ONBI Practicals 3/4/5: Fluorescence and the PSF

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(originally by Ilan Davis and Richard Parton Feb 05. Edited Ian Dobbie 9.04.2009)

Aims of practical 3:

1) Familiarize yourself with the components of the fluorescence path.
2) Be aware of correct alignment and use of different components.
3) Examine different fluorescent specimens.

Aims of practicals 4/5:

4) Prepare a bead slide, observe airy rings, point spread function and aberrations.
5) Learn how to leave the microscope for the next user.

Samples:

Fluorescent fixed material test slides, (either prepared locally or purchased from Invitrogen) will be available. Live fluorescently labeled material can also be prepared as described in practical 1. In addition, slides of Nile Red 210 nm beads and TetraSpec beads dried onto the slide and coverslips and mounted in Vectashield or water have been prepared (see protocol below – Justin Demmerle).

AIM 1) Familiarising your self with the light path:

This practical is for a widefield fluorescence microscope – either upright or inverted. Different microscopes will look very different, however, all widefield fluorescence microscopes are basically the same and share the same major components. More advanced imaging sytems such as Confocal, multiphoton or TIRF systems are often based upon a basic widefield fluorescence stand and will generally have an additional widefield fluorescence excitation light path.

Identify the following parts of your microscope (Refer to Figures 1-4)**:**

The objective lens and eyepiece - The object is viewed by two lenses, known as the objective lens and eyepiece. This arrangement gives two stages of magnification (hence "compound" microscope). In the first stage the objective lens forms an image of the specimen at the intermediate image plane. In the second this image is viewed by the eyepiece (and the eye). Identify the different objective lenses on the microscope.

The excitation light source (fluorescence lamp or equivalent) - Fluorescence microscopy uses bright short wavelength excitation light to elicit weak longer wavelength emission from the specimen.

The condenser lens (fluorescence path)- When correctly set up, light from the source is focused onto the specimen by the condenser lens, to provide high intensity, even illumination so that the highest powers of the microscope can be used to optimum effect – this is known as Koehler illumination. Note: in epi-fluorescence the objective lens acts as the condenser lens so the two are perfectly matched.

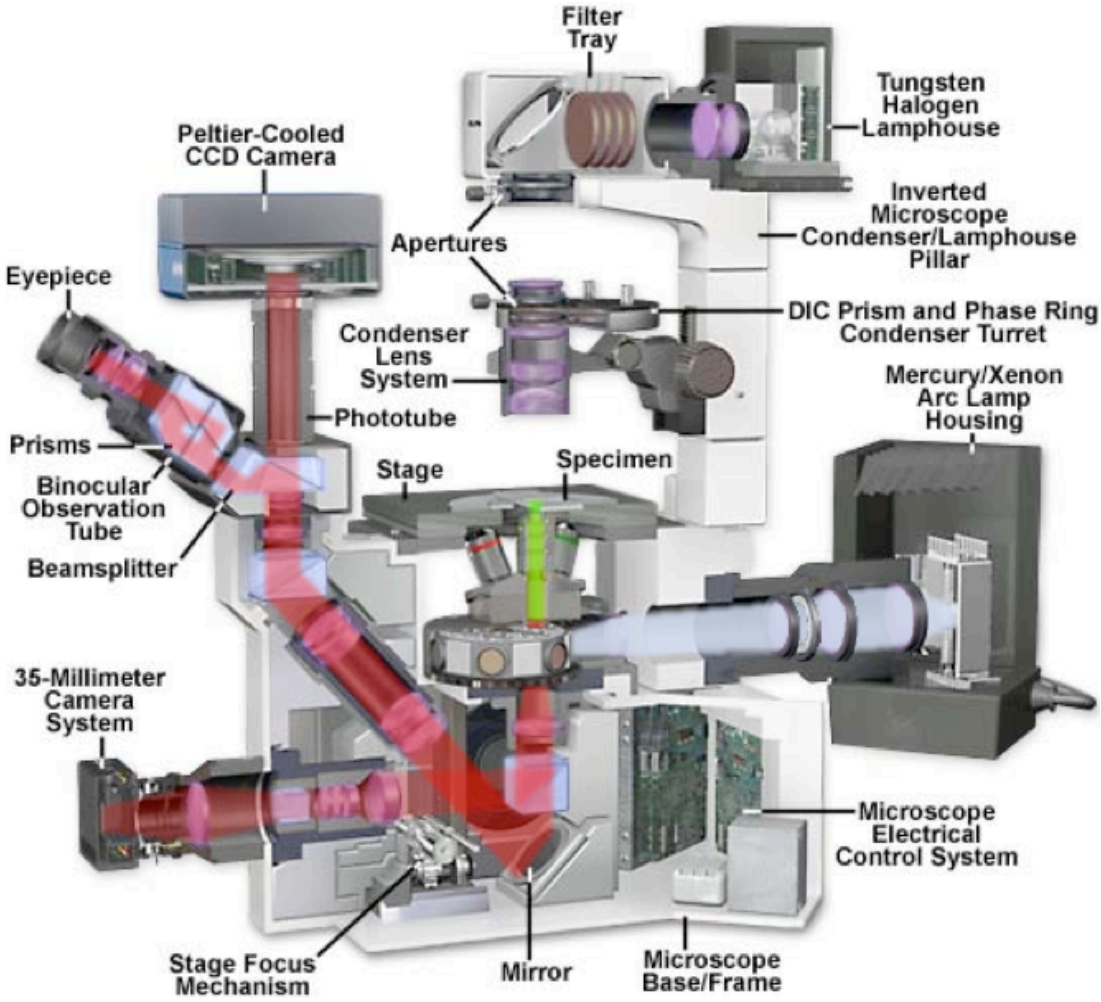
The condenser or aperture iris (fluorescence path - may not be present on all fluorescence microscope stands) - This limits the angle of the light passing from the illumination source through the microscope. The result is reduction in illumination brightness at the specimen and increasing contrast at the expense of resolution due to the fact that resolution is dependent upon the angle of light passing through the microscope).

The field iris (fluorescence path) - This iris restricts the field of illumination at the specimen plane. This increases contrast in the view and reduces photo-damage outside the viewed region of the specimen.

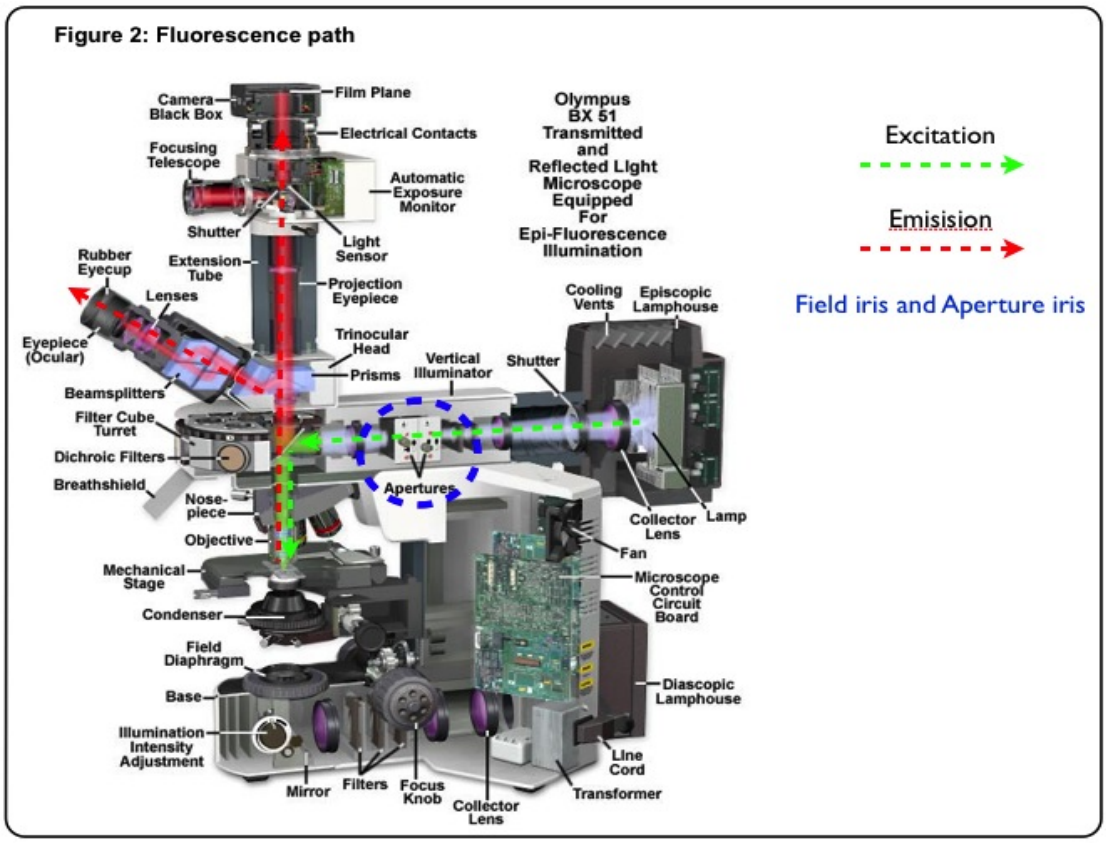
The dichroic mirror (fluorescence path) – Fluorescence microscopy uses bright short wavelength excitation light to elicit weak longer wavelength emission from the specimen. In a transmitted configuration (like bright field) the transmitted excitation light would swamp the emission signal giving poor contrast, even with wavelength discriminating filters. Epi-fluorescence relies on a dichroic mirror that interacts differently with light of different wavelengths. In a simple dichroic, light below a certain wavelength is reflected and above that wavelength transmitted. So – short wavelength excitation is reflected onto the specimen while longer wavelength fluorescence emitted towards the dichroic is transmitted to the detector. Short wavelength excitation light scattered towards the dichroic is reflected away from the detector.

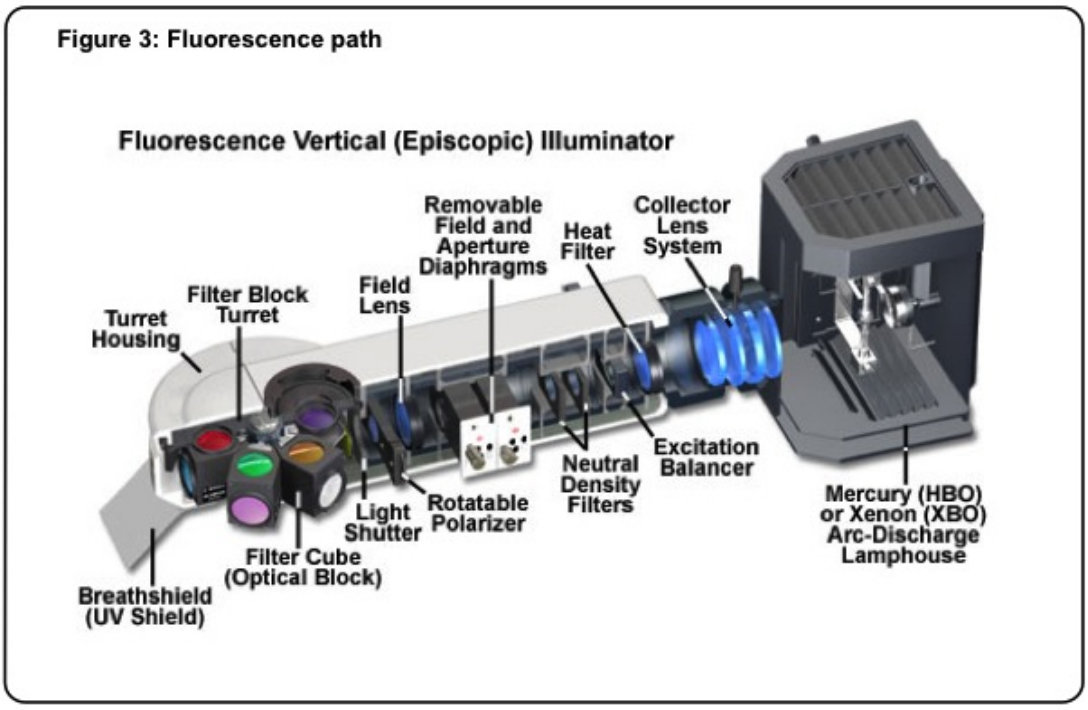
Components of an Inverted Fluorescence Olympus Microscope

Figure 1



Components of an Upright Fluorescence Olympus Microscope





http://www.olympusmicro.com/primer/

http://www.microscopyu.com

AIM2) Be aware of correct alignment and use of different components:

Excitation light source - Mercury or Xenon arc lamp*:

*** Note: many modern systems have alternative illumination sources including LED (light emitting diode) sources**

Open up the mercury arc lamp housing (only if you are familiar with the specific microscope) as if you were to change the mercury bulb (See Figs 2 and 3). Alternatively, find a diagram of a similar housing from the Olympus web site (or print it out in advance). Examine an old bulb. The distance between the electrodes increases with age so that the light source gets bigger and flickers. Re-assemble light source and turn on. **Always turn the mercury bulb on before the microscope and camera electronics. Always turn the bulb off after turning the electronics off.**

Dos and don'ts of arc lamps. Do not turn on and off in rapid succession (otherwise may cause the bulb to explode and release mercury vapour into room) – After turning off, leave at least 30 minutes to allow the bulb to cool before turning on again. When turning on a new bulb for the first time, you should "burn in" the bulb for at least a few hours and preferably half a day to avoid flickering (establishes a consistent arc path between the electrodes). **When changing bulb do not touch the bulb with fingers (use a tissue) -this will reduce the life of the bulb and may cause it to explode.**

Align the bulb – the way you would normally do for your microscope (this varies between makes and models of microscopes). The mirror reflects some additional light from the back to maximize the intensity. Alignment involves: X/Y/Z/focus.

Generally - make a focused image of the bulb (with "angel wings" flanking the electrodes) on a piece of card on the stage (where the specimen normally sits) without an objective in place (the back focal plane of the objective). When an objective is in place, the lamp will be at maximal defocus at the image plane. The exact details will vary between different manufacturers. e.g. Zeiss have an observation side window, DeltaVision bulbs are aligned at point of entry into the fibre optic cable.

Objective lenses:

The most expensive and important components of the microscope. Become familiar with the various lenses and their colour coding (4x red; 10x yellow; 20x green, 40x and x60 blue and x100 white).

Avoid dipping dry lenses in immersion medium and if you do so, tell someone or clean the lens as soon as possible.

Do not leave lots of immersion oil on lenses when not in use and use immersion oil sparingly.

Focusing on a specimen without damaging the objective lens:

If you have a digital or conventional camera, find out how to divert the light path to the eye-pieces. Familiarize yourself with how to use the focus knob (e.g. turning the knob towards you on an Olympus inverted microscope moves the objective towards the specimen). **The stage should be left by the previous user (and by you at the end of the session) with the objective racked away from the stage and with a low powered**

objective in place (e.g. 10x). Always check this before placing the slide on the stage. Check that the slide and cover slip are completely clean. **Never use a slide with leaking medium, dirt, smears or wet nail varnish.** After placing the slide on the stage, find the specimen with low powered objective (e.g. 20x or 40x dry lens) by racking the objective with the coarse, then fine focus towards the cover slip while looking at the slide and objective until almost touching the cover slip. Then use the fine focus to rack the objective **away** from the cover slip while looking through the eye pieces. **Never rack the objective long distances towards the specimen with coarse focus while looking through the eyepieces. Doing so with force so that the objective impacts into the slide can shatter the front element of the objective, damage the spring mount of the objective and shatter the slide.**

To switch to an immersion objective (oil, glycerol or water), first find the specimen at lower power and centre it in the field of view. Rack the objective away from the stage and turn the objective turret to the desired immersion objective. Place a small drop of immersion medium on the cover slip (upright microscope) or on the objective lens (inverted microscope). **Never place more oil than is necessarily and try to avoid creating air bubbles in the oil. Never mix different makes of immersion oils.** Then rack the objective towards the specimen while looking at the objective and slide until almost touching the cover slip. Next slowly rack the objective away from the coverslip while looking through the eyepieces. When you find the specimen in focus, note the appearance of the immersion medium trapped between the objective and specimen. The fastest way to reach almost correct focus, is to recreate the appearance of the trapped immersion medium by eye.

The fluorescence Kohler light path:

In epi-fluorescence the objective itself also acts as the condenser (See Figs 1 and 3). Kohler illumination is important for the same reasons as bright field: to ensure even and intense illumination of the specimen without an image of the mercury light source. However, in most microscopes (with the exception of the DeltaVision) no alignment is required to achieve Kohler illumination. You simply focus on the specimen.

The field aperture is used to reduce out of focus light on the region of interest (pseudo-confocal effect) and stop bleaching outside the region observed. The field aperture has centering screws, but normally should not need adjusting.

Aperture diaphragm (not present on all microscopes) is usually left in the fully open position.

Neutral density filters can be used to reduce the intensity of illumination.

A heat (IR) filter is usually present to avoid damage to living cells (interference filters do not necessarily block IR). Optical elements in light path are usually UV-transmitting to allow excitation in near UV. **UV filter** can be introduced.

Stop slider blocks all fluorescent illumination when not looking at specimen to avoid damage to filters (see below). **Do not have the DIC analyser and other components in place when using fluorescence**: reduces intensity hugely and damages polarising filters. (exceptions to this rule are when signal is very strong and you require simultaneous DIC and fluorescence imaging, although better alternatives exist)

Switching between different fluorescent cubes (or excitation and emission filters, whether manual or motorized):

Take out the filter cube holder (See Fig. 3) and observe the dichroic mirror, emission and excitation filters.

Dos and don't of interference filters:

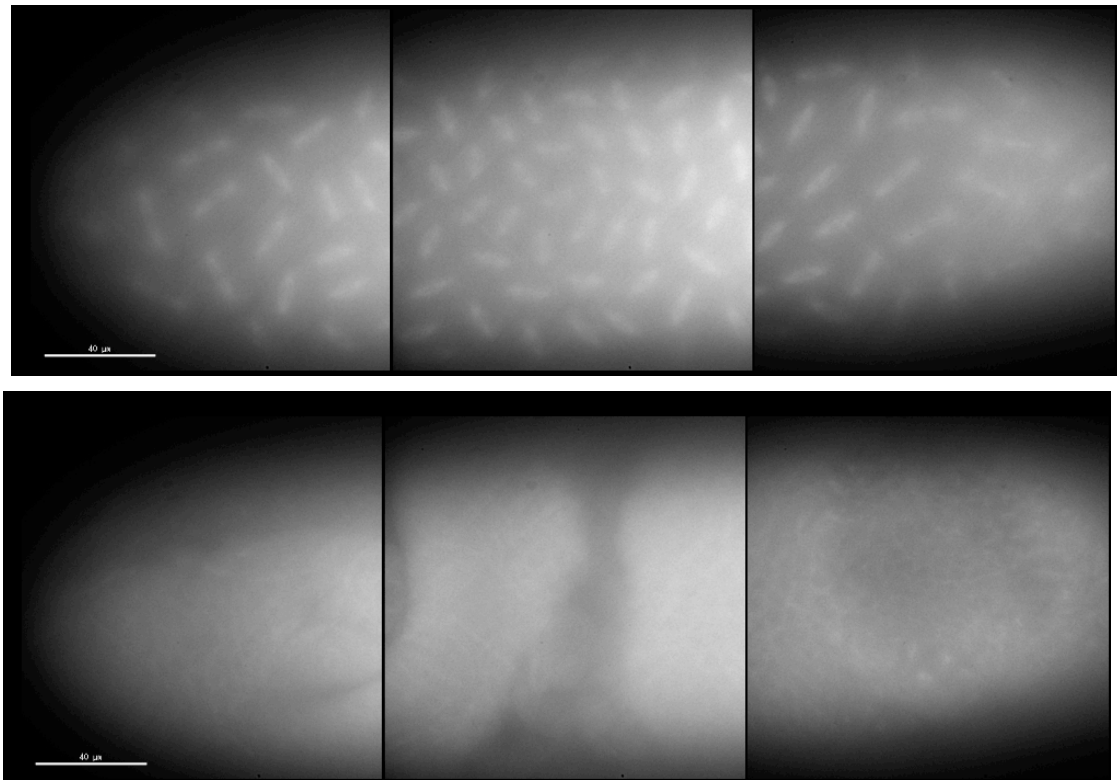
Do not get finger prints or immersion medium on filters and mirrors –almost impossible to clean without damaging, but must be cleaned if you touch them as oil from fingers corrodes the coatings.

Do not leave the excitation light shining on the filters when not observing fluorescence - close the excitation shutter. Do check filters from time to time, as they eventually deteriorate due to oxidation, moisture and through use - they will need replacing every few years for optimal results.

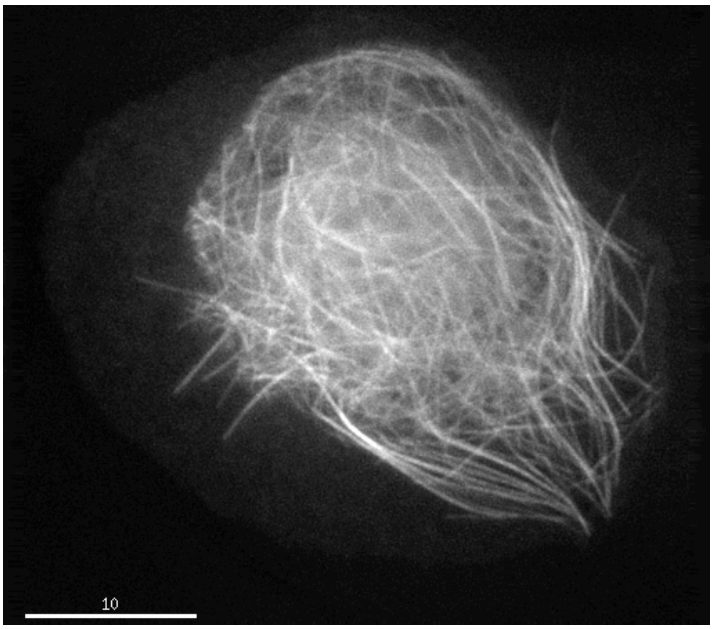
AIM 3) Examine different fluorescent specimens

Examine some of the different fluorescently labeled samples: fixed material slides DAPI, FITC (GFP, Alexa 488), Rhodamine (cy3, Alexa 543, 568); live fluorescent protein tagged *Drosophila* macrophages and embryos. Close the field iris and see how contrast is affected, especially in large densely labeled material. Examine the effects of photobleaching.

***Drosophila* Embryo MT labeled with Jupiter YFP:**



***Drosophila* macrophage MT labeled by Jupiter YFP:**



AIM 4) Observe airy rings and point spread function (Bead slide):

(More experienced microscopists may want to spend most of the time on this and the next part of the practical as long as they have gone through the first part to confirm that they know it well)

Bead sample preparation:

We prefer beads such as the Nile Red 210 nm beads that can be viewed with either FITC (GFP) or TRITC (Rhodamine; RFP) filter sets. To assist in focusing on the beads you can add a small fragment of lens tissue to the drop of mounting medium on the glass slide before covering with the bead-coated coverslip to assist in focusing.

1. Dilute fluorescent beads 1:10^6-10^7 in ddH_2O. To properly assess PSFs, beads should be dilute enough that a single bead can be found without Airy discs from neighboring beads encroaching as you focus up and down.

2. Apply 10-20 µl of bead suspension to a clean coverslip (usually No. 1; 22x22), spread with a pipette tip, and allow to air dry.

3. Apply 10-20 µl of 90% glycerol solution to a pre-cleaned microscope slide then mount the previously prepared coverslip by gently lowering it onto the glycerol drop to avoid air pockets.

4. While most calibrations will be performed using beads on the coverslip, if you want to image structures at >10µm depth into the sample, calibrations may be performed on beads several µm away from the coverslip. In this case, PSF beads may also be deposited on the slide itself, in parallel with depositing them on the coverslip. The distance between slide and coverslip may be adjusted by varying the amount of glycerol used to mount the coverslip.

5. Calibration slides may be produced for various mounting media: e.g. using Prolong Gold, Vectashield, water or Agar instead of glycerol.

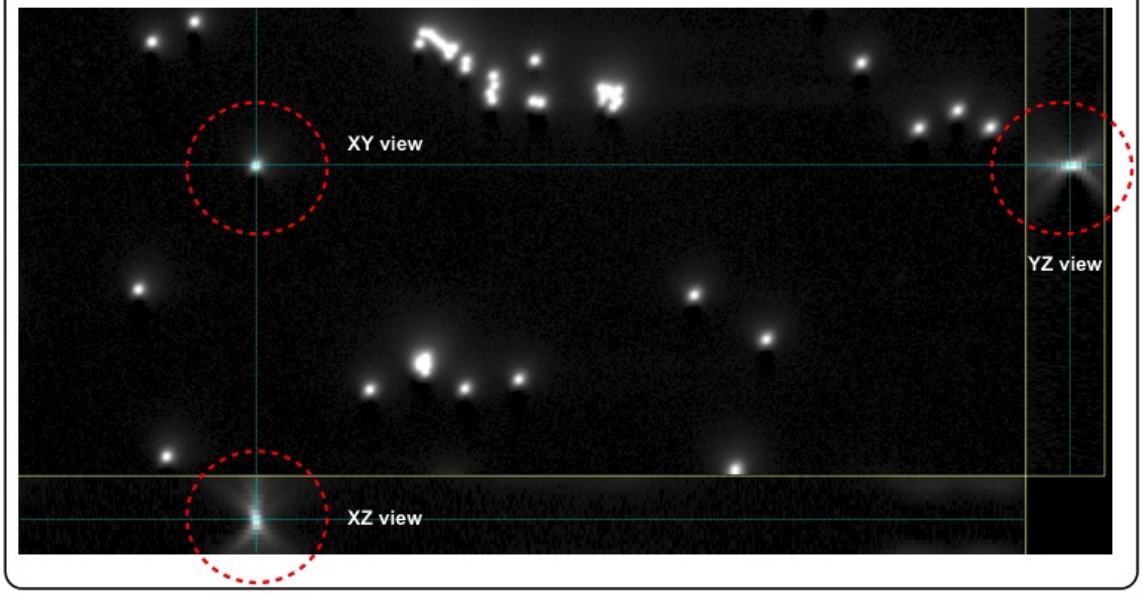
6. Seal the coverslip with clear nail-varnish, wait for it to dry and repeat. Store slides flat at 4 degrees.

Your bead slide should contain either large multi labeled beads or Nile Red 210 nm diameter (sub resolution, red emission) beads or equivalent on the cover slip surface and slide surface. The cover slip is a 1.5 (0.17mm) thickness cover slip. Nile red beads can be viewed with either the FITC (ex 488 em 520) or Rhodamine (ex 540 em 575) filter sets although the latter is far brighter. Use the lens tissue fragment to assist in finding the correct plane of focus.

By focusing up and down on a field of beads with different objectives and changing the fluorescent channel (FITC/Rhodamine) try to observe some of the following:

1) Airy rings of out of focus light above and below the beads (See the supplemental movie). Do the Airy rings differ between the beads on the coverslip and those on the slide?

Figure 4: Fluorescent beads, orthogonal viewer



2) Different pattern of airy rings in the centre of the field of view and at the edges. Compare plan lenses with non-plan lenses.

3) Nile Red has broad emission and excitation ranges – try to observe cross-talk (bleed through) between the channels by observing the Nile Red beads in the Rhodamine, FITC and DAPI channels. The cross degree of cross-talk will depend on the kinds of filters you have and their condition. Filter cubes designed for single wavelengths or ones with multibandpass emission filters will typically be brighter but have more cross-talk than ones designed for observing many channels sequentially.

4) Note that the shape of the Airy rings will vary considerably with the type of objective and make of the microscope.

5) Note that you can spot chromatic aberration (especially in Z on cheap uncorrected lenses) with the Nile Red beads but only if you acquire a digital image and magnify it considerably (Fig 4).

6) You can assess how well your system is performing by comparing your PSF to the theoretically determined resolution limits of the system (see Fig 5).

Figure 5: Empirical assessment of resolution

Theoretical limits:

Resolution in the lateral dimension (XY)

$D_{XY}=0.61\ \lambda/NA_{obj}$ 227

FWHM

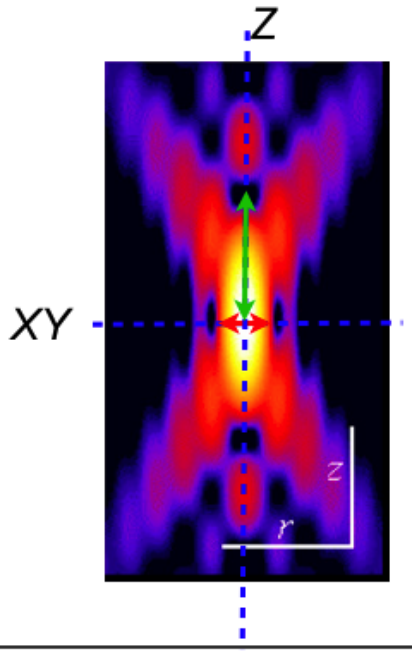
Resolution in the axial dimension (along the optical axis, Z)

$D_Z= 2\ \lambda\eta/(NA_{obj})^2$ 705

(η = refractive index of the object medium)

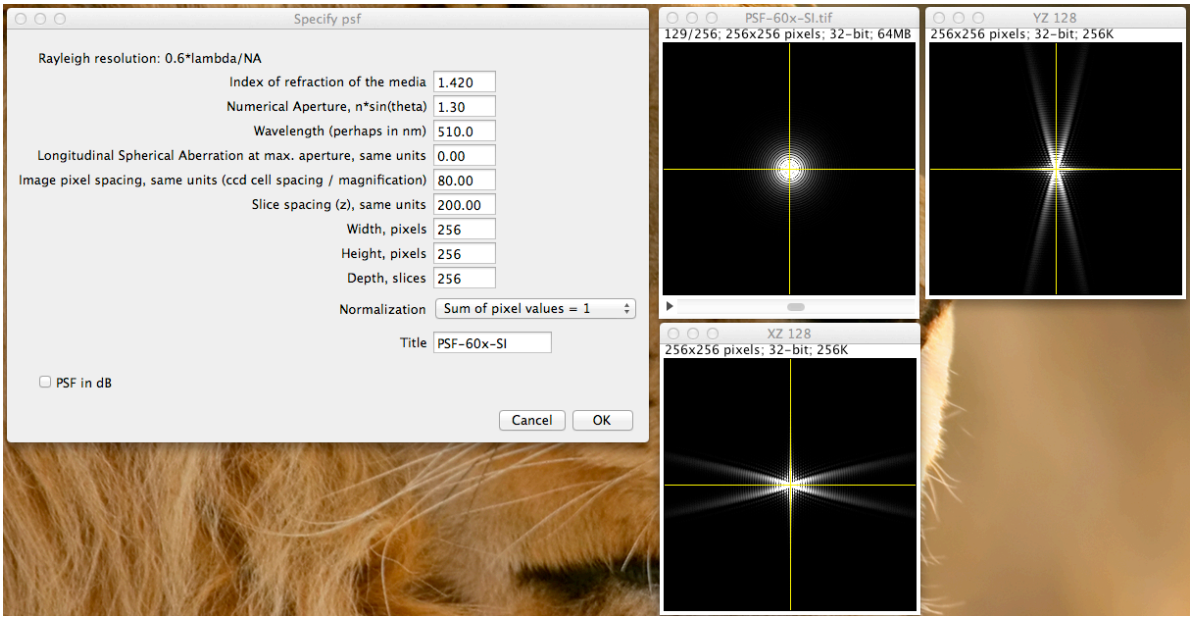
The relationship between the two is:

$D_Z/D_{XY} = 3.28\eta/NA_{obj}$ .... ≈ 3



Theoretical PSF's can be generated based upon simple parameters describing the optical system – for example in Fiji:

http://www.optinav.com/Diffraction-PSF-3D.htm

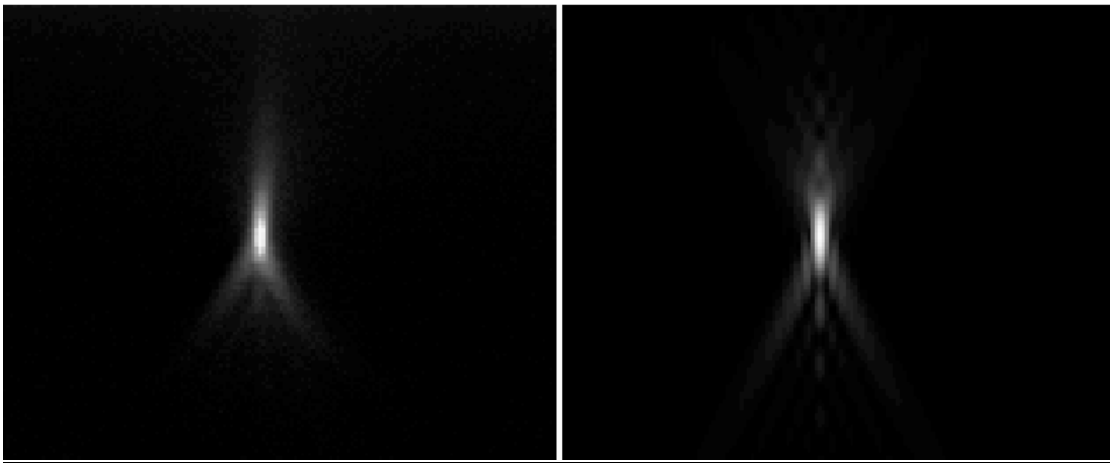


Observe spherical aberration:

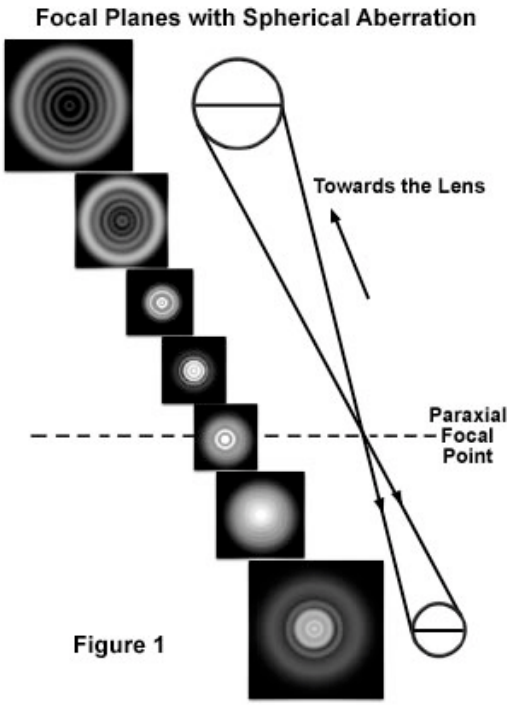
Examination of the PSF will also reveal a variety of aberrations of the system from misalignment, air bubbles in the immersion media, slides which are mounted at an angle and spherical aberration due to refractive index mismatch along the optical path. The latter is one of the most common causes of poor image quality. Try to identify SA in the bead slides. If possible examine ways of correcting SA using objectives with collar corrections, different refractive index oils or lens that use different immersion media.

Aberrated PSFS:

Spherical Aberration



http://bigwww.epfl.ch/teaching/projects/abstracts/frey/index2.html



http://www.olympusmicro.com/primer/java/aberrations/pointspreadaberration/

AIM 5) How to leave the microscope for the next user:

Follow the recommendations of the owner of the microscope for cleaning objective lenses and only clean lenses if you have been shown how to do this safely and are allowed to do so (See the Worksheet1). The slide / cover slip of the sample prep you intend to image must always be clean, and any nail-varnish dry, to avoid damaging the objective.

Make sure that **the dust cover is always on the microscope when not in use** and that the dust cover is not kept on the floor when taken off!

Remove excess immersion oil/glycerol/water by gently wiping the lens with a clean surface of lens tissue (not Kim wipes) folded twice - each time using a new clean surface (this should not require taking the lens off the microscope). Keep lens tissue container closed when not in use to avoid dust accumulating.

To clean an objective lens thoroughly you must first remove it from the microscope and examine the front lens either with an inverted eyepiece or with a dissecting scope.

Above all: do not drop objectives when taking them off or on – they may not be repairable! (scratches on the front element are bad but can be repaired by replacing the front element for a fee of a few hundred pounds). When not on the microscope, lenses should be stored carefully in their correct storage container

If the objective is very dirty or has dirt or tissue stuck to it, then dissolve the dirt away first by pipetting on water, chloroform or ether and wicking the solution off by dragging a clean lens tissue over the surface without applying pressure. Make sure there are no dirt particles on the lens before using a Q-tip (cotton bud). Note that water should be used first if there is a water based solutions/sample or glycerol on the lens.

To remove immersion oil we use chloroform. Ether or ether/ethanol can also be used. Also clean the sides of the objective, less care is needed for this.

Using Q-tips: Purchase only "**lotion free**" Q-tips from a medical or electronics supplier. We use Q-tips with chloroform and rotate the lens while gently twisting the Q-tip and objective around, so that only fresh surfaces touch the front lens surface. Use lots of fresh Q-tips (do not reuse them!) and shake off excess of chloroform after dipping into bottle and before cleaning. **Be gentle - only use very light pressure.**

If you stick to these rules, your objectives will not be damaged and will last for decades, even if used daily.

END OF PRACTICAL -how to leave microscope for next user:

1) Remove specimen

2) Remove excess oil from objective lenses with lens tissue folded twice, several times, fresh surface each time. Only apply very light pressure.

3) Check that there is no contamination of oil on dry lenses or microscope surfaces.

4) Rack the objective lens away from specimen and switch to low powered objective.

5) Turn off electronics

6) Turn off Hg bulb if the microscope has been on for more than 30min and will not be used for at least 1 hour.

7) Cover microscope with the dust cover.

8) Fill out the usage log book.

9) Make sure the area around the microscope is clean and that you have not left any thing behind such as old needles and tubes.