CHAPTER 6

Single-Molecule Imaging of Fluorescent Proteins

Adam D. Douglass and Ronald D. Vale

Department of Cellular and Molecular Pharmacology
University of California
The Howard Hughes Medical Institute
San Francisco, California 94107

Abstract

Single molecule imaging techniques overcome the averaging effects inherent in ensemble measurements and enable characterization of the enormous heterogeneity that exists in biomolecular systems. Though long the domain of a few highly specialized labs, optical imaging of single molecules in living cells is becoming a widely accessible technique. The development of commercially available microscopes, robust analysis tools, and sensitive, low-noise detectors has contributed to this dissemination, as has the ever-growing array of fluorescent proteins. The relative ease with which genetically-tagged proteins can be created and introduced into a cell has largely eliminated more cumbersome and less precise means of particle labeling. A number of special considerations apply when using genetically encoded fluorophores for single molecule experiments, however. We discuss the

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means by which fluorescent proteins can be transfected into living cells to obtain the low particle densities required for single molecule imaging, and consider the limitations that are placed on single molecule analysis by the fluorophore's photophysical properties. We also discuss the types of morphology and subcellular localization that make certain preparations more amenable to single particle imaging than others. Last, we discuss some common pitfalls involved in analyzing single molecule datasets, and consider some of the unique information that can be obtained using these techniques.

I. Introduction

The ability to image single molecules offers substantially new biological information when compared to traditional forms of microscopy. In contrast to ensemble measurements, which average underlying heterogeneity in a population and mask interesting, time-variant behavior, single-molecule visualization provides a direct window into molecular motions, time courses of reactions, and the different properties of subpopulations of molecules. The statistical advantages afforded by these techniques are enabling completely new types of questions to be asked in biochemistry and cell biology.

The first single-molecule measurement occurred over three decades ago with the invention of the patch-clamp technique (Neher and Sakmann, 1976), which allowed researchers to monitor the electrical activity of individual ion channels. While this technique had enormous impact and gave rise to entirely new subdisciplines in the neurosciences, optical measurements of single molecules were relatively slow to follow. The first steps in this direction came from studies of membrane dynamics, where observations of fluorescent tags (Barak and Webb, 1981) or antibody-coated gold particles (Edidin *et al.*, 1991) allowed the movements of individual, cell surface receptors to be tracked by video microscopy. However, while these early attempts were informative, the labeling methods involved made it impossible to unambiguously show that single-molecule sensitivity had been achieved; in many cases, the observed behaviors might have been attributed to small clusters of receptors acting in tandem.

Because of the ambiguities involved in imaging single molecules in living cells, *in vitro* studies came to dominate the single-molecule landscape, and these were largely centered initially around observations of cytoskeletal motor proteins. Howard *et al.* (1989) indirectly measured the processivity of single molecules of the microtubule-associated motor, kinesin, by tracking the movement of fluorescently tagged microtubules that were being translocated by glass-adsorbed motor proteins. Optical trapping was later proven to be a very powerful approach for measuring the forces and steps taken by individual motors (Finer *et al.*, 1994; Svoboda *et al.*, 1993). A direct means of observing single molecules unambiguously was subsequently developed by the Yanagida laboratory (Funatsu *et al.*, 1995), who found that single fluorophores could be observed by total internal reflection

fluorescence (TIRF) microscopy in an aqueous environment, with rapid acquisition speeds (30 fps), and wide-field observation. Although TIRF microscopy was developed many years earlier (Axelrod, 1981), the refinement of TIRF for single-molecule imaging dramatically changed the scope of single-molecule studies. Early studies demonstrated binding and dissociation events of nucleotides to single myosin motors (Funatsu *et al.*, 1995) and tracking of single kinesin motors moving along microtubules (Vale *et al.*, 1996). Shortly thereafter, it was shown that single green fluorescent protein (GFP) molecules could be imaged by TIRF microscopy (Dickson *et al.*, 1997; Pierce *et al.*, 1997), opening up subsequent studies of imaging single GFP molecules in living cells. Because of the precisely defined stoichiometry between a molecule of GFP and the protein to which it is attached, it became relatively simple to show that single-molecule resolution had been obtained. Since that time, a number of groups have used GFP labeling strategies to study single molecules in many different cellular contexts, including signal transduction (Vazquez *et al.*, 2006), cell adhesion (Iino *et al.*, 2001), cytoskeletal dynamics (Watanabe and Mitchison, 2002), and supramolecular patterning at the cell surface (Douglass and Vale, 2005).

In this chapter, we will discuss some of the applications of imaging single fluorescent proteins in living cells and describe the experimental requirements for doing so. Technological improvements over the last decade have made such measurements much less difficult, although there are still a number of nontrivial technical issues that must be addressed. We will focus in particular on the properties of GFP and its variants that pertain to single-molecule imaging, and on methods for delivering fluorescent proteins to cells so as to achieve the low protein densities needed for resolving single molecules. Finally, we will discuss how single-molecule imaging of fluorescent proteins might be expanded in the future.

II. Instrumentation

While it is possible to detect single GFP molecules using other fluorescence microscopy techniques (e.g., confocal imaging), the overwhelming majority of studies have employed TIRF imaging. In TIRF imaging, the illuminating light (usually a laser beam) is steered toward the sample at an angle such that the light totally internally reflects at the interface between the glass substrate and the aqueous sample. In doing so, it creates an exponentially decaying evanescent wave that penetrates a short distance beyond the point of reflection (Fig. 1A). This wave excites fluorophores lying near the substrate, but not those out of the focal plane. Indeed, the utility of this technique stems from the shallowness of the TIRF illumination field, which is typically on the order of 100–200 nm, approximately one-fifth the axial dimension of a typical confocal illumination volume and less than one-tenth that of standard wide-field, epifluorescence illumination. The resulting reduction in out-of-focus fluorescence increases the overall signal-to-noise ratio and greatly facilitates detection of single molecules (Fig. 1B

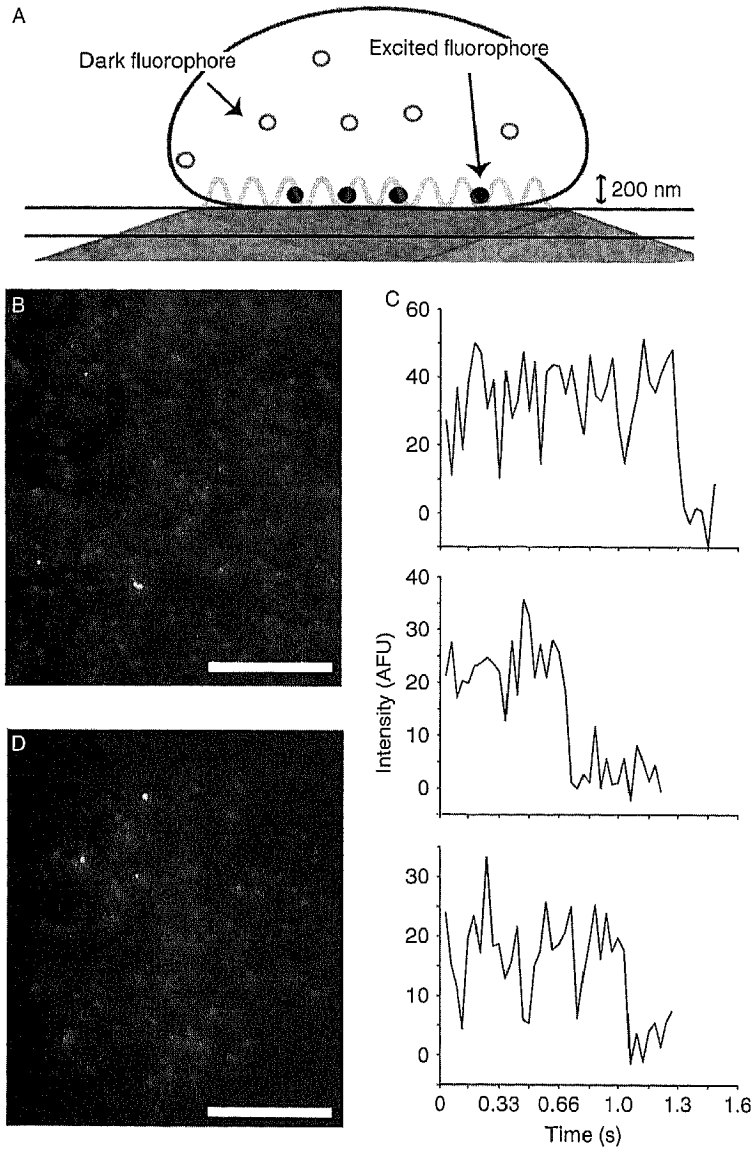

Fig. 1 Single-molecule imaging of GFP by total internal reflection fluorescence microscopy. (A) Reduction in background signal from out-of-focus fluorophores by TIRF illumination. (B) Field of single, purified GFP adhering to a glass coverslip, as visualized by TIRF. Scale bar = 10 μm. (C) Intensity versus time profiles for three representative spots in the field shown in panel B demonstrate single-step photobleaching. (D) Single molecules of CD2-GFP in the surface of a Jurkat T cell adhering to an antibody-coated coverslip, as visualized by TIRF. Scale bar = 10 μm. TIRF images were acquired at 30 fps, using 33-ms exposures.

and D). The depth of penetration of the evanescent wave can be tuned to some extent by varying the angle of incidence of the illuminating light source on the sample.

Not long ago, the difficulty in building a TIRF microscope constituted a barrier for many researchers hoping to conduct single-molecule work. This is no longer the case, as several microscope vendors have marketed their own "turnkey" systems for TIRF imaging. These systems can easily be mounted on conventional, epifluorescence microscope bodies without requiring an optical bench to route the laser beam through the microscope objective at the desired angle. Microscope companies now package several small, powerful diode lasers in housings that are coupled by fiber optics to the microscope. In addition, the major microscope vendors have all developed 100× objectives with numerical apertures of 1.45–1.49, which has allowed through-the-objective TIRF illumination to become standard practice [a substantial improvement in terms of ease-of-use compared with prism-type TIRF microscopy (Axelrod, 2003)]. Microscope companies have also made alignment of lasers for through-the-objective TIRF illumination a relatively trivial operation. Thus, commercial systems now enable any laboratory to perform single-molecule fluorescence imaging. However, for certain custom applications, an optical bench TIRF microscope might be preferable due to their greater flexibility.

In addition to the illumination mode, the sensitivity of the detector is also a critical factor in achieving single-molecule fluorescence imaging. TIRF microscopy, being a wide-field technique, normally uses a CCD camera rather than a point detector such as a photomultiplier tube. If the process being observed occurs on a relatively slow timescale (>500 ms), then lower-sensitivity CCD cameras can be used and the images can be integrated over longer periods to increase the fluorescence signal. This approach has been used to make very precise (a few nanometer resolution) positional measurements of single motor proteins traveling along cytoskeletal filaments *in vitro* (Yildiz *et al.*, 2003). However, many cellular processes cannot be adequately captured at such sampling rates. For instance, the lateral diffusion coefficients of many membrane proteins can approach 1 $\mu m^2/s$, in which case a protein might move 2 μm in a 100-ms time interval. To capture such rapid events, the camera must be able to acquire data at video rate or higher, and must do so with very high sensitivity. There are two types of camera that meet these needs: intensified CCDs (ICCDs) and electron multiplying CCDs (EMCCDs).

ICCD technology has been used in single-molecule imaging for a number of years and derives its high (up to single-photon) sensitivity from the placement of a multichannel plate (MCP) intensifier before a CCD detector. Using such a camera, we have been able to acquire single-molecule images of very rapidly moving particles at frame rates of over 100 fps. While intensifier cooling (to reduce dark noise) and fiber optic coupling between the MCP and CCD have continued to improve the capabilities of ICCDs, these cameras do suffer from several limitations, including a relatively small linear range and image artifacts arising from the intensifier. While changing the intensifier gain settings allows one to tune the

camera's sensitivity over a wide range, at higher gain, the relationship between photon number and pixel intensity becomes nonlinear. This makes quantitative measurements of particle intensity relatively difficult to make, particularly at high frame rates. Image quality also suffers due to imperfections in the coupling of the MCP to the CCD, as small deviations in alignment between these components produce artifacts in the image. This problem becomes more significant as chip resolution increases, and imposes an upper limit on the true resolution attainable with ICCDs.

EMCCDs represent the second camera choice for single-molecule imaging and have become increasingly popular. In these cameras, the signal is multiplied on the CCD chip itself, as the pixel array is read out through a gain register. The sensitivity of these cameras is close to that of an ICCD, but with fewer restrictions on potential resolution and none of the artifacts that result from coupling between the MCP and the CCD. The sensitivity offered by current-generation EMCCDs is sufficient for most applications, and this technology is likely to further develop to allow faster temporal acquisition. These sensors also have very high quantum efficiencies (QEs) of detection—approaching 95%—and dramatically reduce the negative contribution of readout noise.

III. Fluorophores

Genetically encoded fluorescent proteins are widely used for live cell imaging. Genetic fusions of fluorescent proteins have the advantage of a precisely defined stoichiometry; the number of fluorophores attached to a given protein is invariant and directly determined by the engineered DNA sequence. Adverse effects on protein activity can occur in some cases, however, and thus the activity of GFP-fusion proteins is best tested in a genetically tractable system where reconstitution-of-function experiments can be performed.

Whether a process can be visualized or not is determined directly by the intrinsic photophysical properties of the fluorescent tag. In this area, the fluorescent proteins fall somewhat short of the brightest organic dyes. A fluorophore's brightness is determined primarily by its extinction coefficient and quantum yield. EGFP (enhanced green fluorescence protein), with an extinction coefficient of roughly 40,000 and a fluorescence quantum yield of ~0.6, is not as bright as Alexa 488, with an extinction coefficient of 71,000 and a quantum yield near that of EGFP. That said, it has still been possible to image single GFP molecules at or near the plasma membrane. In our experience, EGFP has been too dim only when imaging certain extremely rapidly diffusing membrane proteins, which required acquisition speeds in excess of 100 fps and a concomitant decrease in the number of photons available in a given frame. Other fluorescent proteins have also been employed successfully—EYFP, which is even brighter than EGFP, is a useful tag, as is mCherry and the tandem dimeric version of DsRed. In general, any fluorophore that is approximately as bright as EGFP will be sufficient for most applications, though the continued

development of brighter fluorescent proteins will improve single-molecule imaging and allow faster temporal acquisition.

One serious limitation in using EGFP is its tendency to enter transient "dark" states, during which time the protein does not emit fluorescent light (Dickson *et al.*, 1997). These states can last for hundreds of milliseconds and produce gaps in single-molecule trajectories that can lead to premature truncation of a trace. While conversion to the dark state is not frequent enough to prevent most types of single-molecule measurement, it presents a significant analytical problem that is less problematic for synthetic fluorophores. In the absence of a means of eliminating these states, one must design a particle tracking procedure that can compensate for them by tracking through transient dark periods or simply settle for shorter contiguous traces.

Photostability also is of major importance in single-molecule work. In order to optimize signal to noise, single-molecule images are often acquired at very high illumination intensities. It is sometimes necessary to increase laser power to the point where the fluorophore becomes photosaturated, which will provide the highest possible signal-to-noise characteristics. Under these conditions, photobleaching tends to occur more quickly than in typical ensemble imaging experiments. While EGFP's photostability is reasonable, improvements in this area would enable the position and activity of a single molecule to be tracked over longer timescales. *In vitro* experiments have followed single molecules of the organic dye Cy3 for over 1 min at video rate, whereas comparable measurements of GFP rarely exceed 5–10 s. The development of quantum dots as fluorescent tracers has also led some researchers away from the use of fluorescent proteins, as these dyes are effectively unbleachable (Alivisatos, 2004). On the positive side, photobleaching can actually be useful as a means of demonstrating that single-molecule resolution has been attained. Despite some variability in fluorescence intensity over time, bleaching is an all-or-none process. If a particle's intensity profile suddenly drops to zero, it can be safely inferred that it represented a single fluorophore (Fig. 1C), whereas bleaching in multiple discrete steps or in a slow, continuous fashion would indicate the presence of multiple fluorophores within a diffraction-limited volume.

IV. Reducing Protein Expression Levels

It should be emphasized that single-molecule imaging, when performed using TIRF and without any additional optical or computational manipulation, does not entail an increase in lateral resolution as compared to other wide-field microscopies. This technique simply provides a much greater sensitivity than conventional methods. The lateral dimensions of a single particle will still be subject to the diffraction limit of light, such that molecules lying closer together than about 300 nm will not be resolvable as discrete objects. One ramification of this is that high GFP densities will obscure or completely prevent one from achieving single-molecule resolution, since the diffraction-limited, ~300-nm spots will overlap. Thus, obtaining a low

particle density is a critical factor in single-molecule live cell imaging. Since most gene expression reagents are designed to achieve the highest levels of transgene expression possible, one typically has to make special efforts to achieve low protein expression in order to image single molecules in cells.

There are a number of ways to reduce fluorescence to achieve single-molecule imaging in cells. A simple method is to employ photobleaching. By increasing the power of the excitation source for a short time, it is usually possible to bleach most of the fluorescent particles lying in the evanescent wave. By then turning off the excitation light and waiting for diffusion or active transport to redistribute the remaining particles from the unbleached regions of the cell, imaging at a single-molecule density can then be performed. We have used this technique to achieve the necessary number of adequately spaced GFP molecules for single-molecule tracking in already low-expressing cells (Douglass and Vale, 2005). As with most imaging experiments, however, photobleaching should be avoided if possible. Generation of reactive oxygen species during the bleaching process can harm or, in extreme cases, kill the cell (although this is less of a problem with TIRF than with epifluorescence microscopy). Another factor to consider is that imaging a region very soon after bleaching can obscure the equilibrium behavior of the population being studied. For instance, if a population of molecules equilibrates between slow- and fast-diffusing states, the fast-diffusing class is likely to reenter the bleached region sooner than the slowly diffusing one. Imaging too soon after bleaching would then bias the results to the fast-diffusing state. Thus for some experiments, bleaching should not be used to reduce particle density.

The first step for achieving low fluorophore levels involves the proper choice for transcriptional and translational regulation of the transgene. There are many modifications that can be made to expression constructs to make them produce less protein. In our laboratory, we have seen that the expression of certain genes can be reduced to very low levels by simply omitting a consensus Kozak sequence (or any components of the gene's 5′ untranslated region) in the expression plasmid. Others have demonstrated that deleting large regions of the promoter sequence can produce the desired result (Watanabe and Mitchison, 2002). Another strategy involves the use of inducible promoters, such as those that turn on gene expression in response to the drug tetracycline. By titrating the amount of inducer, it should be possible to find a concentration of drug that drives transgene expression at the desired level. Indeed, basal expression in the absence of the inducer often produces sufficient (or even too much) expression of protein for single-molecule imaging.

Finally, it is also possible to achieve low fluorophore densities through a modified form of transient expression. We have transiently transfected mammalian cell lines with GFP-fused proteins regulated by a standard *Cytomegalovirus* promoter. While expression is very high during the first 24 h, the transgene is not integrated into the host genome in most cells and is steadily lost from the population as the cells divide. By ~72 h after transfection, there are a large number of cells that have presumably lost the plasmid, but retain low residual levels of tagged protein; these cells are suitable for single-molecule imaging. Prior to imaging, this

population of cells can also be enriched by fluorescence-activated cell sorting, though we have not typically found it necessary to do so.

Photoactivation (the converse of photobleaching) holds promise for single-molecule work, although it has not been extensively employed to date. Because the amount of visible fluorophore can be precisely controlled by varying the duration and intensity of the activating light, a wide range of particle densities can be achieved through photoactivation. A number of photoactivatable fluorescent proteins now exist and have fluorescence properties that should make them suitable for single-molecule detection. Among these are PA-GFP, Kaede, and PA-mRFP. Recently, single-molecule photoactivation has been used as the basis of PALM microscopy (Betzig, 2006), a specialized application in which the distribution of fluorescence can be determined with nanometer-scale precision in fixed cells.

V. Biological Preparations

Whether a single-molecule approach is an appropriate avenue of investigation or not depends on both the experimental question and the suitability of the biological preparation for single-molecule imaging. The geometry of TIRF illumination imposes significant restrictions on the types of molecules and structures that can be investigated. In general, because of the short penetration depth of the evanescent wave, only molecules lying in or near the plasma membrane are observable by TIRF. This makes certain systems, such as the cortical cytoskeleton and membrane-proximal signaling networks, very amenable to single-molecule study while other systems are completely inaccessible. Cellular geometry is also quite important; in general, the more the cell can be induced to flatten out against its substrate, the easier it will be to image by TIRF. For this reason, the utility of TIRF in studying plant cells, yeast, and bacteria is relatively limited, though other approaches may still allow single-molecule imaging to work. For instance, increasing the number of GFP molecules attached to a single target molecule through aptamer binding can dramatically increase the signal available and makes it possible to image single molecules using other imaging techniques (Shav-Tal *et al.*, 2004).

We have performed the majority of our single-molecule work in cultured mammalian T cells, adhering to glass coverslips that have been coated with either antibodies directed against cell surface receptors or planar lipid bilayers containing mixtures of receptor ligands. In both cases, the cells adhere to and flatten on the substrate, thereby providing an excellent sample for TIRF imaging (Fig. 1D). The planar bilayer substrate has the additional advantage of being laterally fluid, such that proteins incorporated into the bilayer can diffuse freely in two dimensions. By including an adhesion molecule and a ligand for the T cell receptor in the bilayer, we and others (Varma *et al.*, 2006) have stimulated immune synapse formation in these preparations and have been able to study single-molecule behavior in a context that approximates a real cell–cell contact. Such

bilayer preparations are being adapted to a number of other systems, and all of these should be accessible to TIRF imaging.

VI. Data Analysis and Interpretation

The number of parameters that can be measured in a single-molecule data set is potentially large and diverse. Perhaps the simplest, and often most informative, is measuring the position of a molecule over time. For linearly moving particles, such as many cytoskeletal motor proteins, the position provides a simple measure of a molecule's velocity over time and thus an indicator of the mechanisms driving its movement. The molecular trajectories of membrane proteins also can be used to determine the diffusion coefficients of these particles. We and others have observed dramatic changes in single-molecule diffusion coefficients over time, with periods of free diffusion alternating with periods of complete immobilization (Douglass and Vale, 2005; Suzuki, 2005). By imaging the population distribution of a second protein labeled with a different fluorophore in the same cell, we have been able to assess the single-molecule behavior relative to defined subcellular structures. This relatively simple modification has allowed us to show a strong correlation between a protein's diffusion behavior and its local environment.

Fluorescence intensity can potentially provide information about a particle's oligomerization state. Because fluorescence emission is quantized, the normalized intensity of a diffraction-limited spot can be used to infer the number of fluorophores lying within that spot. While such measurements are relatively simple in certain *in vitro* preparations (Collins *et al.*, 2004), it can be very difficult to make the equivalent measurement in a living cell, for several reasons. For one, the cell membrane is never completely flattened against the substrate, and since the evanescent wave decays in the z-direction, such membrane undulations will give rise to variability in the intensities of single fluorophores. In addition, because these experiments require very low fluorophore densities, it is rare that all molecules of a particular type can be labeled and still provide single-molecule resolution. This means that the majority of oligomerization events will go undetected, as such events will typically involve a single labeled molecule at most. Despite these limitations, a few groups have managed to obtain information on protein clustering from intensity data (Iino *et al.*, 2001; Sako *et al.*, 2000). In general, these measurements are easiest when differentiating between large protein assemblies (i.e., containing tens or hundreds of molecules) and single particles, but it is possible to make finer assessments as well. In an impressive example of this, Leake *et al.* (2006) used intensity and photobleaching measurements to determine the stoichiometry of the MotB protein at the flagellar motor assembly of *Escherichia coli*.

Recently, single-molecule imaging has also been used to measure the duration of associations between cytoplasmic signaling proteins and the cell surface (Vazquez *et al.*, 2006). This is simply another example of measuring position over time, but with the difference that photobleaching must be absolutely avoided or carefully

corrected for. Since particles are effectively invisible when diffusing in the cytoplasm, cell surface residence times can be measured by counting the number of contiguous frames during which a particle is visible. If bleaching is properly controlled for, such measurements can reveal the half-life for dissociation from the membrane, a value that might provide insight into its interactions with other proteins in the membrane. Vasquez *et al.* (2006) used this approach to show that single molecules of the tumor suppressor PTEN spend up to a few hundred milliseconds at a time bound to the plasma membrane, and mapped the relevant interactions to a specific protein domain.

One of the most the most difficult aspects of single-molecule imaging is being able to track single particles in an image sequence. Manual tracking is possible but extremely tedious, and usually less accurate than automated methods. Due to the low signal-to-noise levels inherent in these experiments, image segmentation and centroid tracking are nontrivial. As described earlier, GFP's blinking behavior also can be problematic as transient disappearance of a particle will truncate a trajectory prematurely. Progress continues to be made, however, and a number of computational methods have been developed for single particle tracking. We primarily use a suite of tools written in the IDL language by Weeks and colleagues for tracking colloidal particles in suspension, which we have optimized for single-molecule analysis. One of the useful features of these routines is the inclusion of a "memory" function, which allows one to define a maximal number of image frames over which to continue searching for a particle that has disappeared before terminating the trace. Though artificial truncations do still occur, particularly when analyzing rapidly diffusing particles, the number of particles that can be analyzed in a given data set is often very large. Hundreds of molecules can be tracked in a single cell in just 1 min of acquired data. Because of GFP's relatively low brightness it cannot be localized with particularly high precision, however, and the current generation of fluorescent proteins will need to be dramatically improved to allow the nanometer-scale precision that is currently possible with other dyes (Yildiz *et al.*, 2003).

VII. Future Prospects

As the refinement of imaging technologies has enabled many laboratories to perform single-molecule imaging in living cells, the types of information that can be gained from single-molecule imaging also will undoubtedly expand. For example, the growing list of fluorescent proteins will enable one to track multiple proteins simultaneously. We have used a simple beam splitter to observe single molecules of GFP and tandem dimeric DsRed in the same sample. We also have used a linear unmixing strategy to visualize single GFP and YFP simultaneously. Such approaches enable the spatial-temporal tracking of multiple species at the single-molecule level. Perhaps most exciting, single-molecule FRET—which is relatively straightforward in *in vitro* systems (Tomishige *et al.*, 2006)—can

be used to measure binding events, conformational changes, and enzymatic activity in live cells, taking traditional biochemical measurements out of the test tube and into the complex environment of the cell.

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