MICROSCOPY

Nanoscopic imaging that lasts

Spontaneously blinking membrane probes enable live-cell super-resolution imaging for up to half an hour.

Super-resolution microscopy has established itself as a method of choice for obtaining detailed images of biological structures in fixed cells. However, live-cell super-resolution imaging, especially for extended periods, remains technically challenging. Problems for extended live-cell super-resolution imaging arise from photobleaching of probes, slow image acquisition speeds, and the sometimes toxic light doses needed for generating nanoscopic images.

Alanna Schepartz and Derek Toomre, along with their research teams at Yale University, developed a strategy that enabled long-term super-resolution imaging of membranous structures in living cells. "We were motivated in part by the frustration of not being able to take single molecule switching (SMS) nanoscopy of cell dynamics for very long," recalls Toomre. Their previous work suggested that a combination of high-density labeling of lipids in membranes and mostly dark fluorophores might allow them to image for much longer periods, because this combination would facilitate high-resolution imaging without rapid photobleaching. This insight was key to developing their current imaging strategy.

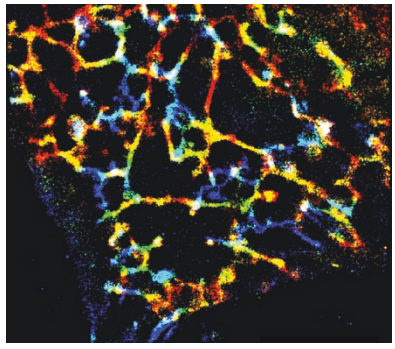
Their extended SMS nanoscopy strategy uses an established silicon rhodamine dye called HMSiR. They found that HMSiR, in the context of a lipophilic membrane environment, would blink spontaneously and with a low ratio of fluorophores in the "on" state; the latter of which is necessary for distinguishing individual fluorophores in the context of densely labeled membranes. This spontaneous blinking, in combination with photostability and optimized imaging parameters, enabled SMS nanoscopy with high spatiotemporal resolution over tens of minutes. By conjugating HMSiR to molecules that targeted a range of subcellular organelles and structures, the team developed a series of high-density environment-sensitive (HIDE) membrane probes.

One such HIDE probe, Cer-HMSiR, labels the endoplasmic reticulum (ER). After labeling with this probe, the researchers were able to generate composite images every 2 s and achieve spatial resolution that allowed clear visualization of ~50-nm ER tubules at the periphery of mammalian cells. This imaging lasted over 25 min—far longer than conventional live-cell super-resolution microscopy, which typically lasts no longer than a few minutes. The team also saw that the majority of cells survived this extended imaging.

The researchers also developed HIDE probes that target the mitochondrial membrane, the plasma membrane, and filopodia. These probes were also useful for prolonged SMS nanoscopy. In addition, the team demonstrated multicolor imaging of HIDE probes and mEos3.2-tagged proteins, as well as volumetric live-cell super-resolution imaging, further highlighting the versatility of their approach.

Although the imaging method now works well, both Schepartz and Toomre recall experiencing bumps along the way. "The biggest challenge was extending the concept to multiple organelles," says Schepartz, mostly due to complicated chemistry involved. She credits Alex Thompson, a graduate student in her lab, for having the idea to modify known organelle-localizing probes to address this issue, a strategy that ended up working well. Toomre also calls 'data deluge' a major issue, and notes that a single movie could represent 2–4 TB of data, which would take a day to analyze.

The steps forward for this work and for the field of live-cell super-resolution microscopy may lie in chemistry, in the form of improved probes. "There is no question that we—and everyone in the field—want to visualize live cells at super-resolution with as many colors as possible," notes Schepartz. To reach this goal, she says that probes with properties such as those found in HMSiR will be needed in different colors.



3D imaging of the mammalian ER with a HIDE probe; colors represent depth. Adapted from Takakura *et al.*, Springer Nature (2017).

Similarly, Toomre and Schepartz agree that improved probes will be needed to extend long-term super-resolution observation beyond large and dense lipid structures to tagged proteins. According to Toomre, the major challenge stems from the fact that individual proteins of interest are not typically found at high densities. He says that real innovation is needed to overcome this challenge and says, "We have some new ideas, but are under no illusion that this will be easy." Schepartz notes that dye photostability will be critical toward extending live-cell imaging, and that new molecular scaffolds and orthoganol reactions will likely be needed to accomplish this goal.

The development of the HIDE probes and the demonstration that combining photostability, low-level stochastic activation, and dense labeling enables extended live-cell super-resolution constitute a major breakthrough that should inspire biologists to ask new questions regarding the structures targeted by the HIDE probes and encourage developers to keep pushing these boundaries.

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RESEARCH ARTICLES

Takakura, H. *et al.* Long time-lapse nanoscopy with spontaneously blinking membrane probes. *Nat. Biotechnol.* http://dx.doi.org/10.1038/nbt.3876 (2017).