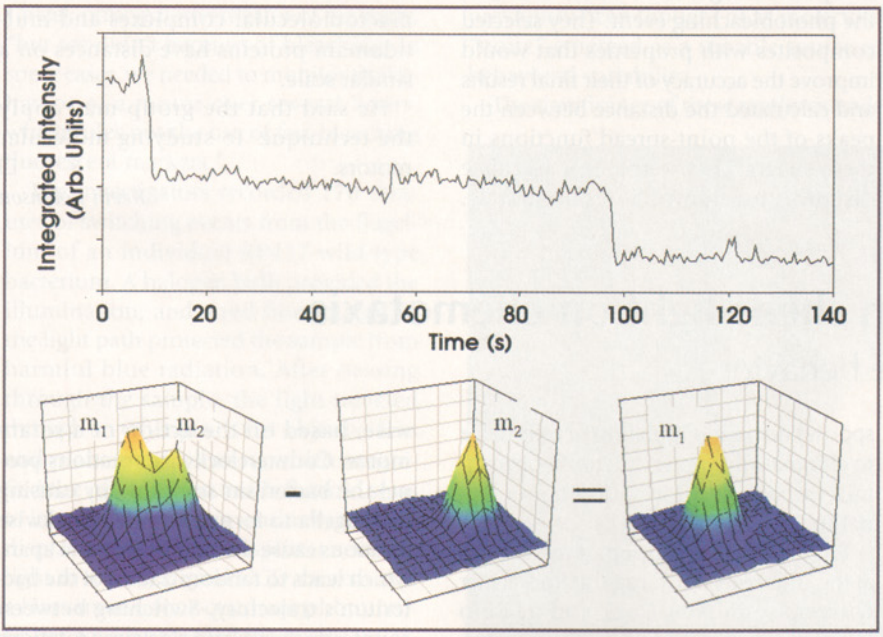
Bridging the resolution gap

Point-spread function technique extended to measure intermolecular distances

As scientists have become more and more interested in the actions of individual molecules, their desire to form images at molecular resolutions has only increased. Unfortunately, the physical properties of light limit the resolution of a visible light microscopic image to about 200 nm. To gain information from smaller distances, researchers use Förster resonance energy transfer (FRET), but this method works only if the two molecules under study are within about 10 nm of each other.

A method based on imaging molecular fluorescence holds the promise of bridging the gap in resolution between FRET and light microscopy. Described in the April 27 issue of *PNAS*, it is based on photobleaching fluorescent molecules. The work comes on the heels of studies by principal investigator Paul R. Selvin of the University of Illinois at Urbana-Champaign that used analysis of a fluorescent molecule's point-spread function to localize the molecule to within 1.5 nm. The group extended the work by using the point-spread function to calculate the distance between two fluorescent molecules, even if they are too close for distinction using conventional optical techniques.

According to lead author Matthew P. Gordon, also of the university, measuring the point-spread function of a fluorescent molecule, at its most elementary, can be likened to pouring sand one grain at a time. If each grain represents a photon, as the pile forms it will be roughly Gaussian in shape. If two mounds are poured sufficiently close to each other, they make one big mound. However, if you could take a photo of both mounds and then somehow subtract all of the grains of sand that make up one of them, you could figure out the distance between the mounds.



By measuring the different photobleaching times of two Cy3 molecules (top) and calculating their point-spread functions (bottom), researchers were able to subtract the photons from molecule 2 (m_2) and leave the point-spread function of molecule 1 (m_1). This allowed them to separate spatially overlapping fluorescence from molecules that are closer to each other than the diffraction limit of light allows an optical microscope to resolve. Reprinted with permission of PNAS.

In the technique, the grains of sand are photons from a fluorescent dye — in this case, Cy3. They pile up, one by one, on a CCD. The key is photobleaching, which occurs when excitation light no longer makes the dye molecule fluoresce. Photobleaching happens in a different fashion for different molecules, even of the same fluorophore. Although this is generally a nuisance in single-molecule imaging, the researchers used it to distinguish between two molecules that photobleach at different times. "So we're really taking a property of fluorescent labels that most people dislike and using it to our advantage," Gordon said. When one molecule of a close pair photobleaches, the effect is tantamount to subtracting the entire pile of sand at once, leaving only one pile.

Photon subtracting

To test the method, Gordon, Selvin and colleague Taekjip Ha used Cy3 to label truncated DNA strands. They imaged fluorescence from the strands with an Olympus microscope and a 60× oil-immersion objective, and an objective-type total-internal-reflection setup was illuminated by a 532-nm, diode-pumped crystal laser from CrystaLaser LC of Reno, Nev. The group used a slow-scan, back-thinned MicroMax CCD camera from Roper Scientific Inc. of Trenton, N.J., because it captures images at 2 Hz with no interframe dead time.

The fluorescently labeled DNA was kept in solution, except to test the effects of drying the sample. A biotin-streptavidin linker attached the DNA to the coverslip to keep it still. The scientists used a DNA concentration that was low enough to reduce the chances of multiple strands clumping together, although some clumping did occur.

Then they turned on the excitation light and began imaging. They made sure to analyze spots with only two fluorophores and not clumps with several. They looked for stepwise intensity changes, with a two-step function indicating the presence of two fluorophores in the point-spread function.

Several factors affect the number of spots available for analysis: premature photobleaching, noise caused by fluorophore blinking, insufficient intensity and DNA clumping. In their study, only about 30 to 40 percent of the fluorescent spots showed a two-step intensity function and could be further analyzed. However, Gordon said the parallel nature of the data collection means that they "can collect a huge amount of data in one sitting — several hundred molecules."

From there, the researchers developed composite images from before and after the photobleaching event. They selected composites with properties that would improve the accuracy of their final results and calculated the distance between the peaks of the point-spread functions in these images. They were able to calculate distances for fluorophores accurately. Three specific examples showed molecules 132.9 nm apart with a standard deviation of 0.93 nm; 72.1 nm apart with a standard deviation of 3.5 nm; and 8.7 nm apart with a deviation of 1.4 nm.

The pixels on the CCD correspond to about 100 nm in real space, and the researchers could determine the center of the point-spread function accurately because, if it shifts by a small amount, it redistributes some of the photons from one pixel to another in a way that they can detect mathematically. "If the pixels were much smaller than the point-spread function, ... the number of photons in each pixel would be very small, [and] the intrinsic noise of the camera would make it impossible to accurately tell how many photons fell on each pixel," Gordon said. "Having the CCD pixels be just about the size of the [point-spread function] width makes this approach work well."

Gordon said that the technique, which is good for measuring distances in the same plane, could measure distances between two points within proteins, such as molecular motor proteins, where head-to-head distances are often on the scale of 20 to 100 nm. Ion channels, some macromolecular complexes and multidomain proteins have distances on a similar scale.

He said that the group may apply the technique to studying molecular motors. □

Kevin Robinson