Super-resolution microscopy: breaking the limits

After a long period of measured development and a recent surge of technical advances driven by physicists, super-resolution fluorescence microscopy emerged in 2008 as a powerful tool for biologists. Kelly Rae Chi reports.

In the summer of 2005, cell biologist Jennifer Lippincott-Schwartz tore down the red lights in her darkroom at the National Institutes of Health in Bethesda, Maryland. Lippincott-Schwartz was making room to host then-unemployed physicists Eric Betzig and Harald Hess. They were working on photoactivation localization microscopy (PALM), a new technology they hoped could dramatically increase the resolving power of fluorescence imaging and be used to see nanometer-scale biology.

Betzig, Hess, and Lippincott-Schwartz's group worked in the tiny room through that winter, wearing their coats and hats in the unheated room and collecting data. Hess admits he and Betzig, now both at the Howard Hughes Medical Institute's Janelia Farm Research Campus in Ashburn, Virginia, didn't know much about biology. For 15 years, they had been thinking about high-resolution imaging. When they learned about photoactivatable green fluorescent protein—which was invented by Lippincott-Schwartz and George Patterson in 2002—they saw it as the missing link in their quest to improve imaging resolution.

"They were so excited," Lippincott-Schwartz remembers. "And I remember the first images. It was hard to know what we were looking at." It was not until she saw the fluorescence images laid over an electron micrograph that Lippincott-Schwartz believed that their method worked. "I thought, 'This is really right.' It was really, truly amazing."

Super-resolution fluorescence microscopy has, in the last few years, hit its stride and allowed researchers to see cellular processes unfolding at nanometer scales. The days of interpreting fuzzy blobs in the 200 to 750 nm size range are over. Though the ideas behind super-resolution microscopy were born and raised in academic institutions starting in the late 1980s, the last few years have seen an explosion of technology, along with the start of commercialization. And now, with dozens of labs setting up their own instruments and tweaking their samples, the most exciting moment, especially for biologists like Lippincott-Schwartz, is seeing it work.

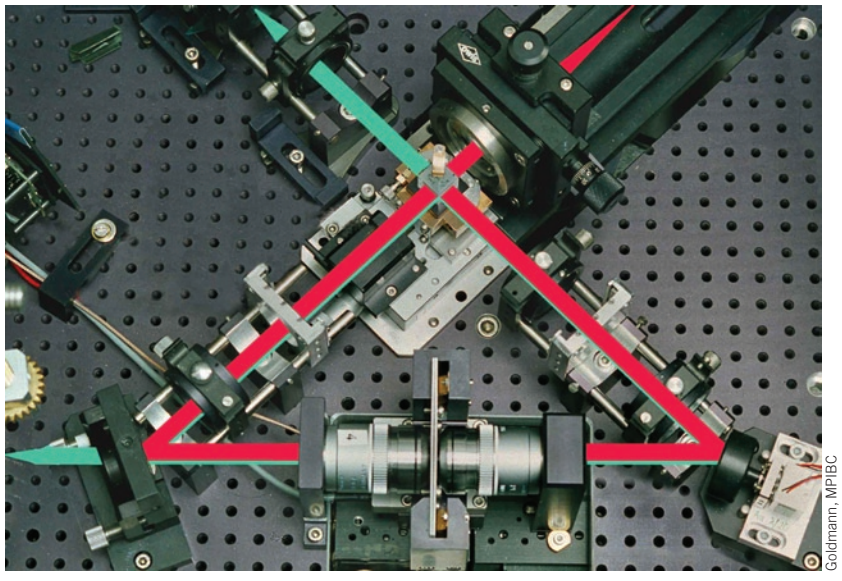
Galonska, MPI BPC

Stefan Hell.

Stefan Hell breaks the barrier

Since 1873, when Ernst Abbe first proposed the rule of diffraction-limited imaging, researchers had believed that the ability to resolve two nearby points was fundamentally limited by the wavelength of light. More than a century later, Stefan Hell, now director of the Max Planck Institute of Biophysical Chemistry in Göttingen, Germany, was the first to show, theoretically and experimentally, that one can use a light microscope to resolve objects on the nanometer scale, below the diffraction limit.

As a graduate student in the mid-1980s, working at the University of Heidelberg in Germany with a low-temperature solid state physicist as his adviser, Hell first realized that it would be possible to improve resolution by having not just a single lens focus the light onto a point but by having two



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The 4Pi microscope, a step along the way to super-resolution imaging.

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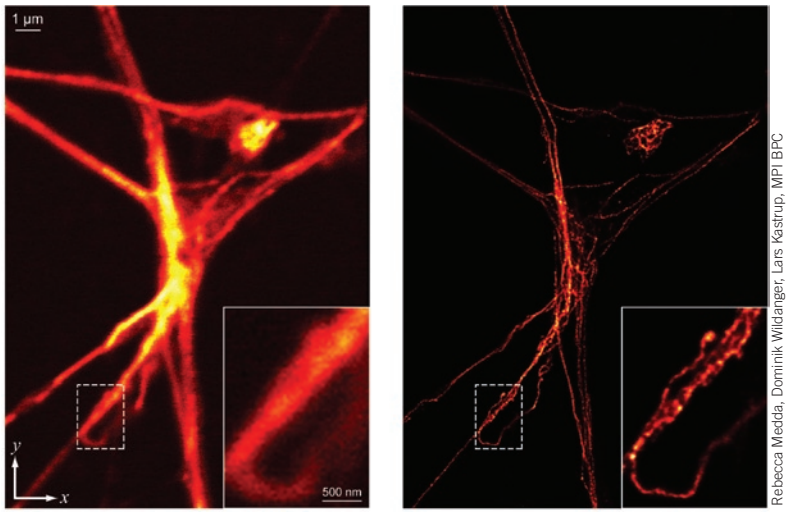


A neuron imaged by confocal microscopy (left) and by STED (right).

large-aperture lenses jointly doing so. Working on his own at home, his fellowship having expired after he completed his PhD thesis in 1990, he devised the 4Pi microscope based on this idea. "I thought, 'How would you realize the idea [of high resolution] using two opposing lenses?'" he recalls. "I conceived that, and I laid it out on paper."

Hell needed a place to show that the principles worked. So, his idea in hand, he went to the European Molecular Biology Laboratory (EMBL) in Heidelberg, where he began work in 1991, supported by a postdoctoral fellowship from the German Science Foundation.

In those early days, many researchers, including prominent physicists, thought that Hell wouldn't get very far in improving resolution. With what little financial independence he had, Hell was taking a risk. But he was tied to the idea of breaking the diffraction barrier. "I stayed in science just because I wanted to improve the spatial resolution," he says.

Sunney Xie, now a professor of chemistry at Harvard University in Cambridge, Massachusetts, met Hell in the 1990s and saw some of his early talks on high-resolution 4Pi microscopy. "He was just so original," Xie says. "He was not afraid to challenge conventional wisdom when he set out to do what he believed in."

Hell's risk-taking paid off. In 1992, he showed for the first time that the 4Pi microscope could improve resolution to three to seven times that of a conventional microscope. But although it improved resolution along the *z* axis, it still did not overcome the limiting role of diffraction.

At his next postdoctoral stint, at the University of Turku in Finland, Hell had an idea one Saturday morning while sitting on his bed in a student dorm and reading a book on the quantum theory of light. He thought that, with the right lasers, he could fluorescently activate a spot and then shrink that spot by depleting the emission in a doughnut-shaped area surrounding it. He would later call the imaging method STED, or stimulated emission depletion. "I instantly went into the lab to make those assessments," he recalls. "That was one of the most exciting moments in my career."

At Turku, "Stefan was working very long days," recalls long-time colleague Pekka Hänninen, who first worked with Hell on the 4Pi microscope while they were both at EMBL. Hell says many thought he was obsessed. But Hänninen remembers lighter moments, too: as the group worked into the evenings, the sounds of Hell's saxophone would sometimes echo through the corridors of the new building.

In 1994, Hell published the theory of STED in *Optics Letters*. But it took several years to show that it worked in practice, a period of Hell's career marked by incremental but necessary steps. During that time, Hell ran out of money and, to continue his work, sold the rights to his 4Pi microscope patent.

But Abbe's rule still loomed large at the time. Many physicists were resistant to Hell's ideas and were focused on other imaging methods. Despite this, in 1997, Hell was offered a five-year contract at the Max Planck Institute for Biophysical Chemistry to show that STED works. In 1999, he sent the resulting manuscript to *Nature* and *Science*. Both rejected it. "They were asking me, 'What kind of new biology have you done?' They didn't see that this could change microscopy," he says. But in 2000, *PNAS* published the data, in which STED was used to produce the first truly nanoscale fluorescence images, and Hell obtained a tenured position at the Institute in 2002, where he continues to develop and apply new imaging methods.

Since this pioneering work, a number of super-resolution techniques have been developed in academic labs. Researchers led by Mats Gustafsson, a physicist and engineer now at Janelia Farm, developed structured-illumination microscopy, or SIM. The idea behind this technology is to illuminate the sample with a series of light patterns that can make an otherwise unresolvable fine structure visible in the form of low-resolution moiré fringes. The information in the fringes can then be extracted through computer processing and used to generate a high-resolution image. In 2000, imaging the actin cytoskeleton in HeLa cells, Gustafsson showed a twofold improvement in the lateral resolution compared with that of a conventional microscope. His group later improved resolution by a total factor of four by exploiting nonlinear effects.

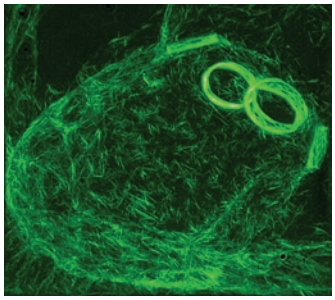
The dash to develop

In 2006, opening a new chapter in the development of the field, three groups, including that of Betzig and Lippincott-Schwartz, simultaneously reported that they had increased resolution using yet another approach.

Samuel Hess's group was one of the three. An assistant professor of physics at the University of Maine, Hess had been talking during the summer of 2005 with chemical and biological engineers at his university about how he might improve resolution to see lipid rafts in live cells.

Then, one night that summer, he awakened to the sounds of a loud party his neighbors were throwing. Half asleep, he went downstairs and sketched a possible setup of lenses and lasers that would allow one to image cells using photoactivatable proteins. "I figured I'll have a good laugh about this in the morning," he recalls. But the next morning, he couldn't find anything wrong with the idea, so he took it around to his colleagues in the physics department. "They couldn't find a problem with it either," he says.

So Hess began working on the microscope, though he was nearing the end of his start-up funding and would need time and



Hesper Rego, Margot Quinlan, Mats Gustafsson

3D SIM image of actin in a fruit fly oocyte, imaged in the intact egg chamber

money to make it work. After assembling the microscope, and having been derailed by the rupture of a steam pipe in the lab, Hess and his colleagues were rushing to acquire data for fear that they would be scooped. In less than two days, researchers from the university's Laboratory for Surface Science and Technology prepared a sapphire crystal that the team could use to demonstrate that the imaging worked. "For them to do it so quickly and go out of their way to help us—it was priceless," Hess says. His group published the data in 2006 in the *Biophysical Journal*, calling the technology fluorescence photoactivation localization microscopy, or FPALM. In 2007, the group showed that FPALM could be used to detect protein clustering in lipid rafts.

Meanwhile, the lab of Xiaowei Zhuang, a Howard Hughes Medical Investigator at Harvard University, was also working on super-resolution imaging. The idea underlying the methods of all three groups—to harness the ability to image single-molecule emitters and compile images of thousands to millions of them to achieve super resolution—was incredibly simple. "We were always wondering whether other people might think of it too," Zhuang says.

In early 2004, Zhuang and her colleagues had made the serendipitous discovery that certain cyanine dyes are photoswitchable; that is, they can be activated to a fluorescent state and deactivated to a dark state at will, by using light of different colors. Since then, Zhuang, who had been developing ways to do single-molecule imaging for years, has worked with two students on these photoswitchable probes, using them to temporally separate the spatially overlapping images of individual molecules and thus to boost resolution. The same basic concept underlies both Eric Betzig's and Samuel Hess's approaches as well, except that they rely on activation and permanent bleaching of photoactivatable probes rather than on photoswitching.

In 2006 Zhuang's group published the idea in *Nature Methods*, calling their approach stochastic optical reconstruction microscopy (STORM) and using it to see DNA and DNA-protein complexes with a 20-nm resolution. At the same time, Lippincott-Schwartz, Betzig, and Harald Hess published their PALM method in *Science*, using it to image cellular focal adhesions as well as proteins in specific organelles.

Seeing it work

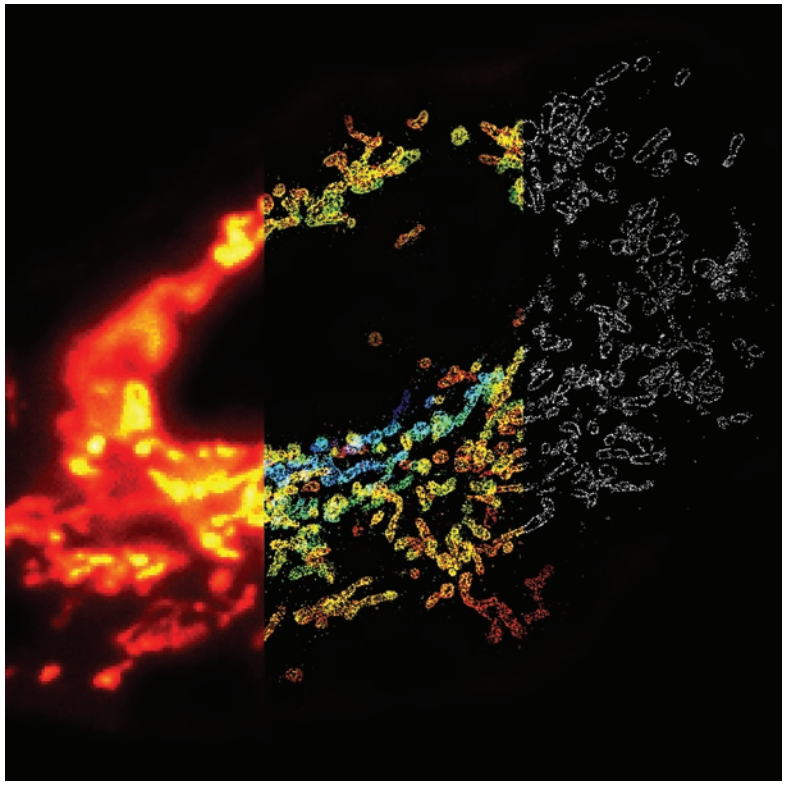
The enthusiasm is palpable among biologists who are beginning to implement super-resolution imaging. Jan Liphardt, a biophysicist at the Lawrence Berkeley National Laboratory, remembers first reading about PALM in Betzig's 2006 *Science* paper. When he saw images of labeled mitochondrial proteins, he immediately thought of how it would apply to his own work on microbes.

"Normally when you would take a fluorescence image of *E[scherichia] coli*, you would have about 20 pixels of information, probably even fewer," Liphardt says. "Now suddenly here was an image containing not 20 pixels, but many thousands."

Liphardt is collaborating with Betzig and others to study chemotaxis in *E. coli*. "The one thing I really never imagined would be this ability of seeing and counting and localizing individual proteins in single cells, comprehensively," he says. "For me, so many of the things I wonder about have to do with which proteins are exactly where, when. There are many indirect approaches for determining that, but for me this has probably been the most thought-provoking technical advance I've seen in a very long time."

Also collaborating with Betzig's group, a group led by Nicholas Barry, an assistant professor of medicine at the University of Colorado Denver, outfitted a commercial Zeiss microscope designed for total internal reflection fluorescence (TIRF) imaging to study how proteins cluster on the apical membranes of kidney cells.

With Zeiss's system and an average computer in hand, the assembly of extra parts was relatively simple, Barry says. They added two lasers at an estimated cost of $30,000. They can assemble an image, using about ten molecules per frame, at 10,000 frames in about eight minutes. The file size is about a third of a gigabyte. They wrote their own software using Perl, a free programming language. "There's a lot of background information [in the single molecule literature]

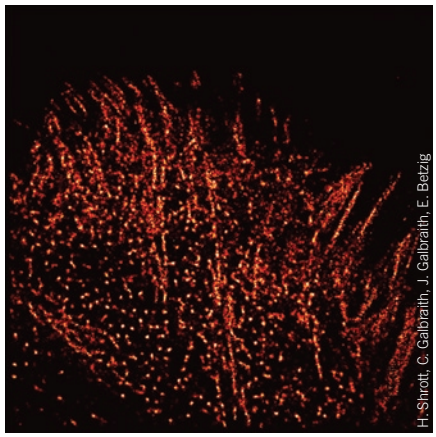


Xiaowei Zhuang

Mitochondrial network in a mammalian cell visualized by 3D STORM. Conventional fluorescence image (left), 3D STORM image with colors denoting *z* location (middle) and single *xy* cross-section from the 3D STORM image (right).

you can use to help calculate a PALM image," he says. Barry bets someone will soon write an algorithm to run as a plug-in in ImageJ, a free image analysis program from the National Institutes of Health. "It's almost certain," he says.

W.E. Moerner, a professor of chemistry and applied physics at Stanford University in Palo Alto, California, who in 1989 performed the first experiment to see a single molecule optically, says the field of super-resolution imaging has undergone remarkable evolution over the years, now topped off by the use of single molecules as nanoscale emitters. "This is a beautiful development, after nearly 20 years of work on single molecules," he says.



Adhesion complexes imaged by PALM in a mammalian cell.

2008 and beyond

Since STORM and PALM came out in 2006, developers have been working feverishly on improvements and new applications. The year 2008 was no exception. Lippincott-Schwartz's group combined PALM with single-particle tracking to detect the movement of membrane proteins in live cells. In *Science*, Zhuang's group demonstrated 3D STORM imaging, with a spatial resolution ten times better than the diffraction limit in all three dimensions, and used the method to image microtubules and other molecular structures within monkey kidney cells, later extending the method to multicolor 3D imaging of whole cells. Gustafsson and others reported using 3D SIM—a method that uses three beams of interfering light instead of two—to see parts of mammalian cell nuclei that go undetected using confocal microscopes. Zeiss, headquartered in Germany, is further developing the SIM and PALM (renamed PAL-M) technologies, and expects to release complete imaging systems by the end of 2009.

Hell's group, early in 2008, used the STED method to show the movement of synaptic vesicles inside living neurons at video rate, the proteins of interest tagged with antibodies. Later in the year, they combined 4Pi and STED methods to produce 3D images of mitochondria in fixed cells, at a resolution of 40 to 50 nm in all dimensions. Most recently, they applied super-resolution imaging to study morphological plasticity in brain slices and reconstructed a 3D image of dendritic spines in a living neuron.

With the 3D and live cell capabilities, the field has exploded in the last few years, Gustafsson says. "Now it is very obvious, with the advent of PALM and STORM, et cetera, and the recent progress, that these techniques will make a lot of things possible that weren't possible," he says.

Though many scientists stand to benefit from the technology, there's room for improving its accessibility. Many labs that have successfully implemented the technique so far have just the right skill set: researchers well versed in physics and biology, who can put together the microscope and get it to work with biological samples. Moerner points out that writing software is not a trivial matter, either: localizing and reporting the photons that you detect requires a careful calculation to define the final resolution.

Sheer cost can limit the accessibility as well. With Leica's TCS STED microscope topping $1 million, it remains a challenge to receive funding, says Bill Betz, director of the light microscopy facility at the University of Colorado, Denver. Betz applied for federal funds for the microscope but was turned down. "It's new and expensive. And in today's world, administrators are trying to spread the money around," he says, adding that they plan to reapply for funding. However, Stefan Hell points out that new laser developments make it possible for researchers to build a STED setup in their own labs for less than $100,000.

Besides getting the methods into the hands of biologists, developers say they are aiming for a broader and more diverse range of fluorescent probes. Better probes will ultimately translate into better resolution and faster image processing. "To work with live mammalian cells, you have to have a whole panel of photoactivatable and photoswitchable proteins," says Vladislav Verkhusha, an associate professor of anatomy and structural biology at the Albert Einstein College of Medicine in New York, whose own work in fluorescent protein development has been accelerated by PALM.

Xiaowei Zhuang, in one of many directions her group is taking, is working with Alice Ting and her lab at the Massachusetts Institute of Technology in Cambridge to develop labeling strategies that allow small and bright photoswitchable probes to be attached to specific proteins in cells, and thus to enable live cell imaging. "A combination of the genetic labeling approach with the small, bright and photoswitchable probes will be ideal for future super-resolution imaging with molecular-scale resolution," she says.

Though developers will continue to work on improvements, the new year will mark the beginning of more widespread use of super-resolution fluorescence microscopy methods, says Harald Hess. "It really goes off into [biologists'] hands," he says. "At the same time, we keep asking them, 'What is the magic killer experiment?' There are a lot of good killer answers."

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