Stochastic Optical Reconstruction Microscopy (STORM)

The multiple imaging modes afforded by widefield, laser point-scanning confocal, and multiphoton fluorescence microscopy permit noninvasive, temporally-resolved imaging of fixed and living cells and tissues with a high level of biochemical specificity. Despite the advantages of traditional fluorescence microscopy, the technique is hampered in ultrastructural investigations due to the resolution limit set by the diffraction of light, which restricts the amount of information that can be captured with standard objectives. In the past few years, a number of novel instrument-based approaches have been employed to circumvent the diffraction limit, including near-field scanning optical microscopy (NSOM), stimulated emission depletion microscopy (STED), saturated structured-illumination microscopy (SSIM), and ground state depletion (GSD). These techniques have all achieved improved lateral (x-y) resolution down to tens of nanometers, more than an order of magnitude beneath that imposed by the diffraction limit, but each method has a unique set of limitations.

Basic Principle of STORM Superresolution Imaging

![Figure 1](image)

The finite spatial resolution limit in optical microscopy arises from what was considered for over a century to be fixed by a fundamental physical law governing the focusing of light into a discrete spot at the image plane. First described by Ernst Abbe in 1873, the ultimate resolution of a microscope is not constrained by the optical quality of the instrument, but rather by the wavelength of light used to gather an image and the angular aperture of the optical system. In formal notation, the lateral and axial resolution limits are mathematically described by the following equations:

\[
\text{Resolution}_{x,y} = \frac{\lambda}{2NA} \quad (1) \quad \text{Resolution}_{z} = \frac{2\lambda}{NA^2} \quad (2)
\]

where \( \lambda \) is the average wavelength of illumination in transmitted light or the excitation wavelength band in fluorescence. The objective numerical aperture (\( NA = n \cdot \sin(\theta) \)) is defined by the refractive index of the imaging medium (\( n; \) usually air, water, glycerin, or oil) multiplied by the sine of the aperture angle (\( \sin(\theta) \)). According to Abbe's theory, images are composed from an array of diffraction-limited spots having varying intensity that overlap to produce the final result. Thus, the only mechanism for
optimizing spatial resolution and image contrast is to minimize the size of the diffraction-limited spots by decreasing the imaging wavelength, increasing numerical aperture, or using an imaging medium having a larger refractive index. However, under ideal conditions, lateral resolution is still limited to relatively modest levels approaching 200 to 250 nanometers (see Equation (1)), whereas axial resolution is even worse (Equation (2)), on the order of 500 nanometers. When attempting to image highly convoluted features, such as cellular organelles, diffraction-limited resolution is manifested as poor axial sectioning capability and lowered contrast in the imaging plane. Furthermore, overall specimen contrast achieved in three-dimensional specimens is generally dominated by the relatively poor axial resolution.

Laser scanning confocal and multiphoton microscopy have been widely used to moderately enhance spatial resolution along both the lateral and axial axes, but the techniques remain limited in terms of achieving substantial improvement. The focused laser excitation coupled with pinhole-restricted detection in confocal microscopy can, in principle, improve the spatial resolution by a factor of 1.4, although this is only realized at a significant cost in signal-to-noise. Likewise, multiphoton fluorescence microscopy takes advantage of nonlinear absorption processes to reduce the effective size of the excitation point-spread function. Once again, however, the smaller and more refined point-spread function is counteracted by the necessity to use longer wavelength excitation light. As a result, rather than providing dramatic improvements to resolution, the primary advantage of confocal and multiphoton microscopy over traditional widefield techniques is the reduction of background signal originating from emission sources removed from the focal plane (out-of-focus light), which enables crisp optical sections to be obtained for three-dimensional volume-rendered imaging.

Although near-field scanning techniques (such as NSOM) can achieve high resolutions without the use of diffraction-limited optics, they are difficult to operate in noninvasive mode, must closely follow the topographic contour of the specimen surface, and are of little use for imaging deep within the cytoplasm of fixed or live cells. STED, GSD, and SSIM are referred to as ensemble focused light imaging techniques, and are based on non-linear optical effects that typically require the application of multiple high-intensity pulsed lasers with specialized modulation filters to control the excitation beam geometry (a technique commonly termed point-spread function engineering). The excessively high cost of pulsed lasers has been addressed with the introduction of CW-STED systems that implement lower cost continuous wave lasers. Even though point-spread function modifying schemes are capable of achieving lateral resolution measured in the tens of nanometers, these methods can potentially damage specimens with high levels of excitation light and require fluorescent probes that are resistant to photobleaching. Furthermore, they rely on highly complex and specialized microscope configurations that are difficult for the average cell biologist to assemble.

Single-Molecule Imaging

With the emergence of total internal reflection fluorescence (TIRF) microscopy as a viable technique for examining fluorescent probes residing within a few hundred nanometers of the coverslip plane, the single-molecule detection capability afforded by this methodology offers new possibilities for obtaining sub-diffraction resolution imaging. Provided that a sufficient number of photons can be collected, the position of single emitters can be determined to an almost arbitrarily high accuracy. Based on this principle, a TIRF-related technique known as fluorescence imaging with one-nanometer accuracy (FIONA) was one of the first methods demonstrated to localize the position of fluorescent probes with high precision by accurately determining the center of their diffraction-limited point-spread function. Unfortunately, the localization accuracy of FIONA does not directly translate into superresolution imaging due to the fact that multiple emitters in close proximity (as would be found in a labeled organelle or membrane) are still difficult, if not impossible, to resolve.

A number of other single-molecule imaging techniques have been employed to localize separate emitters with high precision. By applying photobleaching to selectively remove individual molecules a FIONA-related technique termed nanometer-localized multiple single-molecule fluorescence microscopy (NALMS) was demonstrated to resolve the distances between identical fluorescent probes that overlap within a diffraction-limited region. Likewise, a similar technique related to FIONA known as single-molecule high-resolution imaging with photobleaching (SHRImP) utilizes photobleaching of two or more closely spaced fluorophores to sequentially determine their position starting from the last emitter that has been bleached. A two-color version of FIONA using fiduciary markers to monitor the registration between two detection channels was introduced as single-molecule high-resolution colocalization (SHREC). Another related investigation monitored the binding of diffusible fluorescent probes to a fixed biological target followed by photobleaching for high-resolution imaging, and was termed point accumulation for imaging in nanoscale topography (PAINT). Unlike SHRImP, NALMS, and SHREC, PAINT is not limited in the molecular density of emitters that can be resolved and is more closely aligned with the superresolution techniques that will be subsequently discussed. Other related methodologies involve using quantum dots for localization experiments based on their stochastic and reversible entry into dark states, and fluorophores have also been separated and localized based on differences in their spectral profiles.
The novel single-molecule techniques described above are capable of localizing individual fluorescent emitters with high precision, but they are only useful when the probes are very well isolated from one another at densities ranging between 10 and 50 molecules per square micrometer. The rather large intermolecular spacing is necessary to ensure that the position of each fluorophore can be accurately determined without significant interference from overlapping emitters. In addition, single-molecule methods such as FIONA, NALMS, PAINT, and SHRImP all fail to identify a distinct molecular mechanism that enables the sequential readout of individual emitters from a large ensemble of fluorophores that define a particular sub-cellular structure. Thus, it was not until photoswitching or photoconversion between two distinct fluorescence emission states was applied to heavily-labeled specimens that single-molecule (also referred to as probe-based) superresolution microscopy became a reality. Another defining technological development in supporting probe-based superresolution imaging was the introduction of low-light level electron multiplying charge-coupled device (EMCCD) camera systems that have exquisite single-photon sensitivity.

Illustrated in Figure 2 is one of the commercial implementations of a microscope designed to conduct single-molecule superresolution images in two and three dimensions (which will be discussed in greater detail below). The instrument is capable of achieving a lateral ($x$, $y$) resolution between 20 and 30 nanometers and an axial ($z$) resolution between 50 and 75 nanometers for three-dimensional imaging. The axial range for single-molecule imaging is approximately 500 nanometers, and can be further increased by scanning the specimen stage or objective in the $z$-direction. A focus drift correction system enables researchers to maintain the correct focal plane when conducting single-molecule imaging with objectives in the 60x to 100x range using water and oil immersion techniques. Available lasers for this instrument include a 50 milliwatt 405-nanometer diode unit, as well as individual 200 milliwatt solid-state lasers featuring spectral lines at 488, 561, and 647 nanometers to cover the range of fluorophores that are typically used in single-molecule imaging. Finally, images are recorded using an EMCCD camera system that is interfaced to the microscope control software. A vibration isolation table is required for instrument stability.

**Single-Molecule Superresolution Imaging**

A trio of techniques independently surfaced during a four-month period in 2006 that elegantly demonstrated the sequential and stochastic readout with precise localization of multiple single molecules from specimens that were fluorescently labeled with photoswitchable dyes or optical highlighter fluorescent proteins at densities approaching those commonly employed in widefield and confocal fluorescence microscopy (ranging up to 100,000 molecules per square micrometer). These nearly identical single-molecule superresolution imaging techniques have been termed stochastic optical reconstruction microscopy (STORM; see Figure 1), photoactivated localization microscopy (PALM), and fluorescence photoactivation localization.
microscopy (FPALM). They rely on an activation laser applied at low power to trigger emission switching so that any particular molecule has a small probability of being photoactivated, but the majority of the population remains in the original (dark or native) emissive state. Although the major difference between the methods as they were originally published was simply the nature of the fluorescent probes used to achieve superresolution imaging, these techniques are virtually identical. In fact, the original STORM research report mentioned the use of fluorescent proteins, whereas the first PALM paper discussed the application of caged synthetic fluorophores.

The fundamental principle behind STORM, PALM, FPALM, and related methodology is that the activated state of a photoswitchable molecule must lead to the consecutive emission of sufficient photons to enable precise localization before it enters a dark state or becomes deactivated by photobleaching. Additionally, the sparsely activated fluorescent molecules must be separated by a distance that exceeds the Abbe diffraction limit (in effect, greater than approximately 250 nanometers) to enable the parallel recording of many individual emitters, each having a distinct set of coordinates in the lateral image plane. As will be discussed in detail, the centroids derived from images of the single molecules captured with an area-array detector are used to determine molecular coordinates with a precision that is based on the number of emitted photons (see Figure 3). Images of the emitters are captured using a low power readout laser (usually spanning from 1 to 3 camera frames) until they spontaneously photobleach or re-enter a dark state. Repeating this process for multiple cycles, each generating a stochastically unique subset of activated fluorophores, enables the positions of many fluorophores to be determined and a summed image to be reconstructed at the end of the experiment.

The basic steps involved in creating a STORM superresolution image are presented using a series of cartoon drawings in Figure 1. The target structure illustrated in Figure 1(a) shows a hypothetical densely labeled filamentous network of intracellular structures that can represent cytoskeletal biopolymers constructed with monomeric units of tubulin or actin. In Figure 1(b), a sparse set of the fluorescent probes are activated to produce single-molecule images (represented by orange circles) that do not overlap. After capturing the images with a digital camera, the point-spread functions of the individual molecules are localized with high precision based on the photon output before the probes spontaneously photobleach or switch to a dark state. The positions of localized molecular centers are indicated with black crosses. The process is repeated in Figures 1(c) through 1(e) until all of the fluorescent probes are exhausted due to photobleaching or because the background fluorescence becomes too high. The final superresolution image (Figure 1(f)) is constructed by plotting the measured positions of the fluorescent probes.

The concept of localizing individual molecules with high precision was first described by the German physicist Werner Heisenberg in the 1930s and was more formally addressed with a solid mathematical foundation during the 1980s and 1990s by several research groups. Basically, although the image of a single fluorescent emitter is manifested in a finite-sized spot having dimensions limited by diffraction when captured with a CCD-based camera, the precision with which the exact position of the molecule can be determined can be much higher (in the range of a few nanometers), provided enough photons are captured. This is due to the fact that each captured photon provides an independent measurement of the position, with the ultimate level of precision being directly proportional to the square root of the total number of measurements. Methods for
determining molecular localization coordinates are based on a statistical curve-fitting algorithm of the measured photon distribution to a Gaussian function. The goal of this exercise is to determine both the mean value of the photon distribution ($\mu$) and the standard error in the fitted position (the uncertainty, $\sigma$), according to the equation:

$$\sigma = \sqrt{\left(\frac{s^2}{N}\right) + \left(\frac{a^2/12}{N}\right) + \left(\frac{8\pi s^4 b^2}{a^2 N^2}\right)}$$  \hspace{1cm} (3)

where $s$ is the standard deviation of a Gaussian function approximating the true point-spread function of the emitter, $N$ is the number of photons captured from the fluorescent molecule, $a$ is the pixel size of the imaging detector, and $b$ is the standard deviation of the background (including background fluorescence emission combined with detector noise). The first term under the square root symbol on the right-hand side of Equation (3) takes into account photon shot noise, while the second term includes the effect of finite pixel size of the detector. The last term factors the effects of background noise into the equation. By applying these simple techniques, a localization accuracy of approximately 10 nanometers can be achieved when the measured photon distribution of a fluorophore is approximately 1,000 photons and the background noise is negligible compared to the molecular signal. In the latter case, Equation (3) can be approximated as:

$$\sigma \approx s/\sqrt{N}$$  \hspace{1cm} (4)

As described by Equation (3) and Equation (4), the most critical element in obtaining the high-precision results necessary for precise molecular localization in STORM imaging is to minimize the background noise while simultaneously maximizing photon output from the fluorescent probe. In one of the best case scenarios, if 10,000 photons can be gathered in the absence of background before the fluorophore either photobleaches or is switched off, the center of localization can be determined with an accuracy down to 1 to 2 nanometers. However, as lower numbers of photons are collected, the localization accuracy becomes increasingly less. Thus, if only 400 to 500 photons are measured, the accuracy drops to around 20 nanometers or worse. In STORM imaging, background arises from natural or transfection reagent-induced autofluorescence, as well as from residual fluorescence from surrounding probes that have either not been activated yet or have entered a dark state (but not photobleached). Therefore, the fluorescent probes chosen for STORM imaging should display a high contrast ratio, which is defined as the ratio of fluorescence emission before and after photoactivation or photoconversion. Illustrated in Figure 3 is the localization accuracy of an individual photoswitchable probe. The image profile of a Cy3-Cy5 dye pair during a single cycle is shown in Figure 3(a), whereas the distribution of the centroid positions from many activation cycles after being corrected for stage drift is presented in Figure 3(c). The inset (Figure 3(b)) shows the centroid positions.

A rather large number of variations on the basic concept of STORM, PALM, and FPALM have been reported using a variety of excitation schemes and induced dark states with synthetic fluorophores and fluorescent proteins. Thus, direct STORM (dSTORM) operates in a manner similar to its parent, but instead of paired photoswitchers (such as Cy3 and Cy5), the technique is able to use conventional stand-alone carbocyanine dyes (including Cy5, Alexa Fluor 647, and several of the ATTO series fluorophores). The introduction of dSTORM was an important development because it has become a major driver for research into the development of potential new photoswitchable synthetic fluorophores that can be selectively turned on or off with a single laser. Likewise, in a modified technique known as PALM with independently running acquisition (PALMIRA), the digital camera is operated at high speed without synchronization to the activation laser or the switching cycles of the fluorescent probes. PALMIRA requires the application of either photoconvertable or reversibly photoswitchable fluorophores (either synthetic or fluorescent proteins), and dramatically accelerates image acquisition speeds by up to 100-fold.
Among the other probe-based superresolution methods that were initially reported using synthetic fluorophores is a technique named ground state depletion with single-molecule return (GSDIM), which operates by populating the dark state of a synthetic fluorophore by driving it into a metastable triplet state followed by recording images of the emitters as they stochastically return to the ground state. Many similar techniques have emerged that take advantage of this and related phenomena, including blink microscopy and reversible photobleaching microscopy (RPM), two examples of a seemingly endless stream of technical variants hosting a spectrum of new acronyms. The most significant potential afforded by these new methods is that they are able to generate superresolution images using a subset of standard synthetic fluorescent probes, such as fluorescein, the Alexa Fluors, ATTO dyes, carbocyanines and similar dyes either in simple buffers or coupled to oxygen scavengers and aliphatic thiols. However, it is worth noting that to generate truly high resolution images requires fluorophores with a very low average on time to off time ratio, so that only a small fraction of the molecules is activated at any given time. More specifically, approximately 10 molecules can be localized within a diffraction limited area if the average on-off ratio is equal to 0.1. According to the Nyquist criterion (discussed below), this corresponds to a spatial resolution improvement of only 50 percent over the diffraction limit. Most of the conventional fluorophores do not exhibit a suitable ratio, motivating research into the development of probes with more desirable switching properties.

The series of pseudocolored images in Figure 4 illustrate STORM superresolution imaging of the microtubule network in a mammalian fibroblast kidney cell. The adherent African green monkey kidney cells were fixed with a mixture of paraformaldehyde and glutaraldehyde, and then blocked with 5-percent normal goat serum before being treated with mouse primary antibodies directed to alpha-tubulin. After washing, the specimen was treated with a goat anti-mouse secondary antibody doubly conjugated to Cy3 and Alexa Fluor 647 (a structural analog of Cy5 with similar photoswitching properties). STORM images were generated using a green activation laser (532 nanometers) and a far-red imaging laser (657 nanometers). A conventional widefield fluorescence image of microtubules in a large area of an individual cell is shown in Figure 4(a). The corresponding STORM image gathered from the same area is presented in Figure 4(c). The white boxed region in Figure 4(a) is shown enlarged in Figure 4(b) with the same area resolved using STORM shown in Figure 4(d). Note how clearly the intertwined microtubules are delineated in the STORM image.
Fluorescent Probes for STORM Imaging

Fluorescent probes suitable for imaging with STORM and related superresolution methods are those featuring very high brightness and contrast levels that yield the maximum number of photons per molecule before photobleaching or return to a dark, non-fluorescent state. For all fluorophores, relative brightness is determined by multiplying the molar extinction coefficient and the fluorescence quantum yield, values which ideally should be in the range of 100,000 and 0.75 to 0.90, respectively, in order to achieve the highest precision in localization. In addition, the best probes for STORM imaging should exhibit spectral profiles for the active and inactive species that are sufficiently well separated and thermally stable so that spontaneous interconversion rates are very low compared to the light-controlled activation rate. Due to the fact that many of the fluorophores used in STORM imaging can be photoswitched, these probes should also exhibit high switching reliability, low fatigue rates, and switching kinetics that can be readily controlled. Thus in terms of photobleaching or photoswitching to a dark state, the best probes are those whose inactivation can be balanced with the activation rate to ensure that only a small population of molecules is activated at any particular time.

Aside from the requirements for high brightness levels and photoswitching stability, STORM superresolution probes must be capable of localizing to their intended sub-cellular targets with high precision, and they should exhibit the lowest possible background noise levels. Fluorescent proteins, hybrid systems that combine a genetically-encoded target peptide with a separate synthetic dye component that is membrane permeant, and highly specific synthetic fluorophores (such as MitoTrackers, LysoTrackers, and SYTO fluorophores) are able to selectively target protein assembles or organelles. In contrast, most of the common synthetic dyes and quantum dots must first be conjugated to a carrier targeting molecule for precise labeling. Primary or secondary antibodies can be used with immunofluorescence to target synthetic fluorophores and quantum dots to specific sub-cellular locations, but the proximity of the fluorescent probe to the target is mediated by a 10 to 15 nanometer antibody molecule, and the precise number of actual fluorophore units involved is also uncertain. However, a distinct advantage of immunofluorescence is that it targets endogenous proteins while fluorescent protein fusions often involve over-expression systems that could affect cellular function.

Properties of Selected Probes for STORM Imaging

<table>
<thead>
<tr>
<th>Protein or Name (Acronym)</th>
<th>Ex (nm)</th>
<th>Em (nm)</th>
<th>EC (x 10^-3)</th>
<th>QY</th>
<th>N Photons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photoactivatable Fluorescent Proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA-GFP (N)</td>
<td>400</td>
<td>515</td>
<td>20.7</td>
<td>0.13</td>
<td>70</td>
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<tr>
<td>PA-GFP (G)</td>
<td>504</td>
<td>517</td>
<td>17.4</td>
<td>0.79</td>
<td>300</td>
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<td>PS-CFP2 (C)</td>
<td>400</td>
<td>468</td>
<td>43.0</td>
<td>0.20</td>
<td>ND</td>
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<tr>
<td>PS-CFP2 (G)</td>
<td>490</td>
<td>511</td>
<td>47.0</td>
<td>0.23</td>
<td>260</td>
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<tr>
<td>PA-mCherry1 (R)</td>
<td>564</td>
<td>595</td>
<td>18.0</td>
<td>0.46</td>
<td>ND</td>
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<tr>
<td>photoconvertible Fluorescent Proteins</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>mKikGR (G)</td>
<td>505</td>
<td>515</td>
<td>49.0</td>
<td>0.69</td>
<td>ND</td>
</tr>
<tr>
<td>mKikGR (R)</td>
<td>580</td>
<td>591</td>
<td>28.0</td>
<td>0.63</td>
<td>970</td>
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<tr>
<td>tdEos (G)</td>
<td>506</td>
<td>516</td>
<td>34.0</td>
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<td>ND</td>
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<td>tdEos (R)</td>
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<td>581</td>
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<tr>
<td>mEos2 (G)</td>
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<td>519</td>
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<tr>
<td>mEos2 (R)</td>
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<td>584</td>
<td>46.0</td>
<td>0.66</td>
<td>500</td>
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<tr>
<td>Dendra2 (G)</td>
<td>490</td>
<td>507</td>
<td>45.0</td>
<td>0.50</td>
<td>ND</td>
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<tr>
<td>Dendra2 (R)</td>
<td>553</td>
<td>573</td>
<td>35.0</td>
<td>0.55</td>
<td>ND</td>
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<tr>
<td>Photoswitchable Fluorescent Proteins</td>
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<td>Dronpa</td>
<td>503</td>
<td>517</td>
<td>95.0</td>
<td>0.85</td>
<td>120</td>
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<td>rsFastLime</td>
<td>496</td>
<td>518</td>
<td>39.1</td>
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<td>Padron</td>
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<td>bsDronpa</td>
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<tr>
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<td>488</td>
<td>60.0</td>
<td>0.50</td>
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A compilation of properties for selected fluorescent proteins and synthetic dyes for superresolution microscopy is presented in Table 1. Along with the common name and/or acronym for each fluorophore, the peak excitation (Ex) and emission (Em) wavelengths, molar extinction coefficient (EC), quantum yield (QY), relative brightness, and the number of photons emitted per molecule per switching cycle (N Photons) are listed. C-Rhodamine and C-Fluorescein refer to caged derivatives. The computed brightness values were derived from the product of the molar extinction coefficient and quantum yield, divided by the value for EGFP. The designation ND indicates the values have not been determined. This listing was created from scientific and commercial literature resources and is not intended to be comprehensive. The excitation and emission peak values listed may vary in published reports due in some cases to the broad spectral profiles. In actual fluorescence microscopy investigations, the experimental brightness of a particular fluorophore may differ (in relative terms) from the brightness provided in this table. Among the many potential reasons for these differences are wavelength-dependent variations in the transmission or reflectance of microscope optics and the efficiency of the camera.

STORM was originally developed using a pair of orange and red-emitting carbocyanine dyes, Cy3 and Cy5, combined to form what is referred to as a cyanine switch. In effect, Cy5 can be switched between fluorescent and dark states in a controlled and reversible manner using light of different wavelengths (see Figure 5). Excitation with red laser light (633, 647, or 657 nanometers) generates fluorescence emission from Cy5 and can also photoswitch the dye back to a stable dark state. Exposure to green laser light (around 532 nanometers) will convert Cy5 from the dark state back to a fluorescent state, but the recovery rate depends on the close proximity of a second activator dye, Cy3. The fatigue rate on the Cy3-Cy5 cyanine switch is excellent because Cy5 can be photoswitched on and off hundreds of times before permanent photobleaching occurs. This dye pair was employed to label short double-stranded DNA molecules with multiple switches separated by a well-defined number of base pairs, and STORM was used to resolve the distance between adjacent cyanine switches with a high degree of accuracy.

A variety of other photoswitchable synthetic probes can also be used in STORM imaging. In its simplest form, STORM can be achieved using a single fluorescent dye and continuous wave laser operating with a spectral line that closely overlaps the excitation spectrum of the dye. Thus Alexa Fluor 647 can be imaged with a 657-nanometer laser that accomplishes all three tasks required for STORM, including exciting fluorescence from the probe, driving the probe into a dark state, and reactivating it back once again to the fluorescent state. The fluorophore spends approximately 0.1 to 0.3 percent of its time in the fluorescent state when the system has reached equilibrium, enabling around 6,000 photons to be detected for each switching cycle. The combination of high photon flux and low activation equilibrium affords a significant boost in image resolution, which is markedly improved over the conventional widefield fluorescence image. This technique is well-suited for imaging microtubules, actin filaments, and intermediate cytoskeletal filaments that have diameters well below the Abbe diffraction limit.
Illustrated in Figure 5 are the photoswitching characteristics and molecular structures of carbocyanine activator-reporter dye pairs utilized in STORM imaging. Spectrally distinct combinations of these fluorophores exhibit photoswitching behavior, as shown in Figure 5(a), where the lower panel highlights the fluorescence time traces for three photoswitchable reporters: Cy5 (yellow line), Cy5.5 (orange line), and Cy7 (red line). Each dye is paired with a Cy3 unit as the activator dye. The upper panel in Figure 5(a) traces the green laser pulses (532 nanometers) used to activate the reporter dyes. The far-red laser (657 nanometers) was continuously illuminating the specimen to excite fluorescence from the reporters and to switch them back to a dark state. The molecular structures of suitable carbocyanine reporter and activator fluorophores are presented in Figure 5(b) to demonstrate the similarity of these probes.

Alexa Fluor 647 (Figure 5) is a member of a growing family of photoswitchable carbocyanine and xanthene-based synthetic probes that can be reversibly cycled between a fluorescent and a dark state by exposure to moderate or intense laser illumination, depending upon the experimental parameters. Many of these probes have been used for several decades in preparing specimens for widefield and confocal fluorescence microscopy, and should be equally useful for superresolution imaging. Thus, a wide spectrum of fluorophores that were originally thought to be photostable has since been demonstrated to enter dark states under low oxygen conditions in the presence of aliphatic thiols at moderate laser powers. Others can be imaged using simple inorganic aqueous buffers or tissue culture media with higher laser power. Listed in Table 1 are several of the most studied synthetic dyes that are useful for STORM. These include Alexa Fluors, ATTO dyes, carbocyanines, photochromic rhodamine, and several caged dyes.

In addition to synthetic dyes, fluorescent proteins can also be used for STORM imaging (see Table 1). These unique genetically-encoded fluorescent probes have the intrinsic ability to alter their spectral properties upon exposure to light of a defined wavelength band and are able to target sub-cellular structures with a high level of specificity. Fluorescent proteins have been reported to undergo a variety of novel light-induced photoswitching characteristics, including the generation of distinct emissive and non-emissive states, as well as on-and-off blinking behavior. However, the most useful of these properties are photoactivation, photoconversion, and photoswitching, which are properties that have been collectively termed **optical highlighting**. Thus, photoactivatable fluorescent proteins are capable of being activated from a dark state to a bright fluorescent state upon illumination with ultraviolet or violet light, whereas photoconvertible fluorescent proteins can be optically transformed from one fluorescence emission bandwidth to another. Among the most useful photoactivatable fluorescent proteins for STORM imaging are PA-GFP and PA-mCherry1 (see Figure 6). Photoconvertible fluorescent proteins that have proven effective for STORM and related techniques include tandem dimer Eos (tdEos), mEos2, Dronpa, rsCherry (reversibly switchable), and the photoactivatable cyan variant, PS-CFP2.

The potentially most useful class of optical highlighter fluorescent proteins includes those that are capable of photoswitching between a brightly fluorescent and dark state, such as Dronpa and rsCherry. Unfortunately, these variants have not been particularly useful in single-molecule superresolution investigations due to low photon output in the bright state (see Table 1). It will therefore be useful when improved photoswitchable fluorescent protein variants emerge as candidates for STORM imaging. The brightness issue with fluorescent proteins remains a problem when these probes are compared to synthetic dyes, which generally feature significantly higher photon output per molecule. For example, tdEos yields approximately 750 photons per molecule in contrast to the over 6,000 photons typically observed for the photoswitchable fluorophore Cy5. In
addition, the carbocyanine dyes can undergo tens to hundreds of switching cycles before photobleaching as opposed to a very limited number when using fluorescent proteins. Aside from the brightness issue, new fluorescent protein candidates must be monitored for chromophore maturation rate and monomeric character when expressed as targeting fusions with host proteins to avoid slow development and aggregation artifacts, respectively.

Quantum dots are inorganic semiconductor nanocrystals that exhibit fluorescent properties owing to confined exciton emission. Due to the metallic character of these fluorophores, a passivation layer and hydrophilic coating must be applied to quantum dots for biological applications, and for targeting they must be conjugated to streptavidin, antibodies, or some other bio-active sequence. Among the favorable attributes of quantum dots is their remarkably symmetrical fluorescence emission profile, negligible photobleaching rate, and generally high quantum yields. Additionally, these probes exhibit broad absorption profiles that enable them to be excited over an unusually wide wavelength range. Unfortunately, the exceptional photostability of quantum dots creates a problem for stochastic superresolution imaging, a situation that won't be resolved until quantum dots can be converted into a photoswitchable state. Furthermore, targeting remains a problem with quantum dots, which generally display high background levels and much poorer localization than counterparts labeled with synthetic dyes.

A large library of caged synthetic dyes similar to the photoactivatable fluorescent proteins (PA-GFP and PA-mCherry) would be especially useful as probes for all variations of stochastic superresolution imaging, including STORM. At the present time, however, this fluorophore category has not been extensively developed and the only commercially available candidates are caged versions of fluorescein and rhodamine, which have been demonstrated to be effective in single-molecule superresolution experiments. Caged synthetics can be liberated from a protective ester moiety using irradiation with ultraviolet light to generate a fluorescent species exhibiting excellent contrast that can be localized with high precision and then photobleached. Even though these probes, similar to other synthetic dyes, are hampered by a general lack of specificity and require the assistance of conjugation to antibodies or other targeting peptides, they hold significant promise as high-photon probes for STORM. However, until a larger variety of caged fluorophores emerges, this class will remain limited for many applications.

In choosing probes for STORM imaging, the investigator is faced with the decision of using either synthetic dyes or fluorescent proteins. The downside of using immunofluorescence techniques to label intracellular structures is that the targeting antibodies are too large to permeate membranes and are therefore only useful in fixed and permeabilized cells unless the target antigen is displayed on the outer region of the plasma membrane. Labeling with antibodies is also relatively low in efficiency and potentially can add 10 to 20 nanometers to the localization uncertainty between the label and target. In contrast, fluorescent proteins usually reside within 5 nanometers of the targeting protein. In perspective, however, the use of antibodies to target synthetic probes for STORM and related techniques provides a much higher signal level than fluorescent proteins, which are more useful in live-cell imaging. It should be noted that high concentrations of the oxygen scavengers and aliphatic thiols that are often necessary to produce photoswitching with popular synthetic dyes and single lasers can be incompatible with living cells.

Future efforts in probe development for superresolution microscopy will focus on increasing the specificity and labeling
efficiency of very bright photoswitchable fluorophores while simultaneously decreasing the size of the targeting moieties. Current trends and successes in designing hybrid systems to couple synthetic fluorophores with genetically-encoded targeting sequences imply that one day these probes may be capable of achieving this goal. The highest performance candidates in these systems utilize a small peptide that is expressed in living cells and is capable of recruiting a synthetic fluorophore to generate targeted fluorescence. Other developments center around reducing the size of fluorescent proteins by searching for smaller genetically-encoded stand-alone units, such as the plant phototropin light, oxygen, and voltage (LOV) domain, which contains approximately half the amino acids of jellyfish and coral proteins. Regardless of the progress (or lack thereof) in creating new and better fluorophores, however, superresolution microscopy promises to remain on the cutting edge of optical microscopy for many years to come.

Factors Affecting Resolution in STORM Imaging

Although the theoretical foundation for single-molecule superresolution imaging with techniques such as STORM, PALM, and FPALM has been previously discussed in detail, there are a number of factors involved in determining the actual outcome of any particular investigation using these methods. Among the critical aspects that must be considered are the accuracy of each individual localization measurement, the density of probes that have been localized in the final image (commonly termed molecular density), and the physical size of the labels themselves. The relationship between resolution and the localization precision of a single molecule is readily determined. The ability to resolve two fluorescent molecules as separate entities is limited by the localization precision, which determines the position (and uncertainty) of each molecule and thus the distance between the pair. The localization precision, in turn, is primarily dependent upon the number of photons collected from the fluorescent molecules during a single activation-deactivation cycle, provided the background noise is negligible.

The relationship between molecular density and final image resolution is best described in terms of the Nyquist sampling theory, which requires approximately two data points per resolution unit. In cases where the labeling efficiency (in effect, the fraction of targets that are labeled) of a specimen is insufficient, artifacts such as discontinuity in fine structural details can appear in superresolution images. Thus, for a two-dimensional image having spatial features of size $a$, the minimum required molecular density of localized fluorescent probes necessary to meet the Nyquist criterion is:

\[
\text{Nyquist Molecular Density} \approx \frac{2}{a^2}(5)
\]

Therefore, for example, in order to achieve 20-nanometer resolution in two dimensions, one fluorophore has to be positioned at least every 10 nanometers and an extremely high molecular density of around 10,000 molecules per square micrometer is required. For superresolution in three dimensions, 20-nanometer resolution requires about a million fluorophores per cubic micrometer. In practice, a much lower molecular density is often sufficient when the geometry of the specimen is taken into consideration. Biological structures are often heterogeneous so that even saturation labeling with a relatively high abundance of target sites can result in a relatively low overall density. In general, a sufficient density of fluorescent probes must be present in order to fully map the fine details of a labeled structure. By the same criterion, a sufficient number of these fluorescent probes must be successfully localized during the imaging process.
In single-molecule superresolution techniques that temporally separate the fluorescence emission by employing photoswitchable fluorophores (such as PALM and STORM), the ratio of the on and off switching kinetics is a critical experimental parameter that should be fine-tuned to the molecular density. In the case described above for a molecular density of $10,000$ fluorophores per square micrometer, in order to achieve a final resolution of $20$ nanometers in the lateral plane, approximately $600$ fluorophores must reside in the lateral projection of the point-spread function. Such high molecular densities can be complicated by the fact that the majority of photoswitchable fluorophore are not in their dark state, leading to high background noise. These fluorophores either emit weak fluorescence or they can spontaneously switch to the fluorescent state in the total absence of activation illumination. Nonspecific activation can be either spontaneous or the artifact can be induced by the imaging laser. In situations where the molecular density is very high, a large number of nonspecifically activated fluorophores can generate overlap of single-molecule images, thus compromising the ability to achieve high-precision localization. It is therefore advisable to employ photoswitchable fluorophores that exhibit low dark-state emissions and low nonspecific activation rates.

Presented in Figure 7 is an important concept involving one of the most critical factors influencing spatial resolution in single-molecule superresolution imaging. The resolution versus molecular density is represented in Figure 7 as a series of yellow pixels in a test pattern. Features that might be present in a specimen are imaged at a progressively lower signal-to-noise ratio (fraction of pixels measured) as the molecular density decreases (from the bottom to the top of Figure 7) and these features become unresolvable when the mean molecular separation approaches feature size (the top row in Figure 7). Thus, as described in Equation (5), in order to achieve $N$-fold higher resolution in dimensions $D$, $N^D$-fold more pixels must be acquired. In order to realize such a resolution gain without compromising either the imaging speed or the signal-to-noise ratio, the signal collection rate (photons detected per second) must increase by a factor of at least $N$, which requires an $N^D$-fold higher exposure to the excitation laser for each image required.

In terms of temporal resolution, single-molecule based superresolution approaches do not directly generate an image, but rather are used to map specific molecular localizations that are determined from thousands of individual imaging frames. In this case, temporal resolution is determined by the number of imaging frames that are required to obtain a suitable image. Additionally, the nature of the photoswitchable fluorophores used in PALM and STORM imaging can produce constraints on temporal imaging speed. Fluorescent proteins are excellent for use as genetically-encoded probes in cases where localization to a specific subcellular compartment or biomolecular assembly is required. Unfortunately however, fluorescent proteins exhibit relatively slow photoswitching kinetics compared to many of the synthetic probes, such as Cy5 and Alexa Fluor 647. The latter can exhibit high switching speeds that enable 1-millisecond imaging cycles at a resolution of approximately 30 nanometers, which are several orders of magnitude greater than those obtainable with fluorescent proteins. Switching cycle speeds that help determine the temporal resolution in single-molecule superresolution microscopy will be the ultimate limitation in adapting this methodology to live-cell imaging.

**Two-Color STORM Imaging**

![Molecular Density in Single-Molecule Superresolution Imaging](image-url)
Among the primary advantages of fluorescence microscopy is the capacity to multiply image specimens labeled with more than one fluorophore to generate images featuring two, three, and four colors that help to unravel the relative organization and interactions that occur between different biological structures or molecules. In cases where two or more labeled fluorescent probes reside in the same resolution volume, they can be treated with classical co-localization analysis algorithms to determine the extent of overlap between emission spectral profiles (and thus, yield information about potential in vivo molecular interactions). The ability to conduct investigations in two or more colors is essential for probing interactions between biomolecules and could provide invaluable insight into the nature of many biological processes if it can be successfully applied to superresolution imaging.

At the foundation of fluorescence multicolor imaging lies the requirement to identify several optically distinguishable probes that can be simultaneously applied to a specimen. In STORM, the original carbocyanine dye-pair approach was employed to create a palette of photoswitchable probes each consisting of a reporter probe that can be imaged and deactivated, as well as an activator probe that serves to activate the reporter by absorbing a specific wavelength of light that overlaps with its absorption spectrum. For example, up to nine distinguishable probes can be formed by a combinatorial pairing of three reporter fluorophores (such as Cy5, Cy5.5, and Cy7) having different emission wavelengths and three activator fluorophores with different absorption spectra (including Alexa Fluor 405, Cy2, and Cy3). This strategy was utilized in STORM for the first multicolor imaging demonstrations using DNA molecules immobilized on a surface and two-color imaging of immunofluorescence specimens labeled for microtubules and clathrin-coated pits. STORM imaging was able to achieve a lateral resolution of approximately 25 nanometers using two or three different activators each paired with the same reporter. The primary advantage of using a single reporter dye is that the separate channels are aligned because the localizations are derived from fluorophores imaged along the same optical path.

Presented in Figure 8 are a series of images captured in widefield fluorescence (Figure 8(a) and 8(b)) and two-color single-molecule superresolution (STORM) imaging (Figure 8(c)). The specimen is a fixed preparation of adherent African green monkey kidney cells immunostained with the dye pairs Alexa Fluor 405 and Cy5 to label mitochondria, as well as Alexa Fluor 488 and Cy5 to highlight the microtubule network. During image acquisition, an alternating sequence of 405-nanometer and 532-nanometer laser pulses was used to sequentially activate the probes. The activated probes were then illuminated with a 657-nanometer laser to gather images. Molecular localizations were pseudocolored according to the corresponding activation laser (magenta for 405 nanometers and green for 532 nanometers). Stained cells were imaged in aqueous medium and reconstructed from approximately 500,000 localization points. The white box in the widefield image (Figure 8(a)) is expanded in Figure 8(b) for comparison with the corresponding STORM image in Figure 8(c). Note how the STORM image clearly resolves details of the mitochondria and microtubules even when they are densely packed, allowing a more precise determination of their spatial relation as compared to the conventional widefield fluorescence image.

Multicolor superresolution imaging can also be realized by combining synthetic dyes and fluorescent proteins as labels. For example, using a combination of rsFastLime (a green fluorescent protein photoswitch derived from Dronpa) and the carbocyanine, Cy5, researchers were able to perform two-color imaging of labeled microtubules by sequentially photoswitching the fluorescent protein reporter and the synthetic dye. Multicolor imaging using fluorescent proteins alone has proven to be more difficult because the emission spectrum of the pre-activated state of one fluorescent protein often overlaps with that of the post-activated state of another (due to the very broad absorption and emission spectral profiles of these probes). The first demonstration of two-color imaging with fluorescent proteins coupled the green photoswitch Dronpa with the photocconvertable fluorescent protein tdEos for labeling of actin and focal adhesion complexes, respectively, in fixed cells. The two fluorophores were imaged sequentially, with tdEos being imaged first followed by Dronpa after all of the tdEos molecules were photobleached. Unfortunately, the need to deplete one probe before imaging the other does not allow simultaneous multicolor imaging and thus presents an obstacle for time-resolved multicolor imaging. This problem was overcome by a recently developed blue-shifted Dronpa variant, termed bs-Dronpa, and the red photoactivatable fluorescent protein, PA-mCherry1. Pairing bsDronpa with Dronpa, or PA-mCherry1 with PA-GFP enables simultaneous two-color imaging.
remains, however, a continuing need to develop new fluorescent protein and synthetic dye photoswitches that facilitate rapid multicolor imaging.

Three-Dimensional STORM Imaging

The vast majority of biological structures are three-dimensional entities and thus exhibit both lateral and axial dimensions ranging in the tens of micrometers or more, a feature that is readily addressed by many of the popular imaging modalities in fluorescence microscopy. To extend this capability to the realm of single-molecule superresolution imaging, a mechanism to precisely determine both the lateral and axial positions of activated fluorophores is necessary. Unfortunately, accurate information on the axial position of a fluorophore is difficult to obtain for diffraction limited three-dimensional imaging because the point-spread function is symmetrical in the region near the focal plane. The symmetry renders it difficult to distinguish between the exact \( z \) positions of two molecules that lie a couple of nanometers above or below the focal plane. Furthermore, the point-spread function also contains scant information about the axial position of the fluorophore spanning a larger region around the focal plane (up to a few hundred nanometers) making it difficult to localize molecules residing anywhere near the focal plane of a widefield microscope.

Several innovative solutions have been presented for three-dimensional single-molecule superresolution imaging, including imaging in two different focal planes, interferometry, tilted mirrors, and convolution of the point-spread function Fourier transform with a more complex mathematical model (such as the double-helix point-spread function) using a spatial light modulator. However, one of the simplest and most straightforward techniques involves determining the axial position of a fluorophore on the basis of astigmatism present in the single-molecule emitters as a function of \( z \) depth. Originally developed as an auxiliary system for conducting three-dimensional imaging with STORM, the instrument configuration consists of a weak cylindrical lens that is positioned within the imaging optical train. In operation, the shape (in effect, the ellipticity) of the image for each fluorophore becomes a highly sensitive measure of its distance from the focal plane (along the \( z \) axis), while the centroid of the image serves to localize the lateral \((x, y)\) position.

Three-dimensional STORM imaging achieves a simultaneous lateral resolution of approximately 25 nanometers and an axial resolution approaching 50 nanometers over a range of 600 nanometers in the \( z \) direction without scanning the specimen, whereas implementing a scanning regime allows a specimen several micrometers in depth to be imaged. Illustrated in Figure 9(a) is a simplified diagram of the optical train for a three-dimensional STORM system to diagram the principle for determining the axial coordinate of a fluorescent probe based on the ellipticity of its image by introducing a cylindrical lens into the imaging path. The series of single-molecules images to the right of the optical diagram show the fluorophore at various \( z \) positions. The \( x-y \) view from a three-dimensional STORM image of the microtubule network in a fixed monkey kidney cell is presented in Figure 9(b) with axial position information encoded according to the color-coded bar in Figure 9(d). The white box in Figure 9(b) is shown as an enlargement of this \( x-y \) region in Figure 9(c), with the corresponding \( x-z \) view presented in Figure 9(d) to demonstrate that individual microtubules can be resolved quite nicely in the axial dimension using STORM techniques.

An alternative approach for single-molecule STORM-type three-dimensional imaging takes advantage of multi-focal plane.
imaging to achieve an axial resolution below the diffraction limit. By simultaneously imaging two focal planes in the specimen, the images of activated fluorophores (one overfocused and one underfocused) can be analyzed to fit a three-dimensional point-spread function to determine their spatial coordinates. The ratio of spot sizes is a monotonic function of the axial position and can thus be quantitatively assessed. Termed **bi-plane** axial localization microscopy, this technique is capable of imaging through approximately 800 nanometers in the $z$ direction without scanning and several micrometers with the aid of axial scanning techniques. One of the primary benefits of bi-plane imaging is that the lateral resolution of the image is independent of the axial position. As a test of the methodology, a resolution of 75 nanometers in the axial dimension was demonstrated for three-dimensional imaging of fluorophore-coated beads that were four micrometers in diameter.

Three-dimensional precision localization using single-molecule imaging techniques can be achieved using a widefield microscope that has been modified with a spatial light modulator to generate a double-helix point-spread function. The instrument is configured to include what is termed a 4f image processing section in the detection path that is designed to convolve the standard microscope point-spread function (arising from single-molecule emitters) with the double-helix point-spread function generated by a spatial light modulator. This convolution is performed in an aperture conjugate plane by multiplying the Fourier transform of the standard microscope image with that of the specialized point-spread function (which is a phase-only function). The resulting point-spread function for a single emitter contains two dominant lobes whose angular orientation rotates with the axial position of the emitter. By creating a calibration curve, the axial position of a fluorophore (above or below the focal plane) can be determined with an accuracy between 10 and 20 nanometers (standard deviation), which corresponds to an axial resolution of 25 to 50 nanometers (the full width at half maximum; **FWHM**). The most complex aspect of the double-helix microscope is configuration of the liquid crystal spatial light modulator, which requires several matched auxiliary achromatic lenses.

To date, the highest-resolution demonstration of single-molecule three-dimensional imaging was performed using a combination of interferometry with localization microscopy. Termed **iPALM**, the method is capable of achieving resolutions of approximately 10 nanometers in the vertical and 20 nanometers in the lateral dimensions. Instrumental design is centered on the application of interferometry to measure the differences in two position-dependent pathways taken by a single photon emitted by the specimen after being recombined with a beamsplitter so that the photon can interfere with itself. Among the most critical aspects of iPALM are the special requirements of coherence, instrument calibration, and tolerance of the fluctuating nature of fluorescence, conditions that are met using a specialized multi-phase beamsplitter. In addition, the imaging ($Z$) depth is limited to approximately 200 nanometers. iPALM has been used to resolve the axial dimension in membranes, focal adhesions, microtubules, and the endoplasmic reticulum. Unfortunately, there are no commercial implementations for iPALM and the complexity of the instrumentation (along with the rigorous calibration procedure) may post limits to its widespread application.

**Live-Cell STORM Imaging**

The ability to conduct time-resolved image sequence captures of living cells and tissues is one of the hallmark achievements of fluorescence microscopy, and can be implemented with virtually any contrast or optical sectioning mode, including widefield, laser scanning confocal, spinning disk, total internal reflection, and multiphoton microscopy. Extending this capability to nanoscale resolutions with superresolution microscopy will no doubt provide exciting new insights into many of the basic processes and interactions that occur in live cells. Even though the single-molecule and focused light point-spread engineering approaches to superresolution microscopy are still in their infancy, several breakthrough developments have occurred that show promise for future temporal investigations using these methodologies. As such, the demonstration of video-rate STED imaging of live neurons with 60-nanometer lateral resolution represents a superb example of what the future might hold.

Although single-molecule localization superresolution techniques have generally been thought to require too much time in terms of image acquisition to be of much value in live-cell imaging, several investigations have successfully demonstrated temporal data gathered by this method. As an example, the microstructure of the cell membrane was monitored by tracking the motion of photoactivatable GFP (**PA-GFP**) fused to hemagglutinin molecules with 40-nanometer accuracy. Additionally, single-particle tracking in live cells with nanometer spatial precision has been demonstrated on several occasions, albeit these studies were limited to tracking only one or a few molecules simultaneously and were not used to create superresolution maps. The use of optical highlighter fluorescent proteins, however, enables the motion of numerous protein molecules to be tracked in the same cell to obtain high-resolution information on the underlying cellular structures from particle tracks in both one and two-color experiments.
Superresolution imaging with single-molecule localization precision was demonstrated for focal adhesion complexes in live cells using the photoconvertable fluorescent protein tdEos. Digital videos featuring a lateral resolution of approximately 60 nanometers were obtained at frame rates between 25 and 60 seconds per image (far slower than is possible with many traditional fluorescence imaging modalities) to visualize retrograde transport and elongation of focal adhesions and allowing different morphologies to be observed. Presented in Figure 10 are single-molecule localization images of tdEos fused to the focal adhesion protein paxillin expressed in live Chinese hamster ovary cells. White, yellow, and cyan arrows highlight interactions and elongation of individual adhesion complexes that are observed over the time course (1105 seconds) of image capture. Single-molecule superresolution imaging revealed nascent adhesions that were not visible in widefield fluorescence, and features that appear homogeneous in widefield are revealed at their true size with substantially greater internal detail.

Live-cell imaging has also been demonstrated using direct STORM (dSTORM), which relies on the presence of a naturally occurring thiol-containing agent (glutathione) that is present in animal cells in the reduced state at millimolar concentrations. Because dSTORM takes advantage of inherent photoswitching properties exhibited by common organic fluorophores (such as Cy5, Alexa Fluor 488, and ATTO 655), there is no requirement for precise conjugation to a second fluorophore for activation. Thus, dSTORM can generate images beneath the diffraction limit using living cells labeled with selected synthetic fluorophores at significantly improved resolution. The technique has also been employed to examine translation of actin filaments labeled with Cy5 and Alexa Fluor 647 along myosin II networks absorbed onto glass surfaces in vitro and should be easily transitioned into application with other common biopolymers. Among the limiting factors of using dSTORM in live cells is the difficulty in labeling intracellular targets with synthetic fluorophores. However, development of new cell-permeant dyes coupled to advances in hybrid labeling technology should prove fruitful in future efforts to conduct live-cell imaging assays using dSTORM.

Conclusions

The impressive resolutions thus far reported with STORM and related techniques do not represent the ultimate limit of single-molecule localization microscopy. Spatial resolution using these methods is dictated by the precision and density of molecular localizations, which (provided the instrument is perfectly tuned) are primarily determined by the fluorophore brightness, the residual dark-state fluorescence, the spontaneous activation rate from the dark to the fluorescent state, labeling efficiency, and the size of the label. In cases where there is sufficient probe brightness and molecular density, the expected resolution can be almost arbitrarily high. For example, a bright fluorescent probe such as Alexa Fluor 647 is expected to generate enough photons to enable a localization precision of only a few nanometers, thus promising true molecular-scale resolution. At this level, however, the physical size of the probe becomes a critical factor so that in the ideal case, the fluorophore would be directly coupled to the biomolecule of interest. Fortunately, several recently developed approaches enable specific attachment of small organic fluorophores to cellular proteins through genetic encoding approaches and provide a labeling strategy that could potentially support molecular-scale resolution.
Another critical aspect of STORM imaging that will have a direct bearing on the success of live-cell observation is the data acquisition speed. Due to the intrinsic trade-off between temporal and spatial resolutions, super-resolution imaging is generally relatively slow. More specifically, a STORM image is constructed by plotting the localizations accumulated in a widefield view of the specimen over many imaging frames. Thus, the imaging speed in STORM is limited by the number of frames required to prepare a high-resolution image. In contrast, focused light point-spread function engineering techniques, such as STED, are limited by the time required to scan a small focal point across the specimen. Thus single-molecule techniques are expected to be faster than STED-related methods when imaging a large specimen region but slow when imaging a small area. At the present, a STORM image at the highest resolution typically requires minutes of acquisition time. However, at a spatial resolution between 60 and 70 nanometers, time-resolved images have been gathered with a temporal resolution of a few tens of seconds in living cells. It should be expected that the imaging speed will improve with faster cameras, higher excitation powers, and probes featuring faster photoswitching rates.

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