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Super-resolution Microscopy Approaches for Live Cell Imaging

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ABSTRACT By delivering optical images with spatial resolutions below the diffraction limit, several super-resolution fluorescence microscopy techniques opened new opportunities to study biological structures with details approaching molecular structure sizes. They have now become methods of choice for imaging proteins and their nanoscale dynamic organizations in live cells. In this mini-review, we describe and compare the main far-field super-resolution approaches that allow studying endogenous or overexpressed proteins in live cells.

INTRODUCTION

The decryption of cell functions and subcellular processes has constantly benefited from advances in microscopy. In particular, the developments of fluorescence microscopy and of numerous fluorescent probes allowing the study of specific biomolecules at work in their native environment were instrumental to the advance of live cell mechanism investigations. The optical resolution of microscopes is limited by the diffraction of light, which commonly sets a limit of ~$\lambda/2$ in far-field microscopy. By delivering optical images with spatial resolutions below the diffraction limit, super-resolution fluorescence microscopy offered new promises to study molecular processes with greater detail than with conventional microscopies (1,2). Most of these methods rely on the control of the number of emitting molecules in specific imaging volumes. This can be achieved by controlling local emitter fluorescent state populations or the labeling densities of fluorescing probes at any given time during the image acquisition process. In this mini-review, we will discuss the key features of super-resolution techniques used for live-cell studies. We schematically divide them into three major groups: those based on highly localized fluorescence emission volumes; those based on structured illumination; and those based on single-molecule localizations. A didactic representation of the three families of super-resolution approaches is presented in Fig. 1.

SUPER-RESOLUTION BASED ON HIGHLY LOCALIZED FLUORESCENCE EMISSION VOLUMES

Stimulated emission depletion (STED) and reversible saturable optical fluorescence transition (RESOLFT)

In a far-field confocal microscope, the effective fluorescence volume can be reduced below the diffraction limit (3) by using saturable optical processes that deexcite emitters formerly excited by a focused laser beam. These processes work to prevent fluorescence emission from specific regions of the excitation beam by driving the molecules in these regions between bright and dark states using a depletion light beam. One elegant and efficient strategy consists of using stimulated emission by a high-intensity (>MW/cm^2), doughnut-shaped laser beam superimposed with the focused excitation laser beam, completely preventing fluorescence emission from emitters in peripheral regions of the excitation beam. This process was coined "stimulated emission depletion" (STED) (3). A doughnut-shaped depletion beam is the simplest design; however, in general, any depletion beam featuring a spatial intensity distribution with one or several intensity zeroes can be used to perform STED images.

To generate a super-resolved image with STED based on local excitation volumes, one must scan the excitation/depletion effective volumes over the sample in a deterministic point-by-point manner or by use of parallelized scanning schemes (4,5). STED was successfully applied in several live samples to study slow morphing and movements of organelles such as reticulum endoplasmic or microtubules (6), subcellular organization in live cells (7), and synaptic structures in live samples (7–9). For live cell studies, one should bear in mind that relatively high laser powers are needed in STED, especially when using continuous wave laser beams (e.g., ~MW/cm^2 (10)). Using pulsed excitation beam together with time-gating detection allowed a ~2–3-fold reduction in laser power (11). In addition, photobleaching is a limiting factor for long-term live sample imaging because each fluorescent molecule undergoes a large number of exciting/de-exciting cycles in the depletion beam.

An approach similar to STED using much lower intensities to deplete emitting molecular levels (~kW/cm^2) (12) is based on reversible photoswitching of marker proteins between a fluorescence-activated and a nonactivated state (13–15), whereby one of the transitions is accomplished by means of a spatial intensity distribution featuring a

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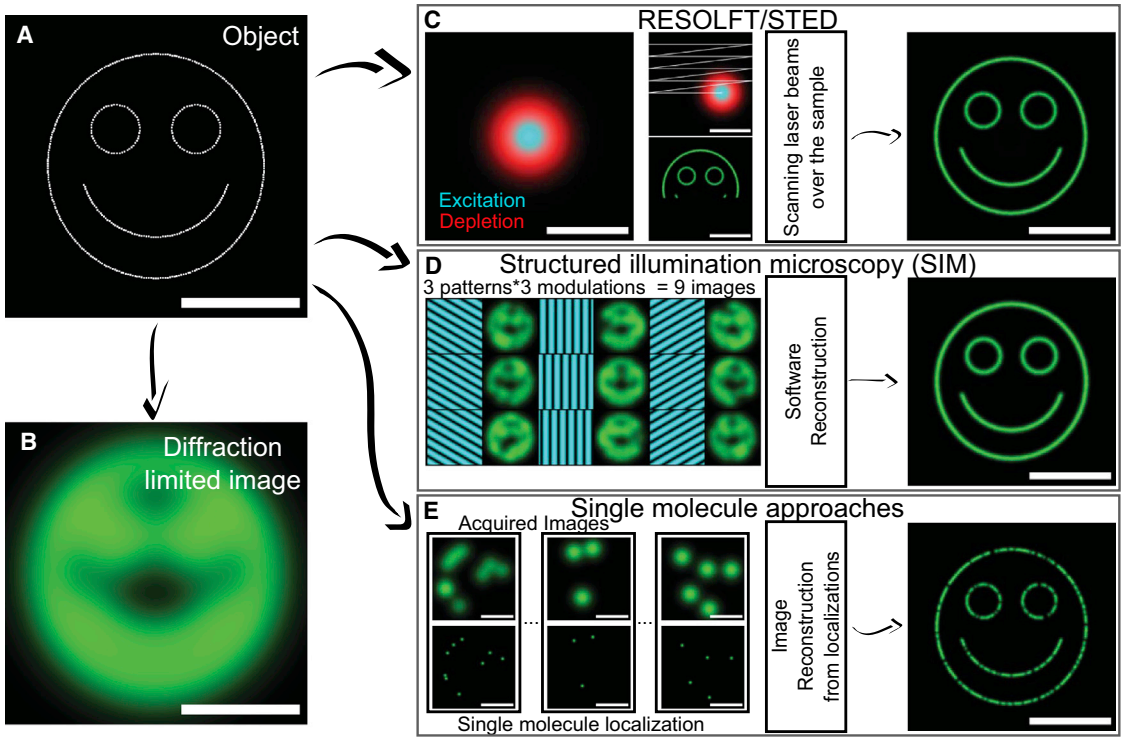

FIGURE 1 Schematic description of the superresolution microscopy approaches. All images for this didactic description are computer-generated. Object to be imaged consisted of fluorescent emitters (*A*) and corresponding diffraction-limited image (*B*). (*C*) In RESOLFT/STED, a focused excitation beam (*cyan*) superimposed with a doughnut-shaped depletion beam (*red*) are scanned over the sample to acquire an image at high resolution (down to ~50–80 nm in live cells). (*D*) In SIM, after the required software reconstruction, multiple wide-field images are acquired using sinusoidal illumination grid patterns to obtain high-resolution images (down to ~50–100 nm in live cells using nonlinear saturated illumination). (*E*) In single-molecule localization microscopy, a large number of wide-field images containing a few isolated single fluorescent emitters are successively acquired. A high-resolution image is reconstructed from the localizations of each individual molecule. Resolutions down to ~50 nm are commonly achieved in live cells. In the example provided, we considered the detection of 80% of the molecules present in the object image. Scale bar represents 1 μm. To see this figure in color, go online.

zero. This generalized approach was named after "reversible saturable optical fluorescence transition" (RESOLFT). Bright photostable switchable fluorophores and fluorescent proteins development were particularly instrumental in the development of these techniques (14–16). Importantly, fluorescent proteins provide specific 1:1 protein labeling and offer the possibility of intracellular live cell imaging.

STRUCTURED ILLUMINATION MICROSCOPY (SIM)

Structured illumination microscopy (SIM) is based on standard wide-field microscopy and is compatible with most standard fluorophores and labeling protocols. It uses nonuniform illuminations with known spatial patterns (e.g., originally a sinusoidal grid, but other illumination distributions can also be used (17)). From multiple acquisitions (e.g., nine images, incorporating three phase shifts for three pattern orientations (18)), high spatial frequency information is retrieved with a dedicated algorithm, comprising a method inaccessible with standard illumination schemes (19). Contrary to standard laser scanning modalities like STED/RESOLFT, SIM allows acquisition of a large field of view over limited times. However, SIM routinely provides only an approximately twofold resolution enhancement of standard wide-field microscopy as compared to other super-resolution methods (19). Nonlinear saturated SIM using fluorophore saturation or photoswitchable proteins as in RESOLFT can achieve higher resolution enhancement (~50 nm), but requires an increased number of image acquisitions (up to 63) and a complex reconstruction process (20,21). SIM has been demonstrated for long-term, live cell imaging in microtubules and other dynamic structures (21–23). Three-dimensional SIM imaging has been further achieved using 15 different pattern acquisitions per axial planes for reconstruction instead of nine images to reject the out-of-focus light (24). Whole-cell volume imaging has been performed using three-dimensional SIM in two colors (25). And, interestingly, fast SIM imaging (11 Hz) has even been developed with a 100-nm resolution for a small field of view (~8 $\times$ 8 μm^2) (18).

SINGLE-MOLECULE LOCALIZATION MICROSCOPY APPROACHES

It is well known that the position of isolated single fluorescent emitters can be determined by image analysis with