Semrock White Paper Series:

Super-resolution Microscopy

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Super-resolution Microscopy

1. Introduction

Fluorescence microscopy has revolutionized the study of biological samples. Ever since the invention of fluorescence microscopy towards the beginning of the 20th century, significant technological advances have enabled elucidation of biological phenomenon at cellular, sub-cellular and even at molecular levels. However, the latest incarnation of the modern fluorescence microscope has led to a paradigm shift. This wave is about breaking the diffraction limit first proposed in 1873 by Ernst Abbe. The implications of this development are profound. This new technology, called super-resolution microscopy, allows for the visualization of cellular samples with a resolution similar to that of an electron microscope, yet it retains the advantages of an optical fluorescence microscope. This means it is possible to uniquely visualize desired molecular species in a cellular environment, even in three dimensions and now in live cells – all at a scale comparable to the spatial dimensions of the molecules under investigation. This article provides an overview of some of the key recent developments in super-resolution microscopy.

2. The problem

So what is wrong with a conventional fluorescence microscope? To understand the answer to this question, consider a Green Fluorescence Protein (GFP) molecule that is about 3 nm in diameter and about 4 nm long. When a single GFP molecule is imaged using a 100X objective the size of the image should be 0.3 to 0.4 microns (100 times the size of the object). Yet the smallest spot that can be seen at the camera is about 25 microns. Which corresponds to an object size of about 250 nm. Thus there is a big disconnect between reality (size of the actual fluorophore) and its perception (image). The image suggests a much larger object size than is actually present.

Why is there a disconnect? The answer lies in the fundamental limitations imposed by the optics used to image such molecules. Conventional fluorescence microscopy utilizes a lens to focus a beam of light onto a spot. For example a lens is used to focus the emission signal onto a CCD detector. Or consider an illumination beam that is focused to a small spot on the sample, such as in a confocal scanning microscope. Performance of all such instruments is dictated by far-field optics (distances >> λ , the wavelength of light). In the far-field regime diffraction plays a dominant role in image formation and in fact limits the smallest spot size that can be obtained at the focal point of a lens (Fig. 1). Because of diffraction, a parallel beam of light is focused by a lens into a three-dimensional *region* near the focal point. This intensity distribution near the focal point is referred to as the point-spread-function (PSF) of a microscope (see Fig. 1) and

forms the basis of resolution of a microscope. The full width at half maxima (FWHM) of the PSF in the lateral (x-y) plane and along the optical (z) axis are given by

$$\Delta x = \Delta y \approx \frac{\lambda}{2n\sin\alpha} \qquad \text{and} \qquad \Delta z \approx \frac{\lambda}{2n\sin^2\alpha}$$

respectively, where λ is the wavelength of light, n is the refractive index of the medium in which light propagates, and α is one half of the angular aperture of the objective lens.

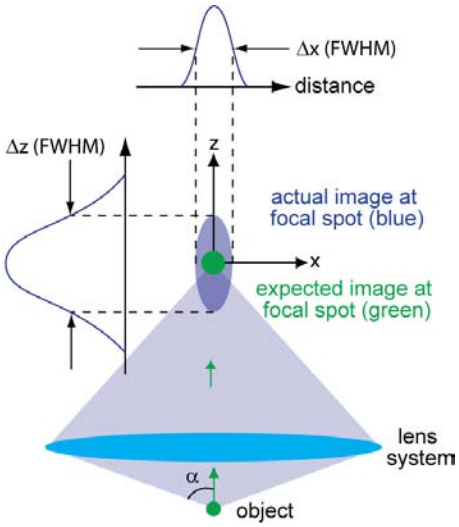

Figure 1: Principle of image formation in a conventional microscope. This schematic depicts the intensity distribution from a very small object (i.e. smaller than the wavelength of light). Note that due to diffraction the actual image (spread over a 3D region, violet) is much larger as well as elongated compared to the expected magnified image (green). The 3D intensity distribution of the actual image is called the Point Spread Function (PSF) of a microscope. A camera placed in the focal plane of the lens system (which includes the objective and the tube lens) captures a cross section of this 3D intensity distribution. The cross section profiles of this intensity distribution along x and z axes are also shown. Also note that due to circular symmetry of the PSF $\Delta x = \Delta y$. α is one half of the angular aperture of the objective lens. Different colors of the expected and actual images are used for the purpose of illustration. Schematic is not to scale.

Due to the non-zero value of the FWHM of the PSF, when two objects are located very close to each other their images cannot be distinguished. Resolution of a microscope is the smallest distance between two objects that can be discerned by the microscope. Ernst Abbe proposed that the lateral resolution (x-y plane) of an optical microscope is given by,

$$d > \frac{0.61\lambda}{n\sin\alpha}$$

which is based upon the observation of a PSF (see Fig. 2). An implication of limited resolution is that fine structures cannot be discerned.

For example, consider microtubules which serve as structural scaffolds within cells and are about 25 nm in diameter. A 250 nm diameter (diffraction-limited) image of a microtubule revealed by conventional microscopy may actually represent a bundle of several microtubules that cannot be distinguished from one another. In this case enhanced resolution would provide additional information about the cellular architecture. Super-resolution also enables the study of

membrane heterogeneity and dynamics of protein assembly – studies that benefit from observing single molecules.

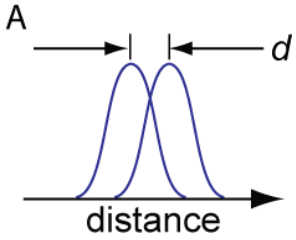

Figure 2: Resolution of an imaging system is based on the observation of its PSF. The smallest distance that can be discerned is given by d (see text). Since the PSF is narrower along the lateral direction than along the axial direction, the lateral resolution is higher than axial resolution. See Fig. 1 for details.

3. Enhancing the resolution

Super-resolution microscopy is about enhancing the diffraction-limited resolution of a microscope.

Near Field Scanning Optical Microscopy (NSOM, Table 1) is a near-field optics based super-resolution microscopy technique. In this technique the surface of a sample is illuminated using a very fine tip (diameter < wavelength of light) and this tip is placed very close (<< wavelength of light, hence the name “near-field”) to the surface such that it collects only the evanescent waves from the sample and therefore avoids the diffraction related issues of far-field optical microscopy. Using this technique a spot size of only a few nanometers can be resolved. However, this technique is limited to the study of surfaces and additionally it is very slow owing to low signal throughput.

Initial attempts at enhancing the resolution of a microscope, utilizing far-field optics, involved designing objective lenses of higher numerical aperture, *NA,* where $NA = n\sin\alpha$. The FWHM of the PSF is reduced (Fig. 1) along both the lateral and the axial directions, thereby enhancing both lateral and z resolutions, respectively. Examples of super-resolution microscopy techniques that utilize this approach include “4Pi” and “I^5M” microscopy (Table 1). Both of these techniques enhance the axial resolution by placing the sample at the focal plane of two opposing lenses. Therefore, the effective numerical aperture of the system is increased (since α , is increased), thereby improving the resolution (Fig. 3B). However these super-resolution microscopy techniques were able to enhance the resolution only along the axial direction compared to the conventional techniques. The lateral resolution remains unchanged.

Changing the PSF is not the only way to enhance the resolution. Fluorescence microscopy has another dimension, called color. Imagine two PSFs of different colors that are located very close to each other (Fig. 3C). Since Abbe’s resolution criterion applies to a given color the resolution of a fluorescence microscope should not be limited when two closely spaced point

sources emit light in different colors. Therefore in theory if all the molecules of a sample could be labeled with a different color then a resolution better than diffraction-limited imaging could be achieved [1]. However, there is a practical limitation of the number of colors that can be used in a given experiment and, more importantly, this method does not allow distinction among the same molecular species, all of which are typically labeled with the same colored fluorophore.

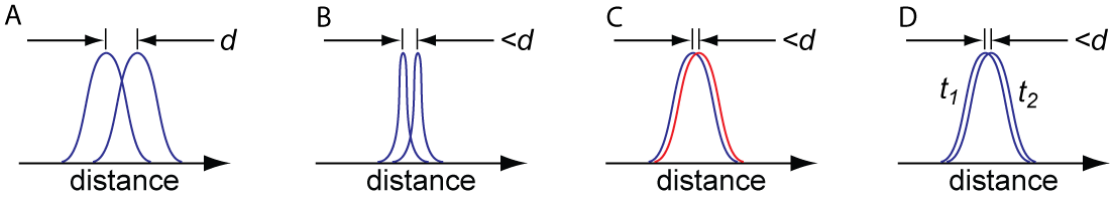

Figure 3: Strategies for enhancing the resolution of an imaging system. (A) Resolution of a conventional microscope. (B) Enhancing the resolution by changing the PSF of the microscope. (C) Abbe's resolution criterion is not limited by color. (D) Time distinction (see text) is a basis of all the current super-resolution techniques. t_1 & t_2 are different instances of time.

Abbe's resolution criterion also does not impose a limit on the resolution when two closely spaced point sources are imaged at different *times.* That is, imagine two PSFs that cannot be otherwise distinguished but each is observed at a different time (Fig. 3D). This methodology of "sequential" imaging at different times forms the basis of the most recent and successful super-resolution microscopy techniques. A table summarizing established and emerging super-resolution microscopy techniques is included at the end of this article for reference (Table 1).

4. Super-resolution microscopy techniques

Depending upon how *time distinction* is achieved, super-resolution microscopy techniques can be broadly categorized into two main approaches [1]. In the first approach, called "targeted switching and readout", the illumination volume in a fluorescent sample is confined to a small region, which is much smaller than the diffraction-limited spot size. Stimulated emission depletion (STED) microscopy is based on this approach (see Fig.5 and Table 1). Knowledge about the illumination spot size is used to generate a super-resolution image. The second approach termed "stochastic switching and readout" utilizes stochastic variation associated with switching fluorophore molecules on or off, under carefully designed experimental conditions, such that a sequence of images acquired at different instances can be used to generate a super-resolution image. In both of these approaches, fluorophores are turned on and off at different time instances, and the images acquired at different instances are combined together to generate a composite image. However, in targeted switching the location of fluorophore molecules that are turned on and off is not stochastic (see Fig. 5).

The mechanism by which *time distinction* is achieved can be explained by the electronic transition states of a fluorophore (Fig. 4). In conventional fluorescence microscopy, a fluorophore absorbs energy from the excitation light and almost instantaneously releases the

emission signal. In order to achieve *time distinction* (which ultimately provides spatial discrimination), nonlinear relationships between excitation and emission of a fluorophore are exploited. By virtue of these nonlinear relationships, specific fluorophore molecules can be switched on or off.

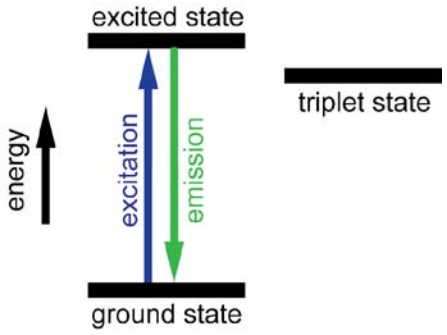

Figure 4: Electronic transition states of a fluorophore. Upon absorption of energy, a fluorophore molecule is excited and almost instantaneously (typically within nanoseconds) releases energy in the form of photons when it relaxes to the ground state. Due to inter-system crossing or (external) photo-physics, an electron in the excited state can also move to the triplet state before returning to the ground state. This nonlinear process can delay (by microseconds to milliseconds) or even quench the fluorescence emission. Fluorophores can also behave nonlinearly by virtue of (external) photo-chemistry; for example, a fluorophore molecule can be rendered excitable (phtoactivation) or non-excitable by altering the molecule into cis and trans isomerization states.

The first super-resolution microscopy technique that utilized this nonlinear relationship is STED microscopy. In conventional point scanning confocal systems, all the fluorophore molecules within a diffraction-limited excitation volume are excited and emit the fluorescence signal simultaneously (Fig. 5). By moving the scanning beam over the sample, a composite image is constructed. The basic implementation of STED microscopy is similar in terms of point-by-point scanning in order to generate a single image. However, in STED microscopy the emission signal is generated from a much smaller volume than that of a conventional confocal scanner. In STED the laser scanning illumination (excitation pulse) spot is overlapped by another beam called the STED beam which has an annular ("doughnut") shape and is of longer wavelength than the scanning beam (and also matches the emission wavelength of the fluorophore). An intense pulse from the STED beam depletes the emission of fluorophores in the annulus region by stimulated emission – in other words, it confines the molecules to the ground state. Immediately after the STED pulse only fluorophores in the central region (the doughnut "hole") are still in the excited state and are thus able to emit fluorescence. Therefore the effective emission spot size is reduced to well below the diffraction limit (10s of nanometers compared to a diffraction limited size of 100s of nm). This means that the PSF for a STED microscope has a much narrower FWHM along the lateral axis (based upon the a priori

knowledge of the illumination spot size and its location), which improves the lateral resolution of the microscope. The axial resolution remains unchanged however.

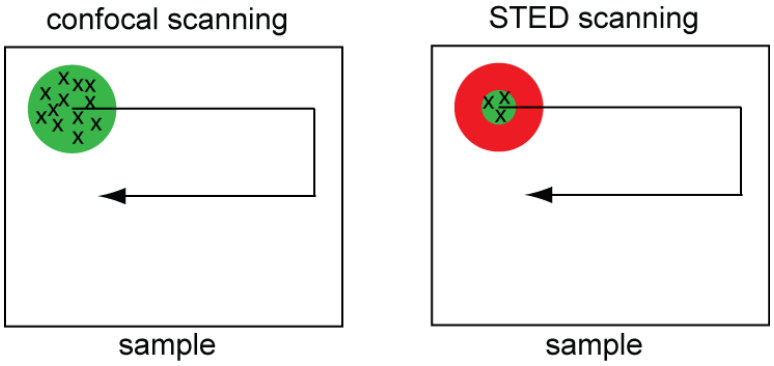

Figure 5: An example of *targeted switching and readout*. In conventional confocal scanning, all the fluorophore molecules (marked 'x') within the diffraction-limited illumination spot simultaneously emit the signal. By exploiting a nonlinear relationship between excitation and emission of a fluorophore, such as in STED microscopy (right panel), a doughnut shaped STED beam (red) confines the molecules to the ground state (which do not fluoresce) and thereby creates a sub-diffraction-limited spot size from which fluorophores emit (green).

Saturated Structured Illumination Microscopy (SSIM) is another example of targeted switching based super-resolution microscopy. However, as opposed to STED this is not a point-by-point scanning method. Instead a periodic illumination pattern is generated on the sample plane by imaging a phase mask (for example a grating with a finely spaced linear pattern) placed in the excitation light path. The phase mask is rotated to scan the entire sample. A sequence of images is acquired in widefield detection mode, each image of the sequence corresponding to a given position of the phase mask. The super-resolution effect in SSIM is achieved by illuminating the fluorophores with patterned excitation light of saturating intensity. This pattern deliberately establishes very narrow regions which contain fluorophores in the off state. Super-resolution information encoded in this "negative data" (off state region of fluorophores) is extracted mathematically. Sophisticated mathematical analysis of the acquired date is used to generate a super-resolution image.

Consider another radically different approach for achieving time distinction. Rather than controlling the illumination spot, what if it were possible to switch single molecules of fluorophores *on* (i.e., to an excitable state that can be imaged) and *off* (a state that cannot be imaged)? At a given time, only a small population of fluorophores is turned on for imaging. Then this subset is turned off after imaging. The premise of this approach is that if the density of the fluorophores in the on state is very low and provided enough photons can be collected from each molecule, then the image of each individual molecule can be resolved (Fig. 6, right). If the molecules that are switched on in any given image are far enough apart so that the diffraction-limited spots associated with each molecule are fully resolvable, then each spot can be

artificially replaced with a much smaller spot (well below the diffraction limited spot). By repeating the process of switching on a small population of fluorophore molecules, imaging and then turning them off, a sequence of images is generated. Finally all these images with artificially smaller spot sizes are super-imposed to arrive at a super-resolution image. In contrast, in widefield imaging by default all the fluorophores are in the on state while imaging and therefore the images of individual molecules cannot be independently resolved (Fig. 6, left). Photoactivation Localization Microscopy (PALM, also synonymously referred to as F-PALM for Fluorescence PALM) and Stochastic Optical Reconstruction Microscopy (STORM) were the first techniques to exploit this principle of stochastic switching of fluorophores to generate super-resolution images.

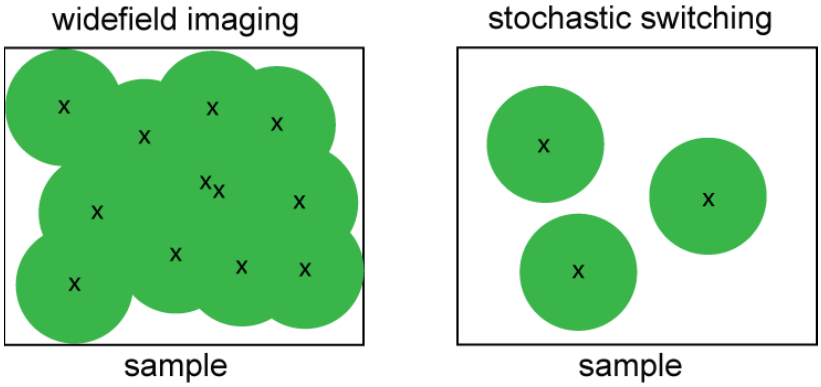

Figure 6: A comparison of widefield imaging with the stochastic switching approach of super-resolution microscopy. The emission signal from a fluorophore (marked 'x') generates a diffraction-limited spot (green circle). In widefield imaging, usually the diffraction-limited imaging spots cannot be distinguished from each other, thereby producing a blurred image (superposition of all the green circles). In "stochastic switching and readout" super-resolution imaging however, at a given time only a few fluorophores fluoresce. The emission signal from each fluorophore molecule still generates a diffraction-limited spot; however, because the population of the fluorophores is so sparse the spots can be independently resolved. Such resolvable individual molecules are imaged, localized (using computational tools) and then switched off (see Table 2). This cycle is repeated to generate a stack of images and the locations of individual molecules (marked 'x') from these images are used to arrive at a rendered super-resolution image.

Specially designed fluorophores are used in PALM and STORM. These fluorophores can be switched on and off with specific wavelengths of light. For example, PA-GFP does not glow with blue illumination light (called the readout beam) unless it is turned on (or activated), by virtue of photo-chemistry using a UV activation beam (Fig. 4) by a UV beam. Once activated, PA-GFP is imaged until it photobleaches (switching off). This is one example of how fluorophores can be turned on and off. Another approach is to use a photoswitchable fluorescent probe, such as Dronpa (Fig. 7, Table 2) which can be activated (with a UV activation beam), imaged (with a green readout beam) and then turned off (also with green illumination). This principle of photoswitching was employed in the development of the STORM technique.

GSDIM (Ground State Depletion followed by Individual Molecule return) is another example of stochastic switching and readout. This technique does not require specialized fluorophores. In this technique, ordinary fluorophores are initially turned off, for example by driving them into the long-lived triplet states (Fig. 4), and then as individual molecules return to the ground state stochastically, a readout scheme similar to PALM and STORM enables super-resolution microscopy.

Due to the numerous possibilities of imaging configurations, the requirements for optical filters in super-resolution imaging are often best met by custom-selecting filters for a given system. A combination of filters that can be used for photoactivation and imaging of Dronpa is shown in Fig. 7. A special characteristic of the dichroic beamsplitter shown in this figure is its wide reflection band that is compatible with both the activation (~405 nm) as well as readout (~488 nm) lasers. High transmission of the emission filter ensures maximum signal collection from a limited population of fluorophores imaged at a given time and thereby enhances overall throughput. An essential step in single-molecule based super-resolution imaging techniques (stochastic switching and readout) is to accurately “localize” individual fluorophore molecules. With higher accuracy of localization higher super-resolution is achieved. Since the accuracy of localization of a given fluorophore increases dramatically with the number of photons acquired from a given fluorophore molecule, highly efficient optical filters play an increasingly important role in super-resolution microscopy.

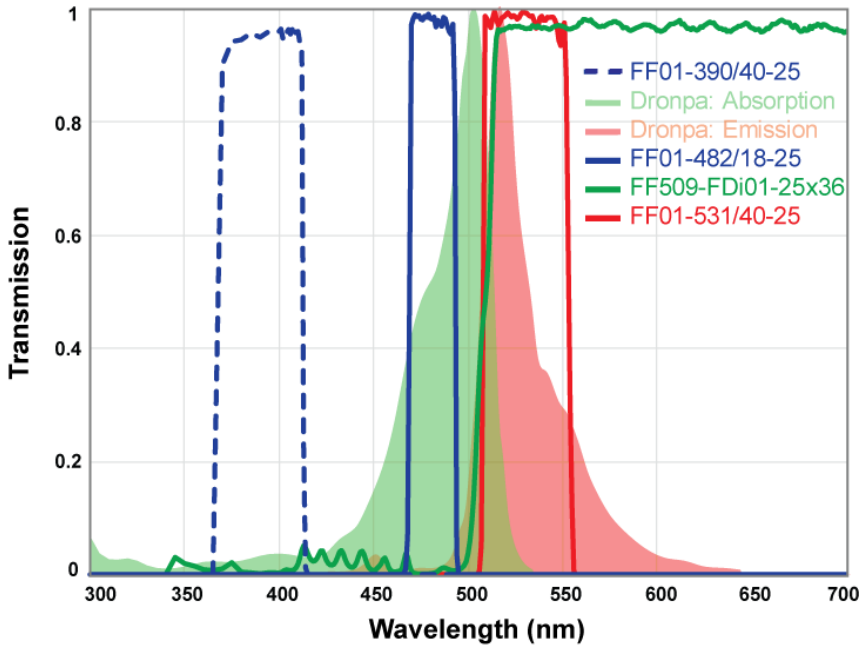

Figure 7: Optical filters for imaging of Dronpa. The wide reflection band of the dichroic ensures that both the activation light (e.g. 405 laser) and imaging beam, 488 nm laser are efficiently reflected and rejected in transmission. A compatible emission filter provides high blocking of both the activation and imaging lasers.

5. Perspective

Excitement about super-resolution imaging techniques is evident from the surprisingly rapid commercialization of “turn-key” instruments. For example, Leica was first to launch a commercially available super-resolution microscope utilizing STED and at the time of writing this article is currently developing a GSDIM system. Zeiss and Nikon are also in the process of launching super-resolution microscopes that utilize PALM and STORM, respectively, as well as variants of SIM (Structured Illumination Microscopy). The availability of commercially available super-resolution instruments will enable widespread research at unprecedented resolution. Research still continues to further enhance the performance of super-resolution techniques in terms of overall throughput so that they can be used even for the study of fast cellular dynamics in live cells. And efforts are underway to enhance the super-resolution not only laterally but also axially. Rather than having to develop and use specialized fluorophores, many researchers have demonstrated the advantages of using standard fluorophores (see table 2), which expands the applicability of super-resolution techniques.

6. References

[1] Special Feature: Method of the year, *Nature Methods*, 6 (1), January 2009.

[2] M. Fernandez-Suarez, and A. Y. Ting, Fluorescent probes for super-resolution imaging in live cells, *Nature reviews, Molecular Cell Biology*, 9: 929-943, December 2009.

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Table 1: Summary of established and emerging super-resolution microscopy techniques. The methodology indicates whether each technique is based upon imaging of single molecules or imaging of an ensemble of molecules (which in the limiting case can also image a single molecule).

Acronym (Name)	Methodology*	Switching mode / detection mode	Comments / limitations
4Pi	Ensemble	NA / Scanning-detection	Enhances the axial resolution, lateral resolution remains unchanged.
I^5M	Ensemble	NA / Widefield detection	Enhances the axial resolution, lateral resolution remains unchanged.
NSOM (Near-field Scanning Optical Microscopy)	Ensemble	NA / Scanning-detection	Lateral and axial super-resolution can be achieved however limited to scanning of surfaces.
STED (Stimulated Emission Depletion)	Ensemble	Targeted switching / Scanning-detection	Enhances the lateral resolution only. The axial resolution can also be improved by combining with 4Pi microscopy (called isoSTED microscopy).
GSD (Ground State Depletion)	Ensemble	Targeted switching / Scanning-detection	Enhances the lateral resolution only and is similar to STED microscopy.
SSIM (Saturated Structured Illumination Microscopy)	Ensemble	Targeted switching / Widefield detection	Allows for highly parallel imaging of the sample. Both lateral and axial super-resolution can be achieved.
PALM (Photoactivation Localization Microscopy) or F-PALM	Single-molecule	Stochastic switching / Widefield detection	Allows for highly parallel imaging of the sample. Enhances the lateral resolution. New developments such as (BP) PALM have allowed 3D super-resolution.
STORM (Stochastic Optical Reconstruction Microscopy)	Single-molecule	Stochastic switching / Widefield detection	Allows for highly parallel imaging of the sample. Enhances the lateral resolution. New developments have allowed 3D super-resolution.
GSDIM (Ground State Depletion followed by Individual Molecule Return)	Single-molecule	Stochastic switching / Widefield detection	Allows for highly parallel imaging of the sample. Enhances the lateral resolution and can utilize conventional fluorophores.
PAINT (Point Accumulation for Imaging in Nanoscale Topography)	Single-molecule	Stochastic switching / Widefield detection	Allows for highly parallel imaging of the sample. Enhances the lateral resolution.

Notes: A variant of STED includes isoSTED that combines STED and 4Pi. SSIM is also referred to as SPEM (Saturated Pattern Excitation Microscopy). RESOLFT (Reversible Saturable / Switchable Optically Linear Fluorescence Transition) is a generalized name for STED or SPEM. F-PALM (Fluorescence PALM) is synonymously used with PALM. Variants of PALM include TL-PALM (Time Lapse PALM), PALMIRA (PALM with Independently Running Acquisition), spt-PALM (Single particle tracking PALM), iPALM (Interferometric PALM) and biplane PALM called (BP) PALM a method that enhances axial super-resolution. Variants of STORM include: direct STORM (dSTORM) based on conventional fluorophores and 3D-STORM.

Table 2: Fluorophores being used in super-resolution microscopy. Activating light makes a fluorophore excitable. Quenching light renders the fluorophore un-excitable; i.e., it does not emit. Note that the readout beam required for imaging a fluorophore is different from the activating beam. Photobleaching is an example of quenching fluorescence. Pre and post colors refer to the fluorescence emission before and after photoshifting or photoactivation, respectively. Additional fluorophores that have been imaged using STED and GSDIM microscopy include several ATTO dyes such as ATTO 532 and ATTO 565. Manganese-doped quantum dots have also been imaged using RESOLFT (Table-1). Fluorophores marked with an asterisk (*) must be used in the presence of another fluorophore called an activator. The choice of activator fluorophore dictates the required activation light. (UV) Ultraviolet. (NA) Not Applicable.

Category	Type of fluorophore	Name	Activating light / quenching light	Abs	Em	Pre color	Post color
Irreversible photoactivatable	Genetically encoded fluorescent proteins	PA-GFP	UV-violet / NA	504	517	Dark	Green
		PA-RFP1-1	UV-violet / NA	578	605	Dark	Red
Reversible photoactivatable		FP595	Green / 450 nm	590	600	Dark	Red
		Dronpa	UV-violet / 488 nm	503	518	Dark	Green
		Padron	Blue / 405 nm	396	522	Dark	Green
Photoshiftable		PS-CFP2	UV-violet / NA	490	511	Cyan	Green
		Kaede	UV-violet / NA	572	582	Cyan	Orange
		KiKGR	UV-violet / NA	583	593	Green	Red
		mEos	UV-violet / NA	573	584	Green	Orange
		Dendra-2	Blue / NA	553	573	Green	Orange
Photoswitcher	Non-genetically encoded probes	Rhodamine B	UV / NA	530	620	Dark	Red
		Alexa Fluor 647*	UV-Blue-Green / red	650	665	Dark	Red
		Cy5*		649	664	Dark	Red
		Cy5.5*		675	694	Dark	Red
		Cy7*		787	767	Dark	NIR
Photocaged		Caged Q-rhodamine	UV / NA	545	575	Dark	Red
		Caged Carboxyfluorescein	UV / NA	494	518	Dark	Green

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