Super-resolution microscopy at a glance

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Advances in microscopy and cell biology are intimately intertwined, with new visualization possibilities often leading to dramatic leaps in our understanding of how cells function. The recent unprecedented technical innovation of super-resolution microscopy has changed the limits of optical resolution from ~250 nm to ~10 nm. Biologists are no longer limited to inferring molecular interactions from the visualization of ensemble perturbations. It is now possible to visualize the individual molecules as they dynamically interact. Super-resolution microscopy offers exciting opportunities for biologists to ask entirely new levels of questions regarding the inner workings of the cell.

The impact of super-resolution microscopy is rapidly expanding as commercial super-resolution microscopes become available. However, super-resolution microscopes are not based on a single technology, and the differences between the individual technologies can influence how suited each approach is to address a specific cell biological question. Here, we highlight the main technologies and demonstrate how the differences between them can affect biological measurements.

Defining super resolution

The resolution limit of conventional light microscopy is ~250 nm in the *x* and *y* direction, and >450–700 nm in the *z* direction. This limit, also called the point-spread function (PSF), is the fixed size of the spread of a single point of light that is diffracted through a microscope; it is also a measure of the minimum-size point source or object that can be resolved by a microscope. Objects that are smaller than the PSF appear to be the same size as the PSF in the microscope, and objects that are closer together than the width of the PSF cannot be distinguished as separate. A commonly used measure of the PSF width is the Rayleigh (R) criterion: $R=0.61\lambda/NA$, where NA is the numerical aperture. Any microscopy technique that overcomes the resolution limit of conventional light microscopy by at least a factor of two is considered to be a super-resolution technique.

Super-resolution techniques break the diffraction limit by temporally or spatially modulating the excitation or activation light. For example, structured illumination microscopy
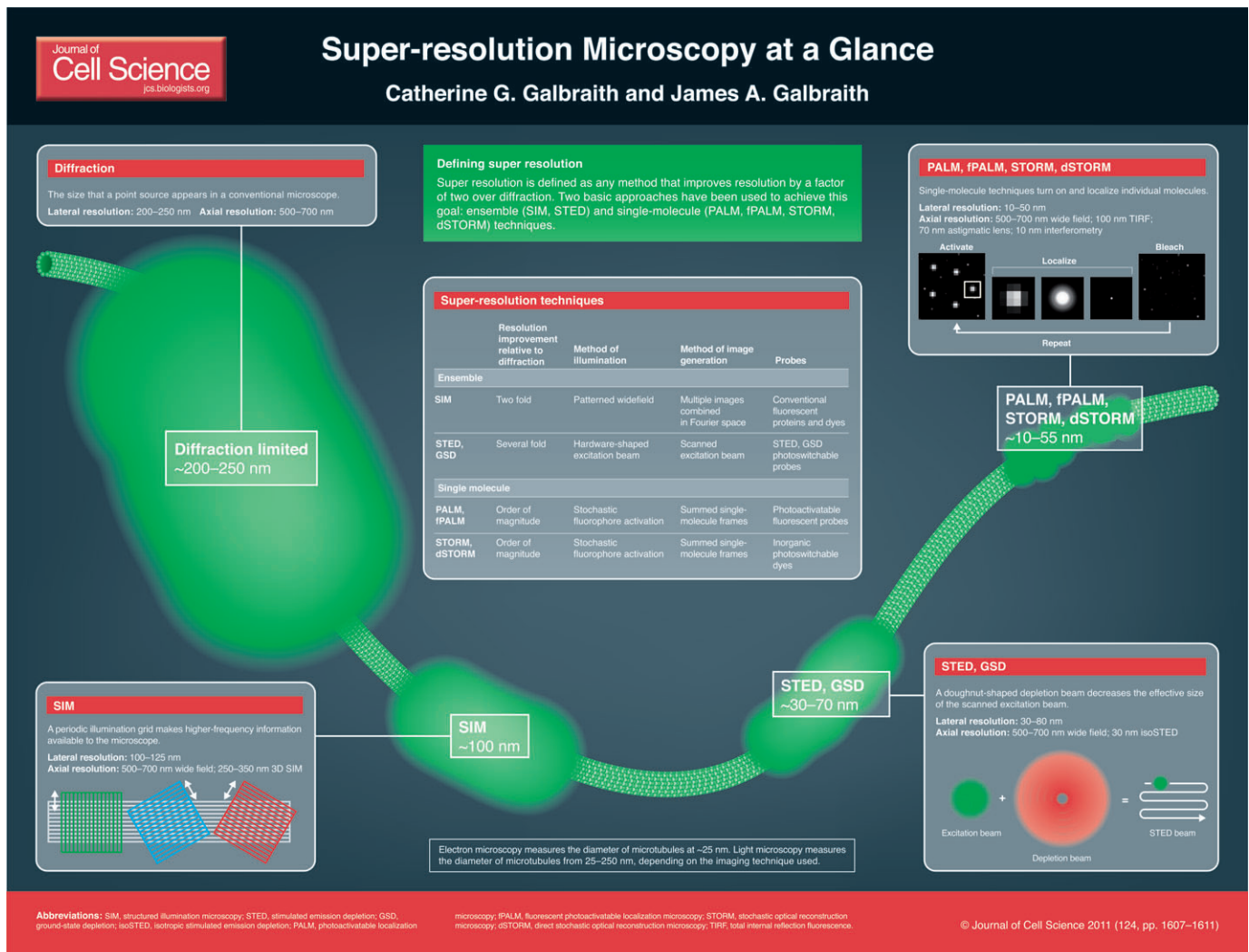
(SIM) illuminates the entire field with a striped pattern of light (Gustafsson, 2000). When this excitation pattern mixes with the spatial pattern of the sample they produce an interference pattern (called a moiré fringe) that is much coarser than either pattern alone and is detectable by the microscope. The excitation pattern is translated and rotated to generate a series of images with different moiré fringes. As the illumination pattern is known, it can be mathematically removed from the moiré to gain access to the normally irresolvable higher resolution information in the sample. SIM increases resolution to ~100 nm in the *x-y* direction and ~400 nm axially (Schermelleh et al., 2008). SIM is limited to this factor-of-two improvement because the periodicity of the illumination pattern is created by diffraction-limited optics and is, therefore, limited by the PSF of conventional microscopy (Gustafsson, 2000).

Other super-resolution imaging techniques modulate the excitation light to exploit the ability to saturate the emission of fluorophores in order to break the diffraction barrier by a greater amount. Saturation can be achieved by using intense illumination to produce a photophysical transition of the fluorophore to a transient dark state that can lead to either a permanently dark state (bleaching) or the emission of light on a microsecond or millisecond time scale, which is much slower than the nanosecond time scale of fluorescence. Alternatively, super-resolution techniques can use light to induce photochemical reactions in photoswitchable or photoactivatable fluorophores, and either transition them between on and off states or change their color. As long as these transitions can be limited to a subset of fluorophores that are spatially separated by the distance of the microscope PSF, the molecules can be located with precision approaching 5 nm. Super-resolution techniques can be separated into two categories depending on whether these effects are exploited at the ensemble level or at the single-molecule level.

Ensemble techniques
Ensemble-based techniques increase resolution by shaping the excitation light, and the resolution of the images obtained by these techniques is determined by the size of the super-resolution PSF. By contrast, single-molecule techniques rely on localization precision, which is the uncertainty in the identification of the center of a molecule's PSF and depends on photon output (Thompson et al., 2002). Importantly, localization precision is not the resolution of the image. Stimulated emission depletion (STED) microscopy and ground-state depletion (GSD) microscopy optically modify

the PSF to reduce its effective diameter. They surround a laser-scanning focal excitation PSF with an annulus of longer-wavelength light that has an intensity high enough to saturate all fluorophores in the annulus to the ground state (STED) (Hell and Wichmann, 1994) or the meta-stable dark state (GSD) (Bretschneider et al., 2007) to suppress their fluorescent emission. Increasing the intensity of light in the annulus expands the zone of saturation to create an increasingly smaller excitation PSF that is smaller in diameter than the diffraction limit of 200 nm. The smaller PSF is then scanned over the sample to generate the enhanced-resolution image. The best two-dimensional (2D) resolution in a biological context for STED or GSD that has been achieved so far is 20–30 nm full width at half maximum (FWHM) across the PSF (Westphal and Hell, 2005). The commercial implementation of STED currently has a resolution of ~70 nm laterally with no increase in axial resolution above diffraction. Although the excitation volume is below the diffraction limit, these techniques only make ensemble measurements because they do not distinguish between individual molecules within an excitation volume.

There are also good prospects for improving the resolution of SIM. The non-linear relationship between excitation and emission can be combined with the illumination pattern used in SIM. This technique – saturated SIM (SSIM) (Gustafsson, 2005) – can be thought of as the inverse of STED, in which sharp dark regions are created instead of sharp bright regions. The resolution of SSIM scales with the level of saturation, and 50 nm lateral resolution has been demonstrated using fluorescent beads (Gustafsson, 2005). The general term for super-resolution techniques that make use of the reversible non-linear switching of the fluorophore state is reversible saturable optical fluorescence transitions (RESOLFT) (Hell, 2003).

Single-molecule techniques
Single-molecule-based techniques overcome the diffraction limit by using light to turn on only a sparse subset of the fluorescent molecules of interest. Even though the visualized molecules that are turned on appear to have the size of a PSF in a conventional microscope, if they are separated from each other by at least 200 nm, then the concept of single-molecule localization can be used to determine their centroids with nanometer-level precision (Gelles et al., 1988; Yildiz et al., 2003). This process is repeated over many cycles, with new molecules turning on and other molecules turning off in a stochastic manner. Super-resolution images are created by assembling all of the localized points. Both pho-

toactivation localization microscopy (PALM) (Betzig et al., 2006) and fluorescent PALM (fPALM) (Hess et al., 2006) use genetically expressed photoactivatable probes to achieve the on–off states of the fluorophore. An alternative approach, stochastic optical reconstruction microscopy (STORM) (Rust et al., 2006), uses pairs of cyanine (Cy) dyes, typically coupled to antibodies up to 15 nm in length, to act as reporter and activator pairs in order to cycle multiple times between the dark and light states. In direct STORM (dSTORM) (Heilemann et al., 2008), several stand-alone synthetic dyes, such as Alexa-Fluor dyes, can also be used in a blinking mode to attain super-resolution images. Despite the technical differences between these techniques, they all localize the position of the molecule of interest to ~20 nm.

The resolution of super resolution
The resolution of any image, conventional or super resolution, depends on the number of points that can be resolved on the structures of interest. According to the Nyquist–Shannon criteria (Shannon, 1949), a structural feature can only be resolved when the distance between two labels is less than half the feature size. Therefore, at least two points need to be resolved within the minimum spatial feature size that is to be imaged. Localizing individual molecules with high precision does not create a super-resolution image when there are not enough labeled molecules within the PSF to identify the spatial and temporal features of the structure. Thus, when a single-molecule-based super-resolution image is reported as having a resolution of 20 nm, the number must refer to structural resolution and not simply indicate that the image is only displaying molecules whose centroids could be identified with ≤20 nm certainty.

Speed of acquisition
Many super-resolution techniques obtain increased resolution at the cost of the speed of image acquisition, simply because they use conventional microscope optics and hardware. It takes longer to scan the smaller PSF of the STED excitation beam across a specimen than it would take to scan the larger PSF of a conventional spot-scanning microscope. The temporal resolution of STED microscopy has been reported as 35 mseconds per image with a 1.8 μm ↔ 2.5 μm field of view, at a 2D resolution of 62 nm (Westphal et al., 2008). However, with a larger field of view, imaging speed slows down dramatically. Acquisition times of 10 seconds per image over a 2.5 μm ↔ 10 μm field of view for a 50 nm resolution have been reported for the very bright fluorescence-

filled dendritic spines in living cells (Nagerl et al., 2008). Similarly, if image acquisition requires moving a grid and collecting as many as 15 images to generate one SIM super-resolution image, then super-resolution images will be obtained at a slower rate than conventional images, i.e. at ~30 seconds per image. However, a recent implementation of SIM using a ferroelectric liquid crystal on a silicon spatial light modulator to produce the patterns has proven to be much faster. Image fields of 32 μm ↔ 32 μm and 8 μm ↔ 8 μm have been successfully imaged at 3.7 Hz and 11 Hz, respectively (Kner et al., 2009). Therefore, it is important to consider whether dynamic biological structures change on a time scale that is slower than the time required to collect an image when choosing a super-resolution method.

The trade-off between speed and resolution is typically more pronounced with single-molecule techniques. Two molecules cannot be turned on within the same PSF at any given time, which limits the speed of the molecular read-out. Although the concept of turning on a sparse subset of labeled molecules has enabled high-speed single-molecule tracking with high molecular density (Hess et al., 2007; Manley et al., 2008), the spatial constraint on molecular proximity has made live-cell super-resolution imaging challenging. However, large fields (28 μm ↔ 28 μm) with a single-molecule localization precision of 20 nm can be obtained every 25–60 seconds, but the images have a spatial resolution of ~60 nm (Shroff et al., 2008). Spatial resolution is normally less than localization precision, not only because of the number of fluorophores localized along a given feature, but also because the structures in living cells translocate, gain molecules and lose molecules during image acquisition. Hence, live-cell single-molecule super-resolution requires that sampling is fast enough – both temporally and spatially – to avoid blurring and to quantify dynamics. It also requires that control experiments that estimate the number of molecules in a structure are used as a guide for parsing the correct number of single-molecule frames into single-molecule super-resolution time series images.

Super-resolution probes

Like all fluorescent techniques, the key to the super-resolution techniques lies in the choice of probes. SIM is the closest to traditional fluorescence microscopy, requiring no special probes but, as multiple intermediate images are collected, photobleaching must be considered. The other super-resolution techniques require fluorescent probes whose state can be controlled. The probes need to be either reversibly or irreversibly switchable between a light and a dark state, or they need to change from one wavelength to another wavelength. The probes should be as bright as possible and should have a high contrast ratio between the two states. Of course, different techniques have different criteria for what makes a good probe.

For STED, dyes need to be easily driven into stimulated emission, have no excitation at the depletion wavelength, and have photostability to withstand high intensities at both the depletion and the excitation wavelengths. ATTO dyes were originally used for STED; however, an extensive list of additional dyes, their resolution, parameters used for depletion and excitation, and references can be found online at the website of the Department of NanoBiophotonics at the Max Planck Institute for Biophysical Chemistry, Göttingen, Germany (http://www.mpibpc.mpg.de/groups/hell/STED_Dyes.html).

All single-molecule super-resolution techniques rely on localization precision, which is approximated as $s/\sqrt{(N)}$, where s is the standard deviation of the PSF, and N is the number of photons detected (Thompson et al., 2002). Therefore, the approximately tenfold higher number of photons detected with an inorganic dye compared with that when using a photoactivatable fluorophore suggest that inorganic dyes provide a far greater localization precision – approaching 5 nm FWHM (Shtengel et al., 2009). However, in biological applications, inorganic dyes are often coupled to antibodies whose size (>10 nm) is two- to threefold larger than the 3–4 nm of genetically expressed fluorescent proteins. The length of the antibody adds uncertainty to the location of the centroid and decreases the higher precision that is inherent to inorganic dyes. 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 used in PALM (Lee et al., 2010), STED (Hein et al., 2010) and STORM (Dellagiacoma et al., 2010). These new technologies promise the possibility of continually increasing localization precision.

As super resolution frequently relies on photophysics, the task of selecting multiple labels requires more consideration than simply choosing spectral separation. For example, multi-color SIM is best achieved with two dyes that have similar excitation spectra but a large enough shift between their emission maxima to spectrally separate the dyes. Similarly, to avoid using pairs of either excitation or depletion lasers, a pulsed-supercontinuum laser was recently used with two-color STED for both the excitation and the STED beam (Wildanger et al., 2008). Multi-color STORM requires either pairing the same activator Cy dye to one of three spectrally different reporter Cy dyes or pairing spectrally distinct activators to the same reporter. In the first scenario, distinct emission spectra are the multicolor read out and, in the second scenario, distinct activation spectra are used to temporally separate the constant emission (Bates et al., 2007). Finally, multi-color PALM schemes originally imaged multiple colors sequentially, by photoconverting one fluorophore from a lower-wavelength to a higher-wavelength color and then reversibly switching a lower-wavelength fluorophore (Shroff et al., 2007). More recently, two-color imaging has been done by using spectrally separated dark-to-light probes (Subach et al., 2010). However, 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 down simultaneous dual-color super-resolution imaging efforts. The continued development of technologies such as dSTORM, which rely on manipulating the photophysics of dyes used in conventional light microscopy, will increase the number of color choices.

Other dimensions

Super-resolution microscopy began by improving resolution in the x and y dimensions, but biological structures are 3D. Although the simplest way to generate 3D super-resolution images is to combine the serial sectioning of tissue with standard lateral super-resolution techniques (Punge et al., 2008), many super-resolution techniques have now been extended to 3D. In 3D STORM a cylindrical lens is simply added 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, and yields a resolution of 20–30 nm laterally and 50–60 nm axially (Huang et al., 2008). Isotropic STED (isoSTED) is a more complicated hardware implementation of STED that uses opposing objectives to create a hollow sphere of light, which replaces the more-cylindrical doughnut-shaped STED, yielding 30 nm isotropic resolution (Schmidt et al., 2009). To date, the greatest axial improvement has been achieved by using opposing objectives to combine axial interferometry with 2D PALM in a technique termed iPALM (Shtengel et al., 2009). iPALM demonstrates 20 nm lateral resolution and 10 nm axial resolution (Shtengel et al., 2009). Previously, structures of this size could only be accurately visualized by electron microscopy (EM), but now the EM resolution can be combined with the molecular specificity
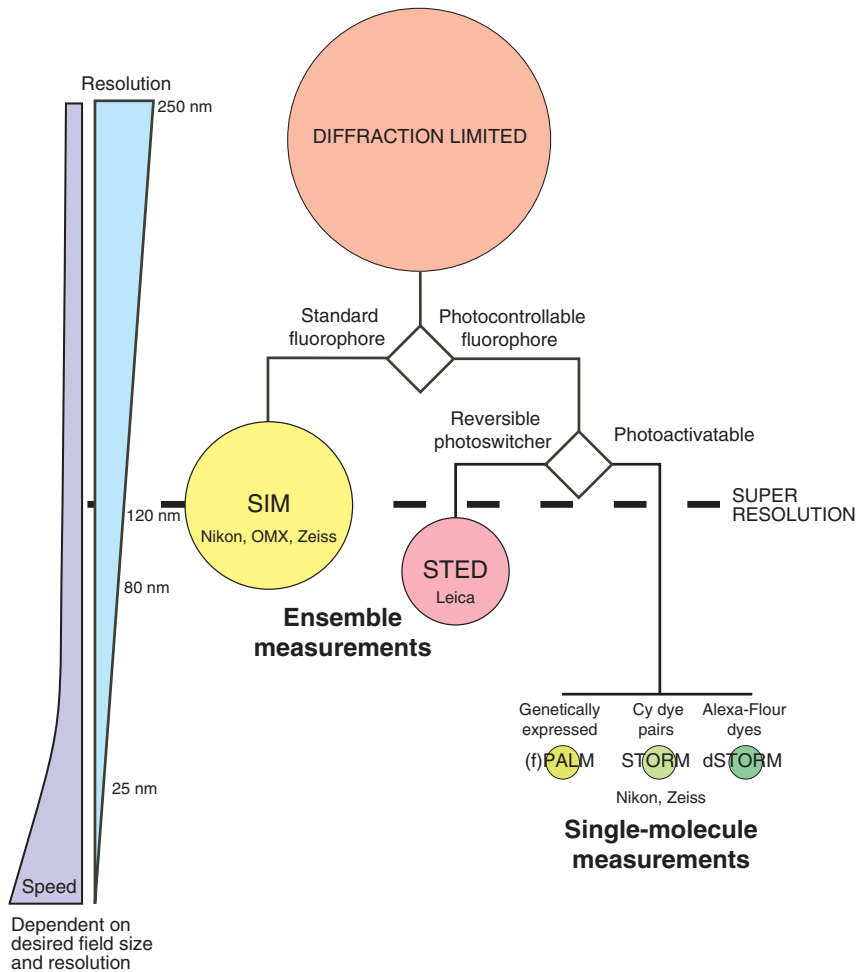
Fig. 1. Pathways to consider when choosing a super-resolution method. Super-resolution microscopy either visualizes ensembles or individual molecules. Within these categories the different technologies require different fluorescent probes. In general there is an inverse relationship between image acquisition speed and image resolution. The key to obtaining a super-resolution image is to understand the trade-offs between probes, speed and resolution. Each of the methods shown is currently available from one or more commercial vendors, and more companies will undoubtedly enter the field.

and high copy number of genetically expressed probes to truly visualize molecules within cells.

Although these techniques are currently limited in the distance from the cover glass that they can penetrate into the specimen, there have been several attempts to image deeper into thicker specimens using two-photon excitation in combination with PALM (Vaziri et al., 2008; York et al., 2011) and STED (Ding et al., 2009). As biological structures are dynamic ensembles, often located deep within tissues, the next challenges in 3D super-resolution microscopy are to adapt the ideas and technologies in order to penetrate further and maintain lateral and axial resolution when confronted with the light-scattering and absorption constraints imposed by animal tissue.

Conclusion

The diffraction limit imposed by conventional light microscopy no longer limits what can be achieved with this tool. Dynamic molecular structures can now be visualized at their correct size, and resolution similar to electron tomography can be obtained with a molecular labeling density that satisfies the Nyquist–Shannon criteria. However, there are still challenges to be faced in embracing these new technologies. Image resolution often comes at the price of acquisition speed. Living cells can potentially move molecules faster than super-resolution microscopes can capture images. Localization precision is frequently much smaller than spatial image resolution. Resolution and localization precisions vary between 10 and 100 nm, depending on the super-resolution technology. There is always a need for more probes – the current color pallet is limited, which may restrict the pace at which super resolution is adopted in biology. As super resolution moves into 3D, the hardware for ensemble techniques can become more challenging, and single-molecule techniques now need to localize millions of molecules to have adequate spatial resolution in a single image. Each of these considerations represents an imaging or a biological trade-off that must be evaluated for each experiment (Fig. 1). Finally, it will be crucial to develop ways to put super-resolution images into context: conventional light or electron microscopy can provide an important background for interpreting the new level of ultrastructure that can be visualized (Watanabe et al., 2011). Super-resolution technologies will continue to evolve, and the exciting commercialization of STED, SIM, PALM, STORM and dSTORM microscopy holds great promise for cell biology.

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