MICROSCOPY

PALM reading

Super-resolution fluorescence microscopy gets a boost in axial resolution from two groups of optics wizards.

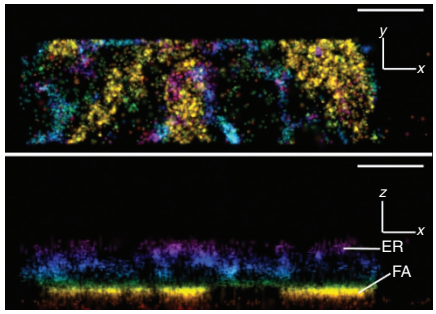
The selection of super-resolution fluorescence microscopy as *Nature Methods*' 2008 Method of the Year marks only the early days of a revolution. Now that the optical diffraction limit has been shattered, scientists are working to tackle the remaining limitations. Two recent papers now describe improved axial resolution in three-dimensional photoactivated localization microscopy (PALM).

PALM takes advantage of brief activation and sensitive localization of single-molecule fluorophores to generate an image. Despite success in lateral-resolution improvement, axial resolution remains 60–70 nanometers. The new methods use different optics to reach even higher axial resolution of 10–20 nanometers.

Harald Hess, of Howard Hughes Medical Institute's Janelia Farm, invented PALM with fellow physicist Eric Betzig. To pursue higher axial resolution, Hess revisited his old days in the semiconductor industry. There, tiny components are routinely localized at 10^{-10}-meter accuracy by interferometry, a method that detects tiny differences in the path length of coherent light beams by measuring light intensity changes resulting from constructive or destructive interference.

Hess exploited the wave property of single photons and used objectives above and below the sample to create two coherent beams from fluorophores. If a fluorophore is closer to one objective, the emitted photon will travel a shorter distance down that path than the other before reaching a custom-made three-way beam splitter where the photon interferes with itself. The interference output signals are used to determine the vertical position of the fluorophore. Hess and colleagues call this technique, which has 10-nanometer axial and 20–30-nanometer lateral resolution, interferometric PALM (iPALM) (Shtengel *et al.*, 2009).

Hess's group tested iPALM in cells expressing fluorescently labeled α-tubulin, and they measured the diameter of a microtubule to be about 25 nanometers. This is in agreement with the estimation of the diameter of microtubule plus the fluorescent probe. They also resolved dorsal and ventral cell membranes, and visualized the spatial arrangement of focal adhesions and endoplasmic reticulum. According to Hess, "the demand [for super-resolution fluorescence microscopy] in biological applications is high. Improving sample preparation and the development of brighter fluorescent probes will make these new methods more applicable to address interesting biological questions."



iPALM images pseudocolored according to the axial position of the PALM signal, from coverslip at the bottom (red) to cytoplasm on the top (purple). Shown are top view (top) and side view (bottom) images of U2OS cells transfected with tandem dimer (td)EosFP-tagged α-integrin. ER, endoplasmic reticulum; FA, focal adhesion. Scale bar, 1 μm. Image courtesy of H.F. Hess and the National Academy of Sciences (Hess, *et al.*, 2009).

Meanwhile, W. E. Moerner of Stanford University, a pioneer of single-molecule imaging, was also exploring ways to improve axial resolution in imaging. He met Rafael Piestun from the University of Colorado, Boulder at a meeting in 2007, where Piestun talked about a concept of making microscopes with a double-helix point spread function (DH-PSF). Together, they realized that the technique could be applied to single-molecule imaging. Piestun sent graduate student Sri Pavani to Stanford to work with another student Michael Thompson, and before long, they built a microscope implementing the DH-PSF (Pavani *et al.*, 2009).

Their approach alters the shape of the focal spot. "Inside a normal microscope, the shape of the focus is like two [opposing] cones; where the tips meet is the focal plane. This shape is called the point spread function," Moerner explains. Changing the point spread function into a double-helix allows axial super-localization. DH-PSF splits a single point emitting source into two spots whose angle in the focal plane depends on the exact axial position of the source. Combining DH-PSF with PALM, they created DH-PALM, which can be used to resolve single-molecule fluorophores with 10–20 nanometer resolution over a 2 micrometer depth of field. The system is "physically easy to implement," says Moerner, and "the depth of field is very large."

Moerner stresses that this work is only a proof of principle. Besides testing it in biological systems, there is still room for improvement. For example, the spatial light modulator used to create the DH-PSF causes a 75% loss of photons. Other optical methods could be applied to reduce the photon loss. Also, this algorithm has not reached the theoretical limit of extracting all information available in the images.

Axial resolution is only one of the many challenges for super-resolution fluorescence microscopy to be widely applied in biology. Other aspects, such as multicolor imaging and temporal resolution, are areas of active development. As super-resolution fluorescence microscopy development enters an exponential growth phase, we can certainly expect these challenges to be met by optical and chemical magic in the near future.

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RESEARCH PAPERS

Pavani, S.R.P. *et al.* Three-dimensional, single-molecule fluorescence imaging beyond the diffraction limit by using a double-helix point spread function. *Proc. Natl. Acad. Sci. USA* **106**, 2995–2999 (2009).

Shtengel, G. *et al.* Interferometric fluorescent super-resolution microscopy resolves 3D cellular ultrastructure. *Proc. Natl. Acad. Sci. USA* **106**, 3125–3130 (2009).