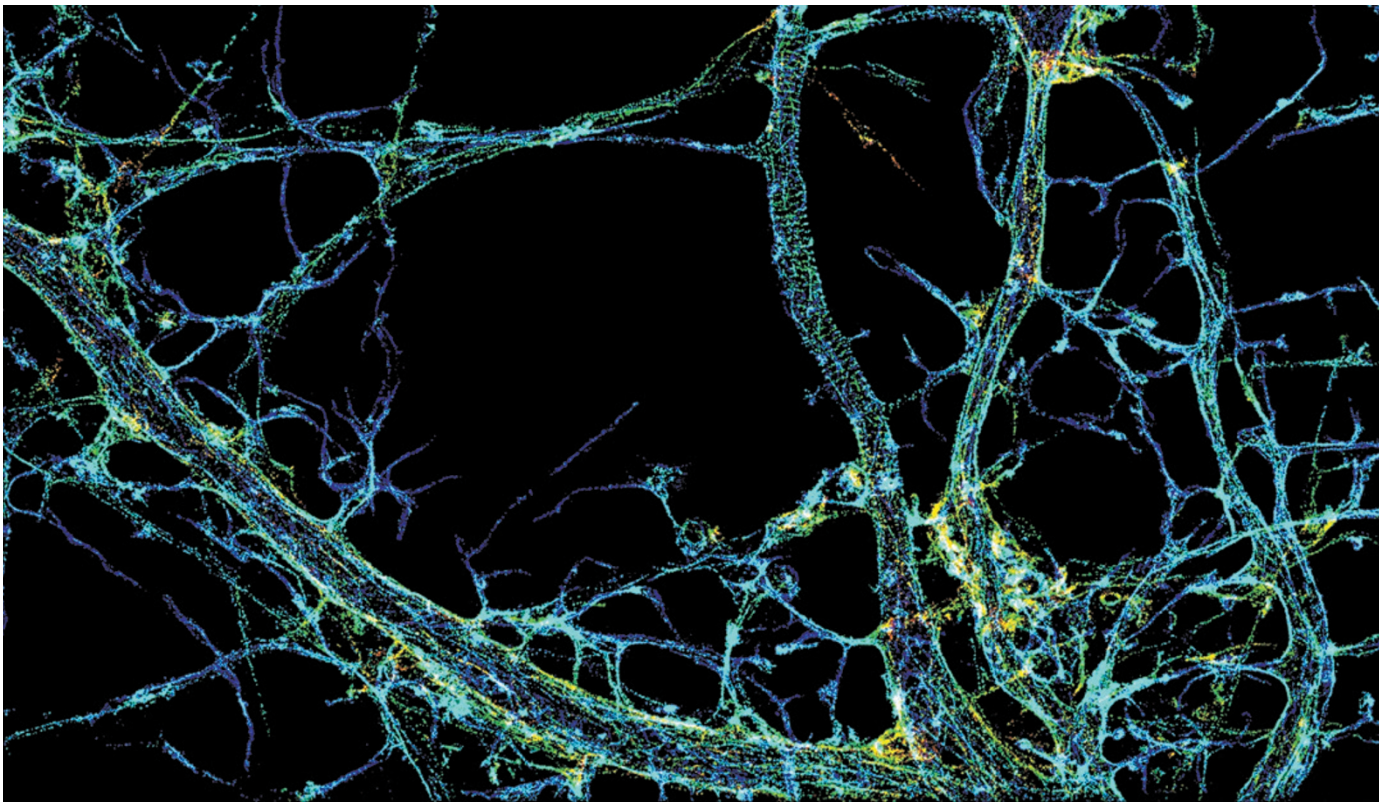
TECHNOLOGY FEATURE

# SUPER-RESOLVE ME: FROM MICRO TO NANO

*Nanoscopes capable of super-resolution offer scientists intricate views of a world beyond the limits of conventional microscopes — but not every technique fits all imaging needs.*

ZHUANG LAB/HHMI/HARVARD UNIV.



**The cytoskeleton in axons was first revealed using super-resolution stochastic optical reconstruction microscopy (STORM) microscopy.**

**BY MICHAEL EISENSTEIN**

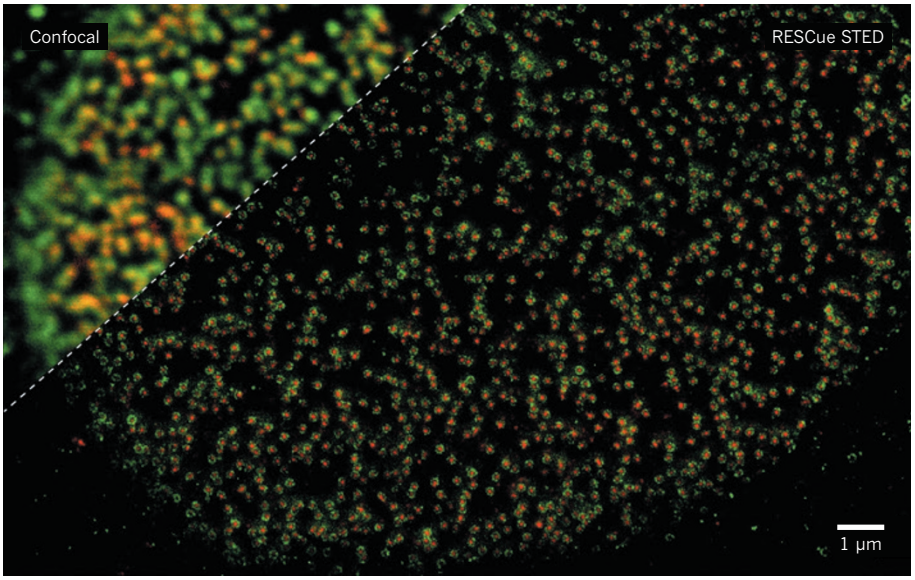
A variety of innovative — even Nobel-prizewinning — approaches to fluorescence microscopy are opening a window for biologists to marvel at structures and processes at the molecular level. Using 'super-resolution' microscopy, life scientists can witness events such as vesicles shuttling loads through the cell membrane or proteins clumping together in Alzheimer's disease — processes that were previously invisible to the human eye.

But scientists who contemplate replicating such dramatic images in their own labs need to anticipate the technical challenges. "A lot of people look at papers that expert labs put out, and everything is gorgeous," says Christine Labno, technical director at the University of Chicago's Light Microscopy Core Facility in Illinois. Careful thought must go into selecting the right technique for the scientific question at hand. "There's quite a bit of education that we have to do about how these techniques actually work."

The good news is that universities and institutes have started to incorporate super-resolution microscopy, or nanoscopy, into shared imaging facilities. And with highly experienced researchers available to offer their wisdom, life scientists have been jumping in. "Our earliest adopters were in neurobiology, looking at synapses that are only about 30 nanometers across," says Labno. "But we've also had immunologists and cytoskeleton researchers eager to start — so it's a pretty diverse bunch."

Super-resolution microscopy tore down a natural wall that, for more than a century, was thought to be impossible to climb. In 1873, German physicist Ernst Abbe described a 'diffraction barrier'. He predicted that because of the fundamental properties of light, optical microscopes would not be able to discriminate individual features separated by fewer than 200 nanometres. In practice, when two details that are closer together light up at the same time, the sample would appear as a blur.

Then came pioneering work in the late 1990s by scientists who would go on to share the 2014 Nobel Prize in Chemistry for devising strategies that broke Abbe's diffraction barrier: Stefan Hell of the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany; Eric ▶




C. WURM/ABBERIOR INSTR. GMBH

**Nuclear-pore complexes on the nuclear membrane seen using conventional and RESCue STED.**

Betzig at the Janelia Research Campus in Ashburn, Virginia; and William Moerner of Stanford University in California.

### MICROSCOPE MATCH-UP

Today, researchers have a suite of super-resolution technologies at their disposal, all with the power to observe molecular-scale details well below the Abbe limit. Neurophysiologist Silvio Rizzoli at the Göttingen Graduate School for Neuroscience, Biophysics and Molecular Biosciences uses a technique called stimulated emission depletion (STED), invented by Hell and his colleagues[1], to study synaptic-vesicle function in nerve terminals. "The best resolution that we get routinely with STED is around 30 nanometres," he says.

STED is relatively simple for experienced fluorescence-microscope users. The method is based on the same principles as a standard confocal instrument, but instead of illuminating the sample with a single light source, it uses two. One beam is set at a wavelength that excites the fluorophores — the fluorescent tags — that are used by researchers to localize and visualize proteins; the other uses a different wavelength that suppresses fluorescence. This beam is doughnut-shaped and overlaps with the first beam, so that only molecules in the central 'doughnut hole' continue to fluoresce.

Obtaining a STED super-resolved image is not very complicated. "You have to play a bit with the parameters to see something nice, but otherwise, you look at it the same way as you would with a confocal," says Jochen Sieber, product manager for super-resolution technologies at Leica Microsystems in Wetzlar, Germany, which manufactures microscopes.

Other super-resolution methods rely on the ability to switch fluorescent labels 'on' and 'off' in a controlled fashion. These 'probe-based' (also known as 'localization-based') techniques carefully tune lighting conditions to ensure that only a few, sparsely distributed individual fluorophores are visible at any given time.

The best known of these methods are photoactivated localization microscopy (PALM), developed by Betzig and his colleagues[2], and stochastic optical reconstruction microscopy (STORM), devised by Xiaowei Zhuang's group[3] at Harvard University in Cambridge, Massachusetts. All fluorescent labels start in a dark state. They are then excited using a controlled pulse of laser light that switches on a tiny fraction of the tags, followed by a second pulse that switches them off again. The process is repeated over and over to generate a series of partial fluorescence images that can be reconstructed into a whole.

With these techniques, cell biologists can achieve remarkable spatial resolution in fixed samples, down to single-molecule imaging. "I trust these approaches for anything below 20-nanometre resolution," says Rizzoli.

But interpreting images at this scale requires a careful labelling strategy to avoid introducing artefacts — inaccurate imaging data that arise from sample staining or processing methods and distort the true structure of the specimen (see 'The antibody problem').

## The antibody problem

It is easy to forget, when performing immunofluorescence on a tissue sample, that fluorescent labels are tethered to their targets with a massive protein intermediary — an antibody. This becomes problematic at ultrahigh resolution: antibodies protrude at distances that disrupt image precision. "How can you resolve a 30-nanometre distance in a sample when the error from labelling is 10–15 nanometres?" asks Helge Ewers, a cell biologist at the Free University of Berlin.

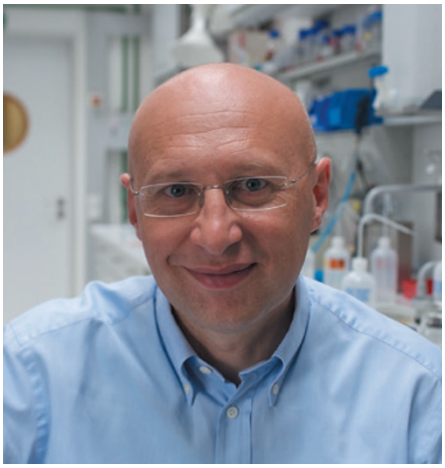
Many labelling protocols exacerbate the problem by using two antibodies — the first recognizes the target, and then a labelled secondary antibody attaches to the first. According to Silvio Rizzoli at the Göttingen Graduate School for Neuroscience, Biophysics and Molecular Biosciences in Germany, this strategy, combined with poor sample preparation, undermined years of STED experiments, revealing apparent patterns of molecular clustering that were actually just cross-linked clumps of antibodies.

To avoid using antibodies, biologists can opt for genetically encoded fluorescent proteins as a label, but these are dimmer and less robust than chemical dyes. As an alternative, Ewers and his colleagues have used dye-tagged 'nanobodies' — camel-derived single-chain antibodies that are roughly one-tenth the size of conventional ones — to reliably label proteins fused to green fluorescent protein (GFP)[9]. "They're chemically defined, they're small and you can produce them in bacteria — I think nanobodies are a tool of the future," says Ewers. His team has since developed more nanobodies for multicolour labelling, and is working with genome-editing strategies to tag endogenous proteins rather than forcing overexpression of cloned genes.

Another alternative is to use 'click-chemistry' approaches, such as SNAP-Tag from New England Biolabs in Ipswich, Massachusetts, or HaloTag from Promega in Madison, Wisconsin. These methods use a simple reaction that forms a permanent link between a short protein tag and a chemically modified dye molecule. "They're useful, because you can choose a membrane-permeable chemical dye and do live-cell experiments," says Rizzoli, "but they do have some problems with non-specific binding."

Yet this requires genetic modification, and so may not be amenable to human tissue samples. Fortunately, there are ways to get reasonable super-resolution images with minimal artefacts — for example, Rizzoli recommends using a fragment for the second antibody to retain the specificity of conventional antibodies with less bulk. M.E.

On the up side, adding PALM or STORM capabilities onto an existing fluorescence microscope is relatively straightforward. Life scientists can also purchase commercial instruments that are designed for probe-based super-resolution, such as the ELYRA from Carl Zeiss Microscopy in Jena, Germany, and the N-STORM from Nikon Instruments in Melville, New York. Amateur users should prepare to invest time in their experimental design (see 'A good way to dye'). "Whenever we start a new project, we spend a lot of effort on optimizing labelling," Zhuang says. "I think ironing this part out takes researchers the most time."



B. SCHULLER/MPI BIOPHYS. CHEM.

**Stefan Hell won a shared Nobel prize for his work.**

## NANO YOUTUBE

Most super-resolution imaging concentrates on fixed cells, but the great promise of nanoscopy is the ability to image dynamic processes in living cells. Cell biologists want to capture molecules and structures as they assemble, adhere and interact. But collecting super-resolution images in real time has a few trade-offs. In probe-based techniques, for example, briefer illumination times mean that fewer fluorophores become activated each round — resulting in an image with much-reduced detail. One way around a weak signal is to use stronger illumination, although pumping too much light into cells can create toxic compounds that jeopardize the sample's viability.

Some researchers are finding that structured-illumination microscopy (SIM), a technique pioneered[4] by Mats Gustafsson at Janelia Farm, offers a good compromise for live-cell super-resolution imaging. "In a good SIM experiment, we're probably down to 100–120-nanometre lateral resolution," says biochemist Jordan Raff at the University of Oxford, UK, who uses SIM to study the structures that help to coordinate cell division.

SIM illuminates the sample with patterned lines of light, which generates fluorescent images that override Abbe's constraints. The method requires little sample preparation and is flexible in terms of fluorophore selection. Like PALM and STORM, SIM is easy to build onto an existing instrument, or users can opt for commercial systems such as the DeltaVision OMX SR from GE Healthcare Life Sciences.

Daniel Davis, an immunologist at the University of Manchester, UK, has found SIM useful for studying how secretory granules move along actin in natural killer cells engaging in an immune response. But Davis also points out that "it doesn't quite get you down to the resolution you can get with other techniques".

STED microscopes can also be adapted for live-cell imaging, despite their reputation for causing severe damage with the intense beams needed for high-resolution pictures. To reduce bleaching, Hell and his team devised a light-limiting technique called RESCue[5], which is available using the STED microscopes made by Abberior Instruments, a company co-founded by Hell and based in Göttingen, Germany. "In some samples, it can reduce light load down to 4% or 5% of traditional STED."

Even PALM and STORM systems, which require comparatively time-consuming collection and reconstruction of multiple images that are produced from individual molecular labels, have ramped up their speeds dramatically to capture data from live cells. Scientists have shrunk the time needed for image collection by using brighter fluorescent labels, and a new generation of detectors can harvest image data from larger numbers of pixels more rapidly than was possible with early methods.

Optical biophysicist Joerg Bewersdorf and his group at Yale University in New Haven, Connecticut, found[6] that combining such detectors with robust image-analysis enabled them to record the movement of proteins on the surface of living cells at up to 32 frames per second — essentially producing super-resolution videos.

Leica offers a probe-based approach that is founded on the principle of ground-state depletion (GSD). This method uses light to force all but a handful of individual fluorophores into an inactive dark state. Live-cell imaging, however, is out of the question. "Building up enough fluorescence events to get a nice-looking image can take 15–20 minutes," says Labno. This is much too long to track a dynamic process.

## LIFE IN 3D

Super-resolution microscopy can also satisfy researchers who crave 3D images. Leica's STED instrument uses two depletion beams, one perpendicular to the other, to generate a 3D-super-resolved zone in the specimen. Alternatively, Abberior's microscopes use a device called a spatial light modulator to achieve an equivalent effect with a single beam.

For probe-based methods, introducing depth measurements is straightforward. Zhuang's group found[7] that adding a cylindrical lens to the light path transforms STORM's light spots into elliptical shapes that can be mapped in 3D. This approach, used in Nikon's N-STORM, can yield a depth ('axial') resolution of 50 nanometres without altering lateral resolution. Zhuang's

team has achieved still further improvements in axial resolution with an iteration of STORM that resolves all three dimensions at 10 nanometres[8].

Imaging success depends on more than just the instruments: the sample itself is also an important consideration. Tissue specimens are especially hard to image because they are dense and tend to scatter photons, generating blurry images and high levels of background fluorescence. As a result, image quality is best near the sample's surface and worsens as the microscope probes deeper into thick samples. It may be possible to overcome this hurdle by using chemical 'clearing' techniques that render tissues transparent.

For now, most researchers find that the simplest solution is to embed fixed samples in plastic, and then sequentially image thin slices shaved off the top. "We're trying to understand the relationship of one synapse with different postsynaptic partners, which requires us to look at thousands of synapses in tissue at high-resolution in parallel," says Bernardo Sabatini, a neurobiologist at Harvard Medical School. "I think that in the short term, this approach plus super-resolution will give you that data quickly."

## PICTURE PERFECT

Even a perfectly executed super-resolution study generally needs some sort of computational processing to produce a high-quality image. For scientists who prefer the simplicity of positioning a sample under a microscope and having the image instantly appear on a computer screen, STED might be best because it generally does not require image processing.

Some scientists use deconvolution tools to sharpen images and eliminate blur, but Hell avoids this whenever possible. "Raw data may not look as fancy, but it's honest, and you know what it means," he says. "For most other techniques, software processing is mandatory." And Davis says of SIM, "You're creating a mathematical model of what the cell looks like based on the fluorescence data. You're not literally seeing it."

Raff notes that many of his early experiences with SIM entailed recognizing that pretty pictures can be deceiving because image-processing algorithms can create artefacts that look every bit as real as the cellular structure of interest. "But if you have people who know what to look for, they can examine the image and tell if something is dodgy," he says.

For PALM and STORM, image-building is like a game of 'join the dots'. The higher the density of the labels, the easier it is for the software to connect those dots, leading to better images. But high density can also cause confusion, by generating overlapping signals that look like single dots — so clever use of powerful image-processing algorithms is essential to make sense of the data.

Given that most super-resolution techniques can be incorporated into existing microscopes, many researchers will probably try their hand at super-resolution imaging in the near future. "In my view, it doesn't make sense for a facility that routinely uses confocal microscopy not to have STED attached to it," says Hell. "You can just stop the STED beam and still have a confocal system."

Those with experience in nanoscopy are helping to train others. Zhuang's team at Harvard University, for example, offers routine STORM workshops. "We go from sample preparation to analysing images with our software," she says. "It's always oversubscribed."

That said, most biologists are still best served by using these instruments in core facilities that provide access to specialists who are familiar with several methods. "As biologists, we're still far away from understanding the physics — and some of us never will," says Raff. "Your best bet is to try multiple different techniques out on your sample in an environment where there are people around who understand it." ■

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## A good way to dye

Microscopist Stefan Hell at the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany, thinks that all super-resolution methods boil down to one crucial element: "The dye is essential," he says. The ideal fluorophore has an extremely bright 'on' state and very dark 'off' state, and the capacity to switch between the two both rapidly and repeatedly.

For live-cell imaging, many researchers prefer to work with genetically encoded fluorescent proteins. Stimulated emission depletion (STED) and structured illumination microscopy (SIM) are highly compatible with standard fluorophores such as green fluorescent protein (GFP). Stochastic optical reconstruction microscopy (STORM) and photoactivation localization microscopy (PALM) need photoswitchable dyes; proteins such as Dendra2 or EosFP, which undergo a laser-induced colour transition, are popular choices.

But fluorescent proteins generally compromise resolution. "They're just too dim," says neurophysiologist Silvio Rizzoli at the Gottingen Graduate School for Neuroscience, Biophysics and Molecular Biosciences in Germany. In STED, "you're taking the laser power and genetic overexpression to the maximum to get a signal".

Organic dyes are a brighter alternative. They tend to be more durable under prolonged illumination. However, they must be linked to another molecule to achieve targeted labelling, and many fluorescent dyes cannot penetrate living cells. For this reason, many researchers still focus on fixed samples. "We'd rather go for the extreme in resolution, and we try to squeeze every single photon out so that we can localize things very accurately," says cell biologist Helge Ewers at the Free University of Berlin. A handful of high-performance dyes can be used with live cells, such as the silicon–rhodamine dyes from the Swiss bioimaging company SpiroChrome, which generate bright-red fluorescence once bound to cytoskeletal proteins.

Things get tricky when one aims to image many targets simultaneously using multicoloured labelling: because each fluorophore responds to a distinct 'on' and 'off' wavelength, researchers may run out of bandwidth to achieve specific detection of more than two or three tags. In principle, probe-based methods can accommodate more labels than STED, but they are also more finicky in terms of experimental conditions. "People often come to us with a combination they want to use, but the dyes have exact opposite needs in terms of buffers," says Christine Labno, technical director of the University of Chicago's Light Microscopy Core Facility in Illinois. Sequential-labelling strategies may offer a more efficient option for conducting larger-scale protein-mapping experiments. For example, a technique known as DNA-PAINT uses DNA tags to selectively conjugate a single dye to different antibodies, enabling stepwise labelling of ten or more protein targets in one super-resolution image. M.E.

## CORRECTION

The Technology Feature 'The cell menagerie: human immune profiling' (*Nature* **525,** 409–411; 2015) misstated the location and research focus of Hedda Wardemann. She is at the German Cancer Research Center in Heidelberg and focuses on single-cell sequencing.