Super-resolution for a 3D world

Joshua W Shaevitz

Technological developments are pushing the emerging super-resolution fluorescence microscopy techniques into the world of three-dimensional imaging.

Scientists have relied upon visual representation of the living world as a means of discovery for centuries. Over time, we have moved from hand-drawn images of animals and tissues to exquisite molecular-scale representations produced by electron microscopy. Much of our knowledge of the inner workings of cells comes from light microscopy studies that identify and localize specific macromolecular structures. In this issue of *Nature Methods*, two groups report the highest-resolution, three-dimensional (3D) light microscopy to date[1,2].

Until the last decade, a hard limit on the scale of objects that could be probed by light microscopy prevented imaging studies from peering into the smallest reaches of the cell. The laws of physics dictate that a wave of light cannot be focused to an arbitrarily small point. Diffraction limits the size of this point to greater than about half the wavelength of light, with the best conventional microscopes achieving a point-spread function (PSF) of 200 nm laterally and 600 nm axially. A wide-field image becomes blurred by about the size of the PSF because the objective lens effectively sees each fluorescent molecule as a point source subject to the resolution limitations described above.

The last few years have seen an explosion in super-resolution techniques that effectively reduce the size of the PSF by an order of magnitude. Although these systems often achieve very high resolution laterally, the axial resolution is often far worse. Biological specimens are inherently 3D objects, and thus these super-resolution techniques fall short of producing a 3D image that is not elongated and blurred along one of the three axes.

Schmidt *et al.* describe an improved version of scanning stimulated emission depletion (STED) fluorescence microscopy to achieve isotropic super-resolution 3D imaging[1]. Single-pixel scanning techniques for fluorescence imaging have been around since at least 1955 (ref. 3). In these techniques, a single-pixel detector collects the emitted photons from a single, focused excitation spot as it is scanned over the sample. The resolution of the resulting image is largely defined by the size of the excitation region, whose dimensions are limited by diffraction.

STED microscopy, which was first introduced in 1994 (ref. 4), greatly reduces the size of the fluorescence excitation spot using a two-laser apparatus. One laser is tuned for fluorescence excitation and a second laser, termed the STED laser, is tuned at the wavelength of an allowable emission transition that lies outside of the normal fluorescence detection band. By inducing stimulated emission of excited fluorophores, the STED laser prevents detection of fluorescence from these molecules. Because the number of fluorescent molecules in the focal volume is finite, this depletion can be saturated, producing very steep spatial gradients of depleted fluorophores. One can create a very small effective excitation volume by separating the excitation and emission laser beams in space.

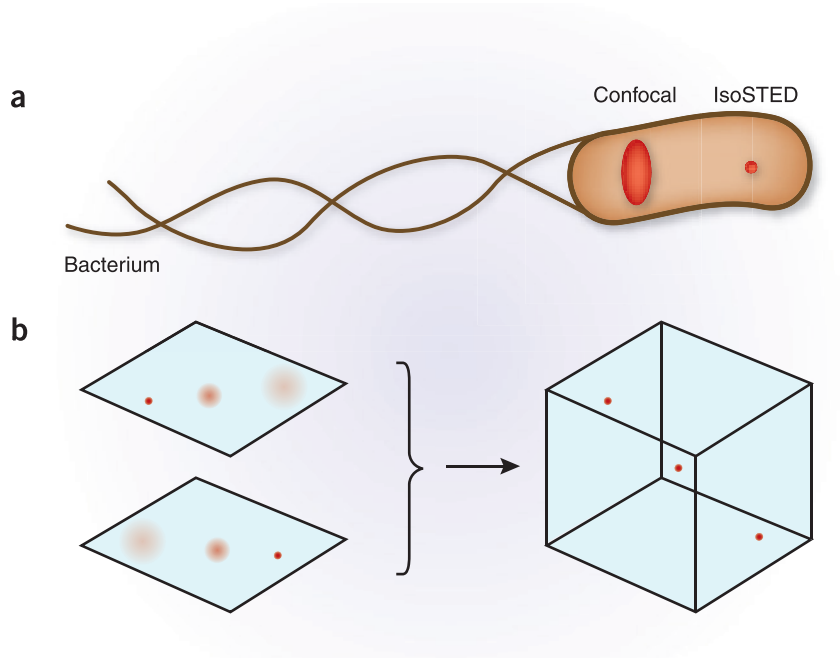

Figure 1 | Two variations on 3D super-resolution imaging. (**a**) Scale representation of a conventional microscope PSF (left) and that achieved in isoSTED microscopy[1] (right) over a bacterium. The tiny spherical shape of the isoSTED PSF allows super-resolution laser-scanning imaging with equal resolution in all three dimensions. (**b**) The use of two imaging planes in the wide-field super-resolution imaging method named biplane FPALM[2] provides super-resolution axial localization in thick samples by analyzing the fluorescence signal in the different planes.

Joshua W. Shaevitz is in the Department of Physics and the Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, New Jersey 08544, USA.
e-mail: shaevitz@princeton.edu

Modern realizations of STED microscopy use pulsed laser beams to take advantage of the time scales for molecular relaxation and the interference of coherent light to produce radially symmetric depletion zones. Digital image deconvolution is then often used to reduce the effect of side-lobes and other PSF artifacts that pollute the measured image, especially along the axial dimension.

Although most demonstrations of this technique produce a reduction of the axial PSF size, the PSF is usually severely asymmetric. Schmidt *et al.* use two STED laser beams along with a two-objective setup to produce separate depletion patterns in the lateral and axial dimensions[1]. This technique, called isoSTED microscopy, produces a tunable, isotropic PSF. The authors demonstrated a 45-nm isotropic PSF (**Fig. 1a**), and this size can presumably be made even smaller by increasing the intensity of the STED lasers. Tuning the PSF size is likely to be important because of the trade-off between the resolution, the signal-to-noise ratio of the resulting image and the amount of time needed to cover an appreciable region of interest. By designing a system with a tunable PSF, one can adjust the PSF size to best suit the application at hand.

Localization-based imaging approaches offer an alternative route to increased resolution[5–7]. These techniques involve the sequential activation and localization of single fluorescent molecules, or fluorophores, in a cell. The lateral position of a lone fluorescently labeled protein can be measured to within a few nanometers. The sequential imaging of many of these proteins individually allows one to build up a representative image of their underlying spatial distribution with nanometer-scale resolution.

Recently, a 3D localization–based technique was introduced that used elliptical astigmatism to quantify the axial position of an activated fluorescent molecule achieving lateral and axial resolutions of 25 and 55 nm, respectively[8]. But this technique is limited to samples less than 1 μm in thickness, and the resolution in the lateral dimensions varies with the axial position of the molecule.

Juette *et al.* now introduce a technique that records images of activated fluorophores in two axially separated planes simultaneously[2]. These datasets can be used to localize individual molecules with 30- and 75-nm resolution in the lateral and axial directions, respectively (**Fig. 1b**). This setup offers two main advantages over the one used previously. First, unlike the astigmatism-based technique, there is essentially no coupling between the lateral resolution and the axial position of the molecule. Second, this method uses epi-illumination, allowing for very deep imaging by scanning the sample axially. By combining the 3D localization results at different depths, the authors achieved 75-nm axial resolution over a range of about 10 μm.

Although the two techniques described here represent advances in super-resolution microscopy, each has its limitations. The localization-based system of Juette *et al.* uses inexpensive lasers coupled into a conventional microscope, but relies on complex photoswitching chemistry to achieve $30 \times 30 \times 75$ nm resolution[2]. In contrast, Schmidt *et al.* use complicated pulsed lasers and a custom, two-objective design with conventional fluorophores to achieve 40-nm isotropic resolution[1]. In principle, however, this technique could be achieved using less expensive continuous-wave lasers, although this has not yet been demonstrated. The jury is still out as to which, if either, of these methods will become widely used by biologists.

We are entering a new era of super-resolution imaging that brings with it a number of challenges. The optical microscope may approach nanometer resolution, but other factors now come in to play when trying to quantify cellular structures at this scale. Genetically encoded fluorophores such as GFP are typically 5 nm in size, and thus make poor probes for features approaching that size. Additionally, even if very small probes are developed, the density at which they can be labeled into the sample will ultimately limit the resolution. These issues aside, it is an exciting time for microscopy as we begin to explore a three-dimensional, super-resolution world.

1. Schmidt, R. *et al. Nat. Methods* **5**, 539–544 (2008).
2. Juette, M.F. *et al. Nat. Methods* **5**, 527–529 (2008).
3. Minksy, M. *Scanning* **10**, 128–138 (1988).
4. Hell, S.W. & Wichmann, J. *Opt. Lett.* **19**, 780–782 (1994).
5. Hess, S.T., Girirajan, T.P. & Mason, M.D. *Biophys. J.* **91**, 4258–4272 (2006).
6. Rust, M.J., Bates, M. & Zhuang, X. *Nat. Methods* **3**, 793–795 (2006).
7. Betzig, E. *et al. Science* **313**, 1642–1645 (2006).
8. Huang, B., Wang, W., Bates, M. & Zhuang, X. *Science* **319**, 810–813 (2008).

Turning fluorescent proteins into energy-saving light bulbs

Gert-Jan Kremers & David W Piston

Screening for photostability in addition to color and brightness creates better fluorescent proteins.

The key properties of fluorescent probes include (i) wavelength of absorption and fluorescence (color), (ii) brightness and (iii) photostability. The established strategies for optimizing fluorescent proteins have primarily focused on color and brightness[1]. Even though poor photostability often limits the temporal resolution of live-cell experiments, this parameter has only occasionally been characterized after a new fluorescent protein is developed. An article in this issue of *Nature Methods* describes an elegant method for screening fluorescent protein variants, with the addition of photostability as a primary selection criterion[2].

The typical approach to fluorescent-protein optimization is the use of random mutagenesis to generate genetic variants expressed in *Escherichia coli* colonies grown on agar plates. Each colony expresses a single fluorescent protein variant, but a single plate can contain hundreds of colonies, each with a protein variant possessing distinct fluorescence properties. The brightest variants of the desired color are usually isolated for further selection or characterization.

Gert-Jan Kremers and David W. Piston are in the Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee 37232, USA.
e-mail: dave.piston@vanderbilt.edu