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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 nano-scale 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.

Keywords (up to 6): super-resolution microscopy, nanoscopy, fluorescence microscopy, live-cell imaging, single molecule detection, localization microscopy.

Running title: Live cell super-resolution imaging

Introduction

The decryption of cell functions and sub-cellular 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 mechanisms investigations. The optical resolution of microscopes is limited by the diffraction of light which commonly sets a limit of $\sim\lambda/2$ 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 details 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, based on structured illumination and based on single-molecule localizations. A didactic representation of the three families of super-resolution approaches is presented in Figure 1.

Super-resolution based on highly localized fluorescence emission volumes.

In a far-field confocal microscope, the effective fluorescence volume can be reduced below the diffraction limit(3) by using saturable optical processes which de-excite emitters formerly excited by a focused laser beam. These processes consist in preventing 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. An elegant and efficient strategy consists in using stimulated emission by a high intensity ($>MW/cm^2$) doughnut-shaped laser beam super-imposed with the focused excitation laser beam. Doing so, fluorescence emission from emitters in peripheral regions of the excitation beam is completely prevented. This process was coined STimulated Emission Depletion (STED)(3). A doughnut-shape depletion beam is the simplest design but 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 which is based on local excitation volumes, it is thus necessary to scan the excitation/depletion effective volumes over the sample in a deterministic point by point or parallelized scanning schemes(4-5). STED was successfully applied in several live samples to study slow morphing and movements of organelles like reticulum endoplasmic or microtubules(6), sub-cellular organization in live cells(7), 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.* $\sim\text{MW}/\text{cm}^2$ (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 ($\sim\text{kW}/\text{cm}^2$)(12) is based on reversible photoswitching of marker proteins between a fluorescence-activated and a non-activated state(13-15), whereby one of the transitions is accomplished by means of a spatial intensity distribution featuring a zero. This generalized approach was named after the acronym REversible Saturable Optical Fluorescence Transitions (RESOLFT). The development of bright photostable switchable fluorophores and fluorescent proteins 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.

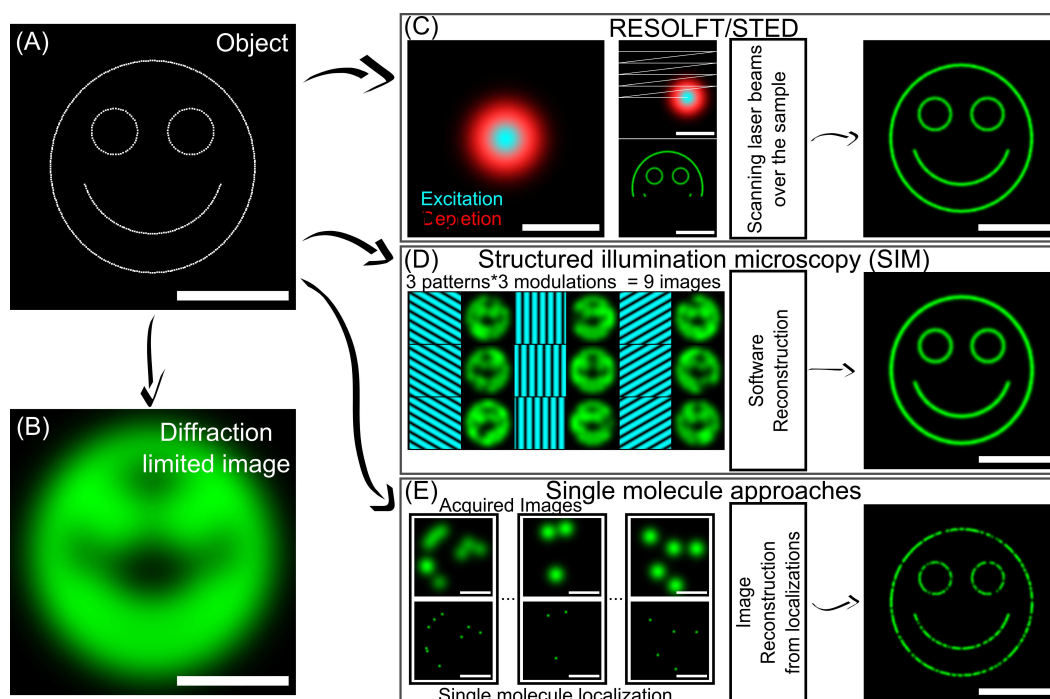


Figure 1: Schematic description of the super-resolution microscopy approaches. All images for this didactic description are computer generated.

Object to be imaged consisting 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, multiple wide-field images are acquired using sinusoidal illumination grid patterns to obtain, after software reconstruction, high-resolution images (down to ~ 50 - 100 nm in live cells using non-linear 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 $1 \mu\text{m}$.

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 non-uniform 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.* 9 images: 3 phase shifts for 3 pattern orientations(18)), high spatial frequency information is retrieved with dedicated algorithm, otherwise inaccessible with standard illumination schemes(19). Contrary to standard laser scanning modalities like STED/RESOLFT, SIM allows acquisition of large field of view in limited times. However, SIM routinely provides only a ~ 2 fold resolution enhancement of standard widefield microscopy as compared to other super-resolution methods(19). Non-linear 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 complex reconstruction process(20-21). SIM was demonstrated for long-term live-cell imaging such as 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 9 images to reject the out-of-focus light(24). Additionally, whole cell volume imaging was achieved using 3D SIM in two colors(25). Interestingly, fast SIM imaging (11 Hz) was also developed with a 100 nm resolution for small field of view ($\sim 8 \times 8 \mu\text{m}^2$)(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 greater precision than the diffraction limit. This feature that has been used for more than twenty years in live cell single particle/molecule studies(26), is key to provide nowadays super-resolved images. Super-resolution methods based on single-molecule localizations simply consist in reconstructing an image from single molecules localizations retrieved from a large number of movie frames (typically thousands of camera frames). The main requirement is that each frame contains the detection of well spatially separated fluorescent emitters(27). Because, a large amount of single molecule detections has to be registered in order to reconstruct a high content super-resolved image, this acquisition process is inherently slow (typically more than a few seconds). We describe below three families of approaches considering

how fluorescent molecules are stochastically isolated from non-fluorescent ones in each camera frame.

Photo-activation localization microscopy (PALM). The development of Photo-Activation Localization Microscopy (PALM)(28-29) is closely linked to the advent of photo-activable proteins(16) which allow to control by light the density of fluorescing proteins in each image. Because PALM takes advantage of the versatility and specificity of genetically encoded fluorescently tagged molecules in cells, it has quickly become the tool of choice for super-resolution live cell imaging. It is however restricted to expression systems which prevents studying endogenous proteins in their native environment. Interestingly, PALM is by design not restricted to biomolecules present at the cell plasma membrane and allows the study of intracellular biomolecules. In addition, by tracking the movement of each individual protein, PALM allows measuring local diffusion properties in living cells on short time scales(30-32) and cellular structural changes in 3D on longer time scales(33).

Stochastic optical reconstruction microscopy (STORM) and Ground state depletion microscopy followed by individual molecular return (GSDIM). STORM(34) and direct STORM (dSTORM)(35) use switchable organic fluorophores placed in specific buffers (*e.g.* with reducing properties) instead of using fluorescent proteins as in PALM. Those probes can be targeted on genetically encoded or endogenous proteins using adequate ligands. STORM was first demonstrated using Cy3-Cy5 pairs(34) but was quickly extended in dSTORM to other synthetic fluorophores that can be stochastically and reversibly switched in the imaging buffers(36-37). STORM is particularly powerful for fixed cells applications(38-40) and can be extended in 3D(38). Yet, live cell experiments should be performed with great care due to possible issues with the use of reducing/oxidizing buffers that can affect cell integrity(41). Interestingly, it was shown that STORM can take advantage of some reducing thiol glutathione, naturally present at millimolar concentrations in bacteria(42) or in specific cell compartments like the nuclei of eukaryote cells using buffers with low cellular toxicity(43-44). Organelles from live cells, like the membrane and mitochondria, were also investigated using multicolor STORM(45-47). Analogously to dSTORM, where stochastic photo-switching is used to control the number of emitting fluorophores, GSDIM is another acronym that covers the techniques

employing the transition between the fluorescent singlet state and a metastable triplet state as the stochastic on-off switch (48 , 49). More precisely, efficient transition to the long-lived triplet state is achieved in such techniques by using high excitation intensities and similar imaging buffer as in STORM to allow obtaining triplet lifetime long enough to leave only a few emitting fluorophore at any time in each image. Under these conditions, GSDIM has been employed for imaging living cells using both fluorescent protein tags (as in PALM) or various organic fluorophores (as in STORM) that selectively bind to tagged proteins(50-51).

Universal point accumulation imaging in the nanoscale topography (uPAINT). In contrast with PALM, STORM and GSDIM that are based on the emitters stochastic photoswitching, the Universal Point Accumulation Imaging in the Nanoscale Topography (uPAINT)(52-55) method captures real-time molecular interactions to control the density of fluorescent emitters suitable for single molecule identification in each image. In the uPAINT approach, target molecules are individually imaged when a specific ligand coupled to a fluorescent dye binds to the target molecule. Indeed, unbound ligands are freely diffusing in the imaging buffer (with typical diffusion constants of the order of tens of $\mu\text{m}^2/\text{s}$) and are not efficiently excited due to an oblique illumination excitation. The unbound ligands are, thus, not detected efficiently by the detector operating at typically video rate on the contrary to bound ligands, which diffuse together with a membrane receptor (with typical diffusion constants of less than $1 \mu\text{m}^2/\text{s}$) in the oblique laser illumination. With uPAINT, any binding entity conjugated to fluorophores having high specificity toward a target molecule (*e.g.* natural/synthetic ligand, antibody) can be used as fluorescent probes to reveal the targeted molecules. Applications included receptors and GPI-anchored proteins diffusing on live cell membranes labeled with antibodies or synthetic ligands. More interesting, it was also applied to image and track endogenous receptors at high densities like glutamate receptors in neurons(54) and epidermal growth factor receptors (EGFRs) in culture living cells(56). Comparison between transfected non-endogenous receptors and endogenous glutamate receptors was also performed(57). A similar approach allowed tracking and imaging by continuously labeling sodium ion channels in live cells, using small fluorescently labeled molecule agents that display reversible binding to sodium ion channel(58). Interestingly, combining single molecule FRET and dual-color uPAINT allowed the specific super-resolution imaging and tracking of interacting receptors activated by their cognate ligand in live cells(56).

This feature stems from the fact that in this uPAINT study, fluorescently tagged ligands are directly used as imaging probes, allowing to extract and image in real time the population of activated functional receptors upon ligand binding.

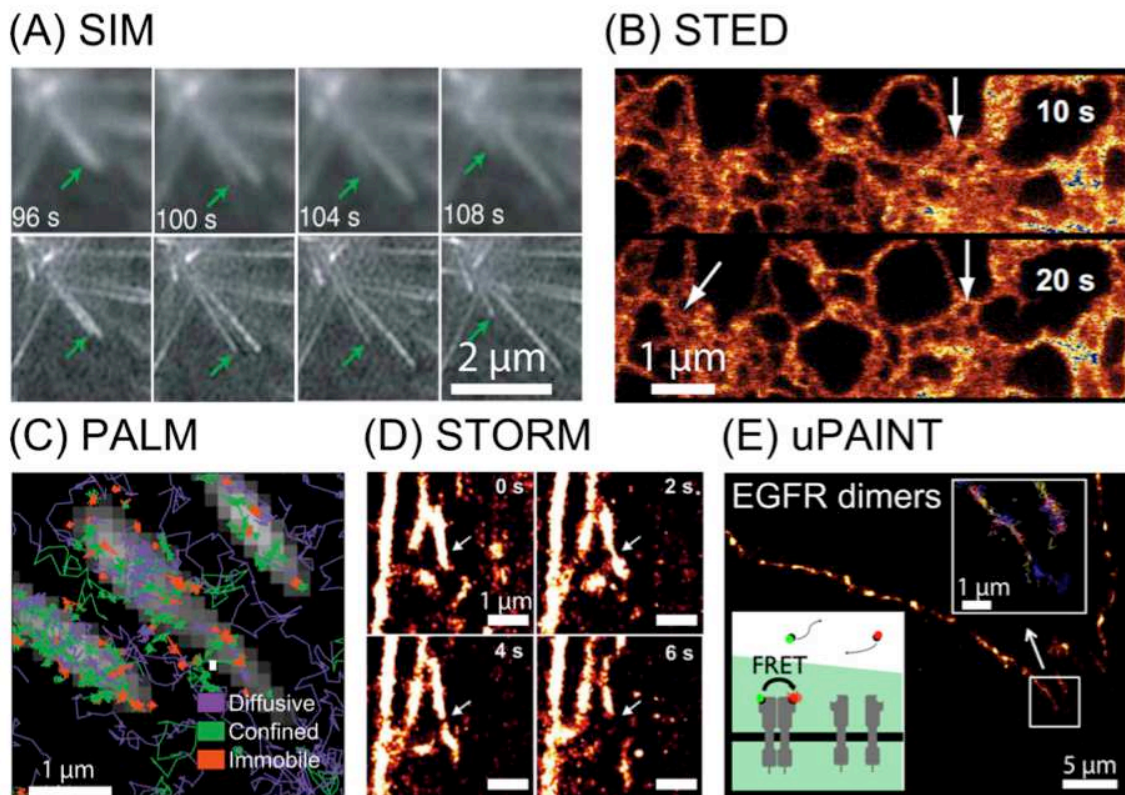


Figure 2: Examples of achievements obtained with super-resolution microscopy in live biological samples.

(A) SIM: Total-internal reflection microscopy image series of eGFP- α -tubulin in a live S2 cell and corresponding SIM images revealing the elongation followed by a rapid shrinking of a microtubule. Integration time of 270 ms per frame. This figure was adapted from(18).

(B) STED: Continuous wave STED images of the yellow fluorescent protein citrine targeted to the endoplasmic reticulum in live cells revealing small tubules (~60 nm). Image sequences show morphing of the endoplasmic reticulum at arrows (pixel size= 20 nm, 10 s recording time per image). Scale bar= 1 μ m. This figure was adapted from(6).

(C) PALM: numerous single trajectories of β 3-integrin fused with mEOS2, obtained on a single MEF cell with PALM, revealing that β 3-integrin undergo slower free-diffusion inside focal adhesions (grey) than outside as well as confined diffusion and immobilization. Figure adapted from(31).

(D) STORM: Spatial dynamics of cortical actin skeleton stained with Lifeact-HaloTag/ATTO655. Each reconstruction was obtained using 1000 frames (2 ms per frame). Scale bar= 1 μ m. This figure was adapted from(47).

(E) uPAINT: Live cell super-resolution imaging of membrane epidermal growth factor receptor (EGFR) dimers based on single-molecule FRET induced by fluorescent ligand activation. Inset highlights preferential cell edge localization of EGFR dimers. In addition, uPAINT provides numerous single molecule trajectories on a single cell allowing extracting the diffusion properties of the EGFR dimer population from the whole ligand activated EGFR population. This figure was adapted from(56).

Discussion

Dynamics and resolution in live cells. In live cells or organisms, supramolecular structures and organelles morph in relatively short time scales often in reaction to stimuli (seconds to minutes and up to hours). To fully understand cell signaling that induce those processes, it is however important to investigate therein the dynamics of molecular (re)organizations. In particular, in order to grasp the full spectrum of mobility behaviors of biomolecules (up to $1 \mu\text{m}^2/\text{s}$ for membrane receptors) fast video rate acquisition is required (20-100 Hz) on large fields of view. Yet, in all super-resolution methods, breaking the diffraction limit on a given field of view comes at the expense of the acquisition time.

In point scanning RESOLFT/STED methods, a compromise between imaging large fields of view and fast acquisition speed has to be made because of the requirement for a dense pixilation. Imaging rates in RESOLFT are rather slow because pixel integration times are limited by the protein photoswitching processes. Being based on stimulated emission, STED is not subject to this fundamental limit. However, increased resolution being achieved with high laser powers, care should be taken to ensure live cell integrity. RESOLFT/STED are able to resolve the movements of the slow structures (typ. 10-30 seconds for 512x512 pixels) like for instance, microtubule networks organizations(22, 59) and neuron morphology dynamics(60-61), both in cell cultures and live animals. Interestingly, by acquiring small fields of views, the dynamics of nanoscale structures can be monitored with higher imaging rates (28 Hz for 60x82 pixels) (62). Recently, large parallelization of the depletion patterns combined with the use matrix detectors drastically increased imaging speed over large field of views in RESOLFT(59) and STED(5).

In its standard form, SIM uses 9 widefield fluorescent images to build a super-resolved image in typically one second. Many applications of SIM were described for imaging subcellular structures in living cells(18, 23, 25). With saturated SIM (the structured illumination approach giving better spatial resolution), additional illumination patterns are needed for the

reconstruction(21) leading to degraded time resolution. Importantly, in SIM techniques any aberration, sample movement or fluorophore photobleaching during the image sequence will induce artifacts that will strongly affect the quality of reconstructed super-resolved images(18).

In single-molecule localization approaches two time-scales are relevant: (i) the raw-images acquisition rate which sets the individual molecules tracking time resolution (1-10's of ms). This rate also sets the single molecule pointing accuracy through its impact on the signal to noise ratio of each molecular detection(63-64). The analysis of single-molecule trajectories provides local mobility maps on live cell regions with high spatiotemporal resolutions(30-32, 54, 57). (ii) the second time scale is set by the total number of images needed to reconstruct a super-resolved image. Indeed, in addition to the pointing accuracy, the local density of single molecule detections obtained from a studied structure also plays a central role in the final spatial resolution (as enounced by the Nyquist theorem). For instance, in order to obtain images with 10 nm resolution, local densities of at least 10,000 detection/ μm^2 are needed. Hence, thousands of image frames are commonly acquired justifying global recording times of the order of seconds to minutes. This time scale directly defines the time resolution at which nanoscale organization of molecular assemblies (e.g. cellular organelles) can be analyzed.

Computer analysis requirements. Conversely to RESOLFT/STED methods, that do not require any post-acquisition analysis, the main source of complexity of SIM is its sophisticated algorithms required for image reconstruction. Similarly for single-molecule localization based techniques, the position of the emitting single molecules has to be retrieved using cutting-edge software as described in a recent comparative study(65).

Labeling strategies and consequences. Expression systems in concert with fluorescent protein engineering provide a method of choice to study with high-specificity sub-cellular organizations in live-cell making of RESOLFT, SIM and PALM essential methods for the applications described in this review. However, despite their wide applicability, one should bear in mind that some signaling and structural artifacts can arise due to the use of fluorescent proteins(66). In this context, SIM, uPAINT, STED and to some extent STORM/GSDIM, using fluorophores conjugated to specific ligands should be considered as they are compatible with studying

endogenous receptors in living cells. Interestingly, by synchronizing single molecule detection and ligand-induced receptor activation, uPAINT is to-date the only super-resolution method allowing studying in real-time specifically activated functional receptors and their interactions at the membrane of living cells(56). Noteworthy, protein number quantification can in principle be performed using PALM and uPAINT since in these methods, photobleaching irreversibly turns off fluorophores after their detection. In STORM/GSDIM however, reversible stochastic switching of fluorophores can bias such quantitative analysis because observing the same fluorescent molecules more than once is plausible. Interestingly, recent advances in self labeling proteins such as SNAP, CLIP and Halo tags allow efficient live cell protein labeling including intracellular ones(46, 51, 67) and provide valuable tools to perform multi-color super-resolution imaging that can be in principle applied to STED, STORM or uPAINT.

Studying structures in three dimensions. Several methods were developed to improve axial resolution in fluorescence imaging. The most widely used strategies are based on single-molecule localization and provide axial information (~20-70 nm) of the position without severely altering radial nor time precision. By shaping the detection point-spread function along the axial position, single molecule position can be precisely determined along the optical axis(38, 68-69). Detecting molecules using two objectives(70-71), by moving the sample in the axial direction(72) or by interfering the signals obtained from two objectives(73-74) could also yield similar resolution improvements along the optical axis. Although, the last approach is the most precise (~20 nm), it is also the most complex to implement. Finally, STED/RESOLFT can also be extended in 3D by scanning distinct axial planes independently(9).

Summary Super-resolution approaches described in this mini-review were proven to grasp information on subcellular organization at different time scales using various labeling probes. Table 1 summarizes the main pros/cons of the approaches discussed here and outlines the different spatiotemporal fundamental limits.

	Single-molecule approaches					
	STED	RESOLFT	SIM	PALM	STORM/GSDIM	uPAINT
Resolution (live samples)	50-70 nm	80-100 nm	50***-100 nm	50 nm	50 nm	50 nm
Toxicity	++	+++	+++	+++	+	+++
Endogenous	+++	-	+++	-	+++	+++
Time for single image (a field of 50µmx50µm is considered for comparison)	10-100 s ~0,1s*	>500 s ~3s**	~1 s	> 2 s	> 2 s	> 2s
Intracellular labeling (live)	+	+++	+++	+++	+	-
Implementation complexity	+	+	+	+++	+++	+++
Reconstruction algorithm	N.A.*	N.A.**	+	++	++	++
Dynamics of large molecular structures	+++	+++	+++	++	++	++
Dynamics of single molecule	+	+	+	+++	++	+++
Multicolor imaging	+	++	+++	++	+++	+++

Table 1: Table comparing the super-resolution approaches presented in this review.

* Parallelization of STED nanoscopy using optical lattices was recently achieved with an imaging rate of 12.5 Hz for a 2.9µmx2.9µm image(5). It requires a simple reconstruction algorithm.

** Large parallelization of the depletion patterns drastically imaging speed over larger field of views in RESOLFT (120µmx100µm in ~3 seconds(59)). It requires a simple reconstruction algorithm.

*** Using non-linear saturated SIM allows accuracy enhancement up to 50nm(21).

Conclusion

In the last two decades, super-resolution approaches have provided new insights into sub-cellular organization at nanometer scale resolutions. Several of these methods have reached a level of maturity which makes them routinely applicable to many biological systems. For live cell imaging, each approach has however its own advantages and drawbacks as described in this mini-review. In this context, combining these techniques can be valuable to tackle specific biological questions taking benefit of each method like for instance, to ensure orthogonal labeling for multicolor imaging(47). Interestingly other recent approaches can also be implemented to increase the image resolution by a factor two to four, by applying software analytical tools to standard experimental imaging data sets. These include deconvolution microscopy(75), Stochastic Optical Fluctuation Imaging (SOFI)(76), Bayesian localization microscopy(77) and compressed sensing(78-80). Furthermore, we foresee that correlating the information gathered by such specific molecular imaging methods with structural information obtained by electron microscopy will provide new insights on molecular subcellular organization and interaction.

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