

# Basics in optical microscopy

Antoine Le Gall  
Centre de Biochimie Structurale

[legall@cbs.cnrs.fr](mailto:legall@cbs.cnrs.fr)

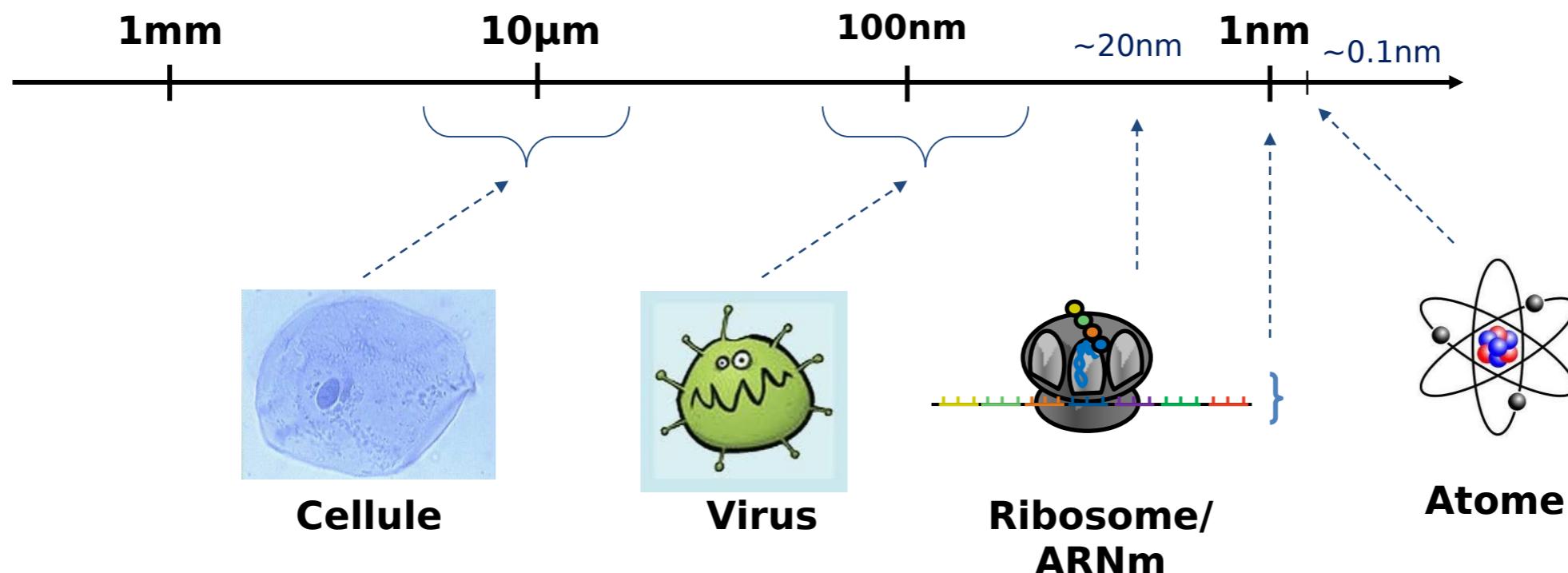
<http://marcnol.weebly.com/>

<http://www.cbs.cnrs.fr/index.php/fr/>

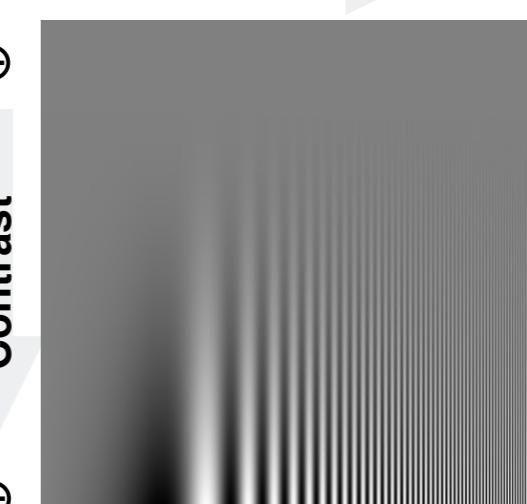
# Introduction

⇒ *Why do we need microscopes?*

## Relevant length scales



⊖ **Details** ⊕



## What can human eyes see?

Spatial resolution  $\approx 100 \mu\text{m}$

Can resolve contrast about 5%

# What does a microscope do?

⇒ ***Why do we need microscopes?***

To observe “small objects”, microscopes main contributions:

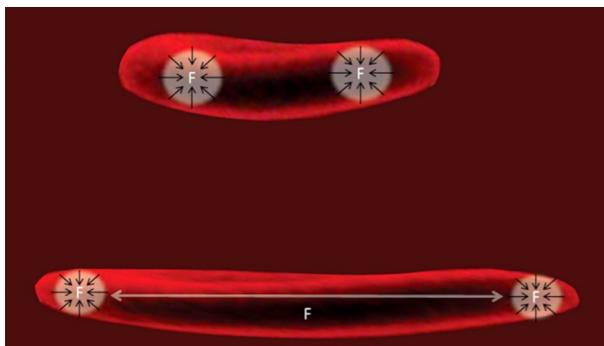
- ⇒ Magnification
- ⇒ Resolution
- ⇒ Contrast

But nowadays, microscopes can do a lot more than that!! see next courses

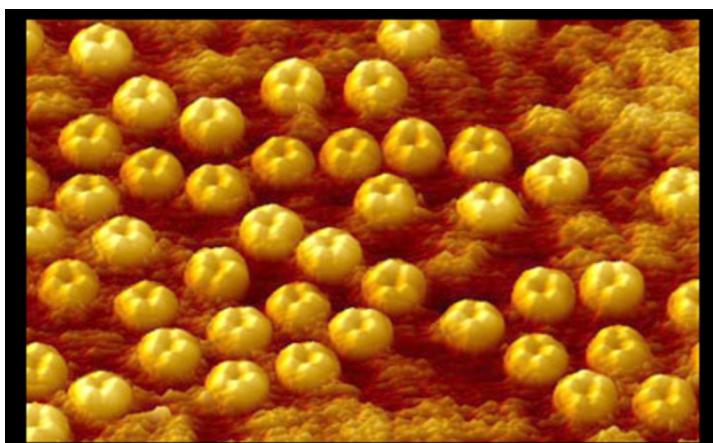
# What else can we do with a microscope?

→ **Association of lasers and microscope led to the implementation of a broad range of new techniques**

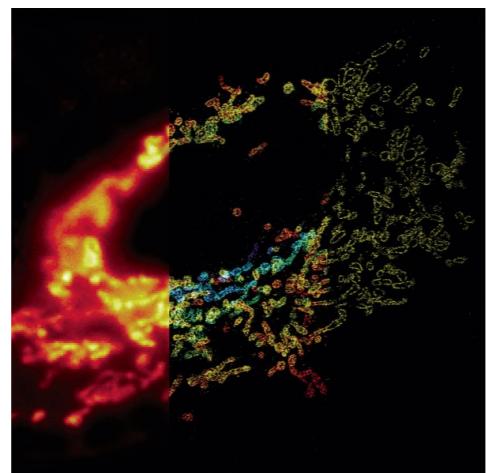
Optical tweezers



Atomic Force Microscopy

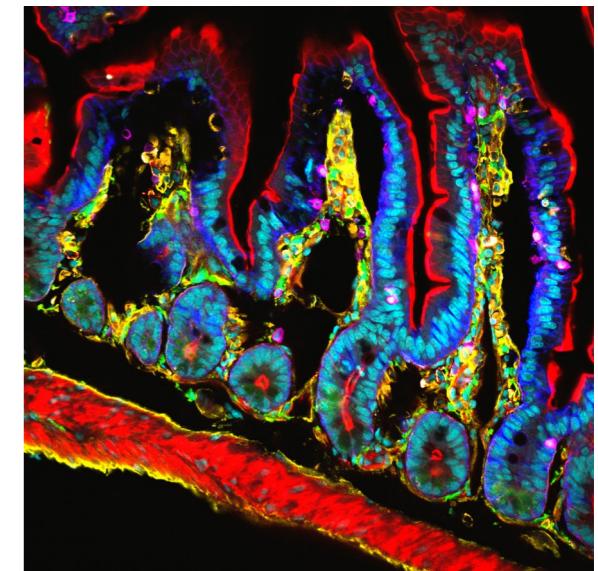


Super resolution microscopy

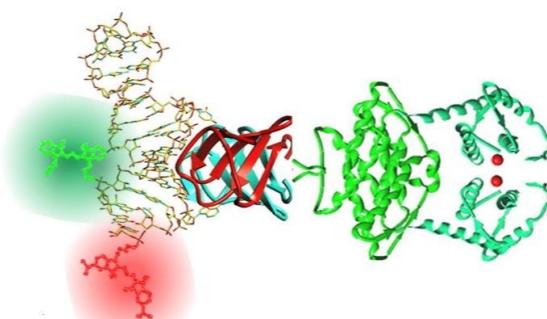
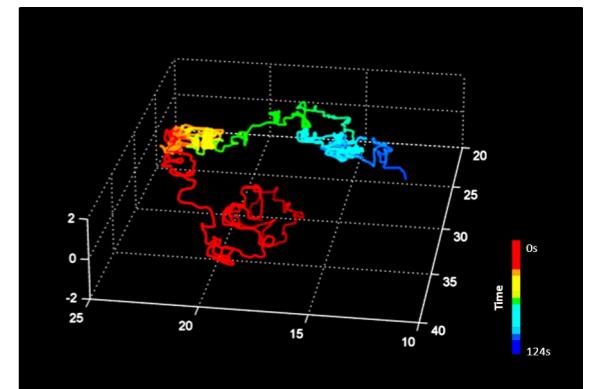


Fluorescence imaging  
Spectroscopy  
Trapping - manipulating  
Measuring forces  
Surface topography  
Particle tracking  
Laser microdissection  
Optogenetics  
...

Fluorescence microscopy



Particle tracking



Forster Resonance Energy Transfert

# Outline

1. Image formation through a lens
2. Diffraction
3. Microscope components
4. Contrasting methods
5. Optical aberrations

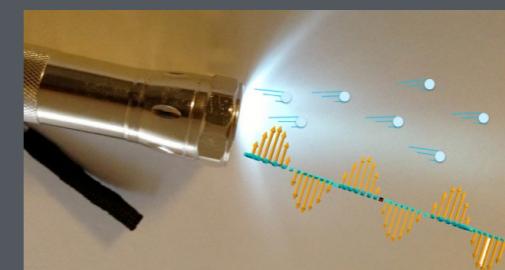
# Outline - Part 1

What is light?

Geometrical optics

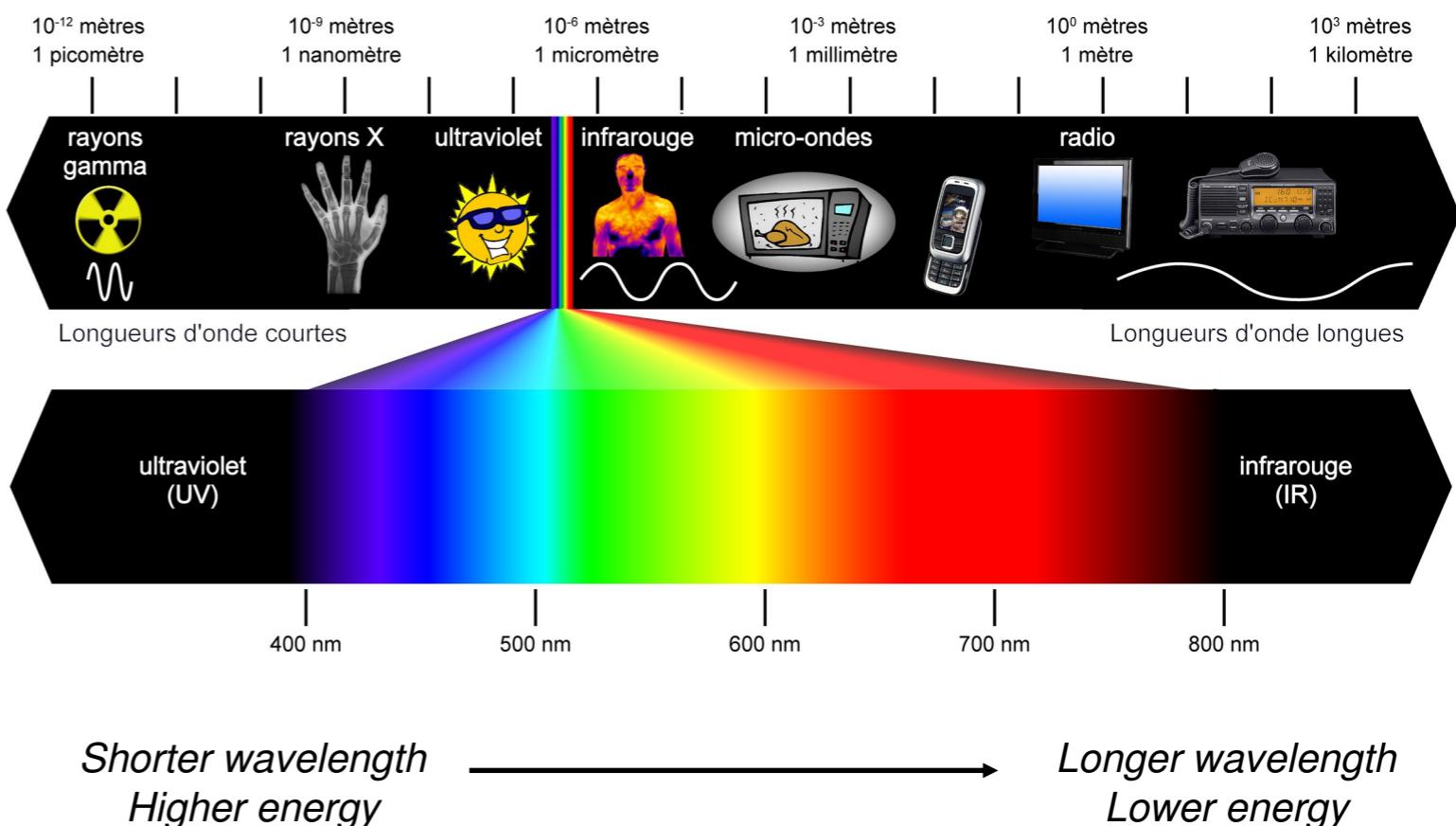
Image formation through a lens

# What is light?



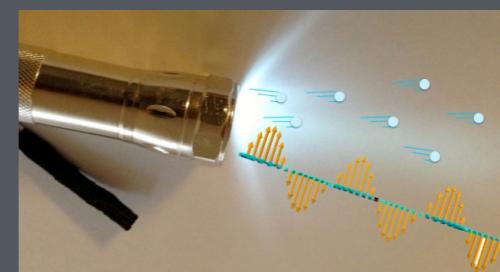
## Light as a wave

- Light is part of the electromagnetic spectrum



## Light as particle

# What is light?

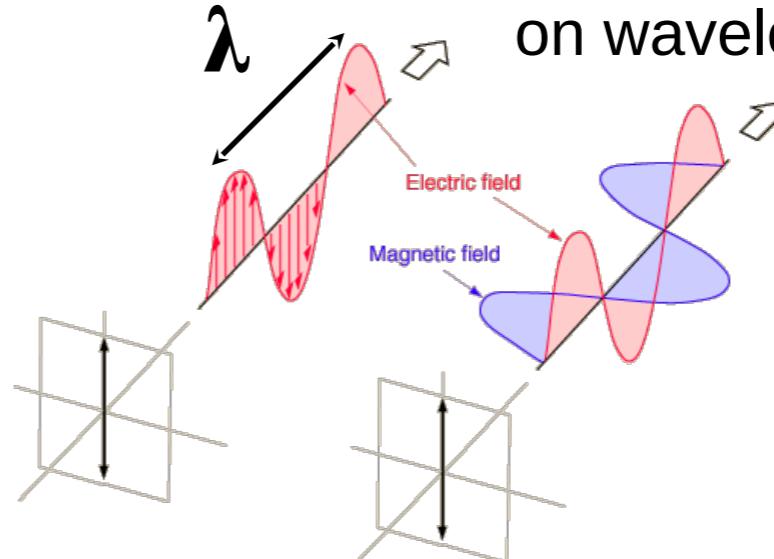


## Light as a wave

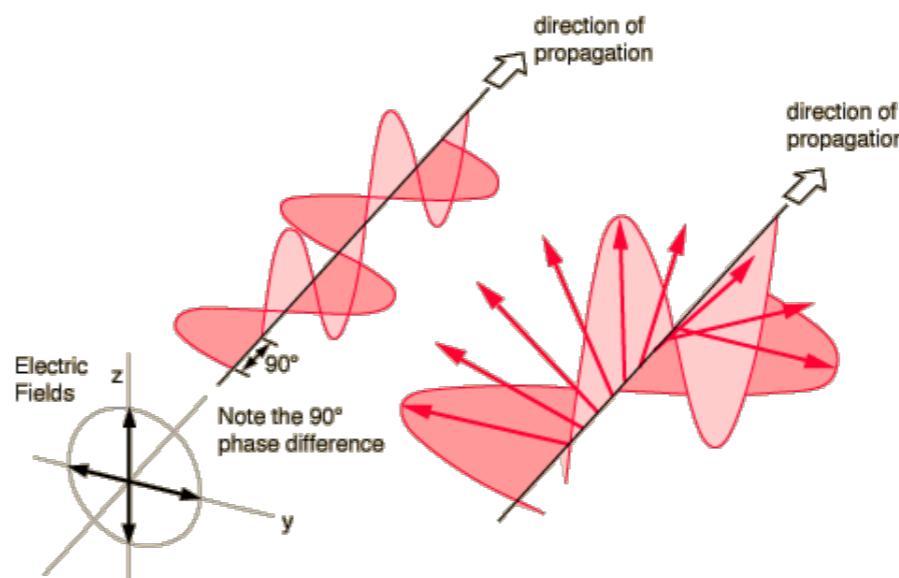
- Light is part of the electromagnetic spectrum
  - ⇒ Intensity
  - ⇒ Frequency or wavelength
  - ⇒ Polarization (*linear, circular, ...*)
  - ⇒ Phase
  - ⇒ Speed  $v=c/n$

Where  $c \approx 3.10^8$  m/s  
and "n" is the index media

⇒ Example : color depends on wavelength ( $\lambda$ )



Linear polarization



Circular polarization

## Light as particle

- A photon is like a particle, but it has no mass

⇒ Example : when light hits a camera sensor, it acts like photons/particles

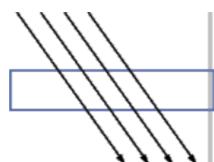
# Geometrical optics

Complete electromagnetic description of light is difficult to apply in practice.

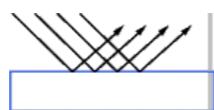
→ Geometrical optics treats light as a collection of rays that travel in straight lines.

**!!! It does not account for certain optical effects such as diffraction and interference !!!**

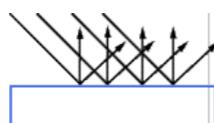
## Light and matter



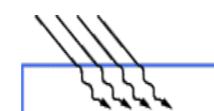
Light can **pass through** the object



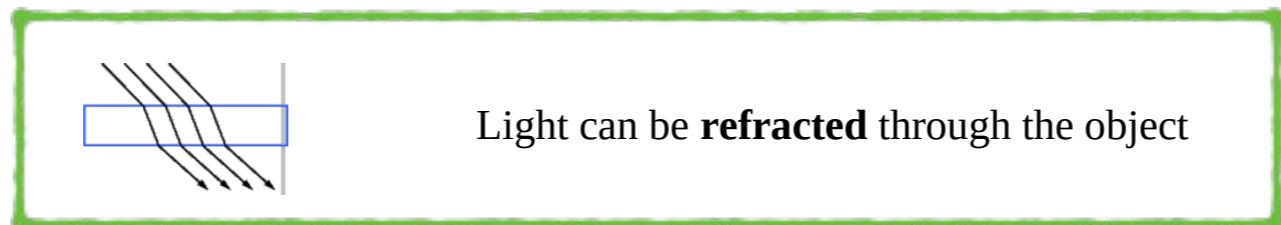
Light can be **reflected** off the object.



Light can be **scattered** off the object.



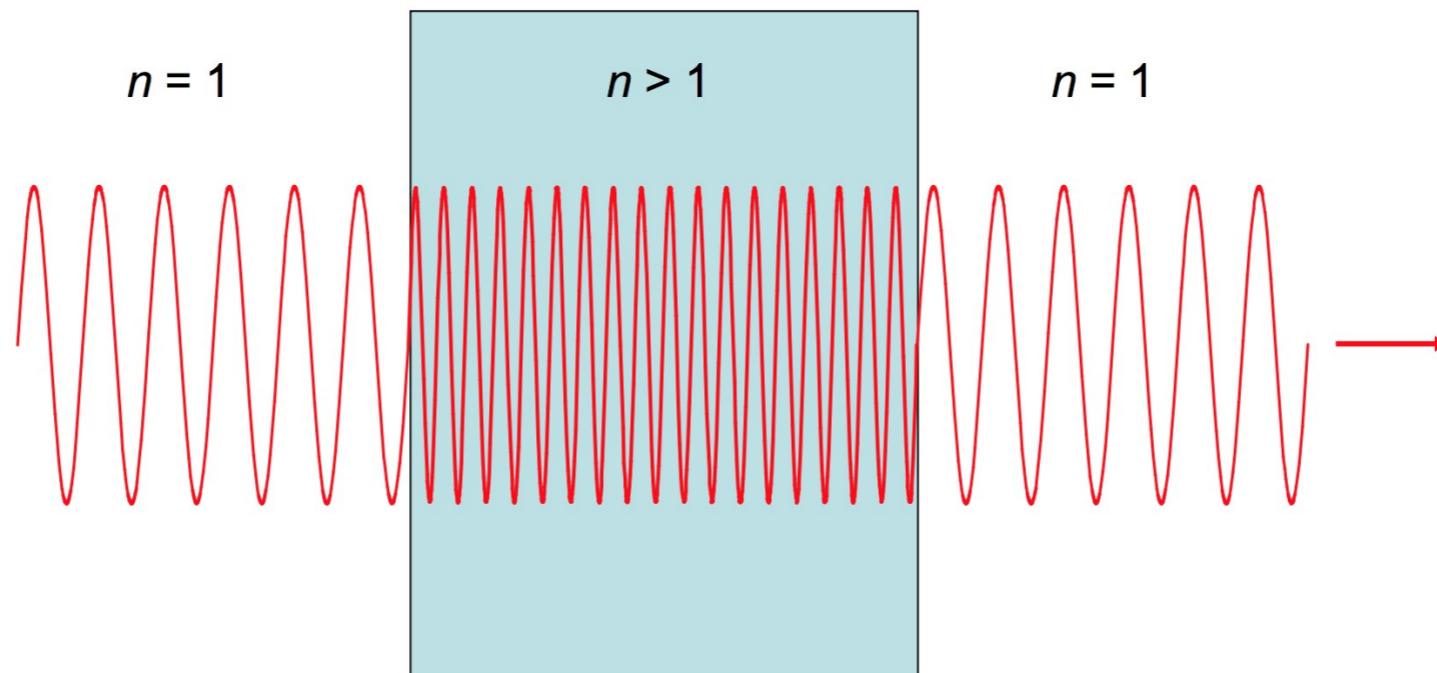
Light can be **absorbed** by the object.



Light can be **refracted** through the object

# Index of refraction

⇒ Light travels more slowly in matter



## Index of refraction :

$$n = c / v$$

$$n_{air} = 1.000293$$

$$n_{water} = 1.333$$

$$n_{glass} \sim 1.5$$

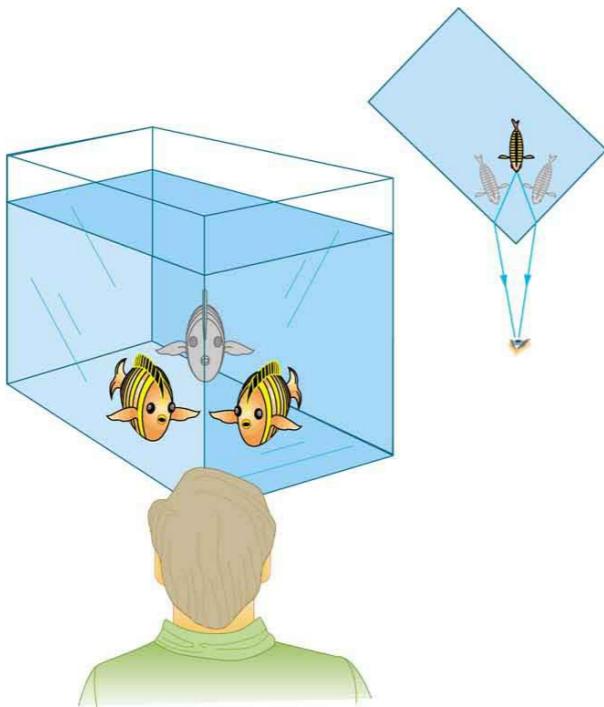
$$n_{cytoplasm} \sim 1.35-1.38$$

v : speed of light in medium  $n$

c : speed of light in vacuum

!!!  $n$  depends on wavelength and temperature !!!

# Refraction of light - Snell's law

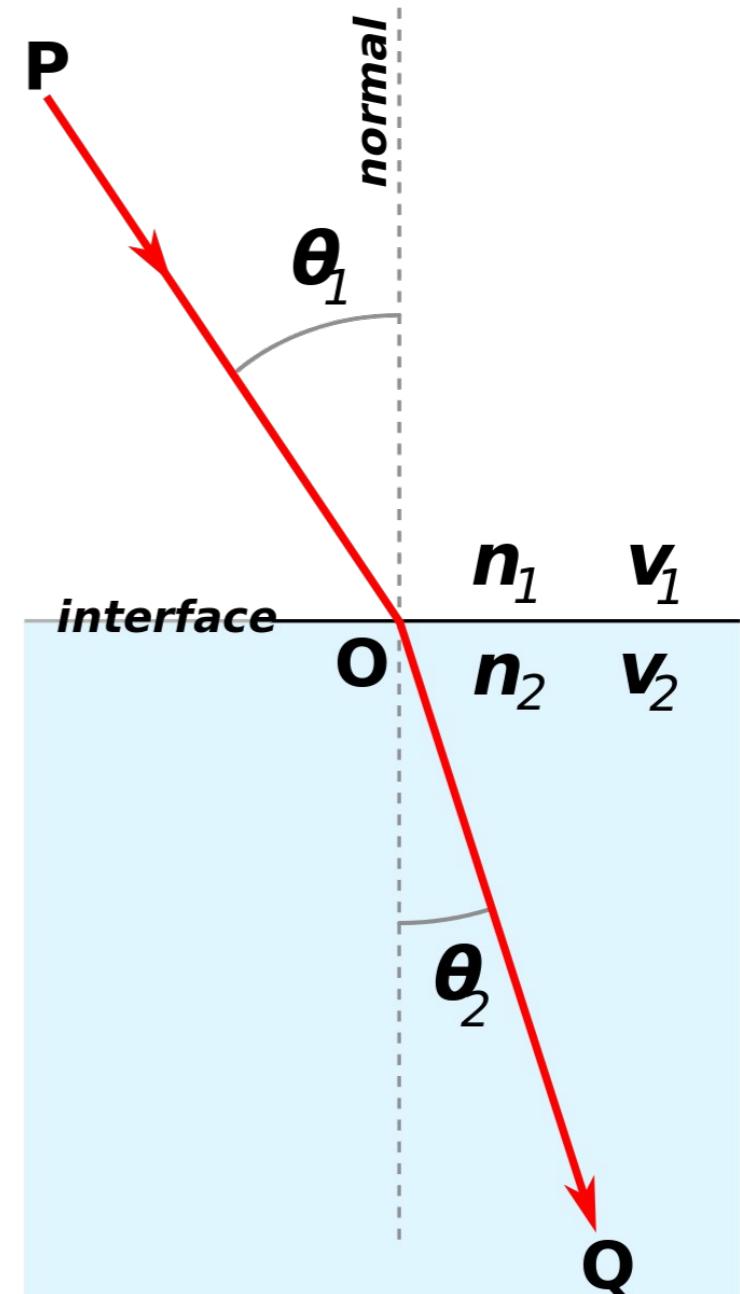


$$\frac{\sin \theta_1}{\sin \theta_2} = \frac{v_1}{v_2} = \frac{\lambda_1}{\lambda_2} = \frac{n_2}{n_1}$$

$$v = c / n$$

v : speed of light in medium  $n$

c : speed of light in vacuum

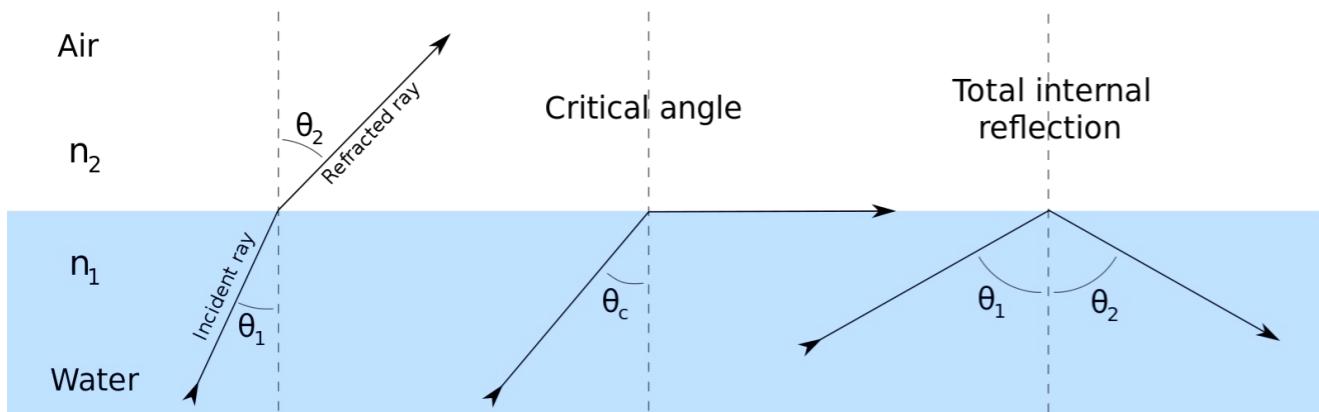


→ Light refraction depends on incident angle and the media indices

# Refraction of light - two important things to keep in mind

## Total internal reflection

Only when going from higher to lower index



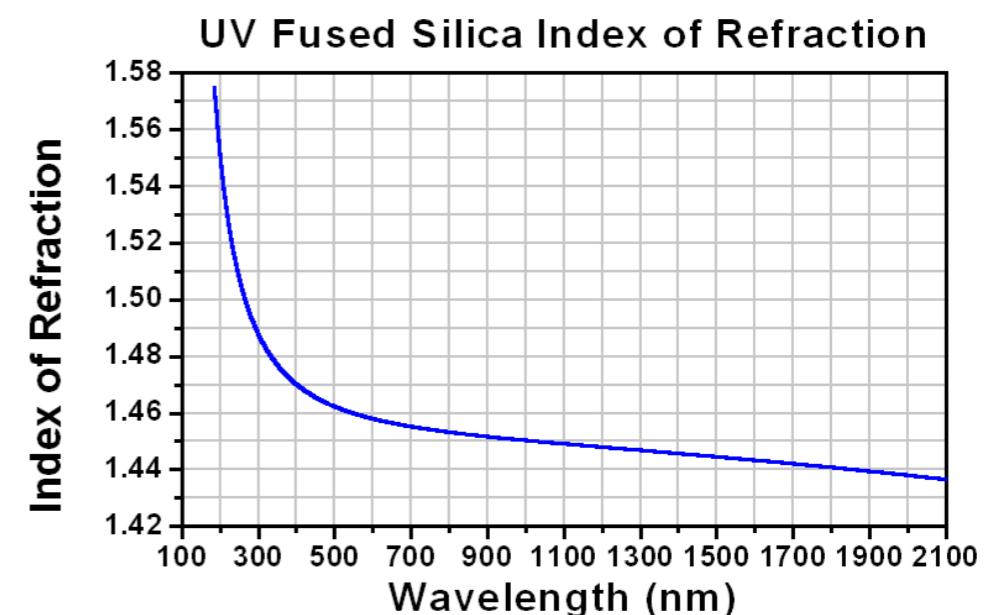
$$n_{air} = 1.000293$$

$$n_{water} = 1.333$$

$$n_{glass} \sim 1.5$$

$$n_{cytoplasm} \sim 1.35-1.38$$

## Chromatism



**APPLICATIONS OF TOTAL INTERNAL REFLECTION**

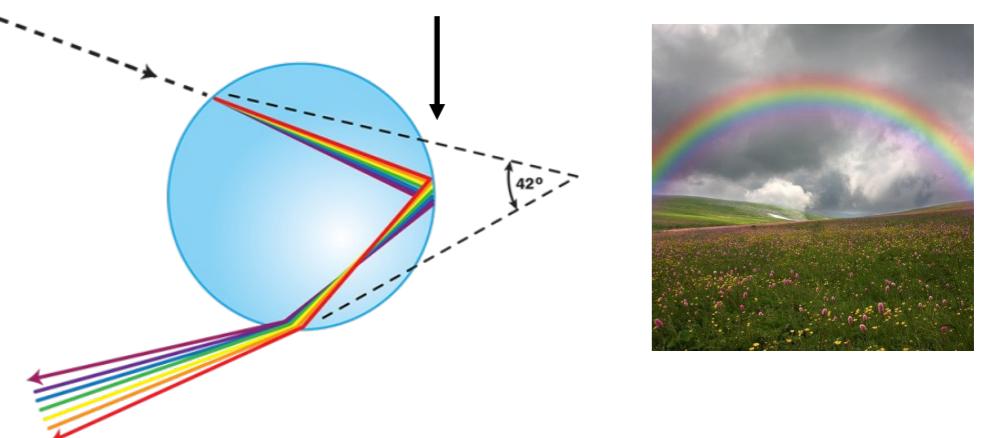
- Binoculars
- Retroreflectors

The diagram shows two applications of TIR. On the left, a binocular prism is shown with light rays entering from the left and reflecting off internal prisms to emerge as parallel rays. On the right, a retroreflector is shown as a triangular prism with an incoming ray from the left reflecting directly back along its path. Labels include "eyepiece", "objective", and "prism".

Sun

Observer

Total internal reflection



Example : rainbows!

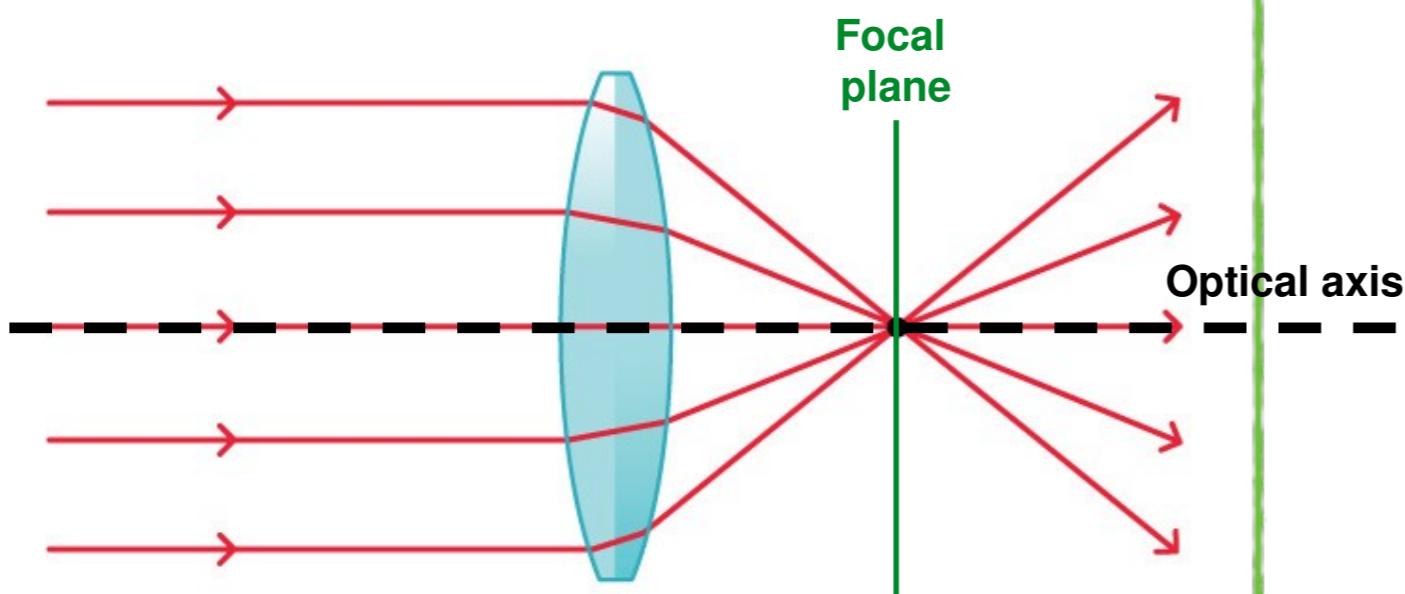
# Parallel rays of light through a lens

**Think of what we can do with a bent interface ?**

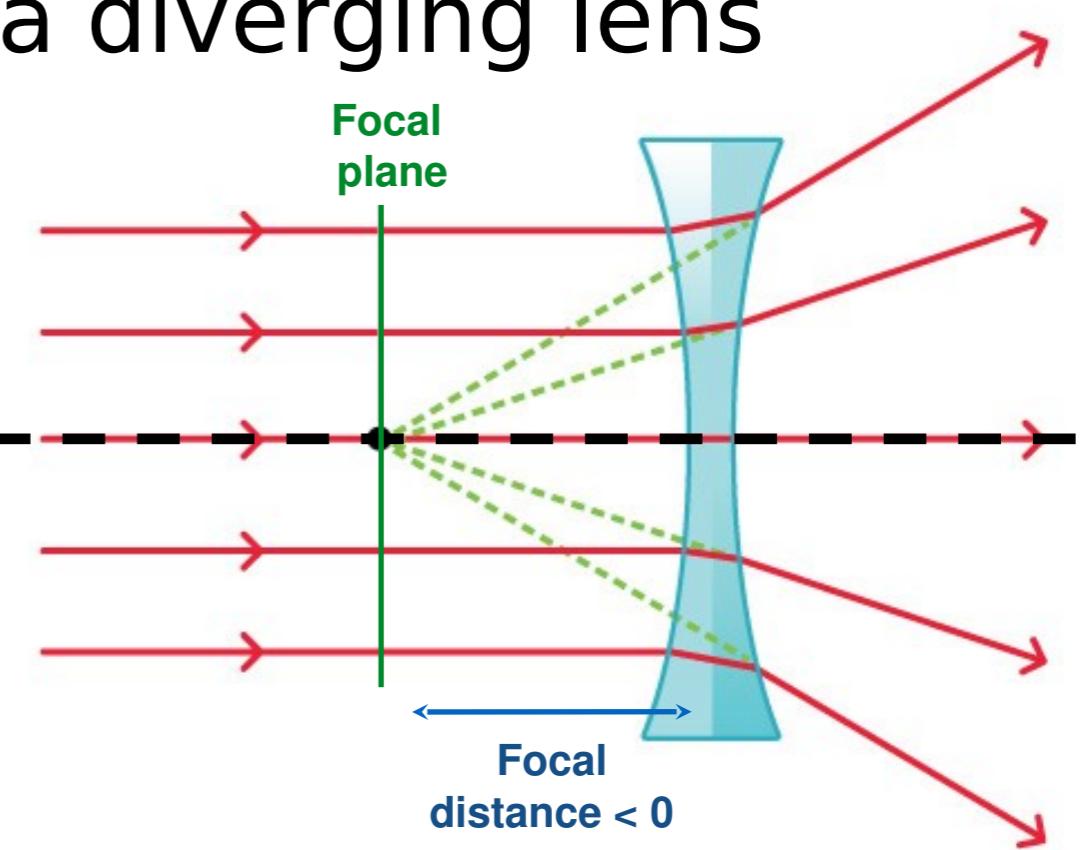
# Parallel rays of light through a lens

Refraction of light through :

a converging lens



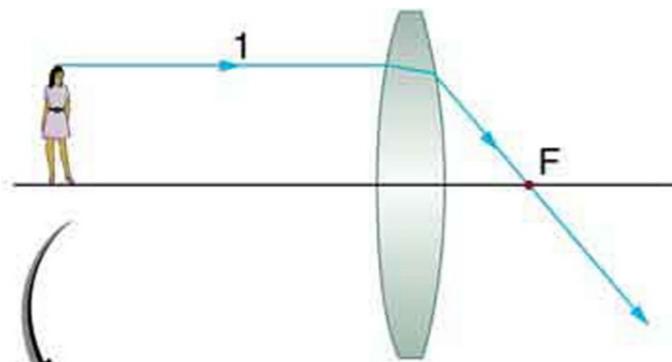
a diverging lens



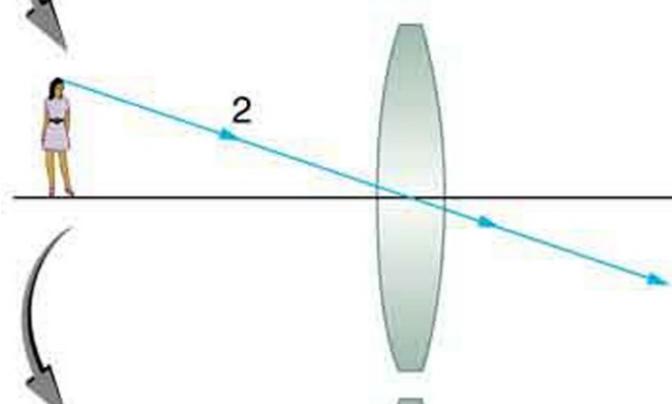
Focal plane  
Focal distance  
Optical axis

# Properties of rays of light through a lens

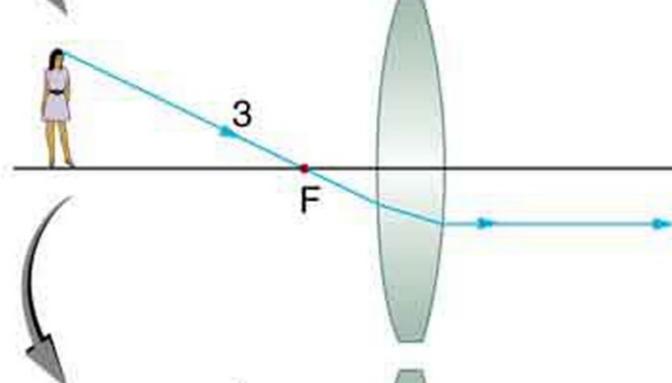
## Three rules:



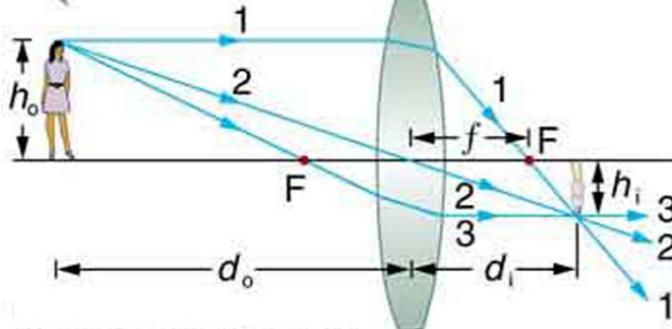
Ray // to optical axis  $\Rightarrow$  ray through focal point



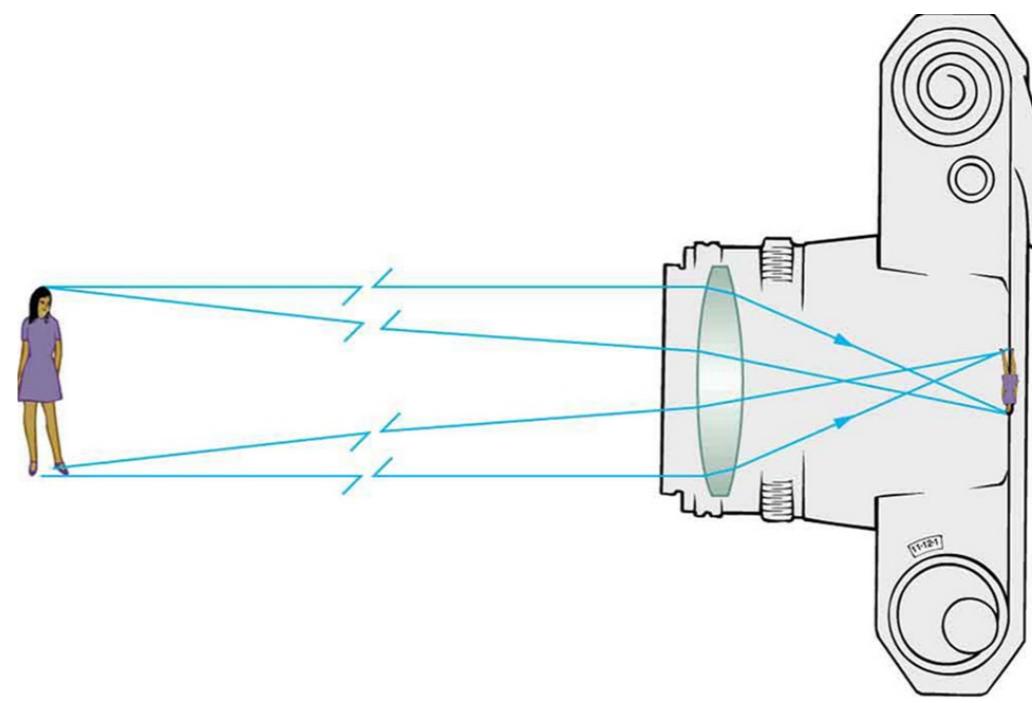
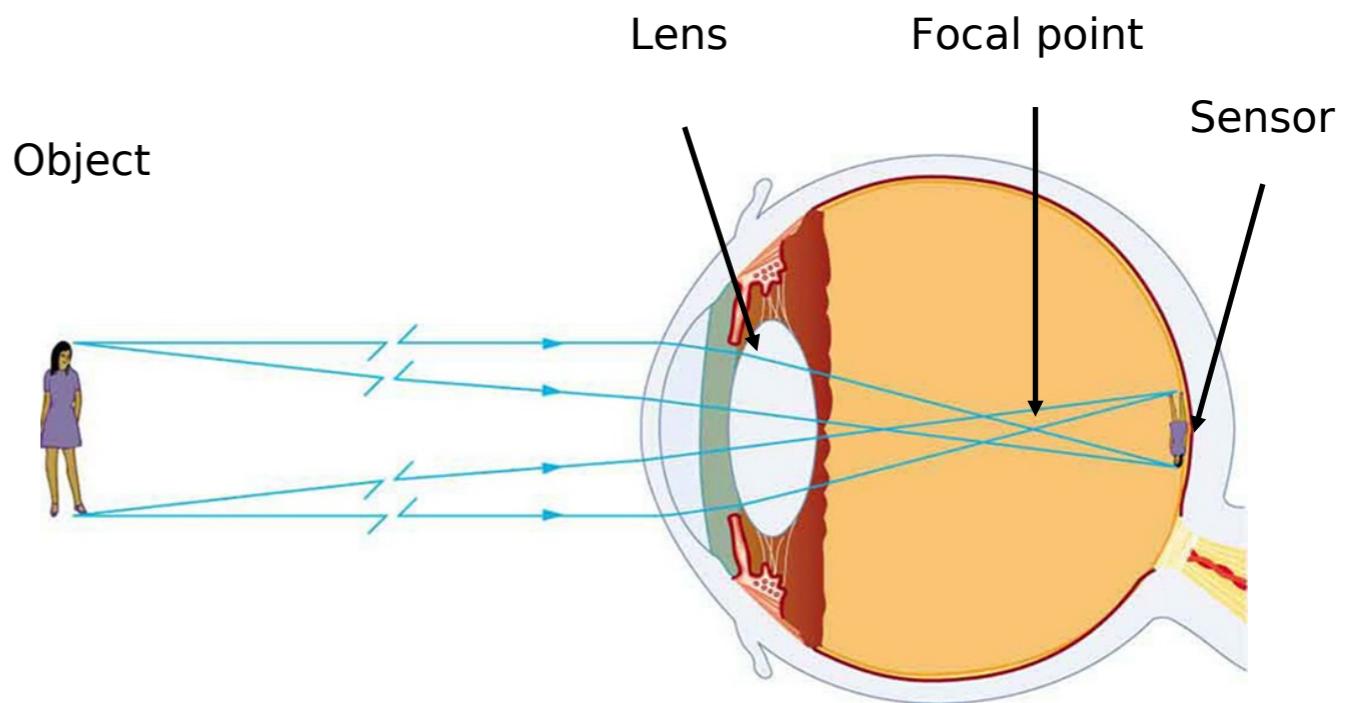
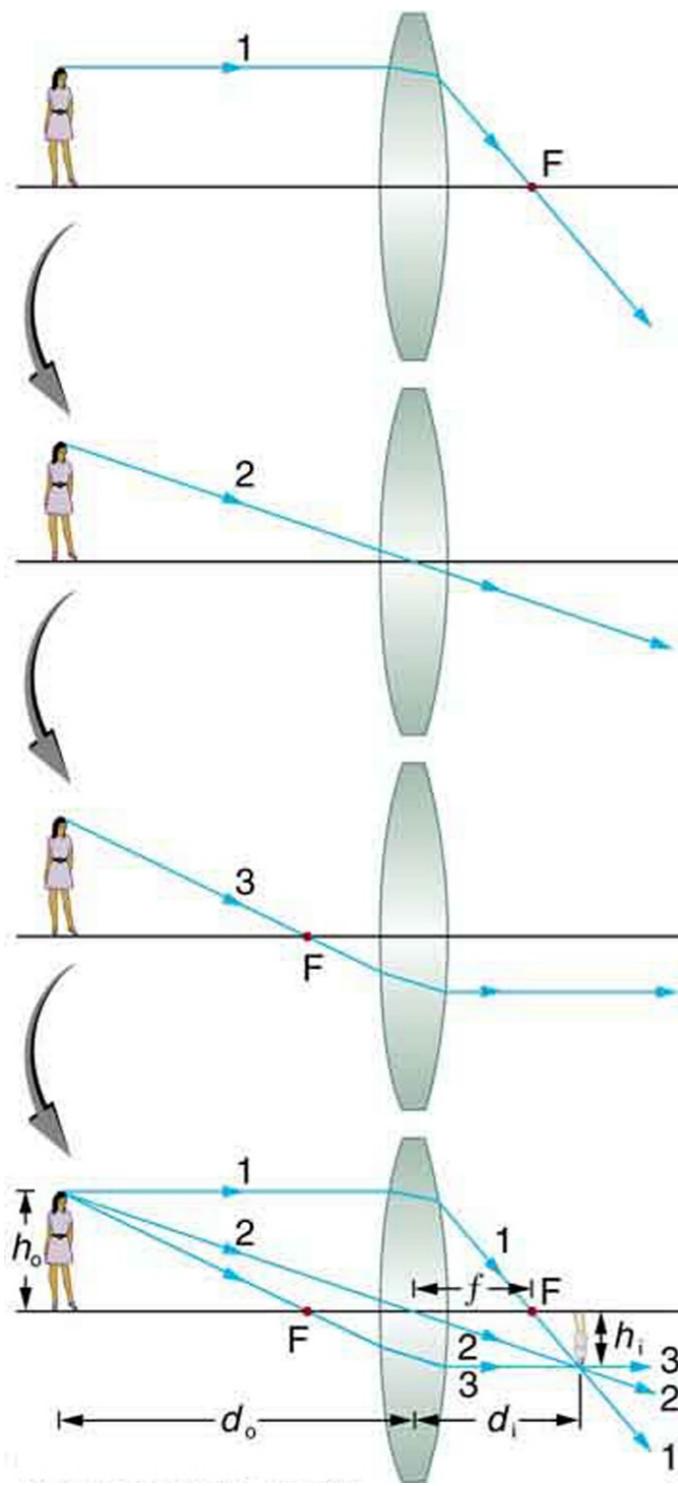
Ray through lens center  $\Rightarrow$  ray unaffected



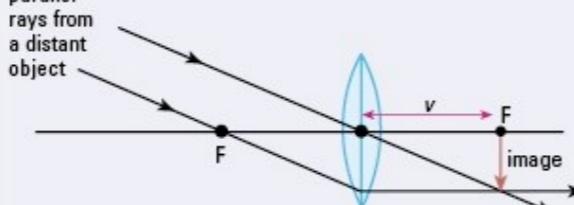
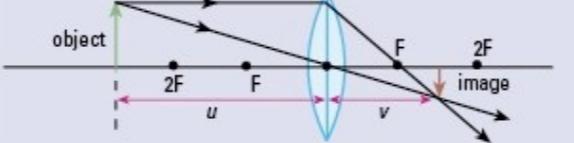
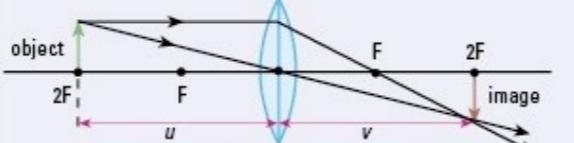
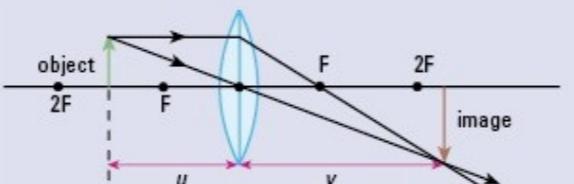
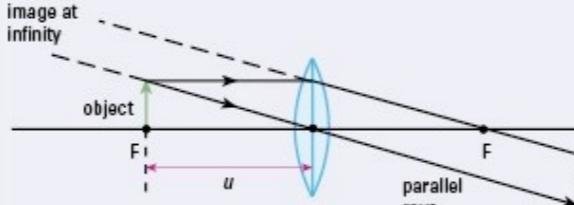
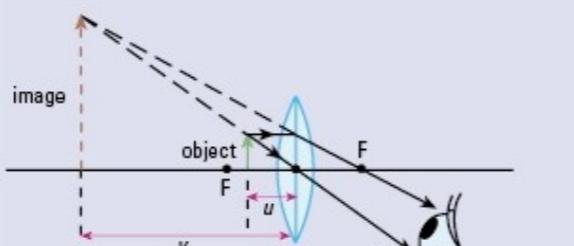
Ray through focal point  $\Rightarrow$  ray // to optical axis



# Image formation through a lens

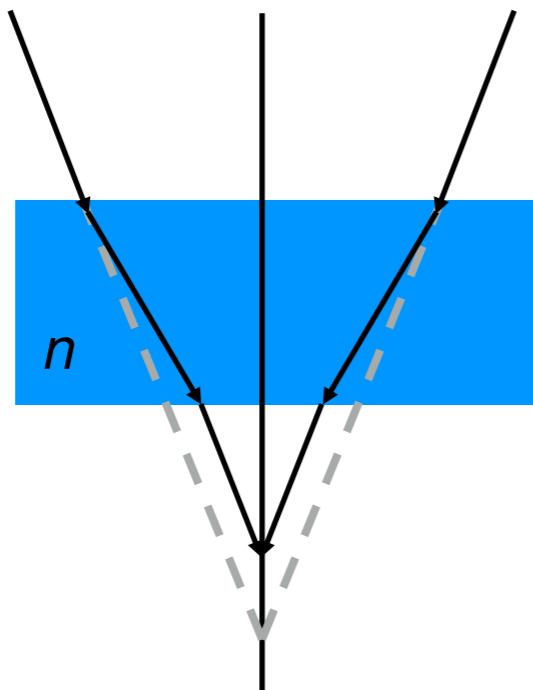


# Properties of rays of light through a lens

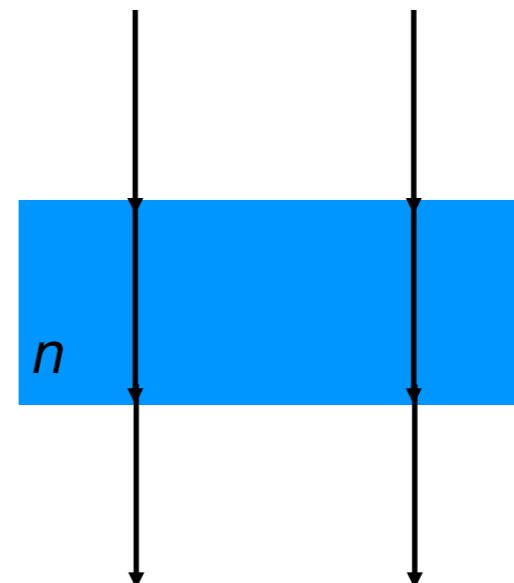
Object distance ( $u$ )	Ray diagram	Type of image	Image distance ( $v$ )	Uses
$U = \infty$		- inverted - real - diminished	$v = f$ - opposite side of the lens	- object lens of a telescope
$u > 2f$		- inverted - real - diminished	$f < v < 2f$ - opposite side of the lens	- camera - eye
$u = 2f$		- inverted - real - same size	$v = 2f$ - opposite side of the lens	- photocopier making same-sized copy
$f < u < 2f$		- inverted - real - magnified	$v > 2f$ - opposite side of the lens	- projector - photograph enlarger
$u = f$		- upright - virtual - magnified	- image at infinity - same side of the lens	- to produce a parallel beam of light, e.g. a spotlight
$u < f$		- upright - virtual - magnified	- image is behind the object - same side of the lens	- magnifying glass

# Disadvantage of working with convergent beams

Convergent beam



Parallel/collimated beam



Converging point (focusing) is shifted  
depending on incident angle

⇒ aberrations

Beam is unaffected

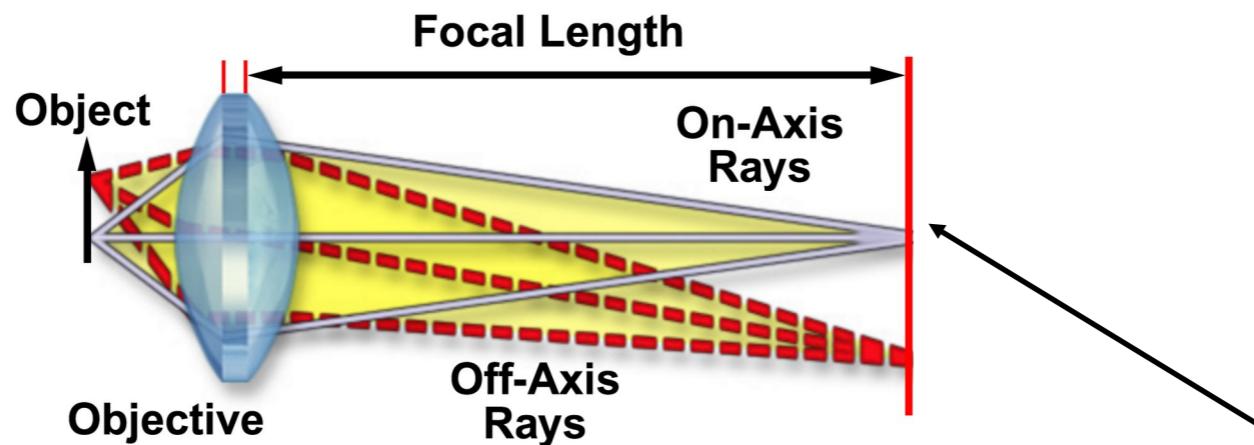
!!! Refraction also depends on  $\lambda$  ( $n$  depends on  $\lambda$ ) !!!

⇒ When possible, work in infinity-focal plane configuration

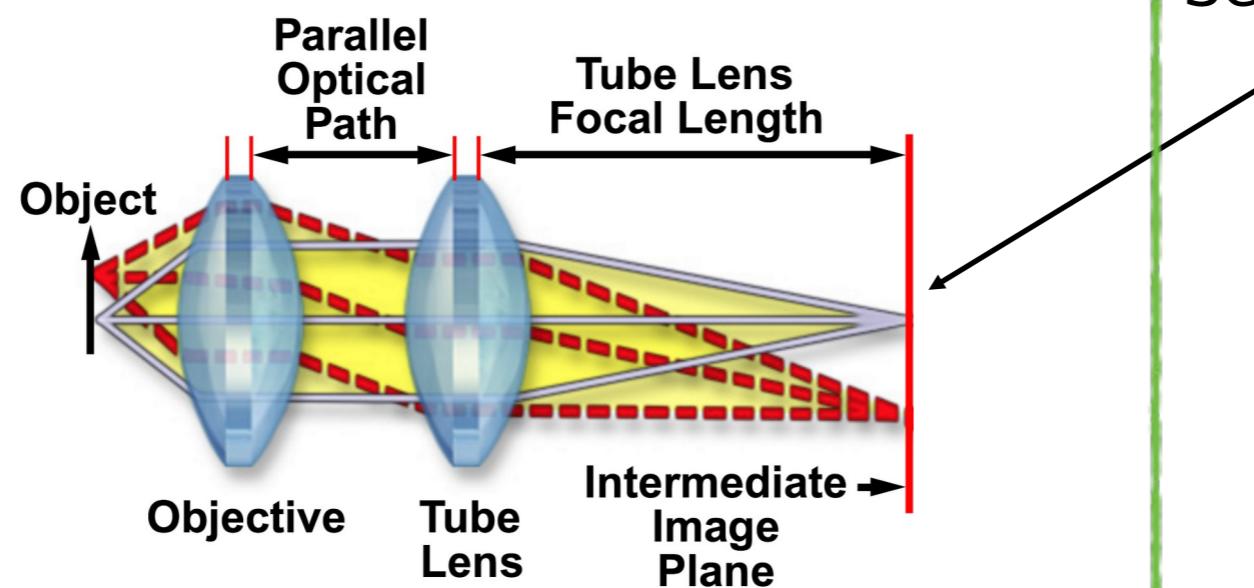
# How to image an object with parallel beams?

Modern microscopes are now infinity optical systems

Finite system  
(old)



Infinity  
system  
(modern)

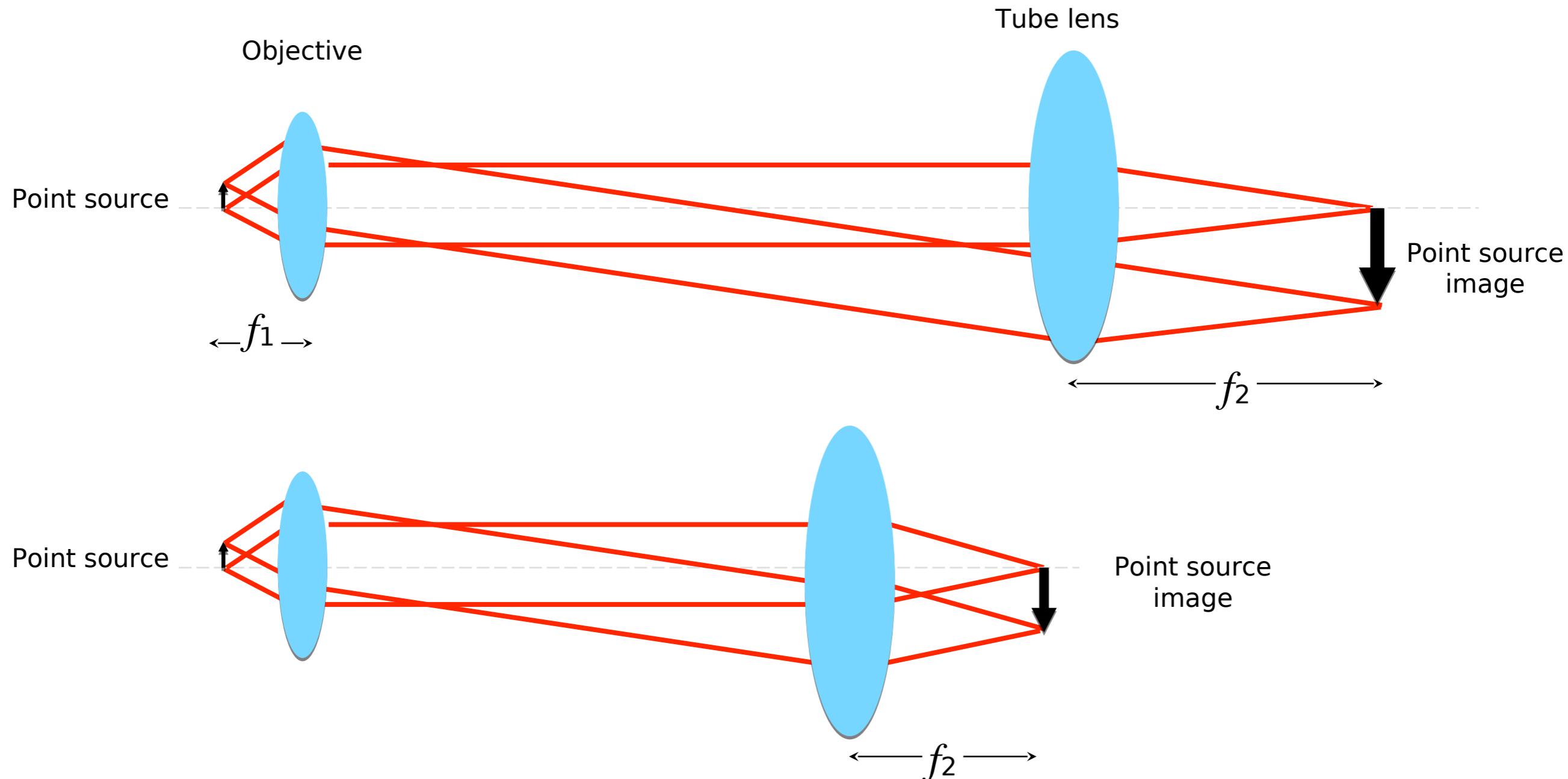


Sensor (camera for ex)

→ introduction of optical components such as filters, polarizers, and beamplitters, additional light sources into the optical path

→ Need at least two lenses.

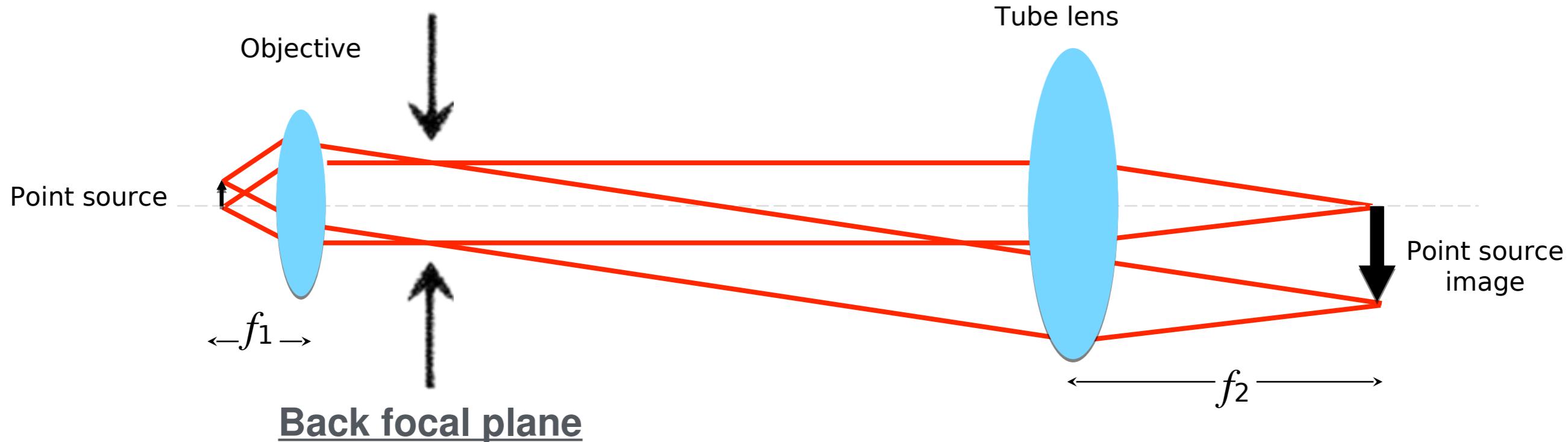
# Magnification



$$\text{Objective magnification : } M = \frac{f_2}{f_1}$$

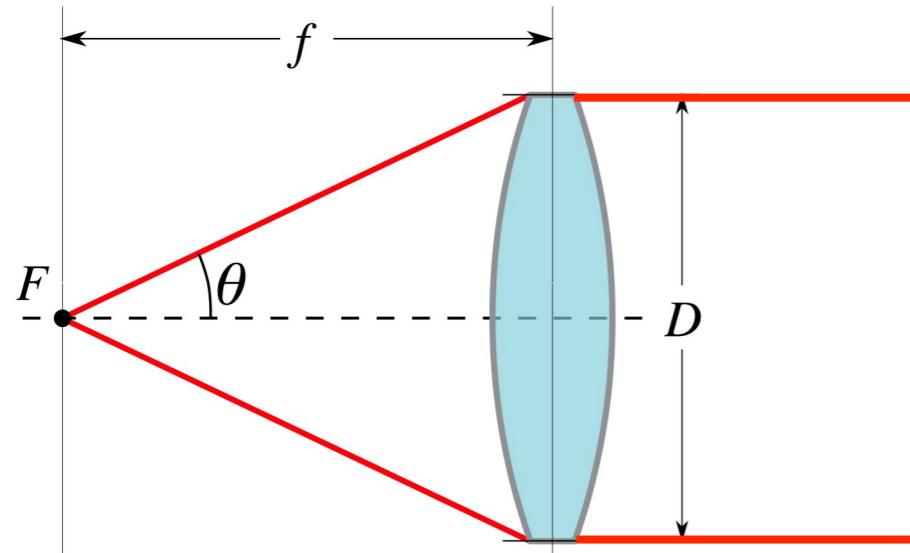
ex : for a 100X with  $f_2 = 165\text{mm}$ , the objective focal length must be  $1.65\text{mm}!$

# Back focal plane



Rays that leave the object with the same angle meet at the objective's back focal plane

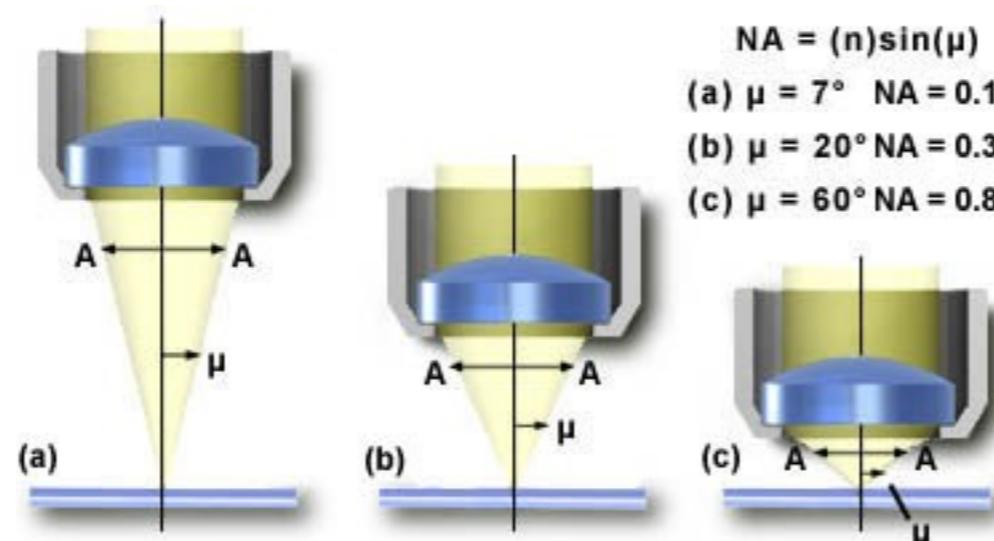
# Numerical aperture



$$\text{NA}_i = n \sin \theta = n \sin \left[ \arctan \left( \frac{D}{2f} \right) \right] \approx n \frac{D}{2f}$$

NB : D is the pupil of the optical system. It is not necessarily (usually it is not) the same as the diameter of the lens.

**Low NA : less light is collected**



**High NA : more light is collected**

The maximum NA is fixed by the index media (1 in air)  
➡ Use immersion media to increase NA

# Keep in mind

Index mismatch induces light refraction

Light refraction depends on incident angle and the media indices

Index of refraction depends on wavelength

Modern microscopes use infinity corrected objectives

Modern microscopes use an objective AND a tube lens

Numerical aperture relates to the collection angles of the microscope

The higher the NA, the more light you collect

The NA cannot be higher than the immersion media (TIR)

# What does a microscope do?

⇒ ***Why do we need microscopes?***

To observe “small objects”, microscopes main contributions:

- ⇒ Magnification ✓
- ⇒ Resolution
- ⇒ Contrast



# Outline - part2

Diffraction

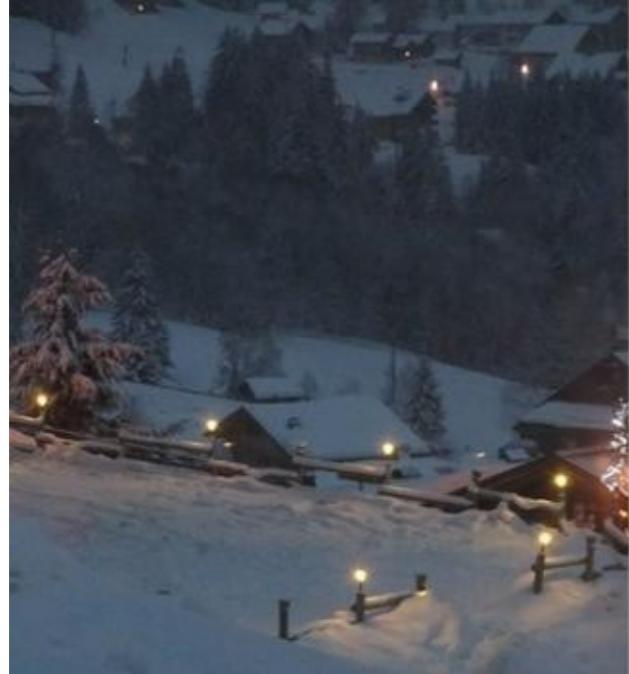
Point spread function

Resolution

Geometrical optics can't explain interferences/diffraction

➡ Let's go back to light as a wave!

# Diffraction



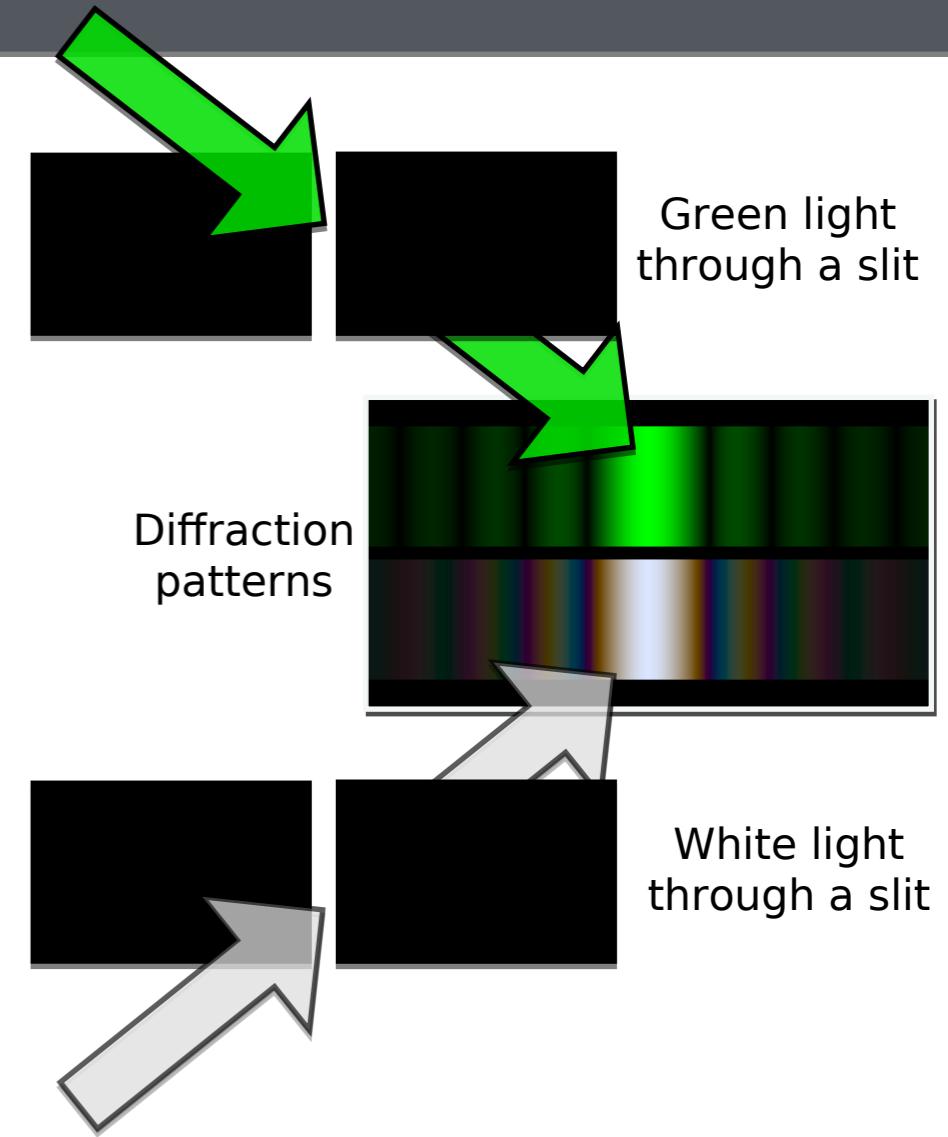
Without curtain



With curtain stitches (square holes)



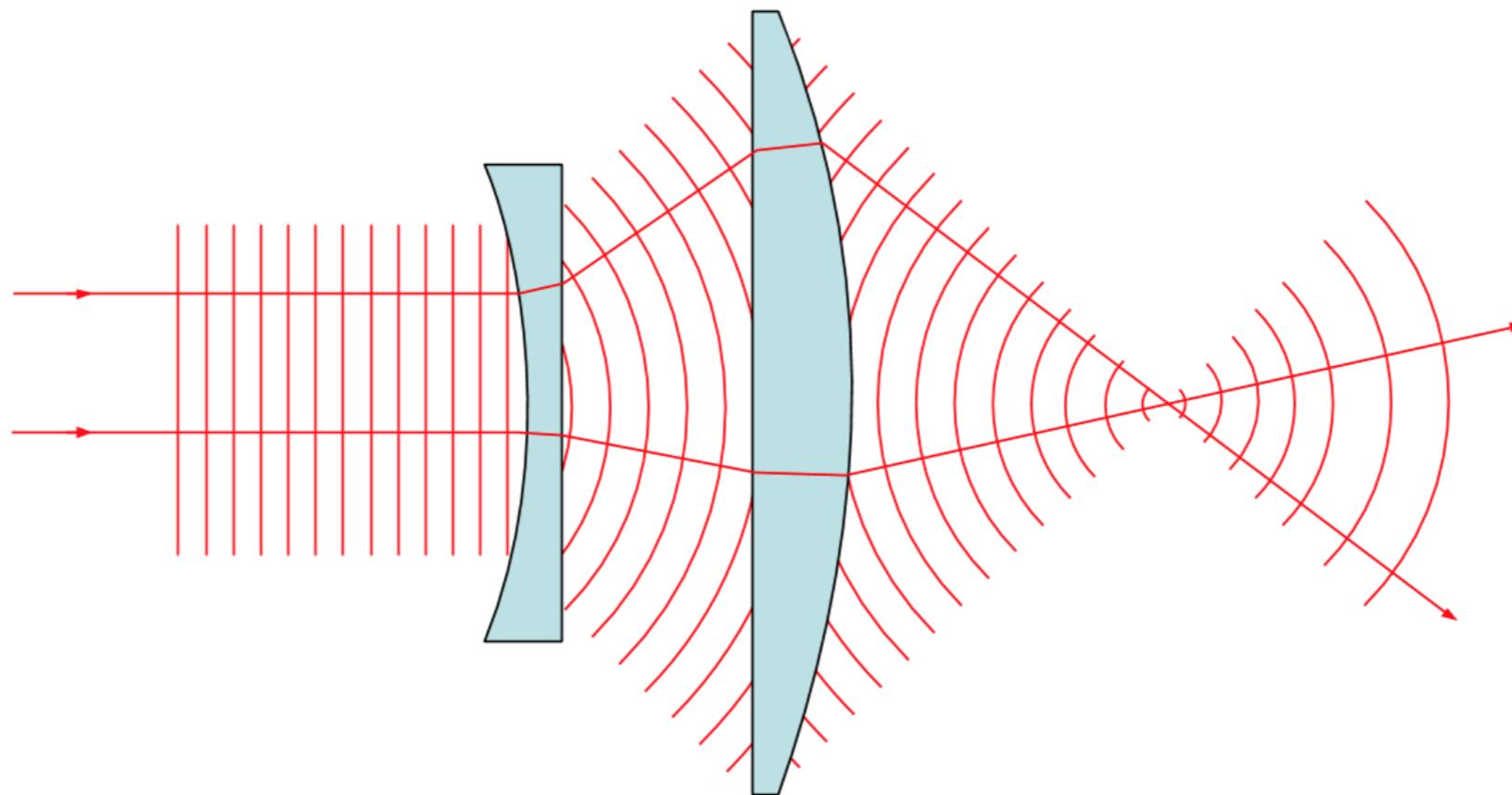
Any wave going through an aperture diffracts.



Diffraction pattern is wavelength and aperture dependant

# Wavefront

Rays are perpendicular to wavefronts

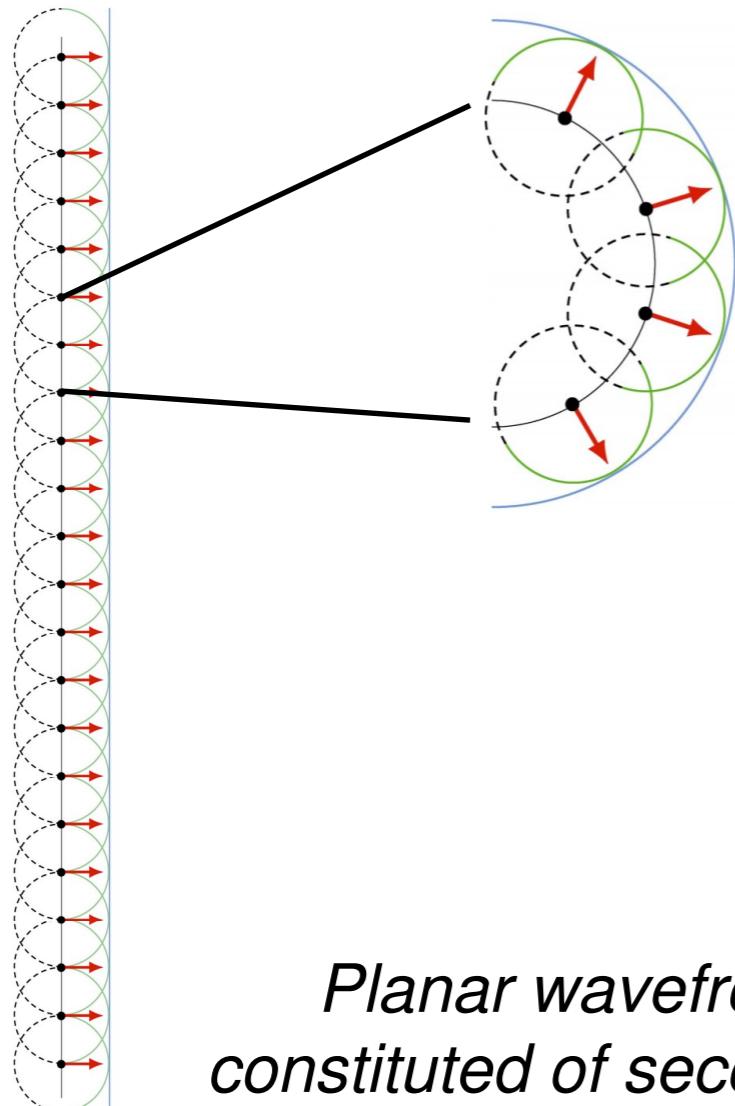


For an electromagnetic wave, a wavefront:

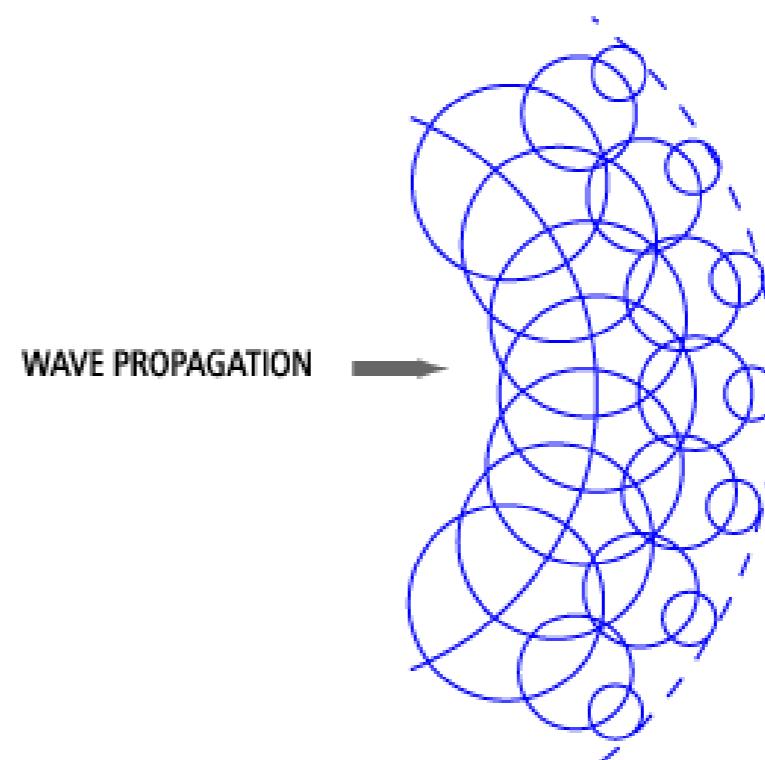
- is represented as a surface of identical phase,
- can be modified with conventional optics. A lens can change the shape of optical wavefronts from planar to spherical as the lens introduces a spatial phase variation across the beam shape.

# Diffraction - Huygens-Fresnel principle

*Every unobstructed point on a wavefront will act as a source of secondary spherical waves.  
The new wavefront is the surface tangent to all the secondary spherical waves.*

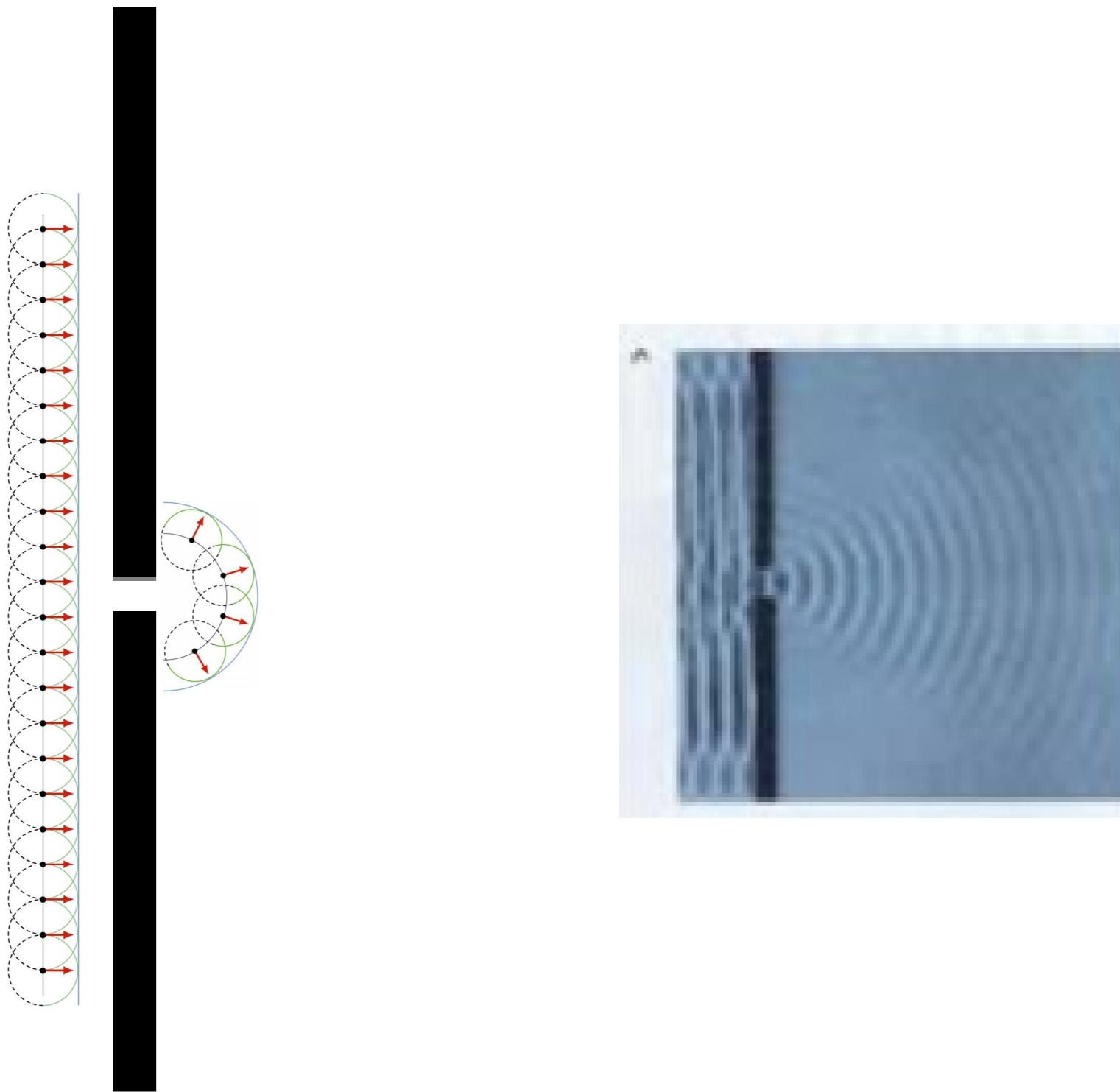


*Planar wavefront  
constituted of secondary  
spherical wavelets*

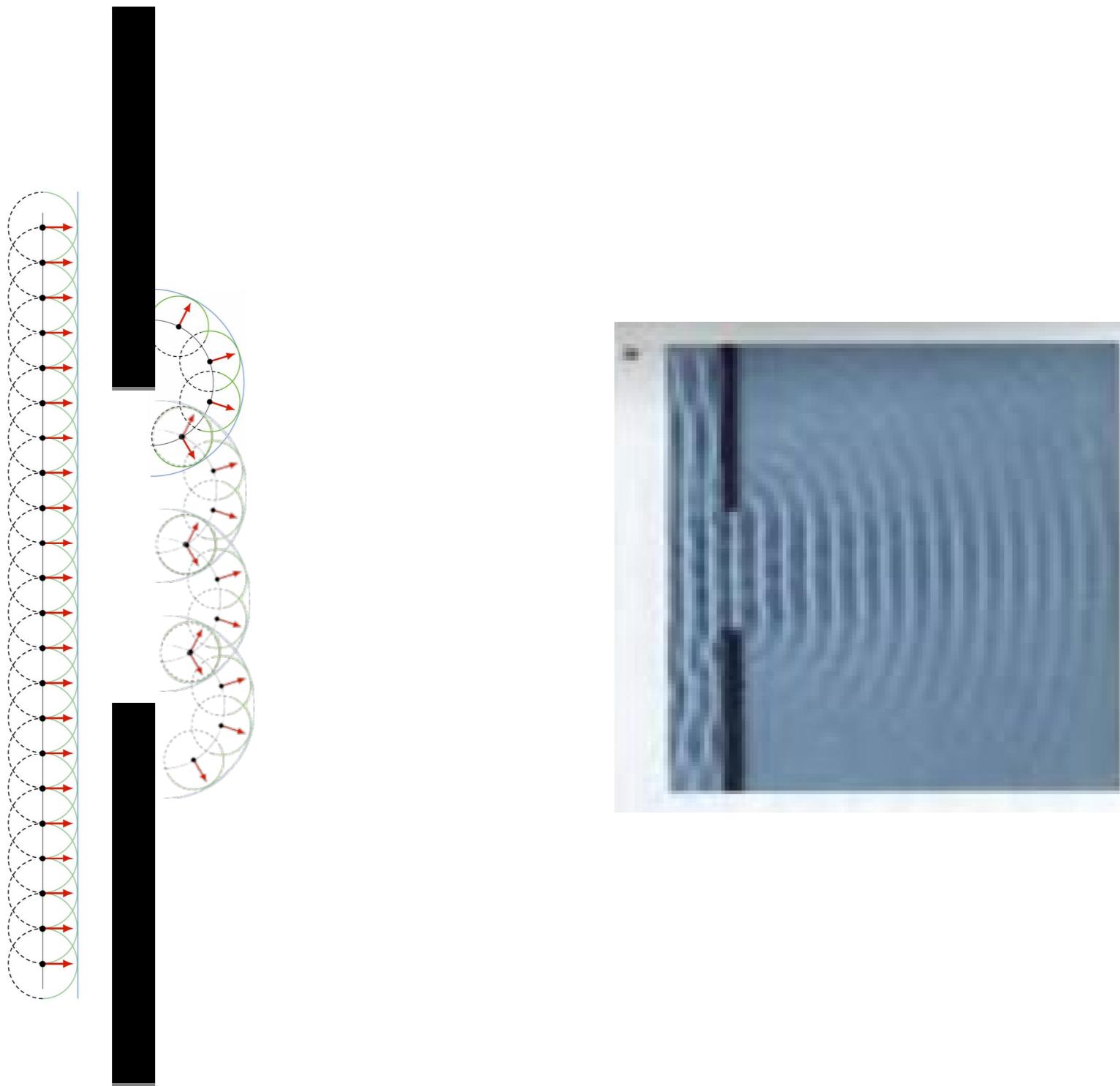


*Spherical wavefront  
constituted of secondary  
spherical wavelets*

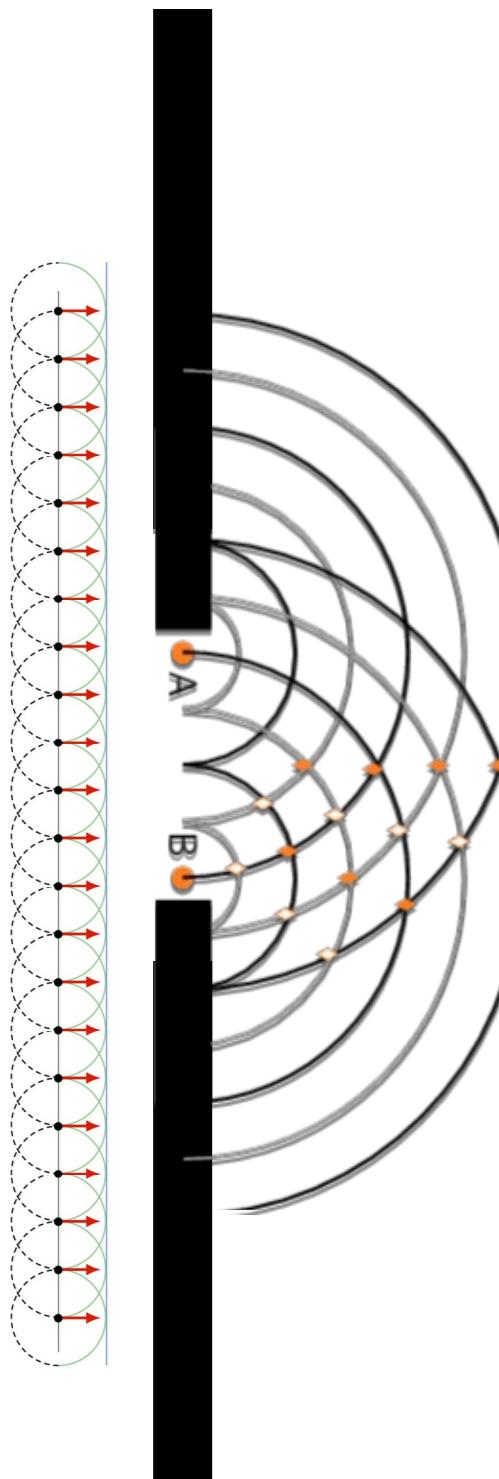
# Light passing through an aperture



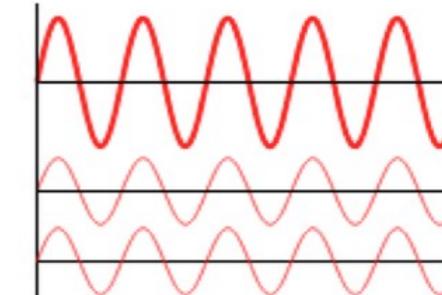
# Light passing through an aperture



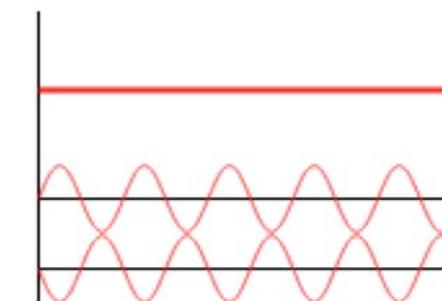
# Light passing through an aperture



The peaks correspond to places where the waves are adding constructively (solid orange diamond) The minima are places where destructive interference is taking place (empty orange diamond)



→ Lines of the same color (grey or black) crossing each other.



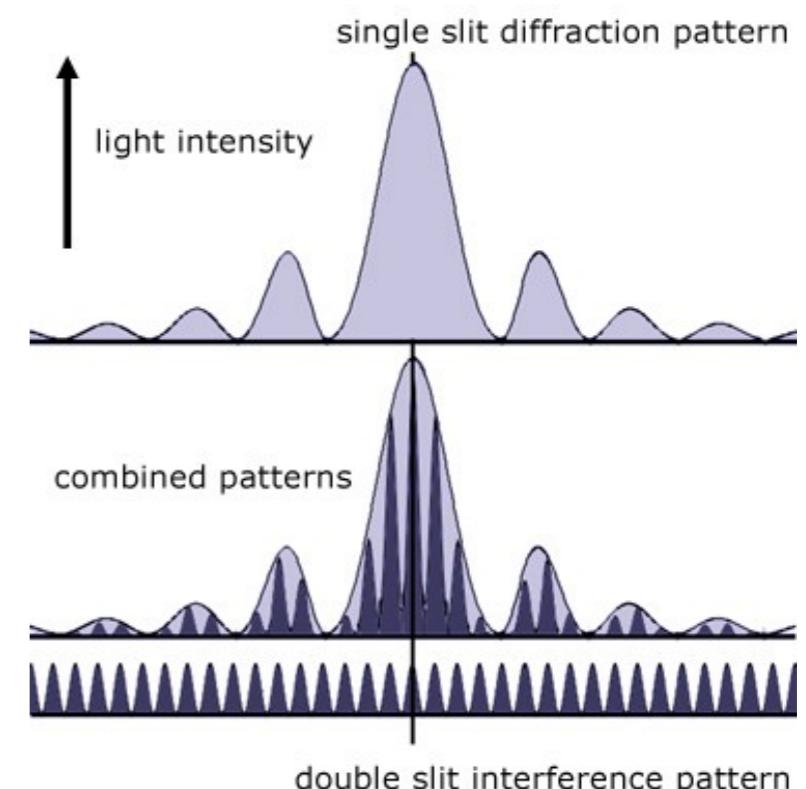
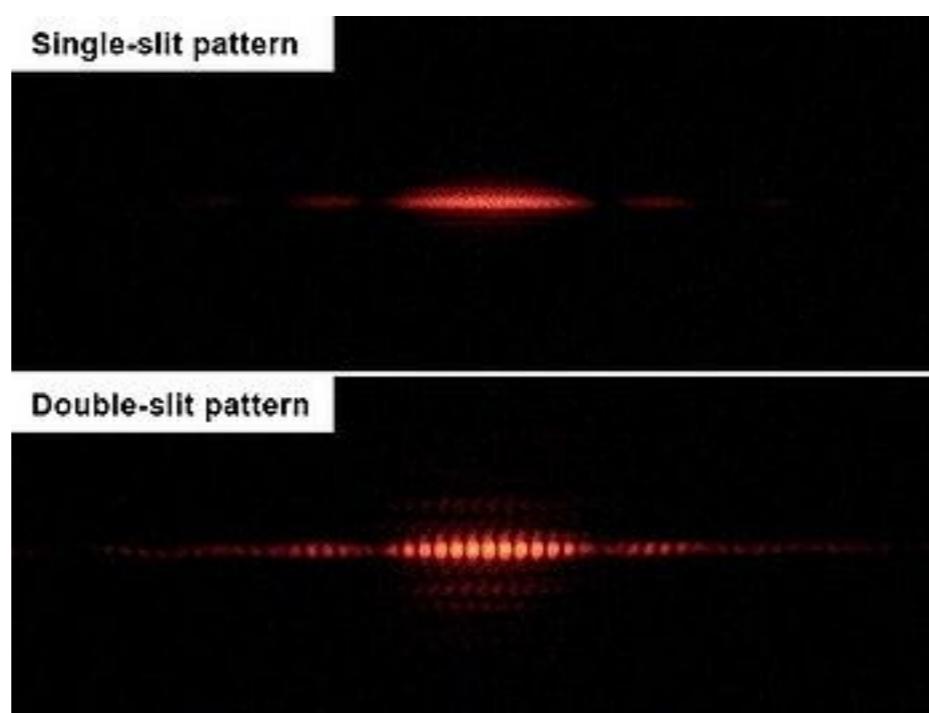
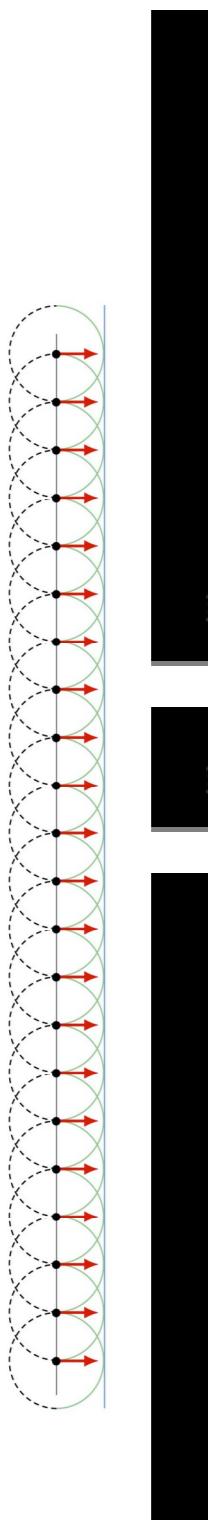
→ Lines of different colors (grey and black) crossing each other.

→ The larger the slit, the narrower the diffraction pattern

# Diffraction or interferences?

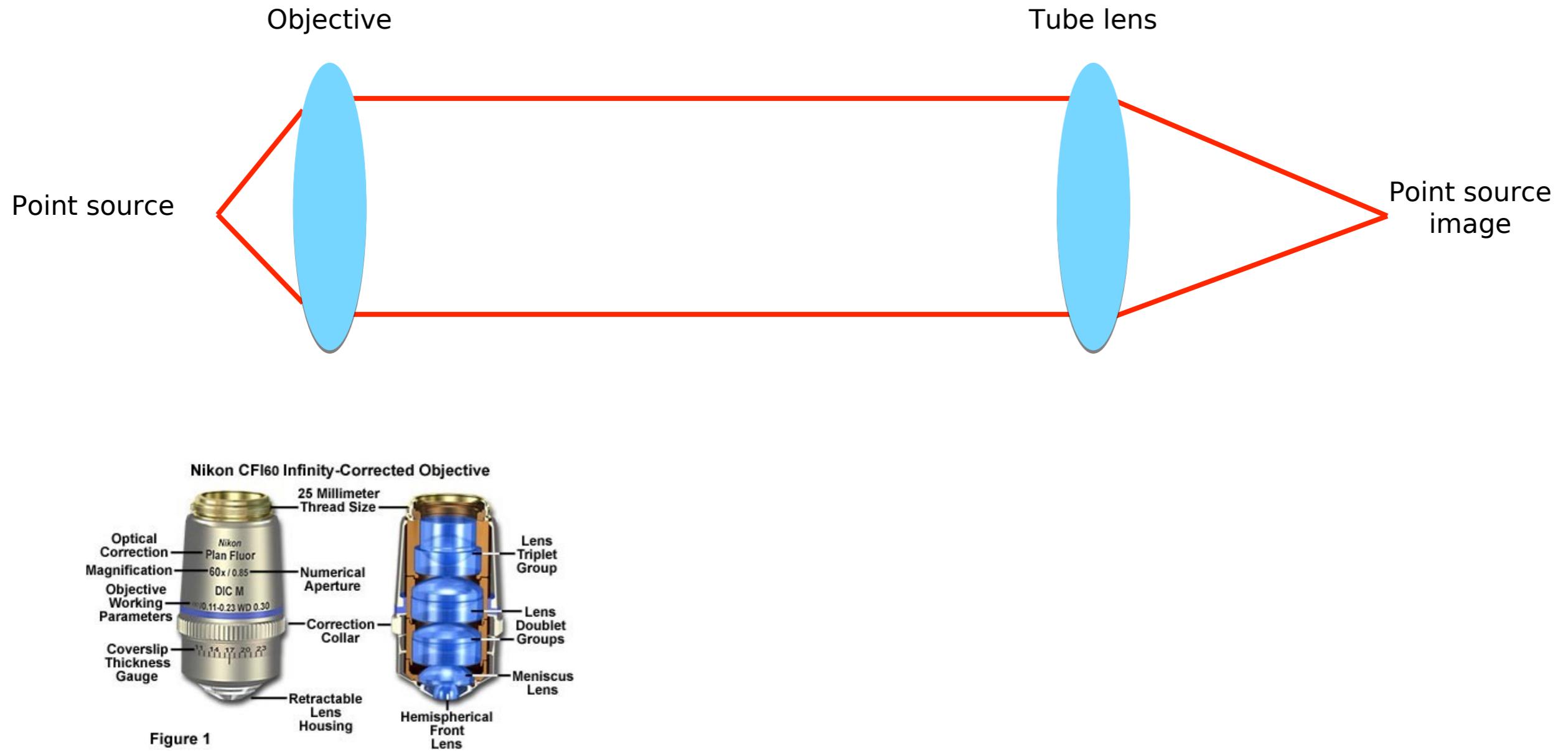
We should point out that there is not much of a difference between the phenomenon of interference and diffraction, indeed, interference corresponds to the situation when we consider the superposition of waves coming out from a number of point sources and diffraction corresponds to the situation when we consider waves coming out from an area sources like a circular or rectangular aperture or even a large number of rectangular apertures (like the diffraction grating).

Ajoy Ghatak



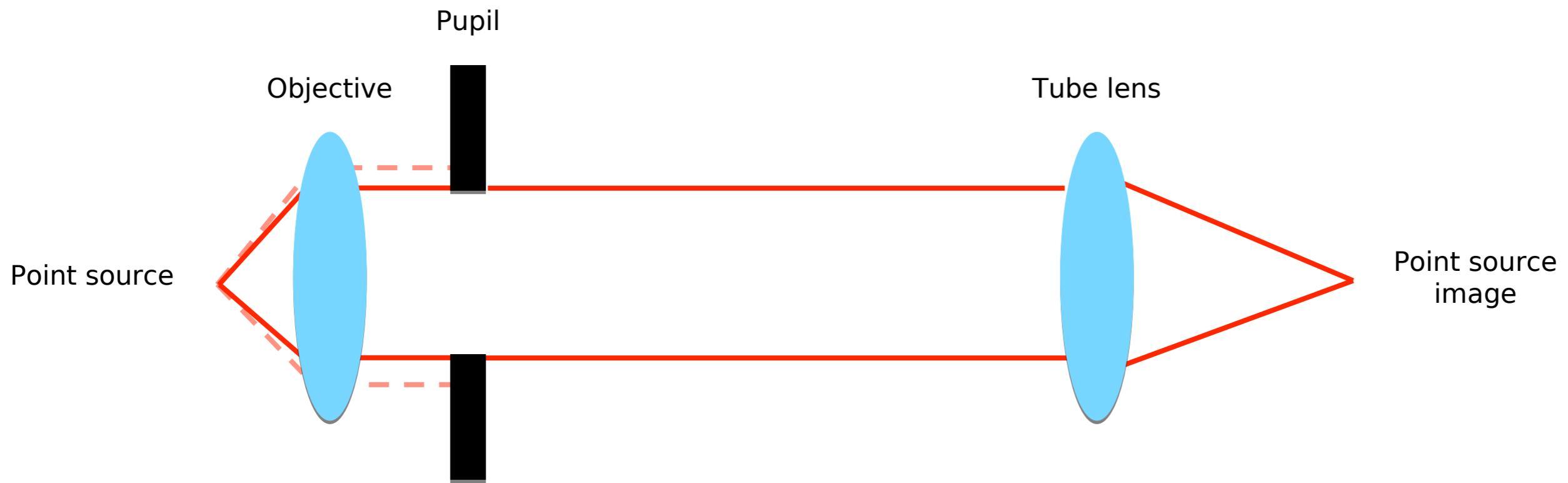
*In a double-slit experiment, the envelope gives information on the size of the slit while the fringes give information on the distance between the slits.*

# Lenses and diffraction



# Lenses and diffraction

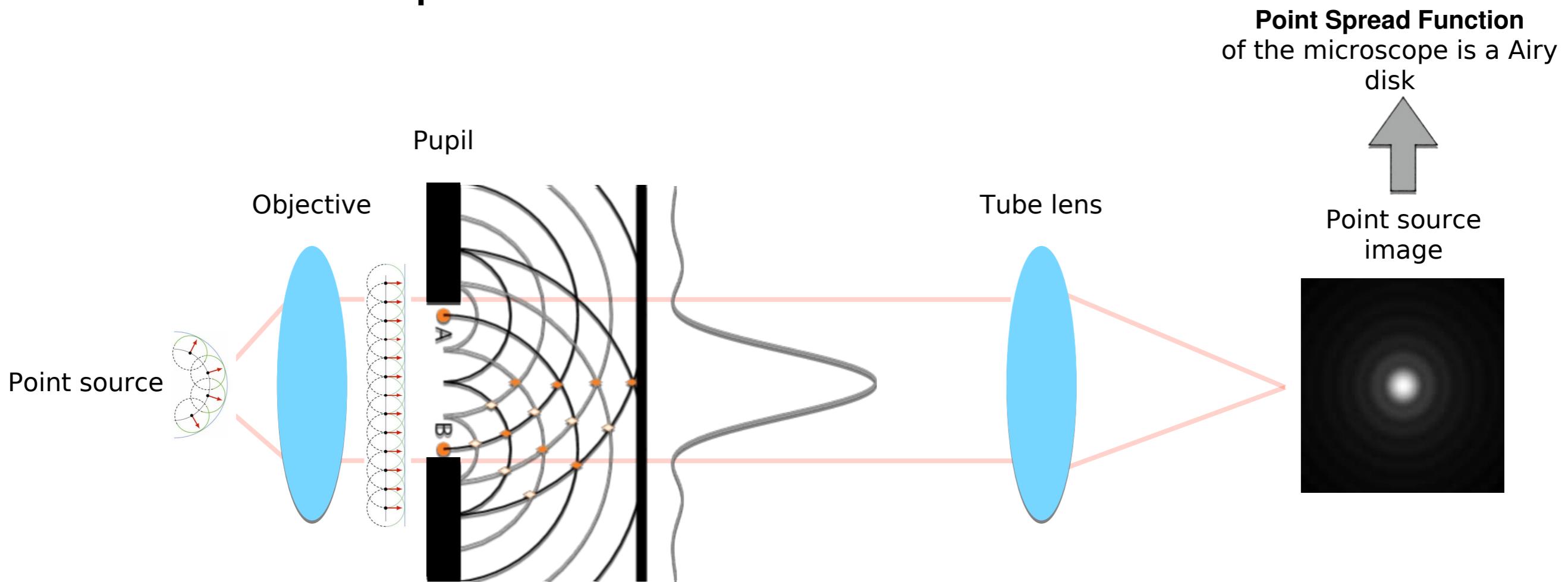
The exit pupil is a virtual **aperture** in an optical system. Only rays which pass through this virtual aperture can exit the system.



The exit pupil of a microscope is usually at the rear of the objective, near the back focal plane.

# Lenses and diffraction

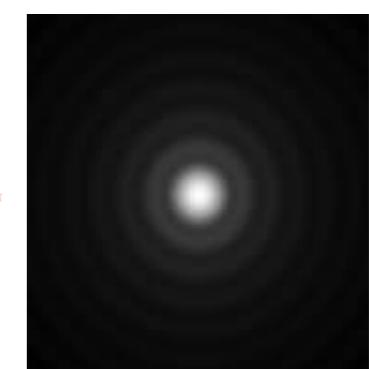
Wave + aperture —> diffraction!



Point Spread Function  
of the microscope is a Airy disk

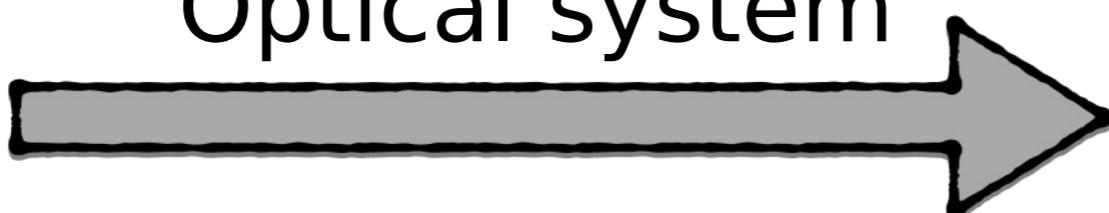


Point source  
image



Teeny  
tiny point

Optical system



Diffraction  
limited spot

# What does it mean???

This means small details of your object will be blurred

This means small details of your object won't pass through the microscope

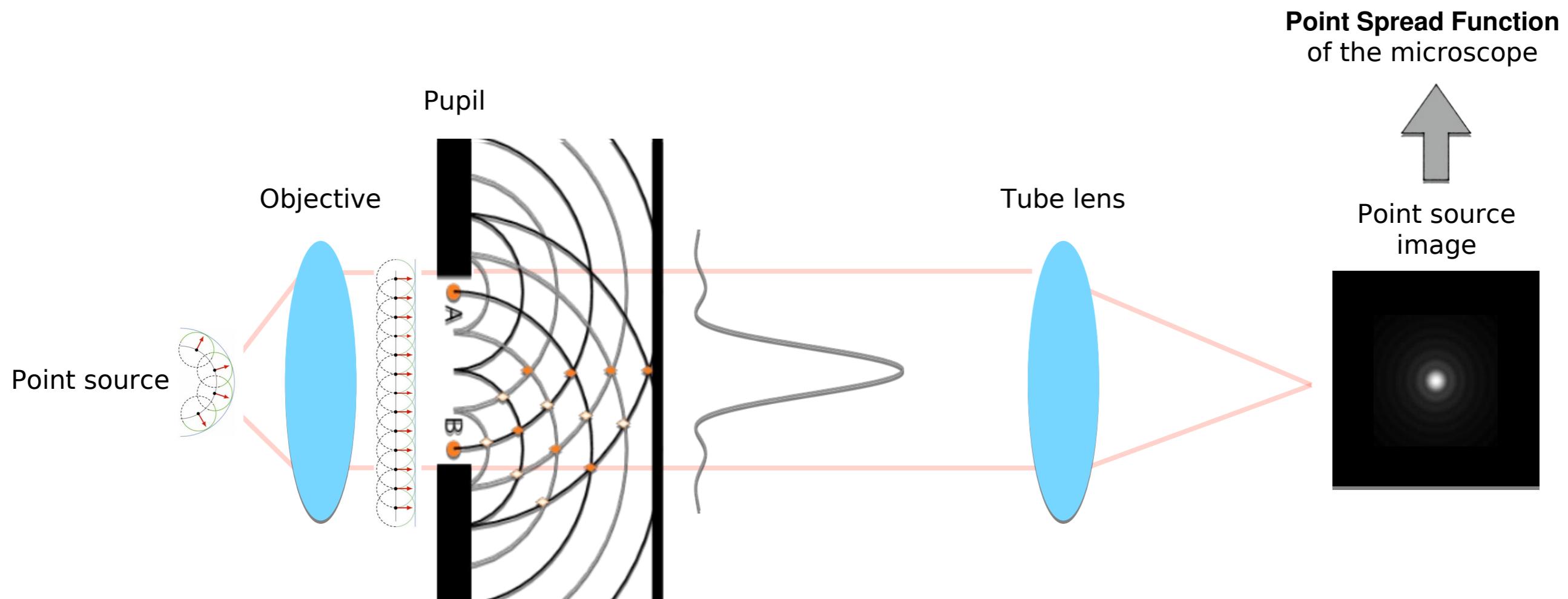
This means a microscope acts as a low pass filter with spatial frequencies.

This means a microscope will always give an image of the object convoluted with the microscope Point Spread Function

This means no matter how big your microscope magnification is, there is an intrinsic resolution limit to the details you can see

# PSF and NA

Wave + aperture —> diffraction!



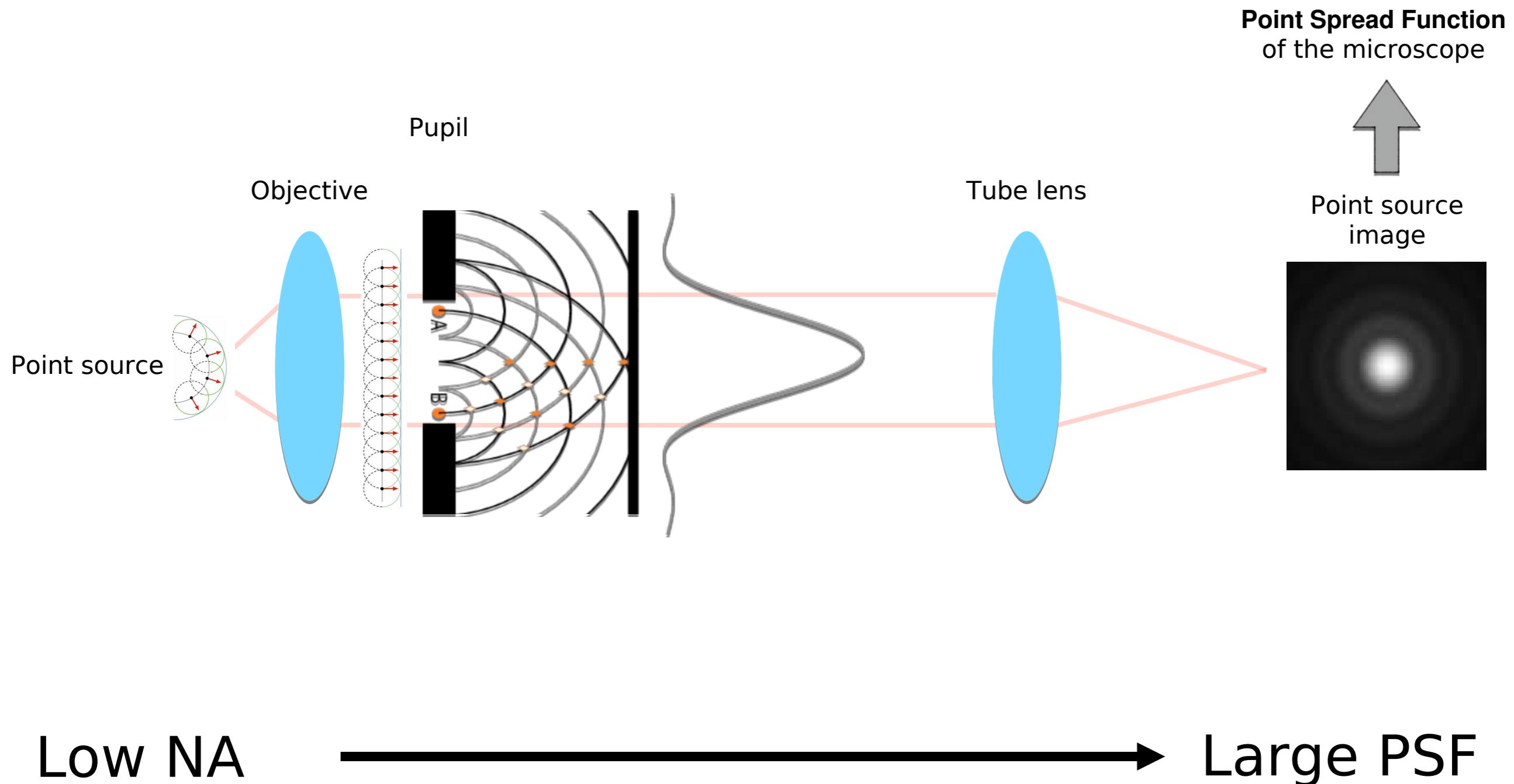
High NA



Small PSF

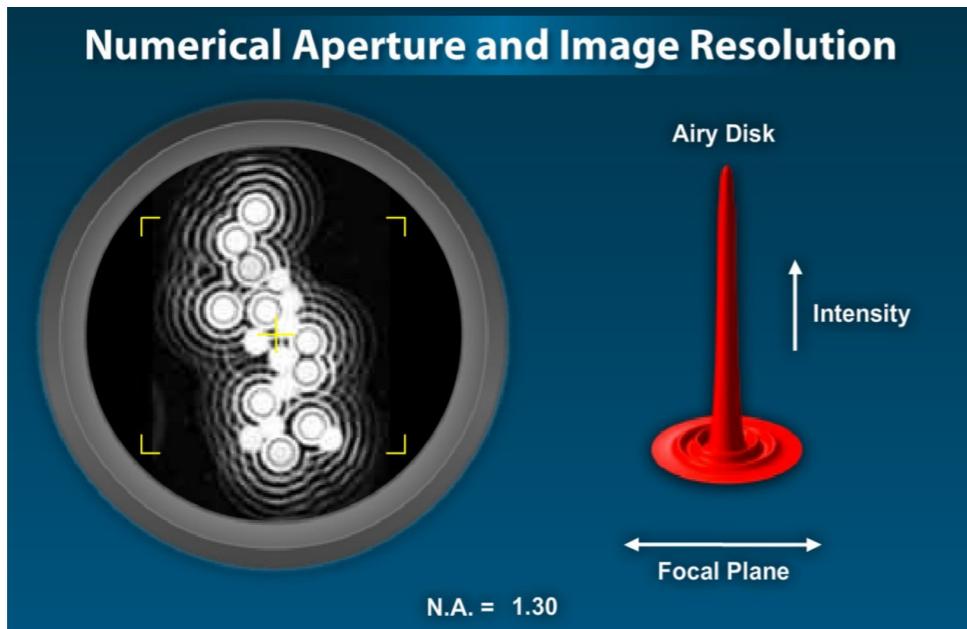
# PSF and NA

Wave + aperture —> diffraction!

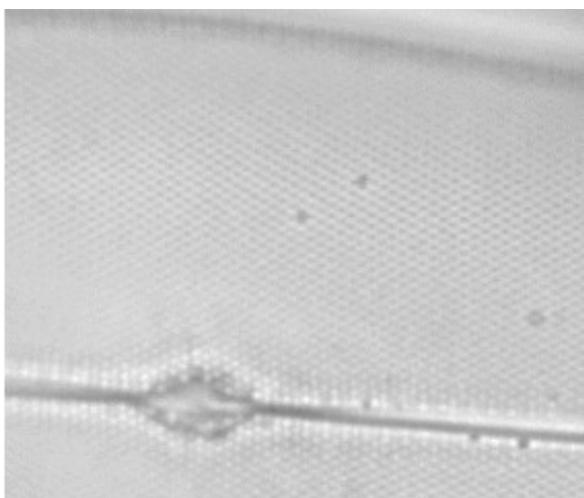
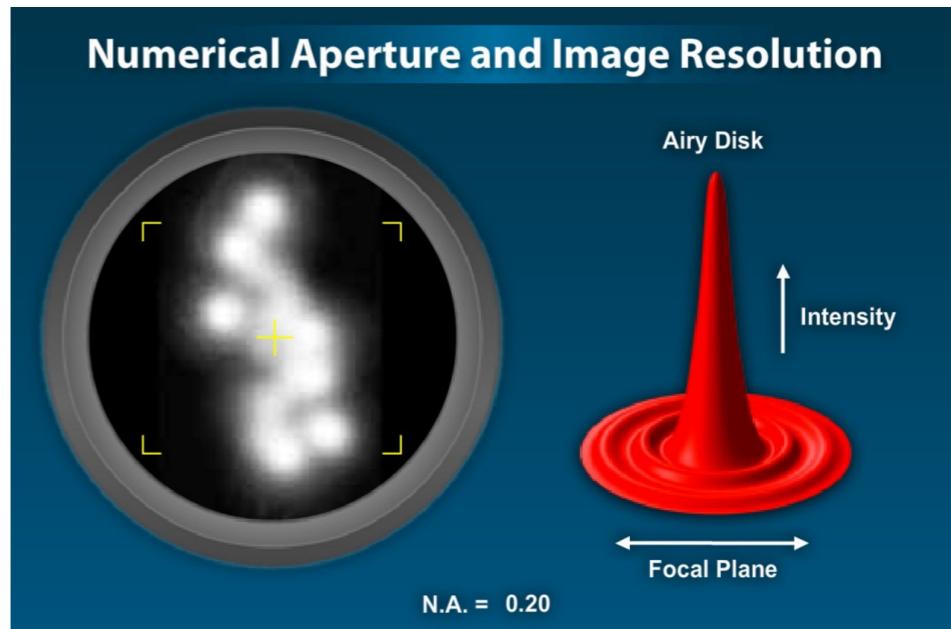


# In other words

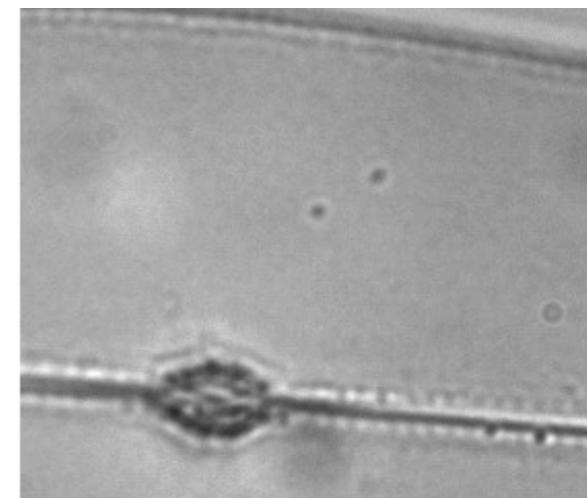
High NA



Low NA

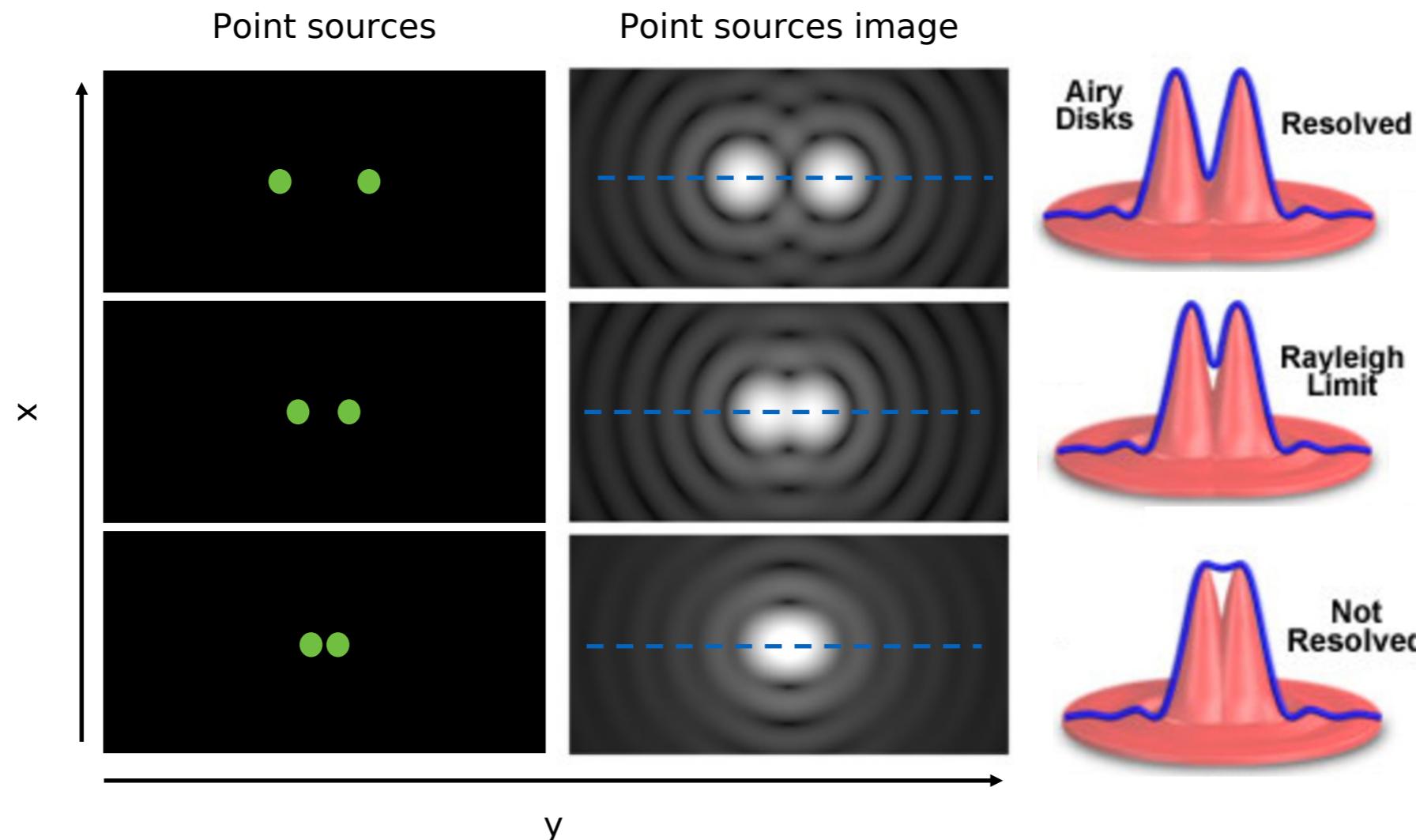


More light  
More details



Less light  
Less details

# The point spread function - resolution



## Rayleigh criterion :

Two objects are said to be just resolved when the maximum of the first Airy pattern falls on top of the first minimum of the second Airy pattern

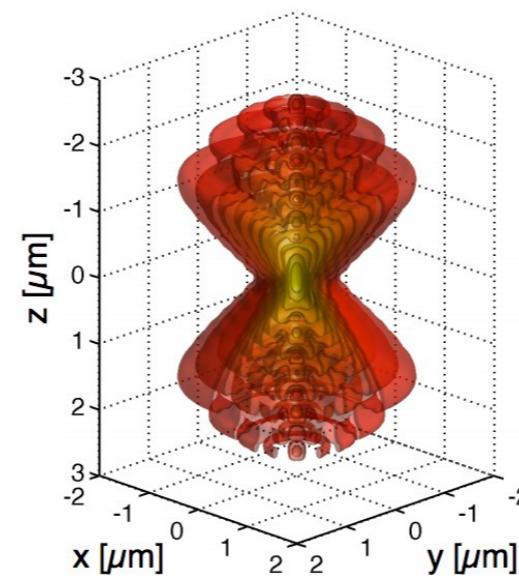
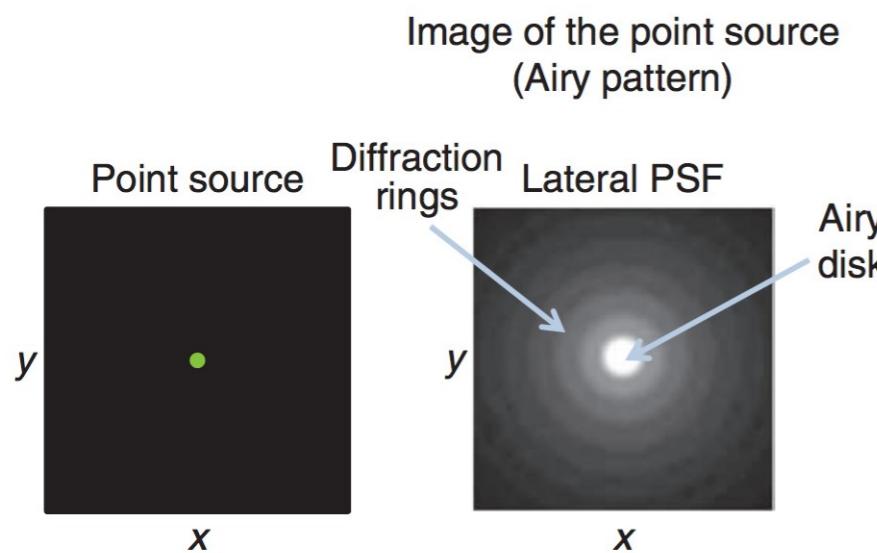
$$r_{xy} = \frac{0.61 \lambda}{NA}$$

ex : a 1.4 NA objective will have a resolution limit of 240nm with green light and 280nm with red light

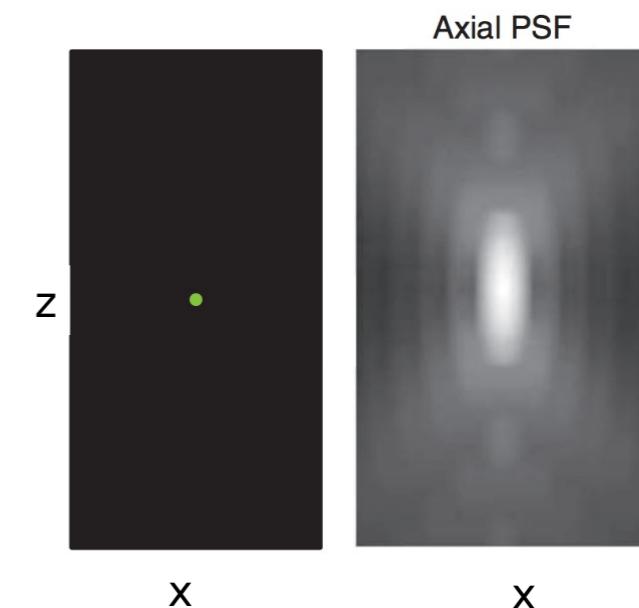
# Notes

The point spread function is three dimensional

## Lateral

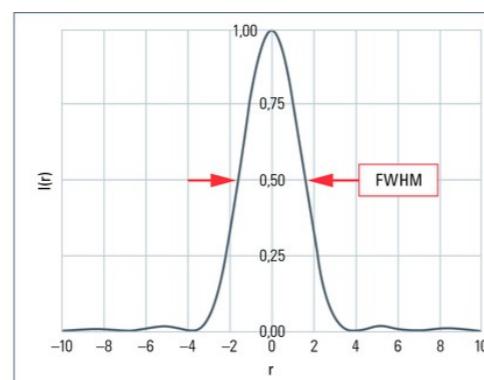
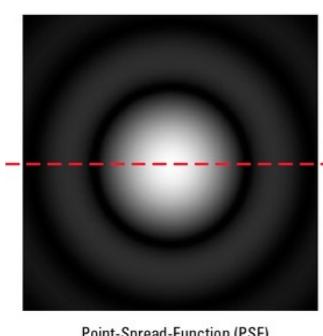


## Axial



In practice we use the Full Width at Half Maximum (FWHM) :

$$r_{\text{FWHM}} = \frac{0.51 \lambda}{NA}$$



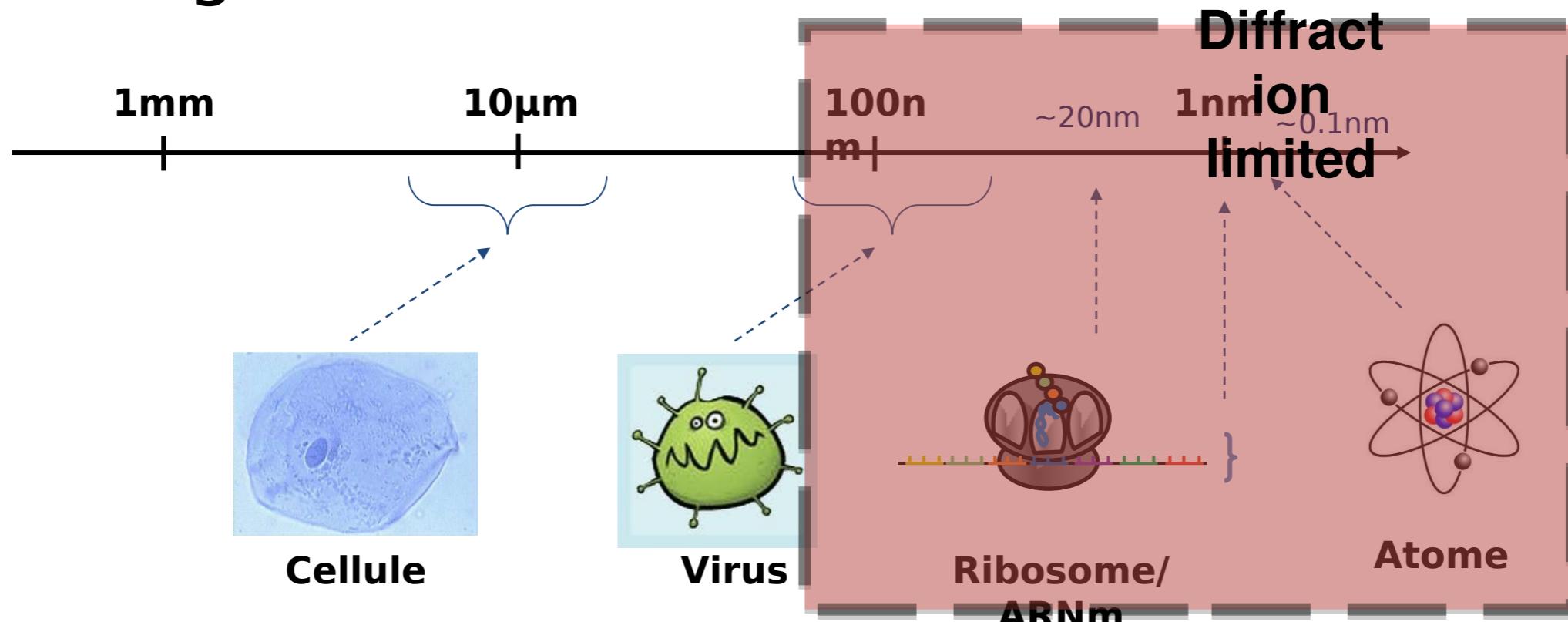
$$r_{xz} = \frac{2 \lambda n}{NA^2}$$

Axial resolution is  $\neq$  from the depth of field which is defined as the thickness region of the specimen that appears in focus in the final image

$$d_{\text{DOP}} = \frac{\lambda n}{NA^2}$$

# Keep in mind

## Relevant length scales



A microscope will always give an image of the object convoluted with the microscope Point Spread Function

The higher the NA, the more details you'll see

The lower the wavelength, the more details you'll see

The higher the NA, the more you can concentrate light (focused light)

The higher the NA, the more light you'll get

# Outline - part3

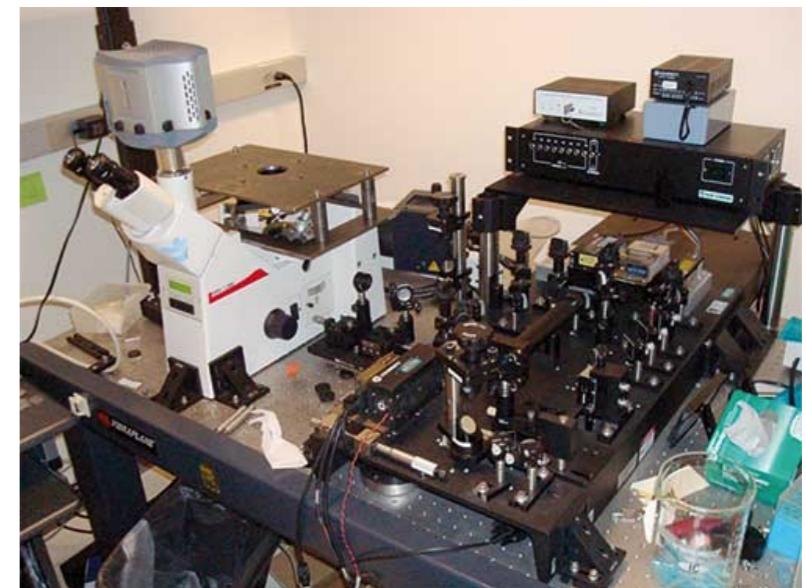
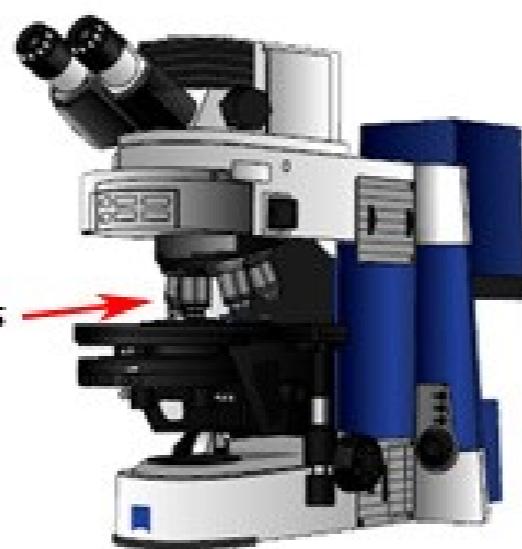
## Microscope components

# Microscope

Inverted

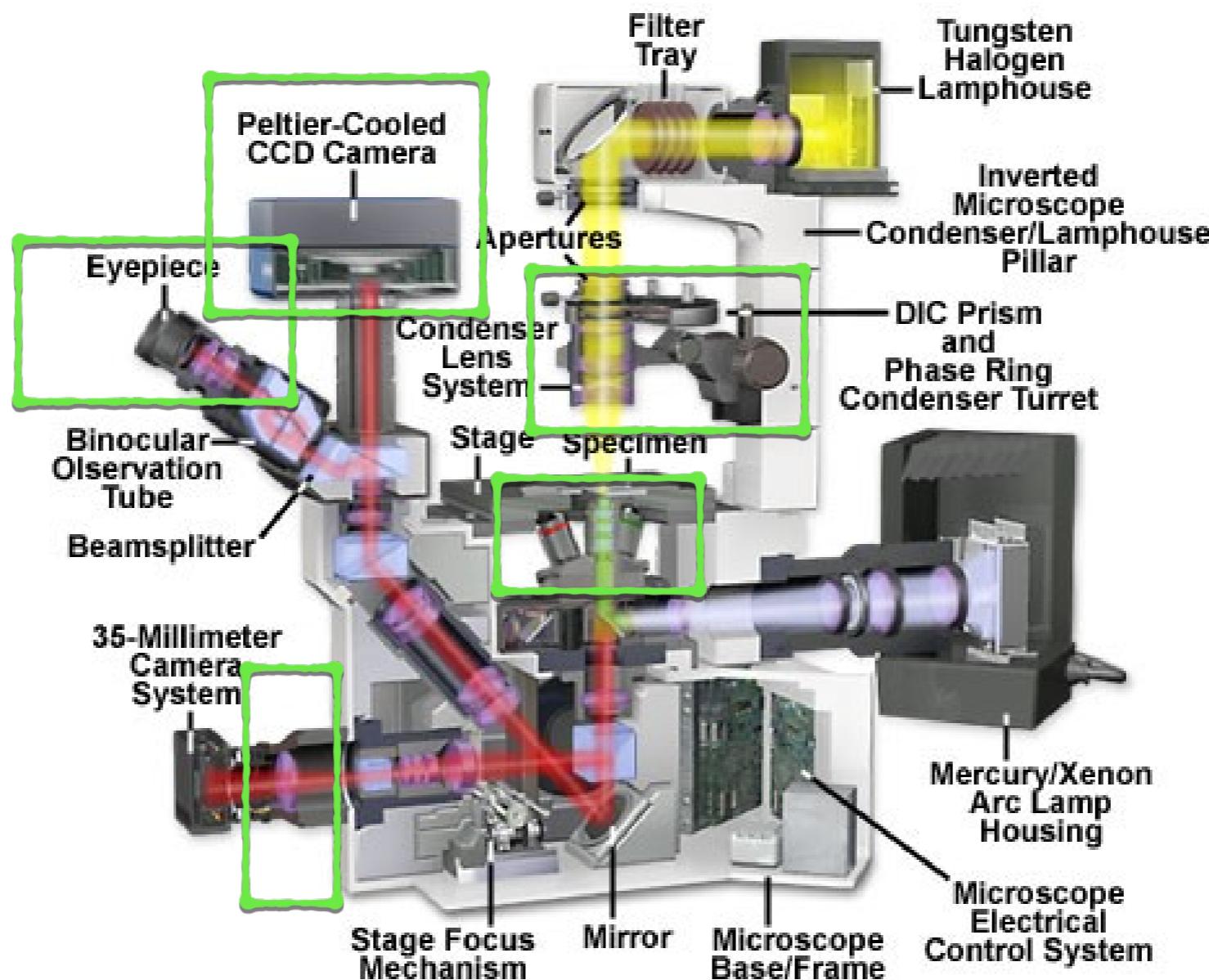


Upright

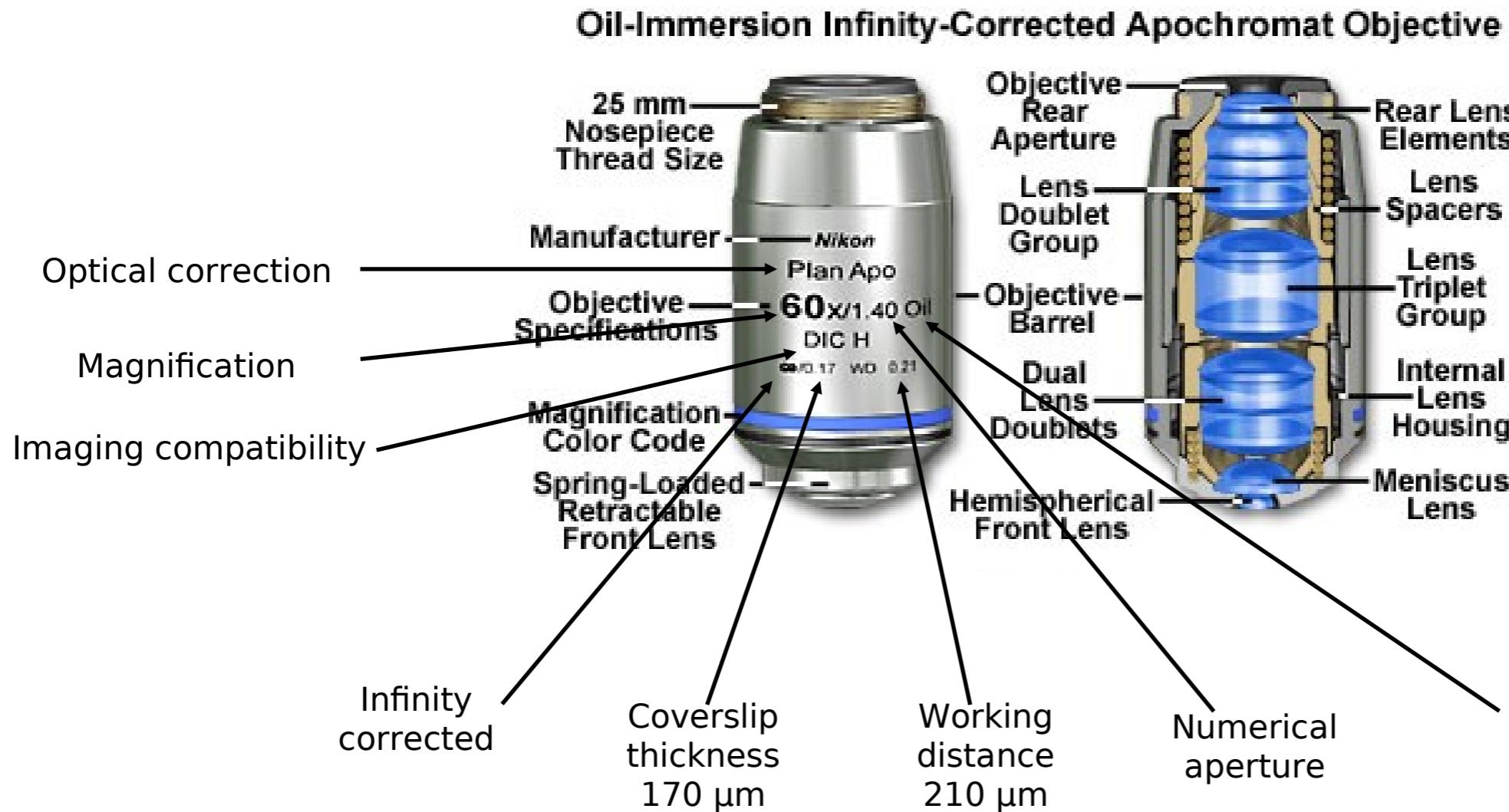


- 1) An inverted microscope gives you greater freedom than an upright one
- 2) Inverted microscopes enable you to look at more samples in a shorter period of time
- 3) With an inverted microscope, you cannot crash an objective into the sample
- 4) Inverted microscopes save you time and money in sample preparation

# Microscope



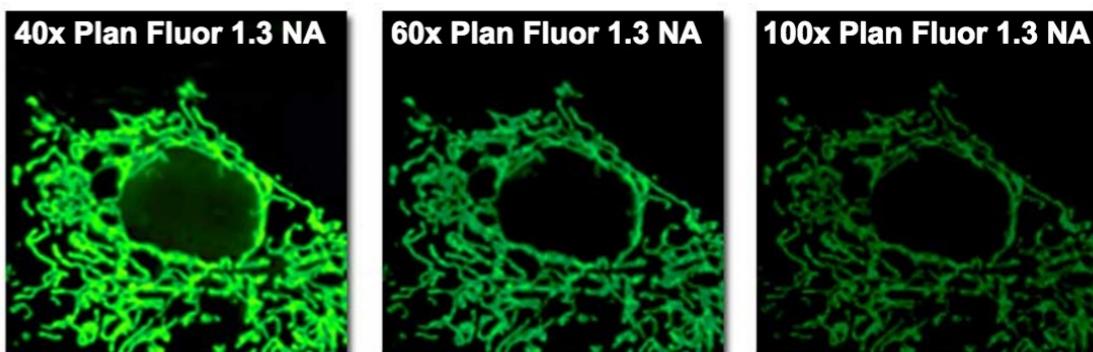
# Objective



Use the right objective for your application!

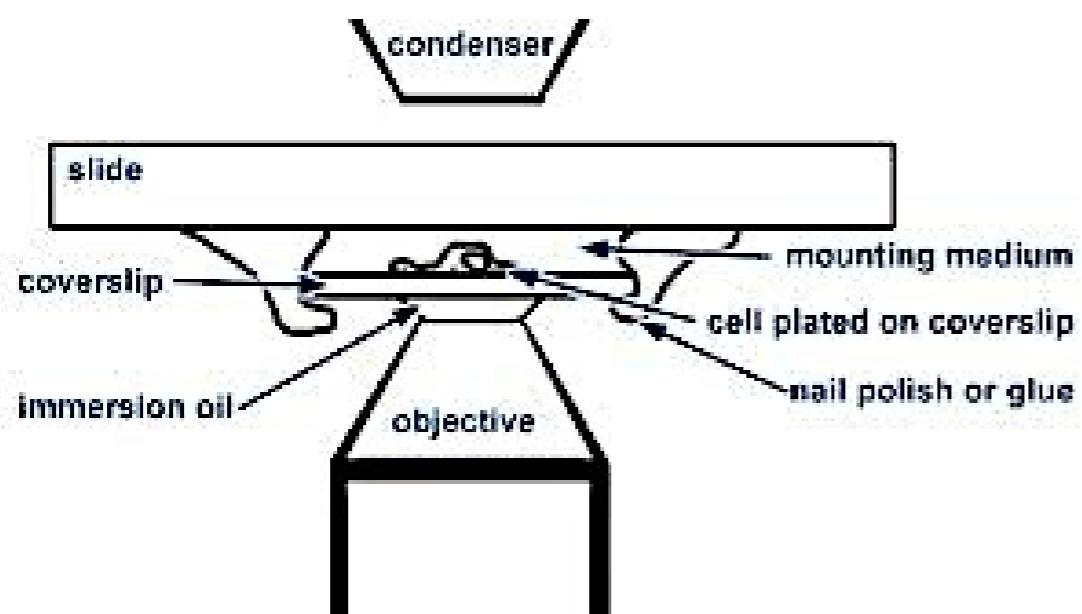
Nomenclature for objective designations:

<http://www.olympusmicro.com/primer/anatomy/specifications.html>



# Coverslip - immersion oils

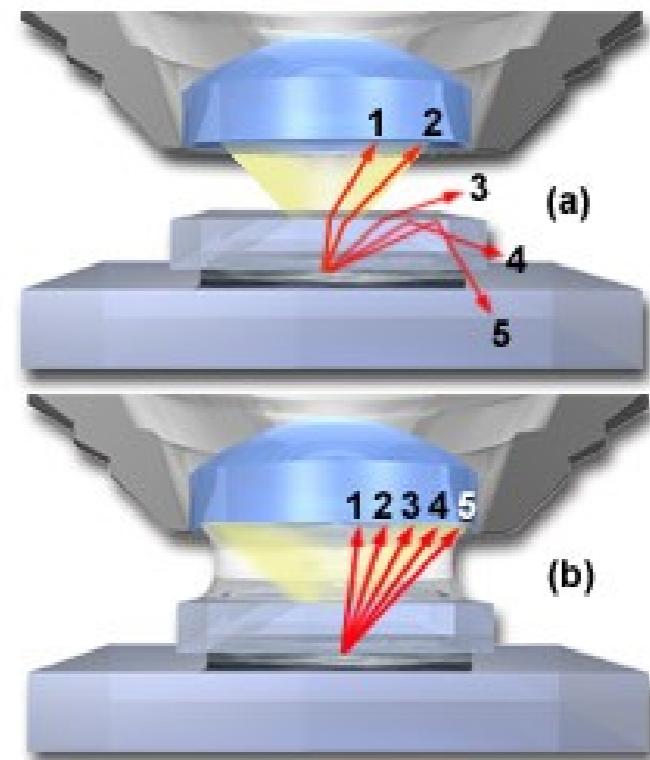
## Use the right coverslip thickness



Typically 170  $\mu\text{m}$  coverslip thickness (#1.5)

## Use the right immersion media (if required)

Oil Immersion and Numerical Aperture

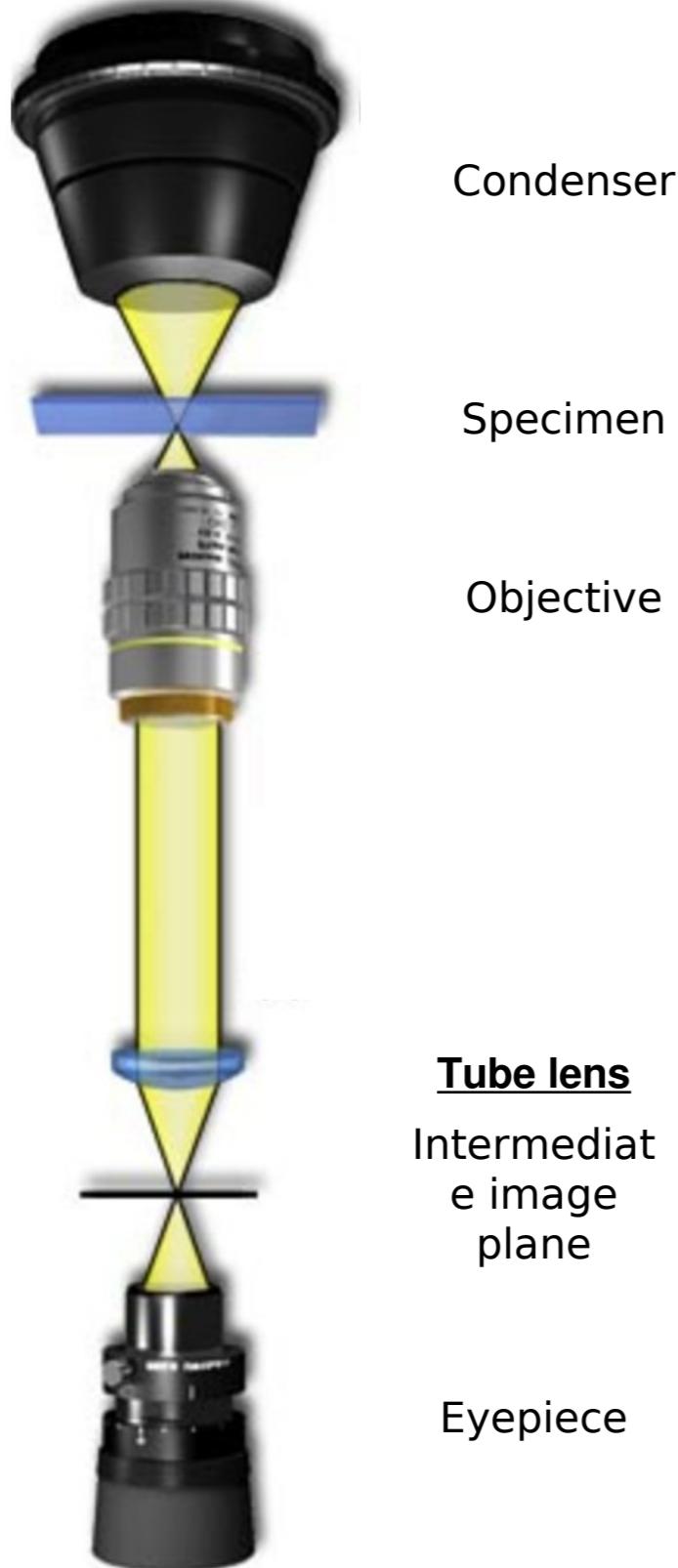


$$n_{\text{air}} = 1.000293$$

$$n_{\text{water}} = 1.333$$

$$n_{\text{glass}} \sim 1.5$$

# Tube lens



Manufacturer	Tube Lens Focal Length (Millimeters)	Parfocal Distance (Millimeters)	Thread Type
Leica	200	45	M25
Nikon	200	60	M25
Olympus	180	45	RMS
Zeiss	165	45	RMS

Objective specifications won't be the same if you don't use it with its corresponding microscope (manufacturer)

*For example the microscope magnification is:*

$$M = \frac{f_{\text{tube lens}}}{f_{\text{objective}}}$$

# Eyepiece



To bring extra confort to the user (extra magnification)

Condenser

Specimen

Objective

Tube lens

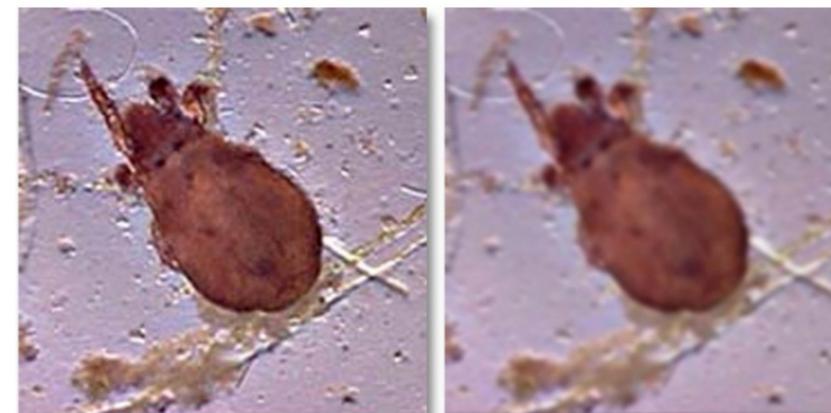
Intermediate  
image  
plane

Eyepiece

$$M_{\text{tot}} = M_{\text{obj}} \times M_{\text{eyepiece}}$$

A simple equation to choose your magnification:

Useful Magnification (total) = 500 to 1000 × NA (Objective)



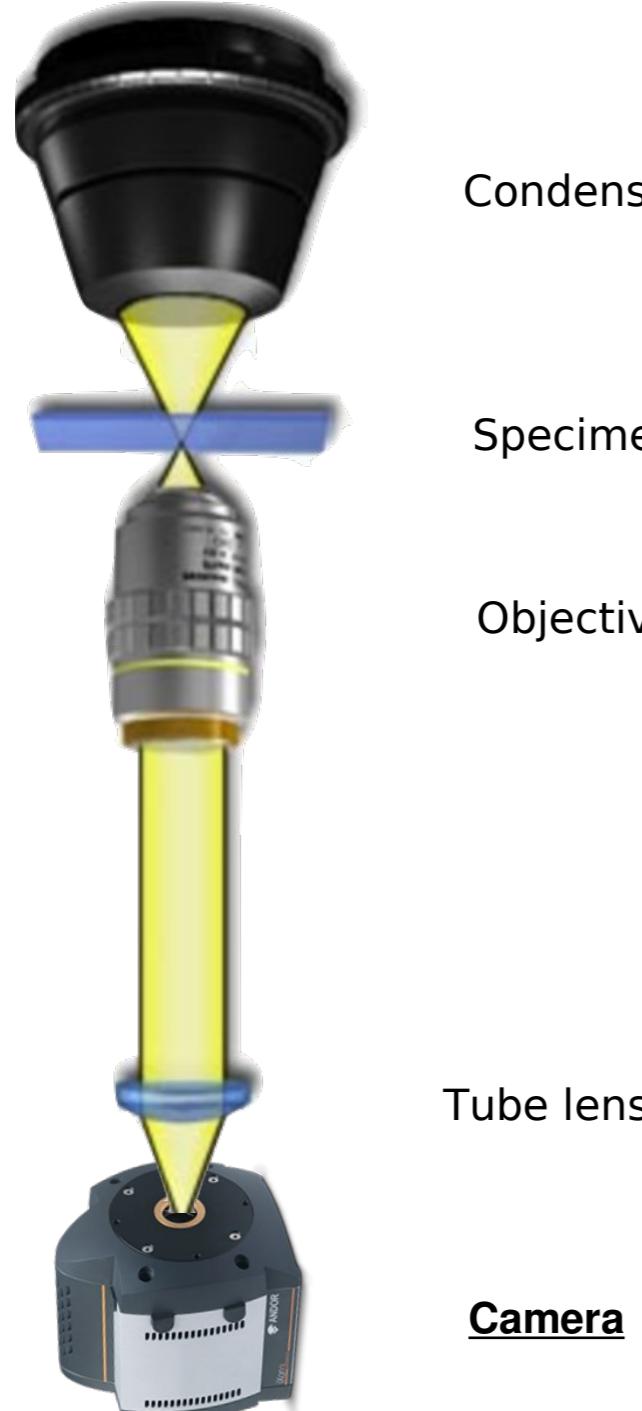
$$D_{\text{fov}} = \frac{\text{F.N.}}{M_{\text{objective}}}$$

D is the diameter of the field of view in millimeters.

F.N. is the field number inscribed on the eyepiece

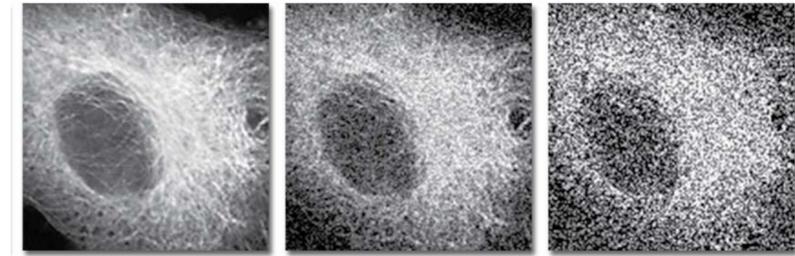
$M_{\text{objective}}$  is the magnification of the objective being used.

# Camera



## Sensitivity (low light conditions)

$$I = \frac{(N.A.)^2}{(Total M)^2}$$

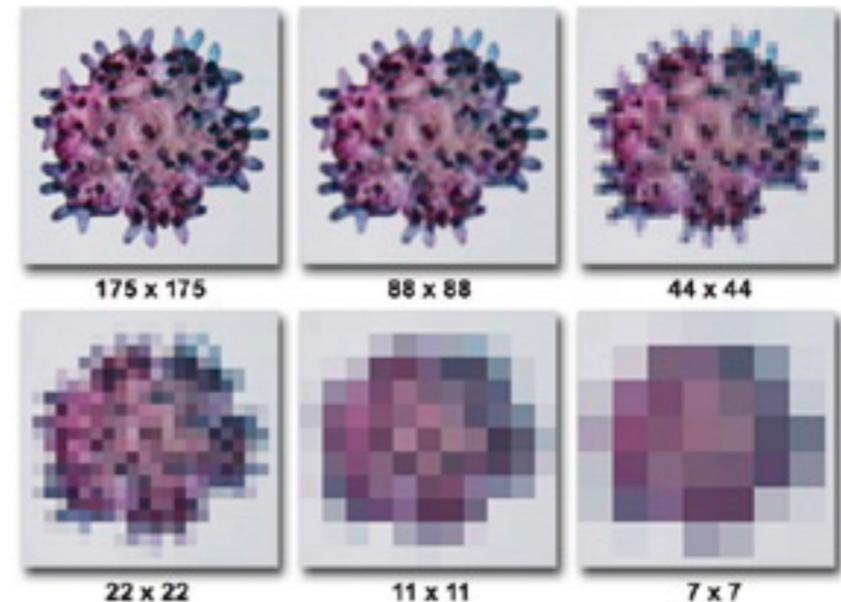


I equals the intensity of the image.

M is the magnification (for transmitted light)

## Pixel size

To capture the full information, the sampling frequency needs to be at least twice the highest sample frequency

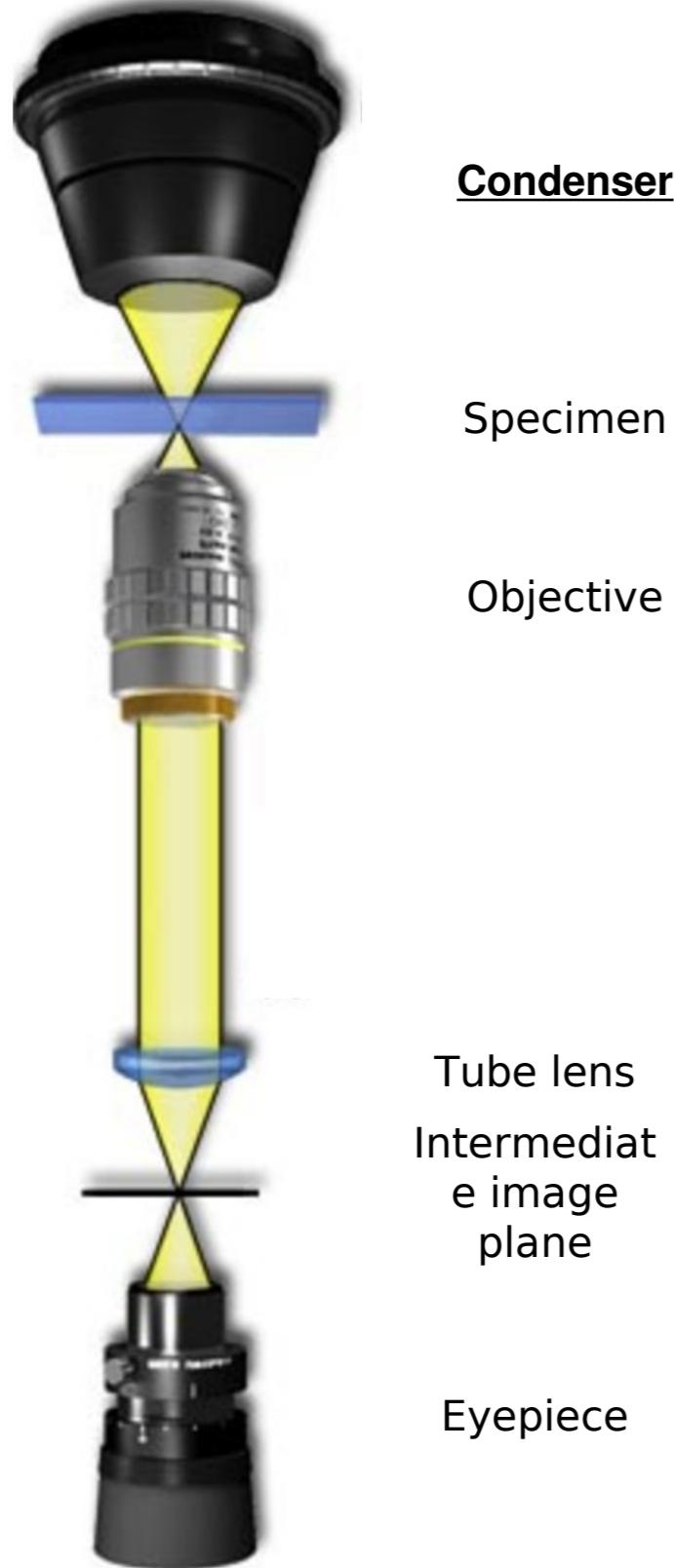


## **Nyquist theorem**

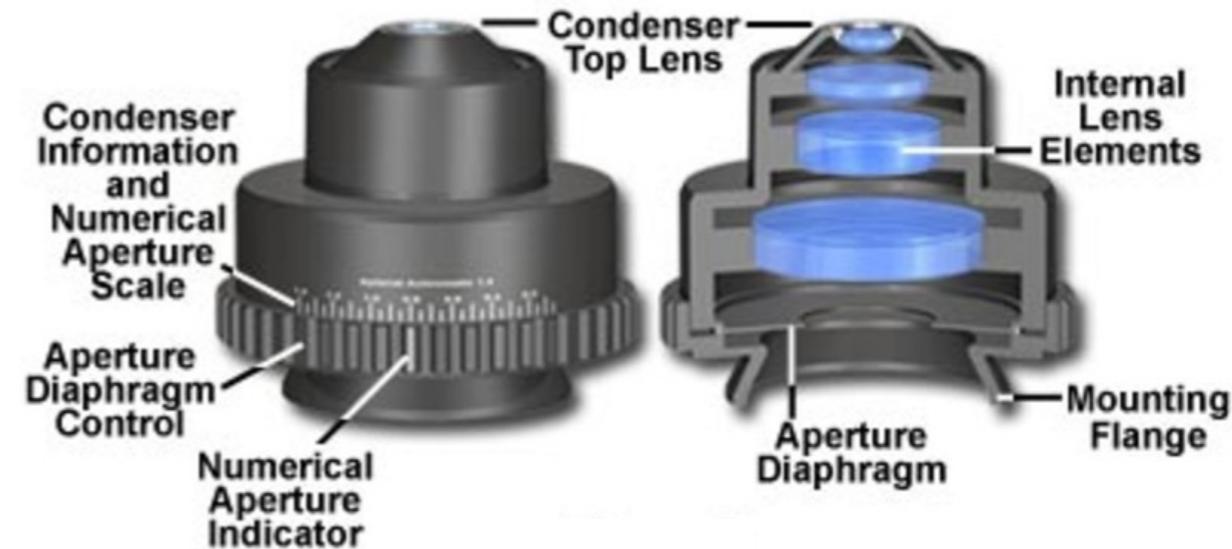
*Ex : for a NA 1.3, 60X objective imaging in the green (540 nm), the camera pixel size should be no more than 6μm*

Acquisition speed, dynamic range, sensor area, etc...

# Condenser



Achromat/Aplanat Condenser (Numerical Aperture = 1.38)

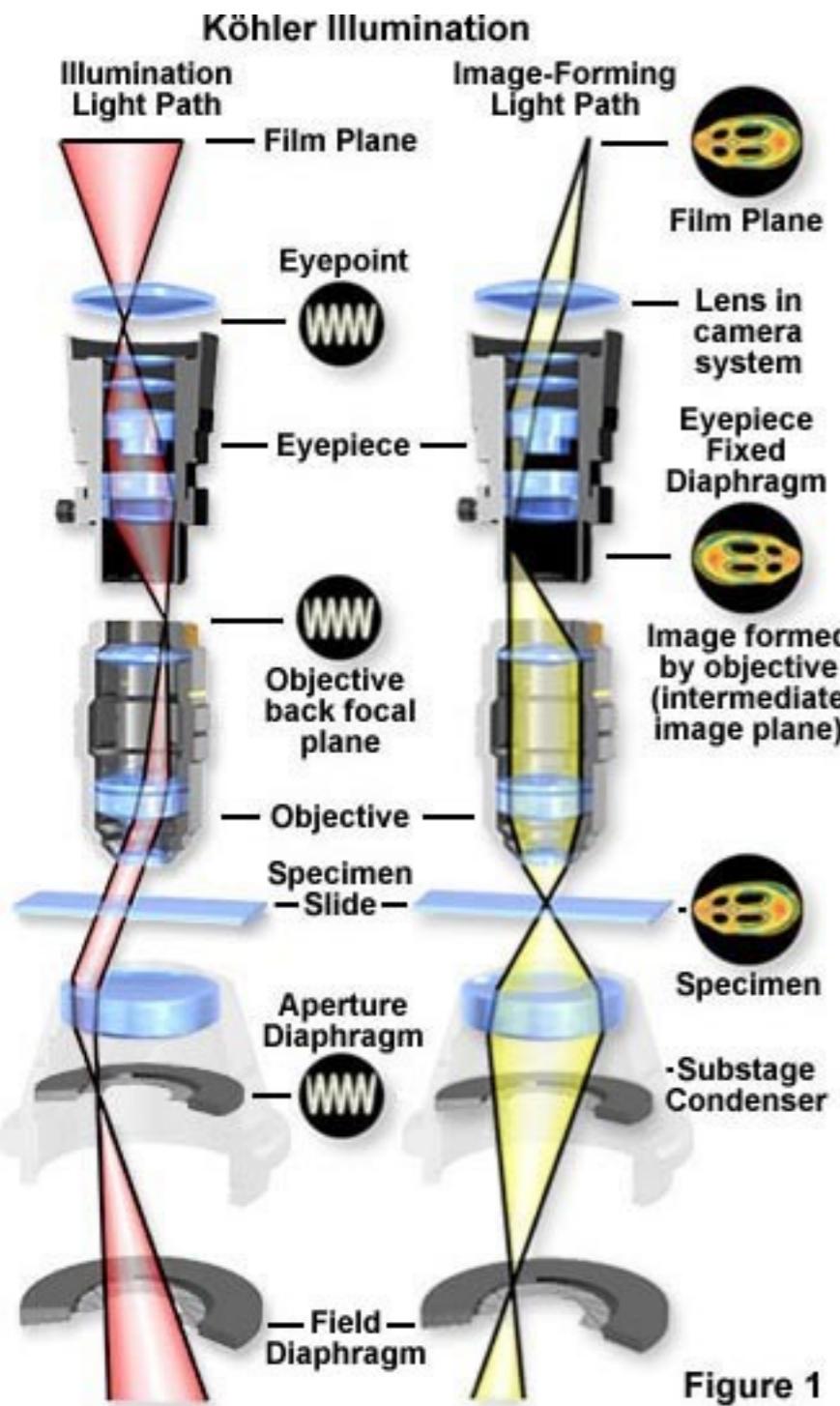


Use the right condenser for your application!

$$r_{\text{rayleigh}} = \frac{0.61 \lambda}{NA_{\text{obj}} + NA_{\text{cond}}}$$

Set it correctly for optimal illumination conditions (kohler illumination)

# Condenser : kohler illumination



## kohler illumination :

- Illumination **uniformly** bright
- Ensures that an image of the illumination source (for example a halogen lamp filament) is not visible in the resulting image.

!! Check your condenser alignment !!

- Even illumination
- Optimal contrast (Aperture diaphragm)
- Optimal resolution (Aperture diaphragm)
- Minimal phototoxicity (Field diaphragm)



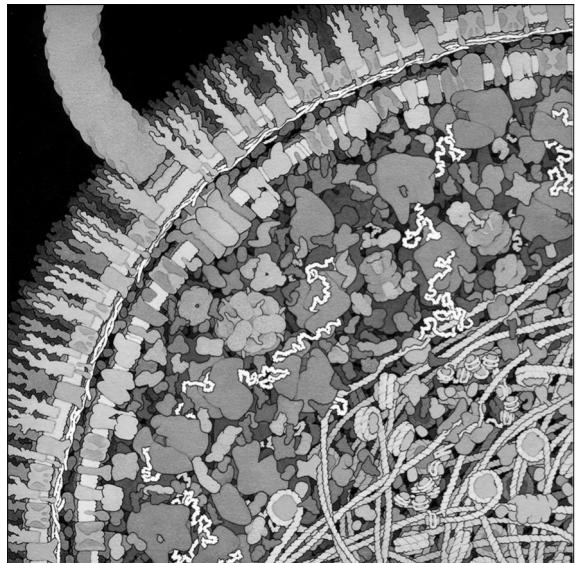
# What does a microscope do?

## ⇒ *Why do we need microscopes?*

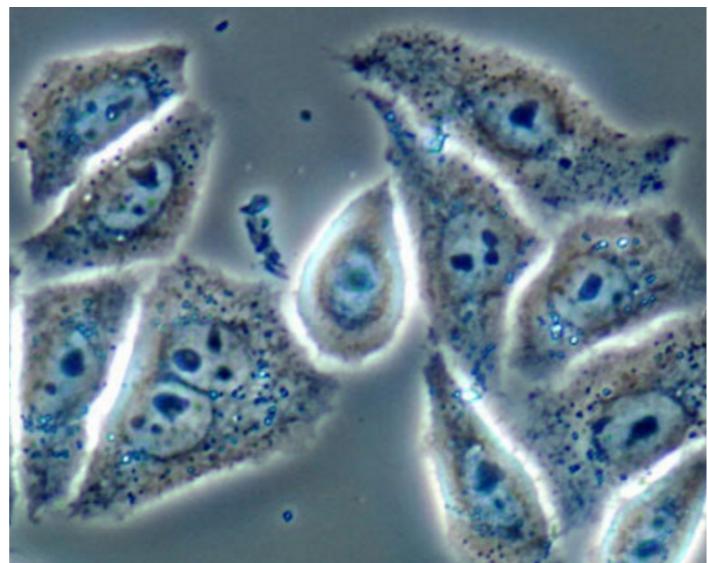
To observe “small objects”, microscopes main contributions:

- ⇒ Magnification ✓
- ⇒ Resolution ✓ Limited to a theoretical limit ~250 nm
- ⇒ Contrast ?

# Part 4 - Contrasting methods



Bright field

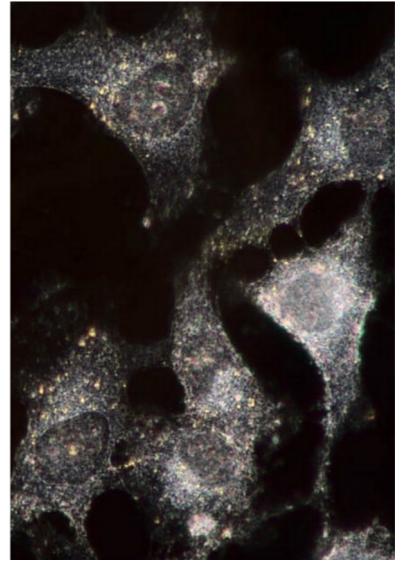


Phase contrast

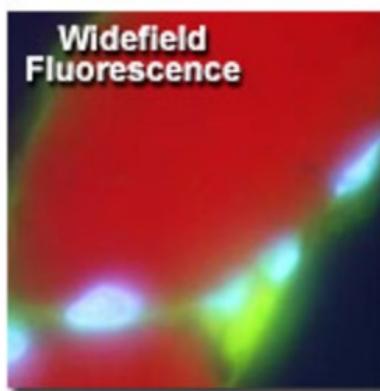


DIC\*

*\*Differential interference contrast*



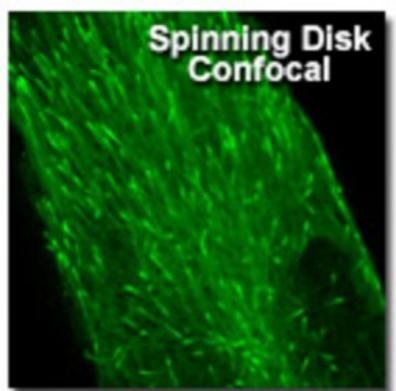
Dark field



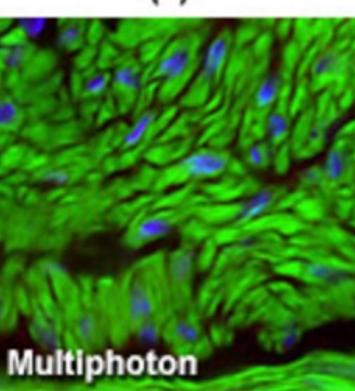
Widefield  
Fluorescence



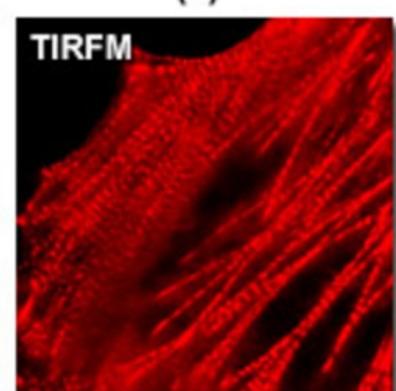
Laser Scanning  
Confocal



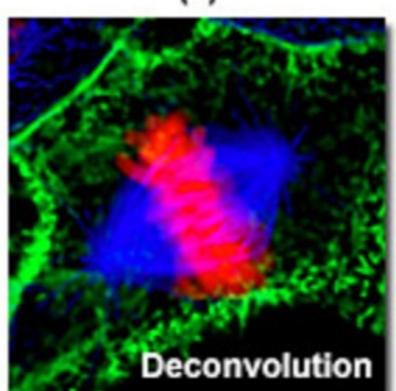
Fluorescence microscopy



Multiphoton



TIRFM



Deconvolution



High contrast  
Specific labelling  
Multiple labelling  
Live compatible  
...

# Outline - part 4

Fluorescence

Excitation sources & filters

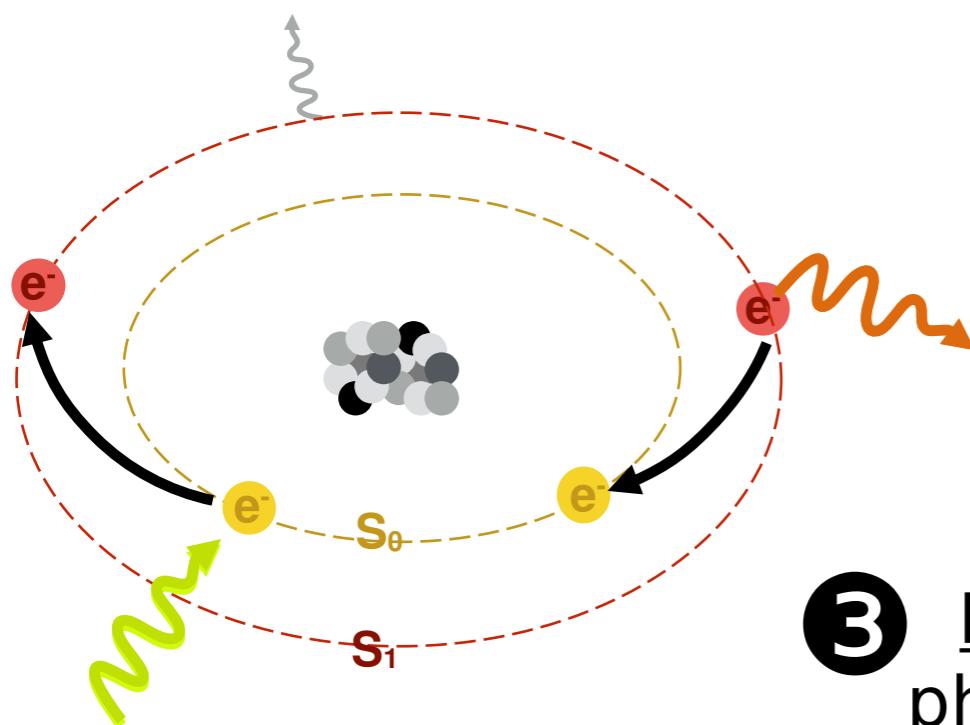
Fluorophores

3 most popular fluorescence techniques

# Fluorescence

Electrons can absorb energy from external sources, such as lasers, arc-discharge lamps, and tungsten-halogen bulbs, and be promoted to higher energy levels.

② **Relaxation**  
energy loss (thermal, kinetic...)

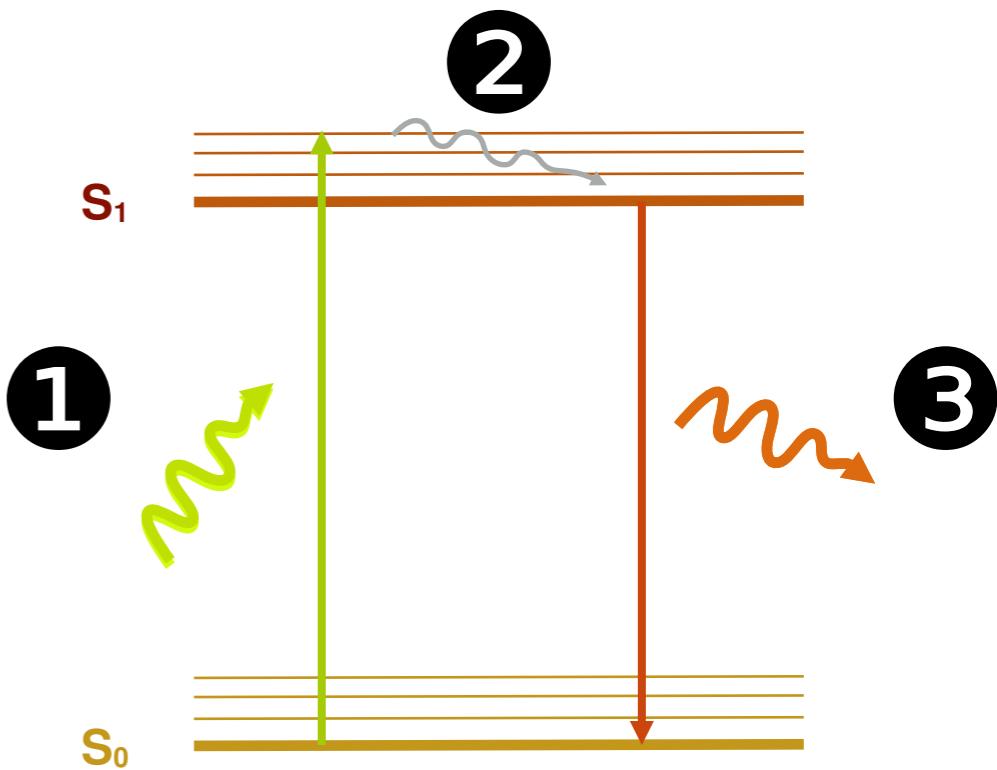


① **Excitation**  
photon absorption

$$E_1 = \frac{hc}{\lambda_1}$$

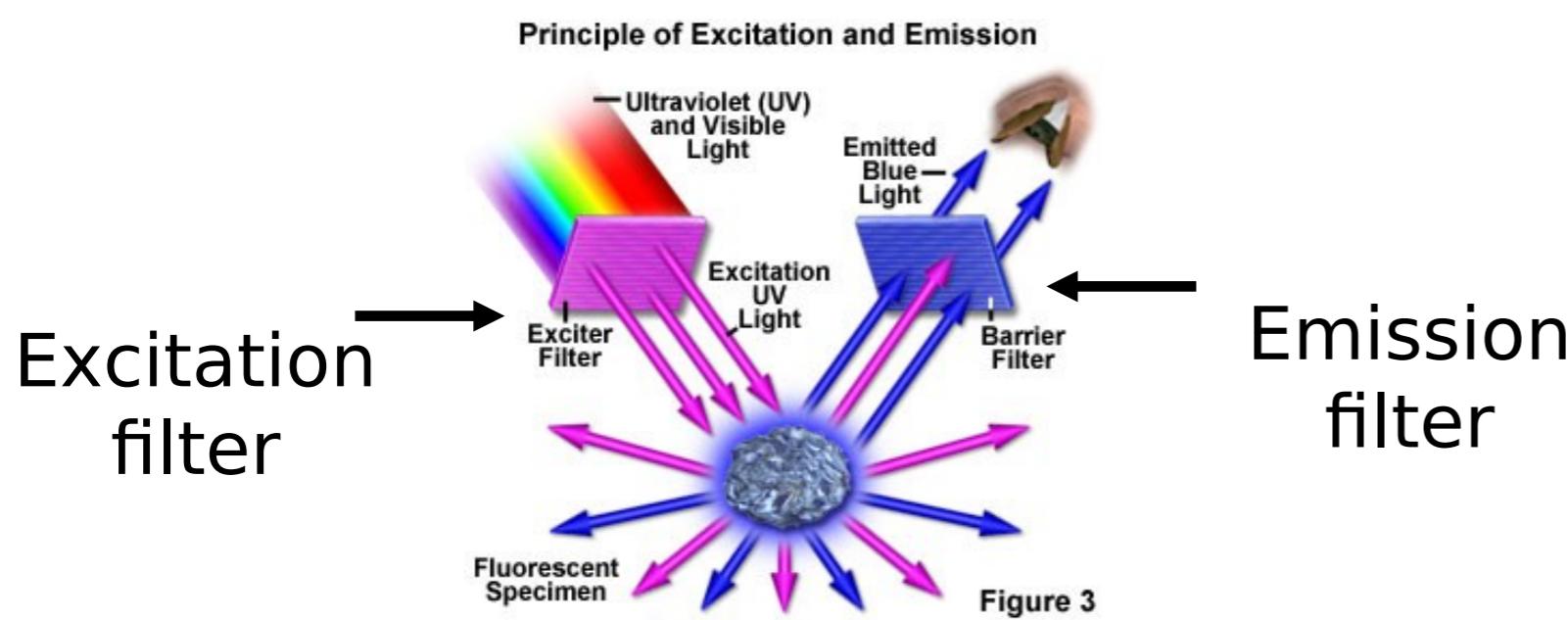
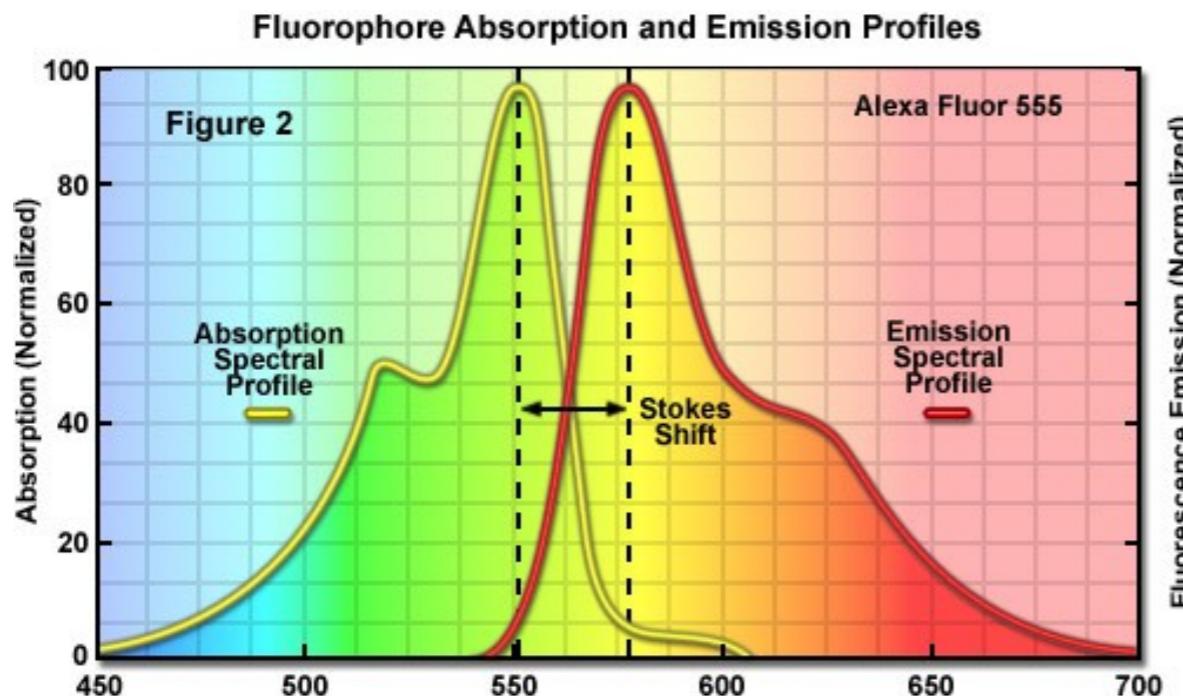
③ **Fluorescence**  
photon emission  
 $E_2 = \frac{hc}{\lambda_2} < E_1$

Jablonski diagram

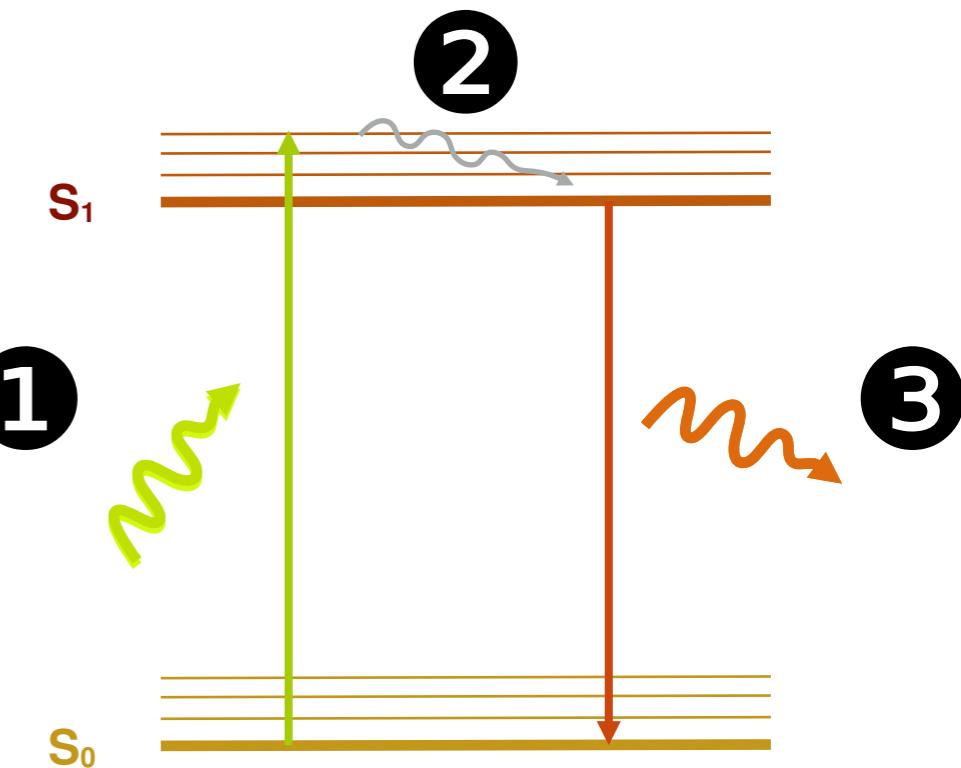


# Fluorescence

Absorption / Emission spectra are shifted (stokes shift)



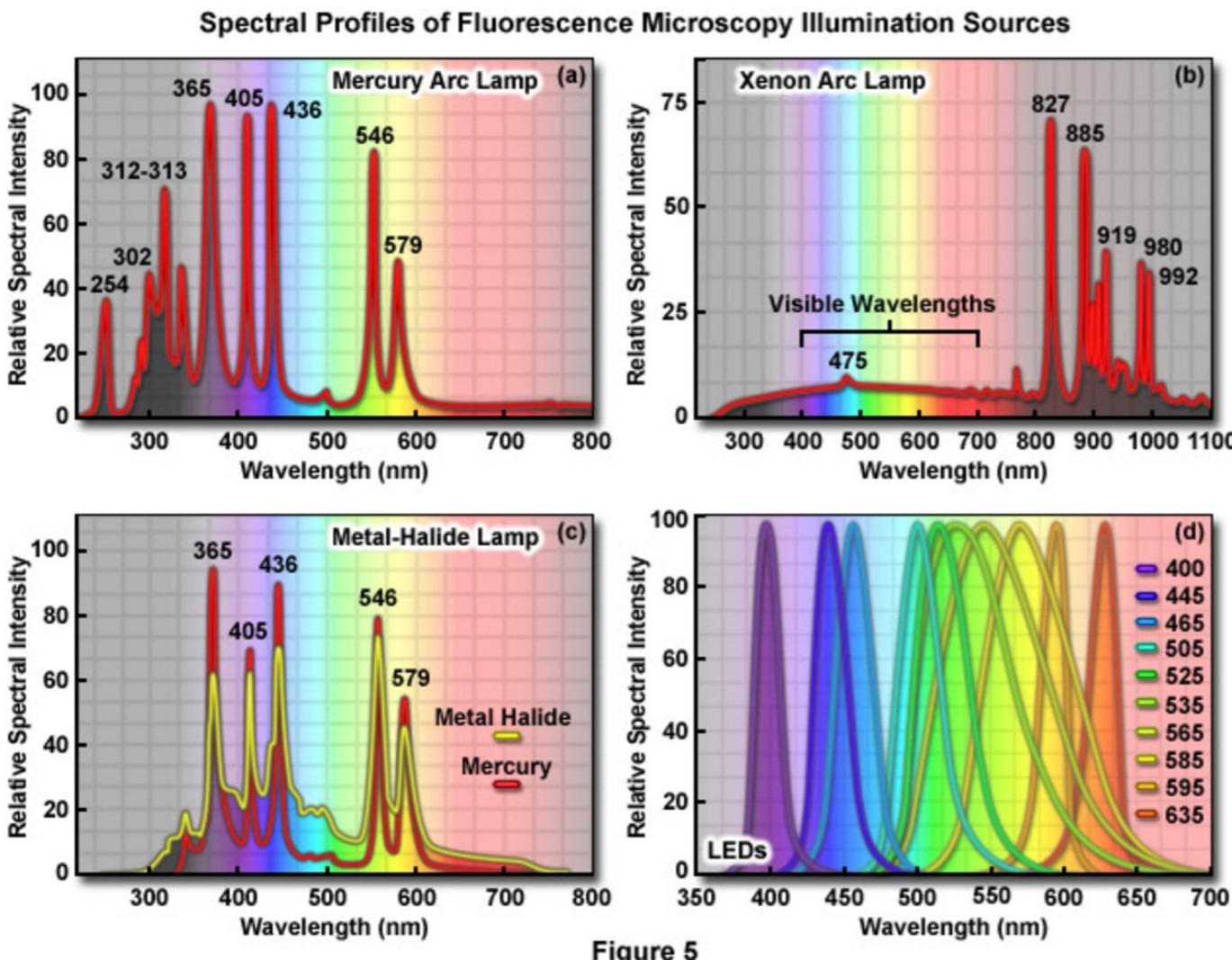
Jablonski diagram



Emission filter

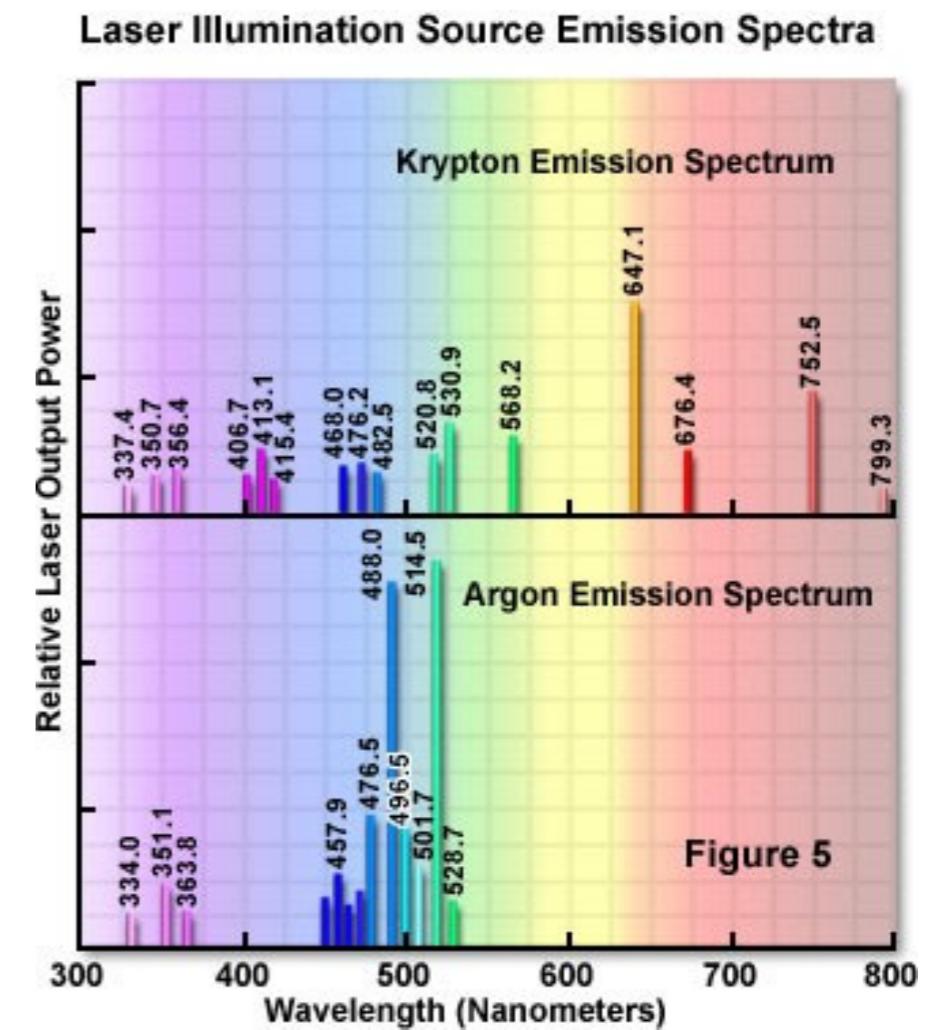
# Excitation light sources

## Broad band light sources



**Broadband**  
sources that require the cost and complexity of extra filters, and presents significant cross-talk challenges when detecting multiple fluorochromes

## Narrow band light sources (LASER)

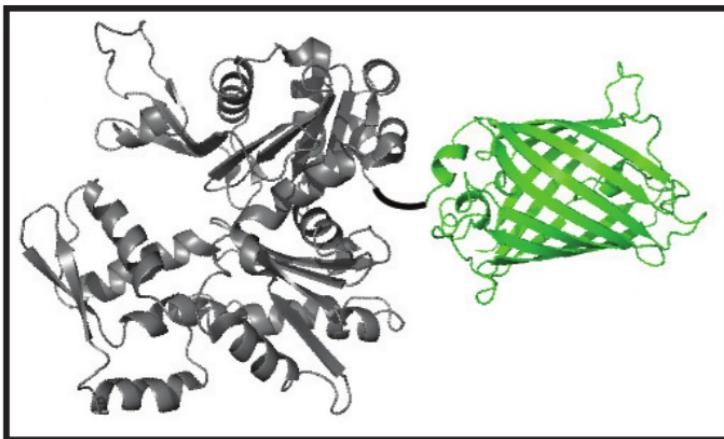


High brightness, stability, longevity and narrow spectral bandwidth, narrow beam divergence, high degree of spatial and temporal coherence, and well-defined polarization properties ...

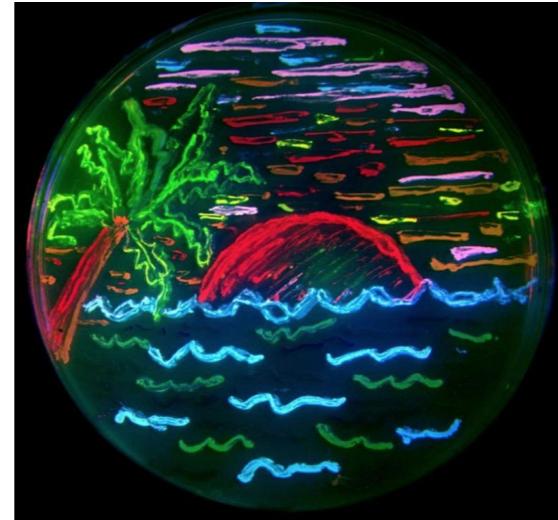
# Fluorophores

→ Specific labelling of a biomolecule of interest

## Fluorescent protein fusion



Fluorescent protein



petri dish

Genetic encoding  
Live imaging

## Synthetic dyes

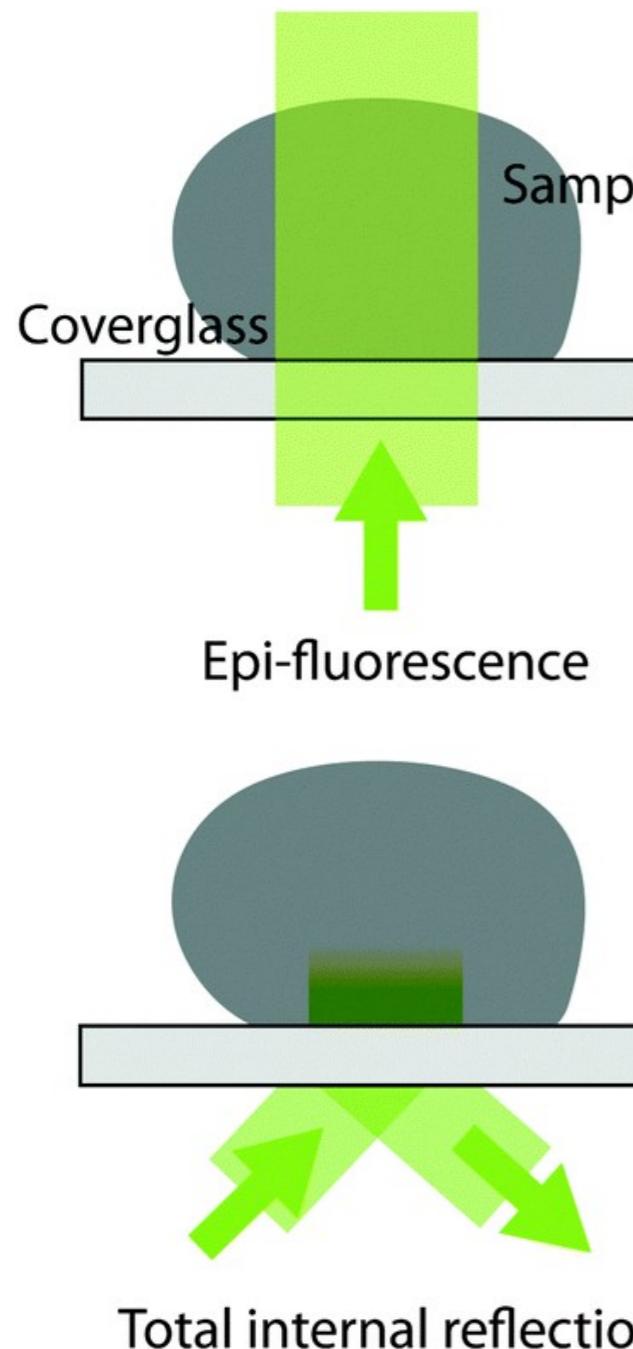


Small size  
Brightness  
Stability  
Bioconjugaison strategies

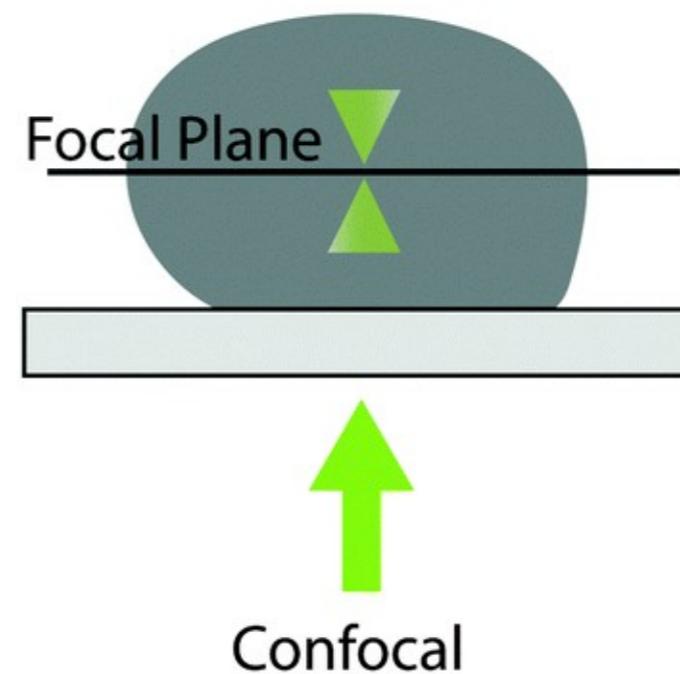
## Quantum dots, fluorescent nanodiamonds, etc...

# Excitation modes

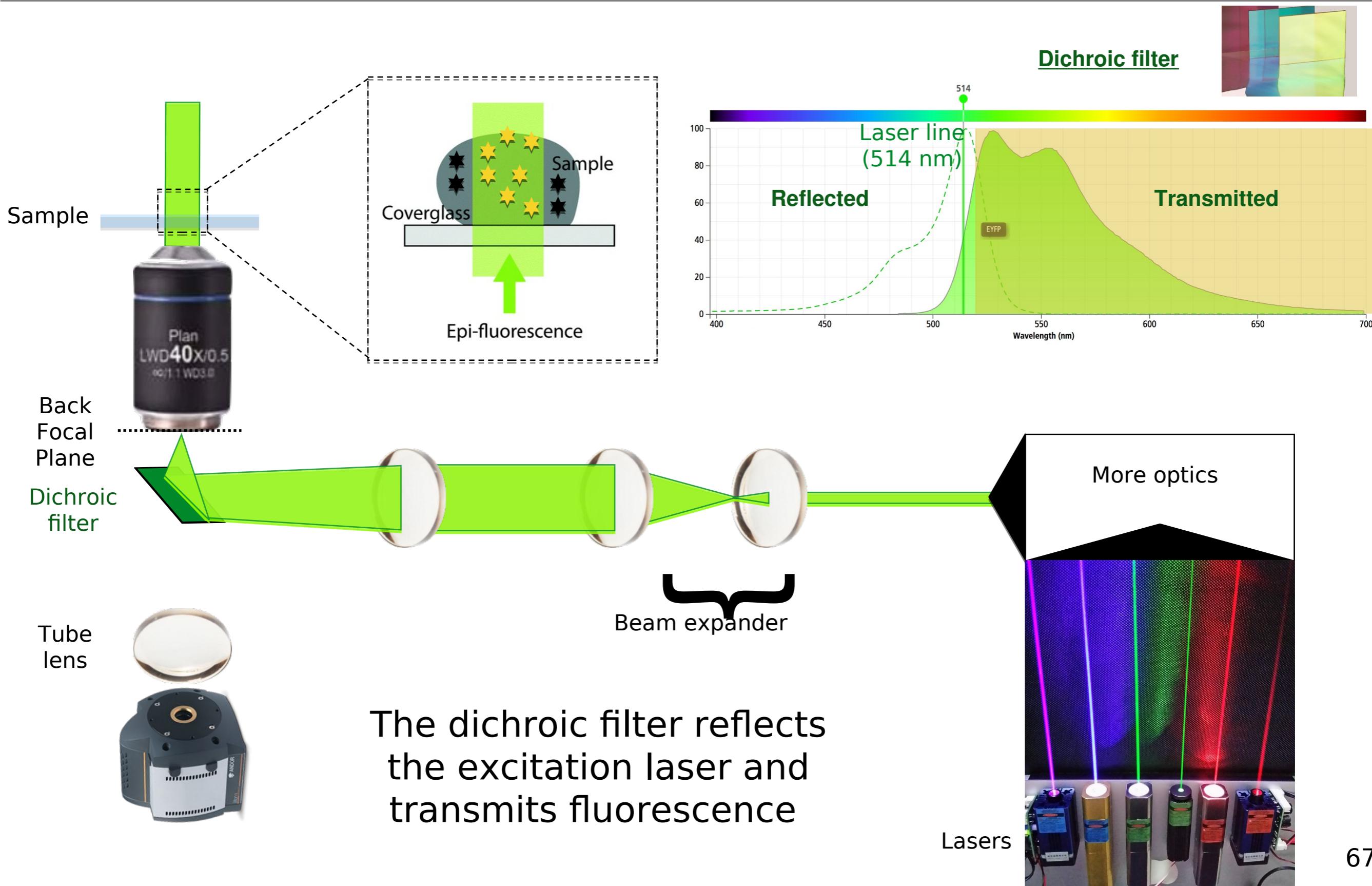
## Wide field imaging



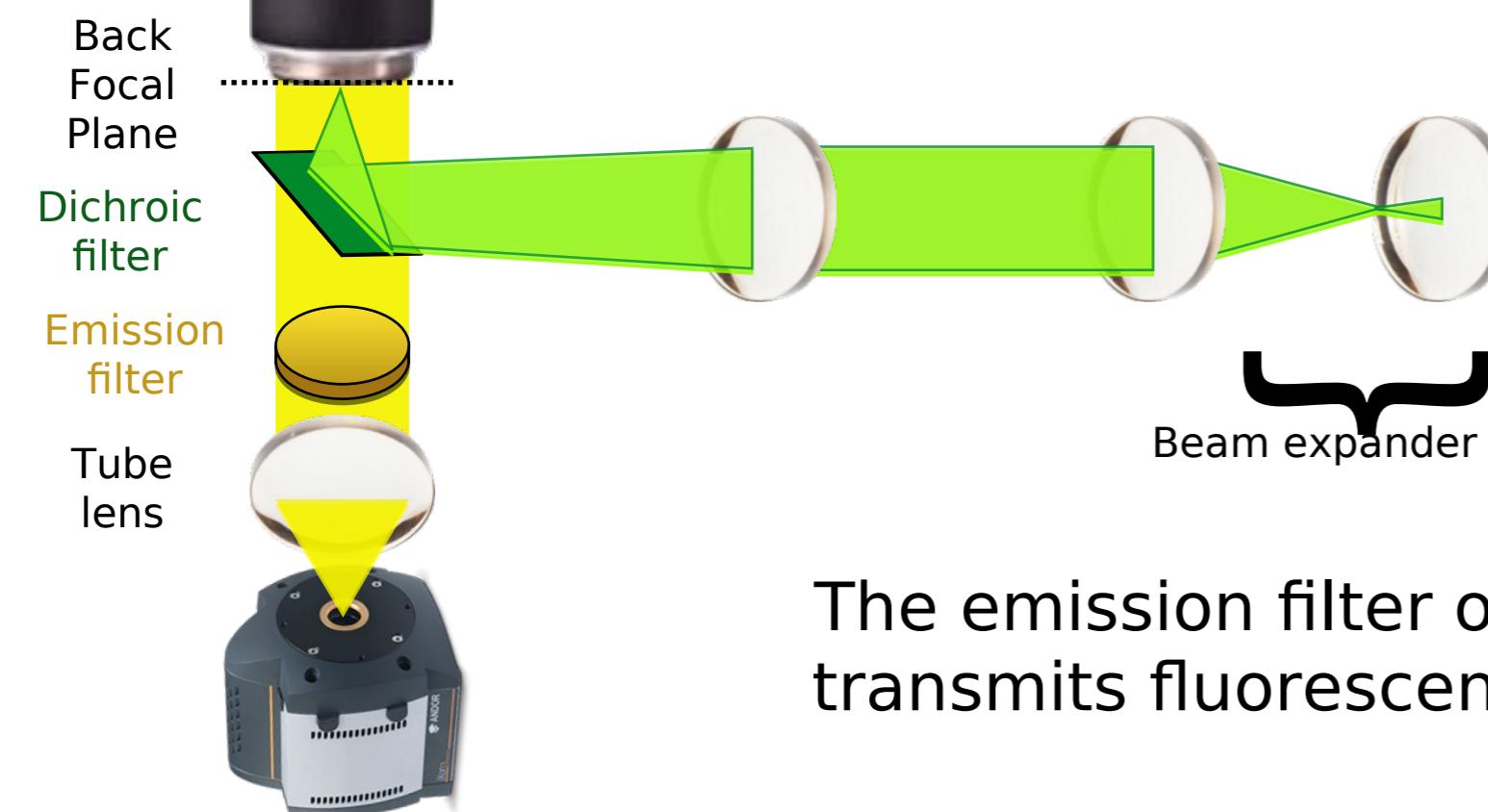
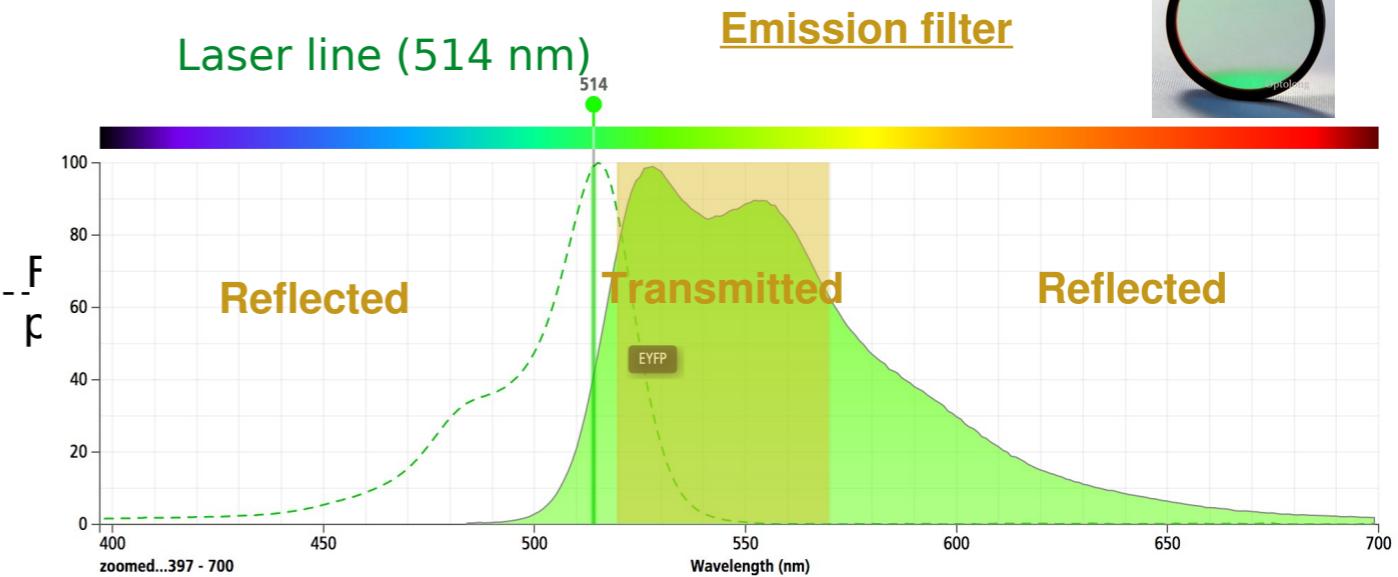
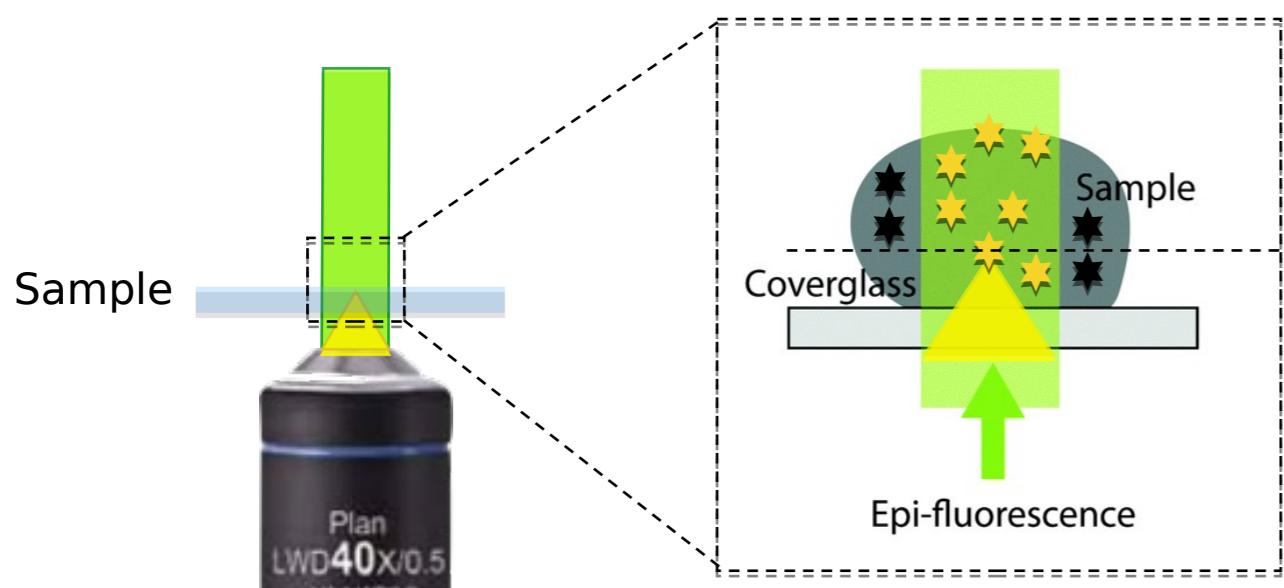
## Laser scanning imaging



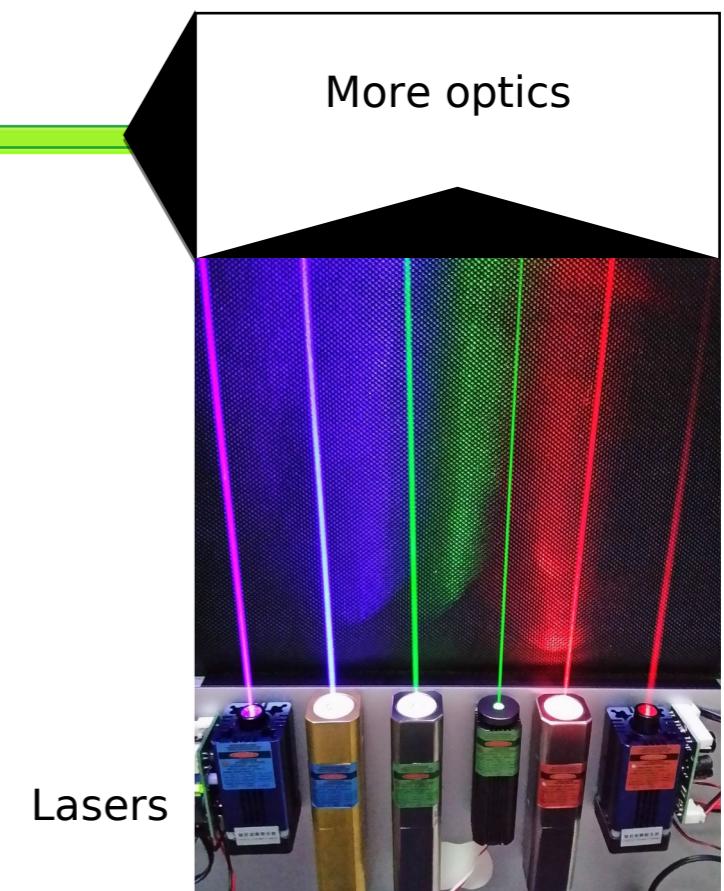
# Wide field fluorescence - Epifluorescence



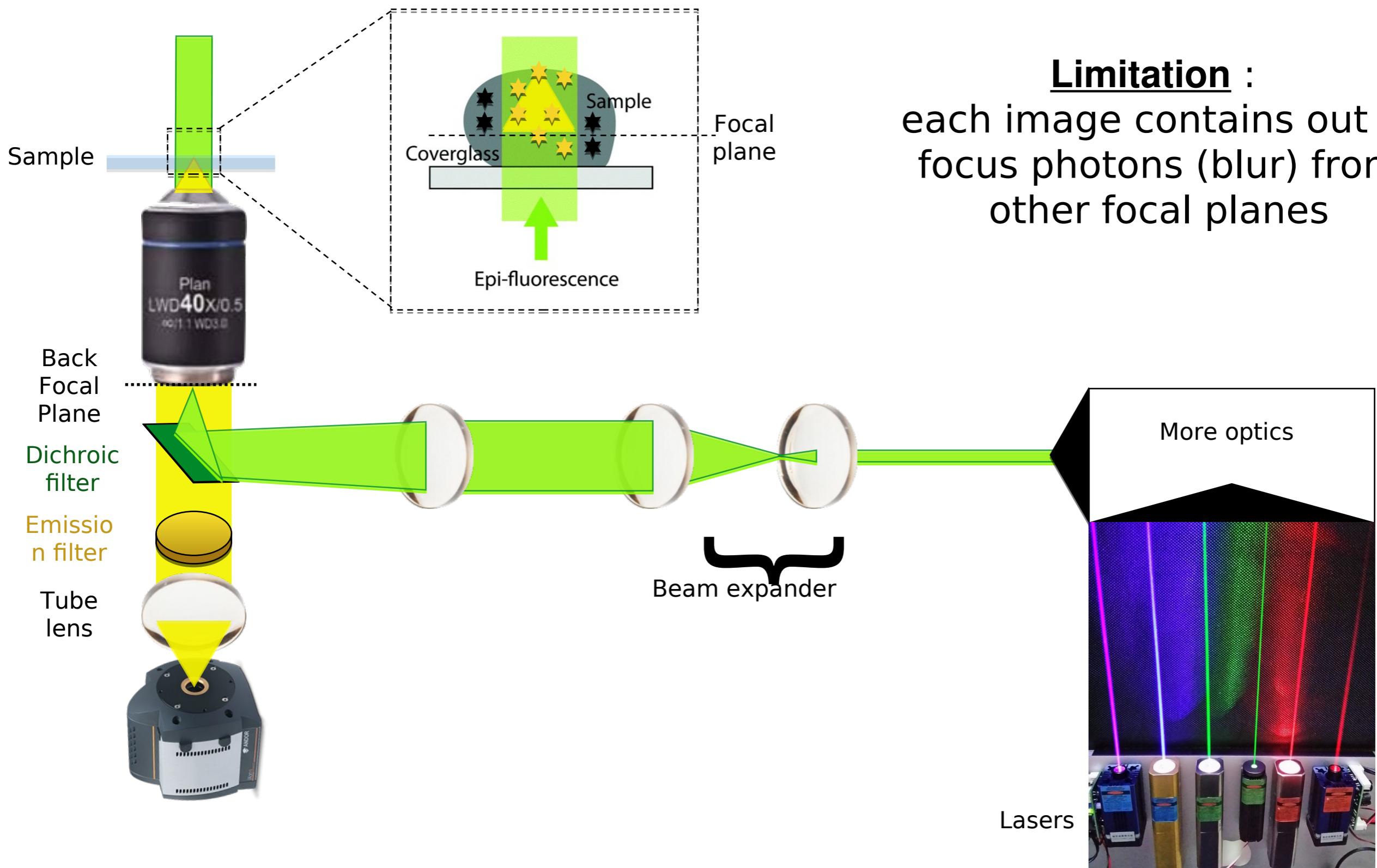
# Wide field fluorescence - Epifluorescence



The emission filter only transmits fluorescence



# Wide field fluorescence - Epifluorescence

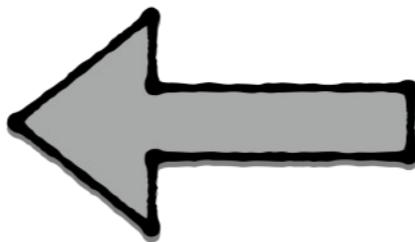


## Limitation :

each image contains out of focus photons (blur) from other focal planes

# Wide field fluorescence - Epifluorescence

**TIRF :**  
Confine excitation within  
the imaging volume



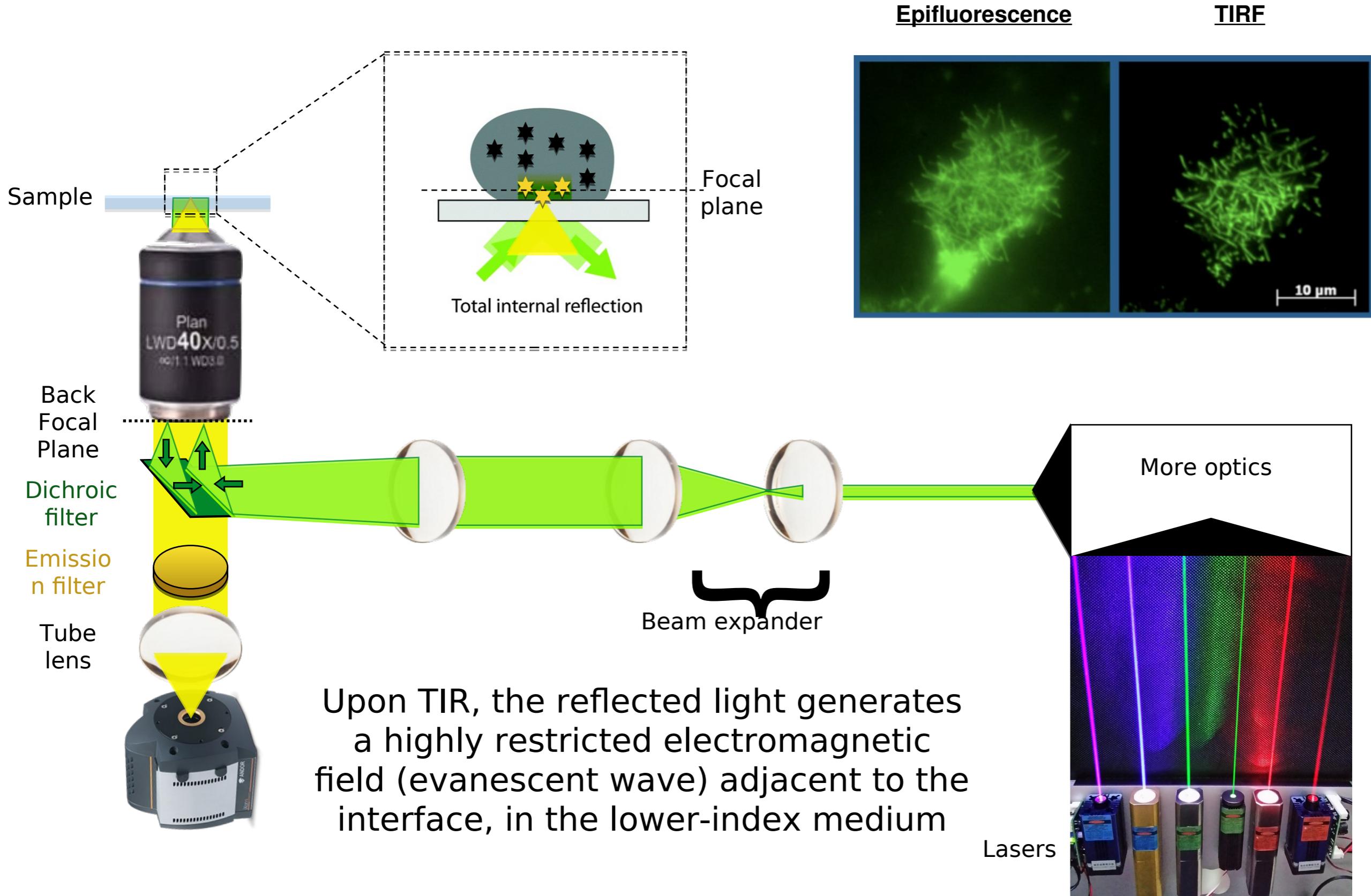
**Limitation :**  
each image contains out of  
focus photons (blur) from  
other focal planes



**Confocal :**  
Confine the collection of  
fluorescence within the  
imaging volume

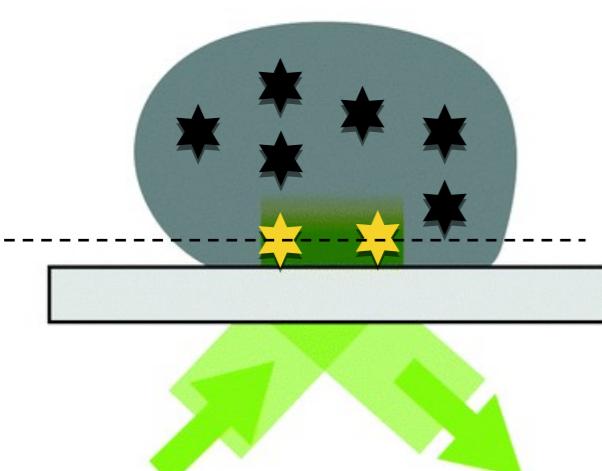
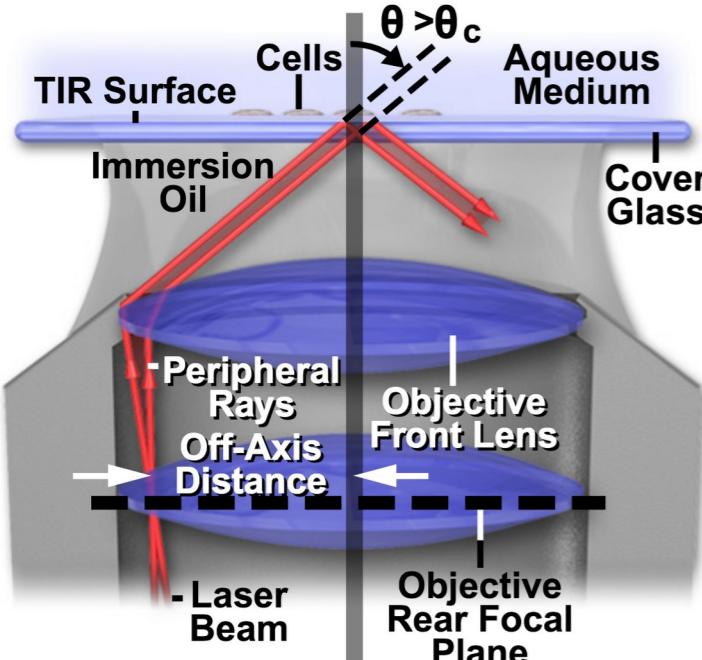
# Wide field fluorescence - TIRF\*

\* Total Internal Reflection Fluorescence



# Wide field fluorescence - TIRF\*

\* Total Internal Reflection Fluorescence



Total internal reflection

## Total Internal Reflection:

$$\theta_c = \sin^{-1}(n_2/n_1)$$

$$n_1 = 1.512 \text{ (coverslip)}$$
$$n_2 = 1.33 \text{ (aqueous buffer)}$$

$$\Rightarrow \theta_c = 61.6^\circ$$

⇒ oil immersion objective required (typically with NA>1.4)

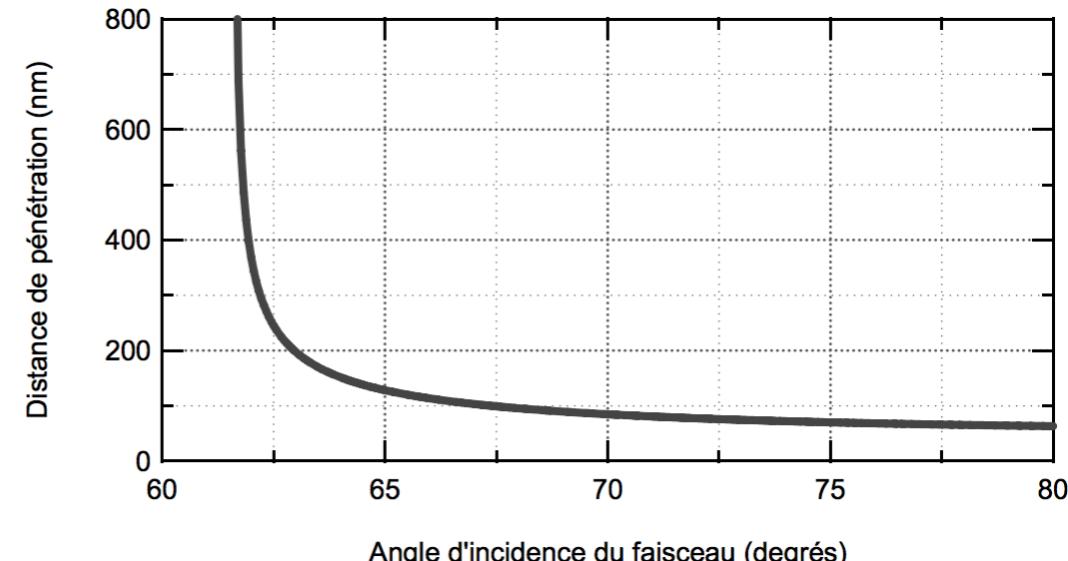
⇒ evanescent wave penetration depth depends on:

- \_ wavelength
- \_ Incident angle
- \_ index media

$$d = \frac{\lambda}{4\pi} (n_1^2 \sin^2 \theta - n_2^2)^{-1/2}$$

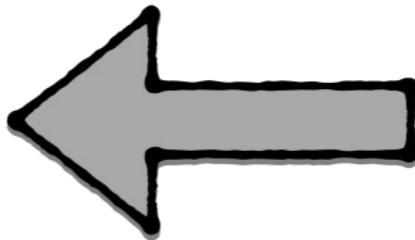
⇒ evanescent wave intensity decays exponentially from the surface:

$$I(z) = I(0)e^{-z/d}$$

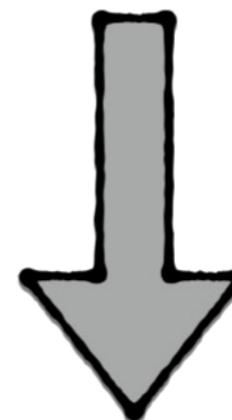


# Wide field fluorescence - Epifluorescence

TIRF :  
Confine excitation within  
the imaging volume

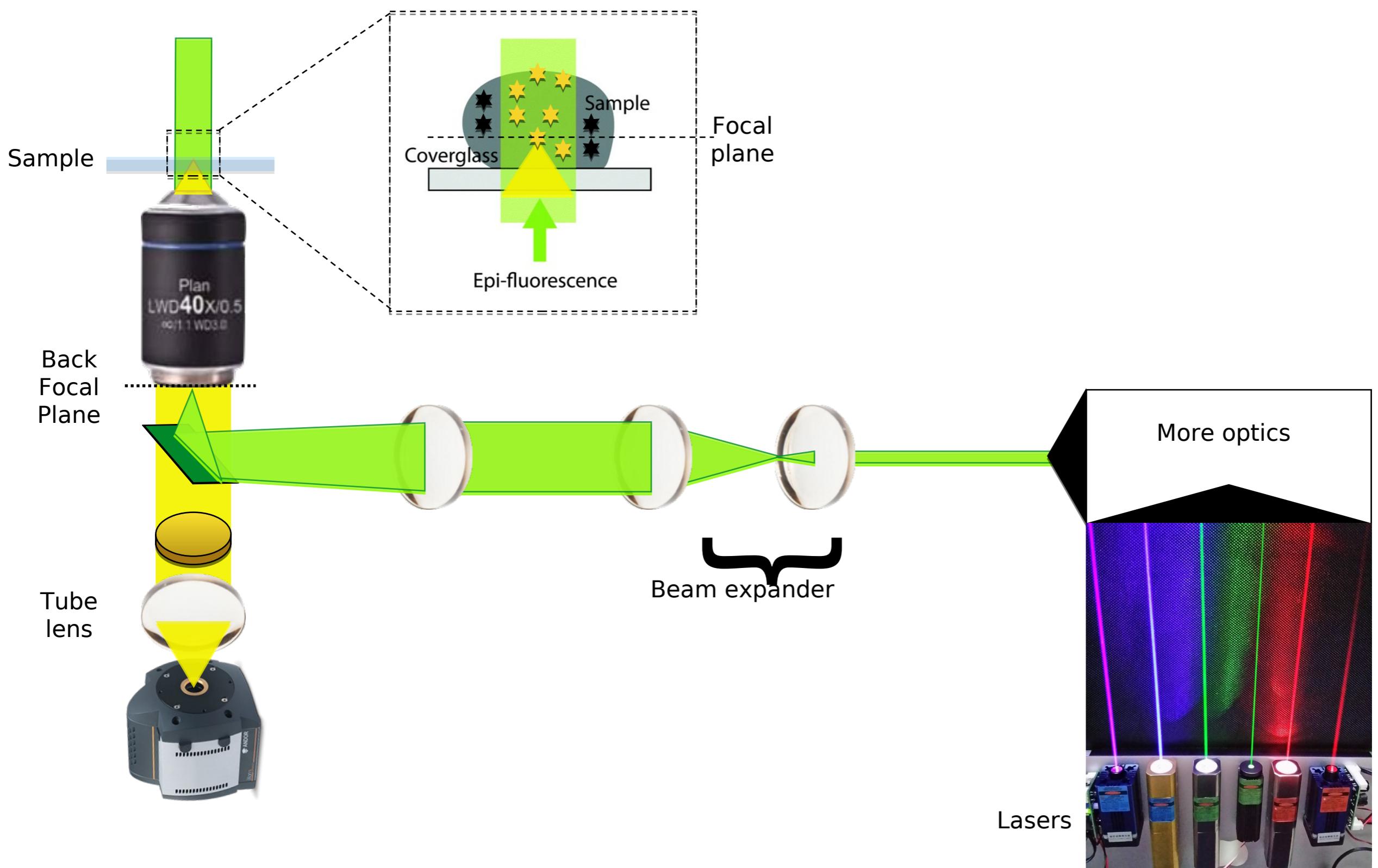


Limitation :  
each image contains out of  
focus photons (blur) from  
other focal planes

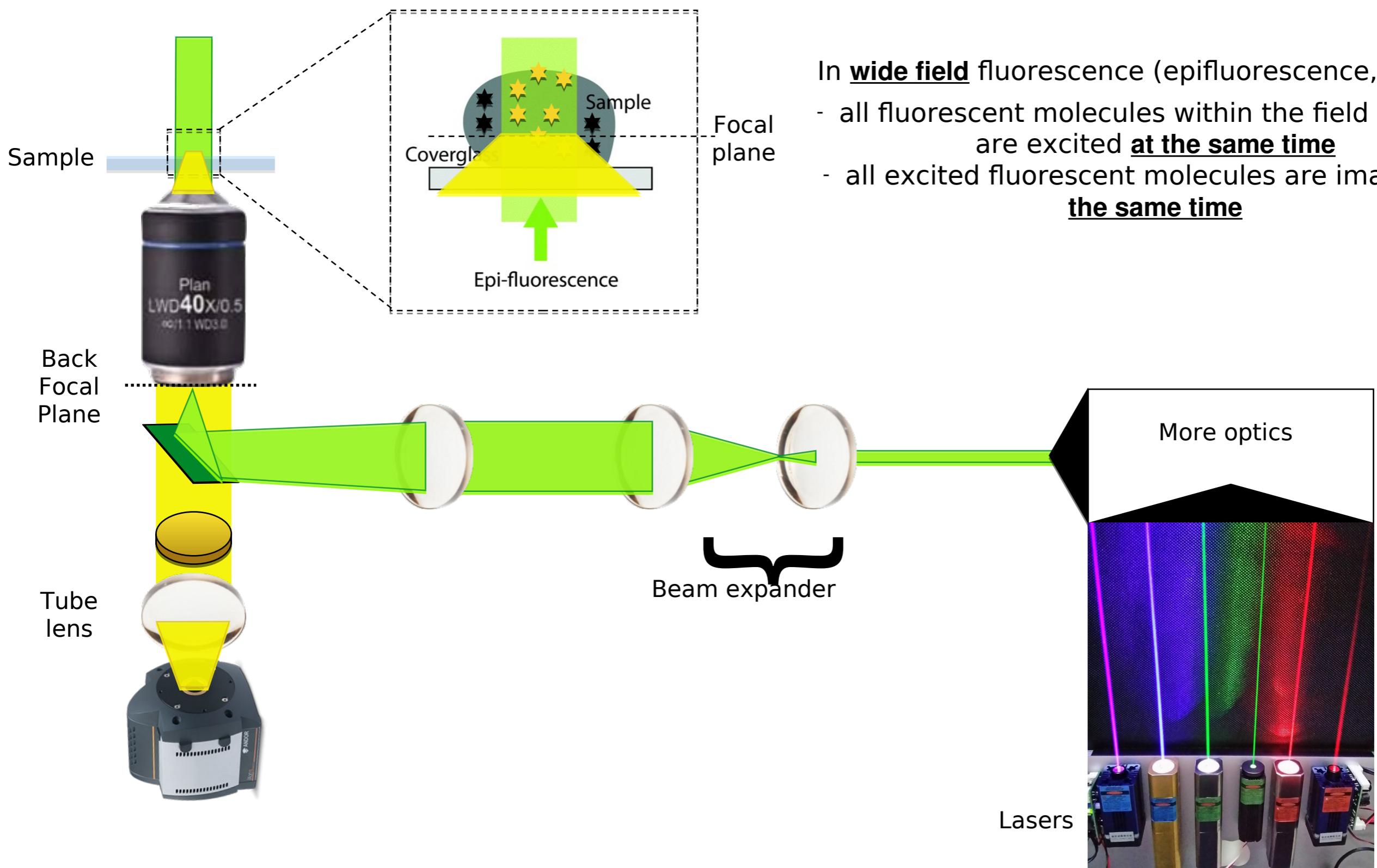


Confocal :  
Confine the collection of  
fluorescence within the  
imaging volume

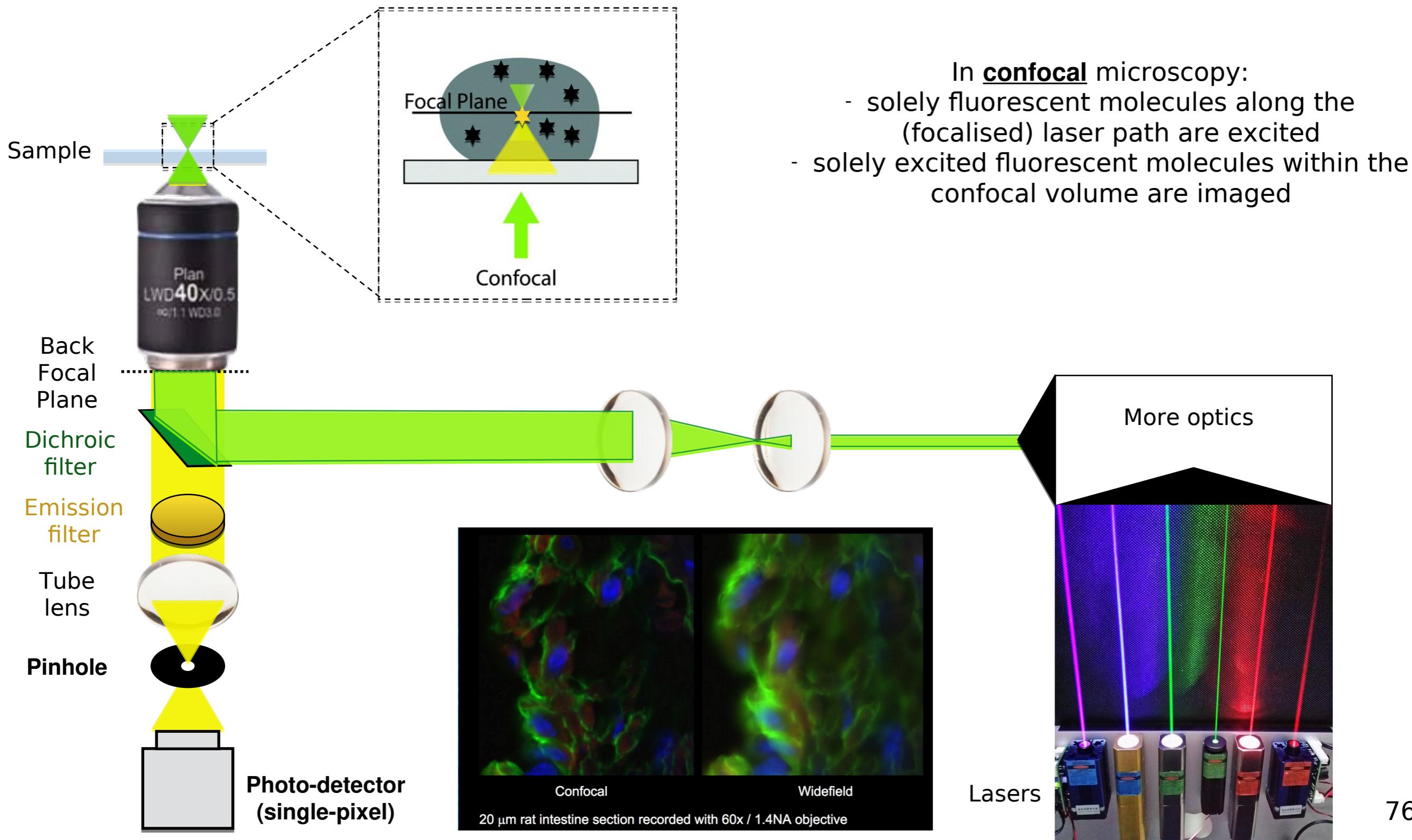
# Wide field fluorescence



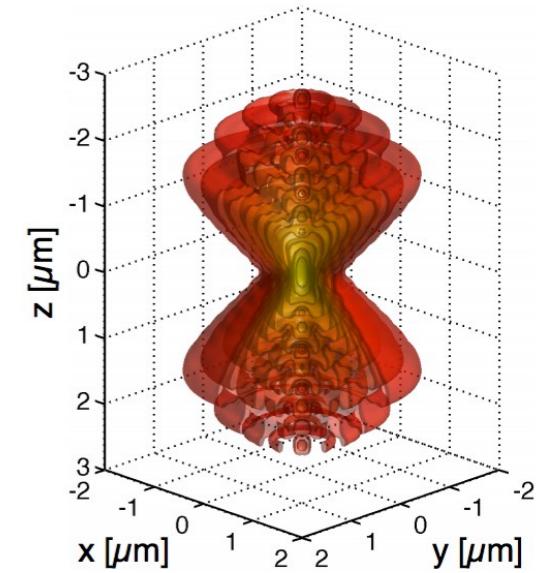
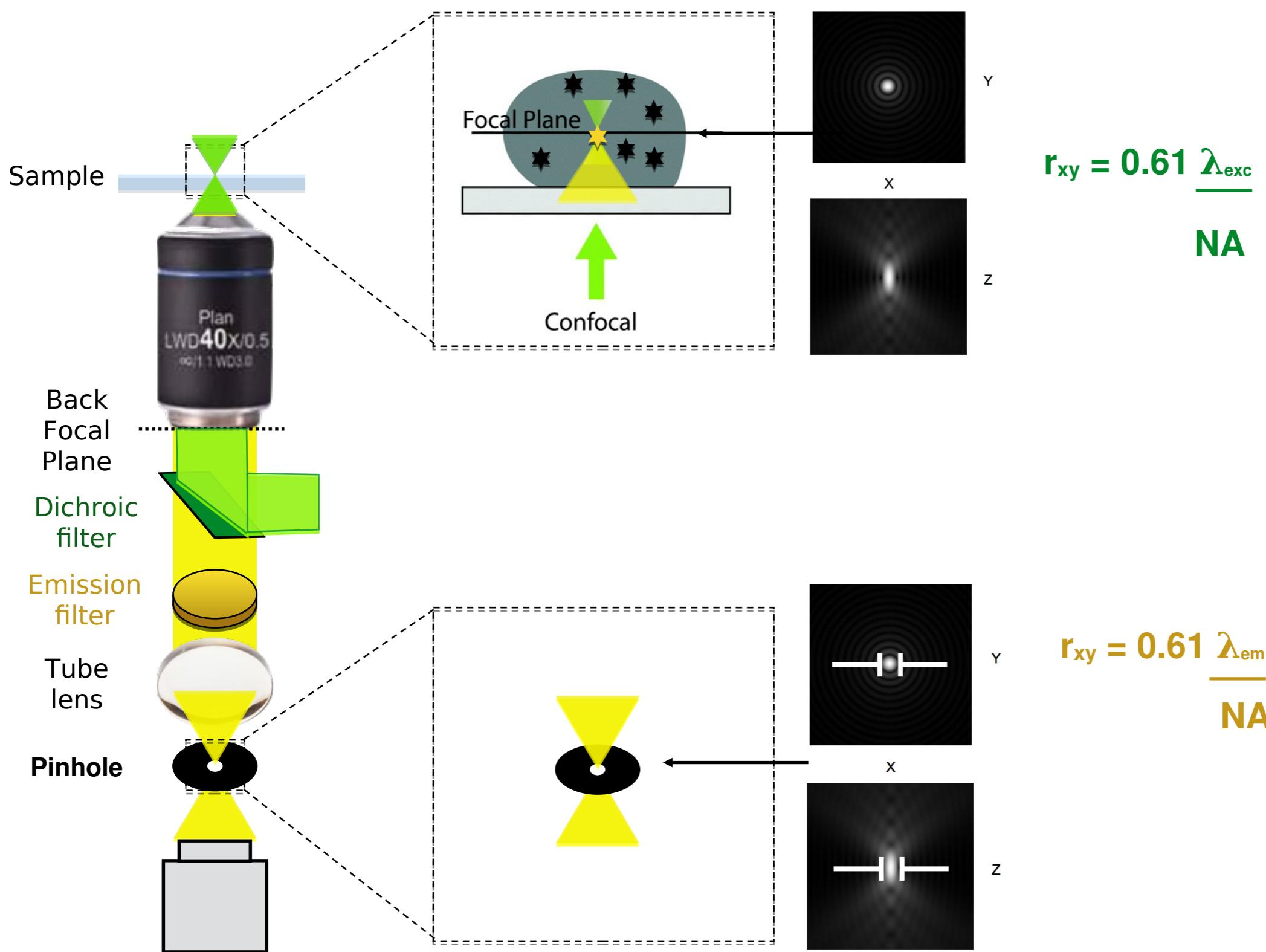
# Wide field fluorescence



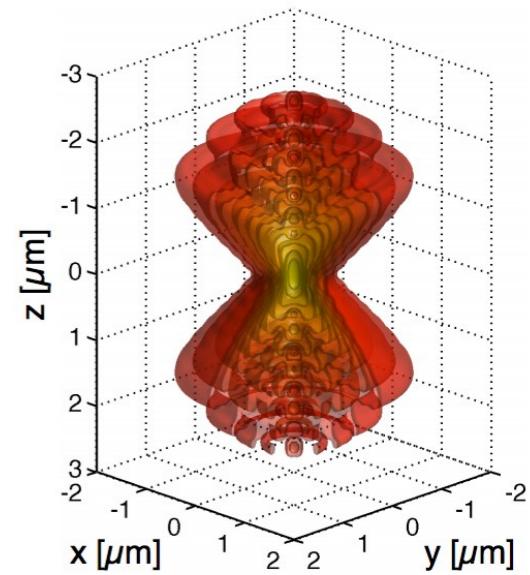
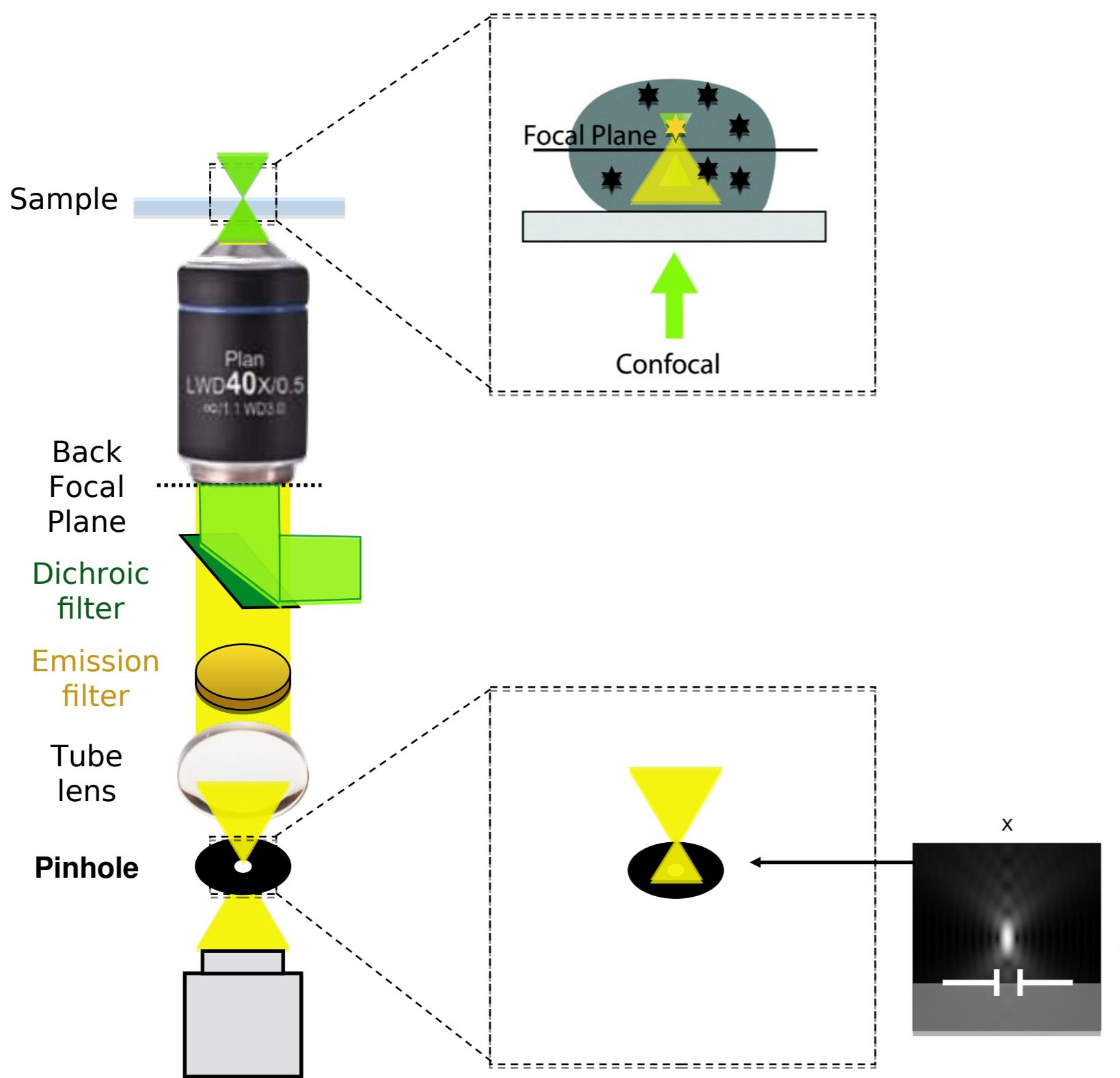
# Confocal microscopy



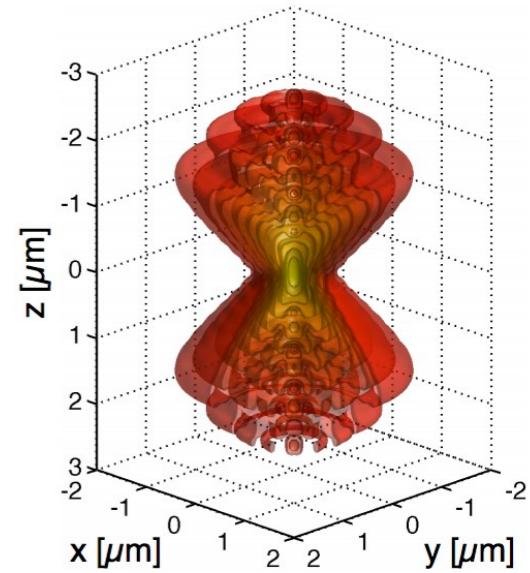
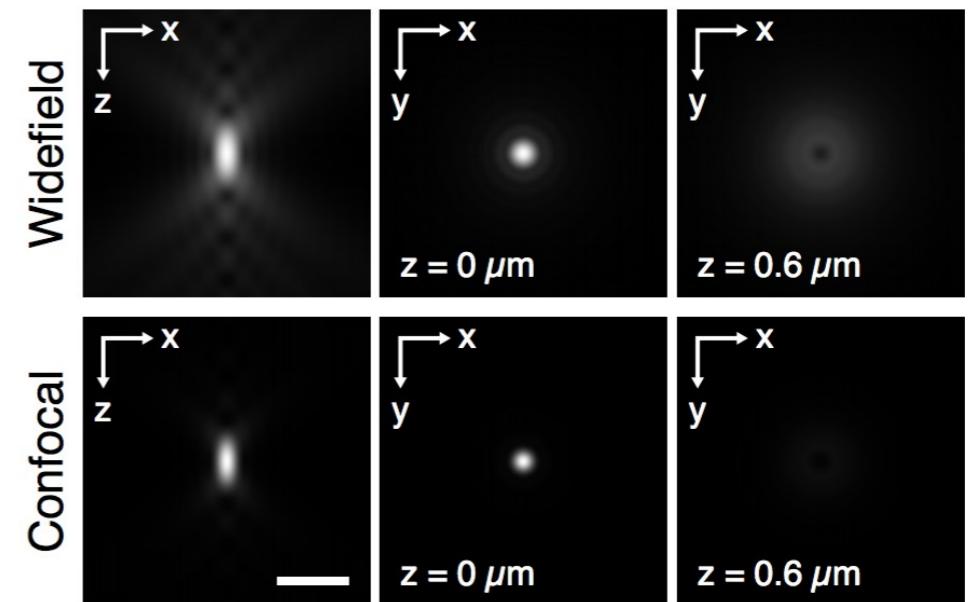
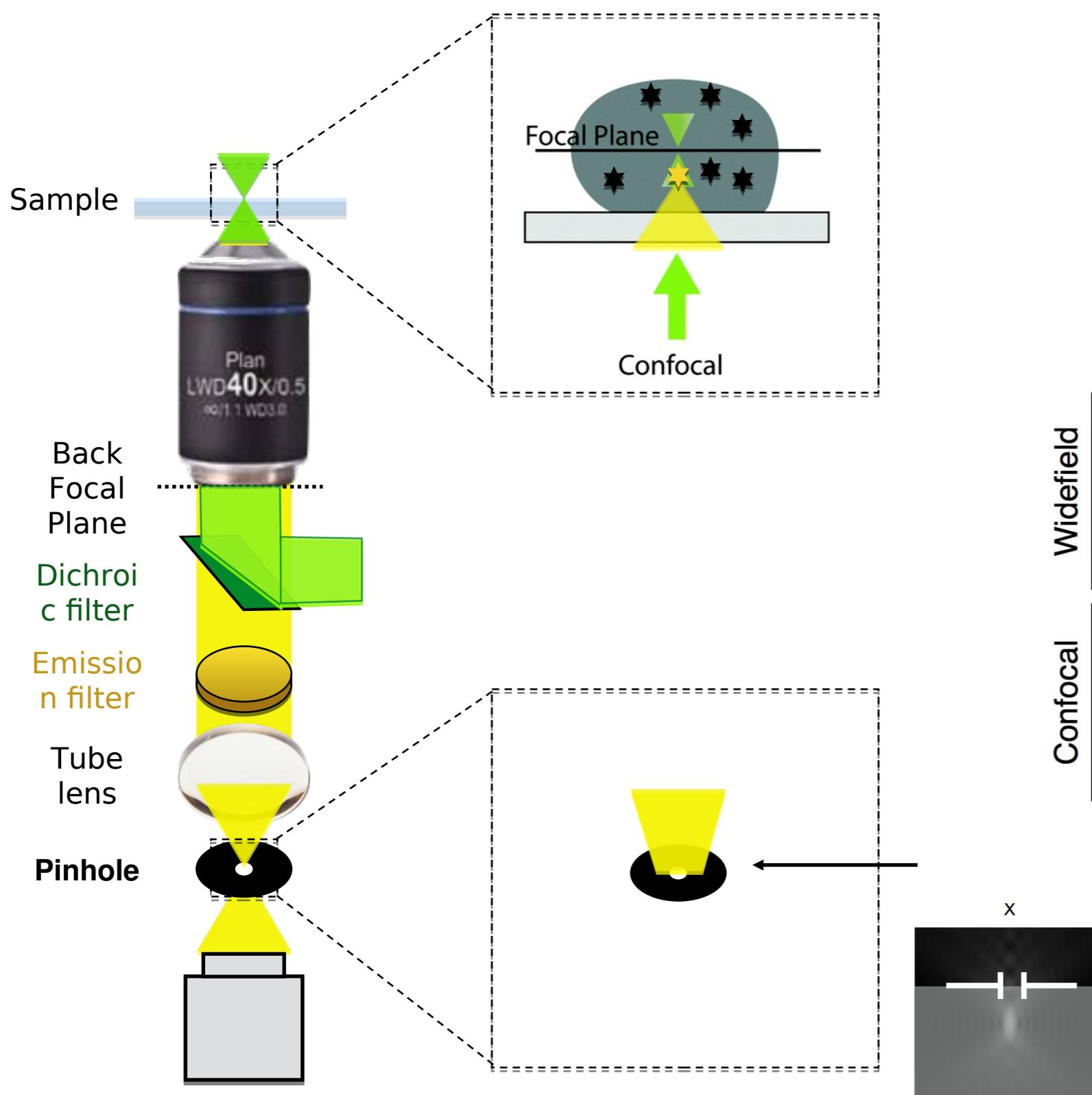
# Confocal microscopy



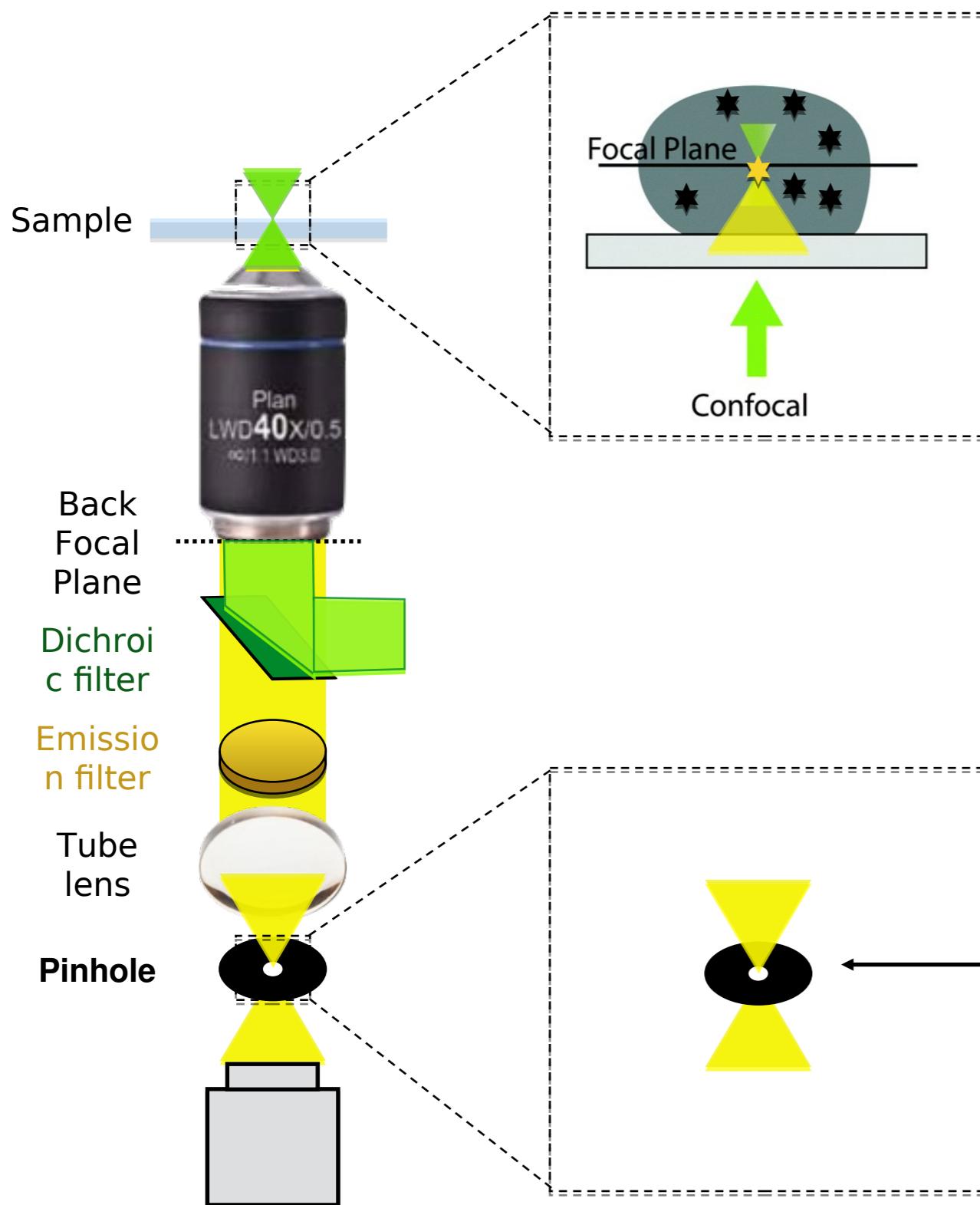
# Confocal microscopy



# Confocal microscopy



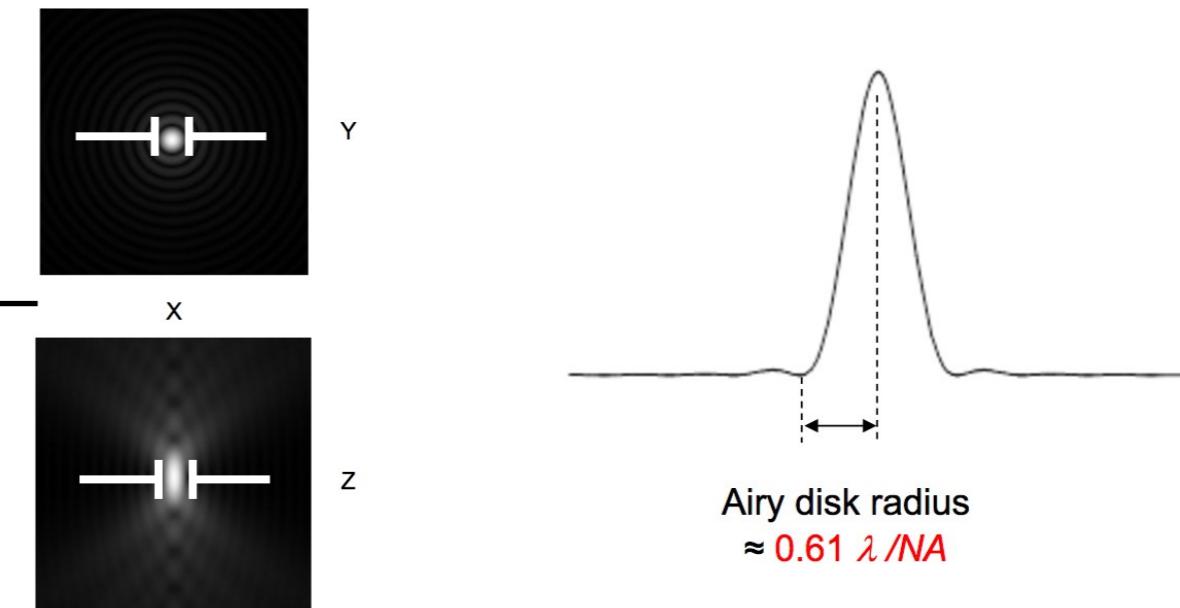
# Confocal microscopy



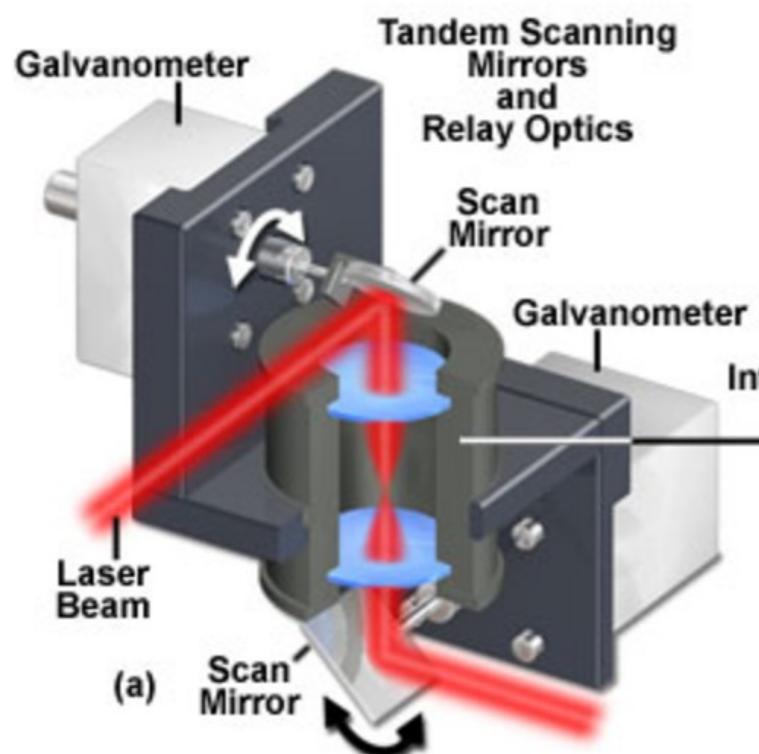
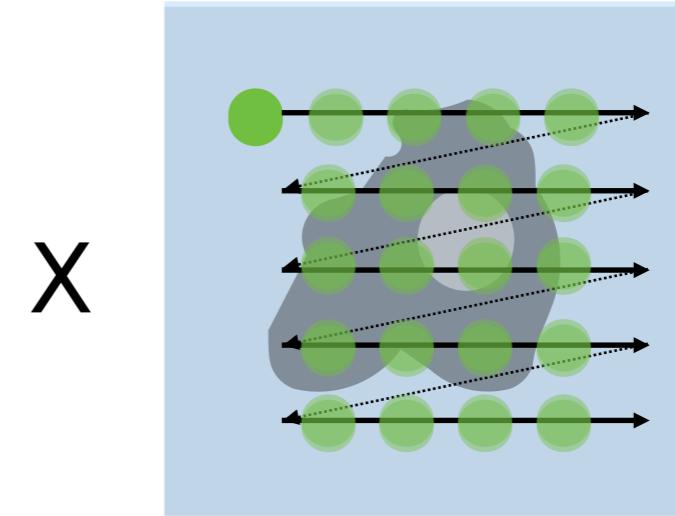
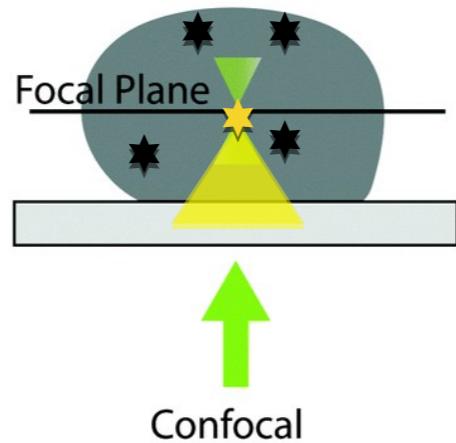
**How to choose the pinhole size?**

Pinhole diameter = microscope resolution x microscope magnification  
x2

100X, 1.4 NA : resolution = 220 nm  $\rightarrow$  Pinhole diameter = 44  $\mu\text{m}$



# Laser scanning - Confocal microscopy



For a  $512 \times 512$  image, you need to take  $512 \times 512 = 262\,144$  measurements

1 measurement (1 pixel)  $\sim 1\text{-}10 \mu\text{s}$

The brighter the sample, the faster you can scan. In practice it takes a few seconds to acquire an image (few tens of ms for wide-field)

# Videos and images galleries

<https://www.microscopyu.com/galleries/fluorescence>

<https://www.microscopyu.com/galleries/confocal/laser-scanning>

# Keep in mind

Both excitation and emission light goes through the objective lens

In wide field microscopy the excitation light is focalised at the entrance (in the BFP) of the objective.

In confocal microscopy the excitation light is focalised at the output of the objective.

In widefield microscopy emission light is collected simultaneously from the different regions of the sample

In confocal microscopy emission light is collected sequentially from the different regions of the sample

Confocal microscopes remove out of focus light from the image (better Z sectioning and more contrasted images), that the widefield system will not

# Outline - part5

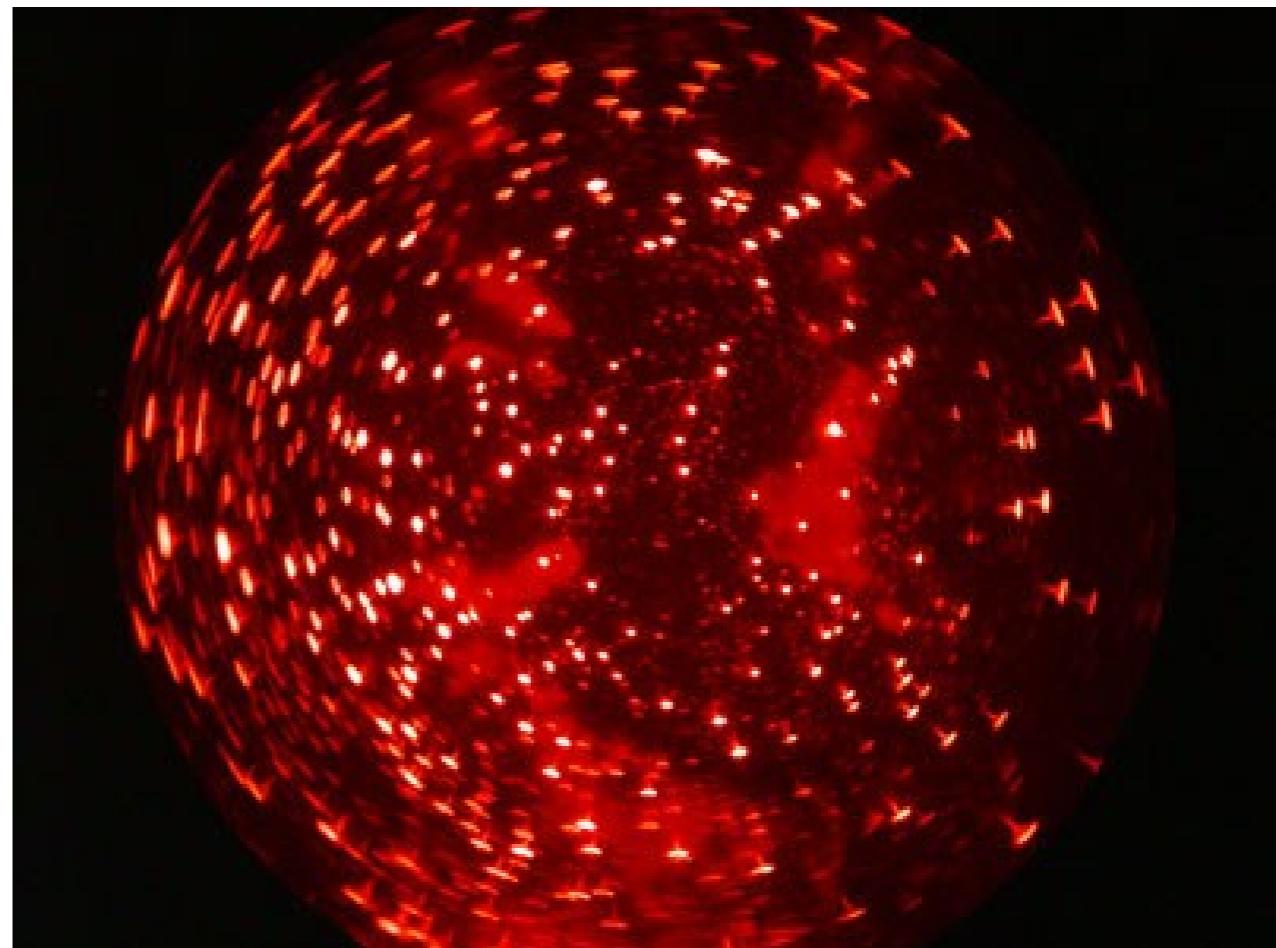
Chromatic aberration

Spherical  
distortion

field curvature

astigmatism

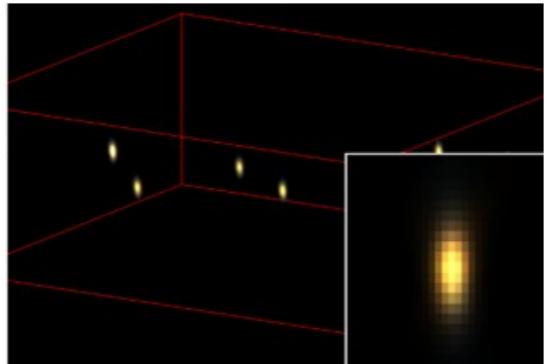
coma



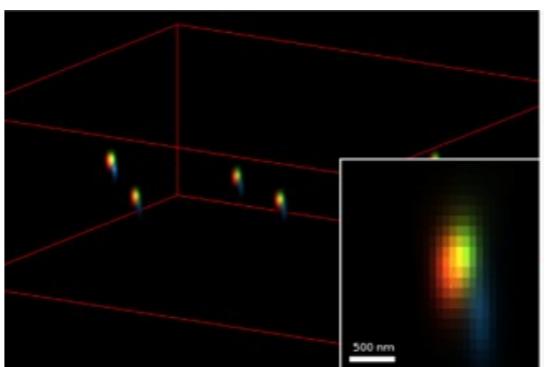
# Chromatic aberrations

- refractive indices for all optical glasses vary with wavelength

No chromatic aberrations



With chromatic aberrations



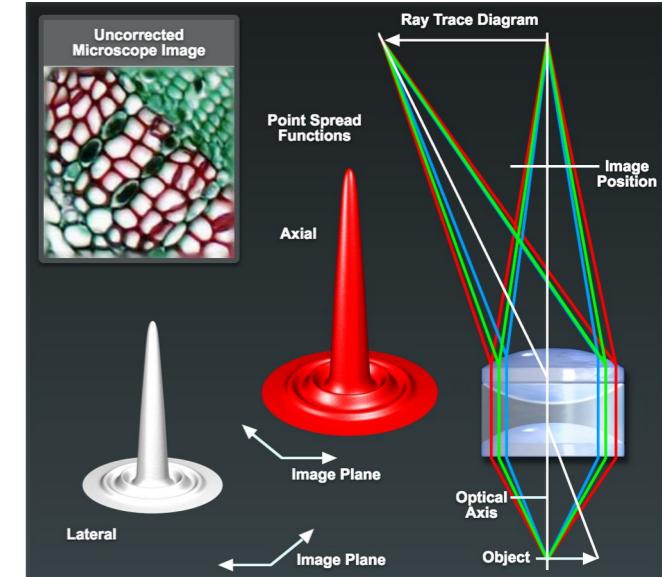
- Shifts between wavelengths

BOTH Lateral AND Axial

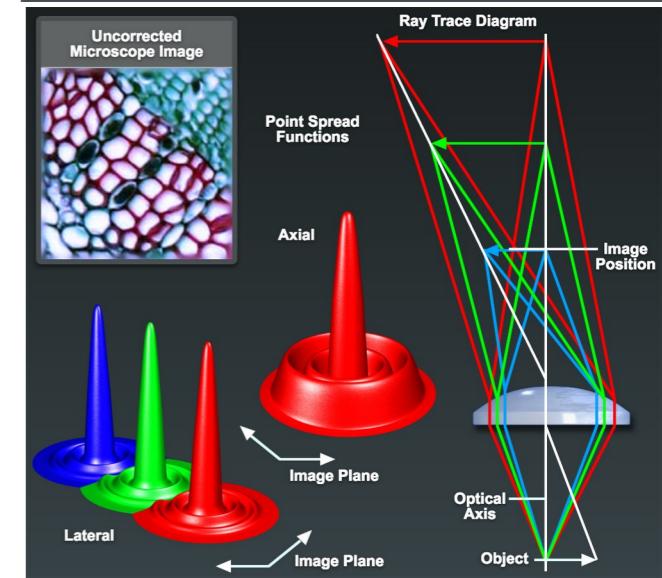
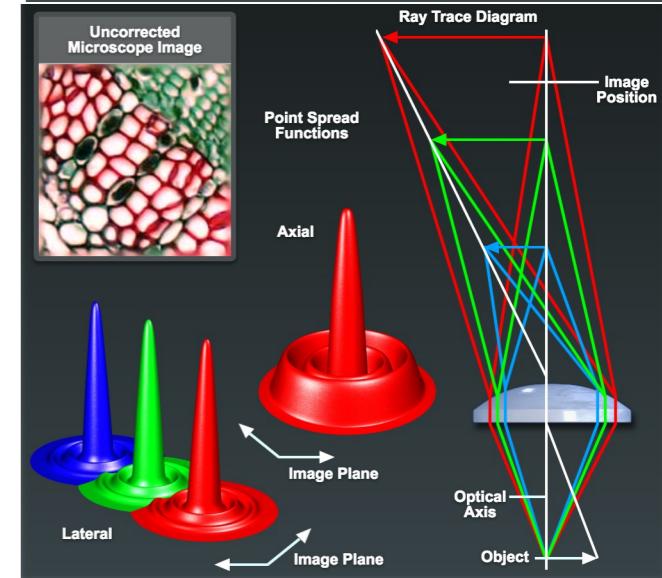
- Because magnification is inversely proportional to focal length, the variation of focal length with wavelength produces a corresponding dependence of magnification on wavelength.

- Invest in a corrected objective (Achro and Achromat, Apochromat, FI, Fluar, Fluor, Neofluar, or Fluotar )

No chromatic aberrations



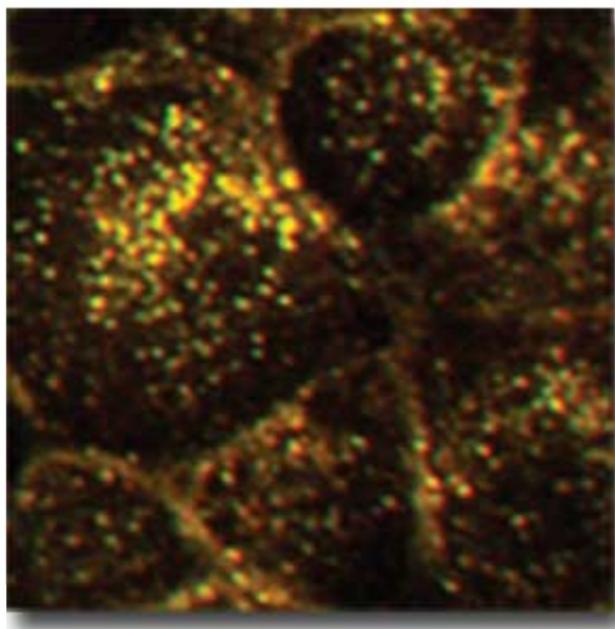
With chromatic aberrations (different focus)



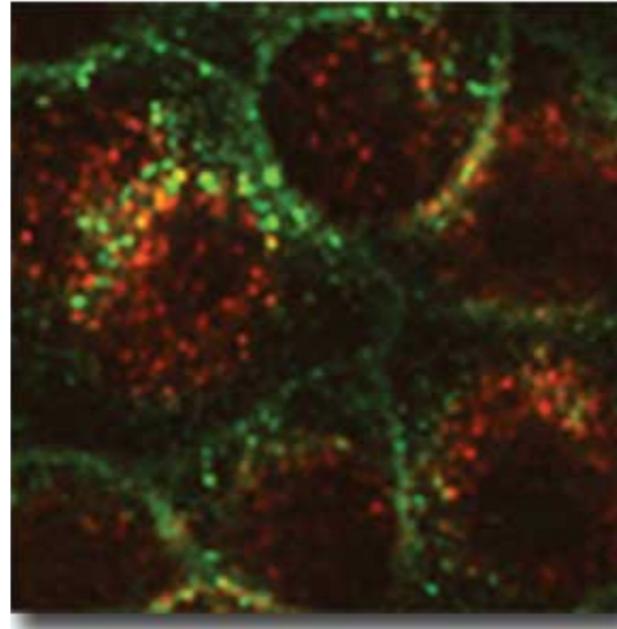
# Chromatic aberrations

Ex : Cells labeled with endosomes conjugated to both fluorescein (green) and rhodamine (red)

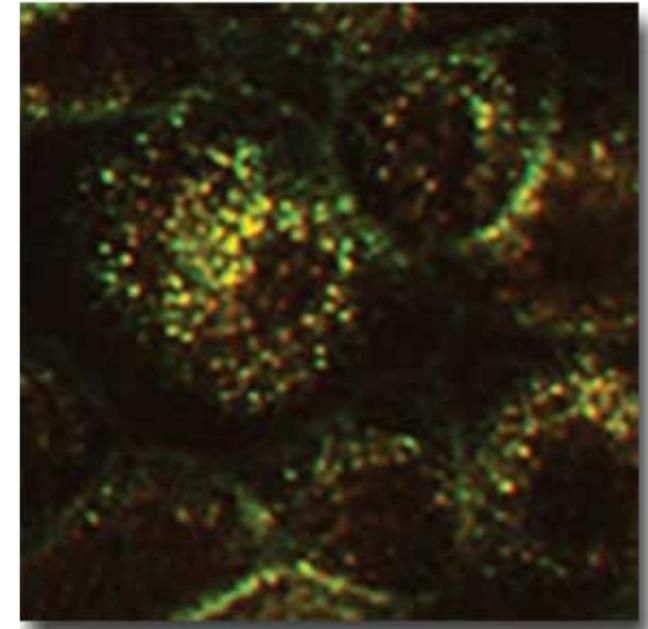
What you would expect without chromatic aberrations : co-localisation



What you get with chromatic aberrations : **NO** co-localisation



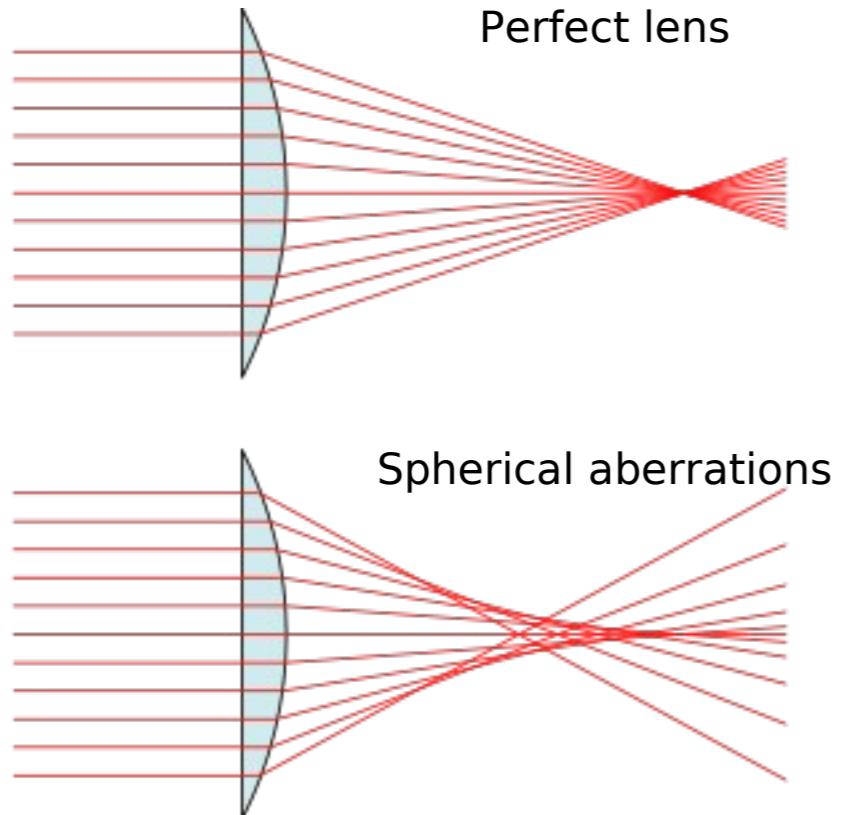
What you get with chromatic aberrations and Z chromatic shift compensation : **partial** co-localisation



Chromatic axial and lateral shifts can be measured using fiducial markers to realign fluorescence images

# Spherical aberrations

- Peripheral rays and axial rays have different focal points
- Image appears fuzzy, poorer contrast and resolution



- Invest in a corrected objective
- Use an objective with a correction collar
- change the immersion media index
- fine tune the oil immersion media index

PSF expands and spreads out asymmetrically from the center along the optical axis

Focal Planes with Spherical Aberration

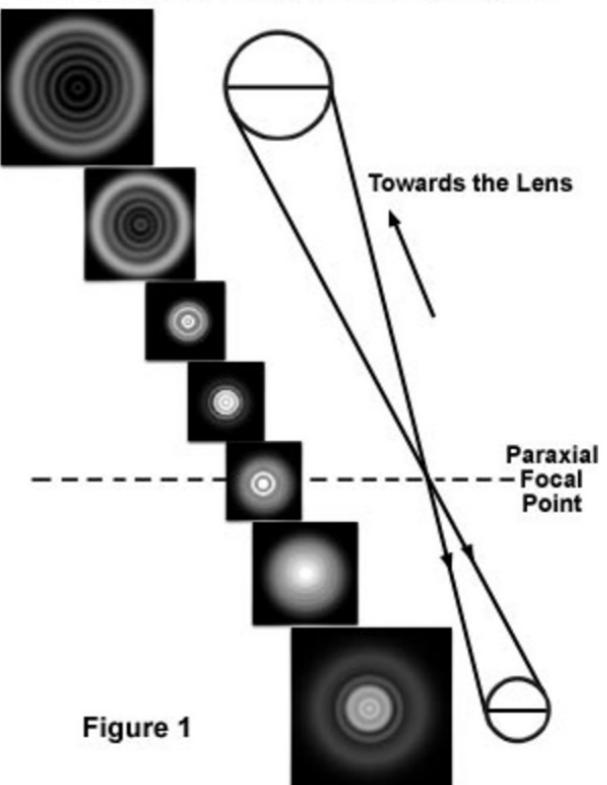
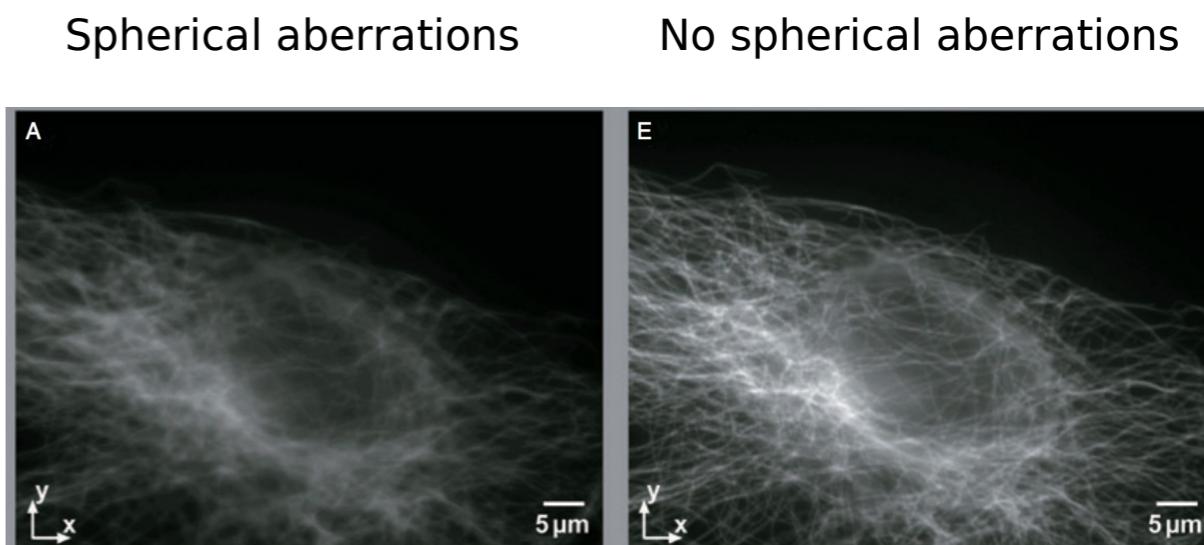


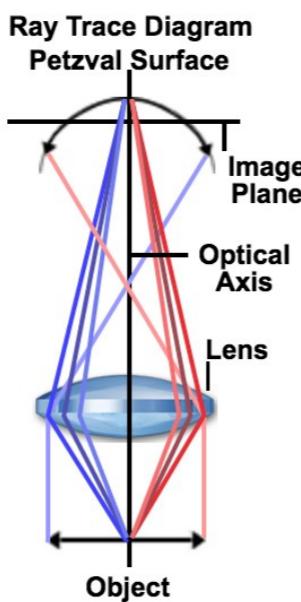
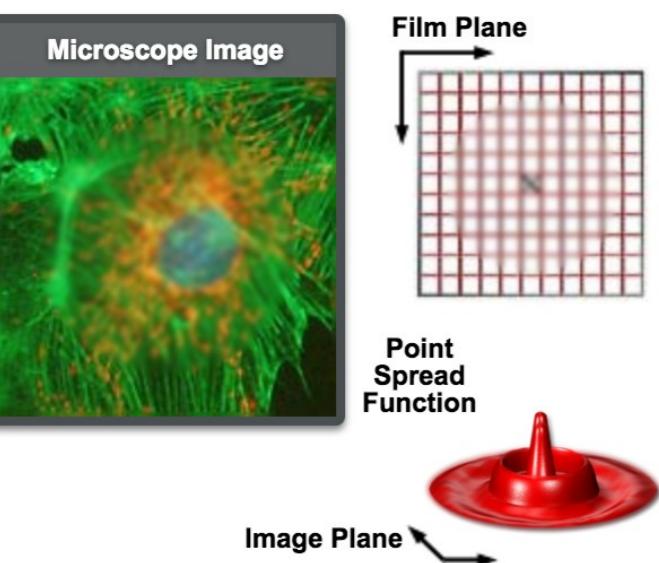
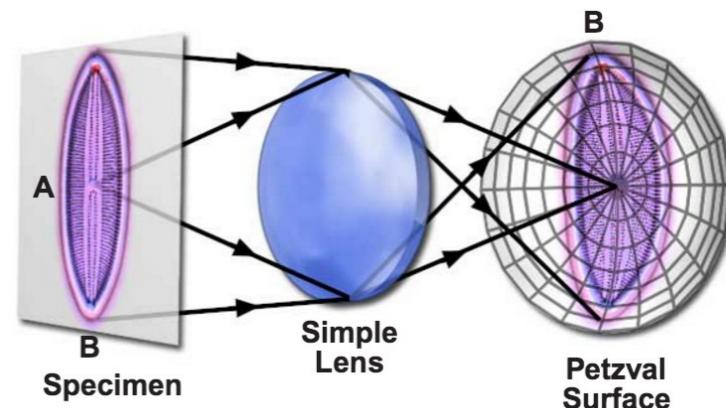
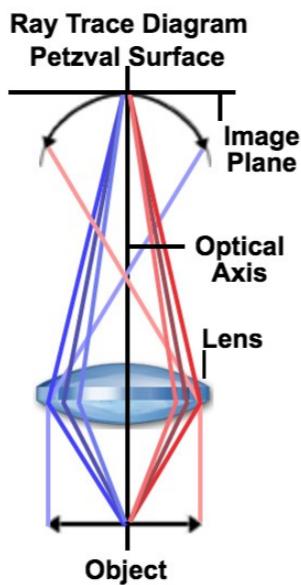
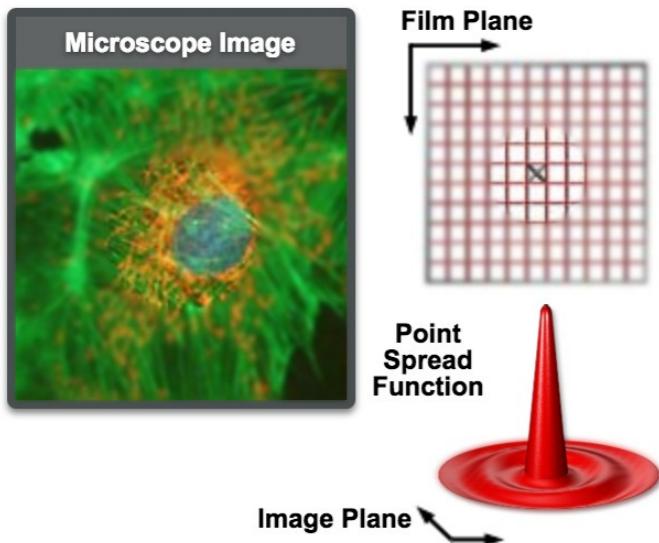
Figure 1



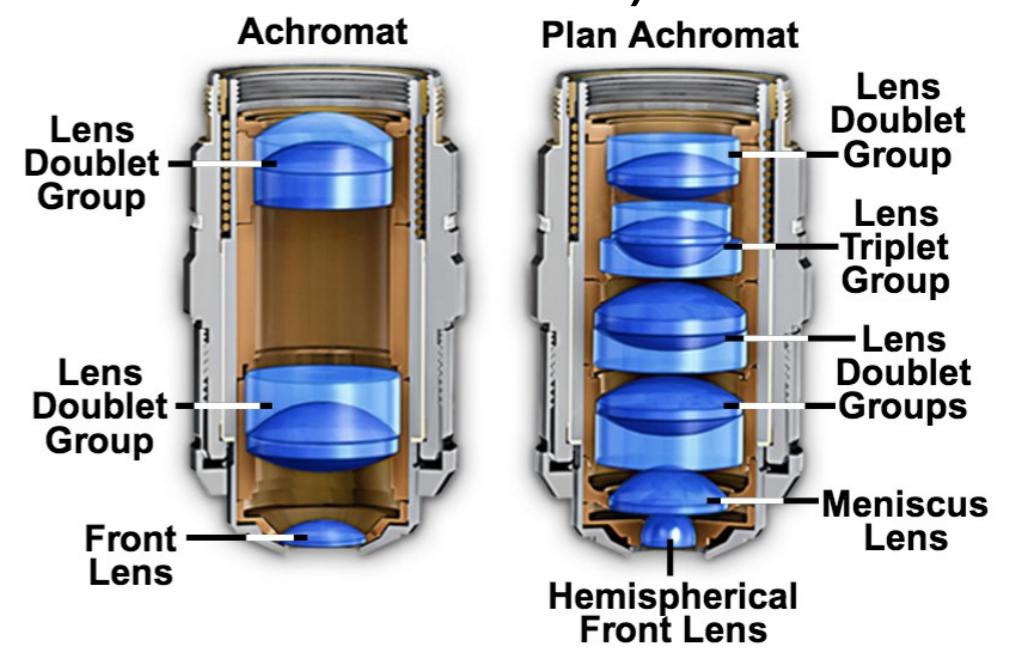
# Field curvature

Planar object imaged onto a spherical surface

→ Can't get the whole FOV in focus

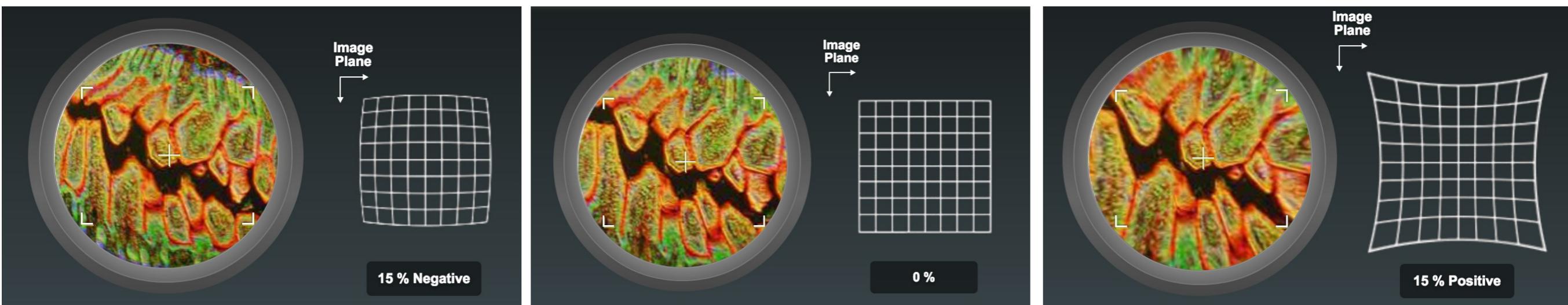


→ Invest in a corrected objective  
( Plan, Pl, EF, Achromat, Plan Apo,  
or Plano.)



# Distortion

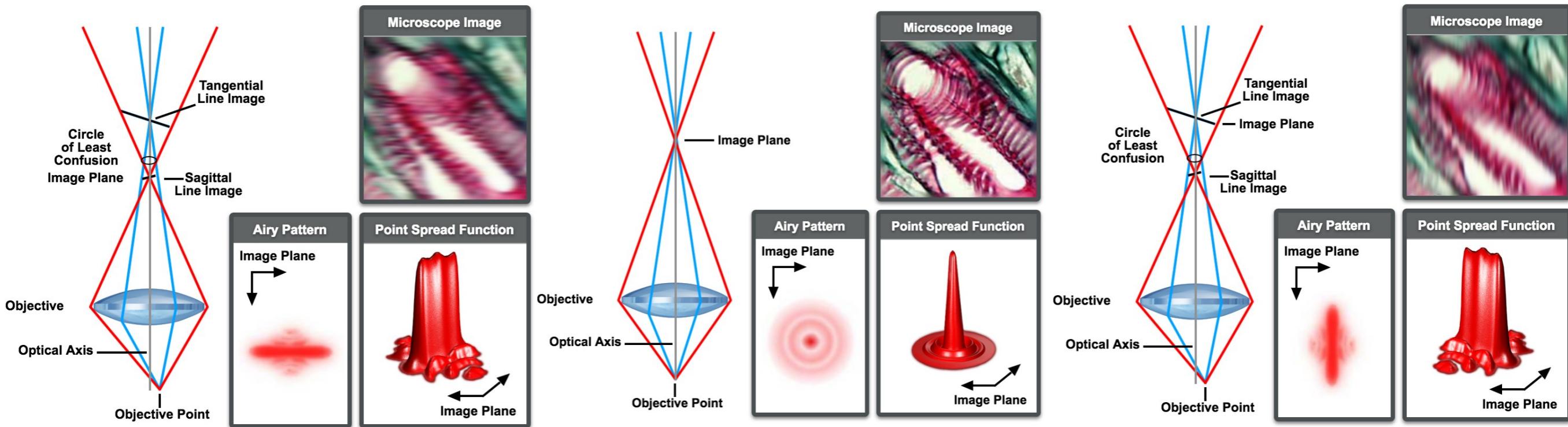
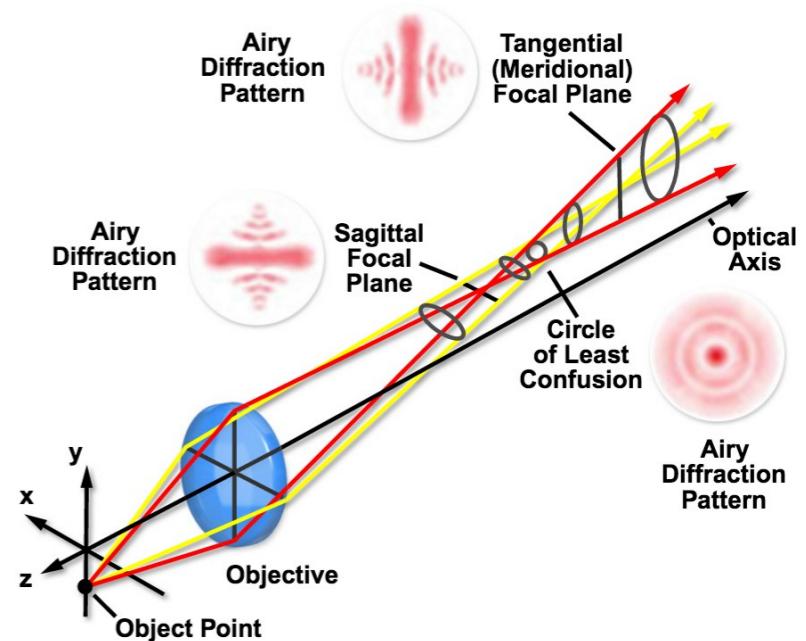
The true geometry of a specimen is no longer maintained in the image



# Astigmatism

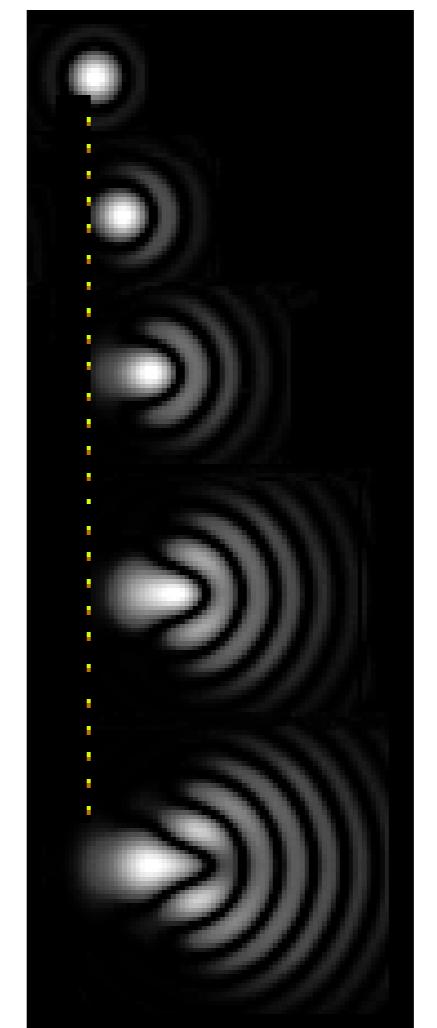
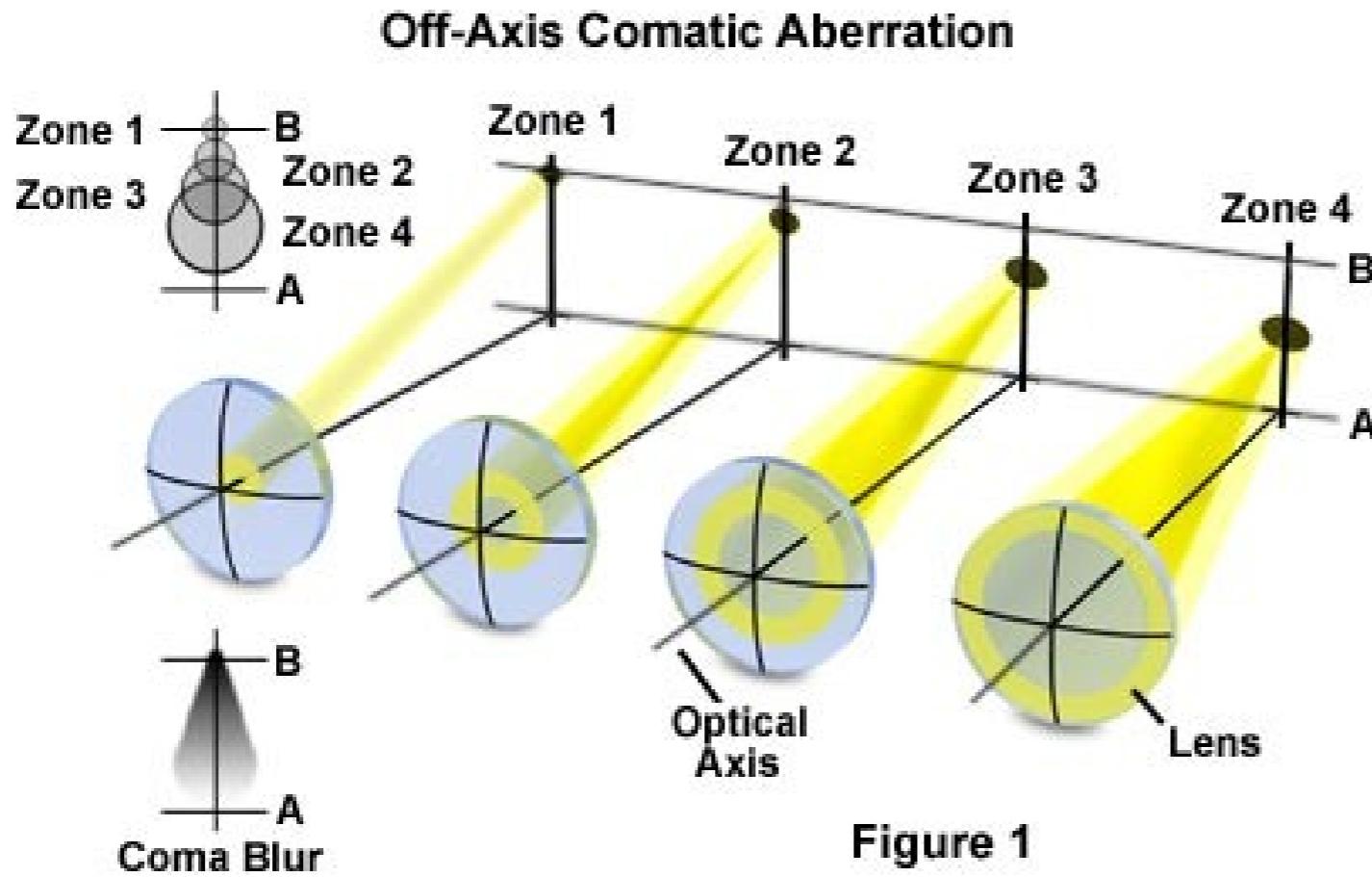
Aberration manifested by the off-axis image of a specimen point appearing as a line or ellipse instead of a point. This is due to different lens curvatures in different planes (lens tilted for ex).

- ➡ Check whether there is a tilted optical component in the optical path



# Coma

Encountered with off-axis light fluxes.  
It results from refraction differences by light rays  
passing through the various lens zones as the  
incident angle increases.  
It may come from microscope mis-alignment



Increasing coma

# Keep in mind

Chromatic and spherical aberrations are the most common

Do not underestimate optical aberrations if you want accurate quantitative measurements

Choose your microscope objective wisely

Beware refractive index mismatch —> choose immersion media, oil index and coverslip wisely

Do not mess up with a microscope alignment

# Online ressources

<https://www.microscopyu.com/>

## Matching Camera to Microscope Resolution

<https://www.microscopyu.com/tutorials/matching-camera-to-microscope-resolution>

## Matching Fluorescent Probes with Fluorescence Filter Blocks

<https://www.microscopyu.com/tutorials/spectralprofiles>

## Choosing Fluorescent Proteins for Dual Labeling Experiments

<https://www.microscopyu.com/tutorials/choosing-fluorescent-proteins-for-dual-labeling-experiments>

...



# Annexes

Achromat

doublet

etc...

# Annexes

Koehler illumination

# Annexes

DIC

Phase contrast

Dark field

etc...

# Annexes

## Deconvolution

