Ludwig Institute for Cancer Research
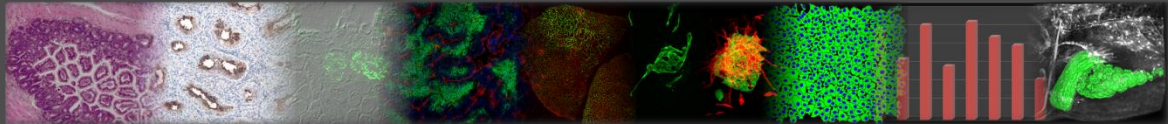
Centre for Advanced Microscopy

Training Manual

Version 2.0
2011

by
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CENTRE FOR ADVANCED MICROSCOPY

LUDWIG INSTITUTE FOR CANCER RESEARCH

Contents

Scope and Purpose

This manual was developed as an adjunct to the tutorials provided to users of the Centre for Advanced Microscopy at the Ludwig Institute for Cancer Research Melbourne – Parkville Branch.

The information in this manual provides basic background information on microscopy imaging techniques, hardware, digital imaging, basic protocols and terminology. Each tutorial has a recommended reading that will enable better quality teaching of new users by providing them with easy to follow information and diagrams.

This manual is not expected to be an exhaustive source of information; further reading on any of the concepts covered in this manual can be provided by the CAM Manager.

Reproduction and Usage

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The CAM facility is managed by an experienced microscopist who provides training and support for all staff and students. While the manager is responsible for the day to day running of the facility, general long term strategic planning is carried out by the CAM manager in consultation with a Microscopy Users Committee. The users committee is made up of lab heads and heavy microscopy users. For administrative and budgetary issues the CAM manager reports directly to the General Manager, who can then raise certain issues with the Ludwig Management Committee.
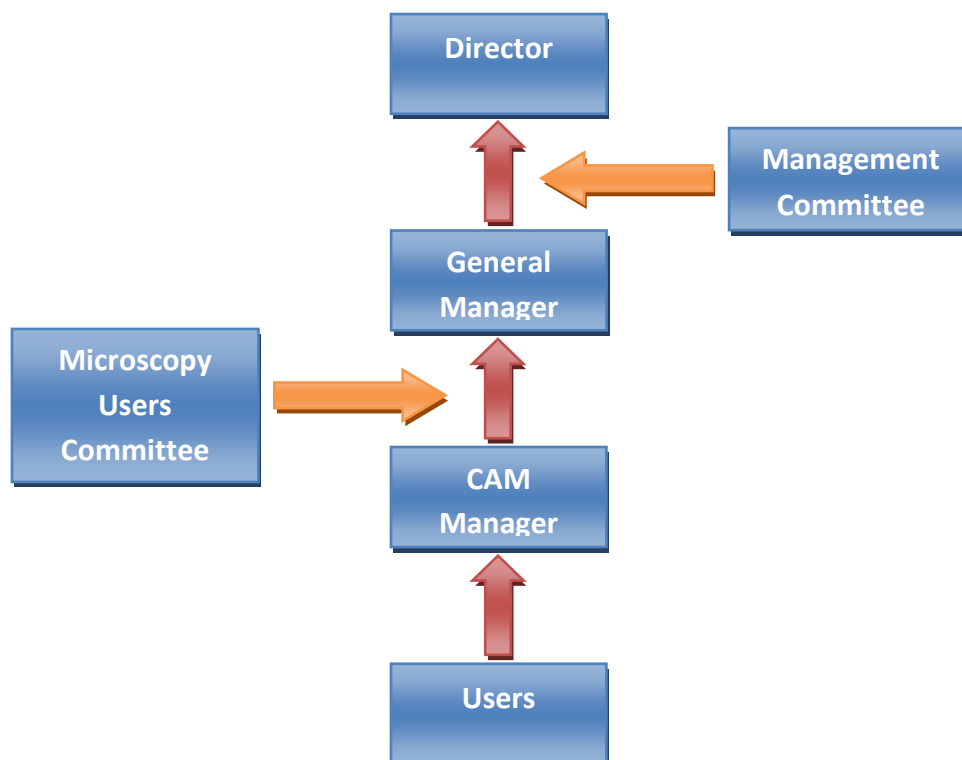
Centre for Advanced Microscopy Usage Policy

1.0 Preamble

The Centre for Advanced Microscopy (hence called CAM) provides a central facility for microscopy imaging and analysis with dedicated support from experienced microscopy professionals.

All equipment in CAM is available for use by all Ludwig Institute for Cancer Research Ltd (Parkville Branch) staff. Staff from external institutions may use the equipment with prior arrangement (see Section 3.0 for more information).

Access to use the equipment is granted following a tutorial (and in some cases a test) performed by the manager of CAM. This ensures users are capable of taking publication quality images that are a true representation of the data.

2.0 Gaining Usage Rights to the Equipment

All staff wishing to use CAM equipment need to have received a tutorial (and any associated test) on the specific equipment they wish to use. With completion of the tutorial/test the staff member is made a registered user of that specific equipment.

Users are only permitted to use equipment they are registered for. Registered users are not allowed to show other non-registered users how to use any of the equipment. Only the CAM manager (or their designated representative) is authorised to train new users.

2.1 Tutorials and Tests

Prior to using any equipment for the first time, the user needs to organise with the CAM manager to have a tutorial. Before a tutorial can be conducted the user needs to speak to the CAM manager and be given a copy of the CAM training manual. The user will be expected to have read the relevant sections of the manual prior to receiving a tutorial. Once the user is ready to receive a tutorial they should contact the CAM manager to organise a mutually suitable time.

All tutorials will be followed up with a test (which requires 100% to pass) to assess the users competency. The content of the test varies depending on the equipment being used. This is essential to ensure the user is collecting valid data and ensures valuable sensitive equipment is not damaged through ignorance.

Tutorials run from 30 minutes to 2 hours depending on complexity. The associated test will be included in the initial tutorial for simple equipment and will take an additional hour for more complicated equipment.

Gaining usage rights to confocal microscopes requires the user to undertake the tutorial, practice for several hours and then take the test. The user will not be given the test unless they can demonstrate the required practice time.

All users are expected to read the sections in the training manual covering digital imaging, image manipulation, experimental design, resolution and objective basics. If the user is doing any fluorescent imaging they are expected to also read the fluorescent basics section.

The following section details the process required to become a registered user on the specified equipment.

2.1.1 Widefield Microscopes

In addition to the compulsory sections, the user is expected to have read the sections of the training manual covering widefield imaging techniques and Kohler illumination.

The available widefield microscopes are:

- Nikon 90i (Upright)
- Nikon Ti-E (Inverted)
- Nikon SMZ-1500 (Stereo)
- Nikon Ti-S (Inverted) – 7th Floor
- Nikon TE-300 (Inverted) – 8th Floor

The tutorial on the widefield microscopes involves the following

- Overview of microscope components and their function
- Alignment of microscope and setting up for Kohler illumination
- Use and alignment if DIC optics, or phase contrast (if required)
- Usage of SPOT camera and software to obtain optimal image quality
    - Flatfield correction – for brightfield
    - Background subtraction – for fluorescence
    - Colour correction – for brightfield
    - White balance – for brightfield
    - Addition of scale bars
- Tips on saving data, image compression and further analysis

Following (and during) the tutorial the user will be tested on the relevant points from above. If it is clear that the user has not read the relevant topics in the training manual the tutorial will be stopped until the user can demonstrate they have done so.

2.1.2 Live Cell Imaging

In addition to the compulsory sections, the user is expected to have read the sections of the training manual covering widefield imaging techniques and Kohler illumination.

To be able to be trained in live cell imaging, the user will need to be a registered user of the required microscope.

The available live cell imaging platforms are:

- Nikon Ti-E
- Nikon C1+ Confocal (room temperature only)
- Olympus FV1000 confocal (single photon)
- Olympus FV1000 confocal (multiphoton)

The tutorial on live cell imaging involves the following

- Setting up gas supply for long term cultures
- Configuring and running the experiment with the relevant software (MetaMorph, EZ-C1, Olympus FV1000) including techniques to prolong cell life, such as minimising exposure time, binning, optimising light intensity and objective choice.
- Setting up multiple stage positions (Ti-E and FV1000 only)
- Review of data sets once acquired
- Image playback including addition of scale bars, time stamps, making moves, light equalisation and so forth. Image analysis is run separately.

Following (and during) the tutorial the user will be tested on the relevant points from above. If it is clear that the user has not read the relevant topics in the training manual the tutorial will be stopped until the user can demonstrate they have done so.

2.1.3 Confocal Microscopes – Single Photon

If the user has no microscopy experience they will be required to undergo a widefield fluorescence tutorial before being able to undergo the confocal tutorial. If a user is already registered for one confocal they only need to complete the tutorial (no test required) for any additional system they wish to learn.

In addition to the compulsory sections, the user is expected to have read the sections of the training manual covering confocal basics, laser function and safety and Nyquist sampling.

To become registered on a confocal microscope the user must have had the initial tutorial, completed several hours of practice using test samples and then have passed the test. The user is expected to take the test within one month of the initial tutorial; if the time is longer the user may be required to take the initial tutorial again.

If the user fails the test they are able to retake it after 24 hours (providing the manager and microscope are free).

The confocal microscopes available are:

- Nikon C1+
- Olympus FV1000 (Inverted)
- Olympus FV1000 (Upright)

The tutorial for confocal microscopes involves the following:

- Overview of confocal microscopy
- Overview of microscope and scan head components and their function
- Procedure for turning on and off the system
- Capturing a confocal image
  - Correct dye setup
  - Correct objective settings
  - Adjusting settings for optimal image capture by avoiding over-saturated and under-saturated pixels
  - Scanning speed and the effect on image quality and intensity
  - Kalman averaging to reduce noise
  - Choosing correct pixel size and Z-slice size to conform to Nyquist sampling theory
  - Collecting a Z-series
  - Projecting a Z-series
  - Adding scale bars
  - Saving data
  - Saving representative images

2.1.4 Confocal Microscopes – Multiphoton

To be able to be trained in multiphoton confocal the user needs to be a registered user of the Olympus FV1000 – single photon.

In addition to the compulsory sections, the user is expected to have read the section of the training manual covering multiphoton confocal.

The multiphoton microscopes available for use are:

- Olympus FV1000 (Upright)
- Olympus FV1000 (Inverted)

The tutorial on multiphoton confocal involves

- Overview of multiphoton imaging
- Starting up of the system
- Alignment of the IR laser
- Safety precautions

Following (and during) the tutorial the user will be tested on the relevant points from above. If it is clear that the user has not read the relevant topics in the training manual the tutorial will be stopped until the user can demonstrate they have done so.

2.1.5 Image Analysis Software

In addition to the compulsory sections, the user is expected to have read the section of the training manual covering image analysis.

No test is required for gaining usage rights to image analysis software, a tutorial is compulsory however.

The software available for use is:

- MetaMorph (Version 7.7.4)
- Imaris (Version 7.0)
- Olympus FV1000 with 3D reconstruction
- ImageJ/Fiji
- Nikon EZ-C1

Tutorials on software will be tailored to suit the individual user's needs. Group tutorials on various aspects of image analysis (cell counting, size measurements, 3D modelling etc.) will be held on a regular basis for all staff to attend.

2.2   Refresher Tutorials

Refresher tutorials are available on request for any equipment within CAM. A refresher tutorial is strongly recommended if you have not used a certain piece of equipment for more than six months.

3.0   Access to CAM Equipment by External Users

External users are able to use CAM equipment if prior arrangements have been made with the CAM manager and the usage does not interfere with Ludwig staff usage. All external users are subject to the same tutorial and test system as Ludwig staff. External users are required to pay for access as per the "CAM external charge rates – Version 1.1" available on request.

All equipment must be booked using the CAM online booking system. It is the responsibility of each user to make sure their usage is accurately portrayed in the online booking calendar.

Any user that does not turn up for their booking within half an hour (without prior notice to other users) will forfeit their booking. Their booking may be taken up by another user on a first come, first served basis. If you are the last user for the day, ensure all equipment is turned off. If equipment is left running after the last user has finished usage will be charged until the time the equipment was turned off.

5.0   Enforcement of Policy

If a user is found to be in breach of this policy (for example; providing tutorials to unregistered users) they will be reminded of their obligation to adhere to CAM policy. Consistently ignoring the policy will result in suspension of access for a given period.

Introduction

Each microscope or software tutorial has a given set of notes the user is expected to have read before sitting down for the tutorial. Additionally there are compulsory tutorials that the user is expected to have read no matter what tutorial is being given. These compulsory tutorials cover basic concepts that are fundamental to all microscopy imaging and analysis.

Compulsory Reading

The following notes/chapters need to be read prior to commencing any tutorial

- Digital Imaging Basics
- Objective Basics
- Resolution Basics
- Nyquist Sampling
- Fluorescent Basics (If doing fluorescent imaging)

Widefield Imaging

The following notes/chapters need to be read prior to commencing a widefield microscope tutorial

- Widefield Imaging Techniques
- Köhler Illumination

Live Cell Imaging

The following notes/chapters need to be read if prior to commencing a widefield microscope tutorial

- Widefield Imaging Techniques
- Köhler Illumination

NOTE: To receive a confocal based live cell imaging tutorial the user must already be a registered user of the required confocal.

The following notes/chapters need to be read prior to commencing a confocal (single photon) tutorial

- Confocal Basics
- Laser Function and Safety

Confocal Imaging – Multiphoton

The following notes/chapters need to be read prior to commencing a confocal (multiphoton) tutorial

- Multiphoton Basics

Additionally a user will not be able to receive a multiphoton tutorial unless they are a registered single photon user

Image Analysis

The following notes/chapters need to be read prior to commencing an image analysis tutorial

- Image Analysis Basics

Digital Imaging Basics

Digital Imaging Basics

Introduction

All modern microscopy uses digital systems, be they cameras or other detectors, to record the analogue data produced by the microscope. There are limitations and pitfalls in digital imaging, but these can be avoided by following some simple rules.

Pixels

Digital images are made up of pixels; a pixel is the smallest representation of the colour and/or intensity information in a digital image. On a microscope each pixel will represent a known size/distance, this allows images to be accurately measured for size, area etc.

Since pixels are digital entities their values are governed by binary logic. At a basic level (1 bit depth) a pixel can be either on or off (black or white). Increasing the bit depth of a pixel gives it the ability to have shades of black and white by adding more combinations of on/offs for each pixel.

Intensity

The intensity, or gray value, represents how bright the pixel is within its given dynamic range. A intensity value of 0 represent black, a intensity value of the maximum of the dynamic range (e.g. 255 for an 8 bit image) represents white; all other values in between represent shades of gray.

Bit Depth (Dynamic Range)

The bit depth of an image governs how many shades of grey can be represented. The higher the bit depth, the more shades of grey are available and hence the greater the dynamic range. Increased dynamic ranges gives greater accuracy when measuring intensity values from an image. For example and 8 bit image has 255 possible shades of grey while a 16 bit image has 65,536 possible shades of grey. It would be much easier to pick up subtle differences in a 16 bit image compared to an 8 bit image.

The table below shows a range of common bit depths and their number of grey levels.

Bit Depth	Shades of Grey
8	255
12	4,096
14	16,384
16	65,536

The number of grey levels in an image is represented by the following formula

$$No. of\ Grey\ Levels = 2^{bit\ depth}$$

The image below shows examples of increasing bit depth

| 1 bit	2 bit	3 bit	4 bit
2 Grey Levels	4 Grey Levels	8 Grey Levels	16 Grey Levels
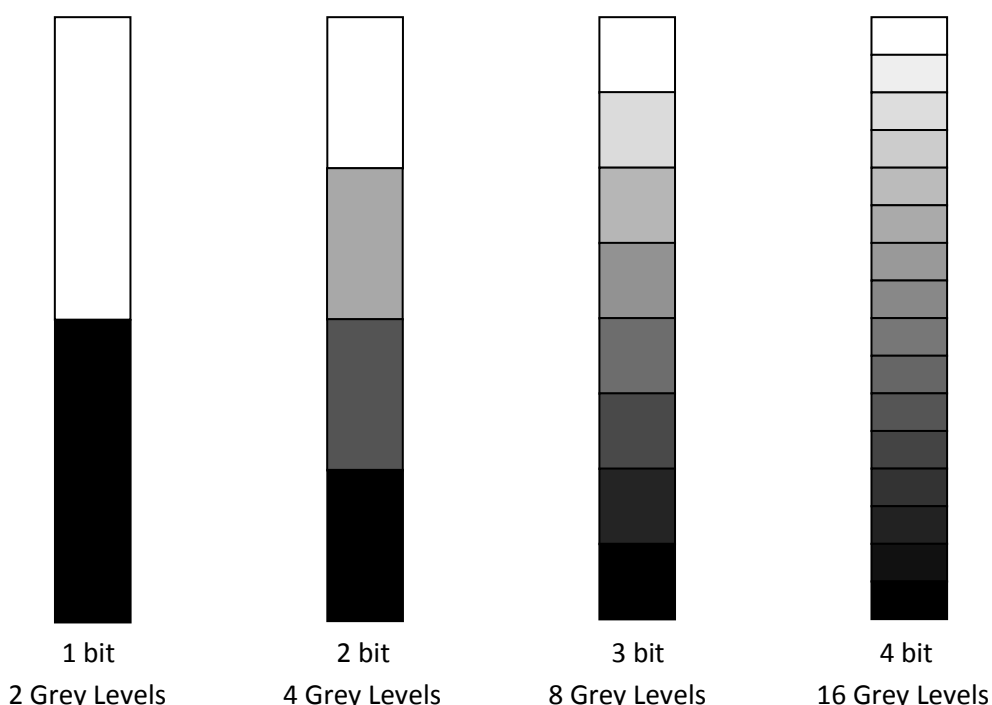
Colour Images

Colour images should only be captured when samples are stained with brightfield dyes (such as H and E, DAB, AEC, Geimsa etc). All fluorescent images should be captured in grey scale and false coloured later. False colouring means changing the look up table (LUT) of the image from one that represents shades of grey, to one that represents shades of the colour wanted. The reason for this is that monochrome cameras are more sensitive and therefore better at picking up fain fluorescent signals. While fluorescence is coloured the end image is a result of several filters that produce a specific emission (see page 28) so a colour camera is not needed.

Colour images, like monochrome images, are made up of shades of grey. The colour image is the result of adding together three monochrome images. These three images represent the intensity of

the three colours of the RGB colour space, namely Red, Green and Blue. Depending on the type of camera used the three colour images are taken through several different means (all essentially involve putting filters of the three colours in front of the camera and taking the respective image).

This is easier to understand if we deconstruct a colour image. Take a colour image and split it into its three component channels (blue, green and red).

Original Image

Blue Channel   Red Channel   Green Channel
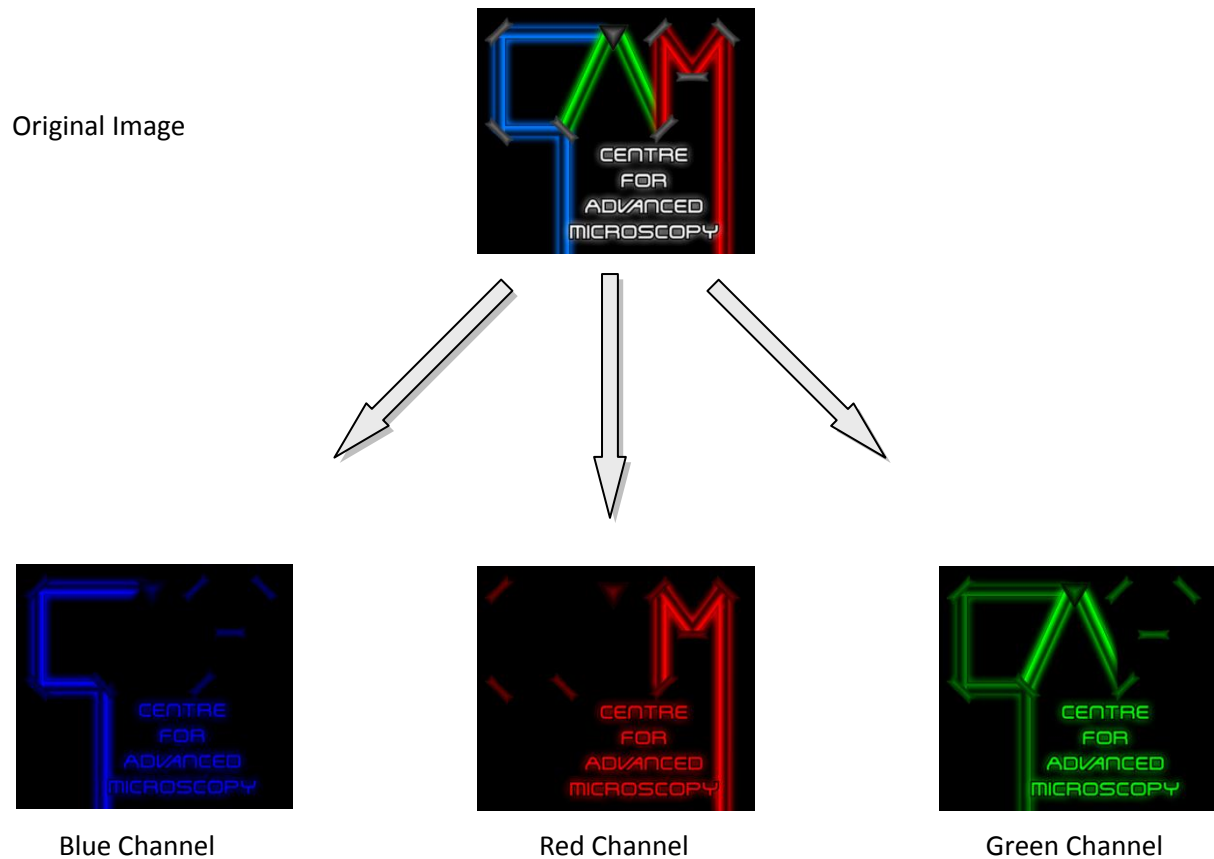
Make each of the channel images grey scale instead of blue, red or green scale. These grey scale images now represent intensities of the colour. The more white there is, the more of that colour will show in the combined image.

Blue Channel   Red Channel   Green Channel

By comparing the resulting intensity images to the original image it can be seen what is contributed by each channel. The letter M is only visible in the red channel as it is made up of only shades of red.

The letter C is visible in both the blue and the green channel because the colour of the C in the final image is an aqua colour (combination of blue and green). The letter C in the blue channel is brighter than the one in the green channel because the C in the original image is more blue than green. The words are equally bright in all channels because in the original image they are white (combination of all the colours).

Image Formats

If the equipment used has a proprietary format (e.g. OIF for Olympus Confocal, ICS for Nikon Confocal etc.) then the data should be saved in that format. This will keep the data in its original format with all notes about the imaging conditions used embedded in the file. If an image is required for analysis (or there is no proprietary format) then the image should be saved in Tagged Image Information Format (TIFF). This format will keep the image in its original form. Images should only be saved in other formats (JPEG, BMP, GIF etc.) if they will be used to email colleagues or present in a PowerPoint presentation. These formats compress the information in the image to make the file size smaller, in doing so they lose information from the image. Images can be compressed to save space using a compression scheme that does not lose any information (known as lossless compression). The effectiveness of lossless compression is dependent on the complexity of the image, more complex images tend to not compress as well, and in some cases can end up bigger than the original.

Objective Basics

Introduction

The objective of the microscope is key to obtaining the best quality image possible. Magnification plays a part in getting a good image, but other factors such as NA, mounting media and immersion media are also just as (and in some cases more) important.

Objective Anatomy

All objectives look very similar and carry a series of markings that tell the user the specific characteristics of the objective. The image below explains these markings.

Plan Apo – The correction that the objective has. In this case the objective is corrected for spherical aberration (Plan) and corrected for chromatic aberration (Apo).

VC – The objective has been violet corrected, chromatic correction for the UV wavelengths like that of DAPI.

100x - The magnification of the objective.

1.40 – The NA of the objective. NA governs the resolution (smallest object discernable) of the objective.

Oil – Immersion media. In this case oil, other objectives may use water or glycerol as well. Not all objectives require immersion media.

∞/0.17 – The objective is for an infinity corrected microscope system and is designed to work with a 0.17mm thick cover slip.

DIC N2 – Contrast optics. In this case the objective is suitable for DIC imaging and requires the N2 condenser prism. Objectives may have a Ph, followed by a number, on them designating it is a phase objective. Not all objectives are suitable for contrast imaging.

When light passes through glass its path is diverted or refracted. In objectives two different types of refraction can occur that will introduce aberrations to the resulting image, these are spherical aberration and chromatic aberration.

Spherical Aberration – Lenses, like those found in objectives, are usually a different thickness at the edge compared to the middle. When light is passed through these lenses it is refracted at different rates at the edge and the middle. The result of this is an image that is not evenly focused (e.g. the edges of the image are blurry when the centre is in focus). Spherical aberration can be corrected for and all modern research grade microscope objectives are usually corrected.
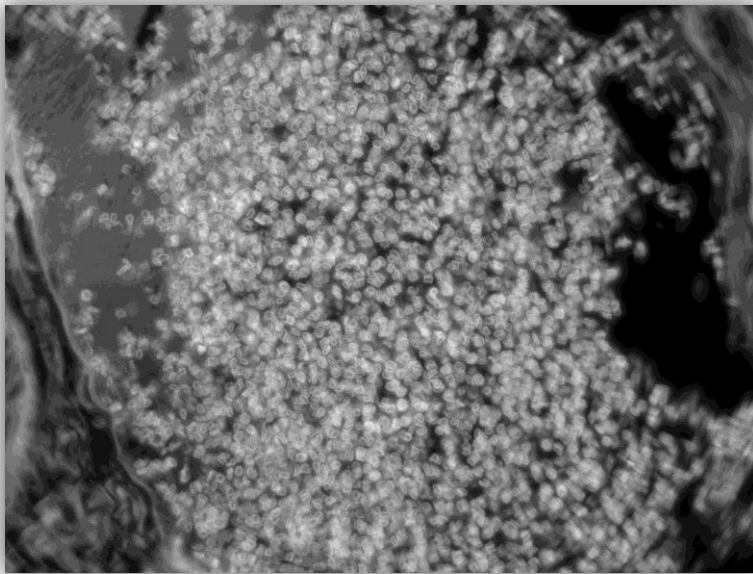
Spherical Aberration – Image collected using a non-spherically corrected 40x objective. Note the corners and edges of the image are out of focus and "streaky". This is the result of spherical aberration.
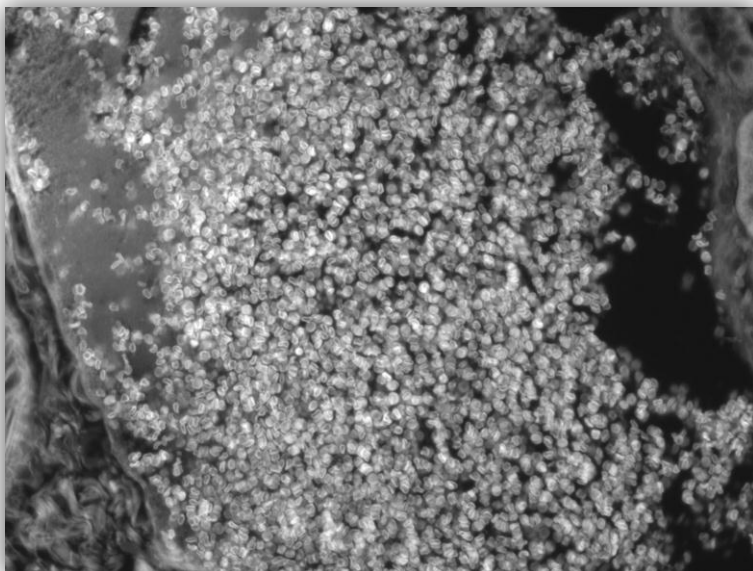
Corrected aberration – Image collected using a Plan objective that is corrected for spherical aberration. Note the whole field is evenly in focus with no blurring or "streaking".

Chromatic Aberration – Light of different wavelengths is refracted differently when passed through a lens. The result is that different wavelengths of light will have different focal planes. So while a blue image maybe in focus the red will not be. This can become a real problem when capturing images of fluorescently labelled samples, as the different stains (colour channels) may not line up with each other. Like spherical aberration this can be corrected and all high quality microscope objectives are corrected for 2 (blue and red), 3 (blue, green and red) or 4 (blue, green, red and far red).

Chromatic Aberration – Example image captured in XZ mode on an Olympus Confocal. Object is a multi-colour fluorescent bead. This image shows that the three colour channels (blue, green and red) do not align in the Z dimension. The objective used is corrected in the XY dimension but not in the Z dimension. **NOTE**: The distance between the blue and the green "bead" is approximately 10µm. This needs to be taken into account in any further interpretation or analysis of the image.

Magnification and NA

For a more detailed explanation of magnification and NA see the **Resolution Basics** training note.

Magnification will govern how much the image of the sample is enlarged and NA will govern the detail of that image. High magnification is useless without high NA to discriminate small objects.

High NA objectives (NA>1) all require immersion media of some sort. Immersion media helps bend the light from the sample at a greater angle, allowing a larger width light cone to enter the objective (see **Resolution Basics**). Water will bend light enough to get an NA of 1.2, glycerol will achieve 1.3 and oil will achieve 1.49 in the right circumstances (the highest NA available).

To achieve the best possible image it is important to match the immersion media to the mountant media of the sample. If the sample is in water (or PBS etc) use a water objective. If the sample is in glycerol or anti-fade reagent (usually glycerol based) use glycerol objective. If the sample is mounted in DPX or similar hard setting mountant use an oil objective.

If immersion and mounting media don't match the image will more than likely still look fine, but the best quality image (in terms of resolution, signal to noise ratio, depth penetration etc.) is achieved by matching them.

Correction Collars

Some objectives have collars on them that can be rotated to adjust the properties of the objective. Most collars allow the user to adjust for difference in coverslip thicknesses (see example on the left below). Others allow the use of different immersion media (see example on the right below). Adjusting the collar is a simple task of rotating it and aligning the desired position with the mark on the body of the objective.

NOTE: The default position for coverslip correction rings should be 0.17mm (or 1.5 on some scales). The correction should only be changed if you are sure the coverslip is different from 0.17mm thick.

The following figure shows the effect of adjusting the correction collar on the quality of the image. Note than when the correction collar is set around 0.1-0.2 (close to the thickness of a coverslip of 0.17mm) the image quality is markedly better than when the collar is set much higher.
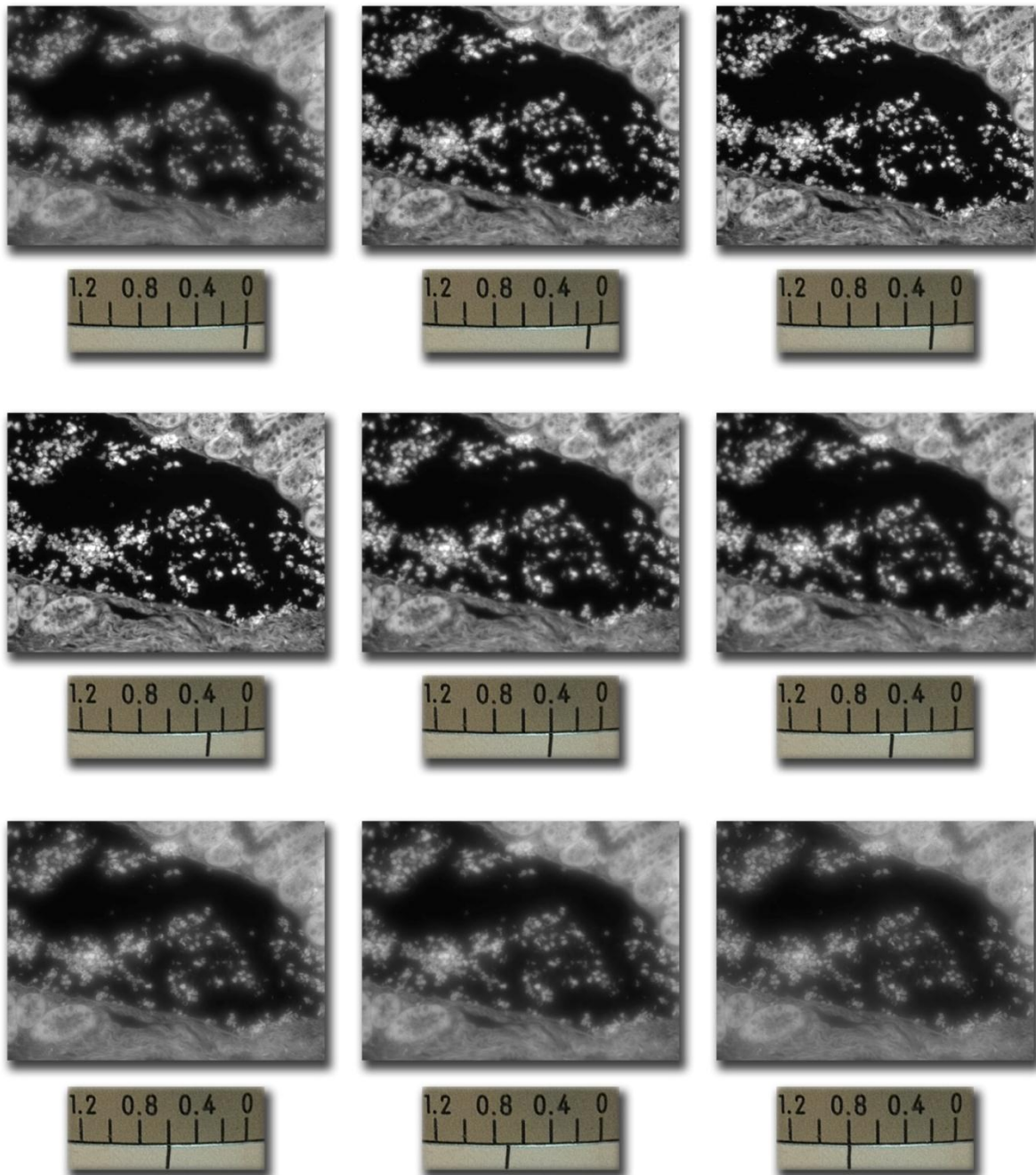
©Cameron J Nowell

Microscope objectives are a very expensive precision optical component, high quality, high NA objective can cost >$10,000. Care must be taken when installing or removing an objective from a microscope.

- Be careful not to touch any of the exposed lens surfaces
- Do not screw objectives in too tight, just loose finger tight is more than enough
- Always clean immersion media off of the objective when finished using 70% ethanol and a KimWipe or lens tissue. Do not use normal facial tissues as they will leave lint on the objective.

Introduction

Resolution is the ability of an objective to definitively resolve an object, or group of objects, as a single entity(s). Magnification plays no part in the ability of an objective to resolve an object of a given size. While magnification will make an image bigger, it will not necessarily make it clearer. Resolution is governed solely by the NA of the objective, the wavelength of the emitted light and for axial resolution, the refractive index of the sample.

For confocal microscopy NA is the most important factor since confocal microscopes have the ability to non-destructively zoom. In widefield microscopy magnification becomes more important as a widefield microscope does not have this function.

Magnification will affect how much light the objective can collect. Higher magnification objectives collect less light, so where possible use the highest NA, lowest magnification objective available.

Numerical Aperture (NA)

The Numerical Aperture (NA) of an objective is the sole discriminator of resolution. The higher an objectives NA, the greater its resolving power. NA is calculated using the following formula:

$$NA = \eta \sin\theta$$

Where η is the refractive index of the immersion medium (1.0 for air, 1.33 for water and 1.56 for oil) and θ is the half-angle of the maximum cone of light that can enter or exit the lens (see diagram below)
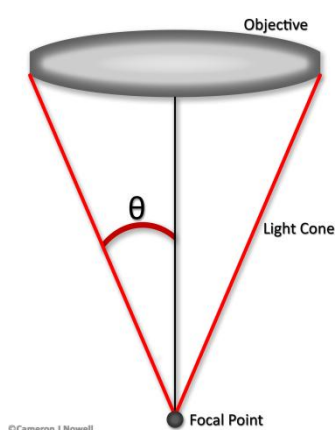
From looking at the above diagram it can be seen that as the objective gets closer to the focal point/sample (this usually happens as the magnification of the objective increases) the angle of θ will likewise increase. This will result in a higher NA value.

The ability of an objective lens to gather light is dependent on both its magnification and its NA. Objectives of the same NA will have decreased light gathering ability as magnification increases. The formula for calculating the light gathering ability (F) of an objective is:

$$F = 10,000 \times \left(\frac{NA^2}{Magnification}\right)^2$$

Where NA is the Numerical Aperture of the objective

The following graph shows a comparison between three objectives (40x, 60x and 100x) and the effect NA has on their light gathering ability.
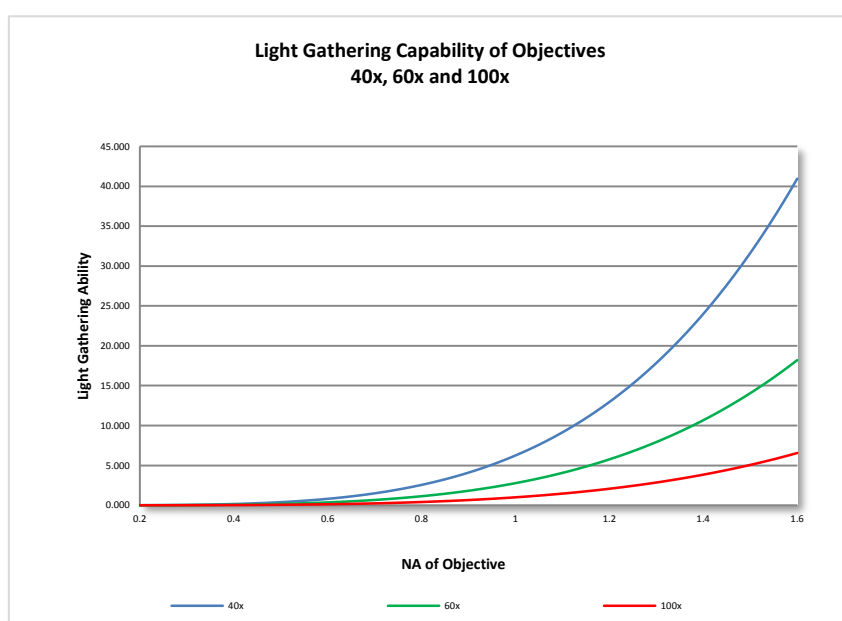
It can be clearly seen that the lowest magnification objective offers the best light gathering capability, while the highest offers the worst. This needs to considered when choosing between a 60x and a 100x objective for confocal imaging. 60x and 100x objectives have the same (or very similar) NA values, but have very different light gathering abilities. A 60x objective will be able to resolve the same detail as a 100x but will collect ~3 times as much light. This would mean less light would have to be applied to the sample to obtain an image, resulting in less photo-bleaching and photo-toxicity.

Lateral resolution is the resolution of an objective in a flat plane (XY dimension). It is determined by the following formulae.

$$r_\text{lateral(confocal)} = \frac{0.4\lambda}{NA} \qquad\qquad r_\text{lateral(widefield)} = \frac{0.61\lambda}{NA}$$

Where λ=wavelength of emission

The formulae above demonstrate that lateral resolution is better in confocal microscopy when compared to widefield microscopy. This is due to the removal of out of focus light from confocal data.

These formulae can be graphed to give an idea of the relationship between NA and resolution. The graphs below clearly demonstrate the increased resolution that comes with a higher NA objective.

Lateral Resolution and NA Relationship - Confocal Imaging

Lateral Resolution and NA Relationship - Widefiled Imaging
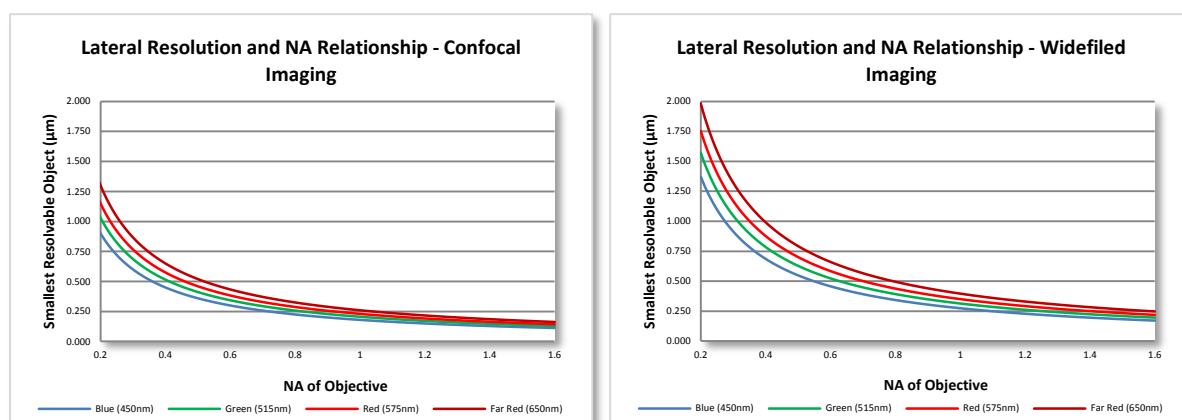
IMPORTANT NOTE: Confocal resolution is only improved over widefield if the pinhole is set to a small size. Any pinhole size bigger than 1 Airy unit will result in resolution equivalent to a widefield microscope. An Airy unit is the size of one Airy Disc, the size of the inner circle of the diffraction pattern of a point light source for that objective and wavelength.

Axial resolution is the resolution of an objective in depth (Z dimension). Objectives are not well suited for resolving objects in the Z dimension; as a result the best axial resolution is usually 2-3 times worse than the equivalent lateral resolution. Axial resolution is also dependant on the mounting media of the sample. This is due to the refractive index of the sample, so air mounted samples will have the lowest axial resolution, while DPX type mountants will give the highest axial resolution. Axial resolution is defined by the following formula:

$$r_{axial} = \frac{1.4\lambda}{\eta \cdot NA}$$

Where λ=wavelength of emission and η=refractive index of the sample

The following graphs demonstrate the relationship between NA, axial resolution and mounting media.
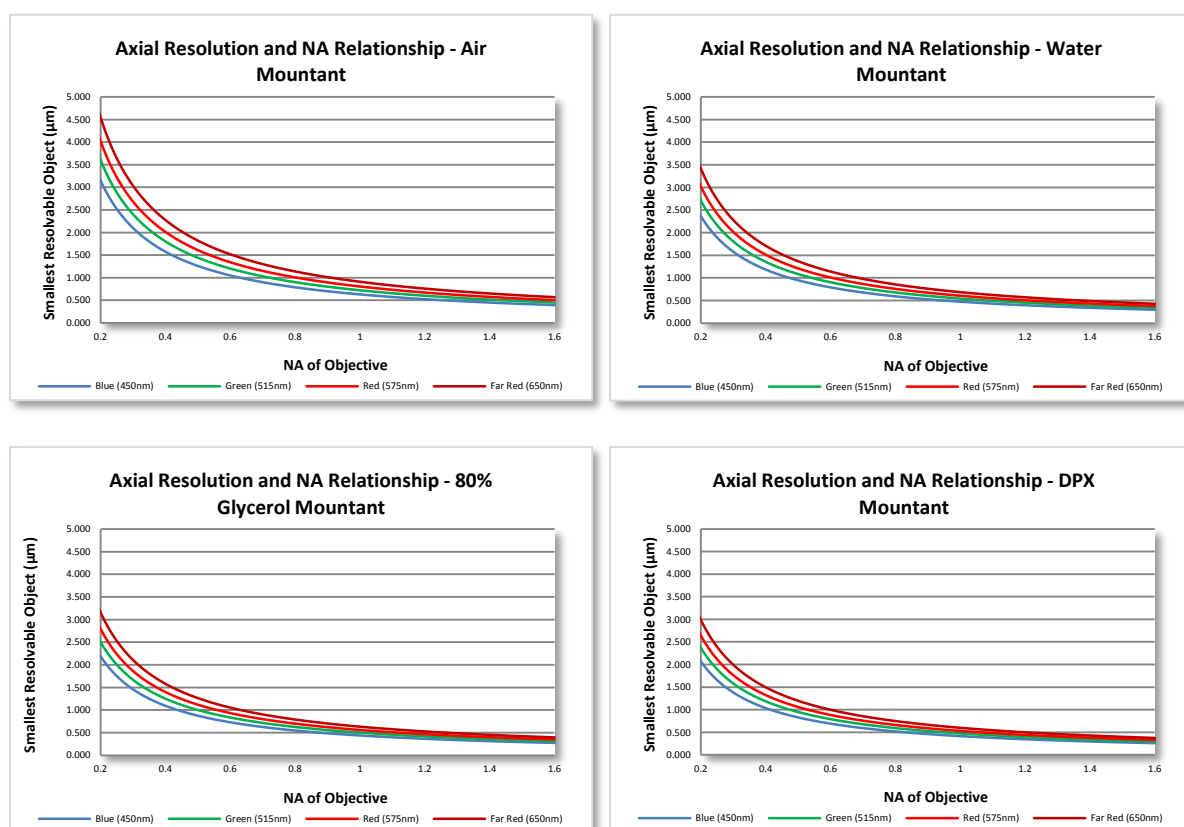
Nyquist Sampling

Introduction

When capturing digital images it is important that they are truly representative of what is being seen by the microscope. It is also important to make sure that what is being described from the images is actually true.

Each microscope objective has a minimum resolution, both laterally and axially. It is important when discriminating small structures that certain criteria are met to achieve an optimal and accurate image. This criterion is referred to as Nyquist Sampling Criteria or Nyquist Sampling Theorem (sometimes referred to as Nyquist-Shannon Sampling Theorem).

Nyquist Sampling dictates that to optimally represent an analogue signal in digital space, the analogue signal needs to be sampled 2.3 times. In microscopy terms this means that the pixel size of an image needs to be 2.3 times smaller than the object that is being resolved.

Why 2.3?

The math behind Nyquist Sampling is beyond the scope of this training note, but the end result is that 2.3 samples need to be taken of an analogue signal to accurately reproduce it. See http://en.wikipedia.org/wiki/Nyquist-Shannon_sampling_theorem for details on the math involved.

The figure on the next page shows an analogue sine wave that has been digitised at various sampling frequencies. The top single image shows the original wave form. The left column shows the original wave form (blue) and the digitised wave form (red). The right column shows the digital waveform converted back to analogue (digital to analogue conversion).

It can easily be seen that low sampling rates (0.47, 0.83 and 1.3 samples) result in sub standard reconstruction of the original wave form, with either no data reconstructed (0.47 samples), to some waves being replicated but others lost (0.83 samples) to a fairly good reproduction with reduced peak heights and widths (1.3 samples).

The wave form that has been sampled 2.3 times results in an accurate reconstruction of the waveform. Sampling at higher rates (10 samples) results in a nicer looking end result but does not add any extra resolution to the data.
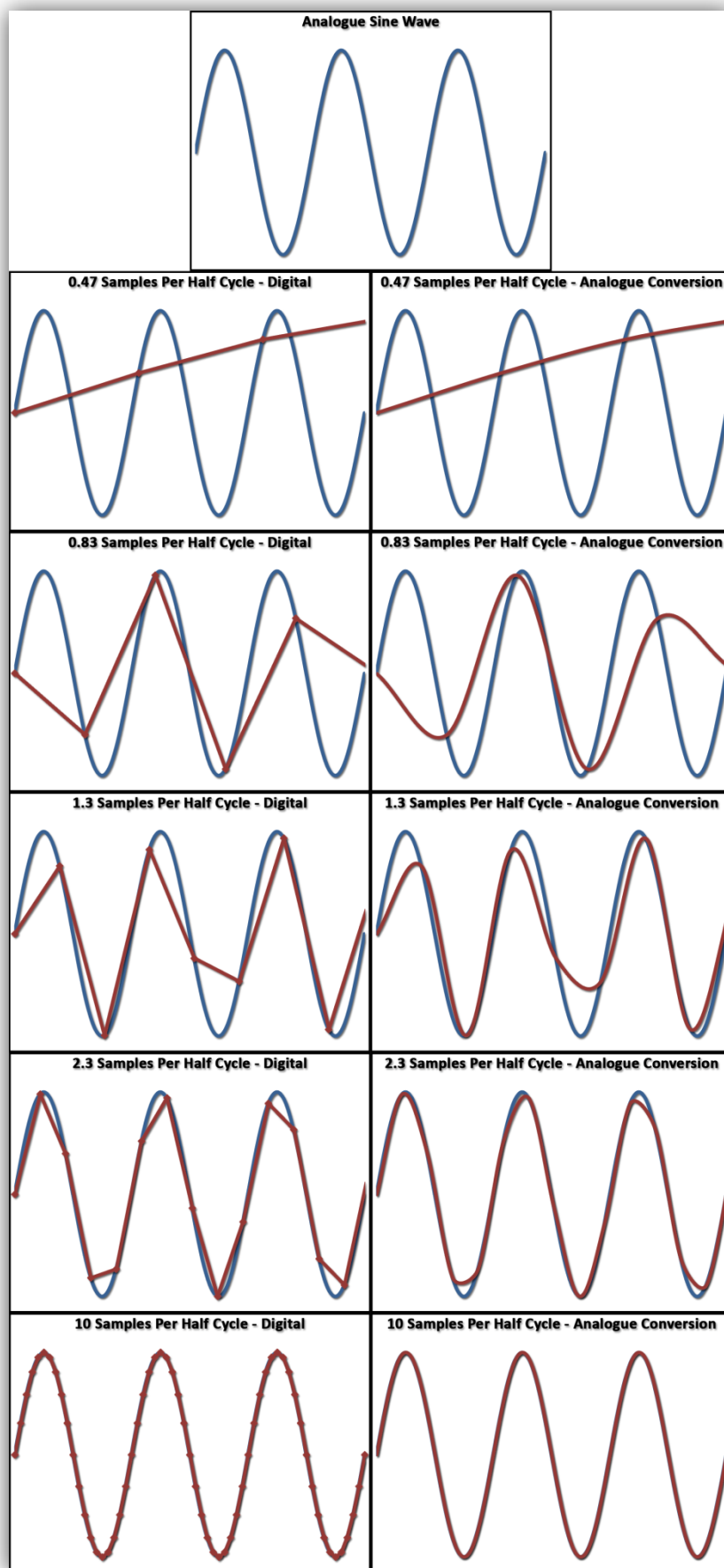
Pixels and Resolution

To optimally resolve an object it needs to be sampled correctly. As the previous example showed an object (or waveform) needs to be sampled 2.3 times to truly resolve it. A digital image is made up of pixels, each pixel representing a known size. So to resolve an object it must have 2.3 pixels in it or fall across it to be resolved. To use an example: if the object you are trying to resolve is mitochondria that is 1μm across, the size of the pixel will need to be 0.43μm (2.3 times smaller than 1μm). If the pixel is bigger than this resolution will not be optimal and there is no way of knowing that what looks like a single mitochondria is actually only one and not many.

Adjusting Pixel Size

On a wide field microscope equipped with a digital camera the pixel size is fixed for each objective and cannot be adjusted. The software used to take an image with the camera will show what the pixel size is, usually it is small enough to achieve the theoretical maximum resolution of the objective. It is important to make sure you are aware of the pixel size for widefield imaging. To use the example from above, if a certain camera/objective combination gave a pixel size of 0.6μm it would not be possible to resolve a 1μm mitochondria.

On a confocal microscope the pixel size can be adjusted. This is achieved by either scanning at a higher image resolution (e.g. 1024x1024 instead of 512x512), if there are more pixels in the image each one has to represent a smaller size. Adjusting the zoom will result in the pixel being smaller as well.

Nyquist and Z sections

When collecting images on a confocal microscope Nyquist Sampling needs to be taken into account for the Z step size (lateral resolution). For example a step size of 1μm will be able to resolve a 2.3μm object in the Z direction.

When capturing in the Z it is important to account for the decreased resolution that objectives have in the Z direction. For example if you want to capture an adherent cell (10 μm thick) at optimal resolution with a 60x NA1.4 objective you would need a step size of approximately 230nm. The optimum axial resolution of the objective is 525nm (mounted in air, with green emission) see page 23). So a step size of 230nm is 2.3 times smaller than the 525nm optimal resolution.

Nyquist and Time

Nyquist sampling should also be applied to time-lapse experiments. For example if it takes 10 minutes for a cell to move one cell width, then the images should be captured every 4.34 minutes (2.3 times shorter than 10 minutes.)

While a structure , be it whole cell, nucleus or mitochondria, may be resolved correctly according to Nyquist Sampling, the resulting image may not be "pretty". To be able to resolve a cell, it technically only needs 2.3 pixels to fall across it, but the resulting image would not look very good. To generate a "pretty" image of the cell there would be many pixels (50-200) pixels falling across it. This well exceeds Nyquist criteria but generates a much nicer image.

The example below shows several images of a smiley face. In the first image it can be seen (or resolved) that it is a face, but it isn't a very clear or "pretty" picture. The second image adds more detail, a smile can be seen. The third and fourth images add more detail again (freckles). So while the first image resolved the face, the image that is more likely to be captured is the third or fourth image.
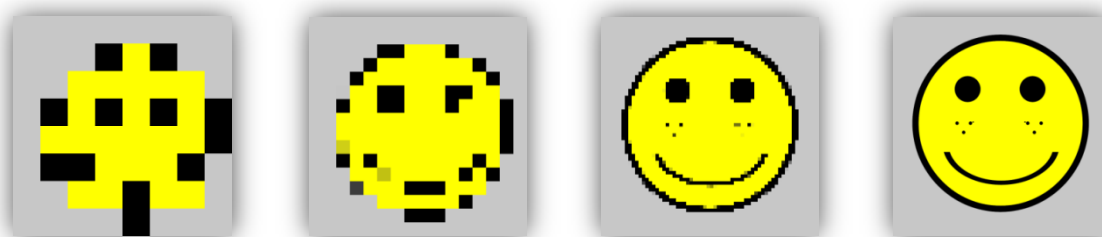
It is worth noting that in the example above the freckles only became visible with a smaller pixel size. So if you were looking for freckles and had the pixel size set as per the first or second image the conclusion could be that there are no freckles present. But they are present but were just not being resolved with the current settings.

Noise

Having noise (background staining, vibration etc) in the image will decrease the resolution. Below are the same four images as above but with noise added to them, notice how much less information can be resolved from the same settings.
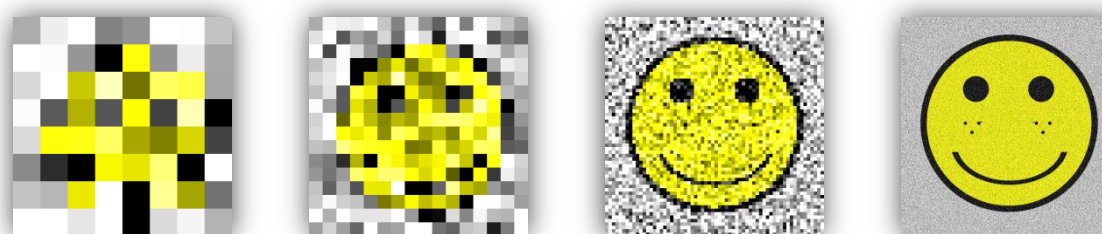
So while you may set the microscope to be able to resolve the structures of interest, it may not be possible with the sample you have due to confounding factors such as background staining or other types of noise.

Fluorescence Basics

Introduction

Fluorescent proteins and dyes provide a powerful toolkit to biological researchers. Fluorescent proteins such as green fluorescent protein (GFP), yellow fluorescent protein (YFP) and red fluorescent protein (RFP) can be tagged to proteins of interest with a cell or organism. This then allows the visualisation and tracking of these proteins in a fixed (dead) or live context. In addition to fluorescent proteins a large range of fluorescent dyes are available to specifically mark cell types, organelles etc. The range of available fluorescent proteins and dyes is growing every year.

Whether a fluorophore is a fluorescent protein or dye the way it produces its fluorescence is the same.

Basic Physics

The fluorescence produced by a fluorophore is a result of electrons within the fluorophore being raised to a higher energy state by the excitation wavelength. When the electrons fall back to a lower energy state fluorescence of a specific emission wavelength is emitted.

©Cameron J Nowell
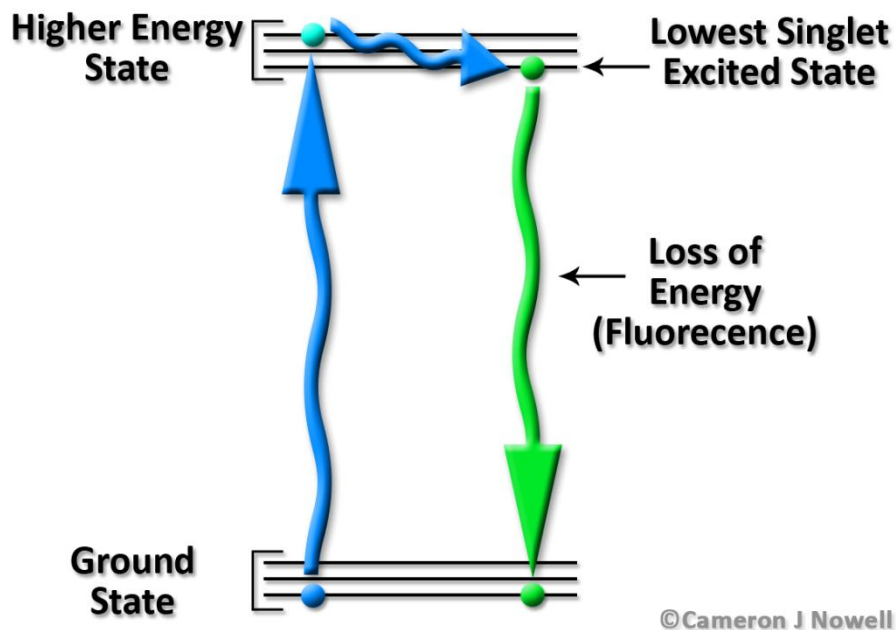
The light emitted (fluoresced) will always be of a longer wavelength than that used for excitation (but still specific). This difference in wavelength is known as the Stokes' Shift. Below is an example fluorescent spectrum for eGFP. Notice the two distinct peaks; the difference between them represents the Stokes' Shift.
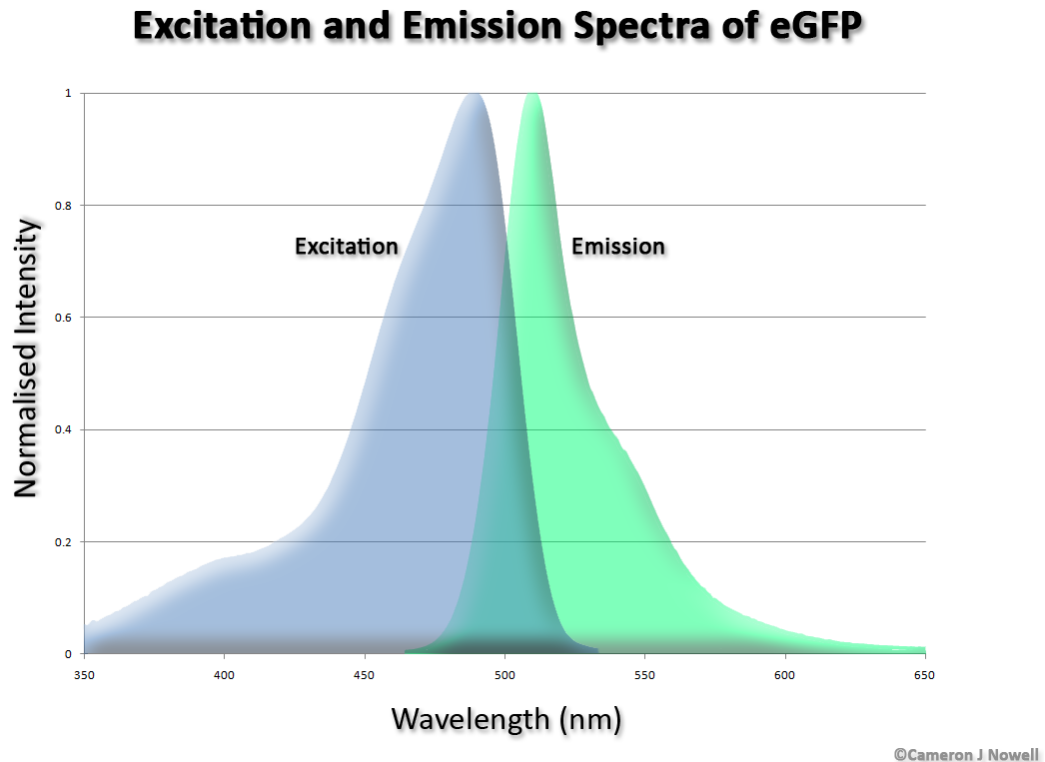

Excitation and Emission

All fluorophores have a maximum excitation and emission wavelength. The maximum excitation wavelength is the wavelengths that will result in the maximum emission of fluorescence. Excitation at other wavelength may result in fluorescence but it will not be optimal. To achieve optimal excitation and emission, filters made from coated glass are used to provide the specific wavelengths required.

Light for excitation is provided from either a mercury or xenon arc lamp (in confocal microscopy a laser of the excitation maximum is used). This light is passed through the microscope to a cube containing three filters; an excitation filter, a dichroic mirror and an emission filter. The following figures show the light path through a filter cube.
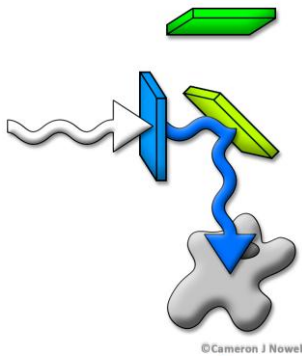
Light from the light source (white arrow) is passed through the excitation filter (blue). The excitation filter only allows light of the specific wavelength of excitation through, in this example blue light. The excitation light is then reflected off of a dichroic mirror (yellow). Dichroic mirrors will reflect some wavelengths of light while allowing others to pass through. The reflected light is passed through the objective of the microscope to the sample
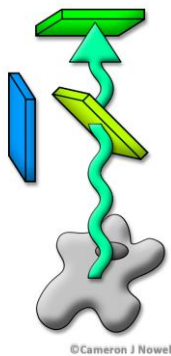

The light fluoresced from the sample passes back through the objective and through the dichroic mirror.
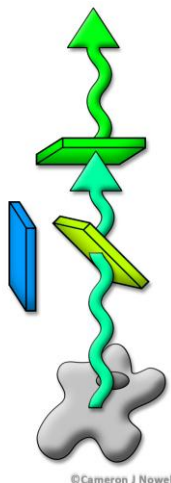
Finally the light passes through the emission filter (green), where it is "cleaned up". The final light is specific for the emission wavelength of the fluorophore and can then be passed through to the ocular of the microscope or another type of detector (e.g. a camera)

These filters can be overlayed onto the previous eGFP spectra, as shown below. Only part of the spectrum is highlighted, the rest is rejected by the filters. This allows for precise illumination and detection of fluorescent molecules. Filter sets exist that can detect two or more fluorophores at the same time. These filter sets still contain the same three components (emission, dichroic and excitation filters) but these filters are created to let multiple bands of light pass through.

Excitation and Emission Spectra of eGFP

©Cameron J Nowell
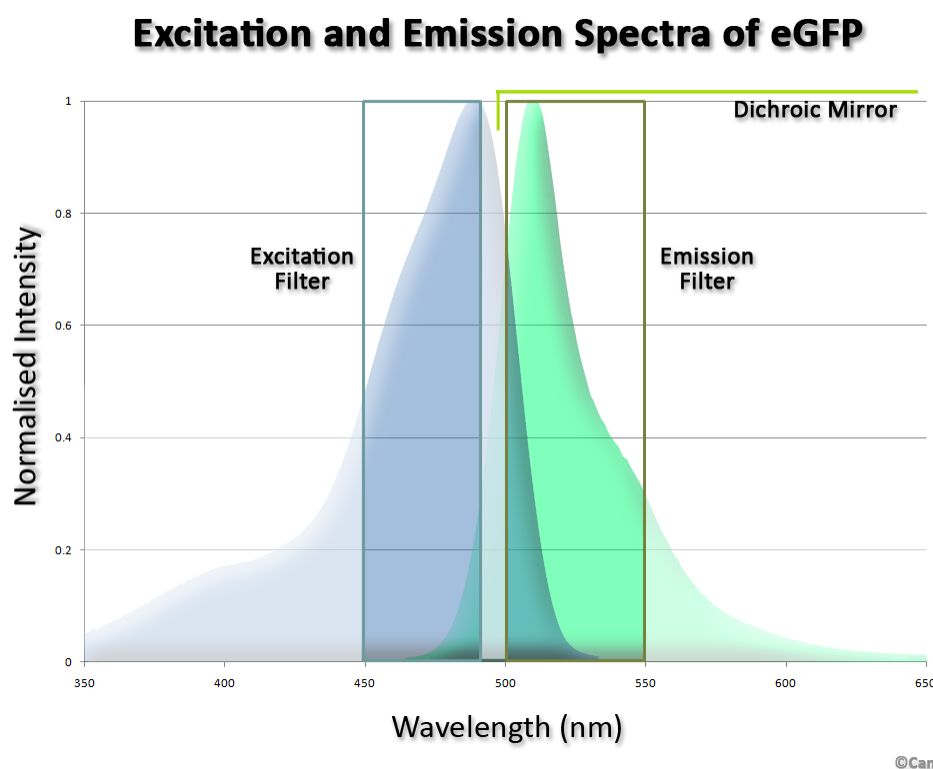
Bleaching

All fluorophores will eventually bleach, this is the result of an irreversible photochemical reaction that destroys the fluorophore and renders it unable to be re-excited. Some fluorophores (such as FITC) will bleach very rapidly while others (such as Alexa dyes) will be more resistant. To limit bleaching of the sample anti-fade reagents can be added to the mounting media (such as Prolong, CitiFluor etc).

To minimise bleaching the length of time the excitation light is shone on the sample should be kept as short as possible. Using high transmittance objectives and sensitive detection systems will help minimise bleaching.

Phototoxicity

When imaging live cells or tissue phototoxicity can become an issue. Free radicals are produced during the fluorescence process; these reactive oxygen species can affect cell viability or kill the cell. The effects of phototoxicity can be reduced, or eliminated, by reducing the amount of fluorescent

light applied to the cells or tissue. Additionally free radical scavengers, such as ascorbic acid or DTT, can be added to the culture media to reduce phototoxic effects.

Introduction

Widefield microscopy provides the user with a powerful array of imaging techniques and instruments. Macro level imaging can be carried out using a stereo microscope using a range of illumination options. Higher magnification imaging can be carried out using a compound microscope (either an upright or inverted), once again with a range of illumination options.

The following sections detail what imaging options are available and which microscopes are best suited to them.

Stereo Microscope – Brightfield

Brightfield images (non-fluorescent) can be captured in three different ways on a stereo microscope. Magnification on a stereo microscope is limited to around 10x.
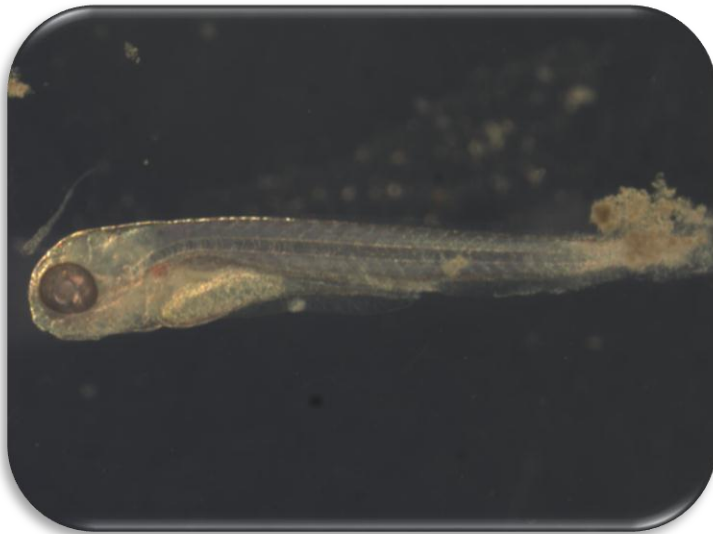
Reflected Light – The most common illumination technique used for stereo microscopes. Light is shone from above the sample, usually using a fibre optic light source. The detail of the surface can be seen, but internal details of transparent specimens may be lost.

Transmitted Light – Light is shone through the sample from below. Only works with transparent or opaque samples. Allows visualisation of internal structures.
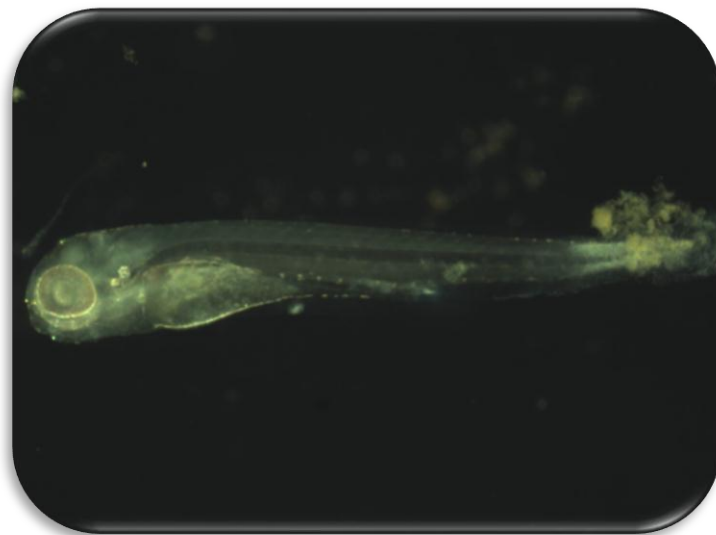
Darkfield Illumination – Light is shone past the specimen from underneath, but it is mostly outside the field of view of the objective. The result is that the sample is illuminated from the side. The resulting image is something like a negative transmitted image, but may show structures that are not visible under transmitted light.

Stereo Microscope – Fluorescence

Fluorescent proteins and dyes can be imaged with a stereo microscope. Due to the low magnification weak signals may not be easily visualised
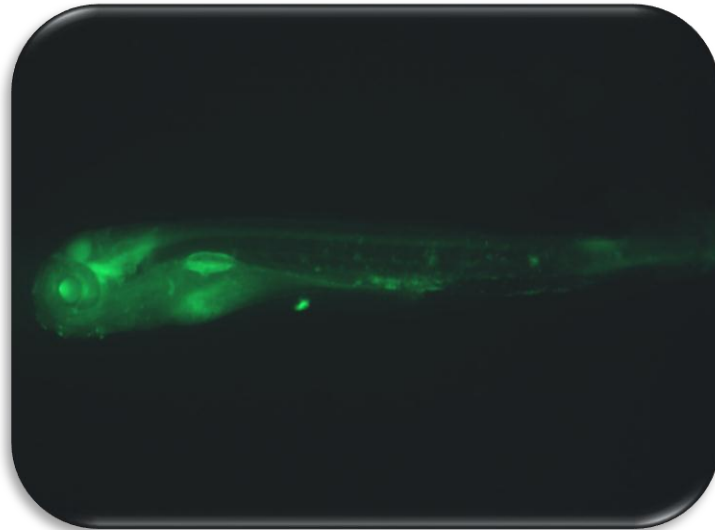
Compound Microscope – Brightfield

All brightfield images taken with a compound microscope are based around transmitted light (light is shone through the sample to the objective). The magnification of the compound microscope allows for more detailed analysis of the sample. Maximum magnification is around 1000x

Stained Samples – Samples that have been stained with dyes or chromagens can be visualised using either an upright or inverted compound microscope. Since most of these samples are mounted on glass slides with a coverslip, an upright microscope is usually the better choice.
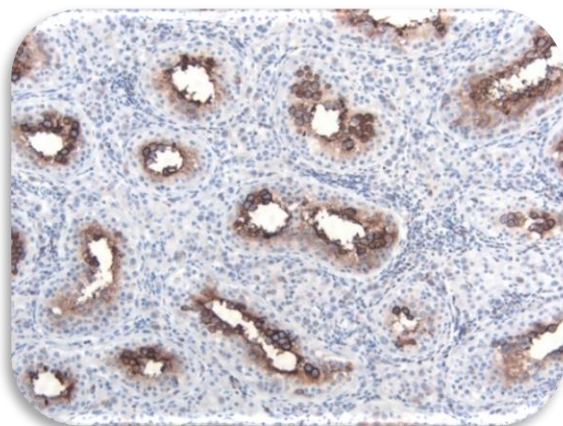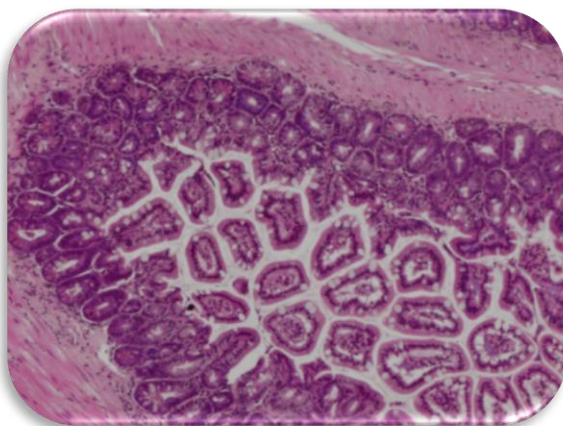
Unstained Samples – If a sample that has no stain is imaged under normal transmitted light conditions it will usually not be visible as there is not enough contrast (see example below).
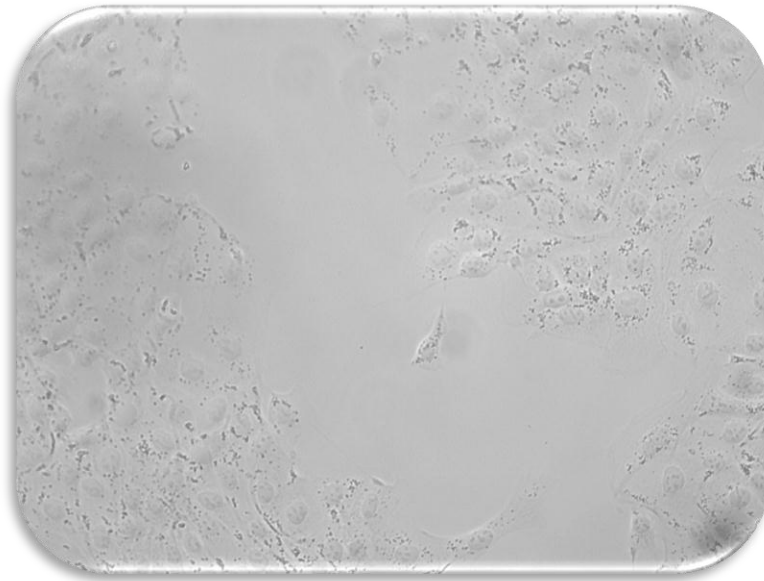
Samples that have no stain in them, such as cultured cells, can be imaged using one of two illumination methods that add contrast.
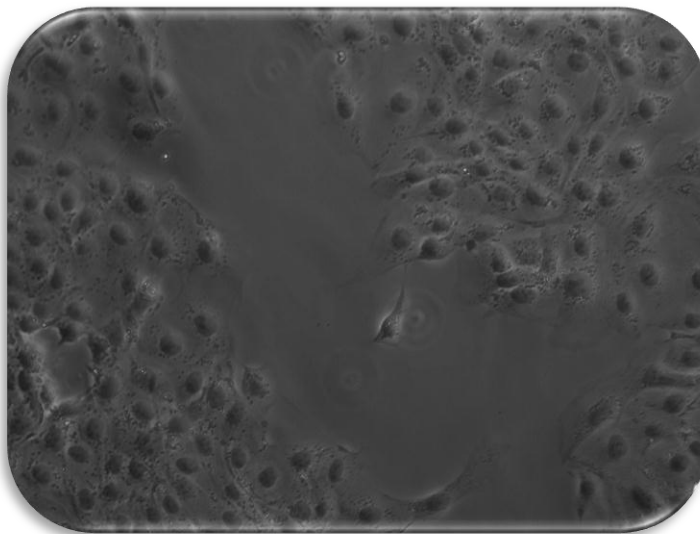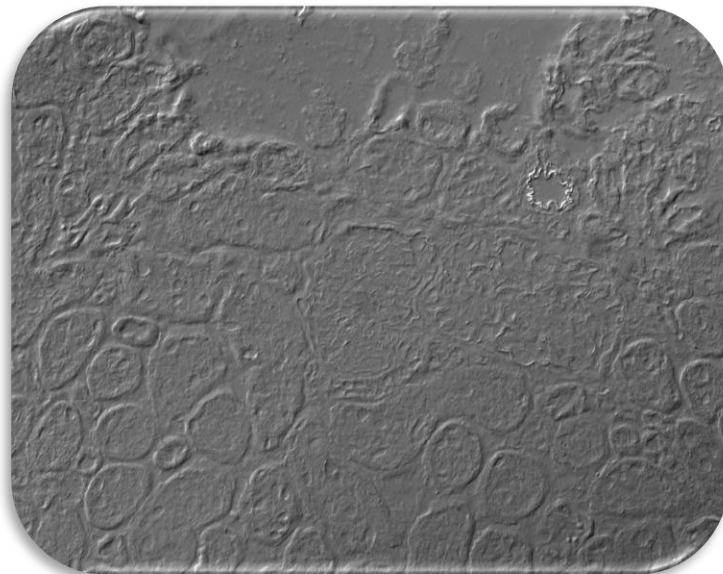

Phase Contrast – Light that illuminates the sample is shone through a small ring in the microscope condenser. This ring of light aligns with an opaque ring inside the objective. This induces a phase shift in the amplitude of the light in the sample, resulting in a contrast type image being generated. Phase contrast imaging works well for cells in plastic tissue culture flasks or plates.
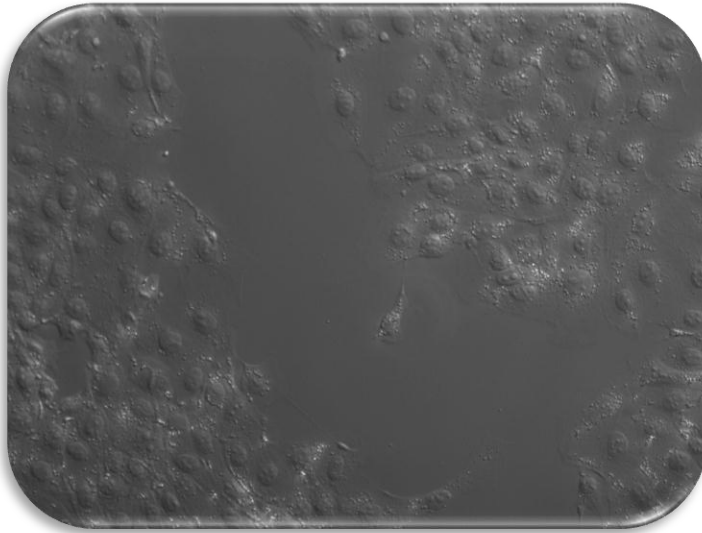
Differential Interference Contrast (DIC) – Also known as Nomarski Interference Contrast relies on two beams of polarised light passing through a sample and interfering with each other. This interference results in a contrast in the sample that has a 3D look to it. DIC only works on samples mounted on glass slides or coverslips. It will not work through plastic culture dishes as the plastic interferes with the polarisation effect.

Generally phase contrast and DIC imaging are carried out on an inverted microscope, though they can both be used on upright microscopes to great effect. The image below shows a DIC image of a tissue section of kidney captured on an upright microscope.

Samples stained with fluorescent dyes, antibodies or expressing fluorescent proteins can be imaged using either an upright or inverted microscope. Upright microscopes are better suited for imaging thin tissue slices mounted on glass slides with a coverslip (left image). Inverted microscopes are better suited for imaging cultured cells or thick tissues (right image).
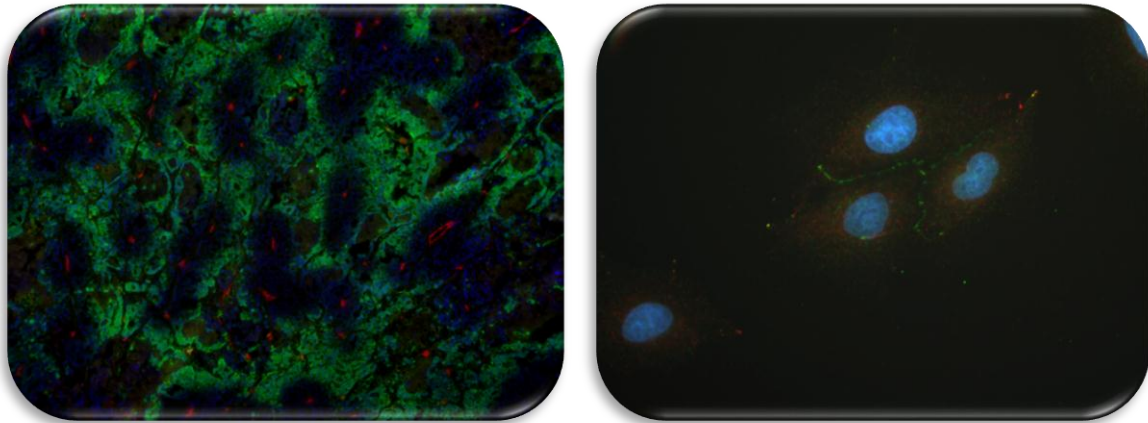
Brightfield and Fluorescence Combined

Brightfield and fluorescent images can be combined to provide more information than each individual image may have provided. The two images below show fluorescent stains overlayed with a DIC image of the sample. The DIC image provides some context as to where the stain is in the sample without the need of another stain.
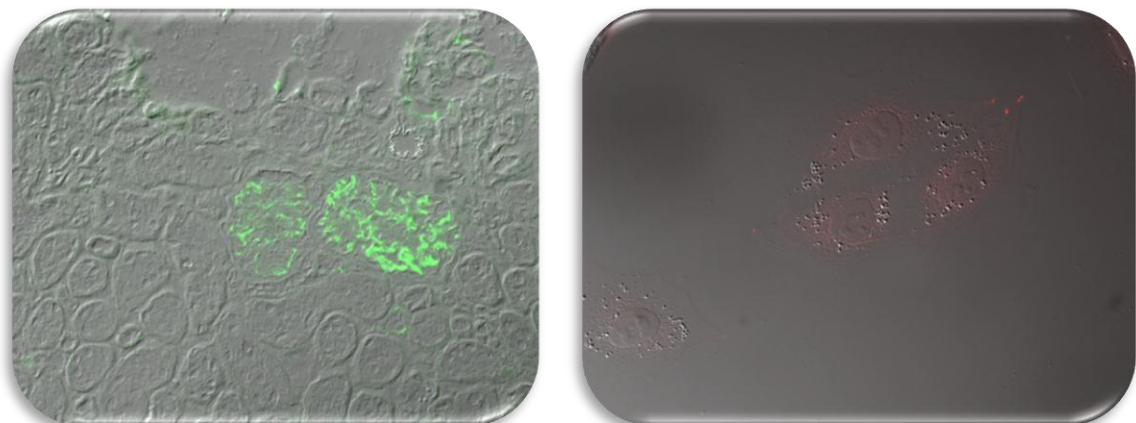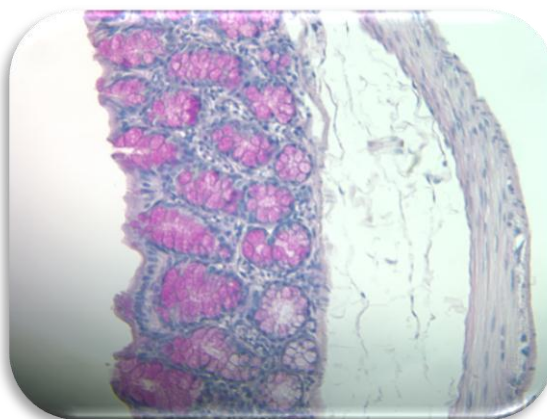
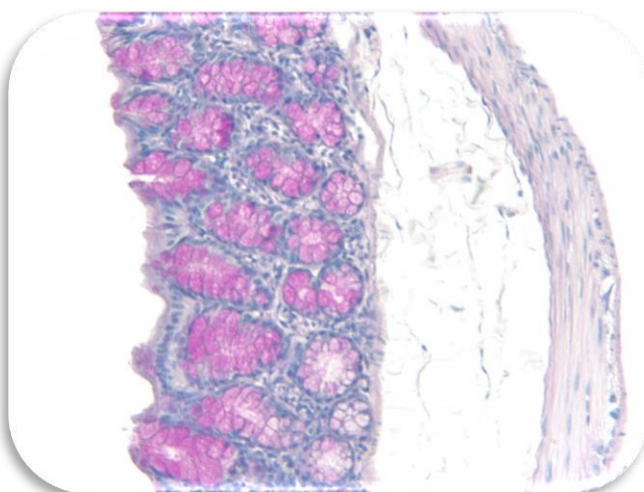# Köhler Illumination

Introduction

When capturing images using transmitted light on a brightfield microscope, it is important to have the illumination system aligned correctly. This system of alignment in known as Köhler Illumination and is essential for obtaining the best quality brightfield image.

Why is it Necessary?

The images below show examples of what happens when the microscope is not correctly setup for Köhler Illumination. The image on the left has the condenser out of focus; the hair and specs that can be seen are not on the sample but are on the condenser. The image on the right has the condenser out of focus and off centre, this creates an uneven illumination that can be easily seen by the grey shading in the bottom left of the image.

Correct alignment of the illumination system will give an image that is even and crisp, as in the example below.
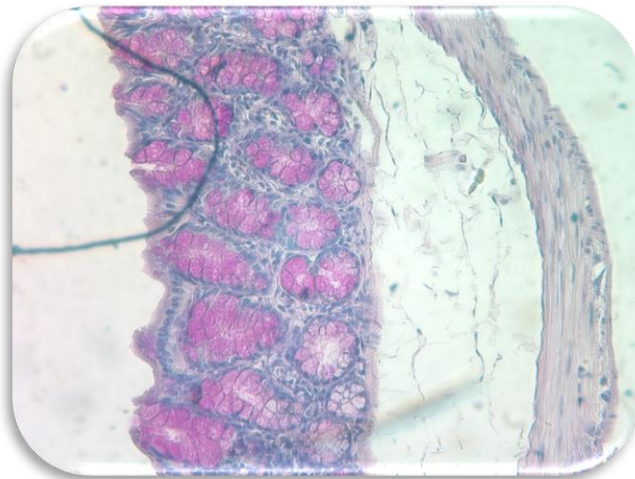
These instruction show how to setup an upright and an inverted microscope for Köhler Illumination. The microscopes used in these examples are a Nikon 90i and a Nikon TE2000-E, but the principles hold true on every microscope, the controls just may be in slightly different places.

Step 1
Focus on the sample

Step 2
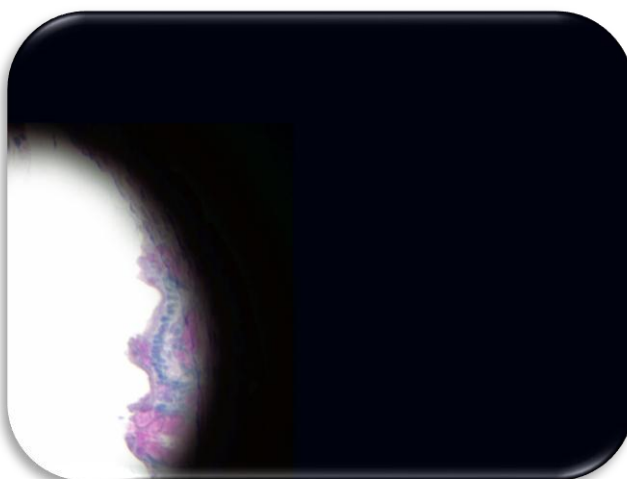Close the field iris (see below) so a ring can be seen.

Nikon 90i Field Iris Control   Nikon TE2000-E Field Iris Control

The ring may be out of focus and off centre.

Step 3

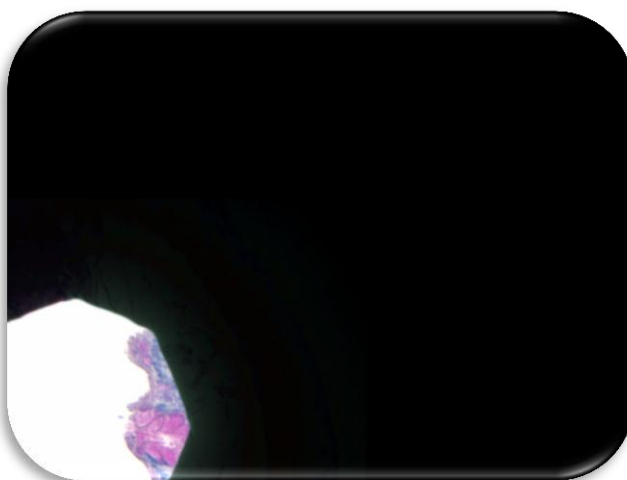Adjust the condenser focus to make the ring sharp.

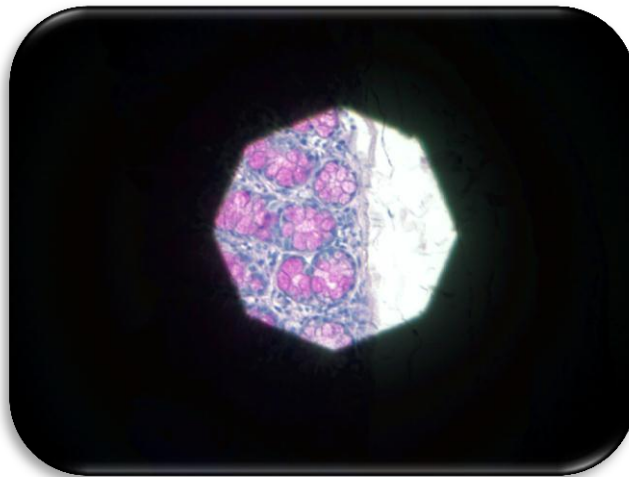Nikon 90i Condenser Focus   Nikon TE2000-E Condenser Focus

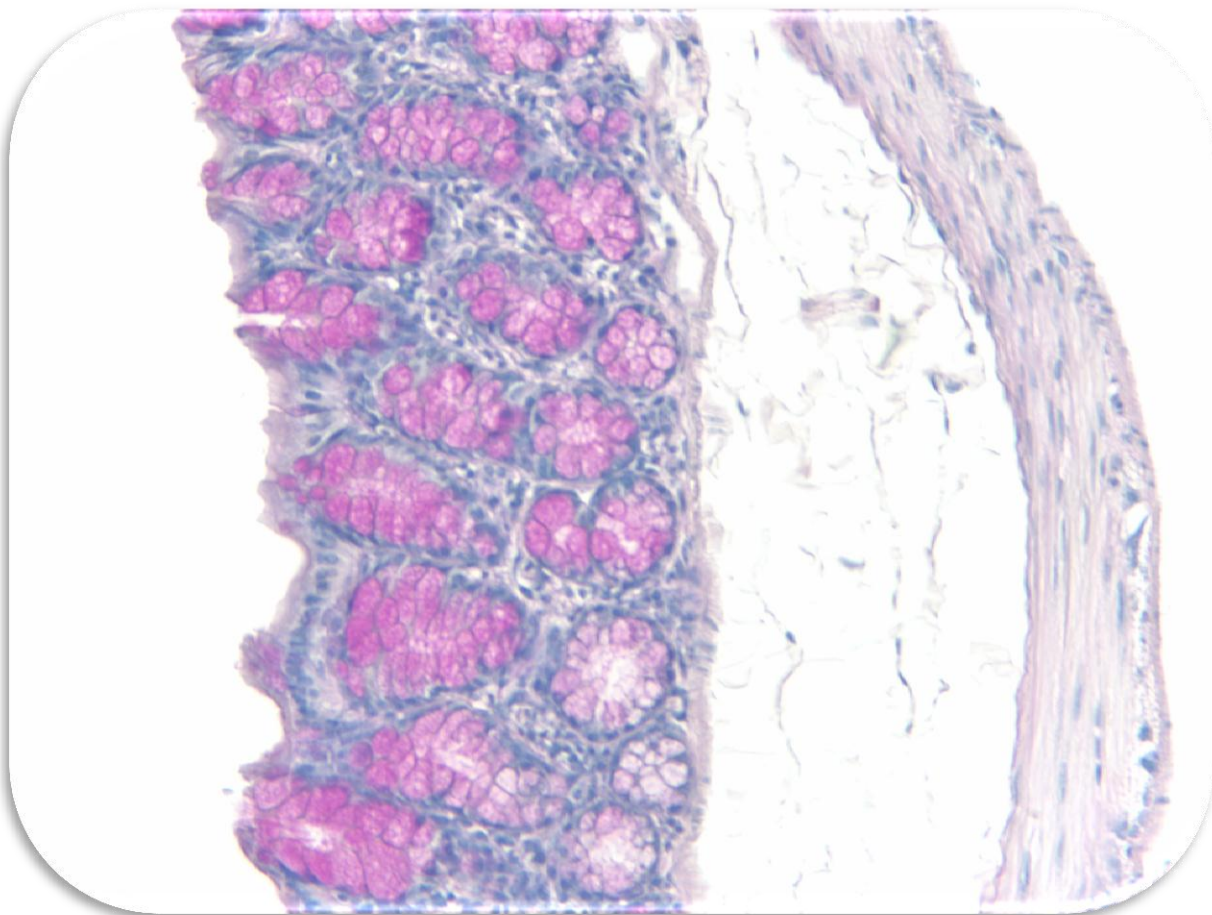When in focus the fuzzy ring should become a sharp octagon.

Step 4

Centre the image of the iris using the condenser centring screws. **NOTE:** The Nikon 90i should not need to be centred by the user.

Nikon TE2000-E Condenser Centring Controls

Step 5

Use the field iris control to open up the iris so that the iris is just outside the field of view.

The microscope is now properly aligned and ready for imaging.

Confocal Basics

Introduction

Confocal microscopy is a powerful tool for taking optical slices though a fluorescently stained sample. Samples are still stained with fluorescent probes (antibodies, fusion proteins or dyes) and are visualised using excitation and emission filter systems. It works on the same principles as standard widefield fluorescence microscopy with three major differences. The first difference is the excitation source, in widefield fluorescent microscopy a mercury or xenon arc lamp is used, for confocal a laser of the specific excitation wavelength required is used. The second difference is that the fluorescent image is collected through a small hole (called the pinhole) that allows only in focus light to pass, resulting in a thin slice of the whole sample being captured. Thirdly the detector is not a camera but a photon multiplier tube. The sample (or the objective) can be moved up and/or down to move the focal plan, resulting in different image slices being generated.

Limitations

Confocal microscopy is a powerful tool for imaging; however there are limitations to what it can achieve. While confocal microscopy can image thick specimens, it is not going to be able to image a whole leg. The depth limit of single photon confocal imaging in normal tissue samples is about 150µm. This depth can be drastically reduced, or increased, by many factors; mounting media, fluorophores used, objective used, laser power, sample type and coverslip thickness to name a few.

Resolution is improved on a confocal microscope compared to a widefield microscope, for example a 1.4NA objective imaging GFP will resolve ~180nm in confocal but only ~220nm in widefield. This is only a small increase and means objects can still be 180nm apart and look like one object. As a result "co-localisation" analysis must always be taken with a grain of salt and not be the only basis for a conclusion.

Confocal microscopy with standard PMT detectors is not highly sensitive. The general rule of thumb is, if you can't see it down the ocular the confocal will struggle to see it as well.

Fluorophores can easily be bleached under confocal scanning. Zooming in or increasing the laser power to high levels to detect a signal will greatly increase the chance of bleaching a fluorophore. Also remember that a confocal captures a series of slices, so while the fluorescence maybe fine at the start of acquisition, by the end of the Z stack it may all be gone.

When a fluorescent sample is excited, excitation and emission does not occur purely at the focal point. Fluorophores above and below the focal point will be excited and emit fluorescence, though not as efficiently as those in the focal plane. When viewed with a widefield microscope these samples look blurred or fuzzy, confocal microscopy removes this by only allowing detection of in focus light.
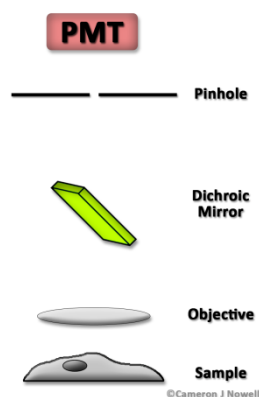

Basic System Layout – The confocal microscope is essentially the same as a widefield fluorescence microscope. Excitation light is passed to the sample through an excitation filter, is reflected of a dichroic mirror, passed back through the mirror, an emission filter and detected. Excitation is provided by a laser of a specified wavelength and detection is carried out by a photon multiplier tube (PMT). A small hole (called the pinhole) is placed in the emission light path to remove out of focus light
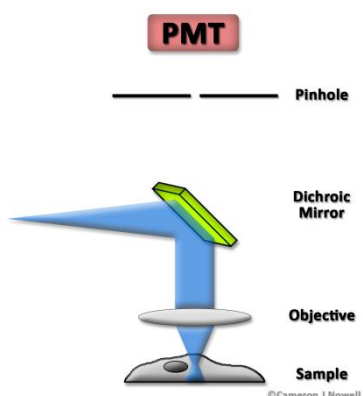
Excitation – Excitation is provided by a laser of a specified wavelength. The laser is passed through an excitation filter (not shown) and reflected off a dichroic mirror. The light is then passed through the objective to the sample. The excitation light is focused by the objective, but not perfectly. Some out of focus light is applied to the sample as well; this can result in excitation of fluorophores outside of the focal plane.
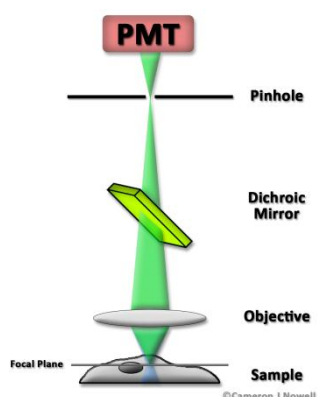

In Focus Light – Light that is from the plane of focus passes through the dichroic mirror, an emission filter (not shown) and finally the pinhole. Only light that is from the focal plane can pass through the pinhole and thus through to the detector. The resulting image shows only in focus light and is therefore a thin slice of the whole sample. Increasing the size of the pinhole results in more light from outside the focal plane being detected, giving a thicker slice. If the pinhole is fully opened the resulting image is essentially the same as that captured with a widefield microscope.
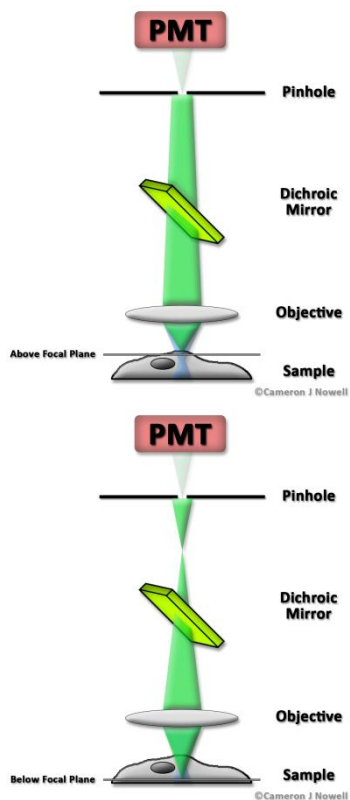
Out of Focus Light – Light that is from outside the plane of focus (either above or below) cannot focus through the pinhole and is therefore not passed through to the detector
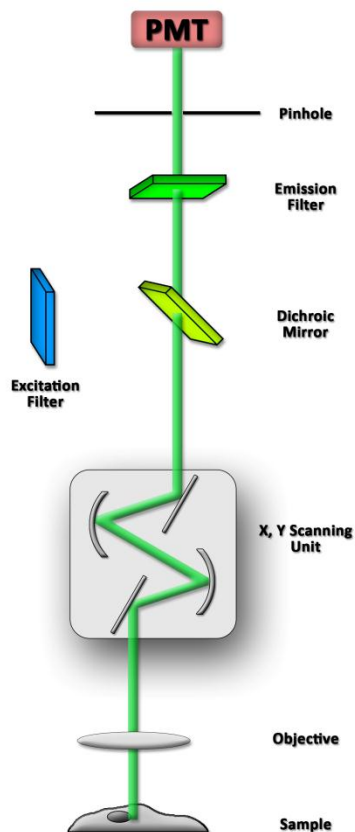
Image Generation

To generate an image the excitation laser is scanned across the sample in a Raster pattern (Raster scanning is the generation of an image one line at a time). The laser sweeps across the sample, is turned off, returned to the starting side of the scan, moved down a set distance and scanned across again. This pattern is repeated until an image of the required size is generated (usually 512 lines).
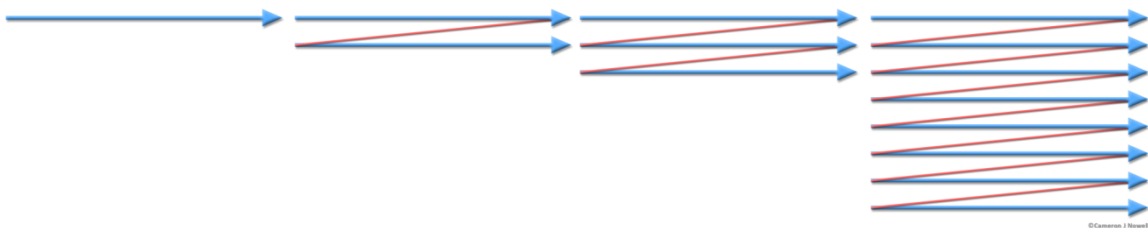
The laser is scanned by an XY scan unit consisting of a pair of galvo mirrors. The galvo mirrors are moved in a precise fashion, deflecting the beam off another set of mirrors allowing the beam point to be moved either horizontally (X) or vertically (Y)

PMT

Pinhole

Emission
Filter

Dichroic
Mirror

Excitation
Filter

X, Y Scanning
Unit
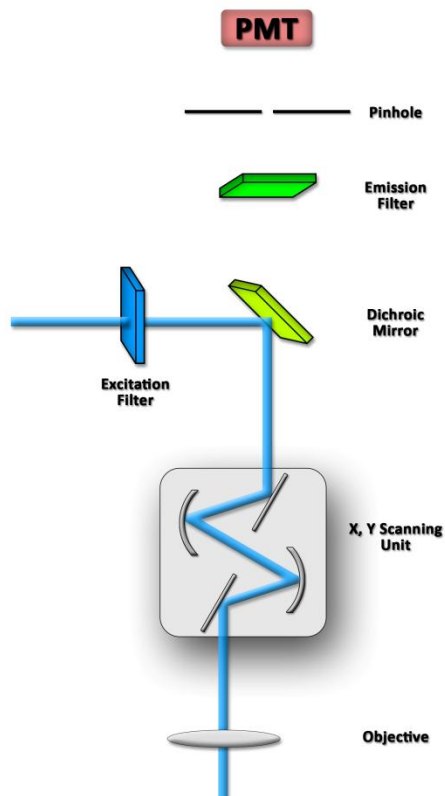
Objective

The excitation light is passed through an excitation filter and reflected off a dichroic mirror as described above. Before reaching the objective the beam is passed through the scan unit, this allows the creation of the raster scan pattern to generate the image.

PMT

Pinhole

Emission
Filter

Dichroic
Mirror

Excitation
Filter

X, Y Scanning
Unit

Objective

Sample

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The excitation light emitted from the sample is passed back through the scanning unit, through the dichroic mirror and emission filter. Out of focus light is rejected by the pinhole and the resulting image is detected by the PMT. This form of detection is known as **de-scanned detection** (meaning the emission light is fed back through the scanning system, or "de-scanned".

NOTE: Multi-photon confocal systems use non-de-scanned detection. The emission light is detected by PMTs that are placed very close behind the objective to increase sensitivity.

Light from the sample (photons) is detected by a photon multiplier tube (PMT). A PMT converts collected photons to electrons by a photocathode. The resulting electron passes through a series of gates called dynodes; each dynode carries an electrical charge (between 500 and 1000 volts). Each time an electron contacts a dynode more electrons are produced. The end result of the cascade is many electrons being detected by the anode as a result of one photon entering the PMT.
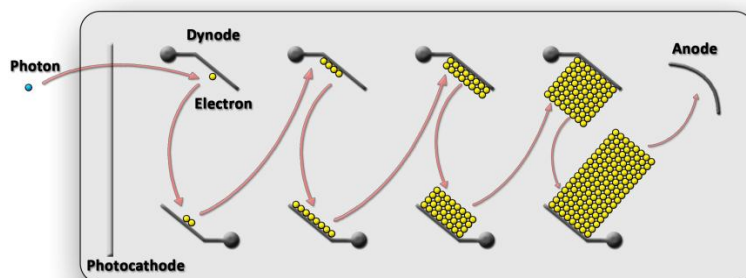

Increasing the voltage applied to the dynode (called gain, smart gain, HV etc by different manufacturers) results in increased sensitivity of detection, but with an increase in noise as well.

PMT are not very efficient, approximately 20% of the photons from a sample will be detected. For comparison a high end CCD camera has an efficiency of nearly 70%, a Electron Multiplying(EM)-CCD camera can be better than 95% efficient.

Pinhole Effects
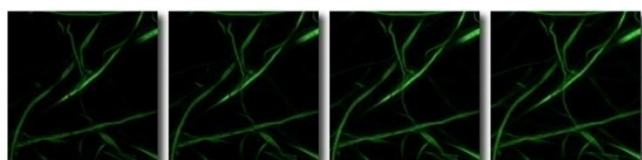
Increasing Pinhole Size
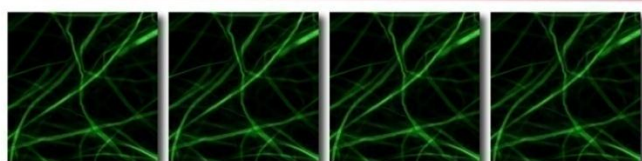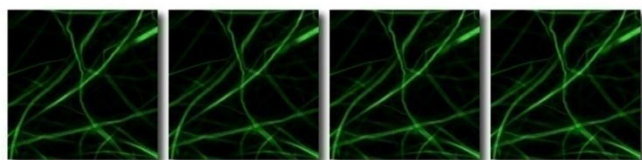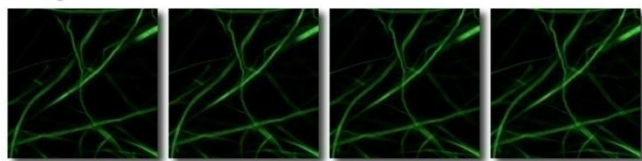
The image to the left demonstrates the effect that opening the pinhole has on the thickness of the optical section captured.

The image is of a piece of fluorescent paper, the individual fibres of which can be easily seen.

The top left image shows only a few fibres that are all in the same plane. As the pinhole size is increased more fibres can be seen above and below the original fibres.

Increasing the size of the pinhole also allows more light to reach the detector, resulting in a brighter image. The example below shows an image generated with the pinhole fully open, and then the pinhole is progressively closed without adjusting for the decrease in brightness. The final image (far right) is barely visible due to the drastically reduced amount of light being detected.
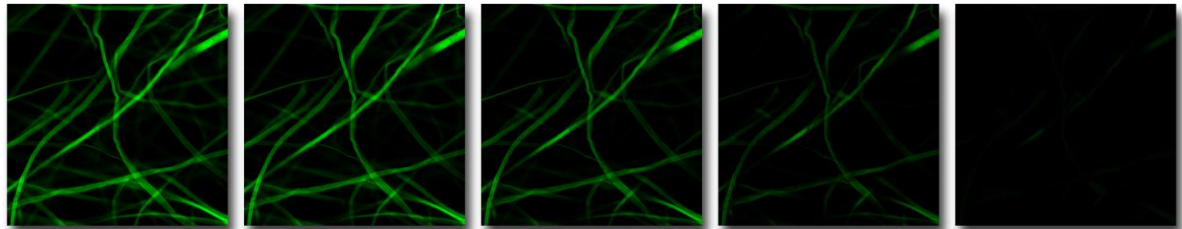
Decreasing Pinhole Size Without Increasing Gain

Slices/Z Planes
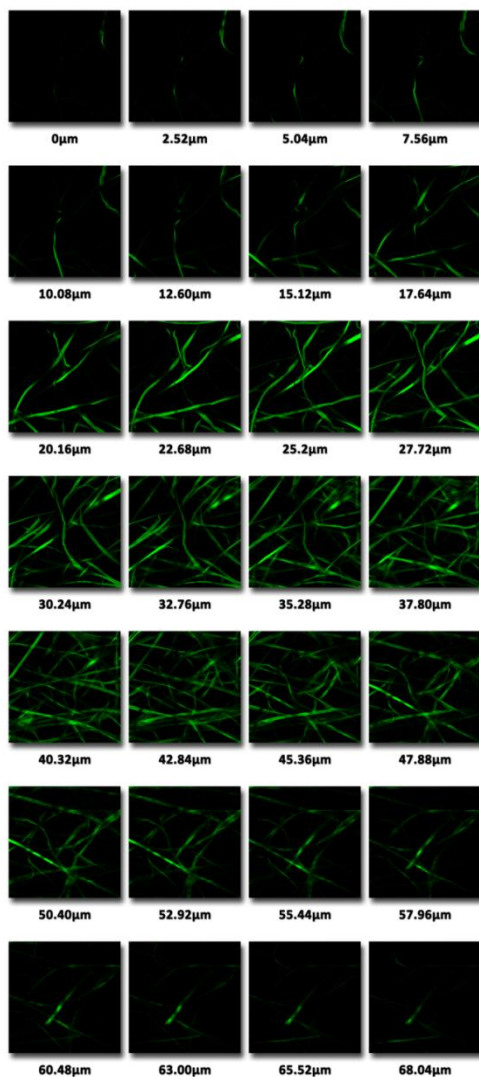

Confocal microscopy data is usually collected as a series of slices commonly called a Z stack. The distance between each slice is determined when the capture is set up. The example to the right shows a series of slices taken through a sample of fluorescent paper. The total thickness of the collected data is 68µm captured in 2.5µm slices.

This data can then be represented in a range of ways.

The vast majority of samples imaged on a confocal microscope will be thick. To be able to effectively image a 3D object the user needs to be aware of what is being captured and what they want to be able to demonstrate with the data.

The example below shows three different slices taken through a sphere, no single slice provides enough information to determine the original objects shape. By looking at all three slices it can be determined that the object is fatter in the middle than on the ends, but what occurs between each of the slices is unknown (there maybe lobes on the object that are not detected).
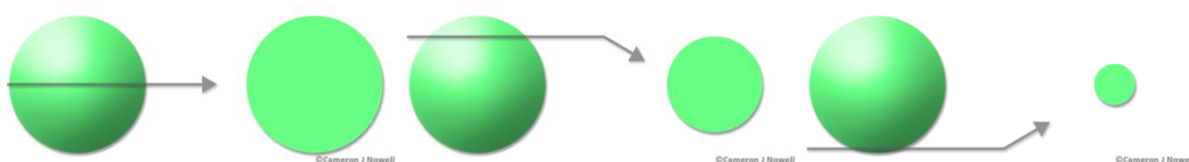

To accurately represent the sample enough slices need to be taken. If the sample is thin and the fluorescence stable enough then slices that are very close together (or overlapping) can be captured.


But if the sample is thick, fluorescence isn't stable or time is important then less slices will have to be captured. As a result fine details and some structures maybe lost.
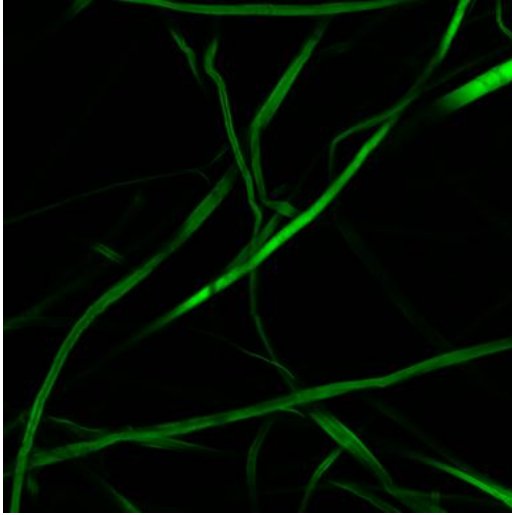
Since confocal data is collected as a series of slices it can be used to generate a range of images. While a single slice, or projection of several slices, may show the result required, sometimes other data representation can be more informative.
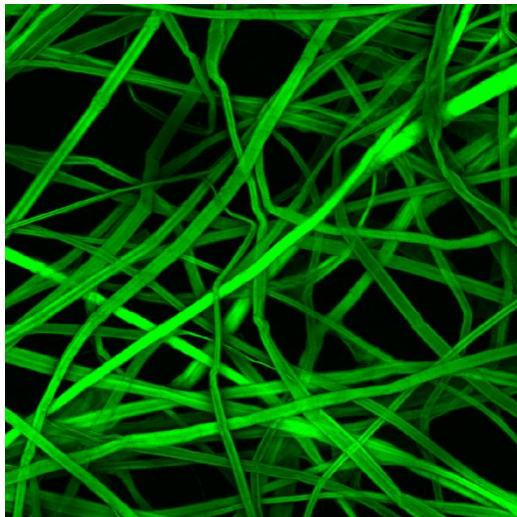
All the slices can be combined to generate a maximum intensity projection. This image draws the brightest pixel from each layer in the final image. The result is somewhat like a widefield microscope image with sharper detail
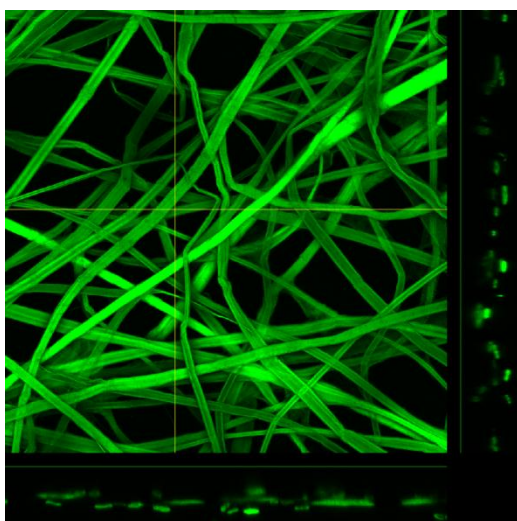
An orthogonal projection can be generated that shows depth information at the selected point. The two thin panels show a view from the right side of the object (YZ dimension) and from the bottom end of the object (XZ dimension). The horizontal and vertical lines in the main image show where the slice has been taken to generate the YZ and XZ views
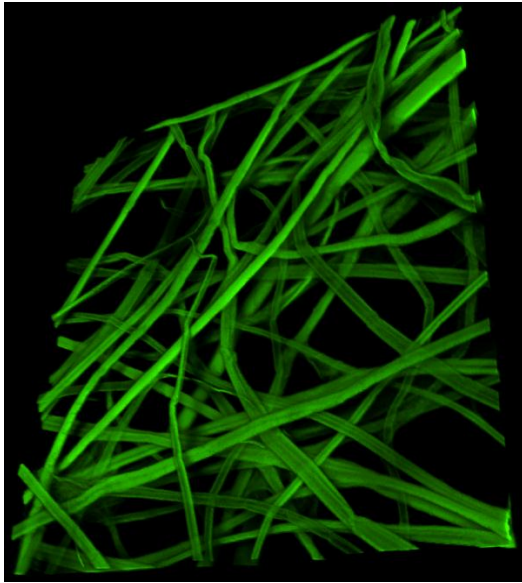
The slices can be combined to generate a 3D (or 4D if it is a timelapse series) model. This model can be rotated to any angle allowing the user to see parts of the data that may otherwise be missed. Once 3D rendered a data set can be measured for volume, distance etc.

Laser Function and Safety

Introduction

Lasers (**L**ight **A**mplification by **S**timulated **E**mission of **R**adiation) provide tightly controlled excitation sources for advanced microscopy such as confocal microscopy. While there are different types of lasers (gas, solid state, semiconductor, chemical etc.) they all operate on the same principle. Energy is supplied (or pumped) to a gain media inside an optical cavity; this media produces and amplifies light. Once the light is of a sufficient energy it escapes the cavity as a beam of laser light.

Lasers can deliver very high power levels and therefore need to be used with care as misuse can result in injury (such as blindness).

What is Laser Light?

Lasers provide a beam of coherent monochromatic light (lasers that emit polychromatic light are also available). This means that the light they produce is of the same wavelength and phase. White light is made of a combination of many different wavelengths that are out of phase with each other.
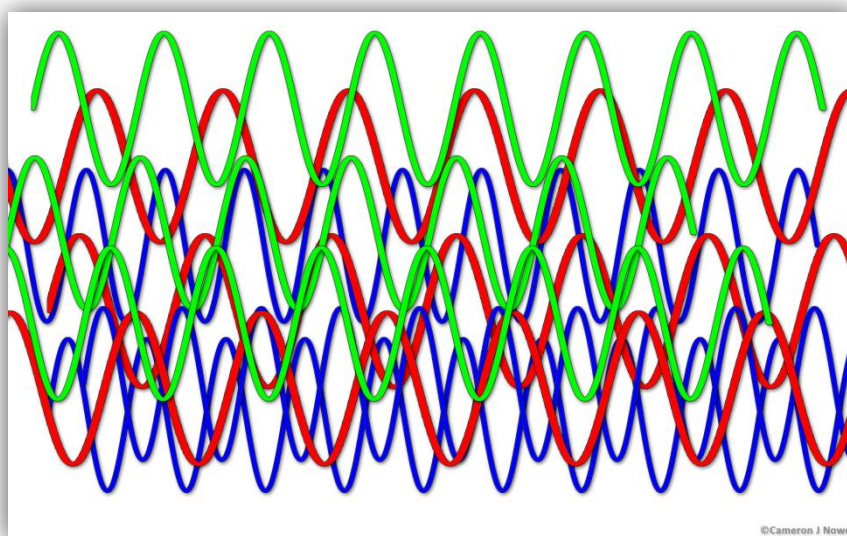
The above image shows a simplified example of white light (made up of only 3 wavelengths; red, green and blue). All the light waves are in different phases (they don't line up) and are of different wavelength, the result is incoherent white light.

If all the light is the same colour as in the example below, it will still not be laser light as it is still incoherent.
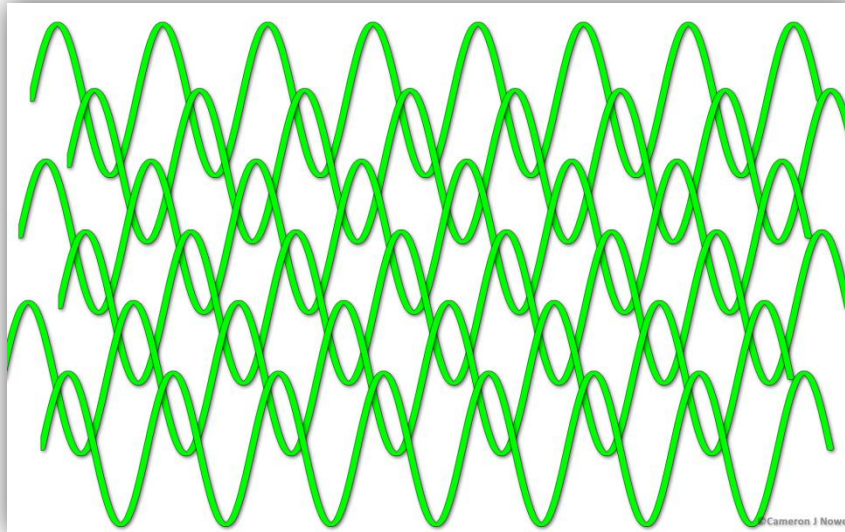
Only once the light is coherent will a laser beam be produced
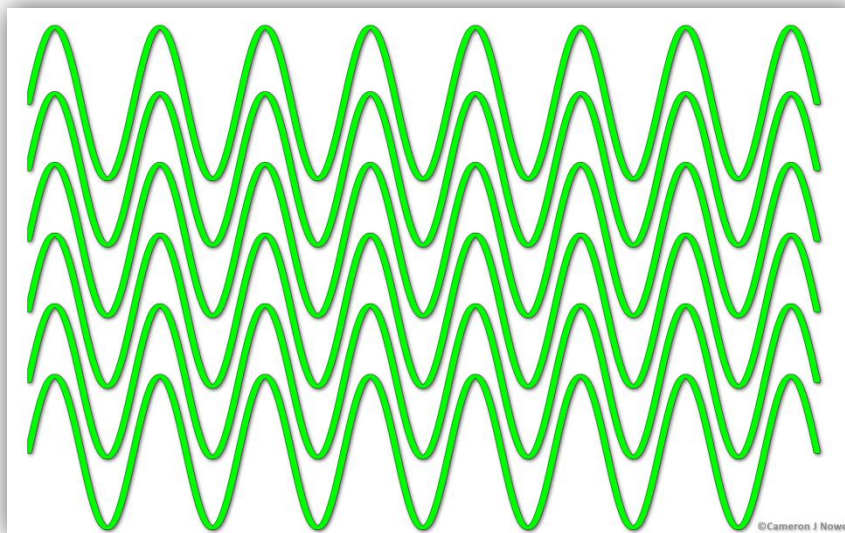
Lasers Used for Imaging

The lasers used in imaging (usually on confocal microscopes) are either solid state/semiconductor or gas types. Solid state lasers are becoming more common due to lower cost, decreased maintenance and longer operational lifetime. They come in a range of wavelengths (from UV through to infra-red) that will match the majority of available fluorophores.

Single and Two Photon Confocal Lasers

Single and two photon confocal imaging use slightly different lasers. Single photon lasers are continuous wave and of reasonably low power (<50mW) while lasers used for two photon imaging are pulsed and of a high power (>3W).

A continuous wave laser produces a constant (or continuous) beam of light. Pulsed lasers fire a beam that is turned on and off (pulsed) rapidly, usually around every 100 femtoseconds (10^{-15} seconds). Due to the pulsing nature of the laser large power levels can be delivered.

Laser Safety

Lasers are classified into classes ranging from 1 through to 4, 1 being the safest. The class system is broken down as follows:

Class 1 – Inherently safe, contained in an enclosure such as a CD player. Something like a CD player can contain a higher class laser, but because it is contained it is classified as a class 1.

Class 2 – Safe during normal use. The blink reflex of the eye is enough to prevent damage. Low powered (<1mW). Laser pointers are an example of Class 2 lasers

Class 3R – Small risk of eye damage within time of the blink response. Up to 5mW. Staring into beam for several seconds will result in minor eye damage.

Class 3B – Can cause immediate eye damage prior to blink response. Up to 500mW. Single photon imaging lasers fall into this category.

Class 4 – Can burn skin and other materials. Usually invisible wavelengths (UV or IR). Reflections from flat surfaces can cause eye damage. Two photon imaging lasers fall into this category.

The lasers used for confocal imaging fall into the two highest laser classes. While the lasers used are potentially dangerous, they are contained within enclosures and have many safety feature built in to minimise the risk of injury. While it is very unlikely that a user could harm themselves using a commercial confocal imaging system the potential still exists. Therefore it is important to never use the system in any way other than the way shown by a trained operator. Do not remove any objective lenses while the laser is firing.

Multiphoton Basics

Introduction

Multiphoton imaging is a variation of standard confocal imaging that uses longer wavelengths of light to excite standard fluorophores. The result of using longer wavelengths (700-1000nm) is that greater penetration of the sample can be achieved. Multiphoton excitation makes use of a pulsed beam (instead of the continuous beam used for single photon imaging) that results in much reduced out of focus excitation of the sample. This means less photo damage and bleaching of the sample.

Terminology

The terms multiphoton and two-photon are usually interchanged. Two-photon excitation refers to the excitation of a fluorophore by two photons. Multiphoton excitation refers to the excitation of a fluorophore by two or more photons.

While three or more photon excitation is possible, the lasers used for microscopy are only capable of exciting fluorophores by two-photon excitation. So in the context of microscopy the terms multiphoton and two-photon excitation are one and the same.

Multiphoton imaging may be referred to as a multiphoton (or two-photon) confocal microscopy. This is technically not true as a pinhole is required to generate a confocal effect (i.e. remove out of focus light). Multiphoton does not generate any out of focus light so does not need a pinhole.

Basic Physics

Single photon excitation relies on the excitation of a fluorophore from its ground state to a higher energy state. This is achieved by one photon of light (at the excitation wavelength) being absorbed by the fluorophore. When the excited electrons fall back to the ground state, fluorescence is emitted. This is demonstrated in the Jablonski diagram below.
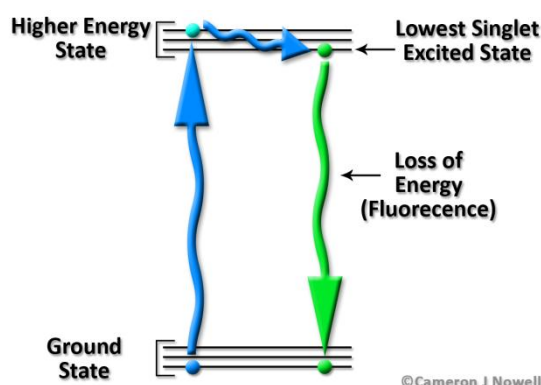
Multiphoton excitation requires the absorbance of two (or more) photons of longer wavelengths to push the electrons from the ground to the higher energy state. The two photons have to hit the fluorophore at exactly the same time, otherwise the excited electron will only reach the middle virtual state and not be pushed to the higher energy state (see diagram below).
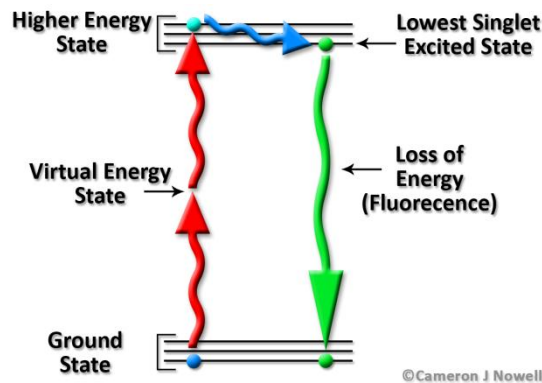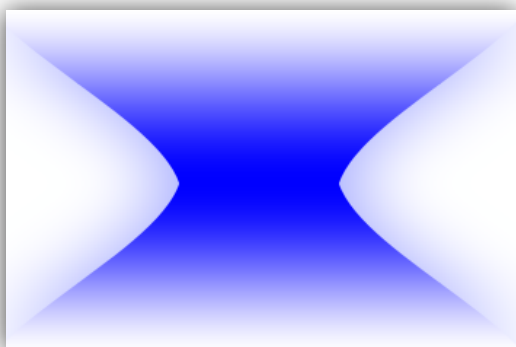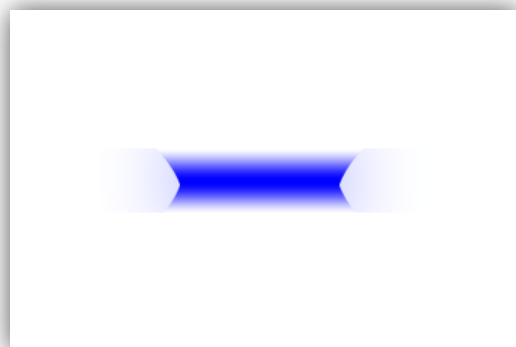

Multiphoton Excitation

The laser used for multiphoton excitation is a high energy infra-red pulsed laser. This means that its emissions are in the far-red to infra red range (700-1000nm) and instead of being fired as a continuous beam the laser is pulsed and off. The pulses are approximately 80 femtoseconds apart. The result of this is that only the fluorophores in the exact focal point of the beam will be excited by the laser.

The excitation of a fluorophore using single photon excitation results in fluorophores above and below the focal plane being excited as well. This can lead to bleaching of the specimen above and below the focal plane. Multiphoton excitation results in very precise focal point with no out of focus excitation of the sample. See the diagram below for a comparison of single and multiphoton excitation.

Single photon excitation profile   Multiphoton excitation profile

The specific wavelength for multiphoton excitation is generally double the single photon excitation wavelength but not always exactly. The following table lists some common single and two photon excitations.

| Fluorophore	Single Photon Excitation (nm)	Two-photon Excitation (nm)
eGFP	492	920
mCherry	587	1030
Quantum Dot (QDot)	405	800
eCFP	439	850

Increased Penetration

Two-photon excitation can lead to increased imaging depth. This is due to the infra-red wavelength used penetrating the sample deeper. It is worth noting though that while the excitation wavelength is better suited to increased penetration, the emission wavelength is still the same as single photon excitation. So while a fluorophore may be excited deeper in a sample, the resulting fluorescence may not be able to exit the sample and so no signal will be detected.

Below is a comparison between single and two photon acquisition. Notice the increased depth of two photon in this case is about 50%.
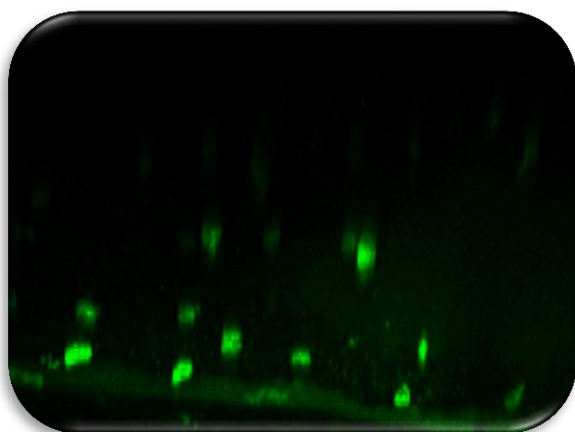
Single Photon   **Two Photon**
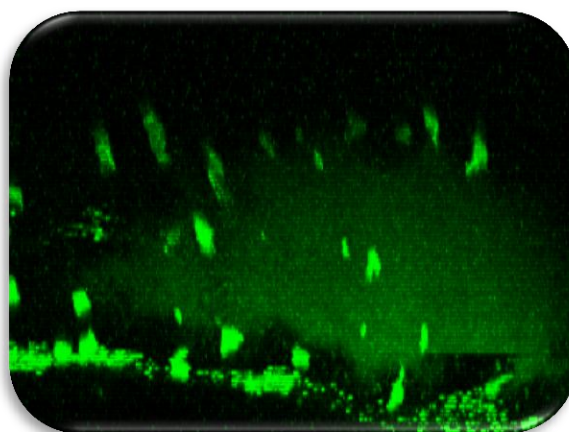
The increase in penetration can range from zero up to ten times. It is highly dependent on the sample, mounting conditions, fluorophore and microscope settings.

While increased depth may not be achieved the quality of the series captured is usually better. This is because under single photon excitation the data collected tends to get dimmer the deeper into the sample it is collected. Under two-photon excitation this does not occur, the data will remain of a similar intensity until maximum depth is reached, after this the signal will fall off to zero very rapidly.

Due to the longer wavelengths of light used for excitation, multiphoton imaging is well suited for live imaging situations. There is less harmful photo damage caused by longer wavelength light, providing the laser power is not set too high.

Image Analysis Basics

Introduction

Analysis of microscopy images can provide useful information ranging from amount of protein present through to cell shape and dimensions. While the range of data produced from image analysis is varied, the basics of how it is generated are the same.

This training note is by no means an exhaustive example of what is possible with image analysis. It is meant as a general overview of what is possible to provide users with a starting point when considering what can be done.

The Golden Rule

The golden rule for all image analysis is "crap in, crap out". If the quality of the image is very low, then the resulting data will be equally low or even impossible to generate.

It is important to remember that while you may be able to see a difference in an image, the software will not always be able to. Image analysis software does not have any advanced pattern recognition like the human brain does, neither can it determine context of a stain or sample.

Bit Depth

The bit depth of an image plays a vital role in image analysis. Higher bit depth images are better for analysis due to the increased range of pixel values available. It is easier to discriminate an object or stain if there are 65,000 shades of grey to use (as in a 16 bit image) compared to 255 shades of grey (as in an 8 bit image).

Thresholding

Almost all image analysis requires a threshold to be set. A threshold is a range of intensities (or colour values) that is used to select the stain, cell, area etc of interest in an image.

Fluorescent Images

Fluorescent images are very simple to threshold as they only contain shades of one colour (usually grey). So a range of grey values can be set to select the stain of interest. The images below show and example of an antibody stain using Alexa488 (green). The image on the left shows the whole stain, the image on the right has been thresholded (defined by the orange colour). The threshold can then be used to make any number of measurements.
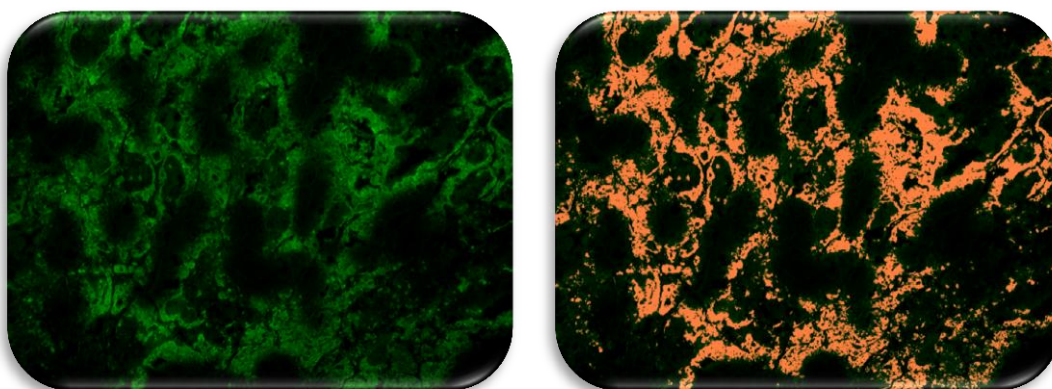

Example measurements that can be made using a threshold as in the example above are:

- Percentage of are stained
- Area, in pixels or calibrated units (μm^2), of stain
- Number of discrete areas of stain
- Intensity of the stain under the threshold

Colour Images

Colour images can be thresholded as well but it may not always work efficiently. While thresholding a monochrome image (like a fluorescent image) requires setting a maximum and minimum intensity, that is not possible with a colour image. To set a threshold for a colour image the user needs to specify what colours, intensities and luminocities make up the colour of interest. If parts of that threshold exist in other places in the image they will be thresholded as well, leading to false thresholding of parts of the image.

Below is an example of a DAB chromagen stained sample that has been thresholded for the brown colour. In this example the brown signal is very strong and easily separated from the rest of the image. If the signal was weak it may not be possible to detect it.
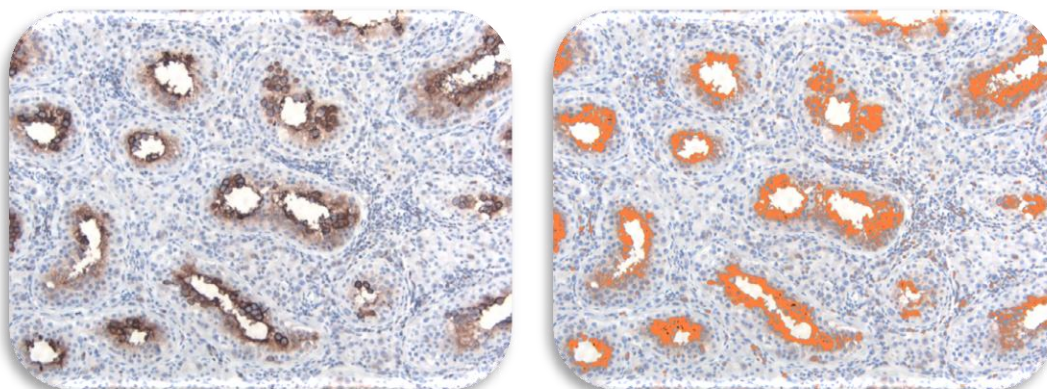
The same information that can be obtained from the previous fluorescent images can be now obtained from this colour image.

Counting Objects

If there are discrete objects in a sample (cells, beads, vessels etc) that can be marked using a fluorophore then they can be automatically counted. If the average size of an object is known then clumps of objects can be counted by treating objects twice the size of the average as two objects, three times the average size as three objects and so on.

The example below shows an image of some 500nm nanoparticles. The image on the right shows the segmentation of the particles, purple dots represent 1 particle while other coloured dots represent larger numbers of particles. The total count for this field was 173 particles.
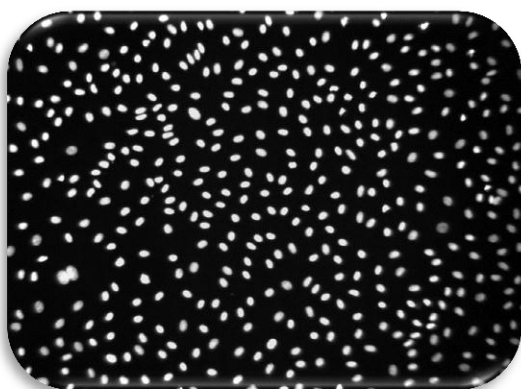
Masks

Masks are sometimes used to delaminate an area or object of interest. Masks are made by thresholding the area or objects of interests and creating a binary image of the threshold. A binary image is made up of only two colours; black and white. These masks can then be used like a threshold, i.e. whatever falls under a mask will be counted/measured and what falls outside will be ignored.

Cell Segmentation

Segmentation is a method of pulling out individual objects, such as cells, from an image. Once these objects have been individually segmented they can be individually analysed. The following example shows segmentation on a sample of cells. For the segmentation to be successful the cells had to be stained with two dyes, one for the nuclei and one for the cell membrane/cytoplasm.

Firstly the two required images are captured

Nuclei	Cytoplasm/membrane
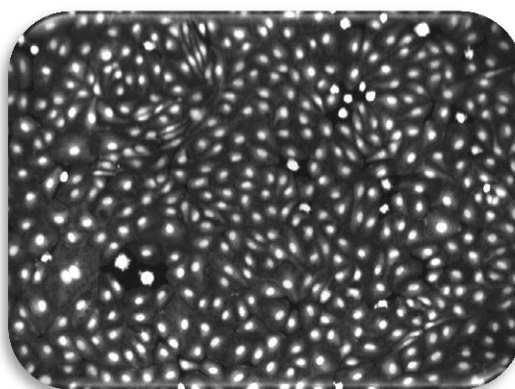
They are then segmented to highlight the individual cells or nuclei.

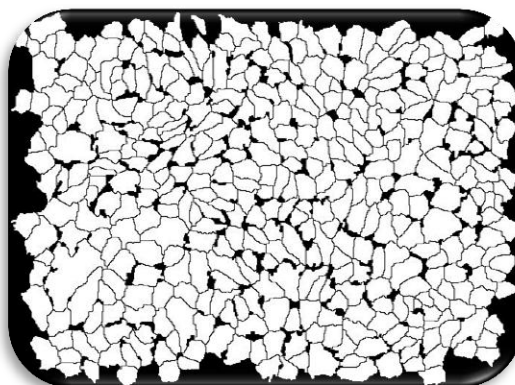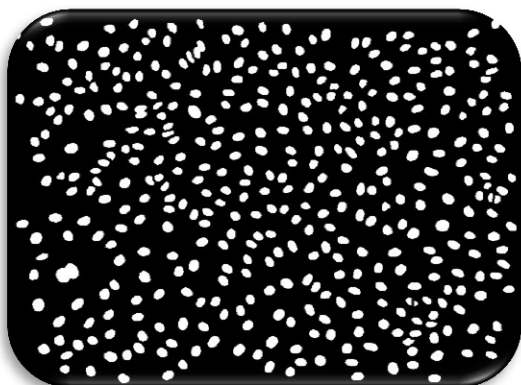The resulting masks can then be used to generate other masks, the example below shows membrane and cytoplasm masks used by doing some subtractive image math to the above images.
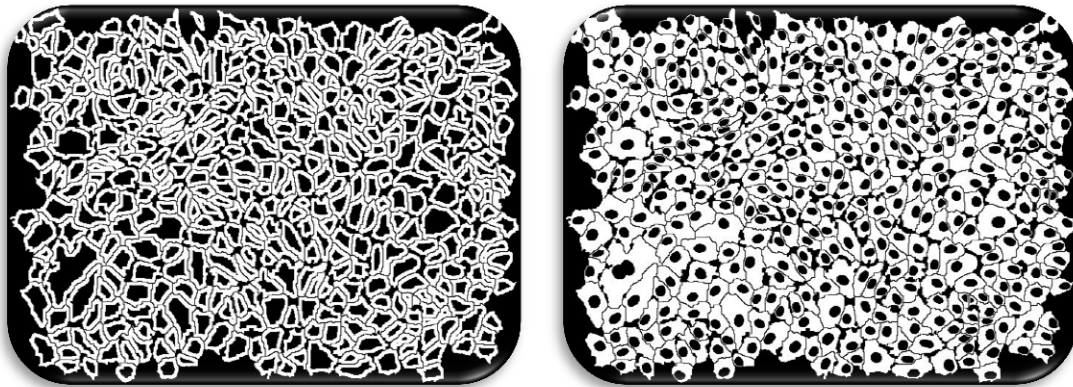
By combining these masks and the original images a wealth of information can be acquired, for example:

- Number of cells
- Area of cells
- Intensity of membrane
- Cytoplasm area
- Shape of cells
- Membrane to nuclei intensity ratio
- Membrane to cytoplasm ratio
- Perimeter of the cell

Conclusion

The above examples just scrape the surface of what is possible with a good quality image and some high end analysis software. It is always important to capture a good image at the start and be aware that what you can see may not necessarily be seen by the software.

Glossary

Airy Disc - The bright, diffuse central spot of light formed by an optical system imaging a point source of light. The size of an airy disc is governed by the diffraction limit of the system and determines the maximum resolution.

Airy Unit – The size of one Airy Disc. Airy Units decrease as NA increases.

Axial Resolution – The resolution in depth or Z dimension.

Bit Depth – The number of possible digital combinations in an image. For example a pixel in an 8bit can represent 255 shades of grey.

Bleaching – The irreversible photochemical destruction of a fluorophore.

Brightfield – Any illumination technique that uses white light.

Chromatic Aberration – Misalignment of colour in final image due to different wavelengths of light having different focal planes.

Confocal – Fluorescence based imaging technique that removes out of focus light using a small hole in the light path.

Darkfield Illumination – An illumination technique that produces an image that looks like an inverted transmitted image.

DIC – Differential Interference Contrast imaging. A technique that uses polarised light to generate an image of an unstained sample such as cells or thick tissue. Does not work with plastic dishes.

Dichroic Mirror – A filter that will reflect some wavelengths of light, while allowing others to pass through.

Emission Filter – A filter (usually coated glass) that filters out only the light of the specific emission wavelength of a fluorophore.

Emission Wavelength – The wavelength of light that is emitted from a fluorophore when it is excited.

Excitation Filter – A filter (usually coated glass) that generates the specific wavelength required for excite a fluorophore.

Excitation Wavelength – The specific wavelength of light that will stimulate a fluorophore to emit fluorescence.

Fluorescence – Light produced from a protein or chemical when excited by a specific wavelength.

Köhler Illumination – Alignment technique used to focus transmitted light evenly on a sample.

Immersion Media – Media (usually liquid) that is used between the sample and the objective to increase the angle of light acceptance, thus increasing NA and resolution. Common immersion medias are oil, water and glycerol.

JPG (JPEG) – Joint Pictures Expert Group. Compressed image format that should only be used for saving images for presentation or for printing in lab books. Data from the original image is list during compression.

Laser – Coherent (usually monochromatic) light source used in confocal imaging to excite a fluorophore

Lateral Resolution – The resolution in XY dimensions

Light Gathering Capability – Number (F) that is a function of the amount of light an objective can collect. Is governed by magnification and NA.

NA – Numerical Aperture, the half angle of the cone of light an objective can accept. NA is the sole discriminator of objective resolution.

Nyquist Sampling – Rule governing the translation of analogue data into digital data. All analogue data needs to be sampled enough (2.3 times) to result in an accurate representation in the digital realm.

Objective – The part of the microscope that magnifies and resolves a sample.

Orthogonal Projection – Way of representing data that shows XZ and YZ projections of a 3D data set.

Phase Contrast - An illumination technique for visualising unstained cells or tissue. Works with plastic. Commonly used on tissue culture microscopes.

Photon – Basic elementary particle of light.

Photon Multiplier Tube (PMT) – Detector used in confocal microscope. Converts photons to electrons to generate an image.

Phototoxicity – The damage caused by exposing living cells or tissue to high intensity light.

Pixel – Smallest representation of colour and/or intensity information in a digital image

Single Photon Excitation – The excitation of a fluorophore by one photon of the specific excitation wavelength.

Spherical Aberration – Degredation of the final image due to light passing through different thickness parts of a lens. Usual result is a blurry image round the edge with infocus centre.

TIFF – Tagged Image Format. Image format that does not use compression. Keeps all original information from the image.

Threshold – A method used in image analysis to highlight the area of interest. Thresholds are set by a maximum and minimum intensity value.

Transmitted Light – Illumination created by shining light through a sample

Two Photon Excitation – The excitation of a fluorophore by two photons, usually double the excitation wavelength of the fluorophore.

Z Slice – A single optical slice generated by a confocal microscope.