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Review of Super-Resolution Fluorescence Microscopy for Biology

Several methodologies have been developed over the past several years for super-resolution fluorescence microscopy including saturated structured-illumination microscopy (SSIM), stimulated emission depletion microscopy (STED), photoactivated localization microscopy (PALM), fluorescence photoactivation localization microscopy (FPALM), and stochastic optical reconstruction microscopy (STORM). While they have shown great promise for biological research, these techniques all have individual strengths and weaknesses. This review will describe the basic principles for achieving super resolution, demonstrate some applications in biology, and provide an overview of technical considerations for implementing these methods.

Index Headings: **Super-resolution; Fluorescence imaging; Saturated structured-illumination microscopy; Stimulated emission depletion microscopy; Photoactivated localization microscopy; Fluorescence photoactivation localization microscopy; Stochastic optical reconstruction microscopy.**

INTRODUCTION

Fluorescence microscopy is currently one of the more powerful and versatile techniques available

for biological studies.¹ The technique uses fluorophores that have large absorption cross-sections at a specific wavelength and emit light at a longer wavelength. Because of the combination of high absorption cross-section and high quantum efficiency, fluorophore-labeled molecules are very bright and readily distinguishable from other background signals. This optical property makes it fairly straightforward to obtain images of the labeled molecules with high contrast. With the development of genetically encoded fluorescent proteins (FPs), it has become possible to image protein expression, localization, and activity in living cells.² Additionally, the combination of fluorescence and confocal microscopies has allowed researchers to render the images in three dimensions (3D).^{3–5} However, optical microscopes have an inherent limitation in spatial resolution because of the wave nature of light. The effect of light diffraction limits the resolution of an optical microscope to approximately half of the wavelength of light used.^{6–8} With the best optics, the resolution of fluorescence microscopy is limited to ~200 nm, which cannot resolve many fine cellular structures.

To visualize cellular structures smaller than 200 nm, researchers have relied on electron microscopy (EM). As the de

Broglie wavelength of an electron is much shorter than visible light, EM has a much higher resolution than optical microscopes. Although atomic resolution has been achieved,⁹ EM has many practical issues that limit its utility for biological studies. The main issue is that electrons carry an electric charge, and they interact with the medium through which they travel. Since electrons interact with molecules in air, samples have to be viewed in vacuum or under low pressure. The interaction between the electrons and the sample prohibits the electrons from penetrating deep into the sample. Therefore, fixation, dehydration, and thin sectioning are required to prepare a sample for EM. With all these sample preparation procedures, one major uncertainty in interpreting images acquired by EM is that these procedures may result in artifacts, such as shrinkage of the specimen and alteration of tissue structures.¹⁰ The low pressure and thin sectioning requirements also make EM problematic for live-cell imaging. Therefore, a microscopic technique that combines both the nondestructive nature of optical microscopy and the nanometer resolution of EM is highly desirable for biological research.

For decades, researchers have been seeking optical techniques that break the diffraction limit of an optical micro-

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scope. One of the most intuitive ways to break the diffraction limit was proposed by Synge in 1928, using a sub-wavelength aperture to image a surface.¹¹ The concept was realized by Ash and Nichols in 1972 using microwaves with a wavelength of 3 cm, and the diffraction limit was overcome for the first time.¹² In 1984, the technique was demonstrated in the visible region,^{13,14} and it evolved into a rapidly expanding field, now known as near-field scanning optical microscopy (NSOM).¹⁵ One limitation of NSOM is that the aperture has to be placed within one wavelength of the light to obtain sub-diffraction-limit resolution before the light spatially diverges. The resolution decreases with increasing distance between the aperture and the sample. Therefore, NSOM is limited to the study of surfaces, and its applicability in biology is limited.

On the other hand, efforts to develop far-field optical microscopy with sub-diffraction-limit resolution have continued. Interference is probably the easiest method by which to obtain a sub-wavelength pattern for far-field microscopy. In 1978, C. Cremer and T. Cremer proposed that a laser-scanning microscope using a point-hologram would produce a spot with a diameter considerably smaller than the wavelength used.¹⁶ In 1993, sub-diffraction-limited axial resolution was demonstrated by using standing-wave excitation.¹⁷ In standing-wave fluorescence microscopy (SWFM), two counter-propagating non-focused laser beams are used to form a standing wave, which creates an excitation field with closely spaced nodes and antinodes to improve the axial resolution. In 1995, a technique called image interference microscopy (I²M) was developed. In I²M, the fluorescence from both sides of a sample is collected using two opposed objective lenses. These two images are combined by a beam-splitter and superposed on the camera to interfere, then images with a higher resolution can be obtained from the interference pattern.¹⁸ I²M can be combined with the incoherent interference illumination (I³) used in SWFM, then called I⁵M microscopy, to achieve a higher resolution. I⁵M has achieved an axial resolution seven-fold better than a typical wide-field microscope.¹⁹

An interferometric technique was also proposed for laser scanning microscopy. In 1992, a detailed theory of the 4Pi confocal fluorescence microscope was published by Hell and Stelzer.²⁰ In a 4Pi confocal microscope the specimen is illuminated by two coherent laser beams through two opposed objective lenses.²¹ The interference of the counter-propagating beams can greatly reduce the size of the focal spot in the axial direction. A seven-fold increase in axial resolution was achieved.²² However, these axial interferometric techniques offered little or no improvement in the lateral resolution and have not been widely adopted among biologists. To improve the lateral resolution for wide-field microscopy, an interferometric technique called structured illumination microscopy (SIM) was proposed in the late 1990s.^{23,24} SIM superposes a known excitation pattern over the unknown sample pattern; then a higher resolution image can be deconvolved from the resulting fringes.²⁵ Using saturated excitation patterns to introduce higher-order terms, saturated structured-illumination microscopy (SSIM) has shown a lateral resolution of < 50 nm on sufficiently bright and photostable samples.²⁶

In 1994, a new type of scanning fluorescence microscope was proposed by Hell and Wichmann to improve the lateral resolution.²¹ The basic principle of the new laser scanning fluorescence microscope is that the size of the fluorescence spot, the point spread function (PSF), can be reduced by employing a second laser beam to stimulate the fluorescent emission in the outer regions of the excitation spot. The second laser beam has a wavelength longer than the detected fluorescence; therefore, the stimulated emission will also emit at a longer wavelength not seen by the detector.²⁷ In this new approach, the shape of the effective fluorescent spot, which determines the spatial resolution, is sharpened by the second laser beam via the stimulated emission. Creating a desired depletion beam profile at the focal point is one of the major challenges to improve the resolution. Between 1994 and 2006, Hell and his co-workers published a series of different designs to manipulate the profile of the depletion beam.^{21,28,29}

The final experimental setup that attracted a great deal of attention was carried out in 2006 using the spatial phase modulation pattern described by Török and Munro.^{30,31} At this point, the potential of stimulated emission depletion (STED) microscopy in biological research became convincing, and commercial STED microscopes have recently become available.

On the other hand, new developments in single-molecule spectroscopy in the early 1990s have enabled a different approach to achieving nanometer-scale optical microscopy.^{32,33} When imaging a single isolated molecule, the size of the molecule that appears in the image, i.e., the PSF, is much larger than the actual size of the molecule because of the diffraction limit. However, the location of the molecule (or the center of the PSF) can be determined with a much higher accuracy. A technique that depends on this effect was proposed by Betzig in 1995.³⁵ In 2006, three research groups independently demonstrated super-resolution microscopy using high-precision localization of single fluorophores, named as photoactivated localization microscopy (PALM),³⁵ stochastic optical reconstruction microscopy (STORM),³⁶ and fluorescence photoactivation localization microscopy (FPALM).³⁷

In this review, we will briefly summarize far-field super-resolution optical microscopy. As SIM, STED, (F)PALM, and STORM microscopy are now becoming commercially available for biologists, their broad impact on biological research can be expected. However, these super-resolution microscopes, which were mostly developed and designed by physicists and chemists, are not as user-friendly as conventional optical microscopes. Currently there is still no ideal system that offers high-speed 3D nanometer spatial resolution with multicolor capabilities for live-cell imaging. Each technique has its strengths and weaknesses. Therefore, researchers need to be informed that there are trade-offs between resolution, sensitivity, and depth of view. Experimental designs are often also limited by the available fluorescence probes. This review will describe the basic principle of SSIM, STED, (F)PALM, and

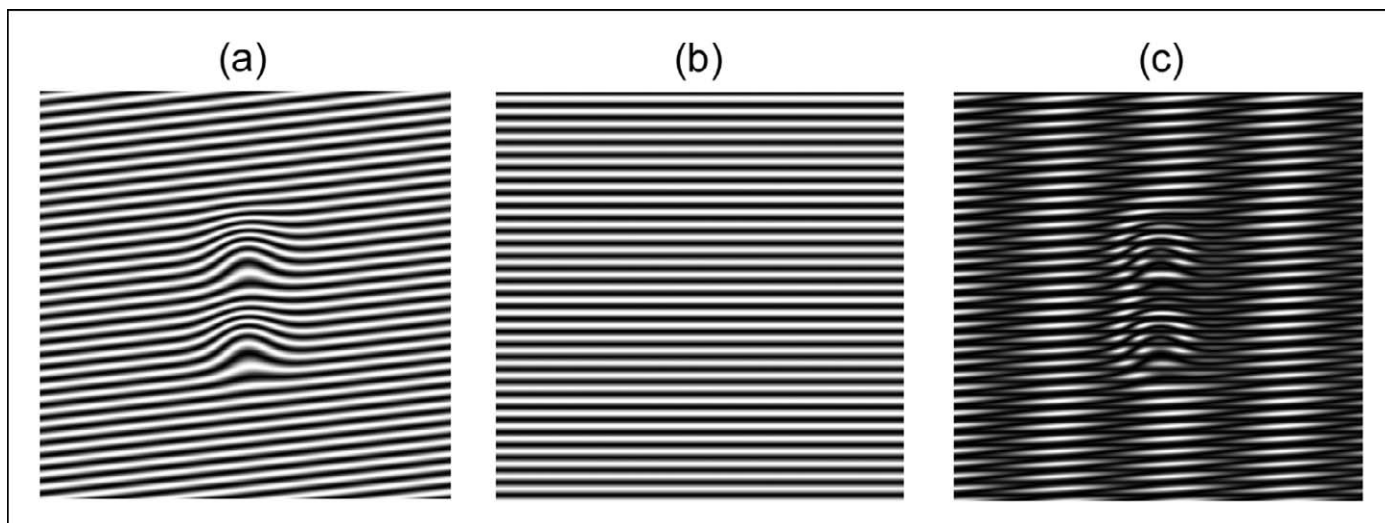


Fig. 1. (a) A sample containing unknown structures. (b) A known structured patterned. (c) The moiré pattern generated by overlaying (a) and (b).

STORM, their applications in biology, and technical considerations for implementing these methods.

STRUCTURED ILLUMINATION MICROSCOPY

The basic concept of structured illumination microscopy (SIM) is to illuminate a sample with patterned light and measure the moiré pattern. A moiré pattern is an interference pattern created by overlaying two grids with different angles or mesh sizes. If one of the patterns is an unknown structure, such as Fig. 1a, and the other pattern is a known pattern shown in Fig. 1b, the moiré fringes (Fig. 1c) obtained by overlapping Figs. 1a and 1b will contain more information about the unknown structure than the original pattern in Fig. 1a. If the known patterns have higher spatial frequencies, the technique will offer better spatial resolution. Unfortunately, the spatial frequencies that one can create optically are also limited by diffraction. Therefore, SIM can only improve the resolution by a factor of approximately 2.²⁵ Figure 2 shows the actin cytoskeleton of a HeLa cell imaged by conventional microscopy and SIM. The full widths at half-maximum (FWHM) of the fibers are 280 nm for a conventional microscope and 110 nm for SIM.

In 2005, Gustafsson further improved the resolution using nonlinear structured

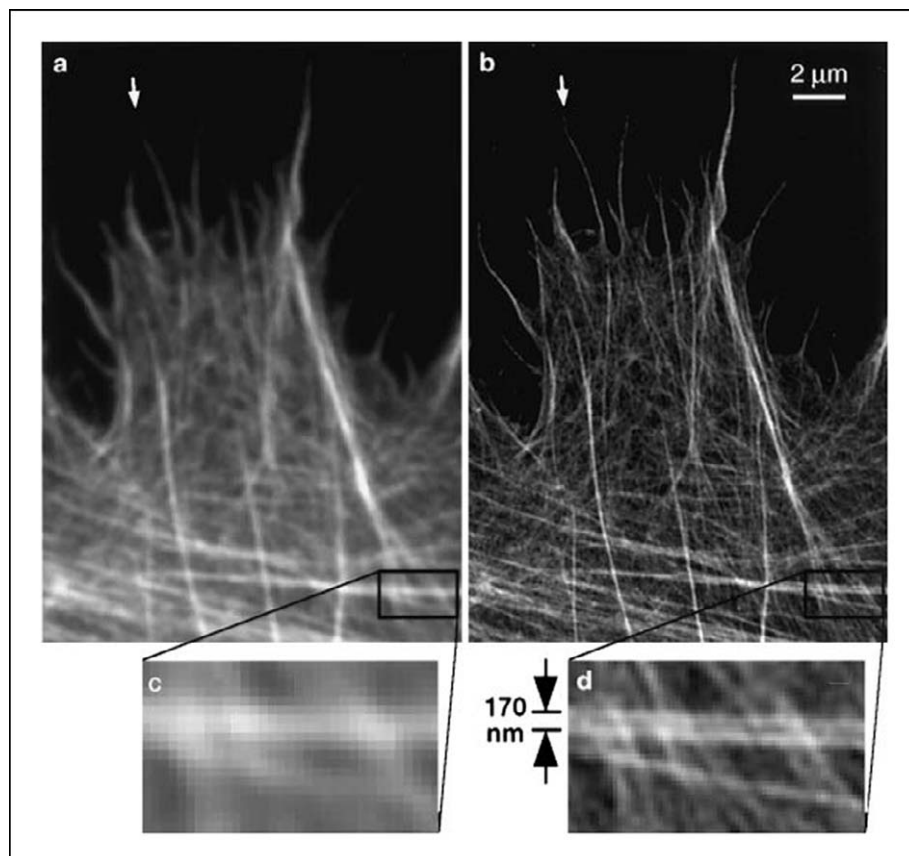


Fig. 2. The actin cytoskeleton of a HeLa cell, as imaged by (a, c) conventional and (b, d) structured illumination microscopy. (c, d) Enlargements of the boxed areas in (a) and (b), respectively. The widths (FWHM) of the finest protruding fibers [small arrows in (a, b)] are 110 ± 120 nm in (b), compared to 280 ± 300 nm in (a). (Reproduced with permission from Ref. 25.)

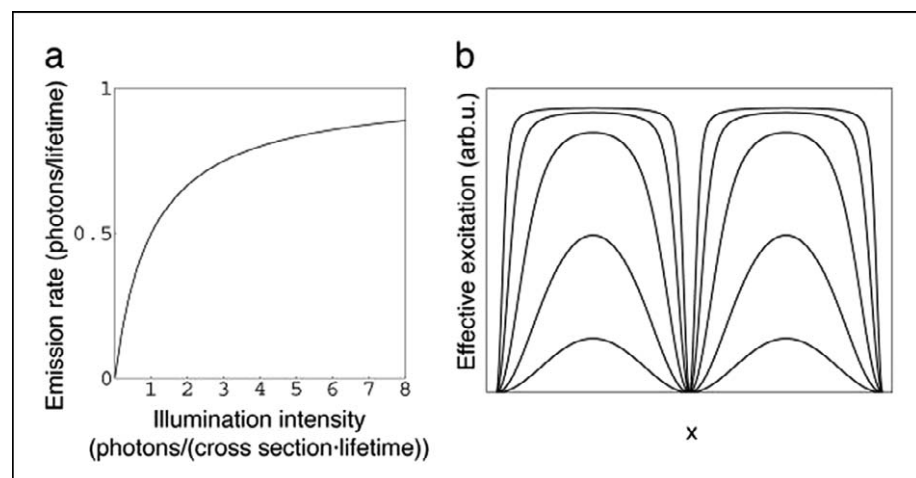


FIG. 3. (a) The nonlinear dependence of the fluorescent emission rate on the illumination intensity in the saturation regime. (b) The effective emission pattern resulting from sinusoidally patterned illumination with peak pulse energy densities of (from bottom to top) 0.25, 1, 4, 16, and 64 times the saturation threshold. (Reproduced with permission from Ref. 26.)

illumination.²⁶ Under intense illumination, the emission intensity can depend nonlinearly on the illumination intensity. As shown in Fig. 3a, the fluorescence emission intensity (rate) will saturate at high illumination intensity. This nonlin-

earity would cause the illumination pattern to saturate at the high-intensity illumination region as indicated in Fig. 3b. With high illumination intensity, the effective illumination pattern in Fig. 3b contains harmonics with spatial frequen-

cies that are higher than the original illumination spatial frequency. These higher spatial frequency components have provided a resolution of <50 nm on bright and photostable samples.²⁶ Figure 4 shows 50 nm fluorescent beads imaged by conventional microscopy (Fig. 4a), conventional microscopy plus filtering (Fig. 4b), linear SIM (Fig. 4c), and saturated structured-illumination microscopy (SSIM) (Fig. 4d), taking into account three harmonic orders in the image processing.

Multicolored 3D-SIM has been reported. 3D-SIM was achieved by illuminating the sample with three beams of interfering light and observing the interference pattern along the x , y , and z axes. As shown in Fig. 5, C2C12 cell nuclei were stained with antibodies against lamin and nuclear pore complex (NPC), and chromatin was stained with 4',6-diaminidino-2-phenylindole (DAPI). The study could image single NPCs that were co-localized with the lamin network and peripheral heterochromatin. Furthermore, double-layered invaginations of the nuclear envelope in prophase was obtained, which were

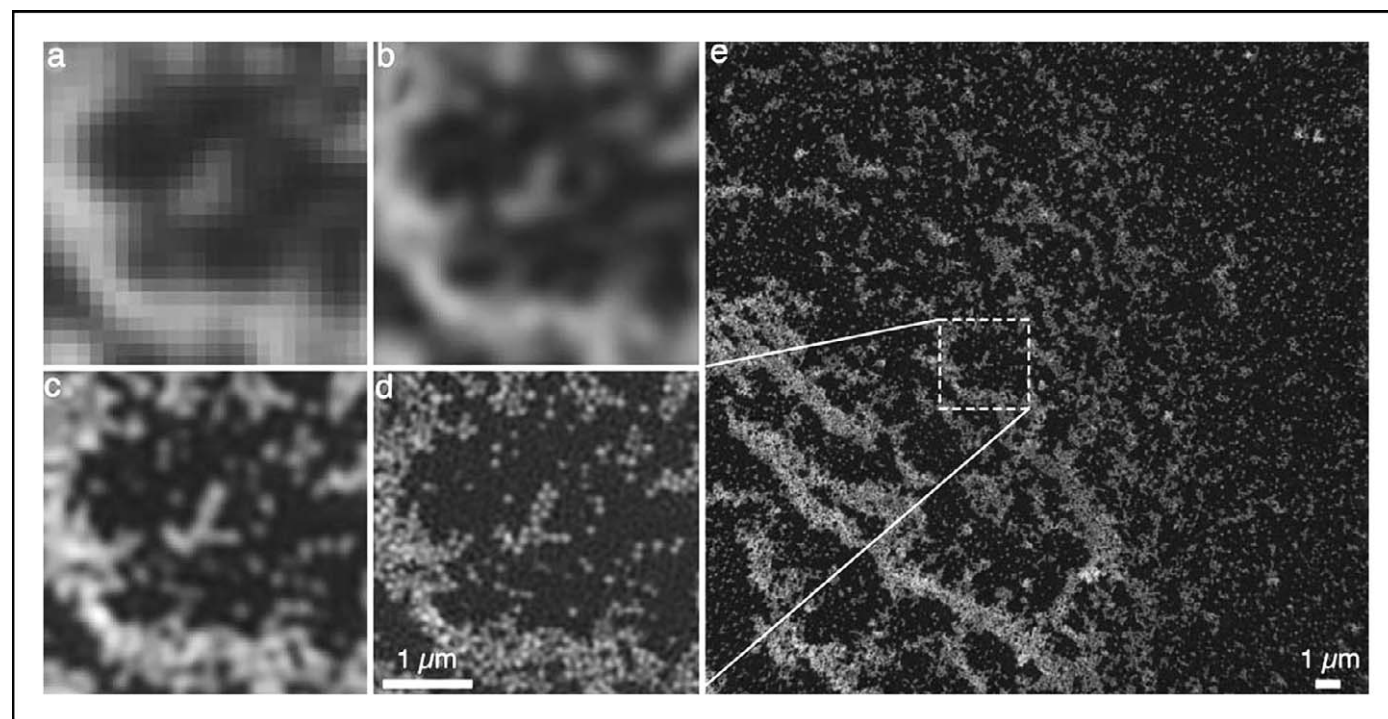


FIG. 4. A field of 50 nm fluorescent beads, imaged by (a) conventional microscopy, (b) conventional microscopy plus filtering, (c) linear structured illumination, and (d) saturated structured illumination taking into account three harmonic orders in the processing. (Reproduced with permission from Ref. 26.)

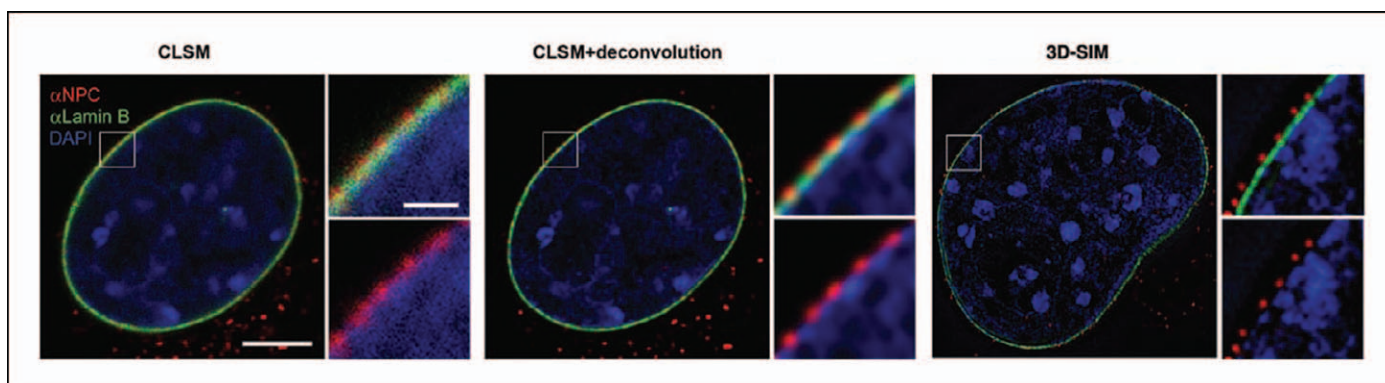


Fig. 5. Simultaneous imaging of DNA, nuclear lamina, and NPC epitopes in C2C12 cells by confocal laser scanning microscopy (CLSM), CLSM + deconvolution, and 3D-SIM. C2C12 cells are immunostained with antibodies against lamin B (green) and antibodies that recognize different NPC epitopes (red). DNA is counterstained with DAPI (blue). With 3D-SIM the spatial separation of NPC, lamina, and chromatin and chromatin-free channels underneath nuclear pores are clearly visible. (Reproduced with permission from Ref. 38.)

previously only acquired via electron microscopy.³⁸ 3D-SIM is currently the 3D super-resolution imaging technique that can easily be expanded to three colors or more, which makes it the most widely accepted super-resolution microscopy technique among biologists.

STIMULATED EMISSION DEPLETION MICROSCOPY

Stimulated emission depletion (STED) microscopy is based on laser scanning confocal microscopy. In a laser scanning confocal microscope, a laser beam is focused by an objective lens into a small focal spot within a specimen; then an image is acquired point-by-point by scanning either the specimen or the laser beam. The point-by-point approach allows an easy 3D reconstruction of a complex object. For biological studies, the specimens are usually fluorophore-labeled. Therefore, the wavelength of the excitation beam should match either the single-photon (1P) or two-photon (2P) absorption (excitation) bands of the fluorophore. Because of the important applications of fluorescent proteins in biological science, the following discussion will use green fluorescent protein (GFP) as an example. Figure 6 shows the absorption and emission spectra of enhanced GFP (EGFP). For STED microscopy, both 1P and 2P excitations have been demonstrated for GFP using excitation beams at 490 nm and 840 nm, respectively.^{39,40} In both cases, a second laser beam near

580 nm was used as the depletion beam to reduce the effective PSF.

Figure 7a shows the energy diagram for a typical STED process. Excitation from the ground state S_0 via 1P or 2P processes quickly relaxes to lower vibrational levels in the S_1 state. Without an external field, the population in S_1 decays to the vibrational ground-state

in S_0 via spontaneous emission, which peaks at 509 nm, as shown in Fig. 6 (green curve). For GFP, a 580 nm beam can effectively stimulate the emission to the higher vibrational states in S_0 .^{21,39,40} Since 580 nm is not within the detection band, the stimulated emission does not enter the detection system, and the spontaneous emission is depleted. Fig-

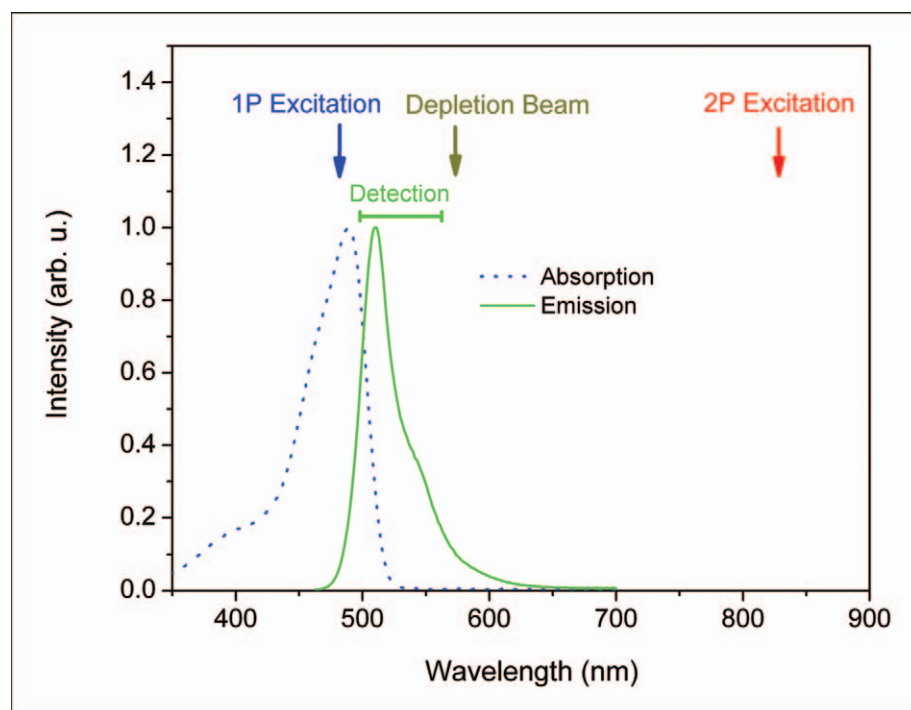


Fig. 6. The absorption and emission spectra of EGFP. The arrows indicate the wavelength for 1P excitation at 490 nm (blue), 2P excitation at 840 nm (red), and depletion beam at 580 nm (dark yellow) for STED microscopy. The detection band is indicated by the detection bar (green).

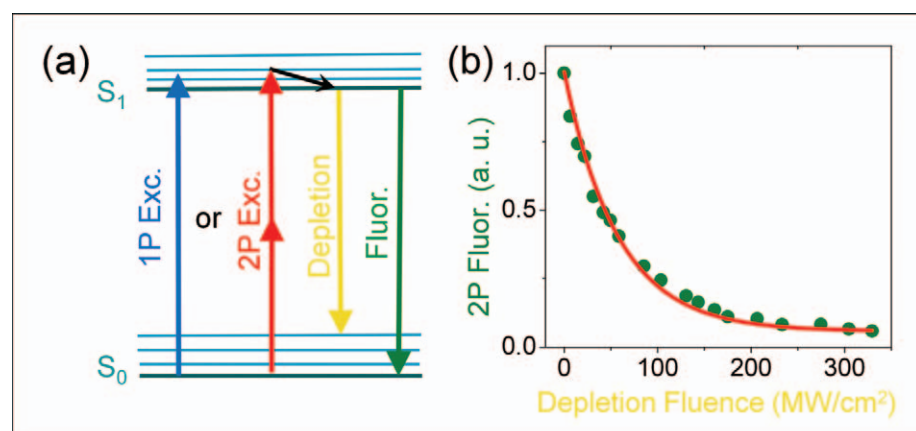


FIG. 7. (a) Energy diagram for the STED microscopy, and (b) measured 2P fluorescence depletion efficiency for GFP. The excitation beam was a 130 fs Ti:sapphire laser with a wavelength of 840 nm. The depletion beam was 200 ps with a wavelength of 580 nm.

ure 7b shows the detected fluorescence of GFP as a function of the 580 nm beam intensity when the excitation and depletion beams are spatially overlapped. Generally, 100% depletion of the spontaneous emission is not achievable but a 90–95% depletion will produce a STED image with an acceptable contrast ratio. In principle, the depletion efficiency will increase if the

depletion beam is blue shifted. However, the depletion beam is also capable of exciting the fluorophore and should not be too close to the absorption band.

The layout of a typical STED microscope is shown in Fig. 8. Two laser beams at different wavelengths are required for STED microscopy. As mass-produced laser diodes are becoming more powerful, 1P excitation STED

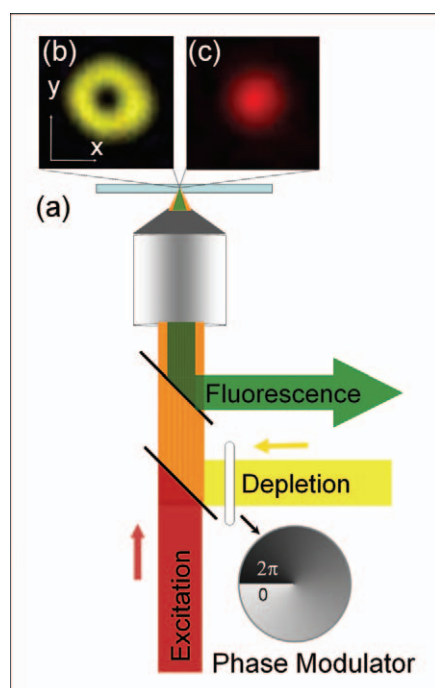


FIG. 8. (a) The layout of a typical STED microscope. (b, c) The intensity profiles of the depletion and excitation beams, respectively, which were recorded by the scattering light from a 100 nm gold particle. (Reproduced with permission from Ref. 40.)

likely can be built with lower cost.⁴¹ However, 2P excitation STED has a deeper penetration depth, which allows imaging of thick tissues.^{42,43} The excitation and depletion beams are combined using a dichroic mirror prior to entering the objective lens. The doughnut-shaped focal intensity profile of the depletion beam (Fig. 8b) can be obtained using a spiral spatial phase modulation pattern proposed by Török et al.³¹ Both pulsed and continuous wave (CW) lasers have been used for STED microscopy. If pulsed lasers are used, these two beams have to be synchronized temporally to maximize the spontaneous fluorescence depletion. With the doughnut-shaped depletion beam, the spontaneous fluorescence emission from the periphery of the excitation spot is inhibited by the stimulated emission, resulting in a fluorescent spot size below the diffraction limit. Because the intensity of the depletion beam is near zero at the center of the doughnut, the spontaneous fluorescence emission at the central region remains unchanged.

Figures 9a and 9b show EGFP-tagged caveolin 1 (Cav1-GFP) in Chinese hamster ovary (CHO) cells imaged by a 2P fluorescence microscope without and with the doughnut-shaped depletion beam, respectively.⁴⁰ Fluorescence spots of various sizes can be seen in Fig. 9 because caveolins exist in different cellular structures in the cytoplasm. The small Cav1-GFP domains are presumed to be caveolar vesicles or protein–lipid complexes, which have diameters of 50 to 100 nm under an electron microscope.^{44,45} Figures 9c and 9d are magnified views of the marked areas in Figs. 9a and 9b, respectively. The regular 2P fluorescence microscope has a diffraction-limited excitation spot with a FWHM of ~ 250 nm. With the doughnut-shaped depletion beam, two caveolar vesicles located within a distance smaller than the 250 nm diffraction limit can be distinguished.

The resolution of the STED microscope can be described by

$$\Delta r \sim \frac{\lambda}{2NA\sqrt{1 + \frac{I}{I_s}}}$$

where NA is the numerical aperture of the objective lens, λ is the wavelength of light, I is the intensity of the STED doughnut maximum, and I_s is the STED intensity used to reduce the fluorescence intensity by one-half.^{46,47} The spatial resolution of STED microscopy depends on the intensity of the depletion beam. As the intensity of the depletion beam increases, the resolution improves. Although there is no theoretical resolution limit, in practice photodamage of the sample usually sets the intensity limit on the depletion beam, particularly for biological samples.

The doughnut-shaped depletion beam works well to deplete the spontaneous fluorescence emission in the lateral direction, but it offers no resolution improvement in the axial direction (z -axis). Figure 10 shows the intensity profiles of the excitation beam, the doughnut-shaped depletion beam, and the resulting effective PSF. As shown in Figs. 10b and 10e, the intensity of the doughnut-shaped depletion beam has little intensity at the center along the z -axis. Therefore, it does not reduce the length of the effective PSF, as shown in Fig. 10f. To improve the axial resolution, a third beam has been used to deplete the spontaneous fluorescence in the axial direction.⁴⁸

Two-color STED microscopy was demonstrated by Donnert et al. in 2007 using green and red fluorescent beads.⁴⁹ Multicolor imaging significantly increases the complexity of the experimental setup as two laser beams with different colors are needed for each fluorophore, one for excitation and one for depletion. Simultaneous imaging of two colors is not straightforward for STED microscopy because four laser beams with four different wavelengths can produce undesired interference. To avoid the interference, Donnert et al. carried out the two-color STED imaging by scanning one color at a time. Recently, Bückers et al. demonstrated a quasi-simultaneous recording of two-color STED by a 40 ns time shift between the pairs of excitation and depletion beams.⁵⁰ Figure 11 shows a three-color STED image by using two dyes with similar spectral properties but different lifetimes (KK 114, ATTO 647N) and a third dye with different

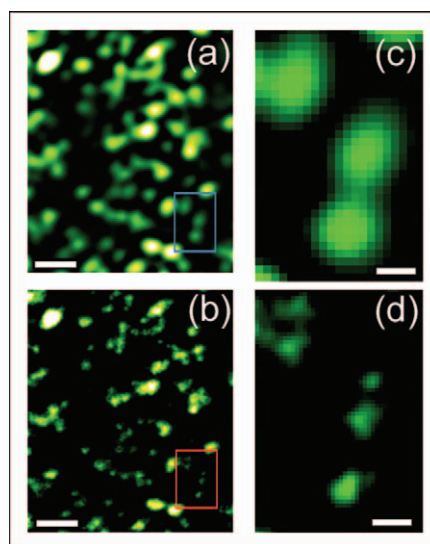


Fig. 9. GFP-tagged caveolin 1 in CHO cells imaged by a 2P STED microscope (a) without and (b) with the doughnut-shaped depletion beam. (c) Magnified view of the marked area in (a). (d) Magnified view of the same marked area in (b). The scale bars are 1 μm in (a) and (b) and 200 nm in (c) and (d). The pixel sizes are 40 nm for (c) and 20 nm for (d). (Reproduced with permission from Ref. 40.)

absorption and fluorescence spectra (ATTO 590) to label the lamin, tubulin, and clathrin in human glioblastoma cells, respectively.⁵⁰

One of the first studies addressed by STED was the outcome of synaptic vesicles employed after synaptic transmission. The main limitation prior to STED was that the 50 nm vesicles were too small and closely packed to be

resolved by conventional fluorescence microscopy. Vesicles that were undergoing endocytosis were labeled by attaching a green emitting dye to antibodies capable of binding to the synaptotagmin protein present on the vesicle membranes.³⁰ Comparison between the membrane-associated and internalized fluorescent dots within cultured hippocampal neurons revealed that

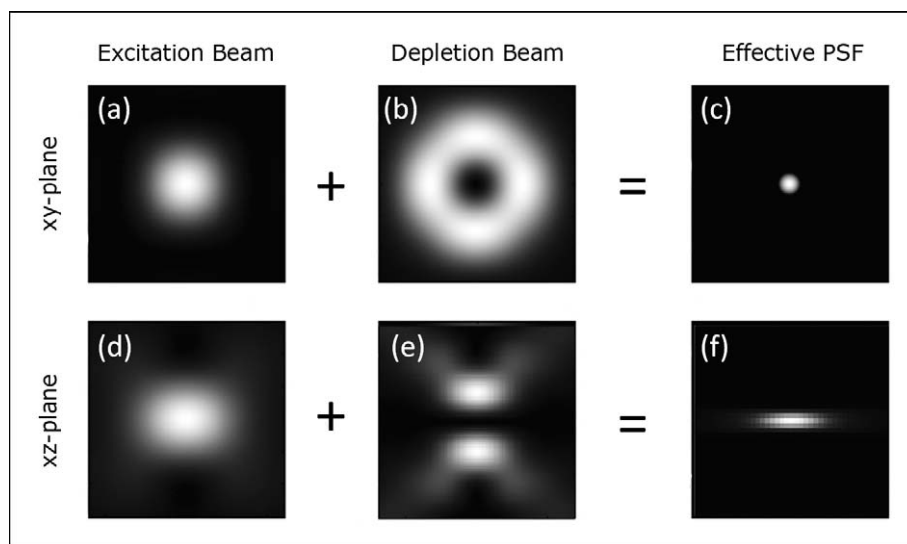


Fig. 10. The intensity profiles of the excitation beam, the doughnut-shaped depletion beam, and the resulting effective PSF in (a–c) the x - y plane and (d–f) the x - z plane (d–f).

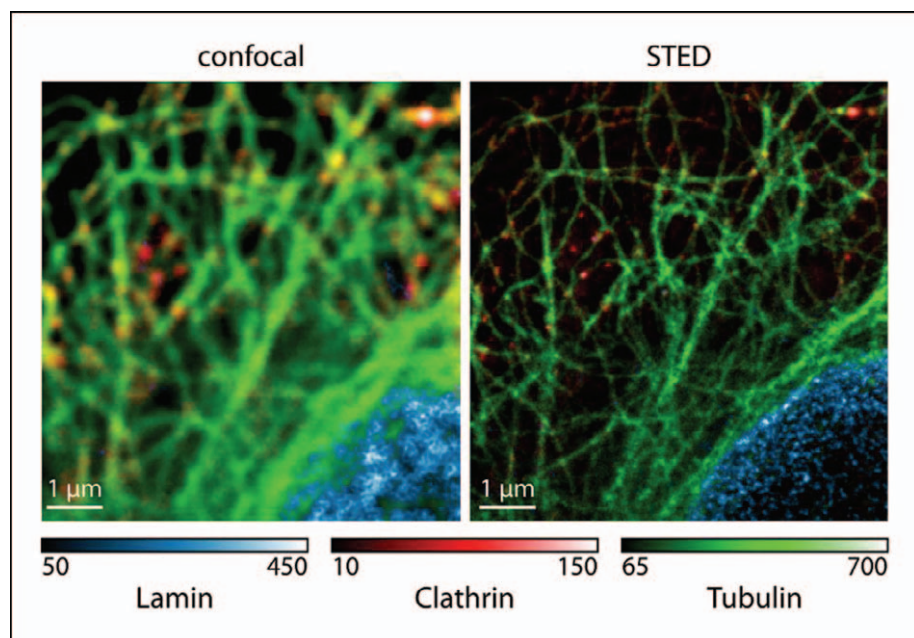


Fig. 11. Lamin (blue), Clathrin (red), and Tubulin (green) imaged with confocal and STED microscopy. (Reproduced with permission from Ref. 50.)

the synaptotagmin protein remains in clusters during recycling and is not diffuse throughout the membrane.

Acetylcholine receptor (AChR) supramolecular aggregates, involved in fast synaptic transmission, were examined with STED microscopy.⁵¹ The study found that the removal of cholesterol from the cell surface changes the organization and distribution of the AChR aggregates. Compared to controls, the decreased cholesterol resulted in fewer AChR aggregates of larger size. This cholesterol-dependent clustering of AChR aggregates was well below the diffraction limit and could not be detected by a conventional optical microscope.

Dendritic spines have been shown to have structural diversity related to potential disease such as depression.⁴² These spines can vary from 0.2 to 2 μm in size and hence are easily assessable by conventional fluorescence microscopy. However, changes in the size and shape of the spines are below the diffraction limit. STED was able to image the dendritic spines of YFP-transgenic mice as time lapse movies at 20 s/frame. With STED, changes in the neck, width, and curvature of the spines could be captured.

STED was also used to investigate the spatial distribution of Human Immunodeficiency Virus (HIV) between infected dendritic cells and uninfected CD4⁺ T cells.⁵² STED was able to resolve fluorescent virus clustering at the synapses with single-virion resolution.

In the last ten years, studies have emerged showing that the cytoskeleton that was previously believed to exist only in eukaryotic cells has bacterial homologs found in almost all prokaryotes.⁵³ The tubulin homolog cell division protein FtsZ helix in fixed cells was imaged with STED, and its structure was found to be patchy and discontinuous, which contrasts sharply with the diffraction-limited conventional fluorescence microscopy, which showed FtsZ to be a smooth structure. Furthermore, STED microscopy showed a much narrower periodic structure of FtsZ (140–200 nm) compared to conventional fluorescence microscopy (550 nm); this tight helix was a new sub-cellular structure imaged for the first time with STED microscopy.

STED has also been used to probe the lipids of cell membranes. Lipid “rafts” have been difficult to detect in living cells due to their small size (5–200 nm) and rapid diffusion.⁵⁴ Sphingomyelin, which is believed to form cholesterol-

mediated lipid nanodomains, and a non-integrating lipid phosphoethanolamine were fluorescently labeled and the diffusion of these single molecules was monitored by both confocal and STED microscopy. While the confocal results showed comparable results for both lipids, the STED measurements revealed phosphoethanolamine with one type of diffusion with fluorescent bursts < 1 ms while sphingomyelin showed heterogeneous bursts ranging from 1 to 50 ms. Depleting the cholesterol in the cell membrane by cholesterol oxidase revealed that the longer transit time of sphingomyelin was found to result from cholesterol-mediated molecular trapping.

Syntaxin 1 and syntaxin 4 clusters, found on the plasma membrane, are involved with docking/fusion of secretory vesicles and caveolae, respectively.⁵⁵ Although it was previously believed that cholesterol was involved in cluster formation, STED microscopy studies revealed that cholesterol alone did not result in correct clustering, but that the SNARE motif (a homologous sequence of 60 to 70 amino acids) involving cytoplasmic protein–protein interactions is required for cluster formation.[†] Using a construct without the SNARE motif of syntax 1A significantly reduced its co-clustering ability with unmodified syntax 1A.

Proteins such as flotillin-1 and flotillin-2, which are associated with the formation of the neurotoxic plaques found in Alzheimer’s disease, have been probed with STED.⁵⁶ It was found that depletion of flotillin-2 resulted in a significant reduction in the amyloid precursor protein (APP) cluster size, while flotillin-1 did not result in varied cluster size or distribution. The results from this study suggest that the presence of flotillin-2 can stimulate APP clustering, potentially promoting endocytosis, which can result in the release of the neurotoxic amyloid-beta peptide.

STED microscopy is also capable of imaging within a living cell. Using fluorescently labeled proteins, the endoplasmic reticulum (ER) of mammalian

[†] SNARE is an acronym for soluble N-ethylmaleimide-sensitive factor attachment protein receptors.

cells was imaged using STED.⁴⁸ Since the ER can be folded in a complex structure, STED was able to profile single stacked ER strands using 150 nm optical sectioning. Furthermore, using a lateral spatial resolution of 50 nm, time-resolved movies (10s frame rate) of ER movement were obtained, revealing ER closure formations and re-openings.

SINGLE-FLUOROPHORE SUPER-RESOLUTION MICROSCOPY

The basic principle of single-fluorophore-based super-resolution microscopy is that the position of a spatially isolated fluorophore can be determined with an accuracy higher than the width of the PSF. Single-molecule detection is the first step in locating the molecule beyond the diffraction limit. Single-molecule spectroscopy was first demonstrated in 1989 at liquid-helium temperatures.³² However, with a modern electron multiplying charge-coupled device (CCD), single-molecule imaging is now easily achievable at room temperature. Given an isolated single molecule, as shown in Fig. 12a, the image size of the molecule on a CCD is diffraction limited, as shown in Fig. 12b. If it is known that there is only one molecule, the position of the molecule can be determined by fitting the image in Fig. 12b with a Gaussian distribution to locate the center of the PSF. The uncertainty in the position of the molecule is determined by the uncertainty in fitting the center of the PSF. Theoretically, the precision scales inversely with the square of the number of detected photons, which is described by

$$\Delta_{\text{localization}} \sim \frac{\Delta_{\text{PSF}}}{\sqrt{N}}$$

where $\Delta_{\text{localization}}$ is the precision of the localization, Δ_{PSF} is the width of the PSF, and N is the number of photons detected from the molecule. Once $\Delta_{\text{localization}}$ is obtained, the image of the molecule can be reconstructed, as shown in Fig. 12c.

For single-fluorophore-based super-resolution microscopy, each molecule must be well separated, or a false image reconstruction will arise, as shown in

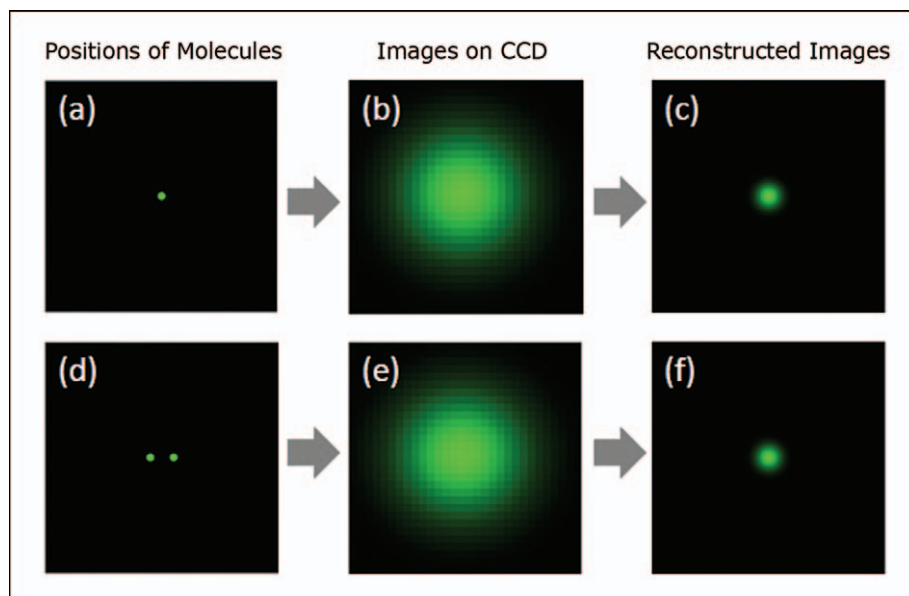


Fig. 12. (a) Location of a single molecule. (b) Image observed by a CCD for the molecule in (a). (c) Reconstructed images of the molecule in (a). (d) Two closely located molecules. (e) Molecules in (d) observed by a CCD. (f) A false image reconstruction that places a molecule near the center of these two molecules.

Figs. 12d through 12f. Figure 12d shows two molecules located within the diffraction limit. These two molecules are not distinguishable on the CCD as shown in Fig. 12e. In this case, only one molecule will be reconstructed, and it will be located near the center of these two molecules. Therefore, a sample with well-separated molecules, which are individually distinguishable on the CCD, is required to avoid a false image reconstruction.

The requirement for spatially distinguishable molecules generally cannot be fulfilled in biological samples because proteins within a cell have very high local density and are not spatially distinguishable on a CCD. This difficulty was overcome in a very clever way using photoactivatable fluorescent labels.^{57,58} Photoactivatable fluorescent labels allow a controlled activation of a subset of the fluorescent molecules. Because the number of activated molecules is small, the chance of having two or more of them located within a diffraction-limited volume is low. Therefore, it is possible to image a subset of well-separated molecules one at a time with high resolution; then the complete image can be obtained by adding all images of the subsets togeth-

er. This approach was independently reported by three research groups in 2006.^{35–37} The methodology and their applications are described as follows.

PHOTOACTIVATED LOCALIZATION MICROSCOPY

Photoactivated localization microscopy (PALM) was first carried out using a photoconvertible FP named EosFP.³⁵ The emission wavelength of photoconvertible FPs can be optically converted from one wavelength to another. Under normal conditions, EosFP emits green fluorescence at 516 nm. Upon irradiation near 400 nm, its fluorescence changes to 581 nm because of a photo-induced modification involving a break in the peptide backbone next to the chromophore.⁵⁹ This optical property makes it possible to convert a subset of EosFPs to emit in the yellow region (581 nm). When the number of converted proteins is small, the proteins will be well separated and can be imaged in the yellow region with high resolution. When this particular subset of EosFPs is photobleached, another subset of EosFPs can be converted and imaged. This process may be cycled 10^4 to 10^5

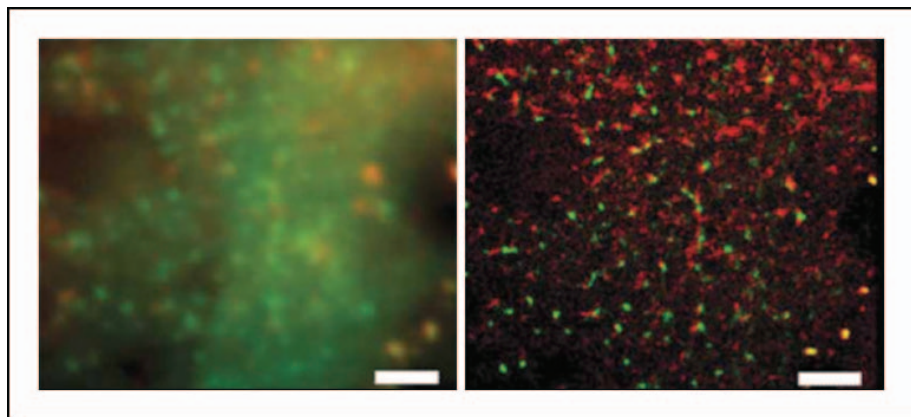


Fig. 13. Distribution of Tfr and CLC using two-color PALM (right) compared to conventional total internal reflection (TIRF) microscopy (left). Tfr clusters are red, CLC clusters are green. Scale bar is 2 μ m. (Reproduced with permission from Ref. 63.)

times until the population of EosFPs is depleted. FPALM utilizes a similar approach and was first demonstrated using photoactivatable GFP.³⁷

Since 2002, many other photoconvertible, photoactivatable, and photoswitchable FPs have been developed. Photoactivatable FPs can be activated from a dark state to a bright state using UV light, and photoswitchable FPs can alternatively be switched on or off with specific illumination. A summary of these proteins can be found in Ref. 60, although not all of them have been proven to be effective for super-resolution microscopy.

Three-dimensional FPALM was achieved in 2008 using biplane (BP) detection.⁶¹ A beam-splitter splits the fluorescence light into a shorter and longer path to form two detection planes for z -position determination. A resolution of ~ 30 nm laterally and ~ 75 nm axially was achieved by BP FPALM.⁶¹ In 2009, interferometric PALM (iPALM) was also introduced and provided sub-20-nm 3D protein localization.⁶²

Two-color PALM was implemented in 2009. COS-7 cells were tagged with transferring receptor (Tfr)-PAmCherry1 and PA-green fluorescent protein (GFP)-clathrin light chain (CLC) and then alternately imaged at 561 nm and 468 nm to excite the red (PAmCherry1) and green (PAGFP) dye, as shown in Fig. 13.⁶³ The results showed < 200 nm clusters of transferring receptors and

clathrin light chains at a lateral resolution of < 20 nm.

PALM was used to study the *E. coli* chemotaxis receptor cluster aggregation at localized poles of the bacteria.⁶⁴ Three proteins (the Tar receptor, CheY, and CheW) involved in chemotaxis were imaged with a resolution of 15 nm. No characteristic cluster sizes were found. The results showed that the receptors were not location specific, but rather diffusion (or stochastic self-assembly) of the receptors can explain the images obtained with PALM.

Dendritic spines protrude from neuronal dendrites and hold postsynaptic molecules.⁶⁵ Changes in the shape of the spine arise from the actin cytoskeleton within the dendritic spines. PALM was used to track a single molecule of F-actin in cultured hippocampal neurons with a resolution of 30 nm. This study found the dynamics of F-actin to be highly heterogeneous. The results propose short actin filaments in the dendritic spine.

The FtsZ protein is involved in prokaryotic cell division and is shaped like a ring, called the Z-ring.⁶⁶ It has been difficult to image the Z-ring with electron microscopy due to the dense cytoplasm. PALM microscopy was able to image the Z-ring in *E. coli* with a spatial resolution of 35 nm and showed that, in addition to a ring structure, the ring exists as a helical conformation. The thickness of the ring is approximately 110 nm and is likely formed by a

loose bundle of randomly overlapped FtsZ protofilaments.

STOCHASTIC OPTICAL RECONSTRUCTION MICROSCOPY

Stochastic optical reconstruction microscopy (STORM) was first introduced using Cy3–Cy5 dye pairs as the optical switch to activate a subset of fluorophores.³⁶ The Cy5 works as the primary fluorophore, which can be reversibly switched between a fluorescent and a dark state in a controlled fashion by 532 nm and 633 nm light, respectively, and the Cy3 is used to facilitate the switching of Cy5.⁵⁸ The optical switch can be cycled on and off hundreds or thousands of times before becoming permanently photobleached. A resolution of 20 nm was demonstrated for RecA-coated circular plasmid DNA.³⁶

Figure 14 shows the comparison between imaging microtubules in a BS-C-1 cell using conventional confocal fluorescence versus STORM. Multicolor STORM was demonstrated for microtubules. Furthermore, the tubules were imaged with clathrin-coated pits, which are involved in receptor-mediated endocytosis.⁶⁷ Cy2-Alexa647 and Cy3-Alexa647 were used to label the microtubules and clathrin, respectively. Lasers with wavelengths of 457 and 532 nm were used to selectively excite the switch pairs. STORM was able to image the microtubules separately from the circular clathrin pits with ~ 30 nm spatial resolution.

Three-dimensional STORM was achieved by introducing astigmatism to the image with a cylindrical lens. Thus, the position of the fluorophore was monitored by examining the ellipticity of the circular fluorophore. Images above and below the focal plane were ellipsoidal and by fitting the image with a 2D elliptical Gaussian function, the peak widths of the x, y coordinates were obtained, which allowed for the calculation of the z coordinate.⁶⁸ Recently, 3D spatial resolution of ~ 30 nm in the lateral direction and ~ 50 nm in the axial direction with time resolution as fast as 1 to 2 s/image have been reported.⁶⁹ 3D STORM has been used to image microtubule networks in green monkey kidney epithelial cells labeled with a Cy3-

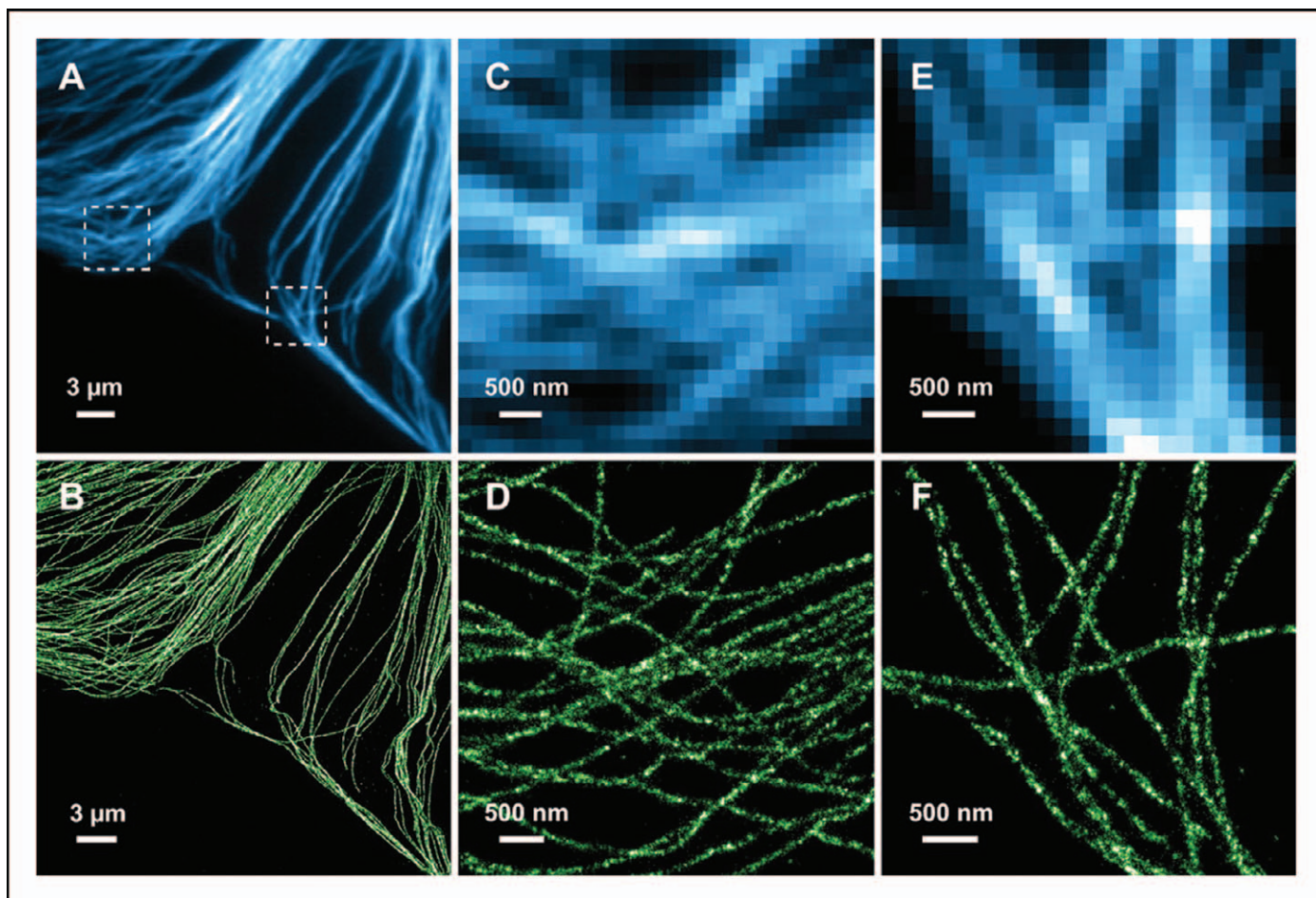


FIG. 14. (B, D, F) STORM and (A, C, E) conventional fluorescence imaging of microtubules in a mammalian cell. (Reproduced with permission from Ref. 67.)

Alexa647 switch. 3D STORM was able to capture multiple layers of microtubule filaments in cell cross-sections and two microtubules 100 nm apart in the z direction were easily separated.

Two-color imaging combined with 3D STORM of tubular mitochondria and microtubules in BS-C-1 cells labeled with A405-Cy5 and A555-Cy5 (or A488-Cy5), respectively, has been reported.⁷⁰ Laser pulses at 405 nm were used to activate A405-Cy5 while laser pulses at 542 nm (or 460 nm) were used to activate the A555/A488-Cy5 dyes. The probes were imaged during the activated state by a 657 nm laser to determine the 3D position of the fluorophores. Compared to conventional fluorescence microscopy, STORM was able to minimize false contacts between the mitochondria and microtubules, showing a 150 nm separation between

microtubule and mitochondria that appeared to be touching in the conventional fluorescence microscopy images.

Recently, Heilemann et al. introduced direct STORM (dSTORM), which uses conventional photoswitchable fluorescent dyes that can be reversibly cycled between a fluorescent and a dark state by irradiation with light of different wavelengths.⁷¹ This approach does not require special fluorophore pairs used in STORM. dSTORM was able to visualize cellular structures with a resolution of approximately 20 nm without the need of an activator molecule.

TECHNICAL CONSIDERATIONS

Photobleaching. Photobleaching is the light-induced destruction of fluorophores. The phenomenon is often an

important limiting factor in imaging biological samples, especially for time-lapse studies. When a fluorophore absorbs a photon, an electron is excited from the ground state to an excited state. When fluorophores are in the excited state, they are more likely to react with other molecules. The mechanism of photobleaching is not fully understood for most molecules, but it is generally assumed to be related to excitations to triplet states as triplet states have longer lifetimes and are more reactive.⁷² There are anti-photobleaching agents that reduce the amount of oxygen in the sample to prevent reactions with oxygen, but many of them are toxic for living cells. Some fluorophores are more photostable than the others. A typical organic dye can emit 10^5 to 10^6 photons before it is photobleached, and a fluorescent protein typically can emit 10^4 to

10^5 photons. Because the directions of the emissions are random, only a fraction of them can enter the detection system. The number of detected photons is a limiting factor for super-resolution microscopy.

In general, photobleaching is more a problem for SSIM and STED than for the other super-resolution imaging approaches as these two techniques require saturated fluorescence excitation and depletion, respectively. For SSIM, photobleaching will cause dimmer subsequent images, causing problems for the reconstruction. For STED microscopy, because the resolution is improved by decreasing the effective PSF, the detected photon counts decrease dramatically as the size of the PSF decreases. Although the photon count per pixel can be increased by increasing the dwell time per pixel, a longer dwell time will increase the photobleaching. For biological studies, the number of fluorophores is predetermined by the nature of the specimens. In these cases, the repetition rate of the excitation can be reduced to reduce photobleaching. It has been reported that a 5- to 25-fold increase in total fluorescence yield for GFP and Atto532 can be obtained when the repetition rate of pulsed illumination is lowered to < 1 MHz.⁷³ This fluorescence signal gain was observed both for 1P and 2P excitation. However, it will significantly increase the image acquisition time. Generally, 50 photons/pixel will produce a good quality image for STED. Because of the low photon count, images obtained by STED are often smoothed or restored by various filters to remove noise.^{30,47,49,74}

For single-fluorophore-based super-resolution microscopy, such as (F)PALM and STORM, the spatial resolution is limited by the number of photons, as indicated in Eq. 2. Unlike STED, which depletes the fluorescence to improve resolution, single-fluorophore-based super-resolution microscopy collects the maximum number of photons emitted from each molecule, and the resolution is determined by the number of photons detected. The number of photons a molecule can emit before it is photobleached (or switched off) varies. For example, EosFP, one of the brighter PA-FPS for PALM, can emit ~ 750 photons

per molecule,⁷⁵ and the switchable fluorophore pair Cy3–Cy5 used for STORM can provide ~ 6000 photons per molecule per switching cycle and lasts ~ 200 switching cycles.^{58,67} A review of fluorescent probes for super-resolution imaging can be found in Ref. 75. Overall, photoswitchable dyes have larger numbers of collected photons per molecule compared with fluorescence proteins.

Quantum dots (QDs) offer excellent photostability for long-term imaging. Several applications of QDs for super-resolution microscopy have been demonstrated.^{76–79} Compared to traditional fluorophores, QDs have a broader absorption spectrum and a narrower emission spectrum, which is beneficial for simultaneous multicolor imaging with the same excitation light.^{80,81} QDs have been estimated to be up to 20 times brighter and 100 times more stable than organic dyes such as rhodamine.⁸² However, for specific labeling, QDs must be conjugated to biomolecules that provide binding specificity.^{83,84} While commercial toolboxes and established labeling protocols are widely available for conjugating dyes to biomolecules, conjugating QDs to biomolecules still remains challenging.⁸⁵

Spatial Resolution. For STED microscopy, the achievable resolution is strongly dependent on the photostability of a sample. A resolution of 5.8 nm has been achieved on color centers of nanocrystals.⁸⁶ For biological samples, a resolution of 50–100 nm can be obtained without causing significant damage. Beside photobleaching, a common issue that reduces the resolution of STED microscopy is a non-ideal depletion beam profile. From a practical standpoint, it is difficult to generate an ideal doughnut-shaped depletion beam with zero intensity at the center. When the wavefront (spatial phase) of the incident beam is distorted or the spatial phase modulation is imperfect, the resulting doughnut will be asymmetric, and there is a finite intensity at the center. The asymmetric depletion profile produces an asymmetric effective PSF, but it is not a critical issue when the size of the effective PSF is smaller or comparable to the scale of interest. One major problem with having a finite intensity at the center of the doughnut

beam is that it also depletes the fluorescence at the center and reduces the number of photons detected. Another problem comes from the aforementioned excitation by the depletion beam. In an ideal case, the fluorescence excited by the doughnut-shaped depletion beam can be mostly excluded from entering the detection system by a spatial filter, such as a pinhole, in front of the detector. However, when the profile of the depletion beam is imperfect, the fluorescence excited by the depletion beam will increase. Typically, the wavefront distortion can be minimized by putting the laser beam through a single-mode fiber or a pinhole, and the depletion beam profile can be checked by measuring the scattering light from a gold nanoparticle to ensure a reasonably good doughnut profile.

For single-fluorophore-based microscopy, such as (F)PALM and STORM, the resolution limit is given by the number of detected photons, as indicated in Eq. 2. Theoretically, a 10 nm resolution is possible with ~ 1000 photons, but practically a resolution of ~ 20 nm in the lateral direction is common, even with 6000 photons.^{36,67} Many other factors can potentially increase the uncertainty in determining the position of a molecule based on the image recorded on a CCD. The mechanical drift of the imaging system is typically in the range of tens of nanometers. For slow imaging, luminescent beads added sparsely to the sample have been used to calibrate the drift during the post-acquisition analysis.³⁵ The location of a fluorophore is usually determined by Gaussian fitting. However, the PSF of a fluorophore may not be a perfect Gaussian function.^{87,88} It has been reported that the fluorescence intensity distribution of a molecule also depends on its three-dimensional orientation.⁸⁹ Additionally, the camera pixels may differ in quantum efficiency and gain.⁹⁰

CONCLUSIONS AND OUTLOOK

Super-resolution fluorescence microscopy developed in the past five years has shown a number of important applications and shows great promise for a better understanding of biology. Although several methodologies have been

developed for sub-diffraction-limit imaging, these techniques have their strengths and weaknesses. The advantage of STED microscopy is that it can be carried out using traditional dyes and fluorescent proteins. The disadvantage is that STED is technically more complicated and the resolution is typically limited to 50 to 100 nm for biological samples. On the other hand, (F)PALM and STORM are technically easier to implement and a higher resolution (~20 nm) is easier to achieve. However, (F)PALM and STORM require special photoactivatable (or photoswitchable) fluorophores. There is still no ideal system that offers user-friendly, high-speed, multicolor, 3D, and multicolor imaging with a nanometer spatial resolution. However, super-resolution microscopy is still in its infancy, and new developments in the next 5 to 10 years can be expected.

Super-resolution fluorescence microscopy for biological research has been a collaborative work among physicists, chemists, and biologists. Further development will continue to rely on new optical methods, new fluorophores, and a better understanding of biology at the nanometer scale. The research will benefit from a more sensitive CCD, faster data processing, and cheaper lasers. Certainly, the development of photobleach-resistant fluorophores will lead to greater advancement in super-resolution imaging. Quantum dots can potentially become excellent labels for super-resolution microscopy as they have great photostability and brightness. In the future, new developments will enable more user-friendly multicolored 3D nanoscale imaging to unlock more biological secrets that still remain hidden.

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