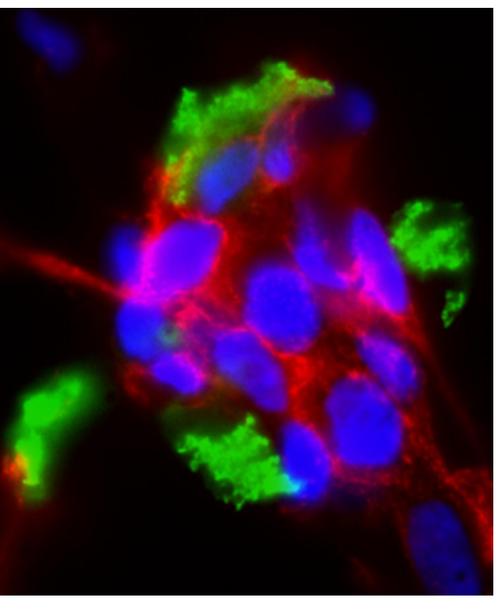
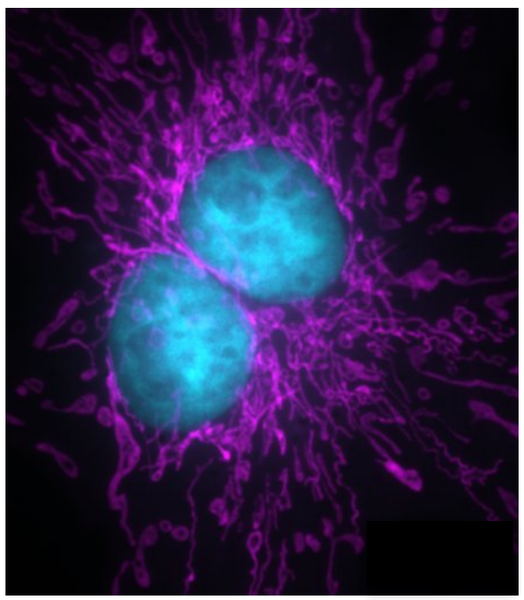
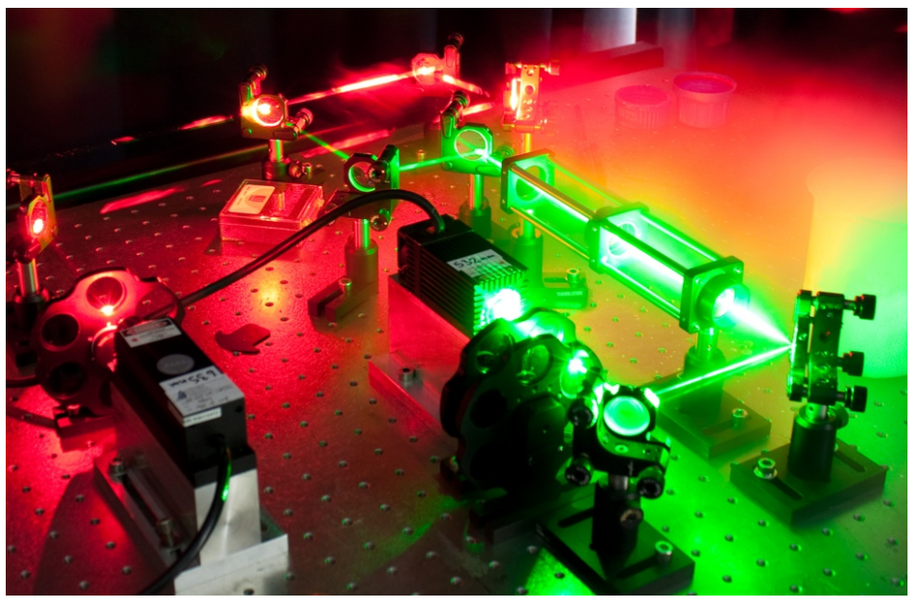
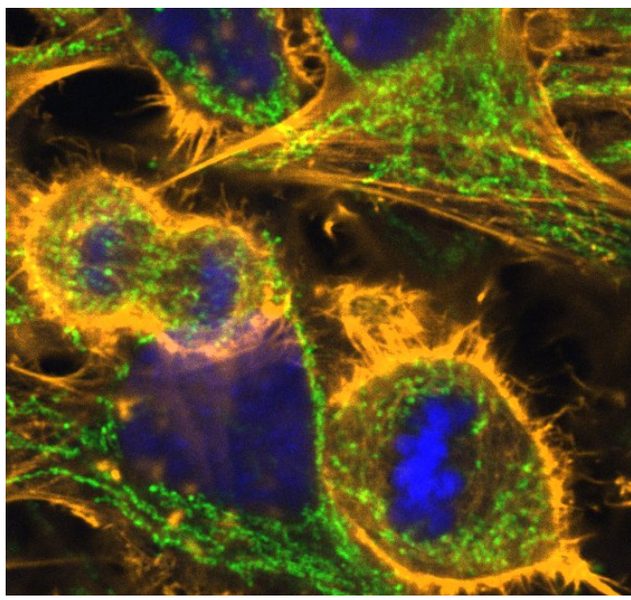
Micron OXFORD

Lecture 10

17th November 2020

Introduction to Fluorescence Microscopy

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@CarinaMonico

Outline

1. What is fluorescence? Fluorescence Spectra
2. Why fluorescence is so commonly used in microscopy?
3. Filtersets for fluorescence imaging
4. Basic principle and components of fluorescence microscopes
    - Dichroic mirror
    - Transmitted vs. Reflected
    - Fluorescent light sources
5. Widefield fluorescent microscopy
6. Deconvolution, PSF, OTF

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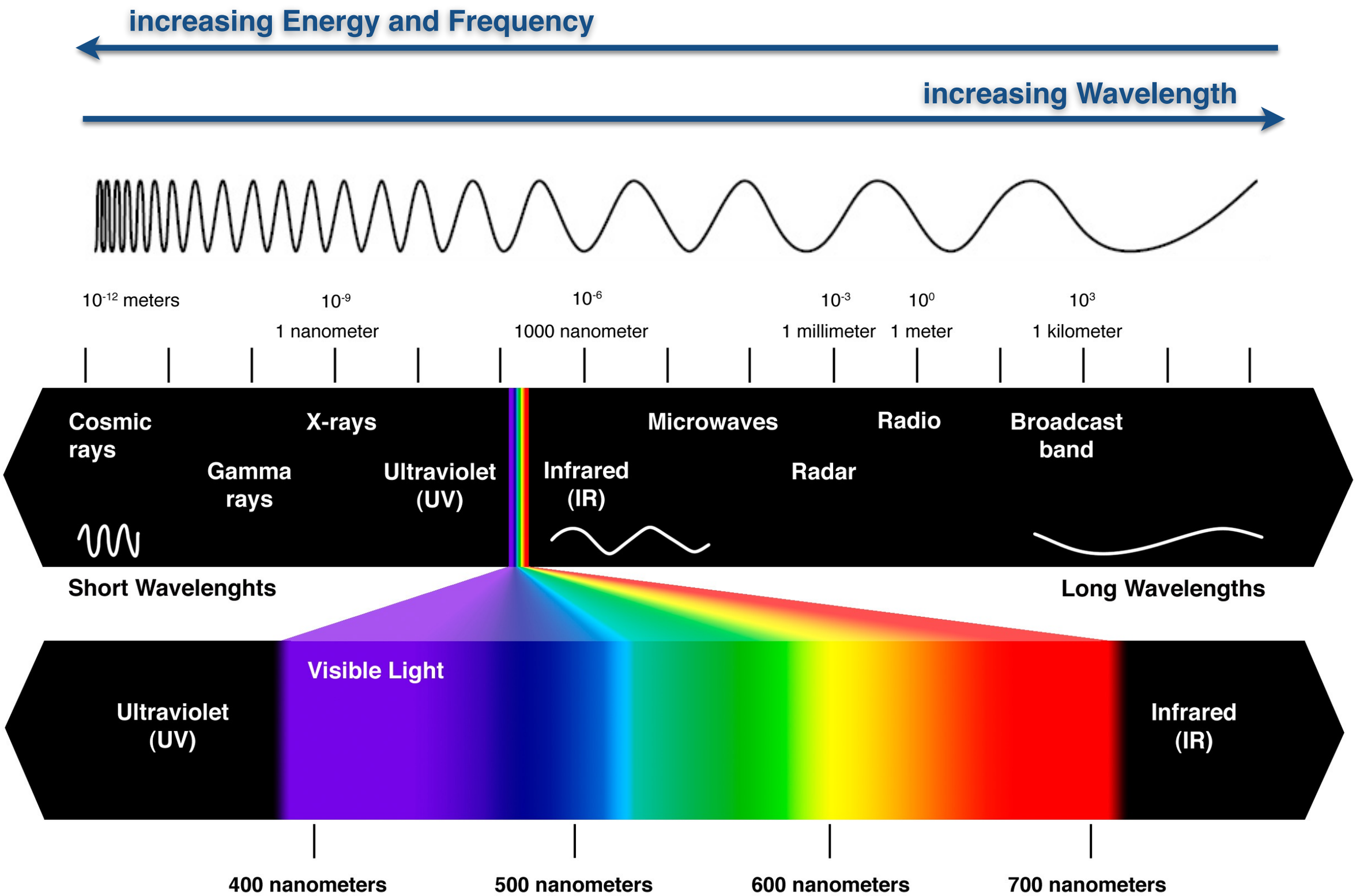
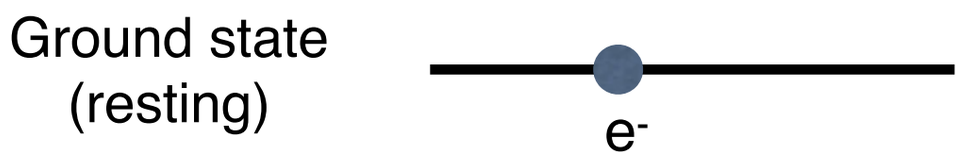
Light: the electromagnetic spectrum



380 - 700 nm visible to the human eye

1. What is Fluorescence?

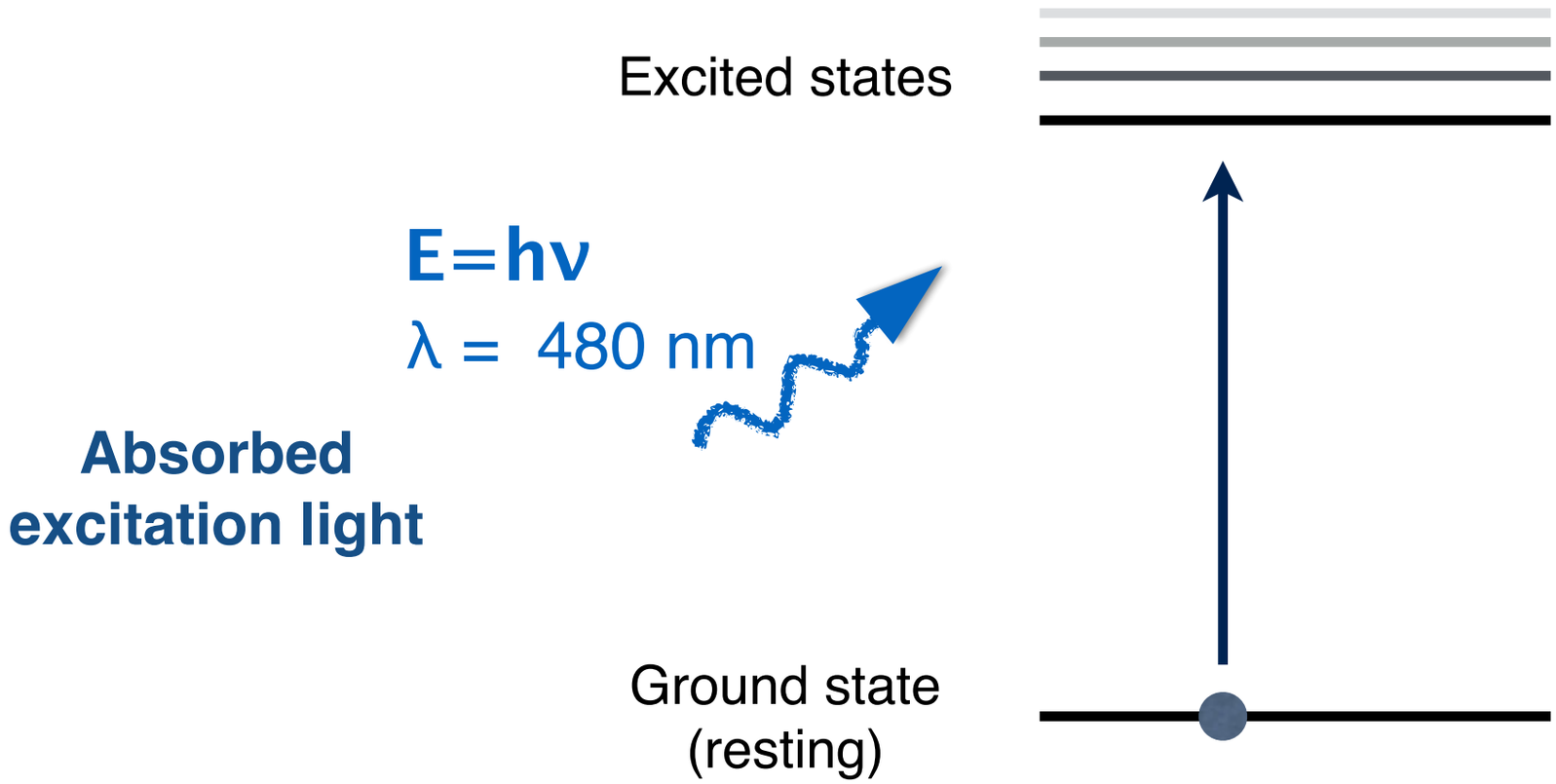
Fluorescence is the emission of light by a molecule that has absorbed light



Molecules have discrete levels of energy

1. What is Fluorescence?

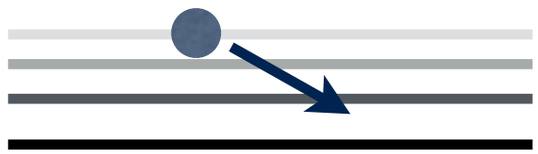
Fluorescence is the emission of light by a molecule that has absorbed light



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

1. What is Fluorescence?

Excited states



non-radiative transition

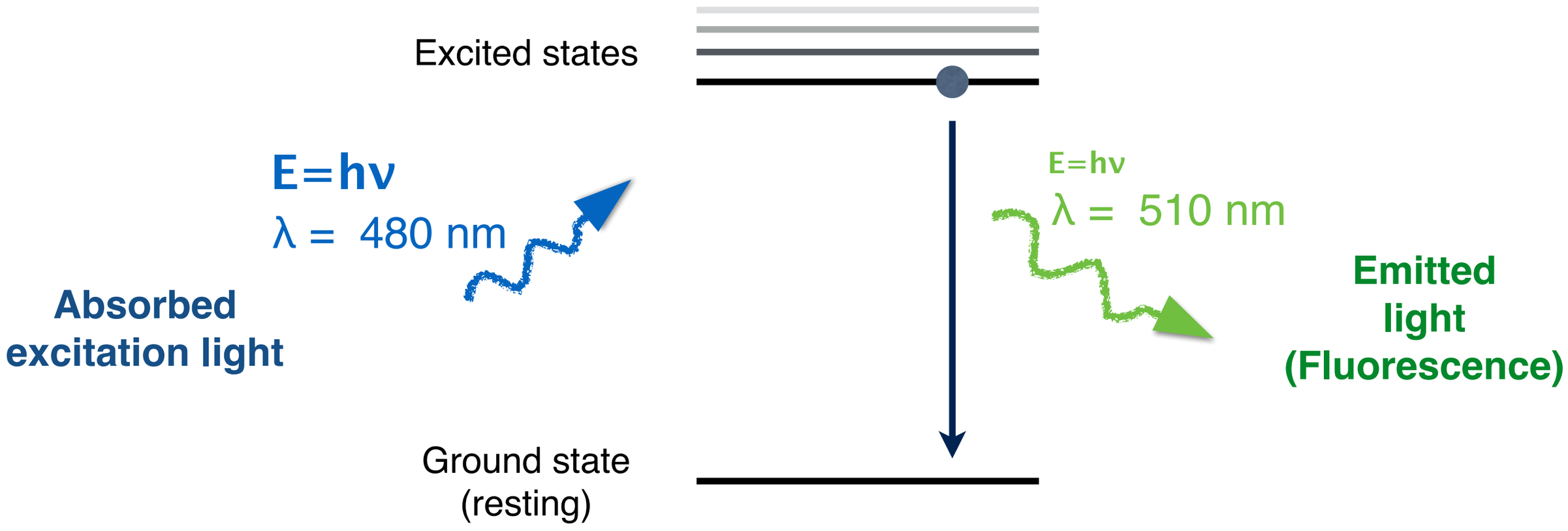
E=hν

λ = 480 nm

Absorbed excitation light

Ground state (resting)

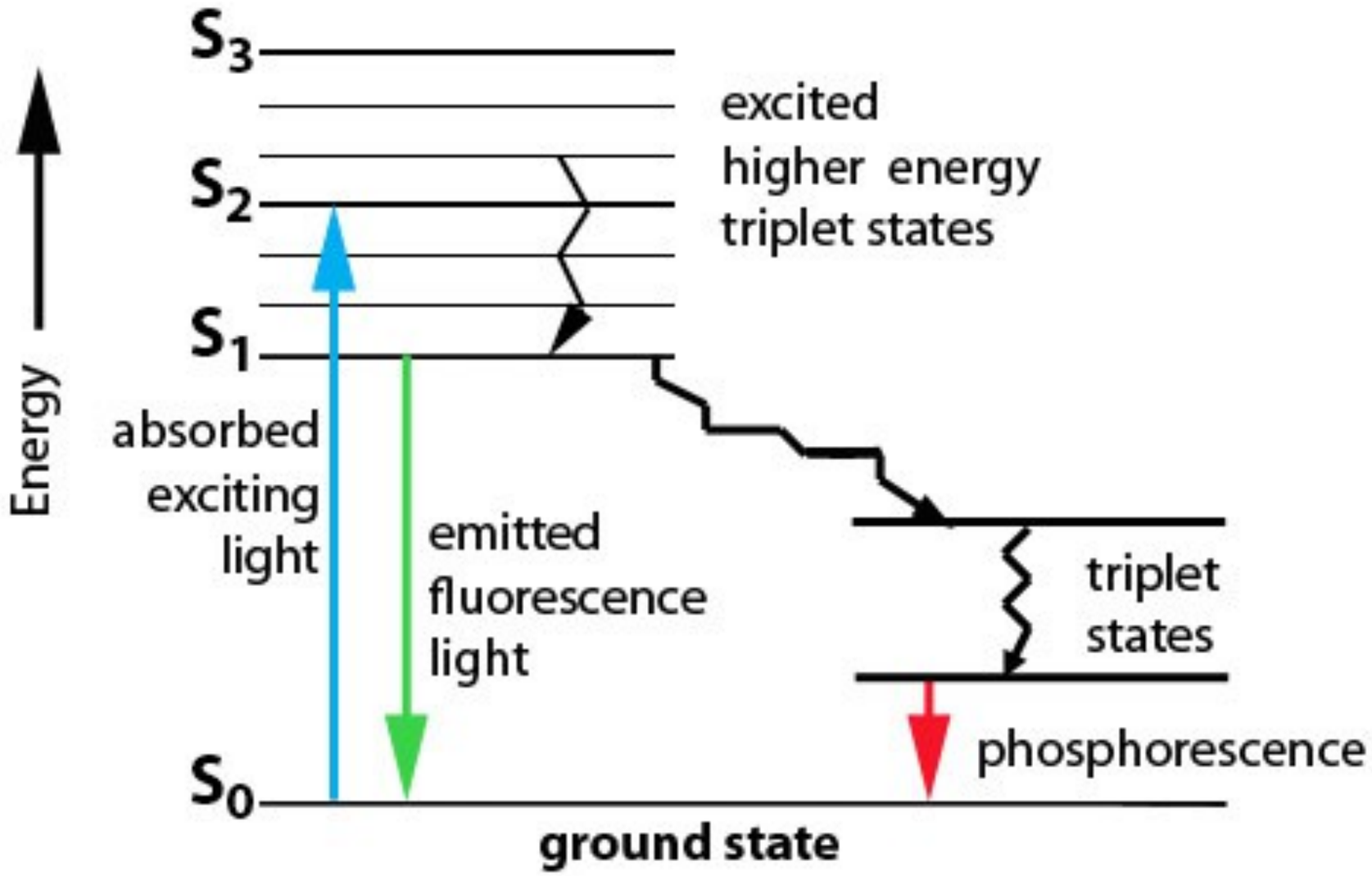
1. What is Fluorescence?



Fluorescence has higher wavelength than absorbed light

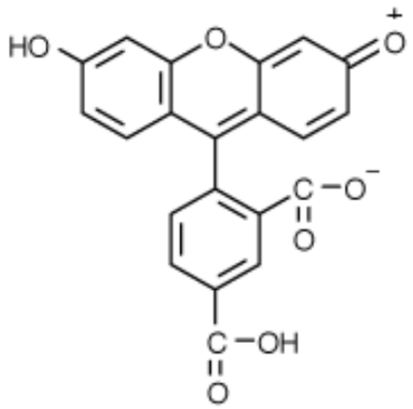
The full picture is represented on the Jablonski diagram…

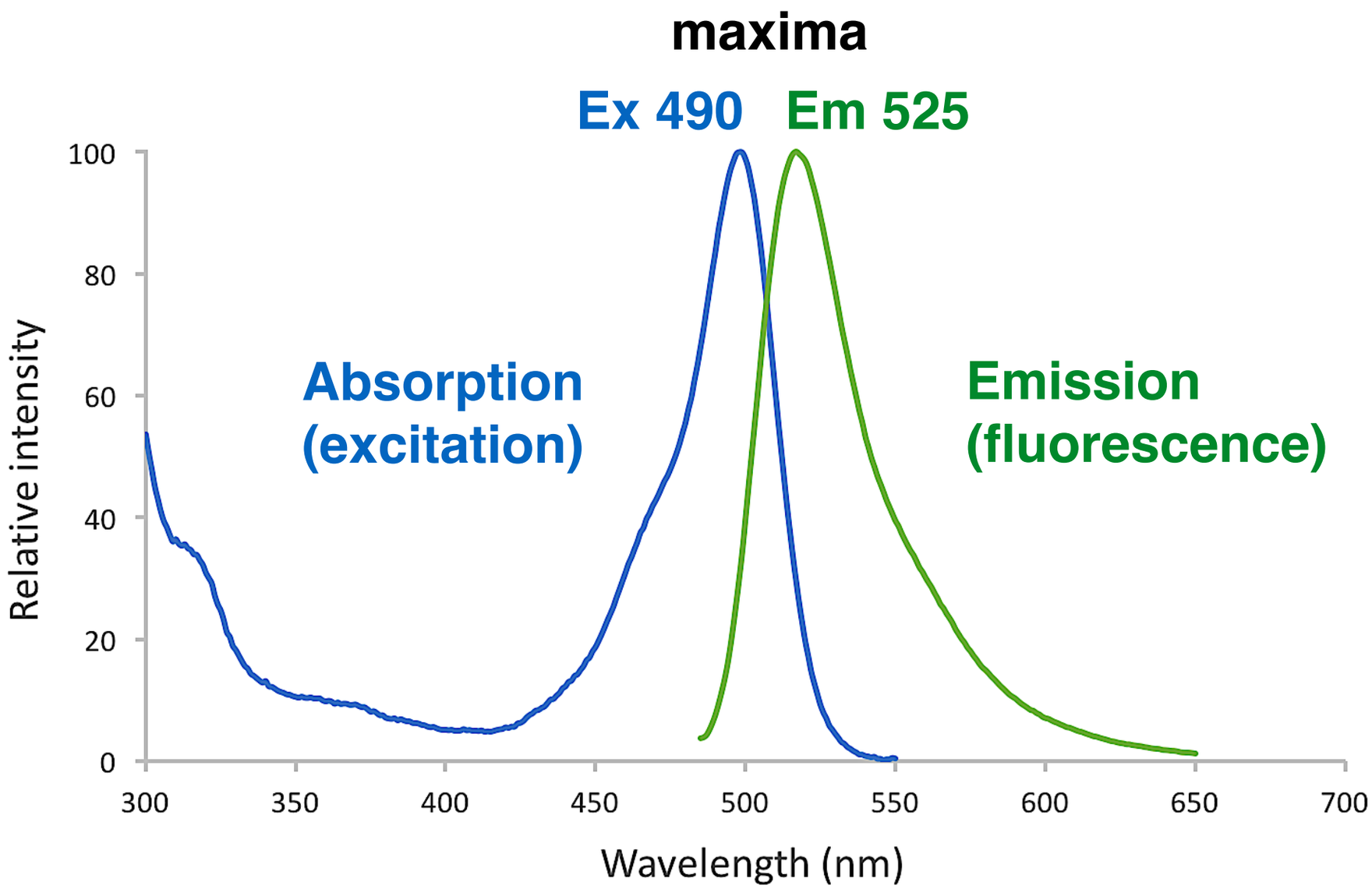
→ Lecture 11



Fluorescence Spectra

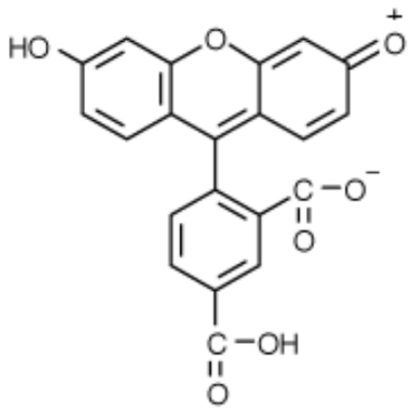
Fluorescein (FITC)





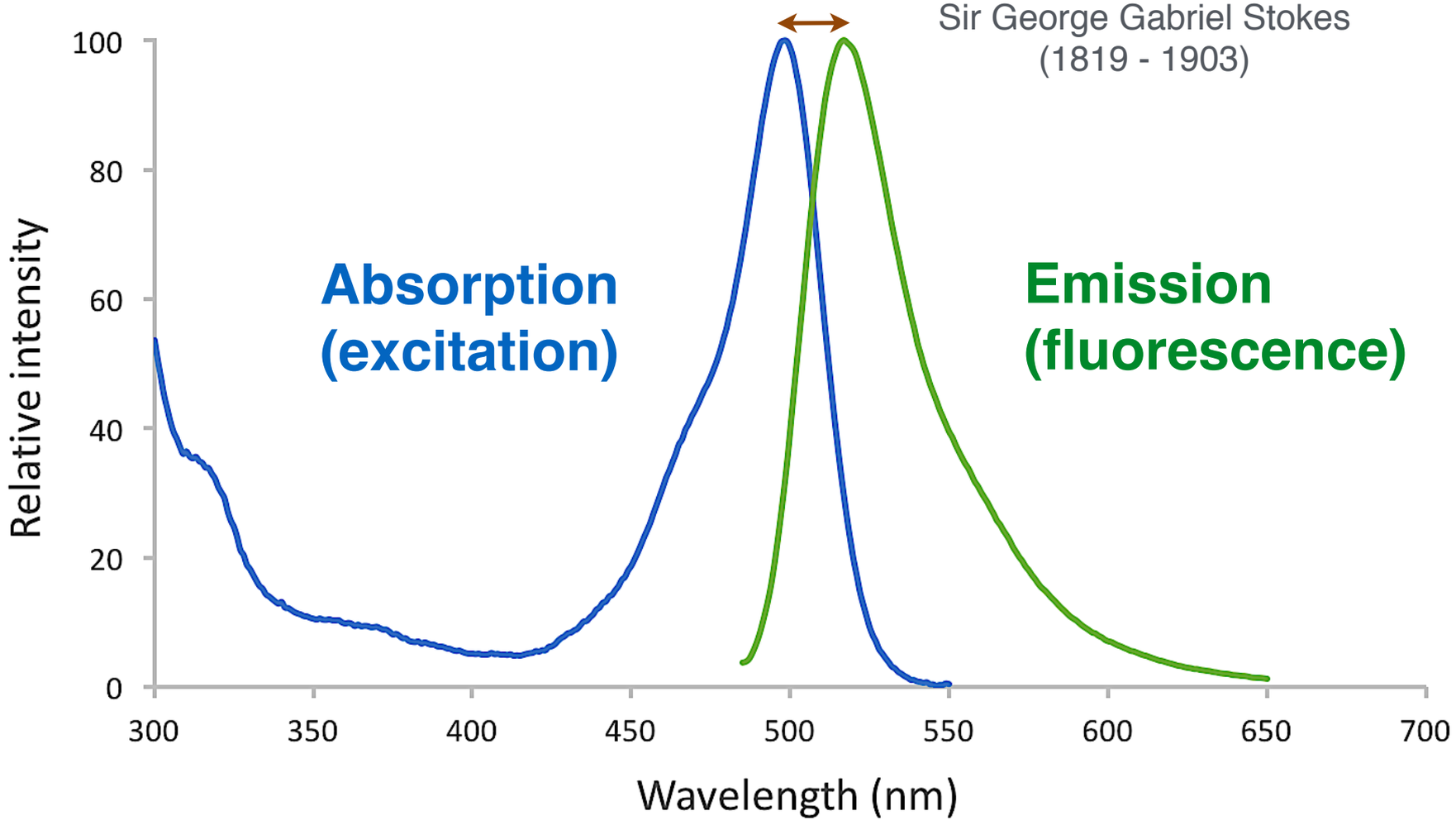
Fluorescence Spectra

Fluorescein (FITC)



Stokes shift

Sir George Gabriel Stokes
(1819 - 1903)



Fluorescent probes for microscopy

→ Lecture 11

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)

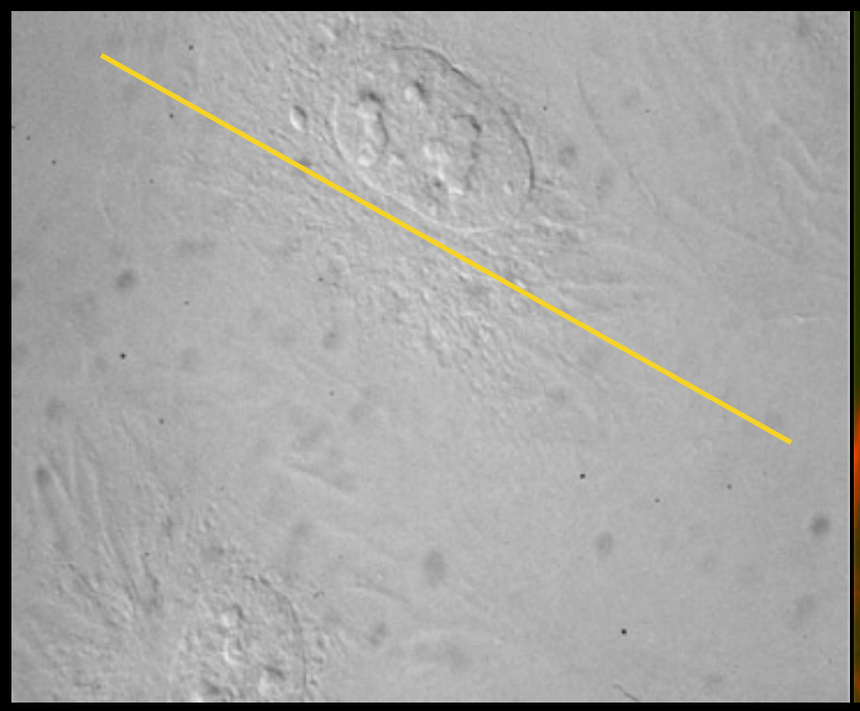
2. Why Fluorescence?

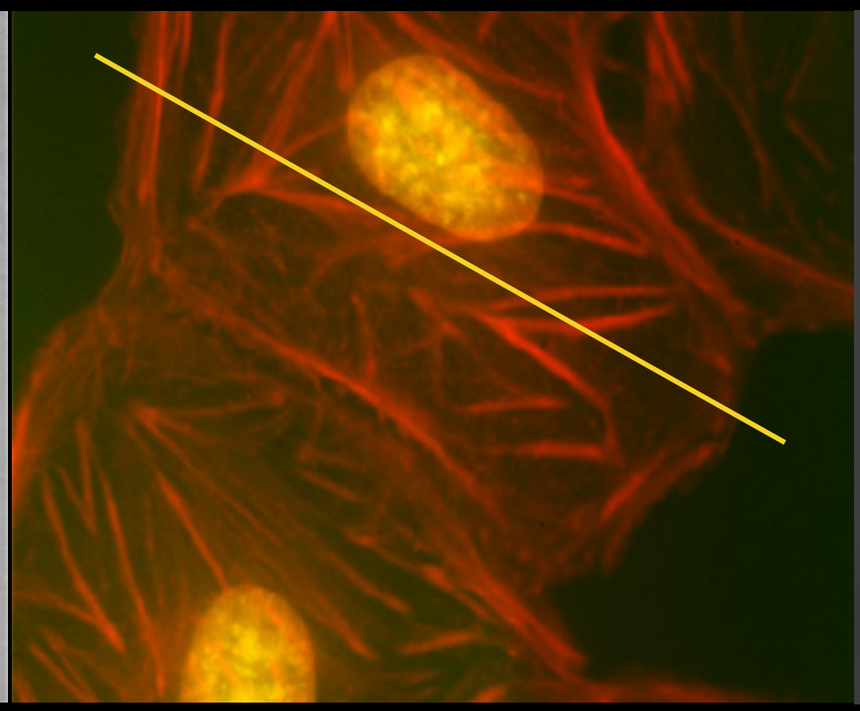
CONTRAST

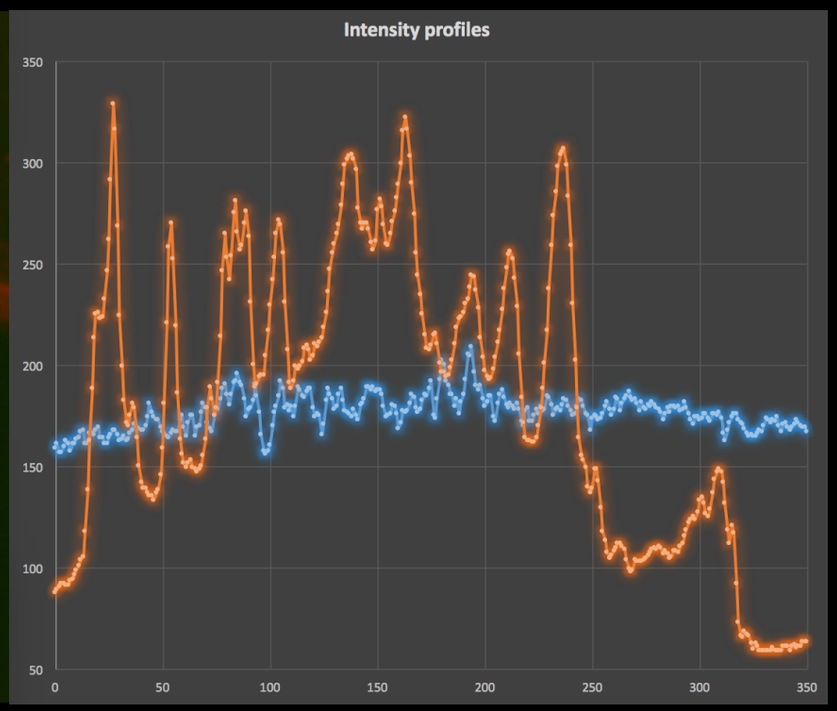
2. Why Fluorescence?

- Weak signal against dark background
- High signal to background - contrast

Bright field (DIC) Fluorescence Intensity profile



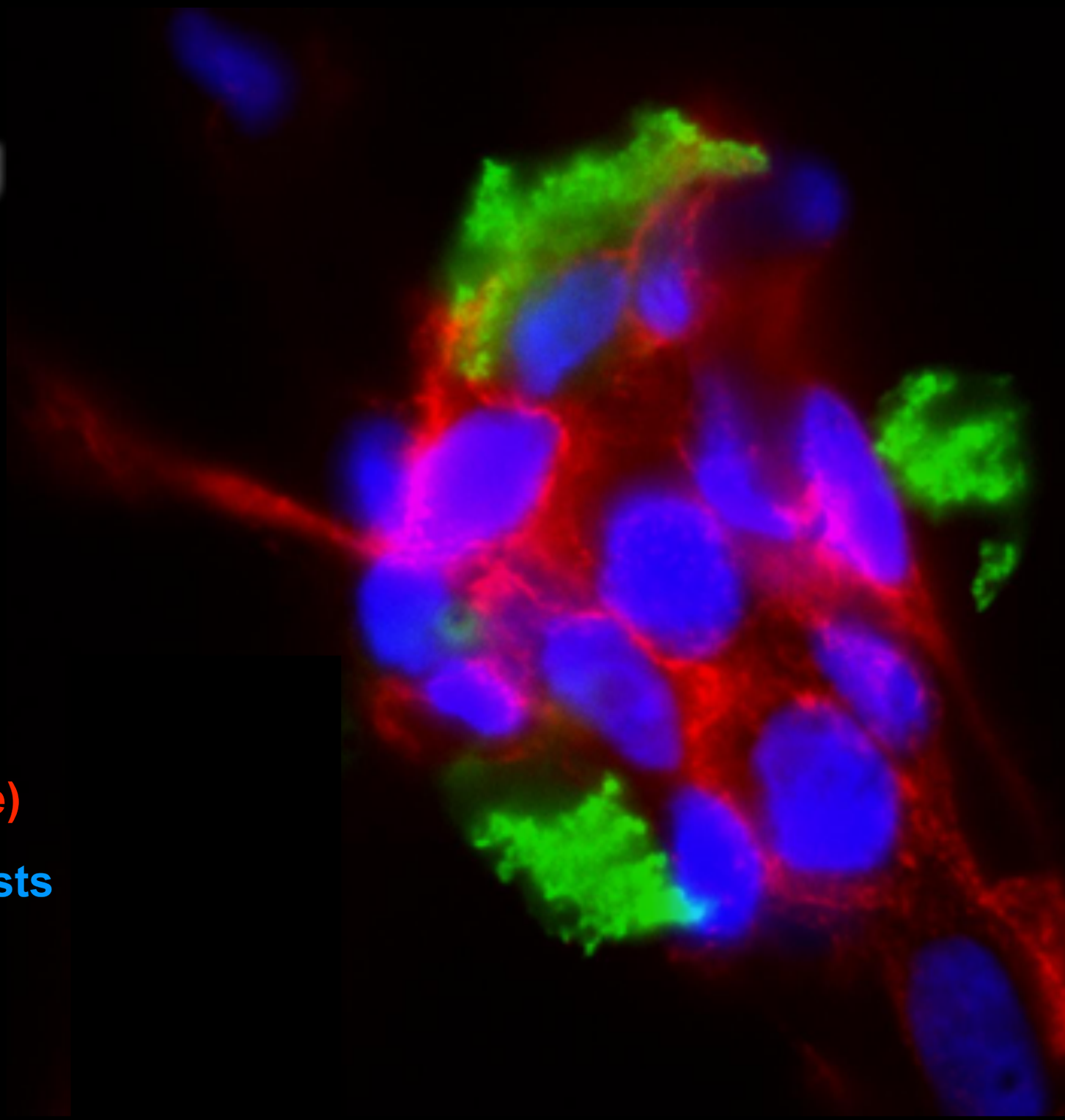




https://www.thermofisher.com/uk/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/imaging-basics/

2. Why Fluorescence?

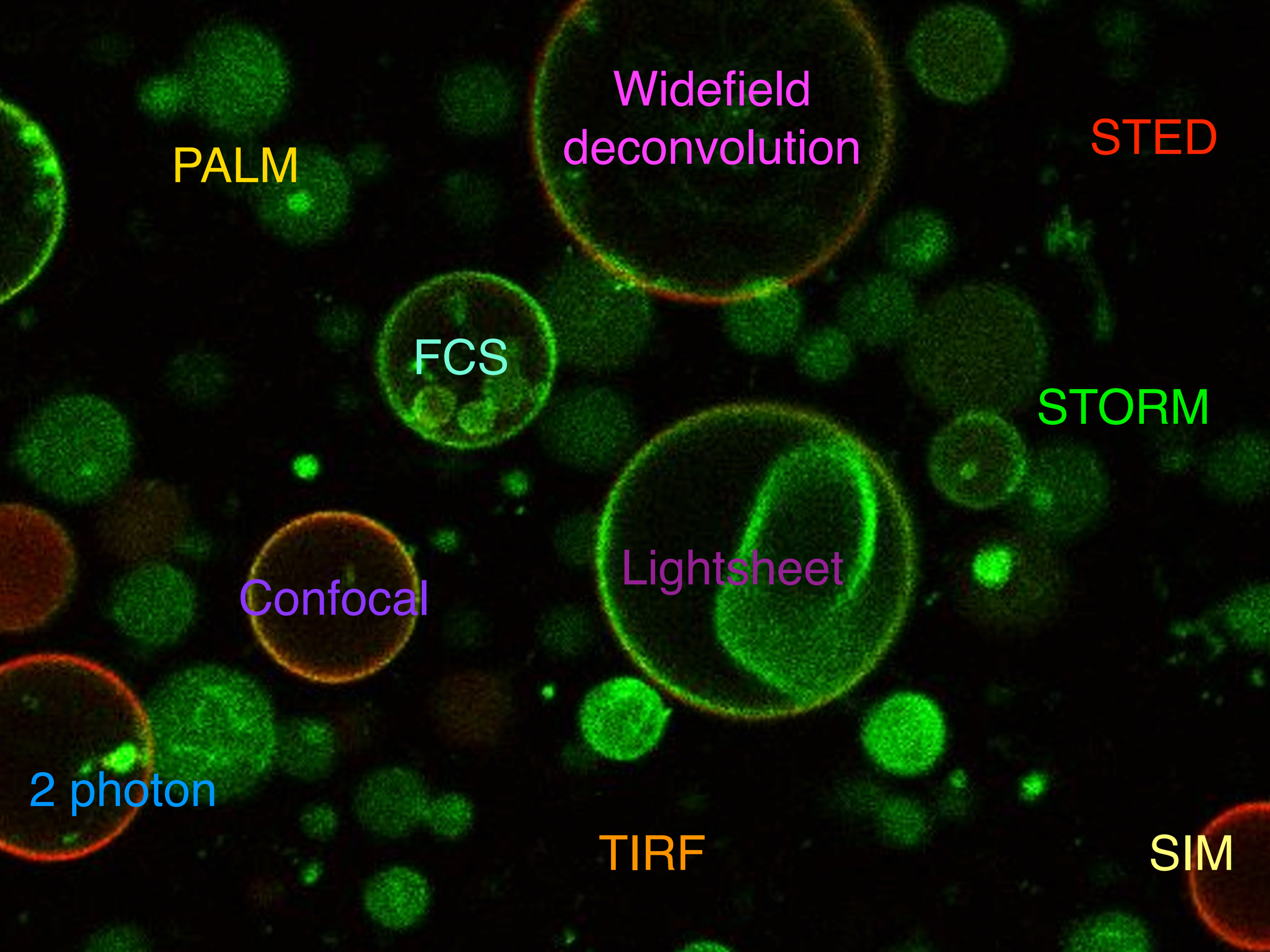
- Selective labeling
- Ease of multiplexing
- Quantitative



HypF-N Amyloid aggregates

Cholera Toxin B (membrane)

DNA (nuclei) - Fibroblasts


PALM
Widefield
deconvolution
STED
FCS
STORM
Confocal
Lightsheet
2 photon
TIRF
SIM

Why is the background black in a fluorescent image...?

3. Fundamental problem in fluorescence microscopy

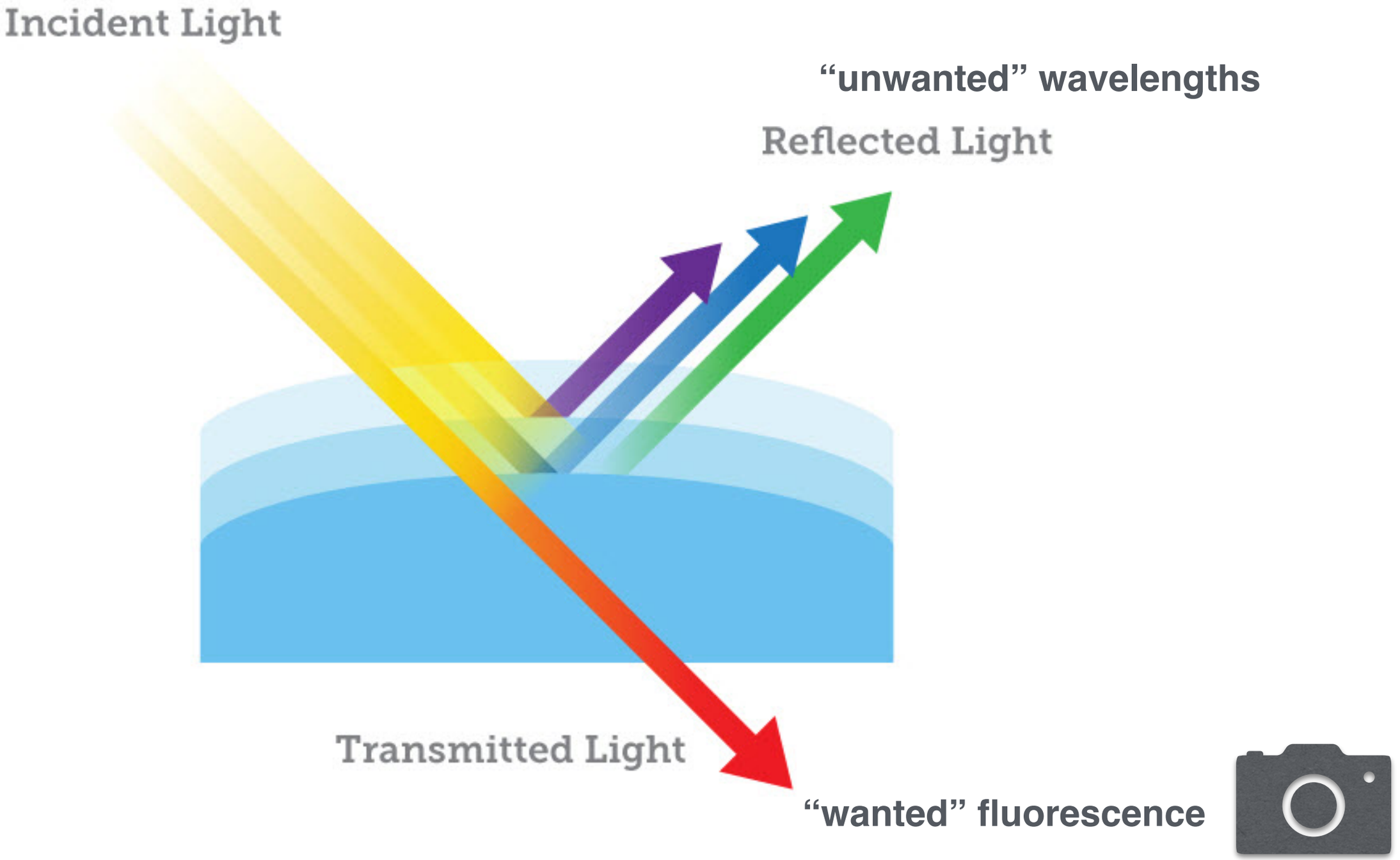
STRONG illumination VS. **WEAK fluorescence signal**

✓ produce high-efficient illumination of the specimen

✓ capture weak fluorescence emission

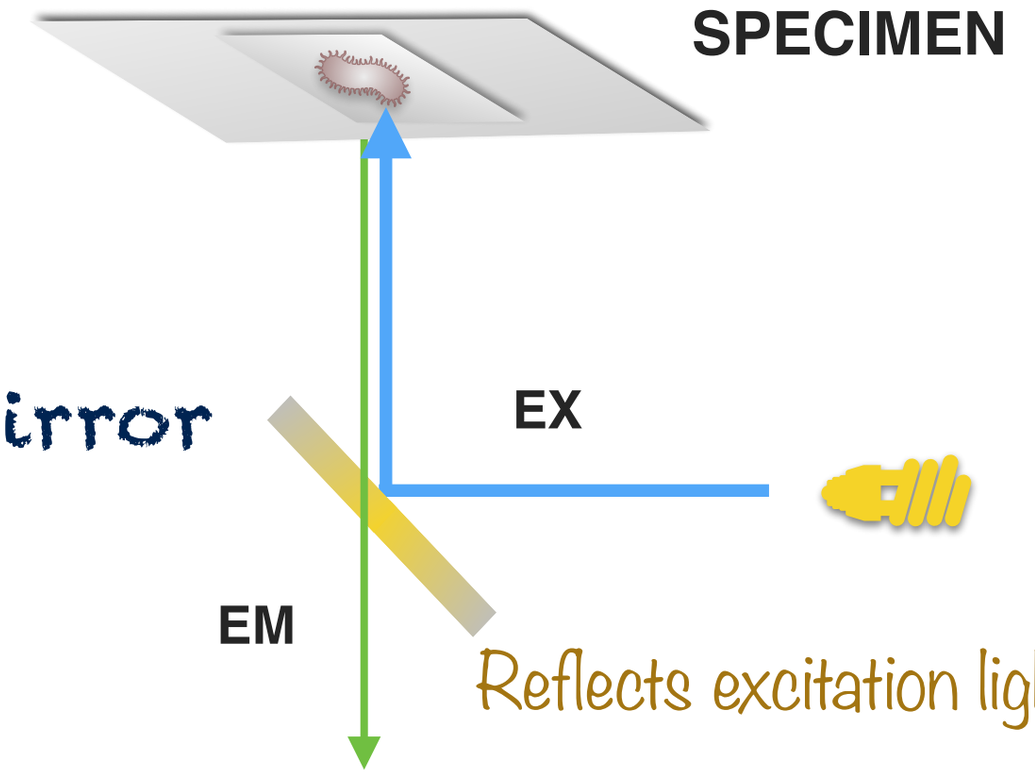
3. Dichroic mirror - at the heart of fluorescence microscopy

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

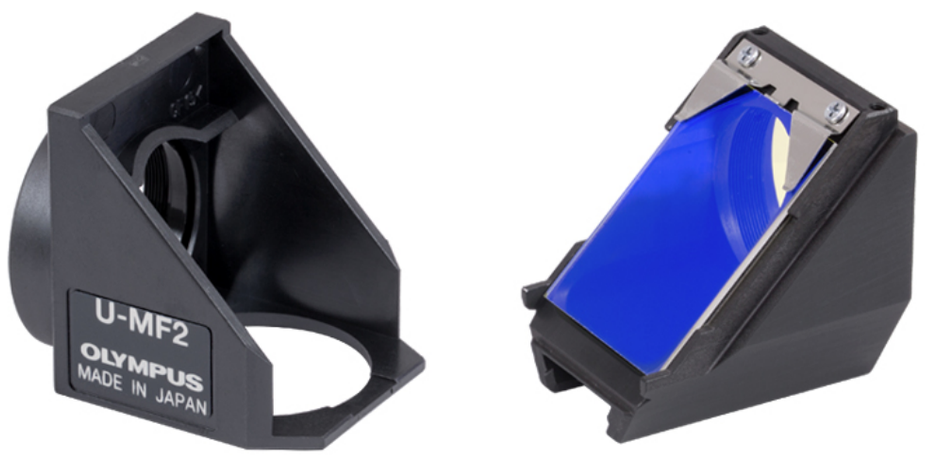


Teledyne imaging (https://possibility.teledyneimaging.com/show-money/)

3. Dichroic mirror - at the heart of fluorescence microscopy

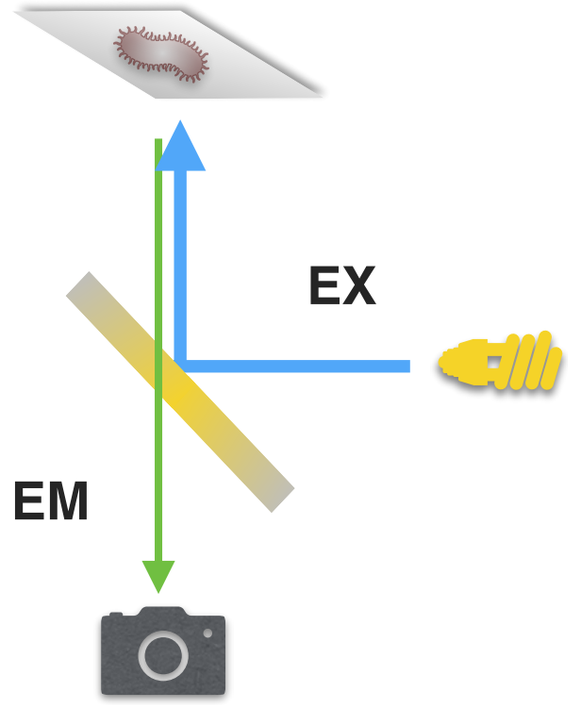


Reflects excitation light and transmits emission



https://www.thorlabs.com/newgrouppage9.cfm?objectgroup_id=5007

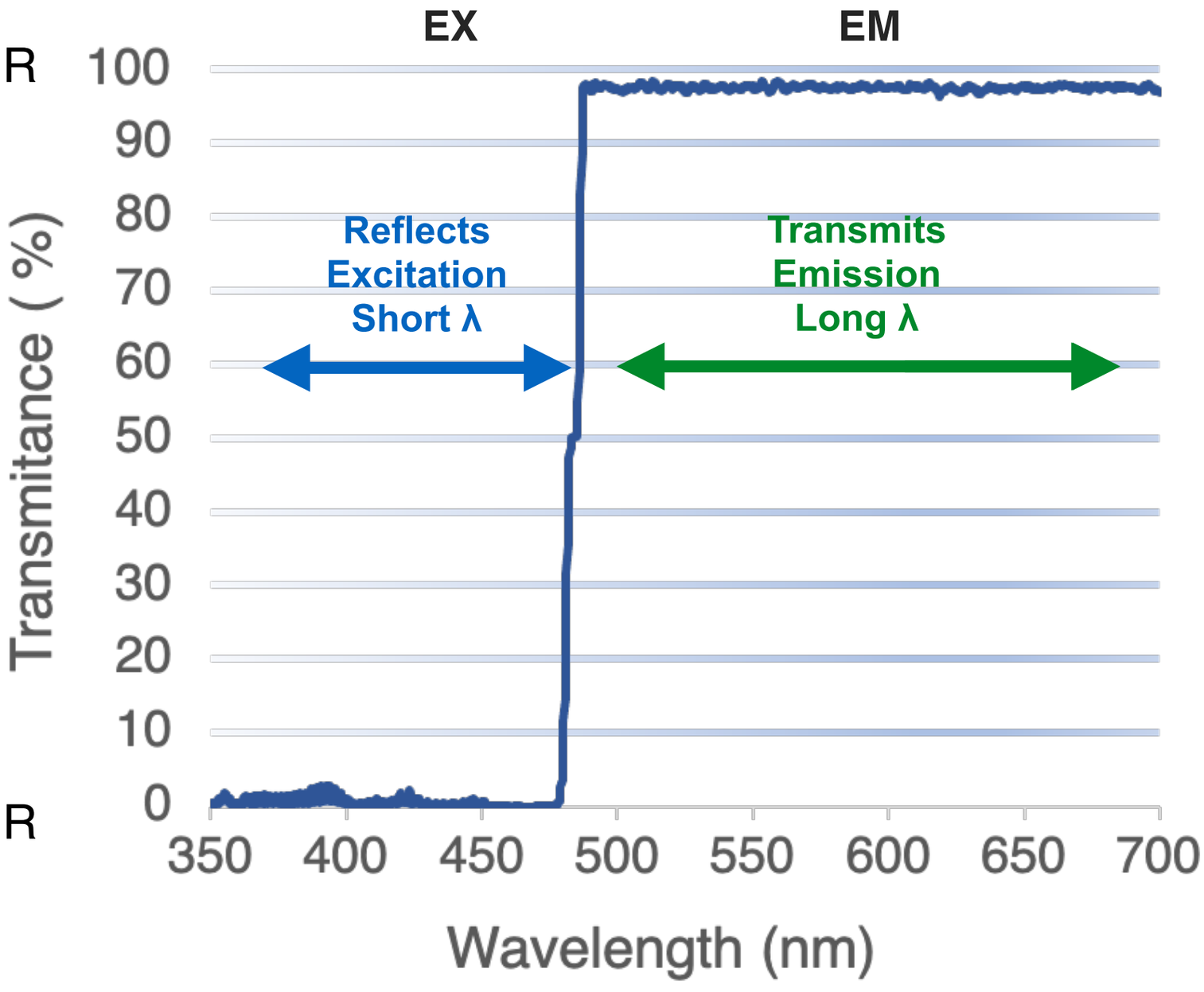
Dichroic mirror - Spectral properties



Dichroic

100% T = 0% R

0% T = 100% R

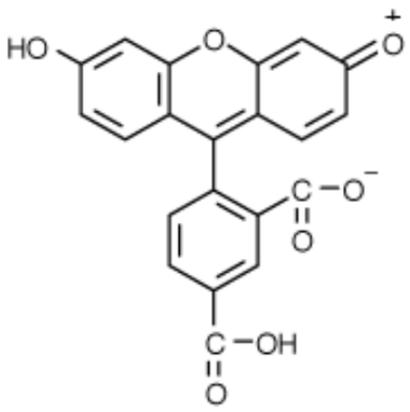


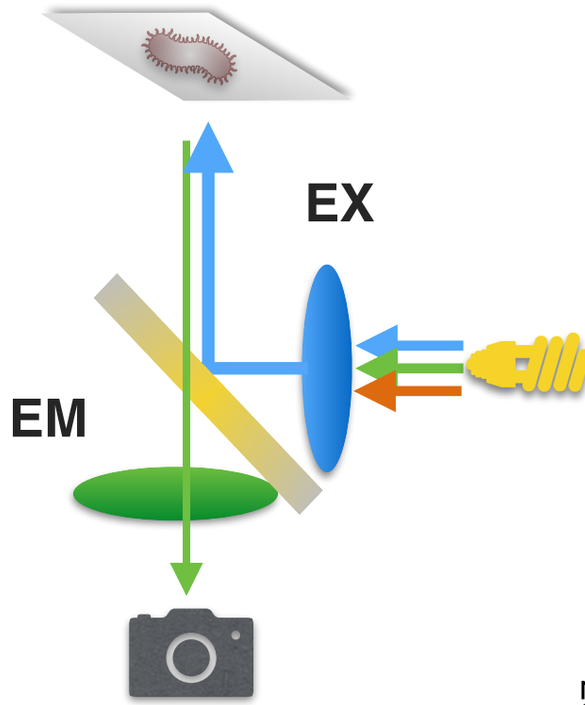
Separates excitation light from emission light

3. Filtersets for fluorescence

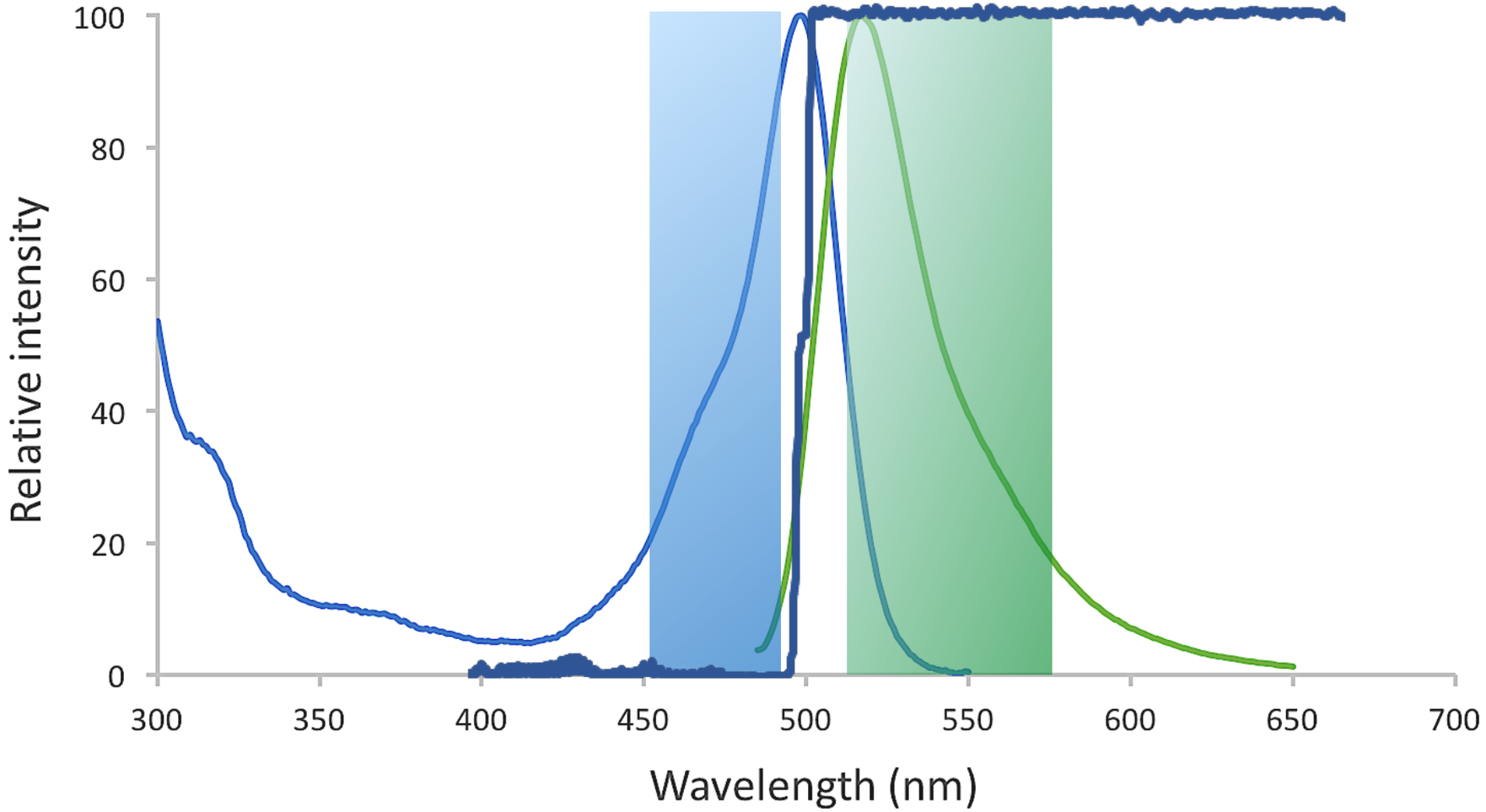
… related to dye spectrum

Fluorescein (FITC)





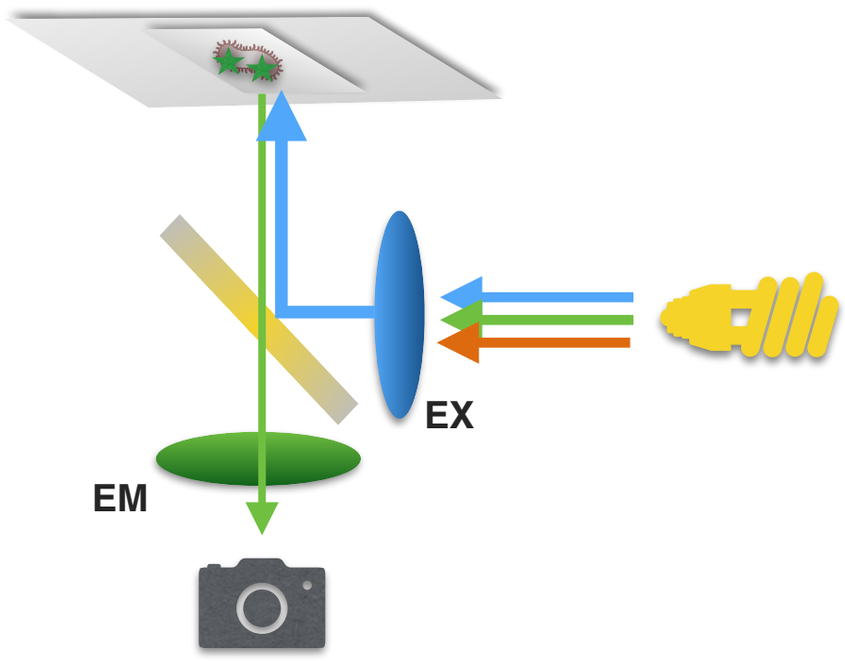
BP470/40 BP550/60



https://www.thorlabs.com/newgrouppage9.cfm?objectgrou
p_id=5007

What about multiplexing…?

Fluorescein (FITC)



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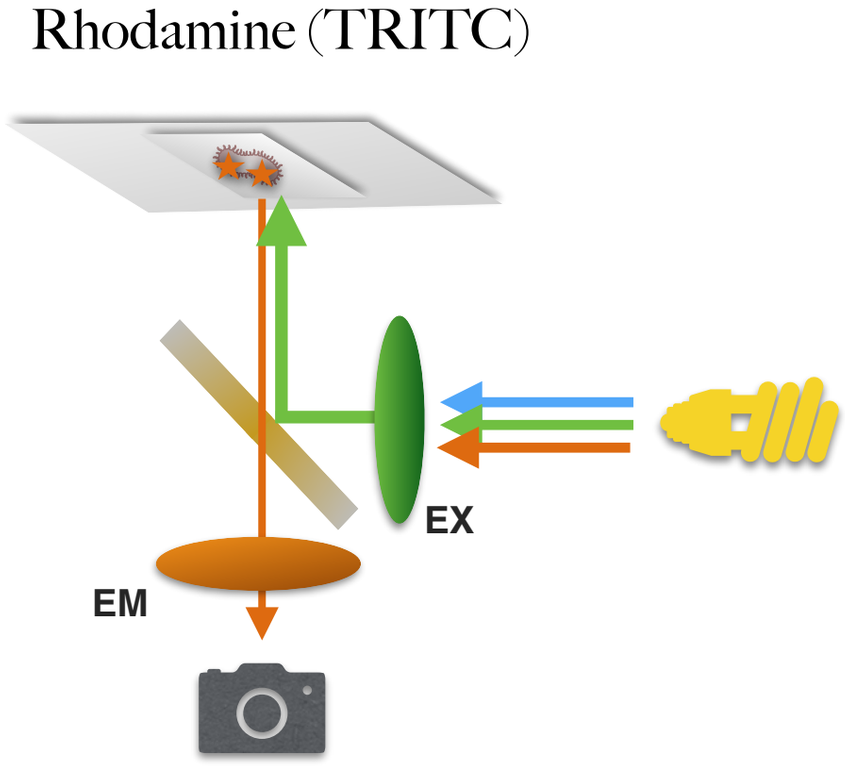
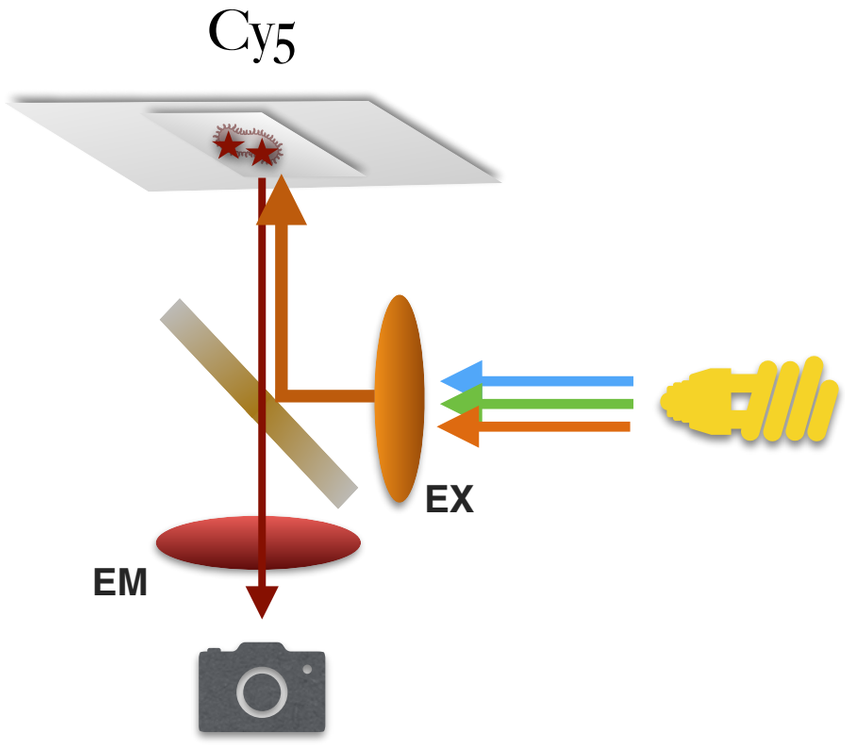


What about multiplexing…?



What about multiplexing…?

Polichroic

EX DAPI 390/18

Ex FITC 475/28

EX TRITC 542/27

EX Cy5 632/22

EM 435/18

EM 525/48

EM 597/45

EM 676/34

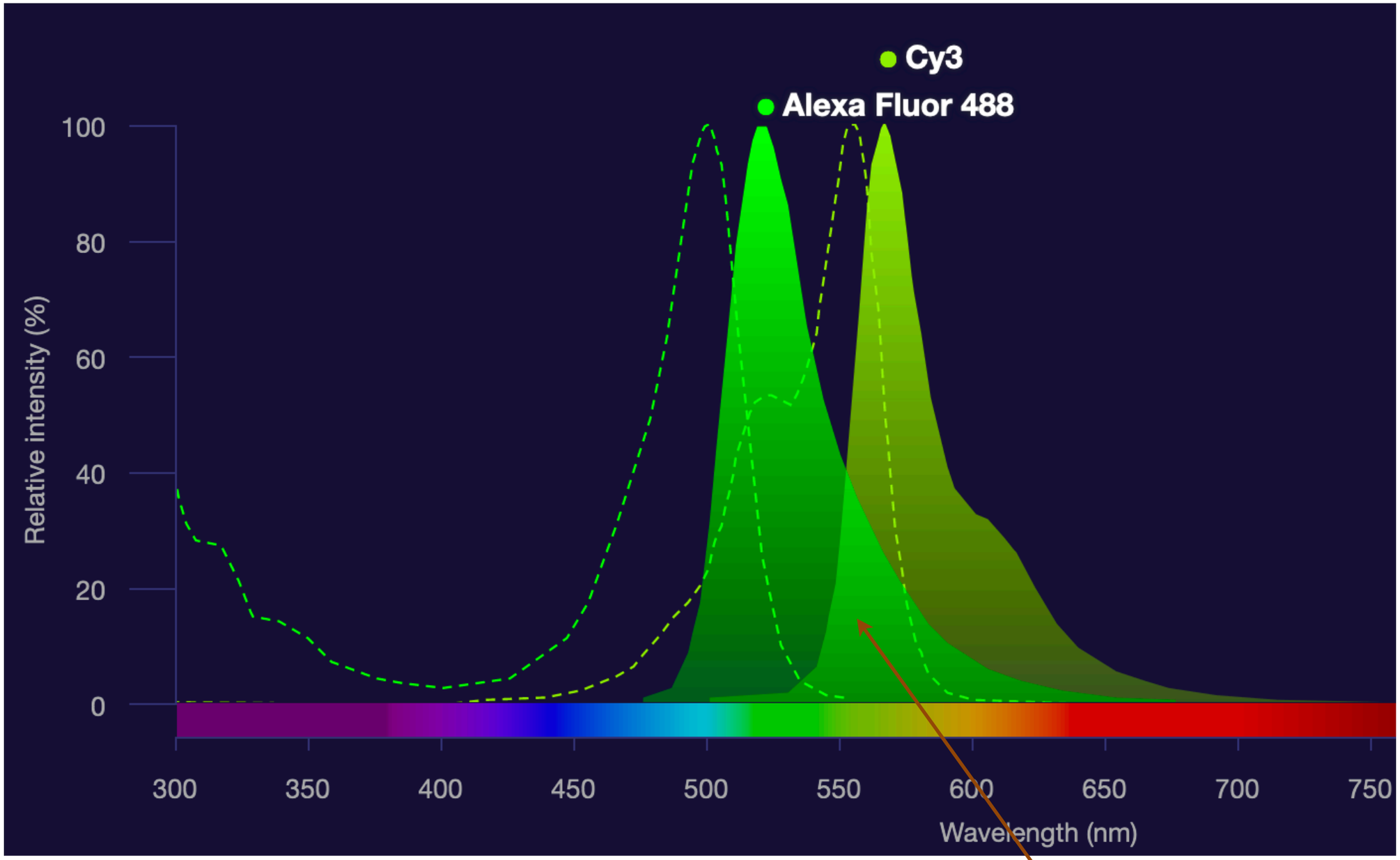
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

300 350 400 450 500 550 600 650 700 750 800

Wavelength (nm)

Care to take when multiplexing

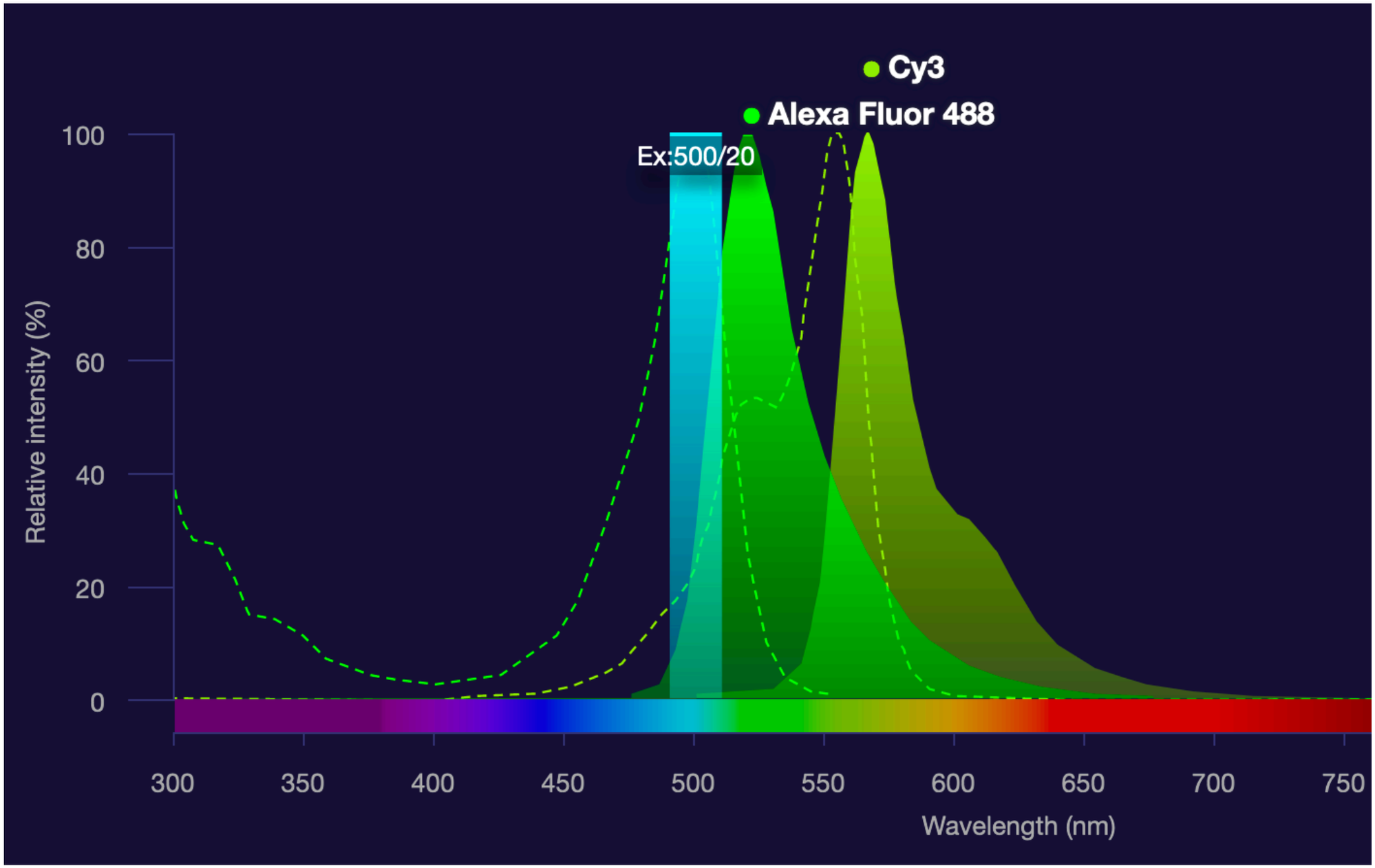
Crosstalk or bleedthrough



Fluorescence SpectraViewer, ThermoFisher scientific

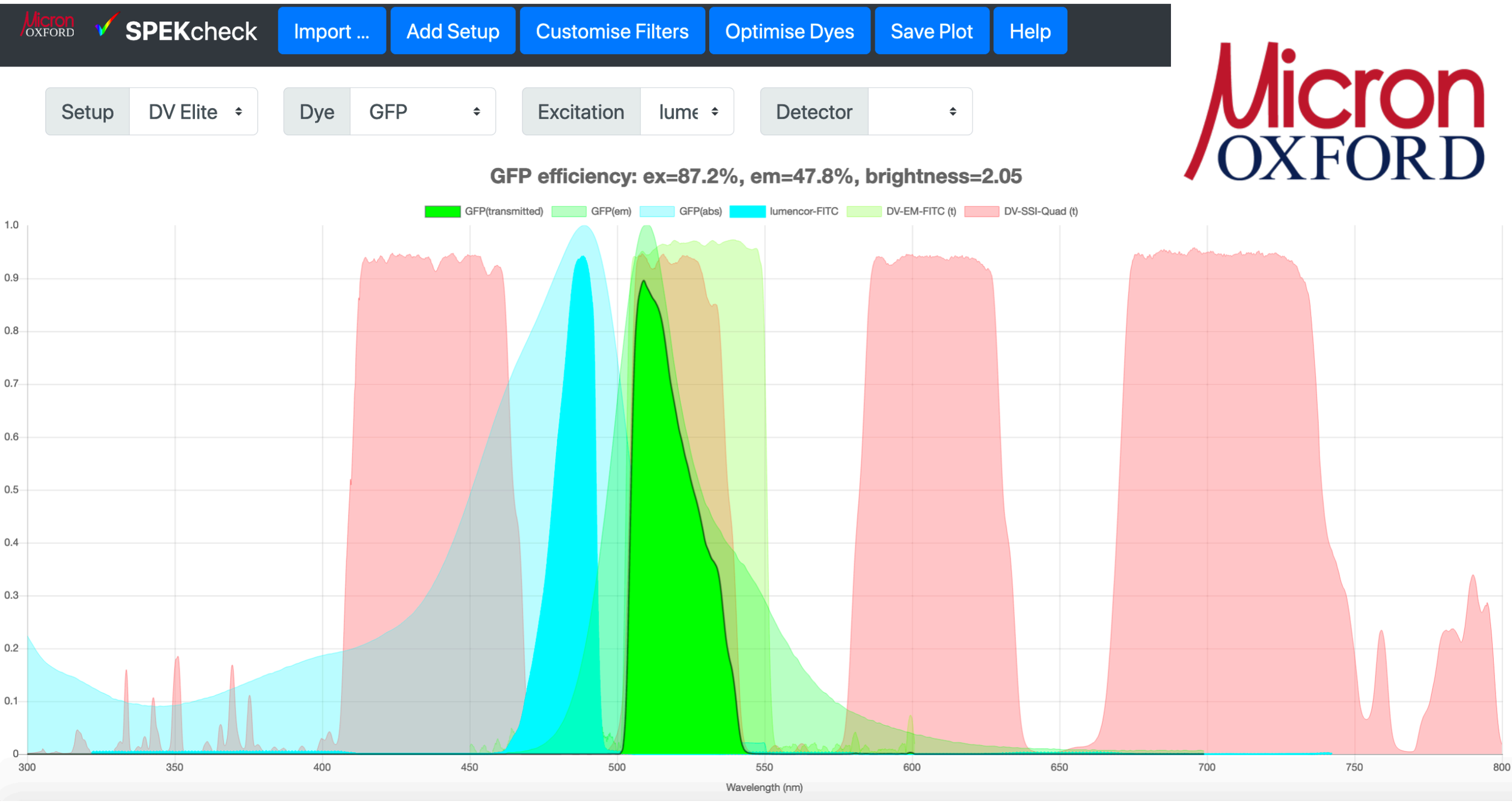
Care to take when multiplexing

Crosstalk or bleedthrough



Fluorescence SpectraViewer, ThermoFisher scientific

SPEKcheck https://www.micron.ox.ac.uk/software/spekcheck/



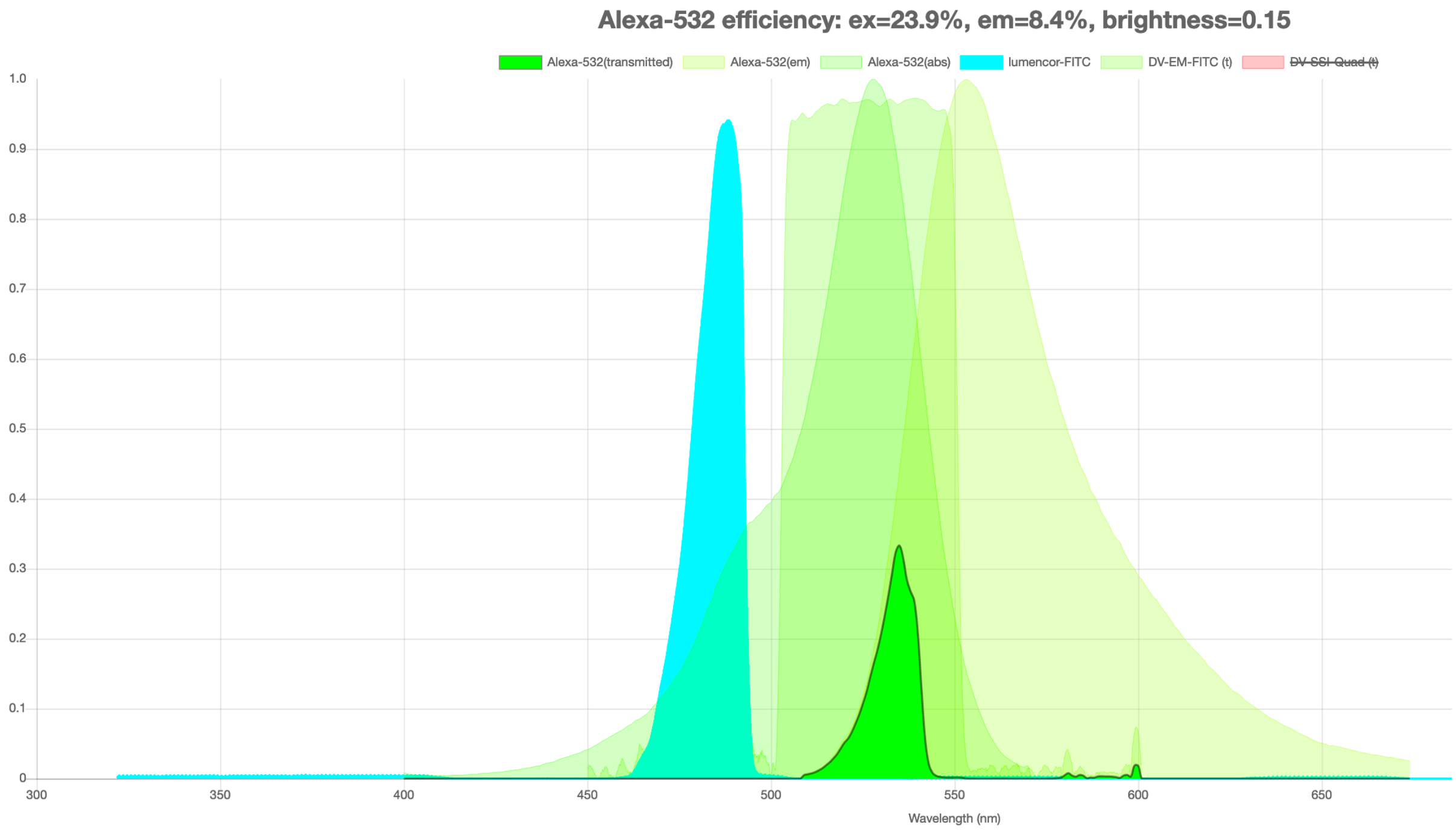
SPEKcheck — fluorescence microscopy spectral visualisation and optimisation: a web application, javascript library, and data resource. Mick A. Phillips, David M. Susano Pinto, Ian M. Dobbie.

Software Tool Article Wellcome Open Research 2018, 3:92

Always check microscope filter sets before designing your experiment

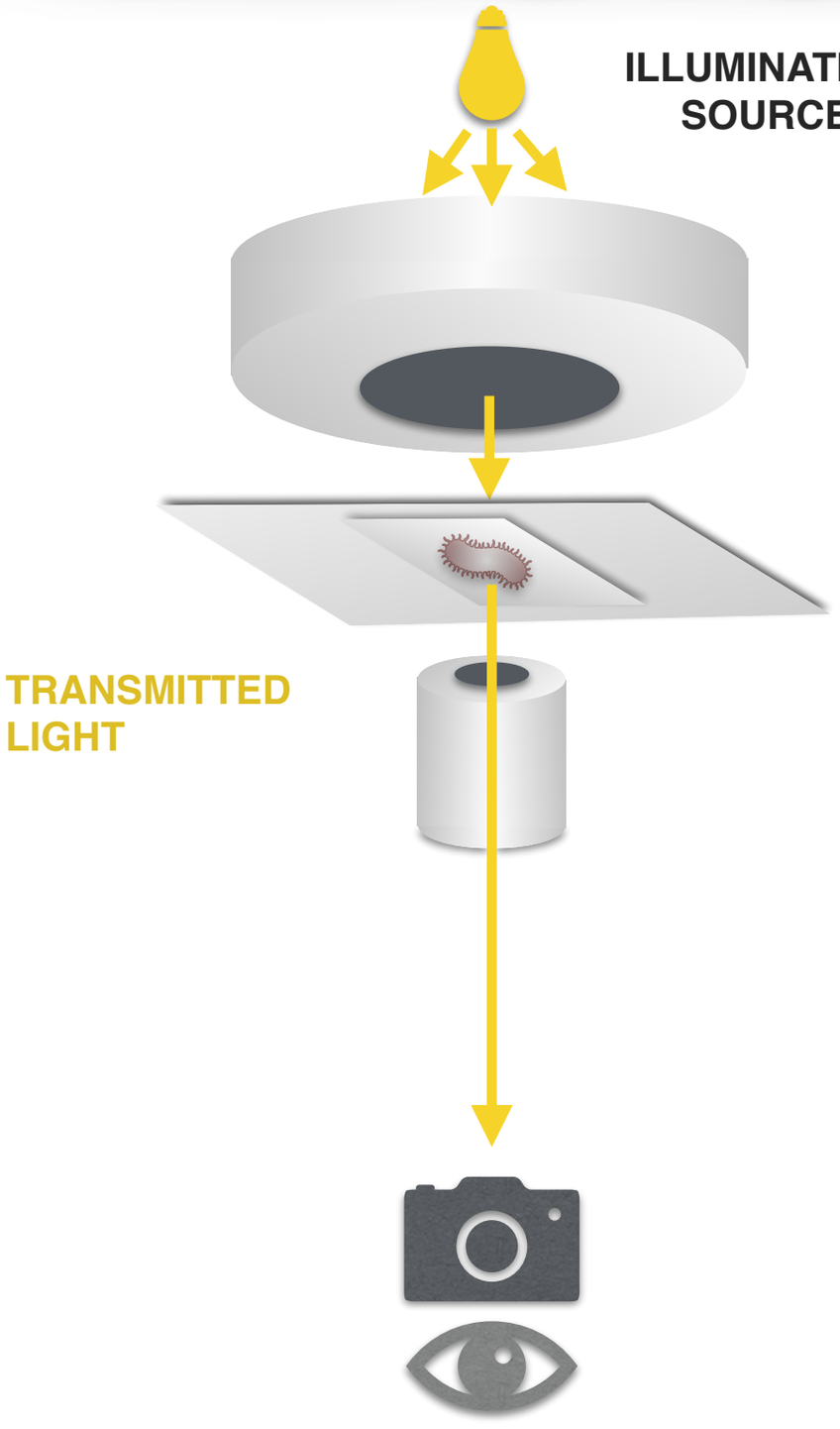
Micron OXFORD SPEKcheck | Import ... | Add Setup | Customise Filters | Optimise Dyes | Save Plot | Help

Setup: DV Elite | Dye: Alexa-532 | Excitation: lume | Detector:



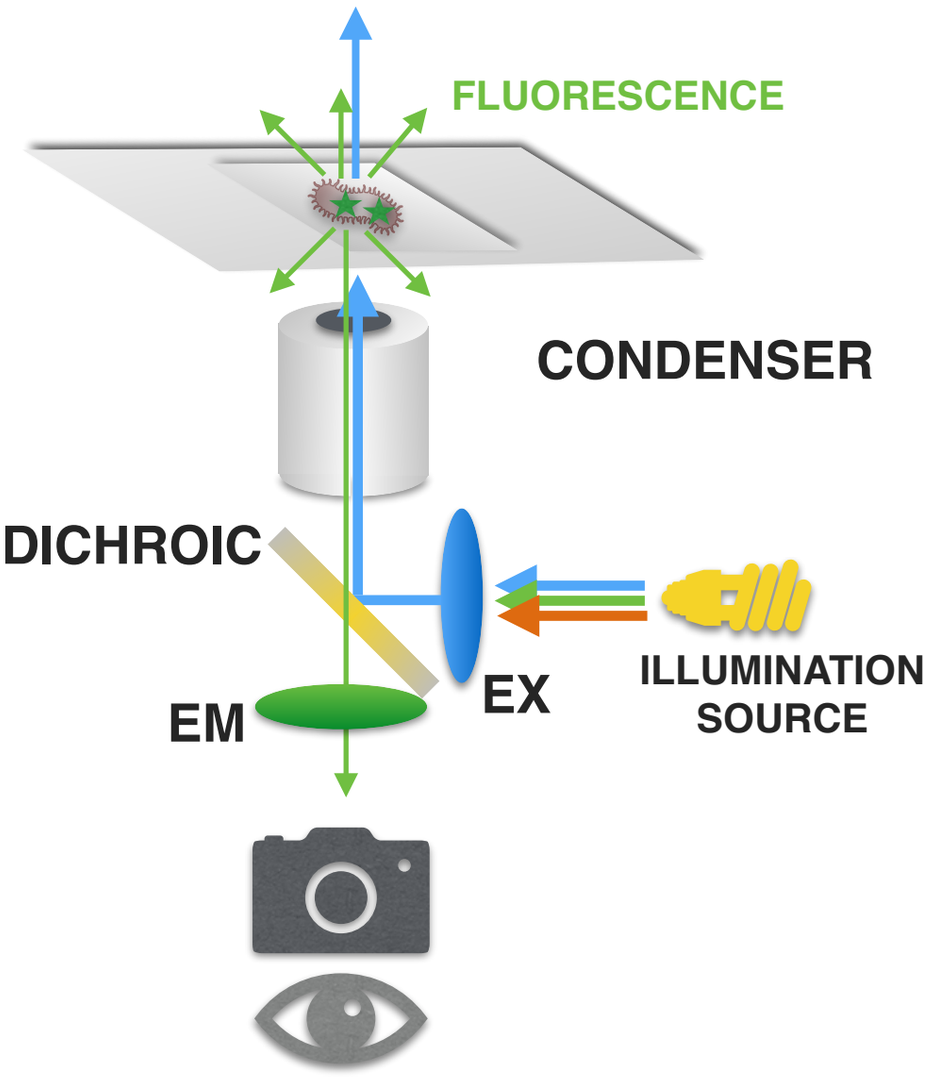
4. Components of a microscope. Brightfield vs. Fluorescence

Transmitted Light (Brightfield)

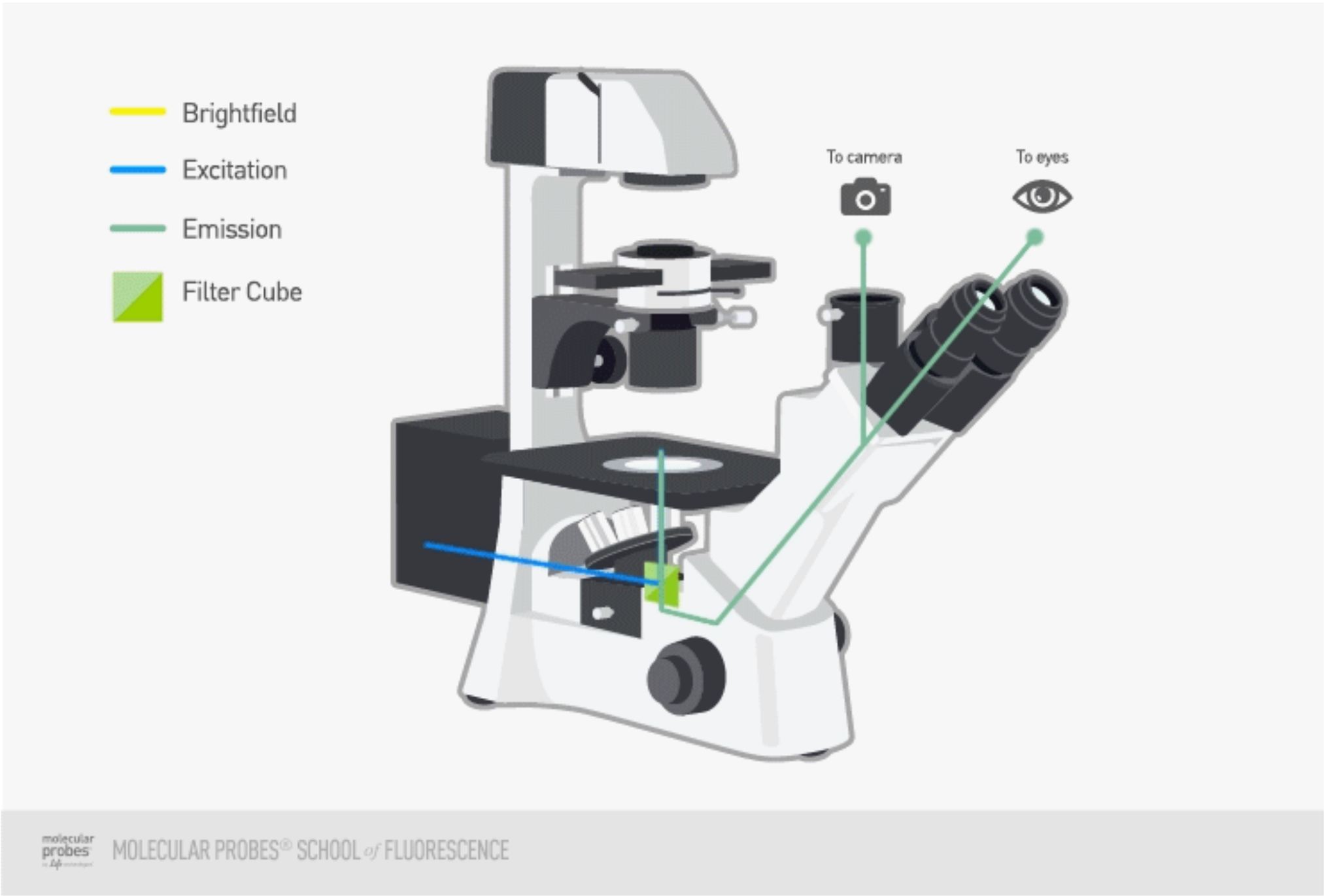


Reflected Light (Fluorescence)

Epifluorescence Widefield



4. Transmitted vs. Reflected light paths (inverted)



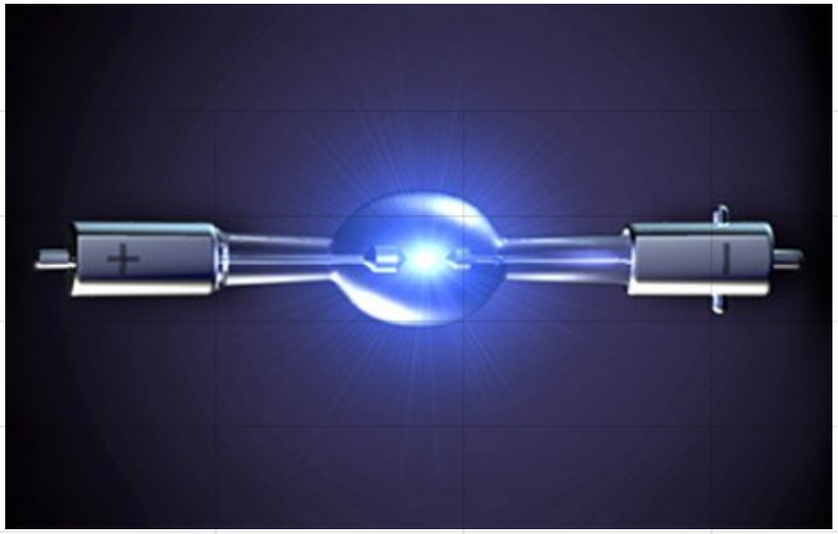
https://www.thermofisher.com/uk/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/imaging-basics/

Illumination sources for widefield fluorescence microscopy

Widefield fluorescence

Mercury Arc Lamp

- 200 h
- hazardous
- *out of use*

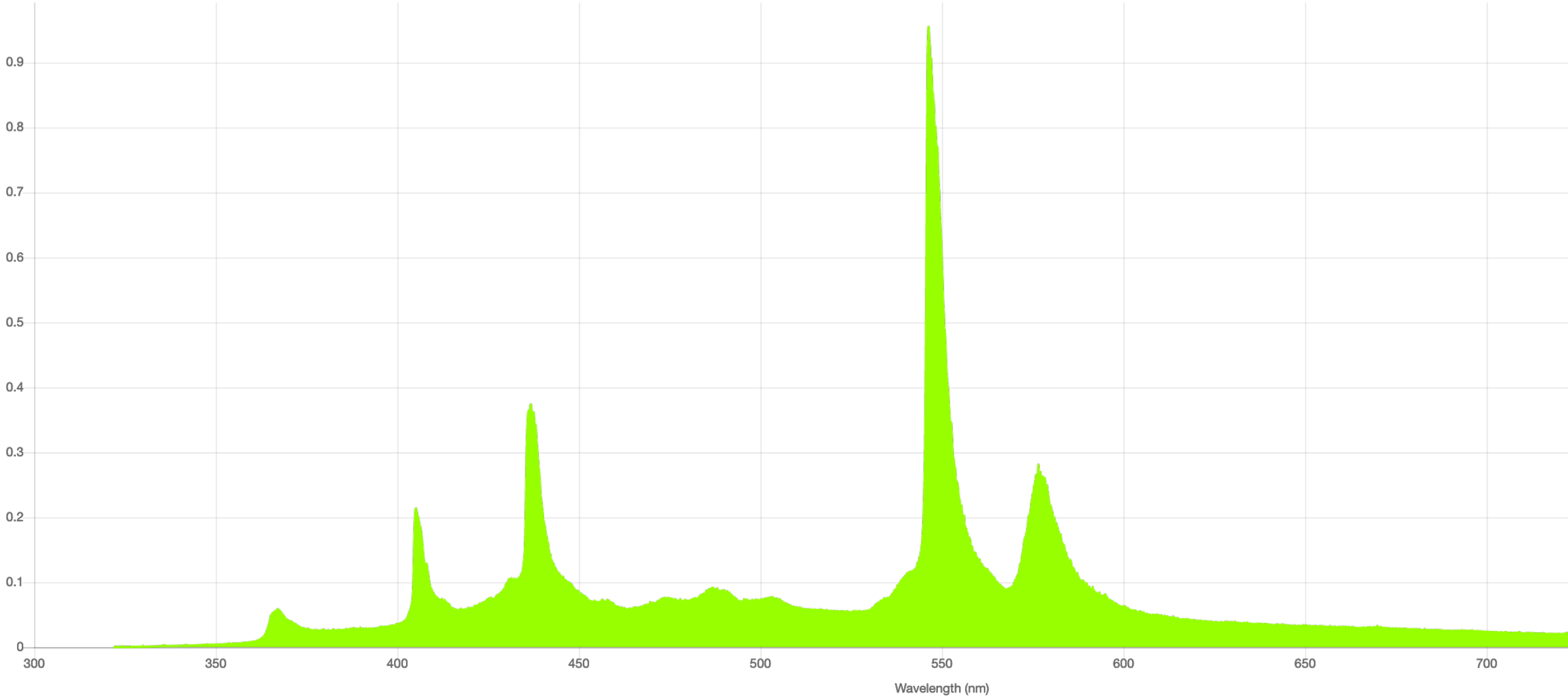


Simultaneous excitation of multiple Fluorophores over a wide wavelength range

Illumination sources for widefield fluorescence microscopy

Widefield fluorescence

Methal halide lamp



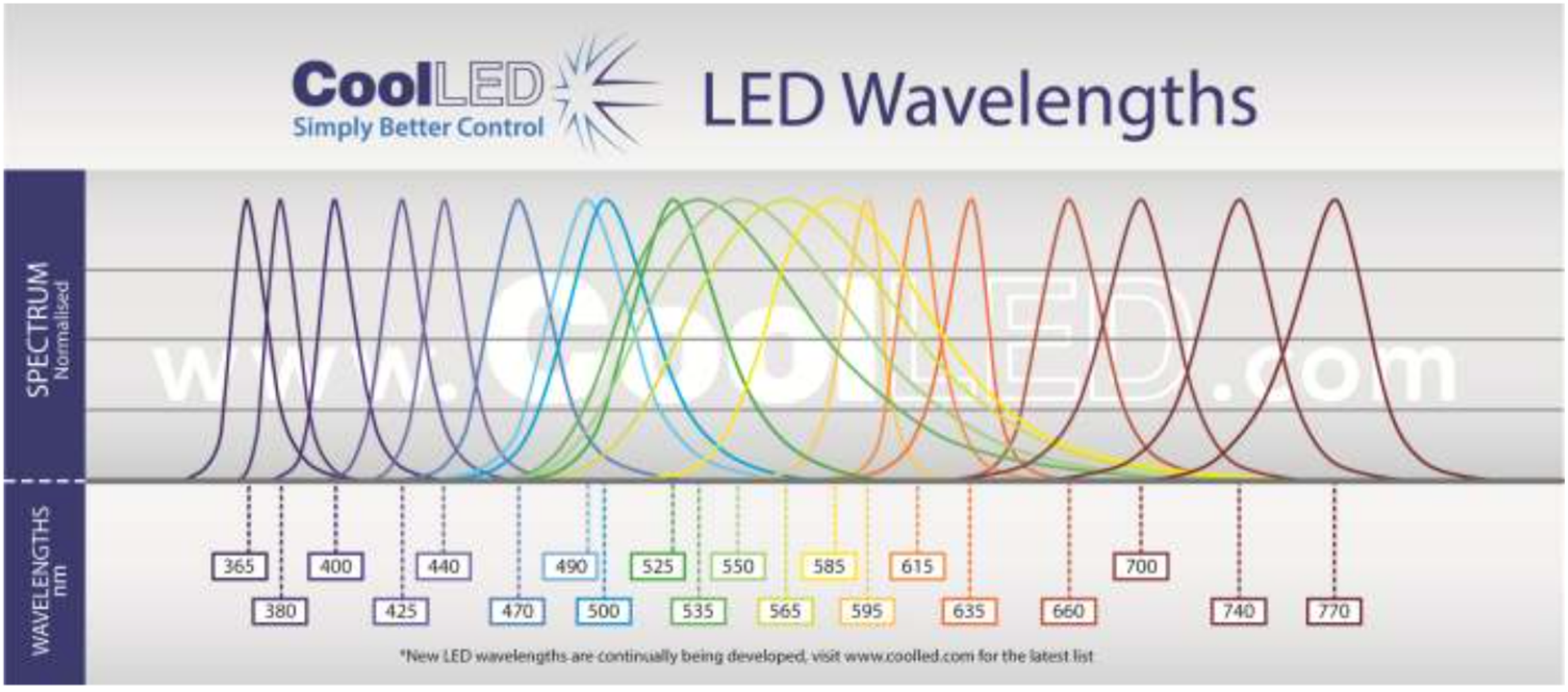
- Coupled with a liquid guideline (fibre)
- Makes noise, takes time to warm up

- No need for alignment
- 2000 h

Illumination sources for widefield fluorescence microscopy

State of the art for widefield fluorescence

LEDs Light Emitting Diodes

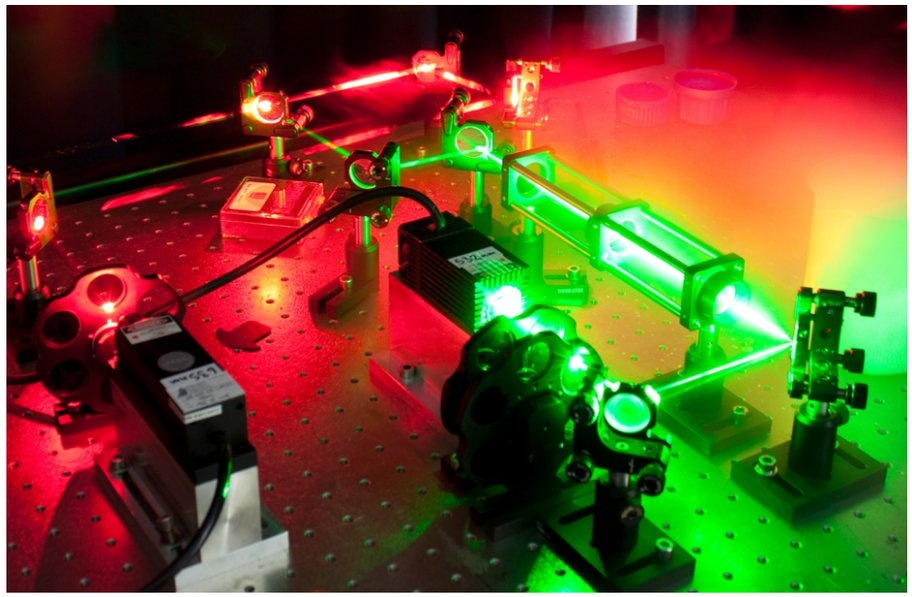


http://www.coolled.com/product-detail/led-wavelengths/

- Wide range of lines available of defined colours
- Stable and bright

- 25,000 h

Illumination sources for fluorescence microscopy



Lasers

(light amplification by stimulated emission of radiation)

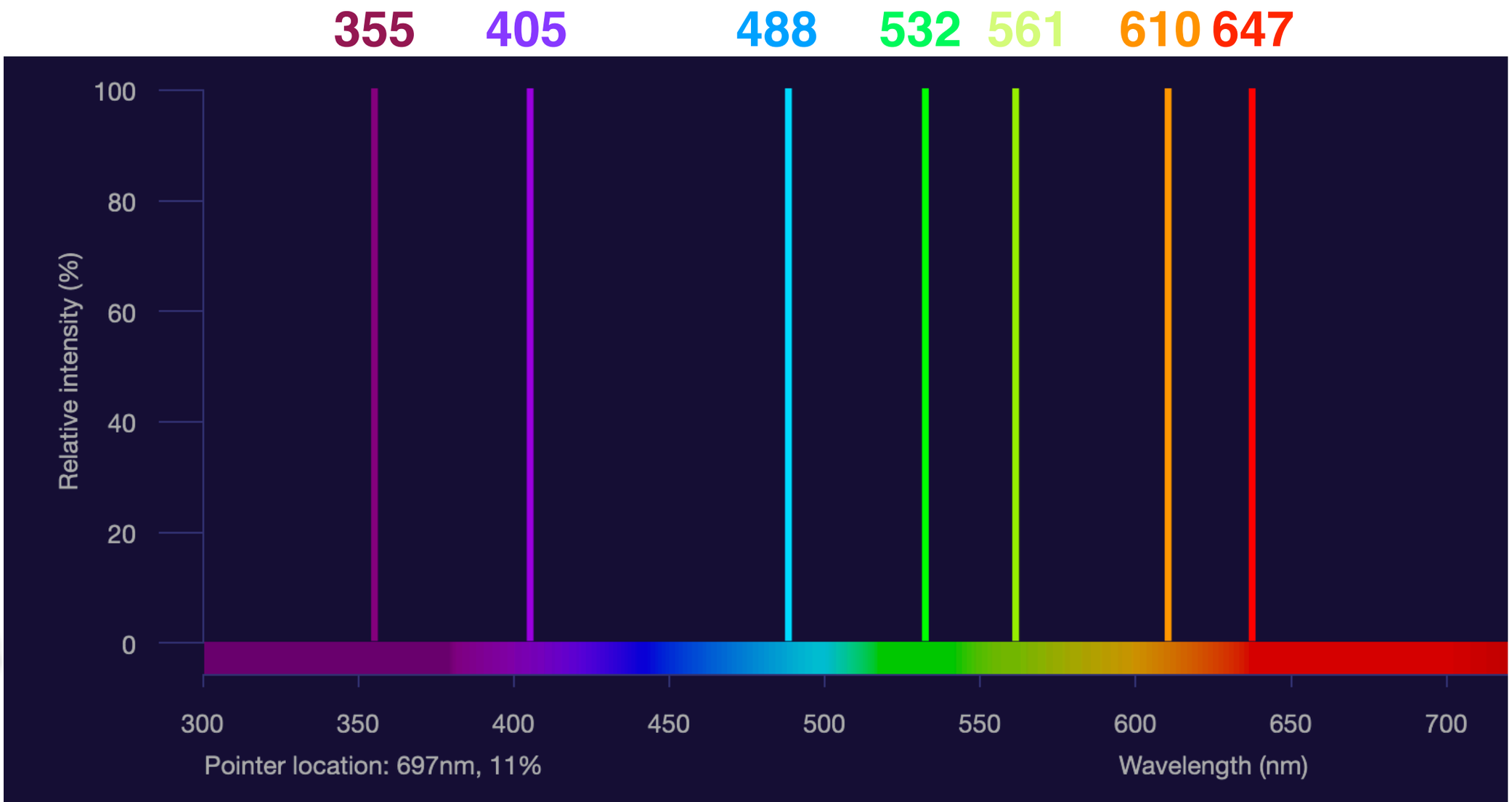
Narrow beams of highly monochromatic, coherent and collimated light

Confocal

2-photon

TIRF

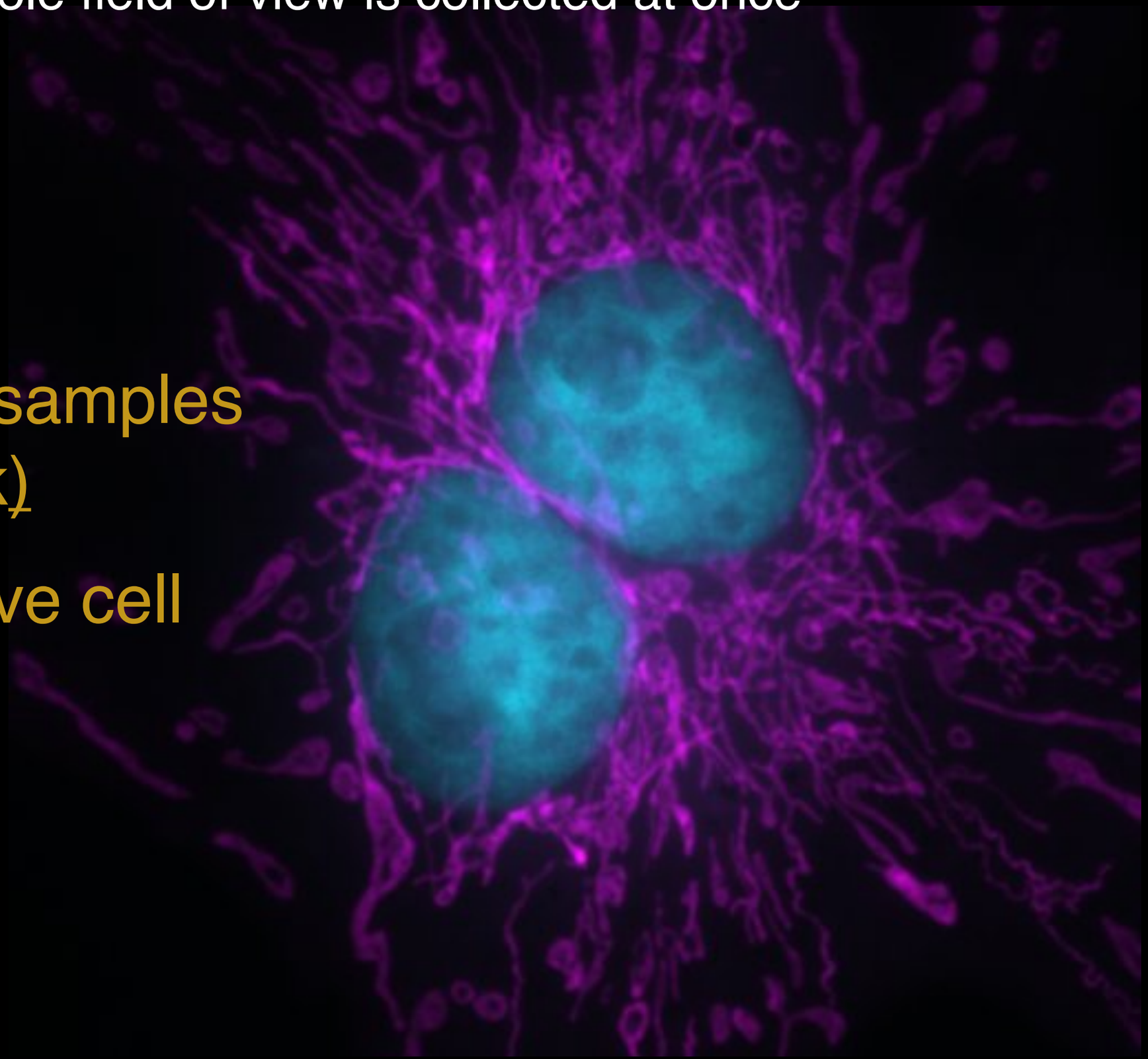
Super-res



5. Widefield Fluorescence Microscopy

The whole field of view is collected at once

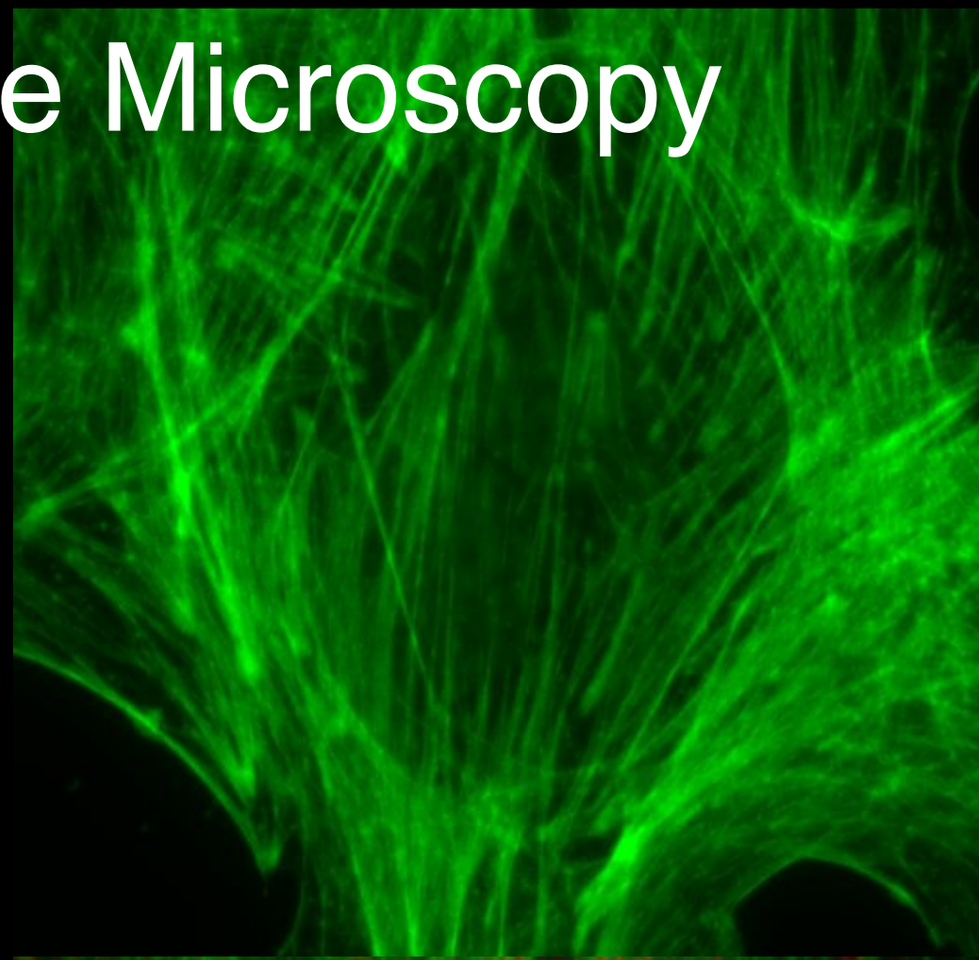
- ✓ Fast
- ✓ Sensitive
- ✓ Ideal for thin samples (~10 μm thick)
- ✓ Suitable for live cell imaging

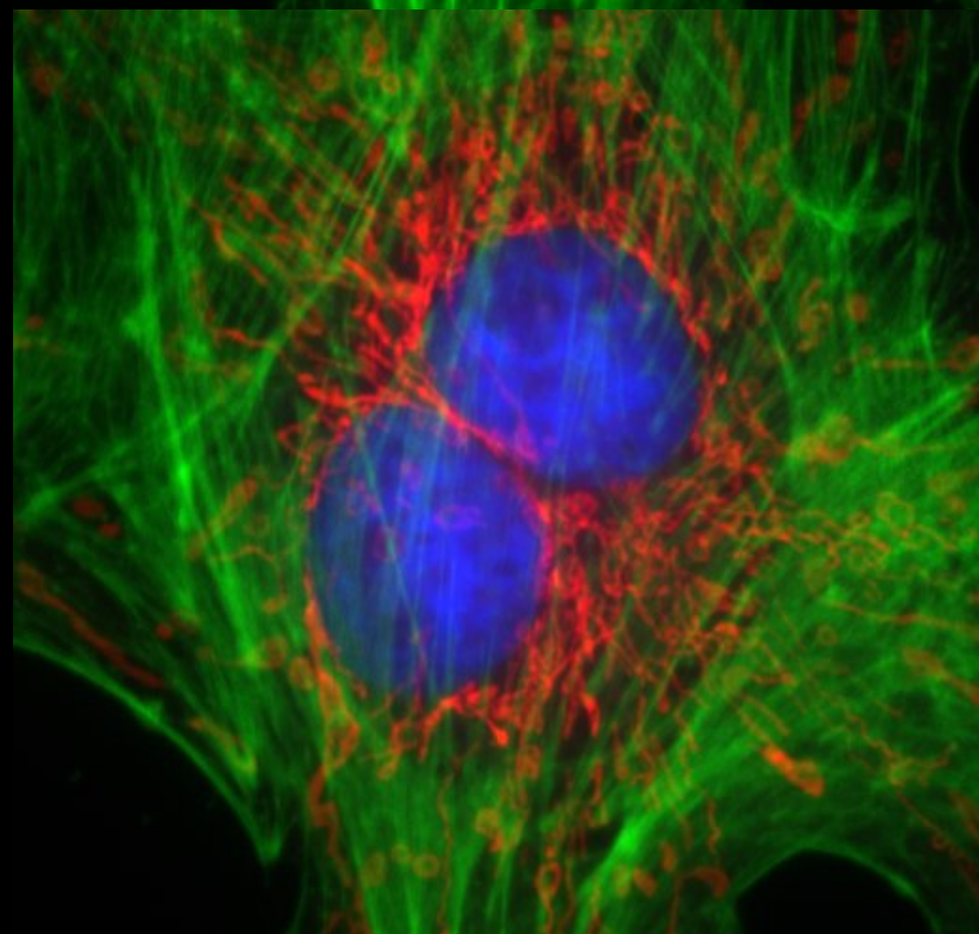


BPAE cells
Mitochondria (Mitotracker Red)
Nuclei (DAPI)

5. Widefield Fluorescence Microscopy

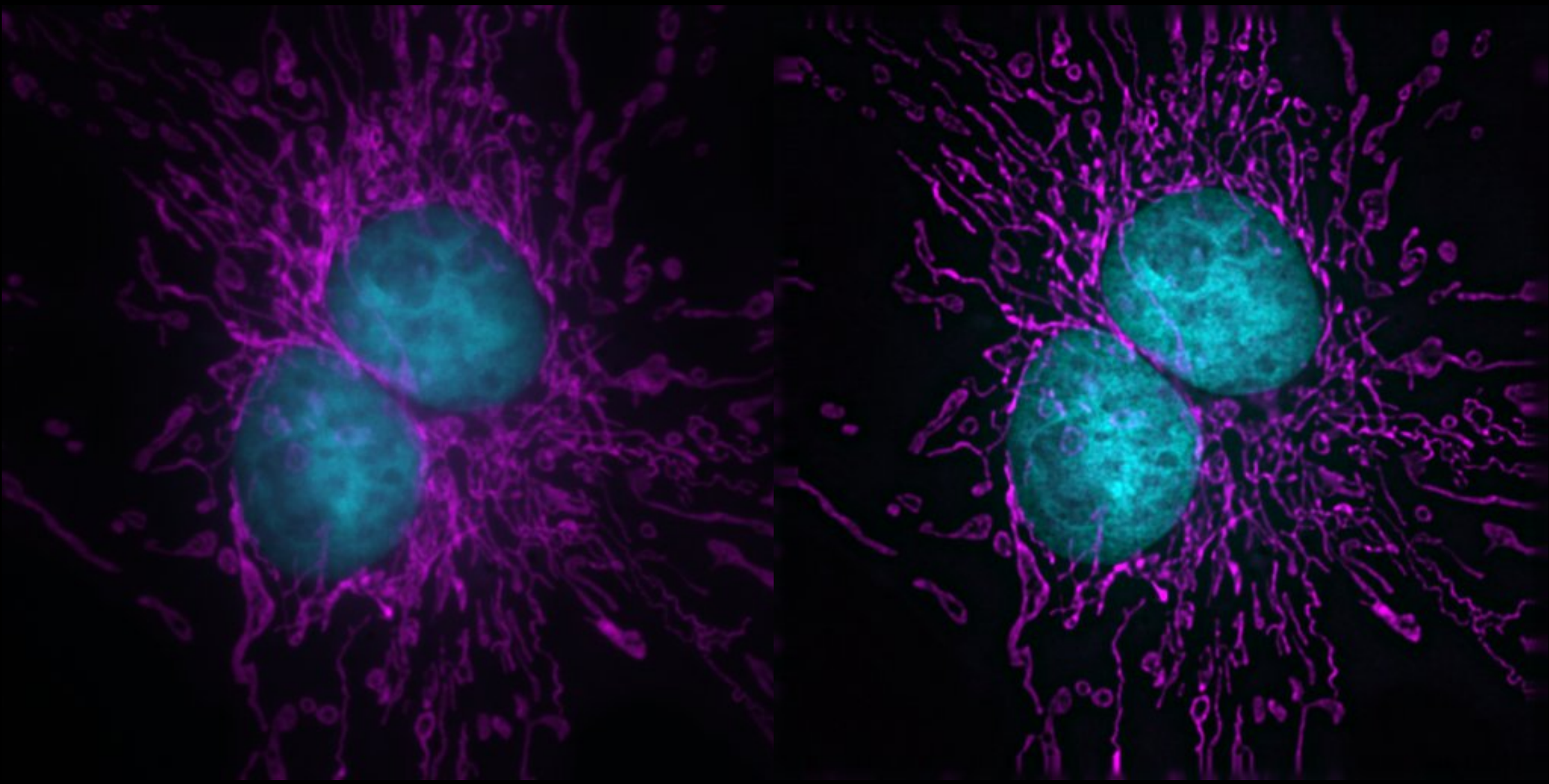
- ✓ Time lapse imaging
- ✓ Multipoint visiting
- ✓ Tiling and stichting
- ✓ Specially powerful when combined with Deconvolution





Widefield

Deconvolution



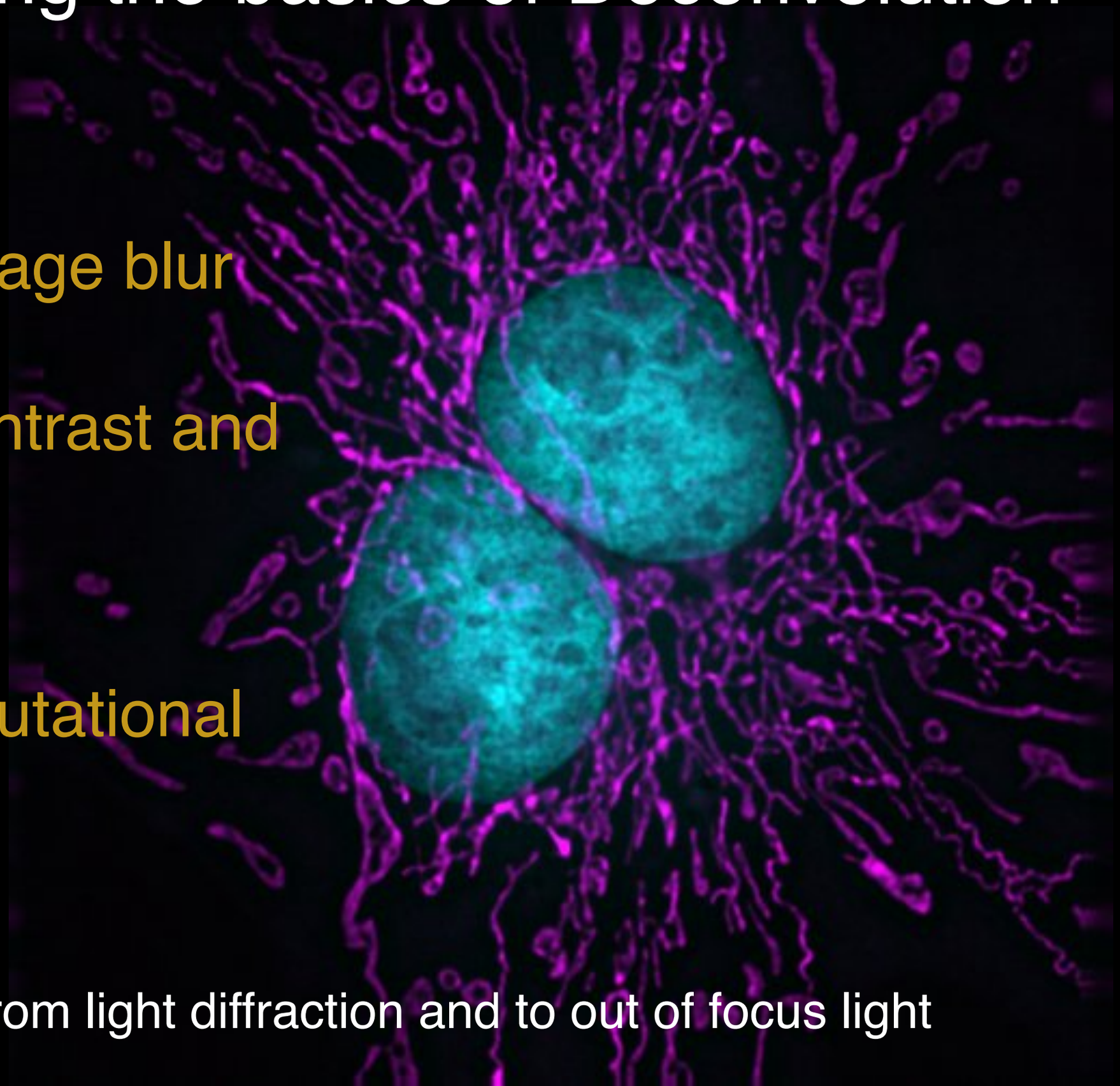
BPAE cells

Mitochondria (Mitotracker Red)

Nuclei (DAPI)

Understanding the basics of Deconvolution

- ✓ Removes image blur
- ✓ Improves contrast and resolution
- ✓ Purely computational



Blur comes from light diffraction and to out of focus light

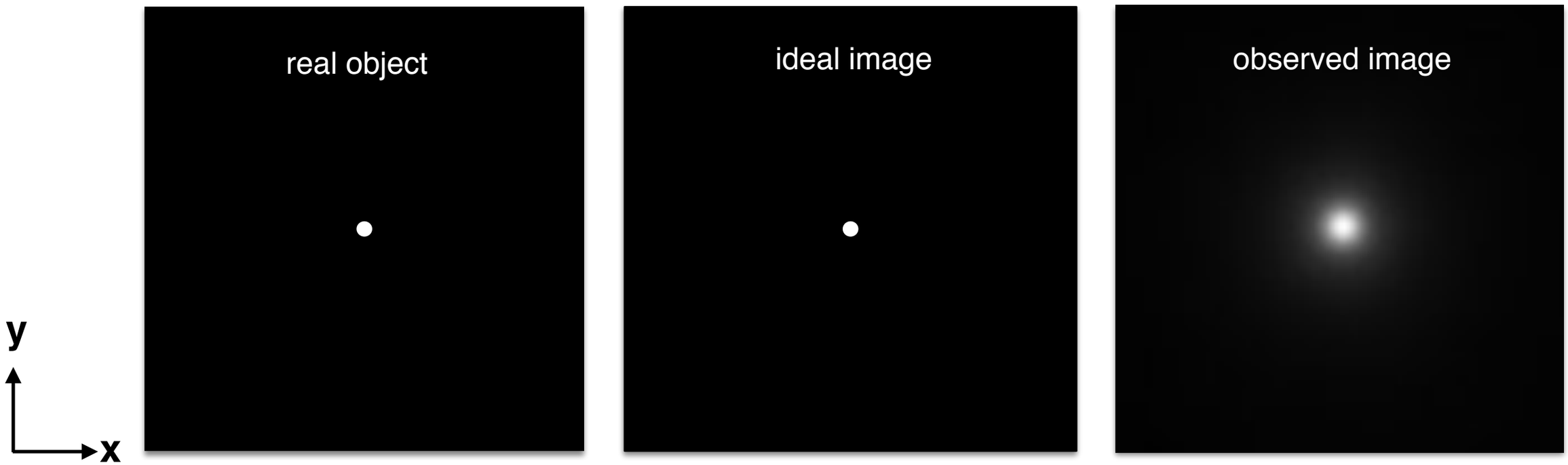
PSF - Point Spread Function

OTF - Optical Transfer Function

6. PSF 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

6. PSF in fluorescence

Point Spread Function

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

- microscope performance
- spherical aberrations

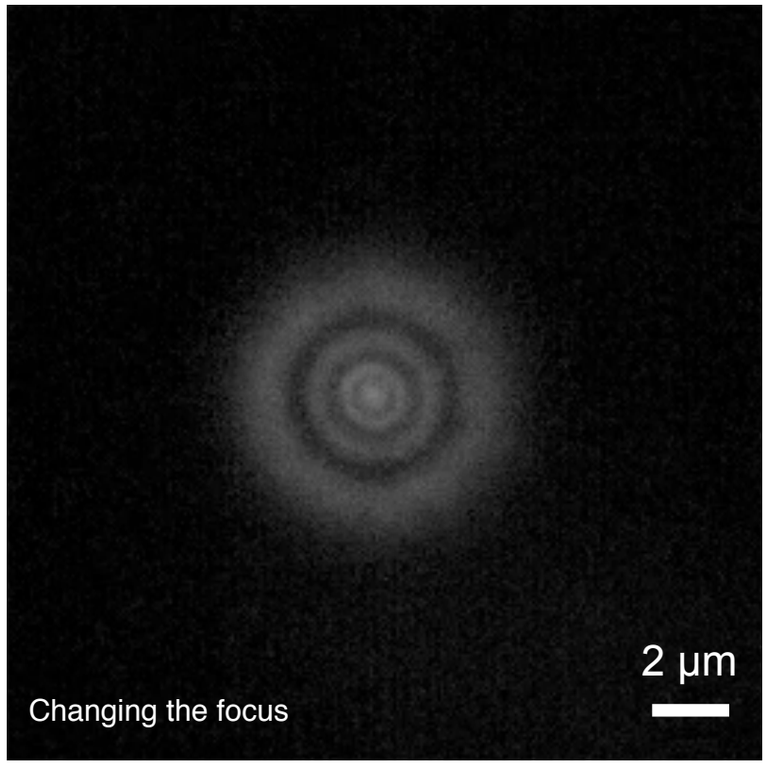
Why is it important?

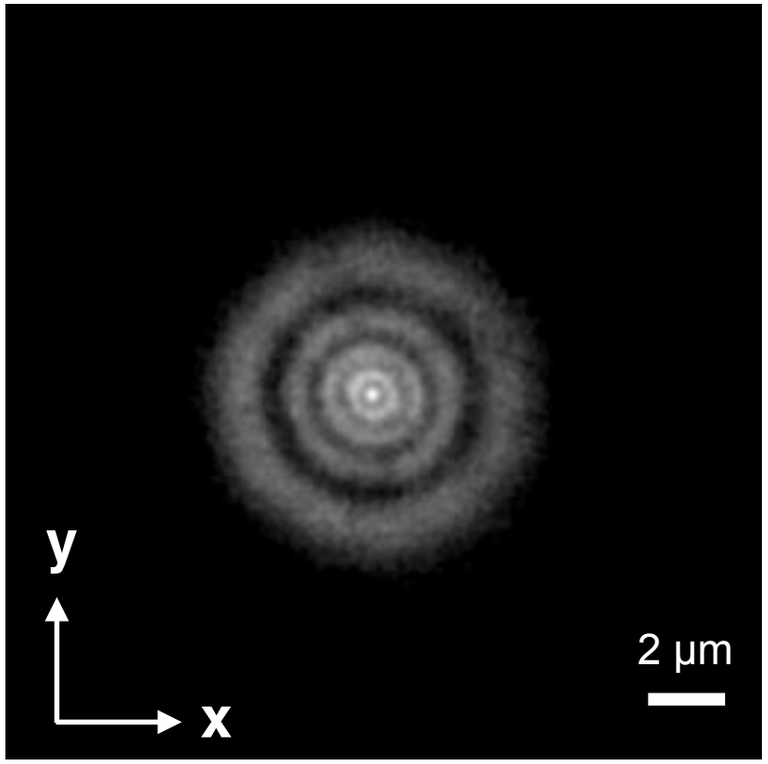
- x, y, z info
- image quality
- alignment
- optical resolution

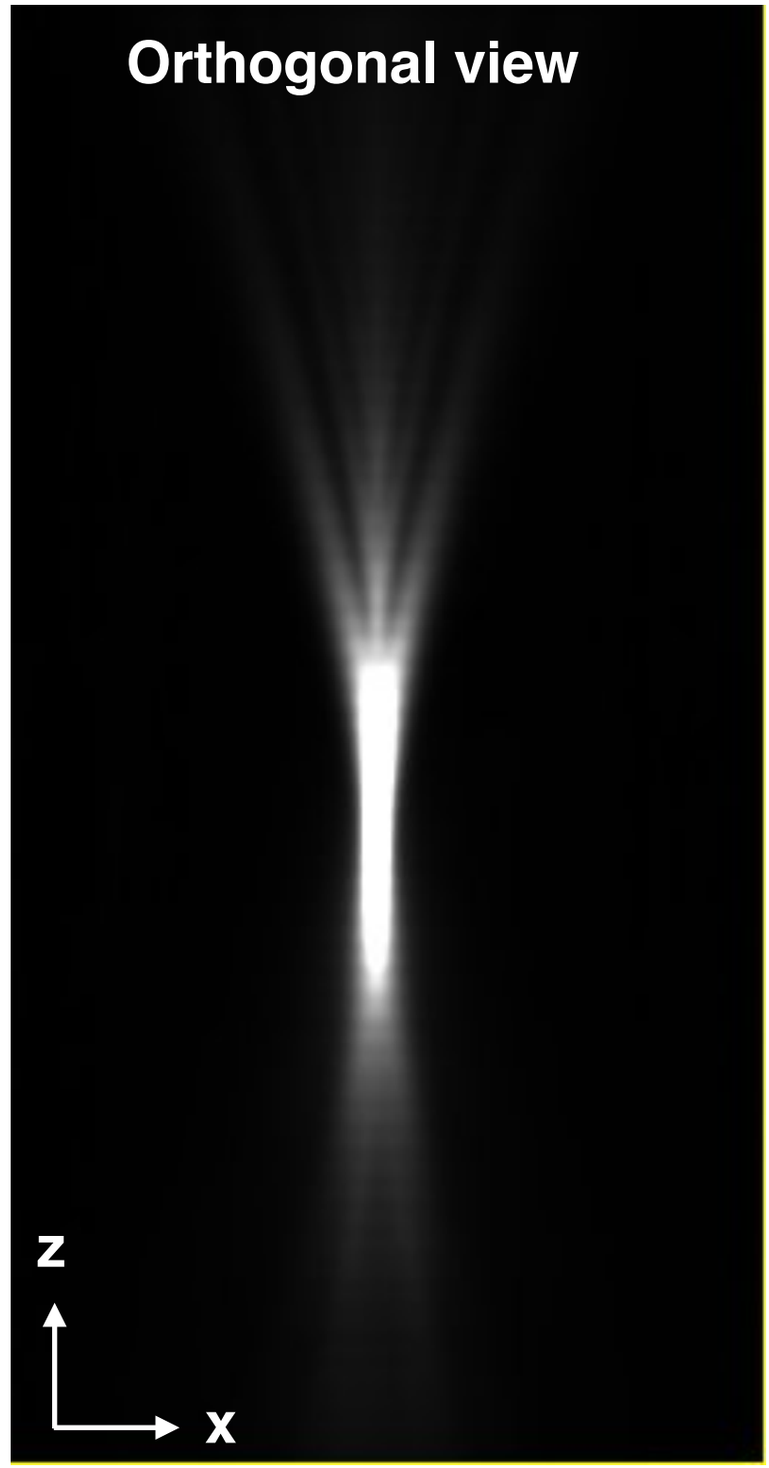
6. PSF in fluorescence

PSF

red fluorescent 170 nm bead







Airy disk diffraction pattern

(concentric rings)

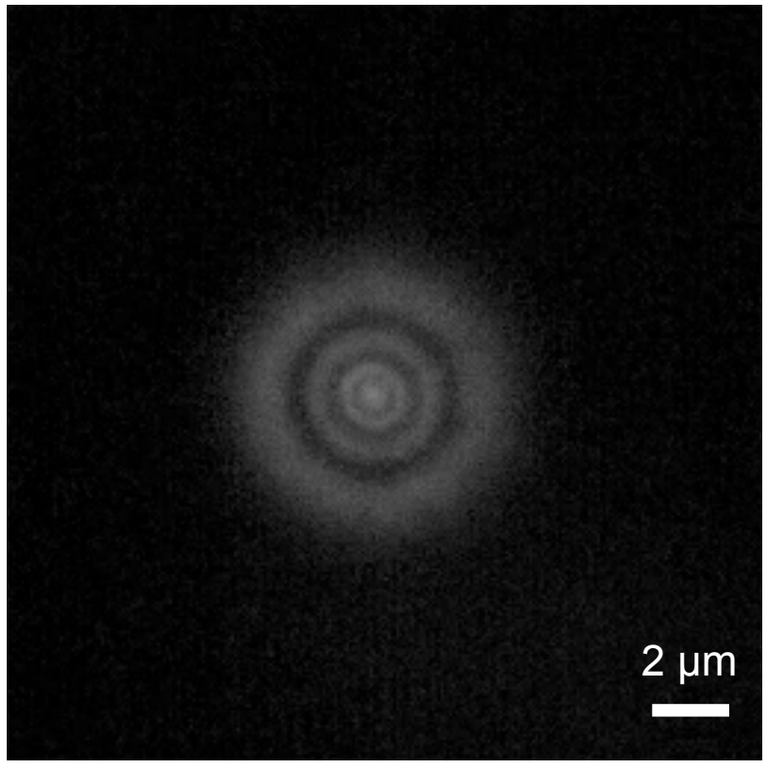
Light waves emitted from a point source are not focused into an infinitely small point by the objective

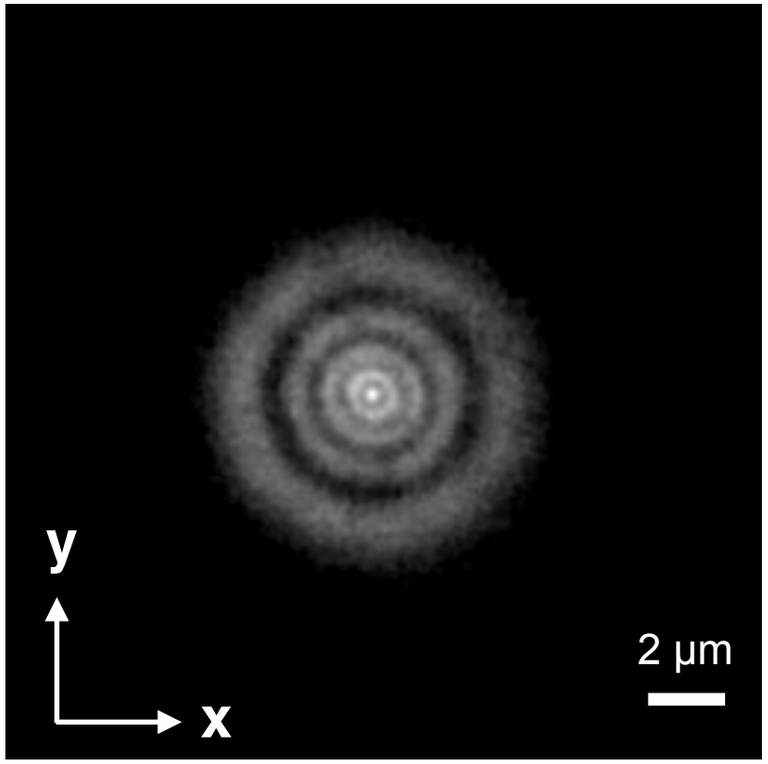
They converge together and interfere in the image plane

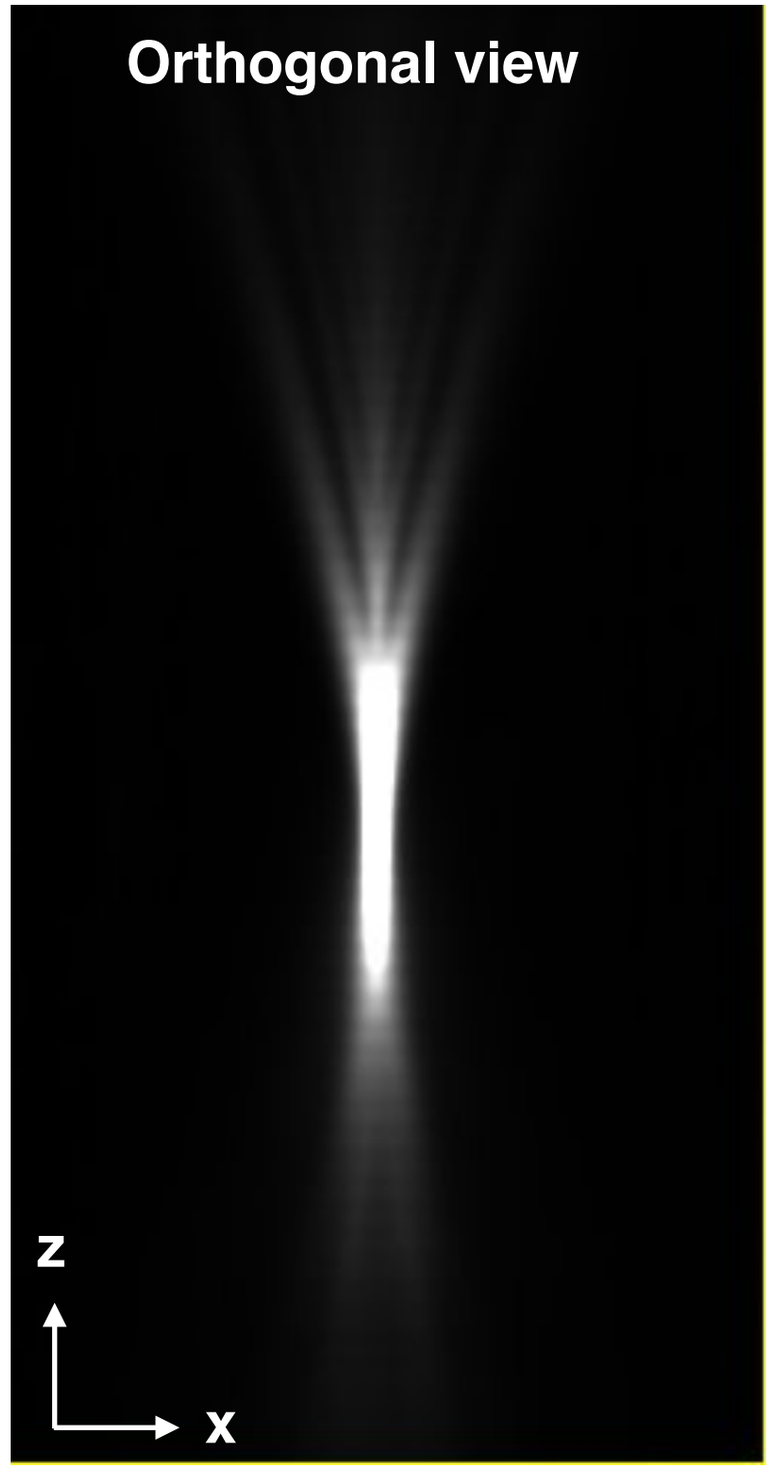
PSF is the 3D image of a point-like object under the microscope

PSF

red fluorescent 100 nm bead





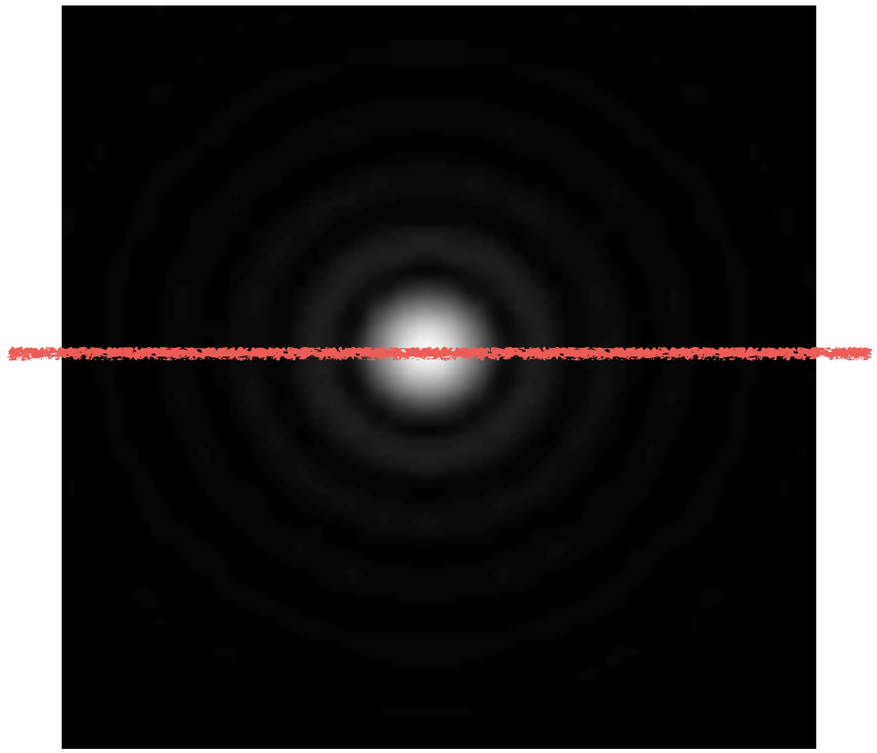


What can we observe?

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

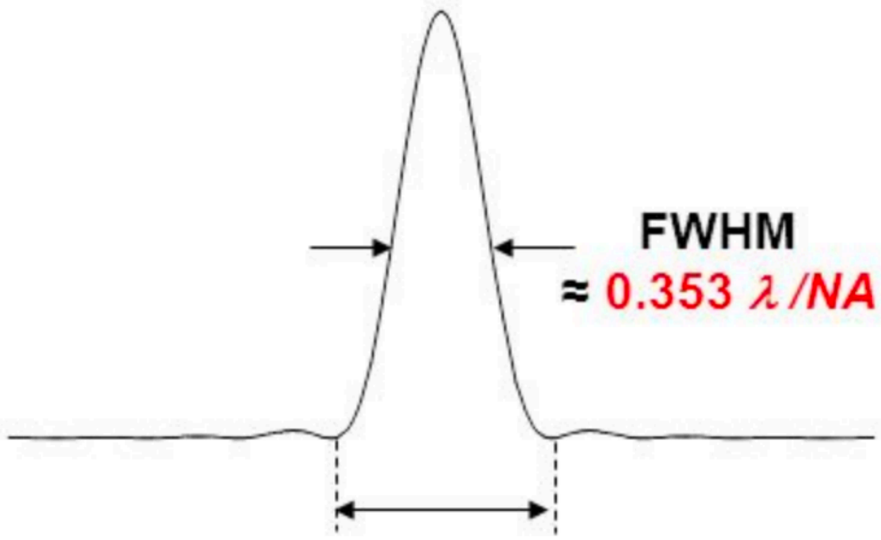
☑ ALIGNMENT, SPHERICAL ABERRATIONS, MISMATCH REFRACTIVE INDEX

PSF is a way to measure resolution



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

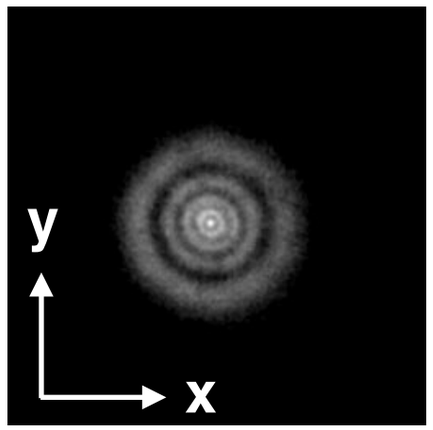
As the diameter of the Airy disk (first dark ring of the PSF)
= “Rayleigh criterion”

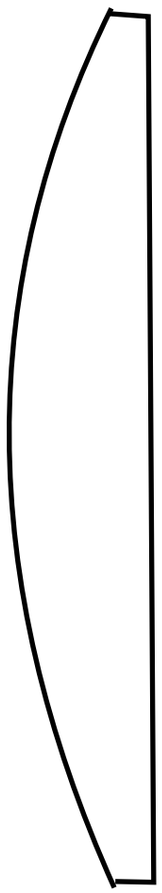


Why is the Airy pattern a distribution of white and black rings?

Objective lens

Image plane

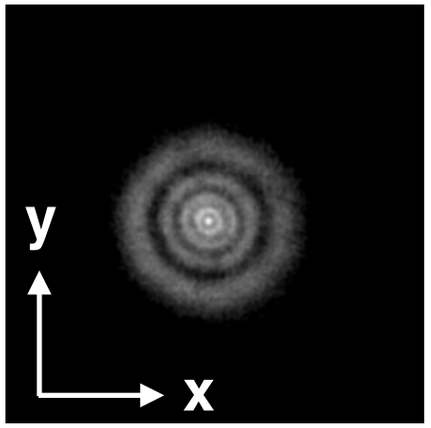


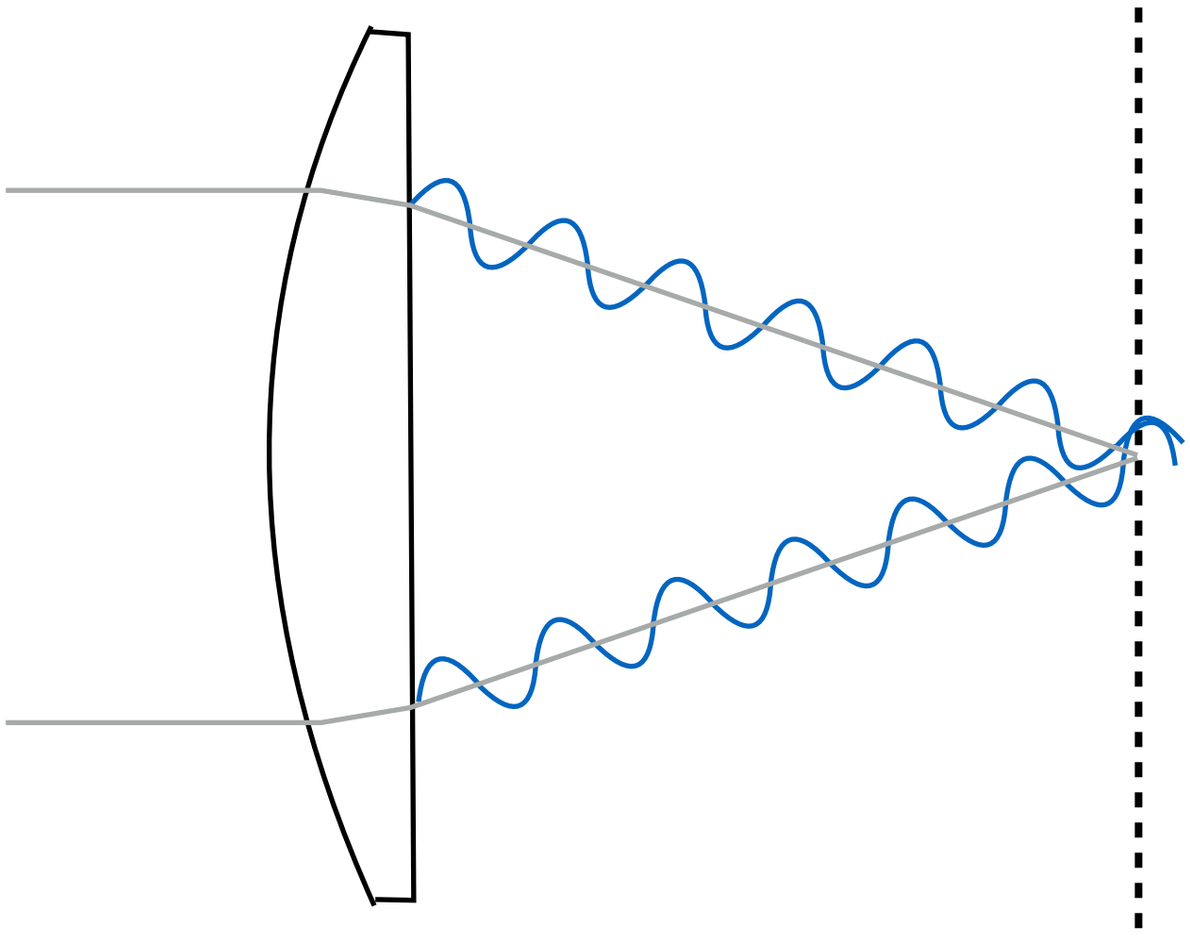


Why blurred and how is the Airy diffraction pattern generated?

Objective lens

Image plane



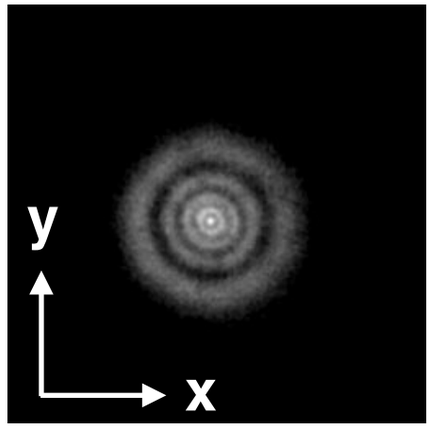


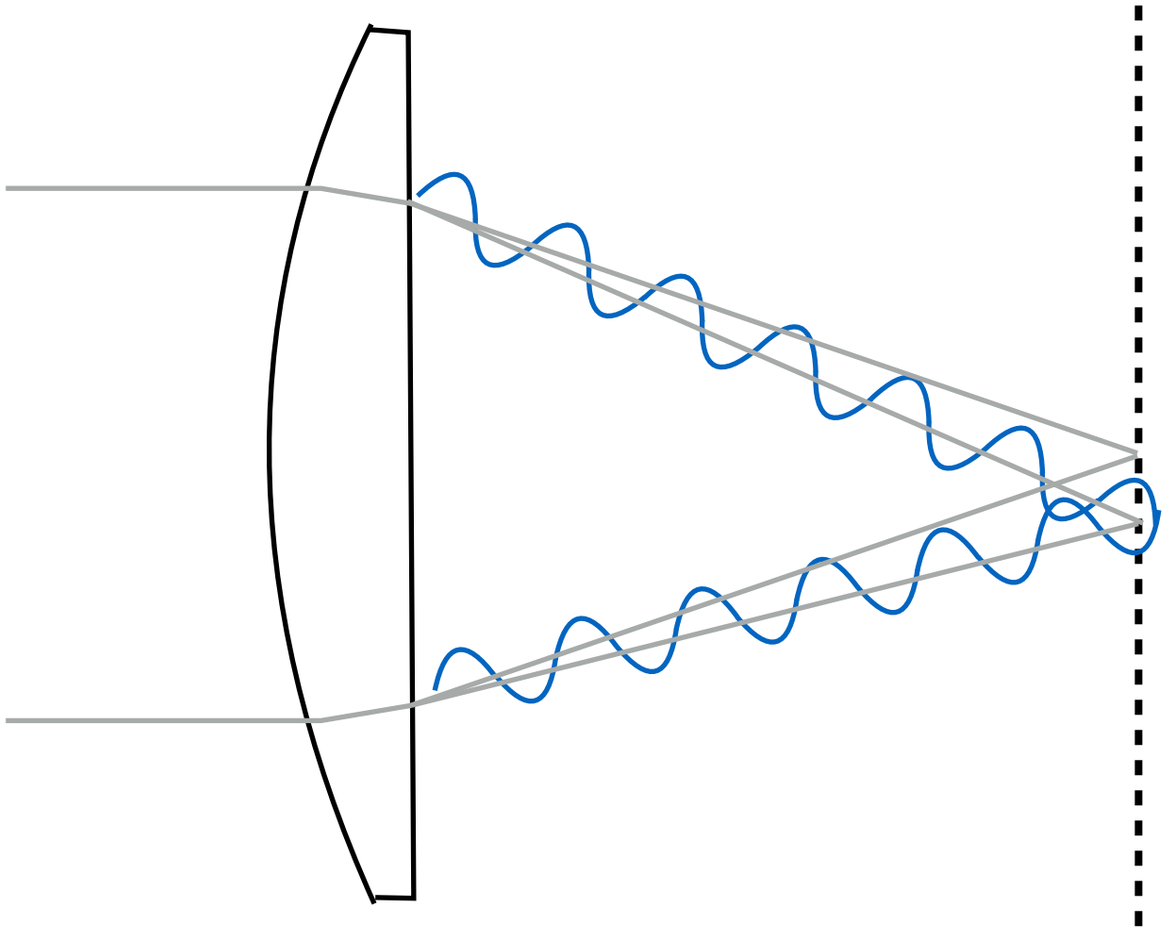
in phase - Constructive interference

Why blurred and how is the Airy diffraction pattern generated?

Objective lens

Image plane





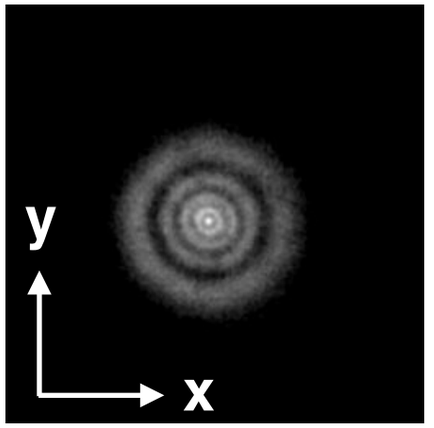
in phase - Constructive interference

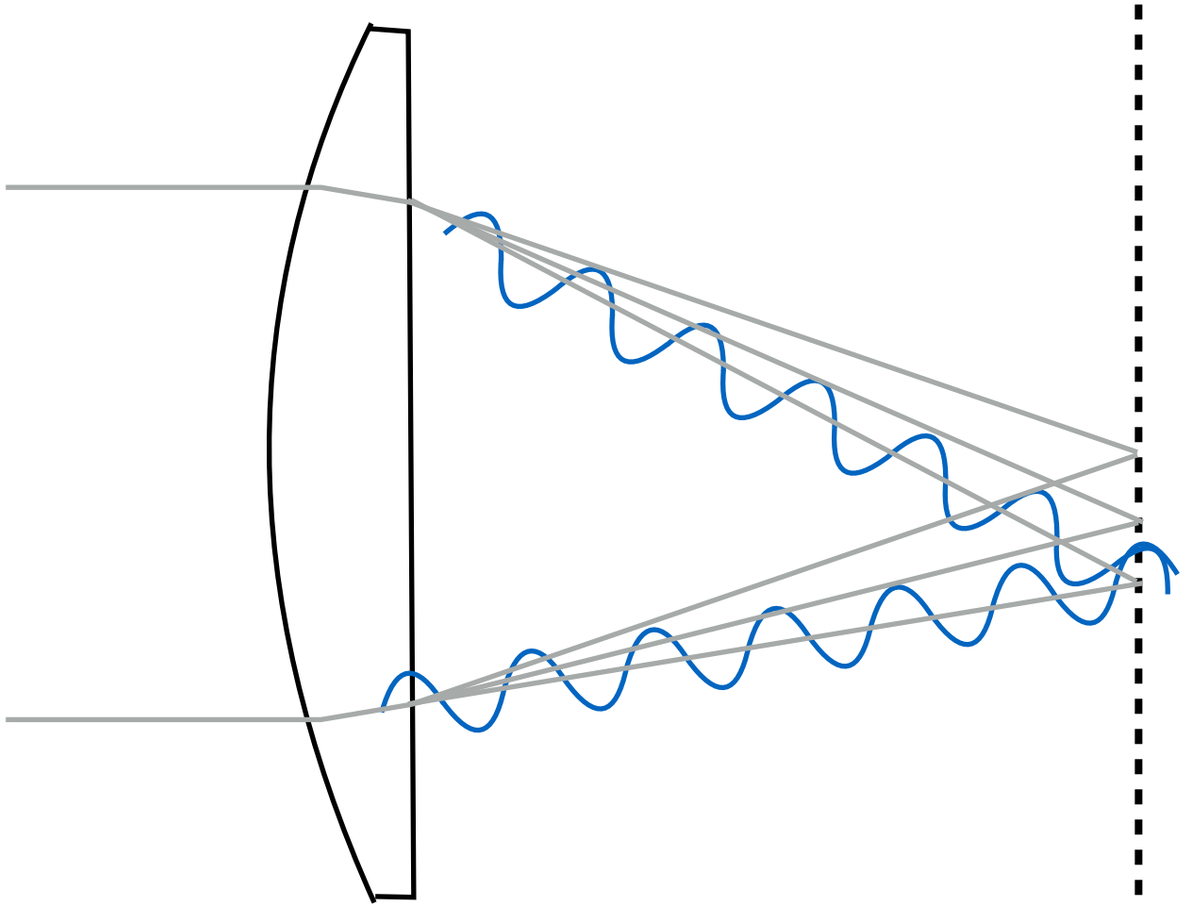
out of phase - Destructive interference

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

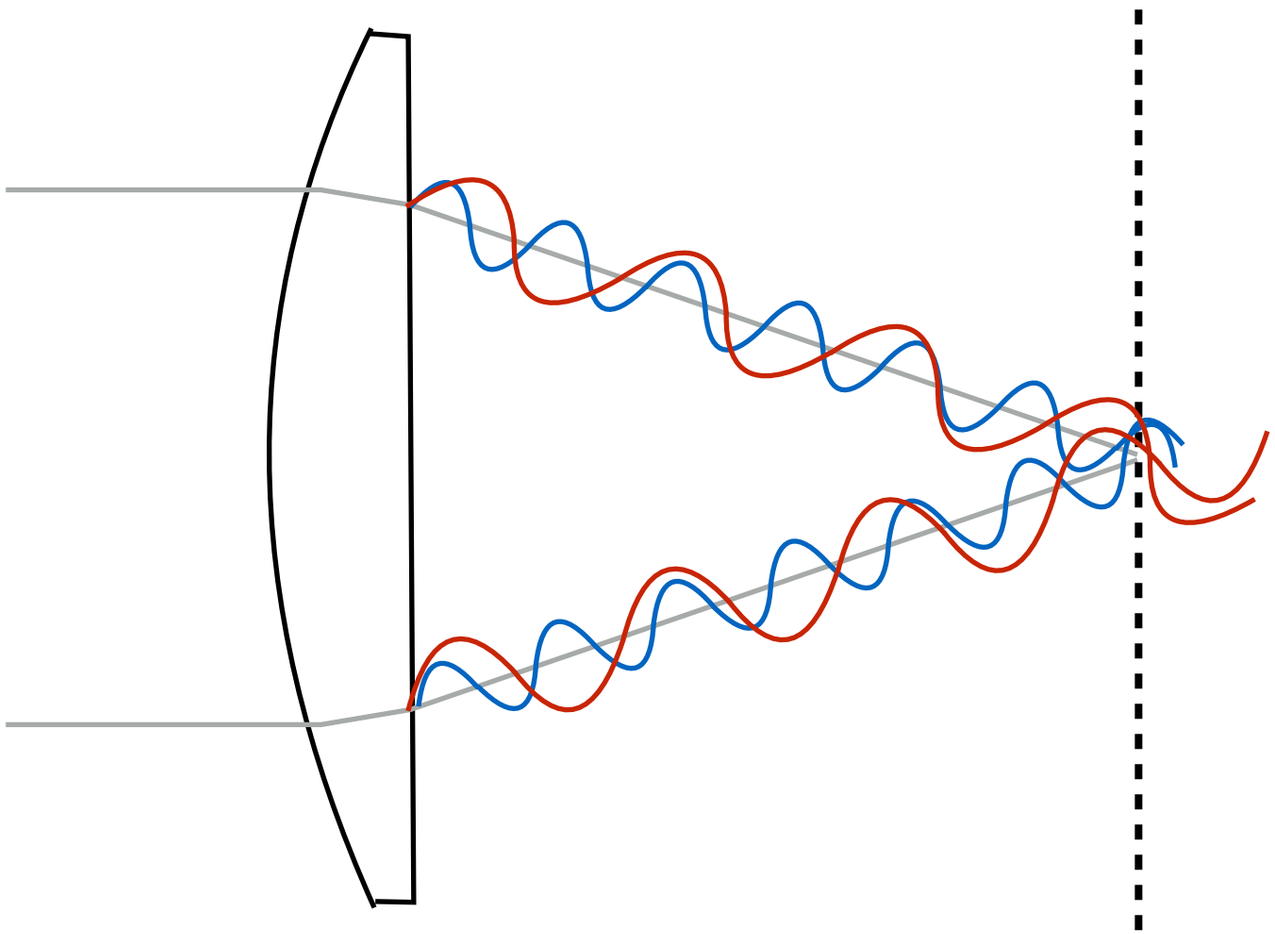
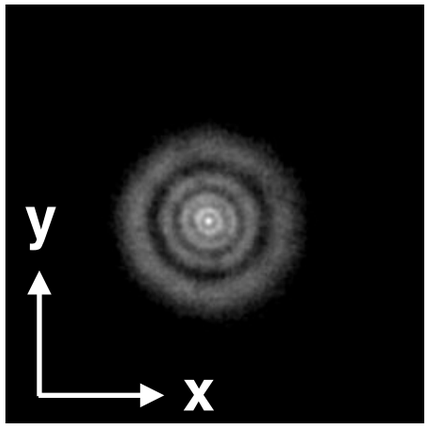


Image plane



wavelength

What does depend on…?

Objective lens

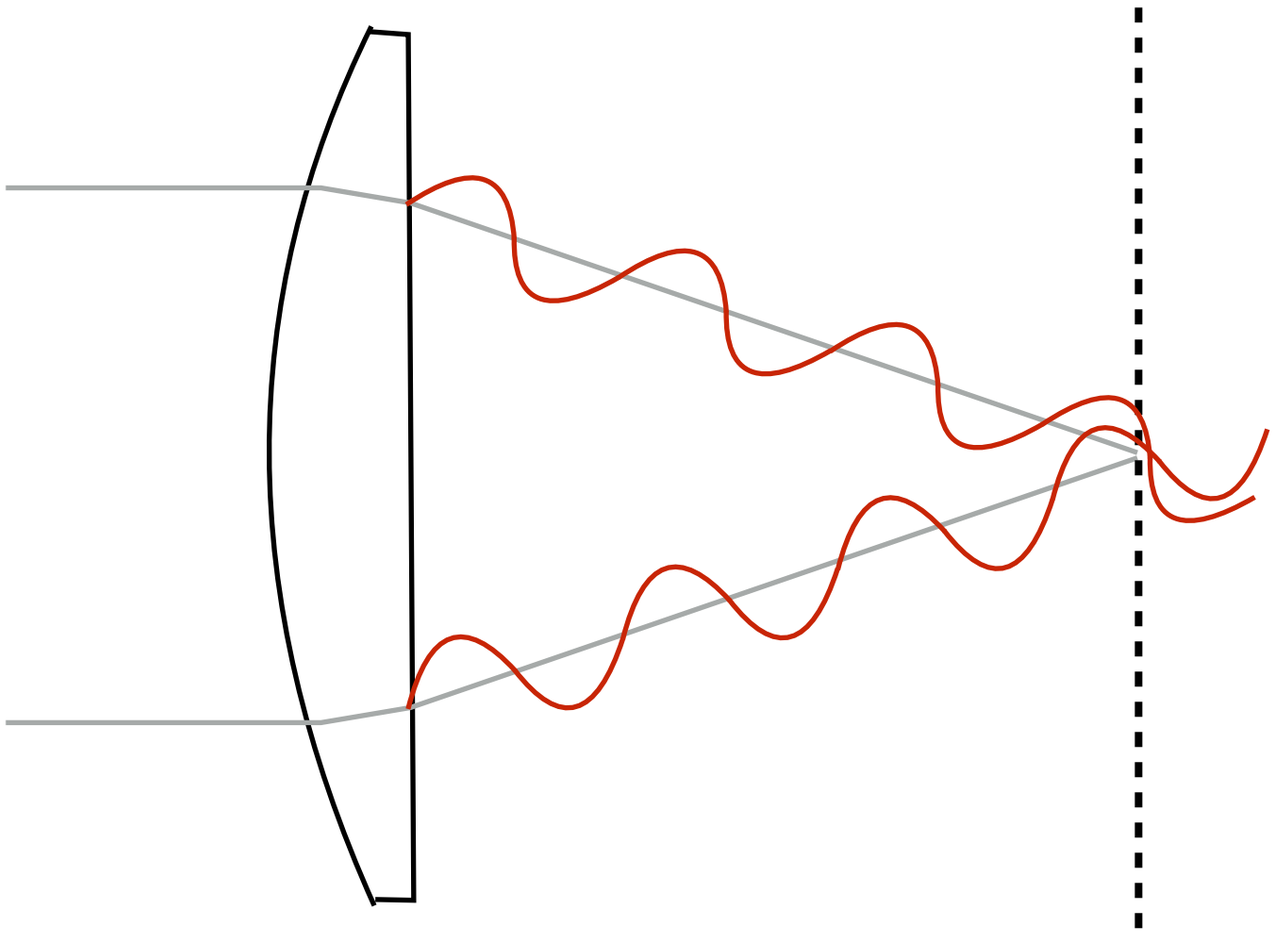
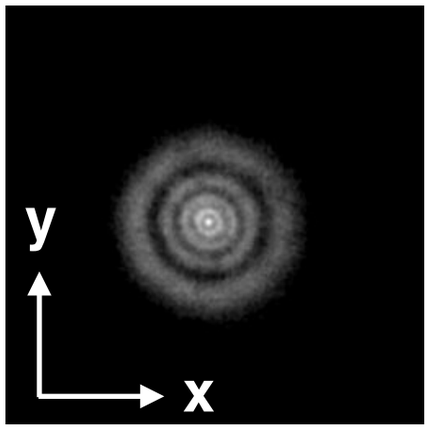
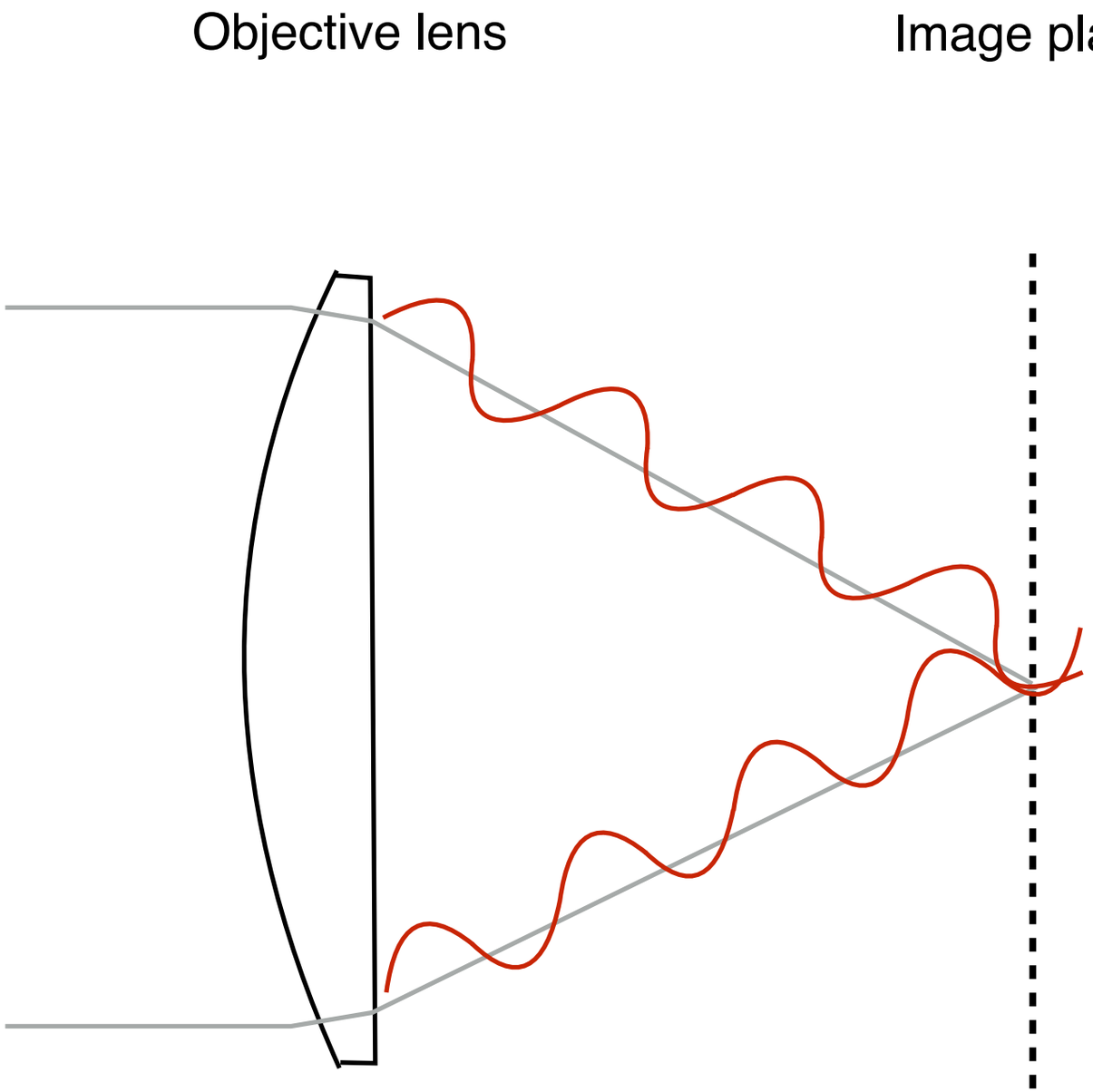


Image plane



Numerical aperture

What does depend on…?

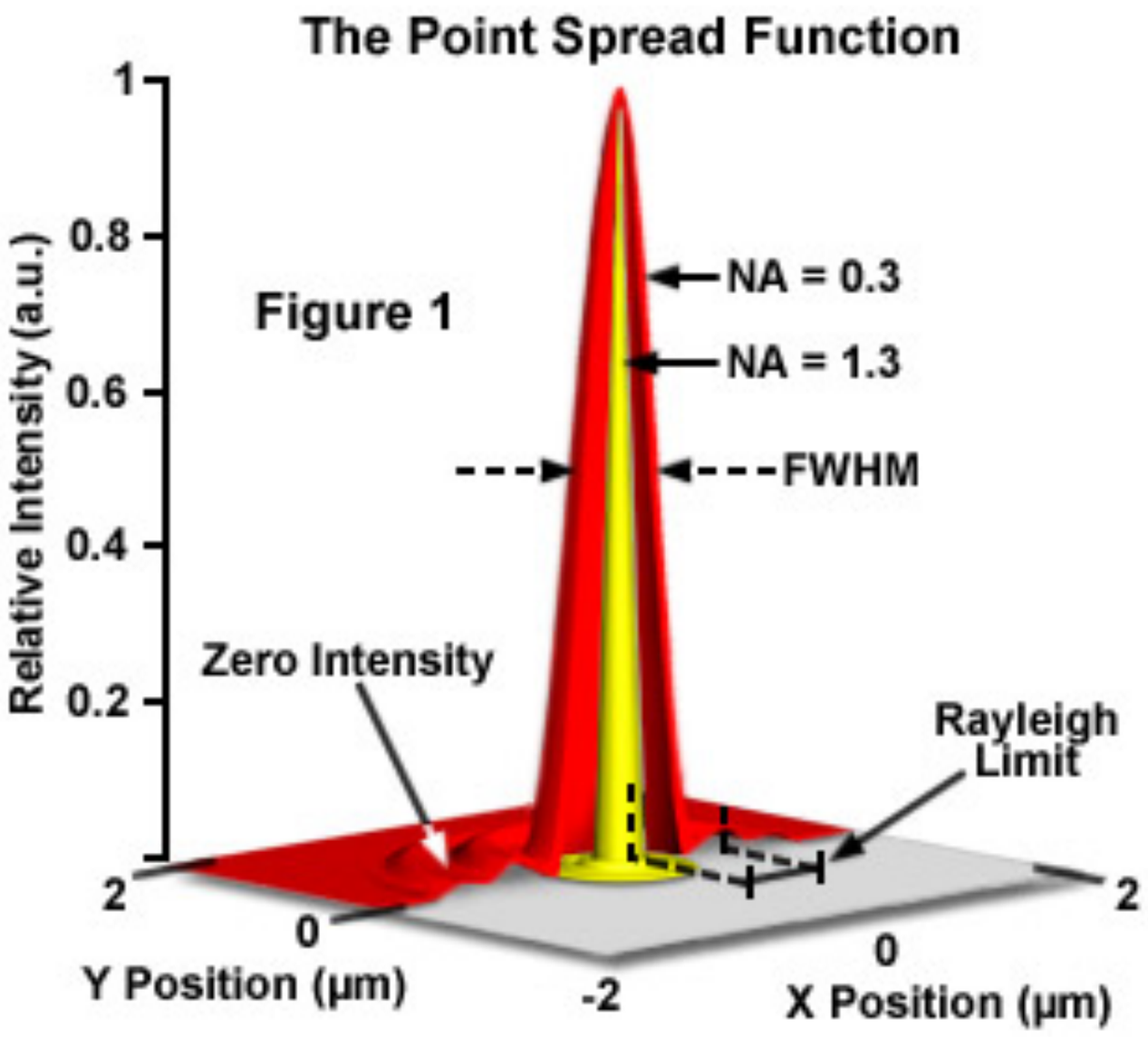


Numerical aperture

☑ Higher numerical aperture, less distortion, higher resolution

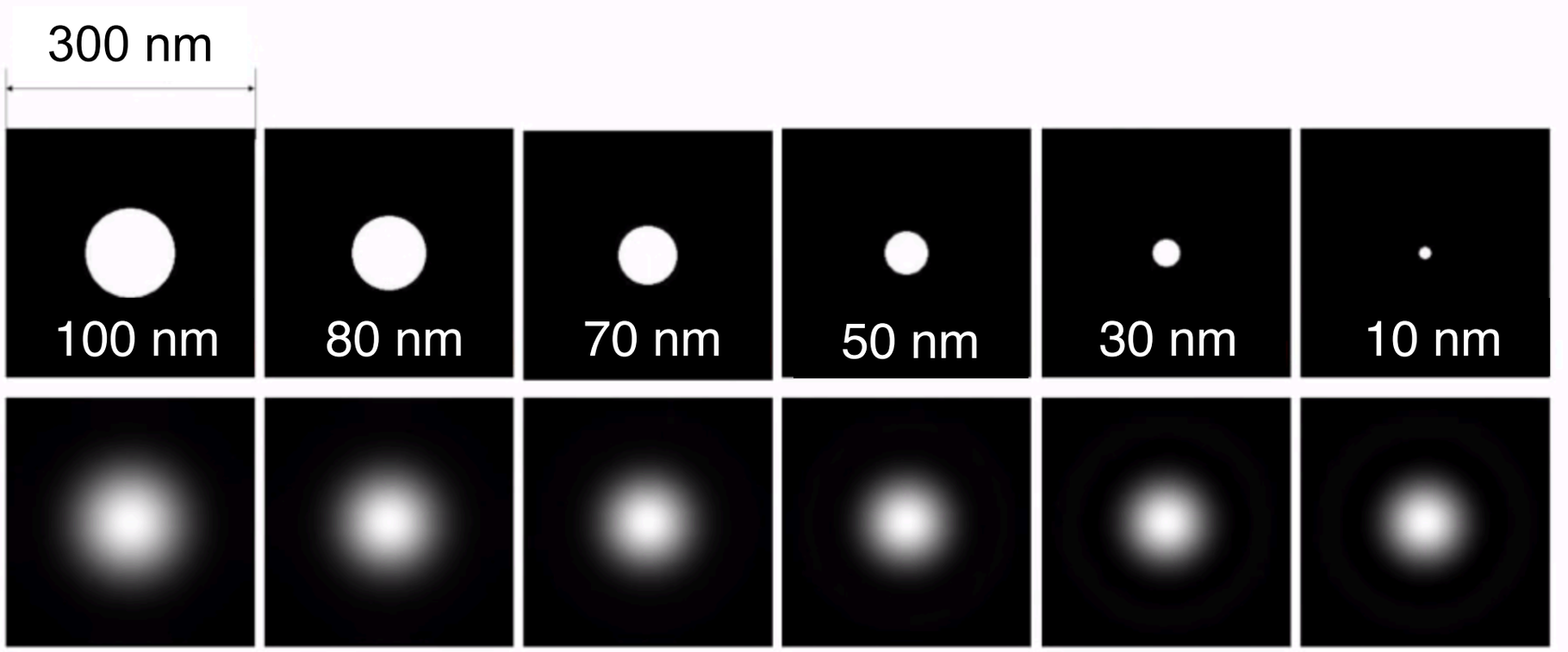
What does depend on…?

Numerical aperture



☑ Higher numerical aperture, less distortion, higher resolution

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

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

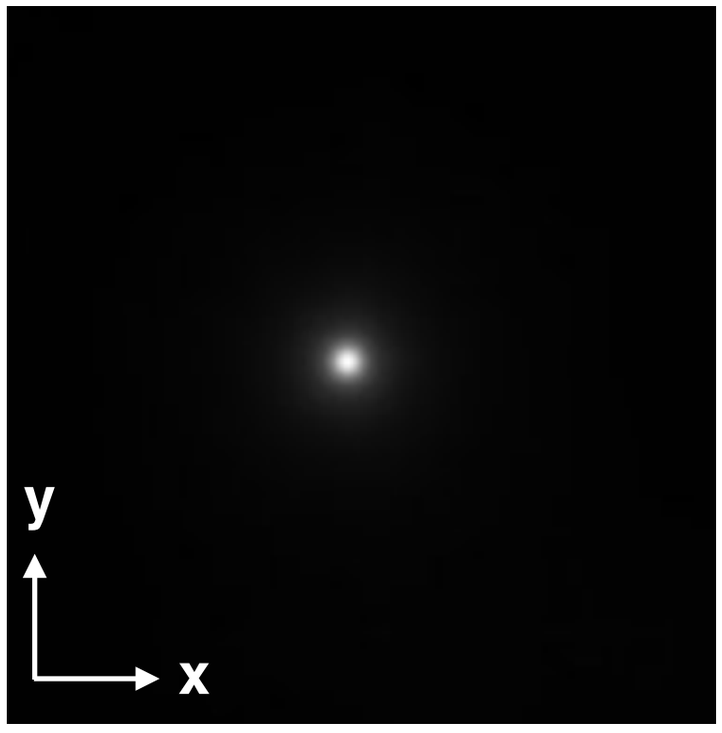
OTF
spatial frequency domain
k_x, k_y, k_z

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

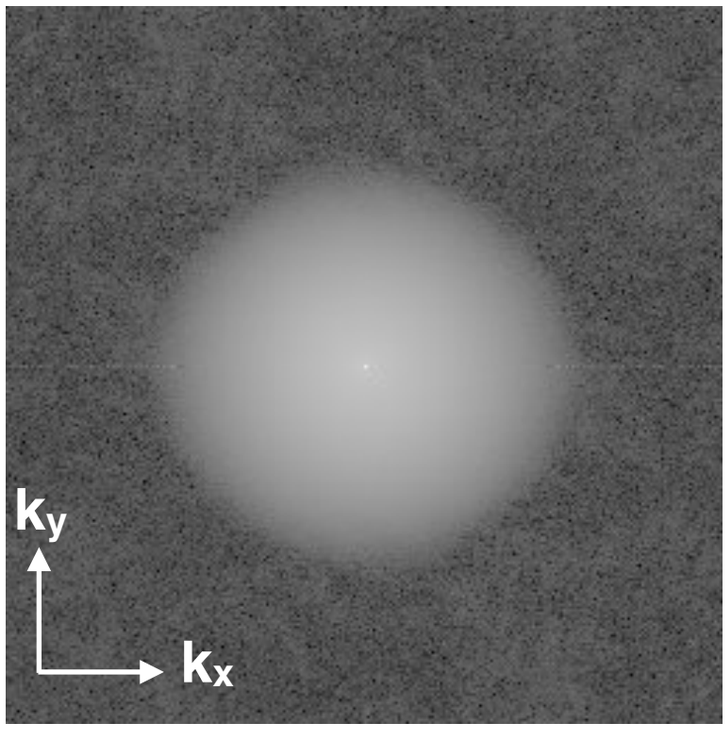
PSF



Fourier transform

Inverse Fourier transform

OTF

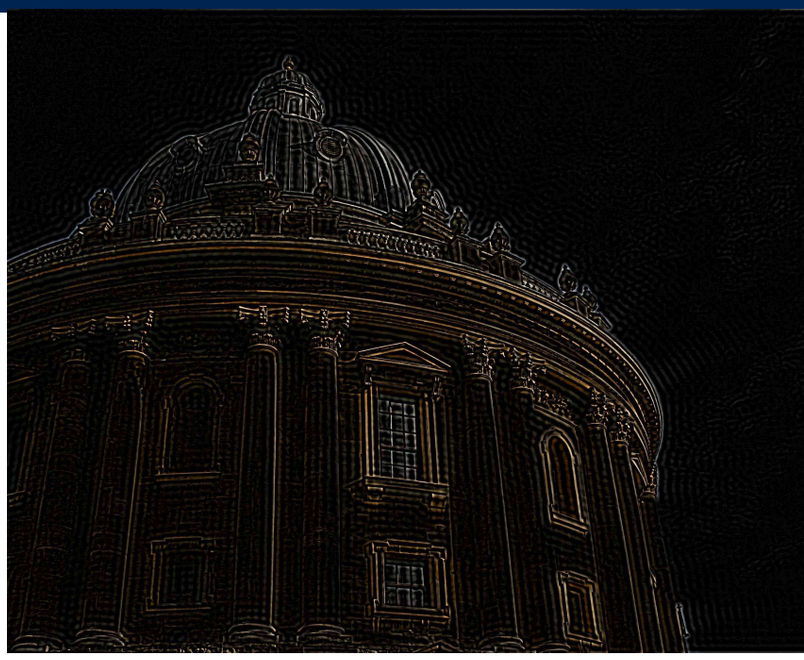


What are spatial frequencies in an image?

Lower frequencies - blurred

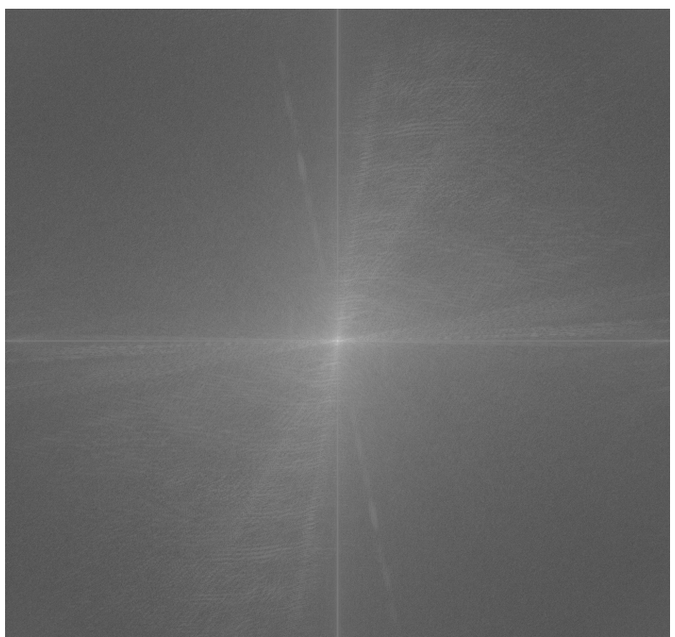
Higher frequencies - sharp

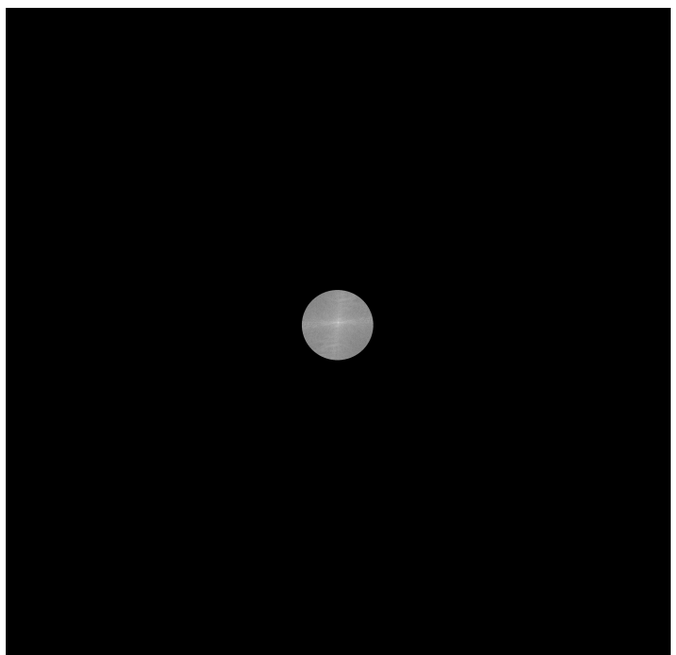
FIJI / Process / FFT

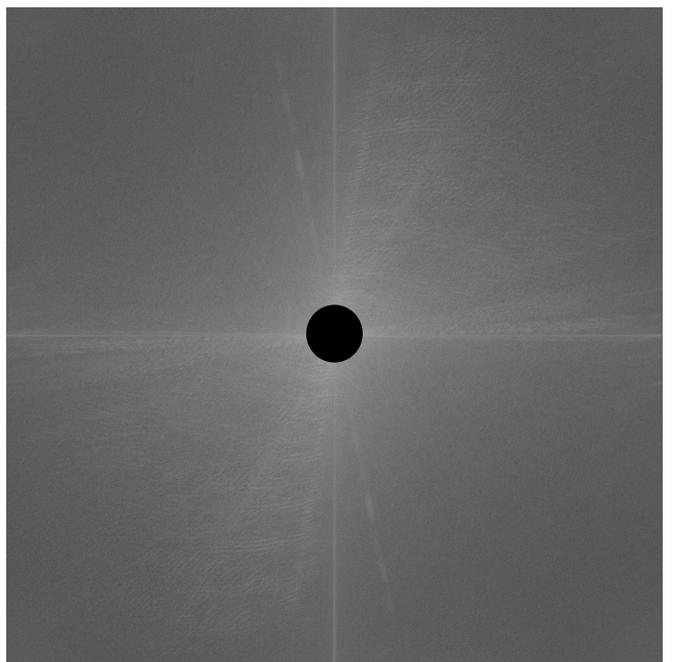


Fourier transform

Inverse Fourier transform

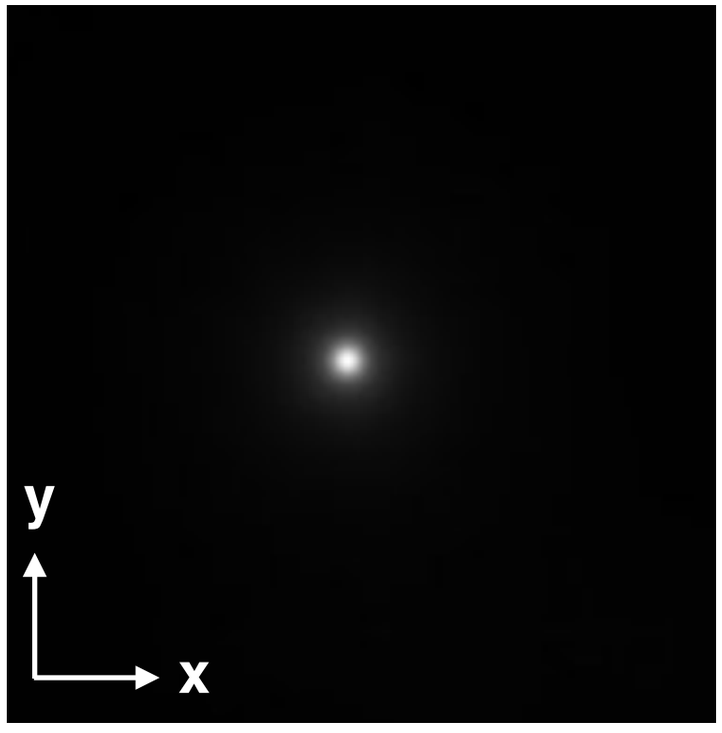






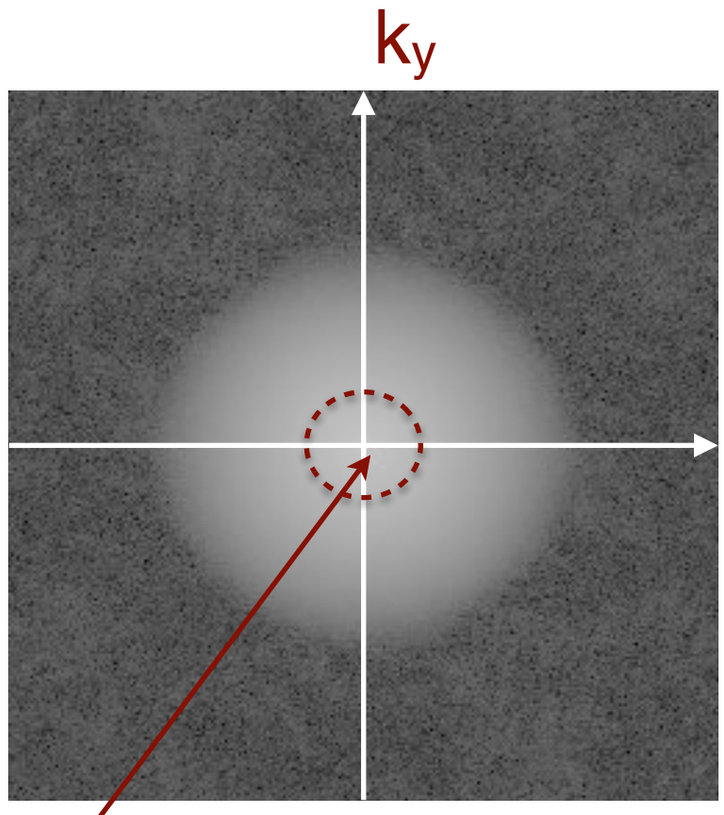
OTF (Optical transfer function) is the Fourier transform of PSF

PSF



Fourier transform

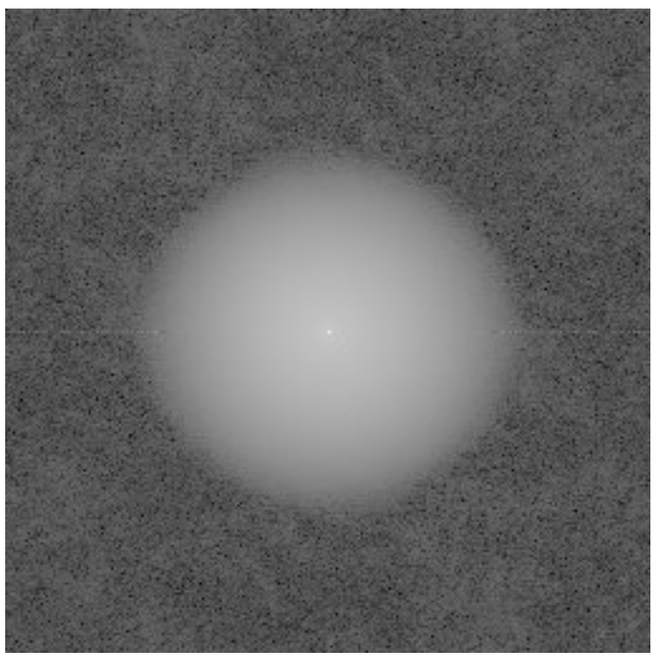
OTF

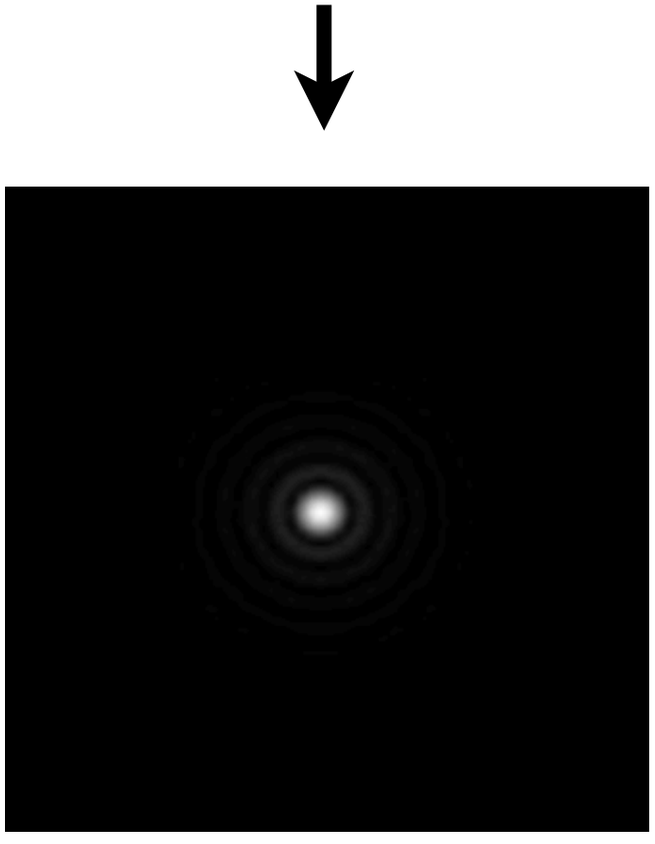


lower frequencies towards the centre

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

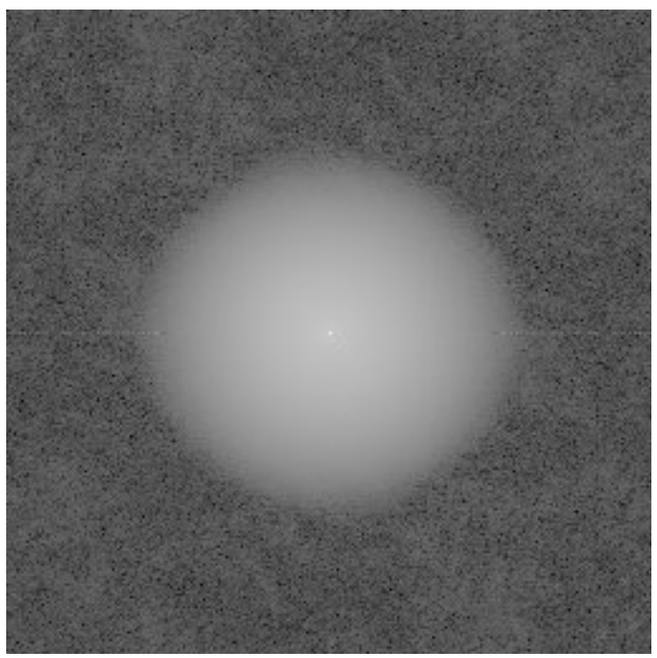
All frequencies



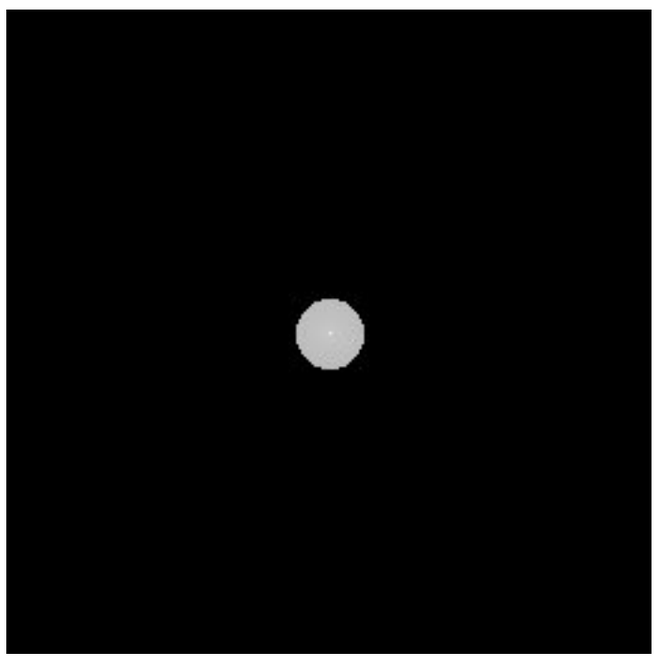


It’s very easy to detect certain features in the frequency domain

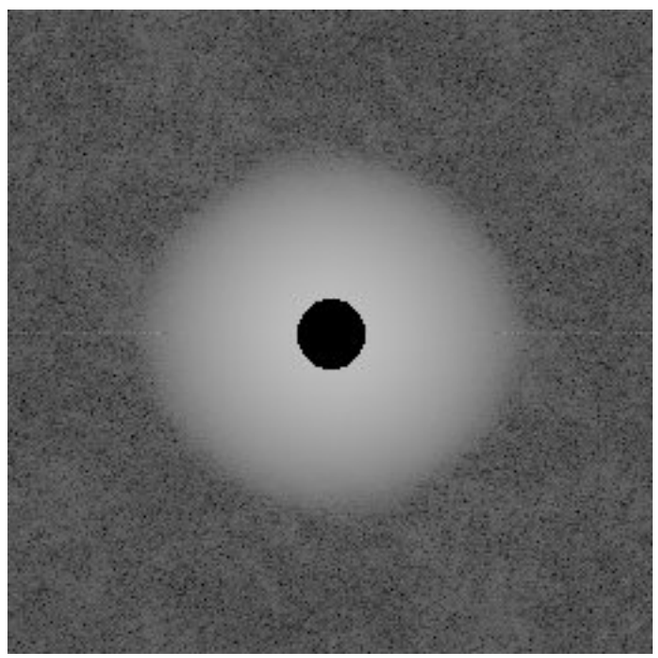
All frequencies



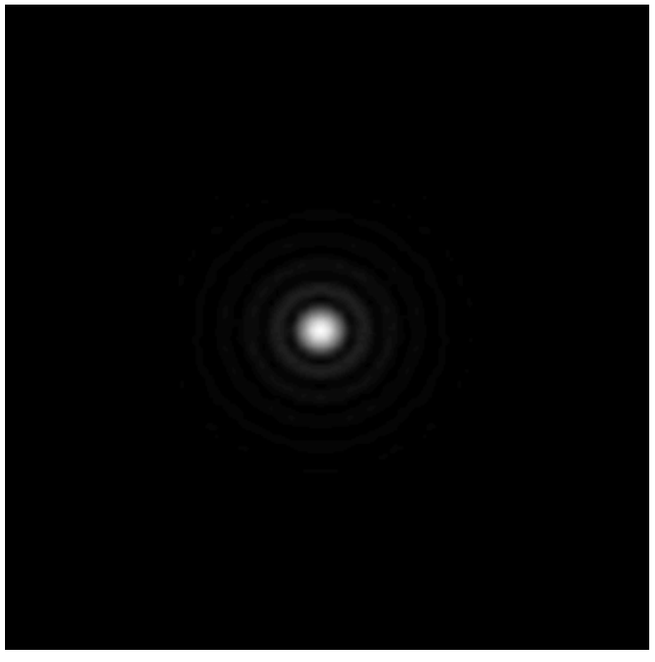
Just lower frequencies

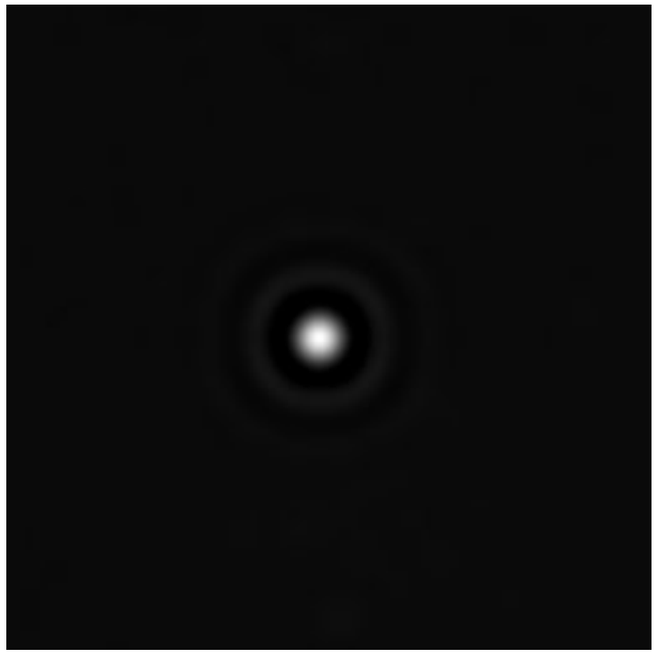


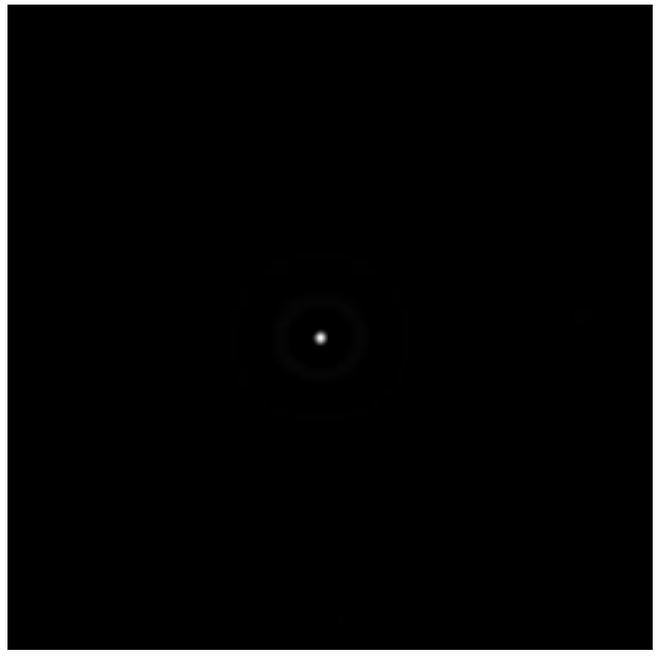
Just higher frequencies



Inverse Fourier transform

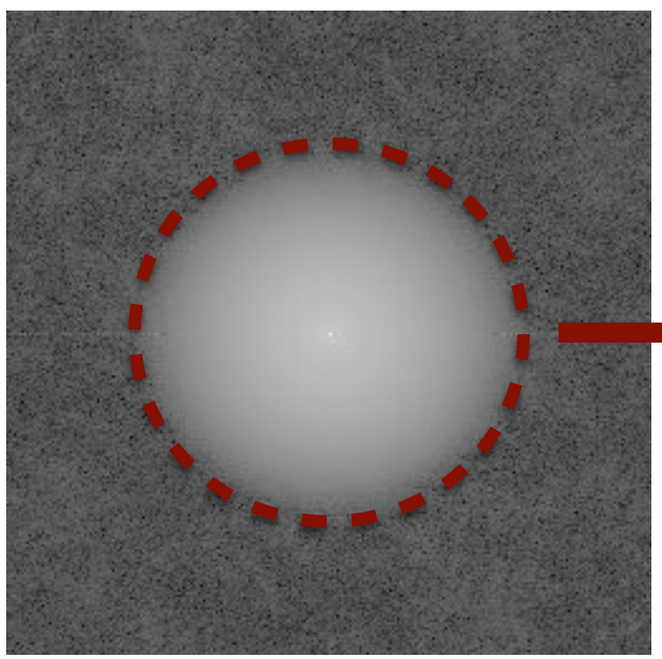






It’s very easy to detect certain features in the frequency domain

All frequencies



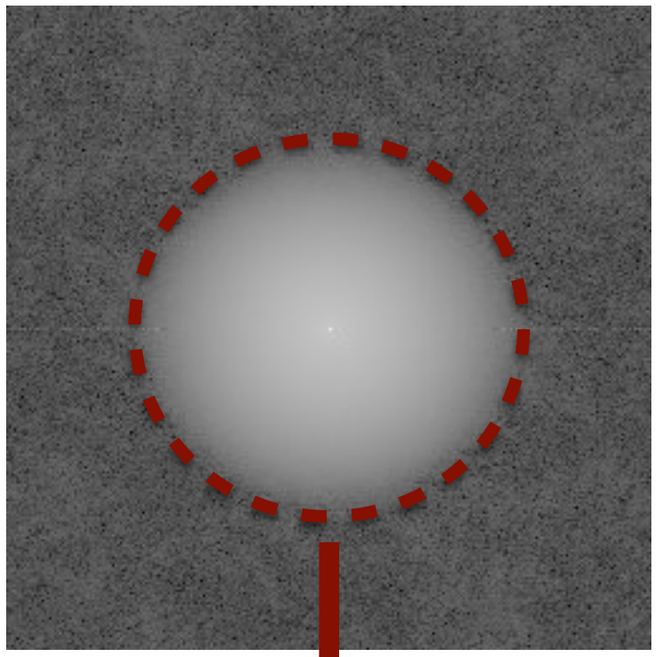
What does it represent?

Back Aperture Objective

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

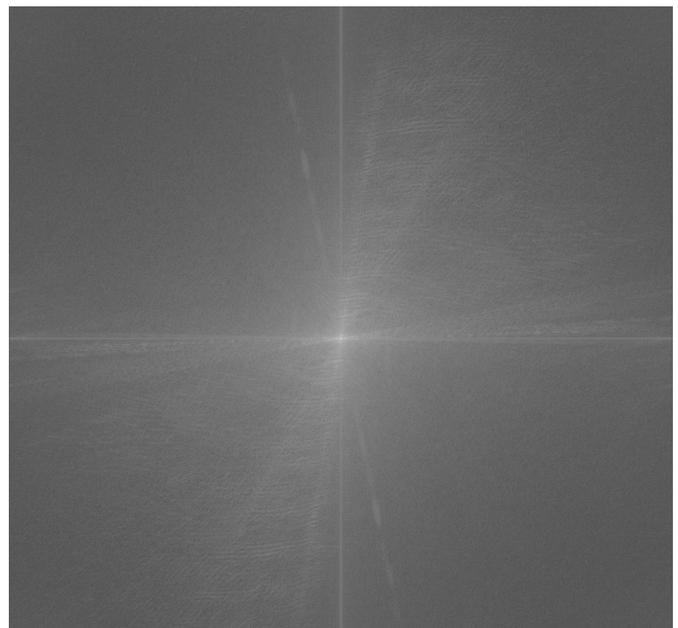
FIJI / Process / FFT

All frequencies

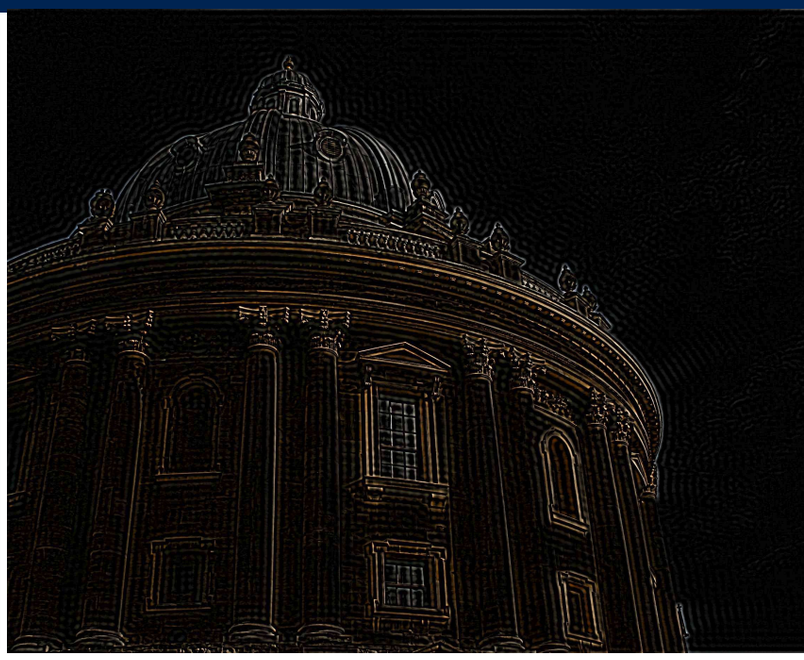


Back Aperture Objective

Fourier transform

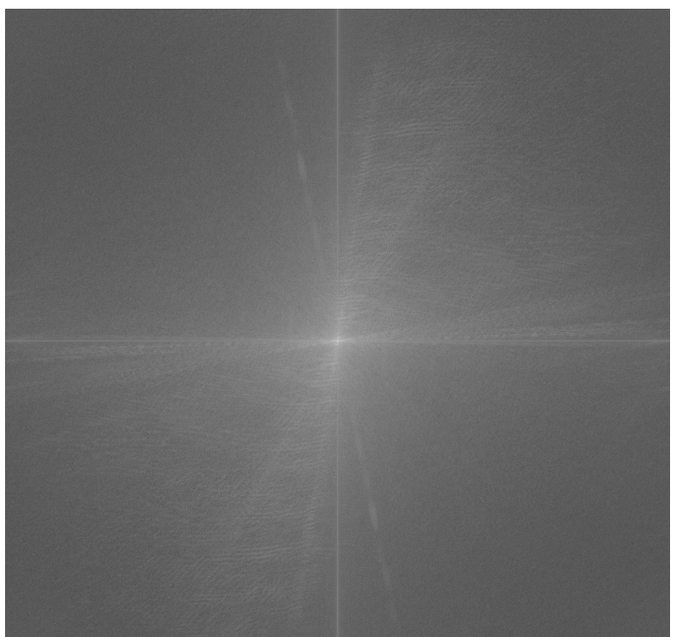


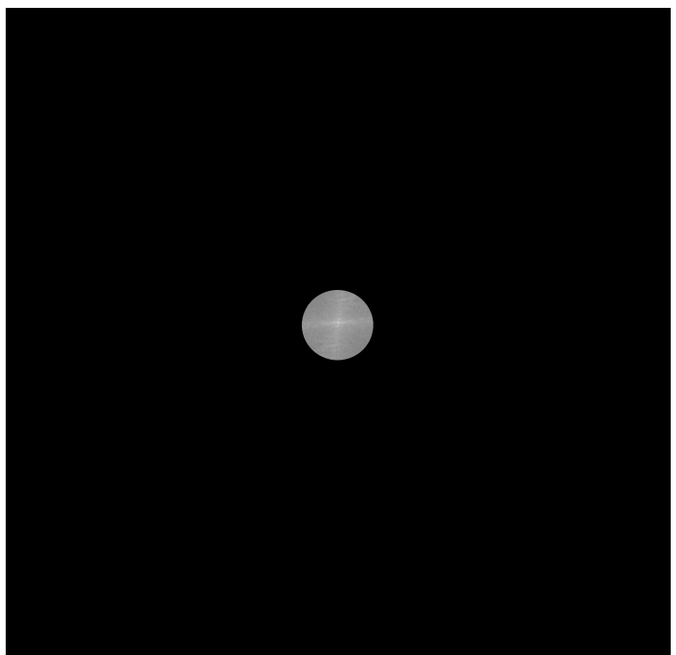
FIJI / Process / FFT

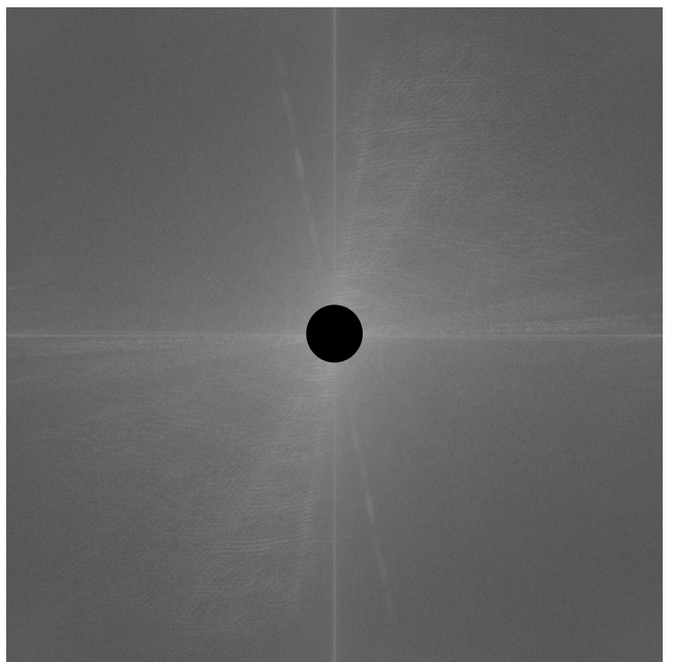


Fourier transform

Inverse Fourier transform

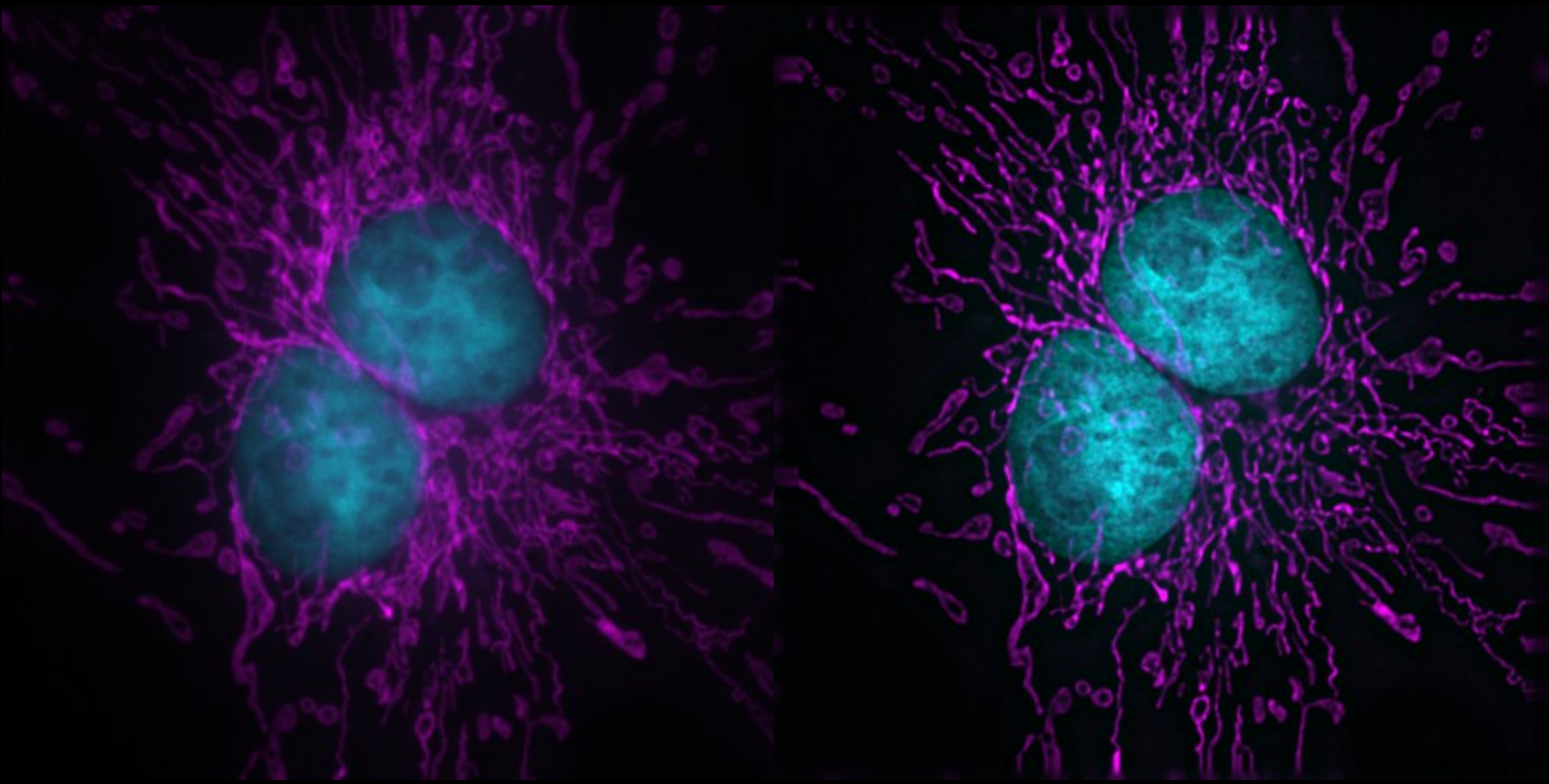






Widefield

Deconvolution



Different methods to deconvolve images → Lecture 22

Conclusions

- Why is fluorescence? CONTRAST
- Dichroic mirror - separates illumination (excitation) from fluorescence (emission)
- Excitation and emission filters to image different fluorophores
- Fluorescence microscope:
  - illumination light is reflected (opposed to transmitted in Brightfield microscopy)
  - objective illuminates and collects fluorescence (both “condenser” and objective)
- 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
- Deconvolution removes blur from microscope images, improving contrast and resolution

Questions?