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# Fluorescence Microscopy

Beniamino Barbieri

| type | feature | appearance | uses |
|---|---|---|---|
| Bright-field | Visible light | Colored/clear specimen<br>Light background | Stained specimen with sufficient color contrast |
| Dark-field | Visible light | Bright specimen<br>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?

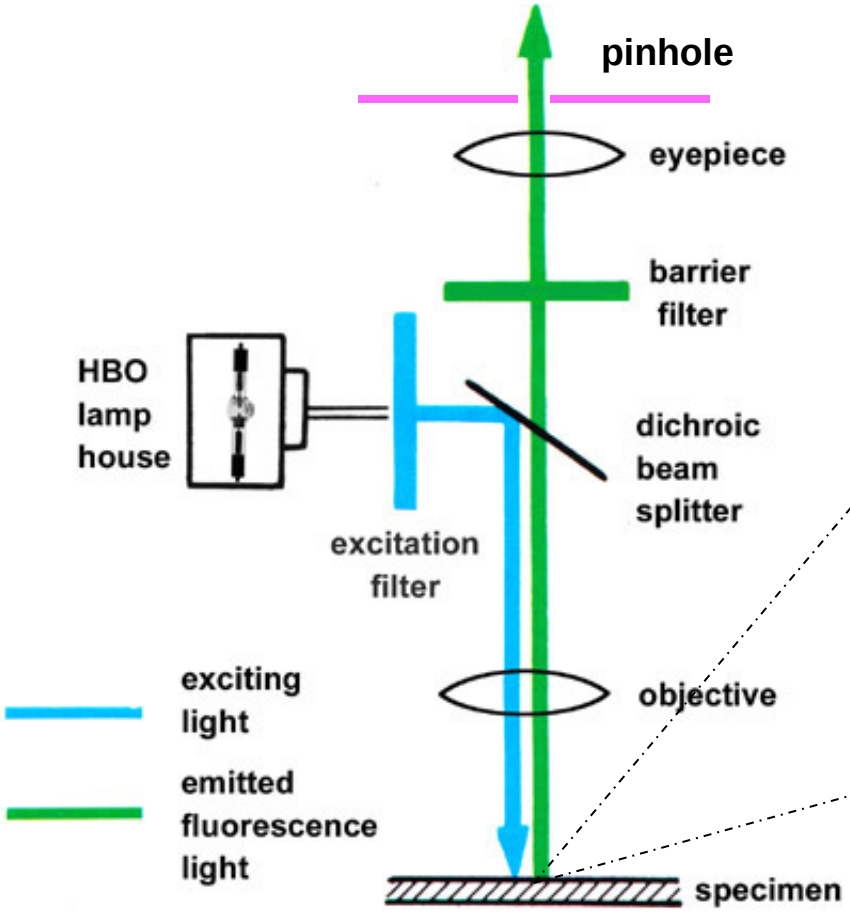
The "fluorescence microscope" refers to any microscope that uses fluorescence or phosphorescence to generate an image.

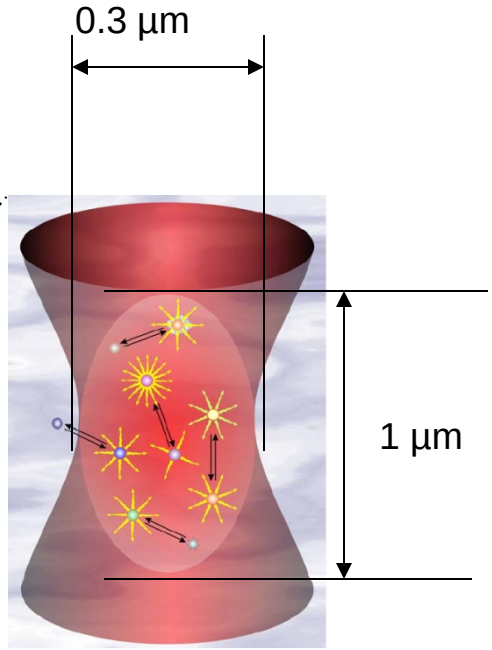
Two geometries:

- upright
- epifluorescence

Two general illumination schemes:

- full field
- confocal




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

**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.

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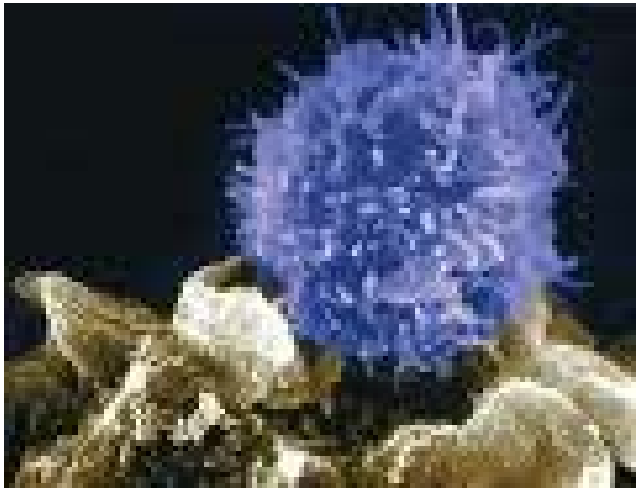
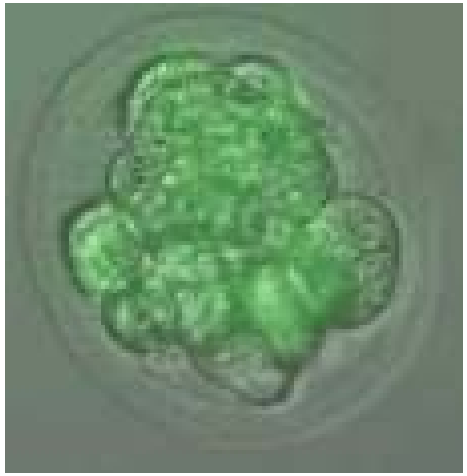
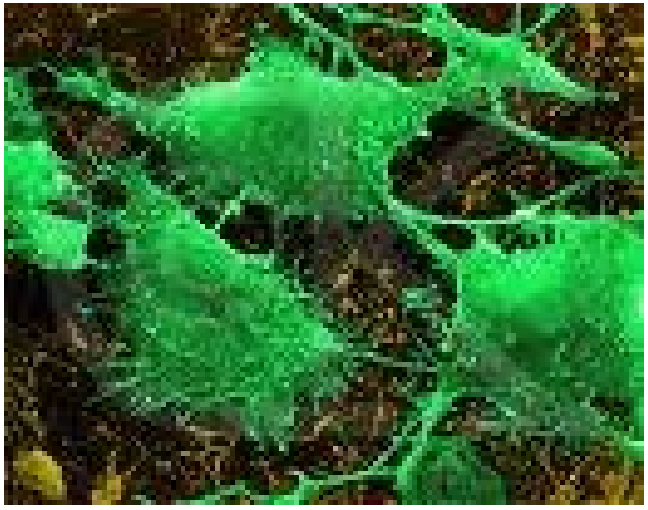
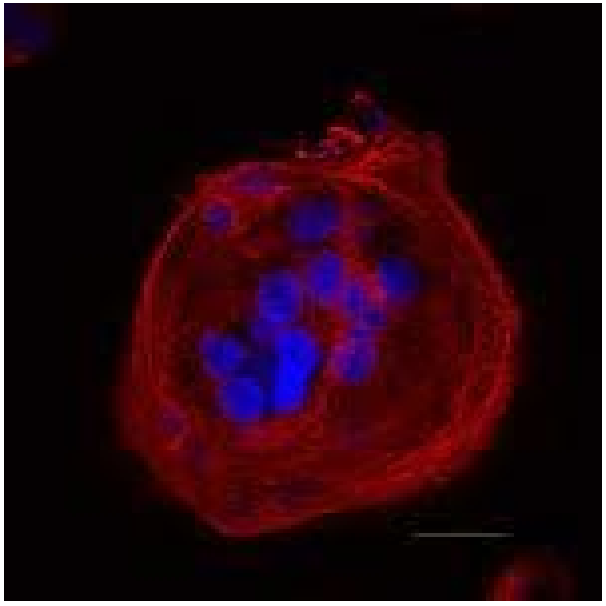
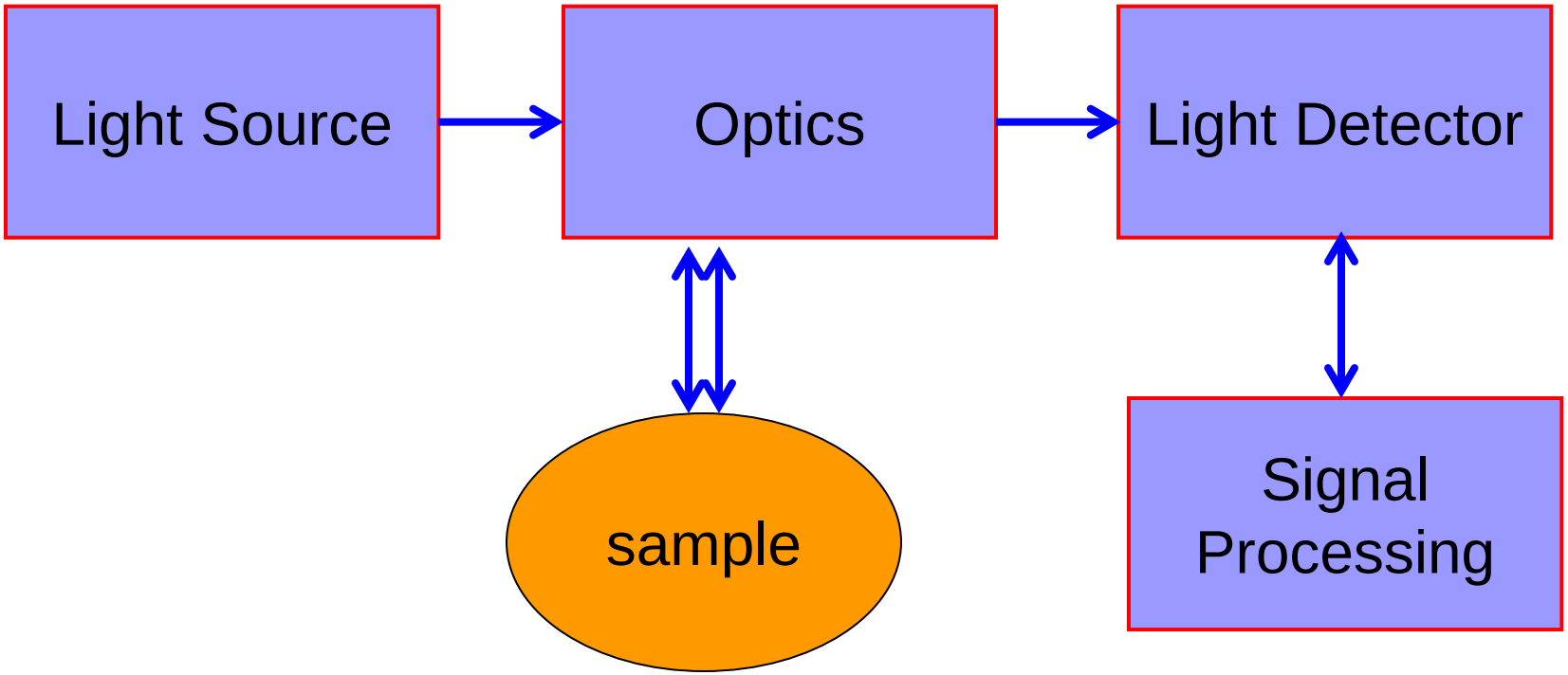




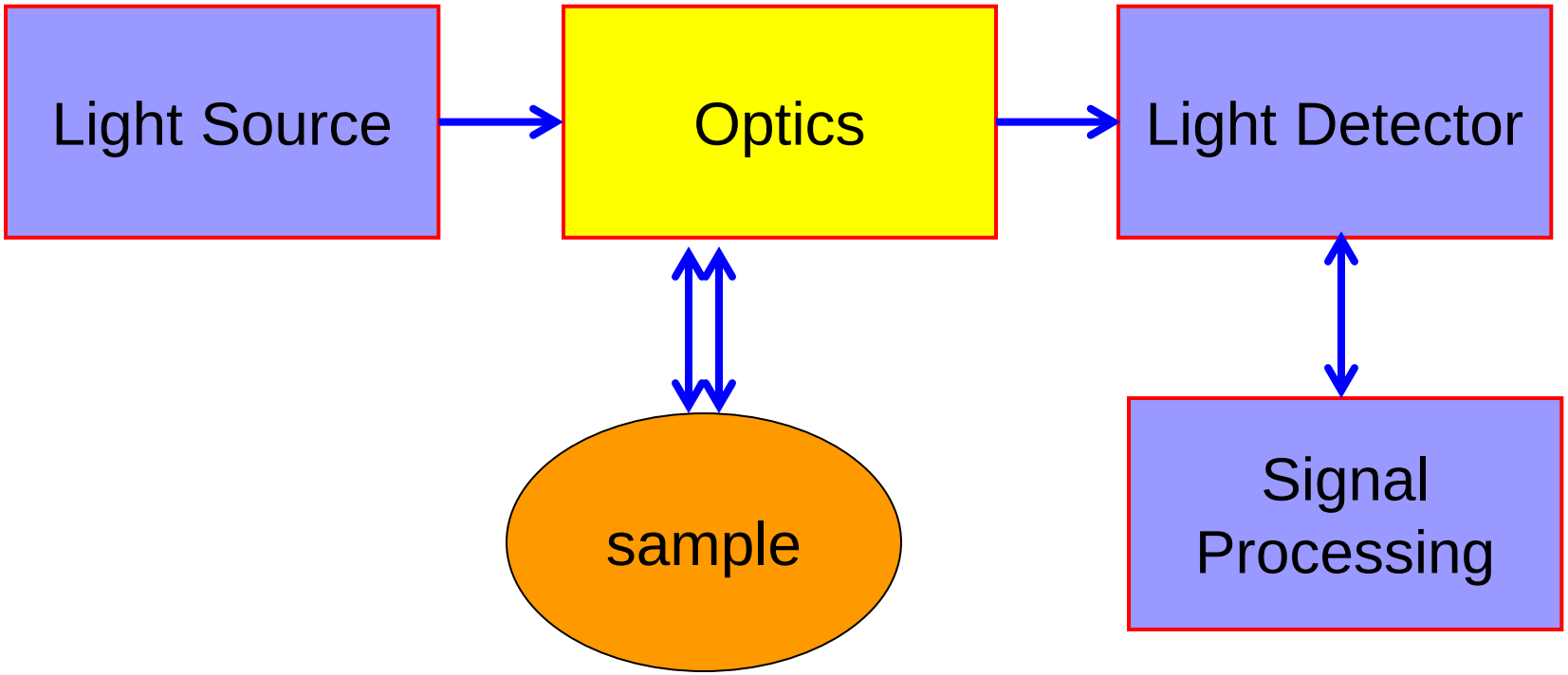




# A typical fluorescence microscopy setup



# A typical fluorescence microscopy setup



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## The Microscope

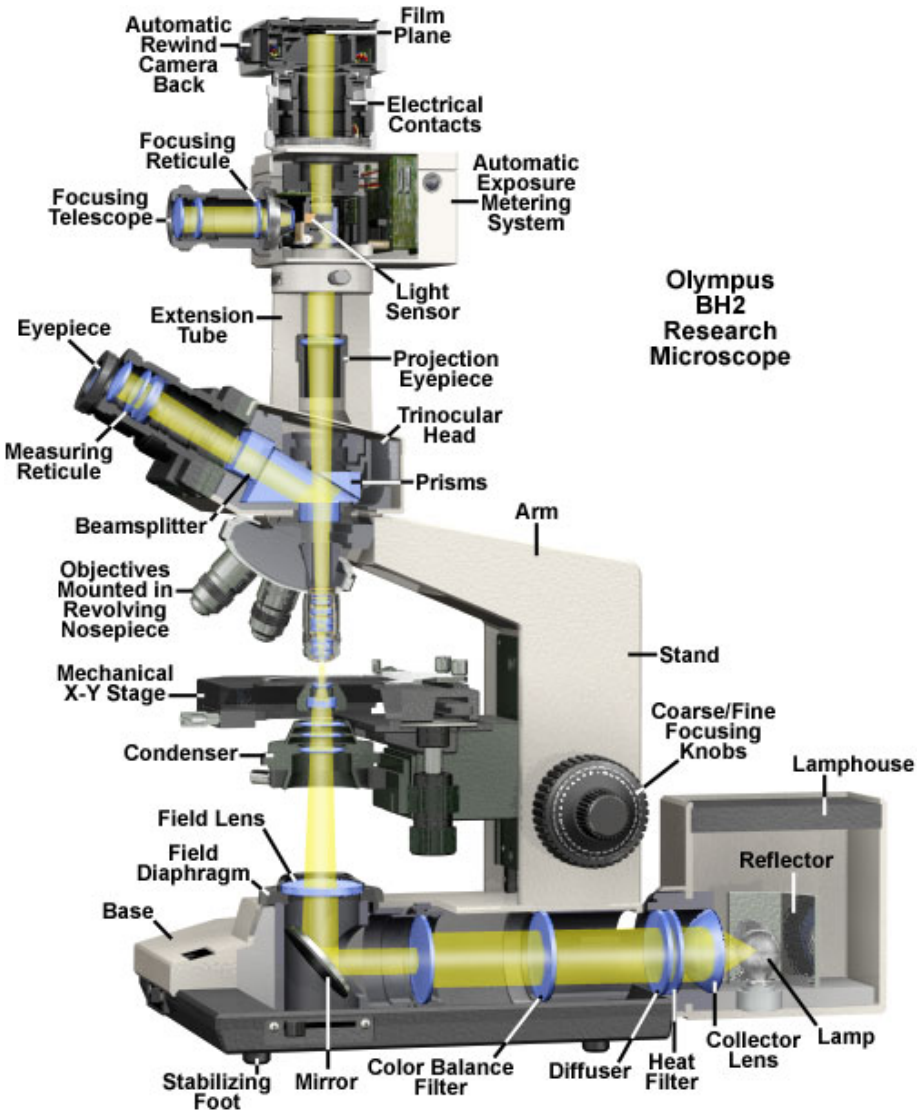
- Structure
- Objectives
- Koehler illumination
- Slides and coverslips
- Contrast and resolution

## Fluorescence Microscope

## Confocal Microscope

- PSF
- Multiphoton excitation

# Olympus BH2 cutaway Diagram




From http://micro.magnet.fsu.edu/primer/anatomy/specifications.html

## Optics:

Lenses, prisms, mirrors

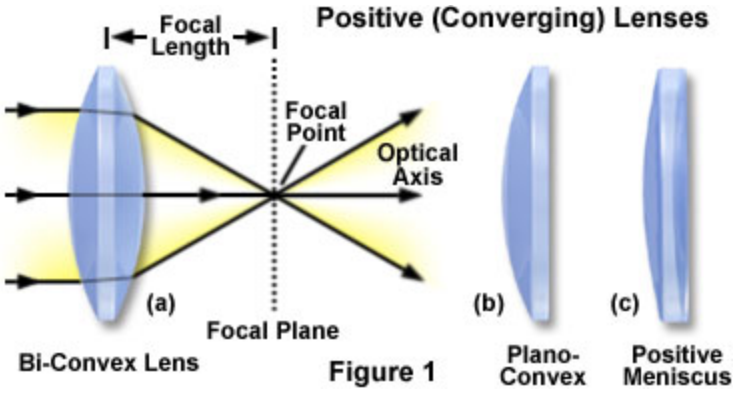


Figure 1

## Some problems need to be addressed:

Chromatic aberrations

Spherical aberration

Coma

Astigmatism

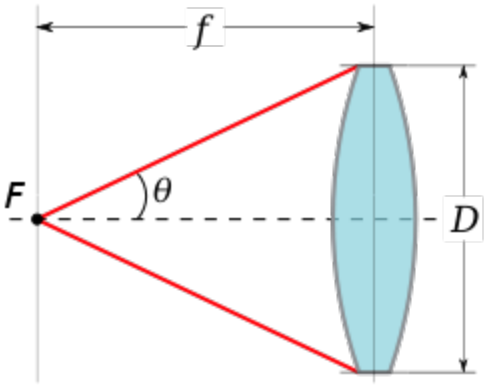
Distortion

From http://micro.magnet.fsu.edu/primer/anatomy/specifications.html

# A few useful parameters:

Numerical aperture $$NA = n \sin\theta = \frac{D}{2f}$$

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




Why are they useful?

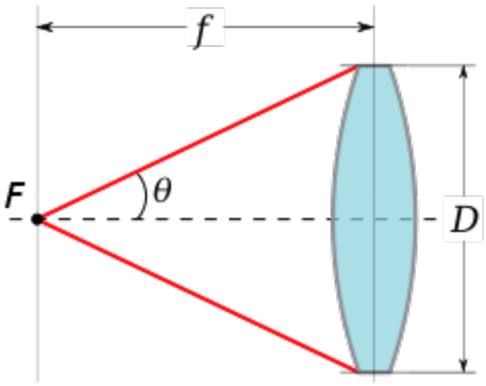
When a lens forms the image of an extended object, the amount of energy collected from a small area of the object is:

**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

From http://micro.magnet.fsu.edu/primer/anatomy/specifications.html

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




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.

From http://micro.magnet.fsu.edu/primer/anatomy/specifications.html

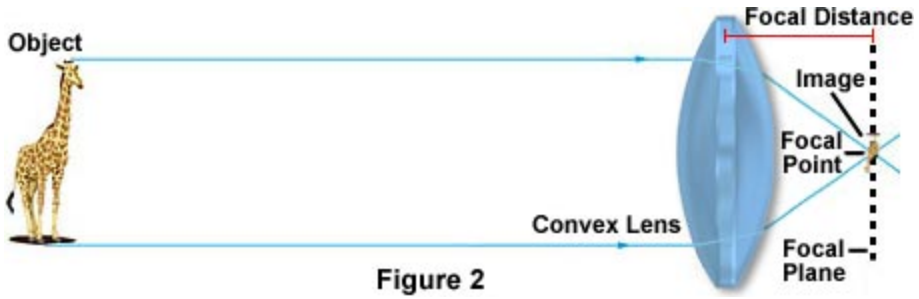


Figure 2

The object is far away

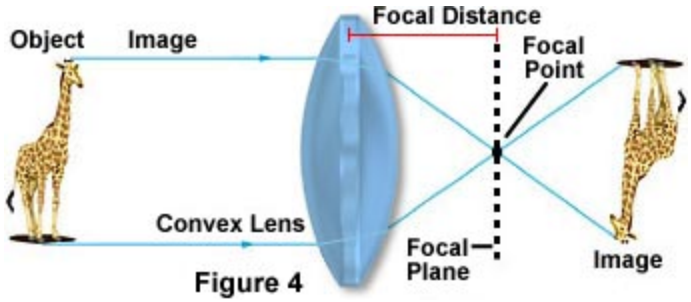


Figure 4

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

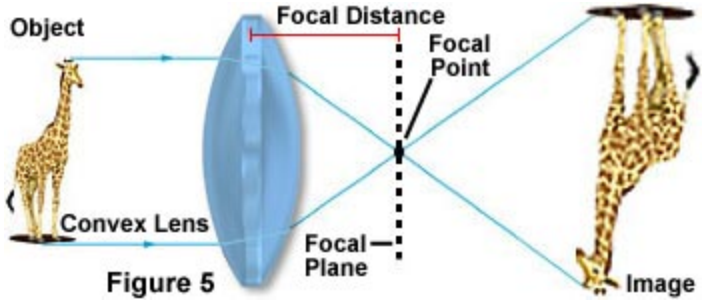
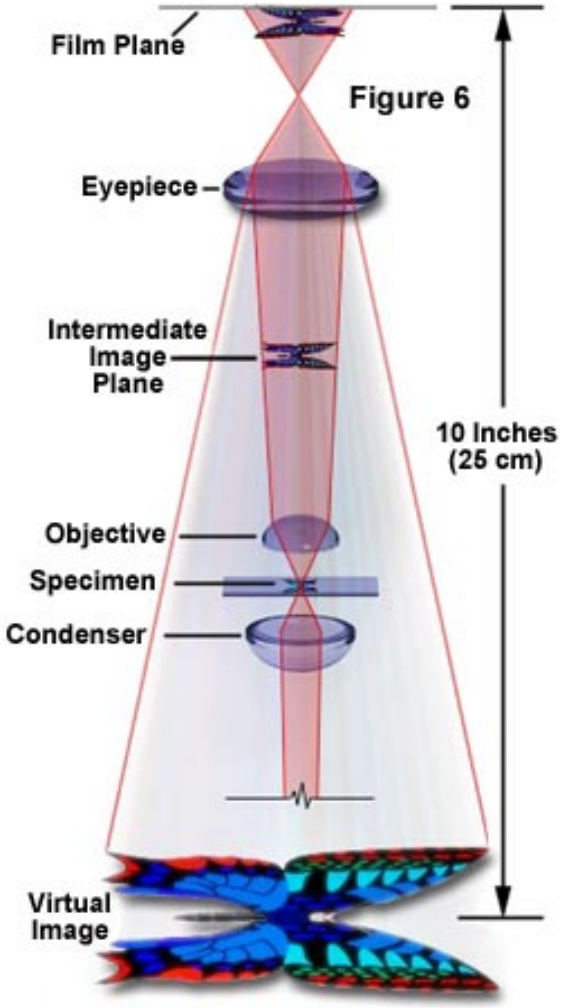


Figure 5

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

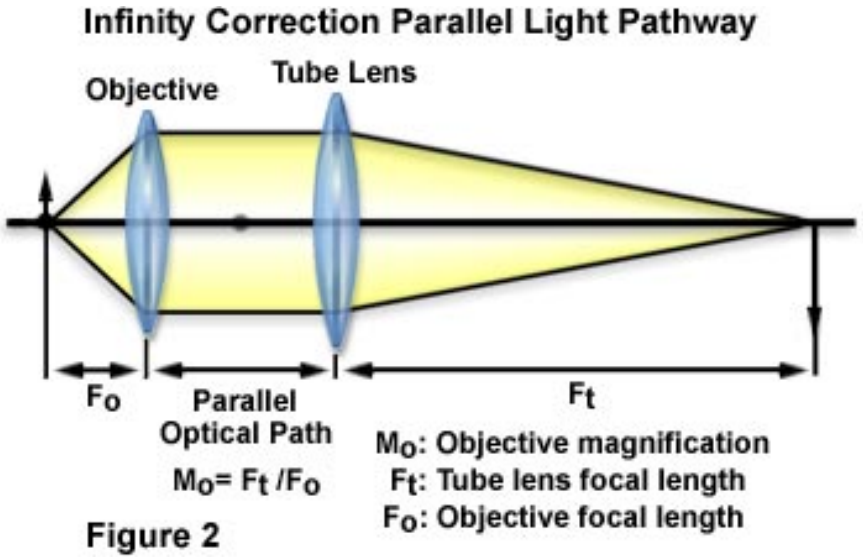



Figure 2

# objectives


Figure 1

From http://micro.magnet.fsu.edu/primer/anatomy/specifications.html

# 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

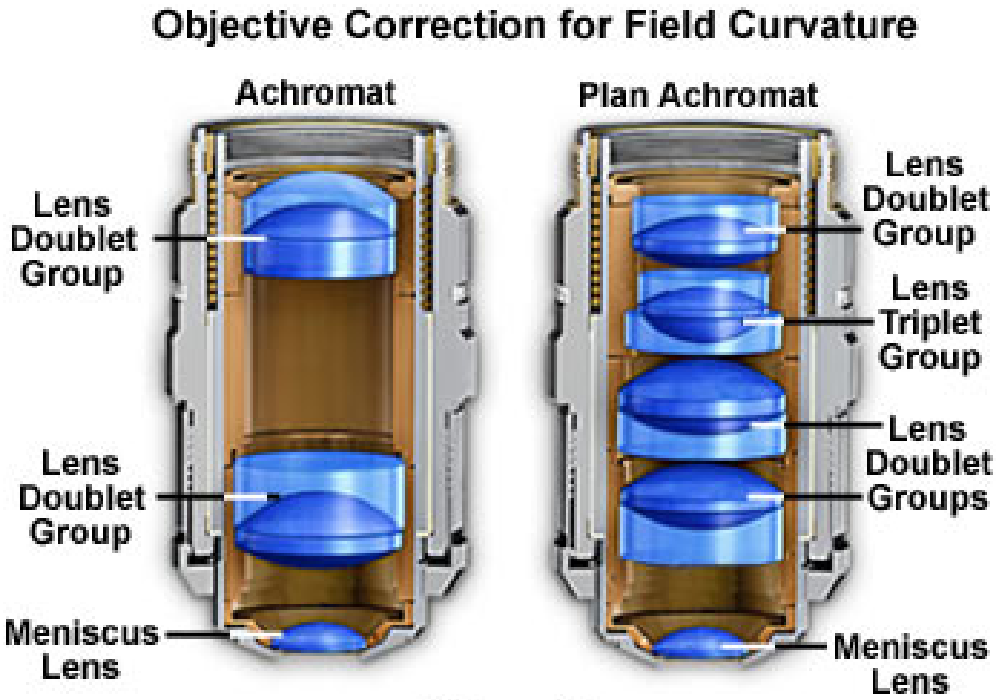


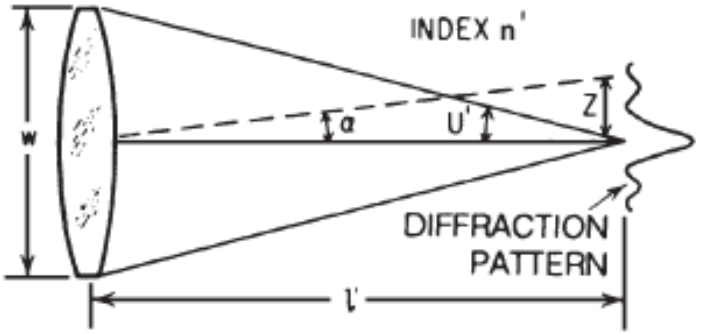

Figure 3

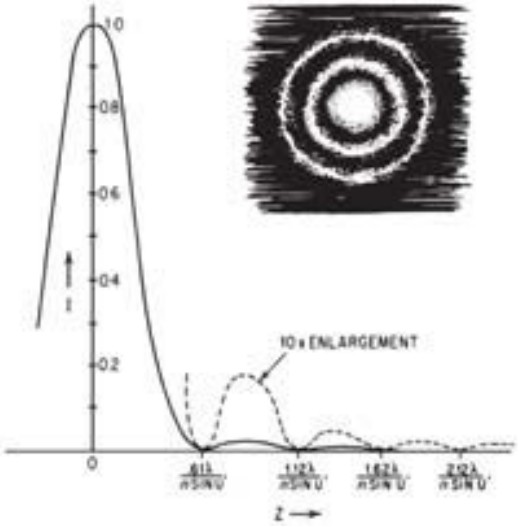
From http://micro.magnet.fsu.edu/primer/anatomy/specifications.html

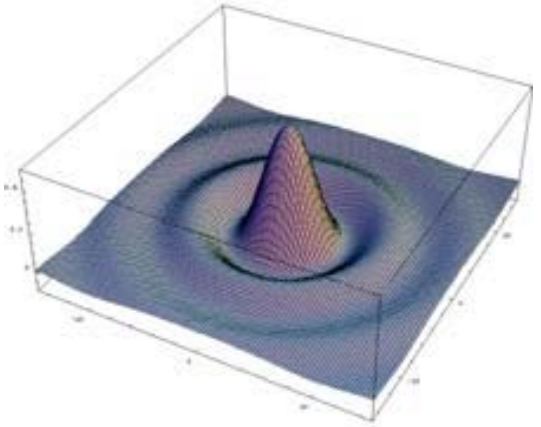
# Resolution of optical systems

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”:

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

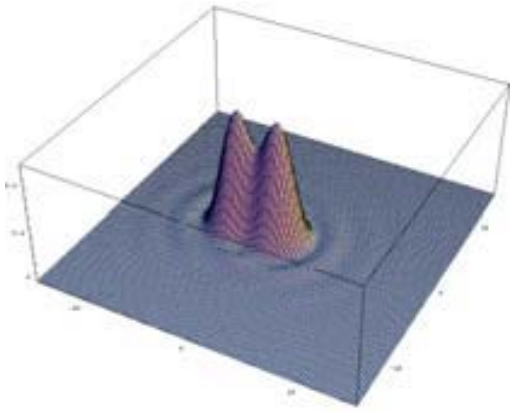


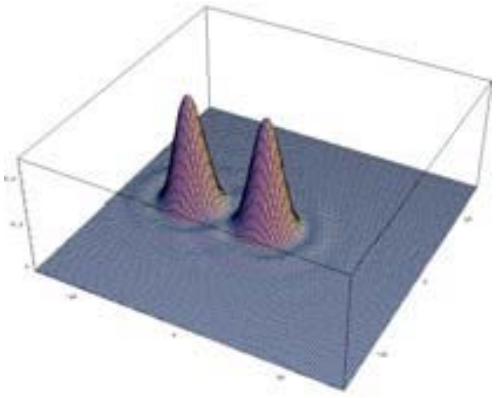



From http://micro.magnet.fsu.edu/primer/anatomy/specifications.html

# 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



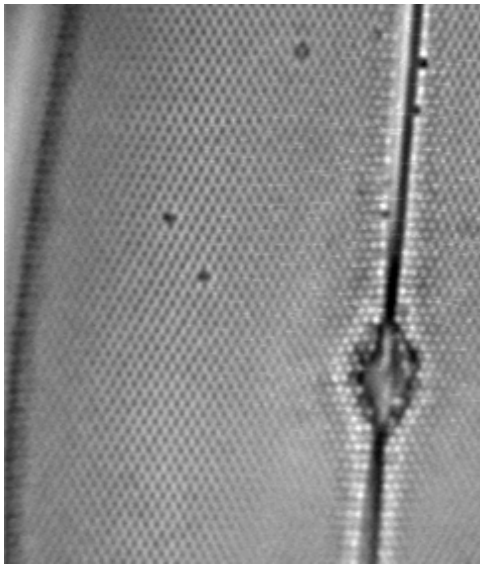
$$\Delta q = \frac{1.22\, r\, \lambda}{D}$$

r –distance from object to image

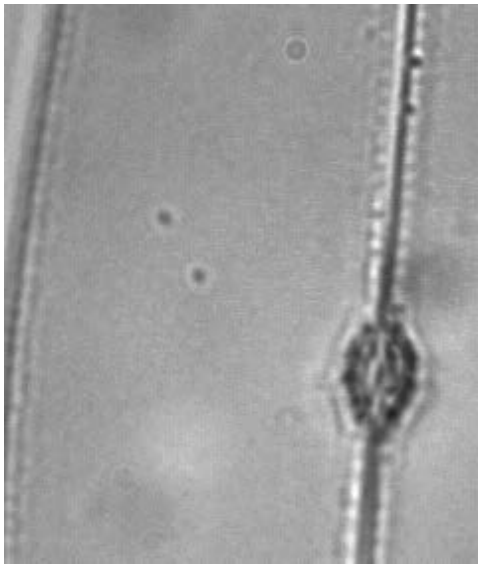
From http://micro.magnet.fsu.edu/primer/anatomy/specifications.html

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

From ED Salmon

Rayleigh Criterion for the resolution of two adjacent spots:

$$P_{lim} = 0.61\ \lambda_o / NA_{obj}$$

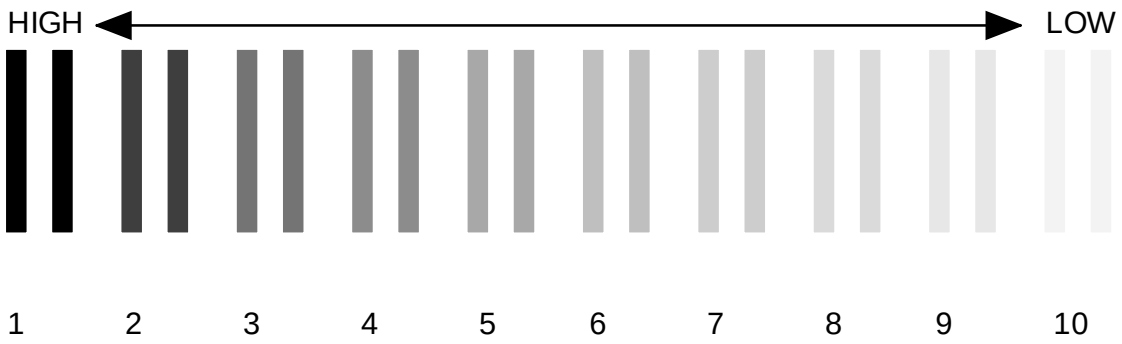
Examples: ($\lambda_o$ = 550 nm)

| | Mag | f(mm) | n | a | NA | $P_{lim}$ (μm) | ($NA_{cond}$=$NA_{obj}$) |
|---|---|---|---|---|---|---|---|
| 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 | |

# Contrast

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

$$\text{CONTRAST} = (I_{sp} - I_{bg})/I_{bg}$$



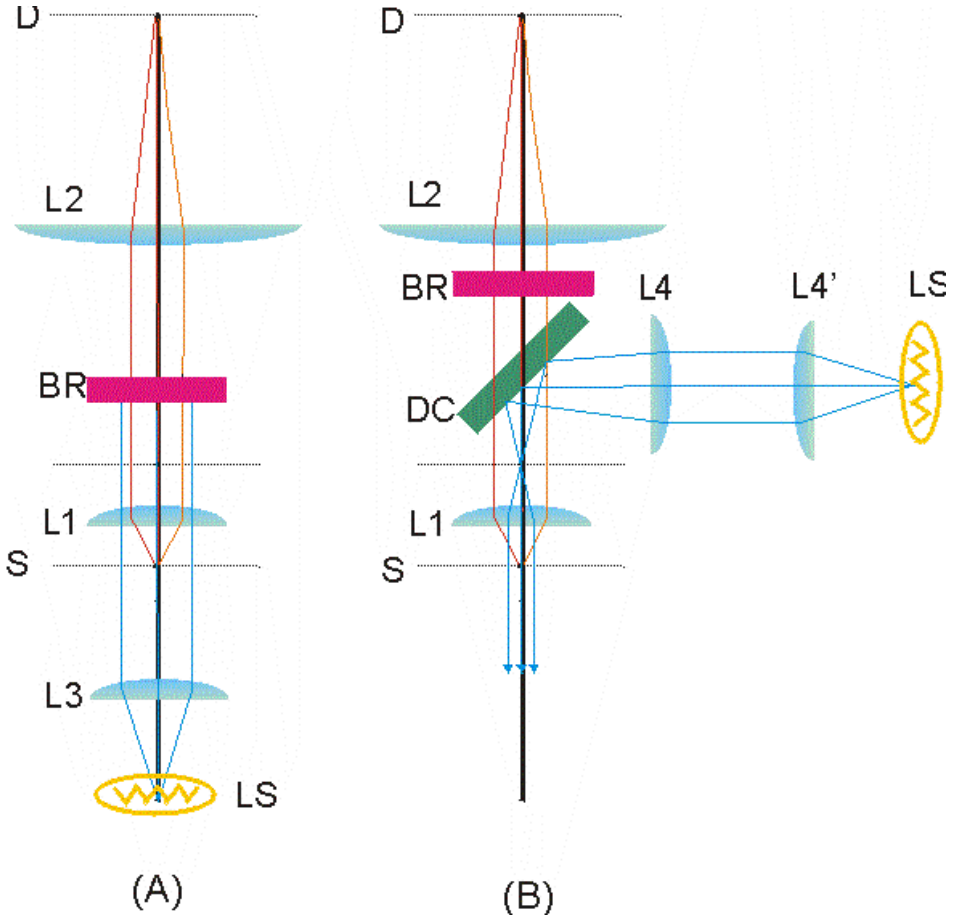

From ED Salmon

Light sources:

- Tungsten lamp
- Halogen lamp
- Mercury
- LEDs
- Lasers

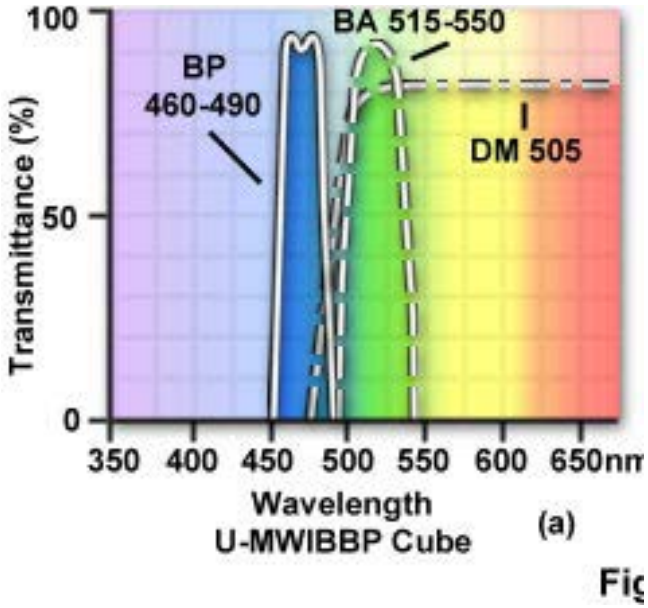
The image of the source is not focused on the specimen

## Köhler illumination




*Courtesy of Dr. Peter So*

# Filters and dichroics



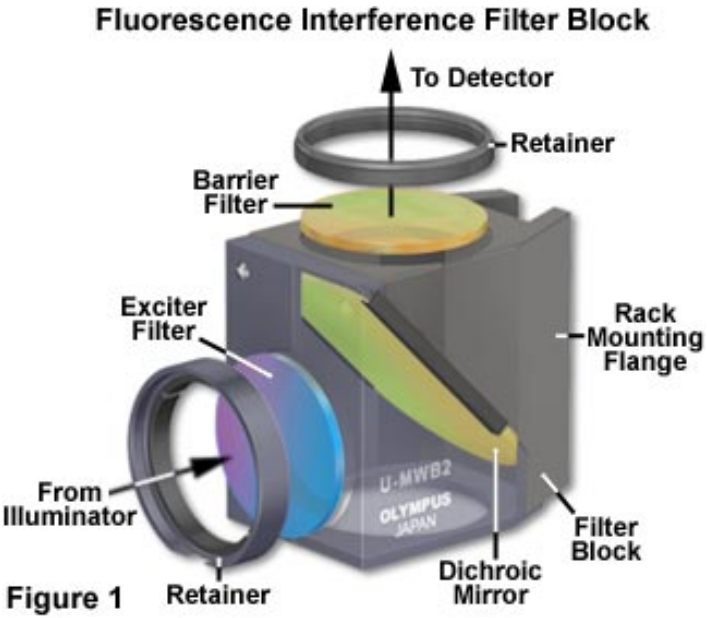




Figure 1

# Microscope cover slips:

Many objectives are designed to be used with standard 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.

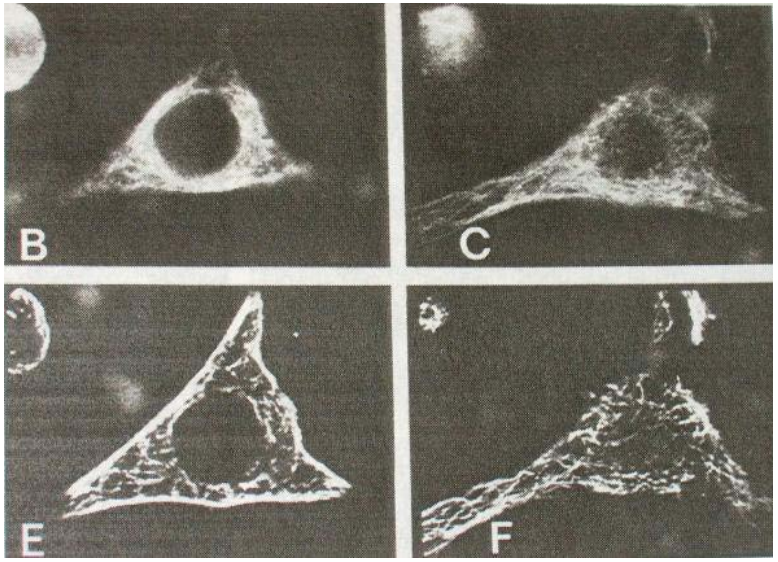
| Grade Number | Thickness (mm) |
|---|---|
| 0 | 0.083–0.13 |
| 1 | 0.13–0.16 |
| 1.5 | 0.16–0.19 (standard) |
| 2 | 0.19–0.25 |

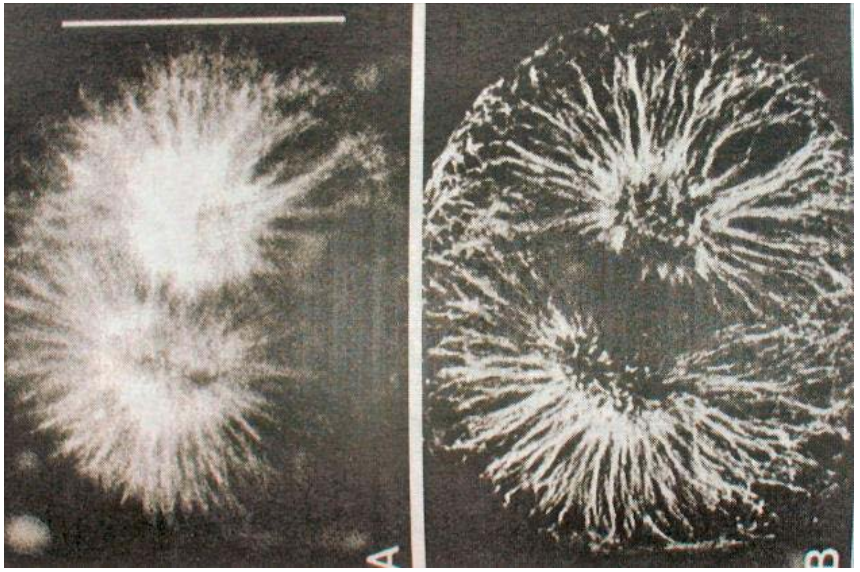
*Courtesy of Dr. Peter So*

# Strengths of Fluorescence Microscopy

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

# Confocal microscopy, early demonstrations

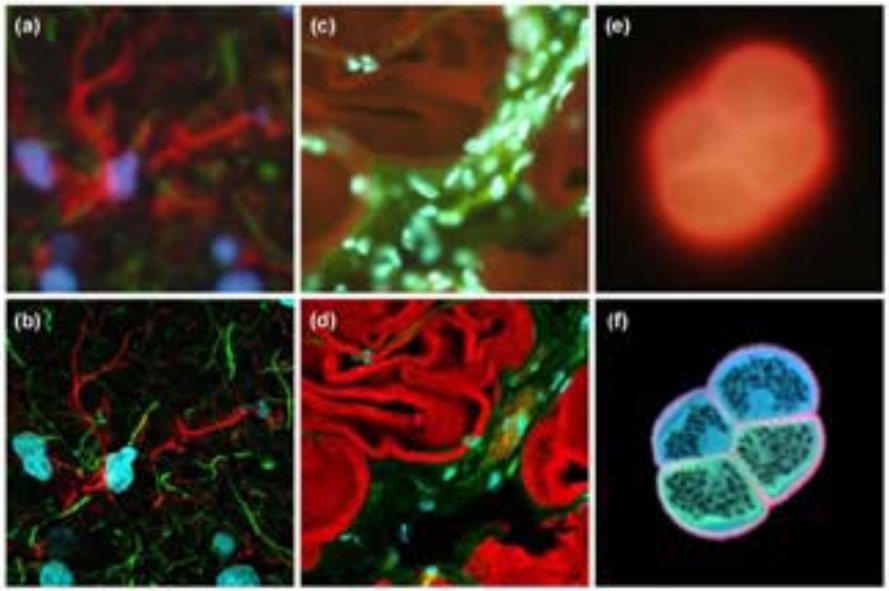


White et al., 1987

# Confocal microscopy; more comparisons

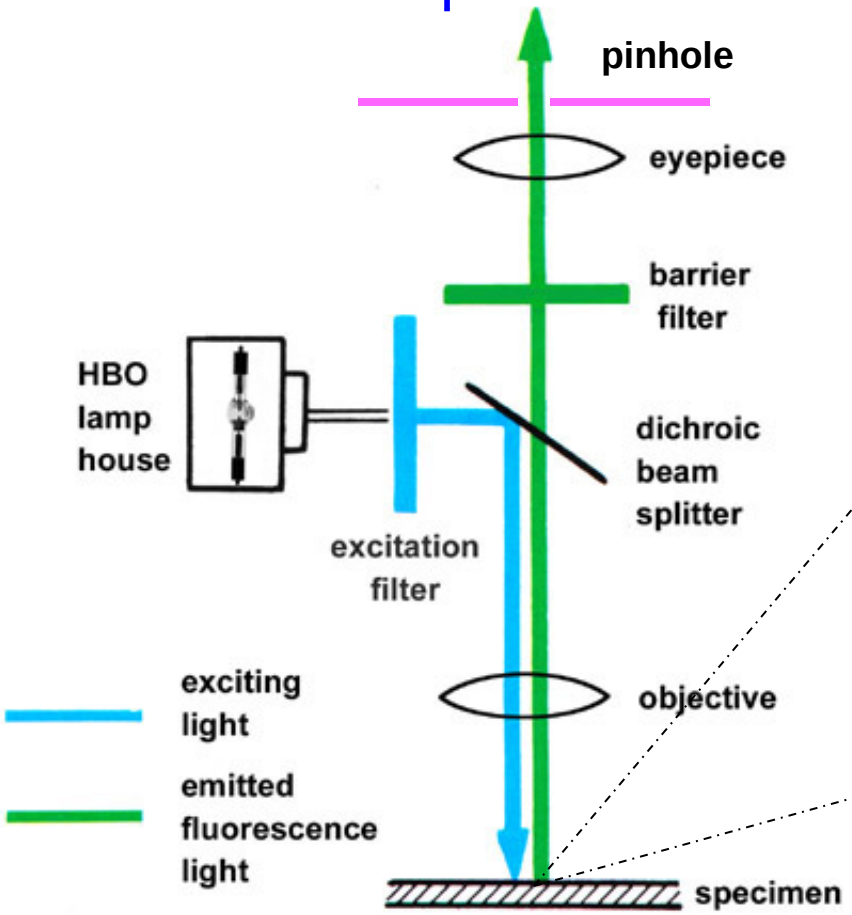
Comparison of widefield (upper row) and laser scanning confocal fluorescence microscopy images (lower row). Note the significant amount of signal in the widefield images from fluorescent structures located outside of the focal plane.

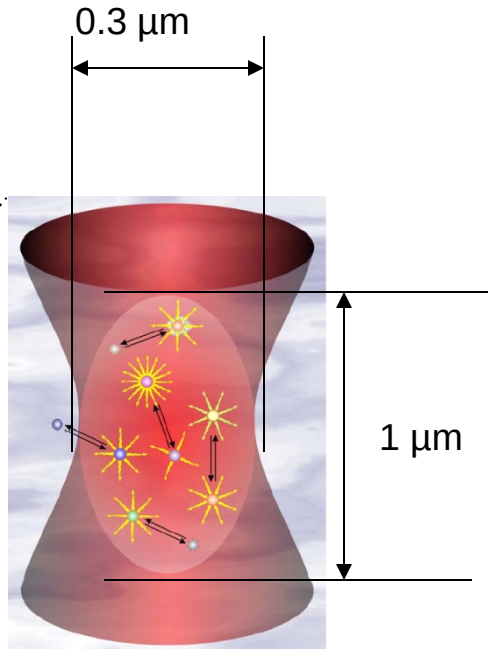



(a) and (b) Mouse brain hippocampus thick section treated with primary antibodies to glial fibrillary acidic protein (GFAP; red), neurofilaments H (green), and counterstained with Hoechst 33342 (blue) to highlight nuclei.
(c) and (d) Thick section of rat smooth muscle stained with phalloidin conjugated 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.

*From R. Clegg lectures*

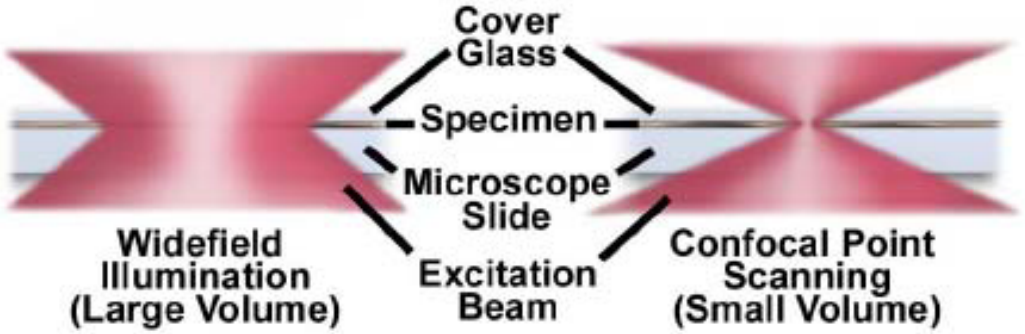
# Confocal Microscope




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

# Confocal microscopy; how to produce a small volume

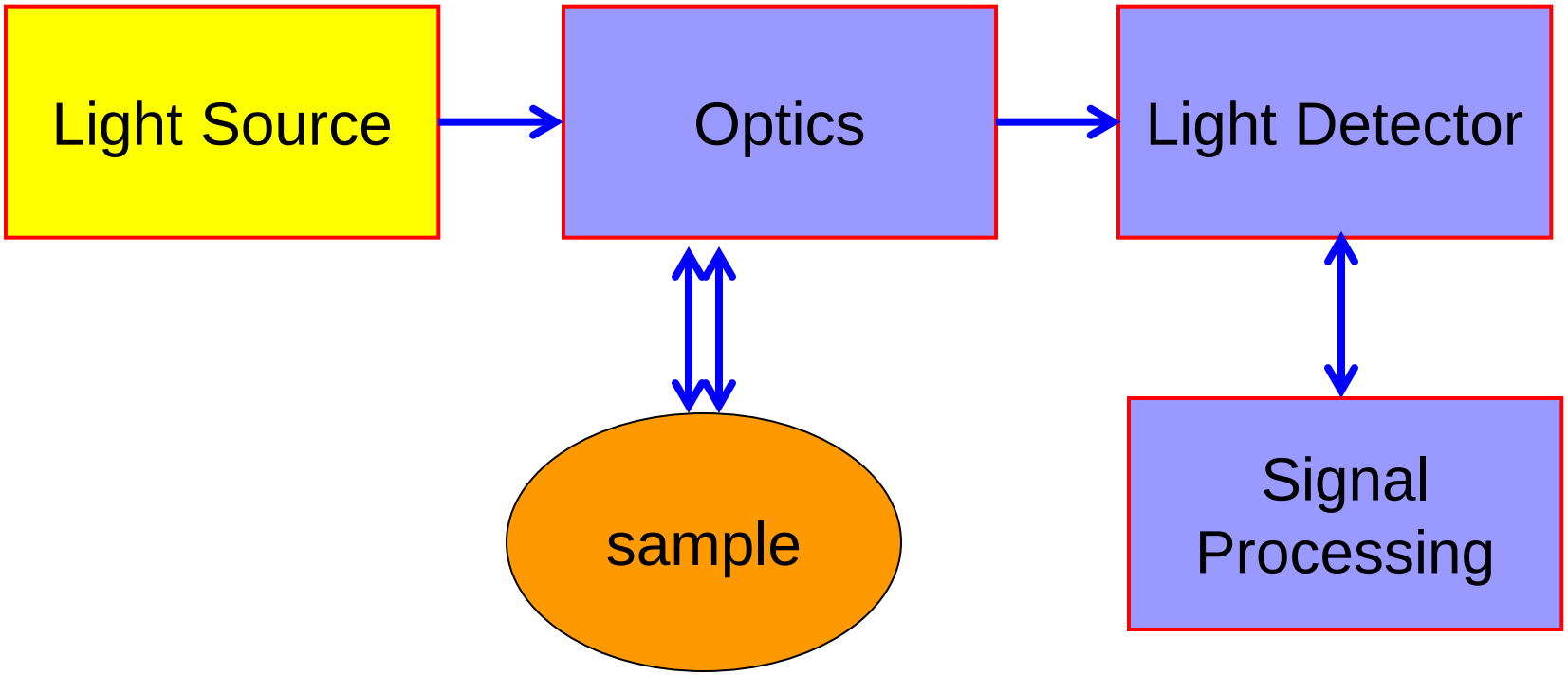



One-photon excitation (confocal pinhole)

Multiphoton effects

- 2-photon excitation
- Second harmonic generation (SHG)
- Four-way mixing (CARS)
- Stimulated emission

# A typical fluorescence microscopy setup



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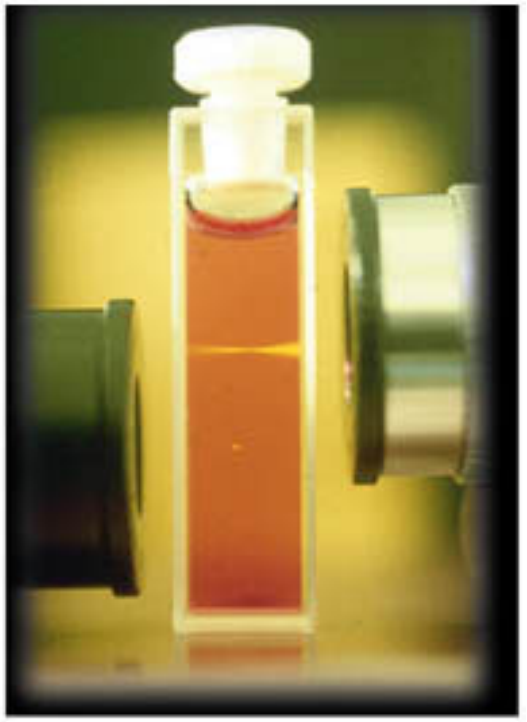
# Laser sources

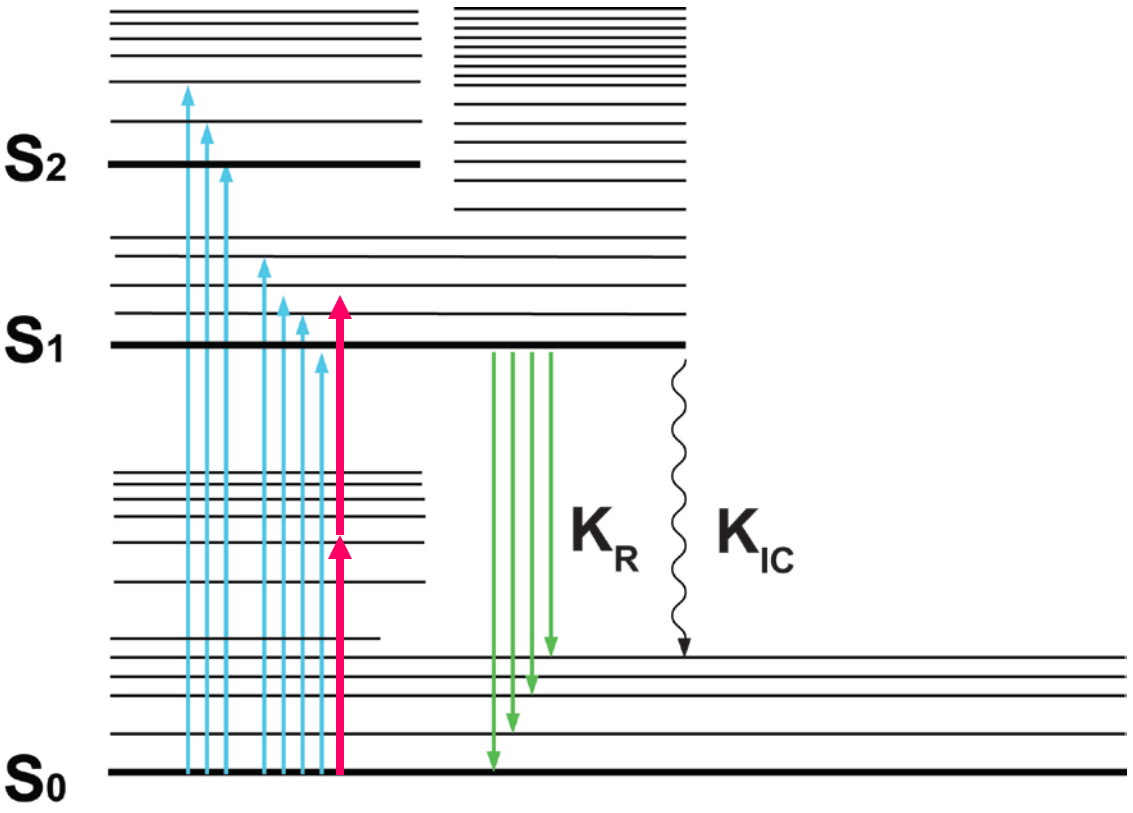
Single photon excitation

Multi-photon excitation



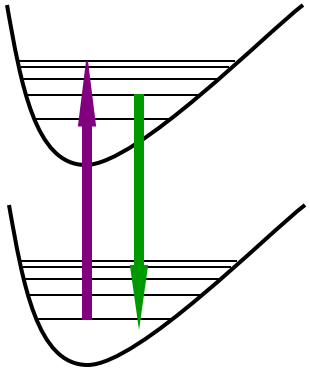
Brad Amos;  MRC, Cambridge, UK

# Perrin-Jabłoński diagram, 2-photon excitation

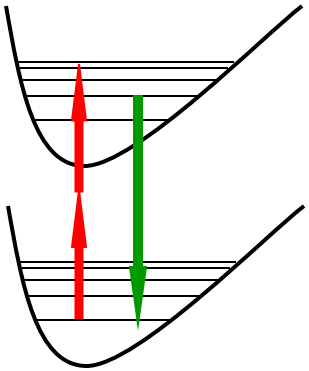


# Two photon excitation

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 $10^{-50}$ $cm^4$ s (defined as 1GM, Göppert-Mayer).

# 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

# Lasers for two-photon excitation

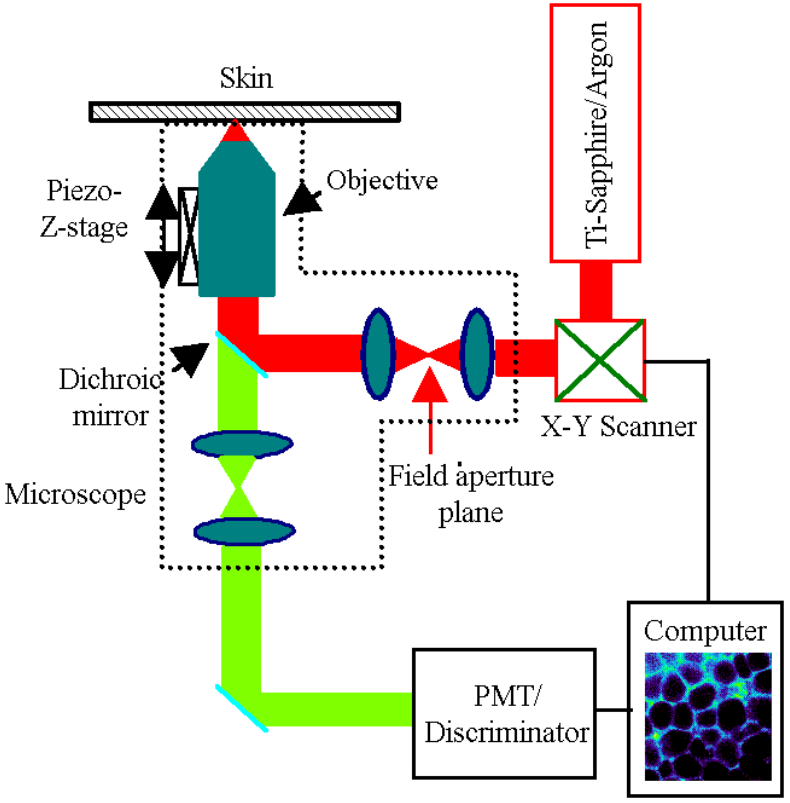
For a δ2 of approximately 10 GM, focusing through an objective of NA >1, an average incident laser power of ≈ 1–50 mW, operating at a wavelength ranging from 680 to 1100 nm with 80–150 fs pulse width and 80–100 MHz repetition rate, one should get fluorescence without saturation.

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

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

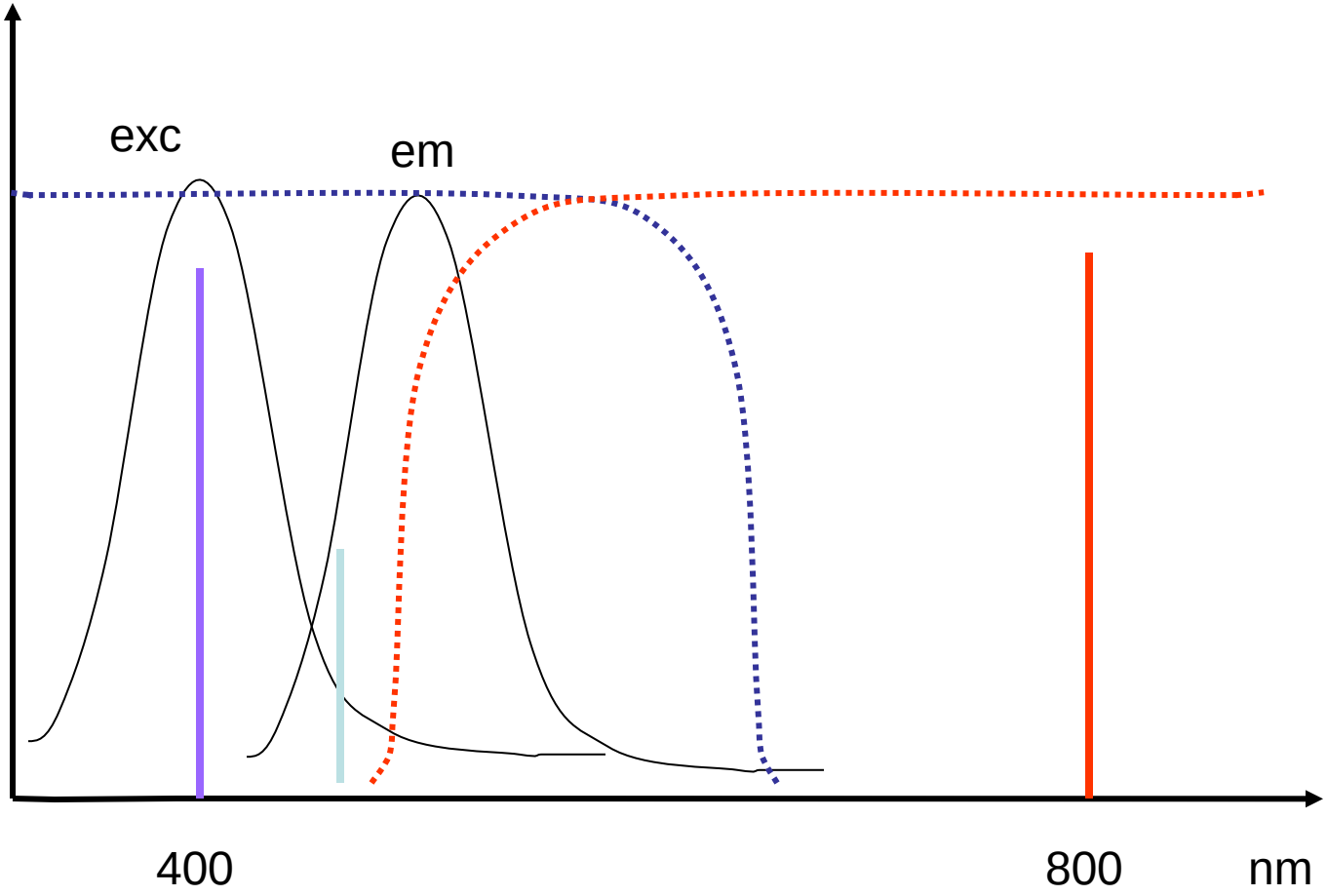
# Design of a two photon microscope

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

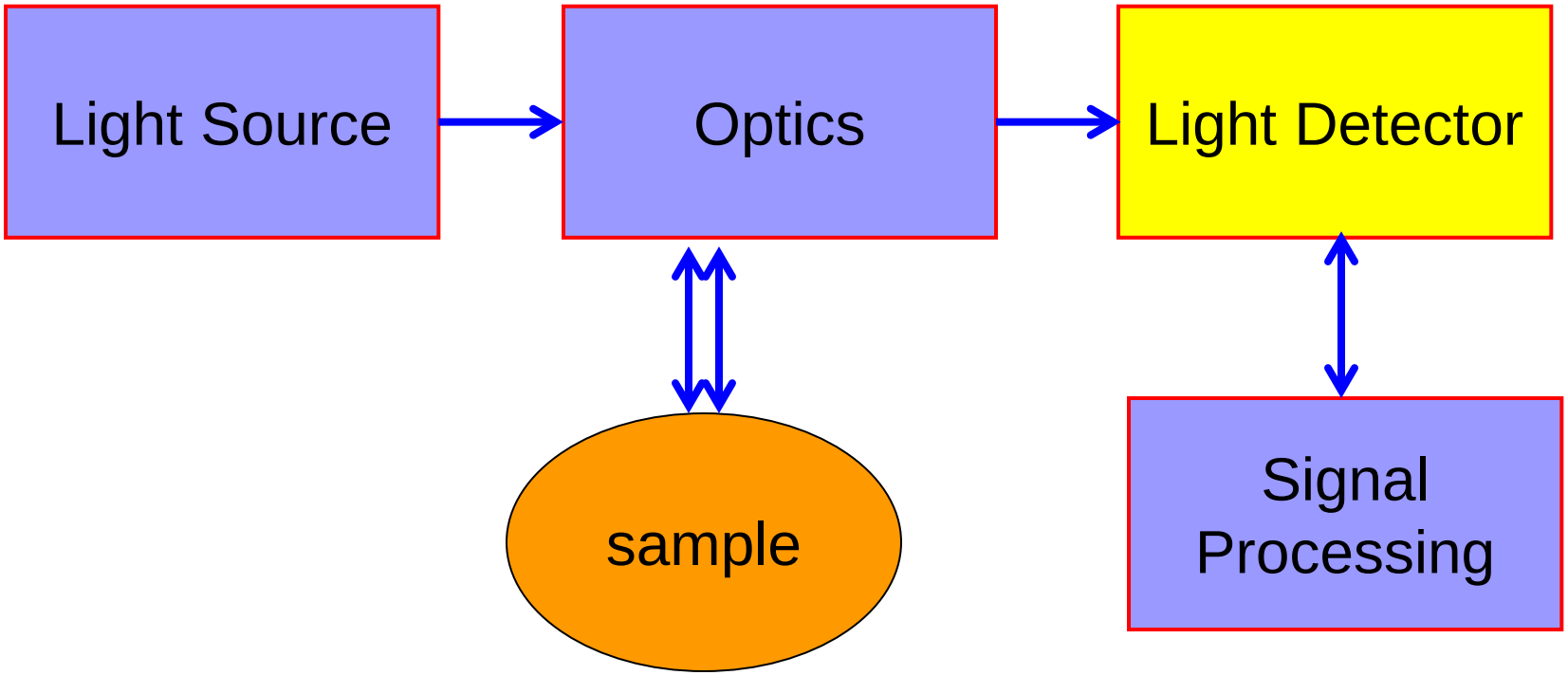



From Peter So lectures

# Separation of excitation and emission



# A typical fluorescence microscopy setup



# Light detectors

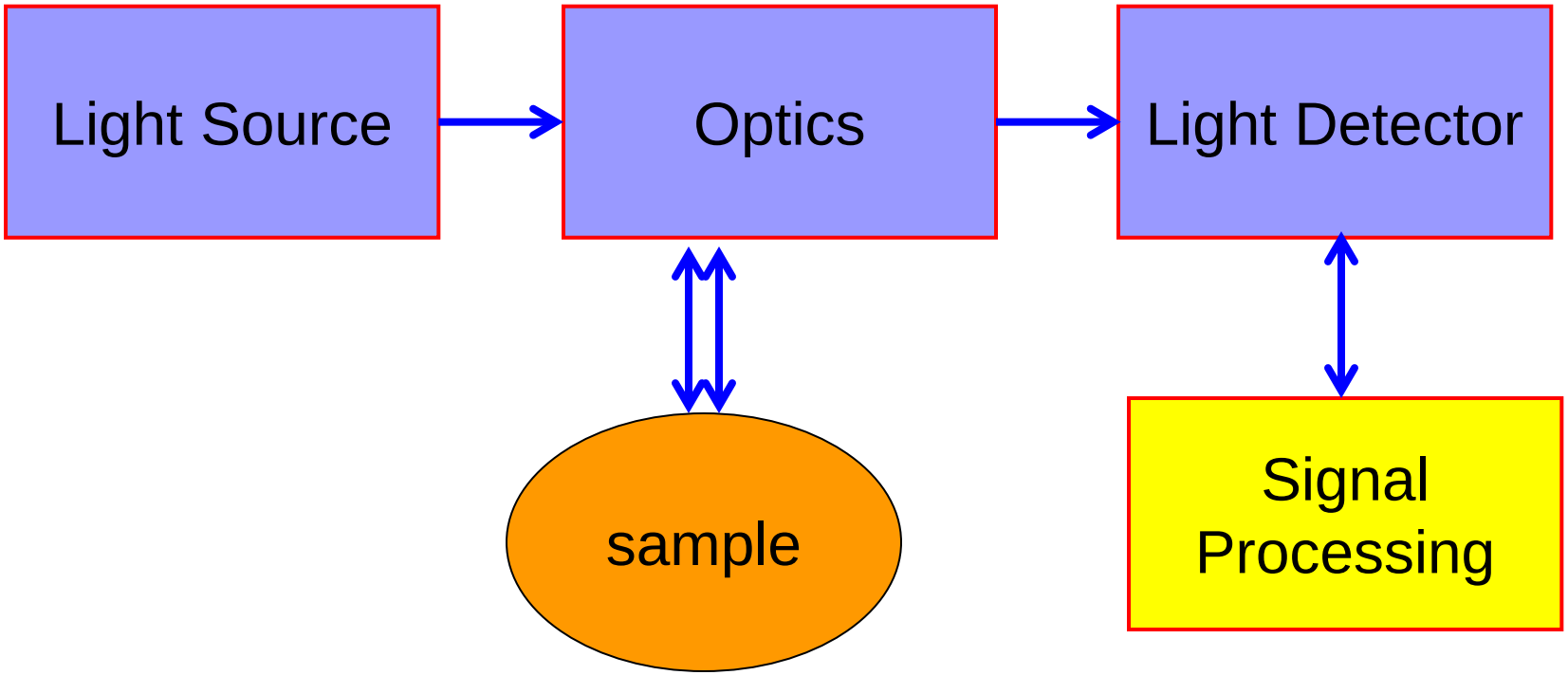
Laser Scanning Microscope

- Photomultiplier tubes (PMT)
- Avalanche Photodiodes (SPAD)
- Hybrid PMT

Full Field (widefield) Illumination

- CCD cameras

# A typical fluorescence microscopy setup



# Signal Processing

Analog

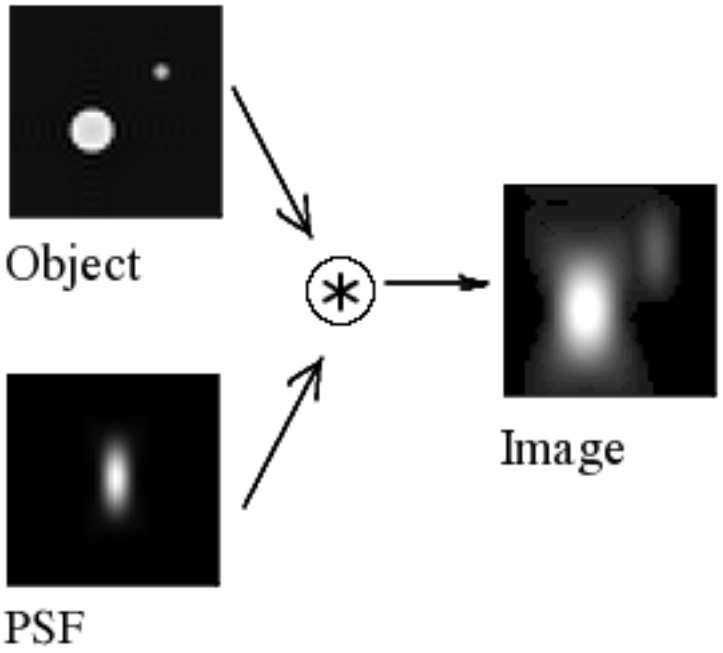
The photons acquired by the light detector are averaged. The signal is provided as <intensity on an arbitrary scale>

Photon Counting

The photons acquired by the light detector are counted. The signal is provided as <photons per second>

# 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

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

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

z-Gaussian

$$I(z) = \frac{1}{1+(\frac{z}{w_{oz}})^2}$$

z-Lorentzian

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

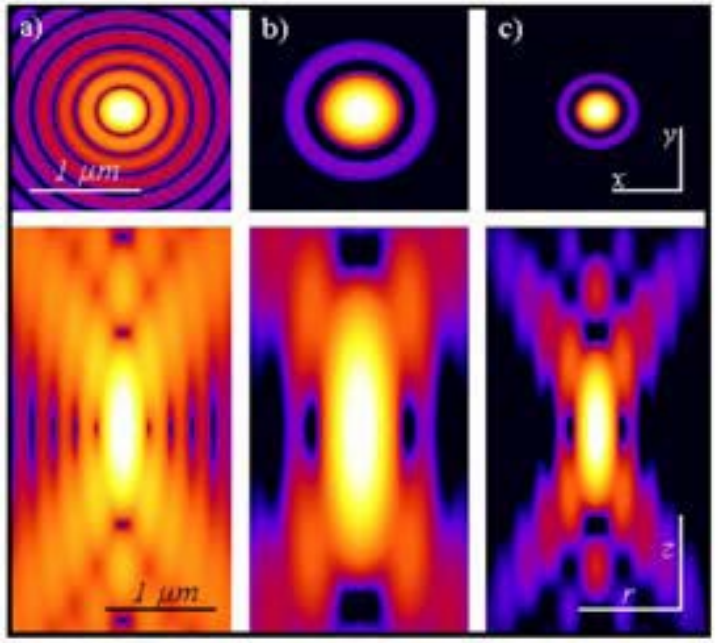
# PSF

a. widefield

b. 2-photon

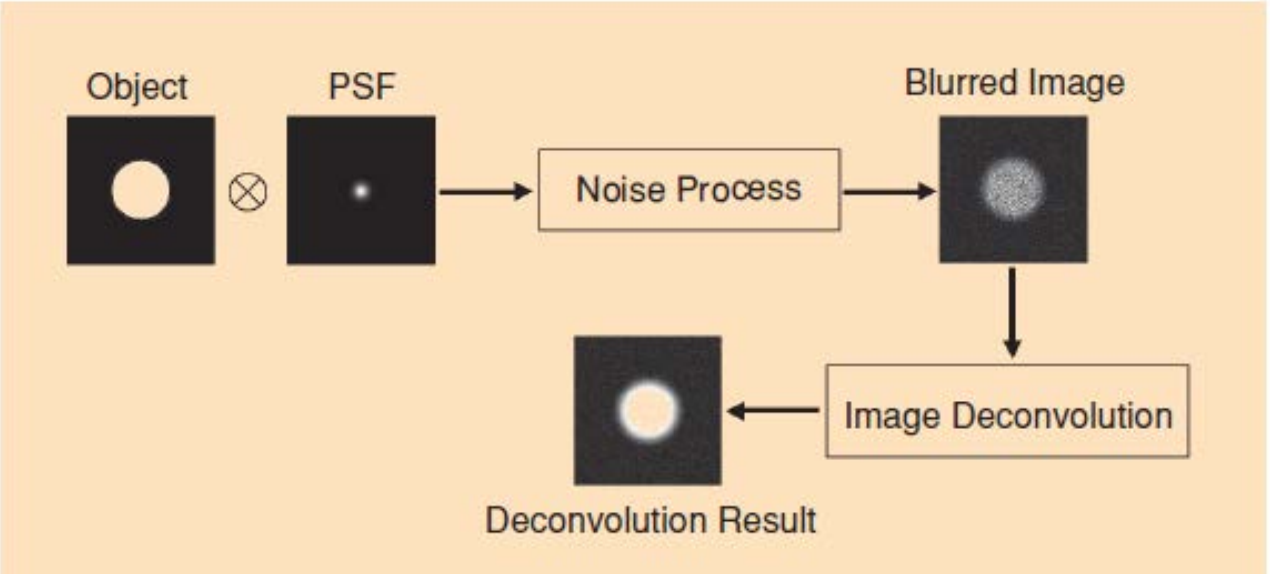
a. 1-photon w/ pinhole

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.




Diaspro et al.; BioMedical Engineering OnLine 5:36, 2006

# 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 $\lambda$=500nm, n=1.5 and $\alpha$=45degrees $\Delta$r=236nm

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

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

The next step

# Nanoscopy

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focus and discover

# Thank you.