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Super-resolution Microscopy for Nanosensing

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Abstract

The recent advances in super-resolution optical microscopy enable the simultaneous visualization of thousands of structural and signaling molecules as they dynamically rearrange within living cells. Super-resolution microscopy offers an unprecedented opportunity to define the molecular mechanisms of nanosensing through direct observation of protein movement. This technology provides a real time readout of how genetically targeted molecular perturbations effect protein interactions. As we strive to meet the challenge offered by the opportunity to ask questions about the workings of as cell that we never thought we could answer, we need to be aware that the new technologies are still evolving. The current limitations of each technique need to be considered when matching them to specific biological questions. In this review, we briefly describe the principles of super-resolution optical microscopy and focus on comparing the characteristics of each technique that are important for their use in studying nanosensing in the cellular microenvironment.

INTRODUCTION

Scientists have been using light microscopes for centuries to visualize structures that are too small to see with the naked eye. The quest to peer into the nanoscopic details of living cells has led to ever increasing technical innovations and molecular labeling strategies. Fluorescent microscopy stands out among the techniques for its ability to visualize intracellular targets tagged with spectrally distinct colors. With the advent of genetically encoded fluorescent proteins (FPs), it has even become possible to study protein dynamics by noninvasively visualizing the interior of cells.

Despite the sweeping impact of live-cell fluorescent microscopy, fluorescence microscopy is fundamentally limited in resolution by the diffraction of light through the optical path. Abbe, Rayleigh, and others^{1,2} identified this constraint as imposing a minimum required spacing between two point sources of light in order for them to be resolved. This spacing is commonly defined as $d = \lambda / (2n \sin \alpha)$, where d is the minimum spacing for resolvability, λ the wavelength of light, n the refractive index of the medium surrounding the sample, and α is the angular aperture of the microscope objective. The diffraction limit represents the point-spread function (PSF) or the size of the intensity profile for a spot measured at the full width half maximum. For a typical fluorescent probe, $d = \sim 200$ nm. Therefore, proteins smaller than 200 nm appear to be 200 nm in size, and proteins closer to each other than 200 nm cannot be distinguished from each other. Clearly, resolution beyond the diffraction limit is needed to define the structure and function of dynamic cellular nanomachines using light microscopy. In fact, it might even be argued that the limitations imposed by diffraction are almost more disadvantageous to fluorescent microscopy than bright field microscopy because we can label individual molecules with different colors, but we cannot optically separate them with conventional microscopy.

The recent revolution in light microscopy has removed the diffraction barrier as a limiting factor to resolution. With the emergence of super-resolution light microscopy, we can now visualize details of cellular and macromolecular structures that were previously impossible to see. Light microscopy now has the resolution to match the level of detail that molecular labeling techniques provide, and molecular specific resolution of 10–20 nm is now routine.^{3,4} Visualizing nanosensing with 60 nm resolution of dynamically reorganizing molecular ensembles in living cells is now an exciting reality.⁵

WHAT IS SUPER-RESOLUTION MICROSCOPY?

Super-resolution microscopy is defined as any method that improves resolution by at least a factor of two over conventional microscopy. The methods that are used to achieve this are frequently distinguished as either hardware or software based on their approach to shrinking the PSF and increasing resolution. In order to provide a logical basis for pairing technologies with specific biological applications, we will limit the discussion in this review to two hardware-based or ensemble methods and two software-based or single molecule methods. However, as improvements and variations of the technologies are emerging as rapidly as the initial evolution of the field, we will also highlight some of the more significant advances to the original methods.

Many super-resolution techniques achieve increased resolution by controlling when and/or where fluorescent molecules are visible, including stimulated emission depletion (STED) microscopy,⁶ ground state depletion (GSD) microscopy,⁷ saturated structured illumination (SSIM),⁸ photoactivation localization microscopy [(PALM),³ fPALM⁹], stochastic optical reconstruction microscopy [STORM⁴ and direct STORM (dSTORM)].¹⁰ One way to turn the molecules on and off is to take advantage of a nonlinear relationship between excitation and emission. For example, intense levels of excitation light can be used to rapidly transition fluorophores to a transient dark state where they can reside for a variable amount of time before they much more slowly either return to the ground state or permanently bleach if additional light is applied (Figure 1). Alternatively, light can be applied to photoactivatable or photoswitchable fluorophores¹¹ to switch them from either an off state to an on state or to switch them from one color to another. Both of these approaches can increase the density of information obtained as long as these transitions meet two criteria: (1) the transitions are limited to a subset of fluorophores in time or space, and (2) the transitions can be detected while the unwanted signals are excluded. The generalized concept of such reversible light-induced transitions has been termed reversible saturatable optical fluorescent transition (RESOLFT) microscopy, and it is crucial to most super-resolution techniques.¹²

Hardware-Based Techniques

Hardware- or ensemble-based techniques rely on spatially patterning the excitation light to achieve the variations between the on and off states for a population of fluorophores (Figure 2(a)). STED⁶ and GSD⁷ microscopy optically modify the excitation PSF to reduce its effective diameter. They surround a laser scanning focal spot that excites fluorescence emission with a doughnut of more intense red-shifted light to bring the fluorophores in the periphery of the excitation PSF to the ground (STED) or the metastable dark state (GSD) and suppresses their fluorescence emission (Figure 1). The higher the intensity of the STED laser, the more saturated depletion can be achieved in the periphery of the excitation PSF, and the smaller the effective excitation region becomes. There is no theoretical limit to how far below the diffraction limit STED or GSD can resolve; however, the best 2D resolution in a biological context that has been achieved to date is 20–30 nm full width at half maximum.¹⁴

Structured illumination microscopy (SIM)¹⁵ is another hardware-based technique that increases resolution by periodically patterning the excitation light. When the excitation pattern mixes with the spatial pattern of the sample, it shifts the high frequency structural information contained within the specimen into the lower frequency detection range of the microscope (Figure 2(b)). In order to obtain an image with increased resolution, a series of images are taken with the excitation pattern positioned at several different orientations, as resolution is maximally improved perpendicular to the axis of light periodicity. These images are mathematically reconstructed; however, SIM can only extend the resolution by a factor of 2 because the periodicity of the illumination pattern is limited by diffraction.¹⁵ If SIM is combined with high intensity light that takes advantage of the nonlinear relationship between excitation and emission in the saturation region of the fluorophore, then much larger increases in spatial resolution can be achieved. This technique, SSIM,⁸ can be thought of as the inverse of STED where sharp dark regions are created instead of a sharp bright region. The resolution of SSIM scales with the level of saturation and 50 nm lateral resolution has been demonstrated using fluorescent beads.⁸

Software-Based Techniques

The software- or single molecule-based techniques are based on the concept of single molecule localization^{16,17} (Figure 2). Although the image of a single fluorophore appears as a diffraction-limited spot, the center of the spot can be determined with precision that is far greater than the ~200 nm diffraction limit. The precision of the centroid determination can be approximated to the first order by $\sigma/\sqrt{N}$ where σ is the standard deviation of the PSF, and N is the number of photons detected.¹⁸ If fluorophores are positioned far enough apart so that their PSFs do not overlap, the position of each molecule can be determined with 1–2 nm accuracy.¹⁷ However, in biological samples, there may be hundreds of thousands of fluorophores within the 200 nm diameter excitation PSF of a conventional microscope. Although STED solves this problem by reducing the diameter of the PSF, in order for software-based techniques to beat the diffraction barrier they need to employ strategies that control the number of fluorophores within a diffraction-limited spot at any given time.

Two different but complementary approaches have been developed to limit the number of fluorophores that are turned on within a given region. In PALM³ and fPALM⁹ only a subset of genetically expressed photoactivatable fluorophores^{11,19} are stochastically activated during each imaging cycle. Once imaged, these molecules are bleached, and the cycle is repeated thousands of times. As new subpopulations are constantly being activated and bleached, an activation intensity is chosen such that the molecular density is low enough to ensure a distance greater than the diffraction limit separates the activated molecules at any given time. Super-resolution images are constructed from the computed localization of the centroid of each fluorophore. STORM⁴ is another software-based technique that is conceptually similar; however, it does not rely on genetically expressed probes. STORM works by switching inorganic fluorescent molecules between on and off states using a number of different combinations of cyanine dye pairs to act as reporter molecules and activator that facilitate turning the reporter to the on state.²⁰ These techniques provide a lateral resolution of 20 nm.^{4,21} A more recent alternative to using a paired cyan dyes is dSTORM, which uses only a single dye and a thiol-containing reducing agent to switch molecules on and off.¹⁰

WHAT IS THE RESOLUTION OF SUPER-RESOLUTION?

It may initially seem paradoxically that all of the software-based super-resolution techniques have similar lateral resolutions. As the localization precision of software-based techniques is dependent upon the number of photons detected, brighter fluorophores should give enhanced localization precision. The ~10-fold higher number of photons detected with an inorganic

dye compared to a photoactivatable fluorophore²¹ suggest that inorganic dyes should provide a far greater localization precision—approaching 5 nm FWHM.¹⁸ However, in biological applications inorganic dyes are often coupled to antibodies whose size of >10 nm is twofold to threefold larger than the 3–4 nm size of genetically expressed FPs.²¹ The uncertainty that the length of the antibody adds to the location of the centroid and decreases the effective higher precision that is inherent to inorganic dyes. Thus, the localization precision of an individual molecule labeled with either an inorganic dye and an antibody or a genetically expressed FP should be comparable for all of the software-based techniques. Note that hardware-based techniques make ensemble measurements and do not localize individual molecules; their resolution reflects the size of the PSF they use to create a superresolution image.

Although the localization precision or the size of the PSF define the position of a molecule, the resolution of any image depends upon the labeling density on the structures of interest. According to the Nyquist-Shannon²² criteria a structure can be accurately resolved only when the distance between labels is smaller than half the feature size. This means that two labels are needed along every feature that is to be resolved. This requirement typically gives the overall resolution advantage in generating a super-resolution image to a genetically expressed molecule tagged with a photoactivatable FP. As has been demonstrated for many years with immuno-electron microscopy techniques—antibodies frequently label a low copy number of antigens. Thus, if the localization precision is similar with the two probes, but the labeling density is far greater with molecular expression, then genetically expressed probes will yield an image with higher spatial resolution.

The techniques discussed thus far have been two-dimensional, but biological structures are three-dimensional (3D). Perhaps the conceptually simplest way to generate 3D super-resolution images is to combine serial sectioning tissue²³ with lateral standard later super-resolution techniques, such as STED.²⁴ However, many lateral super-resolution techniques have now been extended to increase resolution in the axial direction without physically sectioning the specimen. 3D STORM adds a cylindrical lens in the light path to create astigmatic imaging so that the ellipticity of the PSF becomes a sensitive measure of its distance from the focal plane.²⁵ This technique yields a resolution of 20–30 nm laterally and 50–60 nm axially. Several other subsequent 3D imaging methods have obtained similar axial resolutions by using either a double helical PSF²⁶ or by analyzing two spatially separated focal planes of the same image (biplane fPALM)^{27,28} to obtain 30 nm lateral and 75 nm axial resolution. More complicated hardware implementations for shaping the excitation light do not achieve quite as good an axial resolution. 3D STED (43 nm lateral and 125 nm axial resolution) superimposes a lateral depletion pattern (STED doughnut) in conjunction with an axial depletion pattern.²⁹ 3D SIM also utilizes 3D illumination patterns^{30,31} to achieve ~100 nm lateral and ~300 nm axial resolution. isoSTED³² is a more complicated hardware implementation using opposing objectives in a 4-PI configuration to create a hollow sphere of light that replaces the more cylindrical STED doughnut and has achieved 30 nm isotropic resolution. To date the greatest axial improvement has been achieved by combining axial interference with 2D PALM, in a technique termed iPALM.²¹ iPALM demonstrates lateral resolution of 20 and 10 nm axial resolution. Figure 3 compares the axial resolution of several 3D techniques and only iPALM has utilized light microscopy to measure biomolecules such as microtubules at their known size. Previously structures of this size could only be accurately visualized by electron microscopy (EM), but now EM resolution can be combined with the molecular specificity and the high copy number of genetically expressed probes.

Although these techniques are currently limited in how far away from the cover glass they can penetrate into the specimen, there have been several attempts to image deeper into

thicker specimens using two photon excitation (TPE). Using a temporal focusing scheme, it has been possible to PALM a layer $\sim 1.9\ \mu\text{m}$ thick and scan this layer through a $10\text{-}\mu\text{m}$ thick specimen.³⁵ It has also been possible to combine TPE with STED to image dendritic spines $80\text{--}100\ \mu\text{m}$ below the surface in brain slices.³⁶ As biological structures are dynamic ensembles, often located deep within an animal, the next challenges in 3D super-resolution microscopy are to adapt the ideas and technologies to penetrate further and to maintain lateral and axial resolution when confronted with the light scattering and absorption constraints imposed by animal tissue.

MEASURING DYNAMIC PROCESSES WITH SUPER-RESOLUTION TECHNOLOGIES

Fluorescent microscopy has made such a huge impact in biology because of its ability to visualize dynamic processes in living systems. Temporal resolution has been the key for unlocking many biological mechanisms. However, because we rely largely on conventional imaging optics in the implementation of super-resolution microscopy, inevitably the enhanced spatial resolution compared to conventional fluorescence microscopy comes at the cost of temporal resolution. If we are using a software-based technique and do not change the hardware, it takes more time to read out all of the labeled proteins, since we must adjust the acquisition rate to ensure that fluorophores are spatially separated by the diffraction limit. Similarly, if we are using a hardware-based super-resolution technique, it takes more time to move over the entire field at the finer scanning step size required by the Nyquist-Shannon Criteria as we have a smaller excitation PSF. For example, the temporal resolution of STED microscopy has been reported as 35 microsecond per image with a $1.8\ \mu\text{m} \times 2.5\ \mu\text{m}$ field of view at a 2D resolution of $62\ \text{nm}$.³⁷ However, with a larger field of view imaging speed slows down dramatically. Acquisition times of 10 s per image over a $2.5\ \mu\text{m} \times 10\ \mu\text{m}$ field of view for a $50\ \text{nm}$ resolution have been reported for the very bright fluorescently filled dendritic spines in living cells.³⁸

Although SIM and SSIM are widefield techniques, image collection times can also potentially be an issue with them as well because the excitation pattern is rotated to several different orientations to collect images prior to mathematically reconstructing the super-resolution image. Typically the mechanical rotation of the excitation pattern is the time-limiting step. However, a recent implementation of SIM using a ferroelectric liquid crystal on a silicon spatial light modulator to produce the patterns has proven much faster.³⁹ Image fields of $32\ \mu\text{m} \times 32\ \mu\text{m}$ and $8\ \mu\text{m} \times 8\ \mu\text{m}$ have been successfully imaged at $3.7\text{--}11\ \text{Hz}$ with $100\ \text{nm}$ resolution.³⁹

Photobleaching is another limitation that has a significant effect on the duration of imaging, especially for STED and SSIM, where higher laser intensities are used to create the excitation patterns. For software-based techniques, photobleaching can actually speed up the total time required to collect the data for a super-resolution image. As localization precision depends upon the number of photons,¹⁸ it is advantageous to collect the maximum number of photons in the shortest amount of time.

Although nanometer-level precision for tracking single molecules had been demonstrated previously,^{16,17,40} PAFPs allow the motion of many protein molecules to be tracked in the same cell and high resolution information on the underlying cellular structures can be derived from the tracks. The microstructure of cell membranes was first studied with super-resolution by tracking the movement of PAGFP tagged hemagglutinin molecules on the membrane of living cells with $40\ \text{nm}$ accuracy.⁴¹ Similarly, high-density particle tracking of tdEosFP-Gag and VSVG proteins was utilized to distinguish highly motile from largely stationary molecular species.⁴²

Live-cell super-resolution imaging has also been used to visualize structures and not just track single molecules.^{5,43} Adhesion scaffold remodeling has revealed retrograde transport, elongation, and complex morphological interactions between adhesions. The transport of paxillin molecules into and out of the adhesion scaffolds was also directly characterized. These movies of large image fields ($28\ \mu\text{m} \times 28\ \mu\text{m}$) were obtained every 25–60 seconds, and they have a single molecule localization precision of 20 nm, but the image has a spatial resolution of ~ 60 nm (Figure 4a). Recall that localization precision is the accuracy that is used to identify the centroid of a point and spatial resolution is determined by the size of a feature that can be resolved given the number of molecules that could be localized in a given area.⁵ As the rules for accurately sampling in space also apply to time, acquisition rates for a complete image must be fast enough (twice the most rapid event) to capture the phenomena of interest while maintaining a high enough molecular density for structural resolution (Figure 4b,c).

An important consideration in software-based live-cell super-resolution imaging of structures is that the molecular readout is stochastic. Molecules are sampled from all over the structure. However, the structure is remodeling. The structure is typically not just undergoing a simple translocation; the molecules are often rearranging so that the structure is changing shape. Finally, the cell is adding and consuming molecules within the structure. Therefore, in addition to the imaging modality being nonperturbative, it is crucial that both the spatial and temporal sampling rates be fast enough to accurately capture the biological dynamics. Despite these caveats regarding spatial and temporal sampling requirements, we are seeing rapid improvement in the speed of live-cell super-resolution imaging.

HOW MANY COLORS?

The ever-expanding pallet of fluorescent probes^{11,19} has enabled the study of dynamic molecular interactions. It is possible to detect multiple species without significant crosstalk between channels. Of the enhanced resolution techniques SIM is the one whose choice of labels most closely resembles that of conventional fluorescence microscopy. No special photophysics are involved in modulating the excitation light; however, the number of images (as many as 15)³⁹ that are required to construct the image using the patterned illumination do present a photobleaching consideration. In addition, as diffraction is dependent on the wavelength of light, each excitation wavelength needs a uniquely spaced grid. Therefore, multicolor SIM is best achieved using two dyes with similar excitation spectra but with a large enough Stokes shift to separate their emissions. Two-color STED has been implemented using either two pairs of excitation and depletion lasers⁴⁵ or using fluorophores with a large enough Stokes shift so that two different excitation lasers, but only one emission or depletion laser is needed.³² A more recent design that avoids elaborate preparations of laser pulses uses the same pulsed super-continuum for both the excitation and the STED beam.³⁴

The original multicolor implementation of STORM²⁰ utilized cyanine dye activator–reporter pairs to induce photoswitching behavior. Spectrally distinct images were obtained by either using the same activator dye (Cy3) coupled to one of the three different reporter dyes (Cy5, Cy5.5, or Cy7) or using one of the three spectrally distinct activators (Alexa Fluor 405, Cy3, or Cy2) coupled to the same reporter (Cy5). In the first scenario, distinct emission spectra are the multicolor read out, and in the second scenario, distinct activation spectra are utilized to temporally separate the constant emission, suggesting that it would be a slower implementation. With this technique the localization precision of a single fluorophore is ~ 25 nm.²⁰

Other schemes for multiple color super-resolution imaging include combining a photactivatable FP (rsFastLime) with an antibody-labeled inorganic dye (Cy5).⁴⁶ Multicolor imaging using only FPs has been more difficult because the emission spectrum of the unactivated state of one of the PAFPs often overlaps with the activated state of another. The initial attempts to overcome this challenge used an irreversible green to red PAFP (Eos) to convert and image one species of molecules and then subsequently images green reversibly switchable PAFPs⁴⁴ (Dronpa, Figure 5). More recently nearly simultaneous two-color imaging has been achieved on fixed cells with PAGFP and PAmCherry,⁴⁷ and in living cells with PAGFP and PAtagRFP.⁴⁸ Photostability and photon count of the fluorophore can have a profound impact on precision; not all probes yield the same level of super-resolution.⁴⁴ There is still a need for additional probe development, especially for green fluorophores where low photon outputs and the inability to identify transfected cells prior to activation have slowed simultaneous dual color super-resolution imaging efforts. However, new labeling strategies are continuously emerging with the use of Halo, SNAP, and CLIP-tag protein labeling systems that enable specific, covalent attachment of virtually any molecule to a protein of interest and have already been utilized in PALM,⁴⁹ STED,⁵⁰ and STORM.⁵¹ Unlike conventional fluorescence microscopy, super-resolution microscopy requires microscopists to consider the photochemistry of the probe in addition to the spectral characteristics.

CONCLUSION

The diffraction limit imposed by conventional light microscopy is no longer a limiting barrier to what can be achieved in light microscopy. Molecular structures can be visualized at their correct size, and resolution similar to electron tomography can be obtained with the advantage of very high copy number, molecularly specific labeling. However, there are still challenges for us as we embrace these new technologies. We must still make compromises between acquisition speed and image resolution. Precise molecular localization does not necessarily mean adequate resolution of a structure. Living cells can potentially move molecules faster than we can image them. There is always a need for more probes; the current color pallet is limited, and may limit the pace at which super-resolution is adopted in biology. In addition, it will be critical that we strive to put super-resolution images into context; light or electron microscopy can provide an important background for interpreting the new level of ultrastructure we visualize. Finally, the continued development of super-resolution microscopy holds great promise for biology. Super-resolution provides creative scientists with a set of tools for building new levels of understanding about the molecular mechanisms of nanosensing.

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Figure 1.

Super-resolution microscopy relies on the ability to control the light/dark states of fluorescent molecules. If a fluorescent probe is used that has two distinct states that can be reversibly controlled by photoswitching then the technique is known as RESOLFT¹¹. In one state, the probe is fluorescent while in the other state it is dark because it has either been returned to the ground state [stimulated emission depletion (STED) and saturated structured illumination (SSIM)] or shelved to the meta-stable triplet state [ground state depletion (GSD)]. Stochastic optical reconstruction microscopy (STORM) is a RESOLFT technique; it toggles a bistable synthetic dye molecule back and forth between states multiple times. However, PALM and fPALM are not RESOLFT techniques when they irreversibly convert from one fluorescent state to the other, but they do share with STORM the requirement to control conversion so that the molecular density is low enough to avoid overlapping molecules. The transition from ground state to excitation takes $\sim 10^{-15}$ seconds; fluorescence emission requires on the order of 10^{-12} seconds. Molecules can reside in the triplet state variable amounts of time before they give off their phosphorescence and drop to the ground state in $\sim 10^{-6}$ seconds.¹³

Figure 2.

Super-resolution microscopy techniques use either hardware or software to increase resolution. (a) In stimulated emission depletion (STED) microscopy, the excitation point-spread function (PSF) is combined with the PSF of the STED depletion laser to produce a resultant PSF that is smaller than the diffraction limit of light. (b) In structured illumination microscopy (SIM), the excitation light is patterned with on/off transitions. The high frequency periodicity of the illumination pattern combines with the inherent frequency of the image to permit visualization by the microscope. (c) PALM and STORM techniques turn on subsets of molecules that are separated by distances greater than the diffraction limit of light. Mathematical localization of the PSFs is repeated over many frames that are combined into super-resolution images.

Figure 3.

Microtubules provide a molecular yardstick for evaluating 3D super-resolution techniques. Undecorated microtubules have been shown by electron microscopy to have a diameter of ~22–25 nm and antibody decorated microtubules have a diameter of ~55 nm.³³ (a) 3D STED shows microtubules at 100 nm in diameter. (Reprinted with permission from Ref ³⁴. Copyright 2008 Opt Express) (b) 3D STORM shows 66 nm diameter microtubules. (Reprinted with permission from Ref ²⁵. Copyright 2008 Science) (c) iPALM measures microtubules as 25–30 nm in diameter axially, which is the diameter of the undecorated microtubule plus the 2–3 nm of the PAFP. (Reprinted with permission from Ref ²¹. Copyright 2009 PNAS)

Figure 4.

Live-cell PALM demonstrates the molecular evolution of molecules within structural and signaling scaffolds. (a) Boxed regions are shown in higher magnification in (b) and (c). (b) Molecules are added to trailing edge of a large adhesion scaffold over the course of time. (c) Small scaffolds located in the central region of a cell show little size variation. (Reprinted with permission from Ref ⁵. Copyright 2008 Nat Methods)

Figure 5.

Molecular organization reveals that proteins that colocalize at the diffraction limit have a distinct molecular organization. (a) DIC image indicating where adhesion complexes are localized on the cell surface. (b–d) Higher magnification views of super-resolution images indicate that paxillin and vinculin are not colocalized in adhesion molecules. (Reprinted with permission from Ref ⁴⁴. Copyright 2007 PNAS)