University of Queensland
Institute of Molecular Bioscience

Microscopy Workshop

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Background reading

The following webpages will introduce you to basic concepts in microscopy and to the care of microscopy equipment. Reading the following material will help to ensure that you get the most out of this manual.
http://micro.magnet.fsu.edu/primer/anatomy/introduction.html [**1**]
http://micro.magnet.fsu.edu/primer/anatomy/cleaning.html [**2**]

Forming an Image

Introduction

The first optical microscope to be developed was likely in the Netherlands around the 1620s, by Cornelis Drebbel [**3**]. The name microscope was used for the first time for Galileo Galilei's compound microscope in 1625 (Galileo had originally called it the "*occhiolino*" or "*little eye*").

The first detailed account of the interior construction of living tissue based on the use of a microscope did not appear until 1644, in Giambattista Odierna's *L'occhio della mosca*, or *The Fly's Eye*.

It was not until the 1660s and 1670s that the microscope was used extensively for research in Italy, the Netherlands and England. Marcelo Malpighi in Italy began the analysis of biological structures beginning with the lungs. Robert Hooke's *Micrographia* had a huge impact, largely because of its impressive illustrations. The greatest contribution came from Antonie van Leeuwenhoek who discovered red blood cells and spermatozoa and helped popularise microscopy as a technique. On 9 October 1676, Van Leeuwenhoek reported the discovery of micro-organisms [**4**].

Figure 1 - Replica of microscope by Leeuwenhoek [5]

Geometrical Optics

Introduction

In order to understand how a microscope works, a common starting point is through geometrical optics. This is a simplified model of how optical systems work, but it provides us with valuable insight nonetheless. At the heart of this model are the *optical axis* and the *lens*, which cross each other at right angles and describe the plane on which we'll work.

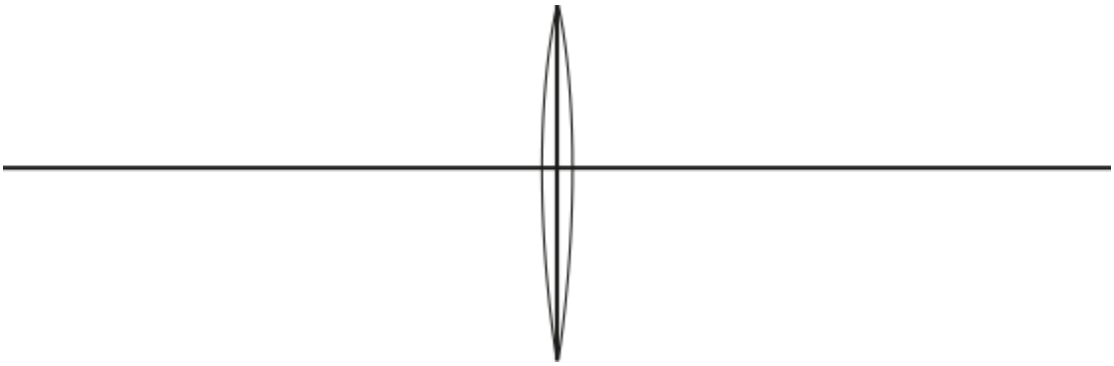


Figure 2 - The optical plane

The lens has two foci, one on either side of the lens. Although not necessarily the case, we'll stipulate that foci are equidistant from the lens. This distance is called the *focal length* (sometimes written *f*).

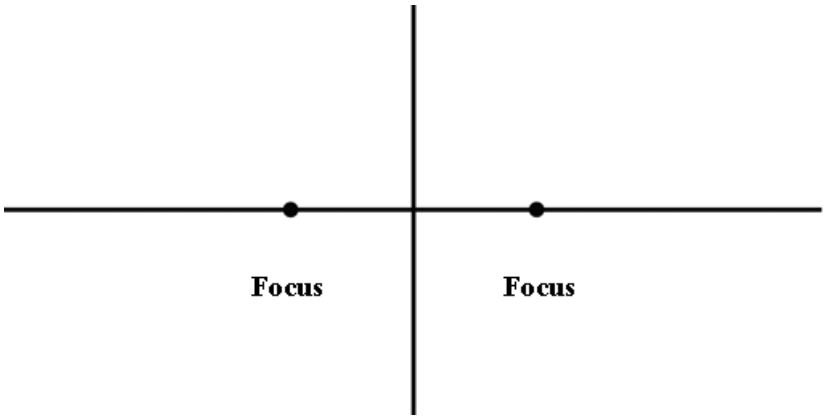


Figure 3 - Focal points

All light rays that approach the lens parallel to the optical axis are bent to pass through the focus on the far side of the lens.

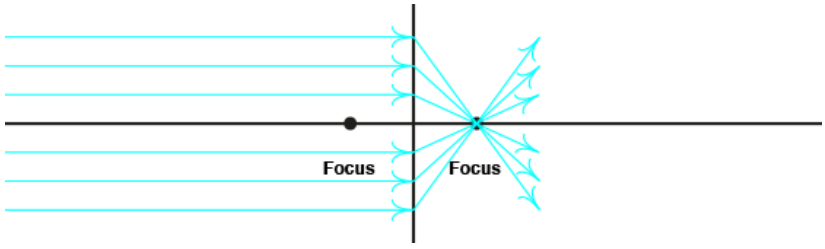


Figure 4 - Parallel light rays converge to the focus on the far side of the lens.

Conversely, all light rays that approach the lens through the focus are bent to emerge parallel to the optical axis on the far side of the lens.

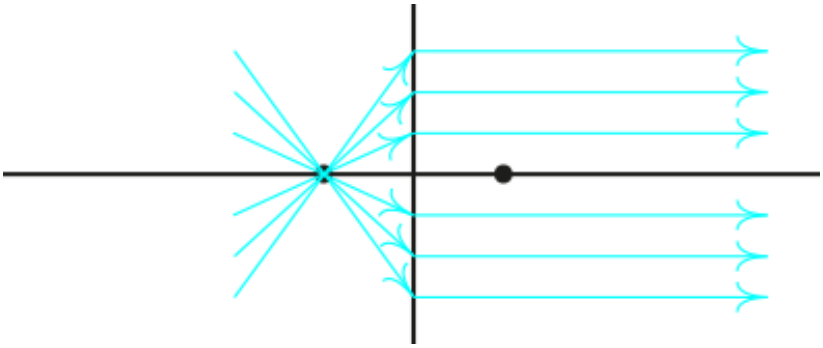


Figure 5 - Rays passing through the focal point emerge from the lens travelling parallel to the optical axis.

Finally, all light rays that pass through the centre of the lens continue straight through to the far side of the lens without bending.

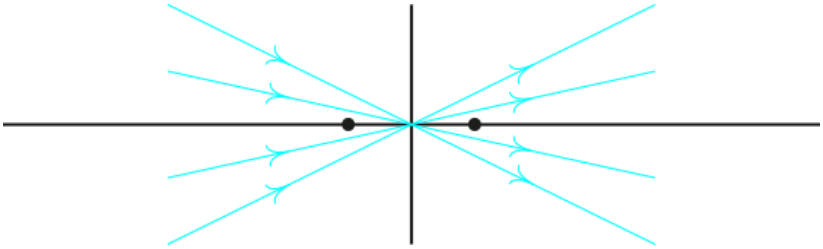


Figure 6 - Rays passing through the centre of the lens emerge undeviated.

These three rules allow us to model how images are formed by simple arrangements of lenses. Lets consider the simplest of these: one lens and one object. Let's place the object to the left of the lens with its base resting on the optical axis. Our object will emit or reflect light rays in all directions, but we'll only be concerned with those travelling towards the lens parallel to the optical axis, those travelling towards the lens and passing through the focus on the way there, and those rays passing through the centre of the lens. To simplify things even further, we'll only concern ourselves with the rays coming from the very top of the object. This means we'll only be concerned with three rays emanating from the top of the object. The ray travelling from the top of the object parallel to the optical axis will be bent by the lens to pass through the focus on the far side of the lens. The ray travelling from the top of the object through the focus on the near side of the lens is bent by the lens to emerge travelling parallel to the optical axis. Finally, the ray travelling through the centre of the lens passes through the lens undeviated.

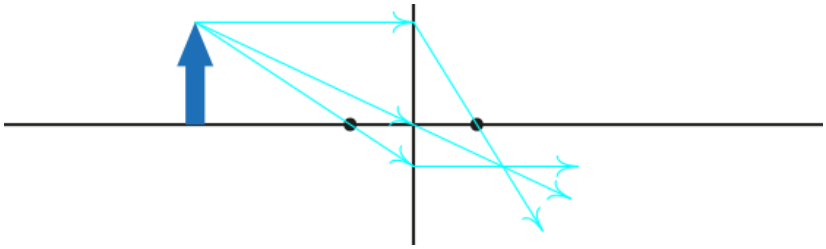


Figure 7 - Reconvergence of rays emanating from one point on the object.

And look what's happened: all of our rays have converged to another point. Here, the light rays from the top of our object have been focussed to produce an image. We could go through this process for all points in the object, but a bit of consideration should suggest that all points will scale and translate similarly – after all, there was nothing *special* about the point we selected – so we can draw our complete image in place.

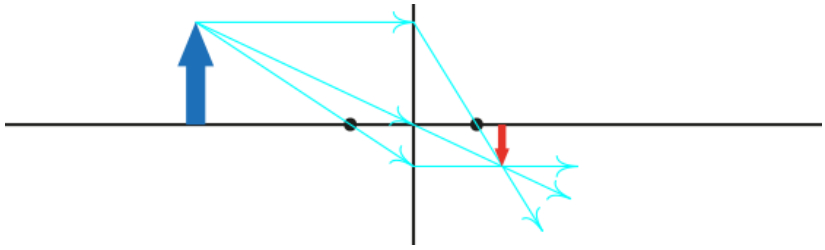


Figure 8 - Real image formation.

Notice that the image is upside down, or *inverted*, with respect to the object; in the full 3-d consideration the image is rotated 180° with respect to the object. This is also what we call a **real image**. Real images can be projected onto a surface or observed directly.

As we move the object towards the lens the image grows larger and moves *away* from the lens; as the object moves in towards the focus on the left side of the lens, the image moves off towards infinity to the right. Note that this is still a real image. This is essentially how modern microscopes magnify images.

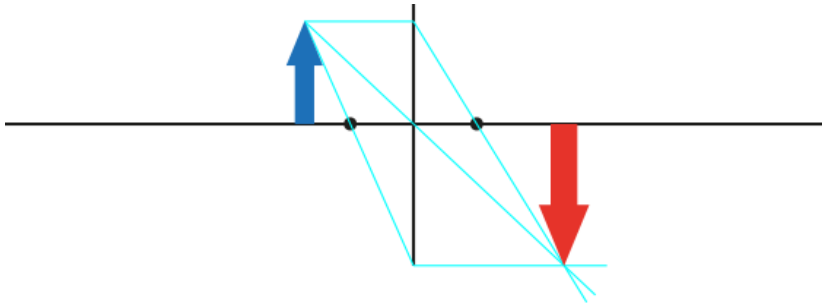


Figure 9 - Magnification of a real image

However, if we move the object to a point in between the focus and the lens something strange happens. We still have our three principal rays and they still behave as normal but they don't converge.

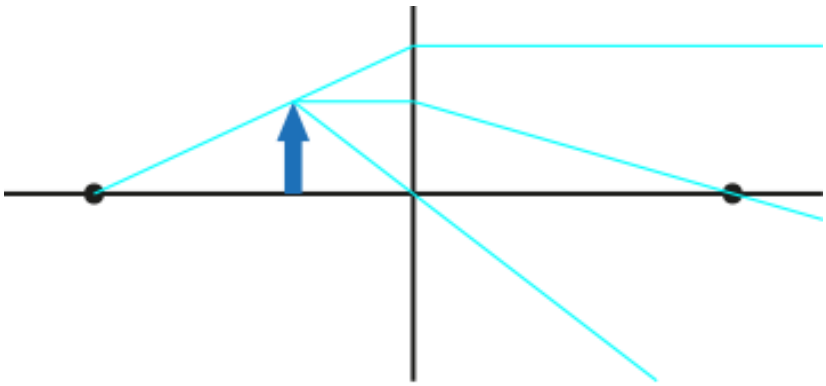


Figure 10 - Light paths for an object between the focal point and the lens.

Instead, if we extend the rays backwards we find that they appear to originate from a point on the object side of the lens.

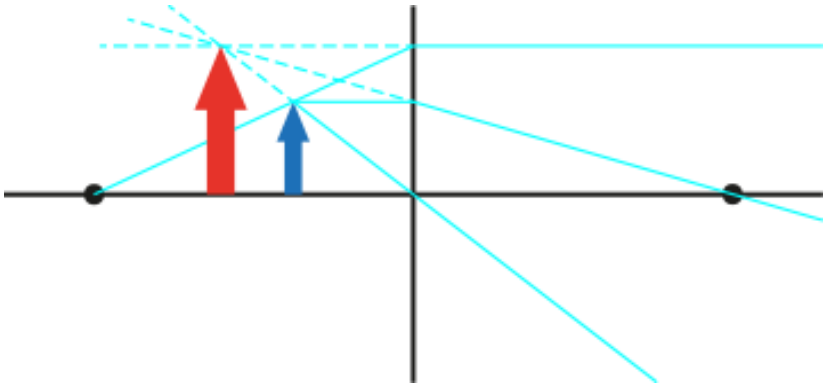


Figure 11 - Virtual image formation.

This forms an image, too, but since the rays only *seem* to converge at the image it cannot be projected onto a surface. The image can be directly observed and is called a **virtual image**. This is the sort of image generally produced by a magnifying glass or a microscope eyepiece. This is also how the Leeuwenhoek microscope works.

Real images

- light rays actually converge to form a real image
- form on the side of the lens opposite the subject
- are *inverted* with respect to the subject
- can be projected
- microscopes form real images at the camera

Virtual images

- light rays only *seem* to converge
- form on the same side of the lens as the subject
- are *upright* with respect to the object
- can be seen but not projected
- microscope eyepieces form a virtual image

A compound microscope produces a real image that is further magnified by an eyepiece to produce a greatly magnified virtual image. In the following figure the object is in blue, the first (real) image is in red, and the second (virtual) image is in green and can be seen to be much larger than the object.

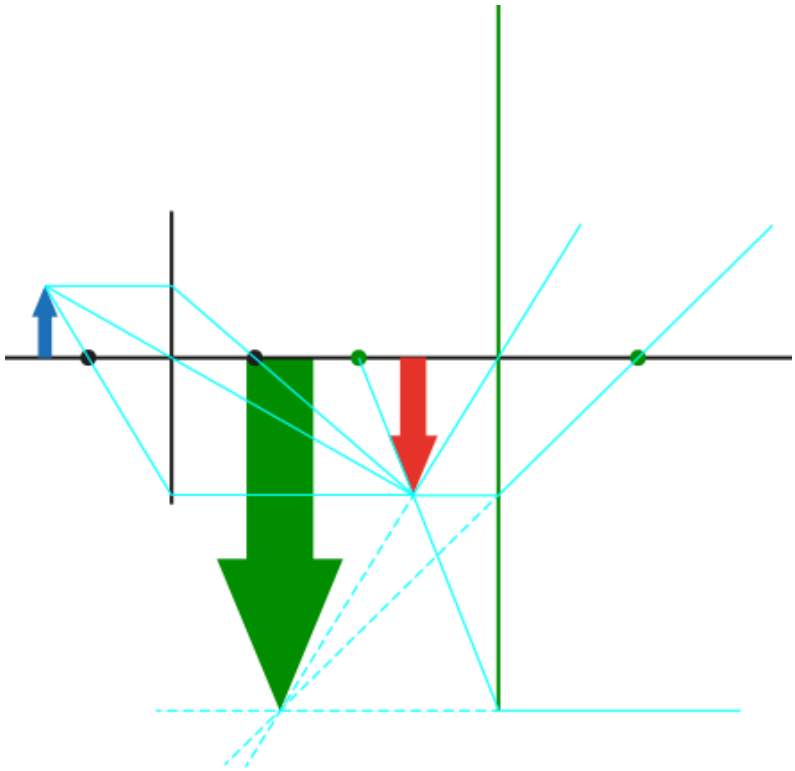


Figure 12 - Geometrical optics representation of a compound microscope.

In reality, most modern microscopes use a system termed **infinity corrected optics** in which the objective produces an image at infinity (where does the object need to be placed for this to occur?) and a separate lens - called a **tube lens** - inside the microscope produces the image we observe magnified through the eyepieces or projected onto our camera sensor. The main benefit of this is that, in the region between the objective and the tube lens, the light collected from the specimen travels in parallel paths and this makes inserting auxiliary items (filters, polarisers, &c.) into the light path of the microscope much easier.

Conjugate Focal Planes

When the microscope is properly aligned using Köhler illumination, several places in the microscope are focussed onto each other. These are called **conjugate focal planes,** and there are two sets of them. In the first set, the condenser field stop, the specimen itself, the image formed by the objective, and the eye's retina or the film/detector plane are all conjugate to one another. In the second set are the light source, the condenser aperture stop, the objective rear focal plane, and the iris of the eye. All of the light that is in focus in one set of conjugate focal planes is perfectly defocussed at the other set of conjugate focal planes. Thus light from the illumination source is spread evenly across the object of interest.

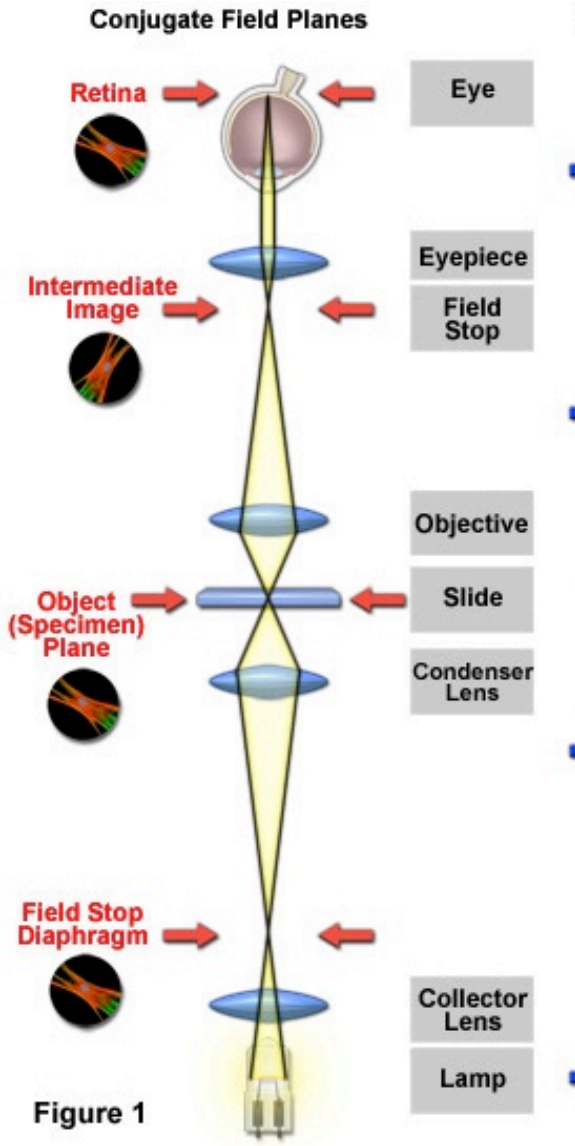


Figure 13 - Conjugate focal planes of the specimen. [6]

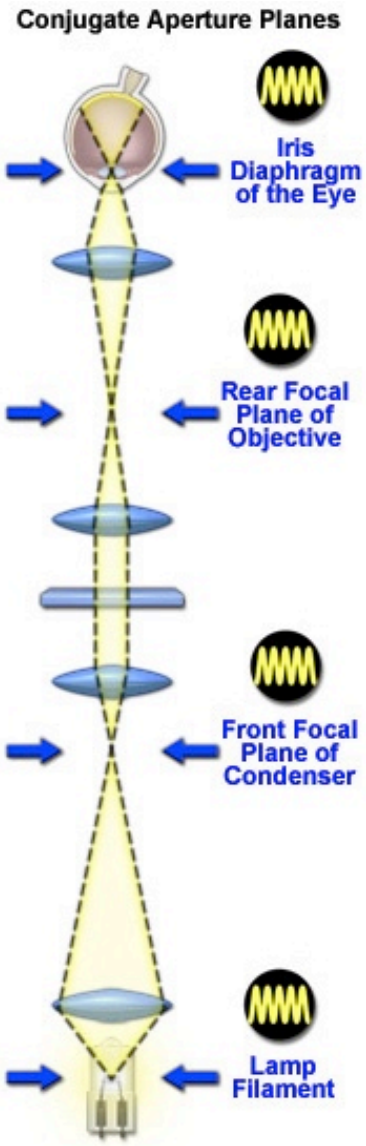


Figure 14 - Conjugate focal planes of the light source. [6]

For additional information on the previous subjects, please see the following web pages:
http://hyperphysics.phy-astr.gsu.edu/hbase/geoopt/conjug.html [7]
https://www.microscopyu.com/articles/formulas/formulasconjugate.html [8]

Magnification

Magnification can be a complex subject, and there are many ways to express how much larger an image is compared to the original object.

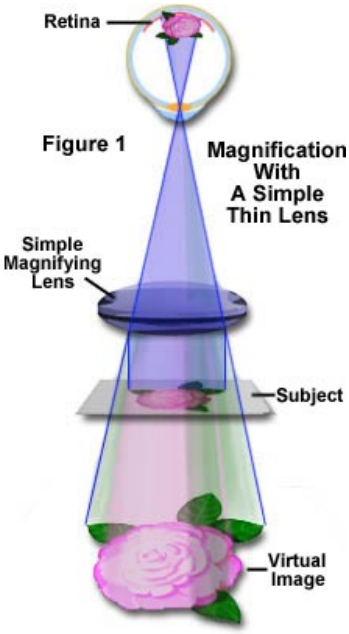


Figure 15 – Magnification of an image. [9]

For all practical purposes in microscopy, the clearest way to report the magnification for an image is to report the magnification and NA of the objective used and to include a scale bar in your image.

Accurate determination of scale is generally performed using a micrometer slide to calibrate the image capture software. This process will generate a µm/pixel calibration value for each objective.

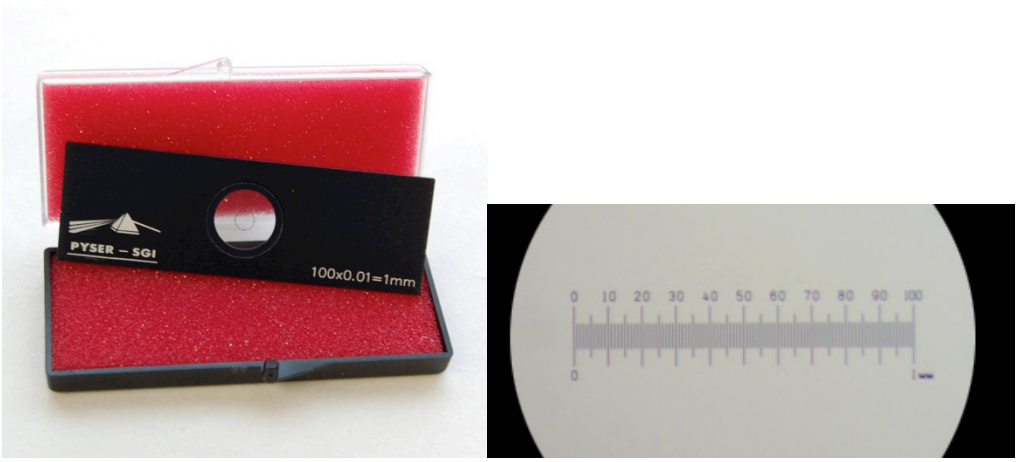


Figure 16 – Micrometer Slide [10]

However in microscopy, magnification is generally referred to as the total magnification of the microscope including the objective lens, tube lens and any other size altering lenses within the lightpath.

For more in-depth information on magnification, please see the following:
http://micro.magnet.fsu.edu/primer/anatomy/magnification.html [**9**]

Optical aberrations

Introduction

An optical aberration is a departure of the performance of an optical system from the predictions of paraxial optics. [**11**] In an imaging system, it occurs when light from one point of an object does not converge into (or does not diverge from) a single point after transmission through the system. Aberrations occur because the simple paraxial theory is not a completely accurate model of the effect of an optical system on light, rather than due to flaws in the optical elements.

Aberration leads to blurring of the image produced by an image-forming optical system. Makers of optical instruments need to correct optical systems to compensate for aberration. [**12**]

For background information on optical aberrations, please see the following links:
http://micro.magnet.fsu.edu/primer/java/aberrations/pointspreadaberration/index.html [**13**]
http://www.olympusmicro.com/primer/anatomy/aberrations.html [**14**]

Field Curvature

Ideally, all points in a plane parallel to our lens would focus to a nice flat plane on the other side of the lens. Unfortunately, in the real world, this doesn't happen for spherical lenses of the sort we have been discussing. Instead, a flat plane in our sample is focussed to a curved plane on the other side of the lens. There are a couple of ways of addressing this. One way is to add more lenses in our system to correct for this. This is complex and possibly adds *more* problems. Alternately, we could just restrict our field of view to a central region of the image where the curvature of the image is not too great. This is a much simpler solution. In actual fact, some combination of the two methods is used. More information can be found at the Molecular Expressions website [**15**]:

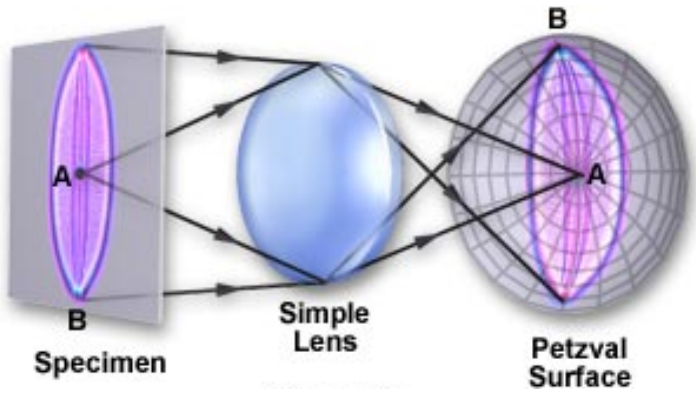


Figure 17 - Field curvature. [15]

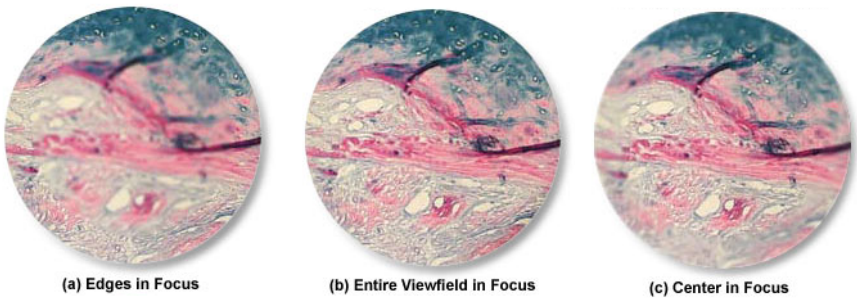


Figure 18 - View of a curved view field through the oculars [15]

Chromatic Aberration

Essentially, lenses are compound prisms and since prisms bend light by varying degrees according to the wavelength of the light, so too do lenses focus light of different wavelengths at different distances from the lens. This leads to artefacts in which images may be ringed by fringes of different colours. Chromatic aberrations may present as both *x-y* and *z* shifts and can prevent the microscopist from elucidating spatial localisation information for different fluorescent markers.

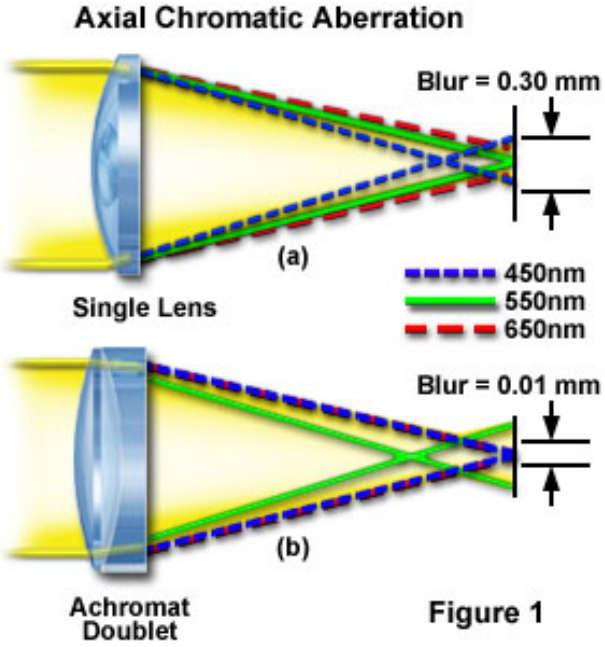


Figure 19 - Chromatic aberration [14]

Spherical Aberration

Light rays of the same wavelength passing through points far from the centre of the lens converge at a point other than that at which rays passing close to the centre converge. Spherical aberrations commonly occur with refractive index mismatches between the sample and the immersion media, causing distortion of objects in the *z*-axis. Spherical aberrations are often exacerbated when imaging deep within a sample. For more information on spherical aberration, please see the Olympus Microscopy Resource Center:
http://www.olympusmicro.com/primer/java/aberrations/spherical/ [**16**]

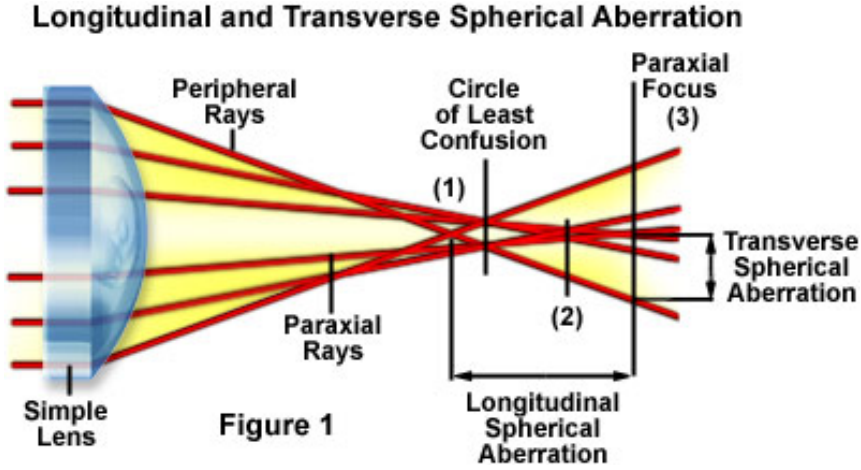


Figure 20 – Spherical aberration [16]

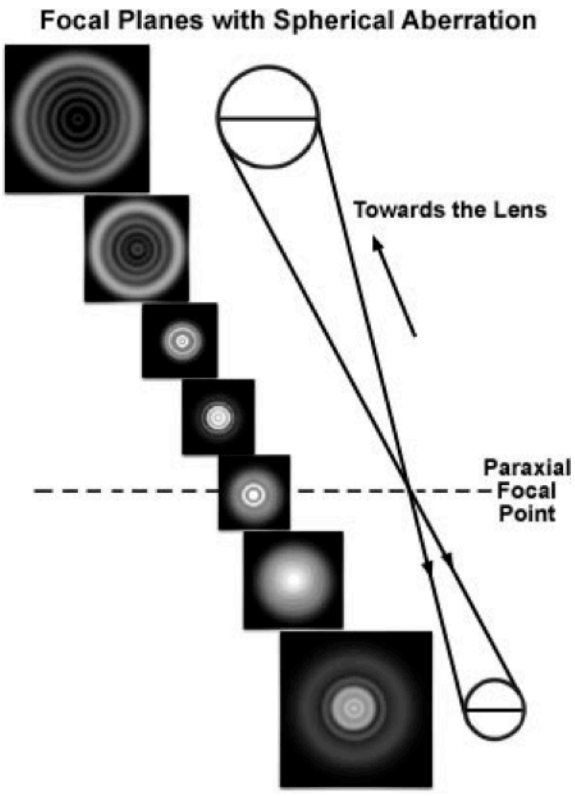


Figure 21 – Focus Depth and Spherical aberration [13]

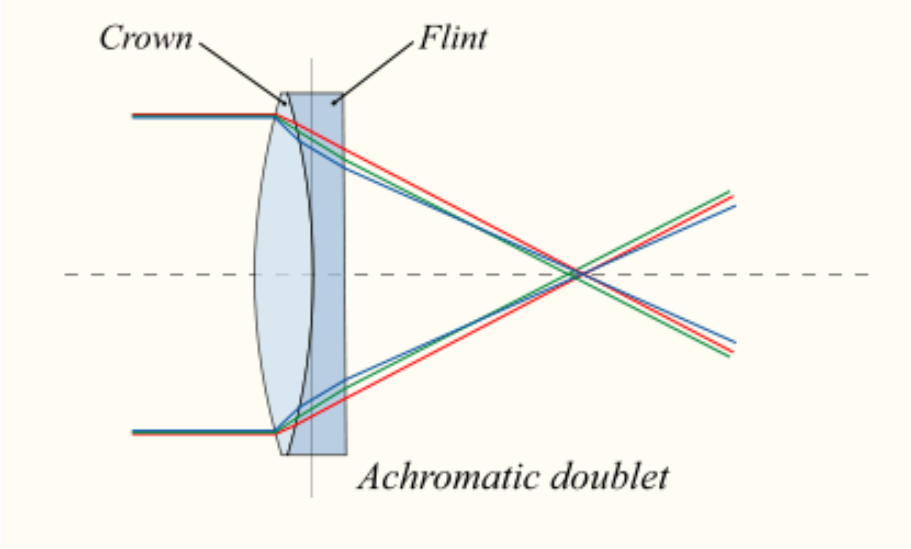


Figure 22 - Correction of chromatic aberration [17]

Both chromatic and spherical aberrations can be corrected either by grinding lenses to have aspherical shapes (which is difficult) or by using more lenses (easier) to correct for these aberrations, along with using mounting media of the correct refractive index. For this reason, the objectives used on our confocal microscopes which are corrected for multiple wavelengths and spherical aberration cost much more than the lower end objectives on tissue culture microscopes.

Objectives

Introduction

Microscope objectives are perhaps the most important components of an optical microscope because they are responsible for primary image formation and play a central role in determining the quality of images that the microscope is capable of producing. Objectives are also instrumental in determining the magnification of a particular specimen and the resolution under which fine specimen detail can be observed in the microscope. The objective is the most difficult component of an optical microscope to design and assemble, and is the first element that light encounters as it proceeds from the specimen to the image plane. Objectives derive their name from the fact that they are, by proximity, the closest component to the object (specimen) being imaged. [**18**]

Numerical Aperture

The numerical aperture of an objective is a description of how wide a cone of light that objective can collect and is defined as:

Equation 1 $$NA = n \cdot sin\boldsymbol{\alpha}$$ [**19**]

where n is the index of refraction of the medium between the sample and the objective and α is the half-angle of the cone of collected light..

Resolution is *inversely proportional* to the numerical aperture (smaller is better):

Equation 2 $$r = 0.61\lambda/NA$$ [**19**]

Thus numerical aperture is an indication of the resolving power of an objective: the higher the numerical aperture, the finer the details an objective can resolve.

	OBJECTIVE TYPE					
	Plan Achromat		Plan Fluorite		Plan Apochromat	
Magnification	N.A	Resolution (µm)	N.A	Resolution (µm)	N.A	Resolution (µm)
4x	0.10	2.75	0.13	2.12	0.20	1.375
10x	0.25	1.10	0.30	0.92	0.45	0.61
20x	0.40	0.69	0.50	0.55	0.75	0.37
40x	0.65	0.42	0.75	0.37	0.95	0.29
60x	0.75	0.37	0.85	0.32	0.95	0.29
100x	1.25	0.22	1.30	0.21	1.40	0.20
N.A. = Numerical Aperture						

Table 1 – Objective Magnification vs Numerical Aperture. [**20**,**19**]

Working Distance

Working distance is the distance from the front of the objective to the coverslip when the subject is in focus at the far side of the coverslip. High numerical aperture objectives inherently have a short working distance compared to low NA lenses.

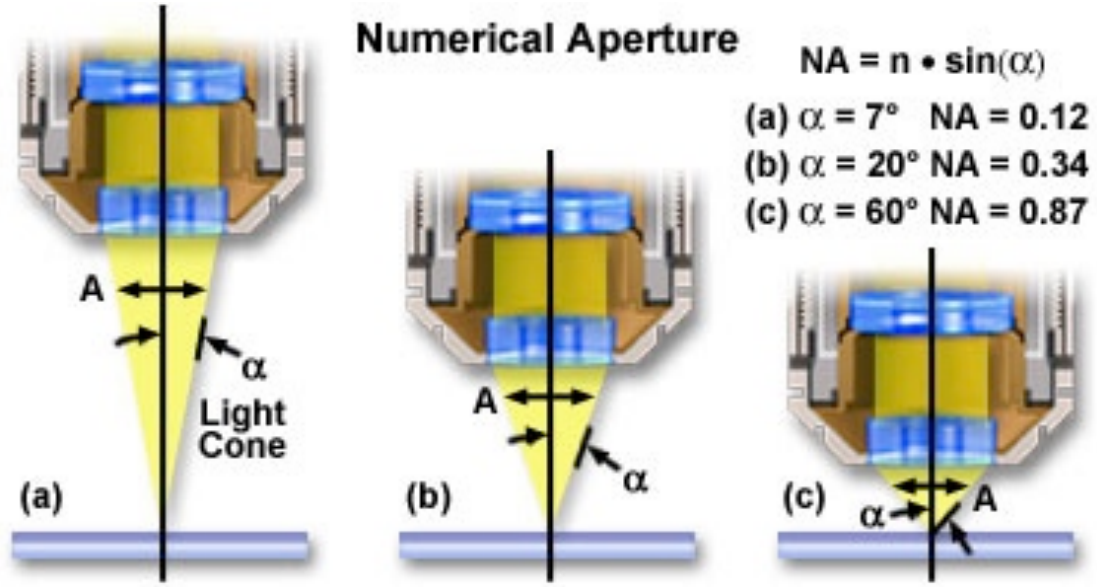


Figure 23 - Relationship between working distance and NA [20]

Manufacturer	Correction	Magnification	Numerical Aperture	Working Distance
Nikon	PlanApo	10x	0.45	4.0 mm
Nikon	PlanFluor	20x	0.75	0.35 mm
Nikon	PlanFluor (oil)	40x	1.30	0.20 mm
Nikon	PlanApo (oil)	60x	1.40	0.21 mm
Nikon	PlanApo (oil)	100x	1.40	0.13 mm

Table 2 – Magnification, working distance and NA [21]

Coverslip Thickness

This is an important topic in microscopy and is usually not given the attention it deserves, even by well-established researchers. Use of a #1.5 (170µm) coverslip and proper preparation of samples will yield better, more reliable images. Different thicknesses of coverslips will produce differing amounts of spherical aberration; most microscope objectives are designed to correct for the spherical aberration imparted by 170µm coverslips. The combination of working distance and coverslip thickness will determine how deep you can image within your sample. For more information on this subject, please see the Nikon and Microscopy UK websites:

http://www.nikon.com/products/instruments/resources/tech/info/microscope_tech/coverglass/index_02.htm [**22**]

http://www.microscopy-uk.org.uk/index.html?http://www.microscopy-uk.org.uk/primer/geomop.htm [**23**]

Correction Collars

Correction collars are generally used to correct for spherical aberration due to variations in coverslip thickness, temperature, wavelength or for the differing refractive indices of different immersion media.

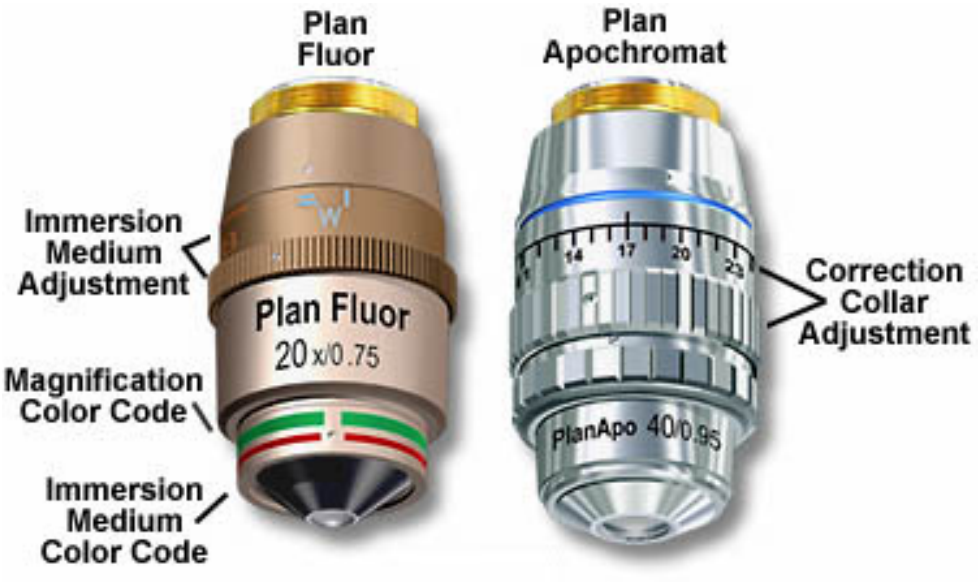


Figure 24 - Objectives with correction collar for coverslip thickness. [24**]**

Immersion Media

Since the numerical aperture of an objective depends on the refractive index of the medium between the objective and the object under inspection, we can increase the numerical aperture (and hence increase the resolution) by using a fluid with a higher index of refraction than that of air (1.00). Common choices include water (n = 1.33), glycerol (n = 1.46), and immersion oil (n = 1.51).

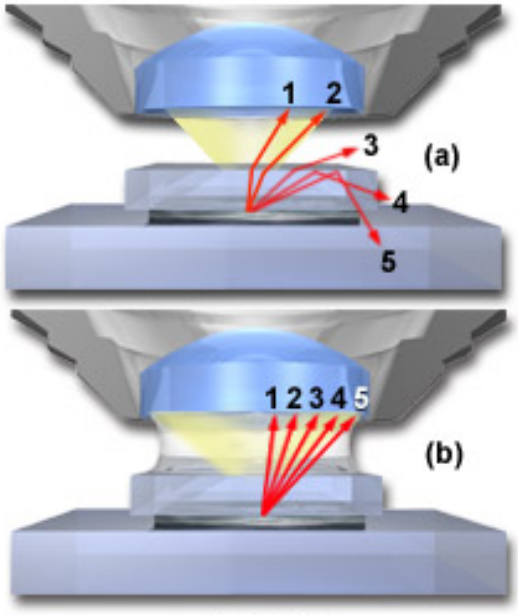


Figure 25 - Effect of immersion oil on the effective numerical aperture of an objective [25]

Field Number

Field number (usually designated as FN on an objective) indicates the size of the field of view available through an eyepiece in millimetres. This number indicates the diameter of the field of view produced by the eyepiece alone.

Equation 3 $$\text{Viewfield Diameter} = \frac{(\text{FN})}{(\text{M(O)x M(T)})}$$ **[26]**

where FN is the field number in millimeters, M(O) is the objective magnification, and M(T) is the tube lens magnification factor (if any). Applying this formula to a typical widefield eyepiece with FN = 22mm, we arrive at the following for a 40x objective with a tube lens magnification of 1.25:

$$\text{Viewfield Diameter} = \frac{(22)}{(63 \text{ x } 1.25)} = 279um$$

However, be aware that almost all microscopes will have some inherent magnification due to the internal optics of the system, not including the objectives. This is known as the instrument factor magnification value, so keep in mind that Eq. 3 is only useful to calculate an approximate value for the Viewfield diameter.

Labelling

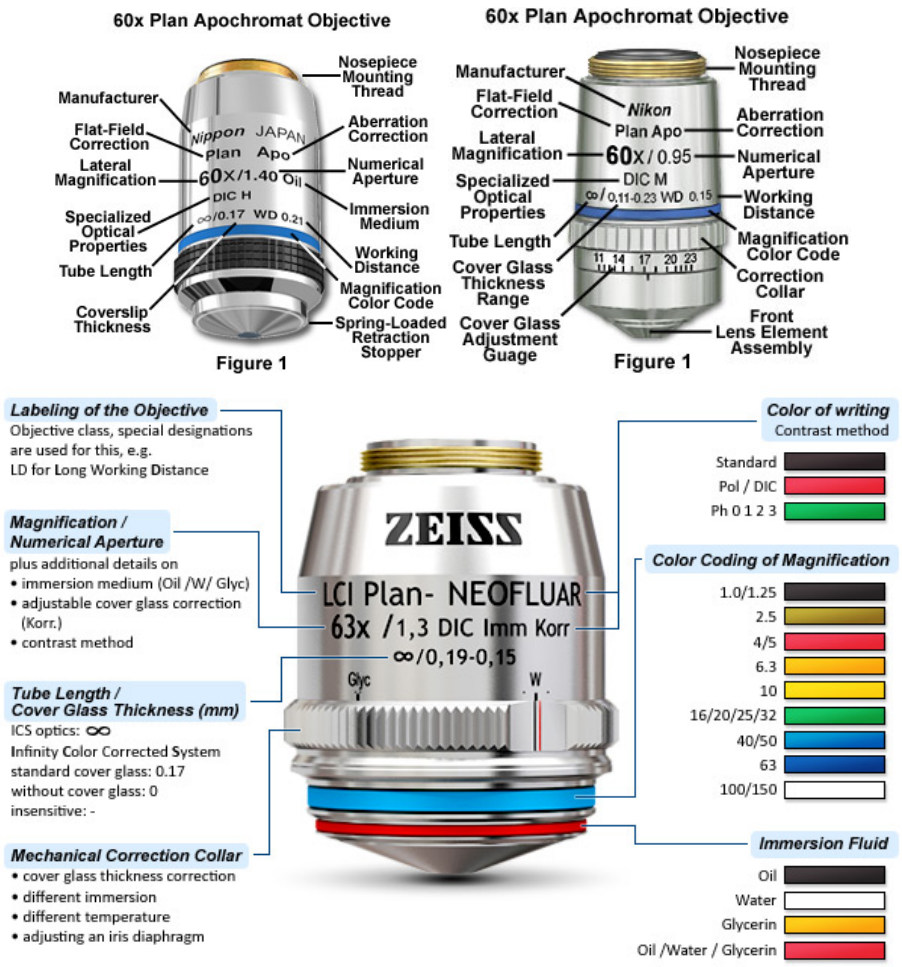


Figure 26 - Objectives with inscribed notation [24] [27] [28] [29]

Objectives are labeled slightly differently by all manufacturers, however they do generally present the most important information such as magnification, NA and immersion media in large text in the centre of the objective. Other useful information may be presented with either text or colour coding. It is important to understand how to read this information so that you may select the best objective for your sample, and to know the limitations of the objective.

Infinity Corrected Optics

Infinity-corrected microscope optical systems, which have overtaken the microscope market, are designed to enable the insertion of auxiliary optical devices, such as vertical illuminators, filter cubes, and intermediate optics such as DIC prisms, into the optical pathway between the objective and eyepieces without introducing spherical aberration, requiring focus corrections, or creating other image problems. In a fixed tube length finite optical system, light passing through the objective converges at the image plane to produce an image. The situation is significantly different for infinity-corrected optical systems where the objective produces a flux of parallel light wavefronts imaged at infinity, which are brought into focus at the intermediate image plane by the tube lens.

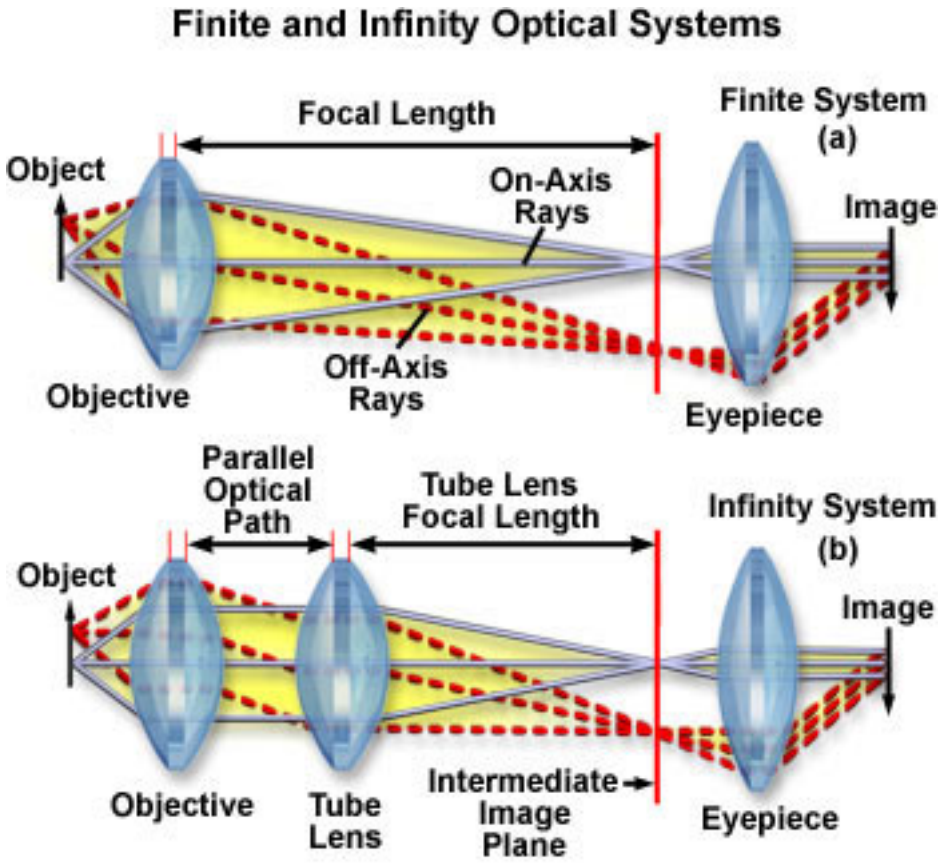


Figure 27 – Finite vs Infinity corrected optical systems [30,31**]**

Transmitted Lightpaths

Introduction

Light microscopes may image specimens through two different methods, transmission (diascopic illumination) or reflection (episcopic illumination). Transmission, as the name suggests, transmits light through the specimen and onto the eyes or detector. This is the simplest, most versatile and cheapest method of imaging samples.

Stereo Microscopes

Stereo microscopes are used for imaging relatively large specimens. The light source in the base of the microscope transmits light through the specimen which is then collected by the large main objective and focussed at infinity (aka Infinity corrected objective). This light is split into two parallel paths, both of which pass through a series of zoom lenses that adjust the amount of magnification. On the stereo microscopes at the IMB, the zoom factor is continuously variable from about 3x to 180x. The light passes through a tube lens to produce an intermediate image, which is rotated by 180° (or *erected*) by a set of prisms before being magnified by the eyepieces.

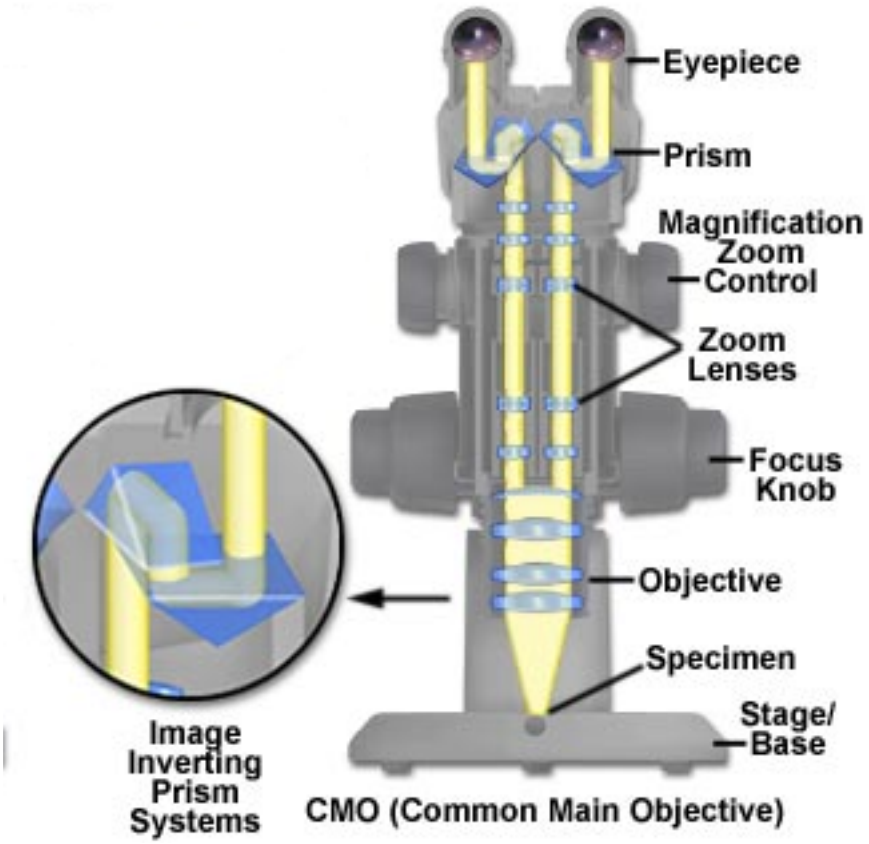


Figure 28 - Stereo microscope lightpath [32]

Upright Microscopes

Upright microscopes are typically used for routine imaging of fixed slides, as they allow easy sample exchange and XY manipulation. On an upright microscope, such as the Olympus BX51's at the IMB, light is provided by a halogen lamp mounted in a housing at the bottom rear of the microscope. The light rays are collimated (made parallel) by the collector lens in the lamp housing, and infrared light is eliminated by an IR filter. The light then passes through a diffuser, to evenly illuminate the specimen, as well as a colour-correcting filter to remove the reddish colour cast from the light. Still in the base of the microscope, the light passes through neutral density (ND) filters to reduce the intensity of the light, if so desired, and is focussed by the field lens, a mirror then directs the light upwards through the field stop and out of the base of the microscope. The field stop limits the field of illumination at the specimen and helps to reduce glare thus preserving contrast in the specimen. The condenser focuses light onto the specimen which is then captured by the objective and collimated (infinity corrected optics) before being focussed by the tube lens onto the oculars and then onto the eyes.

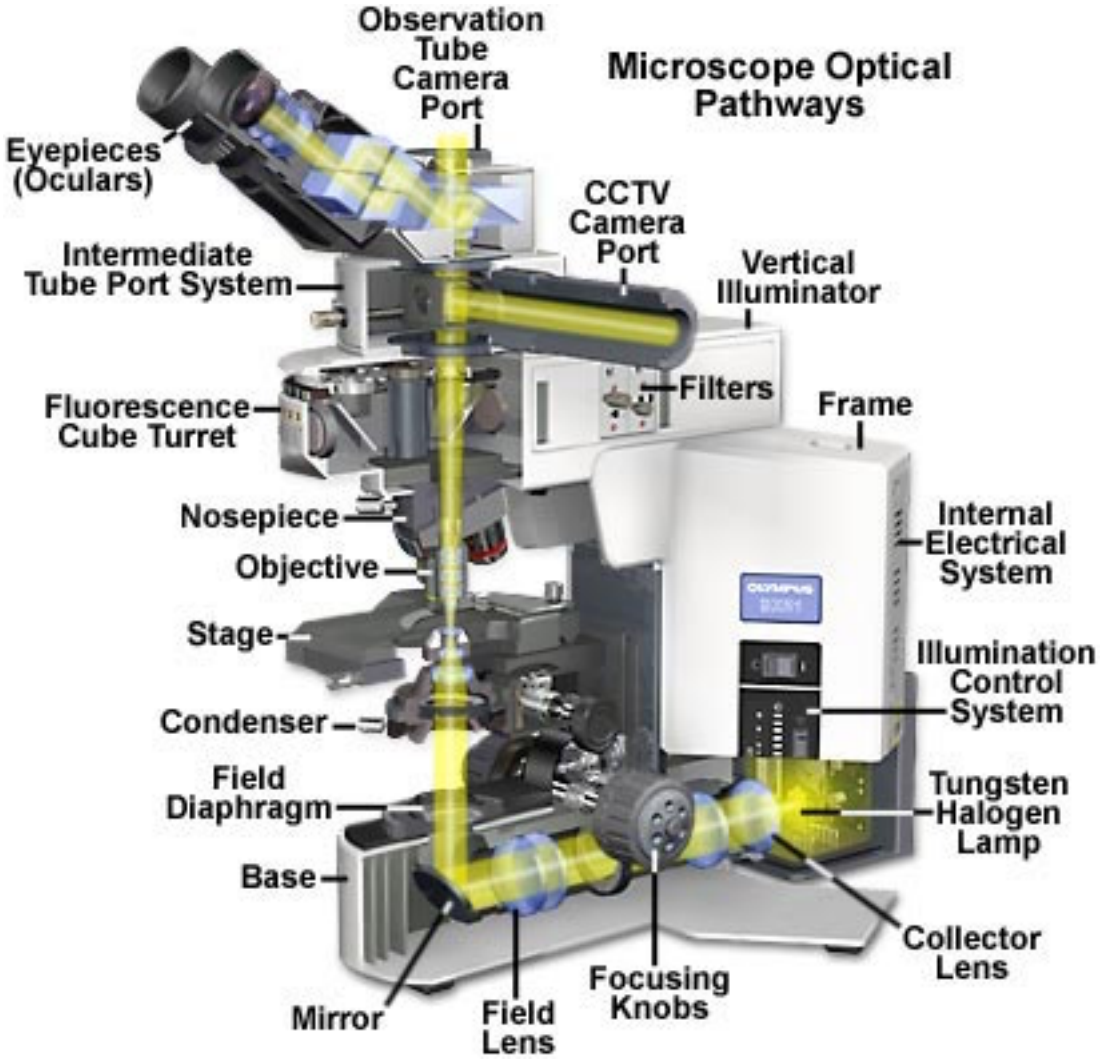


Figure 29 - Upright microscope lightpaths [33]

Inverted Microscopes

Inverted Microscopes are commonly used in biological imaging as they allow samples to be mounted in dishes containing liquids and imaged from below through the coverslip. Inverted microscopes also make it more convenient to change samples without refocussing the objective lens. Inverted microscopes have the same optical components as an upright scope, just upside down; so you will find the lamphouse, field stop and condenser above your sample, whilst the objective and tube lens is situated below the sample.

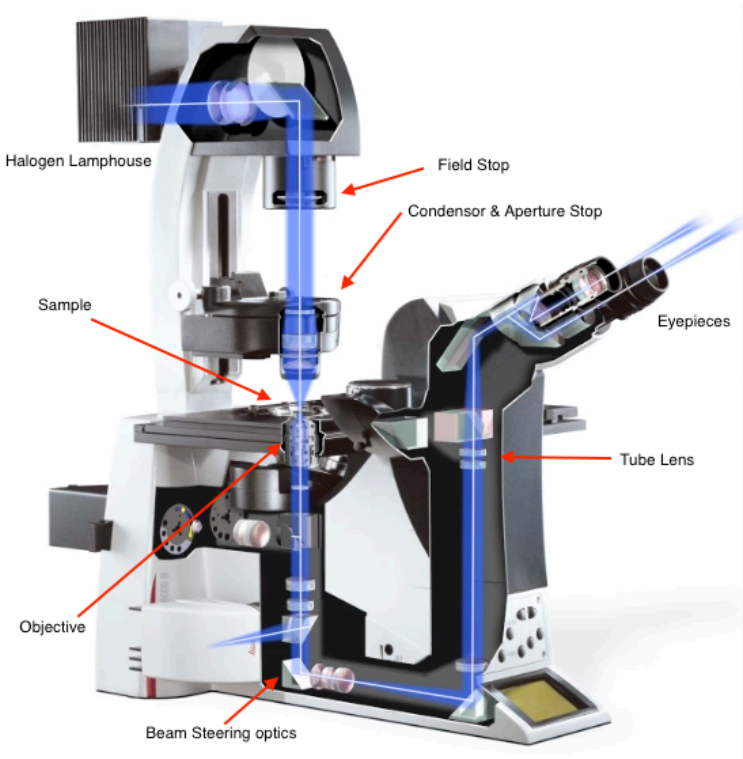


Figure 30 - Inverted microscope lightpaths [34]

Camera Lightpath

Cameras are typically mounted above the eyepieces on an upright microscopes via a trinocular mount or through the side ports of an inverted microscope. The sample image is focused onto the camera by the tube lens and is thus outside of the infinity space of the microscope.

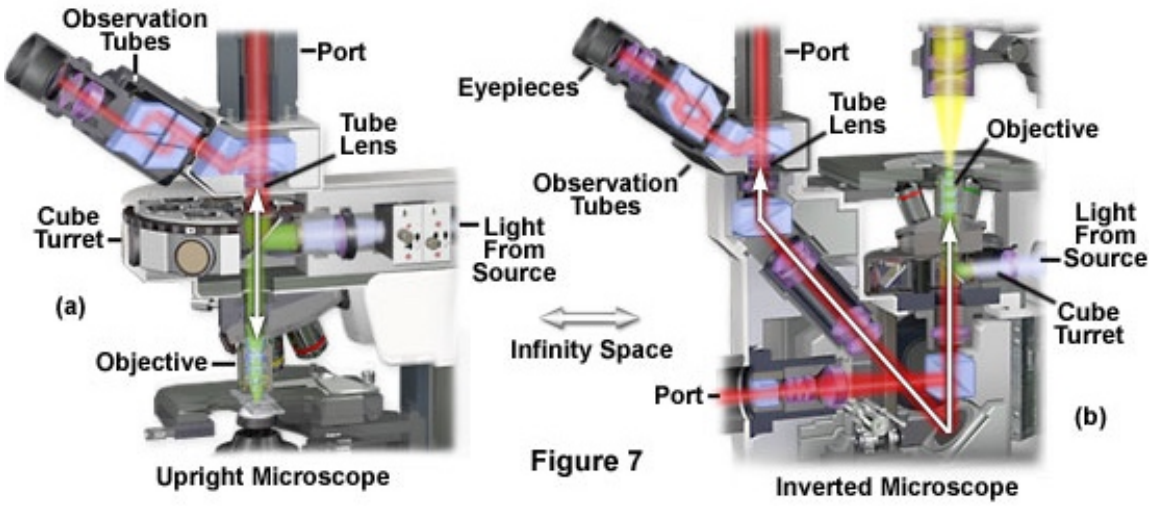


Figure 31 - Infinity space in upright and inverted microscopes [35]

Relay Lens

In order to focus the sample image onto the eyepieces or camera sensor a tube lens is mounted within the microscope body as discussed previously. However the diameter of the light column from this tube lens is typically 22mm in diameter or larger, which is often much larger than the camera sensor. Modern scientific grade cameras come with a variety of sensor and pixel sizes as well as resolutions, so a relay lens is attached to the front of the camera body to focus the light to better match the size of the sensor. By selecting a relay lens with the correct magnification we may also optimize the lightpath to achieve the maximum achievable image resolution as discussed in a later chapter. Most scientific cameras use the c-mount thread standard, so often relay lenses are called c-mounts.

Figure 32 – Camera c-mount incorporating a relay lens [36]

Capturing an Image

Introduction

The first images describing samples viewed through a microscope were hand drawn illustrations made whilst viewing the sample. Over time, improvements in microscope light source stability and brightness as well as optics allowed projection of the magnified image onto a surface so that the illustrations were made over the projected image, improving accuracy and decreasing human bias from the image. With the advent of film based cameras, micrographs were able to be made of the image greatly improving the accuracy of the recorded image, whilst in recent years digital cameras are now able to capture still images as well as record videos of live specimens.

CCD Cameras

A charge coupled device image sensor, or **CCD**, is the most common type of image sensor in use in microscopy today. a CCD image sensor is made of a rectangular array of picture elements, or **pixels**, which generate electrons in response to incoming photons during an exposure – more light means more electrons. These electrons build up in each pixel for the duration of an exposure. Once the exposure is done, the groups of electrons are shifted along the rows of pixels in order to be read out and recorded. The analogy usually used is of a bucket brigade where the buckets are arranged in a regular array and fill up with water as rain comes down on them. Each location in the array is analogous to a pixel and the water is analogous to the electrons that build up as photons rain down on the sensor. To read out the values, the last row of buckets is shifted to the end of the array and, one by one, the buckets are shifted to the end of the row where the amount of water in each bucket is recorded. All the other rows are shifted towards the end of the array by one space, replacing the row that was moved off, and leaving the first row empty. This process is repeated until the amount of water in all of the buckets has been recorded.

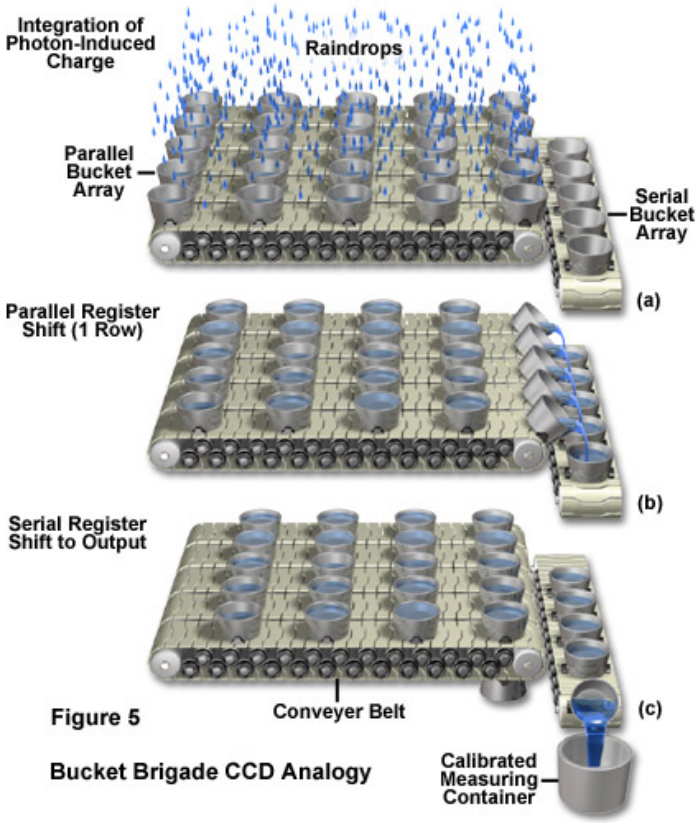


Figure 33 - CCD bucket brigade [37]

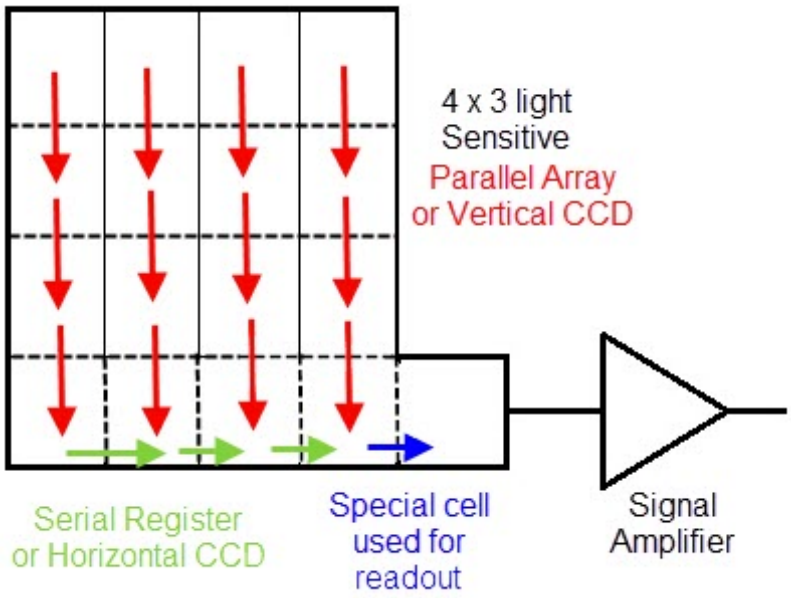


Figure 34 - CCD operation [38]

Additionally, we can imagine that instead of moving all the buckets, we just pour the water from one bucket to another. This will introduce some error, obviously. We can also imagine that all of this is sitting in a turbulent sea that looks like paint on a loudspeaker so that, no matter what, there is always going to be some water splashing into the buckets, even on a cloudless day. This is analogous to the effect of thermal electrons on our signal. Talk about mixing your metaphors!

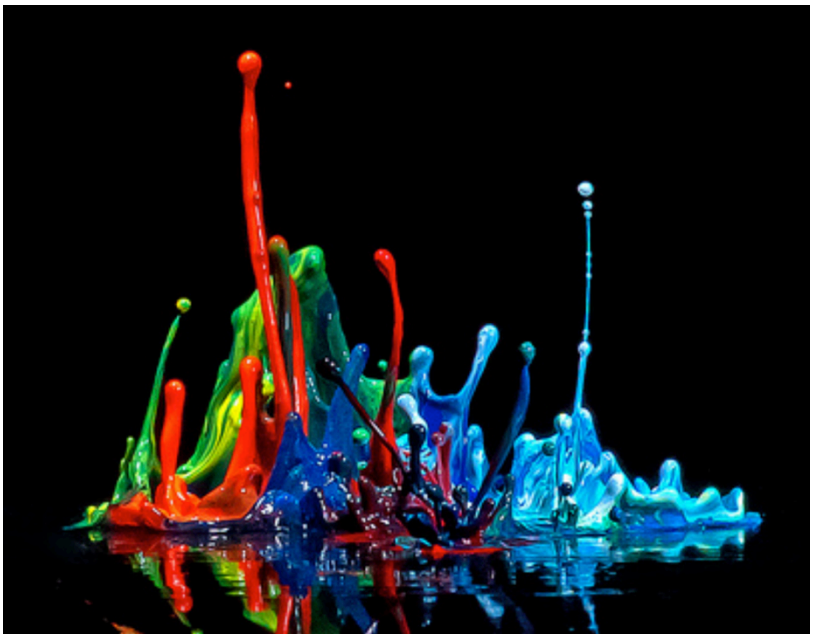


Figure 35 - A metaphor for thermal electrons [39]

Scientific grade cameras use Peltier or water cooling to reduce the thermal electron noise from images.

Colour vs. Mono

Each pixel in a colour camera is generally represented by four pixels in the imaging sensor. As the actual photodiodes detecting the incoming light can't discriminate between different wavelengths of light, filters are laid over them to filter out everything but red, green, or blue light. Since the human eye is much more sensitive to green light which is the strongest component of the Suns emission spectrum, there are two green, one red and one blue filter element. Thus less than the full available area is devoted to detecting signal from each colour band of interest. Monochromatic cameras have no such colour filter and are therefore more sensitive to light from the sample, an important consideration when imaging dim and/or delicate fluorescent samples.

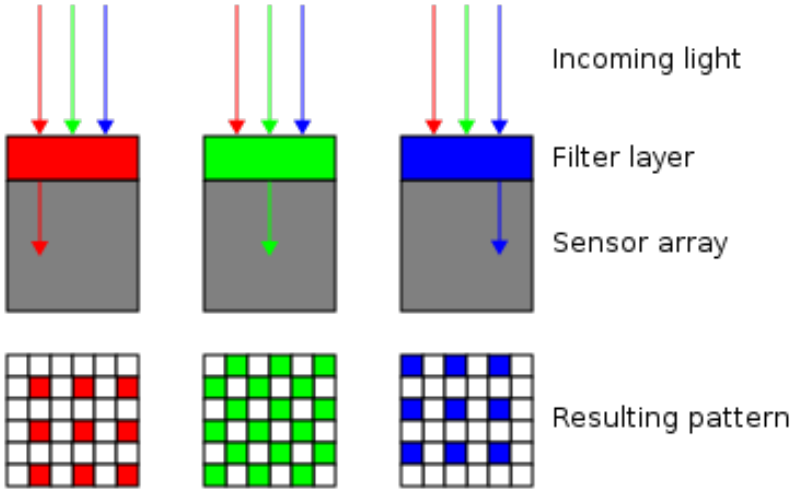


Figure 36 - Bayer filter [40]

Camera Pixel Size

As with everything in microscopy there are trade-offs when it comes to pixel size. Smaller pixels mean more detail but, because they are smaller, they hold fewer electrons and thus have a decreased capacity to register differing light levels.

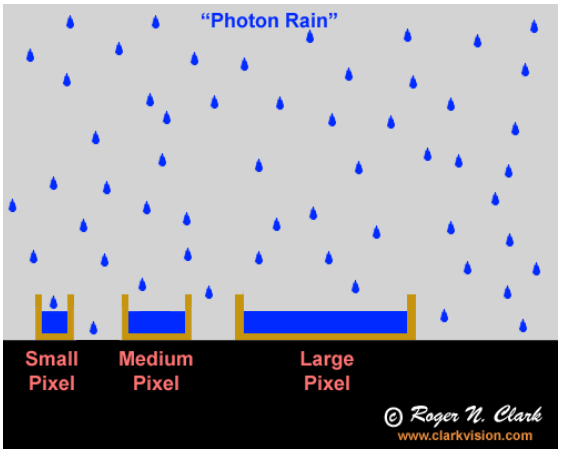


Figure 37 - The effect of pixel size on well depth [41]

Dynamic Range and Well Depth

Dynamic range is the maximum difference in signal intensity that can be represented by a camera system. For CCDs this is often expressed as the ratio of the maximum number of electrons a pixel can hold (the full well capacity) to the readout noise of the CCD. Readout noise is just imperfection in the measurement of the number of electrons in each pixel of the image.

Camera	Pixel Size µm2	Full Well Capacity e-	Read Noise e-	Dynamic Range	Decibels dB	Bits
iXon3 885 EMCCD *EM Amplifier with No Gain (dynamic range higher with EM gain)*	8 x 8	30,000	20 (x3 pre-amp)	1,500	64	11
Luca S EMCCD *EM Amplifier with No Gain*	10 x 10	25,000	15	1,667	64	11
iXon3 897 EMCCD *Conventional Amplifier @ 1MHz (dynamic range higher with alternative pre-amp)*	16 x 16	180,000	9 (x1 pre-amp)	20,000	86	15
Newton 920 CCD *50kHz Readout Rate*	26 x 26	510,000	10 (x3 pre-amp)	51,000	94	16

Table 3 – Comparison of different cameras dynamic range [42]

Saturation

Saturation occurs when camera pixels have collected as much charge as they can. Any additional electrons generated are lost (and thus information about the sample is lost, too). These additional electrons may spill over into neighbouring wells resulting in erroneous measurements including smearing or blooming of the image.

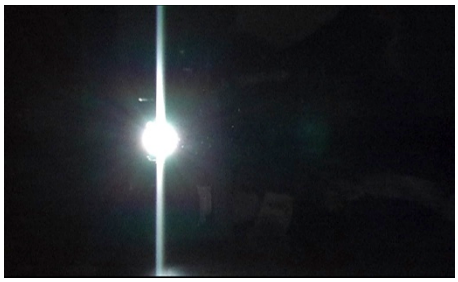


Figure 38 – Pixel saturation of a CCD camera [43] [44]

Bit Depth

Bit depth is an indication of how many grey levels can be encoded in an image: more bits means more grey levels. An 8-bit image uses $2^8 = 256$ grey levels to encode brightness levels, a 12-bit image uses $2^{12} = 2096$ grey levels, and a 16-bit image uses $2^{16} = 65{,}536$ grey levels. Using a greater bit depth results in fewer artefacts after processing your images. Keep in mind that even the best computer monitors can only display 10-bit images. Another advantage of using higher bit depth is that a user is able to capture the same amount of intensity variation information as a lower bit depth camera with less light intensity or exposure time.

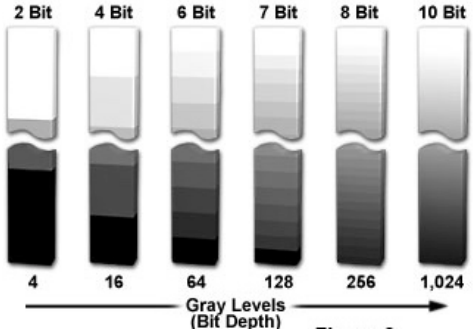


Figure 39 - Bit depth and grey levels [45]

Histogram

An image histogram shows the number of pixels for each grey level in an image and is graphed as number of pixels versus grey value. This can give the microscope operator important information about background levels in an image as well as whether any pixels are overexposed.

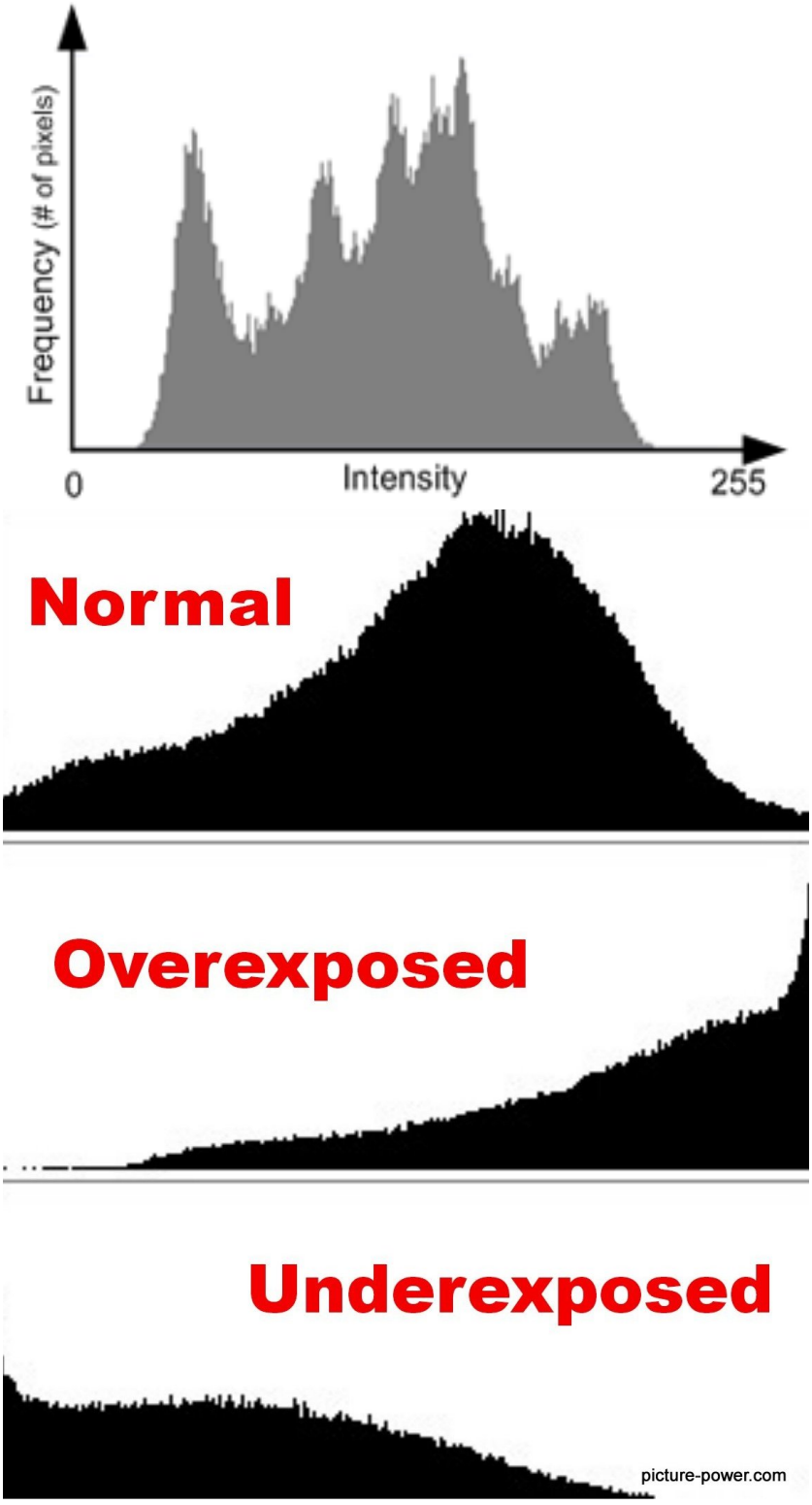


Figure 40 - Image histograms [46]

Gain and Offset

Gain is the amount of amplification placed on the signal coming out of the imaging chip and is analogous to the volume control on a stereo. More gain means greater sensitivity, but it also means more noise (which makes fine features difficult to discern).

A camera chip will always produce *some* signal, if only because the thermal vibrations of atoms in the chip give just enough of a kick to some electrons to push them into the detection well, as well as signal resulting from read noise and other factors. Because of this, we may need to tell our detector how much charge actually correlates to zero signal. This is the offset level.

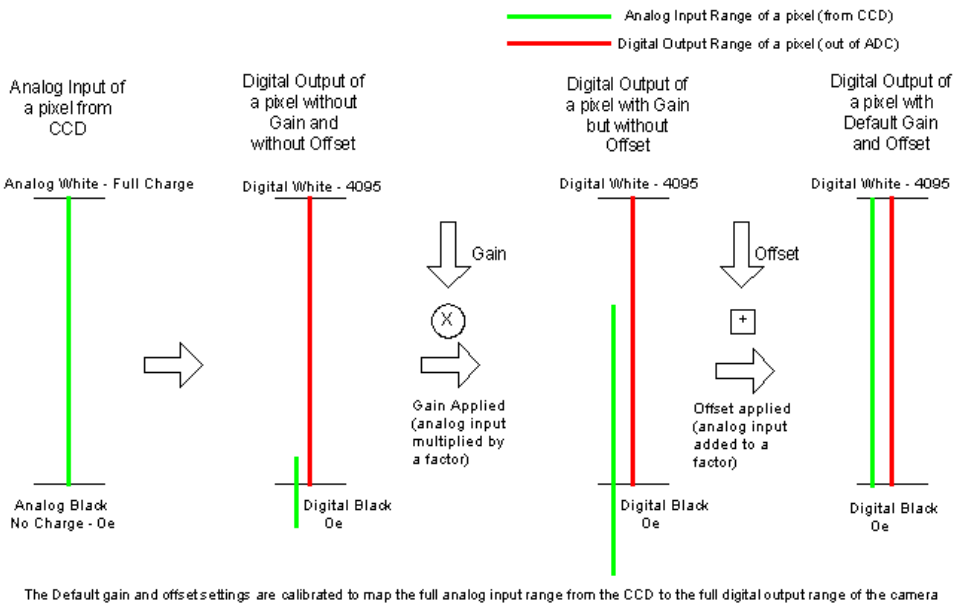


Figure 41 - Gain and offset illustration [47]

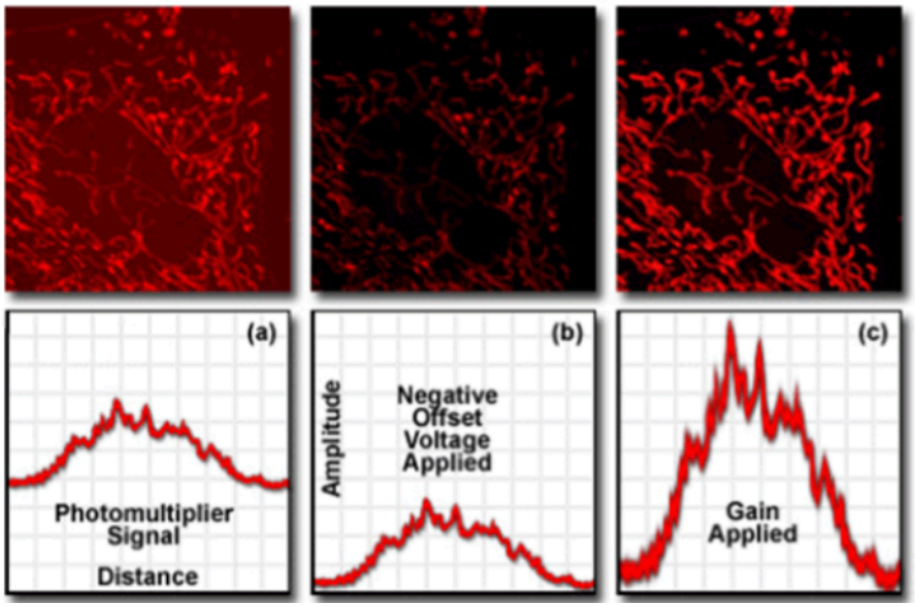


Figure 42 - Gain and offset adjustment of an image [48]

For more information on image detectors, please see the Nikon MicroscopyU and Olympus Microscopy Resource Center websites:
http://www.microscopyu.com/articles/digitalimaging/ccdintro.html [**37**]

http://www.olympus.magnet.fsu.edu/primer/digitalimaging/digitalimagingdetectors.html [**49**]

Contrast Enhancement

Introduction

Contrast is the difference in colour or luminance that makes an object or its image distinguishable from adjacent objects or the immediate background. The human eye is only able to distinguish objects with an apparent contrast of 2% or greater, so for many transparent biological samples a method for contrast enhancement is required to visual structures within the specimen.

Percent contrast is defined as:

Equation 4 $$(C) = ((I(s) - I(b)) \, x \, 100)/I(b)$$ **[50]**

where I(s) = is specimen intensity and I(b) is background intensity.

Process	**Apparent Contrast %**
Transparent specimens in Brightfield	~ 2 to 5
Phase Contrast	~ 15 to 20
Differential Interference Contrast	~ 15 to 20
Fixed & stained specimens in Brightfield	~ 25
Darkfield	~ 60
Fluorescence	~ 75

Table 4 – Apparent contrast of various imaging methods [50**]**

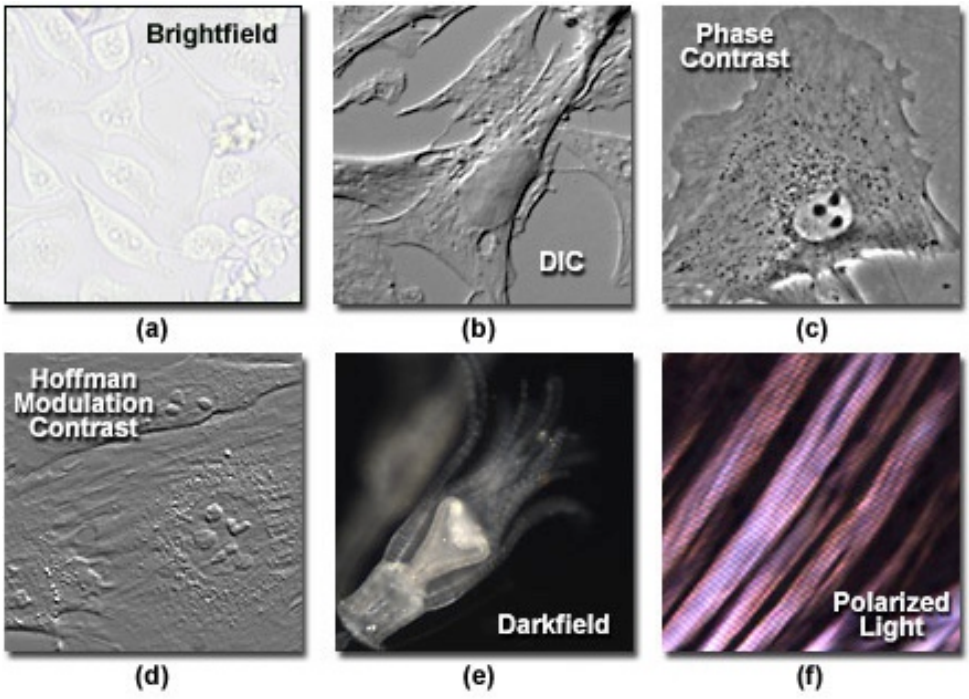


Figure 43 - Images from various contrast enhancement methods [51**]**

Brightfield

Brightfield illumination is a deceptively simple illumination technique. Light is transmitted through the specimen, with differential absorption yielding colour and / or amplitude contrast; a coloured / darkened image against a bright background.

Brightfield is hampered in use by the low contrast inherent in many biological samples. Contrast can be improved during sample preparation through the incorporation of coloured or absorbant dyes binding specific chemicals or structures of interest. Contrast and image quality is, in addition, heavily influenced by characteristics of sample illumination. It was the drive to improve brightfield image contrast through sample illumination that led to the development of Koehler illumination (below) in the late 19th century.

Koehler Illumination

Correctly configured illumination of the specimen is of critical importance in achieving high-quality images in microscopy.

Early efforts at sample illumination typically produced uneven illumination or an image of the light source filament on the sample. Koehler illumination, the configuration of optical elements and their alignment developed by August Koehler in 1893, provides both an extremely even illumination of the sample, and ensures that an image of the illumination source is not visible in the sample image plane. Instead, a defocussed image of the light source appears on the sample plane and its conjugate image planes.

This is achieved through the introduction of a collector lens and condensor system, which produces a set of light source conjugate image planes in addition to the specimen conjugate image planes.

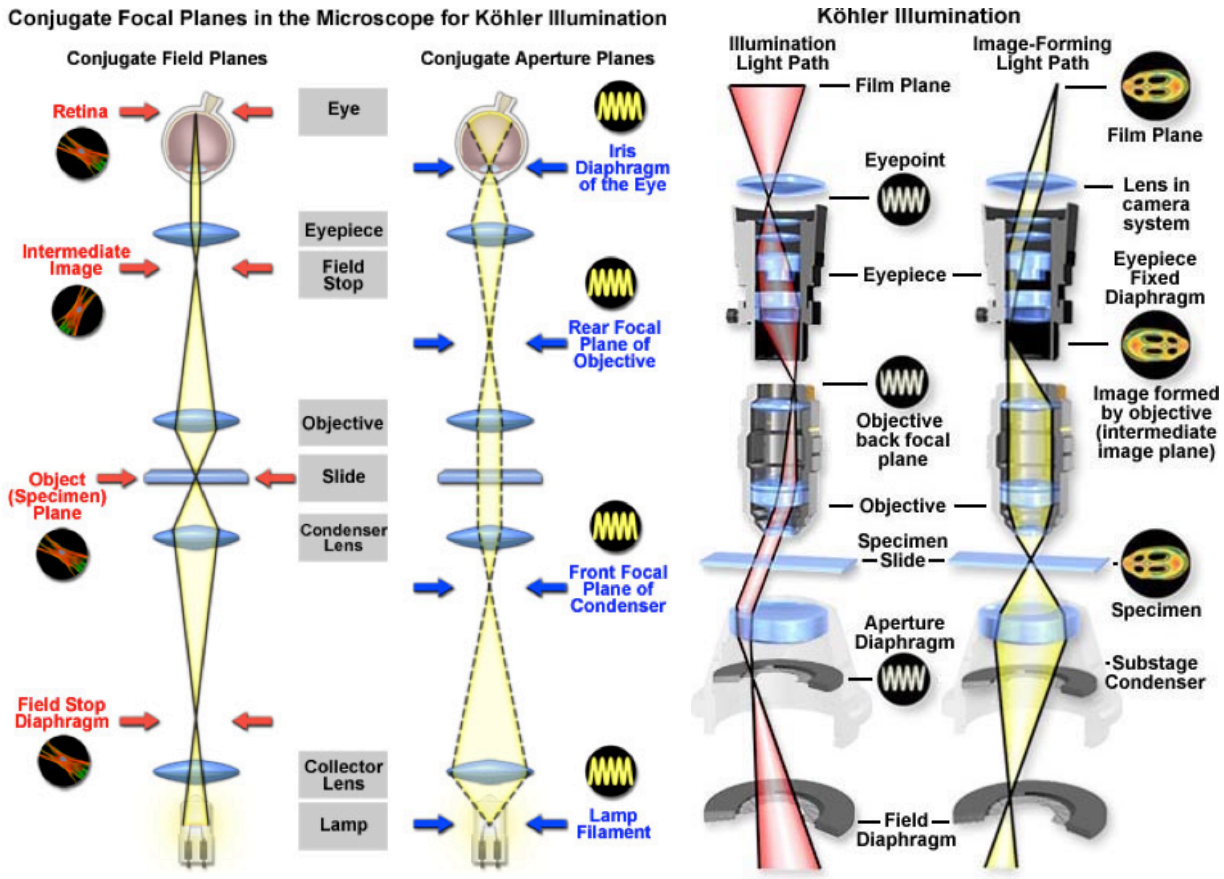


Figure 44 – Specimen and illumination conjugate planes [52,6]

The Koehler system comprises a collector lens, field diaphragm (or field stop), condensor diaphragm (or aperture diaphragm / aperture stop) and condensor lens system, placed in order, respectively, between the illumination source and the sample. Adjustment and positioning of these components is critical to performance. Adjustment of the field diaphragm serves to limit flare derived from excess light, while the aperture diaphragm controls both image contrast and depth of field. Focussing and lateral movement of the condensor system places its optics on the correct conjugate plane and in axial alignment.

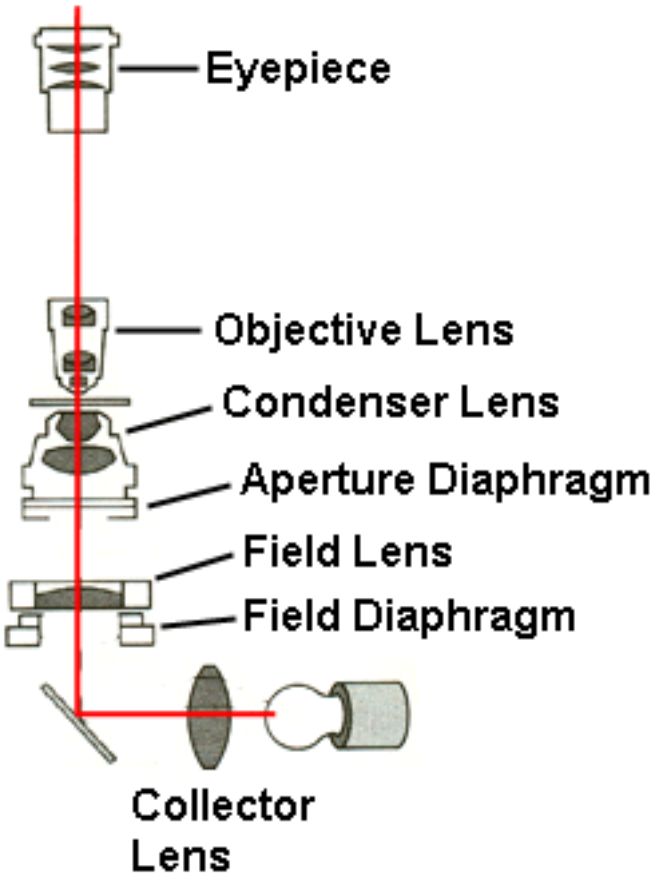


Figure 45 – Brightfield Lightpath [53]

Note that correct set up of Koehler illumination is an essential prerequisite for advanced illumination techniques such as phase contrast and differential interference contrast.

Darkfield

Darkfield illumination is a contrast enhancing technique employing oblique illumination. Specimens under darkfield appear brightly lit against a dark (almost black) background.

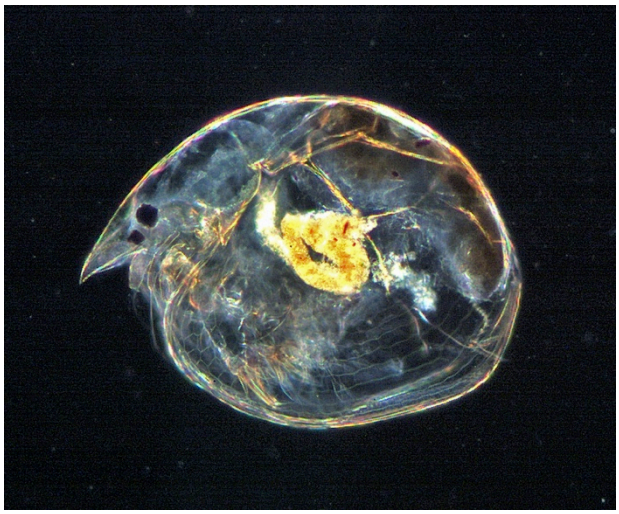


Figure 46 – Darkfield Image of Daphnia, 100x [54]

At its simplest, the darkfield apparatus consists of an opaque disk placed on the optical axis at the front focal plane of the condensor. This blocks the central portion of the illumination beam, and ensures that only a hollow cone of light derived from the periphery of the condensor's field will impinge upon the specimen.

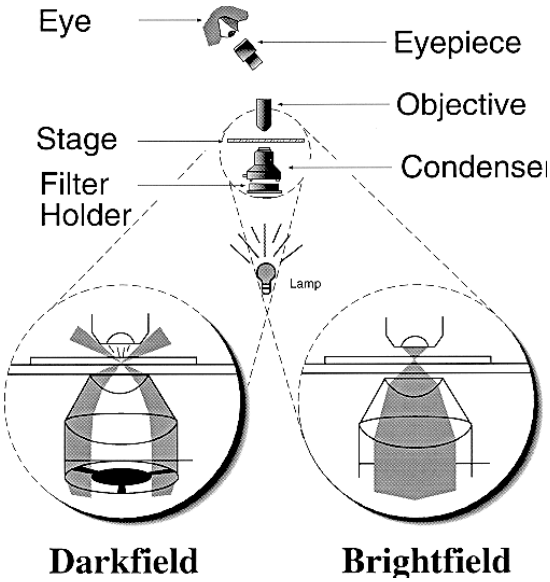


Figure 47 – Light passage through the condensor in brightfield and darkfield illumination [55]

The technique relies upon the numerical aperture of the condensor being greater than that of the objective. In this circumstance and in the absence of a specimen, the oblique illuminating rays cross and fail to enter the objective. The field of view thus appears dark. When a specimen is placed in focus, the oblique rays cross the specimen. Optical discontinuities such as membranes, organelles and cuticular striations reflect, diffract, and/or refract a portion of this light into the objective.

Darkfield specific condensors refine the delivery of the illuminating light yielding a higher effective numerical aperture, in turn allowing use of objectives of higher resolving power.

Amplitude and Phase Objects.

In simple terms, objects that absorb (or otherwise redirect) a portion of transmitted white light passing through them produce a reduction in the intensity, or amplitude, of that light. Wavelength dependent differential absorption (and, for completeness, reflection) produces changes in perceived colour. In microscopy, objects that predominantly influence transmitted light in this fashion are called Amplitude Objects.

In some specimens differences in refractive index between areas or structures within the sample, and between the specimen and its surrounding medium, alter the relative velocity of the transmitted light. The thickness of the structure or specimen also has an effect. The waveform is thus moved out of phase with that of unmodified surrounding light. Objects that predominantly influence transmitted light in this fashion are called Phase Objects.

In practice, most objects influence both the amplitude and phase of transmitted light. Note that the human eye and cameras/detectors see differences in amplitude (as contrast); they can not easily see differences in phase.

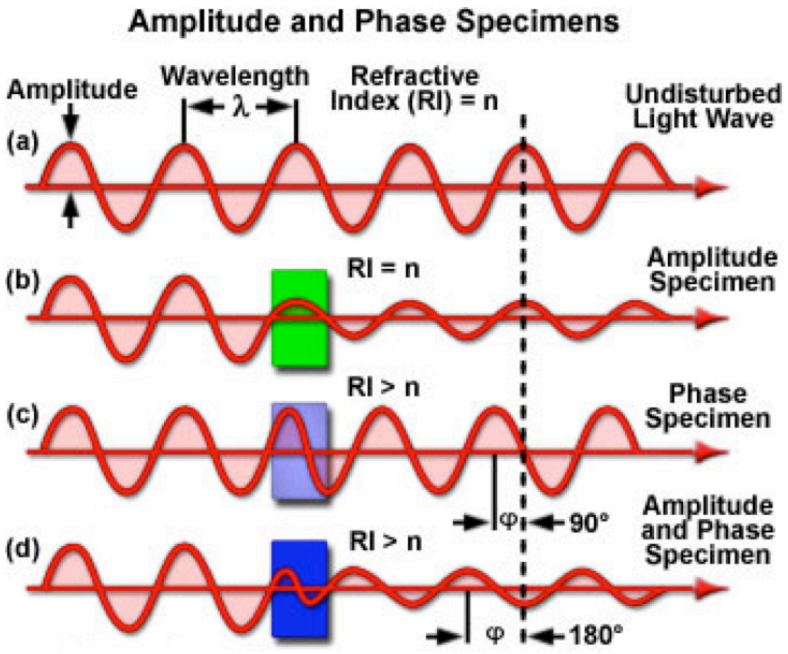


Figure 48 – Specimen influences on amplitude and phase [56**]**

Whilst contrast in many fixed specimens may be increased through use of stains, this is not suitable for some structures. Nor can stains usually be employed for living cells and tissues, which typically have inherently low visible contrast.

Techniques allowing conversion of changes in phase into changes in viewable amplitude have thus proved of value in the observation of features difficult to image with conventional brightfield illumination, and in the observation of living specimens with minimal disturbance.

Phase Contrast.

Phase contrast utilizes phase differences between the various components in a specimen and the surrounding medium together with diffraction by the specimen to form an amplitude image.

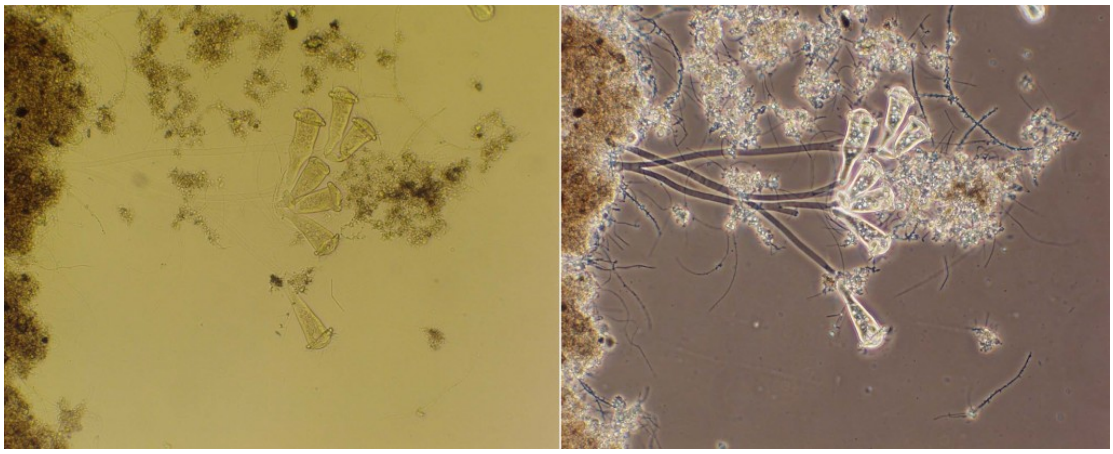


Figure 49 – Brightfield (left) and phase contrast (right) images of protists. [57]

The phase contrast system comprises an annulus, positioned in the front focal plane of the condensor and a complementary phase plate (or ring) positioned in the back focal plane of the objective. In the most common form of phase contrast used today, Positive Phase Contrast, the objective phase plate is of materials that advance light by approximately ¼ wave, and reduce its intensity. Note that the objective phase plate is now not typically a discrete component, but takes the form of a ring etched or deposited upon one the the objective optical elements.

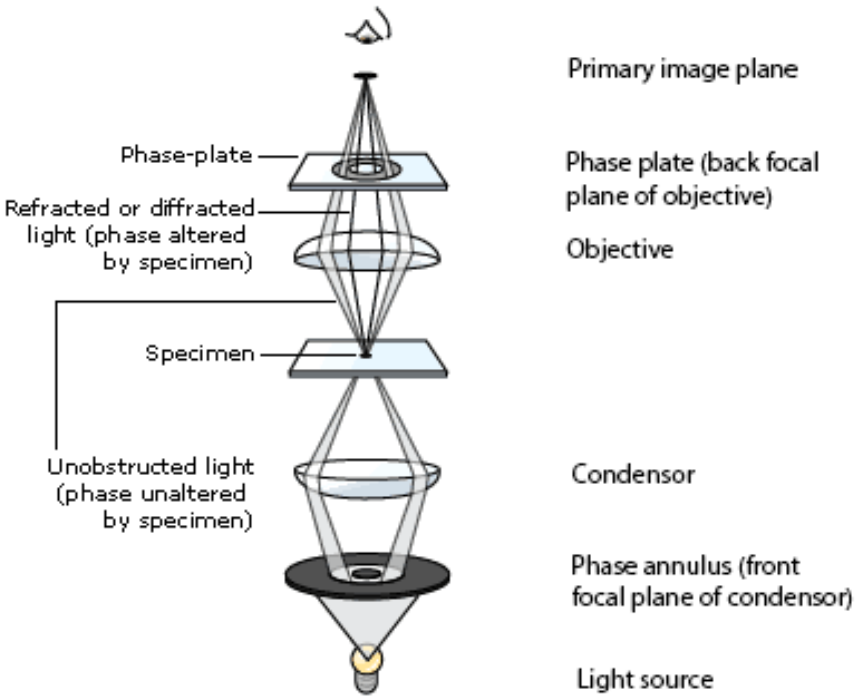


Figure 50 – Schematic view of basic phase contrast components [58]

Light passing through and diffracted by the specimen is typically retarded by up to ¼ wave with respect to light passing through the surrounding medium alone, due to specimen inherent differences in refractive index. It is then focussed by the objective onto the primary image plane. Note, most of the specimen light (specimen wave) bypasses the ring in the objective's back focal plane and is hence not affected by its phase altering properties. However, light passing around the specimen unaltered (the surround wave) is predominantly focused on and passes through the phase ring in the objective, where it is reduced in intensity and advanced by ¼ wave. At the primary image plane the specimen and surround waves, now up to ½ wave out of phase, undergo destructive interference. This destructive interference produces amplitude variations, seen as contrast.

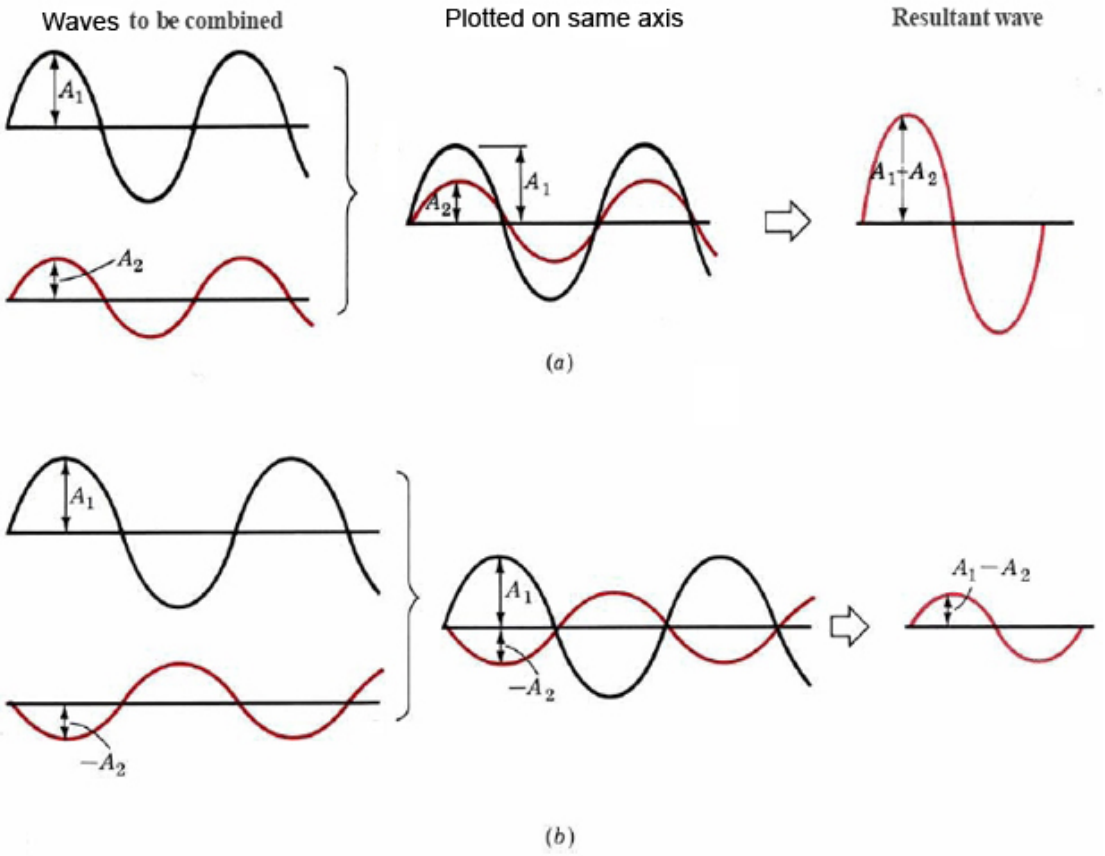


Figure 51 – Constructive (a) and Destructive (b) Interference of Light Waves [59**]**

Phase contrast imaging is not without limitations, and images should be examined carefully for artifacts. Principally, these will be related to the following:

1. Outlines of details and objects are usually surrounded by halos. These sometimes obscure boundaries.
2. Shifts in phase that occur from areas below or above the specimen focal plane tend to confuse the image and distort image detail. This becomes increasingly troublesome with increasing specimen thickness.
3. The phase annuli limit the working numerical aperture of the optical system somewhat, thus reducing resolution.

Halo artifacts are reduced in some modern implementations through the use of an apodized phase plate. This differs from the basic phase plate in that the neutral density component is extended both axially and abaxially of the phase advancing ring itself. The extended areas are of higher density than that upon the phase advancing area, and this serves to dim the interface between the specimen and surround waves and hence, suppress halo formation. The current Nikon phase contrast system is a good example of an apodized phase contrast implementation.

Birefringence.

Birefringence is an optical property of a material where its refractive index depends on the polarization and propagation direction of (the transmitted) light [**60**]. It represents an anisotropy, derived from the structure of the material's chrystalline lattice.

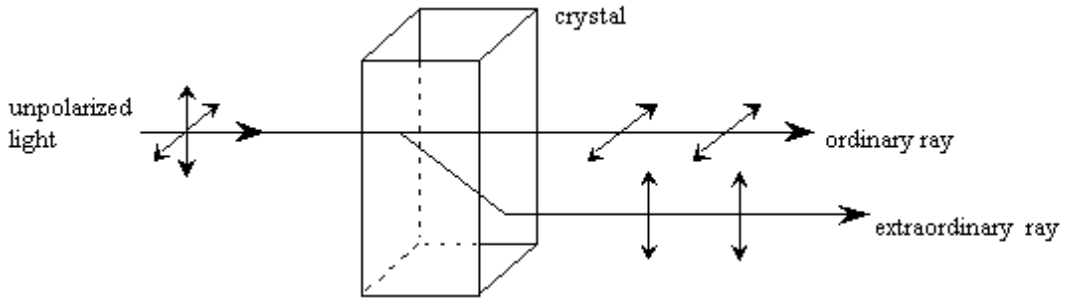


Figure 52 – Birefringence in a calcite crystal illustration [61**]**

Light entering along the optical axis of an anisotropic crystal behaves as in an isotropic crystal and passes through at a single velocity. However, when light enters a non-equivalent axis, it becomes refracted into two rays polarized at right angles to one another, and traveling at different velocities. This can be seen as the production of a double image.

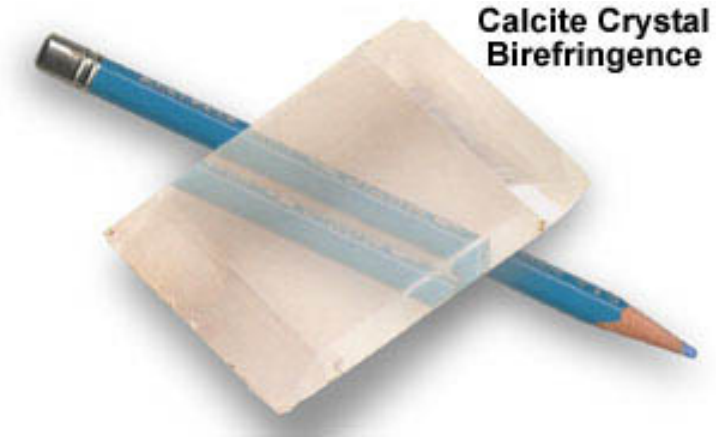


Figure 53 – Birefringent image of a pencil in a calcite crystal [62**]**

The birefringent properties of optical grade calcite is employed in the Wollaston/Nomarski prisms underlying differential interference contrast. Moreover, the birefringent characteristics of some specimens themselves can be exploited to enhance their visualization.

Polarisation

Polarised light microscopy is a method of contrast enhancement particularly suited to birefringent (optically anisotropic) specimens.

The technique requires the addition of a polarising filter within the illumination light path and a second polarising filter, known as the analyser, placed between the objective back focal plane and the image detector (or eye).

The interaction of the plane-polarized illumination with the birefringent specimen produces two wavefronts, now polarized in mutually perpendicular planes. The velocities of these are differentially altered, according to propagation direction with respect to the plane of birefringence. The now phase shifted wavefronts are recombined by the analyser, undergoing constructive and destructive interference to produce visible contrast.

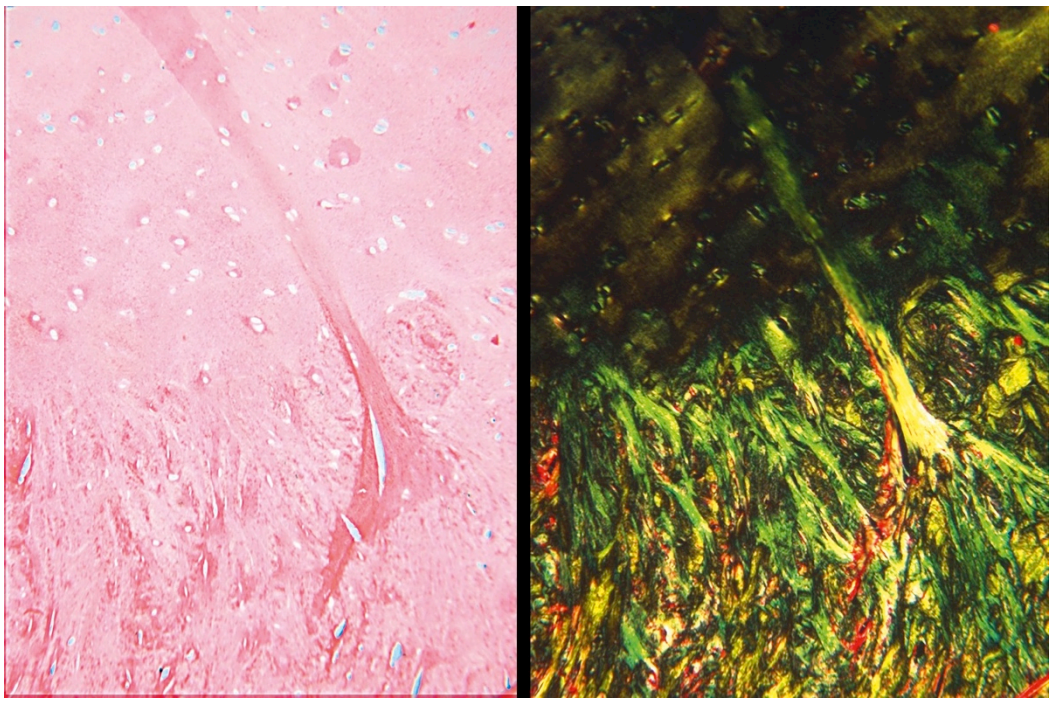


Figure 54 – Brightfield and polarised light views of Sharpey fibres inserting into apophyseal hyaline cartilage [63]

Polarised light microscopy is applicable to a wide range of biological chemicals, macromolecules and structural assemblies, as well as the examination of the structure and composition of natural and manufactured fibres and minerals.

DIC (Differential Interference Contrast)
Differential interference contrast relies on phase gradients to generate image contrast in low contrast (transparent) specimens.

The specimen is illuminated by polarised light, split into two mutually coherent wavefronts spatially displaced (sheared) at the sample plane. The spatial separation of these wavefronts, termed the reference and sample beams, is typically about 0.2 µm. They are, in addition, now orthogonally polarised; ie, rotationally separated by 90°.

As they traverse the sample and/or medium the sample and reference beams may encounter areas differing slightly in optical path length (the product of geometric path length and refractive index), producing a shift in phase of one beam relative to the other. The passage of many such beam pairs and their resultant absorption, diffraction and scattering by the specimen results in the production of two complete images, separated by orthogonal polarisation. Subsequent recombination into a single image with a common plane of polarisation produces interference, generating amplitude differences as a function of optical path length.

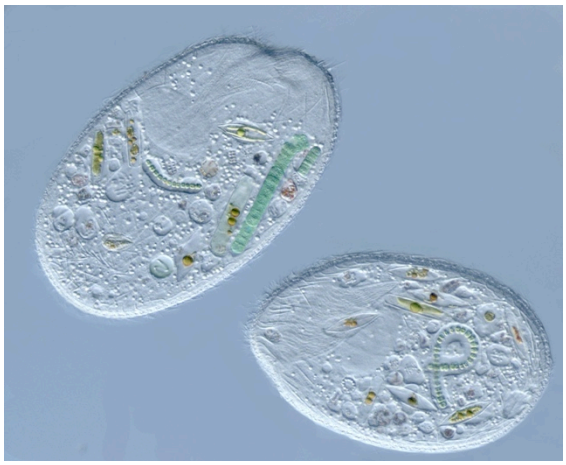


Figure 55 – DIC image of Sonderia sp., by Lipscomb, D. (2012). [64]

The basic DIC system consists of a polarising filter and Wollaston prism inserted into the optical pathway before the microscope's condensor, and a second Wollaston prism and polarising filter mounted after the objective. As devised by Francis Smith in 1955, the Wollaston prisms must be mounted at the front focal plane of the condenser and the rear focal plane of the objective. A modification of the Wollaston prism by Georges Nomarski in the late 1950s allowed mounting away from these conjugate planes. Modern DIC typically utilizes the Nomarski form of the prism; hence DIC is often referred to as Nomarski DIC or Nomarski illumination.

The birefringent Wollaston/Nomarski prisms produce the initial shearing and recombination of the light, and the changes in polarisation seen following initial polarisation. The final polarising filter serves to eliminate directly transmitted light. Modern systems frequently incorporate a translatable upper prism, which allows the addition or subtraction of a constant phase shift or bias value to the overall phase shift distribution. This provides considerable adjustment of final contrast.

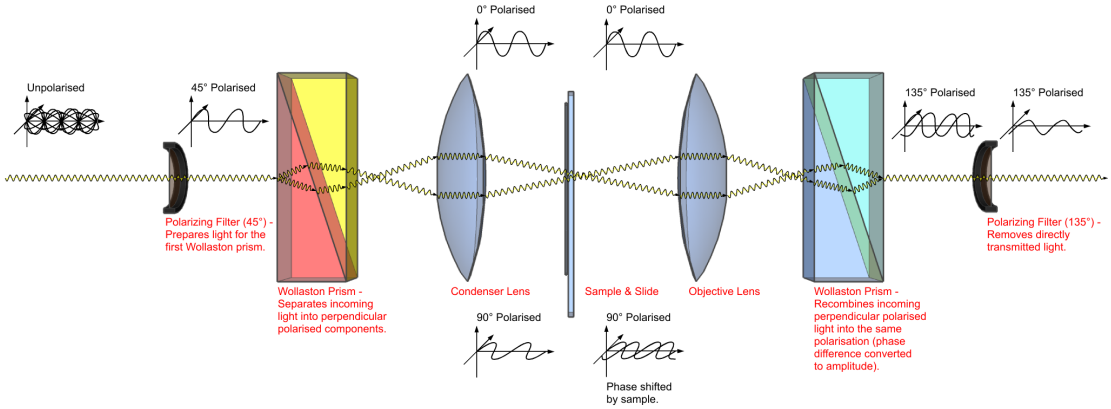


Figure 56 – DIC light path showing principle components. [65]

The DIC image is a representation of the gradient of, or spatial rate of change in, optical path lengths. It presents a striking pseudo three dimensional appearance, but this is wholly illusory; it does not correspond to actual specimen topology. Indeed, the apparent relief of features can be altered or even inverted by rotation of the sample, altering the orientation of structures with respect to the prism's shear plane.

DIC does not restrict system numerical aperture, and images do not exhibit halos around large objects or those with marked refractive index variations, as seen in phase contrast. Images are thus of higher resolution, and detail is often less obscured.

DIC is not suitable for imaging birefringent specimens or specimens within birefringent vessels, such as plastic petrie dishes and plates, etc. This due to the effect of the birefringence upon polarised light. In addition, very thin specimens may yield better images using phase contrast.

Basic Fluorescence

Introduction

Fluorescence is the property of a substance to absorb light at a particular wavelength and, after a brief period, to emit light of longer wavelength. Attaching a fluorescent entity (a fluorochrome) to a suitable probe produces a fluorophore, which can be engineered to bind to specific chemicals, proteins or sites of interest within living and non-living biological samples. With the correct illumination the labelled structures can then, themselves, appear to fluoresce. From an visual perspective fluorescence can thus be seen as another method of contrast enhancement.

Each fluorophore absorbs light within a specific range of wavelengths, and emits light over a specific band of longer, lower energy wavelengths.

Jablonski diagram.

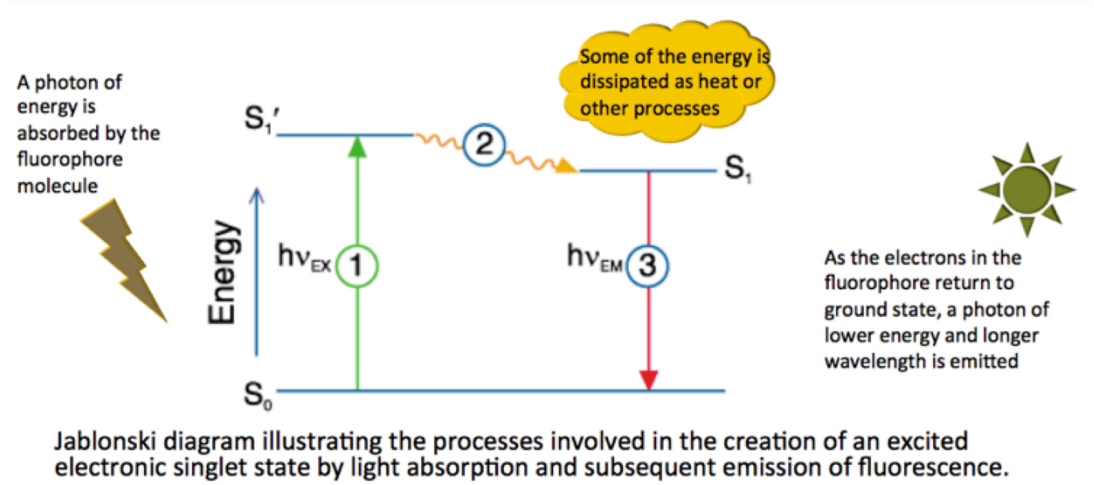


Figure 57 – Jablonski Diagram [66]

The energetic characteristics of fluorescence can be represented by a Jablonski diagram. The simplest way to interpret this diagram is as a representation of transitions in the energy state of one electron in the electron shell of one molecule. As shown, absorption of a photon of the correct energy level raises an electron to a higher state (1) This is excitation. After a period of time (~10^{-7} to 10^{-9} seconds) and the loss of some energy (typically via vibrational relaxation) (2), the electron drops back to the ground state emitting, in the process, a photon (3) This is emission.

Stokes shift.
Note that in simple fluorescence the emission photon wavelength is longer than the excitation; that is, the emitted photon is of lower energy. The difference between the peaks of the excitation and emission wavelength curves is known as the Stokes Shift, and is due to the energy losses in the excited state. The degree of Stokes Shift is fluorophore specific, and can range from a few to several hundred nanometres.

Differing degrees of Stokes Shift can be exploited in multi-fluorochrome imaging. For example; florochromes with similar excitation needs but emission bands sufficiently separated may permit simultaneous two colour imaging with a single laser, whilst those with sufficiently different excitation bands but similar emission may be able to be captured sequentually through a single filter set.

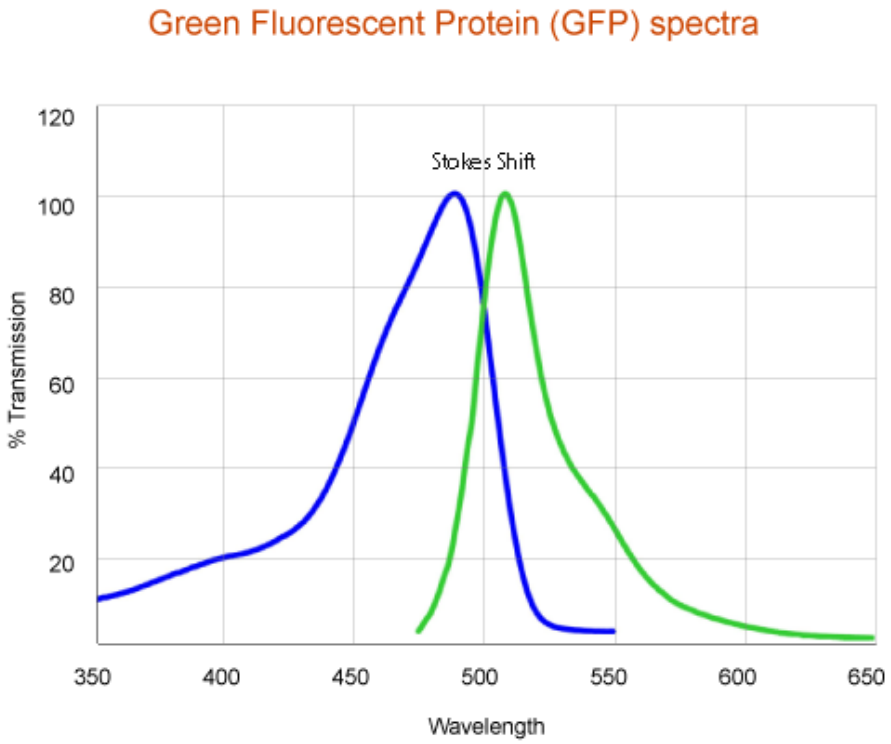


Figure 58 – Excitation and emission spectra of GFP with Stokes shift. [48]

Quantum Yield, Extinction Coefficient and Fluorescence Lifetime.
The extinction coefficient of a fluorophore is a measure of the molecule's ability to absorb light, while quantum yield may be thought of as the probability of the molecule emitting a photon in response to excitation. These are of course influenced by factors such as pH, the medium the fluorescent molecule is suspended in, other molecules it is bound to or in close proximity to, its concentration, and the excitation wavelength used during measurement. The intensity of light output per molecule of fluorophore (ie, its "brightness") is proportional to the product of its quantum yield and extinction coefficient, under the conditions used in their measurement. [**67**] [**68**]

The fluorescence lifetime of a fluorophore is time the molecule remains in an excited state, prior to returning to the ground state. Whilst tending to be characteristic for a given fluorophore, it too can vary over a wide range in response to environmental conditions. Fluorescence Lifetime Imaging Microscopy (or FLIM) exploits the environmental sensitivity of fluorescence lifetimes to derive information on the fluorophore's local environment. This may include aspects such as the pH and concentration of ions in the vicinity of the probe, the presence of photosensitizers (for example, in tumours) or flavanols (in plants), or the interaction of chemicals in cellular structures. [**67**] [**69**]

Fluorophore	Excitation Peak	Emission Peak	Extinction Coefficient $M^{-1}cm^{-1}$	Quantum Yield	Brightness (EC*QY) $(mM*cm)^{-1}$	Fluorescence Lifetime τ(ns)
DAPI + dsDNA	356	455	30600	0.58	18	2.2
mCFP	433	475	32500	0.40	13	>4
EGFP	488	507	56000	0.60	34	3.5
Alexa Fluor 488	495	519	73000	0.92	67	4.1
Fluorescein (pH 8.4)	495	519	75000	0.92	69	4.1
EYFP	514	527	83400	0.61	51	3
CY3	548	562	136000	0.15	20	0.3
Alexa Fluor 546	556	573	112000	0.79	88	4.1
DsRed - Monomer	556	586	35000	0.10	3.5	
Rhodamine B	562	583	88000	0.31	27	1.68
mCherry	587	610	72000	0.22	16	
Texas Red	589	615	84000	0.90	76	4.2
Alexa Fluor 594	590	617	92000	0.66	61	3.9
Alexa Fluor 647	650	668	270000	0.33	89	1

Table 5 – Optical properties of selected fluorophores

Data in this table derived from: Shaner, N., Steinbach, P., & Tsien, R. (2005) A Guide to Choosing Fluorescent Proteins. Nature Methods, 2(12), 905-9; Olenych, S., Claxton, N., Ottenberg, G., & Davidson, M. (2007) The Fluorescent Protein Color Palette. Curr Protoc Cell Biol. 21 (5), 1-34; Nifosı`, R. & Tozzini, V. (2012) One-Photon and Two-Photon Excitation of Fluorescent Proteins. In: Jung, G. (ed.), Fluorescent Proteins I, Springer Ser Fluoresc 11, 3–40; Anon (2016) Lifetime Data of Selected Fluorophores, ISS Data *Tables*; Anon (2016) Fluorescence Quantum Yield Standards, ISS Data *Tables*; Anon (2016) The Alexa Fluor Dye Series. Note 1.1. Thermofischer Scientific *Website*; Anon (2016) Fluorescence Quantum Yields (QY) and Lifetimes (τ) for Alexa Fluor Dyes. Table 1.5. Thermofischer Scientific *Website*; Anon (2016) Cyanine Dyes: CY3, CY5 and Others. Atdbio *Website*; Anon (2016) Long-Wavelength Rhodamines, Texas Red Dyes and QSY Quenchers. Section 1.6. Thermofischer Scientific *Website*; Prahl, S. (2012) 4',6-Diamidino-2-phenylindole, [DAPI]. Oregon Medical Laser Centre *Website*.

Bleaching and Photo Toxicity.
Bleaching is the loss of a fluorescent probe's ability to fluoresce. This occurs due to irreversible changes in the molecule with repeated excitation.

The effect is that as the sample is viewed or imaged, its fluorescence fades.

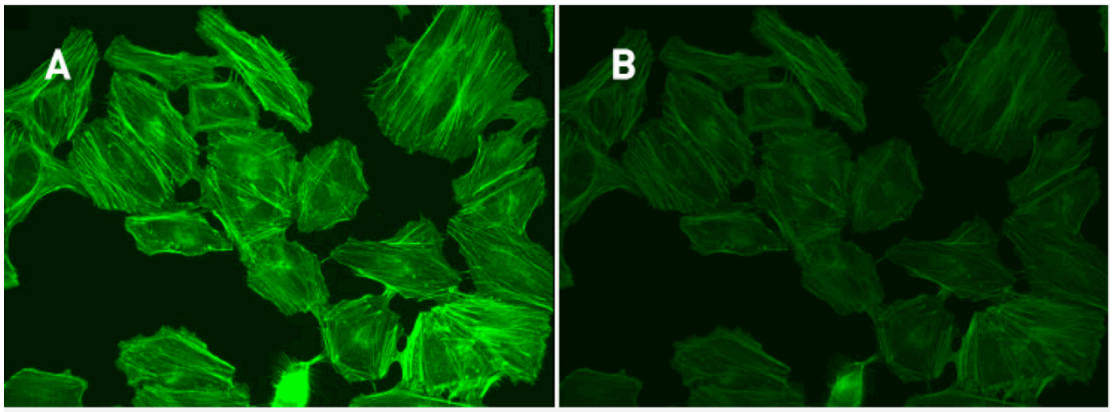


Figure 1. HeLa cells were fixed and labeled with FITC-conjugated phalloidin. Coverslips were mounted in 50% glycerol (in PBS). Panel (A) shows the initial intensity of the fluorophore, while panel (B) shows the photobleaching that occurs after 36 seconds of constant illumination.

Figure 59 – Bleaching of fluorophores [70]

The mechanism of photobleaching is poorly understood. Modification of covalent bonds within the fluorescent molecule, and interactions with surrounding molecules have been implicated. Electronic interactions with light in the presence of oxygen, in addition, can yield singlet state oxygen free radicals that can chemically modify (damage) other molecules in living cells.

Phototoxicity is the damage or death of living samples induced through the interaction of a fluorescent dye and excitation light. In addition to the production of oxygen radicals during photobleaching, many fluorochromes become highly toxic to cells following even relatively short periods of illumination. The illumination of standard culture media components, such as tryptophan and riboflavin, too may be deleterious (Nikon site).

Susceptibility to photobleaching varies with the fluorochrome. Some photobleach quickly, emitting only a few photons, while others can undergo thousands or millions of excitation – emission cycles (Molecular Expressions site). This base rate can degraded by unfavourable environmental variables, such as a high oxygen concentration, temperature, etc.

Photobleaching is an inherent property and thus can be slowed, but not eliminated. Reducing the level of illumination and minimizing exposure time can help to extend viewing time. Fixed samples can be infused with antifading agents (which are essentially oxygen scavengers) during mounting.

Reducing illumination intensity and duration can also help minimize phototoxicity. Choosing fluorochromes excited by the lower energy photons of longer wavelengths is also useful.

Deliberate, localized photobleaching can be exploited to obtain specific information. For example, monitoring the recovery of fluorescence within a deliberately bleached area can provide information on the rate of diffusion of molecules into that area. This technique is called FRAP (Fluorescence Recovery After Photobleaching).

The rates at which fluorophores photobleach can also provide information. Fluorescence Resonant Energy Transfer, or FRET, is a technique used to examine close proximity of one fluorescently tagged molecule to another, tagged with a different fluorophore. The excited donor fluorophore transfers energy to the acceptor through dipole – dipole coupling. This is a non radiative process that takes place over very short distances (1-10nm). FRET efficiencies are calculated from the photobleaching rates of the donor in the presence and absence of an acceptor. (Another method, sensitised emission FRET, uses the degree to which brightness of the acceptor emission is boosted and can provide information on the proximity of the two molecules).

Such measurements permit monitoring of close range molecular interactions, such as protein–DNA interactions, protein-protein interactions, and protein conformational changes.

Note that an excited fluorophore can collide with another molecule to transfer energy in a second type of non-radiative process, quenching, in which no light is emitted.

For further information please follow the links:

http://micro.magnet.fsu.edu/primer/java/fluorescence/photobleaching/ [**71**]

http://www.microscopyu.com/references/phototoxicity.html [**72**]

Excitation sources.

The quantum yield of a fluorophore is the ratio of the number of quanta emitted to the number of quanta absorbed. For most this is quite low, with the result that labelled structures produce relatively few photons for detection. Illumination sources for fluorescence thus need to be intense.

Lamps.

The most common lamps for fluorescence used on widefield microscopes are mercury short arc lamps. These range from approximately 50 to 200 watts and emit a continuous spectrum, punctuated by a number of intense spikes. Mercury arc lamps have been used for this purpose for many years, and a large number of fluorophores have been designed to take advantage of its emission peaks. Brightness, on average, is 10 to 100 times that of a similar wattage halogen lamp.

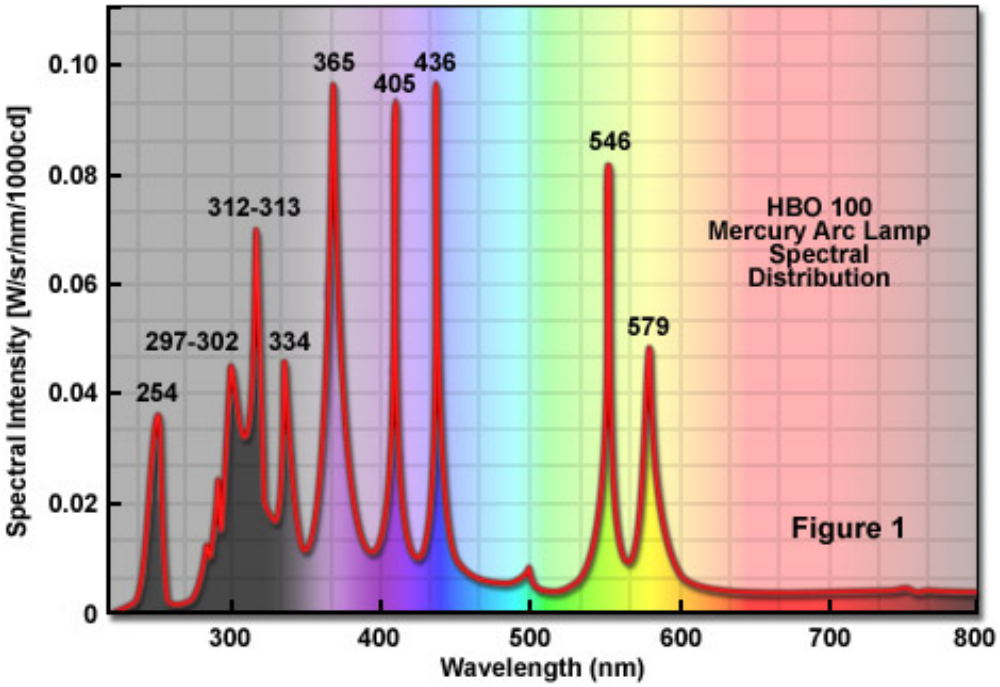


Figure 60 – Mercury arc lamp emission spectrum [73]

Less desirable characteristics of the mercury arc lamp include a short operating lifetime (for some, as little as 100hrs), high cost, critical alignment, specialized enclosure and power requirements, and both a toxic component and potential explosive failure mode. Almost half of the output is at UV wavelengths, and care must be taken to avoid exposure to unfiltered light. Power output falls as the lamp ages. Compared to other fluorescence light sources, mercury arc lamps during routine operation display significantly greater fluctuations in intensity. Finally, they exhibit spatial instabilities in light output, due to arc effects such as wander and flutter. [**73**]

Developments such as xenon arc and metal halide lamps go some way to addressing problems such as lamp life and toxicity, and tend to have a more uniform output spectrum. However, arc related problems remain.

LEDs and Lasers.

The use of Light Emitting Diodes (LEDs) for fluorescence excitation is a promising development. Current devices are beginning to offer usably high output and narrow emission bands, in the order of 20 to 70nm. The latter, in conjunction with an increasing selection of peak output wavelengths, allows LEDs to be used for the individual excitation of wide range of fluorophores. Other advantages include a long operating life, relatively simple power and mounting requirements, and outstanding spatial and temporal stability. [**74**]

Lasers are typically employed as excitation light sources for confocal imaging, where design of the imaging system requires optimal fluorophore excitation. Desirable properties like high brightness, stability, longevity and narrow spectral bandwidth, outweigh high initial costs and complexity in this application. Several other properties unique to lasers, however, such as a high degree of spatial and temporal coherence, narrow beam divergence, and well-defined polarization properties are increasingly finding application in widefield fluorescence. [**75**]

Epifluorescence Lightpaths

Fluorescence microscopy as practiced today is predominantly epifluorescence. In contrast to the most common forms of the techniques examined above, epifluorescence based on an incident light illumination pathway. Illumination is supplied through the imaging objective, which is essentially acting as its own prefocussed and aligned, N.A. matched condensor. The illumination pathway contains field and aperture diaphragms, and the system is capable of Koehler illumination.

Epifluorescence microscopes are commonly available in upright, inverted and stereo (dissecting) configurations.

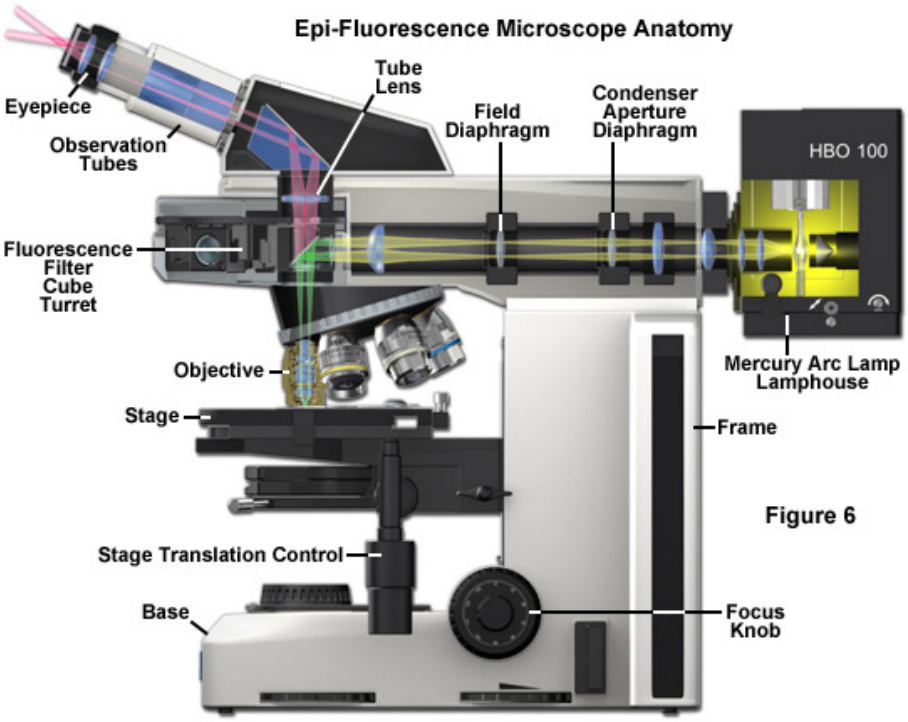


Figure 61 – Sectional diagram of an upright epifluorescence microscope. [76]

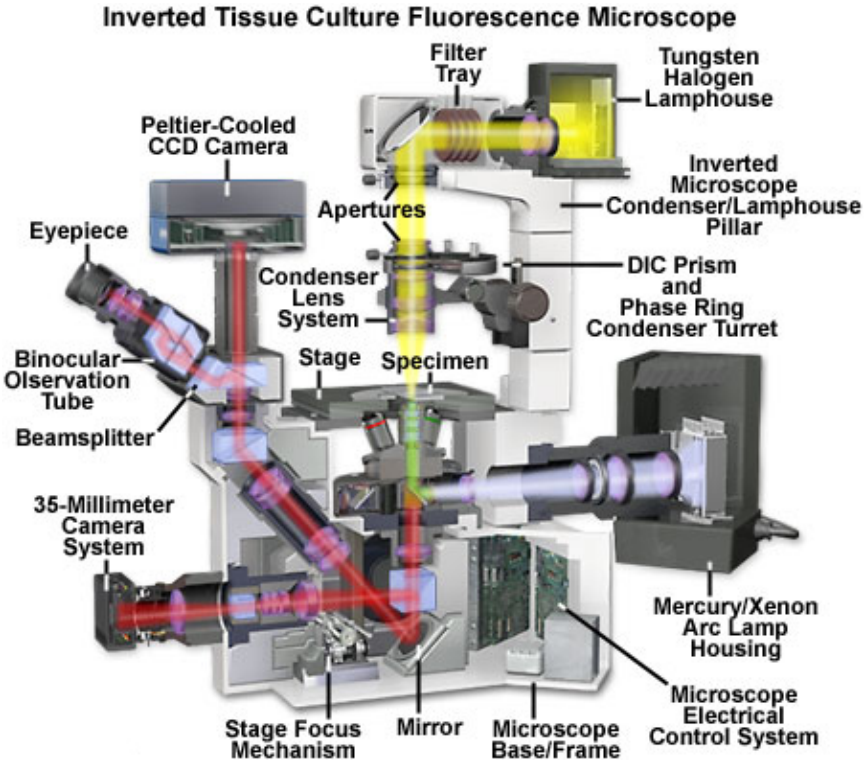


Figure 62 – Sectional diagram of an inverted epifluorescence microscope. [77]

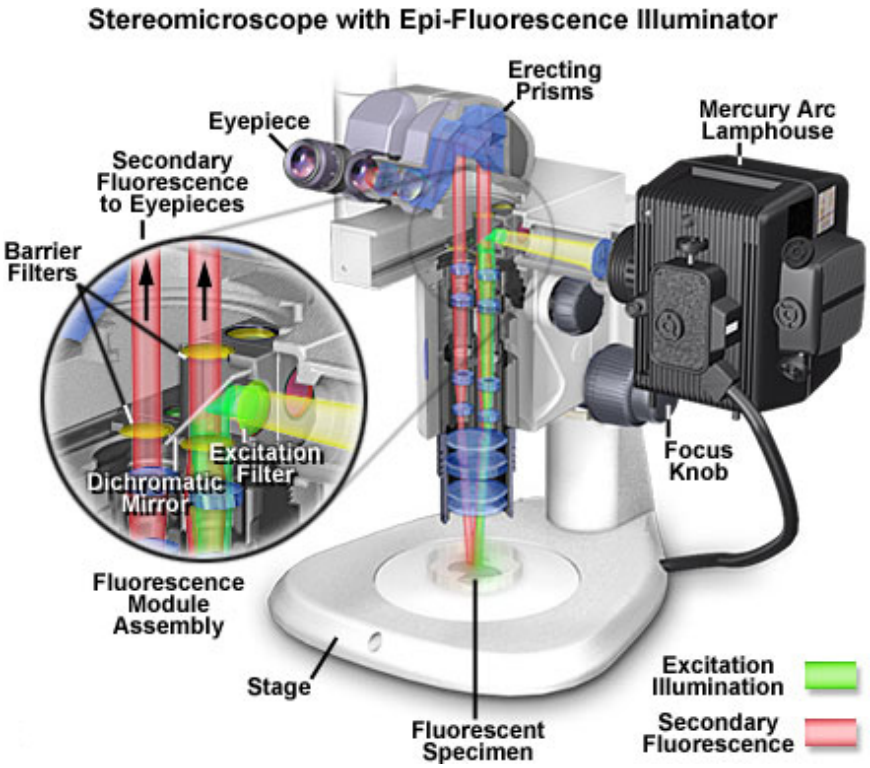


Figure 63 – Sectional diagram of a stereo microscope with epifluoresence illumination. [78]

Note that although the appearance of the microscope varies widely, each of these configurations share the essential feature of illumination through the imaging objective.

Each form also employs excitation filters, dichroic mirrors and emission or barrier filters contained within a filter cube. The role of the excitation filter is simply to differentially pass a suitable band of illumination wavelengths for excitation of the fluorophore, and to exclude others. The dichroic filter reflects this light through the

objective to the specimen. Crucially, the dichroic filter is, however, essentially transparent to the wavelengths in the emission band of the fluorophore, allowing these to pass through to the emission filter. The emission or barrier filter permits a suitable band of emission wavelengths to pass through to the eyepieces or detectors, whilst blocking other wavelengths (including any excitation light that may have passed through the dichroic).

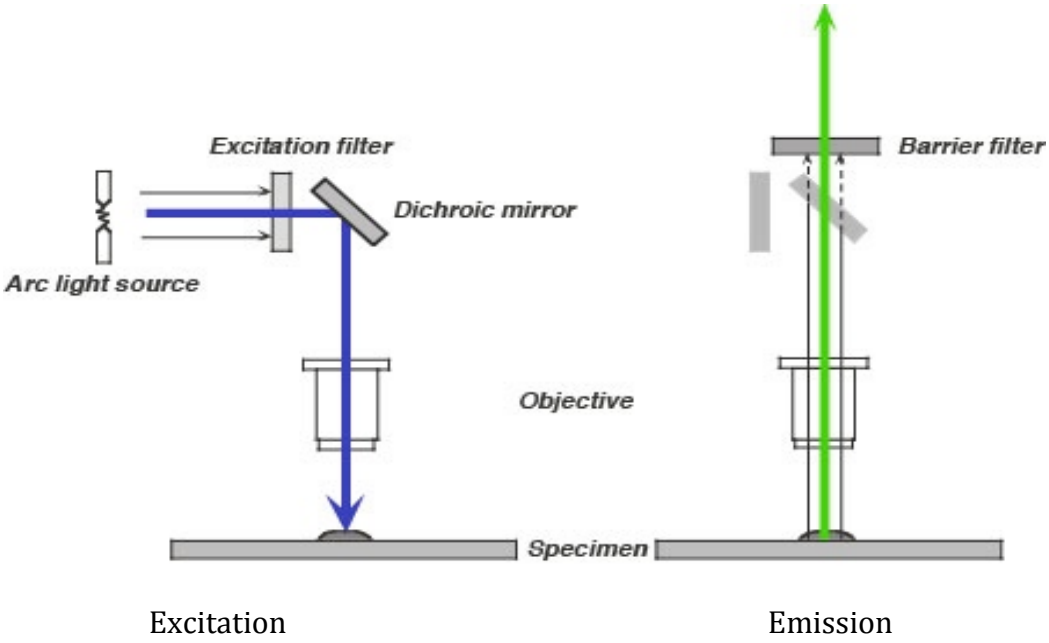


Figure 64 – Light path through the fluorescence filter cube. [79]

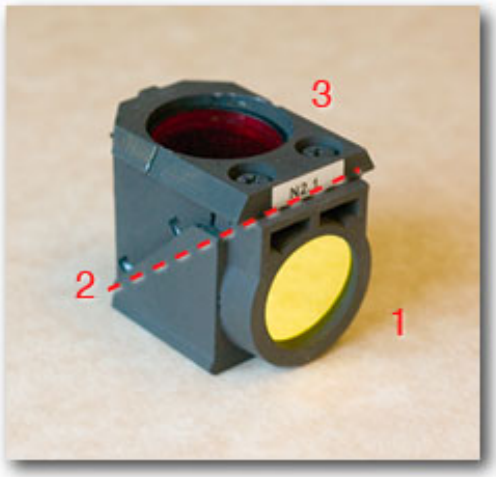


Figure 65 – A typical fluorescence filter cube, as employed in epifluorescence, 1) excitation filter, 2) plane of dichroic mirror, 3) emission filter. [79]

The "suitable band" of excitation and emission wavelengths referred to above is dependent not only on the fluorophore for which the filter set is designed, but also on the intended application. For example, emission bands can be broad, for use when only a single fluorescent probe is to be used, or narrow, for use when the sample contains multiple probes and there is concern that more than one of these may be excited within a given illumination range. Filters can be designed with several discrete pass bands, and dichroic mirrors with several discrete reflection and transmission bands, permitting simultaneous multiple probe imaging. The later are typically used with additional dichroic mirrors mounted after the filter cube, serving to direct the distinct emission bands to separate detectors.

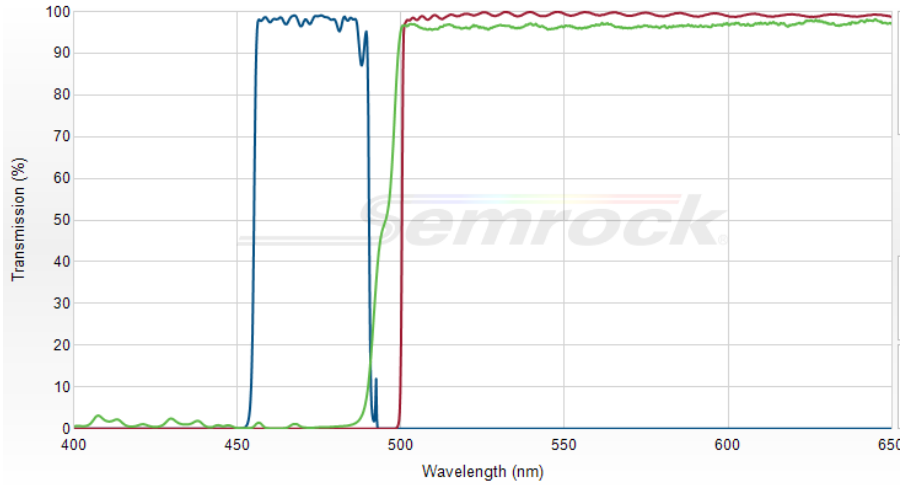


Figure 66 – Transmission characteristics of a single band, long pass filter set designed for GFP. [80]

In the transmission graph for a Semrock single band, long pass filter set for Green Fluorescent Protein (GFP) above, the blue line indicates the excitation band pass, from approx. 455 to 490nm. The green line is the dichroic mirror, reflecting light below approx. 490nm and allowing passage of light above 500nm. The red line indicates the transmission band of the emission filter, in this case from approx. 505nm through to (at least) 650nm.

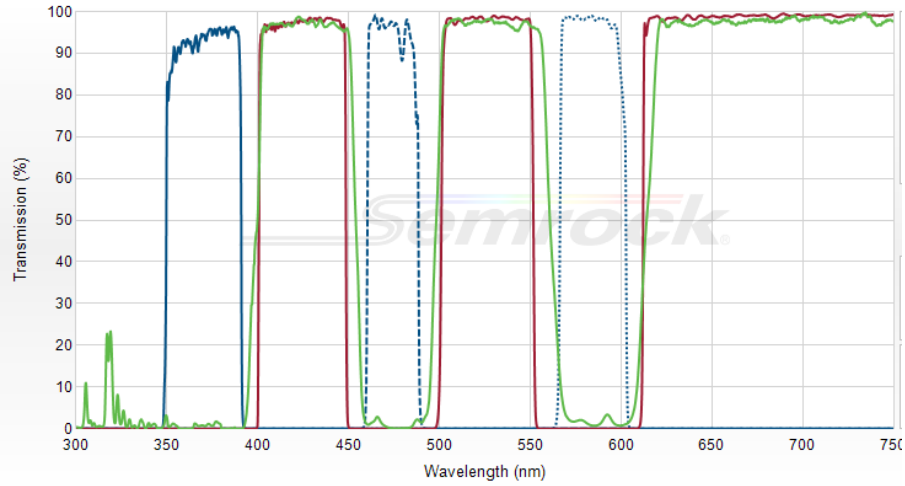


Figure 67 – Transmission characteristics of a triple band filter set designed for BFP, GFP and HcRed. [81]

The triple band filter set designed for simultaneous imaging of BFP, GFP and HcRed fluorophores (above) displays three sets of sharply defined transmission characteristics. As before, blue is the excitation filter, green the dichroic mirror and red, the emission filter.

Choosing a fluorescent marker/filter

Fluorophores are often chosen due to a familiarity with its application, ready availability within a workgroup, or cost. For some targets there are comparatively few choices. Whilst these financial and practical considerations are understandable, failure to consider fluorophore characteristics often leads to poor choices and hence, poor images and/or experimental results.

What should be considered when chosing fluorophores? Some things are obvious; for example a stain that would kill the specimen, such as the nuclear stain DAPI, is not acceptable if the aim is to study dynamic processes in living material.

Some are straightforward; they should possess excitation and emission profiles compatible with the filter sets available in the examining microscope. The emission band should be well clear of the emission wavelengths of any autofluorescence inherent in the specimen. If multiple structures are to be labelled, the fluorophores should have distinct and preferably non overlapping excitation bands, and distinct, non-overlapping (or at least easily separable) emission bands. However, note that fluorophore excitation and emission profiles may be shifted significantly by the sample environment.

Adequate fluorophore separation can be difficult to achieve. Consider the following excitation spectra for some common fluorescent proteins:

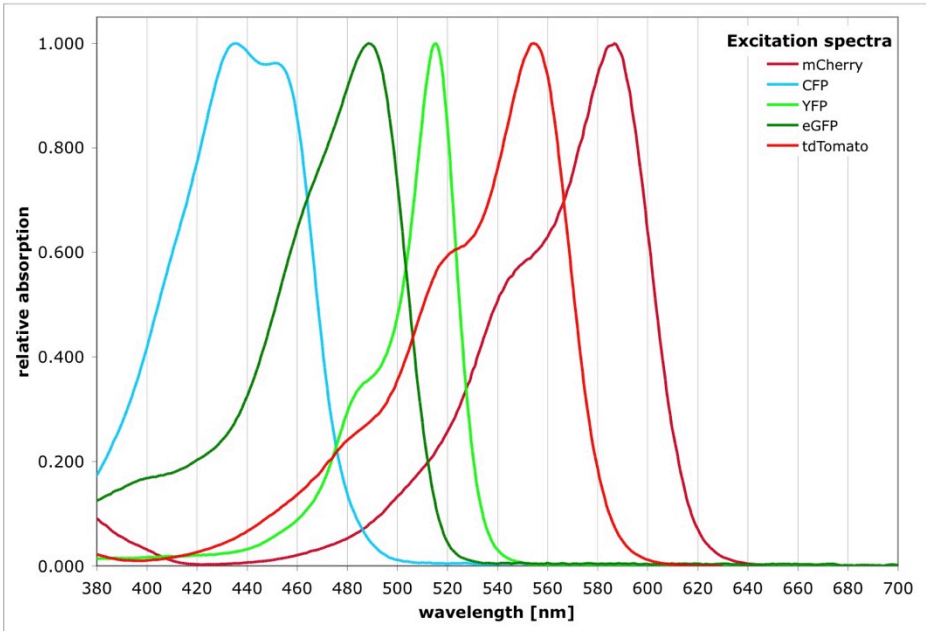


Figure 68 – Excitation spectra of some common fluorescent proteins. [82]

A standard narrow band pass excitation filter for GFP, 460 – 490nm, would also excite CFP, YFP quite well, even tdTomato to an appreciable extent.

The emission bands for these fluorophores also overlap:

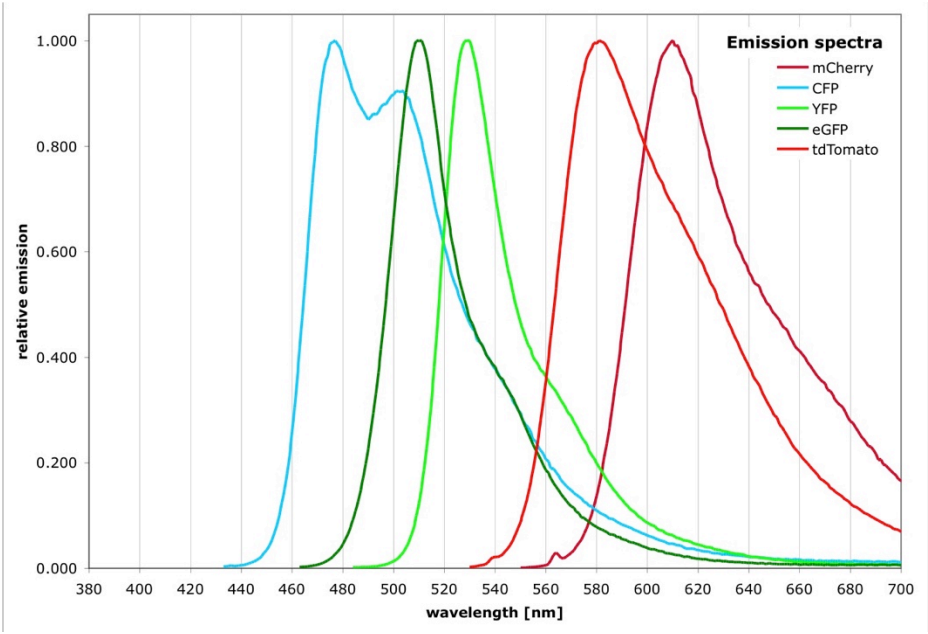


Figure 69 – Emission spectra of some common fluorescent proteins. [82]

A standard GFP narrow band pass emission filter with a transmission range of 500 – 530nm, for example, would capture peak emission for GFP, and very strong emission from both CFP and YFP.

It is clear that were CFP, GFP and YFP to be used together in a sample, it would be very difficult to excite each individually or to distinguish their emission, whether excited individually or not, using general filter sets.

Note that even adequately separated emission bands may produce bleedthrough, should a long emission tail be rendered brightly through an excessive concentration or overexpression of a probe, through very bright excitation or long exposure times.

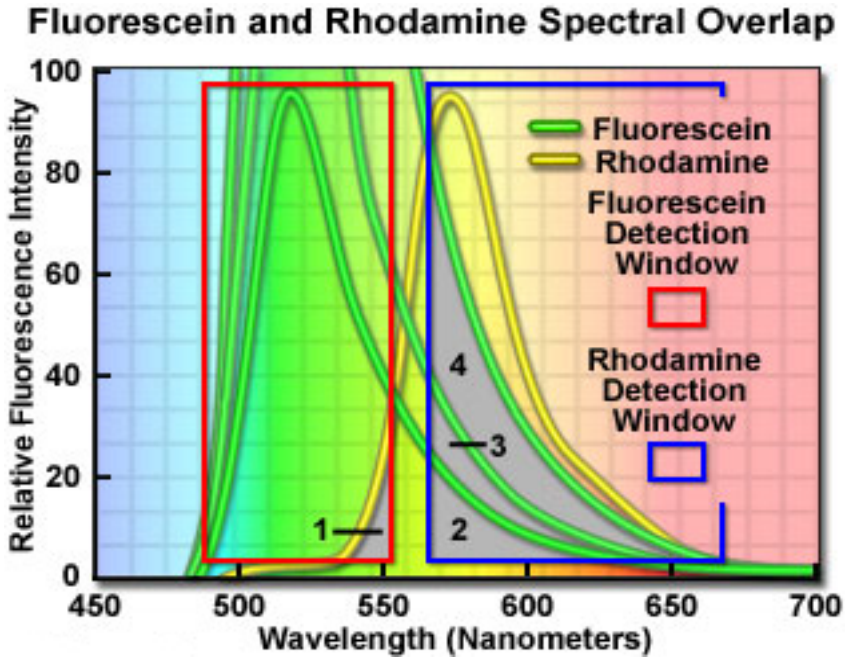


Figure 70 – Effects of fluorescence emission intensity on bleedthrough to rhodamine filter set. [83]

In the figure above equal relative emission intensity produces a relatively minor rhodamine bleed through the fluorescein filter (1), and fluorescein through the rhodamine filter (2). Increased quantity of fluorescein boosts emission brightness, and bleedthrough (3) and (4). Balancing expression can be seen, in this case, as an important step in sample preparation.

In general, the most photostable and brightest fluorophores should be used for least abundant targets. Nevertheless, bleedthrough typically remains in the order of 10 to 15%, unless the emission spectral maxima are separated by at least 100 to 150 nm.

Other considerations influencing flurophore choice may not be so straightforward, or obvious. The fluorescent protein series originally derived from the hydrozoan *Aequorea victoria*, such as eGFP, YFP and RFP, are popular for use in living specimens. These molecules are around 27 kilodaltons in size. How does this compare to the size of the molecule to which it to be attached? β-actin, which is often tagged with GFP, for example, is about 42 kilodaltons. What effects will this have on the normal diffusion or transport of the target molecule, the target's interaction with cellular machinery, and the health and function of the cell as a whole? Has this been considered? What does it mean for the interpretation of observed results?

Indeed, all fluorescent probes have the potential to distort cell behavior, especially if the probe's physical or chemical characteristics are substantially altered by environmental variables, such as pH, ion concentration, and hydrophobicity. [**83**]

Bleedthrough (Emission) and Crosstalk (Excitation)

The broad and asymetrical, typically right skewed emission spectra of many fluorochromes often make it difficult (or impossible) to confine detection to one channel or filter set. The bleedthrough potential of a combination of fluorophores can be assessed through production of a series of control samples, each with one only of the intended probes. Imaging each of these with the filter sets intended for the other probes will reveal unwanted emissions.

Bleedthrough induced artifacts are often confused with effects such as co-localization , FRET, and non-specific background staining [**83**]. **Beware**.

Crosstalk refers to the excitation of not only the intended fluorophore via a particular excitation source, but also of another fluorophore or autofluorescent entity.

As with bleedthrough, this places a limit on the number and spectral spacing of fluorophores that can be usefully used in a sample. Narrow band excitation filters, or laser excitation can assist specificity. The use of spectral controls, as above, is recommended.

Intensity quantification – 39 steps

A large array of uncontrolled variables effectively precludes use of fluorescence intensity as a method of quantifying a parameter of interest, either initially, or following experimental manipulation of said parameter.

Quite simply, brightness **DOES NOT** equal concentration. Read this again. Brightness DOES NOT equal concentration.

Within a given sample or image fluorescence may vary due to position in the cell, local environmental factors such as disolved oxygen and pH, and previous viewing history. These are just a few of the many factors which are known to influence intensity of fluorescence emission. Fluorophores can also be temporarily deactivated by the presence of other chemicals, fluorescent or not, that accept the energy of the fluorophore's excited state. This is called quenching.

If the specimen (for example, a cell line) is then modified to alter expression of the fluorophore tagged substance, can one be certain that none of the factors influencing fluorescence have not also altered? Has there been change (aging, for example) of the microscope's light source or filters, etc.?

The document *The 39 Steps: A Cautionary Tale about "Quantitative" 3D Fluorescence Microscopy* [**84**]is available from the documents menu of your PPMS home page, and details many of the factors that need to be controlled for valid fluorescence quantitation.

A Comparison of Contrast Enhancement Methods

Method	Advantages	Disadvantages
Brightfield	Long history of application – many stains for highlighting specific tissues or cellular components are available. Simplicity of equipment & application.	Low inherent contrast of many living and unstained fixed specimens. Staining typically not suitable for living specimens.
Darkfield	Well suited to live and unstained specimens, such as phyto and zoo planktonic organisms.	Low levels of light transmission inherent in the process necessitates strong sample illumination, which can damage specimens.
Polarised Light	Most commonly used with birefringent specimens.	
Phase Contrast	Suitable for many living specimens.	Numerical aperture of system is restricted by phase equipment, compromising resolution. Phase halos tend to obscure detail at the edges of cells and structures. This is reduced but not eliminated in modern implementations (such as apodized phase contrast).
Differential Interference Contrast	Suitable for many living specimens. No restriction of numerical aperture. No edge obscuring halos. Suitable for thick specimens.	Not suitable for birefringent specimens or sample mounts. Very thin specimens better served by phase contrast.
Fluorescence	Many fluorophores developed for highlighting specific tissues or cellular components.	Bleedthrough (collection of > one fluorophore in a given channel) and Crosstalk (excitation of > fluorophore by a given laser). Persistent errors in common interpretation.

Table 6 – A Comparison of Contrast Enhancement Methods.

Confocal Imaging

Introduction

When a fluorescent sample is excited by a light source through a microscope, excitation and emission does not occur purely at the focal point of the objective. 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 may look blurred or fuzzy due to this out of focus light obscuring features that are in focus. Exciting the sample above and below the focal plane also makes it difficult to extrapolate both the thickness and Axial location of objects within the sample. Confocal microscopy minimizes these issues by discriminating against out of focus light and biasing towards detection of objects within the focal plane of the objective. Confocal microscopy derives its name from the pinhole placed at a **Con**jugate **Focal** Plane within the microscope, which is used to reject this out of focus light.

Excitation

Excitation is provided by a laser of a specified wavelength. The laser is expanded outwards from an optical fiber 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 as can be seen in the image above. This imperfect focus results in excitation of fluorophores outside of the focal plane.

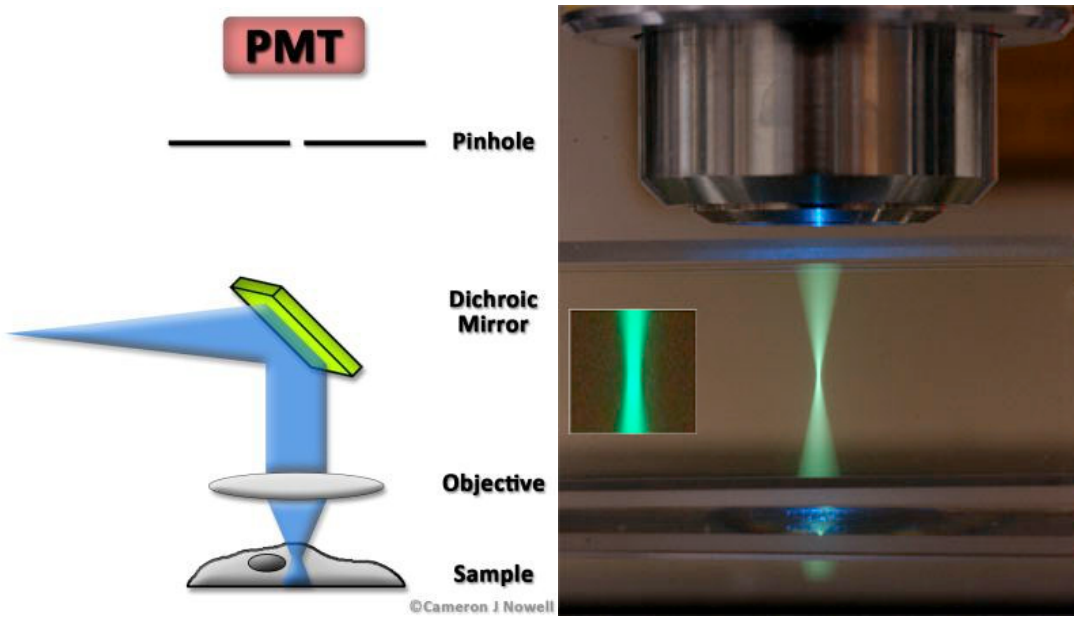


Figure 71 – Simplified confocal excitation lightpath. [85] [86]

Emission

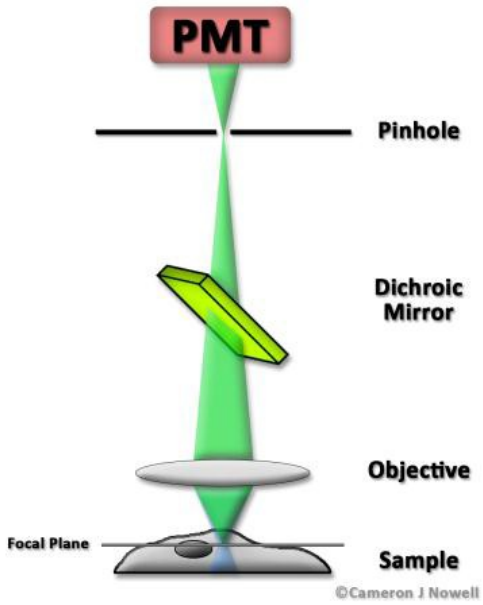


Figure 72 – Simplified confocal emission lightpath [86]

In Focus Light – Emission that is from the plane of focus of the objective passes through the dichroic mirror, an emission filter (not shown) and finally the confocal 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.

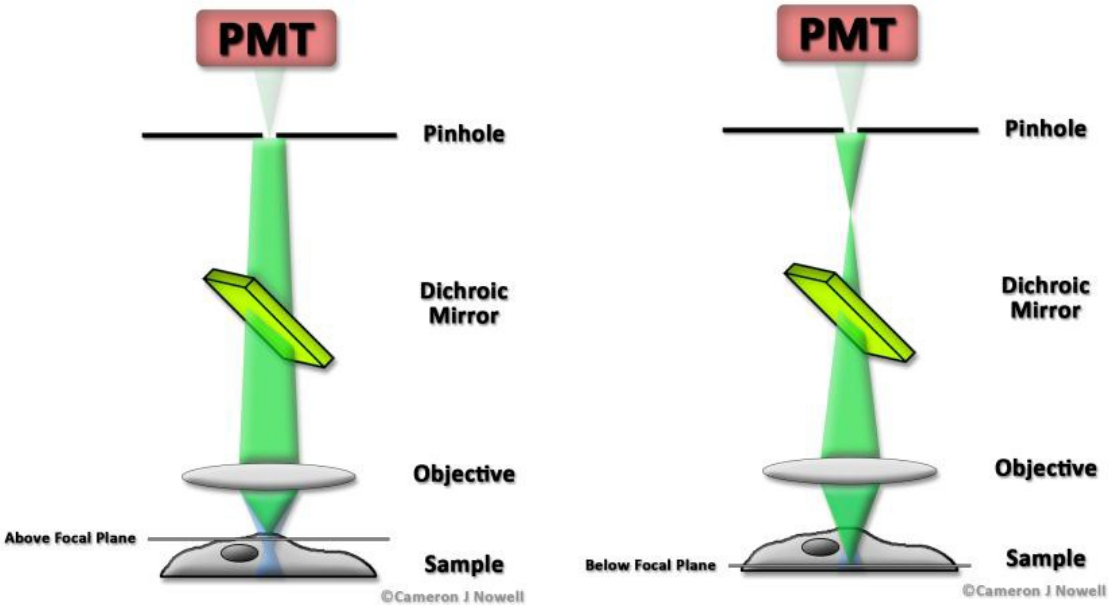


Figure 73 – Out of focus light rejection [86]

Out of Focus Light – Emission 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 with 512 pixels/line).

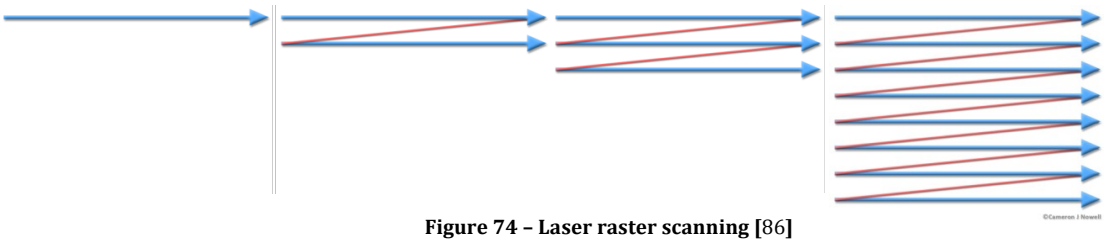


Figure 74 – Laser raster scanning [86]

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 (to alter the angle of light entering the back aperture of the objective) allowing the beam point to be moved either horizontally (X) or vertically (Y). The slower the galvos are scanned across the sample, the greater the so called pixel dwell time or the amount of time the laser spends exciting each area of the sample. The greater the dwell time, the greater the amount of emission detected and hence the brighter the resulting image.

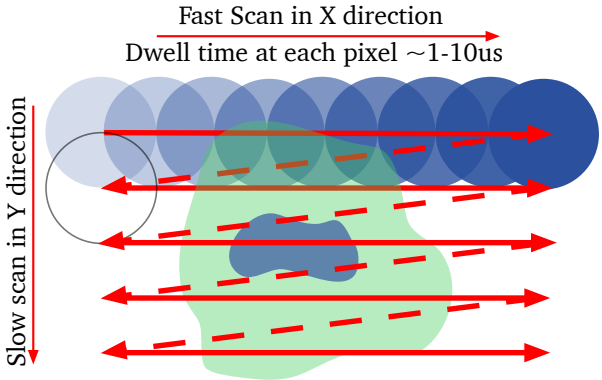
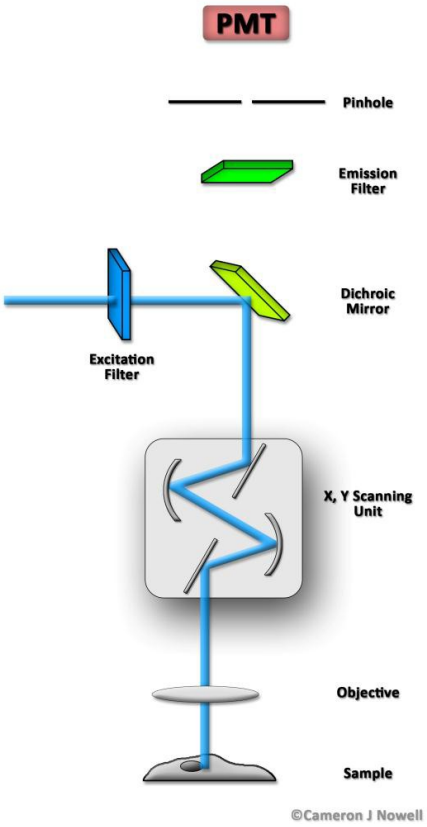


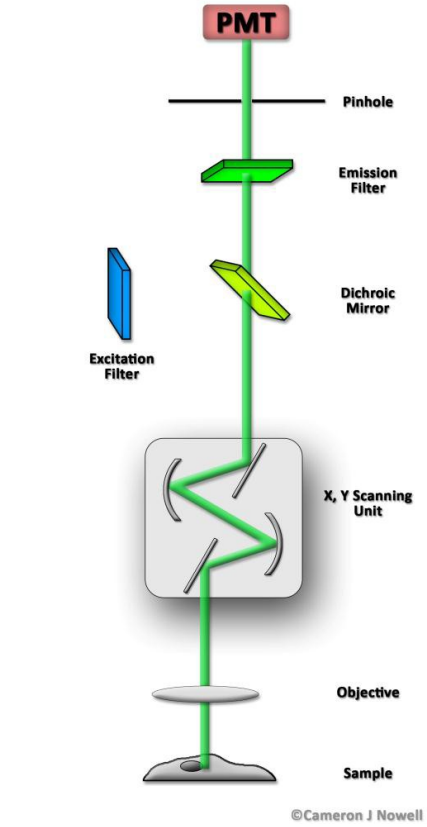
Figure 75 – Laser dwell time during scan [86]

As the laser scans across the sample, a 2D plot will form on the computer monitor, indicating the emission intensity for each sub-volume, this is the confocal image. Typical scan times are ~1 second for a 512x512 pixel image.

To be able to reconstruct all of the information that the given microscope configuration (objective NA, laser wavelengths etc) can collect, the laser step-size (image pixel size) across the sample must be no greater than half of the captured sub-volumes diameter, otherwise information is lost. The resolution in both the XY (Lateral) and Z (Axial) planes of the image is dependent on the NA of the objective as well as the pinhole diameter and excitation wavelength, as described by Rayleigh criterion and not by the pixel size of the image.



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.



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".

Figure 76 – Complete confocal lightpath [86]

Detection

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. It is important to note that the PMT acts like a single monochrome pixel in a CCD camera, it does not able to discriminate the wavelength of light it detects.

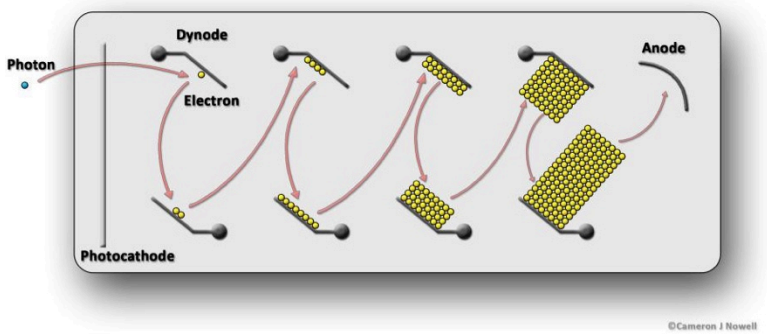


Figure 77 – Photomultiplier tube operation [86]

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. PMTs 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%, an Electron Multiplying (EM)-CCD camera can be better than 95% efficient. Some of the latest confocal microscopes now have a GaAsP PMT detector which have an efficiency of around 40% to 60%.

However as shown in the graph below, each type of detector has a different spectral response, meaning the sensitivity of the microscope is different at different wavelengths. So for instance a GFP emission centred at 520nm will be much easier to detect than a similar intensity Cy5 emission at 650nm.

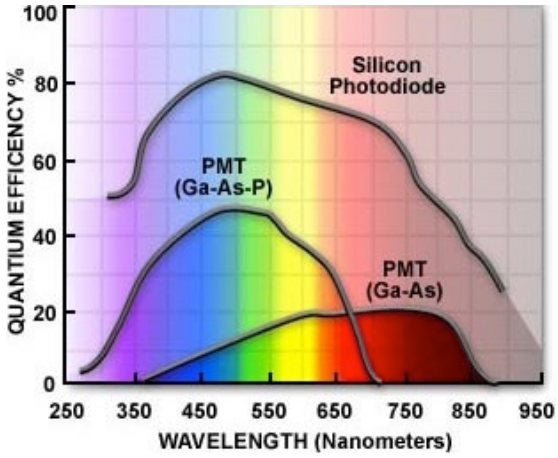


Figure 78 – Electronic Detector Spectral Sensitivities [87]

Pinhole Effects

The image to the below demonstrates the effect that opening the pinhole has on the thickness of the optical section captured.

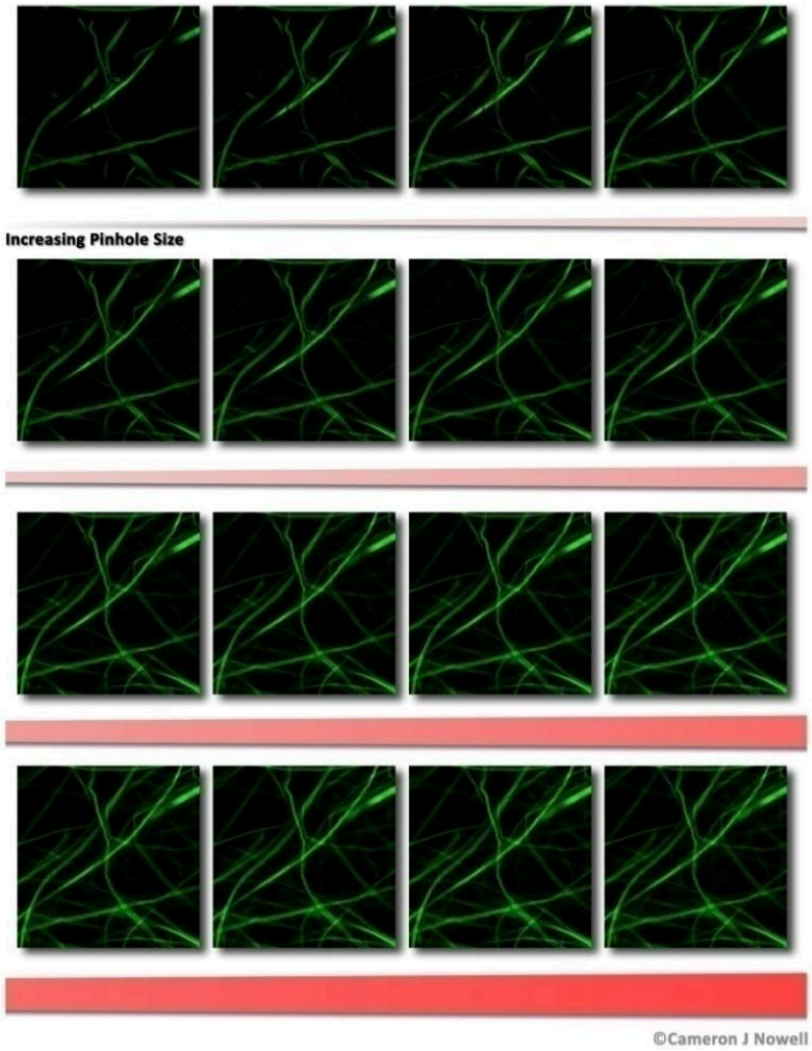


Figure 79 – Pinhole effects on image sectioning thickness [86]

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.

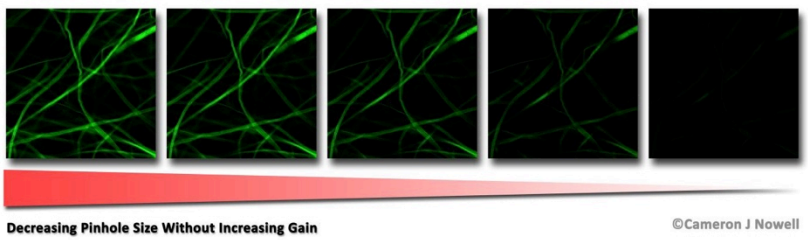


Figure 80 – Pinhole effects on image brightness [86]

Slices/Z Planes

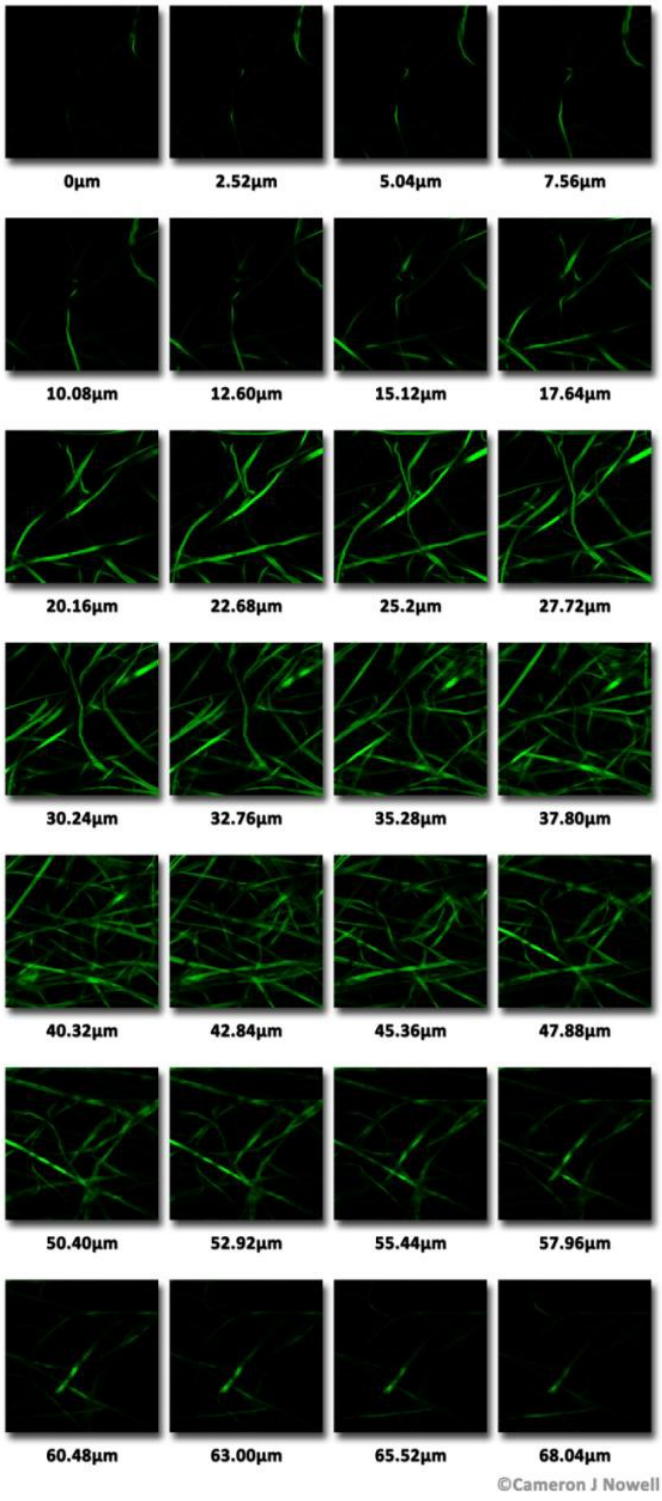


Figure 81 – Z-stack capture [86**]**

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 above shows a series of slices taken through a sample of fluorescent paper. The total thickness of the collected data is 68µm captured with a step size of 2.5µm between each image.

Imaging 3D Objects

The vast majority of samples imaged on a confocal microscope will be thick (>1um). 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).

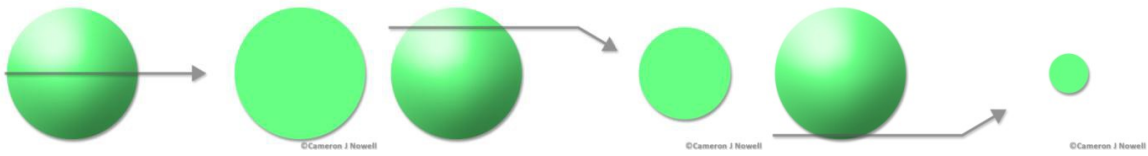


Figure 82 – Slices through a sphere [86]

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.

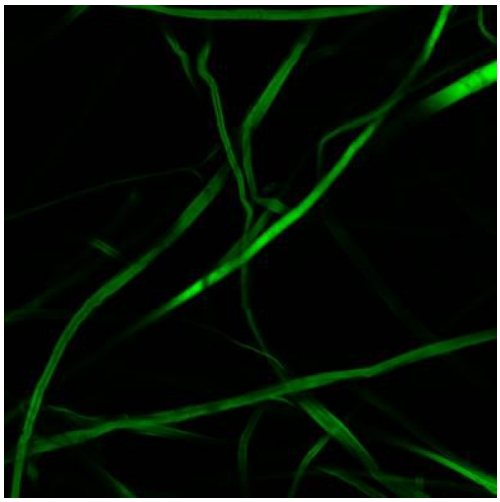
Figure 83 – High sampling rates [86]

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.

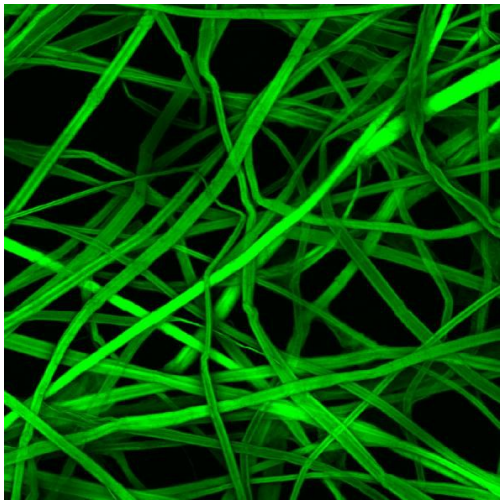
Figure 84 – Low sampling rates [86]

Representing Data

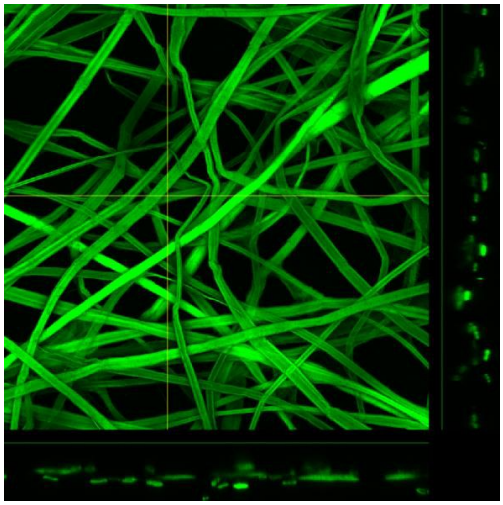
Since confocal data is collected as a series of slices it can be used to generate a range of images.



While a single slice, or projection of several slices, may show the result required, sometimes other data representation can be more informative.

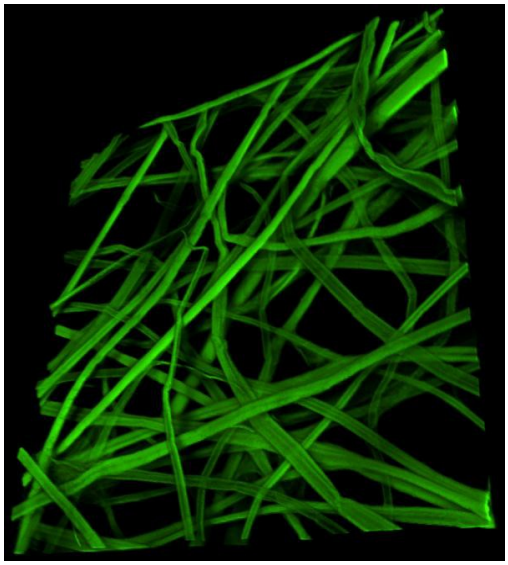


All the slices can be combined to generate a maximum intensity projection. This image draws the brightest pixel from each layer in the final image. The result is somewhat like a widefield microscope image with sharper detail



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

Figure 85 – Representing data through different types of projections, MIP and Orthoview. [86**]**



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.

Figure 86 – Representing data through different types of projections, 3D render. [86**]**

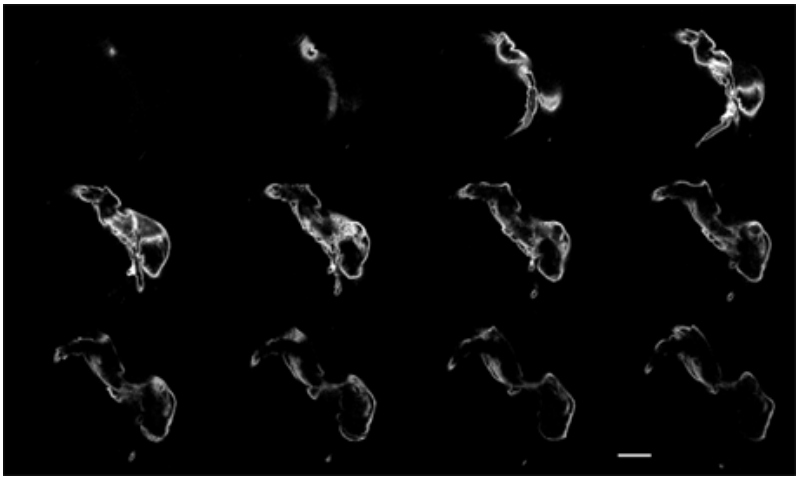


Figure 87 – Serial optical sections through a dust mite (5um steps)

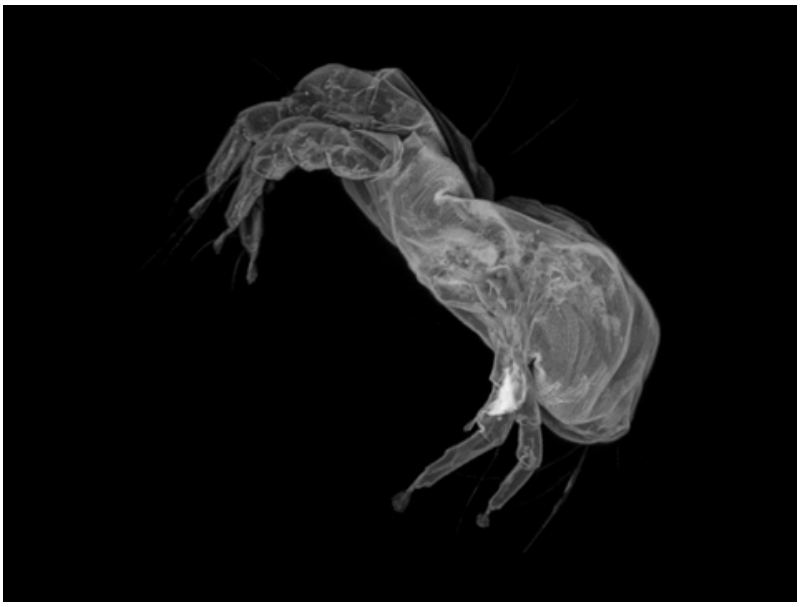


Figure 88 – Maximum Intensity Projection of a dust mite. [48**]**

Light Gathering Capability

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 (M) increases. The formula for calculating the light gathering ability (F) of an objective is:

Equation 5 $$F(trans) = 10^4 \times \mathrm{NA}^2/\mathrm{M}^2$$ [**88**]

Equation 6 $$F(epi) = 10^4 \times (NA^2/M)^2$$ [**88**]

Where NA is the Numerical Aperture of the objective

The following graph shows a comparison between typical objectives and the effect M and NA has on their light gathering ability.

M	NA	$F_{(transmitted)}$	$F_{(epi\text{-}fluorescence)}$
10x	0.45	203	41
20x	0.5	63	16
40x	0.75	35	20
40x	0.95	56	51
63x	1.4	49	97
100x	1.4	20	38

Table 7 – Magnification and NA vs image brightness [88**]**

It can be clearly seen that for the same NA, an increase in magnification will cause a significant decrease in image brightness. Whereas for the same M an increase in NA will cause a significant increase in image brightness. Therefore it is imperative to always choose the lowest magnification and highest NA that will work with you're your sample thickness and mounting method.

63x 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 ~2-3 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.

Resolution

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 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:

Equation 7 $$NA = n \cdot sin\boldsymbol{\theta} \quad [\mathbf{19}]$$

Where η is the refractive index of the immersion medium (1.0 for air, 1.33 for water and 1.515 for oil) and θ or α is the half-angle of the maximum cone of light that can enter or exit the lens (see diagram below)

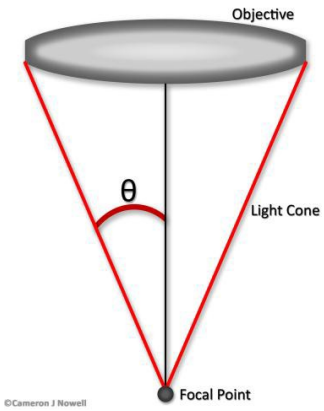


Figure 89 – Light collection cone of a lens [86**]**

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.

Point Spread Function

When coherent (laser) light fills the back aperture of an objective lens, the lens will bring the light to a point focus. The size and shape of this point in 3 dimensions determines the ultimate resolution of the system. This 3D excitation spot is known as the illumination Point Spread Function (PSF_{ill})The figure below illustrates a cross section through the center of the spot in the XY plane (Airy Disk) and through the X Z plane.

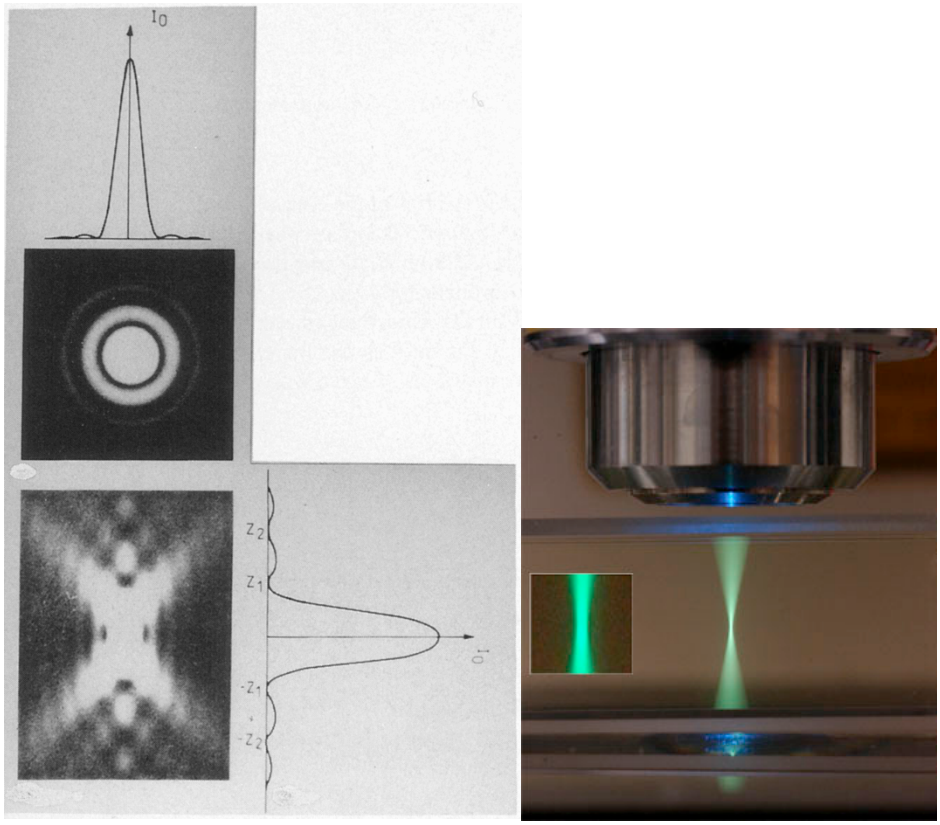


Figure 90 – Illumination Point Spread Function [84] [85]

The resolution in both X and Y is determined by the distance from the center of the Airy disk to the first dark ring. This is referred to as r_{airy}. This distance depends on the wavelength of excitation light (λ) and the Numerical Aperture of the lens (NA).

For a Wide Field (non confocal) system the XY resolution equation is:

Equation 8 $$Rxy\ widefield\ = 0.61\lambda/NA$$ **[89]**

For a confocal system, the pinhole radius may be set somewhat smaller than r_{airy} and thus the XY resolution equation is:

Equation 9 $$Rxy\ confocal\ = 0.4\lambda/NA$$ **[89]**

The resolution in Z (Optical Section Thickness) is determined by the distance from the center of the spot to the edge of the first minimum in vertical space. On the XZ graph this is from the center of the main peak to the edge of one of the first small peaks on either side of it. This distance depends on the wavelength of light, the Refractive Index of the medium between the lens and the specimen (η) and the NA of the lens.

For a widefield system the Z resolution equation is:

Equation 10 $$Rz\,widefield = 2\lambda\eta / NA^2$$ **[89]**

For a confocal system, the pinhole only allows light from a much shorter vertical distance to pass through to the detector so the Z resolution equation is:

Equation 11 $$Rz\,confocal = 1.4\,\lambda\eta / NA^2$$ **[89]**

Objective	Wide Field XY	Confocal XY	Wide Field Z	Confocal Z
10X 0.45 dry	0.66 µm	0.43 µm	4.82 µm	3.37µm
40X 0.75 dry	0.40 µm	0.26 µm	1.74 µm	1.2 µm
40X 1.3 oil	0.23 µm	0.15 µm	0.88 µm	0.61 µm
63X 1.4 oil	0.21 µm	0.14 µm	0.74 µm	0.53 µm

Table 8 – Objective resolutions in widefield and confocal imaging modalities [89**]**

The data in Table 8 is based on the above equations using a wavelength of 488 nm (blue light) a refractive index of 1 for the dry lenses and 1.515 for the oil lenses and a confocal pinhole of 0.7AU.

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.

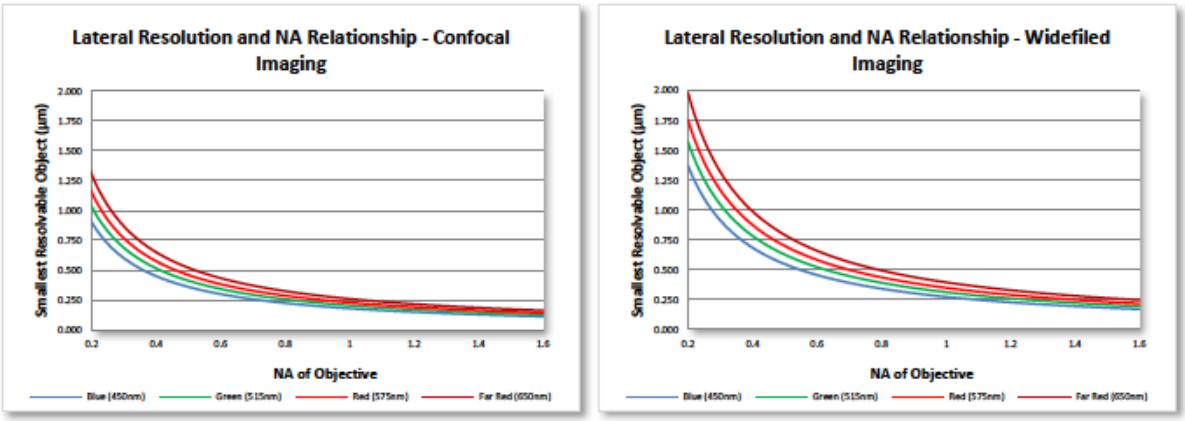


Figure 91 – Lateral resolution vs NA [86**]**

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.

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

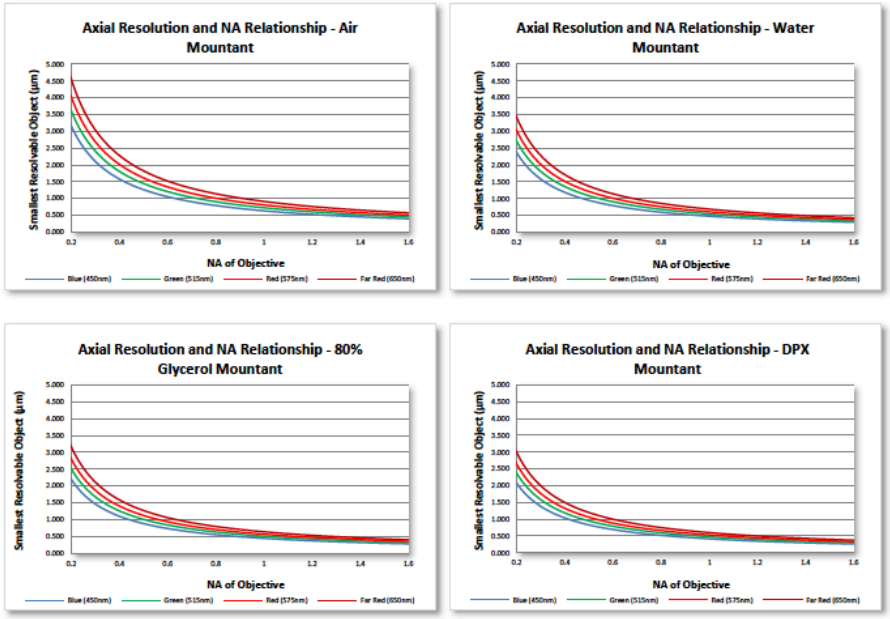


Figure 92 – Axial Resolution vs NA [86]

The Pinhole.

The optics of a confocal microscope are arranged such that the fluorescent light produced in the specimen is focused by the objective lens to an Airy disk that is superimposed on the Pinhole. The diameter of the first minimum of the Airy disk is referred to as one Airy unit. The diameter of the pinhole affects resolution in XY and Z. In the resolution table, the pinhole is set to approximately 70% of the Airy unit. The size of the Airy unit at the pinhole depends on the objective lens NA, the wavelength of the emitted fluorescent light, and any magnification up to the pinhole. Thus a different pinhole size is required for different objective lenses.

Equation 12 $$1\ Airy\ Unit\ = \frac{0.61\ x\ (em.\ wavelength)\ x\ (total\ Mag.)}{NA}$$ **[89]**

This equation gives the size of the pinhole in nm that is equivalent to 1 Airy unit. For any given lens and λ combination, changing the size of the pinhole affects resolution in XY and Z. The following general rules come from practical, experimental work:

1) For pinhole diameters less that about 1/2 Airy unit, the strength of sectioning (Z resolution) remains constant.

2) For pinhole diameters greater than one Airy unit, the Z resolution slowly becomes worse.

3) A pinhole diameter approximately 1/5 Airy unit will produce maximum lateral resolution at the expense of a loss of 95% of the signal.

4) Lateral resolution is more sensitive to pinhole size than axial resolution and rapidly becomes worse as the pinhole is made larger than one Airy unit.

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 [**90**] 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.

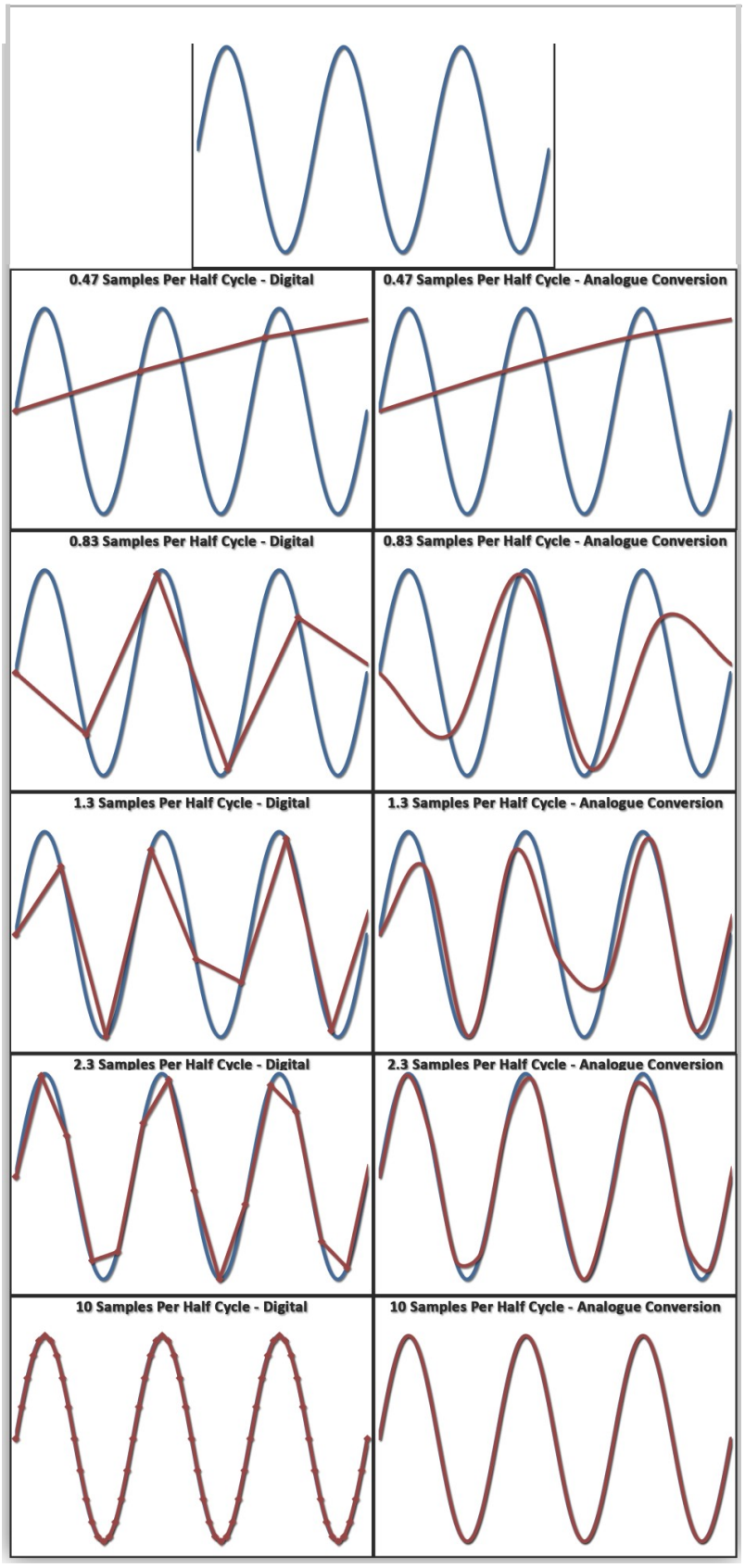


Figure 93 – Various digital sampling rates of an analogue signal [86]

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 atleast 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 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.

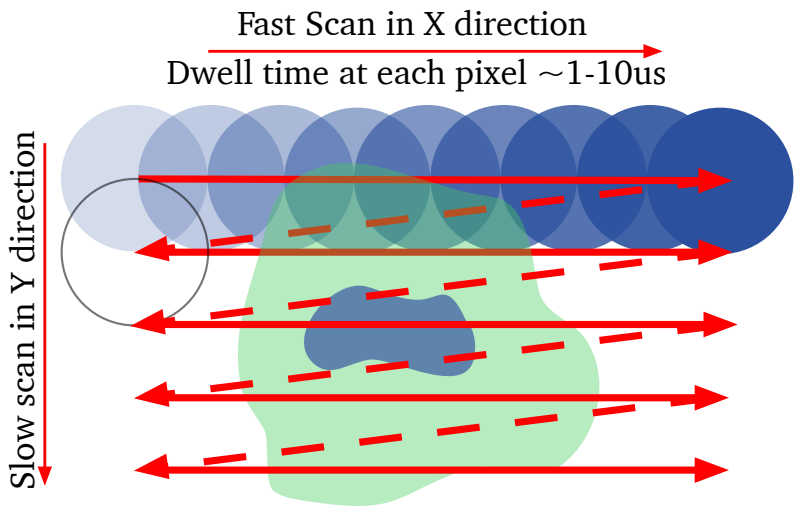


Figure 94 – Confocal pixel size

Nyquist and Z sections

When collecting images on a microscope, Nyquist sampling also needs to be taken into account during collection of Z-stacks. For example if you want to capture an adherent cell (10 µm thick) at optimal resolution with a 60x NA1.4 objective at 488nm excitation on a confocal microscope, you would need to use the following parameters:

Z Stack Range = 10um

Z Section Thickness for a 60x/1.4 lens = 530nm

Therefore, the step size = 530/2.3 = 230nm

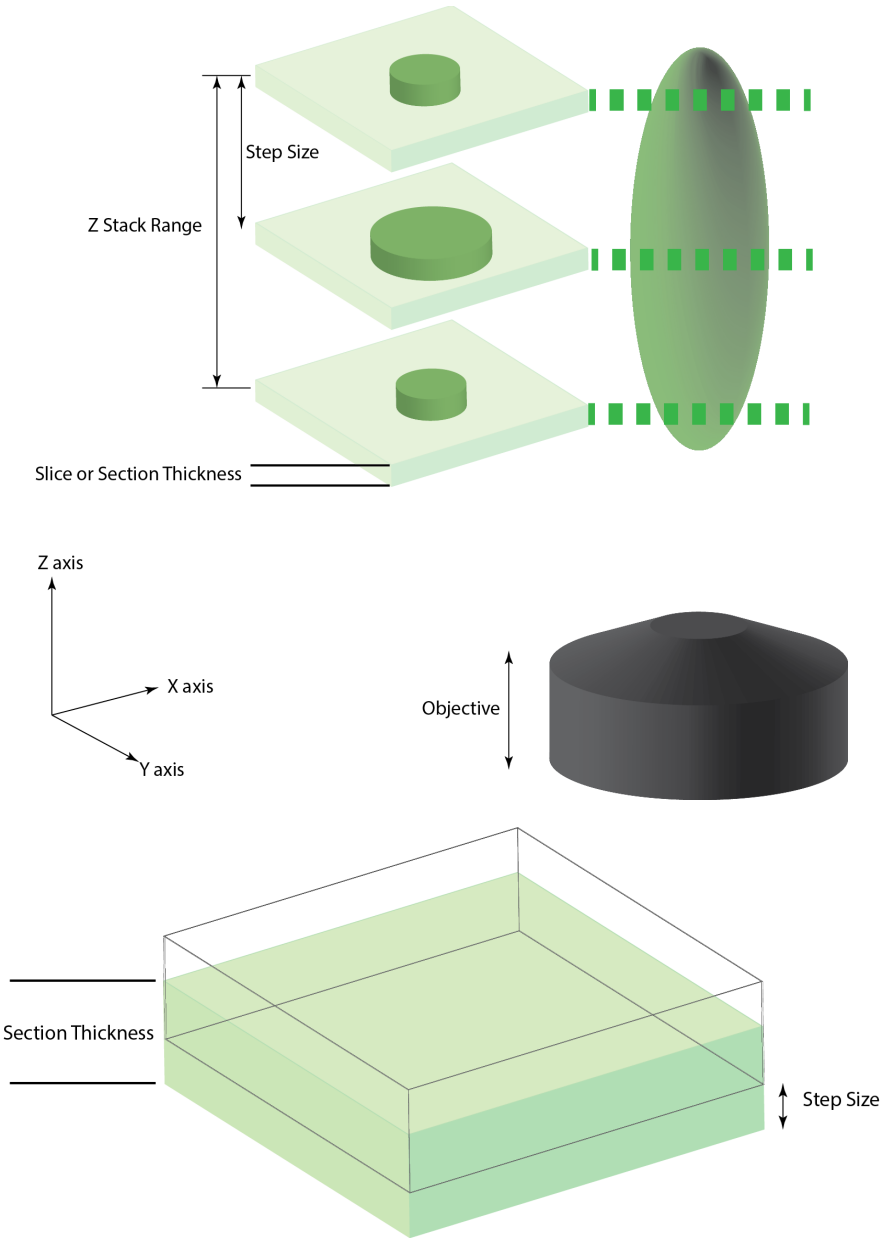


Figure 95 – Z sectioning at Nyquist rates

Step Size needs to be <= (Optical Section Thickness/2.3) to achieve Nyquist sampling rates.

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 length, then the images should be captured every 4.34 minutes (10 minutes / 2.3) so that there is sufficient overlap of the cell image over time to reconstruct it's movements accurately. Both X and Y movement must be accounted for, along with change in morphology over time which may affect the calculations.

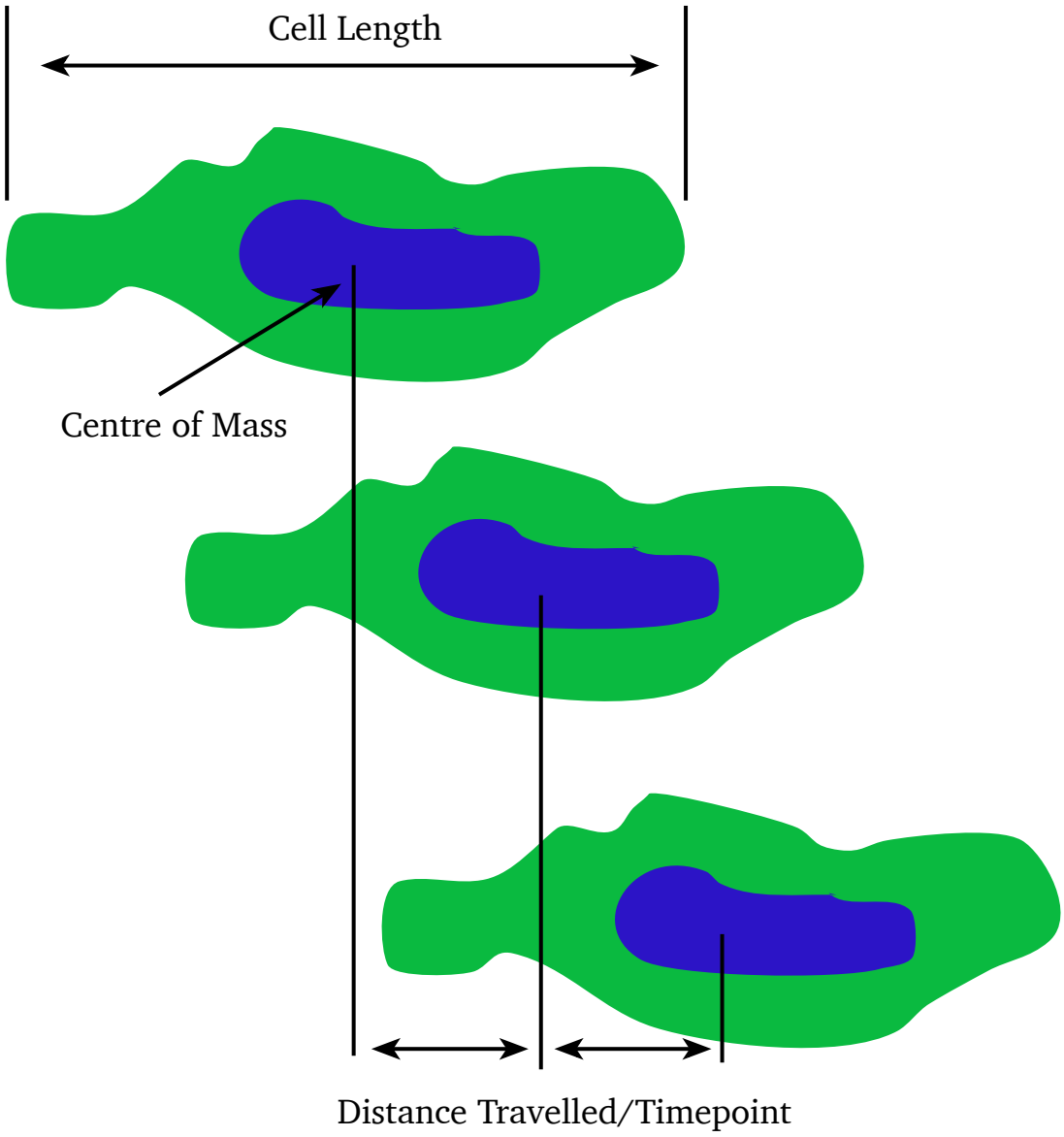


Figure 96 – Time sampling at Nyquist rates

Nyquist Sampling versus Pretty Picture

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.

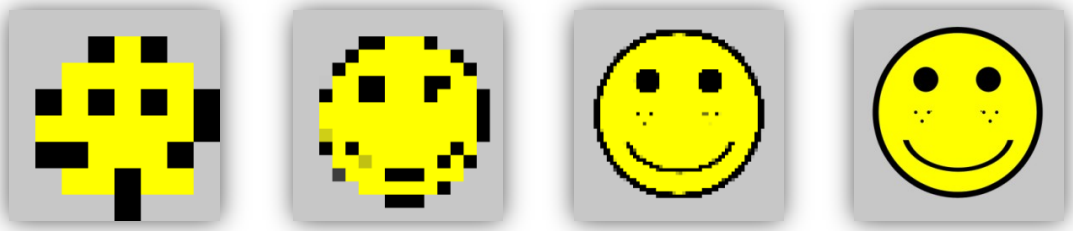


Figure 97 – Nyquist sampling to resolve information within an image [86]

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.

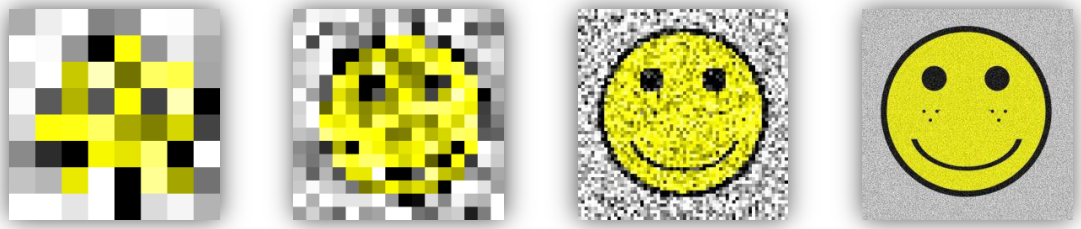


Figure 98 – Effects of noise on Nyquist rates [86]

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.

For the Zeiss Confocals, use the following table to set the Zoom or frame size value to establish Nyquist sampling in X & Y.

Objective	0.7 Airy Unit	1.0 Airy Unit	Zoom	512X512 (µm / pixel)	1024X1024 (µm / pixel)	2048x2048 (µm / pixel)	4096x4096 (µm / pixel)	Line Length (µm)
10X/0.45 Dry	r_{xy} = 0.43	r_{xy} = 0.66	1	1.66	0.83	0.41	0.21	848.5
	r_z = 3.37	r_z = 4.8	2	0.83	0.41	0.21	0.10	424.3
	xy_n = 0.19	xy_n = 0.28	3	0.55	0.28	0.14	0.07	282.8
	z_n = 1.47	z_n = 2.09	4	0.41	0.21	0.10	0.05	212.1
	Z_{thick} = 5.5	Z_{thick} = 7.1	5	0.33	0.17	0.08	0.04	169.7
			6	0.28	0.14	0.07	0.03	141.4
20X/0.8 Dry	r_{xy} = 0.24	r_{xy} = 0.37	1	0.83	0.41	0.21	0.10	424.3
	r_z = 1.07	r_z = 1.53	2	0.41	0.21	0.10	0.05	212.1
	xy_n = 0.10	xy_n = .16	3	0.28	0.14	0.07	0.03	141.4
	z_n = 0.46	z_n = 0.66	4	0.21	0.10	0.05	0.03	106.1
	Z_{thick} = 1.4	Z_{thick} = 1.8	5	0.17	0.08	0.04	0.02	84.9
40X/0.75 Dry	r_{xy} =0.26	r_{xy} = 0.39	1	0.41	0.21	0.10	0.05	212.1
	r_z = 1.2	r_z = 1.74	2	0.21	0.10	0.05	0.03	106.1
	xy_n = 0.11	xy_n = 0.17	3	0.14	0.07	0.03	0.02	70.7
	z_n = 0.53	z_n = 0.75	4	0.10	0.05	0.03	0.01	53.0
	Z_{thick} = 1.5	Z_{thick} = 1.9	5	0.08	0.04	0.02	0.01	42.4
40X/1.3 Oil	r_{xy} = 0.15	r_{xy} = 0.23	1	0.41	0.21	0.10	0.05	212.1
	r_z = 0.6	r_z = 0.87	2	0.21	0.10	0.05	0.03	106.1
	xy_n = 0.065	xy_n = 0.10	3	0.14	0.07	0.03	0.02	70.7
	z_n = 0.27	z_n = 0.38	4	0.10	0.05	0.03	0.01	53.0
	Z_{thick} = 0.7	Z_{thick} = 0.9	5	0.08	0.04	0.02	0.01	42.4
63X/1.4 Oil	r_{xy} = 0.14	r_{xy} = 0.21	1	0.26	0.13	0.07	0.03	134.7
	r_z = 0.53	r_z = 0.75	2	0.13	0.07	0.03	0.02	67.3
	xy_n = 0.06	xy_n = 0.09	3	0.09	0.04	0.02	0.01	44.9
	z_n = 0.23	z_n = 0.33	4	0.07	0.03	0.02	0.01	33.7
	Z_{thick} = 0.6	Z_{thick} = 0.8	5	0.05	0.03	0.01	0.01	26.9
100X/1.46 oil	r_{xy} = 0.13	r_{xy} = 0.20	1	0.17	0.08	0.04	0.02	84.9
	r_z = 0.49	r_z = 0.69	2	0.08	0.04	0.02	0.01	42.4
	xy_n = 0.058	xy_n = 0.09	3	0.06	0.03	0.01	0.01	28.3
	z_n = 0.21	z_n = 0.30	4	0.04	0.02	0.01	0.01	21.2
	Z_{thick} = 0.5	Z_{thick} = 0.7	5	0.03	0.02	0.01	0.00	17.0

Table 9 – Zeiss confocal objective resolutions at various zoom and pixel settings.

Zeiss Confocal Resolution Table Values in BLUE are at the correct zoom setting to satisfy Nyquist criteria.

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.

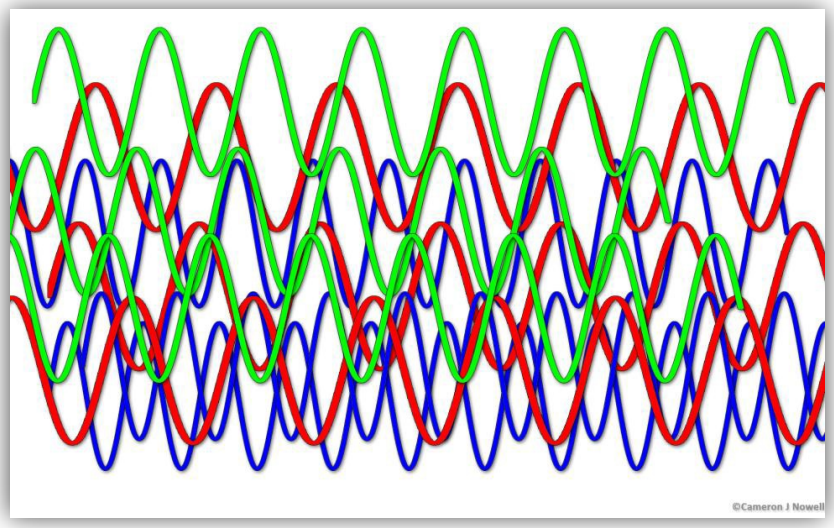


Figure 99 – White light illustration [86**]**

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.

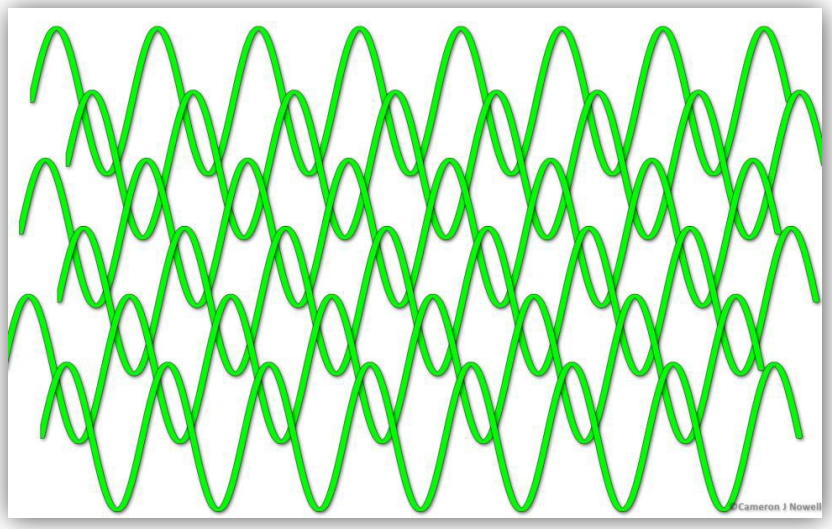


Figure 100 – Incoherent monochrome light illustration [86]

Only once the light is coherent will a laser beam be produced

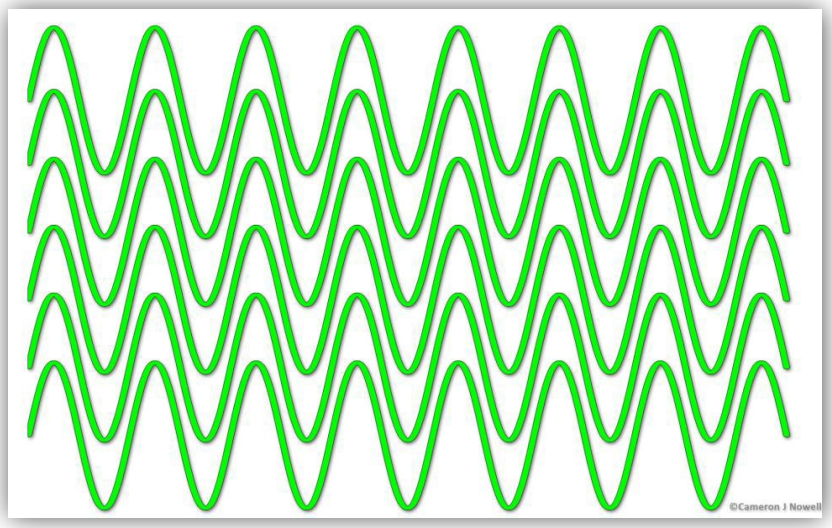


Figure 101 – Coherent laser light illustration [86]

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, this pulse length may vary from seconds to femtoseconds (10^{-15} seconds). Due to the pulsing nature of the laser, large power levels can be delivered which can be extremely dangerous to the human eye.

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 since infrared light isn't absorbed by tissue as easily as visible light. Multiphoton excitation makes use of a pulsed beam (instead of the continuous beam used for single photon imaging) that results in greatly reduced out of focus excitation of the sample. This may also mean less photo damage and bleaching of the sample will occur under the correct conditions.

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.

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 require a confocal 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.

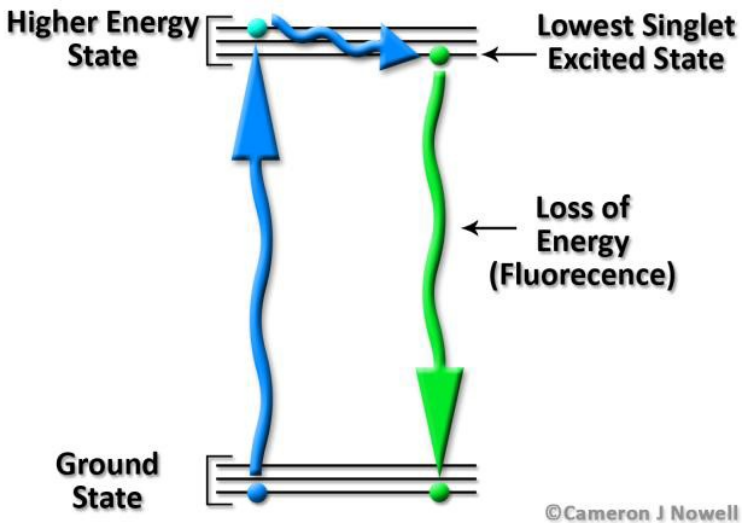


Figure 102 – Jablonski diagram of single photon fluorescence excitation [86]

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 (~10^{-18} seconds or one attosecond), otherwise the excited electron will only reach the middle virtual state and not be pushed to the higher energy state (see diagram below).

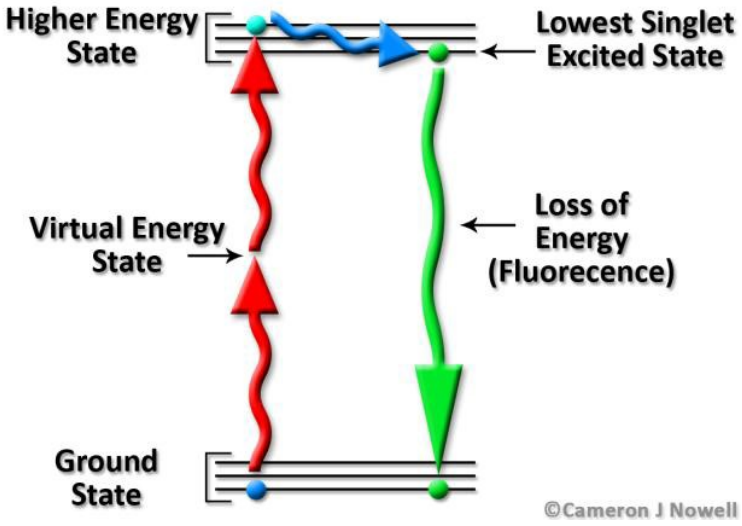
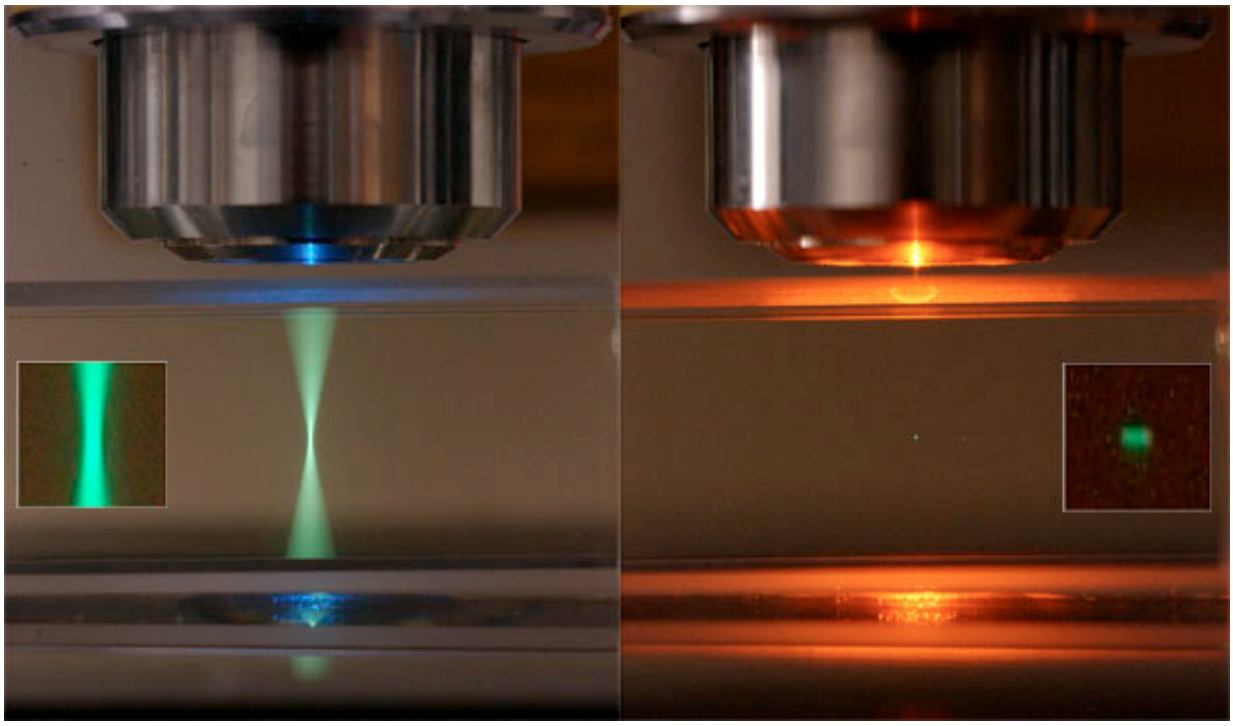


Figure 103 – Jablonski diagram of two photon fluorescence excitation [86]

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 (690-1040nm) and instead of being fired as a continuous beam the laser is pulsed on and off. The ~3 Watt laser pulses are approximately 70 femtoseconds long and 12 nanoseconds apart, resulting in an average power of ~500kW. This is around 25 million times more powerful than the 20mW continuous wave lasers that are used for single photon confocal imaging. When this intense pulse of light containing incredibly large numbers of photons is focused onto the sample, there is a very small probability that a fluorescent molecule will absorb two of these photons at the same time. This probability increases exponentially towards the focal point of the objective as the photon density increases. The result of this is that only the fluorophores in the exact focal point of the beam will be excited by the laser and emit fluorescence.

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 picture below for a comparison of single and multiphoton excitation.



Single photon excitation profile Multiphoton excitation profile

Figure 104 – Single vs Multiphoton excitation of a fluorescent solution [85**]**

Excitation Wavelength

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

Table 10 – Single and two photon fluorophore excitation maxima

Unfortunately due to the longer excitation wavelength, the resolution of two photon imaging is approximately 2x worse than single photon confocal imaging.

Increased Penetration

Two-photon excitation can allow for 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. Multiphoton microscopes usually use non Descanned Detectors (NDDs) for this reason, which are high sensitivity detectors placed directly behind the objective so that as might light as possible is captured.

Single Photon vs Two Photon Imaging

Below is a comparison between single and two photon acquisition. Notice the increased depth of two photon in this case is about 50%.

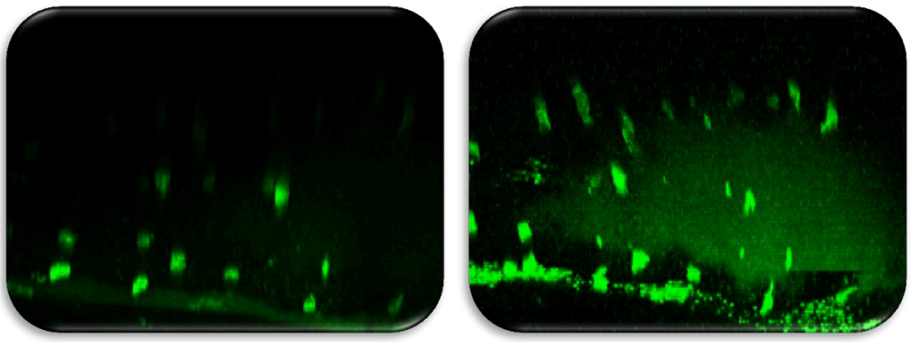


Figure 105 – Single vs Multiphoton depth penetration of a fluorescent sample [86]

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 often 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 as quickly, the data will remain of a similar intensity until maximum depth is reached.

Live Imaging

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.

Spinning Disc confocal imaging is an alternative to galvanometer based point scanning confocal microscopy, where a disc containing thousands of pinholes is utilized to illuminate many points of the sample at once. Combined with a fast and sensitive CMOS or EMCCD camera, these systems may capture a confocal image at 10fps or faster. However the drawback to these systems is that since the pinholes are etched into the disc, a different disc must be used for different Numerical Apertures, this is often not possible due to system design, so the system must be designed around a specific objective.

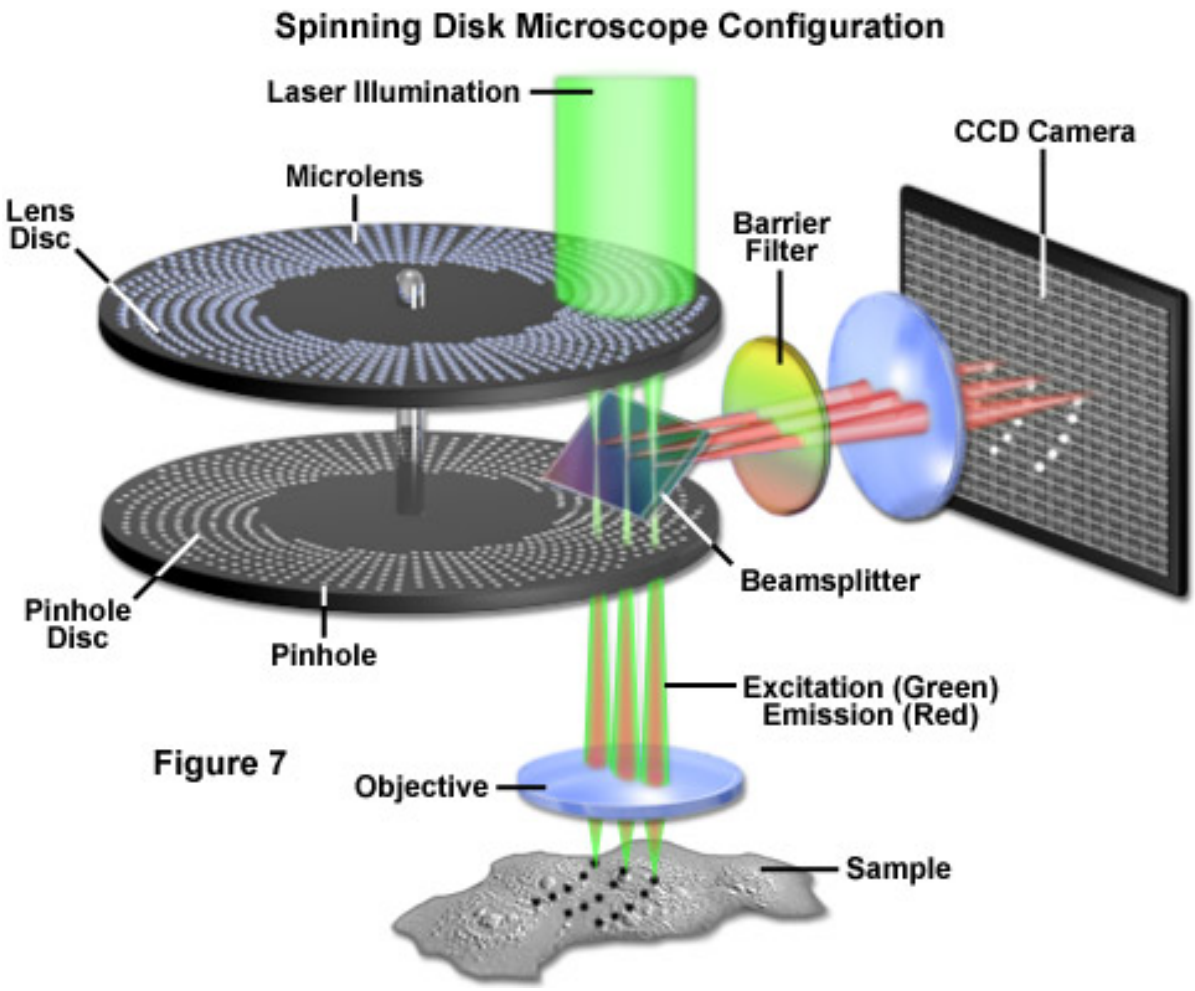


Figure 106 – Spinning disc confocal schematic diagram [91**]**

Spectral Unmixing

Ideally when performing fluorescence imaging, we utilise fluorophores with spectrally separate excitation and emission profiles so that there is no crosstalk or bleedthrough. However sometimes this overlap is unavoidable, such as when there is background auto-fluorescence or when there are 4 or more dyes in your sample or you are performing FRET experiments. In this case we may use a different detector configuration to regular confocal imaging known as Spectral Un-mixing.

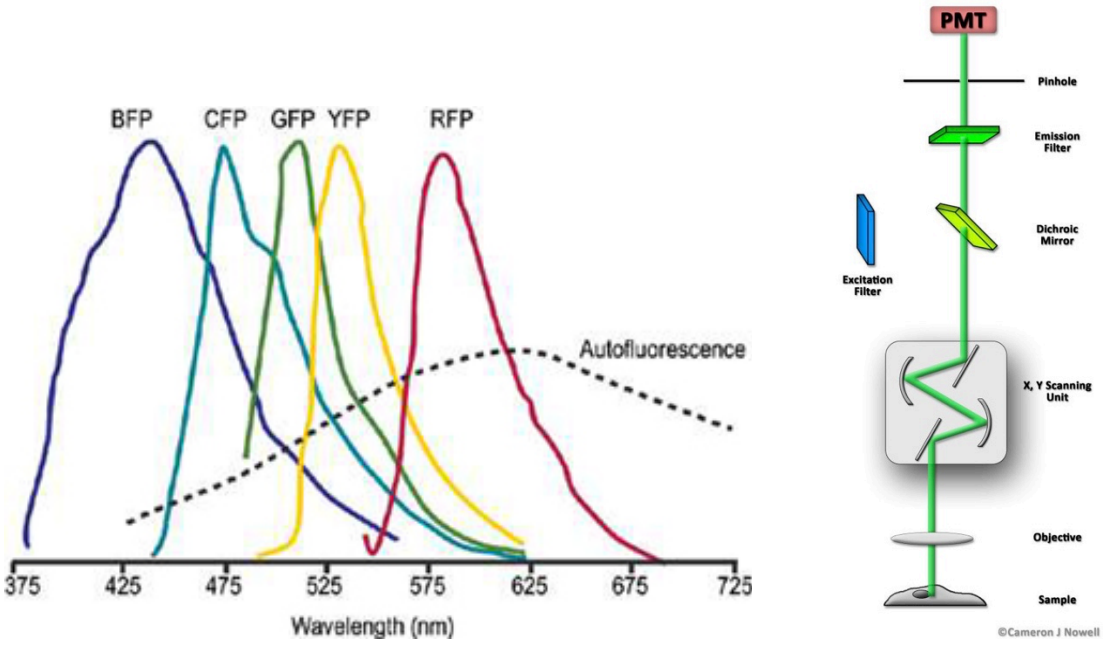


Figure 107 – Excitation spectra of various fluorescent proteins vs autofluorescence [92] [86]

As you can see in the above image, if we were to excite most of these dyes then we would also excite the auto-fluorescence within the sample. Using a typical confocal system, no matter which emission filter we placed in front of the wavelength indiscriminate PMT detector, we would detect the dye as well as the unwanted auto-fluorescent signal.

However if we were to replace the emission filter with a diffraction grating or optical prism, we could disperse the different wavelengths of light spatially and be able to collect the actual emission spectrum of the dyes and the auto-fluorescence.

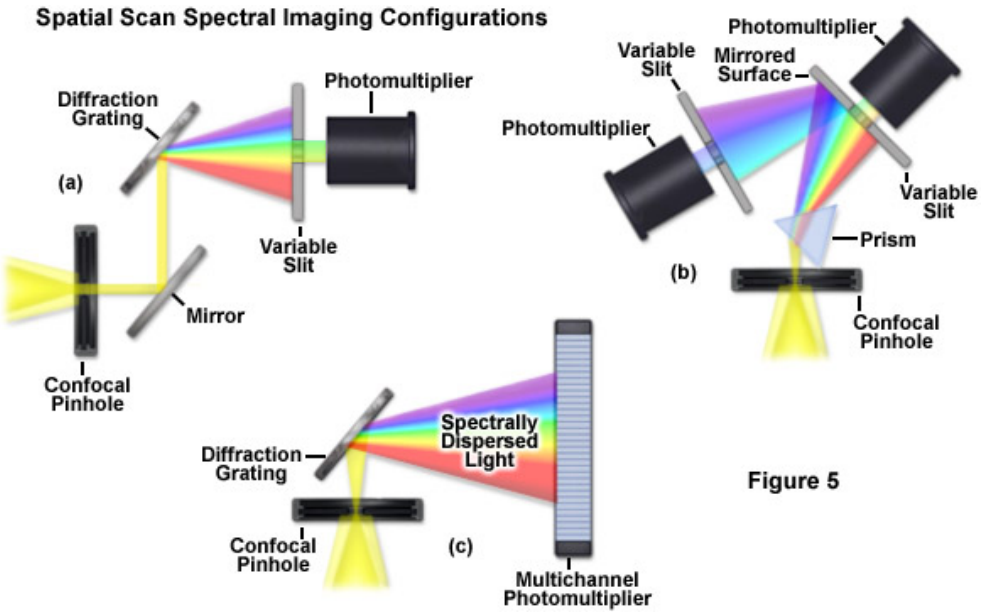


Figure 108 – Spectral Unmixing hardware configurations from different manufacturers [**93**]

By moving the grating or prism to scan the different emitted wavelengths across a single detector or utilizing a multichannel detector to capture all of the emission wavelengths simultaneously, we can produce a stack of images, with each image containing a bandwidth of typically 10nm of the emission spectra. This image stack can be generated for each excitation laser wavelength, producing a very large dataset.

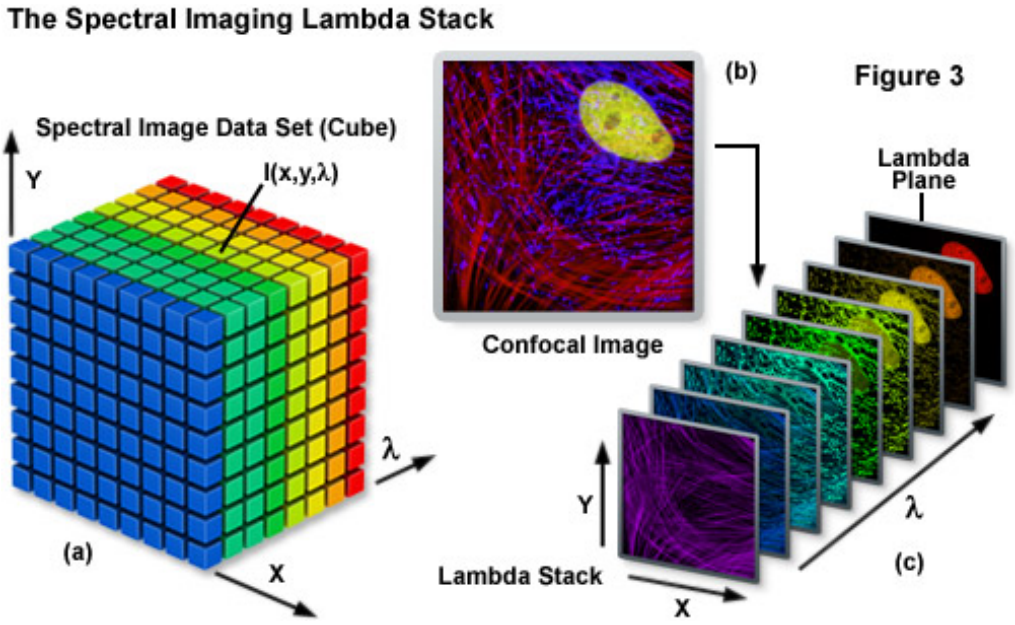


Figure 109 – Spectral imaging lambda stack [93]

Comparing the image stack to a database of fluorophore emission spectra or utilizing mathematical methods allows us to then separate the individual fluorescent spectra at each pixel of the sample.

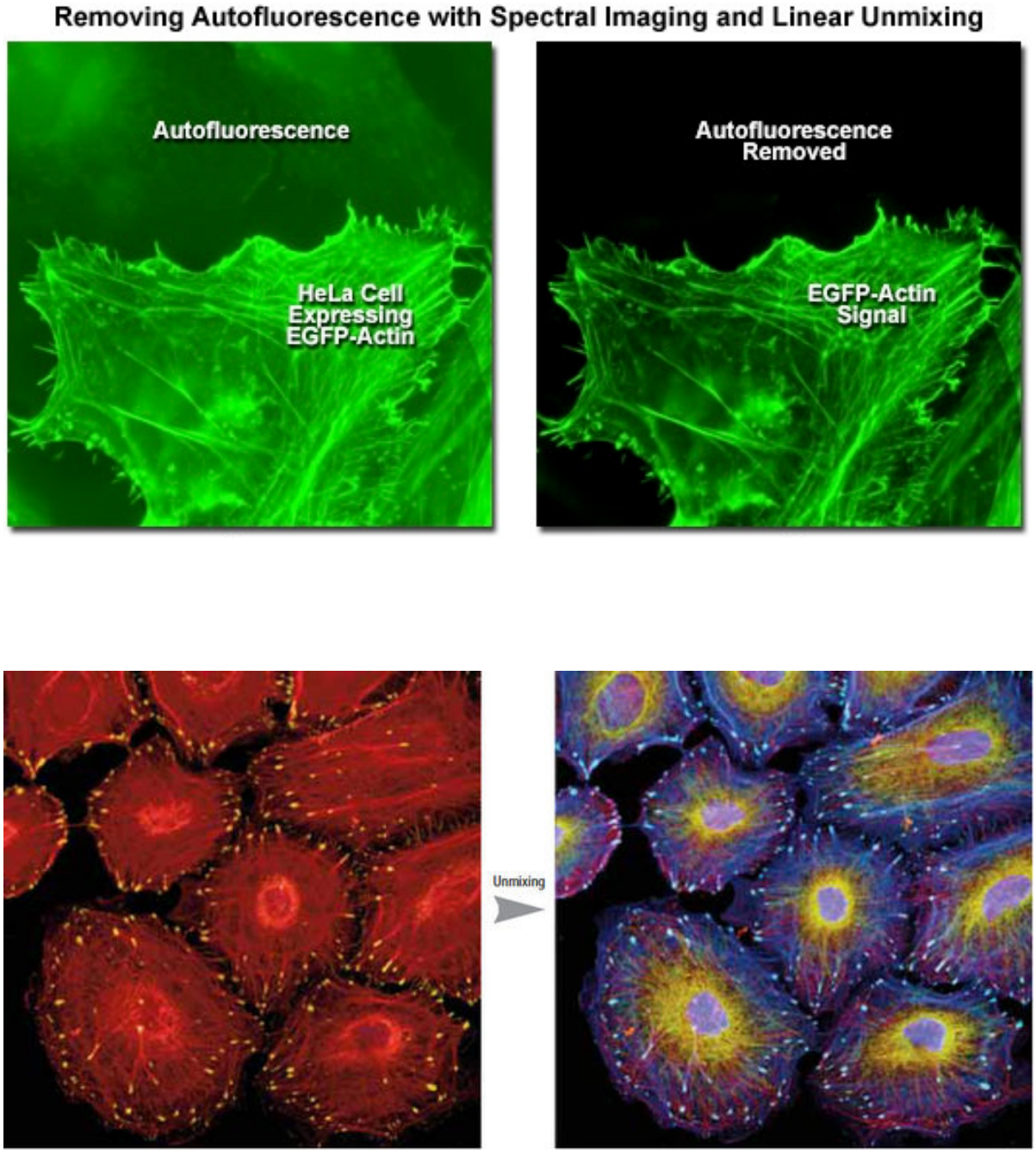


Figure 110 – Spectrally unmixed fluorescent samples [93**]**

Deconvolution

Deconvolution is a mathematical operation used in Image Restoration to recover an image that is degraded by a physical process which can be described by the opposite operation, a convolution. This is the case in image formation by optical systems as used in microscopy and astronomy, but also in many other fields where datasets are captured and require enhancement to resolve information which would otherwise be lost.

In microscopy this convolution process mathematically explains the formation of an image that is degraded by blurring and noise. The blurring is largely due to diffraction limited imaging by the instrument, however another common cause is spherical aberrations due to RI mismatches in the medium and immersion fluid. The noise is usually photon noise, a term that refers to the inherent natural variation of the incident photon flux.

The degree of spreading (blurring) of a single pointlike (Sub Resolution) object is a measure for the quality of an optical system. The 3D blurry image of such a single point light source is usually called the Point Spread Function (PSF).

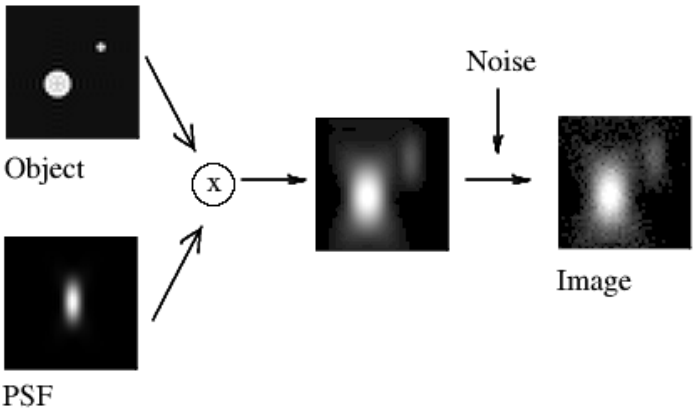
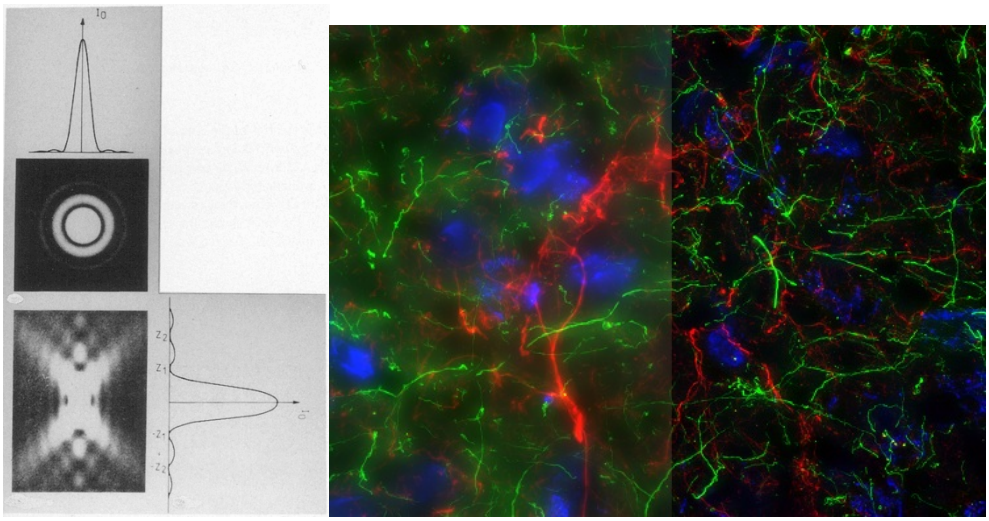


Figure 111 – Convolution of an image [94**]**



PSF *Captured Data* *Deconvolved Data*

Figure 112 – Deconvolution of an image [84**] [**95**]**

In order to deconvolve a dataset, we usually capture a z-stack of our sample at Nyquist conditions for both XY&Z, this allows the algorithm to reassign pixel intensity within both the XY and Z planes to the correct location.

An excellent Nyquist sampling rate calculator can be found Here:

Deconvolution Algorithms can be used on confocal and widefield datasets and work based on three different methods:

- Theoretical PSF, where the user provides information about the microscope and the software generates a theoretical PSF to then deconvolve the image. This achieves a good result at relatively high speeds, but cannot achieve the ultimate resolution improvement possible as many factors such as objective quality are not known precisely.

  Parameters include:

  Sample dimensions

  Pixel size in XY

  Z step-size

  Sample depth from coverslip

  Objective Lens

  NA

  Mounting medium Refractive Index

  Sample Medium Refractive Index

  Excitation Wavelength

  Emission Wavelength

  Back Projected Pinhole Radius (for Confocal-based systems)

  Back Projected Pinhole Distance (for Spinning disc)

  Number of Excitation Photons

- Measured PSF, where the user captures a z-stack of a sub-resolution (<100nm) fluorescent bead and generates an Optical Transfer Function (OTF) file using the Deconvolution software. This OTF file is then used to deconvolve the dataset. This is ultimately the best way to deconvolve a single time point dataset as imaging the PSF of an actual bead captures all of the parameters which cause image degradation.

- Finally, Blind Deconvolution is a method that utilises either a theoretical or measured PSF and then refines the PSF based on the dataset itself. For instance, if a user images an actual PSF it is only done at a single timepoint, so if conditions change in the sample over time, this PSF may no longer be valid. Whereas using blind Deconvolution, the PSF is continuously refined over the course of the experiment, providing the most accurate Deconvolution possible, however the calculations do take longer than the other methods.

2D Deconvolution (also called deblurring) may also be performed on a 2D dataset, however resolution improvements are minimal.

Deconvolution may be performed on any microscope assuming the dataset is acquired under Nyquist conditions and the parameters above are known.

IMB has access to the following Deconvolution hardware and software systems.

Autoquant X3 Software

2D and 3D Theoretical, Measured and Blind Deconvolution

Zeiss Zen Confocal Microscopes and Analysis Software

2D and 3D theoretical and Measured Deconvolution

Deltavision Microscope and Analysis Software

2D and 3D Measured Deconvolution

Nikon Deconvolution Microscope and Analysis Software

2D and 3D Theoretical, Measured and Blind Deconvolution

The Eternal Triangle of Compromise

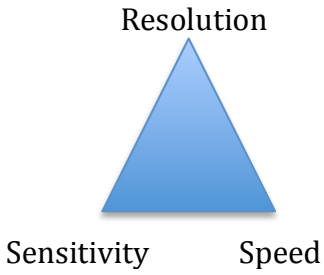


Figure 113 – Eternal Triangle of Compromise

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

Essentially, this means that if one needs to obtain the maximum image acquisition speed in order to capture their samples movements, then either resolution and/or sensitivity must be compromised.

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

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

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

What is important?

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

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

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

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

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

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

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

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

Microscopy Modality Pros and Cons

Equipment	Pros	Cons
Point Scanning Confocal	Good Apparent Resolution (Variable Pin Hole size) Low spatial Crosstalk due to single Pin Hole Typical sample thickness <70um Spectral imaging	Pin hole rejects photons (Quantitation and sensitivity) PMT detectors aren't very sensitive Slow scanning rate (1 – 4fps) Poor cell Viability (not best for Live cell imaging) Poor S/N due to laser stability, PMT detectors
2 Photon Confocal	Typical sample thickness with Two photon <350um	Resolution is approximately 2x worse than confocal imaging Can be destructive in the image plane if high laser powers are used Still a point scanning confocal so many of the same issues as single photon imaging
Spinning Disc Confocal	Fast data capture. CCD Camera based Averaging of images on the fly. Good S/N Fair cell Viability Typical Sample thickness <70um Works with any Microscope 1-100fps	Fixed Pin Hole (50um – 70um) Uneven illumination (15 – 30%) Difficulties with FRAP Spectral crosstalk issues due to Poly-dichroic Spatial crosstalk on thicker samples due to pin hole spacing Not good for highly auto fluorescent samples Not good for weakly fluorescent samples.
Widefield Deconvolution	Fast data Capture. Very Good S/N High Sensitivity Camera No Pin Hole (no light loss) (Good for low light samples) Good Cell Viability White light or LED based (350nm – 1100nm)	Typical Sample thickness <35um Not good for highly auto fluorescent samples Generally single camera, so multicolour acquistions may be slower than required Deconvolution may be slow for large datasets

Table 11 – Microscopy modality comparisons

Credits

This document draws heavily from the Zeiss, Nikon, Olympus and Molecular Expressions microscopy websites, a Berkeley University course page, and the Ludwig Institute for Cancer Research Centre for Advanced Microscopy Training Manual, by Cameron Nowell. Students are directed to these resources for a deeper understanding of this material.

Bibliography

[1] Molecular Expressions. Introduction. [Online]. http://micro.magnet.fsu.edu/primer/anatomy/introduction.html

[2] Molecular Expressions. Cleaning. [Online]. http://micro.magnet.fsu.edu/primer/anatomy/cleaning.html

[3] Wikipedia. Cornelis Drebbel. [Online]. https://en.wikipedia.org/wiki/Cornelis_Drebbel

[4] Wikipedia. Microscope. [Online]. https://en.wikipedia.org/wiki/Microscope#cite_note-Wootton-4

[5] Wikipedia. Antonie van Leeuwenhoek. [Online]. https://en.wikipedia.org/wiki/Antonie_van_Leeuwenhoek

[6] Zeiss. Campus Online. [Online]. http://zeiss-campus.magnet.fsu.edu/articles/basics/kohler.html

[7] Hyperphysics. Conjugate Planes. [Online]. http://hyperphysics.phy-astr.gsu.edu/hbase/geoopt/conjug.html

[8] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/articles/formulas/formulasconjugate.html

[9] Molecular Expressions. Magnifications. [Online]. http://micro.magnet.fsu.edu/primer/anatomy/magnification.html

[10] Proscitech. Calibration slides. [Online]. https://proscitech.com/?navaction=show_page&chapter=s&page=3

[11] Wikipedia. Paraxial approximation. [Online]. https://en.wikipedia.org/wiki/Paraxial_approximation

[12] Wikipedia. Optical Aberration. [Online]. https://en.wikipedia.org/wiki/Optical_aberration

[13] Molecular Expressions. Point Spread Aberration. [Online]. http://micro.magnet.fsu.edu/primer/java/aberrations/pointspreadaberration/index.html

[14] Olympus. Microscopy Resource Centre. [Online]. http://www.olympusmicro.com/primer/anatomy/aberrations.html

[15] Molecular Expressions. Field Curvature. [Online]. http://micro.magnet.fsu.edu/primer/anatomy/fieldcurvature.html

[16] Olympus. Microscopy Resource Centre. [Online]. http://www.olympusmicro.com/primer/java/aberrations/spherical/

[17] Wikipedia. Achromatic Lens. [Online]. https://en.wikipedia.org/wiki/Achromatic_lens

[18] Nikon. Introduction to Microscope Objectives. [Online]. http://www.microscopyu.com/articles/optics/objectiveintro.html

[19] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/articles/formulas/formulasna.html

[20] Olympus. Microscopy Resource Centre. [Online]. http://olympus.magnet.fsu.edu/primer/anatomy/numaperture.html

[21] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/articles/formulas/formulasworkingparfocal.html

[22] Nikon. Coverglass. [Online]. http://www.nikon.com/products/instruments/resources/tech/info/microscope_tech/coverglass/index_02.htm

[23] Microscopy-UK. Microscopy-UK. [Online]. http://www.microscopy-uk.org.uk/index.html?http://www.microscopy-uk.org.uk/primer/geomop.htm

[24] Molecular Expressions. Specifications. [Online]. http://micro.magnet.fsu.edu/primer/anatomy/specifications.html

[25] Molecular Expressions. Immersion. [Online]. https://micro.magnet.fsu.edu/primer/anatomy/immersion.html

[26] Molecular Expressions. Oculars. [Online]. https://micro.magnet.fsu.edu/primer/anatomy/oculars.html

[27] Olympus. Microscopy Resource Centre. [Online]. http://olympus.magnet.fsu.edu/primer/anatomy/specifications.html

[28] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/articles/optics/objectivespecs.html

[29] Zeiss. Campus Online. [Online]. http://zeiss-campus.magnet.fsu.edu/tutorials/basics/objectivecolorcoding/indexflash.html

[30] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/articles/optics/cfintro.html

[31] Zeiss. campus online. [Online]. http://zeiss-campus.magnet.fsu.edu/mobile/tutorials/basics/objectivemagnification/indexmobile.html

[32] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/articles/stereomicroscopy/stereointro.html

[33] Olympus. Microscopy Resource Centre. [Online]. http://olympus.magnet.fsu.edu/primer/anatomy/illumination.html

[34] Leica. Science Lab. [Online]. http://www.leica-microsystems.com/index.php?id=3596

[35] Olympus. Microscopy Resource Centre. [Online]. http://olympus.magnet.fsu.edu/primer/techniques/confocal/confocalobjectives.html

[36] Micropix. Microscope Adaptors. [Online]. http://www.micropix.co.uk/microscope-accessories/microscope-adapters.html

[37] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/print/articles/digitalimaging/ccdintro-print.html

[38] Elprocus. Working Principle of CCDs. [Online]. https://www.elprocus.com/know-about-the-working-principle-of-charge-coupled-device

[39] Marcus Reugels. Fun with Colours. [Online]. https://www.flickr.com/photos/maianer/5346859667

[40] Wikipedia. Bayer Filter. [Online]. https://en.m.wikipedia.org/wiki/Bayer_filter - Fujifilm_.22X-Trans.22_filter

[41] Clark Vision. Does Pixel Size Matter. [Online]. http://www.clarkvision.com/articles/does.pixel.size.matter

[42] Andor. Dynamic Range. [Online]. http://www.andor.com/learning-academy/dynamic-range-and-full-well-capacity-a-definition-of-ccd-dynamic-range

[43] Molecular Expressions. CCD Saturation and Blooming. [Online]. https://micro.magnet.fsu.edu/primer/digitalimaging/concepts/ccdsatandblooming.html

[44] Wikipedia. Charge Coupled Device. [Online]. https://en.wikipedia.org/wiki/Charge-coupled_device

[45] Zeiss. Digital Imaging. [Online]. http://zeiss-campus.magnet.fsu.edu/articles/basics/digitalimaging.html

[46] Picture Power. What is a histogram. [Online]. http://www.picture-power.com/what-is-a-histogram.html

[47] Qimaging. FAQs. [Online]. http://www.qimaging.com/support/faq/faq_details.php?faq_id=6

[48] AMMRF. Fluorescence Microscopy. [Online]. http://www.ammrf.org.au/myscope/popups/confocal/fluorescence.html?iframe=

true&width=800&height=600

[49] Olympus. Digital Imaging Detectors. [Online]. http://www.olympus.magnet.fsu.edu/primer/digitalimaging/digitalimagingdetectors.html

[50] Molecular Expressions. Contrast in Optical Microscopy. [Online]. http://micro.magnet.fsu.edu/primer/techniques/contrast.html

[51] Zeiss. Campus Online. [Online]. http://zeiss-campus.magnet.fsu.edu/print/livecellimaging/techniques-print.html

[52] Molecular Expressions. Kohler Microscope Illumination. [Online]. http://micro.magnet.fsu.edu/primer/anatomy/kohler.html

[53] Histology OLM 4.0. The Light Path. [Online]. http://stevegallik.org/sites/histologyolm.stevegallik.org/htmlpages/HOLM_Chapter01_Page04.html

[54] Microscopy-UK. Darkfield. [Online]. http://www.microscopy-uk.org.uk/mag/indexmag.html?http://www.microscopy-uk.org.uk/mag/artjun07/pw-cameras.html

[55] Charlotte K Omoto. Using Darkfield Microscopy to Enhance Contrast. [Online]. http://public.wsu.edu/~omoto/papers/darkfield.html

[56] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/articles/formulas/specimencontrast.html

[57] BME Lab & Science. Phase Contrast. [Online]. https://www.bmelabandscience.com/new-waste-water-treatment-phase-microscopy-kit/

[58] Nobel Prize.org. The Phase Contrast Microscope. [Online]. http://www.nobelprize.org/educational/physics/microscopes/phase/

[59] ChemEd DL. The Nature of Electromagnetic Radiation. [Online]. http://chempaths.chemeddl.org/services/chempaths/?q=book/General%20Chemistry%20Textbook/Spectra%20and%20Structure%20of%20Atoms%20and%20Molecules/1792/nature-electromagn

[60] Wikipedia. Birefringence. [Online]. https://en.wikipedia.org/wiki/Birefringence

[61] Harvard University. Birefringence. [Online]. http://isites.harvard.edu/icb/icb.do?keyword=k16940&pageid=icb.page93275&pageContentId=icb.pagecontent221674&view=view.do&viewParam_name=indepth.html

[62] Olympus. Microscopy Resource Centre. [Online]. http://olympus.magnet.fsu.edu/primer/lightandcolor/birefringence.html

[63] Michael J Klein and Edward F DiCarlo, "Chapter 13 - Non-neoplastic Diseases of Bones and Joints," in *Silverbergs Principles and Practice of Surgical Pathology and Cytopathology*, 5th ed., 2015, pp. 942-1006. [Online].

http://ebooks.cambridge.org/chapter.jsf?bid=CBO9781139137201&cid=CBO9781139137201A022

[64] Nikon. Nikon Small World. [Online]. http://www.nikonsmallworld.com/galleries/entry/2012-photomicrography-competition/13

[65] Wikipedia. DIC. [Online]. https://en.wikipedia.org/wiki/Differential_interference_contrast_microscopy

[66] James McCracken. Expert Cytometry. [Online]. http://expertcytometry.com/understanding-jablonski-diagram-helps-publish-flow-cytometry-data/

[67] Molecular Expressions. Basic Concepts in Fluorescence. [Online]. https://micro.magnet.fsu.edu/primer/techniques/fluorescence/fluorescenceintro.html

[68] ThermoFisher Scientific. Fluorescence Fundamentals. [Online]. https://www.thermofisher.com/au/en/home/references/molecular-probes-the-handbook/introduction-to-fluorescence-techniques.html

[69] Wikipedia. Fluorescence Lifetime imaging microscopy. [Online]. https://en.wikipedia.org/wiki/Fluorescence-lifetime_imaging_microscopy

[70] ThermoFisher Scientific. Photobleaching in Fluorescence Imaging and ways to prevent it. [Online]. https://www.thermofisher.com/au/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/protocols-troubleshooting/troubleshooting/photobleaching.html

[71] Molecular Expressions. Photobleaching. [Online]. http://micro.magnet.fsu.edu/primer/java/fluorescence/photobleaching/

[72] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/references/phototoxicity.html

[73] Zeiss. Campus Online. [Online]. http://zeiss-campus.magnet.fsu.edu/articles/lightsources/mercuryarc.html

[74] Zeiss. Campus Online. [Online]. http://zeiss-campus.magnet.fsu.edu/articles/lightsources/leds.html

[75] Semrock. Laser based imaging: What you need to know. [Online]. https://www.semrock.com/1mainarticle.aspx

[76] Zeiss. Campus Online. [Online]. http://zeiss-campus.magnet.fsu.edu/articles/basics/fluorescence.html

[77] Olympus. Microscopy Resource Centre. [Online]. http://olympus.magnet.fsu.edu/primer/techniques/fluorescence/anatomy/fluoromicroanatomy.html

[78] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/articles/stereomicroscopy/stereofluorescence.ht

ml

[79] Berkeley University. Fluorescence Microscope. [Online]. http://microscopy.berkeley.edu/courses/tlm/fluor/fluor_micro.html

[80] Semrock. GFP-30LP-B-000. [Online]. https://www.semrock.com/SetDetails.aspx?id=2943

[81] Semrock. BFP/GFP/HcRed-3X-A-000. [Online]. https://www.semrock.com/SetDetails.aspx?id=2716

[82] B.K., Cai, X., & Nebenführ, A. Nelson, "A Multi-color set of in vivo organelle markers for colocalisation studies in arabidopsis and other plants," *Plant Journal*, no. 51, pp. 1126-1136.

[83] Olympus. Microscopy Resource Centre. [Online]. http://olympus.magnet.fsu.edu/primer/techniques/confocal/bleedthrough.html

[84] Jim Pawley, "The 39 Steps: A Cautionary Tale about Quantitative 3D Fluorescence Microscopy," in *BioTechniques*.

[85] Berkeley University. Two Photon Imaging. [Online]. http://microscopy.berkeley.edu/courses/tlm/2P/index.html

[86] Cameron Nowell, *Ludwig Institute for Cancer Research: Centre for Advanced Microscopy Training Manual*.

[87] Olympus. Microscopy Resource Centre. [Online]. http://olympus.magnet.fsu.edu/primer/techniques/confocal/detectorsintro.html

[88] Nikon. MicroscopyU. [Online]. http://www.microscopyu.com/articles/formulas/formulasimagebrightness.html

[89] University of North Carolina. Confocal Laser Scanning Microscopy Tutorial. [Online]. https://www.med.unc.edu/microscopy/files/courses/spring-2013-lm/path-464-class-notes/lm-extras/clsm-tutorial

[90] Wikipedia. Nyquist-Shannon sampling theorem. [Online]. https://en.wikipedia.org/wiki/Nyquist–Shannon_sampling_theorem

[91] Zeiss. Campus Online. [Online]. http://zeiss-campus.magnet.fsu.edu/articles/spinningdisk/introduction.html

[92] Andor. Spectral Imaging. [Online]. http://www.andor.com/learning-academy/spectral-imaging-an-overview-of-andor's-solutions-for-spectral-imaging

[93] Zeiss. Campus Online. [Online]. http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html

[94] Scientific Volume Imaging. Huygens Deconvolution. [Online]. https://svi.nl/HuygensDeconvolution

[95] Media Cybernetics. Autoquant X3. [Online].

http://www.mediacy.com/autoquantx3

[96] Olympus. Microscopy Resource Center. [Online]. http://www.olympusmicro.com/primer/techniques/confocal/resolutionintro.html

[97] Wikipedia. Optics. [Online]. https://en.wikipedia.org/wiki/Optics

[98] Wikipedia. Light. [Online]. https://en.wikipedia.org/wiki/Light

[99] Wikipedia. Image. [Online]. https://en.wikipedia.org/wiki/Image

[100] Wikipedia. Optical Instrument. [Online]. https://en.wikipedia.org/wiki/Image