

Super-resolution Microscopy: A Comparison of Commercially Available Options

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INTRODUCTION

Fluorescence microscopy is currently one of the most powerful and versatile techniques available for biological studies. Fluorophore-labeled molecules are very bright and readily distinguishable from other background signals, making it easy to obtain high-contrast images. With the development of genetically encoded fluorescent proteins, it has become possible to image protein expression, localization, and activity in living cells. However, optical microscopes have an inherent limitation in spatial resolution because of the wave nature of light. In light microscopy, resolution is fundamentally limited by the properties of light diffraction as first described by Ernst Abbe in 1873. This prevents the resolution of structures smaller than approximately half the wavelength of light and, as such, causes sharp point-like objects to appear blurry under a microscope.

Resolution can be quantified by analyzing the point-spread function (PSF) of a microscope. The PSF describes how blurry a single point-like emitter (for example, a single molecule or small fluorescent bead) will appear when diffracted through a microscope and the full width at half maximum (FWHM) value of the PSF is a simple way to characterize resolution. Importantly, resolution does not refer to the ability of a microscope to detect small structures; rather, it denotes the ability to distinguish adjacent objects as separate structures rather than as a single object (Figure 14.1). However, due to the same limitations the true size of objects smaller than the PSF cannot be readily determined. As many structures in biological samples are smaller and/or closer together than the FWHM of the PSF, images from a fluorescent microscope do not always provide a true representation of the sample being visualized.

Practically speaking, the resolution limit of the light microscope depends on two main factors, the wavelength of light (λ) and the numerical aperture (NA) of the objective lens. Visible light ranges between ultraviolet (<400 nm) and infrared (>800 nm), and the NA of an objective refers to the light-collecting ability of the objective lens. Most commercial objective lenses have a NA around 1, and the practical upper limit for a NA is approximately 1.5. Thus, applying a simplified equation based on Abbe's work (where the size of the finest detail that can be resolved is $d = \lambda/2\text{NA}$), 500-nm light and an NA of 1 give $d = 250$ nm. This equation was later refined by Lord Rayleigh in 1896 to give the Rayleigh criterion, defined as the shortest distance at which two point emitters can be distinguished as separate objects: $R = 0.61\lambda/\text{NA}$. The Rayleigh criterion is a commonly used measure of the width of the PSF and is shown in Figure 14.1. This means that light diffraction limits the resolution of an optical microscope to approximately half the wavelength of the light used, usually around 250 nm, and therefore many fine cellular structures cannot be resolved.

As the de Broglie wavelength of an electron is much shorter than visible light, electron microscopy has a much higher resolution than optical microscopy and has long been relied on to visualize cellular structures smaller and/or closer together than 250 nm. However, fixation, dehydration, and thin sectioning are required during sample preparation for electron microscopy, making it technically challenging, prone to artefacts, and incompatible with live-cell imaging. Therefore, microscopic techniques that combine the nondestructive nature of optical microscopy and the nanometer resolution of electron microscopy have been the focus of much research and development in recent years.

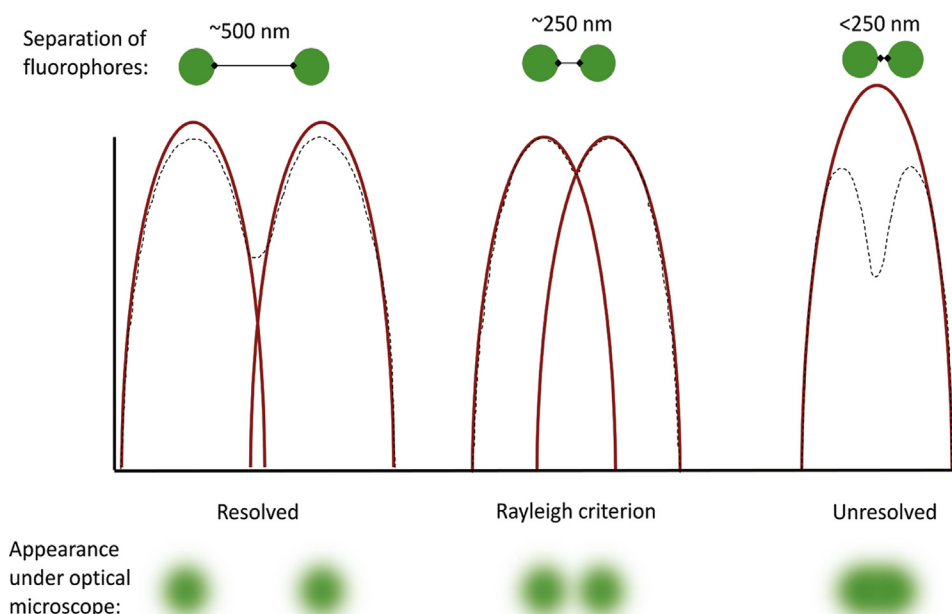


FIGURE 14.1 Definition of resolution and the limits of the ability to differentiate point sources. Point-like objects can be resolved by an optical microscope if they are separated by the Rayleigh criterion but will not be resolved if they are closer together than this distance (<250 nm).

In optical microscopy, the combination of a high-magnification objective lens (e.g., 100 $\times$) and a charge-coupled device (CCD) camera with a small pixel size (e.g., 6 μm) can provide a situation where single diffraction-limited structures can be visualized across multiple pixels (e.g., with an effective final pixel size of 60 nm in this example). Thus, through curve fitting, the center of these objects can be identified with accuracy greater than the diffraction limit (Figure 14.1). However, with densely packed, brightly labeled diffraction limited structures, often the case in biological systems, this may not be possible, particularly if multiple fluorophores share the same pixel space. That being said, this type of centroid identification remains a critical step in some super-resolution approaches (see Single Molecule Localization Microscopy section, below).

Recent advances in fluorescence microscopy have resulted in the development of a series of different super-resolution techniques for breaking the diffraction barrier inherent in light microscopy. This has been achieved by choosing contexts in which Abbe's law does not apply, for example exploiting nearfield effects, selectively switching fluorescent dyes between on and off states, and by localizing the centers of single fluorophores with high precision. Any microscopy technique that overcomes the resolution limit of conventional light microscopy by at least a factor of two is generally considered to be a super-resolution technique. The recent technical innovation of super-resolution microscopy has improved the limits of optical resolution up to nearly

tenfold.¹ Different super-resolution technologies are available, but they are generally built around conventional confocal or widefield fluorescence microscopes equipped with lasers and sensitive cameras—equipment that has already been used for fluorescence imaging and single-particle tracking experiments for decades.

Super-resolution microscopes, however, are not based on one single technology, and several methodologies have been developed independently over the past several years for super-resolution fluorescence microscopy. These include structured illumination microscopy (SIM), stimulated emission depletion (STED) microscopy, photoactivation localization microscopy (PALM), and stochastic optical reconstruction microscopy (STORM). Each of these techniques is not only predicated on a different method for overcoming the diffraction limit but also has inherent advantages and limitations when applied to different biological questions.

In this chapter, we emphasize new developments and applications of super-resolution microscopy. This will provide information about the physical basis for each solution, as well as a consideration of the practical concerns and relative benefits and limitations. Finally, variations on each will be described, as well as future directions in super-resolution microscopy, including newer emerging techniques. As STED microscopy, PALM/STORM, and structured illumination microscopy (SIM) instruments are now commercially available, these are the focus of our discussion. Biologists

TABLE 14.1 Relative Benefits and Limitations of Different Super-resolution Techniques

Modality	Resolution	Illumination	Probes	Acquisition Time	Post-Acquisition Processing	Data Size	Other Concerns
Standard fluorescence microscopy	250 nm	Epi/TIRF/confocal	Conventional fluorescent probes				
SIM	100 nm	Wide-field (epi/TIRF)	Conventional fluorescent probes	Short (seconds)	Yes (Fourier transforms)	Small (9-15 frames)	Out of focus signal and sample thickness
STED	30-70 nm	Laser scanning confocal	Not all conventional probes- see text	Short (seconds)	No	Small (1 image)	Photobleaching
PALM/fPALM	10-55 nm	Wide-field (epi/TIRF)	Photo-activatable fluorescent proteins (e.g. PA-GFP)	Long (minutes)	Yes (centroid identification)	Large (many frames)	Over/under labelling artefacts
STORM/dSTORM	10-55 nm	Wide-field (epi/TIRF)	Photoswitchable dyes (e.g. Cy3/5) Blinking fluorophores (e.g. Alexafluor-647)	Long (minutes)	Yes (centroid identification)	Large (many frames)	Over/under labelling artefacts

who wish to utilize these techniques will need to make informed choices, as there are trade-offs between sensitivity, resolution, field and depth of view, speed, and probe versatility within each technique (Table 14.1).

It should be noted that, owing to space constraints and issues comparing the different commercially available super-resolution solutions, only lateral (x,y) super-resolution is discussed in detail in this chapter. Although axial (z -plane) super-resolution techniques have been developed and applied, these are not covered in any great detail.²⁻⁵

TECHNICAL CONSIDERATIONS

As mentioned above, the differences between the super-resolution technologies can influence how suited an approach is to studying a specific biological question. Some super-resolution microscopes are not always as user friendly as conventional optical microscopes when it comes to imaging biological samples. Currently, there is still no ideal system that offers high-speed, three-dimensional nanometer spatial resolution with multicolor capabilities for live-cell imaging. Thus, the strengths and weaknesses of each technique must be considered with reference to the requirements of the user and the particular sample to be imaged. As

each super-resolution technique has trade-offs in terms of resolution, speed, ease of use, etc., the three techniques discussed in this chapter (SIM, STED, and PALM/STORM) are quite likely to establish their own application niches as they become more widely available. Various factors must be carefully weighed when choosing a technique to use. A few important questions for those wishing to use super-resolution methods include: What resolution is sufficient to observe the structure in question? How fast is the biological process of interest? Is live-cell imaging required or can fixed specimens be used? Additional considerations when selecting a super-resolution technique for visualization of a particular structure include the size and density of the structure, its location within the cell (for example, whether it is primarily in the plasma membrane or internal), whether it can be labeled by expression of a fluorescent-tagged protein or tagged with an antibody or dye, and the signal-to-noise ratio achievable for this label. Some of the trade-offs and technical considerations are dealt with in more detail below.

Acquisition Speed

Many super-resolution techniques obtain increased resolution at the cost of the speed of image acquisition. The trade-off between speed and resolution is typically

more pronounced with single-molecule localization techniques such as PALM and STORM (see Single-Molecule Localization Microscopy section, below). This is because two molecules cannot be turned on within the same PSF at any given time, and multiple rounds of switching fluorophores on and off are required to generate one complete super-resolution picture, limiting the speed of the molecular read-out. As such, live-cell imaging is largely impossible with the single-molecule techniques.

The speed of SIM image acquisition is similarly limited by the need to record multiple frames in order to generate one super-resolution image. However, physical movement of the grating itself takes little time and live-cell imaging with SIM is possible in some samples.⁶

STED is the fastest of the super-resolution techniques. However, because STED is a scanning microscopy technique, its speed depends on the size of the field of view; it is relatively fast when only small fields of view are imaged, making it an excellent choice for video-rate imaging of small areas. As resolution increases, scanning of more and smaller pixels is required, resulting in longer scanning times and high levels of photobleaching, limiting its applicability to live-cell imaging (although live-cell STED is possible).

Fluorescent Probes

Like all fluorescence microscopy techniques, the choice of fluorescent probes in super-resolution imaging is paramount, and each of the different techniques has different criteria for what makes an optimal fluorescent probe. SIM is the only super-resolution technique for which no special fluorescent probes are required. The same fluorescent proteins, antibodies, and dyes used for conventional fluorescence microscopy techniques are all applicable to the technique; however, photobleaching must be considered as multiple intermediate images are required to generate one super-resolution image.

The single-molecule techniques such as PALM and STORM require fluorescent probes whose state can be controlled, either by reversibly or irreversibly switching between a light and a dark state, or by changing from one wavelength to another. In both cases, the probes used must be as bright as possible and require a high contrast ratio between the two states. In the initial configuration, STORM requires pairs of dyes;⁷ however, direct STORM (dSTORM) exploits the blinking phenomenon exhibited by certain dyes such as Alexa Fluor 647.⁸

With most commercially available systems, STED is not achievable with all conventional fluorophores. This is because with a limited selection of depletion wavelengths there will be fluorophores that are actually

excited by the depletion beam and therefore cannot be used. This often means red fluorescent proteins and dyes are impractical, presenting a challenge for dual-color imaging, where green and red fluorophores are typically used.

Photobleaching

Photobleaching is the light-induced destruction of fluorophores, which can be a particular problem for imaging biological samples using time-lapse studies for long periods of time or where high laser powers are required, as is the case with STED microscopy. When a fluorophore absorbs a photon, an electron becomes excited from the ground state to an excited state. When fluorophores are in an excited state, they are more likely to react with other molecules. Although the mechanism of photobleaching is not fully understood for most molecules, it is generally thought that it involves electronic excitations to triplet states because triplet states have longer lifetimes and are more reactive. There are anti-photobleaching agents that reduce the amount of oxygen in the sample to prevent reactions with oxygen, although many of these are toxic to live cells.

Photobleaching can be an issue in PALM/STORM as multiple frames must be acquired to generate a single super-resolution image and the user must decide the point at which to stop imaging after the majority of fluorescent molecules have become photobleached. Photobleaching also presents problems for SIM and STED, as these two techniques require saturated fluorescence excitation and depletion, respectively. For SIM, excessive photobleaching will result in loss of signal between subsequent images in the set of multiple frames required to make one super-resolution image. This can cause problems in reconstruction of the super-resolution image. Some fluorophores are more photostable than others (e.g., Alexa Fluor 488 relative to FITC) and so are preferable when imaging times are longer or when multiple images must be acquired.

Spatial Resolution

The single-molecule localization techniques such as PALM and STORM offer the greatest improvement in spatial resolution when compared to conventional fluorescence microscopy. SIM offers the least improvement in spatial resolution of the super-resolution techniques, often only doubling the resolution achievable. For STED microscopy, the achievable resolution is strongly dependent on the photostability of a sample. For biological samples, a resolution of 50 to 100 nm can be obtained without causing significant damage, which falls somewhere between the high spatial resolution offered by

the single molecule localization techniques and the limited improvement offered by SIM.

SINGLE-MOLECULE LOCALIZATION MICROSCOPY

Single-molecule localization microscopy (SMLM) techniques such as photoactivation localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) differ from all other fluorescence imaging techniques in that an image is built up, as the name suggests, literally molecule by molecule. As mentioned previously, with sparse diffraction-limited fluorescent objects, centroid fitting can be employed to provide more accurate localization than the diffraction limit would normally allow. However, with multiple fluorophores sharing the same pixel space, as is typical in labeled biological samples, this technique breaks down. Thus, SMLM methods can be used to limit the number of active fluorophores in a sample within any single image acquisition.

The SMLM techniques work on the principle that, although the ~ 250 -nm limit of resolution in light microscopy (Figure 14.1) prevents the separation of two objects at distances of less than 250 nm, the centers of individual objects can be determined with nanometer precision.^{9,10} However, by inhibiting the fluorescence emission of the majority of the labels at any one time, only single isolated molecules within a PSF are allowed to fluoresce at a given time. By stochastically switching on and off different single isolated molecules in subsequent camera recordings, a final image with subdiffraction-sized spatial resolution is reconstructed from the summation of all localized spatial positions.

Cells can therefore be imaged at nanometer resolution by determining the exact location of each fluorophore one by one. All single-molecule localization microscopy techniques rely on this temporal separation of fluorescence emission, which is achieved either by switching between a dark and fluorescent state or by consecutive binding of individual fluorophores to the structure. This is achieved by the application of photoconvertible or photoactivatable dyes and proteins. The emission wavelength of photoconvertible dyes or fluorescent proteins (FPs) can be optically converted from one wavelength to another or fluorescence can be turned on and off in the case of photoactivatable proteins. This principle has been published by two independent groups who named the technique PALM and STORM, respectively.

Both PALM¹ and fluorescent PALM (fPALM)¹¹ use genetically expressed photoactivatable fluorescent probes. Under normal conditions, EosFP, the first fluorescent protein to be imaged by PALM, emits green

fluorescence at 516 nm.¹² Upon illumination at around 400 nm, a photo-induced break in the peptide backbone adjacent to the chromophore occurs, causing the fluorophore to emit in the yellow region. If the number of converted fluorophores is small, the proteins emitting in the yellow region will, on average, be well separated and can be imaged with high resolution. When this first subset of EosFPs becomes photobleached, another subset of EosFPs can be converted and imaged, and this process is cycled thousands of times until the entire population of fluorescent proteins becomes photobleached and acquisition is stopped.

Developed in the laboratory of Jennifer Lippincott-Schwartz at the National Institutes of Health, fPALM was originally predicated on the development of the photoactivatable green fluorescent protein (PA-GFP).¹³ Although a detailed description of GFP and the specific physiochemical properties of PA-GFP are outside the scope of this chapter, both originate from the jellyfish *Aequorea victoria* and, as the names imply, fluoresce green when illuminated with blue light. In brief, when initially expressed PA-GFP is essentially nonfluorescent. However, when illuminated with an ultraviolet, or near-ultraviolet (e.g., 405 nm) pulse, PA-GFP enters a bright state in which it can be excited, as with normal GFP, with 488-nm light. Through the precise calibration of the activation pulse and the excitation illumination, the user can cycle a small proportion of the PA-GFP in a sample from dark to bright to photobleached as in PALM. The photobleaching step is technically not essential but aids in preventing the same fluorophores from being imaged multiple times. This reduces both error in fluorophore number measurements and the number of frames that will ultimately have to be acquired to generate the final image.

Because only a few fluorophores in each frame are visualized, large numbers of images are required. Thus, the iterative nature of PALM means that, even with a high-speed electron multiplier CCD (EM-CCD), it can often take several minutes to acquire all the information required to reconstruct a single image. When considering that subdiffraction limit localization is the goal of PALM, this introduces a significant potential for artefacts generated by even small amounts of sample drift during acquisition. For this reason, fiducial markers, such as small fluorescent beads, are often added to samples to be imaged by PALM or other SMLM techniques, and a final image registration algorithm is employed during post-acquisition processing.

Many other photoconvertible, photoactivatable, and photoswitchable fluorescent proteins have been developed. Photoactivatable FPs can be activated from a dark state to a bright state using ultraviolet light, and photoswitchable FPs such as Dronpa can be cycled between light and dark states with specific illumination

wavelengths.¹⁴ A probe that switches only once is advantageous when quantifying the absolute number of fluorescent molecules present within a sample, as each will only be counted once. However, in practice, most organic dyes can be reversibly photoswitched, resulting in the localization of individual molecules occurring multiple times within an acquisition. In addition, a single fluorescent protein will also appear as a cluster of slightly different localizations owing to variable intervals of blinking before irreversible photobleaching eventually occurs.

Another similar SMLM method is STORM, which was developed by Xiaowei Zhuang of the Howard Hughes Medical Institute (HHMI) at Harvard University.⁷ In theory and practice, there are many similarities between PALM and STORM, although STORM uses chemical fluorophores rather than fluorescent proteins. These are typically photoswitchable pairs of cyanine (Cy) dyes coupled to antibodies to act as reporter and activator pairs in order to cycle multiple times between the dark and light states (e.g., Cy3–Cy5 pairs). In direct STORM (dSTORM),⁸ several synthetic dyes, such as Alexa Fluor 647, can also be used in a blinking mode to achieve a similar effect. In the original version of STORM, pairs of fluorophores are used which can be switched on and off by different laser lines. The physiochemical nature of this phenomenon is not easily understood and will not be detailed in this chapter. Otherwise, the iterative nature of the STORM image acquisition process is quite similar to that of PALM and, generally speaking, microscopes that have been developed for PALM can be used for STORM and *vice versa*.

Despite the technical differences between PALM and STORM, they can both localize molecules of interest with ~ 20 nm resolution, depending on the number of

photons collected, and are often known as the single-molecule techniques. In both techniques, subsets of fluorophores are switched on with a brief laser pulse, which is so weak that only a few molecules are stochastically switched on at a time, resulting in such a low density of activated molecules that overlap within diffraction limited resolution is unlikely. Imaging of the on fluorophores is performed until all activated molecules are bleached, and the process is repeated until several hundreds of thousands of molecules are imaged. This process is summarized in Figure 14.2. Once the positions of all fluorophore molecules are identified, a super-resolution image with resolution of up to ~ 20 nm can be reconstructed.

Benefits and Limitations

The chemical fluorophores employed in STORM must be posttranslationally linked to proteins of interest usually through indirect methods such as immunocytochemistry. This introduces other potential concerns such as those regarding sample preparation (see below). However, distances between the target protein and the fluorophore can also raise problems. Antibodies are nearly 15 nm in length, and considering that in indirect immunofluorescence a primary antibody specific to the antigen protein of interest and a fluorophore-tagged secondary antibody are employed, this can result in a relatively large distance between the fluorophore and target. In comparison, most fluorescent proteins have a compact barrel-shaped structure (GFP has a diameter of approximately only 4 nm). Thus, even if short peptide linkers are placed between the protein of interest and the fluorescent protein tag for PALM imaging, antibodies used in STORM can potentially introduce a significant localization error. Furthermore, the requirement of a

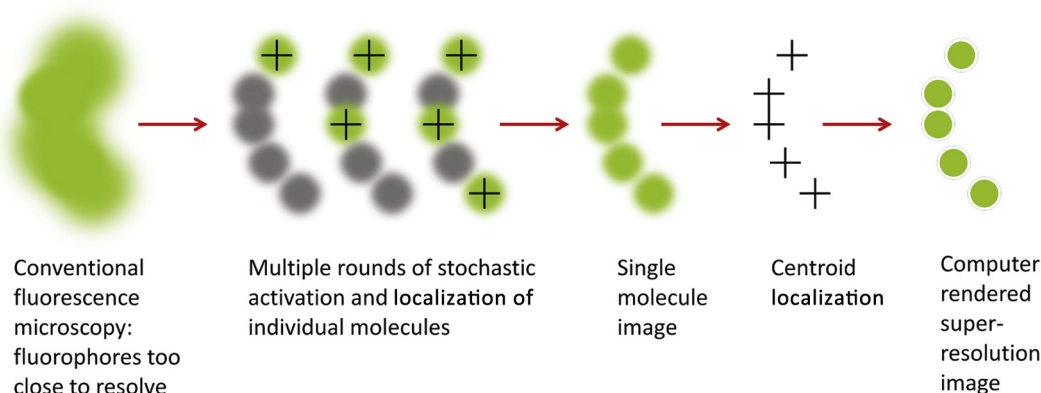


FIGURE 14.2 The process of super-resolution image generation by SMLM techniques. Through iterative identification of the centroids of individual fluorophores, super-resolution images can be reconstructed.

dual fluorophore probe for conventional STORM adds other technical concerns. However, the technique of dSTORM, which is generally considered to be photochemically equivalent to the phenomenon of ground state depletion (GSD), more recently has been gaining prominence. In this case, a single fluorophore (usually Alexa Fluor 647) is shifted between dark and bright states, thus simplifying methodological concerns (Figure 14.3).

In PALM, a cDNA construct of a chimeric fusion protein directly tagging the protein of interest must be introduced into the cell to be imaged. Although fluorescent proteins are generally innocuous tags that do not affect target protein structure, function, or localization, these are potential concerns. In addition, possible overexpression artefacts must be considered, as in conventional microscopy studies.

In SMLM techniques, it is possible to achieve optical resolution so high that the labeling density, the number of fluorophores per target protein, becomes the limiting factor in improving resolution. In fact, labeling density is critical to the ultimate resolution achievable and should not be too great or too sparse. Moreover, as the optical resolution continues to improve toward around 10 nm and beyond, the physical size of the antibodies or fluorescent proteins may start to become the limiting factor for resolution.

The trade-off between speed and resolution in super-resolution microscopy is typically most pronounced with the SMLM techniques. The speed of acquisition is

limited because two molecules cannot be turned on within the same PSF at any given time. This makes imaging live cells extremely challenging, as the structures within live cells move and gain and lose molecules over time. As such, live-cell, single-molecule super-resolution imaging requires that both spatial and temporal sampling are fast enough to avoid the image becoming blurred.

Additionally, as the SMLM techniques build up images literally molecule by molecule, the user must consider how many of these fluorescent molecules are missed or even artificially added during image acquisition. In addition, the user must decide when to stop the acquisition by judging when a significant proportion of fluorophores are bleached, which can introduce error. Moreover, in PALM, an unknown fraction of the fluorescent proteins are actually photoswitchable and the level of replacement of endogenous protein with tagged proteins influence the fraction of fluorescent proteins present.

Single-molecule localization microscopy also faces the problem that if too many fluorescent molecules are missed because a low number of photons are emitted, the resulting image will be incomplete and give an incorrect image reconstruction rather than simply generating a low quality but accurate image. As such, it is not clear if localization techniques can always be used for accurate protein quantification. In contrast, overlabeling can result in images that are improperly rendered owing to the presence of partially overlapping centroids that

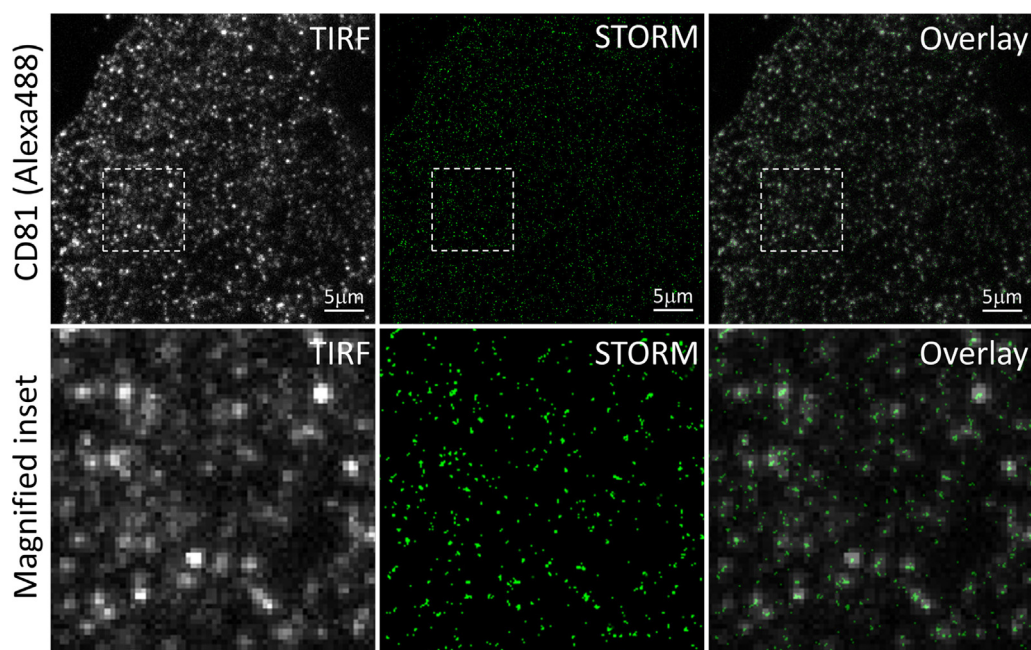


FIGURE 14.3 Example of successful dSTORM imaging. Immunolocalized CD81 labeled with an Alexa Fluor 647 conjugated secondary antibody was visualized first by TIRF microscopy and then through direct stochastic optical reconstruction microscopy (dSTORM), using a Nikon nSTORM microscope.

cannot be resolved. Thus, although extremely powerful, SMLM is not suitable for all applications, and other techniques that are more rapid or that can be used with all fluorophores have been developed.

Data Processing

As described above, once the series of sparsely populated individual frames has been acquired, techniques such as Gaussian fitting can be applied to generate a map of fluorophore centroids, although more recently a significantly faster wavelet approach has also been applied.¹⁵ Thus, when summed together, these individual processed frames provide a super-resolution image. Localization microscopic techniques generate images that are different to those we are used to seeing as microscopists. Our eyes tend to look for patterns and to focus on the largest and brightest structures in a microscopic image. However, STORM and PALM images are generated mathematically, and the size and brightness of the “dots” in the image represent user-selected parameters. There are currently two mathematical approaches commonly used to describe the distribution of molecules in two dimensions: Ripley’s *K* function analysis compares the actual distribution relative to a random distribution and assigns a degree of non-randomness to each molecule to create a cluster map,¹⁶ whereas pair-correlation analysis determines the probability of finding a molecule at a given distance from another molecule compared with the probability expected from a random distribution of molecules.¹⁷ Thus, especially when considering that commercially available software does not provide the user with a great deal of detail into the precise algorithms employed, care must be taken when interpreting and quantifying reconstructed SMLM images.

Application to 3D and Multicolor Imaging

Three-dimensional fPALM has been achieved using a technique known as biplane detection.¹⁸ A beam-splitter splits the fluorescence light into a shorter and longer path to form two detection planes for axial position determination. Additionally, three-dimensional STORM has been achieved by introducing astigmatism to the image using a cylindrical lens. Images above and below the focal plane are ellipsoidal, meaning that the position of the fluorophore can be monitored by examining the ellipticity of the circular fluorophore.³ Two-color PALM has also been implemented. For example, COS-7 cells tagged with transferrin receptor (TfR)-PAM-Cherry1 and PA-green fluorescent protein (PAGFP)-Clathrin light chain (CLC) were alternately imaged at 561 nm and 468 nm to excite the red (PAMCherry1) and green (PAGFP) fluorescent labels.¹⁹ Multicolor

STORM has also been demonstrated. Microtubules were imaged alongside clathrin-coated pits, using Cy2–Alexa Fluor 647 and Cy3–Alexa Fluor 647 to label the microtubules and clathrin, respectively.²⁰ Laser wavelengths of 457 and 532 nm were used to selectively excite the two dye pairs, making it possible to image the microtubules separately from the clathrin-coated pits with ~30-nm spatial resolution. Thus, the inherent power of SMLM is being extended to a variety of new specific applications. However, other super-resolution techniques have been developed that do not suffer from the same limitations as SMLM.

STRUCTURED ILLUMINATION MICROSCOPY

Developed collaboratively between the laboratories of John Sedat at the University of California, San Francisco, and the late Mats Gustafsson at HHMI Janelia Farms, structured illumination microscopy (SIM) also relies upon computational approaches to generate a super-resolution image from a series of acquisitions (Figure 14.4). However, as the name suggests, rather than relying on fluorophore effects, SIM utilizes a structured pattern of illumination light to excite the whole field.²¹ When a grid with high spatial frequency is projected onto a sample, fluorophore emission is blurred when detected. When this excitation pattern mixes with the spatial pattern of the sample, an interference pattern called a moiré fringe is produced, which is much coarser than either pattern alone. Moiré fringes are simply interference patterns created when light passes through regular structures and patterns (shown in Figure 14.4).

If the pattern is moved across the specimen, a characteristic signal variation in the fluorescence response can be observed as a function of time and grid position. If one of the patterns is an unknown structure (the molecules in the sample being imaged) and the other is a known pattern (the grid), the corresponding moiré fringes will contain information about the unknown

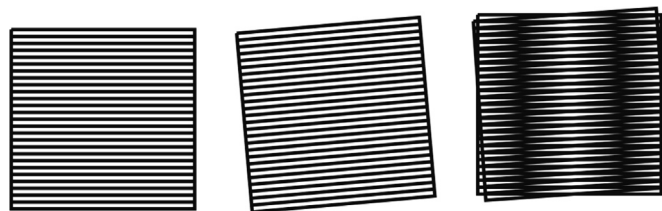


FIGURE 14.4 Generation of moiré fringes by overlaying grids with different patterns. The rotation of two diffraction gratings generates the moiré fringes involved in structure illumination microscopy (SIM).

structure. When the known patterns have higher spatial frequencies than the unknown pattern, the technique will offer an improvement in spatial resolution. This means that, by analyzing the signal variations between images, structural features can be resolved that would not be visible by regular microscopy.²¹ Because structures that have a parallel orientation to the grid would not benefit from this effect, the grid must be not only shifted but also rotated between images to generate a series of several images of the same sample that have different moiré fringes. Thus, in order to restore the sub-resolution information from the moiré fringes, it is necessary to acquire several image frames, each using a different illumination pattern. SIM typically records frames at three different pattern orientations and three to five different positions, generating a total of 9 to 15 frames per final super-resolution SIM image.²² As the illumination pattern is known, this information can be used to generate the super-resolution image.

Data Processing

This method of sequential data collection requires that the final super-resolution image be reconstructed from the raw frames after data collection. Typically, a reconstruction algorithm working in the Fourier domain is used to distinguish sharp image components from signal oscillations to generate a super-resolution image from the frames taken.²¹ This algorithm requires that the images acquired have high-contrast elements, meaning that significant levels of out-of-focus fluorescence can result in a lack of information from which to reconstruct a true super-resolution image. This issue depends on the thickness of the sample being imaged and the localization of the molecules within the sample. This can represent a particular problem for overexpressed proteins tagged with fluorescent proteins such as GFP, where high levels of out-of-focus light are often observed.

This effect can be minimized through the utilization of illumination via total internal reflection fluorescence (TIRF) microscopy. Although a detailed description of TIRF is outside the context of this chapter, TIRF uses oblique illumination to selectively excite fluorophores within a thin (approximately 200-nm) plane above the coverslip to which the sample is adhered.²³ SMLM can also utilize TIRF illumination, and the flexibility of being able to use either TIRF or epi-illumination is one of the shared benefits of these two techniques. However, in the case of SIM, if the target protein is not within 200 nm of the coverslip and is surrounded above and below by fluorophores, proper reconstruction of a super-resolution image can be difficult to achieve.

Once generated, the super-resolution nature of a SIM image can be directly validated by inspection of the

Fourier transform (Figure 14.5). Thus, although artefacts can be difficult to discern in SMLM, in SIM clear quantitative criteria can be employed to determine whether reconstruction was successful. However, it should be noted that most commercial SIM and SMLM systems provide the ability to acquire corresponding conventional non-super-resolution images which can permit direct comparisons and serve as a guide to validate that the reconstructions generated reasonable localizations.

Benefits and Limitations

SIM is arguably one of the most user friendly super-resolution techniques, particularly in terms of sample preparation, as any label used in conventional fluorescence microscopy can be applied to the technique. In addition, conventional excitation routines are applied, resulting in less photobleaching than some of the other techniques (although multiple images of the same sample must be acquired to generate a super-resolution image). SIM is typically a widefield approach, meaning that fast CCD cameras can be used. However, the imaging rate is typically slower than that of conventional microscopy because of the need to acquire several images from different grating positions. SIM allows resolutions of around 100 nm in x , y , and z when biological material is used,⁵ and as such is the least powerful super-resolution technique regarding gained resolution over

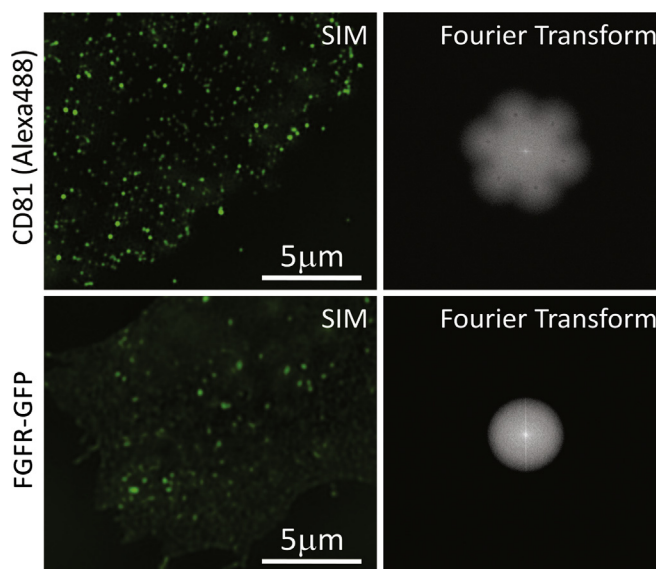


FIGURE 14.5 Examples of successful and unsuccessful SIM imaging. Immunolocalized CD81 labeled with an Alexa Fluor 488 conjugated secondary antibody was successfully visualized by structured illumination microscopy (SIM) and verified by the width and characteristic “petal” shaped of the Fourier transform. GFP-tagged FGFR was not successfully visualized by SIM, as demonstrated by the smaller width and conventional shape of the Fourier transform.

conventional fluorescence microscopy. This is because, unfortunately, the spatial frequencies that are optically created in SIM are themselves limited by diffraction. As such, SIM is generally considered to be limited to this factor-of-two improvement, as it is limited by the PSF of conventional microscopy.²¹

Application to Live-Cell, 3D, and Multicolor Imaging

Multicolor SIM can be easily realized as long as appropriate lasers and correctly aligned diffraction gratings are available. Furthermore, SIM is currently the most widely used super-resolution technique for live-cell imaging and has been applied to a broad range of biological studies, including, for example, live-cell 2D SIM imaging of microtubules.⁶ A difficulty encountered with live-cell SIM is that 9 to 15 recorded frames are required to image the sample. If the object in question moves even slightly during image acquisition, artefacts are created that can prevent reconstruction of the final super-resolution image from the individual frames. 3D-SIM imaging is not limited to regions of interest at the coverslip and can image as far as 10 μm into the sample, and 3D SIM imaging of the nucleus has been used to demonstrate that nuclear pore complexes are adjoined by channels in both the nuclear lamin and peripheral heterochromatin.⁵ However, as stated above, fluorescence signal above or below the plane of focus can limit the ability to successfully reconstruct a SIM image.

STIMULATED EMISSION DEPLETION MICROSCOPY

Both SMLM and SIM employ widefield (either epi or TIRF) illumination and collection through a CCD (or more commonly an EM-CCD), but stimulated emission depletion (STED) microscopy is based on confocal laser scanning microscopy (CLSM) (Figure 14.6). In a laser-scanning confocal microscope, a laser beam is focused by an objective lens into a small focal spot within a specimen. An image is then acquired point by point, by scanning the specimen, using pinhole optics to ensure that only a single focal plane is visualized at a time.

The physical basis for STED microscopy is the generation of an illumination beam with an effective diameter that is smaller than the diffraction limit.²⁴ The technique uses a conventional focused laser beam to stimulate an area of fluorescent molecules that can be several hundred nanometers in diameter. Before the spontaneous emission of fluorescence occurs (within only a few nanoseconds), a second red-shifted doughnut-shaped beam illuminates the sample, depleting the emission of the

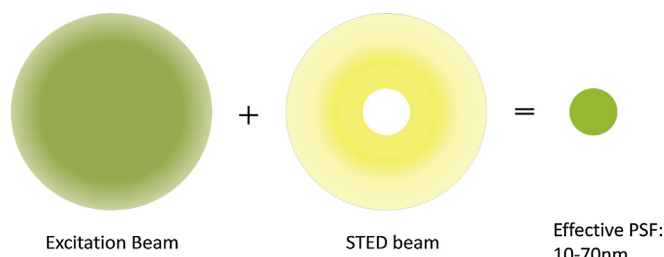


FIGURE 14.6 Generation of an effective PSF smaller than the diffraction limit using a STED depletion beam. Combination of the co-centered excitation and depletion beams creates the phenomenon of stimulated emission depletion (STED), which effectively images only the region inside of the doughnut-shaped depletion beam.

fluorophore outside the central region. Because the depletion beam could also be capable of exciting the fluorophore, it should not be too close to the absorption band. This second beam, known as the STED, or depletion, beam (which in most commercial systems is at 592 nm), forces probe molecules from their excited electronic state back to their ground state by stimulating the emission of a photon of the same wavelength. A bandpass filter excludes these photons, meaning that only the shorter wavelength photons from molecules within the center of the doughnut (which have not been quenched) will be collected. Both the excitation and depletion beams are pulsed lasers, and although they are synchronized, the depletion laser pulse is temporally extended relative to the excitation beam, thus generating “de-excitation” outside the central spot. By overlapping the two beams, fluorescence is only allowed from the center of the spot, essentially switching off a subset of fluorophores and therefore generating a much smaller exciting focal spot, as shown in Figure 14.6. As a smaller effective PSF is generated, this causes less blurring and results in higher resolution. Generally, 100% depletion of the spontaneous emission by the STED beam is not achievable, but 90 to 95% depletion will produce an image with an acceptable contrast ratio. Using this principle, up to 10-fold increases in resolution in one dimension have been achieved.

Increasing the intensity of the doughnut-shaped STED beam dramatically improves the resolution achievable by this technique. This switches off fluorophores even at the inner ring of the doughnut, further sharpening the center fluorescent spot to a size much smaller than the diffraction limit. Typically this requires laser powers around 1000 times that used in conventional confocal microscopy. Although this means that there is no theoretical resolution limit to STED images, in practice photo-damage caused to biological samples by high laser intensities usually sets the intensity limit on the depletion beam for biological applications.

Benefits and Limitations

STED is generally realized as an addition to conventional CLSM, increasing the user friendliness to those already familiar with this widespread technique. The scan times for STED microscopy are quite similar to those of CLSM, apart from an increase proportional to the smaller effective spot size of the scanning laser. If a conventional CLSM can scan a 512×512 image in approximately 1 second with a diffraction limited laser spot, a STED beam tuned to approximately 60 nm would take only about 10 seconds to generate an image. However, as with SMLM, there are fluorophore limitations with STED microscopy (see below), and, as with conventional CLSM, a great deal of emitted light is rejected and never reaches the detector. Thus, STED microscopy can be difficult to employ with weakly fluorescent samples and/or fluorophores that are not particularly photostable.

Although compatible with a large number of fluorescent proteins and chemical dyes, not all fluorophores will successfully display STED. In order to be depleted, a dye should have significant emission in the range of the depletion beam wavelength (e.g., 592 nm). Furthermore, a critical consideration is that the fluorophore is not excited by the depletion beam (Figure 14.7). Thus, as most commercial systems are supplied with a 592-nm depletion beam, many conventional red fluorophores cannot be used for STED imaging. This can create particular issues with multicolor

studies, as a combination of green and red fluorophores is often employed. However, many alternative fluorophores have been identified that are compatible with multicolor STED.

Further concerns about the addition of a STED laser arise from the increased photobleaching of the fluorescent label. This derives from the fact that the STED laser acts on the excited fluorescent label. Light from the depletion beam is not absorbed by the label and does not produce any photoreactive and thus phototoxic species. Rather, stimulated emission shortens the time the fluorophore spends in its excited electronic state and can reduce photoreactions such as photobleaching. Although excited-state absorption of STED light can unfortunately cause severe photobleaching,^{25,26} it was shown that fast scanning and the right choice of STED wavelength can minimize these effects.^{26–29}

The use of fast beam scanners has established STED microscopy as the fastest super-resolution imaging technique available, with recording times of up to 60 to 80 frames per second for observation areas of a few microns in size.³⁰

One key advantage of this technique is that STED microscopy gives instant gratification with a what-you-see-is-what-you-get image similar to conventional confocal microscopy, and it requires no data processing after acquisition. However, as STED is a scanning microscopy technique, its speed depends on the size of the field of view. The technique is relatively fast when only small fields of view are imaged, making it an excellent choice for video-rate imaging of small areas. As resolution increases, scanning of more and smaller pixels is required, meaning longer scanning times and high levels of photobleaching, limiting its applicability to live-cell imaging. However, live-cell STED has been achieved in several cases as detailed below.

Application to Live-Cell, 3D, and Multicolor Imaging

The potential phototoxic effects of the added STED beam were long believed to be incompatible with the study of living cells. (Although the excitation laser power is comparable to that used in conventional confocal microscopy, the STED laser power can be around 1000 times higher.) This has introduced the need to pay attention to heat absorption and potential light-induced toxic reactions. However, live-cell video-rate imaging of synaptic vesicles (28 Hz) has been successfully demonstrated by streamlining the instrumentation and limiting the area imaged to just $\sim 2.5 \times 1.8 \mu\text{m}$.³⁰

Stimulated emission depletion microscopy can be applied to three-dimensional as well as multicolor

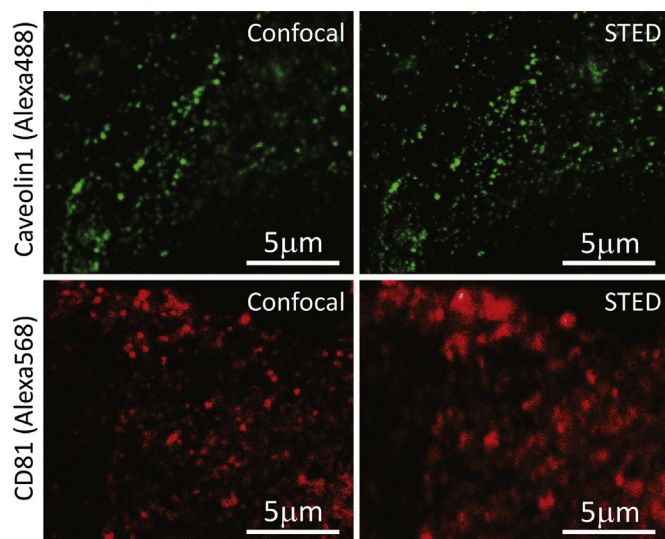


FIGURE 14.7 Examples of successful and unsuccessful STED imaging. Immunolocalized caveolin1 labeled with an Alexa Fluor 488 conjugated secondary antibody was successfully visualized by stimulated emission depletion (STED) microscopy. Immunolocalized CD81 labeled with an Alexa Fluor 568 conjugated secondary antibody was not successfully visualized by STED microscopy due to excitation of the fluorophore by the depletion beam.

imaging. The doughnut-shaped STED beam used in two-dimensional STED works well to deplete the spontaneous fluorescence emission in the lateral direction, but it offers no improvement in resolution in the axial direction. It is possible to combine the improved lateral resolution described above with improved axial resolution if an alternatively shaped STED beam is employed. This STED beam includes axially shifted lobes that quench the axial extension of the PSF.^{2,4}

The complexity of the STED system limits fluorophore choices and can make multicolor imaging difficult. Multicolor imaging represents a challenge to STED microscopy because two laser wavelengths are required for each dye: a conventional excitation laser beam and a red-shifted doughnut-shaped STED beam. This means that for dual-color imaging, four laser beams with four different wavelengths would be needed, which is not only technically challenging but could also easily produce undesired interference and create even greater photobleaching issues. Nevertheless, multicolor STED imaging has been achieved in some cases.^{27,31} To simplify two-color imaging, combining one fluorophore with a second fluorophore that has a similar emission spectrum but a large Stokes shift between excitation and emission can allow single STED laser to be used for both dyes, which simplifies the technique somewhat.³² This approach has also been used for live-cell STED microscopy.³³ Moreover, several recent publications have shown great promise in simplifying the instrument and increasing the availability of additional laser lines.^{28,34,35}

The small effective spot size of STED microscopy also can be exploited to measure nanoscale dynamics in fluorescence correlation spectroscopy (FCS). This has allowed the direct observation that sphingolipids and glycosylphosphatidylinositol-anchored proteins are trapped in cholesterol-mediated sub-20-nm complexes in plasma membranes, supporting the existence of lipid rafts or other microdomains.³⁶

SUMMARY

Among the three main commercially available super-resolution techniques, various relative benefits and limitations exist (Table 14.1), although all require significant attention to issues such as labeling and system alignment. Although SMLM provides the highest spatial resolution, it also takes the longest time to acquire the necessary data, is conducted in fixed cells, and is only compatible with certain fluorophores. SIM can be used with any fluorophore and is relatively rapid, making it useful for live-cell imaging studies, but it does not provide the same resolution gains as SMLM or STED. Furthermore, SIM can be difficult to perform

successfully with samples that have fluorophores above and below the plane of focus. SMLM and SIM share the benefits of being widefield techniques that are compatible with both epi and TIRF illumination, but they also share the limitation that image reconstruction via application of computational algorithms is required. Thus, as in some deconvolution techniques, direct image acquisition is not possible, creating a concern for potential artefacts that cannot easily be discerned. STED is a variant of CLSM and is therefore subject to some of the same benefits and limitations of that technique. STED generally provides intermediate resolution gains between that of SMLM and SIM and is not compatible with all fluorophores; however, STED can be employed, as CLSM, to optically section samples with high fluorophore density within the sample volume. Furthermore, STED is relatively rapid as only a single image is acquired, and as a direct visualization technique no reconstruction is required.

Among the three main commercially available super-resolution solutions, no single technique can be said to be optimal for all applications. Depending upon the specific questions being addressed and the constraints of the experimental system, the user will need to decide which is best. Of course, considering the expense associated with super-resolution systems (each can easily cost in the vicinity of US\$500,000), it may not be practical for each researcher, or even institution, to invest in all three types of systems, so some sort of strategic planning needs to be performed before a system is selected for purchase.

FUTURE DIRECTIONS AND EMERGING TECHNIQUES

Although SMLM, SIM, and STED microscopy represent the main commercially available super-resolution solutions, several other techniques, or variants of these techniques, have been developed. Furthermore, specialized applications of these techniques have recently begun to emerge, and the future will certainly see the emergence of novel microscopy technologies employing innovative methods to break the diffraction barrier. Additionally, in certain cases, three-dimensional and multicolor versions of the existing technologies have had to make use of significant developments to be realized. Other variants of these techniques exist, including, as mentioned above, live-cell TIRF-based SIM and more recently STED compatible with deep penetration into tissue, even in living mice.³⁷ Furthermore, recent developments in computational approaches can provide more rapid SIM reconstruction from fewer images.³⁸ This could mean that reconstruction could be performed during and not after acquisition. Several innovative SMLM

variants have been developed that employ alternative strategies to permit iterative illumination of selective populations of fluorophores. These have included reversible pH quenching and the newly developed technique of universal point accumulation imaging in nanoscale topography (uPAINT), which involves the addition of fluorophores to the sample while imaging.³⁹ uPAINT is particularly useful with fluorescent ligands that will bind to extracellular domains of plasma membrane proteins in living cells; however, uPAINT requires careful fluorophore titration.

Other types of SMLM exist, including relatively simple options that make use of certain inherent properties of particular fluorophores. These include techniques such as Bayesian analysis of blinking and bleaching (3B), which simply tracks these events that can be rare enough to occur only once per pixel during a specific time frame.⁴⁰ However, this can be relatively inefficient and, like other SMLM techniques, subject to labeling density issues. Furthermore, this raises a concern regarding other SMLM techniques such as PALM and STORM, as inherent blinking of a single activated or converted fluorophores could be scored as multiple independent fluorophores, leading to reconstruction and/or fluorophore counting artefacts. Therefore, some SMLM algorithms have been developed with blinking correction functions.

Additionally, single particle tracking (SPT) in live cells can be realized with SMLM.⁴¹ In this case, a small number of fluorophores are selectively visualized through photoactivation or photoconversion and then tracked utilizing very high speed acquisition. This has the benefit over conventional SPT that each molecule of interest can be tracked and can be verified to be a single fluorophore without extensive validation procedures. Furthermore, the entire procedure can be repeatedly performed on individual cells. However, individual tracks can be short owing to fluorophore photobleaching.

Finally, entirely new techniques for achieving super-resolution are being generated. One exciting solution currently in development under the commercial name Bioaxial involves CLSM with the illumination laser passed through a series of polarizing Pockels cells and then into a thin biaxial crystal. The effect of this is, in some ways, a hybrid between SIM and STED. A CCD is employed upon which an iterative series of three to five different diffraction patterns are projected at each point. This technique can generate resolution gains between SIM and STED, uses very low light levels, can be performed without high magnification or high-NA objectives, and is compatible with all fluorophores. However, a large number of images is generated (e.g., $5 \times 512 \times 512$) for a single scan. Thus, although with rapid exposure times acquisition can be relatively quick, this results in a large amount of data to be processed, rendering this technique currently computationally challenging.

In conclusion, this is a very exciting, and confusing, time for super-resolution microscopy. Commercial microscope companies have rapidly responded to user interest with a series of different solutions, each with particular benefits and limitations. It can be difficult for potential adopters to decipher which techniques and technologies are appropriate for their particular applications. Concerns such as fluorophore compatibility, speed of acquisition, and, of course, resolution must all be balanced (Table 14.1). Furthermore, for the facility manager, issues such as cost, flexibility, and user friendliness must also be considered. Thus, two things are clear: (1) no one commercially available super-resolution microscope is the ideal solution for all experimental questions, and (2) more techniques will be developed. Finally, as with all innovations, limitations must be appreciated and not all investigations require super-resolution imaging. Thus, care needs to be taken to avoid the unnecessary purchase and use of these powerful, and often complicated, microscopes.

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