Imaging Single Molecules Using Total Internal Reflection Fluorescence Microscopy (TIRFM)

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INTRODUCTION

Total internal reflection fluorescence microscopy (TIRFM) allows fluorescent molecules to be visualized with an unparalleled signal-to-noise ratio. This is achieved by illuminating only the molecules that are within a thin volume near the coverslip surface but not those that are deeper in solution. Using this technique, fluorescent molecules within ~100 nm of the coverslip can be visualized, and single molecules that are separated by a distance greater than the diffraction limit (~200 nm) can be individually resolved. The application of centroid-tracking methods allows subdiffraction-limited localization precision as low as 1 nm. Additionally, by combining centroid-tracking methods with recent advances in fluorophore technology and imaging methods, even those molecules that are present at high concentrations and closer to one another than the diffraction limit can be individually imaged. TIRF is ideally suited for studying protein dynamics on or near the plasma membrane. Although TIRFM was pioneered in the 1980s, it was not until the mid-1990s that single biological molecules were imaged directly. The explosion of new fluorescent proteins, new organic dyes, and quantum dots (Qdots), along with commercially available TIRFMs, has made this technique increasingly useful and accessible to biologists. In this review, we first describe the theory of TIRFM. We then give a detailed description of important considerations for setting up a TIRFM, based on commercially available systems, and review considerations for purification and labeling of proteins. Finally, we discuss new techniques that allow single molecules to be imaged at cellular concentrations and with super-resolution localization.

RELATED INFORMATION

A protocol for determining the illumination power density at which a dye of interest is saturated can be found in Determining Single-Molecule Intensity as a Function of Power Density (Reck-Peterson et al. 2010a). Use of TIRFM to image single molecular motors is described in Imaging Single Molecular Motor Motility with Total Internal Reflection Fluorescence Microscopy (TIRFM) (Reck-Peterson et al. 2010b).

TIRFM DEFINED

TIRFM utilizes fundamental properties of how light interacts with different transparent optical media to selectively illuminate small volumes of samples or cells on microscope slides. When a beam of light passes from one medium into another with a smaller index of refraction, the interface between the two media causes some of the incident light to be reflected and some of it to continue as a refracted beam at a different angle (Fig. 1A). The angle of the refracted light is dependent on both the angle of incidence of the illumination and the indices of refraction of the two media, a phenomenon described by Snell’s law. As the angle of illumination approaches a critical angle determined by the indices of refraction of the two media, the refracted beam gets closer and closer to being parallel to the interface. When the interface is illuminated at an angle above the critical angle, the refracted beam becomes parallel to the interface, and all of the incident light is reflected back, thus achieving total...
internal reflection (TIR). Above the critical angle, the electric field of the beam creates an evanescent wave that extends into the second medium; however, the intensity of this evanescent wave decays exponentially with distance from the interface and thus only illuminates a thin volume within the second medium. A review by Axelrod (Axelrod et al. 1984) gives a full mathematical treatment of the physics behind TIR.

TIRFM takes advantage of this optical effect to selectively illuminate only a small portion of a sample in solution on a microscope slide. Because the propagation of the evanescent wave into the second medium decays exponentially, the electric field is very weak after ~100 nm and has insufficient intensity for exciting fluorophores beyond that distance. Therefore, only those fluorophores within the evanescent wave receive enough incident illumination to excite fluorescence, leaving all other fluorophores in the sample dark (Fig. 1B). These illumination conditions create excellent signal-to-noise ratios by eliminating significant background.

Several configurations can be used to illuminate a sample with an evanescent wave in a microscope (Stuurman and Vale 2005). A widely used geometry consists of a prism placed on a slide containing the sample. Objective lenses with a very high numerical aperture (NA) have also made it possible to achieve TIR by illuminating through the objective. This illumination geometry is easy to integrate in modern research-grade inverted microscopes and does not obstruct access to the sample; it is therefore the obvious choice for TIRF imaging of live cells.

**SETTING UP A TIRFM**

Commercially Available TIRFMs

Although it is relatively straightforward to assemble a system for TIRFM (Stuurman and Vale 2005), most laboratories involved in live cell or in vitro imaging are likely to buy a commercial system. Currently, all major microscope companies, including Leica, Olympus, Nikon, and Carl Zeiss, sell TIRFMs. These systems use a through-the-objective strategy to achieve TIR and consequently require objectives with NAs of >~ 1.4 (best results are achieved with NAs of ≥ 1.45). Commercial TIRFMs consist of a standard epifluorescence microscope, enhanced with one or more lasers that are coupled into an optical fiber and a TIRF illuminator that guides the light into the microscope such that an evanescent wave can be formed. For single-molecule TIRF imaging, the camera and filters need special attention (see below). Thus, upgrading an epifluorescent microscope to a TIRFM consists of adding a laser-illumination system and TIRF illuminator and possibly upgrading the camera and filters, making TIRFM relatively cost-effective. The commercially available TIRFMs are closer to becoming turnkey systems; however, there are still a number of user adjustments, discussed below, that are critical for achieving good TIRF images.
Laser Alignment

The laser-illumination path is separated in two parts by an optical fiber. In front of the fiber, the output of the lasers is combined (see below for details), and no user adjustments should be necessary (Fig. 2). The output of the fiber is attached to the TIRF illuminator on the microscope. The exit pupil of the fiber must be imaged (focused) onto the back focal plane of the objective lens, resulting in a collimated beam of light exiting the objective. Failure to make this adjustment will allow rays that are below the critical angle to reach the sample, causing wide-field illumination and thus increasing the background. All commercially available TIRFs are inverted microscopes that are focused by moving the objective lens rather than the stage. Consequently, the back focal plane of the objective is not located at a fixed position with respect to the fiber exit pupil, and its position will change whenever the user changes focus. Therefore, all systems have a focusing lens somewhere in the epifluorescent light path that will need to be adjusted by the user after focusing the objective on the sample.

To focus the laser, one must first roughly focus the objective lens on the sample, then project the laser beam on a suitable surface (such as the ceiling) and adjust the laser-focusing lens (located somewhere inside the illumination pathway) such that the laser beam has the smallest possible diameter. Because the location of the back focal plane of the objective is wavelength dependent, different wavelengths of laser light will be focused at slightly different positions; thus, this adjustment should be repeated when changing wavelength (or an intermediate position should be found for multiwavelength experiments). Slight changes in sample height with an accompanying change in the position of the objective will have a negligible effect.

Adjusting the Incident Angle

As described above, TIR is achieved when the incident angle of the incoming laser beam is greater than the critical angle (Fig. 1). To achieve this, the exit pupil of the fiber (which is focused on the back focal plane of the objective) is moved in lateral directions (perpendicular to the direction of the light path), resulting in a lateral movement of the focused spot in the back focal plane of the objective and

![Figure 2](http://cshprotocols.cshlp.org/doi/10.1101/pdb.top73/fig2)
a corresponding change in the angle of incidence. In all commercially available systems, the angle of incidence is changed with micrometers (for both the x and the y directions). There is a wavelength dependence of the angle of incidence, often necessitating different settings at different wavelengths. Some of the newer systems have motorized micrometers that allow incident-angle adjustment for different wavelengths to be computer controlled. For example, the Leica TIRF system is fully motorized and is equipped with a detector for the reflected beam that is used to create a feedback system to automatically set the angle of incidence. To deal with the issue of different angles (and different focus positions) at different wavelengths, some systems (Olympus, also possible with Nikon) employ two or more independent light paths so that the sample can be illuminated at multiple wavelengths that are all perfectly aligned.

Apart from focusing the laser beam and adjusting the angle of incidence, no other alignments are necessary. This makes TIRFM easy to learn for anyone who has worked with an epifluorescent microscope.

ESSENTIAL PERIPHERALS FOR TIRFM

Other components critical to high-sensitivity/high-resolution TIRF imaging are the illumination source, the dichroic mirrors and filters, and the objective lens and camera. Some of these components are discussed in detail elsewhere (see Salmon and Waters [2010]); we will discuss here the aspects that are specifically relevant to TIRFM.

Illumination Source

Laser light is the most widely used light source in TIRFM. However, this is not an absolute requirement, and noncoherent light sources, such as those provided by an arc lamp or light-emitting diode, can also be used. In fact, Nikon offers a white-light TIRF illuminator for TIRFM with a standard epifluorescent light source. However, it is much harder to focus this light such that a usable fraction exits the objective above the critical angle. Consequently, the illumination intensity using white-light TIRF is too low to image single molecules and can only be used for ensemble measurements of relatively brightly stained samples.

Any visible-light laser can be used for TIRFM, including gas (argon, krypton, and argon-krypton), diode, solid-state, and broadband white-light lasers. Parameters that affect the choice of laser include the required wavelength (dictated by the dyes used), output power, size and heat generation of the laser, expected lifetime, and cost. All of the lasers in the system must be combined using dichroic mirrors and launched into an optical fiber that can be either polarization maintaining or nonpolarization maintaining. In some cases, it is essential to use a polarization-maintaining fiber (for instance, the Carl Zeiss TIRFM uses a polarizing beam splitter to direct the beam toward the objective). In many other cases it is unimportant, in part because the commercial systems do not have the capability to manipulate polarization in a manner appropriate for TIRF (for an application of polarized excitation in TIRF, see Sund et al. [1999]).

Some means of shuttering and changing the output power is also required. Diode lasers can be modulated at microsecond timescales and can therefore easily be shuttered using an electrical signal. Other lasers will need either a physical shutter or an acoustical-optical tunable filter (AOTF). The advantage of an AOTF is that it can shutter and modulate output intensities on a microsecond timescale; a disadvantage is that a minuscule amount of light will leak through even when the AOTF is set to shutter the light. The speed of a mechanical shutter is important, because a slow shutter will necessitate a long wait after opening before the image can be taken, resulting in unnecessary illumination that causes photobleaching and phototoxicity. Laser shutters with a switching time of 1 msec can be obtained.

A common question concerning lasers is the laser power needed. This is dictated in part by the light loss between the laser and the sample, and in part by the application, with single-molecule imaging requiring more power than other applications. Typical solid-state lasers produce between 10 mW and 100 mW of output power. When running this through an AOTF and fiber, 40%-70% remains. In our experience, 20%-50% of the light entering the microscope exits the objective (the losses are incurred in part by filters and dichroic mirrors in the light path, but most likely are also caused by the design of the light path, which aims to provide a flat field of illumination). Thus, it is not atypical that only ~8%-20% of the laser light actually reaches the sample.
How much light is seen by dyes in the sample not only depends on the amount of light exiting the objective but also depends on the area over which it is spread out. The parameter of interest is the power density, measured in W/cm². The commercially available TIRF illuminators tend to spread out the light over the whole field of view. The field of view of an objective can be calculated by dividing the field number (the image size at the intermediate image plane in millimeters) by the magnification. When a 100X objective with a field number of 18 is used, the illuminated area will be 180 µm in diameter or 0.0254 mm². Thus, when the sample is illuminated with 1 mW of light, the power density is 3.9 W/cm². Reducing the field number to 11 (as is possible with some of the commercial TIRF illuminators) reduces the illuminated area to a size of 0.0095 mm², and 1 mW of illuminating light now results in a power density of 10.5 W/cm². The field of view on a 1024 × 1024 camera that is magnified to fulfill the Nyquist sampling criteria is ~0.0068 mm²; therefore, reducing the field number (to 11) is sufficient in most circumstances and quite desirable for single-molecule imaging because it increases the power density almost threefold (although it possibly comes at the cost of a less even field of illumination). For a method to determine at what power density a dye is saturated, see Determining Single-Molecule Intensity as a Function of Power Density (Reck-Peterson et al. 2010a).

Dichroic Mirrors and Filters

Because in TIRFM all of the illuminating light is reflected off the coverslip-sample interface and travels back into the microscope, the spectral purity of the illumination source and rejection of excitation light in the path to the camera are of extreme importance in achieving high signal-to-noise ratio images. Thus, even the slightest amount of excitation light that is not rejected will cause a high background in the camera. Because the returning beam is physically separated, it is possible to place a beam stop or mirror in its path, strongly reducing the extreme requirements placed on the filters (most commercial TIRF systems do not implement this design). The following filters are critical for optimal TIRF imaging.

1. Laser cleanup filters. A cleanup filter can be placed immediately in front of the laser. This is needed if the laser emits any other colors than expected and is often essential for diode and gas lasers. In our experience, these are not needed for solid-state lasers.

2. Excitation filters. An excitation filter in the cube inside the microscope (which has positions for an excitation filter, dichroic mirror, and emission filter) can significantly reduce background even in cases where the illuminating laser light is spectrally pure. We suspect this is caused by the excitation filter reducing off-axis light originating from unwanted reflections in the illumination pathway. Because all filters in these systems are of the interference type, which is highly sensitive to the angle of incidence, such off-axis light can find its way to the camera when not rejected by an excitation filter.

3. Dichroic mirrors. As in any fluorescence microscope, the dichroic mirror needs to reflect as much illuminating light as possible and transmit as much of the emitted fluorescence as possible. It is often desirable to use multibandpass dichroic mirrors that reflect multiple wavelengths so that multiple dyes can be imaged without changing dichroic mirrors (which is often the rate-limiting step and in multicolor acquisition will lead to significant shifts in the location of the image on the camera). Although the design of such multibandpass mirrors is complex, great strides have been made in recent years, and dichroic mirrors with impressive properties can now be obtained from multiple vendors, such as Chroma Technology Corp., Semrock, and Omega Optical. For TIRFM, it is extremely important that the dichroic mirror is flat; any curvature in the mirror surface will result in astigmatism in the beam profile that is easily observed when projecting the beam on the ceiling after focusing the beam onto the back focal plane of the objective. Astigmatism in the beam will lead to light entering the sample at various angles and will add a wide-field component to the image. Improper mounting of the mirror in the filter cube can also cause curvature of the dichroic mirror. For instance, springs inside the cube can easily introduce stress in the thin piece of glass used to create the mirror. If the dichroics are purchased already mounted in the cube, this may necessitate testing multiple dichroics.

4. Emission filters. Because the excitation light is totally reflected and the dichroic mirror will transmit at least a few percent of this light, the levels of background in the image are most often determined by how well the emission filter blocks the excitation light. This might not be a limiting factor
when imaging brightly labeled cells, but it can become a significant issue in single-molecule imaging. Transmission of $10^7$ or less is needed to reduce background to manageable levels. It is therefore important to have transmission data for these filters at the excitation wavelengths. It is also important that the emission filter transmits as much light as possible at the wavelength of the dye of interest.

5. Emission filter wheel. When it is impossible to fully block excitation light with the emission filter in the filter cube, or when it is desirable to switch quickly between emission filters, an emission filter wheel can be used. This filter wheel can be located either before the tube lens (inside the microscope) or after the tube lens, just in front of the camera. The latter position is often the only accessible location, but is less desirable because the light is not parallel, causing interference filters to perform suboptimally.

### Objectives

With commercially available TIRFs, all of which utilize a through-the-objective TIR design, it is essential to use an objective with a high NA. The critical angle $\theta$ is given by the equation $\sin(\theta) = n(1)/n(2)$, where $n(1)$ is the refractive index of the sample and $n(2)$ is the refractive index of the coverslip material. For live cells, the refractive index will be slightly higher than the refractive index of water (1.33) and can vary between cell types and even within a given cell, but a number of 1.38 is often quoted in literature. The NA of an objective is given by the equation $\sin(\alpha) = NA/n(2)$, with $\alpha$ being the maximum angle of incidence. Thus, for the angle of incidence ($\omega$) to be larger than the critical angle ($\theta$), the NA should be larger than the refractive index of the sample $n(1)$. It is possible to achieve TIRF using a 1.4-NA objective lens on live cells; however, aligning such a system becomes much easier when higher NA objectives are used. All major microscope objective vendors now sell 1.45- or even 1.49-NA objectives that use standard immersion oil and coverslip glass.

Olympus makes an objective lens with a NA of 1.65. However, to make use of this high NA, it is necessary to increase the refractive index of the immersion medium and coverslip material. To this end, a special immersion oil is used ($n[d] = 1.78$) that is volatile and needs to be handled with extreme care. Special (and relatively expensive) coverslips ($n[d] = 1.78$) must also be used. Even though this objective makes it possible to achieve very steep angles of illumination and therefore an evanescent wave that extends a shorter distance into the sample (achieving even better signal-to-noise ratios), this objective is not widely used, because of the special requirements for coverslips and immersion medium.

Other factors to be considered when selecting an objective lens are the same as in other types of fluorescence microscopy and include transmission at excitation and emission wavelengths, chromatic corrections, and flatness of field. Some of the newer objectives have a spherical aberration correction collar that corrects for spherical aberration induced by imaging samples with a refractive index different from that of the immersion medium (a situation not encountered in TIRF, because imaging takes place at the coverslip sample interface). This collar can also correct for temperature-induced changes in the refractive index of the immersion medium. Careful adjustment of such a correction collar is imperative to achieve high signal-to-noise images.

### Cameras

Criteria for camera selection for TIRFM are identical to those used in other types of fluorescence microscopy (see Salmon and Waters [2010]). However, if one aims to image single molecules, it becomes imperative to select cameras that can image fast enough to track the molecules’ movements. Increasing the readout speed of a camera comes at the cost of increased readout noise. This type of noise is inherent to the camera design and is independent of the exposure time (see Salmon and Waters [2010]). Strategies employed to overcome the problem caused by camera-readout noise include image intensifiers and on-chip electron-multiplication (EM) gain. Image intensifiers multiply the light before it reaches the camera, making the readout noise irrelevant. However, image intensifiers have a relatively low quantum efficiency (the fraction of photons converted into a signal on the camera) and a relatively low dynamic range, and their coupling to the camera causes some blurring of the image. In an EM camera, the signal is amplified on the chip itself through a process called impact ionization. This amplification is a stochastic process (resulting in a new noise factor), making it difficult to amplify all pixels in an image to the exact same level. Nevertheless, cameras using EM gain are the best option when imaging single molecules at a rate faster than ~2 frames/sec. Such cameras are available from vendors such as Photometrics, Hamamatsu, Andor, and QImaging.
Apart from EM gain, it is important to have a camera with the highest possible quantum efficiency. So-called back-thinned charge-coupled device (CCD) chips can reach quantum efficiencies of 95% for large parts of the visible spectrum. All current EM cameras can also be read out as a conventional camera. When imaging at speeds <~2 frames/sec, conventional readout is often beneficial because, at such exposure times, the signal will be significantly higher than the readout noise and would be degraded by the addition of EM-gain-induced noise.

Data Analysis

Although the various data-analysis methods are quite specific for different experiments, several types of analysis are widely used for analyzing single-molecule data. Here we describe the use of kymographs and centroid finding.

Kymographs (Fig. 3) provide a useful method for visualizing the dynamics of single molecules. They provide the complete spatial history through time of a region in the field of view, thus allowing easy visualization and analysis of molecules that traverse that region. The determination of critical single-molecule data parameters such as velocity, direction of motion, and dwell time can be calculated from kymographs. Additionally, they can be used to observe the convergence and divergence of different molecules as well as experimental parameters such as photobleaching rates. The freely available software ImageJ automatically generates kymographs using the function “reslice.”

TIRFM allows for superior signal-to-noise ratio imaging of fluorescent molecules; however, the resolving power of a TIRFM is still subject to the diffraction limit imposed by all conventional optical systems. Because of the wave nature of light, an infinitesimally small point (such as a single molecule) is imaged through a microscope as a spread point with a size determined by the NA of the objective and the wavelength. This point-spread function has a size of ~200 nm for typical microscope systems. When a spatially isolated single fluorophore is imaged, a centroid-finding method can be used to fit its point-spread function and determine its location to an accuracy significantly better than the diffraction limit—as low as 1 nm (Thompson et al. 2002). However, when multiple adjacent fluorophores are within this 200-nm limit, their point-spread functions overlap, and, consequently, it is difficult to separate each fluorophore’s individual fluorescence signal and determine its spatial localization using a centroid-finding technique. This problem is compounded as the density of fluorophores increases. The recently described super-resolution techniques of stochastic optical reconstruction microscopy (STORM) and photoactivatable localization microscopy (PALM), discussed at the end of this article, are data acquisition and analysis methods that create images with spatial resolution below the diffraction limit even when molecules are spaced closer than the diffraction limit (Betzig et al. 2006; Rust et al. 2006).

SINGLE-MOLECULE IMAGING OF MOLECULAR MOTORS

The first single-molecule TIRF experiments were performed on purified, labeled proteins in vitro (Funatsu et al. 1995). This strategy has been very successful for imaging molecular machines as they move and do work. For example, single-molecule experiments using TIRFM have examined the stepping mechanism of all classes of cytoskeletal motors: myosins, kinesins, and dyneins (Yildiz et al.

FIGURE 3. Kymograph of single dynein molecule movements. TMR-labeled dynein is added to microtubules in the presence of ATP. Static images at 0 min, 1 min, and 2 min from a 10-min movie are shown. To build the kymograph, every static image along the microtubule axis in the 10-min movie is stacked horizontally. Single motors that are moving are visualized as a diagonal stripe.
TIRFM has also been used to probe the mechanisms of nucleic-acid-based motors (Zhuang et al. 2000; Joo et al. 2008). In vivo single-molecule experiments employing TIRFM (see Toomre [2010]), because of the limited depth of the evanescent wave, have focused on events on, within, or near the plasma membrane. Such experiments have been important for understanding a number of processes, including signaling events (Sako et al. 2000), cytoskeletal dynamics (Watanabe and Mitchison 2002), and the mobility of proteins within membrane microdomains (Douglass and Vale 2005).

We examine here some general considerations for preparing the protein for imaging (its purification and labeling). Our general imaging protocol is described in *Imaging Single Molecular Motor Motility with Total Internal Reflection Fluorescence Microscopy (TIRFM)* (Reck-Peterson et al. 2010b).

Purification and Labeling of Motor Proteins

The steps for purification and labeling of a protein are usually the most difficult and time-consuming parts of developing any assay and are specific for the protein of interest. In general, expression of recombinant protein is desirable so that specific domains of the protein can be labeled with fluorescent tags. Commonly used tags are green fluorescent protein (GFP) or GFP-related proteins, although these fluorophores are much less photostable and have a lower quantum efficiency than other choices. Cysteine-labeling strategies have been successful for smaller proteins in which native cysteines can be engineered out of the protein (e.g., Rice et al. [1999]).

Self-Labeling Genetic Tags

The recent development of self-labeling genetic tags such as the HaloTag (Promega) and CLIP and SNAP tags (New England BioLabs) has been useful for labeling proteins in vitro with dyes that are brighter and more photostable than GFP and its related fluorescent proteins. The HaloTag is a 33-kDa monomeric hydrolase that has been modified to react with the carbon-halogen bonds found in aliphatic-halogenated substrate compounds, resulting in a covalent bond that links the HaloTag substrate to the protein of interest. HaloTag substrates linked to numerous organic dyes, chemical reactive groups, or biotin are available from Promega. The SNAP and CLIP tags are 19-kDa mutants of the DNA-repair protein O6-alkylguanine-DNA alkyltransferase, which react with benzylguanine or benzylcytosine, respectively, to form a covalent bond. Similar to the HaloTag, numerous SNAP and CLIP substrate-linked reagents are available from New England BioLabs. Commonly used fluorophores for single-molecule studies include Cy3, Cy5, and tetramethyl-rhodamine (TMR). Because these self-labeling genetic tags also allow biotinylation, streptavidin-Qdot attachment is possible. Although Qdots offer unparalleled brightness and stability, they have the disadvantage of being very large (10-15 nm), making it important to perform control experiments to determine whether the presence of a Qdot changes the behavior of the protein being studied. Membrane-permeable variants of the HaloTag, SNAP, and CLIP substrates make it possible to label proteins in vivo (McMurray and Thorner 2008; Svendsen et al. 2008). In the future, coupling these reagents with STORM-imaging techniques (see below) could be a powerful new method for studying single-molecule dynamics in vivo.

STORM AND PALM: SUPER-RESOLUTION SINGLE-MOLECULE IMAGING AT HIGH CELLULAR PROTEIN CONCENTRATIONS

Most single-molecule measurements utilize centroid finding to localize molecules to a resolution better than the diffraction limit. However, this technique becomes difficult, if not impossible, at high concentrations. In these cases, the point-spread functions of the fluorophores overlap, and consequently individual centroids cannot be determined. However, two recently developed techniques, termed PALM and STORM, utilize novel fluorophore and illumination methods to separate the emissions of fluorescent particles in time, thus allowing them to achieve localization results better than the diffraction limit for samples with high concentrations of fluorescent molecules (Betzig et al. 2006; Rust et al. 2006). These methods overcome the problem of overlapping point-spread functions by using photoactivatable dyes with many series of acquired images and, in effect, allow single-molecule measurements of proteins at high cellular concentrations. By activating only a small fraction of the total fluorophores at any given time, individual fluorescent molecules are well separated in each image and can be localized at nanometer resolution by centroid fitting. After bleaching (or switching off) of the...
activated fluorophores, a new set of molecules is switched on and localized at high precision in the next series of images. TIRFM is utilized to reduce background signals and to achieve a high signal-to-noise ratio. Many cycles of sequential activation, localization by centroid finding, and deactivation of individual photoswitchable fluorophores are used to create composite, super-resolution images with localization accuracies on the order of 20 nm. The STORM technique utilizes photoswitchable organic dye pairs to tag cellular objects of interest (Rust et al. 2006), whereas the PALM technique relies on photoactivable fluorescent proteins expressed as fusions with the proteins of interest (Betzig et al. 2006).

Both the STORM and the PALM techniques create composite super-resolution data from hundreds to tens of thousands of individual fluorescence images. Both techniques begin by activating a sparse subset of the total population of fluorescent molecules by illuminating the sample with a brief pulse from an activating laser. This pulse switches on the fluorescent molecules such that they can be excited by standard fluorescence methods (Fig. 4). Because the activation of individual fluorophores is stochastic, the probability of activation is proportional to illumination intensity. The activated fluorophores are then localized by illuminating the sample at the wavelength of the imaging laser.

**FIGURE 4.** STORM and PALM super-resolution microscopy. (A) Schematic of the imaging process utilized in STORM and PALM. A light pulse (green) activates a sparse subset of the photoactivatable fluorophores. A probe pulse (light blue) stimulates the fluorophores’ fluorescence. A more intense pulse of the probe light (dark blue) can be used to deactivate the current set of active fluorophores. For each individual image, the centroid of each fluorophore is found. The individual centroid locations are then compiled into a composite super-resolution image. (B) STORM data of microtubules: (a) Conventional fluorescence microscopy image of a microtubule network; (b) STORM image of the region shown in B(a); (c-f) zoomed images of the boxed regions in B(a) and B(b). (STORM images reprinted from Bates et al. 2007 with permission from the American Association for the Advancement of Science © 2007.) (For color figure, see doi: 10.1101/pdb.top73 online at www.cshprotocols.org.)
At this wavelength, the fluorophores absorb energy and re-emit light at their characteristic fluorescence wavelength. This emission is then recorded on the camera, and the location of each individual fluorophore is determined. Because the fluorophores are only sparsely activated, typically none of their point-spread functions overlap, and thus each individual fluorophore can be localized without ambiguity using the centroid-finding method. Next, the current set of activated fluorophores is deactivated, either by photobleaching (PALM) or through the stochastic deactivation caused by the imaging laser (STORM). The sample is then illuminated again by the activation laser and a different, stochastically chosen subset of fluorophores is activated; then the cycle repeats. Through many of these sequential activation, imaging, and deactivation cycles, localization of each fluorophore is eventually obtained, and a composite image can be constructed Fig. 4(). The clever use of photoswitchable dye pairs or photoactivatable proteins allows both STORM and PALM to be used to generate multicolor images providing the localizations of different species of labeled molecules within the same sample at high protein concentrations.

Because of the great potential of super-resolution localization, both PALM and STORM are attractive techniques for intracellular imaging. However, some technical challenges currently exist that make the application of these two techniques to live cell and intracellular imaging difficult. First, sparse activation of fluorophores is required to ensure that no two individual point-spread functions overlap. However, the need for sparse activation requires that many imaging cycles be executed, leading to acquisition sequences that can take hours (Betzig et al. 2006; Rust et al. 2006). These time constraints generally require that cells be fixed prior to imaging. Second, the data are limited to the volume illuminated by the evanescent TIR illumination wave. Because of this thin illumination volume, fluorescence from molecules within whole cells is only collected for the region of the cells near the coverslip. Alternatively, cells can be fixed and sectioned to provide opportunities for examining the interior of cells. Finally, because STORM relies on dye pairs that are not genetically expressed or cell permeable, current uses of STORM are limited to fixed and permeabilized or sectioned cells and imaging is accomplished using dye-conjugated antibodies that recognize the protein of interest. The self-labeling HaloTag, SNAP, and CLIP tags provide promising methods for using STORM in live cells if dye pair constructs that are membrane permeable can be identified.

More recently, PALM has been used to track freely diffusing membrane proteins that, because of their location on the outside of the cell, are within the TIRF illumination volume (Manley et al. 2008). In this experiment, the dynamic trajectories of multiple diffusing membrane proteins were tracked. The PALM technique was also recently used with a non-TIRF illumination scheme to localize proteins to a lateral accuracy of ~50 nm at varying depths up to 10 µm within cells (Vaziri et al. 2008). In the case of STORM, a recent report demonstrated that an optical modification utilizing calibrated astigmatism could extend the STORM concept into three dimensions. Using this technique, the lateral resolution was maintained at ~25 nm while an axial location resolution of ~55 nm was obtained (Huang et al. 2008). These and other, future extensions of both the PALM and the STORM techniques are likely to allow fluorescence microscopy to achieve resolutions at near-molecular levels and provide important new tools for observing molecular localizations and interactions.

THE FUTURE OF SINGLE-MOLECULE IMAGING

In our opinion, the most exciting future prospects for TIRFM come from the availability of commercial systems that are relatively affordable and easy to use. The advent of these systems within the past few years makes the powerful technique of single-molecule imaging available to many laboratories, allowing the investigation of a wide variety of biological processes using nonensemble methods. The continued development of brighter and more photostable dyes, as well as more affordable lasers and camera systems, is also an important direction for the future that will enable the application of these microscopy techniques to new research areas. The further development of near-TIRF illumination, which extends the illumination depth beyond the limits of the evanescent wave, will allow molecules that are farther away from the coverslip interface to be imaged at signal- to-noise ratios sufficient for single-molecule measurements (Tokunaga et al. 2008). Further development of fluorophore and optical technology as well as novel analysis methods promise to make super-resolution microscopy a standard technique in many laboratories.
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REFERENCES


