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Review

Superresolution imaging for neuroscience

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ABSTRACT

The advent of superresolution fluorescence microscopy beyond the classic diffraction barrier of optical microscopy is poised to transform cell-biological research. A series of proof-of-principle studies have demonstrated its vast potential for a wide range of applications in neuroscience, including nanoscale imaging of neuronal morphology, cellular organelles, protein distributions and protein trafficking.
This review introduces the main incarnations of these new methodologies, including STED, PALM/STORM and SIM, covering basic theoretical and practical aspects concerning their optical principles, technical implementation, scope and limitations. In addition, it highlights several discoveries relating to synapse biology that have been made using these novel approaches to illustrate their appeal for neuroscience research.

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Introduction

Fluorescence microscopy is one of the most powerful and widely used imaging techniques in neuroscience research, owing to the fact that it allows to visualize dynamic processes inside living cells with exquisite sensitivity and specificity.

A series of technological developments played together to facilitate the ascendancy of modern fluorescence microscopy, including laser and detector technology, fluorescent probes and molecular biology. As a result it is becoming increasingly possible to study brain function at the single-cell level under realistic conditions inside intact nervous tissue preparations.

Milestones were the development of confocal microscopy in the 1980s and two-photon microscopy in the 1990s. Subsequently, the green-fluorescent protein (GFP) revolution allowed for labeling of specific proteins and organelles inside living cells, and increases in computing power helped deal with large sets of imaging data.

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More recently, marking a major breakthrough, the classic limit of spatial resolution for fluorescence microscopy, called the *diffraction barrier*, was overcome. The received wisdom was that the spatial resolution of light microscopy is fundamentally limited by the diffraction of light, and that the smallest structures that could be faithfully resolved were on the order of half the wavelength of the light used in the microscope, i.e. typically around 250 nm. This limit has been enshrined as a *de facto* physical law for over a hundred years (Abbe, 1873).

If the diffraction barrier was indeed a hard limit, the study of cell-biological structures and processes occurring on the "mesoscale" of 10–200 nm would essentially remain out of reach for fluorescence microscopy, including macromolecular complexes, many cellular organelles and signaling inside nanodomains.

In contrast to fluorescence microscopy, electron microscopy provides a spatial resolution down to a few nanometers, however, it requires tissue fixation, which is problematic in itself and which impedes understanding dynamic events. In addition, labeling of multiple proteins for electron microscopy is difficult and sampling of cellular volumes (3D reconstruction) is extremely labor-intensive. That is not to say that electron microscopy will now become superfluous, but certainly its future role in neuroscience will be redefined.

The new methods to break this resolution barrier are generally referred to as superresolution microscopy or nanoscopy techniques. This review will go over the basic physical principles and practical implementation of their main incarnations, including stimulated emission depletion microscopy (STED), photo-activated localization microscopy (PALM)/stochastic optical reconstruction microscopy (STORM) and structured illumination microscopy (SIM). In addition, we will discuss their potentials and pitfalls for neuroscience research and highlight several recent applications in neurobiology.

A new wave of imaging

Because of its wave-nature it is in fact impossible to focus light to an infinitesimally small spot. Rather, the smallest spot size that can be achieved by focusing lenses is limited by diffraction, which refers to the phenomenon whereby a wave tends to spread out as it travels through small openings (Born and Wolf, 1999).

However, this does not mean that far-field optical microscopy, i.e. techniques that use focused visible light for image formation, must be limited by diffraction. By exploiting a strong non-linearity between the excitation light and the emitted fluorescence, the superresolution techniques can effectively break the classic diffraction limit (Hell, 2007), without actually getting rid of diffraction.

Thanks to the new techniques, it is now possible to resolve details at the nanoscale (well below 100 nm) in biological specimens without forgoing the inherent benefits of fluorescence microscopy, such as live-cell imaging and bio-molecular labeling specificity.

STED microscopy was the first concrete concept that broke the diffraction limit (Hell and Wichmann, 1994; Klar et al., 2000). Since then, other powerful techniques have been developed for nano-imaging of fluorescent samples, such as PALM (Betzig et al., 2006; Hess et al., 2006) and STORM (Bates et al., 2007), as well as non-linear SIM (Gustafsson, 2005; Heintzmann et al., 2002).

These new superresolution techniques fall into two main categories, those based on single molecule switching and localization (PALM/STORM), and those based on imaging dense ensembles of molecules using patterned illumination (STED/SIM).

Because of differences in design and implementation, the techniques come with specific strengths and weaknesses in terms of temporal resolution, depth penetration, multi-color imaging, instrumentation requirements, practical handling etc. They all have in common that there is in theory no longer a hard resolution limit and it is possible to achieve a spatial resolution as high as a few nanometers under ideal conditions. However, in practice they are limited by signal noise (from drift inherent in samples, particularly in living biological samples, detector noise, etc.) to some tens of nanometers.

STED microscopy

In confocal and two-photon laser scanning microscopy the excitation light is focused by the microscope's objective to a small focal spot that is systematically moved across the specimen in two or three spatial dimensions. Thus, images are reconstructed one pixel at a time by successive spatial sampling of the fluorescence signal. For high-quality imaging the scanning system must be very accurate and the size of the fluorescence spot used for scanning must be small relative to the specimen features to be visualized. Any jitter in the scanning will blur the image and thus degrade spatial resolution.

However, even using a jitter-free scanner and perfectly aligned laser beams, the microscope's objective will not produce an infinitesimally small scanning spot, but rather a blurry intensity distribution, because of diffraction. It is the extent of this blurry spot, called the *point-spread function* (PSF), which defines the spatial resolution of the microscope. It is typically >250 nm wide in the focal plane (i.e. in x and y) for confocal microscopy and even wider for two-photon microscopy (>350 nm) because of the use of longer wavelength light.

The core idea of STED microscopy is to improve the spatial resolution by quenching fluorescence emission on the outer edge of the PSF, so that emission can only occur from a small spot inside, which can be made much smaller than the diffraction limit (Figs. 1A–C). This is achieved by a second laser beam (called the STED beam), which can de-excite fluorescent molecules by stimulated emission at a wavelength that is longer than the fluorescence. By shaping the STED beam like a doughnut in the focal plane, it actively switches off the fluorescence around a circular rim of the PSF and thus only permits fluorescence to occur from the center of the PSF, which coincides with the center of the doughnut (called the null). By saturating the quenching process on the rim of the doughnut, a very steep spatial gradient for molecules that are either 'on' or 'off' is created, which underlies the gain in resolution for STED microscopy. As of now, a spatial resolution of 5.8 nm has been reported using diamond crystals, which is more than two orders of magnitude smaller than the wavelength of light that was used in the experiments (Rittweger et al., 2009).

The classical doughnut-shaped STED PSF does not offer enhanced resolution in the z-axis. However, this can be achieved by shaping the STED beam using another phase mask in a way that delivers STED light above and below the focal plane, squeezing the PSF also along the optical axis (Wildanger et al., 2009).

The maximal speed of STED microscopy is both determined by the imaging hardware and the brightness of the fluorescent sample. Much like confocal and two-photon microscopy there is a trade-off between temporal and spatial resolution, e.g. acquisition speeds up to a few kilohertz can be achieved in line-scan mode, while larger images can take up to several seconds. Using a fast scanning system based on a resonant mirror, STED imaging at video-rate could be performed on small scan areas (Westphal et al., 2008). As a rule of thumb the imaging speed for STED is slightly lower than for confocal or two-photon imaging due to the reduction in signal intensity in the center of the doughnut, which can be compensated by longer pixel dwell-time to collect more photons. Furthermore, the increase in resolution means that the pixel size must be decreased (to satisfy the Nyquist sampling theorem), reducing the field of view or increasing the image acquisition time accordingly. For example, given a five-fold reduction in pixel size, e.g. from 100 nm (confocal case) to 20 nm (for STED), the field of view decreases by a factor of $5\times5=25$.

As STED microscopy uses two separate laser beams, one for fluorescence excitation and another for fluorescence quenching, it is more difficult to incorporate multi-color imaging than for conventional light microscopy. However, several solutions exist for two-color imaging with STED microscopy, relying either on separate lasers for each