Lecture 4: Basic Fluorescence Microscopy

Ilan Davis March 2011

Topics covered

The nature of light and basic physics of microscopy (no maths)
Properties of various common fluorochromes
Fluorescent proteins
How fluorescence works
Properties of fluorochromes and advanced fluorescence methods
How does an epifluorescence widefield microscope work?
Light sources: Mercury, Xenon, laser
Objective lenses
Optical aberrations
The light path in a fluorescence microscope
Kohler illumination / critical illumination / fibre optic delivery
Absorption filters, interference filters and dichroic mirrors

References

Fundamentals of light microscope and electronic imaging
Douglas B. Murphy. Wiley-Liss 2001 ISBN 0-471-25391-X
-probably the best single book for overview if you want to buy one book

Video Microscopy The fundumentals. 2nd Edition
Shinya Inoue and Kenneth R. Spring. KA/PP ISBN 0-306-45531-5
-More details than Murphy on several topics

Optics, 4rd edition (undergraduate Physics textbook)
Hecht ISBN 0-201-838887-7
-for serious deep understanding of optics

http://www.olympusmicro.com/ -free
Very comprehensive and well written very nice diagrams and especially clear java based interactive tutorials (which can take time to download)

http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html

Largest source of fluorescent reagents

The Nature of Light

Electromagnetic radiation - visible light

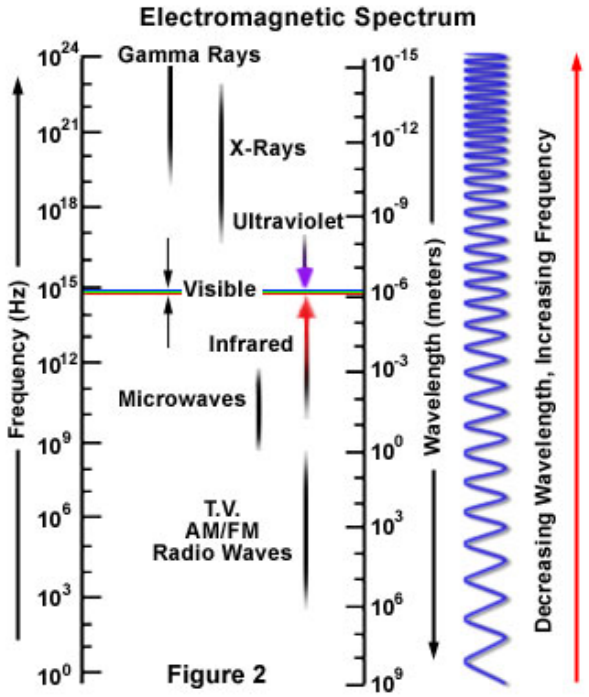


Figure 2

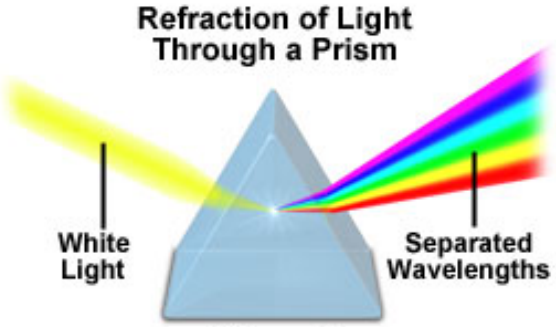
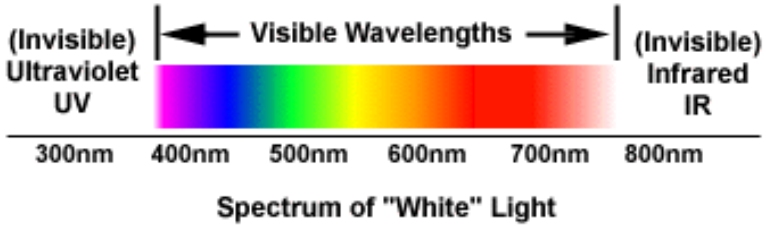
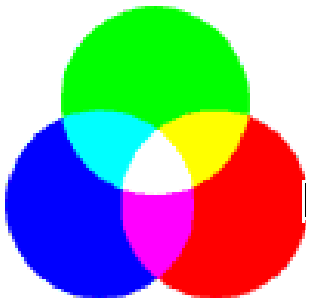


Figure 5



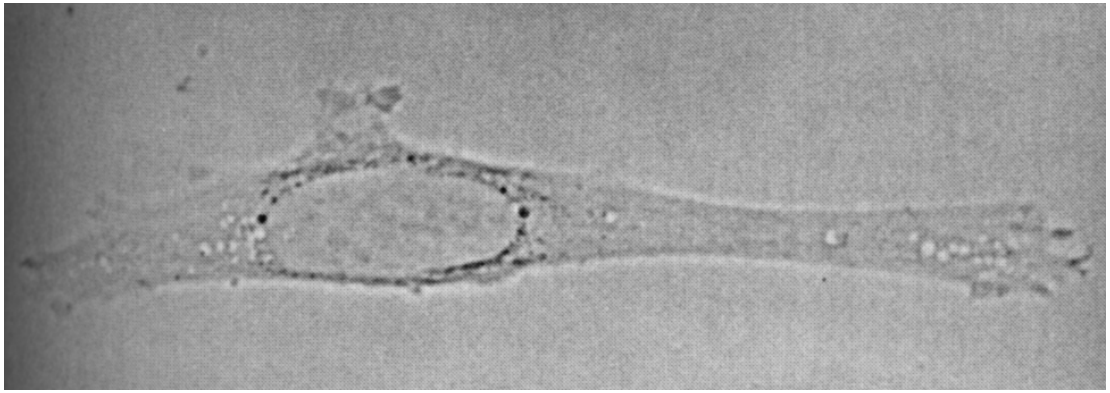
Spectrum of "White" Light


Additive

Wave-like (wavelength)
Particle-like (photons / energy packets=quantum)

By the late 1800s, microscope design was largely figured out, but...

cells are mostly water, and therefore mostly transparent



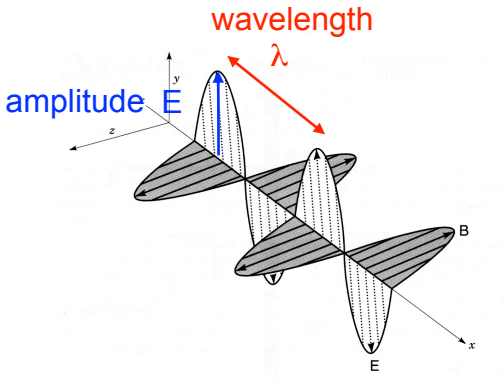
How can you generate <u>contrast</u> to image details in a transparent object?

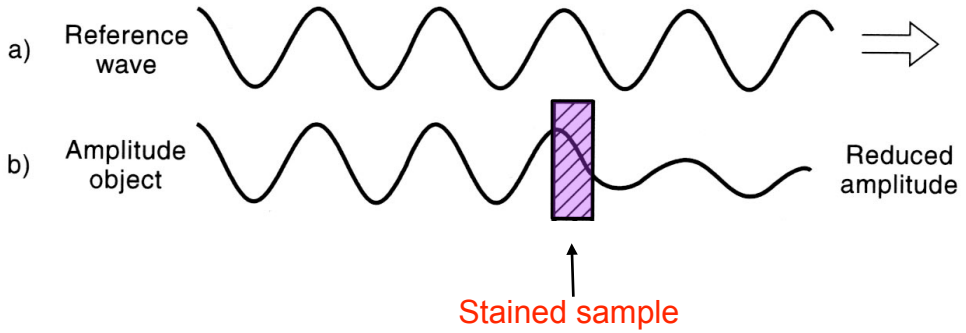
The big advances in microscopy

- Stains and dyes to increase contrast
- Microtomes - thin sections
- Imaging live cells (Phase contrast microscopy: 1930s, Differential contrast microscopy: 1950s).
- Imaging specific molecules inside cells (Immunofluorescence microscopy: 1960s and onwards)
- Imaging specific molecules inside live cells (Fluorescent labelled proteins:1980s, Green Fluorescent Protein: 1990s)

How to image transparent *living* cells?

- How do stained samples generate contrast?
- Now need to think about light as electromagnetic radiation, i.e., waves.
- When stained samples absorb light, they reduce the <u>amplitude</u> of specific wavelengths





Specimen preparation and histochemical stains

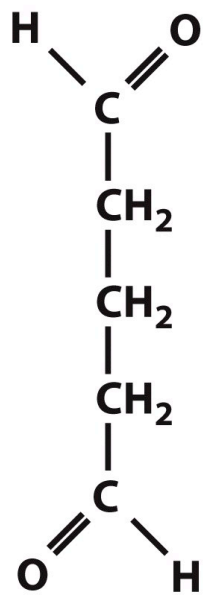
Fixation: to preserve, and to allow specimen to withstand the rigours of manipulation. Acids, alcohols, reactive aldehydes (formaldehyde, glutaraldehyde)

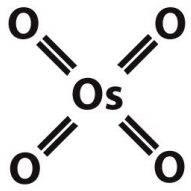
Thin specimens: can be “wholemounted” (bacteria, yeast, nematodes, fly embryos)
Thick speciments: must be sectioned (freeze or embed in wax or resin, usually after dehydration, then sectioned using a microtome or cryostat.

Staining: general stains to visualise transparent structure. For example Giemsa, toluidine blue and many others.

Clearing: dehydration in ethanol series then xylene. Glycerol.

Mounting: resins, glycerol. Coverslip added.





glutaraldehyde **osmium tetroxide**

Figure 9-43 *Molecular Biology of the Cell* (© Garland Science 2008)

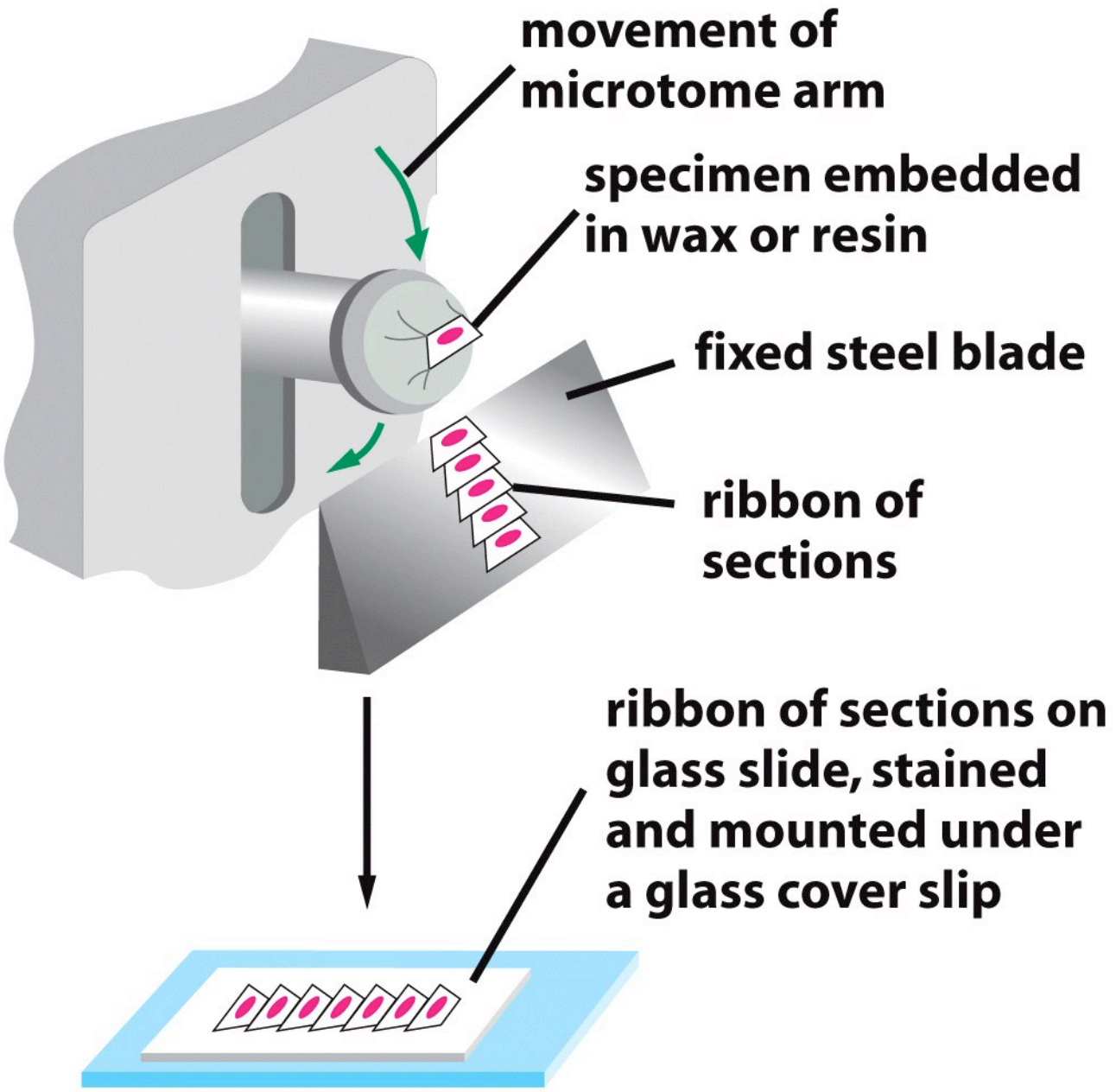
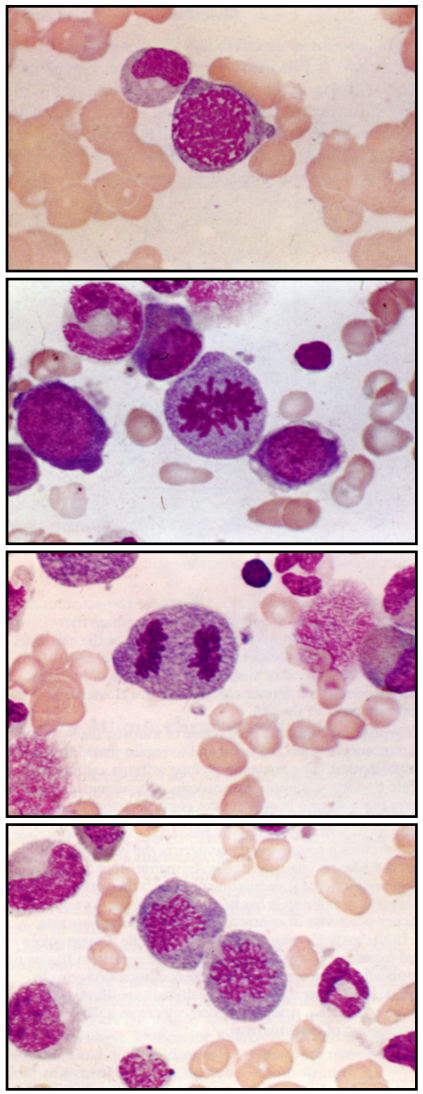
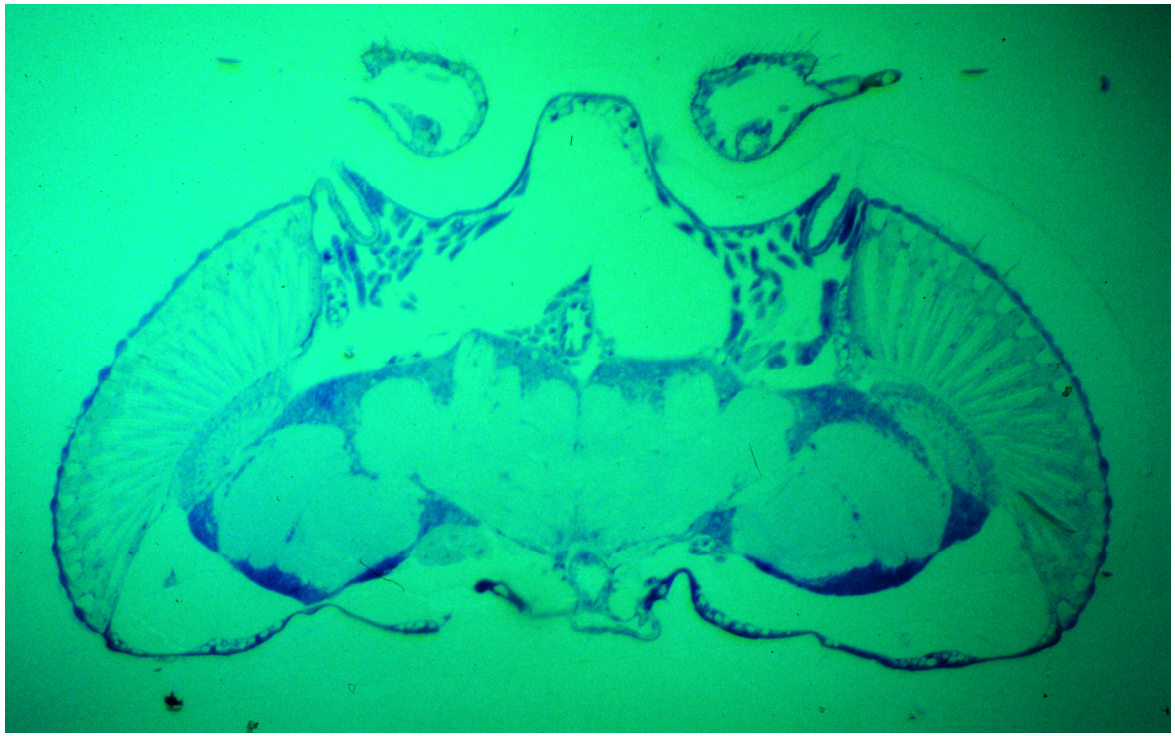


Figure 9-10 *Molecular Biology of the Cell* (© Garland Science 2008)

Visualization of fixed cells and tissues with histochemical stains

Mitosis in white blood cells--
Giemsa stain





2 μm section of *Drosophila* head embedded in resin, stained with toluidine blue

What is Fluorescence?

(not luminescence nor phosphorescence)

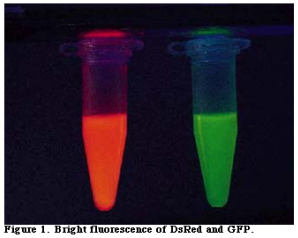


Figure 1. Bright fluorescence of DsRed and GFP.

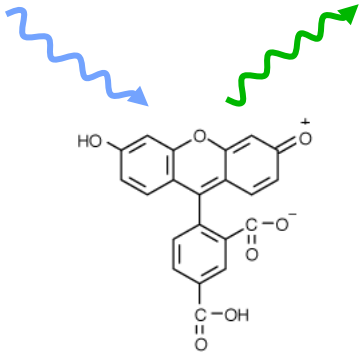
Common Fluorochromes for detecting specific Molecules

DAPI (DNA)
Fluorescein (FITC)
Green Fluorescent Protein (GFP)
Rhodamine (TRITC)
Tetrametyl Rhodamine (TMR)
Bodipy (various colours)
Cyanine3 (Cy3), Cyanine5 (Cy5).
AlexaFluores (most colours) e.g. Alexa Fluor 488, 546, 568
Atto dyes
Quantum dots -available as streptavidin, antifluorescein, anti-DIG

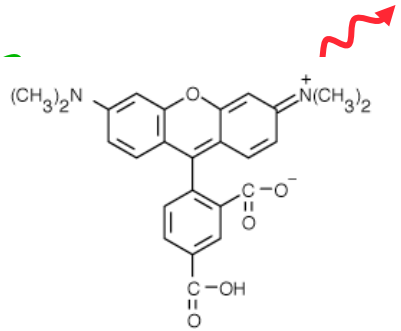
How does fluorescence work ?

http://www.probes.com/handbook/

Fluorescine



Tetramethyl rhodamine



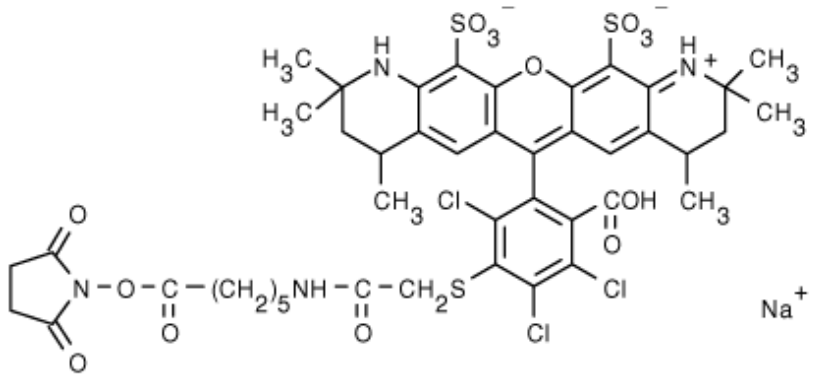
Structure for A-20002

Alexa Fluor® 546 carboxylic acid, succinimidyl ester

Molecular Formula: $C_{44}H_{45}Cl_3N_4NaO_{14}S_3$

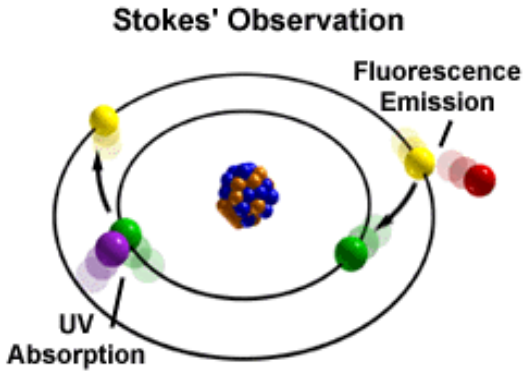
Molecular Weight: 1079.39

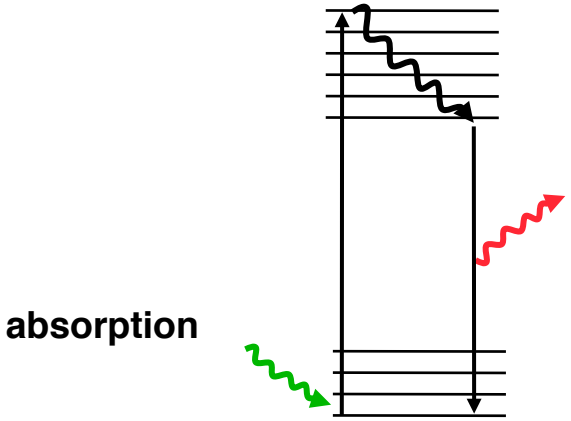
CAS Number/Name: N/A



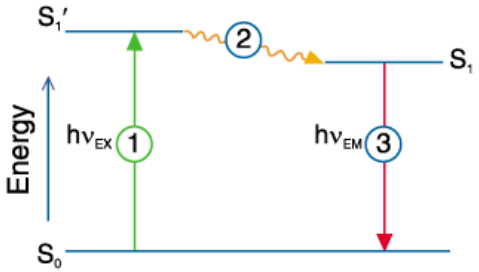
Green Fluorescent Protein (GFP)

Fluorescence





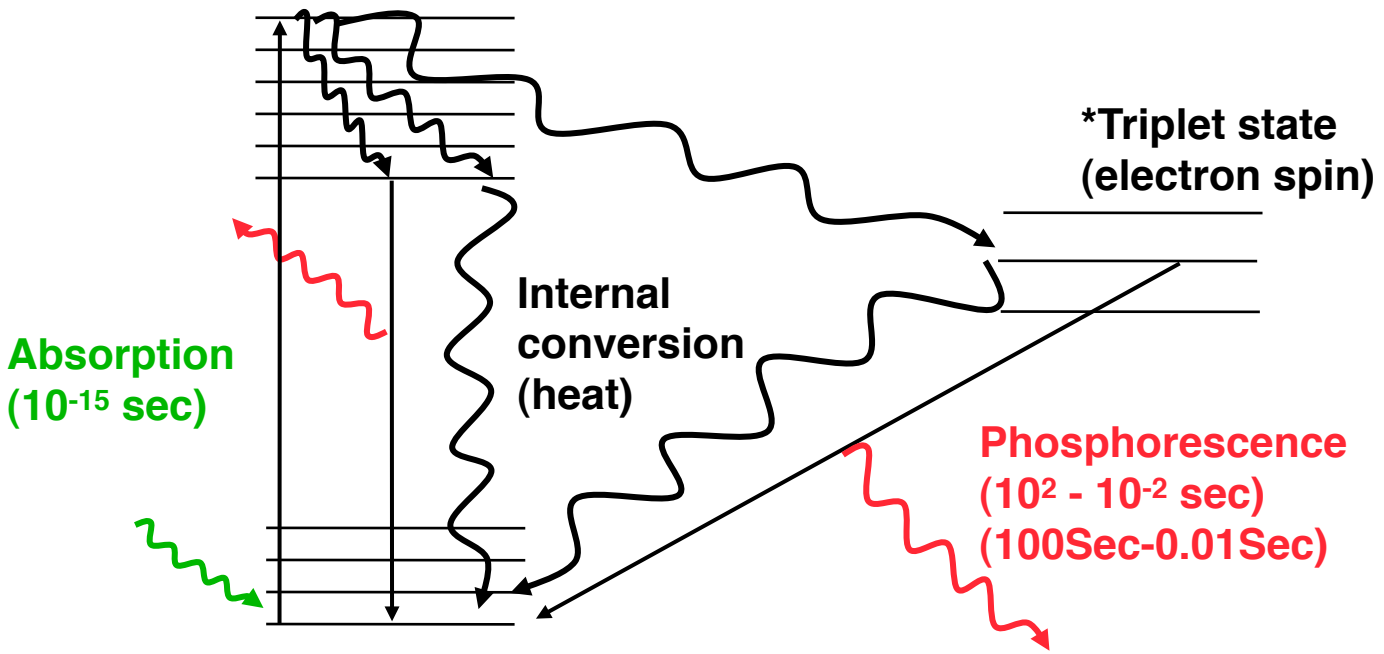
Electron energy levels (orbitals and spin)



Typical fluorochrome:
100,000 cycles per second for 0.1-1 seconds

Energy of a photon = hν
h=Plank's constant
ν =frequency of light (1 / wavelength)

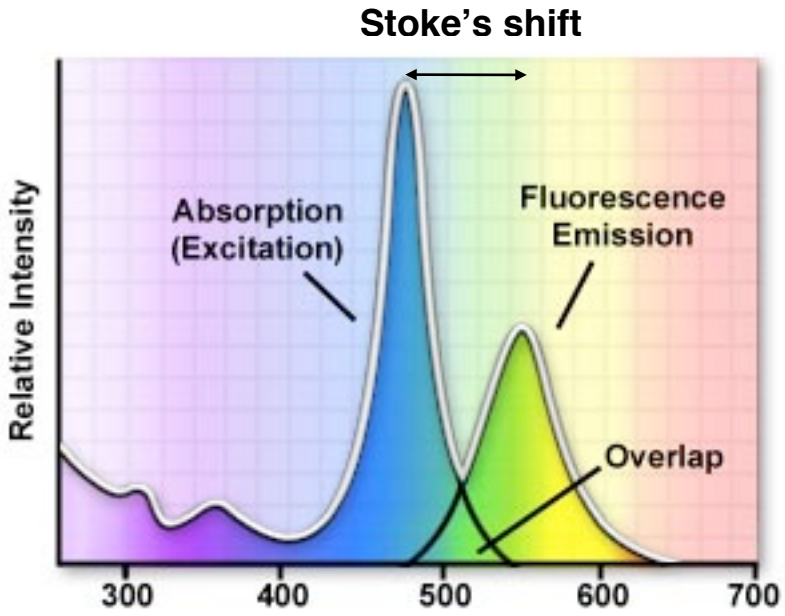
Jablonski diagram



Fluorescence
(10^{-9} - 10^{-12} sec)
(nSec-pSec)

*Triplet state-chemically reactive
Photobleaching reactive damaging
free radicals

FITC Absorption and Emission Spectra



Properties of Fluorescent dyes (fluorochromes)

Excitation/Emission peaks and spectra

Stoke's shift	(e.g. fluorescein only 20nm)
Photobleaching	- Photon induced chemical damage (permanent)
Molar extinction coefficient	- fraction of photons absorbed /M /cm
Quantum Efficiency (QE)	- fraction of absorbed photons which are re-emitted
Quantum yield	- How many photon emitted by a single molecule before it is irreversibly damaged

Environmental sensitivity

- pH
- ionic strength
- solvent polarity
- O2 concentration

-Autoqhenching or change in excitation wavelength

-with concentration

- presence of quenching molecules
- presence of anti bleaching reagents)

-caged fluorochromes (e.g. caged fluorescine)

e.g. Soluble Fluorescein has a Stoke's shift of 20nm.

QE=0.9 at alkaline pH.

QE=0.3-0.6 when conjugated to protein at neutral pH

Special properties used in specific imaging modalities

Quenching, blinking, polarization, resonance energy transfer, bleaching, photoconversion/switching, caging environmental sensors, hydrophobicity / membrane permeability.

Photodamage, Phototoxicity and Photobleaching

Photodamage is caused by the excitation light itself, in the absence of the fluorescent molecule. It is generally not so significant except with the high level infra red irradiation used in mutiphoton imaging or UV light.

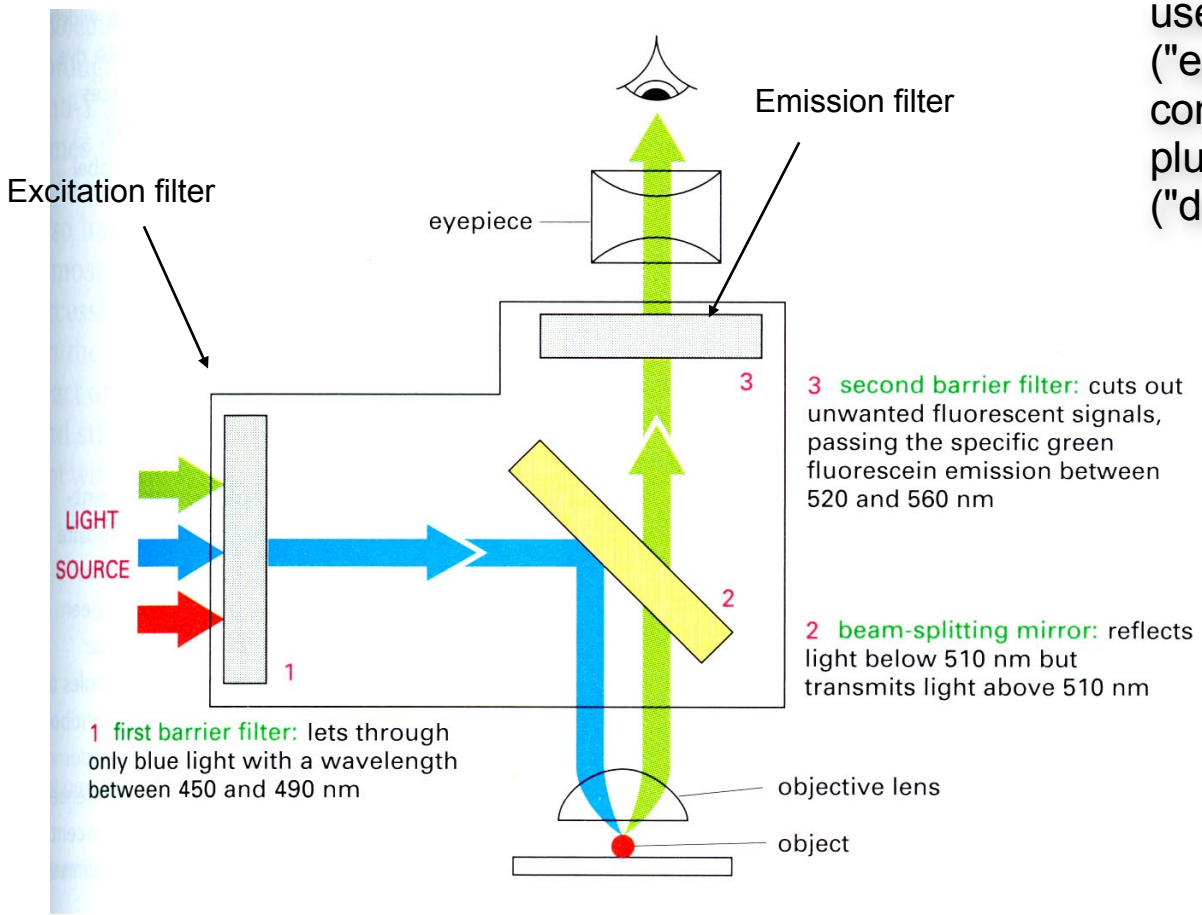
Phototoxicity is caused when the fluorescent molecule itself (upon irradiation) causes the damage, by generating free reactive Oxygen radicals or localised heating. This can be particularly significant and is a major justification for keeping the dye concentration and irradiation to a minimum.

Photobleaching is the process by which the excited light damages the fluorescent molecule so that it is no longer able to emit light

Ways to reduce these processes
Attenuate the excitation power whenever possible:
Neutral density filters, minimize exposure times, maximize the efficiency of the imaging by matching the choice of fluorescent molecule, with the excitation and emission filter sets, light source and the quantum efficiency of the detector and use protective IR and UV filters.

Anti-fade reagents:
Scavange and prevent reactive oxygen species from forming.
Some in living cells some in fixed cells
Home made: 0.3% Diamino benzene (Phenalene diamine) cheap from Sigma OR Propyl Gallate
Vital antifade in fluorescent motility assays: Glucose Oxydase and catalase
Vectashield from Vector Labs, Dabco (proprietary).
Slowfade (proprietary), Molecular probes.

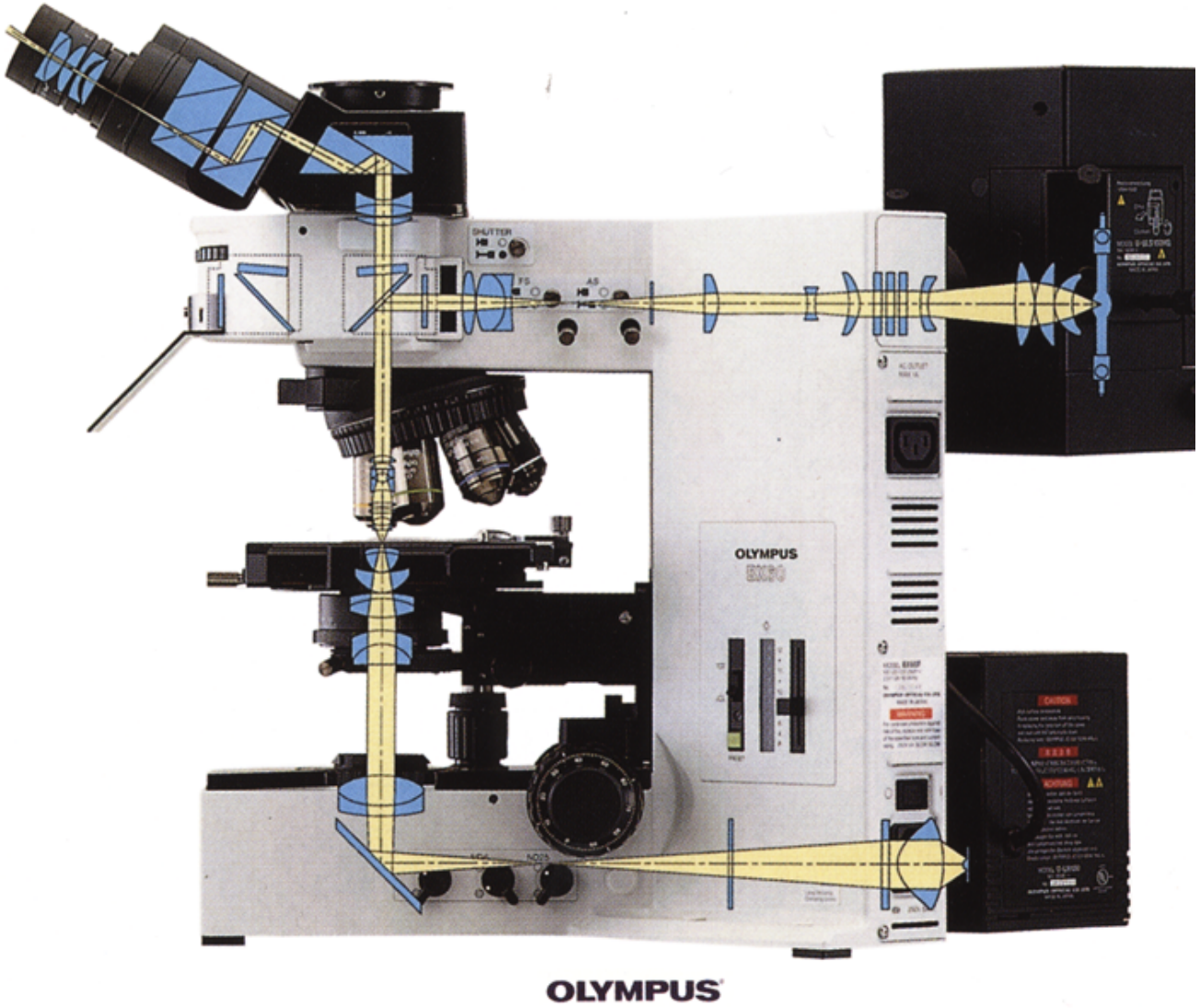
Epifluorescence microscope design



Epifluorescence microscopy uses illumination from above ("epi-") and a special cube containing two coloured filters plus a special beam-splitting ("dichroic") mirror

"Background" fluorescence is very dark!

Olympus upright BX60 Microscope light path

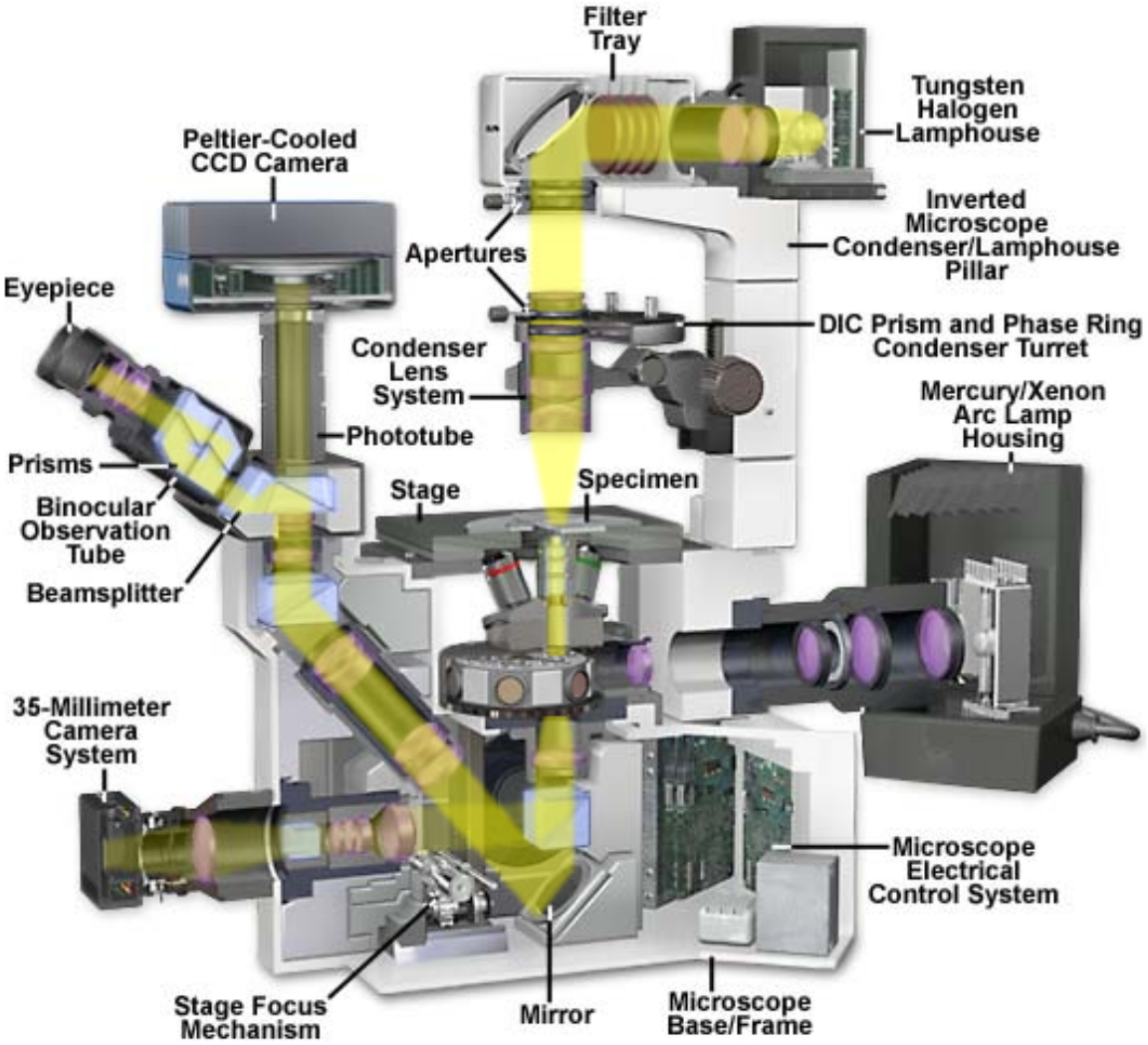


Köhler Illumination

Conjugate Planes

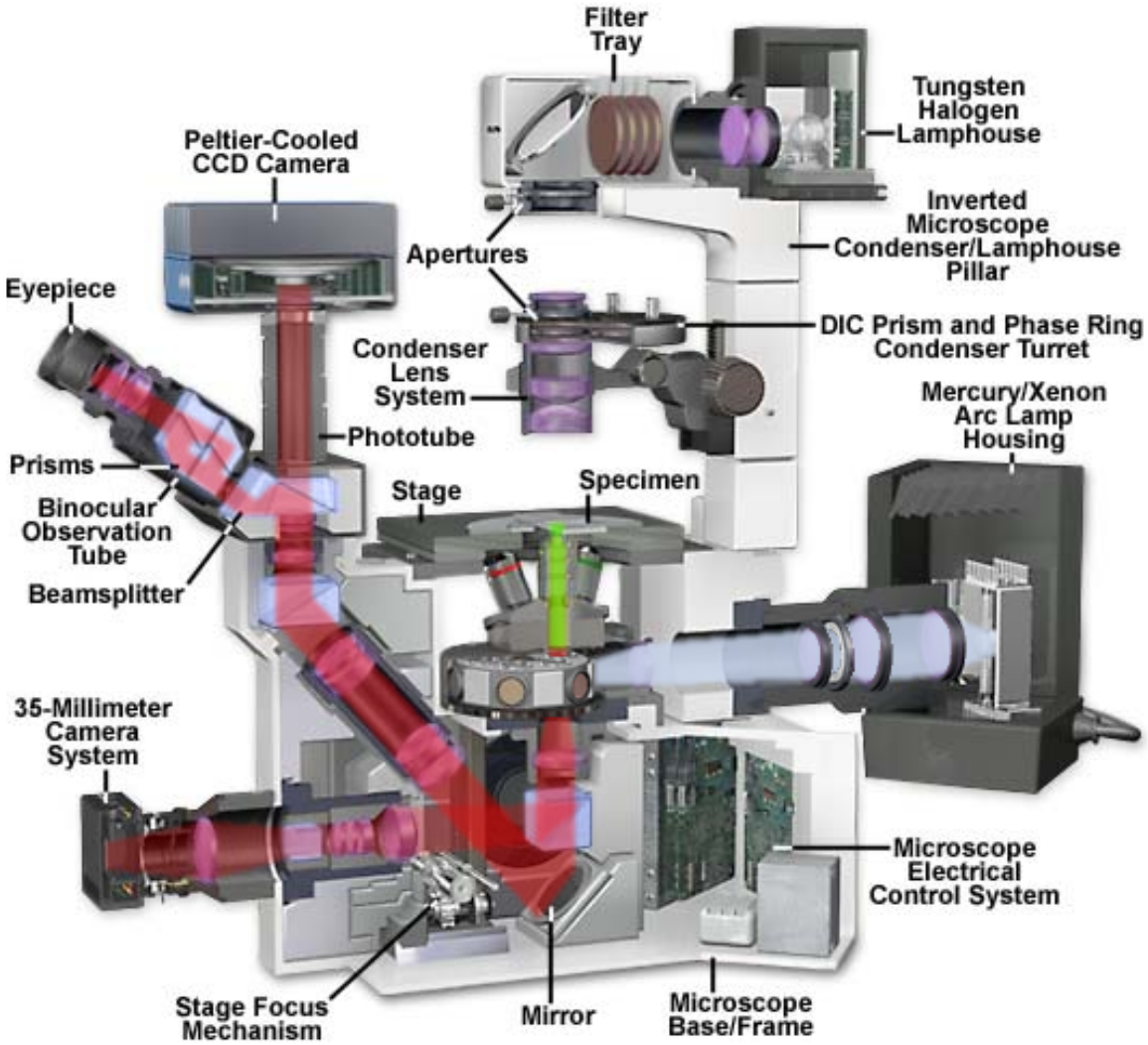
Olympus IX70 Inverted Microscope Light Pathways

Interactive Java Tutorial



Olympus IX70 Inverted Microscope Light Pathways

Interactive Java Tutorial



Go to Java applet
in Microscopy web site

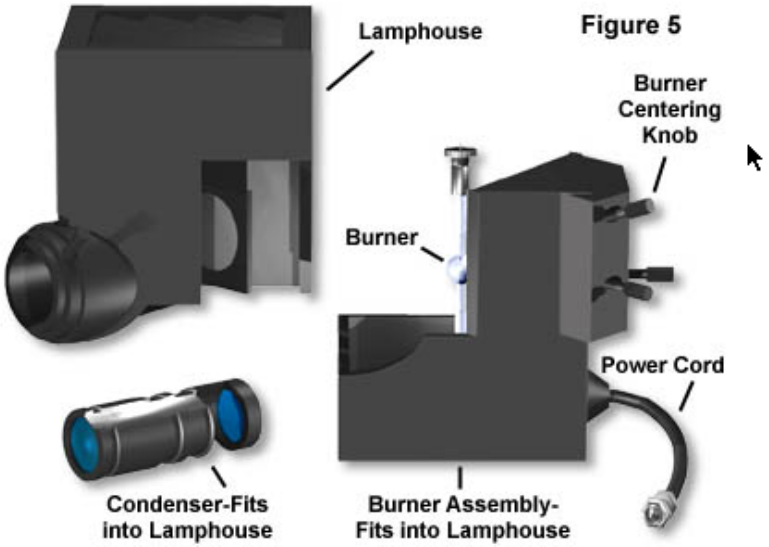


Figure 5

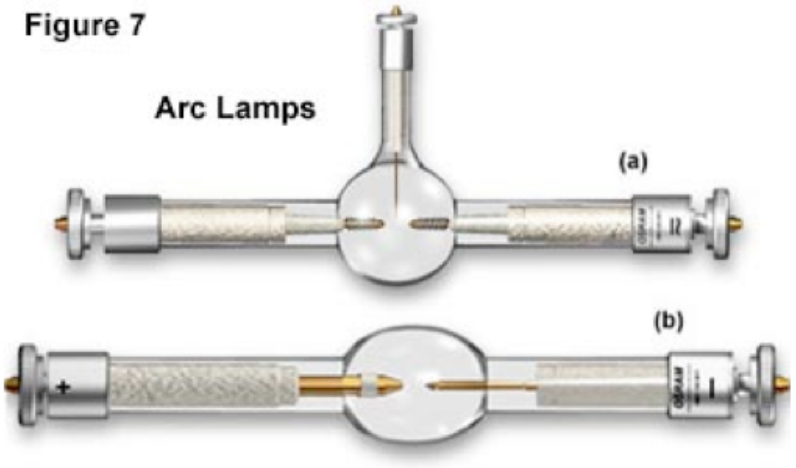


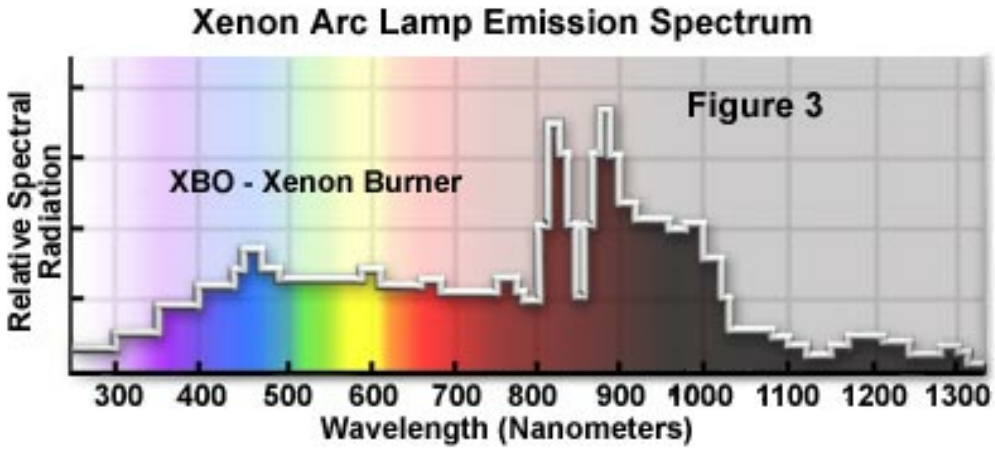
Figure 7

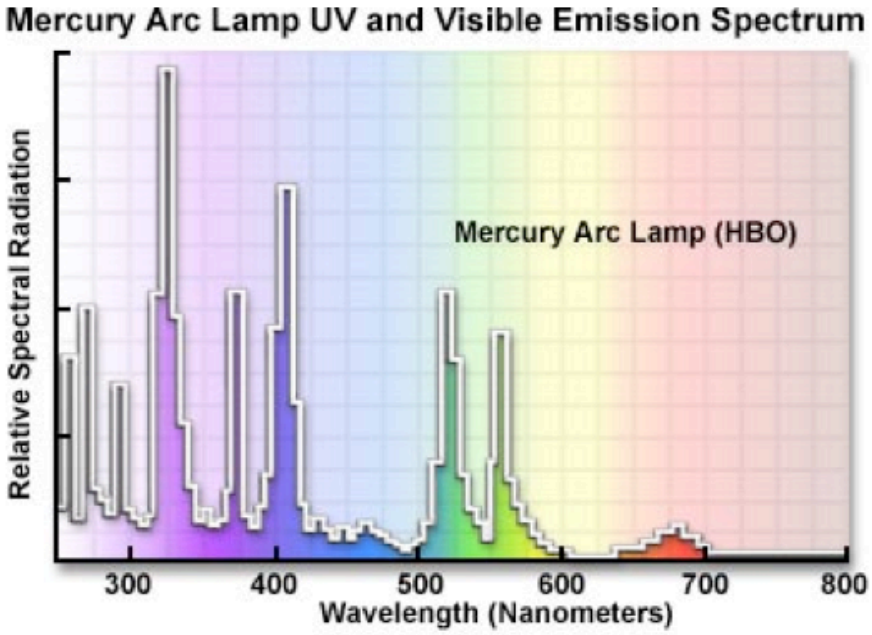
Arc Lamps

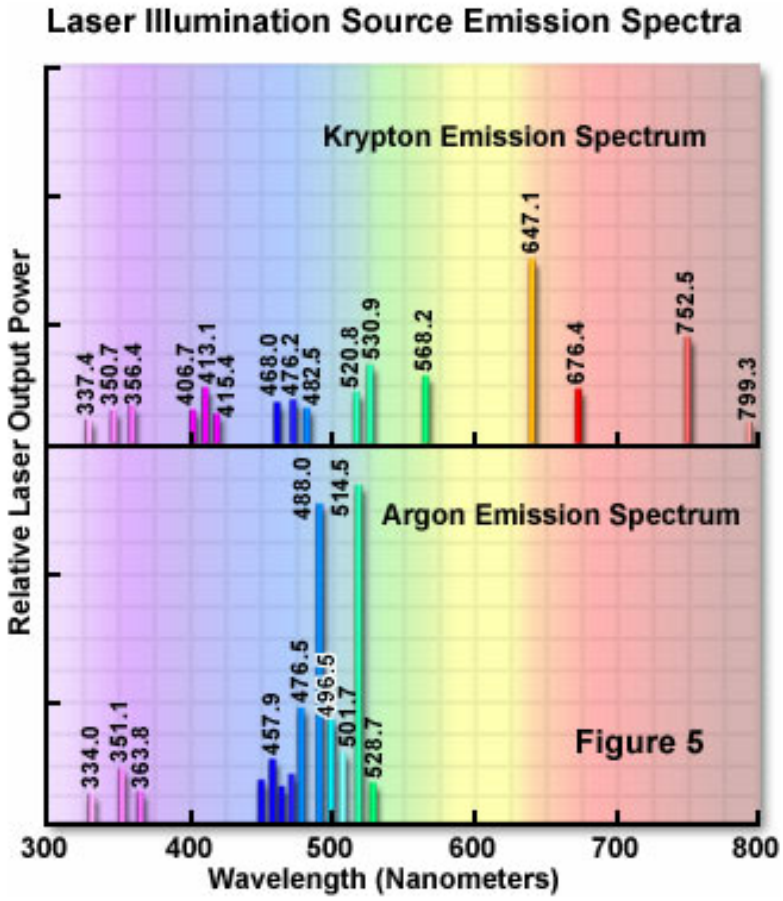
CAUTION!

Mercury and Xenon arc lamps require caution during operation because of the danger of explosion due to very high internal gas pressures and extreme heat generated during use. Never ignite a lamp outside of its housing or observe the lamp directly when it is burning (this can cause serious eye damage). Neither mercury nor xenon lamps should be handled with bare fingers in order to avoid inadvertent etching of the quartz envelope. Change bulbs only after the lamp has had sufficient time to cool. Store lamps in their shipping containers to avoid accidents.

Spectra of various fluorescence light sources







Filter cubes

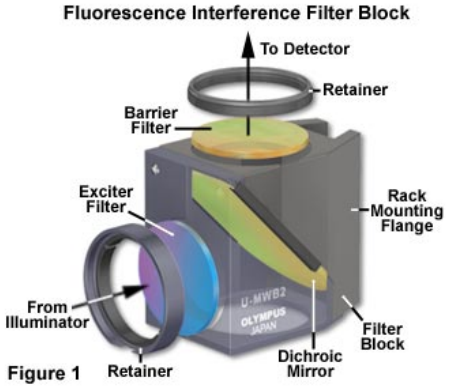
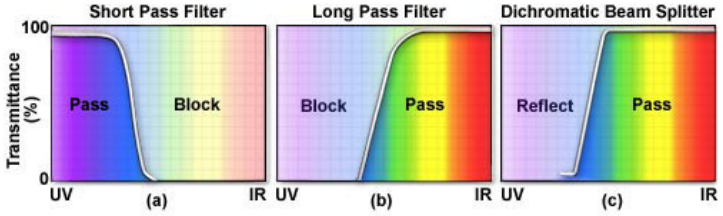


Figure 1



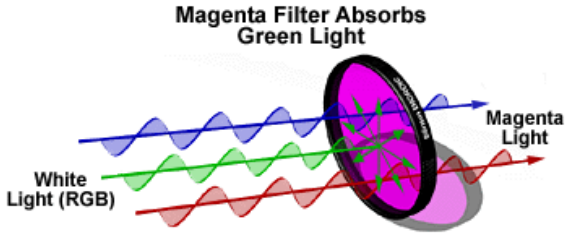


Figure 1

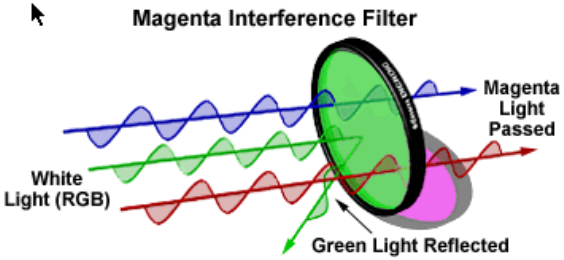
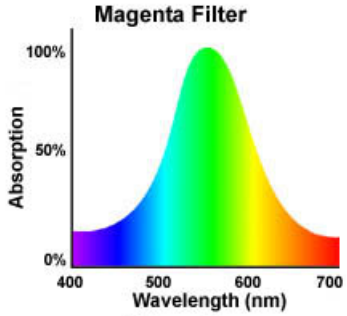
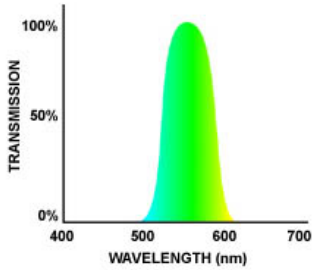


Figure 3





Choosing objective lenses (Olympus)

U Planapos
20X/NA0.75/340nm dry
40X/NA0.95 dry
40X/NA0.65-1.35 oil
60X/NA1.4 oil
60X/NA1.2psf water, symmetric point spread function
60X/N.A.1.3 Silicon immersion oil
100X/NA1.4 oil

Objective parameters affecting intensity (brightness of image)

Absorption of light by glass (wavelength dependent)
Intensity proportional to NA^4
Intensity proportional to 1 / $Magnification^2$

U Plan Apo(chromatic)

60x Plan Apochromat Objective

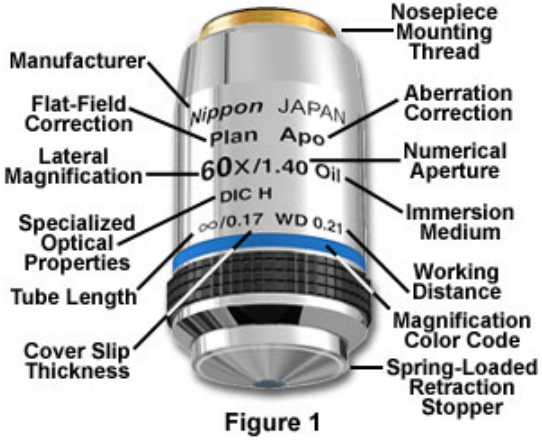


Figure 1

100X Plan Apochromat Objective
With Adjustable Iris Diaphragm

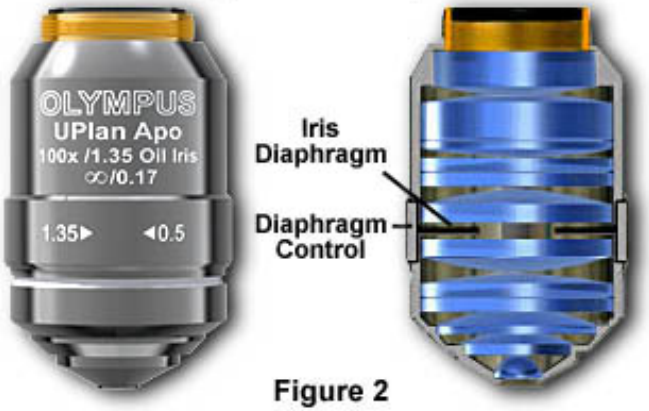
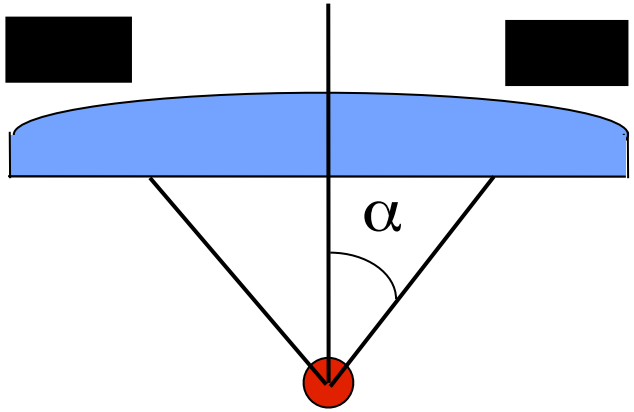


Figure 2

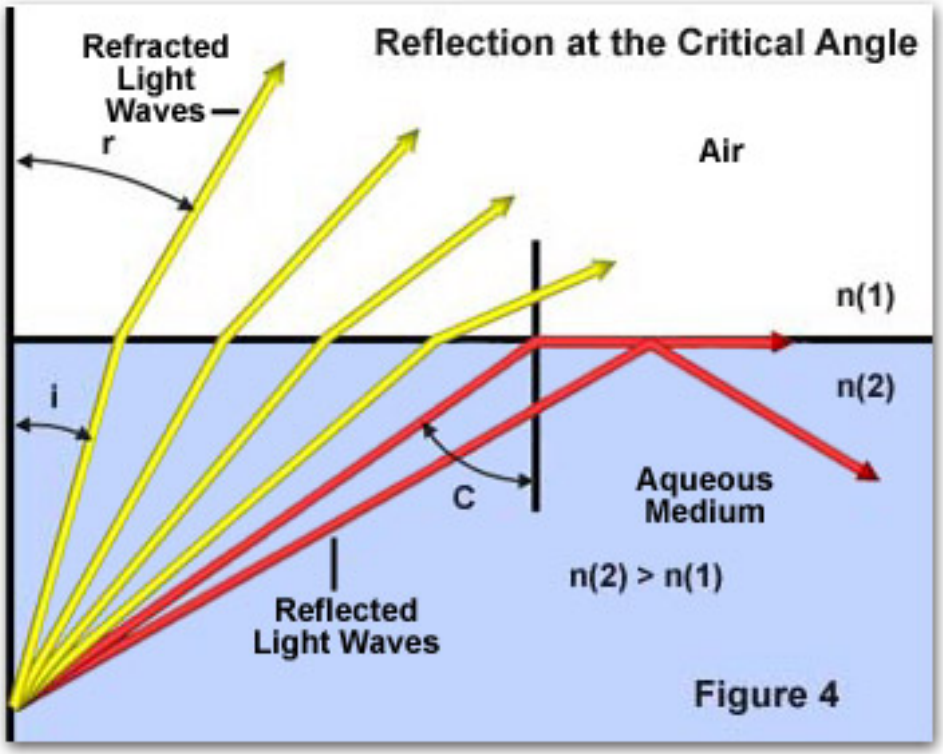
Rule of thumb

NA of lens cannot exceed the refractive index of the medium

Air 1
Water 1.3
Oil 1.515-1.534



NA = R.I. sin α



Single lenses show considerable chromatic aberration

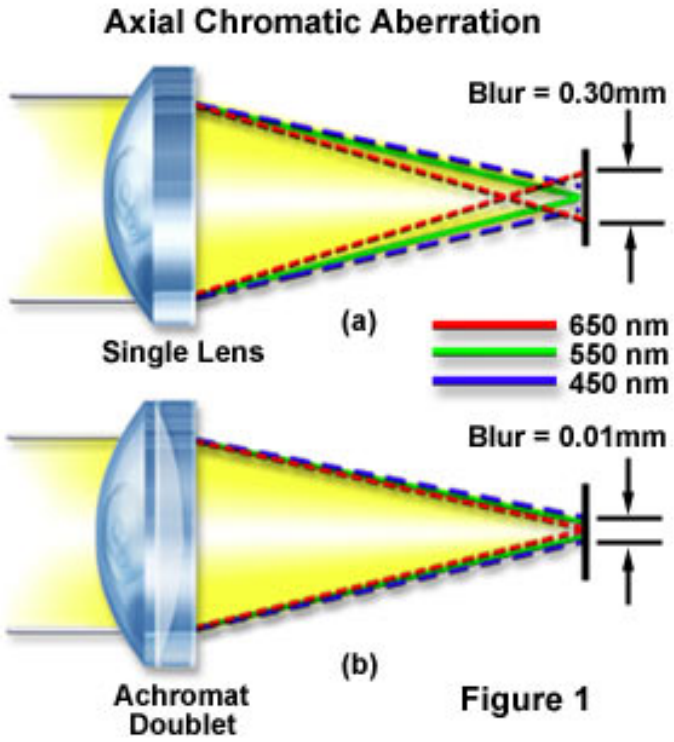


Figure 1

For brightfield imaging
causes blur and
“rainbow appearance”

For fluorescence imaging
Causes lack of registration
Between channels in X,Y and in Z.

Combinations of different kinds of glass (with different refractive indices)

Apochromatic lenses have many elements to correct chromatic aberration to a high degree in

the 4 normal imaging channels (DAPI/FITC/TRITC/Cy5), but there is always some residual small amount of aberration

Simple lenses (single element) have spherical aberration

Objectives are made of many elements to correct spherical aberration.

Most objectives are designed to image correctly at the surface of a cover slip of a particular thickness (usually number 1.5, or 0.17mm (0.15-0.19mm).

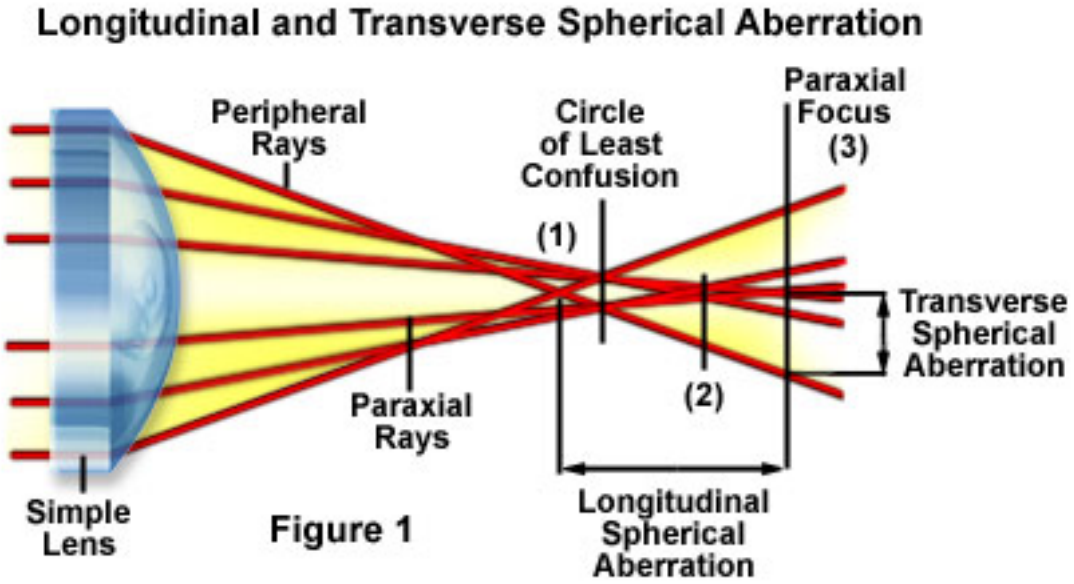
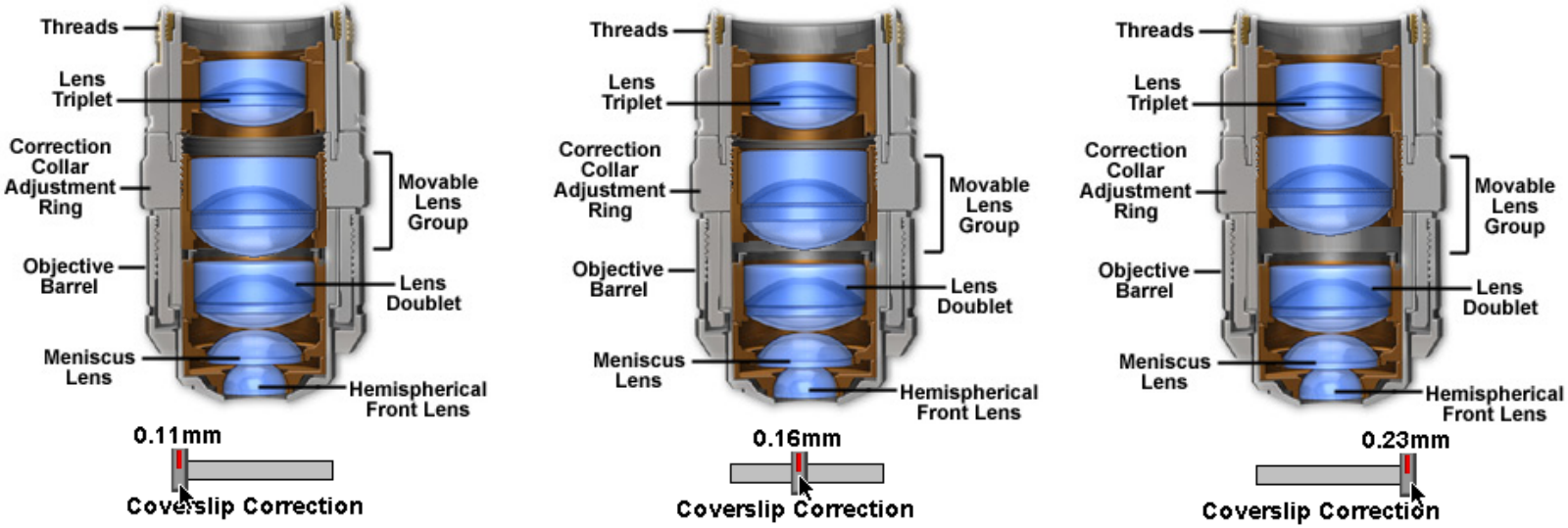


Figure 1

Using (coverslip thickness) correction collars of water immersion objectives to correct spherical aberration When imaging deep sections in thick specimens

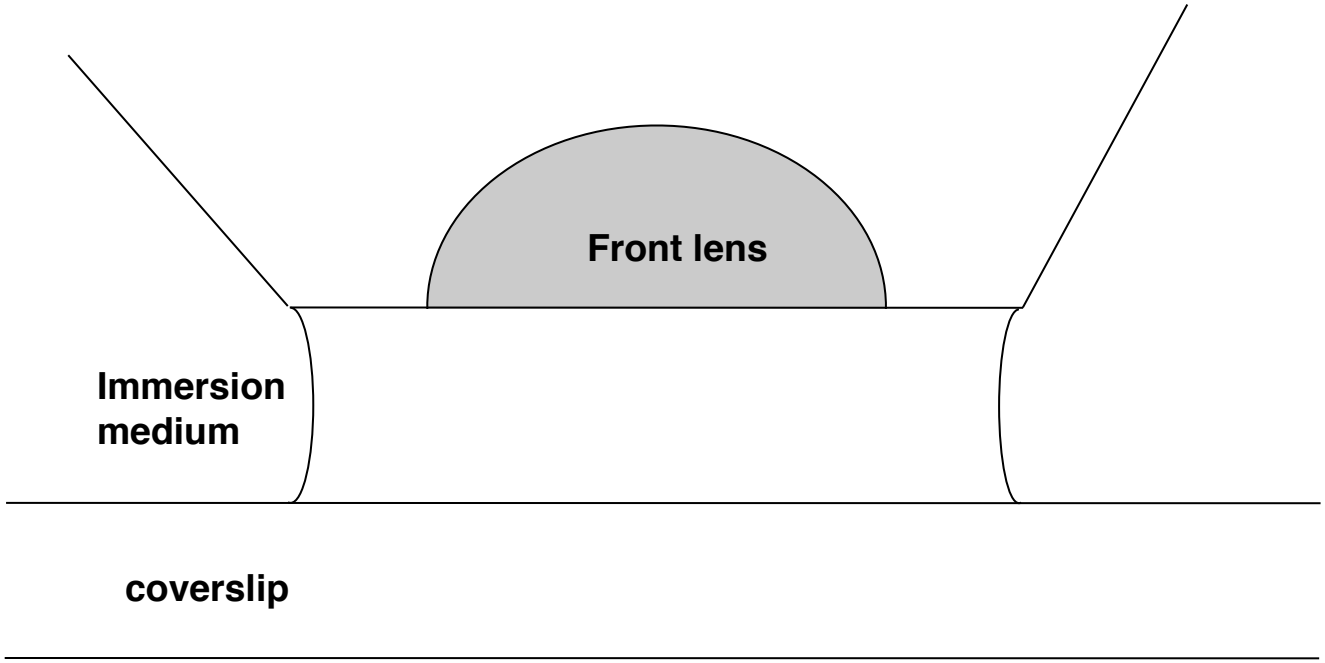


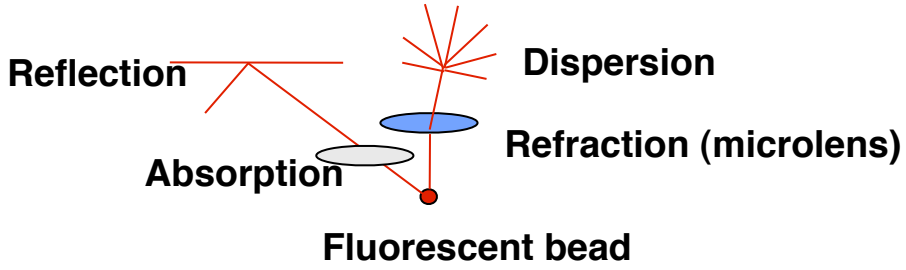
Alternatives:

1) Motorized lens inside microscope (Intelligent imaging-3I).
2) Can use immersion oils of varying refractive indices for oil immersion lenses (RI=1.515 to 1.534 from Cargil).
3) Use total immersion lenses / dipping lenses (no cover slip).

Problems other than spherical aberration

(particulary when imaging deep in thick specimens)





Partly overcome using far red / infrared excitation or multiphoton excitation