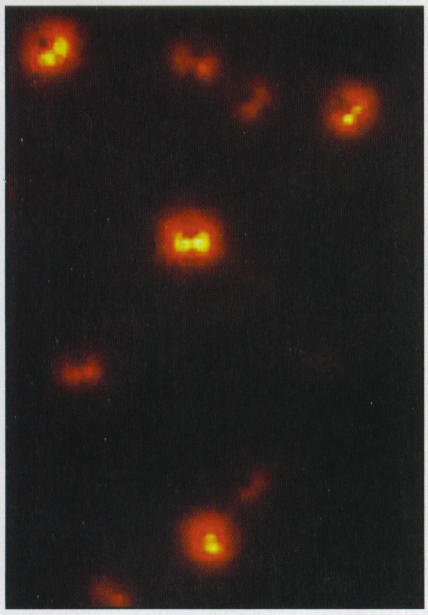
Blurred images bring clarity

Technique reveals rotational as well as translational dynamics of molecules



The intensity distribution of blurred images obtained by defocusing a microscope contains information about the angular distribution of the molecules under observation. Shown here are images of the 3-D orientations of quantum dots acquired with the defocusing technique. Images courtesy of Erdal Toprak.

Consider an actor on a stage. If he takes a few steps to his left, it is fairly easy to determine how far he moves and in what direction — the tools with which to do so are readily available. But then, as he continues, he begins to twist his body and spin his arms. It's a mélange of movement, as revealing of his motivations as the steps he takes. Still, how do you measure that?

Researchers at the University of Illinois at Urbana-Champaign, at Research Institute Jülich in Germany, at Case Western Reserve University in Cleveland, and at the University of Pennsylvania in Philadelphia were faced with a similar problem when looking at the movement of biological macromolecules, such as myosin V, that exhibit both translational and rotational movement. A variety of techniques — for example, optical trapping and fluorescence imaging with 1-nm accuracy — are available for measuring the step sizes of molecular motors.

"But these methods don't tell much about the rotational dynamics of molecular motors ... " said Erdal Toprak, a graduate student at the University of Illinois. "A method that can measure rotational dynamics effectively would be very useful to our lab."

Toprak and colleagues in Paul R. Selvin's group at Urbana-Champaign therefore decided to try the defocused orientation and position imaging technique, which had been successfully demonstrated in 1999 by Andrew P. Bartko and Robert M. Dickson and theoretically solved in 2003 by Martin Böhmer and Jörg Enderlein of Research Institute Jülich. This technique provides information about the angular distribution of a single molecule's fluorescence emission by defocusing the image acquired with a wide-field epifluorescence or a total internal reflection microscope. The intensity distribution of the resulting blurred image contains the relevant information.

The method allows imaging of rotational dynamics, which fluorescence imaging at 1 nm, for instance, does not. At the same time, though, it does not always identify the molecule's position as accurately because the image is spread over a greater number of pixels. For this reason, the investigators came up with a way to switch back and forth between the focused and defocused imaging, providing information about position and orientation with considerable accuracy.

In the April 25 issue of *PNAS*, they reported that they used this combined approach to track the translational and rotational dynamics of fluorescently labeled myosin V as the proteins moved along actin — long, rodlike proteins involved in muscular contraction. Myosin V is a motor protein that hauls cargo along actin in a stepwise, "hand-over-hand" manner, following a helical path around the protein as it does so. It was the perfect choice for demonstrating the technique, Toprak noted, because its "lever arm" — which drags it forward as it moves, alternately leading and trailing — is known to tilt with respect to actin.

The researchers observed 32 molecules during the study, using different patterns of defocused and focused imaging (and 65 molecules using defocused orientation and position imaging only). In one experiment, for example, they measured displacement and 3-D orientation of the molecules with repeated cycles of five consecutive defocused images and three consecutive focused images. They used an exposure time of 0.66 s per frame. In all cases, the sample was moved about 500 nm from the optimal focus position.

In all of the experiments, the scientists used a total internal reflection microscope setup based on an inverted microscope made by Olympus America Inc. of Melville, N.Y., with a 1.6× magnification unit and an infinity-corrected 100×, 1.45-NA oil-immersion objective. A 532-nm diode-pumped Nd:YAG laser made by CrystaLaser LC of Reno, Nev., provided excitation, and a back-thinned CCD camera from Andor Technology of South Windsor, Conn., captured the images. They controlled the distance between the sample and objective (and thus defocusing) with a piezoelectric Z-axis sample stage made by Mad City Labs Inc. of Madison, Wis. Image acquisition and defocusing were synchronized using custom-written software.

The researchers unveiled several features of the lever arm dynamics and translocation of the myosin, showing, for example, that all of the moving myosin exhibited lever arm tilts and that the tilting and stepping events occurred simultaneously. "We can distinguish the trailing and leading lever arm states of myosin," Toprak said. "I believe that this may help to understand how the two lever arms communicate with each other."

The technique could be used to study any number of biological molecules that rotate, and possibly even membrane and

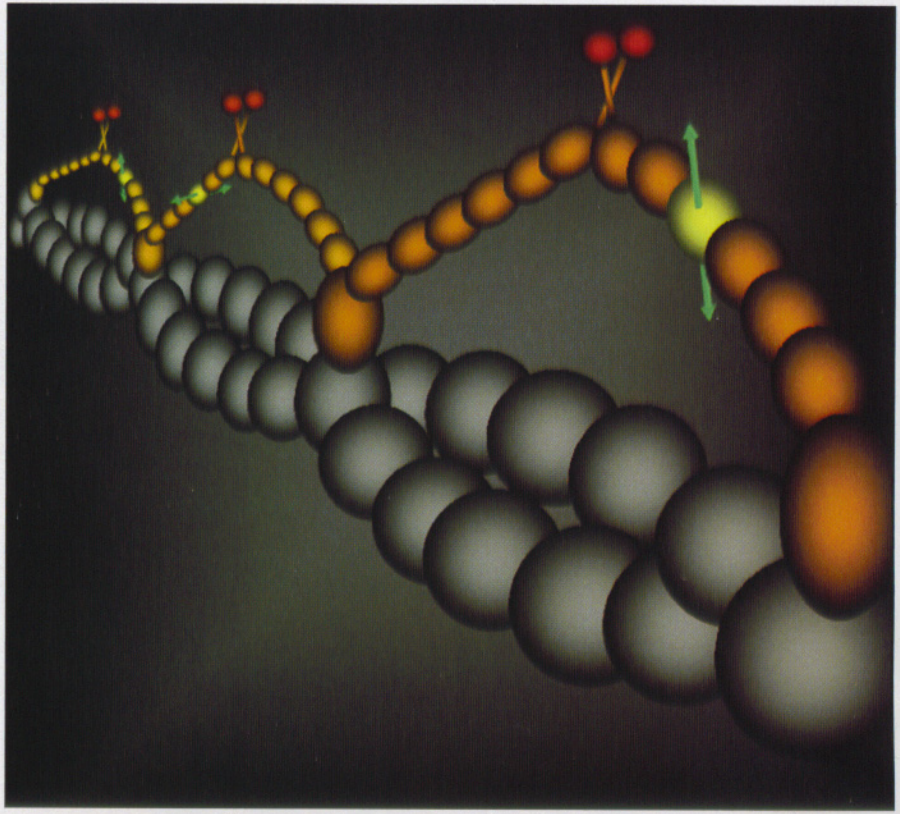
motor proteins. It might also be applied to visualize the alignments of fluorescent nanocrystals.

Other methods, such as single-molecule fluorescence polarization microscopy, can measure the 3-D orientations of fluorophores with a time resolution of about 40 ms. With normal excitation powers, defocused orientation and position imaging offers a resolution of 100 ms for quantum dots and 0.5 s for conventional fluorescent dyes.

However, these other methods generally have angular degeneracy issues. For example, single-molecule fluorescence polarization microscopy detects the 3-D orientation of dipoles based on their absorptions and emissions. However, dipoles with orientations 180° apart can yield exactly the same absorption and emission and thus can be impossible to distinguish. Also, the setups are often costly and difficult to build. "Our method is relatively easy and [inexpensive] to use, and we have no angular degeneracy," Toprak said.

The researchers continue to develop the technique, working to increase its time resolution and positional accuracy. They also are planning to apply it to other motor proteins. □

Gary Boas



The defocusing technique provides information about the translational and rotational dynamics of molecules based on defocused images, shedding light on the dynamics of a myosin V molecule as it moves across actin.