Fluorescence microscopy watches proteins at their own scale

Visible light can track molecules with nanometer and millisecond resolution, even amid the complexity of a living cell.

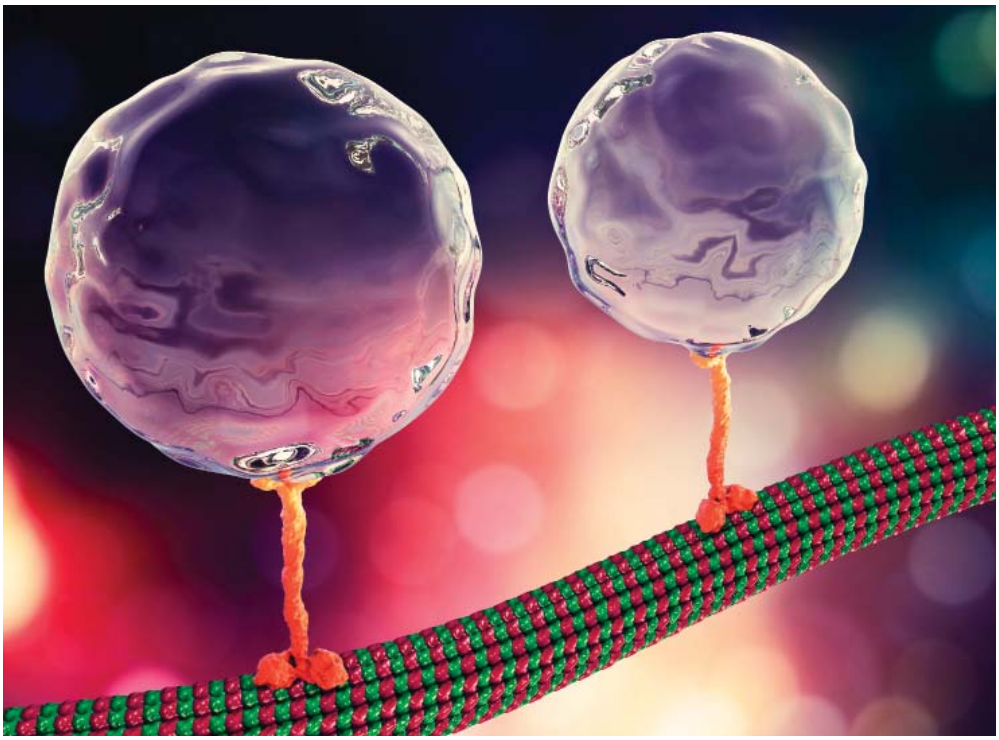

FIGURE 1. KINESIN, shown in this artist's illustration in orange, is a motor protein that carries cargo (transparent spheres) around inside a cell. The kinesin walks along a microtubule (part of the cell's cytoskeleton, shown in green and red) by moving its feet alternately, one in front of the other. But until now, its movement was too fast to be directly studied in detail. (Image by Kateryna Kon/Shutterstock.com.)

Sometimes what looks like a fundamental physical limitation isn't so insurmountable after all. An example is the diffraction limit: Light can't be focused to a spot smaller than half its wavelength, so it might seem to be impossible to use visible light to image features smaller than 200 nm. But researchers took on the diffraction limit and won, as highlighted by the 2014 Nobel Prize in Chemistry (see Physics Today, December 2014, page 18). The three laureates—Eric Betzig, Stefan Hell, and William Moerner—and others developed ingenious ways to use optical fluorescence microscopy to obtain images with resolution of around 20 nm. And the resolution revolution was just getting started.

One can see a lot at 20 nm resolution. In biological systems—the most appealing imaging target because of all their unknown nanoscale complexity—that's the scale of organelles, protein complexes, and many other supramolecular structures (see, for example, Physics Today, May 2015, page 14). But a lot remains unseen. Many important features are an order of magnitude smaller, including the shapes and conformations of individual proteins. And still images completely leave out what's arguably the most important thing about living systems: the way they change over time.

In the years since the Nobel, superresolution researchers have pushed to improve the resolution in both space and time. They've now reached the point of achieving nanometer and millisecond precision simultaneously—good enough to watch the motions of proteins in real time—as shown in new work by two groups in Heidelberg, Germany, one led by Jonas Ries of the European Molecular Biology Laboratory (EMBL)[1] and the other led by Hell at the Max Planck Institute for Medical Research.[2] Both groups used MINFLUX, a technique Hell and colleagues unveiled[3] in 2017, and both studied the motor protein kinesin, illustrated in figure 1, whose job it is to carry cargo from place to place inside a cell.

The exquisite precision comes with a trade-off: The researchers don't obtain a complete moving image of kinesin and all of its surroundings. Instead, they track the position of just one fluorescent molecule, called a fluorophore. But through judicious choice of where to attach the fluorophore to the kinesin, they can obtain detailed insights into how the molecule moves—and perhaps a similar understanding of other proteins that operate on the same time scales.

Why so slow?

One of the key insights that make superresolution imaging possible is that although a dense bunch of fluorophores blur together, the position of a single isolated fluorophore, with no other light sources around, can be pinpointed precisely. There are various ways of doing that, the simplest being just taking a picture with a camera. The fluorophore appears as a diffraction-limited blob, hundreds of nanometers across. But the center of the blob—and thus, presumably, the position of the fluorophore—can be determined with much greater precision.

Single-fluorophore localization predates the Nobel-winning superresolution work by several decades (and in fact was the basis for early work on the kinesin mechanism, described below). The Nobel Prize honored the development of a suite of techniques for building up large numbers of single-fluorophore detections into complete images of complicated structures. Unsurprisingly, the imaging takes a long time—on the order of

minutes. That's far too slow to study any kind of biological dynamics.

But localizing even a single fluorophore from its diffraction-limited blob can still be impractically slow, and its spatial precision in practice falls far short of the theoretical ideal. To pinpoint the center of a blob of light with nanometer precision, one needs to know its shape extremely well, which requires collecting thousands of photons. All those photons are fluorescence emissions from the same fluorophore, which must be cycled thousands of times between the same two quantum states.

Not all fluorophores can even endure that much cycling—they get damaged by the laser or fall into a nonfluorescing quantum state long before they've emitted enough photons—and for those that can, the process takes tens to hundreds of milliseconds. Localization can be performed faster and more gently on fluorophores, but at the cost of spatial resolution.

Furthermore, camera-based localization has a systemic limitation, no matter how many photons are detected. Fluorophores absorb and emit light as dipoles, so their fluorescence doesn't emanate equally in all directions. If a fluorophore doesn't rotate freely over the exposure time, the center of the fluorescence blob on camera may be several nanometers removed from the fluorophore's true location, which hampers the imaging resolution.

Other techniques are capable of studying biomolecules with high spatial precision. There's cryoelectron microscopy (which garnered its own Nobel Prize; see Physics Today, December 2017, page 22) and its cousin cryoelectron tomography, but they require specimens to be frozen. Tethering a molecule to a bead held in an optical trap can track molecular motions on scales of nanometers and microseconds (see Physics Today, June 2016, page 14), but there's no guarantee that the force exerted by the tether doesn't interfere with the molecule's natural movement. The appeal of fluorescence imaging is that it's comparatively benign and noninvasive, so it's compatible with living cells. But it hadn't yet reached its potential for studying cellular machinery in motion.

In the dark

With MINFLUX, Hell's idea was to detect fluorophores not from the photons they emit, but from the ones they don't. "You still need a lot of photons to localize a molecule to within a nanometer," he says. "That follows from the uncertainty principle, and you can't get around it. But we can put the burden of the required number of photons on the laser, not the fluorophore. And there's no shortage of laser photons."

In the original MINFLUX (not an acronym, but an abbreviation for "minimal fluorescence photon flux"), a molecule is illuminated by a laser beam with a doughnut-shaped profile.[3] When the molecule is exactly in the center of the doughnut, it emits no fluorescence. Even one fluorescence photon is enough to show that the molecule is slightly off-center.

It's a little tricky to determine in what direction it's off-center. But Hell and colleagues developed an algorithm, a version of which is shown in figure 2a, to scan the beam's position and home in on the molecule quickly and efficiently: The localization precision scales exponentially with the number of fluorescence photons detected. (In contrast, the resolution of a camera-based localization scales with the square root of the number of fluorescence photons.) "That's a major thing," says Hell. "That's why this is so effective."

MINFLUX could provide complete—but slow—images of whole specimens labeled with many fluorophores. But it was also well suited, for the first time, for tracking single fluorophores with simultaneous nanometer and millisecond precision.

The MINFLUX apparatus is specialized and a bit complicated, and at first it existed only in Hell's lab. But he quickly commercialized the technology through his spin-off company, Abberior Instruments, to make the technique available to the broader community. The first commercial prototype went to EMBL, where Ries had already collaborated with Hell on MINFLUX applications in biology. "We were looking for an application that wouldn't be possible with any technology other than MINFLUX," says Ries, "and quickly we identified motor proteins in living cells, specifically kinesins."

Hell, meanwhile, was also studying kinesin, not in living cells, but as a test system for a new version of MINFLUX his group had developed. As shown in figure 2b, the new MINFLUX microscope replaces the doughnut beam with a linear interference pattern, which is shifted left and right to localize a fluorophore in the horizontal direction. The process needs to be repeated with an orthogonal interference pattern to find the molecule's vertical position. But because the intensity profile is better suited than the doughnut beam to fast and precise localization, the result is an improved resolution in space and time—and an unprecedented look at how kinesin moves.

Walk like a kinesin

From illustrations such as figure 1, it's hard not to see kinesin's resemblance to

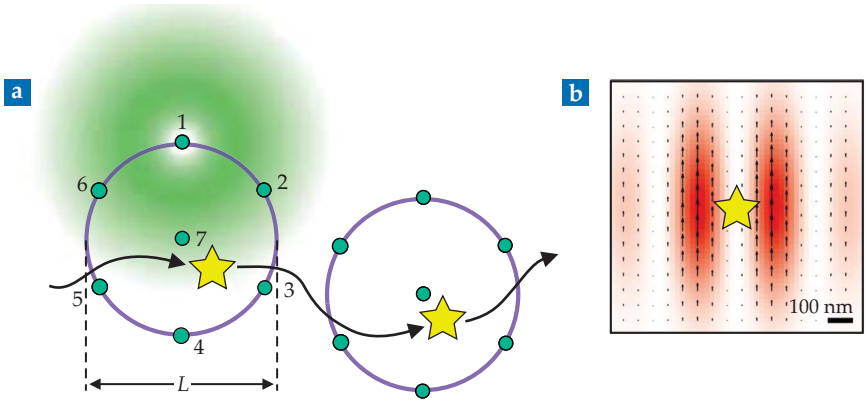

FIGURE 2. MINFLUX, an optical fluorescence technique, finds the positions of fluorescent molecules (yellow stars) quickly and precisely, using minimal fluorescence photon flux. In its original, recently commercialized version **(a)**, a doughnut-shaped light beam (green) probes several positions around a circle that's thought to contain the molecule. The circle diameter *L* is iteratively reduced to find the molecule with nanometer precision. In a newly developed version of the technique **(b)**, the doughnut beam is replaced with a linear interference pattern (red). Even though the molecule must now be localized in the horizontal and vertical directions separately, the localization is faster and more precise. (Panel a adapted from ref. 1; panel b adapted from ref. 2.)

a stick figure. The molecule has two feet that walk along a microtubule (part of the cell's cytoskeleton) and two hands that grasp cargo to carry it around the cell. But the resemblance in form doesn't necessarily mean that kinesin moves like a human does. For a long time, researchers debated whether kinesin really walks—taking alternate steps with its two feet, putting each in front of the other—or whether it shuffles along like an inchworm, with the same foot always leading.

That question was first answered in 2004 by the University of Illinois at Urbana-Champaign's Paul Selvin and colleagues using a technique called FIONA (which stands for "fluorescence imaging with one-nanometer accuracy," although it doesn't produce complete images, and the resolution in practice is coarser than 1 nm). They attached a robust fluorophore to a kinesin foot and collected the requisite thousands of photons to localize its position. It was already known from other methods that the kinesin body moves 8 nm with each step. Selvin and colleagues found that the foot moved 16 nm at a time, a clear sign that the feet moved alternately in a walking motion.[4]

To get that result, they had to slow the kinesin down by starving it of fuel. Kinesin walking at full speed can take tens of steps per second, whereas a single FIONA localization took 0.33 seconds. Like most cellular machinery, kinesin runs on adenosine triphosphate (ATP). In their *in vitro* experiment, Selvin and colleagues supplied an ATP concentration a thousandth of that found in cells, which reduced the kinesin speed to less than one step per second, slow enough for them to measure.

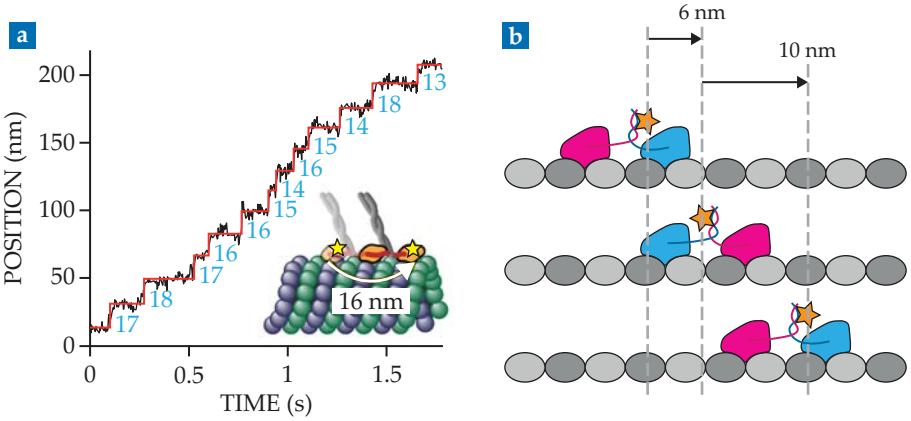

FIGURE 3. ATTACHING FLUOROPHORES (yellow stars) to different parts of a kinesin molecule probes different aspects of the molecule's movement. **(a)** A fluorophore on the kinesin foot reveals the average stride length of 16 nm. Because the kinesin body moves 8 nm with each step, the stride length is a clear sign that the kinesin feet step alternately. The data shown here were recorded in a living cell. **(b)** A fluorophore on the side of the kinesin stalk shows that the stalk rotates 180° between steps. The finding addresses, but doesn't definitively resolve, the question of whether kinesin walks facing forward or twirls like a ballerina. (Panel a adapted from ref. 1; panel b adapted from ref. 2.)

Merely knowing that kinesin steps with alternate feet doesn't explain everything about its mechanism. Does it really walk facing forward, with the right foot staying on the right and the left foot on the left, or does it twirl like a ballerina, with each foot passing the other on the same side? It's questions like that, among others, that Hell and colleagues hoped to address with their improved higher-resolution MINFLUX measurements.

In a twirling kinesin, the stalk would rotate 180° with each step, with the front of the stalk becoming the back and the back becoming the front. The stalk itself is too thin for even MINFLUX to resolve its front from its back. But because a fluorophore has nonzero size, the technique can distinguish a fluorophore attached to the front of the stalk from one attached to the back. As shown in figure 3b, a

fluorophore on a rotating kinesin stalk would be seen to take alternate steps of 6 nm and 10 nm, even though the stalk itself advances by a steady 8 nm each time.

When Hell and colleagues performed that experiment, the alternating step sizes are just what they found. The result isn't definitive evidence of a twirling mechanism—kinesin could still walk facing forward while twisting from side to side—but it doesn't rule it out.

Next steps

Both groups' MINFLUX experiments focus on tracking the position of a single fluorophore, with no information about the dynamics of anything else in the system. That capability works for studying kinesin, because the microtubules don't move much on the time scales of the experiments, even in living cells. But a more powerful tool would be one that simultaneously tracks the positions of two fluorophores that fluoresce in different colors. A two-color experiment could monitor protein interactions or conformations; with one fluorophore on each foot of a kinesin, it could settle the question of whether kinesin walks or twirls. "That's exactly what we're working on establishing next," says Ries.

For Hell, the next MINFLUX frontier is pushing the technique's resolution even further. The spatial resolution is already as good as it can be, because it's limited by the size of the fluorophore, but the temporal precision still has room to run. "The limiting factor is the fluorophore brightness," says Hell. A molecule that fluoresces more efficiently provides the same positional information in much less time. "With brighter fluorophores, we could get from one nanometer per millisecond down to one nanometer per ten microseconds," he says. "There's no reason that shouldn't be possible."

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References

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Trapped-atom analysis pushes calcium-41 onto the radiometric dating scene

With recent advances in laser technology and cold-atom methods, the technique's sensitivity to the isotope has reached environmental levels.

When Willard Libby and colleagues developed radiocarbon dating in the late 1940s, they faced an experimental obstacle: No radiation detection tool was sensitive enough to detect carbon-14 at its expected environmental concentrations—about 1 out of every 10^{12} carbon atoms.

To overcome that obstacle, Libby and coworkers built a sample chamber surrounded by multiple Geiger counters and significant shielding. It removed some background signal and allowed them to calibrate for still more, thereby enabling them to pick out the ^{14}C signal.

As the field of radiometric dating has expanded to include isotopes with longer half-lives, lower abundances, and trickier contaminants, so has the range of experimental challenges. Calcium-41 was identified in the late 1970s as a potentially useful radioactive tracer for studying biochemical and geochemical cycles because of its prevalence in both biological organisms and Earth's crust. And because ^{41}Ca has a longer half-life than ^{14}C—nearly 100 000 years rather than around 5700 years—it could date older specimens. But its low natural baseline abundance, about 1 out of every 10^{15} Ca atoms, kept ^{41}Ca dating out of experimental reach.

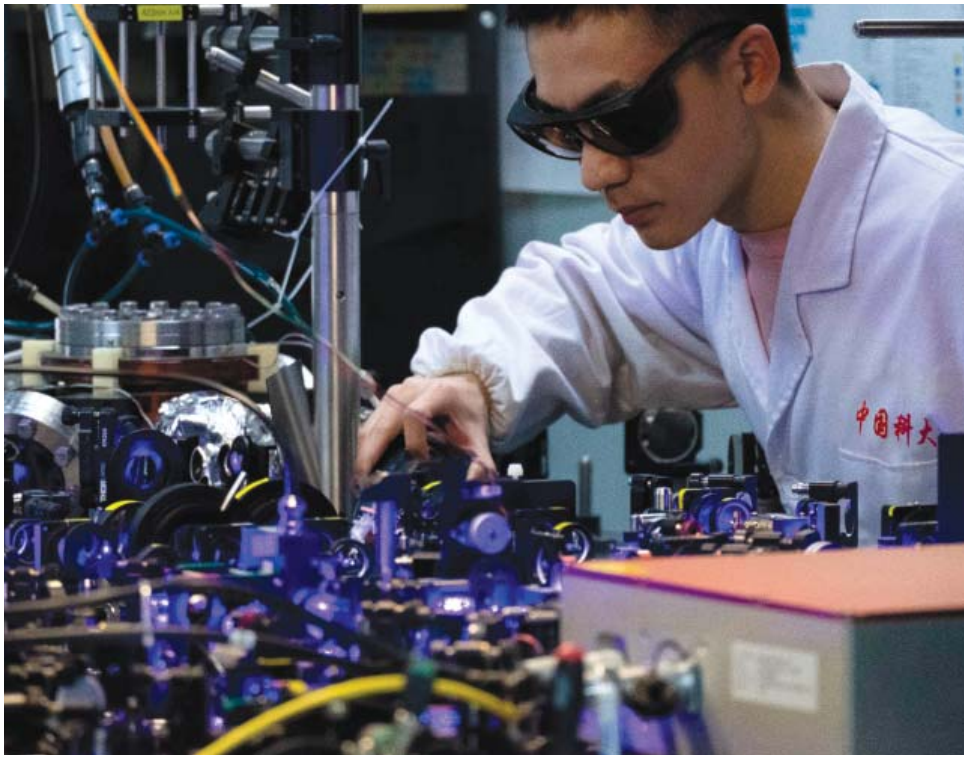

FIGURE 1. WEI-WEI SUN, a graduate student in the Laser Laboratory for Trace Analysis and Precision Measurements at the University of Science and Technology of China, adjusts the blue laser that slows and traps calcium atoms in the group's atom-trapping setup. (Courtesy of Wei-Wei Sun.)