Micron OXFORD

Advanced Bioimaging Unit

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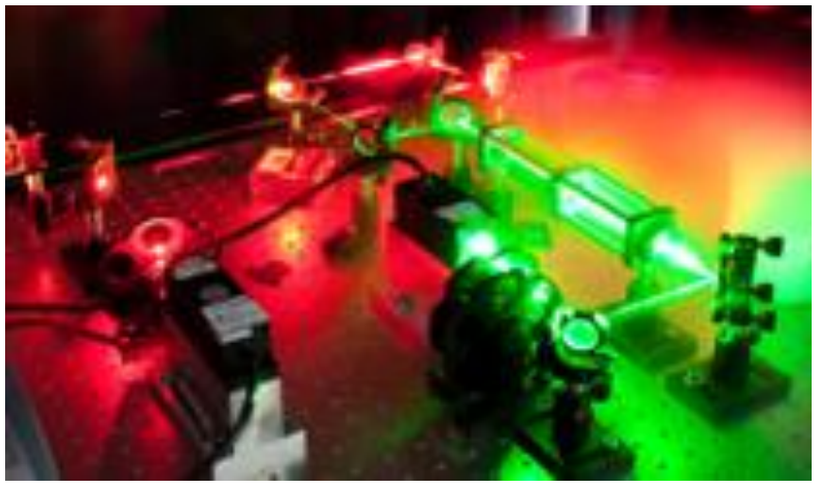
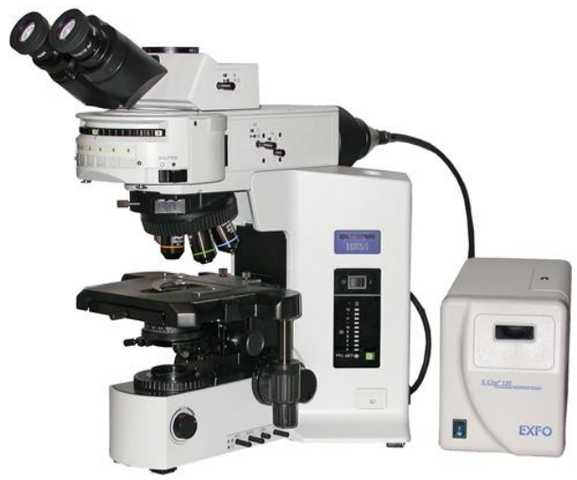
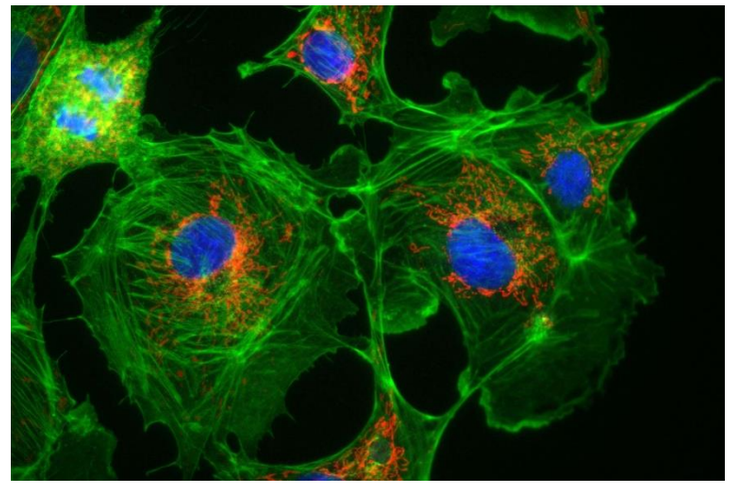
UNIVERSITY OF OXFORD

Lecture 4
Understanding and applying fluorescence microscopy







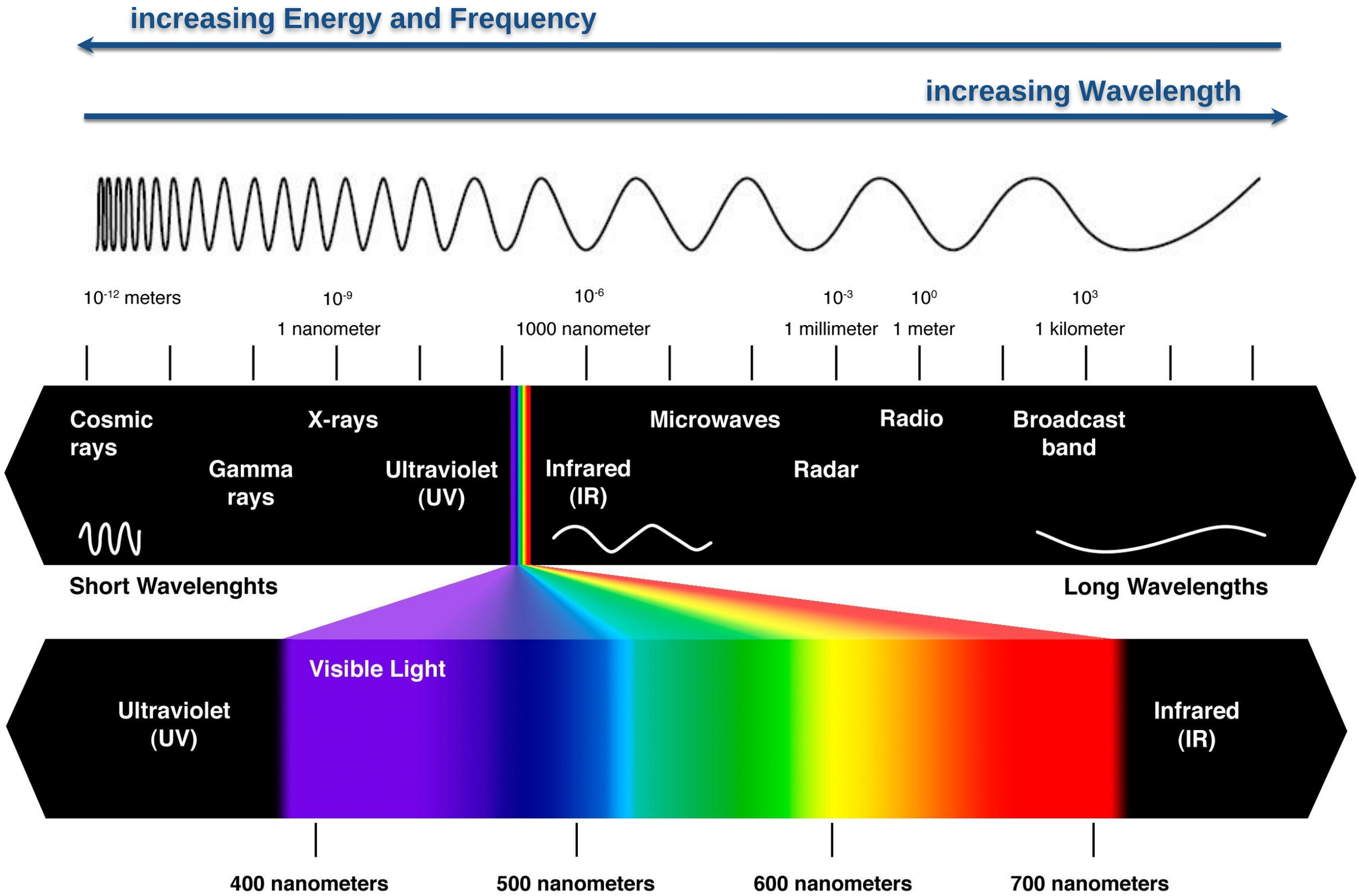
Andrew Jefferson
Micron Advanced Bioimaging Unit

Outline

1. What is fluorescence?
2. Why fluorescence?
3. Principle and components of the fluorescence microscope
4. Fluorescent light sources
5. Point Spread Functions
6. Fixation for light microscopy

1. What is fluorescence?

The electromagnetic spectrum



380 – 700 nm visible to the human eye

What is Fluorescence?

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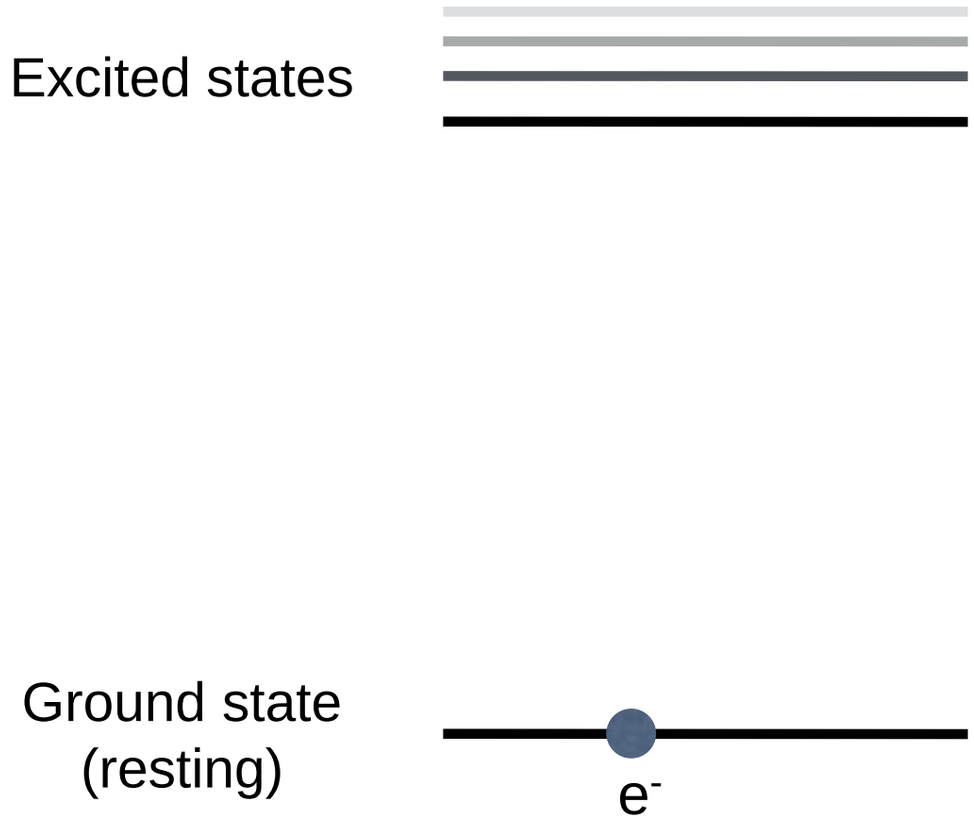
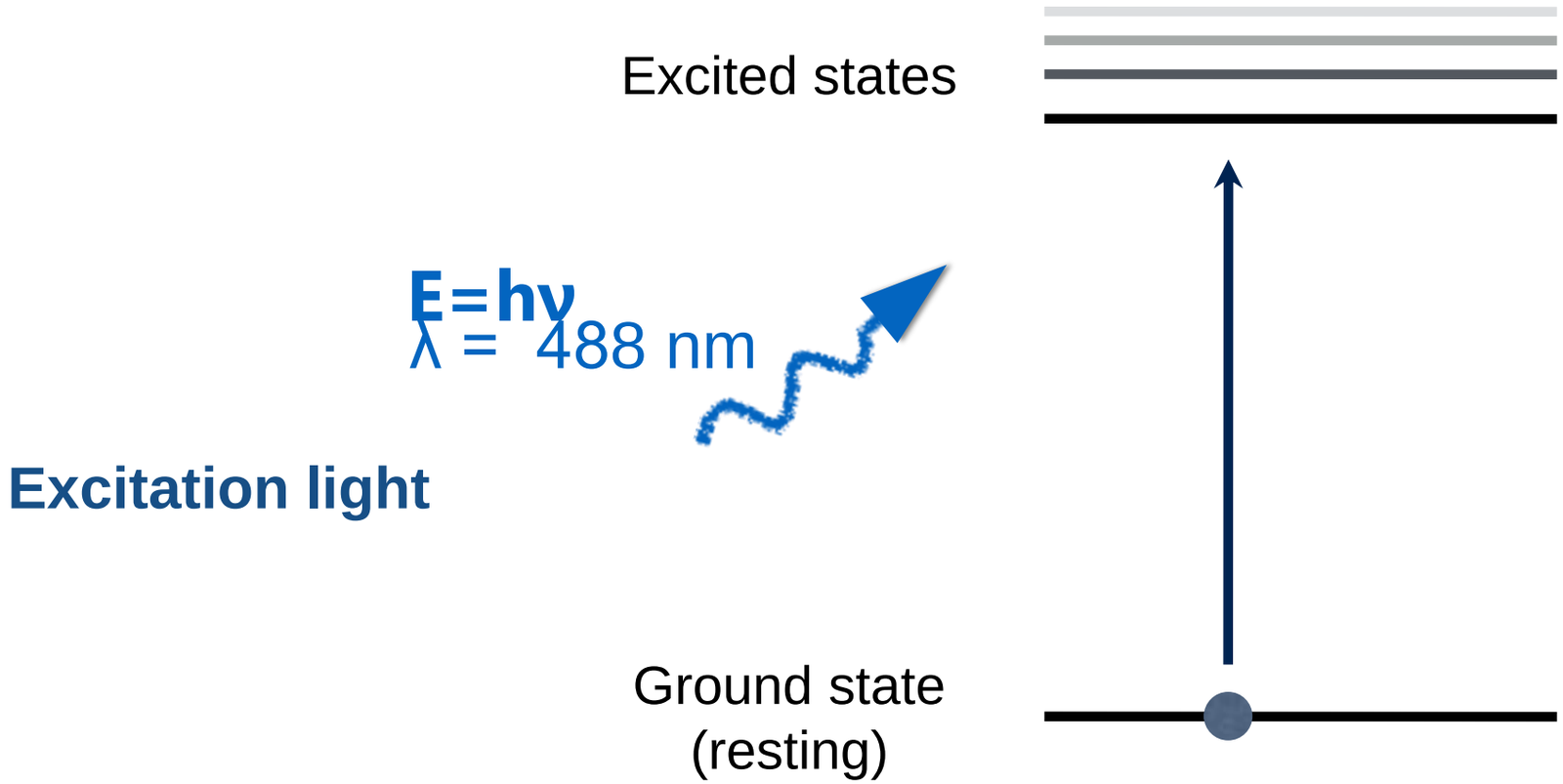
"**Fluorescence** is the emission of light by a substance that has absorbed light"

https://en.wikipedia.org/wiki/Fluorescence



Molecules have discrete levels of energy

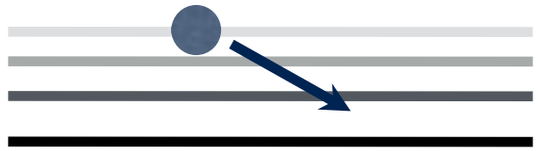
What is Fluorescence?



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

What is Fluorescence?

Excited states



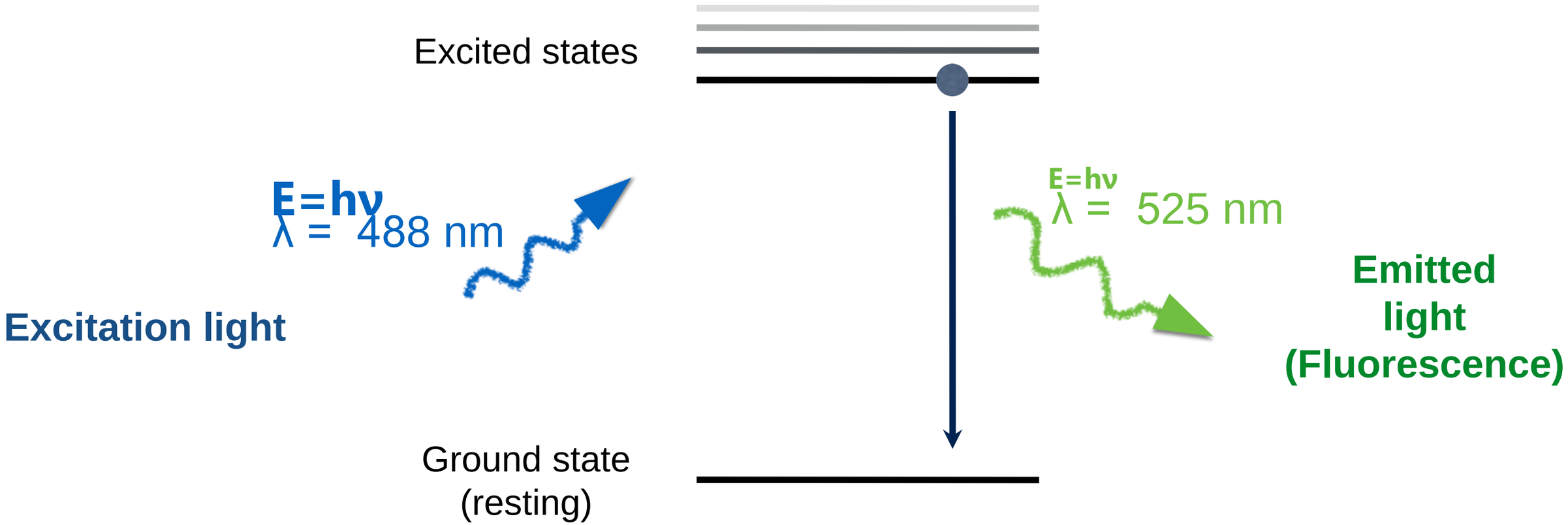
$E = h\nu$

$\lambda = 488$ nm

Excitation light

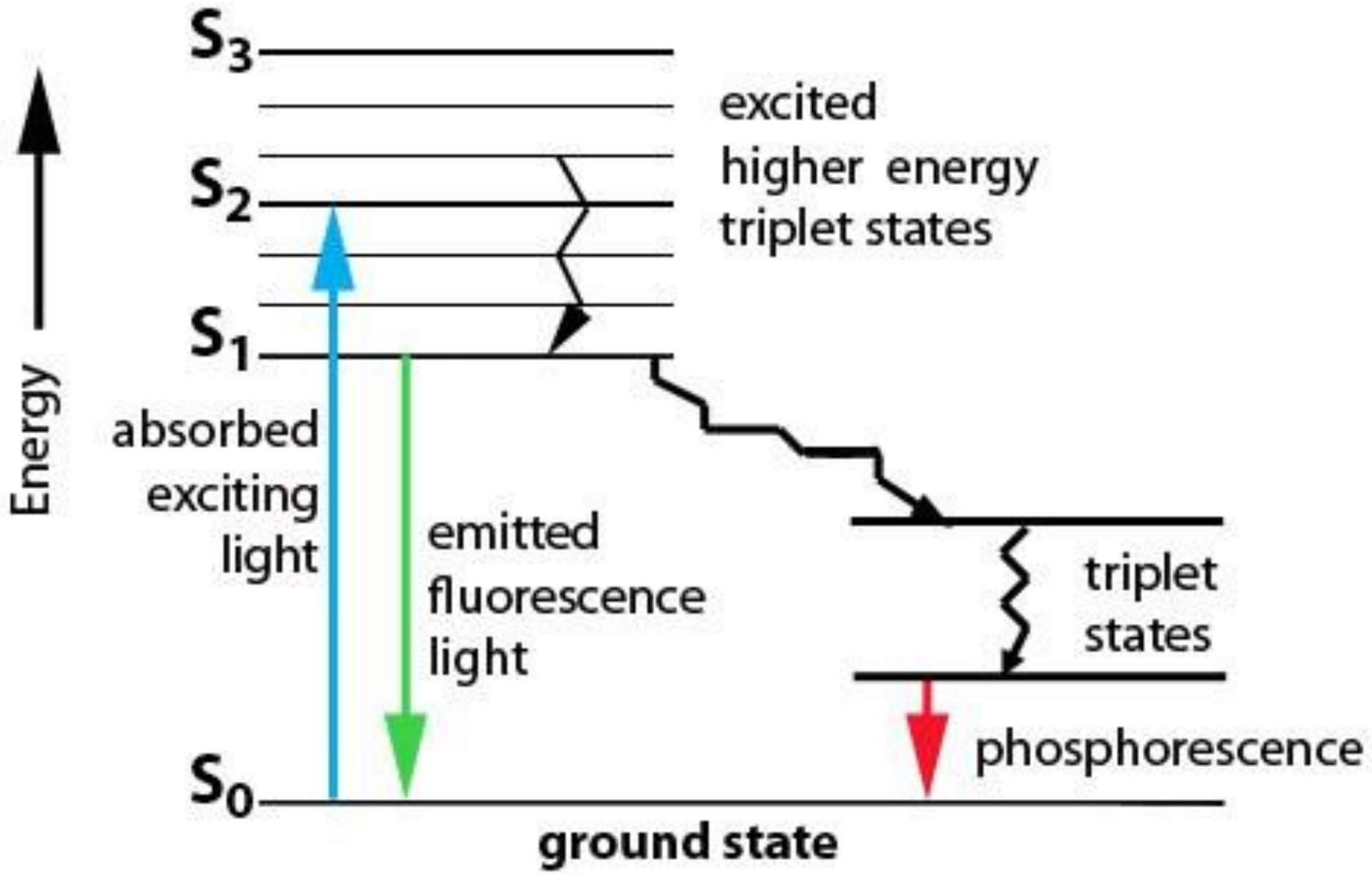
Ground state
(resting)

What is Fluorescence?



The full picture is represented by the Jablonski diagram…

→ Lecture 5



Fluorescence Spectra

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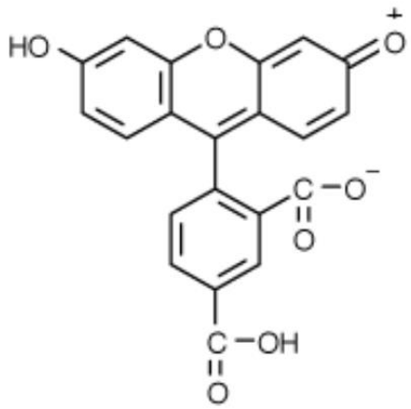
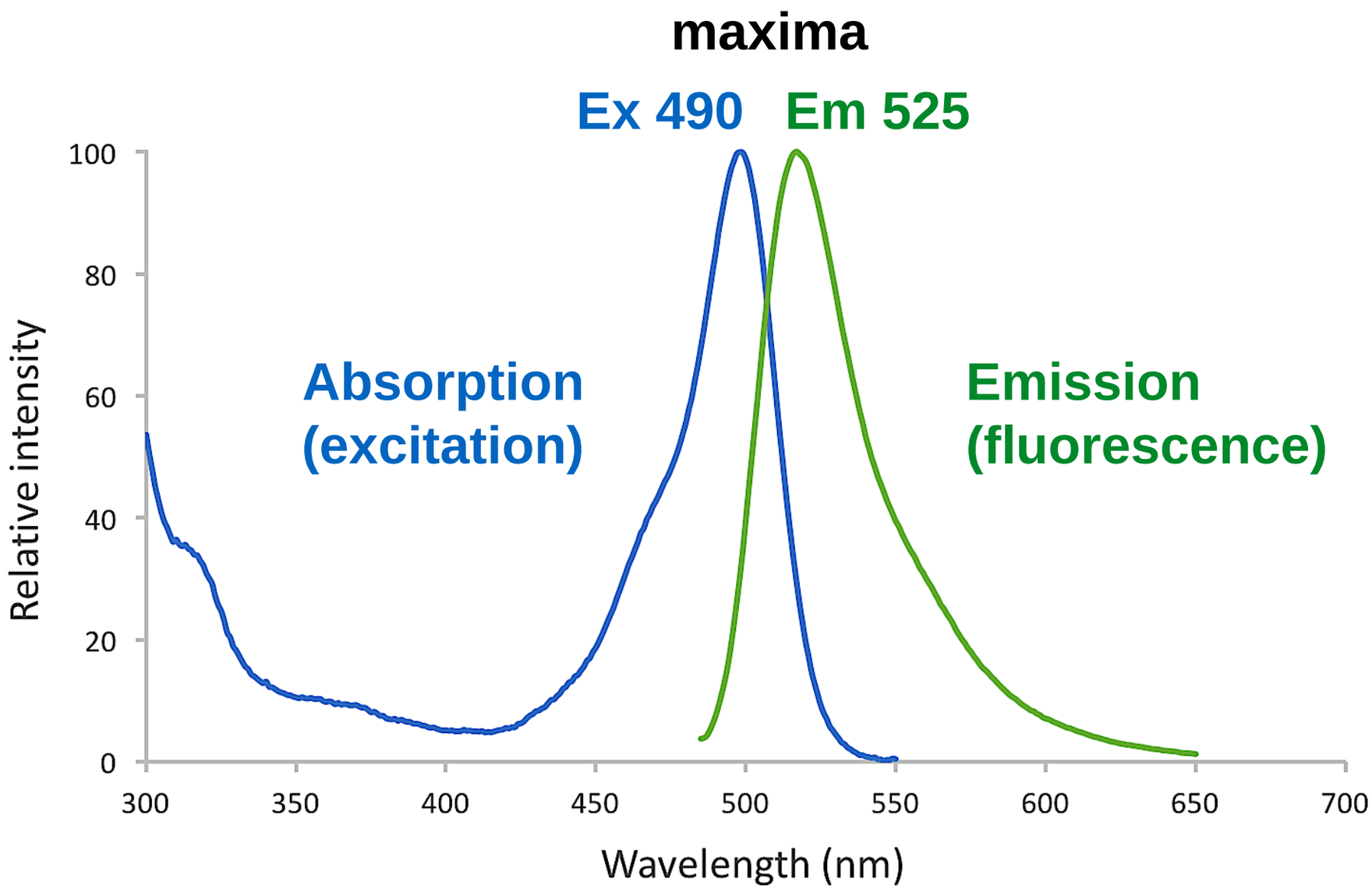
Fluorescein (FITC)





Fluorescent probes for microscopy

→ Lecture 5

Genetically encoded fluorescent proteins

- GFP, YFP, mCherry

Organic dyes

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

Inorganic dyes

- Quantum Dots

Endogenous species

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

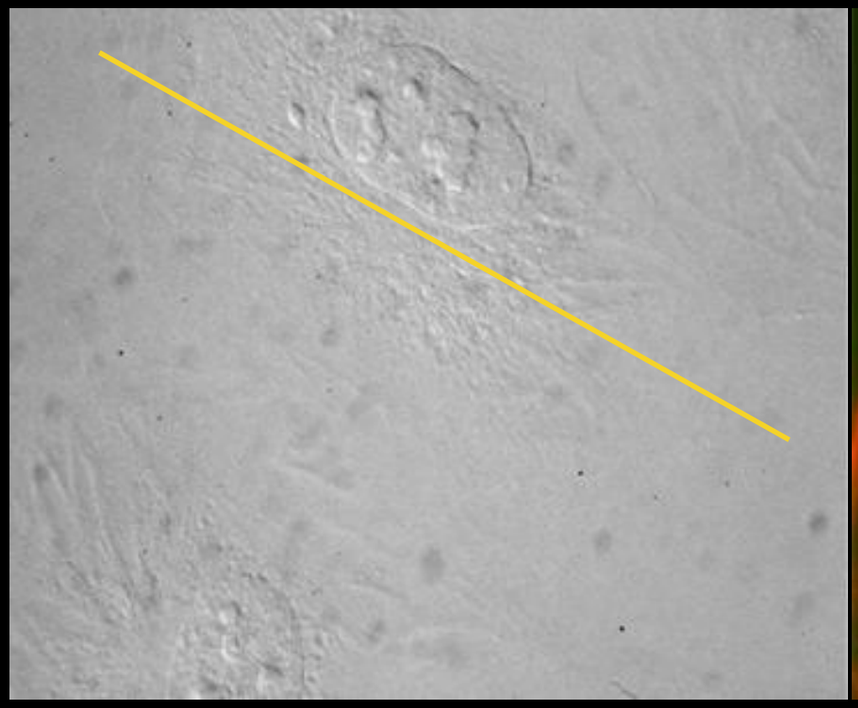
2. Why fluorescence?

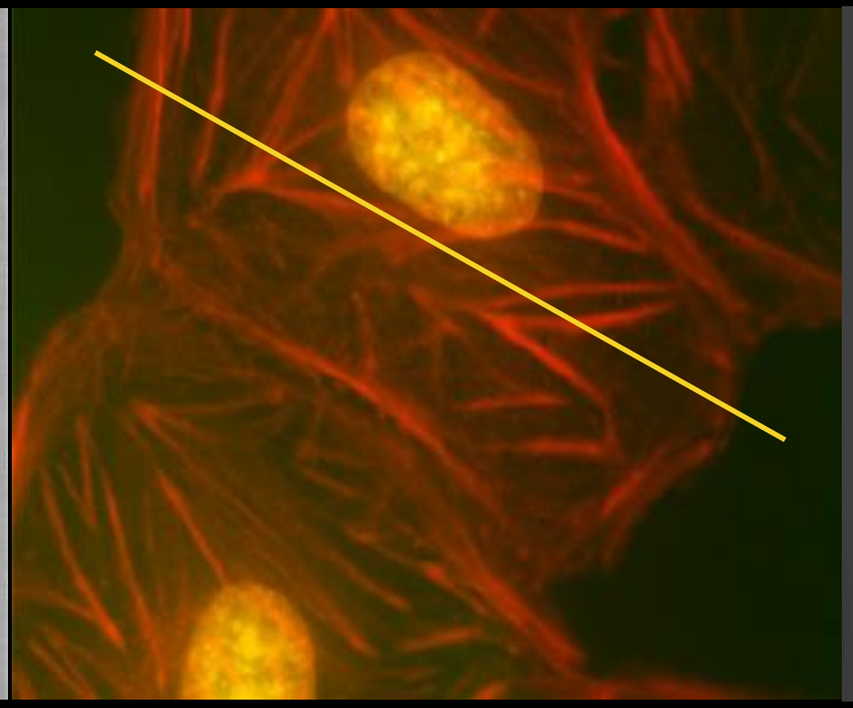
Why Fluorescence?

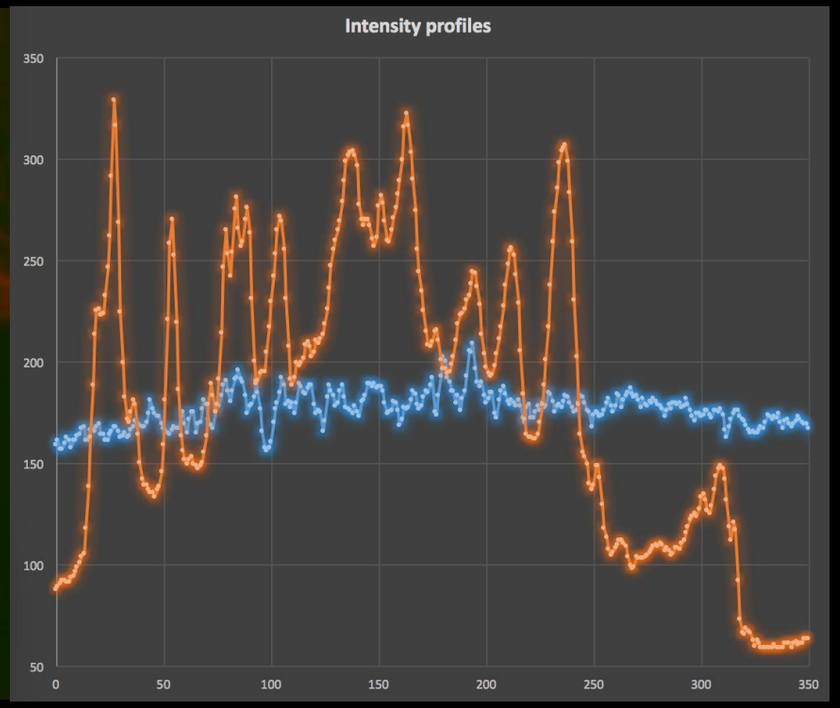
Chris Teren: https://www.youtube.com/watch?v=PhclTQ3g0s8

CONTRAST

Why Fluorescence?





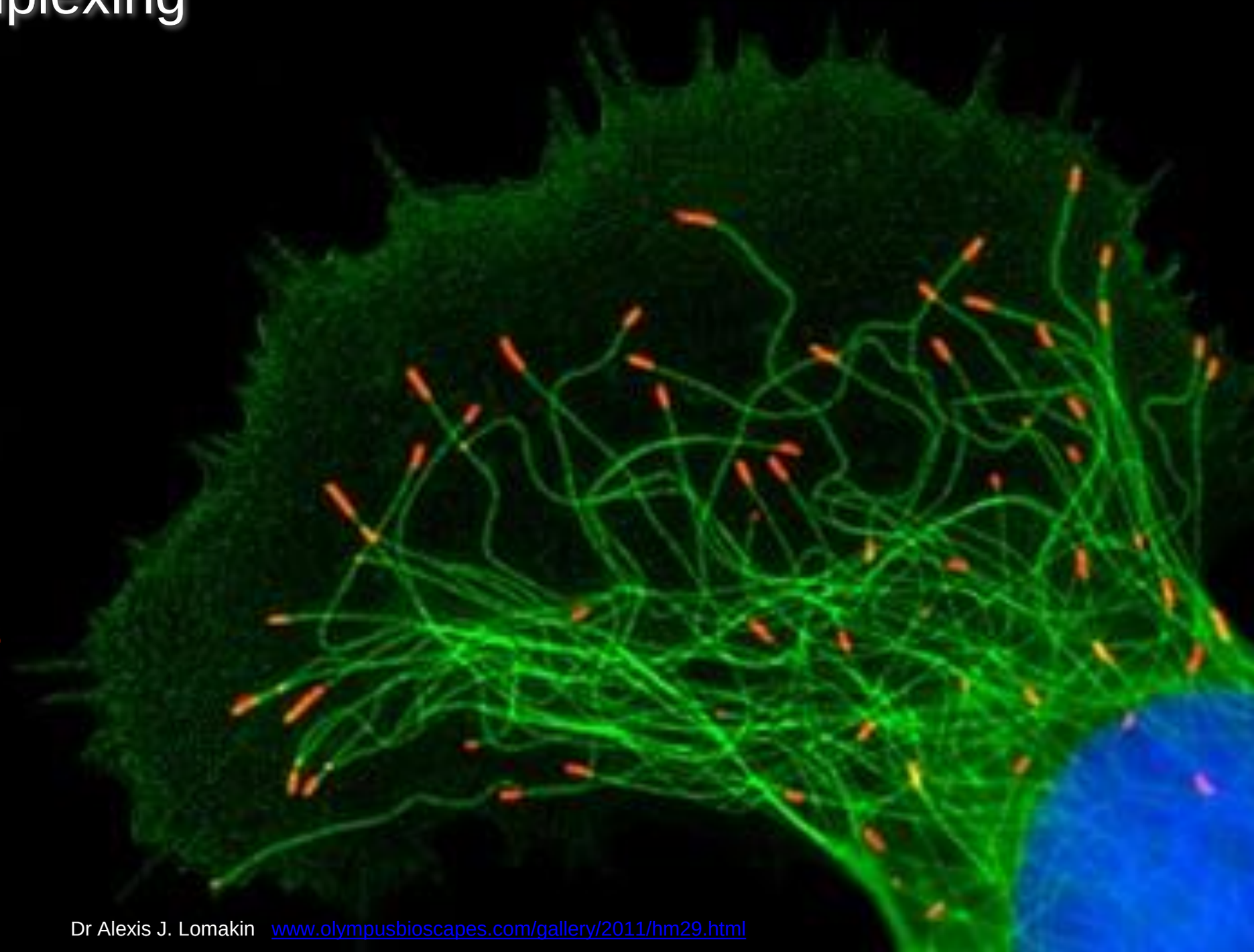


bright field (DIC) fluorescence Intensity profile

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

Why Fluorescence?

- Selective labeling
- Ease of multiplexing
- Quantitative



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

How do we get a black background….?

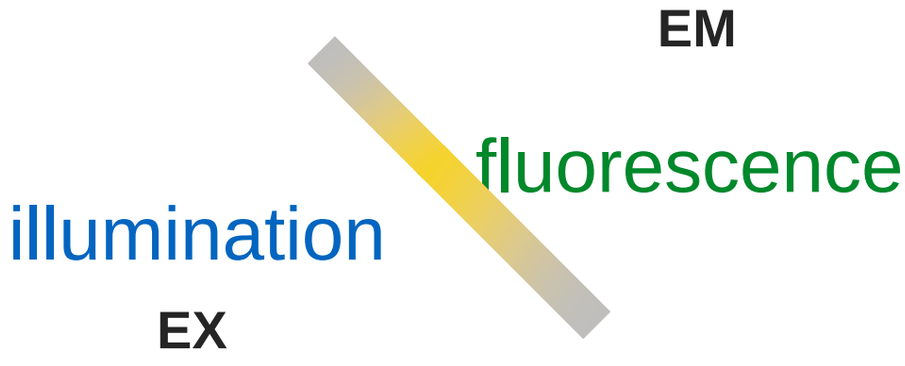
Fundamental problem in fluorescence microscopy

STRONG illumination

WEAK fluorescence signal

→ produce high-efficiency illumination of the specimen

while simultaneously capturing weak fluorescence emission



3. Principles and components of the fluorescence microscope

Components of a fluorescence microscope

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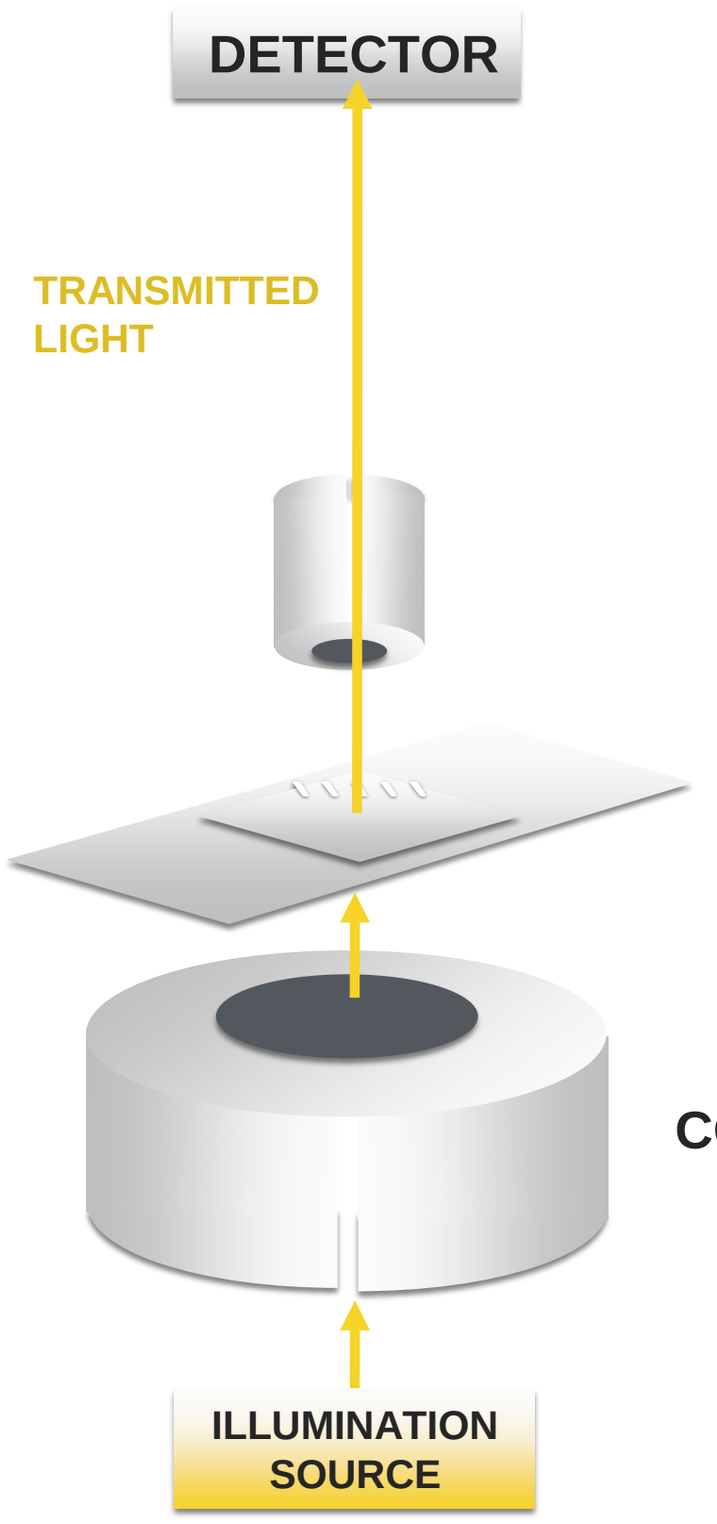
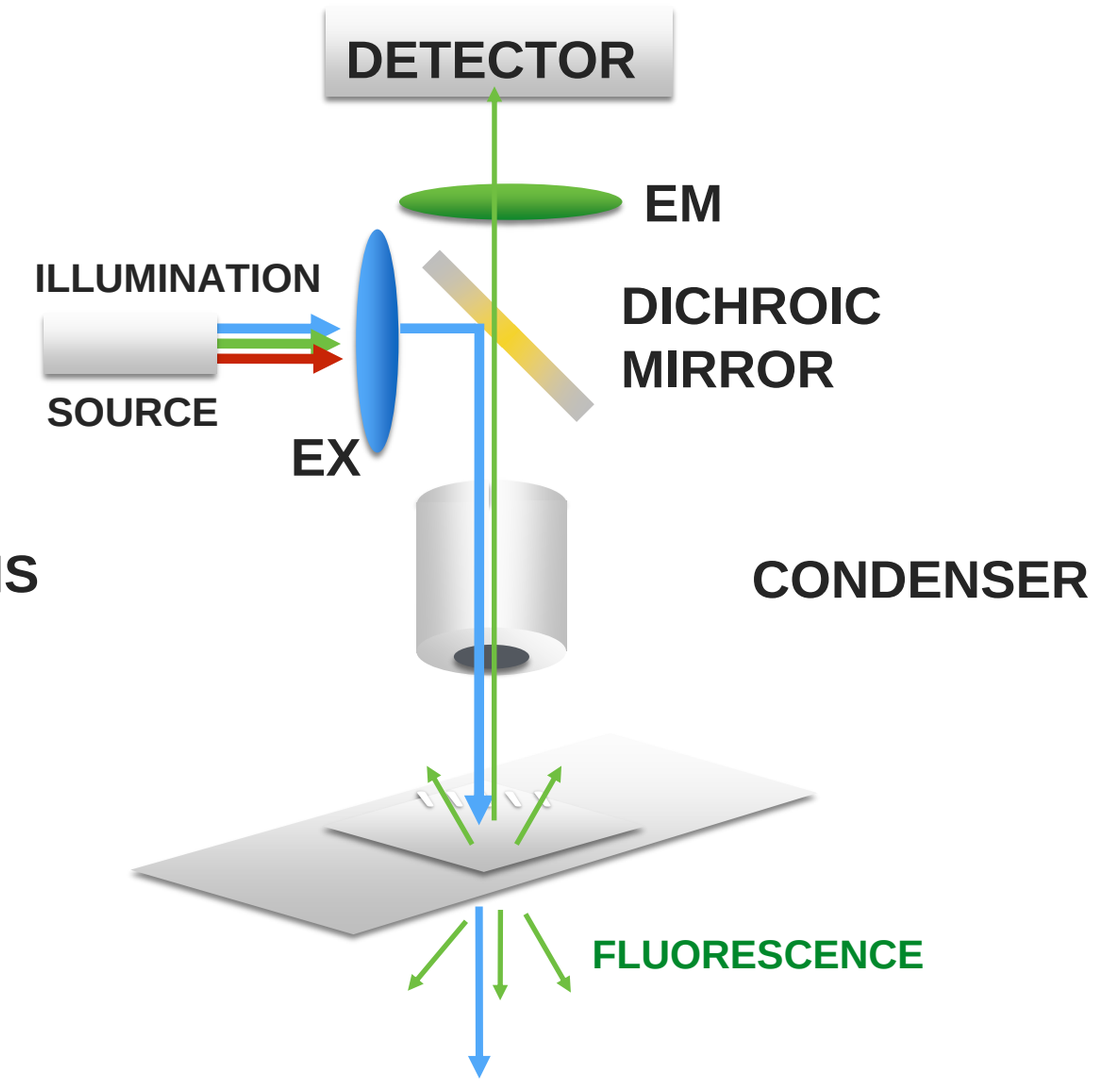
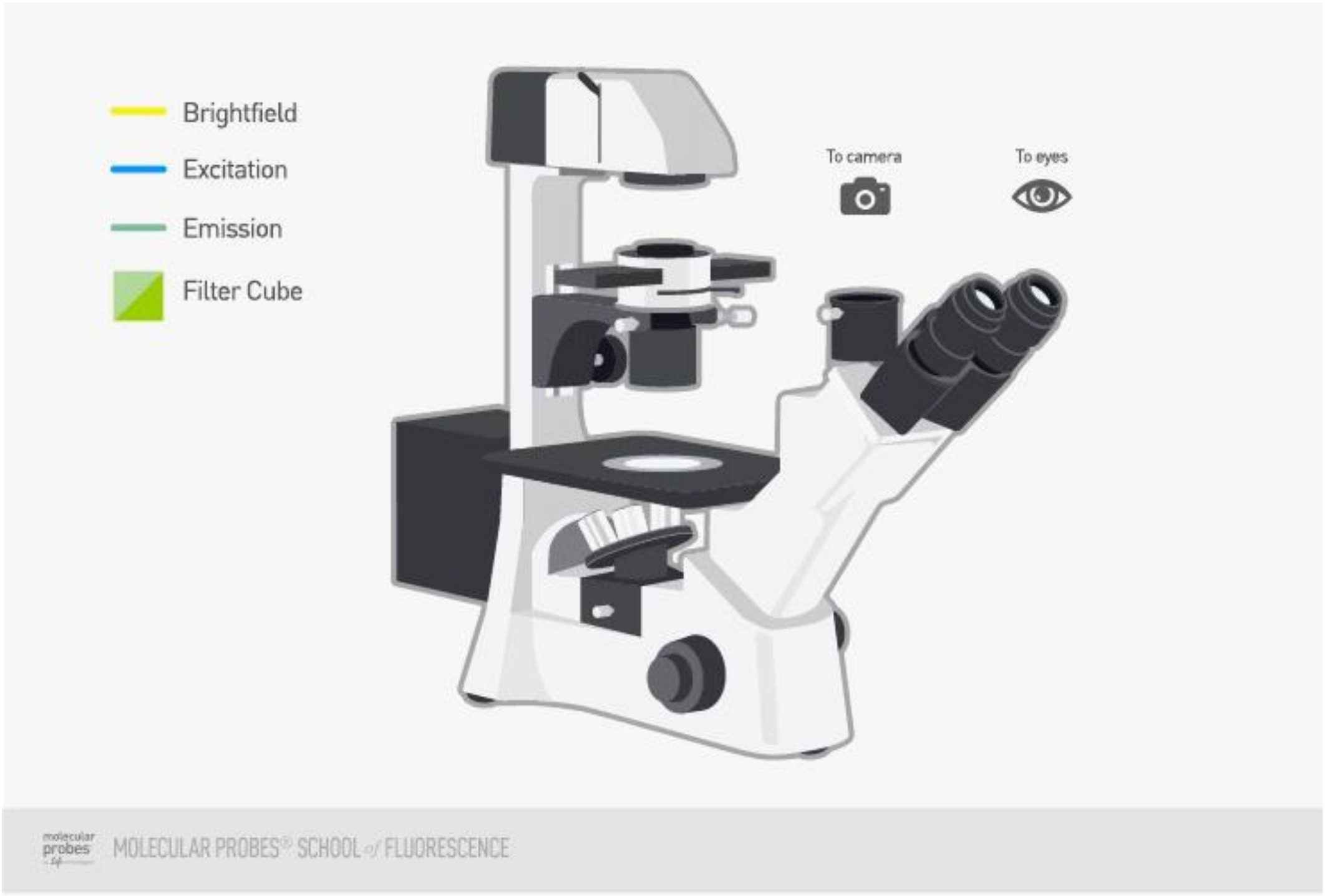
Transmitted light (Brightfield)



Reflected Light (Epifluorescence)



Epifluorescence vs Transillumination light paths (inverted)



Dichroic beamsplitter - at the heart of fluorescence microscopy

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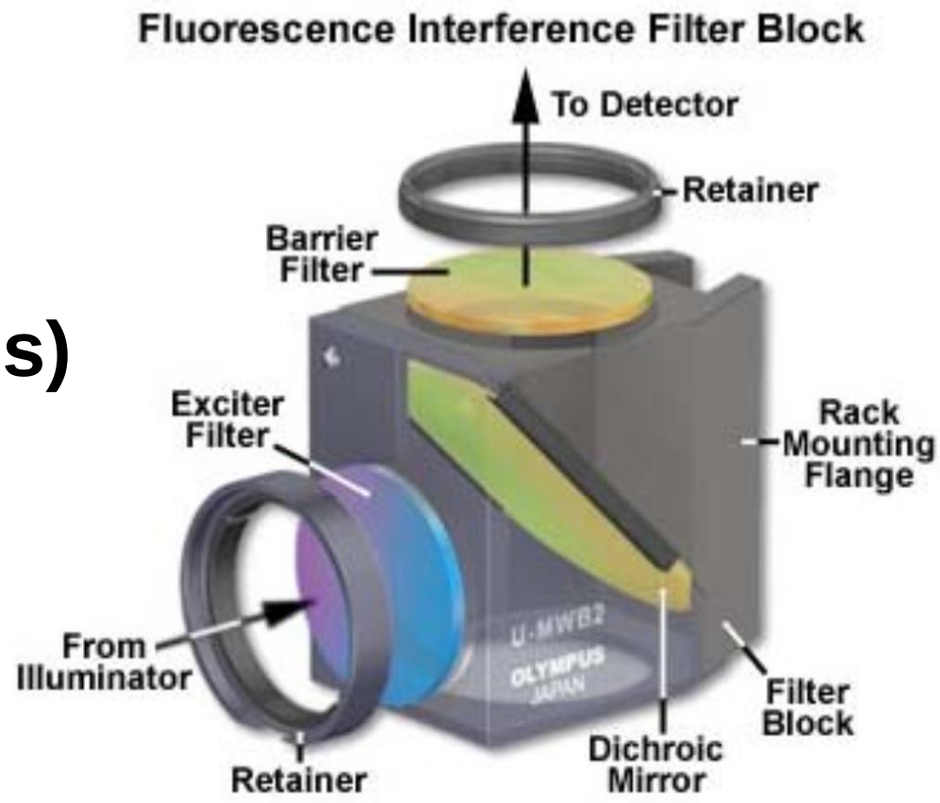
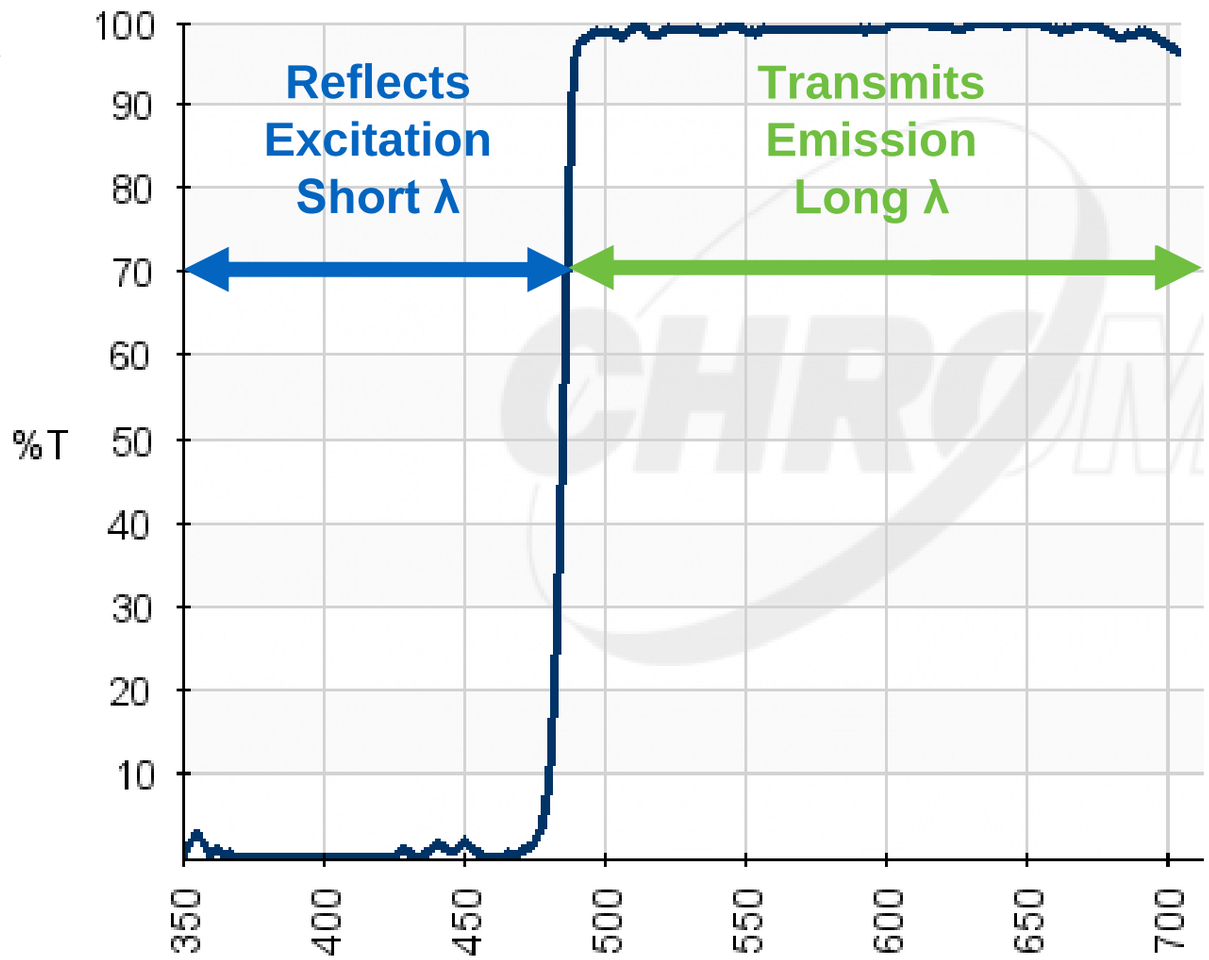
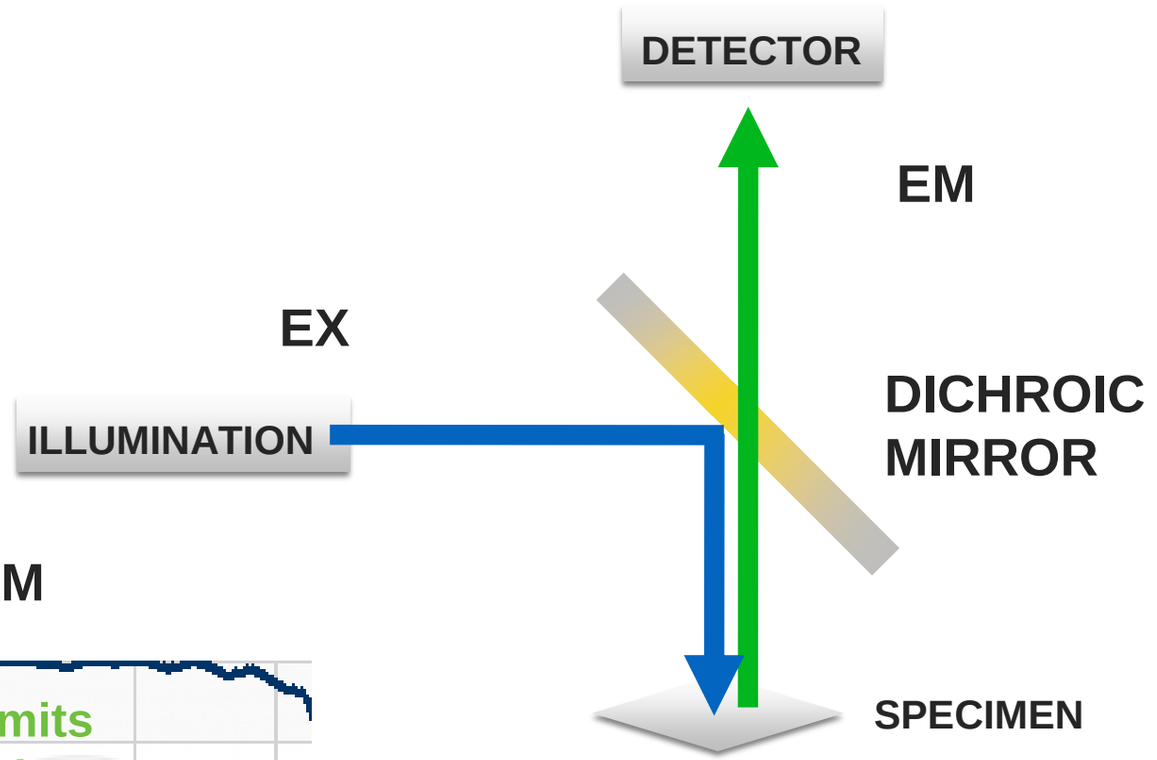
typical Dichroic (spectral properties)



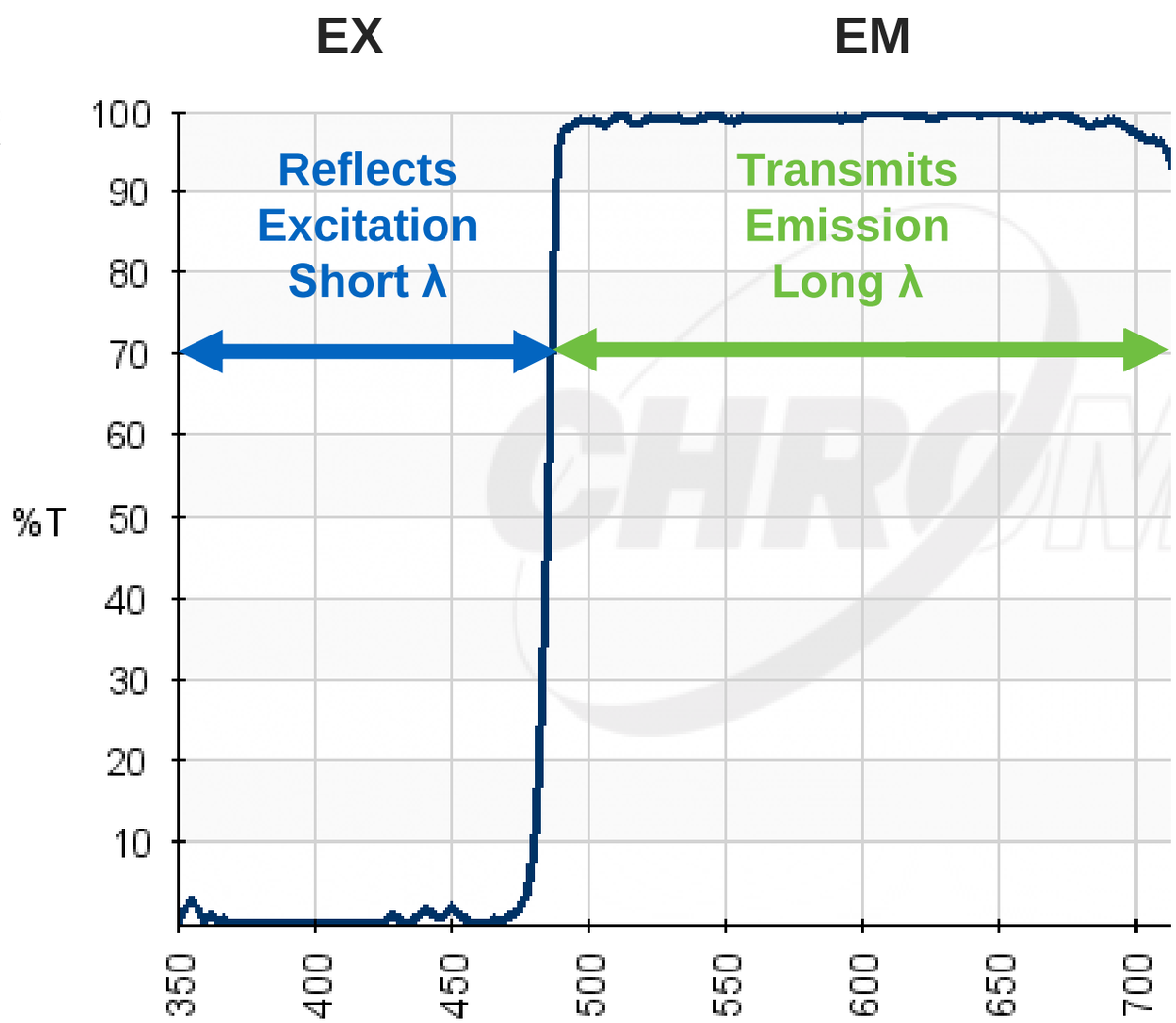
100% T = 0% R



Dichroic beamsplitter - at the heart of fluorescence microscopy



100% T = 0% R



Dichroic beamsplitter - at the heart of fluorescence microscopy

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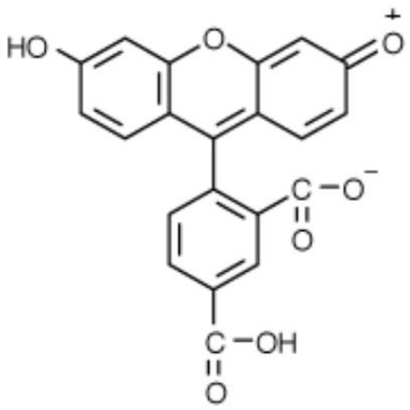
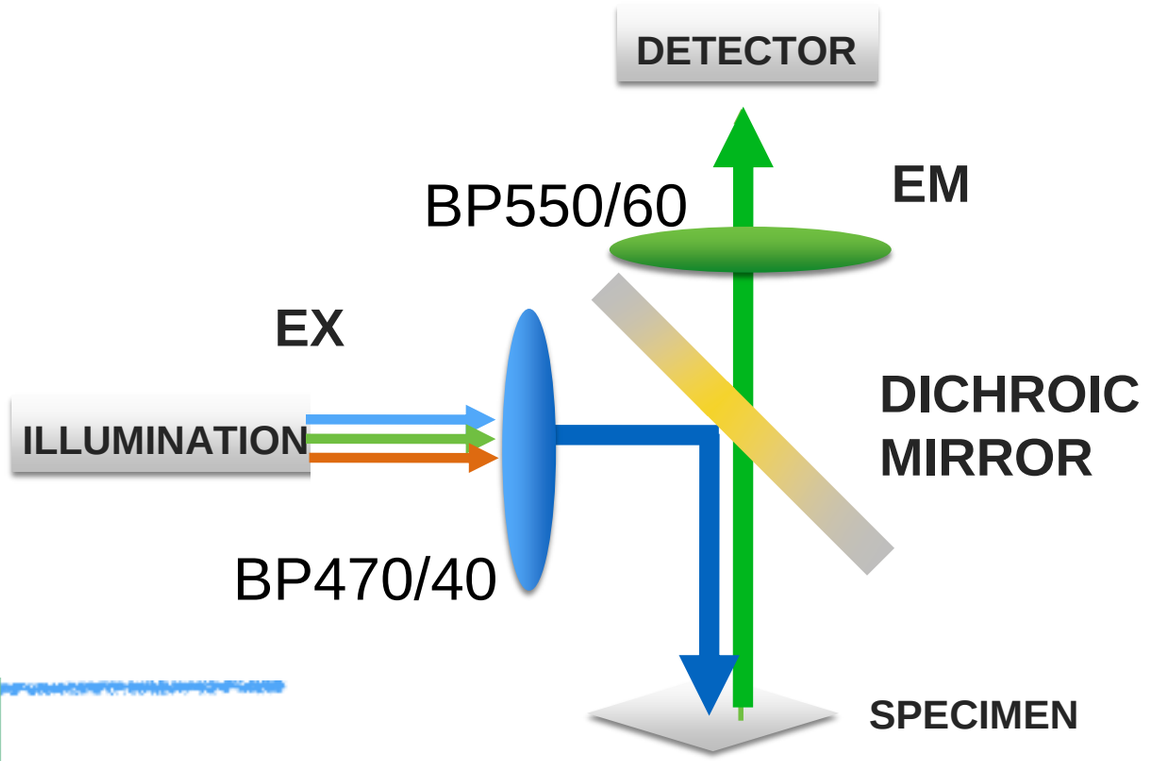
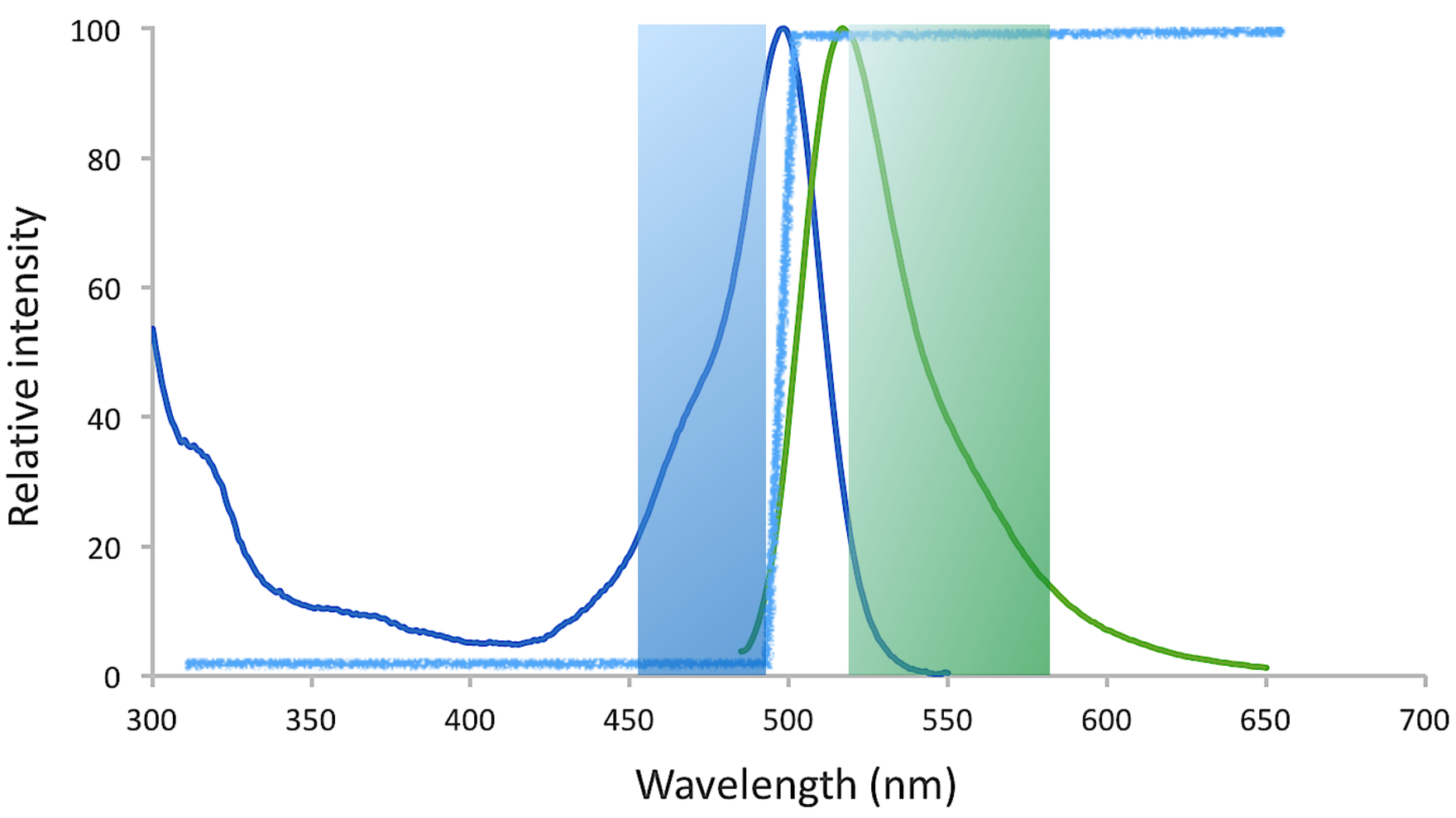
… relate to dye spectrum

Fluorescein (FITC)

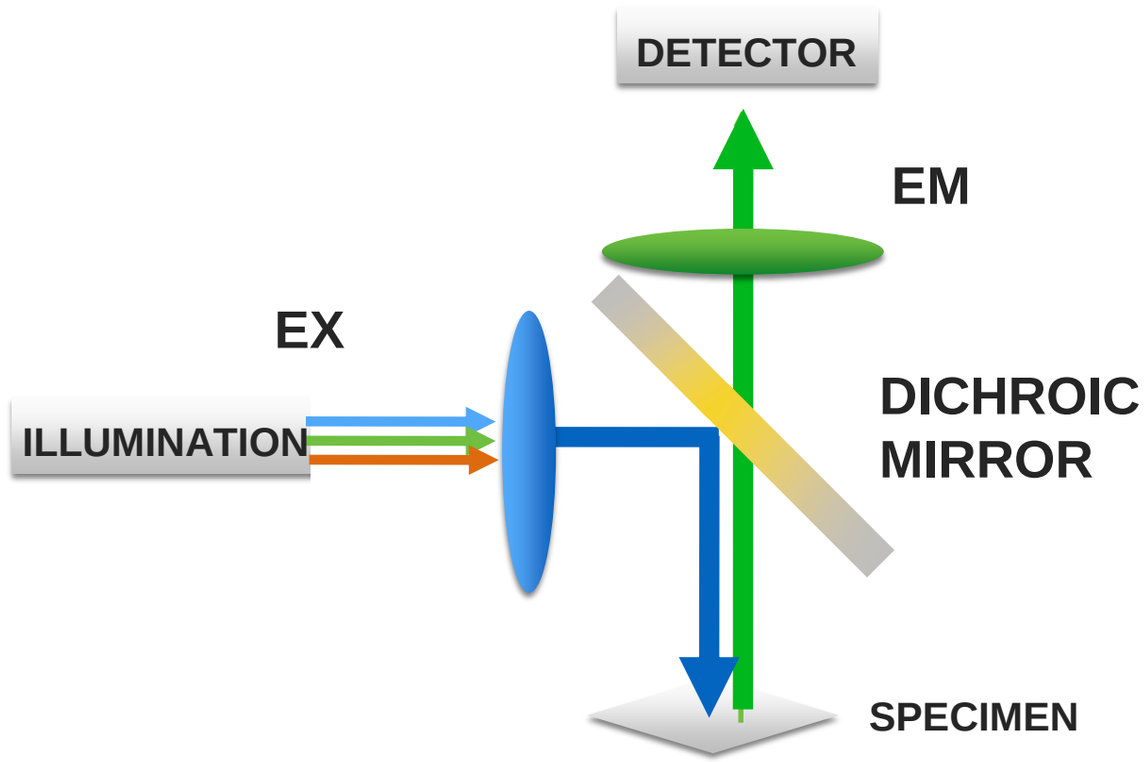






Dichroic beamsplitter - at the heart of fluorescence microscopy

… what about multiplexing…?



http://www.olympusmicro.com/

Dichroic beamsplitter - at the heart of fluorescence microscopy

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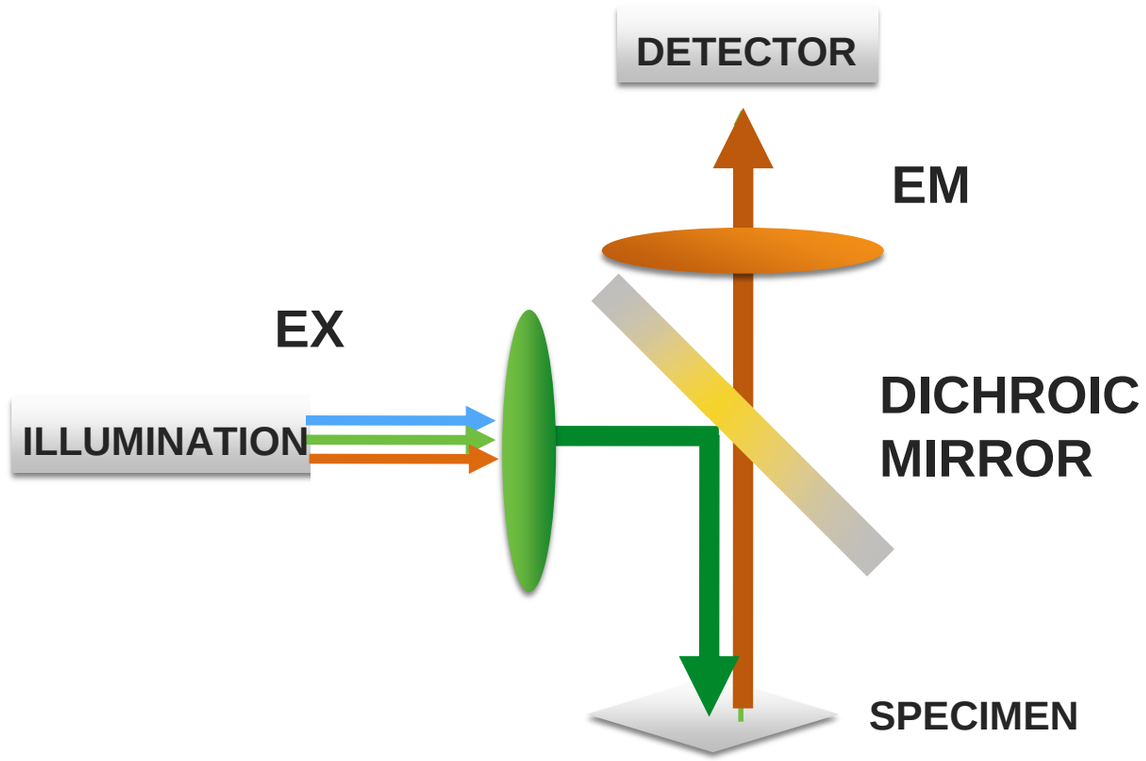

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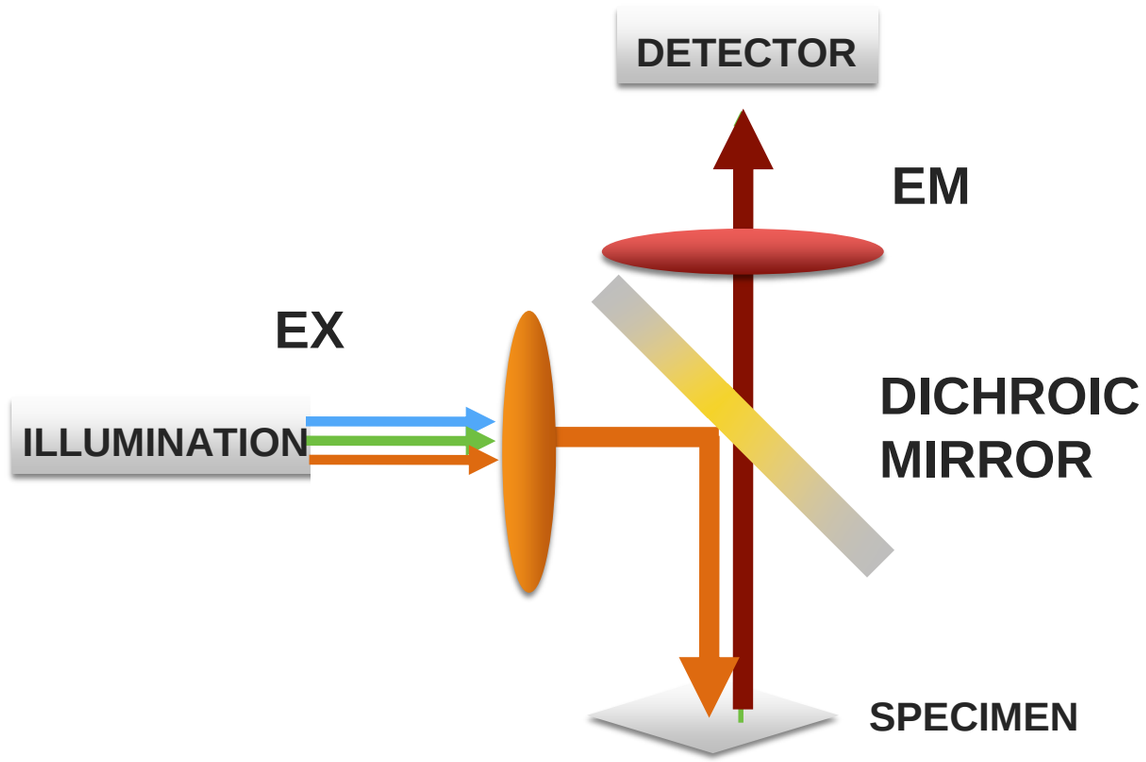
Dichroic beamsplitter - at the heart of fluorescence microscopy

… what about multiplexing…?



http://www.olympusmicro.com/

Dichroic beamsplitter - at the heart of fluorescence microscopy

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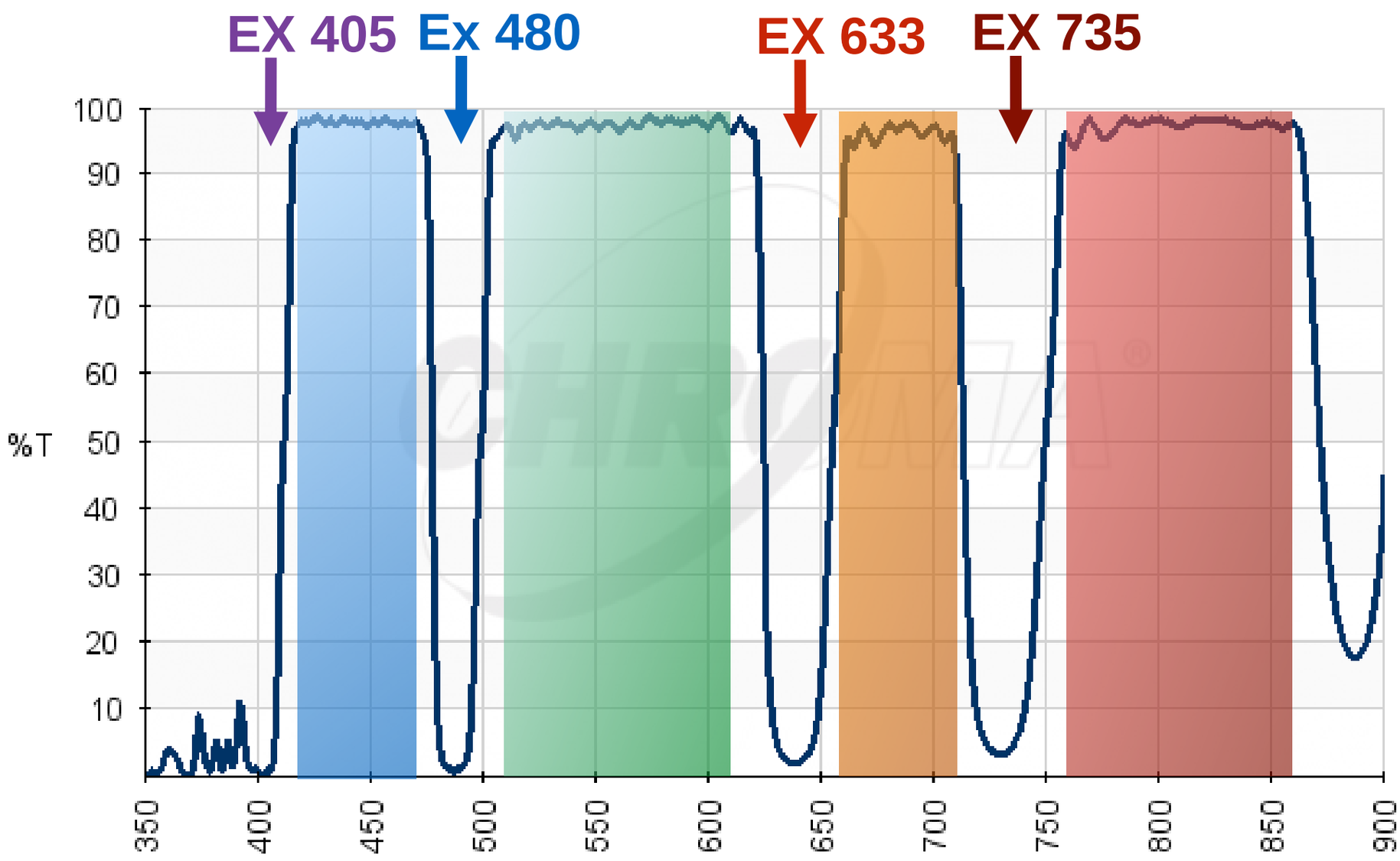
Polychroic



4. Fluorescent light sources

Illumination sources for widefield fluorescence microscopy

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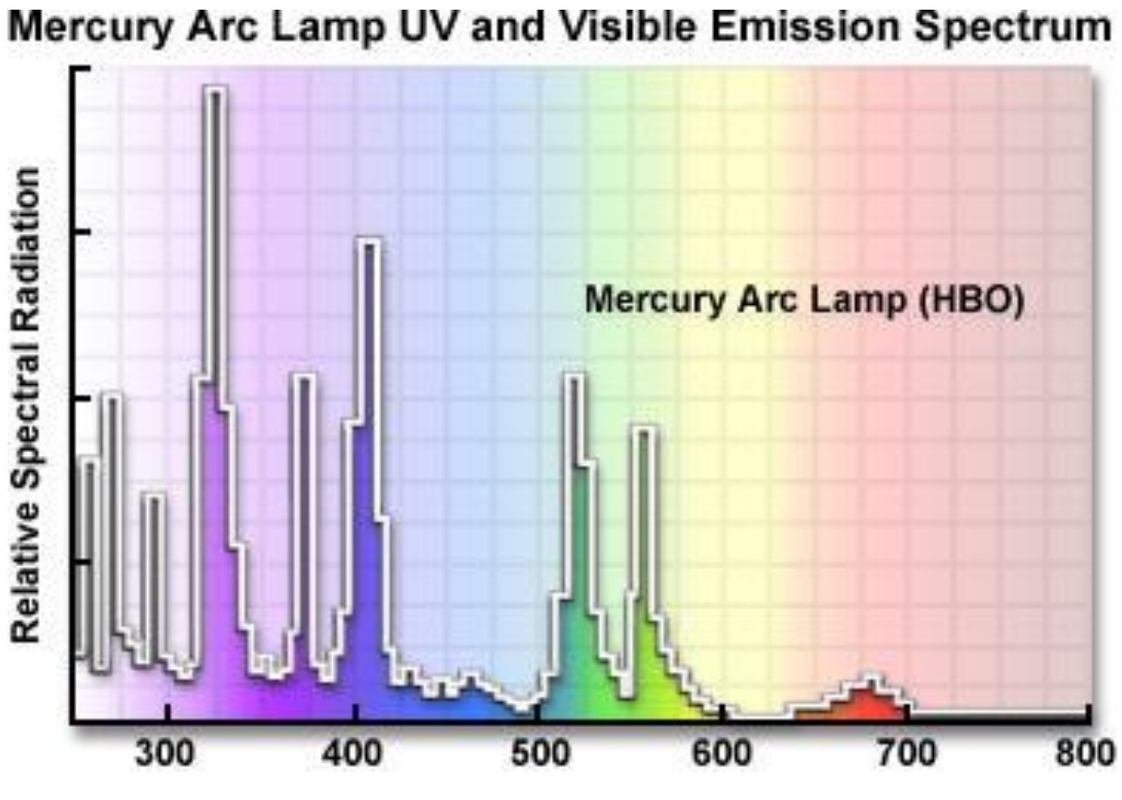
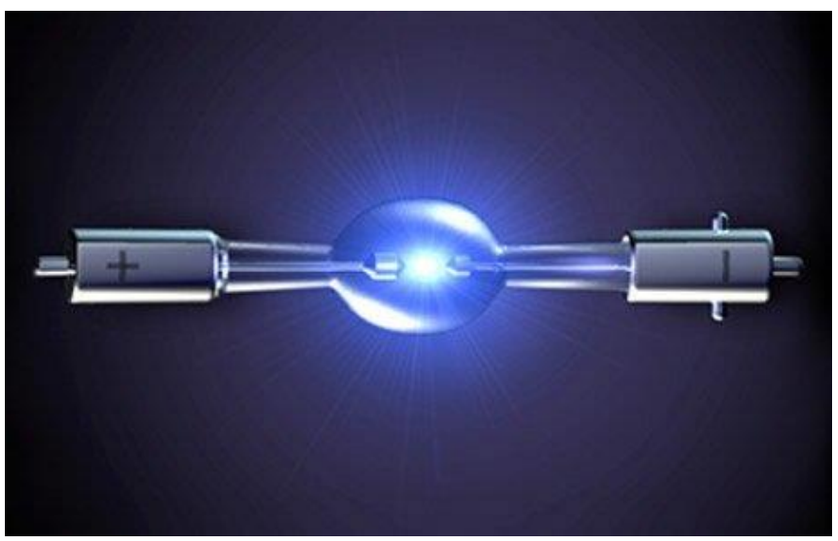
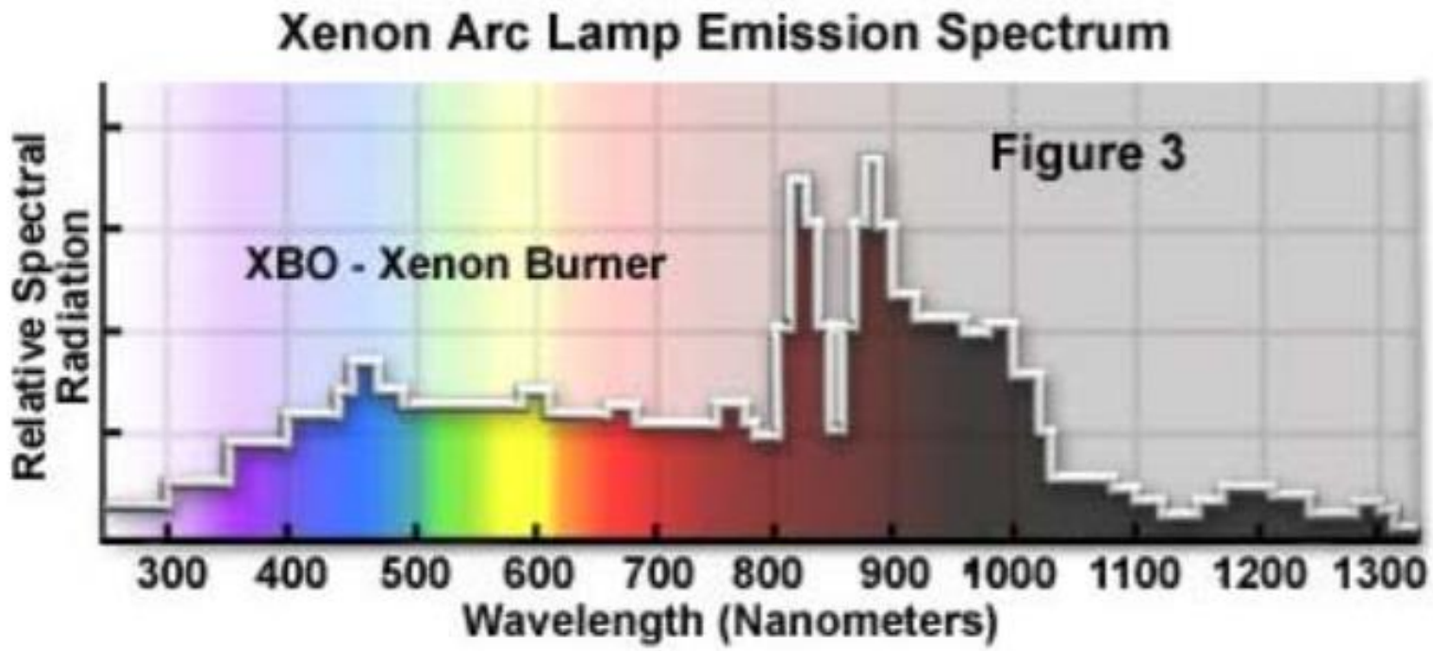
Metal arc lamps

- 200-300h lifespan
- Hazardous
- Generate heat
- *Generally out of use*





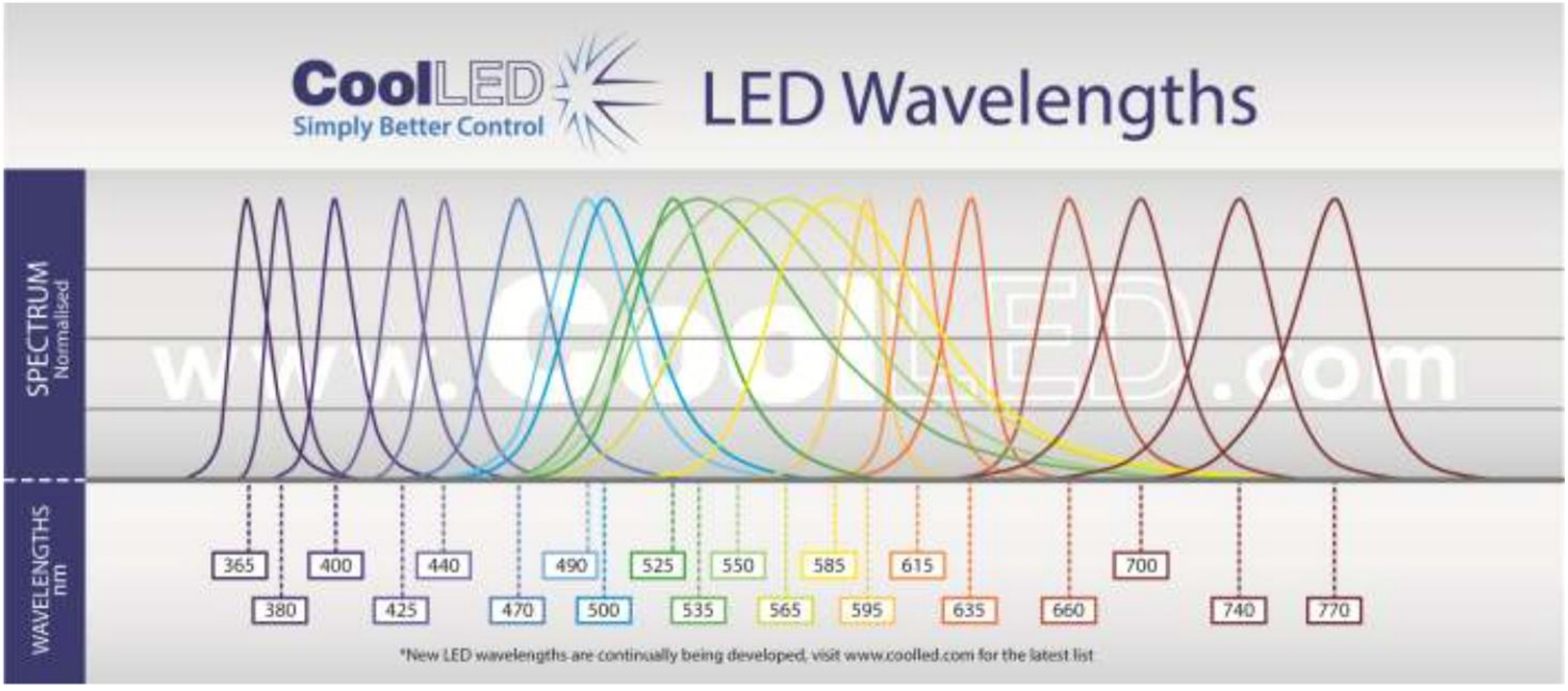


simultaneous excitation of multiple fluorophores over a wide wavelength range

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

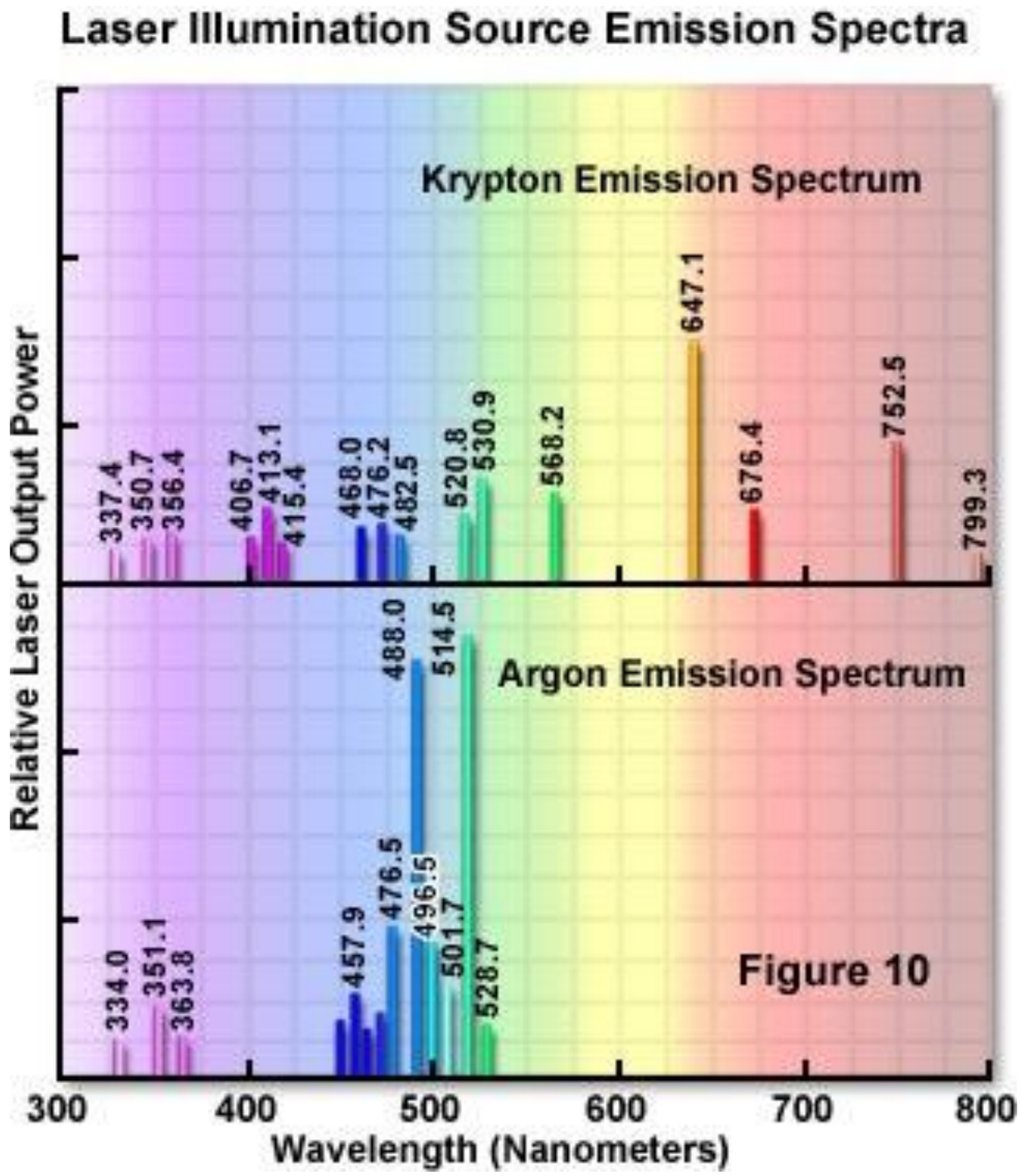
Illumination sources for widefield fluorescence microscopy

*** Diode lasers * Solid State lasers**

*** Gas lasers**

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

5. Point Spread Functions

PSF (Point Spread Function) in fluorescence

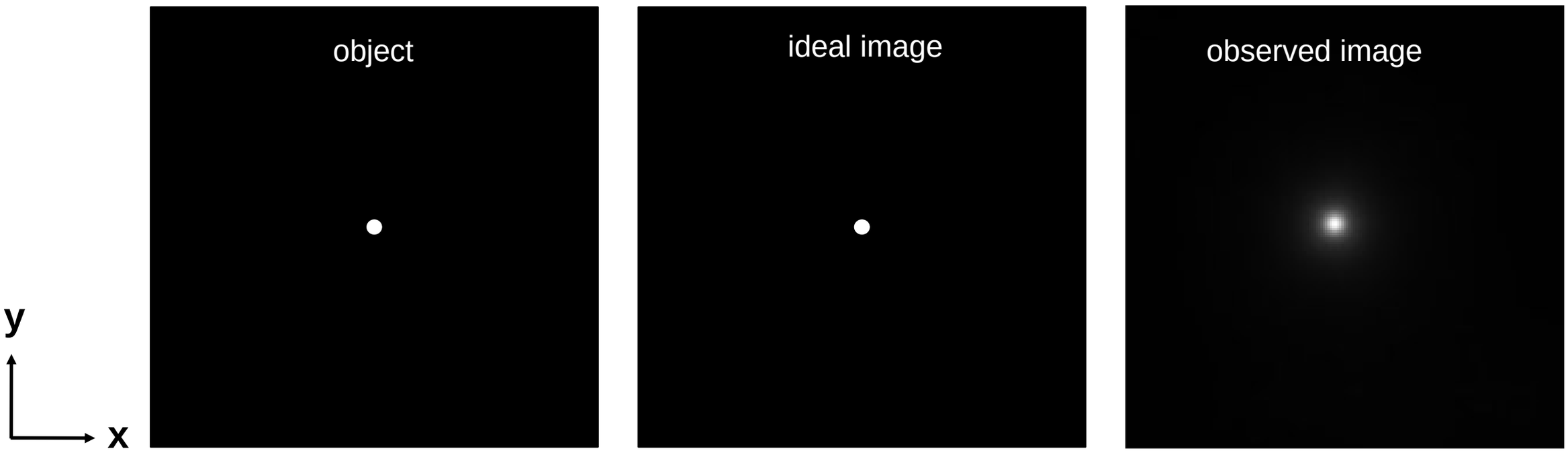
PSF is a measure of the microscope response to a point source of light

Why is this important?

- Microscope performance
- x, y, z information
- Image quality
- Alignment
- Optical resolution

PSF (Point Spread Function) in fluorescence

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

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

PSF (Point Spread Function) in fluorescence

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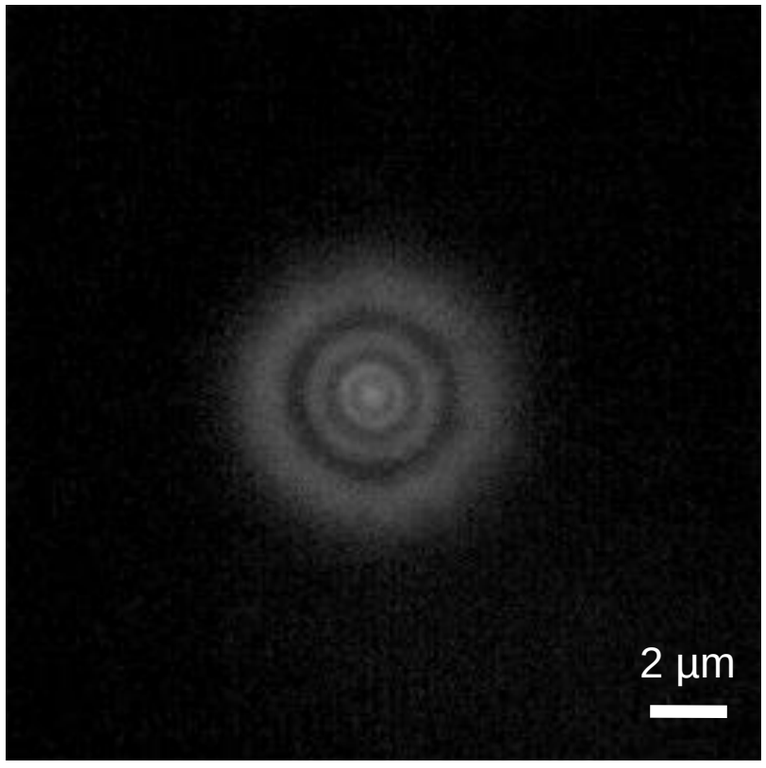
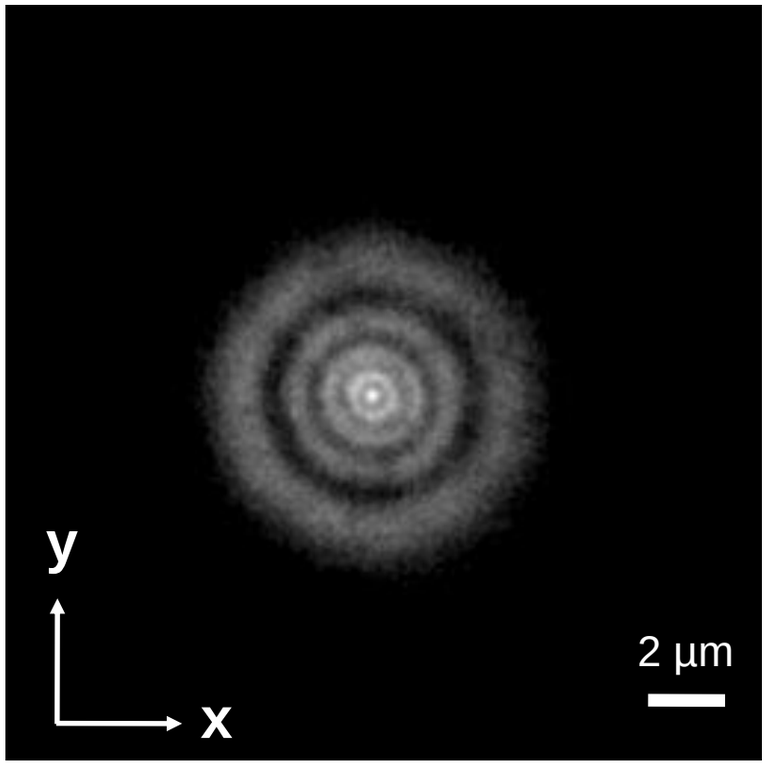
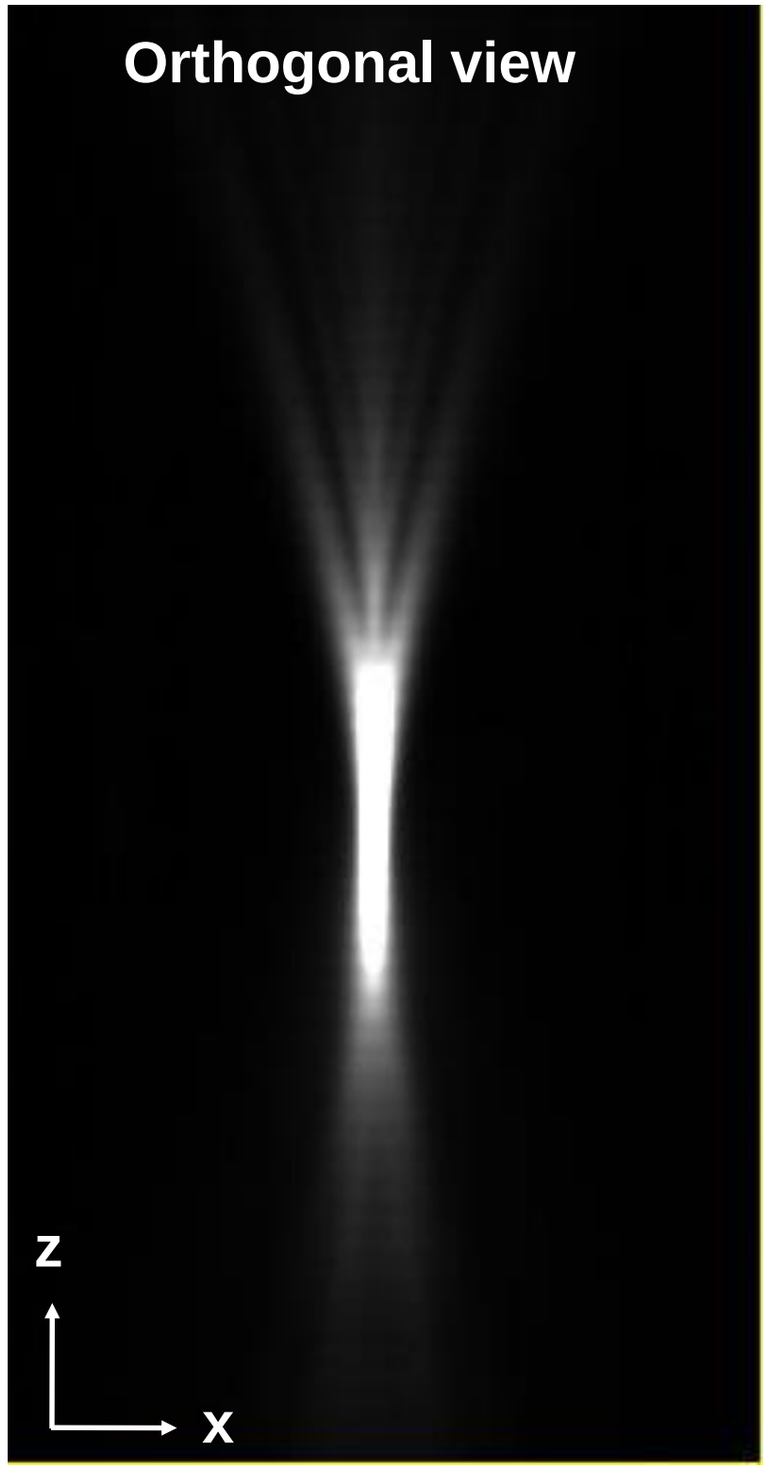
red fluorescent 170 nm bead







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 (Point Spread Function) in fluorescence

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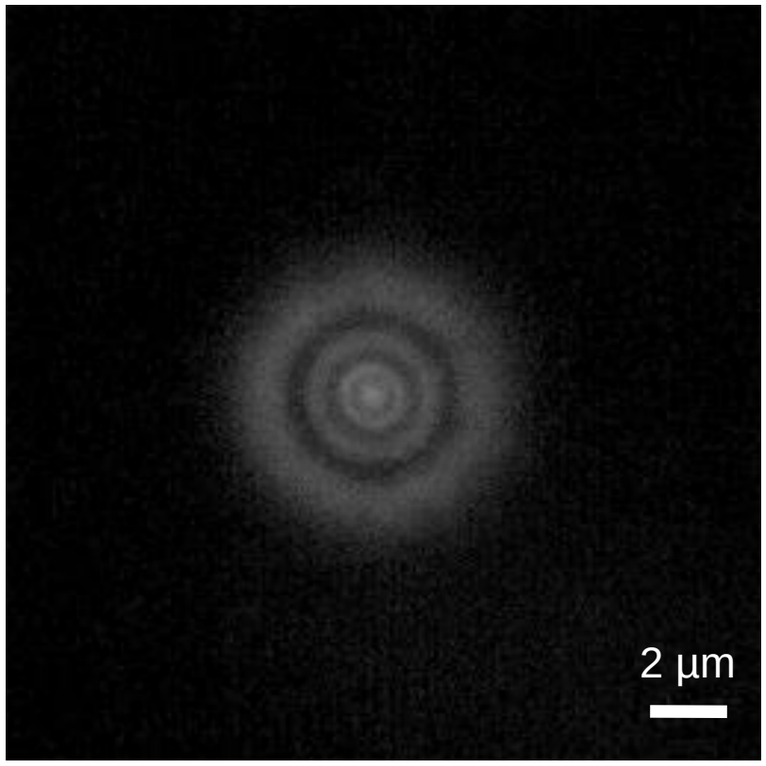
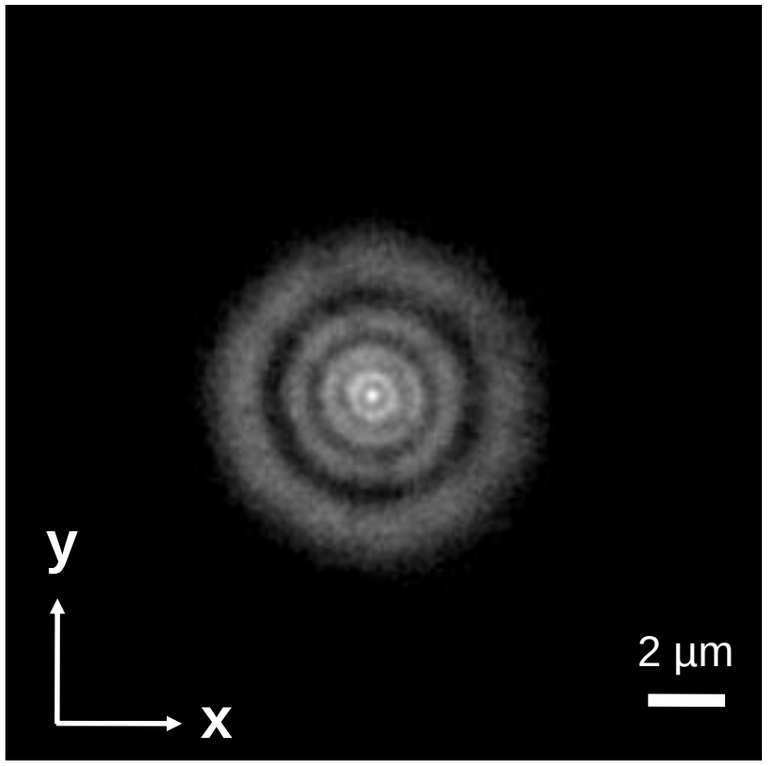
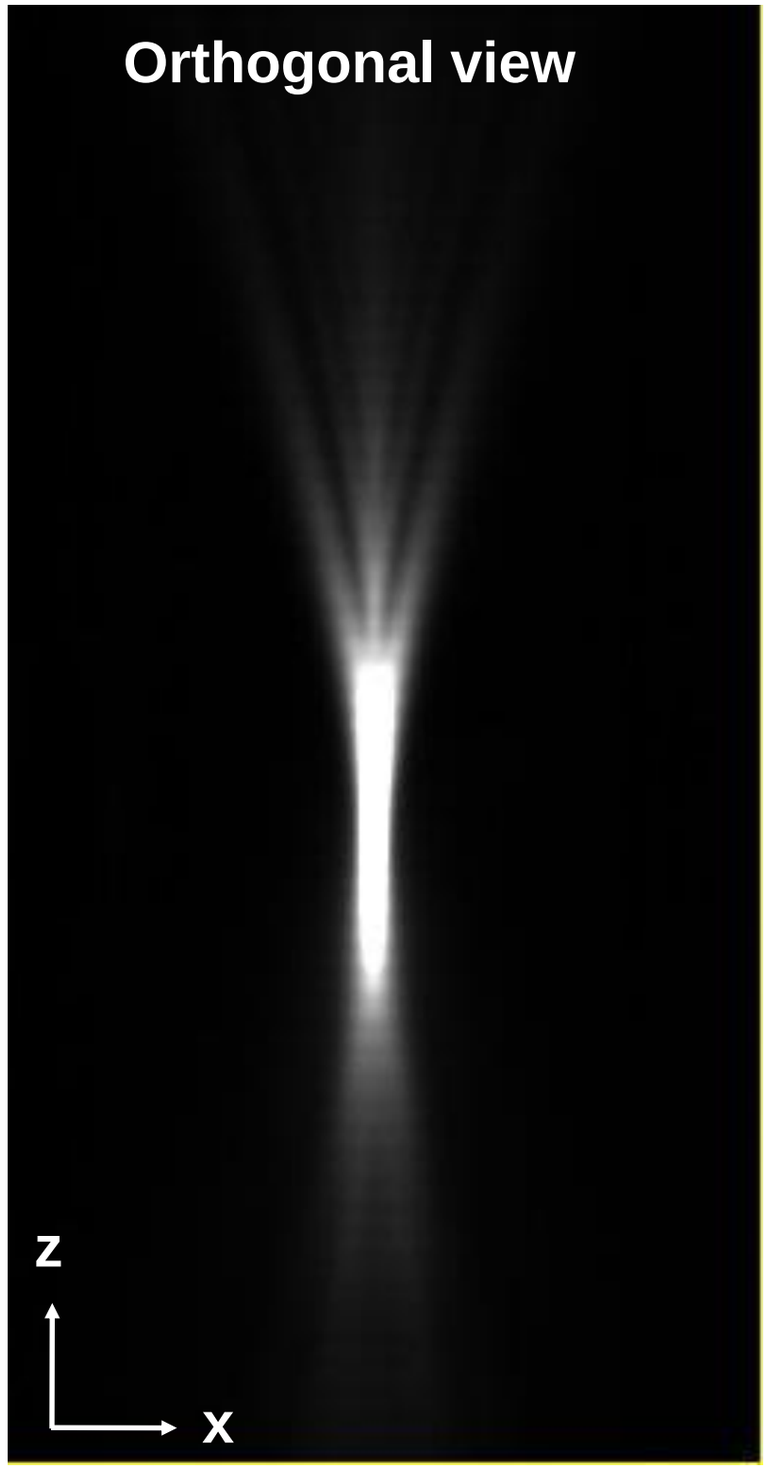
red fluorescent 100 nm bead





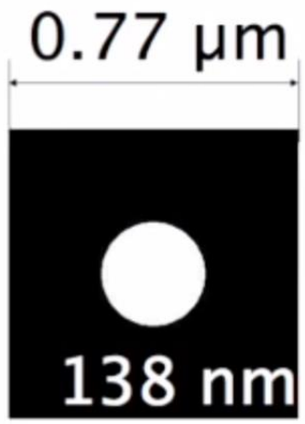


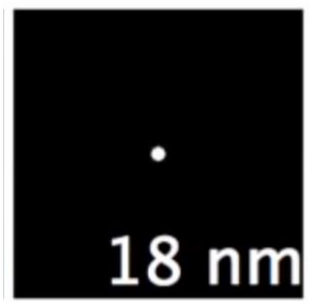
What can we observe?

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

ALIGNMENT, SPHERICAL ABERRATIONS, MISMATCH REFRACTIVE INDEX

PSF of a small object





1.4NA objective

$\lambda = 0.48\ \mu m$

Image from Jeff Lichtman

6. Fixation for fluorescence microscopy

What is fixation?

- Fixation is the preservation of the state or structure of a biological sample
- It can be achieved a number of ways, including:

  Chemical cross-linking

  UV cross-linking

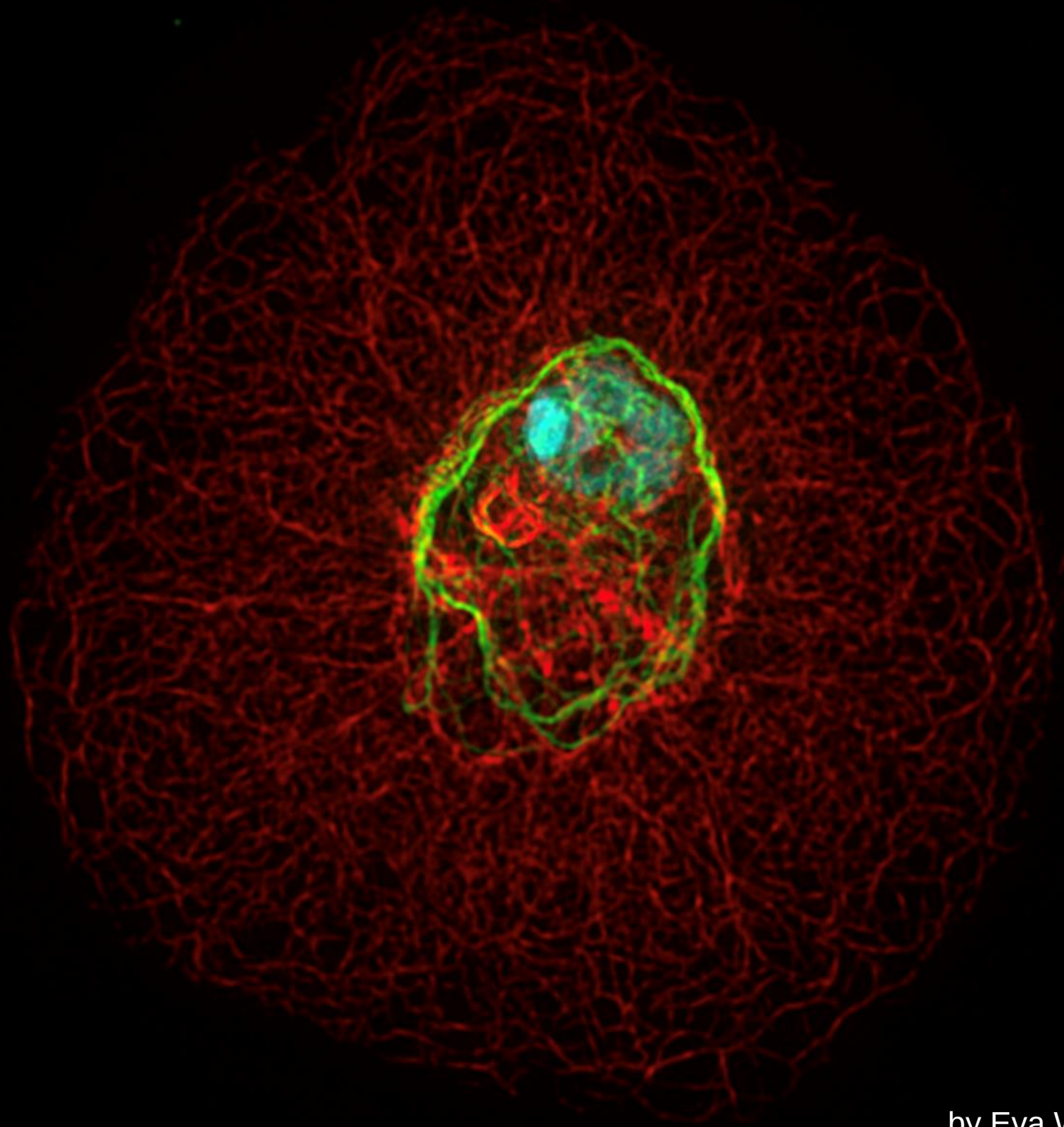
  Dehydration

Why fix biological samples?

1. Convenience
2. Throughput
3. Widely applicable molecular labeling:
   - Immunofluorescence (cells or tissue)
   - Fluorescence *in situ* hybridization (FISH)
4. Ease of multiplexing bright, relatively stable fluorescent labels

Typical Immunocytochemistry Protocol

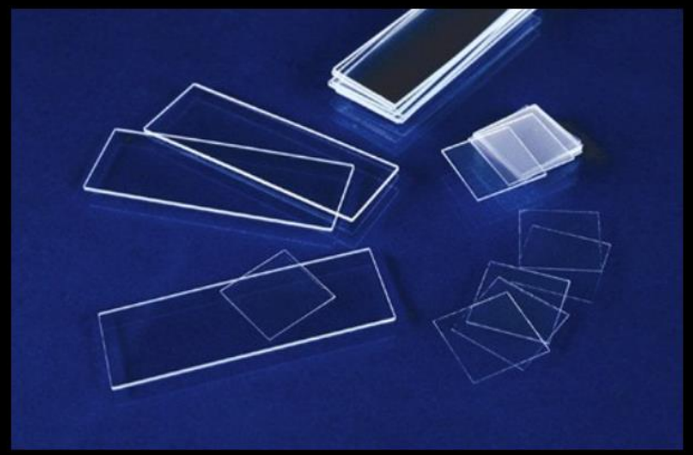
Fixation
Permeabilisation
Washes
Blocking
1° antibody
Washes
2° antibody
Washes
Mounting

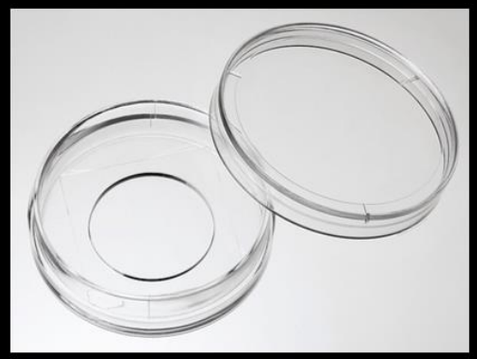


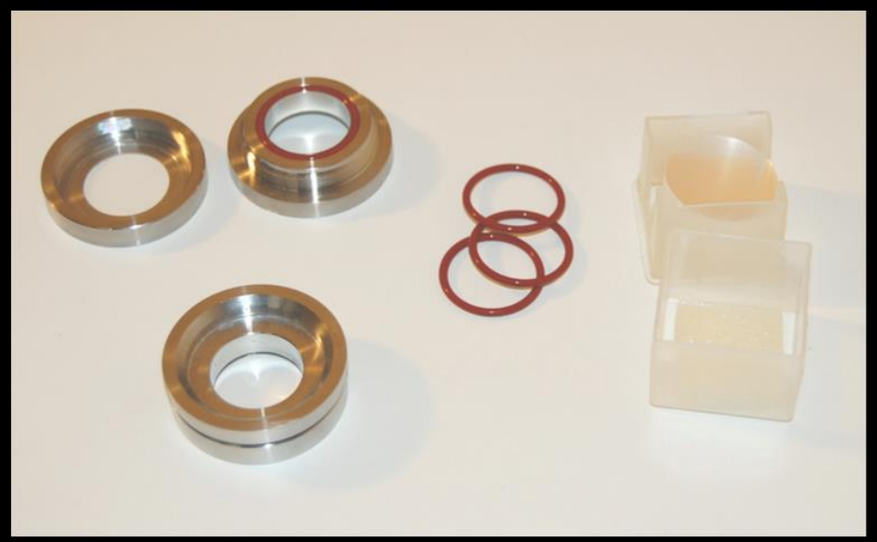
by Eva Wegel

Immobilising the specimen

Sample holder must be suitable for imaging





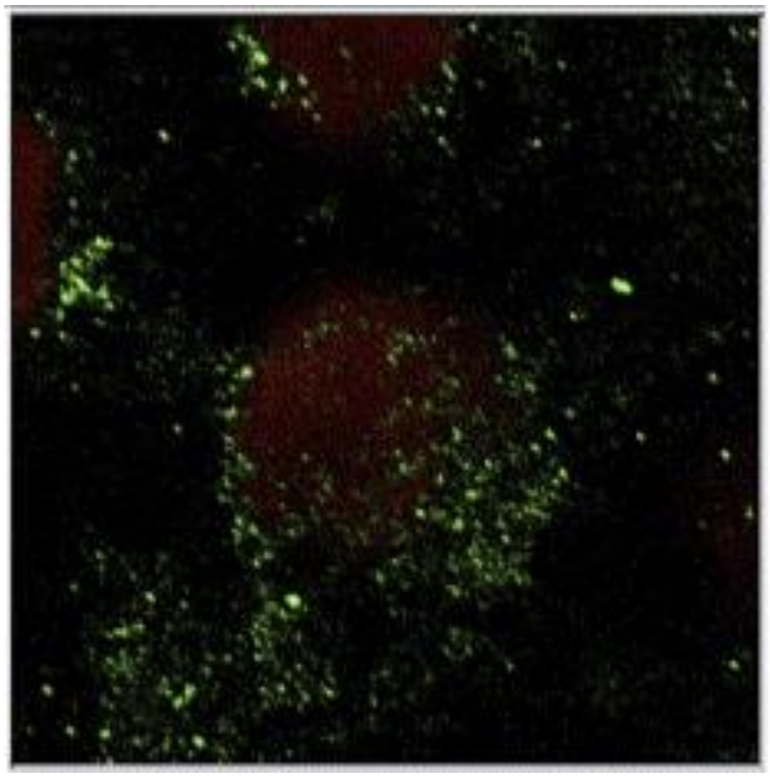


by Esther Garcia

Types of Fixation

Denaturing fixation:

Cold methanol

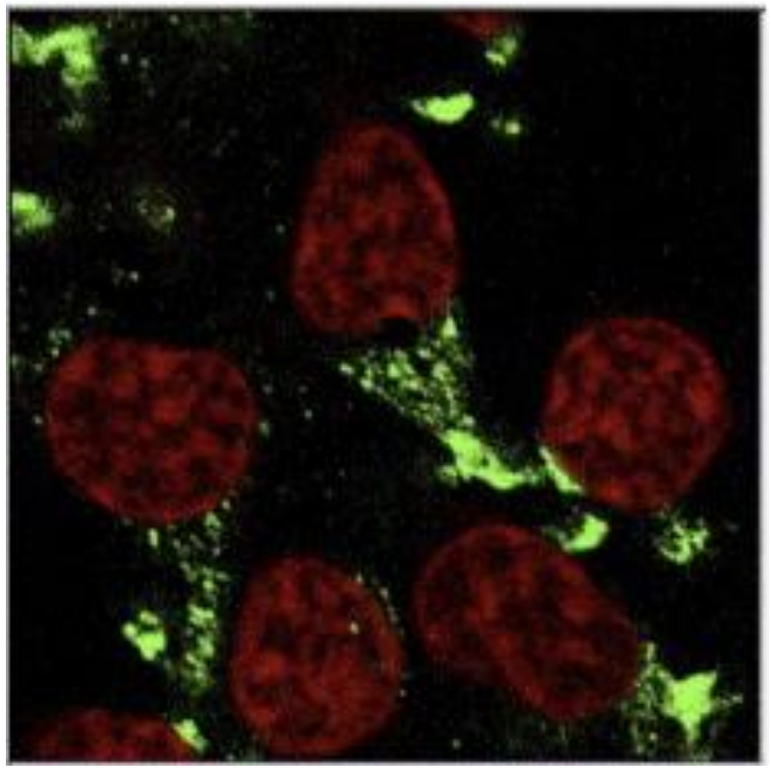


destroys 3D protein structure

dissolves lipids into micelles

Cross-linking fixation:

Formaldehyde (PFA)



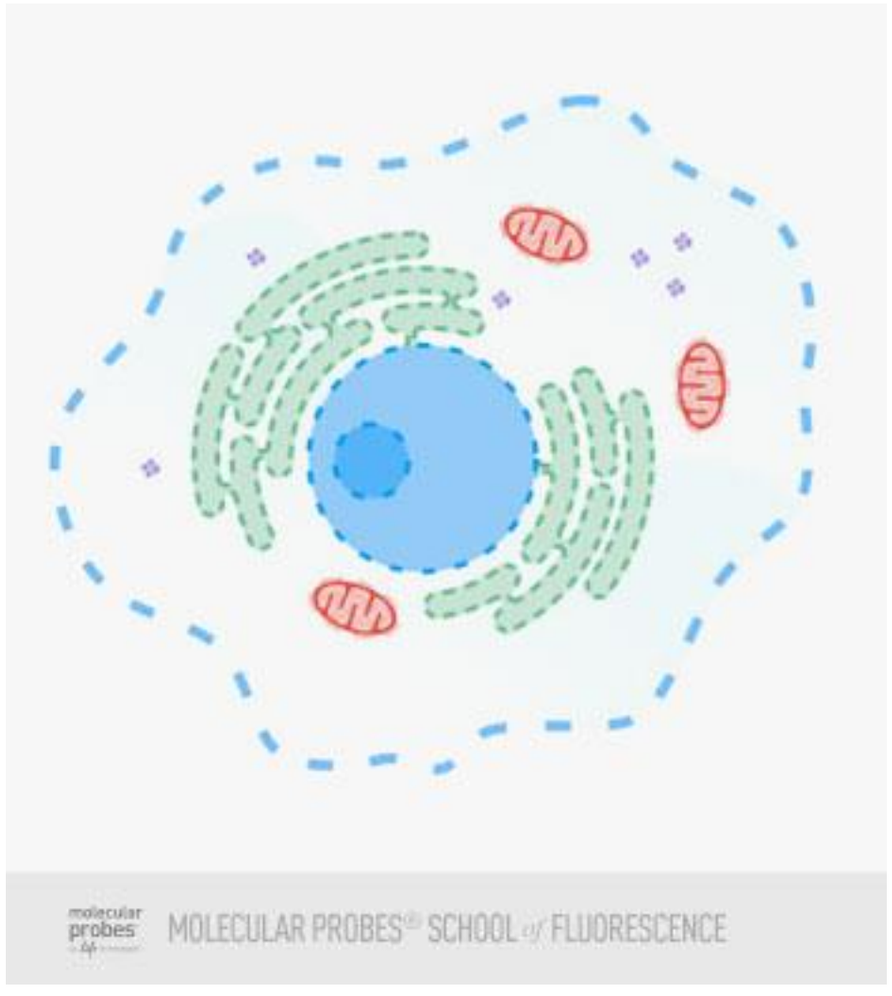
binds to proteins and some lipids,

but not RNA, DNA or most sugars

Sometimes a combination of both is necessary …

Permeabilisation

done by removing some lipids with detergents



Tween 20

Triton X-100

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

Reduction of nonspecific “background” 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)

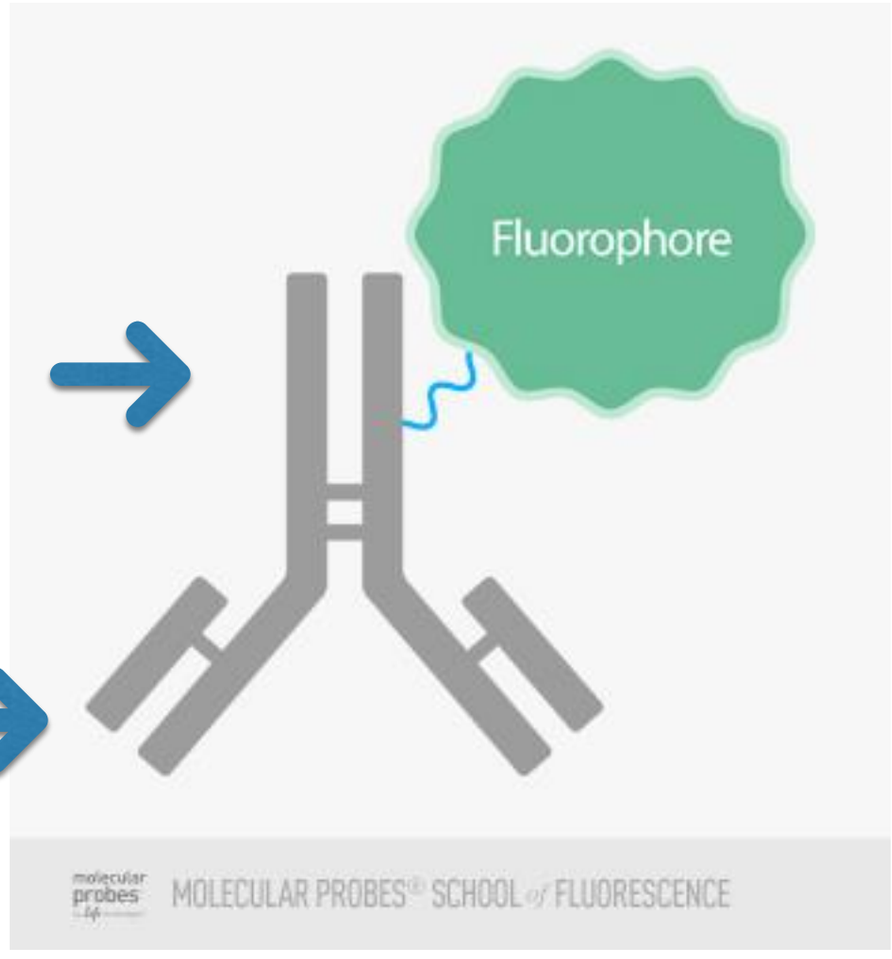
Antibody (large Y-shaped protein called immunoglobulin)

produced by the immune system, found in the blood or other body fluids of **vertebrates**.

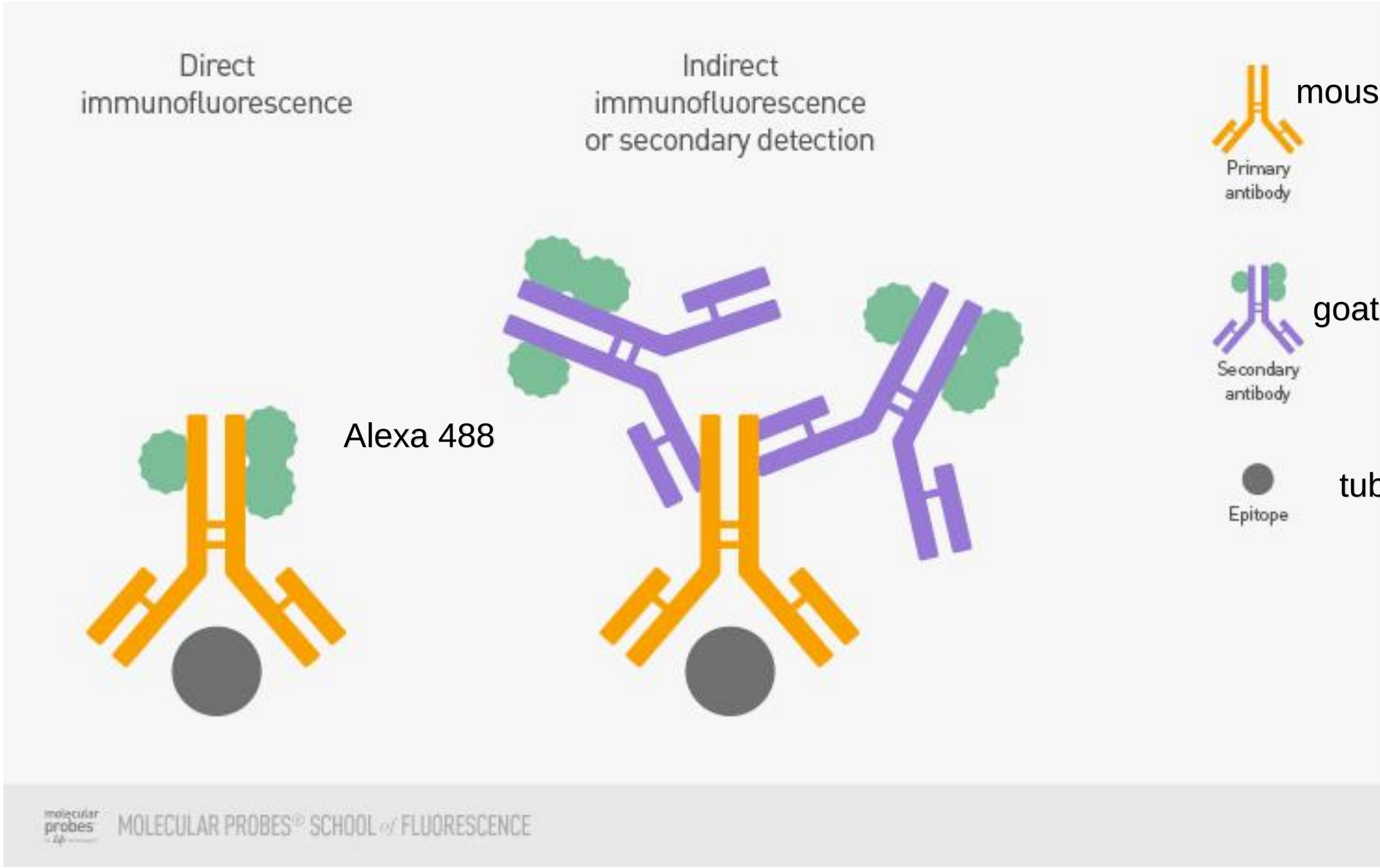
The **antibody** recognises unique parts of the foreign target called an **antigen**.

Conserved among species

VARIABLE
Antigen binding site



Immunolabeling (antibodies)



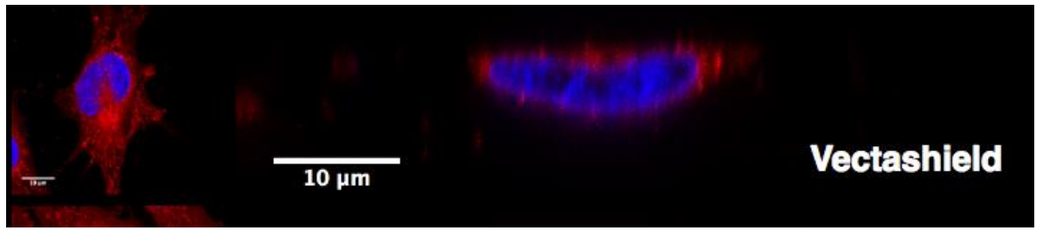
mouse anti-tubulin

goat anti-mouse

tubulin

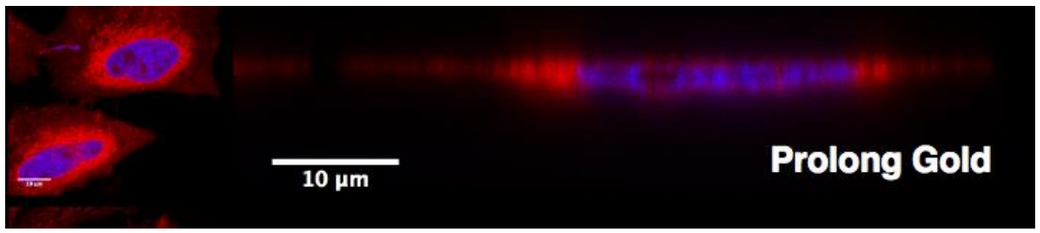
Mounting

- Non-hardening

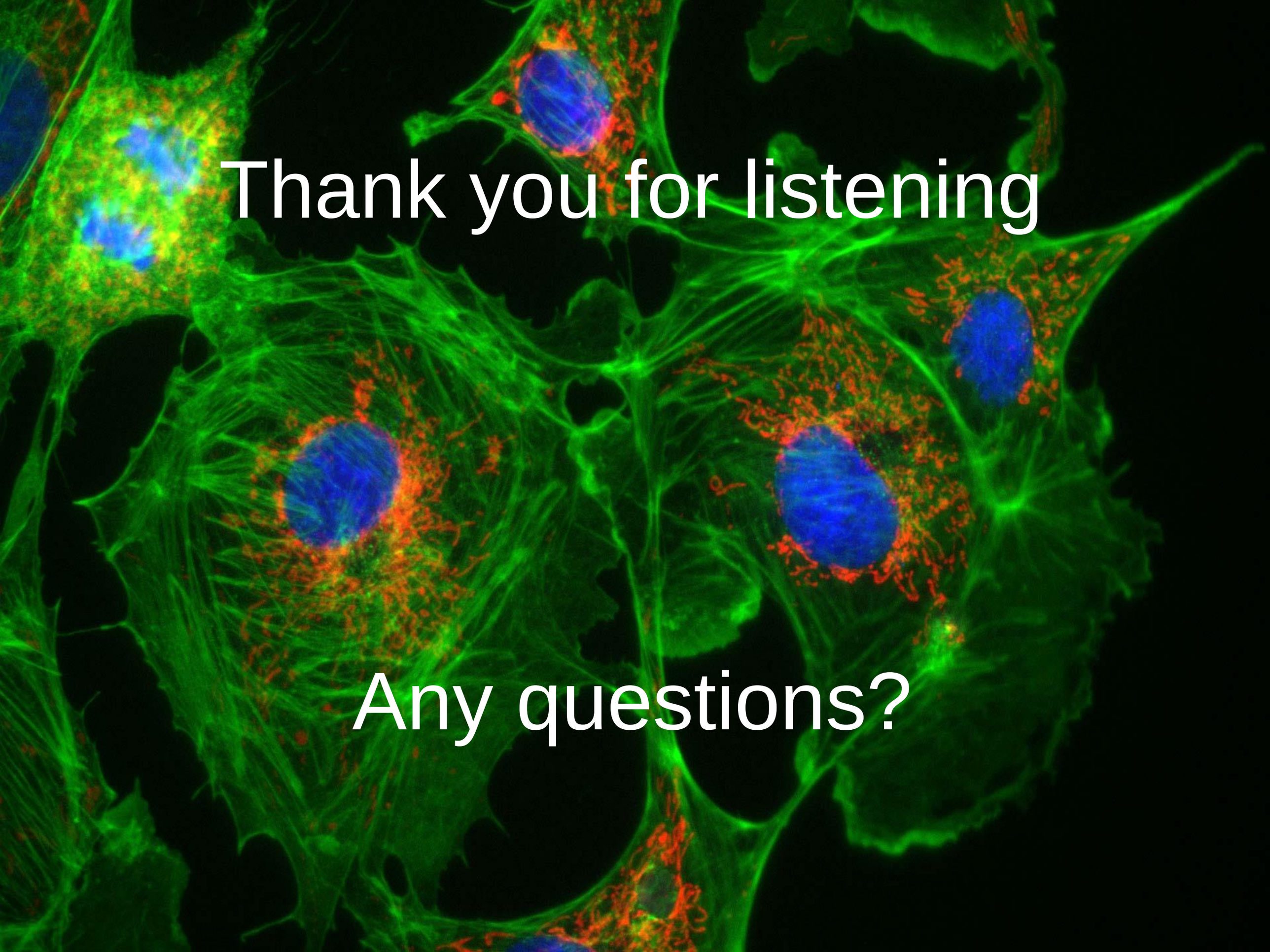


  - Short-term storage (days to few weeks)
  - **Dabco, Glycerol, Vectashield** (antifading agent, but does not work with FarRed dyes)

- Hardening



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



Thank you for listening

Any questions?