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Nano-imaging with Storm

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Abstract

Multicolour, three-dimensional stochastic optical reconstruction microscopy now makes it possible to image cellular structures with near molecular-scale resolution.

When combined with a large repertoire of fluorescent probes and biochemically specific labelling techniques, multicolour fluorescence microscopy allows the direct visualization of molecular interactions and processes in living organisms. The diffraction-limited resolution of fluorescence microscopy, however, leaves many biological structures too small to be observed in detail. Recent years have seen the development of super-resolution imaging approaches that break the diffraction limit. These methods can be divided into two categories: (1) methods that use spatially patterned illumination to sharpen the point-spread function of the microscope, such as stimulated emission depletion (STED) microscopy (see also the Progress article on page 381 of this issue¹) or saturated structured-illumination microscopy (SSIM) (see also the Commentary on page 362 of this issue²), and (2) a method based on the high-precision localization of individual fluorescent molecules, which has been referred to as stochastic optical reconstruction microscopy (STORM)³ or (fluorescence) photoactivation localization microscopy ((F)PALM)^{4,5}. Here I focus on this latter approach.

STORM, PALM and FPALM rely on the detection⁶ and localization^{7–9} of single fluorescent molecules. Although the image of a single fluorescent emitter has a finite size due to diffraction (roughly 200–300 nm in a lateral direction and 500–800 nm along the axial direction), the position of the emitter can be determined to a much higher precision depending on the number of photons detected⁸. This can be accomplished simply by fitting the image to find its centroid position. Fitting an image consisting of N photons can be viewed as N measurements of the fluorophore position, each with an uncertainty determined by the width of the image. The final precision thus equals the width of the image divided by $\sqrt{N}$. For example, the position of a single-dye molecule can be determined to accuracy as high as ~ 1 nm (ref. ⁹). This high-precision localization, however, does not directly translate into sub-diffraction image resolution, as the position of nearby emitters with overlapping images would still be difficult to determine.

A super-resolution imaging method based on the sequential localization of photo-switchable fluorescent probes has recently been invented^{3–5}. These photoswitchable probes can be optically switched between a fluorescent and a dark state. Their fluorescence emission can thus be controlled over time, such that different molecules are turned on during different time windows. This additional control in the time domain allows molecules with spatially overlapping images to be separated in time, and consequently allows their positions to be precisely determined. Specifically, in the imaging process, only a subset of probes is activated to the fluorescent state at any given time (for example by exposure to light of a certain wavelength and intensity) such that the images of individual molecules do not typically overlap. By fitting these isolated images, the positions of the activated molecules can be localized with high precision. This process is then repeated to allow more molecules to be localized. Once

enough localizations have been accumulated, a high-resolution image can then be constructed from the measured positions of the probes (see Fig. 1). The resolution of the final image is no longer limited by diffraction, but by the precision of each localization. This method has been variously named STORM³, PALM⁴ or FPALM⁵. For simplicity, I will use the term STORM to refer to this category of method for the remainder of the article.

STORM can be realized using a variety of photo-switchable probes, including dyes and fluorescent proteins. In its simplest form, STORM can be achieved using a simple fluorescent dye and a single colour continuously illuminating laser. Figure 2a shows such an example, in which the microtubules in a cell are immunostained with Alexa Fluor 647 and imaged with a red laser (657 nm). The red laser accomplishes all three tasks required for STORM: exciting fluorescence from Alexa Fluor 647, deactivating it to the dark state and reactivating it to the fluorescent state. At equilibrium, the fluorophore spends about 0.1% of its time in the fluorescent state, allowing around 6,000 photons to be detected per switching cycle. The combination of high photon flux and low activation equilibrium affords a high image resolution. Indeed, the STORM image shows a marked improvement in resolution over the corresponding conventional fluorescence image, as can be seen in Fig. 2b,c. In the regions where microtubules are densely packed and unresolved in the conventional image, individual microtubule filaments are clearly resolved by STORM. Alexa Fluor 647 is a member of a family of photo-switchable cyanine dyes that can be reversibly cycled between a fluorescent and a dark state by exposure to light^{10–12}. This family includes other red cyanine dyes suitable for STORM, such as Cy5, Cy5.5 and Cy7 (refs ^{3, 12, 13}). STORM has also been implemented using other switchable dyes, such as photochromic rhodamine¹⁴, caged dyes⁴ and blinking dyes^{15,16}.

In addition to dyes, photo-switchable fluorescent proteins can also be used for STORM imaging. Figure 2d and e shows the comparison of conventional and STORM images of mEos2-labelled vimentin in a cell, again showing substantial resolution improvement by STORM. mEos2 is a monomeric variant of the Eos fluorescent protein that can be photoactivated from a green-emitting form into a red-emitting form^{17,18}. The STORM image was taken by activating mEos2 with a 405-nm laser and imaging the activated mEos2 with a 568-nm laser. Other photo-switchable fluorescent proteins, such as PA-GFP, Kaede, tdEos, KikGR, Dronpa, rsFastLime, bsDronpa, psCFP2, Dendra2, EYFP and PAmCherry, have also been demonstrated for superresolution imaging^{4,5,19–23}.

One of the main advantages of fluorescence microscopy is its capacity for multicolour imaging. Co-localization analysis with multicolour imaging has been widely used to map interactions between different biological structures, but the accuracy of co-localization is inherently limited by the image resolution. Taking advantage of its high resolution, STORM provides a more precise picture of molecular interactions. Various combinations of the aforementioned photo-switchable dyes and fluorescent proteins have been used for multicolour super-resolution imaging^{12,20,21,23–25}. For example, the distinct emission spectra of Cy5, Cy5.5 and Cy7 allow multicolour imaging by distinguishing their emission colours. Another interesting property of these cyanine dyes is the adjustable nature of their activation wavelength. The wavelength of light that efficiently activates these dyes can be adjusted almost at will by pairing them with an ‘activator’ dye. For example, when the photo-switchable ‘reporter’ Cy5 is paired with Cy3, Cy2 and Alexa Fluor 405, it can be selectively activated by a green, a blue and a violet laser, respectively¹². This colour-specific activation allows a second approach for multicolour STORM: distinct probes can be differentiated by the colour of their activation light. Combinatorial pairing reporters and activators could offer even more colours. Figure 3 shows two colour STORM images of microtubules and clathrin-coated pits, an important endocytic machinery in cells¹². The false co-localization between clathrin-coated pits and microtubules

shown in the conventional image (Fig. 3a) is now clearly resolved in the STORM image (Fig. 3b and c).

Most cellular structures have a three dimensional (3D) architecture, and resolving these structures requires high resolution in all three dimensions. This can be accomplished by high-precision 3D localization of fluorophores. The lateral position of a molecule can be determined from the centroid of its image, and the shape of the image contains information about the axial (z) position of the molecule. High localization precision along the z axis can be achieved by a variety of approaches. The astigmatism approach was first used to achieve 3D STORM²⁶. In this approach, a cylindrical lens is introduced into the imaging path to create a different focus in the lateral (x and y) directions, such that the image of a molecule appears elliptical. The ellipticity of the image varies with the z position of the molecule, and thus can be used to derive the value of z with high precision. Using this approach, we have resolved cellular structures with lateral and axial resolutions of 20 nm and 50 nm (full width at half maximum, FWHM), respectively²⁶. Both the standard deviation (SD) and FWHM have been used to describe resolution in the literature. Here we define resolution in FWHM (equal to 2.2 SD for a Gaussian distribution), as it provides a better measure of the closest distance between two objects that can be resolved.

The high resolution allows STORM to resolve the 3D morphology of nanoscopic structures in cells. Figure 4 shows the example of clathrin-coated pits, which typically have a diameter of 100–200 nm (ref. ²⁶). In a conventional image, clathrin-coated pits appear as diffraction-limited spots with no discernable structure (Fig. 4a). In stark contrast, the 3D STORM clearly resolves the half-spherical shell morphology of coated pits (Fig. 4b–d). STORM allows a sample several hundred nanometres in thickness to be imaged in 3D without any scanning. But as the sample gets thicker, the image of a molecule becomes more blurred, ultimately limiting the localization precision. A coarse stepping of the sample stage can be combined with high-precision 3D location for imaging thicker samples, such as a mitochondria network in an entire cell²⁵. Several other approaches have also been used for 3D STORM, such as defocusing light²⁷, using a double-helical point-spread function²⁸ or using interferometry²⁹. A z resolution as high as 10–20 nm has been reported for the latter approach²⁹.

Although impressive, the resolutions mentioned above do not represent the ultimate limit of STORM. Its spatial resolution is determined by the precision and density of localizations in the image. These two quantities are in turn determined by several practical factors: (i) the brightness of the probe, (ii) the residual dark-state fluorescence, (iii) the spontaneous activation rate from the dark to the fluorescent state, (iv) the labelling efficiency and (v) the label size³⁰. Given sufficient probe brightness and labelling density, resolution can be almost arbitrarily high. For a bright probe, such as Alexa Fluor 647, the number of photons detected allows in principle a localization precision of a few nanometres, promising true molecular-scale resolution. At this level, the physical size of the label becomes an important factor. Ideally, one would like to couple a small and bright fluorophore directly to the molecule of interest. Several recently developed approaches allow specific attachment of small organic fluorophores to cellular proteins through genetic encoding approaches³¹, providing a labelling strategy that could potentially support molecular-scale resolution.

Another important aspect of imaging is the data acquisition speed. Because of the intrinsic trade-off between time and spatial resolutions, super-resolution imaging is relatively slow. Specifically, a STORM image is constructed from localizations accumulated in a wide field over many imaging frames. The imaging speed is thus limited by the number of frames required to construct a high-resolution image. This speed-limiting mechanism is in contrast to that of STED, in which the scanning of the small focal point across the sample limits the imaging speed. Thus STORM is expected to be faster than STED when imaging a large sample area

but slower than STED when imaging a small area³⁰. Currently, a STORM image at the highest resolution typically requires minutes of acquisition time. At a spatial resolution of 60–70 nm, time-resolved images have been acquired with a temporal resolution of a few tens of seconds in living cells³². We expect the imaging speed to improve further with faster cameras, higher excitation power and probes with faster switching rates.

In summary, the STORM/PALM/FPALM approach provides a new platform for high-resolution imaging and shows great promise in the study of cellular processes at the molecular scale. Future development of the imaging technology itself, as well as new fluorescent probes and labelling methods, will continue to improve the power and versatility of this imaging approach. We can expect broad applications of super-resolution imaging in many areas of life science.

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References

1. Hell SW, Schmidt R, Egner A. *Nature Photon* 2009;3:381–387.
2. Heintzmann R, Gustafsson MGL. *Nature Photon* 2009;3:362–364.
3. Rust MJ, Bates M, Zhuang X. *Nature Meth* 2006;3:793–795.
4. Betzig E, et al. *Science* 2006;313:1642–1645. [PubMed: 16902090]
5. Hess ST, Girirajan TPK, Mason MD. *Biophys. J* 2006;91:4258–4272. [PubMed: 16980368]
6. Moerner WE, Orrit M. *Science* 1999;283:1670–1676. [PubMed: 10073924]
7. Gelles J, Schnapp BJ, Sheetz MP. *Nature* 1988;331:450–453. [PubMed: 3123999]
8. Thompson RE, Larson DR, Webb WW. *Biophys. J* 2002;82:2775–2783. [PubMed: 11964263]
9. Yildiz A, et al. *Science* 2003;300:2061–2065. [PubMed: 12791999]
10. Bates M, Blosser TR, Zhuang X. *Phys. Rev. Lett* 2005;94:108101. [PubMed: 15783528]
11. Heilemann M, Margeat M, Kasper R, Sauer M, Tinnefeld P. *J. Am. Chem. Soc* 2005;127:3801–3806. [PubMed: 15771514]
12. Bates M, Huang B, Dempsey GT, Zhuang X. *Science* 2007;317:1749–1753. [PubMed: 17702910]
13. Heilemann M, et al. *Angew. Chem. Int. Ed* 2008;47:6172–6176.
14. Fölling J, et al. *Angew. Chem. Int. Ed* 2007;46:6266–6270.
15. Fölling J, et al. *Nature Meth* 2008;5:943–945.
16. Steinhauer C, Forthmann C, Vogelsang J, Tinnefeld P. *J. Am. Chem. Soc* 2008;130:16840–16841. [PubMed: 19053449]
17. McKinney SA, Murphy CS, Hazelwood KL, Davidson MW, Looger LL. *Nature Meth* 2008;6:131–133.
18. Wiedenmann J, et al. *Proc. Natl Acad. Sci. USA* 2004;101:15905–15910. [PubMed: 15505211]
19. Egner A, et al. *Biophys. J* 2007;93:3285–3290. [PubMed: 17660318]
20. Shroff H, et al. *Proc. Natl Acad. Sci. USA* 2007;104:20308–20313. [PubMed: 18077327]
21. Andresen M, et al. *Nature Biotechnol* 2008;26:1035–1040. [PubMed: 18724362]
22. Biteen JS, et al. *Nature Meth* 2008;5:947–949.
23. Subach FV, et al. *Nature Meth* 2009;6:153–159.
24. Bock H, et al. *Appl. Phys. B* 2007;88:161–165.
25. Huang B, Jones SA, Brandenburg B, Zhuang X. *Nature Meth* 2008;5:1047–1052.
26. Huang B, Wang W, Bates M, Zhuang X. *Science* 2008;319:810–813. [PubMed: 18174397]
27. Juetten MF, et al. *Nature Meth* 2008;5:527–529.
28. Pavani SRP, et al. *Proc. Natl Acad. Sci. USA* 2009;206:2995–2999. [PubMed: 19211795]

29. Shtengel G, et al. Proc. Natl Acad. Sci. USA 2009;106:3125–3130. [PubMed: 19202073]
30. Huang B, Bates M, Zhuang X. Annu. Rev. Biochem 2009;78:993–1016. [PubMed: 19489737]
31. Fernandez-Suarez M, Ting AY. Nature Rev. Mol. Cell. Biol 2008;9:929–943. [PubMed: 19002208]
32. Shroff H, Galbraith CG, Galbraith JA, Betzig E. Nature Meth 2008;5:417–423.

Fig. 1. The principle of STORM

For a densely labeled cellular structure shown in **a**, only a sparse set of fluorescent labels are activated at any given time such that their images (green circles) do not overlap (**b**). **b**, The position of each activated fluorophore (red crosses) is then determined from its image. **c**, After a sufficient number of fluorophores have been localized, a high resolution image is constructed by plotting the measured positions. Modified with permission from ref. ³⁰. © 2009 Annual Reviews.

Fig. 2. STORM imaging of cells with photo-switchable dyes and fluorescent proteins

a, STORM image of microtubules in a BS-C-1 cell. The microtubules are immunostained with antibodies that are labeled with photo-switchable Alexa Fluor 647. **b** and **c**, Conventional (**b**) and STORM (**c**) images corresponds to the boxed region in **a**. **d** and **e**, Conventional (**d**) and STORM (**e**) images of vimentin in a BS-C-1 cell. The vimentin filaments are labeled with a photo-switchable fluorescent protein mEos2.

Fig. 3. Multicolour STORM

The microtubules and clathrin-coated pits in a BS-C-1 cell are immunostained with Cy2-Alexa 647-labeled and Cy3-Alexa 647-labeled antibodies, respectively. **a**, Conventional fluorescence image. **b**, STORM image of the same area. **c**, Magnified view of the boxed region in **b**. Parts b and c adapted with permission from ref. ¹². © 2007 AAAS.

Figure 4. Three-dimensional STORM

a, Conventional image of clathrin-coated pits in a BS-C-1 cell. Clathrin is immuno-stained with Cy3-Alexa 647-labeled antibodies. **b**, x - y cross-section of the corresponding 3D STORM image. **c**, Serial x - y and x - z cross-sections of a coated pit. **d**, An x - y and x - z cross section presented in 3D perspective. Parts a–d from ref. ²⁶. © 2008 AAAS.