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Super-resolution microscopy by nanoscale localization of photo-switchable fluorescent probes

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A new form of super-resolution fluorescence microscopy has emerged in recent years, based on the high accuracy localization of individual photo-switchable fluorescent labels. Image resolution as high as 20 nm in the lateral dimensions and 50 nm in the axial direction has been attained with this concept, representing an order of magnitude improvement over the diffraction limit. The demonstration of multicolor imaging with molecular specificity, three-dimensional (3D) imaging of cellular structures, and time-resolved imaging of living cells further illustrates the exciting potential of this method for biological imaging at the nanoscopic scale.

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Introduction

Fluorescence microscopy is one of the most widely used tools in modern biomedical research. Inherently non-invasive, fluorescence imaging enables the observation of specific components or processes in living cells, tissues, and whole organisms. Fluorescent probes are available with a range of colors that span the visible spectrum, and a variety of labeling techniques such as immunofluorescence and *in situ* hybridization allow fluorophores to be specifically coupled to molecules of interest, enabling the simultaneous visualization of multiple targets by multicolor imaging. Furthermore, the revolutionizing development of fluorescent proteins and other genetically encoded fluorescent labels has allowed specific proteins in living cells to be observed in real time [1].

The limited resolution of fluorescence microscopy, however, leaves many biological structures too small to be studied in detail. Subcellular structures span a range of length scales from micrometers to nanometers, while the light microscope is classically limited to a resolution of ~200 nm in the lateral direction and ~500 nm in the axial direction. Other imaging techniques such as electron microscopy (EM) have achieved much higher spatial resolutions [2], and the ability of these methods to visualize biological samples with molecular resolution has had a tremendous impact on our understanding of biology. The use of electron-dense tags for molecule-specific labeling in EM, however, has limitations such as low labeling efficiency and the small number of species that can be simultaneously observed, making it difficult to map out molecular interactions in cells. Moreover, the sample preparation methods used for EM currently preclude the imaging of live samples. To achieve image resolutions comparable to EM but with the labeling specificity and live-cell compatibility provided by fluorescence microscopy would open a new window for the study of the nanoscale structure and dynamics of cells and tissues. With this goal in mind the classical limit of optical resolution has been tested, giving way in recent years to a number of new ideas which we collectively refer to as 'super-resolution' imaging techniques. In this review we focus primarily on a newly developed concept for super-resolution imaging that is based on the nanoscale localization of photo-switchable fluorescent probes.

The diffraction limit of resolution

It was recognized by Abbe in the 19th century that the spatial resolution of optical microscopy is limited by the diffraction of light [3]. 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 Δx, $\Delta y \approx \lambda/(2NA)$, and $\Delta z \approx 2\lambda\eta/(NA)^2$, respectively, where λ is the wavelength of the emitted light, η is the index of refraction of the medium, and NA is the numerical aperture of the objective lens [4]. Two identical fluorophores separated by a distance less than the PSF width will generate substantially overlapped images, rendering them difficult or impossible to resolve. The resolution of the microscope is thus limited by the width of the PSF. For visible light ($\lambda \approx 550$ nm) and a high numerical

aperture objective ($NA = 1.4$), the resolution limit is ~200 nm laterally and >500 nm along the optic axis.

A number of fluorescence imaging techniques have pushed the boundaries of optical resolution. Among these, confocal and multi-photon fluorescence microscopies improve the resolution while simultaneously attenuating out-of-focus light to allow optical sectioning and three-dimensional (3D) imaging [4,5]. Methods employing two objective lenses, such as 4Pi and I^5M microscopy, effectively increase the numerical aperture of the microscope and substantially improve the axial resolution [4,6,7]. Digital deconvolution algorithms have also been developed to produce a sharpened image with resolution improvement [8]. Each of these methods achieves an increase in the lateral and/or axial resolution, extending the resolution of the microscope to the 100–200 nm range.

Optical resolution beyond the diffraction limit

The first demonstration of optical image resolution substantially below the diffraction limit was by near-field scanning optical microscopy [9–11], though the requirement of a near-field scanning probe has limited the application of this technique to imaging near the sample surface. The idea of far-field light microscopy with diffraction-unlimited resolution is a relatively recent one. Hell and co-workers introduced Stimulated Emission Depletion (STED) fluorescence microscopy and related concepts for subdiffraction limit imaging in the mid-1990s [12]. STED microscopy takes advantage of the non-linear depletion of fluorophores from the excited state by stimulated emission. In addition to a focused excitation laser beam which pumps the fluorophores to their excited state, a second red-shifted STED beam, typically with a donut-shaped intensity profile, is used to trigger stimulated emission of the excited fluorophores in a region surrounding the excitation spot, forcing them to the ground state. This effectively confines fluorescence emission to a small region at the center of the donut, thereby reducing the size of the PSF and increasing the resolution. Experimental realizations of the STED microscope have achieved image resolutions previously thought to be unattainable. Different implementations of STED have demonstrated resolutions as high as ~20 nm in the lateral directions, 30–40 nm in the axial direction, and 40–45 nm in all three dimensions simultaneously when lateral and axial STED beams are combined in a 4Pi geometry for 3D super-resolution imaging [13,14]. Variations of STED have also been introduced, using other Reversibly Saturable Optical Fluorescent Transitions (RESOLFT) [15]. Recently, in an experimental *tour de force*, the first example of video-rate subdiffraction limit imaging was demonstrated using STED to study synaptic vesicles in live neuronal cells with 60 nm resolution [16].

Another approach to effectively sharpen the PSF is by Structured Illumination Microscopy (SIM) [17,18]. When a sample is illuminated by wide-field periodically patterned light, the excitation pattern mixes with the spatial information in the sample and shifts high frequency structural information to within the detection range of the microscope. SIM effectively expands the frequency space detectable by the microscope, thereby reducing the PSF size. This method increases the resolution by up to a factor of two, as the periodicity of the excitation pattern is itself limited by diffraction. In combination with a dual objective lens imaging geometry (I^5M), a 3D image resolution of ~100 nm has been demonstrated with this technique [19]. When structured illumination is used at saturating intensities, excitation patterns with arbitrarily high spatial frequencies may be generated, extending the resolution of SIM significantly beyond the diffraction limit [20,21]. The experimental realization of Saturated Structured Illumination Microscopy (SSIM) attained ~50 nm in-plane resolution [21].

In the past several years a new concept for super-resolution microscopy based on the localization of individual fluorescent molecules has emerged [22–24]. Rather than modify the excitation light pattern to yield a smaller PSF, as in STED and SSIM, image resolution below the diffraction limit may be achieved by precisely determining the positions of the fluorophores labeling the sample. The use of photo-switchable fluorescent probes allows the overlapping images of many individual fluorophores to be separated in time and enables precise fluorophore localization on densely labeled samples. The positions of the fluorphores are then plotted to construct a high-resolution image. In the remainder of this review we focus on the recent development of techniques based on this concept. The reader is referred to another recent review article for a more detailed description of other subdiffraction limit imaging methods [15].

Super-resolution imaging by single molecule localization

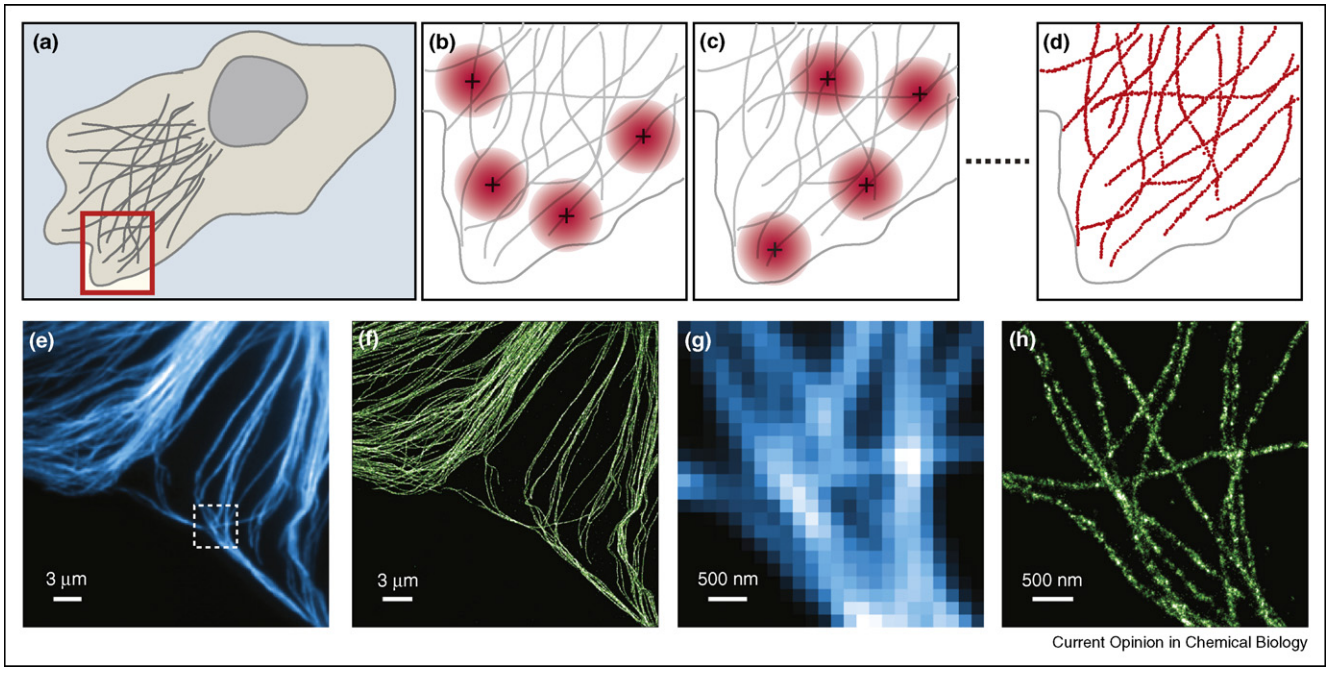
The position of an isolated fluorescent emitter, although its image appears as a diffraction-limited spot, can be precisely determined by finding the centroid of its image. The precision of this localization process is given approximately by $s/\sqrt{N}$, where s is the standard deviation of the PSF and N is the number of photons detected [25]. This concept has been used to track small particles with nanometer-scale accuracy [26,27]. Recently it has been shown that, even when the emitter is a single fluorescent dye molecule, its position can be determined with a precision as high as ~1 nm [28]. 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. In order to distinguish the fluorescence signal from nearby fluorescent emitters, several approaches have been used, based on differences in emission wavelength [29–31], the sequential photobleaching of each fluorophore [32,33], or stochastic blinking [34]. These methods have obtained high accuracy localization for several closely spaced emitters, but are difficult to extend to densities higher than 2–5 fluorophores per diffraction-limited area. A fluorescently labeled biological sample, by contrast, may be labeled with hundreds or thousands of fluorophores per diffraction-limited region.

A method for super-resolution imaging based on fluorophore localization has recently been developed by taking advantage of the properties of photo-switchable fluorescent molecules, which can be switched between a non-fluorescent (dark) state and a fluorescent (bright) state by exposure to light of particular wavelengths. The imaging process consists of many cycles during which fluorophores are activated, imaged, and deactivated (Figure 1). During each cycle, 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. This process is repeated until a sufficient number of localizations have been recorded, and 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. This concept, independently developed by three research groups, has been given the names Stochastic Optical Reconstruction Microscopy (STORM) [22], Photoactivated Localization Microscopy (PALM) [23], or Fluorescence Photo-activation Localization Microscopy (FPALM) [24].

The initial results obtained with this method yielded images with extraordinary spatial resolution and employed a number of different fluorescent probes. Using a reversibly switchable synthetic dye pair, Cy3–Cy5 [35], Rust *et al.* achieved an experimental localization precision

Figure 1



Super-resolution imaging by high precision localization of photo-switchable fluorophores. **(a–d)** The imaging concept. (a) Schematic of a cell in which the structure of interest (grey filaments in this case) are labeled with photo-switchable fluorophores (not shown). All fluorophores are initially in the non-fluorescent 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 have been localized over the course of multiple activation cycles, 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 precision of each fluorophore localization and by the number of fluorophore positions obtained. **(e–h)** Comparison of conventional immunofluorescence images of microtubules in a BS-C-1 cell (e and g) and STORM images (f and h) of the same areas. In the STORM images, each localization is rendered as a Gaussian peak whose width corresponds to the theoretical localization precision. The areas shown in (g) and (h) are expanded views of the region defined by the dashed box in (e). The microtubules are immunolabeled with photo-switchable Cy3–Alexa 647 dye pairs. Panels e–h reproduced from [50] with permission.

of 8 nm (standard deviation) for each switchable probe, corresponding to an image resolution of ~18 nm in terms of the limit of resolvability for two adjacent probes (the FWHM of the localization probability distribution) [22]. DNA and DNA-protein complexes were imaged *in vitro* using these probes and fluorophores separated by ~40 nm were clearly resolved. Betzig *et al.* demonstrated imaging of fixed cell samples expressing target proteins fused with the photo-switchable fluorescent proteins Kaede [36] and EosFP [37]. A variety of targets were labeled and the super-resolution images revealed cellular structures with sizes well below the diffraction limit [23]. Hess *et al.* imaged photo-activatable Green Fluorescent Protein (PA-GFP) [38] on a surface, achieving a spatial resolution of several tens of nanometers [24]. Collectively, these results demonstrated a resolution improvement of an order of magnitude over conventional imaging, while requiring no specialized setup other than a standard fluorescence microscope, low power continuous wave (CW) lasers, and a sensitive CCD camera.

A number of photo-switchable proteins and organic dyes have been applied to these techniques (summarized in Table 1), and variations in the image acquisition process have been demonstrated. In addition to those mentioned above, other photo-switchable fluorescent proteins have been used for super-resolution imaging including KikGR [39], Dronpa [40] and its mutants Dronpa-2, Dronpa-3, and rsFastLime [41,42], photo-switchable CFP2 [43], and other photo-switchable organic dyes including caged rhodamine [23], caged fluorescein [44], and a newly developed photochromic rhodamine compound [45]. Increased imaging speeds were demonstrated by using asynchronous activation and matching the camera frame rate with the switching kinetics of the fluorophore [46], and also by stroboscopic illumination [47]. In certain cases, the dynamic binding or translational motions of fluorescent molecules have also been used to produce high-resolution images of cellular structures without requiring the use of photo-switchable probes [48,49].

Multicolor imaging

One of the major advantages of fluorescence microscopy is its capacity for multicolor imaging, allowing the relative organization and interactions between different biological structures or molecules to be visualized, for example, through the colocalization of differently colored probes [1]. This aspect of fluorescence imaging is essential for probing interactions between biomolecules and could provide invaluable insights into biological processes if realized in super-resolution. Multicolor imaging requires multiple, optically distinguishable probes. By generalizing their cyanine dye-pair approach, Bates *et al.* have created a palette of photo-switchable probes, each consisting of a coupled dye pair: a 'reporter' dye that can be imaged and deactivated by red light, and an 'activator' dye that serves to activate the reporter by absorbing a specific wavelength of light corresponding to its absorption spectrum [50]. For example, as many as nine distinguishable probes can be formed by a combinatorial pairing of three reporter dyes with different emission wavelengths (e.g. Cy5, Cy5.5, and Cy7) and three activator dyes with different absorption spectra (e.g. Alexa Fluor 405, Cy2, and Cy3). With these probes, three-color imaging of DNA molecules immobilized on a surface and two-color imaging of immuno-labeled microtubules and

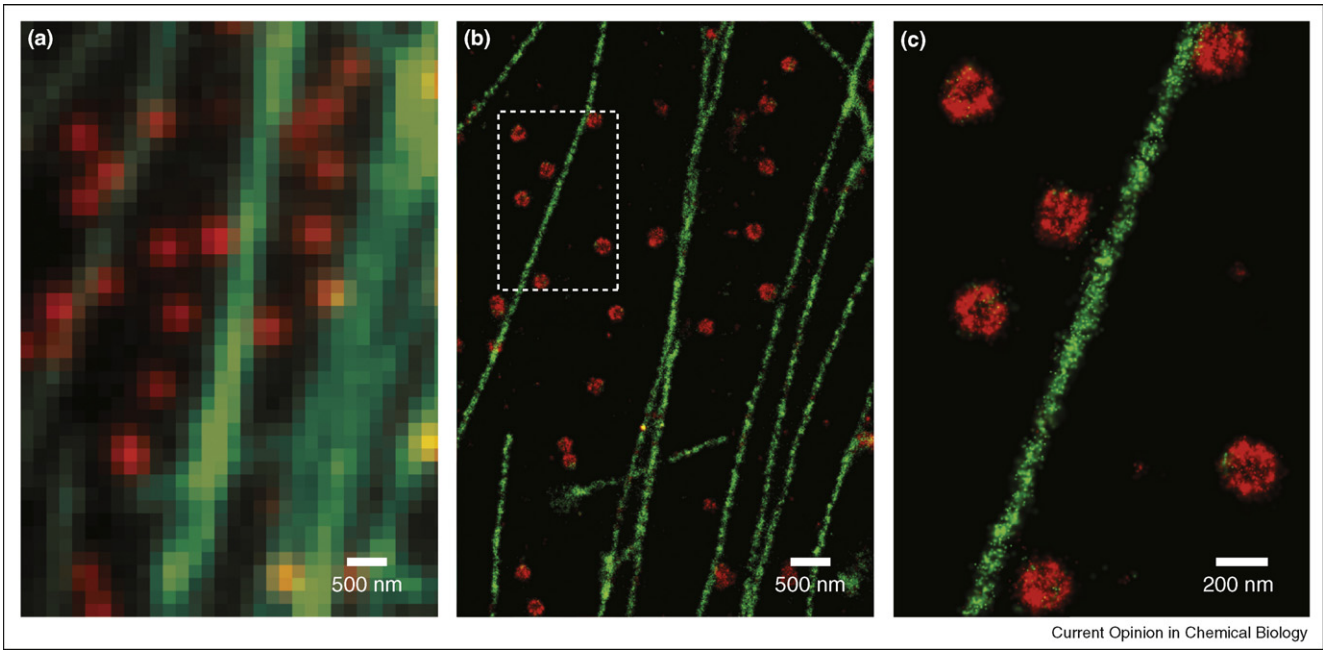
Table 1

Spectral properties of photo-switchable organic dyes and fluorescent proteins, including the fluorescence excitation and emission wavelengths of the pre-activation and post-activation states, and the wavelength used for activation of the fluorophore

Fluorophore	Activation wavelength (nm)	Pre-activation		Post-activation		Reference
		Absorption maximum (nm)	Emission maximum (nm)	Absorption maximum (nm)	Emission maximum (nm)	
Fluorescent proteins:						
Kaede	405	508	518	572	582	36
EosFP	405	506	516	571	581	37
KikGR	405	507	517	583	593	39
PA-GFP	405	400	515	504	517	38
PS-CFP2	405	400	468	490	511	43
Dronpa	405	–	–	503	518	40
Dronpa-2, Dronpa-3	405	–	–	486	513	41
rsFastLime	405	–	–	496	518	42
Organic dyes:						
Cy5	350–570[a]	–	–	649	670	50, GE Healthcare
Cy5.5	350–570[a]	–	–	675	694	50, GE Healthcare
Cy7	350–570[a]	–	–	747	776	50, GE Healthcare
Alexa Fluor 647	350–570[a]	–	–	650	665	50, Invitrogen
Photochromic rhodamine B	375	–	–	565	580	45
Caged Q rhodamine	405	–	–	545	575	Invitrogen
Caged fluorescein	405	–	–	497	516	Invitrogen

[a] Dependent upon the activator dye if present.

Figure 2



Multicolor imaging. **(a and b)** A comparison of two-color conventional immunofluoresence (a) and STORM (b) images of microtubules (green) and clathrin-coated pits (red) in fixed BS-C-1 cells. The antibodies used for microtubule staining were labeled with Cy2 and Alexa 647 (a structural analog of Cy5) as the activator and reporter, respectively. For clathrin labeling the antibodies were labeled with Cy3 and Alexa 647. **(c)** A further magnified view of the boxed region shown in (b). Panels b and c reproduced from [50] with permission.

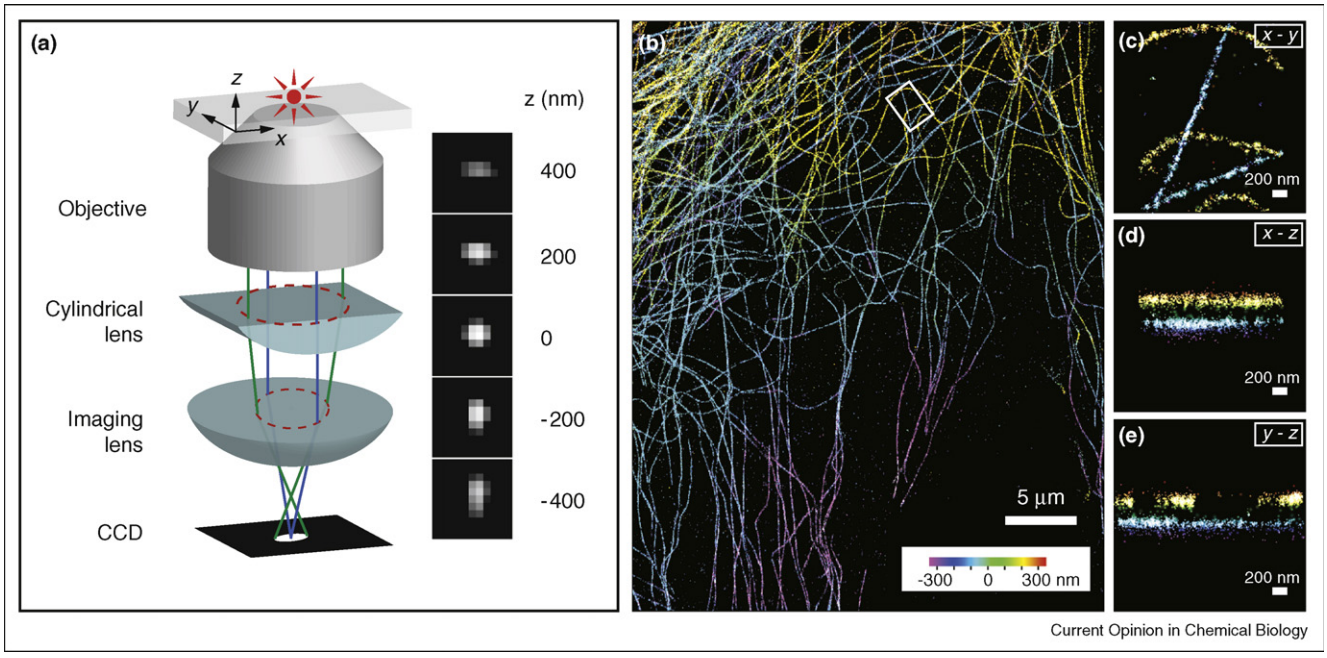
clathrin-coated pits in a fixed cell was demonstrated (Figure 2), achieving a lateral resolution of ~25 nm using two or three different activators each paired with the same reporter [50]. An advantage of multicolor imaging using a single reporter dye is that the different color channels are naturally aligned because the localizations are derived from the same type of fluorophores imaged along the same optical path.

Multicolor super-resolution imaging has also been achieved using a photo-switchable fluorescent protein (rsFastLime) and a photo-switchable organic dye (Cy5) as labels. By sequentially imaging these two fluorophores at different excitation and emission wavelengths, Bock *et al.* have demonstrated two-color imaging of microtubules in cells [51]. Multicolor imaging using fluorescent proteins alone is 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 (Table 1). Shroff *et al.* have circumvented this problem by using a reversibly switchable green fluorescent protein (Dronpa) and an irreversibly switchable green–orange fluorescent protein (EosFP) for two-color imaging of actin and adhesion complexes in fixed cells [52]. The two fluorophores are imaged sequentially, with EosFP being imaged first followed by Dronpa, which is imaged after all of the EosFP have been photobleached. The necessity for sequential imaging of each color, however, would present an obstacle for time-resolved multicolor imaging which requires structures labeled with different colors to be monitored over multiple time points. This illustrates the continued need for the development of new photo-switchable fluorescent proteins that allow simultaneous multicolor imaging. During the publication of this article, such a fluorophore was reported by Andresen et al. who demonstrated two-color imaging using a newly developed blue-shifted Dronpa variant together with the original Dronpa [53].

Three-dimensional imaging

Among the most useful aspects of fluorescence microscopy is its ability to provide a 3D image of the sample. To extend this capability to super-resolution imaging by fluorophore localization, a means to determine the lateral as well as the axial positions of the activated fluorophores is required. Such a method has been demonstrated by Huang *et al.*, in which the axial position of the fluorophore is determined on the basis of astigmatism in the image, as illustrated in Figure 3a [54]. Specifically, a cylindrical lens is inserted into the imaging path so that the shape (the ellipticity) of the image of each fluorophore becomes a highly sensitive measure of its distance from the focal plane [55], while the centroid of the image provides the lateral position as before. This method achieves a simultaneous lateral resolution of ~25 nm and an axial resolution of ~50 nm over a range of 600 nm in the z direction without scanning the

Figure 3



Three-dimensional imaging. **(a)** Simplified optical diagram illustrating 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 inset panels show images of a fluorophore at various *z* positions. **(b)** Three-dimensional STORM image of microtubules in a cell. The *z*-position information is color-coded according to the color scale bar. **(c–e)** The *x*–*y*, *x*–*z*, and *y*–*z* cross-sections of a small region of the cell outlined by the white box in (b). Figure reproduced from [54] with permission.

sample. This technique has been used to visualize the 3D organization of microtubules in a cell (Figure 3b–e) and to resolve the structure of clathrin-coated pits, which have a diameter of ~150 nm [54]. In combination with optical or physical *z*-sectioning, thicker samples of several micrometers in depth could be imaged in this manner.

Another approach for 3D imaging was subsequently reported by Jurette *et al.* [44] that takes advantage of multi-focal plane imaging [56] to achieve subdiffraction limit axial resolution. By simultaneously imaging two focal planes in the sample, the images of activated fluorophores can be fit with a 3D PSF to determine their spatial coordinates. This technique is capable of imaging 800 nm in the *z* direction without scanning, and several micrometers with the aid of *z*-scanning. A benefit of this approach is that the lateral resolution of the image is independent of axial position. A resolution of 75 nm in *z* was demonstrated for 3D imaging of fluorophore-coated beads 4 micrometers in diameter.

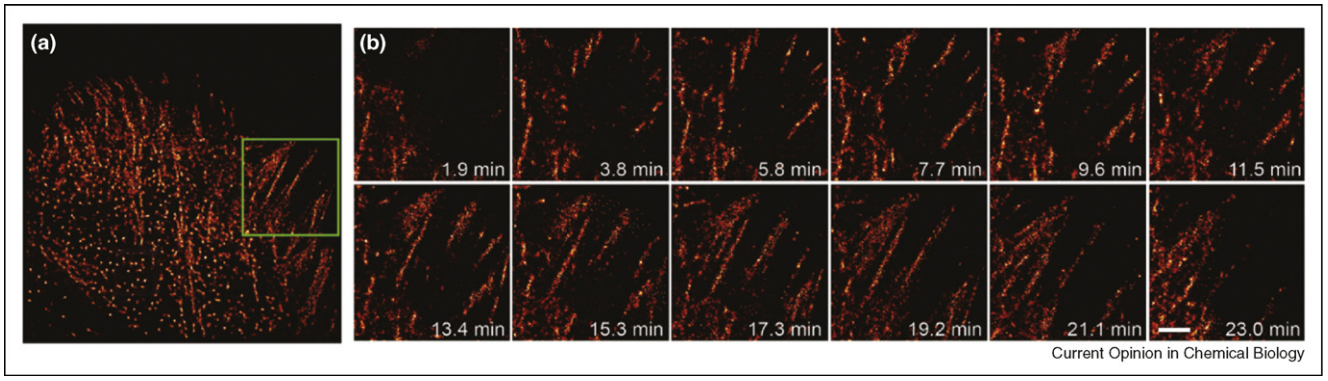
Several other techniques could prove useful for 3D super-resolution imaging. For example, defocused imaging methods used for 3D particle tracking may also be suitable for the 3D localization of activated fluorophores [57]. Conventional optical sectioning methods such as confocal or two-photon microscopy, which allow for only a thin (500–800 nm) layer of fluorophores in the sample to be observed or activated [4,5,45], may also be used to improve 3D imaging of thick samples by minimizing the fluorescence signal originating from outside the focal plane.

Live-cell imaging

An important advantage of fluorescence microscopy is its capacity for time-resolved imaging of living organisms. Extending this capability to nanoscale resolutions will provide exciting new insights into many basic processes of the cell. In this respect, the recent achievement of video-rate STED imaging of a live neuron with 60 nm lateral resolution represents a major advance [16].

Several advances have been made toward live-cell super-resolution imaging by fluorophore localization techniques. Hess *et al.* have studied the microstructure of the cell membrane by tracking the movement of PA-GFP labeled hemaglutinin molecules on the membrane of a living cell with 40 nm accuracy [58]. While single-particle tracking in live cells with nanometer spatial precision has been demonstrated previously [27,59,60], the use of photo-switchable fluorescent probes allows the motion of numerous protein molecules to be tracked in the same cell and high-resolution information on the underlying cellular structures to be derived from the particle tracks. Using a similar approach, Manley *et al.* have achieved

Figure 4



Live-cell imaging **(a)** PALM image of a wide area of the cell, showing elongated and punctate adhesion complexes in a CHO cell expressing paxillin fused to EosFP. Scale bar 5 μm. **(b)** A higher magnification view of the green boxed region in (a) illustrating adhesion complex initiation and elongation over a time course of 23 min. Scale bar 3 μm. Figure reproduced from [62] with permission.

impressively high density particle tracking of EosFP-labeled Gag and VSVG proteins in a single cell, allowing a high-resolution map of the local membrane diffusion coefficient for each protein to be generated [61].

Finally, Shroff and co-workers have demonstrated live-cell super-resolution imaging of adhesion complexes using photo-switchable EosFP [62]. Movies with a resolution of ~60 nm were obtained at a rate of 25–60 s per image, revealing the retrograde transport and elongation of adhesion complexes and allowing different adhesion complex morphologies to be observed (Figure 4). The transport of paxillin molecules into and out of the adhesion complex was also characterized. Although the imaging speed of 25–60 s is substantially slower than that of video-rate STED, a much larger sample area is imaged in this work. Considering the relatively short history of the STORM/PALM/FPALM techniques, we expect that the imaging speed will improve significantly with continued development.

Image resolution and probe development

The resolution of an image constructed from the localization of many individual fluorescent labels is dependent on several factors, including (*i*) the accuracy of each localization, (*ii*) the density of localizations obtained in the image, and (*iii*) the physical size of the labels themselves. The relationship between resolution and localization precision is fairly straightforward. The ability to resolve two labels as separate entities is limited by the precision with which the position of each label can be determined. As discussed above, the localization precision is dependent primarily on the number of photons collected from the fluorophore during a single activation–deactivation cycle.

The relationship between image resolution and the localization density can be thought of in terms of Nyquist sampling theory: for a d-dimensional image, in order to resolve a structure having spatial features of size α, the minimum required local density of localizations is $\approx(2/\alpha)^d$ [62]. This requirement typically depends on the particular geometry of the sample, however, as many biological structures are heterogeneously distributed in cells and a high local labelling density can often be achieved from a relatively low overall label density. In general, a sufficient density of fluorophores must be present in order to fully map out the fine details of the labeled structure. By the same criterion, a sufficient number of these fluorescent labels must be localized in the imaging process.

These factors place important constraints on the selection of photo-switchable fluorophores for super-resolution imaging. Photo-switchable organic dyes and fluorescent proteins may have a finite rate of fluorescence emission in the dark state, or a finite rate of spontaneous activation from the dark state to the bright state, both of which can result in an undesired background signal during image acquisition. Since accurate fluorophore localization depends on low background fluorescence and high photon flux from the molecules of interest, the properties of a fluorophore most relevant to super-resolution imaging are the number of photons detected per activation cycle and the contrast ratio between the fluorescent and the dark state (i.e. the emission intensity ratio between the fluorescent state and the dark state, or equivalently the fraction of time the fluorophore spends in the dark state versus the fluorescent state in the absence of specific activation). Fluorophores that emit a larger number of photons before switching off allow each localization to be performed with greater accuracy. Fluorophores with a higher contrast ratio effectively allow the sample to be labeled at a higher density without generating problematic background fluorescence, allowing for a higher resolution in the final image. The best of both aspects may not be found in the same fluorophore. For example,

while the Cy5 and Alexa Fluor 647 dyes are very bright, with ~6000 photons detected per activation cycle [50], they also have a relatively high spontaneous activation rate and hence a relatively low contrast ratio of ~1000. By comparison, photochromic rhodamine and EosFP are substantially dimmer (~900 and 400 photons detected, respectively) and yet they exhibit a much higher contrast ratio (10^4–10^7 and 10^5, respectively) [45,52]. The optimal choice of fluorophore will differ for different applications.

Provided a sufficiently large photon flux and labeling density, the image resolution achieved by STORM/PALM/FPALM can be almost arbitrarily high. For example, the 6000 photons detected in the case of Cy5 corresponds to a theoretical localization precision of only a few nanometers [25]. Such an accuracy points to the possibility of achieving true molecular-scale image resolution. At this level of resolution, the physical size of the label also becomes an important factor. Commonly used labels for fluorescence imaging of biomolecules include antibodies (~10 nm in size), Fab fragments of antibodies (~5 nm) and fluorescent proteins (~4 nm), all of which may significantly affect resolution at the molecular level. Alternatively, certain peptide sequences have been demonstrated which show high affinities to specific chemical groups [63,64], or which can be recognized by ligases for covalent attachment of chemical groups [65,66]. These peptides, when fused to target proteins, allow small organic dyes to be linked directly to specific molecules of interest. This type of genetically encoded small molecule labeling strategy may prove useful for pushing the limits of optical image resolution to the molecular scale.

Finally, time-resolved super-resolution imaging of live samples would benefit in particular from the further development of reversibly switchable probes. In contrast to probes that undergo a single activation cycle before permanent photobleaching, reversibly switchable fluorophores can be switched on and off many times. This property significantly benefits time-resolved imaging as the same fluorophore can be localized at different time points in the imaging process, and the pool of activatable fluorophores is depleted more slowly, allowing higher time resolution and longer movies.

Conclusions

Recent years have witnessed rapid progress in subdiffraction limit fluorescence imaging, facilitated by the development of fluorescent probes with novel properties such as photo-switchable fluorescence emission. In this review we have focused on a method of super-resolution imaging based on the high accuracy localization of individual fluorophores. This method has yielded fluorescence images with spatial resolution an order of magnitude finer than the classical diffraction limit of optical microscopy, and has the advantage of ease of implementation, requiring no specialized apparatus apart from a fluorescence microscope and a sensitive CCD camera. The demonstration of multicolor, 3D, and live-cell super-resolution imaging capabilities illustrates the great potential of this approach. Together with other subdiffraction limit imaging techniques such as STED and SSIM, and considering the rapid pace of development in the field as a whole, we expect that super-resolution fluorescence microscopy will be broadly applied to biological research and bring about new insights into life at the nanometer scale.

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