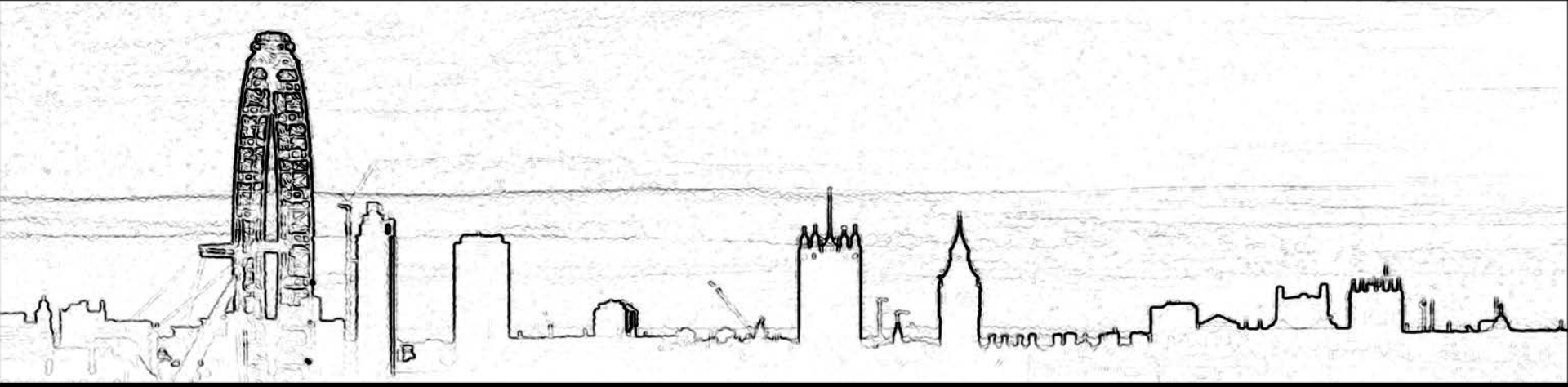


Basics of Optical Microscopy

Nicolas Olivier
King's College London

Imperial College London

Facility for Imaging by Light Microscopy

FILM

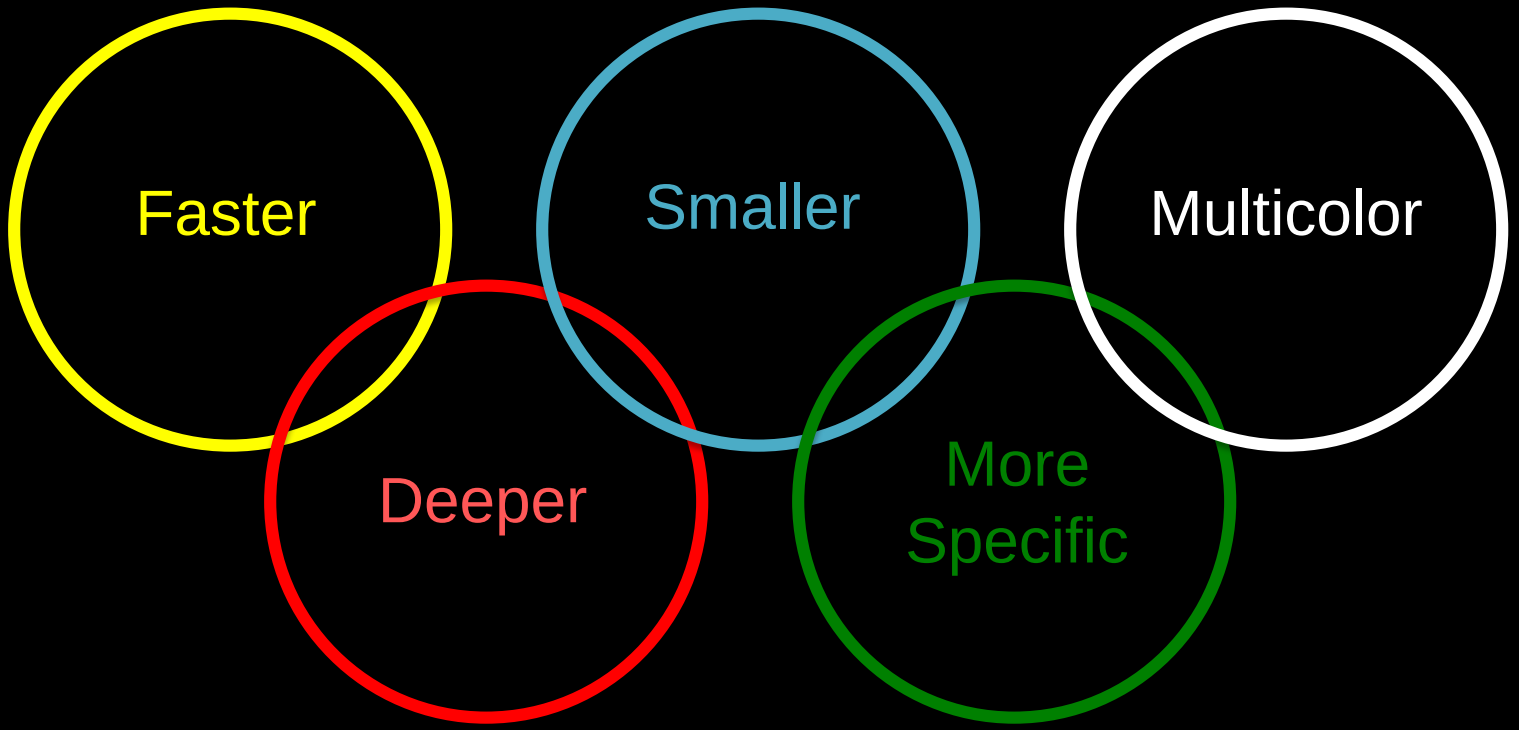
Observing Life As It Happens

Microscopy Day
FILM Facility
Imperial College
June 23rd 2015

Why use microscopes ?

Aim: observe the position of each molecule,
in a living organism in real time

« Microscopy Olympics »

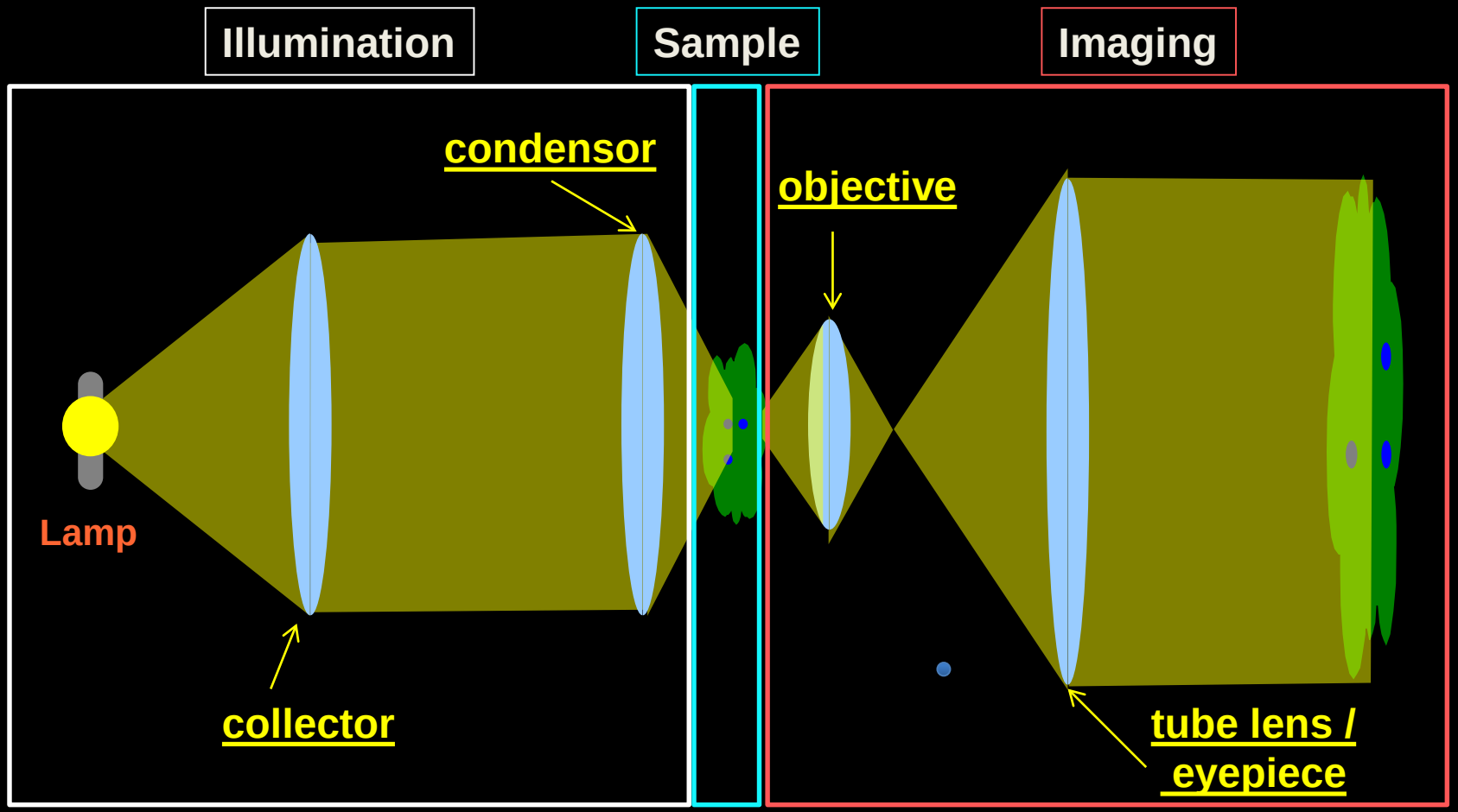


→ This talk: Smaller, More specific, (Deeper)

→ Other talks: All other aspects

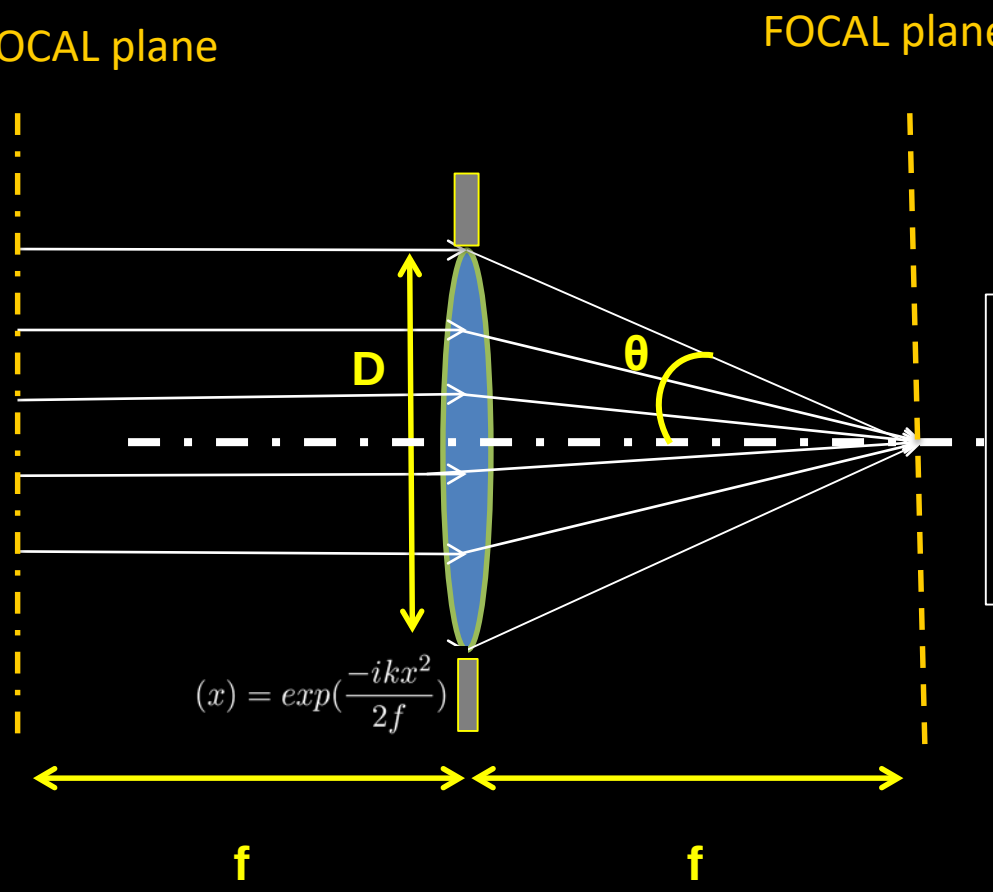
Anatomy of a microscope

(Lots of) More complex designs exist, but we'll first stick to this ☺



A microscope is ~ a light source, a detector, and some lenses in between

Anatomy of a lens



The angles at which the rays are emitted by a point in the focal plane can be seen as position in the back facal plane

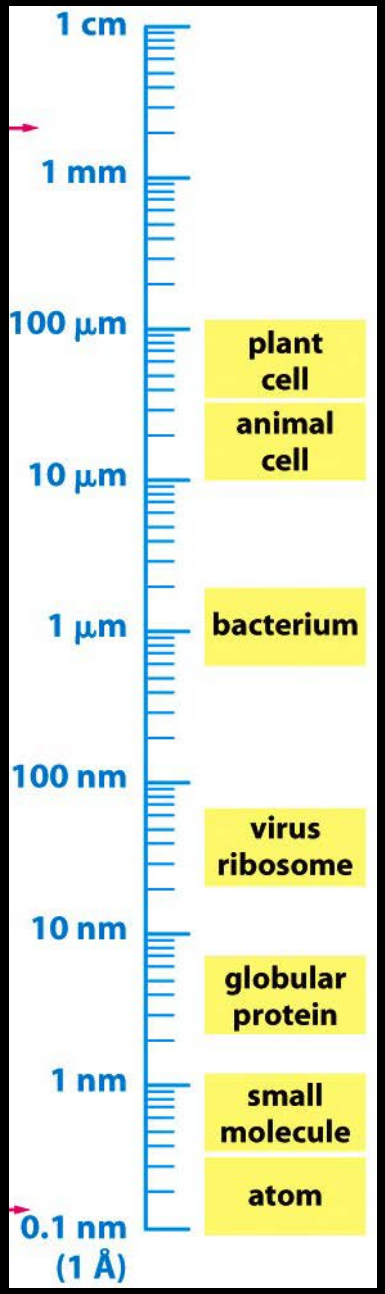
A lens focuses parallel rays at the back focal plane to a single point in the focal plane

A lens converts angular information into position information

Looking at small objects: Magnification and Resolution

~Eye

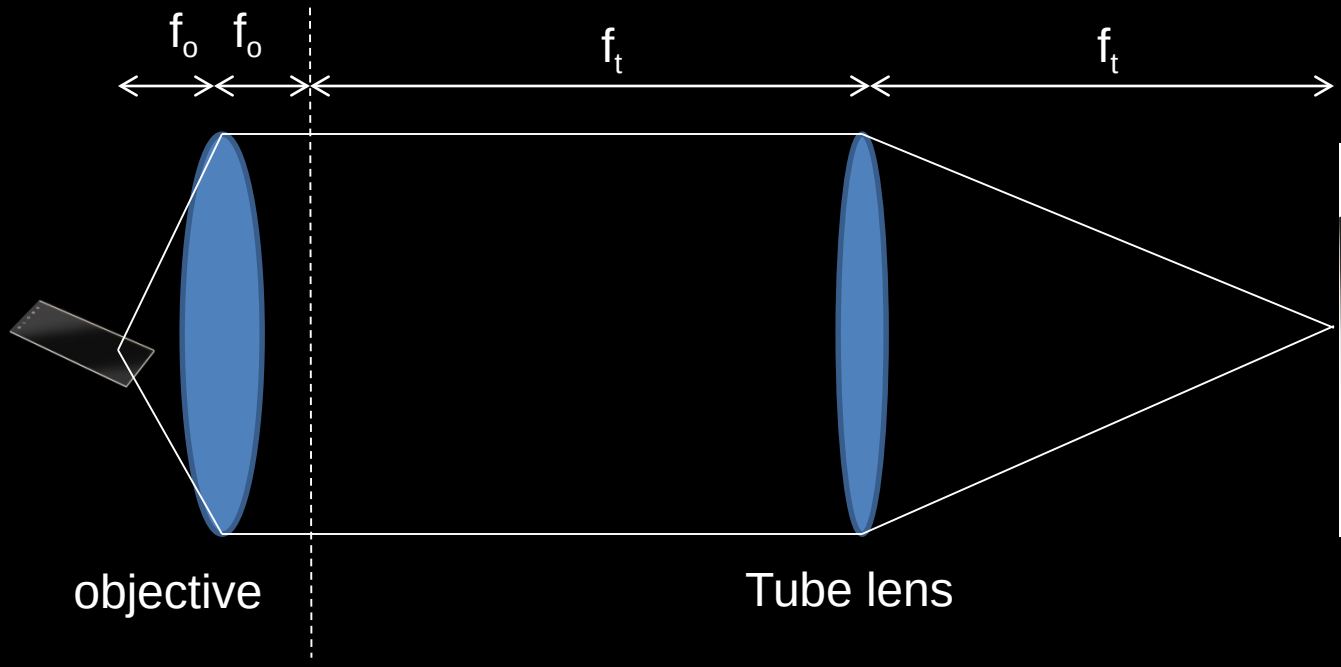
hair



Optical Microscopy

What is a microscope?

2 lenses system creating a magnified real image

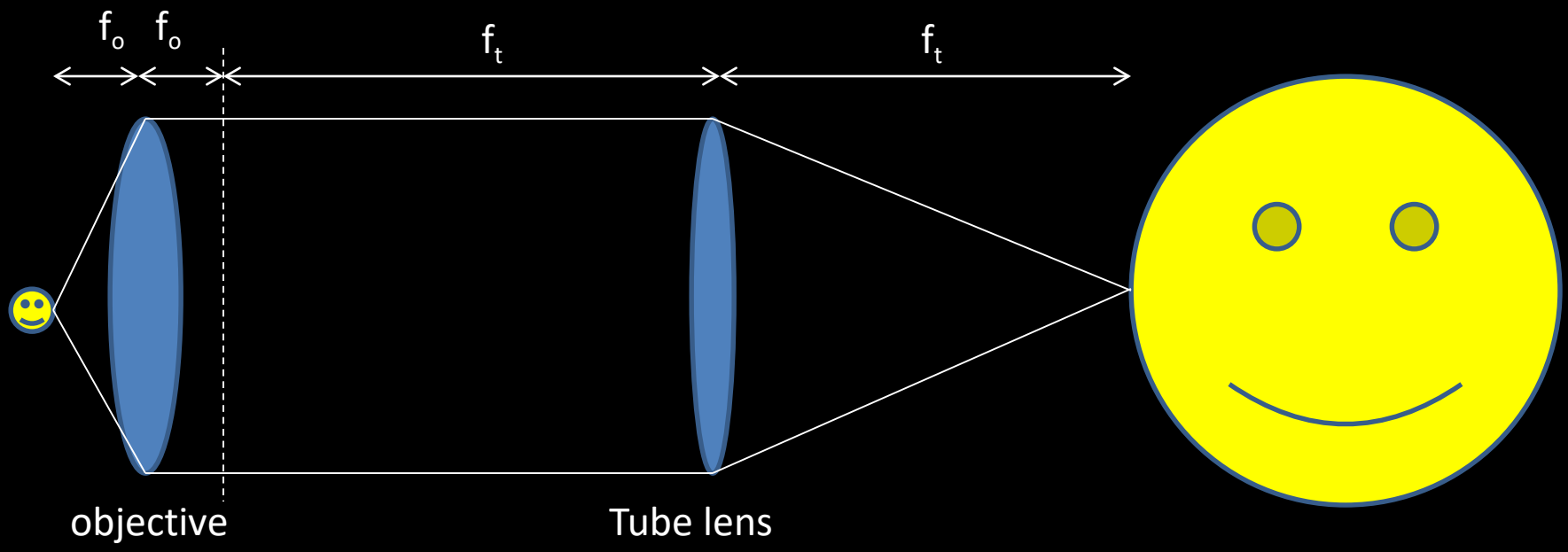


camera

Optical Microscopy

What is a microscope?

2 lenses system creating a magnified real image

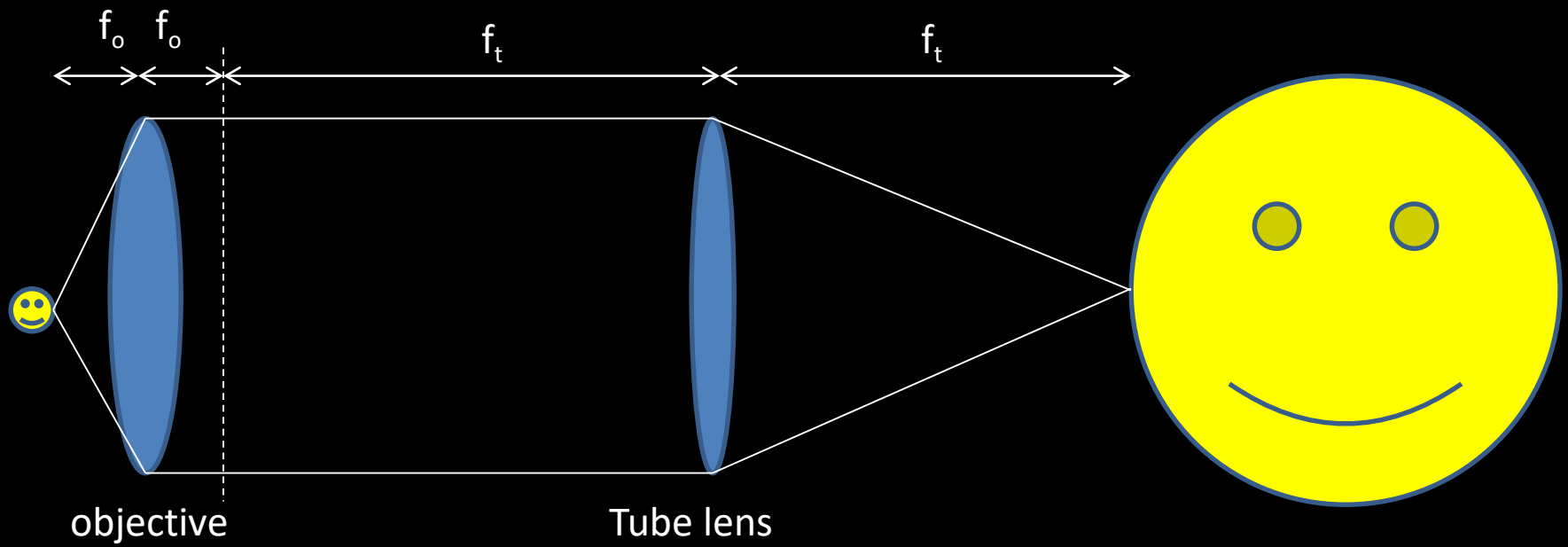


Magnification: Geometric optics ($M = f_t/f_o$)

Since the tube lens of a microscope is standard (for each company), microscope objectives are typically defined by their magnification instead of their focal length: a 10x objective means $f_0=f_t/10$

Optical Microscopy

2 lenses system creating a magnified real image



How far can we increase the magnification ?

⇩

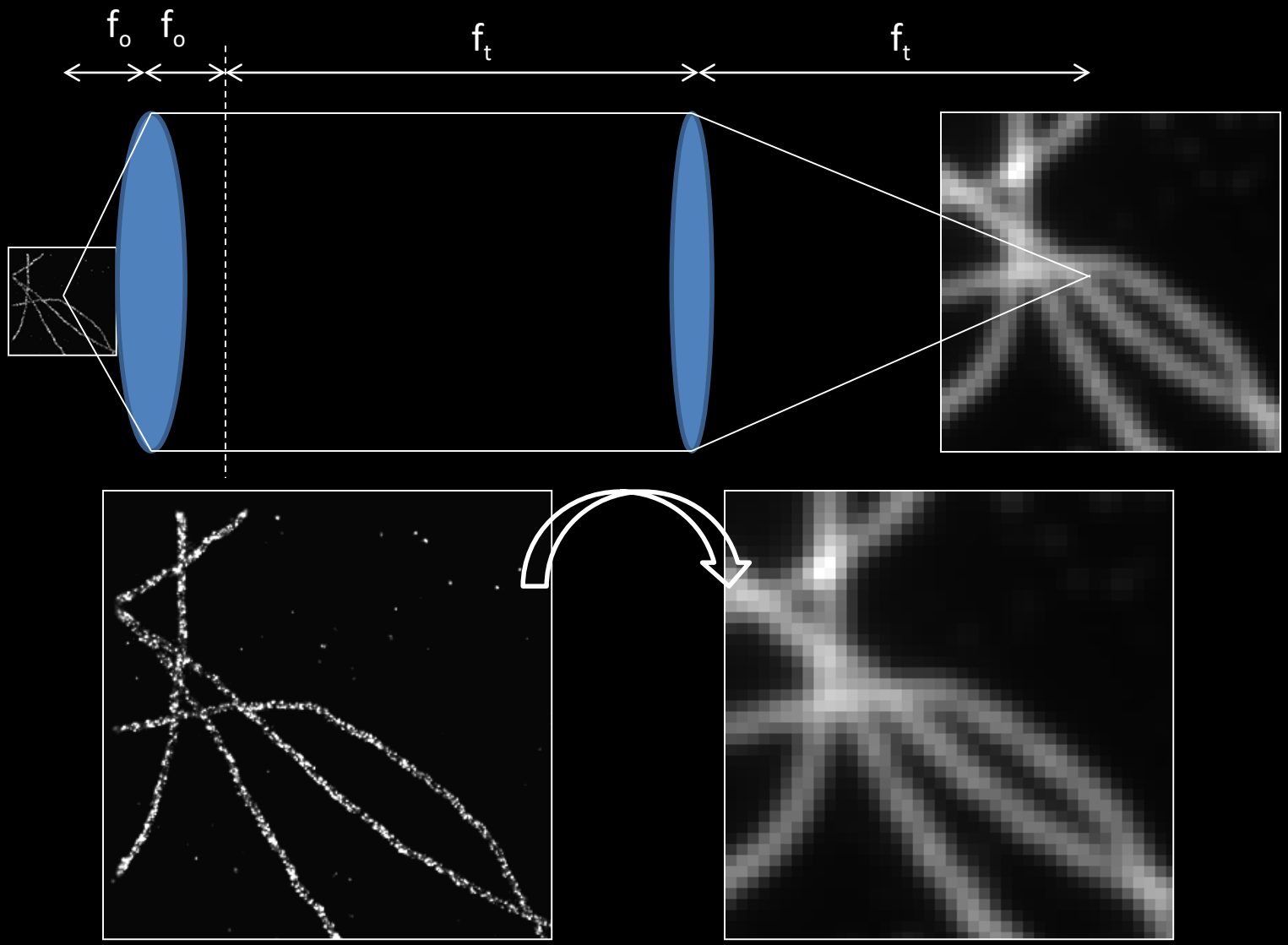
As much a we want !

Typical high magnification obj ~100x : just need to stack 2 x100 microscopes to reach x10.000 !

With a typical camera and 10.000 M, one pixel would be ~ 1nm →why not ?

Optical Microscopy

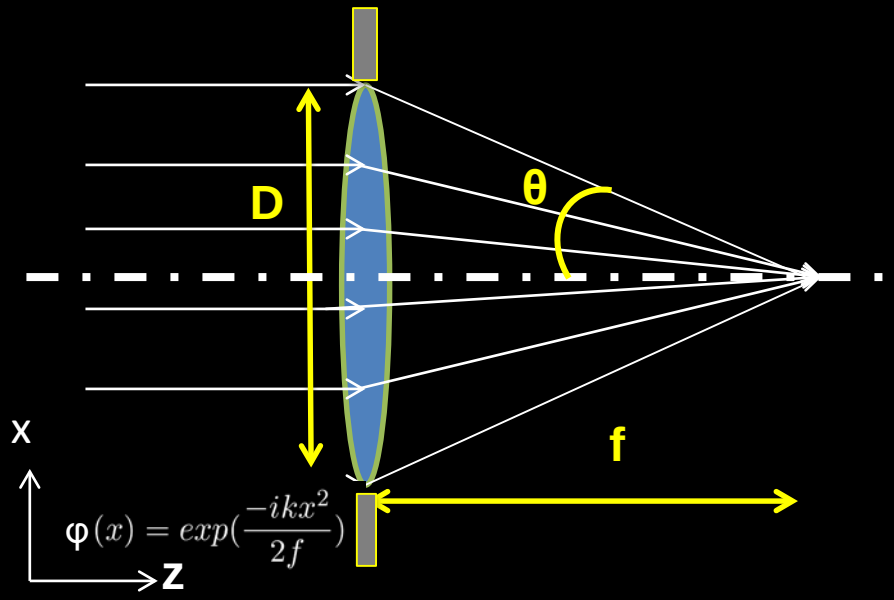
A microscope magnifies, but at the same time it blurs images

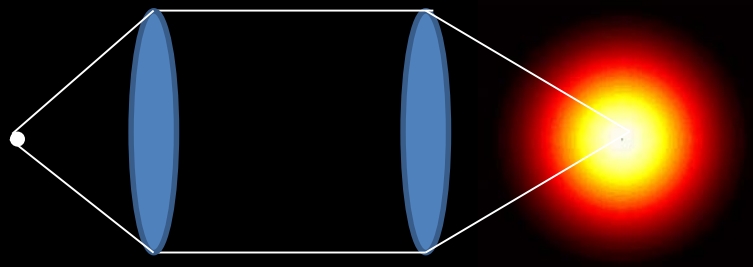


Light as a wave: Diffraction

The blurring comes from the wave nature of light

→ Image of a point by 2 lenses is a (~Gaussian) spot





Size of the spot:
function of the numerical aperture

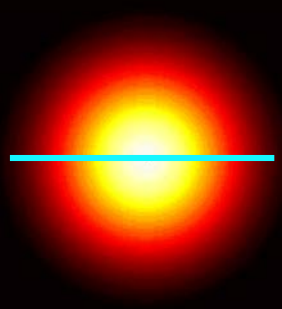
NA = n.sin(θ)=n.sin(arctan(D/2f))

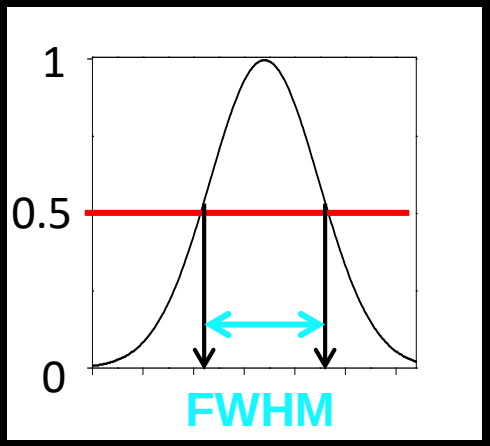
n: optical index

FWHM ~ 0.51λ/NA

⇩

FWHM > 0.51λ/n

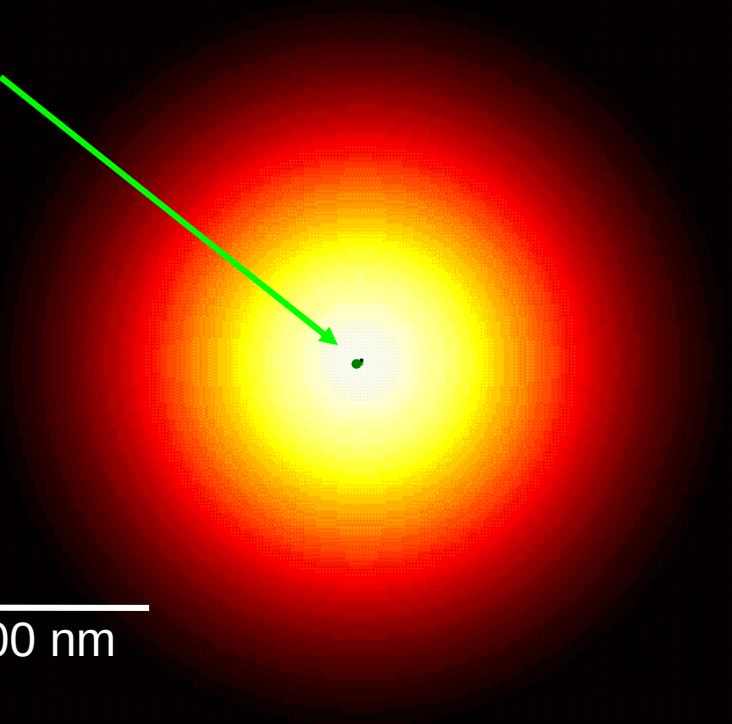




Diffraction: consequences

Image of a point source

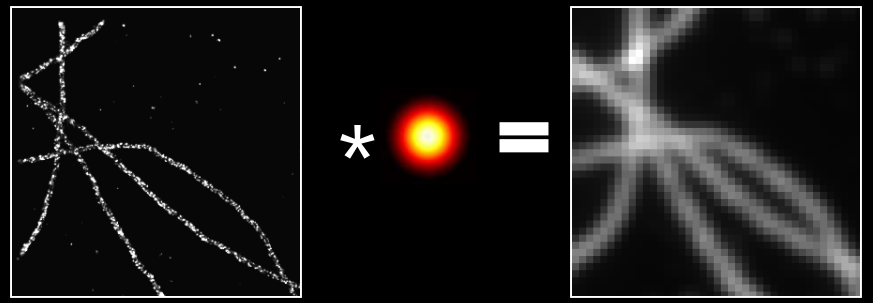
GFP
3 nm



100 nm

Diffraction limit
~ 100x size of a molecule

Linear system:



All the information smaller than the difraction limit is lost

Resolution

In terms of convolution:

The image is the original image magnified and convoluted with a Gaussian function of size:

FWHM ~ 0.51λ /NA

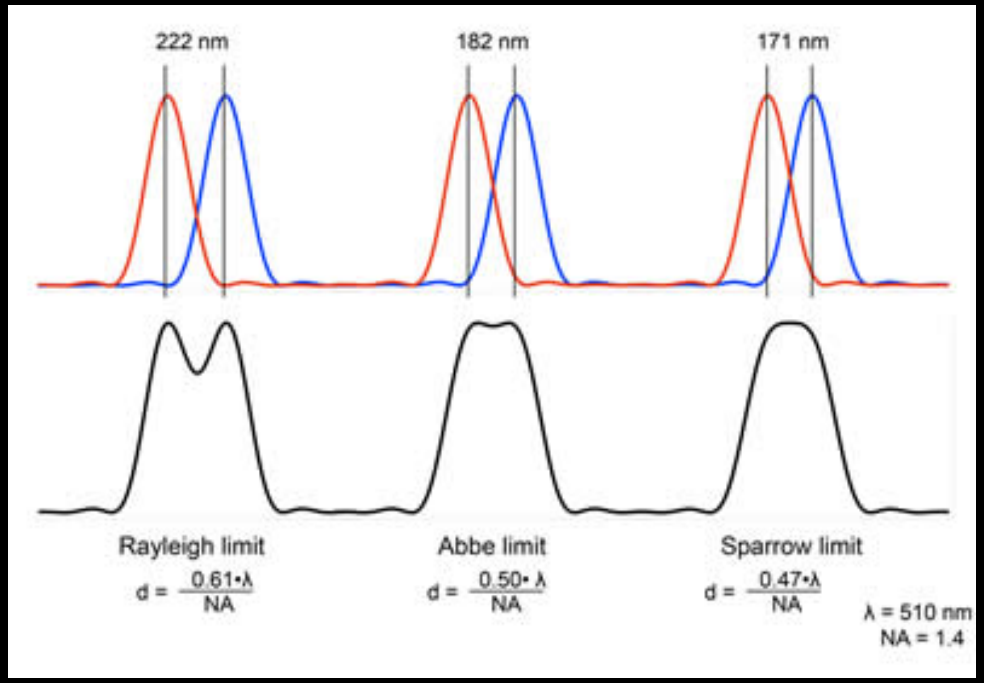
GFP (~520nm), NA=1.42

FWHM~178nm

In terms of « Resolution »

Can we distinguish two neighboring objects ?

Rayleight	Abbe	Sparrow
Distance max - first zero	Min distance to resolve 2 peaks	Min distance for inflexion point



Resolution in Widefield

Linear dependence on wavelength and Numerical aperture:

FWHM ~ 0.51 λ /**NA**

	λ (400nm)	λ (500nm)	λ (600nm)	λ (700nm)
NA=0.8 (air)	255	320	385	450
NA=1.2 (water)	170	215	255	300
NA=1.4(oil)	145	180	220	255

Super-”regular” resolution

FWHM ~ 0.51λ /NA

Decrease **λ**

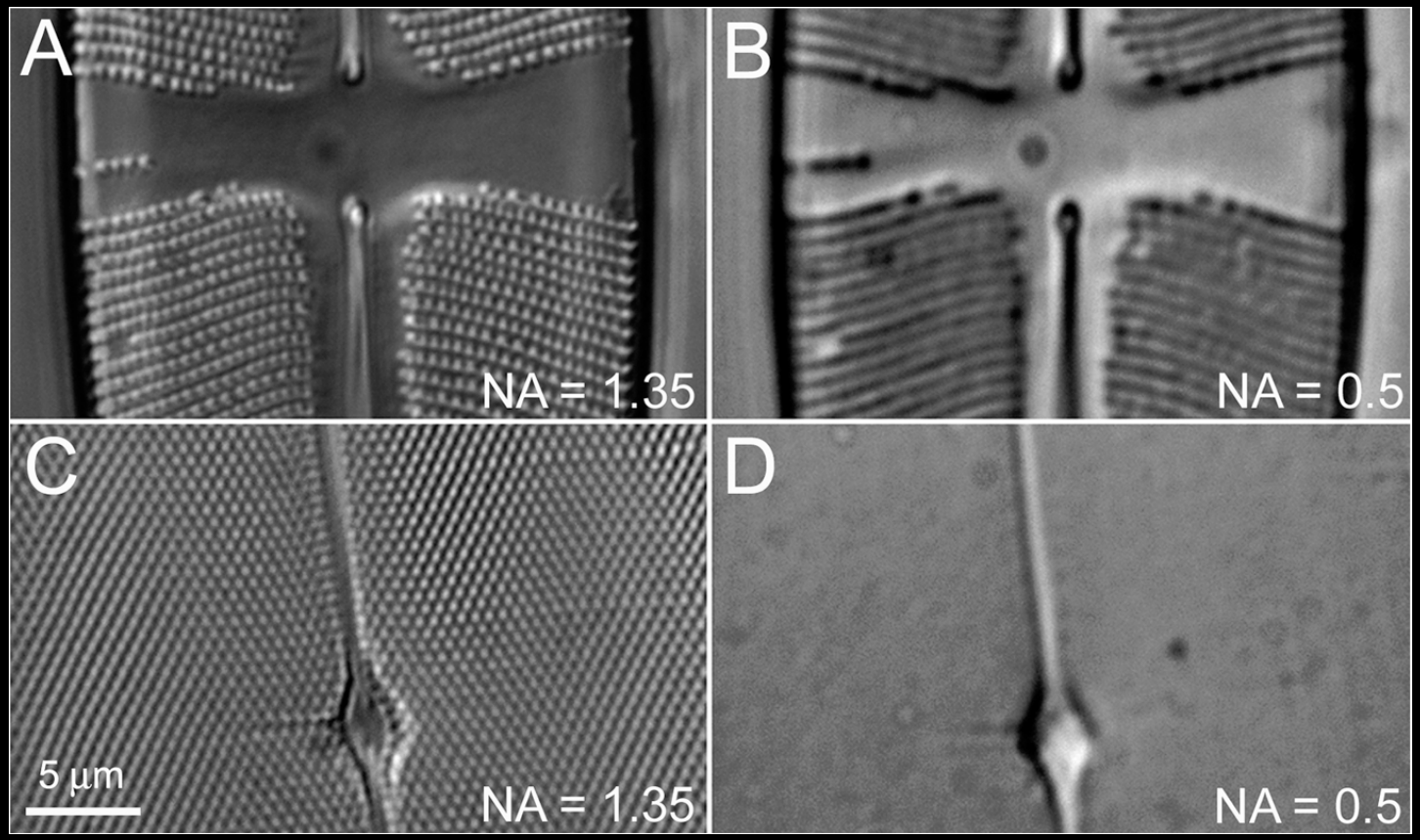
Increase NA
NA=n.sin(**θ**)

Increase n

Increase **θ**

Resolution in Widefield

- Linear dependence on Numerical aperture: FWHM (r) ~ 0.51 λ /**NA**

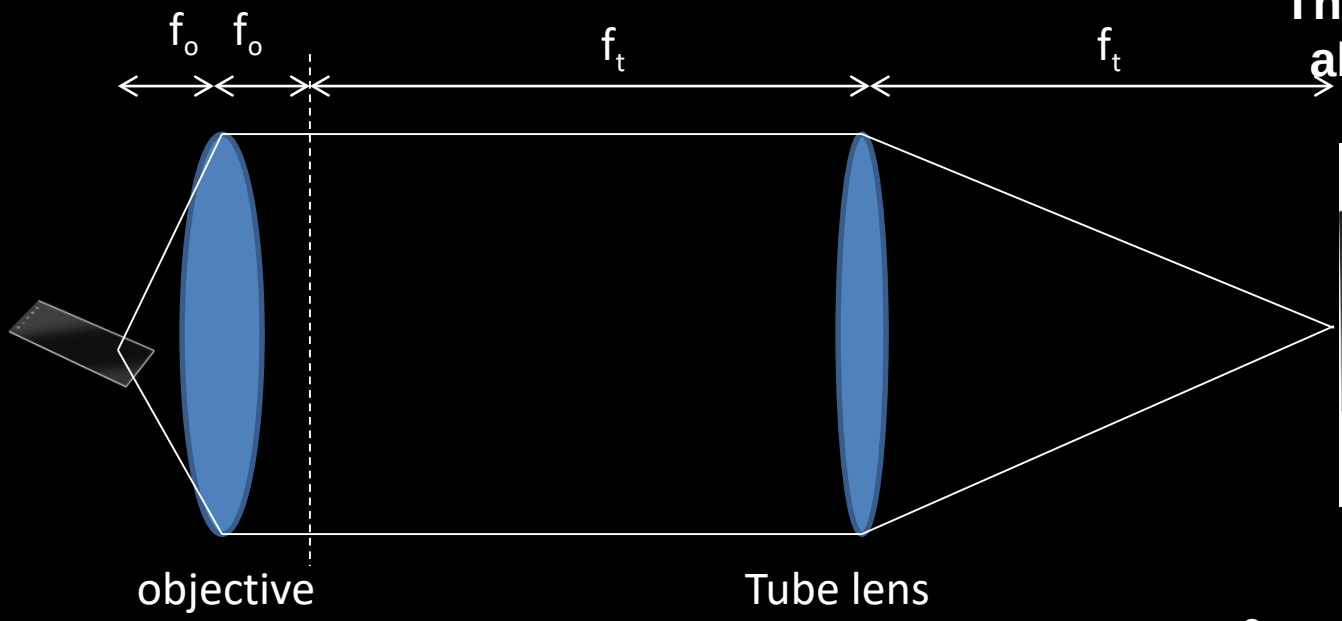


U Plan-Apo 100 objective lens with an adjustable NA (0.5–1.35)

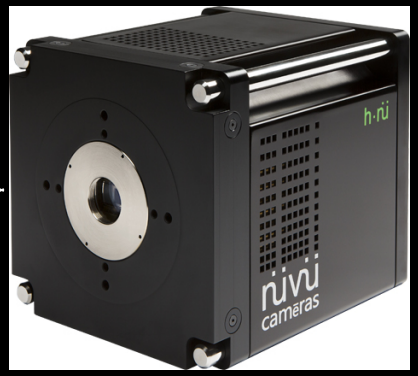
North A, Journal of Cell Biology, 2006

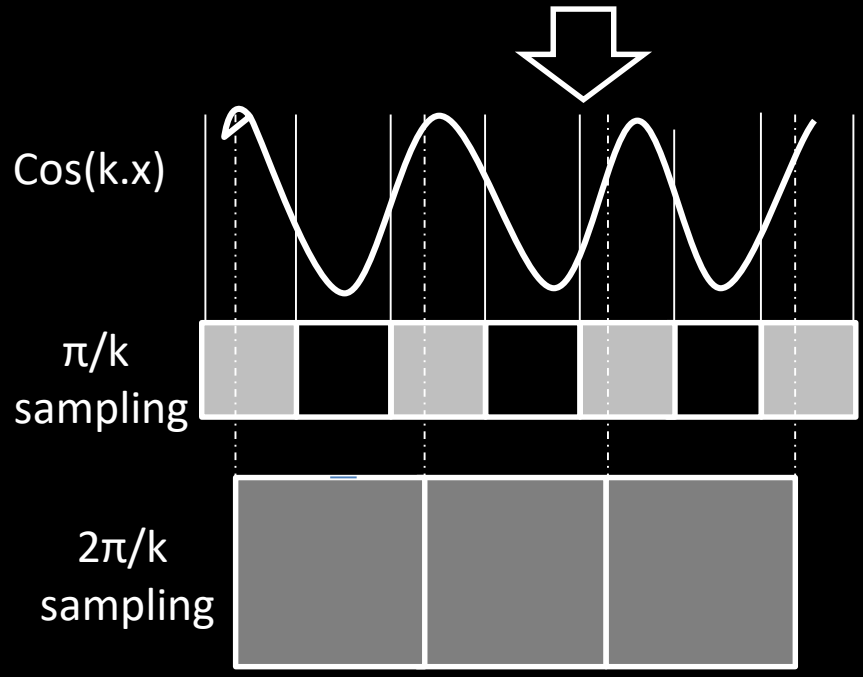
The NA, not magnification determines the resolving power of the objective lens !

Camera and Sampling



The camera needs to be able to capture all the information





Nyquist theorem

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

Pizel size < ½ * resolution~ 0.25λ/NA

Looking at small objects: Magnification and Resolution

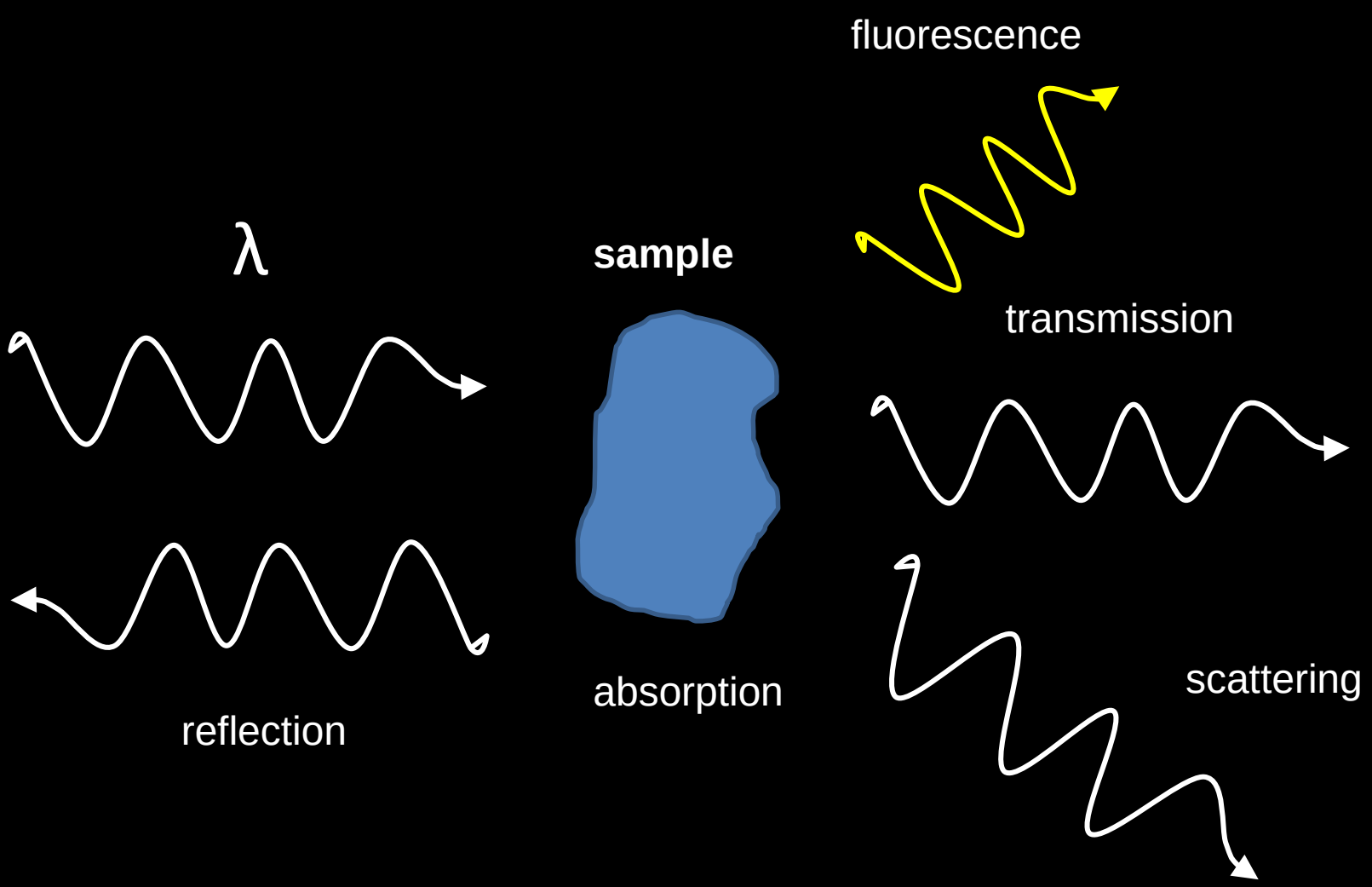
Resolution is limited by diffraction (bluring), not magnification

Commerial microscope fully characterized by magnification and Numerical aperture of objective lens

Camera needs to fulfill Nyquist criterion otherwise some information will ne lost

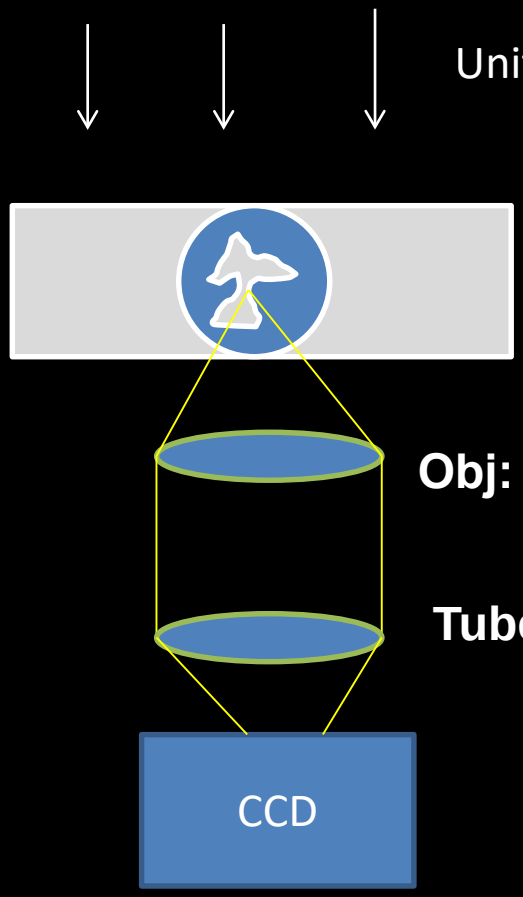
Looking at something specific: contrast mechanisms

Light matter interaction

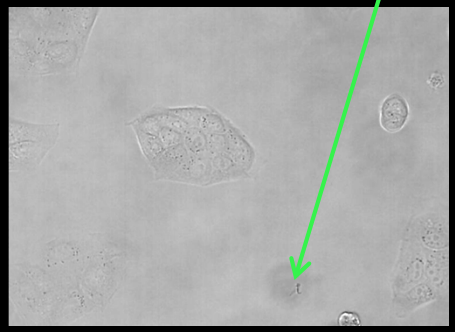


These interactions can be probed using different geometries

Brightfield microscopy (transmission)



Requires good illumination

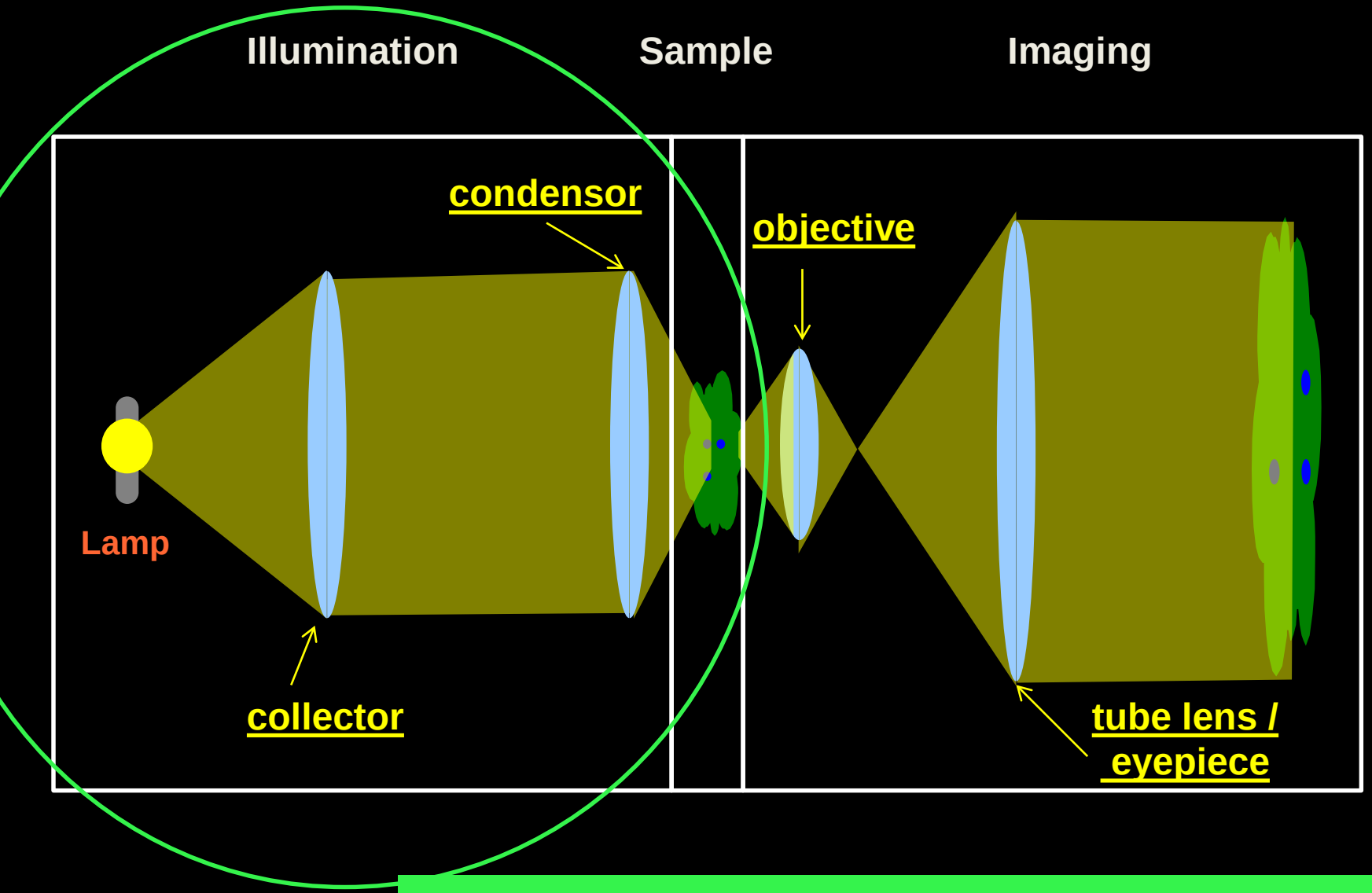


Low contrast on cells ☹

Widefield transmission microscopy:

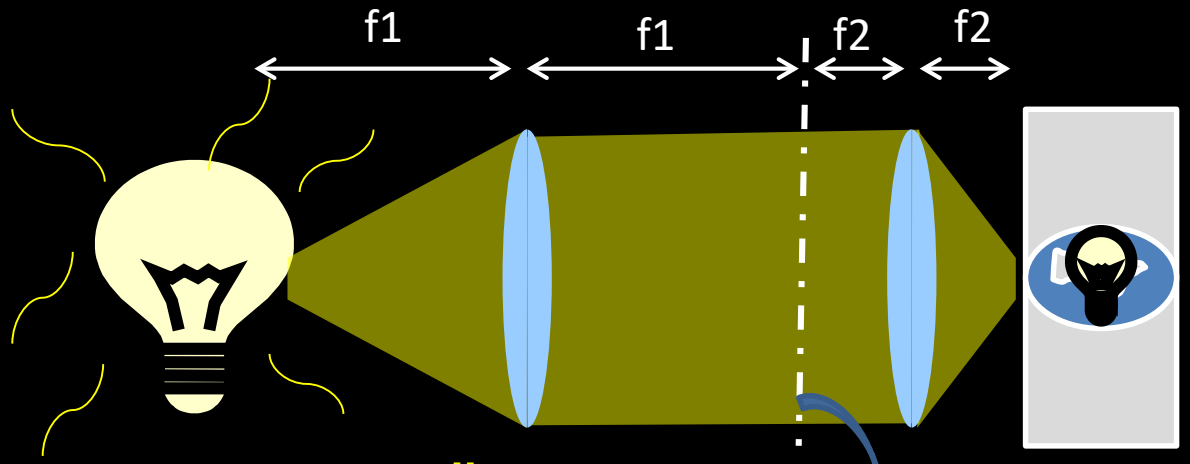
sensitive to absorption, reflection and some scattering (absorbance)

Achieving uniform illumination



Aim : prevent image formation of light bulb in sample plane

Achieving uniform illumination

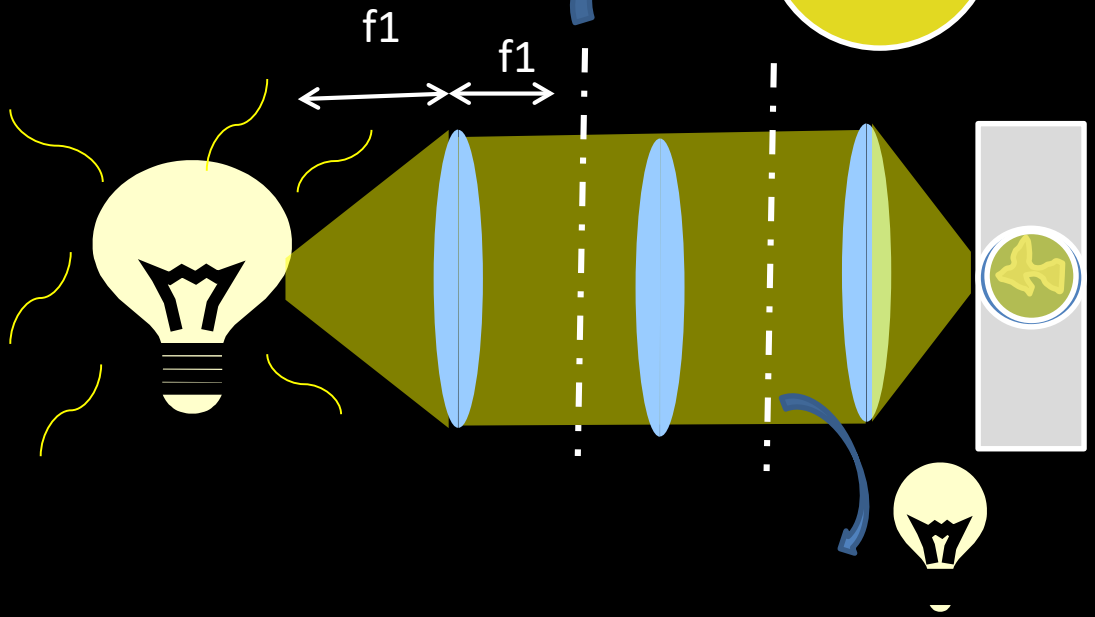


In this simple configuration, the lightbulb is imaged onto the sample !

collector

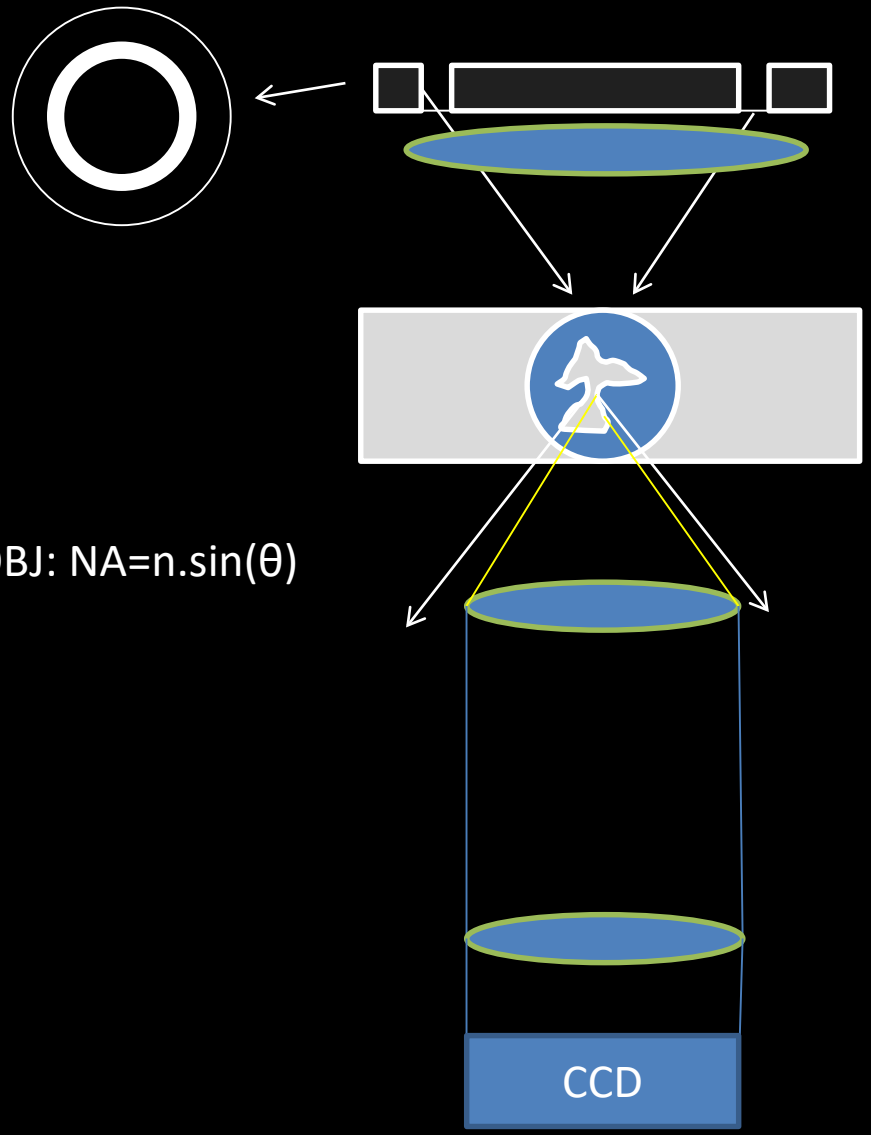
condensor

Since the light bulb emits light in every direction, the intensity in this plane is uniform



Köhler Illumination: the light bulb is imaged in the Fourier plane

Looking at scattering: dark field



OBJ: NA=n.sin(θ)

Illumination:
only allow one angle Φ

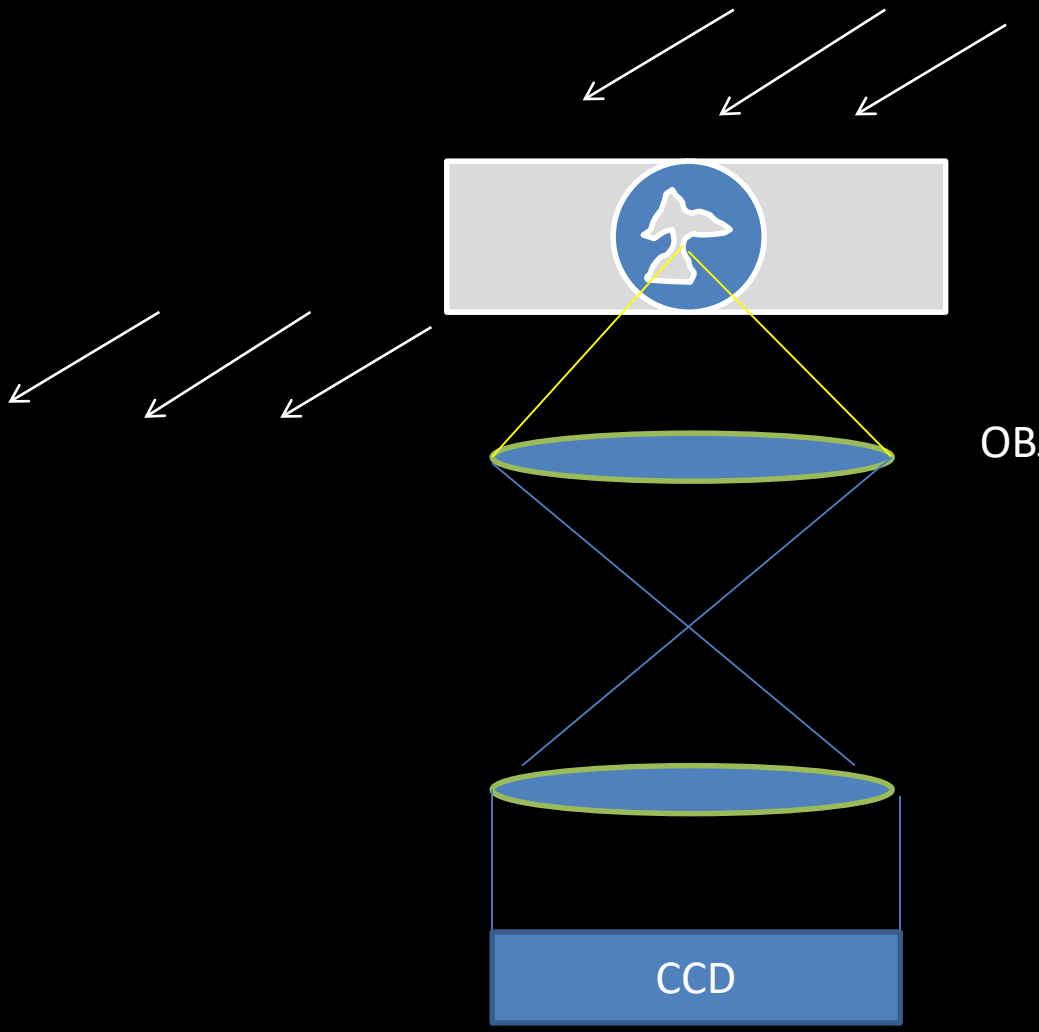
Φ>θ

Detection:
block light transmitted
at the illumination angle Φ:

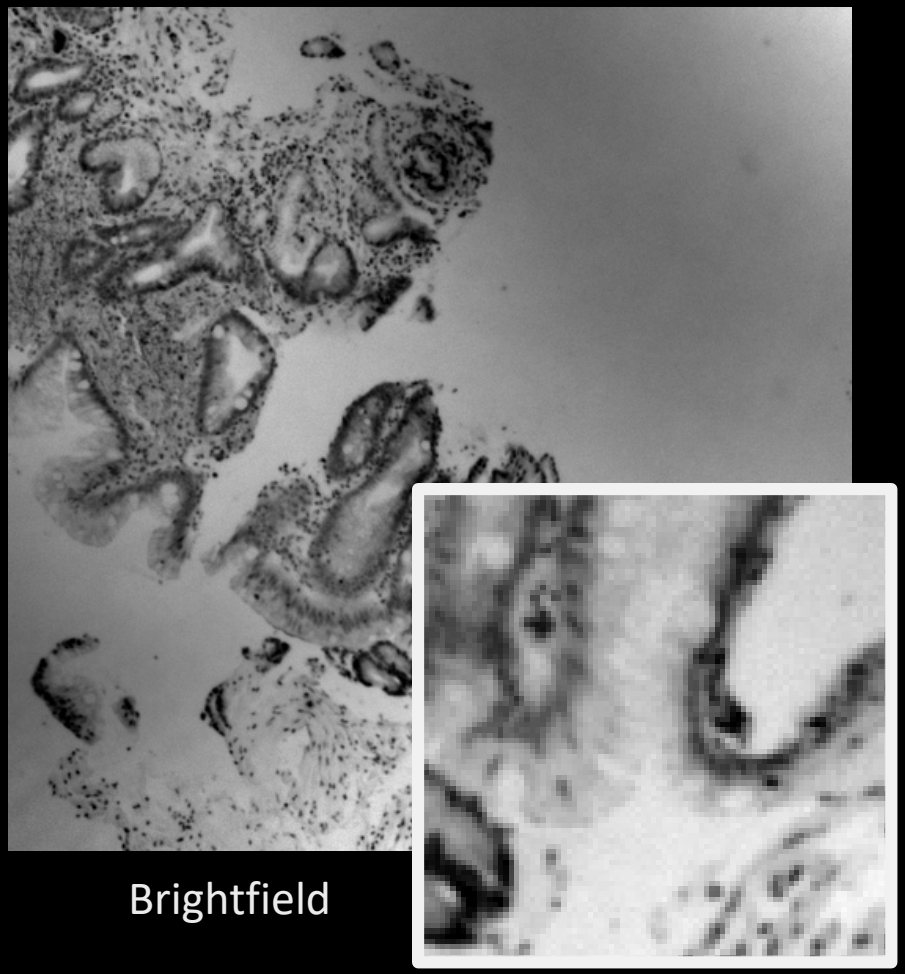
Points where light is scattered appear bright on a dark camera image

Looking at scattering: dark field

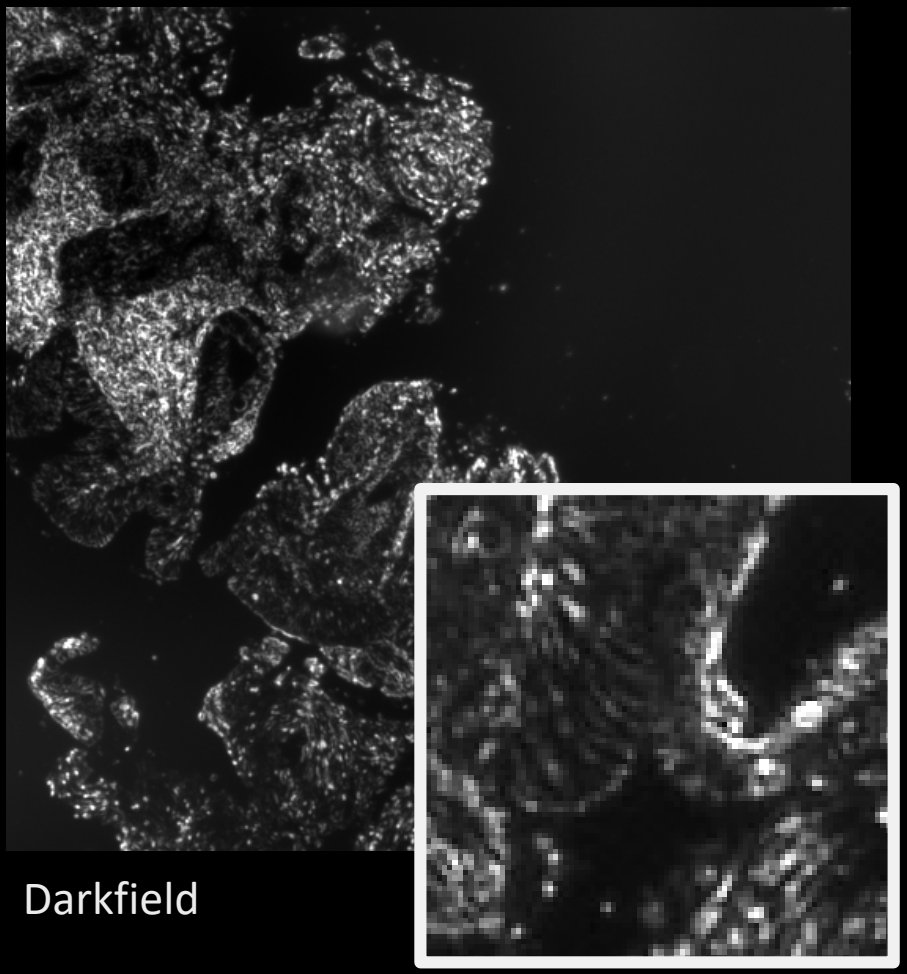
Simple darkfield:



Looking at scattering: dark field



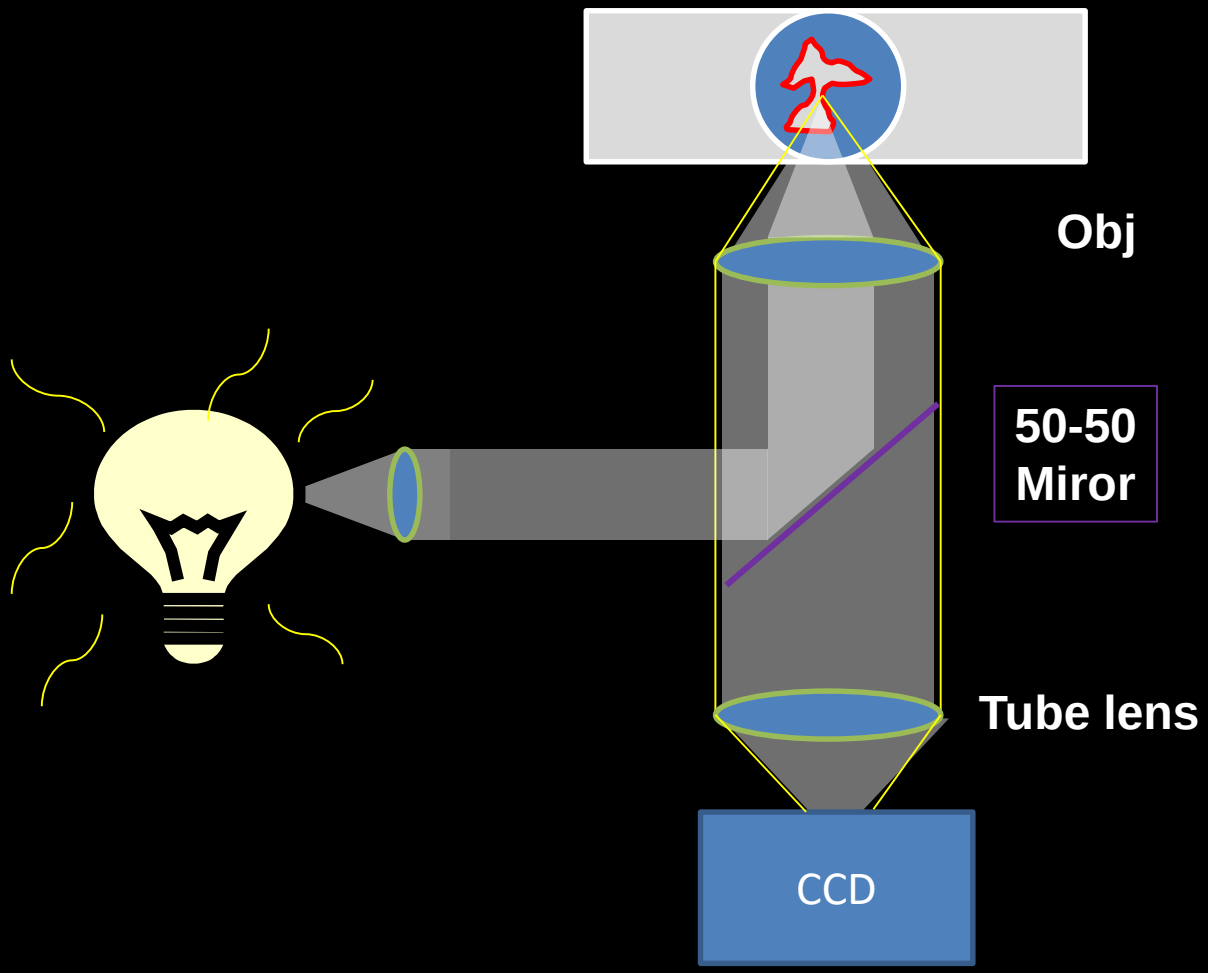
Brightfield



Darkfield

Martin Spitaler

Reflection microscopy

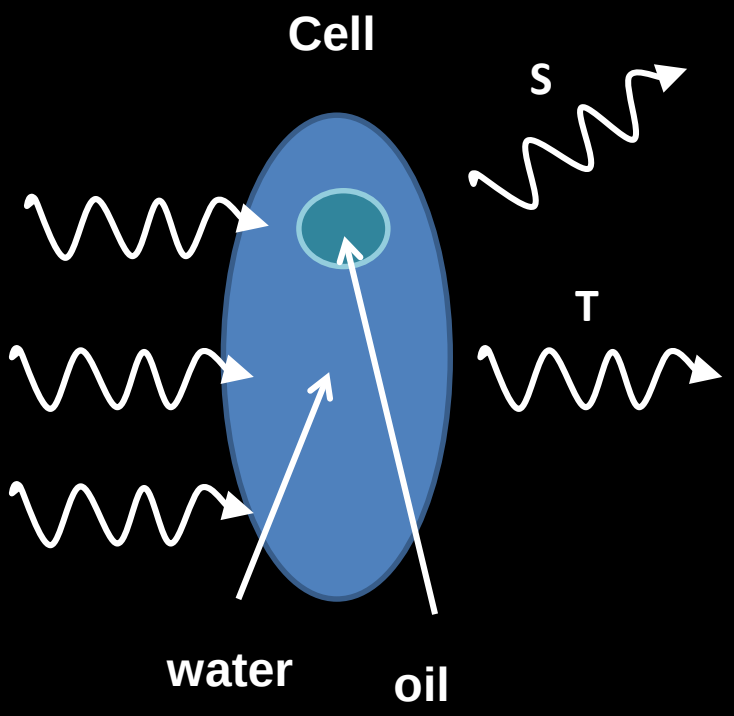


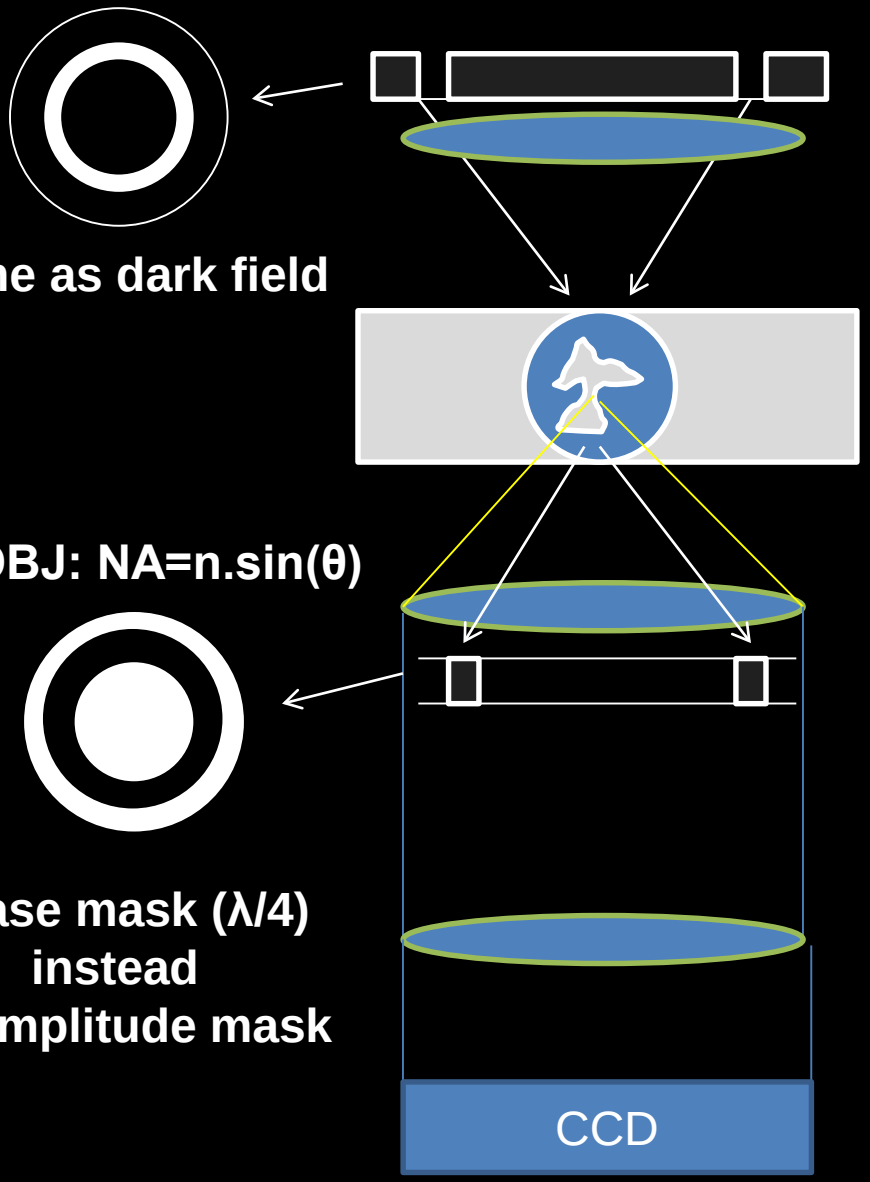
Very usefull when sample is non tranparent !

Looking at optical indices: phase microscopy

Zernike 1930s

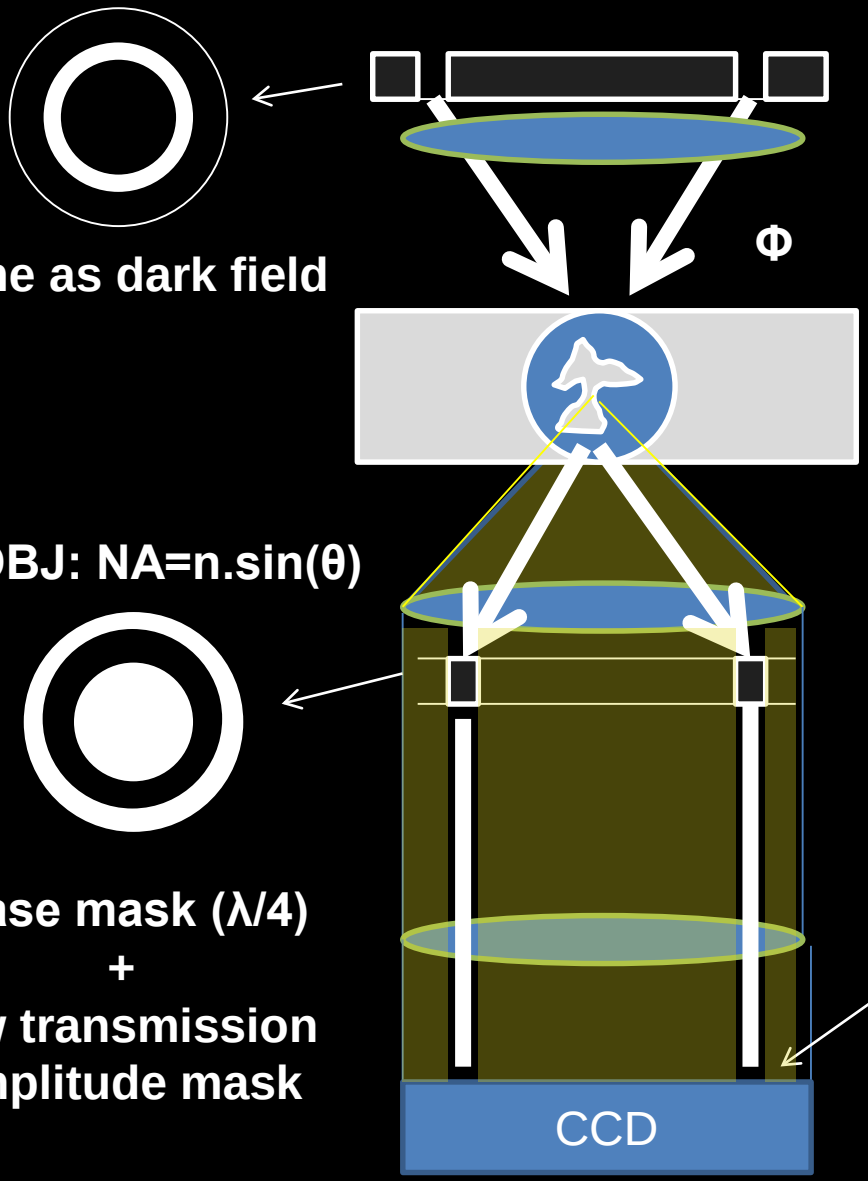
Both water and oil are transparent
BUT
Different indices of refraction
Water: 1.33
Oil:1.51





Looking at optical indices: phase microscopy

Zernike 1930s



2 image:

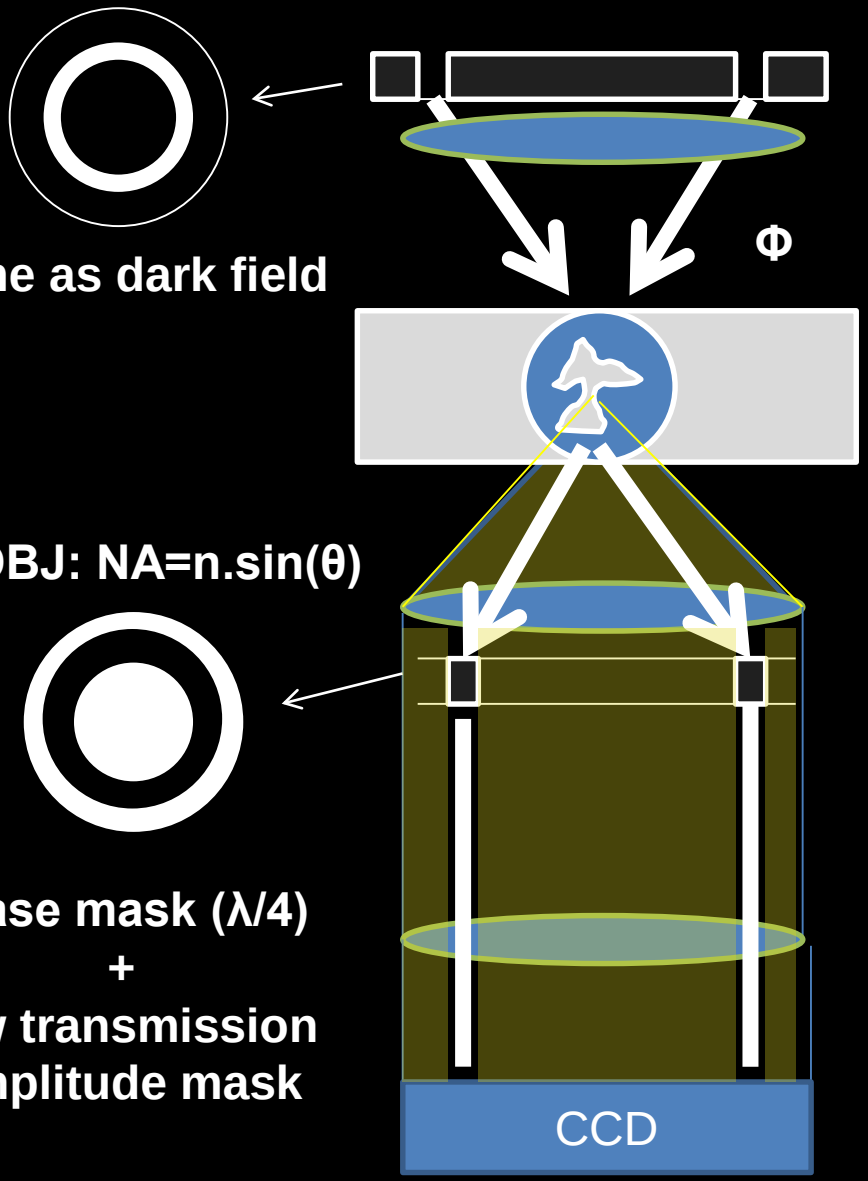
-one with transmitted light (~same as brightfield)

-one with scattered light (~same as darkfield)

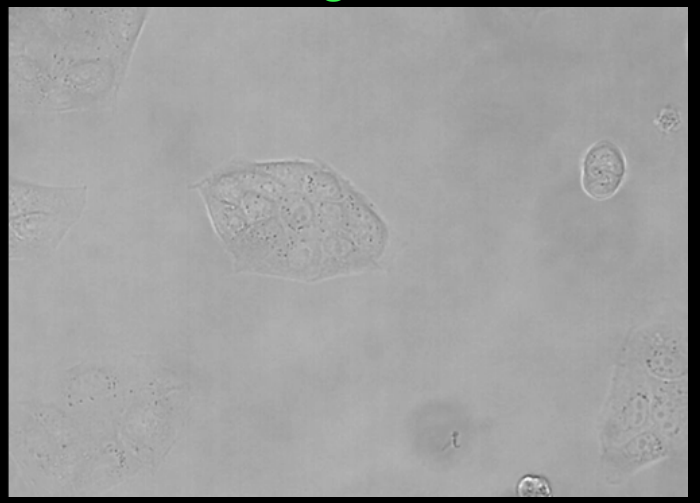
Both images interfere because of the phase mask

Looking at optical indices: phase microscopy

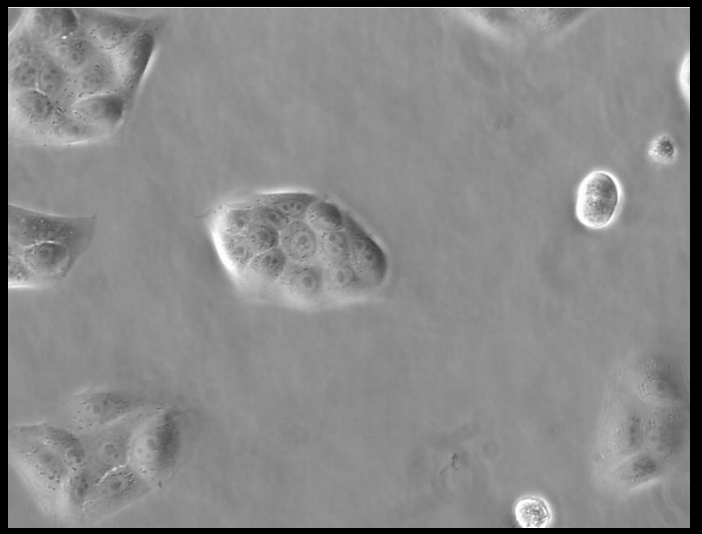
Zernike 1930s



Brightfield



Phase contrast

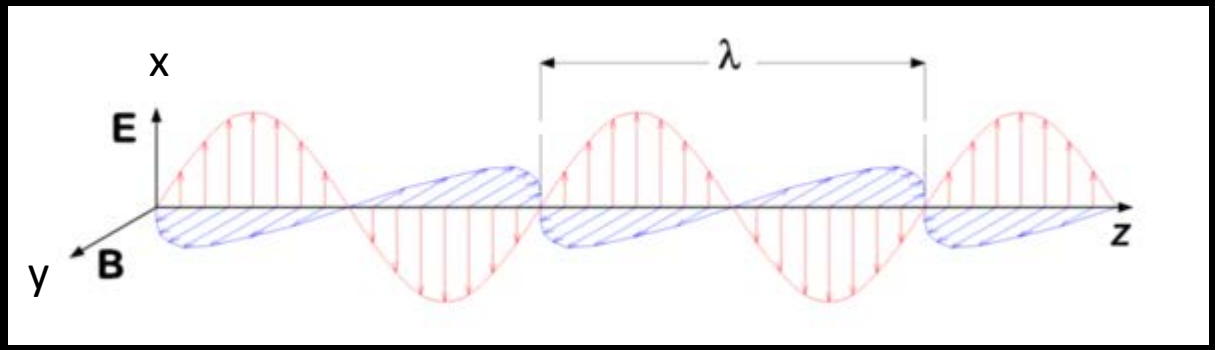


http://www.leica-microsystems.com/science-lab/phase-contrast/

Looking at optical indices: polarization microscopy

So far we've only looked at the intensity and phase of light, but it also has vectorial properties

The electric field can propagate along a given axis: its polarization axis



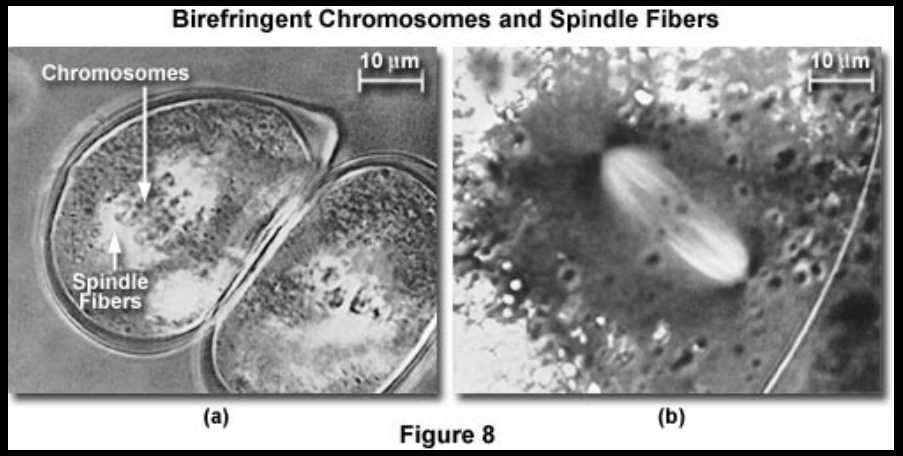
A polarizer can select a given polarization

wikipedia

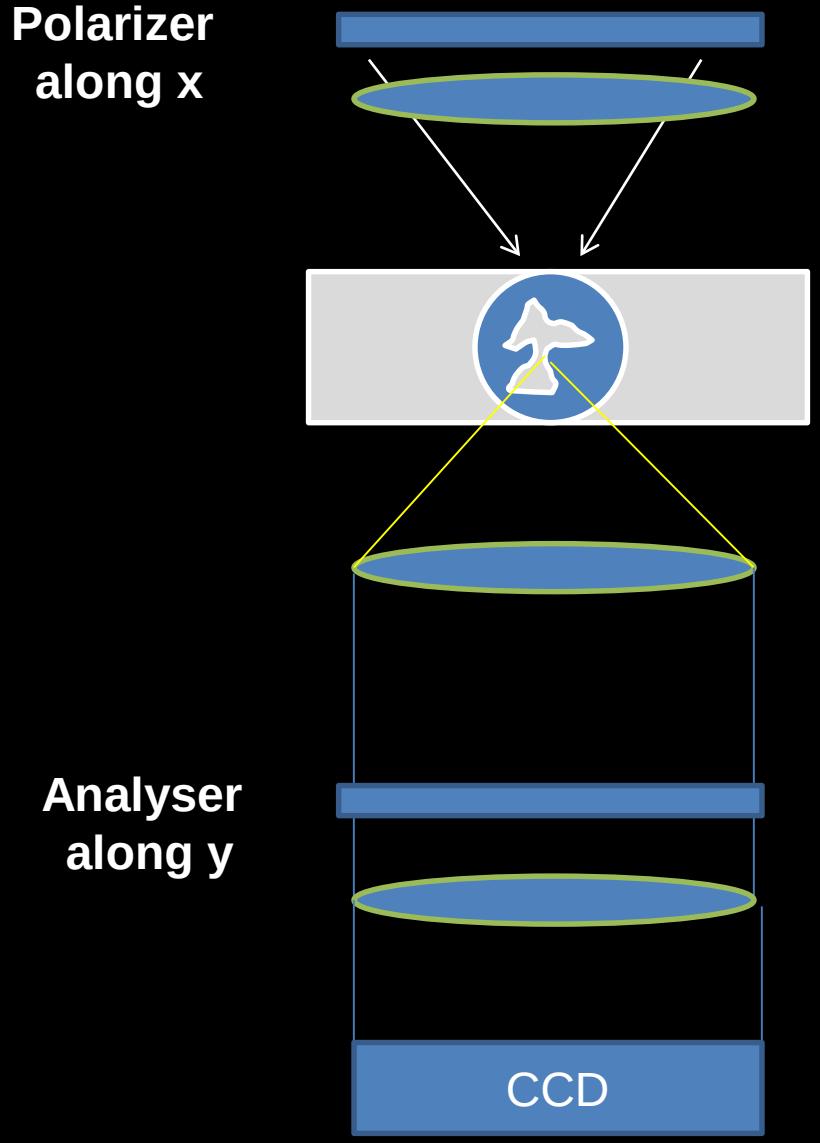
Looking at optical indices: polarization microscopy

Some samples are birefringent: they can rotate the polarization of light !

Only the parts of the sample that can rotate the polarization appear bright

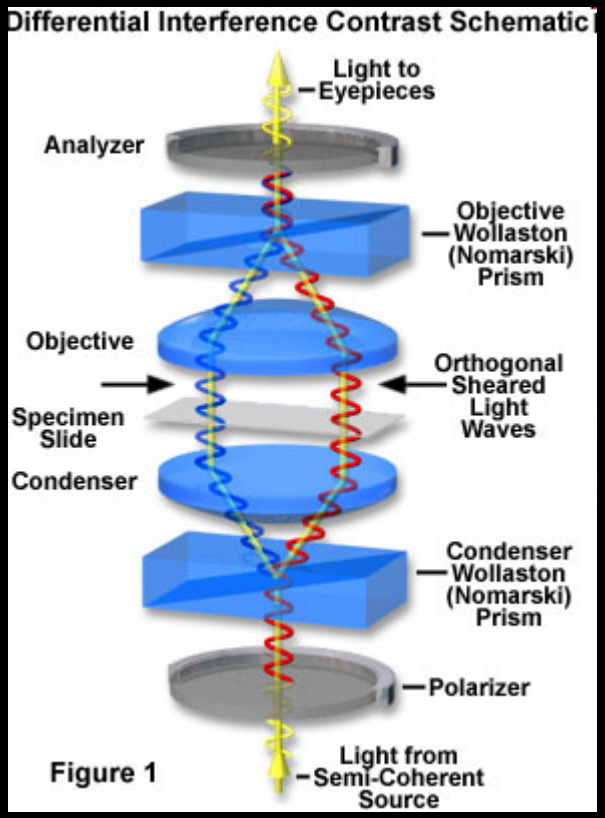


http://micro.magnet.fsu.edu/



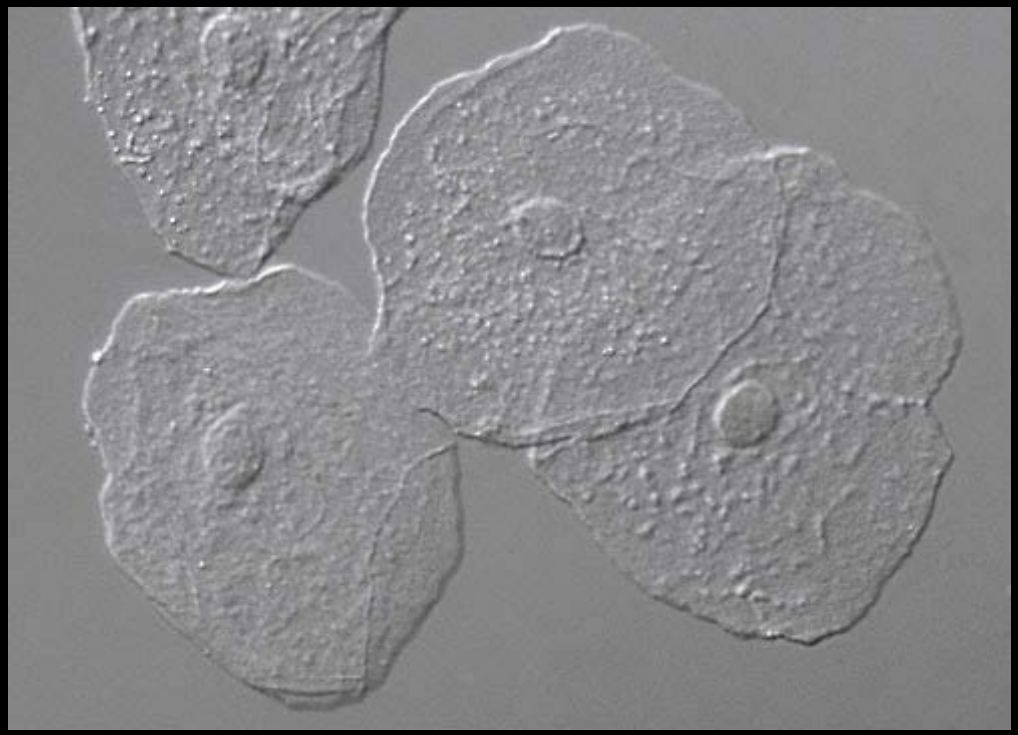
Looking at optical indices: DIC microscopy

Differential Interference Contrast (DIC)



from 'microscopy primer' (http://micro.magnet.fsu.edu)

DIC microscopy is an extension of phase contrast where two different polarizations are used

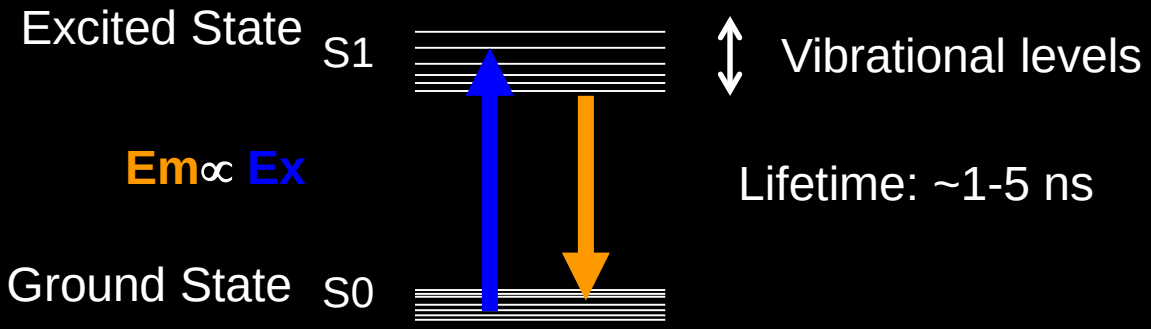


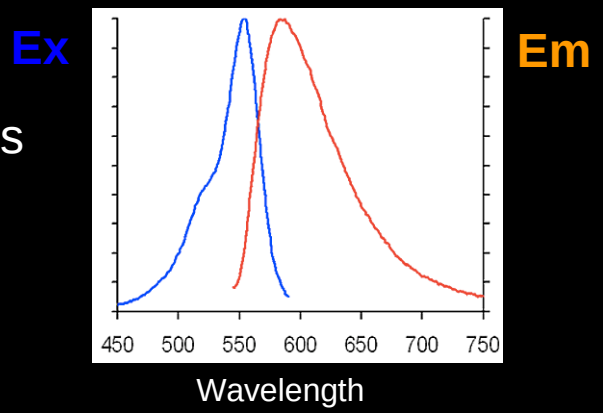
Nikon MicroscopyU

Fluorescence microscopy

Simplest and most powerful ☺

~ two level system

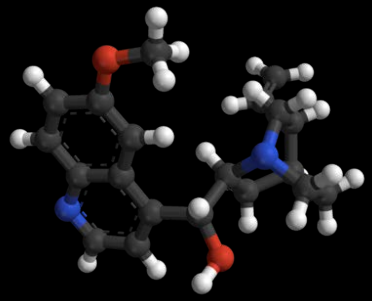




Organic dyes (XIX)

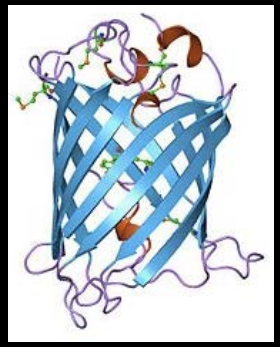
Ex: Rhodamine 6G

$C_{28}H_{31}N_2O_3Cl$



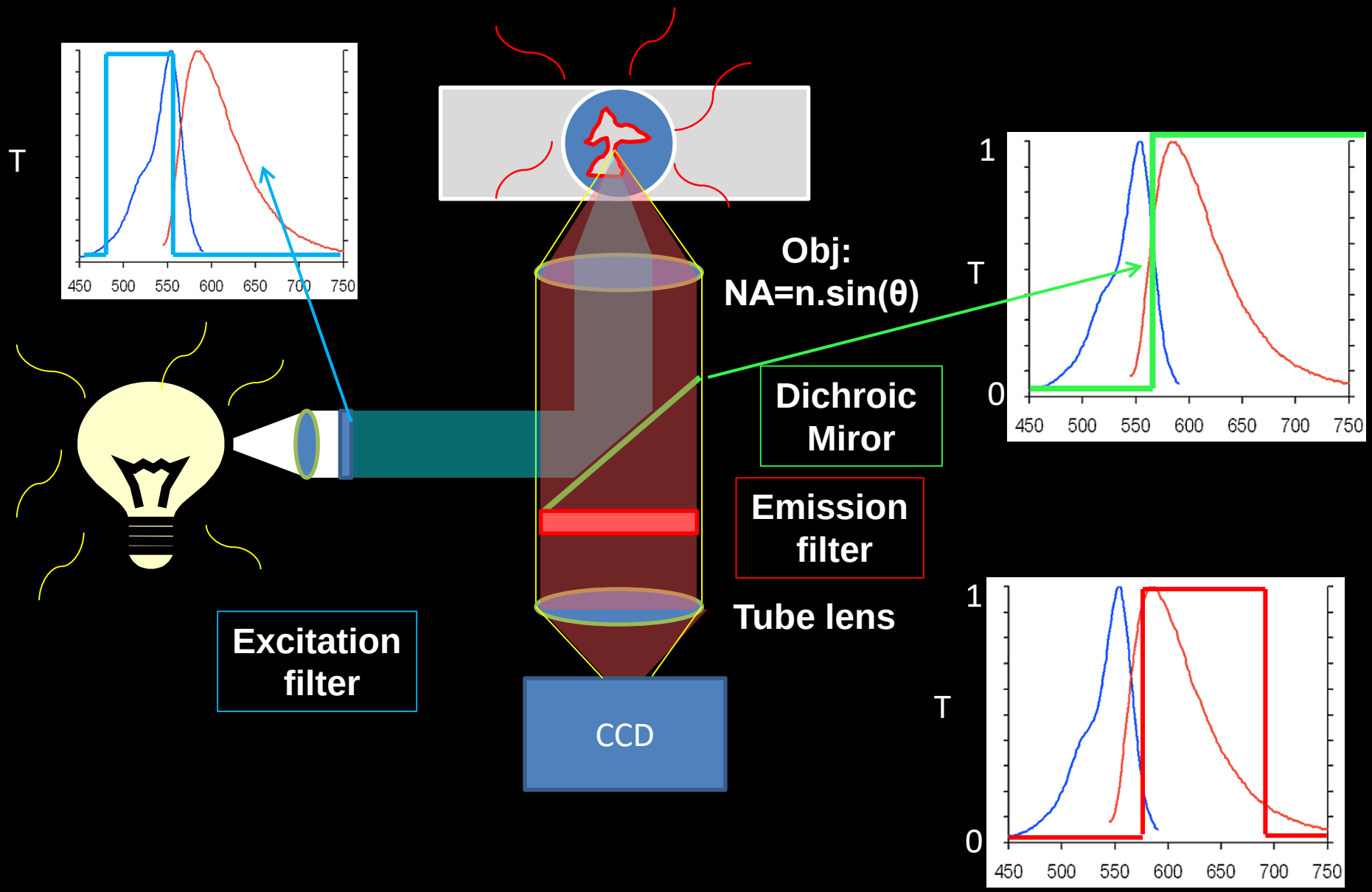
Proteins: GFP : 1994-1997

Nobel Prize Chemistry 2008
Tsien, Chalfie, Shimomura

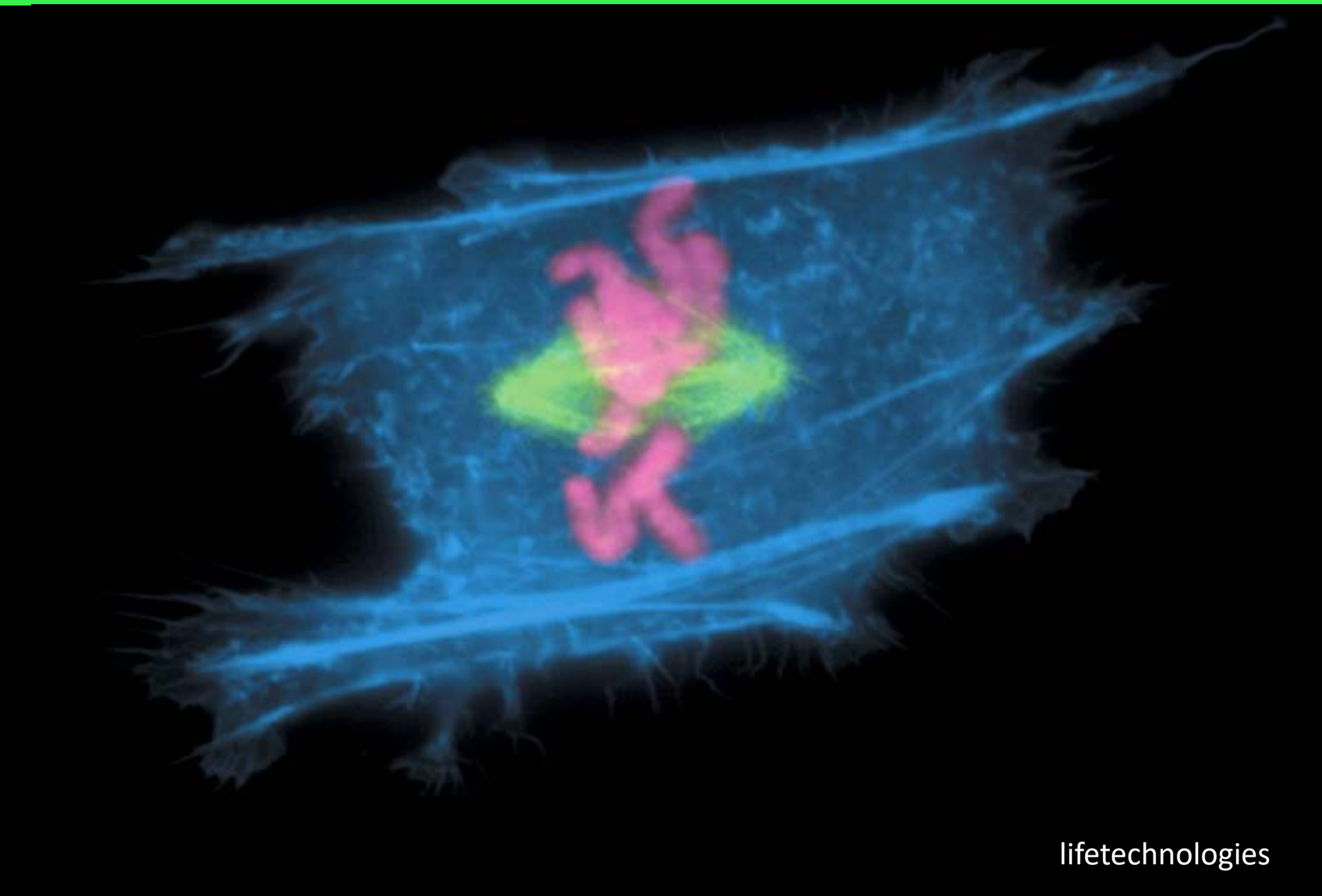


can target specific protein
in living cells

Fluorescence microscopy



Fluorescence microscopy



Looking at something specific: contrast mechanisms

Brightfield microscopy is easy, but has low contrast on cells

Darkfield, Phase contrast and DIC help improve the contrast through more complex optical implementations

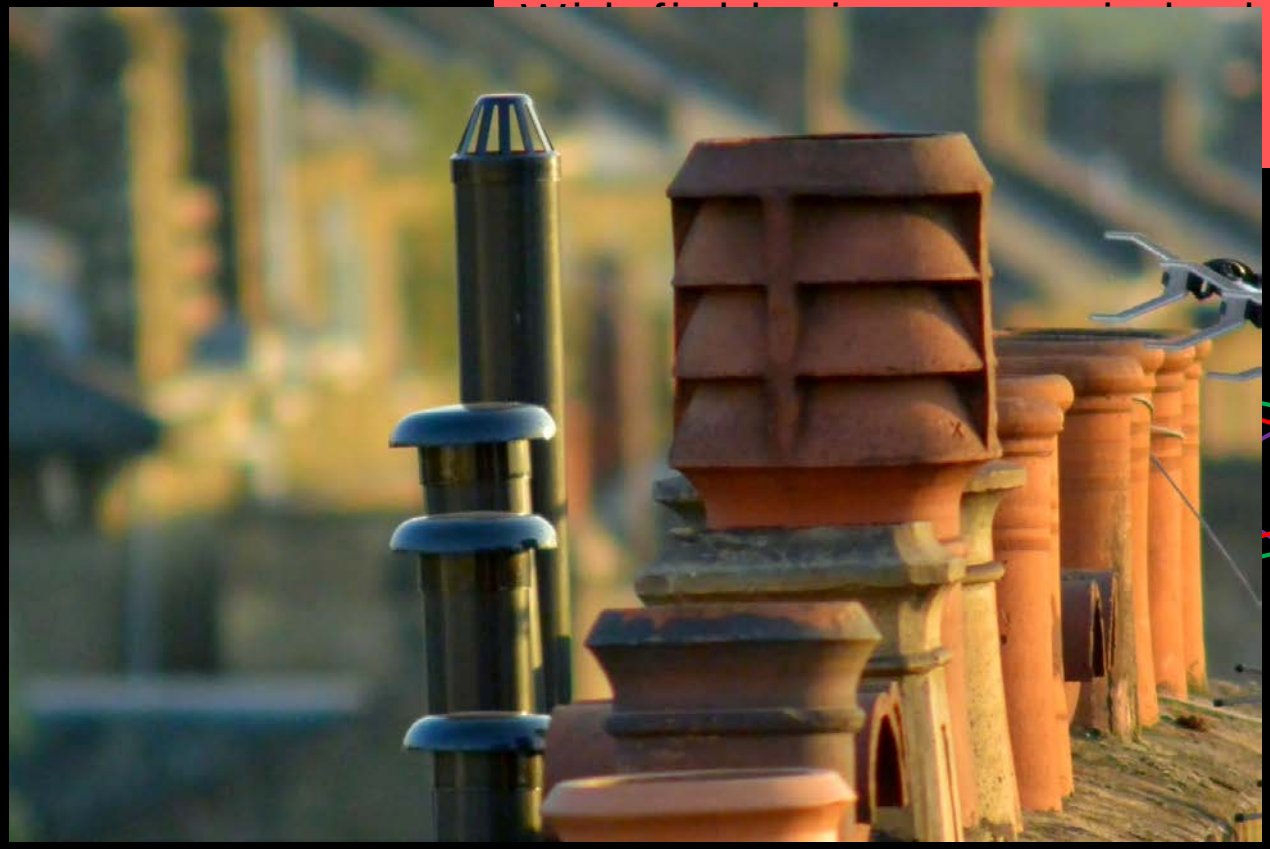
Fluorescence allows simple high contrast specific imaging (+ lots of exciting properties : next talk, super-res)

More contrast mechanisms exist (nonlinear microscopy, …)

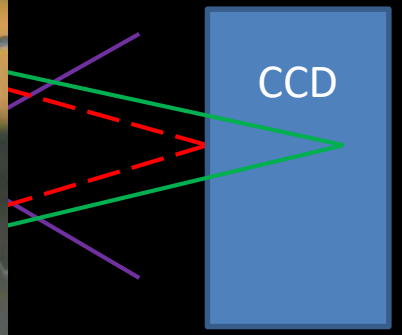
3D imaging, and tissue imaging

Confocal Microscopy

for

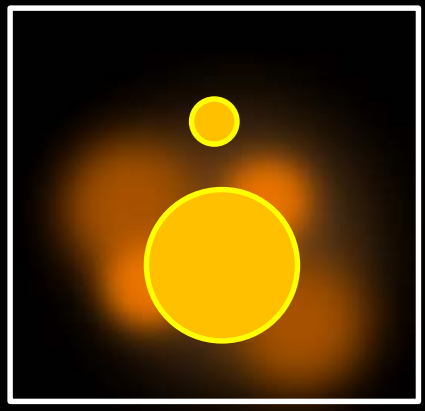


Camera sums all the different signals



Out of focus **Out of focus**

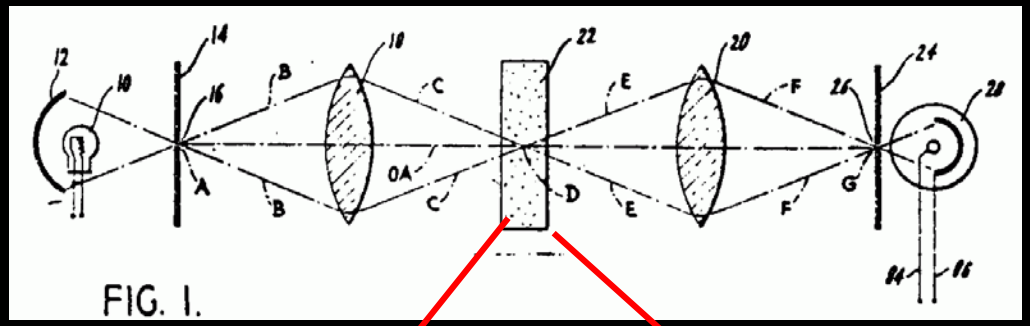
Same as photography



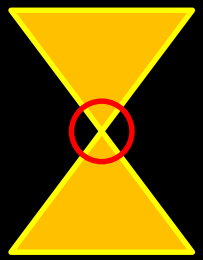
Confocal Microscopy

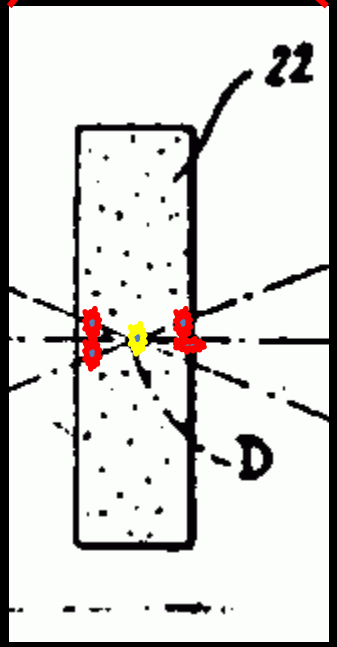
Confocal Microscopy: point illumination & point detection

Minsky 1957



Illumination: excites cone of light in sample





Detection: select only light coming from one point

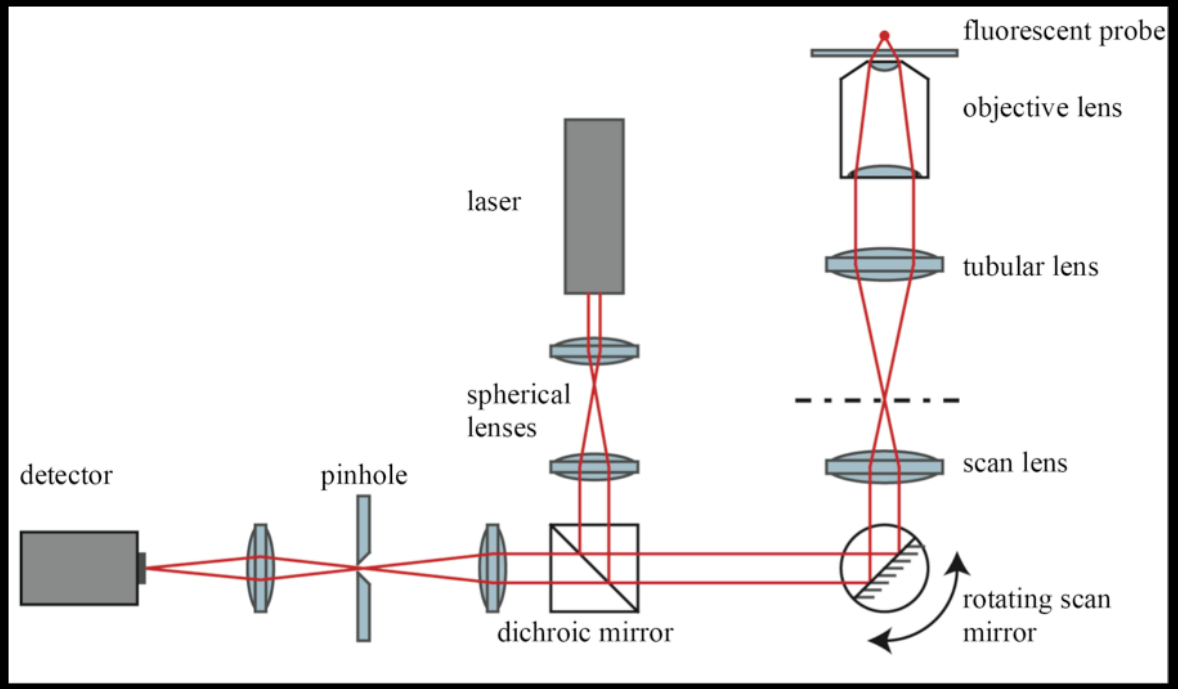
Idea: ONLY signal created at the focal point is detected

Instrument response = Excitation response x Detection response

Point-scanning technique: originaly sample, now laser beam

Laser-scanning Confocal Microscopy

Sheppard, Wilson, Cremer, Brakenhoff…



-Allows 3D resolution
- Increases 2D resolution (up to 40% but loss of signal)

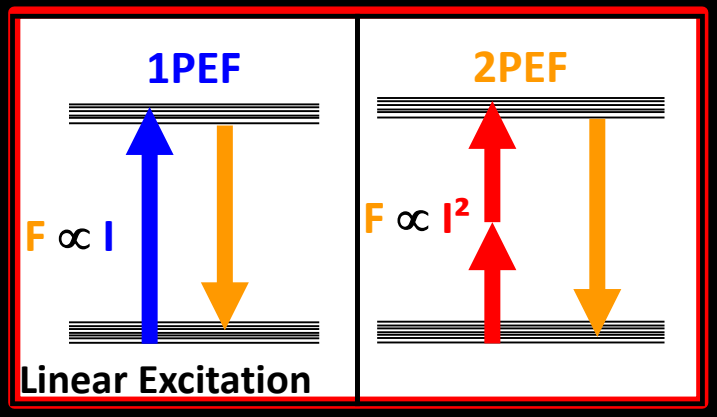
http://malone.bioquant.uni-heidelberg.de/methods/imaging/imaging.html

Nonlinear Microscopy

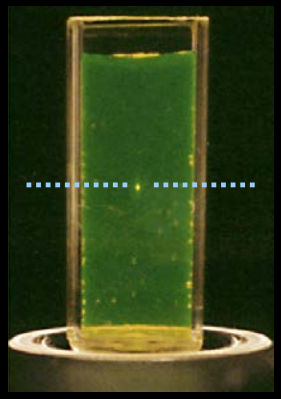
Imaging technique that relies on optical nonlinear effects which intensity depends nonlinearly on the excitation intensity

⇒ The nonlinear effects are confined in the focal volume

Example: 2PEF
2 photon Excited fluorescence



S ∝ I
⇒ not confined



Zipfel & Webb (Cornell University)

S ∝ I^2 ou I^3
⇒ **confined excitation**

⇒ Intrinsic point-scanning technique

3D imaging, and tissue imaging

Confocal microscopy allows 3D imaging of biological structures

Nonlinear microscopy extends the range of confocals for deep tissue imaging

Light-sheet microscopy uses a completely different approach to achieve the same goal

Basics of Optical Microscopy Conclusion

Lots of different microscopy methods, choose the most adapted to your sample

Magnification and resolution are different: increase resolution by decreasing λ and increasing NA

Fluorescence based-methods are very powerful as you'll see in next talk ☺