MYSC**O**PE
MICROSCOPY TRAINING

Light &
Fluorescence
Microscopy

Train for advanced research

Welcome

MyScope was developed by Microscopy Australia to provide an online learning environment for those who want to learn about microscopy. The platform provides insights into the fundamental science behind different microscopes, explores what can and cannot be measured by different systems and provides a realistic operating experience on high end microscopes.

We sincerely hope you find our website: www.myscope.training an enjoyable environment. In there you can explore the microscopy space and leave ready to undertake your own exciting experiments.

Module Contents

Credits

Introduction - Basics

Optical microscopy uses visible light, and its performance is inherently limited by the wavelength of light. This ranges from 400nm (violet) to 650nm (red). There are two different ways of calculating resolution, according to whether the specimen is illuminated externally (Abbe calculation) or is effectively self luminous as in fluorescence microscopy (Rayleigh calculation). Both give similar results, the difference lies in how to set up the microscope for best performance. With an ideal lens, diffraction limits the resolution to about half the wavelength of light, and our best objectives come within 95% of this.

What makes an objective good?

The most important quality of a lens is not its magnification, essential though that is, but its numerical aperture. This is defined as the sine of the maximum angle (from the vertical) at which light can enter. It is 'numerical' because it is a ratio, the actual size of the lens makes no difference. It could be, and usually is, a small lens very close to the slide, but where a large working distance is required it can be larger and further away (and probably more expensive).

30°
NA 0,5

72°
NA 0.95
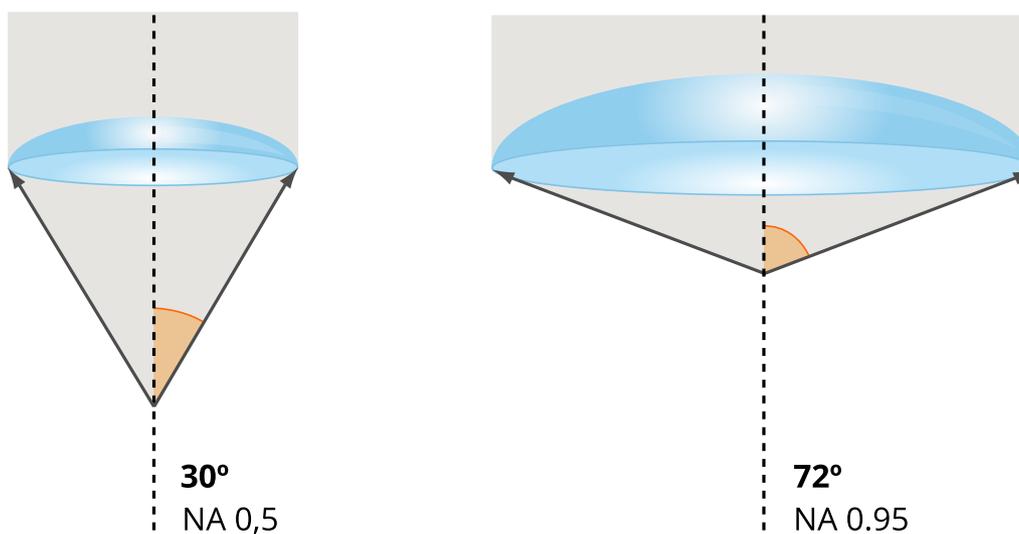
The largest acceptance angle we can get, in practice, is about 72°, which gives us a numerical aperture (NA) of 0.95. Considering that even if we put the lens flat on the specimen the NA would only be 1, it's clear there is little room for improvement. NA 0.75 would be the highest 'dry' lens found on most microscopes, and that will give a resolution roughly equal to the wavelength of light. Why 'dry'? Because if our sample is sitting in something with a higher refractive index than air, we should in principle be able to get better resolution, since the wavelength of light becomes shorter the higher the refractive index.

The snag is that we must keep the refractive index constant the same all the way to the objective, or the highest-angle rays will be bent away and won't reach the lens. So if our specimen is in a permanent mounting medium of refractive index (n) ~ 1.5, we put immersion oil of the same index between the coverslip and the lens. The NA is now conventionally given as the sine of the acceptance angle multiplied by n, and an oil immersion objective can get up to an NA of 1.4. This will now give us a resolution of about half the wavelength of light, which is pretty impressive when you think about it.

Glass: n=1.5

Oil: n=1.5

Glass: n=1.5
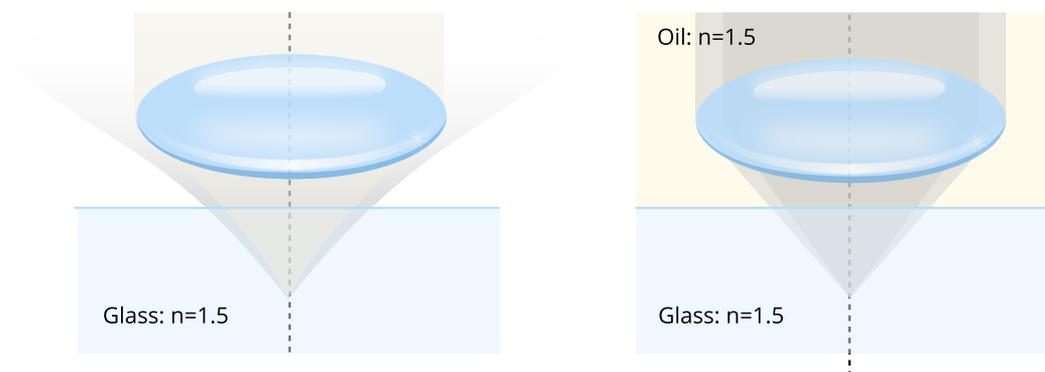
With living samples in water (n = 1.3) we have to use a water immersion objective, and the resolution boost will be a bit less.

Aberrations

So far it all seems easy - we just make a lens which is very wide compared to its working distance. Unfortunately lenses are not perfect, and two imperfections are particularly problematic. Spherical aberration is an inherent property of a simple lens - the lens is more powerful at the edge that in the centre, so the image will not be sharp.
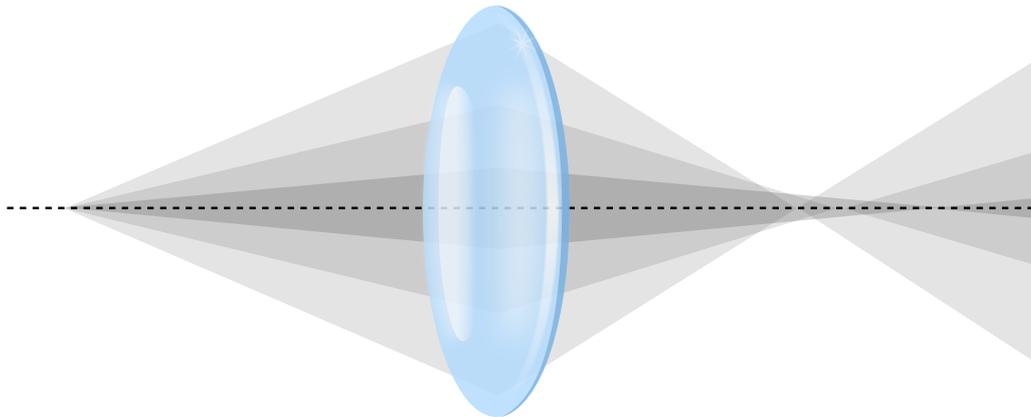
Spherical Abberation

Chromatic aberration is an inherent property of glass - it has different refractive indices at different wavelengths. That is why a prism splits white light into a spectrum. This means that we cannot get all colours in focus at the same time. We can overcome this by using just one colour, and this works well, for example, when looking at living cells under phase contrast. But in the wider picture, we would be throwing out one of the great advantages of light microscopy - the ability to show different structures or substances in different colours.
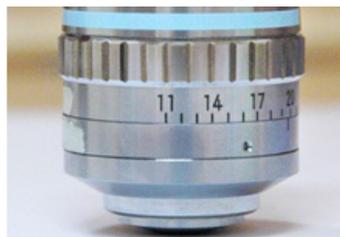
Unfortunately, both these aberrations get worse with increasing NA, and they do so very much more rapidly than the resolution increases. Without correcting these aberrations we cannot hope to make a usable high-NA lens. Fortunately we can correct them, but it requires multiple optical elements, which is why high-NA objectives are expensive. However, spherical aberration can only be corrected for one precise set of optical conditions and, for example, using the wrong thickness of coverslip, or using an oil-immersion lens on a sample in water, will spoil the correction. It becomes so tricky that very high NA objectives often have a correction collar to adjust for different coverslip thicknesses (in a dry lens) or temperature and salinity (in a water lens).

Chromatic correction come in various grades, from achromat (basic correction) through fluorite (better) to apochromat (best). Best correction is not always best for your experiment, though, since apochromats contain a lot of glass and will therefore absorb more light than fluorites. Some apochromats also do not transmit UV very well, which can be a problem in fluorescence. Also, lenses are only corrected for a particular range of wavelengths - usually blue to red. Their performance can be very bad outside this range, and with the increasing use in microscopy of violet and UV at the short end and far-red and near IR at the long end, this can be a problem. Apochromats with different correction ranges have become available to meet this need.

Finally, the objective is only one of several optical elements in a microscope, and manufacturers design all of these to work together. Swapping objectives between different brands of microscope is therefore not a good idea.

Light microscopy - The complete microscope

The traditional layout of a complete 'compound' microscope is shown here. The objective forms a real, magnified and inverted image because the sample is further from the lens than its focus. The image is 'real' because it can be projected on a screen - a slide projector produces a real image in this way (and we therefore have to put slides in upside down). The eyepiece is placed quite close to this real image - too close to form a real image of it. Instead the rays which reach the eye appear to come from a magnified 'virtual image' located further away. The virtual image is not inverted, so in the end we always see an inverted image of the specimen.

Modern research microscopes modify this layout a bit. The problem with the simple arrangement is that the distance between objective and eyepiece must be absolutely fixed, since spherical aberration can only be corrected for one position of the image. If we want to add in components for fluorescence, polarization and so on we are in trouble. Modern objectives put the specimen at the focus of the lens, so they will form an image 'at infinity' - that is, the rays from any one point on the sample leave the lens parallel to each other. This won't form an actual image, so an additional lens, the tube lens brings the rays to a focus just in front of the eyepiece, as before. The diagram below shows this layout, and indicates where each component is in the actual microscope.

The great advantage of this plan is that it doesn't matter (within reason) what the distance is between the objective and the tube lens - the rays are parallel and so the SA correction is unaffected. There is a limit, of course, or rays from objects at the edge of the field of view will get cut off. Nevertheless the few centimetres of free space we gain are very valuable.
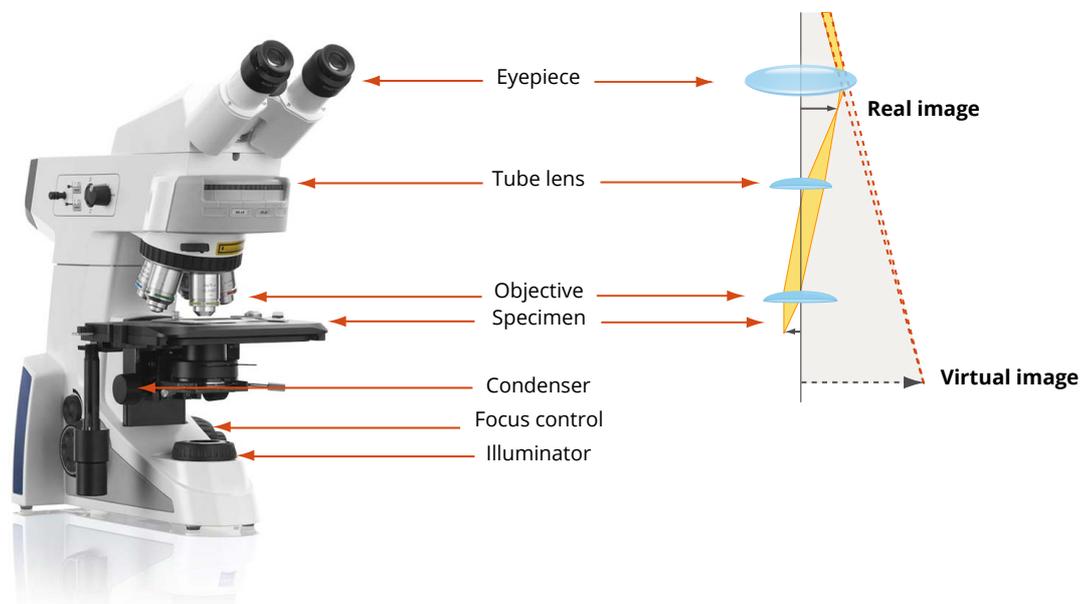
Microscope photo by ZEISS Microscopy [CC BY 2.0 (https://creativecommons.org/licenses/by/2.0)], via Wikipedia Commons

The other feature of a modern research microscope is that the illumination system is built in. Abbe showed that when we view an object with transmitted light diffraction at the sample, not just the objective, limits our resolution. We therefore need a condenser lens to illuminate the sample with an NA matching that of the objective. Since the illuminator has to be aligned with the condenser it makes sense to build this into the microscope as well.

Just because the condenser and illuminator are built on does not absolve the user from the need to adjust them correctly, and the next section explains how to optimise the system. Do not fall into the trap of assuming that if you are just doing fluorescence or confocal you don't need this. You will almost always want to capture a phase or DIC image to match your fluorescence, and if you are going to use the (non-confocal) transmission detector built into most confocal microscopes the condenser and illuminator must be accurately set up or the image will be terrible.

Koehler illumination

Koehler illumination was first introduced by August Koehler in 1893 to provide optimal contrast and resolution in light microscopy that complement the numerical aperture of the objective lens. The process involves aligning and focusing the light path, and adjusting the apertures. Koehler illumination has to be performed every time objective lenses are changed.

The light path and microscope

There are two sets of conjugate planes in Koehler Illumination. In the first set, the field diaphragm, objective front focal plane (specimen), the intermediate image plane and the retina are in conjugation with one another. In the second set, the filament, condenser focal plane, objective back focal plane and the iris of the eye are in conjugation. The latter is best viewed by removing the eyepice and inserting an eyepiece telescope or Bertrand lens.

Set 1: Conjugate Field Planes
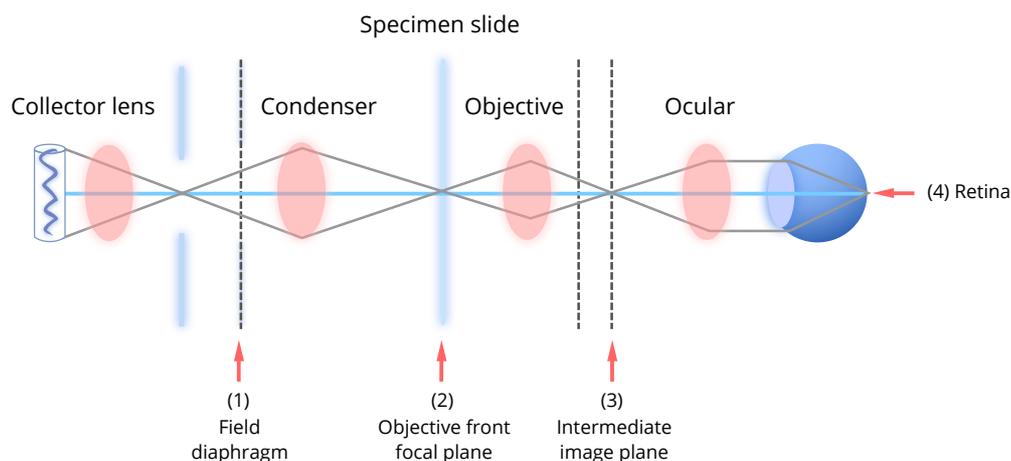
Set 2: Conjugate Aperture Planes
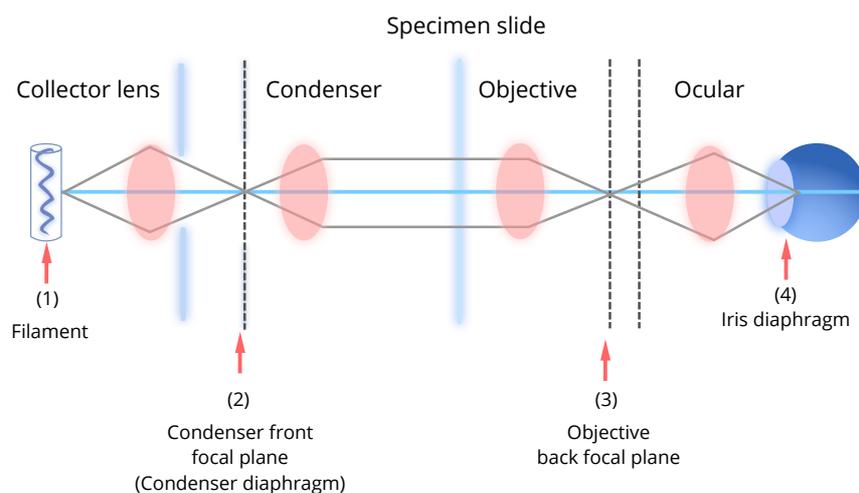
The positions of conjugate planes in light microscopy. These planes are located where the light rays crossover. Note that there are two sets of conjugate focal planes in a light microscope adjusted for Koehler illumination.

The collector lens is located between the lamp and the field diaphragm. It gathers the light from the lamp, and magnifies and focuses an image of the filament at the front focal plane of the condenser (Set 2). This can be achieved by focusing the condenser using the condenser focus dial.

The field diaphragm is located in front of the condenser. It is used to adjust the illumination field reaching the specimen and should not exceed the capacity of the objective lens. Illuminating extraneous objects can cause light to scatter into the lens and cause glare. This will in turn, reduce contrast and resolution.

The condenser focuses light onto the specimen plane. This light then spreads from the specimen onto the objective lens (Set 1). The condenser also forms an image of the field diaphragm (Set 1).

The condenser diaphragm is adjusted so that the light achieves an angle that sufficiently fills the back focal plane of the objective lens (Set 2). This is important to achieve good resolution in the image. Removing the eyepiece allows you to view the back focal plane of the objective where an image of the condenser diaphragm appears. Adjusting the condenser levers focuses the image (Set 2).

The objective lens focuses the specimen image onto the intermediate focal plane (Set 1). This lens should also form an image of the filament at its back focal plane. (Set 2).

The ocular focuses the cone of light emerging from the image of the filament at the objective's back focal plane onto the eye's iris (Set 2).

The eye lens focuses the diverging rays of light from the ocular onto the retina where an image of the specimen is formed (Set 1). The filament, however, cannot be focused but is viewed as a field of light.

Performing Koehler illumination

This is a brief summary of the steps involved when performing Koehler illumination:

1. Place specimen on stage and focus.
2. Focus the field diaphragm by adjusting the condenser levers.
3. Open the field diaphragm to the edge of the field of view and centre using the condenser centering controls.
4. Adjust the condenser diaphragm until it is 2/3 open.

Brightfield (BF) Imaging -
What is Brightfield imaging?

Brightfield (BF) microscopy is the simplest type of microscopy and is generally used with compound microscopes. The specimen is usually dark and contrasted by the surrounding bright viewing field, hence the name. BF microscopy has many applications, both in biological and materials sciences. It can be used to view fixed samples or live cells. For transparent samples, as is often the case for biological samples, various staining methods must be applied to create contrast that allows the sample to be visible under the microscope. BF microscopy can be expanded to different complimentary techniques that allow for increased contrast and visualisation of unstained samples. These techniques include differential interference contrast (DIC), Phase, Polarised and Dark Field (DF) imaging methods.
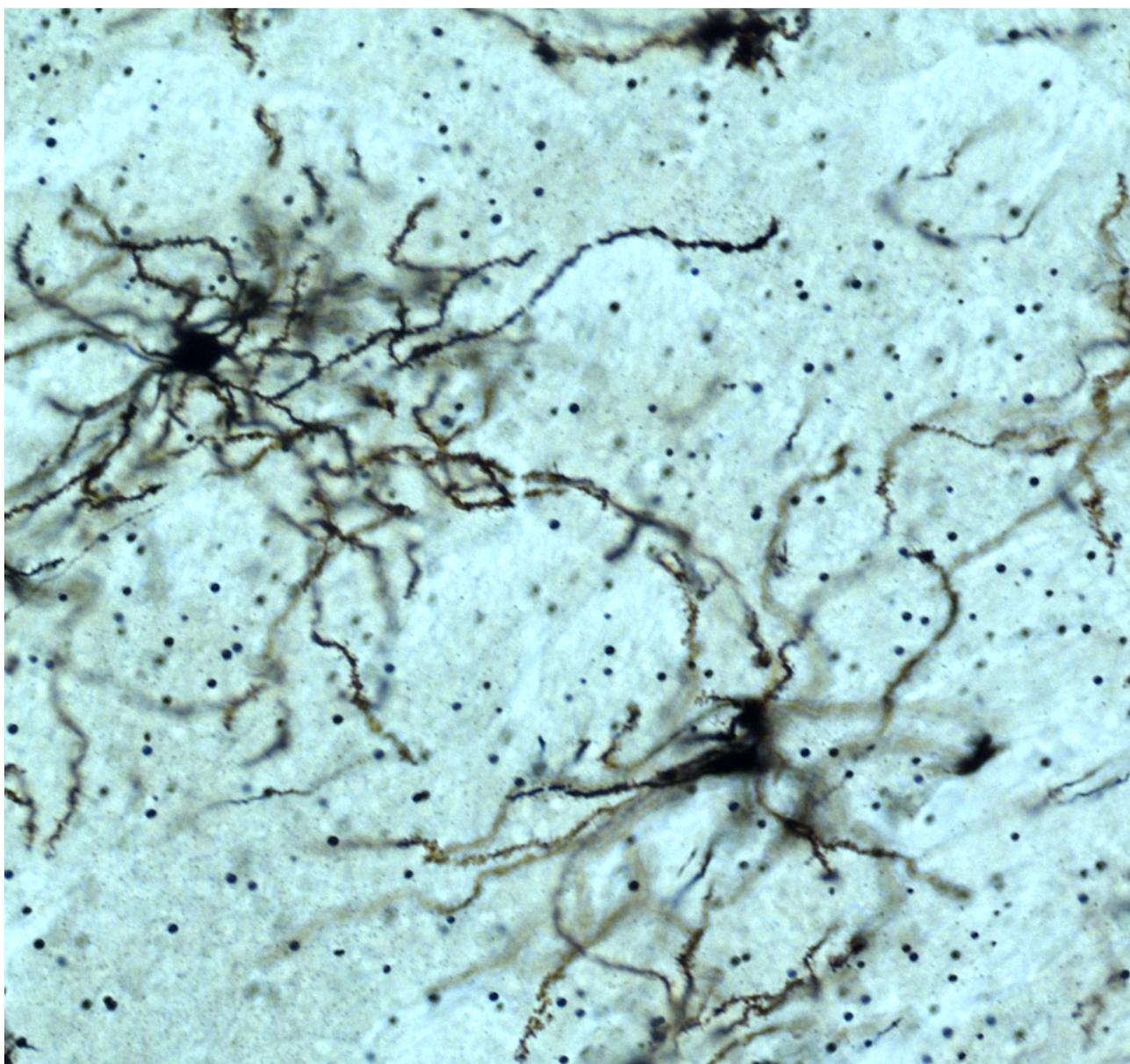
BF image of astrocytes labeled with DAB

Microscope components for transmitted light imaging

To achieve proper illumination of the sample for a given imaging modality, a specific array of optical components must be aligned in the microscope. These components are described below.

Light Source

A halogen lamp is the standard light source for BF imaging

Condenser

A condenser lens focuses the light onto the sample (figure_below, white arrow). In upright microscopes, it is located below the sample (and vice versa for inverted microscopes). There are different types of condensers based on their correction for optical aberrations, the most common and simplest being the Abbe condenser (this condenser is not corrected for any aberrations).
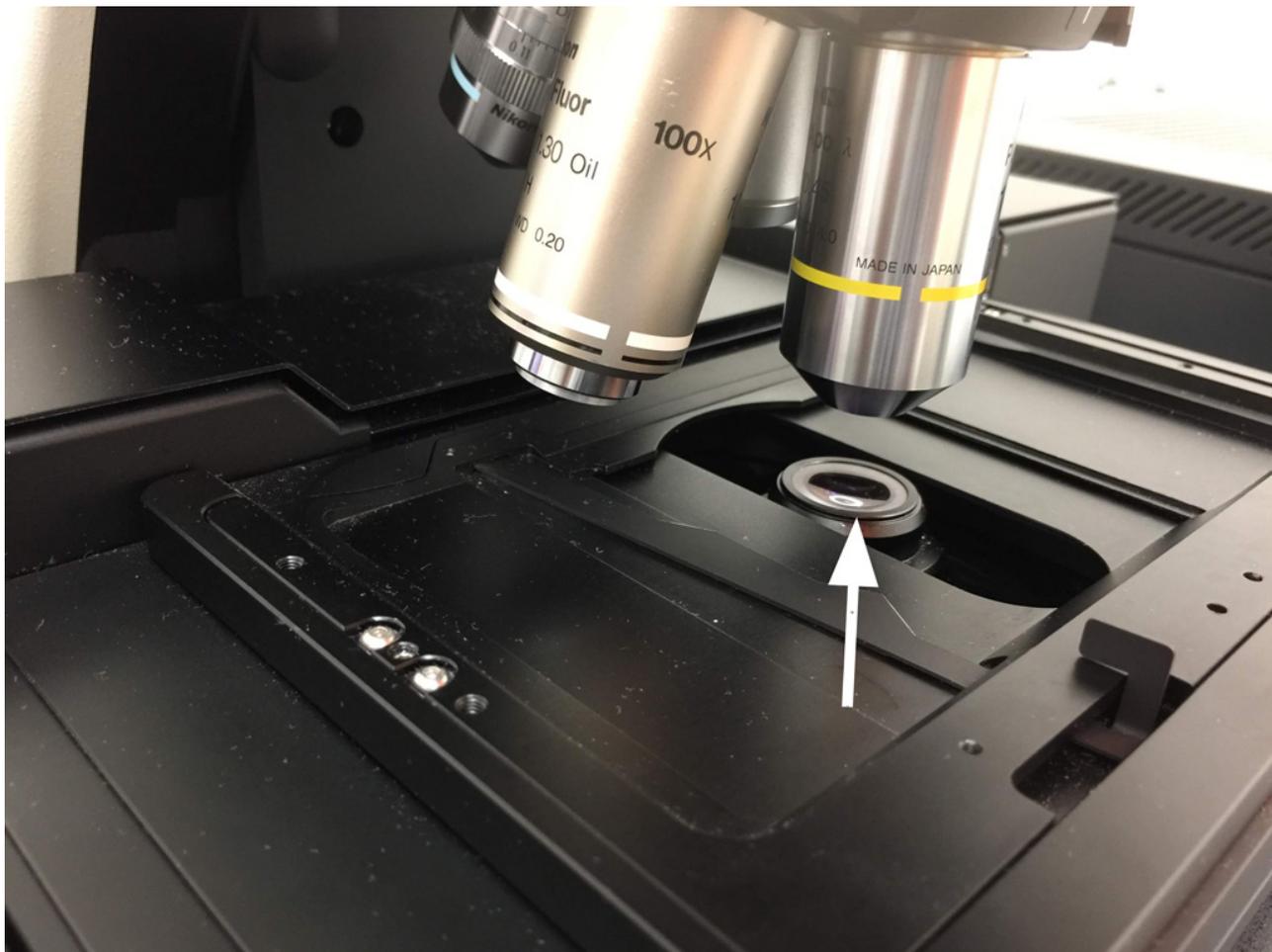
The condenser lens is situated below the stage on an upright microscope as shown here (white arrow)

Field Diaphragm

Controls the diameter of the light beam emitted by the light source before it enters the condenser. This should be adjusted to match the aperture of the condenser.

Objective

Different types of objectives can be used for BF imaging, from low magnification (e.g., 2.5x) to high (e.g., 100x). The chosen magnification depends on the information required from the sample, for example visualising broad anatomy or looking at smaller details within a cell. Specialised BF microscopy, like DIC or Phase, can only be performed with objectives designed for such purposes. This can be deteremined by checking objective specifications, which are usually listed on the objective itself. The figure_below shows a DIC crapable objective (green outline).

This objective is capable of DIC imaging, as specified on the objective (green outline).

Oculars and/or Camera

The sample may be visualised through the oculars or imaged with a camera. BF microscopy utilises colour cameras, like normal photography, to capture RGB images. Most of these cameras are capable of collecting 8 bit (per channel), 12 bit and sometimes 16 bit images. The 8 bit/channel = 32 bit images are usually sufficient in terms of the information collected for image analysis and may be opened by most software.

Analyser

The analyser is placed between the objective lens and the eye-piece. It is not required for basic BF imaging. However, for DIC and other types of imaging where polarisation of light is required, the analyser should be inserted into the light path.

Polariser

This prism is placed in the light path, beneath the specimen, to work together with the analyser in polarising light for Polarised microscopy. The analyser and polariser can be rotated to achieve omnidirectional light waves.

Nomarski DIC Prisms

For DIC imaging, the light beam is split into two beams below the sample and then converged after passing through the sample. For this, a Nomarski prism is installed into the condenser (below the sample) and above the objective (above the sample). The figures below show the prism that is inserted above the objective.

The DIC Nomarski prism is inserted above the objective to converge the light after it passes through the sample

Annulus Aperture and Phase Plate

Phase microscopy allows visualisation of transparent, unstained samples and is based on the phase shift of light passing through a specimen. The key elements for phase contrast microscopy are an annulus aperture and a phase plate. The annulus aperture is placed in the condenser and limits the angle of the penetrating light waves. The phase plate lies in the objective and has a phase ring made of a material that dims the light passing through it and changes its phase.

Light Path for BF microscopy

The general light path in BF microscopy is as follows: Light enters the condenser and travels towards and through the specimen and into the objective. From there, it travels through the filter turret (which may or may not have a specialised filter in place) and towards a viewing method, i.e., oculars or camera.

The five techniques

There are 5 main types of transmitted light imaging, where light passes through the sample: basic bright field, differential interference contrast (DIC or Nomarski), phase, polarised, and dark field. All these techniques can be conducted on a standard light microscope fitted with the required accessories. Köhler illumination is required to optimise the light path for all these techniques, just as for basic bright field.

Each of these transmitted light techniques is described below and has specialised applications.

Some types of samples that are not thin or transparent, such as whole insects, or pieces of rock or minerals, require the light to be reflected off the sample. Reflectance microscopy will be discussed in the next section.

Bright field

Basic bright-field imaging directs a focussed beam of white light through the sample. The transmitted light is collected after it has passed through the sample and an image in natural colour is produced, which you can see down the eyepieces or on the computer screen.

Bright-field microscopy is extremely useful for stained or naturally pigmented samples, and ones where thin slices can be prepared. However, these conditions may not be suitable for viewing your particular sample. Staining is not always convenient or possible, especially when you want to observe living cells. There are some stains you can use in living cells, but they serve a different purpose than the wide range of stains used to highlight different tissue types in fixed specimens. In unstained living cells or tissue sections the light passes through the transparent or translucent samples with little or no interaction. Therefore, very little definition of the component structures can be generated. To address these issues the techniques described below can provide a route to useful imaging by the introduction of contrast.

Applications of bright-field microscopy include research and diagnostics to view fixed and stained (e.g. with H&E) tissue sections or cell films/smears, and for immunohistochemistry (IHC) where antibody-coupled enzyme reactions give rise to coloured precipitates (e.g., DAB).
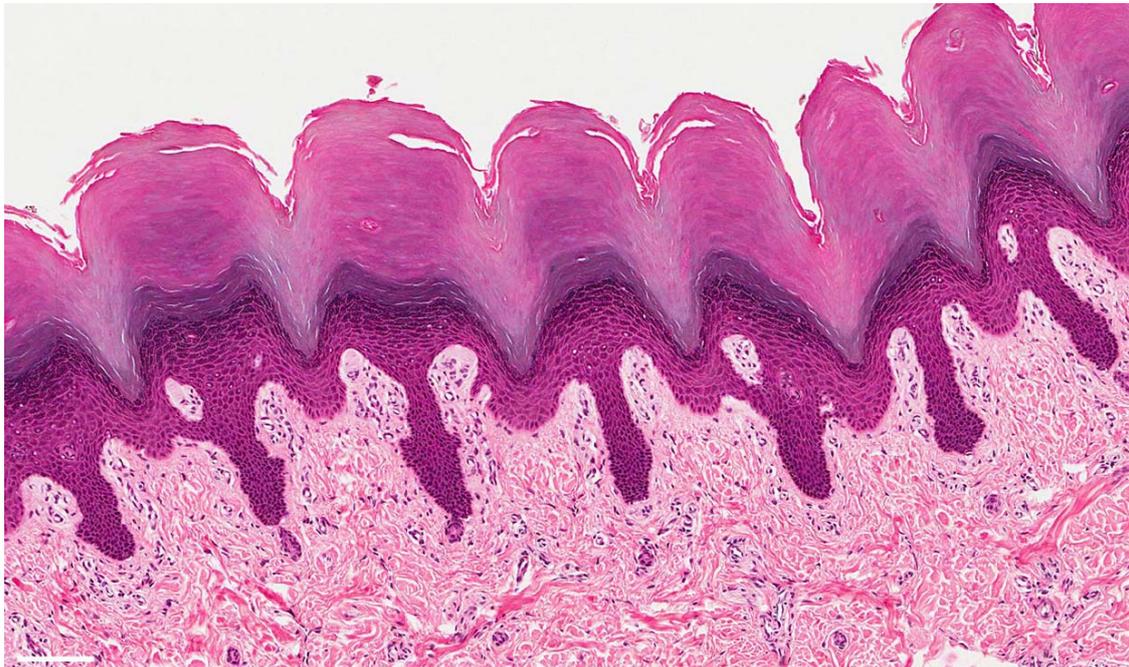
Skin sample stained with H&E - BF image of skin.
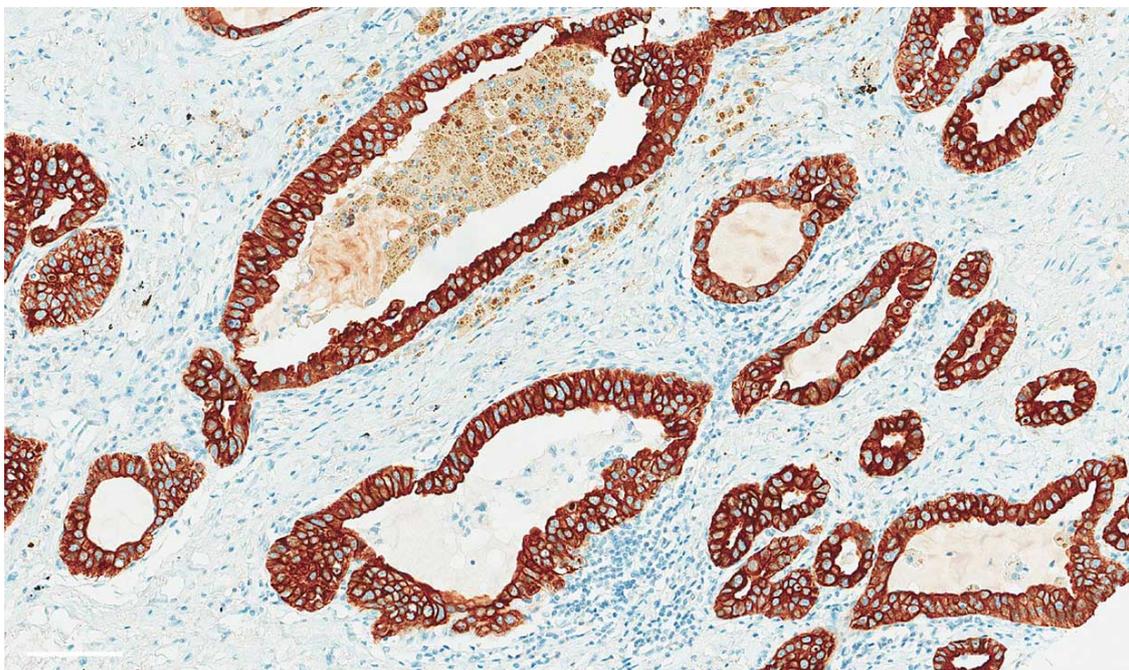
Liver sample showing imunocytochemistry cytokeratin – detected with DAB. Counter stained with methylene blue.

Dark field microscopy

This is the oldest technique for looking at unstained samples using diffracted light. A hollow cone of light is produced by replacing the condenser diaphragm with a ring-shaped aperture or by using a fibre-optic ring illuminator. You can also get condensers that concentrate light into ring rather than losing it. To achieve a dark field, these devices must ensure that all the light is directed outside the acceptance angle (θ) of the objective lens so if left undeflected it will not enter the objective. Light scattered up to an angle of 2θ can enter the objective.

Put simply, this means that if there is nothing in the sample to deflect the light, the light beam will continue out past the edge of the objective and that part of the image will appear dark. Where something in the sample scatters the light, the light is deflected so it can enter the objective and that area appears bright.
Darkfield is very sensitive to dirt and contamination, as these features tend to scatter more light than specimen.

Objective

θ

Condenser

Ring stop
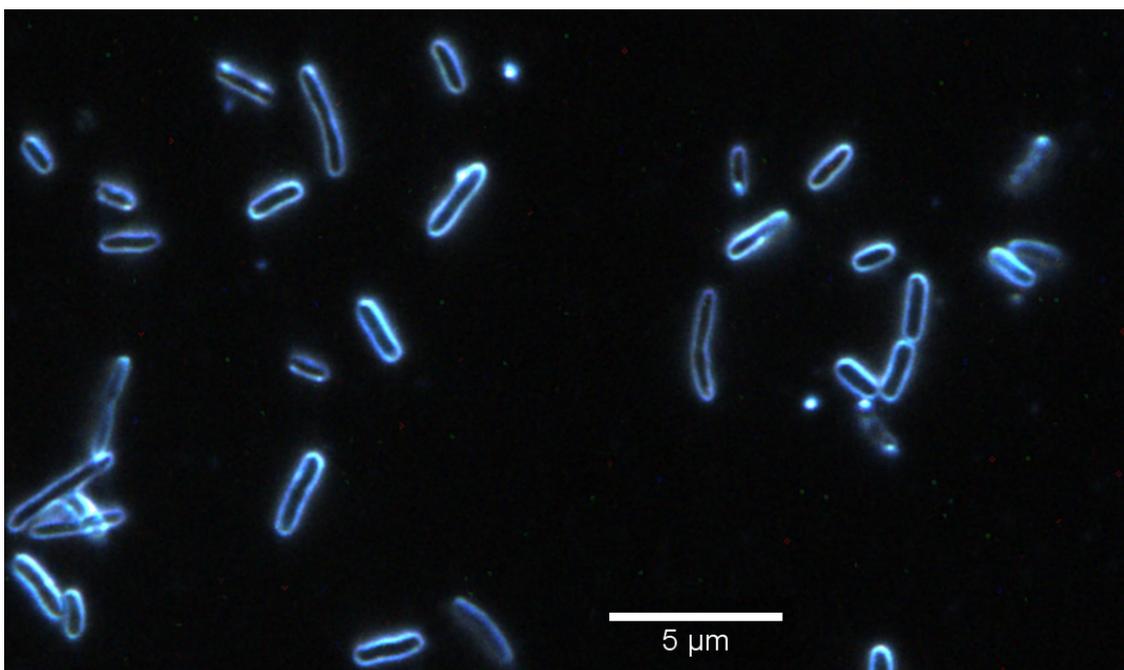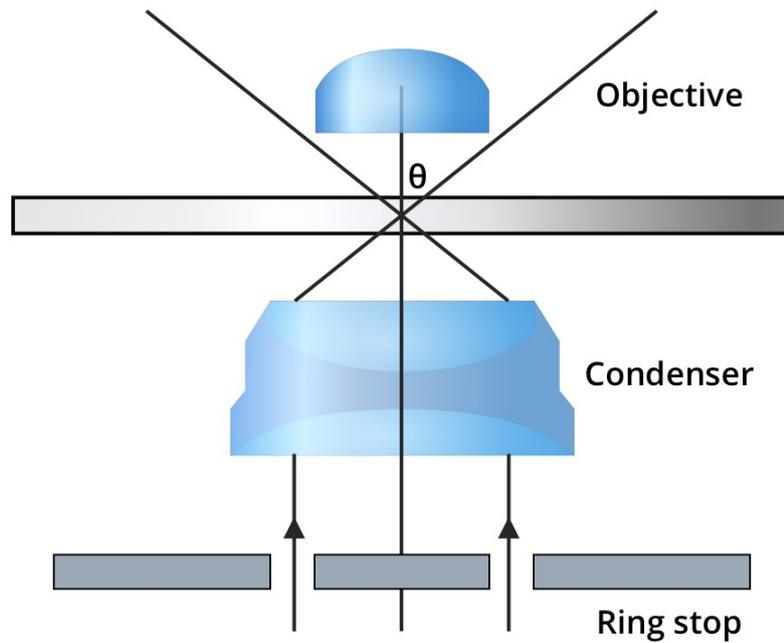
Transmitted darkfield image of bacteria by Daryl Webb.
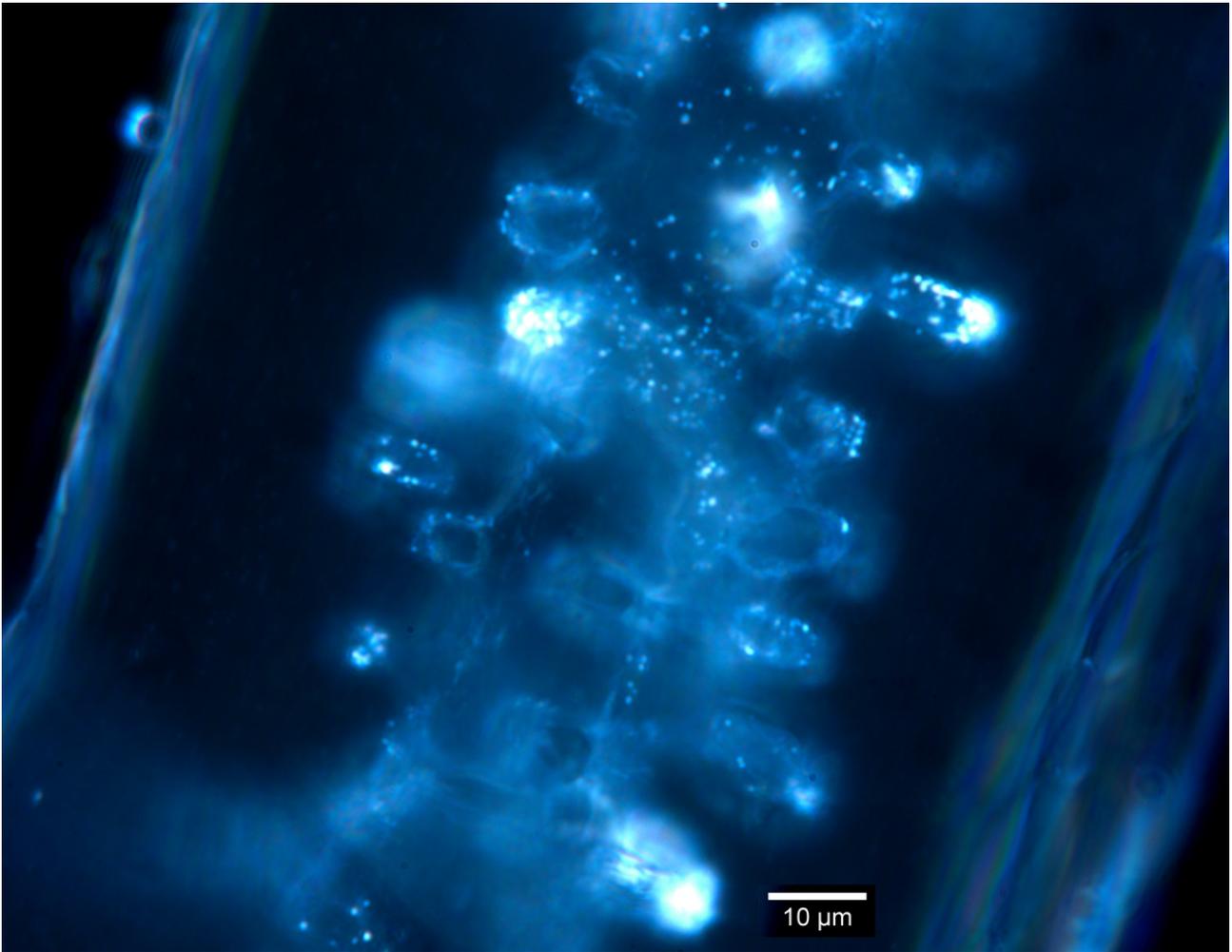
Transmitted darkfield image of an animal hair by Daryl Webb.

Phase contrast

Phase contrast enhances contrast between structures in a sample (e.g., cellular components) based on differences of their refractive index. Hence, phase contrast is ideal for viewing thin unstained samples. Phase contrast also works through plastic, whereas DIC does not. This makes phase contrast ideal for monitoring and imaging live cells growing in plastic culture dishes and flasks and it is widely used for this purpose. It is also useful for monitoring unstained thin sections and a host of other applications.

Phase contrast works by making the diffracted rays half a wavelength out of step with the direct rays, so that anything in the specimen that scatters light will appear darker than its surroundings in the final image.

To break this down, the direct rays that have not been scattered by the sample still enter the objective to give a light background to the image, which is more familiar to our eyes and gives an image that is easier to interpret.

Since the structures in the sample that scatter light are of higher refractive index than those that don't, the scattered light will be a bit behind the direct light – but by less than half a wavelength (λ). We cannot know by exactly how much, but the general compromise is to assume that the scattered light will be about ¼λ behind. If we then slow it by a further ¼λ we will bring the total path difference to ~ ½λ.

This is done by inserting a phase plate with a circular groove, into the objective, aligned to the back focal plane of that objective. The groove is ¼λ deep, which for green light (the centre of the visible spectrum) is 550/4 = 137.5 nanometres. Imaging with a green filter will therefore give the best contrast. The groove is in line with the direct light that has passed through the sample unscattered. The rest of the light, which has been scattered by the sample, will be bent to different extents and will therefore pass through the central, thicker part of the phase plate. This results in an image like the following. There is also a neutral density layer in the groove to reduce the brightness of the direct light to make it more similar in brightness to the scattered light.
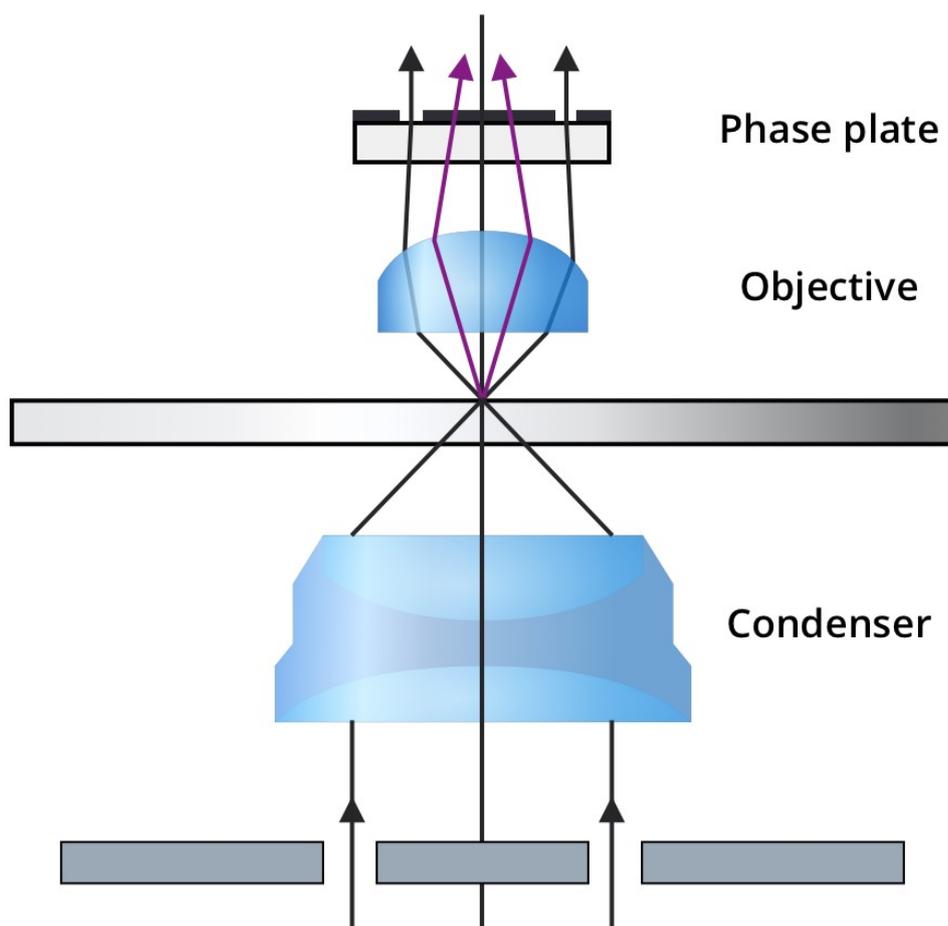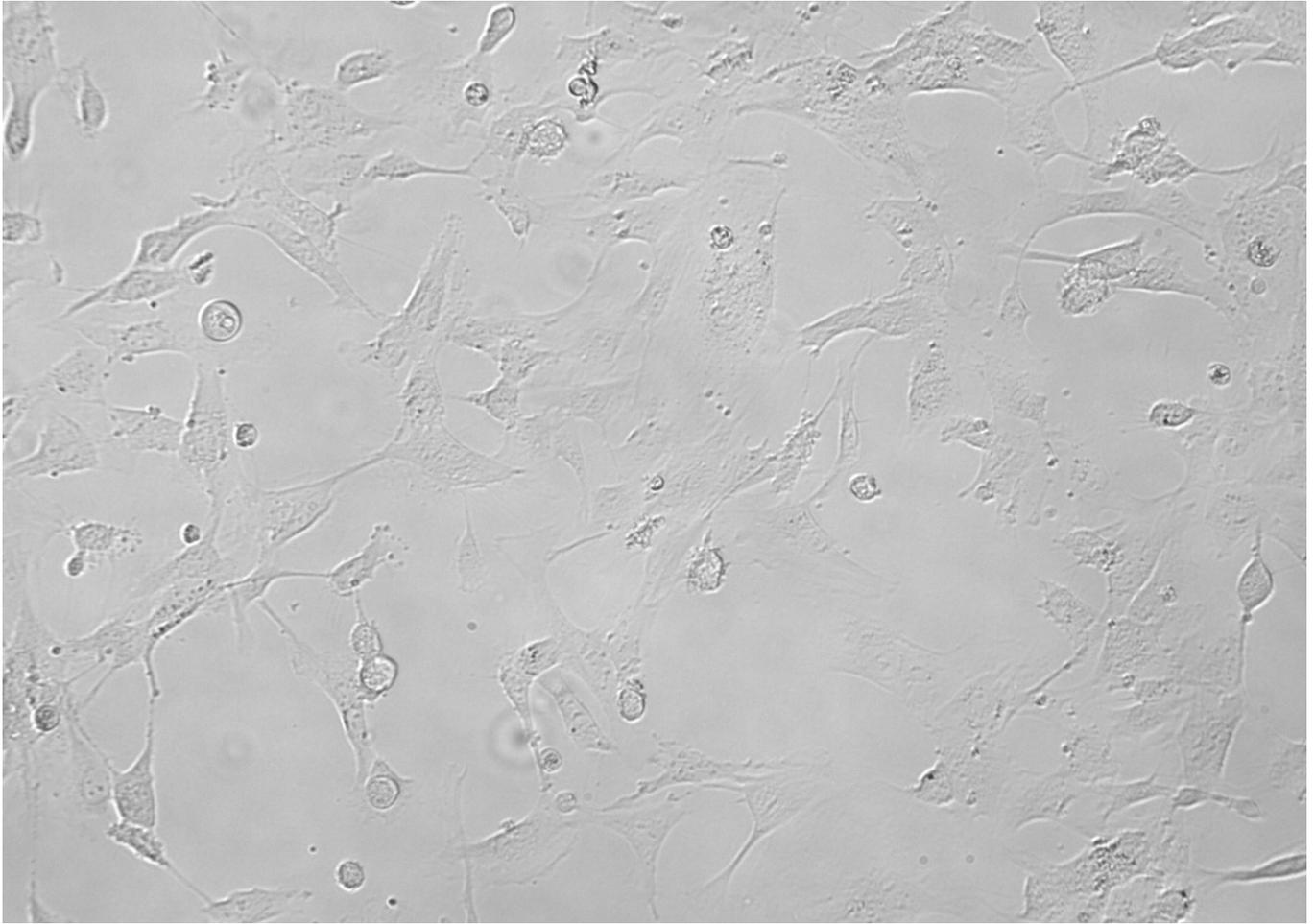
Phase contrast image of cultured cells by Louise Cole.

Differential interference contrast imaging (DIC or Nomarski imaging)

Unlike phase contrast, which is based on total refractive index, DIC is based on the local change in refractive index. This contrast enhancing method also allows visualisation of unstained samples, eg. unstained tissue or cells, but is suitable for thicker samples than phase contrast. It is based on light refraction differences of different parts of the transparent specimen. This effect is achieved by making each ray of light interfere with another passing through the specimen a very small distance away from it.

If the refractive index of the specimen is changing, there will be a path difference between the two rays, if it is uniform, there won't be.

The contrast we see in the final image will depend on the local rate of change of refractive index in the specimen - hence the name differential interference contrast.

DIC is often used as an overlay channel with fluorescent labels and tends to give a somewhat 3D effect. It is also widely used in microinjection techniques where it is necessary to see clearly defined, intracellular structures such as nuclei.

DIC will only work through glass, so if you want to use this technique with living, cultured cells, you must set up your cultures in dishes with glass bottoms and lids.
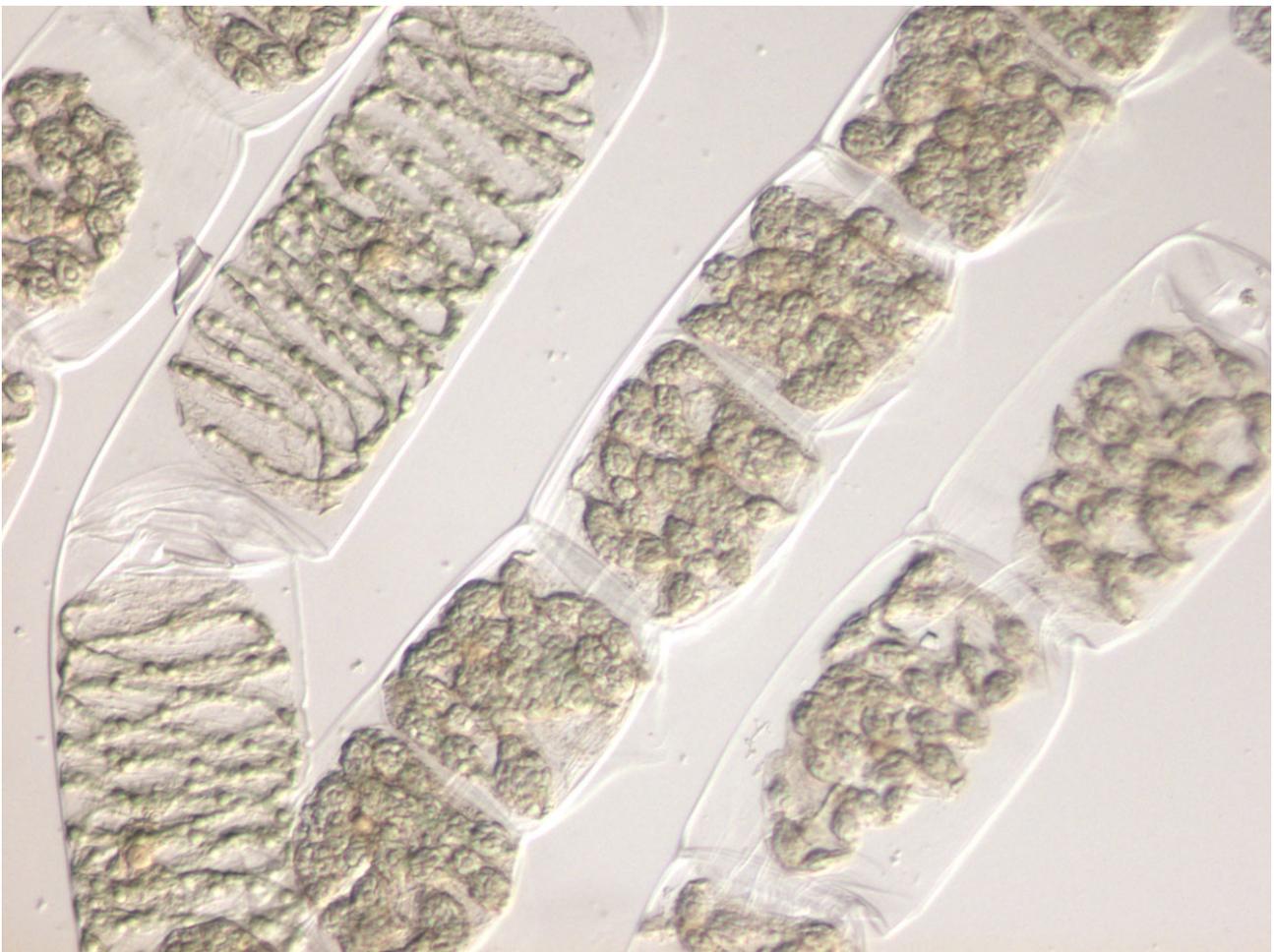
DIC image of Spirogyra by Dr Louise Cole
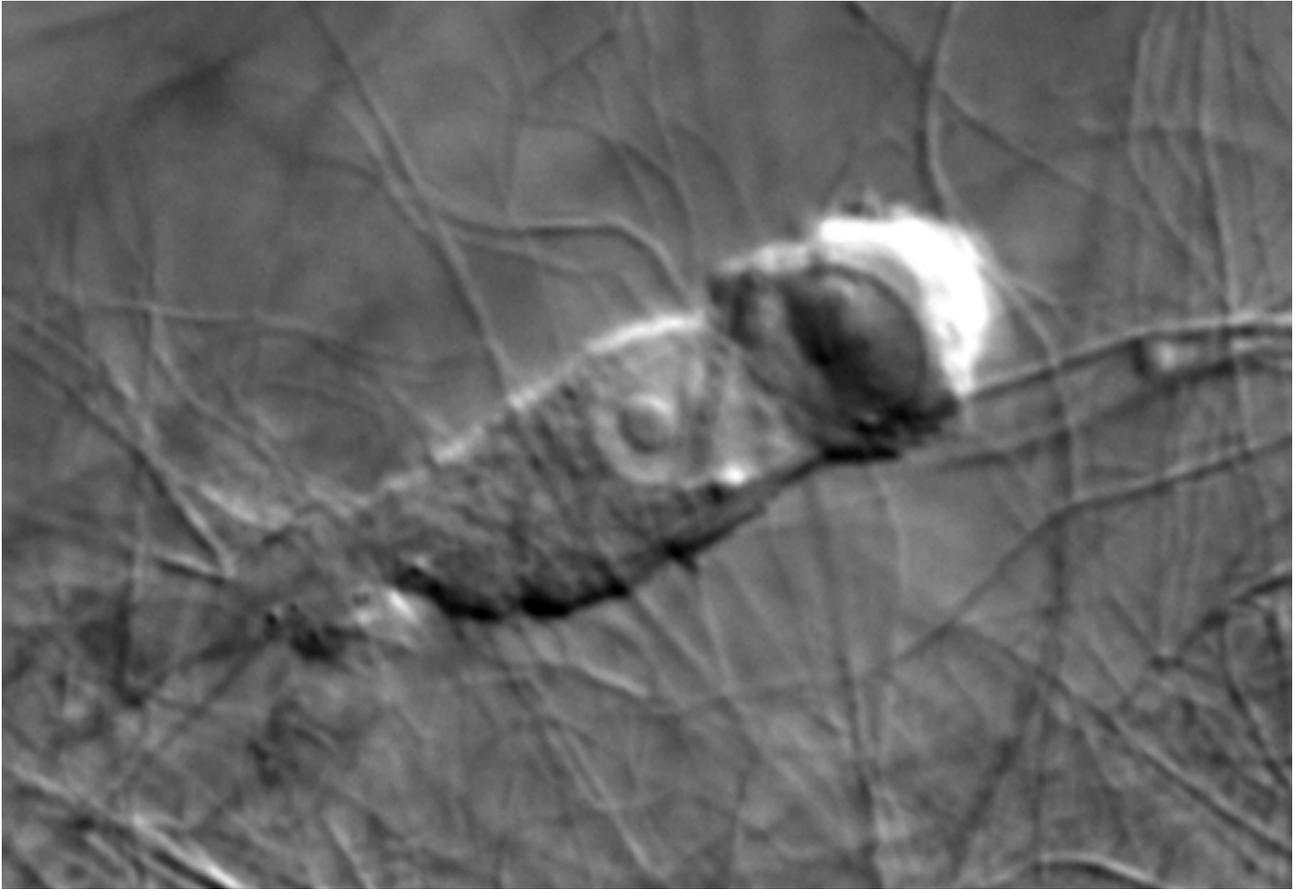
DIC of a breast cancer cell in a collagen matrix by Sandra Fok
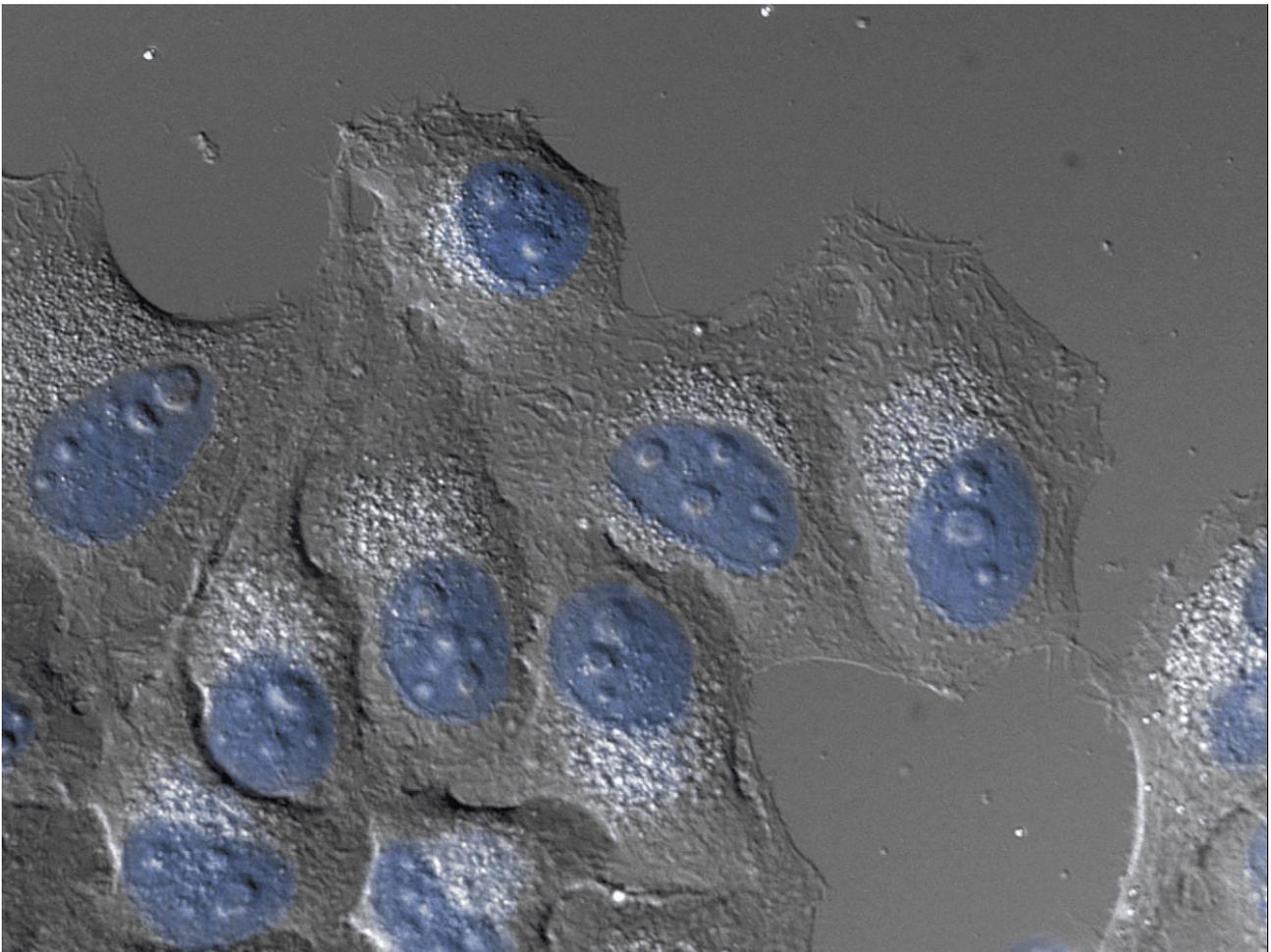
Hela cells in culture stained with Hoechst 33342 to label nuclei and imaged with both fluorescence and DIC. There are a range of Hoechst stains that be used with living cells. By Ying Ying Su.
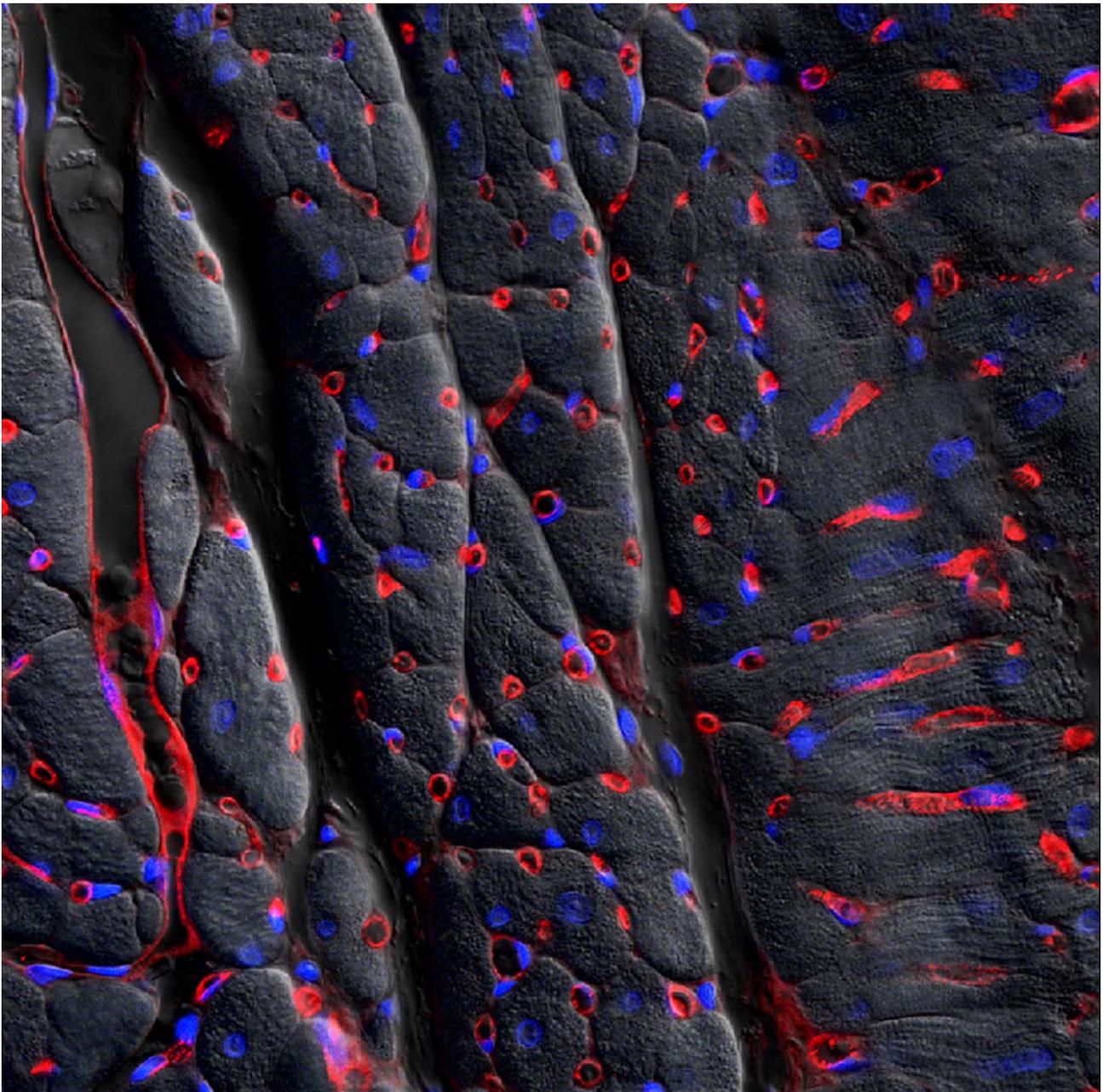
Heart muscle visualised using confocal microscopy superimposed with differential interference contrast microscopy by Dr Paul Monaghan, CSIRO.

The microscope is set up as shown in the diagram

- A polariser sits below the condenser to generate a beam of polarised light.
- A Wollaston prism sits at the position of the condenser aperture. This is made of two quartz wedges, with their crystal axes at right-angles, cemented together. Quartz is birefringent so it will resolve the incoming polarised light into two rays emerging at slightly different angles.
- The λ/4 plate puts a quarter wave path difference between them. This can be either above or below the objective-specific Wollaston prism.
- The condenser lens will focus these two rays to slightly different points on the specimen. These points must be closer together than the resolution of the microscope, so that you don't see a double image.
- As the rays emerge from the sample, they are recombined in a second Wollaston prism past the objective although the two rays will have different lengths from passing through different parts of the sample. This recombined beam is then depolarised by passage through a second polariser, also called an analyser.
- Positive or negative bias can then also be introduced to cause features to appear as either raised or depressed. This can be very valuable for optimising the appearance of particular features of interest.

Analyser

Nosepiece prism

Objective

Specimen

Condenser

Objective-specific prism

Quarter waveplate

Polariser

Light source (non-polarised light)
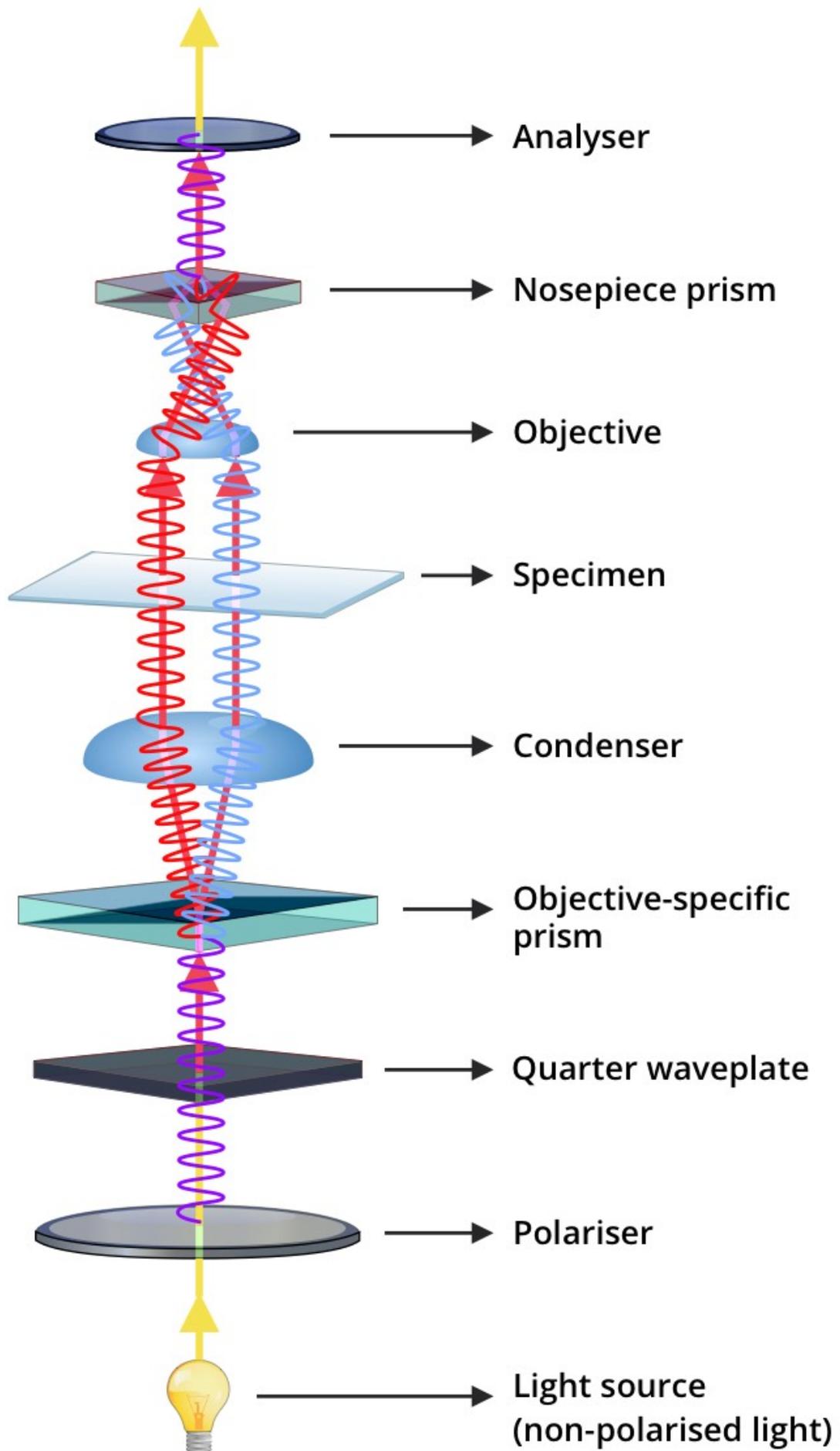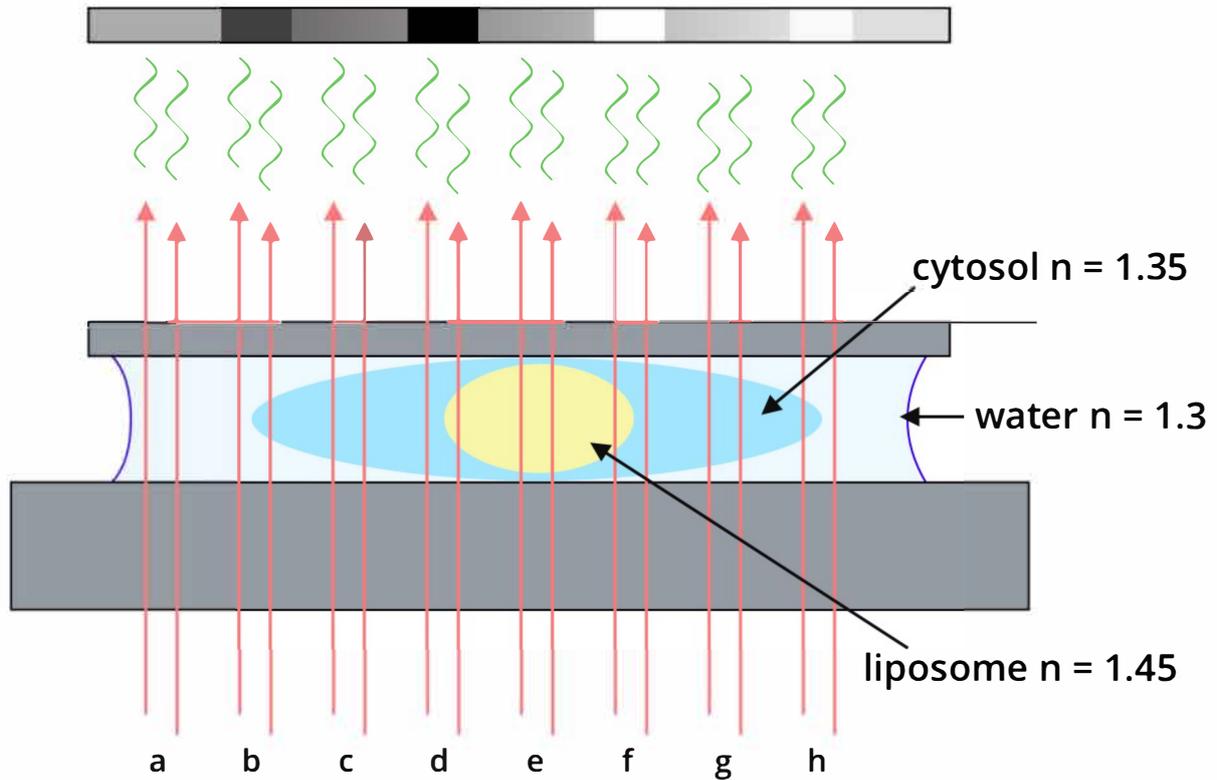
a. both rays in water - no phase difference

b. one in water, one cytosol - phase difference

c. one sees more cytosol - small phase difference

d. one in cytosol other in liposome - large phase difference

e. both in liposome + cytosol - no phase difference

f. one in liposome and one in cytosol so the leading wave is retarded bringing them both into phase

g. one sees more cytosol – small phase shift

h. one in water one in cytosol – small phase shift to retard leading wave – nearly in phase
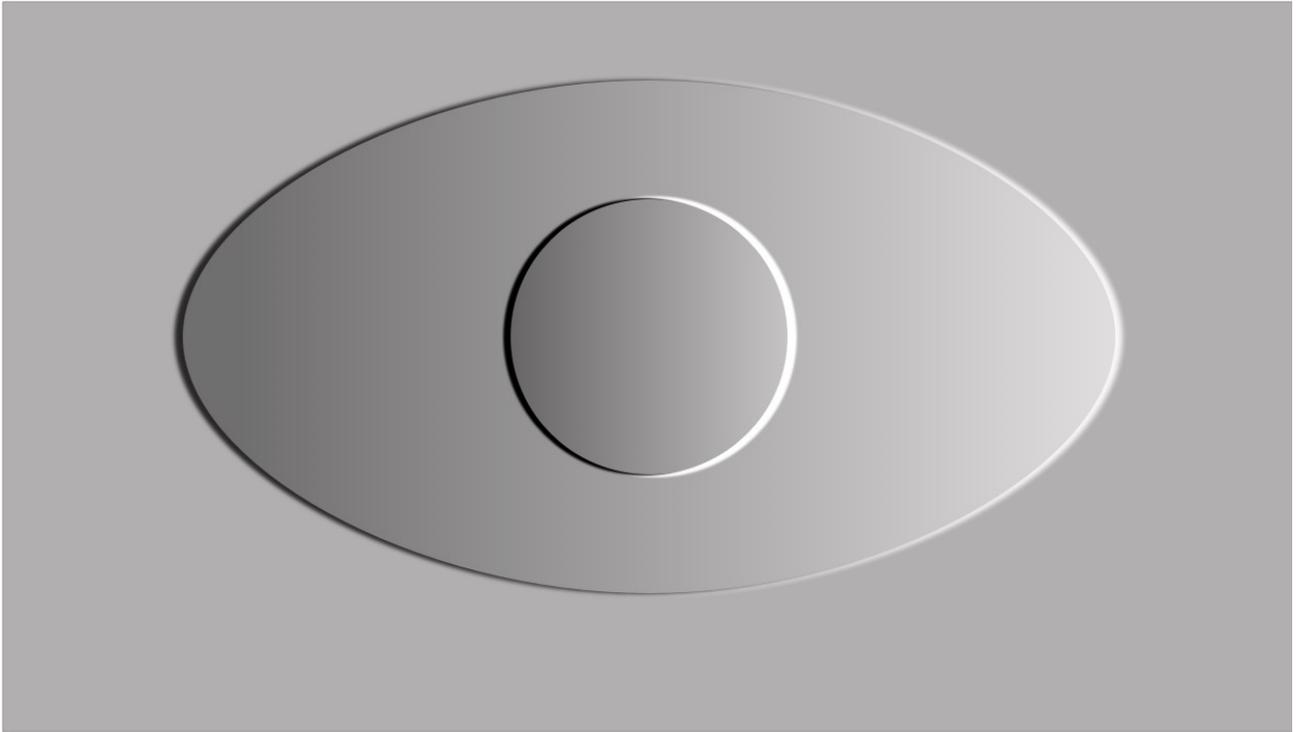
A 'relief' effect – striking but entirely artificial

Polarised light microscopy

Many crystalline and fibrous materials are birefringent. This means that the crystallographic orientations within the material vary so that their refractive index is different for two directions of polarisation. These differences allow polarised light to bend in different directions. Polarised light microscopy provides information about the material structure by enhancing contrast within such samples.

Non-polarised light vibrates in all directions but when it passes through a polariser it becomes constrained to only vibrate in one direction.

In polarised light microscopy, polarised light is generated from a polariser located below the sample and is used to illuminate the birefringent sample. When this beam passes through the sample it separates into two components whose directions and velocities are determined by the crystal orientations within the sample. These two out-of-phase divergent beams are then recombined as they pass through a second polariser (often called an analyser) with either constructive or destructive interference leading to bright, differently coloured or dark regions to be visible in the sample.
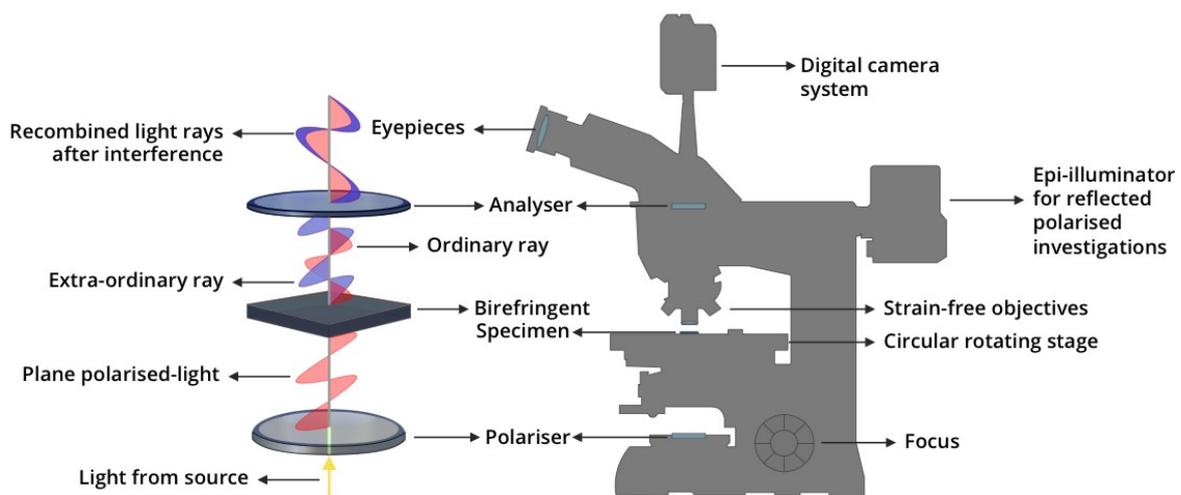
The way in which materials interact with polarised light can provide information about their structure and composition. This information cannot be achieved with isotropic materials as the crystalline structure of these is unidirectional. Polarised light microscopy is common in geological research to identify and study minerals, in other material sciences, and for certain biological and archaeological applications. For example, it can be used to examine collagen formation and to identify ancient starch grains. In petrological work, the polariser can be rotated during visualisation to measure the degree of birefringence, thus helping to identify individual minerals.

From Geoff Clarke
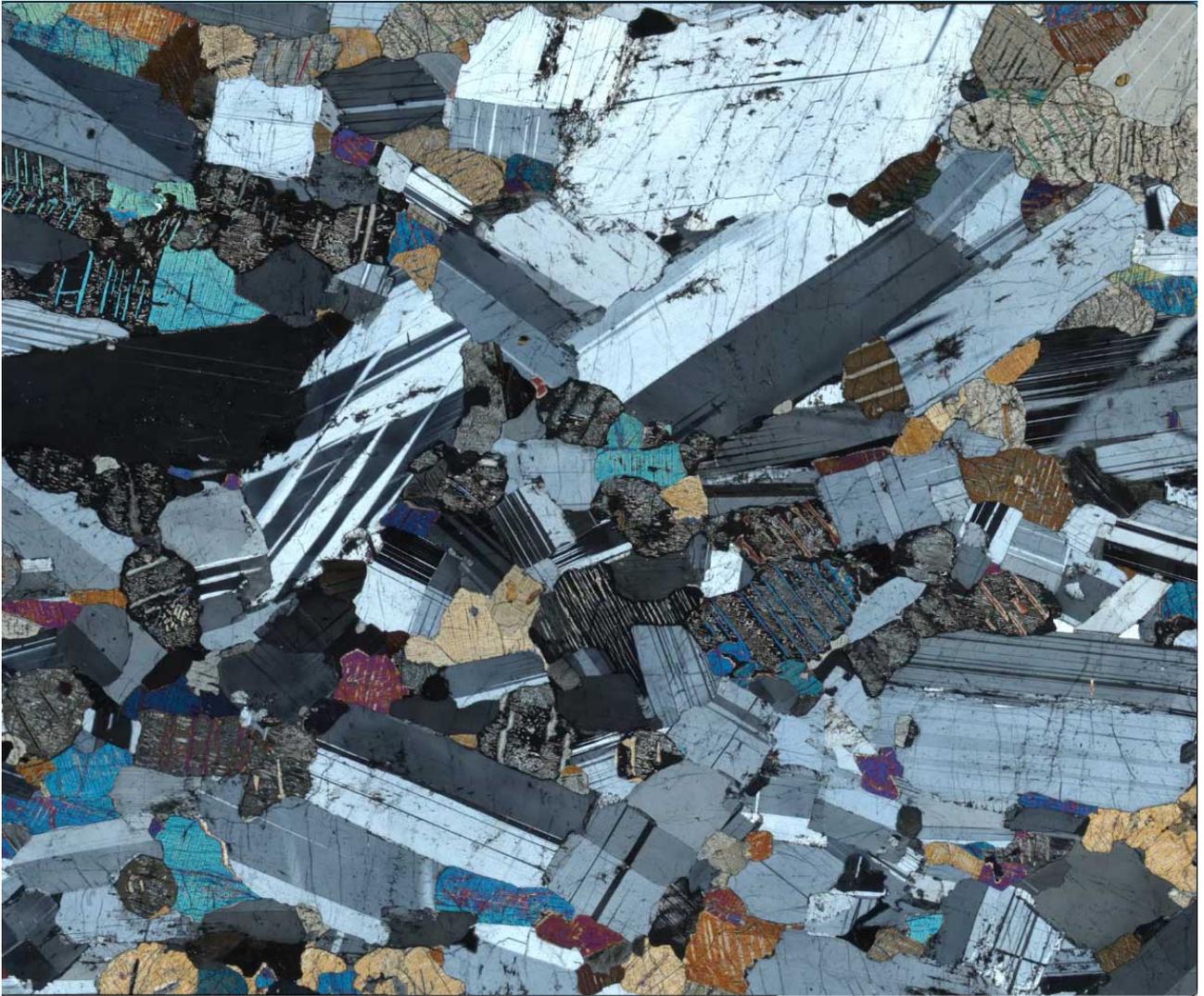
Protein crystal in a hanging drop – Paul Rigby

Reflected light imaging

Reflected light microscopy is used to examine opaque samples that are not suitable for examination with conventional transmitted light techniques. These samples need to be illuminated with reflected light instead. This is how we normally see the world around us, where light rays bounce off the objects and into our eyes. This gives us a view of the surfaces of these objects. So it is for reflected light microscopy.

Reflected light microscopy is also known as incident light, epi-illumination, or metallurgical microscopy. It would be the technique of choice for samples including insects, pollen, rocks and minerals, archaeological finds, metals and electronic components, wood, plastics and composites, ceramics and paper, to name just a few.

In a reflectance upright microscope, the sample is illuminated from above through the objective. The beam is deflected by a partially silvered mirror, so it passes down through the objective onto the sample. The reflected light then passes back up through the objective and up through the same mirror to the eyepiece or camera. This partially silvered mirror allows some of the light from the lamp to be reflected down onto the sample but also allows the light reflected off the sample to pass up through the mirror to the eyepiece or camera.
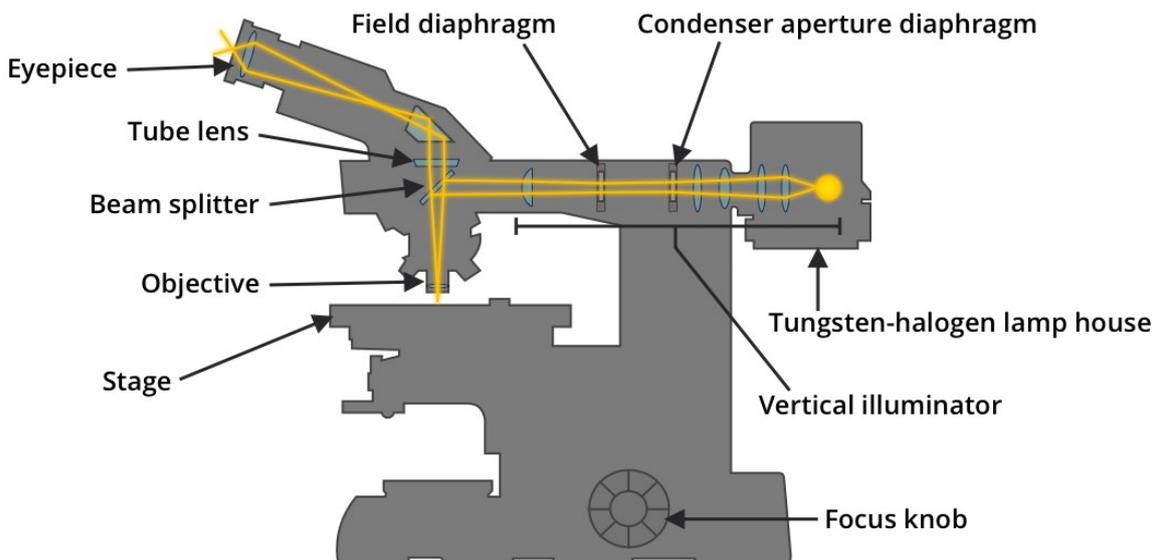
Image showing light path in an upright microscope and an inverted reflectance microscope.

Many upright light microscopes have two lamps, one positioned to illuminate the sample from below in transmittance mode and another to illuminate from above in reflectance mode.

Reflected/Transmited Light Microscope
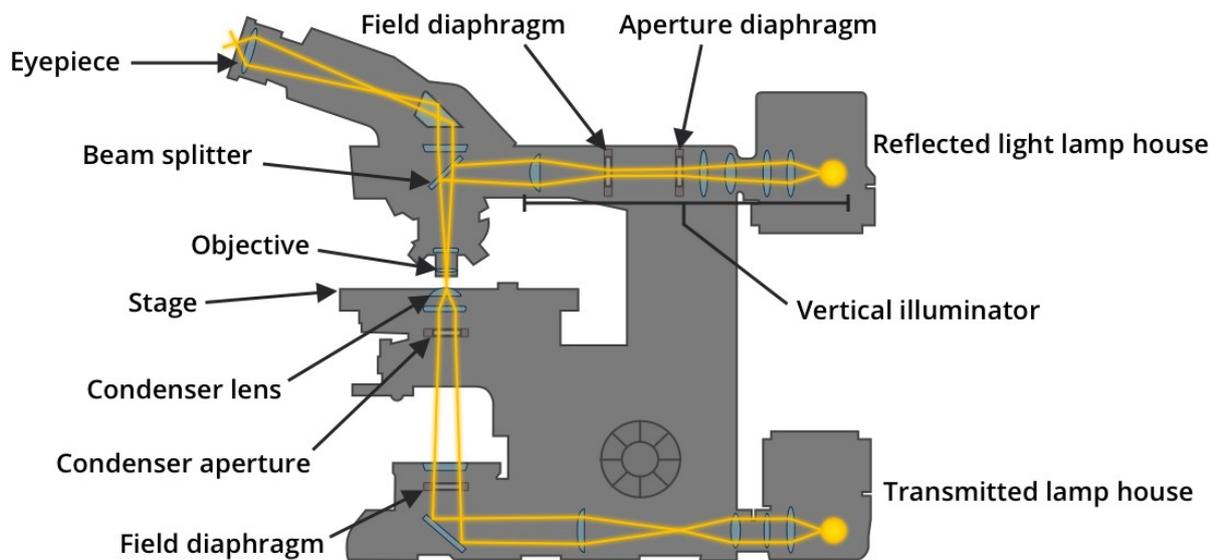
Combined showing light paths.

Reflected light can also be used with inverted microscopes, allowing for the close inspection of larger samples that would not fit beneath the objective of an upright microscope. This microscope design has the advantage that the specimen's surface is always very close to the focal point of the microscope, however, it is only appropriate for flat specimens that can sit flush with the sample stage.

Inverted Reflected Light Microscope
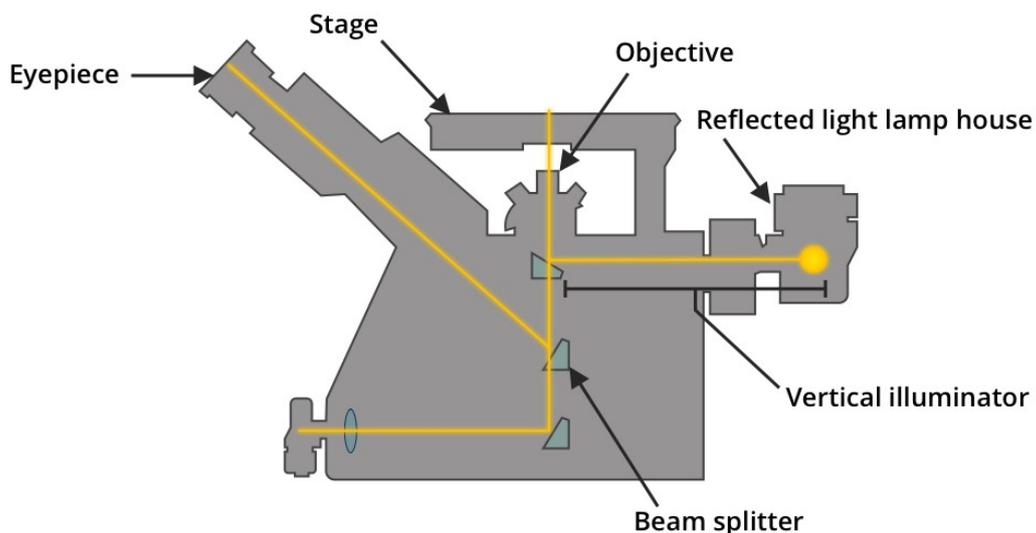
Inverted reflected microscope.

As well as bright-field, reflected light microscopes also allow the contrast techniques of darkfield, DIC and polarisation, described above for transmitted light, to be applied in reflectance mode. In these cases, the arrangement of the essential components is adapted to the different arrangement of the light path.

In reflected light DIC, it is the difference in height that is measured rather than the difference in the refractive index.

It is also worth noting that reflected light objectives are not corrected to take account of the refractive index of the cover slip. Therefore, objectives should not moved between transmitted and reflected light microscopes.

Reflected Brightfield

Tube lens

Beamsplitter

Objective

Specimen

Reflected Polarised Light

Tube lens

Analyser

Beamsplitter

Polariser

Objective

Specimen

Reflected Darkfield

Tube lens

Ring stop

Mirror

DF objective

Specimen

Reflected DIC

Tube lens

Analyser

Beamsplitter

Polariser

Nomarski's prism

Objective

Specimen

Relief feature
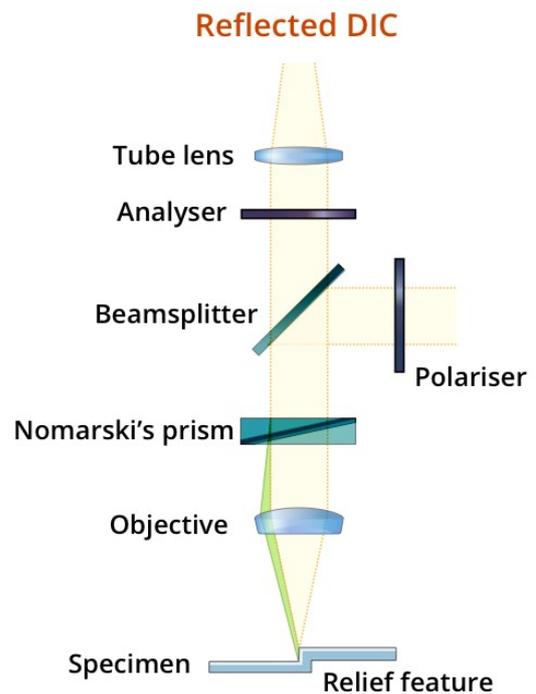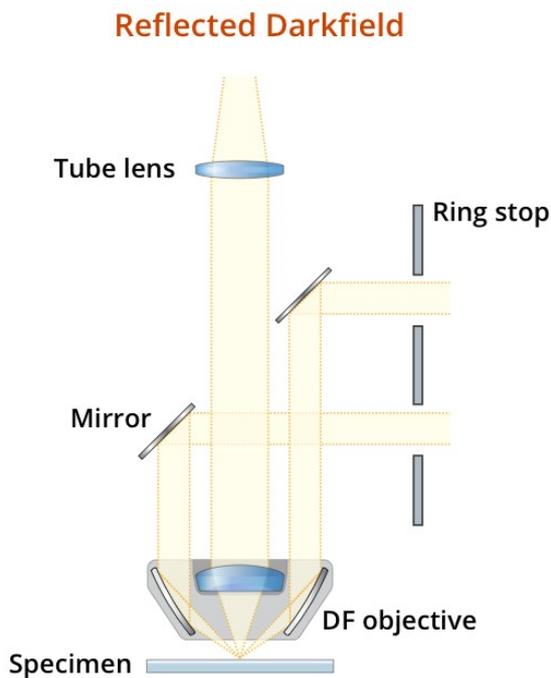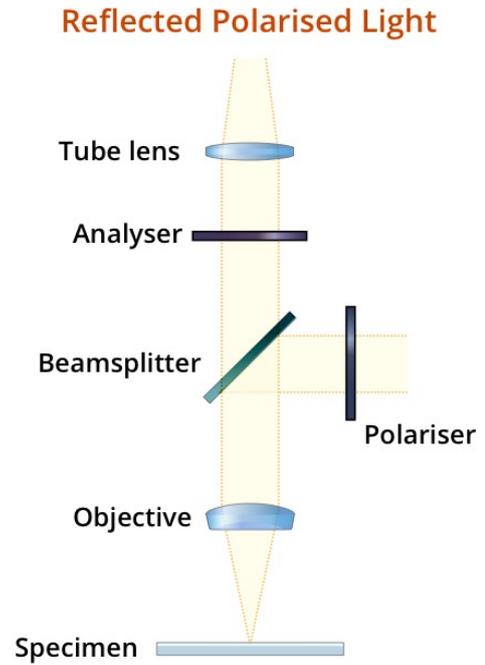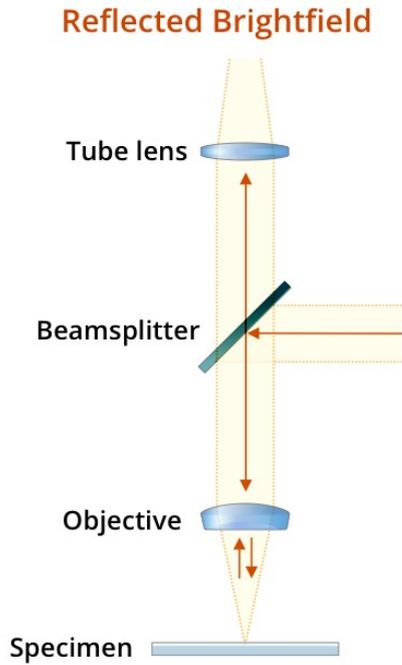
Köhler illumination is also required in reflected light microscopy to optimise the resolution and contrast of the system.

Köhler Illumination in Reflected Light Microscopy

50 µm
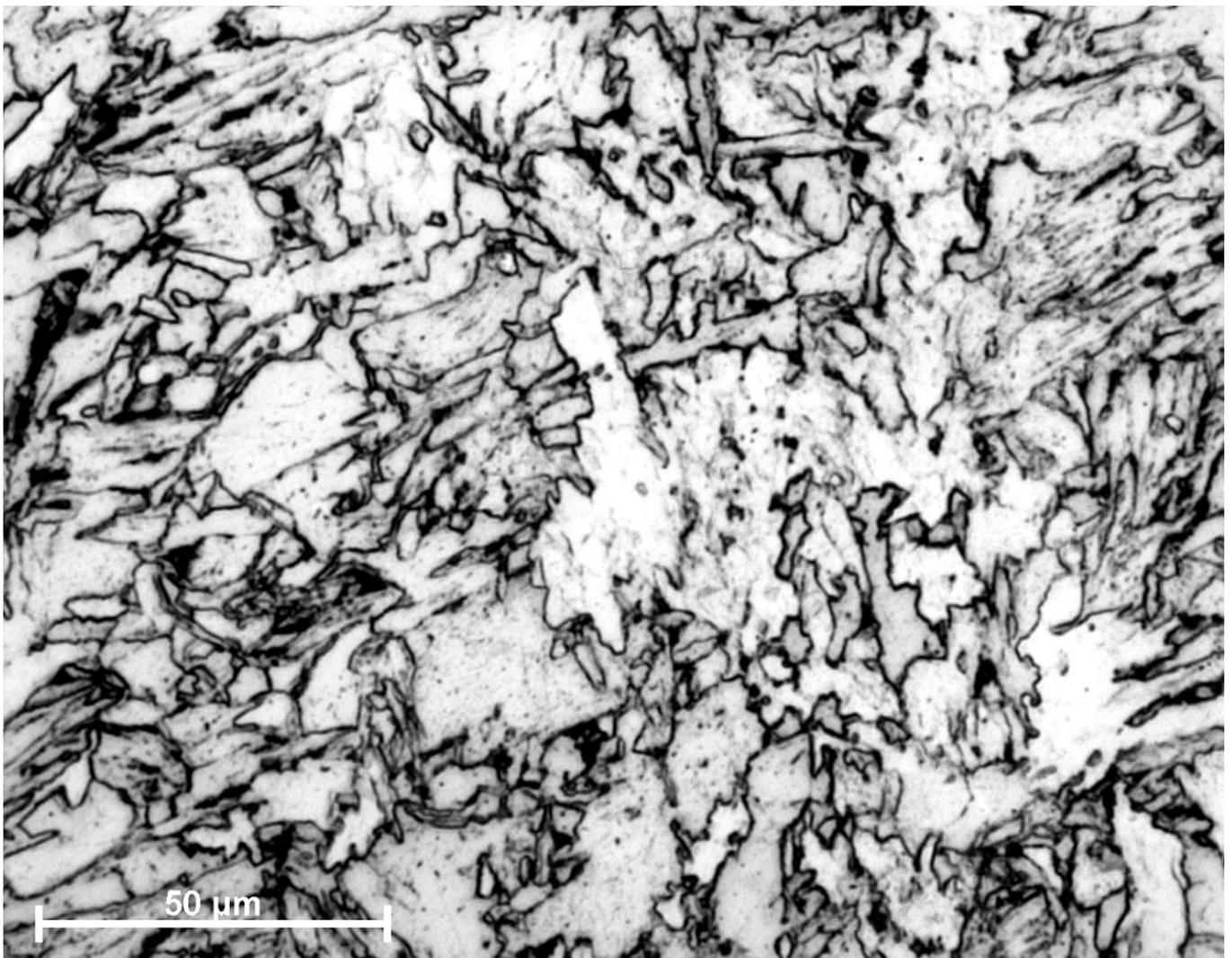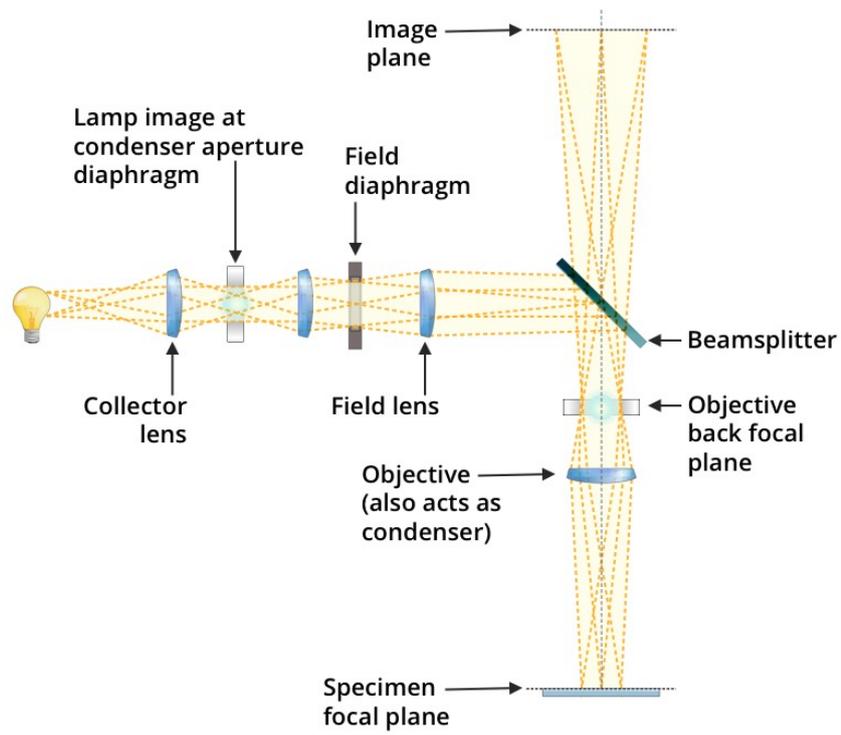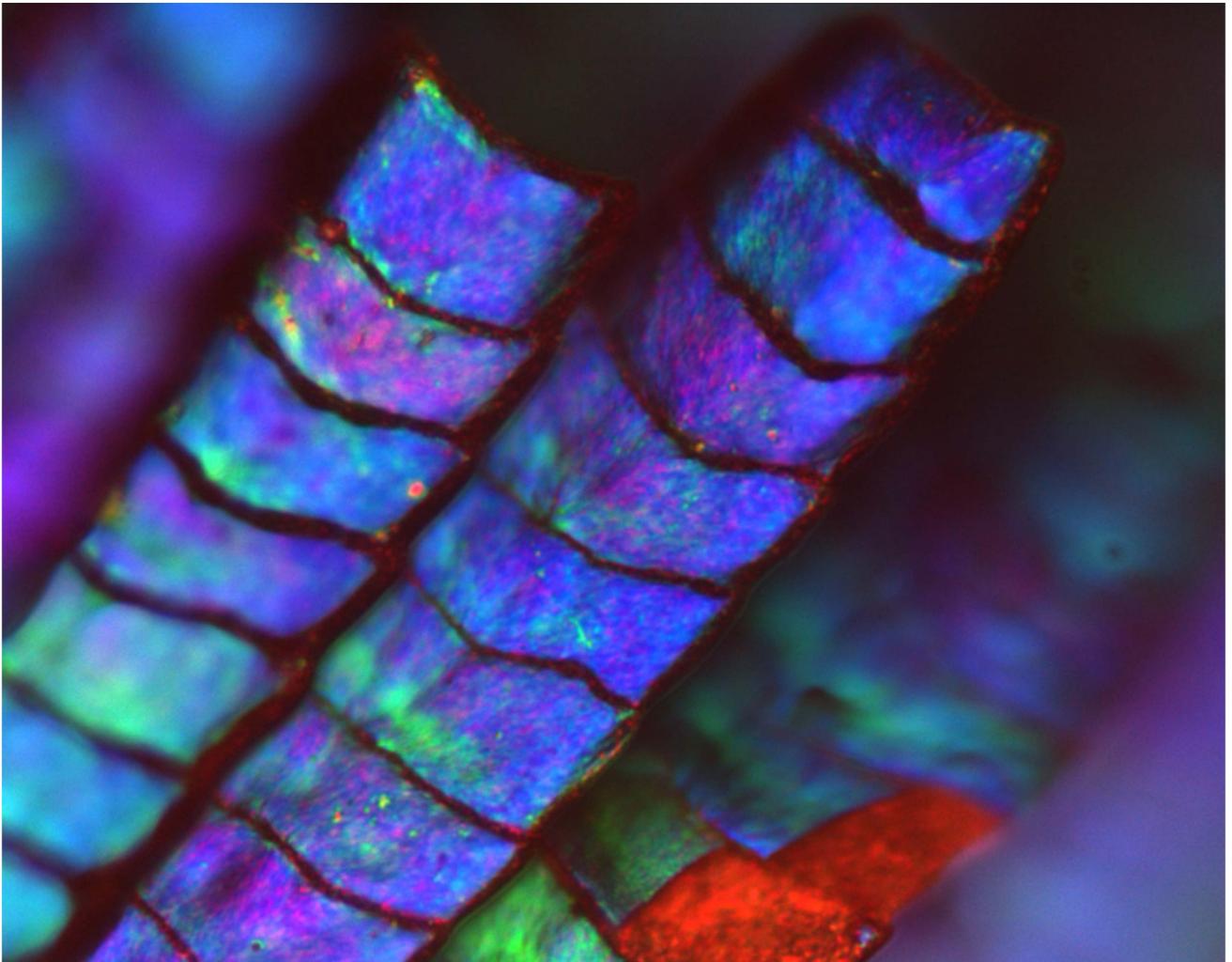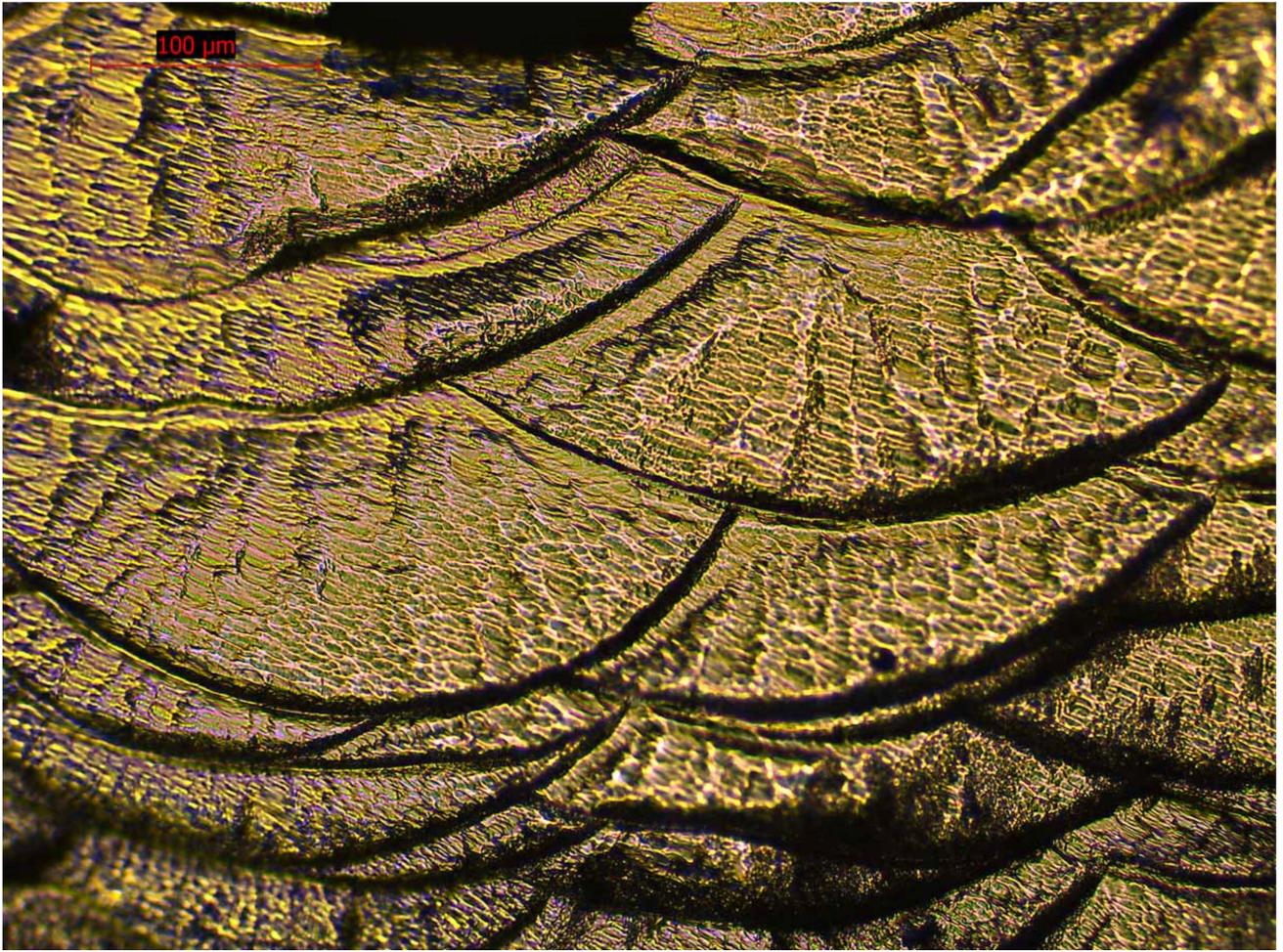
Fluorescence microscopy -
What is Fluorescence imaging?

Some cell components (such as chlorophyll and phenolic compounds) are inherently fluorescent (autofluorescence). In general, however, fluorescence microscopy involves labelling the molecule or feature of interest with a fluorescent dye or fluorophore. There are many ways of achieving this, but the two commonest are immunolabelling and using fluorescent proteins. Immunolabelling involves raising an antibody to the molecule of interest, and coupling that antibody to a fluorescent dye such as fluorescein, rhodamine or one of the new commercial dyes such as the Alexa series. The antibody latches on to its target molecule, which thereby becomes fluorescent. Fluorescent proteins are actually produced by the cell, expressing introduced DNA coding for the protein typically attached to the gene for the target molecule. The best known such protein is the green fluorescent protein (GFP) from the jellyfish Aequoria victoria, but now a complete spectrum of such proteins is available.

Each fluorochrome has a specific set of spectra so that optimal excitation and detection can only be achieved within a small bandwidth of light wavelengths. When excited, the fluorochrome absorbs photons leading to the shuttling of electrons to higher energy states (see the Jablonski diagram). The electrons quickly return to the ground state and in the process lose energy and emit light. The emitted light is always of a lower energy and longer wavelength compared with the excitation light. A sample is expressing eGFP, which has excitation (Ex) and Emission (Em) peaks of 488 and 509 nm. The peaks in the spectra indicate the optimal wavelengths to excite the sample and to collect the emitted light. The Jablonski diagram illustrates the energy states of the fluorophore and excitation and emission paths. Note that the blue wavelength light is shorter than that of green light.
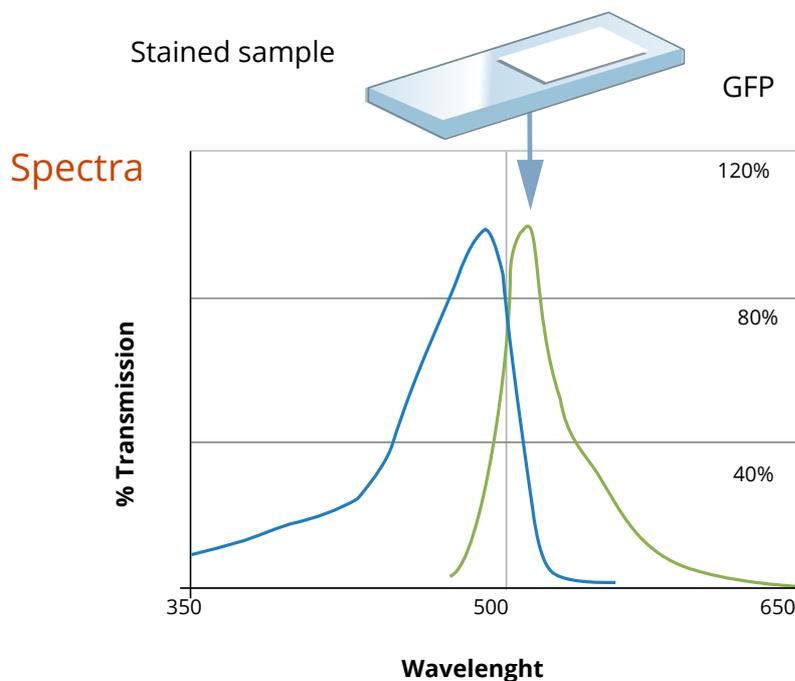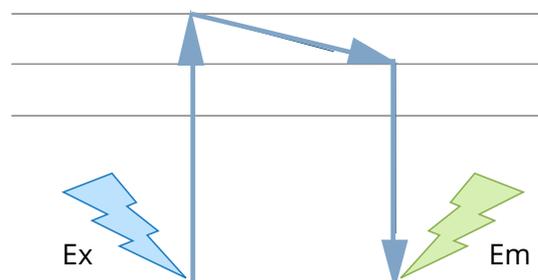
The light path and microscope parts

The basic requirements for fluorescence microscopy are the abilities to produce fluorescence from the sample, separate the excitation and emission light, resolve microscopic structures and acquire an image. To achieve these goals, the following microscope parts are necessary.

Light source

Lamps available for fluorescence microscopy may emit over a broad spectrum of light and/or produce discrete wavelengths of light. A high-pressure short arc mercury lamp, for example, has excellent lines for excitation in the green, violet and UV range, albeit blue is relatively weak.

Filter turret

The filter turret contains one or more sets of filter cubes that can separate the spectra of various fluorophores or flourescent proteins or dyes. The turret can be rotated in turn when capturing more than one emitted wavelength of light. Each filter cube contains a set of filters known as an excitation filter and a barrier filter, which selects for the transmission of excitation light and emitted light, respectively. A dichroic mirror further separates the excitation from the emission by reflecting shorter wavelengths of light (excitation) and transmitting longer ones (emission). The diagrams below illustrate the orientation of the filters within a filter cube and how they work together in fluorescence microscopy.

Objective lens

In addition to imaging fluorescent entities in the specimen by their emitted fluorescence, the objective lens in epi-illumination is also responsible for the transmission of light to the sample.

Detection system

There are many camera systems available that can be tailored to the needs of the instrument, whether it may be for rapid acquisition or high-resolution. Most use similar CCD technology to consumer cameras, but the more advanced cameras today utilise EMCCD technology. The advantage of EMCCD cameras is that they have essentially overcome an intrinsic weakness in conventional CCDs where speed and sensitivity are not compatible, and thus have enabled greater sensitivity without compromising speed.

Detector

515LP
barrier filter

Ex filter
450-490

Lamp

505 dichroic mirror

Filter cube

Objective lens
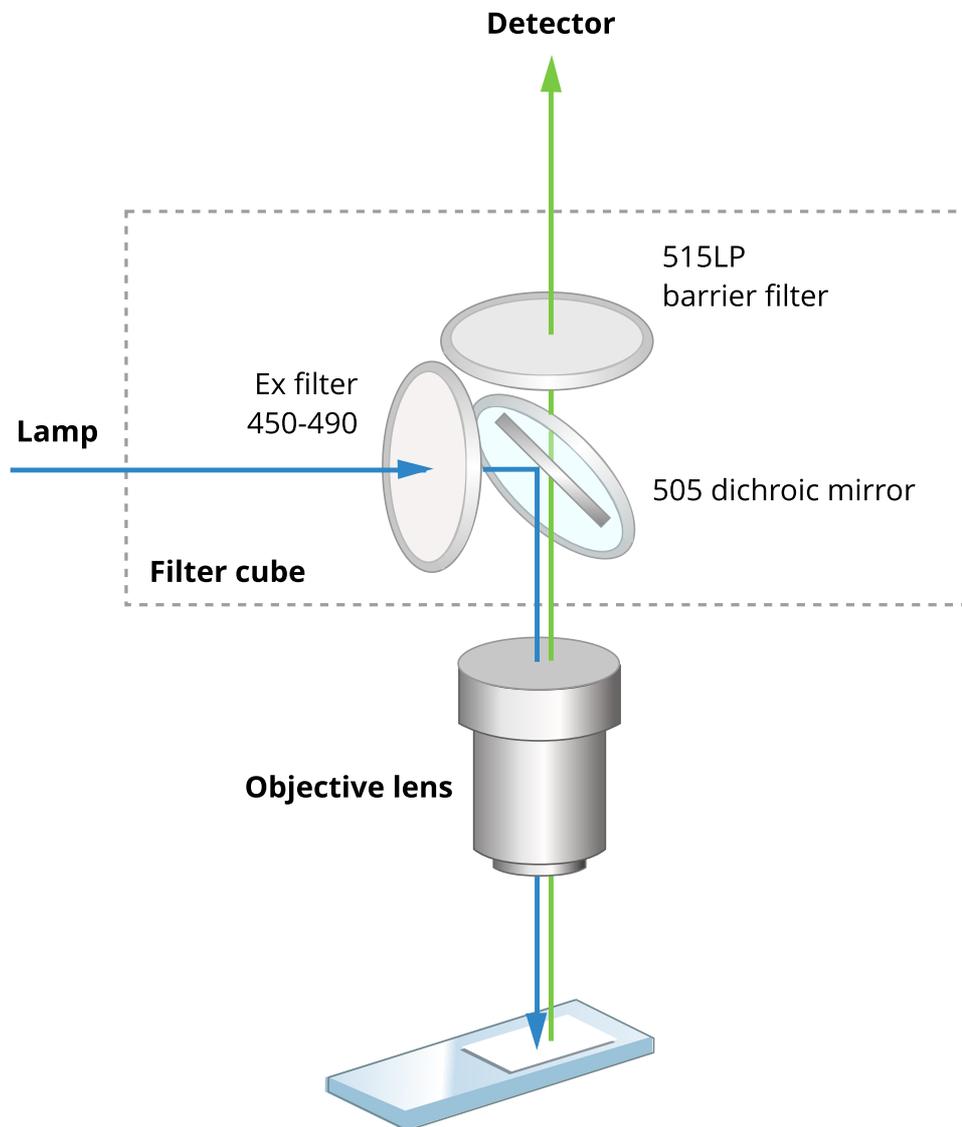
Components and light paths of a fluorescence microscope. The excitation light is filtered by an excitation filter, which allows only a narrow band of wavelengths to enter. The dichroic mirror reflects this light allowing it to pass through the lens onto the specimen. The light emitted by the sample returns via the same path and is transmitted by the dichroic mirror. A barrier filter further eliminates any excitation light allowing only emitted light to reach the detector

Separation of excitation and emission by filters. The arrangement of the filters in the filter cube results in the transmission of a band of excitation light in the 450-500 nm range and transmission of light from 500-550nm to the eyepieces. This set-up neatly segregates the excitation and emission light so that the former reaches the sample and the latter reaches the detector.

Green Fluorescent Protein (GFP) spectra
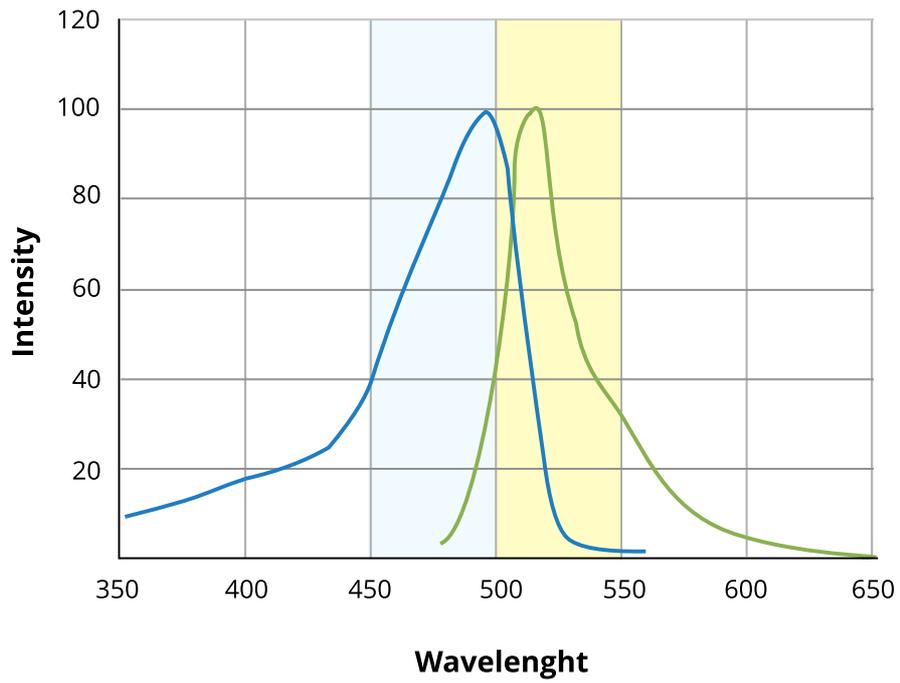
Confocal microscopy -
What is Confocal imaging?

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.

Beamsplitter

Pinhole

Laser

Focal plane
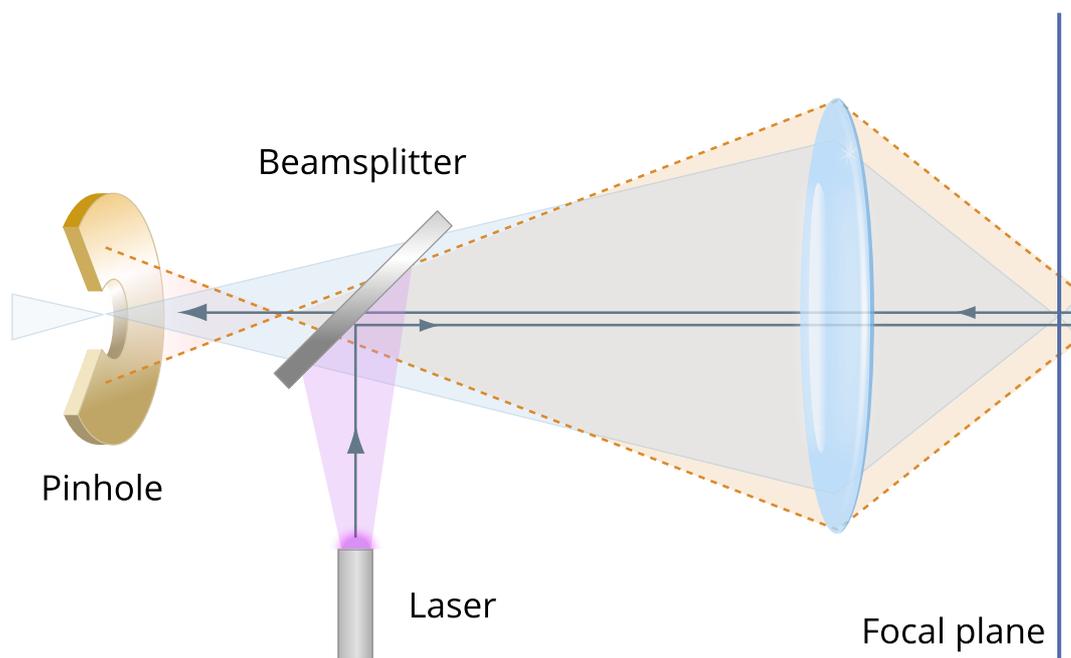
These ray-paths are shown in blue. Outside the plane of focus we still excite fluorescence, so in a conventional microscope we would see blurred objects or just an overall haze spoiling the image quality. In the confocal microscope, light from outside the focal plane (red dotted lines) is smeared out 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 square of the NA. An oil lens (NA 1.4) will give a Z resolution of about 500nm, while a dry NA 0.7 objective will be 4 times worse, at 2µm

Since we are collecting our image point by point we have to consider whether we have enough pixels to actually capture this resolution. The usual rule (the Nyquist criterion) is that to obtain Rayleigh resolution we need a minimum of 2.3 pixels within our expected resolution. So for 250nm resolution we need our pixel size to be ~110nm. To give a margin of error we might go to 3 pixels (~80nm) - but going any further is pointless. There is nothing to be gained, and we will just increase the bleaching of the sample.

A practical confocal microscope

Here we see a schematic of a simple confocal microscope. The laser beam enters (usually from an optical fibre) and is deflected down into the microscope by the primary beamsplitter - usually a dichroic mirror. Since a simple dichroic will need to be changed for different laser wavelengths, some makers use a double or triple dichroic which reflects two or three wavelengths while passing the rest of the spectrum. Another alternative is a polarizing beam-splitter which reflects polarized light (the laser beam) while passing non-polarized light (the fluorescence).
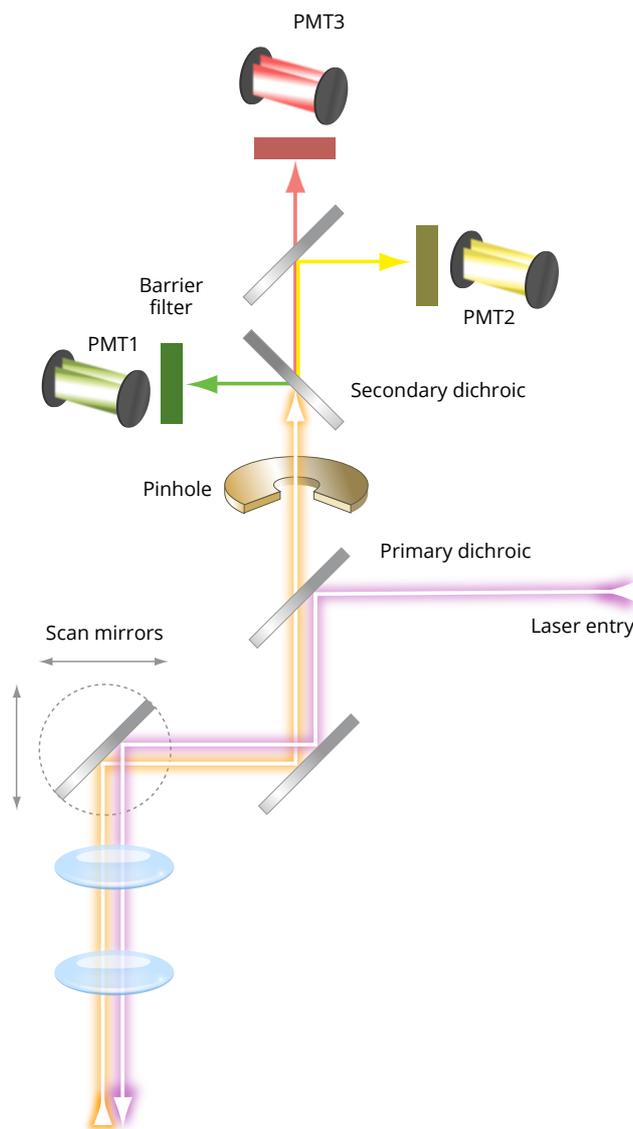
From there the beam passes via other mirrors and maybe lenses to the scanning mirrors which scan the beam across the sample. These have to be at a point conjugate with the back focal plane of the objective. (This means that a change in angle of the beam at the mirror will accurately translate into a change in position on the specimen).

Then the beam enters the microscope itself via a 'photo eyepiece' just like the one used for taking photographs from the microscope. The returning fluorescence is de-scanned - returned to a stationary spot - by the scan mirrors, passes through the dichroic and arrives at the pinhole. This must be accurately located at the image plane so that all the in-focus light will be at one spot and will pass the pinhole, while out of focus light is blocked. It follows that any chromatic or spherical aberration will seriously affect performance.

Beyond the pinhole the different wavelengths of the signal are sent to the appropriate detectors. In this schematic dichroic mirrors are used, and commercial systems offer anything from one to five detectors in such an arrangement. Barrier filters in front of the PMTs provide additional blocking of any stray laser light, and allow us to be more selective about which wavelengths we detect - we don't have to take everything reflected by the dichroic mirror.

A more versatile alternative, which is now becoming popular, is to disperse the returning light into a spectrum with a prism or diffraction grating and direct the region or regions we are interested in to particular detectors. We are then no longer limited by what filters are installed, and we have the added bonus that we can actually measure the spectrum of the fluorescence and then decide how to detect it most effectively.

PMTs have a very wide dynamic range, but just as in any method of image recording we need to get the exposure right, and the microscope will have a false-colour palette to assist with this. Always reduce the laser power to the minimum needed to get a good image before making the final adjustments to the PMT gain. Acquiring a confocal 3D stack will always be harder on your specimen than recording a single wide-field image, but taking time to optimise imaging conditions will go a long way towards minimising damage.

Components of the confocal microscope - Laser

Light Amplification by Stimulated Emission of Radiation (Laser). As stated, lasers are used because they are an intense coherent monochromatic source of light, capable of being expanded to fill an aperture or focused to a spot. The laser beam is usually linearly polarized. The main drawback in using lasers is that to cover a large excitation range you will need several lasers.

Below is a list of the most commonly used lasers.

- GAS Argon - (453) (476) 488, 494, 514 nm
- Argon-krypton - 488, 568 and 647 nm
- Krypton - 568 and 647 nm
- DIODE 640 nm red, 480 nm blue,440 nm blue/violet, 405 nm violet
- Helium-neon - 633 nm
- Green HeNe - 544 nm
- Frequency doubled DPSS 488 nm blue, 532 nm green, 561 nm yellow/green
- Multiphoton tunable lasers. 700 to 1040 nm.

However a single laser line may not optimally excite your molecule. The table below gives you an example of which laser lines to use and the efficiency of excitation for different probes.

Another thing to note is that each laser line is of different strengths. Therefore if you have similar intensities of probe you may use 10% of one line and 5% of another to achieve the same emission strength.

FLUOROCHROMES	STAINS	Ex LASER	Ex PEAK	Em PEAK	405*	422*	488*	514*	561*	640*
Blue Em Fluorochromes	Alexa Fluor 405	405	401	442	96	0	0	0	0	0
	DAPI	405	359	461	10	0	0	0	0	0
Green Em Fluorochromes	Alexa 430	442	430	550	69	95	25	0	0	0
	Alexa Fluro 488	488	500	519	0	8	72	51	0	0
	Alexa Fluro 514	514	519	542	0	0	40	95	0	0
	CY2	488	489	506	0	17	97	0	0	0
	GFP	488	395/470	509	19	36	99	16	0	0
	Rhodamine green	488	502	527	0	11	79	41	0	0
Orange Em Fluorochromes	CY3	561/514	550	565	0	0	15	50	87	0
	Alexa Fluro 555	561	553	568	0	0	13	41	80	0
	TRITC	561	554	576	0	0	9	31	84	0
	Rhodamine phalloidin	561	560	576	0	0	10	33	98	0
Red Em Fluorochromes	Alexa Fluro 568	561	576	602	0	0	0	15	61	0
	Propidium iodide	514/561/488	536	617	0	0	8	16	13	0
	Texas red	561	587	602	0	0	14	28	61	0
Far Red Em Fluorochromes	Alexa Fluro 635	640	654	670	0	0	0	0	13	81
	Bodipy 650/665	640	646	665	0	0	0	0	11	94
	CY5	640	650	670	0	0	0	0	11	73
	Nile blue	640	629	662	0	0	9	14	25	94

*% percent excitations. Table developed by Chris Johnson PerkinElmer.

Filters

There are basically 4 types of filters, short and long pass, bandpass (narrow) and beamsplitters (dichroics). In fluorescence microscopy these are usually combined in a filter block as is illustrated below.
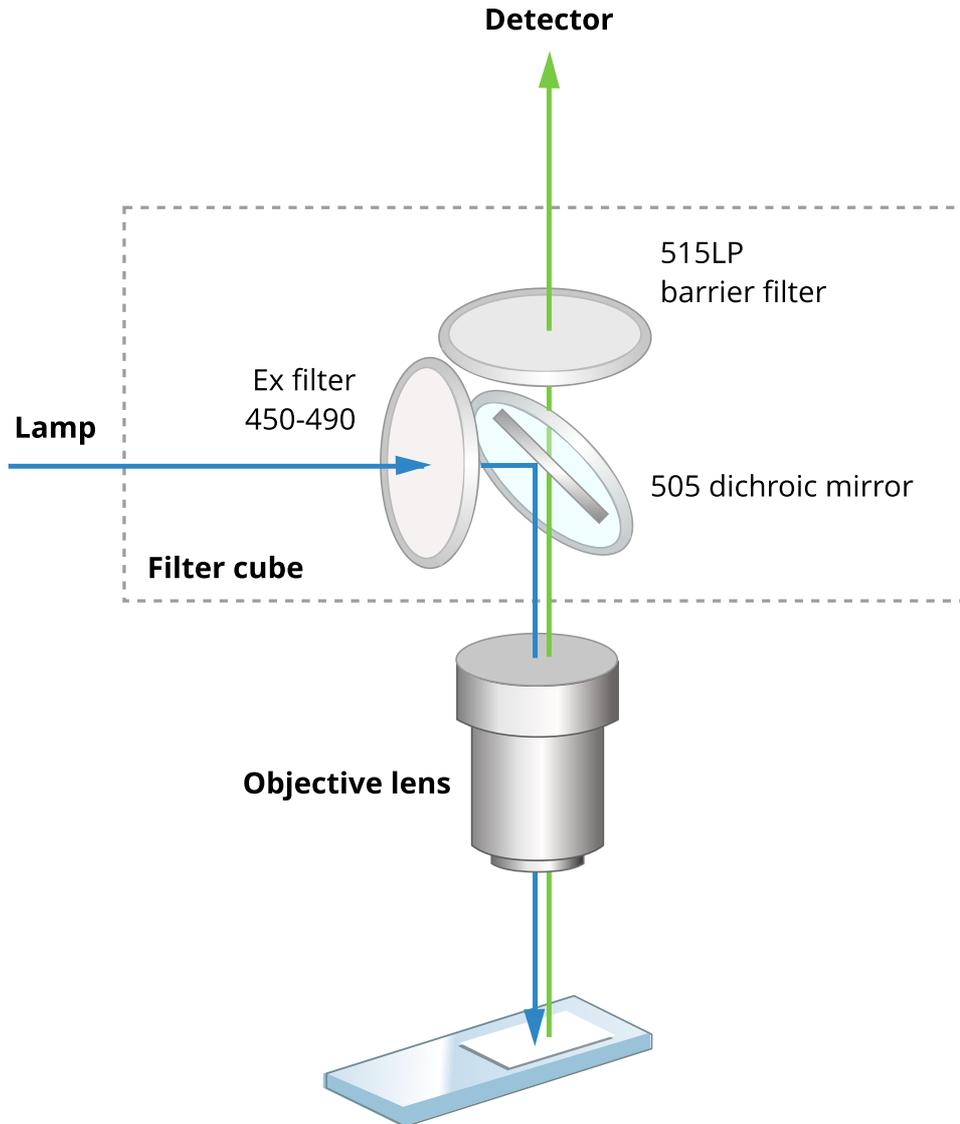
The excitation and barrier filter can be either a short, long or bandpass. It's purpose is to only allow the required excitation light to pass to the specimen. The dichroic is positioned at an angle of 45 degrees and will reflect down the excitation light to the specimen and then allow the longer fluorescent light to pass through it to the detector. The final filter which is known as a barrier filter can once again be any of the short, long or bandpass. It's purpose is to block any of the excitation light and only pass the required emitted fluorescent light.

Below is an example of a transmission curve of a filter block that could be used for a UV excitable dye. The blue curve shows you the excitation light the green curve shows you the dichroic which will reflect most of the excitation light but block its transmission and the red curve is the barrier filter which is a bandpass filter only allowing light between 430 and 490 nm to pass to the detector.

Example of a transmission curve of a filter block
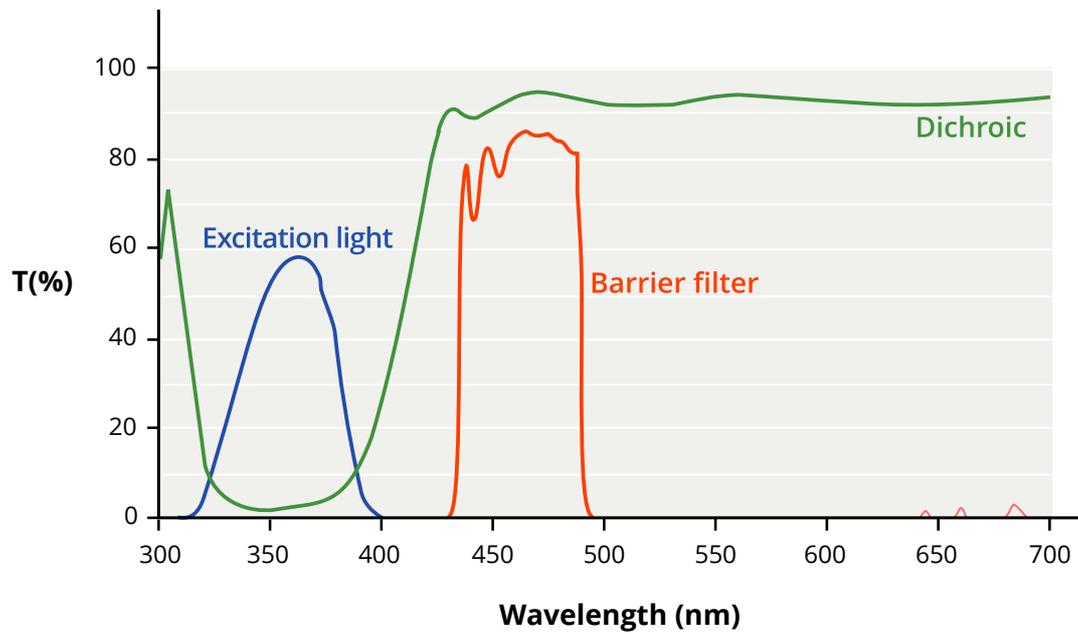
In today's modern confocals the emissions are able to be separated with even finer precision. Each confocal achieves this slightly differently.

Photomultiplier tubes (PMTs)
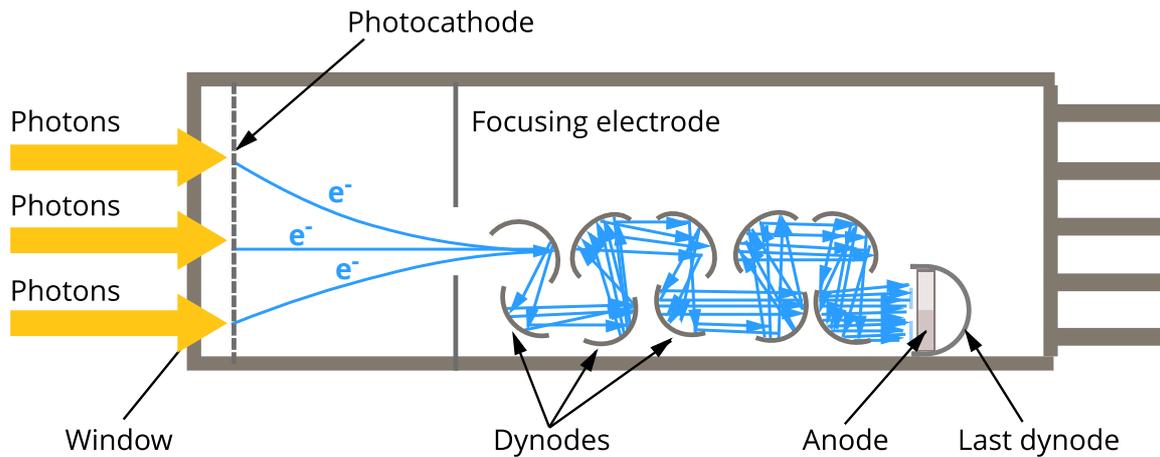
Photomultiplier tubes (PMTs) are the most common detectors in point scanning confocal microscopes. An incoming photon liberates an electron from the photocathode which starts a chain reaction so that a cascade of electrons finally reaches the anode.

The main reason that PMT's have been used in confocal microscopy is they are very sensitive.

There are various types of PMT's as illustrated with the diagram below. From this diagram you can see that they have different sensitivities over different ranges.

Photocathode spectral responses
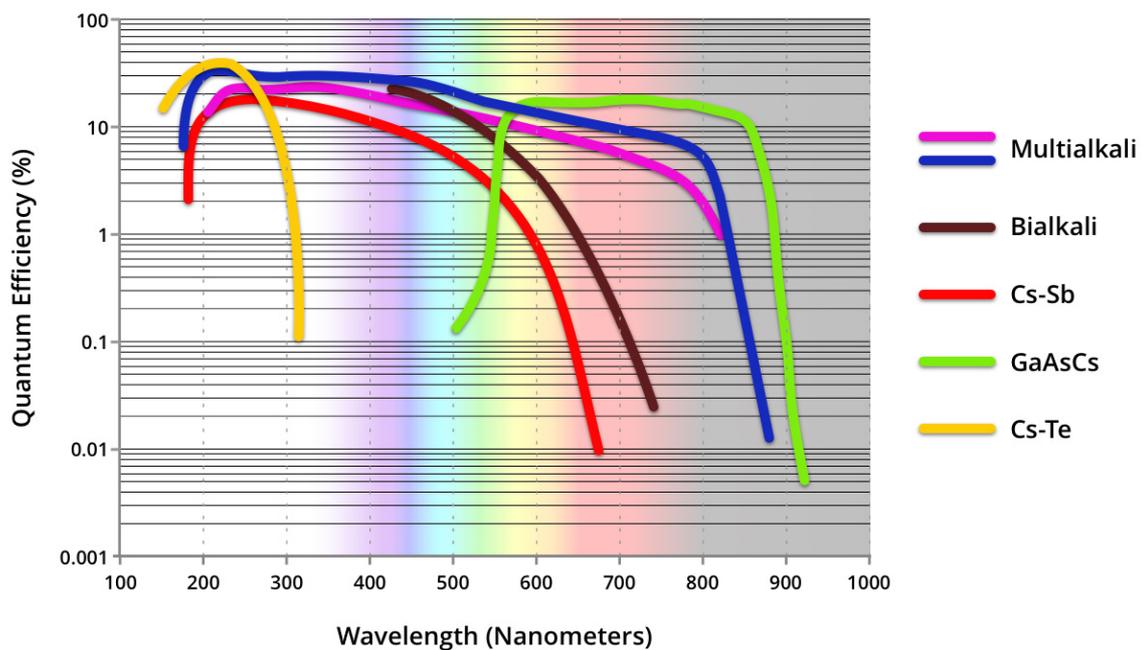
Practical image acquisition - Adjustments

Perhaps one of the most confusing aspects of starting to use a confocal microscope is selecting and adjusting the controls - which ones should one adjust to get the best image? And by how much?

Before attempting to answer these questions, ask a couple of different questions - why are you collecting an image on the microscope and what do you expect to see in your image? More specifically, are you collecting images for quantitation? Is your image to be used for the cover of a journal? Do you have co-localisation? Is the protein localised in the nucleus or in the cytoplasm? By answering questions like these, which microscope controls to adjust (and by how much) will become more obvious.

One comment for new users of confocal microscopes: 'you get nothing for free'. This means when one adjusts one control to improve an image (or the data in the image), something else will get worse. Good confocal microscopy is all about understanding the limitations and advantages of each of the adjustments in relation to the questions you are asking without compromising the integrity of your specimen.

The eternal triangle
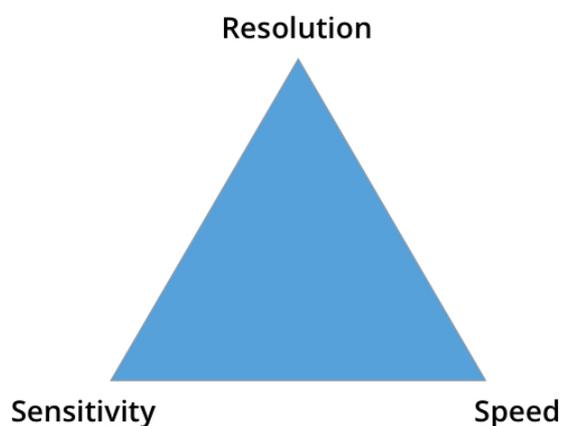
If maximum Speed, Resolution and Sensitivity are represented at the apexes of an equilateral triangle, then all possible combinations of these three parameters are contained within the space occupied by that triangle.

Essentially, this means that if one needs to obtain the maximum image acquisition speed, then either resolution and/or sensitivity must be compromised. This might be necessary for imaging fast moving objects in the specimen.

If maximum sensitivity is required to image weakly fluorescent or faint specimens, then either speed and/or resolution must be compromised.

If the objects of interest are very small and maximum resolution is required, speed and/or sensitivity must be compromised.

Unfortunately, this is the nature of confocal microscopy. It is up to you, the microscopist, to decide which parameters are most important and which ones are of least significance to the questions you are asking about your specimen. This will then guide you towards choosing the best image acquisition parameter values for imaging your specimen.

What is important?

For whatever reason you are using a confocal microscope, it is essential that, by using the microscope, you are not altering the very thing you are trying to measure. Intense laser light has the ability to alter biological molecules and structures; local heating from the laser may modify the environment and cause the sample to move; live cells can respond to light and some organelles (chloroplasts) and cells in the retina are designed to react to light. It is essential that experimental design takes into account these possible effects.

1. Photodamage: Except in special circumstances, photodamage of biological molecules must be avoided at all costs. This will often require lowering the illuminating laser power or using a laser wavelength that minimises any photodamage.

2. Photobleaching: Photobleaching is the irreversible destruction of a fluorescent molecule so that it no longer fluoresces. Photobleaching results when a fluorescent molecule is in its excited semi-stable state and is then hit by a second photon causing permanent damage to the molecule. This mostly occurs when using higher laser powers for longer times. Apart from decreasing the strength of any fluorescent signal, biologically reactive molecules are often produced as a consequence of photobleaching, thereby potentially altering the very events one is trying to measure. Reducing the laser power (and pixel dwell time) can minimise photobleaching. It has been calculated that 150μW of laser power at the sample is sufficient to fully saturate all fluorescent molecules in that sample (Pawley Handbook), thereby rendering them more likely to be damaged irreversibly.

3. Signal: Noise Ratio To image a specimen in the confocal microscope, a minimum number of photons must be collected at the detector. These detected photons are converted to photoelectrons and that signal is then amplified and displayed as a pixel intensity value in our image. These values are usually referred to as our **signal**. Inherent in this process are electrons that are also produced within the detectors and amplifiers. However these electrons are not directly related to the photons emanating from our specimen and are, therefore, usually referred to as **noise** within our microscope system. While the absolute signal is important, it is the ratio of signal to noise (often shown as S/N ratio) that is critical. Sometimes it is easier to reduce the noise in a detector system (perhaps by cooling the detector) than to increase the absolute signal emanating from our sample. Such a system would give a higher S/N ratio and, therefore, more robust data and a better image.

4. Controls: One of the most difficult tasks when teaching confocal microscopy is to convince trainees of the importance of using appropriate control samples. Such samples are **critical**, especially when using new antibodies or looking at new specimens. Why is this?

○ Simply by adjusting the amplifier microscope controls, it is possible to make a negative sample look positive. A good procedure to guard against this possibility is to image a 'positive' sample and then, without adjusting any microscope controls, image a suitable 'negative' control. Open both images on the same computer monitor, side by side, and compare the images. If the 'positive' looks bright and the 'negative' image appears dim, then it is likely you have some specific signal. If the images look of similar intensity, then it is unlikely any specific signal exists in the 'positive' sample. Comparisons like this also allow a very unbiased means to evaluate labelling protocols when titrating antibodies.

○ Many samples will show some level of autofluorescence. It is essential, at least when starting with a new specimen, to image a totally unstained sample. By imaging this unstained sample together with a 'positive' and 'negative' sample all collected at the same microscope settings, the levels of autofluorescence, non-specific signal and specific signal can all be evaluated.

○ If no signal can be seen in a 'positive' sample, it may be necessary to label and image a sample **known** to contain the molecules or proteins of interest (a positive control).

The Confocal Pinhole

The major function of the confocal pinhole is to block or reject out-of-focus light from reaching the detector. If the confocal pinhole is small, out-of-focus light emanating from just above and from just below the focal plane is rejected by the pinhole. If the pinhole diameter is increased in size, more out-of-focus light from above and below the focal plane can reach the detector. If the pinhole is opened to its maximum value, the confocal microscope can produce images that are similar to those produced by a widefield, epifluorescence microscope.

One of the most difficult tasks in confocal microscopy can be actually focussing on your sample. It is always good practice to first focus on the sample using brightfield illumination. Additionally, using widefield, epifluorescence microscopy is helpful because out-of-focus light is usually easily seen even if the sample is grossly out of focus. However, when in confocal mode, if the sample is just slightly out of focus and the confocal pinhole is closed to a small diameter, it is possible that no light at all will reach the detectors. When no signal is visible it is nearly impossible to decide what the problem is - (1) Which direction to adjust the focus controls? (2) Is there any fluorescence on the sample? (3) Do the detector amplifiers need to be increased? or (4) Has the sample moved out of the scan area? Simply opening the confocal pinhole to the maximum diameter will allow any out-of-focus light to be visible and will increase the total signal reaching the detector. When some signal can be seen, it is then simply a matter of refocussing and then improving the quality of the image by adjusting other controls. When this has been done, slowly close the pinhole while continuing to adjust other controls.

Good confocal imaging is usually achieved by starting with some signal and then gradually improving the quality (and often the intensity) by making small, incremental adjustments.

Most commonly, to achieve the best resolution, a confocal pinhole diameter of 1.0 Airy Units is used for imaging. Reducing the pinhole size to less than 1.0 Airy Units will give better resolution. However, because of the significantly lower signal level, this is rarely done when imaging biological specimens.

Scanning and resolution -
How scanning works

In a confocal microscope the laser light is usually focussed by the objective lens to a diffraction limited spot on the specimen. As the light hits the specimen, fluorescence photons are emitted and some enter the objective lens. Some of these photons eventually pass through the confocal pinhole and reach the detector. By placing a small mirror (which is rapidly rotated by a galvanometer motor) in the light path, the spot of light can then be moved across the specimen. Moving the galvanometer mirror in one direction (usually the x direction) would result in a line of points on the specimen being illuminated and the photons emanating from those points being detected.

If the light that is reflected by the x direction galvanometer mirror is directed onto another galvanometer mirror rotating at right angles to the first mirror (ie in the y direction) and if the timing of both galvanometer mirrors is adjusted correctly, a **raster scan** can be performed over the specimen. (This is similar to the way the electron beam is moved across a cathode ray tube like in older televisions.) The result is a two-dimensional image made up from many individual points forming lines and many lines forming the image. Each individual point usually corresponds to a pixel in the final digital image.

The time the laser beam illuminates a point on the specimen (and the signal from that point is detected) is referred to as the **pixel dwell** time (the time the laser dwells on each pixel position). The longer the pixel dwell time, the more laser light excites fluorophores and the more emitted photons are detected giving a brighter signal. This is good.

However, the longer the pixel dwell time, the more likely it is that another laser photon can hit an already excited, semi-stable fluorescent molecule and result in permanent damage to that fluorescent molecule causing photobleaching. Shorter pixel dwell times usually result in less photobleaching - this is also good. However, shorter pixel dwell times give less emitted photons and therefore less signal - not so good.

So, a reminder - 'you get nothing for free'. It is up to you to decide what is important - more signal or more bleaching? Common pixel dwell times are usually around a few microseconds.

The number of pixels in an image can be important and is discussed later. However, as a general guide, quick scanning is commonly done on 512 x 512 (or smaller) pixel arrays while slower but higher resolution imaging uses at least 1024 x 1024 pixels. Remember, more pixels means a longer total scan time. Remember the eternal triangle!

As was mentioned in the Introduction it is the Numerical Aperture (NA) that is most important in resolution not the magnification. This is shown in the table below. Note the 40x 1.25 lens has better resolution than the 40x 0.75 .

MAGNIFICATION	NUMERICAL APERTURE	LATERAL RESOLUTION (μm)*
10x	0.3	0.666
40x	0.75	0.266
40x	1.25 oil	0.160
63x	1.2 H2O	0.166
63x	1.3 Gly	0.153

*@ 500nm

The resolving power of a lens is ultimately limited by diffraction effects. The lens' aperture is a 'hole' that is analogous to a two-dimensional version of the single-slit experiment; light passing through it interferes with itself, creating a ring-shaped diffraction pattern, known as the Airy pattern, that blurs the image. As can be seen from the diagram below the pinhole improves resolution. The above calculations have been made with the equation $r = 0.4\ \lambda\ /NA$.
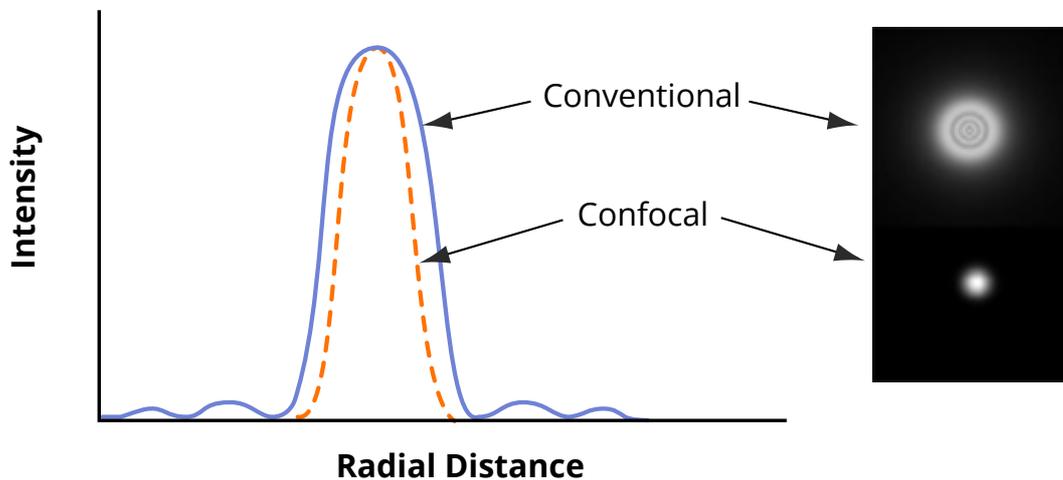
$$r_{conventional} = 0,61\lambda/NA \qquad r_{confocal} = 0,41\lambda /NA$$

The above figure shows the intensity profile of a point source of light. With a pinhole inserted much of the out of focus light is removed and the intensity profile is sharper. This shows an increase in resolution.

Scan areas and relationship to pixels and resolution

Confocal microscopes collect digital images and in modern day confocal microscopes we can vary the number of pixels and the scan area over a wide range. Therefore, for the rest of this discussion we will be comparing a 512 x 512 pixel scan versus a 1024 x 1024 pixel scan. Note that the scan area and pixel size will be dependent on the particular confocal microscope you are using as all microscopes will likely have slightly different configurations. Each microscope needs to be calibrated.

MAGNIFICATION	NUMERICAL APERTURE	SCAN SIZE (pixels)	SCAN AREA (µm2)	PIXEL SIZE (µm)	LATERAL RESOLUTION (µm<)
10x	0.3	512 x 512	1555	3.03	0.66
		1024 x 1024		1.52	
40x	0.75	512 x 512	387.5	0.758	0.266
		1024 x 1024		0.378	
	1.25 (oil)	512 x 512		0.758	0.16
		1024 x 1024		0.378	
63x	1.2 (H2O)	512 x 512	246.03	0.481	0.166
		1024 x 1024		0.24	
	1.3 (Gly)	512 x 512		0.481	0.153
		1024 x 1024		0.24	

As can be seen from the above table, scanning your image onto a 512 x 512 image or 1024 x1024 image, the scan area does not change. However the resolution per pixel does. For publication purposes it is usually important to have higher resolution scans.

Zoom

As was mentioned in the Introduction the confocal does allow you to zoom thus decreasing the scan area and increasing the magnification. However when doing this you must remember that there is a maximum zoom above which it is not advisable to go. The simple calculation for this is the lateral resolution divided by 2.3 which giving you the minimum pixel size. Higher zooms usually cause unnecessary bleaching of your sample without any improvement in resolution.

In the table below you can see the zoom at which you reach Nyquist sampling for this particular microscope, at the stated NA and wavelength.

MAGNIFICATION	NUMERICAL APERTURE	SCAN SIZE (pixels)	SCAN AREA (µm2)	PIXEL SIZE (µm)	LATERAL RESOLUTION (µm<)	NYQUIST PIXEL SIZE (µm)	ZOOM TO OBTAIN NYQUIST
10x	0.3	512 x 512	1555	3.03	0.66	0.289	10.54
		1024 x 1024		1.52			5.24
40x	0.75	512 x 512	387.5	0.758	0.266	0.115	6.57
		1024 x 1024		0.378			3.28
	1.25 (oil)	512 x 512		0.758	0.16	0.069	10.88
		1024 x 1024		0.378			5.43
63x	1.2 (H2O)	512 x 512	246.03	0.481	0.166	0.072	6.6
		1024 x 1024		0.24			3.34
	1.3 (Gly)	512 x 512		0.481	0.153	0.066	7.22
		1024 x 1024		0.24			3.6

Detection parameters -
Laser power

One golden rule: **"Use the lowest laser power to give an acceptable image."** When an acceptable image can not be obtained and all other parameters have been adjusted to the point they are causing problems with the image, only then is it usual to increase the laser power. Remember, excessive laser light can alter your specimen and cause photodamage and photobleaching. Using the lowest possible laser power for the shortest possible time will best preserve the fluorescence or biological activity in your samples.

Laser powers are given using quite different scales on microscopes by different manufacturers. Sometimes percentage of maximum power is used, sometimes values from 0 to 255 are used, and sometimes actual laser power is measured at some point in the optical path (which is not necessarily the same as at the specimen focal plane). Additionally, different lasers are used with quite different power outputs. So, it is nearly impossible to compare laser power from one confocal microscope to another confocal microscope, even if manufactured by the same company.

So how much power should one use? **"Use the lowest laser power to give an acceptable image"**. (Do you think someone is trying to tell you something here?)

Adjusting the Image and Detector Controls

Gain or HV

Gain (also called HV or high voltage) is a relative measure of the amplification one applies to the photon detection system. Usually, higher gain settings result in brighter images (increased signal). However, as one increases the gain, increasing noise becomes evident in the image. (This is visible as a 'speckle' overlying the image data.) So, at some point as the gain is increased (and the overall image gets brighter), the signal/noise ratio (S/N) starts to decrease. The overall aim is to get the highest S/N, **NOT** the brightest image.

Offset or Black Level

Offset (also called black level) is an adjustment on the amplifiers of the detectors that resets the baseline values of the detectors.
By increasing the offset, the darkest areas of the image get brighter.
By decreasing the offset, the dark areas of the image get darker.
Caution: by decreasing the offset too far, very faint data will actually be lost - it is critical that this is not allowed to happen. New users are often tempted to decrease the offset value to try to eliminate background or non-specific signal. This should **NEVER** be done.

Dynamic Range

In order to collect the very best images, it is essential to capture both the very darkest values in our data as well as the brightest values. The difference between the brightest and darkest values is termed the dynamic range.
Correct adjustment of the gain and offset values is **ESSENTIAL** to obtain the highest dynamic range (and, therefore, the best looking image).

Look Up Tables (LUTs)

Look up tables (sometimes called Hilo) are simply coloured overlays that sit over your display to indicate the brightness of individual pixels. Many microscope manufacturers use specific LUTs to indicate when pixels in an image are saturated - where brightness (laser power or gain) is set too high, or undersaturated where offset is set too low.
It is critical, especially for newer confocal microscopists, to use LUTs to correctly set the gain and offset, thereby collecting the very best range of values (dynamic range) and therefore the very best image. Remember, the data in the image that you collect on the microscope is the very best it will ever be. Later image processing to improve the appearance of your image usually throws some data away.

Gain and offset adjusment in confocal microscopy
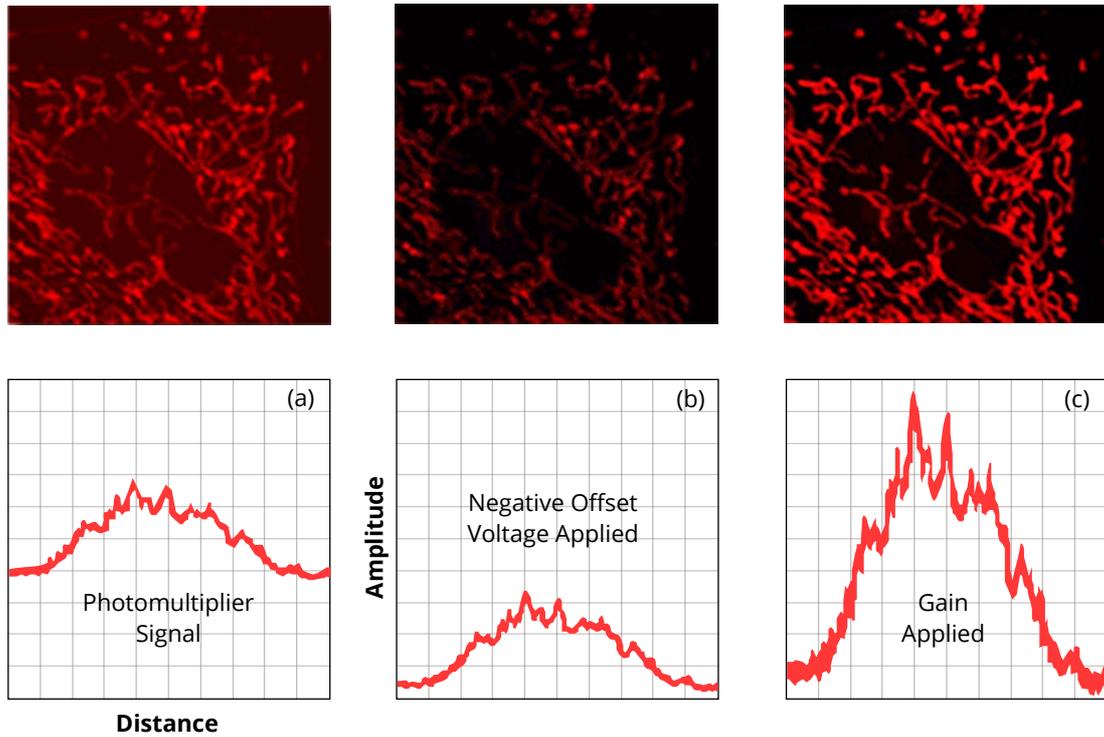
To explore the effect of photomultiplier settings, consider the three images and photomultiplier outputs above. In Figures a) and b) the offset on the PMT is raised or lowered to make the image brighter or darker. However, the images are both somewhat washed out with a) being overall too bright and b) being overall too dark. In Figure c) the PMT gain has been increased, which results in an image with better contrast, more dynamic range and is overall a much clearer image. PMT offset and gain are analogous to brightness and contrast in photography. Both have to be set correctly to get the optimal result.

Averaging

Averaging is used to reduce noise which is mostly generated by electronic components of the detection amplifier. For all intentional purposes, noise is generated randomly and is seen in an image as bright and dark spots of varying intensity scattered in a random pattern over the image. This makes it difficult to see fine image detail (the signal) in a noisy image.

A good analogy is to think of the old analogue television sets where the aerial lead has been removed. What do you see? The "snow" that is visible on the screen is simply noise generated by the signal amplifiers working hard to show an image where we have removed the signal coming from the aerial.
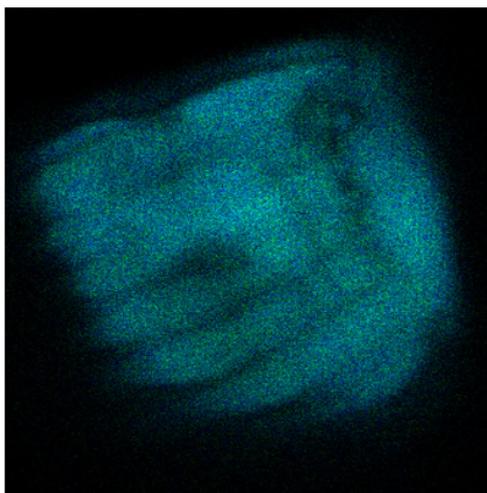
Autofluoresence of a pollen grain imaged using a high detector amplifier gain setting. Notice the "speckled" appearance and the loss of surface detail.

Noise in an image is reduced by averaging multiple images together, pixel by pixel, and calculating an "average" pixel value for each x,y position in the image. There are numerous algorithms for performing this calculation, but one of the most common is the Kalman estimator.

Frame averaging is often done by collecting one noisy image, then collecting a second noisy image, adding these images together numerically, pixel by pixel, and then dividing the resulting image values by two. The resulting image will show less noise. By repeating this process several times, noise can be averaged enough to allow fine detail (signal) to be seen.

Line averaging is performed in a similar manner, except that data from a single scan line is collected several times and averaged before the next scan line is collected.

How many images should be averaged to get a good image? This is primarily determined by the detector amplifier gain setting (higher gain settings generate more noise so more averaging is required). However, it should be noted that improvement in image quality usually gets less with each additional scan. This means there is a practical limit to the number of frames that should be averaged, beyond which improvement in quality is minimal. Remember, for each additional scan, more laser energy reaches the sample potentially increasing the chances of photodamage and photobleaching.

Frame averaging should usually not be performed on moving specimens (a motion-blurred image would result). However, line averaging might be suitable for slowly moving objects, albeit resulting in a dimensionally distorted object image.

Sequential and simultaneous imaging -
Using multiple dyes

When collecting fluorescence images it is essential to understand the characteristics of the fluorescent dyes one is using and the configuration of the lasers and filters in the confocal microscope.

When imaging a single fluorescent dye it is usually only necessary to (1) select the laser which most efficiently excites the fluorophore and (2) select the emission filter that most efficiently allows the emitted fluorescent light to reach the detector. However, when attempting to image multiple fluorescent probes in the same sample, it is essential that excitation laser wavelengths and emission filters are carefully chosen. In order to do this, researchers must have a more thorough understanding of the excitation and emission spectra of the fluorescent dyes they are using.

Fluorescence Spectra

There are a range of wavelengths (colours) of light that can excite a fluorescent probe, with some wavelengths being more efficient at excitation than other wavelengths. A graph of all excitation wavelengths (and the efficiency of excitation) represents the excitation spectrum for that particular probe. The excitation spectrum for the DNA stain DAPI is shown below in the dotted line.

When DAPI is excited by the appropriate wavelength of light, the molecule enters a transient excited state for a short period of time (the "lifetime" which, for many probes used in confocal microscopy, is usually between 1 and 10 nanoseconds), before it may decay back to some resting energy state and emitting the previously absorbed energy as a photon of light. The emitted light can have a range of wavelengths of varying intensities and a graph of these values is called the emission spectrum. The emission spectrum for DAPI (when bound to DNA) is shown as the shaded area in the graph below:
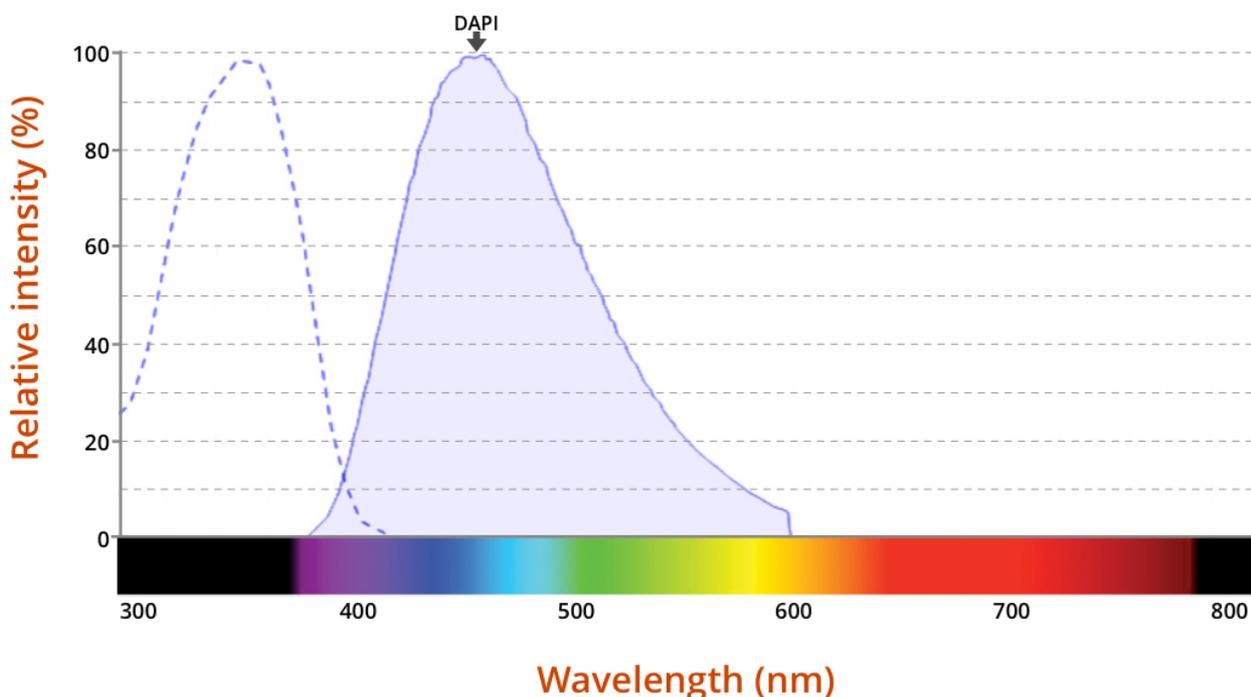
Excitation (dotted line) and emission (solid line) spectra for DAPI. Note the range of wavelengths emitted when DAPI fluoresces (from approximately 400nm (violet) to 600nm (red)) and the maximum value at approximately 440nm.

To image DAPI on a confocal microscope, it is best to excite DAPI with a laser with a wavelength close to the excitation peak (approximately 350nm (UV)). However, UV lasers are expensive and can easily alter biological molecules, so they are seldom used. Instead, since DAPI fluorescence is usually very strong, a cheaper 405nm (violet) laser (which excites DAPI with about only 10% efficiency) is more commonly used. DAPI fluorescence is usually detected through a 440nm longpass filter. However, if more than one fluorescent probe is used in the same sample, it is more common to detect DAPI fluorescence through a 450/50nm bandpass filter.

Fluorescence Spectral Overlap

When two (or more) fluorescent probes are used within the same sample, it is possible that the emission spectra of the two probes overlap (as seen below).
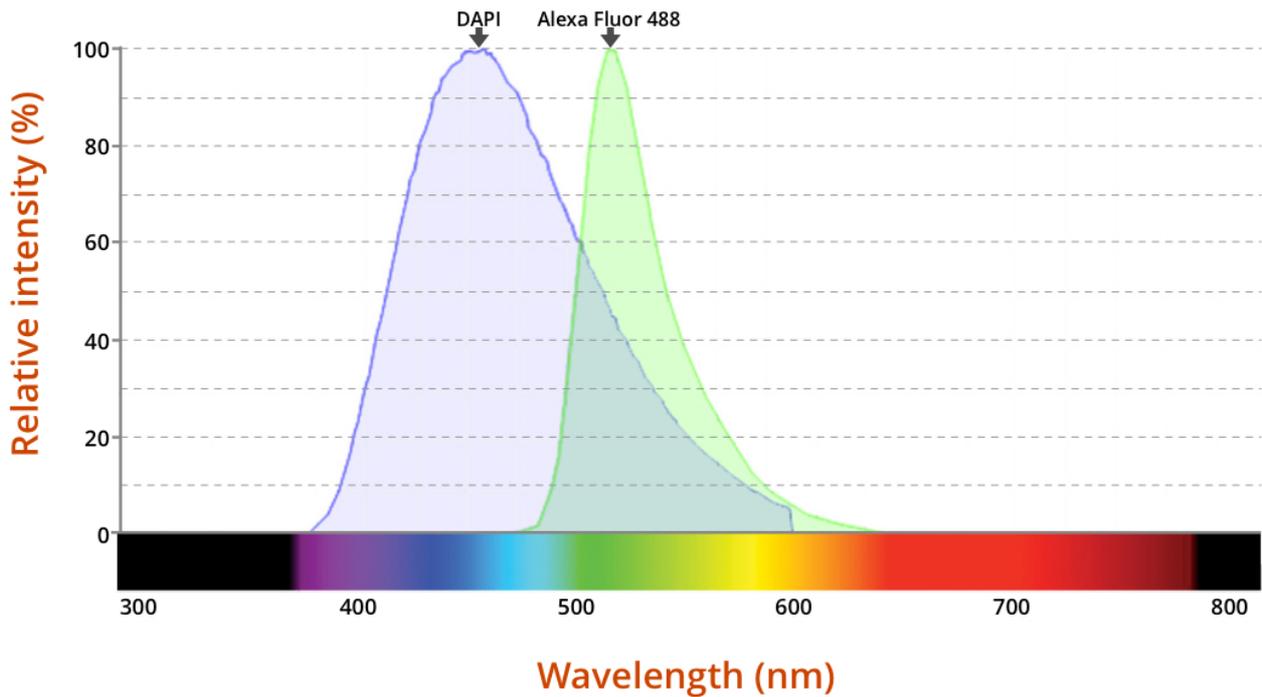
Emission spectra for DAPI (blue) and AF488 (green). Note the overlap in the spectra between approximately 500nm and 600nm.

Since the fluorescent signal is usually only detected as an intensity of photons passing through an emission filter, separating the signals from each probe can become particularly difficult.

Simultaneous Imaging

Imaging more than one fluorescent probe at the same time (exciting with all required lasers and detecting the signal through all filters at the same time) is usually referred to as simultaneous image collection.

In a typical experiment simultaneously imaging the fluorescent probes DAPI and AlexaFluor 488 (AF488) involves exciting both fluorophores with both the 405nm and 488nm laser at the same time:
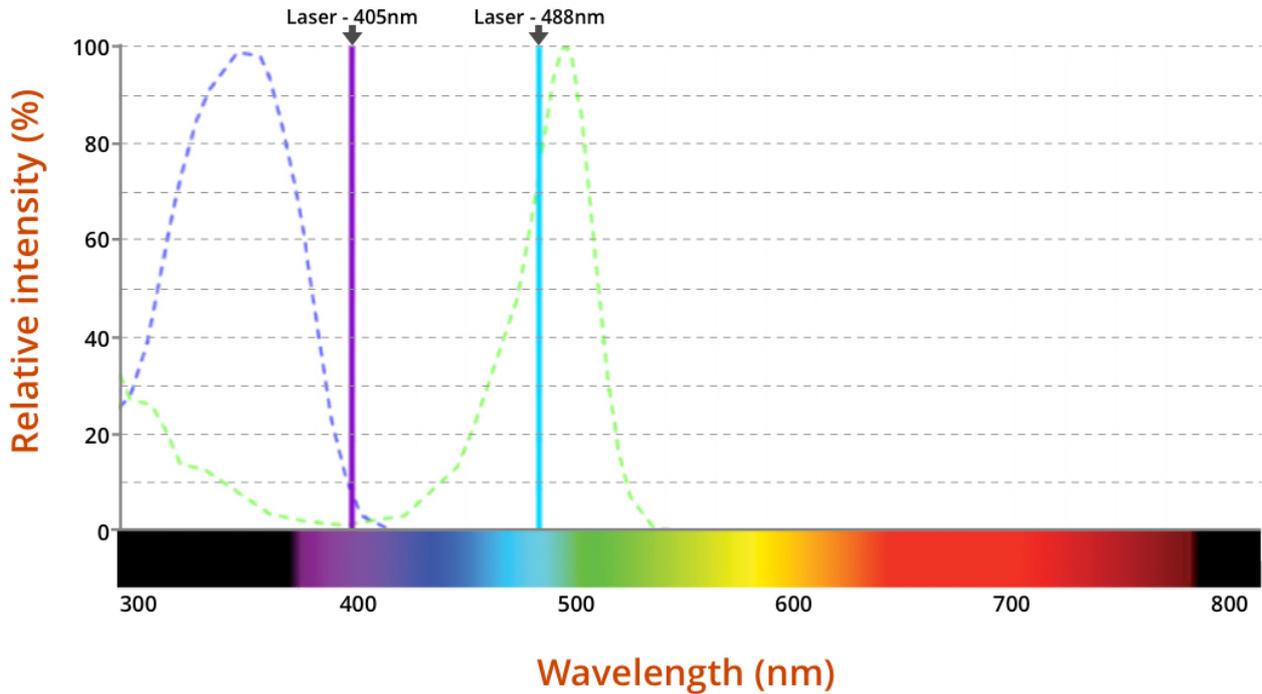
Simultaneous excitation of both DAPI and AF488.

The fluorescence signals resulting from dual excitation of these probes are usually collected in two separate detectors through two separate emission filters. The DAPI signal is collected through a 450/50nm bandpass filter. This signal should have very little signal emitted by the AF488 fluorophore (since the AF488 emission will not pass through the 450/50nm filter).
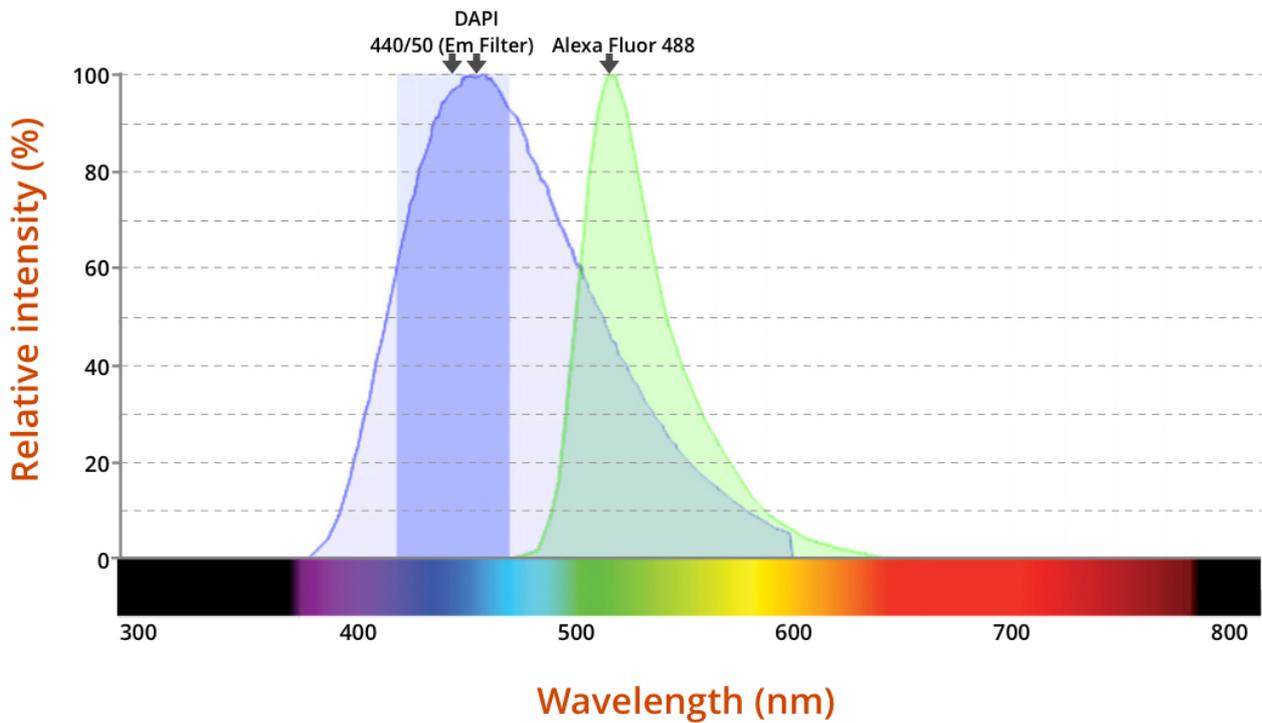
Emission spectra for DAPI and AF488. DAPI fluorescence is detected through a 450/50nm bandpass filter usually into the first detector

The AF488 signal is collected through a 525/50nm bandpass filter. However, as can be seen in figure 5, at least some of the fluorescence emission from both DAPI and AF488 will pass through the 525/50nm filter. In this example, almost 30% of the total signal detected in the second detector will originate from DAPI. Detection of more than one fluorescence signal in a single detector is usually referred to as spectral bleedthrough and it can be very difficult to separate the different fluorophore signals from each other.
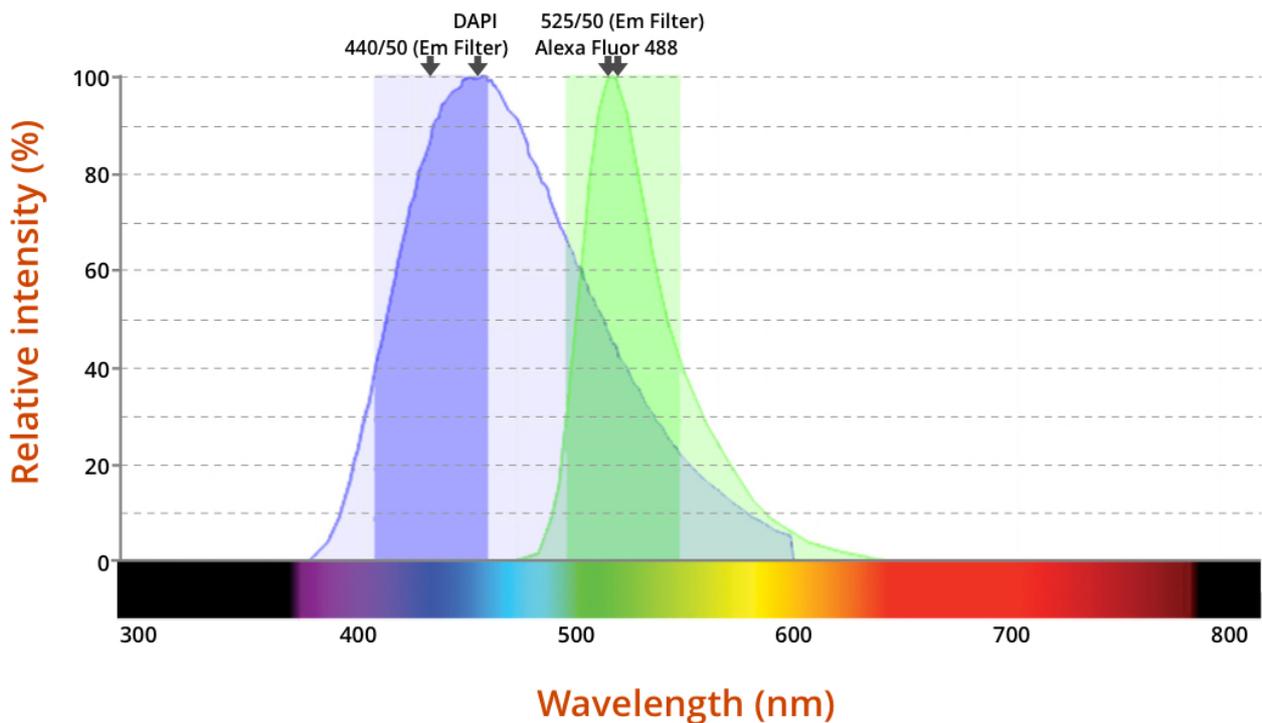
Emission spectra for DAPI and AF488. DAPI fluorescence is detected through a 450/50nm bandpass filter (usually into the first detector) while AF488 fluorescence is detected through a 525/50nm bandpass filter (usually into the second detector). However, note that almost 30% of the signal seen in detector 2 originates from DAPI fluorescence.

Simultaneous imaging of more than one fluorescent probe has the advantages of more rapid image collection and, for live cell imaging, no temporal displacement between the two images. Good experimental design using adequately spectrally separated fluorophores can avoid (or at least minimise) spectral bleedthrough. An example of this would be imaging DAPI and AlexaFluor 647 (AF647)
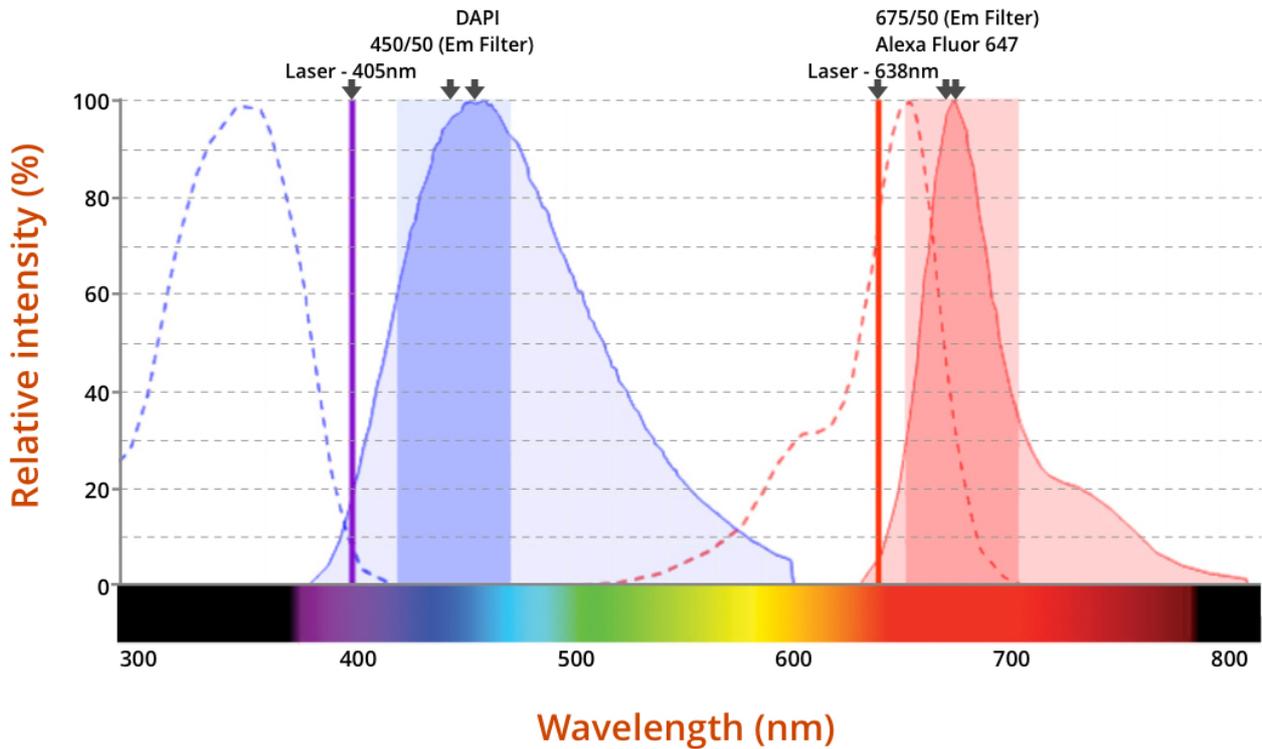
Simultaneous imaging of DAPI and AF647 in a dual labelled specimen is possible without spectral bleedthrough because of minimal cross-excitation or emission of the two fluorophores.

Sequential Imaging

Sequential imaging involves exciting fluorophores on the specimen with only one laser at a time and collecting fluorescence photons emitted by the excited fluorophores. Then, by swapping to another laser wavelength and detecting photons emitted from another fluorophore, spectrally separated signals can be collected. Provided each laser line excites only one fluorophore, all emitted photons will be derived only from the relevant fluorophore with no spectral bleedthrough. When sequentially imaged a specimen that has been dual labelled with both DAPI and AF488, a single laser is used for excitation and the emitted photons collected (image A). Then the other laser is used for excitation and the relevant emission photons collected (image B).

Image A
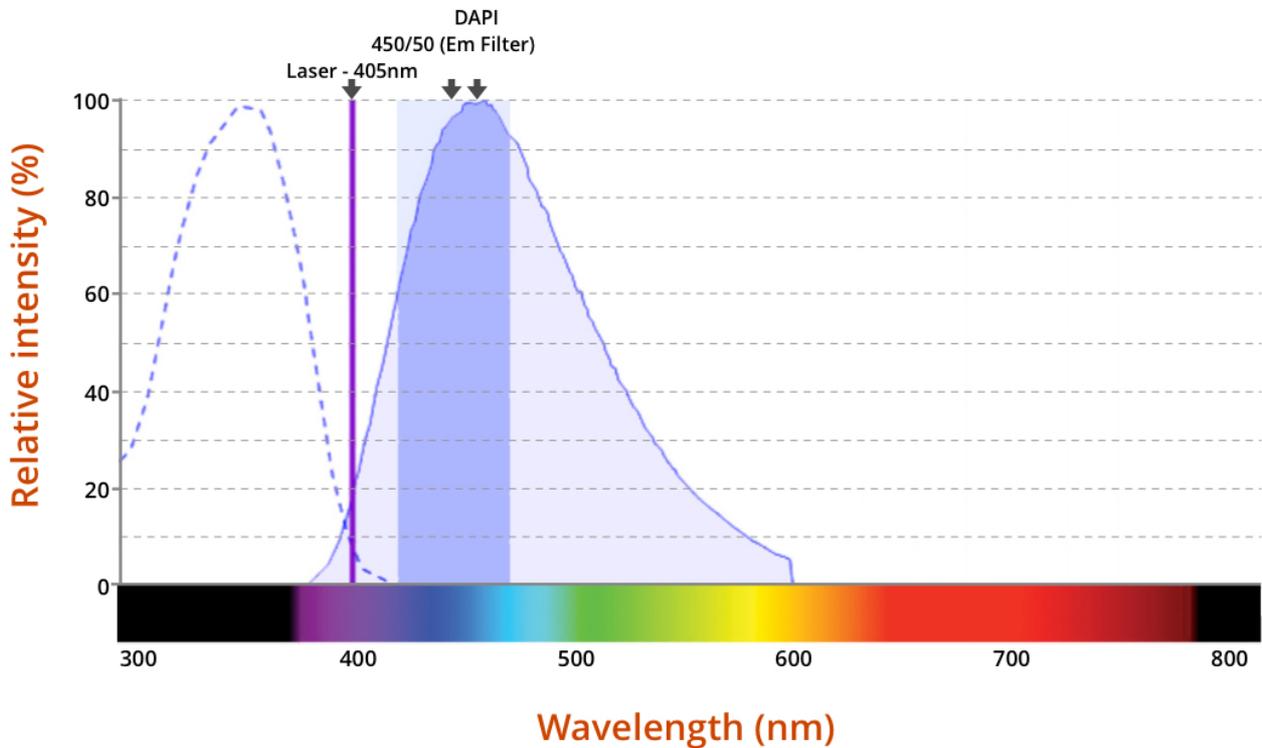
Sequential imaging of DAPI and AF488 in a dual labelled specimen. In this example, DAPI is imaged first (and the 488nm laser is turned off during image collection).

Image B
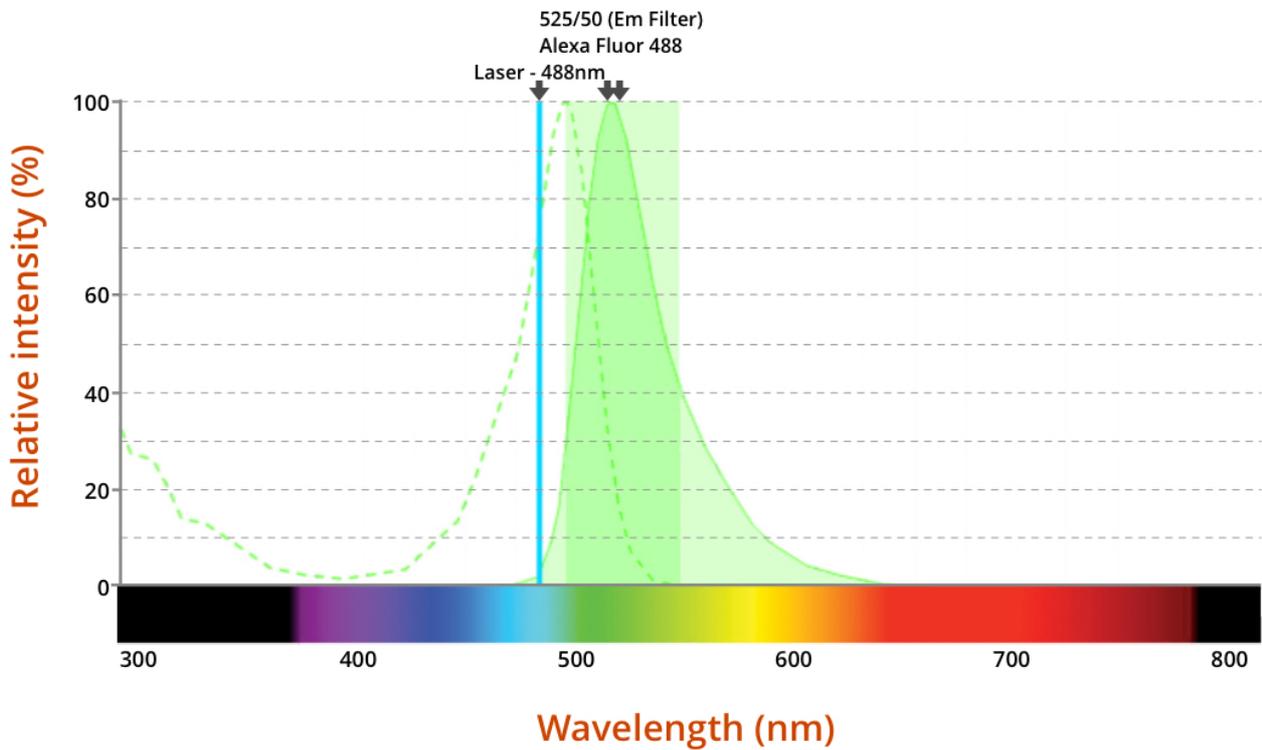
Sequential imaging of DAPI and AF488 in a dual labelled specimen. When imaging the second fluorophore (AF488), the 405nm laser is turned off during image collection and the only signal collected is derived from AF488.

Simultaneous imaging three or four fluorophores in the same specimen will almost always produce significant spectral bleedthrough (as can be seen below). Careful selection of fluorophores combined with sequential imaging can usually eliminate (or at least minimise) spectral bleedthrough.
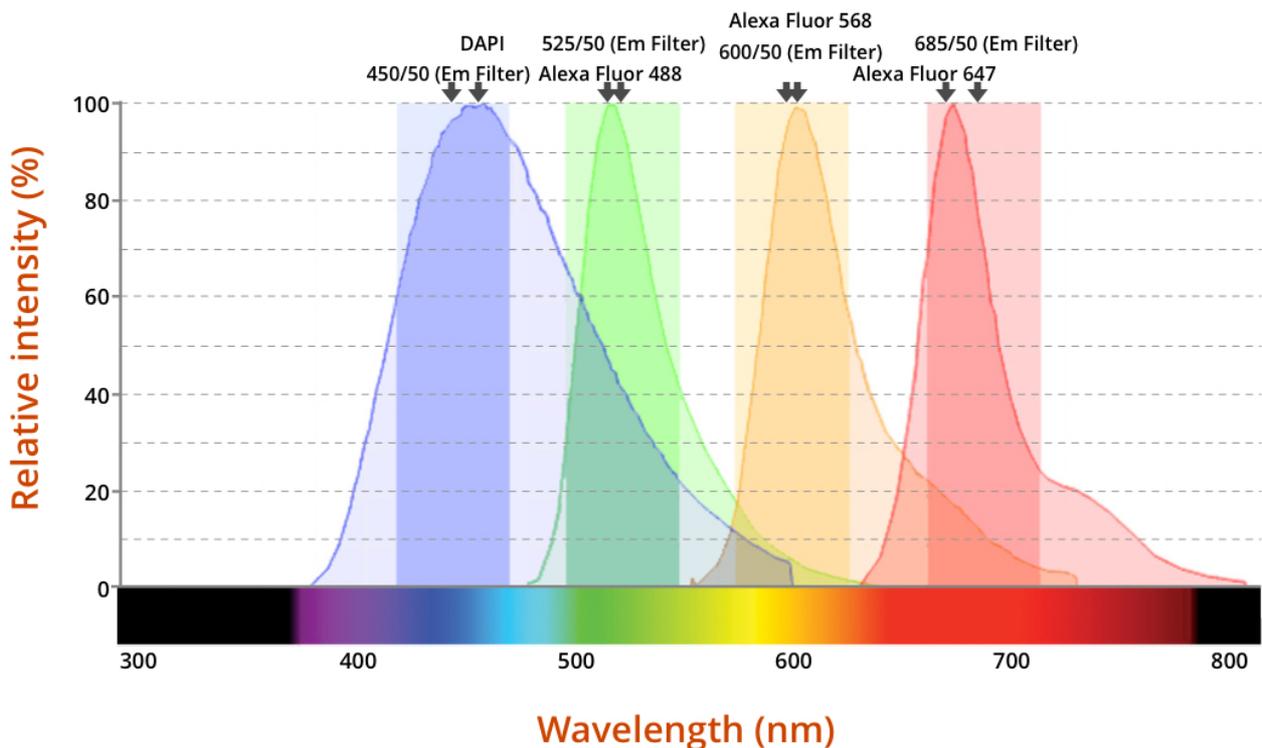
Sequential imaging of DAPI, AF488, AF568 and AF647 in a four fluorophore labelled specimen can minimise spectral bleedthrough.

Sequential imaging has the advantage of minimising spectral bleedthrough and, in most cases, should always be used when performing analyses of colocalisation of multiple fluorophores. However, sequential imaging of four fluorophores means image collection time will take at least four times longer. Additionally, the temporal separation between each fluorophore image using sequential imaging usually precludes using this technique to image rapidly moving live samples.

Some Possible Fluorophore Combinations

FLUOROPHORES	SIMULTANEOUS	SEQUENTIAL
DAPI	✔	-
AF488	✔	-
AF586	✔	-
AF647	✔	-
DAPI + AF488	✘ 1	✔
DAPI + AF568	✔ 2	✔
DAPI + AF647	✔	✔
DAPI + AF488 +AF568	✘ 3	✔
DAPI + AF488 +AF647	✘ 4	✔
DAPI + AF568 +AF647	✘ 5	✔
DAPI + AF488 +AF568 +AF647	✘ 6	✔

1. DAPI bleedthrough into AF488 channel.
2. DAPI bleedthrough into AF568 channel if DAPI signal is very strong.
3. DAPI bleedthrough into AF488 channel and, if DAPI signal is very strong, also into AF568 channel.
4. DAPI bleedthrough into AF488 channel but not AF647 channel.
5. Minimal DAPI bleedthrough into AF568 channel; significant AF568 bleedthrough into AF647 channel.
6. Spectral bleedthrough into all channels except DAPI channel.

Collecting Z stacks -
What is a Z-stack?

As you have already seen, one particular advantage of confocal microscopes is the ability to take optical sections and create three dimensional representations of a specimen without actually cutting or sectioning the specimen. This allows a greater understanding of the 'overall' structure of our samples.

So how can we generate three dimensional images? If we change the focus of the microscope by a known amount, we can collect another, different, optical section and save it to a file. By moving the microscope focus another known amount, we can collect another section and save it and do this again and again... If we know the dimensions between each point in the x and y directions (we know this by calibrating the microscope) and we know how much we moved the focus (ie the z dimension), we have complete three dimensional data for our specimen. This data can then be used by computer programmes to generate three dimensional images.
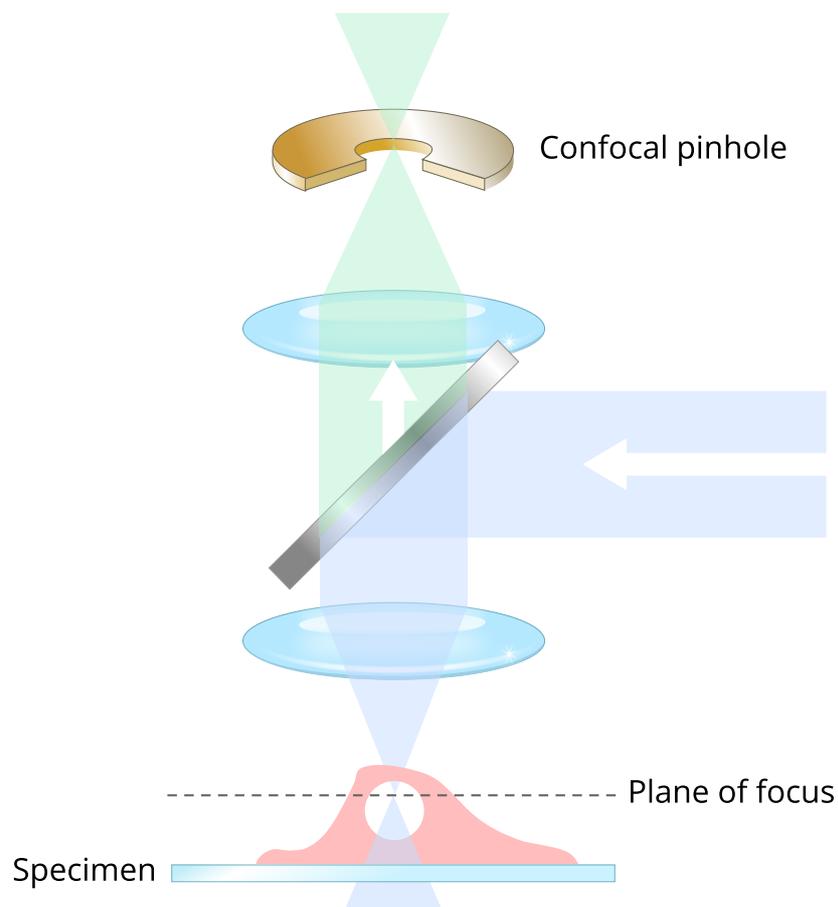
Remember that when changing the focus of the microscope, the relative positions of the plane of focus and confocal pinhole do NOT change in relation to each other - only the relative position of the specimen is changing. It is sometimes best to think of changing the focus as simply moving the specimen up or down (along the optical or z axis.)

Optical Section Thickness

The thickness of an optical section is predominantly determined by the numerical aperture of the objective and the diameter of the confocal pinhole (and, to a lesser extent, the wavelength of the light.) The following table lists the optical section thickness (OST) for sections collected with different objectives and different pinhole sizes.

Below is a table that shows you the optical section thickness for various objectives and Airy units (AU).

MAGNIFICATION	NUMERICAL APERTURE	OST (µm) 1AU	OST (µm) 2AU	OST (µm) 4AU
10x	0.3	11.08	19.96	38.74
40x	0.75	1.772	3.19	6.19
	1.25 (oil)	0.969	1.745	3.387
63x	1.2 (H2O)	0.921	1.659	3.221
	1.3 (Gly)	0.856	1.54	2.99

OST = optical section thickness.

Nyquist Sampling for Z stacks

If information is to be preserved in all three dimensions, it is important that the images are collected with appropriate x, y and z dimensions. These dimensions are determined by the Nyquist sampling theorem which, in essence, says that, to resolve an object, we must sample that object 2.3 times more frequently than its dimensions. Another way to say this is that our voxel size needs to be a minimum of approximately 0.4 x the dimensions of that object.

For example, if the theoretical resolution limit in the lateral (x,y) plane is 0.2μm, then we must use a pixel dimension of 0.2μm x 0.4 = 0.08μm (or 80nm) in that plane. Similarly, if the z dimension resolution limit is 0.5μm, then our best z step should be 0.5μm x 0.4 = 0.2μm (200nm). This means our final microscope setup should be collecting images with voxels that have dimensions of 0.08μm (x) x 0.08μm (y) x 0.2μm(z).

As a guide, use the table detailing optical resolution and the above table to determine what dimensions should be used to collect the very best detail in an image.

Under and Over Sampling in Z Stacks

Frequently it may be necessary to collect confocal image slices at z intervals that are other than optimal. Sampling with voxel dimensions greater than required for full resolution is termed 'undersampling' and sampling with voxel dimensions smaller than required is termed 'oversampling'.

Instances where undersampling might be necessary include
1. Where the specimen is weakly fluorescent or is very photolabile and sensitive to photobleachin
2. The specimen is moving slowly, and registration between the first and last images would be poo

Undersampling results is less information in one or more dimensions and may compromise the collected data. It is often tempting to undersample specimens because of the extra time required to collect full resolution data sets. Before undersampling, first consider how long it has taken to perform your experiment and prepare your sample. Is the extra 10 or 15 minutes needed for good imaging worth compromising your data? Remember, the data in the image you collect on the microscope is "as good as that data will **EVER** be" - subsequent image processing may make the picture look better but it will have no more data (which can not be added after images are collected).

Oversampling (where one or more voxel dimensions are smaller than necessary) gives **no more information** in the image. When oversampling is used, one is essentially just magnifying blur, and is usually, potentially creating more photobleaching potential in the sample.

Projections

Projections are techniques used to display three dimension information as some form of two dimensional image. One commonly used technique is a maximum intensity projection (MIP), also sometimes called an extended focus image.

The following diagram represents three optical sections taken through a specimen. Each image or section contains some information. However, from any one (or even a few) sections it might be difficult to visualise what the complete structure looks like.

Projection or extended focus image

Section 1
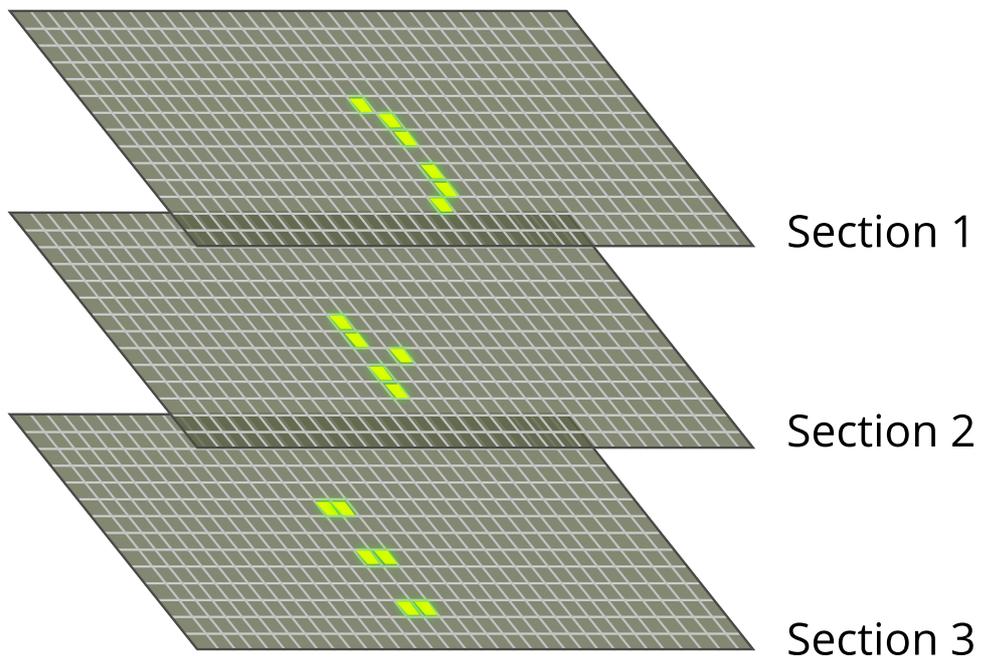
Section 2

Section 3

If we were to reproduce each single section onto a clear and transparent plastic sheet, then put all the sheets together into a "stack", hold the stack up to the light and look through it, we would see a representation of the entire data set. This representation might give us a better understanding of what is really in our data. We call this type of representation a projection or an **extended focus image**. (The term extended focus image is used, since each image or optical section comes only from the plane of focus and, therefore, must be in focus. Therefore, the data from a projection of multiple in focus images must, similarly, all be in focus.)

Projection or extended focus image
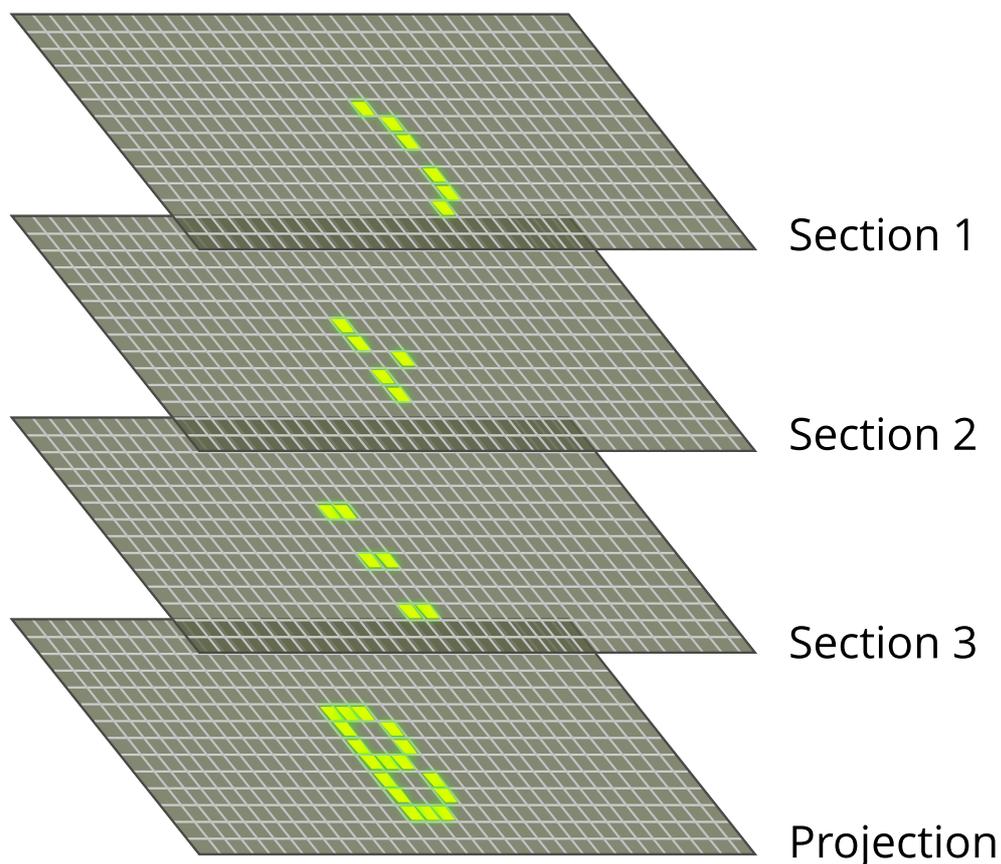
The following images show four optical sections taken through a house dust mite and the resulting projection of that data set. Note that while there is information in each section, a better understanding of the structure of the entire house dust mite can be gained from the projected image.
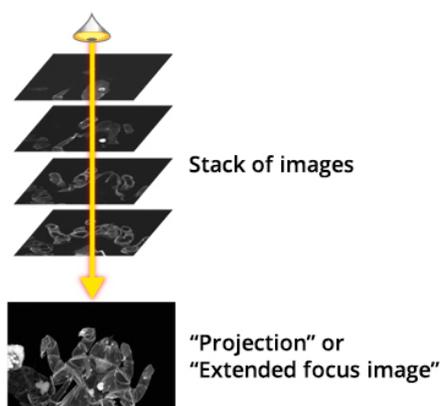
The following images were each taken from a data set where the focus (z dimension) was moved by 5μm between images. These optical sections taken through a house dust mite each show some information about the mite.
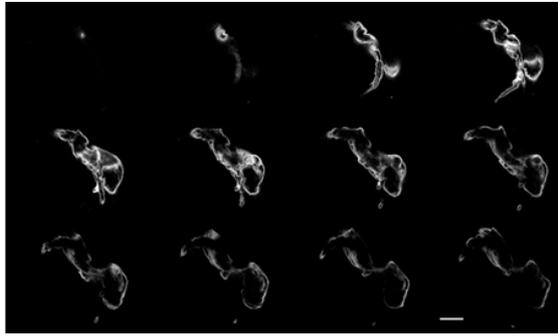
Serial optical sections through a dust mite (5μm steps)

But a significantly better appreciation can be obtained by viewing a projection of the entire data set.

The image on the left (below) shows a single optical section taken through a paramaecium (a unicellular aquatic organism). The paramaecium has been immunofluorescently labelled with an antibody directed against tubulin, which can be seen localised in several internal structures as well as in the extensive cilia that surround the organism. In the image on the right, a projection of the entire paramaecium data set is shown. Notice that the internal structures are no longer visible. Depending upon how the projection is created, emphasis can be placed on different structures to highlight those features of particular interest. This particular projection was done to highlight the surface features of the paramaecium.
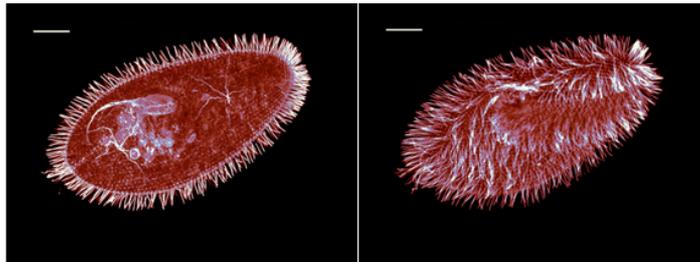
Image rotations

In a confocal microscope image data stack, we have information about all three dimensions. This means that we do not always have to do projections along the optical or z axis. We can generate a projection at some arbitrary angle or view through the data stack (this is simply like moving our viewpoint and looking through the transparent sheets when they are each separated by the correct z step).

We can then change our viewpoint (or angle from the optical axis) and generate another projection. We can do this multiple times and generate many projections.
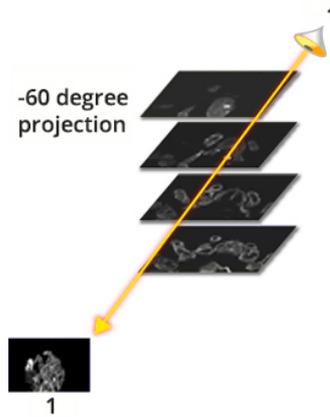
(Remember, that moving your viewpoint on a stationary object and generating projections is exactly the same as keeping your viewpoint the same and moving the object you are viewing.)

If we then "play" these projections in sequence, we generate a movie of the multiple viewpoints.
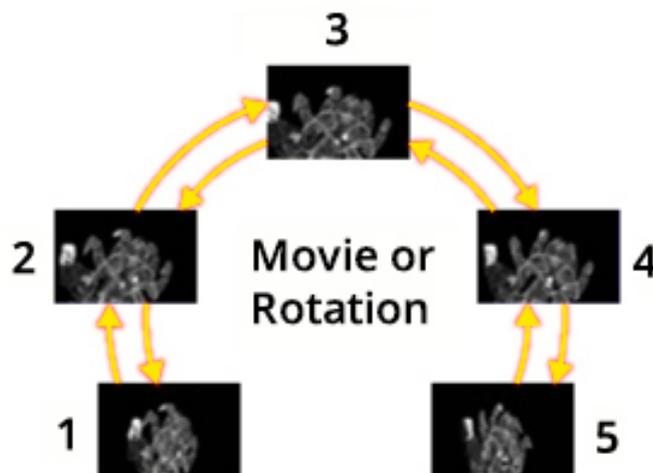
We (as humans) use three dimensional and motion information every day and are highly skilled and efficient at interpreting that data. We are not so good at interpreting multiple two dimensional data and relating that to a three dimensional structure. Motion and 3D views may help us to extract more information from the mountain of data we have already collected.

Axial resolution and Optical section thickness

With the insertion of a pinhole we are now able to optically section the sample. However the axial resolution is worse than the lateral resolution. In fact the airy disk is elliptical. See diagram below.

Horizontal PSF   **Axial PSF**

Full Widht at
Half Maximum
(FWHM)
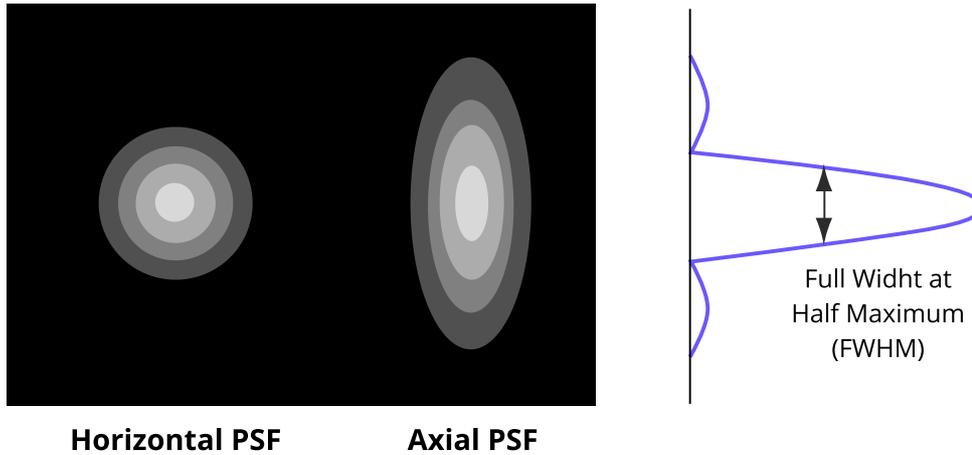
Axial resolution can be up to 4x worse than horizontal resolution, depending on NA:

$$R_{axial} = 1{,}67\lambda\,/(NA)^2$$

The calculation for axial resolution is $R=1.67\lambda/(NA)^2$

To collect a 3 dimensional image you must know your optical section thickness (OST). Most confocals calculate this for you now and all their formulas are slightly different.

Super–Resolution Microscopy -
The power of Super–Resolution

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 to Ernst Abbe is defined as:

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

with
λ = wavelength of light.
NA = numerical aperture of the objective lens.

The typical achievable resolution with standard light microscopes is typically limited to ~250 nm laterally and ~600-700 nm axially. With super-resolution microscopy, image resolution of less than 40 nm can be routinely achieved and below 10 nm has been demonstrated.

In terms of resolving power super-resolution light microscopes are only superseded by electron microscopes, which are limited to non-living specimen in high vacuum.
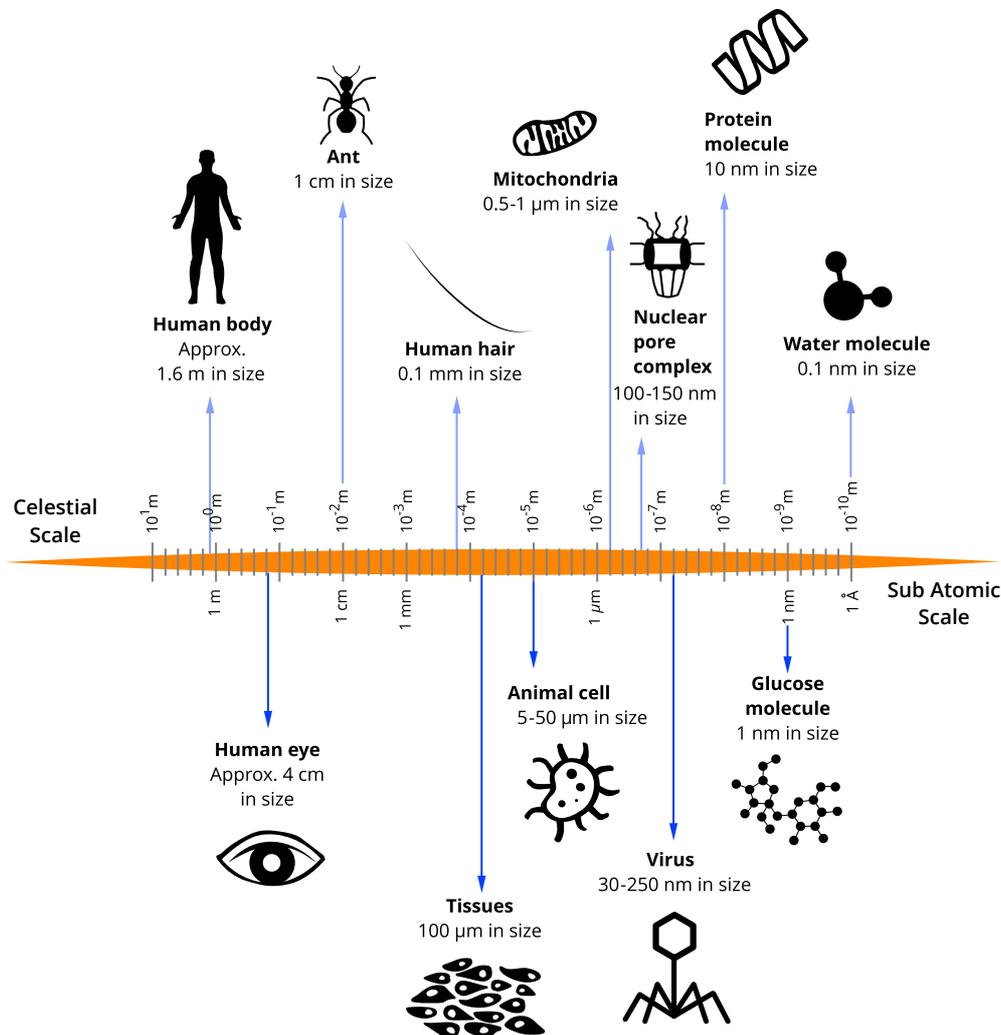
Scales in Biology. Biological structures and specimen span a wide range of sizes. Smaller entities require tools for magnification and detection. These tools are physically limited in their maximum resolution.

Which is the resolution barrier defining a super-resolution microscope? Currently, multiple definitions are used. All of them have in common that the resolution of the super-resolution microscope needs to exceed the Abbe limit.

The term nanoscopy is currently used in equivalent to super-resolution microscopy if the resolution of the microscope breaks the diffraction barrier fundamentally and enables in theory an unlimited resolution.

Criteria for optical resolution

Full-width-half maximum (FWHM)	**Sparrow's criterion**	**Dawes' limit**	**Rayleigh's criterion**
		(5% dip)	(20% dip, maximum of one spot coincides with first minimum of other)
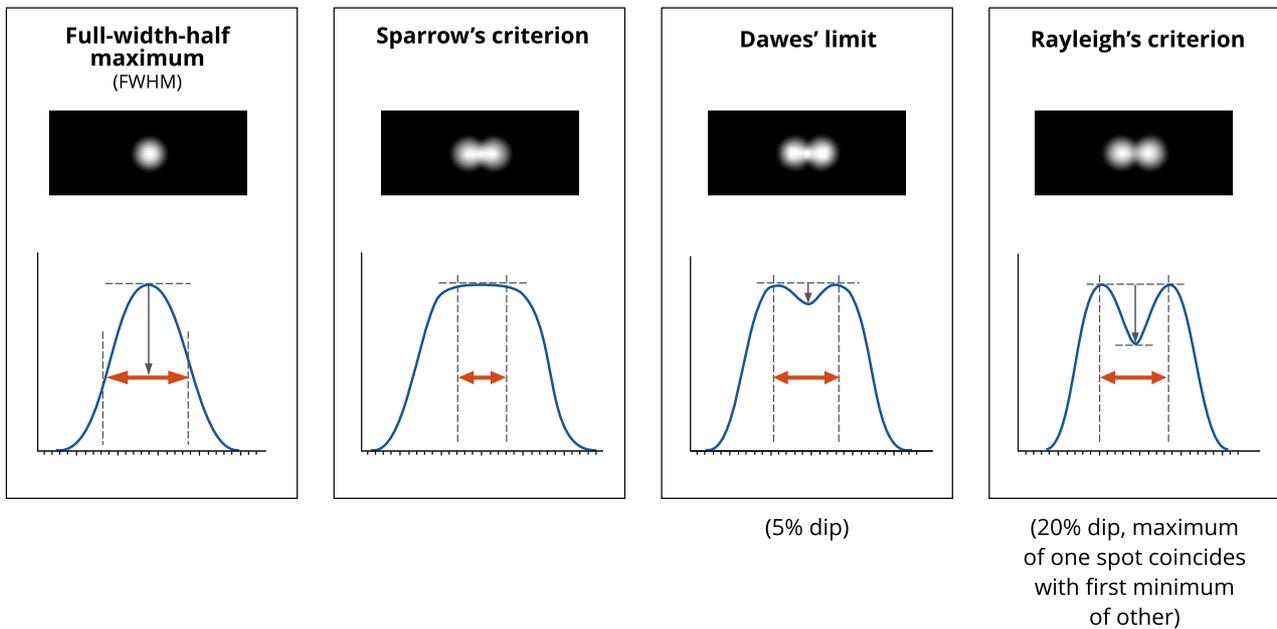
Optical resolution criteria. Measuring resolution from one (A) or two (B-D) point emitters. Dependent on the criteria applied, different distances between the two emitters are formally needed to resolve them.

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 its technical feasibility.

A number of criteria have been defined in the past to express the resolution of a microscope and thereby highlighting the difficulties to define such measures universally:

The Sparrow's 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 Dawe's limit or Rayleigh's criterion, a dip in intensity between the point-emitters of approx. 5% or 20% is required, respectively.

Another approach is measuring the Full Width at Half Maximum (FWHM) of a single point emitter's airy disc, which is often used as an approximation of the optical resolution of a microscope.

Biological samples are more complex and of higher emitter (fluorophore) density than the ideal two-point-emitters depicted above. Consequently, a two to three fold higher resolution (than the Abbe-limit) is often required to resolve the structures of interest reliably. If two structures of interest are for example 60 nm apart, only a resolution of 20-30 nm will deliver a sufficient 'dip' in the intensity between them to unambiguously separate them in the complex environment of the cell.

Advantages of higher resolved images

A simulated example for co-localisation

Higher resolution can always be considered as an advantage. However, it can change the view on standard image analysis concepts, as for example measuring co-localisation of fluorescently labelled proteins. The figure displays the same simulated structure (ground truth) as imaged at different resolution. In addition, it reports the commonly used Pearson coefficient as one possible measure for co-localisation: In a standard confocal image the structure seems to be partially co-localised which is also reflected by a Pearson coefficient of 0.6. By applying deconvolution or other means of increasing the resolution moderately into a range of 140 nm, the Pearson coefficient drops to 0.17 and the structure seems to consist of two concentric rings with different diameters. If nanoscopy (e.g. STED microscopy) is applied with a resolution of 30 nm, the rings can be subdivided into dots that do not co-localise at all (Pearson coefficient: 0.0).

The concept of co-localisation should therefore be always seen (and reported) as being dependent on the resolution applied in the imaging process. It needs to be noted that the two fluorophore-labelled proteins can of course never occupy the exact same space and that they only appear to do so due to the resolution limit and subsequent blurring coming from the imaging technology applied.
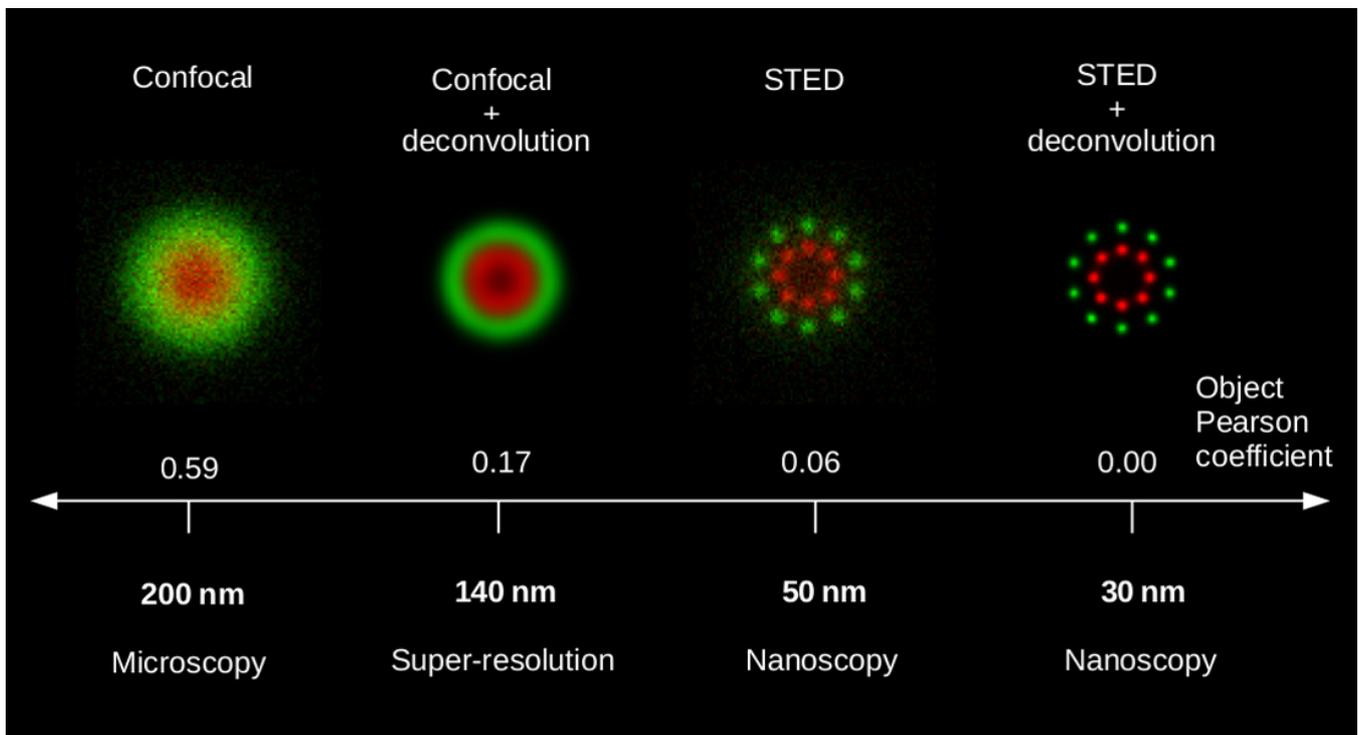
Microscopy image simulation of red and green diffraction-limited spots organized in two rings as seen at different levels of resolution, and the effect of resolution on co-localisation analysis (Object Pearson coefficient). Image simulation and deconvolution powered by Huygens Professional. Image used with permission. Courtesy of Remko Dijkstra, Scientific Volume Imaging (www.svi.nl).

STED/RESOLFT techniques - Overview to STED/RESOLFT

The optical resolution of a light microscope is limited by the diffraction of light, a fundamental physical law that restricts the resolution of all conventional microscopes. In super-resolution microscopy, the interaction of light with the complex properties of the fluorophore can be used to gain additional information and thereby to fundamentally improve the resolution by several folds beyond the diffraction limit.

Employing the properties of many fluorophores is possible since fluorophores can occupy different photophysical states. Using distinguishable states, fluorophores can be separated: a) in bulk within the diffraction-limited volume of a confocal microscope (e.g. in Stimulated emission depletion microscopy (STED) microscopy) or b) as individual, well-separated fluorophores in a widefield microscope (e.g. in Single Molecule localisation Microscopy (SMLM)). In addition to the photophysical properties of the fluorophore, the resolution increase of these microscopes requires clever illumination and detections schemes, which will be discussed in the following chapters.

In theory, any pair of separable states of a fluorophore (e.g. fluorescent vs. non-fluorescent, changes in emission wavelength etc.) can be employed. Stimulated emission depletion microscopy (STED) was the first experimental demonstration of the utilization of two separable states within a diffraction-limited volume to realize super-resolution microscopy beyond the Abbe resolution limit.

STED - Introduction to STED

STED is the abbreviation for Stimulated Emission Depletion. It employs the fact that an excited fluorophore can be spontaneously switched off by illuminating it with light of a wavelength which is within the emission spectrum of the respective fluorophore. The resting time of the molecule in the excited state is very short (usually in the range of nanoseconds) and the likelihood of stimulated emission is low. Therefore a relatively high light intensity has to be used to switch off (or deplete) the excited molecule. STED microscopy is based on a confocal microscope and therefore it is typically implemented with single-point scanning confocal microscopes.
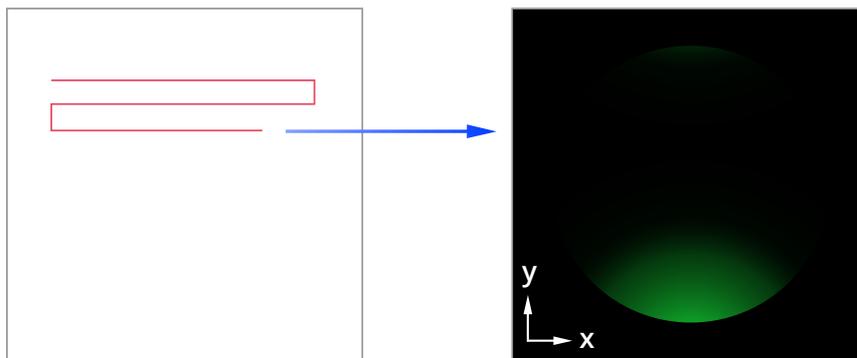
Principle of confocal scanning: A Gaussian-shaped excitation beam (right) derived from a focused laser is scanned over the sample in a line-wise fashion (left).

By controlling the off-switching of the excited fluorophores spatially, a higher resolution can be achieved. 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 their excited state in the nanosecond range before returning to the ground state by emitting a photon. However, if a STED beam is applied, the molecules return much faster to the ground state (are switched off). As long as the depletion beam is of the same shape as the excitation beam, this does not provide any advantage and the molecules are only switched off faster (and are practically invisible since they emit photons of the same wavelength as the powerful depletion beam and can therefore not be distinguished from the light by the depletion beam). However, if the depletion beam is shaped in a different way, e.g. like a donut, the following 'trick' can be applied: First, a laser spot (of Gaussian shape) is used to excite the fluorophores. Second, the STED beam is formed into a donut with no light intensity in the center which is then projected on top of the excitation beam. Molecules which are hit by the intense depletion beam are switched off practically immediately when sufficiently high depletion laser power is used. This leaves only the fluorophores in the center to emit photons with their normal lifetime effectively decreasing the size of the excitation spot. Consequently, the fluorescence is now emitted from a much smaller spot than the diffraction-limited excitation spot: super-resolution is achieved.

Diffraction Limit Diffraction Limit Super-resolution

EXCITATION DEPLETION Detecting only the fluorescence from this spot!

y
x
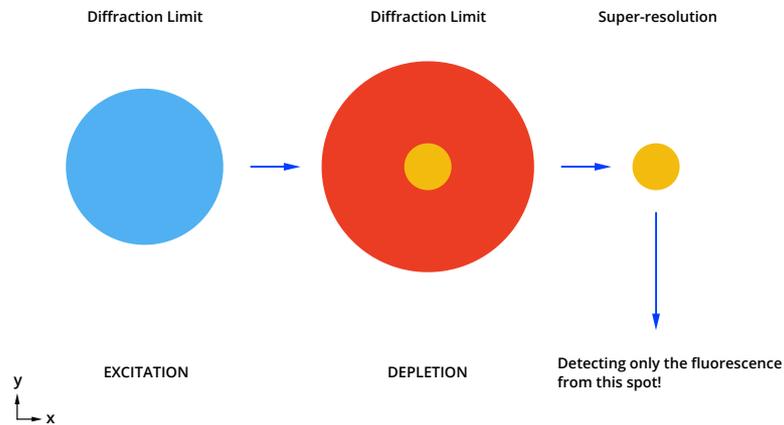
Simplified, schematic principle of STED microscopy: The Gaussian excitation beam is overlaid by the donut-shaped STED beam which is 'switching off' the excited fluorophores. The remaining fluorophores are emitting fluorescence which is detected. The detected fluorescence is emanating from a region that is much smaller than the original area of excitation. Scanning a sample with this effective sub-diffraction-sized detection point spread function (PSF) enables recording an image with a resolution much below the diffraction barrier.

The Photo-physical Principle

A STED microscope is usually based on a point scanning confocal fluorescence microscope and employs reversible ON (excited S_1) and OFF (non-excited ground S_0) states of fluorophores. We will first discuss how a fluorophore can be switched off using stimulated emission. The technical implementation on the microscope will be explained in a separate chapter. The absorption of a photon (within an appropriate energy window) 'catapults' an electron from the ground state (S_0) to the first excited state (S_1). The electron usually returns spontaneously to the ground state in the time scale of nanoseconds, which is often accompanied via the emission of a photon. 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 and returns to the ground state (S_0) by emitting a photon at exactly the same energy (or wavelength) as the photon causing the stimulated emission. The abbreviation STED stands for Stimulated Emission Depletion and highlights the fact that stimulated emission is used to deplete the excited state and to thereby switch excited fluorophores back to the ground state (off) much faster than it would occur by normal fluorescence. This reversible and transient OFF-switching imposed on excited fluorophores within the diffraction-limited excitation volume forms the basis of STED microscopy. Please note, that the allowed transitions between S_1 and S_0 for stimulated emission are identical with the energy levels shaping the emission spectrum of a given fluorophore. The lifetime of an excited fluorophore is short (in the nanoseconds range) and high photon fluxes delivered by a high-power laser are required to ensure an efficient 'depletion' of the fluorescence.

S_1

Excitation Fluorescence STED

S_0

Excitation / Emission

1.0

Excitation STED

0.5

0

350 400 450 500 550 600 650

Wavelength (nm)

Excitation Detection STED
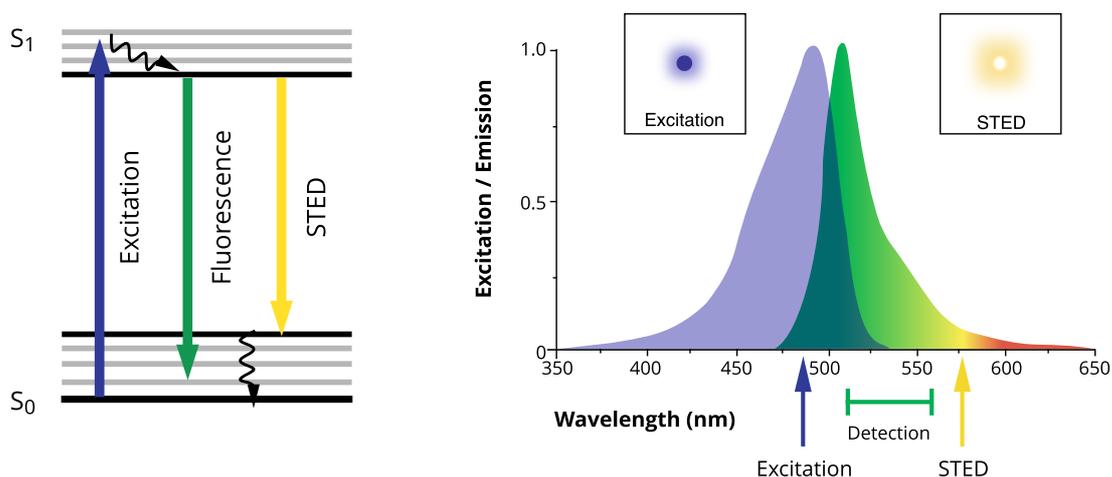
Photo-physical principle of stimulated emission depletion. Simplified Jablonski diagram (left): Upon excitation, an electron of a fluorophore is transferred from the ground state (S_0) to the first excited state (S_1). The electron can return to the ground state either via the emission of fluorescence or by being 'forced' via stimulated emission depletion (STED). For stimulated emission depletion, a photon with the same energy difference of the excited state to the ground state is interacting with the excited molecule and stimulating it to relax by emitting an additional photon of the same energy than the incoming photon. Choice of 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 spectrum but a) not overlap with the excitation spectrum and b) not reduce the detection window for fluorescence too much. The wavelength of the STED beam is typically located in the red-shifted 'tail' of the emission spectrum.

Resolution in STED microscopy

The theoretical resolution of a conventional fluorescence microscope can be calculated by a formula originally described by Ernst Abbe in 1873.

$$D = \lambda/(2\, n{*}\sin\alpha) = \lambda/(2\, NA)$$

With:
D : resolution.
l : wavelength of the emission light.
NA : numerical aperture of the objective lens.
n : refractive index of the immersion medium.
α : half-angular aperture of the objective lens.

The theoretical resolution of a STED microscope can be calculated by a modified Abbe formula.

$$D = \lambda/(2n \sin \alpha \sqrt{(1 + I/I_{sat})})$$

With:
D : resolution.
l : wavelength of the emission light.
n : refractive index of the immersion medium.
α : half-angular aperture.
I : peak intensity of the STED laser.
I_{sat} : saturation intensity.

Whereas I is proportional to the laser power of the STED beam, the parameter I_{sat} is dependent on the fluorophore: The lower the saturation intensity of a given fluorophore, the easier it is to switch molecules from the excited state (ON state) to the ground state (OFF state). I_{sat} is the characteristic saturation intensity of a fluorophore 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 lasers have power ratings of 1 W power output or higher). Tuning of the resolution in STED images is usually achieved by modulation of the STED laser power after selecting optimal dyes for STED microscopy.

By shrinking the volume in which fluorophores are allowed to emit photons 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.
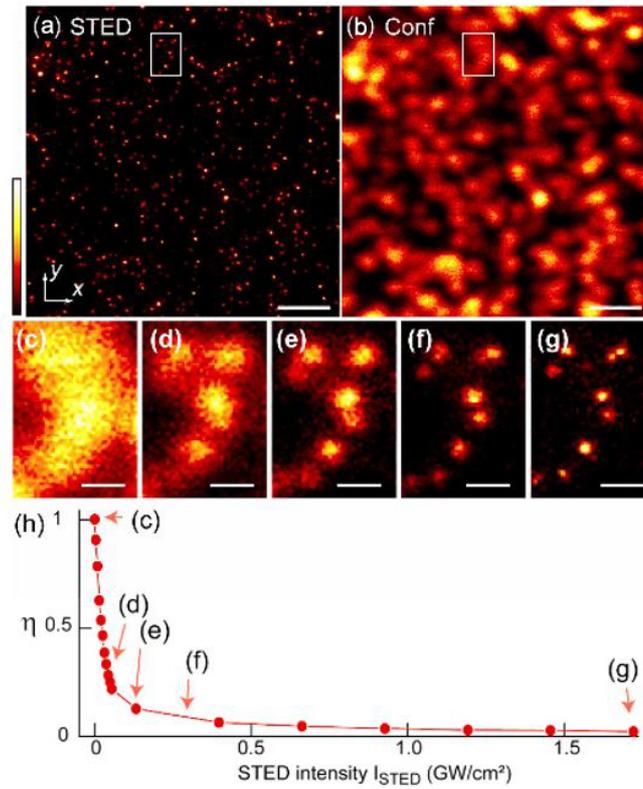
Resolution enhancement as a function of the STED laser power. Fluorescent beads were used to demonstrate the resolution enhancement of STED (a) over classical confocal microscopy (b). The resolution in the STED image can be tuned by changing the laser power of the STED beam and thereby altering the power density in the donut (c-g and h).

Adapted with permission from The Optical Society (OSA). (Benjamin Harke, Jan Keller, Chaitanya K. Ullal, Volker Westphal, Andreas Schönle, and Stefan W. Hell, 'Resolution scaling in STED microscopy' Opt. Express 16, 4154-4162 (2008))

RESOLFT

Similar as in STED microscopy, RESOLFT (Reversible Saturable Optical Linear Fluorescence Transitions)-microscopy utilizes an induced state transition (e.g. ON - OFF) of specialized fluorophores to overcome the diffraction barrier in fluorescence microscopy. RESOLFT microscopy is based on reversibly switchable fluorescent proteins (RSFPs) which can be switched between a fluorescent (ON) and a non-fluorescent (OFF) form by using light of a certain wavelength. The ON- and OFF-states are metastable with lifetimes from seconds to hours or even days at room temperature. Using X-ray crystal structures of RSFPs in their fluorescent and non-fluorescent form, the molecular mechanism of the switching was revealed. The switchable fluorescence behavior of the RSFPs is based on a *cis/trans* isomerization of the chromophore.
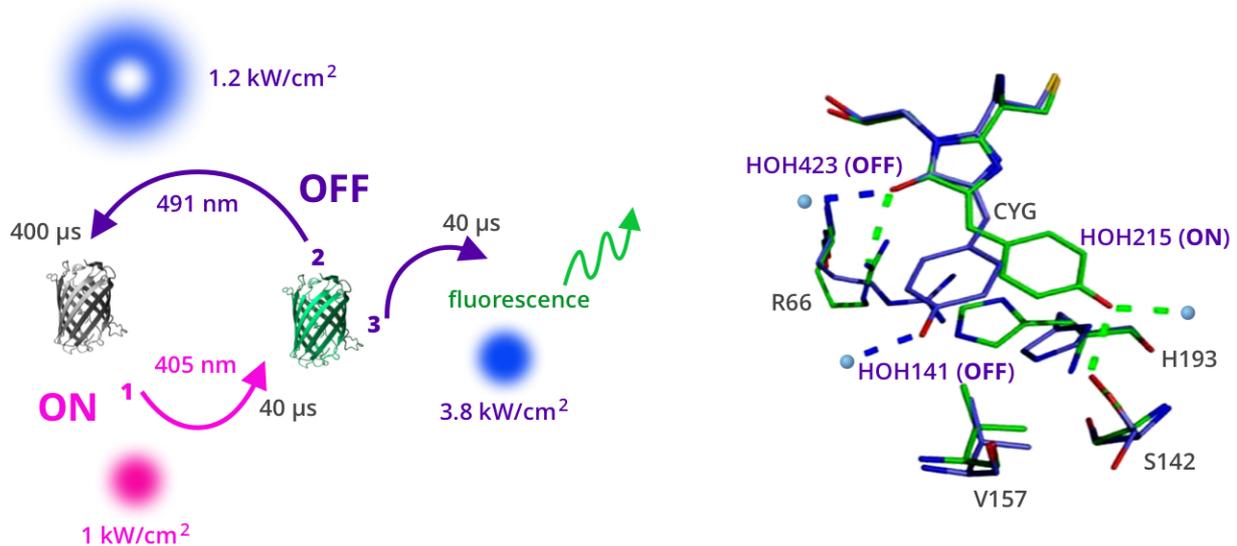
Switching mechanism of RSFPs. (Left) Switching model of the RSFP rsEGFP2. Illumination of the fluorescent (on-state) fluorophore leads to fluorescence emission and switching to the off-state. To switch the fluorophores back to their fluorescent form a short activation pulse with UV-light is needed. The switching is reversible and can be repeated up to several thousand times based on the utilized RSFP. (Right) Stick model illustration of the cis/trans isomerization of the chromophore. The cis-conformation is depicted in green whereas the trans-conformation is depicted in blue. Dotted lines illustrate water bridges of the chromophore in the On- and Off-state. Upon switching to the trans-state stabilizing water bridges are reduced and the chromophore is slightly tilted. The reduction in planarity of the chromophore leads to the strong reduction of fluorescence of the trans chromophore.

In a point scanning RESOLFT microscope, imaging with RSFPs is based on executing multiple illumination steps at each (scanning) position to trigger the on- or off-switching of RSFPs at the focal spot. After an initial on-switching step, the molecules located in the periphery of the excitation volume are switched off via a donut-shaped beam which deposits no energy in the center. Subsequently, the molecules which were located before in the center of the off-switching beam are still fluorescent and can be read out. Thereby, the recorded fluorescence originates from a sub-diffraction sized area which is confined in its size by the light intensity of the donut-shaped off-switching beam. By repeating these switching steps at all scan positions (e.g. every 25 nm), a super-resolution image of the sample can be obtained.

The switching speed of established RSFPs is limiting the achievable frame-rate of point scanning RESOLFT imaging to typical dwell times in the range of 100-500 µs leading to 10x-100x longer image acquisition times compared to regular confocal microscopy. Thus, if fast imaging is required, STED microscopy might be of advantage.

In STED microscopy fluorophores are switched between a fluorescent and a non-fluorescent state by stimulated emission (see also STED microscopy). Here the stimulated emission has to compete with the spontaneous fluorescence emission of the fluorophores which is typically in the time range of a few nanoseconds. Thus, the switching in STED can be seen as practically instantaneous. However, to achieve a saturated off-switching via stimulated emission of molecules in the periphery of the excitation volume, very high peak intensities (MW-GW / cm^2) are needed which might cause photo-stress in living cells. In RESOLFT microscopy the fluorophores are switched between two metastable states with a long lifetime and, thus, the transition can be driven with low light intensities. In RESOLFT microscopy light intensities of few W-kW / cm^2 are used, which are in the range of regular confocal imaging. In light sensitive specimen RESOLFT microscopy might be of advantage compared to other super-resolution microscopy techniques.
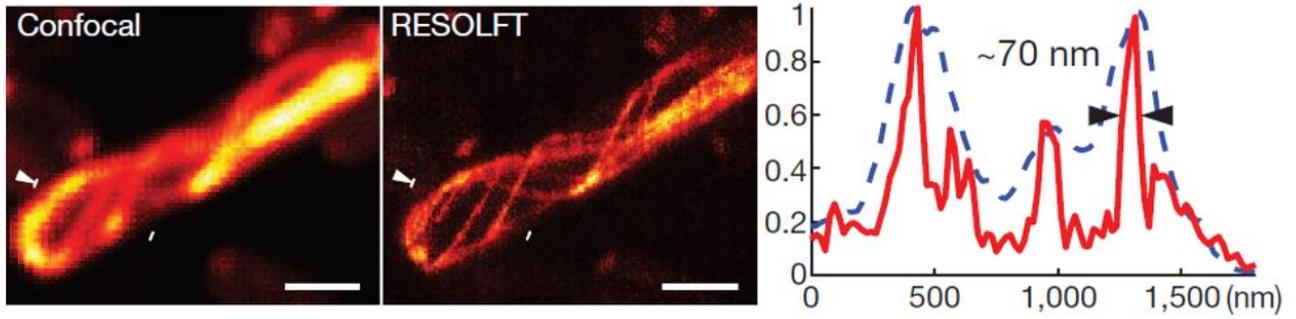
RESOLFT microscopy using rsEGFP as a fluorescent label. (Left) Diffraction-limited confocal and super-resolution RESOLFT image of rsEGFP-MreB expressing E. coli. (Right) Line profile across the indicated position in the confocal and RESOLFT image, showing the resolution enhancement in the RESOLFT imaging mode.

Single molecule localisation techniques - One point at a time

Single Molecule localisation Microscopy (SMLM) is mostly based on Widefield-microscopes often by using laser-based illumination in TIRF- or Widefield illumination mode in combinations with cameras able to detect the emission from single fluorophores (EMCCD- or sCMOS-cameras).

Long-time before images with SMLM have been acquired by detecting the fluorescence of single molecules, scientists 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 can be calculated with very high precision (achieving nanometer precision), e.g. by fitting the signal recorded on the camera chip (typically an EMCCD- or sCMOS-camera) with a Gaussian-function as a sufficiently accurate approximation of the PSF of a single emitter. The human vision is excellent in doing the same while watching a myosin molecule 'walking' along a myosin-filament *in vitro* (Fig. 10) - 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 the step size could be.
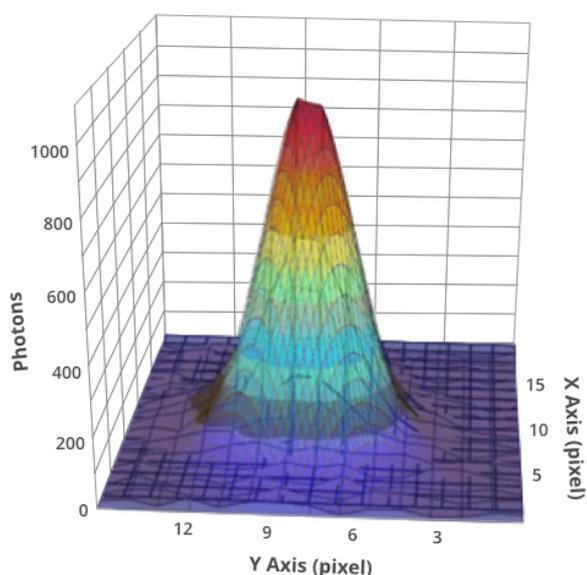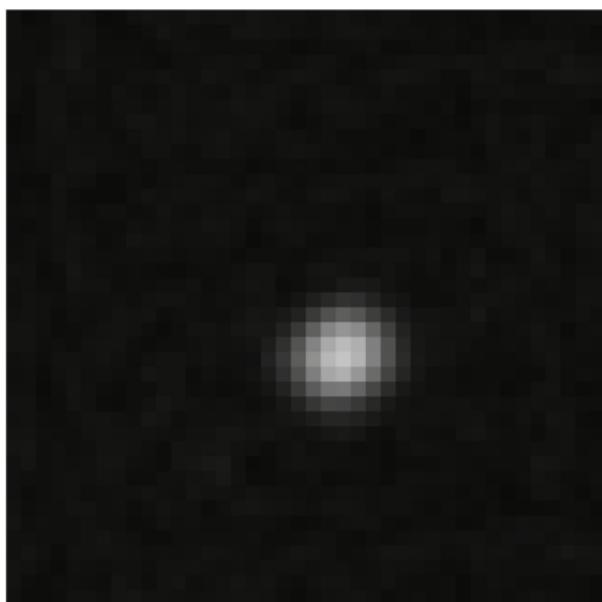
Example of diffraction limited, single molecule detection of a myosin molecule labelled with a single fluorophore moving along an actin filament in vitro. Please note in the movie, that also small steps can be identified by eye (left). Fitting 3D-Gaussian function to a diffraction limited spot of the image sequence allows precise localisation 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. Therefore, the point spread functions of each single fluorophore emitting light are overlapping to such an extent that they cannot be separated by a typical diffraction limited standard microscope.

Again a 'trick' is needed: instead of looking at all molecules at once, the molecules are observed sequentially and thereby separated not in space but in time. To localise all molecules marking a densely labelled structure, each of these molecules has to be turned on and off in order to ensure that the position of all molecules is detected at least once. After having recorded the coordinates of all fluorophores, an image with improved resolution can be created by this information. The super-resolution image can feature a smaller pixel sizes to display the increased resolution without 'empty magnification' (typically 20 nm or below).
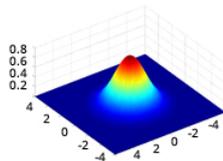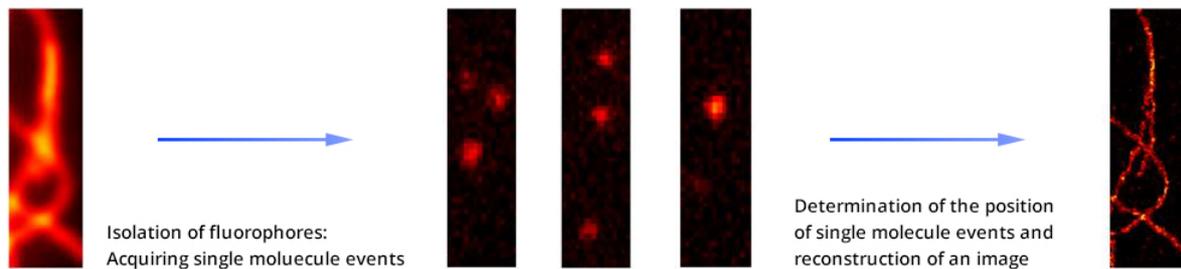
Principle of Single Molecule localisation Microscopy (SMLM): A densely labelled fluorescent sample is depicted on the left. By reducing the number of active fluorophores, single molecules can be detected in different images over time. After Gaussian fitting, the precise coordinates of each fluorophore are translated back into a (super-resolved) image (right).

Each of the signals ('blinks') in one image of the time-lapse originates from a burst of hundreds to thousands of photons derived from a single active fluorophore. Or in other words, during the active period, the fluorophore is cycling hundreds to thousands of times between the ground and first excited state (S_1), before returning to an off-state or bleaching.
The localisation precision σ in SMLM can be estimated using the following formula (Mortensen et al., 2010):

$$\Delta x = \sqrt{\frac{\sigma^2 + a^2/12}{N}\left(\frac{16}{9} + \frac{8\pi(\sigma^2 + a^2/12)^2 b^2}{a^2 N}\right)} \approx \frac{\sigma}{\sqrt{N}}$$

With:
N: number of collected photons.
a: pixel size of the detector.
b: average background signal.
σ: standard deviation of the point spread function.

However, if there is only low background signal and the fluorophore emits multiple hundreds to thousands of photons per frame, the formula can be simplified to:

$$\sigma = d/\sqrt{N}$$

With:
σ: localisation precision.
d: diameter of FWHM of the PSF.
N: number of detected photons.

PALM

The first method applied to singularize fluorophores in an otherwise densely labelled sample was coined Photoactivated Localisation Microscopy (PALM). A special kind of fluorescent proteins was used to control the number of (simultaneously) 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 positions of the fluorophores are estimated by e.g. Gaussian-fitting for the reconstruction of the Super-resolution image.

Please note: that fluorescent proteins can also exhibit some kind of blinking by transiently occupying an off state. Quantitative approaches like counting molecules should integrate this behavior in the analysis model.

To capture a two-color PALM image e.g. two fluorescent proteins like mEos and Dronpa are used. First mEos labelled structures are imaged by photo-conversion of a small subset of mEos proteins via illumination with light of 405 nm wavelength. If ideally all mEos proteins are localised the Dronpa proteins are off-switched via a strong illumination with light of 488 nm. By a simultaneous illumination of the sample with light of 488 nm at high intensity and light of 405 nm with very low intensity, the majority of Dronpa proteins are kept in their non-fluorescent form, while few Dronpa proteins are stochastically activated and registered and finally reconstructed as an image.

Photoactivatable dyes, e.g. paGFP

UV-Light (405 nm) for activation

Detection and bleaching

Photoswitchable dyes, e.g. EOS-family (tdEOS, mEOS..)

UV-Light (405 nm) for activation

Detection and bleaching
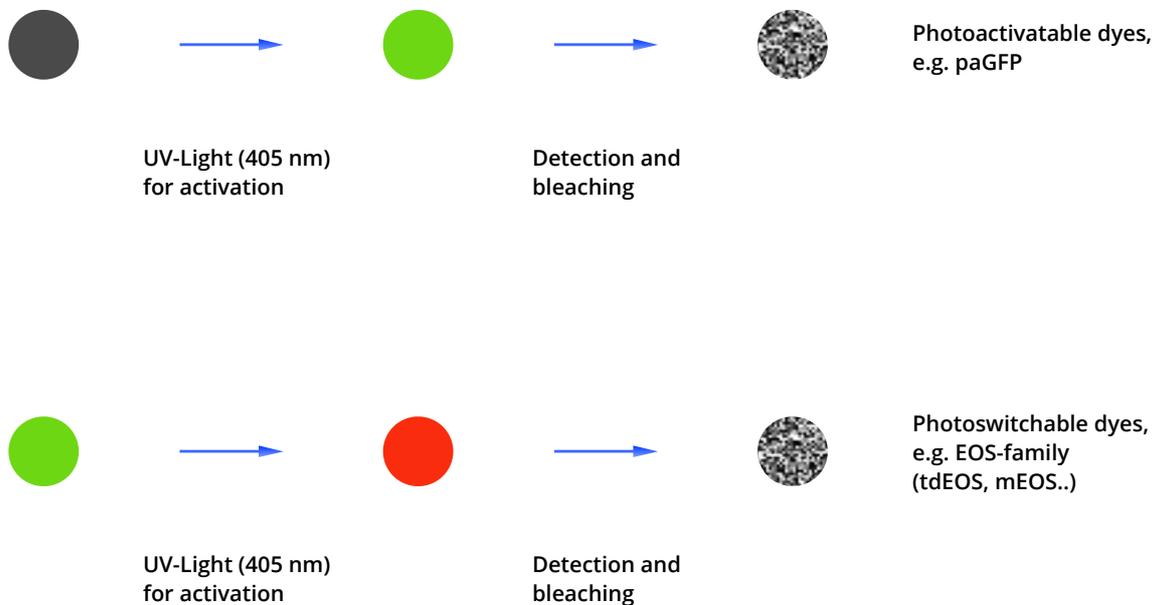
Types of PALM-capable fluorescent proteins: Photo-activatable fluorescent proteins like PA-GFP are non-fluorescent in their native state, but can be converted into a fluorescent species upon UV-irradiation. During the imaging procedure, these molecules are bleached and subsequently new fluorophores can be activated (top). It has been proven easier for the researcher to see the structure of interest - even diffraction limited - and be able e.g. to focus on it, and not navigating blindly in the sample. To this end, photo-convertible fluorescent proteins, like the mEos-family, are commonly used. They are fluorescent emitting green fluorescence 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.

dSTORM / GSDIM

The most common SMLM technique is direct stochastic optical reconstruction microscopy (dSTORM). In dSTORM, molecules are separated in time just like in PALM (or any other SMLM technique) but by applying a distinct photophysical mechanism. dSTORM is based on using organic dyes (like Alexa Fluor or ATTO-dyes) as photoswitchers. By illuminating a labeled cell using a substantially high laser power, the majority of fluorophores are transitioned into a non-fluorescent so-called dark state. The imaging buffer contains millimolar concentrations of a reducing compound such as dithiothreitol (DTT) or glutathione (GSH), which quenches the fluorophore's triplet state and enables a more stable non-fluorescent dark state. While the majority of fluorophores rest in this dark state, single molecules return into their emissive state spontaneously or upon activation with UV-light. Subsequently, these molecules are excited and their fluorescence is recorded by a sensitive camera (EMCCD or sCMOS). From these single stochastic fluorescent events recorded, the position of the molecule is calculated by Gaussian-fitting (or another mean of fitting the location of the fluorophore).
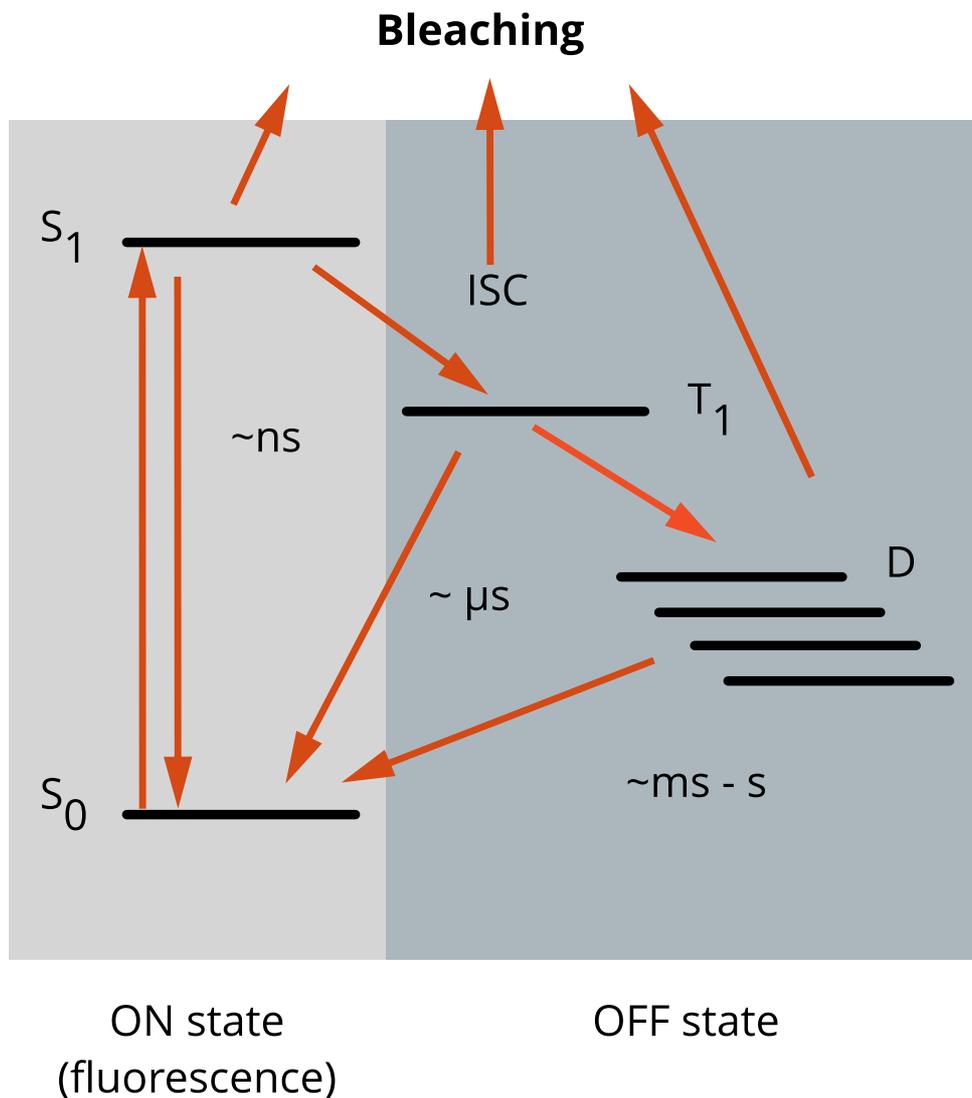
Simplified Jablonski diagram. Upon excitation, an electron of the fluorophore is elevated from the ground state (S_0) to the first excited state (S_1). The electron can return to the ground state via emitting a photon within the time range of nanoseconds. Alternatively, from the excited S_1 state the fluorophore can transition into a Triplet state (T_1) via Intersystem crossing (ISC). From this Triplet state a fluorophore can bind to reducing agents contained in the buffer. The resulting molecule is non-fluorescent relatively unstable. It spontaneously or upon UV irradiation splits again. This effectively creates long lived dark states (D) with lifetimes of several milliseconds to seconds.

Again, the position of the fluorophore is estimated by fitting and finally, the super-resolution image is reconstructed from the calculated positions of the fluorophores.

It is very important that the labelling density of the sample is appropriate to display all features of the investigated structure. Further, a sufficient number of fluorescent events from each singularized fluorophore have to be collected to guarantee a good signal to noise level.

Since dSTORM and GSDIM are using the same buffers and imaging schemes, there is no practical difference between the methods. The only difference is in the proposed models and which dark states are employed by the fluorophores, e.g. is it only the triplet state or are more dark states including some radical states involved in switching the molecules 'off'.
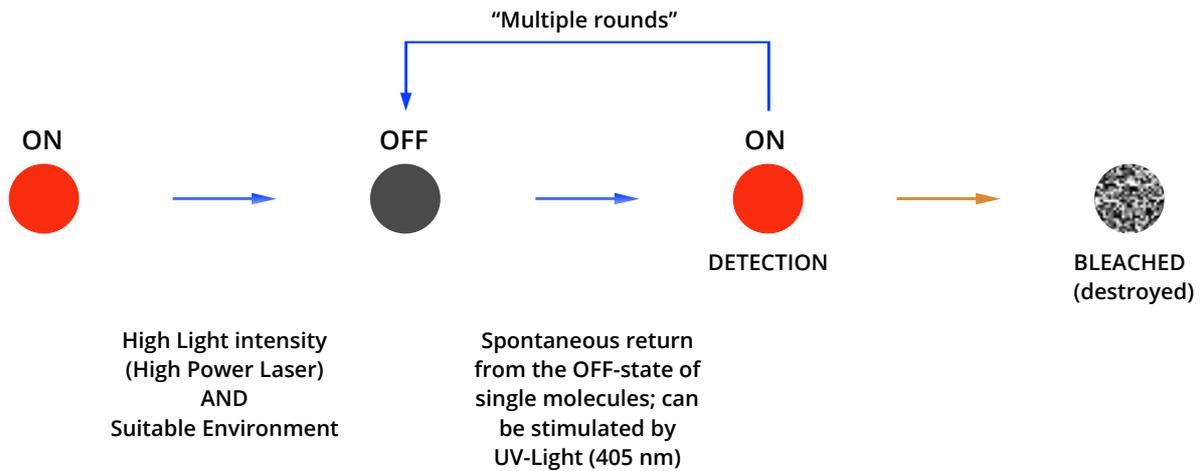
Principle of dSTORM imaging. Fluorescent organic dyes are photoswitched from a fluorescent ON-state to a non-fluorescent OFF-state via illumination with high light intensity. From this transient dark state, single molecules return to their fluorescent ON-state spontaneously or via activation by UV-light. These ON-switched molecules are detected and localised. By accumulating thousands of single molecule returns, a super-resolution image can be reconstructed.

STORM

Stochastic optical reconstruction microscopy (STORM), another SMLM technique based on Widefield and TIRF-microscopy, was reported independently of PALM (in 2006) prior to GSDIM/dSTORM (in 2008).
In STORM, like in any other SMLM technique, only a subset of fluorophores of a densely labelled sample are emitting fluorescence at one detection time point, thus their fluorescence can be separately registered on the detector of the microscope (e.g. a camera). In comparison to dSTORM / GSDIM blinking is achieved by photoswitchable dye-pairs (activator-reporter pairs e.g. Cy3-Cy5 or Cy2-Cy5) which allow for an activation of single fluorophores. These fluorophore pairs are in close proximity to each other e.g. on the same secondary antibody. From the recorded single detection events, the position of the fluorophore is analyzed similar to other SMLM techniques by calculating e.g. the centroid position of the recorded fluorescence. Subsequently, the fluorophore is deactivated by bleaching and another subset is activated and recorded. These activation, detection and deactivation steps are repeated until ideally all fluorophores have been registered which allow the reconstruction of a super-resolution image depicting the localised positions of the fluorophores. PALM and STORM microscopy deploy the same principle to achieve a super-resolution image. However, the techniques differ in their utilized fluorophores. PALM relies on photoactivatable fluorescent proteins, whereas STORM and its variants, is based on single or pairs of organic dyes. The achievable spatial resolution in STORM microscopy is typically in the range of 20-30 nm in the lateral dimension.

Fluorophores
too close to resolve

Stochastic activation
and localization
of individual molecules

Super-resolution
image reconstructed
from localizations
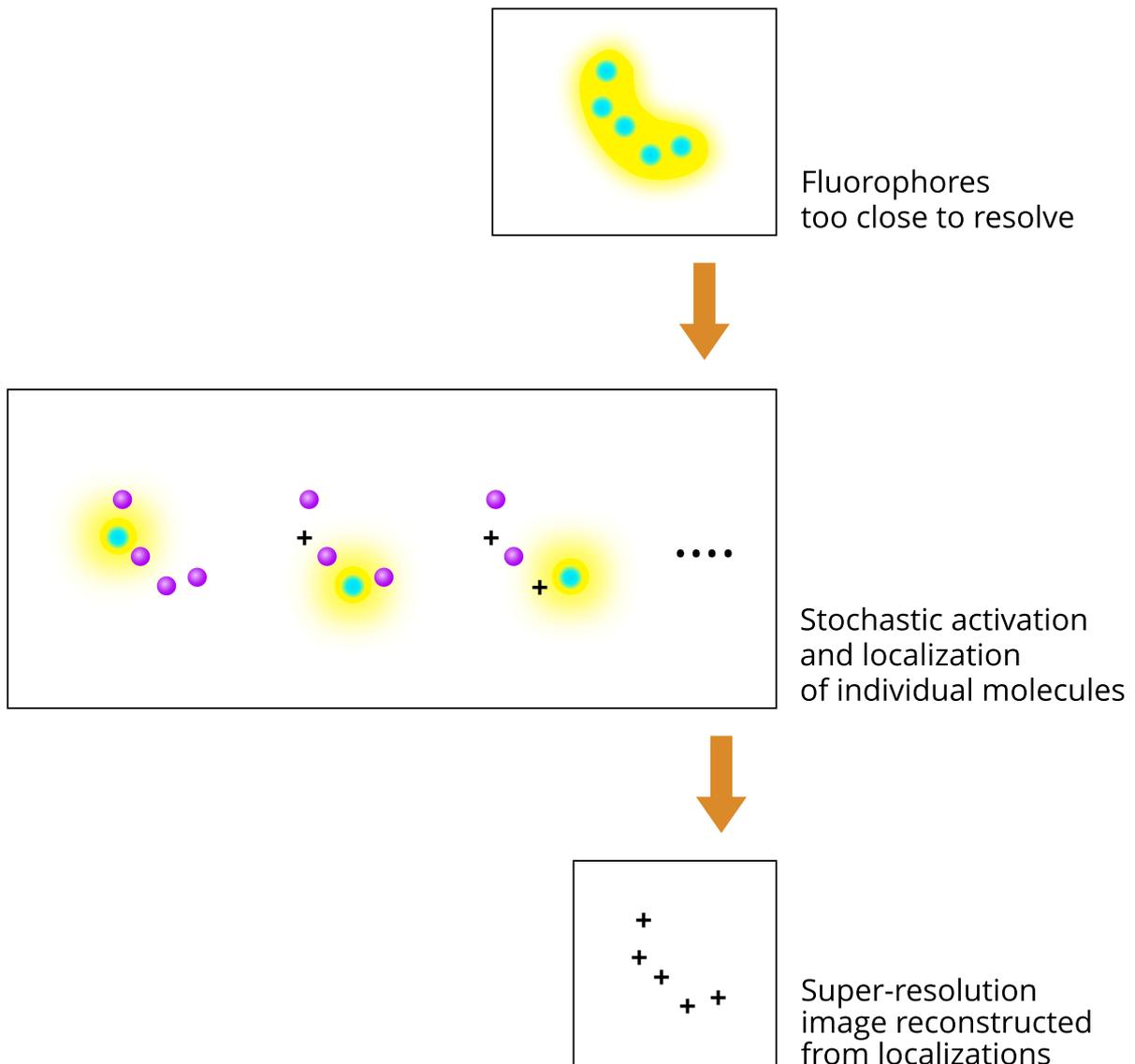
STORM principle. Single fluorophores are activated and readout stochastically and single molecules are localised and used to reconstruct a super-resolution image which displays the calculated positions of the fluorophores.

PAINT and DNA PAINT

Point Accumulation for Imaging in Nanoscale Topography (PAINT) is based on the principle of observing singularized fluorescent events and is therefore another SMLM technique. In this technique the used fluorophores are typically not detected in an unbound form because of fast diffusion in the sample or the used aqueous mounting solution. Upon binding to their target-motif the molecules rest at a given position and their fluorescence is recorded by the detector for a short time until they are subsequently bleached or the probe has dissociated from the target.

The wide range of probes used for PAINT imaging feature all a relatively low affinity to their target, which results in a dissociation after binding to their target after a certain time. The probes include low affinity antibodies, nucleic acid probes like Hoechst-JF646 or membrane binding dyes like Nile Red. The recorded signal is used to determine the centroid position of the fluorophore based on the center of mass or by Gaussian-fitting as in the other SMLM techniques. The calculated positions of the fluorescent events are used to reconstruct a super-resolution image. PAINT microscopy is often coupled to TIRF- (Total Internal Reflection Fluorescence) or HILO- (Highly Inclined and Laminated Optical sheet) illumination to lower the background signal. Here the sample is excited from an oblique angle leading to the generation of an evanescent wave of the excitation light close to the cover-slip and cell-interface. This restricts the excitation and detection of fluorescence to a thin region of the specimen and thus reduces the background signal dramatically.

DNA-point accumulation for imaging in nanoscale topography (DNA-PAINT) uses DNA-oligonucleotides to create the blinking needed for stochastic super-resolution microscopy. These labelled oligonucleotides are transiently binding to their target and are immobilized for few hundred milliseconds allowing detection. Non-bound fluorophore coupled DNA-strands are not detected by the camera as they diffuse rapidly through the cell during the duration of a single camera frame and only giving rise to a high background signal.

Universal PAINT (uPAINT) can be applied to a broad set of targets on the surface of living cells, since it allows the usage of any fluorescent labelled specific ligand (e.g. a dye-coupled antibody). The fluorescent labelled ligand binds to its target motif and enables a localisation of the protein of interest. In living cells trajectories of proteins of interest bound by the ligand can be tracked with sub-diffraction resolution with this method until the fluorophore is finally bleached. However, a background fluorescence signal of unbound ligands hampers detection of single fluorescent ligands, which are bound to their target. Therefore, uPAINT is normally performed using TIRF- or HILO-illumination of the sample to restrict the excitation to the plasma membrane of the cell.
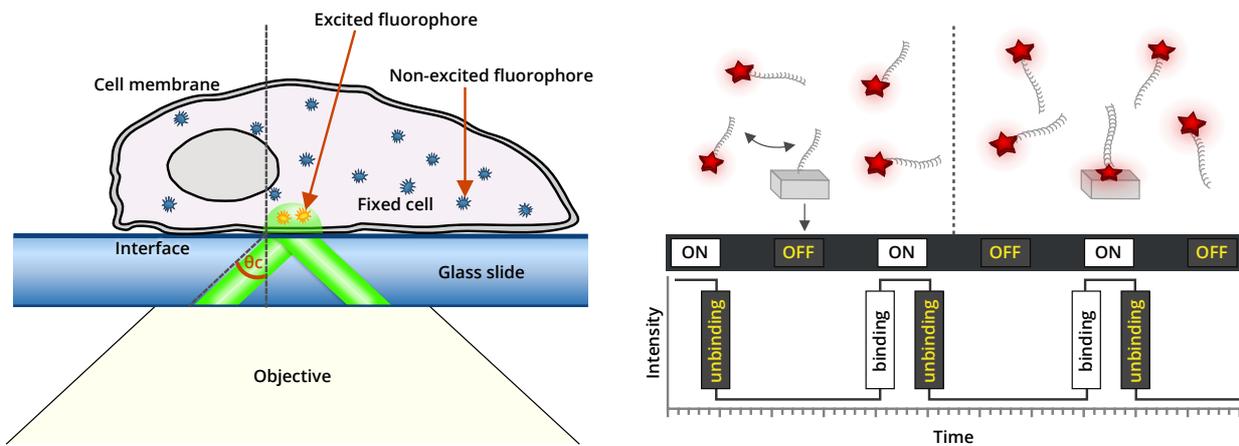
Principle of uPAINT and DNA-PAINT. (Left): If a dye-coupled ligand binds to the target, fluorescence is emitted at the position of the molecule, while unbound ligands move very fast and the fluorescence emitted only contributes to the background signal. TIRF illumination restricts the detection of fluorescence to a small region at the cell-coverslip interface reducing the background signal (green region in the cell). (Right) DNA-PAINT: Single small dye-coupled oligonucleotides are diffusing through the cell and are not detected by the camera. If the dye-labelled oligonucleotides bind to their target motif, their fluorescence is recorded and the position of the fluorophore is calculated.

3D-SMLM

In SMLM the excitation light is focused by an objective lens to a single focal plane and the lateral position of the fluorophores is localised. However, the cigar-shaped symmetry of a single emitter's point spread function impedes the calculation of the axial position based on a single camera frame, because there are always two positions which feature the same intensity on a camera frame. To obtain information about their axial position, multiple frames at different depths have to be recorded.
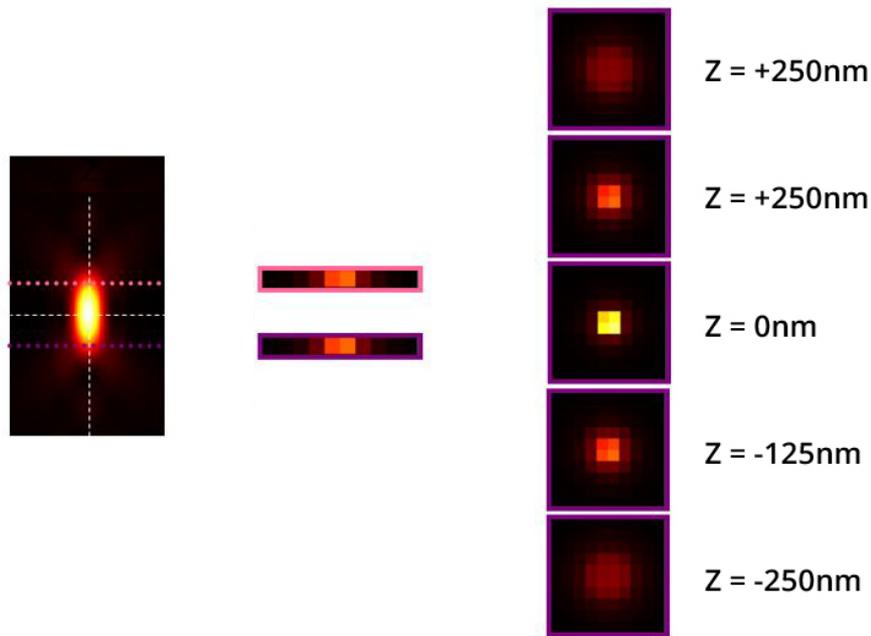
Schematic illustration of the intensity distribution of an ideal point spread function. (Left) Because of the cigar-shaped symmetry of the PSF the signal intensity at two positions equally distant from the center feature the same intensity. (Right) Signal intensities of the PSF at different depths of the focus.

To circumvent this limitation many different experimental modifications of the light microscope have been invented to extract the axial position of an emitter while imaging single planes.

One possibility is to record two planes simultaneously. The simultaneously recorded images are used to calculate the axial position of the single emitter in the three-dimensional point spread function based on the signal intensities at different foci. This approach was termed bi-plane localisation microscopy and can be used to reconstruct volumes with a depth of about 1-2 μm along the axial axes with one level of bi-plane acquisition. Thicker stacks can be covered by sequentially taking bi-plane acquisitions in different z-levels.

z (nm)

400

200

0

-200

-400
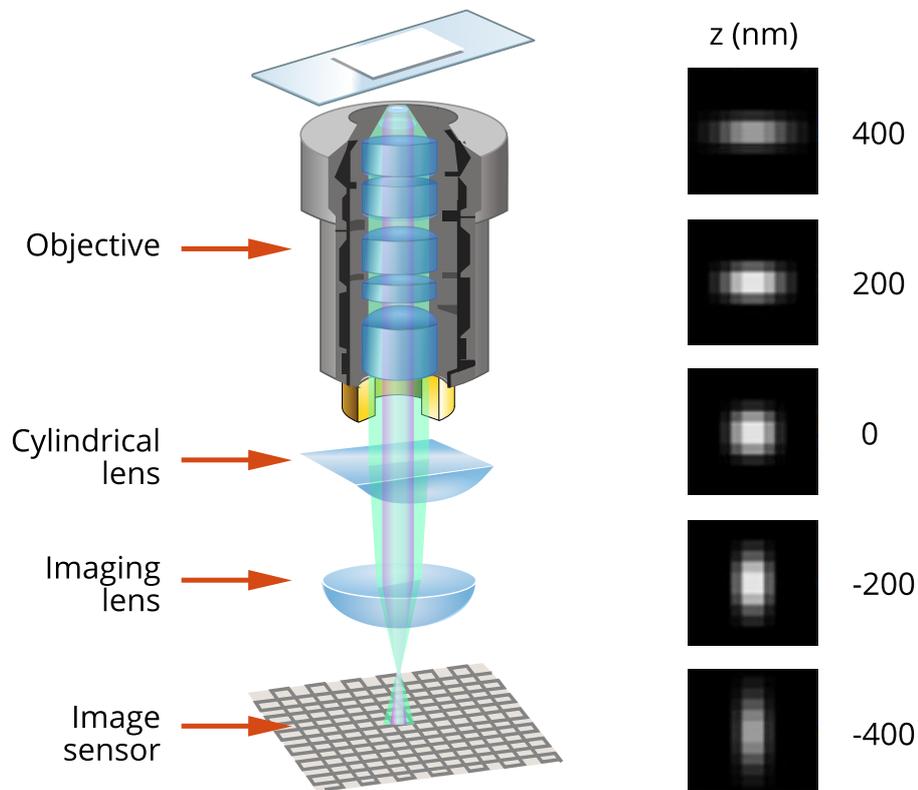
Principle of optical astigmatism encoded axial localisation. By integration of a cylindrical lens into the beam path, the PSF of the microscope is either elliptically elongated horizontally or vertically depending on the axial distance to the central focus position.

A second approach to map the axial position of a fluorophore in SMLM is based on optical astigmatism. A cylindrical lens is inserted into the emission beam path of an SMLM microscope. Depending on the axial position of the fluorophore within the excitation volume, the recorded signal of a single emitter is modified by astigmatism and the resulting elliptic shape can be used to calculate the axial position. The introduced astigmatism leads to an ellipticity of the recorded fluorescence events of emitters that are not localised in the center of the focal plane. Depending on the axial deviation from the center, the recorded PSF is elongated vertically or horizontally and thus allows for a precise calculation of the actual axial position up to 50-60 nm along the axial axis.

A third approach which is based on a modification of the PSF of an emitter was termed Double helix-point spread function (DHPSF)-SMLM. Here a Spatial light modulator (SLM) is used to convolute the emitted fluorescence signal from a fluorophore with a double helix point spread function. As a result, the image of an emitter contains two lobes encoding the axial position of the emitter by the orientation of the lobes to each other. DHPSF-SMLM achieves up to ~ 20 nm axial resolution.

Over the years, even more approaches have been developed to determine the axial position of a fluorophore in SMLM including interferometric PALM (iPALM) or super critical angle fluorescence nanoscopy (SAF). A detailed explanation of all of them would go beyond the scope of this chapter and we would like to encourage you to read up in the literature if you would like to know more.
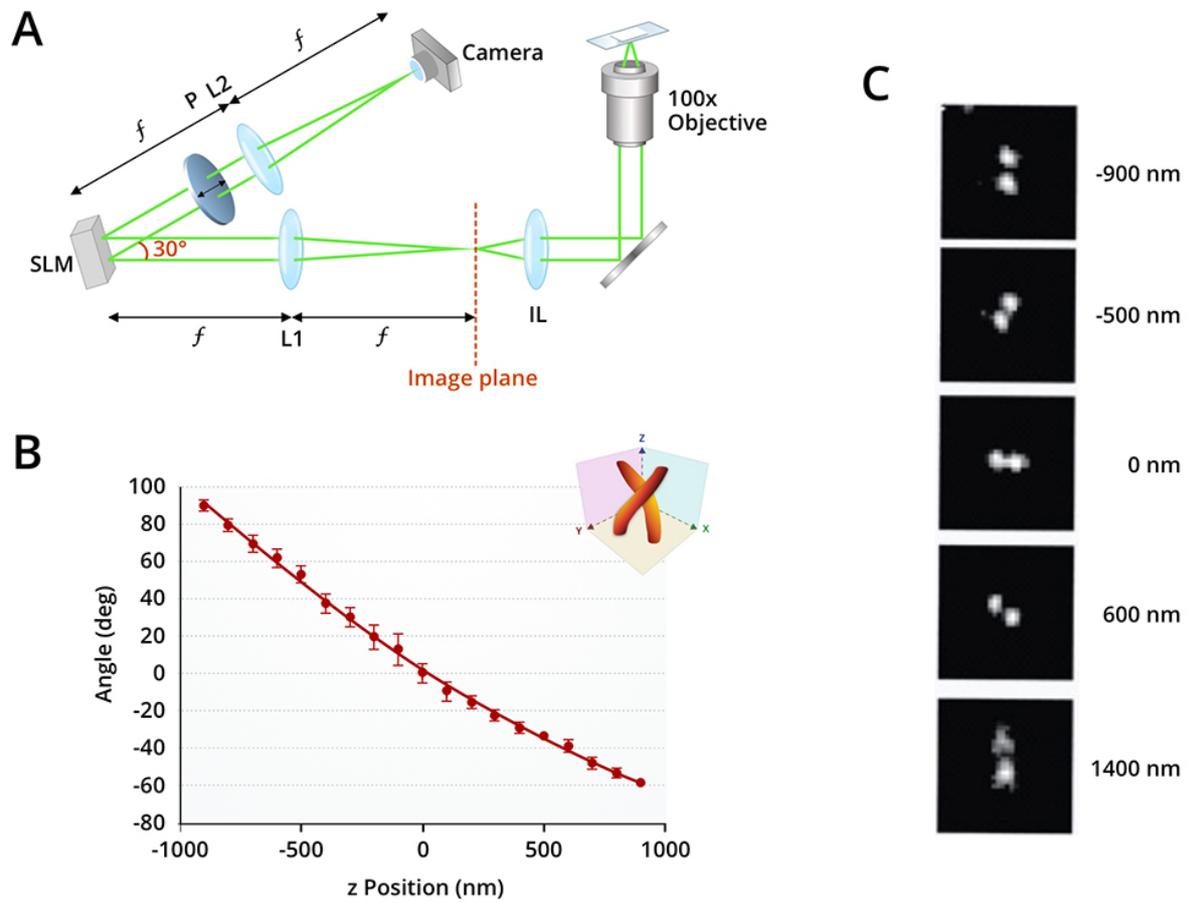
Principle of double helix-point spread function (DHPSF) SMLM. (A) Drawing of a typical beampath of a DHPSF-microscope. A spatial light modulator (SLM) is used to generate a double helix shape of the PSF. (B) Graph depicting the angle to z-position relationship of the generated Double helix PSF. (C) measured examples of the Double helix PSF at various depths.

Sample preparation for Super-Resolution Microscopy - General Considerations - Sample preparation

Many super-resolution microscopy techniques require specialized fluorophores to enable optimal imaging with the highest possible resolution. Further, the performance of the individual techniques varies if different fluorophores are used. Fluorescent molecules are characterized by many photophysical properties like brightness, photostability, fluorescence lifetime, water solubility, blinking behaviour and much more. A researcher should consider these characteristics and ideally choose the appropriate dye for each respective super-resolution microscopy technique. However, even without the optimal dyes, often a sufficient resolution enhancement can be achieved.

In addition to the dye itself, many other labelling parameters can determine the quality of a super-resolution microscopy experiment, e.g. the label density of a sample has to be appropriate to ensure a complete labelling of the target structure. Further care has to be taken, if the aim is to quantify the images afterwards or to determine the exact (!) size of a structure. Please note, that the measured size of a structure often increased by adding large labels or (more often) by the insufficient resolution of the (eventually diffraction limited) microscope.

Sample fixation

Fixation retains the cellular and subcellular structure by crosslinking and immobilizing of cellular proteins. The fixation leads to a stabilization of the cell morphology and e.g. the preservation of tissue architecture. Fixation of cells is mostly based on crosslinking reagents (such as Paraformaldehyde or Glutaraldehyde) typically used in concentrations of 0.1-8 %. Depending on the sample (single cells or tissues) or the structure of interest (membrane bound or located in the cytosol) the fixation conditions have to be adjusted to enable an appropriate fixation together with a sufficient staining of the respective target structure. Small amounts of Glutaraldehyde (0.05 to 0.2 %) in addition to the Paraformaldehyde/Formaldehyde strongly improves structure preservation. However, quenching of increased background fluorescence (with ammonium, NaBH4 etc.) might be necessary.

Next to crosslinking of proteins, cells can be fixed using draining reagents such as ethanol and methanol at -20 °C. However, this removes a substantial amount of lipids and membranes of the cell and leads to a loss of the cytoplasm. Fixation of cells will immobilize antigens thus enable the labelling of cellular proteins via Immunohistochemistry (IHC). However, the strength and duration of chemical fixation has to be evaluated, because very long or strong fixation (over-fixation) can alter antigens and thus lower the staining efficiency. Depending on the antibodies themselves and the sensitivity of the epitope, the fixation method and conditions have to be optimized to enable a specific staining of the target protein. To enable a labelling of cellular proteins with antibodies, cells are normally first fixed and then permeabilized using detergents like Saponin, Trition-X or Tween-20 (which differ in their permeabilization capability) to allow antibodies to enter the cell through the permeabilized membrane.

Properties of labels

Size and brightness for crosslinked samples

In fluorescence microscopy a fluorophore is attached to the protein of interest, which is then detected by a fluorescence microscope. The position of the fluorophore is used as a proxy for the position of the protein of interest. In super-resolution microscopy, the applied labelling technique needs to be taken into consideration in the analysis of the recorded data, if the resolution is in the range of the error caused by the size of the fluorophore and its distance to the target structure.

Frequently, Immunofluorescence (IF) is used to label a protein of interest in a fixed cell. Therefore, the cell is incubated with a primary antibody, which is subsequently recognized by several dye-coupled secondary antibodies. The size of cellular structures can appear significantly enlarged in IF samples, since a standard IgG-antibody has a size of ~10 nm, an antibody pair of primary and secondary antibodies adds about ~15 nm in size. However, the combination of one primary with multiple dye-coupled secondary antibodies leads to a signal enhancement improving the signal-to-noise ratio in the image. Dye-coupled primary antibodies can be used to lower the distance of the fluorophore to the target protein. Instead of large IgG antibodies, smaller antigen binding (Fab)-fragments can be used which is particular helpful if the resolution of the microscope used is in the range of the label size itself. Smaller labels also improve the labelling in dense areas where large IgGs might be excluded. However, smaller Fab-fragments and directly labelled primary antibodies normally feature less fluorophores and thus the fluorescence signal is reduced and might be limiting in super-resolution imaging due to decreased signal-to-noise ratio (S/N).

Antibodies	**Flourescent Proteins**	**Small tags**
~ 10 nm	~ 3 nm	~ 1 nm

~ 7 per AB

(e.g. click chemestry)

~ 6000 photons   < 1000 photons   ~ 6000 photons
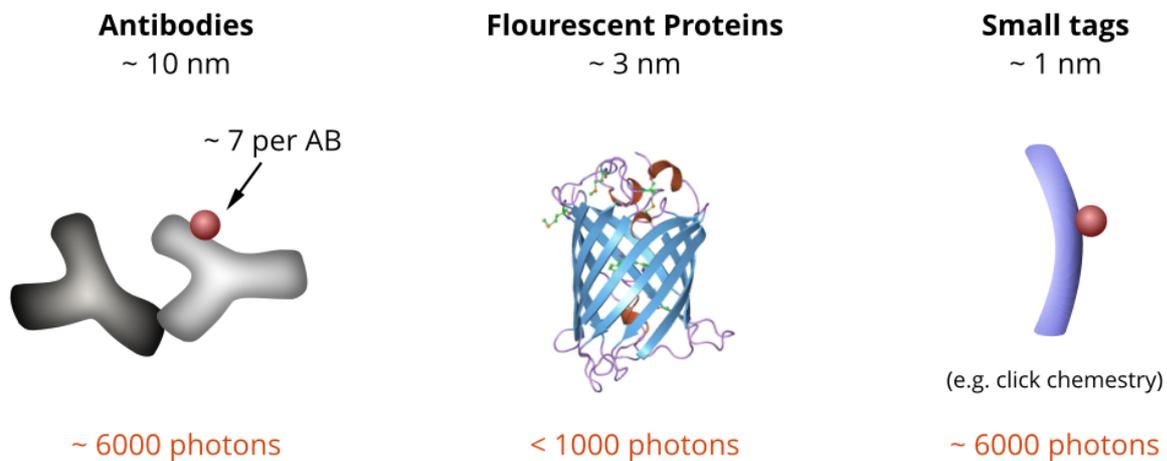
Comparison of label size and number of fluorophores of different labelling techniques.

Live-cell Imaging

Labelling a protein of interest for live cell imaging experiments can be achieved in many different ways. The most common way is the usage of genetic labelling by generating a genetic fusion construct of the protein of interest and a fluorescent protein (FP) like eGFP or mCherry. Fluorescent proteins feature a relative small size of ~ 3 nm and form autocatalytically a fluorescent chromophore. Since the original discovery of green fluorescent protein (GFP) many different variants and new FPs have been generated extending the spectral range of fluorescence microscopy from 350 nm to 700 nm. The concurrent usage of spectrally distinct FPs allows multi-color imaging to study several proteins of interest at the same time.

However, FPs can be limited in their photophysical properties, including high frequency blinking, low brightness or photostability. For some special fluorescence applications such as for super-resolution microscopy, organic dyes are often advantageous.

Instead of generating a FP-fusion construct, the protein of interest can be genetically linked to a self-labelling protein-tag. Self-labelling enzymes or tags are useful tools for live-cell imaging experiments, since it is possible to label cellular proteins inside living cells using membrane permeable organic dyes.

Self-labelling proteins like SNAP-, Click- or Halo-tag are of about similar size like a GFP-Tag and catalyse the covalent attachment of an organic dye to the fusion protein inside living cells. The SNAP-tag is based on the human AGT (hAGT) protein, an O6-alkylguanine-DNA alkyltransferase (AGT) which is normally involved in DNA repair. The SNAP proteins accept O6-benzylguanine derivatives instead of alkylated guanine derivatives in damaged DNA. SNAP catalyzes the irreversible transfer of benzylguanine to a free cysteine residue of the AGT protein. By using modified O6-benzylguanine substrates with e.g. fluorescent molecules attached, a fusion protein consisting of the protein of interest and the SNAP protein can be labelled specifically with an organic dye.

The CLIP-tag is another protein based on a mutated hAGT protein which uses modified O2-benzylcytosines as a substrate. This allows for the usage of the SNAP- and CLIP-tag simultaneously to label two proteins of interest with different orthogonal fluorescent ligands.

Another self-labeling enzyme is the Halo-tag, which is based on a different coupling reaction. The Halo-tag is a bacterial haloalkane dehalogenase that catalyzes the removal of halides from hydrocarbon chains via a nucleophilic displacement, in this reaction a covalent ester is formed between the hydrocarbon chain and an aspartate-residue of the Halo-protein. In the native bacterial Halo-protein this bound is reduced and the ligand is then released. However, in the Halo-tag the responsible histidine residue is mutated and the covalent bound between the Halo-tag and the ligand stays.
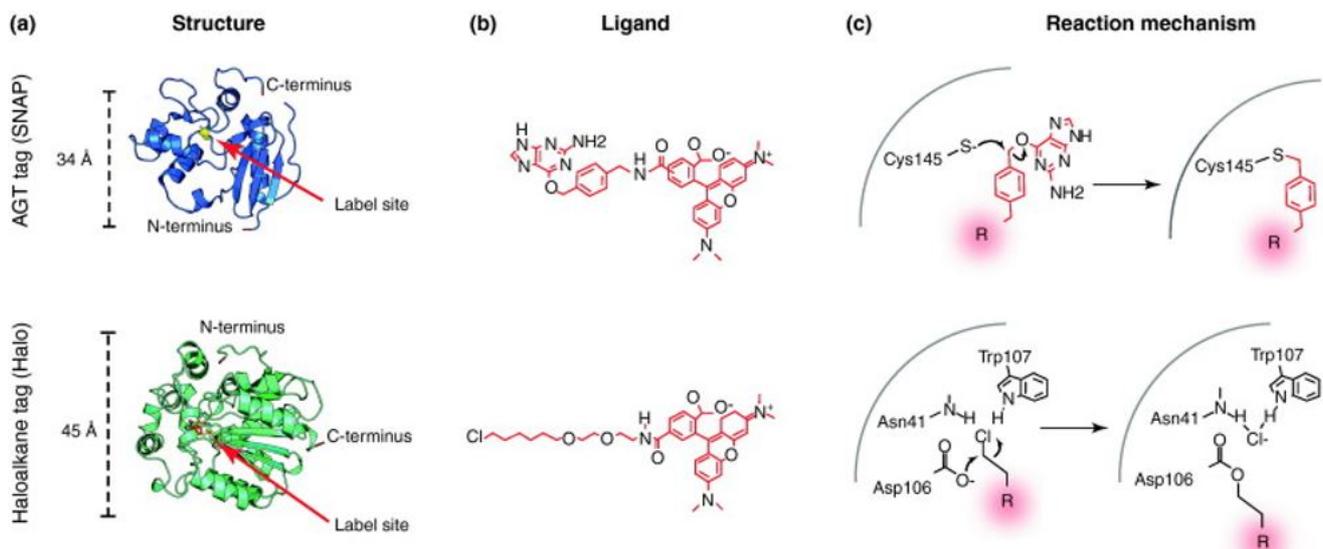
Principle of SNAP- and Halo-tag labelling. The SNAP and Halo protein self-catalyzes the labelling reaction with modified synthetic ligands (e.g. benzylguarine and haloalkane labelled with TMR) leading to a covalent bound between the protein itself and the label (R).

Genetic labelling via fusion of the protein of interest with a self-labeling enzyme is one possibility to label a protein of interest with an organic dye. Another one is Genetic code expansion in combination with so-called 'click chemistry' a site specific labelling of the protein of interest with organic dyes. Here, the gene coding region of the proteins of interest is modified by the insertion of an Amber stop codon (UAG) into the amino acid coding sequence. Additionally, an appropriate pair of an orthogonal tRNA and their respective aminoacyl-tRNA synthetase has to be expressed. During translation, the orthogonal tRNA recognizes specifically the modified codon sequence and an unnatural amino acid (UAA) featuring an alkyne group is incorporated at this integration site. The alkyne group can be coupled to a modified dye via a copper based 'click-reaction'. However, the usage of copper aggravates the application to living cells because of toxicity, leading to the development of copper-free click reactions.

Labeling via affinity probes

Another approach to reduce the size of a fluorescently label is the use of small affinity probes (if they are available for the protein of interest). Here structure specific ligands are used to target an organic dye to cellular structures. One example is Docetaxel, which is a chemotherapy drug that features a strong affinity to microtubule filaments. If Docetaxel is combined with a fluorescent organic dye, it can be used to label the microtubule network inside living cells with organic dyes. Next to small ligands to tubulin filaments, many various fluorescently labeled ligands are available which target cellular structures like e.g. actin filaments. Variants of affinity probes in combination with specialized fluorescent dyes (SiR-tubulin, SiR-actin, SiR -DNA or 580CP-LTX) have been successfully used in super-resolution microscopy applications. Since these reagents can alter cell dynamics, the useful concentration needs to be carefully determined for each application.

Sample preparation for STED microscopy - Fluorophores and strategies for fixed samples

STED microscopy is not limited to specialised fluorophores as stimulated emission is a photo-physical process which can be observed among a broad set of fluorophores. However, to achieve a super-resolution STED image featuring a good signal-to-noise ratio, the fluorophores have to be chosen carefully. A prerequisite for stimulated emission is that the emission spectrum of the fluorophore has to overlap with the wavelength of the depletion laser. In multi-colour experiments, the fluorophores ideally have spectrally separated emission spectra while all fluorophores are susceptible for the same STED beam.
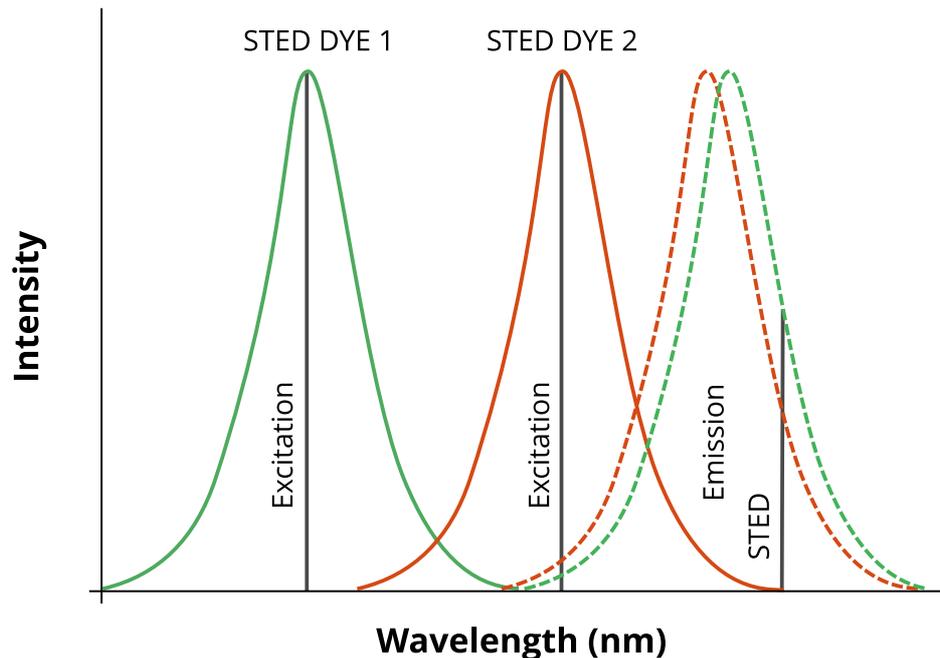
Illustration of excitation and emission spectra for two dyes used for STED imaging. The dyes feature different excitation spectra as well as different emission spectra. The STED wavelength overlaps with both emission spectra.

The high light intensities employed in STED microscopy (STED intensities of 1-200 mW/cm^2) possess a particular challenge for the photostability of the fluorescent molecules used for labelling. To achieve a super-resolution image, very high STED intensities have to be used. Thus, photobleaching of the fluorescent labels limits the achievable resolution. Organic dyes proved to be much more photostable compared to fluorescent proteins, resulting in a better achievable resolution.
List of recommended STED dyes:
Fixed samples:

FLUOROPHORE	EXCITATION WAVELENGTH (nm)	STED WAVELENGTH (nm)
Alexa Fluor 488	488	592
CELL	532	592/660
Abberior Star 580	560	755
Atto 590	590	755
Alexa Fluor 594	594	755
Atto 594	594	755
Abberior Star 635P	635	755
Atto 647N	647	755

List of recommended fluorophores for STED imaging of fixed samples.

Live-cell imaging:

FLUOROPHORE	EXCITATION WAVELENGTH (nm)	STED WAVELENGTH (nm)
eGFP	488	592
mNeonGreen	506	592/660
mCitrine	516	592/660
mStrawberry	574	660
Abberior LIVE 580	580	775
SiR-Dyes	635	775

List of recommended fluorophores for live-cell STED imaging.

Live-cell STED Imaging

STED imaging in living cells can be performed with various labels. Normally fluorescent proteins or orthogonal tags (SNAP, Halo or CLIP) are used which are encoded genetically and expressed as a fusion with the protein of interest. Fluorescent proteins are fluorescent after their maturation time and thus highlight the protein of interest without any further treatment. Orthogonal tags need a fluorescent ligand to become fluorescent and thus to enable a visualization of the protein of interest. The fluorescent ligand has to be cell-permeable to enable a labelling inside living cells. However, the dye has to be chosen carefully to not influence living cells and to not label unspecific cellular compartments.

The achievable resolution in STED microscopy is inherently coupled to the photophysical properties of the label used. As mentioned before, fluorescent proteins are not as photostable as organic dyes and thus are prone to bleaching if high STED intensities are used. However, to obtain a super-resolution STED image, very high STED intensities and small scanning steps have to be used. Thus, there is always a trade-off between the achievable resolution and the number of frames to record before the fluorescent labels are bleached. If a high number of frames or a high resolution is needed, organic dyes might be the better choice for a live-cell STED experiment.

In addition to photobleaching, the general brightness of a label is very important when it comes to high resolution live-cell imaging. If the label is rather dim, the pixel dwell time has to be increased to obtain a sufficient number of photons from each scan position. However, this elongates the recording time of a single frame and limits the achievable framerate in time-lapse series. Furthermore, by increasing the STED intensity, the number of excited molecules at the very center of the excitation volume is decreased and, thus, the signal to be detected comes closer to the background noise. At a certain point the signal emanating from the molecules at the very center of the STED donut is not anymore detectable over the background noise and, thus, the achievable resolution is limited.

By performing live-cell STED microscopy, the high light intensities can cause photodamage in the cell. If possible, a far red-shifted depletion laser should be used (e.g. wavelength of 775 nm) which is less absorbed by the cells, thereby reducing the risk of photodamage during image acquisition.

Sample preparation for PALM - Fluorophores and their properties

For PALM imaging, specialized fluorophores are needed. Therefore, the protein of interest has to be labelled via genetic labelling with photoactivatable or photoconvertible fluorescent proteins. PALM imaging is mostly based on fixed samples. Photoactivatable fluorescent proteins frequently used in PALM microscopy are e.g. mEos or mMaple, which are convertible after fixation and can be also used for live cell imaging. mEos is a very good fluorescent protein for PALM since it features a very good contrast ratio between its native state and the converted form. This enables PALM imaging of e.g. very densely packed clusters. Further, mEosFP emits a sufficient number of photons before it is bleached and thus enables a high localisation precision up to 10 nm.

The experimental design of PALM experiments allows for the tracking of single molecules in living cells. In these (single particle tracking-PALM) experiments, ideally only a single fluorophore (in a given region) is converted or activated and tracked inside living cells until it is finally bleached. Then new fluorophores are converted until sufficient data for statistical evaluation is recorded.

Useful Fluorescent proteins for PALM microscopy:

FLUORESCENT PROTEIN
mEos3.2
Dendra2
PA-mCherry
PA-GFP

Useful Fluorescent proteins for PALM microscopy.

Sample preparation for dSTORM - Fluorophores and strategies for fixed samples

The most commonly used sub-type of SMLM is dSTORM / GSDIM. Any suitable 'switchable' fluorophore, which can be switched between a fluorescent ON and OFF-state, can be used for dSTORM / GSDIM imaging. So far, dSTORM / GSDIM was demonstrated to work with organic dyes, fluorescent proteins and quantum dots.

Choosing the right fluorophore for dSTORM / GSDIM microscopy is mostly based on two properties of fluorescent molecules. First, how many photons are emitted during one ON/OFF-cycle and second, the fraction of time a molecule rests in the fluorescent ON-state (duty-cycle). ON and OFF refers here to the switching of one molecule from the OFF (or dark) state to the ON- state (or fluorescent state) and should not be confused with the multiple cycles of the electron between S_0- and S_1-states during the ON-time, which gives rise to hundreds to thousands of photons.

The number of emitted photons is crucially important to determine the position of the molecule accurately, because the localisation precision depends inversely to the square root of the number of detected photons of a molecule. Thus, a high number of photons per molecules is needed, otherwise the achievable resolution might be limited. If a high label density is expected, a low duty cycle is beneficial since the maximum number of fluorophores that can be individually detected at one time point is limited by the diffraction and is inversely proportional to the duty cycle. Meaning the lower the duty cycle of a fluorophores, the closer the molecules can be located to each other at a densely labelled structure. Both properties, the total number of emitted photons and the duty cycle of a fluorophore, are limiting the achievable resolution in dSTORM / GSDIM imaging.
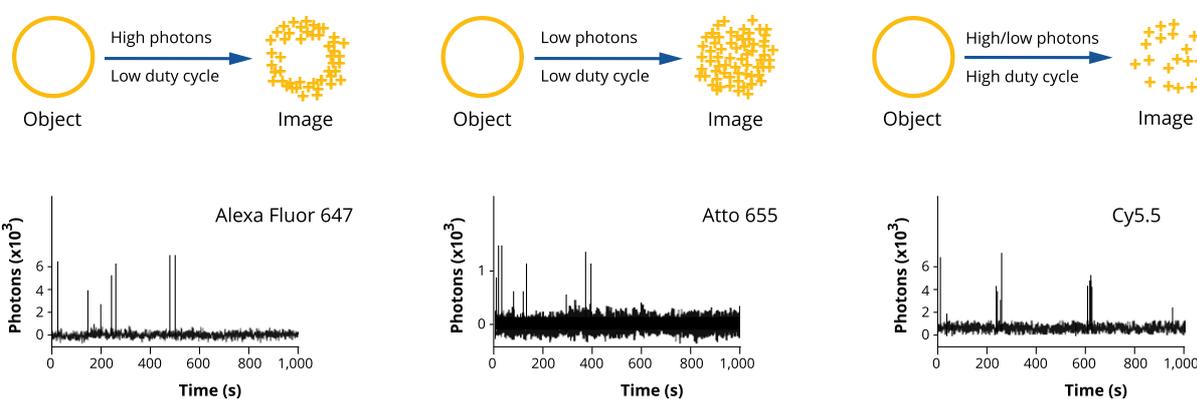
Influence of Photon number and duty cycle of fluorophores in the image quality of SMLM. a) A high photon number together with low duty cycle allows to detect and locate single fluorophores to map an underlying structure. b) and c) the combination of low photons with low duty cycle or high photons with a high duty cycle hampers the precise localisation of the single emitters thus the underlying structure cannot be resolved.

In the easiest imaging mode, a blinking fluorophore (like Alexa 647) can be excited continuously with a laser, each of so-called 'blinks' is a bursts (during which the molecule is in the ON-stat and 'blinks') of a few thousands photons, which are detected on a camera and used to reconstruct a super-resolution dSTORM image.

The most common dSTORM buffers contain catalase, glucose and glucose oxidase (GLOX) as an oxygen scavenging system combined with reducing agents like β-Mercaptoethanol (BME) or mercaptoethylamine (MEA) in slightly varying concentrations. Oxygen in the imaging buffer reduces the time that a fluorophore rest in the OFF-state. A too short time in the OFF-state impairs the imaging, since too many fluorophores will return to their fluorescent ON-state and interfere with the detection of singularized fluorophores. Therefore, the imaging buffers should also stabilize the OFF-state of a fluorophore which is often achieved by a reducing agent like BME or MEA. In addition, these buffers reduce the bleaching rate of the fluorophores.

A typical GLOX (Glucose Oxidase based) imaging buffer composition is listed exemplary below optimized for maximum performance with Alexa 647:

0.5 mg/ml glucose oxidase
40 µg/ml catalase
10% w/v glucose
50 mM Tris-HCl, pH 8.0
10 mM NaCl
10 mM β-Mercaptoethylamine

Based on the imaging buffer - dye combination, the final image resolution and perceived image quality might differ. Therefore, the buffer conditions should be optimized for each specific dye or dye combination. E.g. the typical GLOX buffer is very well suited for Alexa 647 but less for other dyes. Alexa 647 can work well with e.g. Alexa 532 in dual color experiments. In contrast Alexa 488 performs comparably poorly in the GLOX buffer, but is performing well in OxEA buffer. OxEA is also providing a good performance with Alexa 647, and therefore enabling dual color experiments with this dye

combination. Even triple color labelling is possible by combing the Oxea buffer with A568 as the third label in addition to Alexa 488 and Alexa 647.

More buffer has been described in the literature and dependent on the needs of the researcher also Borohydrate based buffers can be employed for maximum number of photons per bursts, but Borohydrate is toxic.

Dye		BUFFER CONDITION
		GLOX and thiol
Blue-absorbing	Atto 488	++
	Alexa Fluor 488	++
	Atto 520	++
	Fluorescein	+
	FITC	+
	Cy2	-
Yellow-absorbing	Cy3B	++
	Alexa Fluor 568	+
	TAMRA	+
	Cy3	+
	Cy3.5	+
	Atto 565	+
Red-absorbing	Alexa Fluor 647	++
	Cy5	++
	Atto 647	+
	Atto 647N	+
	Dyomics 654	++
	Atto 655	++
	Atto 680	++
	Cy5.5	+
NIR-absorbing	DyLight 750	++
	Cy7	++
	Alexa Fluor 75C	+
	Atto 740	+
	Alexa Fluor 79C	+
	IRDye 800CW	-

Cells were immunostained for microtubules using each of the dyes. The thiol used was MEA. Dyes were assigned a "-","+" or "++" if rapid bleaching (no image), low to moderate photoswitching (low to moderate quality image) or robust photoswitching (high quality image) was observed, respectively. Whereas lower image quality (+) was observed for Atto 647N and Alexa Fluor 750 in the 'GLOX and thiol' condition, reasonable image quality (++) was observed for these two dyes when βME was used instead of MEA.

Dyes and their 'blinking' properties in the above-mentioned GLOX buffer.

Super-Resolution Image Acquisition - STED -
The optical path of a STED microscope

A STED microscope is based on a confocal scanning microscope. To enhance the lateral resolution in a STED microscope, typically a donut-shaped STED beam with no intensity in the center (a 'zero') is employed. A donut-shaped beam can be created by placing a so-called phase mask 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. Using galvo-scan mirrors, these two combined beams are coincidentally scanned over the sample.
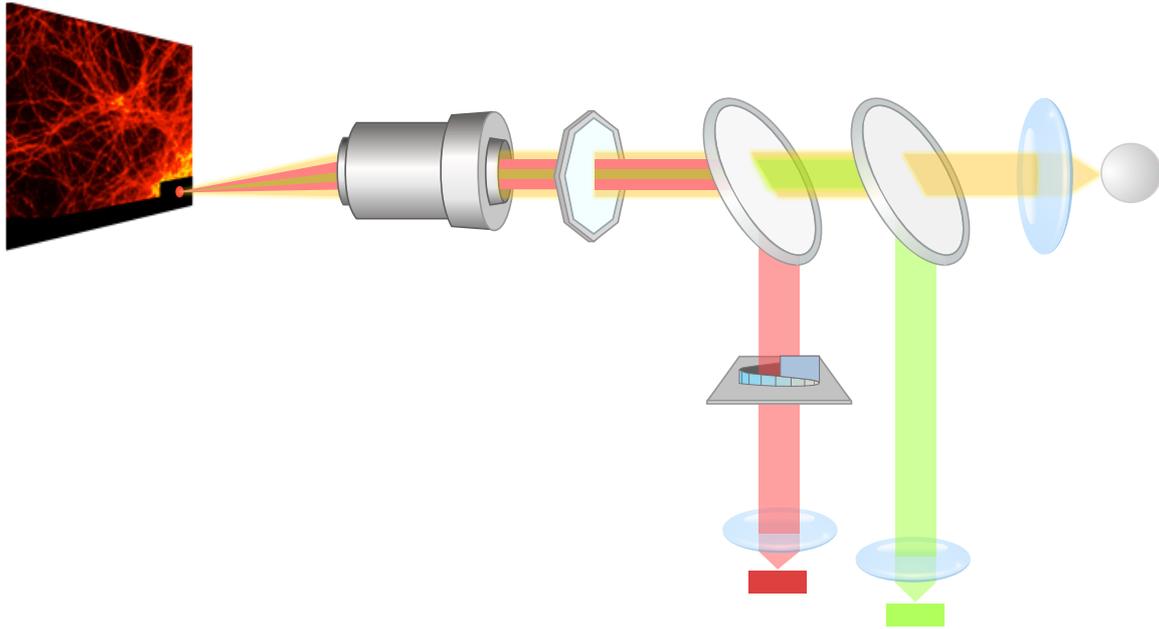
Incorporation of the STED laser beam into a single-point confocal scanning microscope: The excitation laser of the conventional laser scanning microscope and the STED beam are combined via a dichroic mirror and then scanned coincidentally over the sample. Please note the lambda/4 plate placed in the beam path close to the objective.

The donut-shaped STED beam is generated via integration of a phase plate into the laser beam path. The applied phase mask modifies the light passing through the phase plate, leading to destructive interference at the very center of the beam and generating a local 'zero' intensity area of the beam.
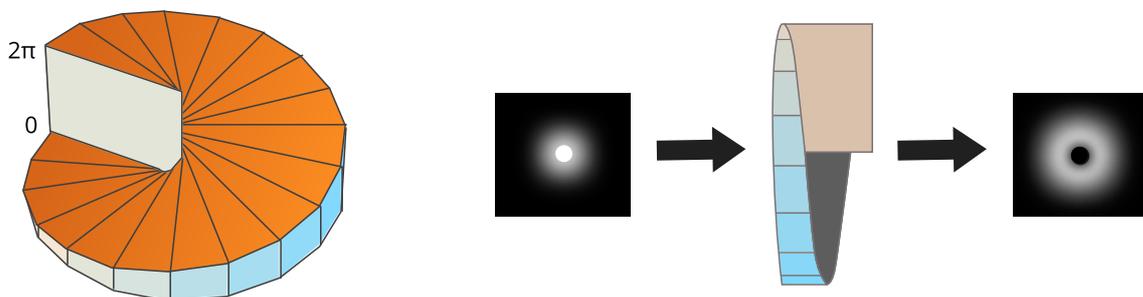
Schematic drawing of a phase plate (also called phase mask): The phase mask is a stepwise increasing polymer staircase delaying the beam for up to 2pi (top). The phase mask is placed in the laser beam, converting a Gaussian-shaped profile into a donut-shaped profile in the sample plane (drawn schematically, bottom).

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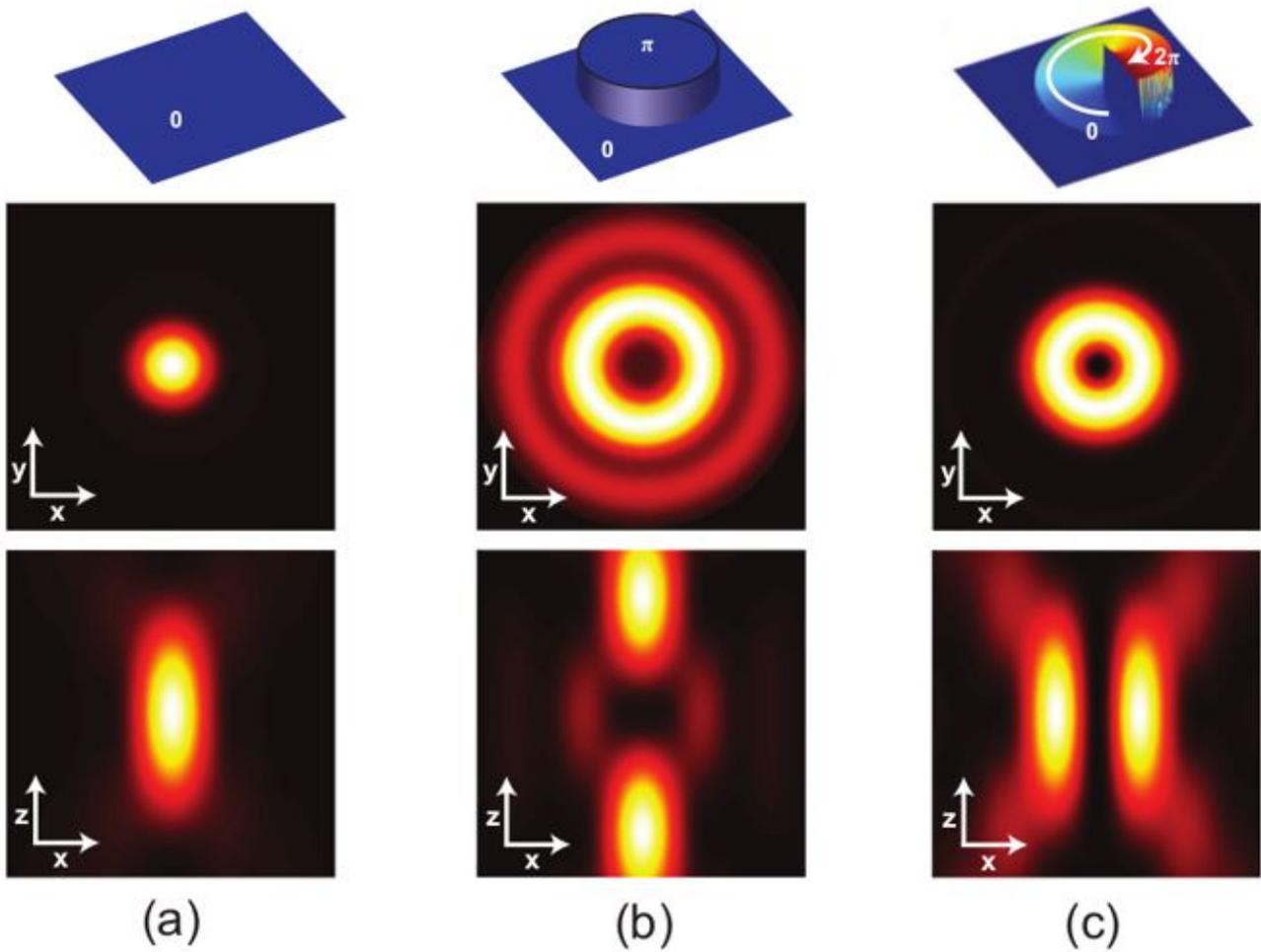
Effect of different phase masks on the shape of the STED laser beam: no phase mask displays the typical Gaussian beam (left), a staircase 0-2pi phase mask creates a donut, increasing the lateral but not the axial resolution (right). A single step of pi creates a depletion beam that is enhancing the lateral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

STED Imaging

STED microscopy offers the possibility to optimize imaging parameters (e.g. dwell time or STED- and excitation-power) during the measurement to optimize the STED image, because the recorded STED raw image already features the high resolution information intrinsically. The excitation power or STED intensity can be lowered or increased while imaging and thereby optimized.

To record a STED image one should record a regular confocal overview image first to set the focus of the objective lens to the optimal focal plane. From this region of interest (ROI), the final super-resolution STED image can be recorded by only changing a few parameters. Because of the higher optical resolution, the pixel size has to be set to smaller pixel sizes (following the Nyquist criterion), compared to regular confocal imaging. The pixel size should be adapted to the increased optical resolution: typically a pixel size of 20 nm is chosen and sufficient to record images with approximately 50 nm optical resolution. It is still good practice, to record a regular confocal image besides STED image to compare the achieved resolution enhancement.

Second, as a rule of thumb, the excitation power for the STED image can be increased to a factor of three. Next, a super-resolution STED image can be recorded. Hereby the STED power should be set to a low intensity first and can then be gradually increased until the aimed resolution is achieved (always compare the STED image to the corresponding confocal image).

If strong bleaching becomes apparent while imaging, first the excitation power and second the STED intensity should be decreased. Furthermore, the pixel size and the dwell time of the STED channel can be decreased to reduce the number of ON-to-OFF-transitions resulting in less bleaching.

After recording the STED image, it is of advantage to record again an overview image. This gives the information if the imaged area has been bleached during STED imaging.

SMLM -
The optical path of a SMLM microscope

An SMLM microscope is based on a typical Widefield microscope. Here the excitation light is provided by a laser and is focused by the objective lens to the focal plane. The field of view depends on the objective lens and camera used and is typically in the range of 20-50 μm for a 100x (or 160x) oil objective lens. Typically, a pixel size of around 100 nm in the sample plane is the theoretical optimum and therefore most microscope manufactures try to achieve this number. An electronic camera typically records the fluorescence, either using an electron multiplying charge-coupled device (EM-CCD) or a scientific complementary metal oxide semiconductor (sCMOS) camera. Both detectors have advantages and disadvantages. Modern sCMOS detectors feature a high-quantum efficiency and a low pixel noise. These detectors are faster than EM-CCDs, which enables a data acquisition at much higher rates compared to an EM-CCD detector. However, sCMOS detectors have a different noise behavior per pixel and thus to avoid image artefacts, compensation of the recorded raw data is needed.

EM-CCD feature a very high photon efficiency and thus are very suitable to detect weak fluorescence signals but with the expense of a slower acquisition time. Furthermore, no compensations of individual pixel noise and gain are necessary. SMLM is often performed in standard Widefield mode. However, dense samples or imaging the basolateral plasma membrane benefit from specialized illumination schemes to decrease the background while imaging. Using TIRF- (Total Internal Reflection Fluorescence) or HILO- (Highly Inclined and Laminated Optical sheet) illumination of the sample, the excitation light is restricted to a certain plane of the sample. In TIRF-SMLM-imaging the sample is excited from an oblique angle leading to the generation of an evanescent wave of excitation light close to the cover-slip media interface. This restricts the excitation and detection of fluorescence to a thin region of the specimen above the glass surface and thus reduces the background signal dramatically.

Imaging strategies for dSTORM - Blinking fluorophores

Single molecule localisation microscopy (SMLM) is relying on 'blinking' fluorophores which are well separated in space and time allowing sufficiently precise fitting with a theoretical function (e.g. a Gaussian-fit) and thereby revealing the most probable location of each fluorophore. Most commonly SMLM using dSTORM / GSDIM is used to create the 'blinks', which themselves are short bursts of a few hundred to a few thousand photons hitting the camera within the exposure time of one frame. The final image quality is not only dependent on the number of photons detected (which determine the localisation precision) but also on the capability of the detection algorithm to perform a sufficient fit. For example, high background and overlapping PSF of single fluorophores can prevent the algorithm from detecting the correct position of the fluorophore. More sophisticated fitting algorithms, which can separate overlapping fluorophores are nowadays available and therefore can improve the localisation in datasets with overlapping simultaneous emitting fluorophores. However, if the parameters are not set properly, artefacts can occur and therefore optimizing the blinking frequency first is a preferable strategy.

Ideally, the fluorophores can still be separated by eye and allowing even the simplest type of fitters, e.g. a center of mass determination, to find a decent approximation of the fluorophore position. A number of means can optimize the blinking and the parameters should be tested systematically.

Laser power

Increasing the laser power (and thereby increasing the power density in the sample plane) often improves the blinking of standard fluorophores like Alexa 647.

Focusing the laser onto a smaller sample area increases the power density and is available on some microscopes.

TIRF imaging: In TIRF imaging, it is usually beneficial to perform the initial 'switching off' light pulse or pump pulse with epi-illumination to ensure that also fluorophores in larger distance to the coverslips are efficiently switched off before starting the recording in TRIF illumination. A vastly decreased background is the typical result.

Buffer

Increasing the pH of a standard GLOX buffer improves the switching off of Dyes like Alexa 647 and therefore can be an alternative to increasing the laser power.

Reducing agent concentration: Reducing agents like β-Mercaptoethylamine (MEA) stabilize the off states and therefore are key players in determining the blinking performance. Dependent on the dye, GLOX buffer with higher (or lower) MEA concentrations could be tested. Some fluorophores do not benefit from the Oxygen Scavenger system at all and therefore MEA in concentration range between 10-100 mM in PBS (pH adjusted to 7.4) can be a sensible alternative.

New buffer recipes are constantly published and evaluated; therefore a careful literature search should be also included in the optimization step. Microtubules have been proven to be a relatively easy to prepare sample to evaluate the performance of different imaging conditions and buffers. Continuity and width of the filaments (measured with the Full Width at Half Maximum (FWHM)) are the major parameters to score the performance of the imaging condition tested.

UV Light

With longer recording time, the number of active fluorophores ('blinks') is decreasing. By applying small amounts of UV light during acquisition (pulsed or CW), fluorophores can be converted from the off state back to an on state and thereby increasing the number of 'blinks'. Ideally, the number of fluorophores actively 'blinking' is just under the threshold before fluorophores start overlapping. Using the UV laser appropriately can speed up the imaging significantly without a decrease in image quality.

Exposure time

Ideally, the individual fluorophore 'blinks' contain a few thousand photons and all the photons detected in one or two frames. This reduces the contribution of the camera readout noise to the final image and increases the localisation precision in each frame (since a larger number of photons is recorded).

Optimization strategy:

By inspecting the time-lapse recording of the fluorophore blinking (see also movie) and switching between individual images, try to see if the majority of fluorophores are emitting only in one or two frames and not e.g. 4 or more. If the latter is the case, the exposure time can be easily extended by a factor of 1.5-2. If almost all fluorophores emit in one frame, a shorter exposure time might be beneficial. By a shorter exposure time, the number of events might be decreased per image allowing a shorter total acquisition time by employing UV light to increase the number of active molecules (see also below) and maximizing the number of well-separated events again.

Grouping of detect events in the analysis software over time should not lead to a vast decrease in the number of events in the super-resolution image recording. If this is the case, the exposure time can be extended. If this function has practical no effect, the exposure time can be most likely shortened but a manual inspection of the time-lapse is still recommended.
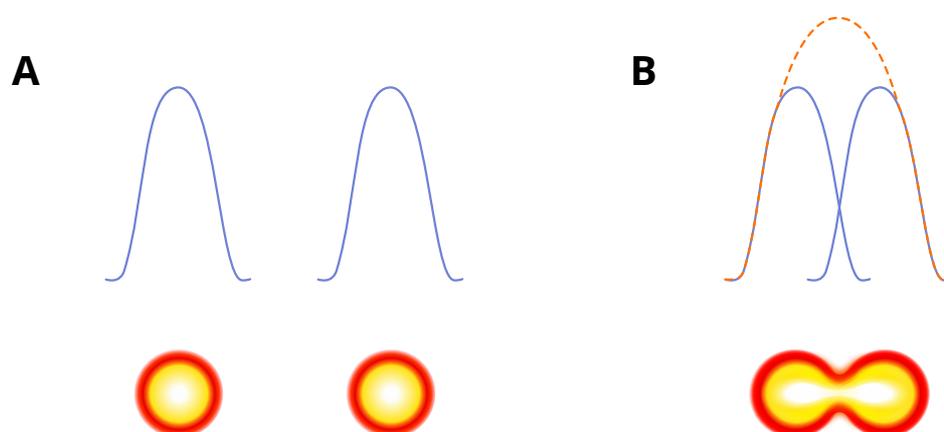
Best conditions for Single Molecule position localisation: (A) Well-separated fluorophores in space can be easily fitted with practical all algorithms available. (B) Overlapping fluorophores cannot be properly fitted by simple center-of-mass algorithms (often leading to a position reported in center between the two fluorophores (depicted) or the rejection of the two events). Overlapping fluorophores can lead to reconstruction artefacts which can be avoided by well-tuned multi-emitter fitters, which still can separate two or more overlapping fluorophores.

The number of localisations is not necessarily a measure of image quality and should be therefore handled with care. A high blinking frequency can lead to a number of bursts which contain only a few hundreds of photons each and thereby decrease localisation precision and lead to a scattering of the localisations around the true position ultimately leading to a blurrier (or lower resolved) image than necessary.

It is a tricky question to determine after how many ('blinking') frames recorded the acquisition should be stopped. One strategy can be to look at an online reconstruction of the super-resolution image and stop the acquisition when the image looks sufficiently well-resolved and detailed. The strategy can be risky since in later analysis steps the structure of interest might not be sufficiently covered by fluorophore localisations. More time consuming but certainly on the safe side is the following approach: Record the blinking timelapse until the UV laser is ramped up to full power and only a few events are still visible in the live images. Thereby, almost all accessible fluorophores have been recorded. A premature termination of the image acquisition could cause under-determination and result in a bad image quality. However, this is certainly the more time consuming approach.

Image reconstruction from SMLM data

The result of the single molecule localisation routine e.g. via Gaussian-fitting is not an image, but a table with the coordinates of the events. An event is the fluorophore localised in a given frame. Please note that a fluorophore can emit photons in multiple frames and therefore can give raise to multiple events. The localisation precision (derived from the number of photons) is reported in the table amongst some other parameters for data handling (e.g. an event ID).

This table needs to be converted into an image or in other words the super resolution image needs to be reconstructed. Two common approaches will be briefly described here. One approach is the histogram (based) reconstruction. In this case, a new image is created with a smaller pixel-size than the original camera image to account for the increased resolution in the super-resolution image. Typically, a pixel size of 20 nm is chosen, but the operator of the software is free to use other values (to match the Nyquist criterion) on the one hand and still retain a usable S/N ratio). The algorithm is allocating every event to a pixel just by determining if the x, y, (z) position is within the area (volume) covered by the pixel (voxel). The number of events per pixel is then displayed as the brightness of the pixel.

The Gauss-reconstruction can be explained easiest in a slightly simplified manner: The algorithm turns every event into a gauss function representing the localisation precision (sigma) by its width. The 2D Gaussian-function for each event is then placed at the x, y position of the event in the reconstructed image. Finally, an (adjusted) sum projection is performed to create the final image. Usually, a pixel size of 5 nm is selected to be able to appreciate the details of the Gaussian-shape of the events reconstructed.
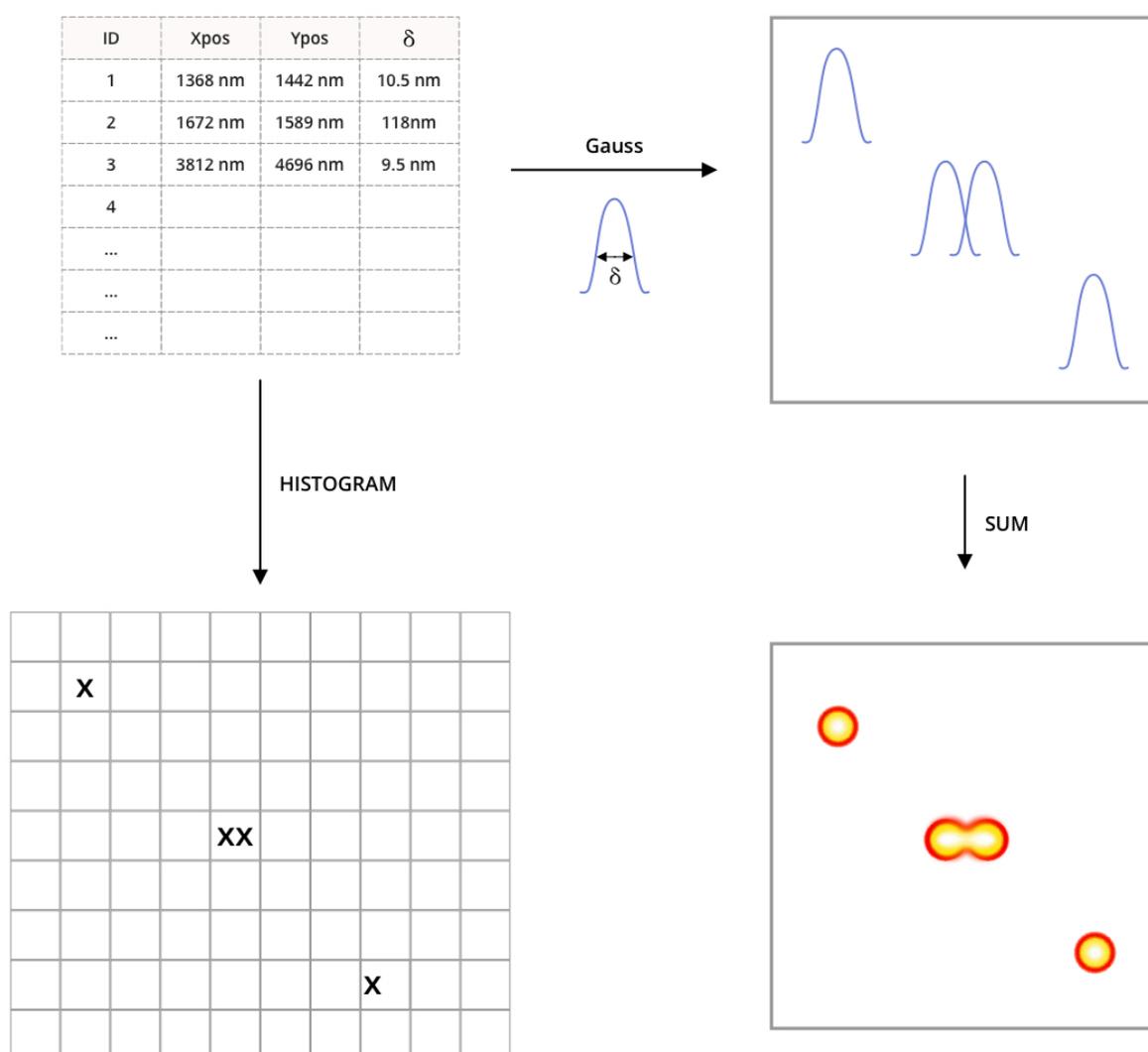
Schematic drawing of image reconstruction of SMLM images. From the localised single fluorophores a high resolution image can be reconstructed in the Histogram or in the Gauss-mode.

Credits

Microscopy Australia acknowledges the huge input of time and expertise by the many staff members and associates who have contributed to the development of MyScope over the years.

For Light and Fluorescence we thank: Ellie Kable, Pam Young, Ying Ying Su, Minh Huynh, Melanie Rug, Daryl Webb, Agatha Labrinidis, Gwen Mayo, Paul Rigby, John Murphy, Filip Braet, Lilian Soon, Courtney Wright, Marko Lampe, Liisa Hirvonen, Guy Cox, Louise Cole, Geoffrey Clarke, Jenny Whiting.