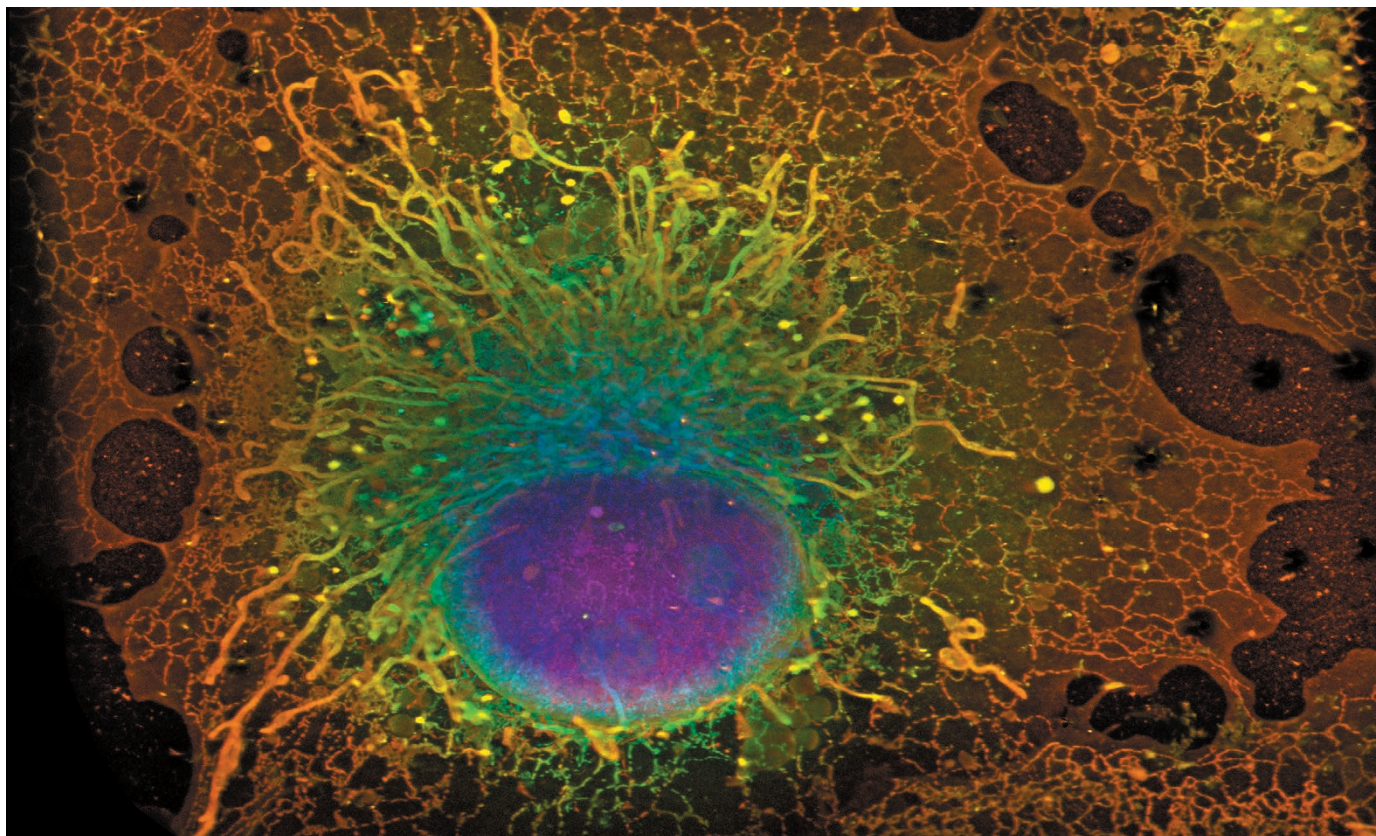


ILLUMINATING LIFE'S BUILDING BLOCKS

A suite of tools now enables scientists to see proteins at work in living cells at the single-molecule level.

WESLEY R. LEGANT



A combination of fluorescent labelling and light-sheet imaging yields a super-resolution view of structures inside a fixed (non-living) cell.

BY MARISSA FESSENDEN

Biophysicist Joerg Bewersdorf says that 2006 was fluorescence microscopy's *annus mirabilis* — a 'miraculous year' as momentous in its own way as 1905, when Albert Einstein revolutionized physics in the realms of relativity, quantum theory and atomic physics. In microscopy's case, the revolution consisted of three papers¹⁻³ that, for the first time, gave scientists the power to peer down into the cell and track the behaviour of individual molecules.

"Every molecule is a machine, a little nano-machine," Bewersdorf says. Proteins, in particular, are complex molecules that twist, flex,

open and shut in a multitude of ways to perform the reactions necessary for cell metabolism and growth, sending messages and providing structure. "That is what we are ultimately interested in understanding," says Bewersdorf: "How do all these little machines work together for the global function of the cell?"

Until scientists could observe that world, however, they had only the cloudiest idea of how to answer that question. Light microscopes were no help; beyond a certain magnification, diffraction causes light waves to spread out instead of converging to form an image. Any features closer together than about 200 nanometres, or about 40 times the width of a typical cell membrane, become a hopeless blur. Images

made using electron microscopy can resolve fine structures — but they are static and almost impossible to obtain from a live cell.

The three laboratories that independently circumvented the 'diffraction barrier' in 2006 adopted a similar strategy: studying the sample with specialized fluorescence probes that can be selectively switched on, a few at a time, until all of the probes are captured in a series of images. Combining the data from those images builds a picture, in a similar way to an impressionist painter building up a scene with dots of colour (see 'Connecting the dots'). The three techniques — photoactivated localization microscopy (PALM)¹, fluorescence PALM (FPALM)² and stochastic optical ►

► reconstruction microscopy (STORM)³ — can differentiate between points just 20 nanometres apart, producing the sharpest-ever fluorescent images at the single-molecule level. Researchers have rushed to take advantage of these capabilities. At Bewersdorf's lab at Yale University in New Haven, Connecticut, for example, he has filmed proteins moving across the surface of living cells⁴.

In the decade since 2006, the three techniques have inspired a wave of technological and methodological innovations. Researchers are designing fluorescent probes that shine brighter and are robust enough to image cellular processes as they unfold. They are also developing methods that cause less disruption to living cells. Several illumination strategies seek to reduce the visual noise caused by background fluorescence, whereas computational methods and strategies are allowing researchers to combine multiple imaging approaches to see molecular interactions in real time.

"The big excitement over these past few years is that these technologies have become doable in living cells," says Jennifer Lippincott-Schwartz, a cell biologist at the Howard Hughes Medical Institute Janelia Research Campus in Ashburn, Virginia. "The time is definitely ripe for being able to image individual proteins using fluorescence."

LASTING LONGER, SHINING STRONGER

All three of the super-resolution microscopy techniques invented in 2006 rely on the light emitted by probe compounds such as green fluorescent protein (GFP), which was first isolated in a bioluminescent jellyfish. The genes for these probes can be inserted into the DNA coding for a cellular protein of interest. Then, when that protein is produced, it will have the fluorescent compound attached, and will reveal its presence by glowing.

But these techniques have some severe limitations. A big one is that many of these probes can emit only a finite number of photons before they are irreversibly damaged by the intensity of the lasers that are used to excite

them into emitting light. Even before this photo-bleaching effect takes hold, the probes are quite dim when imaged individually.

Synthetic versions of these probes known as organic (that is, carbon-containing) dyes are brighter, but they cannot be genetically encoded to their target and manufactured inside the cell. Instead, they are often linked with antibodies that can seek out the protein of interest. However, that combination can make the probes too large to pass through the cell membrane or bulky enough to interfere with a protein's function.

"Probes are really limiting us and really defining to some extent where this technology can go," says Bewersdorf.

"The time is definitely ripe for being able to image individual proteins using fluorescence."

Fortunately, alternatives are emerging. Bewersdorf's group is working with two 'clickable chemistry' probes: SNAP-tag, from New England

Biolabs in Ipswich, Massachusetts, and HaloTag, from Promega in Madison, Wisconsin. These technologies involve a short target sequence that can be encoded into the protein of interest and a dye molecule that clicks into place with its target protein through a simple chemical reaction. Bewersdorf and his colleagues demonstrated that the two tags can be used with organic dyes in living cells to achieve a resolution below 50 nm⁵ — almost as precise as the 20-nm resolution of the original techniques, with the advantage that they combine the specificity and leanness of genetically encoded probes with the brightness of synthetic dyes.

Researchers have also turned to quantum dots, nanoscale semiconductors that are not only bright and stable for a month or more, but can also link to biological molecules. Diane Lidke, a biophysicist at the University of New Mexico in Albuquerque, uses quantum dots in her lab's work on cell signalling. For her, the benefits outweigh the major disadvantage of quantum dots — their size. Commercially available quantum dots are surrounded by a shell that can link the dot to other

molecules but that expands the dot's diameter to 15–25 nm. "They are quite large and bulky," she says, at least when compared with a fluorescent protein, which can be just 4 nm wide.

That pitfall means that researchers have difficulty getting quantum dots into the cell or other tight spaces, but they work well for the kind of extracellular, membrane-bound proteins that Lidke targets. In collaboration with her husband Keith Lidke, a physicist at the same university, she has developed a multiple-colour, fast, single-molecule tracking method that uses quantum dots to produce images on a custom microscope⁶.

Still, much of the cell's internal processes remain locked inside the membrane, difficult to reach with fluorescent probes.

CRACKING OPEN THE CELL

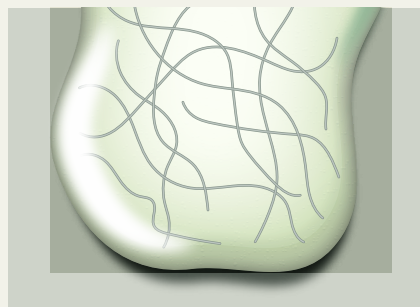
Getting past the cell's membrane is one of the most daunting hurdles that fluorescence microscopy faces. "Even though it is only five nanometres thick, [the cell membrane] has had a few billion years of evolution to separate the inside of the cell from the outside, and it does this amazingly well," says Paul Selvin, a biophysicist at the University of Illinois at Urbana-Champaign.

Selvin's lab has developed its own version of quantum dots that are smaller, closer to 9 nm in diameter⁷. That size reduction helps him to slip quantum dots into the approximately 20–40 nm gap between nerve cells, the synapse, where signalling molecules pass on messages to nearby nerve cells. Once in that cleft, the quantum dots can bind to and advertise the presence of receptors that facilitate memory formation. Selvin hasn't sent these smaller quantum dots inside living cells yet, but he says that this could be possible.

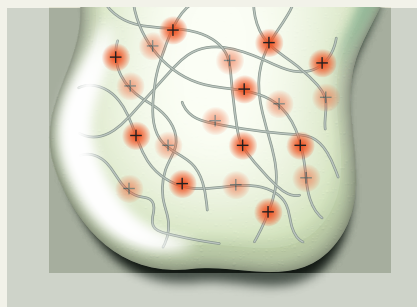
Selvin's lab is also working on a strategy to punch holes in the plasma membrane and then quickly reseal them so that the cell isn't disturbed. "We effectively drill little microscopic pores into the membrane that are about five nanometres," he says, using a bacterial enzyme called streptolysin O. That's just wide enough

CONNECTING THE DOTS

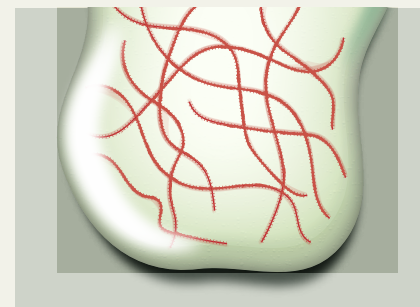
Super-resolution microscopy based on fluorescent probe molecules can reveal cellular structures that are much smaller than the wavelength of light.



The cellular structures of interest, shown here as grey strands, are labelled by fluorescent probes that are invisible until they are illuminated.



Low-intensity laser light repeatedly excites individual probes at random, causing them to emit isolated blobs of fluorescent light (red).



Each probe molecule is mapped to the precise centre of its blob. Over time, these points build up a map of the original structures.

to let a fluorescent protein, even one linked to an antibody, slip inside and find its intracellular target. The method, which Selvin has yet to publish, then patches up those holes within 20 minutes.

Yet there is always the concern that the added probe could interfere with the target protein's typical function. An alternative strategy that doesn't impair the protein comes from Jie Xiao, a biophysicist at Johns Hopkins University in Baltimore, Maryland. Her probe molecules are genetically encoded, but instead of hanging on to the molecule of interest, they are cleaved by an enzyme as soon as they are produced and scurry off to a particular part of the cell membrane. That means that they no longer carry any information about the target molecule's position, but they are in a position where Xiao can count them precisely and thus get an exact tally of the proteins produced, while the proteins themselves are free to go about their business unencumbered. She calls the method co-translational activation by cleavage (CoTrAC)⁸.

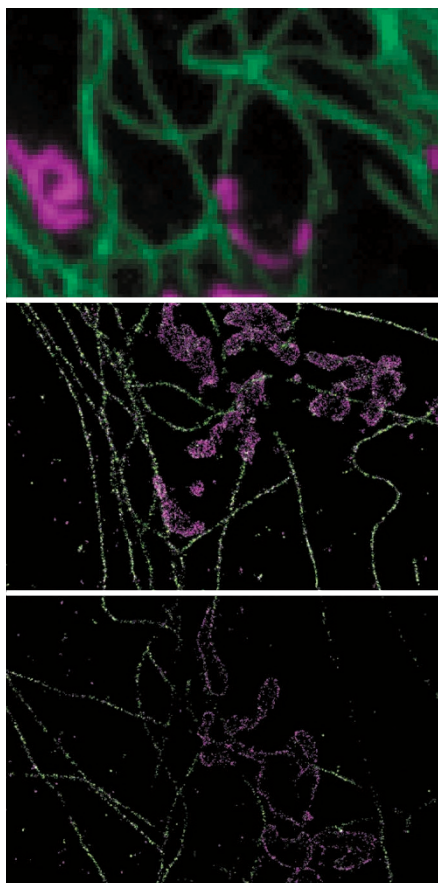
"Being able to quantify the absolute protein level in a living cell is very important," Xiao explains. "Most of the time people use the fluorescence to indicate relative change." However, only a few of the gene-regulation proteins she studies are produced at a time, which makes them difficult to image with most super-resolution techniques. Furthermore, small changes in the exact number of those proteins can determine whether the cell changes states or not. The advantage of CoTrAC is that, by gathering probes for different proteins at different locations on the cell membrane, this technique can be used to count multiple protein products using the same fluorescence molecule and colour. This is a crucial ability when producing different probes can take varying amounts of time and may obscure the timing of cell processes.

LIGHTING THE WAY

Crisper, striking images can come from brighter probes, but another way to make the probes stand out is to reduce the background light. "You have many different planes that you observe simultaneously but you can see only one plane sharply — that in the focus plane of your camera," says Ulrich Kubitschek, a biophysical chemist at the University of Bonn in Germany. "But you still have the diffused background of everything else in the cell."

Non-target proteins can even have their own, natural, dim fluorescence that contributes to this noise. If that background can instead be kept in the dark, the contrast and clarity of images can be enhanced.

Therefore, researchers are constantly improving their illumination strategies. Kubitschek's lab uses light-sheet microscopy to generate a very thin beam of precisely focused light that slices through the sample from the side. "We have a glass chamber that is transparent from below and the sides," he says.



Fluorescently labelled microtubules (green) and mitochondria (magenta) are blurred when seen through a conventional microscope (top). They are sharper when stochastic optical reconstruction microscopy (STORM, middle) is used — and sharper still (bottom) when an advanced version of STORM reveals structures in 3D.

By shining light through the side rather than the top of the sample, his group illuminates a section only 200–300 nm thick and observes the sample from below. In this way, the group has seen RNA molecules as they are exported out of the nucleus through a protein complex called the nuclear pore and into the cytoplasm, where they go on to instruct protein synthesis⁹.

Microscopes equipped to carry out light-sheet imaging are commercially available from companies such as Leica Microsystems in Wetzlar, Germany, and Carl Zeiss in Oberkochen, Germany. Groups with the necessary expertise can even do as Kubitschek's team has done and build their own, custom microscopes.

The next iteration of such selective plane illumination microscopy is called lattice light-sheet microscopy and hails from the lab of Eric Betzig, a physicist at Janelia who also developed PALM microscopy. The technology can generate an illumination plane 300–500 nm thick, says collaborator Zhe Liu, a cell biologist at Janelia — but the real advantage of the method is the structure that the light takes. The lattice forms a three-dimensional grid that moves, illuminating successive sections through the sample.

“You can image for a much longer period of time because the [out-of-focus] molecules are being preserved,” Liu says. Capturing 3D images is also possible. Liu first started using the technology three years ago, while Betzig was still developing it, to examine the organization of protein clusters that are necessary for maintaining a stem cell’s ability to self-renew¹⁰.

Lattice light-sheet microscopy is not yet commercially available, although Carl Zeiss is working on a microscope. Until this goes on sale, groups that are interested in using the technology need to assemble their own custom microscopes. “Alignment is tricky,” Liu cautions, but Betzig and others provide workshops to help those willing to tackle the challenge.

MOTION IN MINIATURE

These probes and illumination strategies can be combined for truly novel insights. “Typically, the field has defined the structures in cells,” says Lippincott-Schwartz. “Now we are getting a handle on the underlying mechanisms that allow these structures to move and interact.”

In her lab at Janelia, Lippincott-Schwartz and her colleagues are leveraging lattice light-sheet microscopy — which she calls a “truly transformative technology” — to look at the way cell organelles and proteins interact. She and her colleagues have watched enzymes repeatedly interact with the endoplasmic

reticulum (ER), a network of membranes in the cell where proteins are synthesized and folded¹¹. Canonically, the ER has been thought of as only a site of protein secretion, but these observations have “really made me start thinking very differently about the primary or major function of this organelle”, she says. The ER might be communicating with other structures more than was previously thought.

This kind of work requires expertise even beyond that needed to align and use the microscopes properly. Lippincott-Schwartz explains that it requires the appropriate algorithms to reconstruct the image from the data acquired by the microscopes. “This is not just something you can pick up,” she says. Typically, labs without substantial experience in super-resolution imaging and the statistical methods it relies on can turn to the specialized knowledge held by experts in the shared imaging facilities that some institutions have. But she also sees a need for standards related to image acquisition and analysis to be set for the field. “Otherwise, there will be information that will be improperly interpreted,” she says.

The complexity of single-molecule imaging means that clean data, controls and proper analysis are invaluable. “If I could recommend something to people entering the field,” says Antoine Triller, a neurobiologist at the Institute of Biology of the École Normale Supérieure in

Paris who studies the movement of molecules in the synapse, “it would be either to have a good background in statistical physics or to work with people with a very good knowledge in the field.”

Making this effort is worth it, however, to gain access to the ‘black box’ of molecule-scale life occupying the space between classic light microscopy at the microscale and electron microscopy at the nanoscale. Single-molecule approaches in living cells offer a way to find new biological parameters to measure and observe, Triller says. “These parameters will allow us to develop new theoretical fields and a new understanding of living matter.” ■

Marissa Fessenden is a freelance writer in Bozeman, Montana.

1. Betzig, E. *et al.* *Science* **313**, 1642–1645 (2006).
2. Hess, S. T., Girirajan, T. P. K. & Mason, M. D. *Biophys. J.* **91**, 4258–4272 (2006).
3. Rust, M. J., Bates, M. & Zhuang, X. *Nature Methods* **3**, 793–796 (2006).
4. Juette, M. F. & Bewersdorf, J. *Nano Lett.* **10**, 4657–4663 (2010).
5. Bottanelli, F. *et al.* *Nature Commun.* **7**, 10778 (2016).
6. Cutler, P. J. *et al.* *PLoS ONE* **8**, e64320 (2013).
7. Ma, L. *et al.* *J. Am. Chem. Soc.* **138**, 3382–3394 (2016).
8. Hensel, Z., Fang, X. & Xiao, J. *J. Vis. Exp.* **73**, e50042 (2013).
9. Spille, J.-H. *et al.* *Nucleic Acids Res.* **43**, e14 (2015).
10. Liu, Z. *et al.* *eLife* **3**, e04236 (2014).
11. Sengupta, P. *et al.* *Proc. Natl Acad. Sci. USA* **112**, E6752–E6761 (2015).