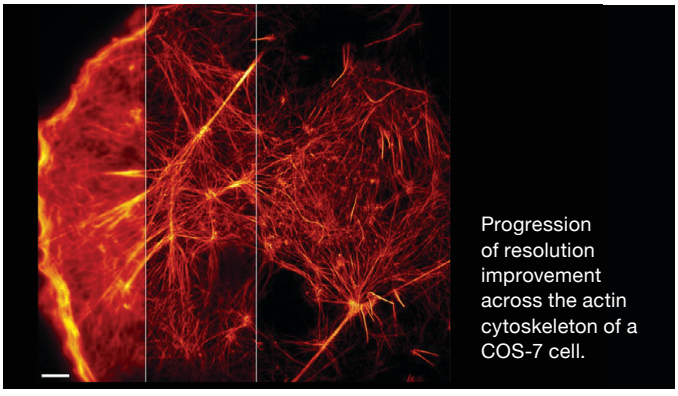

Progression of resolution improvement across the actin cytoskeleton of a COS-7 cell.

Superresolution microscopy

From van Leeuwenhoek to the new millennium, microscopy was governed by one seemingly unbreakable principle: The ability to resolve two objects is constrained by the wavelength of the light used to view them. But in 2000, researchers showed this so-called diffraction limit could be broken, unveiling over the next decade an alphabet soup of superresolution techniques from GSDIM and PALM to SIM, STED, and STORM. The resulting images are both beautiful and revealing, documenting biological phenomena and structures that researchers never even knew they were missing. **By Jeffrey M. Perkel**

No, there hasn't been a breakdown in copy editing, this sentence really does end with two periods.. Your brain caught that apparent mistake because the lenses in your eyes have sufficient resolving power to distinguish objects spaced that close together. Microscopes, of course, can resolve much finer details, but not infinitely fine. The so-called Abbe (or diffraction) limit constrains the microscope's power, and at some point, two objects will appear as one.

In 2006, Xiaowei Zhuang's team at **Harvard University** first described the superresolution technique known as stochastic optical reconstruction microscopy (STORM), a single-molecule localization method whose 20-nm lateral (*xy*) resolution beats the diffraction limit by a full order of magnitude.

Over the following decade, Zhuang's team steadily refined the technique to make it multicolor, higher resolution, and 3D.

"Almost all biological structures are three-dimensional," says Zhuang, a Howard Hughes Medical Institute Investigator and the David B. Arnold Jr. Professor of Science at Harvard. "So if you break your diffraction limit in only [the] *xy* [plane] and not in *z*, you're quite limited."

To overcome that limitation, Zhuang's team inserted a cylindrical lens in the microscope's optical path. In that configuration, light arising from a single molecule produces not a circle but an ellipse, which indicates the molecule's axial position with 50-nm resolution. Later, the lab boosted resolution still further by positioning objectives above and below the sample, which when combined with the cylindrical lens yielded axial positions with better than 20-nm resolution.

When her team applied that technique to fixed rat hippocampal neurons, they discovered something completely unexpected, Zhuang says. Under conventional fluorescence microscopy, actin in the axons of these neurons appears as a uniform, featureless green. But when they used STORM to zoom in, just beneath the plasma membrane of the axonal shaft they discovered a literal cytoskeleton: periodic rings of green actin 180 nm apart, separated by magenta spectrin spacers (see page 851).

"It's a beautiful structure," Zhuang says, providing both mechanical flexibility and organizational scaffolding. With it, the cell can precisely position key membrane proteins, such as ion channels, to ensure the efficient propagation of neural impulses. "Having these very important signaling molecules being anchored on the structure gives you the possibility that this is the case," she says, adding, "It was really one of those STORM images that I'm most proud of."

Published in 2013, Zhuang's study is "a seminal contribution," agrees Joerg Bewersdorf, Associate Professor of Cell Biology and Biomedical Engineering at the **Yale University School of Medicine**, who builds superresolution microscopes (including one now commercialized by **Bruker**) in his lab. "Nobody had seen these actin rings before, even though people had looked with the electron microscope for 20 years to understand the actin organization in axons."

Superresolution explained

According to Professor Stefan Hell of the **Max Planck Institute for Biophysical Chemistry**, who was the first to break the diffraction barrier in 2000, all superresolution methods rely on the same fundamental principle.

"If you have freely propagating light, that light will always be diffracted. You can't focus it more sharply than to an area that is about half the wavelength of light across. Full stop," Hell explains. As a result, objects that are closer together than about 200 nm will be illuminated simultaneously and fluoresce in concert. Long story short: multiple objects bleed together into a single, indistinguishable blur. "The solution to that problem was to make sure that not all objects that are flooded with excitation light are in the end capable of emitting fluorescence."

STORM, photoactivated localization microscopy (PALM), and ground state depletion microscopy with individual molecule return (GSDIM)—commercialized by **Nikon**, **Zeiss**, and **Leica**, respectively—are stochastic, localization-based methods, using photoactivatable or photoswitchable fluorescent proteins or dyes to switch on a few fluorophores at random while the majority remain dark. Under those conditions, researchers can be relatively certain that each fluorescent point corresponds to only a single active molecule, thus making it possible to assign their positions with nanometer precision. Those fluorophores then turn off, another handful turns on, and the cycle repeats until a complete dataset has been collected.

"Basically we take advantage of what I typically call the 'fourth dimension,'" explains Zhuang. If multiple, otherwise

IMAGE: L. DONG *ET AL.*, *SCIENCE* **349**, 944 (2015).

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indistinguishable objects are closely positioned in space, she says, "we don't allow them to overlap in time."

Stimulated emission depletion (STED), reversible saturable optical fluorescence transitions (RESOLFT), and structured illumination microscopy (SIM), conversely, use "patterns of light … that determine where the molecules are active and where the molecules are off," says Hell, who developed both STED and RESOLFT and cofounded a company called **Abberior** to commercialize fluorophores for those and other superresolution methods. STED, for instance, is usually implemented as a point-scanning approach that uses a "donut" of light, superimposed on the excitation beam, to force fluorophores at the periphery of the illumination volume to stay dark via a process called "stimulated emission," thereby shrinking the emitting region to the donut's center. (RESOLFT uses photoswitchable fluorophores to achieve the same effect.)

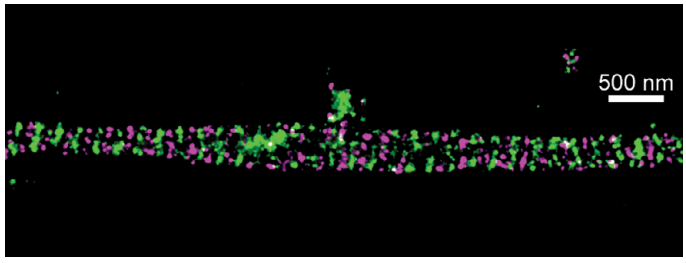
According to Jochen Sieber, STED and GSDIM microscopy product manager at Leica Microsystems, localization systems typically offer sharper resolution than STED or RESOLFT, but take far longer as they require the acquisition and merging of thousands of frames to build a single image. (Indeed, **National Institutes of Health** Distinguished Investigator Jennifer Lippincott-Schwartz, in whose lab PALM was invented, notes that those first images took hours to collect; today the typical frame rate is about 5 minutes.)

SIM—commercialized by Nikon, **GE Healthcare Life Sciences**, and Zeiss—is generally regarded as the fastest approach available and also the easiest, as it requires no special sample preparation. But at 100 nm, it also yields the poorest resolution.

Recently, **Janelia Research Campus** Investigator Eric Betzig, who with Hell and William E. Moerner of Stanford University won the 2014 Nobel Prize in Chemistry for his work on superresolution, demonstrated that even that limit is not insurmountable. In late 2015, Betzig and his team documented two strategies, including "nonlinear SIM with patterned activation," to increase the method's resolution to about 45 nm. "My personal feeling is that SIM is the superresolution method that is likely to answer the most biological questions in the next decade," Betzig says, laughing. "And yet it's the one that wasn't involved in the Nobel Prize."

Raman Das, Medical Research Council (MRC) Career Development Fellow at the **University of Manchester**, United Kingdom, answered one such question in 2014 when he used SIM (among other methods) to document a new form of cell subdivision called "apical abscission." Das was interested in how progenitor cells in the embryonic spinal cord differentiate into neurons and exit the tissue. By using time-lapse diffraction-limited fluorescence microscopy and SIM, Das's team discovered that the cells actually prune off the cellular "tip," removing those proteins in one shot—a process that Das likens to closing a draw-string purse. "This results in an acute loss of cell polarity in these cells," he explains, leading to the formation of functional neuronal architecture.

Perhaps the simplest, and certainly least expensive, superresolution option is expansion microscopy. Developed by Ed Boyden of the **Massachusetts Institute of Technology**, the technique improves imaging resolution by encasing samples in a "swellable polymer" gel that causes the sample to grow isotropically about fivefold larger. Functionally, that's the same as increasing resolution from 200 nm down to 40 nm. "It's kind of like an optical zoom," explains Silvio Rizzoli, a professor at the **University of Göttingen**, Germany, who uses this technique (as well as STED and STORM) in his neural synapse research.



When [Zhuang's team] used STORM to zoom in, just beneath the plasma membrane of the axonal shaft they discovered a literal cytoskeleton: periodic rings of green actin 180 nm apart, separated by magenta spectrin spacers.

IMAGE: XU *ET AL.*, *SCIENCE* **339**, 452 (2013).

Fluorophore on, fluorophore off

Stefan Jakobs, head of the Mitochondrial Structure and Dynamics Research Group at the Max Planck Institute for Biophysical Chemistry, uses superresolution methodologies to study the role mitochondria play in cell death or apoptosis. One protein, called Bax, was known to induce mitochondrial membrane disruption in response to apoptotic signals. What wasn't clear was how the protein does that.

Using fixed-cell STED, Jakobs's team showed that multiple Bax molecules assemble into rings in the mitochondrial outer membrane, like diaphanous green halos crowning the red membrane. That structure explains how Bax translocation during apoptosis can cause the mitochondrial contents to spill, Jakobs says. "It appears that these rings represent pores," he explains. Yet they were impossible to discern via conventional microscopy, in which the rings appear as "a smear."

To see those rings, Jakobs used anti-Bax antibodies labeled with a fluorescent dye. Yet good fluorescent probes for superresolution microscopy can be hard to come by. "You want to have fast image acquisition and high resolution; you want to have many colors and low phototoxicity. None of the techniques is currently at the point where all of these parameters are entirely fulfilled," he says.

Jakobs has developed reversibly switchable fluorescent proteins (RSFPs)—variants of green fluorescent proteins (GFPs), such as rsEGFP2—that can be pushed repeatedly from a fluorescent to nonfluorescent state and back again using specific wavelengths of light. Such proteins are amenable to RESOLFT and STED live-cell applications, he notes, as they require no external antibody for labeling. But it is critical not to overexpress these proteins, he says, as that can lead to artifacts. So, Jakobs's team has used the gene-editing system known as CRISPR/Cas9 to couple rsEGFP2 to protein-coding genes in order to ensure endogenous expression levels. Such work is "challenging," he admits, "but I think it is very important for the field that we do that."

Another genetic labeling option involves tagging genes with self-labeling protein tags, such as the SNAP-tag and CLIP-tag (available from **New England BioLabs**) or **Promega**'s cont.>

Featured Participants

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www.abberior.com

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www.epfl.ch

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www.gelifesciences.com

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www.harvard.edu

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www.icfo.eu

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King's College London
www.kcl.ac.uk

Leica
www.leica-microsystems.com

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www.mit.edu

Max Planck Institute for Biophysical Chemistry
www.mpibpc.mpg.de/en

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www.neb.com

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www.nih.gov

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www.nikoninstruments.com

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University of Göttingen
www.uni-goettingen.de/en

University of Manchester
www.manchester.ac.uk

Yale University School of Medicine
medicine.yale.edu

Zeiss
www.zeiss.com

HaloTag, which allow researchers to specifically label proteins of interest in live cells by adding cell-permeable dyes linked to the appropriate substrate. The trick, of course, is that such dyes must be both bright and cell-permeable, and few current fluorophores meet that standard, says Kai Johnsson, professor at the Institute of Chemical Sciences and Engineering at **École Polytechnique Fédérale de Lausanne** in Switzerland.

Janelia Research Campus researcher Luke Lavis recently reported a collection of cell-permeable, high-quantum-efficiency rhodamine variants, which are compatible with HaloTag chemistry. Johnsson has likewise developed rhodamine variants that he calls "SiR dyes" (carboxylated silicon-rhodamine) that are commercialized by **Spirochrome**.

By coupling these dyes to targeting ligands such as the DNA fluorescent stain Hoechst or the cancer drug taxol, Johnsson created probes capable of specifically lighting up genetic material and microtubules respectively, in live-cell STED and SIM (but not PALM or STORM).

In living color

Because of the extended time required to compile images in localization superresolution microscopy, the method can be largely incompatible with live cells.

One solution is replacing traditional charge-coupled devices (CCDs) with high-speed scientific complementary metal-oxide semiconductor (sCMOS) cameras, which can capture thousands of frames per second. In 2013, Bewersdorf and colleagues demonstrated noise-handling algorithms that allowed an sCMOS camera to record superresolved images at up to 32 frames per second, for instance. Today, Leica's GSDIM instrument includes an sCMOS camera that reduces data acquisition times to "well below a minute," says product manager Peter Laskey, thus putting live-cell applications "within reach." So too does Nikon's new N-STORM 4.0, which also boasts a brighter laser to "get the blinking on-off rates extremely high," according to general manager Stephen Ross.

Researchers also have devised alternative strategies to circumvent the speed problem. Melike Lakadamyali, a group leader at the **Institute of Photonic Sciences** in Barcelona, Spain, for instance, studies microtubule vesicle trafficking, specifically how the motor proteins transporting these vesicles can "evade roadblocks and traffic jams in the crowded environment of a living cell."

To answer that question, Lakadamyali plays to her system's strengths. By recording a live-cell movie of the vesicles at high temporal (but low spatial) resolution, her team can track vesicle motion, overlaying those trajectories on a STORM image of microtubule positions.

The researchers found that vesicles tend to pause at microtubule intersections; the pausing "correlates with tight intersections in which the separation of the two microtubules is small," says Lakadamyali. But they also found that motors can eventually navigate through the intersection. The group is now using 3D particle tracking to focus on microtubule-associated protofilaments that may provide a kind of highway shoulder for skirting roadblocks.

Susan Cox, Royal Society University Research Fellow at **King's College London**, is similarly interested in live-cell localization microscopy. Working with Lippincott-Schwartz, Cox has developed an algorithm, called "Bayesian analysis of blinking and bleaching (3B)," that allows researchers to image more fluorophores simultaneously, even some that are actually spatially overlapping, thereby reducing the number of images required per experiment by about an order of magnitude. "We've looked at systems like cardiac myocytes, [and] been able to image them between the 'beats' of the cells," Cox says.

Still, for all that high resolution can teach, microscopy is about trade-offs. In talks, Betzig says he typically displays a tetrahedron whose axes represent different goals: resolution, speed, low phototoxicity, and imaging depth. "You can't have it all," he tells the audience. For Betzig, speed is paramount, and he focuses his efforts on squeezing every last photon from his samples. That's why he has edged away from pure superresolution toward ultrahigh-speed imaging with a new (albeit superresolution-compatible) design called "lattice light-sheet microscopy," soon to be commercialized by Zeiss and **3i**.

Others may interpret the tetrahedron differently, of course. But the bottom line is this: When it comes to microscopy, the future has never looked brighter—or sharper.

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