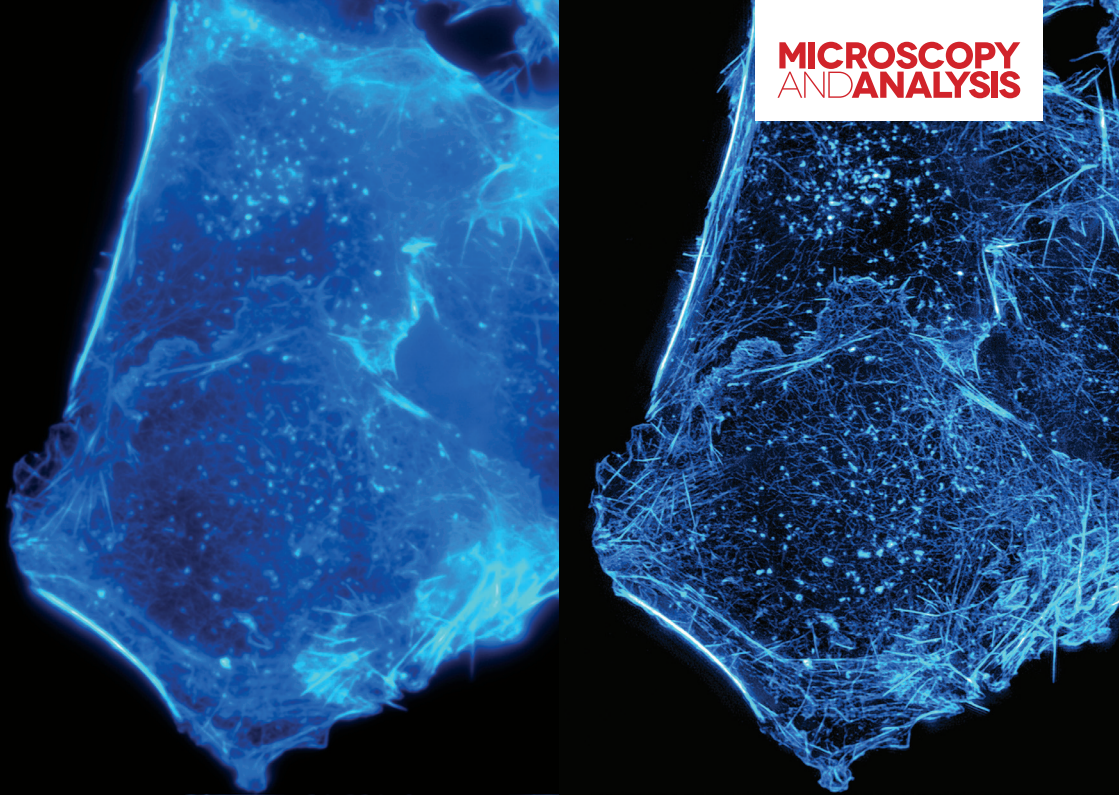

MICROSCOPY AND ANALYSIS

Super-resolution Microscopy

Essential Knowledge Briefings

Third Edition, 2019

ZEISS

Cover Image: Actin labeled with Phalloidin. Comparing the widefield image (left) and Lattice SIM image (right) demonstrates the two fold resolution improvement of structured illumination microscopy. The images were acquired on ZEISS Elyra 7 with Lattice SIM.

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01 INTRODUCTION

Super-resolution microscopy in optical microscopy encompasses techniques that allow acquisition of images with a resolution better than the limit imposed by the diffraction of light – typically between 10 and 150 nm. (Definitions from Wikipedia and other online sources). These techniques are mainly based on, but not restricted to, fluorescence microscopy.

Optical microscopes are the tools of choice when it comes to visualizing the structures and dynamic processes inside cells or within virtually any specimen. They have been essential in life sciences, biology and biomedicine, from the characterization of tissues and microorganisms to drug development, diagnosis and treatment of diseases. The aforementioned limited resolution of these methods – to around 240 nm in the focal plane (xy) and around 600 nm along the optical axis (z) make individual details, such as sub-cellular structures (e.g. membranes, vesicles and organelles), appear blurred out, lacking finer detail. Compare this with, for example, electron microscopy where resolution of few nm is commonplace, however, requiring substantial sample preparation (metal coatings, freeze fracture, etc.) and extreme imaging conditions (high vacuum, bombardment by charged particles, etc.).

Circumventing the resolution limit while keeping the minimally invasive nature of optical microscopy: This is what super-resolution microscopy offers. Observing and characterizing life in its finest structures, down to resolving molecular details. Cellular mechanisms become quantifiable, for example the reorganizing of actin and microtubulin filaments, the firing

at individual synapses in a live neuron. Super-resolution techniques can uncover mechanistic details in biological processes and will therefore play a major role in life sciences.

As of writing, the scientific community is at a point where various super-resolution techniques have become commercially available. The methods have left the field of specialized research, in which every piece of equipment required high levels of maintenance, and have rapidly reached the mainstream of scientific research. It is thanks to companies like ZEISS and other manufacturers, that the scientific community now can routinely employ super-resolution techniques to answer their questions.

This Essential Knowledge Briefing provides a general overview of the field of super-resolution microscopy. It will explain in a simplified way how the various techniques work and give examples of how scientists successfully employed these techniques in their research.

02 HISTORY AND BACKGROUND

The driving force in the development of optical microscopy techniques lies in keeping its minimally invasive nature, while removing the limitations in optical resolution.

Advances in optical microscopy, historically, focused on improving contrast and resolution with illumination/detection schemes such as differential interference contrast (DIC), phase contrast, etc. A strong push forward came with fluorescence microscopy, digital imaging and the possibility of specific labeling (e.g. genetically, using fluorescent proteins like GFP or using antibodies in immunolabeling). Fluorescence provides high experimental contrast – as only the structures that are of interest become visible.

The combination of fluorescence techniques with confocal laser scanning microscopy (LSM) in the mid-1980s, proved extremely fruitful for biological and biomedical research. It allowed scientists to characterize specimens with the benefit of fluorescence specificity at the diffraction limit, with an in plane (lateral, xy) resolution of about 240 nm and vertical (axial, z) resolution of around 600 nm (cf. numerous reviews and tutorials on confocal microscopy).

Super-resolution techniques emerged as a logical extension in this process. For a more thorough historical context, see [Cremer13]. The four approaches in super-resolution microscopy presented here are the ones that emerged as robust and commercially viable, all of which are based on fluorescence microscopy:

Widefield-based approaches:
- Super-resolution through Structured Illumination (SR-SIM, Lattice SIM)
- Localization microscopy (dSTORM, PALM, PAINT, etc.)

Point-scanning approaches:
- Confocal based Airyscan
- Stimulated Emission Depletion (STED)

Figure 1 gives a schematic overview. The techniques, their advantages / limitations and practical considerations, e.g. on sample preparation, are given in the next chapter.

There is no final statement asserting that one technique is superior to another. Performance and usability depend strongly on the question at hand and on the specimen that is studied. The most important points will be laid out in the following sections in order to allow for a more balanced comparison. Nevertheless, one will often find a comparison in terms of resolution as shown in figure 2. The point spread functions (PSF) of the individual techniques, which describes the apparent size of an infinitely small point of light are placed side-by-side showing the middle section along the optical axis. The lateral (r) and axial (z) diameters of the ellipsoids correspond roughly to the best obtainable resolution. Often times this is interpreted as "the smaller the better". While the most significant resolution improvement is obtained with 3D-STED and Localization microscopy, these techniques have some limitations in sample preparation. On the other hand, Airyscan and SR-SIM are broadly employable and deliver high resolution – albeit not as

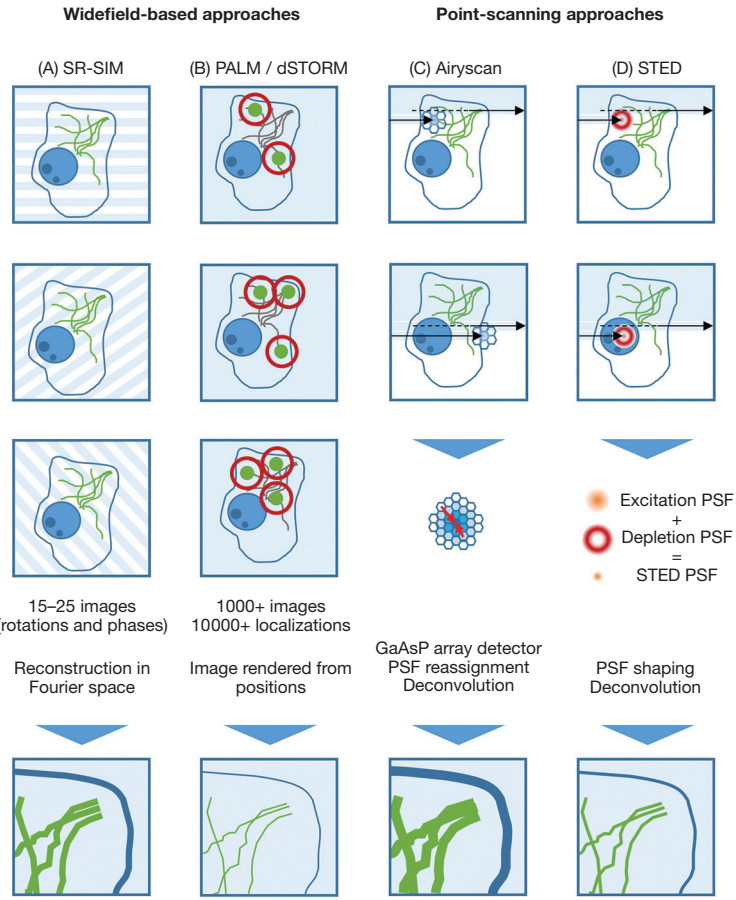


FIG 1: Overview schematic of the super-resolution techniques. The image acquisition principle, processing details, and the final super-resolution image are arranged from top to bottom (object of interest highlighted green - note the difference in levels of detail). From left to right: (A) SR-SIM. Structured illumination sequences (orientations and phases) yield images with resolution down to 100 nm (xy) and 250 nm (z). (B) Localization microscopy (e.g. PALM, dSTORM) identifies and localizes individual molecules yielding a final image with typical resolution of 20 nm (xy) and 60 nm (z). (C) Airyscan combines raster scanning with an array detector. Reconstruction yields resolution down to 120 nm (xy) and 350 nm (z). (D) STED combines raster scanning with a combination of imaging and depletion beam. The opening of the central depletion beam determines the resolution of the final image. Typically 60 nm (xy) and 100 nm (z).

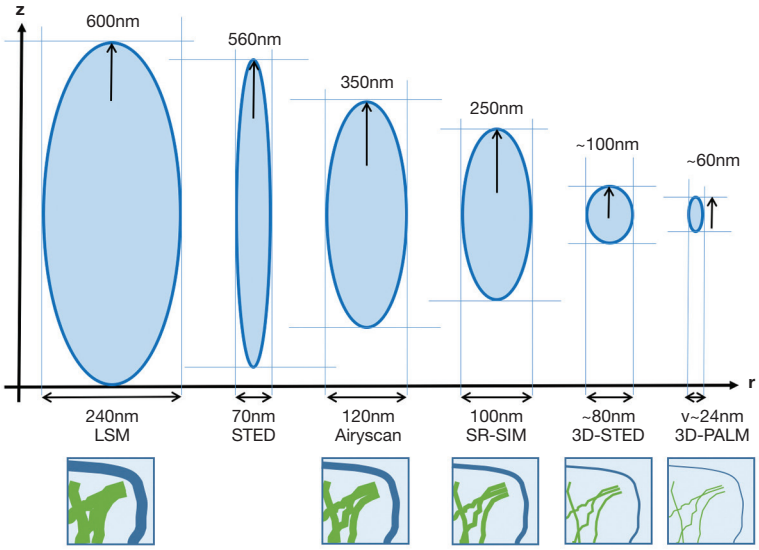


FIG 2: Point spread functions (PSF) of the various techniques represented by ellipsoids with a lateral (r) and axial (z) diameter equivalent to the most typically quoted resolution. Drawn to scale. The individual advantages and drawbacks of the techniques are not represented in this graph. Numbers were taken from [Schermelleh10] and may not represent individual manufacturer's specifications.

high as the one obtainable with localization microscopy (e.g. 3D-PALM, dSTORM, PAINT etc.).

It is important to emphasize that the quality of the samples will affect the attainable resolution and that the values reached in practice, for example from a noise-limited biological sample, may be significantly (10-20%) larger than the numbers given in figure 2. The resolution of a technique or of an instrument is often characterized using very clean and bright technical samples (patterns, beads, etc.). As a recommendation for choosing the technique that would be best suitable for a given study or specimen, the researcher should consider many aspects besides the resolution – like sample preparation and temporal resolution.

03 IN PRACTICE

Super-resolution with structured illumination microscopy (sr-sim, lattice sim)

Super-resolution with Structured Illumination Microscopy (SR-SIM) combines fluorescence, widefield-based structured illumination and digital image reconstruction. The structure, a sequence of known grating patterns, leads to the reconstruction of the image with up to two-fold improved resolution (or 120 nm in xy and 300 nm in z). SR-SIM emerged in the 1990s, for example by Mats Gustafsson, at the University of California San Francisco.

The raw images in structured illumination (figure 3, top row) show the lattice patterns (constant and consistent over

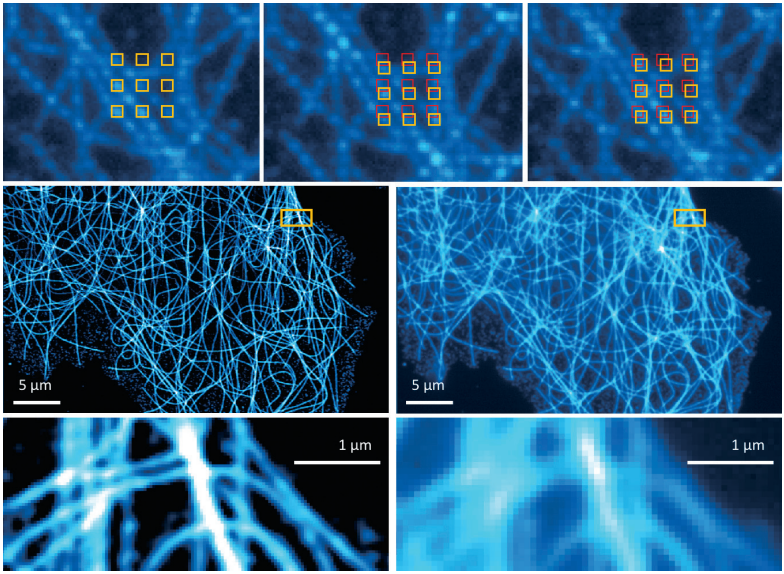


FIG 3: SR-SIM acquisition process on Alexa-561 labeled tubulin structures. Top row shows a subset of raw images with differently shifted and oriented Lattice SIM patterns (using ZEISS Elyra 7). Middle row shows the reconstructed super-resolution image on the left and, as a comparison, the same area imaged with confocal microscopy. Bottom row shows zoomed-in views of the indicated orange squares.

the entire image), which are ideally adapted to the optics of the microscope (e.g. numerical aperture, laser wavelength, etc.). Acquisition and reconstruction (left panels of figure 3 middle and bottom rows) are automated on modern super-resolution microscopes (e.g. matching of grating to optics, adaptive reconstruction filters, etc.).

SR-SIM uses the information contained in the known illumination pattern (i.e. the spacing of the lattice and its position). The resulting patterned images are a product of the lattice overlapping with the structure of interest (e.g. the tubulin mesh in a cell). This overlap creates distinct patterns of coarser lines running across the image (or moiré, e.g. as seen in daily life through folded curtains). The combined image with the coarse pattern can be separated into individual components (in the frequency domain) and the known illumination pattern can be accounted for. Small objects (high frequencies) contribute to the coarse patterns (i.e. they have been down-transformed), while large objects (low frequencies) remain unaffected. Once separated and cleaned from the lattice, the frequencies can then be transferred back to their original positions, and this is then regenerated as the proper image of the small object. The net result is an image with improved resolution up to a factor 2.

The pattern separation is at the heart of SR-SIM: Accurate separation that leads to high quality images requires high contrast. Ultimately, this is why the pattern needs to be swept across the image at different positions to generate a high precision image throughout the specimen. Presently, one will find that there are two pattern variants, the conventional lin-

ear SIM pattern (with differently oriented stripe gratings) and a more modern and light-efficient Lattice SIM pattern (as shown in figure 3). A typical single image in SR-SIM is reconstructed from between 9 to 25 individual images (depending on the system used). Modern SIM instruments can acquire a full set of raw images at 100 or more frames per second.

Main advantages and limitations of sr sim

Versatility and live-cell imaging: SR-SIM is compatible with live cell imaging conditions and is not restricted to specific wavelengths or laser powers.

Acquisition speed: SR-SIM can acquire images from a large area (e.g. 80 x 80 μm) at 100 fps or more. Simultaneous acquisition of multiple colors is possible when using multiple cameras and appropriate beamsplitters.

Resolution in 3D and depth: The resolution improvement (i.e. 100 nm in xy / 300 nm in z) is typically obtained from images taken up to 20 μm distance from the coverslip surface. Lattice SIM allows for a better modulation contrast at depth in comparison to conventional SIM and can reach to 100 μm, depending on sample properties.

Data analysis: Data analysis, like co-localization or distance measurements are straightforward. Noise is visible as texture and can be affected by reconstruction filter settings. Texture can be greatly minimized by increasing the number of grating angles (e.g. from 3 to 5) or stabilizing jitter, drift and photobleaching on the image acquisition.

SR-SIM needs reconstruction algorithms. Reconstruction artifacts (textures) can appear as intensity fluctuations and de-

pend on the quality of the initial "raw" data. Poor image quality can have various origins: From instrument alignment issues to poor sample preparation. As of writing, a number of SR-SIM data verification protocols and software packages (e.g. SIM-Check, for ImageJ) can be used to help or guide the user.

Localization microscopy (palm / dstorm)

Localization microscopy, like photoactivatable localization microscopy (PALM) and direct stochastic optical reconstruction microscopy (dSTORM) (more details e.g. in [Blom17]), relies on the possibility to determine the position of individual fluorescent molecules located at a structure of interest, rather than resolving them optically. The positions can be determined with a precision of the order of 10nm. If thousands of such positions are gathered and superimposed, then it is possible to generate an image of a structure with improved resolution. The resolution depends on the size and density of molecules and the obtainable signal-to-noise ratio. It is therefore theoretically unlimited. Typical images, however, provide 10-fold improved resolution in comparison to conventional microscopy (20 nm in xy and 60 nm in z).

In localization microscopy, the challenge lies in detecting molecules on a one-by-one basis in order to resolve the structure of interest (e.g. a tubulin fiber). In most cases, this is achieved on the time axis, distinguishing between individual molecules via their on-off behavior (roughly: from a dark state to an emissive state – see the strongly simplified Jablonski-Perrin Diagram fig. 4 top row). In a sequence of images, the individual molecules are visible as diffraction limited patterns that

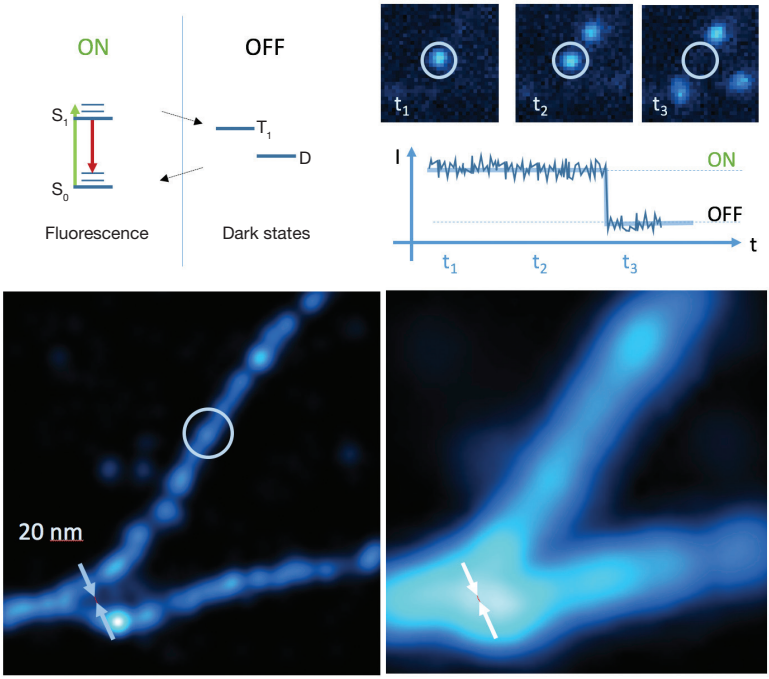


FIG 4: Localization microscopy. Upper row illustrates the typical on/off behavior of single molecules as seen in a time-series (left a simplified Jablonski-Perrin Fluorescence diagram). Individually molecules are visible as diffraction limited patterns that switch on and off in time (the last off step is usually irreversible). The graph shows a typical intensity transient for a single molecule. The two images below show segments of microtubules as reconstructed from the individual molecule positions (left) with around 20 nm resolution and the corresponding widefield image (right) for comparison (Sample using standard Alexa 561 immunolabeling and embedding).

appear and disappear (on/off) as the sequence progresses (see fig 4 top row, right). The sequences of (typically 10,000 and more) frames are acquired using, for example, widefield illumination or total internal reflection approaches (TIRF) as well as sensitive, fast, camera detection. Analysis of the sequence gives the individual molecule's positions, which are plotted in the final image with improved resolution. Fig 4 bottom row left

shows a reconstructed image in which the individual positions of each spot (or molecule) are superimposed.

The two most common approaches that allow observation of individual molecules via their on/off behavior are PALM and dSTORM – the difference lies in the switching mechanism: PALM, developed by Eric Betzig and colleagues at Howard Hughes Medical Institute in Virginia, uses photoactivation to switch the molecules. In a sequence of images, the activation can be set so that only few molecules appear in each frame of the sequence. They can therefore be easily identified and distinguished from one another in the final sequence. dSTORM, relies on the physico-chemical interaction of fluorescent dye molecules with its immediate surroundings, which cause the molecules to switch on and off (hence the term "stochastic"). Under proper conditions (e.g. pH value, redox states, etc.) only few molecules are on during the acquisition of each frame and therefore easily distinguishable from one another in the sequence. For completeness, we would also like to mention a third approach: PAINT (Point Accumulation for Imaging in Nanoscale Topography). It does not rely on a direct on/off switching of the dye molecules, but rather their appearance and disappearance from the images due to binding and unbinding. When the dyes bind they remain immobilized and are visible as spots that can be localized, when they unbind they diffuse rapidly and therefore remain undetected).

For the final reconstruction step – i.e. the recognition of individual molecules and mapping them out on a final image - it is of little relevance whether they were photoactivated

(PALM), if they were blinking passively (dSTORM), or if they were only appearing and disappearing from the images through some other process (PAINT). There is some difference however, between the two switching approaches from the experimental side:

In **dSTORM** label molecules emit at random times due to chemical reactions or interactions in their immediate vicinity (e.g. cis-trans-isomerism, complexation with reactive oxygen species, etc.). There is little exterior influence or control over the experiment except for adding chemical reagents that will have to reach the immediate vicinity of the dye molecule (this is also not trivial if one considers compartmentalization or hydrophobicity, for example). On the positive side, the organic dye molecules are usually very bright and are better adaptable to the experimental circumstances (e.g. using far-red dyes, such as Cy5, that emit outside of the autofluorescence spectrum).

PALM employs photoactivatable dyes (predominantly switchable fluorescent proteins, like photoswitchable GFP, tdEOS, etc.). The switching of the individual molecules is still random, but the rate with which the molecules switch on or off can be controlled by increasing or decreasing the intensity of the switching laser (e.g. 405 nm). Fluorescent proteins are around 5 nm in size and are genetically encoded into the structure of interest (they can be used in vivo, have a higher specificity and do not require fixation and permeabilization of the specimen). On the negative side, fluorescent proteins can exhibit maturation issues; they can disturb the expression levels of the protein of interest and are comparatively dim.

An important question in localization microscopy is: "How many molecules are needed to get a good image?" The answer: It depends on the size of the structure itself. Roughly, one would need (at least) one fluorescent molecule in every 20 nm. This is comparable to the size of a large protein. Thus, obtaining the required labeling density is not a trivial task. In the same vein, the labeling molecules themselves (including functional groups or primary and secondary antibodies) are in a size regime comparable to that of the positioning accuracy. So: While the positioning accuracy for a single fluorophore can be smaller than 20nm, this is of little use if the structure was labeled only with few molecules, and if the labeling molecules themselves (e.g. primary and secondary antibodies) were too large.

Advantages and limitations of palm / dstorm

Versatility and live-cell imaging: dSTORM is generally not considered to be an adequate technique for live cell imaging because the samples are generally prepared via immunolabeling (fixed cells). Localization microscopy, however, has been used to image living specimens with meaningful data from the perspective of molecule or particle tracking [Hellriegel 2011], [Nan 2013].

Acquisition speed: PALM and dSTORM are considered slow because collection of a typical image sequence (>1000 frames) takes upward of 10s, typically minutes.

Resolution in 3D and depth: In practice, PALM / dSTORM deliver the highest resolution of all presented super-resolution methods (theoretically unlimited - typically 20 nm in xy / 60 nm in z) and can deliver molecular detail. Best

results are obtained from transparent and well-prepared specimens near the coverslide surface (ca. 10 μm from the coverslip surface)

Data analysis: Classic data analysis (distance measurements and 3D reconstruction) is possible. The capability to detect individual molecules, however, opens a wealth of data analysis possibilities that are not easily accessible via other methods (cluster analysis, intermolecular distances, etc.). PALM/dSTORM images are rendered from a table of localized molecules (accurate channel alignment and drift compensation needs to be in place). Quantitation requires careful calibration and controls.

The biggest challenge for PALM/dSTORM is the need for photoswitchable molecules or addition of chemistry to bring the labels into an adequate "blinking" regime. Also, PALM and dSTORM have limited in vivo applications. Long term stability is a crucial concern for PALM/dSTORM equipment.

Airyscan

Airyscanning is a super-resolution approach, which strongly relates to confocal laser scanning microscopy (LSM). LSM raster-scans a focused excitation beam over the specimen. Fluorescence originating at that focal spot is then separated from off-focus fluorescence with a pinhole. This rejection and the accompanying increase in resolution depends on the pinhole size. A small pinhole increases the rejection, and therefore, increases the resolution (like the tip of a sharp pencil in comparison to a larger, blunt tip). When the pinhole size matches the illumination spot diameter (1 Airy

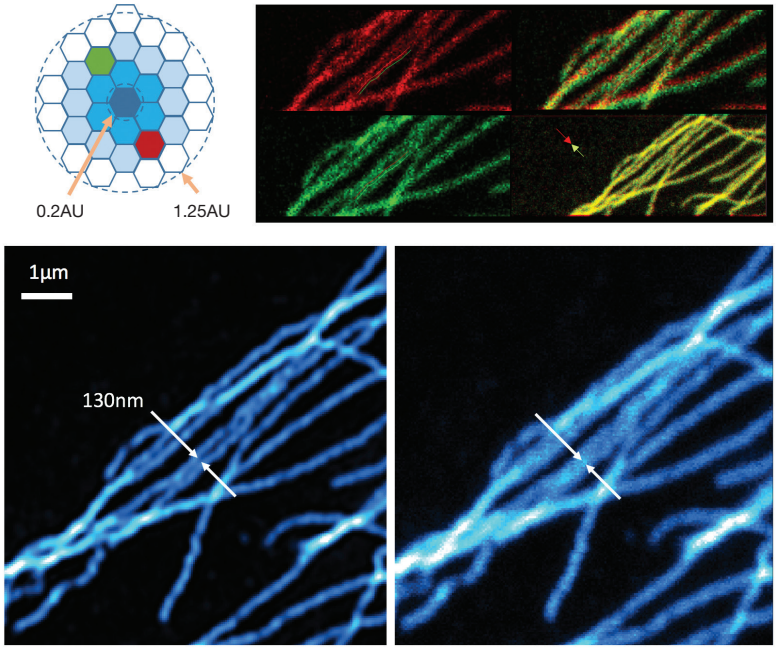


FIG 5: Top row shows the Airyscan detector array. It consists of individual detectors that correspond to a pinhole closed to 0.2 Airy Units. The individual images are offset and need to be rearranged (right panel top row shows the images from two individual detectors of the array). The final result is shown in the bottom row images. Left: The reconstructed Airyscan image, right confocal image for comparison. Microtubules labeled with Alexa 561 (as in the previous figures)

Unit, AU) most signal is collected at optimal rejection of the off-focus light. The resolution of conventional LSMs is given for a 1 AU opening of the pinhole, about 240 nm in xy, and around 600nm in z.

The pinhole opening can be made arbitrarily small - e.g. to a fifth of the illumination spot diameter (0,2 Airy Units - AU). It turns out that the accompanying resolution improvement will be limited (depending on the wavelength and optics used) to a factor 1.4 enhancement (to around 180 nm)

in comparison to the classical diffraction limit. More importantly, however, is the strong rejection of signal. At 0.2 AU the rejection is 20-fold, and this proves prohibitively impractical for biological samples (and even more so for live specimens).

The key element in Airyscanning is the detector design, which acts as an array of closed pinholes, each providing the aforementioned resolution increase, but collectively not suffering from a high rejection of signal. This is so because the detectors are adjacent to each other: The light rejected by one detector sub-unit is collected by its neighbors. This simple and elegant idea was formulated early in the development of LSMs [Sheppard 1982], and re-visited experimentally more recently [Manders 2013]. A fast enough, sensitive and low-noise detector array became commercially available in 2014.

Airyscan addresses the challenge of imaging biologically relevant samples (low expression levels, and keeping low laser dosage) without signal rejection at closed pinhole. It is a new detection standard for LSM imaging. It allows entering the realm of super-resolution. It is compatible with any form of LSM, including 2-photon microscopy, adding tremendously to the versatility and applicability of the approach.

The data collected with an Airyscan detector array requires pixel reassignment (or deconvolution, a computational approach broadly employed in LSM) which is a simple computational step. In total, the resolution improvement can reach 120 nm laterally (xy) for a single plane image. In 3D images it is typically 140 nm laterally (xy) and 400 nm axially (z).

Advantages and limitations of airyscan

Versatility and live-cell imaging: Of all presented super-resolution techniques, it is the most robust under live-cell imaging conditions. Airyscan is not restricted to specific wavelengths, laser powers or special objective lenses.

Acquisition Speed: Depends on the scanned area. Small areas (e.g. 10 x 10 µm) allow speeds upward of 30 frames per second, large areas (e.g. 60 x 60 µm) require few seconds per image.

Resolution in 3D and depth: The 3D resolution improvement is approximately 2-fold in comparison to LSM (e.g. 140 nm in xy / 350 nm in z) but can reach 120 nm lateral resolution for a single plane. Best quality images are readily obtained from transparent and well-prepared specimens (up to 20 µm from the coverslip surface). However, Airyscan can also be used in conjunction with large working distance objectives and 2-photon excitation conditions and can be used to reach deep (millimeters) into tissue preparations.

Data analysis: The reconstruction of the images is straightforward and fast on modern computers. Airyscan, just as confocal LSM allows for quantitative data analysis (e.g. imaging and quantifying calcium bursts, photorecovery in diffusion, colocalization, etc.).

Of all presented super-resolution methods, it provides a resolution improvement comparable to that of SR-SIM. It is comparatively slow at scanning a full FOV. However, since it is an LSM-based approach it is tremendously flexible (e.g. zoomed-in scanning, photomanipulation, combination with 2-photon imaging, etc.) and less sensitive towards changes in refractive index and inhomogeneity in the specimen.

Stimulated emission depletion microscopy (sted)

Stimulated emission depletion (STED) microscopy is related to confocal laser scanning microscopy (LSM) in the sense that it, too, is a point scanning method – however, instead of using a pinhole to reject off-focus light (see previous section "Airyscan"), it suppresses off-focus emission (via stimulated emission) right at the illumination spot on the sample. To accomplish that a second, high intensity donut-shaped depletion beam is superposed with the focused imaging beam. The size of the "opening" of the depletion donut therefore determines the obtainable resolution – the smaller this opening the higher the resolution (as, in analogy, the sharper the pencil, the smaller the tip). This elegant approach was pioneered in the

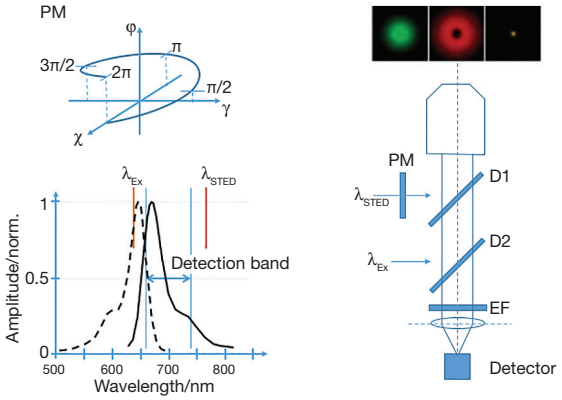


FIG 6: Simplified schematics of a STED microscope (right panel). The excitation and STED beams are superimposed by dichroic mirrors (D2, D1) and focused in the sample. A helical phase mask (PM) in the STED beam path creates a doughnut-shaped STED focus in the sample (red pattern, PM schematics in top left). Fluorescence is collected by the objective and focused onto a detector (D). Images are generated via raster-scanning. Left, bottom, shows a hypothetical excitation (dotted line) and emission spectrum of a fluorophore. Adapted from: Fluorescence Microscopy: From Principles to Biological Applications, First Edition. Edited by U. Kubitscheck 2013 Wiley-VCH Verlag GmbH & Co.

mid-1990s in the group of Stefan W. Hell, now at the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany (cf. [Eggeling15]). A particularly interesting aspect of STED is that the donut "opening" could, in principle, be made arbitrarily small. Therefore the resolution is, in principle, not limited (see fig. 5 for schematic illustration). The depletion beam can be shaped in 3D in order to enhance resolution also along the z-axis. In practice, the typical resolution given is of the order of 50 nm in xy and 80 nm in z.

The image formation is analogous to the image formation in confocal LSM, i.e. via raster scanning the beam across the specimen and detecting (and mapping) the fluorescence intensity in the final image. The key element (and the key difference) in STED (in comparison to confocal LSM) is the presence of the second beam for depletion via stimulated emission: The aforementioned donut shaped beam, that is centered with the first (say, conventional) imaging beam.

From the perspective of experimental usability, the challenges associated with STED are threefold: (1) Maintaining a stable and precise shape of the donut, perfectly centered with the imaging beam. (2) Conceiving the experiment so the used dyes and labels are within the spectral and photophysical confines of the depletion beam (not all dyes can be depleted efficiently) (3) Adapting the depletion beam intensity to minimize the dosage on the specimen.

Staying within the confines of the available spectral windows, brings the necessity to use "depleteable dyes". Not all dye molecules perform in the best possible manner for STED studies but the list is increasing at a fast pace. Coping with

very high intensity laser (increased bleaching, increased toxicity, heating, photodamage) brings further restraints into the experimental planning, but newest technology in STED (e.g. rescue STED) is also minimizing that problem. Commercially STED is available, for example, as a stand-alone system or as an add-on setup for various microscopy stands (e.g. through Abberior Instruments, Göttingen, Germany).

Advantages and limitations of sted

Versatility and live-cell imaging: STED is generally not considered the most adequate due to the high power required for the depletion beam. Live cell measurements have been demonstrated, however.

Acquisition Speed and FOV: As a point-scanning based method, the frame rate of STED depends on the used FOV, and the resolution under which it is operated. For small zoomed in regions (10µm x 10µm, for example) speeds in excess of 50 frames per second can be acquired. Larger fields of view require few seconds. Simultaneous acquisition of multiple colors is possible.

Resolution in 3D and depth: The typical resolution is 50 nm in xy and 80 nm in z, but theoretically unlimited. Additional image analysis (and deconvolution) allow for further resolution improvement. The depletion beam can also be shaped along the z-axis, giving resolution in z of about 80 nm (at a slight expense of lateral resolution). STED becomes challenging when going into thick, over-labeled, noise-rich, scattering specimens.

Data analysis: All data analysis, such as co-localization, size and distance measurements are possible with STED images.

Also, highly quantitative fluorescence correlation techniques (FCS) have been demonstrated using STED-based setups. (Eggeling15)

The most frequently found point of criticism of STED is its requirement for high power laser and the concomitant photo-toxicity and increased photobleaching rate. Most efforts that are put into STED and STED related technology is aimed at keeping the depletion laser stable, and as low as possible.

Considerations on sample preparation for all methods

Super-resolution microscopy requires thorough sample preparation. Image quality is rapidly affected by impurities (dust grains, bubbles, unspecific staining, etc.) on the specimen being studied - for sample preparation see e.g. [Allen2013]. In essence, the message is simple: Care must be taken that all parts of the system, from the coverglass to the mounting or embedding medium are clean and well-defined (e.g. uniform thickness, clean mounting, labeling specificity, etc.).

Dyes and labels play an important role in fluorescence microscopy. Fluorescent proteins (e.g. GFP, mCherry, tdEOS, etc.) and organic dyes (rhodamines, cyanines, etc.) are available in various different colors and functionalities (e.g. antibodies, succinimide esters, etc.) allowing scientists to visualize cellular processes or interactions between organelles by labeling two or three groups of proteins (or DNA, lipids, etc.) with different color labels. The labeling approach depends strongly on the specific topic that is being studied. Also, not all super-resolution techniques can be used in conjunction with all available dyes. In the following we present a few re-

marks on the topic of labeling specific to each technique presented in this guide:

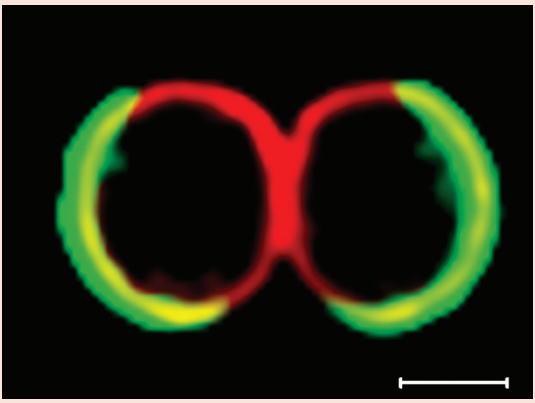
Airyscan and **SR-SIM** can work with the same fluorescent labels as conventional fluorescence microscopy. These techniques impose the least amount of restriction when it comes to choice of fluorescent labeling.

STED requires a careful combination of fluorescent dyes considering the used depletion laser wavelengths. The list of dyes that have been used and tested in STED is rapidly growing, but it is, in comparison, still much smaller than the list used for Airyscan, SR-SIM. Labels for STED are exposed to high intensity light and therefore need to be stable and robust enough to withstand repeated excitation.

PALM / dSTORM require labels that can be chemically or optically switched so that they only fluoresce for a short time – not all dyes do that and not all recipes are guaranteed to work (for example, if the dye molecule is not accessible to the chemical reagents due to compartmentalization or solubility issues). The list of dyes (and preparation protocols) for PALM or dSTORM is rapidly growing.

CASE STUDY 1: SR-SIM

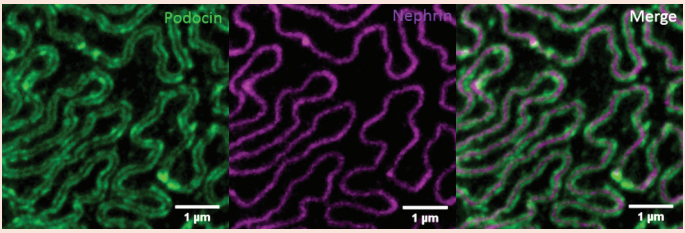
SR-SIM was recently employed by the research group of Prof. Mariana Pinho from the Laboratory of Bacterial Cell Biology, Instituto de Tecnologia Química e Biológica, Portugal, to reappraise the questions surrounding bacterial cell-division. In their publication [Monteiro 2015] they state that it was the small-size of bacterial cells that called for studies employing super-resolution methods. With super-resolution they were able to draw a clear picture of asymmetry generated at the cell division of S. Aureus bacteria.



***S. aureus* COL cells were labelled for 5 min with WGA-488 (peripheral cell wall dye, green), washed and stained with Nile Red (membrane dye, red). Cells were then placed on an agarose pad, allowed to grow at room temperature and imaged by SR-SIM. Upon division, the old cell wall preserved the green WGA-488 signal while the new surface is labelled only in red. Scale bar 500nm. Images taken with a ZEISS Elyra PS.1 microscope using a Plan-Apochromat 63x/1.4 Oil DIC M27 objective. Images were acquired using five grid rotations.**

CASE STUDY 2: STED

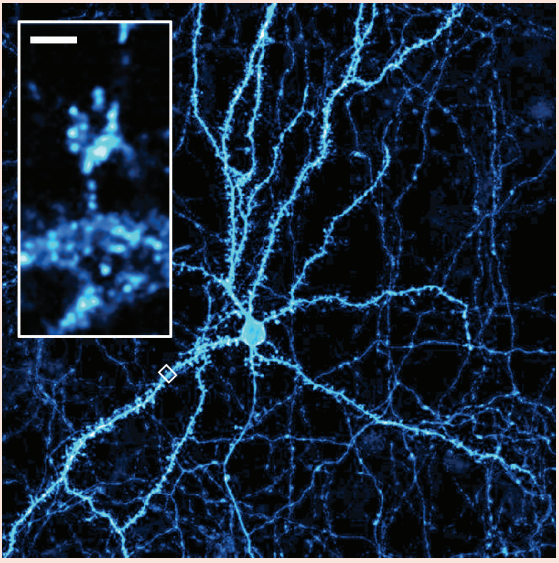
Stimulated emission depletion microscopy (STED) with its high resolution capabilities and optical sectioning is well suited to image the nanoscale in tissue or organisms. In their publication researchers Unnersjö-Jess and colleagues show that STED imaging is well suited to resolve the filtration slit in cleared kidney tissue [Unnersjö-Jess 2016]. The glomerular filtration barrier, consisting of podocyte foot processes with bridging slit diaphragm, glomerular basement membrane, and endothelium, is a key component for renal function and tools that allow for their 3D structural characterization in health and disease highly desirable. It was the combination of clearing techniques and deconvolution applied to STED imaging that provided to the required resolution and signal quality.



Top-view of the filtration slit in podocyte foot-processes from a cleared kidney biopsy where the two slit proteins podocin (green) and nephrin (mangenta) were immunochemically labeled with antibodies carrying Alexa594 and Abberior-STAR635 and super-resolved with a 775 nm STED laser. Scale bar 1 µm.

CASE STUDY 3: PALM

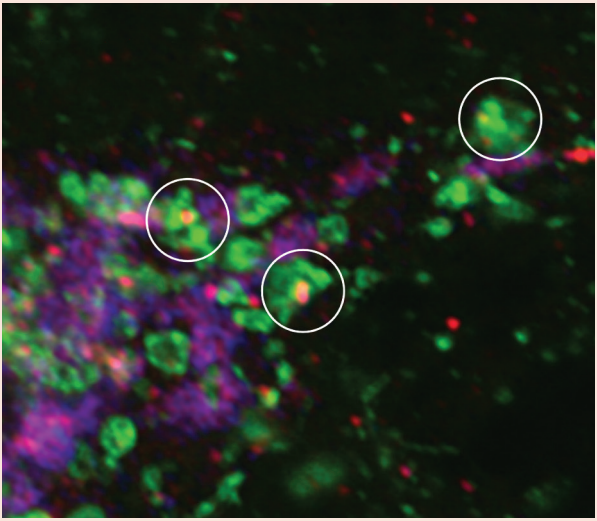
The dimensions of neuronal synapses, of the order of 100 nm, suggest that optical super-resolution imaging methods are necessary for thorough investigation of protein distributions. In their publication [Liebmann 2013] scientists from the Karolinska Institutet in Sweden applied localization microscopy techniques (PALM) to resolve synaptic protein topology and subsequent single molecule quantifications of the neuronal sodium-pump. Results revealed a compartmentalized distribution of sodium pumps in dendritic spines, with several nanoclusters of pumps typically found in the spine head and fewer in the spine neck.



Overview of a cultured hippocampal neuron genetically expressing the neuronal sodium-pump (a3 isoform) labelled with the fluorescent protein PA-eGFP. The inset shows a PALM generated pointillistic single molecule image of the sodium-pump topology in a dendritic spine (blue square). The image was acquired on a ZEISS Elyra PS.1 with 488 nm excitation and 405 nm photoactivation. Scale bar 300 nm.

CASE STUDY 4: AIRYSCAN

Most neuronal function is regulated via membrane trafficking for which the transmembrane proteins Nsg1 and Nsg2 play a critical role. Nsg1 has been previously has been identified as helping regulate endosomal recycling and sorting. However, in contrast to previous conclusions, the lab of Dr. Bettina Winckler in the Department of Cell Biology at the University of Virginia has demonstrated with Airyscan technology that Nsg1 and Nsg2 proteins are not resident endosomal proteins but traffic rapidly between the cell surface and lysosomes. [Yap 2017]



Cells were fixed in 2% paraformaldehyde/3% sucrose/PBS in 50% conditioned medium at room temperature for 30 minutes, quenched in 10 mM glycine/PBS for 10 minutes. The fixation conditions used do not introduce holes into the overwhelming majority of cells. Coverslips were then blocked in 5% horse serum/1% BSA/PBS ± 0.2% TritonX-100 or 0.1% saponin for 20 minutes. Antibodies were diluted in 1% BSA/PBS and incubated for 1 hour.

04 PROBLEMS AND SOLUTIONS

With the availability of super-resolution microscopes, the capability of achieving resolutions in the range between 10-150 nm, it is not surprising that the scientific community rapidly incorporated the techniques to their research. Super-resolution techniques are presently in widespread use. Despite this success, however, and as mentioned above, all techniques have certain constraints. In this chapter, we present these constraints again as a side-by-side comparison, and point (when possible) towards strategies to overcome, or minimize certain limitations.

Versatility / live cell imaging

Airyscan: It is perfectly compatible with live cell imaging. It even outperforms conventional confocal or 2-photon LSM, in terms of gentle imaging conditions (laser power reduced by a factor 3 – 10). There is no restriction to specific laser wavelengths or specific objectives. In other words, it is straightforward to acquire 4 channel images across the visible spectrum (from blue to far red) in an incubated glass-bottom petri dish, with the cell culture in conventional (but phenol-red free) culture medium, at 37°C using, for example, a 63x/1.2 water immersion objective.

SR-SIM: SR-SIM is compatible with live cell imaging; there is no requirement for particularly high laser power or specific dyes to achieve super-resolution. It is straightforward to acquire 4 channel images across the visible spectrum (from blue to far red) in an incubated glass-bottom petri dish, with the cell culture in conventional (but phenol-red free) culture medium, at 37°C using, for example, a 63x/1.2 water immersion

objective. Lattice SIM, in addition, enhances modulation contrast, which translates into lower laser dosage, placing SIM among the most gentle fluorescence imaging techniques.

PALM/dSTORM: Is not considered compatible with live cell imaging. While there is no requirement for particularly high laser power to achieve super-resolution, it is very difficult to acquire meaningful data in terms of super-resolution images and in the context of live imaging. Photoactivation of individual fluorescent proteins (PALM) and other types of switching (e.g. passive) can be achieved in live cell imaging, but the choices of fluorophores are limited.

STED: STED brings the highest irradiation dosage to the specimen, because of the high intensity depletion beam. It is therefore considered the least gentle super-resolution imaging technique. It has been demonstrated early on in the context of live cell imaging [Nägerl08], but it still remains a challenging modality for STED microscopy. Moreover, it is restricted to the dyes that can be depleted by the offered laser wavelengths. Considerable effort is being put into development of STED microscopes that utilize less power in the depletion beam (e.g. using pulsed lasers and gated detection). Alternatively, one can also deliberately choose to decrease the obtainable super-resolution (to levels similar to those of Airyscan or SR-SIM) by reducing the laser power of the depletion beam.

Speed / live cell imaging

SR-SIM: Delivers the highest acquisition speed at full field-of-view (FOV)**.** As a camera-based method, SR-SIM acquires a large FOV (e.g. 66 µm with around 1200 x 1200 pixels) in a

very short time (around 100 fps - limited essentially by the frame rate of the camera). Simultaneous acquisition of multiple colors is possible when using multiple cameras (e.g. using dual camera adaptors) and appropriate beam splitters.

Airyscan: As a point-scanning based method, the frame rate of Airyscan depends on the used FOV. For small zoomed in regions (e.g. 10 x 10 μm) speeds in excess of 30 frames per second can be acquired. Large fields of view, however, require few seconds.

STED: As a point-scanning based method, the frame rate of STED depends on the used FOV, and the number of pixels. For small zoomed in regions (e.g. 10 x 10 μm) speeds in excess of 30 frames per second can be acquired. Large fields of view require few seconds. Simultaneous acquisition of two colors and fluorescence correlation spectroscopy (FCS) is possible.

PALM / dSTORM: These techniques are not considered fast. While a large FOV (e.g. 66μm) is acquired in a single shot of few (10 or so) ms duration, there is also the necessity to acquire thousands of individual images. This brings the temporal resolution of PALM/dSTORM images to the time scale of minutes. This is sufficient temporal resolution for the observation of very slow cellular processes. Fast processes, however, on the molecular scale need to be examined via tracking individual molecules – while this approach does not deliver super-resolution images per se, the information from an individual molecule (in the form of its trajectory) brings a tremendous amount of dynamic information. Simultaneous acquisition of multiple colors is possible when using multiple cameras (e.g. using dual camera adaptors) and appropriate beam splitters.

Depth

Airyscan: Airyscan is the technique of choice when it comes to imaging in deep tissue, or thick specimens, in particular the possibility to combine 2-photon LSM with Airyscan detection offers an interesting way to reach deep into thick tissue preparations, or living organisms. Airyscan is less sensitive towards changes in refractive index, scattering and inhomogeneity in the specimen and it can outperform all other super-resolution techniques when used deep (tens of microns) inside tissue.

SR-SIM: SR-SIM performance depends on the accurate (and high contrast) projection of the illumination pattern into the specimen. While this is easily achieved in transparent and well-prepared specimens (e.g. cell cultures, bacteria, thin plant roots, C.elegans, zebrafish embryos and, in general, objects close to the coverslip surface) it rapidly becomes challenging when going into thick, over-labeled, noise-rich, scattering specimens; or also very sparse, dim and quickly bleaching structures. Typically, best results are obtained from images taken up to 20μm distance from the coverslip. Lattice SIM (in contrast to conventional SIM) utilizes a pattern that generates higher modulation contrast and is therefore less susceptible to suffer from detrimental depth effects.

PALM / dSTORM: Best results in PALM / dSTORM are obtained from regions close to the coverslilde surface (up to 20μm) and this is achieved easily in transparent and well-prepared specimens (e.g. fixed cell cultures, bacteria, thin plant roots, objects close to the coverslip surface). While PALM/ dSTORM can operated under TIRF illumination conditions

(and therefore delivering very high contrast) this aspect will be omitted here as there is virtually no depth information in this mode of operation. In terms of depth PALM/dSTORM becomes more and more challenging when going into thick, over-labeled, noise-rich, scattering specimens.

STED: The problems of STED with respect to depth of imaging are twofold. First, the high intensity depletion beam needs to generate a well-shaped donut in order to provide the resolution enhancement. Projecting a well-defined donut through a thick specimen is difficult and the quality rapidly decays with penetration depth. Second, the high power depletion beam has to cross the entire specimen, placing a substantial laser dosage also on the image planes that are not being imaged.

05 WHAT'S NEXT?

Optical super-resolution methods have reached the mainstream of scientific research thanks to the commercial availability of robust and well-designed microscopy platforms. The main driving force for the further development and improvement of these methods, however, comes from a vibrant and active community (of networked users and experts) that incessantly pushes forward for newer, improved or alternative techniques and analysis methods, benchmark tests and protocol collections.

The main goal is to apply the techniques to live cell imaging situations. Here, the specimen is usually, sensitive, dim and mobile in comparison to fixed samples (bright and immobile). The challenge, therefore, is to attain the highest possible super-resolution, with high contrast, acquired faster and with less laser power, in thick scattering tissues, with minimal labeling and on-line functional data processing (at the same time, of course, the equipment should be economically viable and easy to use by non-experts):

SR-SIM: The bottleneck, currently, lies in the acquisition of 15-25 raw images per final reconstructed super-resolution image. For this reason, one of the main challenges in SR-SIM is to produce microscope platforms that allow minimizing the acquisition time in a robust manner (for example by changing to different, more time-efficient, illumination patterns). Another aspect in SR-SIM is related to data reconstruction and data handling, which presently is an additional post-acquisition step on most platforms. Faster and more robust, easier-to-use reconstruction software (that would perform raw data quality

check as it is being acquired, for example) is highly desirable.
Airyscan allows for fast imaging over small areas, for example 100ms per image (10 fps). The speed drops dramatically for large fields-of-view to few seconds per frame. The most promising path to address this problem is to parallelize acquisition by using multiple foci or modulating the PSF (ZEISS' Airyscan FAST is a step in that direction).
STED (as a point-scanning based technique) allows for fast scanning over small fields of view, however – the high resolution obtainable with STED requires more (smaller) pixels to be scanned for a given field of view. This means it can take 10–30 seconds to produce a single STED image. Parallelization (using e.g. multiple foci) seems challenging, as it would require also multiple depletion beams. Gating and pulsed beam approaches, different forms to shape (also in 3D) the depletion beam could improve this situation. Significant effort is also being channeled into the development of adequate dyes for STED.
PALM/dSTORM, even with the latest multi-emitter algorithms, the time scale per image depends on the number of detected molecules, labeling density, etc. The tradeoff, invariably, is, therefore, temporal resolution versus sufficient number of molecules. As of writing, there is considerable effort being put into particle fluctuation and particle tracking approaches that are complementary to "conventional" PALM / dSTORM imaging.

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