TRICKING ABBE
SUPER-RESOLUTION MICROSCOPY

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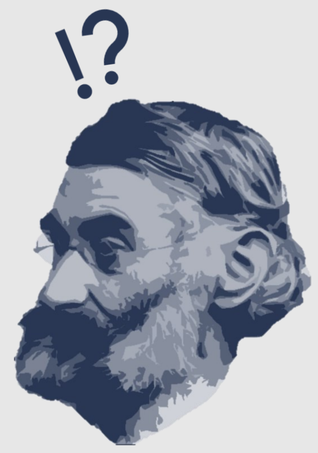

The famous diffraction limit according to Abbe represents a physical boundary on the optical resolution, which settles at around half of the used wavelength. Formerly thought to be unbreakable, advances have been made in the field of microscopy that have led to super-resolution microscopy techniques. With these, it is possible to circumvent the Abbe limit or to evade its validity and thus also map subcellular structures, for example. In 2014 Betzig, Hell and Moerner were awarded the Nobel Prize in Chemistry for the development of super-resolved fluorescence-based microscopy, which not least underpins the topicality of the subject.
Here, we therefore provide an introductory overview of this kind of microscopy and the super-resolution that can be achieved with it. We show qualitatively that the usage of a confocal setup with the Airyscan technique leads to a high gain in resolution. The non-diffraction limited STED technique enables an even higher resolution improvement by a factor of approximately 8. In addition, Fluorescence Correlation Microscopy is shortly discussed, which allows an evaluation of achievable resolutions, here especially for STED microscopy. This is completed by a demonstration of live cell imaging. For this and additionally for a z-scan of a cell we provide supplementary videos.

SETUPS

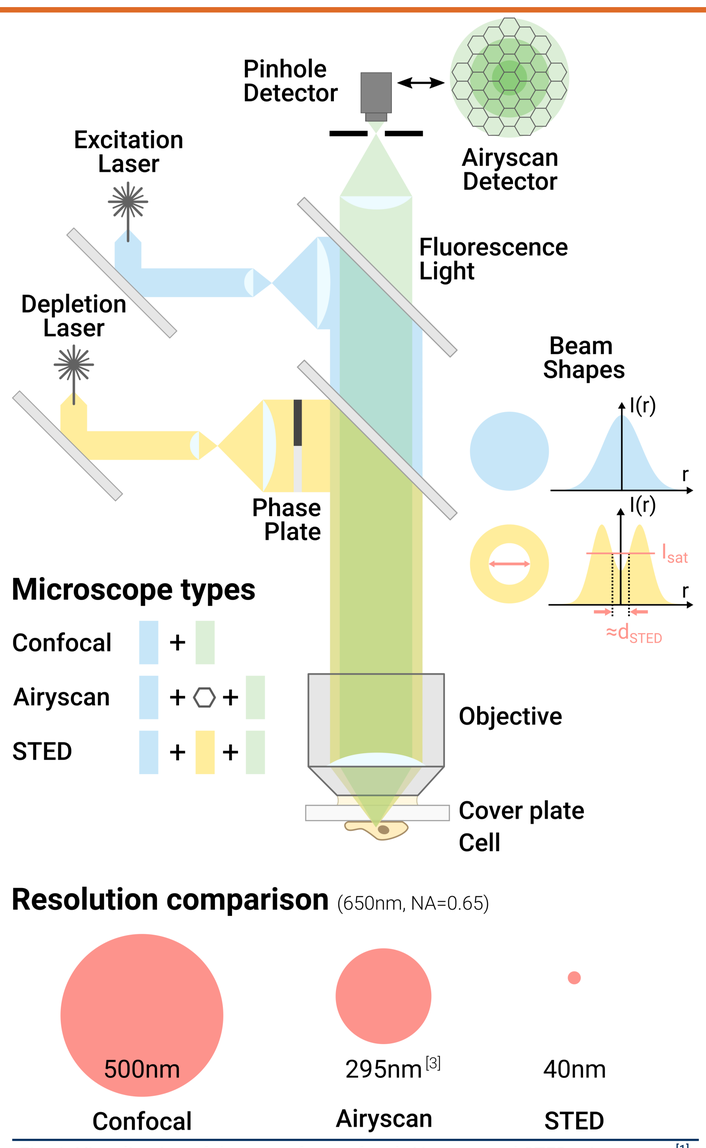

Fig. 1: Visualisation of the setup for confocal, Airyscan and STED microscopy.[1]

FLUORESCENCE MICROSCOPY

Basic Concept: The phenomenon of fluorescence can be used to generate images with high contrast compared to transmitted light microscopes. In the process, fluorophores, groupings of atoms that absorb photons and in turn emit photons of lower energy, are attached to a specific part of the specimen. The light of a longer wavelength emitted in this way can be separated from the excitation light by spectral filtering and subsequently detected. The process of fluorescence is illustrated in Fig. 2: After absorption-induced excitation and non-radiative transition, the fluorophore returns to the ground state under emission.

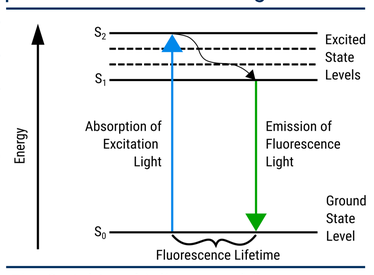

Fig. 2: Jablonski diagram of fluorescence.

Labelling: Fluorescent labelling is the process of binding fluorescent dyes to specific functional groups contained in biomolecules so that they can be visualised by fluorescence imaging. Such labels are highly sensitive even at low concentrations, stable over long periods of time and do not interfere with the function of the target molecules.[2] This allows the visualisation of the distribution of specific biomolecules over the entire specimen. The biomolecule of interest can be targeted by immunofluorescence, e.g. Alexa Fluor 488 and Abberior Star 580 attaching to mitochondria, which uses the specificity of antibodies to their antigen, which is principally shown in Fig. 3. Other techniques take advantage of the specificity of toxins, e.g. Phalloidin StarRed binding to actin, or bond-related properties, e.g. DAPI which intercalates into the structure of DNA and becomes fluorescent due to spatial restriction.

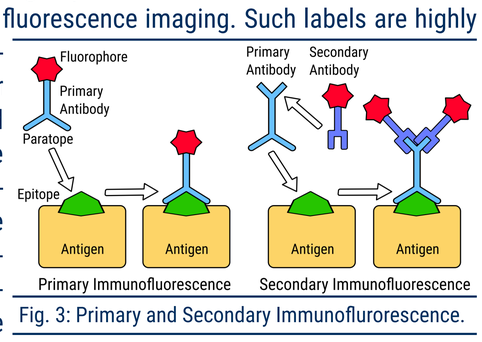

Fig. 3: Primary and Secondary Immunofluorescence.

Resolution: Resolution can be defined as the distance that two structures must have in order to still be perceived separately from each other. This distance is naturally subject to a limitation by diffraction, which is called the Abbe limit. The calculation is done by the famous Abbe formula:

$$d = \frac{\lambda}{2n \sin \alpha}.$$

Ordinary confocal microscopy is always still diffraction limited, using only a smaller pinhole in detection to block light from outside the focus and thus obtain an improvement in optical resolution by scanning the sample. By improving parameters that are not considered in the Abbe limit, it can be deliberately tricked. For example, the Airyscan method works with an improved detection principle. With STED, spontaneous fluorescence light is emitted forcibly only from a central area that is smaller than the diffraction-limited excitation focus.

AIRYSCAN MICROSCOPY

The pinhole has a considerable effect on the resolution. If its size is reduced, the resolution increases, but this is accompanied by a larger SNR. Airyscan microscopy uses a 32-channel gallium arsenide phosphide area detector, which collects a whole Airy disc, while each channel represents a pinhole with a size of 0.2 AU (Airy Units).[3] Thus it can take advantage of the improved resolution and still get a good SNR. This can be seen in Fig. 4, for which Phalloidin StarRed (red) and Alexa 488 (green) were used.

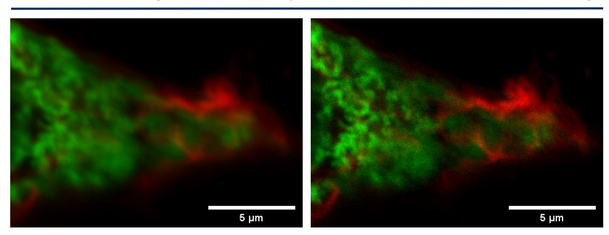

Fig. 4: Direct comparison of confocal and Airyscan microscopy.

STIMULATED EMISSION DEPLETION MICROSCOPY

STED microscopy uses the illumination of a specimen by two synchronised lasers. One delivers an excitation laser pulse of smaller duration, which is followed by a red-shifted depletion laser pulse (STED beam) from the other. The STED beam is generated using the interference of coherent light to create a vanishing intensity at the center of the focal point, which gives the beam a torus-like shape. Thus the fluorophores are first excited and then selectively certain fluorophores are re-excited by stimulated emission through the depletion pulse, effectively narrowing the point spread function. This results in a significantly improved resolution that exceeds the diffraction limit.[4] The resolution can be calculated with a modified Abbe formula[5]:

$$d_{STED} \approx \frac{\lambda}{2n \sin \alpha \sqrt{1 + \frac{I}{I_{SAT}}}},$$

Under the root is the ratio of the used STED intensity and the fluorophore-specific saturation intensity. In Fig. 5, fluorescent beads with a size of 40nm were used to estimate the resolution. For high powers of the STED beam, the values converge with the calculated ones. Lower values show a high deviation caused by the evaluation method. In Fig. 6, the same cell was imaged once confocally and once with the STED method. The fluorophores used are Alexa Fluor 488(green, confocal), Abberior Star 580 (green, STED) and Abberior StarRed (red, both).

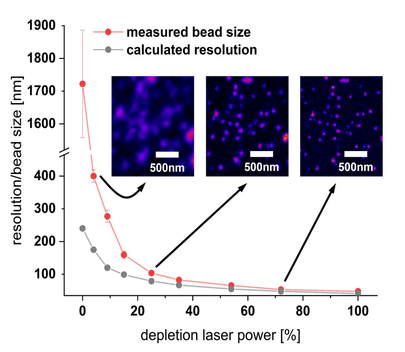

Fig. 5: Comparison of calculated and measured resolution, depending on the STED laser power.

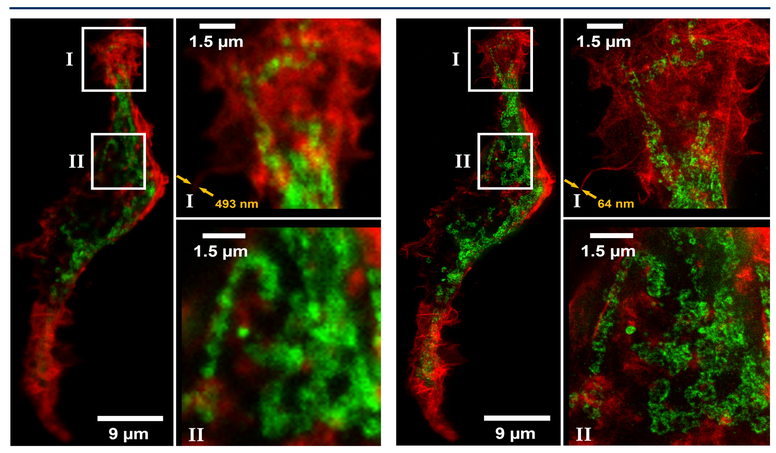

Fig. 6: Direct comparison of the resolution of confocal and STED microscopy.

LIVE CELL IMAGING

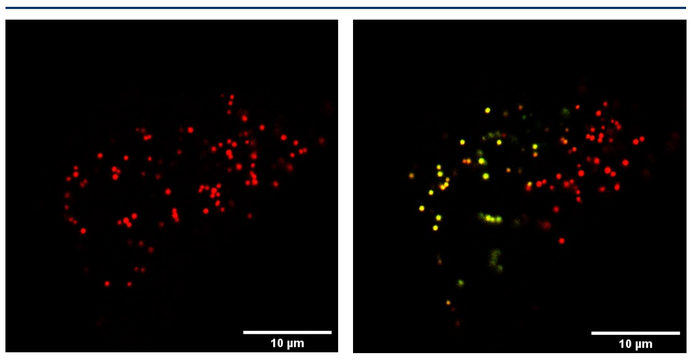

Fig. 7: Comparison of the peroxisomes before and after the photoactivation.

Microscopy of living cells enables the study of their dynamics, e.g. by using time series. This leads to a better understanding of the biological functions of the cells. Fluorescence microscopy has a great impact here because it allows to study specific organelles or even single proteins. In Fig. 7, the peroxisomes are labelled by transient gene expression with the fluorophores mCherry (red) and PA-GFP (green) and subjected to confocal microscopy. PA stands for photoactivatable, which means that the fluorophore must be activated with 400nm light before it can fluoresce. This helps to follow the motion of the peroxisomes. As visible in the supplementary movie 2, there are three types of motion: rest, vibration and fast directed motion.

FLUORESCENCE CORRELATION SPECTROSCOPY

Stationary fluctuations of the intensity of fluorescence in a specimen can be used for statistical analysis. FCS is based on the autocorrelation function:

$$G^{(2)}(\tau) = \frac{\langle \delta I(t) \delta I(t+\tau) \rangle}{\langle I(t) \rangle^2}, \delta I(t) = I(t) - \bar{I}(t).$$

Fitting the correlated data for a STED microscope properly, provides a parameter to derive the resolution:

$$d_{STED} = d_{CONF} \cdot \sqrt{\frac{\tau_{STED}}{\tau_{CONF}}}.$$

This leads to Fig. 8, where the calculated values for the resolution are presented together with the data of two spots, which were analysed with FCS. Overall, the curve could be reproduced, but there are deviations in detail, which can be attributed to too few measurements. However, FCS is more commonly used to measure the diffusion coefficient of lipid membranes or comparable structures.

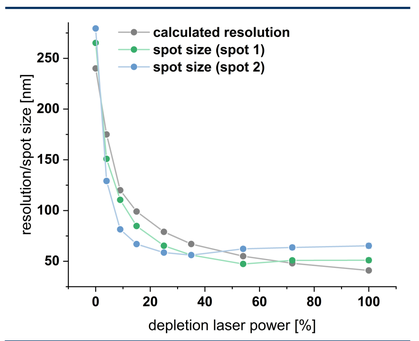

Fig. 8: Comparison of calculated and FCS-determined resolution for STED microscopy.

References and supplementary videos can be viewed via the QR code.
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