

# Single-molecule mountains yield nanoscale cell images

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Methods to simultaneously localize the positions of multiple single fluorophores by precisely determining their individual positions are now yielding impressive gains in fluorescence microscopy resolution.

Optical microscopy has been a mainstay of cellular biology ever since van Leeuwenhoek used Hooke's microscope to observe the strange biological objects he called "animalcules." Using light for biological microscopy takes advantage of its relatively noninvasive character, and with high magnification one can work at a distance, observing small

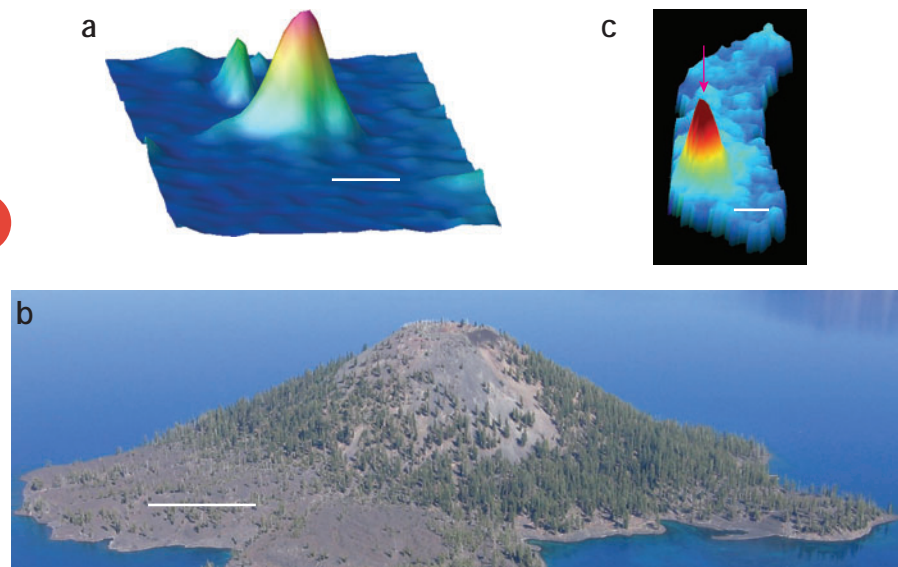
objects. Fluorescence microscopy depends upon a variety of labeling techniques to light up different structures in cells, but the price often paid for using visible light is the relatively poor resolution compared to X-ray or electron microscopy. The basic problem is that fundamental diffraction effects limit the resolution of standard optical microscopy

to a dimension of roughly the optical wavelength divided by two,  $\lambda/2$ , that is,  $\sim 250$  nm for visible light of 500 nm wavelength. In recent work, the light from single fluorescent molecules about 1–2 nm in size has provided a way around this problem.

How can single molecules help? Since 1989, researchers worldwide have been using optics to detect and measure individual molecules in solids, liquids and biological systems. For example, **Figure 1a** is a 1991 image of single molecules of pentacene in a crystal<sup>1</sup>. Each single molecule acts as a nanoscale light source, where the light is generated by pumping the pentacene molecules one at a time, each of which is about 2 nm in size. But owing to diffraction effects (and the large laser spot in a cryostat), scanning a laser spot over a single molecule yielded the much wider mountain-like peaks shown and actually provided a way to measure the laser spot size (here about 5  $\mu\text{m}$ ).

Recently, several researchers have begun to take advantage of the nanoscale size of single-molecule emitters more directly. The basic idea is illustrated in **Figure 1b**. Everyone knows that a hiker can walk to the top of a mountain with a global positioning system device to nail down the position of the peak quite accurately. In optical microscopy, the observed 'peak' from a single point source of light is called the point-spread function (PSF) of the microscope. **Figure 1c** illustrates this PSF for emission from a single molecule of the bacterial actin protein MreB (the mountain, labeled by fusion to yellow fluorescent protein) in a bacterial cell<sup>2</sup>. Simply by measuring the shape of the PSF, the position of its center can be determined much more accurately than its width. This idea, digitizing the PSF, a form of simple deconvolution, has been known for many years, but deconvolution alone can generate spurious features in the presence of noise. The knowledge that a single small object is producing the PSF helps tremendously in determining the centroid position with nanometer-scale accuracy, and this idea was first applied with single beads<sup>3</sup> and then to single-molecule images<sup>4–6</sup>.

In the last two years, a plethora of acronyms has been proposed for biological imaging beyond the diffraction limit based



**Figure 1** | Measuring mountain peaks for superresolution imaging. **(a)** Position-frequency image of single molecules of pentacene in a crystal<sup>1</sup>, as a pumping laser beam is moved across the sample and the fluorescence is recorded. **(b)** It is easily possible to walk to the top of the cinder cone in Crater Lake to find the position of the peak accurately. **(c)** Similarly, in a living bacterial cell (blue)<sup>2</sup>, the image of a single fluorescent protein molecule appears as a wide spot due to fundamental diffraction effects, but one can determine its position (pink arrow) to much less than the width of the "mountain." By turning single-molecule light sources like this on and off in a cell, researchers have recently obtained superresolution images. Scale bars: **a**,  $5 \times 10^3$  nm; **b**,  $\sim 120 \times 10^9$  nm; **c**, 250 nm.

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on digitizing the PSF. In Selvin's FIONA<sup>7</sup>, the position of single labeled myosin molecules was determined to an accuracy as small as a few nanometers, limited now by the number of photons before photobleaching (which controls the signal-to-noise ratio) rather than diffraction effects. But there is still a problem for imaging of complex structures in cells: keeping the single molecules more than ~500 nm apart so that their mountains do not overlap.

The solution to this (apparently arising from work both from Hell *et al.*<sup>8</sup> and Qu *et al.*<sup>9</sup> in the method termed NALMS) was to photoswitch the little single-molecule emitters on and off in various ways. In the PALM method of Betzig *et al.*<sup>10</sup>, light-induced photoactivation of GFP mutant fusions was used to randomly turn on only a few single molecules at a time in fixed cell sections or in fixed cells. In a tour-de-force effort, a few photoactivated molecular PSFs were imaged in detail to find their positions to ~20 nm, then they were photobleached so that others could be turned on, and so on; after 2–12 h of imaging and many thousands of molecules, a high-resolution image was extracted that correlated well with a TEM image.

Lidke *et al.*<sup>11</sup> showed that superresolution beyond the diffraction limit can also be achieved with the blinking of fluorescent semiconductor quantum dots, and these emitters have allowed observation of time-dependent, nanometer-sized steps in cells by the Xie group<sup>12</sup>. In another approach, the STORM method of Zhuang<sup>13</sup>, a single photo-switchable molecule on DNA or an antibody is turned on and off, again and again, to measure its position accurately with ~20 nm resolution. This method uses a Cy3-Cy5 pair in close proximity, which shows a novel property: restoration of Cy5's bleached emission by brief pumping of the Cy3 molecule. The controllability of a reversible photoswitch based on these good single-molecule emitters is appealing, but since these molecules cannot be genetically encoded in cells, the way autofluorescent proteins are encoded, other methods of implanting properly formed Cy3-Cy5 pairs in cells need to be developed.

Finally, in an alternative approach, Hell's group is using a powerful but sophisticated laser method called STED microscopy<sup>14</sup> to make the PSF itself much smaller, a step that will improve, in principle, all the other super-resolution methods at the cost of somewhat higher complexity.

All of these tantalizing new approaches to superresolution in biological imaging have

advantages and disadvantages, and which method will achieve widespread use and useful time resolution to observe nanoscale cellular dynamics is yet to be determined. Better single-molecule emitters for cellular labeling would certainly help, as all superresolution methods have accuracy limitations arising from the finite number of photons available from a single molecule before photobleaching. It is an exciting time for single molecules as nanoscale light sources, indeed!

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## The inside track on HIV

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The combination of appropriate labeling and a new imaging software allows researchers to follow the progress of individual HIV particles within infected cells with outstanding precision.

On page 817 of this issue, Pierre Charneau's group at the Pasteur Institute report a remarkably detailed analysis of the dynamics of HIV behavior during infection<sup>1</sup>. By fluorescently labeling and tracking individual viral particles on their way to the nucleus of living cells, the team has documented distinct modes of viral motility and for the first time demonstrated the movement of HIV within the nuclei of infected cells. Using high-resolution live-cell microscopy combined with a sophisticated new tracking software to follow dozens of viral particles, the group identified distinct mechanisms of viral transport based on their characteristic speed, directionality and distance traveled.

All viruses must gain access to the interior of the host cell to propagate and many must enter the nucleus before initiating a new round of infection. HIV is a member of the class of retroviruses known as lentiviruses, which can infect nondividing cells by entering the nucleus through nuclear pore complexes. This is one of the features that

makes HIV a useful tool for gene therapy as well as a sneaky foe, able to slip into quiescent cells without alerting the host antiviral response.

Until recently it was thought that nuclear entry occurred by diffusion through the cytoplasm and import through the nuclear pore complex. It turns out, however, that viral particles are much too large to freely diffuse in the dense cytoplasmic milieu and therefore must engage the transportation machinery inside the cell. For other viruses, this usually means either actin- or microtubule-based motility is involved, and in some cases both major cytoskeleton transport systems are used (reviewed in ref. 2). Moreover, once a viral particle reaches the nucleus, it is not at all apparent how the relatively large viral structure gets through the smaller nuclear pore.

In the case of HIV, many high-profile articles have been published defining the key regulators of its nuclear import and an equal number of publications refute each finding, so that our understanding of the

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