Light Microscopy Core Facility
DUKE UNIVERSITY AND DUKE UNIVERSITY MEDICAL CENTER

CMB551 1A:

Microscopy and Image Analysis in Cell Biology

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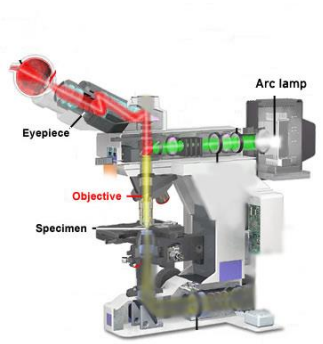
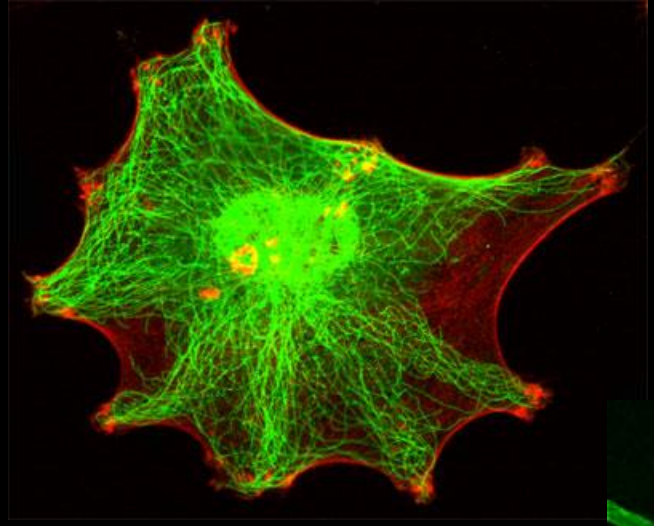
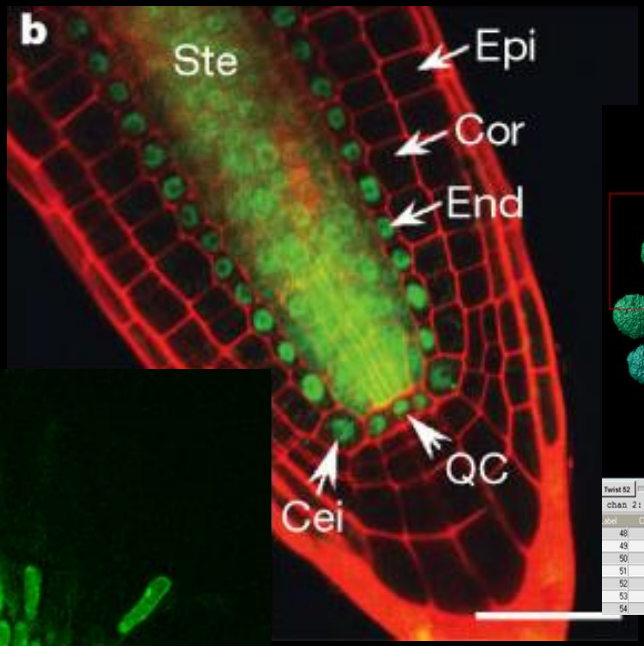
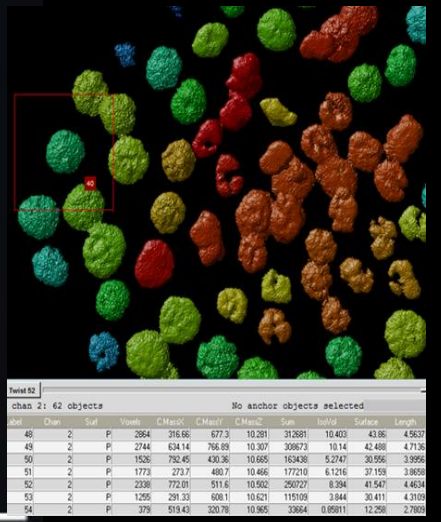
Rationale of the microscopy module

1 2 3	**Principles of microscopy** - Fluorescence, core concepts, objectives, cameras, filters, transmitted light and contrast. Optical sectioning: confocals and 3D imaging - The confocal principle, lasers, scanning, SNR improvements, detectors, sampling, point-spread function. limitations of point scanning confocals, spinning disks, TIRF, multiphoton, SPIM. Review and comparison. Super resolution overview - three families of techniques that improve the resolution beyond the diffraction limit.
Any	**Techniques** - Immunofluorescence, fluorophores, live cell imaging, fluorescent proteins, photokinetics, protein-protein interactions, photocontrol, reporters, miscellaneous.
4 5 6	**Images and analysis** - Principles and examples using FIJI/ImageJ: image formats, histogram, scaling and digital contrast, display and visualization. Bring your computer and follow along with the exercises.





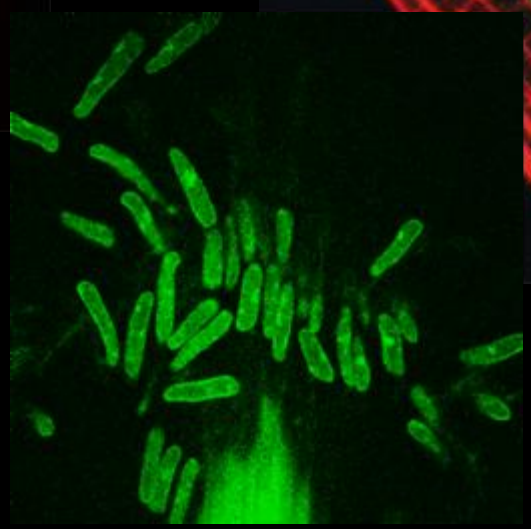


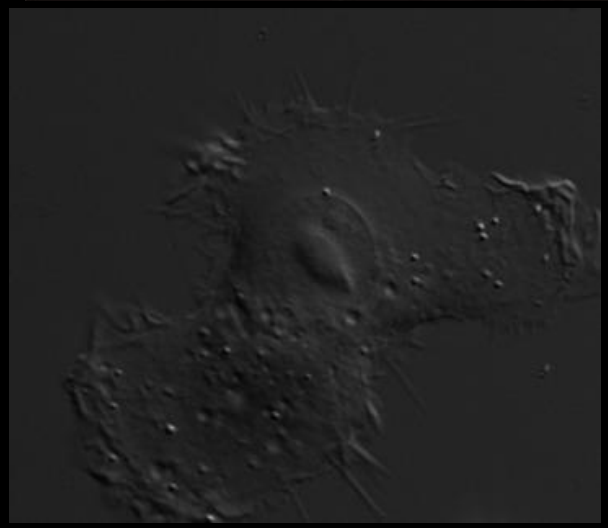


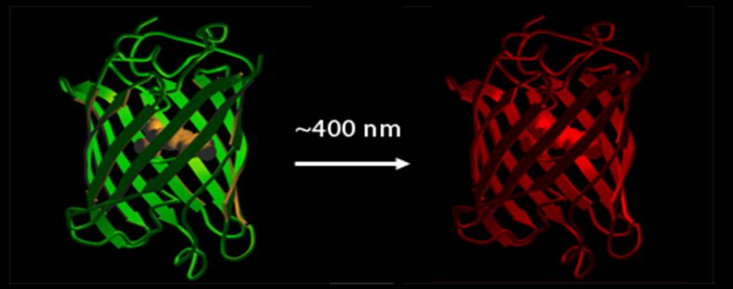
Twist 52

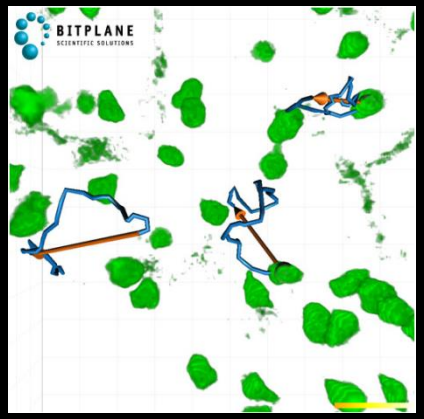
chan 2: 62 objects — No anchor objects selected

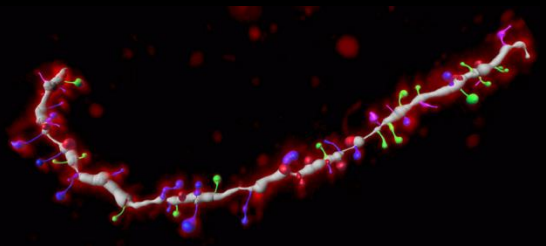
Label	Chan	Surf	Voxels	C.MassX	C.MassY	C.MassZ	Sum	IsoVol	Surface	Length
48	2	P	2864	316.66	677.3	10.281	312681	10.403	43.86	4.5637
49	2	P	2744	634.14	766.89	10.307	308673	10.14	42.488	4.7136
50	2	P	1526	792.45	430.36	10.665	163438	5.2747	30.556	3.9956
51	2	P	1773	273.7	480.7	10.466	177210	6.1216	37.159	3.8656
52	2	P	2336	772.01	511.6	10.502	250727	8.394	41.547	4.4634
53	2	P	1255	291.33	608.1	10.621	115109	3.844	30.411	4.3109
54	2	P	379	519.43	320.78	10.965	33664	0.85811	12.258	2.7809

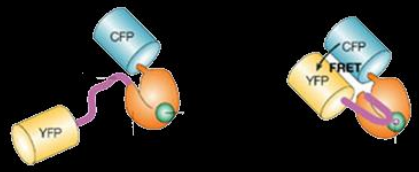


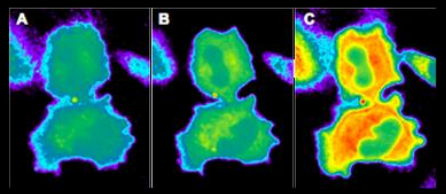




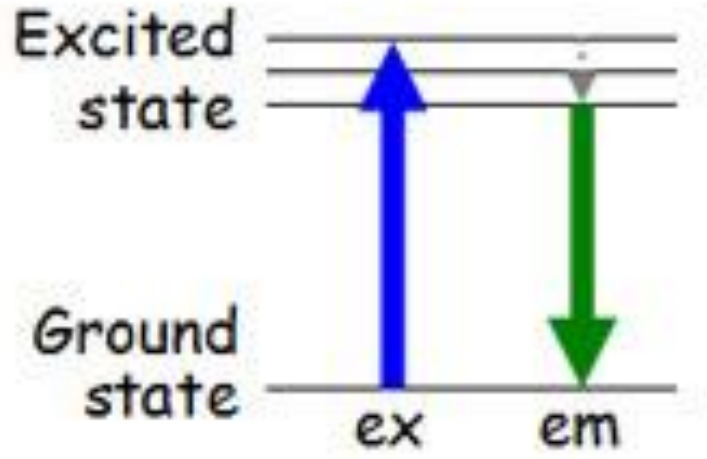


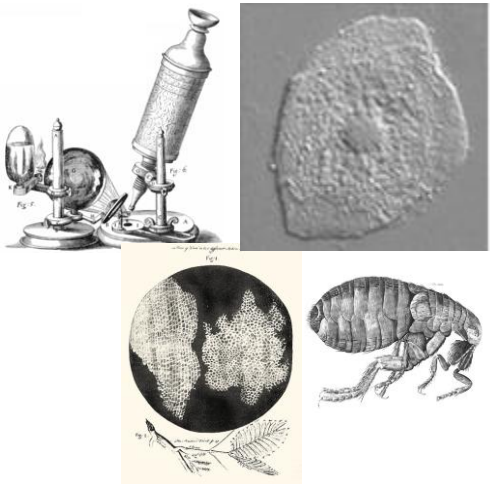




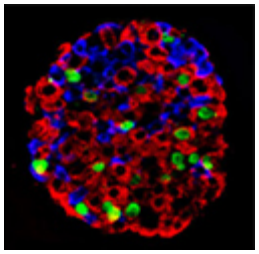


Fluorescence





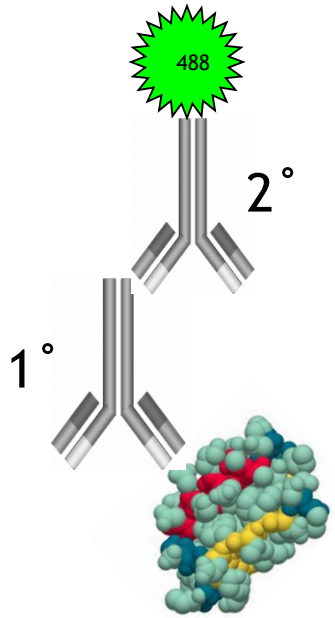
Relies on intrinsic contrast, little specificity

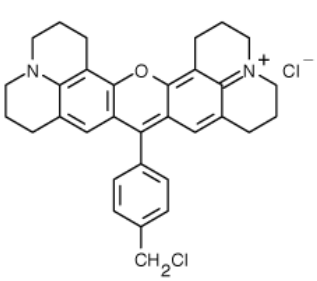


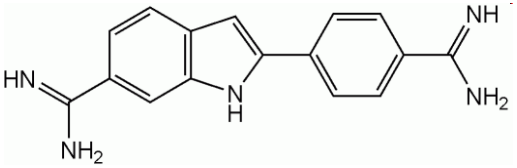
High-contrast, multi-channel imaging.

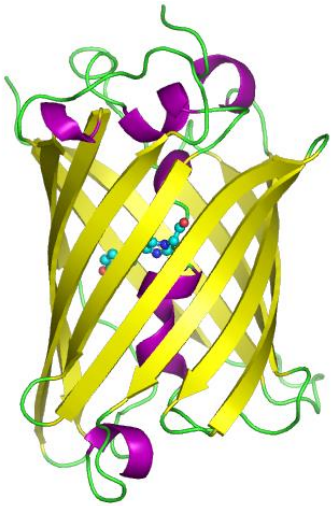
Allows many modes of imaging – confocal, TIRF, 2-photon, single-molecule, fluorescent proteins= live cell imaging, FRAP, photoconversion, FRET, FCS, super resolution. . .

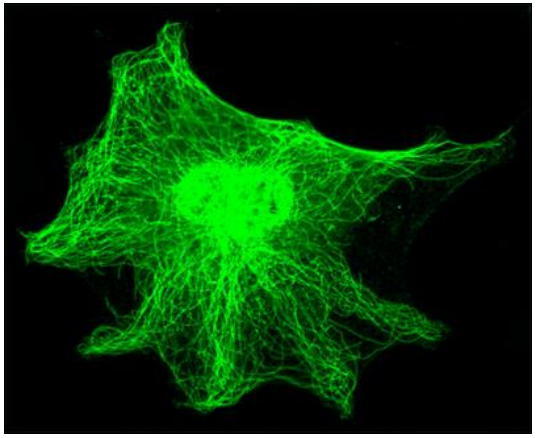
Fluorescence can be . . .

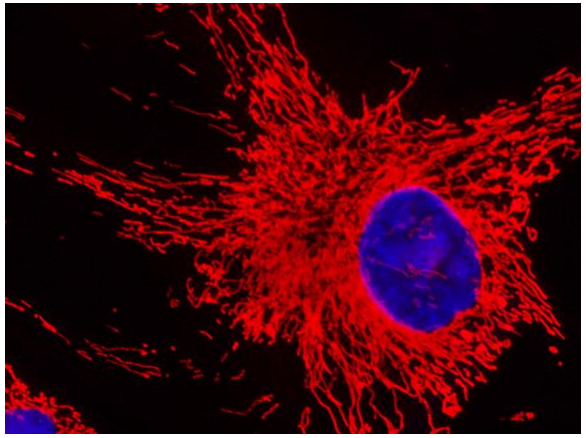


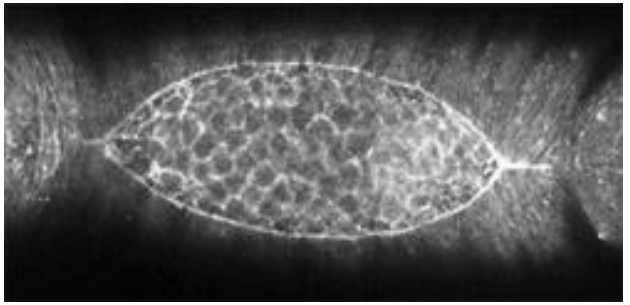




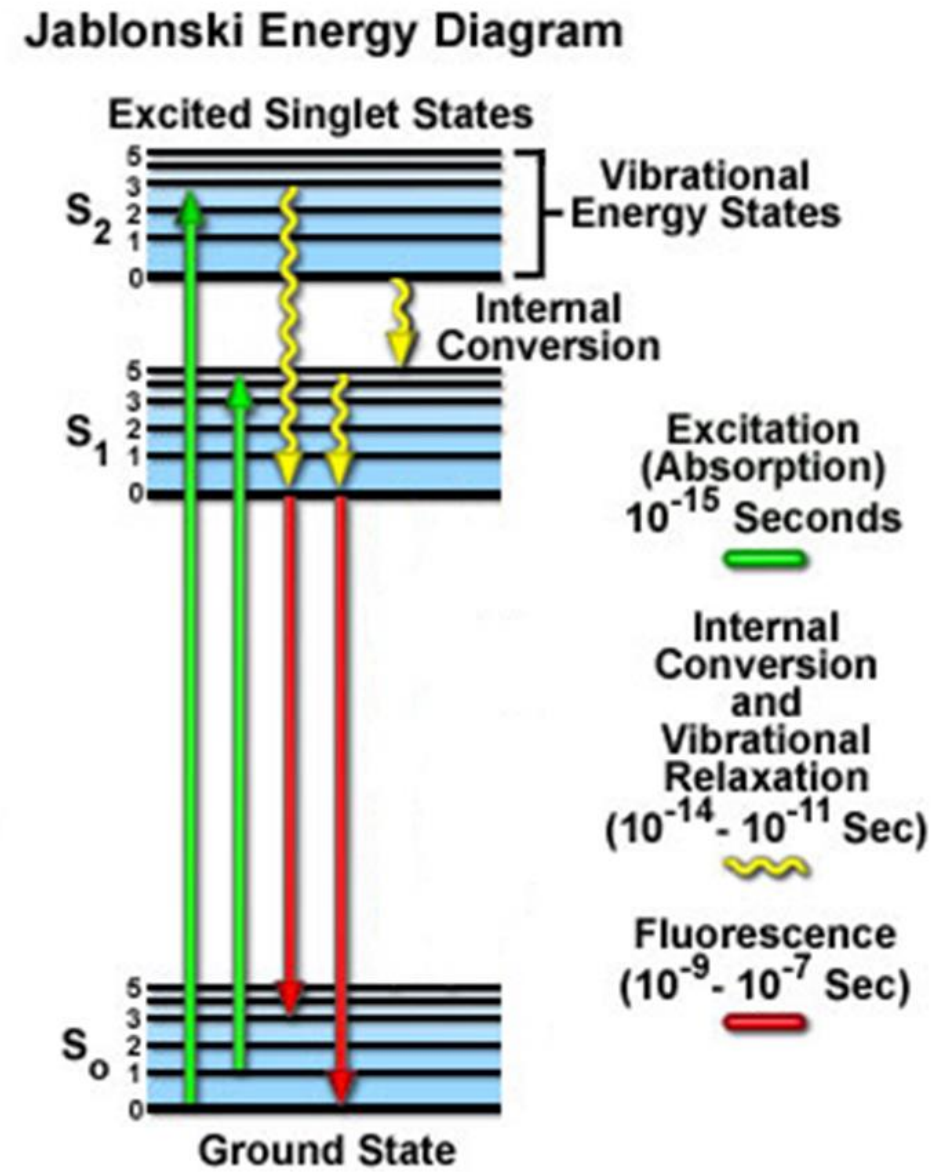






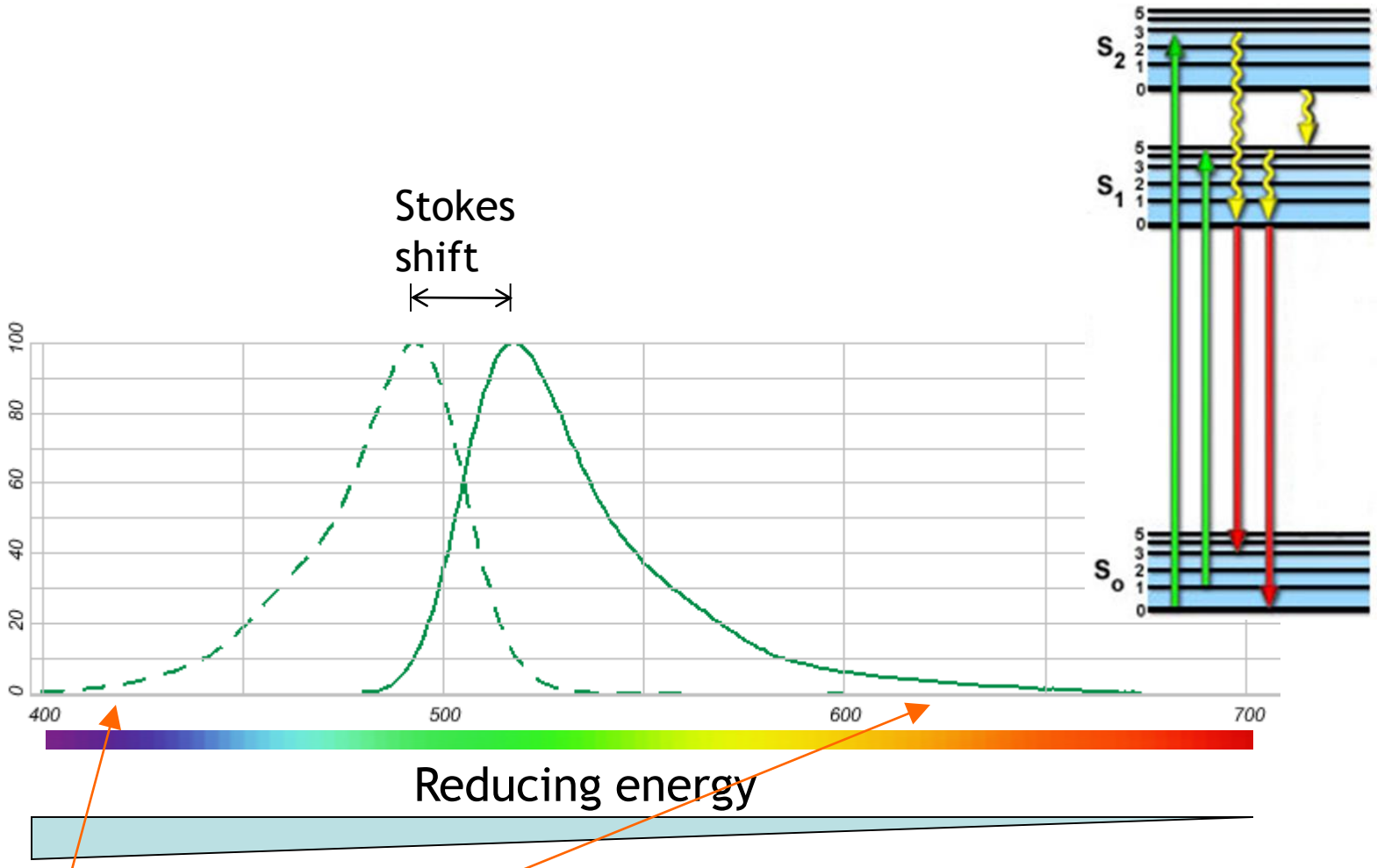


Fluorescence in more detail



http://www.olympusmicro.com/primer/java/jablonski/jabintro/

Excitation and emission



Notice the “tails”

http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html

Emission is excitation-independent

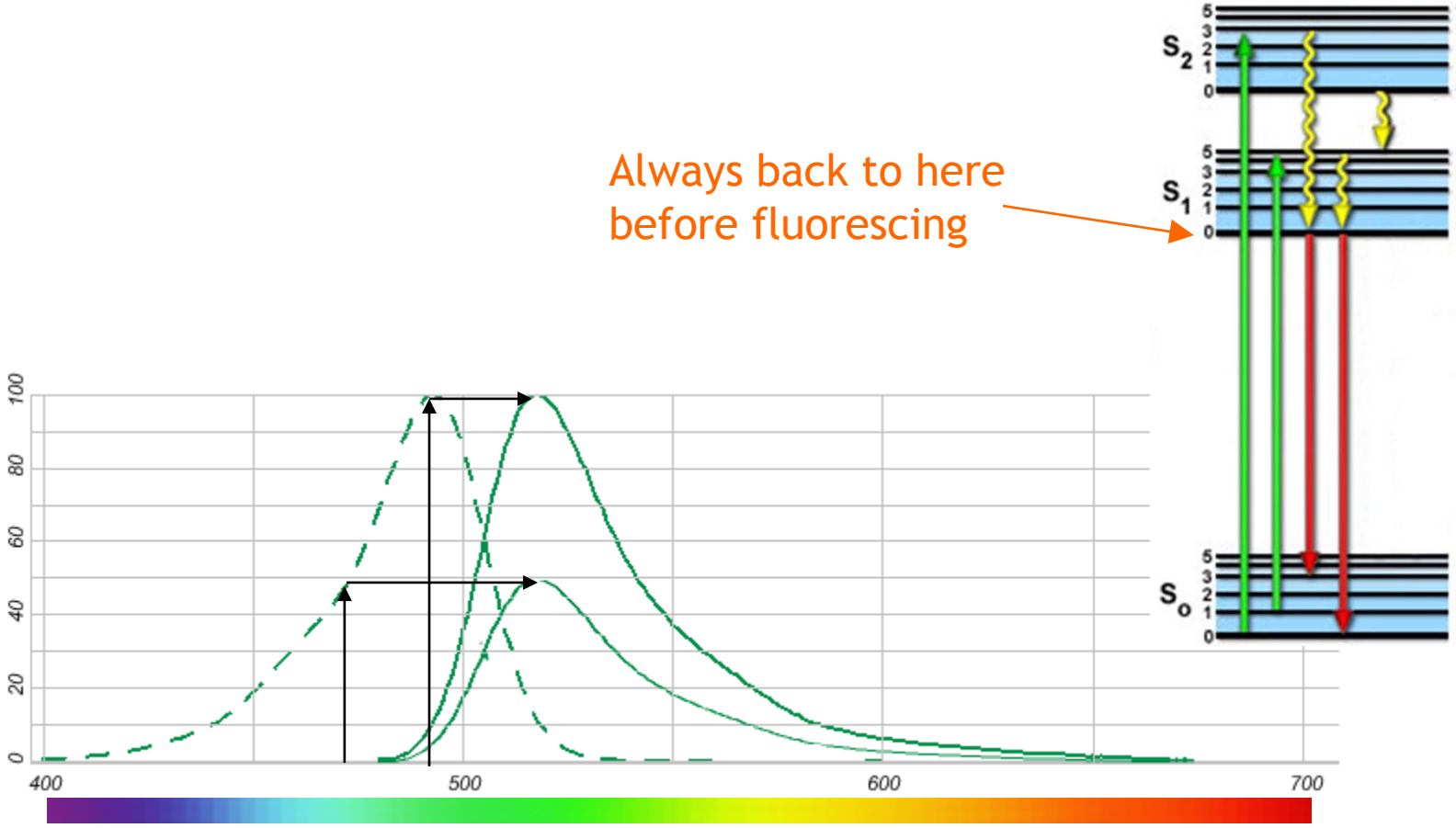


Photo-bleaching

Chemical reaction causing irreversible loss of fluorescence

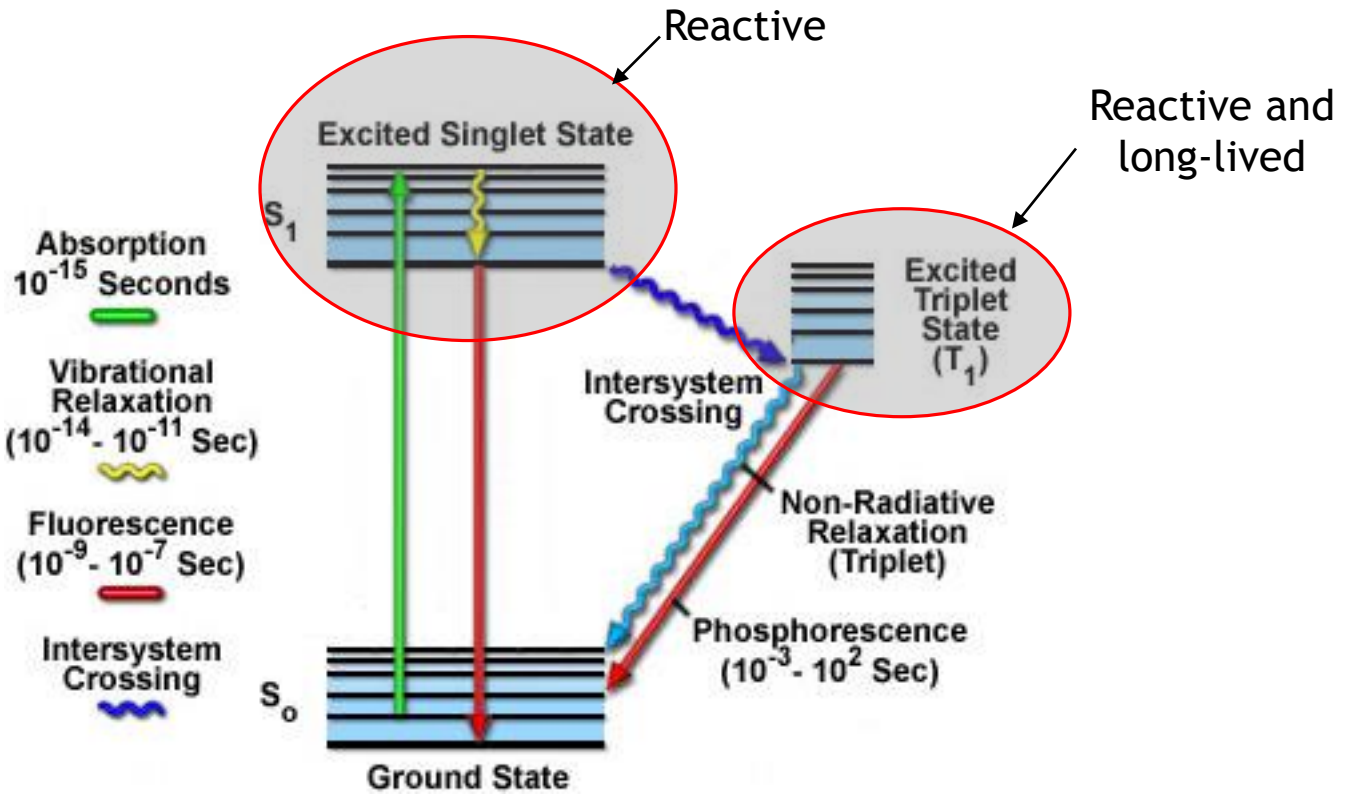
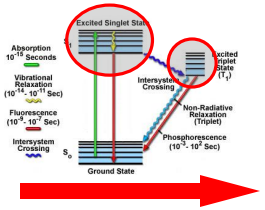


Photo-toxicity

Same principle as photo-bleaching but this time it's bad for the cell not just the fluorophore

Oxygen

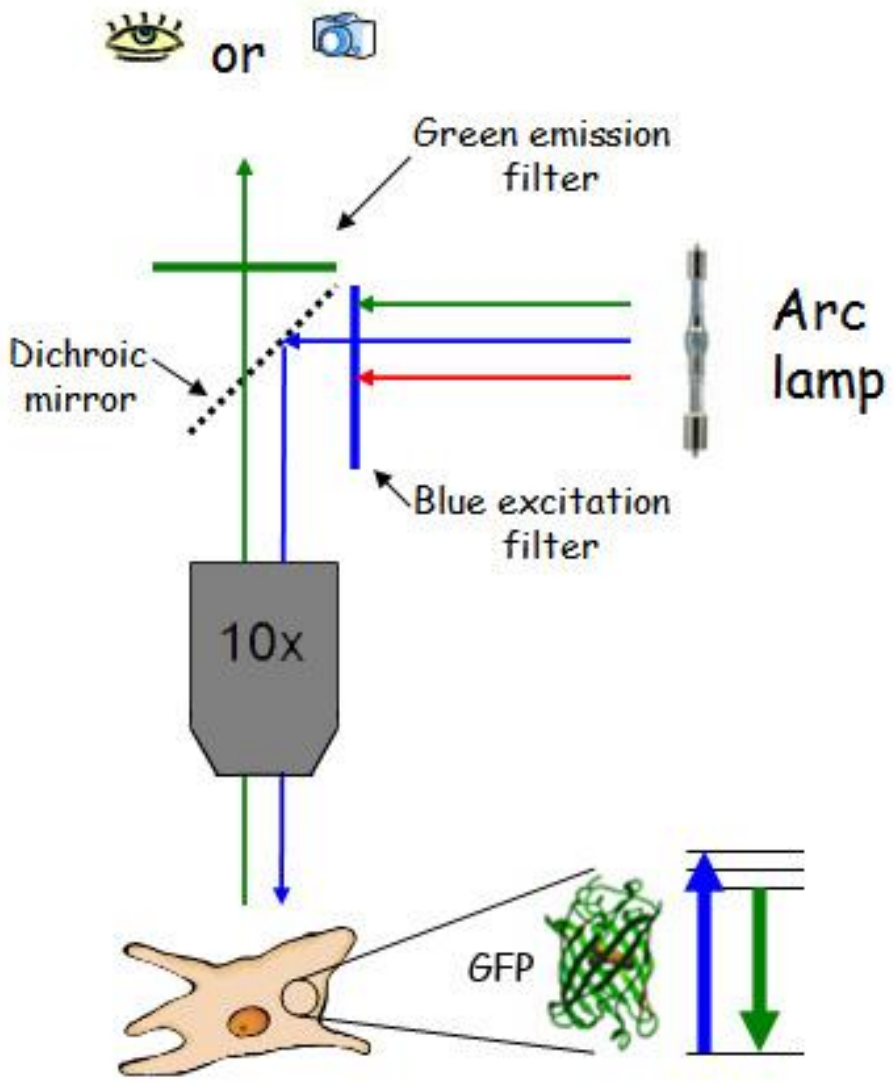


Radicals

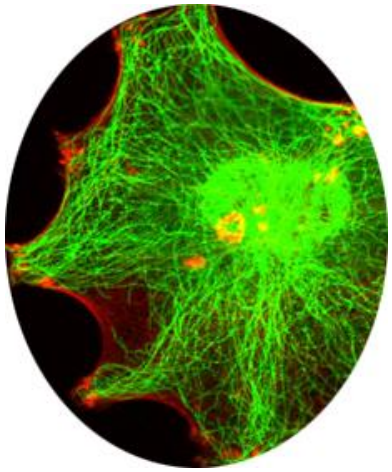
Generally UV light is more toxic than far-red wavelength, with or without any exogenous fluorophore around

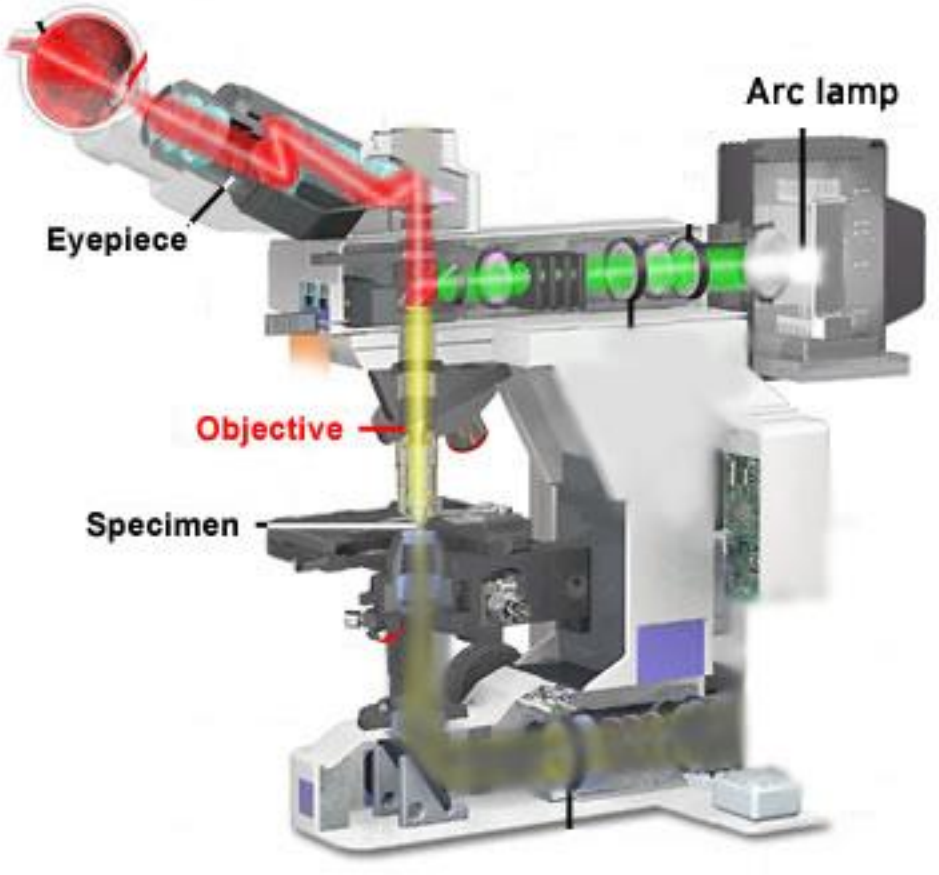
Modalities | Photon budget

Basis of all fluorescence scopes



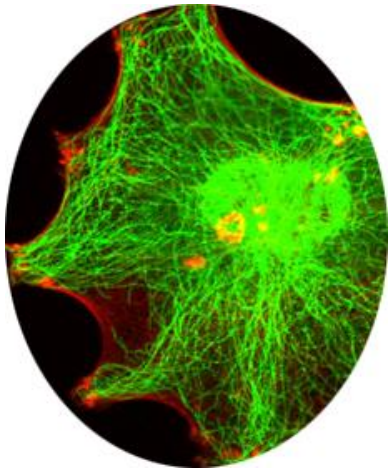
The insides of a microscope: Fluorescence

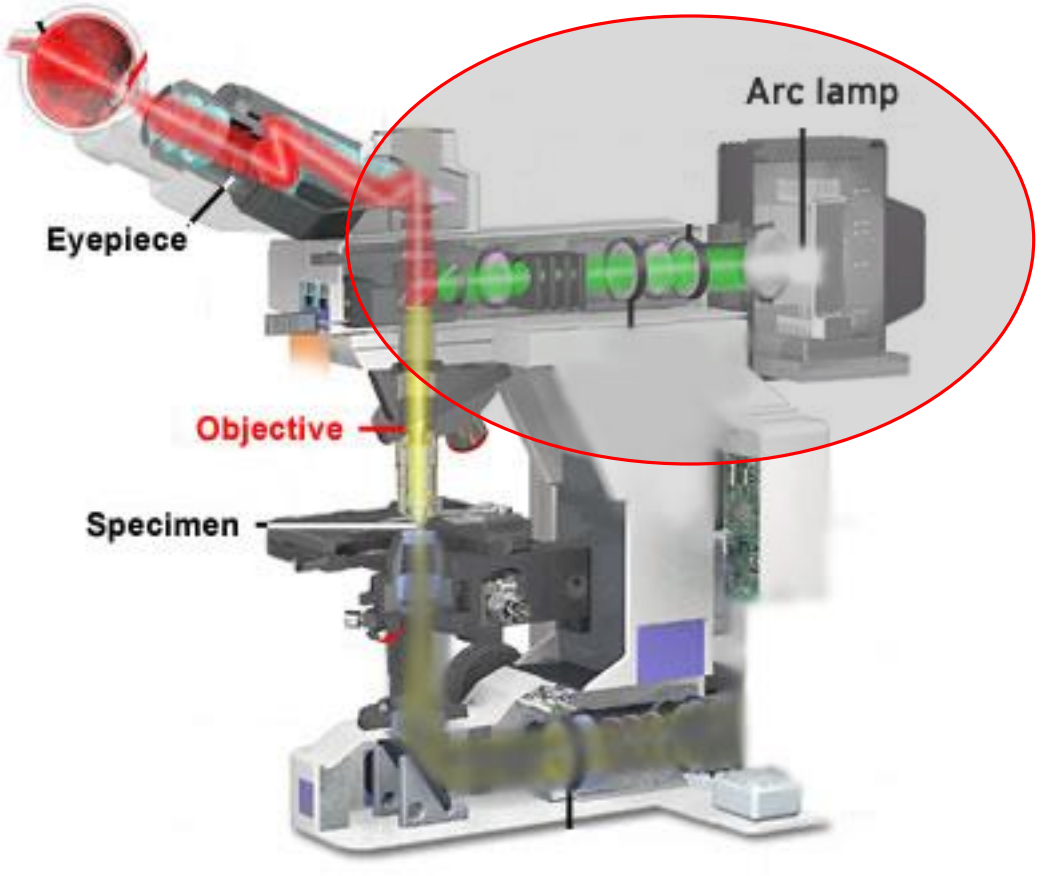




Adapted from http://www.microscopyu.com/articles/formulas/formulasconjugate.html

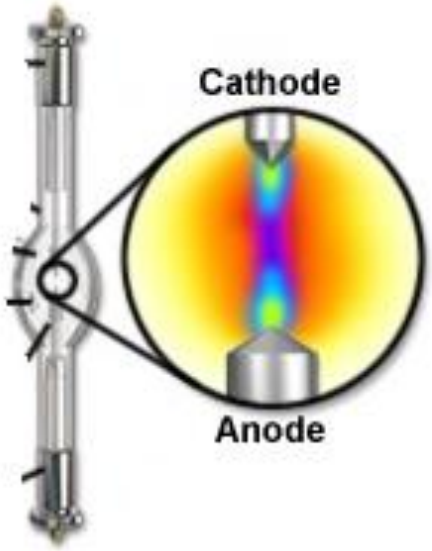
The insides of a microscope: Fluorescence

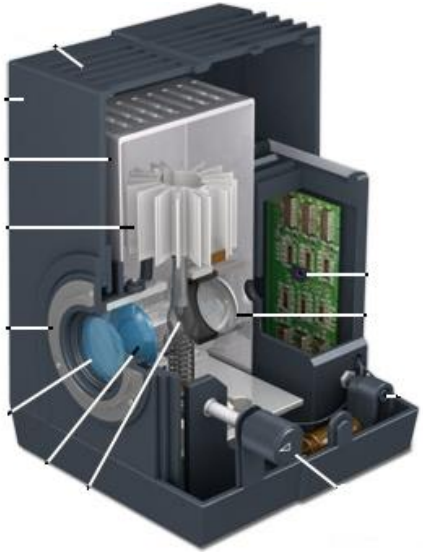




Adapted from http://www.microscopyu.com/articles/formulas/formulasconjugate.html

Illumination sources





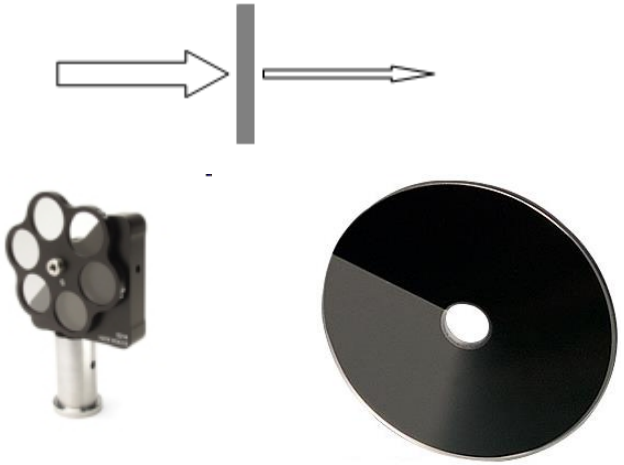
Arc lamp

Metal Halide

http://zeiss-campus.magnet.fsu.edu/articles/lightsources/mercuryarc.html

Regulation of exposure to excitation

Arc lamps tend to bright, unregulatable and slow to turn on/off (30 min minimum to extend life)

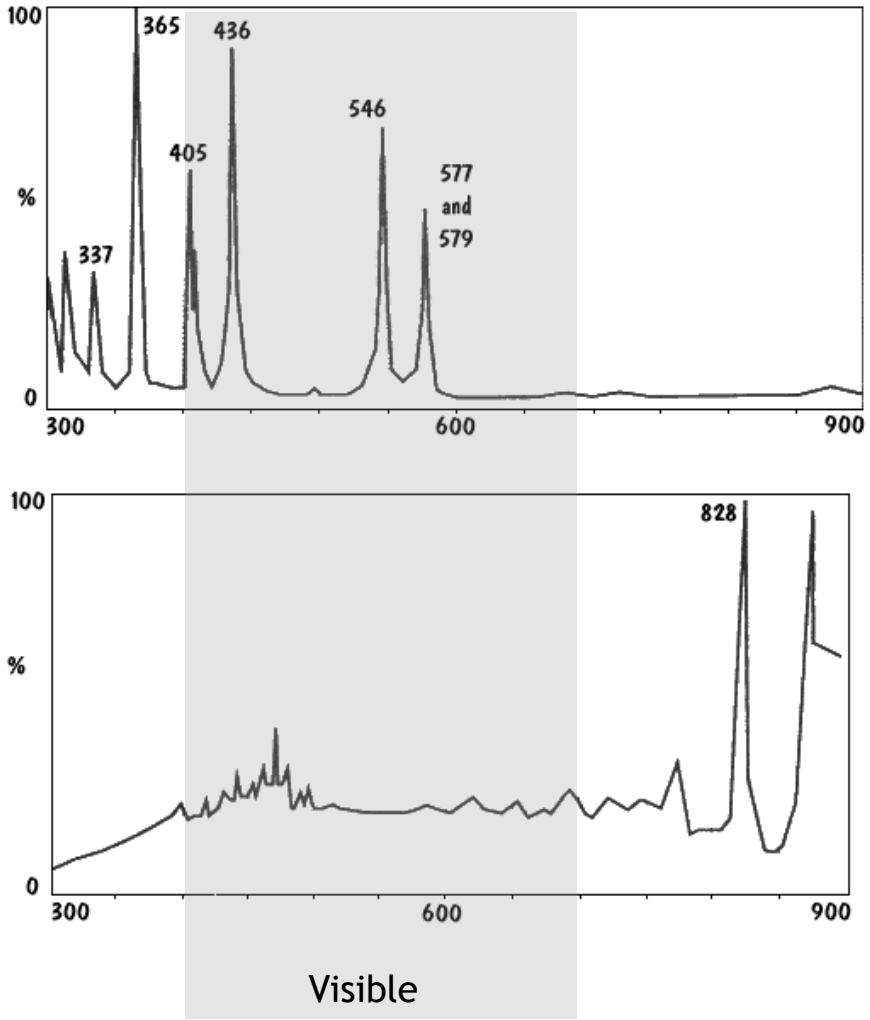


- Fast electromechanical shutter
- About 10 mSec cycle

- Neutral Density
- Usually expressed as a transmission
- ND20 = 20% of light goes through

Illumination sources: Arc lamps and similar

Mercury
Arc lamp



Spectra uneven and much in UV

Xenon
Arc lamp

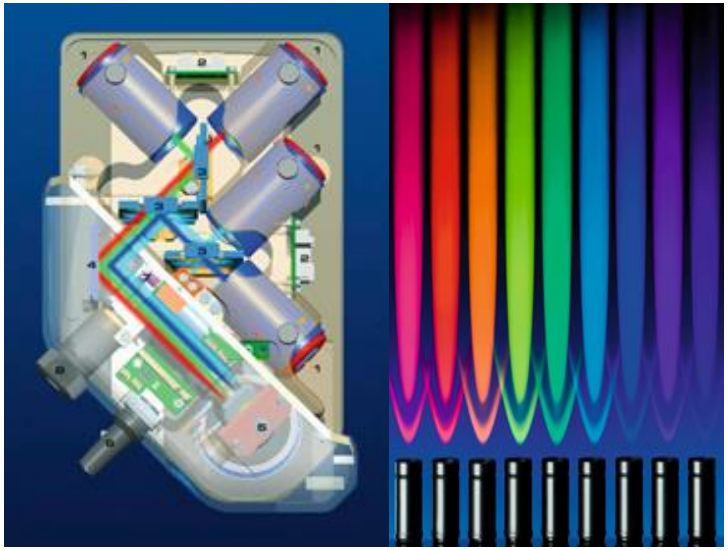
Spectra more even, weak in UV

Illumination sources: LEDs and similar

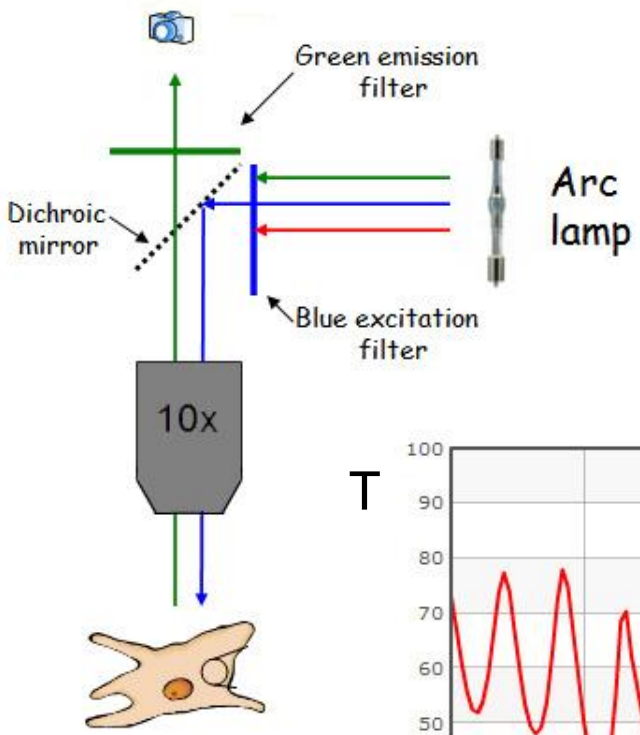
Last ~forever (50 000 hr)

Fast switching and power regulation

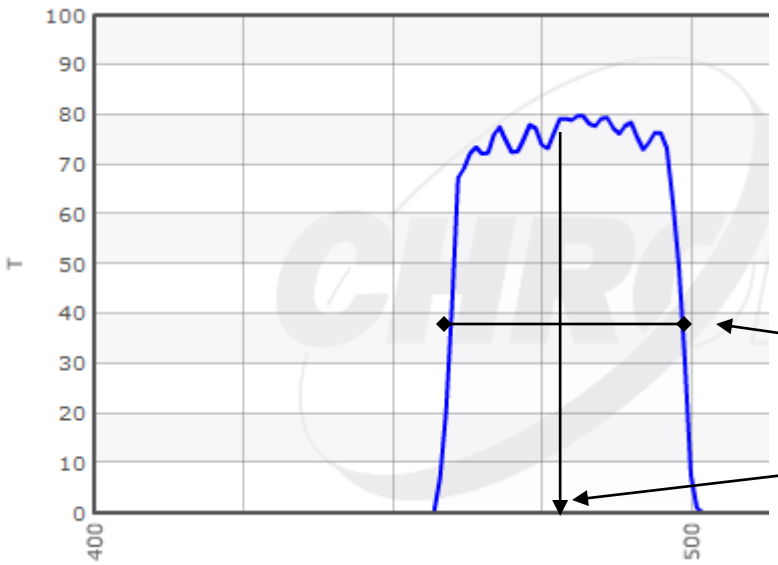
Taking off for the future - cheaper, brighter, more λ . . .



Filters and dichroics



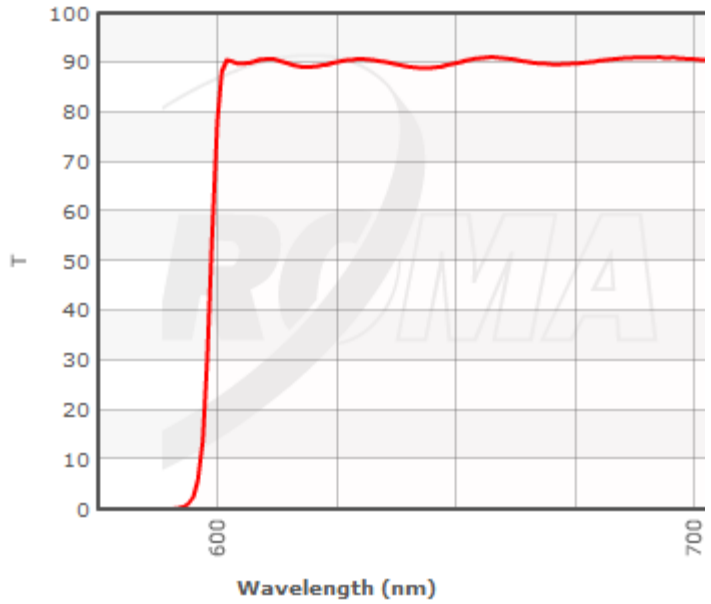
Filter terminology



Bandpass (BP) – 480/40
CWL/bandwidth

Full-Width at Half Maximal (FWHM)

Central wavelength



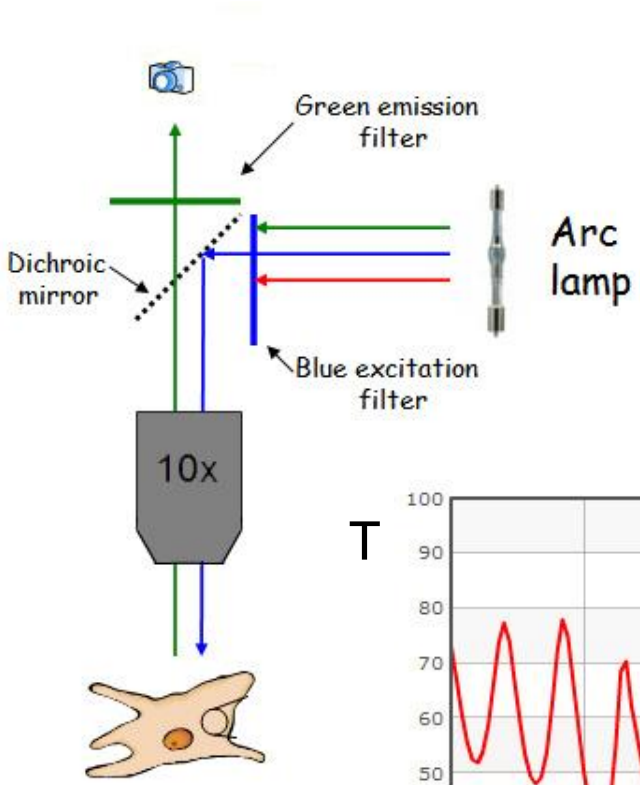
Longpass – LP600

(Defined around center of cut-on λ)

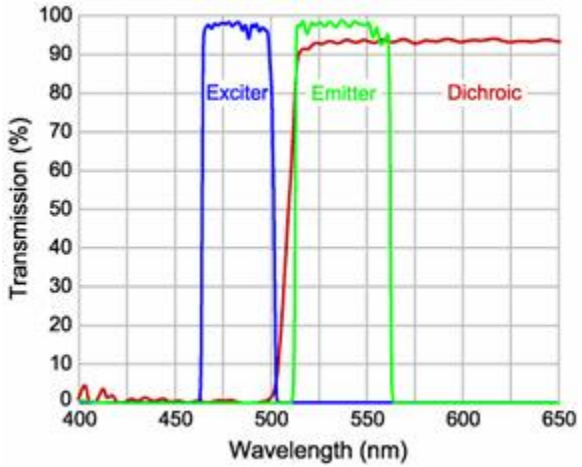
(guess what, say, Short Pass 670 means)

http://micro.magnet.fsu.edu/primer/techniques/fluorescence/filters.html

Filters and dichroics



Even better filter set



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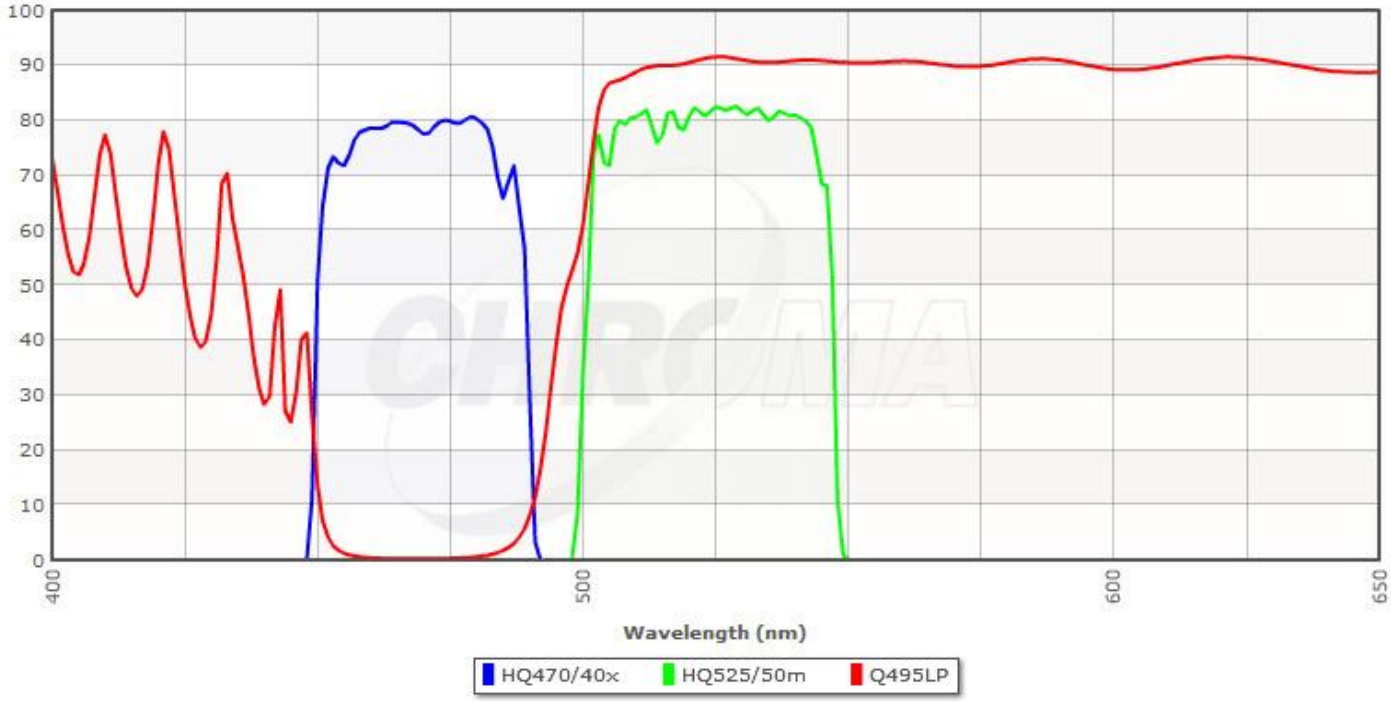
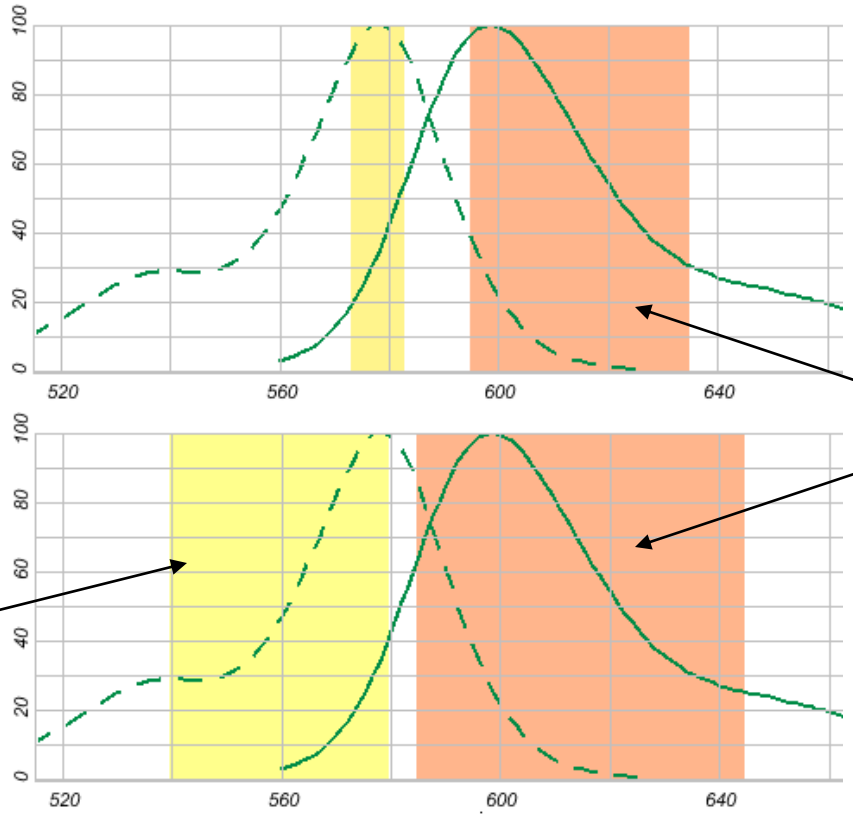


Filter choice and efficiency

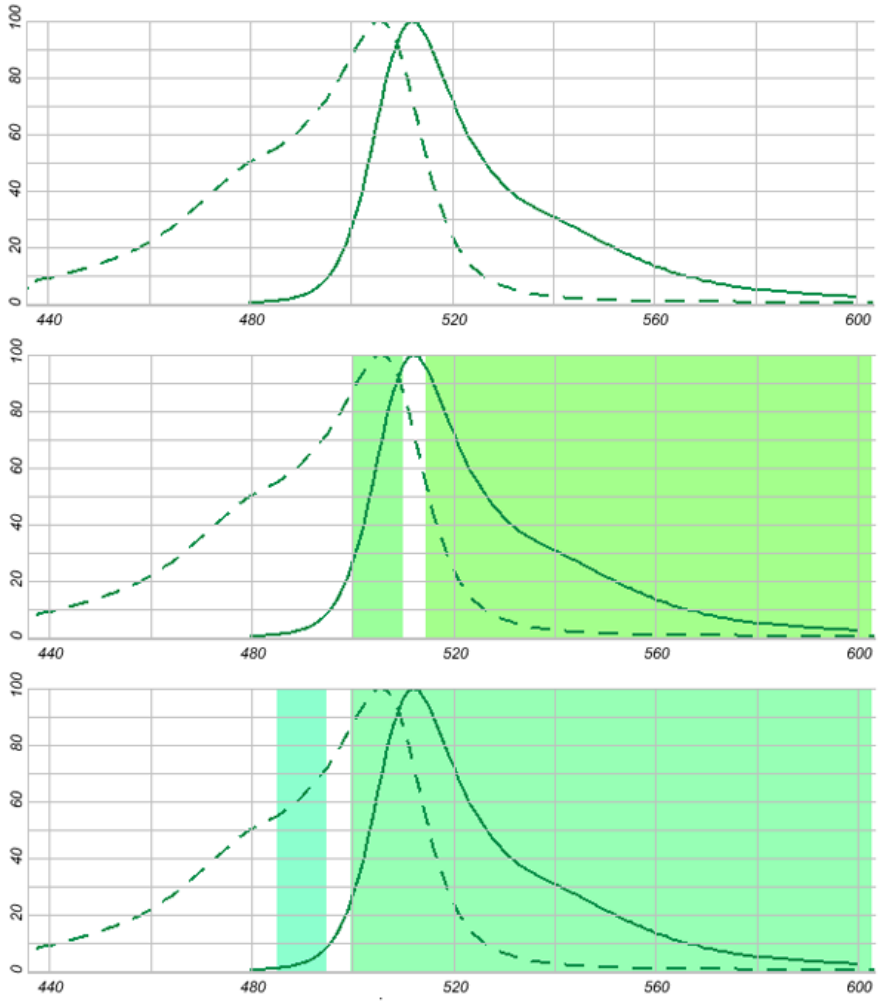
Choose filter to match your fluorophore (tools to help)



More photons = better

Does this help?

Filter choice and efficiency



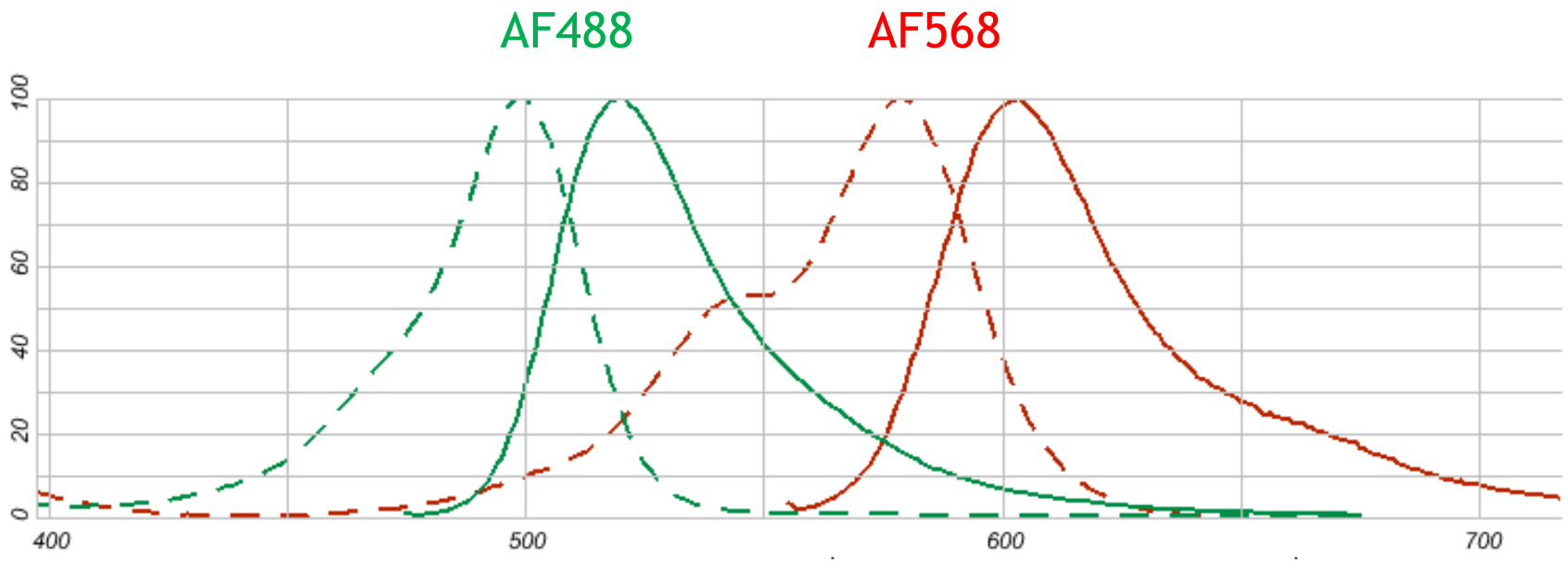
A fluor' with a fairly small stokes shift

Best excitation

Better emission

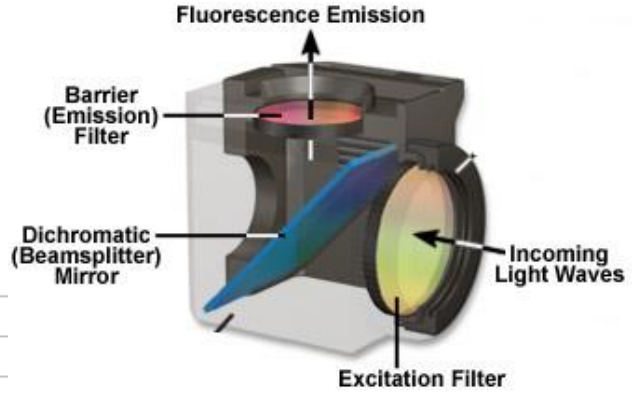
Multi-channel imaging

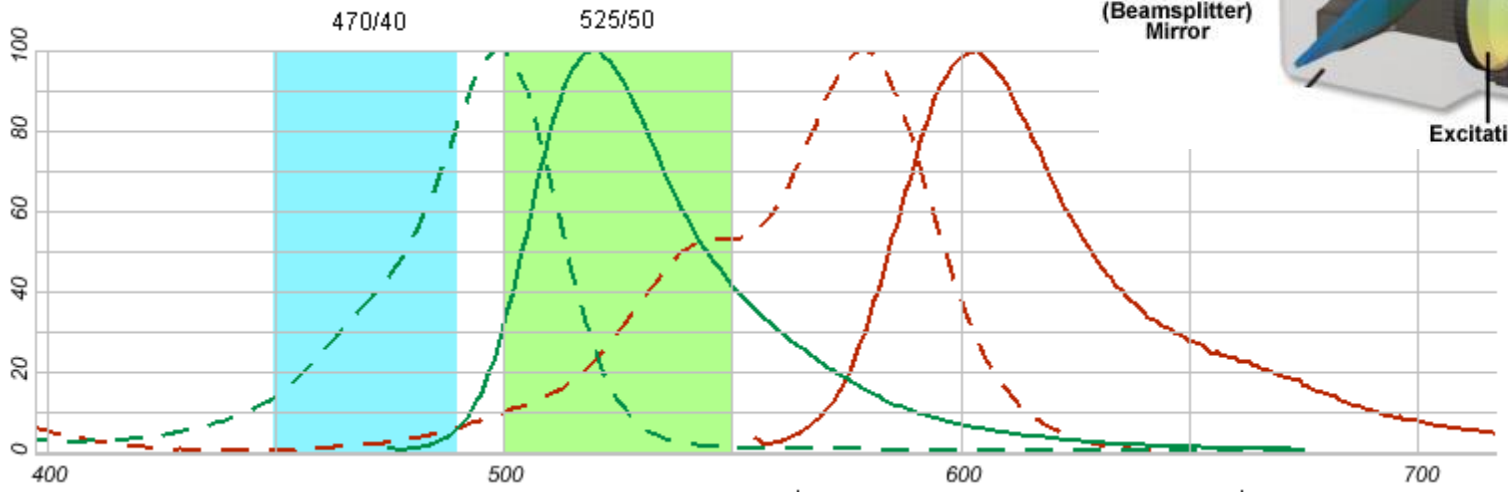
More than one color; more than one fluorescent protein, probe, antibody . . .

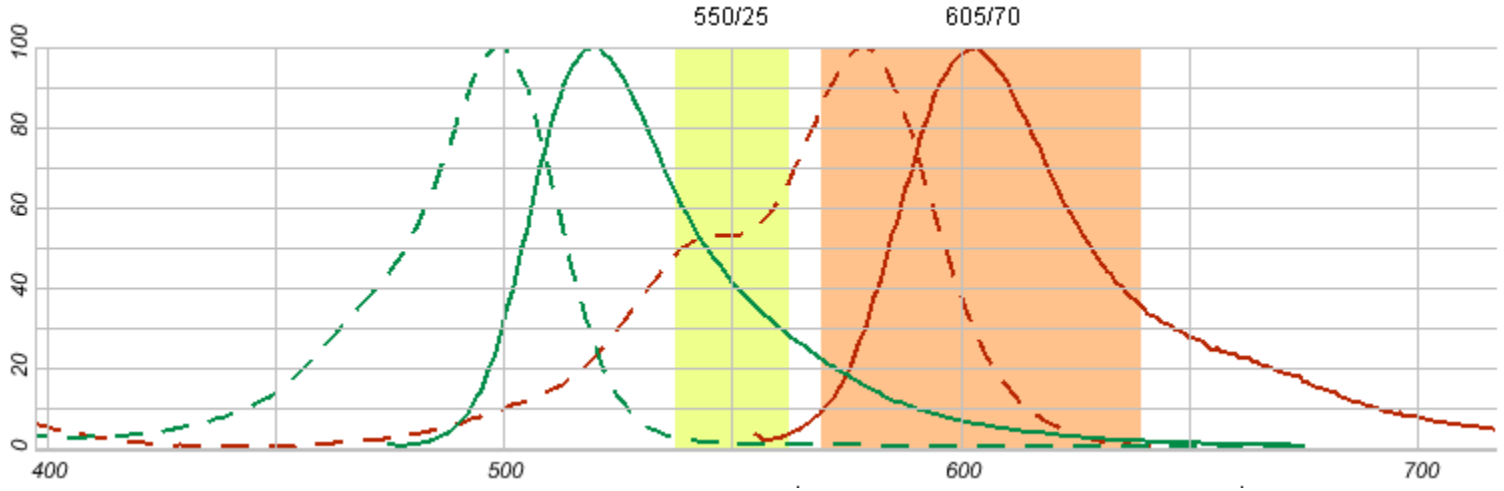


Multi-channel imaging

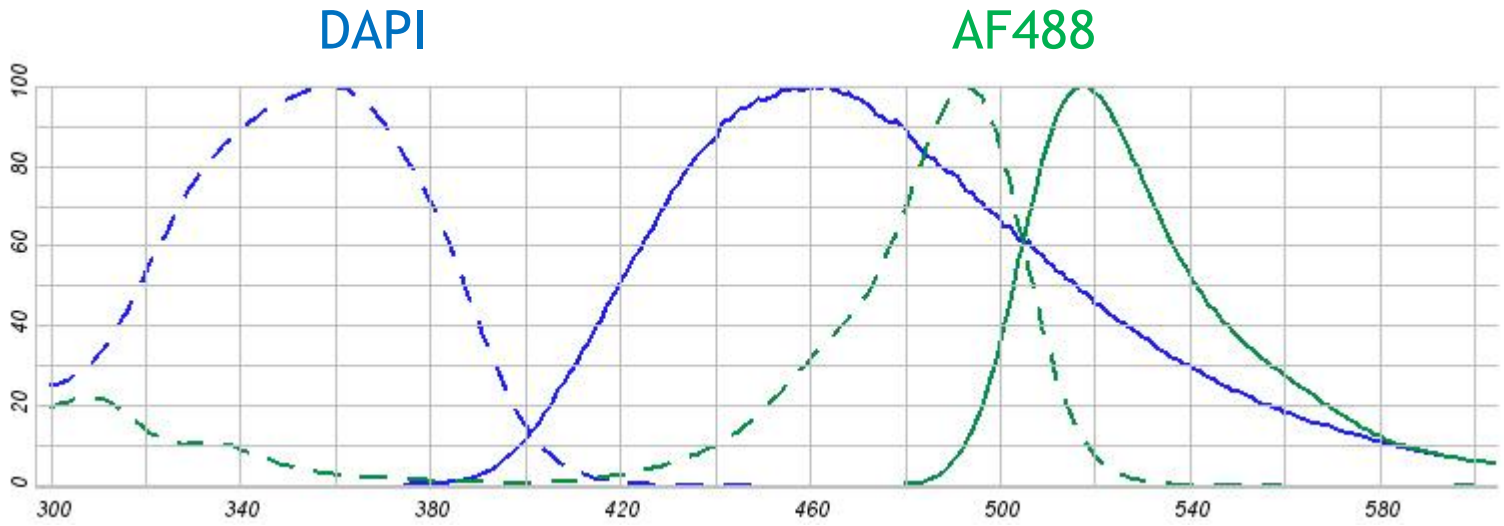
Filter cubes are an easy way of switching excitation filter, dichroic and emission filter







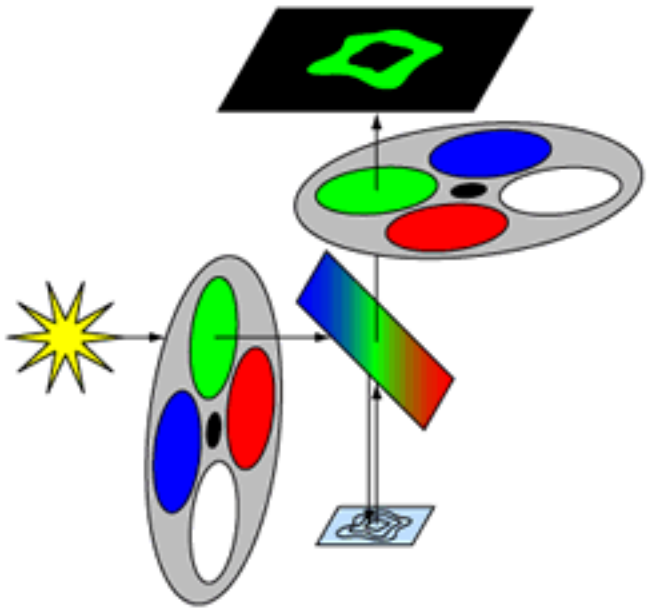
Bleedthrough



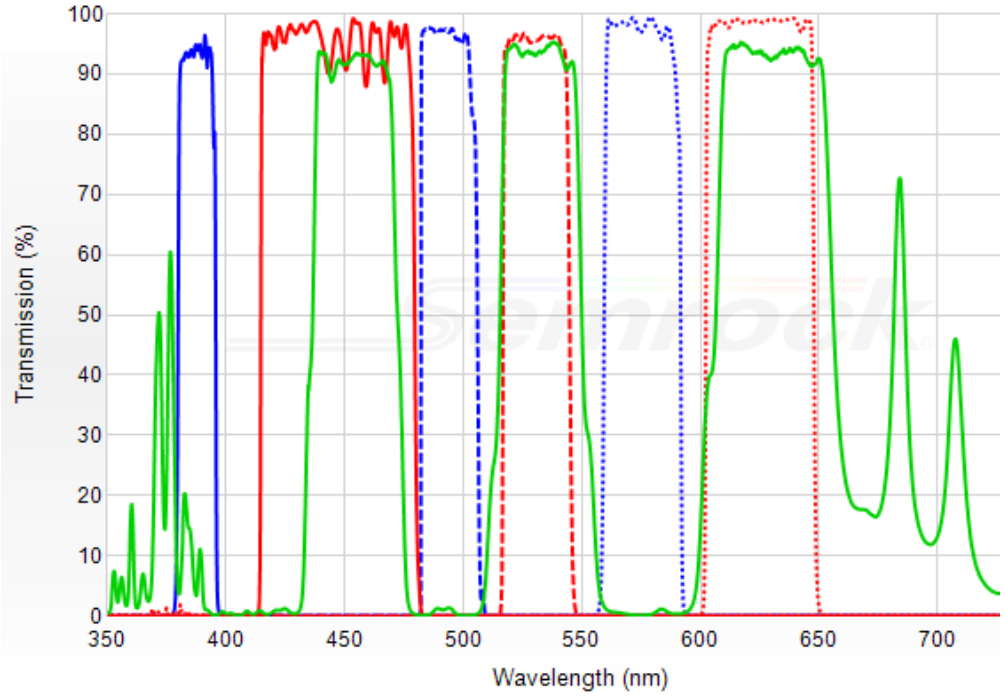
- Down the spectrum (1st law of thermodynamics)
- Worse when intensities are unbalanced

Other multi-channel methods

"Sedat" Configuration
(Multiband dichroic, single-band exciters, & single-band emitters)



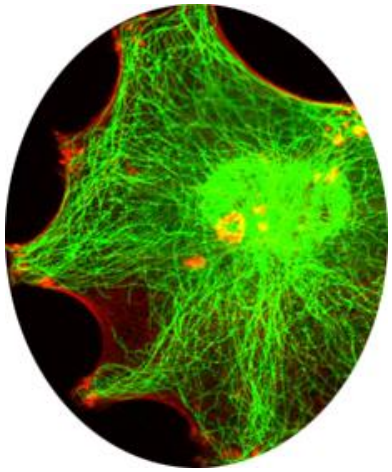
DAPI GFP AF568

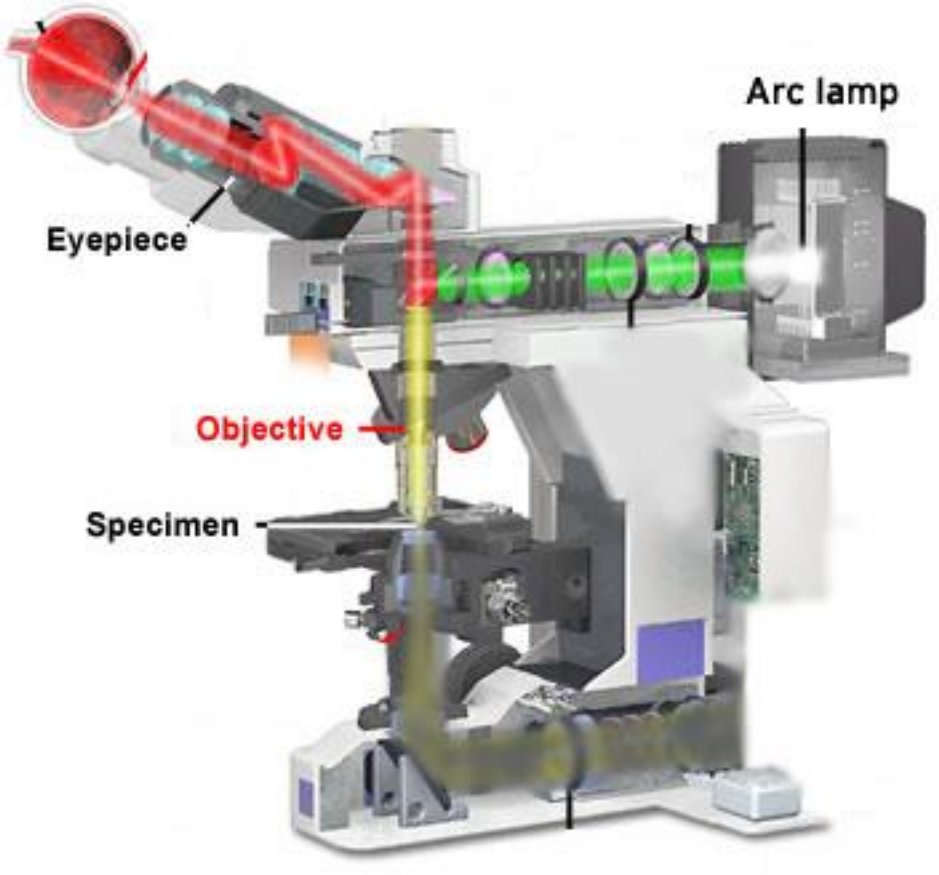


Can be faster
Better registration
Some disadvanatges

Exciters Dichroic Emitters

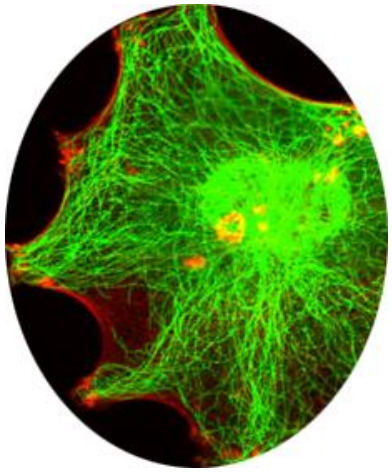
The insides of a microscope: Fluorescence

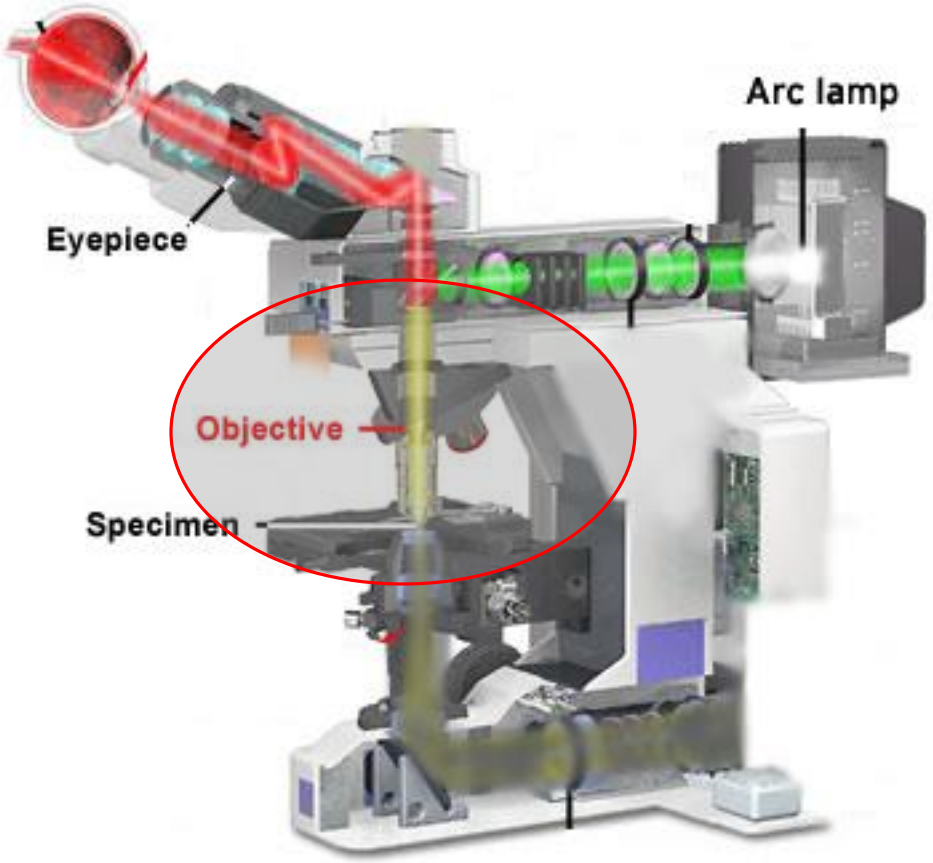




Adapted from http://www.microscopyu.com/articles/formulas/formulasconjugate.html

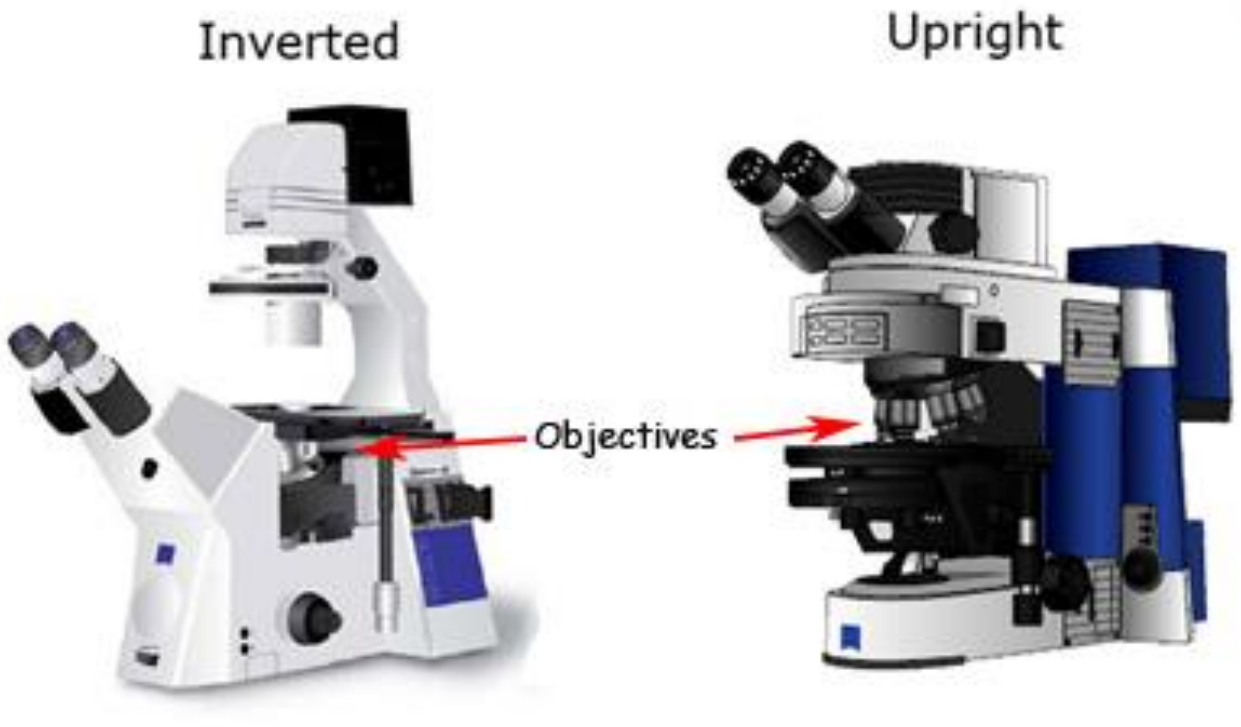
The insides of a microscope: Fluorescence





Adapted from http://www.microscopyu.com/articles/formulas/formulasconjugate.html

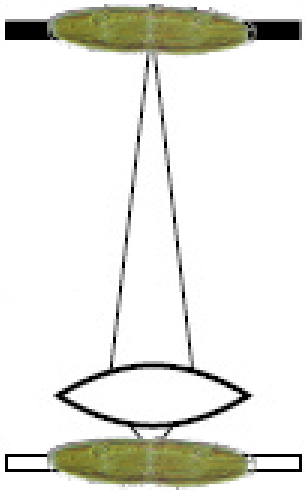
Microscope configurations



The objectives point in different directions but the optical principles are the same

The objective

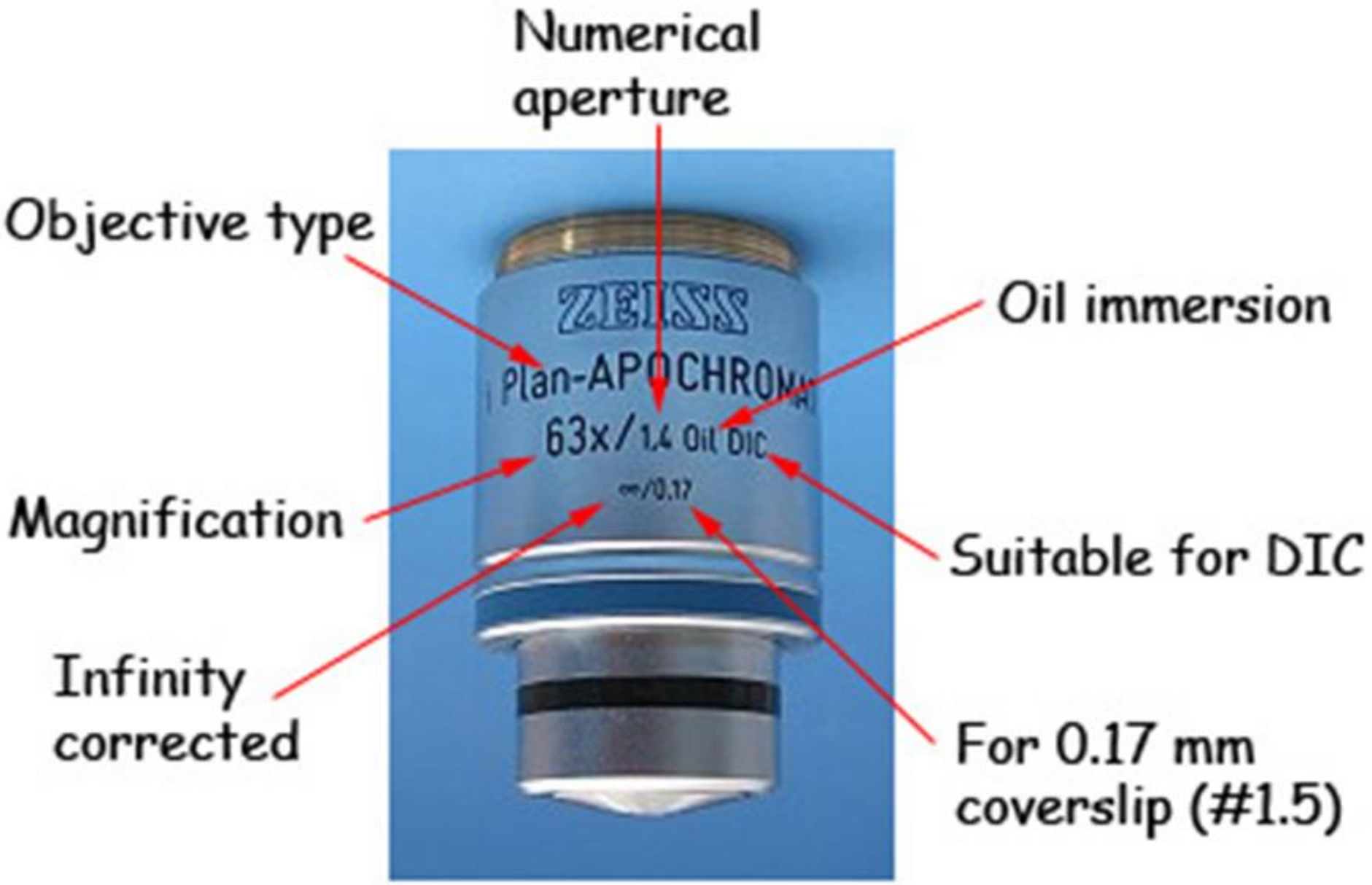
Collects light from the sample and forms an image up the microscope near the eyepieces



Intermediate image

SAMPLE

Objectives are the most important parts

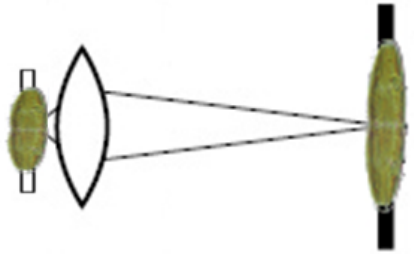

Numerical aperture
Objective type
Oil immersion
ZEISS
Plan-APOCHROMAT
63x/1.4 Oil DIC
∞/0.17
Magnification
Suitable for DIC
Infinity corrected
For 0.17 mm coverslip (#1.5)

Three important concepts in microscopy

- Magnification
- Resolution
- Contrast

Magnification

"How many times bigger the image is than the object"



Magnification is not a very useful concept on its own

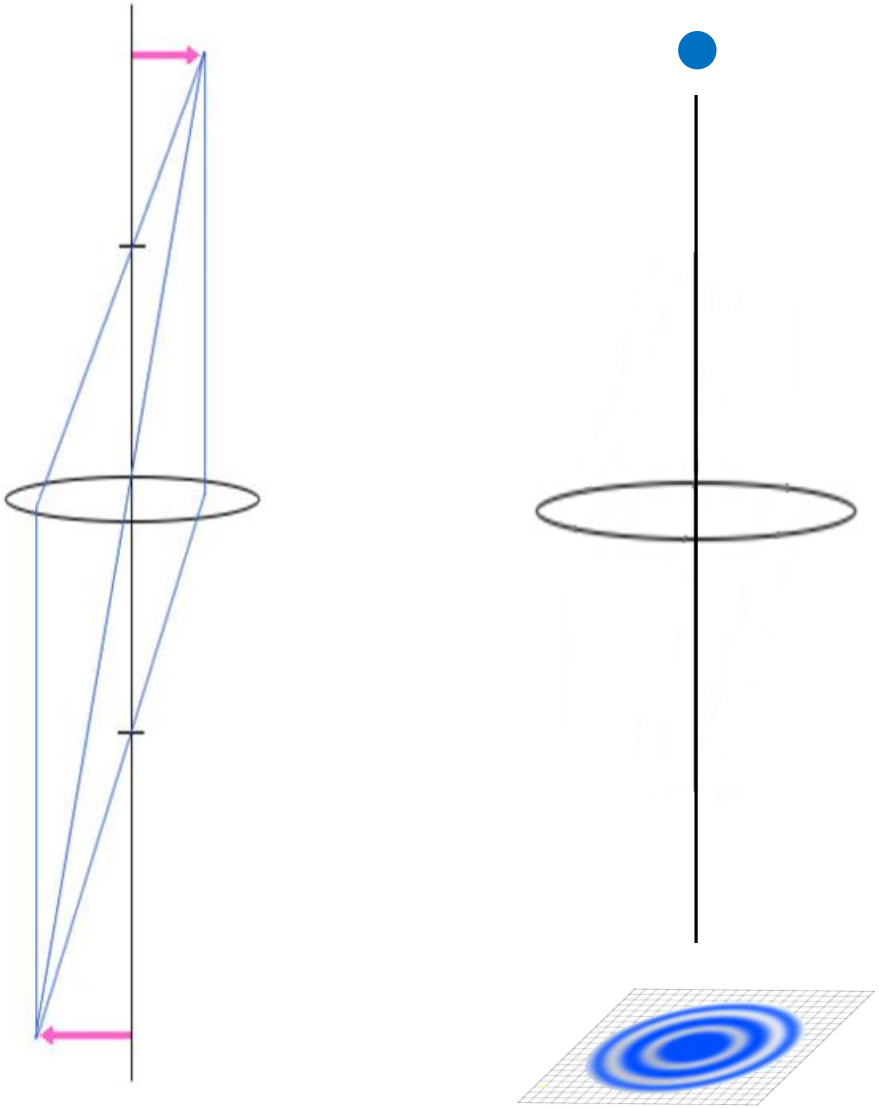
Resolution

"The smallest distance between two objects that can be observed as two objects"

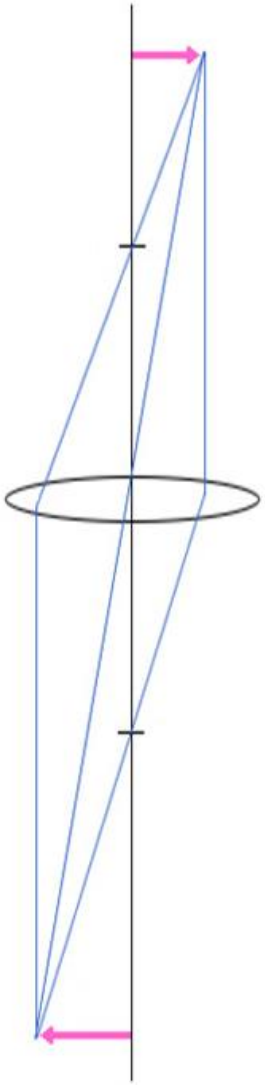
It doesn't mean anything else
(e.g. how nice the image looks)

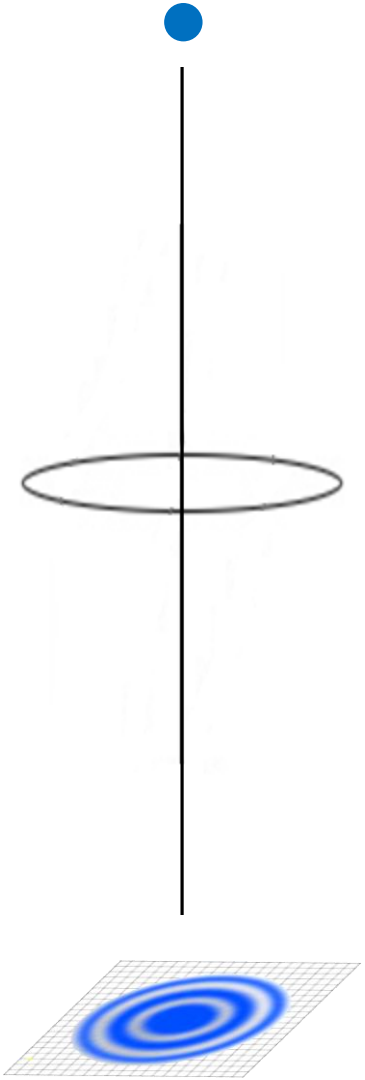
Does the resolution also limit the smallest object we can see?

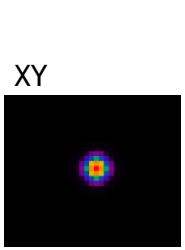
Imaging a tiny fluorescent object

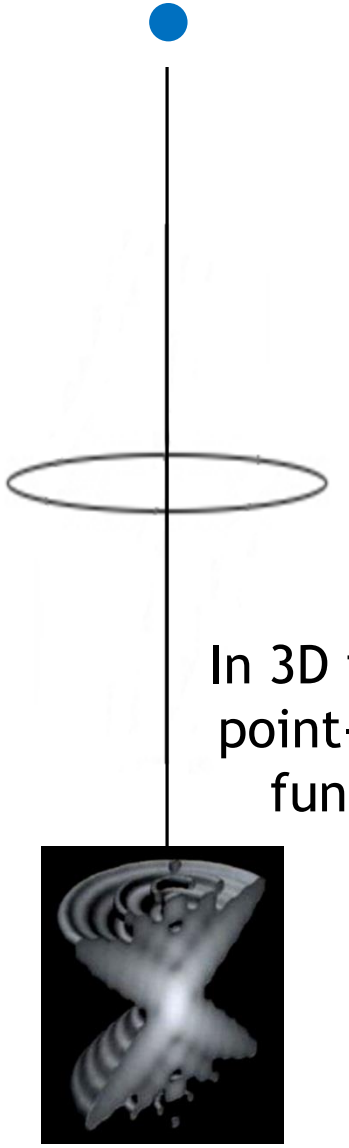


Imaging a tiny fluorescent object

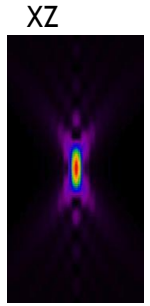








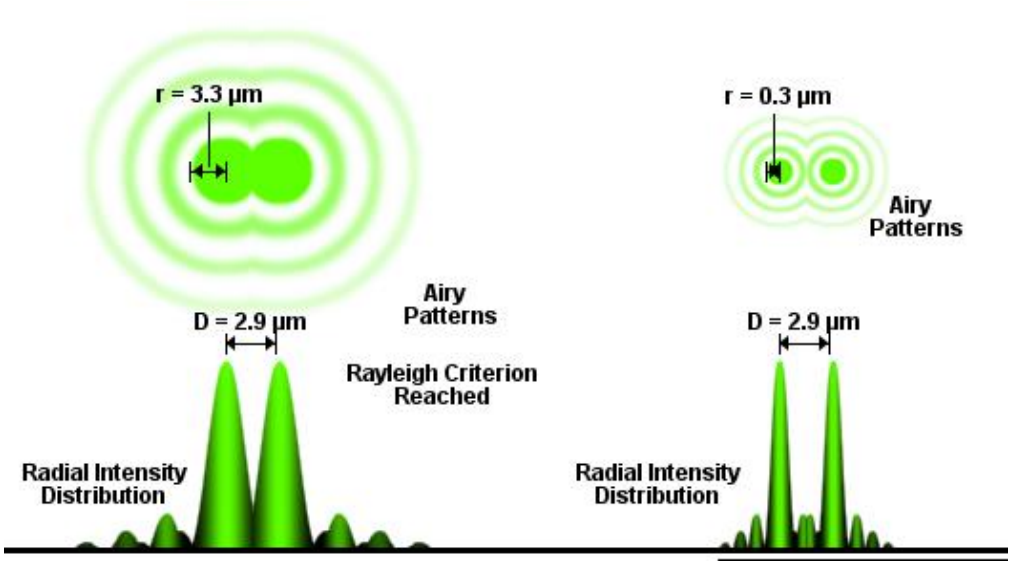
In 3D this is a point-spread function

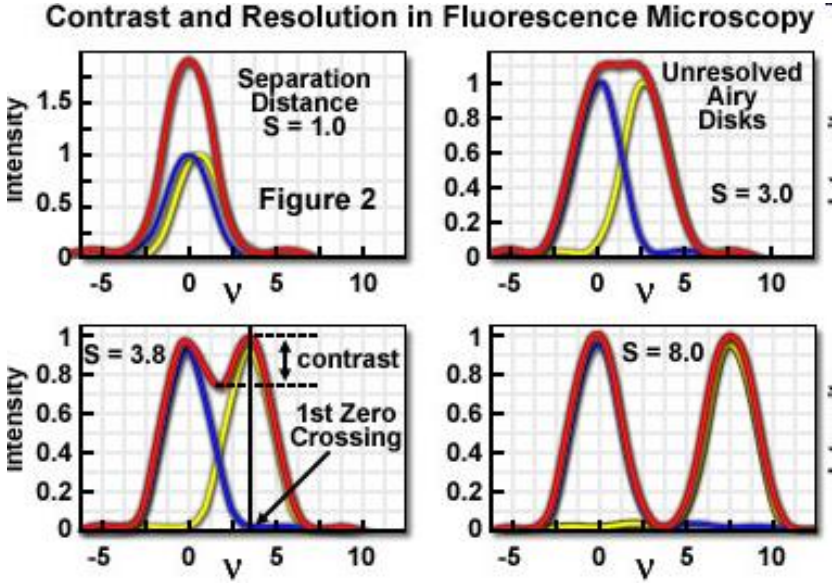


Resolution in fluorescence terms

Object

Airy disk





http://www.olympusmicro.com/primer/java/imageformation/rayleighdisks/index.html

Resolution limit of Light Microscopy

About 200 nm

- Naked eye = 100 μm
- Electron microscopy = <1 nm

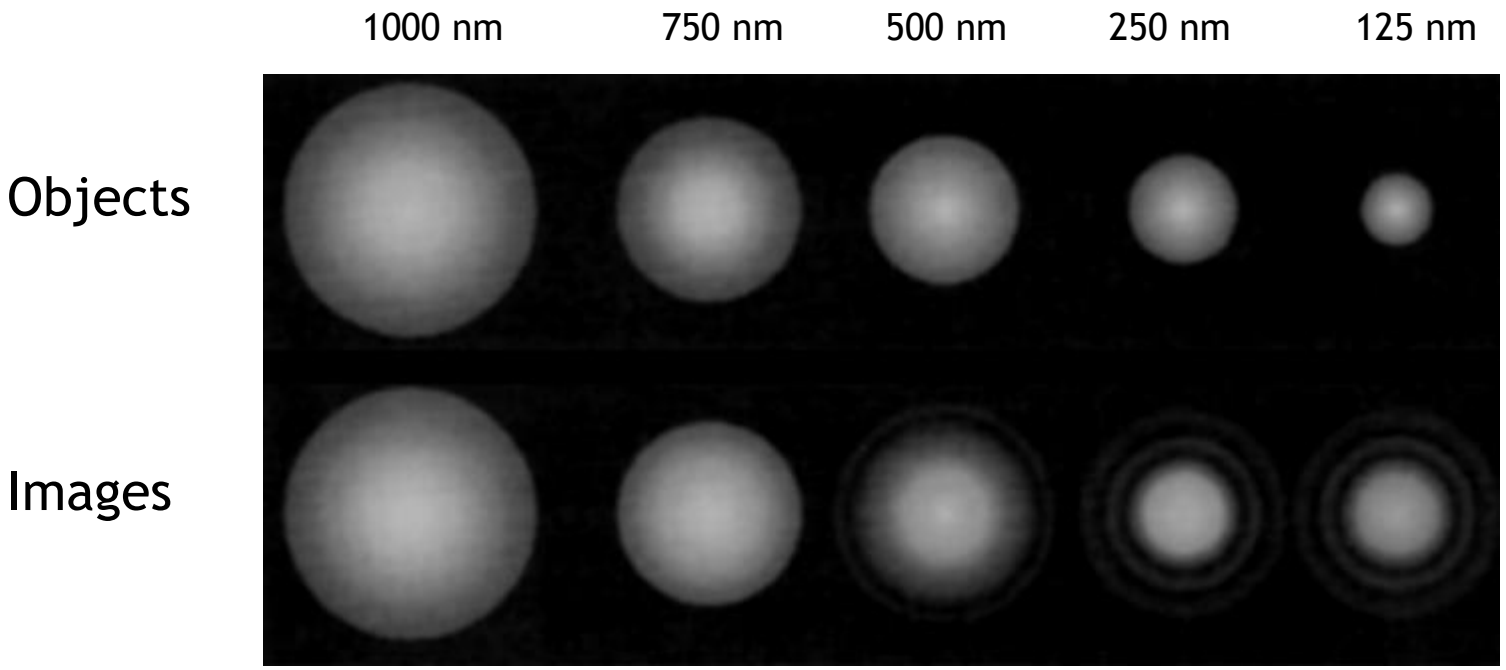
http://learn.genetics.utah.edu/content/begin/cells/scale/

Reference sizes

- Diameter of an adherent fibroblast 100 μm
- Mammalian cell nucleus 10 μm
- Red blood cell 7 μm
- Bacteria 1 μm
- Virus 50 nm
- Ribosome 20 nm
- Globular protein 2 nm

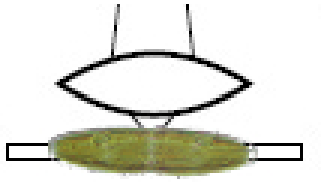
Resolution in fluorescence terms

Imaging green fluorescent beads of different sizes



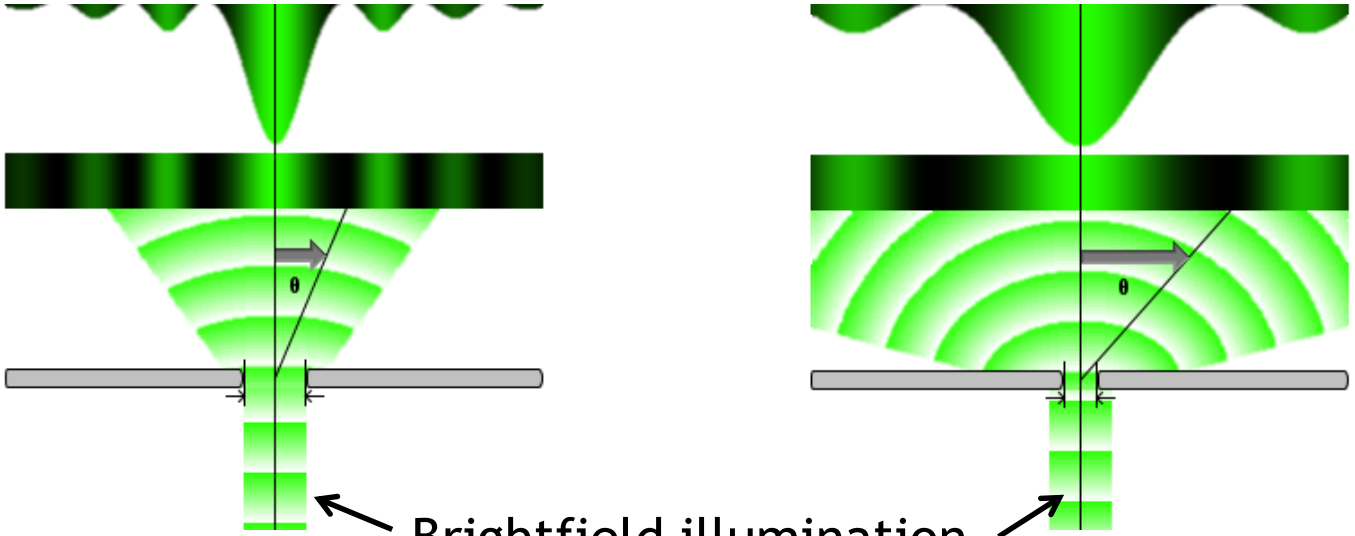
Resolution in transmitted light terms

With brightfield TL you don't see things below the resolution



Ok

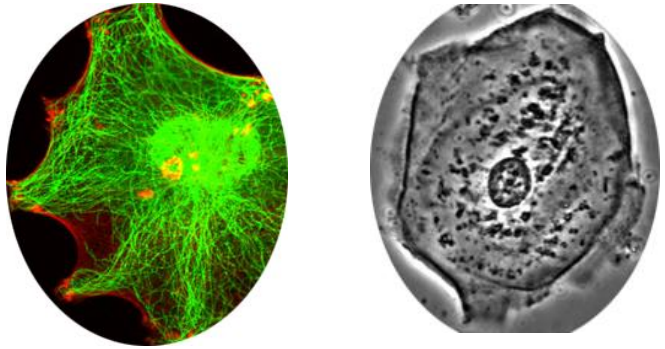
Small objects diffract light at a greater angle, not captured by the lens



Contrast

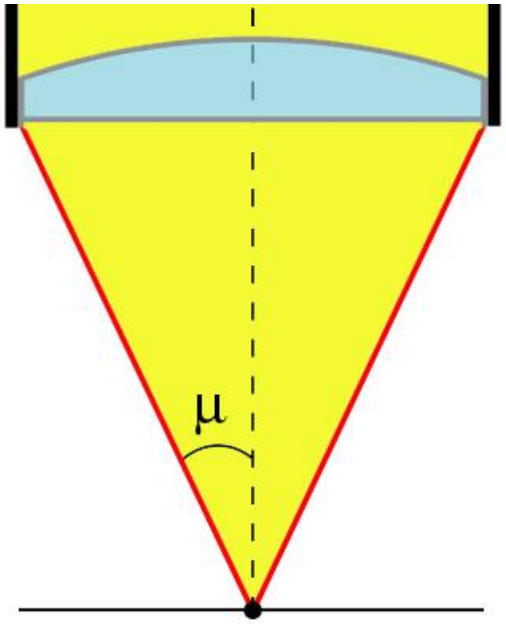
Contrast is needed in the image to be able to resolve anything

$$\text{Contrast} = \frac{\Delta I}{\bar{I}}$$



Signal to noise is a related concept

Numerical Aperture



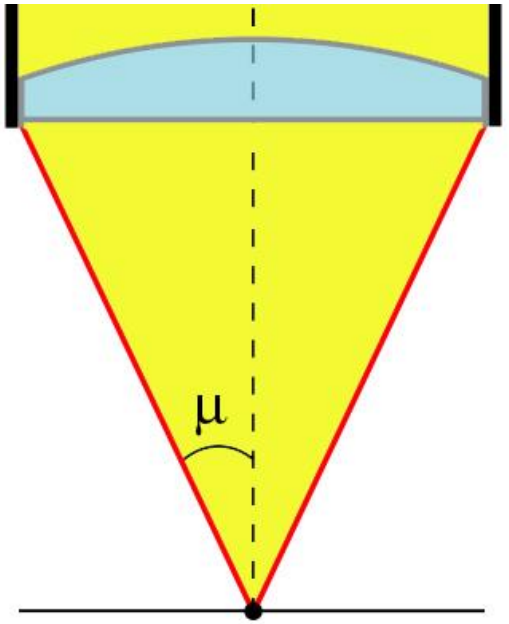
$$NA = n \sin(\mu)$$

Numerical Aperture determines. . .

Brightness = NA^4 / Mag^2 (epi)

= NA^2 / Mag^2 (trans)

Resolution = 0.61 λ / NA



Example set objectives for fluorescence

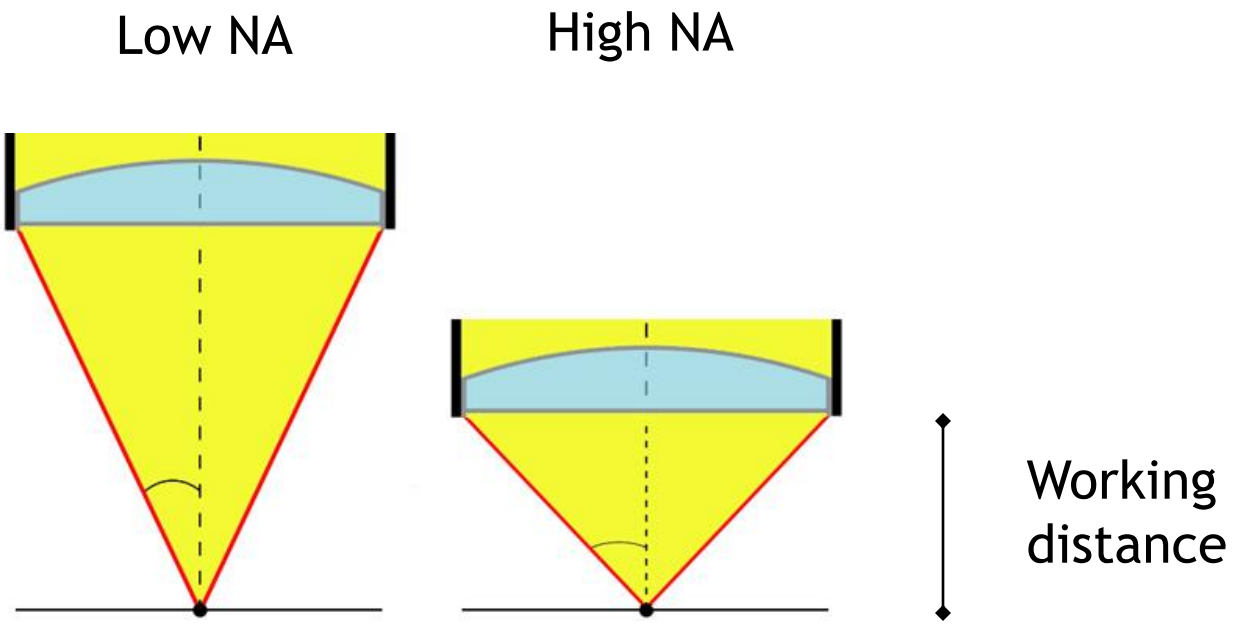
Brightness $= NA^4 / Mag^2$ (epi)

$= NA^2 / Mag^2$ (trans)

Magnification	NA	Immersion	Resolution (nm)	Relative brightness
5x	0.15	DRY	2033	1.0
10x	0.30	DRY	1017	4.0
20x	0.50	DRY	610	7.7
40x	0.75	DRY	407	10
63x	1.40	OIL	218	48
100x	1.40	OIL	218	19

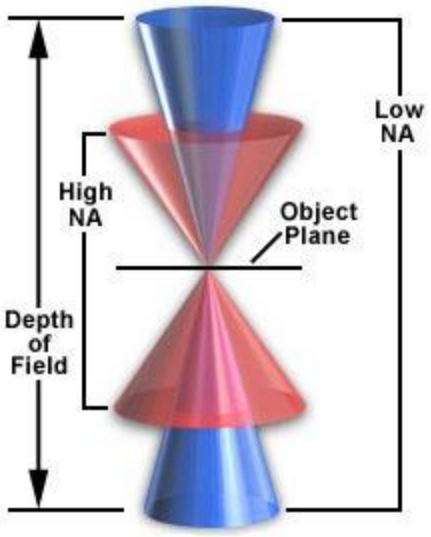
This formula ignores transmission efficiency

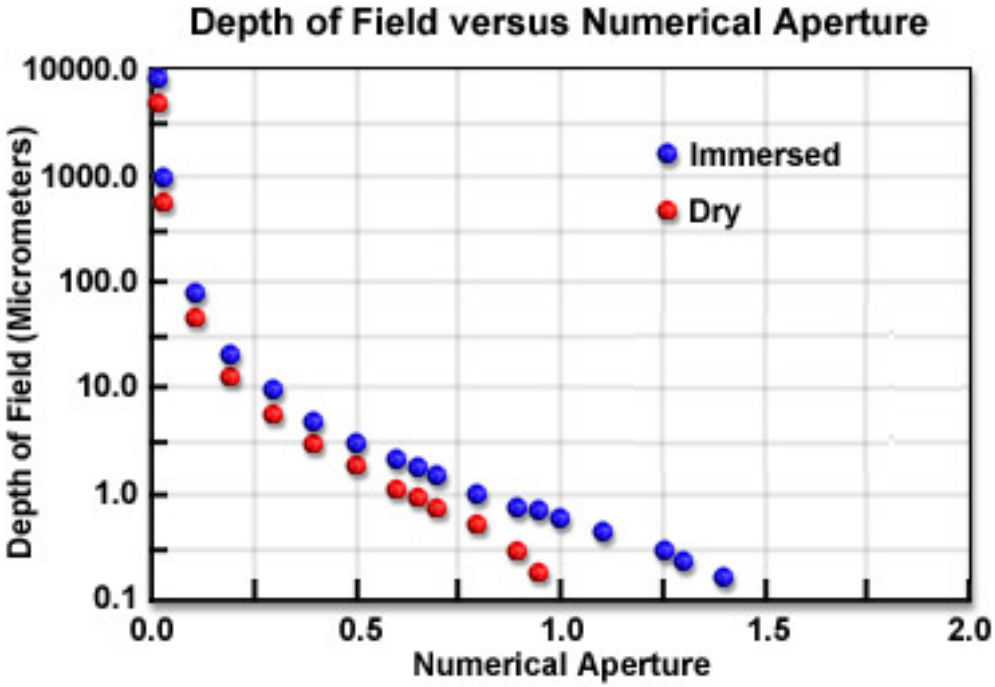
Numerical Aperture and working distance



NA and depth of . . .

- **Depth of field -** Z- range of image that is sharp
- **(Depth of focus -** Range focus can be moved and image still sharp, more a photography term but sometimes used interchangeably)





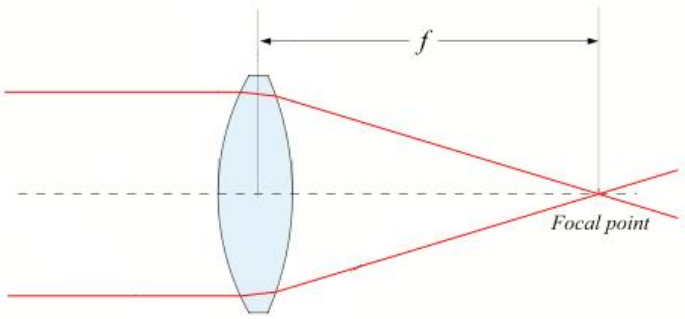
Optical cost comparison

$3.29

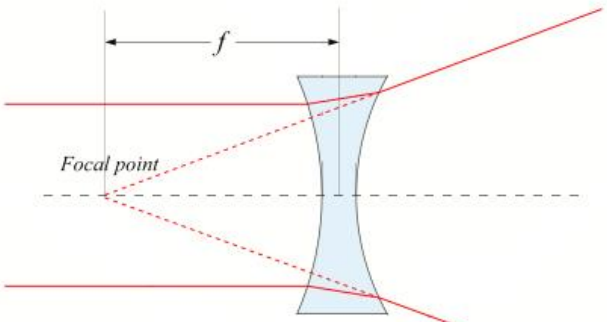
$11,822

Aberrations

Simple lenses

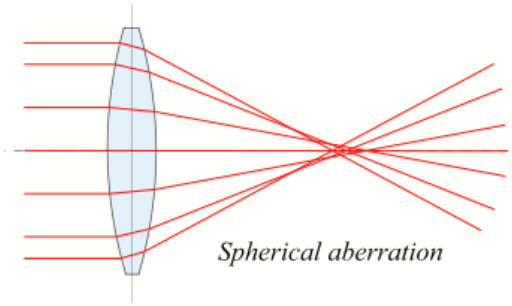


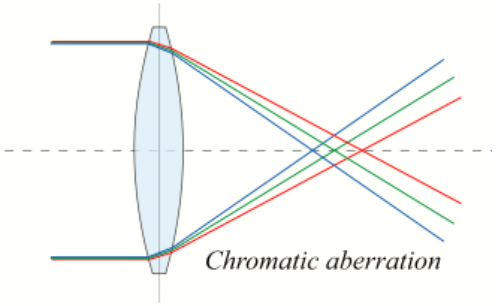
Positive (converging) lens

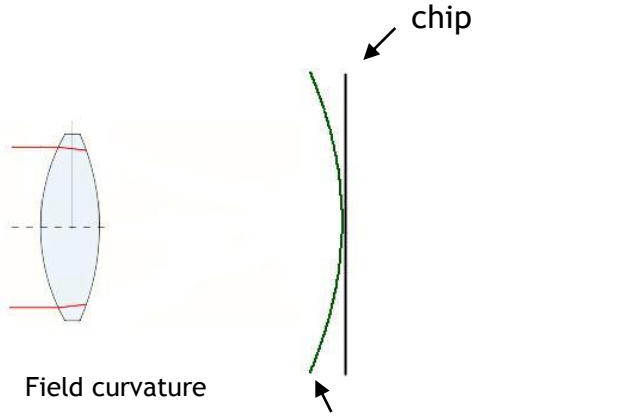


Negative (diverging) lens

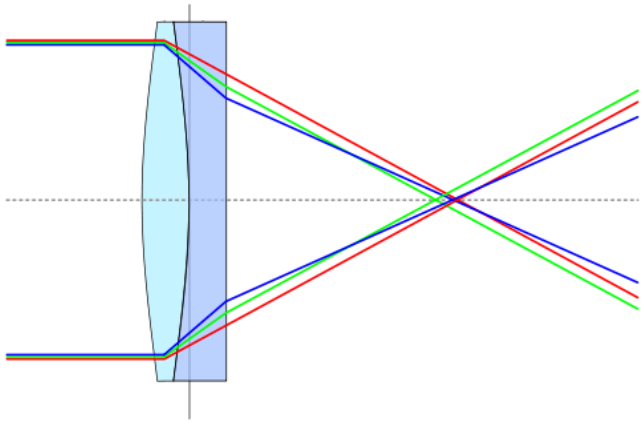
Problems . . .

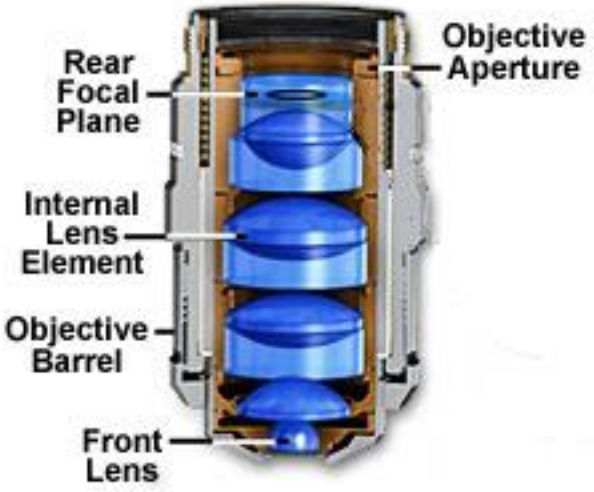






Objectives correct for some aberrations



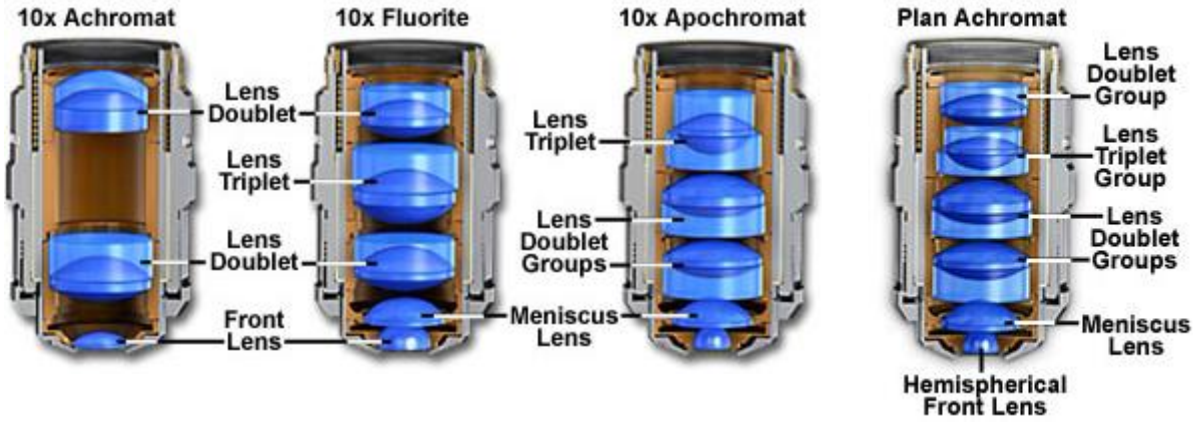


Good optics are relatively free of aberrations.

Microscopes are expensive because high magnification requires the best optics.

Many types of objective

Achromats –	Limited color correction	$
Fluorite -	Good all round objectives, good transmission, NA up to 1.3	
Apochromats –	Highly color corrected	
Plan Apochromats –	Additional correction for field curvature	$$$



http://www.olympusmicro.com/primer/anatomy/objectives.html

Many types of objective immersion

Dry

Oil

Water

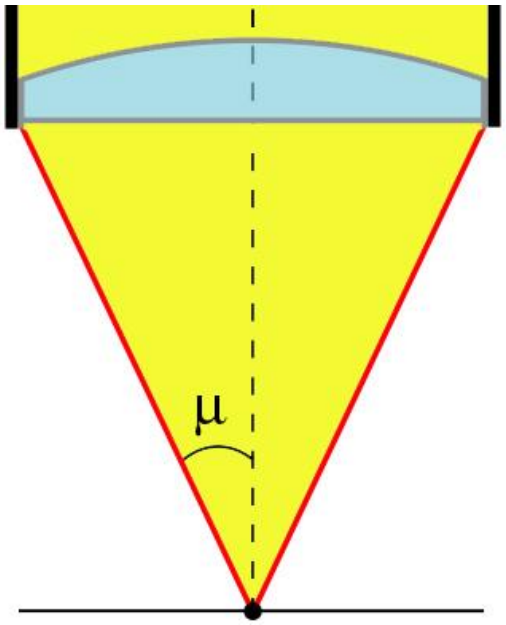
Dipping

Silicon
n=1.4

Glycerc

Multi

Why use oil or water objectives?



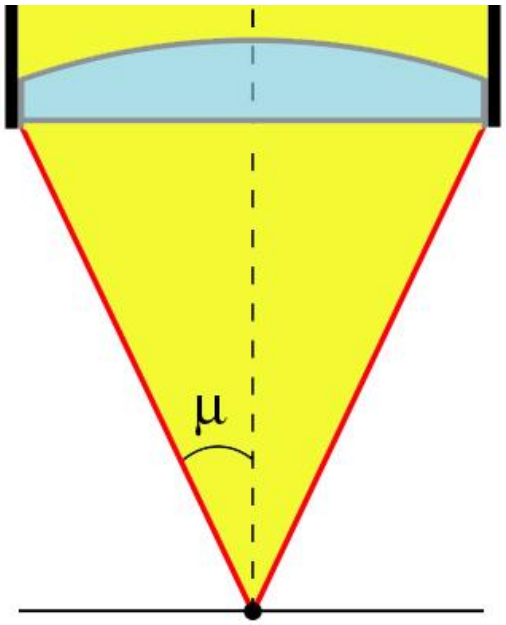
Higher NA = more resolution and brightness

$$NA = n \sin(\mu)$$

(sin (90) = 1)

n = refractive index	Effective limit of NA
Air = 1.0003	~0.95
Water = 1.33	~1.2
Glass = 1.515	~1.4

Why use oil or water objectives?



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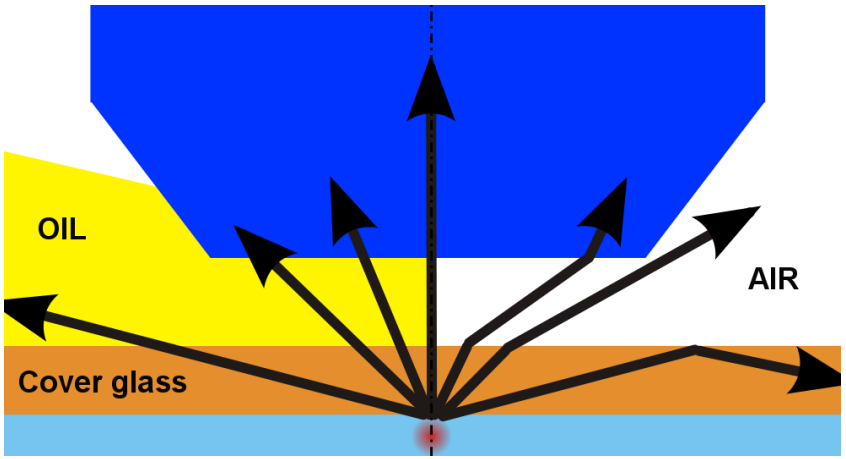
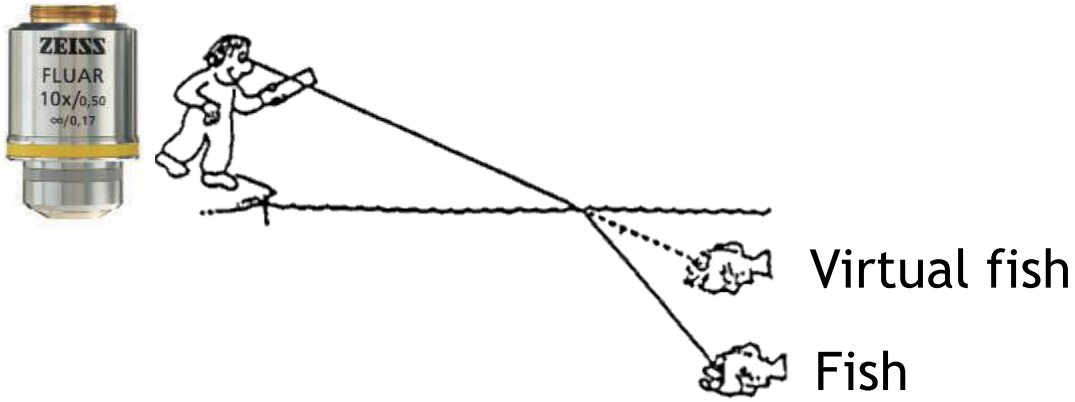
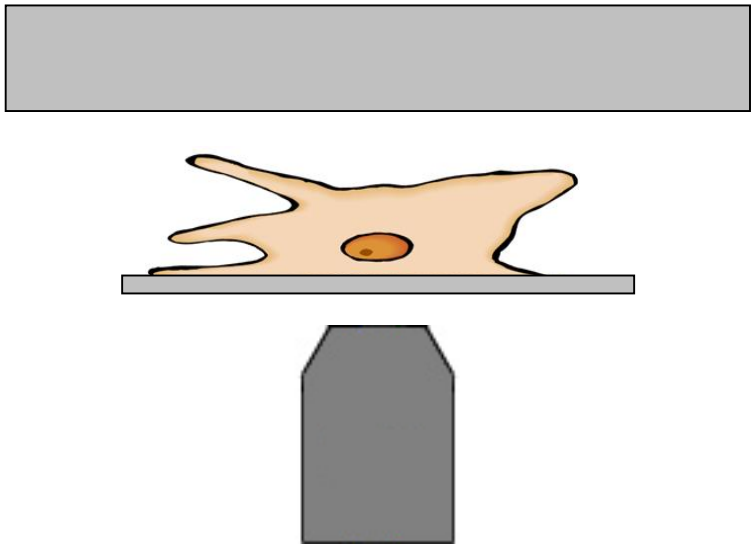


Why use oil or water objectives?



Matching the refractive index of the sample and the objective immersion helps keep aberrations and degradation to a minimum

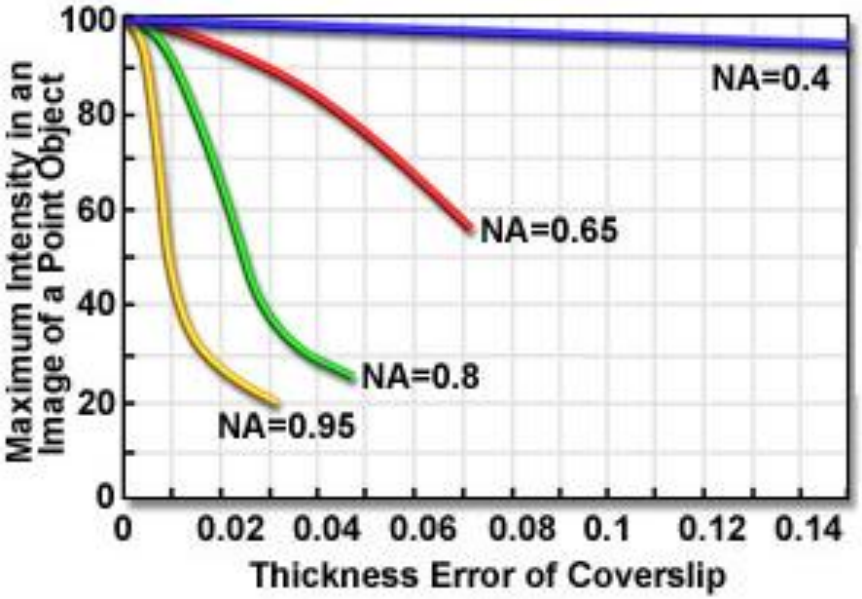
Coverslips



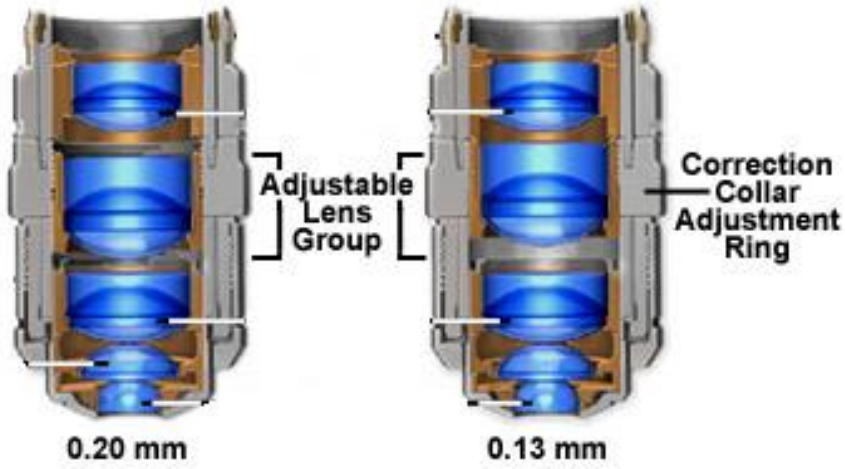
Number	Ideal thickness	Range
#0	100 µm	80-130 µm
#1	150 µm	130-170 µm
#1.5	**170 µm**	**160-190 µm**
#2.0	220 µm	190-250 µm

http://microscopy.duke.edu/coverslip.html

Coverslip thickness is very important



Worst for high NA dry objectives

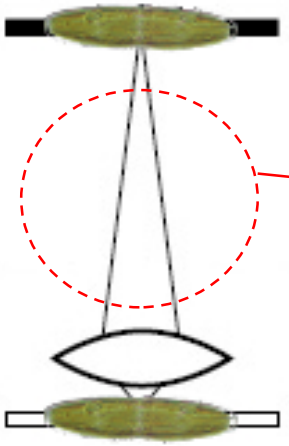


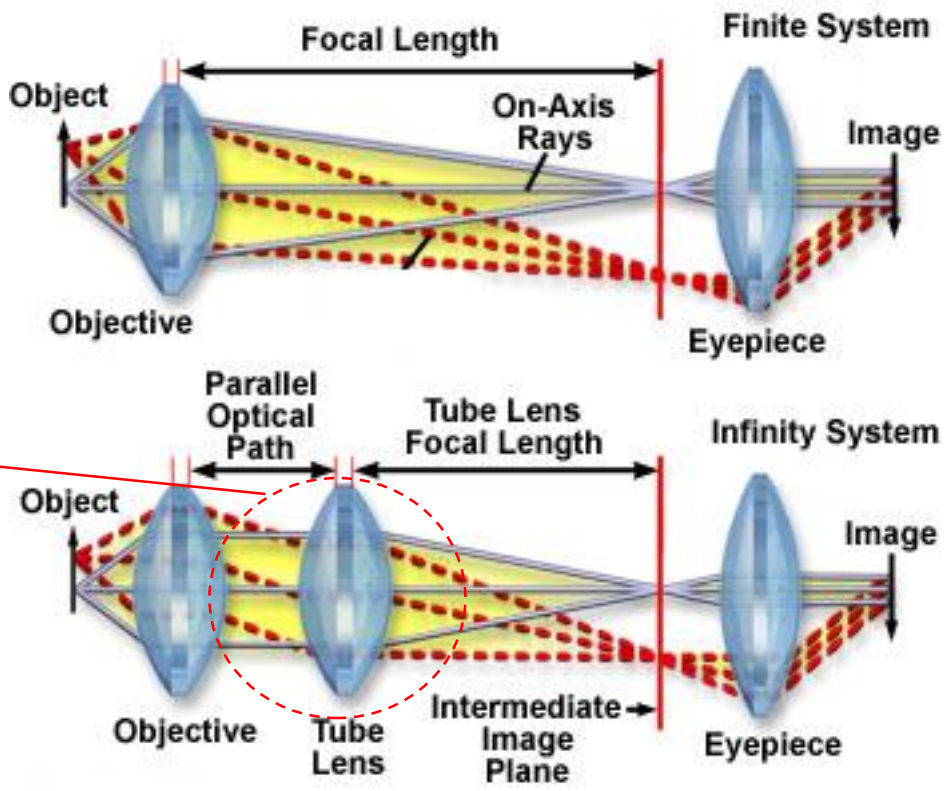
Adjustable correction collar to minimize spherical aberration and light loss

http://www.microscopyu.com/articles/formulas/formulascoverslipcorrection.html

Infinity optics

A missing bit from a few slides ago



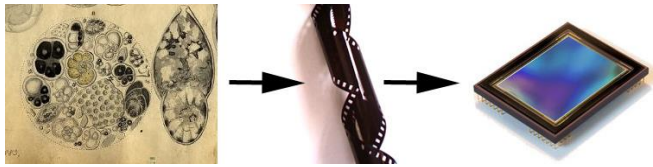


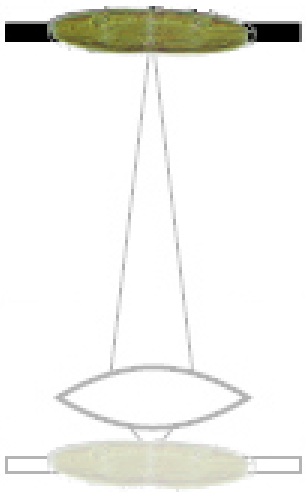
Infinity advantages - Easier to add extra components (eg filters)
Can focus by moving the objective

http://www.olympusmicro.com/primer/anatomy/infinityintro.html

/The objective

Collects light from the sample and forms an image up the microscope near the eyepieces

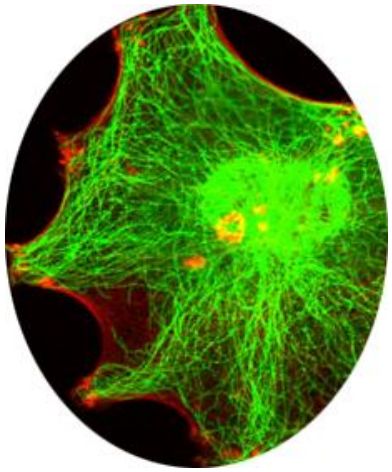


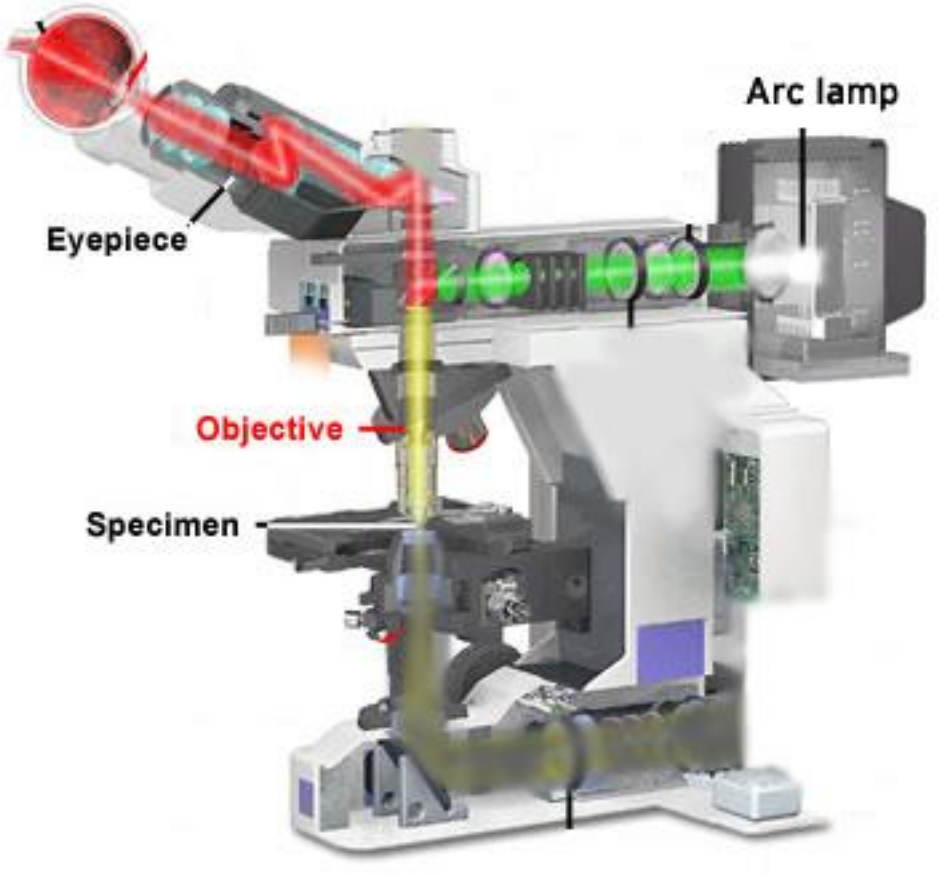


Intermediate image

SAMPLE

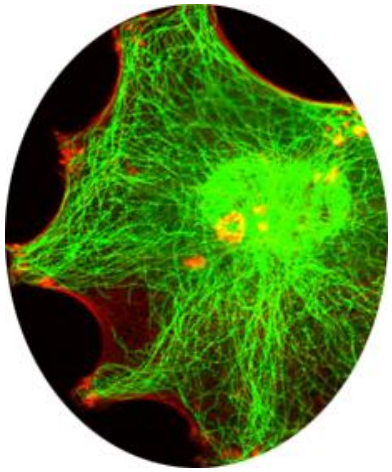
The insides of a microscope: Fluorescence

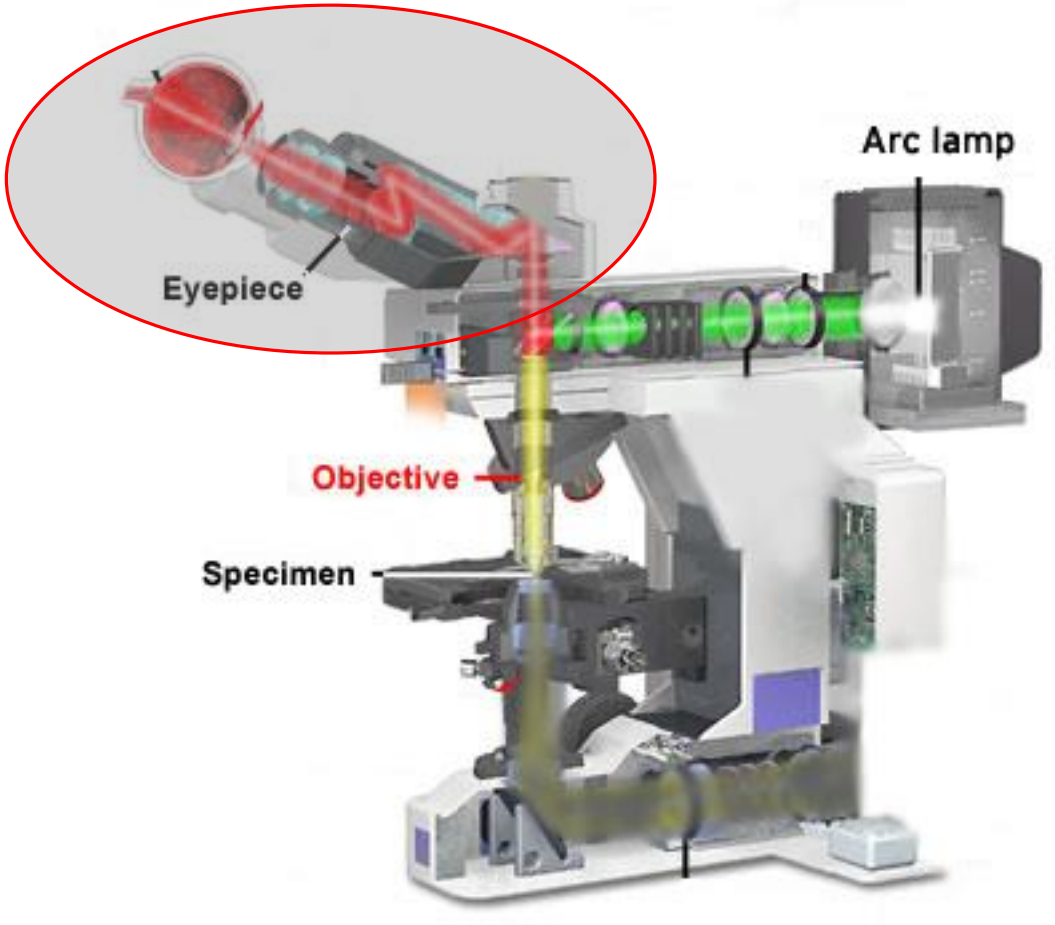




Adapted from http://www.microscopyu.com/articles/formulas/formulasconjugate.html

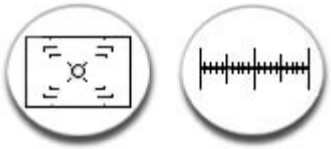
The insides of a microscope: Fluorescence

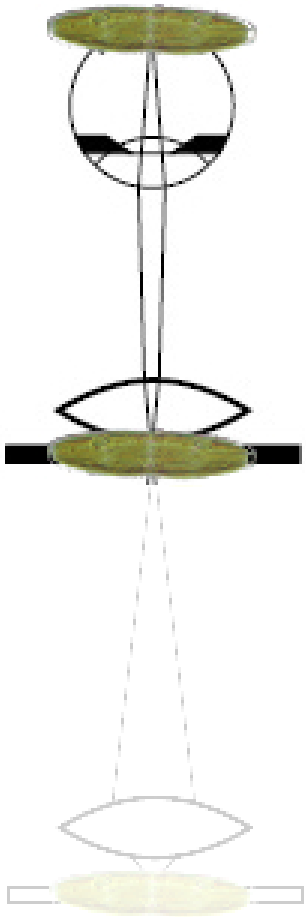




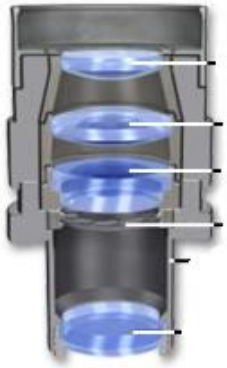
Adapted from http://www.microscopyu.com/articles/formulas/formulasconjugate.html

The eyepiece (s)





Intermediate image

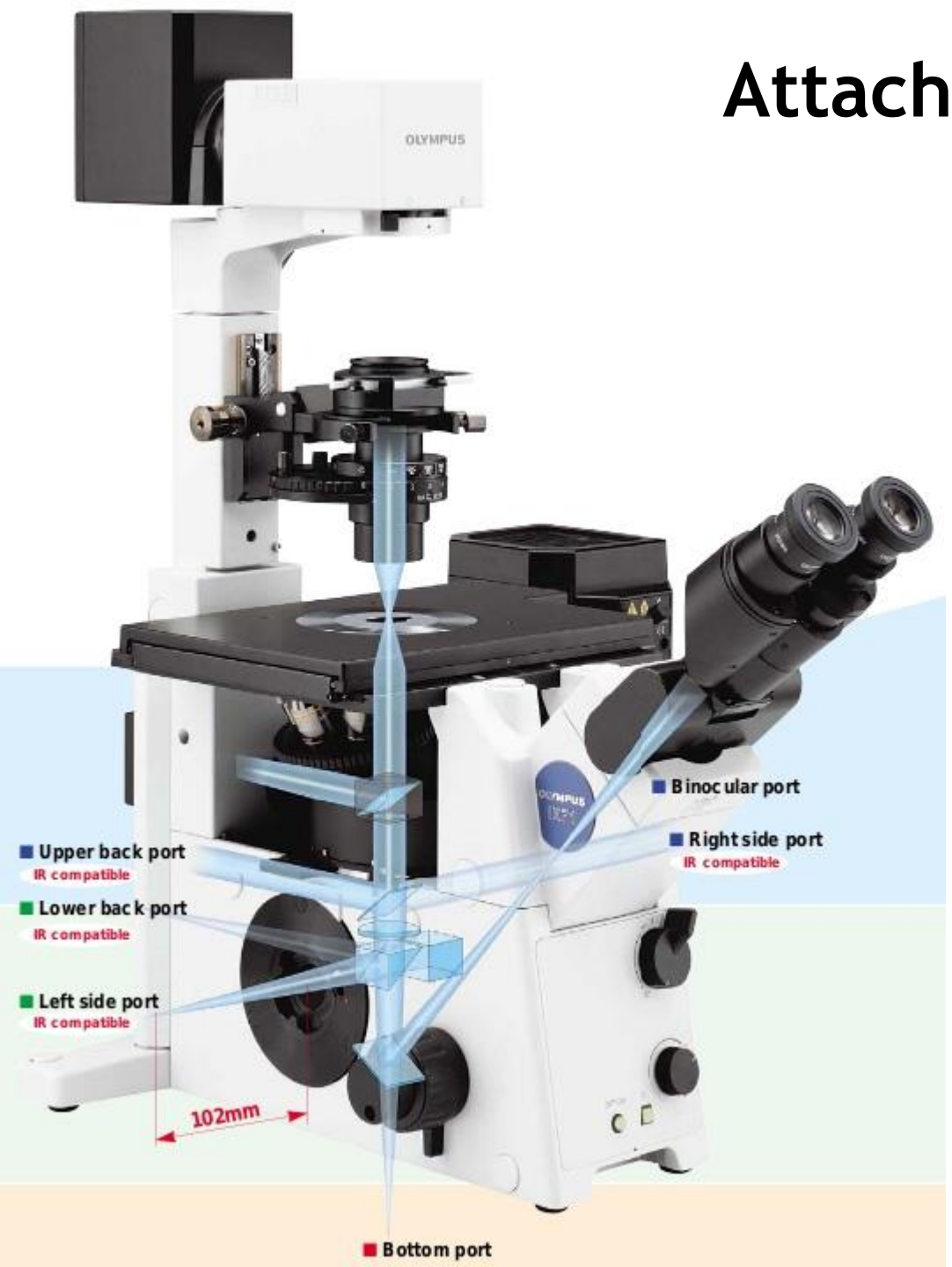


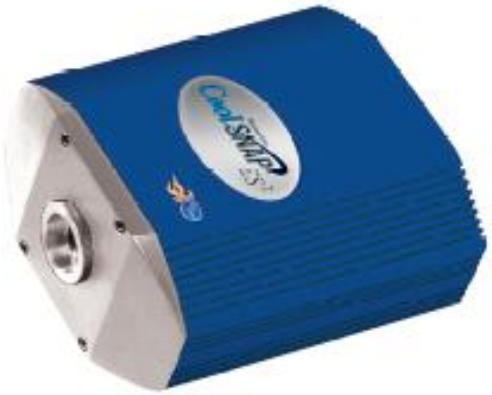
Magnifies the intermediate image and allows us to see the image

SAMPLE

Total magnification = Mobjective x Meyepeice

Attaching a camera to a scope





1.4 Mpixels

~$12 000 from Photometrics

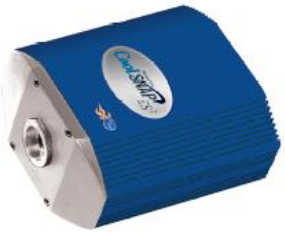
12 Mpixels

~$80 from BestBuy

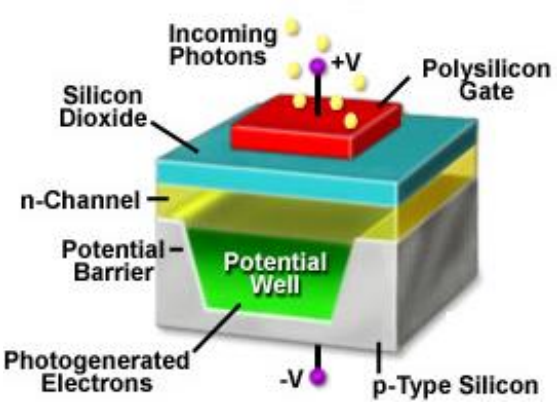
The details required for microscopy cameras

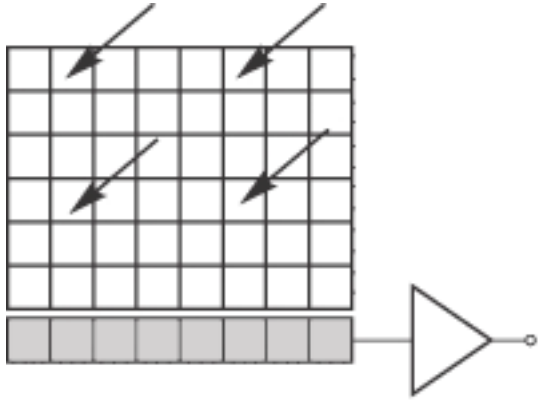
- **Quantum Efficiency** – Chance of a photon being recorded
- **Dynamic range** – Full well capacity (how many electrons each pixel holds)/noise
- **Cooling** – Low light levels, noise is significant and cooling helps.
- **Noise** – Cooling reduces dark noise a lot. Electronics optimized for low read noise.
- **Frame rate** – 100 fps can be useful sometimes.
- **Grade** – No (or few dead pixels)

CCDs, charge-coupled devices



Photons → Charge → Pixels

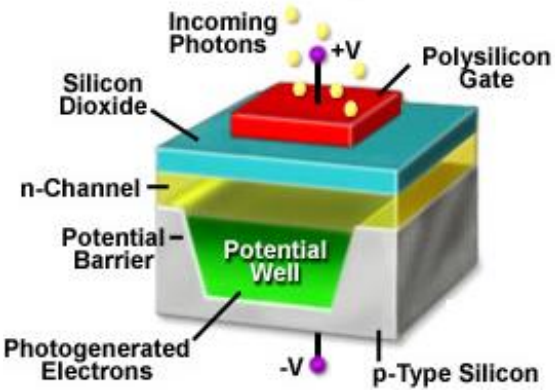


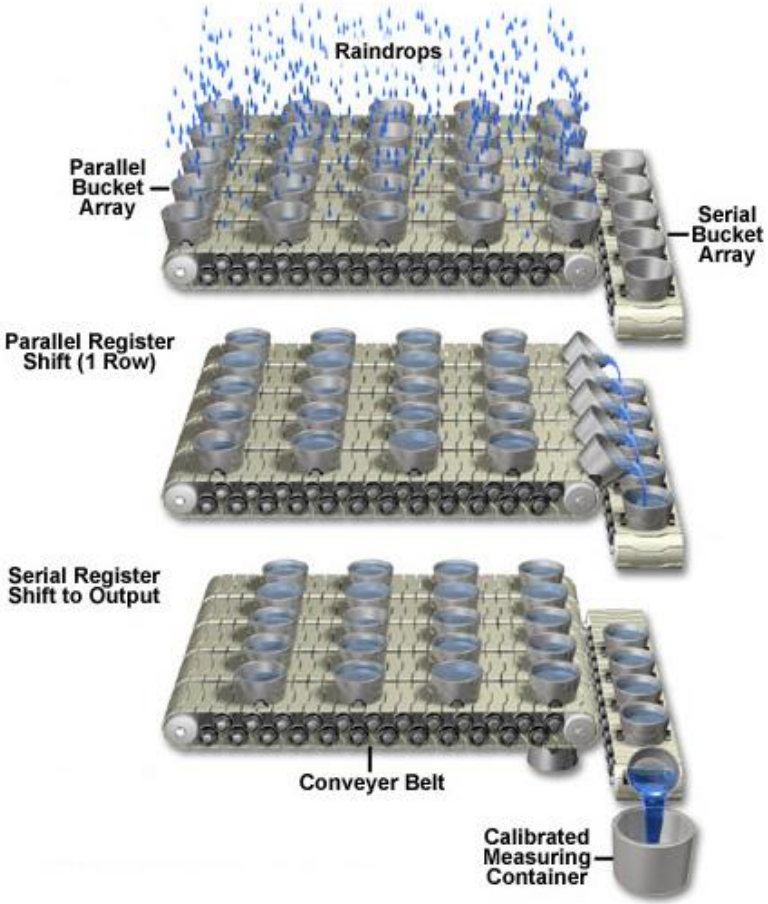


http://www.microscopyu.com/articles/digitalimaging/ccdintro.html

CCD sensors

Photons → Charge → Pixels

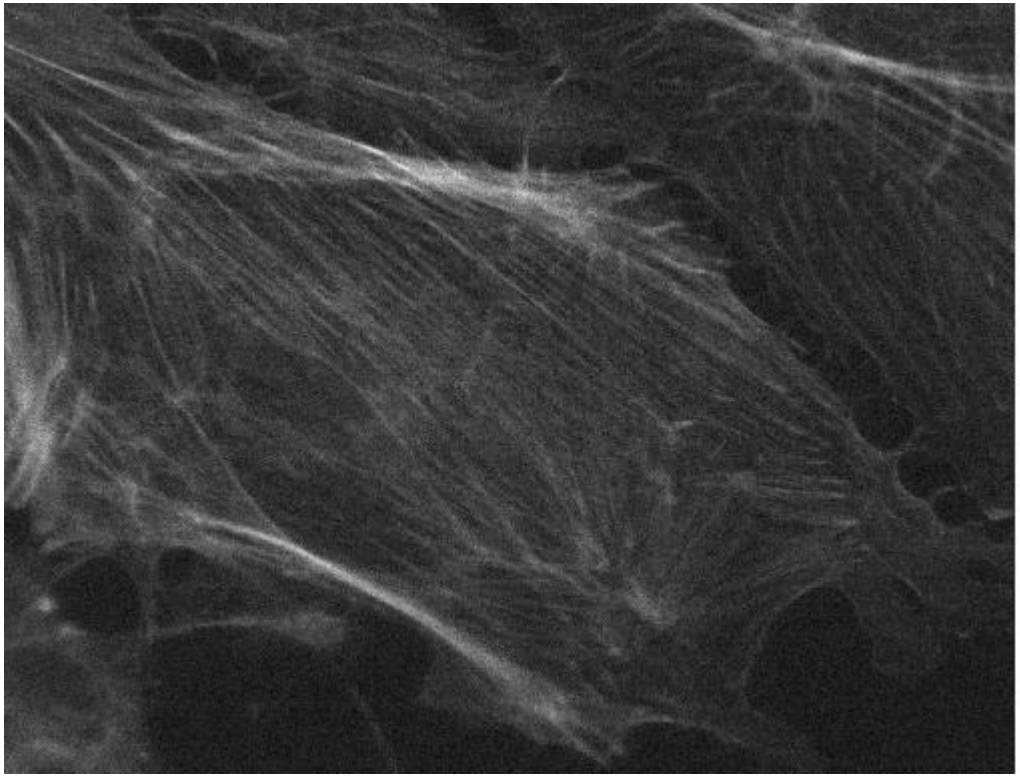




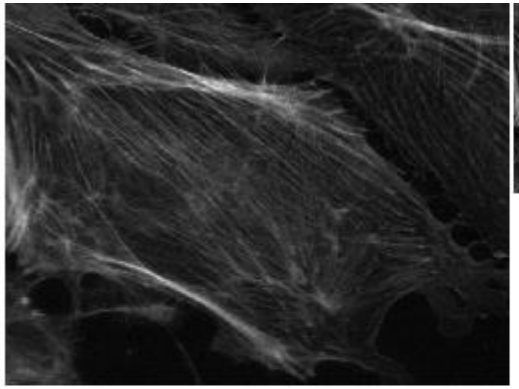
http://www.microscopyu.com/articles/digitalimaging/ccdintro.html

Binning

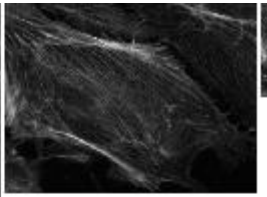
Combining pixels on the CCD chip to make superpixels



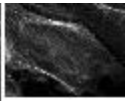
Bin 1: 1344x1024



Bin 2: 672x512

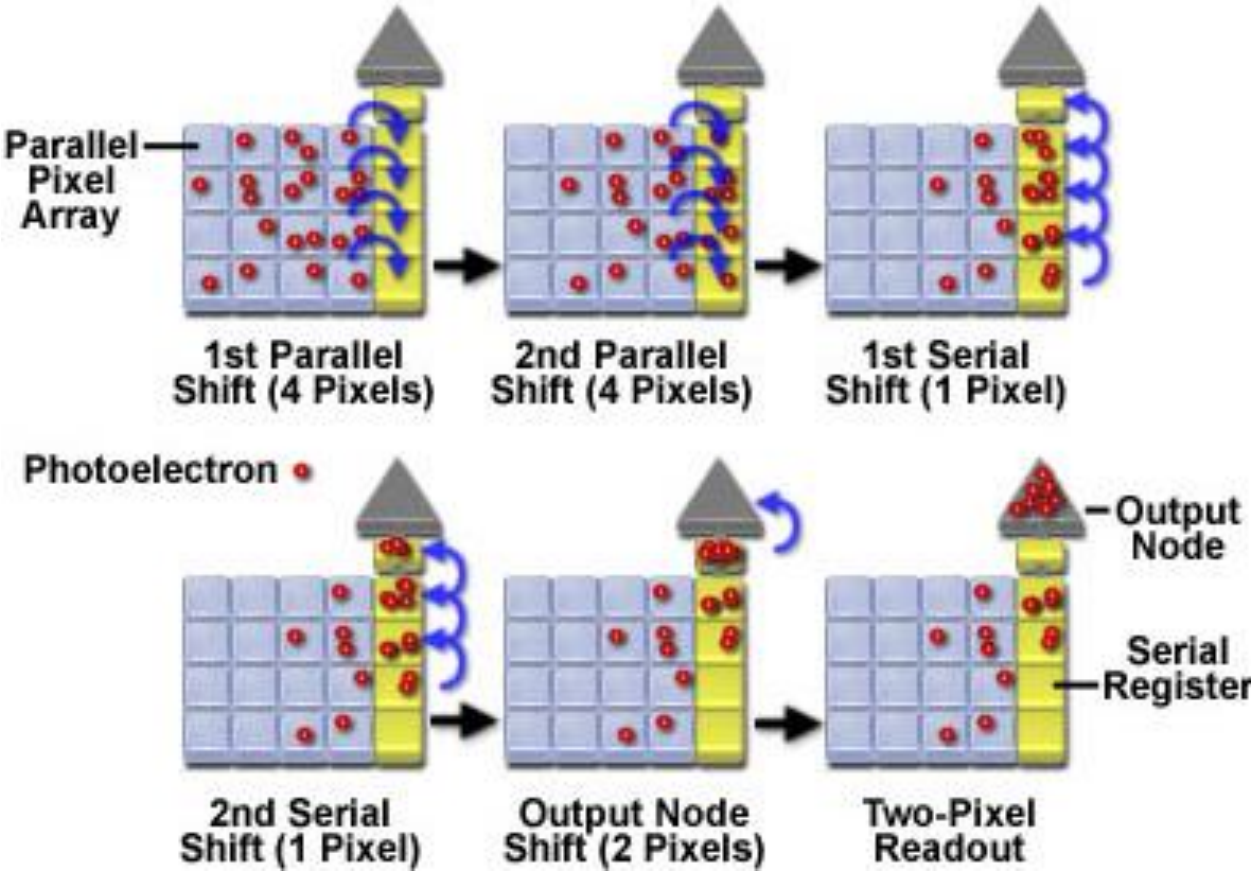


Bin 4:



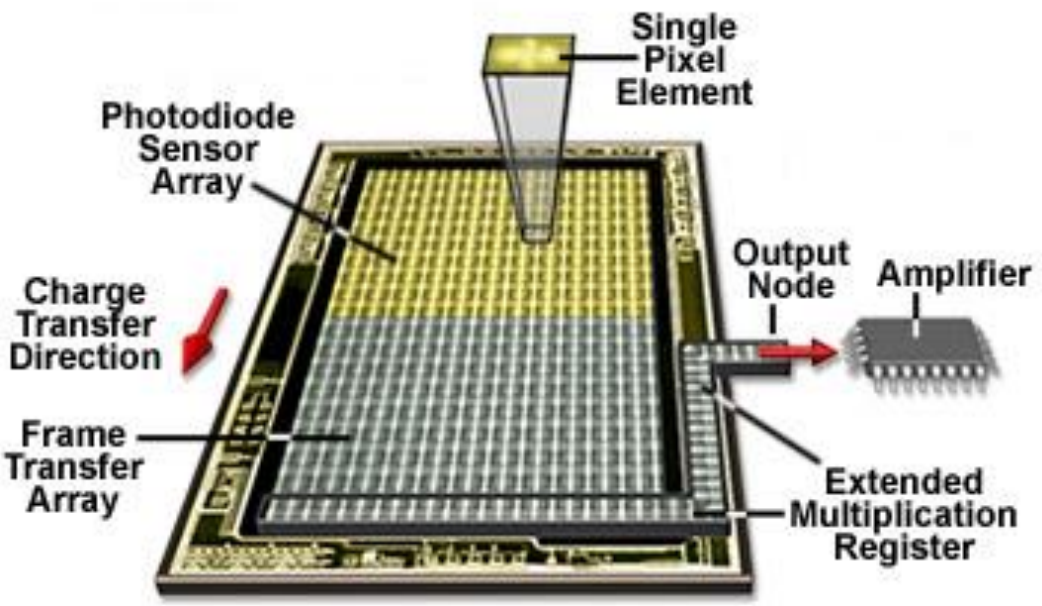
Bin 8:

Binning



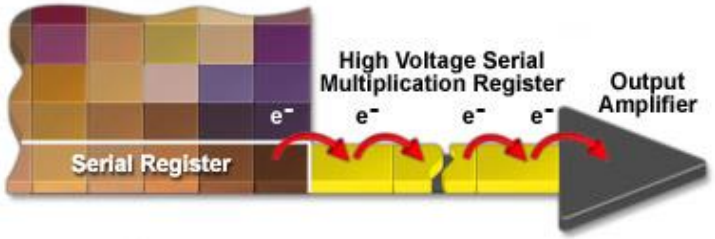
More photons collected in each output pixel

Electron Multiplying-CCD cameras



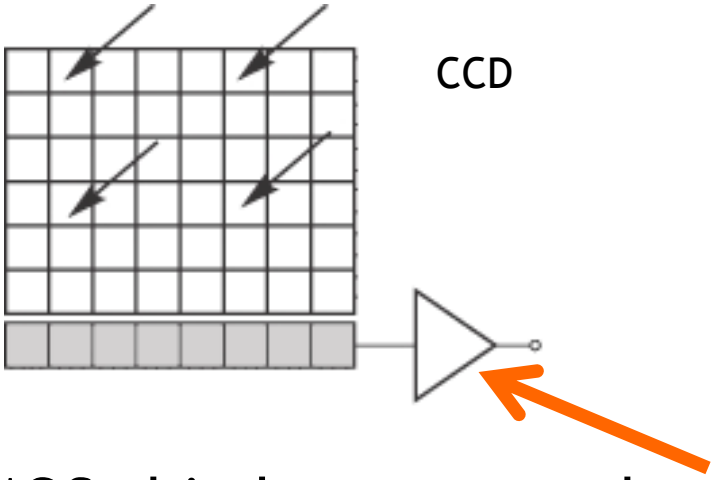
Also tend to be built for highest QE – **peak ~95%** (cf ~70% normal)

Good for very fast, dim or photosensitive samples



http://learn.hamamatsu.com/articles/emccds.html

CMOS



Each column in a CMOS chip has own readout structure

Possible to read the image faster
(and use a larger chip = larger fov or more resolution)

2048 x 2048 pixels at 100 fps

~50 GB/min!

Quiz

Which one of these statements is false?

1. Magnification and resolution are related but not the same.
2. You might be able to image fluorescent objects smaller than the resolution.
3. You can always increase the resolution by magnifying the image more.
4. You can fail to benefit from the full extent of resolution by not magnifying enough.
5. Resolution limits the smallest object you can see in brightfield imaging.
6. Very low contrast or signal to noise ratio can limit resolution.

Spectraviewer Homework

http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html

Use a spectra viewer to choose which of these filter sets you would prefer to use for a sample labelled with AF488 and AF568

Set A: Cube1 - x480/25 dichroic 495 m520/40 Cube2 - x570/20 dichroic 585 mLP590
Set B: Cube1 - x500/25 dichroic 515 m540/30 Cube2 - x595/10 dichroic 610 mLP615