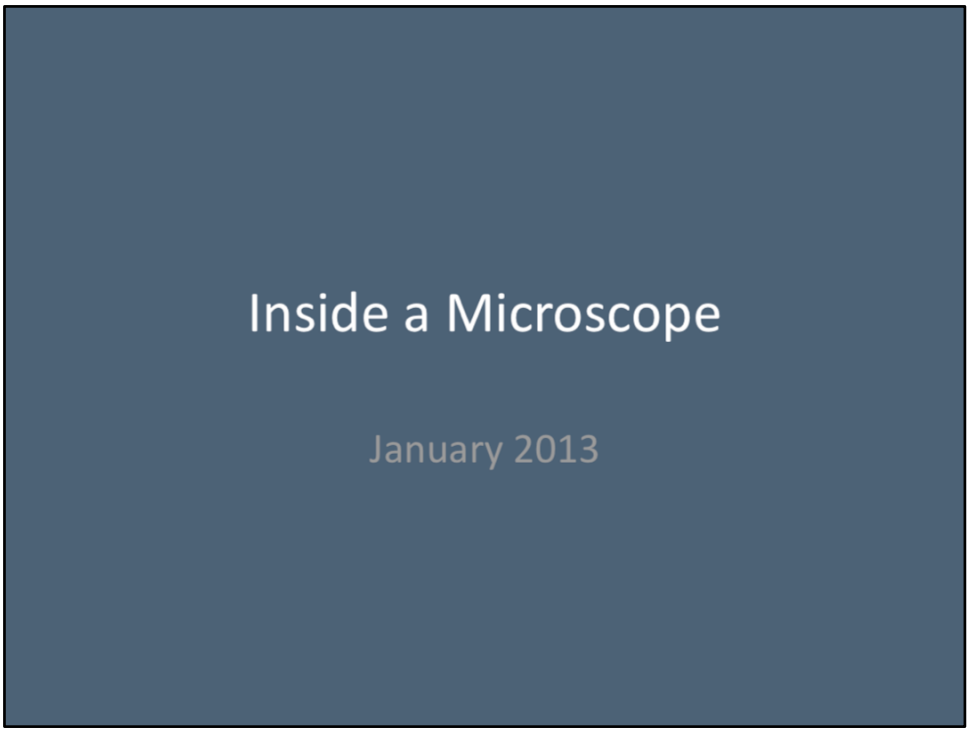
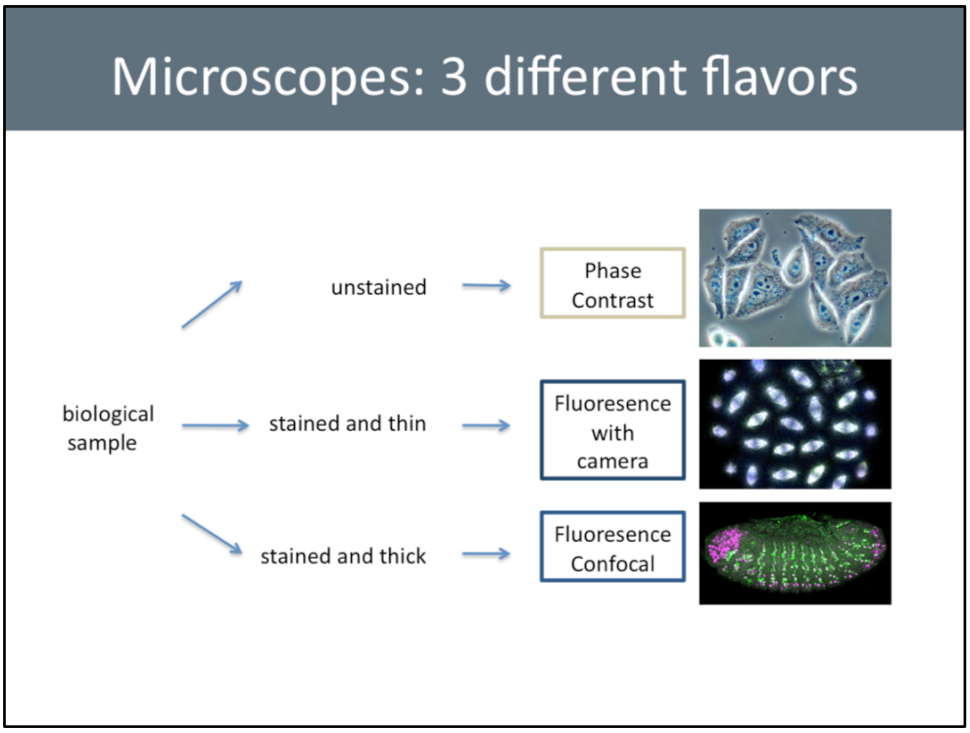


This is going to be a more practical description of the different components of Light, fluorescence, and confocal microscopes, focusing on the microscopes that are available in the Dunn School Bioimaging Facility.

This lecture will include a more hands on session where participants will be able to take apart a microscope to investigate it's inner workings.



The three different microscopes that we will be covering are Phase contrast and fluorescence microscope (These are both carried out on the same microscope body) and confocal microscope.

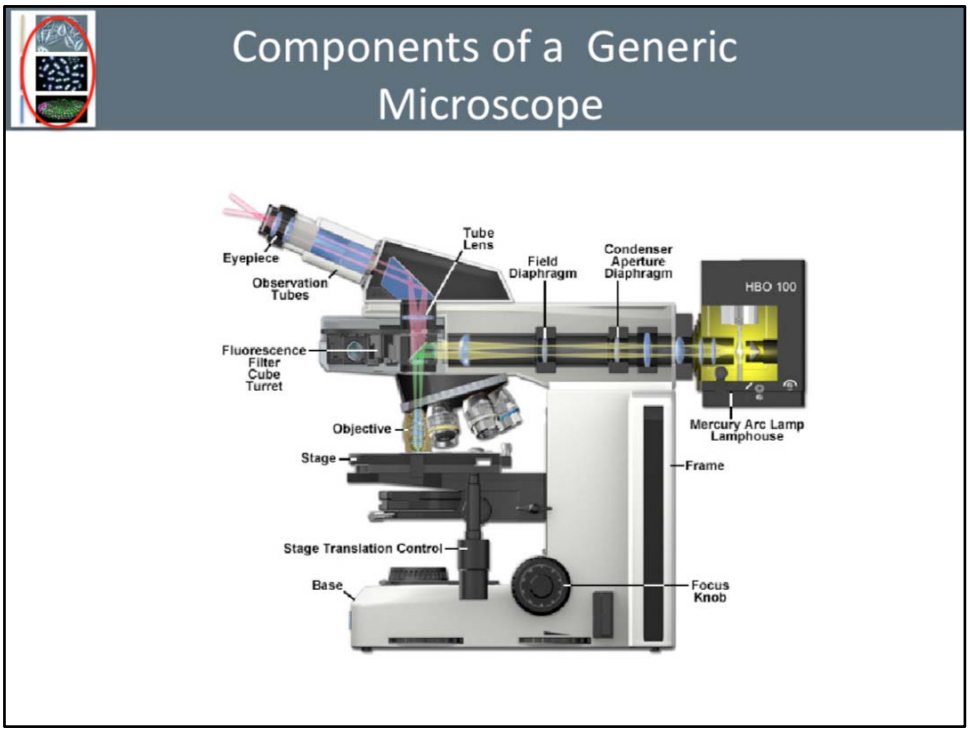
Each of these microscopes is useful depending on the biological sample that you wish to look at:

Phase contrast microscopy is used for looking at fairly thin unstained samples such as tissue culture cells. A particular application of phase contrast is to combine it with fluorescence.

Fluorescence microscopy is useful when looking at fairly thin samples fixed and stained with various fluorophores.

Confocal microscopes are useful for looking at thicker samples, and can be useful to assess details in the Z axis through Z-stack generation and 3D reconstructions.

We will look at these 3 different microscopes in turn, with the main focus being on fluorescence microscope.



Components of a Generic microscope

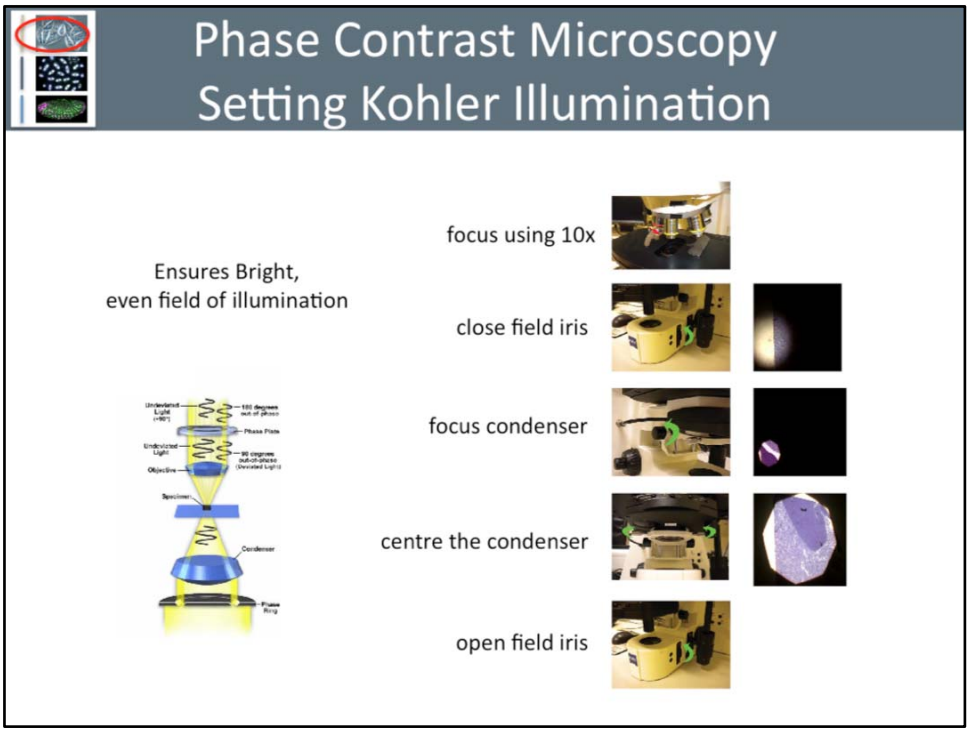
Microscopes come in all kind of shapes, sizes and complexity. They can be inverted or upright, have brightfield or fluorescent illumination (or both) have eyepiece, camera or PMT detector for observing the sample.

All microscopes have the following components:

A light source.
Various lenses and condensers to focus the light onto the specimen.
A lens to capture light from the specimen and magnify the image.
A stage to enable the focus point of the sample to be changed.
Some way of observing the image.

Phase Contrast

Phase Contrast is the most widely used technique for looking at unstained specimens. Interior cellular details are readily seen, but features (especially thick round structures) are surrounded by a halo which is unavoidable and can be distracting.

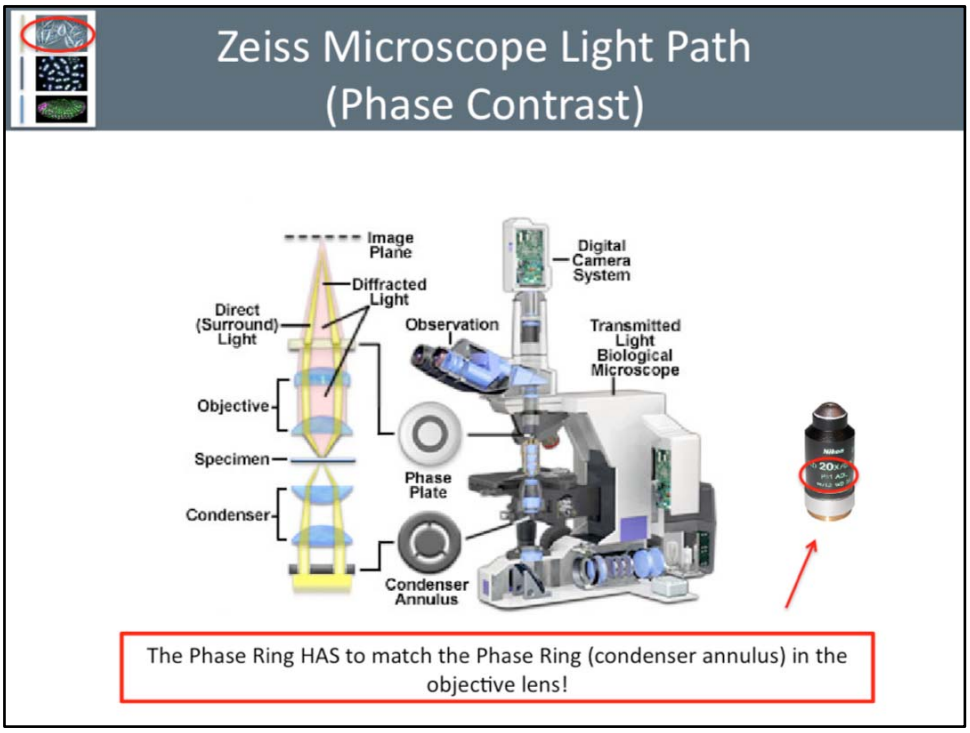


Kohler Illumination

Kohler Illumination gives bright, even lighting across the whole field of view, even at low magnifications. It is essential starting point for setting up a microscope.

1.) Focus on a specimen using a 10x objective
2.) Close the field iris diaphragm.
3.) Focus the condenser to get a 'sharp edged' image of the iris blades.
4.) Centre the condenser, opening the iris as you go.
5.) Set the aperture iris diaphragm to 20%

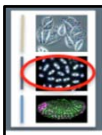
When changing objectives the condenser does not need re-centering, but adjust the field iris to just outside the field of view, and reset the aperture iris to match the objective



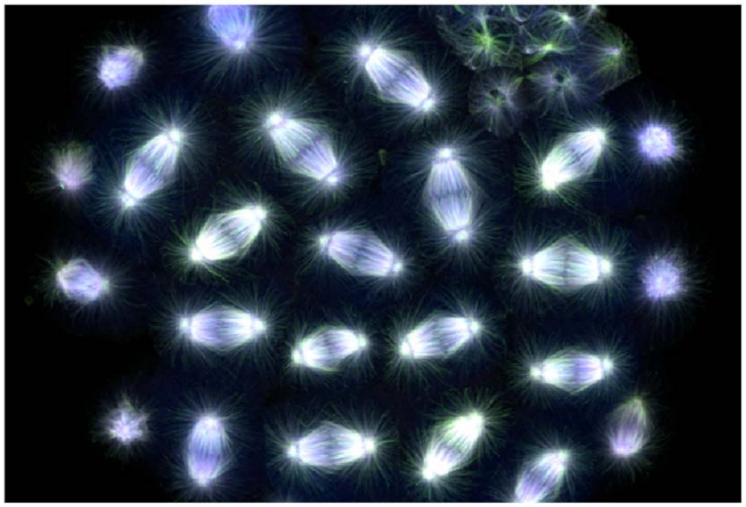
Phase Contrast Microscope

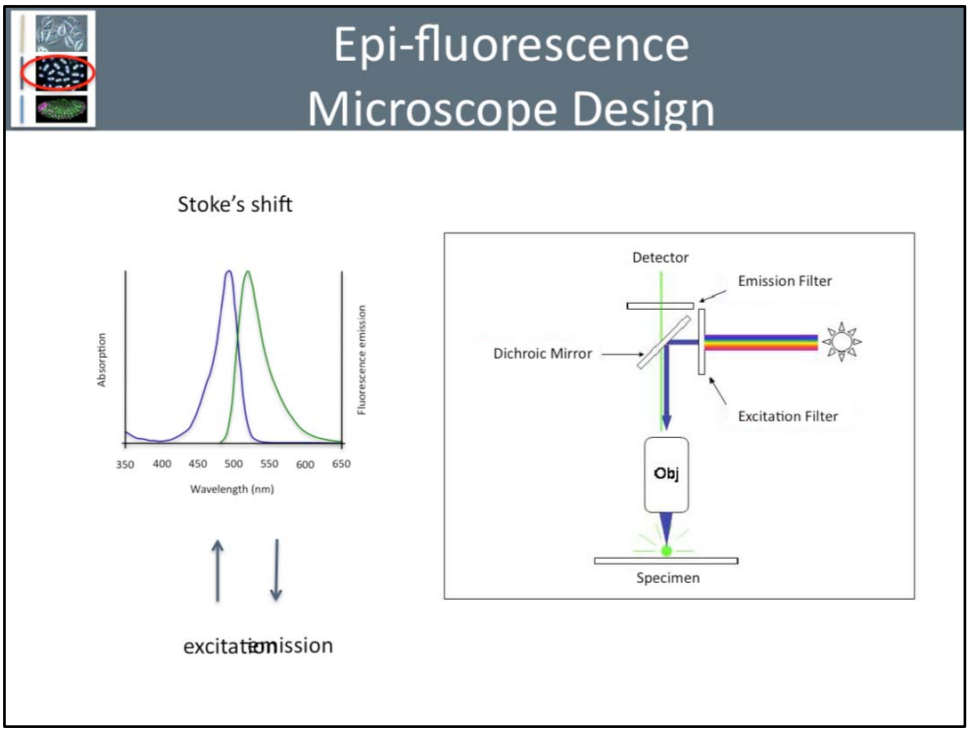
As phase contrast is created by phase shifts in the light path the best effect is seen if monochromatic light is used, and as the eye is most sensitive to green, a green glass filter is recommended.

Remember: Bring the relevant condenser annulus into position. If in doubt the number on the the condenser annulus (eg. Ph1) must match that written on the objective lens.



Fluorescence Microscope

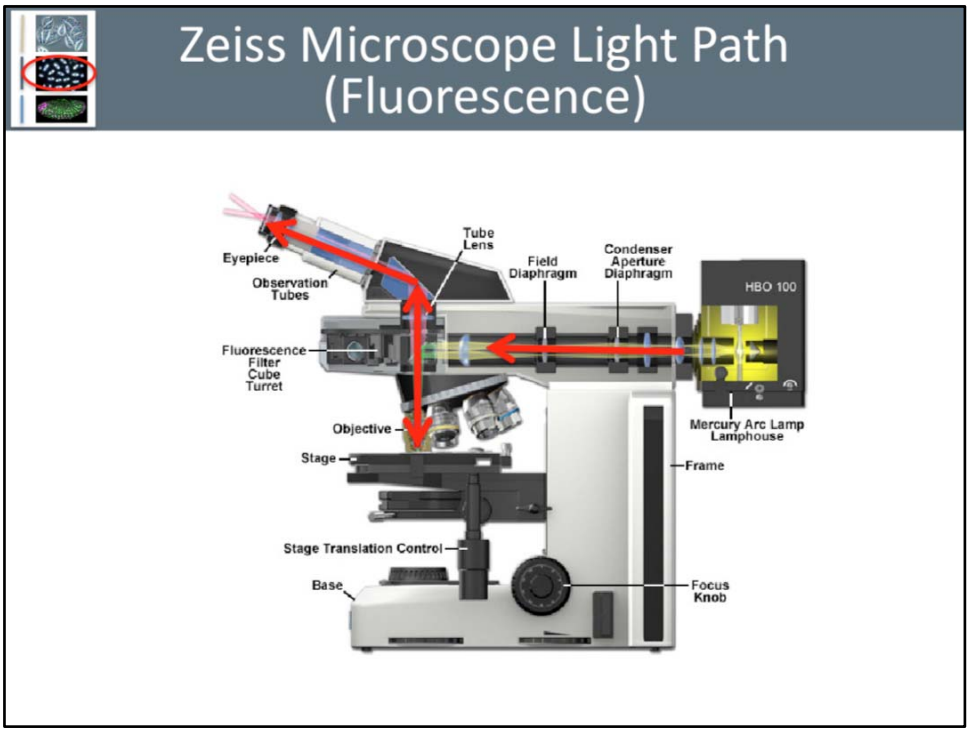




Fluorescence Microscope

The design of a fluorescence microscope is based on the Stokes shift of excitation light to a longer wavelength of the emitted light during fluorescence. This enables a dichoric mirror to separated exitation and emitted light.

The dichroic mirror is at the heart of the fluorescence microscope. This 'mirror' reflects light below a certain wavelength and allows light above this wavelength to pass through.

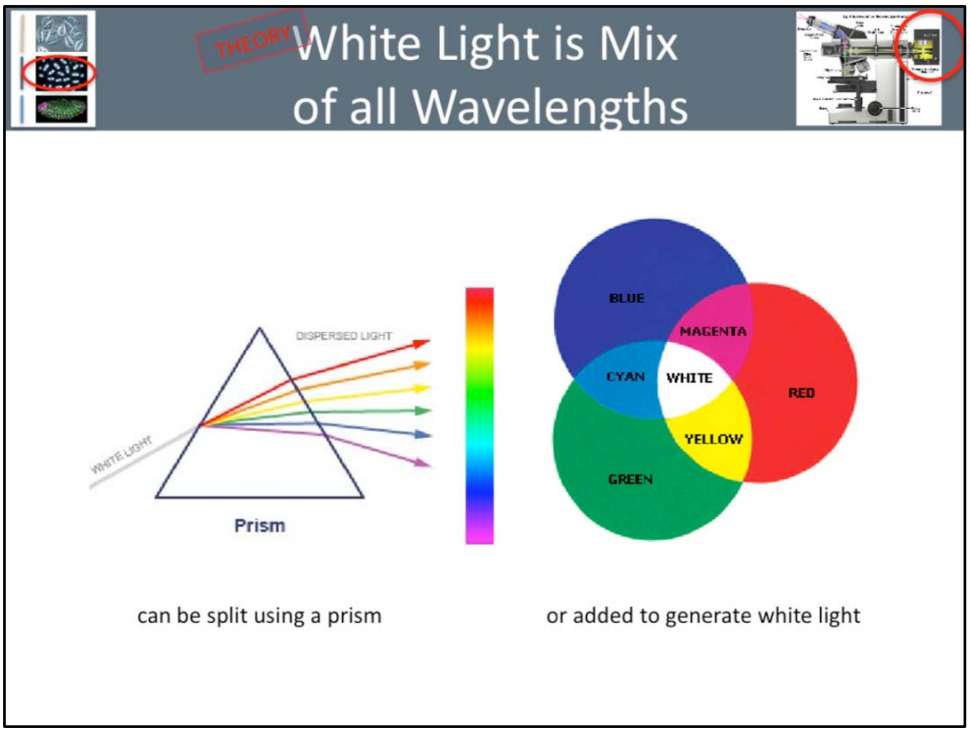


Fluorescence Microscope

In addition to the dichroic mirror, a basic fluorescence microscope has various other components. If we follow the light path, these are in turn:

1.) The Light Source (In this case a Mercury Arc lamp)
2.) The Filter cube, which is composed of excitation and emission filters in addition to the dichroic mirror.
3.) The Objective Lens

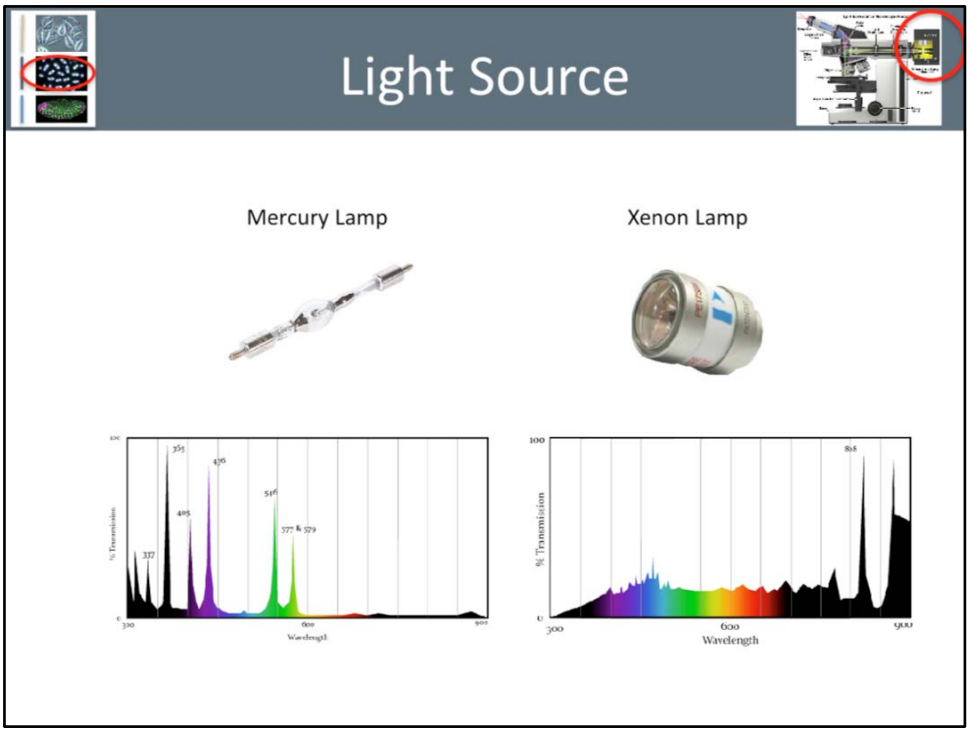
We will take each of these in turn and cover some of the details that are important to consider to generate high quality images.



White Light

Firstly we will consider the Light Sources used on fluorescence microscopes.

Just a bit of theory here to confirm that white light is a mix of all wavelengths of visible light. This is routinely seen either in a rainbow, or when passing light through a glass prism. The various component wavelengths are diffracted to differing extents.



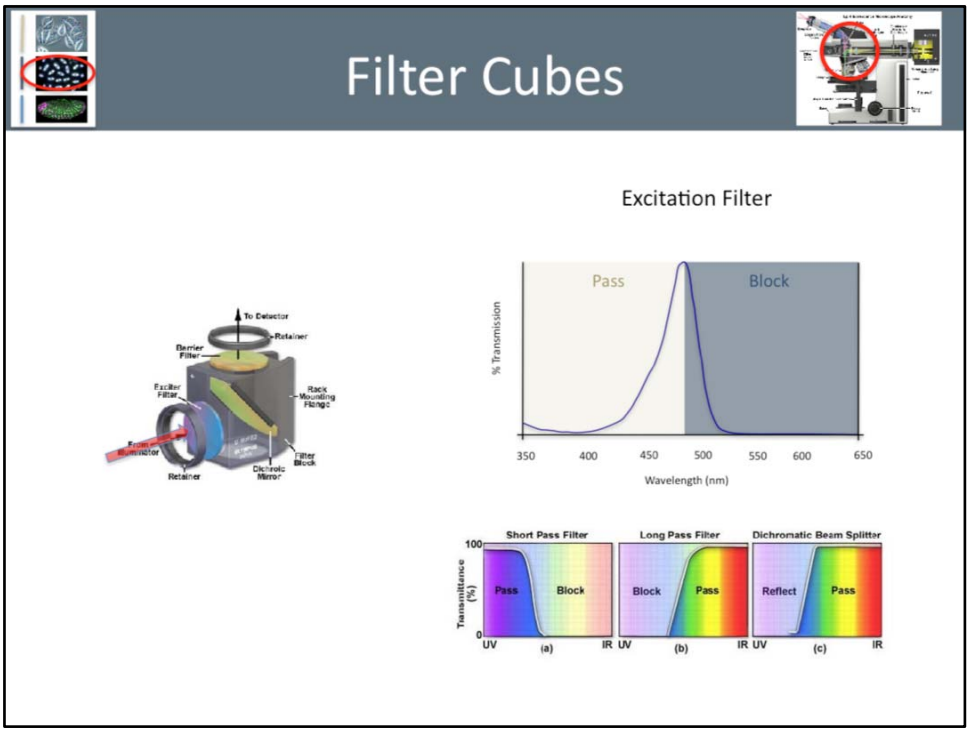
Fluorescent Light Sources

So commonly used Light sources for fluorescence microscopes are:

Mercury Vapour burners: These produce an intense but peaky light. They are good for routine light as the peaks are around blue light (though not so good if you hope to use far red fluorophores).

Xenon burners: These give a smoother emission curve over the spectrum of visible light, and are particularly good at the red end of the spectrum.

(standard 100w halogen bulbs are not sufficiently bright enough to use for fluorescence microscopy)



Filter Cubes

Three key components: excitation and emission filters and dichroic mirror.

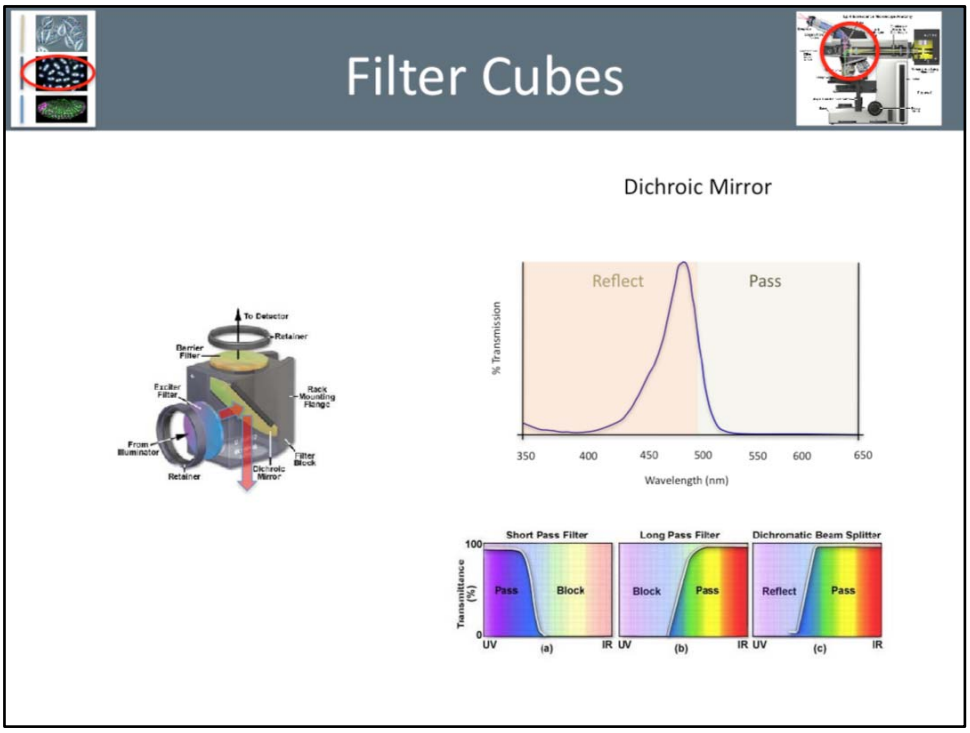
In each filter cube, the filter sets are matched to standard chromophores:
[ie for viewing DAPI (em blue), GFP (em green), FITC (em green), Cy3 (em red), Cy5(em far red) .]

can be either shortpass (wavelengths less than a certain value pass through, but those longer are blocked).

Longpass (wavelengths higher than a certain value pass through, but those shorter are blocked).

Bandpass (wavelengths between two values pass through, but all pther wavelengths are blocked).

The dichroic mirror reflects light below a certain wavelength and allows light above this wavelength to pass through.



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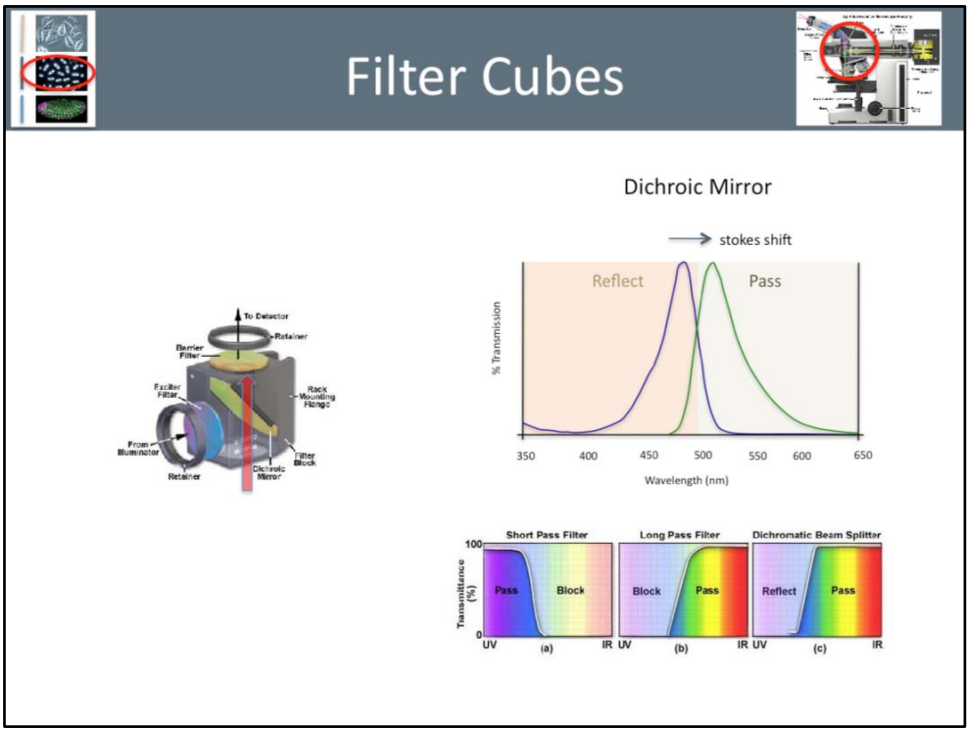

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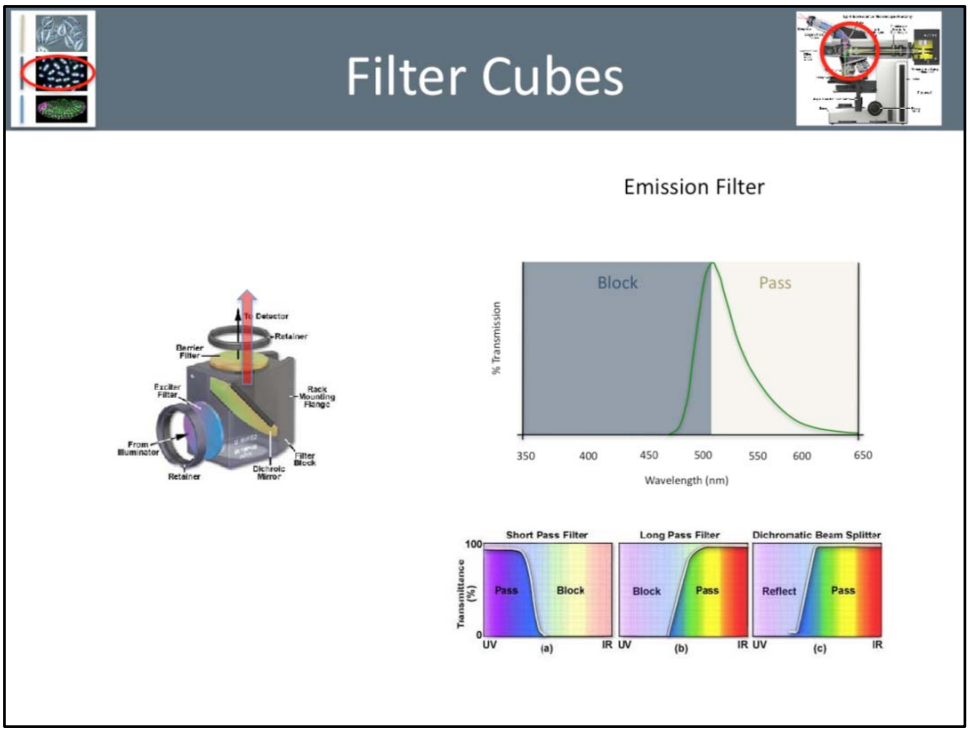

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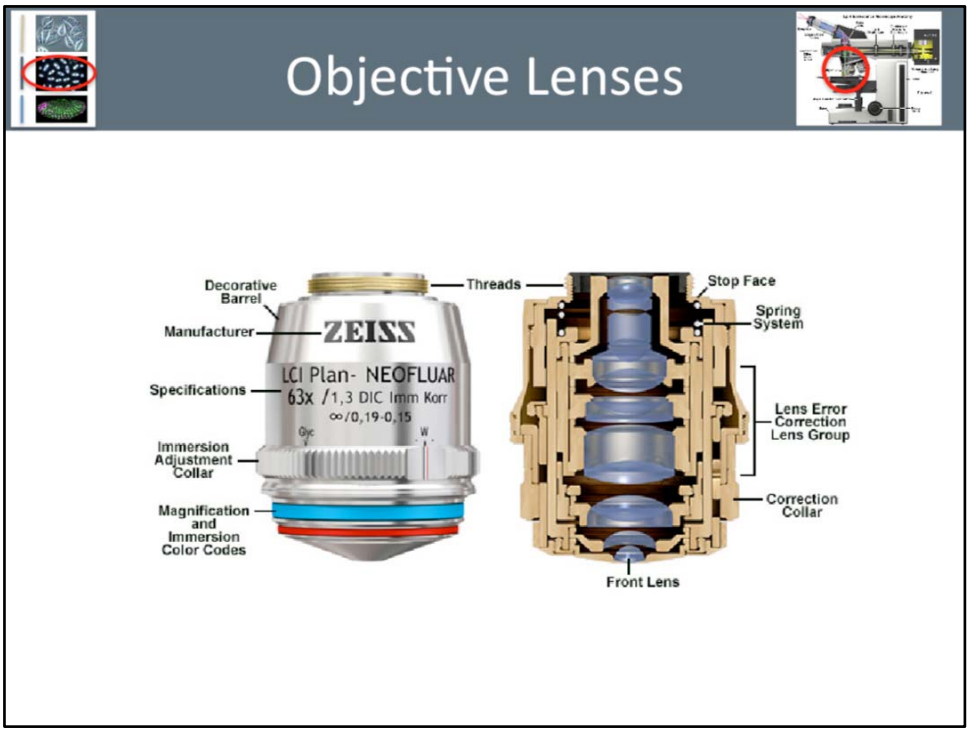
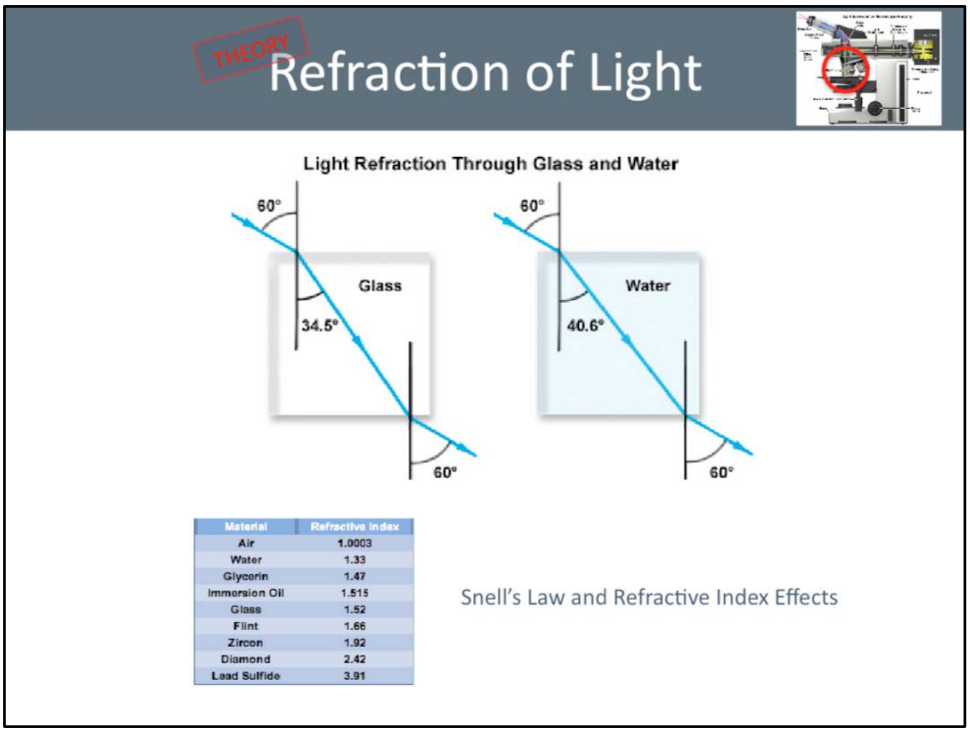


Objective Lenses

MAGNIFICATION: Important to read this along with the NA (lateral magnification 63x)
NA : 1.3
Abberation Correction: covered later
Coverslip thickness -: in this example it is 0.19-0.15

These are the most precious part of the microscope, made up of many glass elements and should be treated with the care that is due to them!



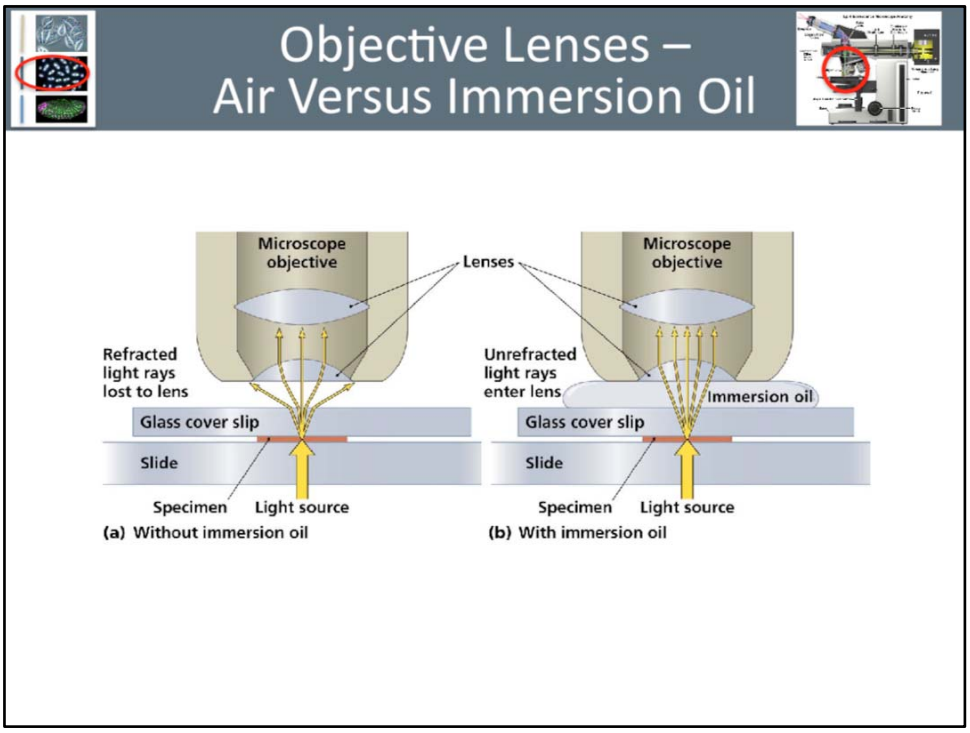
Refraction of Light

Light travels in straight lines. Light can be 'bent' or refracted – this is the phenomenon describing how the angle of a light path changes when passing from one medium into another (ie from air into glass).

Light travels at different speeds in different media (eg faster in air (=1) and slower in glass (=1.52)

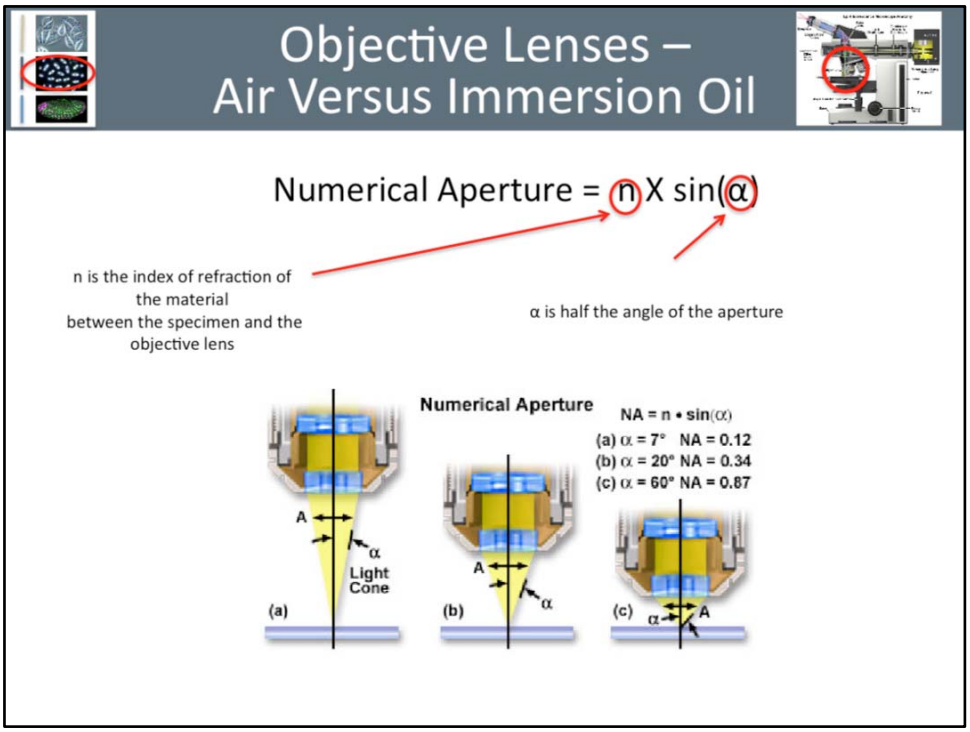
When light passes from glass to air, if the angle is too great then the rays do not emerge but are totally internally reflected.

When light passes from glass (RI=1.515) into immersion oil (RI=1.515) , the rays are not refracted since the refractive indexes are the same!



Oil Immersion

In order it increase the Numerical Aperture of a lens it is necessary to place oil between the coverslip and the objective lens



Numerical Aperture

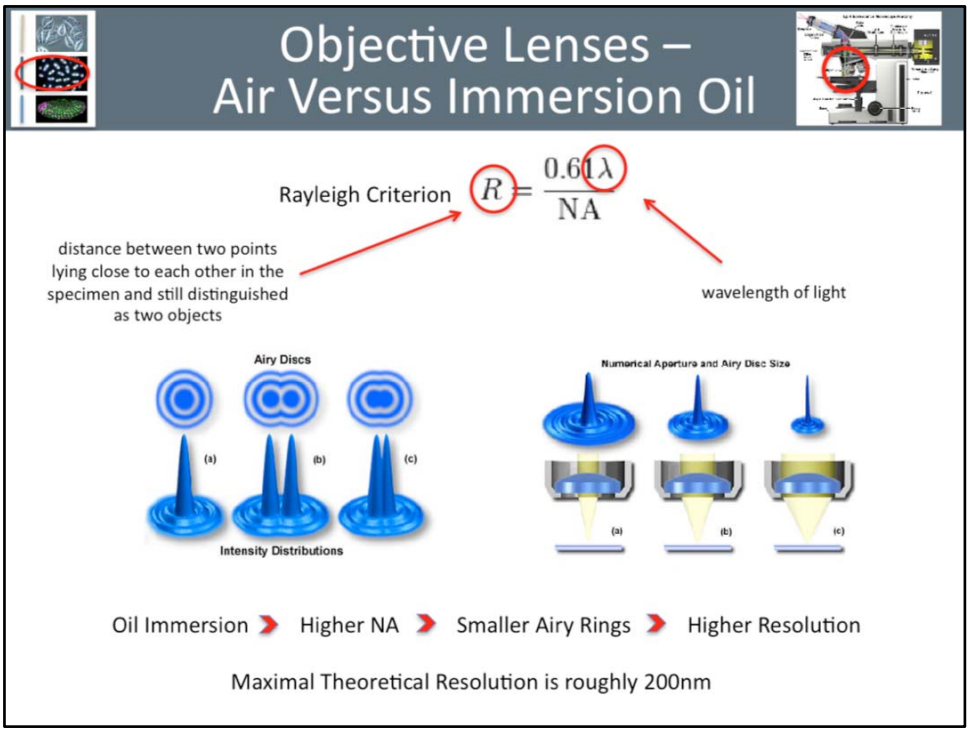
Numerical Aperture = n x sin(α)

n is the index of refraction of the material between the specimen and the objective lens

since α can't exceed 90°, the sin of α must be one or less.

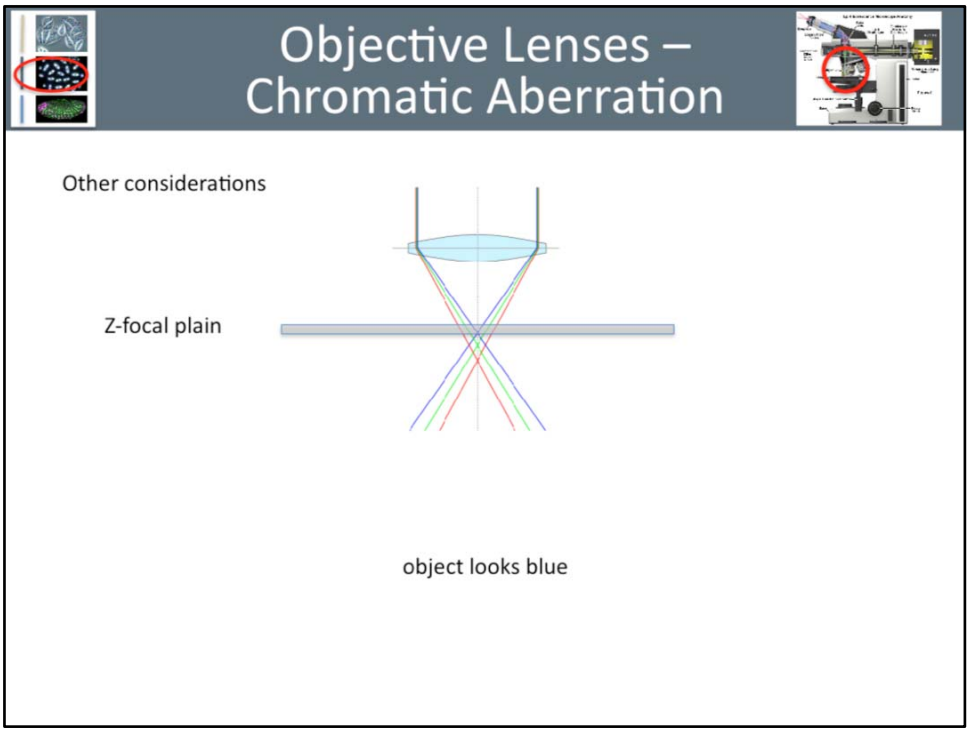
Therefore the maximum theoretical NA for a dry lens is 1 (n=1 for air) ; but in practice it is no more than 0.95

the maximum theoretical NA for an oil lens is 1.5 (n=1.515 for oil); but in practice it is no more than 1.4



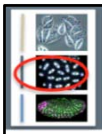
Numerical Aperture and Resolution

Abbe, Rayleigh, Airy et al, realised that when you look at light from a very small point (bead) it appears as small discs rather than a point – known as Airy discs. This phenomenon is caused by diffraction and scattering as light passes through the specimen. The smaller these Airy discs, the finer the detail that is discernable because the discs are less likely to overlap with one another. Objectives with higher NA produce smaller Airy discs and therefore be able to resolve finer detail.



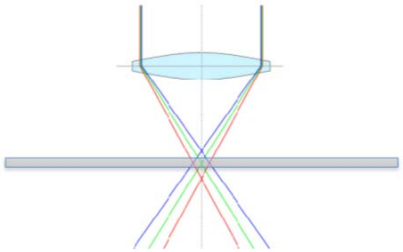
Chromatic Abberation

With simple lenses you can have a problem of chromatic abberation where light of different wavelengths comes from slightly different depths within you sample.

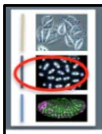


Objective Lenses – Chromatic Aberration

Other considerations

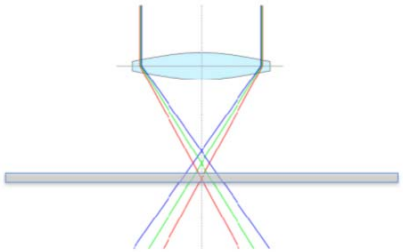


object looks green

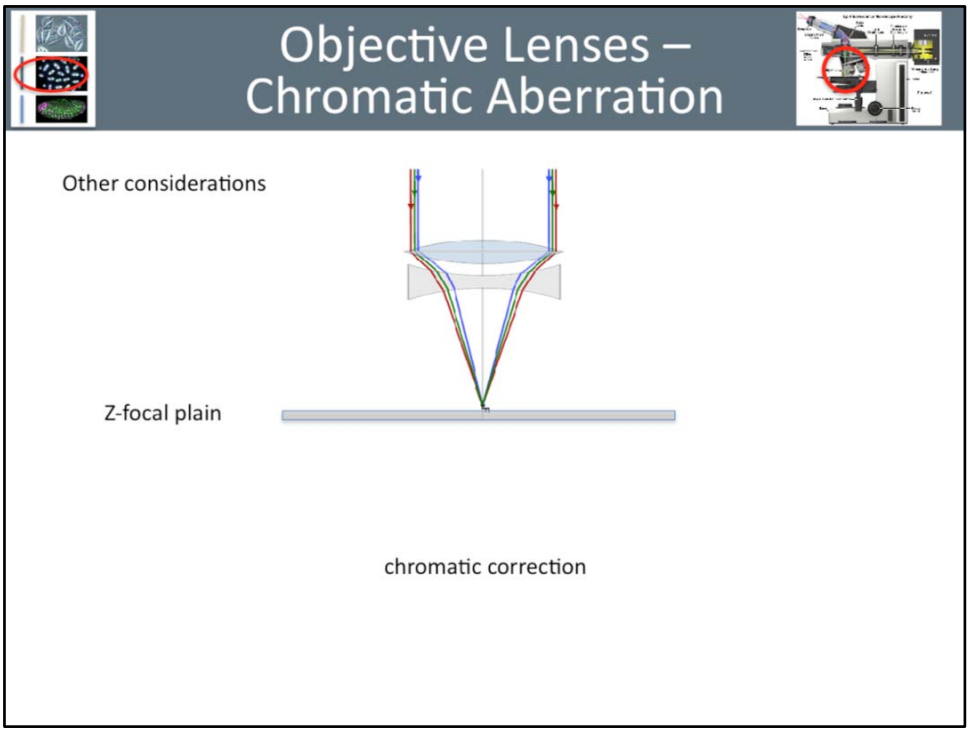


Objective Lenses – Chromatic Aberration

Other considerations



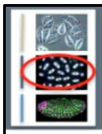
object looks red



Chromatic Abberation

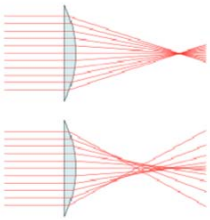
With simple lenses you can have a problem of chromatic abberation where light of different wavelengths comes from slightly different depths within you sample.

In most lenses used in microscopy this is corrected for so that all colors lights have the same focal point



Objective Lenses – Spherical Aberration

Other considerations

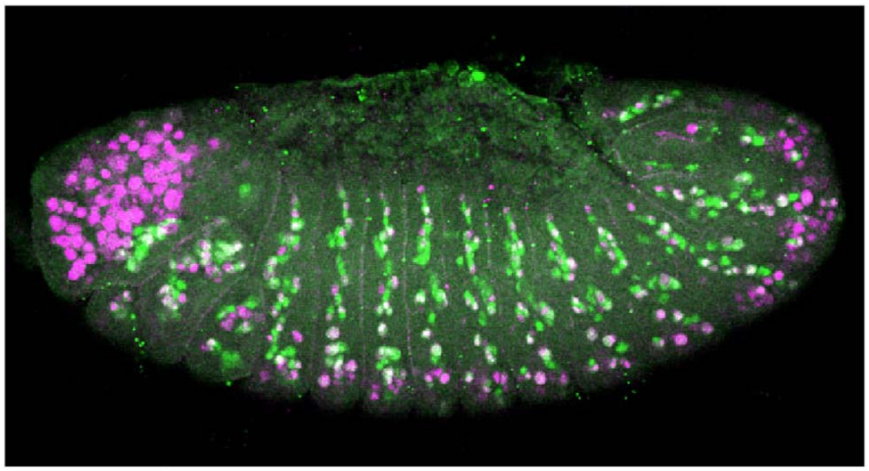


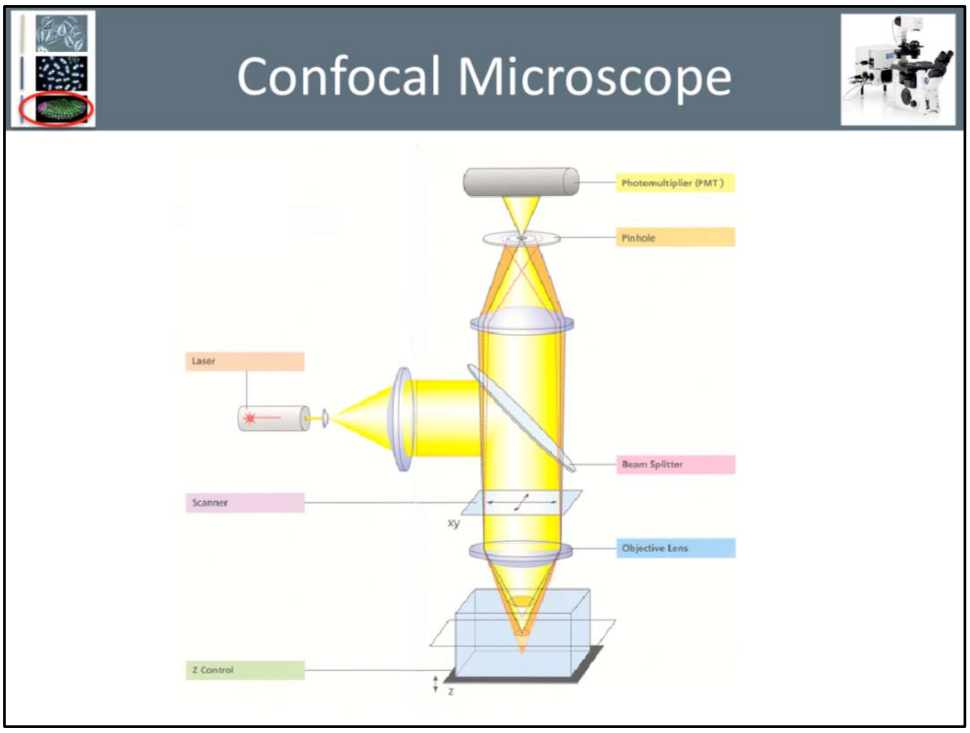
Due to the shape of the objective lens; resulting in light passing through the lens center being focused at a different distance to light passing through the outer portion of the lens

Result: Blurred image

I can be serious!
- Hubble telescope launched with a spherical abberation.

Confocal Microscope





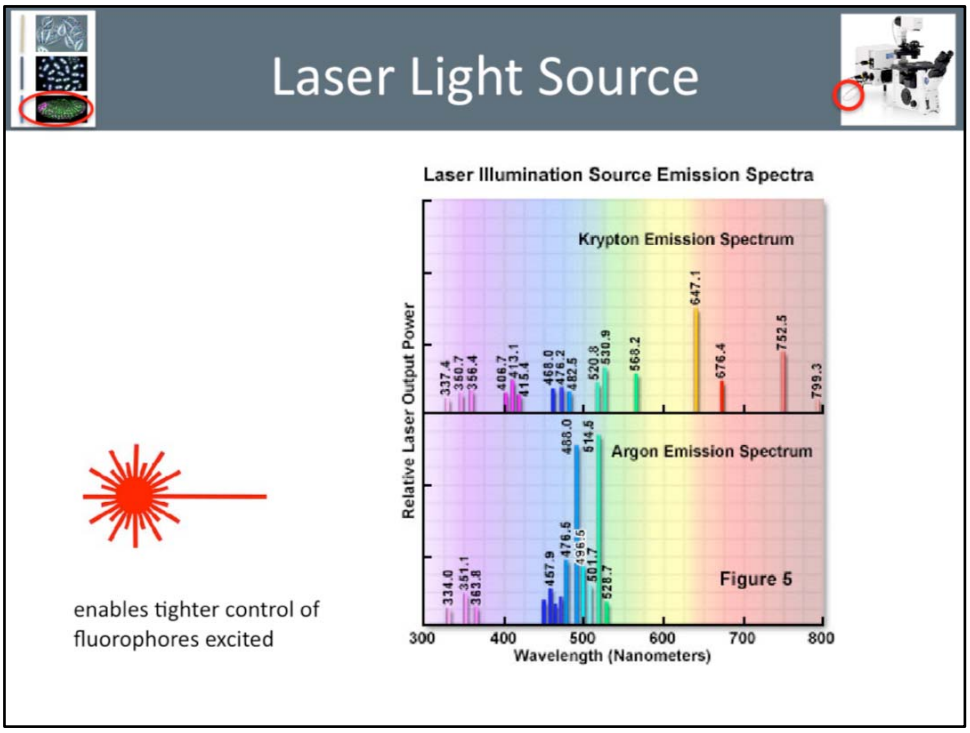
Confocal Microscope

Another type of microscope that is commonly used here in the Dunn School are confocal microscopes. These have two main features that set them apart from standard fluorescent microscopes.

Firstly they use lasers as the light source
and secondly they have a pinhole in front of the PMT detector.

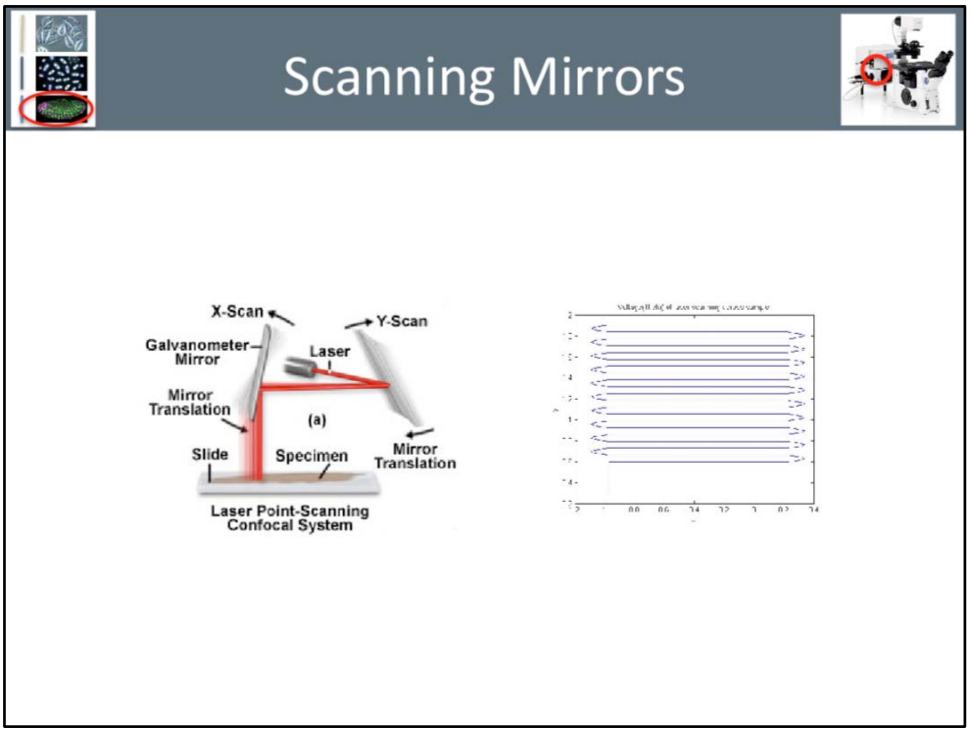
Excitation light passes through the whole thickness of the sample. On a standard fluorescence microscope light from above and below the focal plane still enter the detector but are out of focus and therefore make the image blurry.
On a confocal the pinhole blocks this out of focus light from entering the detector.

Light entering the detector therefore comes from a very thin ‘optical section’ within the sample.



Lasers

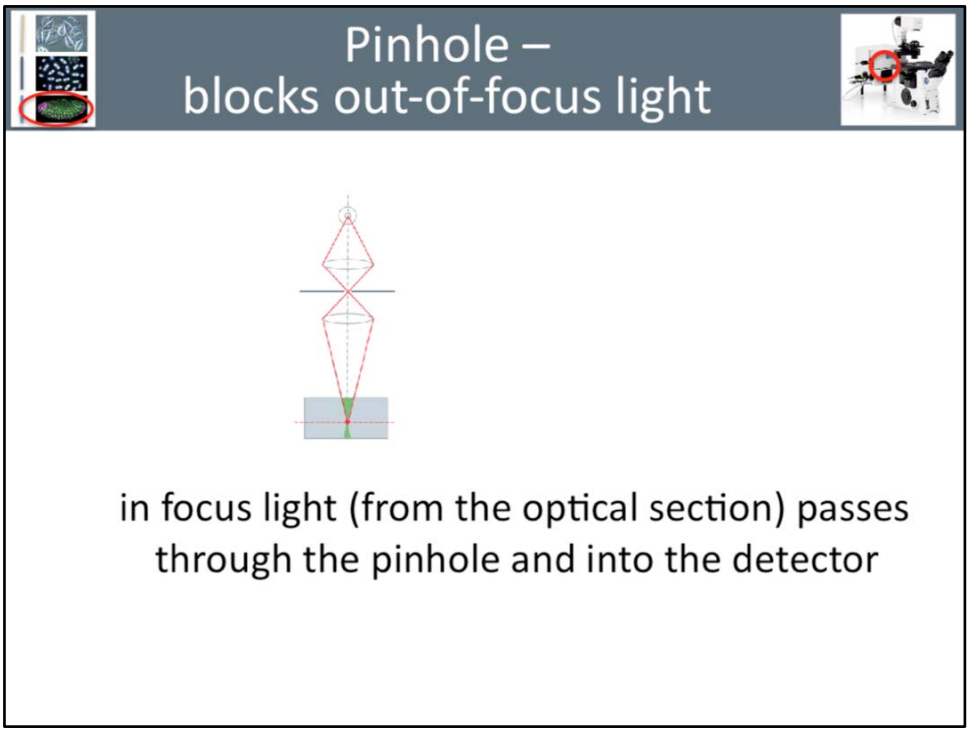
A common laser used in confocal microscopy is an Argon laser. This emits strongly at 488nm (and also more weakly at 514nm) which is idea for exciting GFP / FITC / alexa 488 (and YFP)



Scanning Mirrors

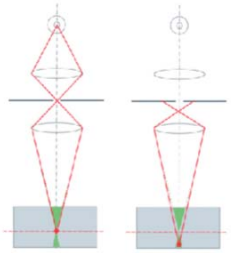
Confocal microscopes use a point of light in order to illuminate a sample. Therefore in order to illuminate a biological specimen (which is 3D) this laser light has to be scanned over the sample.

This is achieved using 2 scanning 'galvanometer' mirrors one which scans the laser in an x-direction (left to right) and a second at right-angles which scans the laser in a Y-direction (up and down).



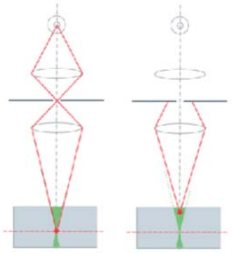
in focus light (from the optical section) passes through the pinhole and into the detector
light from below the optical section crosses infront of the pinhole and doesn't pass through the pinhole aperture
light from above the optical section also doesn't pass through the pinhole aperture

Pinhole – blocks out-of-focus light



light from below the optical section crosses infront of the pinhole and doesn't pass through the pinhole aperture

Pinhole – blocks out-of-focus light



light from above the optical section also doesn't pass through the pinhole aperture

Summary

Although they should be treated with much care microscopes are relatively simple pieces of equipment made of a series of rather clever components.

microscope ≠ black box of magic