enthusiasm, particularly for large membrane complexes. "X-ray crystallography is very powerful," he says. "CryoEM has the same kind of potential, it's just 30 years behind. That makes it really fun to be in because new methods are being invented every day."



M. CAFFREY LAB.

Martin Caffrey mixes lipids, detergents and proteins to stabilize crystallizing membrane proteins.

Perhaps the most surprising technique to be applied to membrane structural biology is mass spectrometry. Proteins analysed by mass spectrometry are usually broken down and studied as fragments, but Carol Robinson, a chemist at the University of Oxford, UK, applies the technique to large protein complexes, under controlled conditions that cause subunits to separate from the main complex. In 2008, she and her colleagues showed that the technique could be used to study a membrane-protein transporter complex called $BtuC_2D_2$ (ref. 13), which imports vitamin B12 into the cell. Since then, Robinson has applied mass spectrometry to four more membrane transport complexes, each with differing subunits. She is now applying the technique to larger complexes, with up to 20 subunits.

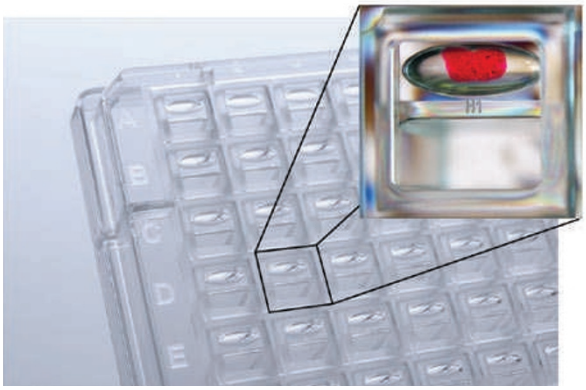
But mass spectrometry, cryoEM and X-ray crystallography share a problem: they cannot show the dynamics of protein movement. "Drugs probably don't work by stabilizing a single state but an ensemble of states, and we need to understand what those are," says Kobilka.

To gain this understanding, many researchers are turning to technologies borrowed, with a lot of tweaking, from studies on soluble proteins. Stevens subjects membrane proteins to a short burst of deuterium, then fragments the proteins and uses mass spectrometry to identify which peptides are most heavily deuterated, and thus are most mobile in solution. Kobilka is using fluorescence quenching to try to learn how far apart bits of the protein are in various drug-induced conformations.

Researchers are also resorting to NMR, in solid state or in solution. The advantage of solid-state NMR is that it can be performed with proteins of any size, in conditions very similar to those of the cell membrane. However, solid-state NMR is technically challenging and expensive. It is often carried out at cold temperatures and so may not show how proteins move in living cells. Solution NMR is more commonly used, but it can follow only small proteins or parts of proteins. These proteins must be encased in protective groups called micelles, which add to the protein's weight and dampen the NMR signal. It does, however, offer the ability to look at different parts of the protein at once and show how they move in response to drugs.

"NMR can really show us the dynamics. We're beginning to appreciate the value of that kind of study," says Kobilka. "It was such a difficult task for so many years that many people were unwilling to undertake it. It isn't such a high-risk prospect any more." In fact, it's possible that the study of membrane-protein movement could follow a similar course to the one that membrane-protein structures are currently on: a series of tiny steps from impossible-to-get to essential-to-have. ■

Monya Baker is technology editor for *Nature* and *Nature Methods*.



QIAGEN

QIAGEN offers a crystallization plate for growing crystals in lipidic cubic phase. The red dye is added for visualization.

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