SAPIENZA Università di Roma
Laurea magistrale in Ingegneria delle Nanotecnologie
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Biophotonics Laboratory Course

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Applications of optics and photonics

Microscopic Techniques
- Conventional Wide-Field Fluorescence
- TIRF
- FLIM
- FRET, FRAP
- Confocal
- Two-Photon
- Second Harmonic
- Super-resolution (SNOM, STED, PALM, STORM)

Non-Microscopic Label-free
- Surface plasmon Polaritons (SPP)
- Photonic crystals (PC)
- Raman , CARS
- Quantum dots

Non-Microscopic Techniques
- Cytofluorimetry
- ELISA
- DNA-Chip
- Cycle-sequencing
- SOLID

Other non Microscopic Techniques
- Southern
- Western
- Northern

All of them make use of the emission of luminescent markers (labels)

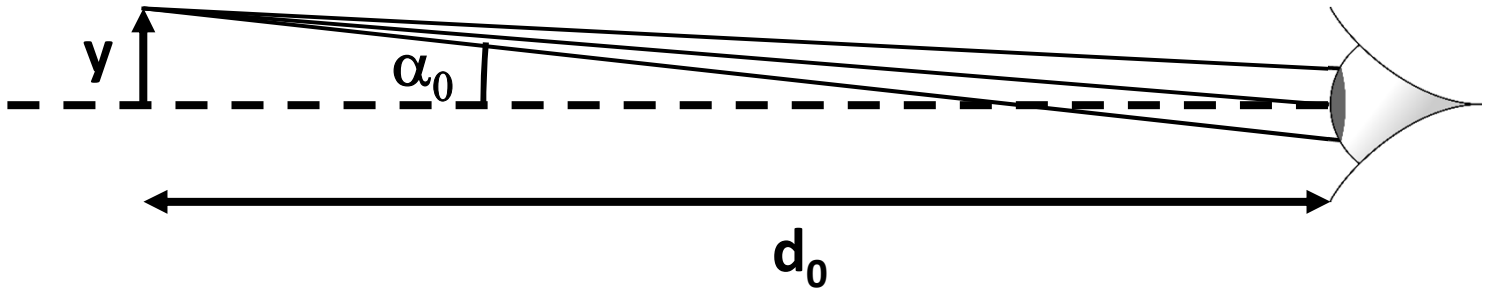
LECTURE 4

Wide-Field Fluorescence Microscopy and Confocal Microscopy

Basics of Optical Microscopy

Direct Human vision

- The appearent dimension of an object depend on the dimension of the image that is formed on the eye's retina, which is proportional to the angle of view, which is the angle under which an object is viewed from the eye.
- To explore the details one should bring the object as close as possible to the eye.
- There is a minimum distance $\mathbf{d_0}$ where one can bring the object and where the eye is still able to focus it on the retina.
  - Kid (10 years) $\mathbf{d_0}$ = 7 cm
  - Adult man $\mathbf{d_0}$ = 25 cm
  - Aged man $\mathbf{d_0}$ > 25 cm (presbiopy)

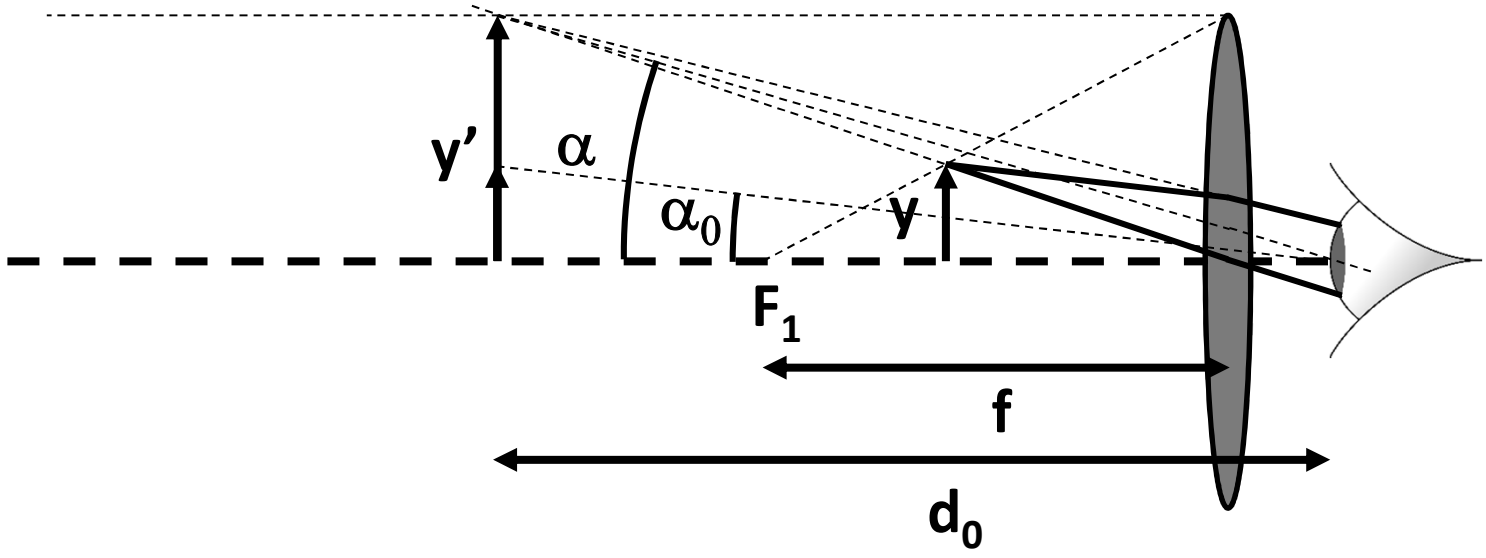


- Maximum angle in the direct vision $\boldsymbol{\alpha_0 \approx tg\alpha = \frac{y}{d_0}}$

Basics of Optical Microscopy

Human vision by means of a simple microscope (magnifying lens)

- A thin convergent lens provides a positive and virtual image of an object that is closer to the lens than the focal length f.
- One can put the object in a position that is such that to provide an image that is at a distance $\mathbf{d \geq d_0}$ from the eye, which under these conditions can focus on th retina without any problem.

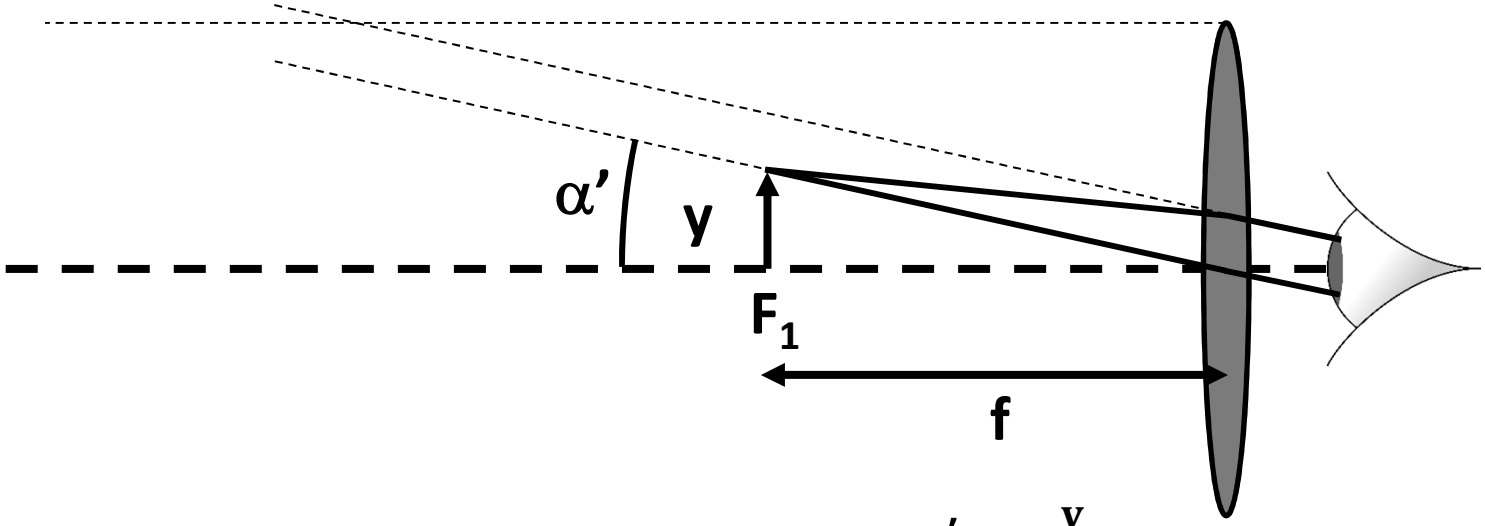


- Maimum angle in the vision $\alpha \approx \frac{y'}{d_0} > \alpha_0$

Basics of Optical Microscopy

Human vision by means of a simple microscope (magnifying lens)

- The maximum angular dimension of the image is obtained when the object is in the focal plane of the lens.
- In such a situation the eye operates under completely relaxed conditions, i.e. it is not stressed as it focused an object that is at infinite distance.

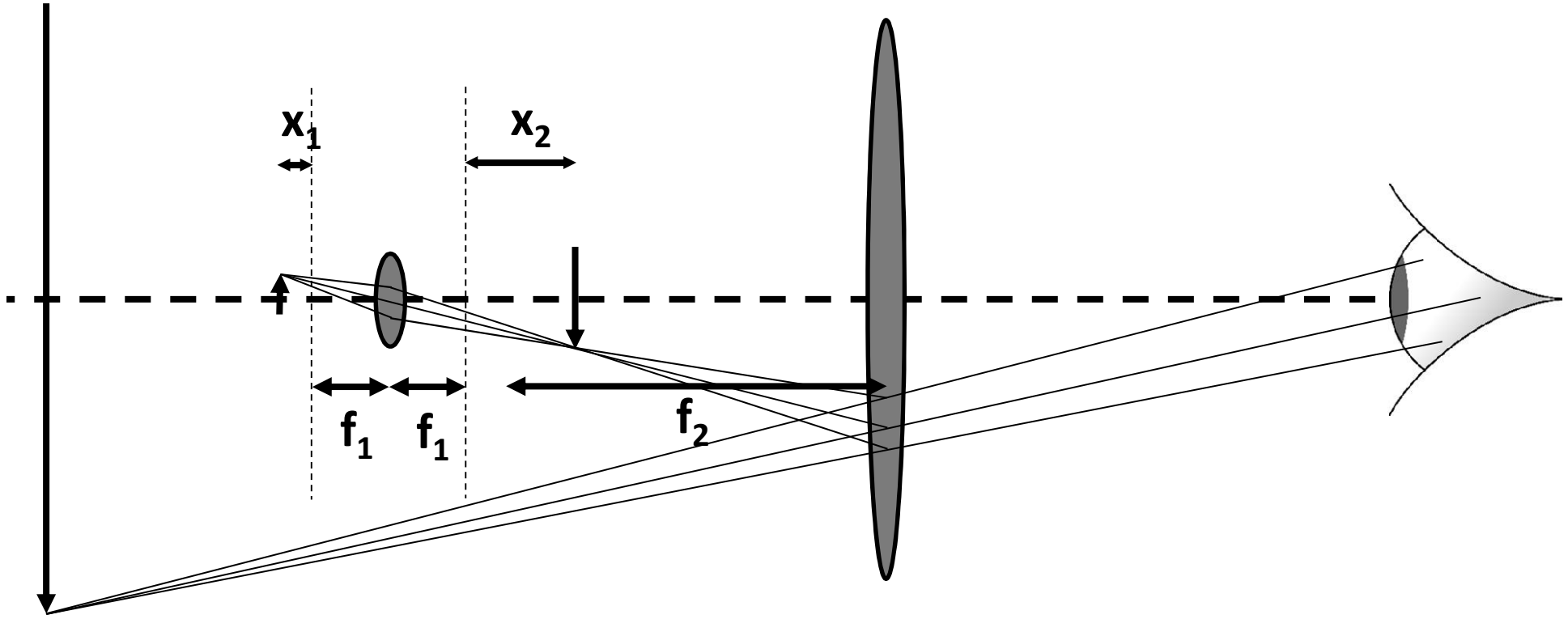


- Maximum angle in the vision $\alpha' \approx \frac{y}{f}$
- Angular magnification $M = \frac{\alpha'}{\alpha} = \frac{d_0}{f} = \frac{25}{f(cm)}$
- Typically $M = 5 \div 10$

Basics of Optical Microscopy

Human vision by means of a composite microscope

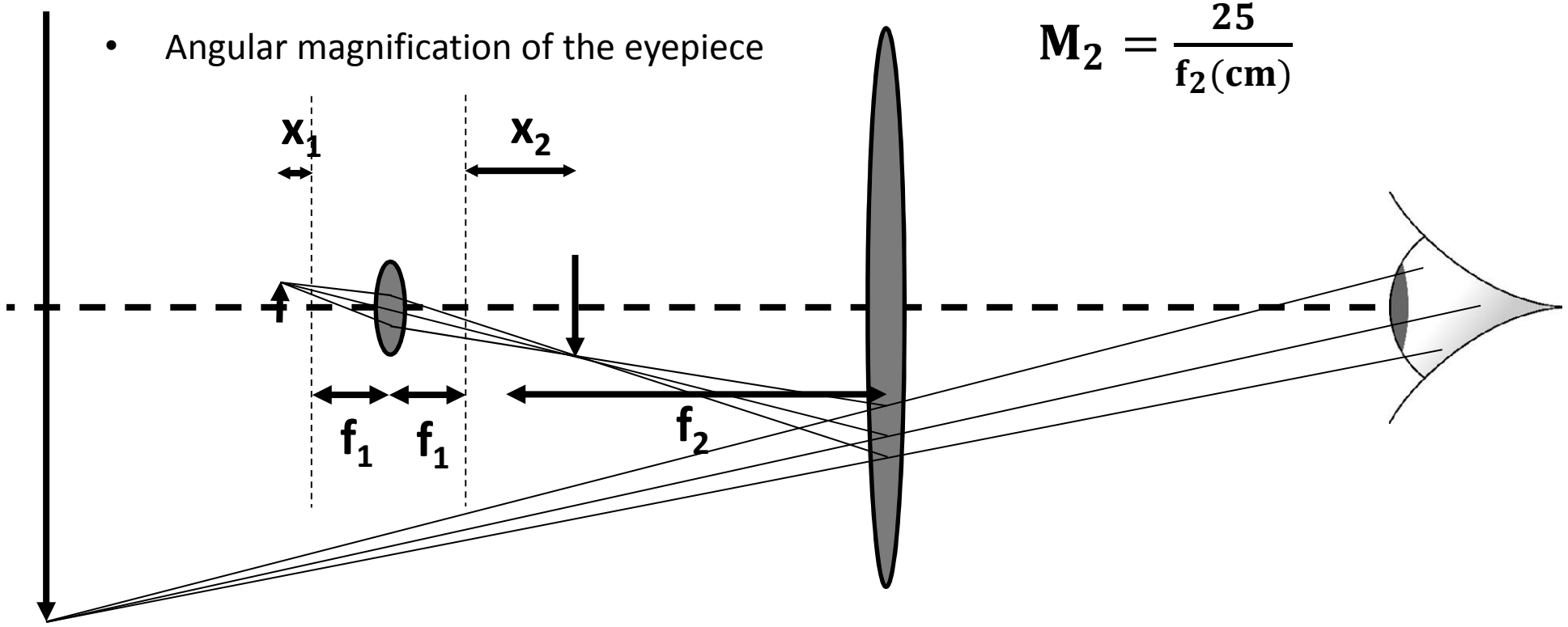
- Based on two systems of corrected lenses.
- The first (**objective**) has a very short focal length $\mathbf{f_1}$ and is used to provide a real and magnified image of the object.
- The second (**eyepiece**) has a longer focal length $\mathbf{f_2}$ and is used to observe such an image with the eye in the best conditions.



Basics of Optical Microscopy

Human vision by means of a composite microscope

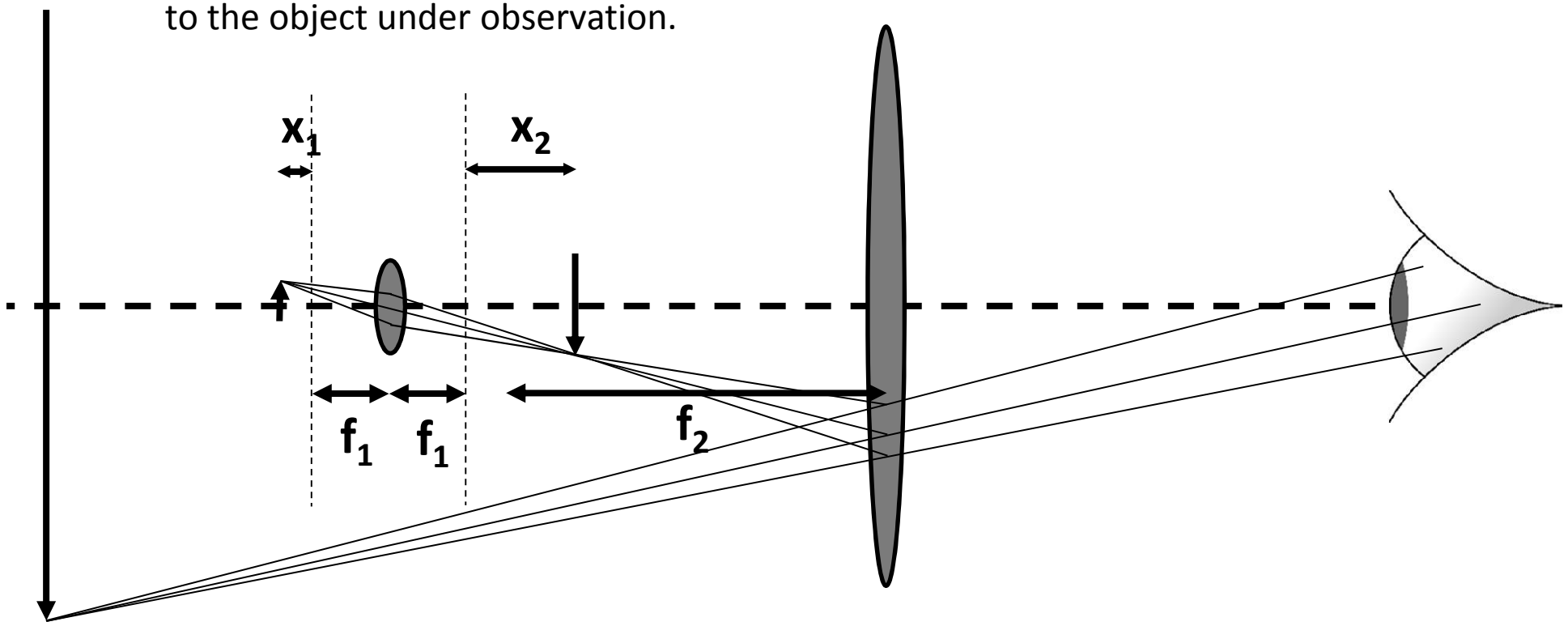
- The total angular magnification of the composite microscope is the product of the linear magnification $\mathbf{m_1}$ of the objective and the angular magnification $\mathbf{M_2}$ of the eyepiece.
- Linear magnification of the objective $$\mathbf{m_1 = -\frac{p}{q} = -\frac{x_2 + f_1}{x_1 + f_1} \approx -\frac{x_2}{f_1}}$$
- Angular magnification of the eyepiece $$\mathbf{M_2 = \frac{25}{f_2(cm)}}$$



Basics of Optical Microscopy

Human vision by means of a composite microscope

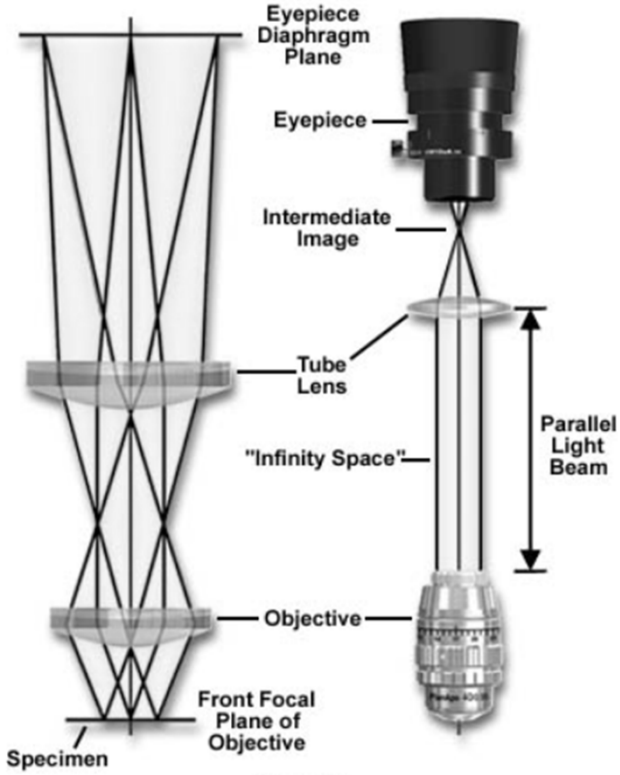
- Total angular magnification $$M = -\frac{x_2}{f_1}\frac{25}{f_2}$$
- Tipically one gets M<1000.
- In common microscopes the eyepiece/objective distance is fixed, as they are mounted in a tube, and focusing is carried out by shifting the tube with respect to the object under observation.



Basics of Optical Microscopy

Human vision by means of a composite microscope

- Almost all manufacturers design optical microscopes that make use of objectives that are corrected for observation at infinite distance.
- To create the image, the tube must contain a supplementary lens, the **tube lens**.
- The **tube lens** provides an image located on the diaphragm plane of the eyepiece (**intermediate image plane**).
- The systems that are corrected for infinite observation:
  - Show a region where the beams are parallel;
  - Eliminate the ghost images due to the transit of converging beams through planar optical elements;
  - Permit to introduce between the objective and the tube lens complex optical systems. This is particularly useful in wide-field and confocal epifluorescence microscopy and their most advanced derivatives.



Fluorescence Microscopy

Scheme of a Fluorescence microscope in reflection configuration (Epifluorescence)

- ***Light sources***
  - Lamps(Hg, Xe)
  - Laser(Argon Ions)

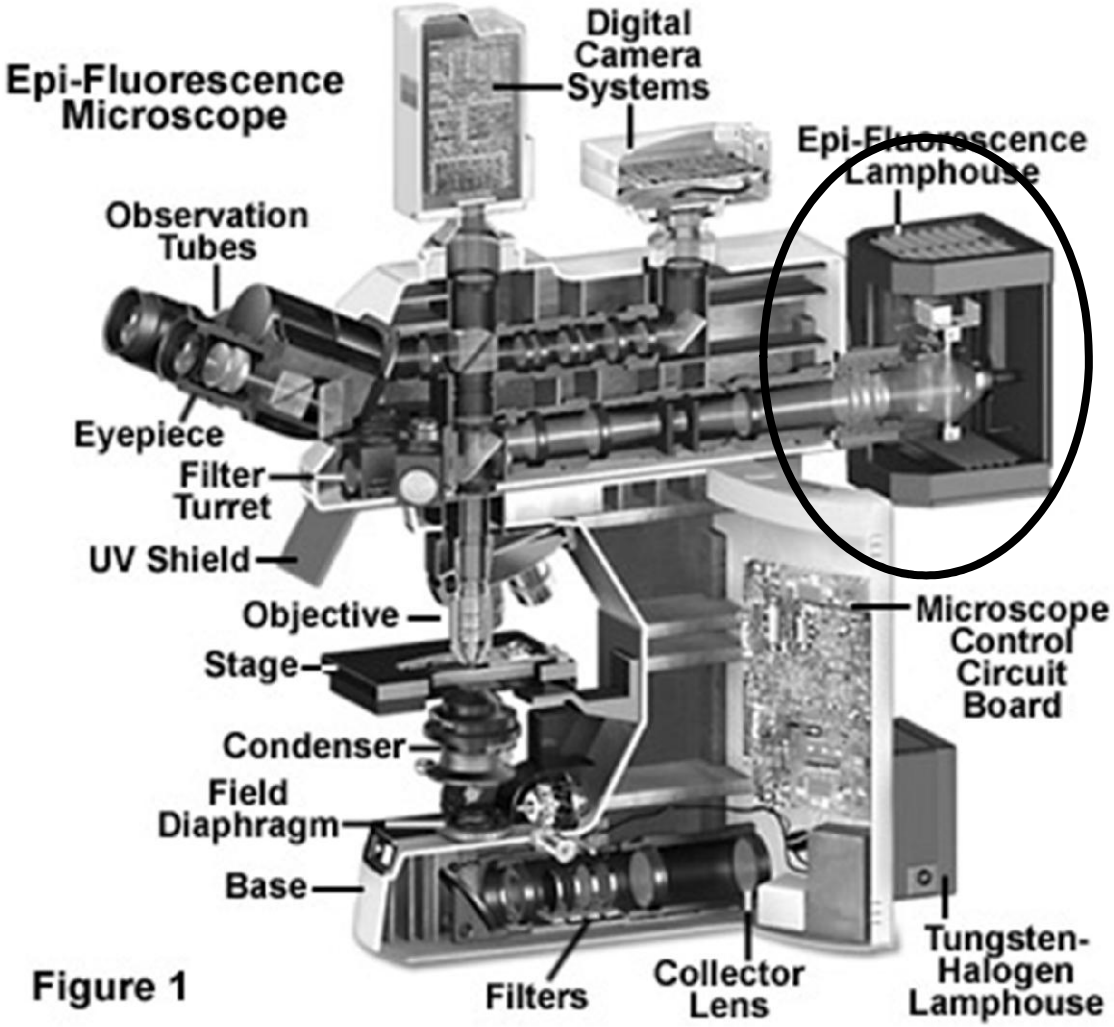
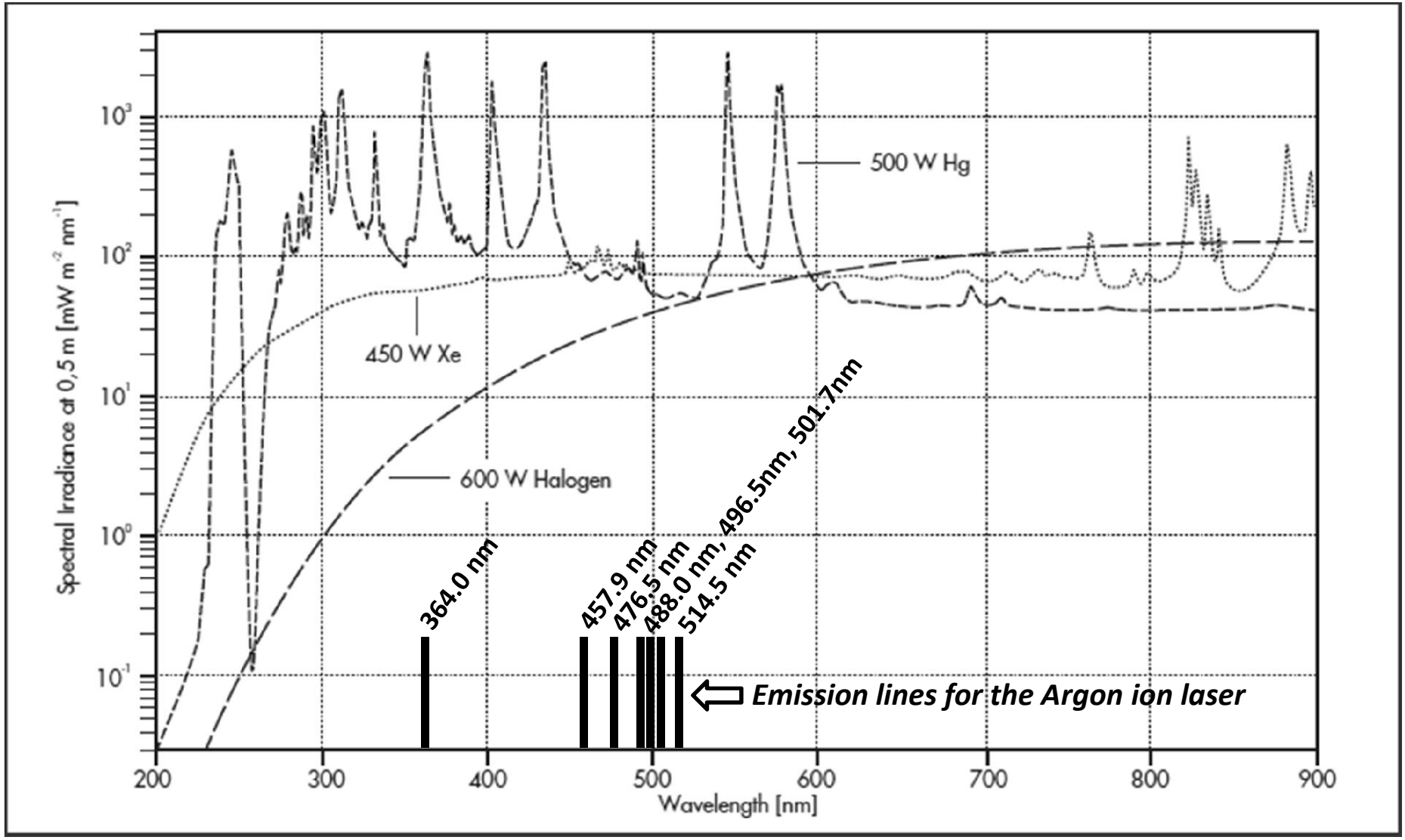


Figure 1

Fluorescence Microscopy

Light sources



Fluorescence Microscopy

Scheme of a Fluorescence microscope in reflection configuration (Epifluorescence)

- ***Light sources***
  - Lamps(Hg, Xe)
  - Laser(Argon Ions)
- ***Filters block and dichroic mirror***

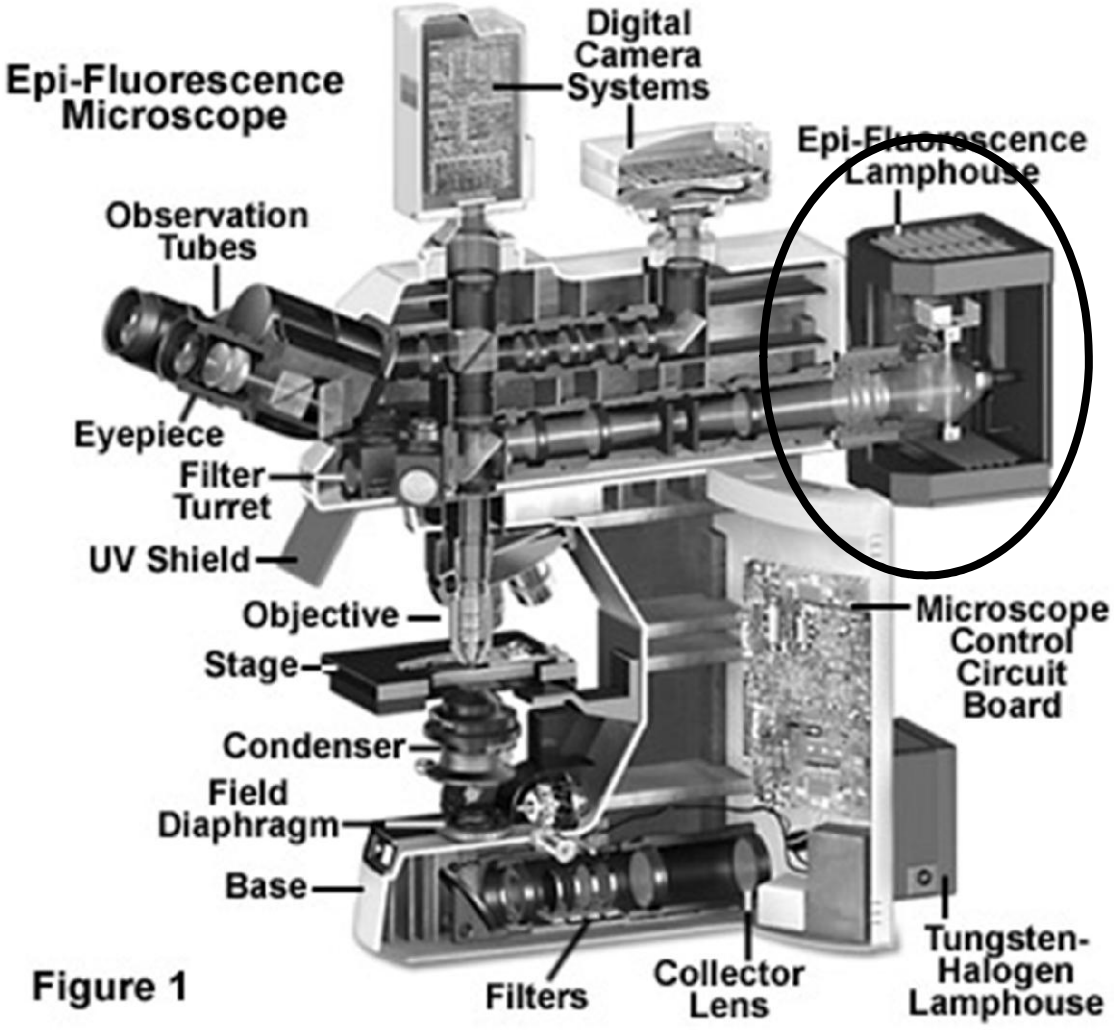
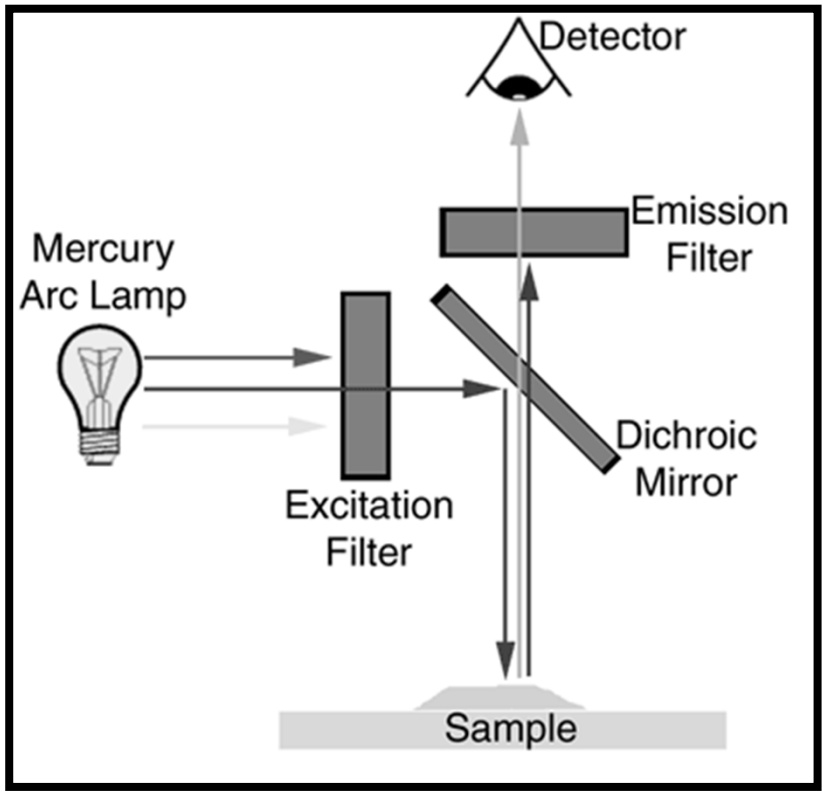


Figure 1

Fluorescence Microscopy

Filters block and dichroic mirror



- ***Excitation filter***

Selects a portion of the spectrum of the lamp in correspondence of the absorption spectrum of the particular chromophore that we are using

- ***Dichroic mirror***

Reflects the excitation radiation and transmits the radiation emitted by the chromophores

- ***Emission filter***

Selects the portion of the spectrum corresponding to the emission spectrum of the particular chromophore we are using

Fluorescence Microscopy

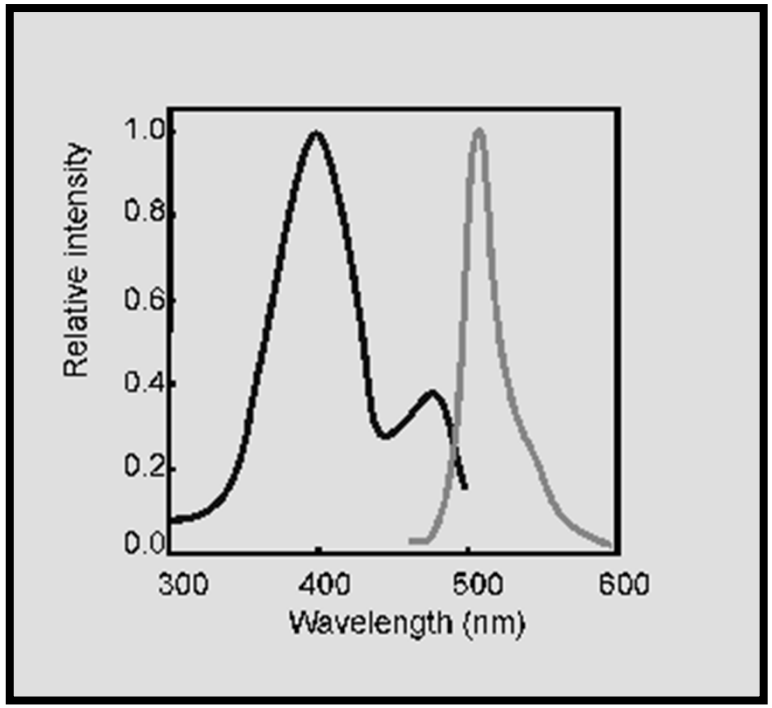
Filters block and dichroic mirror

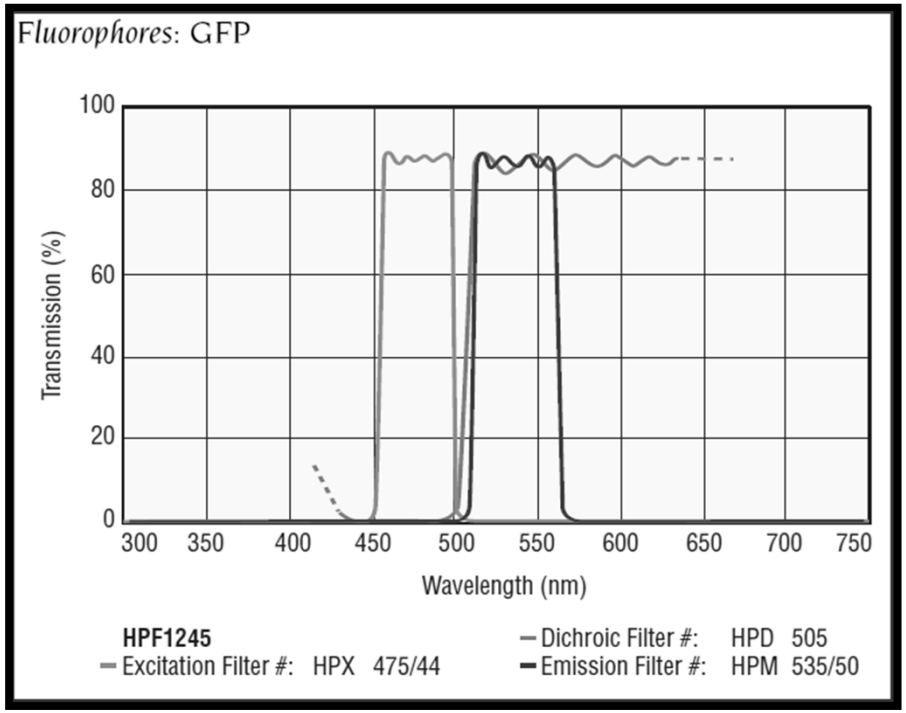
Newport HPF™ Filters for Microscopy Applications

- In commercial fluorescence microscopes the three filters that are needed to observe a particular chromophore are inserted in a single block.
- The microscopes are equipped with several blocks that can be changed by means of a revolver charger.
- Almost all filters are interferential filters. They are periodic multilayers constituted by high and low refractive index materials.

Fluorescence Microscopy

Filters block and dichroic mirror
Chromophore: Green Fluorescent Protein (GFP)





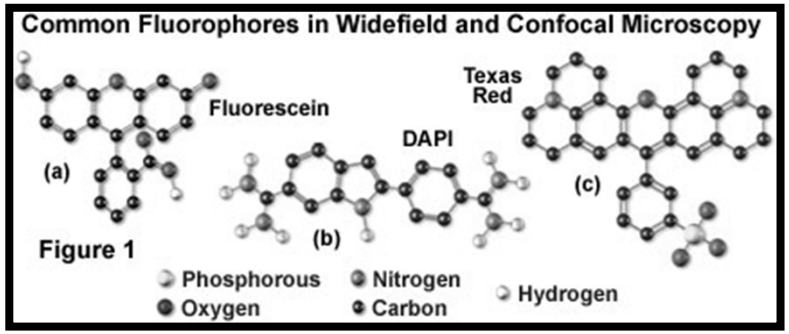
The principal characteristics that a filter must show are:

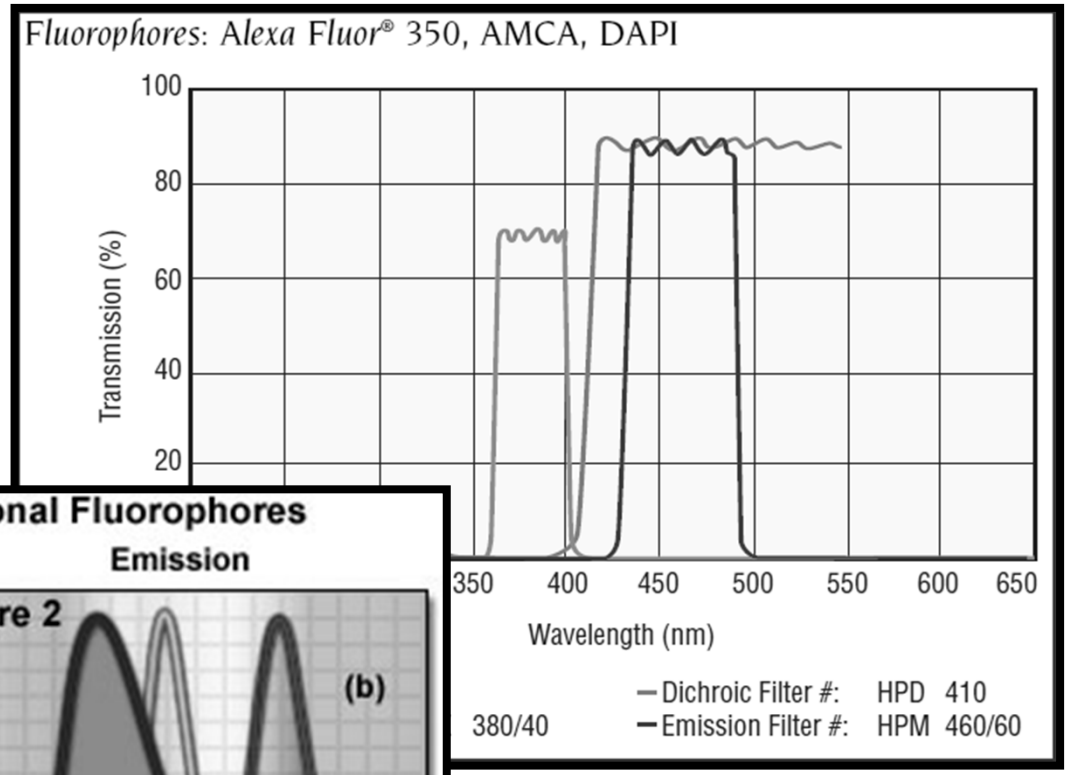
- Minimum values of autofluorescence
- Sharp spectral transitions between transmittance and reflectance regions
- Maximum transmittance in the transmission window
- Maximum signal to noise ratio

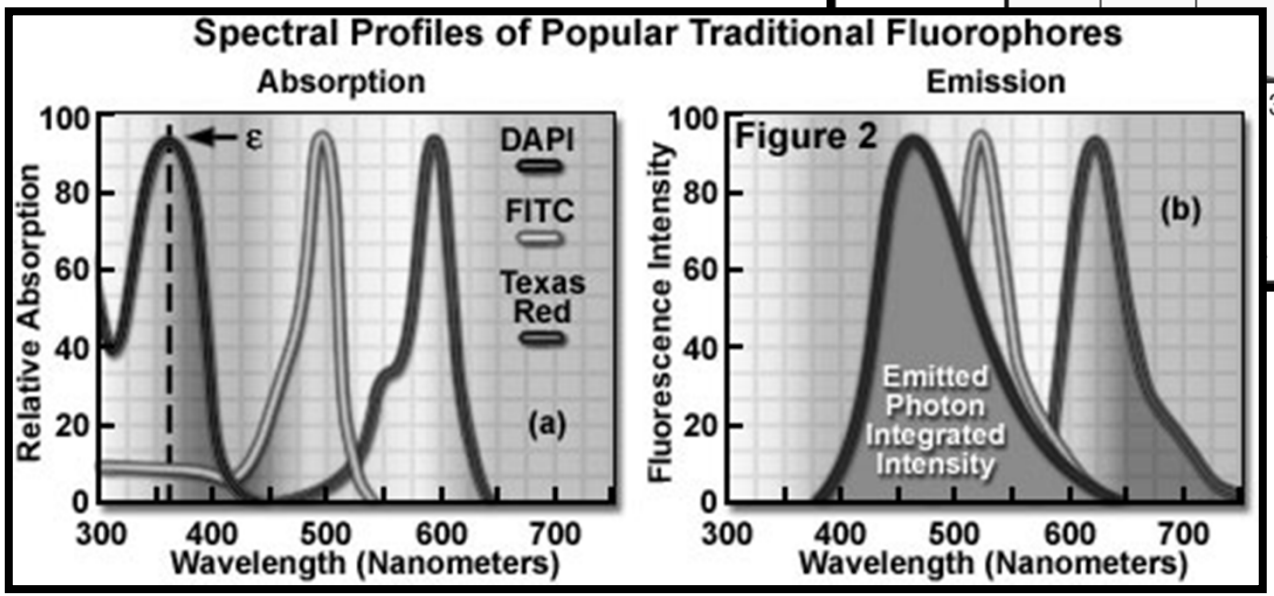
Fluorescence Microscopy

Filters block and dichroic mirror

Chromophore: 4',6-diamidino-2-phenylindole (DAPI)

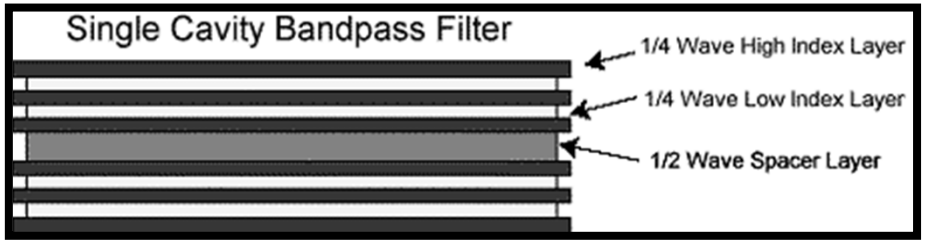


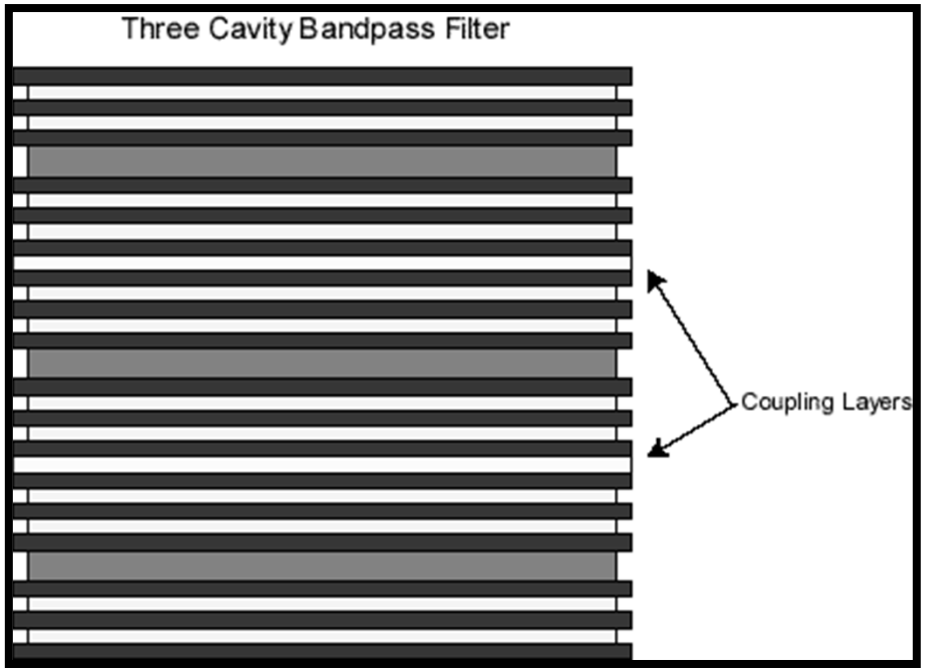


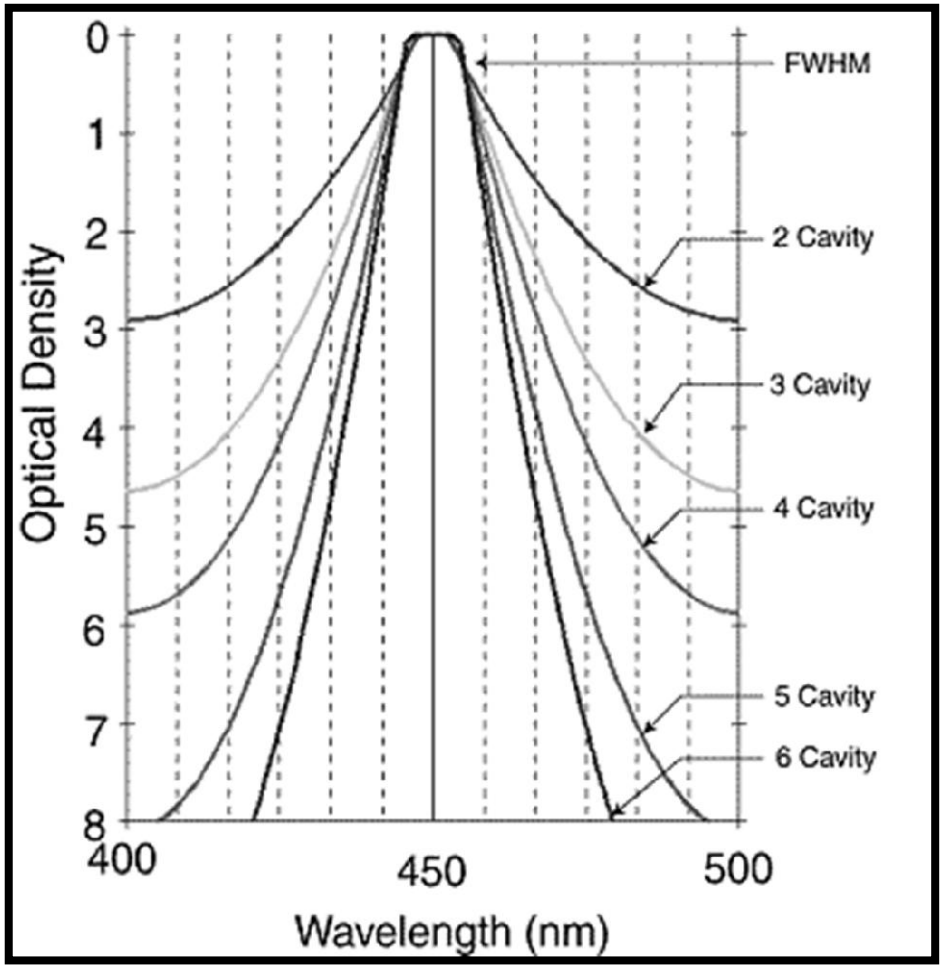


Fluorescence Microscopy

Interferential Filters







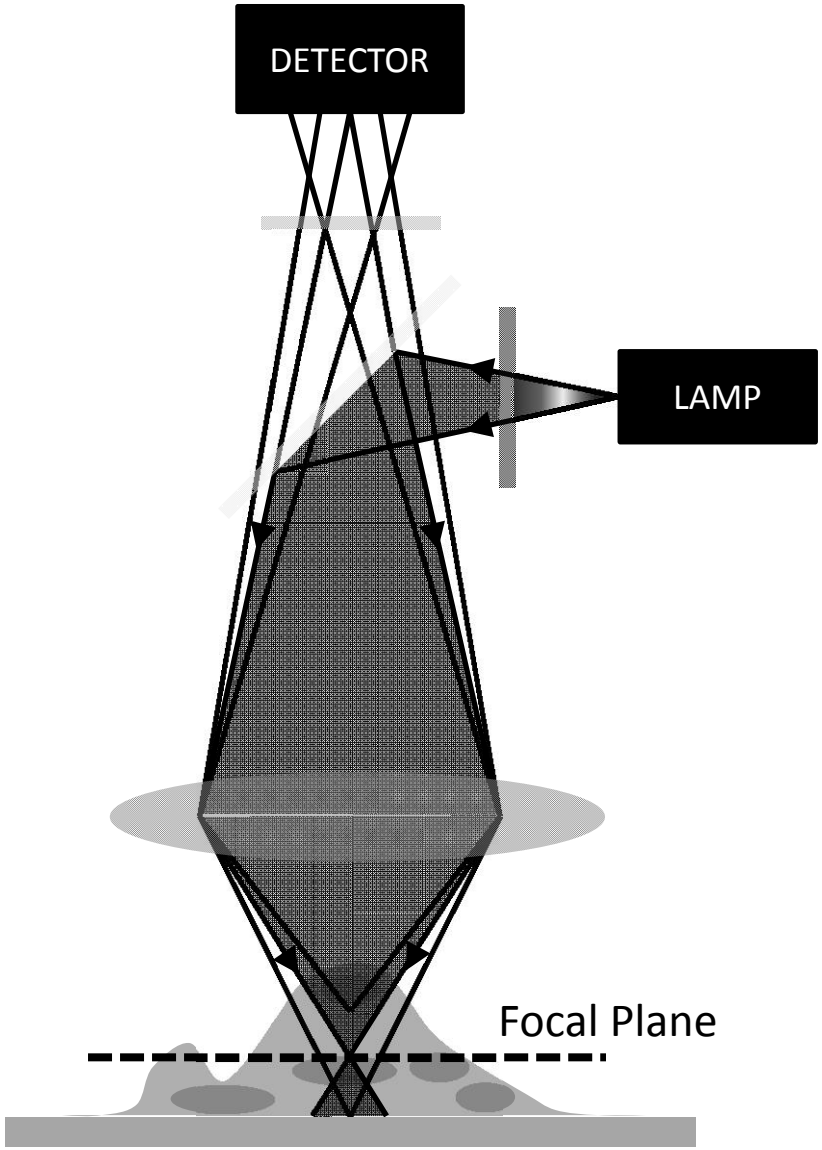
Fluorescence Microscopy

Problems

- Fluorescence that comes from regions of the samples that are outside the focal plane is anyhow collected by the detector. This deterirates the contrast (blur).

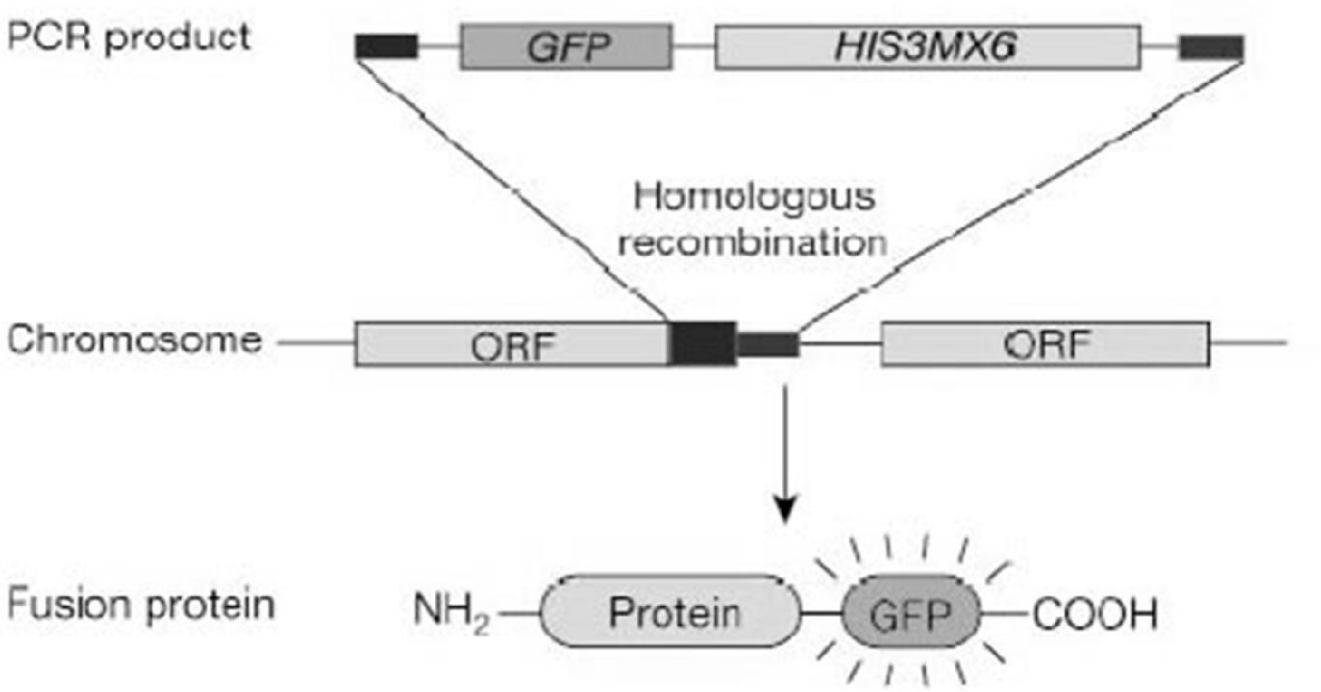
- The thickness of the samples is limited by the focal depth of the objective that is used.

- Detection of a very low chromphores' concentration is limited by the signal/noise ratio, i.e. by the capacitiy of the emission filter to completely eliminate the phtons of the excitation beam.



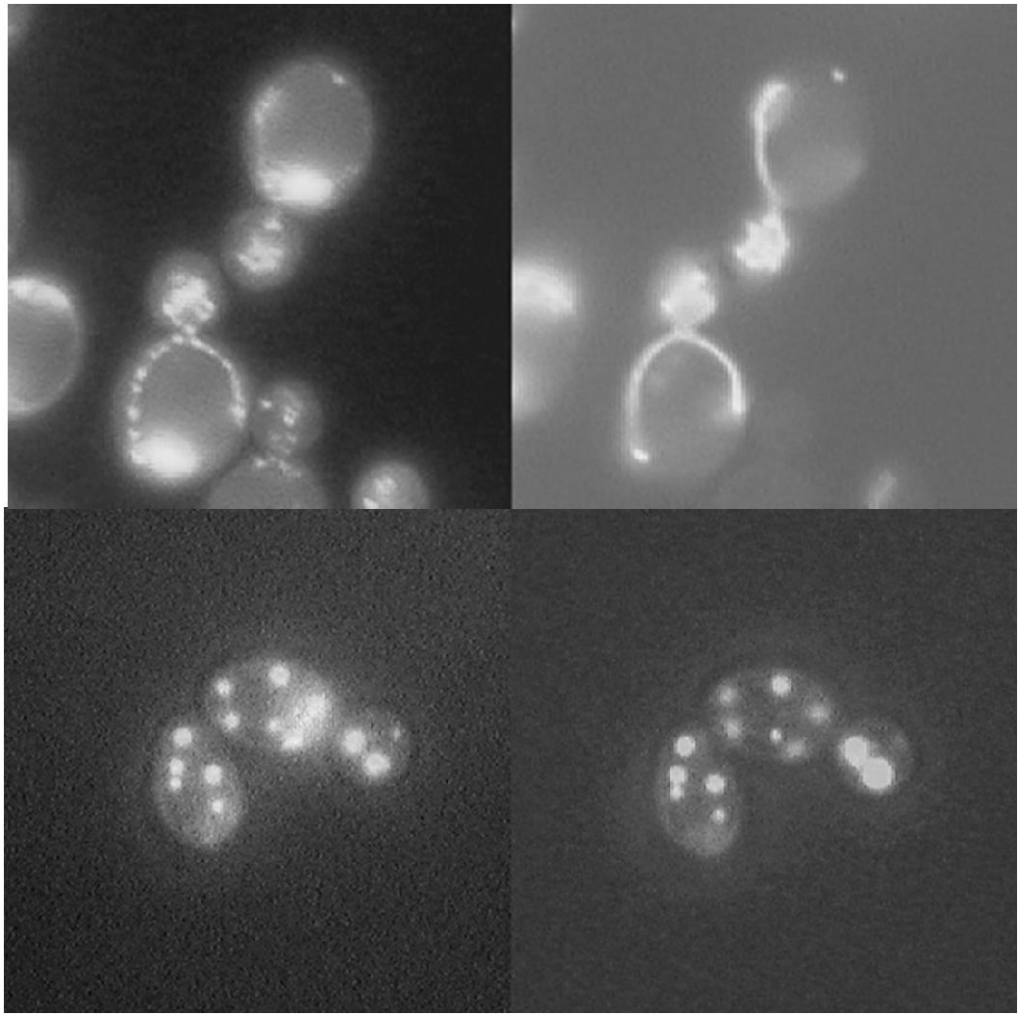
Fluorescence Microscopy

GFP fusion



Fluorescence Microscopy

The rpn11-m1 mutant shows a mitochondrial morphology defect

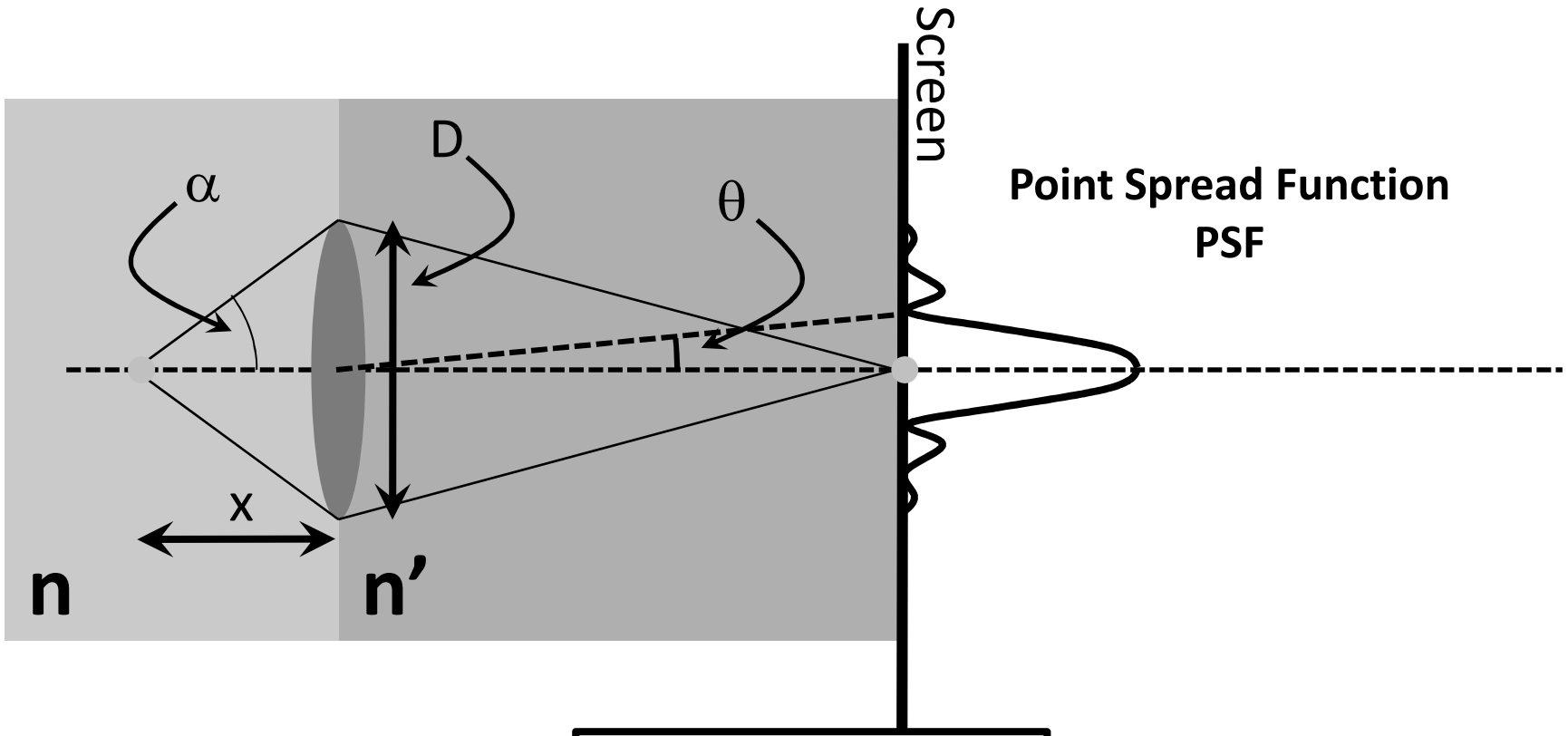


Wild-type

rpn11-m1

Fluorescence microscopy images; DAPI staining and MitoGfp

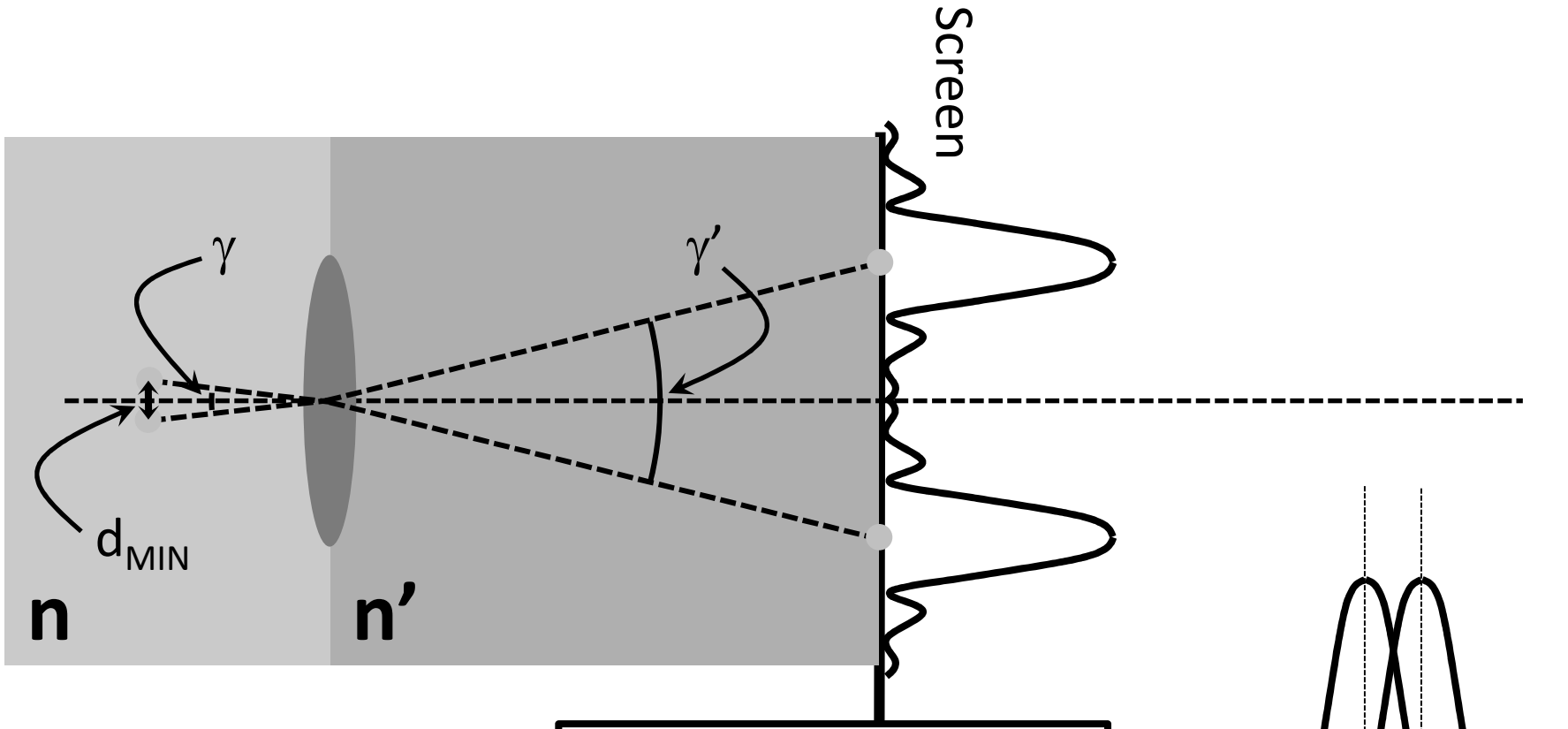
Lateral resolution of a wide-field microscope



Diffraction (Airy) → $$\sin\theta \approx \theta \approx 1.22\frac{\lambda}{D}$$

Numerical aperture → $$N.A. = n \cdot \sin\alpha \approx n \cdot \alpha \approx n \cdot \frac{D}{2x}$$

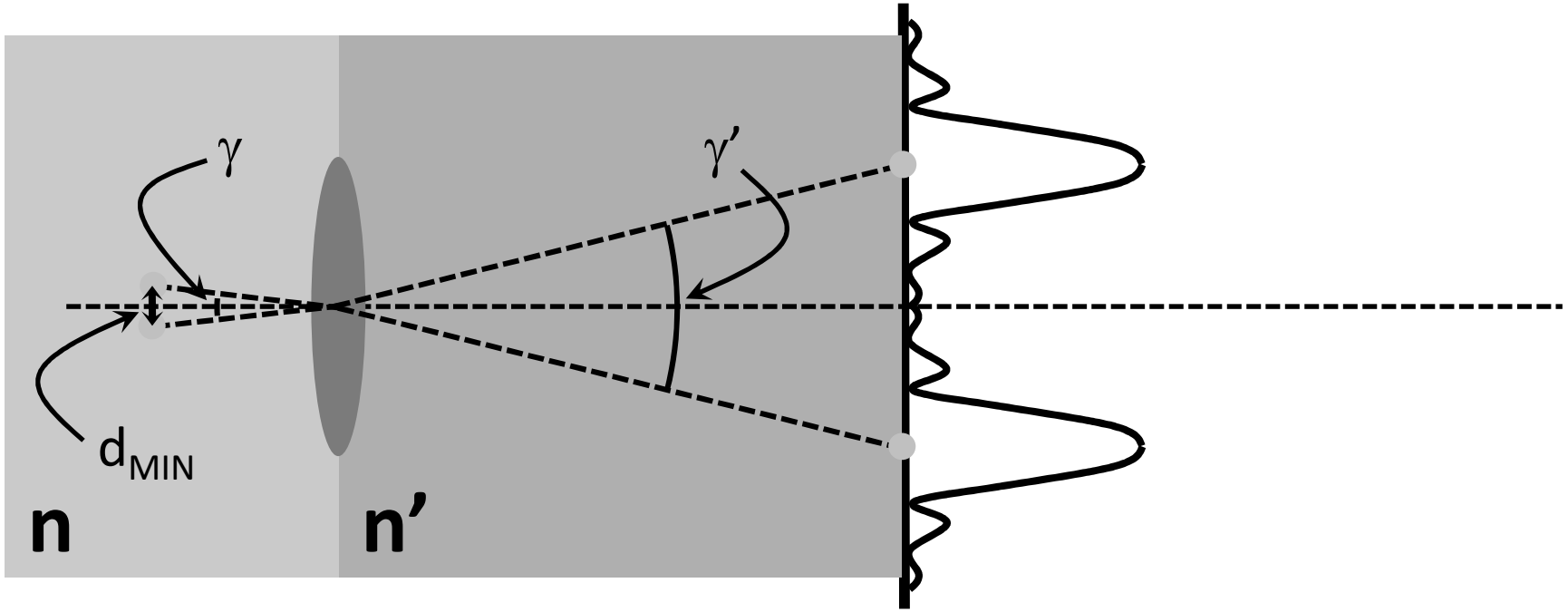
Lateral resolution of a wide-field microscope



Rayleigh → $\gamma'_{MIN} = \theta_{AIRY} = 1.22\frac{\lambda}{D}$

Snell → $n\sin\frac{\gamma}{2} = n'\sin\frac{\gamma'}{2} \qquad \sin\frac{\gamma}{2} \approx \frac{\gamma}{2} \qquad n\gamma \approx n'\gamma'$

Lateral resolution of a wide-field microscope



$$d_{MIN} \approx \gamma \cdot x \approx \frac{n'}{n}\gamma' x \approx \frac{n'}{n} x \cdot 1.22 \cdot \frac{\lambda}{D} \approx \frac{n'}{n} \cdot \frac{D}{2\alpha} \cdot 1.22 \cdot \frac{\lambda}{D} \approx \frac{0.61 \cdot \lambda \cdot n'}{n \cdot \alpha} \approx \frac{0.61 \cdot \lambda \cdot n'}{N.A.}$$

Abbe → $$d_{MIN} = \frac{0.61 \cdot \lambda \cdot n'}{N.A.} = \frac{0.61 \cdot \lambda}{N.A.} \quad \text{where} \quad n' = 1$$

Lateral resolution of a wide-field microscope

Lateral resolution of a conventional wide-field microscope.

Objective	N.A.	d_{MIN} @535nm	d_{MIN} @670nm
20x	0.5 (air)	652.7nm	817.4nm
40x	0.75 (air)	435.1nm	544.9nm
60x	1.4 (oil)	233.1nm	291.9nm

Confocal Microscopy

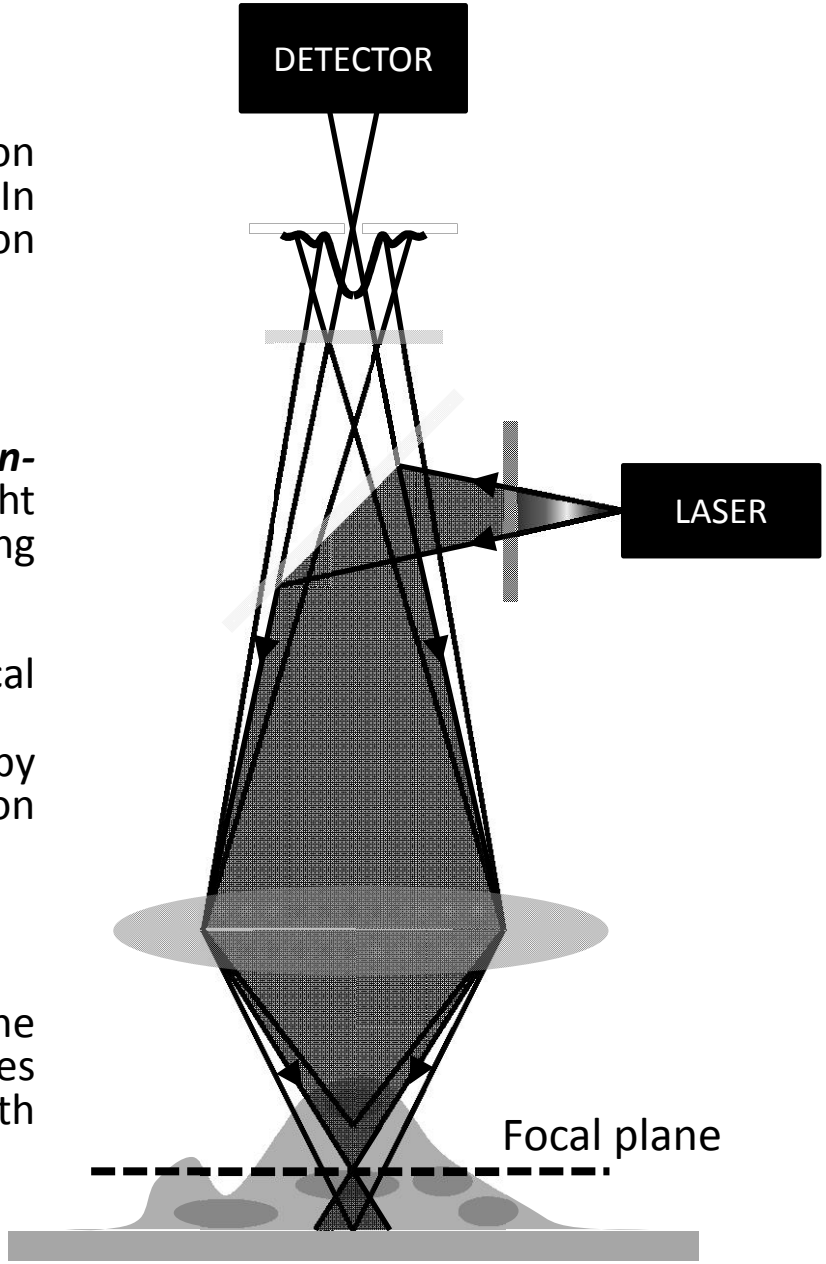
Characteristics

- The confocal microscope uses the same configuration of the wide-field fluorescence microscope. In particular the block with excitation and emission filters and dichroic mirror.
- The light source is normally a single line CW laser.
- The fundamental and novel issue is the use of a ***pin-hole*** that is positioned in the point where the light rays coming from the focal plane are converging (***conjugated point***). The pin-hole:
  - Elimnates the radiation coming from the off focal plane planes;
  - Permits to increase the lateral resolution by filtering the secondary maxima of the diffraction pattern:

$$d_{MIN} = \frac{0.4 \cdot \lambda}{N.A.}$$

- By shifting the sample along the vertical direction one can obtain images of the fluorescence that comes from all horizontal sections inside the sample with axial resolution:

$$d_{ASS} = \frac{1.4 \cdot \lambda \cdot n}{(N.A.)^2}$$



Confocal Microscopy

Lateral and axial resolution of a confocal microscope.

Objective	N.A.	d_{MIN} @535nm	d_{MIN} @670nm	d_{ASS} @535nm	d_{ASS} @670nm
20x	0.5 (air)	428.0nm	536.0nm	2.996μm	3.752μm
40x	0.75 (air)	285.3nm	357.3nm	1.332μm	1.668μm
60x	1.4 (oil n=1.5)	152.8nm	191.4nm	0.382μm	0.479μm

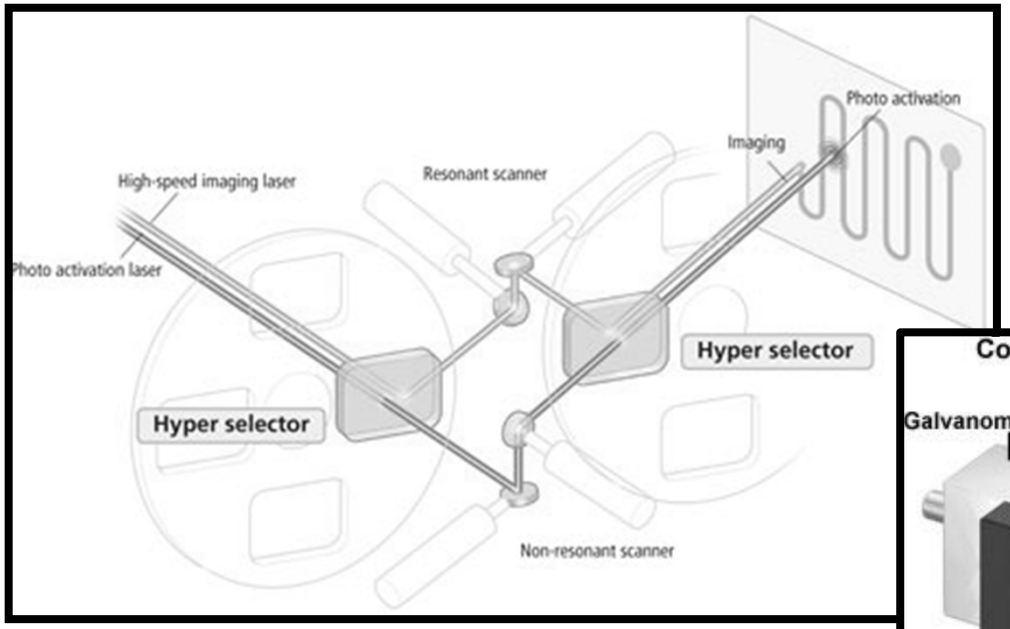
Optical Microscope

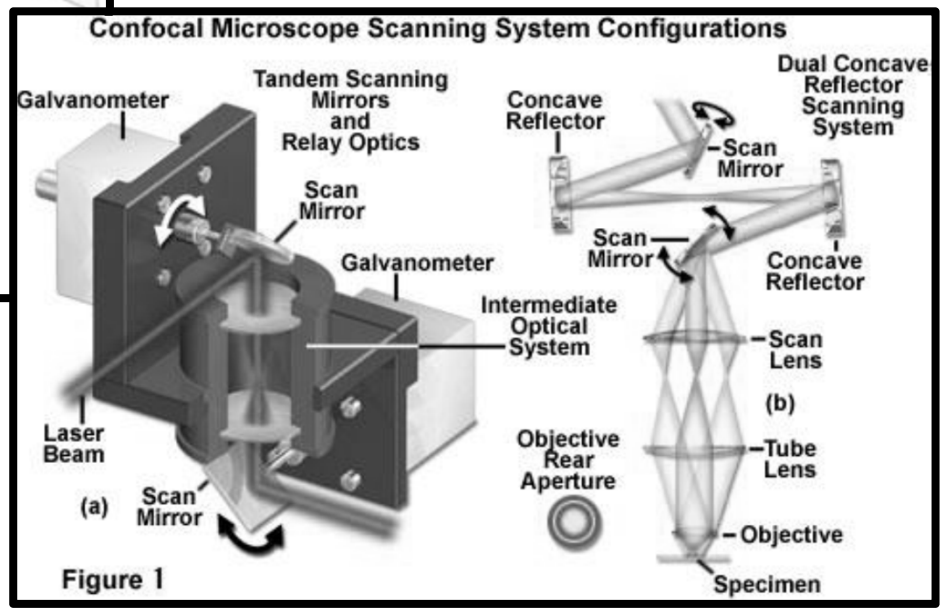
Objective	N.A.	d_{MIN} @535nm	d_{MIN} @670nm
20x	0.5 (air)	652.7nm	817.4nm
40x	0.75 (air)	435.1nm	544.9nm
60x	1.4 (oil)	233.1nm	291.9nm

Confocal Microscopy

Characterisitcs of the «Scanning systems»

The images are obtained by scanning the excitation laser point by point over the sample by means of a system of mirrors mounted on galvanometric actuators (***scanner***). Light is collected point by point by the pin-hole/detector system.





Confocal Microscopy

Characteristics of the «Spinning Disk Systems»

- The images are obtained by using a Nipkow disk associated to an array of microlenses.
- Such systems can use disks conteining arrays with up to 20000 microlenses.

- Every microlens focuses a portion of the excitation laser beam in the respective pin-hole. This increases the laser intensity on the sample to up 70%.
- By illuminating up to 1000 points simultaneously (multi-point scanner), it can reach a scanning speed larger than 2000 fps.
- It is therefore a key instrument to study living cells in real time.

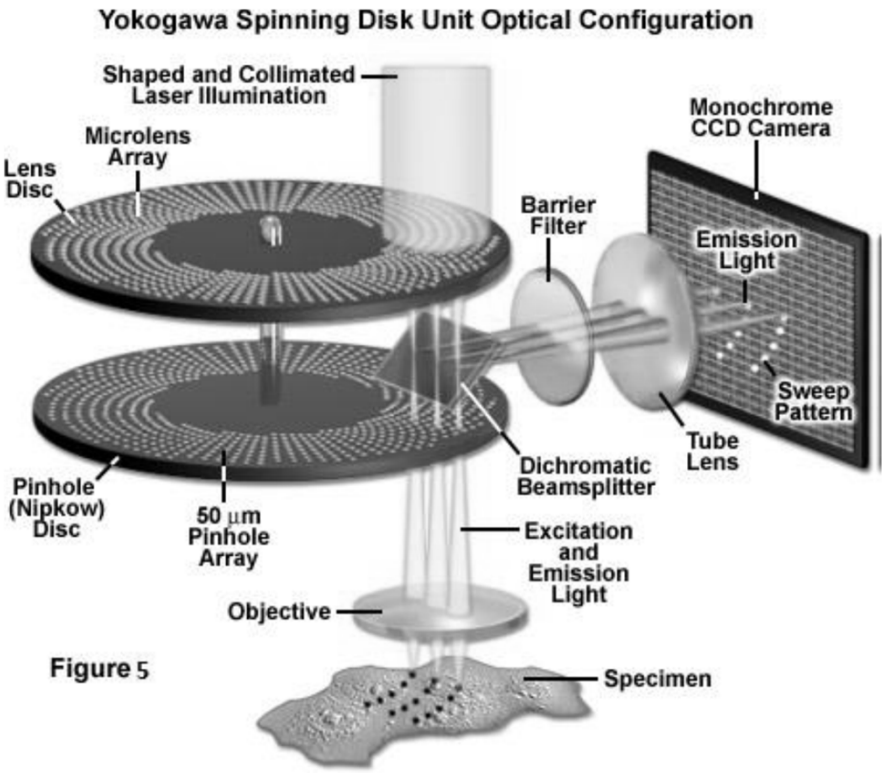


Figure 5

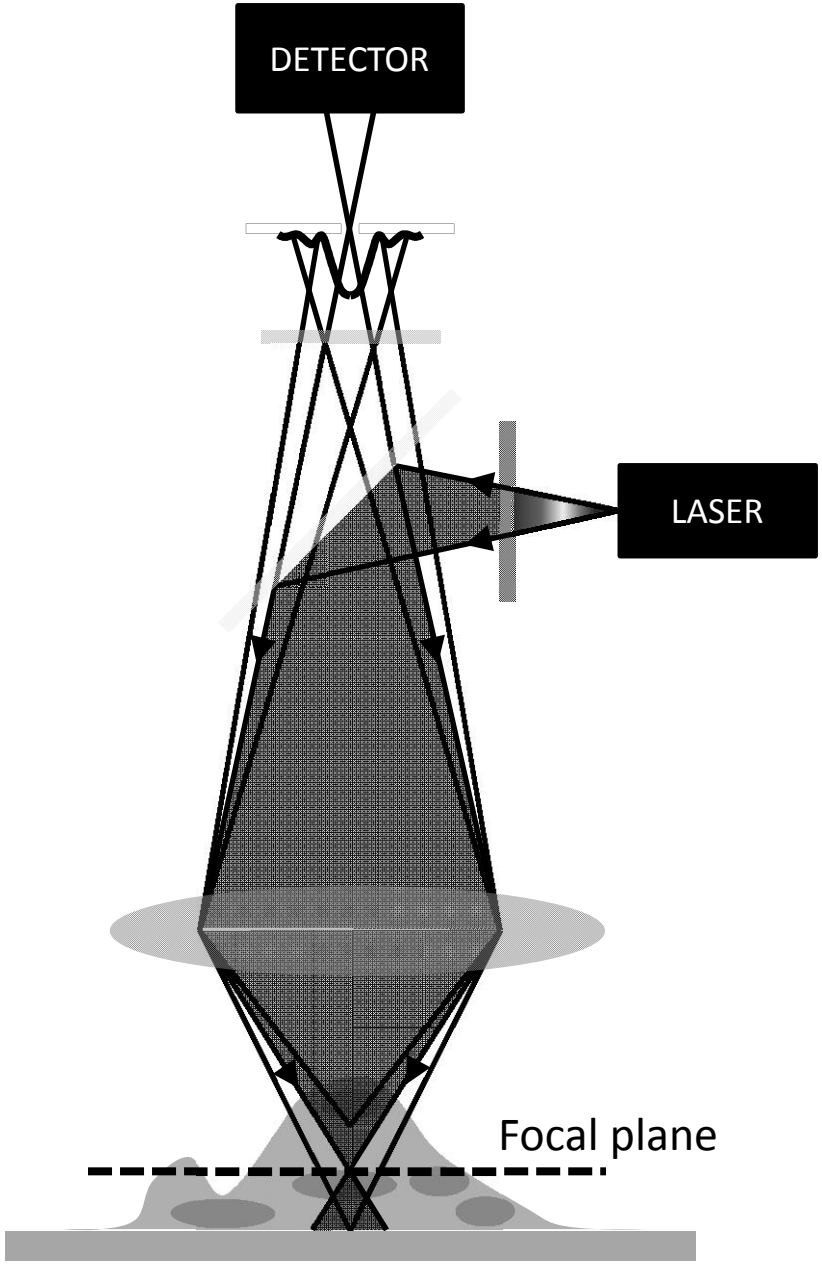
Confocal Microscopy

Problems

- Using the pin-hole considerably reduces the brightness of the images. It is therefore necessary to increase the sensitivity of the detectors (photomultiplier tubes), the intensity of the excitation laser or the detector's integration time.

- In all cases we increase the dose of the illumination radiation and the ***photo-bleaching*** of the chromo-phores is accelerated.

- Confocal microscopy cannot therefore be used with chromophores that show low thresholds for the photobleaching process, such as ***DAPI*** for example.



Confocal Microscopy

LAB WORK

- Study of yeast cells labelled with Green Fluorescent Protein (GFP). Comparison between images obtained by white-light wide-field microscopy and confocal microscopy.