Superresolution Microscopy

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Advanced article

Article Contents

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More than a century after Ernst Abbe defined the optical diffraction limit and thus seemed to rule out the accurate visualisation of subcellular details below its threshold forever, several approaches now achieve resolutions significantly below it. Structured illumination microscopy, stimulated emission depletion microscopy and single-molecule localisation-based methods use different strategies to separate and visualise the cellular and molecular details that until now were lost in the blur of diffraction. This opens up new possibilities in the observation of biological processes at the smallest scales. The new field is only developing and still poses exciting challenges that require new instrumentation and new labeling strategies.

Introduction

More than 140 years ago, the German microscopy pioneer Ernst Abbe defined an optical formula so simple and powerful that it had to be considered as absolute:

$$d = \frac{\lambda}{2\mathrm{NA}}$$

The minimal resolvable distance (d) between two objects is defined by the wavelength of the light (λ) used for observation and two times the numerical aperture (NA) of the observation system.

Even though the absolute values for the minimal distance may vary according to the contrast that is considered necessary in between two overlapping diffraction patterns (Lauterbach, 2012), all applicable definitions (Abbe's limit, the Rayleigh Criterion and the Sparrow limit) describe a minimal distance beyond which two objects cannot be perceived as separated that is defined by the observation light and the observation system. They thus set a limit for resolvable structures in light microscopy around approximately 200 nm, based on the wavelengths of the visible light spectrum (400–700 nm) and the finite angles provided by microscope objectives for light collection. This limit was optically reached already at the turn of the twentieth century. The improvements provided by immunofluorescence, fluorescent proteins, confocal microscopy and other forms of advanced light microscopy in the past decades provided specificity, speed and multidimensionality and with this spectacular new forms of imaging, but they did not further increase resolution. They were, however, the building blocks with which in the past few years a range of methods were created that overcame the optical resolution limit and allowed to visualise structures up to 10 times below that limit.

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Superresolution Light Microscopy

Several recent methods have established a new form of light microscopy called superresolution microscopy and can be distinguished into two categories:

1. Methods that shift the resolution beyond Abbe's diffraction limit but are still directly linked to it. This is mainly represented by structured illumination microscopy (Gustafsson, 2000) which doubles the resolution relative to the diffraction limit but can be considered to also include opposing illumination interference-based methods such as 4Pi (Hell *et al*., 1994) and I5M (Gustafsson *et al*., 1995) microscopy that significantly increase axial resolution.
2. Methods that move resolution beyond the diffraction limit and are not anymore linked to it. These are not purely based on optics but require the complicity of the sample to uncouple the image resolution from the optical resolution of the imaging system. This seems to be the definition adopted by the Swedish Academy of Sciences when it awarded the 2014 Nobel Prize for Chemistry for superresolution microscopy, which implies a fundamental role of the (fluorescence) label

and did not include the resolution improvement provided by structured illumination microscopy. These methods are stimulated emission depletion (STED) microscopy (Klar *et al*., 2000) and the localisation-based microscopy methods described further in the following paragraphs.

Whether still linked to the diffraction limit or not, none of the currently described superresolution methods violate the optical principles defined by Abbe (1873). They have, however, extended resolution by further sharpening the local contrast of structures (in the optics based methods of category I) or found ways of circumventing them by tapping into label-based image modalities aside from resolution to generate spatial information (in the fluorescence label-based methods of category II).

Major progress in widefield-based superresolution microscopy was only made in the past 25 years and in aspects of applicability mainly in the past decade.

Theoretical solutions for approaches that were not linked anymore to the diffraction limit (category II) were initially proposed in the middle of the 1990s for STED (Hell and Wichmann, 1994) and localisation microscopy (Betzig, 1995) but could only be executed years after (Klar *et al*., 2000; Betzig *et al*., 2006) owing to the technical challenges that needed to be overcome first. All currently described methods for superresolution imaging share the feature that they can only be executed with light sources, detectors, labels and computing at performance levels that have only been reached in the past years. This explains their recent implementation and the long rule of diffraction-limited imaging as defined by Abbe.

In the three main forms of currently used superresolution microscopy (Category I: structured illumination microscopy, Category II: STED microscopy and localisation microscopy), the 'on' and 'off' states of fluorescence that provide the local contrast between structures are created in three different manners which lie at the heart of their operation. **See also**: **Far-field Light Microscopy**

Structured Illumination Microscopy

This method (Gustafsson, 2000; Schermelleh *et al*., 2008) is based on the spatial variation of illumination of the sample in a sinusoidal pattern (**Figure 1a**). This variation generates alternating bright and dark areas in the sample image, and the interference between the illumination pattern and the sample structures generates Moiré effects that encode information below the diffraction limit while technically remaining inside the available optical transfer function of the microscope. The subdiffraction information can be retrieved by creating an image series in which the sinusoidal pattern is shifted over the image in several orientations. This allows the reconstruction of the subdiffraction structures from the Moiré patterns based on the knowledge of the contributing sinusoidal illumination pattern. In its currently linear implementation (i.e. the excitation and emission intensities are directly proportional), this method allows an improvement of the resolution by a factor of 2 relative to the diffraction limit and thus remains directly linked to it (category I). Nonlinear variants of structured illumination have been described and are being developed and would be independent of the diffraction limit (Heintzmann *et al*., 2002; Gustafsson, 2005). They do, however, only achieve this in conjunction with suitable labels (e.g. highly photostable fluorophores or photoswitchable proteins) which makes them category II methods that require sample complicity.

STED and RESOLFT Microscopy

STED microscopy (**Figure 1b**) makes use of a general property of fluorophores. An excited molecule can be induced to return to the ground state through the interaction with a photon of a wavelength inside the fluorophore's emission spectrum. It will during this process emit a photon at the same wavelength as the one that stimulated the emission. This principle also lies at the heart of a laser (light amplification through stimulated emission of radiation) but serves a different purpose here: It gives control over how a fluorophore will return to the ground state, namely at which wavelength it will emit and when it will do so. Given a high enough amount of photons to induce stimulated emission, this transition to the ground state can be saturated and thereby ensure the (almost) complete depletion of excited fluorophore states in the illuminated area. This spectrally controlled return to the ground state is equivalent to inducing 'off' states even though the fluorophore emits a fluorescence photon at the stimulation wavelength. If the stimulation wavelength is chosen outside the spectral range of the detection channel, the stimulated fluorophore will not be perceived. Stefan Hell showed that partially overlaying an excitation light beam with a depletion beam will reduce the volume from which detectable fluorescence is emitted and thus increase resolution (Hell and Wichmann, 1994; Klar *et al*., 2000). The beam arrangement used in STED is to centre an excitation beam and a suitably chosen depletion beam on the same spot. The introduction of a phase plate in the depletion beam causes through destructive self-interference a central minimum (and thus no light) in the depletion beam's centre that is surrounded by a concentric ring of strong illumination reminiscent of a doughnut. The combination of a focused excitation beam with the phase-shifted depletion beam creates a central excitation volume that is surrounded by a concentric ring of depletion light that will make fluorophores in its area undetectable. This creates a significantly reduced effective detection volume with which a sample can be scanned at resolutions below the diffraction limit. The increase in resolution is set by the intensity of the depletion beam as higher intensities will extend the area of saturated fluorophore depletions by stimulated emission inwards and thereby progressively decrease the effective detection volume. Resolution becomes potentially infinite and is described by the following extension of Abbe's formula:

$$\Delta_{\min} = \frac{\lambda}{2\mathrm{NA}\left(\sqrt{1 + {}^{I_0}/_{I_{\mathrm{sat}}}}\right)}$$

where $\Delta_{\min}$ is the detectable region of fluorescence, I_0 the intensity of the depletion beam and I_{sat} the intensity at which a specific fluorophore is 50% depleted. The square root in the term shows that the resolution increase is nonlinear and that sufficiently high

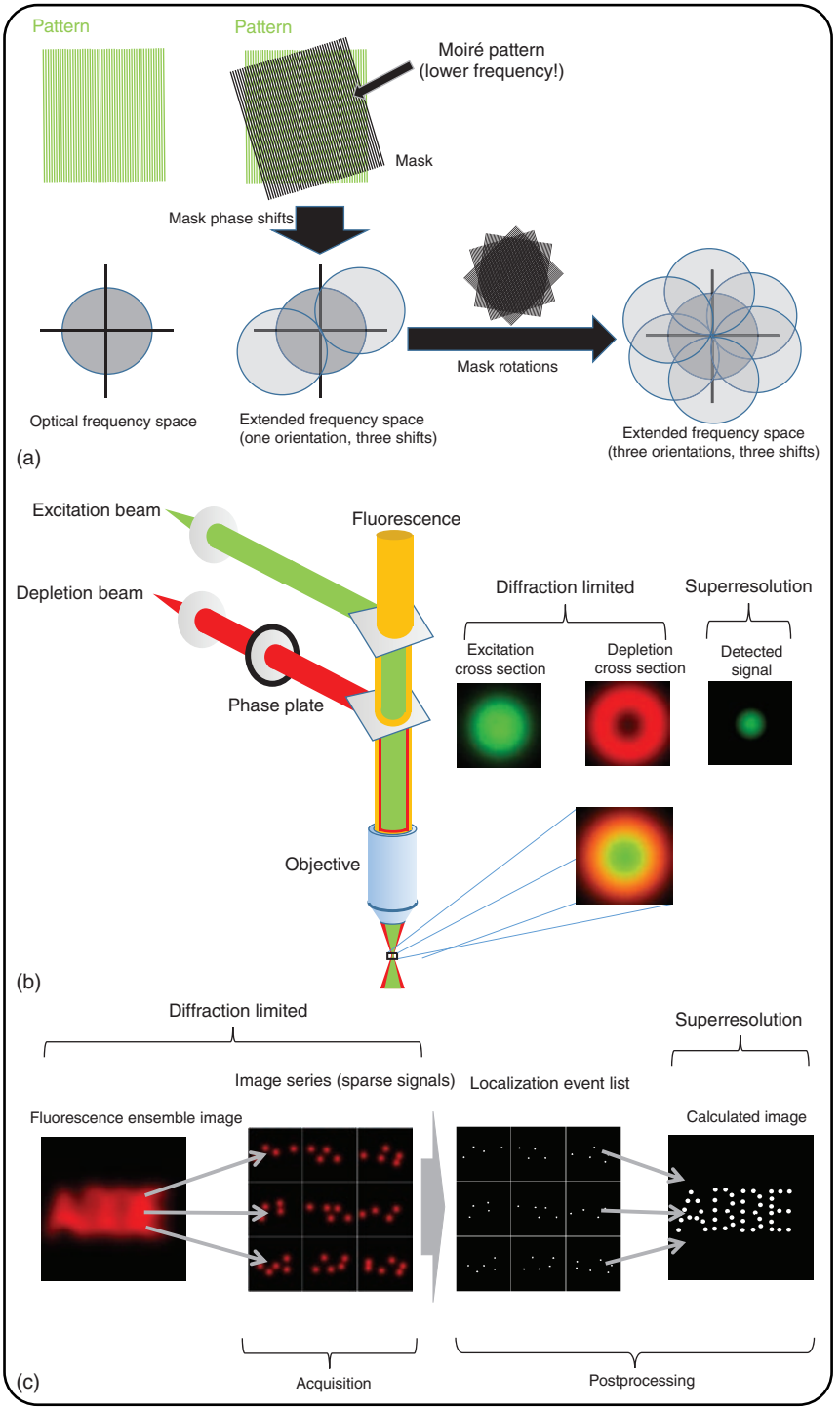

Figure 1 Schematics of the three main forms of superresolution light microscopy. (a) In structured illumination microscopy, a sinusoidal pattern of illumination is projected into the focal plane of the microscope image. The interaction of the illumination pattern with the sample generates Moiré effects that are displayed in lower optical frequencies than the details that they encode. Phase shifting and rotating the illumination pattern generates an image series that can be used to calculate an image with twice the optical resolution by taking into account the known illumination pattern to extract the image components from the Moiré fringes. (b) Lightpath of a stimulated emission depletion (STED) microscope with a fluorescence excitation beam (green) that is overlaid with a depletion beam (red) at a wavelength that can induce stimulated emission of the fluorophore. The depletion lightpath contains a phase plate that generates a central minimum in the focal plane (shown in the depletion cross section). As seen in the cross section of the focal plane, excitation and depletions overlap in a manner that detectable fluorescence (orange) is only generated in the centre of the diffraction-limited beams. (c) For localisation-based microscopy methods, series of sparse single-molecule signals are transformed into a list of highly accurate coordinates for every detected event that can be combined into calculated image based on the coordinates and not on the diffraction patterns of the molecules.

I_0 intensities or suitably low I_{sat} properties will increase resolution independent of the diffraction limit. With STED, structures significantly below the diffraction limit can be resolved, but given the nonlinearity of the resolution increase, there are physical and chemical limitations on both I_0 and I_{sat} that make the theoretically unlimited resolution in practice limited. This is an active field of research that has already seen significant progress in efficient fluorophore depletion (Vicidomini *et al*., 2011) and fluorophore optimisation (Lukinavicius *et al*., 2013) but that is still facing a lot of challenges. STED can be considered as a component of a group of methods described by a more general concept, reversible saturable optical fluorescence transitions (RESOLFTs, Hell *et al*., 2006) that can be applied to switchable fluorophores or suitable fluorophores under saturated excitation. This concept also comprises possible nonlinear structured illumination microscopy methods mentioned in the previous section (Heintzmann *et al*., 2002; Gustafsson, 2005) and, through the concept of optical shelving of molecules away from the fluorescent ground state (Ground State Depletion, GSD, Hell and Kroug, 1995) also is related to the localisation microscopy methods described in the following section (Hell, 2007). In conjunction with reversibly photoswitchable fluorophores (Grotjohann *et al*., 2011), RESOLFT can be implemented at significantly lower illumination intensities than STED (Hell *et al*., 2006) and can be parallelised with multiple detection volumes or structured illumination patterns. However, the requirements on the possible number of switching cycles and the speed of the switching event are high and still need further fluorophore developments.

Localisation-based Superresolution Microscopy

It has long been known that the accuracy with which the position of a subresolution structure can be determined is much higher than the resolving power of the imaging optics used. Fitting a Gaussian function on the central maximum of the diffraction pattern of a single point source can determine its centre with an accuracy that is determined by the brightness of this point source relative to its background (i.e. its contrast) and can be an order of magnitude higher than the resolution. Normally, this cannot be applied in imaging as many diffraction patterns overlap to form an image and single point sources cannot be distinguished from their neighbours. The label has to be very scarce to be detectable. This has already been used for quite some time in single-particle tracking applications, where very diluted labels made it possible to follow single molecules on their trajectories (Barak and Webb, 1982), and speckle microscopy (Waterman-Storer *et al*., 1998) where the incorporation of a sparse subpopulation of labelled molecules into polymers allowed to study the dynamics of cytoskeletal actin and microtubule structures. By their very nature of incomplete labelling, these methods, however, cannot provide a complete picture of the whole structure. High label density and single point localisation by sparse signals are mutually exclusive unless large parts of a label population can be made invisible so that only single members stand out. If it is possible to generate a large series of sparse and randomly distributed visible subpopulations then, given enough repetitions, the sum of all subpopulations would represent the whole label population and thus the structure of interest. For every cycle, the positions of single visible molecules can be determined with high accuracy and for the whole series of several thousand cycles, a position list of every detected single event could be created and reconstituted into an image with a resolution up to 10 times above the optical resolution (**Figure 1c**). When Electron multiplying CCD (EM-CCD) technology made it possible to conveniently record the fluorescence of single molecules at high frame rates, and the postprocessing of image series consisting of tens of thousands of images could be handled by fast computers in reasonable time, recent advances in photoswitchable fluorophores (Patterson and Lippincott-Schwartz, 2002; Habuchi *et al*., 2005) and the description of long-lived reversible dark states of fluorophores (Dickson *et al*., 1997; Heilemann *et al*., 2005) led to the description of localisation-based superresolution microscopy by three separate groups within the same year. Eric Betzig and his colleagues presented photoactivation localisation microscopy (PALM, Betzig *et al*., 2006), Samuel Hess fluorescence photoactivation localisation microscopy (FPALM, Hess *et al*., 2006) and Xiaowei Zhuang stochastic optical reconstruction microscopy (STORM, Rust *et al*., 2006).

The resolution of localisation-based microscopy methods is based on the precision of localisation of single point sources. The localisation error Δ_{min} associated with the fitted position of a point thus determines the image resolution and is defined by the following formula:

$$\Delta_{min} = \frac{\Delta}{\sqrt{N}}$$

which contains the full width half maximum of the Point Spread Function (PSF) (Δ) and the number of photons (N) collected from a single blinking event. As a category II method, localisation microscopy requires compliant labels with inducible dark states, and its resolution is determined (and ultimately limited) by the amount of photons a single blinking event provides inside an image frame. This amount depends on the fluorophore and the buffer composition used with the sample, and optimisation of these factors is an active field of research (Dempsey *et al*., 2011; Olivier *et al*., 2013; Nahidiazar *et al*., 2016).

Localisation-based microscopy was quickly extended to contain also information on the axial (z) position of the detected signals by introducing systematic distortions in the microscope point spread function (Huang *et al*., 2008; Backlund *et al*., 2013) or by the simultaneous acquisition of more than one focal plane (Juette *et al*., 2008).

The following years saw a generalisation of the principle to chemically enhanced light-induced blinking of fluorophores in the concept of direct stochastic optical reconstruction microscopy (dSTORM, Heilemann *et al*., 2008) and ground-state depletion followed by individual molecule return (GSDIM, Folling *et al*., 2008), which was based on initial considerations of Stefan Hell from 1995 (Hell and Kroug, 1995) on (scanning-based) optical shelving of molecules away from the ground state in which they are excitable and visible.

Instead of single-molecule blinking, sparse transient binding events can also be used for the generation of accurate localisation maps. Point accumulation for imaging in nanoscale topography

(PAINT) was already described in the same year as the PALM and STORM techniques based on the transient binding of Nile Red to lipid molecules on a membrane surface (Sharonov and Hochstrasser, 2006). The concept was extended to universal point accumulation for imaging in nanoscale topography (uPAINT, Giannone *et al*., 2010) to provide single-molecule tracks of endogenous molecules at the surface of cells that interact with fluorescently labelled ligands in the sample buffer. In DNA (deoxyribonucleic acid)-PAINT (Jungmann *et al*., 2014), complementary DNA motifs on antibodies transiently bind fluorescently labelled DNA fragments in the sample solution to generate shortlived localised signals on the structures labelled by the antibodies. Here, no blinking needs to be induced by high light intensities in a suitable chemical environment, and the localisations get repeated infinitely as the fluorescence signal continuously gets replenished by newly bound fluorescent markers present in the buffer. Binding/unbinding does, however, take longer than molecule blinking so that the sequence acquisition takes longer and thus requires high sample stability or fiducials in the sample to compensate sample drift.

A Comparison of Strengths and Weaknesses

While all described forms of superresolution light microscopy are able to create images with significantly improved resolution, they have different strengths and shortcomings. These need to be well understood to choose the right approach for a specific biomedical problem or experiment.

No special labels are needed for structured illumination methods. The resolution improvement was early on applied to the lateral (x, y) as well as axial (z) resolution of the sample (Schermelleh *et al*., 2008) and can generate highly resolved data sets of whole cells or other small structures. This for some time set it apart from other solutions for resolution improvement and even though the resolution is only doubled, a thus improved z-resolution may be more informative than a higher lateral resolution without z-improvement that is applied to a complex 3D topology or a densely packed structure such as the nucleus. **See also**: **Chromatin in the Cell Nucleus: Higher-order Organisation**

Drawbacks of the method are the limitation to a doubling of the resolution, the need for recording of the structured illumination pattern in multiple phases, orientations and focal planes to provide the necessary information (especially for a 3D improvement) and the need for data set postprocessing to extract the additional resolution information. Dynamic structures (where the information may change during pattern acquisition) are therefore problematic, as well as deep and scattering samples that degrade the illumination pattern. Structured illumination can be efficiently combined with non-Gaussian optics and light-sheet-based imaging (Gao *et al*., 2012; Chen *et al*., 2014) which overcomes some of the speed and penetration limits and opens a range of new imaging possibilities, albeit on optically complex setups.

Although STED requires a compliant sample, it is the only superresolution method that is all-optical and does not require postprocessing of the data. As soon as the depletion beam is activated, resolution is instantaneously improved in the image. As a scanning-based imaging method, it can, in principle, be applied to any experiment that can be done on a confocal microscope. Owing to the immediate local resolution improvement and the confocal single-beam scanning principle, STED imaging should be applicable to *in vivo* imaging without local movement artefacts and in thick samples. Both are possible but not necessarily straightforward. The amounts of light needed to create the STED depletion are significant and easily affect the sample. Additional interactions of the fluorophore with the intense depletion light can lead to a rapid disappearance of the signal. This is a problem for repeated imaging as needed for timelapse series and the acquisition of 3D stacks in living or fixed samples. Technical developments allow to reduce the amount of depletion illumination needed (Vicidomini *et al*., 2011) and *in vivo* dyes in spectral ranges where the damage to the sample is reduced (Lukinavicius *et al*., 2013) have been described. The resolution improvement by STED is strongly affected by refractive index mismatches between the sample and the immersion objective. In the imaging of living cells in watery medium or deep inside tissues, the focused beams can get distorted and the resolution affected, but using properly matched objectives and lightpaths, STED can be applied deep inside living tissues (Urban *et al*., 2011). Dedicated STED objectives have been developed by the microscope manufacturers that are specifically suited to the refractive indices found in *in vivo* and tissue imaging.

Owing to the high light levels needed for depletion, restrictions on the realistically achievable resolution in the image are often posed by the maximum powers of the lightsources, the ability of the sample to sustain strong illuminations and the behaviour of the fluorescence labels under strong illumination. Under ideal conditions, it can go up to the levels achieved with localisation methods, but for many applications, it is somewhere between that of structured illumination and that of localisation methods. Much progress has been made in optimising STED dyes over the past years. The main requirements are as follows:

- brightness to provide sufficient signals even in reduced detection volumes
- photostability
- overlap with the chosen depletion wavelength at the ‘red’ end of its emission spectrum
- no anti-Stokes excitability by the selected depletion light or susceptibility of excited or dark states to that light

Molecular properties are hard to predict and vary even among spectrally very similar dyes. By now, suitable STED dyes do exist for all wavelength ranges. As resolution can be improved laterally and axially in STED systems, high sampling rates need to be applied to efficiently image a volume with superresolution pixel sizes, especially for 3D stacks. This requires robustness of the chosen fluorophores as mentioned earlier, but often additional compromises in the amount of excitation light used. So, even though STED is an optical effect that generally does not require postprocessing, signal to noise (and through this local contrast and resolution) can be significantly improved by deconvolution postprocessing. As STED PSFs are not Gaussian,

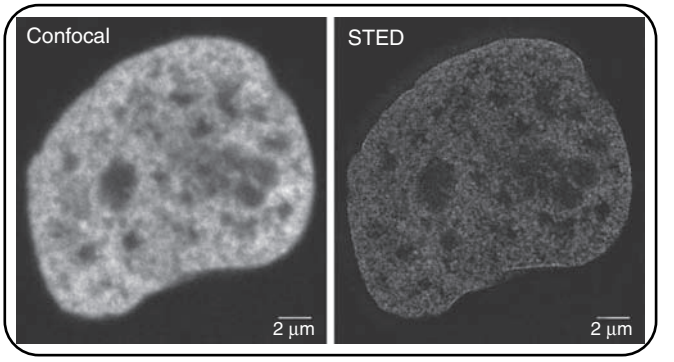

Figure 2 Comparison of a confocal and a deconvolved STED image of Histone2B labelled with a SNAP-tag and Silicon-Rhodamine (SiR)-SNAP in a fixed Hela cell nucleus. The STED images were taken with pulsed 775 nm depletion with a water immersion objective.

specific deconvolution modules are required for this. **Figure 2** shows a comparison of the optical resolution achievable with confocal microscopy and the resolution of the same sample as a postprocessed STED sample. Multichannel imaging in STED can be problematic if more than one depletion wavelength is used. In that case, the channels more to the red end of the spectrum must be imaged first as they will be irrevocably destroyed by the depletion light used for shorter wavelength fluorophores. Alternatively the same depletion laser can be used for two fluorophores, but this requires overcoming the increased overlap between them as they have to be in a more constrained spectral range to efficiently interact with the chosen depletion laser.

Localisation-based methods have major differences to the other two described methods as they are based on single molecules instead of molecule ensembles. They depend on the absence of interfering overlapping signals and detect the very weak signals of single molecules one (or rather several molecules at different image locations) at a time. Background in such measurements is frequently suppressed by imaging with strongly inclined illumination light below or slightly above the critical angle for total internal reflection of the excitation light. This limits the imaging depth, as well as the fact that single-molecule detection deeper in the sample would be affected by aberrations that reduce its quality. Localisation microscopy can, however, be combined with spinning disk microscopy or with lightsheet-based imaging (Zanacchi *et al*., 2011; Chen *et al*., 2014) to overcome some of the limitations. The sparse single signals per acquired image also mean that single frames contain basically no information on the structure of interest which is only revealed when the series is analysed as a collective. For this, series of several thousand images are needed and the acquisition of such amounts of frames takes several minutes as well as generating GB-sized sets of raw data. The raw image data and the final calculated image are very distinct from each other in localisation microscopy, and care must be taken in the transformation of the one into the other. The calculated superresolution images are based on single point fits that are strongly affected by the label quality, label density and the measurement conditions (Dempsey *et al*., 2011; Nahidiazar *et al*., 2016) in ways that may not be readily understood by inexperienced users. Caution is therefore essential as the fitting software may generate images that imply sense and structure (e.g. clusters) where there is none. In addition, different software packages may provide different results for the same data sets (Sage *et al*., 2015).

Localisation microscopy works well for fixed samples (**Figure 3**), but even though there are ongoing efforts to image dynamic structures this poses severe limitations on generating images that are not affected by movement artefacts. The long exposure to intense excitation light during series acquisition is also quite damaging to living cells and some wavelengths that are more permissive than others have been identified (Waldchen *et al*., 2015).

As the produced images are displayed in extremely high resolution, even slight movements such as sample drift affect the result. Laterally, this can be corrected with fiducials or by cross-correlation of recurring localisation patterns throughout the data series, but *z*-drift can be extremely detrimental. Laser-based focus stabilsation, image-based stabilisation (McGorty *et al*., 2013) or specially constructed stages in a temperature-stable environment can overcome this, in conjunction with properly selected sample containers (Nahidiazar *et al*., 2016).

Imaging of multiple fluorophores can be performed using spectrally distinct fluorophores (Nahidiazar *et al*., 2016) or activator-emitter systems for STORM that read out into one channel but are activated at different wavelengths (Bates *et al*., 2007). For spectrally distinct fluorophores, chemical buffer conditions that are equally suitable for all labels must be chosen. For activator–emitter combinations, spontaneous emitters need to be taken into account.

The Labelling Challenge

In fluorescence microscopy, we see the label but not the structure being labelled. This leads to an inevitable slight discrepancy between the position of the structure of interest and the position of its attached label(s). In indirectly immunolabelled samples, the target structure is labelled by fluorophores conjugated to secondary antibodies, several of which are connected to the target epitope by an unlabelled primary antibody that provides the specificity of the labelling. This is not a problem as long as the spatial discrepancy between the positions of structure and label is smaller than the spatial resolution of the imaging

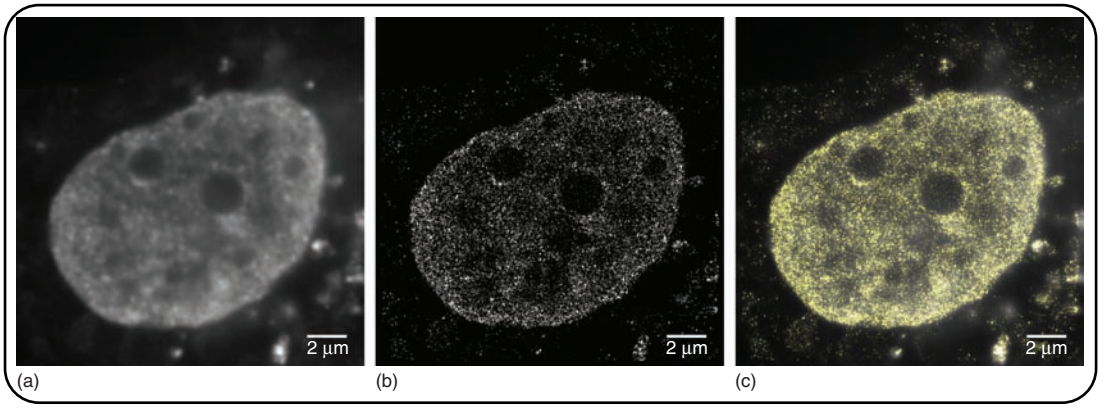

Figure 3 dSTORM/GSDIM images of a fixed nucleus with Histone2B labelled with a SNAP-tag and TMR-STAR-SNAP. Image (a) is the diffraction-limited sum of all fluorescence signals collected during the acquisition of the blinking events and Image (b) is the superresolution image reconstituted from the list of localisations. (c) The superresolution image is overlaid in yellow over the grey diffraction-limited image.

Table 1 Overview of labelling approaches suitable for superresolution microscopy

	Label size	Spectral flexibility	Brightness	*In vivo* compatible
Exogenous				
Indirect antibody labelling	+++	+++	+++	No
Primary antibody labelling	+	++	+	No
Labelled molecules/proteins	+	++	++	Yes
Genetically encoded				
Fluorescent proteins	+	++	+++ − +	Yes
Tags (SNAP, Halo)	+	++	++	Yes*
Nanobodies	+	+++	++	No

*Depending on the conjugated fluorophore.

system. This is the case for classical light microscopy but not for superresolution approaches under optimal conditions. In a regular structure such as microtubules with a known diameter of approximately 25 nm, antibody labelling adds approximately 10 nm in every direction so that the labelled structure is perceived as around 50 nm, and the interior tubular structure is separated by two peaks on both sides that can be measured as approximately 38 nm apart (Zurek-Biesiada *et al*., 2016). The label is thus not anymore representing the true distribution of the structure. Such issues can be alleviated or overcome by some of the label strategies with smaller distances listed in **Table 1**. Among the genetically encoded tags, fluorescent proteins work for many superresolution approaches, but tags for which the bound fluorophore can be exchanged without modifying the genetic construct such as SNAP- (Keppler *et al*., 2003; Lukinavicius *et al*., 2015) and Halo-tags (Peterson and Kwon, 2012) hold great potential for adaptation to new methods and multichannel imaging. Nanobodies that specifically recognise fluorescent proteins (Ries *et al*., 2012) are another way to combine the advantage of the comparably small genetic tags with spectral flexibility.

For STED and localisation microscopy methods, the properties of the fluorophores influence the achievable resolution and the efforts to create ever better labels are ongoing. In addition to the properties of the label, the increased image resolutions that are now possible also require improved feature conservation during sample preparation as fixation artefacts become more visible and unspecific background signals mask the available information and may not be distinguishable from the relevant features.

Conclusion and Outlook

The past years have seen the arrival of methods for superresolution light microscopy that reveals the structures in biological samples in unprecedented detail and bridge the gap to the improved resolution of electron microscopy. Improvements in instrumentation and labelling lead to ever brighter and more accurate labels and decreased light budgets needed for detection. One of the latest outcomes is MINFLUX, the fruitful combination of the stochastic activation of single molecules of localisation microscopy with the RESOLFT-like beam scanning principle of a local minimum beam that increases the achievable resolution into the range of single nanometers (Balzarotti *et al*., 2016).

As important as further technical developments will be that biomedical researchers embrace the potential of these technologies in a responsible manner and apply them to their research.

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Further Reading

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