Specialty probes give super-res imaging that special blink

Users of super-resolution imaging describe how they match probes to imaging modalities

Vivien Marx

Fluorescent probes light the way to cellular detail, but light can also get in the way. Because of the diffraction limit, structures closer to one another than 200 nanometers (nm) or so cannot be discerned. Unless you use probes with super-resolution imaging. These techniques, such as reversible saturable optical linear fluorescence transitions (RESOLFT) or photoactivated localization microscopy (PALM)/stochastic optical reconstruction microscopy (STORM), use specialty probes, dyes and fluorescent proteins (FPs) that can switch from dark to light and from one color to another[1–4]. "We need the labels in combination with the microscope to overcome the diffraction barrier," says Stefan Jakobs, who develops probes at University Medical Center Göttingen and the Max Planck Institute for Biophysical Chemistry. In structured illumination microscopy (SIM), labs routinely achieve 100-nm resolution, he says. Scientists using stimulated emission depletion microscopy (STED), RESOLFT, PALM or STORM reach beyond 50-nm resolution. In principle, he says, the methods are diffraction unlimited.

Photoswitchable and photoconvertible FPs drive the development of super-res microscopy imaging technology, says Pingyong Xu, a probe and imaging methods developer at the Institute of Biophysics of the Chinese Academy of Sciences in Beijing. The probes have enabled PALM/STORM, nudged SIM to nonlinear (NL)-SIM and moved STED to RESOLFT, which, in his view, offers better resolution than STED and less phototoxicity.

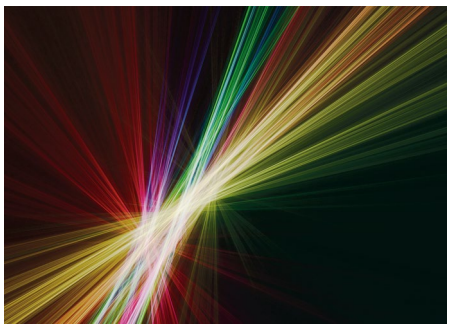
Label types

Photoactivatable FPs can be turned on with light; photoconvertible FPs start with green fluorescence and can be switched to red. Reversibly switchable FPs (rsFPs) can switch between non-fluorescent and fluorescent states many times, behavior based generally on the chromophore's *cis–trans* isomerization, says Jakobs. Illuminated probes emit light, but finding signal amid noise is the daily battle that determines resolution. An important factor in this battle is the difference in fluorescence intensity between probe on and off states, says Xu. "Brightness is also important, but sometimes high brightness does not mean a high signal-to-noise ratio," he says. Single-molecule brightness is critical in PALM/STORM. Working with a bright FP in SIM and RESOLFT means less light is needed, lowering phototoxicity risks in live-cell imaging. The switching kinetics of switchable and convertible probes matters, too, since this determines how long one can image, says Xu. Theoretically, the more switching cycles the better, but this depends on the biology being studied. Switching kinetics affects signal-to-noise ratio and spatial resolution: probes with slower on/off switching emit more photons and fast on/off kinetics can mean a signal is too dim for good image reconstruction.

A lab might optimize for switching cycles, but it needs the other parameters to be in a narrow, favorable regime as well. "It's a combination of properties," says Jakobs. Labs need favorable signal-to-noise ratios, bright probes to harvest many photons and probes capable of switching thousands of times, which in RESOLFT microscopy relates to the attainable resolution.

Jakobs and colleagues, including Nobel laureate Stefan Hell, started characterizing super-res imaging probes as they emerged, for example, from the Lukyanov lab at the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, the Verkhusha lab at Albert Einstein College of Medicine and the Miyawaki lab at RIKEN. Among the early probes are asFP595, switchable EGFPs, and the green fluorescent protein Dronpa and its variants. Jakobs' personal favorite is rsEGFP2, developed in his lab, but a favorite is not better than others in all respects, he says. For example, rsEGFP2 does not switch off as readily as Dronpa variants, which he and his team also like.

We need the labels in combination with the microscope to overcome the diffraction barrier, says Stefan Jakobs. (Max Planck Institute for Biophysical Chemistry)

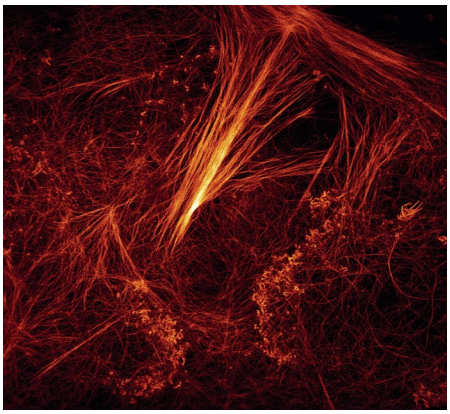


It takes light from fluorescent probes to reveal cellular detail and dynamics. Credit: Mmdi/Stone/Getty

To select FPs, says Xu, scientists will want to consider available imaging setups, whether they are imaging live or fixed samples, the biological processes and where their protein of interest is located. A label should not interfere with the protein's 'natural localization'.

To University of Osaka probe developer Takeharu Nagai, the important photophysical traits include switching kinetics, photon budget, switching fatigue and bleaching. His lab's probe, Kohinoor[5], has been used with RESOLFT, in NL-SIM in fixed cells and in live cells using SPoD-ExPAN, polarization demodulation and excitation polarization angle-narrowing techniques.

Well-matched imaging systems and probes maximize the strengths of both. For PALM/STORM, Xu prefers mEos3.2. For live-cell single-molecule-guided Bayesian localization microscopy, SIMBA, a technique developed in his lab, mEos.3.2 is best and Skylan-S[6], also from his lab, is suitable for super-resolution optical fluctuation imaging (SOFI). Skylan-NS pairs well with the



Shown here, Skylan-NS matched with PA NL-SIM for live-cell super-res imaging of actin in COS-7 cells. Adapted with permission from ref. [6]

extended-resolution structured illumination method known as PA NL-SIM, and the probe achieves better results than rsEGFP2 and QMars-Q. "The biggest advantage of rsEGFP2 is its high stability against bleaching," he says, which makes it suitable for extended RESOLFT imaging. Skylan-S and Skylan-NS work well with RESOLFT, too. Researchers will need to compare probes for their experimental particulars, such as the target molecule of interest, biological system, sample preparation method and optical imaging settings, says Xu.

Cellular signaling dynamics are quick and need short image acquisition times. But super-resolution acquisition times tend to be long, so he prefers spinning disk confocal imaging, says Joachim Goedhart, a probe developer at the University of Amsterdam. With probes, including the rsFPs, he advises labs to select and characterize a few candidates. Testing on purified protein is advisable, but the "important properties need to be scrutinized in cells," he says. Ideally, the comparison involves well-known, established variants. After all, probe developers are unlikely to have tested the probes in a user's exact experimental setup.

Imaging live cells

The majority of super-res microscopy papers "by far" have involved fixed cells, says Jakobs. It can be easier to use techniques that take more time, such as PALM/STORM, with fixed cells, he says. But independently of imaging modality, researchers want to learn from live cells. "We don't want a protein, which is perfect on a glass coverslip, we want to have it in a living cell," says Jakobs. It needs to tag well, to mature quickly at 37 °C and to not dimerize.

When illuminated at 488 nm, Dronpa fluoresces green, and extended illumination turns it off. Light at 405 nm turns it back on. The Jakobs lab developed a partner for Dronpa called Padron[7]. Illumination at 488 nm induces its fluorescence, the shorter wavelength 405 nm will turn it off and 488 nm will turn it back on. Dronpa and its variants have been extensively characterized to study switching, says Jakobs, but the uses of the original Dronpa in super-res imaging are limited. He likes Dreiklang[8], a YFP-based FP from his lab in which, unlike DronSpa and rsEGFP, the on/off switch is decoupled from fluorescence readout. Dreiklang works in live mammalian cells but is not yet as good in terms of fatigue and ease of use as Dronpa variants and rsEGFP.

Going red

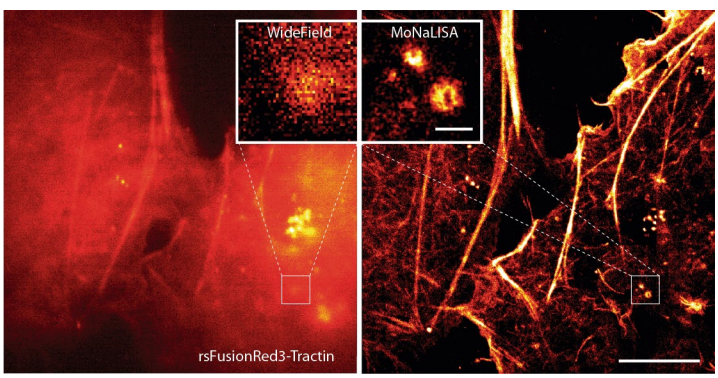
The existing green rsFPs perform well, but "the red ones lag behind," says Jakobs. Xu agrees and says that red probes matter for multicolor imaging. His lab has tried rsTagRFP, rsCherry and rsCherryRev, but not found them well suited to their needs. Because of its low quantum yield, rsCherry is hard to use in super-res microscopy, says Nagai. Xu is optimistic about the potential performance and applications of rsFusionRed from the Testa lab. Both Xu and Nagai are eager to see these probes evaluated in comparison with others.

Because rsFPs are mainly green, their switching wavelengths tend to be rather toxic in extended live-cell imaging. That motivated Ilaria Testa to develop rsFPs based on FusionRed, which is photoswitchable with green-orange light[9]. Testa is a researcher at the KTH Royal Institute of Technology in Stockholm and part of SciLifeLab, a collaboration of four universities in Stockholm and Uppsala. She performed single-molecule detection with PALM/STORM and then worked more with STED/RESOLFT. She developed a super-res imaging approach called molecular nanoscale live imaging with sectioning ability (MoNaLISA) to accompany rsFP development and enable super-res imaging of cells in low light.

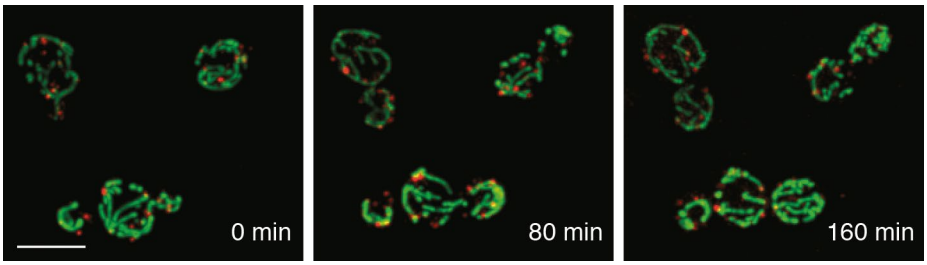
Important photophysical traits include switching kinetics, photon budget, fatigue and bleaching, says Takeharu Nagai.

Super-res imaging labs navigate a triangle of temporal resolution, spatial resolution and photodamage, says Testa. STED is fast, but its high-intensity light means it cannot be used for long with live cells. STORM's resolution is high, but the method is quite slow. With MoNaLISA, she sought a way to be faster than STORM without needing high light intensity, says Testa. The imaging system has a large field of view in which 4,000 microlenses survey the entire cell. A multi-focus light pattern switches rsFusionRed on, a second light pattern turns the molecules off and a third multi-focus orange light pattern reads out on-state fluorescence. She is advancing the system for use in multicolor, multiplexed experiments.

The three FusionRed variants from her lab show good photoswitching behavior with MoNaLISA and RESOLFT, says Testa. They are bright monomers, they work well with protein fusions and they are highly specific, she says. Red is kinder to cells than other wavelengths and penetrates deeper, which opens experimental options with zebrafish and *Caenorhabditis elegans*, or imaging the cortex of live mice. By developing imaging modalities around such probes, "you can really aim to bring super-resolution there,



Switching wavelengths for rsFPs can be toxic to live cells. That's when red comes into play. Here, rsFPs based on FusionRed are imaged with MoNaLISA. Credit: Testa lab, KTH Royal Institute of Technology



The Jakobs lab tracked two proteins in live budding yeast cells expressing rsFastLime and Padron by using reversible switching. Adapted with permission from ref. [7]

into tissue, living tissue," she says, to study fast-developing events in living systems.

SIMBA and Bayesian analysis of blinking and bleaching (3B) are super-res imaging methods based on single-molecule localization that leverage different FP characteristics, says Xu. SIMBA uses the on/off characteristics of photoconvertable FPs, while the 3B method uses blinking, much like the on/off of conventional FPs such as mCherry. The on/off switching is more probable with photoconvertable FPs than mCherry's blinking, which renders SIMBA more likely to localize a single molecule than 3B. SIMBA uses actual positional information for detected single molecules to establish the initial model in the calculation, while 3B uses random selection points that may or may not contain positioning information for the initially established model. Thus, Xu says, SIMBA calculates faster and more accurately.

In terms of instruments and illumination patterns, SIMBA is simpler than RESOLFT, says Xu, and its spatial-temporal resolution is comparable. In his view, SIMBA is more suitable for biologists less familiar with microscopy methods.

Dyes versus FPs

Photoswitchable dyes are much brighter than FPs, "but they require a lot of skill and expertise to use them well," says Xu. Some issues include label specificity, membrane permeability and background problems with unbound dyes. The "good news," he says, is that many dyes have been developed that only emit light when they bind to their target proteins. This can reduce the background and improve the specificity of the label, he says, and it's an important future direction for dye development.

Jakobs, too, sees dyes as powerful probes, including switchable ones such as those from the labs of Luke Lavis at Janelia Research Campus and Vladimir Belov at the Max Planck Institute for Biophysical Chemistry. But dyes must be brought into the cell where they also need to avoid interfering with cellular housekeeping. For now, he says, FPs outperform dyes for RESOLFT-type applications. In the future, labs will likely not just use one label type or imaging modality. "We need to have different labels, different methods" to empower the field, he says.

Jakobs is partial to FPs. "I like my barrel," he says, referring to FP structure. He likes that FPs can be expressed in a genetically modified cell or that an animal can carry a genetically encoded FP. The strategy for making FPs is powerful. Labs can screen 100,000 variants to select the best one.

Cameras

In super-res imaging with rsFPs, both electron-multiplying charge-coupled device (EM-CCD) and scientific complementary metal-oxide-semiconductor (sCMOS) cameras can be used. The EM-CCD read speed is around 70 frames per second, a limitation in super-res microscopy, says Xu. For experiments that call for high imaging speed, he prefers sCMOS cameras. Unlike EM-CCDs, noise for sCMOS cameras is pixel dependent and must be computationally removed. The Xu lab has developed Hessian-SMLM[10], a noise-calibration algorithm that eliminates pixel-dependent readout noise well from sCMOS cameras in single-molecule localization microscopy, he says.

At the University of Bielefeld, Thomas Huser and colleagues have tested and compared cameras for single-molecule localization microscopy. High-end cameras are typically only needed for dim samples. For quick sample inspection to evaluate labeling efficiency or blinking rates, his lab has equipped two microscopes with lower-cost industry-grade cameras. In preliminary experiments with photoswitchable proteins and samples with low labeling density, industry-grade cameras work but the performance is borderline, says Huser, who is also affiliated with the University of California, Davis. That's when the more expensive but lower-noise sCMOS and EM-CCD cameras shine. A lab's choice will depend on the application. For low-speed, extremely low-noise applications, an EM-CCD is currently the camera of choice. For higher-speed, low-noise imaging, sCMOS is the right choice, he says.

FPs, and photoswitchable FPs in particular, are not as brightly fluorescent and as long-lived as organic fluorophores, says Huser. For applications using immunostaining with organic fluorophores and fixed samples, it almost doesn't matter whether an EM-CCD, sCMOS or industry-grade CMOS camera is used for imaging. With live-cell imaging, however, involving fusions with fluorescent or photoswitchable FPs against a high background of autofluorescence, the less noise the camera contributes to imaging, the better, he says.

Camera noise is shaped by a detector's quantum efficiency, which is the percentage of photons that are converted to electrons. Back-illuminated EM-CCD cameras have the highest quantum yield and reach close to 95% quantum efficiency, says Huser. To maintain this excellent low noise, these cameras need to be cooled to –75 °C. Back-illuminated sCMOS cameras have recently become available, he says, and it's too early to determine the improvement they deliver.

The demand for cellphone and mobile cameras has accelerated CMOS chip development, says Huser. This gives camera manufacturers broader choices with detectors and chips. Camera manufacturers develop the electronics for data transmission and work with external companies that develop camera chips. Calibrating CMOS cameras can be more work than with EM-CCDs, mainly because noise can vary from pixel to pixel, requiring different analysis and calibration routines. "This is, however, now also well published and developed," he says.

To camera manufacturers, it's important to work on noise issues so users don't miss a low signal, says cell biologist Stephanie Fullerton at camera manufacturer Hamamatsu. CCDs and CMOS cameras both use arrays of photodiodes, and both convert photons to electrons. In CCDs, the electron charge moves from one pixel to the next and ultimately delivers an intensity

For higher-speed, low-noise imaging, sCMOS cameras are the right choice, says Thomas Huser.

(N. Langohr, University of Bielefeld)

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value. "Every single pixel goes out through that same electronic circuit," says Fullerton.

With CMOS, each pixel basically has a built-in circuit, including amplifiers. "I always joke, but our scientific cameras have gotten better because people wanted to take pictures in bars where it was dark," says Fullerton. Scientific developments have propelled consumer devices, but it's the inverse with CMOS cameras. The consumer market led sensor manufacturers to "nail down the uniformity of pixel to pixel," she says. Not pulling a signal off of a sensor fast enough can mean missing that flash of calcium and not getting the desired temporal resolution. When an electronic circuit runs fast in a CCD, the signal is noisier. A quick-running CMOS, however, with parallelized signal readout, can keep the noise levels low.

Hamamatsu's engineers measure read noise as electrons root mean square, or electrons r.m.s. With CCDs, the company's best values have been 6–8 electrons r.m.s.; CMOS drops that number to around 1.6, says Fullerton. Its newest camera, the ORCA-Fusion, has a read noise of 0.7 electrons r.m.s. It was designed with "low-light super-resolution people" in mind, says Fullerton, to help them be confident about single-molecule measurements, and can work with switchable FPs that don't emit many photons. When calculating signal-to-noise ratio, read noise enters the equation squared, which means that small differences in read noise make a big difference. With dim samples, a low signal becomes more readily detectable by pulling the noise floor down, she says. What also matters is having a CMOS camera with close similarity between pixel readouts to ensure precise single-molecule localization data.

Some camera manufacturers address noise by optimizing quantum efficiency. Fullerton acknowledges that some cameras reach higher quantum efficiency levels than Hamamatsu's. Better quantum efficiency helps with handling low signal, but "you still need low read noise to detect it," she says.

One issue with EM-CCD cameras is their large pixels, which can diminish resolution in super-res imaging, says Fullerton. EM-CCD cameras also have a smaller field of view than CMOS cameras. But cellular imaging benefits from a wide field of view. The new camera has fewer pixels that can disrupt the computation needed in imaging experiments. A camera that can detect in low-light imaging cuts down on light needs, reducing phototoxicity and photobleaching risks. Even when their dyes are bright, scientists do not want extended exposures for live-cell imaging.

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Red dyes are generally dimmer, says Fullerton, and camera sensors are typically less sensitive in the red. Longer wavelengths can pass through a sensor undetected. To deal with such issues, manufacturers work on sensors at the wafer level. The choice of silicon, its thickness and the way it is doped will all affect the number of detected red photons. If a wafer's detection layer is too thick, resolution can be lowered. There is likely no one best camera for a lab. "The ideal is to get a camera that covers the majority of your applications and gives you a lot of versatility," she says.

View from here

A number of labs have worked with switchable FPs in cold temperatures. For example, the Moerner lab has used PAmKate, a red photoactivatable FP from the Verkhusha lab, to do single-molecule localization with super-res techniques in vitrified samples. In one study the localization precision was four times what they could achieve at room temperature: the probe had longer on times. "Cold is good because it reduces photobleaching," says Jakobs. Labs can correlate findings in multiple modalities with probes that work across techniques: they might begin with a fluorescence-based method and move to cryo-electron microscopy. "I see a lot of potential here," he says. More generally, he hopes that super-res imaging with live cells can become routine. That will take advances with probes and imaging modalities, to reduce photobleaching and increase speed and the number of images recorded from living cells. He is partial to RESOLFT given its lower light intensities relative to STED or conventional PALM modalities. Xu sees much room for developing photoswitchable and photoconvertible FPs in super-res imaging to, for example, localize different proteins simultaneously in the cell. FPs are needed for multicolor experiments with excitation at different wavelengths, such as red and far red/far infrared photoswitchable FPs. Non-linear optical effects hinder deep cellular observations into the nuclei of cells or into tissues. New photoswitchable and photoconvertible FPs could be matched with deep imaging techniques such as light-sheet and two-photon microscopy, he says. For Nagai, adapting switchable FPs to be functional indicators that spy on nanoscopic physiology remains challenging. Innovative design approaches could let labs use photophysical traits directly as sensor readouts. When labeling nanodomains within organelles, properties such as pH tolerance and monomeric behavior will become ever more important, he says. FPs with replenishable chromophores will matter for long-term super-res functional imaging, and novel types of switching FPs, chromophores and dyes that excel in such traits are likely to gain more attention. ❐

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