IMAGING

Pointillist microscopy

The proteins used as fluorescent markers in cellular imaging are only a few nanometres in size, yet the image resolution is typically diffraction-limited to one hundred times this scale. Now, a new strategy exists for imaging intracellular structure and dynamics with 10 nm resolution.

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Viewed from a distance, Georges Seurat's masterpiece *A Sunday on La Grande Jatte* is an impressionist scene of repose by the Seine. Upon close inspection, however, the painting is revealed to be constructed from thousands of millimetre-size dots. Although a closer view reveals the secret of the art, the picture must be viewed as a whole to understand the context and meaning of the dots.

This sort of 'pointillist' microscopy is the essence of a new approach to nanoscale microscopy developed by Eric Betzig of the Howard Hughes Medical Institute and co-workers[1]. In this work, termed photoactive localization microscopy (PALM), fluorescently labelled proteins are localized to better than 2 nm, beating the diffraction limit by nearly two orders of magnitude. An important advance over similar techniques, PALM yields high-resolution images even when the proteins are closer together than the resolving power of the fluorescence detection optics. The secret of the art in this case involves imaging only a small number of the proteins at a time and then summing the 'dots' to get one complete picture.

A long-standing objective of optical microscopy, which typically involves imaging fluorescently tagged molecules, is to obtain ever-better resolution. In principle, fluorescently tagged proteins provide an intrinsically high resolution because they are only a few nanometres in size. But the well-known 'diffraction limit' in optical and electron microscopy is set by the wavelength of light and numerical aperture of the imaging lens or microscope objective. For visible light, the diffraction limit sets the resolution at about 200–300 nm, much larger than many important features in biological systems.

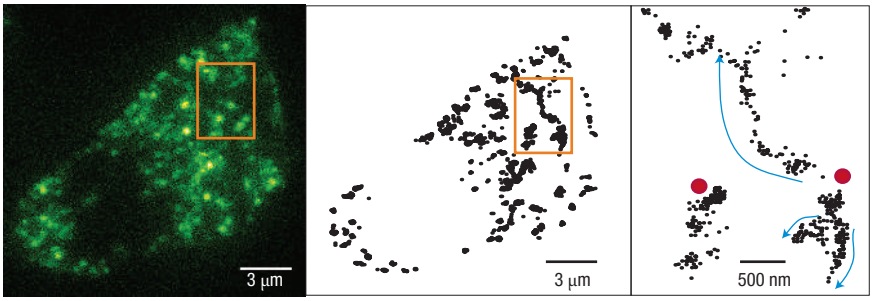

Figure 1 Pointillist microscopy techniques can be applied to the study of intracellular dynamics. Left: A fluorescence image shows a MIN6 insulin-secreting cell. The insulin is labelled with E-GFP as a fluorescent marker. The bright spots are the 250-nm granules that contain the insulin. Centre: The same cell after centroid analysis at 30 nm resolution. The image is obtained by summing over 100 consecutive image frames. The granules move over the time it takes to form the composite image (100 s), which can therefore be used to study the motion of the granules. The blow-up of this image (right) at 12 nm resolution for the centroid points indicates the diversity of the dynamics. The blue arrows indicate rapid transport of some of the granules, and the red markers indicate the more localized diffusion of other granules[11].

Although improvements in imaging resolution are possible, they often place stringent requirements on the stability of the microscope optics and mirrors[2–4]. So-called 'super-resolution' can be achieved by nonlinear microscopies that use multiple laser beams for excitation and depletion[3]. Although it is possible to achieve a remarkable resolution of around 30 nm with these techniques, they require highly specialized optics and extremely stringent alignment. Furthermore, the high laser power density that is required results in accelerated photobleaching, or 'fading', of the fluorescent molecules.

A gentler way to improve the resolution that works well for low densities of fluorescent molecules is to fit the 'centroid', or the centre of the distribution, of the photons emitted from a single molecule[5]. The accuracy of localization is determined by the number of photons detected and can be better than 2 nm (ref. 6). Centroid localization has been used to determine the individual positions of five or more single molecules within the laser excitation spot by taking the difference between the distribution of the photons before and after single-molecule photobleaching events[7,8]. This technique has been applied to a number of nanoscale imaging issues in biology, including mapping the locations of specific nucleotide base sequences in DNA[8]. Perhaps most famously, this technique has been used to determine the step size of motor proteins along actin filaments and microtubules[9], and to establish the quasi-one-dimensional search mechanism of single proteins along DNA[10].

The drawback of this technique is that it only works well when the proteins are well isolated from one another, at a density of about 10–100 molecules μm^{-2}. This ensures that the centroid distribution of each of the molecules can be accurately fitted. By contrast, the results obtained by PALM show that the technique can be used to study dense concentrations of proteins, of the order of 10^5 μm^{-2}, while still achieving a resolution of less than 10 nm.

Betzig *et al.* study mammalian cells that are prepared so that they express fluorescently tagged proteins. A near-ultraviolet laser is used to activate the proteins and make them fluorescent. The key is to make the measurements at very low laser power, so that any single molecule has

a small probability of being photoactivated. A second laser (whose wavelength is in the visible at 561 nm) is then used for excitation of the activated proteins and the fluorescence is detected. As a result, at any point in time, the image contains a random distribution of photoactivated proteins that are far enough apart from one another that their centroids can be accurately fitted at high resolution. Following the inevitable photobleaching of each single protein, the authors are able to photoactivate and replenish a new set of fluorescent proteins at low enough density that only one (at most) is in the resolution volume for each image acquired. Measured all at once, the cumulative image of all of these fluorescent proteins is, of course, diffraction-limited. But fitting the centroids of the randomly excited proteins and then summing the series of more than 10^4 images of the centroids results in a 'super-resolution' optical image with 10 nm resolution.

The technique poses an experimental challenge, as the cells must remain fixed over the 2–12 hours needed to acquire the summed image. The length of time depends on the brightness of the molecules and how long it takes for the proteins to photobleach. Therefore, the authors went to great lengths to minimize thermal and mechanical drift of the sample during these extended image acquisition times.

Comparisons of PALM and transmission electron microscope images of mitochondria containing the protein cytochrome-C oxidase show that this protein is distributed no closer than about 20 nm from the outer membrane of the mitochondria. Transmission electron microscopy alone cannot provide this information, as it is unable to determine protein identity. Nor can conventional optical imaging yield this type of resolution, even with image deconvolution. Ultimately, Betzig and co-workers suggest that the technique may be used to determine the orientation of a single molecule. PALM is thus a considerable advance in nanoscale structure determination.

One important extension of this pointillist microscopy is imaging dynamics. This would entail some trade-off in resolution, because the time for image acquisition must be short compared with the intrinsic molecular motion. The key factor is to keep the number of fluorescent objects within the resolution volume to less than one to permit an accurate determination of the centroid. Figure 1 shows a conventional resolution microscopy image[11] of a MIN6 insulin-secreting cell, and two images obtained with the centroid imaging technique. The insulin-containing vesicles, termed granules, are labelled with the fluorescent protein E-GFP. The magnified image on the far right shows the centroid locations of the granules at 12 nm resolution summed from 100 images in a movie. The high spatial resolution is needed to resolve details of the granule motion and determine whether they are simply diffusing or being actively transported from one region of the cell to another.

Combining the centroid analysis with a microscopy that permits a study of the motions of the individual specks would allow probing of cellular dynamics as well as structure[12], giving a more complete picture of the cellular function.

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NANOTRIBOLOGY

Bringing friction to a halt

Controlling the friction between two moving surfaces — and possibly even reducing it to zero — is one of the outstanding challenges in modern tribology. Two recent discoveries may make this dream come true.

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The increase in the surface-to-volume ratio that occurs when devices are scaled down in size makes friction increasingly problematic in miniature instruments, such as micro- and nanoelectromechanical systems. Indeed, the devices that work reliably usually have designs that avoid sliding contacts. Systems with moving components that come into contact with each other, on the other hand, suffer enormous problems due to stiction, friction and wear. Lubrication is not an option because the lubricant would be too viscous on the nanoscale and, moreover, the adhesion forces introduced by liquids are strong enough to damage tiny devices. However, the development of two new methods that allow the amount of friction to be varied could lead to greater control over this most troublesome force[1,2].

The typical approach to reducing friction is to optimize the properties of the surfaces that come into contact with each other. The idea is to make friction low by the appropriate choice of chemical composition, crystal structure, surface roughness, electrostatic interactions and other properties. Considerable progress has been made over the past decade with special coatings, such as the family of diamond-like carbon films[3].

More delicate but perhaps less practical ways to reach ultralow friction involve either an extreme reduction of the contact pressure[4] or a cancellation of lateral forces. This latter goal can be achieved by making use of the non-periodicity of quasicrystals[5] or by introducing a deliberate lattice mismatch between the two sliding crystal surfaces (a mechanism referred to as superlubricity[6]). What these techniques all have in common is that they change the energy 'landscape' of the interaction between the surfaces at the atomic scale.