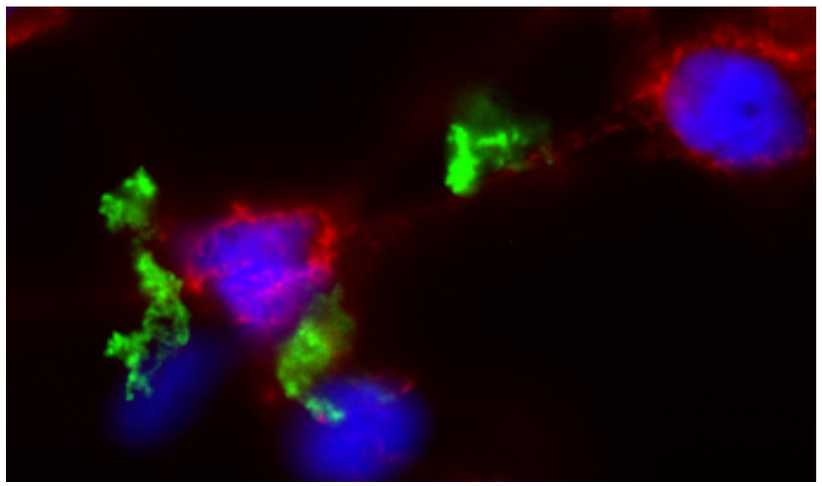
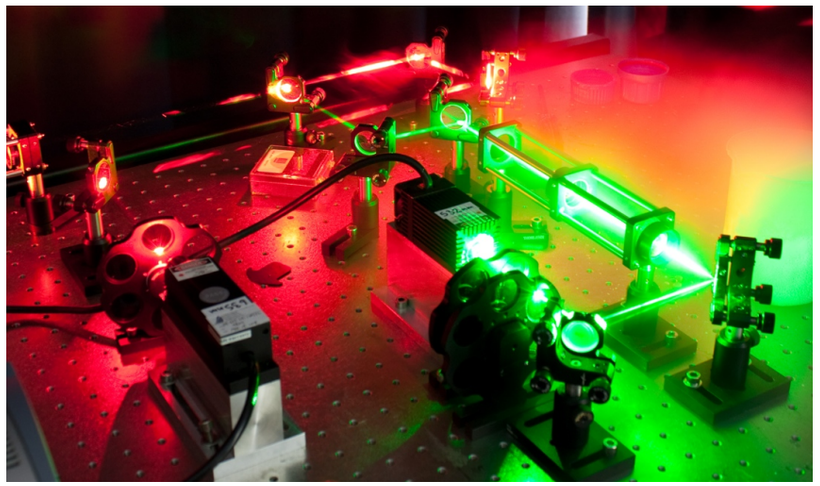
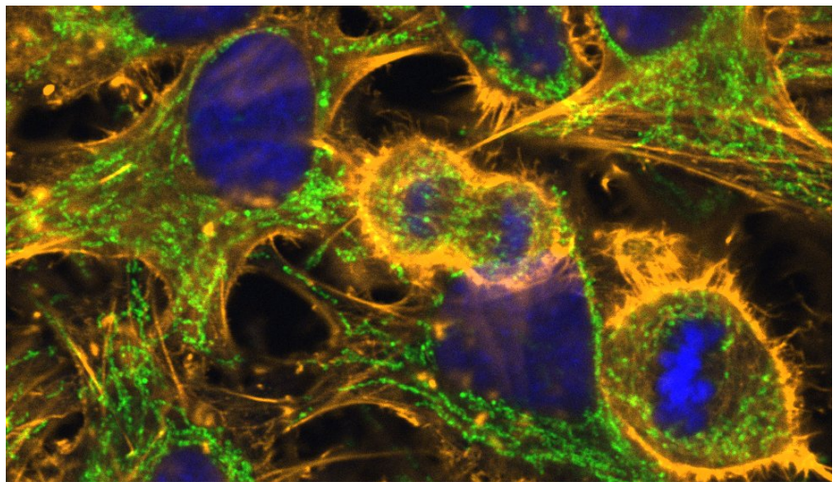
Understanding and Applying Fluorescence Microscopy







Carina Mónico

Micron assistant manager

Outline

Part I:

What is fluorescence?

Why fluorescence?

Fundamental problem in fluorescence microscopy

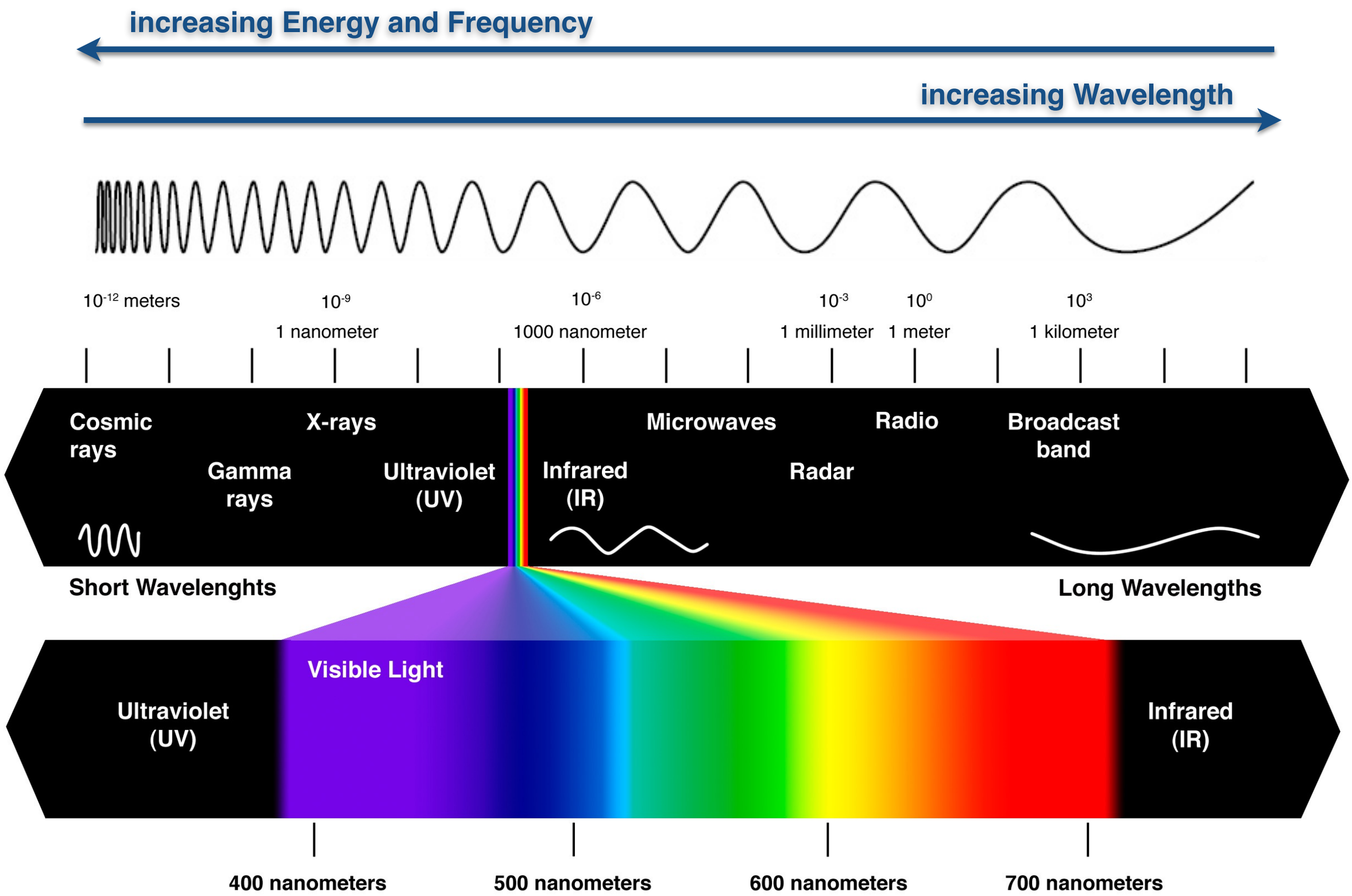
Components of the fluorescence microscope: dichroic mirror

Fluorescent light sources

Part II: Tips on sample preparation - Fixed samples

Part III: Point Spread Function and Optical Transfer Function

Light: the electromagnetic spectrum



380 – 700 nm visible to the human eye

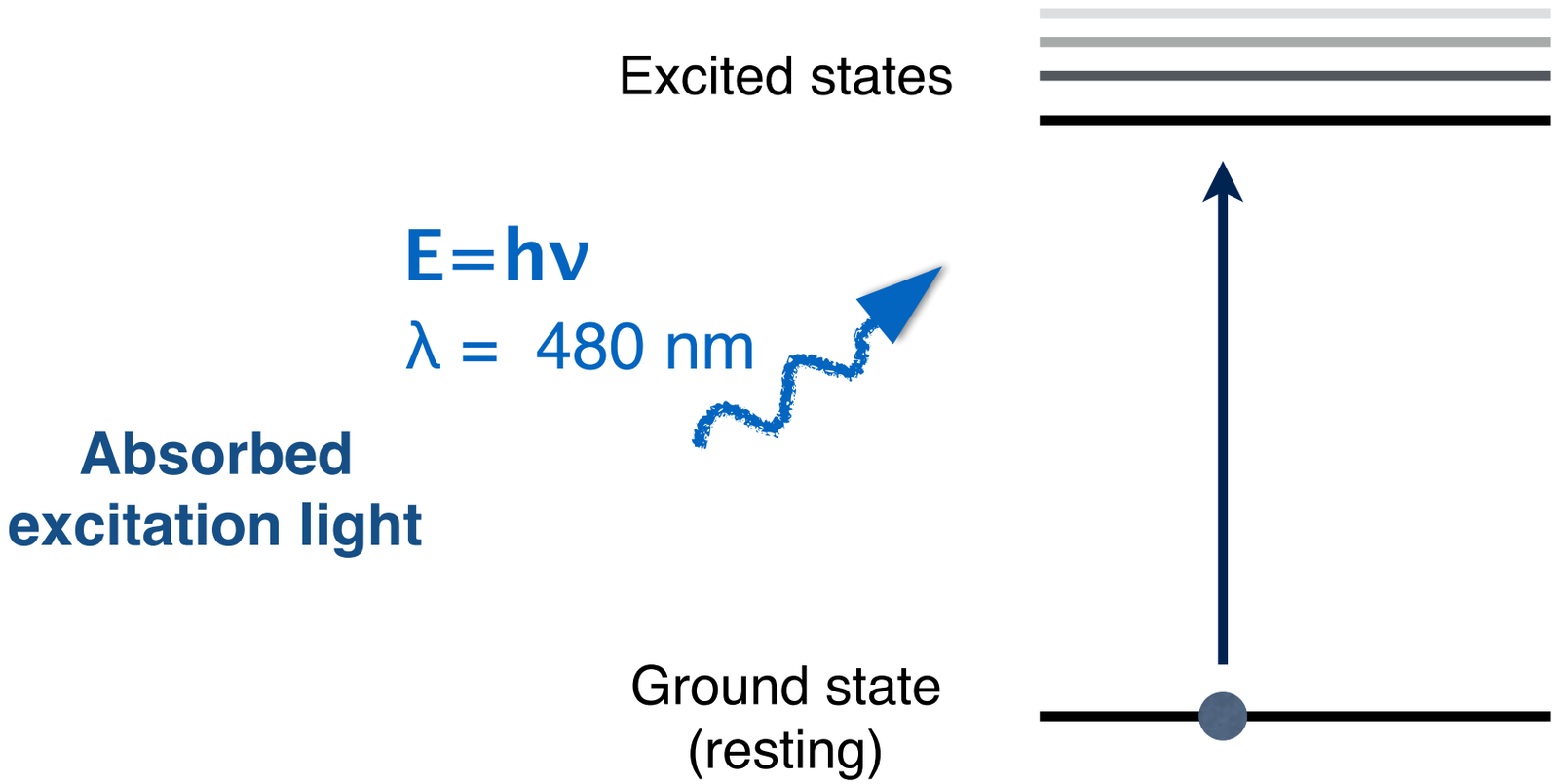
What is Fluorescence?

Fluorescence is the emission of light by a molecule that has absorbed light

Molecules have discrete levels of energy

What is Fluorescence?

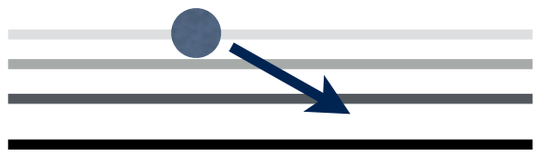
Fluorescence is the emission of light by a molecule that has absorbed light



A photon is the energy unit for light to interact with matter

What is Fluorescence?

Excited states



non-radiative transition

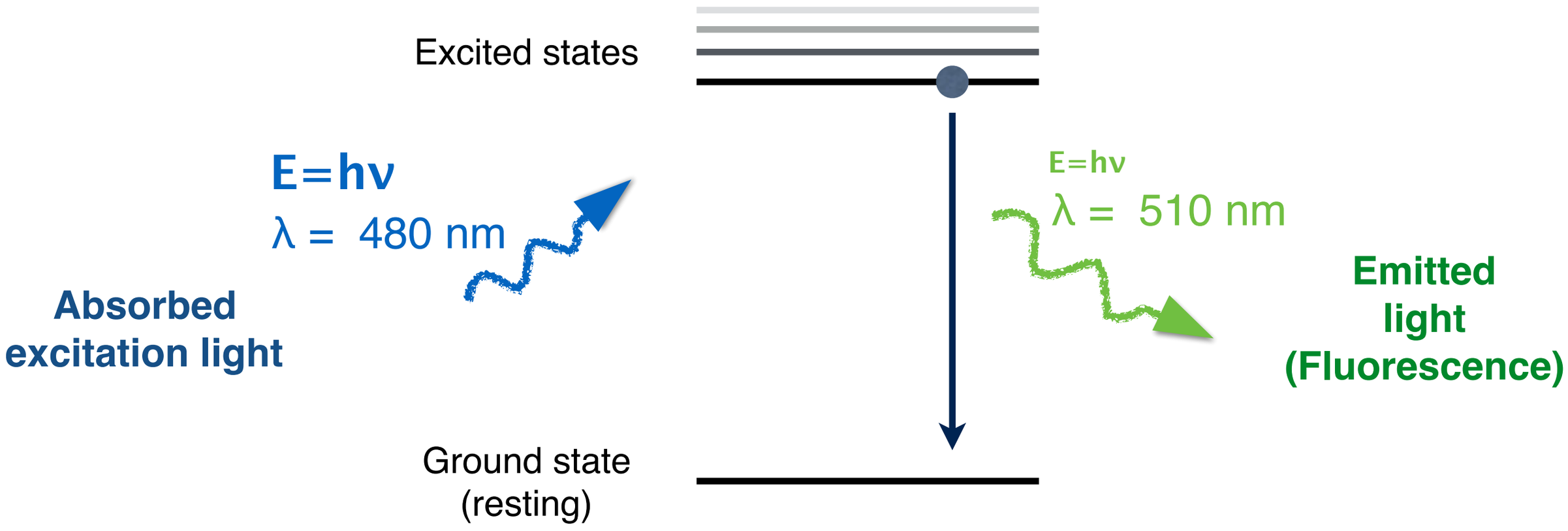
E=hν

λ = 480 nm

Absorbed excitation light

Ground state (resting)

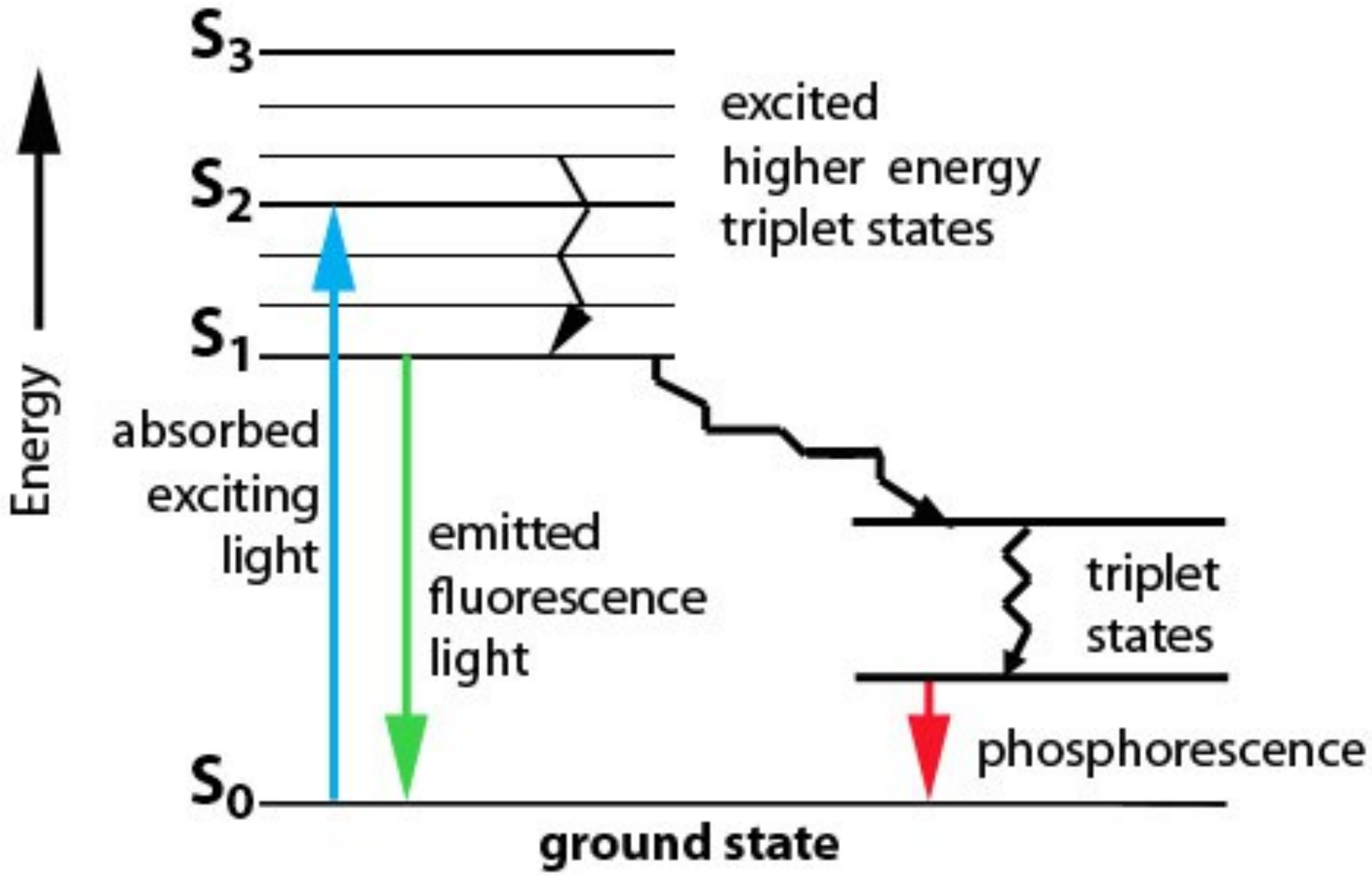
What is Fluorescence?



Fluorescence has higher wavelength than absorbed light

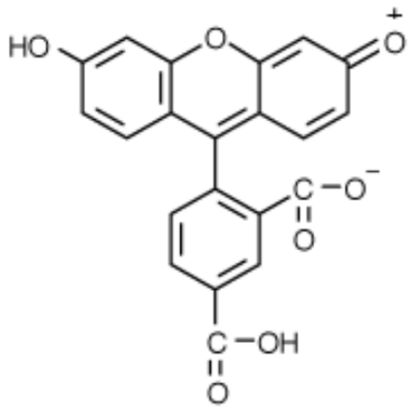
The full picture is represented on the Jablonski diagram…

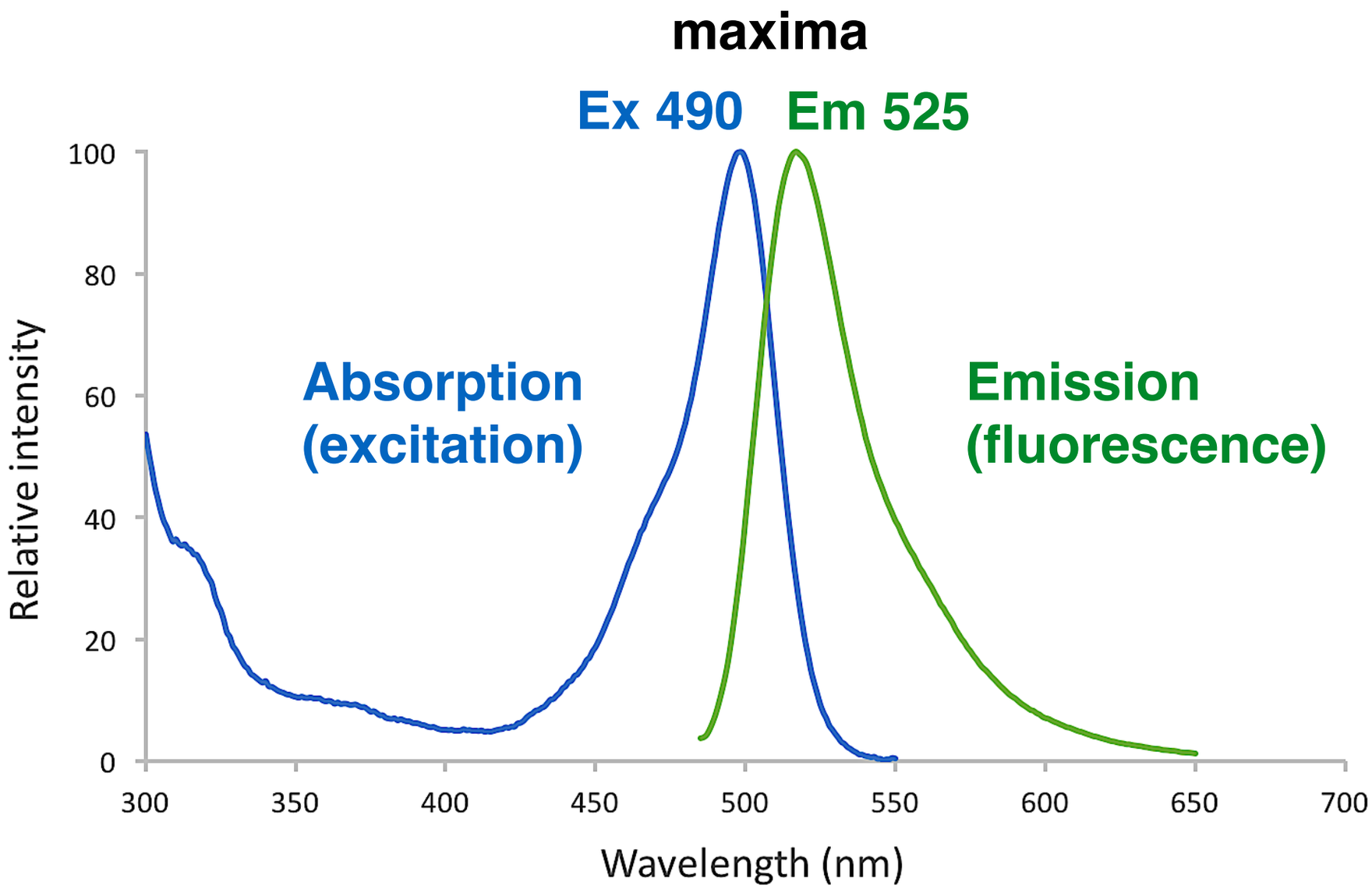
→ Lecture 6



Fluorescence Spectra

Fluorescein (FITC)





Fluorescence Spectra

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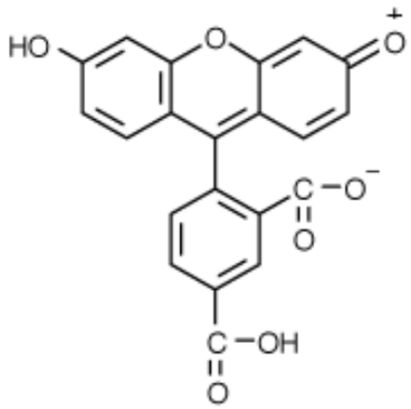
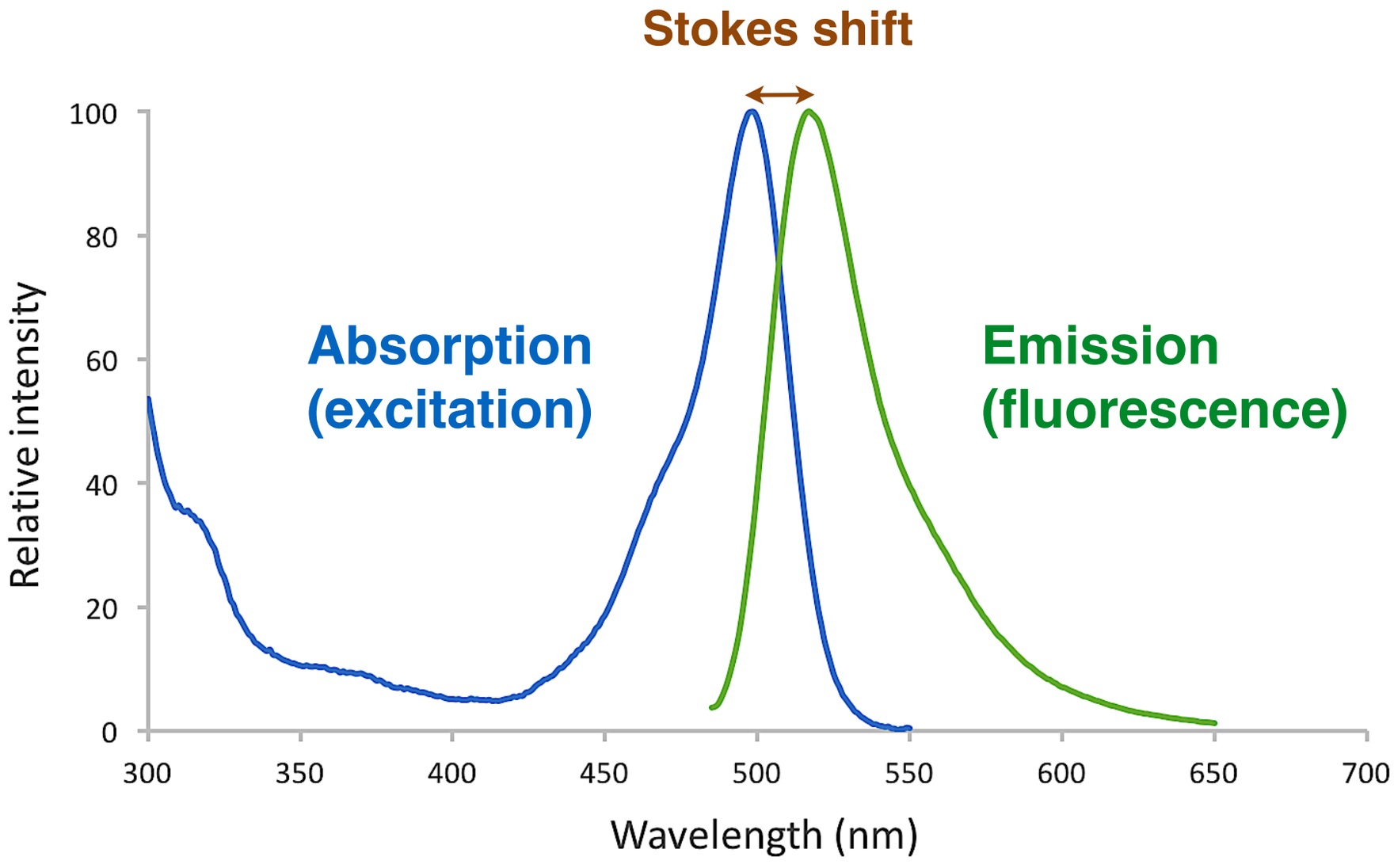




Fluorescent probes for microscopy

→ Lecture 6

Genetically encoded fluorescent proteins

- GFP, YFP, mCherry

Organic dyes

- Alexa, ATTO, Fluorescein, DAPI, Cyanine (Cy3, Cy5)
- Fluorescent labelled antibodies (immunofluorescence)

Inorganic dyes

- Quantum Dots

Endogenous species

- Elastin, collagen, metabolic coenzymes (NADH, FAD)

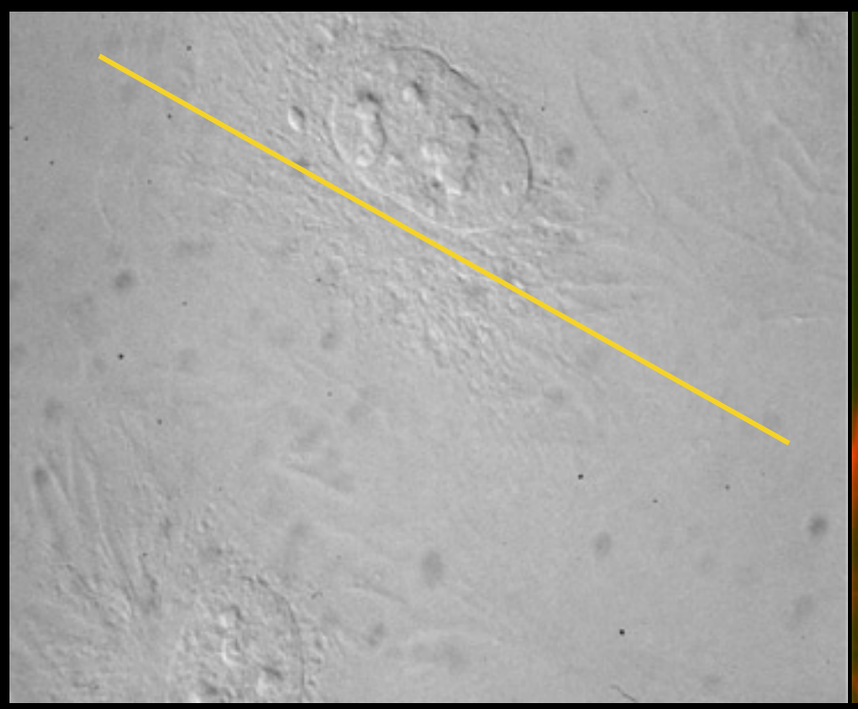
Why Fluorescence?

Chris Teren: https://www.youtube.com/watch?v=PhclTQ3g0s8

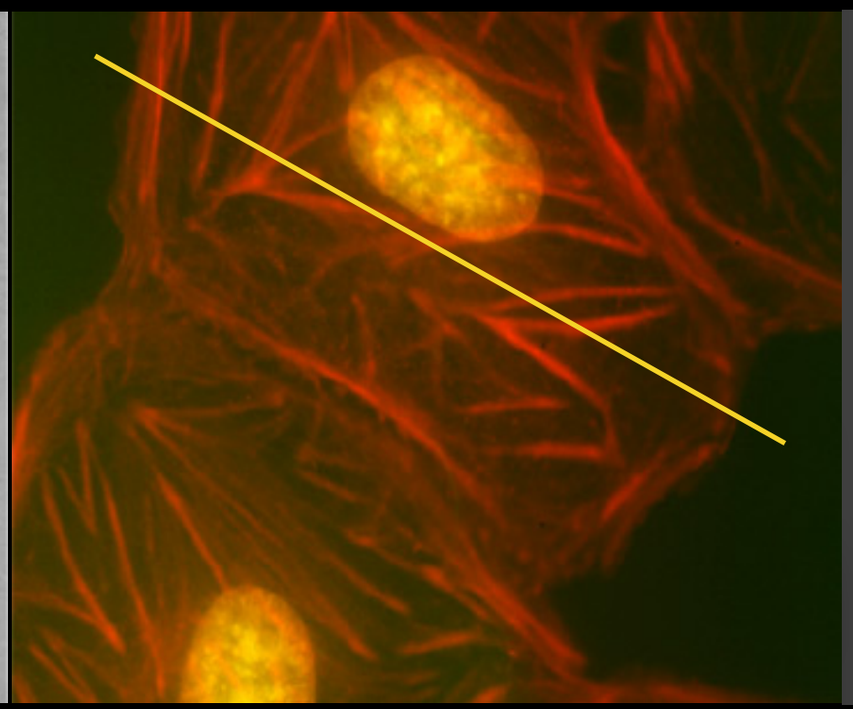
CONTRAST

Why Fluorescence?

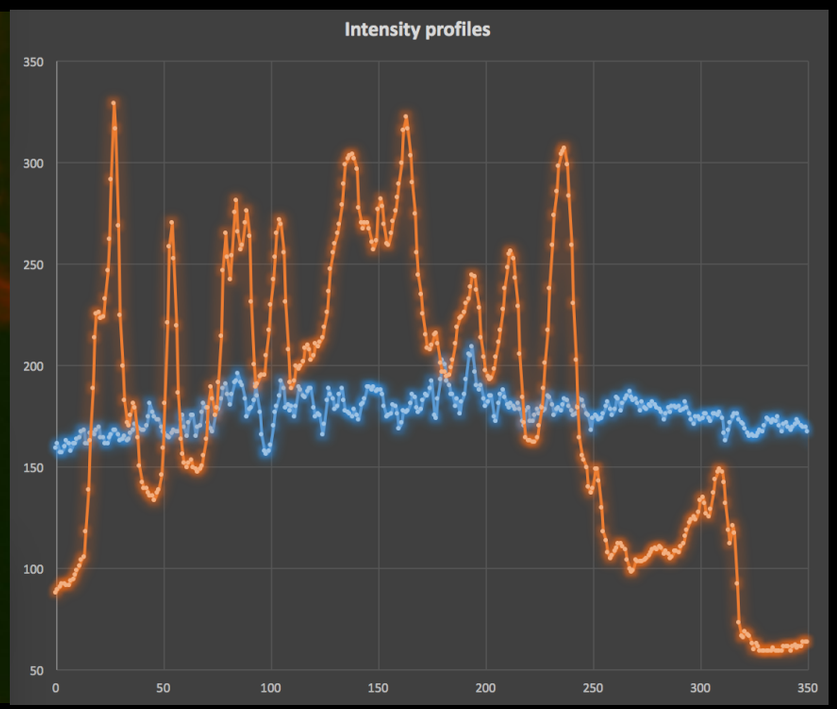
- Weak signal against dark background is easier to measure
- High signal to background - contrast



bright field (DIC)



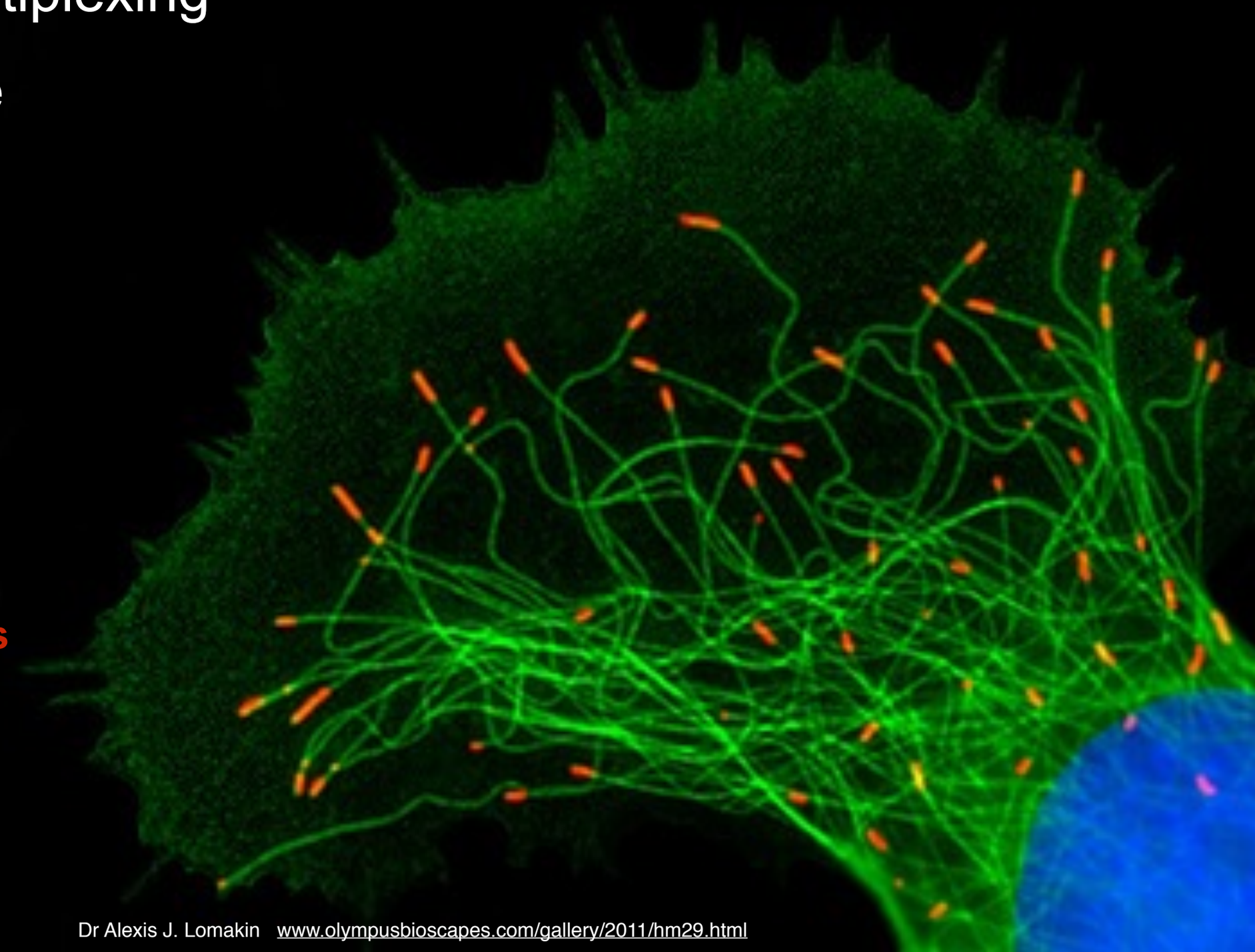
fluorescence



Intensity profile

Why Fluorescence?

- Selective labeling
- Ease of multiplexing
- Quantitative



Dr Alexis J. Lomakin www.olympusbioscapes.com/gallery/2011/hm29.html

Why is the background black In a fluorescent image ….?

Widefield deconvolution

Confocal

TIRF

FCS

dSTORM

PALM

Multi-photon

STED

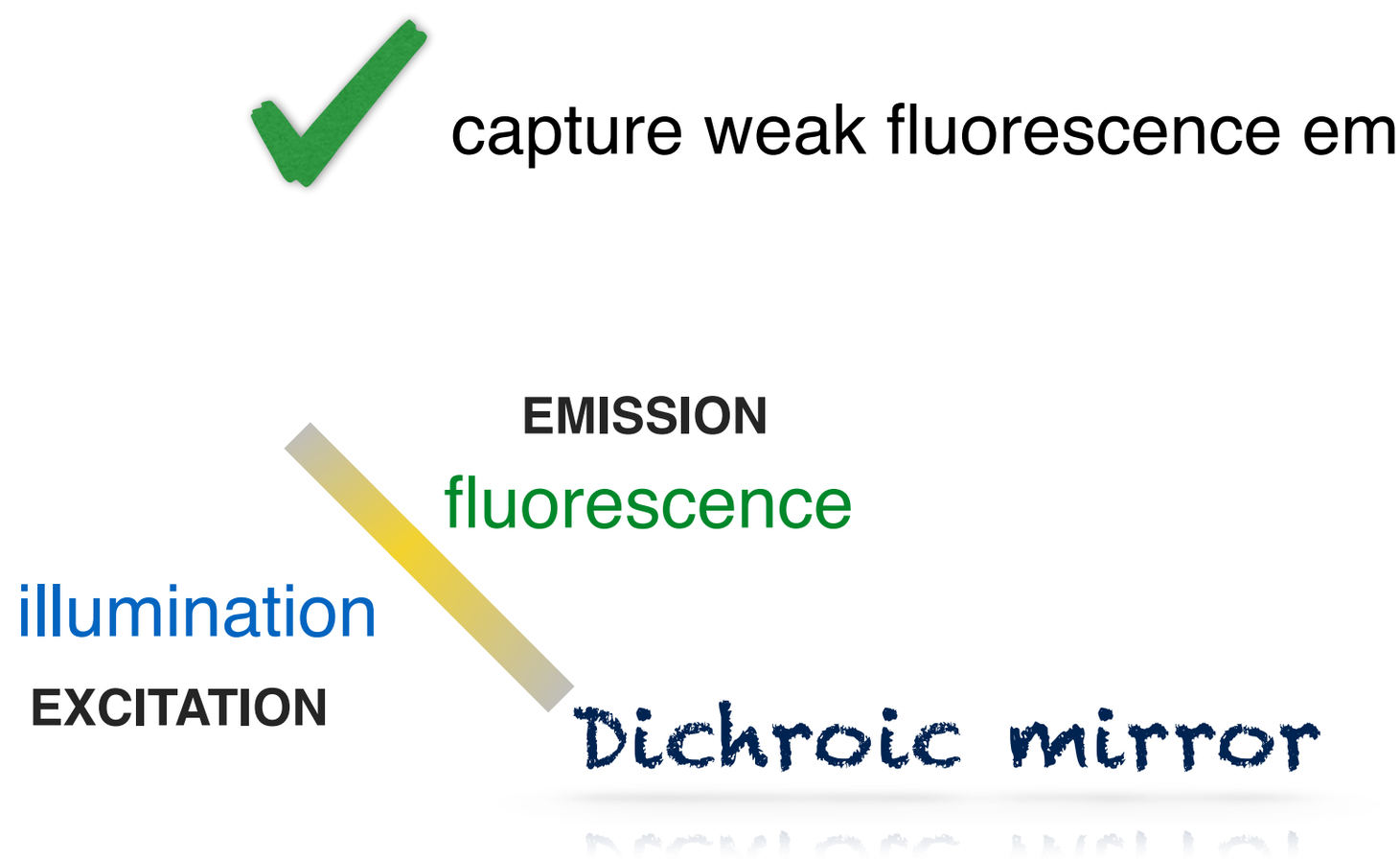
3D-SIM

Fundamental problem in fluorescence microscopy

STRONG illumination VS. **WEAK fluorescence signal**

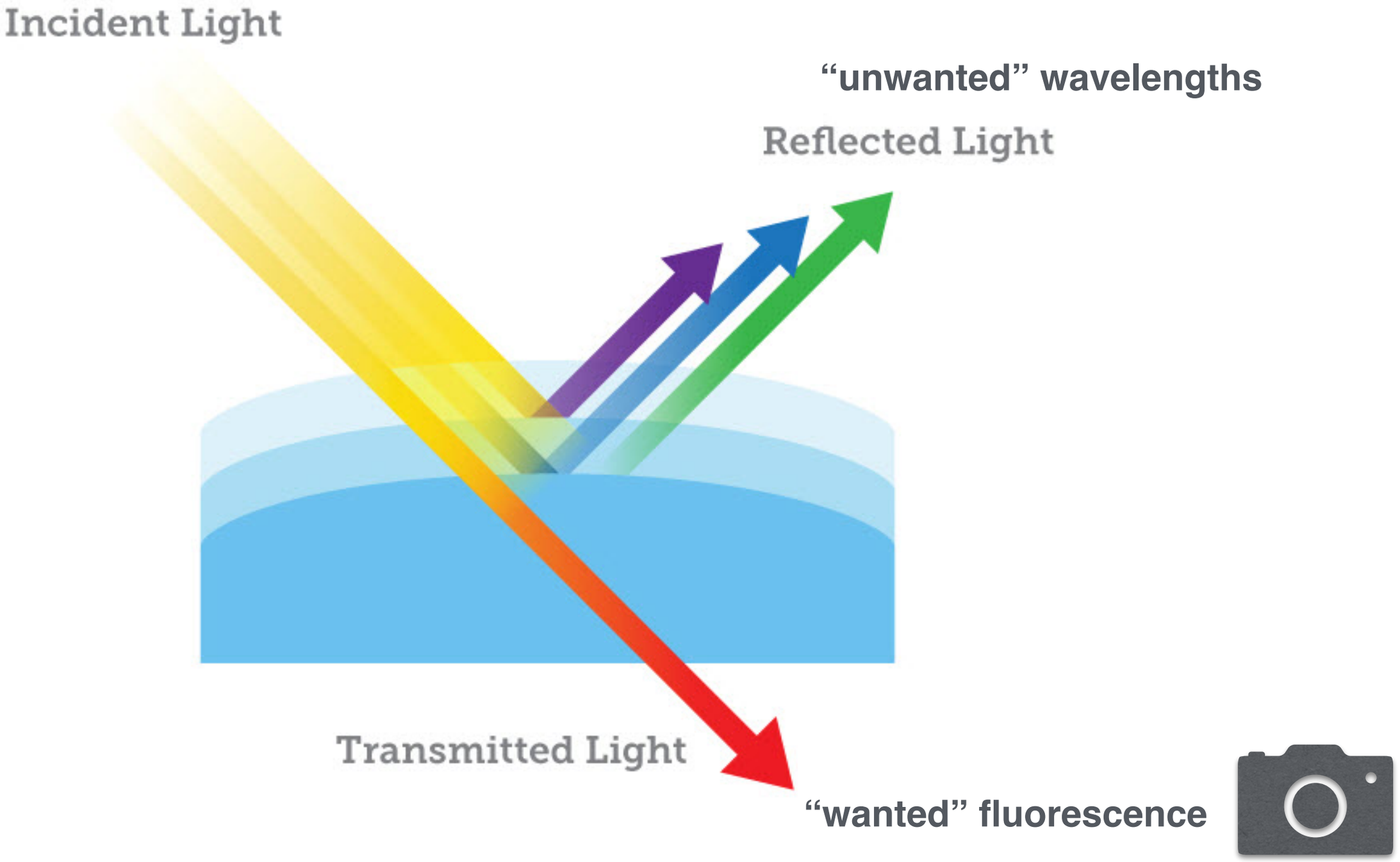
✓ produce high-efficient illumination of the specimen

✓ capture weak fluorescence emission

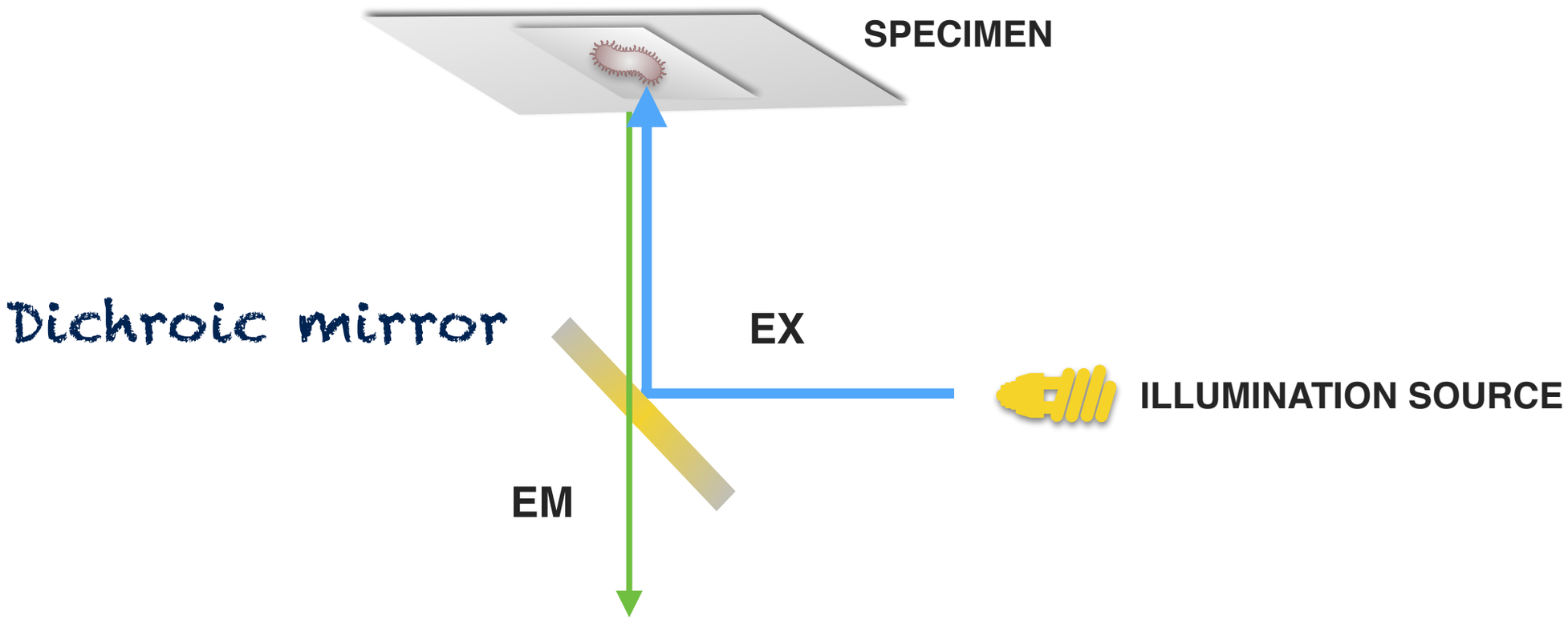


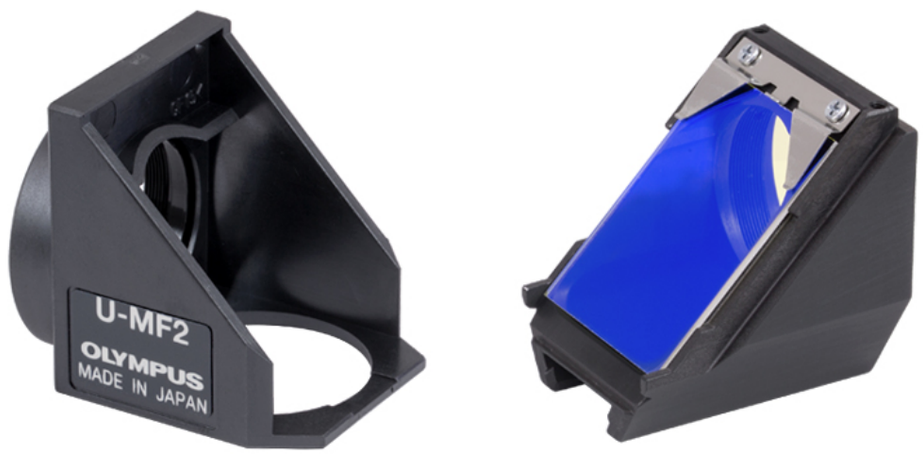
Dichroic mirror - at the heart of fluorescence microscopy

Dichroic mirrors are made by coating a glass substrate with a series of optical coatings

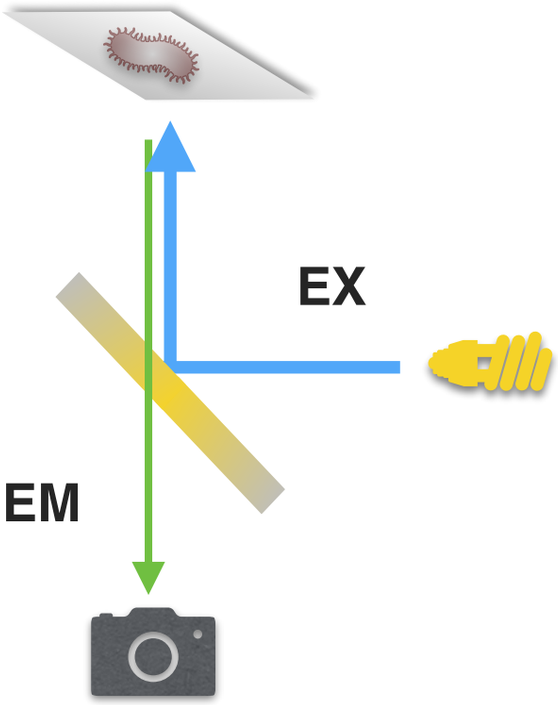


Dichroic mirror - at the heart of fluorescence microscopy



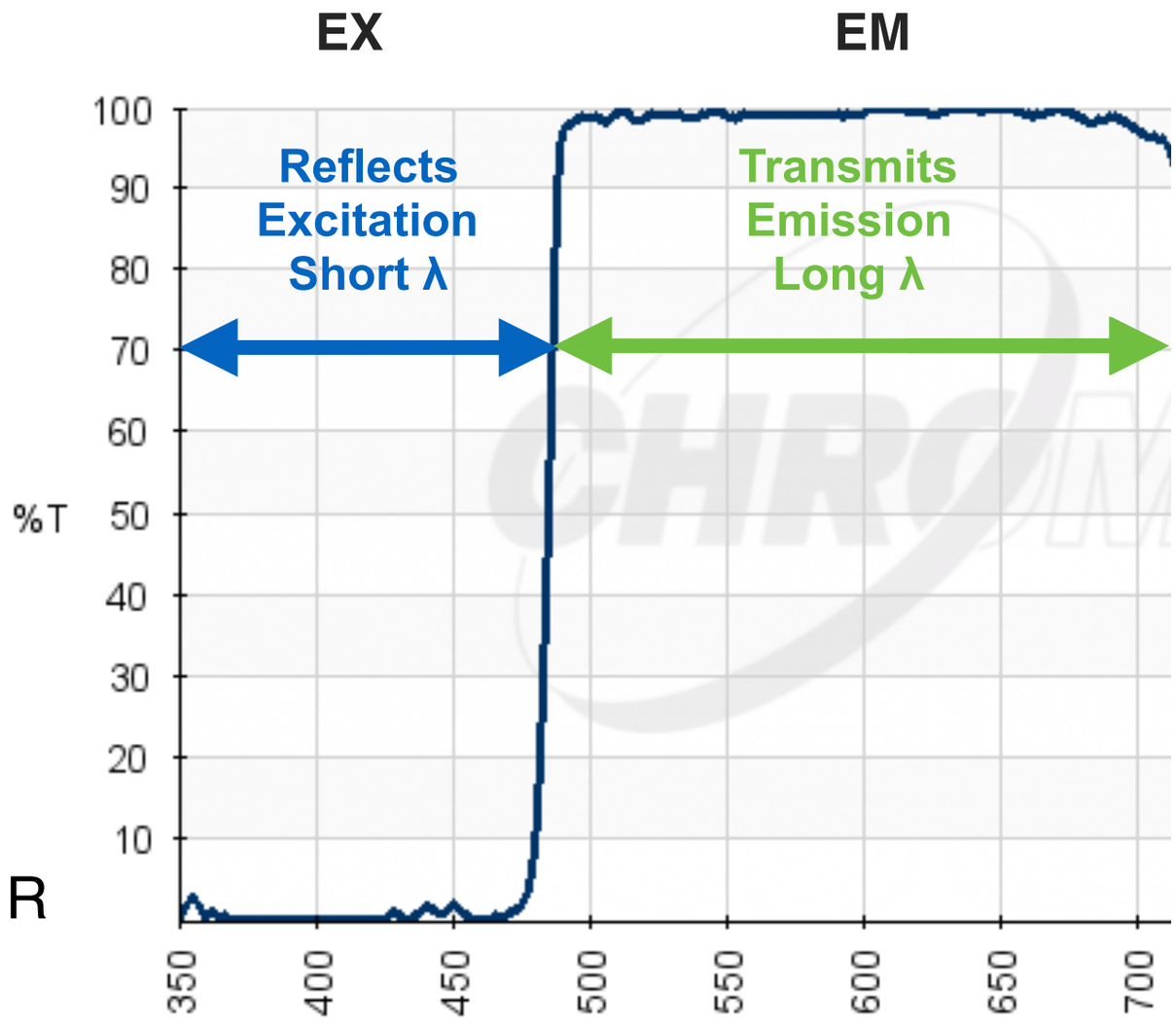


Dichroic mirror - Spectral properties



Dichroic

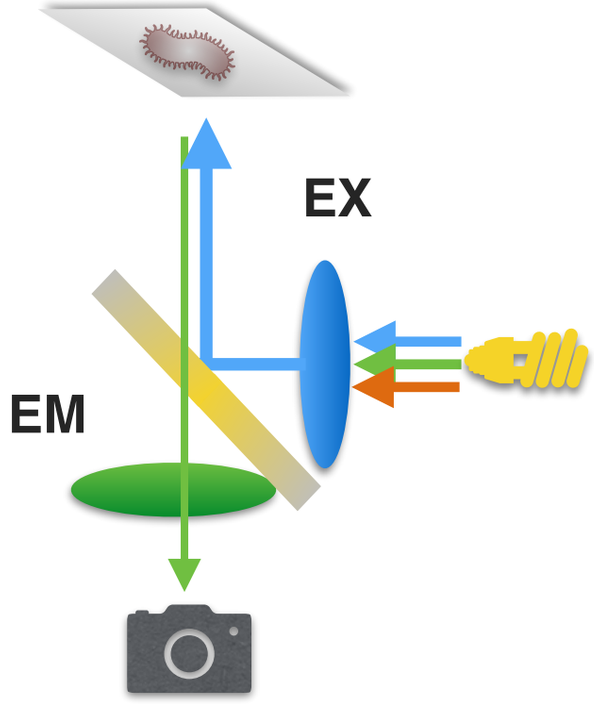
100% T = 0% R



0% T = 100% R

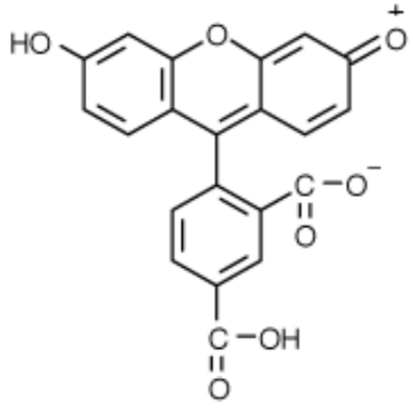
Separates excitation light from emission light

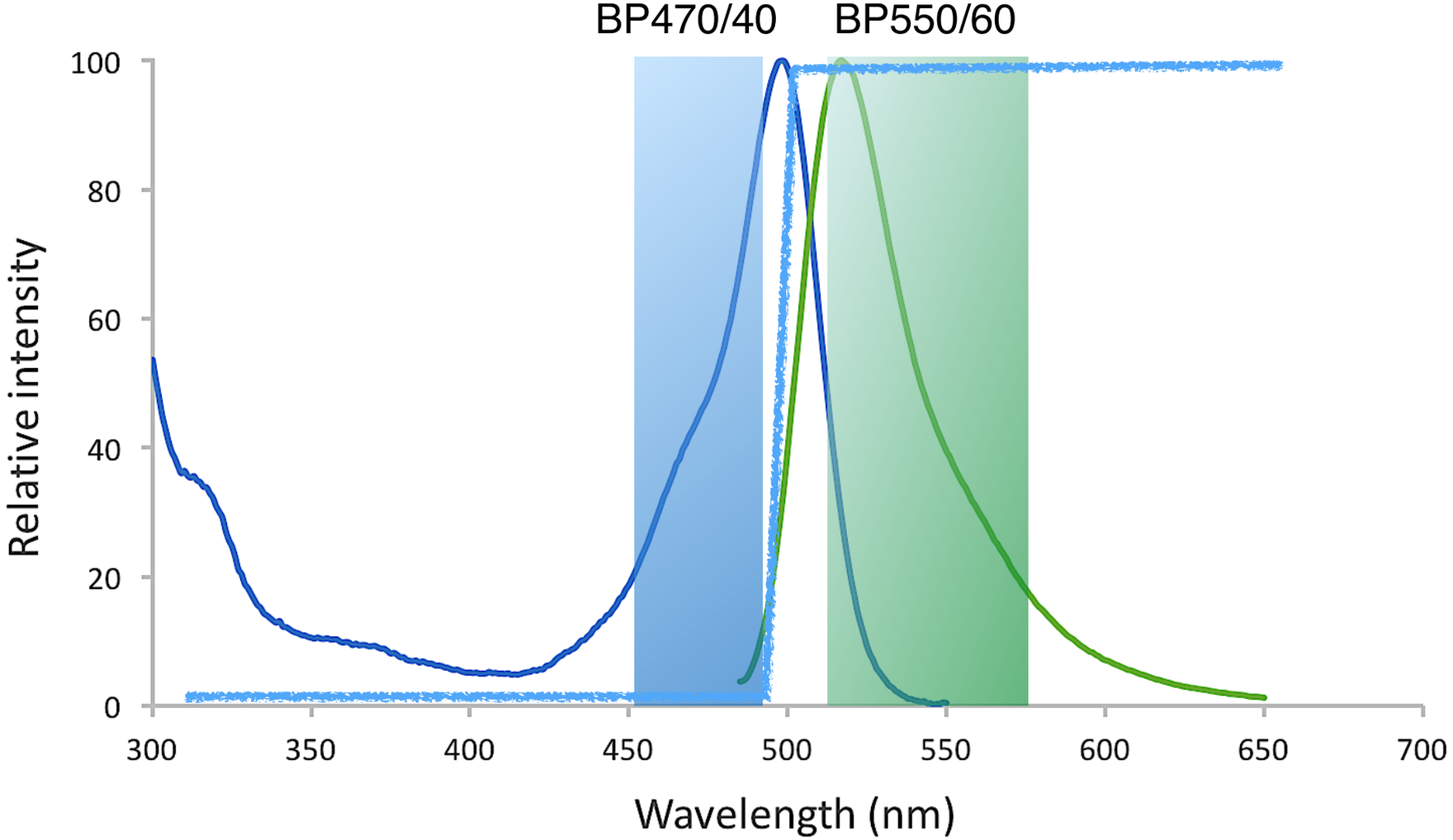
Dichroic, excitation and emission filters



… related to dye spectrum

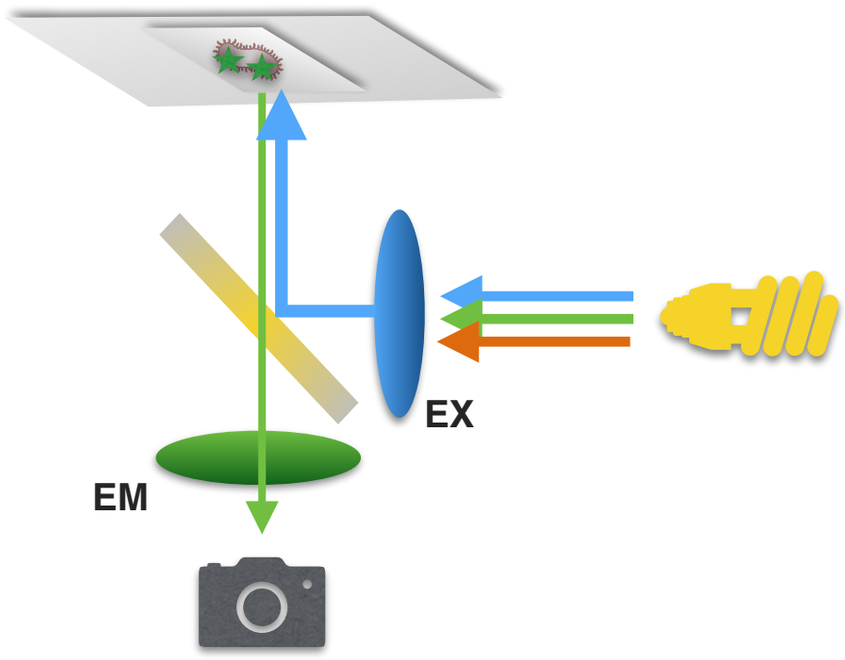
Fluorescein (FITC)





What about multiplexing…?

Fluorescein (FITC), GFP



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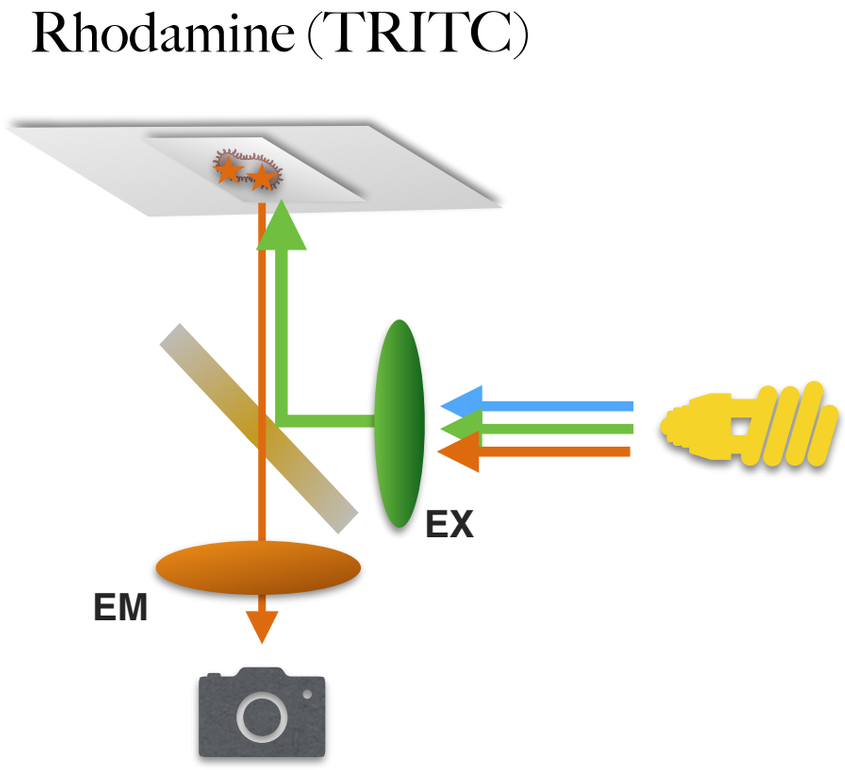
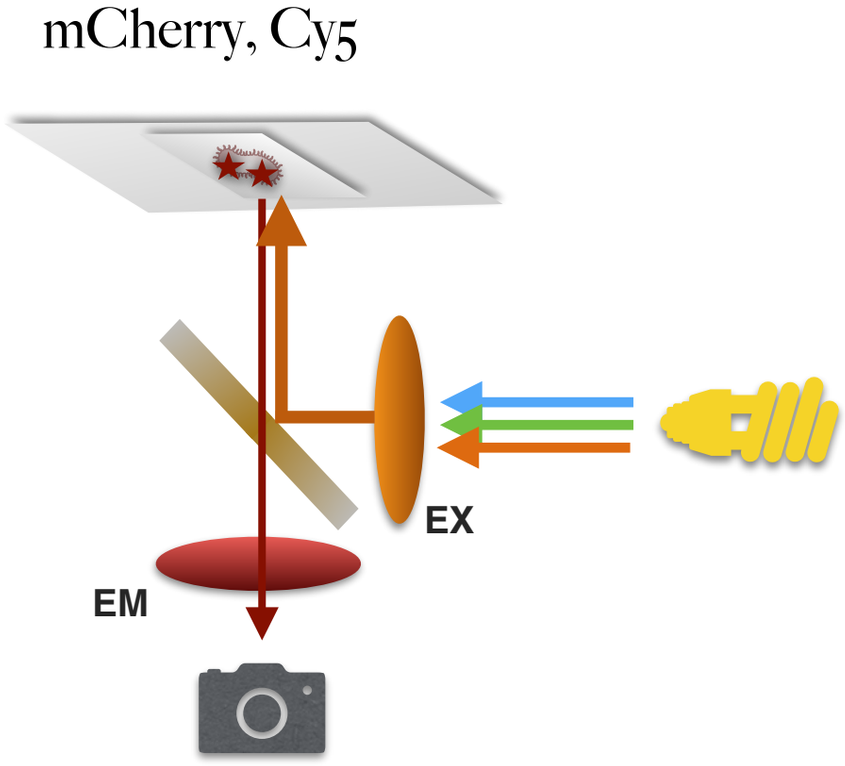
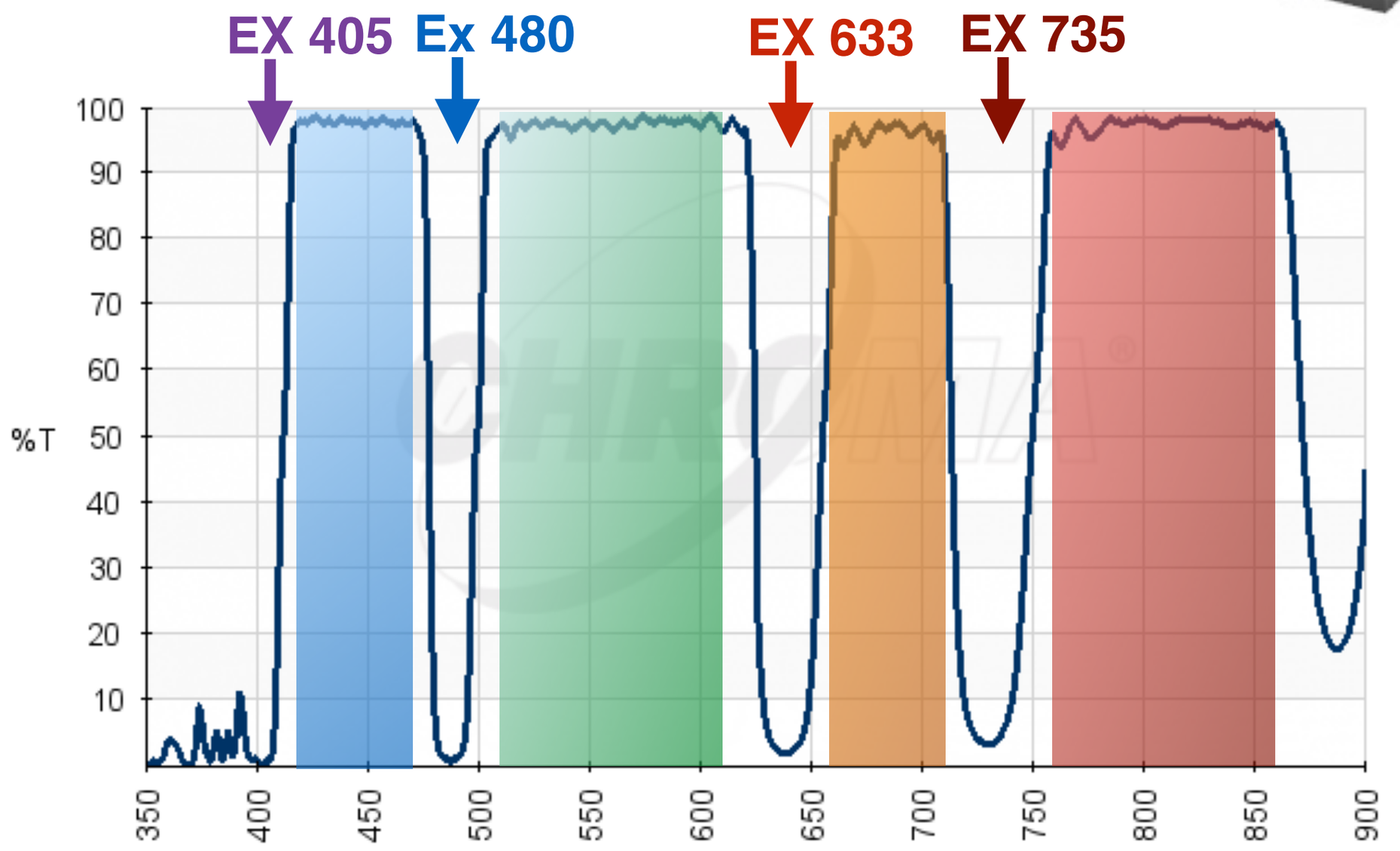


What about multiplexing…?



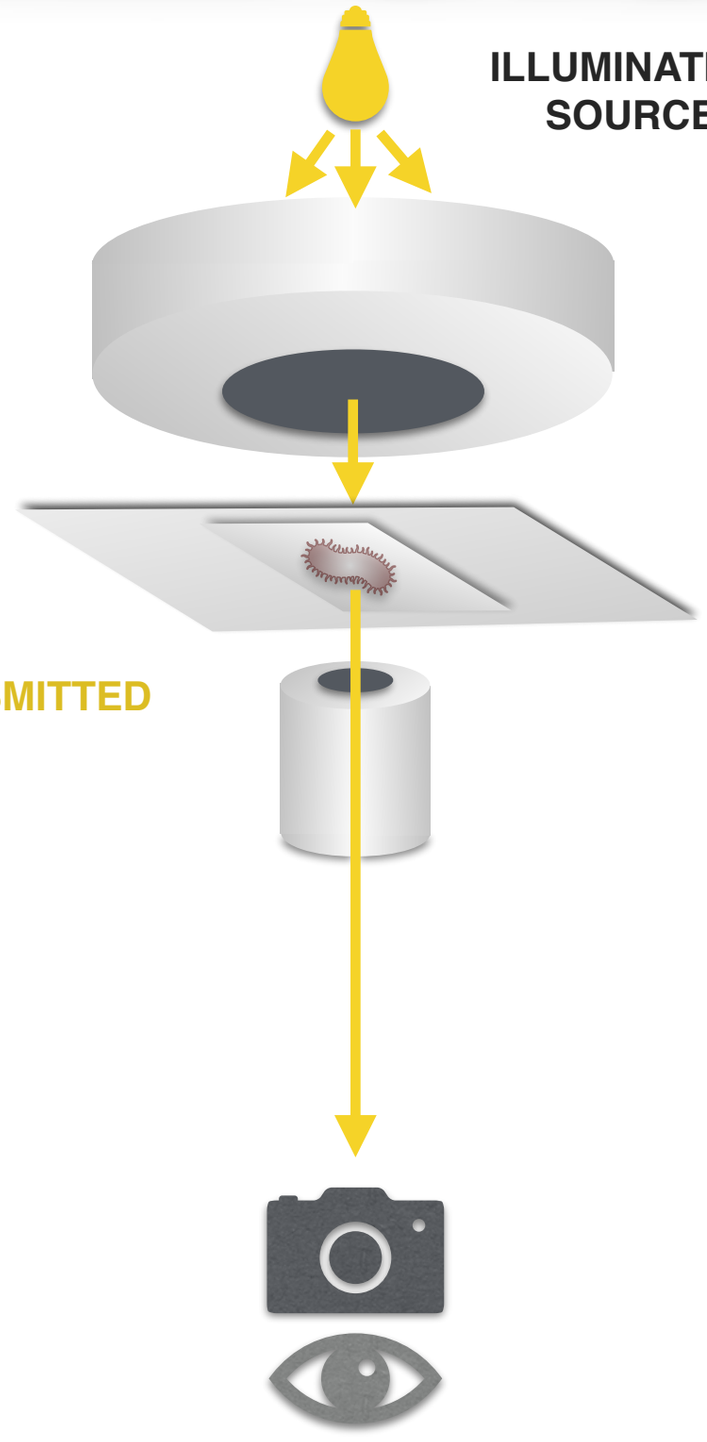
What about multiplexing…?

Polichroic



Components of a microscope: Brightfield vs. Fluorescence

Transmitted Light (Brightfield)



CONDENSER

SPECIMEN

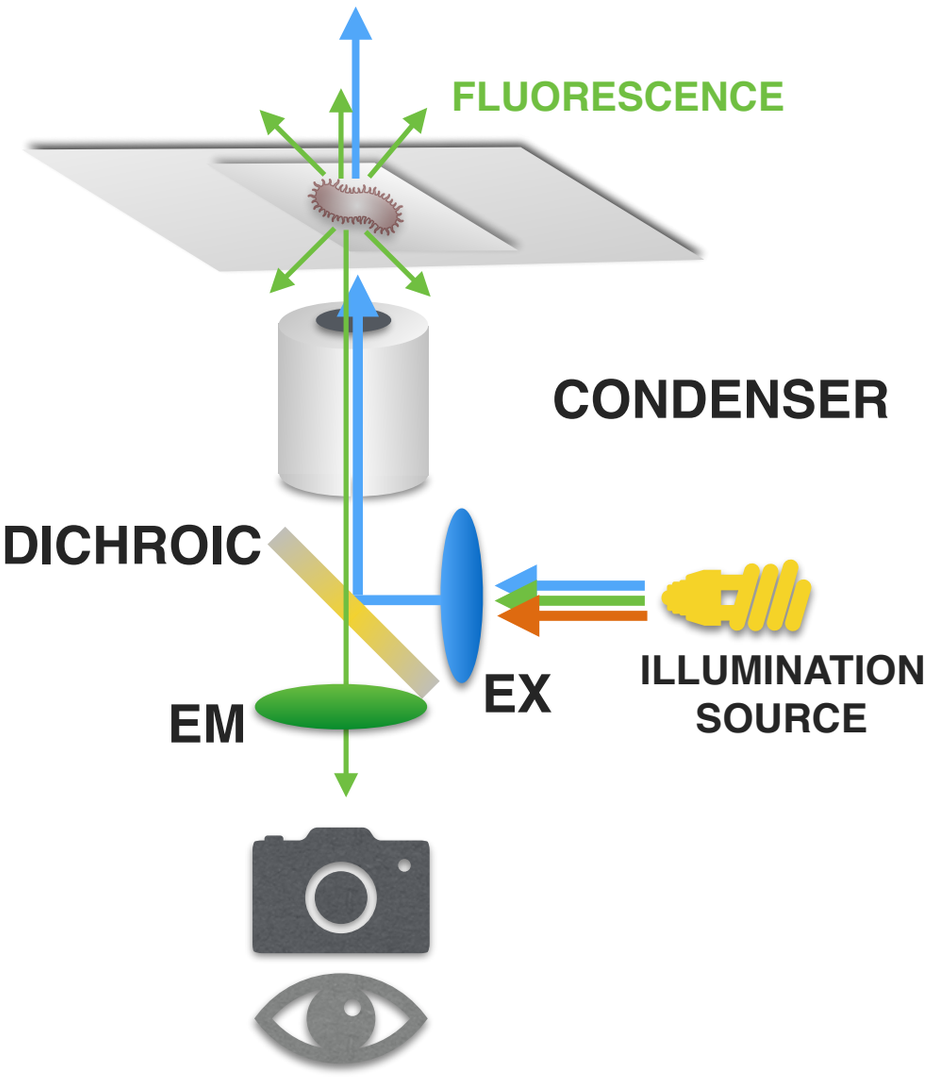
OBJECTIVE LENS

DETECTOR

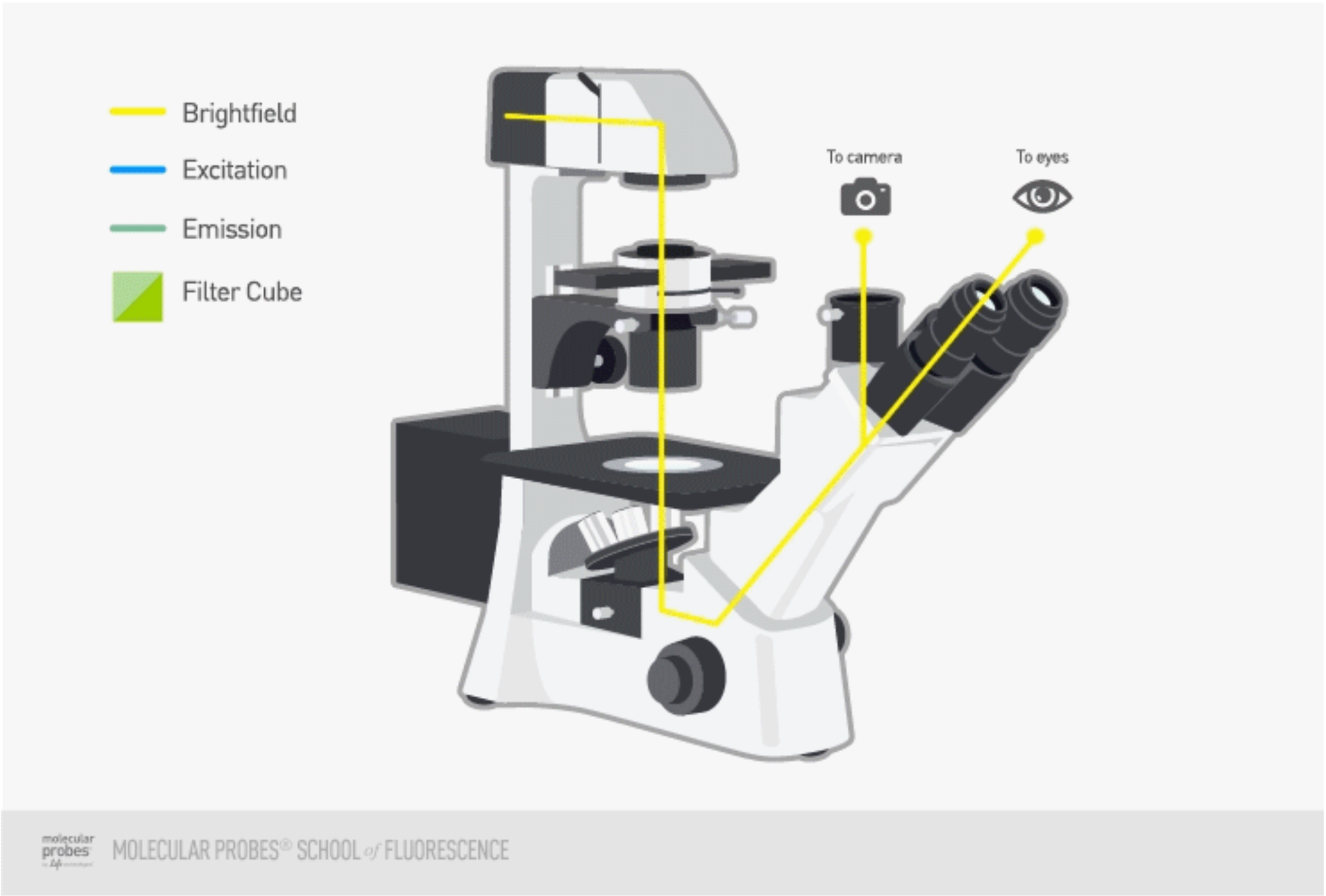
EYEPIECE

Reflected Light (Fluorescence)

Epifluorescence Widefield

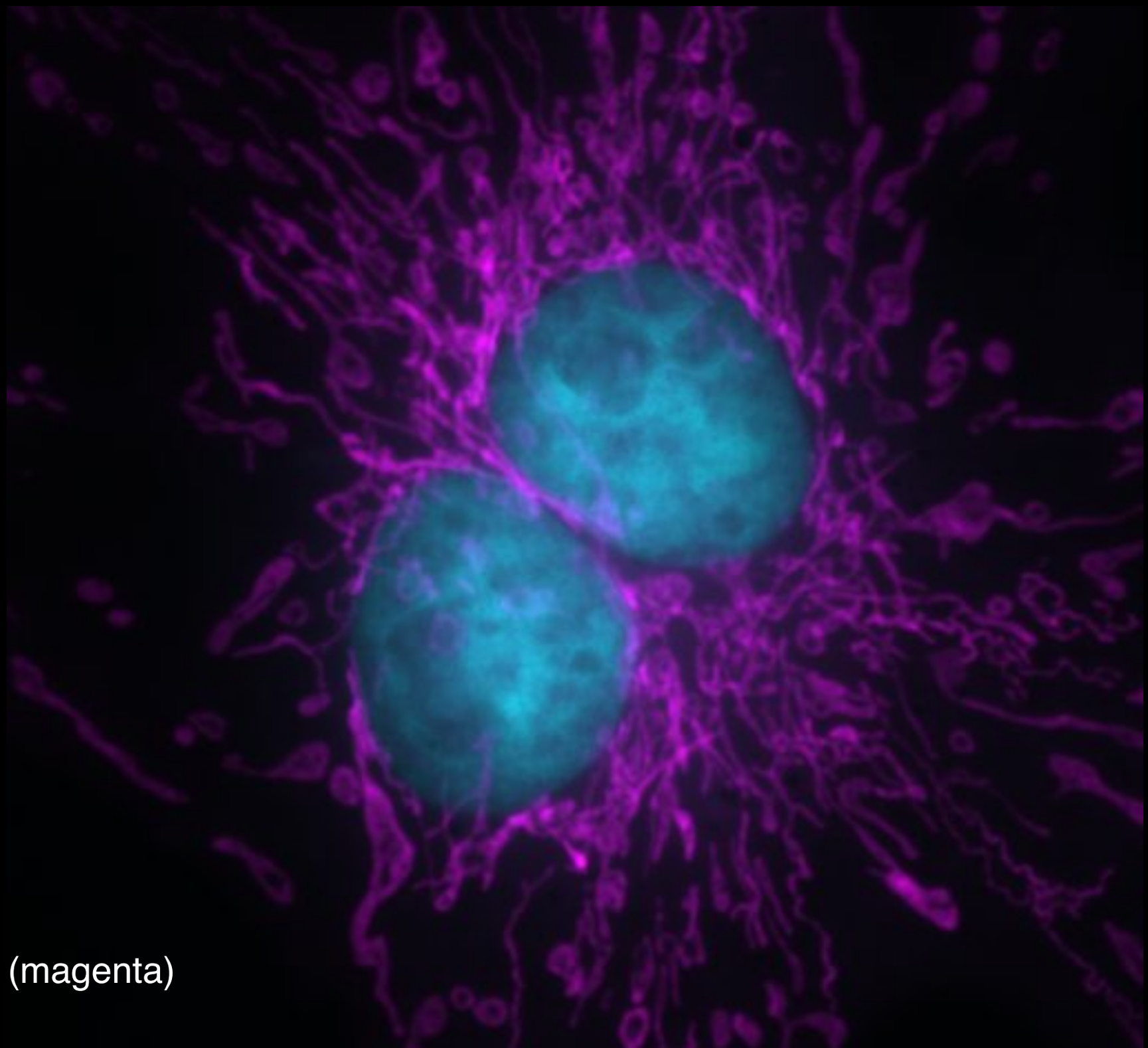


Epifluorescence vs Brightfield light paths (inverted)



Widefield Fluorescence Microscopy

The whole field of view is collected at once



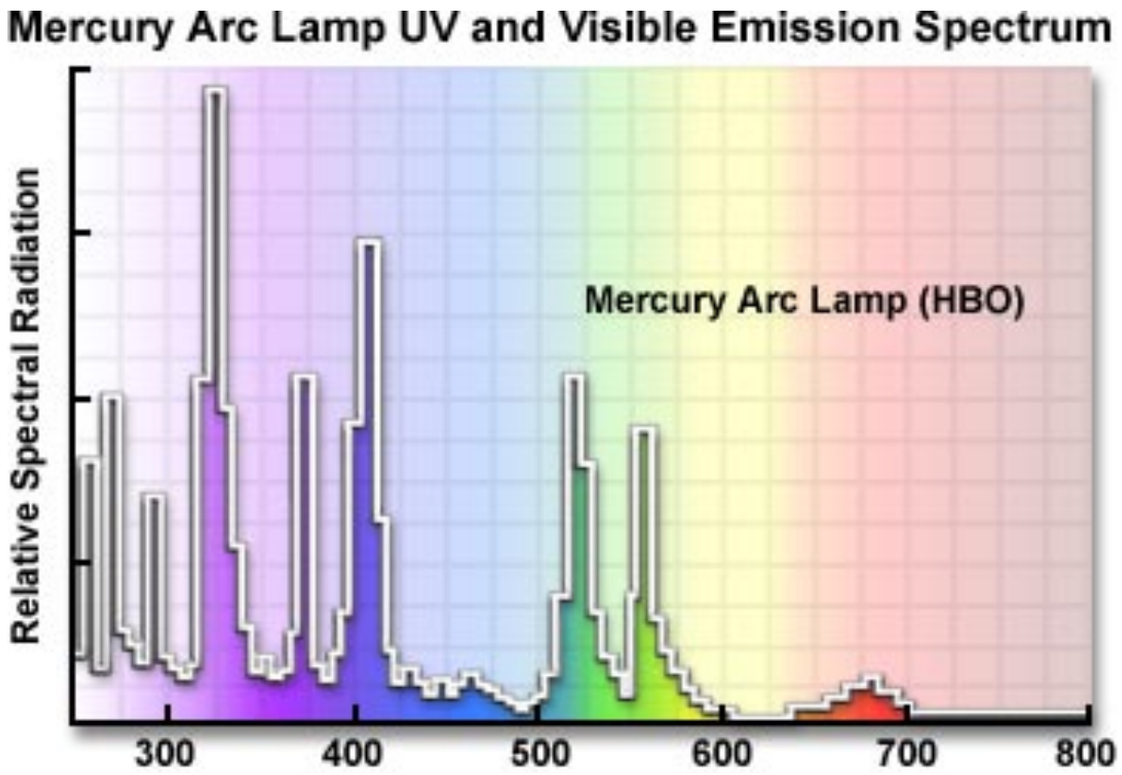
BPAE cells
Mitotracker Red (magenta)
DAPI (cyan)

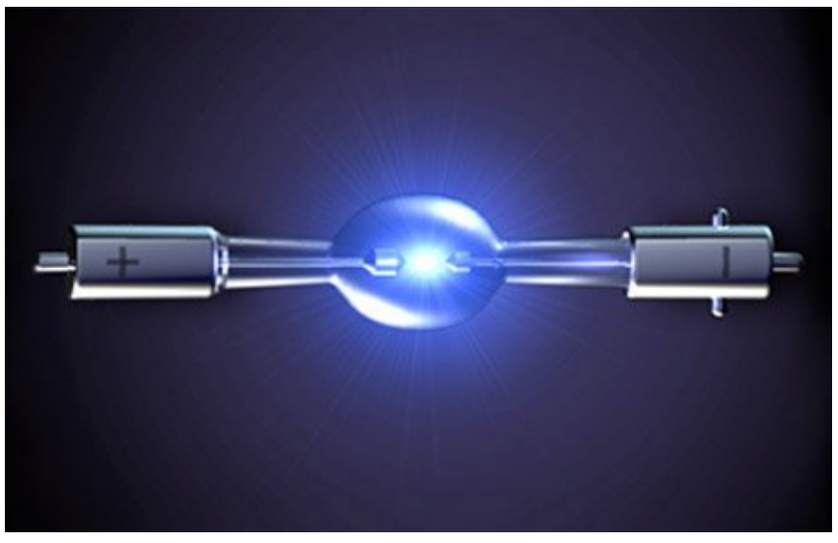
Illumination sources for widefield fluorescence microscopy

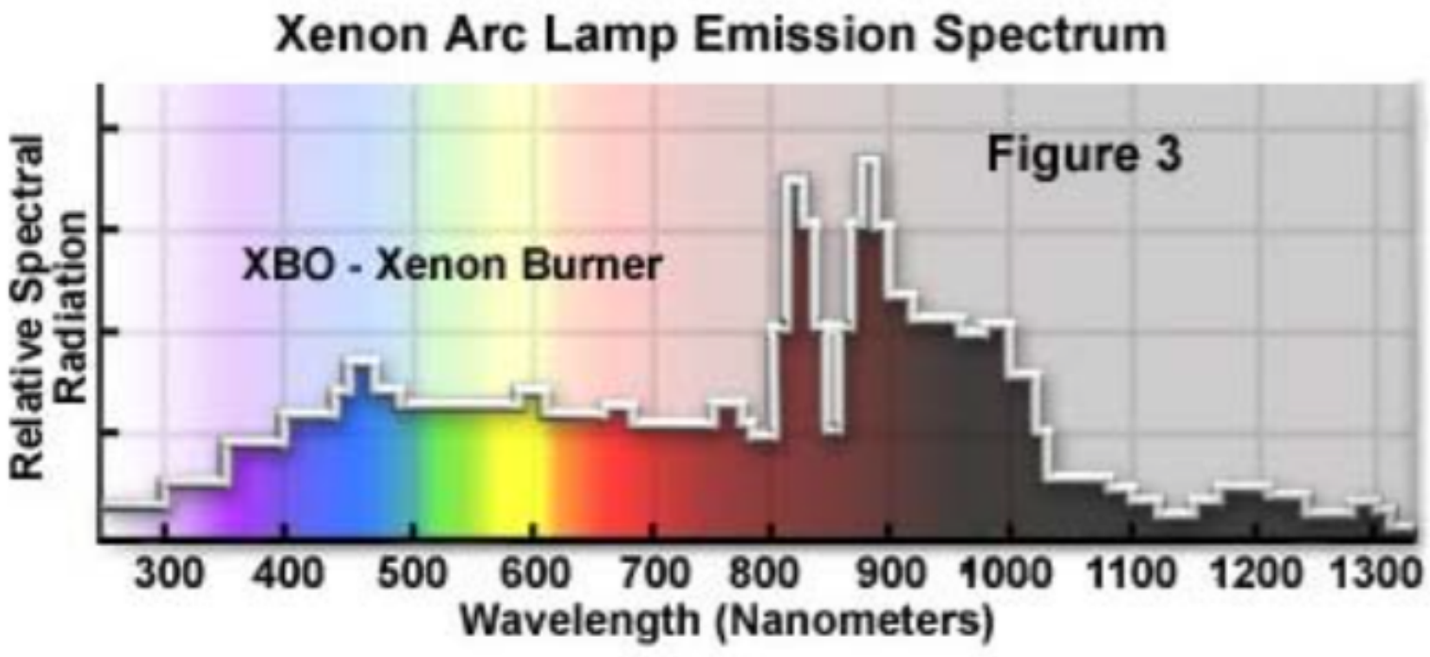
Widefield fluorescence

Arc Lamp Mercury

- 200h
- hazardous
- *out of use*





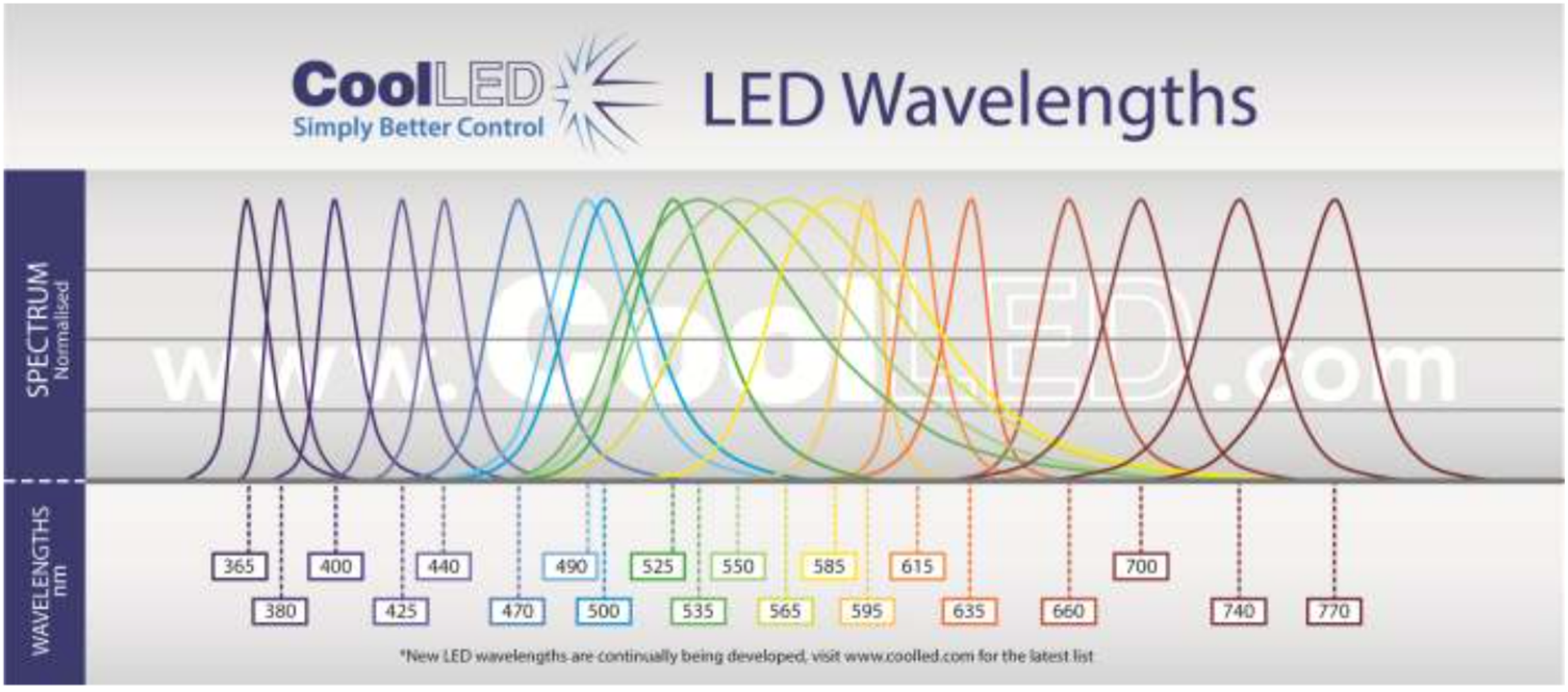


simultaneous excitation of multiple fluorophores over a wide wavelength range

Illumination sources for widefield fluorescence microscopy

State of the art for widefield fluorescence

LEDs Light Emitting Diodes



http://www.coolled.com/product-detail/led-wavelengths/

- Wide range of lines available
- 25,000 h

Illumination sources for fluorescence microscopy

Confocal

2-photon **TIRF**

Super-resolution

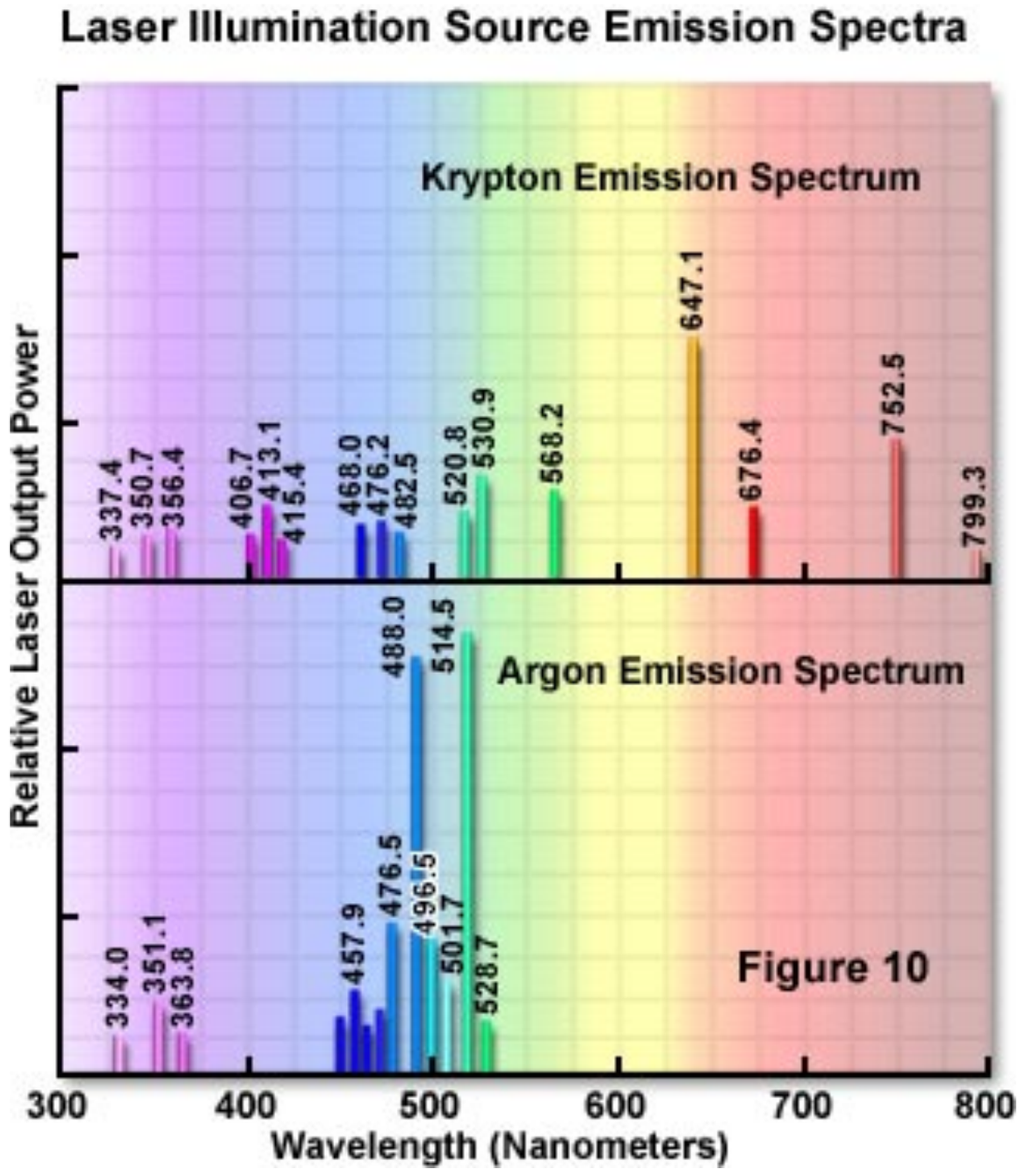
Only discrete lines!

lines	Alexa dye	
405	405	440
440	430	540
488	488	515
514	514	540
561	568	605
633	633	645

Narrow beams of highly monochromatic, coherent and collimated light

Lasers

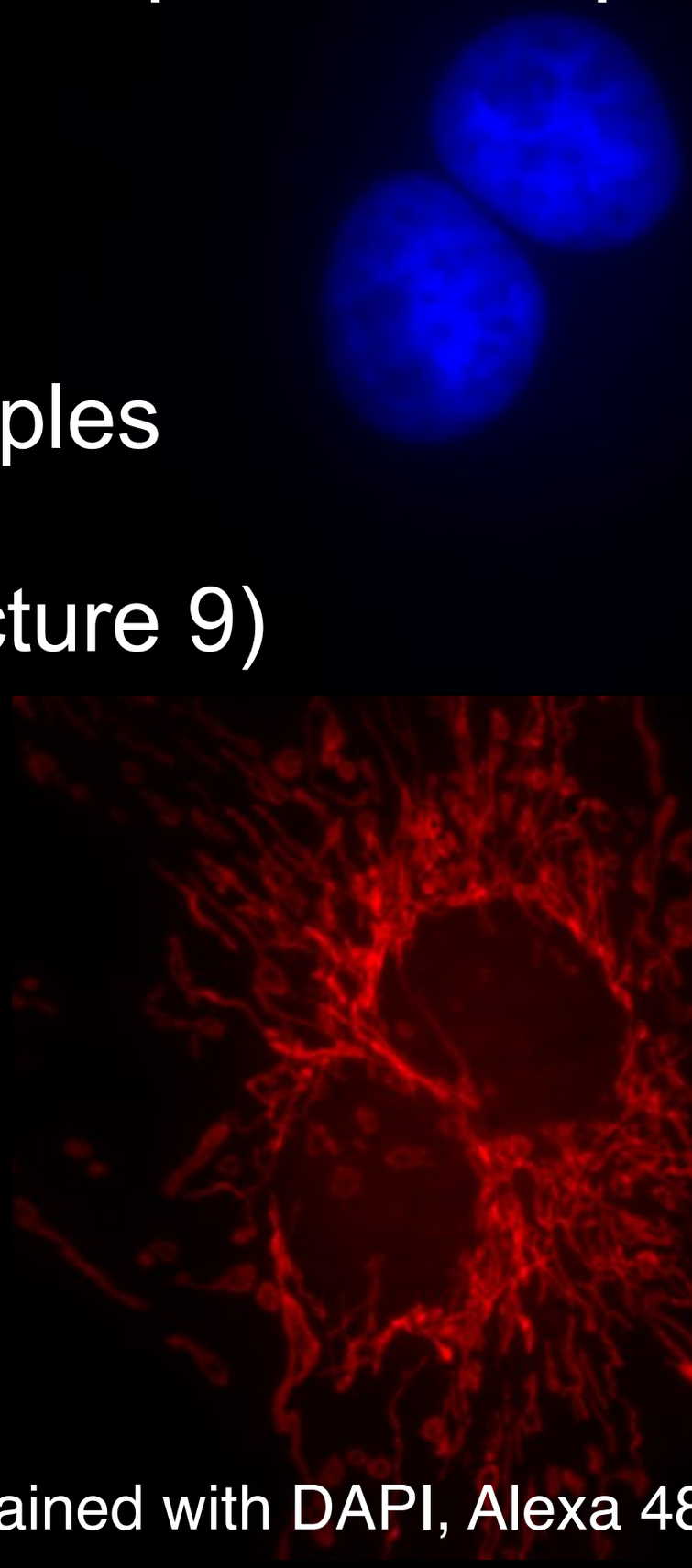
(*light amplification* by stimulated emission of radiation)

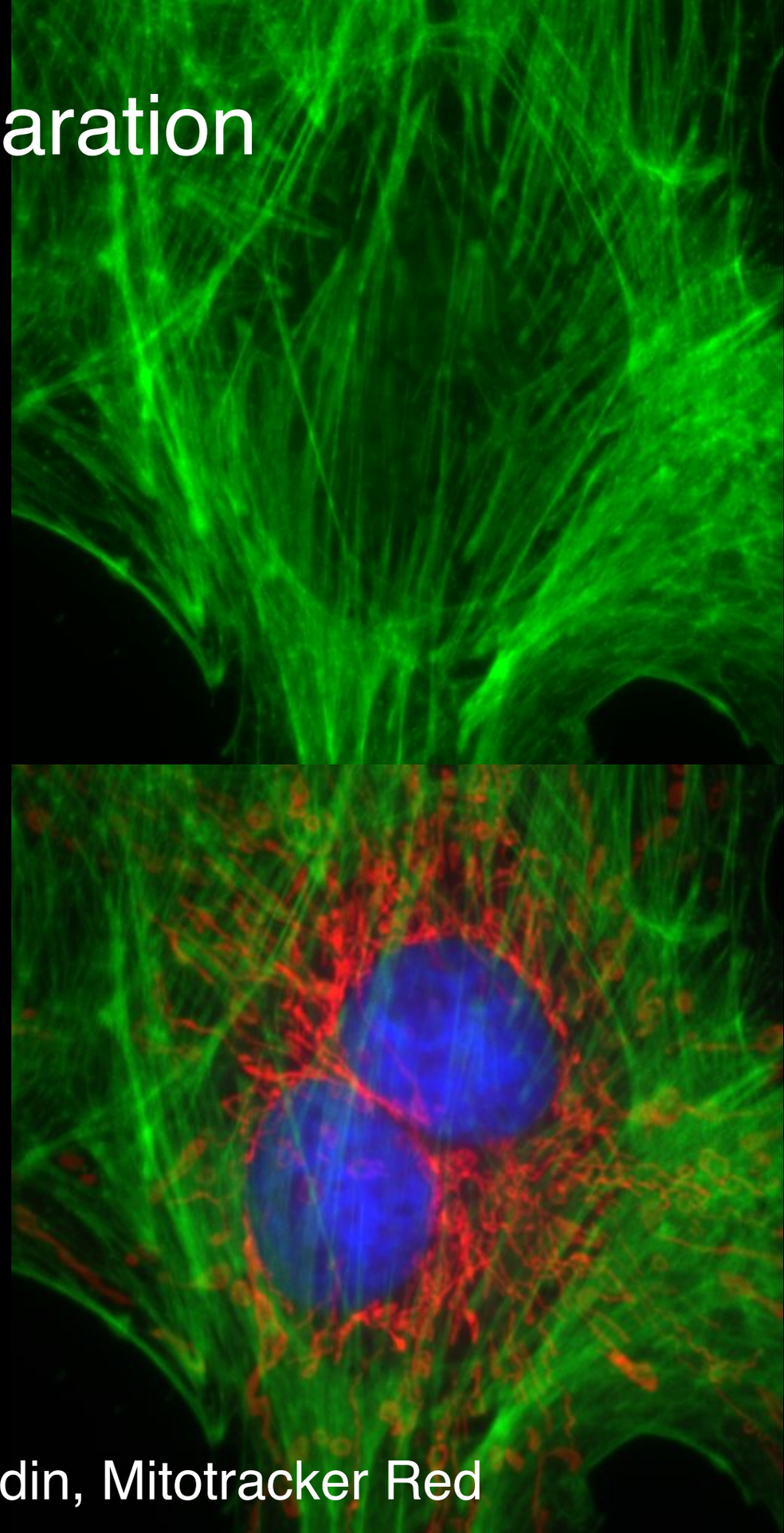


Part 2 Tips on sample preparation

Fixed samples

(*in vivo* lecture 9)





BPAE cells stained with DAPI, Alexa 488 phalloidin, Mitotracker Red

Why work with fixed material?

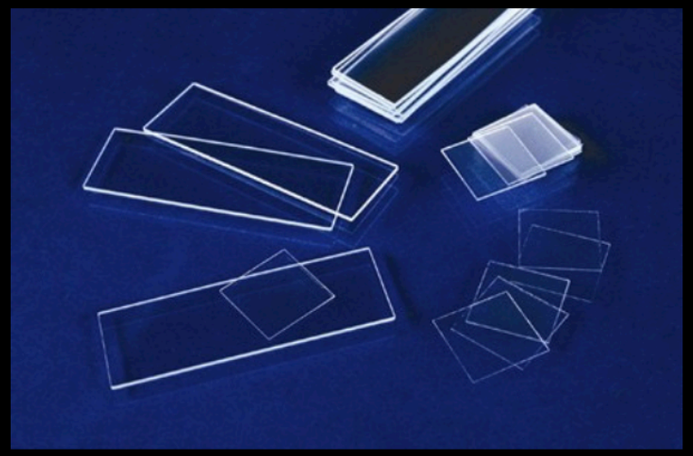
1. Convenience / Throughput

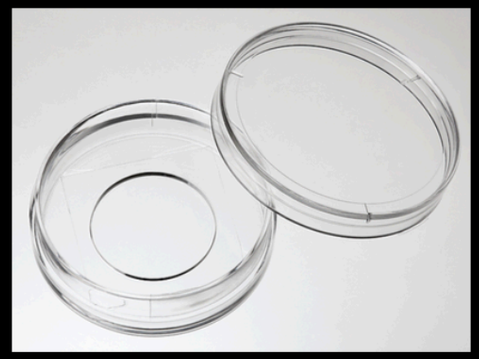
2. Widely applicable molecular labeling:
   Immunofluorescence
   FISH

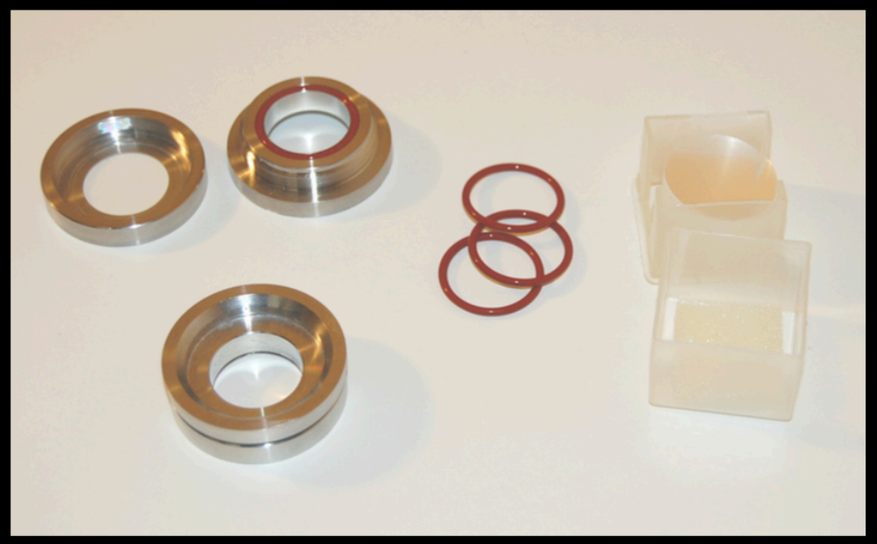
3. Ease of multiplexing bright stable labels

Immobilising the specimen

Sample holder must be suitable for imaging



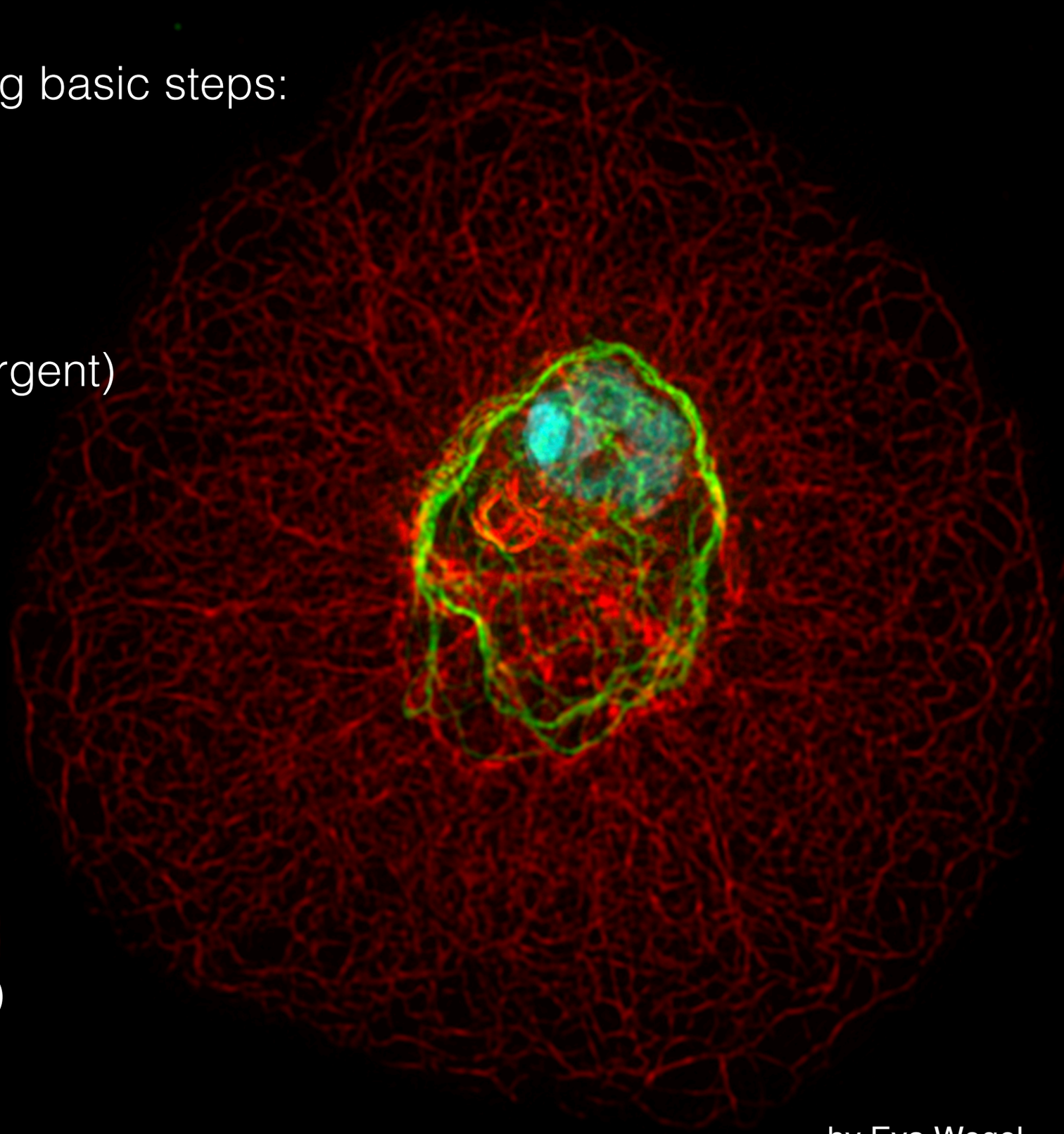




Typical Immunocytochemistry protocol

Most are variants of the following basic steps:

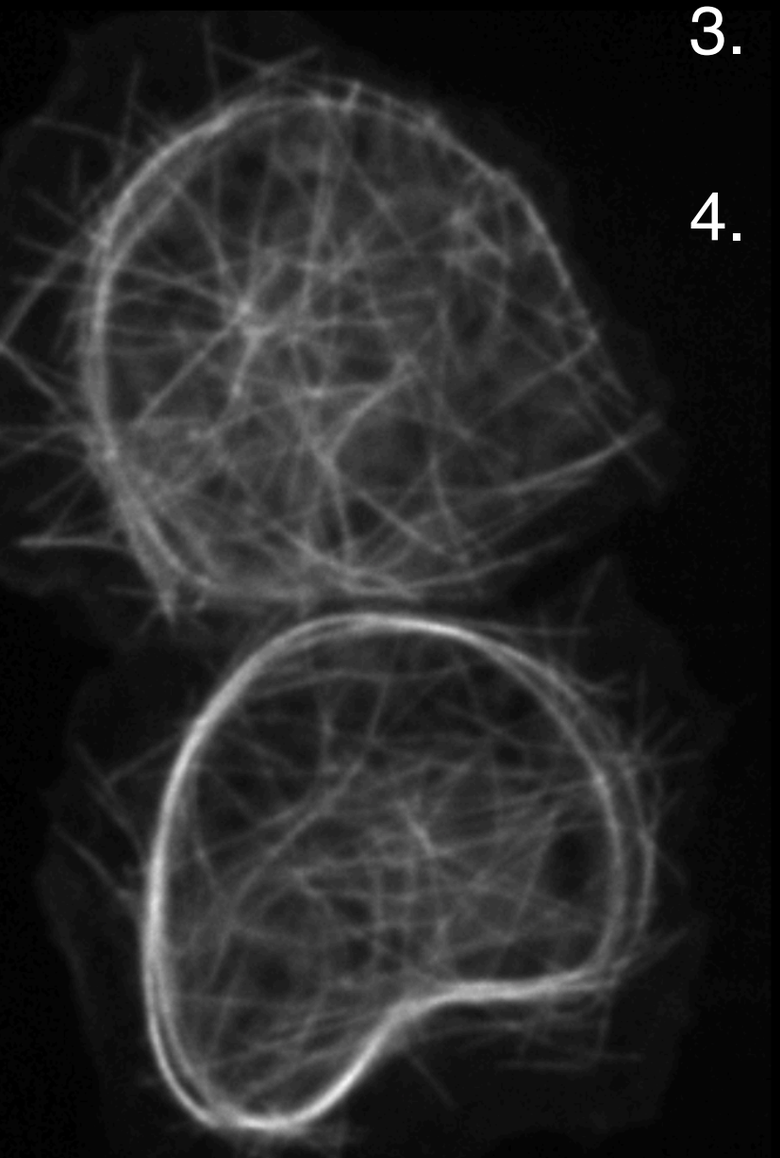
1. Fixation (e.g. PFA)
2. Permeabilisation (e.g. detergent)
3. Washes (e.g. PBS)
4. Blocking (e.g. serum)
5. 1^0 antibody
6. Washes (e.g. PBS)
7. 2^0 antibody
8. Washes (e.g. PBS + H_2O)
9. Mounting (e.g. Vectashield)



by Eva Wegel

Fixation: preservation of cells or tissue in a life-like state

1. Preserve structural features
2. Uniform fixation throughout the sample
3. Enable dye labeling
4. Reduce background fluorescence

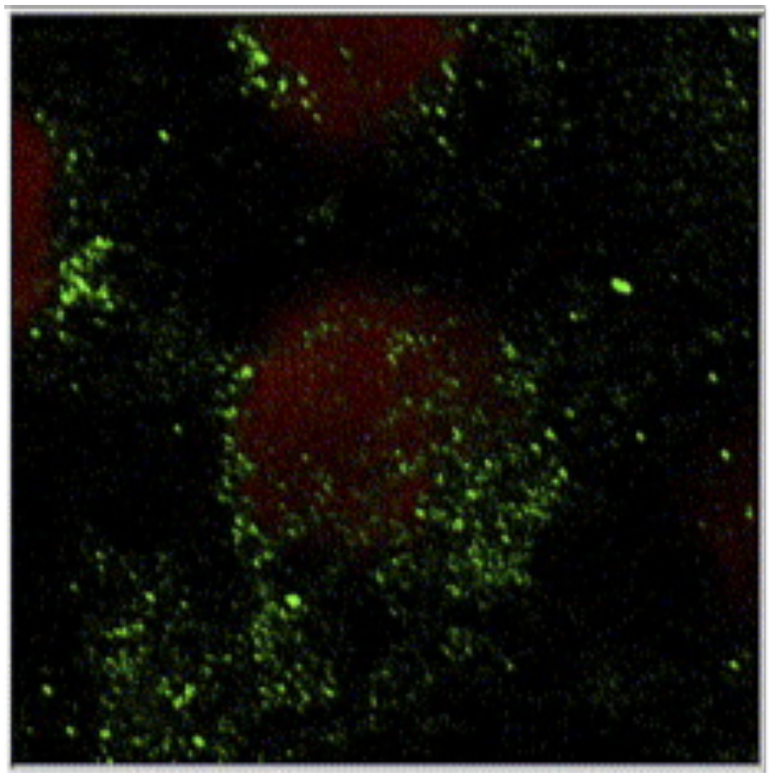


Microtubules in Drosophila macrophages
Left :
Live cells expressing Jupiter-GFP
Right:
PFA fixed cell stained with anti-tubulin antibody and Alexa Fluor 488

Types of Fixation

Denaturing fixation:

Cold methanol

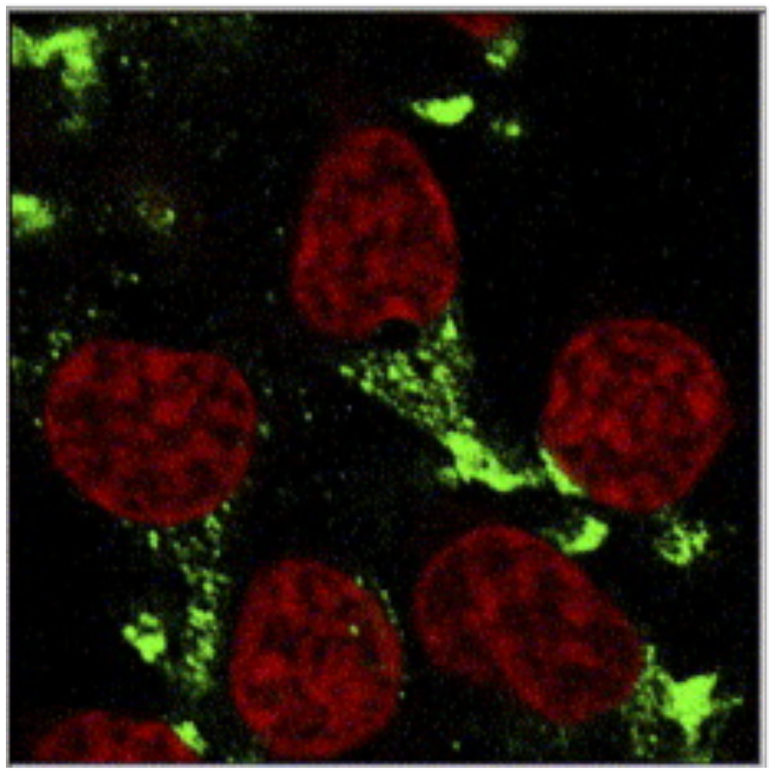


destroys 3D protein structure

dissolves lipids into micelles

Cross-linking fixation:

Formaldehyde (PFA)

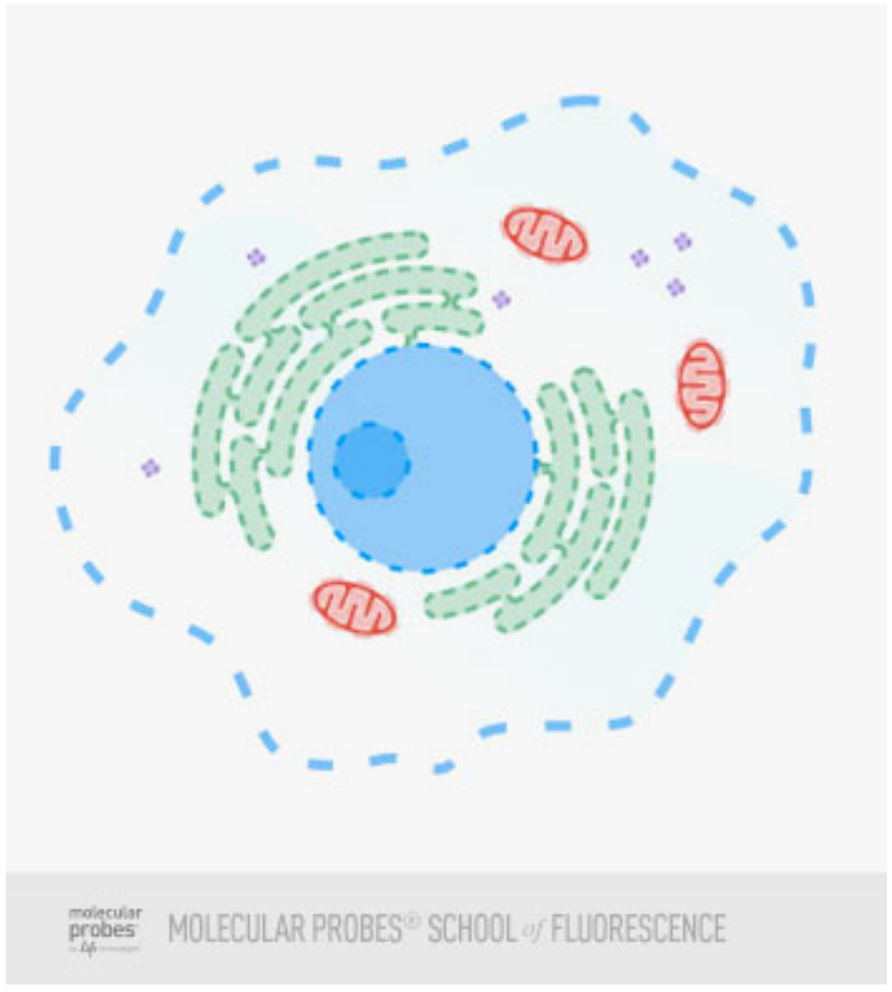


binds to proteins and some lipids,

but not RNA, DNA or most sugars

Sometimes a combination of both is necessary …

Removal of some lipids with detergents



Tween 20

Triton X-100

Goal: to allow large labels (antibodies) to penetrate fixed cells/tissue

Blocking

Reduction of nonspecific staining

done with a solution containing excess of protein

Bovine Serum Albumin (BSA)

Casein (or non-fat dry milk)

blocking proteins prevent low-affinity antibody interactions elsewhere in the sample

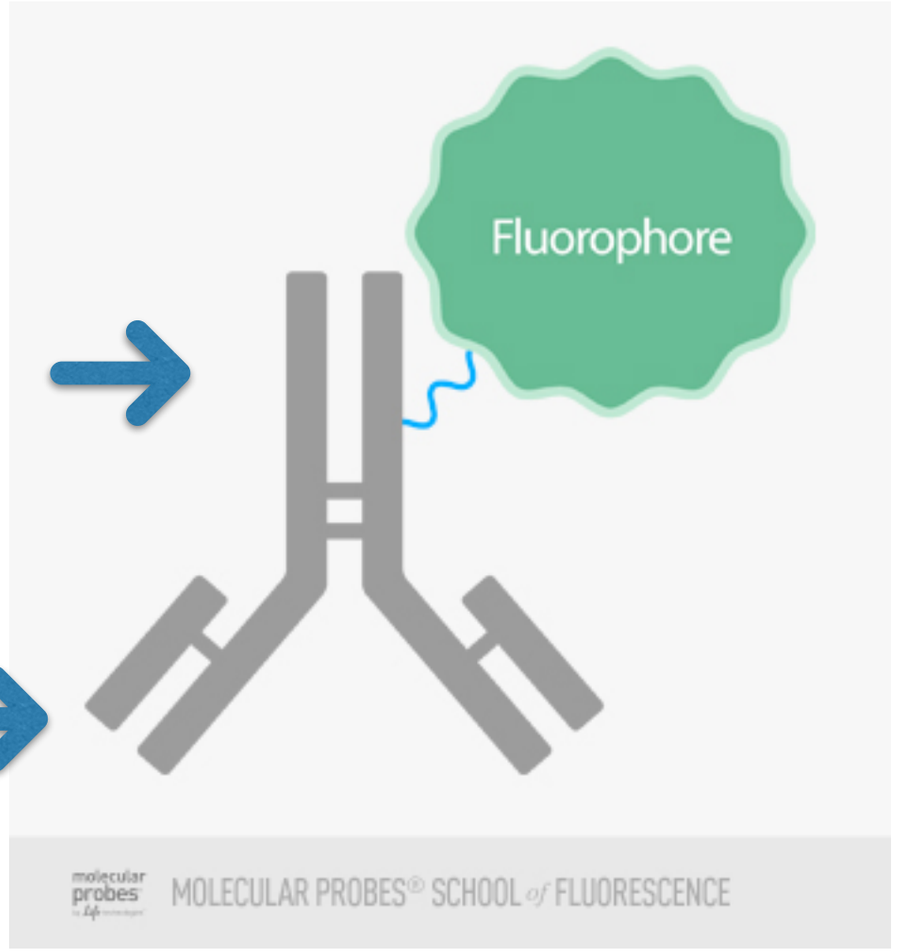
Immunolabeling (antibodies)

Antibody (large Y-shaped protein called immunoglobulin) produced by the immune system, found in the blood or other body fluids of **vertebrates**.

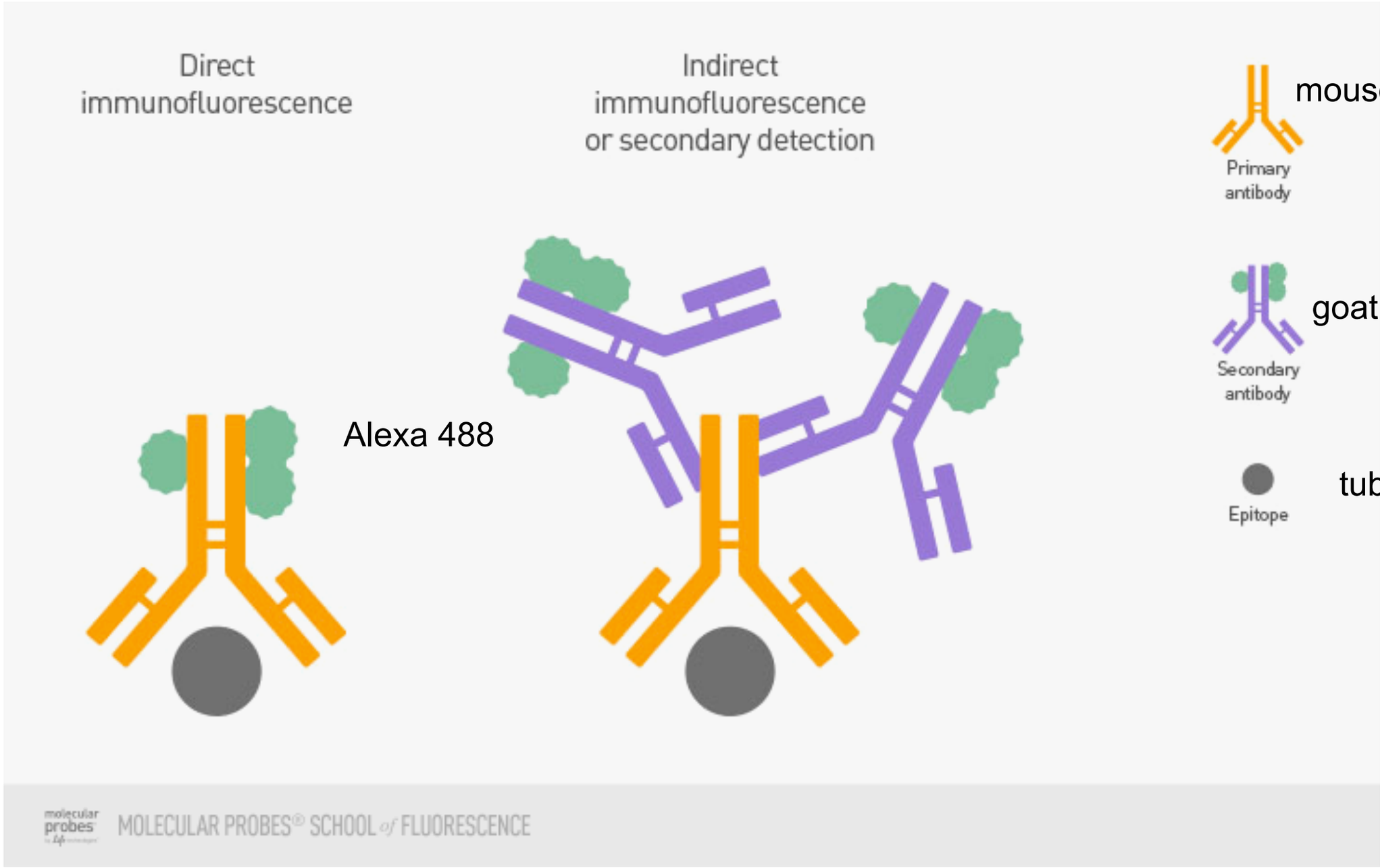
The **antibody** recognises unique parts of the foreign target called an **antigen**.

Conserved among species

VARIABLE
Antigen binding site



Immunolabeling (antibodies)



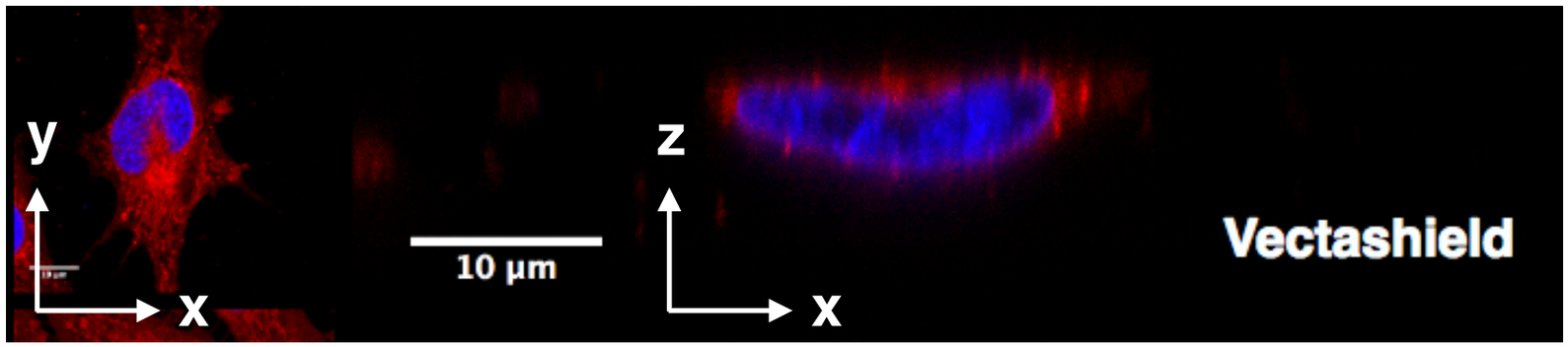
mouse anti-tubulin

goat anti-mouse

tubulin

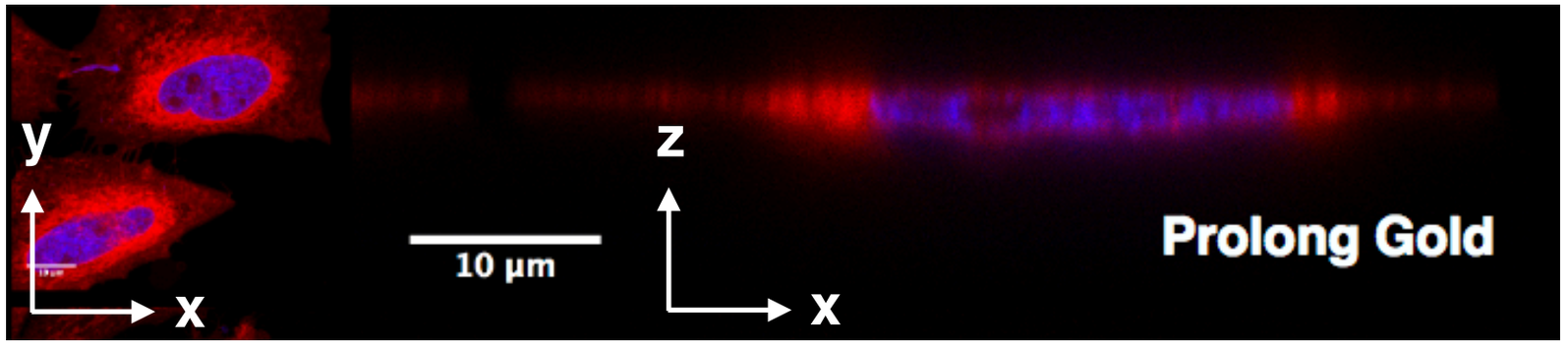
Mounting

- Non-hardening



  - Short-term storage (days to few weeks)
  - **Dabco, Glicerol, Vectashield** (antifading agent, but does not work with FarRed dyes)

- Hardening



  - Long term storage (months)
  - It can flatten the cell if polymerises too fast
  - **Prolong Gold, Prolong Glass, Vectashield harset**

Experimental controls: the key for reliable results

- ☑ No primary or secondary antibody (autofluorescence)
- ☑ Incubate with secondary but not primary antibody
- ☑ Check cross-talk between dyes and microscope filterset
- ☑ Test specificity in knock-out /knock-down cells

Part 3

PSF and OTF

PSF (Point Spread Function) in fluorescence

Point Spread Function

PSF is a measure of the microscope response to a point source of light

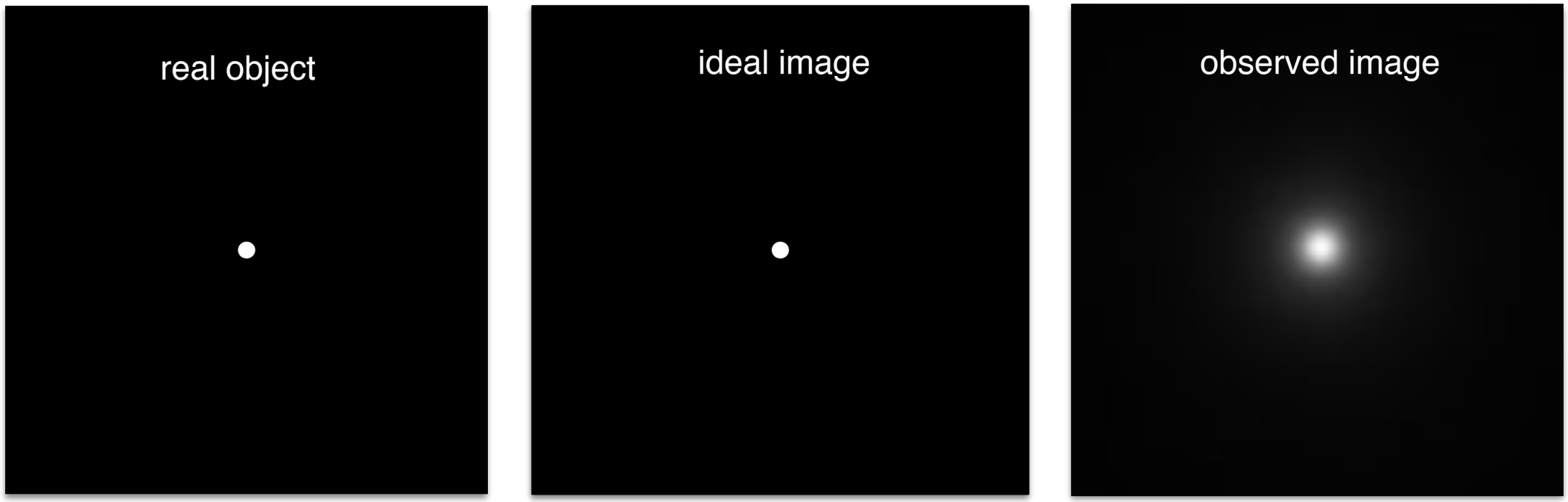
Why bother?

- microscope performance
- spherical aberrations
- x, y, z info
- image quality
- alignment
- optical resolution

PSF (Point Spread Function) in fluorescence

Point Spread Function

How does light spread out from a single point?



Light is emitted in all directions

If all light was collected and if light would travel in straight lines

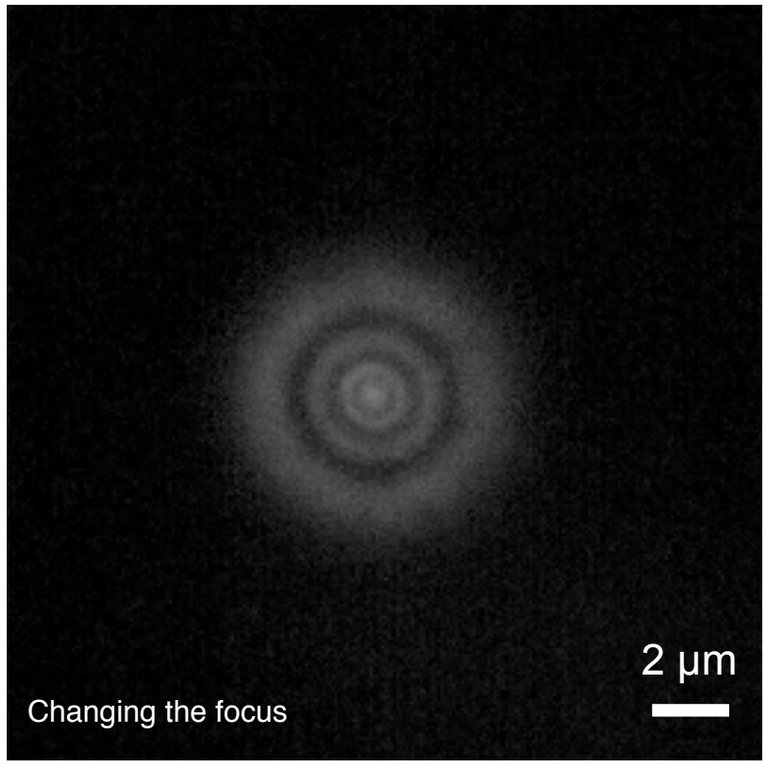
But the point actually looks blurred / distorted because of diffraction (Airy diffraction pattern)

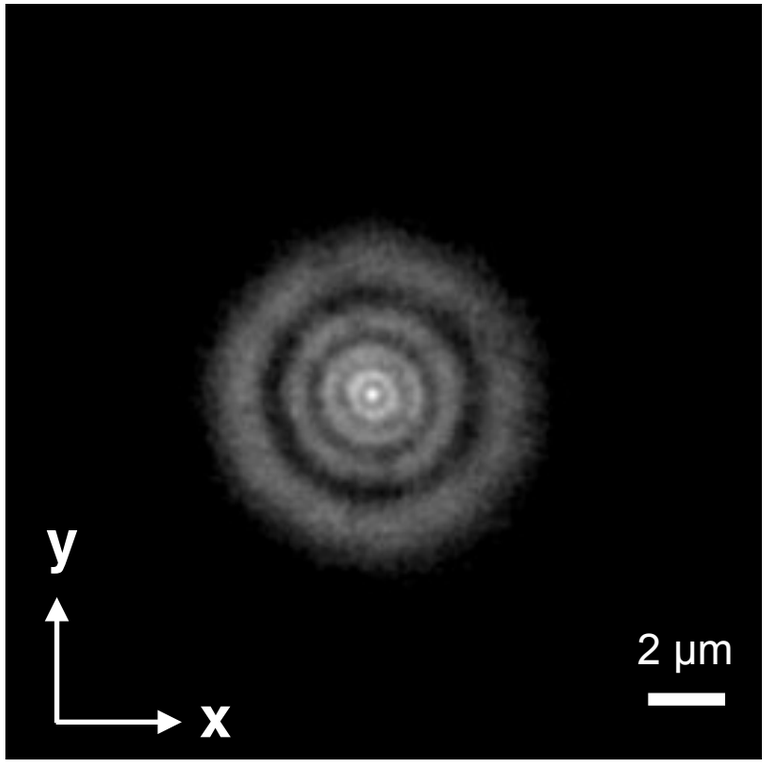
Fluorescent bead, single dye, or a fluorescent protein as a point source of light

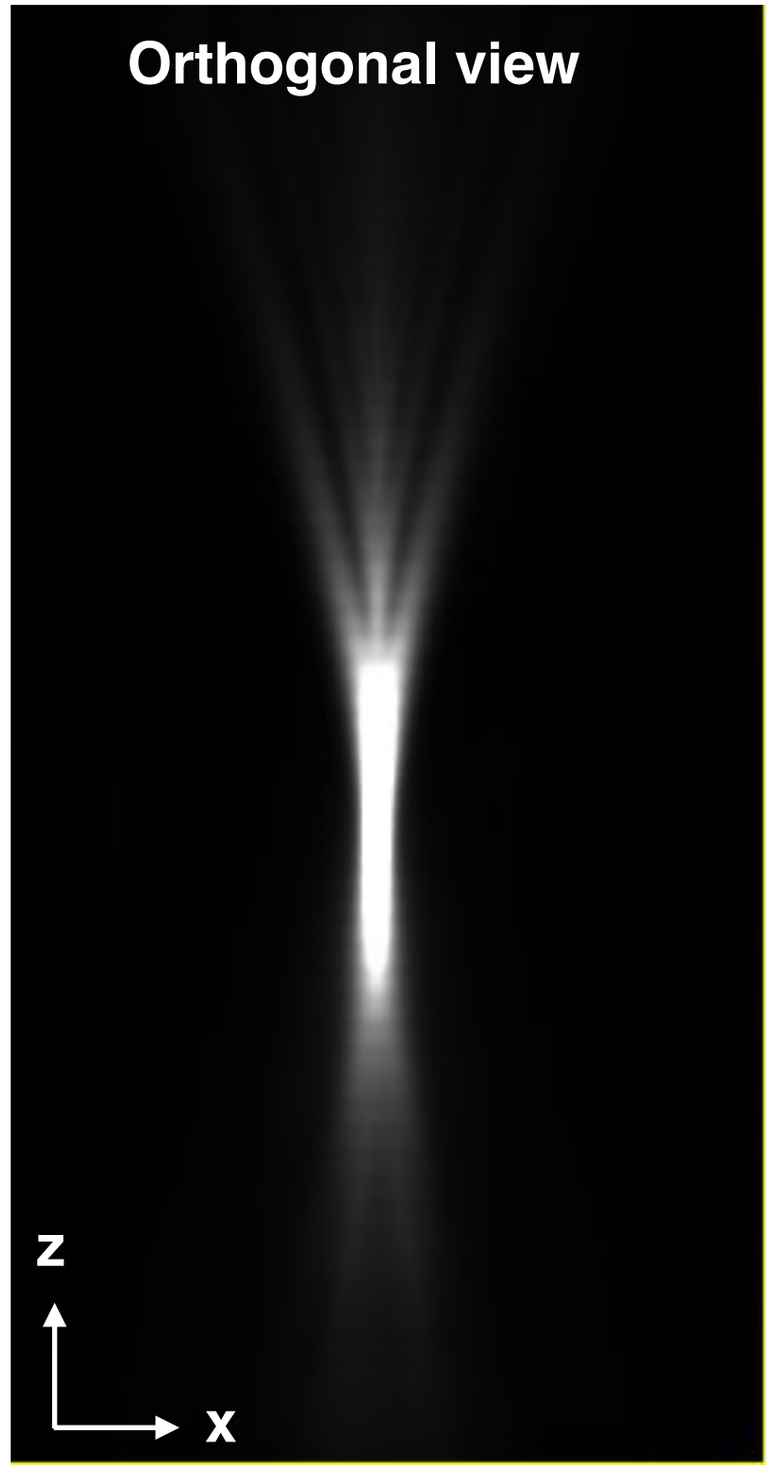
PSF (Point Spread Function) in fluorescence

PSF

red fluorescent 170 nm bead







Airy disk diffraction pattern

(concentric rings)

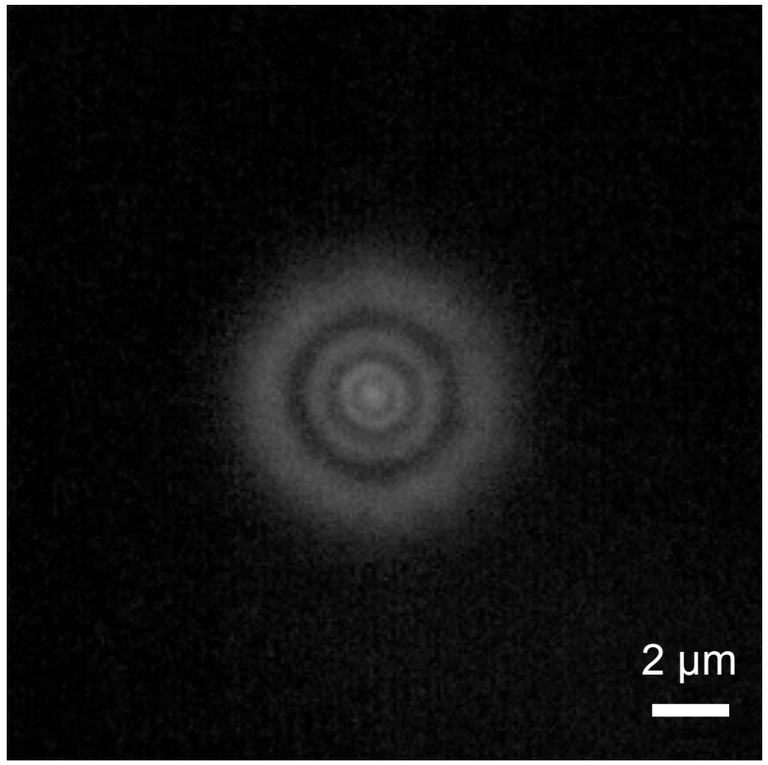
Light waves emitted from a point source are not focused into an infinitely small point by the objective

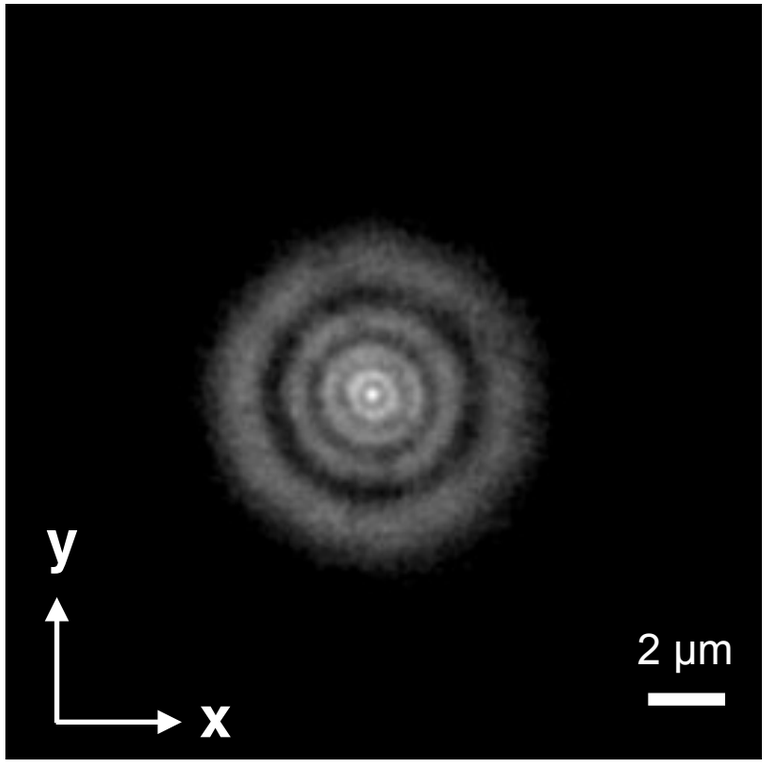
They converge together and interfere in the image plane

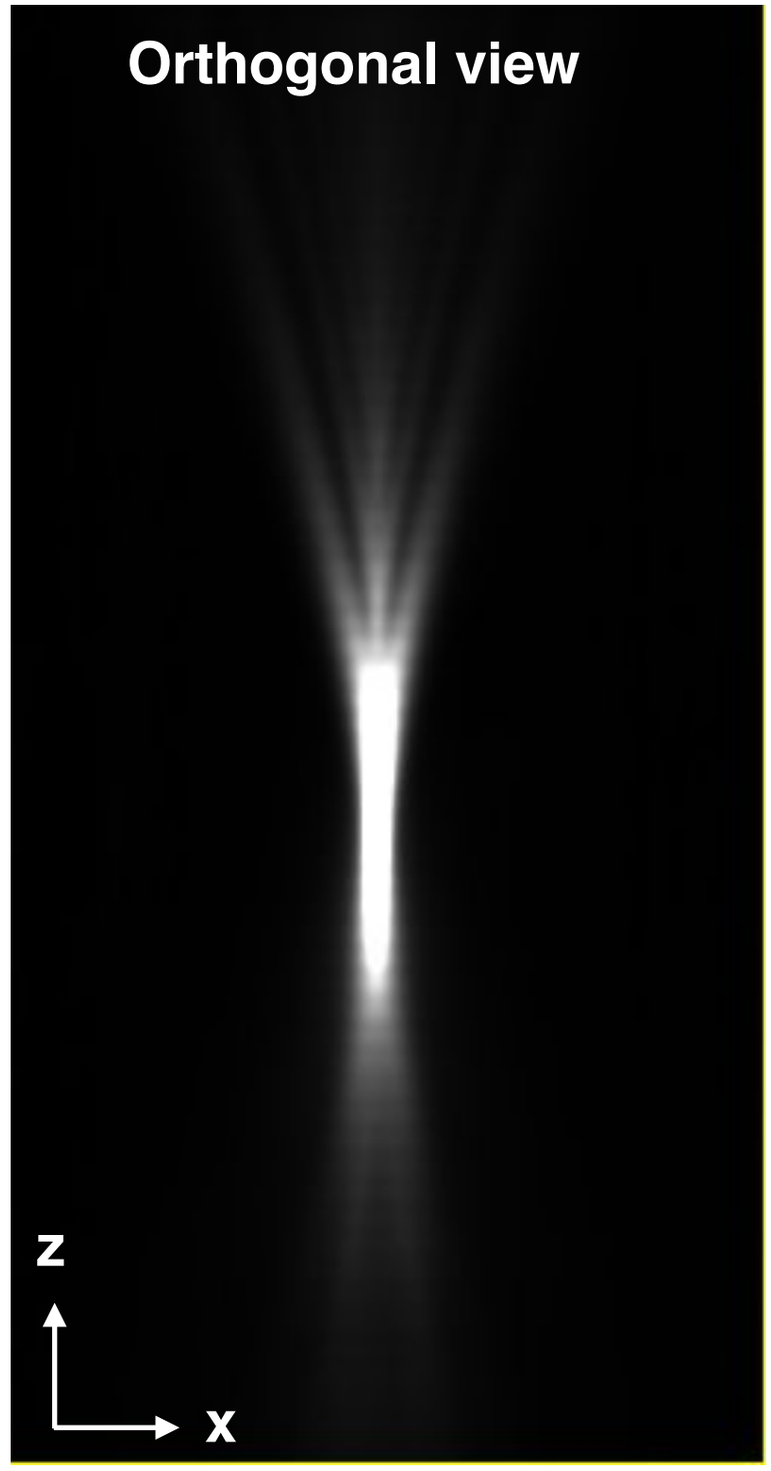
PSF is the 3D image of a point-like object under the microscope

PSF

red fluorescent 100 nm bead







What can we observe?

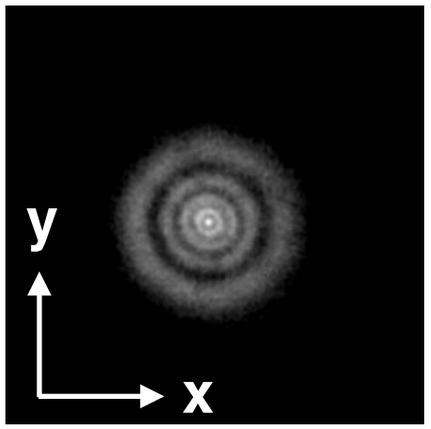
- Blur is broader in z than xy ☑ *RESOLUTION*
- How symmetric is the distribution

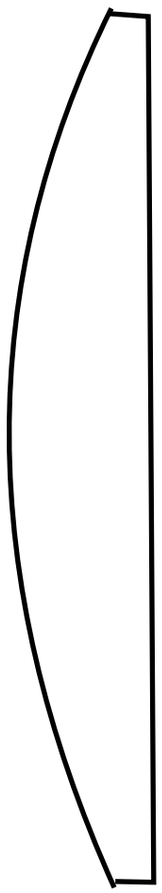
☑ ALIGNMENT, SPHERICAL ABERRATIONS, MISMATCH REFRACTIVE INDEX

Why blurred and how is the Airy diffraction pattern generated?

Objective lens

Image plane

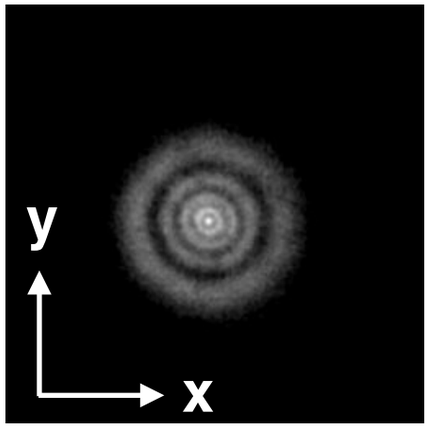


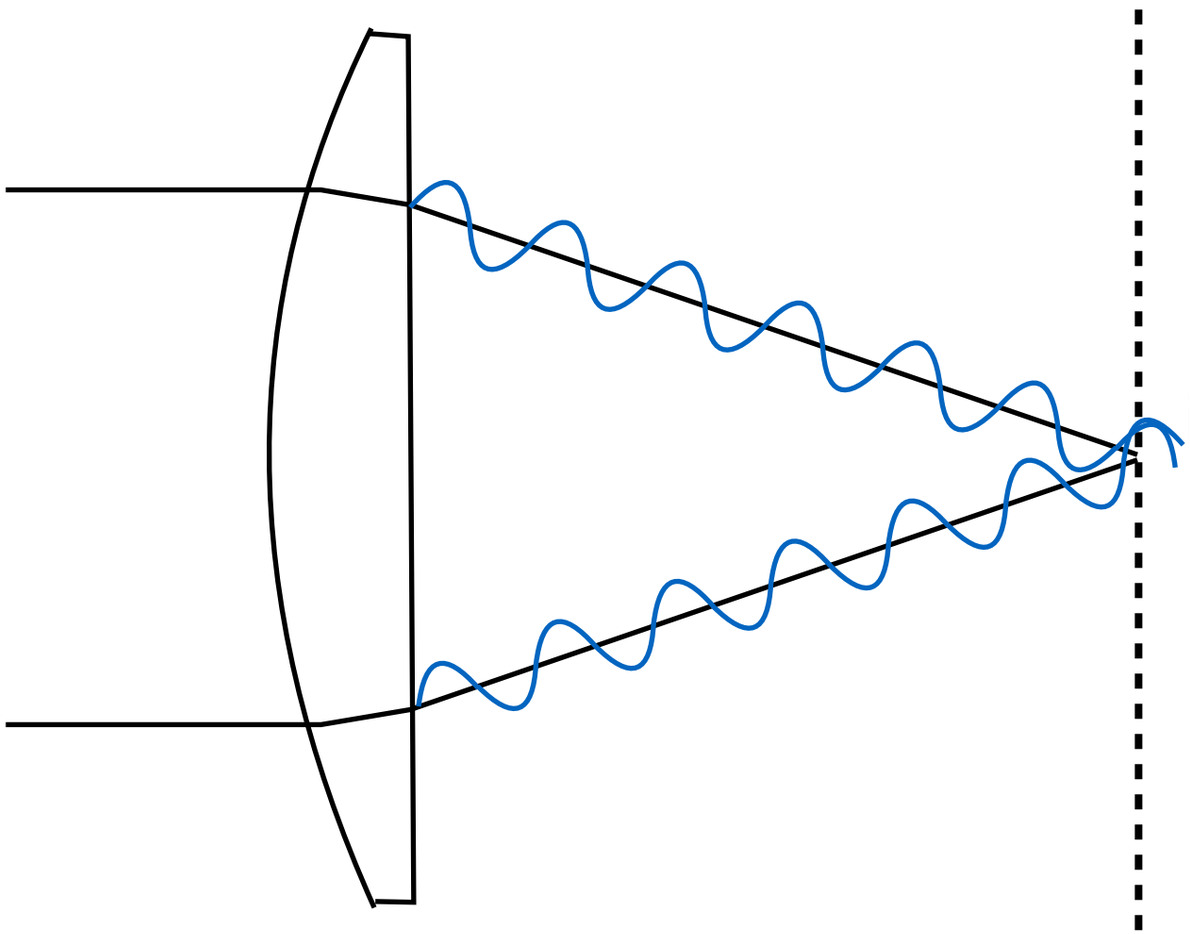


Why blurred and how is the Airy diffraction pattern generated?

Objective lens

Image plane



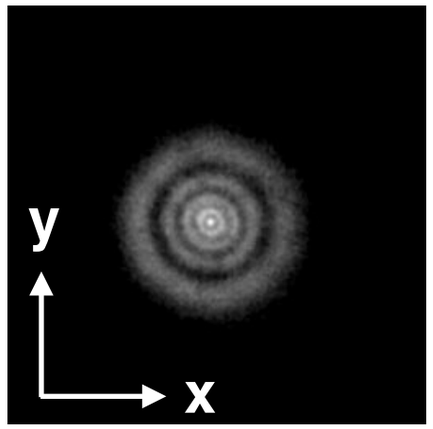


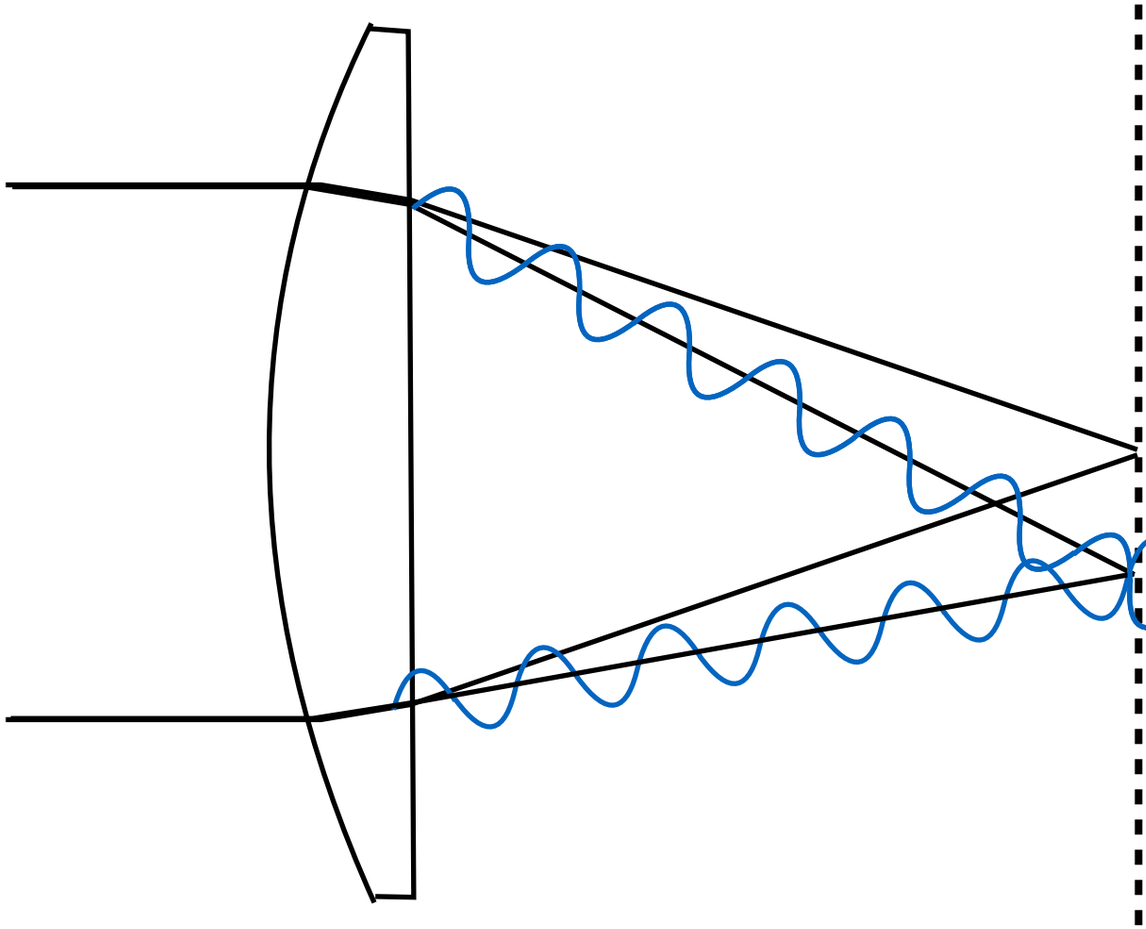
in phase - Constructive interference

Why blurred and how is the Airy diffraction pattern generated?

Objective lens

Image plane





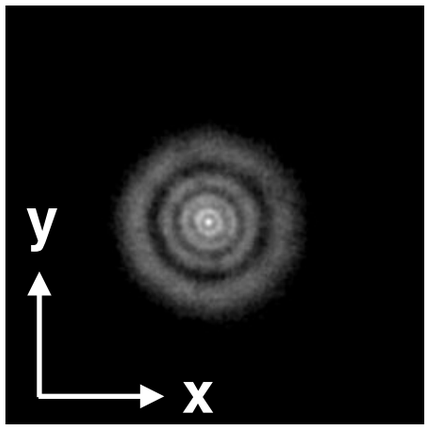
in phase - Constructive interference

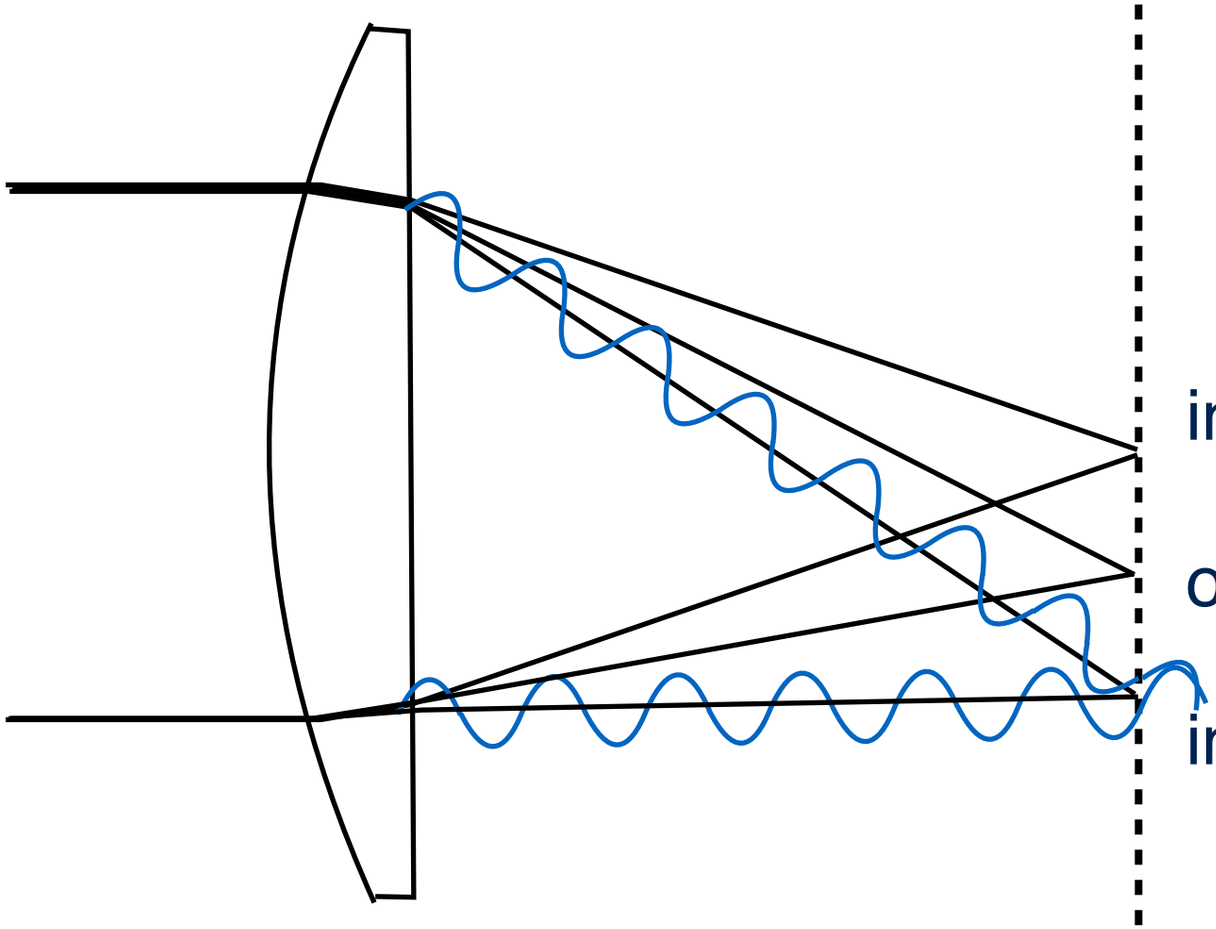
out of phase - Destructive interference

Why blurred and how is the Airy diffraction pattern generated?

Objective lens

Image plane





in phase - Constructive interference

out of phase - Destructive interference

in phase - Constructive interference

What does depend on…?

Objective lens

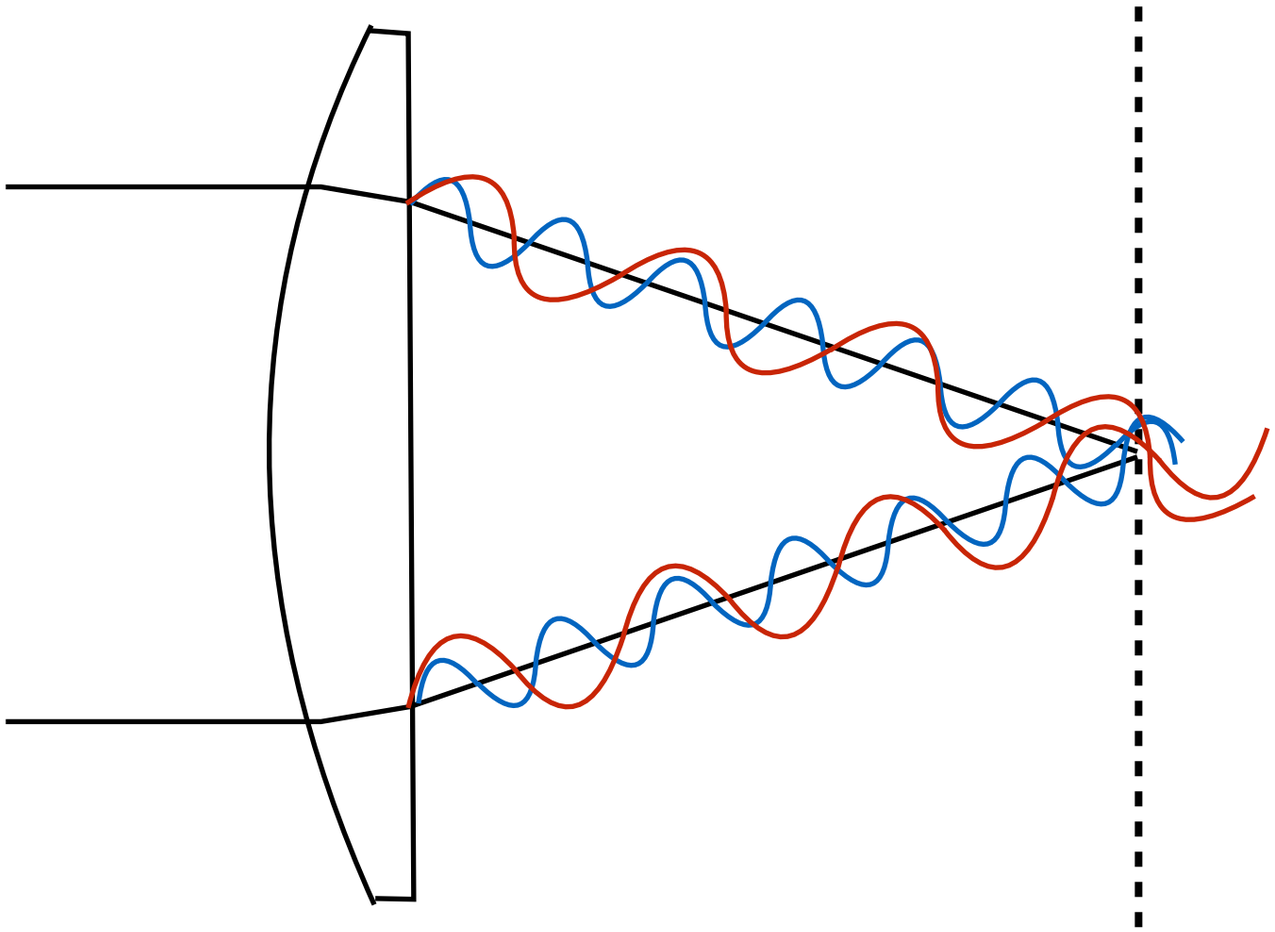
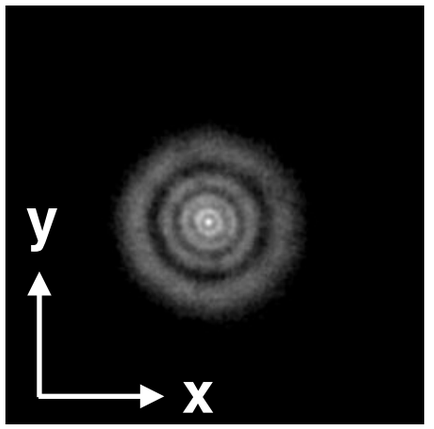


Image plane

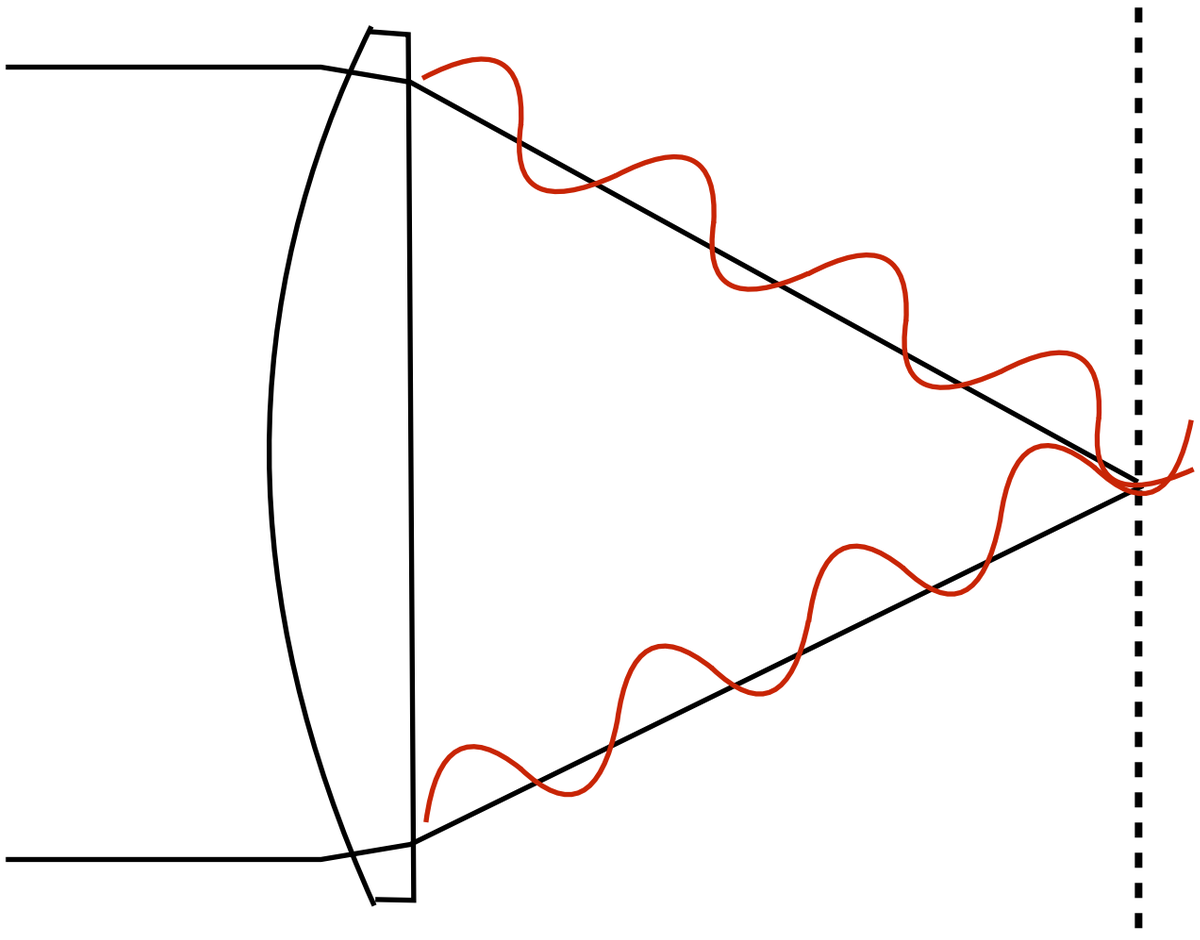


wavelength

What does depend on…?

Objective lens

Image plane

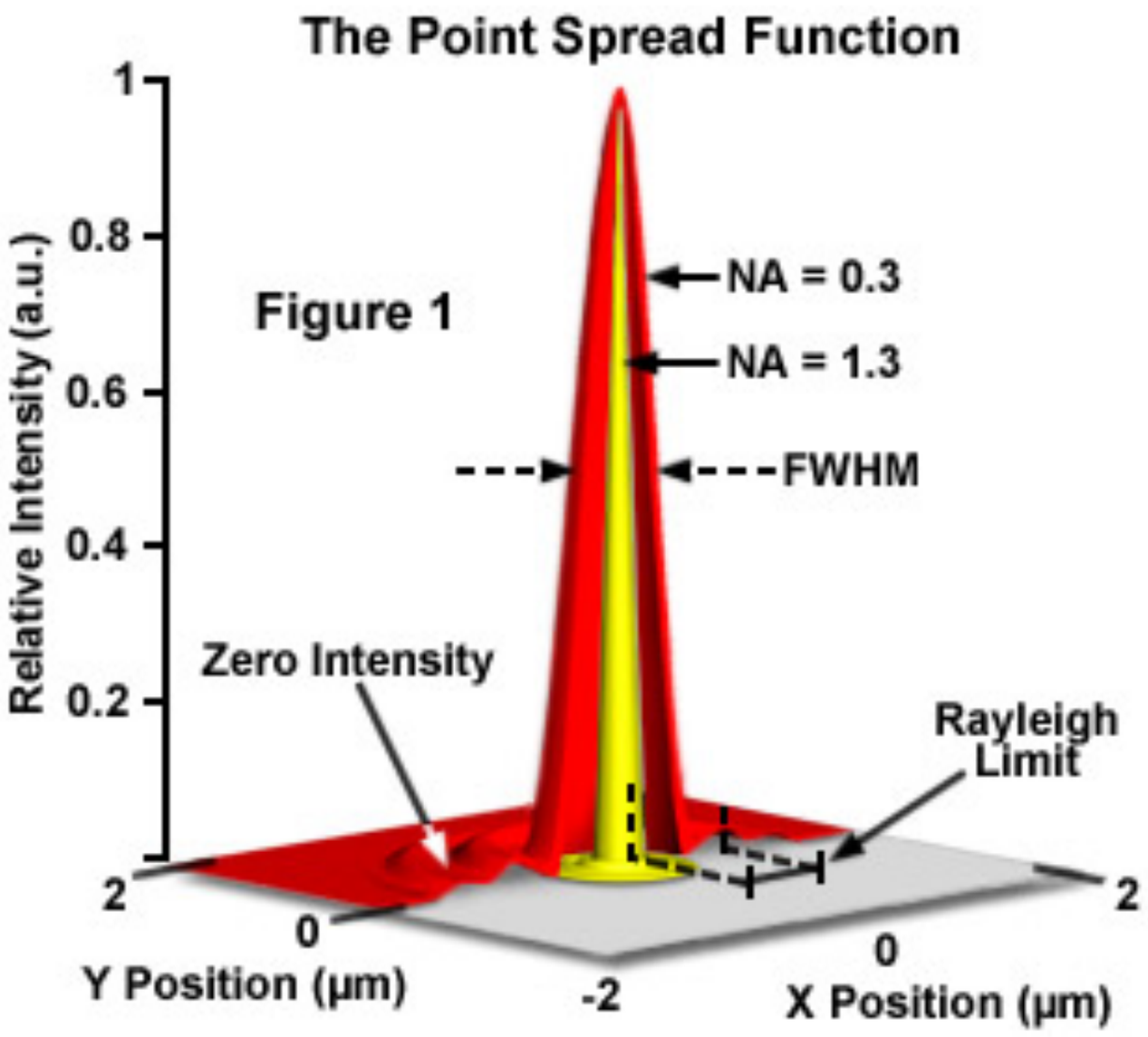


Numerical aperture

☑ Higher numerical aperture, less distortion, higher resolution

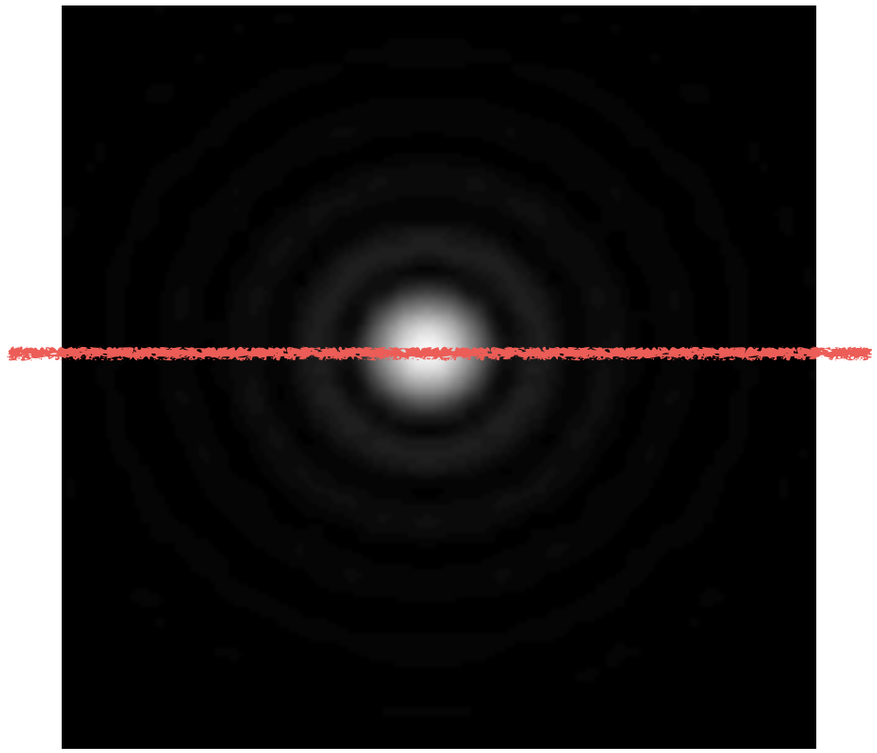
What does depend on…?

Numerical aperture



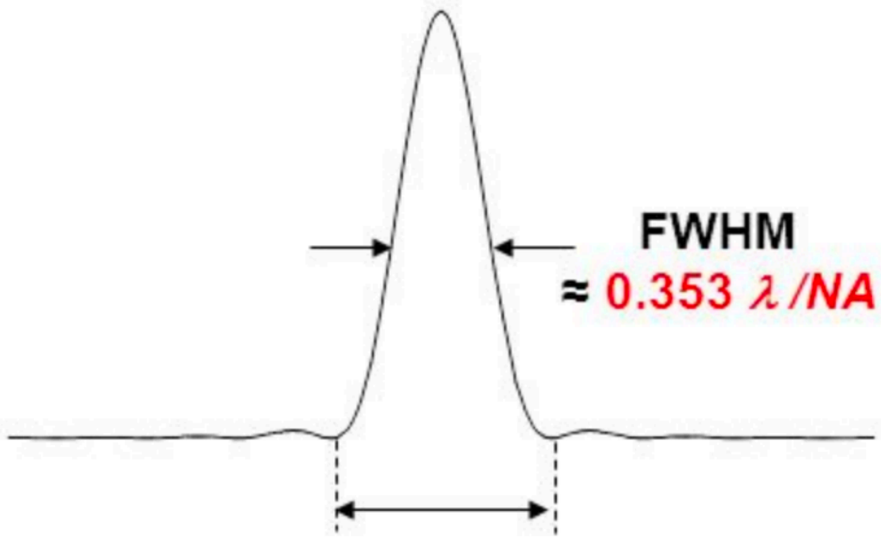
☑ Higher numerical aperture, less distortion, higher resolution

PSF is a way to measure resolution

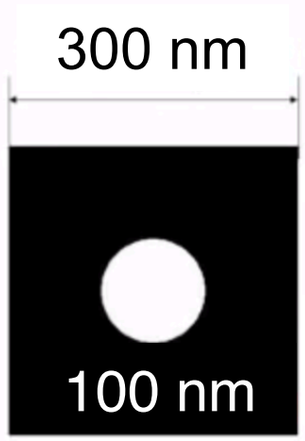


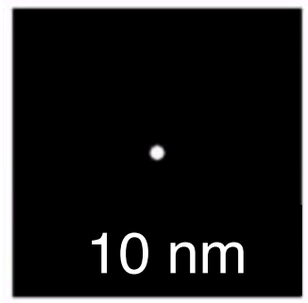
As the Full Width at Half Max (FWHM) of the PSF

As the diameter of the Airy disk (first dark ring of the PSF)
= “Rayleigh criterion”



How is the PSF of a small object?

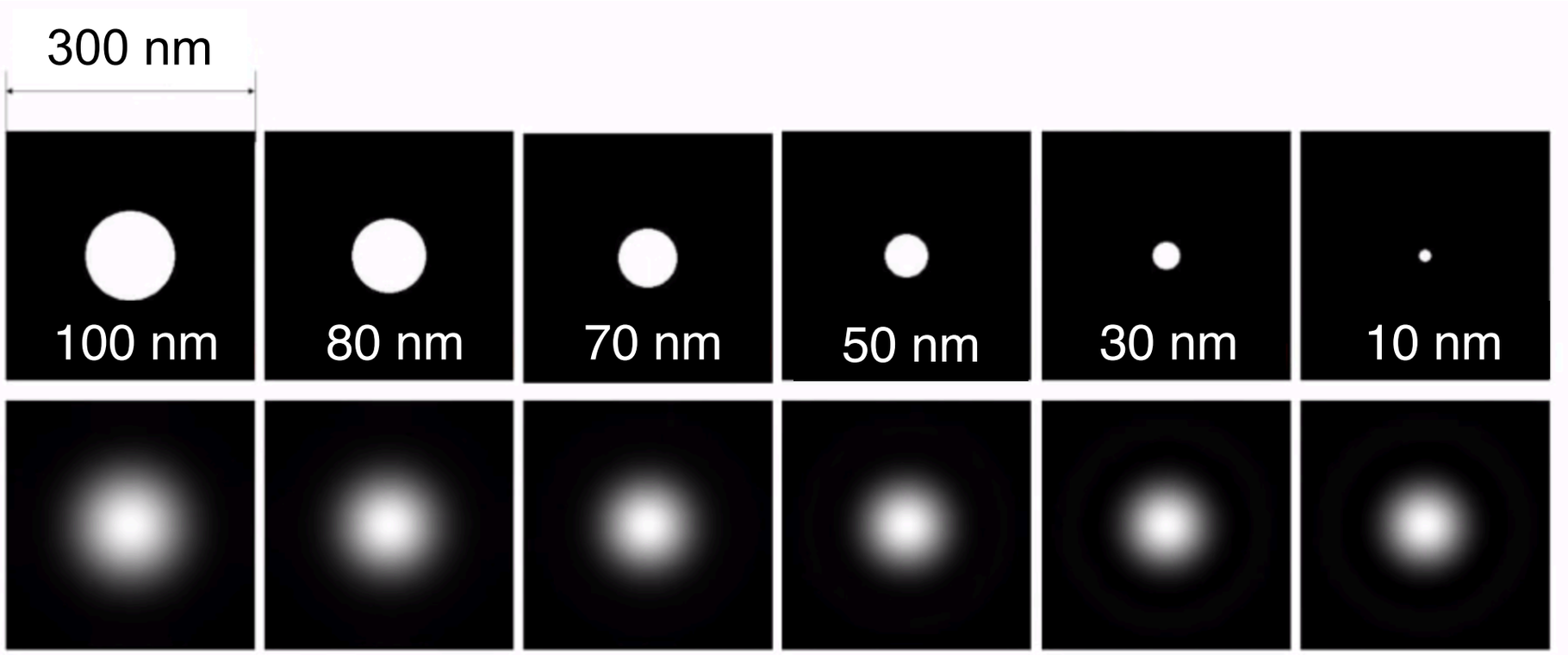




1.4NA objective

λ = 0.48 μm

How is the PSF of a small object?



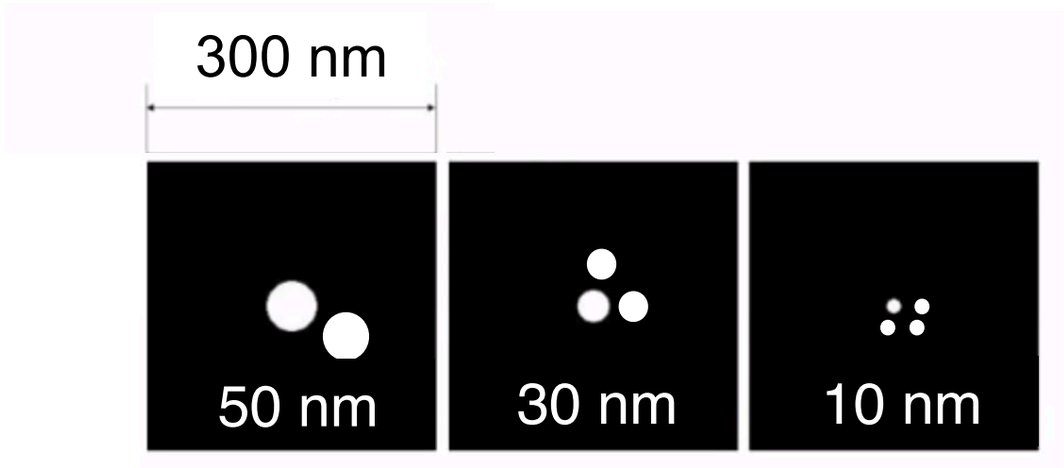
1.4NA objective

$\lambda = 0.48\ \mu m$

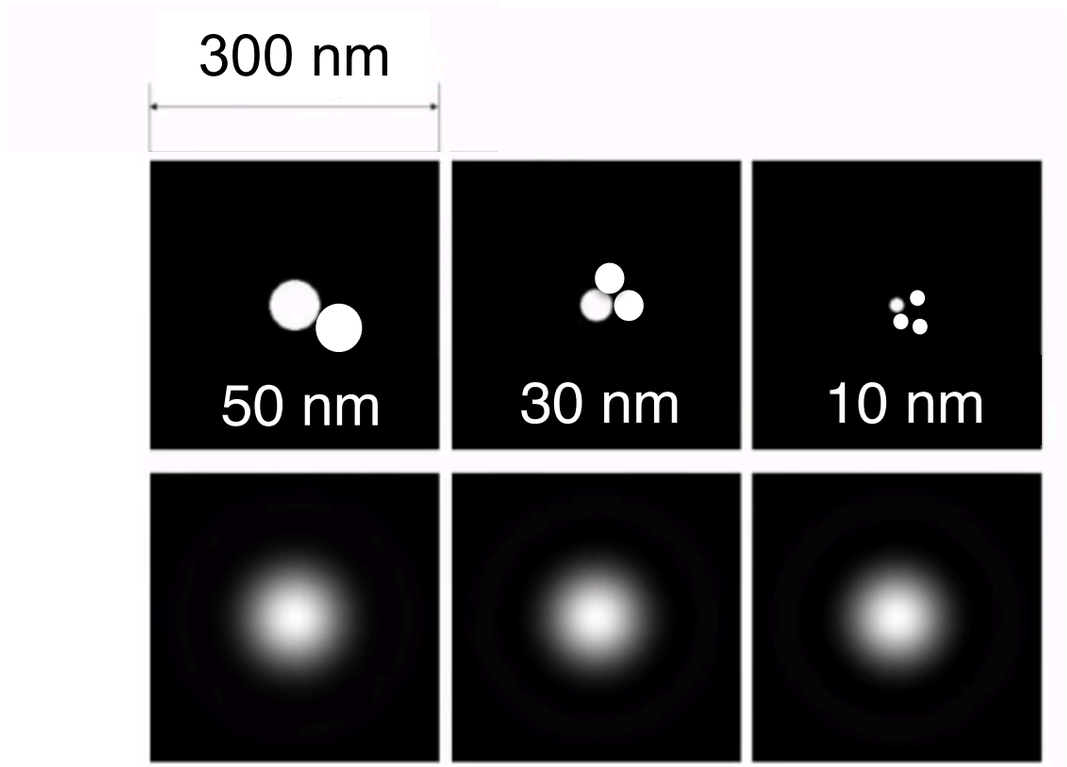
$$d = \frac{\lambda}{2\mathrm{NA}} \sim 170\mathrm{nm}$$

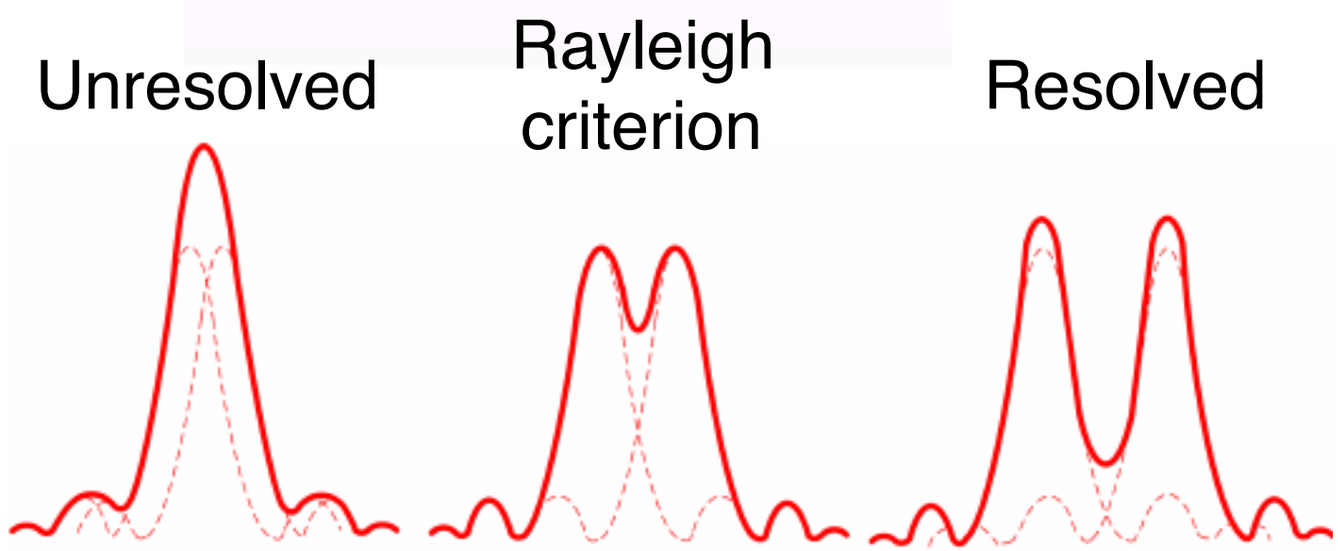
Abbe's diffraction limit

How is the PSF of many small objects?



PSF of many small objects





OTF (Optical transfer function)

Used in widefield-deconvolution and Super-resolution (SIM)

OTF is the **Fourier transform** of PSF

FT algorithm computes

a signal into its

frequency domain

PSF
spatial domain
x, y, z

Fourier

transform

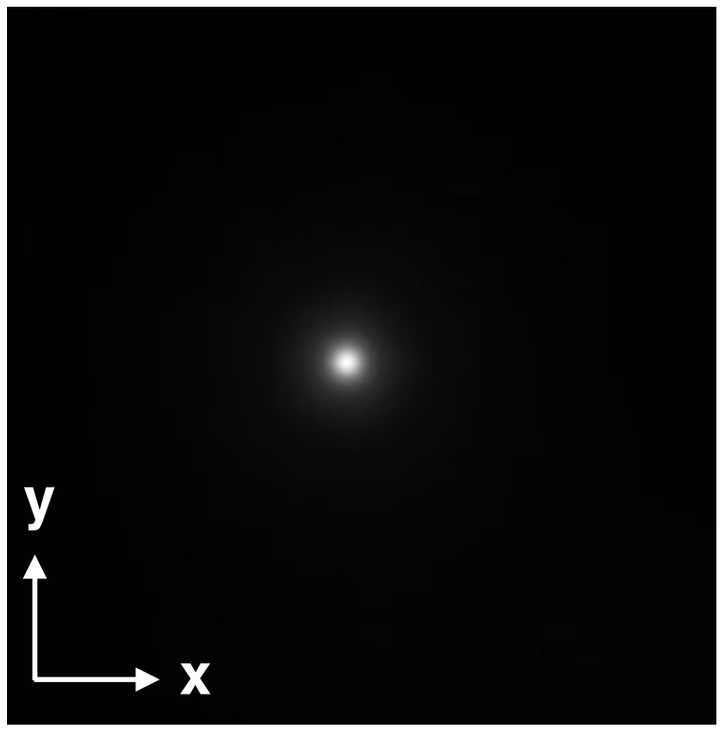
OTF
spatial frequency domain

OTF represents how spatial frequencies are handled by the optical system

How often it happens in space?

OTF (Optical transfer function) is the Fourier transform of PSF

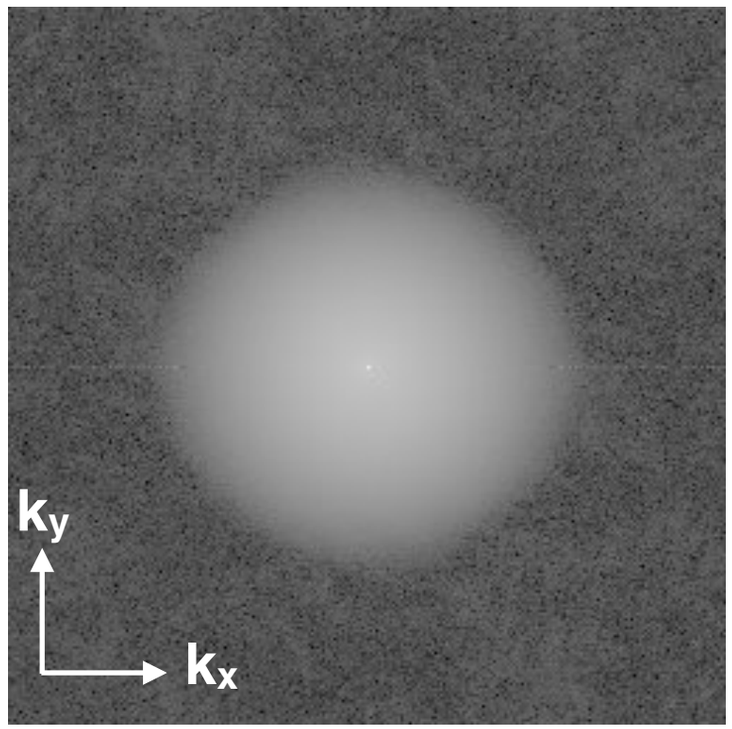
PSF



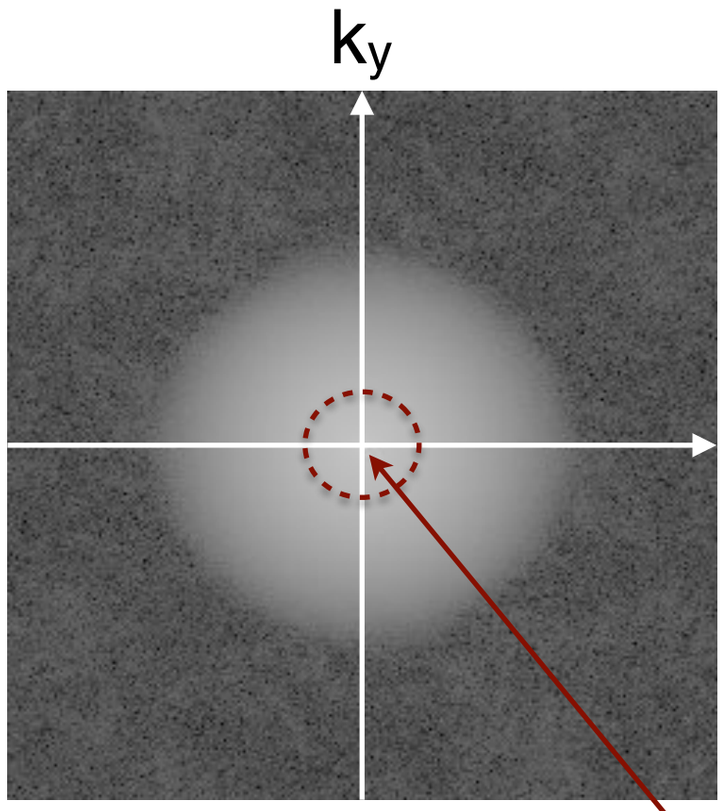
Fourier transform

Inverse Fourier transform

OTF



OTF (Optical transfer function) is the Fourier transform of PSF



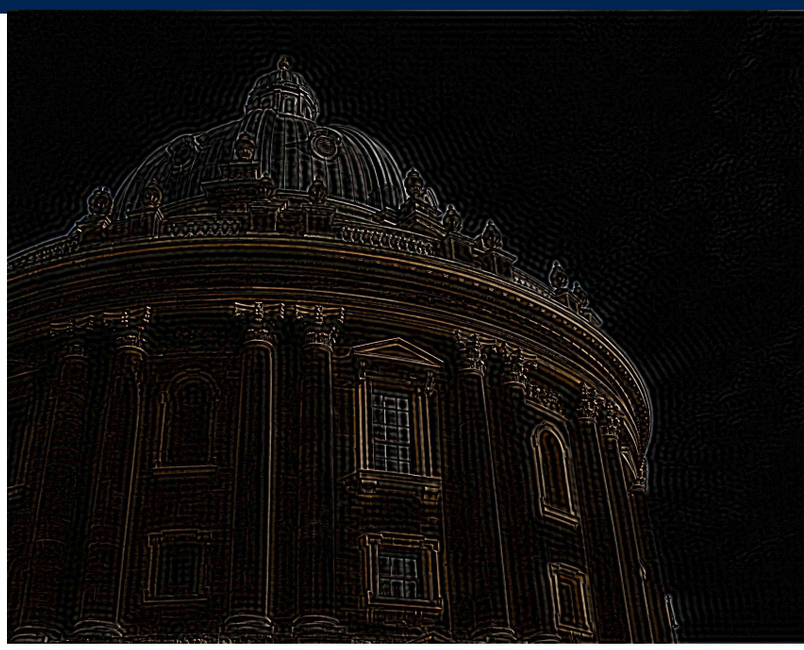
Lower frequencies
towards the centre

What are spatial frequencies … in an image?

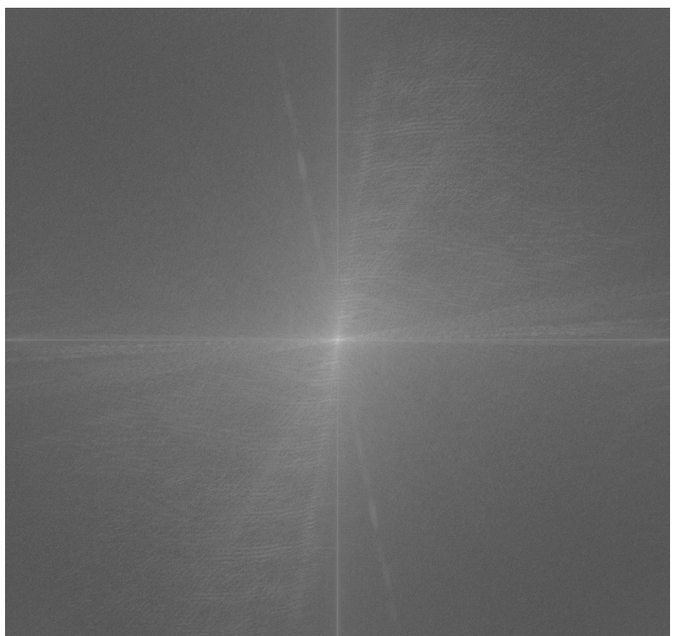
Lower frequencies - blurred

Higher frequencies - sharp

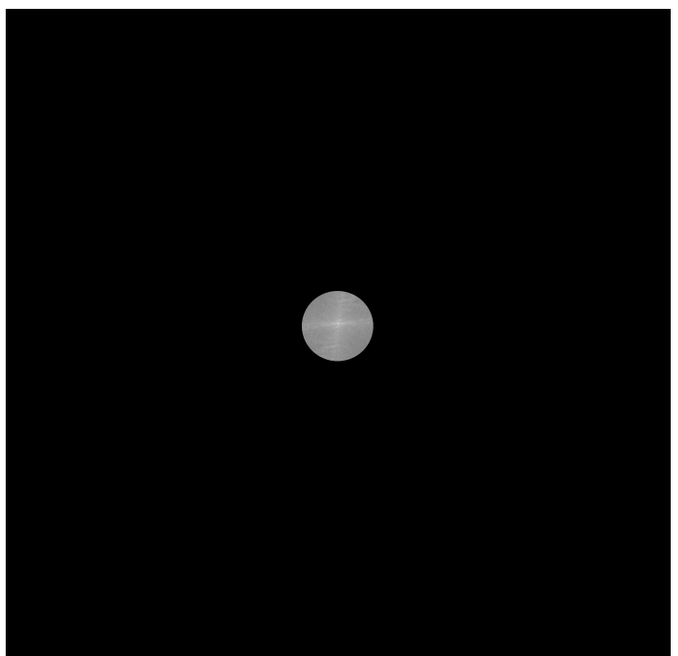
FIJI / Process / FFT

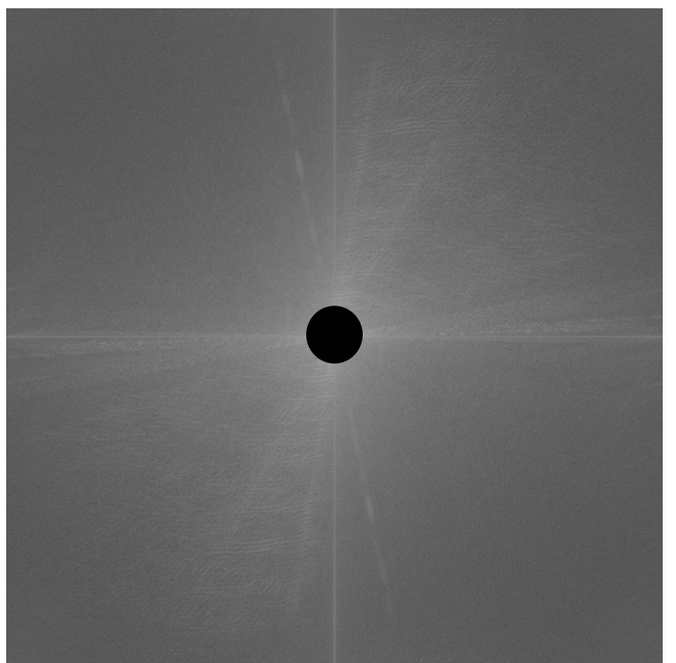


Fourier transform



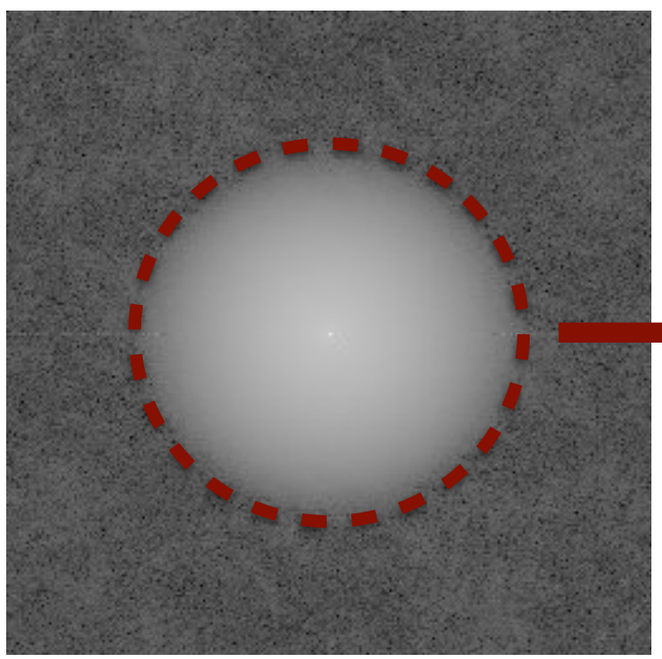
Inverse Fourier transform





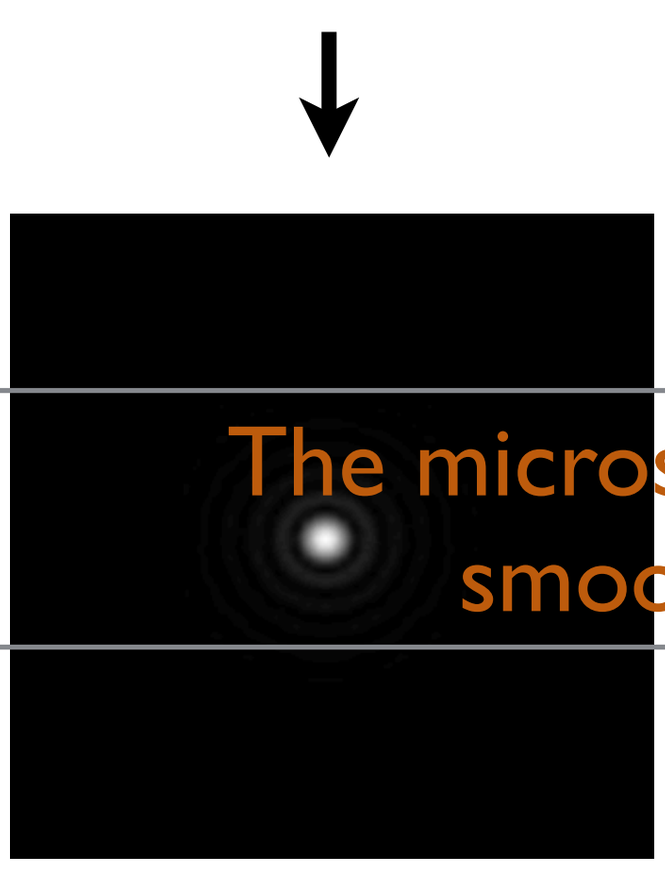
It’s very easy to detect certain features in the frequency domain

All frequencies



What does it represent?

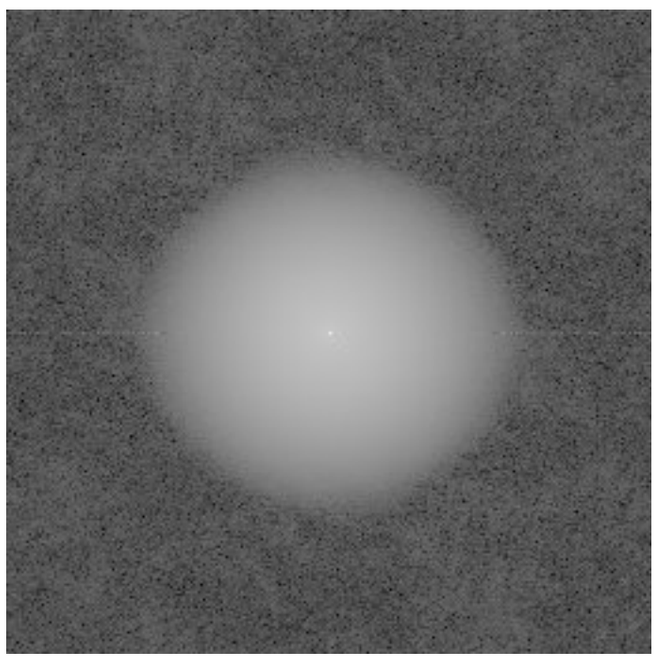
Back Aperture Objective



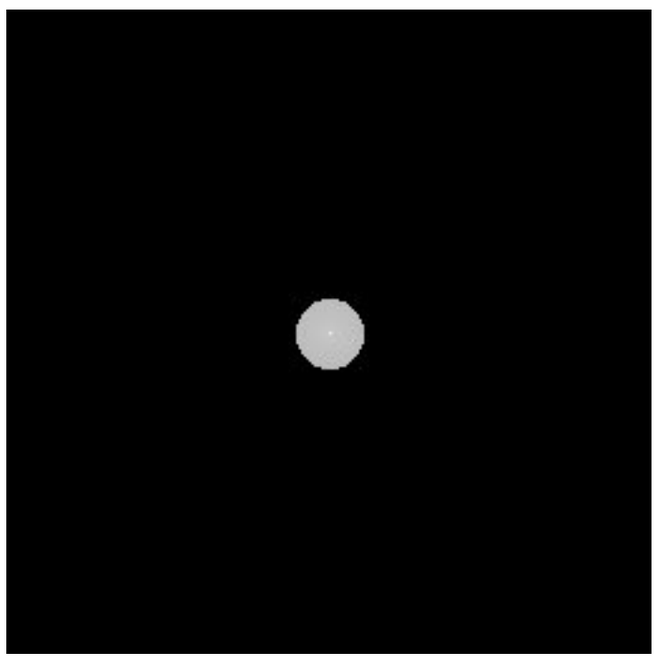
The microscope passes low frequencies (large and smooth) and excludes high frequencies

It’s very easy to detect certain features in the frequency domain

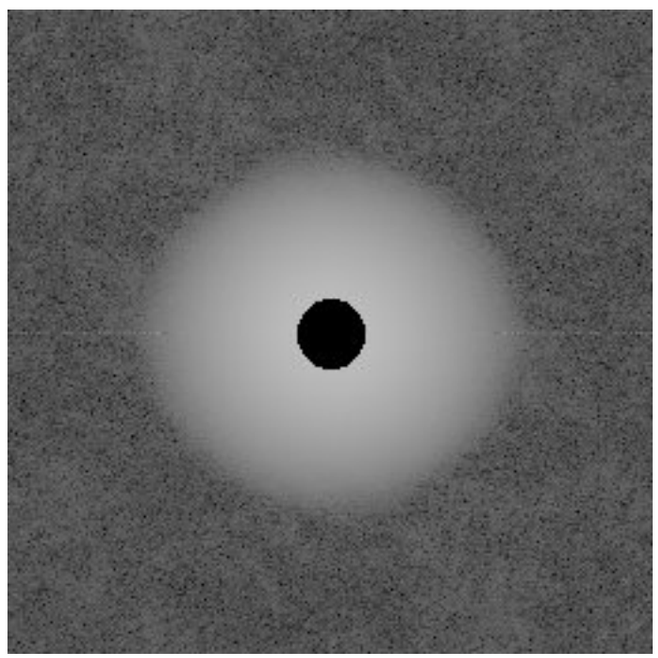
All frequencies



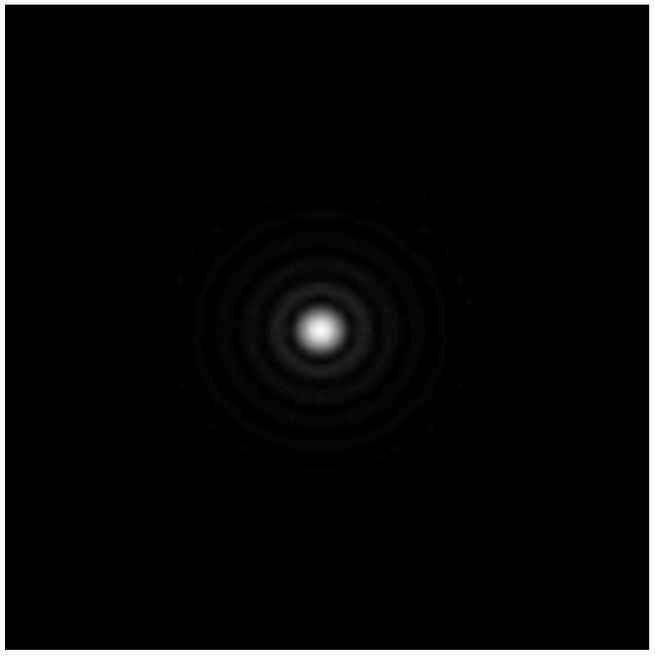
Just lower frequencies

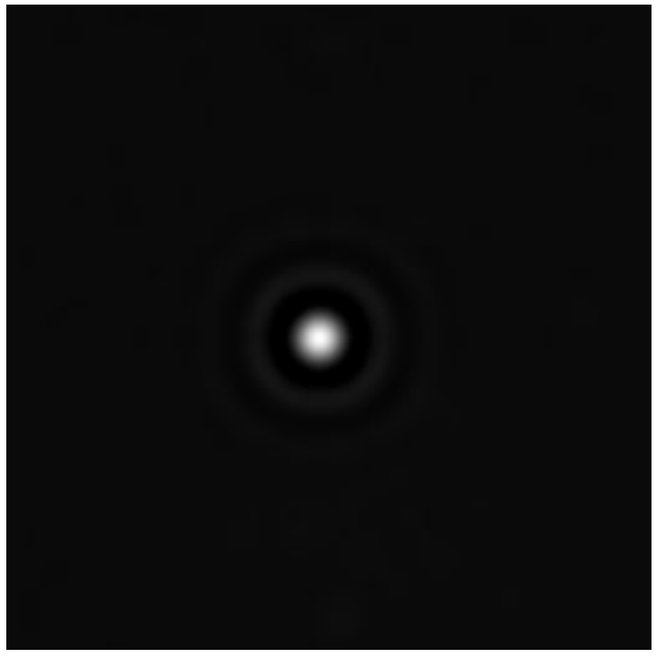


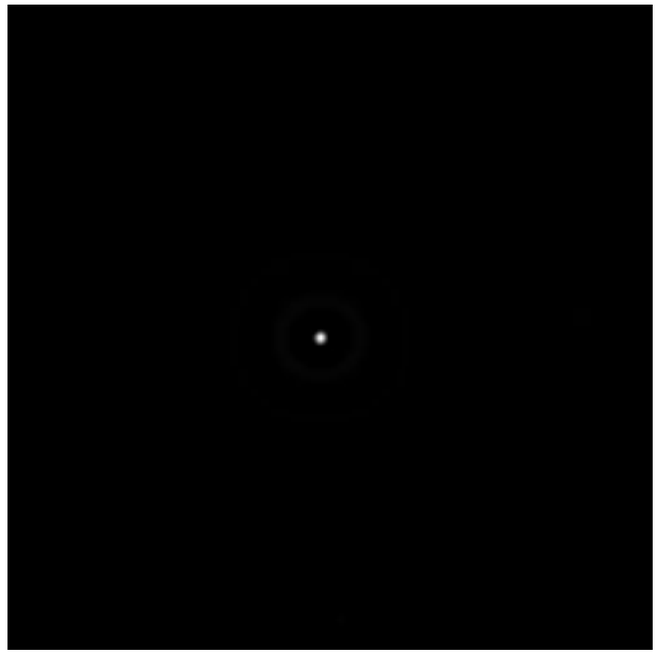
Just higher frequencies



Inverse Fourier transform

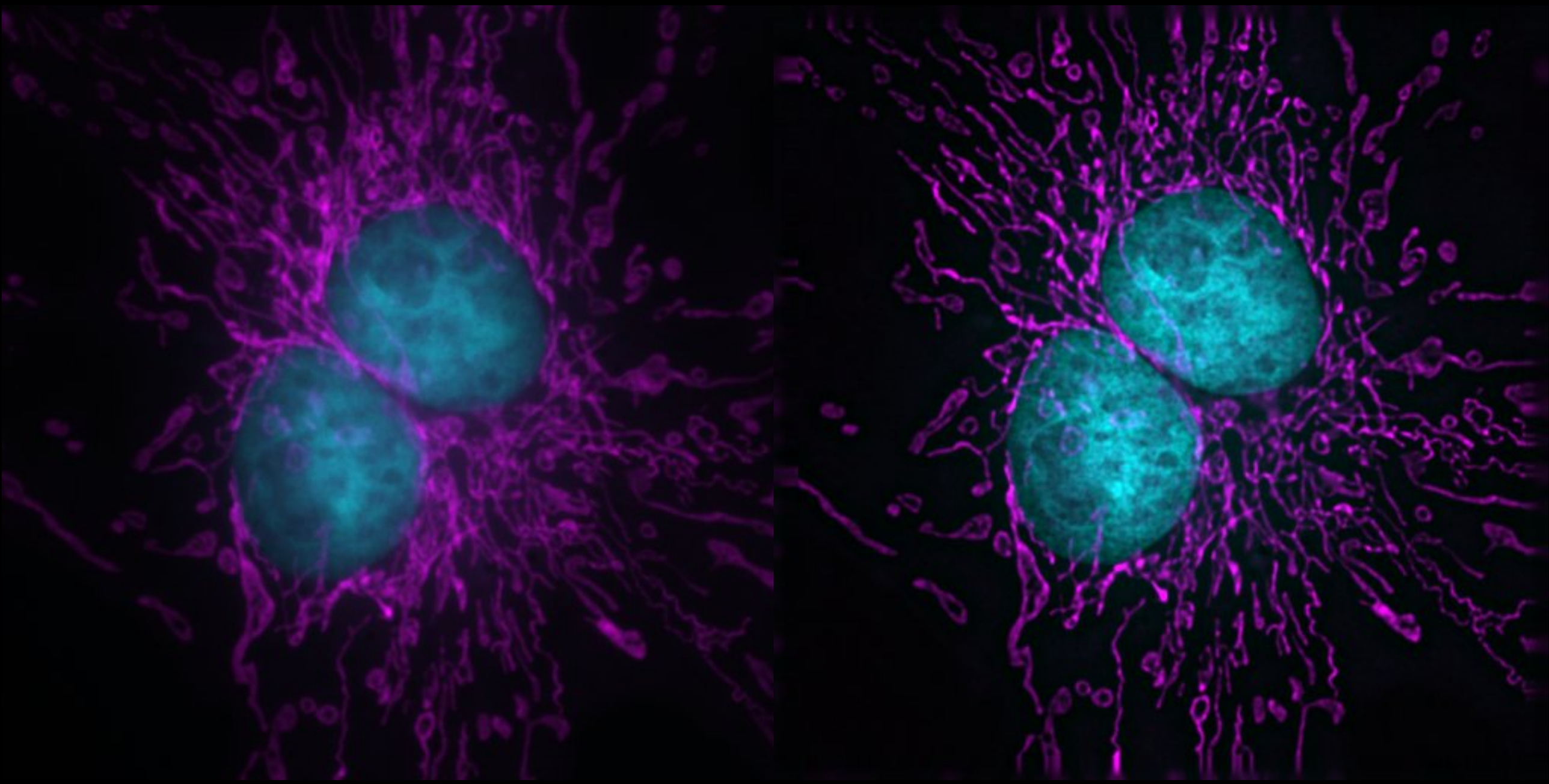






Widefield

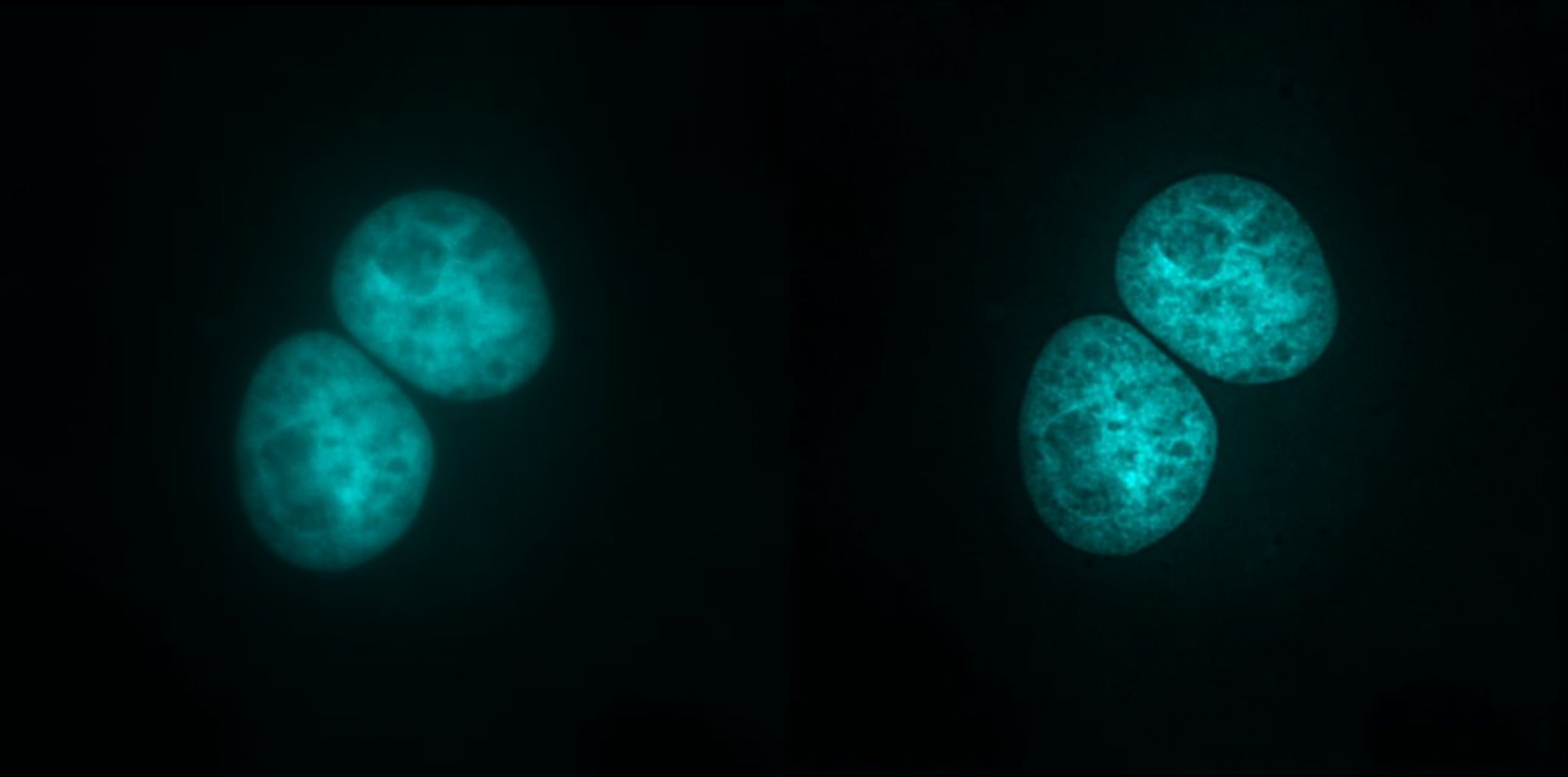
Deconvolution



BPAE cells with Mitotracker Red (magenta) and DAPI (cyan)

Widefield

Deconvolution



Nuclei from BPAE cells stained with DAPI (cyan)

Conclusions

- Why is fluorescence? CONTRAST
- Dichroic mirror - separates illumination (excitation) from fluorescence (emission)
- Fluorescence microscope:
    - illumination light is reflected (opposed to transmitted)
        - objective illuminates and collects fluorescence (both “condenser” and objective)
- Tips on fixed sample preparation
- Point Spread Function and Optical Transfer Function
- Widefield fluorescence microscopy collects the whole field of view at once; it’s fast and very sensitive and you can have deconvolution for free

Questions?