

Global BioImaging Project

D3.2 Report on test-run of virtual platform for training material

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Abstract

D3.2 reports on the successful development of a prototype virtual training module for super-resolution microscopy to be integrated into the new Myscope platform (developed and hosted by the Australian Microscopy and Microanalysis Research Facility AMMRF). [MyScope](#) is an online suite of education tools for teaching and learning in the area of microscopy and microanalysis.

During the Exchange of Experience workshop EoE I in Heidelberg (M7), WP3 together with the represented international experts defined the strategy for developing online training material on innovative imaging technologies, and the GBI partners assembled an international committee of senior imaging and training experts to prepare for the realization together with Euro-BioImaging and AMMRF. It was decided to work on two different virtual training modules for imaging technologies: a) confocal microscopy and b) super-resolution microscopy. In the following steps, WP3 partners and AMMRF discussed the mode of implementation and workflow, and decided that the Australian partners would work on the module 'confocal microscopy', whereas WP3 partner EMBL would lead the work on 'super-resolution microscopy'. This report summarizes the content of the prototype for super-resolution microscopy.

Table of Contents

1. Introduction	Page 3
2. Towards a common virtual platform for training material	Page 3
3. Overview of virtual training module "Super-resolution microscopy"	Page 5
4. Summary and next steps	Page 5
5. Annex	Page 7

1. Introduction

The international partners in Global Biolmaging WP3 are establishing a virtual platform (repository) for training material on innovative imaging technologies in close collaboration with the existing initiative “MyScope” (developed and hosted by the Australian Microscopy and Microanalysis Research Facility AMMRF). [MyScope](#) is an online suite of education tools for teaching and learning in the area of microscopy and microanalysis. It comprises several instrument based modules that enable flexible learning including self guided tutorials with videos, animations and glossary to prepare students with knowledge and specialist language; virtual instrument platforms to practice use of instrumentation; and online competency testing to demonstrate readiness for hands-on experience. MyScope has been successfully deployed at all the nodes of the AMMRF, however, uptake of the open access platform has been most remarkable internationally with more than **100,000 visits per annum**. Researchers from UK, Germany and France have been active users of MyScope with these three EU member states in the top ten accessing the platform.

The availability of MyScope and the experience of the AMMRF in developing online training tools are the foundation for the outcomes of WP3 of the Global Biolmaging Project. As part of the project, the AMMRF offers the successful MyScope platform as a building block for a Global Biolmaging Project virtual training environment. The GBI WP3 partners and AMMRF further develop Myscope to incorporate additional modules on advanced imaging technologies and enabling its integration into global online resources for bioimaging research infrastructures.

D3.2 reports on the successful development of a prototype virtual training module for super-resolution microscopy to be integrated into the new Myscope platform, which is currently completely refurbished by the AMMRF for launch in 2018.

2. Towards a common virtual platform for training material

First WP3 engaged with the international stakeholders and GBI partners, to understand the expectations and requirements for building a common virtual platform for training material. As outlined in the grant agreement, the existing *MyScope* eLearning tool developed and hosted by the AMMRF was reconfirmed as the strongest and most suitable platform to integrate new eLearning modules for cutting-edge imaging technologies. The collaboration between AMMRF and GBI WP3 is building upon the existing Collaboration Framework between the AMMRF and Euro-Biolmaging, to advance cutting-edge education in microscopy. The collaboration between Euro-Biolmaging and the AMMRF was strengthened by commonly signing a Memorandum of Understanding, which defines the development of the online learning material building on the existing Australian platform “*MyScope*”.

The work started in the first reporting period of GBI as part of the WP3 meeting during the Exchange of Experience workshop EoE I in Heidelberg (M7). After defining with the represented international experts a strategy for developing an international virtual platform for training material on innovative imaging technologies, the GBI partners assembled an international committee of senior imaging and training experts to prepare for the realization together with Euro-Biolmaging and AMMRF. A first conference call

of this committee took place in M10. Here, it was decided to work on two different virtual training modules for imaging technologies: a) confocal microscopy and b) super-resolution microscopy. In the following, WP3 partners and AMMRF discussed the mode of implementation and workflow, and decided that the Australian partners would work on the module 'confocal microscopy', whereas WP3 partner EMBL would lead the work on 'super-resolution microscopy'.

The development of the modules started directly after the call in M10, and is now being finalized parallel to the complete refurbishment of the Myscope platform by the Australian partners.

The new modules will mutually benefit users and staff of the AMMRF, Euro-BioImaging, and Global-BioImaging Project partners, in providing national and international infrastructures to support research in the areas of biological and medical sciences. The resulting modules aspire to:

- Increase user knowledge in confocal microscopy and super-resolution microscopy
- Support core facility staff by reducing on-instrument training time
- Provide best practice in the areas of user training for instrument operation

To prepare for the development of the modules, WP3 organized the following meetings and conference calls:

- Meeting of GBI WP3 together with GBI WP4 and EuBI Preparatory Phase II WP1, WP3, WP5, WP6, WP7 on synergies on training activities: overview of planned activities in both projects and first discussion on content of international training course for core facility staff (CFS) (M3, 24-25 Feb 2016, EMBL Heidelberg)
- Meeting of international training experts within the *Exchange of Experience I* workshop: first proposal of a program for the international training workshops for CFS; launch of the WP3 organizing committees 'CFS training course' and 'eLearning' (M7, 09-10 Jun 2016, EMBL Heidelberg)
- Conference call WP3 organizing committee 'eLearning' (M10, 05 Sep 2016)
- 2 Conference calls WP3-AMMRF 'eLearning' (M11, 10 Oct 2016 and 26 Oct 2016)
- Conference call WP3 organizing committee 'eLearning' (M12, 21 Nov 2016)
- Presentation of GBI WP3 activities including elearning at 'Focus on Microscopy 2017' (M17, 12 Apr 2017, Bordeaux, France)
- Regular internal conference calls of WP3 partners, technical officers and AMMRF representatives
- Continuous written communication by email between technical officers in Europe and Australia, also using other virtual means (e.g. skype).

3. Overview of virtual training module “Super-resolution microscopy”

The super-resolution module shall familiarize students and postdoctoral researchers with the basic knowledge of the Nobel-Prize winning technology and prepare them to apply the technology to their research fields by interactive training. In addition to the physical concepts, a special focus is therefore on the preparation of biological samples for super-resolution microscopy and the operation of super-resolution microscopes (commercially available by many manufacturers) by providing them a unified, virtual training environment. Training on state of the art analysis and quantification of super-resolution images will conclude the module.

Intensive discussion between eLearning and microscopy experts lead to the successful assembly of a comprehensive outline for super-resolution microscopy (see Annex), starting with the super-resolution techniques which are utilized the most in the field of biological imaging and yield the best resolution (nanoscopy methods: STED and SMLM). After the module was outlined, a literature search was conducted and experience from successful super-resolution microscopy courses at EMBL incorporated. A draft version of content (text and figures/graphics) was created and internally discussed. The constructive discussions lead to a number of improvements. As a result, the introductory parts of the super-resolution modules were optimized and converted to the format required by MyScope according to its content and programming guidelines.

To practically test the workflow with AMMRF, a prototype super-resolution module consisting of the introductory parts (mentioned above and see outline) was created in 2017 employing the existing workflow of the AMMRF by creating a master excel document for the programmer. The test for content integration into MyScope was successfully concluded. The subsequent parts of the super-resolution module are currently in production and the schedule of production is aligned with the available resources at the AMMRF to ensure a timely integration into MyScope in 2018.

Examples of the general layout of the re-launched and upgraded MyScope website are included in (annex). The new MyScope will improve on all aspects of the learning experience building on the foundations of the previous version and also improving compatibility with modern computer and software technology while eliminating legacy software plug-ins. Therefore, the super-resolution module will be state of the art in terms of content, e-learning experience and implementation of code.

4. Summary and next steps

The concept of a virtual training platform for confocal microscopy and super-resolution microscopy has been developed over the reporting period with a number of actions including all stakeholders in the GBI consortium. The production of the super-resolution module has been started and the implementation procedure of prototype content into MyScope was tested successfully.

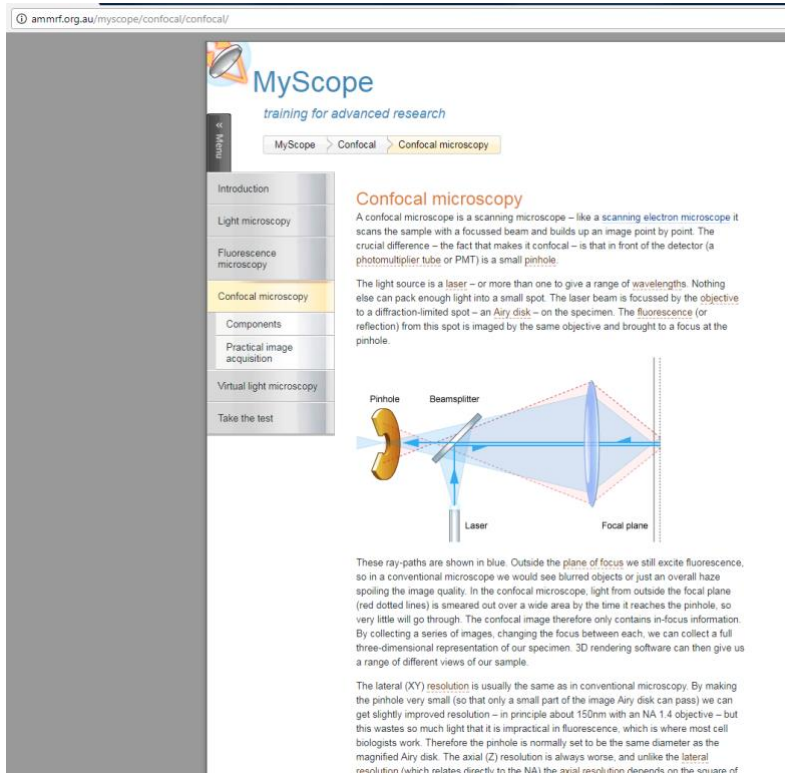
The AMMRF is currently working on the re-launch and improvement of the existing MyScope eLearning tool. As soon as the confocal module is integrated, the super-resolution module will follow. The schedule

for content production, quality review and integration into MyScope will be tightly aligned with the AMMRF and the schedule will be constantly updated to ensure the launch of the final version in 2018.

5. Annex

Screenshots of AMMRF's MyScope

amrf.org.au/myscope/confocal/confocal/



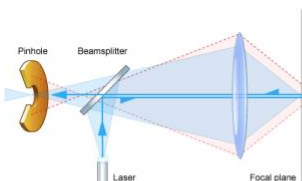
MyScope
training for advanced research

MyScope > Confocal > Confocal microscopy

Confocal microscopy

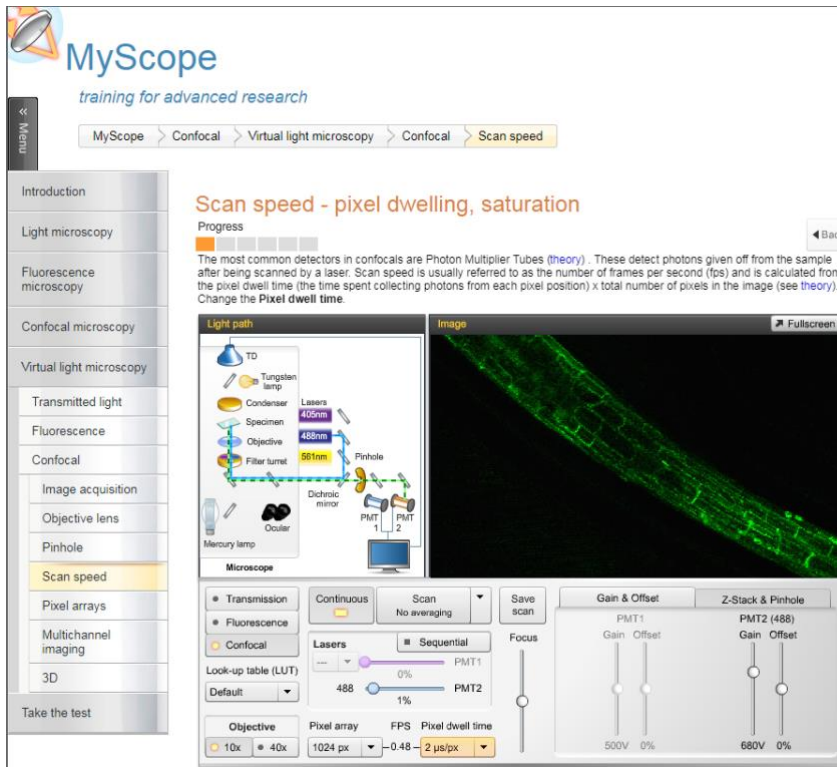
A confocal microscope is a scanning microscope – like a scanning electron microscope it scans the sample with a focussed beam and builds up an image point by point. The crucial difference – the fact that makes it confocal – is that in front of the detector (a photomultiplier tube or PMT) is a small pinhole.

The light source is a laser – or more than one to give a range of wavelengths. Nothing else can pack enough light into a small spot. The laser beam is focussed by the objective to a diffraction-limited spot – an Airy disk – on the specimen. The fluorescence (or reflection) from this spot is imaged by the same objective and brought to a focus at the pinhole.



These ray-paths are shown in blue. Outside the plane of focus we still excite fluorescence, so in a conventional microscope we would see blurred objects or just an overall haze spoiling the image quality. In the confocal microscope, light from outside the focal plane (red dotted lines) is smeared out over a wide area by the time it reaches the pinhole, so very little will go through. The confocal image therefore only contains in-focus information. By collecting a series of images, changing the focus between each, we can collect a full three-dimensional representation of our specimen. 3D rendering software can then give us a range of different views of our sample.

The lateral (XY) resolution is usually the same as in conventional microscopy. By making the pinhole very small (so that only a small part of the image Airy disk can pass) we can get slightly improved resolution – in principle about 150nm with an NA 1.4 objective – but this wastes so much light that it is impractical in fluorescence, which is where most cell biologists work. Therefore the pinhole is normally set to be the same diameter as the magnified Airy disk. The axial (Z) resolution is always worse, and unlike the lateral resolution (which relates directly to the NA) the axial resolution depends on the choice of



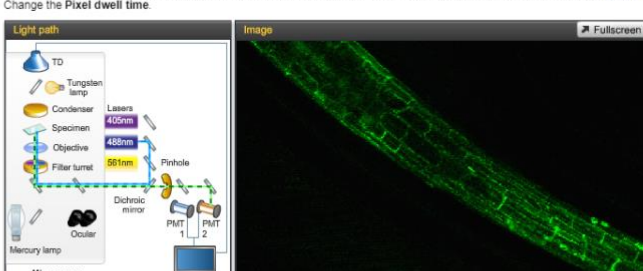
MyScope
training for advanced research

MyScope > Confocal > Virtual light microscopy > Confocal > Scan speed

Scan speed - pixel dwelling, saturation

Progress

The most common detectors in confocals are Photon Multiplier Tubes (theory). These detect photons given off from the sample after being scanned by a laser. Scan speed is usually referred to as the number of frames per second (fps) and is calculated from the pixel dwell time (the time spent collecting photons from each pixel position) x total number of pixels in the image (see theory). Change the **Pixel dwell time**.



Microscope

Transmission Continuous Scan No averaging Save scan

Lasers Sequential

Look-up table (LUT) Default

Objective Pixel array FPS Pixel dwell time

10x 40x 1024 px -0.48 2 μs/px

Gain & Offset Z-Stack & Pinhole

PMT1 Gain Offset 500V 0%

PMT2 (488) Gain Offset 680V 0%

-DRAFTED TEXT FOR THE E-LEARNING MODULE PROTOTYPE-

(This text might not or only partially be included in the final version.)

Outline of the eLearning module “Super-Resolution Microscopy”

- Green parts have been used for testing the workflow –

1. Super Resolution Microscopy

1.1. Introduction to Super-resolution Microscopy

1.1.1. Conceptual advantages of higher resolved images – a simulated example for co-localization

1.1.2. How to measure resolution?

1.2. Overview of Super-resolution microscopy concepts

1.2.1. STED

1.2.1.1. STED – the Photo-physical Principle

1.2.1.1.1. Resolution in STED microscopy

1.2.2. Single Molecule Localization Microscopy (SMLM)

1.2.2.1. PALM

1.2.2.2. dSTORM / GSDIM

1.2.2.3. STORM

1.2.2.4. PAINT and DNA PAINT

1.2.3. SIM- Structured Illumination Microscopy

1.2.3.1. [Bessel Beam]

1.2.4. RESOLFT

1.2.5. [RE-SCAN, Airyscan, SOFI, SRRF and other variants]

1.3. Sample preparation for Super-Resolution Microscopy

1.3.1. General Considerations

1.3.1.1. Fixation of samples

1.3.1.2. Properties of labels – size and brightness for crosslinked samples

1.3.1.3. Live-cell Imaging (Fluorescent proteins, SNAP-, HALO-Tag or Click Chemistry)

1.3.2. STED

1.3.2.1. Fluorophores and strategies for fixed samples

1.3.2.2. Live cell STED Imaging

1.3.3. PALM

1.3.3.1. Fluorophores and their properties

1.3.4. dSTORM / GSDIM

1.3.4.1. Fluorophores and strategies for fixed samples

1.3.5. [RESOLFT]

1.3.5.1. Fluorophores and strategies for living samples

1.3.5.2. Fluorophores and strategies for fixed samples

1.4. Super-Resolution Image Acquisition

1.4.1. STED

- 1.4.1.1. The optical path of a STED microscope and its critical components
- 1.4.1.2. Setting and optimization strategies for STED Imaging
- 1.4.1.3. [Microscope simulator for STED]
- 1.4.2.SMLM
 - 1.4.2.1. The optical path of a typical SMLM microscope and its critical components
 - 1.4.2.2. Imaging strategies for PALM and dSTORM /GSDIM
 - 1.4.2.3. [Microscope simulator for SMLM with dSTORM]
 - 1.4.2.4. Image reconstruction from SMLM data
- 1.5. [Image analysis and quantification of Super-Resolution Images]
 - 1.5.1.Genreal Differences between Super-resolution images and diffraction limited data
 - 1.5.1.1. Analysing STED images
 - 1.5.1.2. Analysing SMLM images
 - 1.5.1.2.1. Cluster analysis for interaction studies
- 1.6. Glossary
- 1.7. Literature index
- 1.8. Test / (Self-) Evaluation

[]: optional – advanced content which might be produced (dependent on the decision of the advising committee)

1 Super-Resolution Microscopy

1.1 Introduction to concepts of Super-Resolution Microscopy

Super-resolution microscopy has revolutionized the field of light microscopy. For the first time it became possible to visualize structures beyond a physical limit: the diffraction limit which was governing and limiting the maximum resolving power of microscopes.

The diffraction limit (d) according Ernst Abbe is defined as:

$$d \approx \lambda / 2NA$$

with

λ : wavelength of the light

NA : numerical aperture of the objective

The typical maximal achievable resolution with standard light microscopes is limited to a practical achievable value due to technical constraints and typically in the range of ~250 nm laterally and ~500-700 nm axially. With Super-resolution microscopy, image resolution of less than 40 nm can be routinely achieved and below 10nm has been demonstrated.

Super-Resolution Light Microscopes are only superseded by electron microscopes in resolution which require to image non-living specimen in high vacuum.

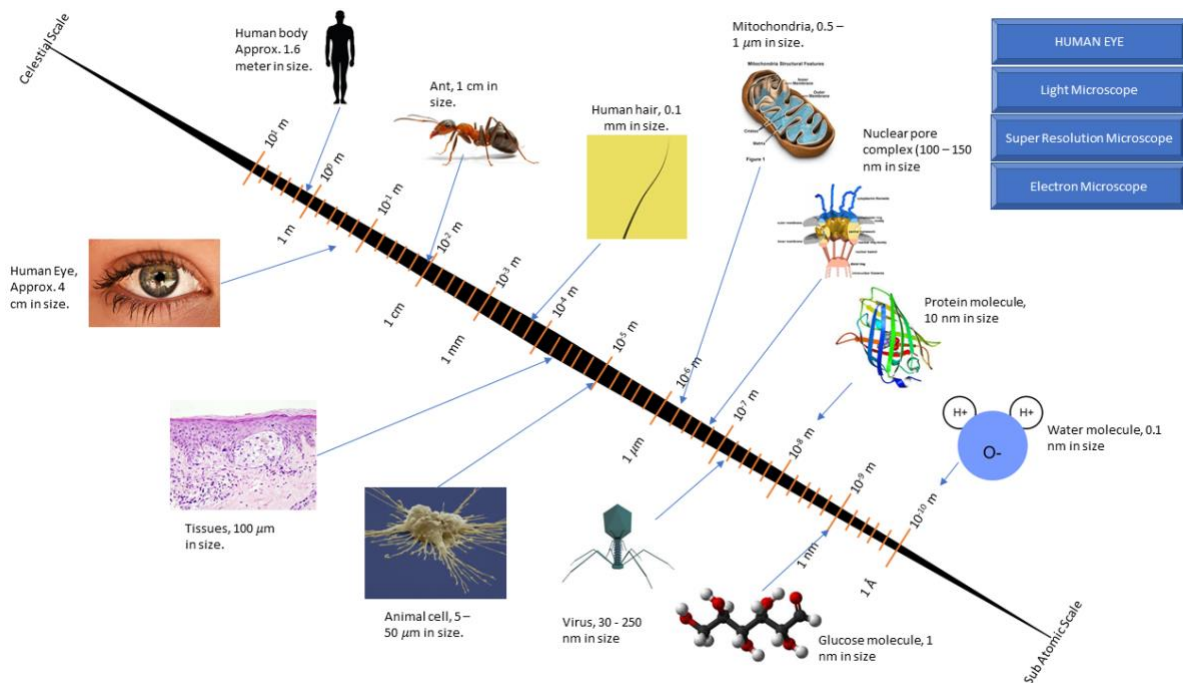


Figure 1: Scales in Biology: Biological Structures and Specimen span a wide range of sizes. Smaller entities require tools for magnification and detection. These tools have physically limited maximum resolution. Please explore the animation panel to the top right.

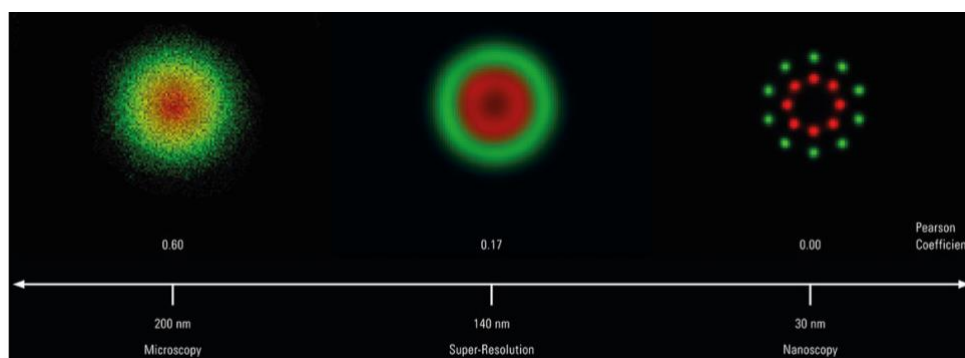
Which is the resolution barrier defining a Super-resolution microscope? Currently, multiple definitions are used. Common of all of them is that the resolution of the Super-resolution microscopes needs to exceed the Abbe limit. However, some set the Abbe limit as the threshold and other require at least a 2-times enhancement over the Abbe limit as the barrier which needs to be overcome to use the term super-resolution.

The term nanoscopy is currently used in equivalent to super-resolution microscopy if the resolution of the microscope surpasses 80nm.

1.2 Conceptual advantages of higher resolved images – a simulated example for co-localization

Higher resolution can be considered always an advantage, but it can change the view on standard image analysis concepts, for example measuring co-localization of fluorescently labelled proteins. Fig. 2 displays the same simulated structure (ground truth) as they would be imaged with differently resolving microscopes and also reports the Pearson Coefficient as measure for co-localization: In a standard confocal image the structure seems to be partially co-localized which is also reflected by a Pearson coefficient of 0.6. By applying deconvolution or other means of increasing moderately the resolution into a range of 140nm, Pearson drops to 0.17 and the structure seems to consist of two concentric rings of different diameter. If nanoscopy (e.g. STED microscopy) is applied with a resolution of 30nm, the rings can be subdivided into dots that do not co-localize at all (Pearson: 0.0).

The concept of co-localization should therefore be always seen (and reported) as being dependent on the resolution applied in the imaging process. Please note that the two fluorophore-labelled proteins can of course never occupy the exactly same space and they only appear to do so due to the resolution limit and subsequent blurring coming from the imaging technology applied.



Simulation of red and green entities organized in two rings as seen at different levels of resolution. Data courtesy of Remko Dijkstra, Scientific Volume Imaging Deconvolution powered by Huygens - SVI

Figure 2 Simulated image to demonstrate the effect of resolution on co-localization measurements. Confocal image on the left, deconvolved confocal image with higher resolution in the center and STED super-resolution images on the right. Please note that not only the appearance of the structure is changing with the resolution but also the Pearson coefficient. (Image courtesy: SVI, NL)

1.3 Criteria for resolution and resolution measurements?

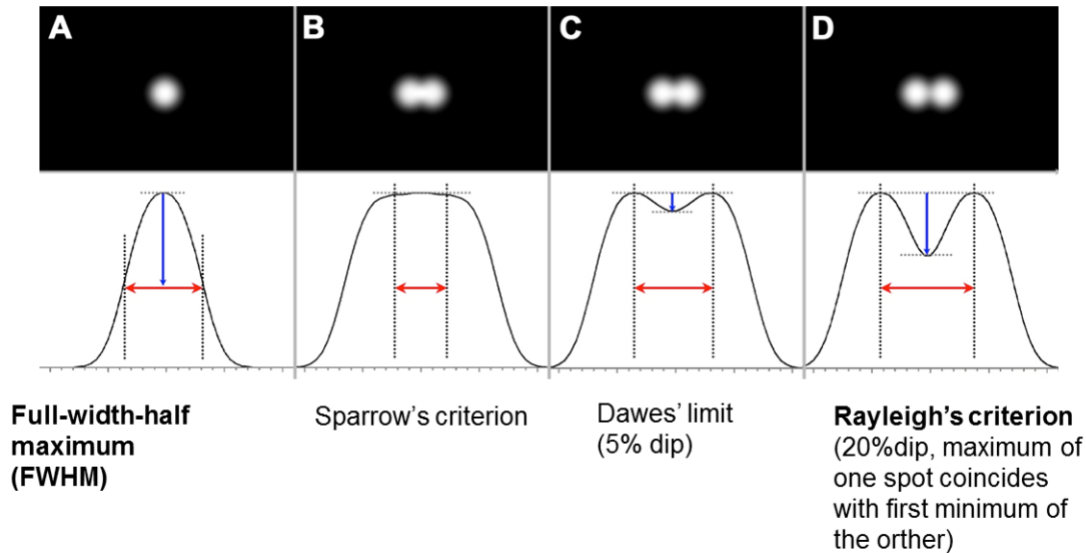


Figure 3 Measuring resolution from one (A) or two (B-D) point emitters. Dependent on the criteria applied, depicting different distances between the two emitters are formally needed to resolve them.

The resolution of microscopy is classically defined by the capacity to separate (visually) two objects. This can become practically important when the necessary resolution for a biological experiment needs to be estimated to assess the technical feasibility.

A number of criteria have been defined in the past to express the resolution of a microscope and the practical importance for a biological experiment should be considered. Sparrows criterion defines two-point emitters as resolved, as soon as a plateau is visible in the middle between them instead of a single peak. By applying the Dawes limit or Rayleigh criterion, a dip in intensity between the point-emitters of approx. 5 or 20% is required, respectively. Another approach is measuring the Full-width-half-maximum (FWHM) of a single point emitter. The calculated Abbe resolution is close to the Dawes limit and approx. 2/3 of the Rayleigh value.

Biologically samples are more complex and of higher density than the ideal two-point-emitters depicted above. As a consequence, a higher resolution (than the Abbe-limit) is often required to resolve the structures of interest reliably. In practice, using an approx. 3 times higher resolution provides enough information in the image to achieve reproducible and consistent data (e.g. if the structures of interests are 60 nm apart, an optical resolution of the microscope between 15-25nm delivers sufficient detail for analysis).

2 Overview of Super-resolution microscopy concepts

2.1 Stimulated emission Depletion (STED) microscopy

STED is the abbreviation for Stimulated Emission Depletion. It employs the fact that an excited molecule can be spontaneously switched off by illuminating it using a wavelength which is within the emission spectrum of the respective molecule. The duration of the molecule in the excited state is very short (usually in the range of ns), therefore a relatively high light intensity has to be used to switch off (or in other words deplete) the molecule. STED microscopy is based on a confocal microscope and therefore typically implemented with single-point scanning confocal microscopes.

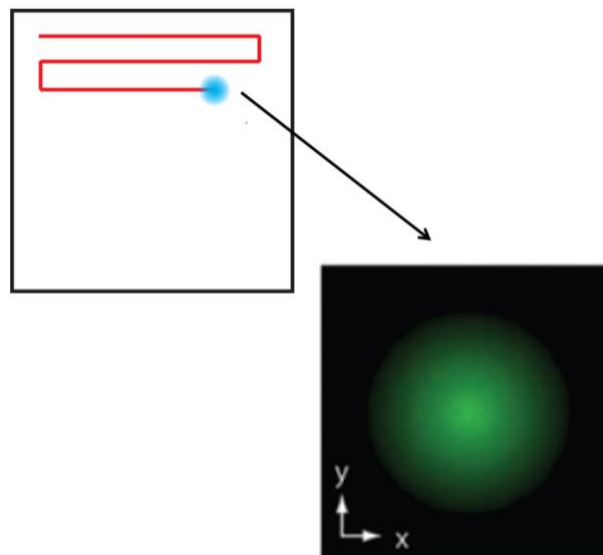


Figure 4 Principle of confocal scanning: A Gaussian shaped excitation beam (lower right) derived from a focused laser is scanned over the sample in a line wise fashion (upper left).

By controlling the switching off of the excited fluorophores spatially, a higher resolution can be achieved: First, a diffraction limited laser spot (in the absorption range of the given fluorophore) excites the fluorophores in the area of the spot. Fluorophores have a lifetime in the ns range in the excited state before returning to the ground state by emitting a photon. However, if a STED beam is applied, the molecules can return to the ground state (be switched off) much faster. This does not provide any advantage as long as the depletion beam is of the same shape as the excitation beam. The molecules are only switched off faster (and practically invisible since they emit photons of the same wavelength as the powerful depletion beam and cannot be distinguished from the light by this beam). If the depletion beam is shaped in a different way, e.g. like a donut, the following “trick” can be applied: a laser spot (of Gaussian shape) is used to excite the fluorophores; the STED beam is formed to a (larger) donut with no light intensity in the center and projected on top of the excitation beam (and therefore the molecules in the excited

state). Molecules which are hit by the intense depletion beam are switched off practically immediately. This leaves only the fluorophores in the center to emit photons with their normal lifetime and to be detected. The fluorescence is now emitted from a much smaller spot than the excitation spot and can be therefore more precisely registered in the pixel-based image by using smaller pixel sizes (typically 20nm instead of the diffraction limited optimum of ~120nm): super-resolution is achieved.

Stimulated Emission depletion (STED)

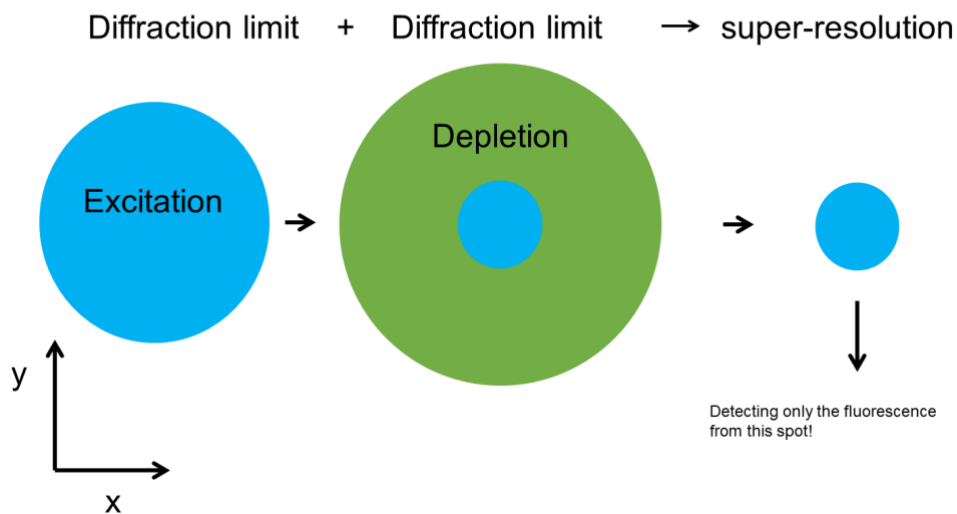


Figure 5 Simplified, schematic principle of STED microscopy: The Gaussian excitation beam is overlaid by the donut shaped STED beam, which is “switching off” the excited fluorescence molecules. The remaining fluorophores are emitting with their normal lifetime and are detecting. The region of emission is much smaller than the original area of excitation and therefore the PSF is smaller and the resolution is increased.

2.2 STED – the Photo-physical Principle

A STED microscope is usually based on a point scanning confocal fluorescence microscope and employs reversible ON (Fluorescent-) and OFF (Non-fluorescent)-states of fluorophores.

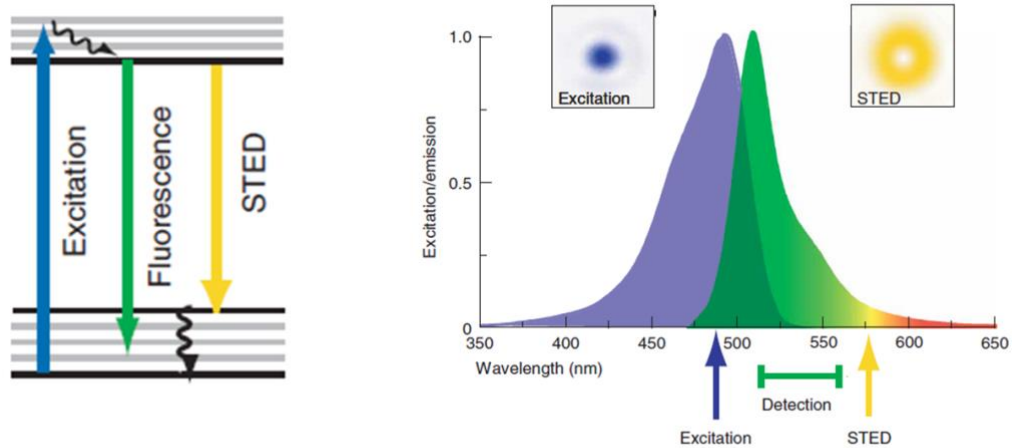


Figure 6 Photo-physical principle of stimulated emission depletion. Simplified Jablonski diagram (left): Upon excitation, the electron of the fluorophore is transferred from the ground state to the first excited state. The electron can return to the ground state via fluorescence or “forced” to the ground state via stimulated emission depletion. For stimulated emission depletion (STED), a photon with the same energy difference of the excited state to ground state is interacting with the excited molecule and forcing the molecule to spontaneously relax and emit an additional photon of the same energy than the incoming photon. Choosing the wavelength of light for fluorophore excitation and stimulated depletion (right): The excitation wavelength is chosen in the standard way like for any other conventional fluorescence microscopy technique. The STED wavelength should efficiently overlap with the emission beam, but a) not overlap with the excitation spectrum nor b) minimize the detection window for fluorescence. The wavelength of the STED beam is often located in the “tail” of the emission spectrum.

How can fluorophore switching be achieved? One way is to employ stimulated emission: If a fluorophore is excited by absorption of a photon, an electron is “catapulted” from the ground state (S_0) to the first excited state (S_1). It would autonomously return to the ground state after a few ns – the exact lifetime in the excited state is fluorophore and environment depended and is often reported in the literature. However, if the fluorophore in the excited state interacts with another photon of the same energy as a possible transition from S_1 to S_0 state, the fluorophore is immediately relaxed by emitting a photon at exactly the same energy (or wavelength) as the photon causing the stimulated emission and the electron returns to the ground state. The allowed transitions between S_1 and S_0 for stimulated emission are the same that form the emission spectrum of a given fluorophore.

The lifetime of an excited fluorophore is short and to ensure a “depletion” of the fluorescence, high photon fluxes delivered by a high-power laser are required. Therefore, it is possible to turn

excited molecules selective and defined OFF by using stimulated emission to deplete the fluorescence: **STimulated Emission Depletion**.

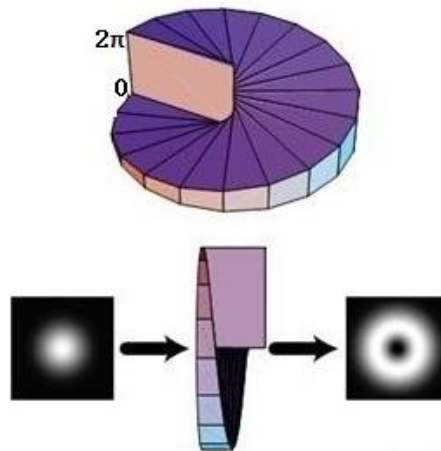


Figure 7 Schematic drawing of a phase plate (also called phase mask): The phase mask is a stepwise increasing polymer staircase delaying the beam for up to 2π (top). The phase mask is placed in the laser beam, converting a Gaussian shaped profile into a donut profile in the sample plane (schematically, bottom).

To enhance the lateral resolution in a confocal microscope, typically a donut shaped STED beam with no intensity (a “zero”) in the center is employed. A donut shaped beam can be created by placing a so-called phase mask (Fig 7) in the beam path of the STED laser. In the excitation path of the confocal microscope, the STED donut beam is centered on top of the Gaussian shaped excitation beam. These two combined beams are scanned with the galvo-scan mirrors over the sample.

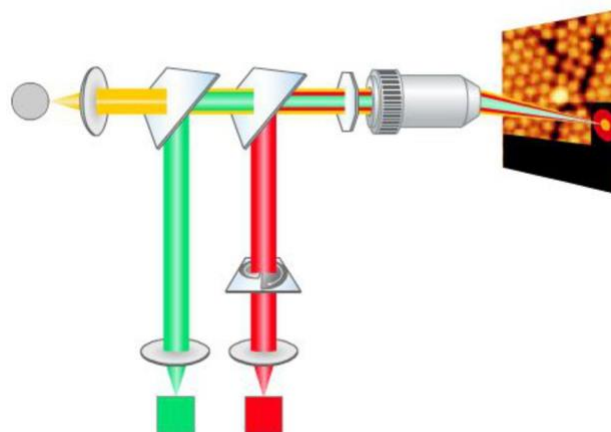


Figure 8 Incorporation of the STED laser beam into a single point confocal scanning microscope: The excitation laser of the conventional laser scanning microscope and the STED beam are combined via a dichroic mirror and then scanned coincidental over the sample. Please note the $\lambda/4$ plate placed in the beam path close to the objective.

The use of different phase masks can create different STED depletion beam shapes (fig). The most common ones are the 2D-STED-donut, only improving the lateral resolution over the confocal microscope resolution, and the 3D-bottlebeam, predominantly improving the axial resolution leading to a near isotropic PSF.

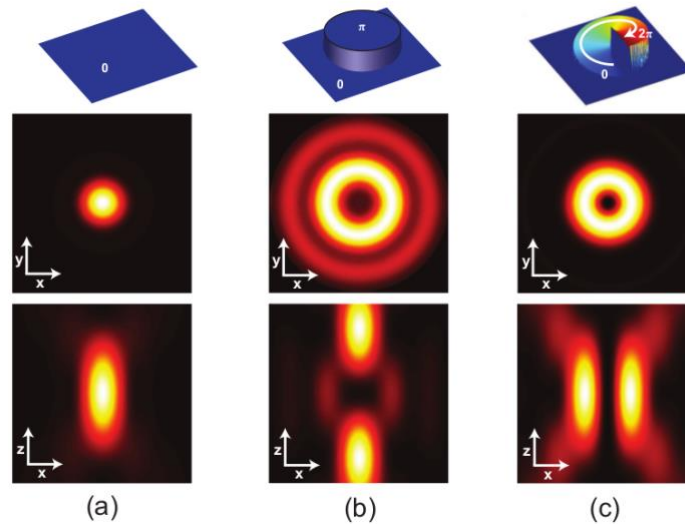


Figure 9 Effect of different phase masks on the shape of the STED laser beam: no phase mask displays the typical Gaussian beam (left), a staircase 0-2pi phase mask creates a donut, increasing the lateral but not the axial resolution (right). A single step of pi creates a depletion beam that is enhancing lateral and axial resolution and can create a close to isotropic resolution of <100nm (middle).

2.3 Resolution in STED microscopy

The theoretical resolution of STED microscopy can be calculated by a modified Abbe formula (extension in blue).

$$D = \frac{\lambda}{2n \sin \alpha \sqrt{1 + \frac{I}{I_{\text{sat}}}}}$$

With:

D: resolution

λ : Wavelength of the emission light

N: refractive index of the medium

α : half-angular aperture

I: intracavity intensity / peak intensity of the STED laser

I_{sat} : saturation intensity

Whereas I is proportional to the laser power of the STED beam, I_{sat} is a fluorophore dependent parameter: The lower the saturation intensity of a given fluorophore the easier to switch the molecules from the excited state (ON state) to the ground- or OFF-state. I_{sat} is defined as the laser intensity at which 50% of the molecules can be switched off. The resolution can therefore be enhanced by carefully selecting the optimal fluorophores.

In addition, the resolution can be improved by increasing the STED laser power (I) (typical STED laser have power ratings in excess of 1W power output). The scaling of the resolution in STED images is usually achieved by altering the STED laser power after selecting optimal dyes for STED microscopy.

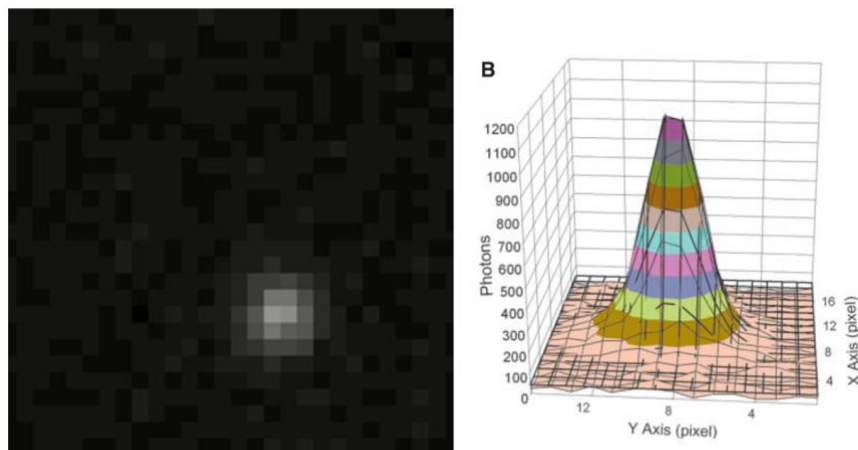
By shrinking the volume in which fluorophore are allowed to emit photons (PSF) the signal-to-noise (S/N) ratio is decreased as a consequence. This leads to noisier images if no measures are taken to increase the number of photons emitted by the reduced volume, e.g. by using accumulation of multiple images of the same region or simply by increasing the laser power for excitation.

2.4 Single Molecule Localization Microscopy (SMLM)

Single Molecule Localization Microscopy (SMLM) is based on Widefield-microscopes often by using laser based illumination in TIRF- or Epifluorescence mode.

Long-time before images with super-resolution have been acquired by detecting the fluorescence of single molecules, scientist were already detecting e.g. motor-proteins labelled with single dyes to address their mode of action *in vitro*. These scientists were taking advantage of the feature that the fluorophore is always located in the center of any diffraction limited spot derived by this (single) fluorophore.

The center of the diffraction limited spot be calculated with extreme precision (achieving nanometer precision), e.g. by fitting the signal recorded on the camera chip (typically an EMCCD- or sCMOS-camera) with a Gauss-function. The human vision is pretty good in doing the same while watching a myosin molecule “walking” a long a myosin-filament *in vitro* (Fig.Z) – although the spot is much larger than any of the steps, it is quite apparent where the center of the spot is located and what step size could be.



Movie

Figure 10 Example of diffraction limited, single molecule detection of a myosin molecule labelled with a single fluorophore and moving along an actin filament *in vitro*. Please note in the movie, that also small steps can be identified by eye (left). Fitting 3D-Gaussian function to a diffraction limited spot of the image sequence allows precise localization of the position of the fluorophore (right).

In contrast to a single myosin molecule labelled with a single fluorophore, typical biological samples consist of millions of labelled (with a similar or even higher number of fluorophores) proteins in close proximity. As a consequence the point spread function of each single fluorophore emitting light are overlapping to such an extent that they cannot be separated by a typical diffraction limited standard microscope.

To localize all molecules marking a densely labelled structure, any of these molecules has to be turned on and off in a certain manner to ensure that the position of all molecules is detected at least once. In other words: instead of looking at all molecules at once, the molecules are observed sequentially and thereby separated not in space but in time. After having recorded the coordinates of all of this fluorophores, an image with improved resolution can be created by this information. The super-resolution image can feature a smaller pixel size without “empty magnification” (typically 20nm or below).

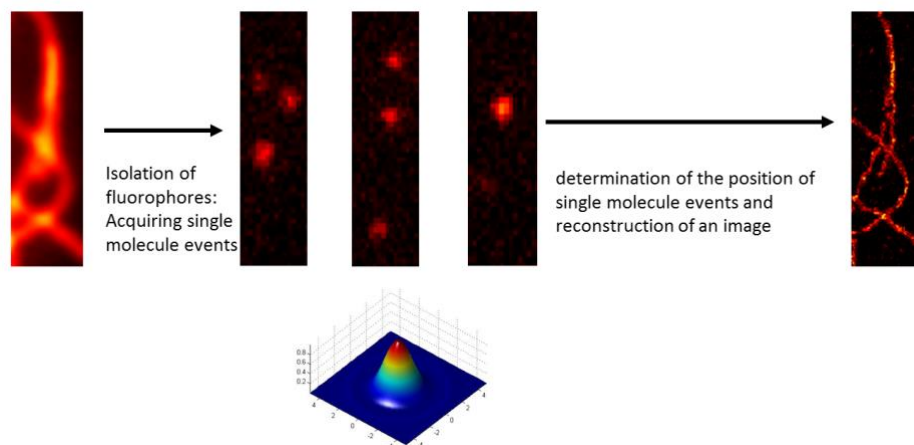


Figure 11 Principle of Single Molecule Localization Microscopy (SMLM): A densely labelled fluorescent sample is depicted on the left. By reducing the number of active fluorophores, single molecules can be detected in different images over time. After Gaussian fitting, the precise coordinates of each fluorophore are translated back into a (super-resolved) image (right).

2.5 PALM

The first method applied to singularize fluorophores in an otherwise densely labelled sample was coined Photoactivated Localization Microscopy (PALM). A special kind of fluorescent proteins was used to control the number of emitting molecules. These can be activated from a non-fluorescent state (like PA-GFP) or switched from one fluorescent state to another (e.g. mEOS from green to red fluorescence) upon activation with UV light. Single molecule detection could be achieved by tuning the activation light to such a low level that in a given area only one fluorophore is activated. After a while, the active fluorophore is bleached and a new fluorophore can be activated in the same area. Images are recorded in fast time-lapse until all molecules have been imaged and the individual fluorophores are localized by Gaussian-fitting for the reconstruction of the Super-resolution image.

PALM: Photoactivated localization microscopy

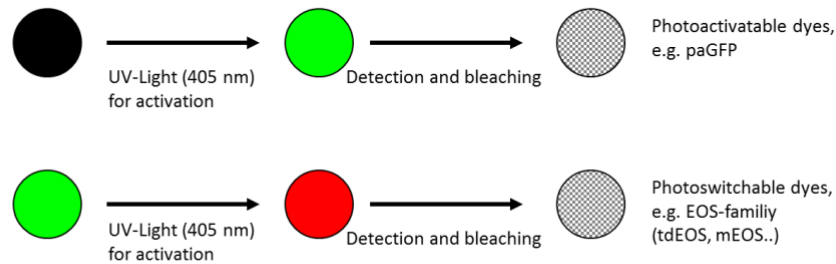


Figure 12 Types of PALM-capable fluorescent proteins: Photo-activatable fluorescent proteins like PA-GFP are non-fluorescent at the native state, but can be converted into a fluorescent species upon UV-irradiation. During the imaging procedure, these molecules are destroyed by bleaching and therefore new fluorophores can be activated (top). It has been proven easier for the researcher to see – even diffraction limited- the structure of interest, e.g. for focusing, and not navigating blindly in the sample. To this end, photo-switchable fluorescent proteins, like the mEos-family, are commonly used. They are fluorescent from the start and can be converted to a red-shifted fluorescent species upon UV-light exposure. Only the red fluorescent species is detected in PALM and the same detection and fitting scheme applies as for PA-GFP.