Leading Edge
Essay
Cell

Microscopes for Fluorimeters: The Era of Single Molecule Measurements

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As transforming as the first atomic resolution view of myoglobin in the late 1950s, scientists can now use a suite of single molecule technologies to watch protein macromolecular machines executing their functions "in real time." This Essay highlights applications and challenges of single molecule studies in structural biology, cell biology, and biotechnology.

Valuable information is often lost when data from a large population are examined in aggregate. To illustrate this, let us consider the recent and historic presidential election, where the popular vote favored Barack Obama (53%) over John McCain (46%). These numbers convey the outcome, but little more. However, progressively greater insight into American sociology and culture can be derived from the voting records of each of the 50 states, from individual counties within these states, and finally from exit polls of individual voters.

In biology, measurements of individuals also can reveal new information that would otherwise be lost in ensemble averages. Biochemists and biophysicists have traditionally used fluorimeters or spectrophotometers in their trade, which at best detect the activity of 50 million proteins (~a femptomole). However, many are now turning toward microscopes that can measure outputs from single molecules. In addition to enabling researchers to study vanishingly small amounts of material, single molecule technologies permit analysis of heterogeneous molecular populations or complex kinetics and dynamics of chemically identical molecules. Furthermore, single molecule measurements have allowed the study of protein outputs that previously were very difficult to explore (e.g., forces and steps of molecular motors). Applying a similar logic on a larger scale, cell biologists are uncovering new information about how cells make decisions and integrate information from their environment by measuring outputs from individual cells.

The broad impact of single molecule technologies also illustrates how rapid advances can be made through physicists, biologists, and chemists working together to solve important problems in the biological sciences. Physicists and biophysicists have contributed by developing new microscopes that can measure outputs of single molecules with stunning accuracy. Biologists and chemists have participated in this partnership by engineering molecules and developing fluorescent dyes for single molecule assays. As pioneers continue to push the limits of what can be measured at the single molecule level, they have left behind a "wake" of mature and readily accessible technologies and methods. Indeed, many single molecule measurements have become relatively easy to perform. One goal of this Essay is to encourage more laboratories to incorporate these techniques into their routine toolkit for studying macromolecules both in vitro and in vivo.

A Historical Perspective

A brief history of single molecule measurements in biology illuminates the trajectory of the field and the diverse methodologies that have emerged. The development of patch clamp recordings by Neher and Sakmann in 1976 represented the birth of single molecule measurements. This revolutionary technique, capable of measuring the 10^{-12} amps of current flowing through a single ion channel, enabled researchers to measure individual opening and closing events, reflecting the underlying conformational changes in the pore of the ion channel. Despite the enormous benefit of patch clamp measurements to neurophysiology and the biophysical understanding of ion channels (and awarding of the Nobel Prize to Neher and Sakmann in 1991), it took many years before single molecule measurements spread to other biological disciplines. Perhaps ion channels were viewed as a "special case," given that the signal (current) from a single molecule could be greatly amplified. However, outputs of single molecules can be measured with clever assays that do not require sophisticated technologies. Probably more likely, biochemists in the 1970s and 1980s did not yet envisage a compelling reason to invest their energies in making single molecule measurements. The spectrofluorimeter still ruled the kingdom.

One of the first "single molecule" experiments to follow ion channels was the measurement of individual microtubules (Mitchison and Kirschner, 1984). The polymerization of tubulin into cylindrical 25 nm diameter microtubules was routinely measured by detecting the scattered light using a spectrophotometer. When the polymerization reaction reached a steady state (polymer mass remaining constant), little change in microtubule length was expected, as the rates of subunit addition and dissociation should be equal. By looking at individual microtubules by immunofluorescence and measuring their length at steady state, Mitchison and Kirschner discovered surprisingly that some of the microtubules in the population were growing

longer, whereas others were becoming shorter. From these single microtubule observations, the investigators correctly postulated that this unexpected "dynamic instability" was due to hydrolysis of GTP by tubulin after it became incorporated into the microtubule. At about the same time, the relatively new technology of video microscopy (acquiring images with a camera rather than by eye) allowed Horio and Hotani (1986) to watch and record the "live" growth and shrinkage of single microtubules by dark field video microscopy, illustrating the power of observing individual macromolecules over time (Movie S1 available online). Today, dynamic instability is easily appreciated by watching single GFP-tagged microtubules grow and shrink in living cells using time-lapse microscopy. These experiments on single microtubules provide marvelous examples of how new mechanistic insights can emerge from studying individuals rather than populations.

Single microtubule observations continue to yield new surprises and discoveries. For example, differences in the growth and shrinkage kinetics of single microtubules in living cells versus that measured for pure tubulin in vitro are leading to discoveries of how microtubule-associated proteins modulate dynamic instability. The tale of microtubule dynamics provides a paradigm in which the biochemistry of a molecular machine is dissected in vitro and then the regulation of this process is understood in the context of living cells, with the observations of single macromolecules contributing prominently to both levels of investigation.

The next round of single molecule measurements centered around cytoskeletal motor proteins. Early observations of organelle transport in reconstituted assays suggested that few or possibly one kinesin motor might suffice to transport cargo. By systematically varying the density of purified kinesin adsorbed onto a glass slide surface, a single kinesin molecule was indeed shown to be capable of moving processively along a microtubule for several microns (Howard et al., 1989). Here, a relatively large object (a microtubule) that could be easily imaged by conventional microscopy provided a means for measuring the output of a much smaller, "invisible" molecule (a kinesin motor). Using a similar general strategy, the rotation of the central γ-polypeptide subunit in the F1-ATPase was observed using an attached fluorescently labeled actin filament (easily visualized by conventional microscopy) (Movie S2) (Noji et al., 1997). This experiment elegantly proved, beyond any doubt, the Nobel Prize winning hypothesis of rotating catalysis championed by Boyer and Walker. Thus, very successful single molecule experiments have been and still can be performed with simple, conventional types of microscopy, provided that one has the right type of assay.

Protein Machines: Forces, Steps, and Conformational Changes

With the realization that purified enzymes could be assayed at the single molecule level, researchers began to measure more challenging outputs, such as the steps and forces produced by molecular motors. Huxley and Simmons (1971) first tackled this problem, not by measuring single molecules but by attempting to synchronize the power strokes of the thousands of myosins in a muscle fiber. By quickly releasing the tension in an isometrically contracting muscle fiber, they measured the force recovery in the ensuing few milliseconds, a time during which attached, "primed" myosin motors could complete a force-producing conformational change but too fast for new motors to engage and go through another round of ATP hydrolysis. The Huxley and Simmons estimates of the force and step size of single myosins were remarkably close to currently accepted values, but this heroic measurement was difficult to perform and could not be extended to other force-generating systems that could not be studied with a tensiometer.

Two decades passed before technologies and assays enabled the direct measurement of forces and steps from single motors. The first studies used thin microneedles with attached actin filaments that were cast onto awaiting myosin filaments. Like a fishing rod being bent by a powerful fish, the deflections of the microneedle reported the forces exerted by myosin upon the filament (Kishino and Yanagida, 1988). A few years later, optical traps (which exploit forces generated as a result of focusing a powerful laser beam upon a small ~1 μm bead) were used to measure the pN forces and nanometer steps taken by single kinesin and myosin motors (Svoboda et al., 1993; Finer et al., 1994). The sensitivity of optical trapping microscopes is amazing, given that the forces produced by single motors (usually ~5 pN) are several orders of magnitude smaller than the gravitational attraction occurring between the reader and this issue of *Cell* (assuming that you are one of the rare individuals who is actually reading a hard copy of the journal). Optical trapping has proven to be a powerful and versatile technology for understanding the forces produced by proteins as well as how these machines respond to an applied force. Other single molecule nanomanipulation methods such as atomic force microscopy and magnetic tweezers also are now widely used for such purposes.

From the Physics Laboratory to the Biology Laboratory

Single molecule measurements were originally restricted to laboratories that had the expertise to build their own optical bench microscopes. For most biologists, these experiments appeared sufficiently daunting that they did not consider such work. However, this situation is changing. Consider the trajectory of single molecule fluorescence measurements. Detection of single fluorescent dye molecules was first achieved in 1989, and their imaging was advanced a few years later using near-field scanning microscopes. These pioneering experiments were influential for photophysics and illustrated what was possible to achieve, but the early technology was complex and not applicable to biological samples (performed under a vacuum, often at very low temperatures). Only a few years later, new methods brought single molecule fluorescence experiments into the realm of biology. In particular, Yanagida and colleagues realized that recently developed cameras were sufficiently sensitive to detect the photons emitted from a single fluorophore and that the key to their detection was to eliminate background from autofluorescence and out-of-focus fluorescence dyes, which swamped the sig-

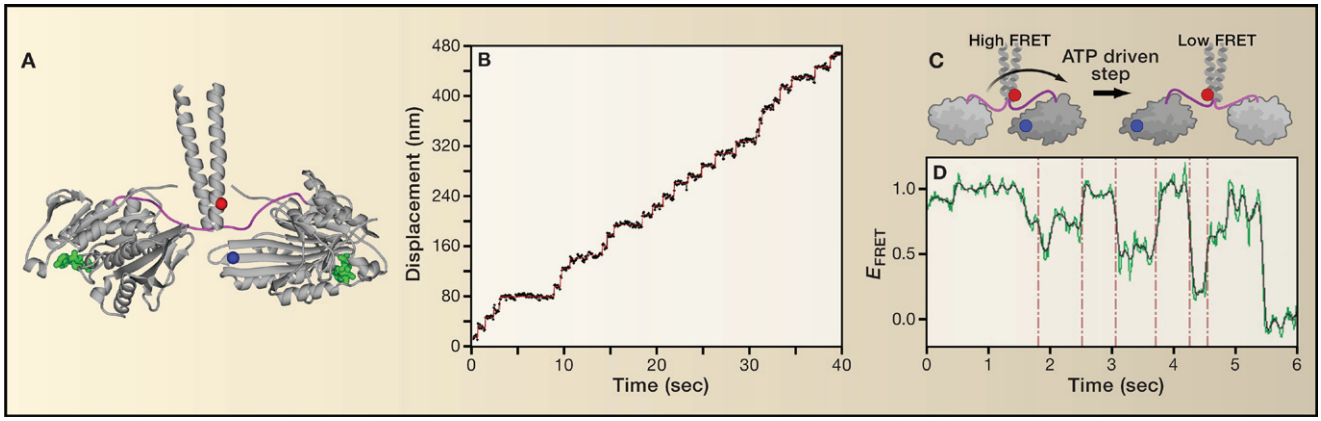

Figure 1. Bridging Two Worlds

There is a need to link atomic-resolution structures obtained by X-ray crystallography and "dynamic" information obtained by single molecule measurements. (A) A ribbon diagram of a kinesin dimer obtained by crystallography (protein database file name 3kin). Such structures provide a wealth of information about protein chemistry, but these static images do not satisfy our curiosity about the dynamics of protein conformational changes during motility. In addition, a structure obtained through crystallization in solution may not correspond precisely to a state adopted by a kinesin motor executing motility on a microtubule.

(B) Nanometer precision tracking of kinesin moving along a microtubule can be obtained by fluorescence imaging or optical trapping (shown is the position of a fluorescent quantum dot attached to a single motor domain of the kinesin dimer; data courtesy of Ahmet Yildiz). This experiment provides a great deal of information on dynamics (step sizes and "dwell" time between steps) but not on protein structure.

(C and D) By attaching different fluorophores to two residues of a protein (location of residues shown in panel A; one dye, depicted in blue, is on the main enzyme core, the other dye, depicted in red, is on the mechanical element of kinesin called the neck linker, pink), one can determine if particular elements of a protein undergo relative movement with respect to one another using single molecule FRET. (D) Panel shows abrupt FRET efficiency changes, which report on ATP-driven conformational changes of the neck linker as a single kinesin molecule walks along a microtubule (Tomishige et al., 2006). However, this experiment provides only limited information on the many conformational changes in the motor domain that are likely to occur during kinesin motion.

nal from the single fluorophore (just as stars cannot be discerned amidst the "background" light of the noontime sun). These investigators showed that single molecule detection was achievable by confocal imaging with some effort, but they also discovered that the less widely used method of total internal reflection fluorescence (TIRF) illumination was a particularly effective and simple method for imaging single fluorophores (Funatsu et al., 1995). Moreover, this wide-field imaging method could be performed in aqueous buffer and with rapid (e.g., 30 frame/s) acquisition, conditions that were familiar and friendly for biological experimentation.

Now, single molecule fluorescence imaging can be readily achieved in virtually any laboratory, provided that one has ~$200,000 in financial resources. Several commercial vendors package laser-based TIRF systems with outstanding objectives (1.49 N.A.) that optimize photon collection and make alignment of total internal reflection illumination relatively easy. Improved dichroic mirrors and new high-sensitivity cameras (particularly the new EM-CCD cameras) offer exceptionally high sensitivity and very low noise. In the Woods Hole Physiology Course, it is possible to set up a commercial single molecule TIRF system during the day and have students make movies of single molecules in vitro or in vivo by dinner time (Movie S3). In addition, new fluorescent dyes (e.g., Cy3) and quantum dots produce brighter signals with less photobleaching than earlier dyes. Optical traps also are becoming easier to build and commercial microscopes with optical trapping microscopes recently have entered the market. One can dispel the myth that single molecule imaging is "too hard" for most biology laboratories to undertake.

This is not to say that all single molecule experiments are easy! As some experiments have become easier, researchers are constantly pushing single molecule technologies. As an example, Block and colleagues (Abbondanzieri et al., 2005) have refined their optical trap so that it can measure polymerase stepping at the level of individual DNA bases (3.4 Angstroms; approximately 3-fold the diameter of a hydrogen atom). However, achieving this resolution required a high degree of vibration isolation, including enclosing part of the microscope in an atmosphere of helium. The codon-by-codon movement of a ribosome along an mRNA also has been measured, providing the first dynamic picture of protein translation (Wen et al., 2008). These are just two examples of many remarkable experimental efforts to extract fine details of how molecular machines work. Although such initial breakthroughs often involve complex instruments and assays, a natural progression is to make these measurements easier for the next generation of PhD students and postdoctoral fellows within a lab and subsequently have the techniques disseminated to other laboratories (sometimes through commercialization).

Molecular Machines: Watching Biochemistry in Action

Single molecule measurements have made their biggest impact in studying the mechanisms and properties of biological macromolecules in vitro. Steady-state measurements of large numbers of molecules can obscure interesting behavior (e.g., the example of microtubules discussed above). Pre-steady-state kinetics measurements tend to be more informative, but rather difficult synchronization methods (e.g., millisecond mixing of a ligand with an enzyme or the mechani-

cal release of muscle fibers) are required to tease out time-dependent reactions in large populations. Measuring single molecules removes these constraints, as one can follow time-dependent changes directly with very small amounts of material and without confounding signals of "out-of-step" molecules. Moreover, one can build up statistics from measurements on many molecules, allowing determination of rate constants that have been measured by population pre-steady-state kinetics. Biochemical kinetics can now be performed with a microscope instead of a spectrofluorimeter.

But single molecule measurements are not just a new way of performing traditional biochemistry, they are providing insights into macromolecules that simply were not accessible by earlier methods. Using optical tweezers, magnetic tweezers, glass needles, or atomic force microscopy, it is possible to poke, bend, twist, pull, or otherwise torture single molecules with infinite variation and creativity. In addition to the inherent joy of being able to master something so small, these techniques are opening up entire new fields, allowing biophysicists to understand how proteins generate and respond to force and how physical interactions hold macromolecules together (e.g., protein and nucleic acid folding).

A great deal of information, however, is still missing from most single molecule measurements, namely the precise structural changes that occur as a protein moves, catalyzes a reaction, or responds to force. In contrast, X-ray crystallography provides the necessary atomic resolution information that one seeks, but alas, the atomic model is an average of large numbers of "nondynamic" proteins in a crystal. Perhaps the biggest challenge is to bridge the information gap between high-resolution models derived from crystallographic techniques and the lower resolution, dynamic measurements made by single molecule microscopes (Figure 1). It would be wonderful to watch a "movie" of a single protein executing its activities at near atomic resolution or see how its structure deforms under tension. But the "premier showing" of such a movie is a long way off, largely due to the inherent limitations in pushing spatial and temporal resolution simultaneously.

However, single molecule fluorescence resonance energy transfer (to measure nanometer-scale changes in protein structure; Figure 1) and fluorescence anisotropy (to measure orientation changes in protein domains) provide windows into protein structure, albeit a fuzzy and partial view. These experiments are still quite laborious, usually limited by one's ability to label molecules with fluorescent dyes in specific locations. A very productive hour on a single molecule microscope might represent the culmination of a year's effort of protein engineering and chemical labeling. Thus, improving methods for obtaining readouts of structure and conformational changes represents an important challenge for the single molecule field.

Would such a technological push be worth the effort? The answer is likely "yes," as we still have much to learn about the dynamics of protein machines that cannot be gained from X-ray crystallography. For example, tension sensing is becoming increasingly interesting to cell biologists and biophysicists. By combining "nano manipulations" (e.g., optical and magnetic traps, and atomic force microscopes that apply tension to individual proteins or larger complexes such as ribosomes or focal adhesions) with conformational FRET sensors or readouts of chemical states (e.g., the nucleotide state of an enzyme; Adachi et al., 2007; Sakamoto et al., 2008), new insights might be gained about how proteins respond to tension. Single molecule measurements also might revise our standard textbook portrayal of protein "machines" as invariant robots that proceed through a series of stereotypical chemical and structural states. A few studies with single molecule probes have found that proteins can rapidly fluctuate between conformations (even with the same chemical intermediate in the active site) or can show slower time-dependent variations in output. For example, work by Xie and colleagues reveals that a single cholesterol oxidase enzyme fluctuates between bursts of fast turnovers and then a series of slower turnovers, which they attribute to the enzyme shifting between different conformational states (Lu et al., 1998). Time-dependent changes in dynein's step size also have been observed, which might reflect different conformational states (Gennerich et al., 2007). Variations between single molecules in, for example, the velocity of one motor protein compared with another may reflect different posttranslational modifications or perhaps even long-lived structural states (loosely analogous to distinct conformational states of a prion protein). To address such questions, improved tools are needed for connecting single molecule output to an understanding of protein structure. We also need to train a new generation of investigators who can think and work fluidly with classical structural methods and single molecule techniques.

Single Molecules in Living Cells

Single molecule studies in vitro have stimulated a new frontier of single molecule measurements within cells, mainly using fluorescence techniques. However, living cells pose several additional challenges compared with in vitro observations, such as higher intrinsic background fluorescence and greater difficulty in fluorescently tagging macromolecules. For eliminating background fluorescence and achieving single molecule detection, total internal reflection illumination provides a powerful means of imaging single fluorescently tagged proteins at or near the plasma membranes of living cells. Such methods have allowed, for example, the diffusional motion of plasma membrane proteins and protein-protein interactions within membrane microdomains (Douglass and Vale, 2005), the turnover and stoichiometry of protein complexes (e.g., bacterial flagella; Leake et al. 2006), and the dynamics of actin filaments (Watanabe and Mitchison, 2002).

TIRF is effective for imaging single molecules near the plasma membrane but cannot be used to probe deeper structures. However, other strategies have allowed researchers to image the actions of single molecules in the interstices of the cell. One recent method, called highly inclined thin illumination (which can be readily implemented by most TIRF microscopes), has been used to image single green fluorescent protein (GFP) molecules at the nuclear envelope (Tokunaga et al., 2008). Another strategy is to use quantum dots, which shine above the background fluores-

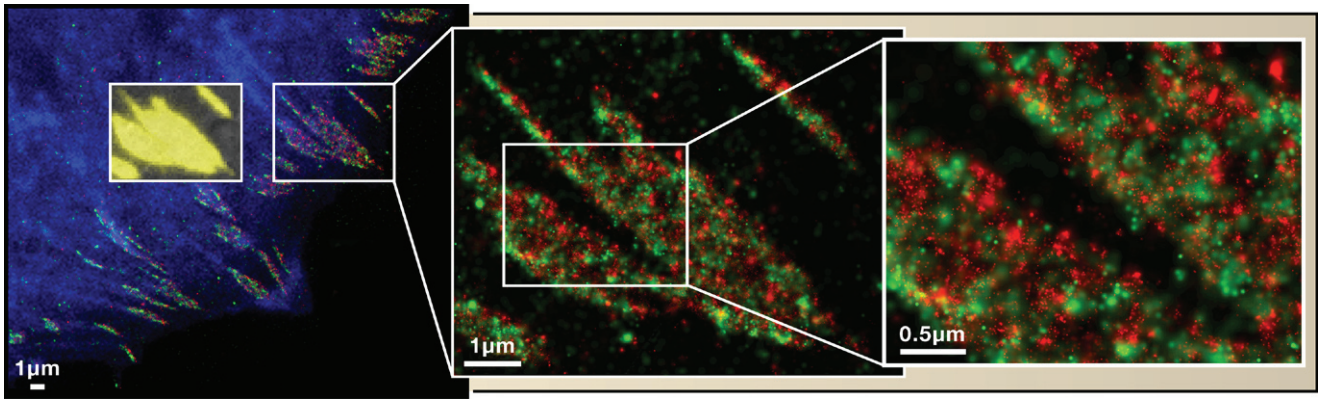

Figure 2. High-Resolution Fluorescence Imaging using Single Molecule Detection
New techniques like PALM, fPALM, and STORM build up images from repeated centroid measurements of individually resolved single fluorophores. This PALM image shows two different photoactivatable fluorescent proteins fused to paxillin (green) and vinculin (red) at peripheral adhesion complexes, with the boxes showing increasing magnifications. The PALM image shows that these two proteins occupy different regions of the adhesion complexes. A lower resolution conventional wide-field fluorescence image (yellow image, left, resulting from overlay of the green and red images) cannot resolve such details. Image courtesy of H. Shroff and E. Betzig (Shroff et al., 2007).

cence of the cytoplasm. But delivery of quantum dots to the inside of cells is problematic, unless they are taken up by the cell through endocytosis. An alternative strategy is to increase signal by attaching several fluorophores to one molecule. As an example, tracking of single ribonucleoprotein (RNP) particles within the nucleus was achieved by targeting several GFP fluorophores to a single mRNA (Shav-Tal et al., 2004). In some cases, one can amplify the output of a single molecule. For example, Cai et al. (2006) could detect bursts of gene expression leading to the production of one or a few β-galactosidase molecules using enzymatic activity to produce fluorescent molecules from a nonfluorescent substrate.

Single molecule fluorescence detection also has been used to "beat" the diffraction-limited resolution barrier of the light microscope. When the light emitted from a single molecule is collected by an objective lens, the energy spreads into a Gaussian-shaped "point spread function" (psf). When the broadened images of two fluorophores begin to overlap on a detector, they merge into a single profile and are no longer discernable as two molecules, thus limiting resolution. However, the central position of the psf can be determined with the precision of a few nanometers, provided that sufficient numbers of photons are collected. Such "centroid" determination of psf has been used to track the motion of fluorescently labeled motor proteins in vitro and in vivo, enabling observation of individual motor-driven steps (Yildiz et al., 2003). Now, new methods such as PALM (PhotoActivated Light Microscopy; Betzig et al., 2006), the related fPALM (Hess et al., 2006), and STORM (Stochastic Optical Reconstruction Microscopy; Bates et al., 2007) take advantage of this strategy to build high-resolution fluorescent images of molecules, mainly in fixed cells (Figure 2). In PALM and STORM, brief laser pulses are used to turn on a subset of the photoactivatable fluorophore-tagged proteins, photons are collected until they are photobleached or switched off, and the centroids of these single molecules are measured at ~20 nm resolution. By repeated photoactivations of new fluorophores, a high-resolution spatial map of the protein emerges from the centroid positions of many single molecules. Both photoswitchable cyanine dye pairs (STORM; different pairs allow for multiple wavelength excitation) and photoactivatable fluorescent proteins (PALM) have been used to implement this method.

The recent flurry of single molecule measurements in cells foreshadows what is likely to become an exciting research area, but there are still many technical barriers to be addressed. The unstable fluorescence signal and photobleaching rate of GFP are problematic for single molecule measurements. Improved dyes and new ways of incorporating such dyes into genetically encoded proteins (e.g., HaloTag) will likely extend experimental possibilities. Quantum dots provide ample signal for making single molecule measurements in cells and even in tissues, but their relatively large size and multivalent chemistry creates problems for retaining native protein function in living cells. However, new methods for delivering quantum dots and monovalent chemistry (Howarth et al., 2008) could extend their utility in cell biology. PALM and STORM are very promising and are likely to receive considerable attention in the next few years. PALM will benefit from more photoactivatable fluorescent proteins (currently two are available, but three- or four-color imaging will add more possibilities as is the case with STORM). Meanwhile, STORM could find new cell biological applications if ways are developed to attach the dyes directly to genetically encoded proteins (instead of through antibodies). Both methods will benefit from improved microscope automation to "build up" sequential single molecule images into high-resolution maps. But such advances are happening rapidly, as evidenced by a recent study demonstrating time-lapse PALM in living cells (still only useful for slowly changing cellular structures) (Shroff et al., 2008). The utility of such methods for high-resolution 3D imaging also has been demonstrated (Huang et al., 2008). These techniques are being incorporated into commercial instruments for cell biology labs.

Single molecule work in cells also awaits a next generation of assay development. Most live-cell studies currently just "follow the bouncing ball." By tracking single molecule movement, one can determine if the motion is purely diffusional, restricted (reflecting boundaries or interactions with a "static" structure), or super-diffusional (reflecting directed transport). From such measurements, one can derive insight into the environment that the molecules experience inside the cell. Even direct observation of individual motor steps inside living cells is possible with sufficiently bright probes. In addition to "tracking" single fluorophores in living cells, new assays will be needed to measure outputs of single protein molecules, e.g., phosphorylation and dephosphorylation events, ligand binding, and changes in protein conformation (e.g., relief of autoinhibition). Many of these events can be measured with FRET sensors (possibly using new quantum dots as donors; Howarth et al., 2008), although other readouts are possible. By following the "biochemical histories" of single molecules, one can potentially gain new insights into spatial-temporal events and stochastic properties of signaling pathways.

Single Molecule Detection and Biotechnology

Single molecule detection also is likely to have a considerable impact in biotechnology. A recent advance has been the sequencing of single DNA molecules. Single molecule DNA sequencing has been long considered as a means of achieving higher fidelity (since error-prone PCR amplification is avoided) and potentially lower cost than other sequencing methods. Recently, this goal has been realized by investigators at Helicos Biosciences who have reported single molecule DNA sequencing of a viral genome (Harris et al., 2008). Their technique involves attaching short fragments of genomic DNA to a surface at low enough densities so that individual DNA fragments can be distinguished by light microscopy. Then, one of the four nucleotides labeled with a fluorescent dye is added and DNA polymerase incorporates the dye-tagged nucleotide into the subset of DNA fragments that have a complementary nucleotide in the primed position. The slide is rinsed, hundreds of thousands of DNA fragments are imaged by fluorescence microscopy, and then the fluorescence dye is cleaved off to allow a new round of fluorescent nucleotide incorporation and imaging. By scoring the nucleotide cycles when the DNA fragment is "bright" or "dark," its nucleotide sequence can be read. The next goal will be applying this method to the large human genome with sufficient coverage to enable rare mutations to be identified with confidence. This method potentially could be used to obtain information on epigenetic modifications (e.g., by using other fluorescent antibodies to methylated DNA). A second method for single molecule DNA sequencing (Greenleaf and Block, 2006) determined the precise positions of RNA polymerase pausing when one of each of the four bases was at low concentration. Although unlikely to become a low cost, high-throughput method, it provides a clear example of how watching the motion of an enzyme can provide detailed structural and chemical information about the substrate.

Proteomics also will no doubt benefit from miniaturization and single molecule detection. Huang et al. (2007) have taken an important first step in this direction by building a device to count the number of molecules of a specific protein present in a single cell. Their technology involves a microfluidic chip that can capture, lyse, and label the released proteins with a fluorescently labeled antibody. The protein-antibody complexes are then electrophoretically separated and individually counted by an intensified CCD camera as they flow through an observation channel. Other types of single molecule proteomic technologies will likely be developed in the future.

Conclusions

Single molecule measurements are certainly technological highlights of the past two decades. These methods have played a prominent role in developing an understanding of kinesin, myosin, F1-ATPase, and RNA polymerase, providing paradigms of what it is possible to learn using these approaches. But this only represents a very small subset of the interesting proteins that deserve to be explored by single molecule techniques, leaving much to be done by the next generation of biophysicists. In living cells, researchers are still exploring how single molecule approaches might best be used to answer a large range of questions in biology. In an era of research characterized by a proliferation of standard kits, assays, and reagents, formulating the right question and developing the proper assay and instrumentation to peer into the lives of single molecules is a challenging but extremely rewarding undertaking. There is a lot left to explore, and scientists from many disciplines can contribute to such efforts.

Supplemental Data

Supplemental Data include three movies and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01438-4.

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