

Fluorescence Microscopy

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I am greatly indebted for this presentation to Professor Enrico Gratton.

type	feature	appearance	uses
Bright-field	Visible light	Colored/clear specimen Light background	Stained specimen with sufficient color contrast
Dark-field	Visible light	Bright specimen Dark background	Unstained or difficult to stain specimen
Phase contrast	Visible light / phase shifting	Different degrees of brightness and darkness	internal structure of specimen
Differential Interference contrast	Visible light out of phase	Nearly 3D image	Fine details of internal structure of unstained specimen
Fluorescence	UV-IR light	Fluorescence specimen	Detection of molecules, organisms, antibodies in clinical specimens
Transmission Electron Microscope	Electron beam / magnetic lens	High magnification	Internal structures; viruses
Scanning Electron Microscope	Electron beam	3D view of surfaces	External or internal surfaces of cells
Scanning Tunneling	Wire probe over surfaces	3D view of surfaces	Observation of external surfaces of atoms or molecules

What is a Fluorescence Microscope?

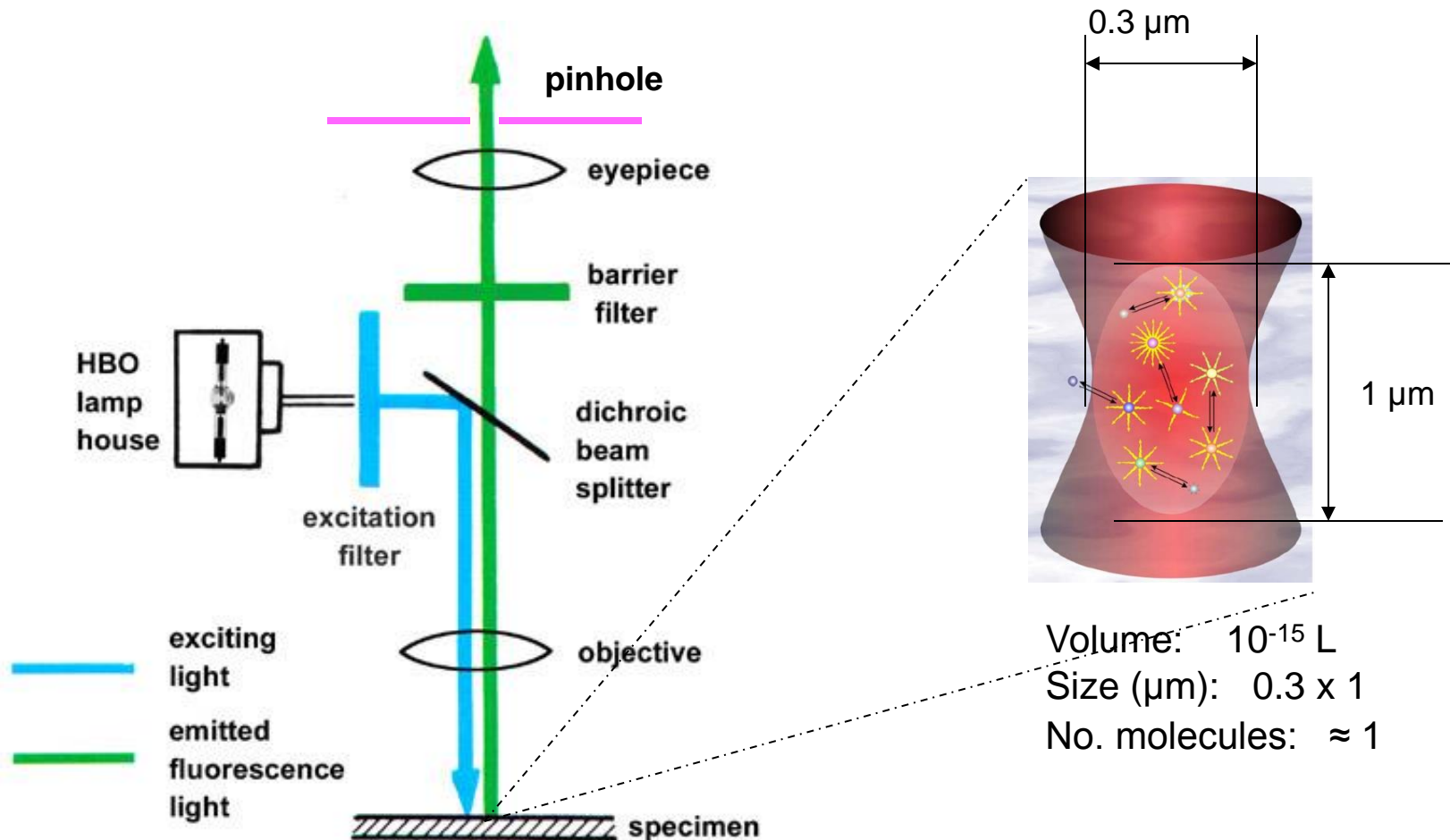
The "fluorescence microscope" refers to any microscope that uses fluorescence or phosphorescence to generate an image.

Two geometries:

upright
epifluorescence

Two general illumination schemes:

full field
confocal





Frits Zernike

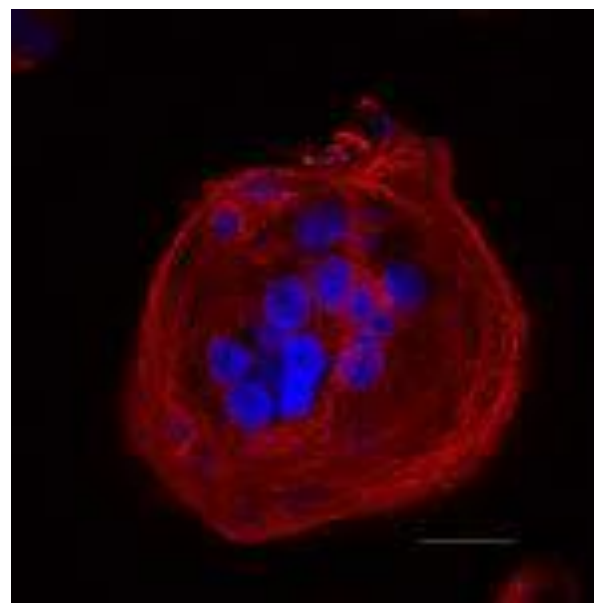
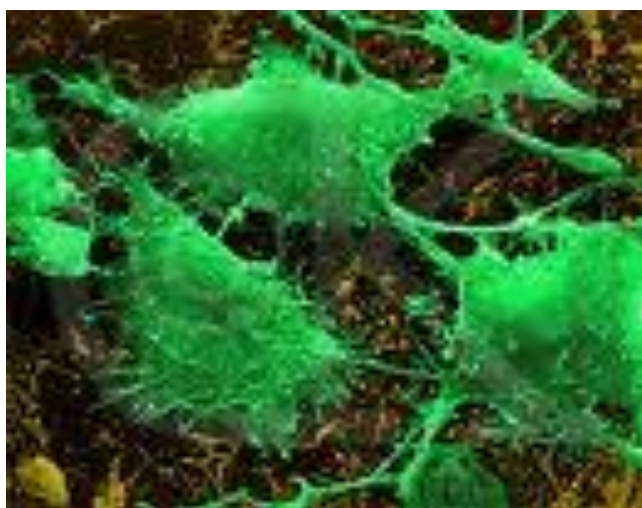
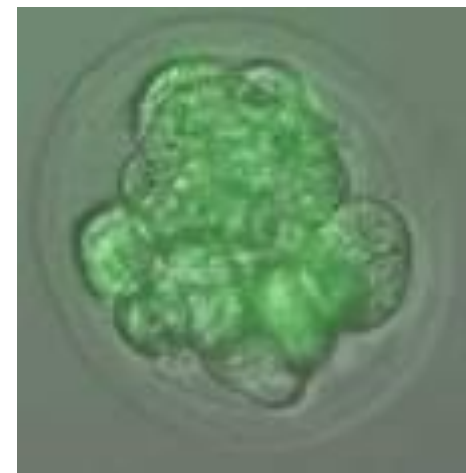
The Nobel Prize in Physics 1953

How I Discovered Phase Contrast

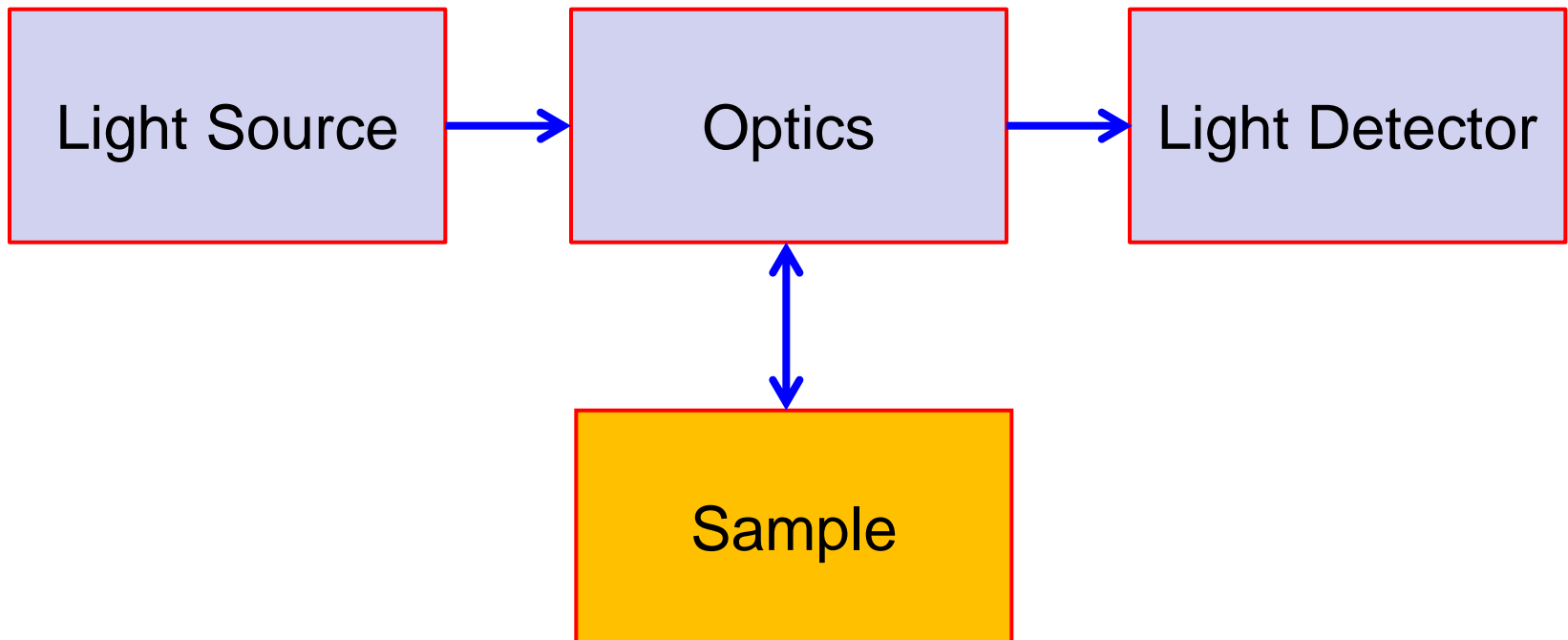
F. Zernike

Department of Physics, University of Groningen, Netherlands

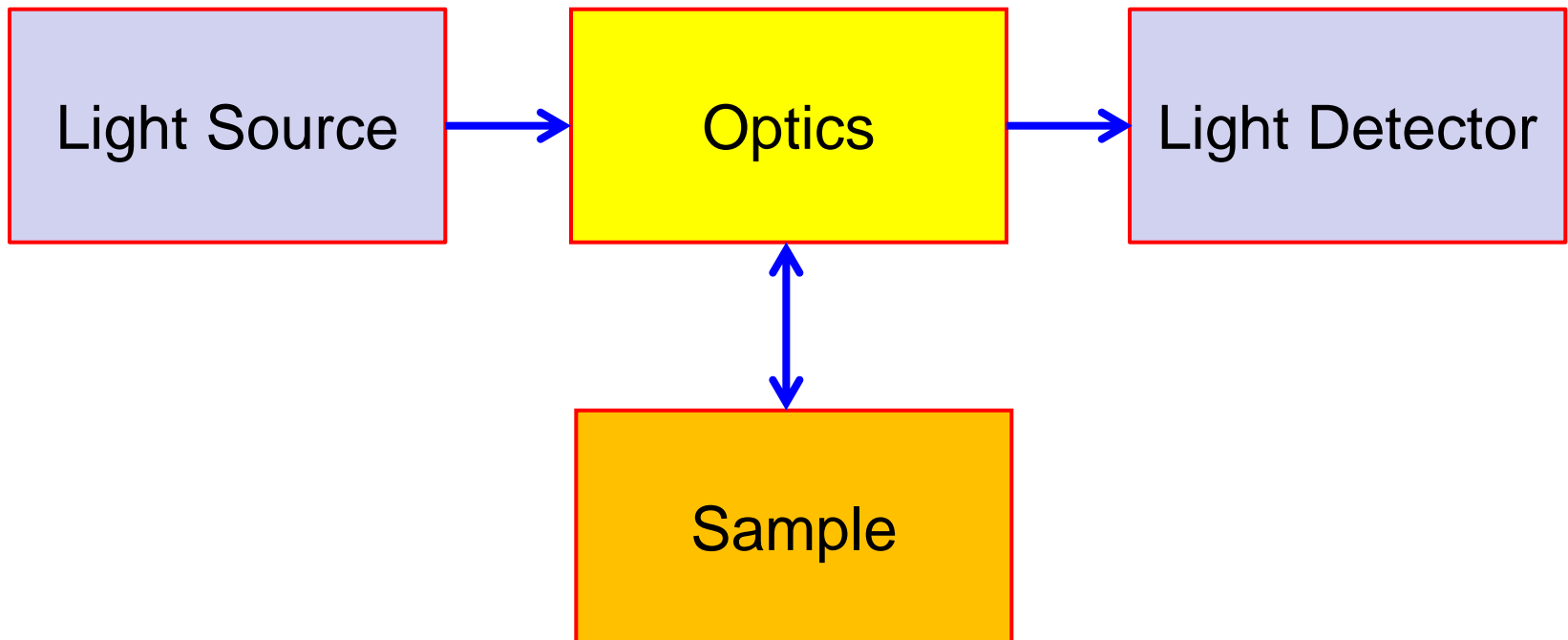
On looking back to this event, I am impressed by the great limitations of the human mind. How quick we are to learn – that is, to imitate what others have done or thought before – and how slow to understand – that is, to see the deeper connections. Slowest of all, however, we are in inventing new connections or even in applying old ideas in a new field.



A typical microscopy setup



A typical microscopy setup



The Microscope

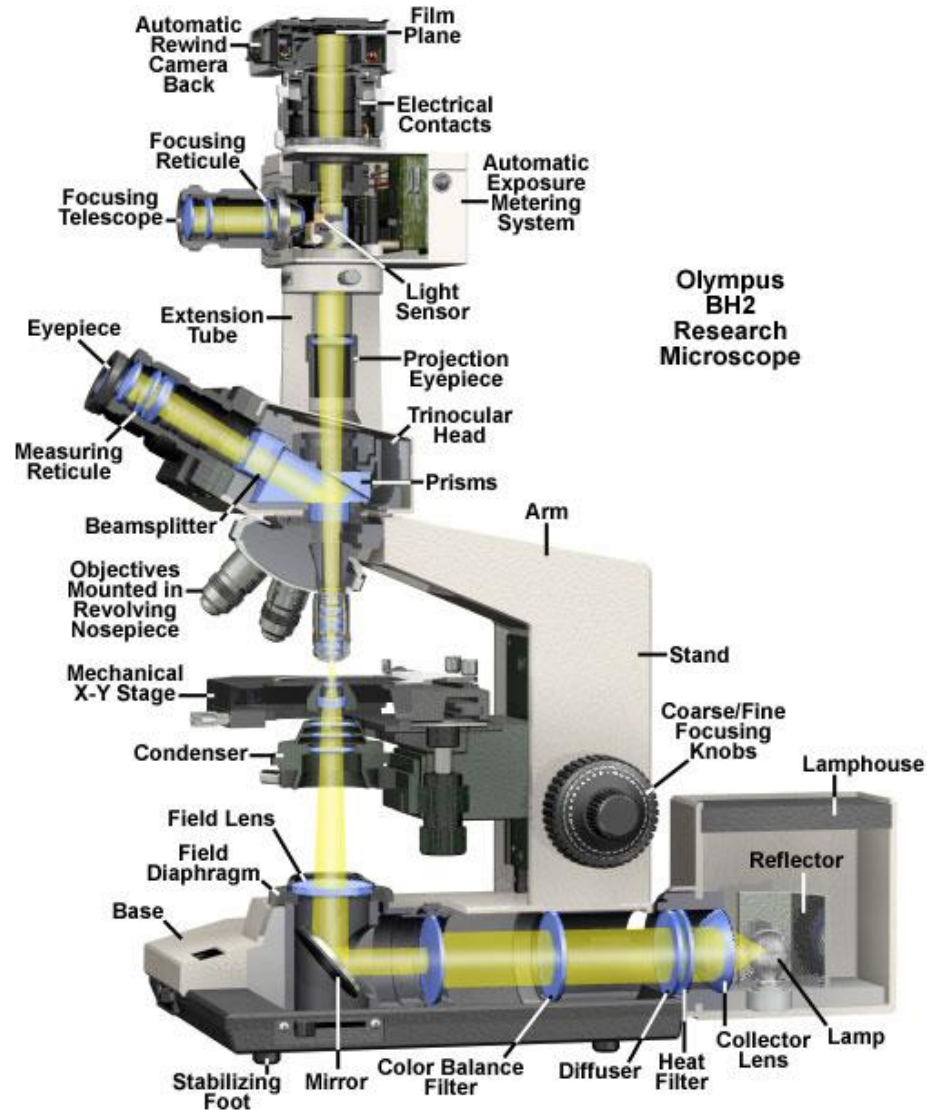
- Structure
- Objectives
- Koehler illumination
- Slides and coverslips
- Contrast and resolution

Fluorescence Microscope

Confocal Microscope

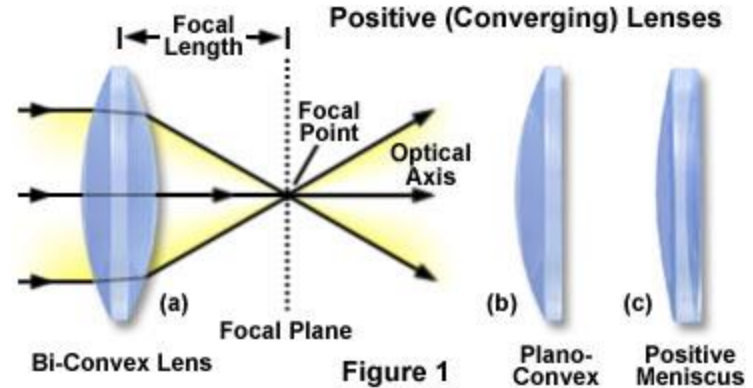
- PSF
- Multiphoton excitation

Olympus BH2 cutaway Diagram



Optics:

Lenses, prisms, mirrors



Some problems need to be addressed:

Chromatic aberrations

Spherical aberration

Coma

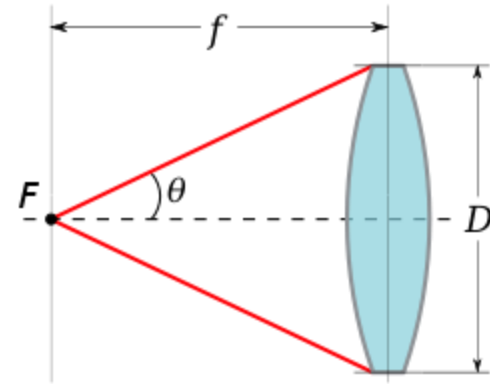
Astigmatism

Distortion

A few useful parameters:

Numerical aperture $NA = n \sin \theta$

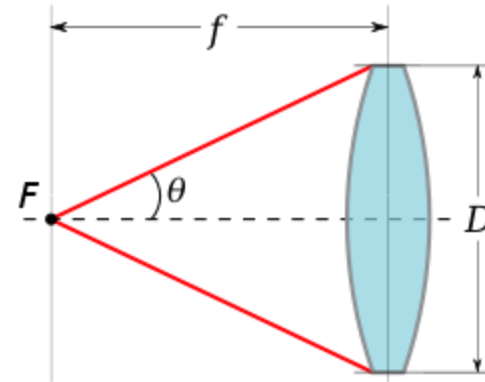
F-number $f / \# = \frac{f}{D} = \frac{1}{2 NA}$



Why are they useful?

When a lens forms the image of an extended object, the amount of energy collected from a small area of the object is **1) directly proportional** to the area of the **clear aperture**, or entrance pupil, of the lens. **2) At the image**, the illumination (power per unit area) **is inversely proportional** to the **image area** over which this object is spread. The aperture area is proportional to the square of the pupil diameter, and the image area is proportional to the square of the image distance, or square of the focal length

$$f - number = \frac{f}{D} = \frac{1}{2 NA}$$



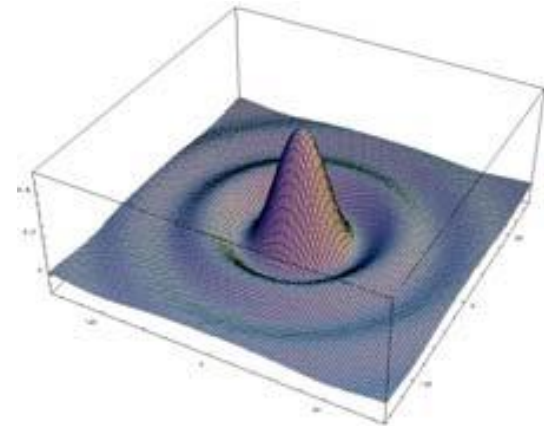
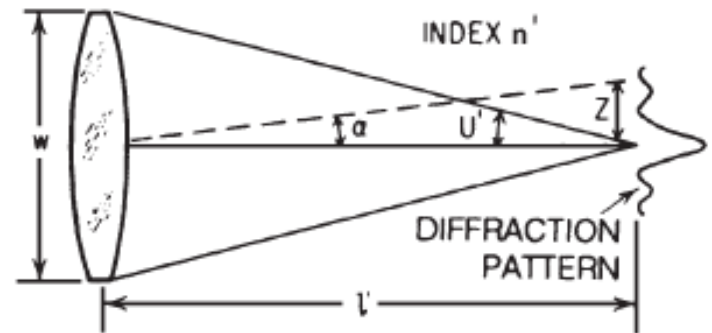
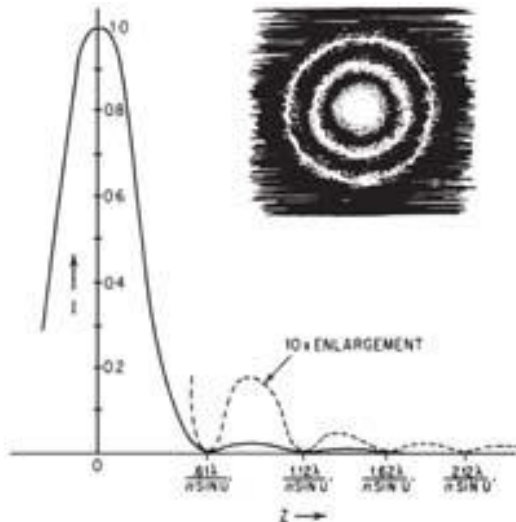
The above relationship is only true for aplanatic systems (i.e., systems corrected for coma and spherical aberration) with infinite object distances. The terms “fast” and “slow” are often applied to the f-number of an optical system to describe its “speed.” A lens with a large aperture (and thus a small f-number) is said to be “fast,” or to have a high “speed.” A smaller aperture lens is described as “slow.” This terminology derives from photographic usage, where a larger aperture allows a shorter (or faster) exposure time to get the same quantity of energy on the film and may allow a rapidly moving object to be photographed without blurring.

A system working at finite conjugates will have an **object-side numerical aperture** as well as an **image-side numerical aperture** and that the ratio NA/NA' (object-side NA)/(image-side NA) must equal the absolute value of the magnification.

Resolution of optical systems

When a bright **point source of light** is imaged, an Airy disk with a perfect lens, encircling rings appear in the image plane. The distance from the center of the Airy disk to the first dark ring is “z”:

$$z = \frac{1.22}{2} \frac{\lambda}{NA}$$



Two bright spots

Light from any point in a sample that passes through a circular aperture is diffracted and this diffraction distribution in the image sets the limit of resolution on an optical device such as a microscope or telescope.

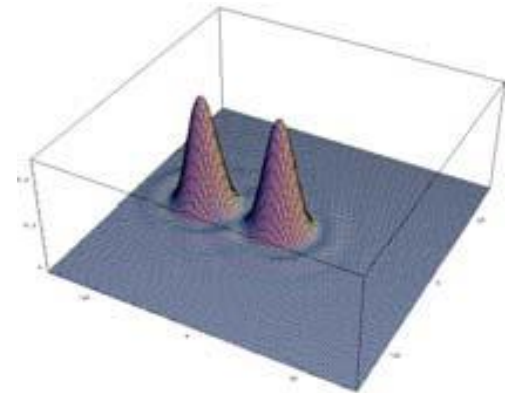
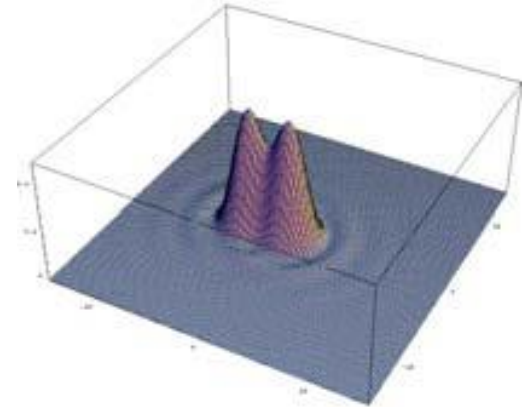
The limit of resolution between two sources is when the central spot of one Airy disk is on the zero of the other Airy disk. This is known as the Raleigh criterion.

This is the most used criterion for resolution. The limits are:

Diffraction limits for resolution of two sources

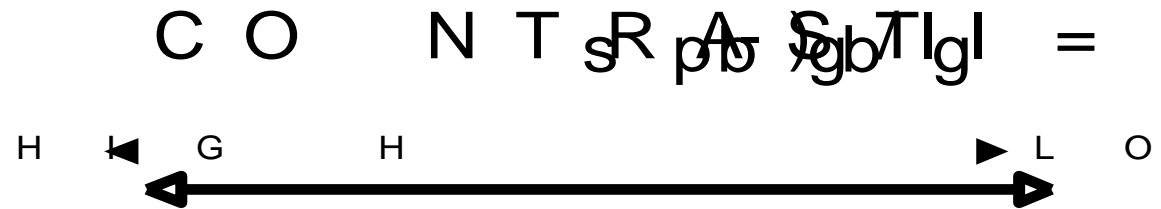
$$\Delta q = \frac{1.22 r \lambda}{D}$$

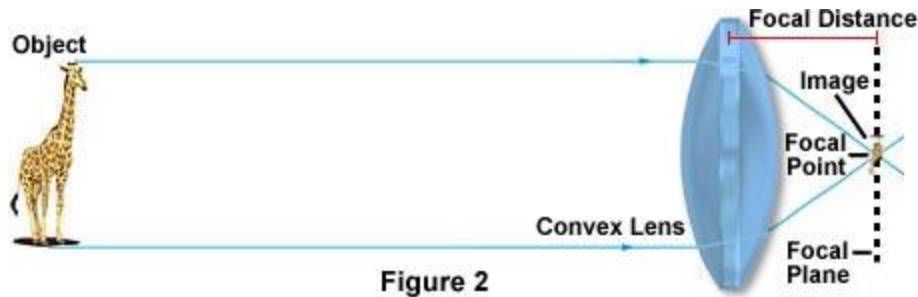
r –distance from object to image



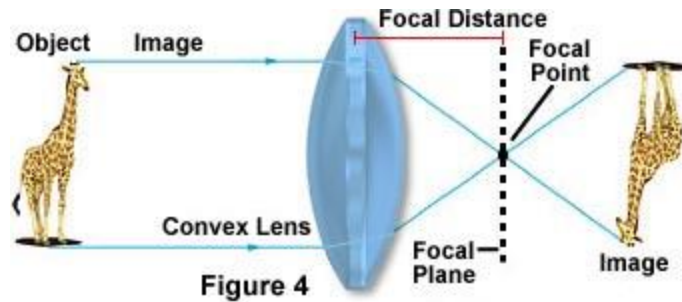
Contrast

All the resolution in the world won't do you any good, if there is no contrast to visualize the specimen.

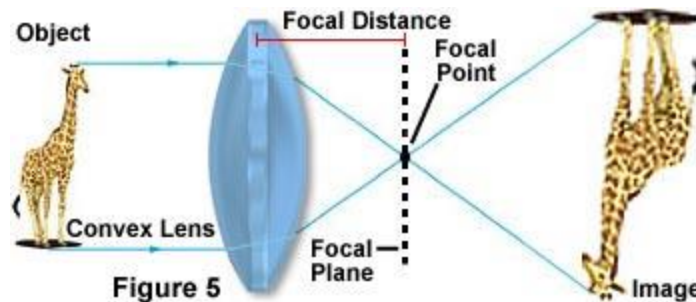




The object is far away

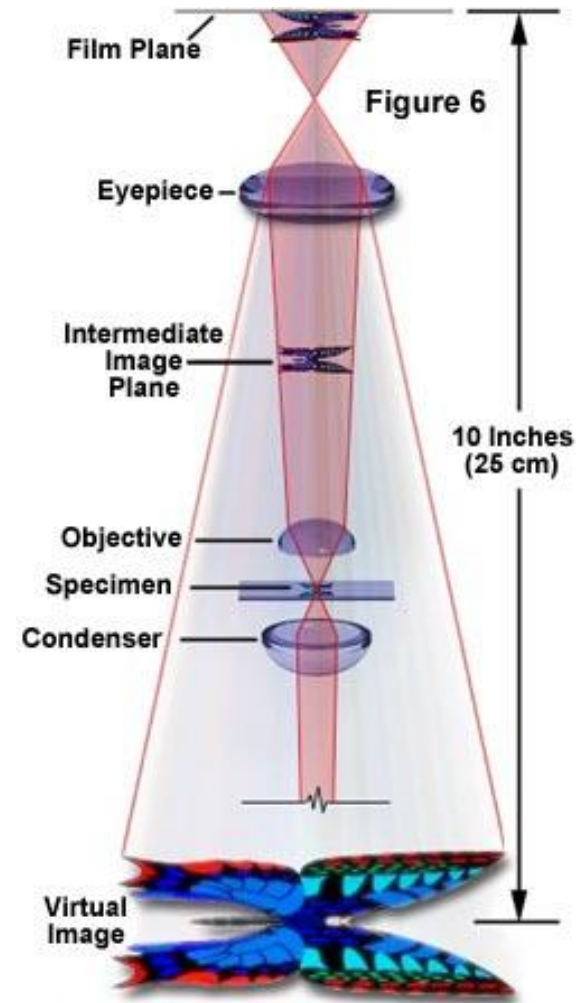


The object is at $2f$ from the lens. The image is at $2f$ beyond the focal length; it has the same size as the object, it is real and inverted



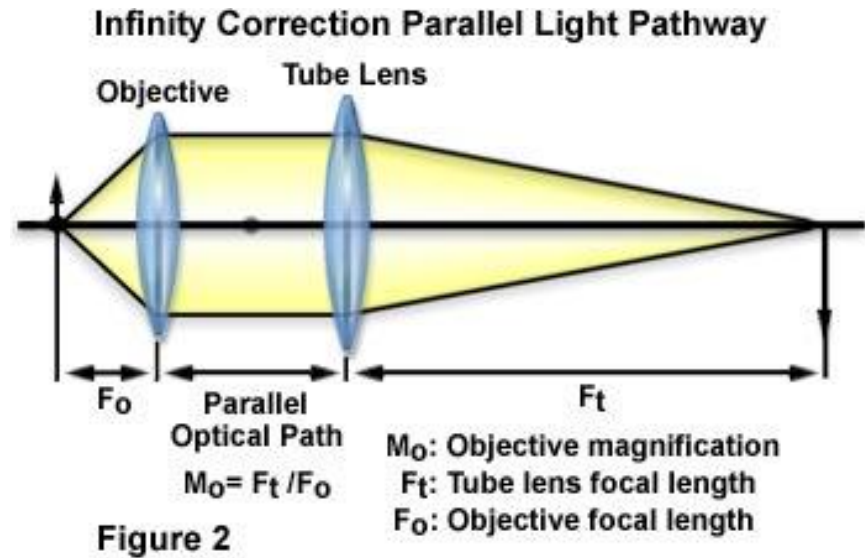
The object is at $2f < O < 1.5f$ from the lens. The image is magnified, inverted and real.

The last case listed above describes the functioning of the observation eyepiece of the microscope. The "object" examined by the eyepiece is the magnified, inverted, real image projected by the objective. When the human eye is placed above the eyepiece, the lens and cornea of the eye "look" at this secondarily magnified virtual image and see this virtual image as if it were 10 inches from the eye, near the base of the microscope.



Infinity corrected optics

Tube lens: 200 / 180 / 160 mm



objectives



Optical corrections

Achromatic: corrected
for chromatic aberrations
red/blue

Fluorites: chromatically
corrected for red, blue;
spherically corrected for 2
colors

Apochromatic:
chromatically corrected for
red, green, blues; spherically
corrected for 2 colors

Plan-: corrected for field
curvature

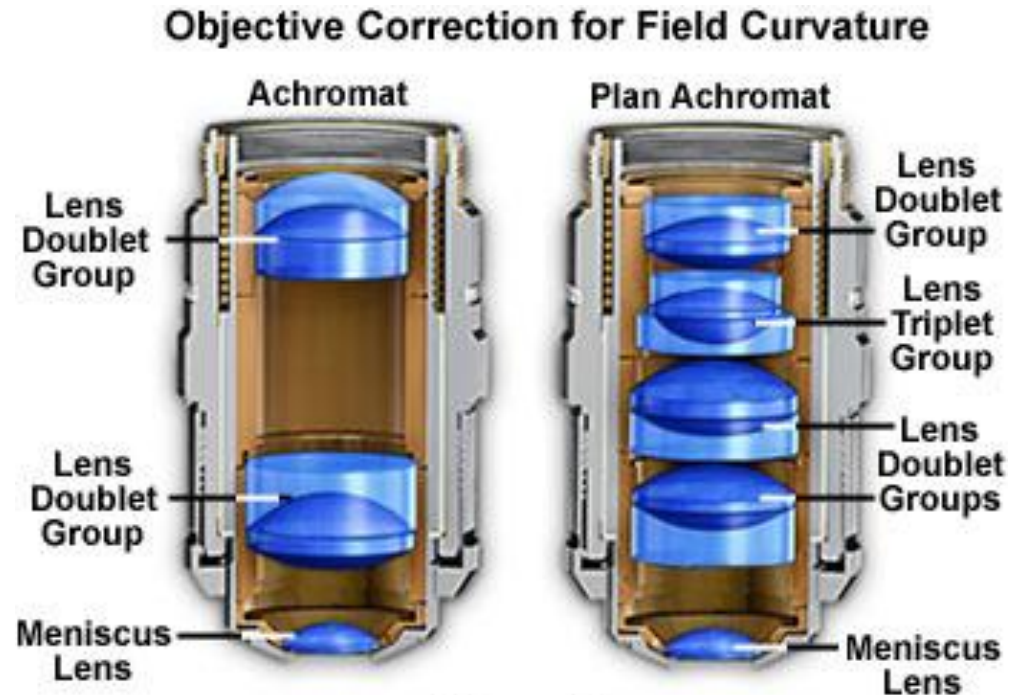
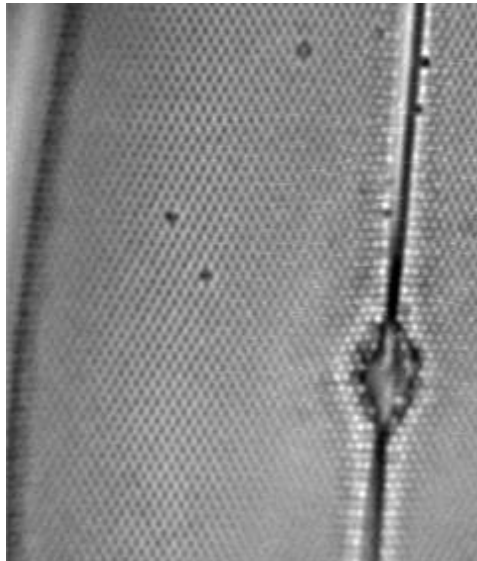


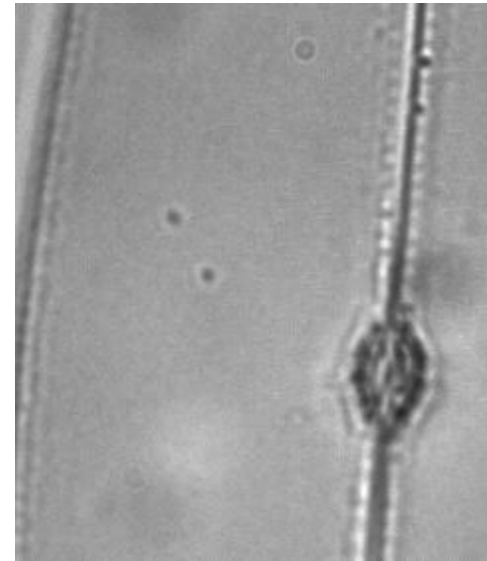
Figure 3

Resolution is better at shorter wavelengths, higher objective NA or higher condenser NA

$$r = \frac{1.22 \lambda}{2n \sin \theta} = \frac{0.61 \lambda}{NA}$$



High NA and/or shorter λ



Low NA and/or longer λ

Rayleigh Criterion for the resolution of two adjacent spots:

$$P_{\text{lim}} = 0.61 \lambda_o / \text{NA}_{\text{obj}}$$

Examples: ($\lambda_o = 550 \text{ nm}$)

	Mag	f(mm)	n	a	NA	$P_{\text{lim}} (\mu\text{m})$	($\text{NA}_{\text{cond}} = \text{NA}_{\text{obj}}$)
high dry	10x	16	1.00	15	0.25	1.10	
	40x	4	1.00	40	0.65	0.42	
oil	100x	1.6	1.52	61	1.33	0.204	
	63x	2.5	1.52	67.5	1.40	0.196	

Light sources:

Tungsten lamp

Halogen lamp

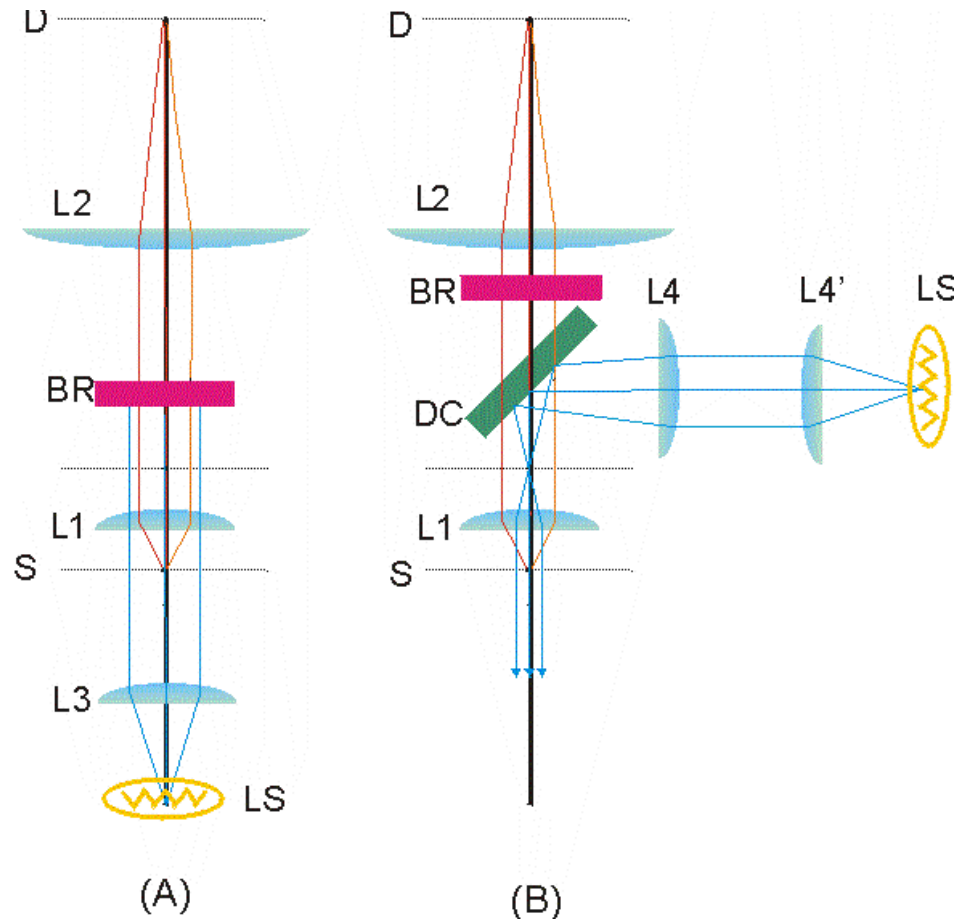
Mercury

LEDs

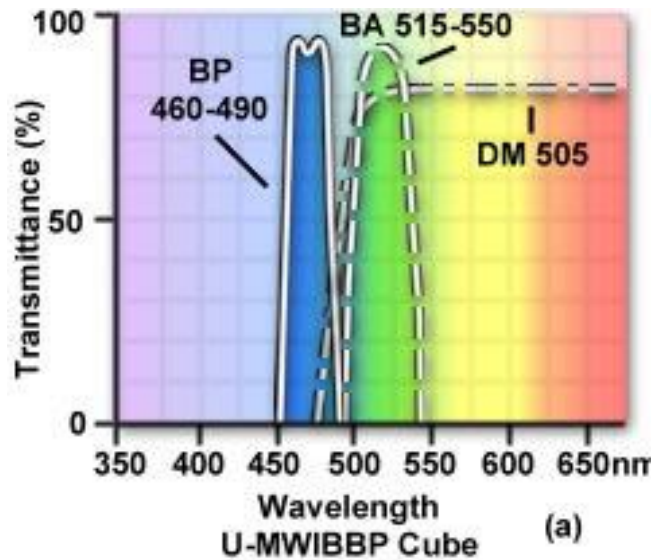
Lasers

The image of the source is not focused on the specimen

Köhler illumination



Filters and dichroics



Fig

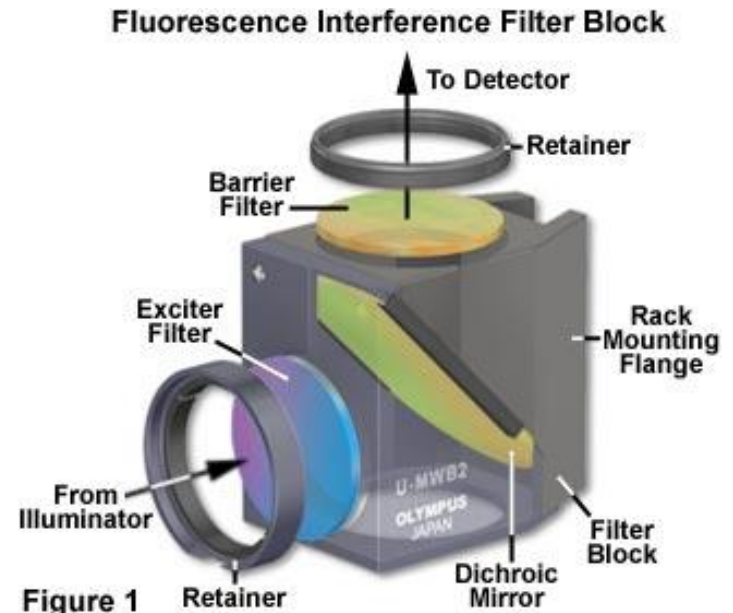


Figure 1

Microscope cover slips:

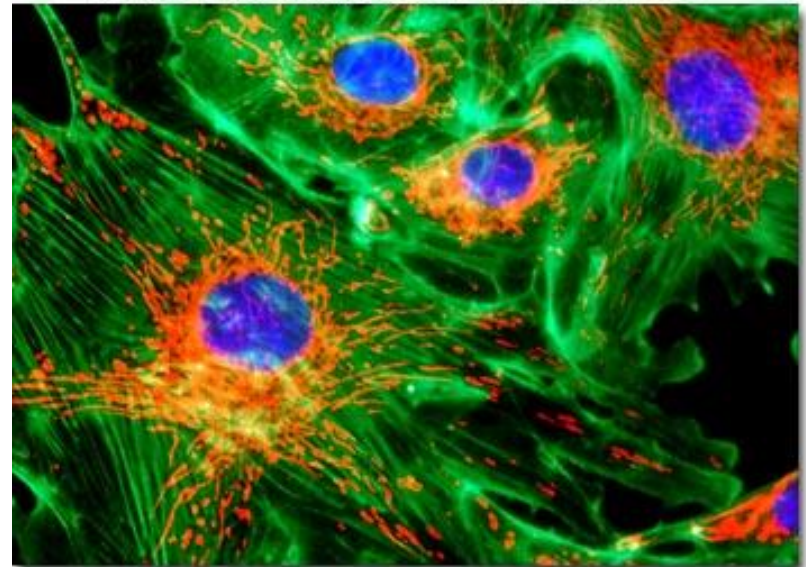
Many objectives are designed to be used with standard (1.1 mm thick) glass slides and coverslips of a certain thickness, usually 0.17 mm, which corresponds to thickness grade 1.5. Other coverslip thicknesses induce spherical aberration and give poorer performance, especially when used with high, dry lenses above 40. For lenses with an NA 0.4, coverslip thickness is not particularly important.

Grade Number	Thickness (mm)
0	0.083–0.13
1	0.13–0.16
1.5	0.16–0.19 (standard)
2	0.19–0.25

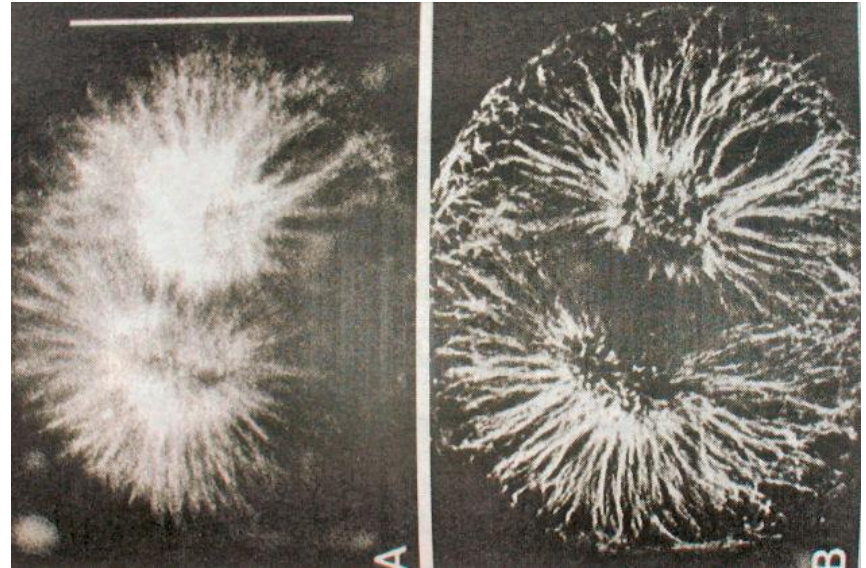
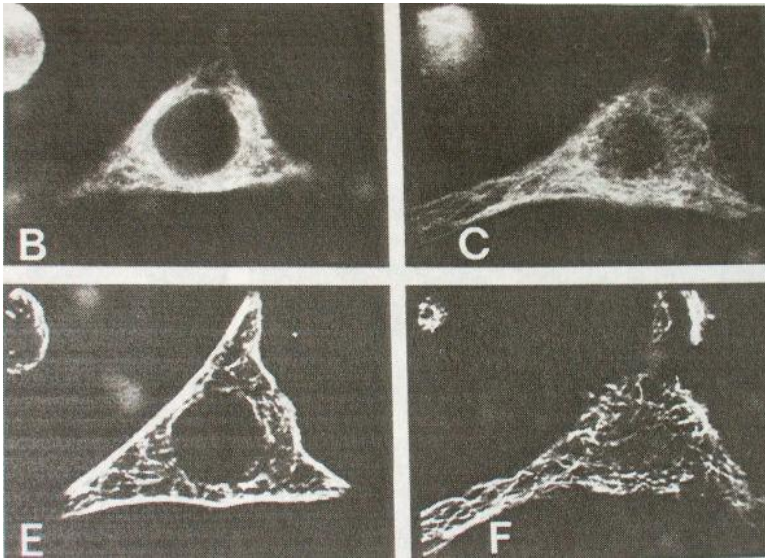
Strengths of Fluorescence Microscopy

- Enhanced contrast mechanism
- Specificity – individual structural components can be tagged based upon their biochemical differences
- Molecular sensitivity
- Monitor microenvironmental changes

Bovine Pulmonary Artery Epithelial Cells

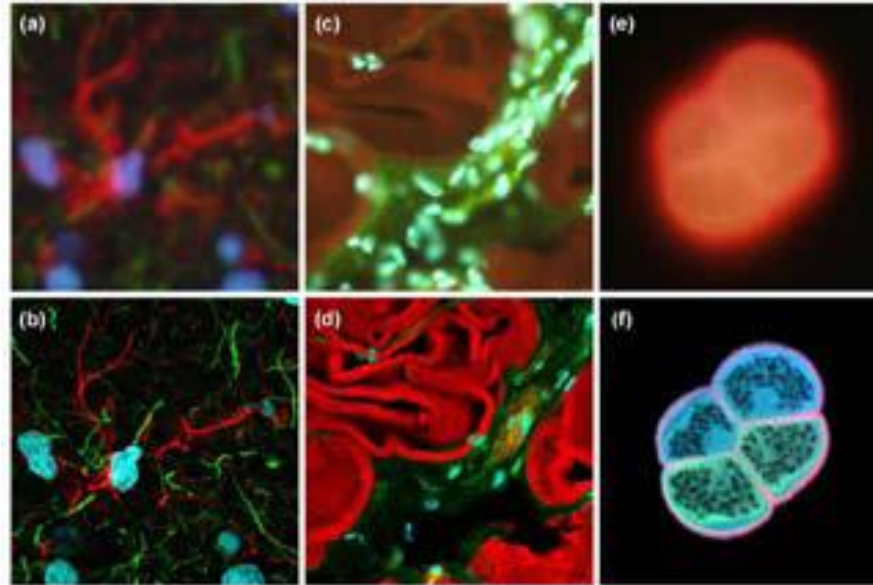


Confocal microscopy, early demonstrations



Confocal microscopy; more comparisons

Comparison of widefield (upper row) and laser scanning confocal fluorescence microscopy images (lower row). Note the significant amount of signal in the widefield images from fluorescent structures located outside of the focal plane.

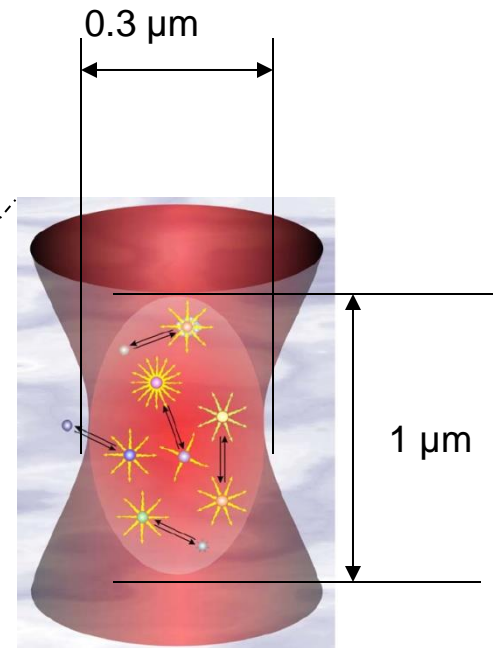
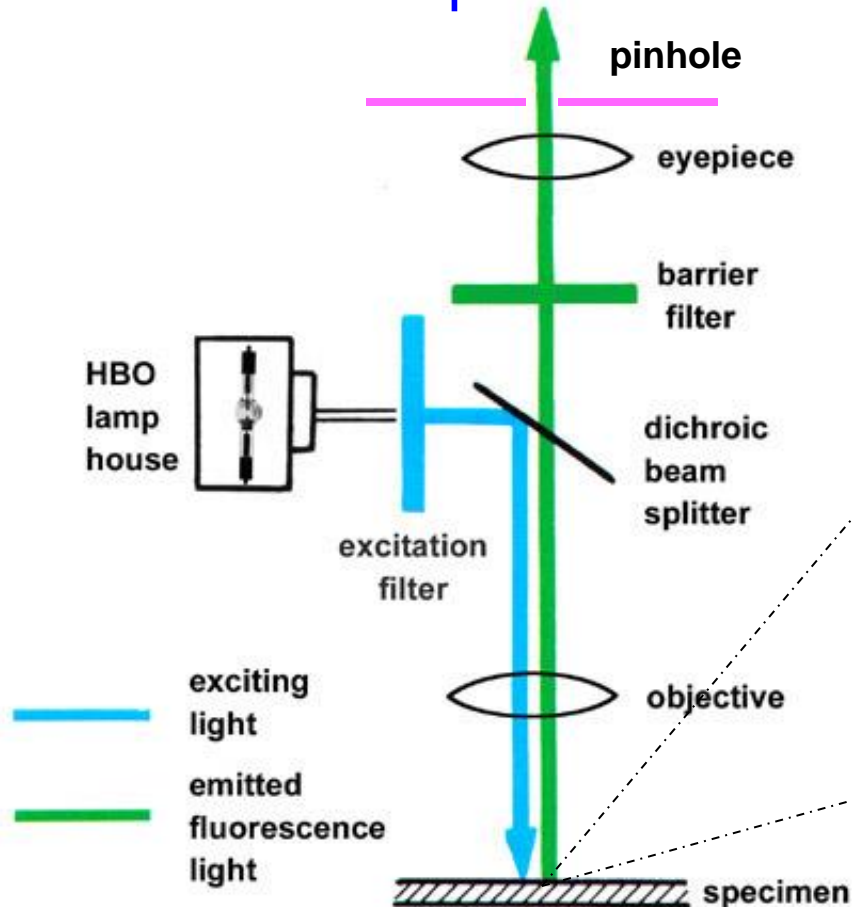


(a) and (b) Mouse brain hippocampus thick section treated with primary antibodies to glial fibrillary acidic protein (GFAP; red), neurofilaments H (green), and counterstained with Hoechst 33342 (blue) to highlight nuclei.

(c) and (d) Thick section of rat smooth muscle stained with phalloidin conjugated to Alexa Fluor 568 (targeting actin; red), wheat germ agglutinin conjugated to Oregon Green 488 (glycoproteins; green), and counterstained with DRAQ5 (nuclei; blue).

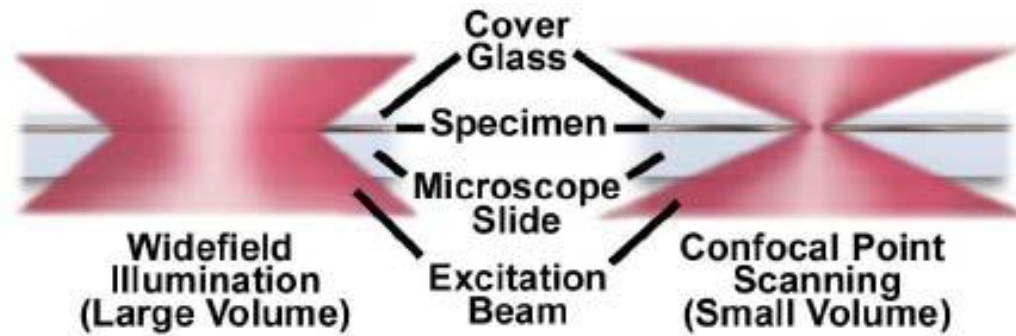
(e) and (f) Sunflower pollen grain tetrad autofluorescence.

Confocal Microscope



Volume: $10^{-15}\ \text{L}$
 Size (μm): 0.3×1
 No. molecules: ≈ 1

Confocal microscopy; how to produce a small volume



One-photon excitation (confocal pinhole)

Multiphoton effects

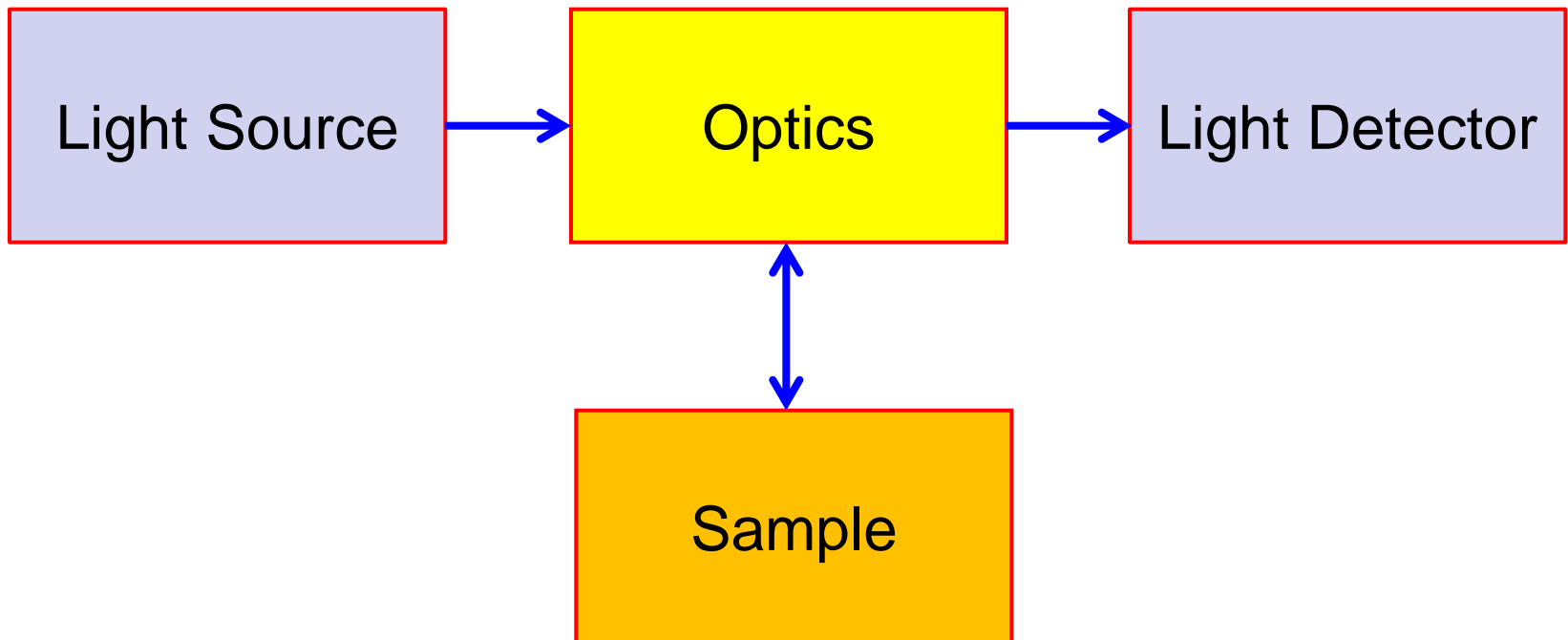
- 2-photon excitation

- Second harmonic generation (SHG)

- Four-way mixing (CARS)

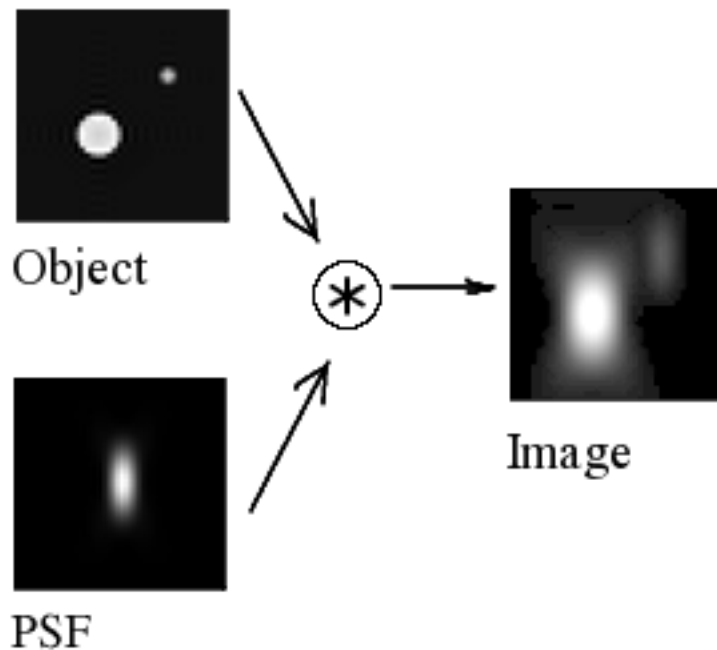
- Stimulated emission

A typical microscopy setup



Point Spread Function (PSF)

The PSF describes the response of an imaging system to a point source or point object. The **confocal point spread function (PSFCF)** is a combination of the **PSFS of the illumination** (light source) and the **PSFP of the pinhole**.



The PSF is generally complex. It is measured using nanoparticles and/or fluorescent beads.

Point Spread Function (PSF) for the source

Laser beam profile

$$F(x, y, z) = I_0 I(z) e^{-\frac{2(x^2 + y^2)}{w_0^2}}$$

$$I(z) = \text{Exp} \left[-\frac{2z^2}{w_{0z}^2} \right]$$

z-Gaussian

$$I(z) = \frac{1}{1 + \left(\frac{z}{w_{0z}} \right)^2}$$

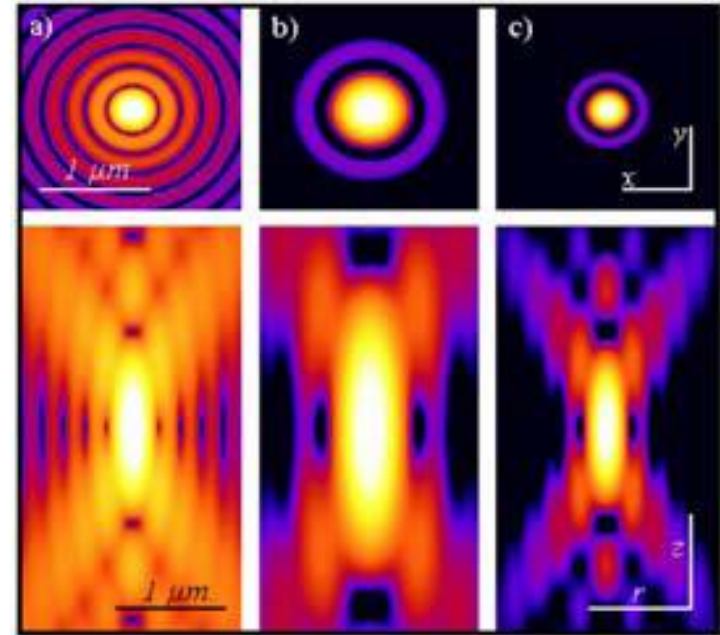
z-Lorentzian

The PSF is generally complex. It is measured using nanoparticles and/or fluorescent beads.

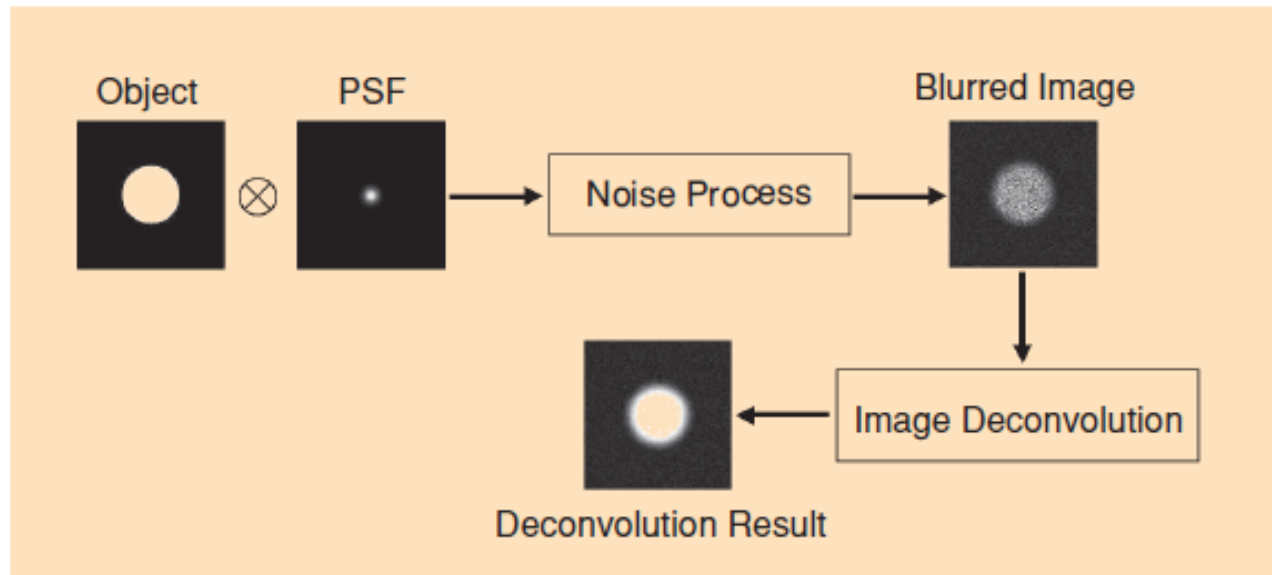
PSF

- a. widefield
- b. 2-photon
- a. 1-photon w/ pinhole

Optical conditions: excitation wavelengths are 488 nm and 900 nm for 1PE and 2PE, respectively; emission wavelength is 520 nm; numerical aperture is 1.3 for an oil immersion objective with oil refractive index value set at 1.515.



Deconvolution techniques



$$i(x, y, z) = N \left(\iiint O(x', y', z') h(x, y, z, x', y', z') dx' dy' dz' \right)$$

is the **acquired image**, **o** the **object acquired**, **h** the **PSF** and **N** the **noise**.

Resolution in Confocal microscopy

The maximum resolution that can be achieved using light of wavelength λ is given by:

$$\Delta r = \frac{\lambda}{2n \sin(\alpha)}$$

(Abbe limit)

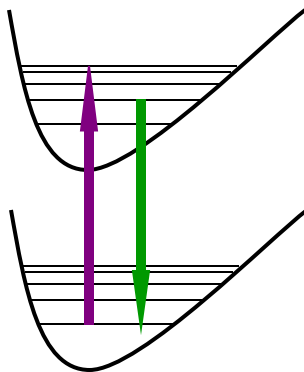
For $\lambda=500\text{nm}$, $n=1.5$ and $\alpha=45\text{degrees}$ $\Delta r=236\text{nm}$

NA can be as large as 1.50, so that a resolution of $\lambda/3$ can be achieved!

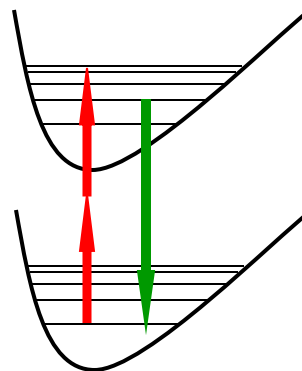
We call super-resolution any method that goes beyond the Abbe's limit

Two photon excitation

1-photon



2-photon



Since two-photon absorption is a second-order process involving the almost Simultaneous interaction of two photons with one fluorophore, this process has a small cross-section, on the order of $10^{-50} \text{ cm}^4 \text{ s}$ (defined as 1GM, Göppert-Mayer).

Lasers for two-photon excitation

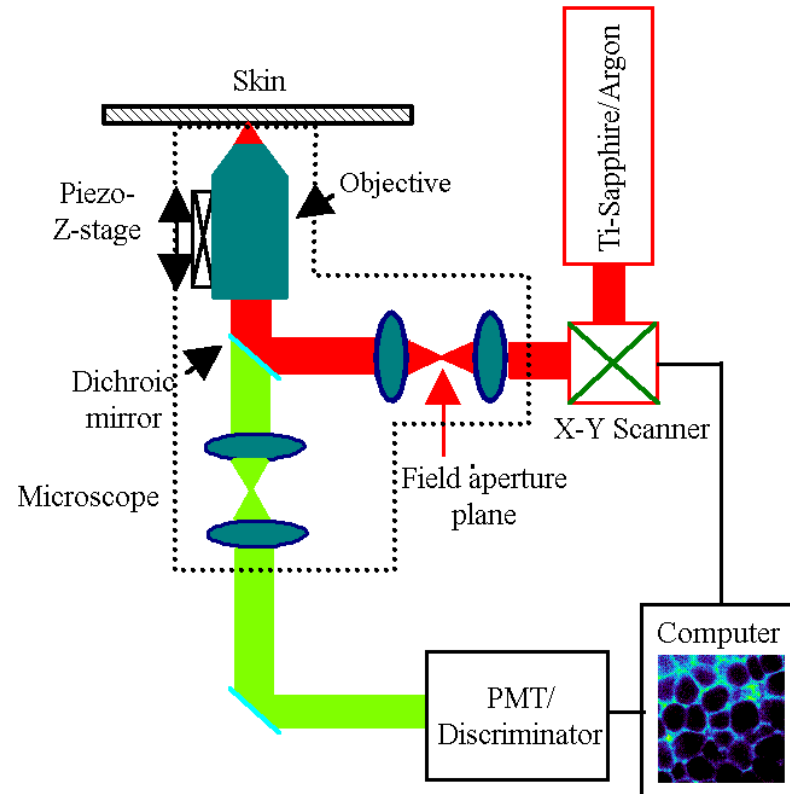
For a δ_2 of approximately 10 GM, focusing through an objective of $NA > 1$, an average incident laser power of $\approx 1\text{--}50$ mW, operating at a wavelength ranging from 680 to 1100 nm with 80–150 fs pulse width and 80–100 MHz repetition rate, one should get fluorescence without saturation.

n_a , is the probability that a certain fluorophore simultaneously absorbs two photons during a single pulse; in the paraxial approximation this is given by

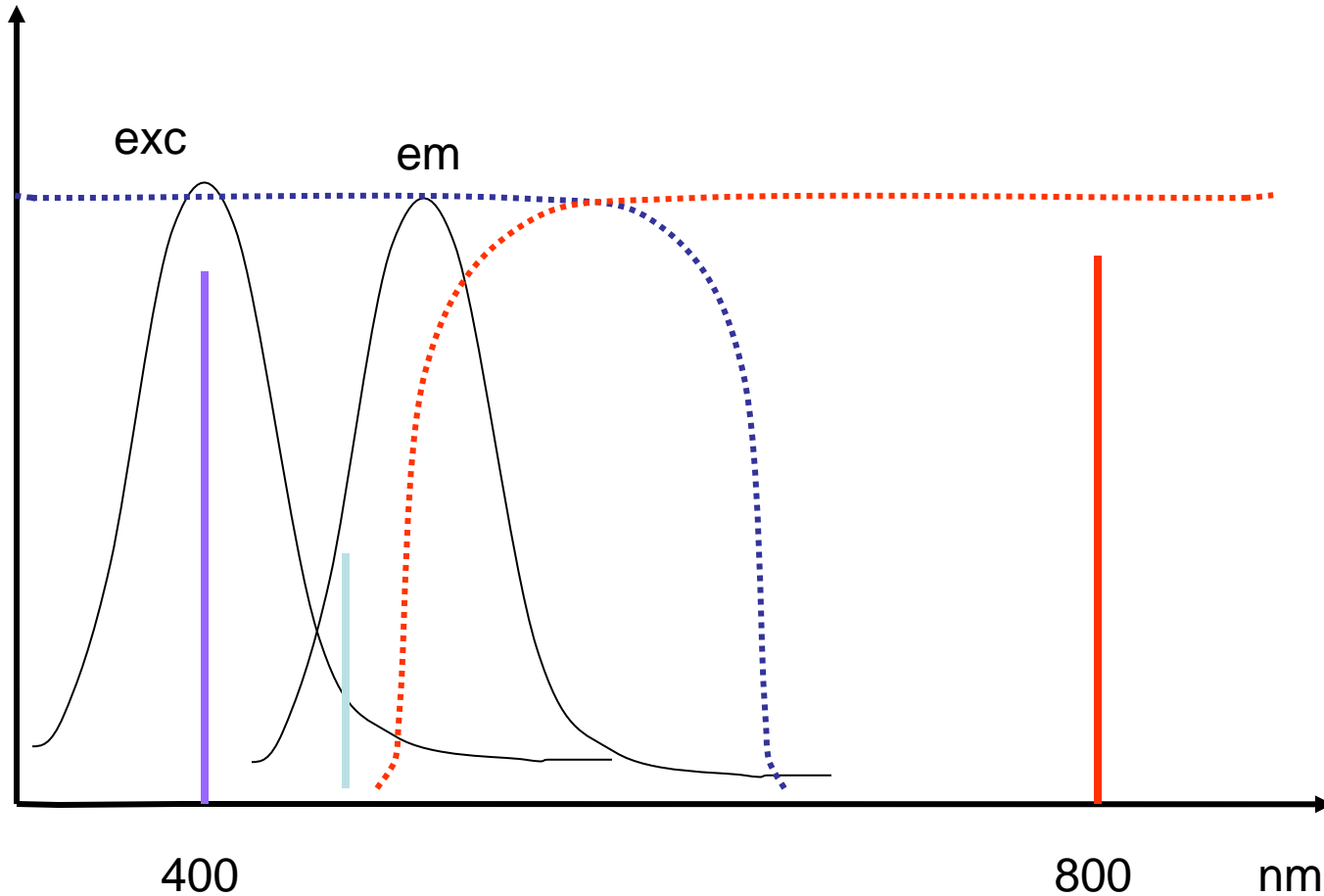
$$n_a \propto \frac{\delta_2 P^2}{\tau_p f_p^2} \left(\frac{NA^2}{2hc\lambda} \right)$$

Design of a two photon microscope

Two-photon microscope design is actually significantly simpler than that of confocal microscope and has much in common.



Separation of excitation and emission



Advantages of 2-photon excitation

- 3-D sectioning effect
- Deep penetration in tissues
- No Raman from the solvent
- Absence of photo bleaching in out of focus regions
- Large separation of excitation and emission
- Single wavelength of excitation for many dyes
- High polarization



Brad Amos; MRC, Cambridge, UK

Maximizing the sensitivity

- Use High Objective NA and Lowest Magnification: $I_{fl} \sim I_{il} NA_{obj}^4 / M_{tot}^2$
 - Buy the newest objective: select for best efficiency
- Close Field Diaphragm down as far as possible
- Use high efficiency filters
- Use as few optical components as possible
- Match magnification to camera resolution:
$$M_{Max} = 3 * \text{Pixel Size of Detector} / \text{Optical Resolution}$$

E.g.: $3 * 7 \mu\text{m} / [0.6 * 520\text{nm} / 1.4] = 91X$
- Reduce Photobleaching
- Use High Quantum Efficiency Detector

Confocal microscopy

Confocal microscopy

Confocal microscopy

Thank you.