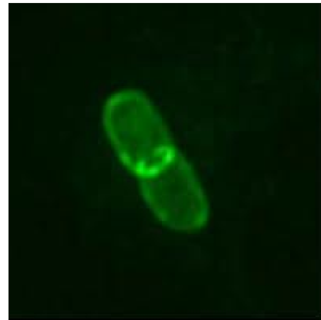
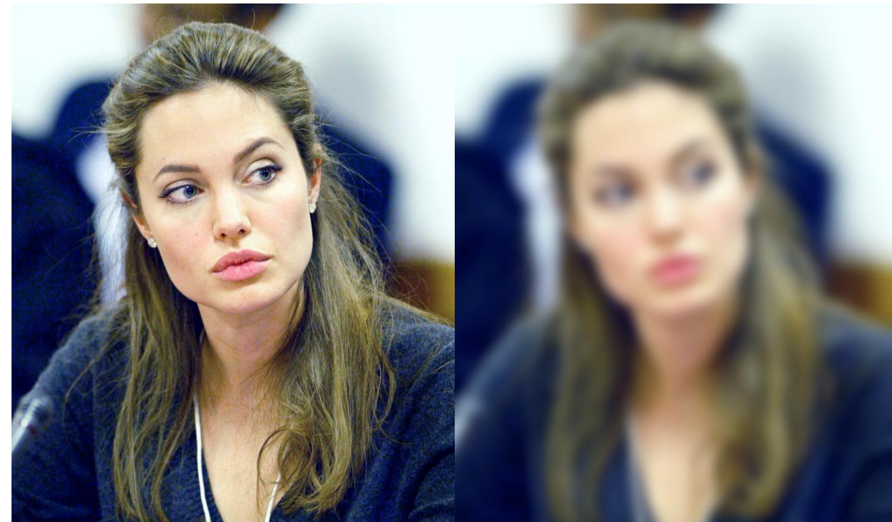
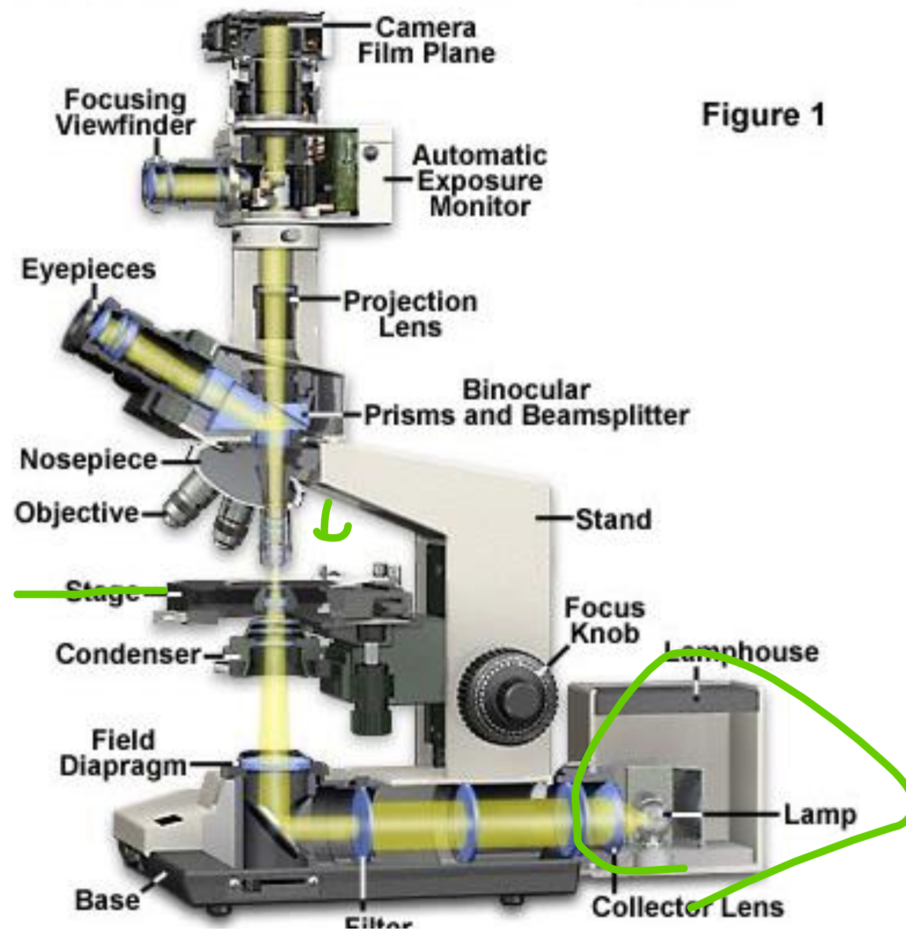
Microscopy – Magnification and Resolution

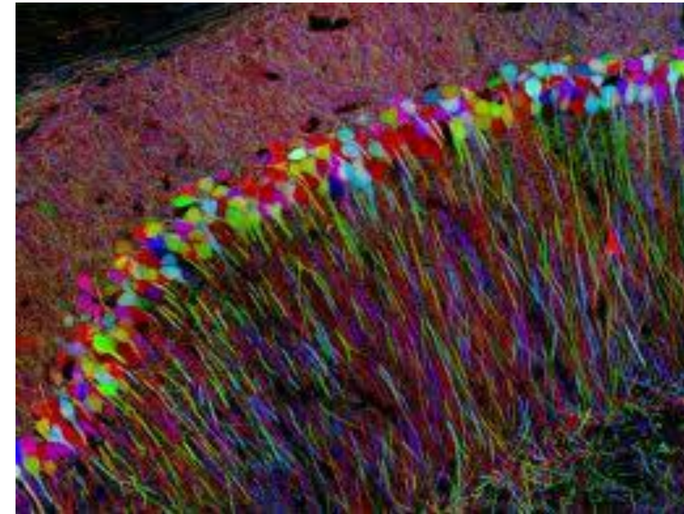
Magnification

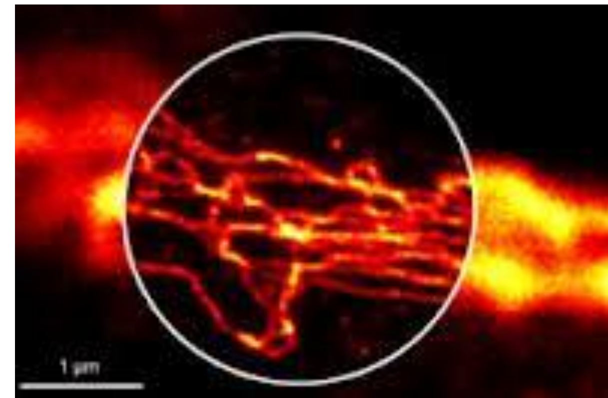




Microscopy is an instrument to see small objects



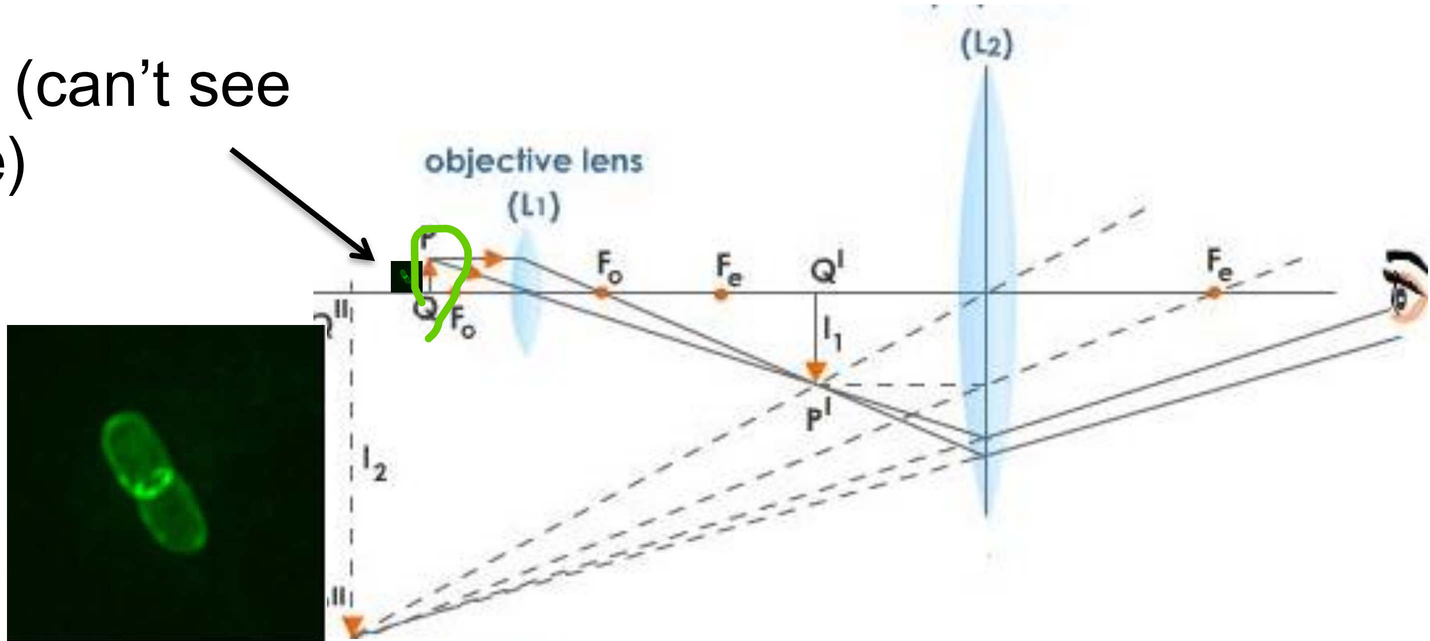




Magnification of an image

Real
Image (can't see
by eye)

Virtual
Image

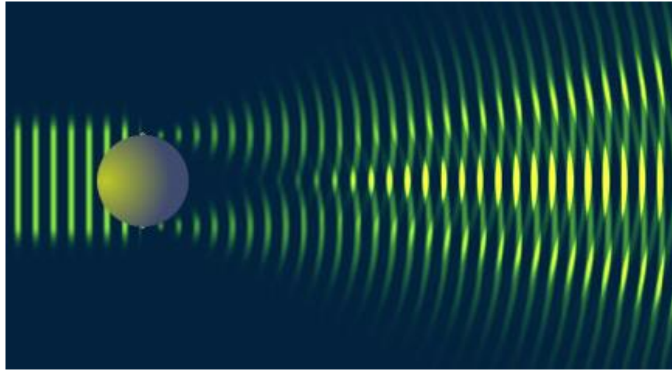


Mag = Length of the object from eye piece / Real length of the object

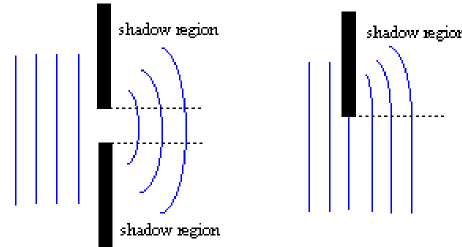
Mag = $Mag_{objective}$ x $Mag_{eyepiece}$ x $Mag_{extralens}$

Bacteria is magnified to the objective and observed at a larger size by the eye piece of the microscope

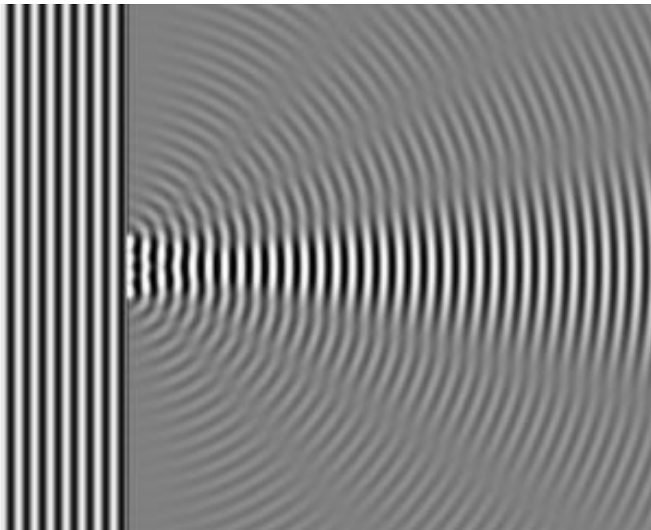
Diffraction of light: Bending light with materials

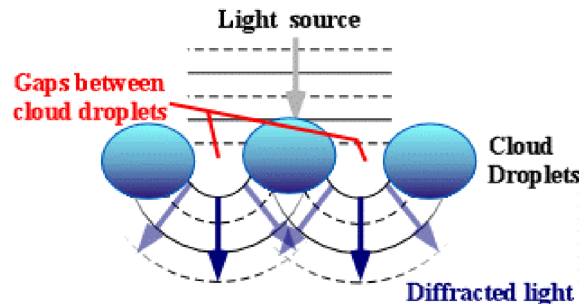


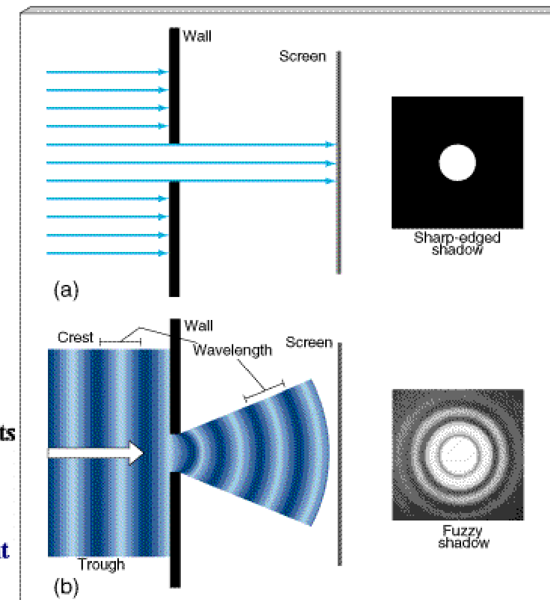
When light passes around a small object it appears to bend



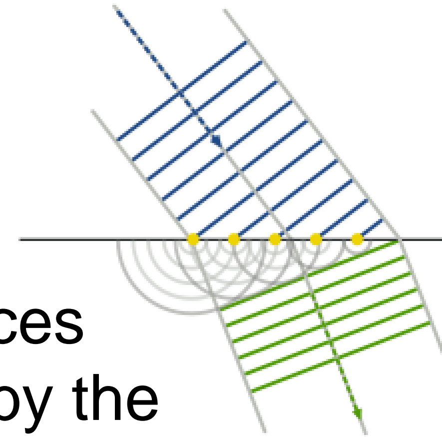
When the light passes through a small opening it spreads out.
If the wavelength is comparable to or bigger than the size of an aperture or an obstacle then significant diffraction takes place.



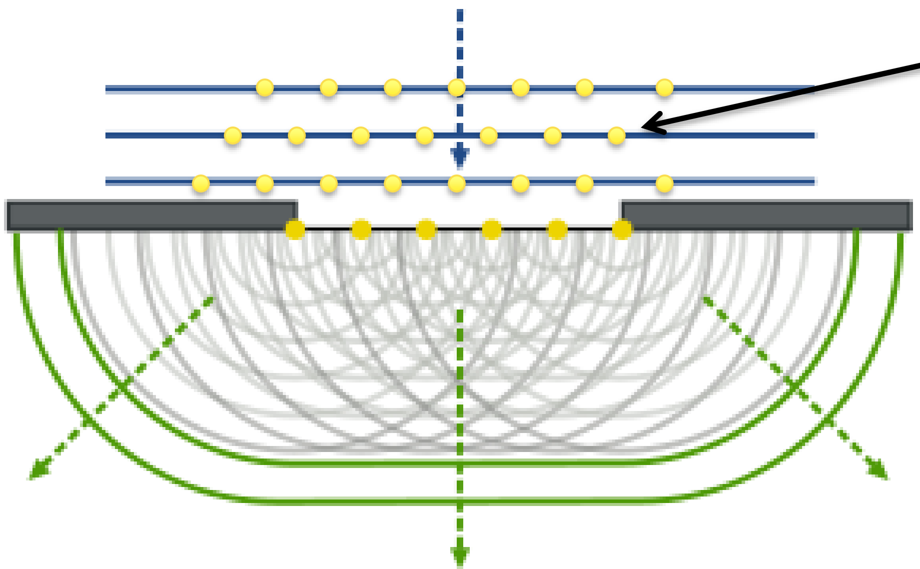




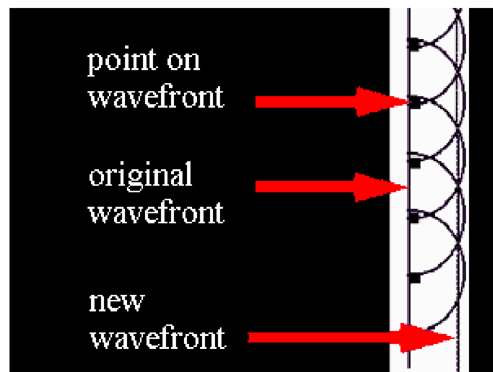
Huygens' Principle



Every wave front acts like a new source.



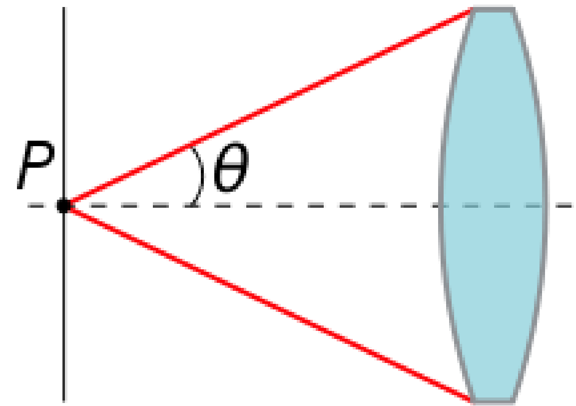
This point sources are eliminated by the slit, therefore can not contribute as a constructive wave on the screen.



You see light as if it is bending where the slit are. Point sources at edges are removed because of slit. Point sources at the corners of slit do not have any contribution to form the constructive patterns on the screen.

Numerical aperture

- $NA = n\sin(\theta)$



$$R = \lambda/2n(\sin(\theta))$$

R is the separation distance
λ is the illumination wavelength
n is the imaging medium refractive index
θ is one-half of the objective angular aperture

Abbe's theory

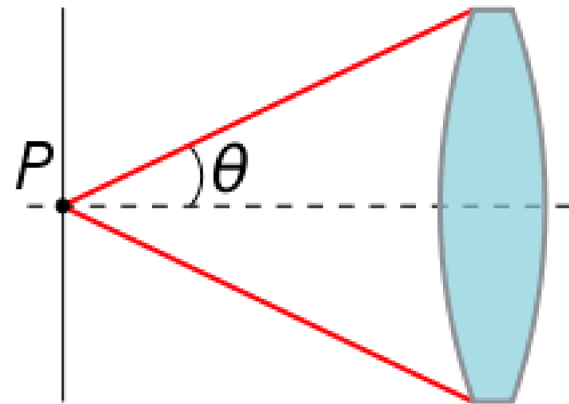
Ernst Abbe is the person who first established the theoretical framework for image formation in a microscopy. Any imaging system has a finite limit of resolution that is the capacity to generate distinguishable images of two close objects.

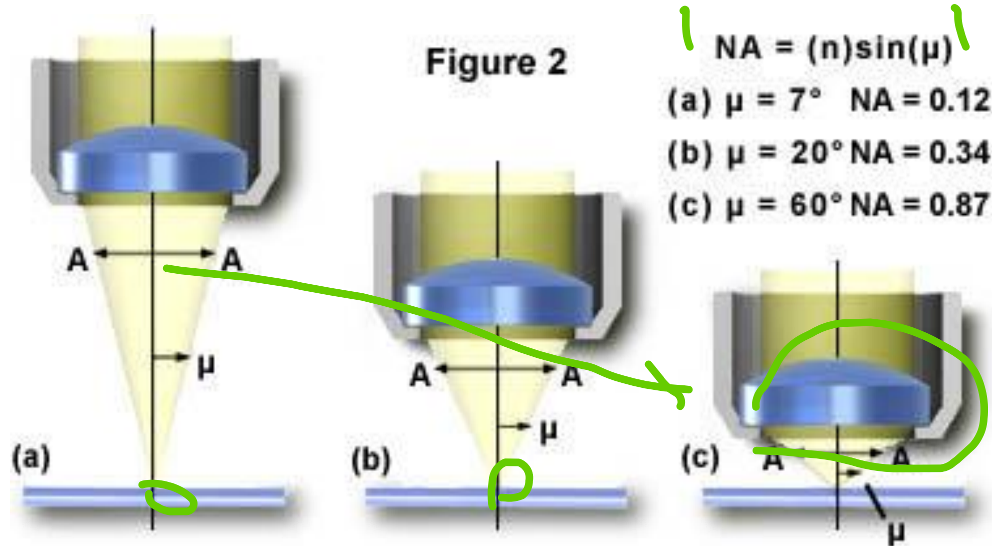
Diffraction of light waves is the principal reason limiting the resolution.

diaphragms and lens edges restricts light rays, leading to each infinitely small point being imaged as a diffraction spot of a finite size. Diffraction spots from nearby points may overlap with each other and become indistinguishable. The present experiment studies the diffraction resolution limit of a microscope objective.

$$d = \frac{\lambda}{2NA}$$

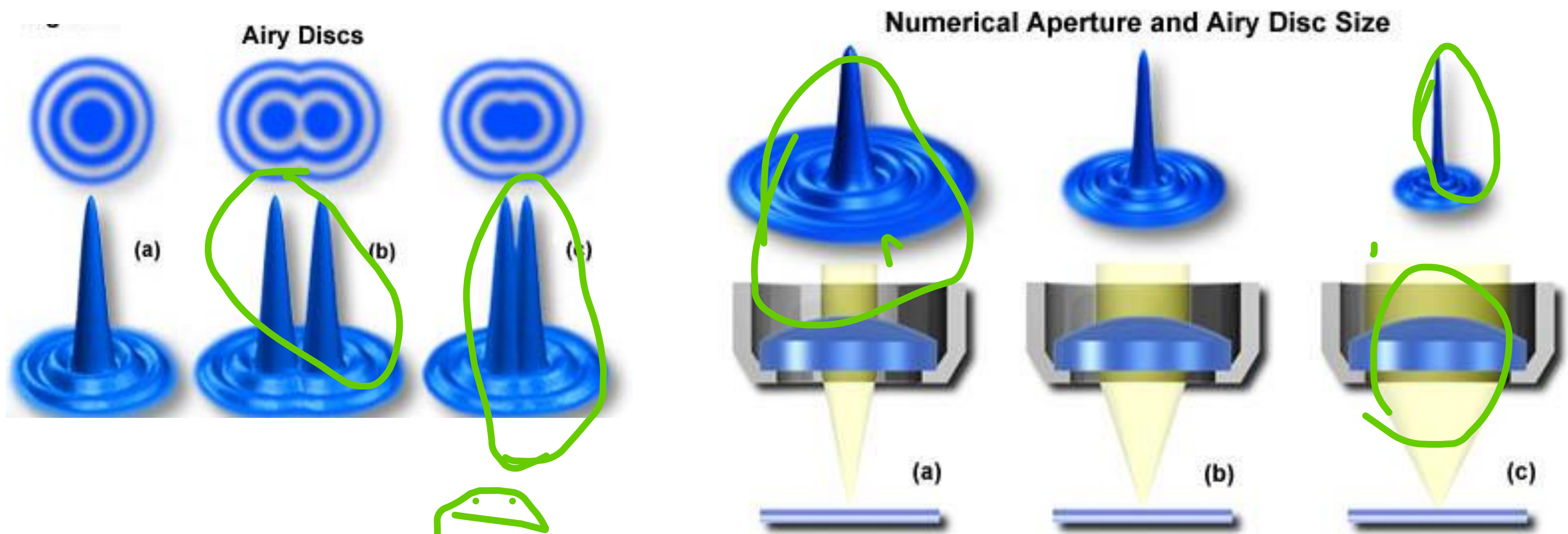
Objectives with different NA





Airy Disc

Airy disc formation is caused by the diffraction or scattering of the light as it passes through the small spaces in the specimen and the circular back aperture of the objective.



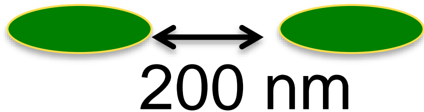
You can think of less resolution in small NA as the beam pass through a small slit therefore very wide zero order peak at the center of the airy disc.

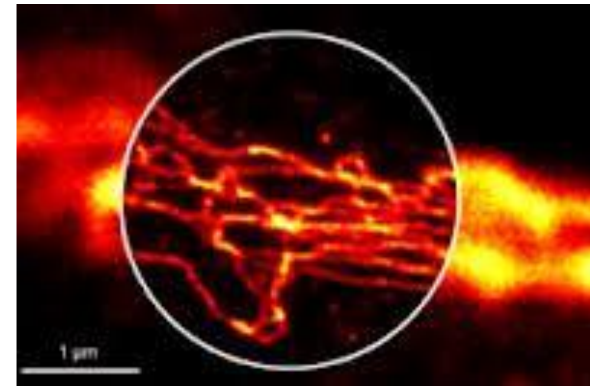
http://www.walter-fendt.de/ph14e/singleslit.htm

Nyquist sampling theorem for microscopy

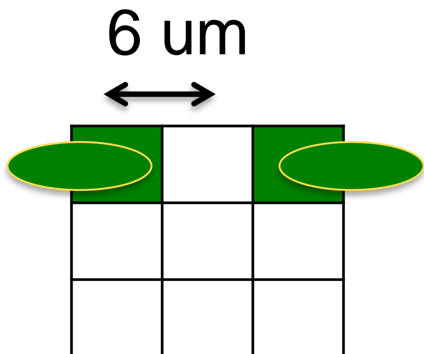
- Consider the smallest resolvable distance in the image is 200 nm, if you use 60x objective the distance is 12 um. The frequency is 1/12.
- The pixel sampling frequency should be 1/6, twice the ratio of distance frequency.

60 x objective = 12000 nm = 12 um

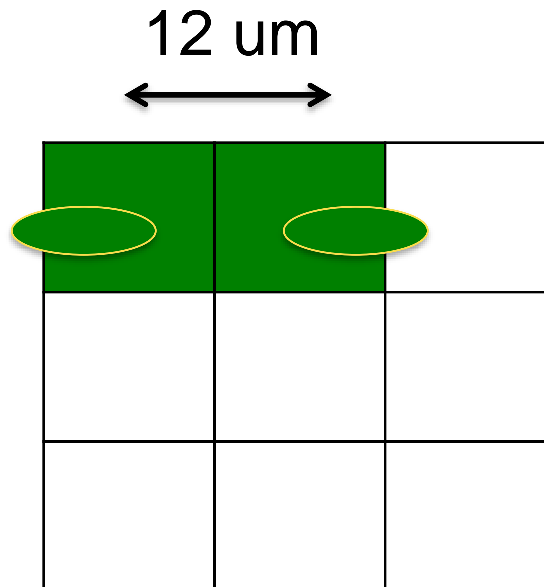




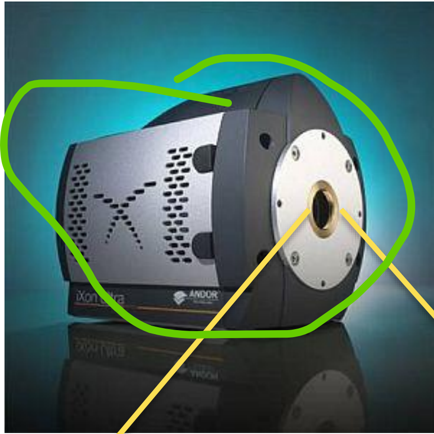
Pixel size matters

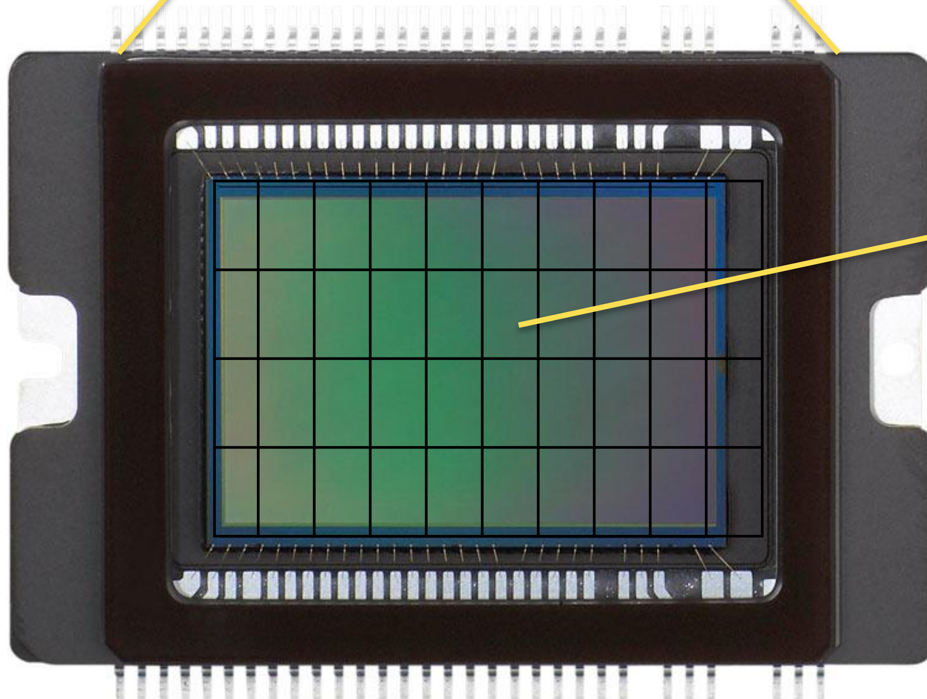


Can be resolved



Cannot be resolved!





Pixels on the camera

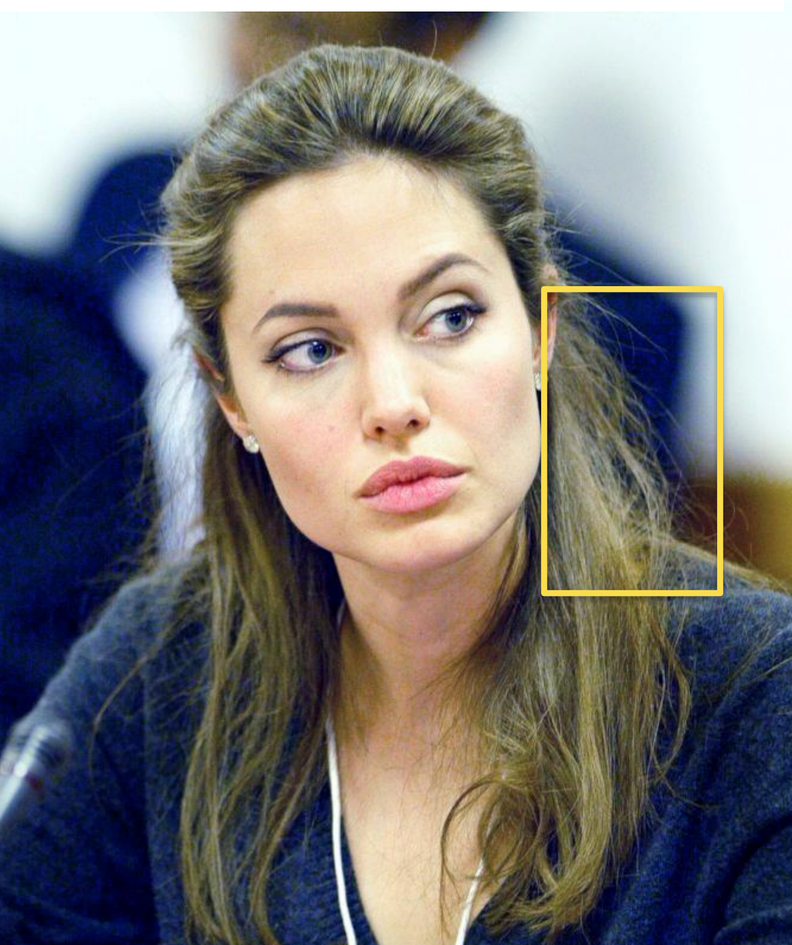
10 Megapixel CCD,
Each pixel is 10 um.

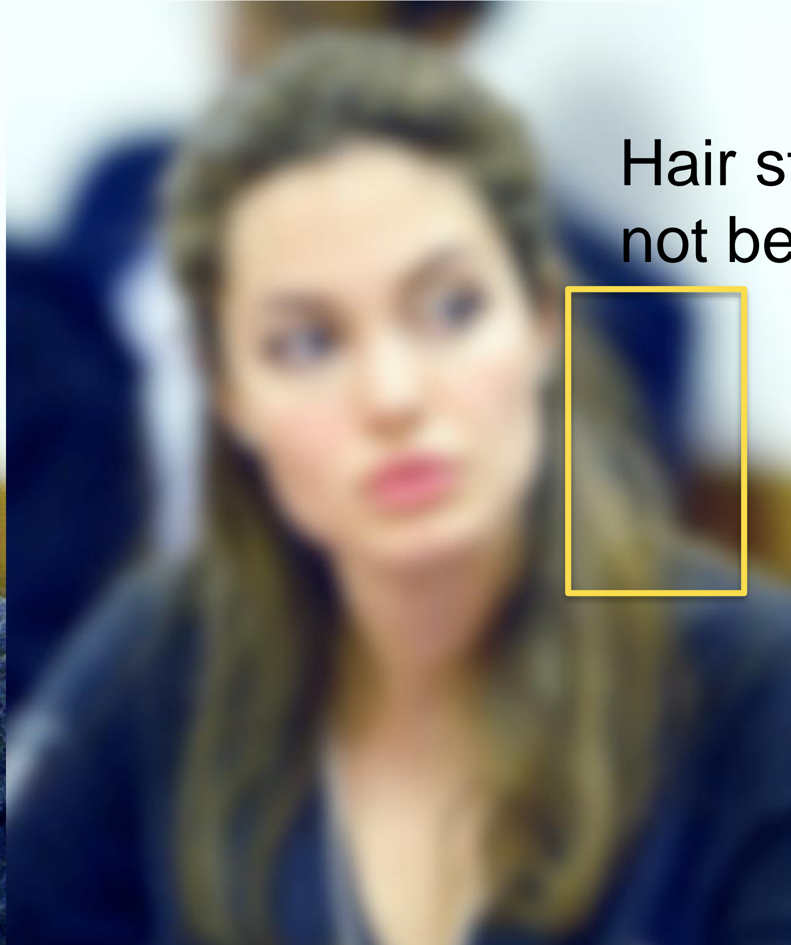
Pixel size and resolution

Small pixel size

vs.

Large Pixel size



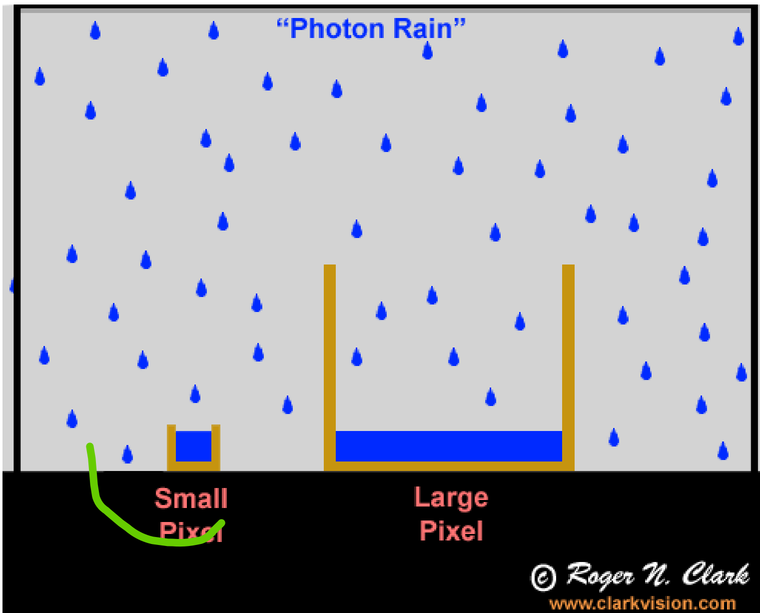


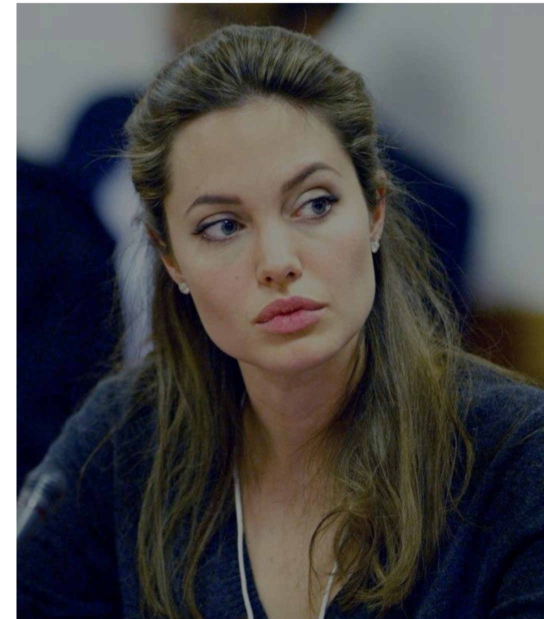
Hair strands can not be resolved!

what if the camera has a very small pixel size?

- Small pixel size will collect less photons, So you will need a longer exposure times for the brightness of image.
- There is a trade-off between the resolution and image brightness.

2 um pixel size > 1 um pixel size





200 ms exposure = 200 ms exposure

Objective lenses

Figure 2

Figure 1

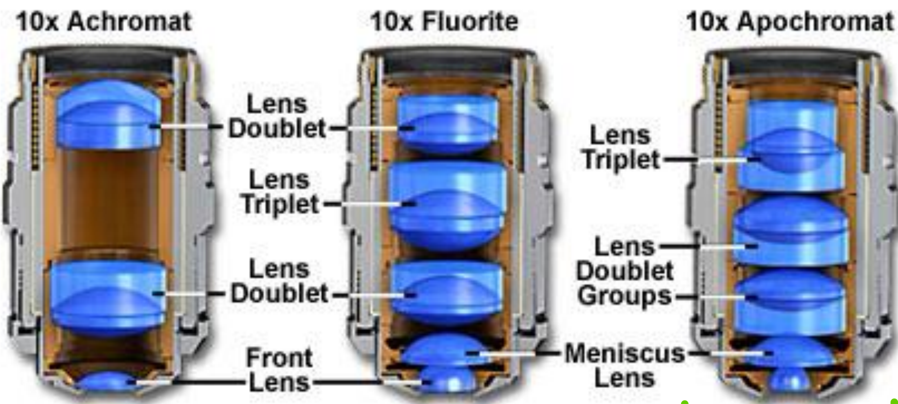


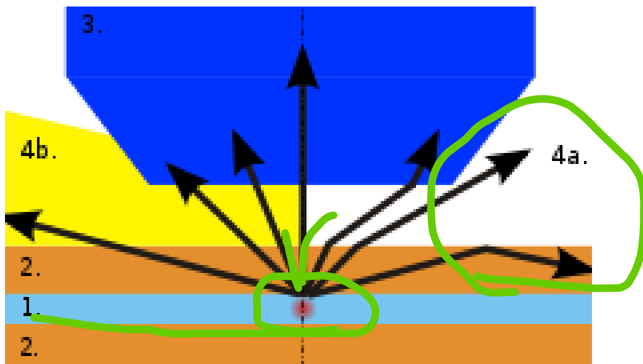
Figure 2

Plan Fluorite Correction		
Magnification	Numerical Aperture	Working Distance (mm)
4x	0.13	17.10
10x	0.30	16.00
20x	0.50	2.10
40x	0.75	0.72
40x (oil)	1.30	0.2
60x	0.85	0.3
100x (dry)	0.90	0.30
100x (oil)	1.30	0.20
100x (oil with iris)	0.5-1.3	0.20

Plan Apochromat Correction		
Magnification	Numerical Aperture	Working Distance (mm)
2x	0.10	8.50
4x	0.20	15.70
10x	0.45	4.00
20x	0.75	1.00
40x	0.95	0.14
40x (oil)	1.00	0.16
60x	0.95	0.15
60x (oil)	1.40	0.21
60x (Water Immersion)	1.20	0.22
100x (oil)	1.40	0.13

Immersion objectives

Immersion objective should be use with an oil or water that depend on the NA of the objective. Oil or water is added between the front lens and the coverslip. These objectives are very small working distance but high NA number.



1. Oil immersion, refractive index 1.51
2. Water immersion, refractive index 1.33

Objective working distance

It is defined as the distance from the lens of the objective to the surface of the coverslip when the specimen is in sharp focus.

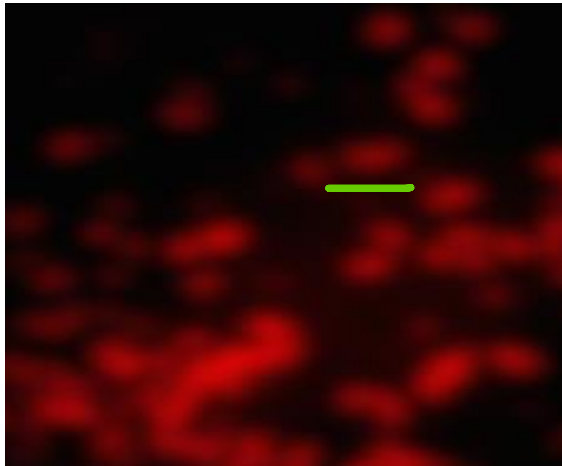
Generally, working distance decreases in objectives as the magnification and numerical aperture increase.

Common Objective Working Distances

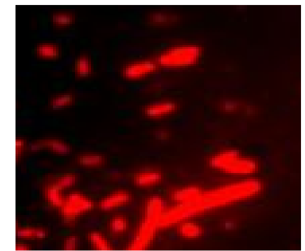
Manufacturer	Correction	Magnification	Numerical Aperture	Working Distance
Nikon	PlanApo	10x	0.45	4.0 mm
Nikon	PlanFluor	20x	0.75	0.35 mm
Nikon	PlanFluor (oil)	40x	1.30	0.20 mm
Nikon	PlanApo (oil)	60x	1.40	0.21 mm
Nikon	PlanApo (oil)	100x	1.40	0.13 mm

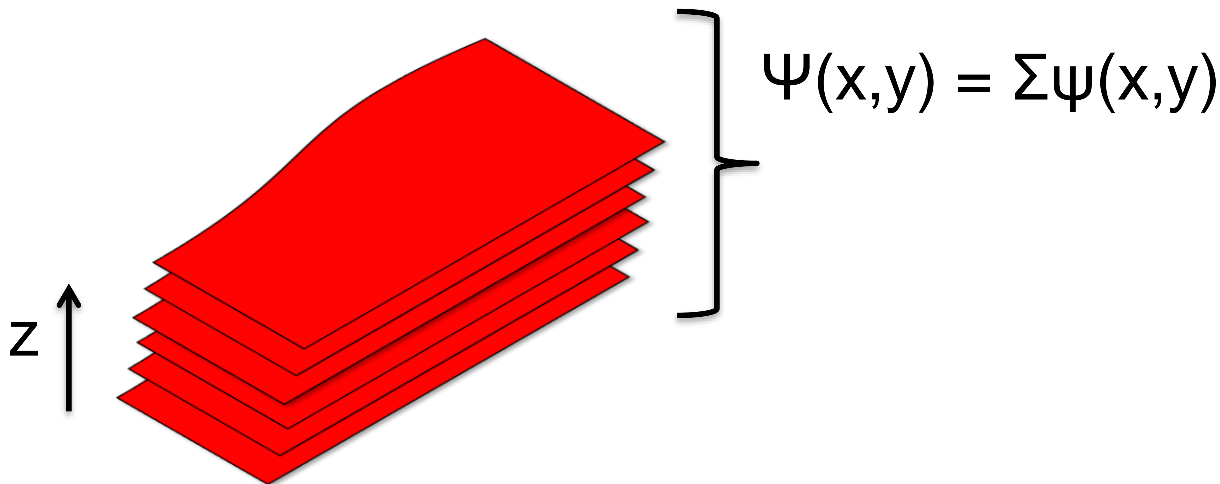
Depth of Field

- You can get sharp images with High NA objective theoretically. It is good if there is a backgrounds signal from other planes along the z axis. However field of gets smaller.

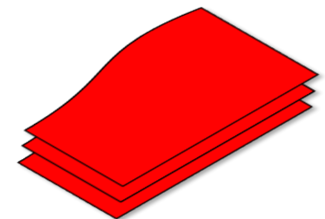


Average image of all layers. Waves from each layer contribute to the image.

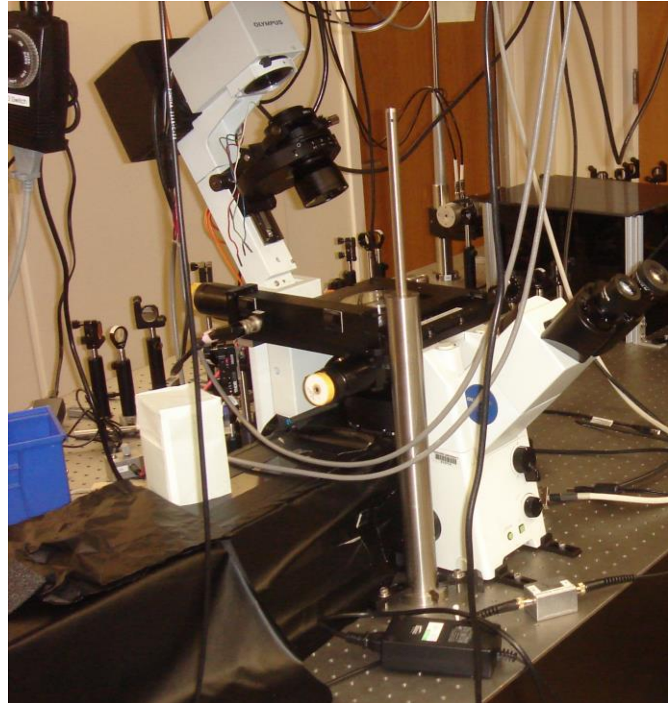


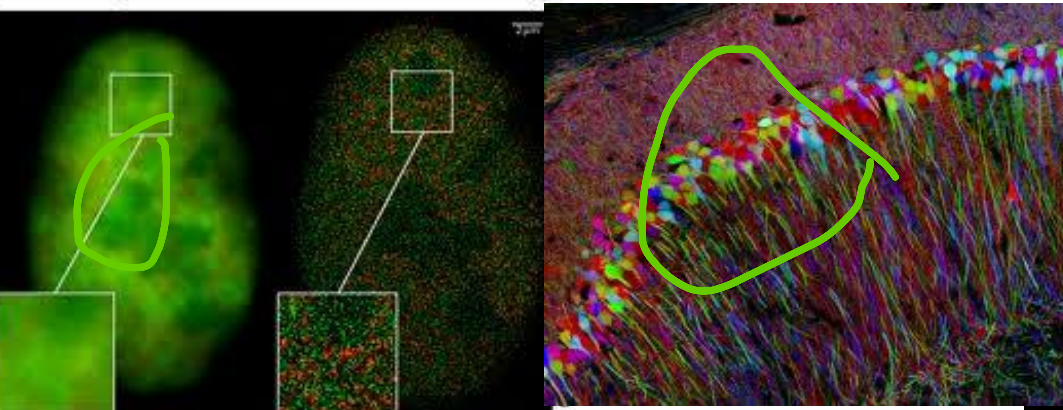


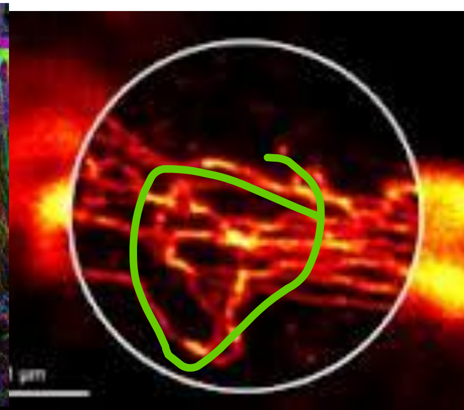
$$\Psi(x,y) = \Sigma\psi(x,y)$$

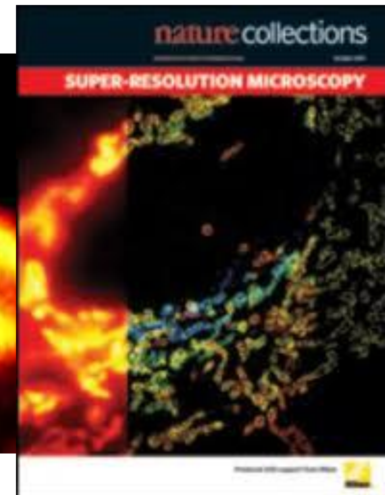


Fluorescence Microscopy Principles

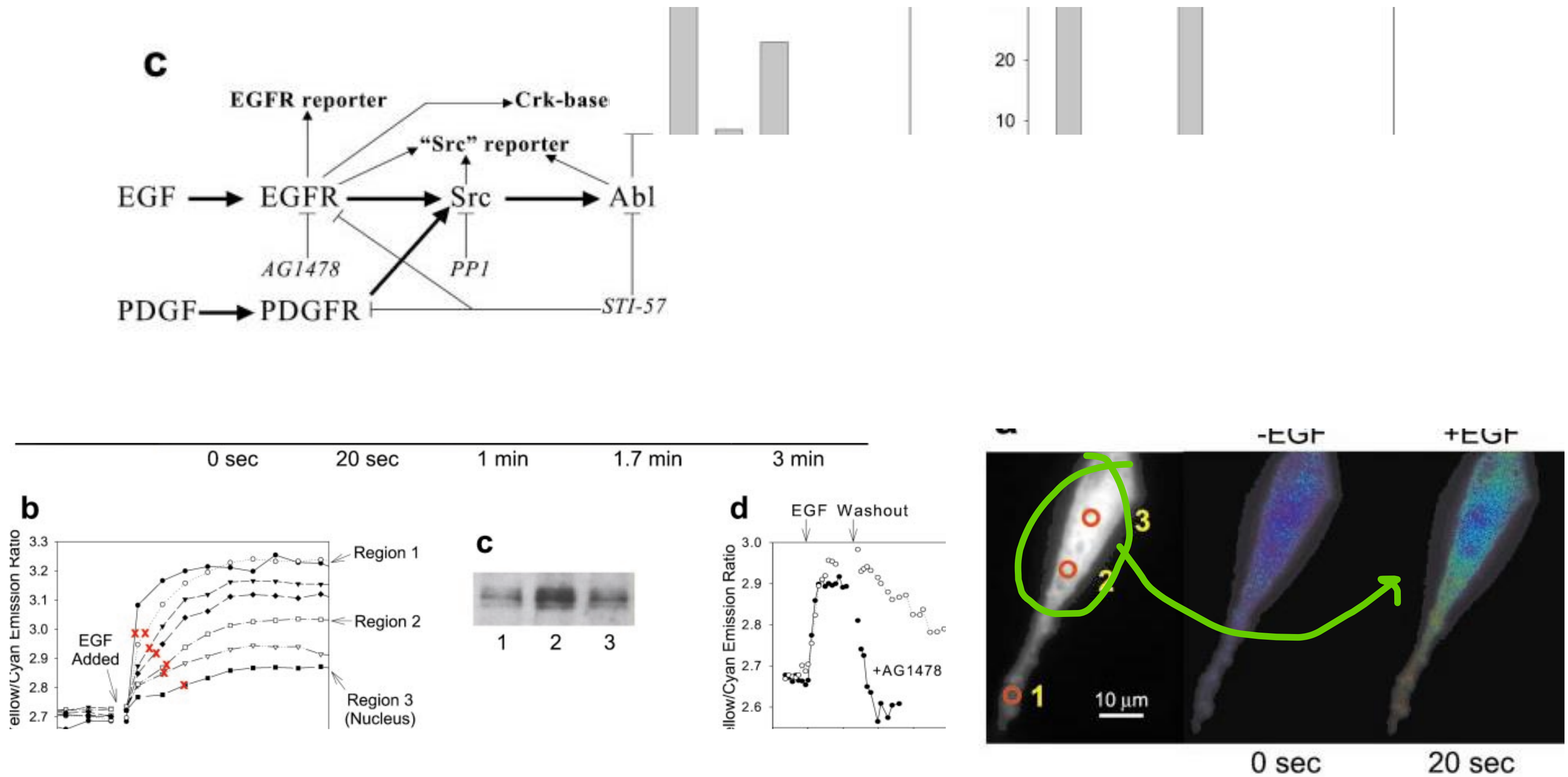








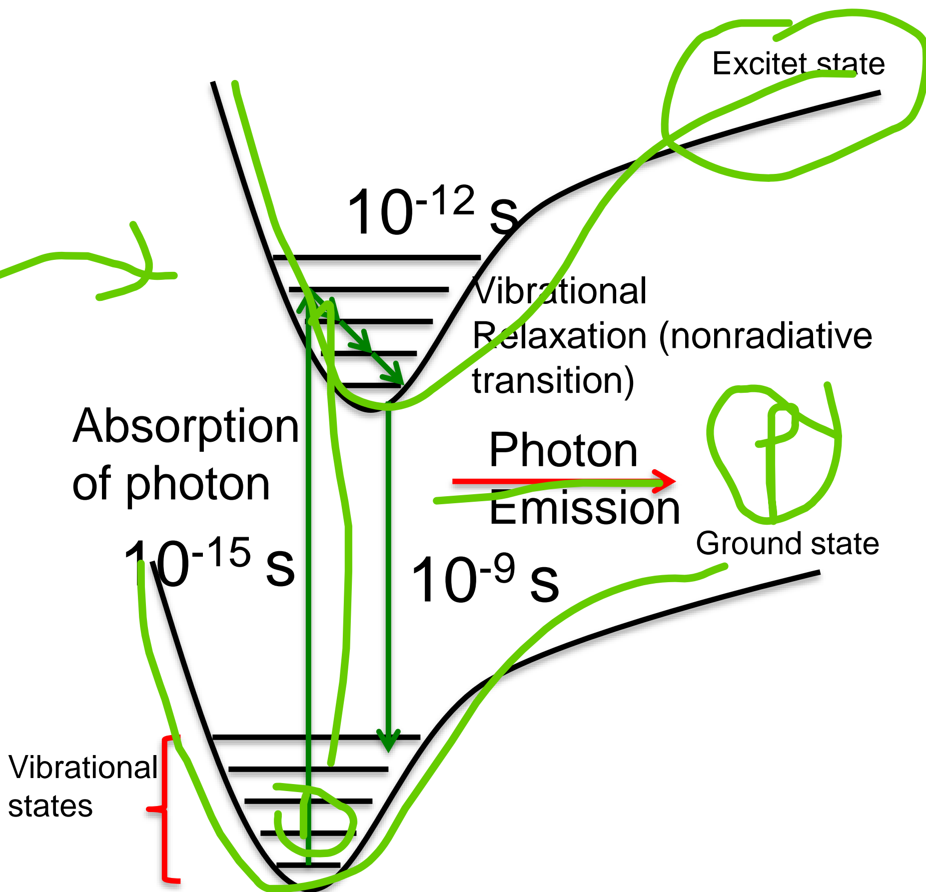
Fluorescence Assay to monitor kinase activity in cells

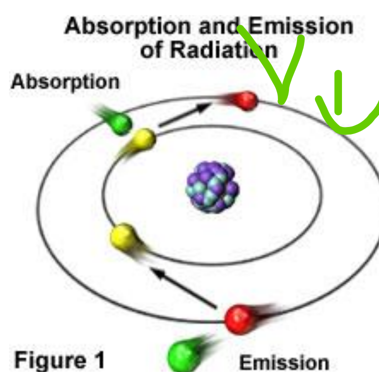


Alice Ting et. al. PNAS, 2001, 98, 15003

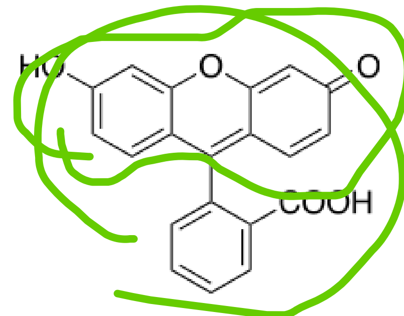
Fluorescence

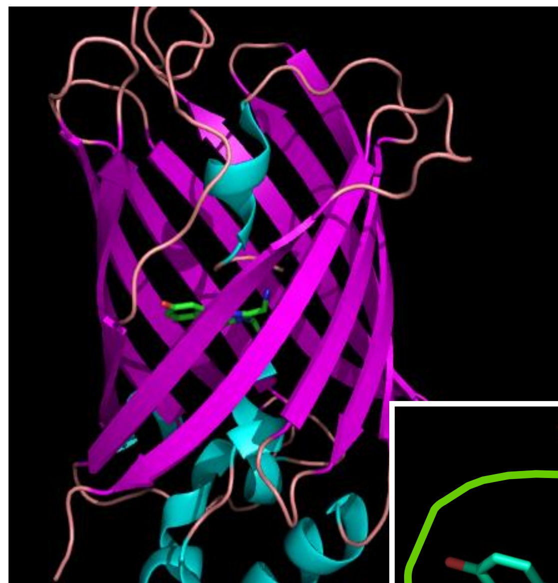
Electronic transition during fluorescence



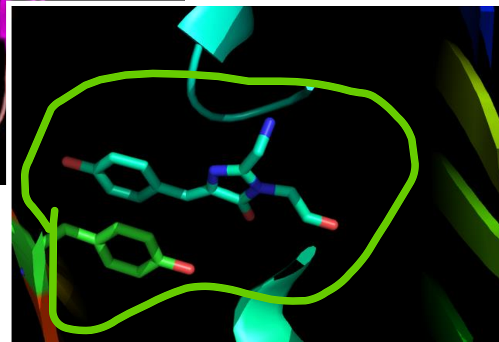


Fluorescein

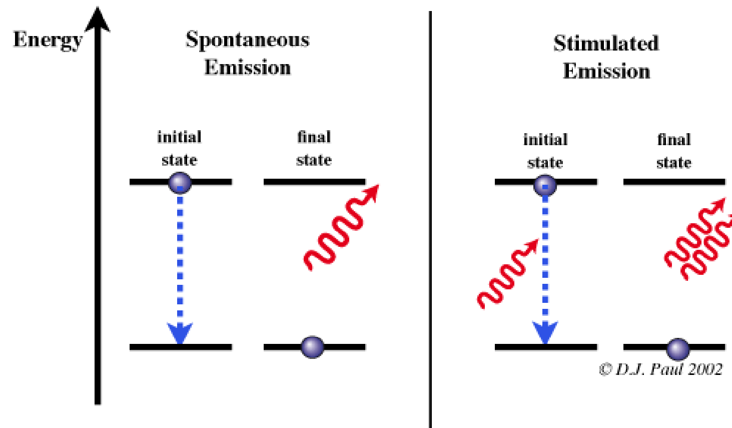




Green Fluorescent Protein



Spontaneous vs. Stimulated Emission



Fluorescence is a spontaneous emission process. The decay happens randomly.
In stimulated emission, just before the photon emitted during the decay another photon arrives and the dipoles of these two photons will start oscillate the the same phase.
Remember that the decay is slow 10^{-9} s. And excitation is very fast
The second photons can not excite the electron because it has already in the excited state.

Stimulated emission can be explained by dipole-dipole interactions among photons.

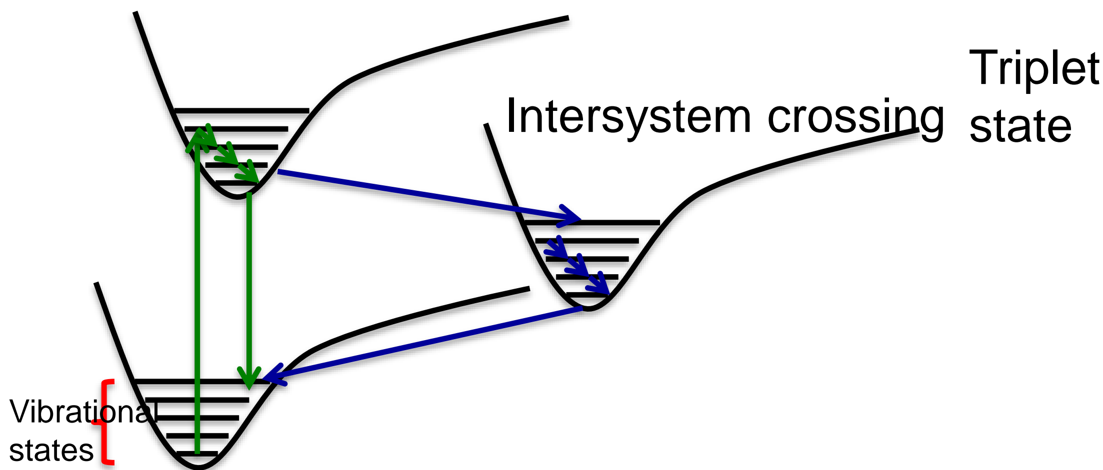
Quantum yield of fluorophore

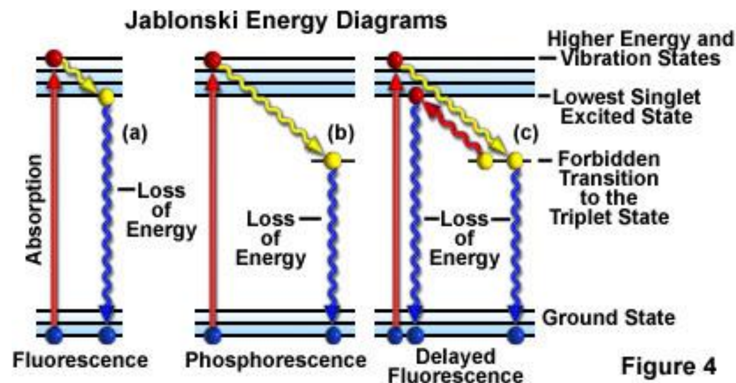
The fluorescence quantum yield (Φ_F) is the ratio of photons absorbed to photons emitted through fluorescence.

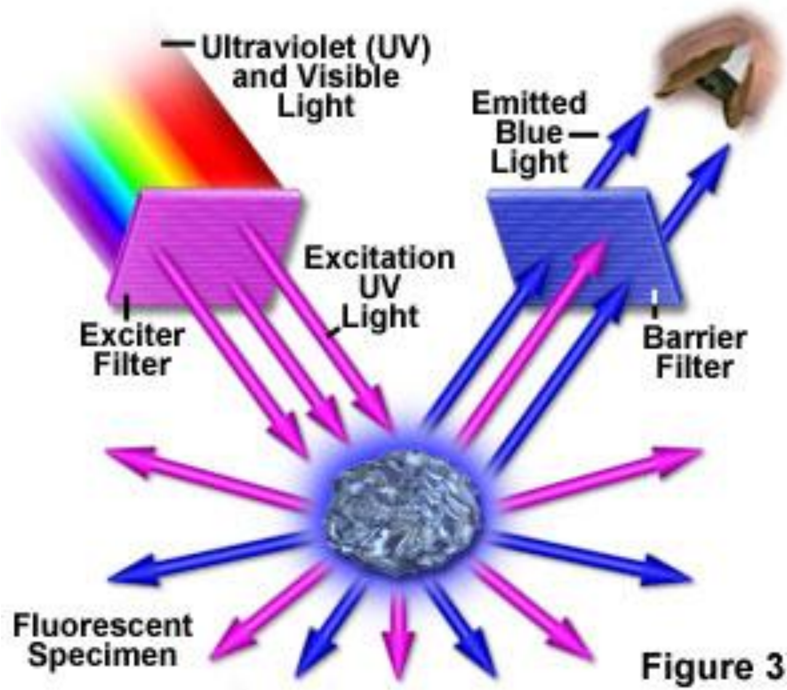
$$F = \frac{numberofphotonsemittted}{numberofphotonsabsorbed}$$

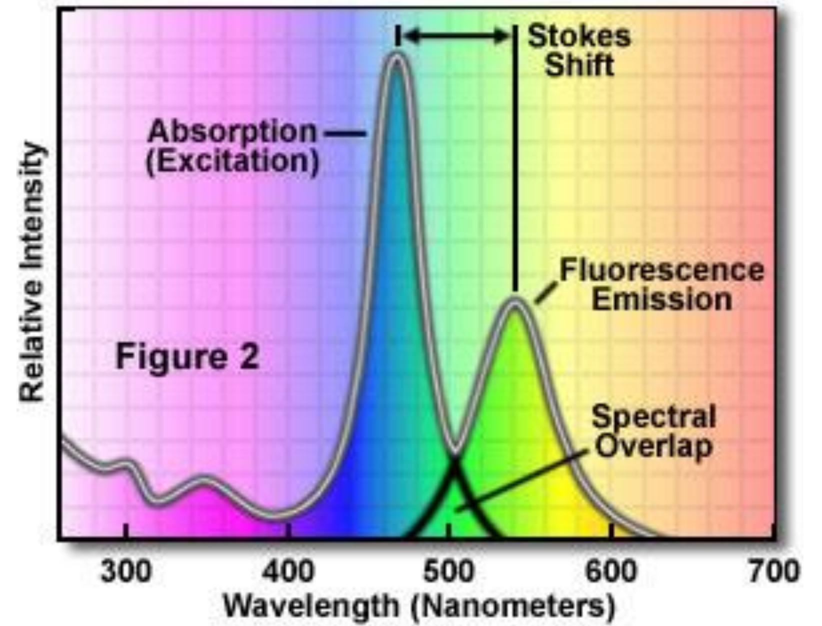
$$F = \frac{k_f}{k_f + k_{nr} + k_{isc}}$$

k_f = rate of fluorescence
k_i = rate of nonradiative decay
k_{isc} = rate of intersystem crossing

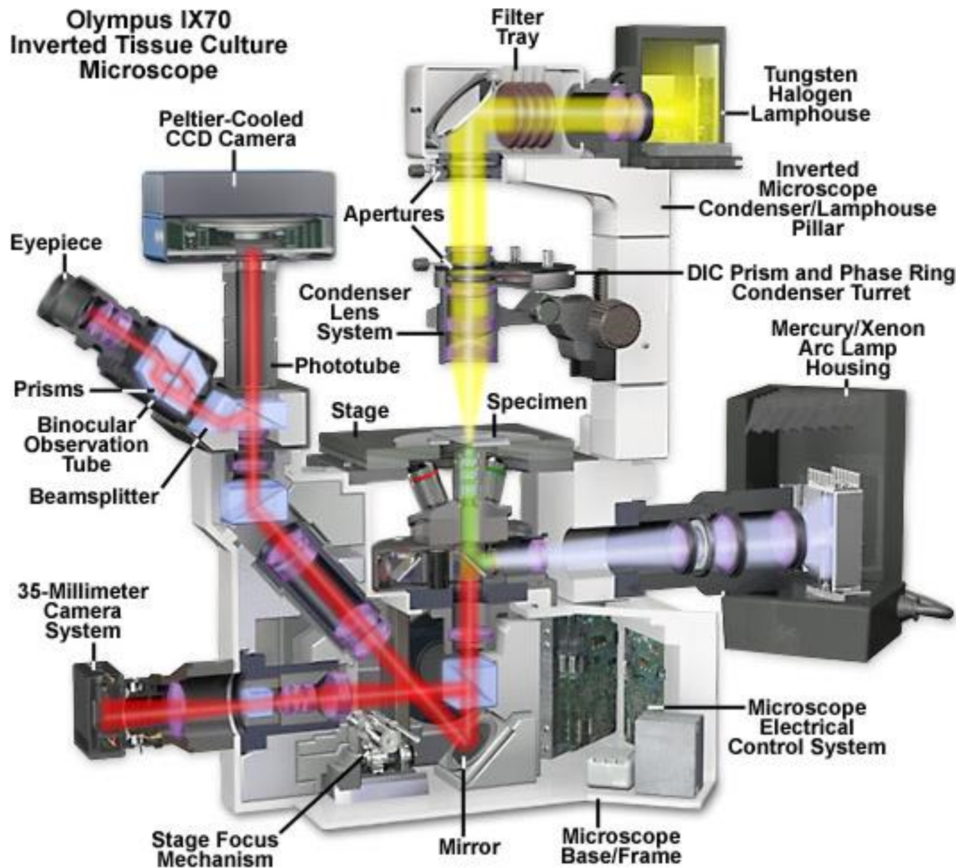




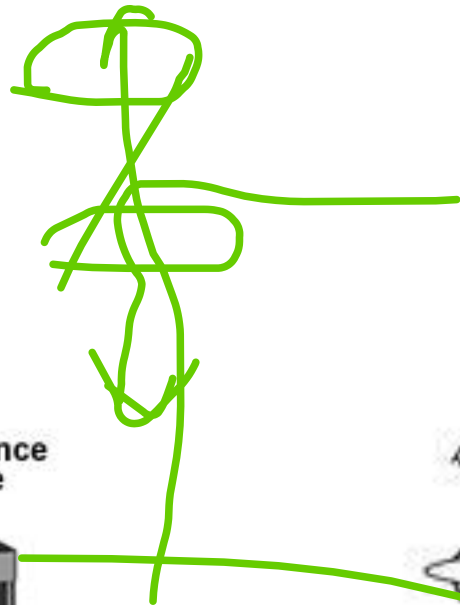

Principle of Excitation and Emission
Ultraviolet (UV) and Visible Light
Emitted Blue Light
Excitation UV Light
Exciter Filter
Barrier Filter
Fluorescent Specimen
Figure 3


Excitation and Emission Spectral Profiles
Stokes Shift
Absorption (Excitation)
Fluorescence Emission
Figure 2
Spectral Overlap
Relative Intensity
300
400
500
600
700
Wavelength (Nanometers)

Components of inverted microscope



Upright Microscope



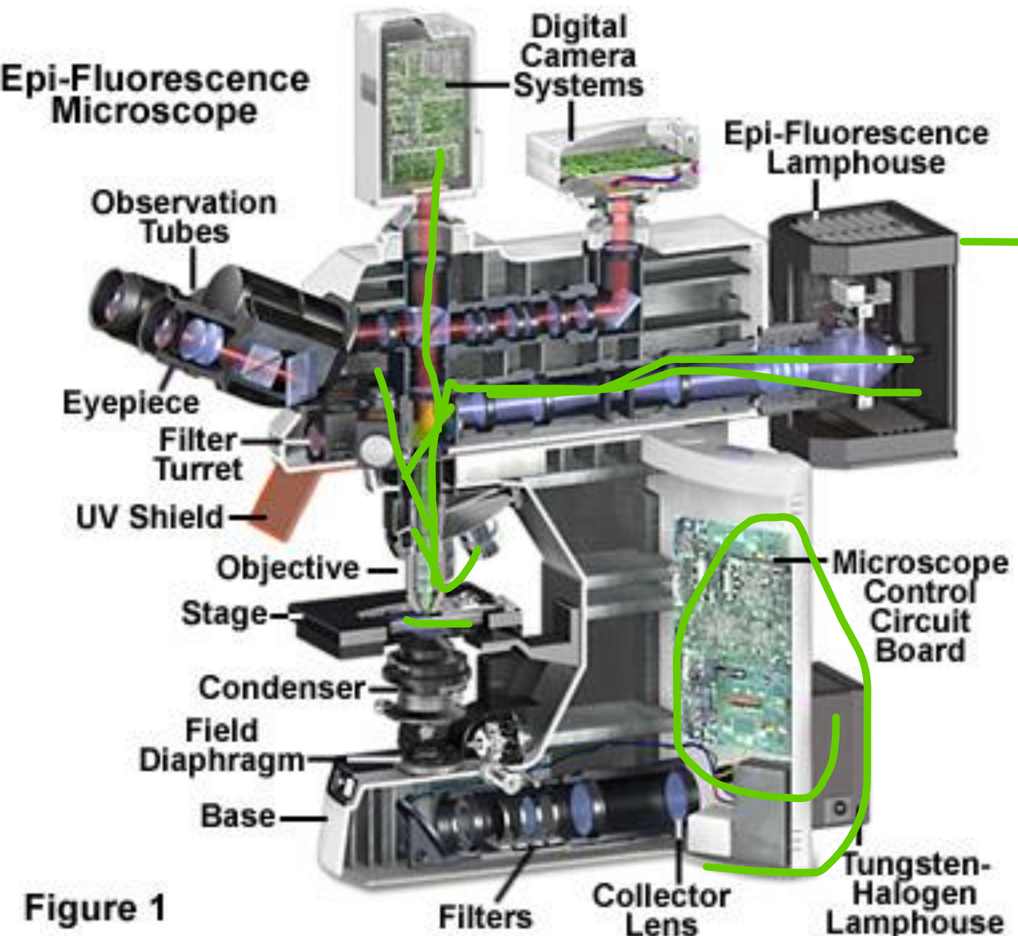
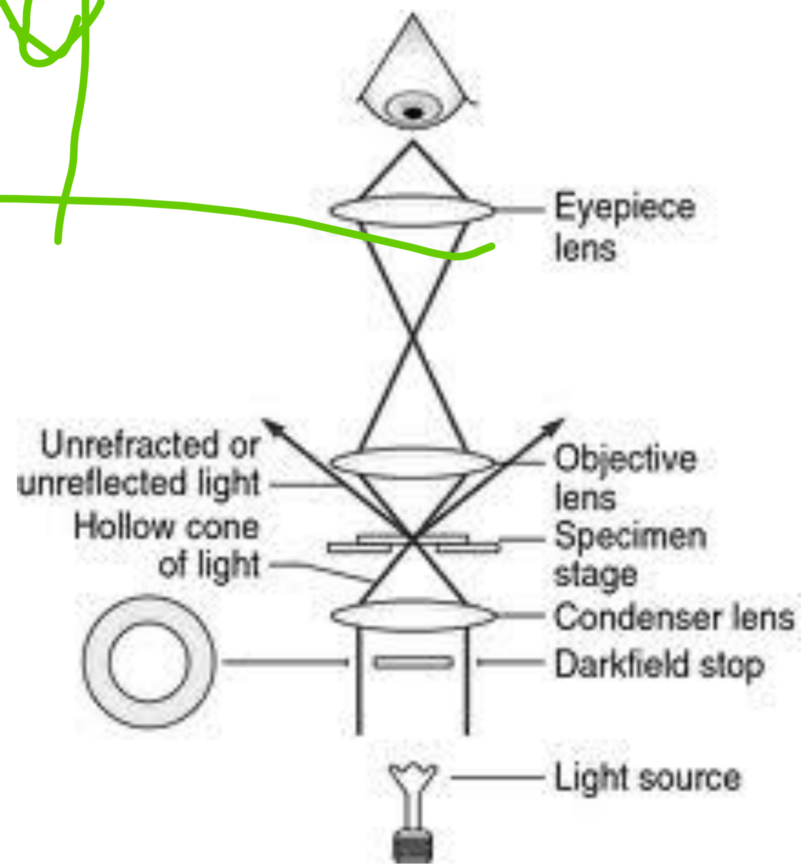
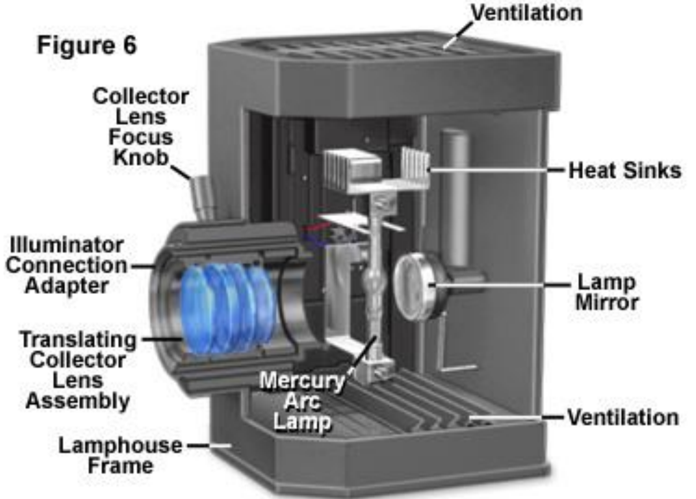
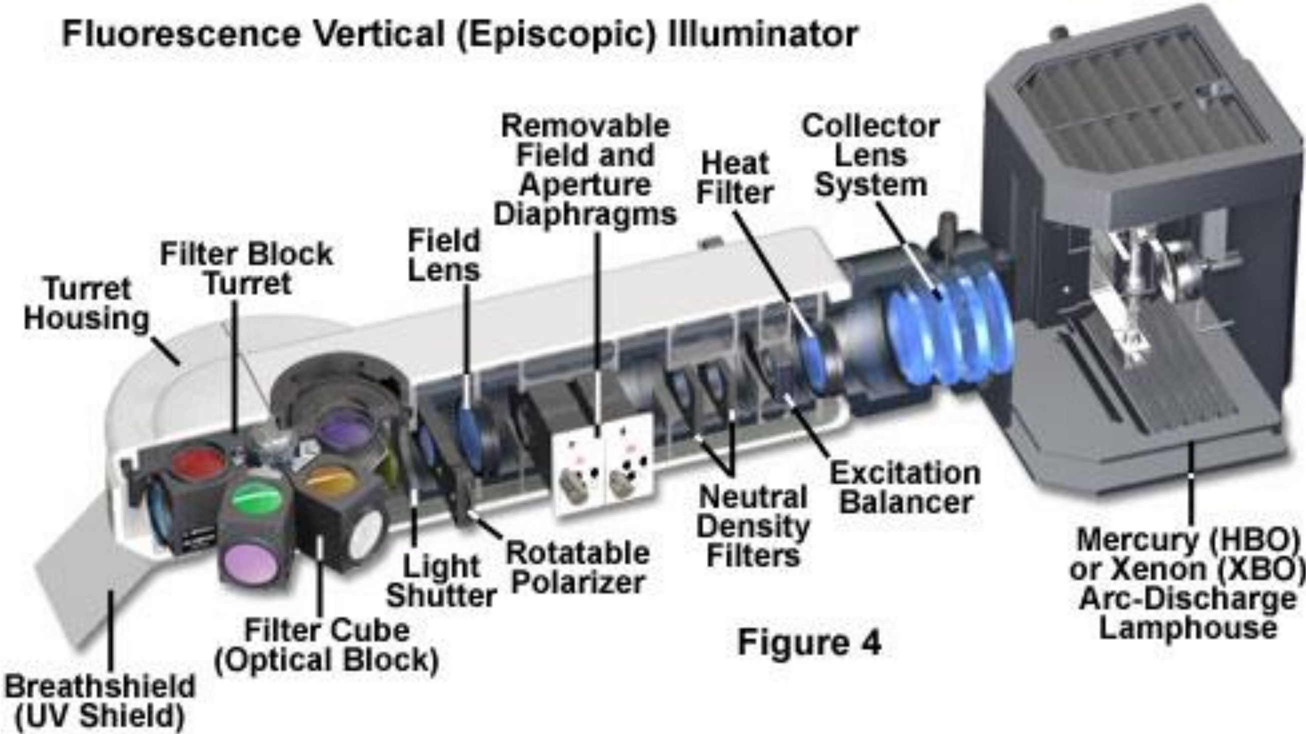


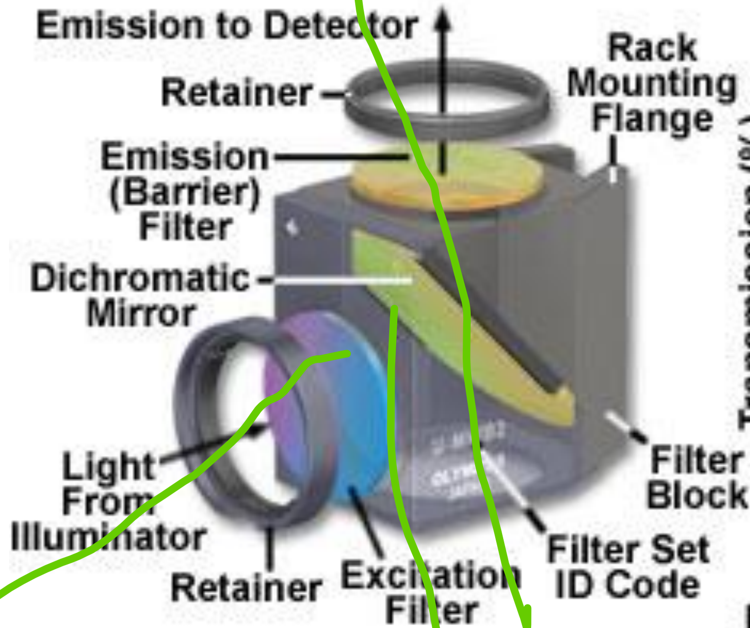
Figure 1

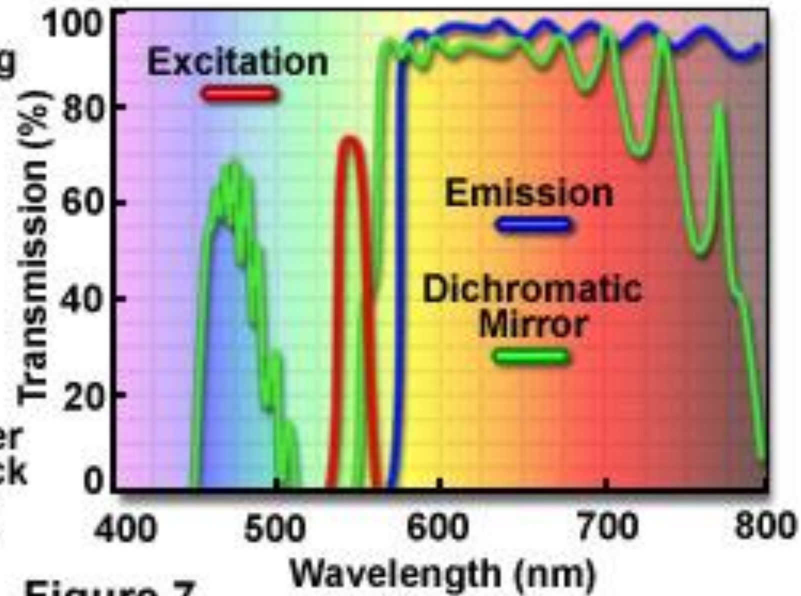



Fluorescence Microscope Arc-Discharge Lamp Housing
Figure 6
Ventilation
Collector Lens Focus Knob
Heat Sinks
Illuminator Connection Adapter
Lamp Mirror
Translating Collector Lens Assembly
Mercury Arc Lamp
Ventilation
Lamphouse Frame


Fluorescence Vertical (Episcopic) Illuminator
Removable Field and Aperture Diaphragms
Collector Lens System
Heat Filter
Field Lens
Filter Block Turret
Turret Housing
Excitation Balancer
Neutral Density Filters
Rotatable Polarizer
Light Shutter
Mercury (HBO) or Xenon (XBO) Arc-Discharge Lamphouse
Filter Cube (Optical Block)
Figure 4
Breathshield (UV Shield)

Fluorescence Filter Cube (Block) and Associated Spectra

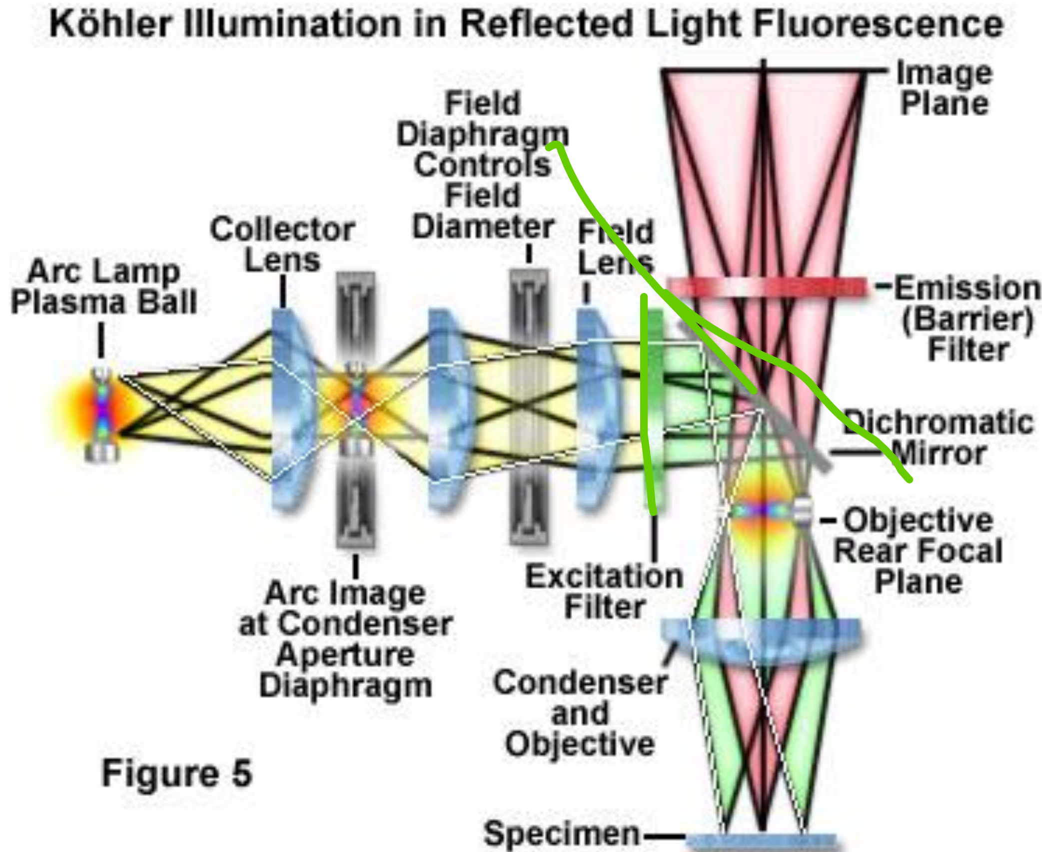


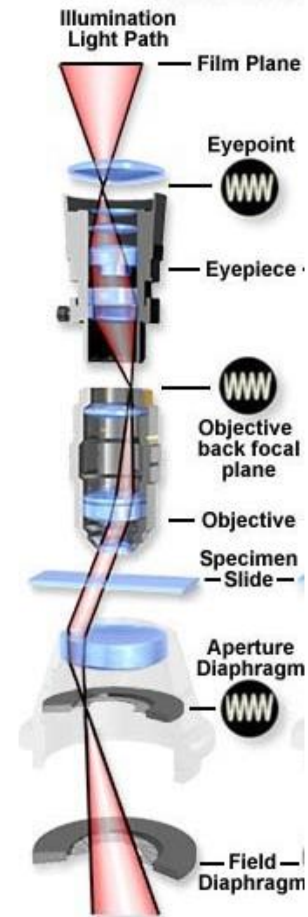


http://www.microscopyu.com/tutorials/flash/spectralprofiles/index.html

http://www.semrock.com/fluorophore-table.aspx

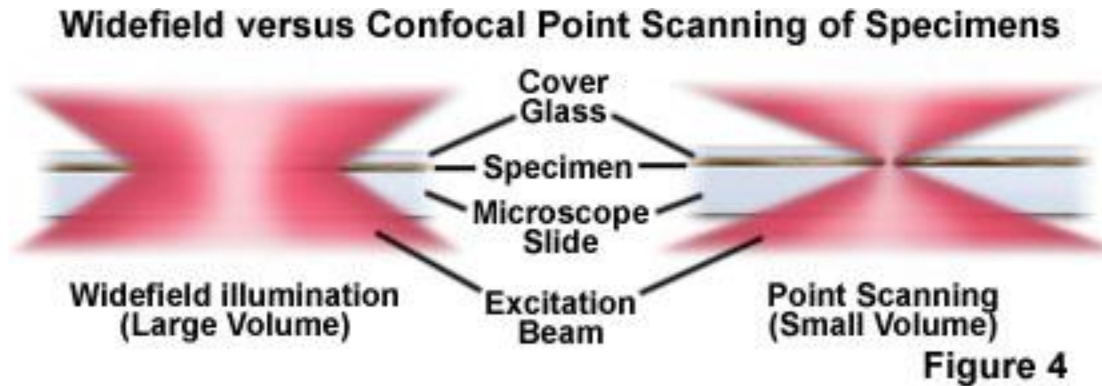
Epi-Fluorescence-Widefield

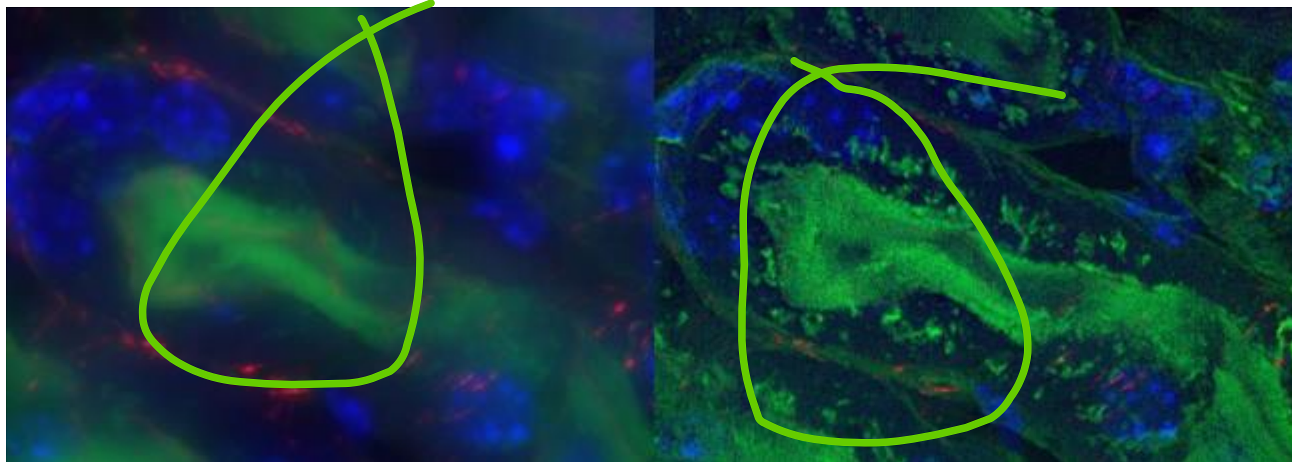




Transmitted Light Illumination Path

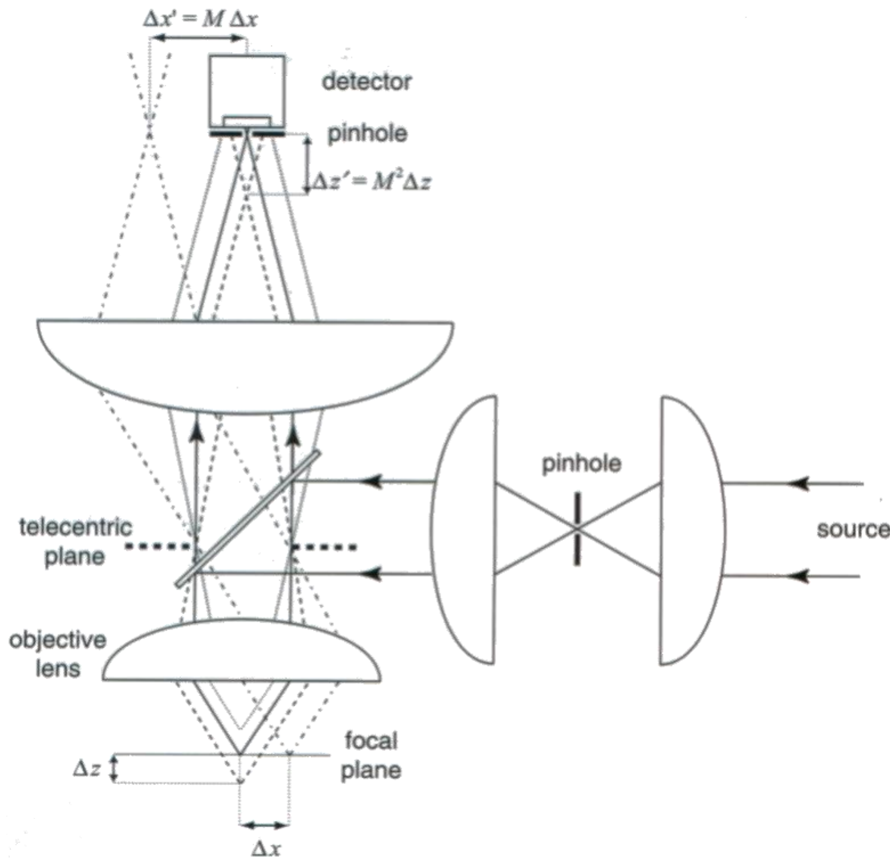
Widefield vs. Point Scanning Confocal





spatial filtering technique eliminate out-of-focus light or glare in specimens whose thickness exceeds the dimensions of the focal plane.

In confocal system: The whole field is not excited all at once.



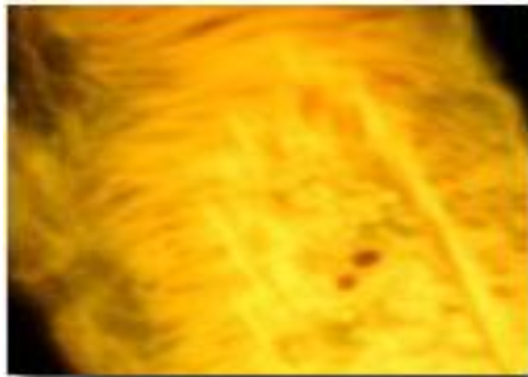
The shape of the excitation light is in the diffraction limited points, which can be scanned over the sample.

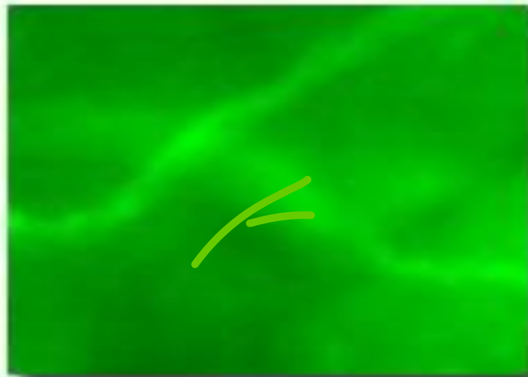
A point source of light is collected by some intermediate lens and is transmitted as parallel rays.

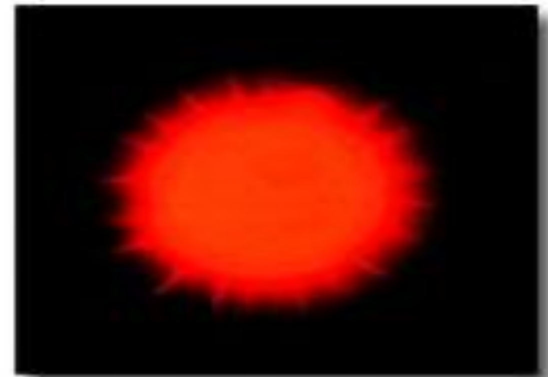
The parallel rays enters the objective back aperture and is focused by the objective as a single point on the sample.

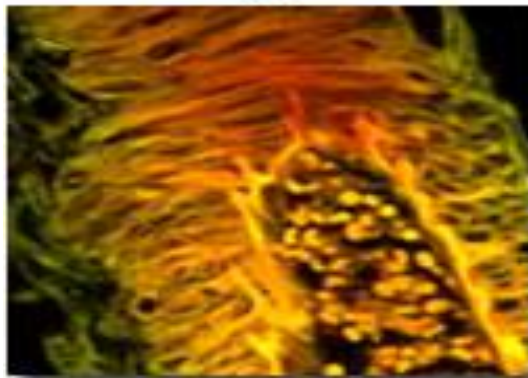
Changing the angle of parallel light changes the location of the point source at the sample plane.

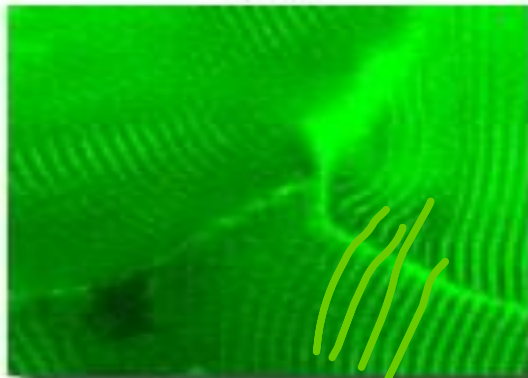
Confocal vs Epifluorescence Images


(a)


(b)


(c)


(d)


(e)

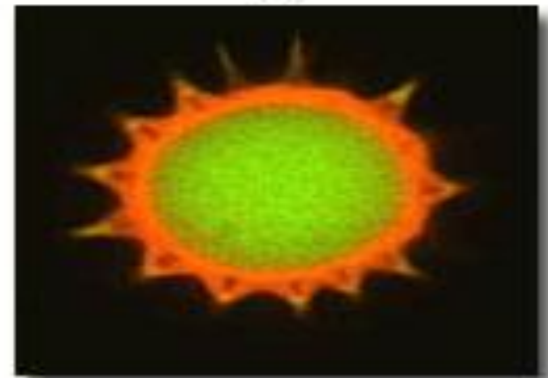
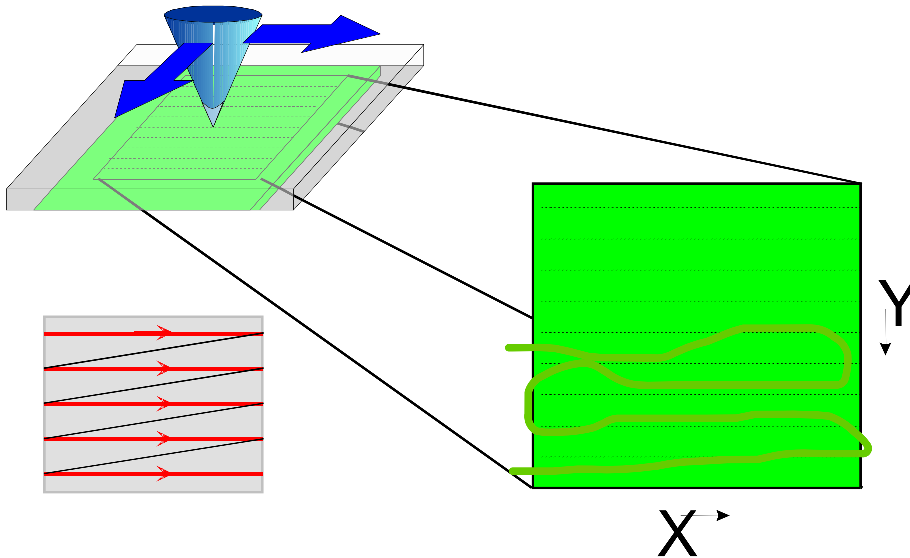
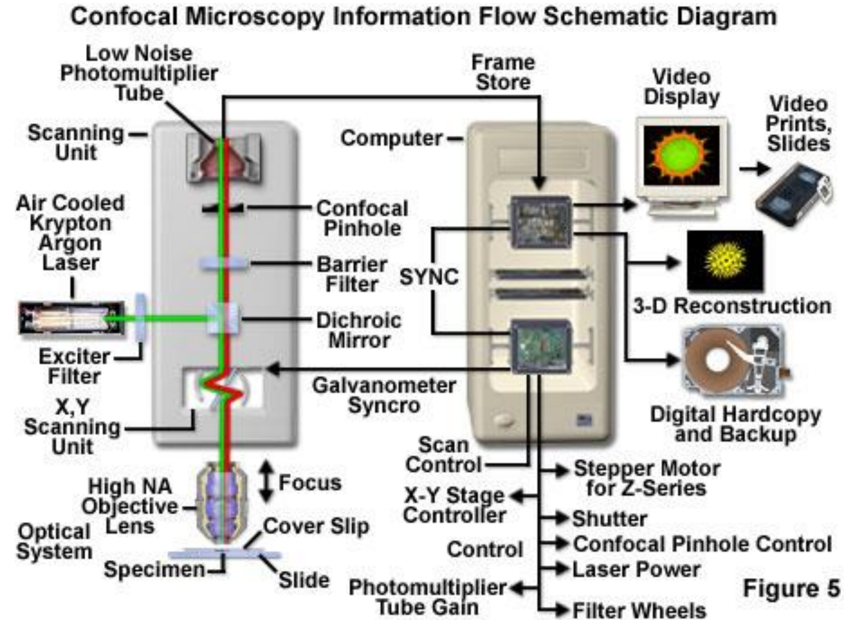

(f)

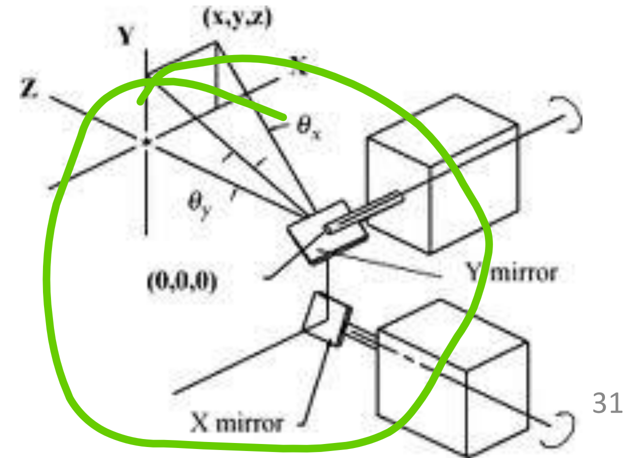
Figure 1

Point Scanning Confocal





The point is scanned using mirrors along the sample

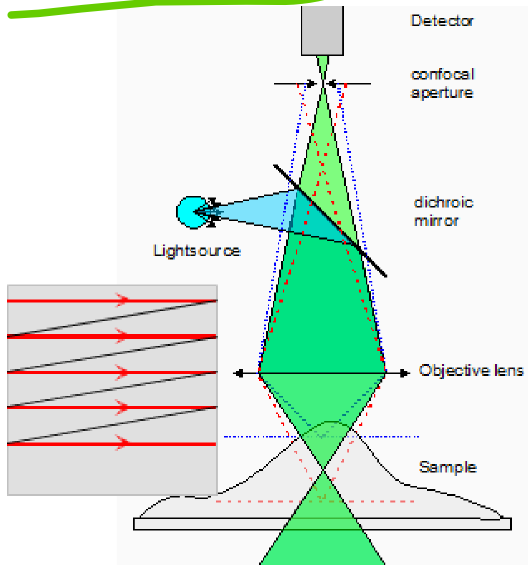


Three Optical Sectioning Methods

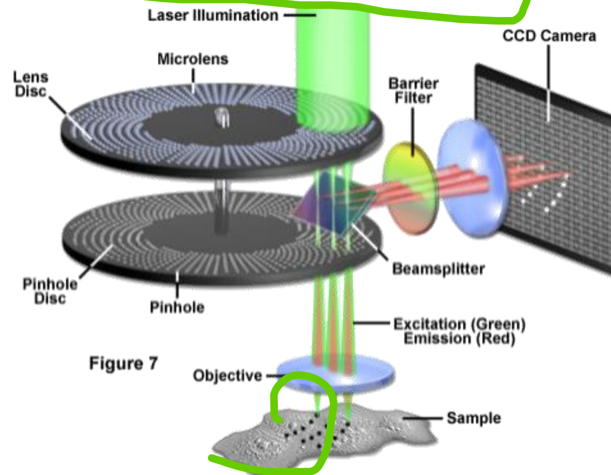
Compare Sectioning Methods with Widefield Fluorescence Methods

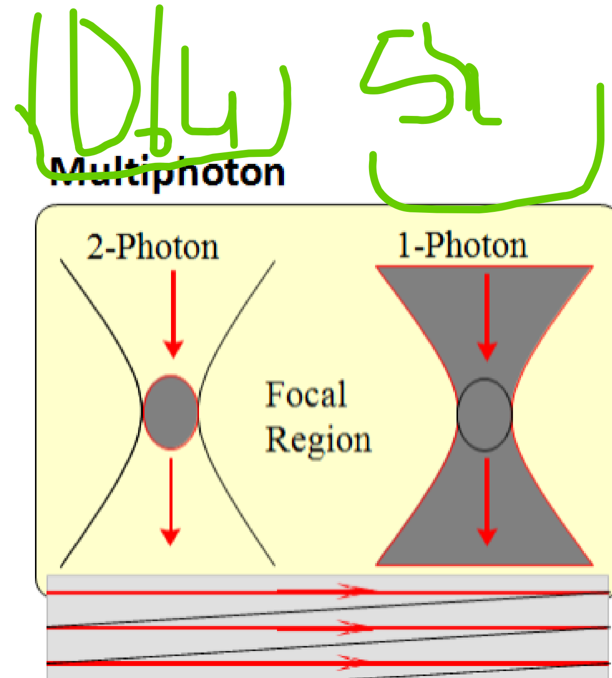
1. Optical scanning using galvano mirrors
2. Spinning disc confocal microscope
3. Multi-photon confocal microscopy

Point Scanning Confocal

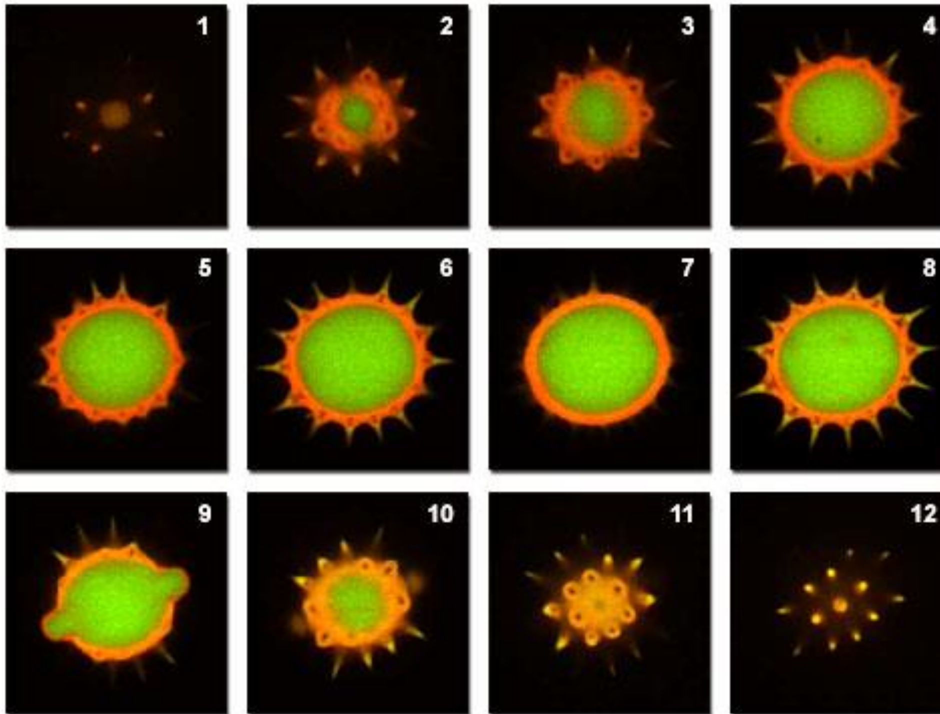


Spinning Disk Confocal





Z- axis imaging using confocal microscopy



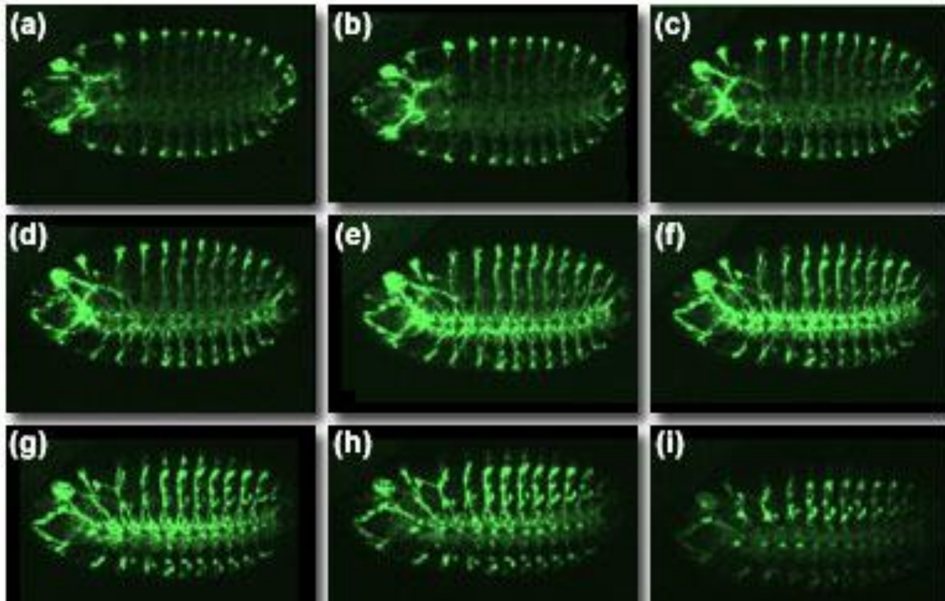
Z axis stack of optical sections through a sunflower pollen grain. It reveals internal variations in autofluorescence emission wavelengths.

They represent about 3 μm distance of focal planes.
It is about the size of 25 - 40 microns in diameter.
12 * 3μm = 40 μm approximately.

sunflower pollen grain revealing internal variations in autofluorescence emission wavelengths

Z-Series and Three-Dimensional Imaging

In confocal microscope, images in Z axis are collected by coordinating step-by-step changes in the fine focus of the microscope with sequential image acquisition at each step.



Computer-controlled stepping motor that changes focus by predetermined increments therefore the focusing on the sample plane can be adjusted.

In summary

- In wide field microscopy, images are formed by lenses from all specimen points simultaneously. Disadvantage is that image is formed by both in-focus and out focus light. Remember every objective comes with certain range of depth. Deconvolution technique could be used by collecting series images through different depths and reconstructing the image.

1/9	1/9	1/9
1/9	1/9	1/9
1/9	1/9	1/9

-1	-1	-1
2	2	2
-1	-1	-1

Horizontal lines

-1	2	-1
-1	2	-1
-1	2	-1

Vertical lines

-1	-1	2
-1	2	-1
2	-1	-1

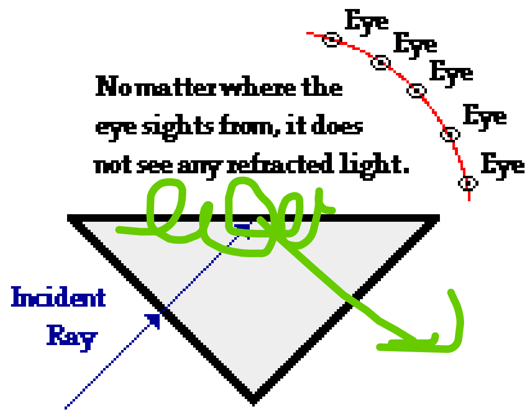
45 degree lines

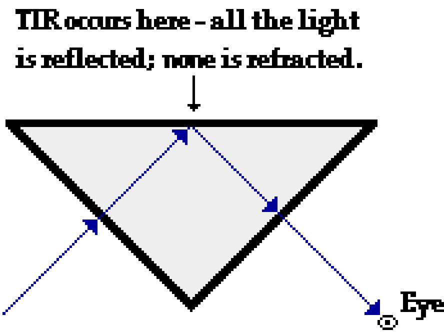
2	-1	-1
-1	2	-1
-1	-1	2

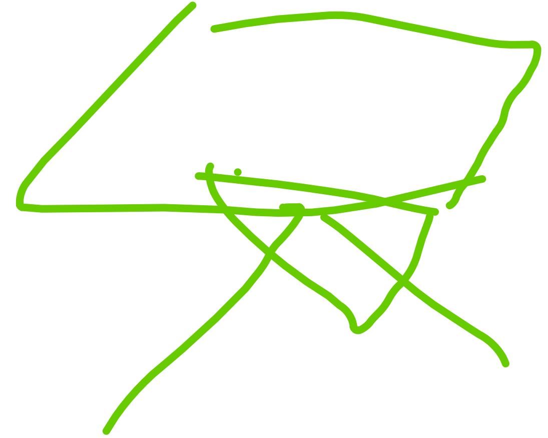
135 degree lines

TIRF MICROSCOPY

The Discrepant Event - Total Internal Reflection







Air

n_2

n_1

Water

θ_2

Refracted ray

Incident ray

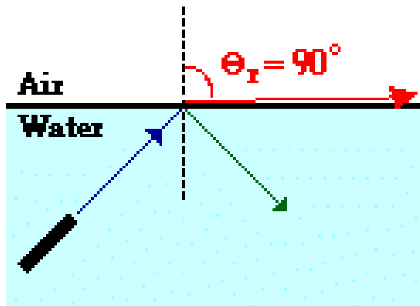
θ_1

Critical angle

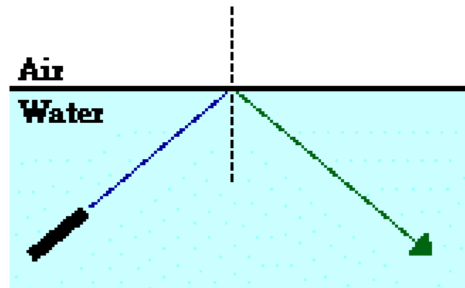
θ_c

Total internal reflection

θ_1 θ_2

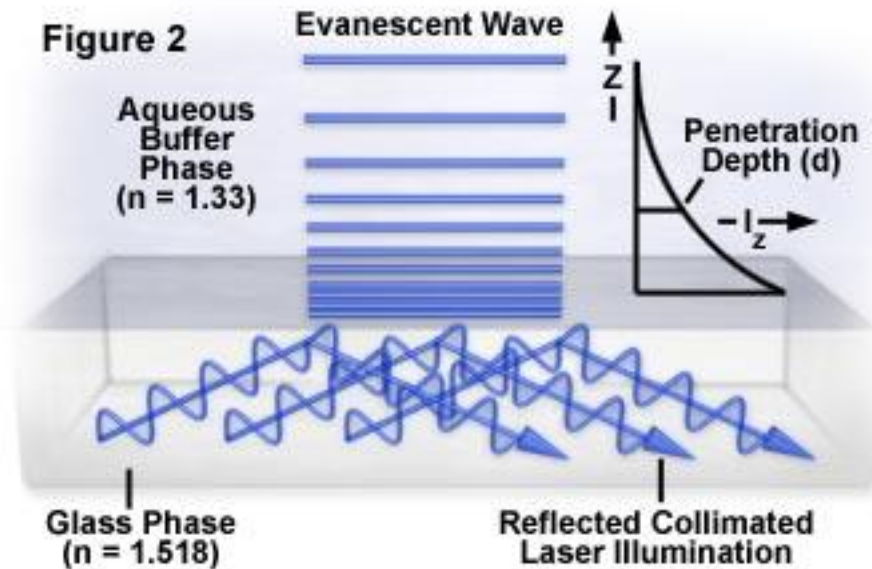


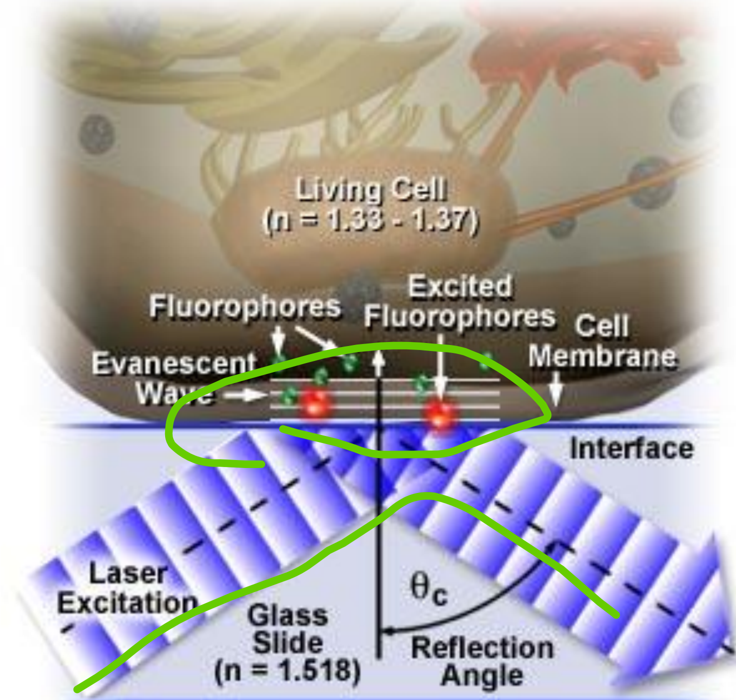
When the angle of incidence equal the critical angle, the angle of refraction is 90-degrees.



When the angle of incidence is greater than the critical angle, all the light undergoes reflection.

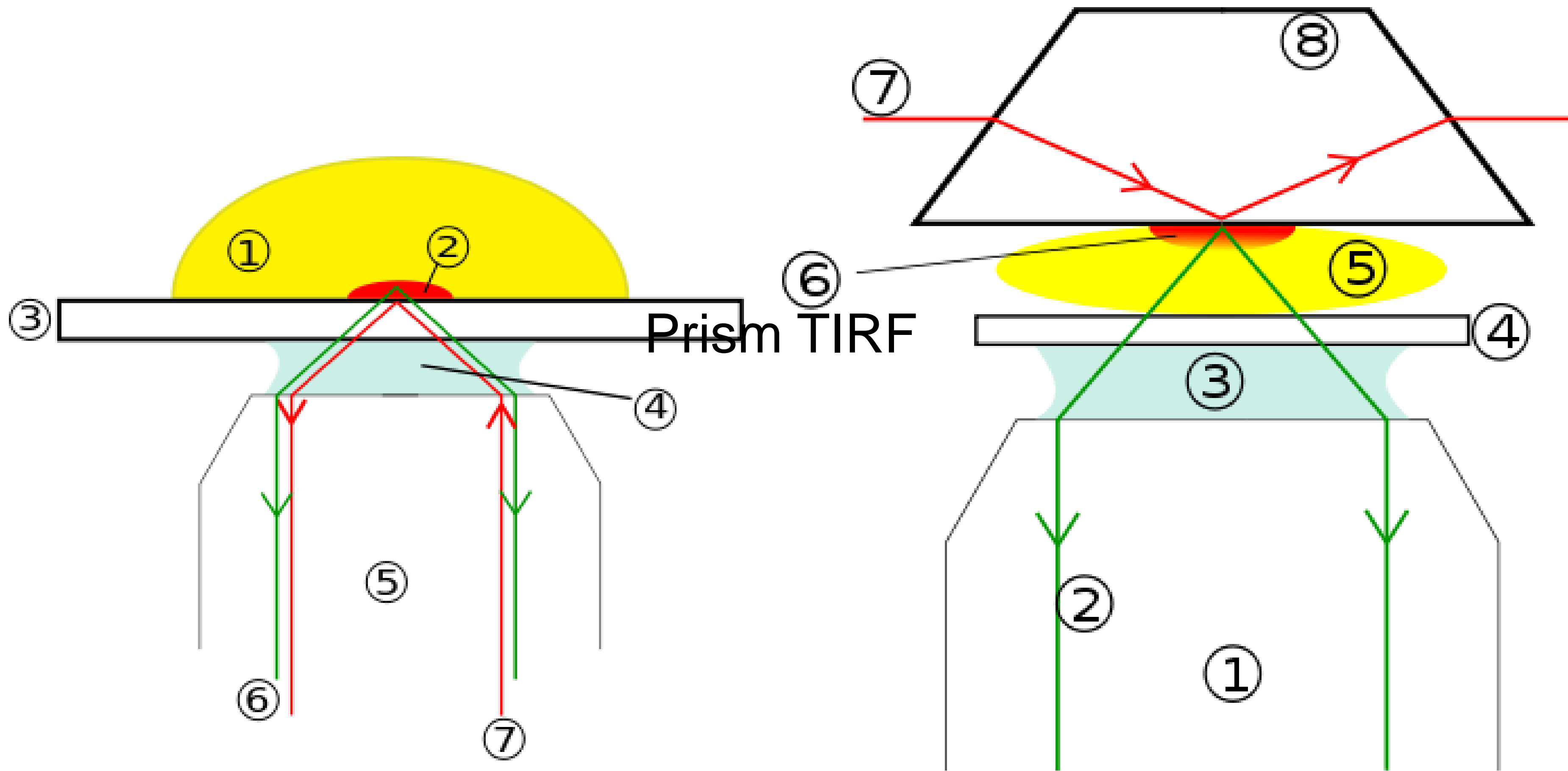
Distance (Nanometers)	Relative Intensity
1	0.99
10	0.92
100	0.43
1000	0.0002





Objective vs. Prism TIRF

Less than 150-200 nm thick evanescent field can be observed through tirf system.



Polarization of light

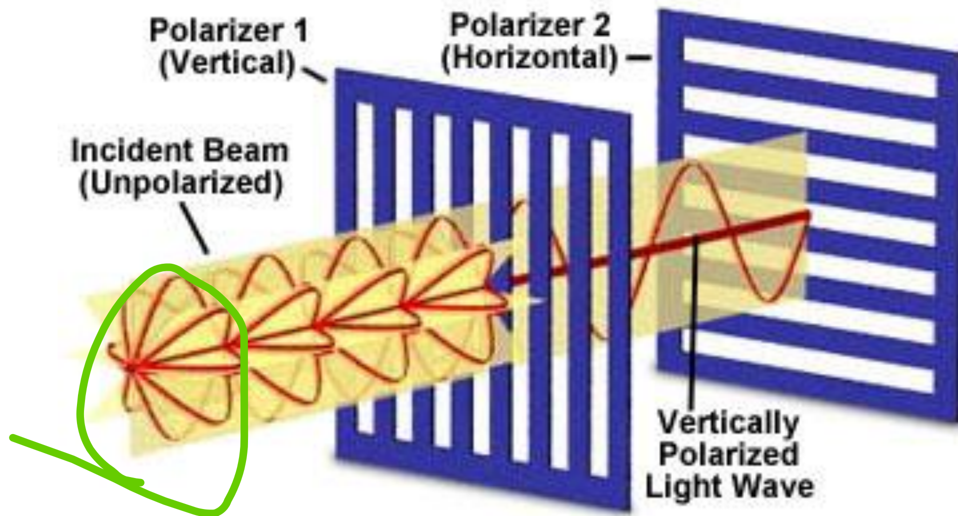


Figure 1

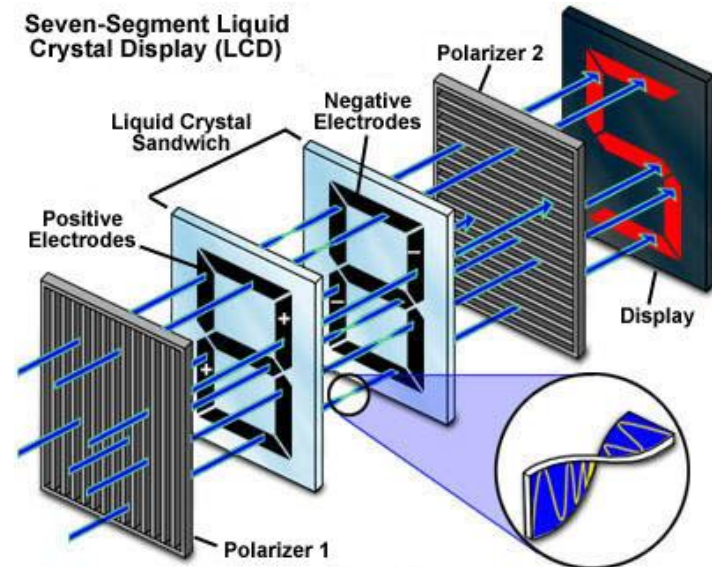
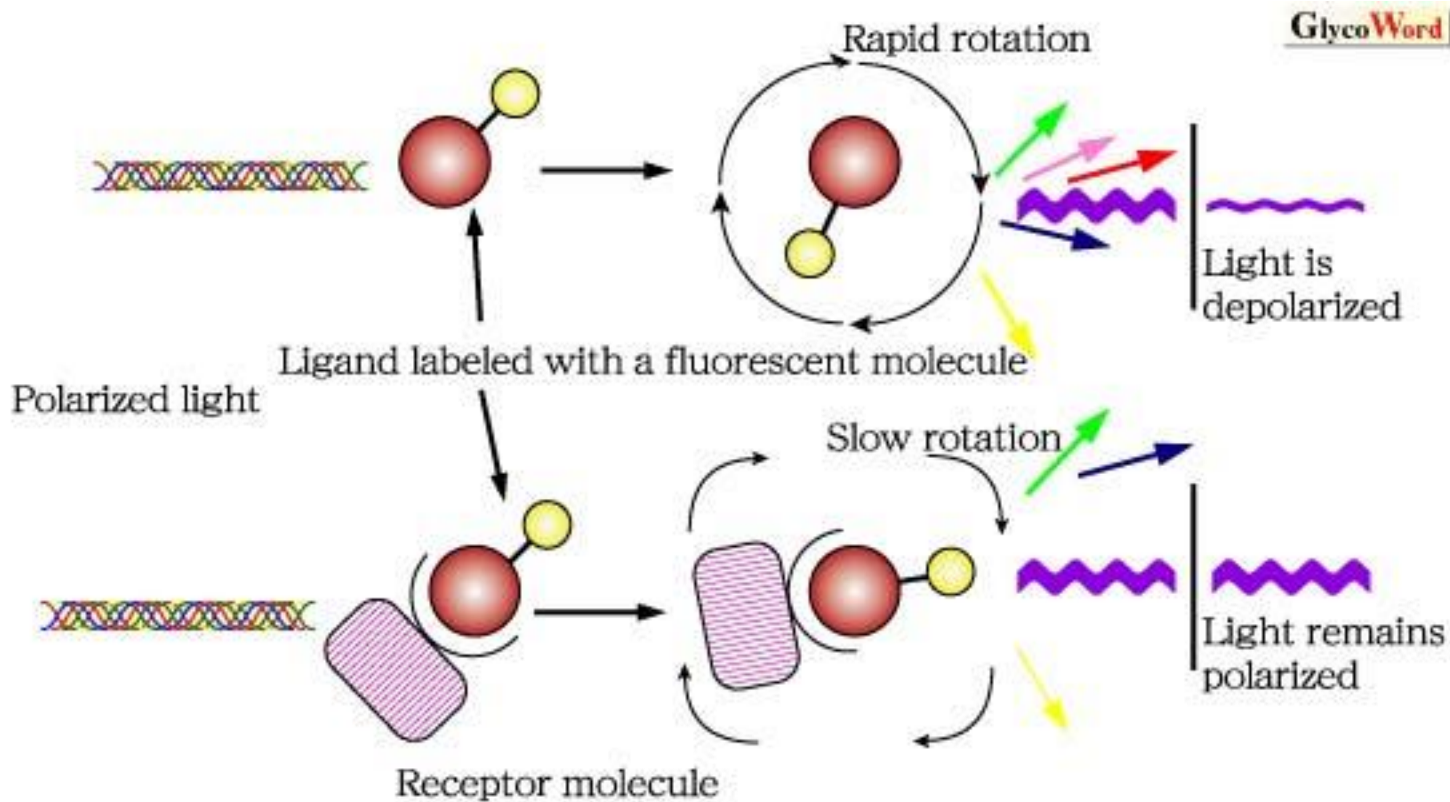


Figure 3

Excited with a plane-polarized light, emit light into a fixed plane if the molecules remain stationary during the fluorophore's excitation.



However, the molecule will emit light into a different plane if the molecule rotates and tumbles during the fluorophore's excitation. Therefore, when a fluorescent molecule binds to a large molecule such as protein, the emitted light is obviously less depolarized

The fluorescence polarization (P) of a labeled macromolecule depends on the fluorescence lifetime (τ) and the rotational correlation time (Tc)

$$Tc = nV/ kT$$

Or

$$Tc = (nMW/RT)\ (V+h)$$

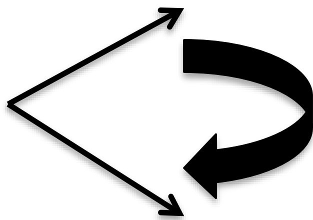
R is ideal gas constant
K is boltzman constant
n is viscosity
V molecular volume
MW molecular weight
H is hydration
V is specific volume

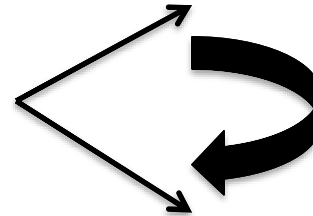
Lets consider a small protein

If Tc >> Tf ro / r >> 1 very low anisotropy

Tc = nV/ RT

Tc = n Vlow/ RT Tc is very short





Tc is very short

Tc is very long

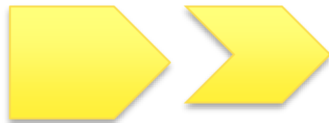
Fluorescence polarization is very important for high throughput assay where many drugs can be tested for a given target molecules. Fluorescence polarization (FP) is a technique for analyzing the interaction of molecules.

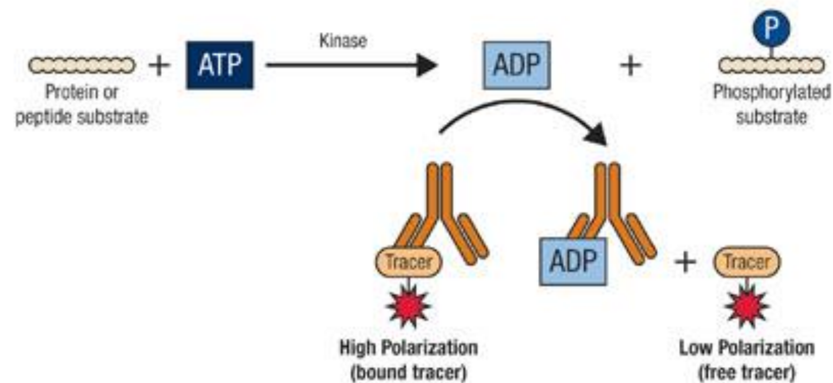
Small fluorescent ligand + Protein target $\rightleftharpoons$ PL

Fast rotation
Low R

Slow rotation
High R

Fluorescence Polarization Immunoassay
Patented by Abbott labs = $ million

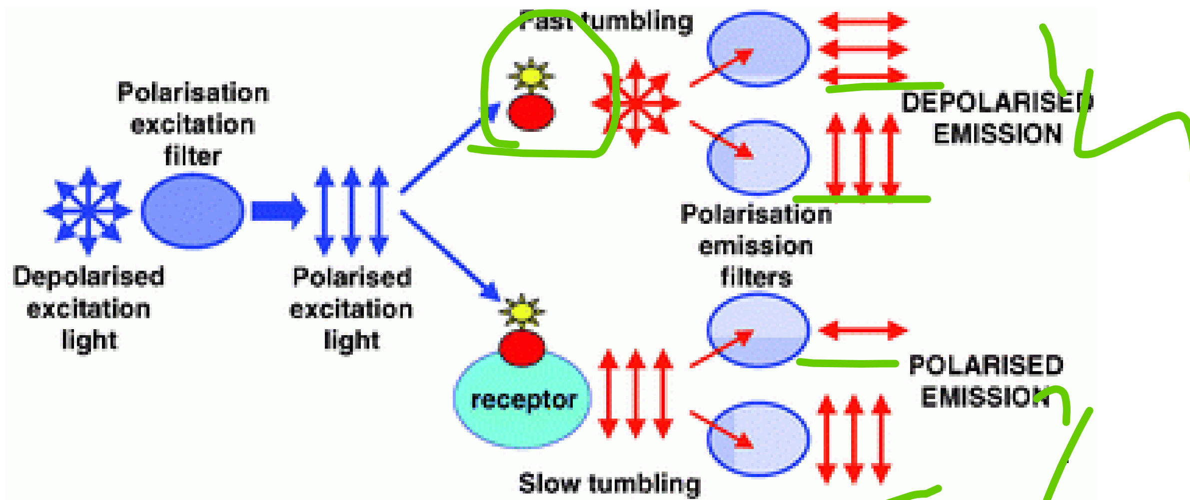


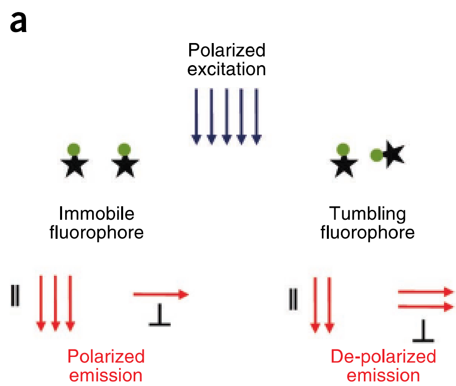


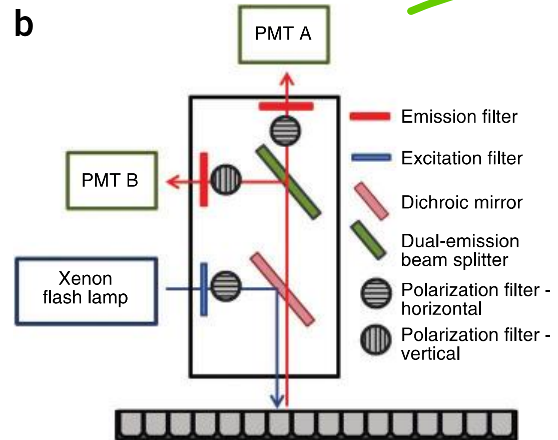
When a drug molecule tagged with a fluorophore binds to the target molecule, polarization changes

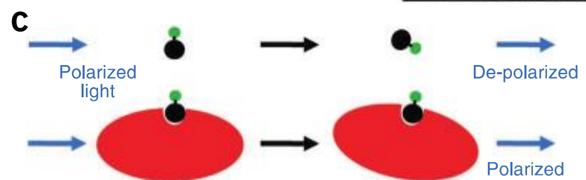
it does not require immobilization or washing step, and it is used in immunoassay, and therapeutic drug monitoring in clinical pharmacy.

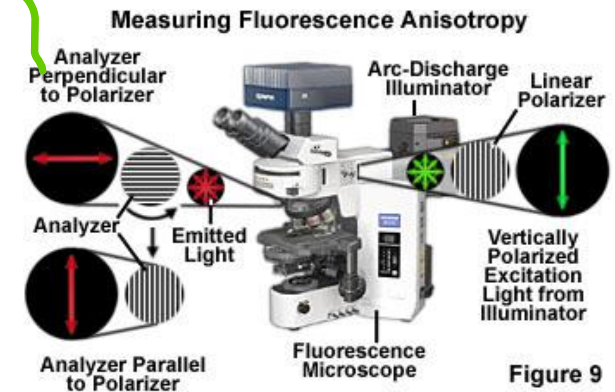
Remember that faster rotation of molecules





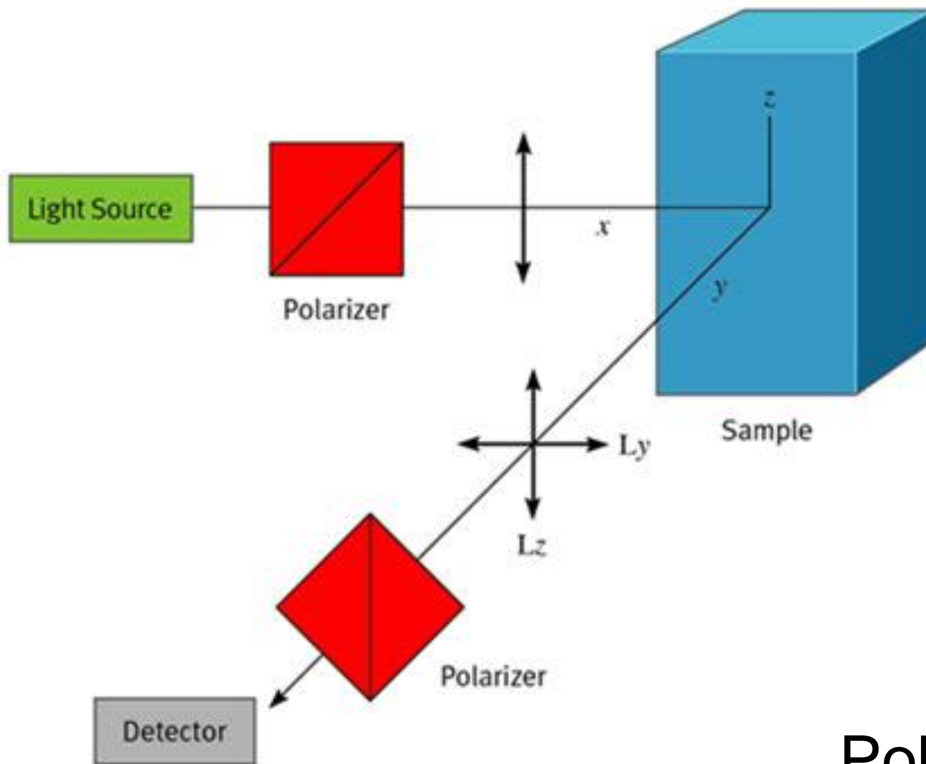






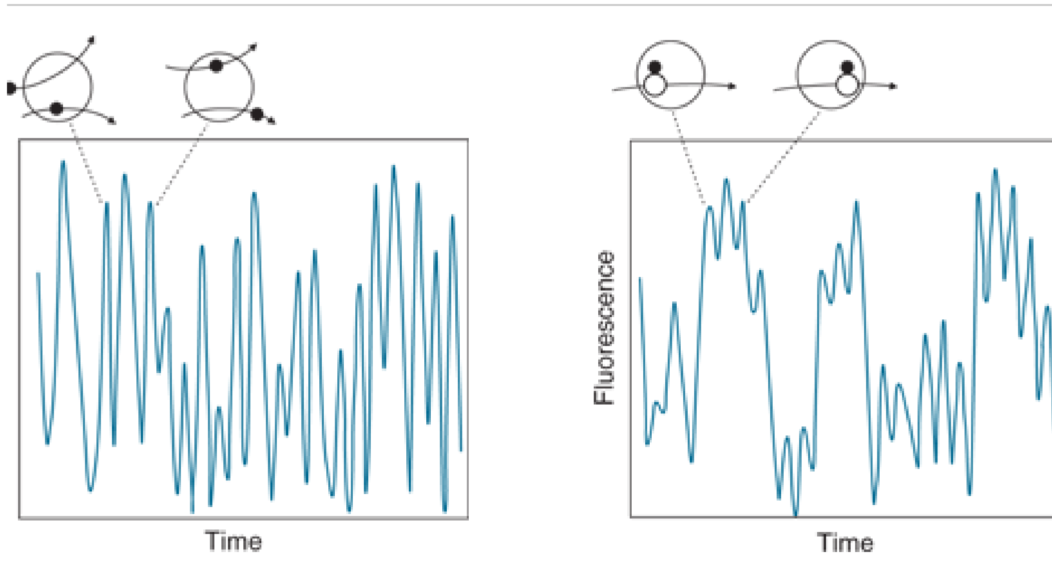
Optical Setup for polarization microscopy

In a typical experiment the sample containing the fluorescent probe is excited with linear polarized light and the vertical and horizontal components of the intensity of the emitted light are measured and the polarization (P) or anisotropy (r) are calculated using the following equations:

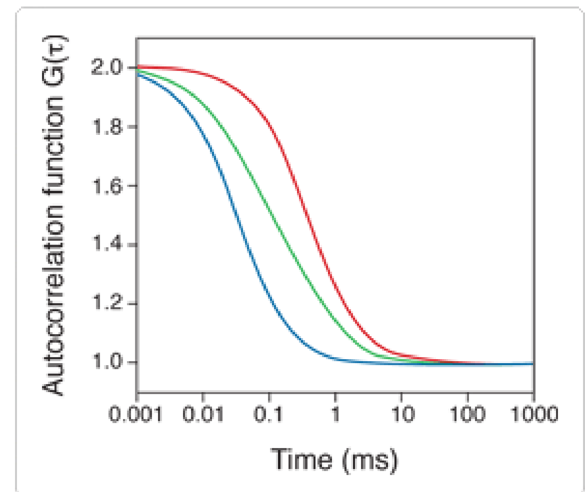


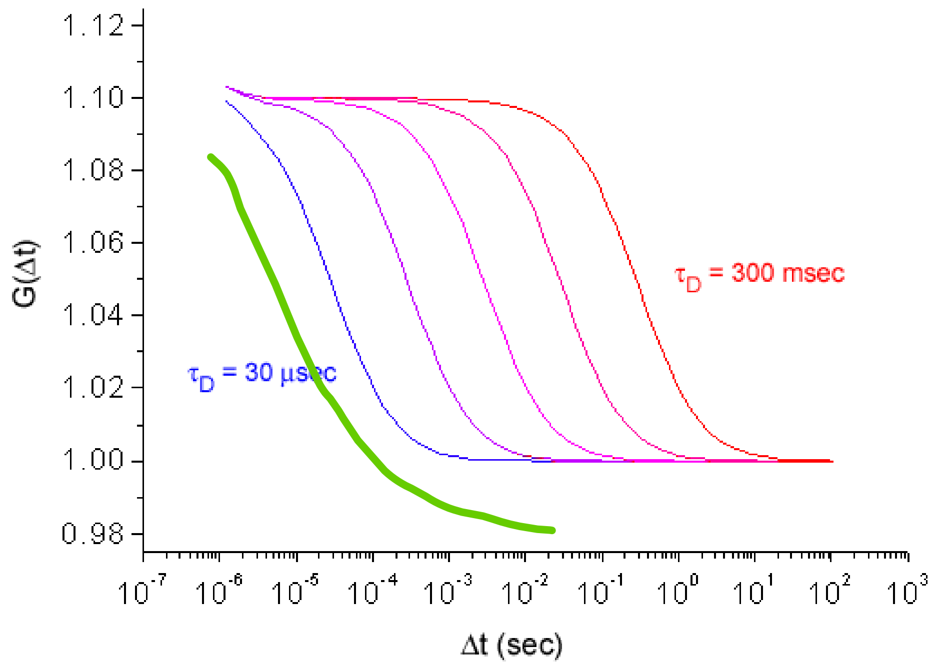
Polarization (P) = $(I_v - I_h) / (I_v + I_h)$ Anisotropy (r) = $(I_v - I_h) / (I_v + 2I_h)$

Fluorescence Correlation Spectroscopy

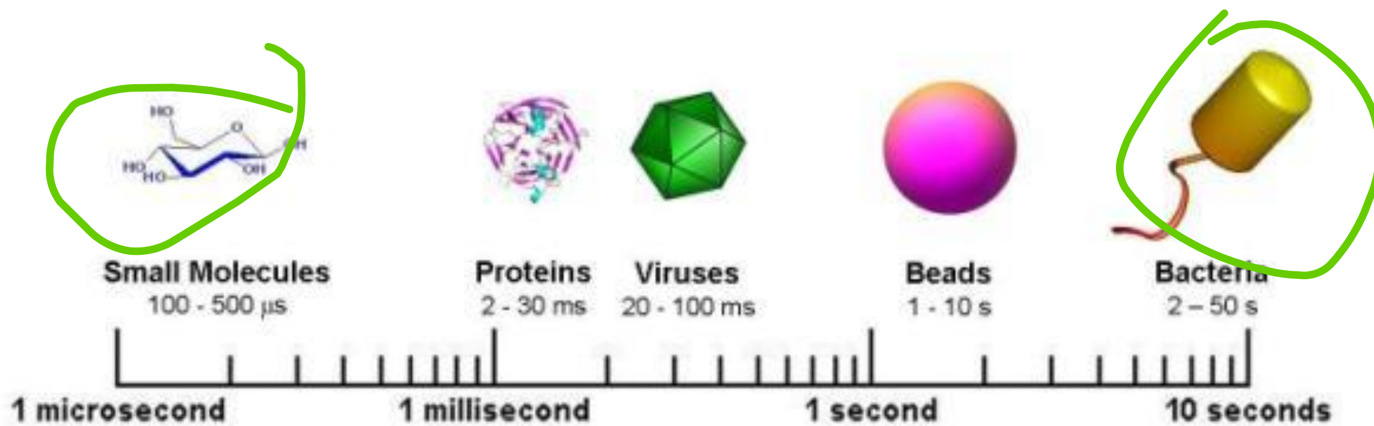


$$G(\tau) = \frac{\langle \delta F(t) \bullet \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$





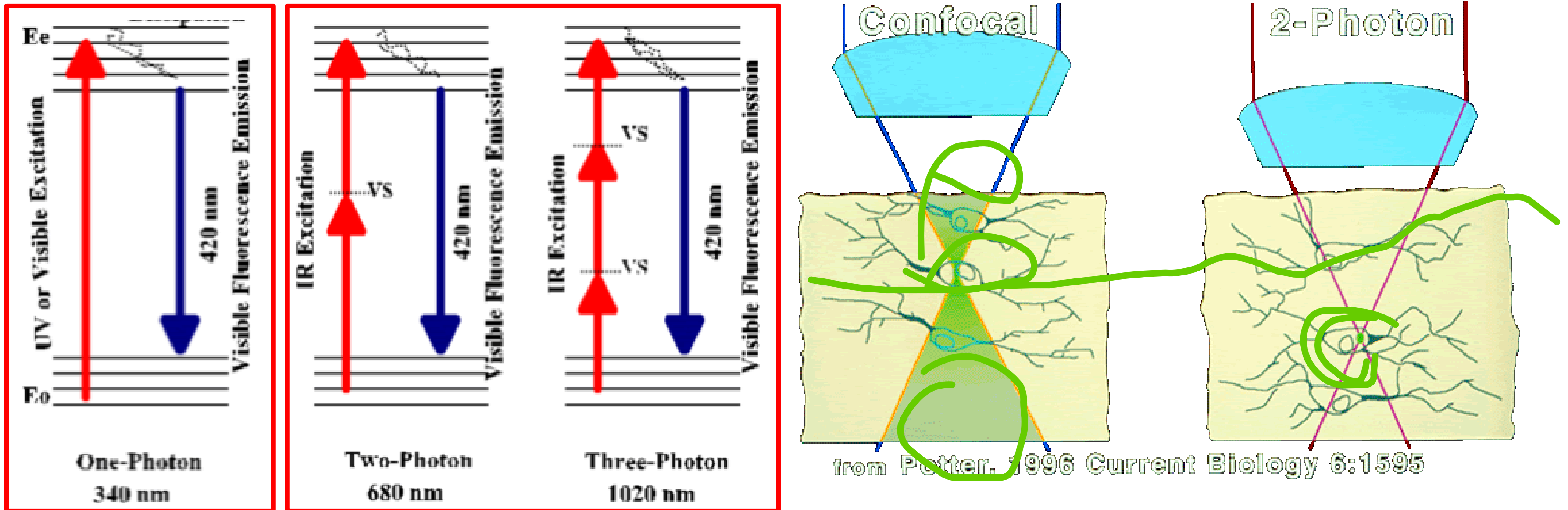
Correlation persists longer for slowly diffusing particles and decays quickly for rapidly diffusing particles



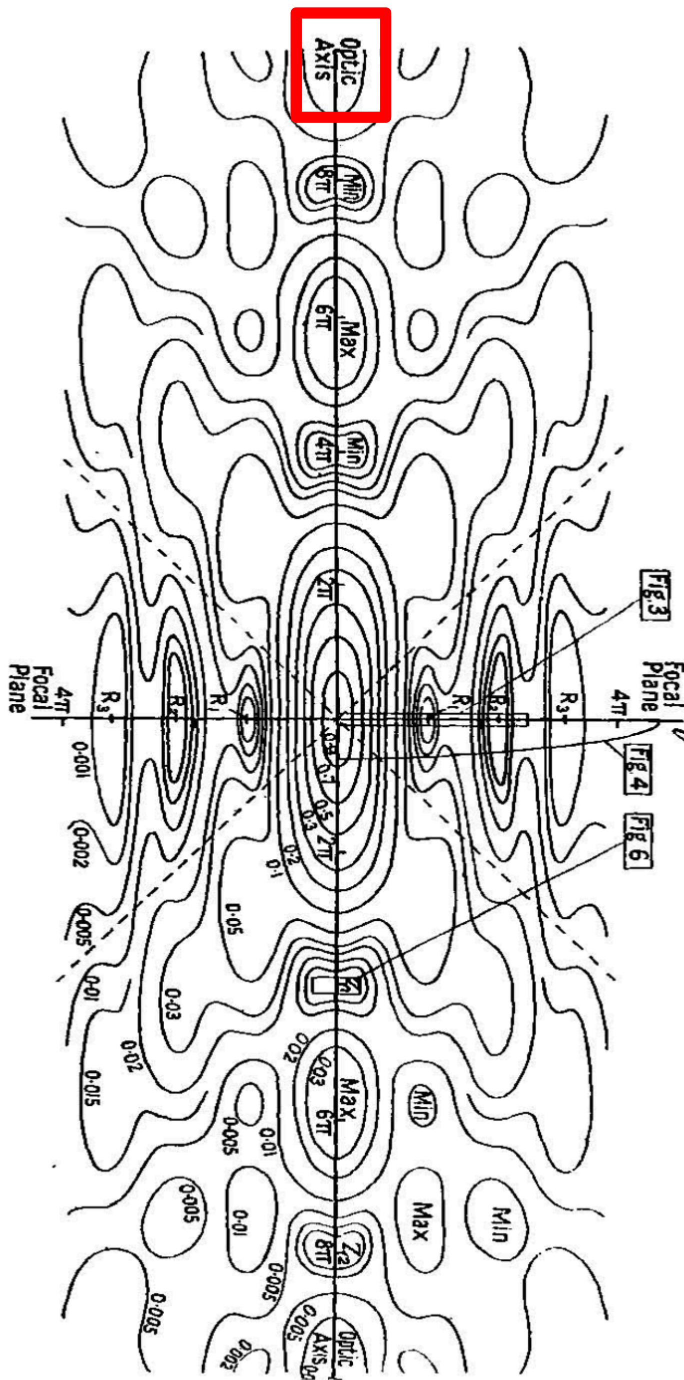
Two-Photon Excitation

Two-photon excitation requires the absorption of two photons of theoretically double the wavelength usually required for the excitation, within the tiny time interval of about one femtosecond (10-15 s). In order to get a reasonable probability of such three-particle events, the photon flux must be extremely high. This means, that not only a high output power is required, but usually also pulsed excitation is used, to get an even higher photon density per pulse relative to the average output power. The joint probability of absorbing two photons per excitation process is proportional to the mean square of the intensity. This results in inherent depth discrimination such that only the immediate vicinity of the objective's focal spot receives sufficient intensity for significant fluorescence excitation.

Confocal v. Multi-Photon



Optical sections do not require pinhole.



Point Spread Function (PSF) sample

We learned in class that a real point object, once captured by a lens, produces an image that is only an approximation of the object. The lens transforms the object of discreet dimensions and the result is shown to the left.

The point is no longer a point and the resultant object is called a point spread function (PSF). Since an object of any form and size is just a collection of points, we should understand how a lens (microscope) distorts a single point. We will use the fluorescent microscope to observe the PSF and compare it to the PSF formed through optical sectioning methods.

The sample is a fluorescent latex bead, 175nm +/- 5nm in diameter and embedded with a fluorescent dye. The excitation/emission wavelength is 505 nm/515 nm.

Airy disc PSF in the image plane is produced by an point source in the object plane