Topic Introduction

Stochastic Optical Reconstruction Microscopy (STORM): A Method for Superresolution Fluorescence Imaging

Mark Bates, Sara A. Jones, and Xiaowei Zhuang

The relatively low spatial resolution of the optical microscope presents significant limitations for the observation of biological ultrastructure. Subcellular structures and molecular complexes essential for biological function exist on length scales from nanometers to micrometers. When observed with light, however, structural features smaller than $\sim 0.2 \mu\text{m}$ are blurred and are difficult or impossible to resolve. In this article, we describe stochastic optical reconstruction microscopy (STORM), a method for super-resolution imaging based on the high accuracy localization of individual fluorophores. It uses optically switchable fluorophores: molecules that can be switched between a nonfluorescent and a fluorescent state by exposure to light. The article discusses photoswitchable fluorescent molecules, STORM microscope design and the imaging procedure, data analysis, imaging of cultured cells, multicolor STORM, and three-dimensional (3D) STORM. This approach is generally applicable to biological imaging and requires relatively simple experimental apparatus; its spatial resolution is theoretically unlimited, and a resolution improvement of an order of magnitude over conventional optical microscopy has been experimentally demonstrated.

INTRODUCTION

Fluorescence microscopy is a versatile technique widely used in molecular and cell biology. The noninvasive nature of light allows biological specimens to be imaged with little perturbation, enabling researchers to observe dynamic processes as they occur in living cells and tissues. Fluorescent labeling techniques such as immunofluorescence, fluorescence in situ hybridization, and genetically encoded fluorescent tags (e.g., green fluorescent protein [GFP]) make it possible to label and to image specific biochemical components of a sample (Giepmans et al. 2006; Spector and Goldman 2006). The availability of a broad range of fluorophores enables the simultaneous imaging of multiple targets by multicolor labeling with spectrally distinct fluorescent probes. Furthermore, three-dimensional (3D) images may be generated, for example, by focusing into the sample at different depths.

Despite these advantages, conventional fluorescence microscopy is limited by its spatial resolution, leaving many biological structures too small to be studied in detail. Subcellular structures span a range of length scales from micrometers to nanometers, whereas the light microscope is limited by diffraction to a resolution of $\sim 200 \text{ nm}$ in the lateral direction and $>500 \text{ nm}$ in the axial direction (Pawley 2006). Other imaging techniques such as electron microscopy (EM) have obtained much higher spatial resolutions, and the ability of these methods to visualize biological samples with molecular resolution has had a tremendous impact on our understanding of biology (Koster and Klumperman 2003). To achieve image resolutions comparable to EM but with the labeling specificity and live cell compatibility provided by fluorescence microscopy would create a new opportunity to study the nanoscale structure and dynamics of cells and tissues.

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Over the past several years, this goal has been met with the introduction of a number of new methods, which we collectively refer to as superresolution imaging techniques. In this article, we present one such technique: stochastic optical reconstruction microscopy (STORM). Here, we review the theoretical basis of this method and focus on practical aspects of its implementation. Requirements for experimental apparatus and data analysis procedures are presented in detail, with the goals of facilitating the implementation of STORM and illustrating its capabilities as a tool for sub-diffraction-limit microscopy. For overviews of the superresolution fluorescence imaging field and descriptions of the various techniques, the reader is referred to several review articles (Hell 2007; Bates et al. 2008; Fernandez-Suarez and Ting 2008; Heintzmann and Gustafsson 2009; Hell et al. 2009; Huang et al. 2009; Zhuang 2009).

THEORY AND CONCEPT OF STORM

The Diffraction Limit of Resolution

It was recognized by Abbe in the 19th century that the spatial resolution of an optical microscope is limited by the diffraction of light (Abbe 1873). It is due to diffraction that a point source of light, when imaged through a microscope, appears as a spot with a finite size. The intensity profile of this spot defines the point-spread function (PSF) of the microscope. The full-width at half-maximum (FWHM) of the PSF in the lateral ($x-y$) and axial (z) directions is given approximately by $\Delta x, \Delta y = \lambda/(2NA)$ and $\Delta z = 2\lambda\nu/(NA)^2$, respectively, where λ is the wavelength of the emitted light, ν is the index of refraction of the medium, and NA is the numerical aperture of the objective lens (Pawley 2006).

The diffraction limit of resolution can be understood by considering the image formed by two identical fluorophores spaced some distance d apart in the $x-y$ plane. When $d \gg \Delta x$, two well-separated spots are observed in the image plane, each with an intensity profile corresponding to the PSF of the microscope. As the fluorophores are moved closer together, however, the images of the two fluorophores begin to overlap significantly when $d \approx \Delta x$. This is the minimum separation distance at which the two fluorophores are resolvable as separate entities. As the distance between the fluorophores decreases further, the images are fully overlapped, and the two fluorophores are not distinguishable.

The resolution of the microscope is thus limited by the width of the PSF. This line of reasoning extends to any sample imaged with an optical microscope: Features smaller than the PSF are blurred and cannot be resolved. Some improvement in resolution can be obtained from reducing the size of the PSF by using shorter-wavelength light or an objective lens with a higher NA. In practice, for visible light ($\lambda \approx 550$ nm) and a high-NA objective ($NA = 1.4$), the resolution of the conventional light microscope remains limited to ~ 200 nm laterally and >500 nm along the optical axis.

Nanoscale Localization of Single Fluorophores

The position of an isolated fluorescent emitter, although its image appears as a diffraction-limited spot, can be precisely determined by finding the centroid of its image. For example, shown in Figure 1 is the image of a single fluorescent molecule bound to a surface. Despite the nanoscale size of the fluorophore, the image appears as a diffraction-broadened peak several hundred nanometers in width, reflecting the PSF of the microscope. Although the width of the PSF limits the spatial resolution of a conventional optical microscope, it does not prevent the precise determination of the fluorophore position when the image of the fluorophore is isolated from those of the other molecules. The position

FIGURE 1. Fluorescence image of a single Cy5 fluorophore. The emitted light (wavelength ~ 670 nm) is imaged onto a CCD camera. Although the fluorophore is only nanometers in size, its image appears as a broad spot with an approximately Gaussian intensity profile. The position of the fluorophore can be determined with nanometer precision by fitting the image to find its centroid.

of the fluorophore corresponds to the center position of the peak, and this can be measured with a high degree of precision. By fitting the image to find the peak centroid, the position of the fluorophore can be determined with a precision limited only by the number of photons collected. The precision of this localization process, expressed in terms of the standard deviation of the position measurement (σ), is given approximately by

$$\sigma \approx \sqrt{\frac{s^2 + (a^2/12)}{N} + \frac{4\sqrt{\pi s^3 b^2}}{aN^2}},$$

where s is the standard deviation of the PSF, a is the edge size of the area imaged on each charge-coupled device (CCD) pixel, b is the standard deviation of the image background, and N is the number of photons detected from the fluorophore (Thompson et al. 2002). This concept of high-precision localization has been used to track small particles with nanometer-scale accuracy (Gelles et al. 1988; Ghosh and Webb 1994). Recently it has been shown that, even for a single fluorescent dye molecule under ambient conditions, its position can be determined with a precision as high as ~ 1 nm (Yildiz et al. 2003).

Nanoscale precision in single-molecule localization does not, however, translate directly into image resolution. When multiple fluorophores are positioned close together such that they are separated by a distance less than the PSF width, their images overlap, and this prevents accurate localization of each of the fluorophores. To distinguish the fluorescence signal from the nearby fluorescent emitters, several approaches have been used, based on differences in the emission wavelength (van Oijen et al. 1998; Lacoste et al. 2000; Churchman et al. 2005), the sequential photo-bleaching of each fluorophore (Gordon et al. 2004; Qu et al. 2004), or quantum dot blinking (Lidke et al. 2005; Lagerholm et al. 2006). These methods have obtained high accuracy localization for several closely spaced emitters but are difficult to extend to densities of more than a few fluorophores per diffraction-limited area. A fluorescently labeled biological sample, in contrast, may be labeled with hundreds of fluorophores per diffraction-limited region.

Stochastic Optical Reconstruction Microscopy

A solution to the problem of localizing multiple fluorescent molecules positioned in close proximity presents itself in the form of optically switchable fluorophores: molecules that can be switched between a nonfluorescent and a fluorescent state by exposure to light. The fluorescence emission of such molecules can be controlled such that, at any point in time, only a single one, or a sparse subset, of the fluorophores in a given region of the sample is in the fluorescent state. This sparse condition ensures that the images of the individual molecules are not overlapping, allowing each to be localized with high precision. If the fluorescent molecules are then deactivated to the dark state, and a new subset is activated to the fluorescent state, these newly activated fluorescent molecules can also be localized. By repeating this cycle of activation, localization, and deactivation, the positions of an arbitrary number of closely spaced fluorophores may be determined.

An imaging method based on this principle is illustrated in Figure 2. Beginning with a fluorescently labeled sample, the goal of the method is to determine the positions of the fluorescent labels and to plot them to form an image. The imaging process consists of many cycles during which fluorophores are activated, imaged, and deactivated, as described above. The density of activated molecules is kept low by using a weak activation light intensity such that the images of individual fluorophores do not typically overlap, thereby allowing each fluorophore to be localized with high precision. As this process is repeated, a stochastically different subset of fluorophores is activated in each cycle, enabling the positions of many fluorophores to be determined. After a sufficient number of localizations has been recorded, a high-resolution image is constructed from the measured positions of the fluorophores. The resolution of the final image is not limited by diffraction but by the precision of each localization and the localization density. In this sense, the resolution of the image is limited only by the number of photons collected from the fluorophores and the number of fluorophores labeling the sample. We termed this method “stochastic optical reconstruction microscopy” (STORM). This

FIGURE 2. The STORM imaging procedure. (A) Schematic of a cell in which the structures of interest (gray filaments in this case) are labeled with photoswitchable fluorophores (not shown). All fluorophores are initially in the nonfluorescent state. The red box indicates the area shown in panels B–D. (B) An activation cycle: A sparse set of fluorophores is activated to the fluorescent state such that their images (large red circles) do not overlap. The image of each fluorophore appears as a diffraction-broadened spot, and the position of each activated fluorophore is determined by fitting to find the centroid of the spot (black crosses). (C) A subsequent activation cycle: A different set of fluorophores is activated, and their positions are determined as before. (D) After a sufficient number of fluorophores has been localized, a high-resolution image is constructed by plotting the measured positions of the fluorophores (red dots). The resolution of this image is not limited by diffraction but by the accuracy of each fluorophore localization and by the number of fluorophore positions obtained. (Adapted, with permission, from Bates et al. 2008, ©Elsevier.)

concept was independently developed by multiple research groups, and it is also referred to as photo-activated localization microscopy (PALM) or fluorescence photoactivation localization microscopy (FPALM) (Betzig et al. 2006; Hess et al. 2006; Rust et al. 2006). For ease of reference, we will refer to the method as STORM in this article.

PHOTOSWITCHABLE FLUORESCENT MOLECULES

A fluorescent molecule is termed photoswitchable, photoactivatable, or photochromatic when its fluorescence emission or other spectral characteristics can be converted between two or more distinct states under the control of an external light source. Fluorescence switching was first reported for a variant of the fluorescent protein, YFP (yellow fluorescent protein), which was shown to cycle reversibly between a fluorescent and a dark state by alternating exposure to blue and violet light (Dickson et al. 1997). Similar effects have been shown with synthetic organic fluorophores (Kulzer et al. 1997; Irie et al. 2002). One such example is the red cyanine dye Cy5, which can be reversibly converted between a fluorescent state and a nonfluorescent state (Bates et al. 2005; Heilemann et al. 2005). When viewed at the single-molecule level, Cy5 appears to switch its fluorescence on and off. In contrast, the fluorescent protein Kaede undergoes an irreversible spectral change from green fluorescent to red fluorescent emission when exposed to ultraviolet (UV) light (Ando et al. 2002). Photoswitchable fluorophores, such as these, have found increasing application for superresolution imaging and also as optical highlighters in which a specific region of a sample can be marked by photoconverting the fluorophores in a localized area (Elowitz et al. 1997; Marchant et al. 2001). A wide variety of photoswitchable synthetic fluorophores and fluorescent proteins are now available in a range of colors, a selection of which is summarized in Table 1.

Any of the photoswitchable fluorescent proteins and organic dyes listed in Table 1 may be applied to STORM. Some fluorophores are certainly more suited than others, however. Generally, organic dyes tend to be brighter than fluorescent proteins, allowing for higher localization precision. Fluorescent proteins, on the other hand, have the advantage of being genetically encoded, making it easier to label intracellular proteins in living cells. In the following discussion, we will describe the cyanine dyes as a specific example; however, the reader should be aware of the many options available among the fluorescent proteins and other synthetic fluorescent dyes.

Cyanine Dyes

As mentioned above, the red cyanine dye Cy5 can be converted between a fluorescent and a non-fluorescent state under the control of an external light source. This photoswitching behavior is

TABLE 1. Spectral properties of photoswitchable fluorescent proteins and organic dyes

Fluorophore	Activation (nm)	Before activation		After activation		Reference(s)
		Absorption max (nm)	Emission max (nm)	Absorption max (nm)	Emission max (nm)	
Fluorescent proteins						
PA-GFP	405	400	515	504	517	Patterson and Lippincott-Schwartz (2002)
PS-CFP2	405	400	468	490	511	Chudakov et al. (2004)
Dendra-2	405	490	507	553	573	Gurskaya et al. (2006)
Kaede	405	508	518	572	582	Ando et al. (2002)
EosFP, mEos2	405	506	516	571	581	Wiedenmann et al. (2004), McKinney et al. (2009)
mKikGR	405	507	517	583	593	Tsutsui et al. (2005), Habuchi et al. (2008)
Dronpa	405	—	—	503	518	Habuchi et al. (2005)
Dronpa-2	405	—	—	486	513	Ando et al. (2007)
bs-Dronpa	405	—	—	460	504	Andresen et al. (2008)
rsFastLime	405	—	—	496	518	Stiel et al. (2007)
eYFP	405	—	—	514	529	Dickson et al. (1997), Biteen et al. (2008)
PA-mCherry	405	—	—	570	596	Subach et al. (2009)
Synthetic dyes						
Cy5	350–570 ^a	—	—	649	670	Bates et al. (2005, 2007), Heilemann et al. (2005), Dempsey et al. (2009a)
Cy5.5	350–570 ^a	—	—	675	694	Bates et al. (2005, 2007), Dempsey et al. (2009a)
Cy7	350–570 ^a	—	—	747	776	Bates et al. (2005, 2007), Dempsey et al. (2009a)
Alexa Fluor 647	350–570 ^a	—	—	650	665	Bates et al. (2005, 2007), Dempsey et al. (2009a)
Photochromic rhodamine B	375	—	—	565	580	Folling et al. (2007)
Rhodamine spiromides	375	—	—	537–591 ^b	555–620 ^b	Belov et al. (2009)
Azido-DCDHF	—	—	—	570	613	Lord et al. (2009)

PA, photoactivatable; GFP, green fluorescent protein; PS, photoswitchable; CFP, cyan fluorescent protein; eYFP, enhanced yellow fluorescent protein; azido-DCDHF, azido-2-dicyanomethylene-3-cyano-2,5-dihydrofuran.

^aDependent on the activator dye, if present.

^bDependent on the specific compound.

dependent on the presence of a thiol-containing molecule in the imaging solution such as β -mercaptoethanol (β ME), β -mercaptoethylamine (MEA), or cysteine (Bates et al. 2005; Heilemann et al. 2005; Dempsey et al. 2009a). When single molecules of Cy5 are exposed to red light, they are initially fluorescent before switching into a stable dark state. If this sample is then exposed to UV light, a significant fraction of the molecules rapidly return to their fluorescent state. This is the hallmark of a photoswitchable fluorophore: that it can be optically converted between distinct fluorescent states. It is worth noting that the photoswitching behavior of Cy5 is observed over a broad range of thiol concentrations, including low concentrations at which living cells remain viable—this feature allows the cyanine dyes to be used with live cell STORM imaging.

The fluorescence reactivation of Cy5 by visible light is made significantly more efficient when Cy5 is paired with a second fluorophore that absorbs visible light such as the green fluorophore Cy3. When Cy3 and Cy5 are positioned in close proximity (e.g., on a doubly labeled antibody as illustrated in Fig. 3), the Cy5 fluorophores switch off as described earlier when exposed to red light in the presence of a thiol-containing reagent. However, when exposed to a low-intensity green excitation light source ($\lambda = 532$ nm), the Cy5 molecules rapidly recover their fluorescence. The rate of fluorescence reactivation by green light is $\sim 10^3$ times greater when Cy5 is paired with Cy3, as compared with an isolated Cy5 molecule. One can think of the pair of dye molecules Cy3 and Cy5 as acting together to form a fluorescent switch. With red illumination, Cy5 emits fluorescence and is converted to the dark state. When exposed to green light, Cy3 absorbs a green photon and causes Cy5 to be reactivated to the fluorescent state through a short-range interaction (Bates et al. 2005). This process is repeatable and is robust, with the dye pair undergoing, on average, more than 100 switching cycles before permanent

FIGURE 3. A cartoon representation of the photoconversion of Cy5 between a fluorescent and a nonfluorescent state. In this diagram, an antibody molecule is labeled with the green fluorophore Cy3 and the red fluorophore Cy5. When Cy5 is exposed to red light, it emits fluorescence and then switches to a dark nonfluorescent state. Illumination with green light, however, causes Cy5 to revert to its fluorescent state by means of a short-range interaction with the neighboring Cy3. Under illumination conditions allowing single-molecule detection, this single-molecule fluorescent switch can be cycled on and off for hundreds of cycles before permanent photobleaching occurs.

photobleaching occurs. We refer to the dyes as an activator–reporter pair in which Cy3 is the activator because it serves to activate Cy5 to the fluorescent state and Cy5 is the reporter because its fluorescence emission reports the state of the switch.

Activator–Reporter Dye Pairs for Multicolor Imaging

A useful property of the activator–reporter concept is the ability to create multicolor spectrally distinct probes using different combinations of activator and reporter dyes. In general, the activation wavelength for photoswitching is determined by the absorption spectrum of the activator dye. If a blue fluorescent dye (e.g., Cy2) is substituted in place of Cy3, the Cy2–Cy5 pair shows photoswitching as before, but efficient reactivation occurs only in response to blue light. Similarly, a violet fluorophore (e.g., Alexa Fluor 405) may be used as the activator dye, and, in this case, the Alexa 405–Cy5 construct is reactivated efficiently when exposed to violet light. Each combination represents a spectrally distinct photoswitchable probe that is distinguishable by the wavelength of light required for activation (Bates et al. 2007). This property enables multicolor imaging schemes, as illustrated later in this article in the discussion of multicolor STORM.

Characteristics of Photoswitchable Fluorophores

Several properties of photoswitchable fluorophores determine their suitability for superresolution imaging using the STORM method. In particular, the brightness of the fluorophore is crucial for single-molecule detection and precise localization. Also, non-ideal switching characteristics such as residual fluorescence emission from the dark state and spontaneous reactivation from the dark state to the fluorescent state can present significant challenges when imaging densely labeled samples.

Fluorophore Brightness

The precise and accurate measurement of the position of each fluorescent label is an essential part of the STORM method. The precision of each position measurement is primarily determined by the number of photons collected from the activated fluorophore during a single cycle of switching on and off. Photoswitchable fluorescent proteins and organic dyes vary widely in this respect, with the number of photons detected per activation event ranging from a few hundred for some of the relatively dim fluorescent proteins to as high as 6000 for Cy5 and Alexa Fluor 647 (Hess et al. 2006; Bates et al. 2007; Shroff et al. 2007). Among the photoactivatable fluorescent proteins, EosFP is one of the brightest with approximately 1000 photons detected per activation event (Shroff et al. 2007).

Contrast Ratio and Spontaneous Activation

An essential step of the STORM imaging procedure is to activate only sparse subsets of the fluorescent labels at any one time such that each fluorophore is optically resolvable from the rest and can, therefore, be localized with high precision. In most cases, this means that the number of fluorophores in the dark state is many times higher than the number in the fluorescent state. In this scenario, two common characteristics of photoswitchable fluorophores, dark-state fluorescence and spontaneous activation, can have adverse effects on the imaging procedure.

Dark-state fluorescence refers to residual fluorescence emission from fluorophores that are in the deactivated state. This is commonly observed in photoactivatable fluorescent proteins and is characterized by the contrast ratio, defined as the ratio of a fluorophore's brightness in the fluorescent state to that in the dark state (Betzig et al. 2006). In the case of a fluorescent protein with a contrast ratio of 10^3 , for example, a single fluorophore in the fluorescent state is equal in brightness to 10^3 fluorophores in the dark state. When the density of fluorophores in the sample is greater than 10^3 per diffraction-limited region, the background fluorescence emitted by the dark-state fluorophores begins to degrade the ability of the analysis procedure to detect and to calculate the location of the individual fluorophores in the fluorescent state.

Spontaneous activation (also called nonspecific activation or blinking) is another effect commonly observed with photoswitchable fluorophores. This refers to the activation of a fluorophore in the absence of specific exposure to the activation light source. In the case of the Cy3–Cy5 dye pair, for example, Cy5 will occasionally be activated when no green light is applied, seemingly blinking on and off on its own. This effect is caused by a relatively small but finite rate of activation of Cy5 by the red imaging laser itself, which is also used to excite fluorescence from the dye and to switch it off. Both the rate of switching off (k_{off}) and the rate of reactivation by the red laser (k_{blink}) are found to be linear in the illumination intensity. Consequentially, at equilibrium under red illumination, a fraction of the fluorophores present on the sample equal to $k_{\text{blink}}/(k_{\text{off}} + k_{\text{blink}})$ will be in the fluorescent state because of spontaneous activation. For Cy5, this fraction has been measured to be ~ 0.001 – 0.005 depending on the imaging condition such as the thiol reagent used in the imaging buffer. For densely labeled samples, the population of fluorophores activated due to this effect may violate the requirement for sparse activation and thereby, may interfere with the detection and the localization of individual fluorophores.

STORM MICROSCOPE DESIGN AND IMAGING PROCEDURE

The basic setup for STORM is very similar to that for conventional wide-field fluorescence microscopy, with a few modifications. In this section, the major components of the microscope are described, along with a general outline of the experimental procedure.

Microscope Components

Microscope Frame and Objective Lens

A schematic of the STORM microscope is shown in Figure 4. Although a microscope frame is not an essential component, we have found it convenient to base our microscopes on a standard inverted fluorescence microscope frame. The microscope frame and sample stage should provide a stable mount for the sample and the objective lens, and the focusing mechanism must also be relatively stable. Because the raw data used to generate a STORM image are collected over a period of time, sample drift during the measurement degrades the image resolution and must be corrected for in postprocessing. At the nanometer scale, drift of the microscope is inevitable, but any steps taken to minimize drift will improve image quality such as thermal and mechanical isolation of the microscope.

Drift of the focus mechanism is particularly problematic because this effect is difficult to correct for in the analysis procedure. We use an active focus-lock system based on a weak IR laser beam, which

FIGURE 4. A schematic of the STORM microscope. (A) The microscope is based on a standard inverted fluorescence microscope. Laser sources are combined into a single beam, which is expanded and focused to the back focal plane of the microscope objective. A translation stage allows adjustment of the incidence angle of the illumination light. The detected light passes through an emission filter and is imaged onto a CCD camera. The specific laser wavelengths shown here are an example of activation and imaging wavelengths that may be useful for STORM. Other wavelengths may be required depending on the choice of photoswitchable dye or fluorescent protein. The cylindrical lens (CL) is used only for astigmatic imaging in 3D STORM applications. (B) The geometry of the focus-lock system. An infrared (IR) laser beam is projected through the objective lens from a second dichroic mirror (not shown in panel A). The beam reflects off the sample–cover-glass interface and back through the objective to where its position is detected on a quadrant photodiode (QPD). (Adapted from Dempsey et al. 2009b.)

passes through the objective lens, is reflected off the sample–cover-glass interface, and is then projected onto a quadrant photodiode (QPD), as illustrated in Figure 4B. The position of the beam on the diode reports any relative motion between the objective lens and the sample, and this signal is fed back to a piezoelectric z stage, which corrects for the motion.

The objective lens should be chosen to maximize light collection efficiency and to minimize aberrations and background fluorescence. We typically use an oil-immersion lens such as the UPlan-SAPO 100 $\times$ NA 1.4 microscope objective from Olympus, which allows for both epifluorescence- and objective-type total internal reflection fluorescence (TIRF) illumination. Other similar objective lenses may also be used.

Illumination Geometry

For cell imaging, a through-the-objective illumination geometry (Fig. 4) is convenient, allowing cell culture plates to be imaged from underneath such that the cells can remain in solution during imaging. This scheme also allows for the use of short-working-distance high-NA objective lenses, increasing light collection efficiency significantly.

To bring the illumination light to the sample, the imaging and activation lasers are combined into a single path using dichroic mirrors and then are expanded and collimated using a beam expander. This beam is focused to the back focal plane of the objective. A translation stage allows the beam to be shifted toward the edge of the objective so that the light emerging from the objective reaches the sample at a high incident angle near, but not exceeding, the critical angle of the glass–

water interface. In this manner, a region of the sample several micrometers in thickness is illuminated by the angled beam. For thinner regions of the sample, adjusting the incident angle allows a narrower depth of illumination and lower background fluorescence (Cui et al. 2007; Tokunaga et al. 2008). For imaging near the cell surface, the incidence angle of the illumination light can be adjusted to greater than the critical angle to allow total internal reflection and to further reduce the background signal.

Detection Optics and CCD Camera

Because STORM relies on the detection of single fluorescent molecules, the optical detection path of the microscope should be optimized for maximum light collection efficiency, and the CCD camera must be suitable for low-light fluorescence imaging. In our experiments, we have used thermoelectrically cooled cameras incorporating electron-multiplying CCD (EMCCD) technology, a form of on-chip signal amplification that raises the signal well above the CCD read noise and dark current levels (Jerram et al. 2001).

The degree of magnification of the image is chosen for optimal fluorophore localization. If the magnification is too low, the image of the fluorophore is smaller than a camera pixel, and no sub-diffraction-limit position information can be determined. If the magnification is too high, the photons collected from the fluorophore are spread over many pixels, and the noise generated as each pixel is read out degrades the localization accuracy. As studied by Thompson and colleagues, the localization process is most precise when the magnification is chosen such that the FWHM of the image of the PSF is equal in size to ~ 2.4 camera pixels (Thompson et al. 2002).

To relate the STORM data to real distances, the image magnification must be carefully calibrated. For example, the system may be calibrated by imaging a micrometer slide that has a pattern of lines spaced 10 μm apart.

For 3D STORM imaging, a long focal-length cylindrical lens is inserted as the final optical element before the camera (Huang et al. 2008b). This will be discussed further in the sections below.

Light Sources

Laser illumination allows for highly efficient excitation of fluorophores and adds the ability to block the excitation light from entering the detection channel using spectral filters, thereby maximizing detection of the fluorescence signal and minimizing background. For imaging Cy5 or Alexa Fluor 647, a laser source at ~ 650 nm with a power output >50 mW is sufficient to enable short switching times and rapid data collection. Higher laser powers enable faster fluorophore switching and, hence, faster data collection rates. When imaging a photoactivatable fluorescent protein such as EosFP, a laser source of similar intensity with a wavelength of 561 or 568 nm may be used.

One or more additional lasers may be used for activation of the switchable fluorophores. As described above, multicolor photoswitchable activator–reporter pairs may be activated using various laser wavelengths (e.g., 405, 457, or 532 nm) when the activator dye is Alexa Fluor 405, Cy2, or Cy3, respectively. For photoactivatable fluorescent proteins, 405 nm is the most commonly used activation wavelength.

Finally, the addition of a conventional mercury arc lamp enables the user to quickly image standard fluorophores such as DAPI (4',6-diamino-2-phenylindole), GFP, or the Cy3 signal from Cy3–Cy5 labeled samples, before starting STORM data collection.

Electronically Controlled Laser Shutters or Acousto-Optical Modulator

A system for switching the laser illumination on and off in synchrony with the data acquisition allows switchable fluorophores to be activated with a predetermined timing. This facilitates multicolor STORM imaging using fluorophores that are activated by distinct wavelengths of light (see the discussion of multicolor STORM methods later in the article). If the laser exposure times are synchronized with the frame rate of the camera, the data analysis software may keep track of which frames

of the recorded data correspond to which illumination color. In this manner, the time at which a fluorophore switching event appears in the data set may be correlated with a particular activation wavelength. Rapid switching of the laser illumination may be achieved with an acousto-optical modulator (AOM), an acousto-optical tunable filter (AOTF), or mechanical shutters. To synchronize the laser switching with the CCD exposures, the clock signal provided by the CCD camera can be used as the timing source to control the AOM, the AOTF, or the shutters.

Computer Control

When recording STORM data, a computer equipped with a multifunction data acquisition card records image data continuously from the camera, streaming it directly to a disk. The computer also switches the laser modulators/shutters in sync with the camera frames according to a preset laser pulse sequence, and interfaces with the QPD and the piezo-stage controller to maintain the sample focus.

Laser Intensity Control

A means for controlling the laser intensity, in particular, the activation laser intensity, is required for STORM imaging. As discussed above, the fraction of activated fluorophores must be maintained at a low enough level such that the images of individual fluorophores are not typically overlapping. To achieve this condition, the intensity of the activation laser must be adjusted. A neutral-density filter wheel provides a convenient coarse laser intensity adjustment. Fine adjustments can be made by passing the laser through a half-wave plate followed by a polarizing beam-splitter cube. Rotating the wave plate rotates the laser polarization, which, in turn, modulates the amount of light passing through the beam splitter. Intensity control can also be provided by an AOM or an AOTF.

STORM Experimental Procedure

Sample Preparation

As in any fluorescence imaging experiment, the sample must first be fluorescently labeled. There are several approaches to labeling, including immunofluorescence techniques in which the sample is labeled with fluorescent antibodies and fluorescence in situ hybridization in which the probes are fluorescent nucleic acids (Spector and Goldman 2006). Any synthetic fluorophore or genetically encoded fluorescent protein may be used provided they are photoswitchable. The easiest approach to introduce photoswitchable fluorescent proteins into cells is by transfection, although the generation of stable cell lines or transgenic animals expressing fluorescent proteins may also be used and may be preferred because overexpression can adversely affect the sample. A method for transfection of genetically encoded photoswitchable probes is described in **Transfection of Genetically Encoded Photoswitchable Probes for STORM Imaging** (Bates et al. 2013a). The preparation of antibodies labeled with a pairing scheme of synthetic fluorophores is described in **Preparation of Photoswitchable Labeled Antibodies for STORM Imaging** (Bates et al. 2013b). The cell or tissue samples may be imaged in either live or fixed conditions.

In general, the staining procedure must be optimized to ensure that the ultrastructure of the sample is not disrupted, to maximize the density of fluorescent labels bound to the structure of interest, and to minimize non-specific labeling. These requirements are more stringent for super-resolution fluorescence imaging than they are for conventional microscopy. The high spatial resolution of methods such as STORM must be complemented by a high density of fluorescent labeling to image the sub-diffraction-limit structural features of the sample. Any fixation procedure used must also preserve the ultrastructure of the sample. Fixation protocols previously developed for EM imaging, such as rapid freezing, freeze substitution, and fixation with glutaraldehyde, may be useful in this respect (McIntosh 2007).

Imaging Medium

When using cyanine dyes as photoswitchable probes, certain components are required in the imaging medium. In particular, the imaging buffer should contain a thiol compound, such as β ME (~ 140 mM) or MEA (~ 100 mM), to enable photoswitching of the dyes. The use of MEA results in a $2\times$ increase in the rate of switching off of Cy5 and a corresponding decrease in both the number of photons collected per switching event and the steady-state fraction of spontaneously activated fluorophores (fluorophores activated by red imaging light, as discussed above). A significantly lower concentration of β ME or MEA (~ 70 mM and ~ 6 mM, respectively) is used for live cell imaging to keep cells viable under this condition.

An oxygen scavenger system that reduces photobleaching also benefits STORM imaging with photoswitchable fluorescent dyes. A commonly used scavenger system contains glucose oxidase (~ 0.5 mg/mL), catalase (~ 40 μ g/mL), and glucose ($\sim 10\%$ w/v). For live cell imaging, a lower concentration of glucose ($\sim 1\%$ w/v) is used to maintain cell viability.

Data Collection

The general procedure for STORM imaging is illustrated in Figure 2, which shows a cell in which the cytoskeleton has been labeled with switchable fluorophores. Initially, the fluorophores are prepared in the dark state (by exposure to the red laser, for example). Imaging then proceeds as follows.

1. *Activate a sparse set of fluorophores.* A brief exposure to the activation light source (e.g., a green 532-nm laser) causes some of the fluorophores on the sample to switch to the fluorescent state. Alternatively, exposure to the imaging light source can serve this purpose (see the discussion of spontaneous activation above).
2. *Image the fluorophores, and switch them off.* The sample is exposed to the imaging light source (e.g., a red 647-nm laser), and the activated fluorophores emit light, as illustrated in Figure 2B. The density of activated fluorophores is low enough that the images of the fluorophores do not overlap. The duration of the imaging light pulse is set to be long enough that most of the activated fluorophores will switch off before the next activation pulse.
3. *Activate a new set of fluorophores, and repeat.* As illustrated in Figure 2C, a different set of fluorophores is stochastically activated by the following activation light pulse. These are imaged, and the process is repeated until a sufficient number of fluorophore positions has been determined to map out the high-resolution structure of the sample.

This simple sequence of activation and imaging is useful for illustrating the basic concept of STORM, but any experimental procedure that allows for sparse subsets of fluorophores to be independently imaged over time may be used. For example, as an alternative to Steps 1–3, the imaging light source and the activation light source may be kept on continually so that imaging and activation are happening throughout the data collection. In this case, the activation light intensity is adjusted to be sufficiently low such that only a small fraction of the fluorophores in the field of view are activated at any moment in time and their images are not overlapping.

In general, the intensity of the imaging laser determines the rate at which the fluorophores switch off. To maximize the signal-to-noise ratio of the data, the camera exposure duration is adjusted such that, on average, the fluorophore on-time is equal to one exposure time of the camera. In this manner, almost all of the light from each fluorophore switching event is collected in a single camera frame, minimizing the noise associated with reading out a CCD image (Bock et al. 2007). In practice, the data collection speed is limited by either one of the maximum camera frame rate or the maximum rate at which the fluorophores can be cycled on and off.

The intensity of the activation laser is adjusted to control the density of fluorophores activated during each cycle. As the experiment proceeds and some fluorophores are permanently photobleached, the activation light intensity may be increased to maintain the activated density at an optimal level.

Spontaneous activation of the fluorophores (e.g., activation by the imaging laser) results in a baseline density of activated fluorophores that depends on the total density of fluorophores bound to the sample (see above). One can take advantage of spontaneous activation to record a STORM data set without the requirement for a separate activation light source—the sample may simply be exposed to the imaging light source, and the switching events may be recorded over time (Zhuang 2009). However, in cases of very concentrated labeling, the density of spontaneously activated fluorophores may be high enough that the images of the activated fluorophores are constantly overlapping, preventing accurate localization. In this situation, it may be necessary to bleach some fraction of the fluorophores by exposure to intense illumination or to reduce the density of labeling during sample preparation.

STORM DATA ANALYSIS

During the STORM experiment, a series of images is recorded showing individual fluorophores switching on and off in time. A STORM image is typically generated from a sequence of between 500 and 100,000 image frames, 256×256 pixels each, recorded at a rate of 20–500 Hz. The large volume of data generated in these experiments necessitates efficient automated data analysis software. For this purpose, a custom data analysis and visualization software package was written, and this section describes its principal algorithms. Additional analysis steps for the generation of multi-color or 3D images are described in later sections.

Peak Identification and Fitting

The first task of the analysis software is to identify the fluorescent molecules in each image of the movie and to fit them to determine their precise locations. This task is very similar to the well-studied problem of automated star analysis in astronomical data sets; hence, we can take advantage of algorithms that have already been developed (Stetson 1987). The problem is made simpler because the intensity profile of each fluorophore's image is identical, corresponding to the PSF of the microscope.

Image Filtering and Peak Identification

Each image frame is first convolved with a Gaussian kernel: a unit height Gaussian having the same width as the expected PSF and lowered to have a zero integral (Stetson 1987). This convolution removes high-frequency noise and low-frequency background intensity variations. The image is then thresholded and is searched for local maxima, which are identified as peaks. Figure 5 shows a portion of an image frame taken from a STORM movie. The left panel shows the raw data, and the right panel shows the same image with the identified peaks boxed in white.

Peak Fitting

A square region of $n \times n$ pixels ($n = 5-7$) centered on each peak is passed to a nonlinear least-squares fitting algorithm, which attempts to fit a Gaussian function to the data. The data are weighted assuming Poisson noise for the counts in each pixel. Two consecutive fits are performed for each peak. First, the data are fit to a continuous ellipsoidal Gaussian:

$$I(x, y) = A_0 + I_0 \exp \left\{ -\frac{1}{2} \left[\left(\frac{x'}{a} \right)^2 + \left(\frac{y'}{b} \right)^2 \right] \right\},$$

where

$$x' = (x - x_0) \cos \theta - (y - y_0) \sin \theta,$$

FIGURE 5. A portion of an image frame taken from a STORM movie. (A) The bright spots in the image correspond to the fluorescence emission of individual fluorophores. Most of the fluorescent labels are switched off such that the fluorescent molecules are well separated and distinguishable. (B) An automated peak-finding algorithm is used to identify the peaks. The identified peaks are marked by the white boxes. The areas enclosed by the white boxes indicate the regions of the image that will be fit to determine the positions of the fluorescent molecules that give rise to the peaks.

$$y' = (x - x_0) \sin \theta + (y - y_0) \cos \theta,$$

Here, A_0 is the background fluorescence level, I_0 is the amplitude of the peak, a and b define the widths of the Gaussian distribution along the x and y directions, x_0 and y_0 describe the center coordinates of the peak, and θ is the tilt angle of the ellipse relative to the pixel edges. Based on this seven-parameter fit, we compute a peak ellipticity parameter defined as $\max(a,b)/\min(a,b)$. This measure of the ellipticity is used to identify and to reject peaks corresponding to the overlapping images of two or more closely spaced fluorophores, as the image of a single fluorophore is expected to have circular symmetry (i.e., $a \approx b$). This constraint is relaxed for 3D STORM analysis, as described in the section on 3D STORM.

The second fit step is used to determine the peak center position, the amplitude, and the width. The data are fit to a rotationally symmetric Gaussian function:

$$I(x, y) = A_0 + I_0 \exp \left\{ -\frac{1}{2} \left[\left(\frac{x - x_0}{\sigma} \right)^2 + \left(\frac{y - y_0}{\sigma} \right)^2 \right] \right\},$$

where σ is the width of the peak. The total number of counts collected in the peak is calculated as $2\pi\sigma^2 I_0$, and this number is then converted to photoelectrons and, thus, the number of photons detected, based on the calibrated camera gain settings used during imaging.

Filtering

A fraction of the peaks identified for peak fitting will correspond to two or more activated fluorophores located in very close proximity such that their image appears as a single peak. This fraction is minimized by activating only a sparse set of fluorophores on the sample at any one time; but, nonetheless, further measures are necessary to avoid including in the final image the incorrect fluorophore positions resulting from fitting peaks corresponding to multiple fluorophores.

For this purpose, several of the fit parameters are stored for each peak and are subsequently used to filter out the erroneous peaks. For each identified peak, the following fit parameters are stored: center position, width, ellipticity, and number of photons detected. These values are compared against a set of filtering criteria, and any peak falling outside the range of acceptable width, ellipticity, and brightness is rejected from further analysis because it is unlikely to yield a precise fluorophore localization.

Trail Generation

The time at which a fluorophore is activated until the time it switches off will often span multiple image frames, generating multiple sets of fit results. Grouping these data together allows a more precise estimate of the fluorophore position. Peaks appearing in consecutive image frames with a relative displacement smaller than one camera pixel are considered to originate from the same fluorescent molecule, and their center positions are grouped into a data structure that we refer to as a trail. The final position of the molecule is calculated as the average of the center positions across the entire trail, weighted by the number of photons detected in each frame.

Drift Correction

An important factor that limits the resolution of a STORM image is sample drift. If the fluorescence image of the sample shifts with respect to the CCD camera pixels during the course of the experiment, the relative positions of fluorophores localized at different points in time are no longer accurate. Any source of mechanical drift in the microscope, such as thermal expansion and contraction of the microscope frame or vibration of the sample stage, can result in such an image shift.

A means of measuring sample drift allows the localized positions of the fluorophores to be corrected during data analysis. We have found two methods to be particularly useful in correcting for mechanical drift. The first method involves adding fiducial markers (e.g., fluorescent beads or gold particles) to the sample, which bind to the glass substrate and are visible in the fluorescence image. These spots can then be tracked for the duration of the movie, and their averaged motion as a function of time is then subtracted from the measured localizations, yielding a drift-corrected image.

The second method involves determining the drift from the STORM data set itself. The data set is divided into a number of equal-time segments, and the STORM image for each time slice is rendered. By calculating the correlation function between the STORM images generated from consecutive time slices, the sample drift during that time window may be determined. After the drift as a function of time has been calculated for all time slices, the drift is then subtracted from each localization to generate the drift-corrected image. This latter approach is useful for fixed samples but not for live samples with time-dependent variations.

STORM Image Rendering

After the data analysis is complete, the final result is a list of fluorophore positions that have passed the peak filtering criteria. The processed data are stored in this form, and STORM images of the data are dynamically generated as the user pans and zooms the image to a given region and magnification level. Dynamic rendering avoids the problem of having to render and to store the entire image area at the highest magnification: a $40 \times 40\text{-}\mu\text{m}$ sample region rendered at 1 nm per pixel would yield a $40,000 \times 40,000$ -pixel image, requiring 4.5 Gbyte of computer memory to view.

When the image is generated, each localization is assigned as one point in the STORM image. These points are either represented either by a small marker (e.g., a cross) or rendered as an intensity peak. For example, each peak can be represented as a Gaussian intensity profile with unit volume, and its width can be scaled to correspond to the localization uncertainty (Thompson et al. 2002), based on the number of photons collected for that switching event. In this sense, each intensity peak corresponds to a probability density function for finding the fluorophore at that position in space (Betzig et al. 2006). The summed peaks from all of the localizations then form an intensity image of the sample.

STORM IMAGING OF CULTURED CELLS

Fluorescence imaging of tissue cells in culture is a widely used method for cell and molecular biology (Spector and Goldman 2006), which would benefit significantly from increased spatial resolution. Many parts of the cell have structural features spanning a range of length scales from nanometers to

micrometers, and much of this range is beyond the resolution capabilities of conventional microscopy. Moreover, nanometer-scale image resolution would enable precise colocalization analysis for biomolecular complexes.

As an example of fixed cell imaging with STORM, microtubules were labeled by indirect immunofluorescence (Bates et al. 2007). Microtubules are filamentous components of the cytoskeleton of the cell, important for many cellular functions including cell division and intracellular cargo trafficking. Microtubule filaments extend throughout the cell, appearing as long and narrow fibers in fluorescence images. These well-defined structures provide an ideal test sample for demonstrating STORM image resolution. In the following example, Green monkey kidney epithelial (BS-C-1) cells were immunostained with primary antibodies and then with activator-reporter-labeled secondary antibodies. Cy3 was used as the activator and Alexa Fluor 647, a cyanine dye with very similar structural, spectral and photoswitching properties to Cy5, was used as the reporter. The STORM images were obtained by activating and deactivating the Alexa Fluor 647 fluorophores with repeated pulses of green (532 nm) and red light (647 nm).

As illustrated in Figure 6, the STORM image shows a substantial improvement in the resolution of the microtubule network as compared with the conventional fluorescence image. In the regions in which microtubules were densely packed and undefined in the conventional image, individual microtubule filaments were clearly resolved by STORM.

To determine the image resolution more quantitatively, we identified point-like objects in the cell, appearing as small clusters of localizations away from any discernible microtubule filaments. These clusters likely represent individual antibodies not specifically attached to the cell. The width (FWHM) of the localization clusters was 24 ± 1 nm, corresponding to a standard deviation of 10 nm, a measure of the fluorophore localization precision in this experiment. In terms of resolution, therefore, two fluorophores spaced 24 nm apart are resolvable. This represents a resolution improvement of an order of magnitude over the conventional optical microscope.

FIGURE 6. STORM image of microtubules in a large area of a BS-C-1 cell. (A) The microtubules are immunostained with Cy3-Alexa 647-labeled antibodies. Each fluorophore localization is plotted as a yellow peak with a Gaussian intensity profile and width scaled according to the photon-number-dependent localization precision. The total number of localizations plotted in the image is $\sim 2.4 \times 10^6$. A magnified view of the boxed region is shown in B and C. (B) Conventional fluorescence image of the boxed region in A. To obtain the conventional image, the cell was illuminated with a mercury arc lamp, and the Cy3 channel was imaged directly. (C) STORM image of the boxed region in A. In comparison with the conventional image in B, the STORM image reveals a substantial improvement in spatial resolution.

FIGURE 7. STORM imaging of vimentin filaments labeled with photo-switchable fluorescent proteins in a mammalian cell. (A) Conventional image of vimentin in a BS-C-1 cell. (B) STORM image of the same region. The vimentin filaments are labeled with the photoswitchable fluorescent protein mEos2. (Adapted from Zhuang 2009.)

Another widely used method to fluorescently label cellular structures takes advantage of fluorescent proteins, which can be genetically fused to proteins of interest. For STORM imaging of cellular structures using this labeling approach, the target protein in cells may be labeled with photoswitchable fluorescent proteins (Betzig et al. 2006). Figure 7 shows a comparison of conventional and STORM images of mEos2-labeled vimentin in a cell (Zhuang 2009). The protein mEos2 is a monomeric variant of the Eos fluorescent protein that can be photoactivated from a green-emitting form into a red-emitting form (McKinney et al. 2009). The STORM image was taken by activating mEos2 with a 405-nm laser and imaging the activated mEos2 with a 568-nm laser. Again, a substantial resolution improvement is observed for the STORM image as compared with its corresponding conventional image.

MULTICOLOR STORM

Conventional multicolor fluorescence imaging relies on the spectral separation of the emission spectrum of each type of fluorophore present in the sample. This approach to multicolor imaging is equally applicable to STORM, using photoswitchable fluorophores with distinct emission wavelengths. For example, among the cyanine dyes, three red-emitting dyes have been shown to display photoswitchable fluorescence: Cy5, Cy5.5, and Cy7 (Bates et al. 2007). Because of the separation of their emission spectra, these dyes can be used for multicolor STORM imaging using a multichannel fluorescence detection scheme. Similarly, other photoswitchable fluorescent dyes and fluorescent proteins are available for multicolor imaging applications (see Table 1). In the case of photoswitchable fluorescent proteins, two-color imaging has been shown using combinations of mEos and Dronpa (Shroff et al. 2007), PA-mCherry and PA-GFP (Subach et al. 2009), and Dronpa with bs-Dronpa (Andersen et al. 2008), for example.

Photoswitchable fluorophores, however, offer a second parameter for multicolor detection: the activation wavelength. The example below shows a multicolor STORM imaging scheme based on the distinct activation wavelengths of different photoswitchable fluorophores. Fluorescent probes with identical emission spectra can be distinguished by the wavelength of the light that causes them to be activated to the fluorescent state.

Experimental Procedure and Data Analysis

When using the activation wavelength as a means to distinguish different photoswitchable fluorophores, the time point in the data set at which the fluorophore is switched on is used to assign a color. For this multicolor imaging scheme, STORM data are acquired by alternately exposing the sample to pulses of activation light and imaging light. In a two-color experiment, for example, the sample may be exposed to the following repeating pattern of illumination pulses: green laser on for a duration of one camera exposure (frame), red laser for three frames, blue laser for one frame, and red laser for three frames. Fluorophores are switched on during the green or blue activation pulses, then imaged and switched off when the sample is exposed to red light. If the fluorophore is first detected in an image frame that immediately follows an activation pulse, the fluorophore is assumed to have been activated by that pulse, and a color is assigned accordingly. If detected in a frame not immediately following an activation pulse, the fluorophore may have been activated spontaneously by the imaging laser, and it is

impossible to know which activator dye is paired with the reporter. In this case, the color information is not known, but the localization may be assigned to a nonspecific category because its position can still aid in discerning the structure of the sample.

Color Cross Talk

Any incorrect color identification of fluorophores results in cross talk between the color channels. When the activation wavelength is used to distinguish between different types of photoswitchable fluorophores, color cross talk arises mainly because of spontaneous activation of the reporter dye by the imaging laser. Fluorophore activation induced by the imaging laser can be identified if the switching event was first detected in a frame that does not immediately follow an activation pulse. A spontaneous activation event may also occur during the frame immediately following an activation laser pulse, however, and, in this case, the color may be incorrectly assigned.

The frequency of these incorrect assignments will depend on the frequency of nonspecific activation events as compared with color-specific activation events that occur in response to an activation pulse. In practice, this will depend, in part, on the local density of fluorophores in the sample because at higher densities, the activation light intensity must be reduced to maintain the activated fluorophores at a spatially sparse level. In general, this source of cross talk is minimized when the camera frame rate is matched to the average on-time of the dye, and the activation laser intensities are adjusted to maximize the number of specifically activated dyes without increasing the density of activated fluorophores beyond the point in which their images are overlapping. A typical cross-talk level is 10%–30% and can be further corrected statistically in the image-processing stage (Bates et al. 2007).

Multicolor STORM Example

As an example of multicolor STORM imaging, Figure 8 shows a two-color image of microtubules and clathrin-coated pits (CCPs), cellular structures involved in receptor-mediated endocytosis (Bates et al. 2007). Microtubules and clathrin were immunostained with primary antibodies and then with activator–reporter-labeled secondary antibodies. The activator–reporter pairs used were Cy2–Alexa Fluor

FIGURE 8. Two-color STORM imaging of microtubules and CCPs in a mammalian cell. (A) STORM image of a large area of a BS-C-1 cell. The secondary antibodies used for microtubule staining were labeled with Cy2 and Alexa Fluor 647, whereas those for clathrin were labeled with Cy3 and Alexa Fluor 647. Two laser wavelengths, 457 and 532 nm, were used to selectively activate the two pairs. Each localization was falsely colored according to the following code: green for 457-nm activation and red for 532-nm activation. (B) STORM image corresponding to the boxed region in A shown at a higher magnification. (C) A further magnified view of the boxed region in B. (Adapted, with permission, from Bates et al. 2007, ©AAAS.)

647 for microtubules and Cy3-Alexa Fluor 647 for clathrin. Two different laser wavelengths, 457 and 532 nm, were used to selectively activate the two pairs. Cross talk between the two-color channels was subtracted from the image after statistical analysis (Bates et al. 2007).

The green channel (457-nm activation) revealed filamentous structures as expected for microtubules. The red channel shows predominantly circular structures, characteristic of CCPs and vesicles. Interestingly, many of the CCPs appeared to have a higher density of localizations toward the periphery, which is consistent with the two-dimensional projection of a 3D cage structure. The size distribution of CCPs can be measured directly from the image, yielding a mean size of ~ 180 nm, which agrees quantitatively with results obtained using EM (Heuser and Anderson 1989).

3D STORM

Although the techniques described thus far yield high-resolution information in two dimensions, most organelles and cellular structures have a 3D morphology. Three-dimensional fluorescence imaging is most commonly performed using confocal and multiphoton microscopies, the axial resolution of which is typically in the range of 500–800 nm, two to three times worse than the lateral resolution (Torok and Wilson 1997; Zipfel et al. 2003).

In this section, we introduce a method for 3D STORM imaging. Although the lateral position of a particle can be determined from the centroid of its image, the shape of the image contains information about the particle's axial (z) position. Nanoscale localization precision in the z dimension has been previously achieved by introducing defocusing (van Oijen et al. 1998; Speidel et al. 2003; Prabhat et al. 2006; Toprak and Selvin 2007) or astigmatism (Kao and Verkman 1994; Holtzer et al. 2007) into the image without significantly compromising the lateral position measurement. The STORM technique can be extended to 3D imaging by making use of these methods to determine the 3D coordinates for each fluorescent label with high precision.

The examples given here illustrate applications of the astigmatism imaging approach. Other methods for 3D fluorophore localization with application to nanoscale imaging have also been shown, including multifocal-plane imaging (Juetten et al. 2008), PSF engineering to create a double-helix PSF (Pavani et al. 2009), and interferometric methods using two objective lenses (von Middendorff et al. 2008; Shtengel et al. 2009).

Astigmatism Imaging: A Method for 3D Fluorophore Localization

The first implementation of 3D STORM uses the astigmatism imaging method to localize each fluorophore in 3D space (Huang et al. 2008b). This involves the insertion of a weak cylindrical lens into the imaging path to create two slightly different focal planes for the x and y directions as illustrated in Figure 9A. As a result of this modification to the imaging optics, the images of individual fluorophores typically appear elliptical, and the ellipticity varies as the position of the fluorophore changes in z . When the fluorophore is located in the average focal plane approximately halfway between the x and y focal planes, the image appears round; when the fluorophore is above the average focal plane, its image is more focused in the y direction than in the x direction and, thus, appears ellipsoidal with its long axis along the x axis; conversely, when the fluorophore is below the focal plane, the image appears ellipsoidal with its long axis along the y axis. The peak center position corresponds to the $x - y$ position of the fluorophore as before, and a measurement of the peak widths along the x - and y -axes w_x and w_y , allows the z coordinate of the fluorophore to be unambiguously determined.

The calibration curves of w_x and w_y as a function of z , shown in Figure 9B, are experimentally determined for single Alexa Fluor 647 fluorophores bound to a glass surface and scanned in z using a piezo-driven sample stage. These curves are used in the data analysis procedure to determine the z coordinate of each photoactivated fluorophore by comparing the measured w_x and w_y values of its image with the calibration data. In addition, for samples immersed in aqueous solution on a glass

FIGURE 9. Three-dimensional localization of individual fluorophores. (A) This simplified optical diagram illustrates the principle of determining the z coordinate of a fluorescent object from the ellipticity of its image by introducing a cylindrical lens into the imaging path. The *right* panel shows the images of a fluorophore at various z -positions along the optical axis. (B) The calibration curve of the image widths w_x and w_y as a function of z obtained from single molecules of Alexa Fluor 647. (Adapted, with permission, from Huang et al. 2008b, ©AAAS.)

substrate, a small correction is applied to the z localization to quantitatively account for the refractive index mismatch between glass and water (Egner and Hell 2006; Huang et al. 2008a,b).

The 3D localization precision obtained by this method, although dependent on many factors unique to an individual microscope setup, is normally sufficient for STORM over a range of ~ 300 nm above and below the focal plane. This allows high-resolution imaging of samples in a region several hundred nanometers in thickness (Huang et al. 2008b). When combined with z stepping of the sample stage relative to the objective, the imaging depth can be increased to several micrometers (Huang et al. 2008a). Another important aspect to consider is the spherical aberration generated by the refractive index mismatch between the sample and the objective. Generally speaking, oil-immersion objectives are preferable for STORM imaging because of their high NA. Oil objectives may pose a problem when imaging a sample in an aqueous medium, however, as spherical aberration becomes an increasing concern higher above the glass–water interface. Methods have been described to correct imaging aberrations up to $\sim 3 \mu\text{m}$ into the sample (Huang et al. 2008a). Beyond this limit, it is advisable to switch to a water-immersion objective or to use index-matched media with the oil-immersion objective, despite the other disadvantages these options may present such as decreased light collection efficiency in the former case or incompatibility with live cell imaging in the latter case.

3D STORM Imaging Examples

Three-dimensional STORM images of CCPs provide a clear demonstration of the ability of this method to resolve a sub-diffraction-limit sized cellular structure with a complex 3D morphology. For 3D imaging of CCPs, a direct immunofluorescence strategy was used in which primary antibodies against clathrin were doubly labeled with Cy3 and Alexa Fluor 647. As shown in Figure 10A, when imaged by conventional fluorescence microscopy, all CCPs appeared as nearly diffraction-limited spots with no discernible structure. When imaged using STORM, the 3D structure of the pits becomes apparent (Figure 10B–D). Figure 10B shows an $x - y$ cross section of the data, taken from a region near the opening of the pits at the cell surface. The circular ring-like structure of the pit periphery is unambiguously resolved. Moreover, consecutive $x - y$ and $x - z$ cross sections of the pits (Figure 10C) clearly reveal the half-spherical cage-like morphology of these structures. The spatial resolution of this image was determined to be ~ 20 nm in the $x - y$ -directions and ~ 50 nm in the z -direction (Huang et al. 2008b).

FIGURE 10. Three-dimensional STORM imaging of CCPs in a cell. (A) Conventional direct immunofluorescence image of clathrin in a region of a BS-C-1 cell. (B) An $x-y$ cross section (50-nm thick in z) of the 3D STORM image of the same area, showing the ring-like structure of the periphery of the CCPs at the plasma membrane. (C) Serial $x-y$ cross sections (each 50-nm thick in z) and $x-z$ cross sections (each 50-nm thick in y) of a CCP. (D) An $x-y$ and $x-z$ cross section presented in 3D perspective, showing the half-spherical cage-like structure of the pit. (Adapted, with permission, from Huang et al. 2008b, ©AAAS.)

Whole-Cell 3D STORM Imaging of Mitochondria

The imaging depth of a single 3D STORM image is typically several hundred nanometers in the z dimension, as the localization precision of fluorophores at large distances from the focal plane is not sufficient to produce a high-resolution image. However, a typical mammalian cell may span several micrometers, or more, in height. To obtain the STORM image of the entire cell, one can combine the STORM imaging process with z stepping of the sample stage relative to the objective lens. Figure 11 shows a 3D STORM image of the whole mitochondrial network of a mammalian cell, which extends throughout the cell body. Each localization is colored by its z height as indicated by the color bar. The cell was immunostained with a primary antibody against a mitochondrial outer membrane protein Tom20 and a secondary antibody labeled with Alexa Fluor 405 and Cy5. To achieve the imaging depth

FIGURE 11. Whole-cell 3D STORM imaging of the mitochondrial network in a BS-C-1 cell. (A) A 3D STORM image of the mitochondrial outer membrane. Axial stepping of the objective was used to obtain an imaging depth of 3 μm . The z -dimension position is encoded according to the inset color bar. (B) Magnified vertical cross sections of several mitochondria from the image in A. Localizations from each z slice are uniquely colored by the slice in which they were originally recorded with the color index bar shown on the right. The arrows indicate regions of overlap between adjacent z slices of the mitochondrion in which the alignment error in z is estimated to be ~ 18 nm after correction for spherical aberration. (Adapted from Huang et al. 2008a.)

of 3 μm , the sample was scanned in z with $\sim 300\text{-nm}$ steps, and STORM images were taken at each scan step. As the imaging depth increases, the effect of spherical aberration caused by the refractive index mismatch between the imaging medium and the oil-immersion objective becomes more severe. Only localizations within a range of 350 nm below the focal plane were accepted to minimize the effect of spherical aberration, as the localization precision above the focal plane is subjected to substantially larger degradation because of spherical aberration as compared with that below the focal plane (Huang et al. 2008a). These image slices were then combined to create a 3D image of the entire cell. As shown in Figure 11B, the hollow shape of the outer mitochondrial membrane is clearly visible in the STORM image. The arrows highlight the alignment of regions with significant overlap that were originally recorded in different z slices, illustrating the accuracy of the image alignment and stacking. Other strategies to reduce spherical aberration include the use of a water-immersion objective or an index-matched imaging medium, as discussed above.

SUMMARY

Recent years have witnessed rapid progress in sub-diffraction-limit fluorescence imaging, facilitated by the development of fluorescent probes with novel properties such as photoswitchable fluorescence emission. In this article, we have discussed the theory and the implementation of STORM, a method for superresolution imaging based on the high accuracy localization of individual fluorophores. This method yields fluorescence images with spatial resolution an order of magnitude finer than the classical diffraction limit of optical microscopy, and even higher image resolution is possible. STORM requires no specialized apparatus apart from a fluorescence microscope and a sensitive CCD camera. Furthermore, the demonstration of multicolor and 3D STORM illustrates the potential to use this approach for biological imaging applications. Although not discussed in detail here, STORM can also be used to image live cells, enabling the observation of the nanoscale dynamics of cellular structures, with both photoswitchable fluorescent proteins (Biteen et al. 2008; Shroff et al. 2008) and photoswitchable dyes (M. Bates, S.A. Jones, and X. Zhuang, unpubl. data). Superresolution techniques have also found application in prokaryotic biology, in which the small size of bacteria makes these samples difficult to visualize with conventional optical microscopy (Kim et al. 2006; Biteen et al. 2008; Greenfield et al. 2009). Finally, photoswitchable fluorescent probes also facilitate high-density particle tracking measurements in live cells (Hess et al. 2007; Manley et al. 2008). Together with other high-resolution imaging techniques, we expect that superresolution fluorescence microscopy will be broadly applied to biological research and will bring about new insights into life at the nanometer scale.

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