MyScope
training for advanced research

Confocal Microscope
Training module

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Introduction

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).

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.

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 aberration](image)

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.

![Correction collar to adjust for coverslip thickness.](image)

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 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.

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

www.ammrf.org.au/myscope/confocal/light
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. Below are a description of the microscope parts that are important for the process and a brief summary of the steps involved. For virtual Koehler illumination, please click on this link.

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 (Fig 1). The latter is best viewed by removing the eyepiece and inserting an eyepiece telescope or Bertrand lens.

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

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.
Figure 1. 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.
Fluorescence microscopy

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 fluorescent 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.

![Components and light paths of a fluorescence microscope.](image-url)

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.

Resources and references

A Spectra Viewer courtesy of Invitrogen


Confocal microscopy

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

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

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

The lateral (XY) resolution is usually the same as in conventional microscopy. By making the pinhole very small (so that only a small part of the image Airy disk can pass) we can get slightly improved resolution – in principle about 150nm with an NA 1.4 objective – but this wastes so much light that it is impractical in fluorescence, which is where most cell biologists work. Therefore the pinhole is normally set to be the same diameter as the magnified Airy disk. The axial (Z) resolution is always worse, and unlike the lateral resolution (which relates directly to the NA) the axial resolution depends on the 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 2 µm.

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 before 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.

This image shows how they differ in size and shape. www.olympusmicro.com/primer/techniques/confocal/laserintro.html

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. (This table was developed by Chris Johnson PerkinElmer.)

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<th>Ex Laser</th>
<th>Ex Peak</th>
<th>Em Peak</th>
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<th>422*</th>
<th>488*</th>
<th>514*</th>
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*% percent excitations

**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. Its 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. Its purpose is to block any of the excitation light and only pass the required emitted fluorescent light.

Below are examples of these filter types.

http://www.olympusmicro.com/primer/techniques/fluorescence/filters.html

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 to pass to the detector.
In today’s modern confocals the emissions are able to be separated with even finer precision. Each confocal achieves this slightly differently. For further reading see the websites below.

- [zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html](http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html) Zeiss
- [www.olympusmicro.com/primer/techniques/confocal/bleedthrough.html](http://www.olympusmicro.com/primer/techniques/confocal/bleedthrough.html) Olympus

### 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

![Diagram of Photomultiplier tubes (PMTs)](image-url)
different sensitivities over different ranges.

www.olympusmicro.com/primer/techniques/confocal/pmtintro.html
Practical image acquisition

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

Remember we mentioned that you “get nothing for free” when doing confocal microscopy. The “Eternal Triangle” describes this conundrum particularly well:

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 150?W 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 objective 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

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.

<table>
<thead>
<tr>
<th>Magnification</th>
<th>Numerical Aperture</th>
<th>Lateral Resolution ((?m))*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x</td>
<td>0.3</td>
<td>0.666</td>
</tr>
<tr>
<td>40x</td>
<td>0.75</td>
<td>0.266</td>
</tr>
<tr>
<td>40x oil</td>
<td>1.25</td>
<td>0.160</td>
</tr>
<tr>
<td>63x H2O</td>
<td>1.2</td>
<td>0.166</td>
</tr>
<tr>
<td>63x Gly</td>
<td>1.3</td>
<td>0.153</td>
</tr>
</tbody>
</table>

*@ 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 \( r = 0.4 ? /\text{NA} \).
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.

<table>
<thead>
<tr>
<th>Magnification</th>
<th>Numerical Aperture</th>
<th>Scan size (pixels)</th>
<th>Scan area (μm²)</th>
<th>Pixel size (μm)</th>
<th>Lateral resolution (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x</td>
<td>0.3</td>
<td>512 x 512</td>
<td>1555</td>
<td>3.03</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1024 x 1024</td>
<td></td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td>40x</td>
<td>0.75</td>
<td>512 x 512</td>
<td>387.5</td>
<td>0.758</td>
<td>0.266</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1024 x 1024</td>
<td></td>
<td>0.378</td>
<td></td>
</tr>
<tr>
<td>1.25 (oil)</td>
<td></td>
<td>512 x 512</td>
<td></td>
<td>0.758</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1024 x 1024</td>
<td></td>
<td>0.378</td>
<td></td>
</tr>
<tr>
<td>63x</td>
<td>1.2 (H2O)</td>
<td>512 x 512</td>
<td>246.03</td>
<td>0.481</td>
<td>0.166</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1024 x 1024</td>
<td></td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>1.3 (Gly)</td>
<td></td>
<td>512 x 512</td>
<td></td>
<td>0.481</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1024 x 1024</td>
<td></td>
<td>0.24</td>
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</table>

As can be seen from the above table, scanning your image onto a 512 x 512 image or 1024 x 1024 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.

<table>
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<th>Scan area (μm²)</th>
<th>Pixel size (μm)</th>
<th>Lateral resolution (μm)</th>
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</table>
Detection parameters

Laser power

One golden rule: “Use the lowest laser power to give an acceptable image.” When an acceptable image cannot 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” overlaying 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 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 adjustment 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 lower 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.

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.

Images of a pollen grain autofluorescence collected using identical gain values but using different line averaging values. The corresponding graph indicates the pixel intensities along the magenta line traversing the pollen grain surface. Note the high level of noise in the unfiltered images which make the surface details of the pollen grain difficult to see. Increasing numbers of averages reduces the noise and makes surface detail clearer.
Sequential and simultaneous

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:

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 (Fig 2).

Fig 2: 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:
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).

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.

Fig 5: 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) (Fig 6).

Fig 6: 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 (Fig 7a). Then the other laser is used for excitation and the relevant emission photons collected (Fig 7b).

**Fig 7a:** 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).

**Fig 7b:** 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 in Fig 8). Careful selection of fluorophores combined with sequential imaging can usually eliminate (or at least minimise) spectral bleedthrough.

Fig 8: 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

<table>
<thead>
<tr>
<th>Fluorophores</th>
<th>Simultaneous</th>
<th>Sequential</th>
</tr>
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<tbody>
<tr>
<td>DAPI</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AF488</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AF586</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AF647</td>
<td>-</td>
<td>-</td>
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<td>DAPI + AF488</td>
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</tr>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>DAPI + AF568 + AF647</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>DAPI + AF488 + AF568 + AF647</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

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.
All graphs on this page were generated on the Fluorescence SpectraViewer from the Life Technologies website:
Collecting Z stacks

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.

<table>
<thead>
<tr>
<th>Magnification</th>
<th>Numerical Aperture</th>
<th>OST (?m) 1AU</th>
<th>OST (?m) 2AU</th>
<th>OST (?m) 4AU</th>
</tr>
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<tbody>
<tr>
<td>10x</td>
<td>0.3</td>
<td>11.08</td>
<td>19.96</td>
<td>38.74</td>
</tr>
</tbody>
</table>
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 0.2?m, then we must use a pixel dimension of 0.2?m x 0.4 = 0.08?m (or 80nm) in that plane. Similarly, if the z dimension resolution limit is 0.5?m, then our best z step should be 0.5?m x 0.4 = 0.2?m (200nm). This means our final microscope setup should be collecting images with voxels that have dimensions of 0.08?m (x) x 0.08?m (y) x 0.2?m(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 photobleaching,
2. The specimen is moving slowly, and registration between the first and last images would be poor.

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.
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.)

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.

But a significantly better appreciation can be obtained by viewing a projection of the entire data set.
Here the legs, antennae and even some of the internal organs can be clearly seen in the mite. (As an aside, this data was derived by collecting the autofluorescence signal emitted predominantly by the chitin exoskeleton when excited with 488nm (blue) laser light.)

The image on the left (above) 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.
Exercise: While the movie is playing, what do you notice about the back of the mite? Can you see the indentation? This specimen was freeze-dried and the back has collapsed in the processing. Now, the important thing to note is that this movie was generated from exactly the same data set that we have seen previously. Did you notice the indentation in the earlier images of the mite? Go back and look more closely.

This exercise highlights an important feature when examining three dimensional data. 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.

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

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.

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

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<td>38.74</td>
</tr>
<tr>
<td>40x</td>
<td>0.75</td>
<td>1.772</td>
<td>3.19</td>
<td>6.19</td>
</tr>
<tr>
<td></td>
<td>1.25 oil</td>
<td>0.969</td>
<td>1.745</td>
<td>3.387</td>
</tr>
<tr>
<td>63x</td>
<td>1.2 H2O</td>
<td>0.921</td>
<td>1.659</td>
<td>3.221</td>
</tr>
<tr>
<td></td>
<td>1.3 Gly</td>
<td>0.856</td>
<td>1.54</td>
<td>2.99</td>
</tr>
</tbody>
</table>

OST = optical section thickness.
Virtual light microscopy

- Transmitted light microscope
- Fluorescence microscope
- Confocal microscope
Transmitted light microscope

Interactive simulation of a confocal microscope.

Adobe Flash player is required to use this activity.

Reference material in this activity
Fluorescence microscope

Interactive simulation of a confocal microscope.

Adobe Flash player is required to use this activity.

Reference material in this activity
Confocal microscope

The following activities are available:

- Image acquisition (single scan)
- Objective lens - magnification versus resolution
- Pinhole - effects of resolution
- Scan speed - pixel dwelling, saturation
- Pixel arrays - dimensions versus pixel size
- Multichannel imaging - simultaneous, sequential and transmitted light
Image acquisition (single scan)

Interactive simulation of a confocal microscope.

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Objective lens - magnification versus resolution

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Reference material in this activity
Multichannel imaging

Adobe Flash player is required to use this activity.

Material referenced in this activity:
3D

Adobe Flash player is required to use this activity.

Material referenced in this activity: