Chemical & Engineering News

Cover Story

June 4, 2007
Volume 85, Number 23
pp. 13-17

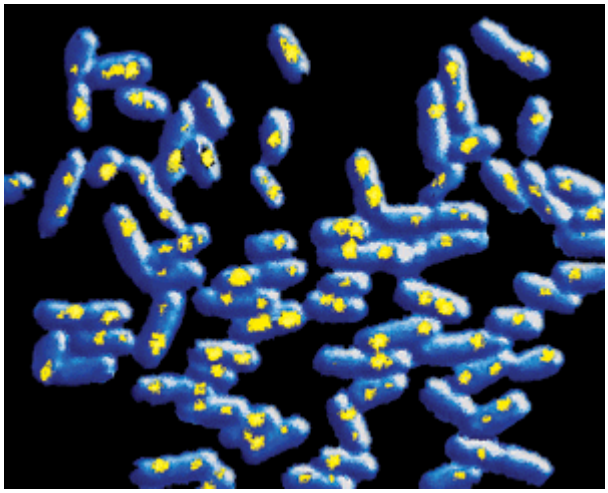
Peeking Into Live Cells

Fluorescent imaging of single molecules in live cells uncovers details of gene expression, structural proteins, and molecular motors

Celia Henry Arnaud

AVERAGES CAN sometimes mislead. Just looking at averages, an observer who didn't know better could conclude that every person is half male and half female. Only by looking at individuals does it become obvious that a person can only be one or the other.

In biological research, ensemble measurements can lead to the same sort of problem. Measurements that capture only the average of a population of molecules blur the differences between individual molecules, and important information is lost.



Courtesy of Gene-Wei Li and Peter Sims, Harvard University

Be specific Single transcription factor molecules bind to specific sites on DNA in single bacterial cells. The picture is graphically enhanced from the overlay of a fluorescence image of the labeled transcription factors (yellow) on the simultaneous transmission image of *Escherichia coli* cells (blue).

"One of the reasons for exploring single molecules is to ask whether there is different behavior from one copy of a molecule to the next copy," says W. E. Moerner, a physical chemist at Stanford University. "We want to see if there's any hidden heterogeneity." Each molecule experiences its own environment that influences its behavior. In addition, some molecules, such as enzymes, have different behavior at different times, something that gets smeared out if one measures only ensemble averages.

For some biological questions, single-molecule methods aren't just desirable, they're a necessity. For instance, at the cellular level, gene expression is inherently a single-molecule problem. Any cell contains at most two copies of the genetic DNA, depending on the stage of the cell cycle. The number of copies of a particular messenger RNA is small. And many of the final products—the proteins—are produced in small quantities, sometimes only a single copy.

Single-molecule methods are well-established for in vitro measurements. But now scientists are using these tools to probe live cells, where they can see biological molecules in context, and they are being rewarded with a new view of the workings of cells.

Most single-molecule work has relied on purifying and reconstituting individual components of a system outside the cell. Eventually, however, "you will reach a point of diminished return," at which the effort required to add another component to the system won't yield commensurate benefits, says Taekjip Ha, a biophysicist at the University of Illinois, Urbana-Champaign.

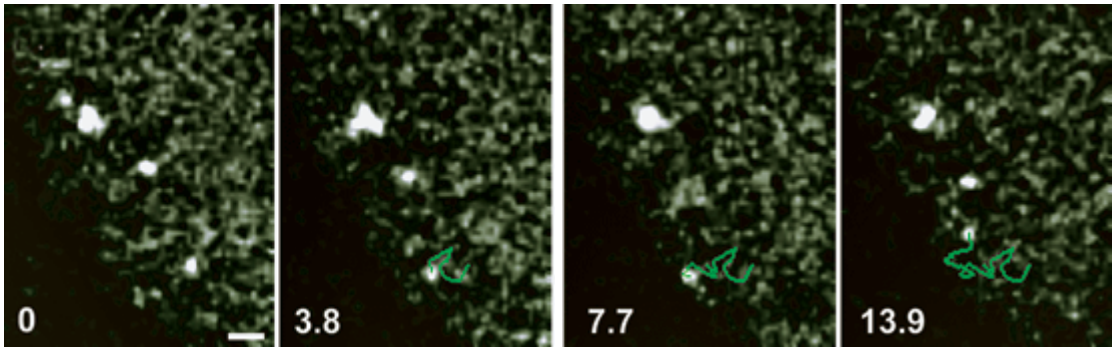
"With in vitro, you have a reductionist approach," says X. Sunney Xie, a chemistry professor at Harvard University. "If you really have all the parts, that's fine, but maybe we're missing something."

Nothing is left out when biomolecular processes are viewed in the context of their natural environment. Unfortunately, such single-molecule live-cell imaging measurements are still challenging because of shortcomings in probe design, methods for labeling the target protein with the probe, and live-cell imaging techniques.

The most common biological single-molecule experiments involve attaching a fluorescent probe to the molecule of interest. The ideal probe is small enough that it doesn't perturb the system, is bright and long-lived so it can be seen above the cell's background fluorescence for a long time, and is easily delivered to the molecule of interest in its natural environment. Unfortunately, no currently available probes meet all these criteria, so scientists are making do with what they have and improving probes for the future.

The most common probes are the rainbow of fluorescent proteins such as green fluorescent protein (GFP) or yellow fluorescent protein (YFP). These probes have the advantage that they can be genetically encoded as a fusion protein attached to the target protein, thereby circumventing the delivery challenge. However, their signals are relatively weak, they blink, and their color quickly fades when exposed to light. Most scientists have learned to live with these drawbacks.

ULTIMATELY, the semiconductor nanoparticles known as quantum dots may turn out to be the best probes, according to several scientists. But quantum dots currently fall short, too. The main problem with them is their size: They're too big. The nanoparticle itself is about the same size as GFP, but to make quantum dots water soluble and to attach them to biomolecules, they must be coated with various agents that nearly double their size. "They become so large that what you can do with them is limited," Ha says.



Moving message In live cells, green trace marks diffusional pathway of a single mRNA molecule (bright spots). Numbers indicate elapsed seconds; bar = 1 µm.

To solve this problem, Alice Y. Ting and Moungi G. Bawendi of Massachusetts Institute of Technology have synthesized quantum dots that are half the hydrodynamic diameter of those that are commercially available. Ting can use these smaller quantum dots to study synaptic receptors; the commercial quantum dots don't fit in the neuronal synapse.

But quantum dots are difficult to get to the desired location in the cell. "Most high-throughput methods to introduce quantum dots into cells tend to end up with the quantum dots engulfed in organelles with membranes around them. They're not really introduced in a uniform way into the cytosol," says Shimon Weiss, a physical chemist at the University of California, Los Angeles. "That's a big problem."

Targeting remains a challenge for all types of probes, particularly quantum dots and synthetic dyes. In the past, scientists have gotten the probe to the right place by using a designed protein tag that binds the probe specifically and can be fused to the intracellular protein of interest. It would be better to identify

smaller peptide tags that have the same specificity for the probe. But "it's extremely difficult to do that just by rationally designing a chemical structure and complementing it with a peptide recognition sequence," Ting notes.

Ting is tackling the problem with enzyme-mediated site-specific protein labeling. Kai Johnsson at the Swiss Federal Institute of Technology in Lausanne has pioneered similar methods.

Ting uses bacterial labeling enzymes that have been tweaked to recognize a particular peptide sequence that she adds to the protein of interest. The labeling enzyme installs a given probe at the peptide sequence, thereby labeling the protein of interest. "We want the labeling method to be as general as possible, where we have the ability to label any protein of interest with any fluorophore or probe," she says.

Researchers are pushing the sensitivity and accuracy limits of imaging methods so that they can pinpoint the location of each fluorescent probe in live cells. To achieve the single-molecule sensitivity needed for in vivo studies, Xie uses an approach that he has characterized as detection by confinement.

In this method, which he also calls LIVE (local in vivo enhancement), fluorophores become visible when their motion is slowed down enough that they stay in one place. Their fluorescence is then strong enough to be seen above the background rather than blurred across the whole cell.

Paul R. Selvin, a biophysicist at the University of Illinois, Urbana-Champaign, coined the name FIONA (fluorescence imaging with one nanometer accuracy) to describe a high-accuracy method for fluorophore detection. By collecting enough photons, the centers of even fairly large fluorophores can be located with 1-nm accuracy in live cells. The method is used to track the movement of individual particles rather than resolve multiple particles.

Scientists are starting to use these single-molecule imaging methods to look at a variety of biological questions.

To get a better look at one part of the gene expression process, Robert H. Singer of Albert Einstein College of Medicine has used single-molecule methods to track single mRNA molecules in live cells as they make their way from the nucleus to the cytoplasm (*Science* **2004,** *304,* 1797). Singer first looked at single RNA molecules in fixed cells nearly a decade ago, but such an approach proved dissatisfying. "If you're looking at a dead cell, you are only looking at things that have been stopped. You have no idea what forces are acting on those molecules to allow them to get where they are," he says.

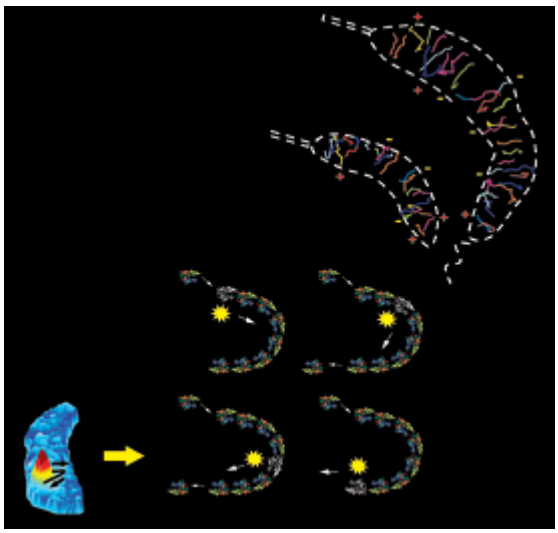
In order to see the mRNA in live cells, Singer needed to make the molecules bright enough to see above the cells' natural fluorescent background. He played a bit of a trick by labeling individual RNA molecules with many copies of a YFP fusion protein that binds to stem loops on the RNA via a noncovalent interaction.

By looking at single RNA molecules in live cells, Singer and coworkers determined that diffusion governs their movement. "That was a big revelation because some people thought these RNA molecules moved out of the nucleus through specific highways," Singer says. "The truth is they just bang around until they accidentally find their way out. Over the small distances of a cell, diffusion is probably the most effective way to move things around."

Last year, Xie's group reported real-time observations of protein synthesis resulting from gene expression, one molecule at a time, in *Escherichia coli* cells (*Science* **2006,** *311,* 1600). Recently, Xie, postdoc Johan Elf, and grad student Gene-Wei Li have used single-molecule methods to observe another part of the gene expression process-the binding of a protein transcription factor to DNA. Transcription factors are proteins that control the formation of an RNA copy of a gene. "Nobody has seen this binding and unbinding in a live cell in real time," Xie says.

Previously, Paul Blainey, a joint graduate student of Xie and his Harvard colleague Gregory L. Verdine, observed the nonspecific binding of a DNA repair enzyme to DNA during in vitro experiments (*Proc. Natl. Acad. Sci. USA* **2006,** *103,* 5752). In that case, the enzyme binds to the wrong spot and then slides along

the DNA looking for the right spot. Now, they have seen the same binding behavior in transcription factors in live cells (*Science* **2007,** *316,* 1191).



Courtesy of W. E. Moerner

On the treadmill The dynamics of the bacterial cytoskeleton of a living *Caulobacter crescentus* cell can be observed by single-molecule fluorescence imaging of a fusion of yellow fluorescent protein with the bacterial actin MreB (actual image lower left). As treadmilling occurs in a curved MreB filament, the single fluorescently labeled protein moves through the filament across the cell (black line in the actual image, schematic in center). Various tracks taken by single treadmilling MreB proteins show the shape and length of filaments in *C. crescentus* cells in different stages of life (upper right).

Dynamic information allows them to distinguish between specific and nonspecific binding. "We found that the transcription factor spends 90% of the time on DNA nonspecifically bound," Xie says. The other 10% of the time is spent diffusing through the cytoplasm. They observed that the transcription factor spends a few milliseconds on the DNA before it falls off. When the transcription factor stumbles on the right spot, it camps out there for about 40 minutes. When it pops back off, RNA polymerase starts transcribing the gene.

"We were able to see the nonspecific binding using a technique similar to strobe photography," Xie says. The laser pulses used to excite the fluorescence are very short relative to the frame rate of the camera. During each pulse, the protein nonspecifically bound on DNA doesn't move far, making it visible above the background. With shorter pulses, the researchers can even see the freely diffusing protein in the cytoplasm.

In addition to gene-expression applications, researchers are also using single-molecule methods to study cytoskeletal proteins and molecular motors that move cargos around the cell. Most studies of such molecules have been in vitro, but some scientists have started to look in live cells, too.

Selvin is studying the workings of dynein and kinesin, the motor proteins that transport cargo along the microtubule highways in the cytoskeleton. He wanted to know whether the two proteins, which move in different directions along the microtubules, cooperate with one another or are in a slugfest for control of cargo.

In collaboration with Vladimir I. Gelfand of Northwestern University School of Medicine, Selvin watched kinesin and dynein move organelles called peroxisomes around the cell (*Science* **2005,** *308,* 1469). Using FIONA, they achieved 1.5-nm accuracy and 1-millisecond time resolution, which allowed them to work at physiological adenosine triphosphate (ATP) concentrations. ATP is the molecule that provides the energy the motor proteins need to move. To study these motors' movement in vitro, scientists typically must use only subphysiological concentrations of ATP.

In another study, Selvin and Gelfand tracked kinesin- and dynein-toting melanosomes—organelles that carry the pigment melanin—around the cell (*Proc. Natl. Acad. Sci. USA* **2007,** *104*, 5378). Because they are darkly colored, individual melanosomes can be seen by bright-field imaging rather than fluorescence

imaging. An unusual kind of kinesin called kinesin-2 is associated with the melanosomes. The two legs of kinesin-2 are not identical, but it still walks with the same 8-nm steps as kinesin-1, which has identical legs. In addition, they were able to see kinesin hand the melanosome off to another motor protein called myosin V, which moves along actin filaments.

Xie is also studying molecular motors. His graduate students Xiaolin Nan and Peter A. Sims started out by labeling them with quantum dots (*J. Phys. Chem.* B **2005,** *109*, 24220), but more recently, they have labeled the cargo with gold nanoparticles. The key to studying the molecular motors is high time resolution, Xie says. "When the concentration of ATP is low, it's easy to follow the individual steps," Xie says. "Inside living cells, the ATP concentration is high. One millisecond time resolution is not enough to resolve the individual steps."

Xie's team achieved the necessary time resolution by using a detector called a quadrant photodiode, which gives them 25-microsecond resolution. "We were able to resolve individual steps of the cargos even at the highest cargo velocities," Xie says. Kinesin moved with the expected 8-nm steps dictated by the lattice spacing on the microtubules. Dynein, which is larger than kinesin, was able to take longer steps. They found that the motors take smaller steps when they carry larger cargos.

Researchers are also using single-molecule methods to study structural proteins. Moerner, working with graduate student So Yeon Kim and microbiologist Lucy Shapiro of Stanford, uses single-molecule approaches to observe the bacterial actin MreB, a cytoskeletal protein, in the bacterium *Caulobacter crescentus* (*Proc. Natl. Acad. Sci. USA* **2006,** *103,* 10929). These bacterial proteins polymerize to form filaments, with monomers being added at one end and falling off at the other, a process termed treadmilling. "People used to think that bacteria were just bags of enzymes, so for there to be any cytoskeleton is an exciting new area," Moerner says.

Moerner's team labels just a few of the MreB molecules with YFP in a background of unlabeled MreB. The labeled molecules are visible only when they are part of the filament. Otherwise, the protein moves too quickly to be seen. By watching when a labeled MreB joins the filament and falls back off, the researchers can trace the length of the filament.

Now that single-molecule techniques have moved from the test tube to the cell, the next logical step is tissues and even whole animals. Such work is only in the preliminary phases, but researchers such as Weiss and Thomas Schmidt of the University of Leiden, in the Netherlands, are starting to look at single organelles in zebrafish. In unpublished work, Weiss and Laurent Bentolila of UCLA have observed single vesicles trafficking inside a single cell within the fish. "In the vesicle, you can have more than one quantum dot, but the fact that you can see a small organelle in a whole animal is quite impressive," Weiss says.

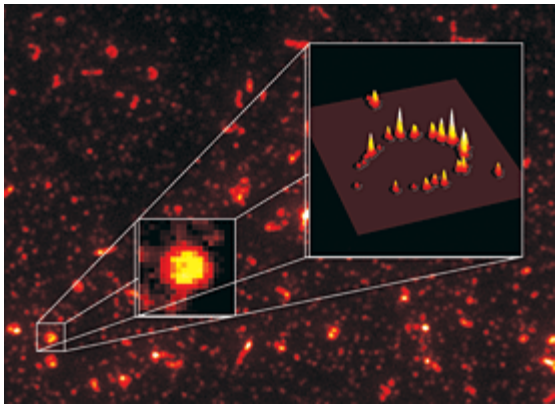
Single-molecule live-cell imaging is still in its early days, and opportunities are plenty to keep many scientists busy for a long time. Originally, single-molecule work was restricted to a handful of researchers, but it will become "an industry," Weiss predicts. "Over time, it will get easier, because you will eventually have better probes and push-button machines that will make things simple enough for everybody to use it."

Ting hopes to see a day when single-molecule imaging can be easily used in any cell biology lab. "By making super-good probes, I'm hoping there will be less of a need for specialized equipment," she says.

Xie thinks that single-molecule imaging is already easier than many people imagine. He notes that both the microscope and image sensor his team uses are commercially available. "It's easy to do, and there is so much to do. I really think this is becoming a powerful tool for biological discovery."

Zooming In

Super-resolution Techniques On The Horizon


Courtesy of Xiaowei Zhuang

Storm Front Not yet a live-cell technique, the super-resolution technique STORM (stochastic optical reconstruction microscopy) captures individual fluorophores on a ring of DNA coated with RecA, an enzyme involved in DNA repair.

In the future, super-resolution methods that push the limits of single-molecule imaging could revolutionize the study of individual molecules in live cells.

These methods have catchy acronyms such as STORM (stochastic optical reconstruction microscopy) and PALM (photoactivated localization microscopy) (C&EN, Sept. 4, 2006, page 49). They are based on the principles of single-molecule microscopy. These methods and other super-resolution microscopy techniques have not yet been used for live-cell imaging, but researchers in the field say they are close to making this possible.

"We're still developing the technology, and applying it to fixed cells makes it easier to figure out problems and solutions," says biophysicist Xiaowei Zhuang of Harvard University, leader of the STORM team. Both STORM and PALM rely on photoswitchable fluorophores such that only a subset of fluorophores well-separated from one another can be turned on at one time.

These super-resolution microscopy methods have improved the spatial resolution of imaging by at least one order of magnitude compared with conventional optical imaging, which is diffraction limited to about 300 nm, Zhuang notes. She envisions that these methods will eventually make imaging cells and tissues at molecular resolution possible. These methods are currently too slow for most live-cell imaging. However, "we are improving the imaging speed and expect that live-cell imaging with STORM will be possible," Zhuang says.

Scientists such as Alice Ting at Massachusetts Institute of Technology and Shimon Weiss at the University of California, Los Angeles, are excited by the prospects for the super-resolution techniques. "They can potentially give the spatial resolution of electron microscopy but be done on live cells," Ting says.

Cover Story

[Top of Page]