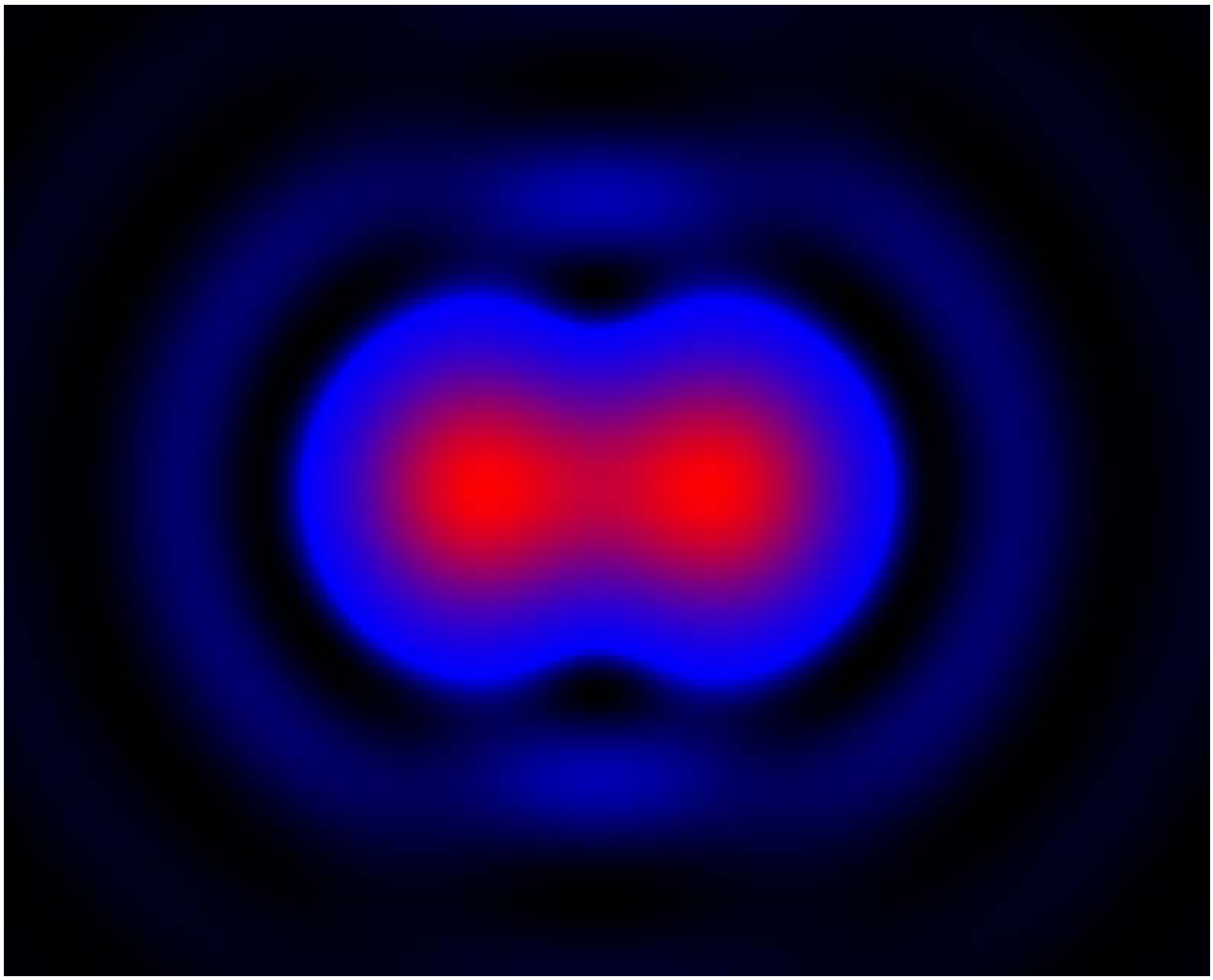
TECHNOLOGICAL READINGS

Super-Resolution

On a Heuristic Point of View About the Resolution of a Light Microscope



Dr. Rolf T. Borlinghaus, Leica Microsystems

If you can't explain it simply, you don't understand it well enough.

Albert Einstein

1. What is resolution?

In our context, resolution is the opposite of fusion. When something appears resolved, we can distinguish the discrete components. In microscopy, three main methods are used to describe resolution. One is the Abbe formula, named after Ernst Abbe. He studied linear structures in transmitted light (1).

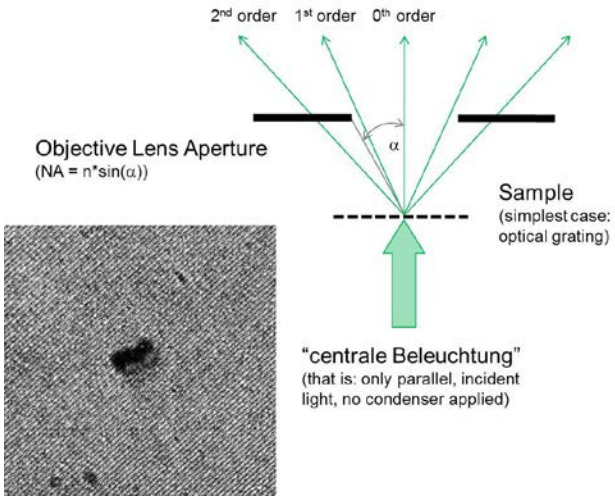


Fig 01: Ernst Abbe's definition of resolution. Assuming the sample to represent a periodic structure, it is necessary to collect at least the first diffraction order to create an image. Therefore, the lens aperture must be large enough: $n*\sin\alpha = NA \geq \lambda/d$, with d the spatial period.
The image shows an optical grating (with some dust on it), recorded with a variable aperture lens, adjusted to just resolve the structure.

For illumination with a condenser, the smallest distance resolved arrives at

$$d_A = \frac{0{,}5 * \lambda}{NA}$$

The other is the Rayleigh criterion according to John W. Strutt, who studied point-shaped emitters and defined two point-images as optically resolved if the maximum of the diffraction pattern of one emitter coincided with the first minimum of the diffraction pattern of the second(2). This leads to

$$d_R = \frac{0{,}61 * \lambda}{NA}.$$

In this case, there is a minimum brightness between the two maxima that corresponds to approximately ¾ of the maxima intensity.

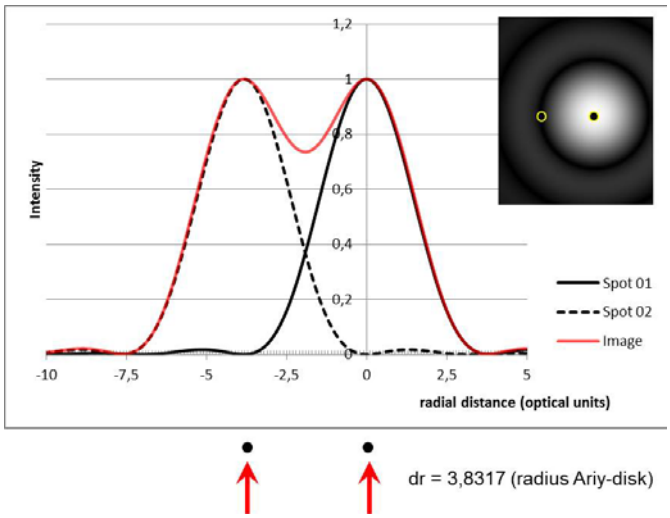


Fig 02: Rayleigh criterion. Two spots are regarded as resolved, if the center of one spot falls into the first zero of the other spot's point spread function (PSF).
The criterion is only valid for Airy-like PSFs. In case of e.g. a Gauss profile, there is no zero at all, and the criterion is not applicable. Other criteria, such as the sparrow criterion will work with any profile type, as it refers to the distance, where the first derivative halfway between the patterns disappears ("plateau criterion")

It is obvious that points can still be differentiated when this drop in brightness is even less pronounced. Therefore, the generally discussed resolution values are arbitrary and cannot be put down to a law of nature, no matter how much math and diffraction optics theory is applied.

The third, more practical approach is to describe the full width at half maximum (FWHM), (3) of an optically unresolved structure. This value is relatively easy to measure with any microscope and has therefore become a generally accepted comparison parameter. The theoretical value is

$$d_H = \frac{0{,}51 * \lambda}{NA}.$$

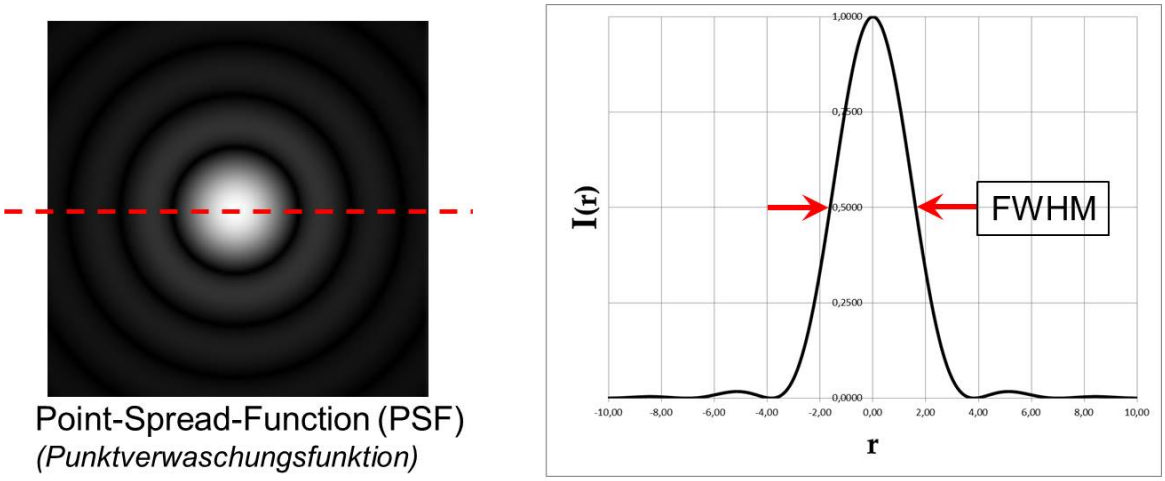


Fig 03, left: Point spread function by a circular aperture (the intensity is nonlinearly enhanced to make the dim rings visible). The inner spot is called an Airy-disk. Right: profile through the center of the circular diffraction pattern. A good and measurable feature is the full width at half the maximum intensity (FWHM =: d).
The advantage of this criterion is, that it is easily measurable in microscopic images of sub-resolution features. For calibration or limit-measurements often fluorochromed latex beads of various diameters are applied and measured.

As can be seen, all these values – although acquired with very different assumptions – deviate by less than 10% from a mean value. This makes the discussion considerably easier: we know that we are not making a major error if we simply take the FWHM as the observable resolution parameter – which is comparably simple to measure and is close enough to

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

These resolution values, when derived from physical and mathematical assumptions, are theoretical estimates. They assume perfect imaging systems and a light point in a vacuum or a fully homogeneous substrate as the specimen. Naturally, this is never the case in real life, let alone in daily laboratory practice. One particularly serious assumption is that light is available in infinite supply. In reality it is not, and the measureable resolution depends significantly on the signal-to-noise ratio (SNR). It is also obvious, that typical biological samples such as brain slices do optically not behave as friendly as a vacuum.

Basically, the measurement results are therefore always inferior to the *optical* resolution of a microscope. This is a particularly important point to remember when, for instance, examining thick and weakly stained tissue sections! Moreover, the microscope image is formed by the interference of many diffraction patterns, not just one or two. To be taken seriously, resolution measurements must always contain a large number of readings at different positions (and in different samples, if possible) which then give a mean value with an error. It is also obvious, for example, that the claim "We have attained a resolution of 197.48 nm" is a fib, and it would surely be more honest to call it "200 nm".

2. And what is super-resolution?

The prefix "super" comes from Latin and means "above" or "beyond". Super-resolution is therefore used to describe techniques that enhance the resolution of the microscope image. And this immediately leads to confusion: does it refer to an improvement of the (theoretical) *optical* resolution or an improvement of the *measurable* resolution when an image is recorded, or both? Or does it refer to completely different techniques that allow higher resolution using other methods than those of classic optical theory? To a certain extent, all such techniques could justifiably be termed "super-resolution". Whether a technique is actually called "super-resolution" or not is then a matter of philosophy or intended to control significance. But let us leave this discussion aside here in favor of a comprehensive mention of the most significant techniques. Here too, the line between "resolution" and "super-resolution" is arbitrary and therefore discretionary.

Image Restoration (Deconvolution)

An optical instrument, e.g. a microscope, visualizes an object in a different form. It is the job of a microscope to magnify small structures that cannot be distinguished with the naked eye so that we can see them. Unfortunately, something is always lost when such an image is produced – we cannot keep increasing the magnification in the hope of seeing smaller and smaller structures. This is because the microscopic representation of objects, however small, is principally governed by the laws of diffraction.

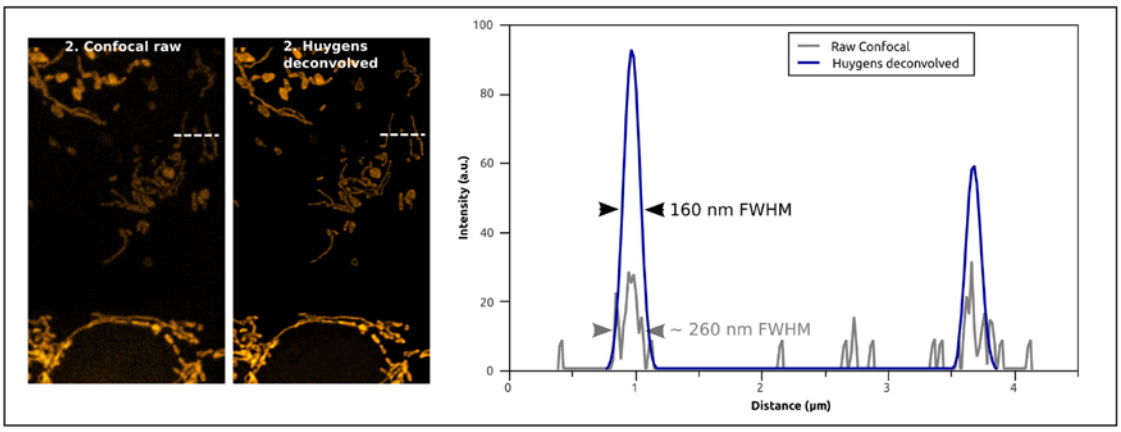


Fig 4: Image comparison of a confocal optical section, featuring cellular compartments with the deconvolved result of that image. The dotted lines indicate the intensity profiles shown on the right side. The apparent resolution improvement by comparison of the FWHMs is 1.6-fold. Also note the noise reduction and increase of peak intensity of the signal.

A point-shaped object is therefore imaged as a diffraction pattern. This diffraction pattern is the "point spread function", a three-dimensional description of what the microscope has made of this point. The spread by the optical system is called "convolution". It is possible to calculate such point spread functions. Point spread functions which are calculated under the assumption of ideal optics and samples look very brilliant. However, it is better to measure them in a real sample, as all the optical aberrations of the imaging instrument and sample influences are then detected as well. The idea of deconvolution is to apply one's knowledge of the point spread function to a three-dimensionally recorded image data set in order to restore the original light distribution in the object.

As this method indeed improves object separation in actually recorded images, deconvolution is sometimes referred to as a type of super-resolution technique. Improvements of just under 2x in lateral (x and y) direction and slightly better than 2x in axial (z) direction are claimed (4).

Confocal Microscopy

In confocal microscopy, only one point is illuminated at a time, and the emitted light from this point is threaded through a small pinhole onto the detector, the pinhole having the effect of a virtually point-shaped detector. Roughly speaking, one can already surmise from this approach that this type of system is inherently less prone to convolution interference: When a whole field is illuminated and observed simultaneously, the data of all the recorded pixels contain components of other spatial elements. Indeed, confocal imaging leads to extremely thin optical sections, limited by diffraction properties. For the optical conditions encountered in normal practice, the FWHM in z is roughly twice the value for xy. A conventional microscope – however large – has no possibility of discriminating information in axial direction.

To get the best results from a confocal microscope, a pinhole diameter that corresponds to the inner disk of the diffraction figure of round apertures (Airy disk) is used. This gives a section thickness close to the diffraction limit without losing too much light. It is not possible to improve lateral resolution under such conditions.

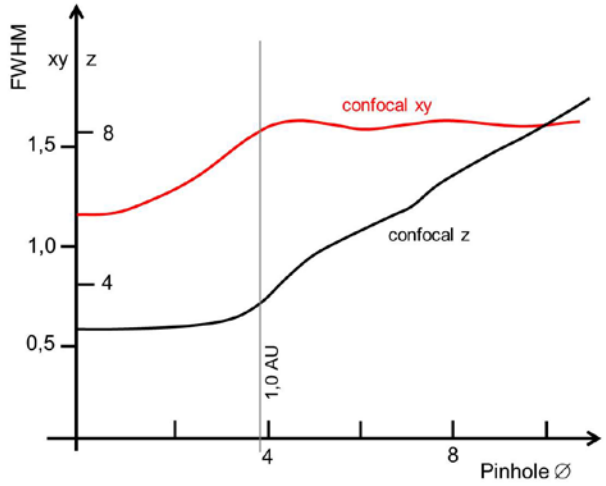


Fig 05: Resolution performances in a true confocal (single spot) microscope as a function of pinhole diameter (curves adapted from (5)). The optical sectioning performance is shown in black, the lateral resolution in red. If the pinhole has the size of the inner disc of the diffraction pattern (indicated by the grey line at 1 AU), further closing does not improve sectioning but increases lateral resolution. At pinhole zero, the section thickness will assume the diffraction limit, and the lateral resolution is better by a factor of √2 as compared to the widefield diffraction limit.

So, classic confocal images are not super-resolution images as regards the lateral resolution. However, lateral resolution is improved by a further narrowing of the pinhole diaphragm. For the (admittedly only theoretical) case of a pinhole with a diameter of 0, an improvement of around 1.4x could be expected (5) as shown in Fig 05. In between (sub-1 AU-confocal), improvements are possible. The notoriously sensitive fluorescence samples, rely on high transmission of the optical components (AOBS and SP Detector) and a sensitive sensor (here, HyD is the choice). The advantage is that no other modifications are required, apart from the classic confocal microscope (provided the design meets the above mentioned criteria).

Resolution in the above defined sense, can additionally be enhanced by subsequent deconvolution. Here, high efficiency and detector sensitivity have a positive effect, too, as deconvolution algorithms expect an appropriately high signal-to-noise ratio.

Image Scanning Microscopy

Another idea for improving the resolution of confocal microscopes was given the name "Image Scanning Microscopy" or "Re-scan confocal microscopy (6)". This method takes advantage of the fact that the FWHM of the point image in a confocal microscope is slightly narrower outside the central diffraction disk than in the center. Basically, this is equivalent to the observation that a poorly centered pinhole leads to slightly better resolution than a well centered one – although this comes at immense cost to intensity.

Theoretically, one can expect a gain in lateral resolution of about 1.5x if recording the whole diffraction image in many channels and then distributing the intensities to the "right" pixels. However, this only applies for an infinite number of detectors over an infinitely large area. In practice, this factor is significantly smaller. If there is a claim of

improvement by more than 1.5x (e.g. 1.7x), they are using a combination of image scanning and deconvolution. Incidentally, such a microscope loses the capability to generate optical sections, as the diffraction pattern as a whole is no longer cut. If one wants to recover the optical sectioning ability, one has to confine the detection to the area of the diffraction pattern, to e.g. 1.25 AU. However, that is nearly the same as an ordinary confocal microscope with 1.0 AU. In particular, the intensity component between 1.0 and 1.25 AU is only 2%, as a zero point is crossed at 1 AU; above and below it there is not much intensity.

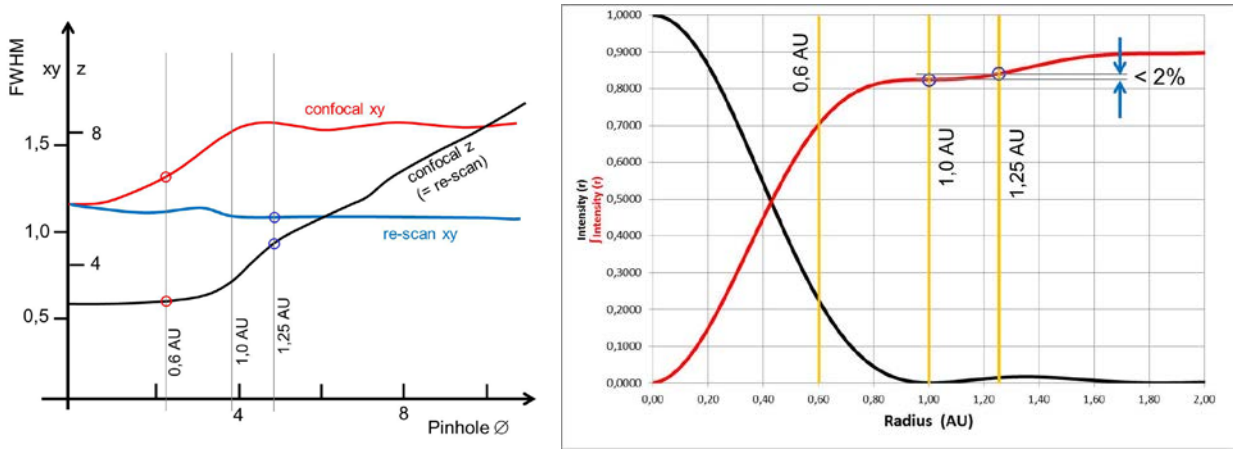


Fig 6, left: Comparison of lateral and axial performance of confocal (red and black curve) and re-scan (blue and black curve). Re-scan data adapted from (6). If a fraction covering 1.25 AU of the diffraction pattern is used for re-scanning (blue circles), the lateral resolution is improved a little, but the sectioning is significantly deteriorated. When recording confocal at 0.6 AU (red circles), the lateral resolution is well improved, and the sectioning performance is close to diffraction limited. Right: Radial intensity in a circular PSF (black) and integrated energy in an area with increasing radius (corresponding to pinhole diameter). The focal energy from 1 AU vs 1.25 AU fractions differs only by some 2%.

Additionally, the design of such instruments is often fraught with other losses resulting from the segmentation of the recording pixels. These losses easily add up to 1/3 of the overall intensity and are therefore greater than ordinary confocal microscopes, for instance, with a pinhole diameter of ≈ 0.6 AU!

Structured Illumination

Yet another different approach is a technique using structured illumination. This can be understood by looking at so-called Moiré patterns, which are formed by projecting two stripe patterns on top of one another at different angles. If one knows one of the stripe patterns and measures the Moiré pattern, it is possible to calculate the other stripe pattern. This is exactly what happens in structured illumination microscopy. The known stripe pattern is the illumination, the pattern formed when the illumination is folded with the object structures can be measured with a camera. The two pieces of information are then taken to reconstruct the third, namely the structural information. To do this, however, one has to record images in at least three different illumination directions and three phases. Better results are achieved with 5 directions and 5 phases, which means 25 image recordings altogether. Naturally, this takes some time and also subjects the samples to considerable exposure. The gain in resolution is approximately two-fold (7).

The methods described so far all show a potential improvement of detail visibility, achieving double the resolution at the most. So assuming a value of about 200 nm for conventional microscopy (using green light and an objective with an aperture of 1.3) the best one can hope for with this method is a resolution of 100 nm. The following methods are in principle unlimited. The resolutions actually achieved only depend on the parameter settings, the efficiency of the sample and the size of the emitter itself.

Localization microscopy

The image of a point is described by the diffraction pattern. In the case of a microscope with circular apertures, this is the Airy pattern. If one can be reasonably certain that a point of light comes from a single emitter ("single molecule

microscopy"), one can measure the resulting Airy figure and deduce the emission focus. One determines the center of the fluorescing electron system, therefore.

There are various methods for ensuring that truly separate emitters are measured. If the diffraction figures overlap but are still distinguishable as such, they can be localized with separation algorithms. They can be recognized as separate entities by color coding, for example, or different blinking frequencies. A separation in time is the most frequent method, for which the emitters are switched on or off. There are also various switching options for this: bleaching (switching off only), stochastic return from a dark state (8), stochastic encounter of two non-emitting partial molecules, extinguishing by another dye molecule (9), active switching with different photon energies (10) etc. The result is always an (at least temporarily) isolated emitter whose fluorescence forms an Airy pattern on a camera chip.

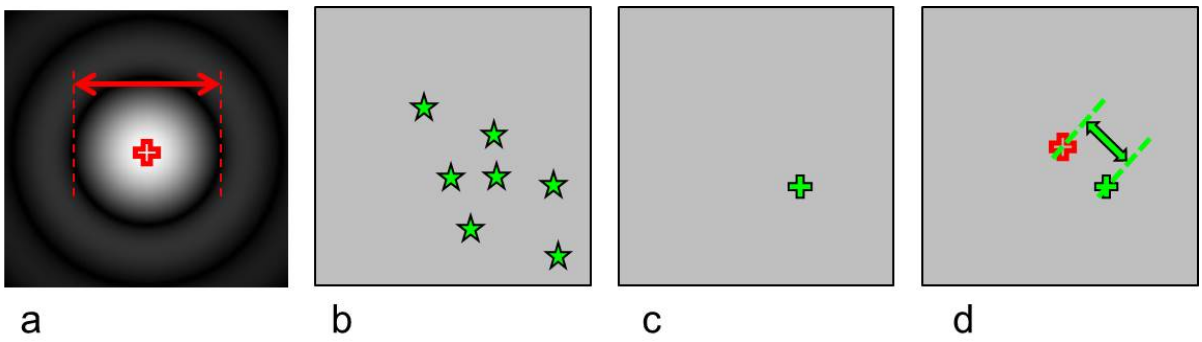


Fig 7: Accuracy of localization of a single emitter. a) theoretical PSF of a single emitter (red cross) in the center of the square. The double arrow indicates the size of the distribution (r), given by the diffraction pattern. b) a series of individual emission-collections, c) coordinate of the center of the fittest PSF –> (green cross), d) measurement error of the example shown here. The mean error is inversely proportional to the number of photons contributing to the measurement.

The accuracy with which the center of the diffraction figure can be determined again depends on the size of the diffraction pattern itself (determined by the emission wavelength and the numerical aperture of the objective) and on the number of photons that can be collected during the recording of a single image (11).

The higher the number of photons, the better the accuracy, in fact it is theoretically possible to achieve infinite accuracy (12).

$$d_{xy} \approx \frac{r}{\sqrt{N}}$$

So, there is no physical limit for a position accuracy given an infinite amount of light. The coordinates of such position measurements are transferred to an image memory and the measurement is repeated very often (several thousands of images) with emitters switched on at random in order to obtain a coherent image of the fluorescence molecule distribution. Multiple measurements of the same emitter (with different results) cannot be ruled out. The resolution in such an image is then determined by the above-mentioned position accuracy.

STED Microscopy

The first technique to describe theoretically unlimited resolution uses a phenomenon called "stimulated emission". Here, a trigger photon activates the transition of a fluorochrome from the excited to the ground state. Every laser takes advantage of this phenomenon. As described in 2.2, a confocal laser scanning microscope illuminates only a diffraction-limited area at any one time. This area is the cause of emission and its size determines the resolution. Consequently, reducing the size should theoretically lead to higher resolution. With the stimulated emission technique, excitation states can be extinguished before the emission process takes place. So, when light that is suitable for triggering stimulated emission is directed to the area with the excited emitters, the excitation states at this

position can be extinguished or prevented. To benefit from this technique, one has to make sure that the depletion laser is focused in a ring shape around the center of the Airy pattern. Otherwise, of course, all the fluorochromes will be affected and no more images can be recorded. Circular diffraction patterns of this type are comparatively easy to achieve by inserting phase plates into the illumination light path (13).

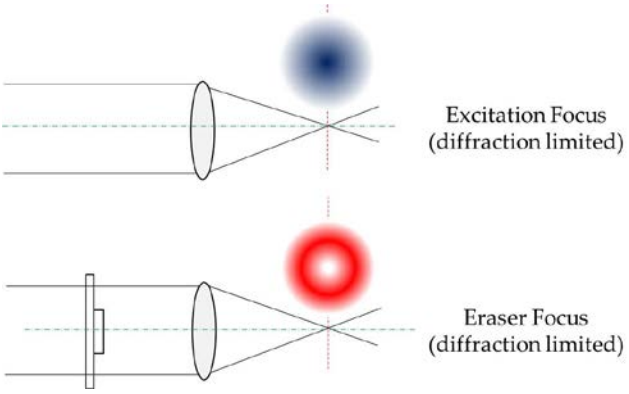


Fig 08: Excitation of a diffraction limited spot (top graph) in a STED microscope. The blue area is illuminated by a diffraction limited circular optics that generates an area of excited molecules. Illumination with a toroid focus at a stimulated emission triggering wavelength erases the outer features of the excited area, leaving a small area for emission which results in increased resolution.

The residual area now depends on the ratio of the excitation area to the "thickness" of the extinguishing ring. This dimension is again determined by the diffraction parameters wavelength and numerical aperture. In addition, however, it is also determined by the energy applied to this ring-shaped focus. The energy in this focus is ruled by the power of the depletion laser. Theoretically, the laser energy might assume whatever value – there is only a limit by current technological development. The STED technique is therefore not limited by diffraction.

The parameter that actually decides the efficiency of resolution increase at a given depletion laser energy, is the saturation intensity I_{sat}. This is a parameter is controlled by the photophysics of fluorochrome. The ratio I/I_{sat} implemented in the denominator of Abbe's formula models appropriately the effect of the depletion.

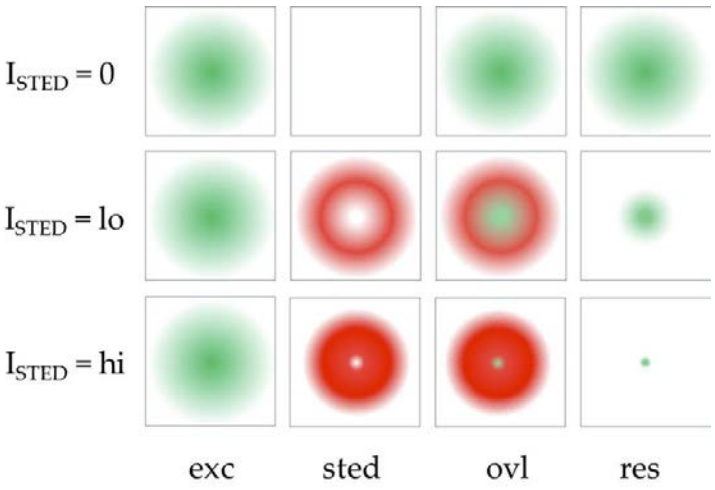


Fig 09: Impact on increasing the depletion laser in a STED microscope. First column: excitation area. This is constant for all examples, as the excitation intensity is not altered. Second column: view of the diffraction pattern of the depletion laser for increasing intensities from top to bottom. Third column: overlay of excitation and depletion. Fourth column: residual excitation area, which decreases with increasing depletion power. Theoretically offering diffraction-unlimited resolution

$$d = \frac{\lambda}{2 \cdot NA \cdot \sqrt{1 + I / I_{sat}}}$$

STED offers a number of advantages, which makes it the ideal tool for modern medical and biological research. First of all: it is an instant method. The images are generated in one sweep – no recording of thousands of images with subsequent number crunching as it is the case with localization techniques. This is crucial for life imaging at high frame rates, a must when attempting to do physiological relevant experiments. Furthermore, it is possible to combine a series of different fluorochromes, a prerequisite for correlation of signals in space and time. Although the system is not a small-scale microscope, this has a good reason. As a derivative of a confocal scanning microscope, confocal microscopy is inherently included as an alternative imaging method.

References:

1. Abbe E K (1873). "Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung". Archiv für Mikroskopische Anatomie 9 (1) 413–468.
2. Rayleigh Lord (1879). "Investigations in optics, with special reference to the spectroscope". Philosophical magazine 5 8 (49) 261–274.
3. http://en.wikipedia.org/wiki/Full_width_at_half_maximum retrieved Oct. 10th, 2014
4. http://www.svi.nl/ExpectedResolutionImprovementImpFaq retrieved Oct. 10th, 2014
5. Wilson T (1990). "Confocal Microscopy", Academic Press
6. De Luca G M R et al. (2013) "Re-scan confocal microscopy scanning twice for better resolution". Optics Express 4, 2644-2656
7. Gustafsson M G L (2000). "Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy". Journal of Microscopy 198 / 2, 82-87.
8. Hell S W, Kroug M (1995) "Ground-state-depletion fluorescence microscopy: a concept for breaking the diffraction resolution limit". Appl. Phys. B 60, 495-497
9. Rust M J, Bates M and Zhuang X (2006). "Sub diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM)". Nature Methods 3 (20), 793–796
10. Betzig E, G. H. et al (2006). "Imaging Intracellular Fluorescent Proteins at Nanometer Resolution". Science 313 (5793) 1642–1645
11. Thompson R E, Larson D R and Webb W W (2002). "Precise Nanometer Localization Analysis for Individual Fluorescent Probes". Biophysical Journal 82 2775-2783
12. Mortensen K I et al, (2010). "Optimized localization analysis for single-molecule tracking and super-resolution microscopy". Nature Methods 7 (5) 377–381
13. Hell S W and Wichmann J (1994). "Breaking the diffraction resolution limit by stimulated emission." Opt. Lett. 19(11) 780-782.

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