Micron Advanced Light Microscopy Course Introduction to Microscopy 2016

Understanding and Applying Fluorescence Microscopy

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University of Oxford

Fluorescence Microscopy:

- What is fluorescence?
- Why fluorescence?
- Principle and components of the fluorescence microscope
- Fluorescent light sources for basic wide field
- Fluorescence PSFs and OTFs
- Fixation for light microscopy

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

What is Fluorescence?

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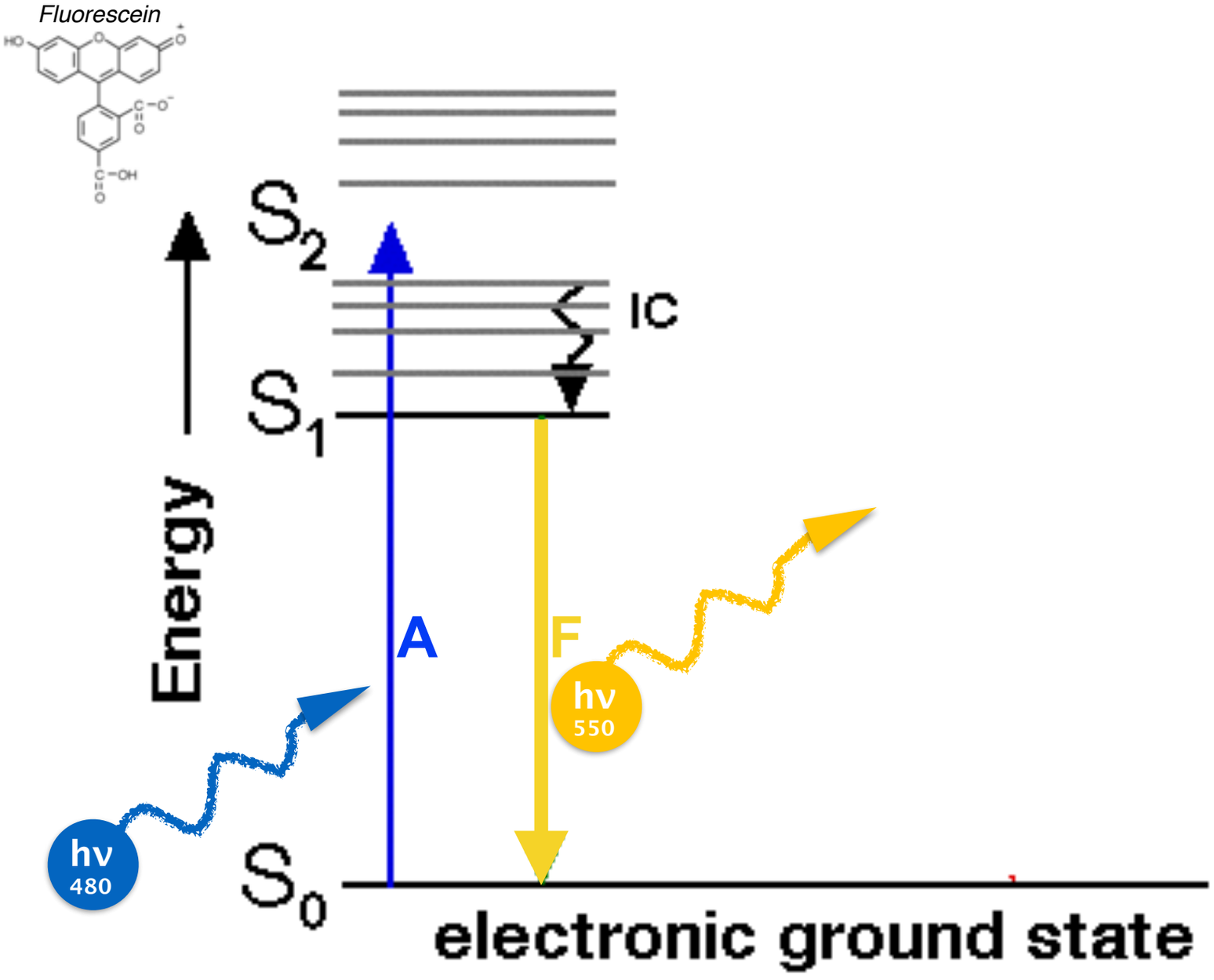
Fluorescein



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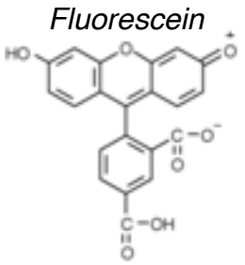
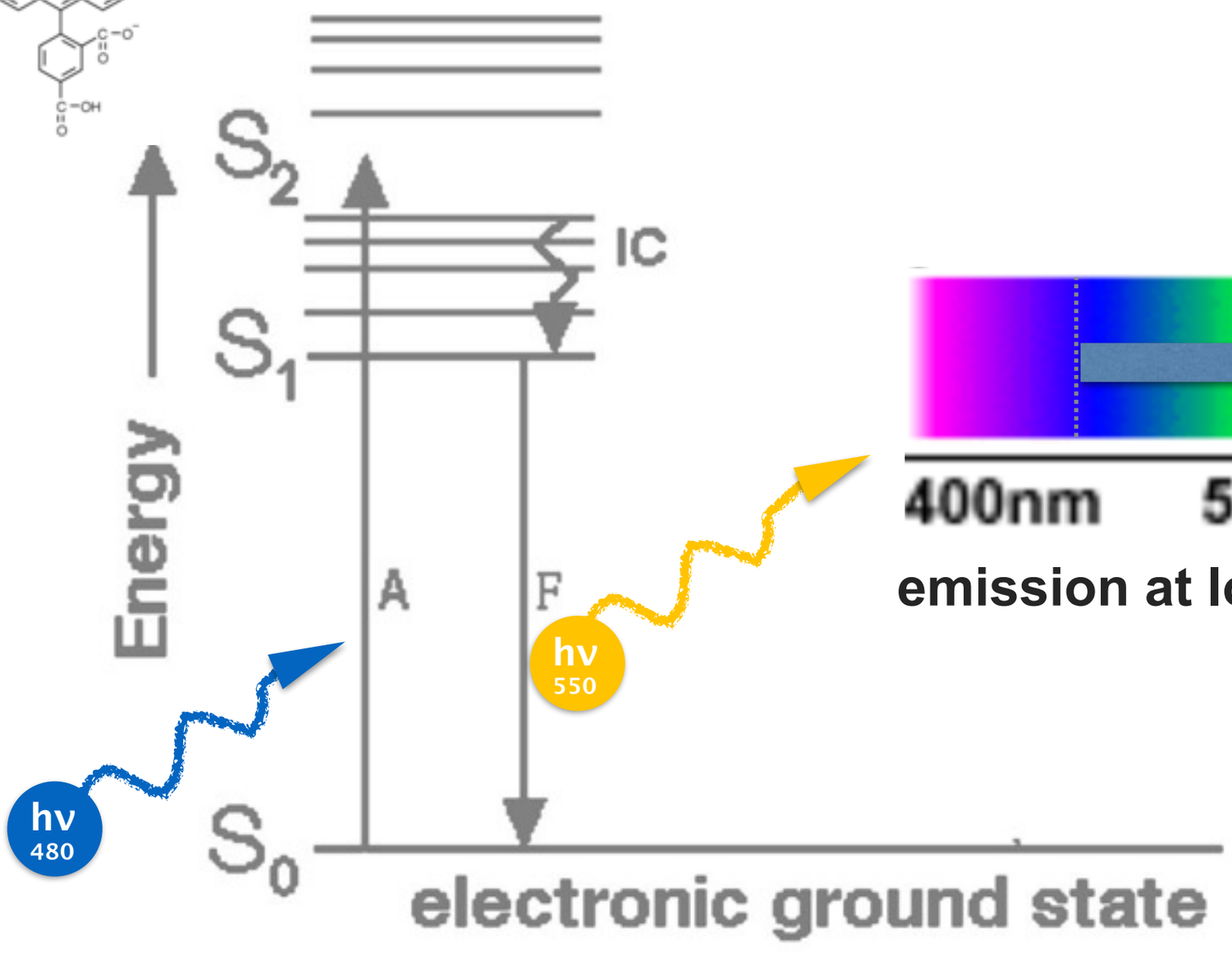
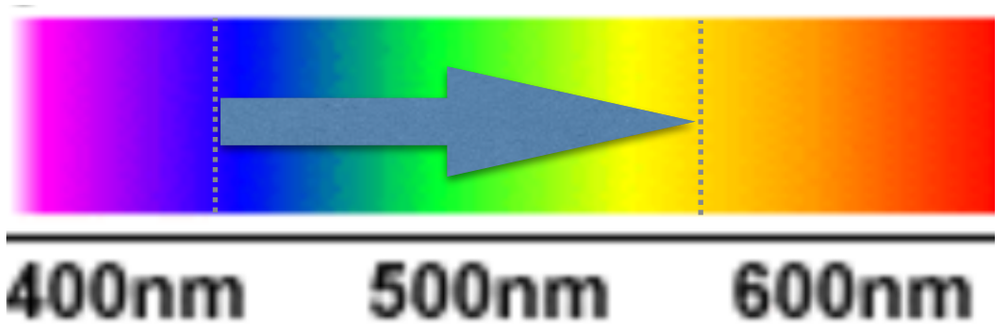
Fluorescein



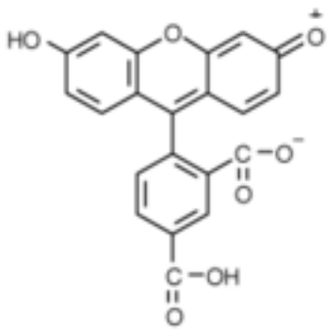




emission at longer wavelengths
= lower energy

What is Fluorescence?

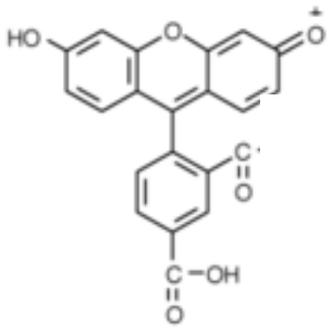
Fluorescein



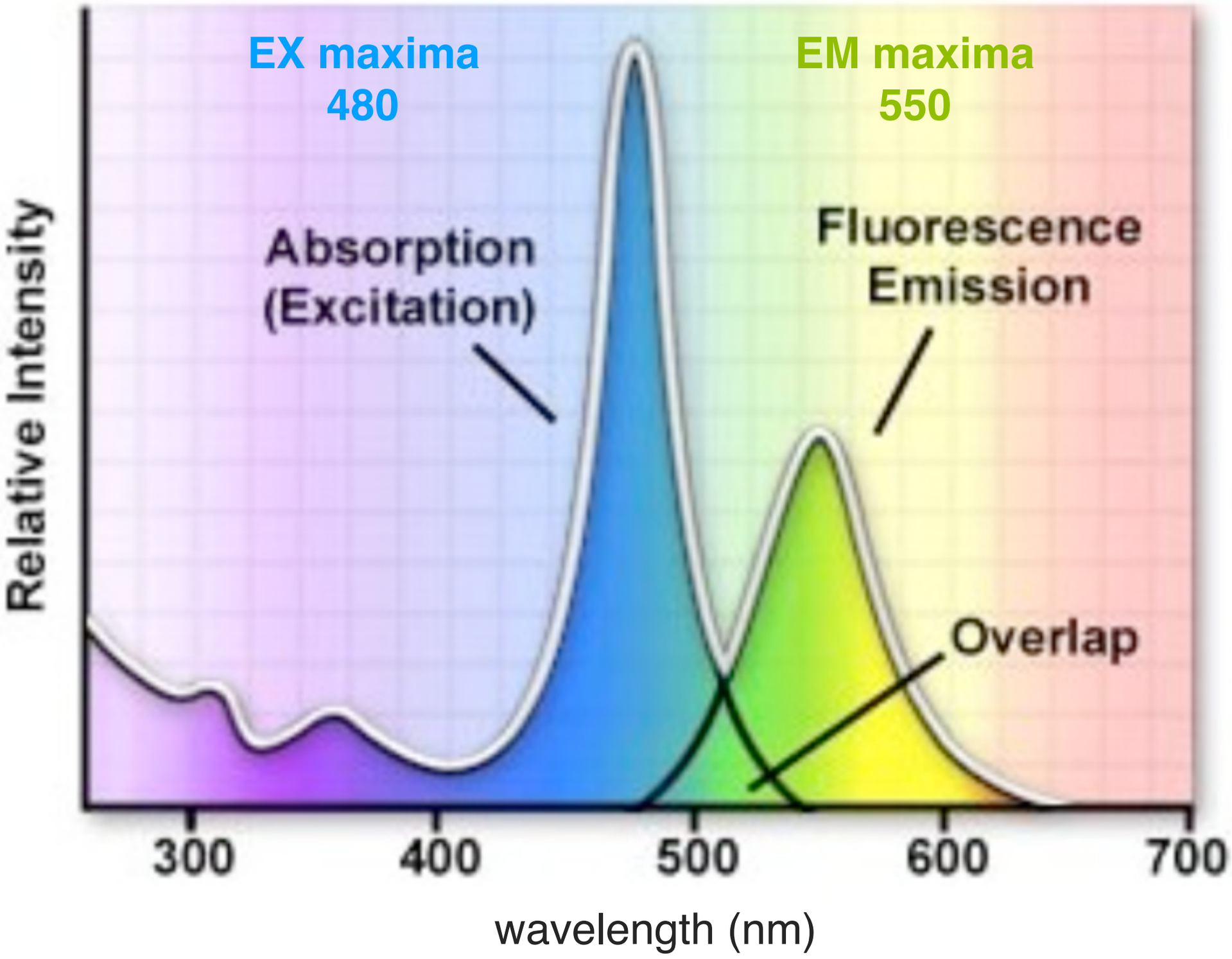
Dye Spectral Characteristics

What is Fluorescence?

Fluorescein



Dye Spectral Characteristics



Fluorescent labels for microscopy (Lecture 5)

- Autofluorescence (~background)
- Selective vital stains
- Fluorescent protein tags (GFP's etc)
- Inorganic fluorescent reporters
- Fluorescence labeled antibodies

The definitive guide:

https://www.thermofisher.com/uk/en/home/references/molecular-probes-the-handbook.html

Why Fluorescence?

Why Fluorescence?

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

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CONTRAST

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- High signal to background

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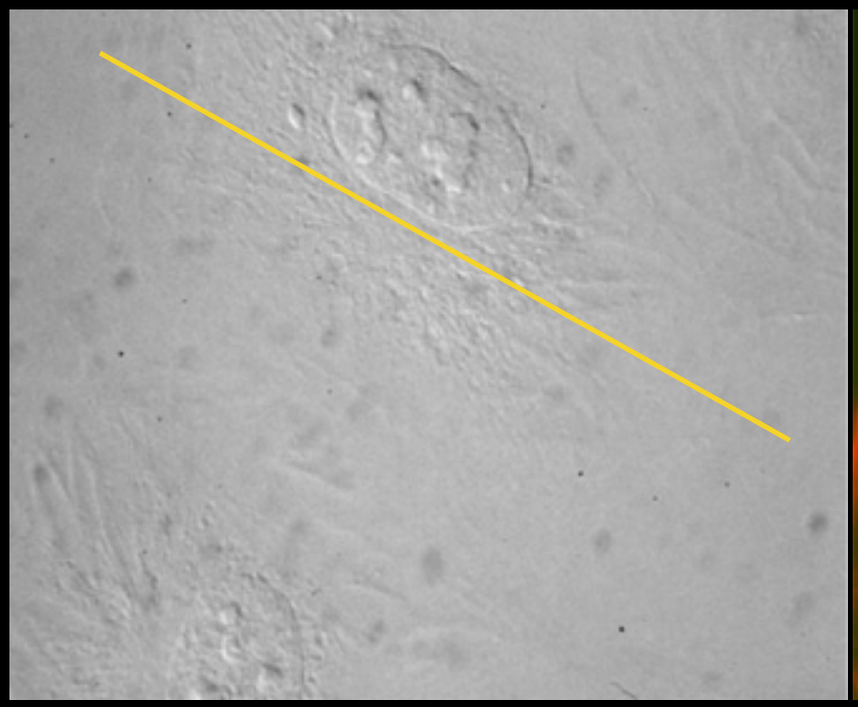
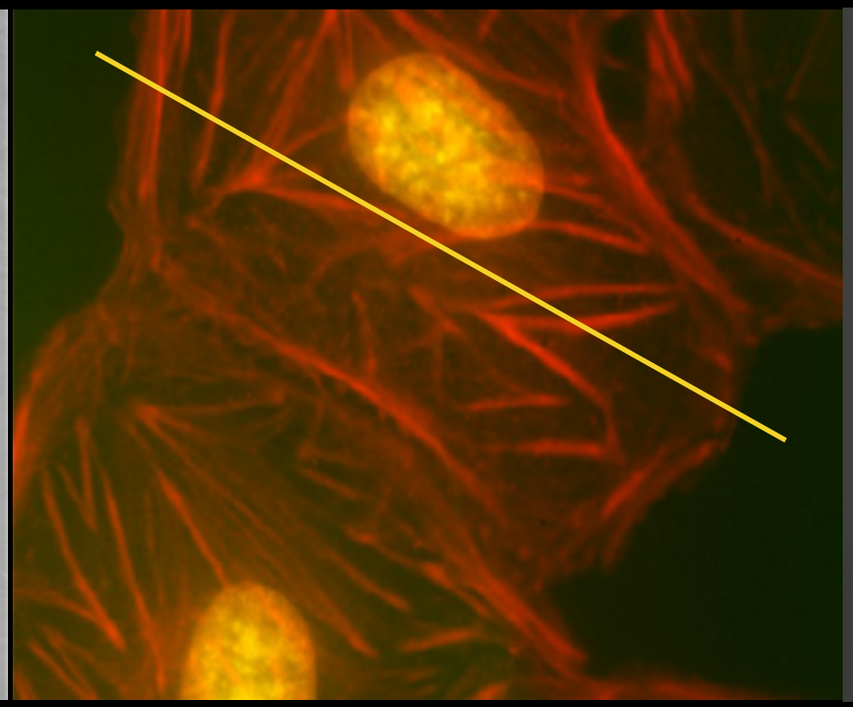
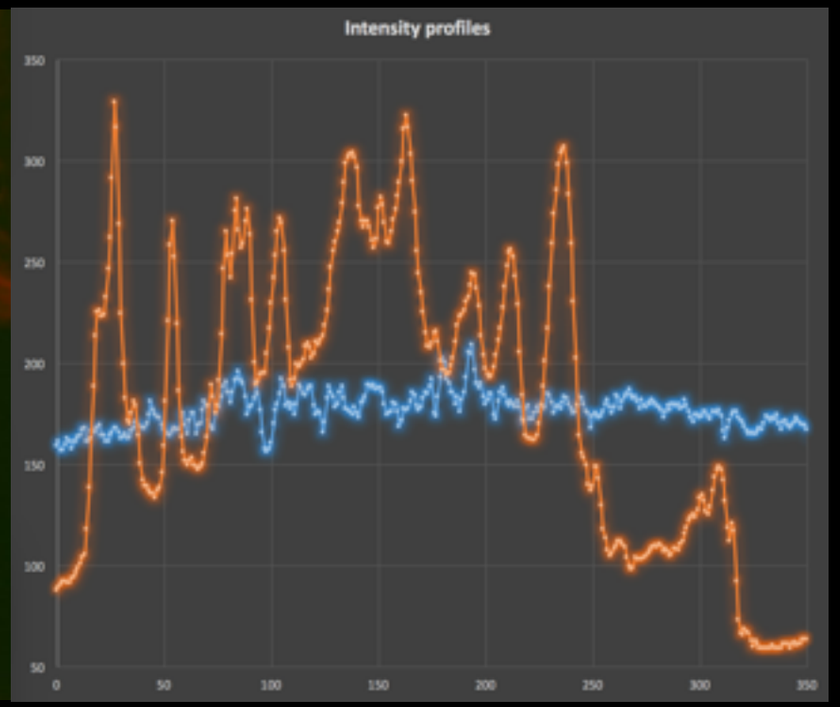


bright field (DIC)



fluorescence



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- High signal to background
- Selective labeling
- Ease of multiplexing

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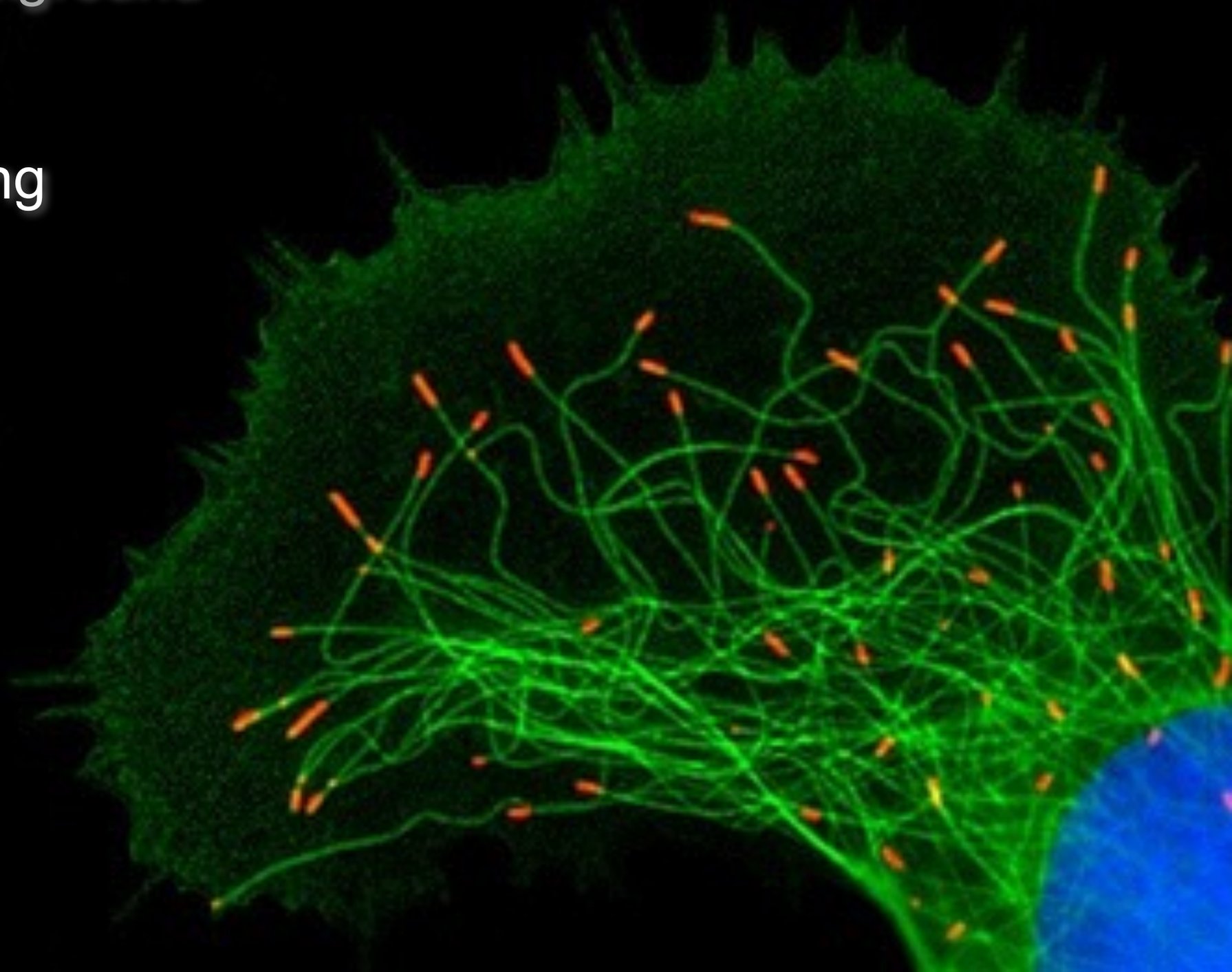
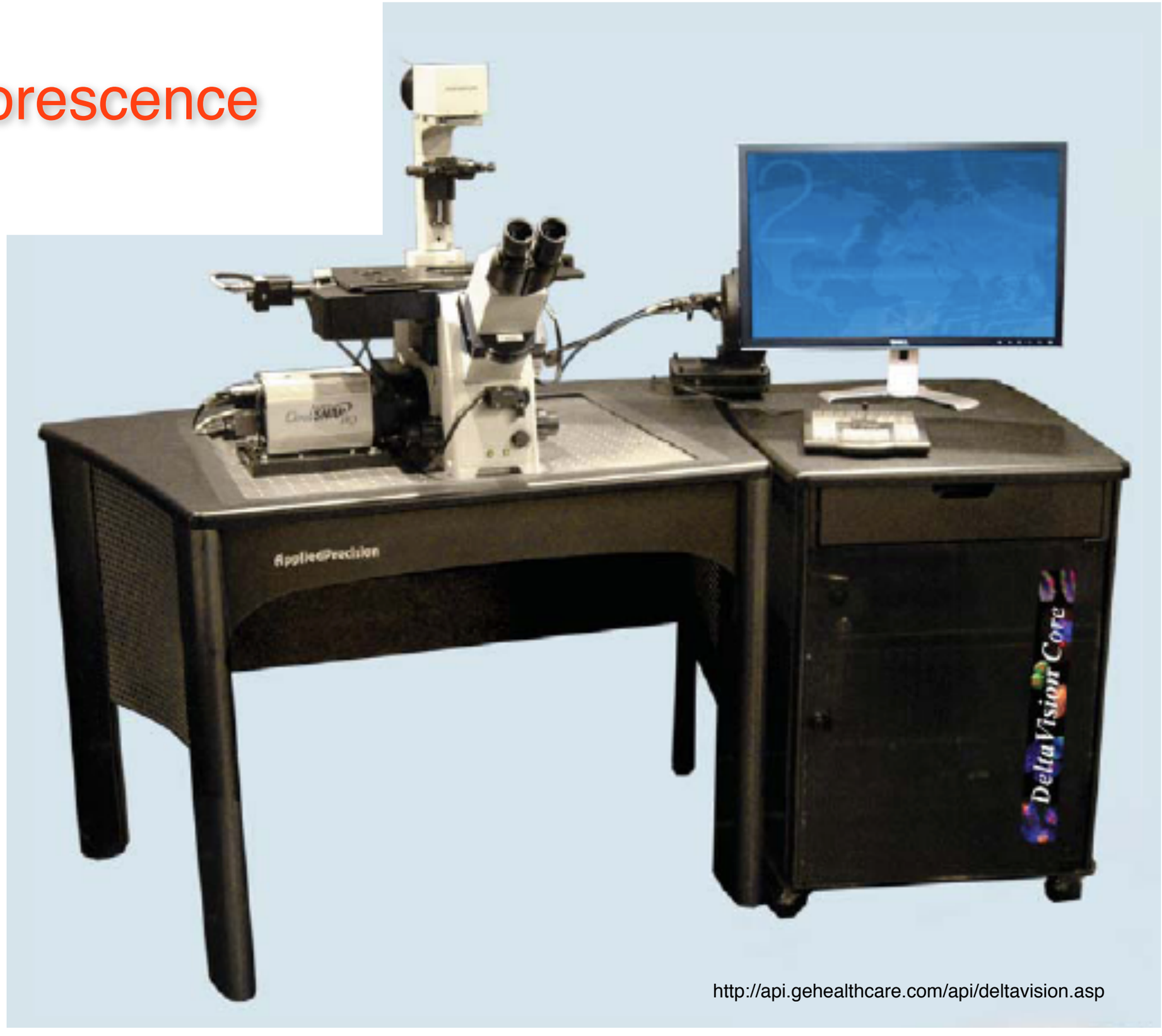


Principle and components of the fluorescence microscope

Epi-Fluorescence



http://api.gehealthcare.com/api/deltavision.asp

Widefield deconvolution

Confocal

TIRF

FCS

dSTORM

PALM

Multi-photon

STED

3D-SIM

The Epi-Fluorescence Microscope

Transmitted light (e.g. BF)

Epi-Fluorescence

The Epi-Fluorescence Microscope

Transmitted light (e.g. BF)

DETECTOR

LENS

SPECIMEN

Epi-Fluorescence

DETECTOR

The Epi-Fluorescence Microscope

Transmitted light (e.g. BF)

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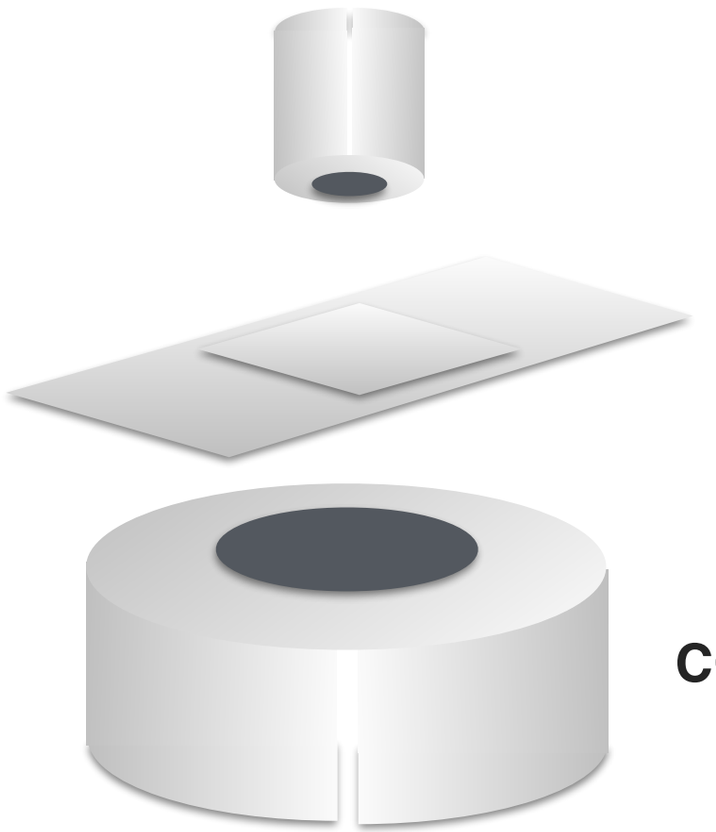

LENS

SPECIMEN

CONDENSER

Epi-Fluorescence

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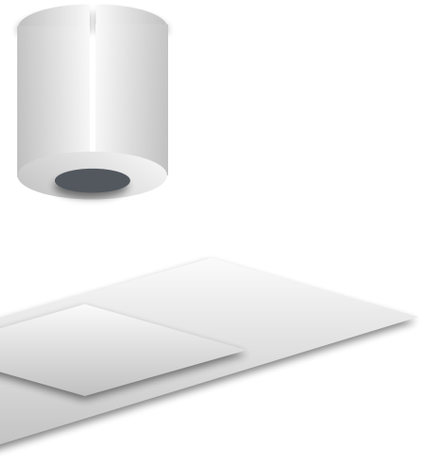

The Epi-Fluorescence Microscope

Transmitted light (e.g. BF)

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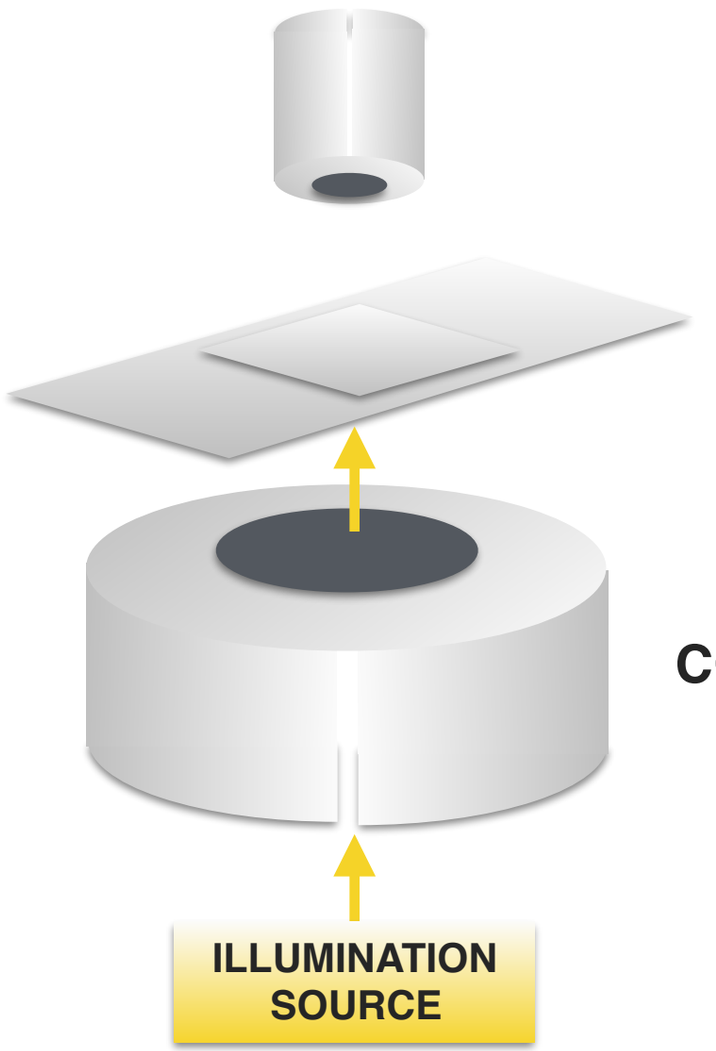


LENS

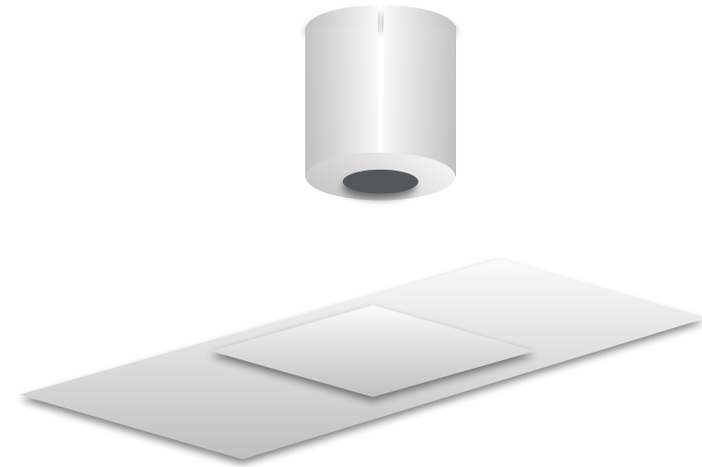
SPECIMEN

CONDENSER

ILLUMINATION SOURCE

Epi-Fluorescence

DETECTOR



The Epi-Fluorescence Microscope

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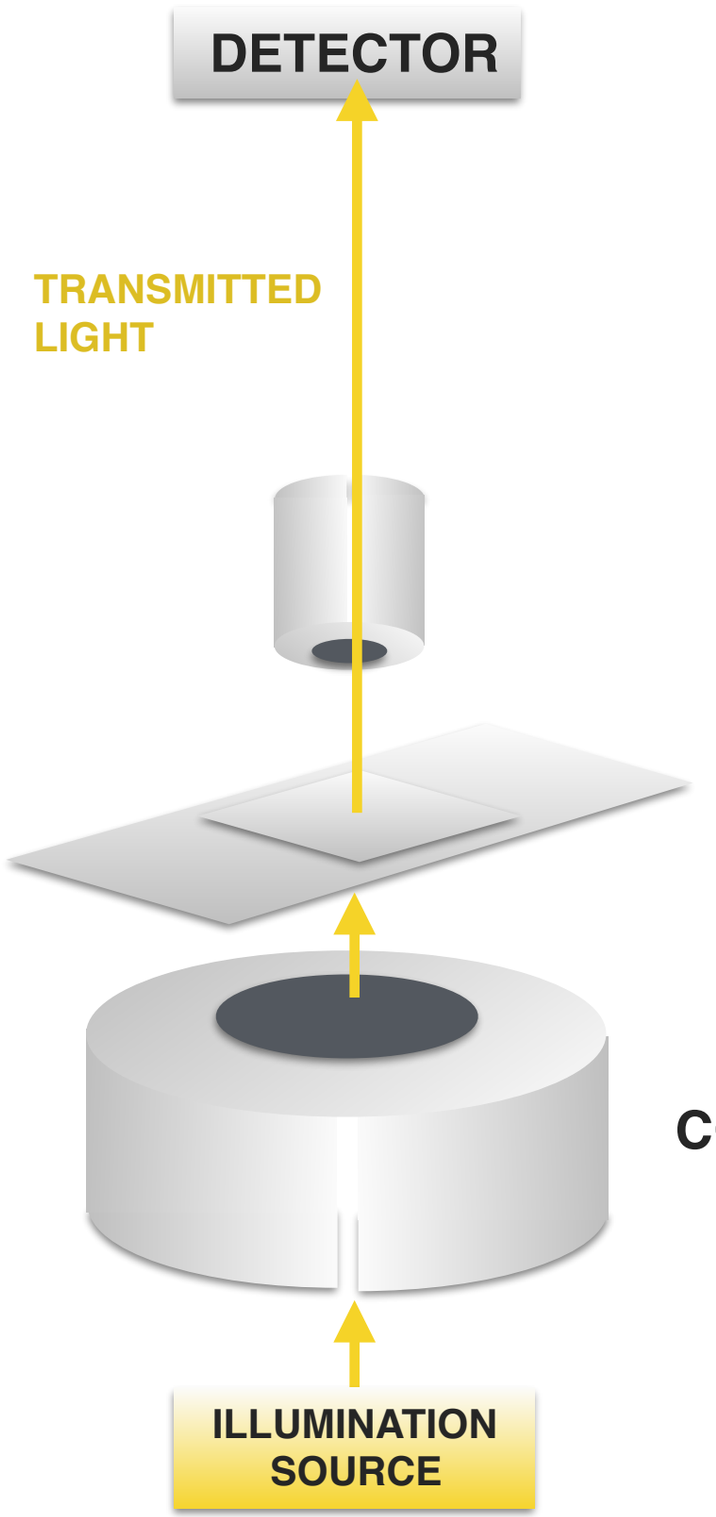

Epi-Fluorescence

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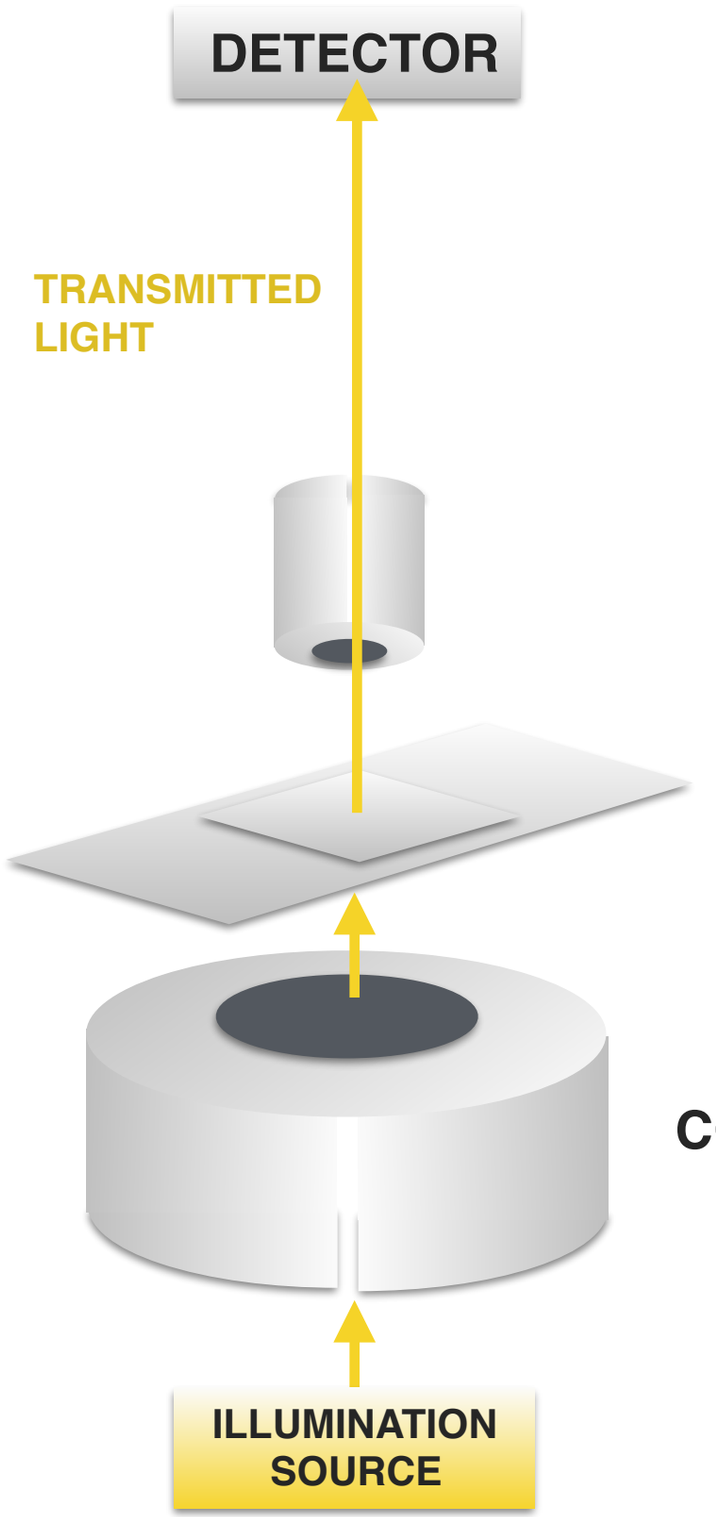

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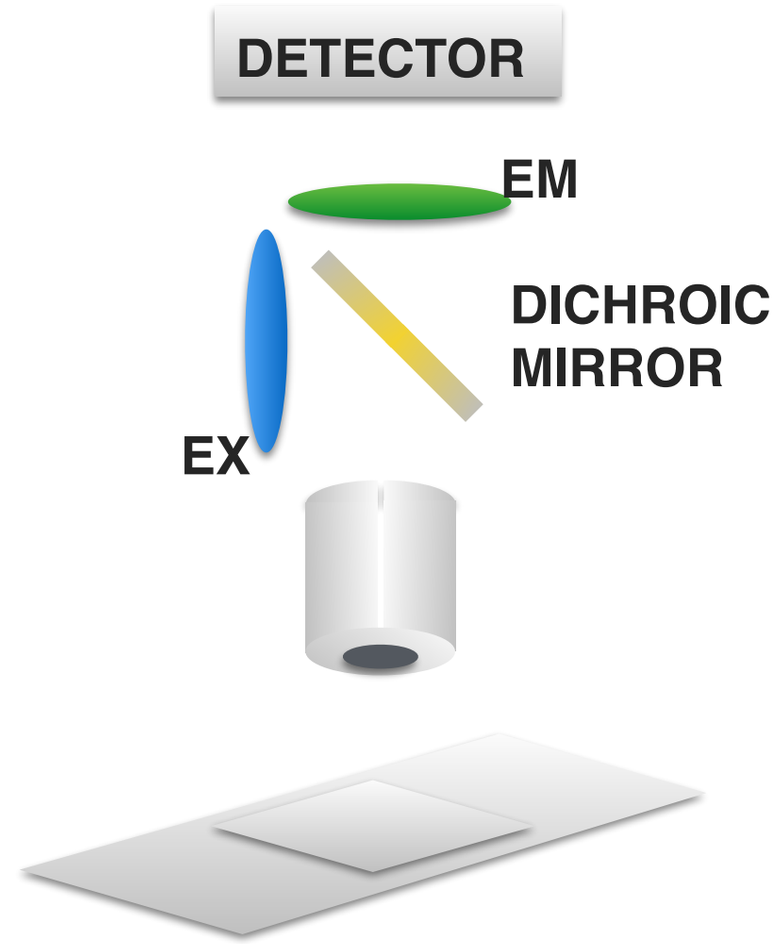

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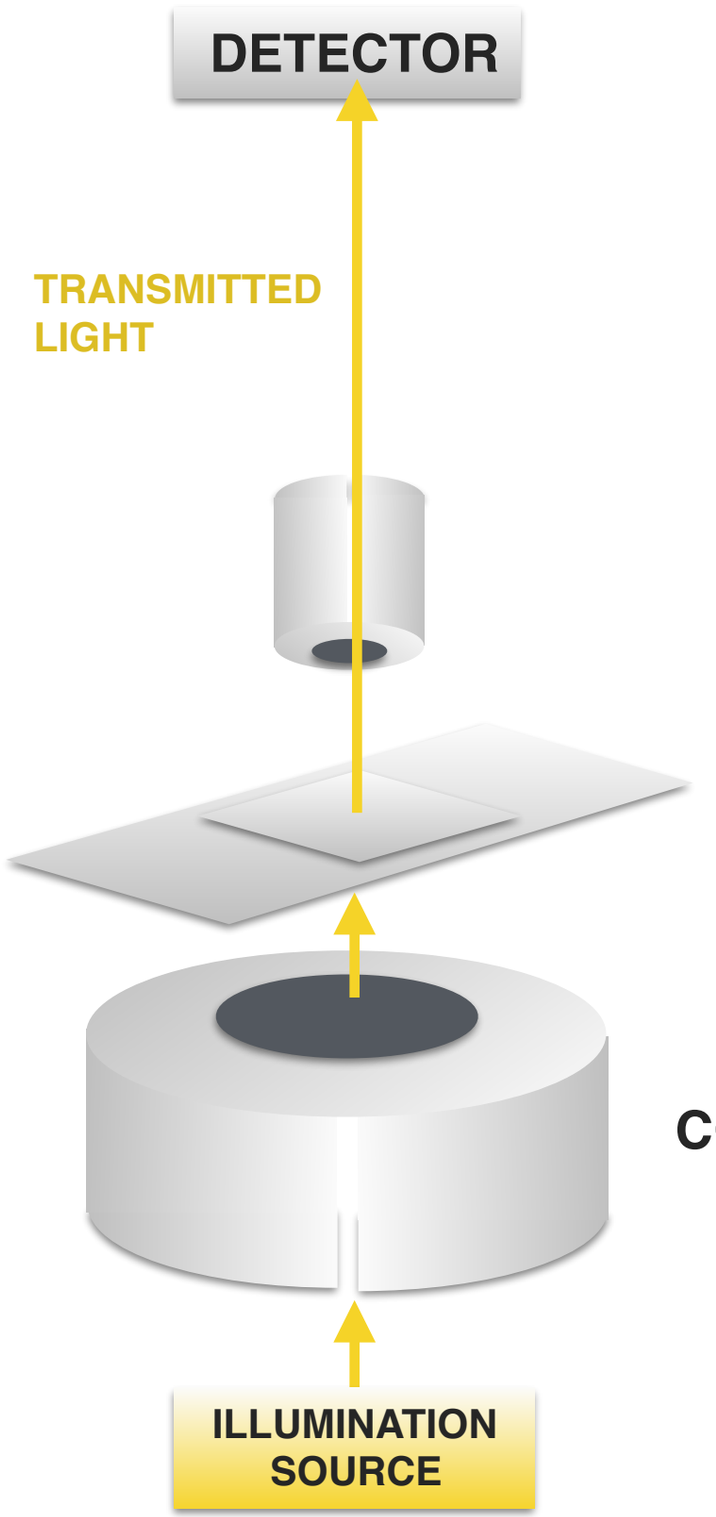

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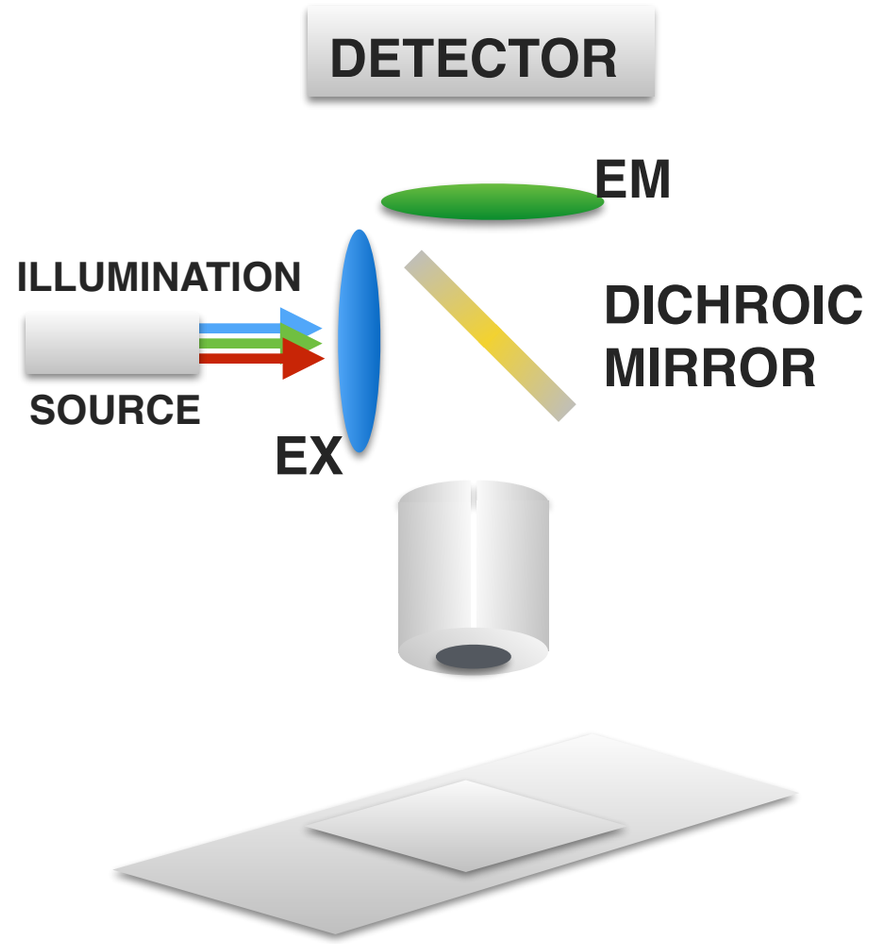

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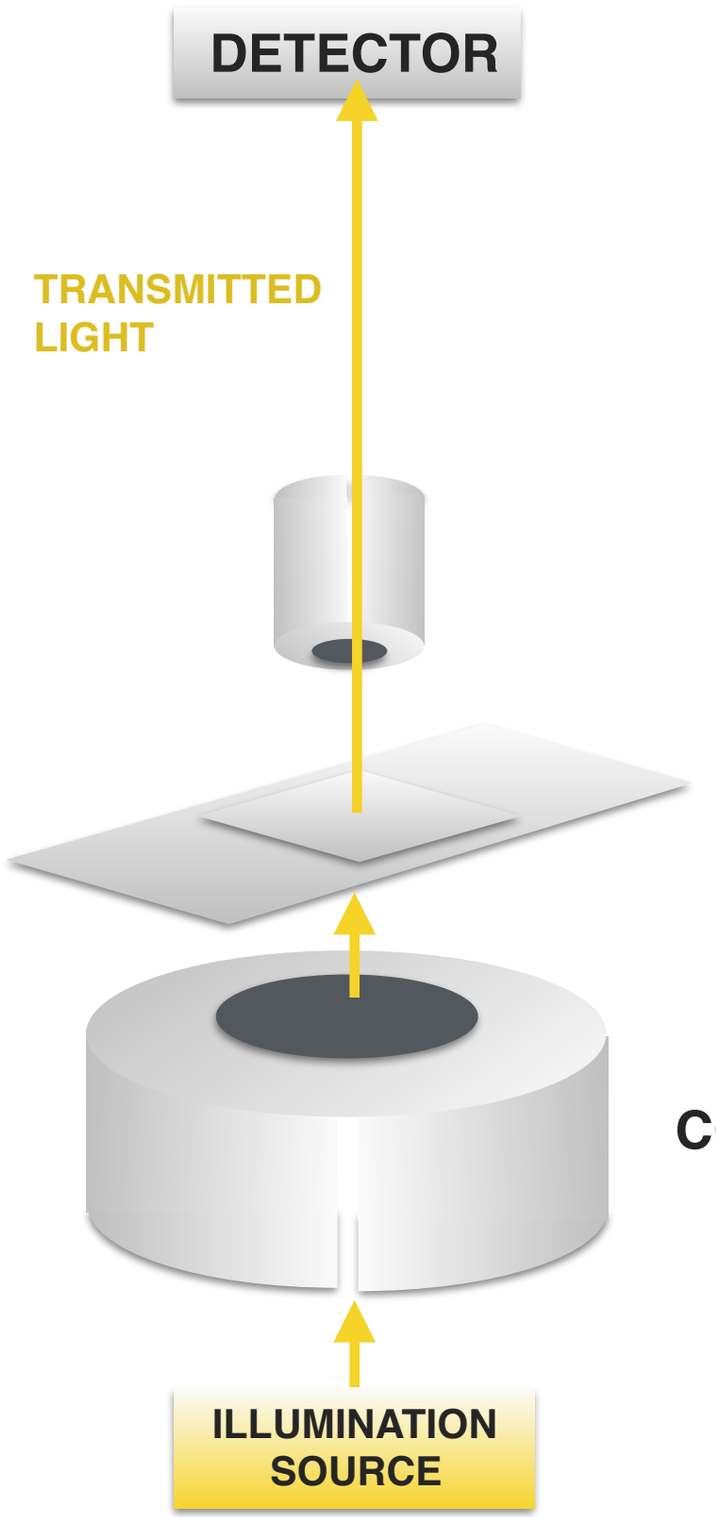

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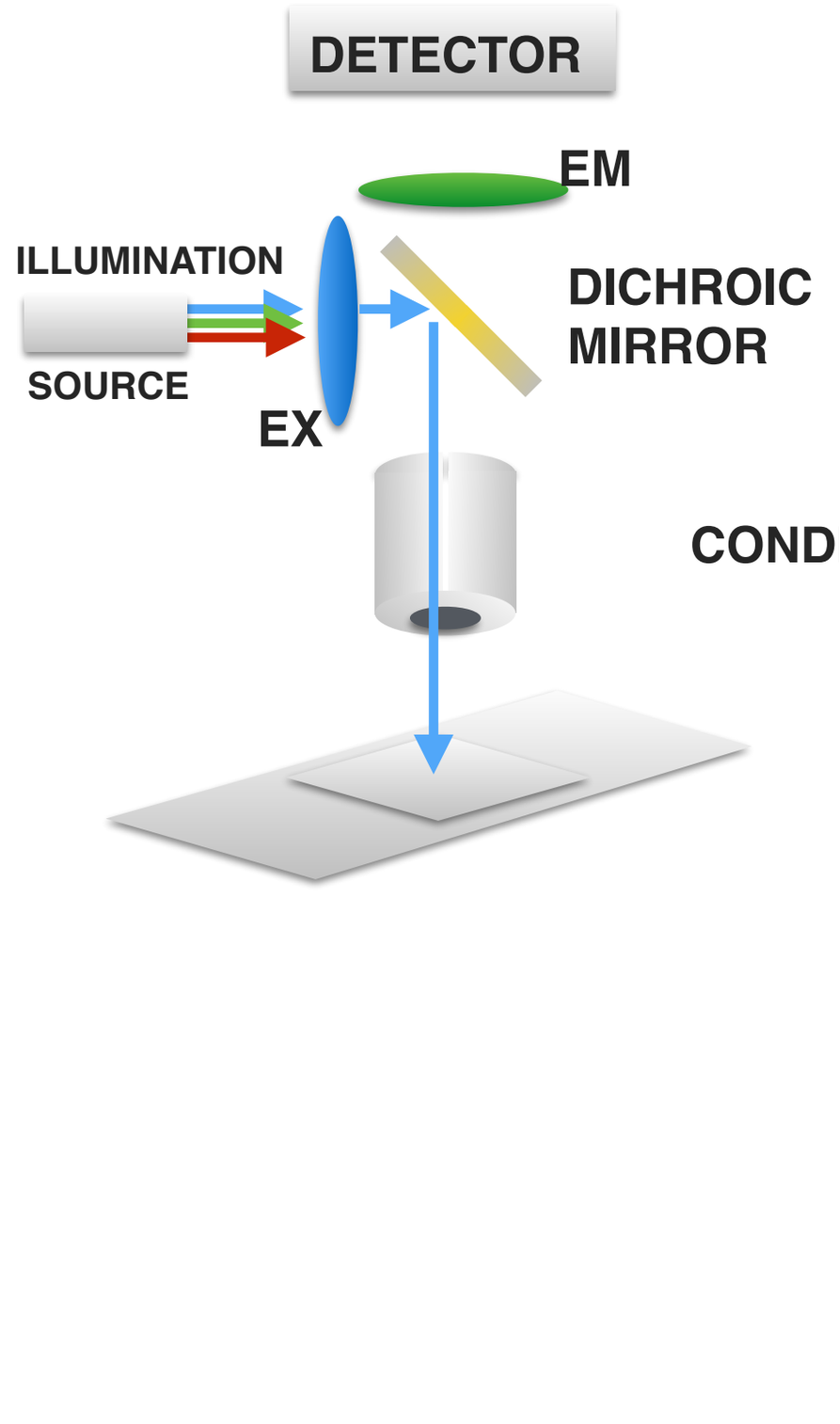

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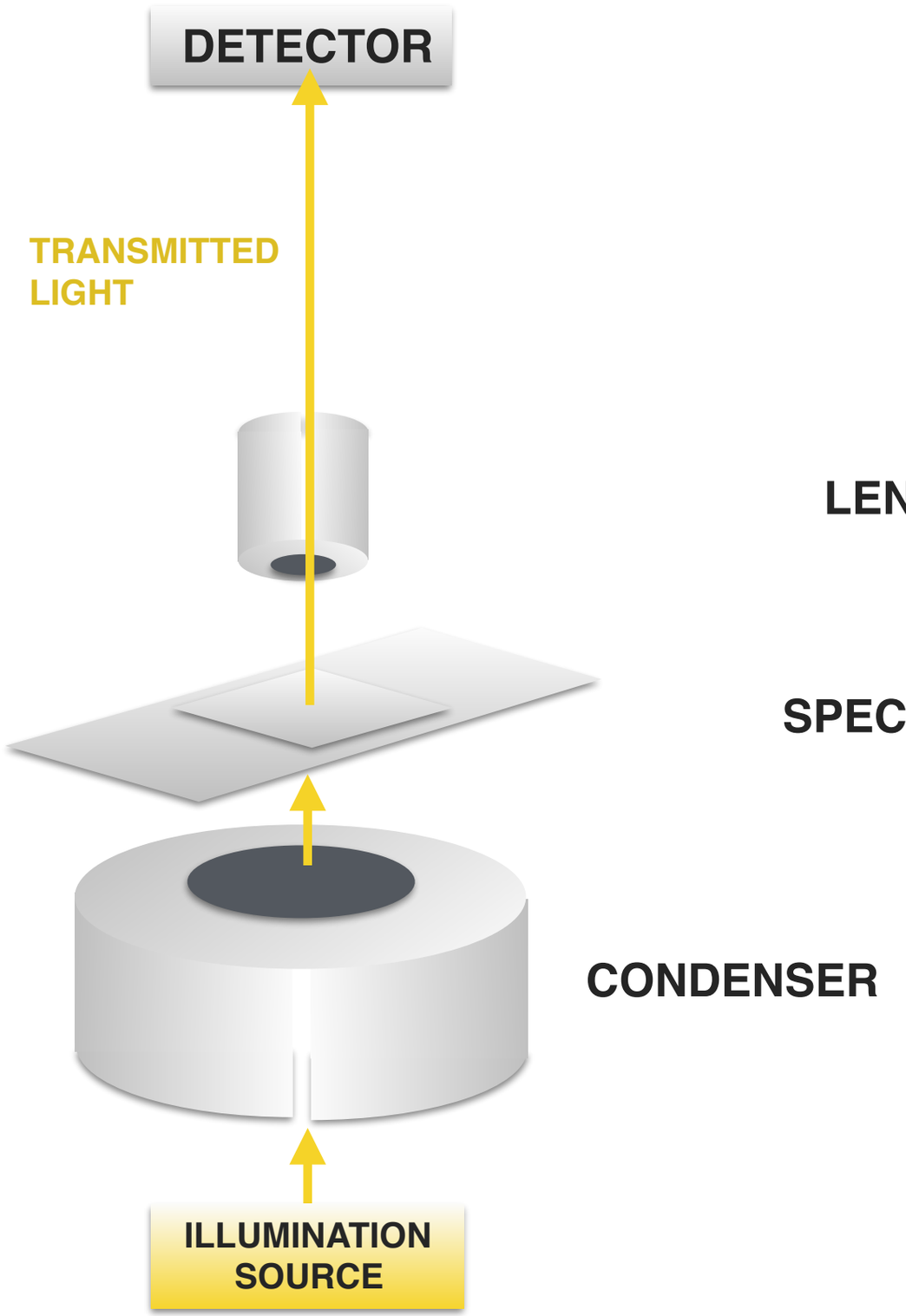

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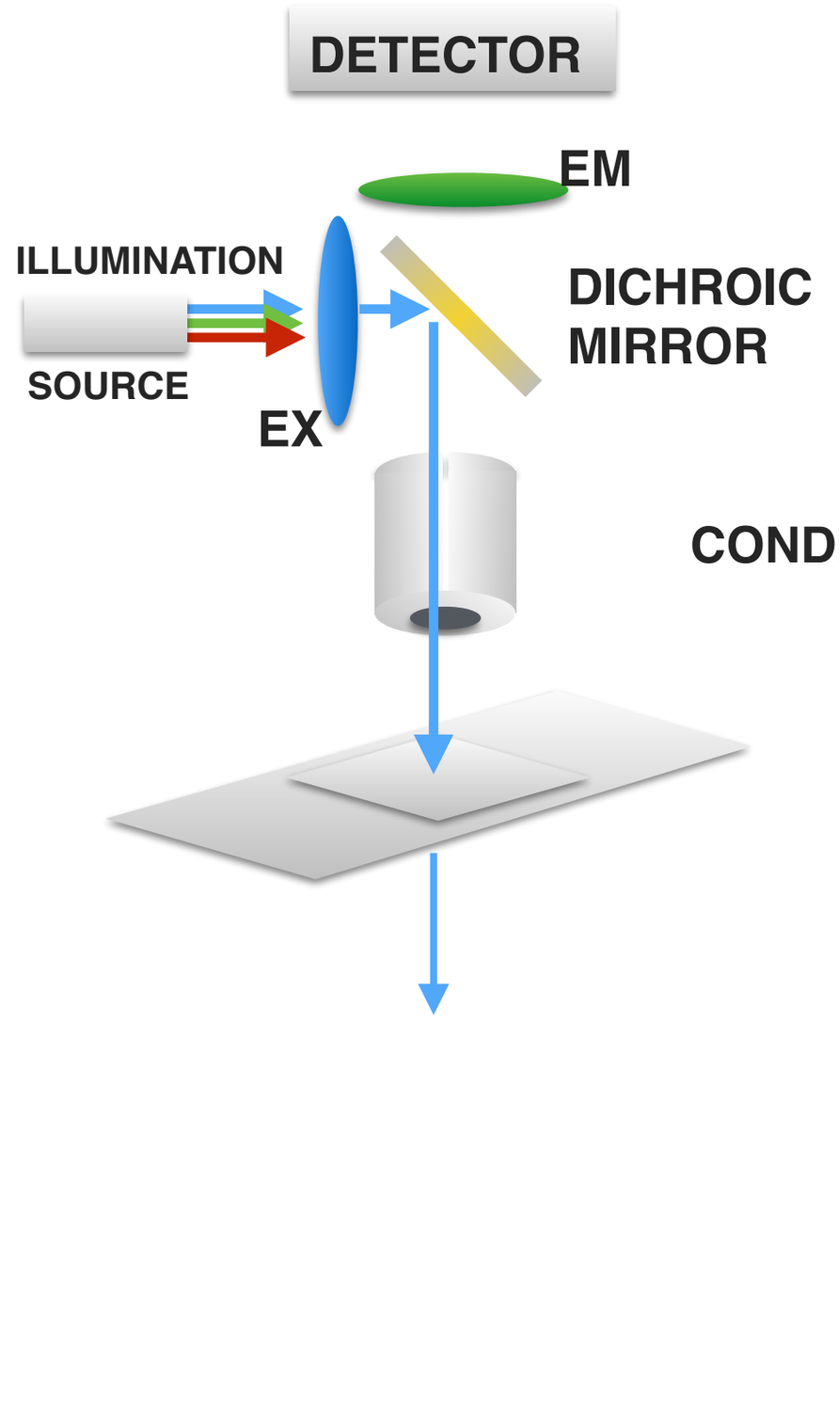

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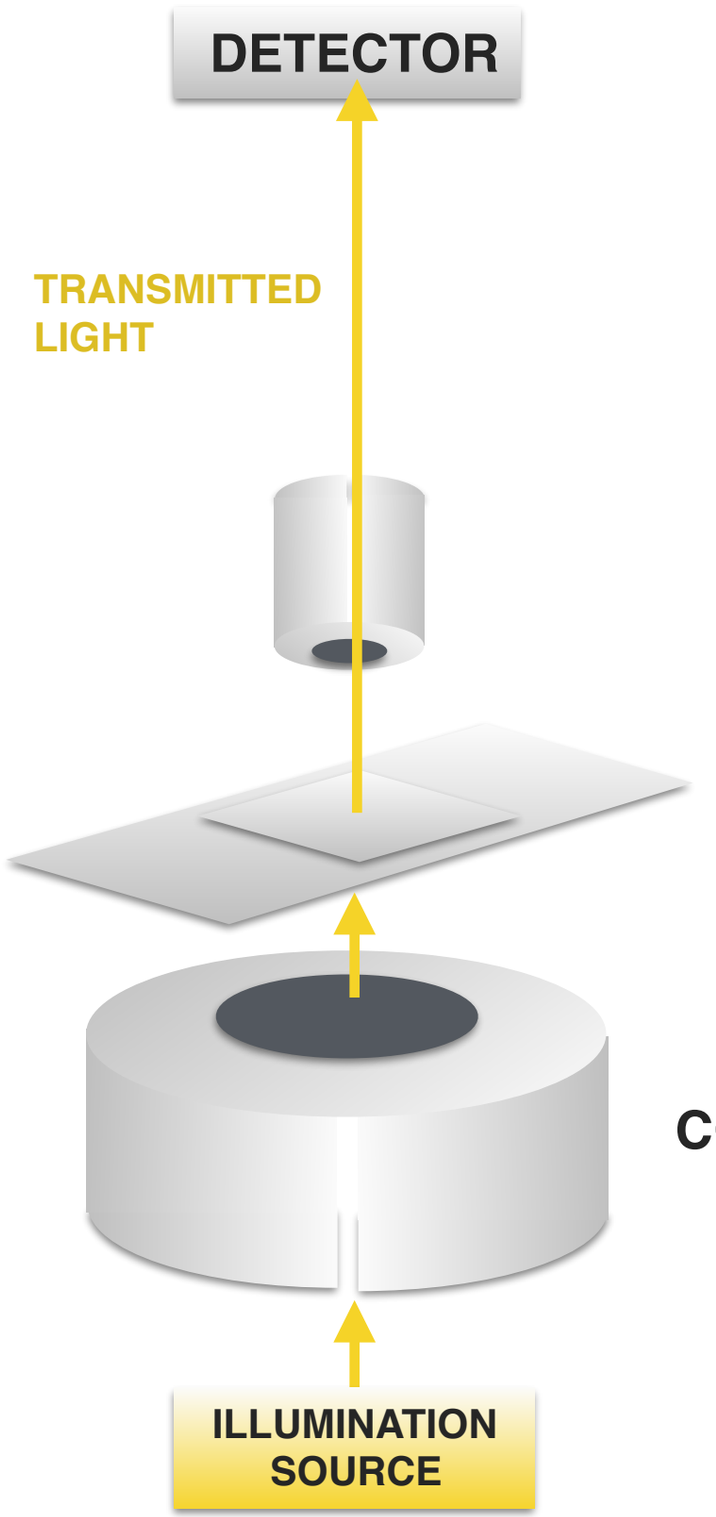

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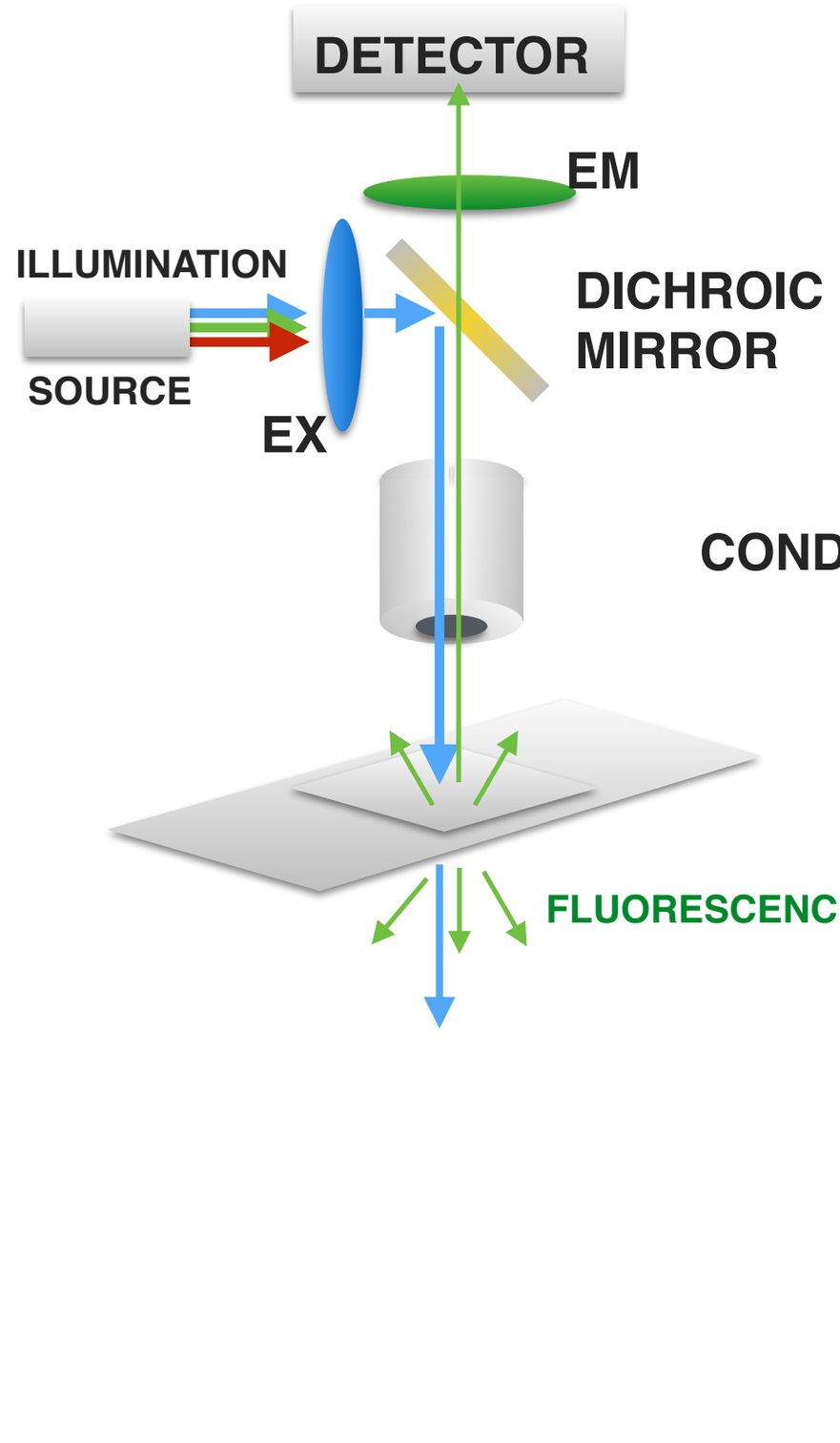
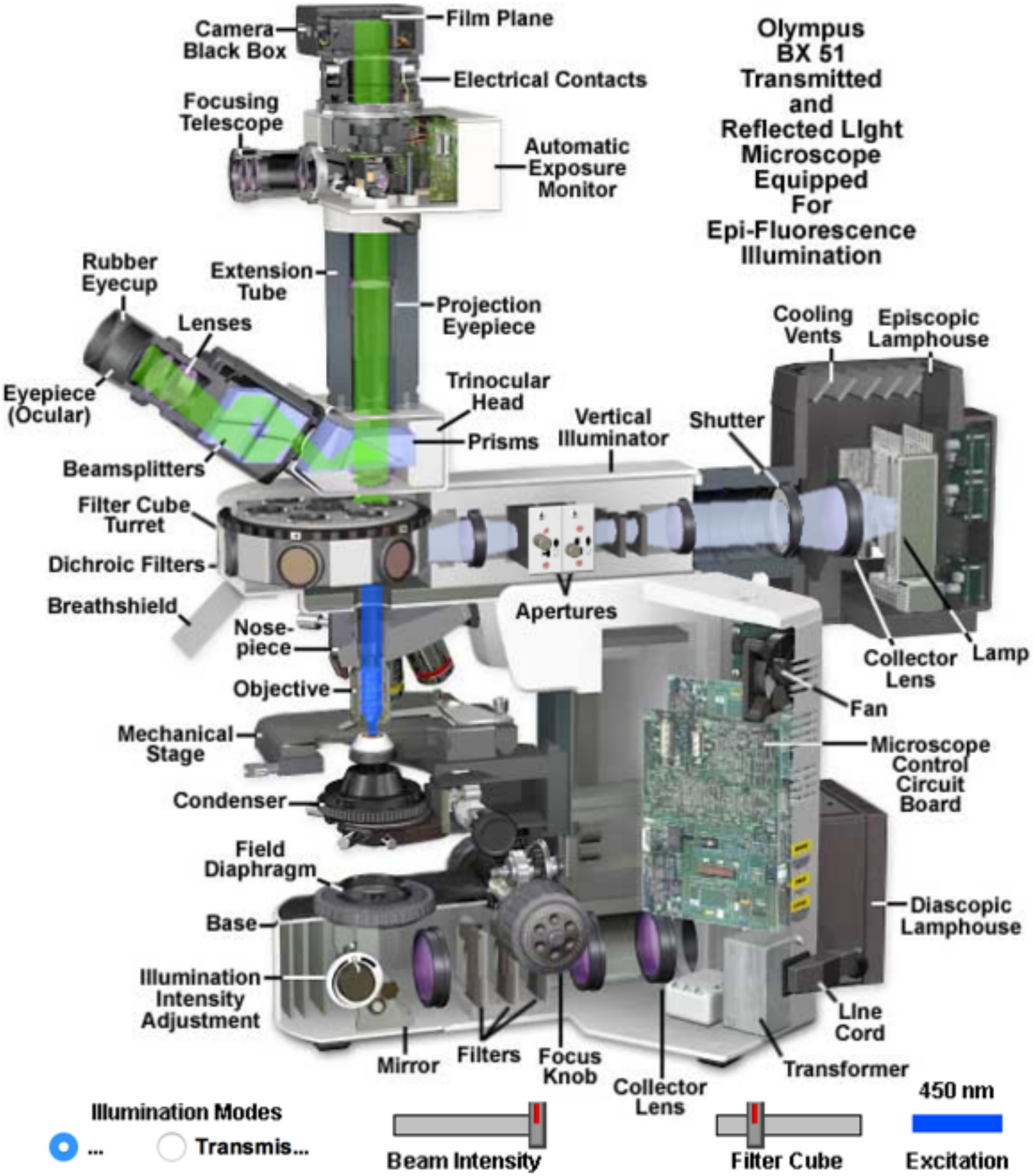


The Epi-Fluorescence Light Path (upright)



http://www.olympusmicro.com/

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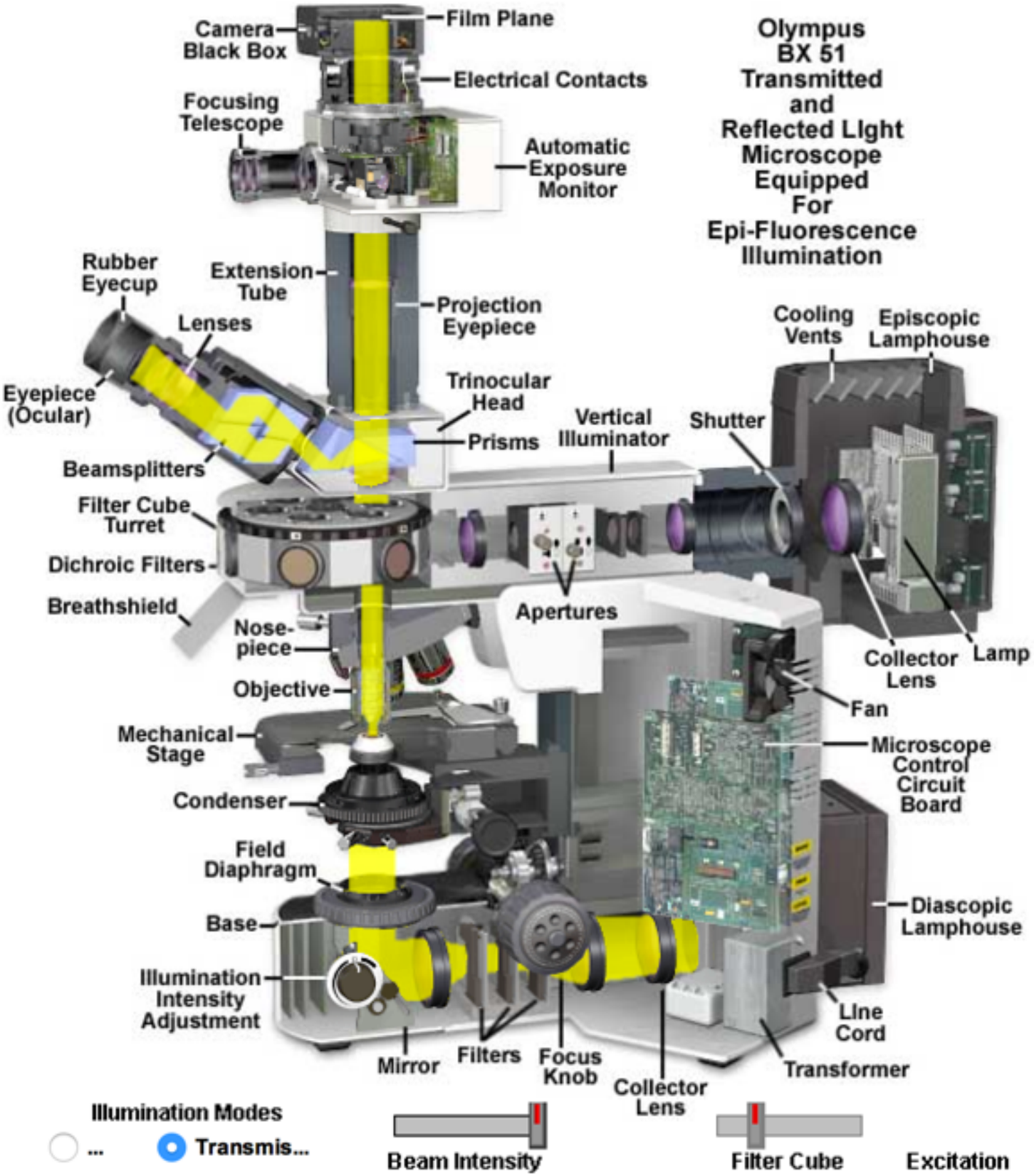

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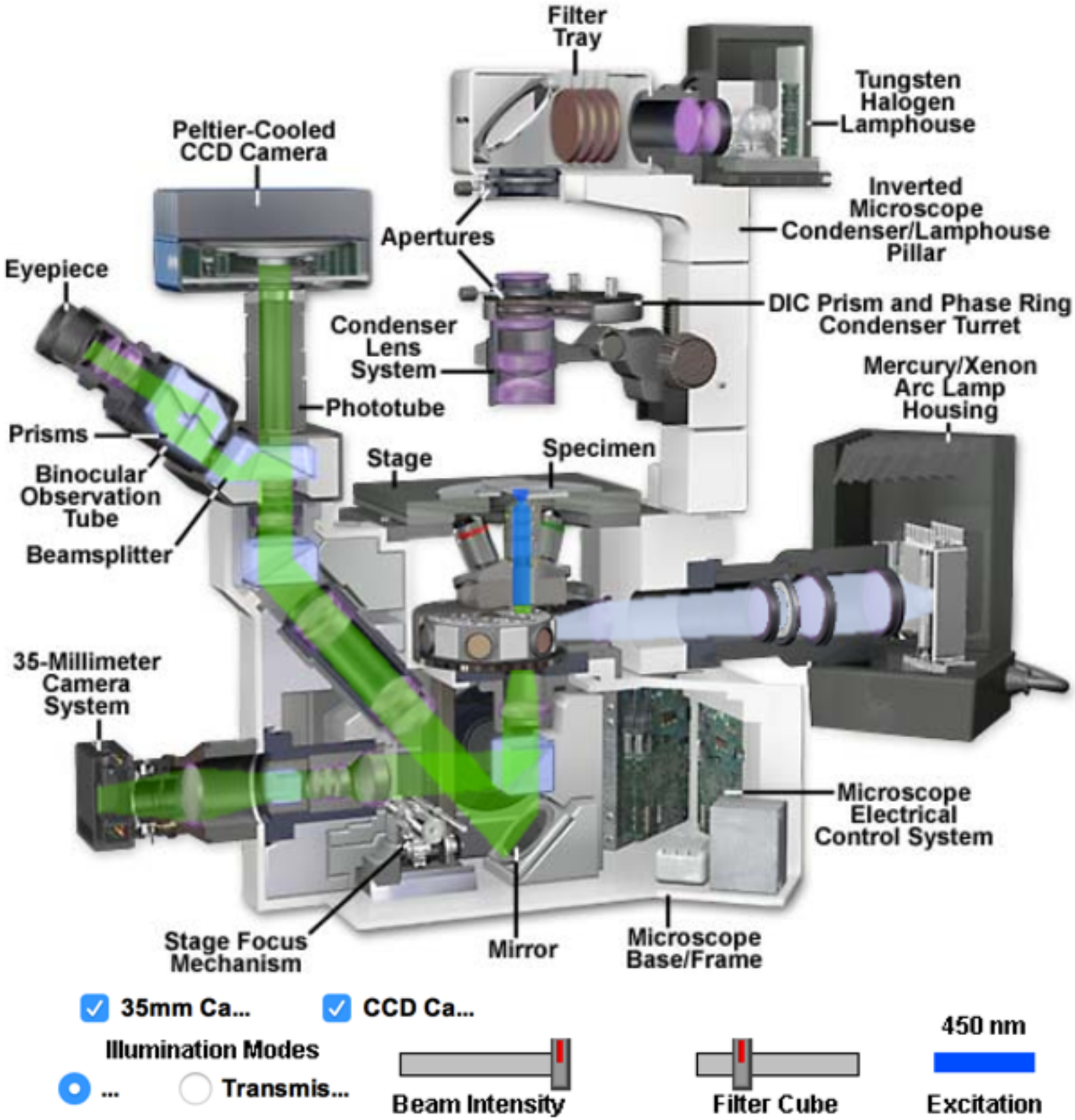
The Epi-Fluorescence Light Path (inverted)



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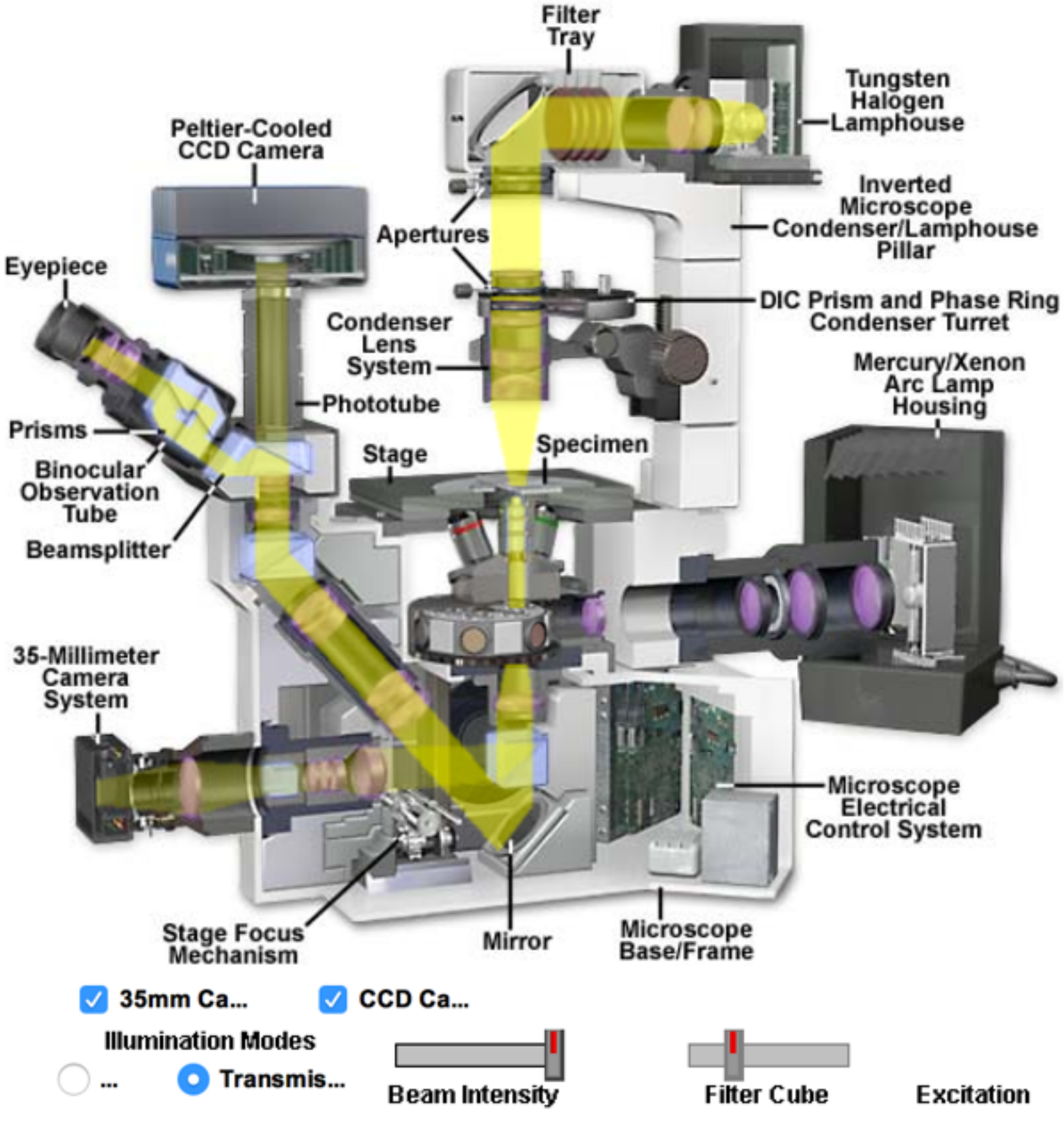

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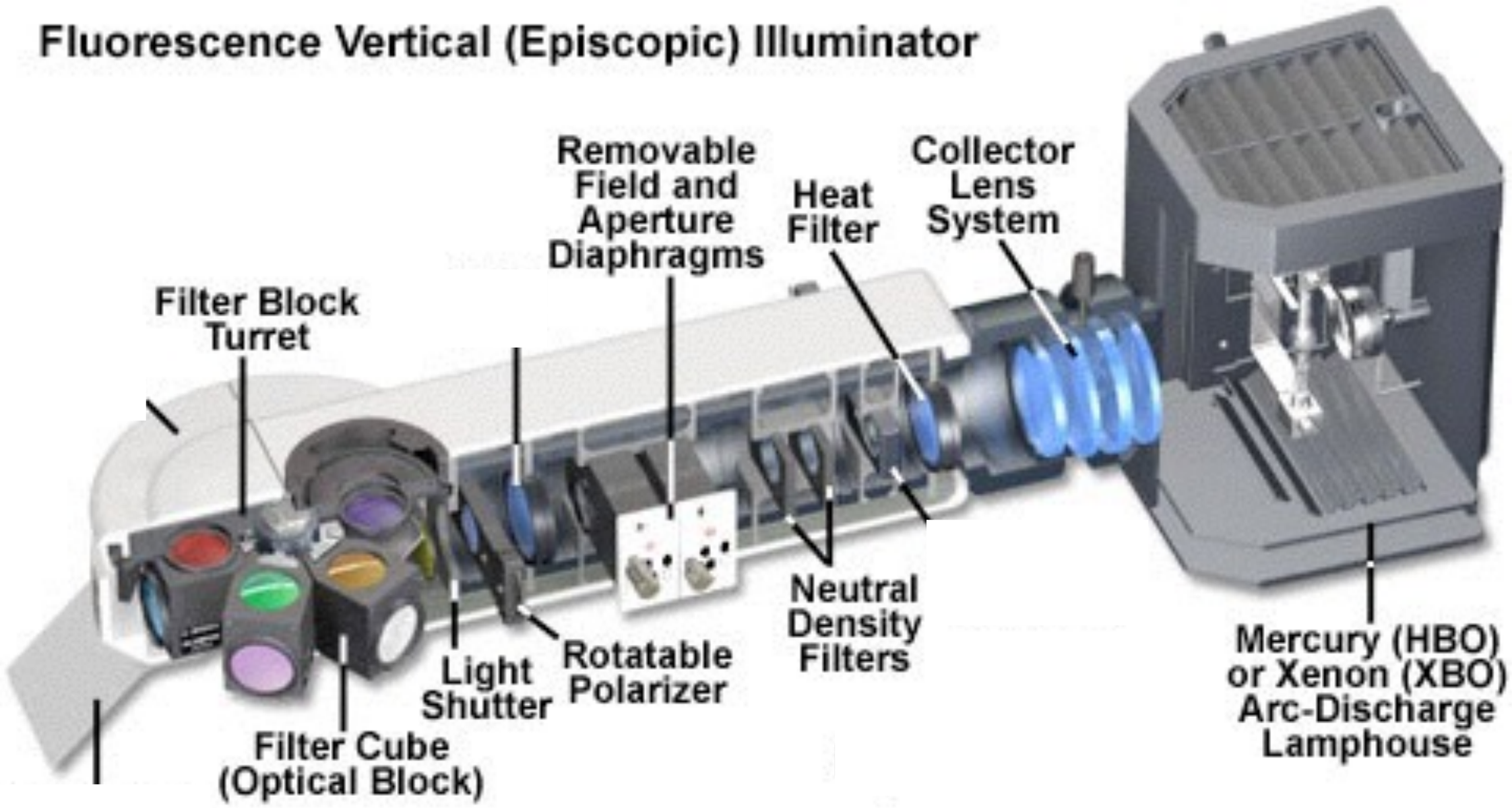

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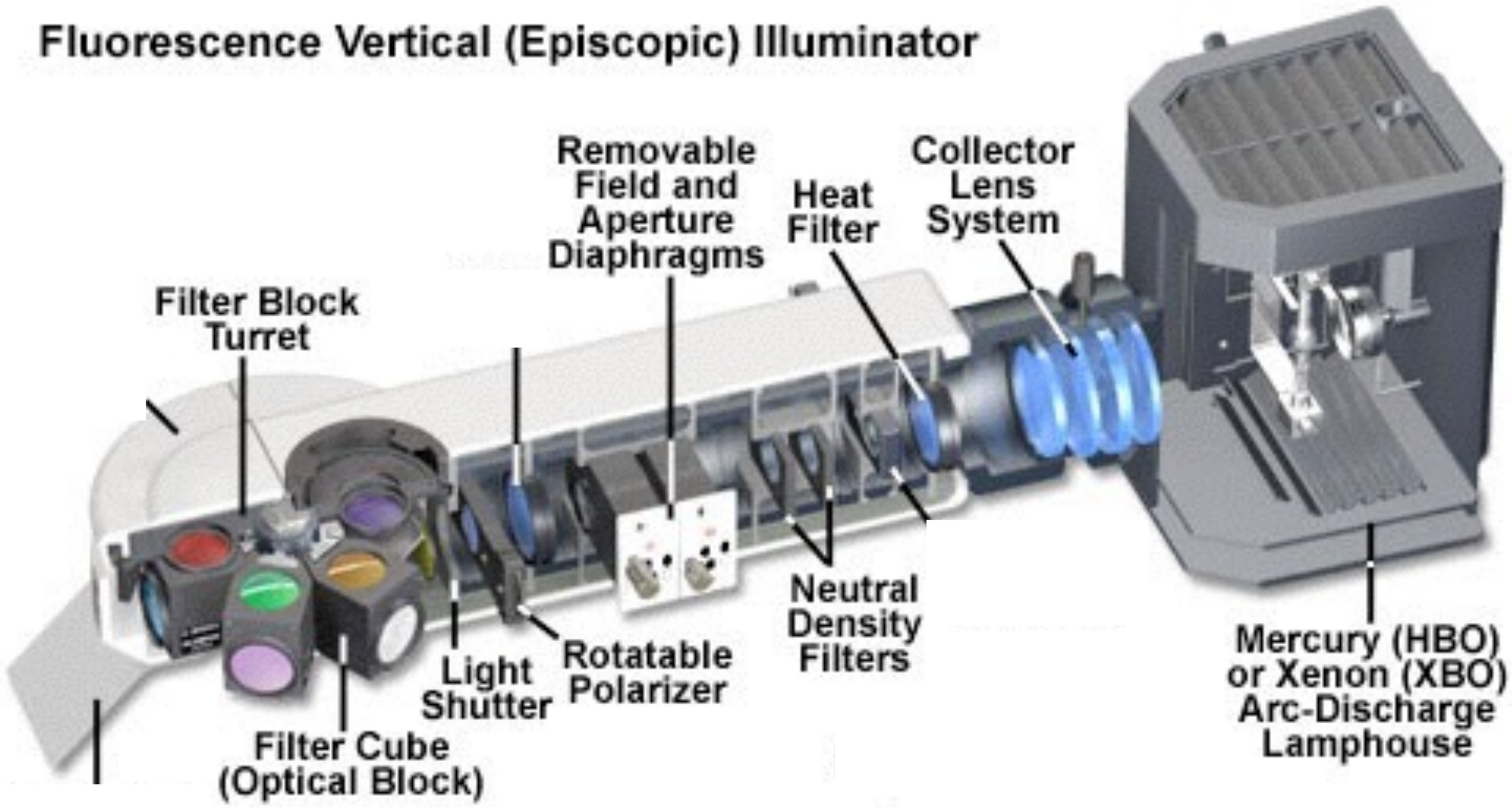

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The Epi-Fluorescence Light Path - excitation

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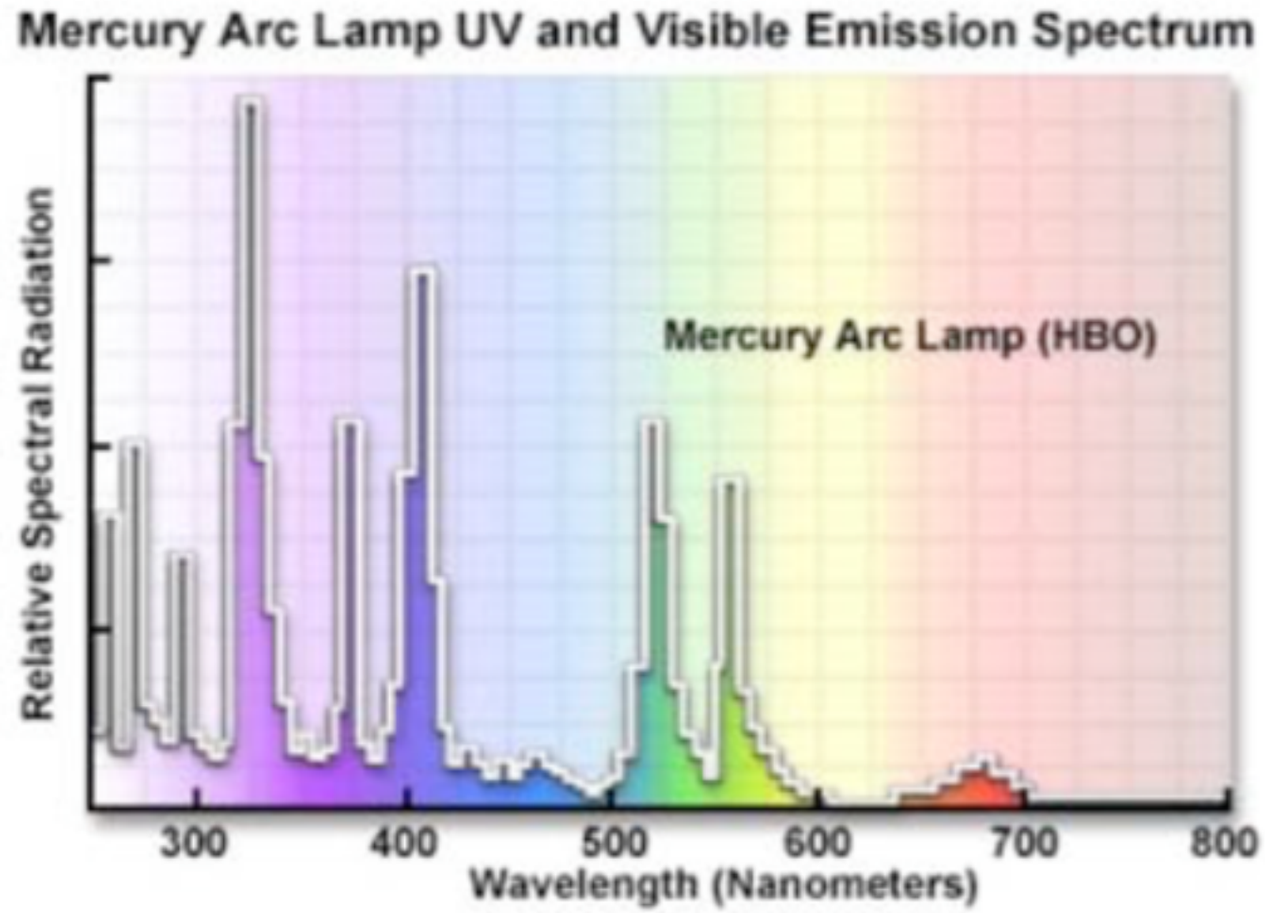
Fluorescence Arc Lamp (Wide field)



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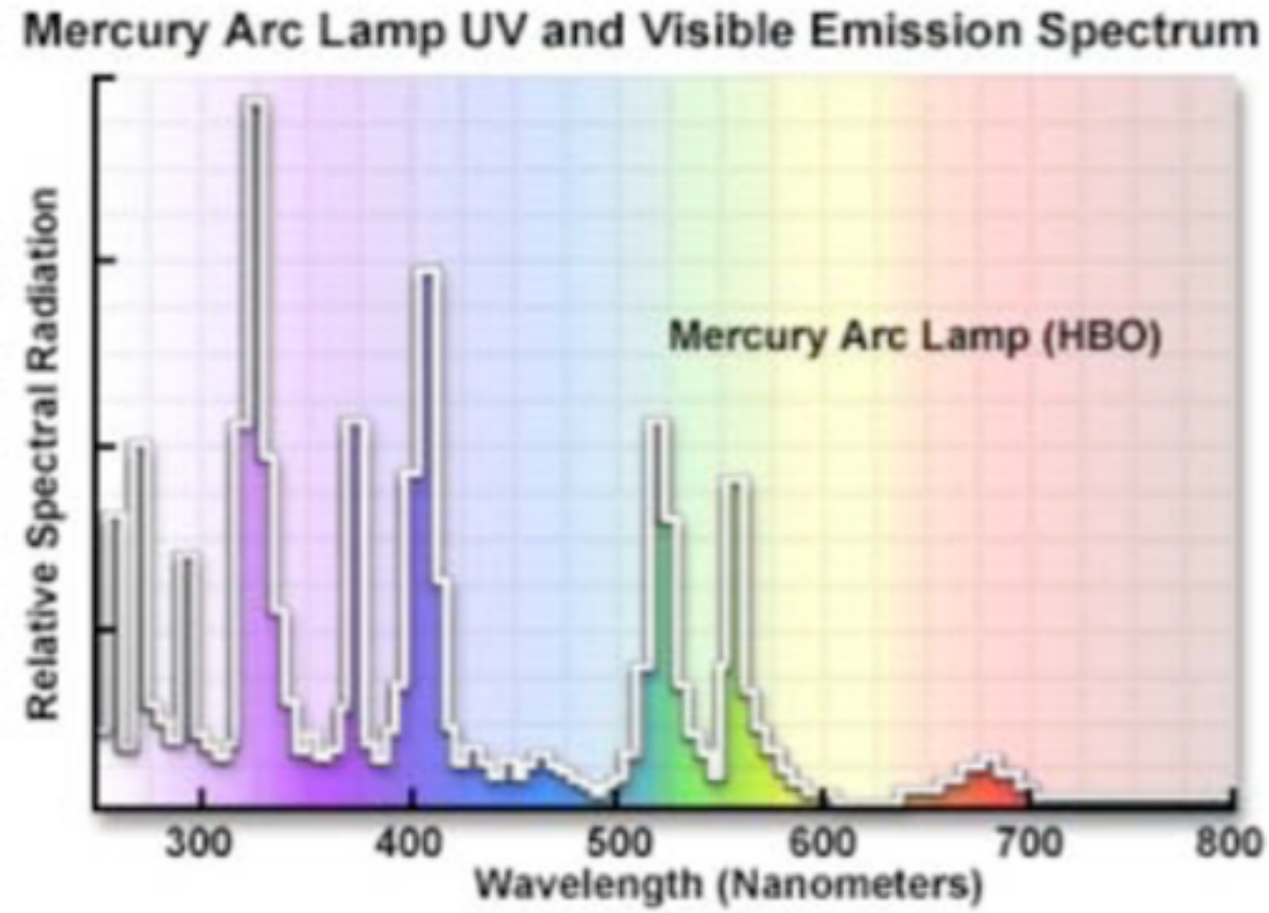

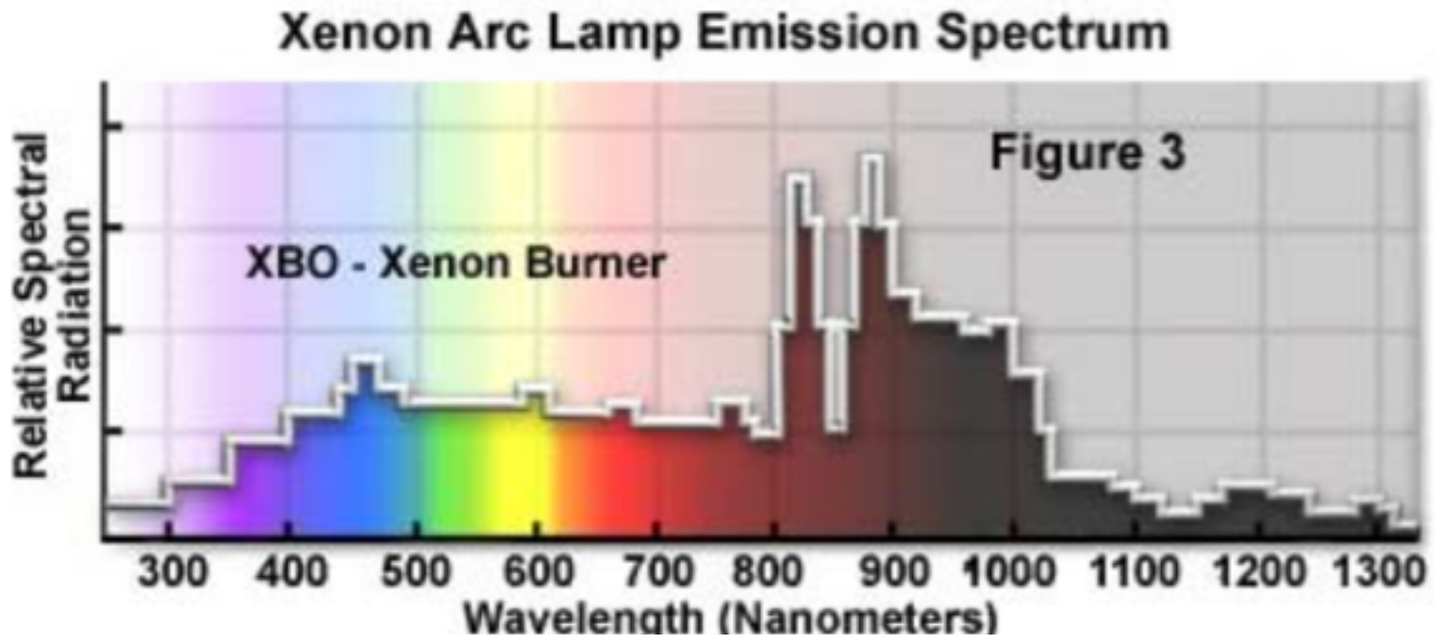

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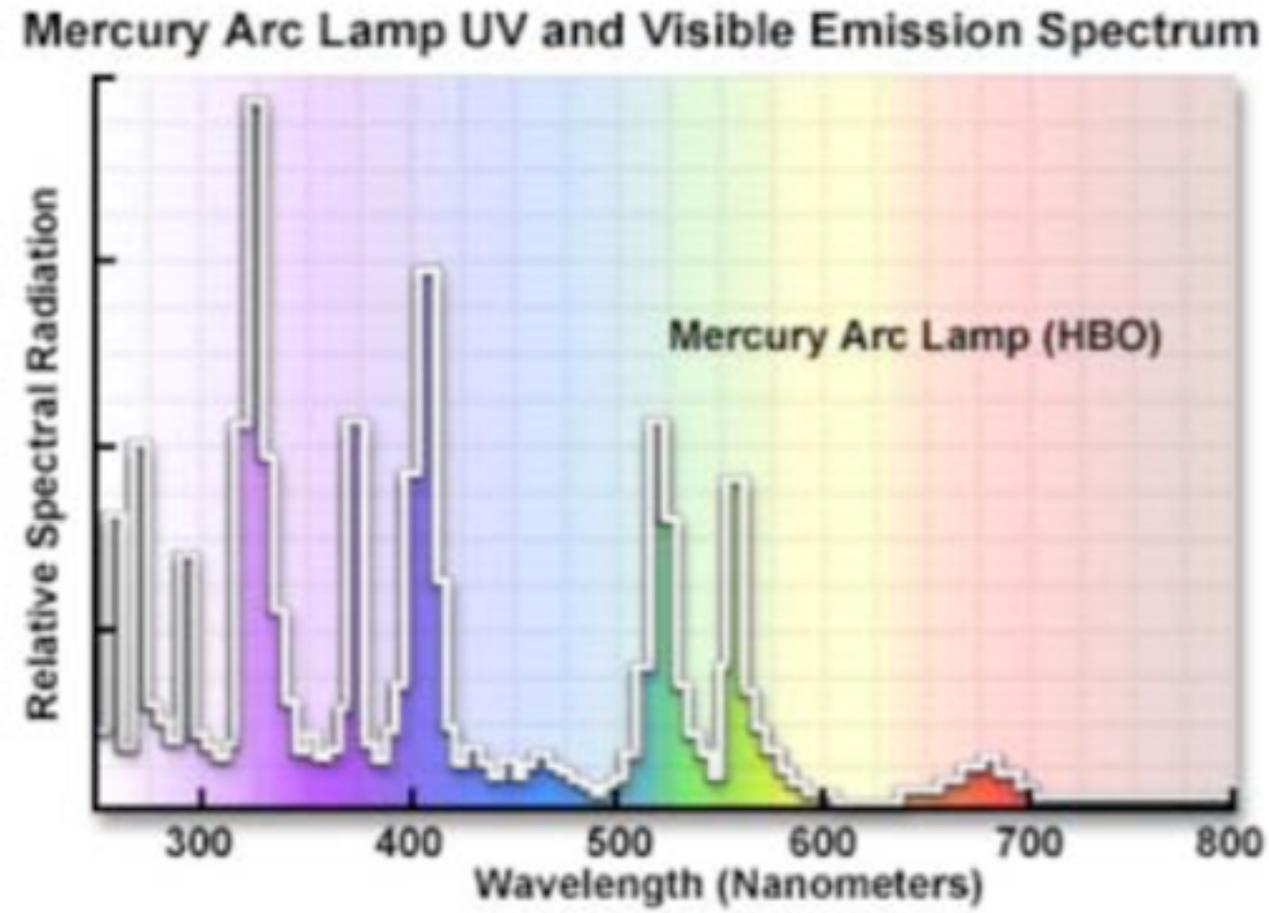
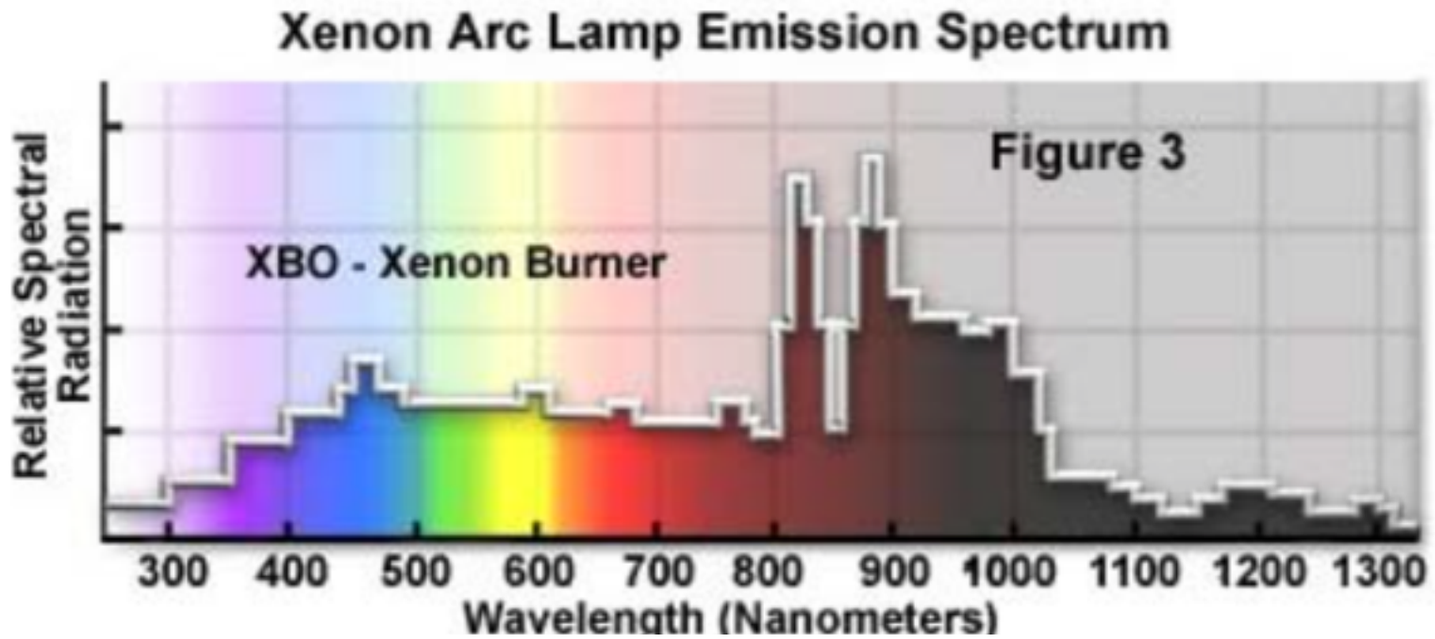


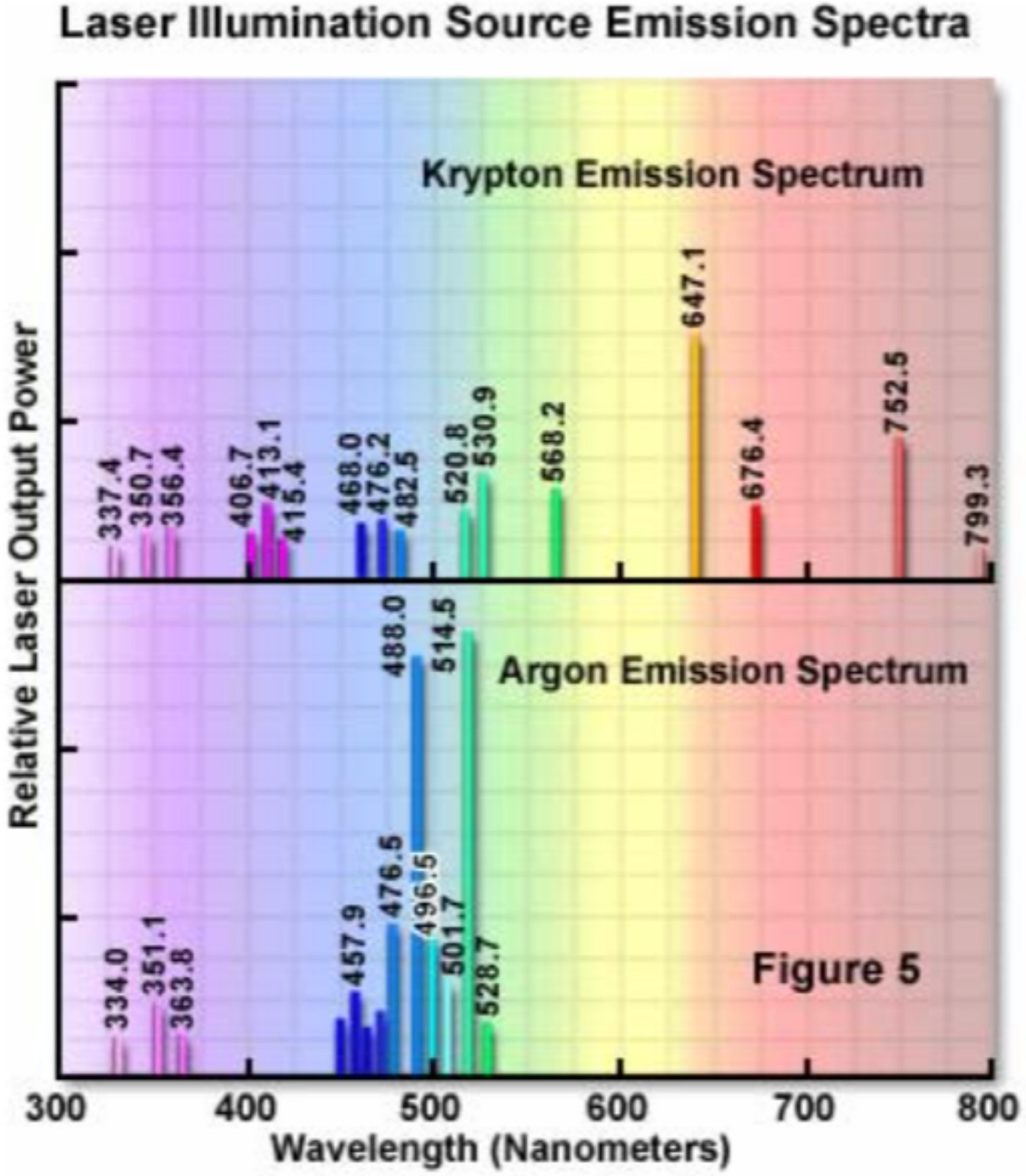
Uneven Spectral Output!



The Epi-Fluorescence Light Path - excitation

The Epi-Fluorescence Light Path - excitation

Lasers
(Confocal / Wide Field)



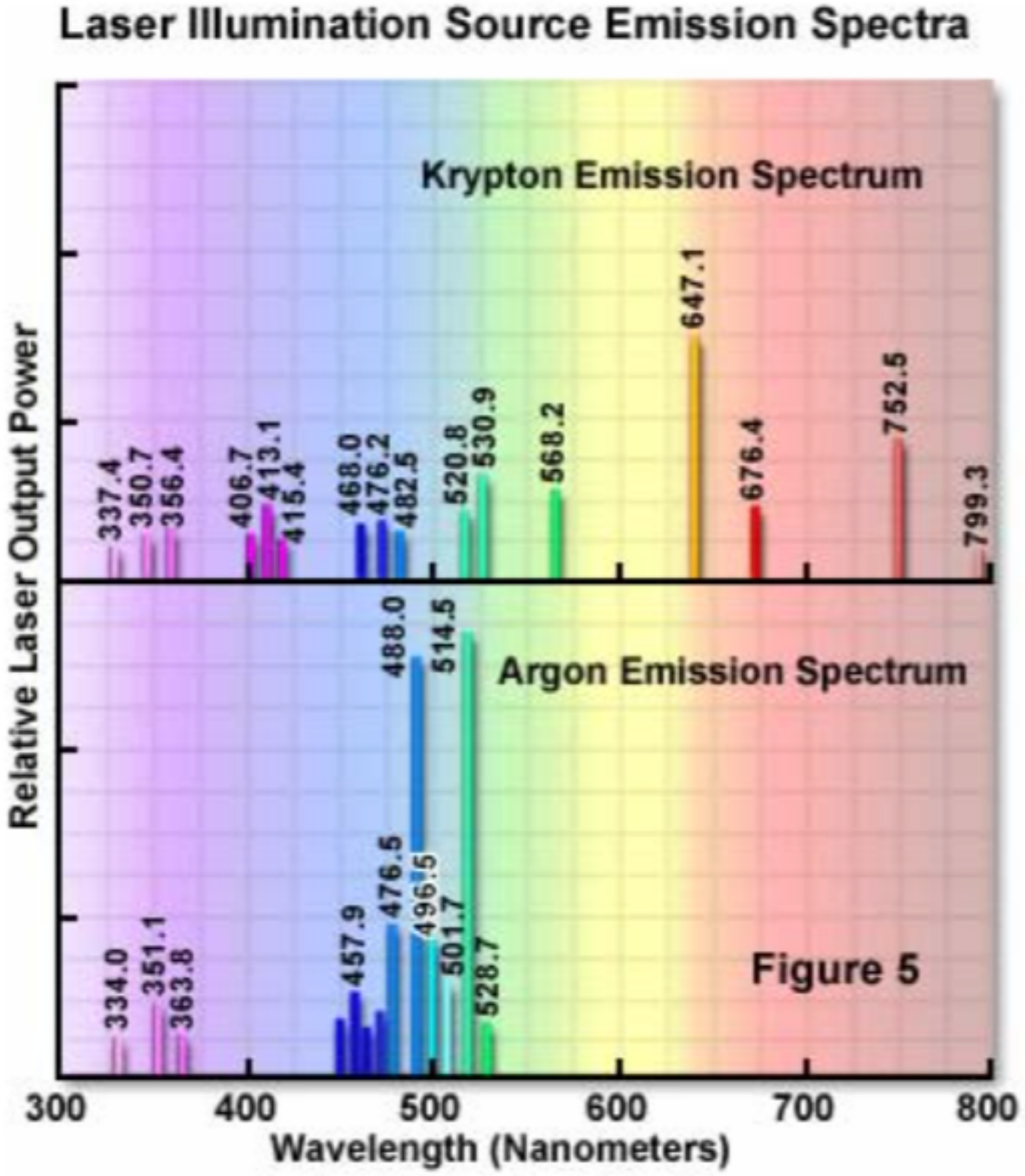
The Epi-Fluorescence Light Path - excitation

Lasers
(Confocal / Wide Field)

Only discrete lines!

lines

405
440
488
514
561
633

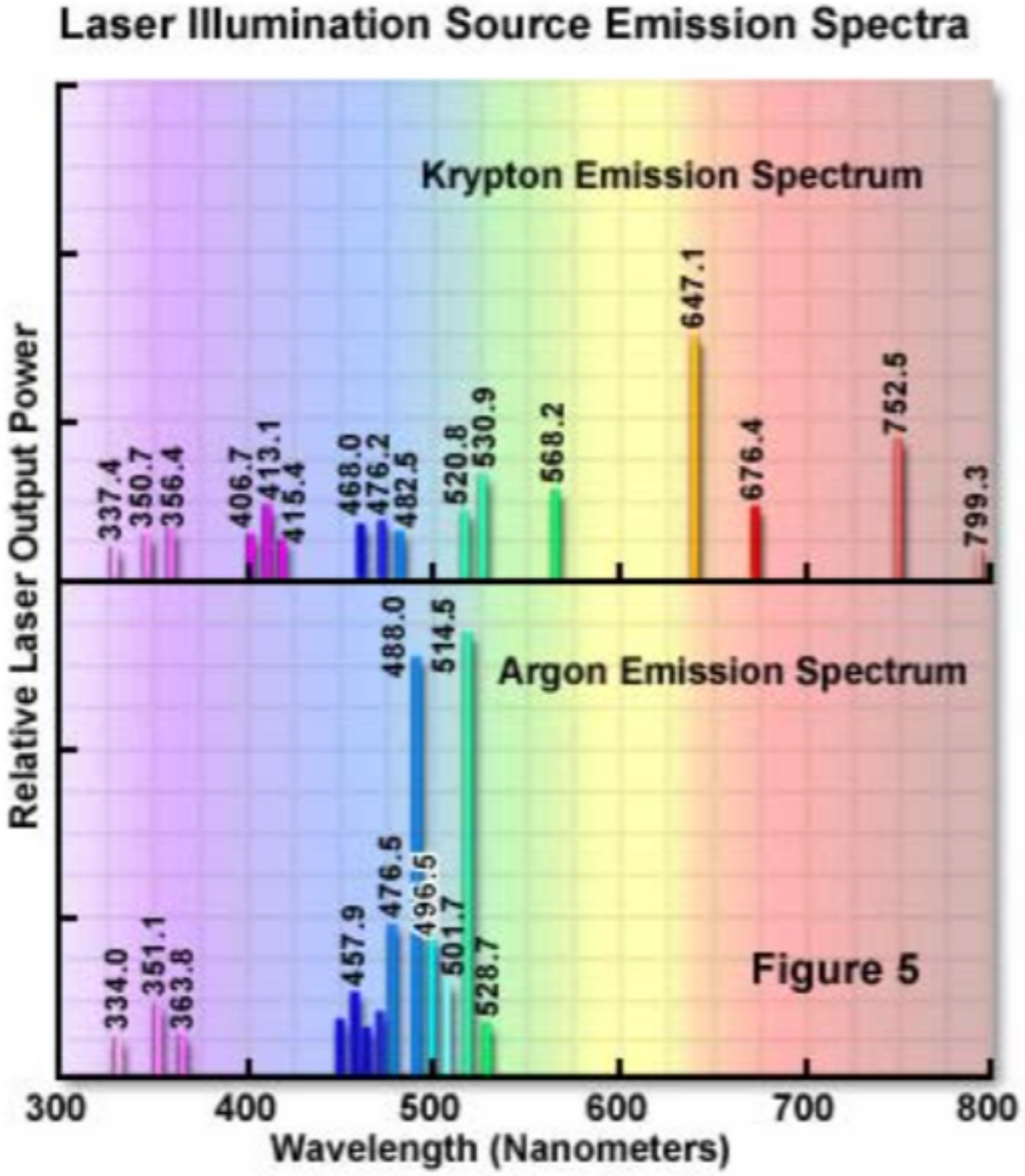


The Epi-Fluorescence Light Path - excitation

Lasers
(Confocal / Wide Field)

Only discrete lines!

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



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Lasers
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Only discrete lines!

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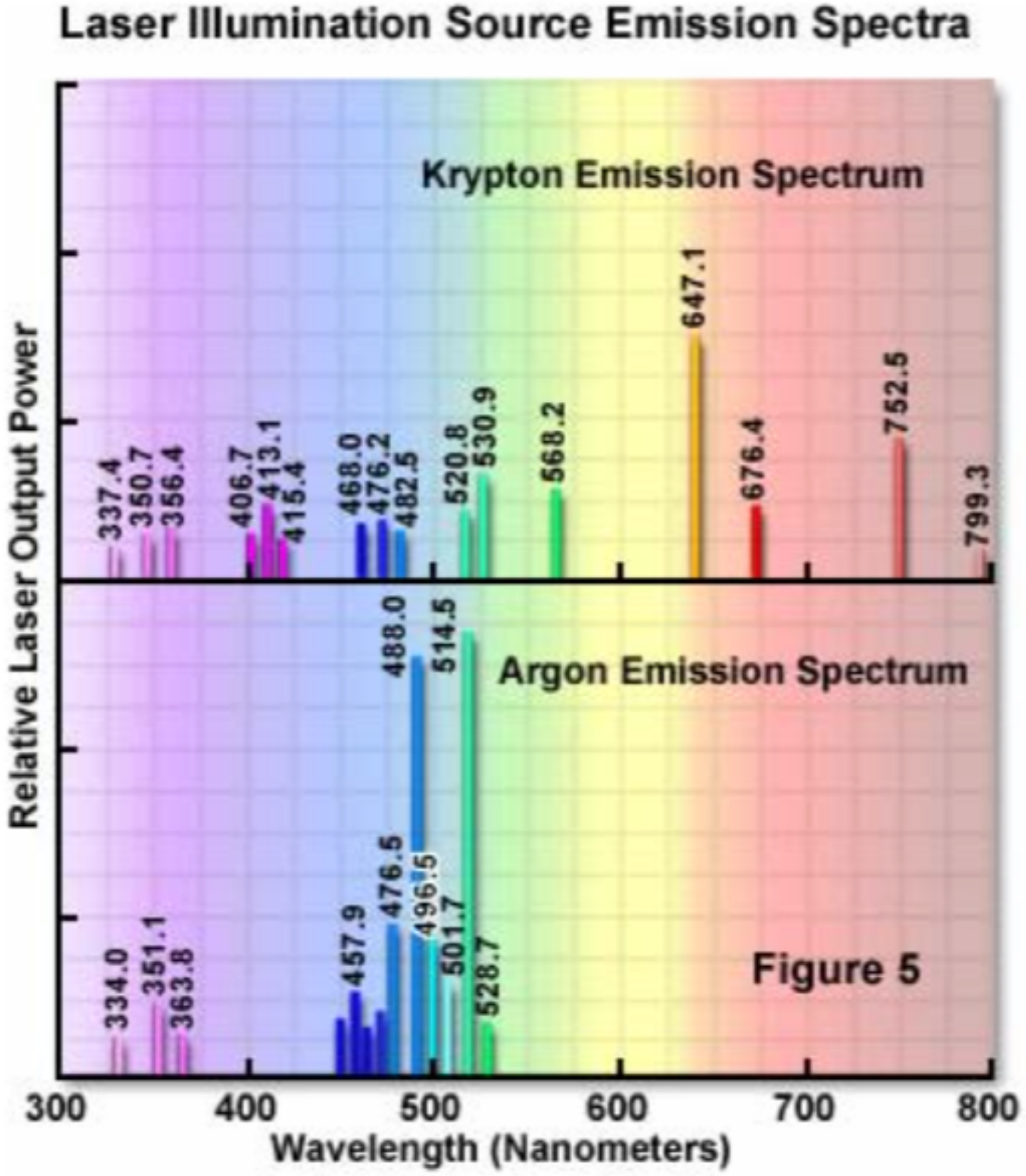
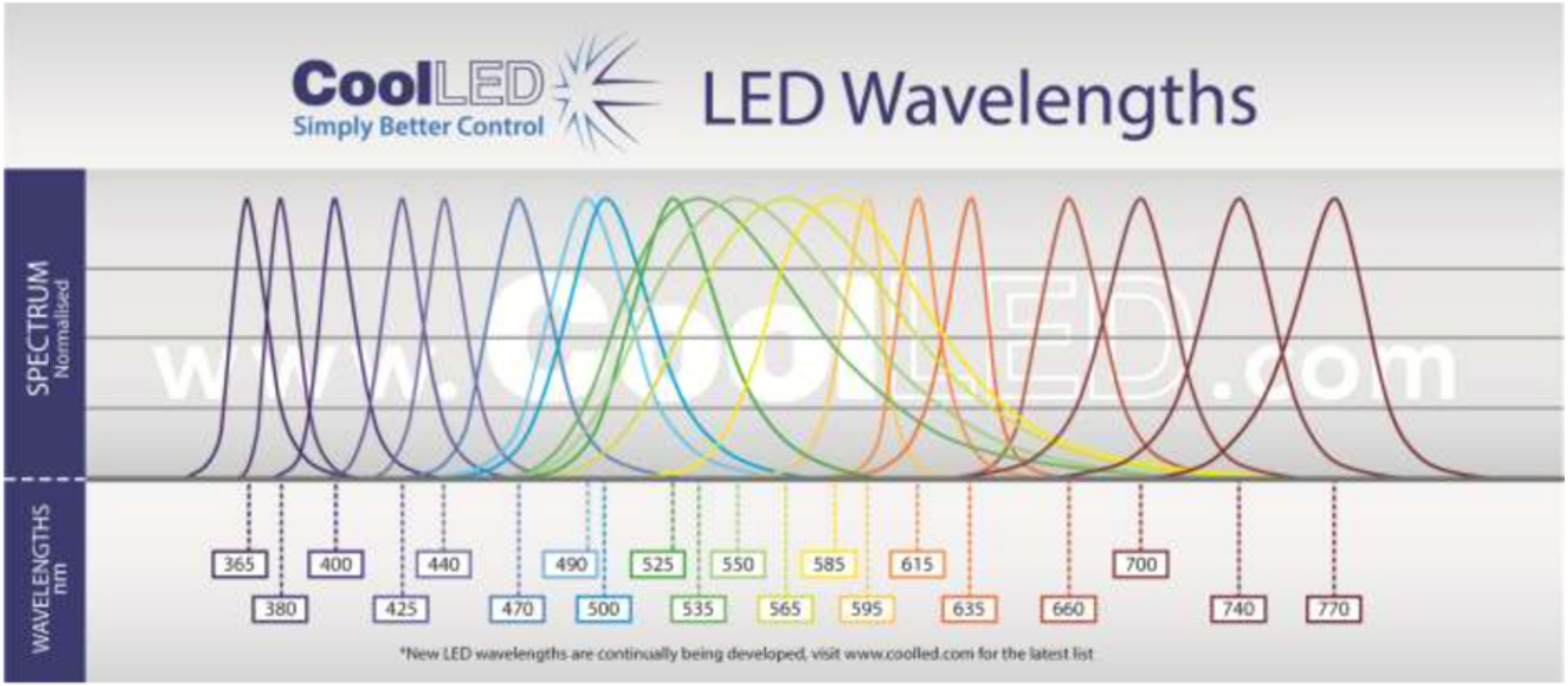
*** Solid State Lasers**



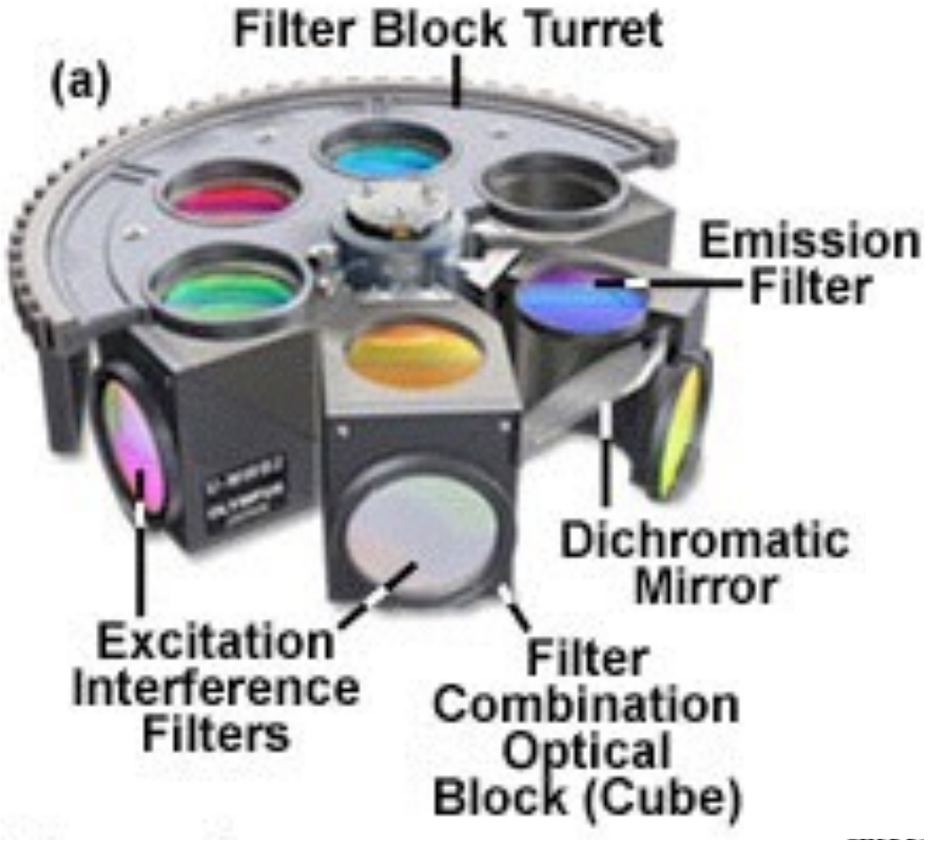
The Epi-Fluorescence Light Path - excitation

Diodes - Wide range of lines available!
(Wide field)



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

The fluorescence filter set



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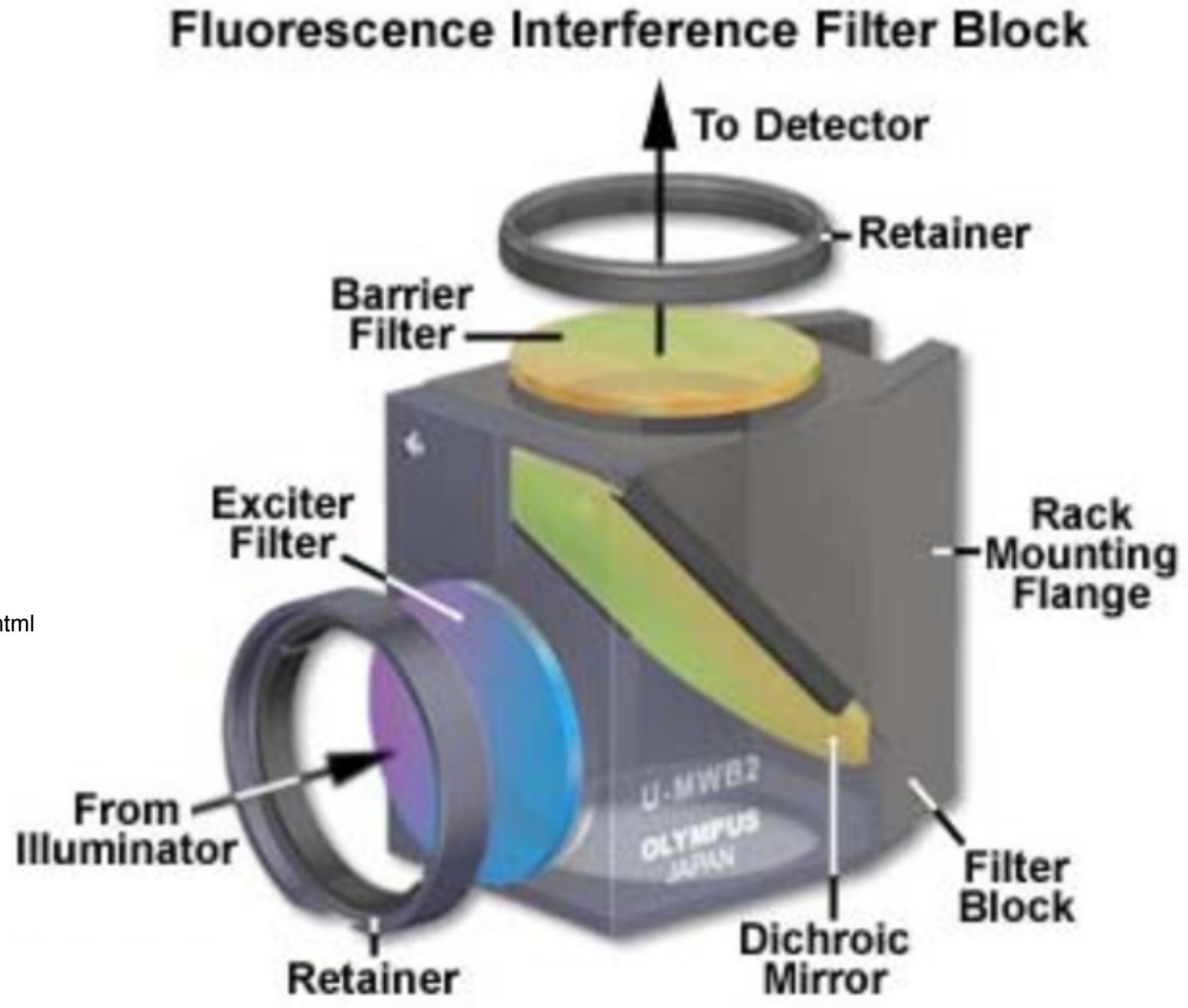

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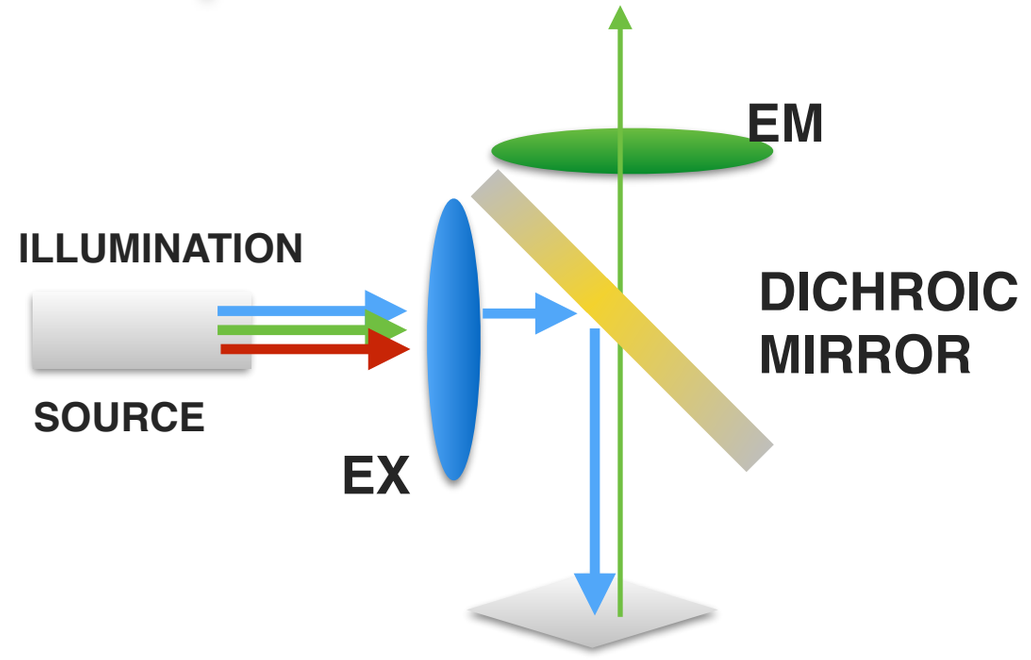
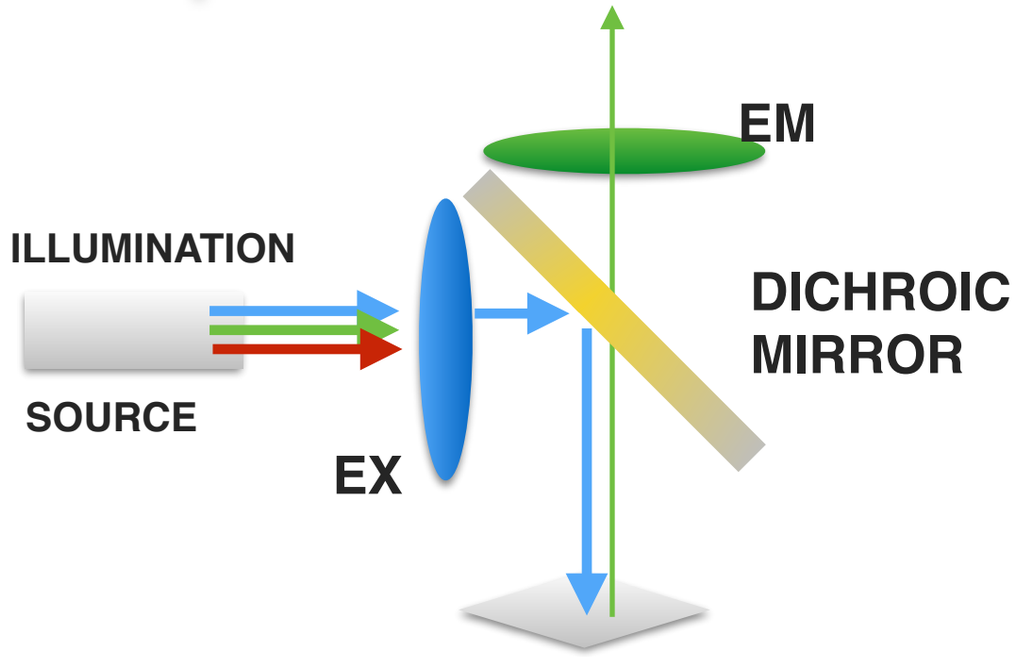
The Dichroic mirror - at the heart of epifluorescence



The Dichroic mirror - at the heart of epifluorescence

Separates Excitation from Emission



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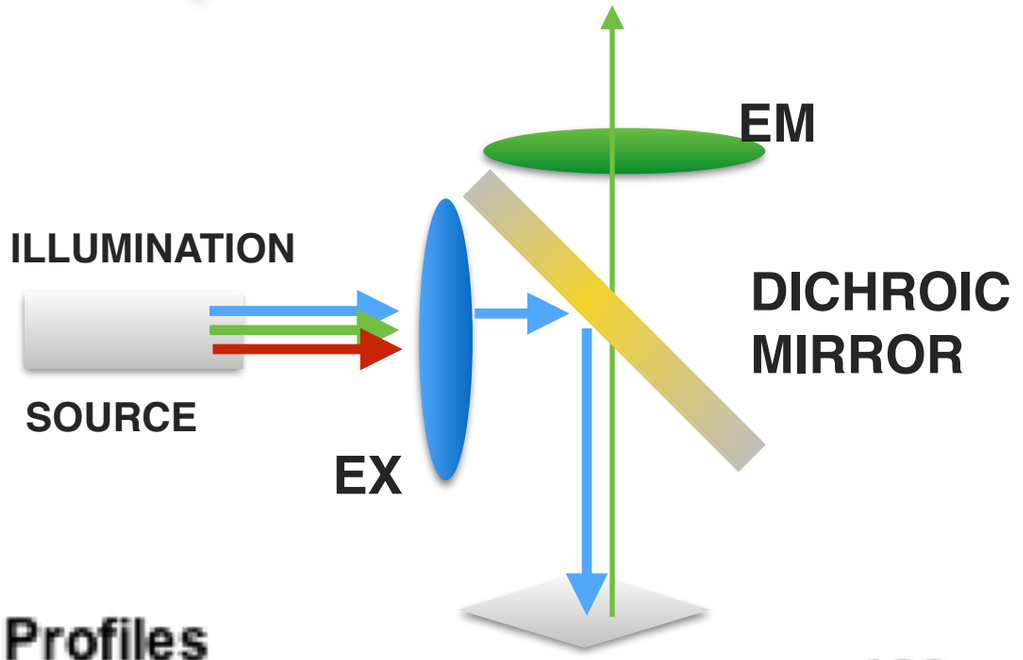
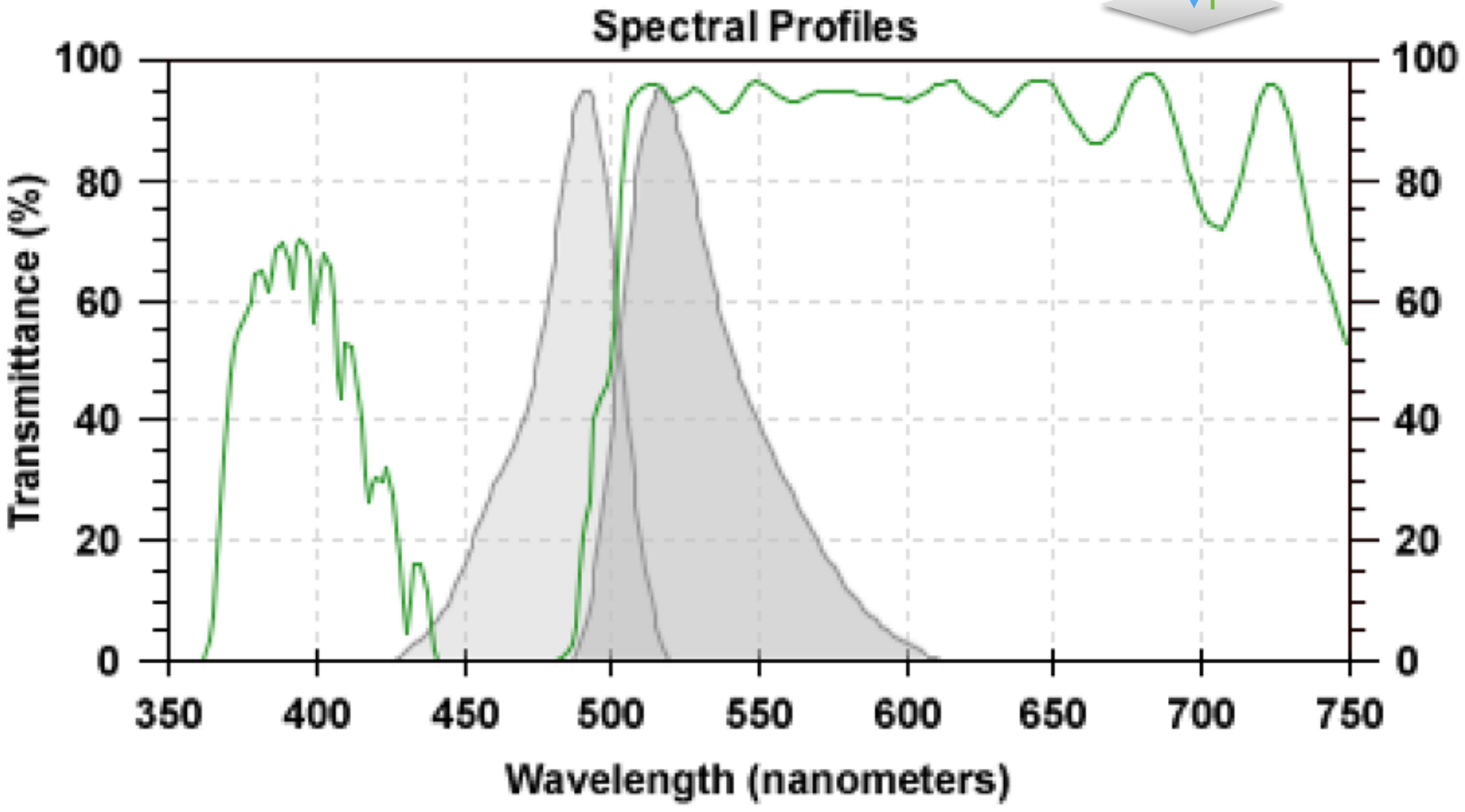
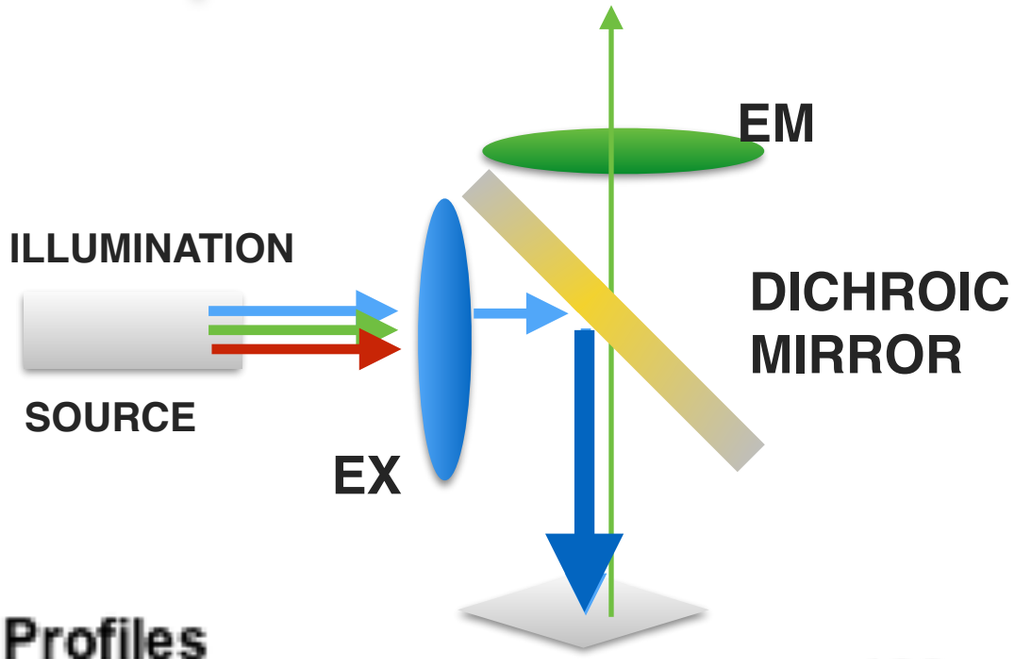
Separates Excitation from Emission

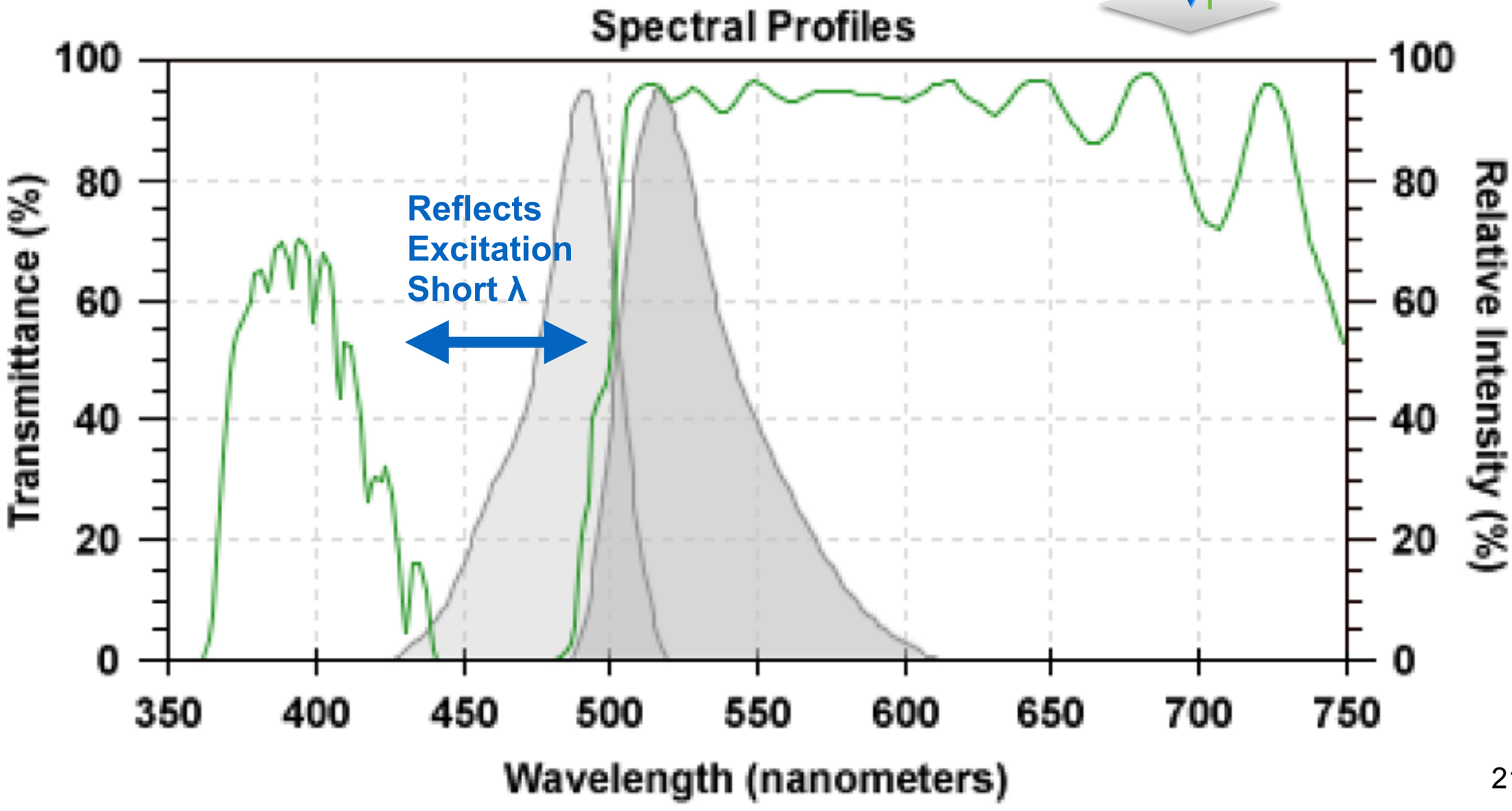




The Dichroic mirror - at the heart of epifluorescence

Separates Excitation from Emission





The Dichroic mirror - at the heart of epifluorescence

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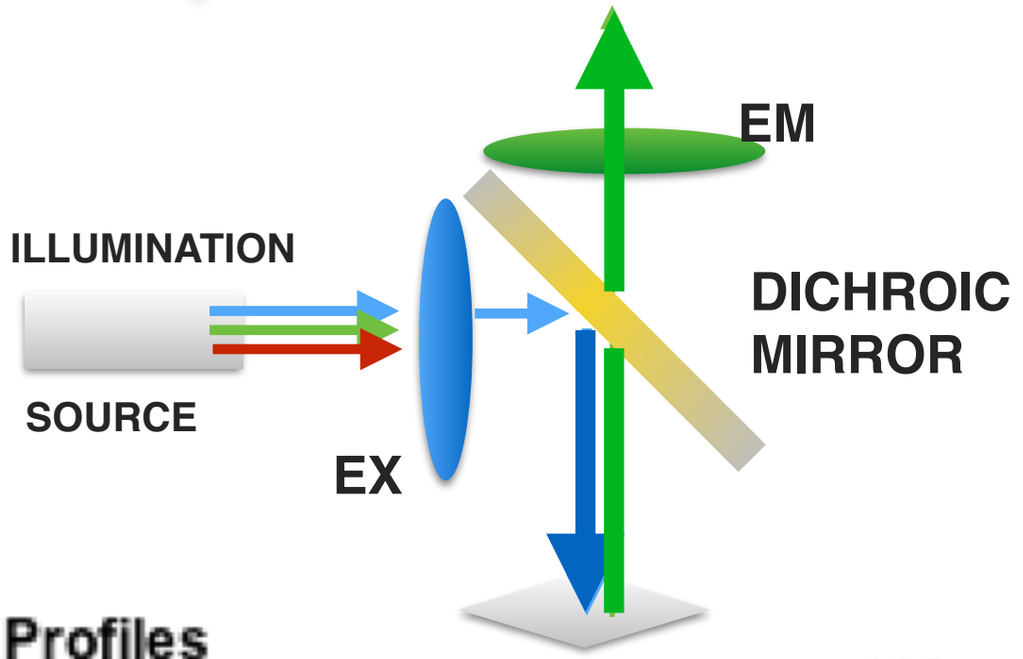
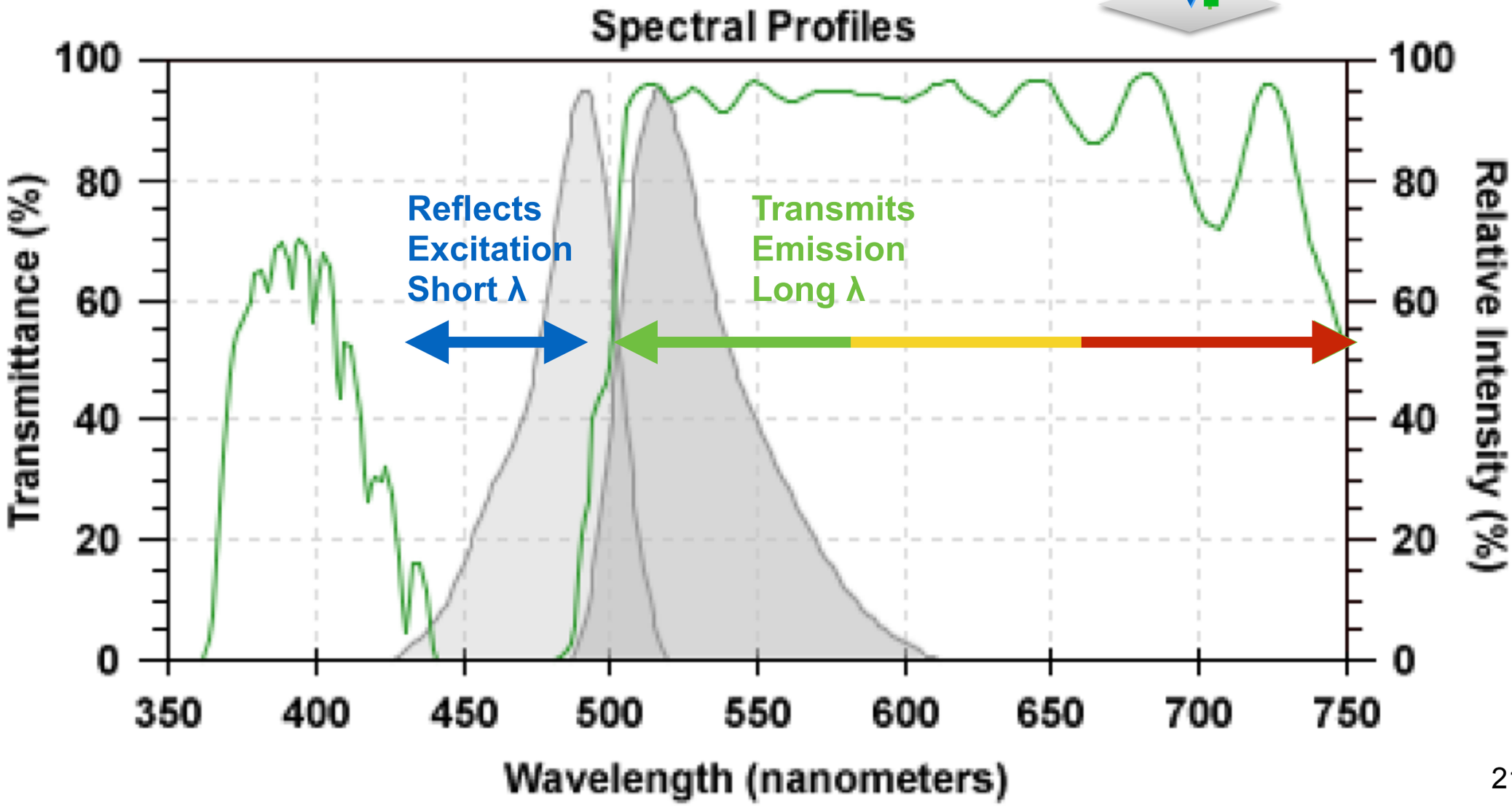
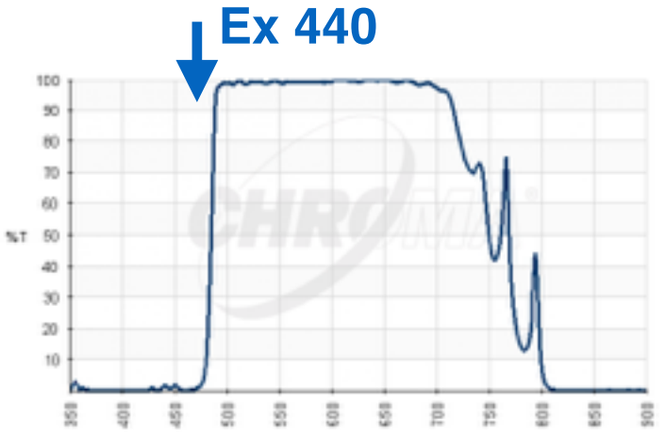




The Dichroic mirror - at the heart of epifluorescence

Typical Dichroic



The Dichroic mirror - at the heart of epifluorescence

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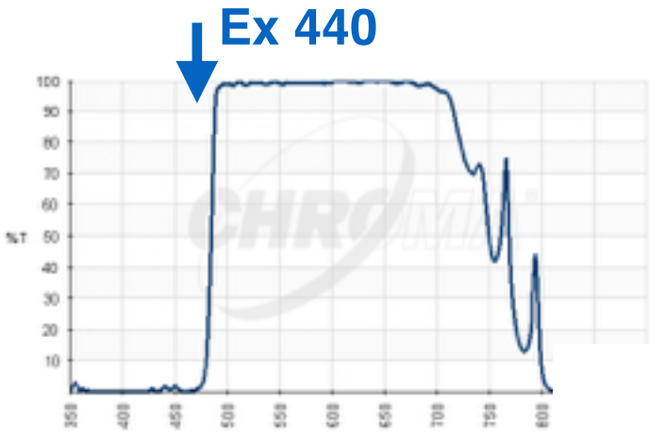
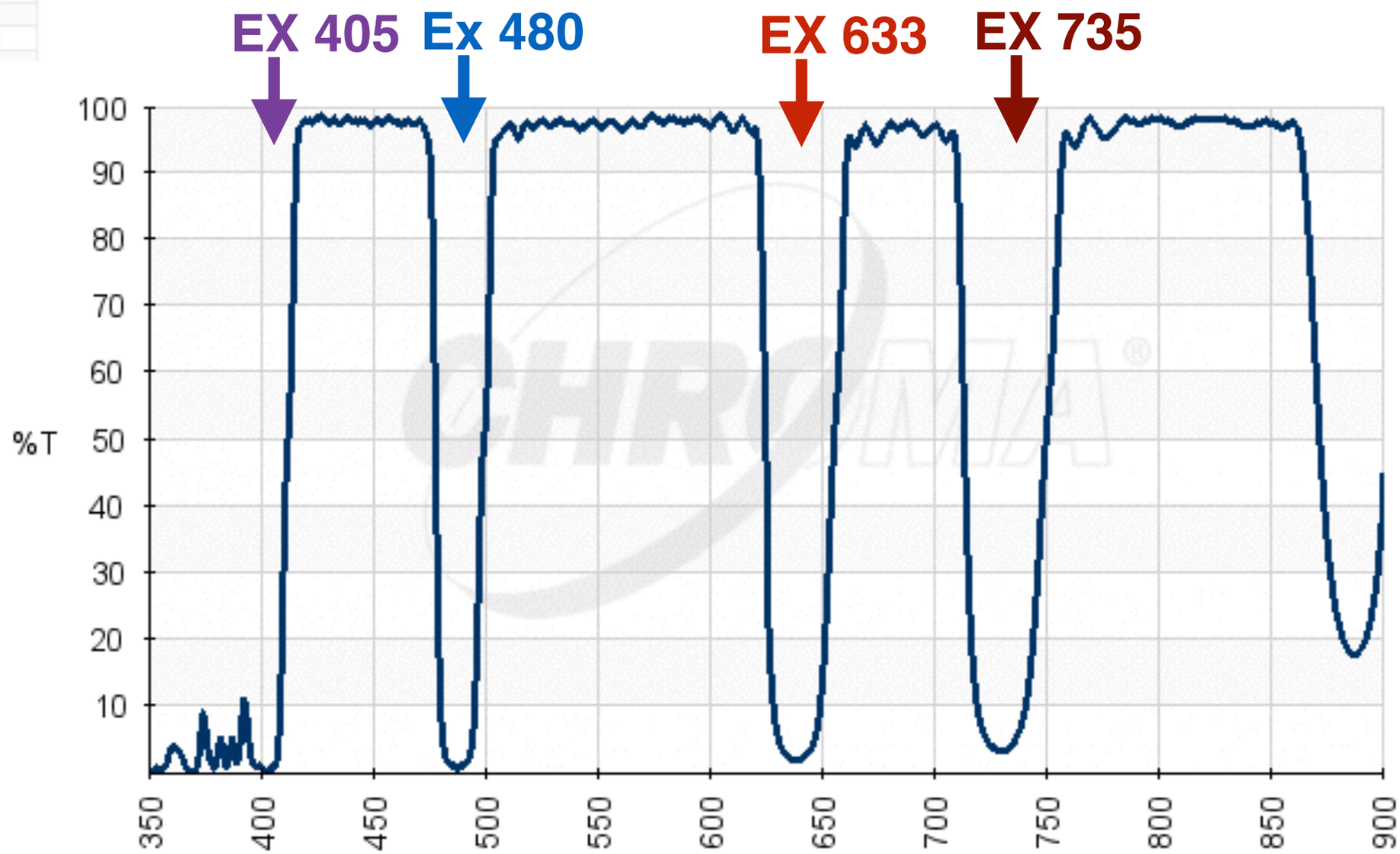
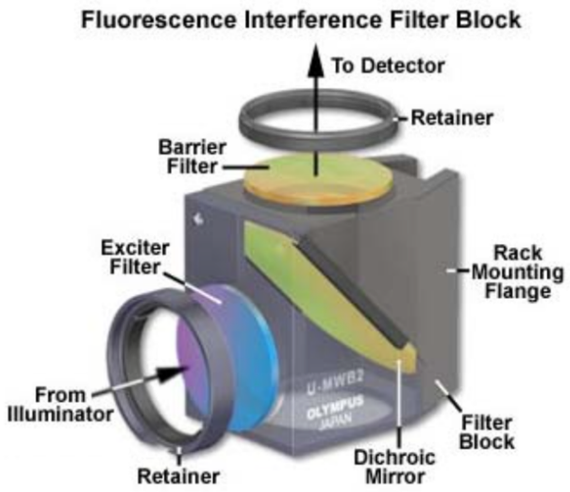


Also Polychroic!

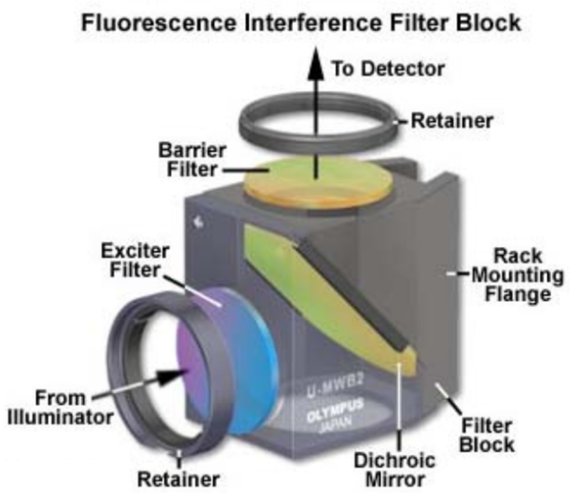


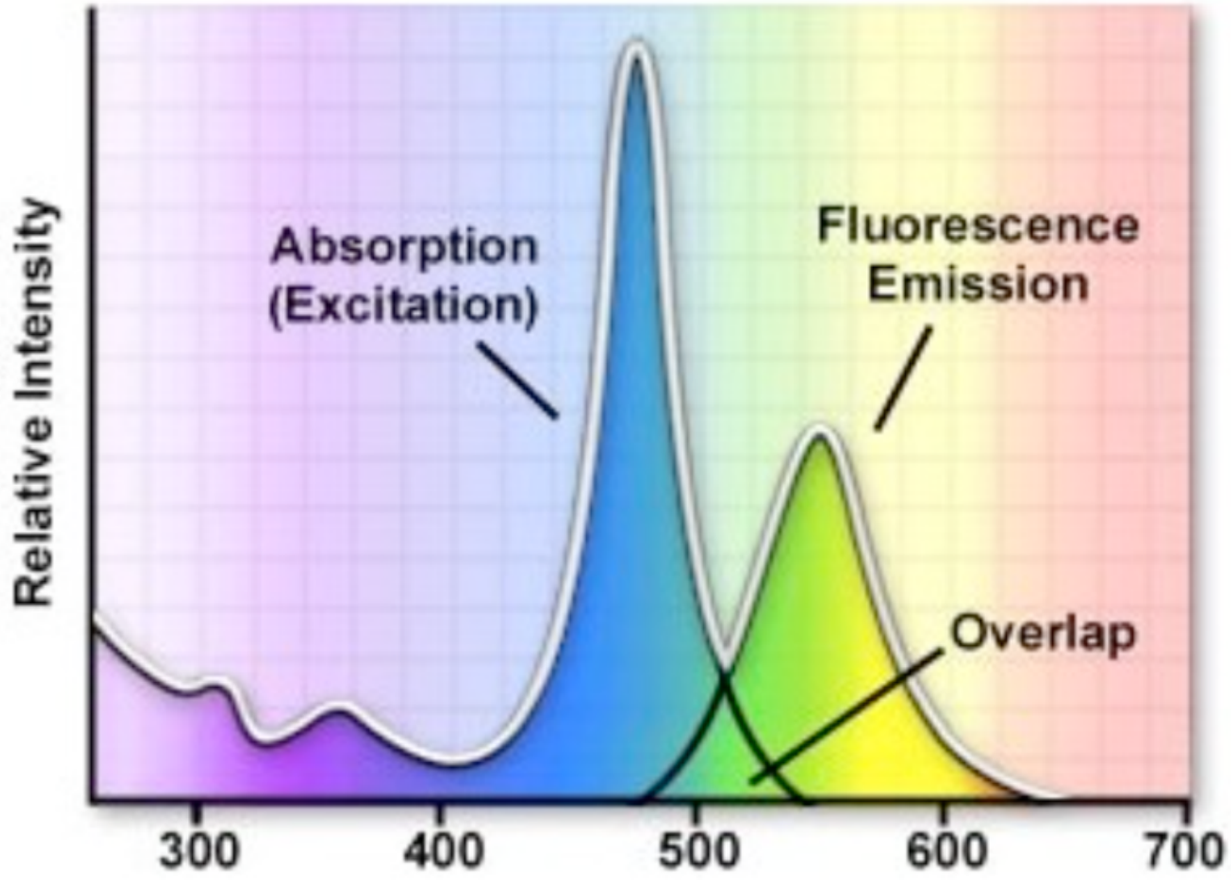
Choosing / configuring a fluorescence filter set



Choosing / configuring a fluorescence filter set

……..Relate to dye spectrum





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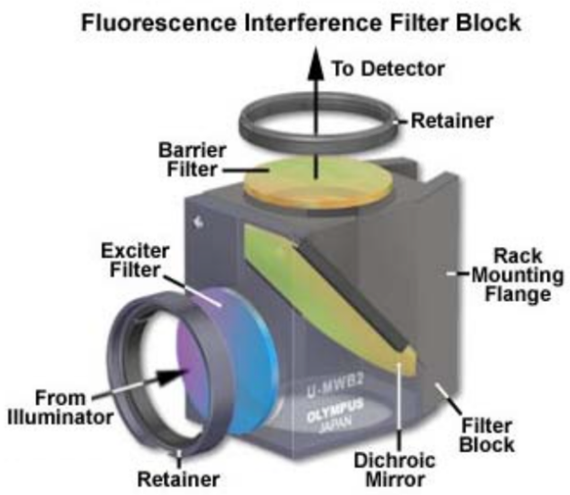
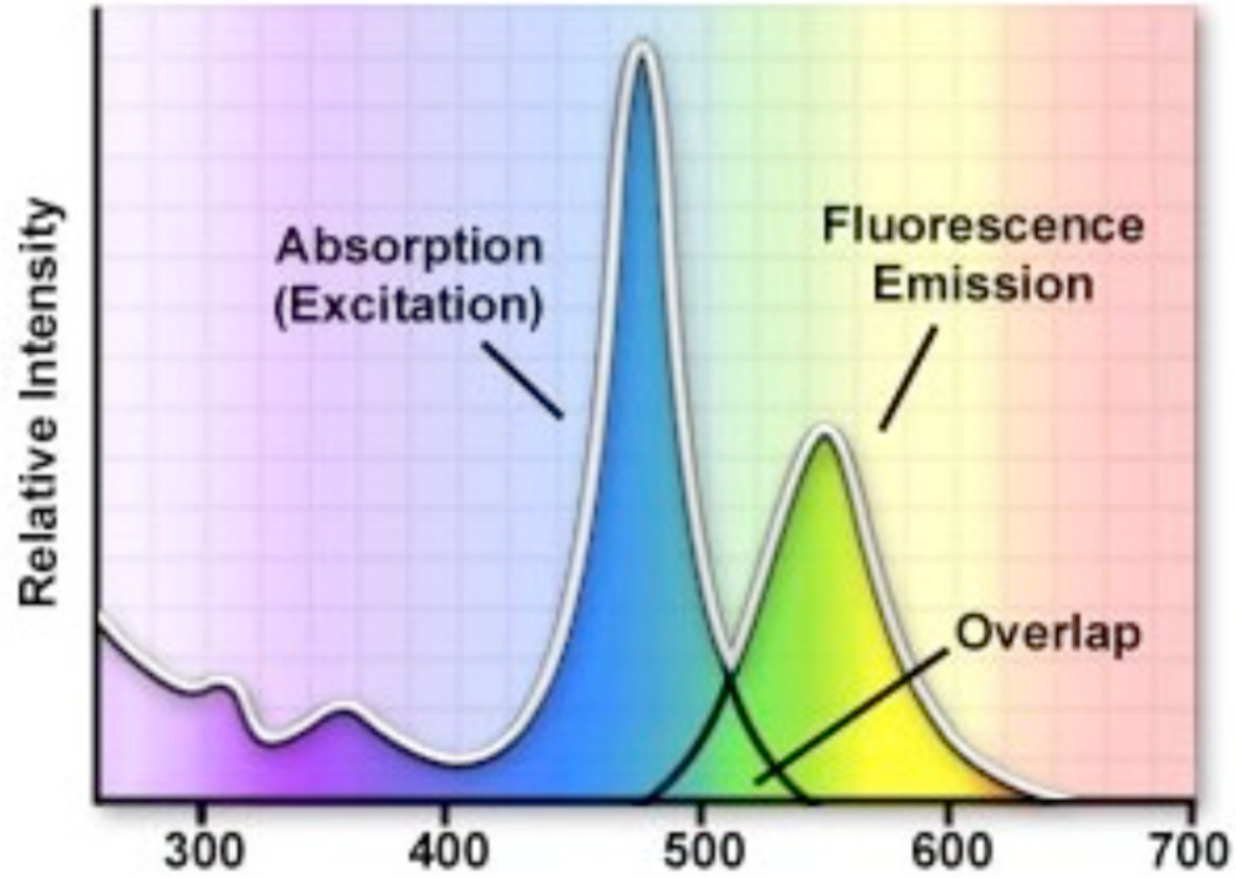
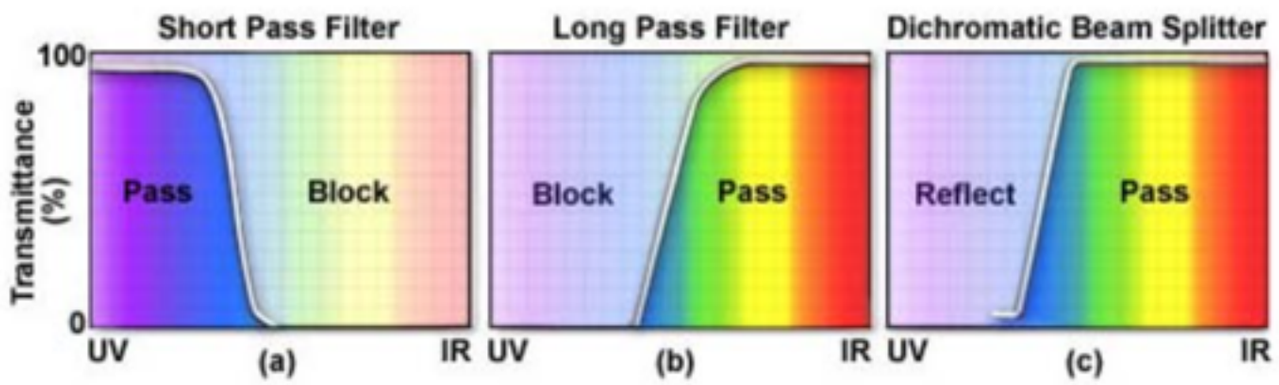


……..Relate to dye spectrum

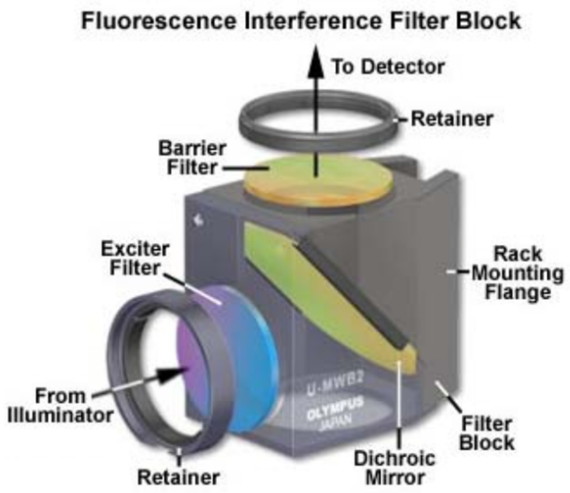


EX filter **EM filter** **Dichroic**



http://olympus.magnet.fsu.edu/primer/java/fluorescence/matchingfilters/index.html

Choosing / configuring a fluorescence filter set



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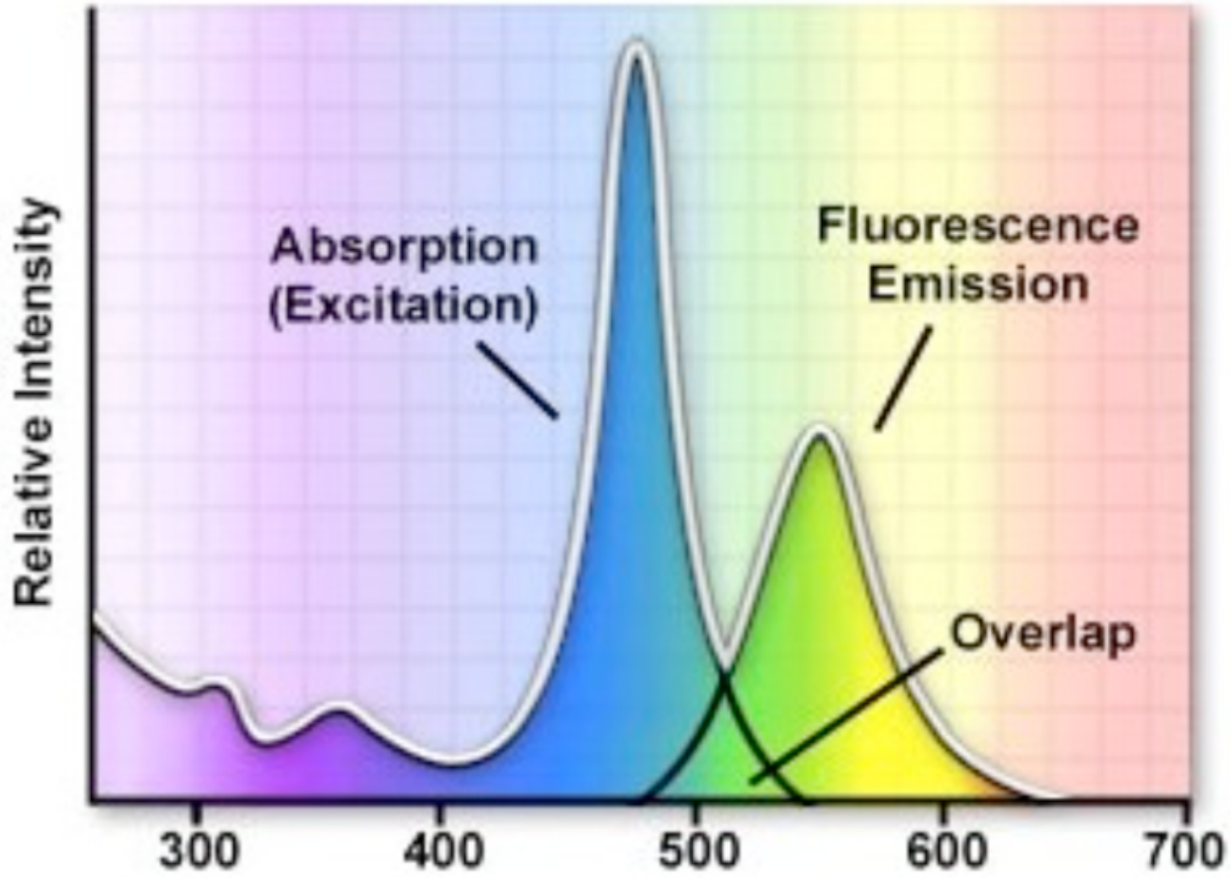
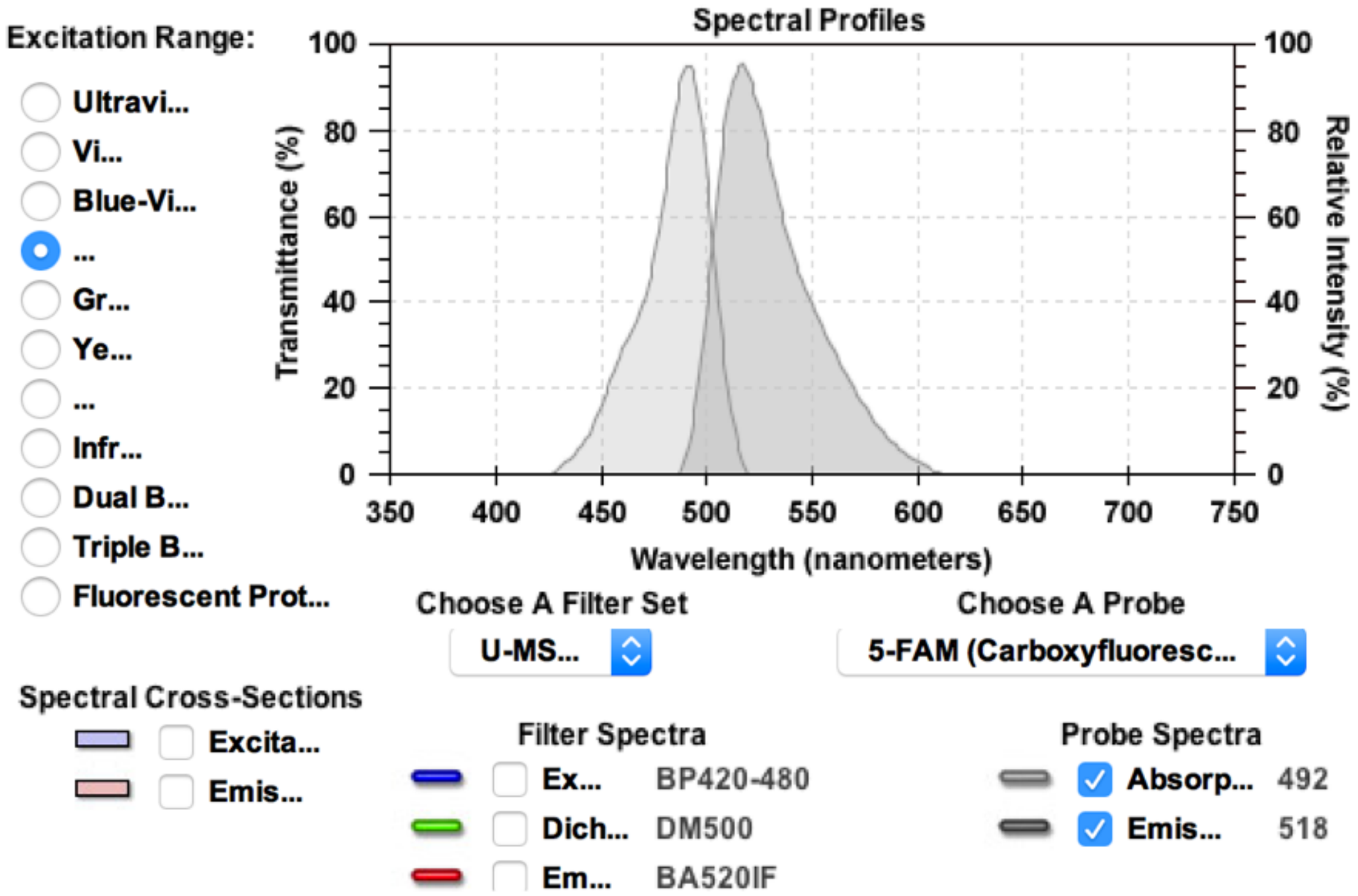


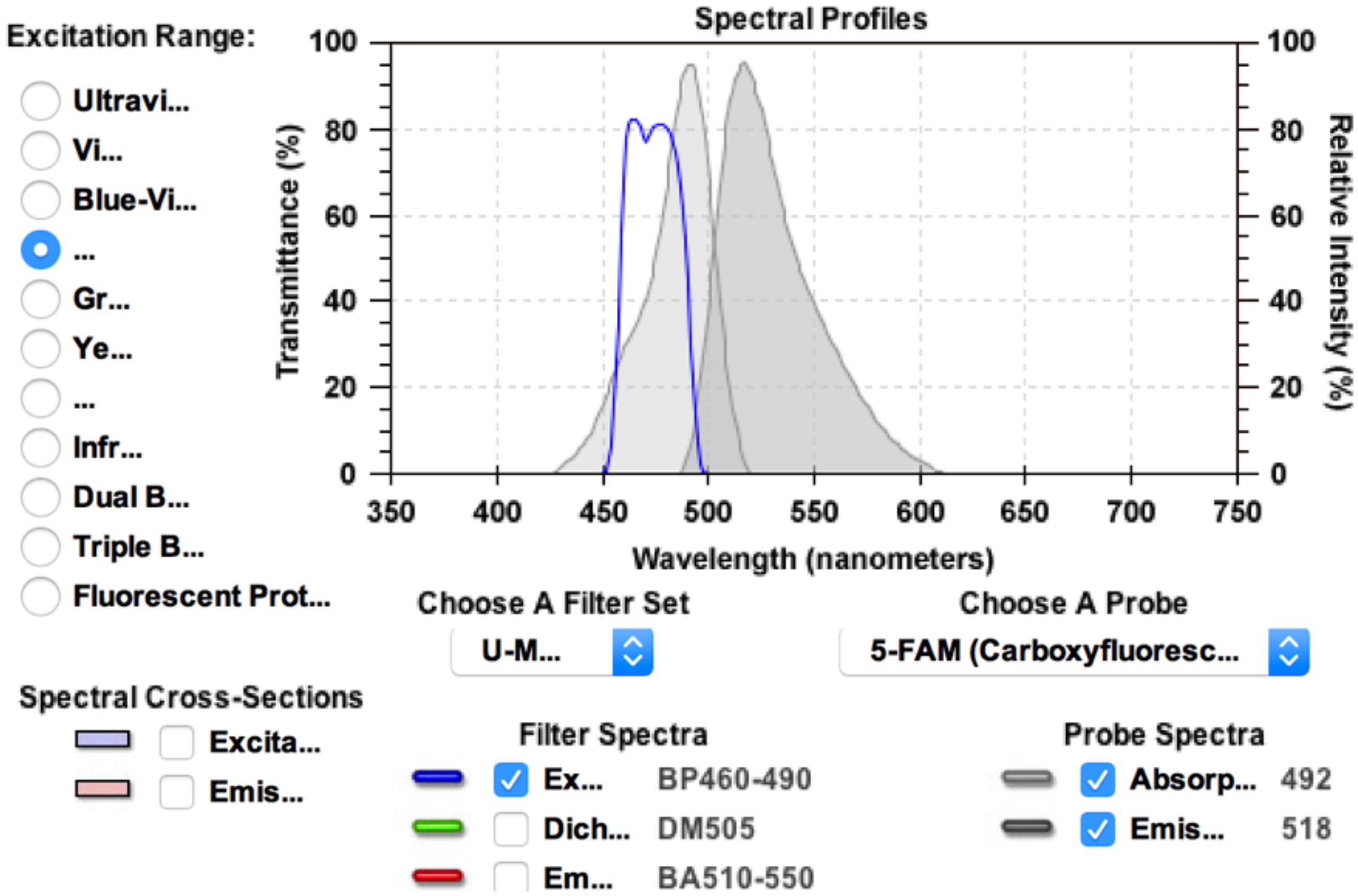
optimise signal and separation!

Identify your dye:



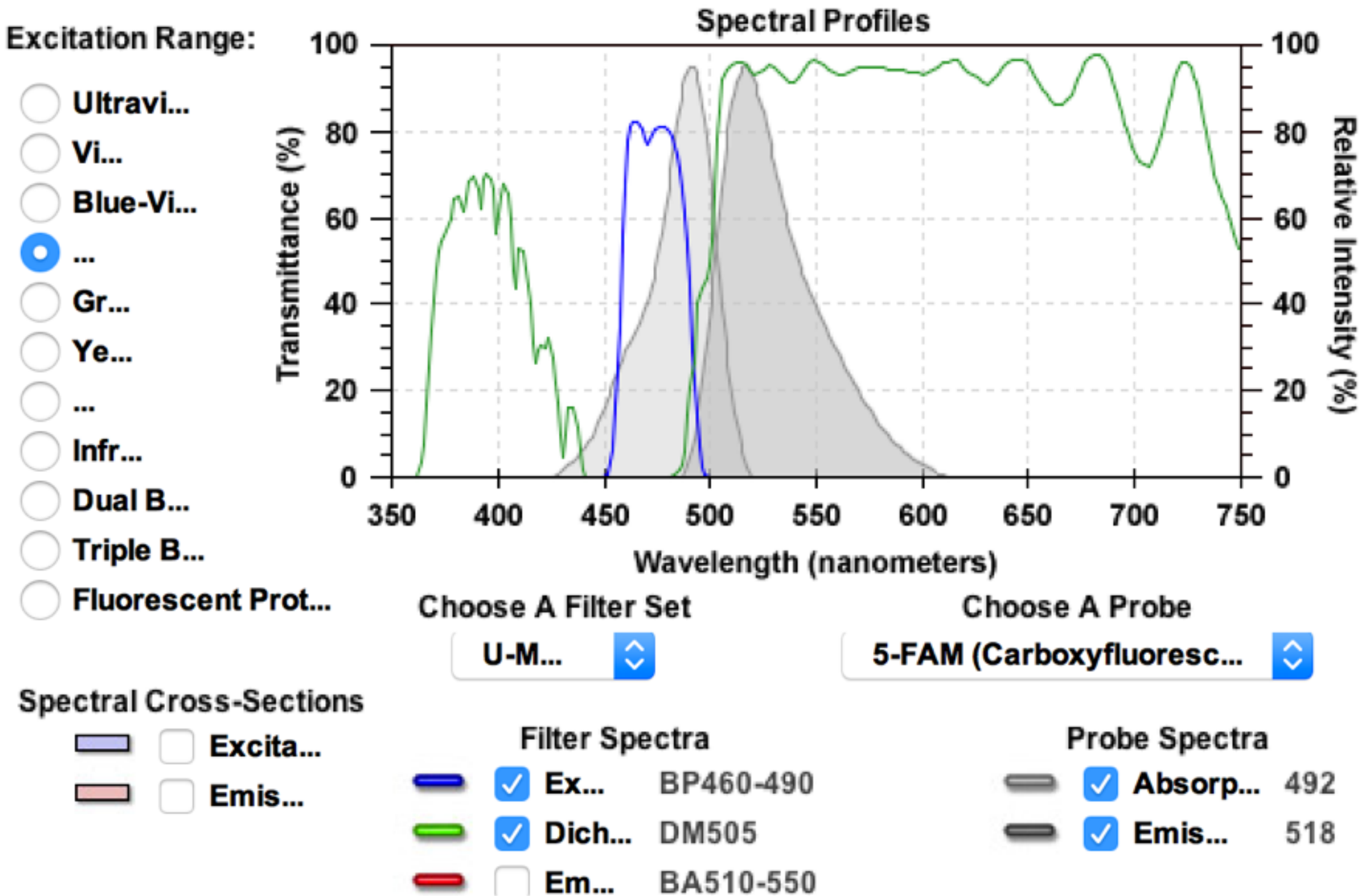
http://olympus.magnet.fsu.edu/primer/java/fluorescence/matchingfilters/index.html

Identify your excitation:



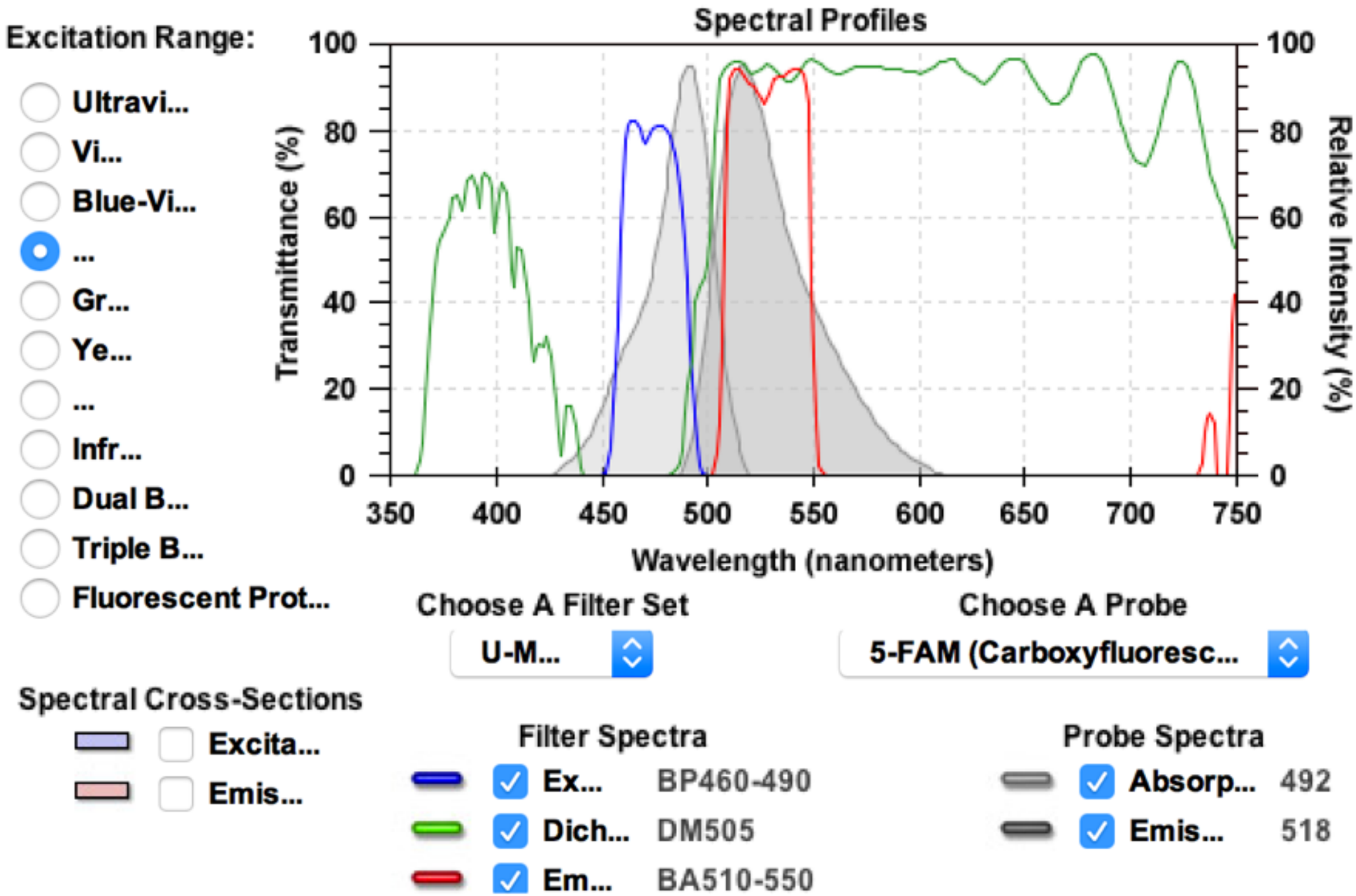
http://olympus.magnet.fsu.edu/primer/java/fluorescence/matchingfilters/index.html

Identify your Dichroic:



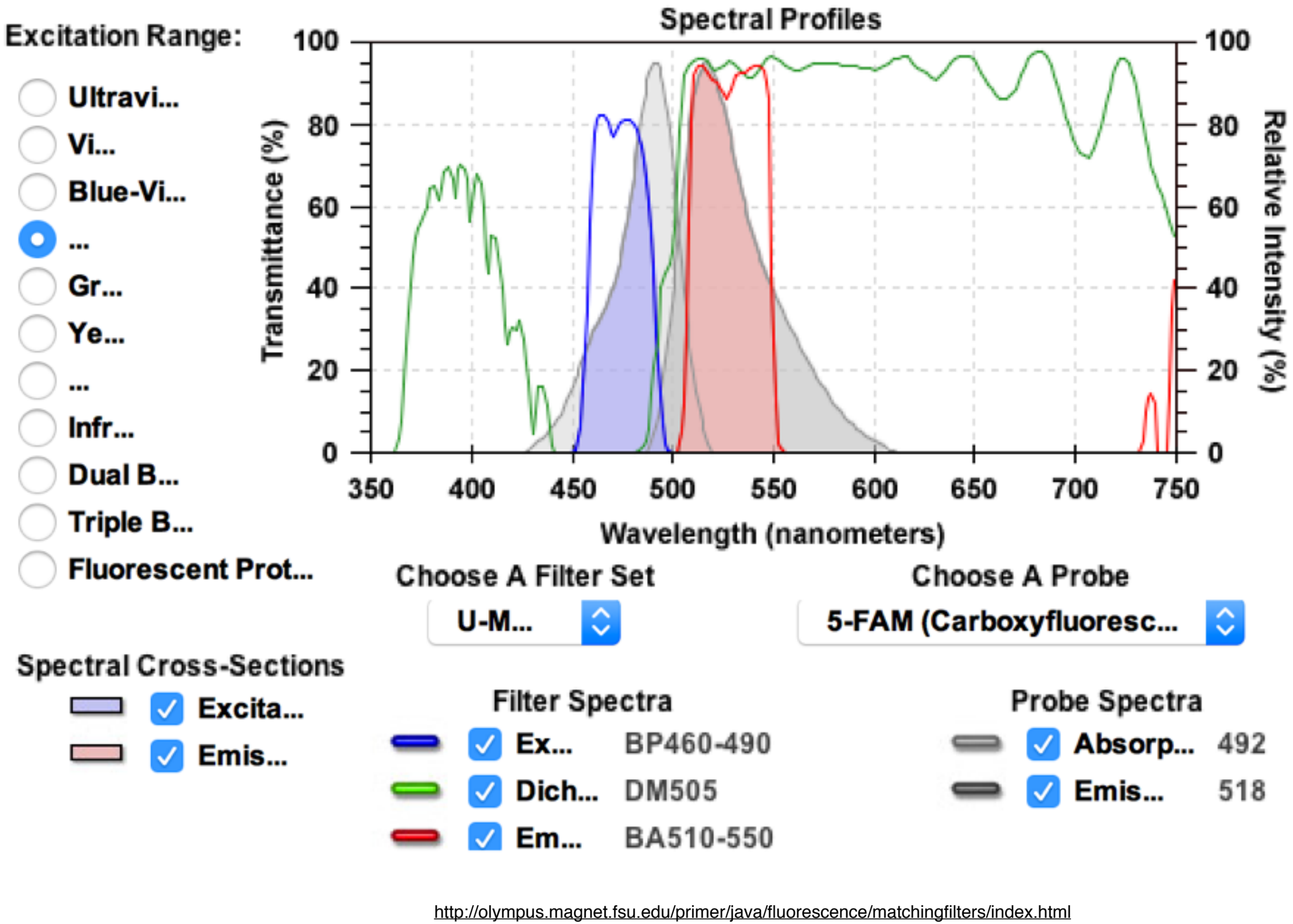
http://olympus.magnet.fsu.edu/primer/java/fluorescence/matchingfilters/index.html

Identify your emission:



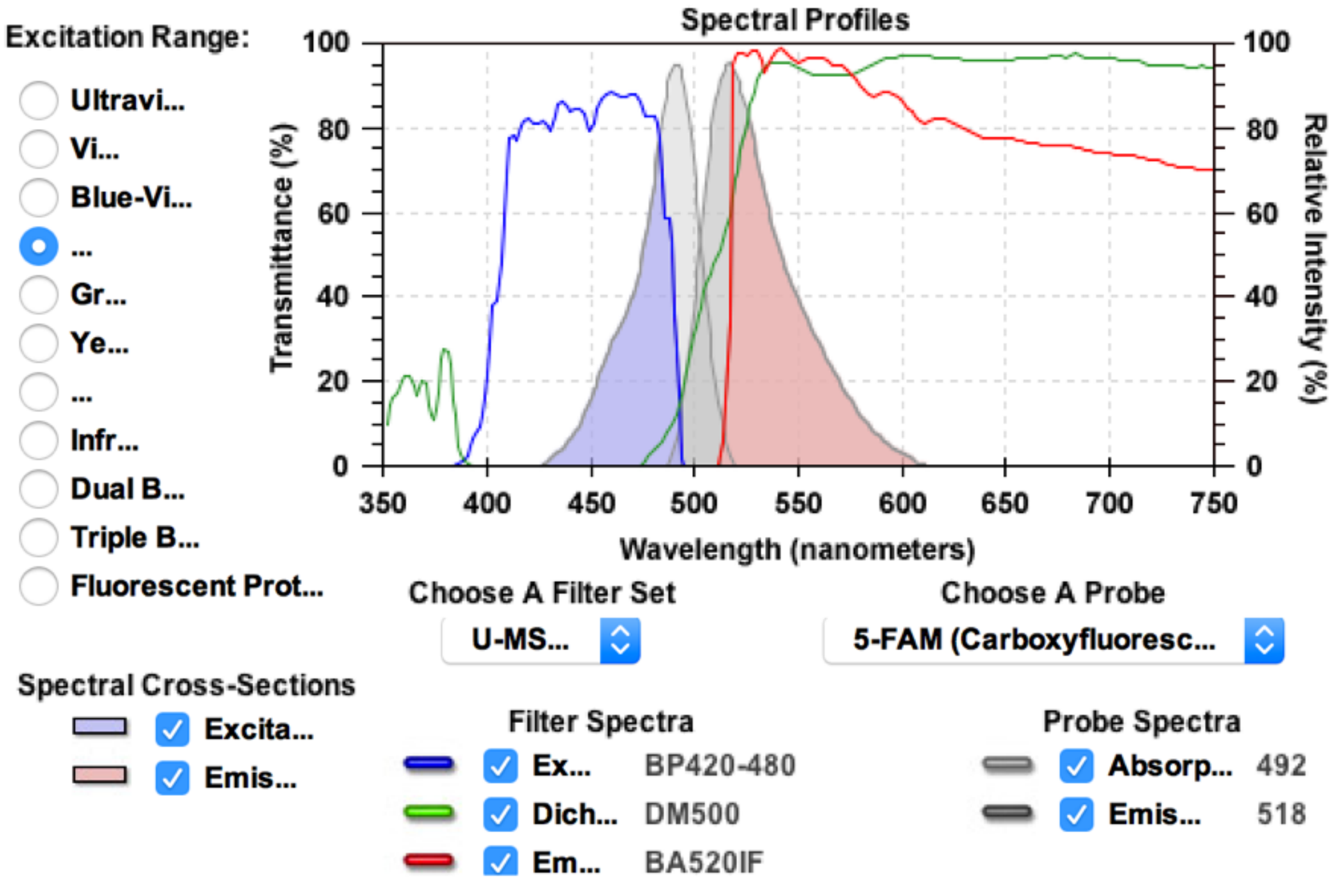
http://olympus.magnet.fsu.edu/primer/java/fluorescence/matchingfilters/index.html

Assess your optimisation:



http://olympus.magnet.fsu.edu/primer/java/fluorescence/matchingfilters/index.html

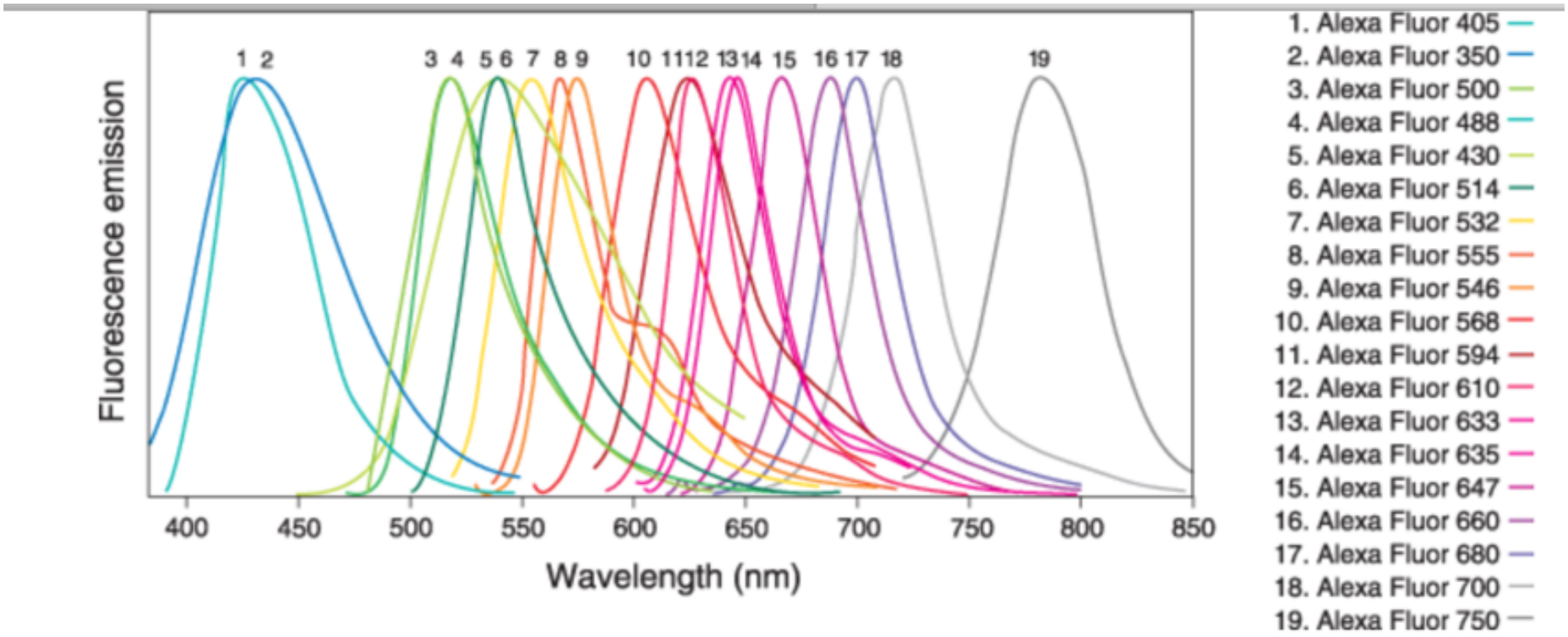
Consider alternatives:



http://olympus.magnet.fsu.edu/primer/java/fluorescence/matchingfilters/index.html

Effective Multiplexing

….know your dyes!



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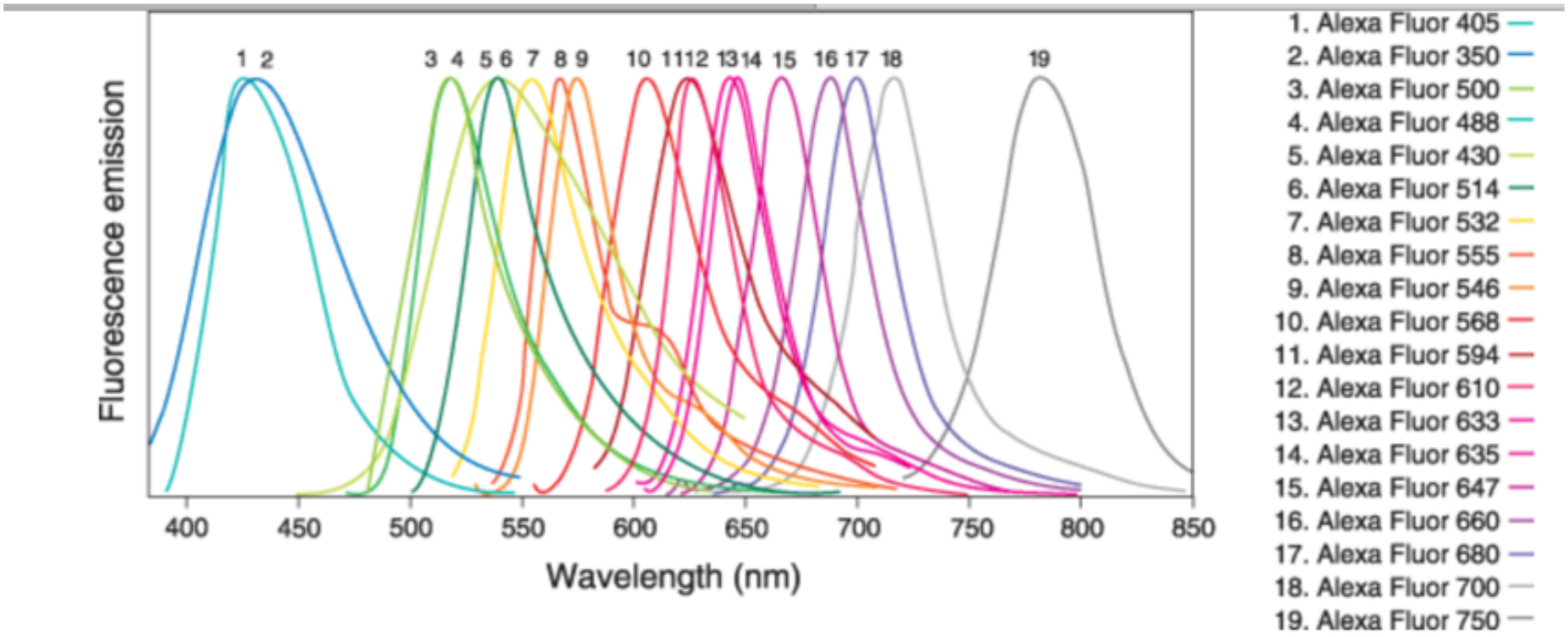
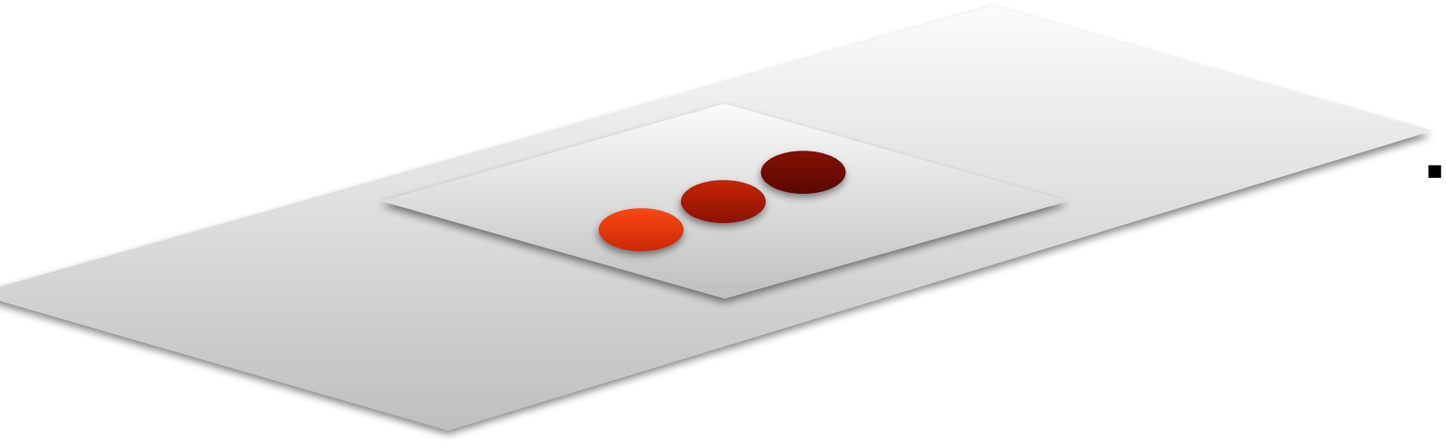




….test your dyes!

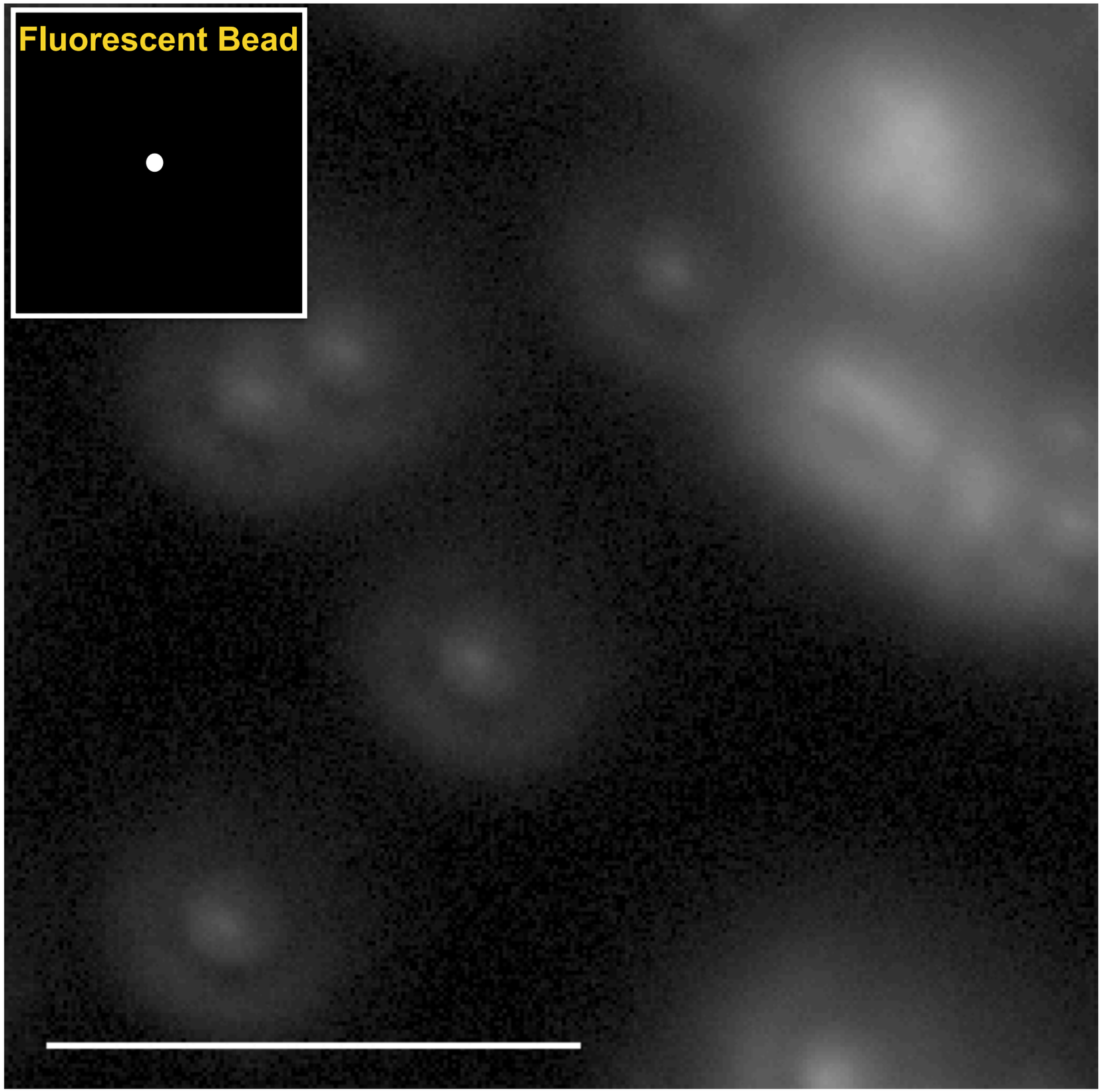
PSF / OTF In fluorescence

PSF / OTF In fluorescence:

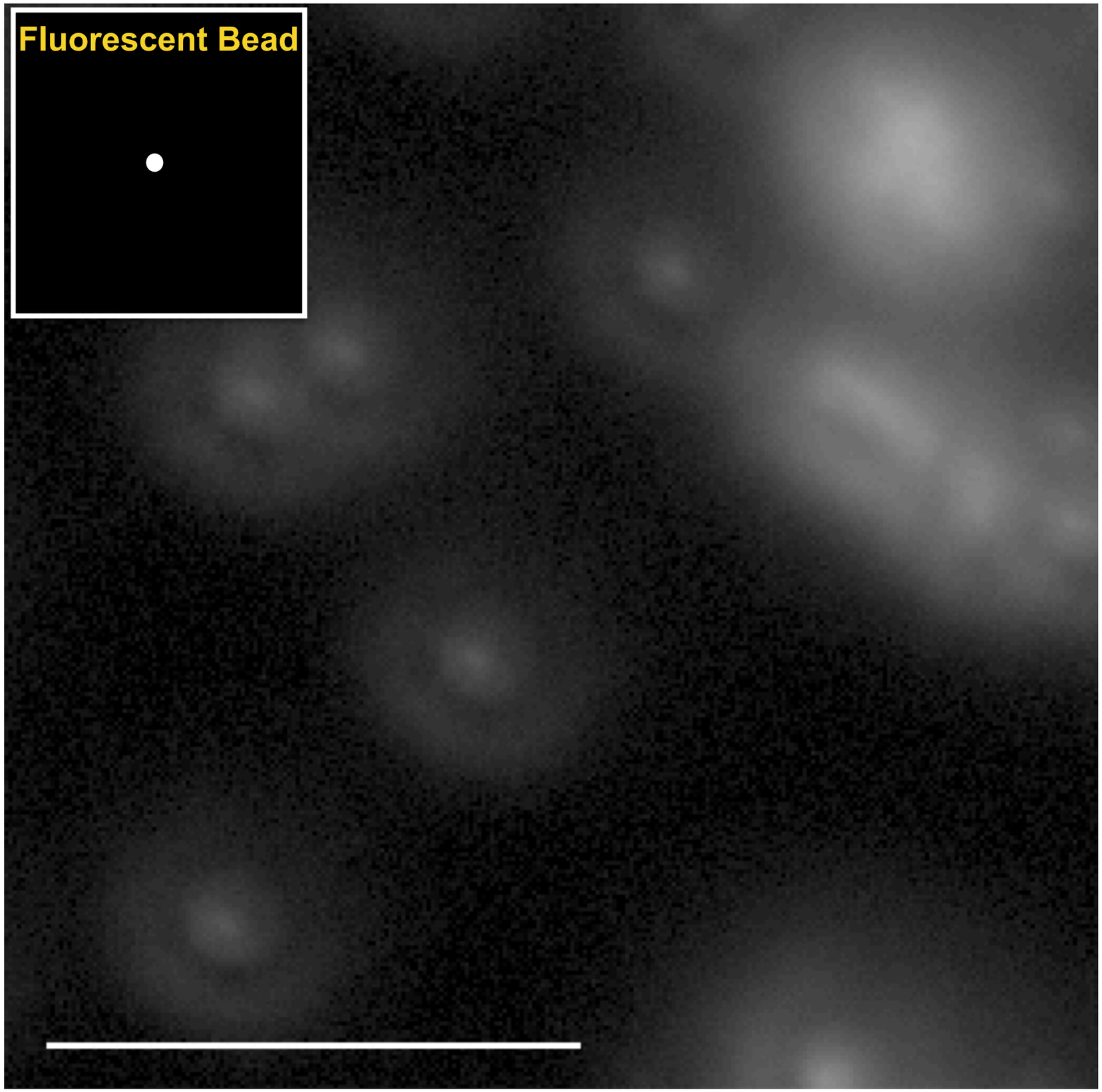
PSF / OTF In fluorescence:

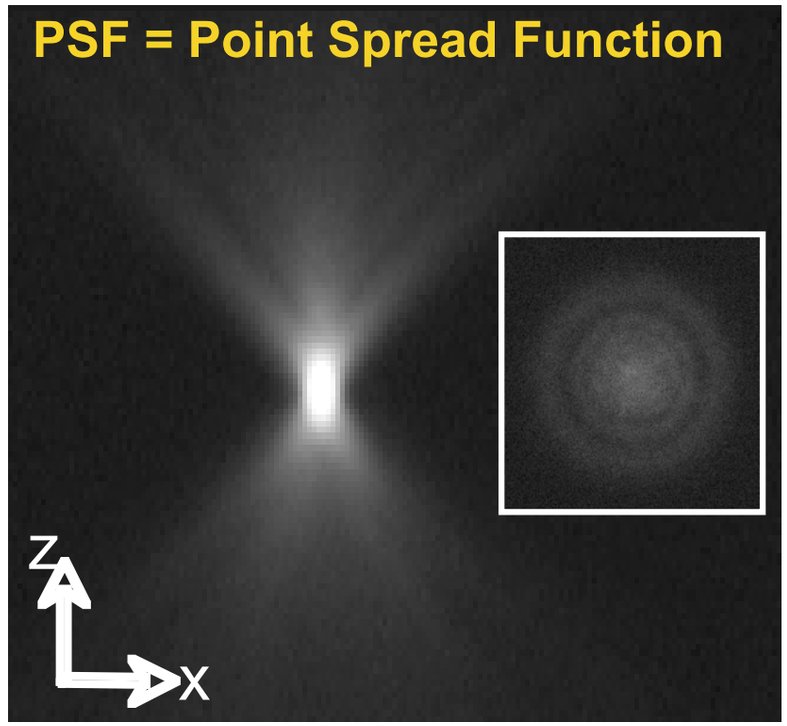
Imaging a point-source of light

PSF / OTF In fluorescence:

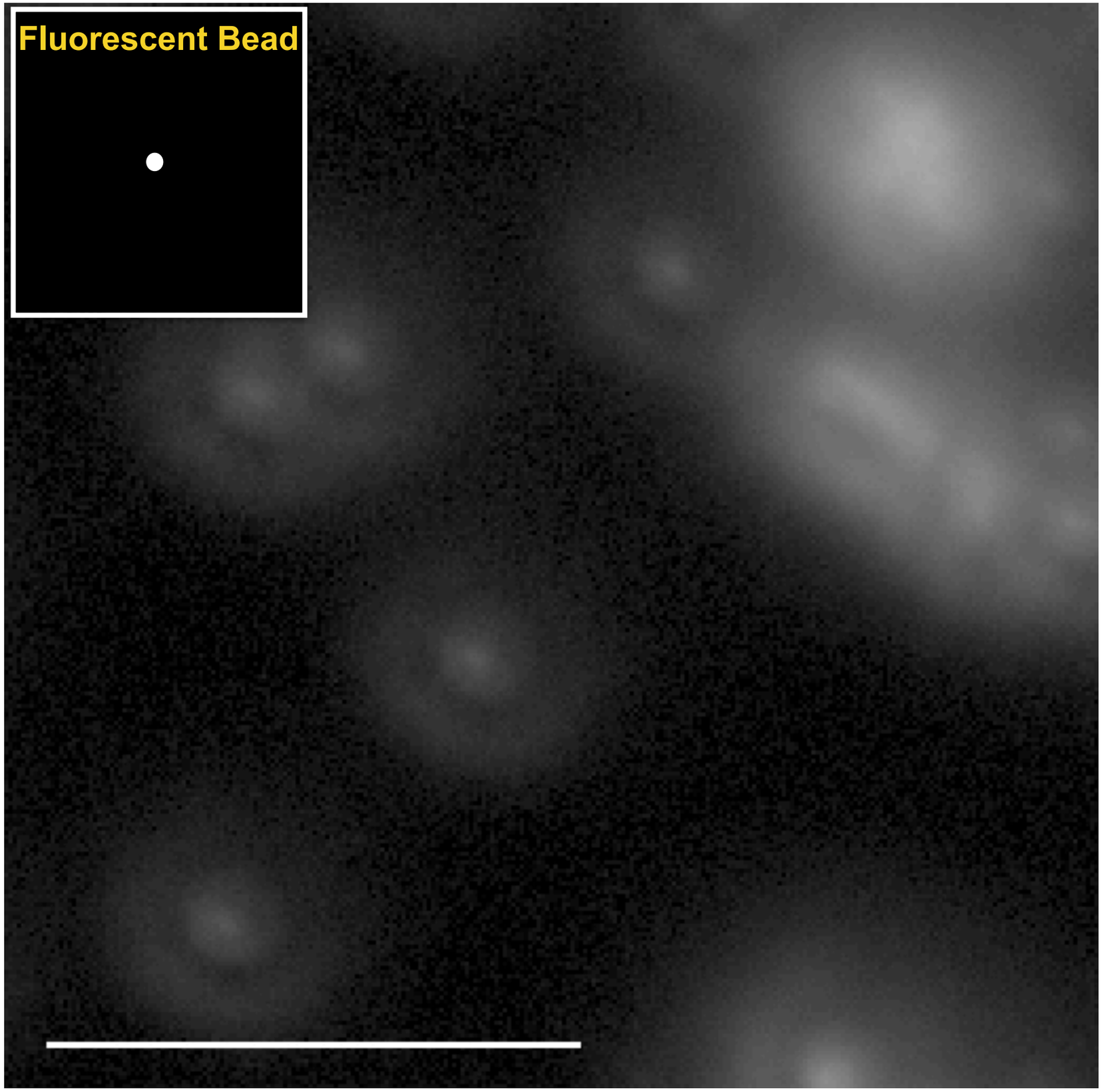


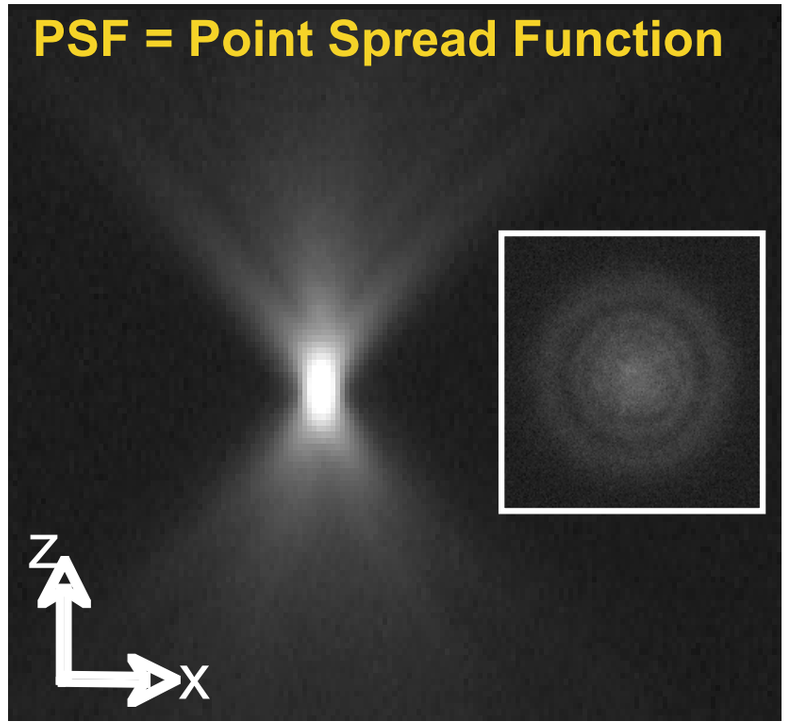
PSF / OTF In fluorescence:

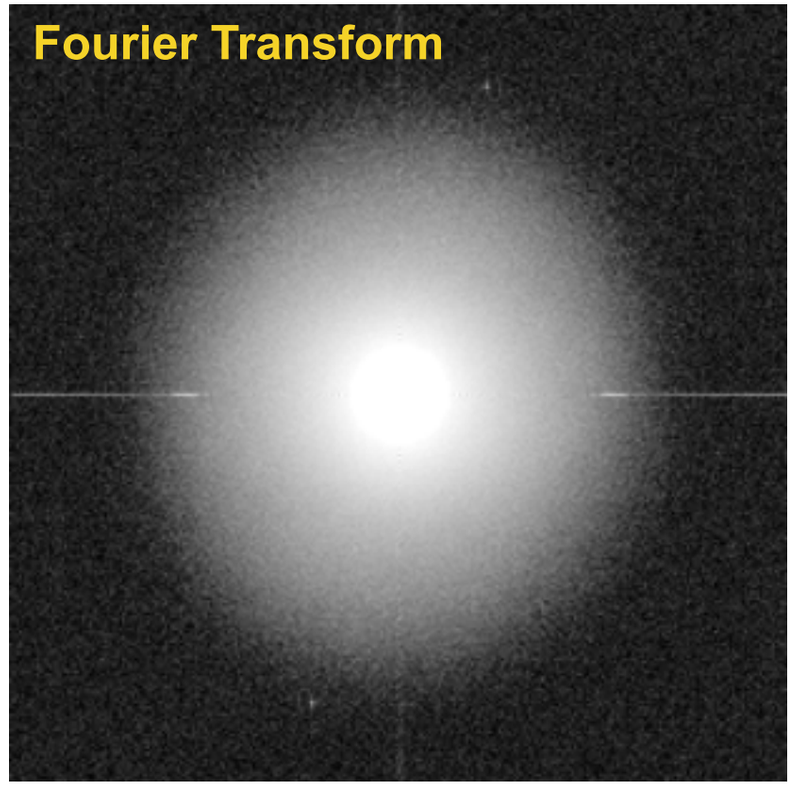




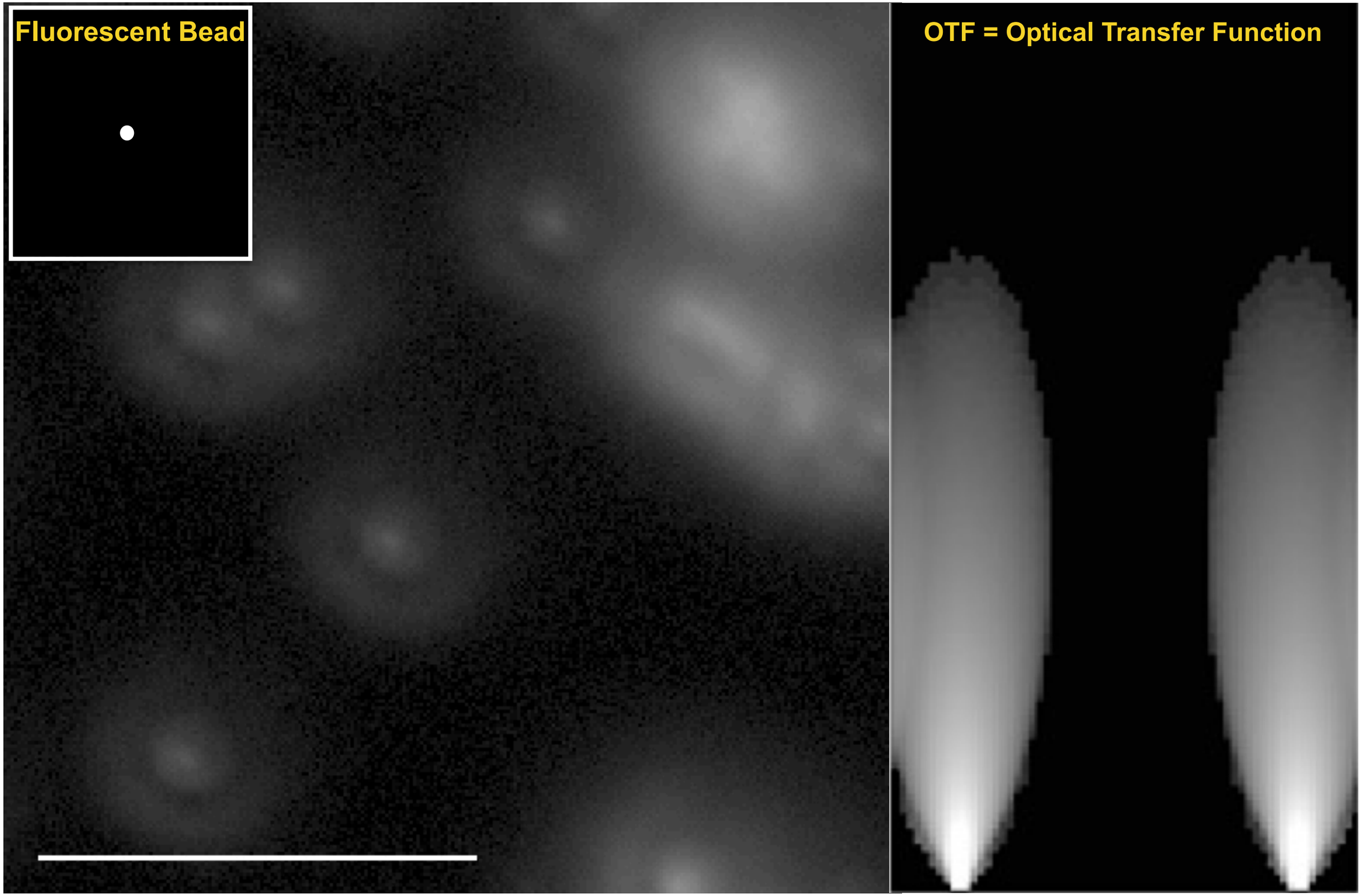
PSF / OTF In fluorescence:







PSF / OTF In fluorescence:



Basics of fluorescence Labeling

Labeling Fixed Material

1. Why work with fixed material?
2. Fixation procedures
3. Labeling

Why work with fixed material?

1. Convenience / Throughput
2. Widely applicable molecular labeling:
   Immunofluorescence
   FISH
3. Ease of multiplexing bright stable labels

Fixation procedures - important considerations

1. Preserve structural features
2. Preserve antigenicity (Immuno)
3. Enable penetration
4. Reduce background fluorescence
5. Preserve endogenous labels (e.g.GFP)

Basic sample prep

Many protocols exist covering most samples and situations!

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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
7. 2^0 antibody
8. Washes
9. Mounting e.g. Vectashield

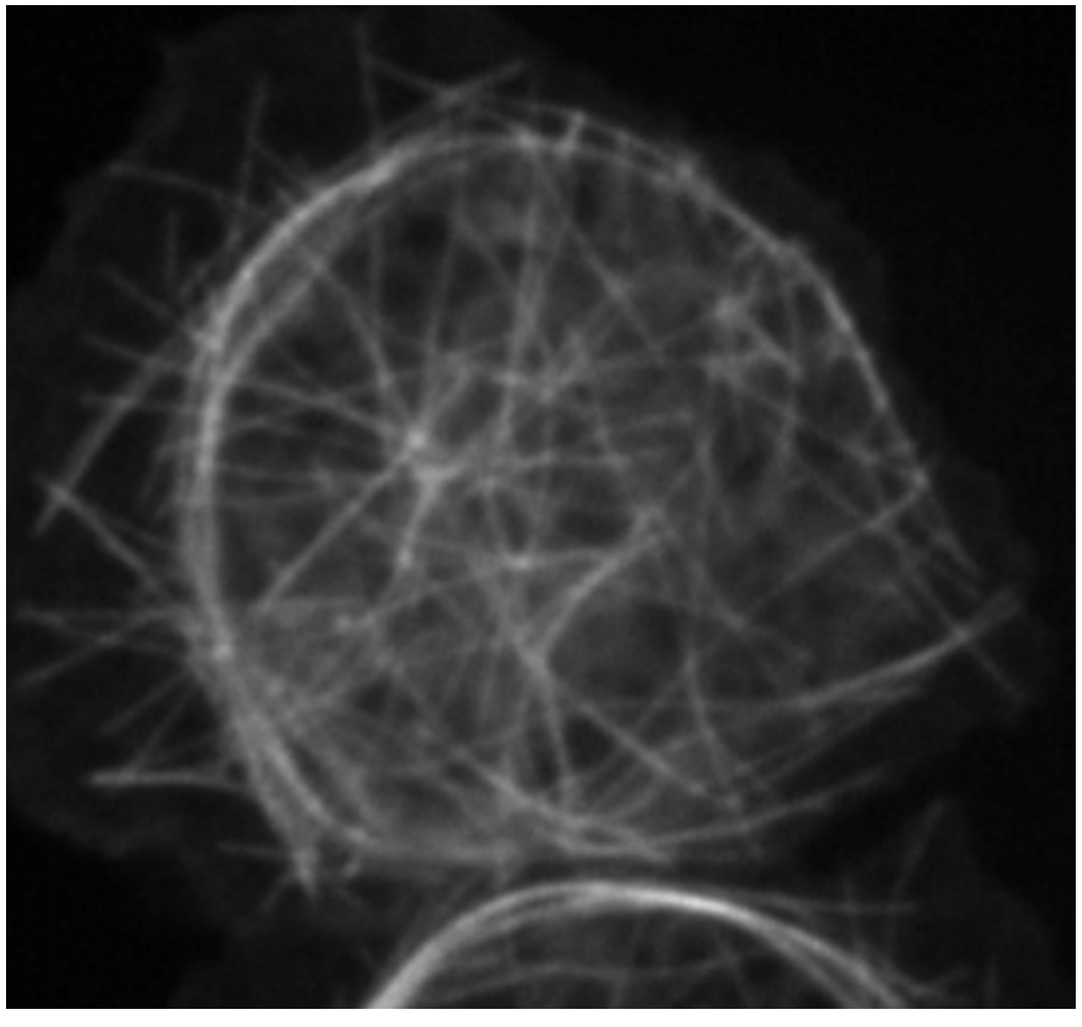
Fixation procedures - important considerations

1. Preserve structural features

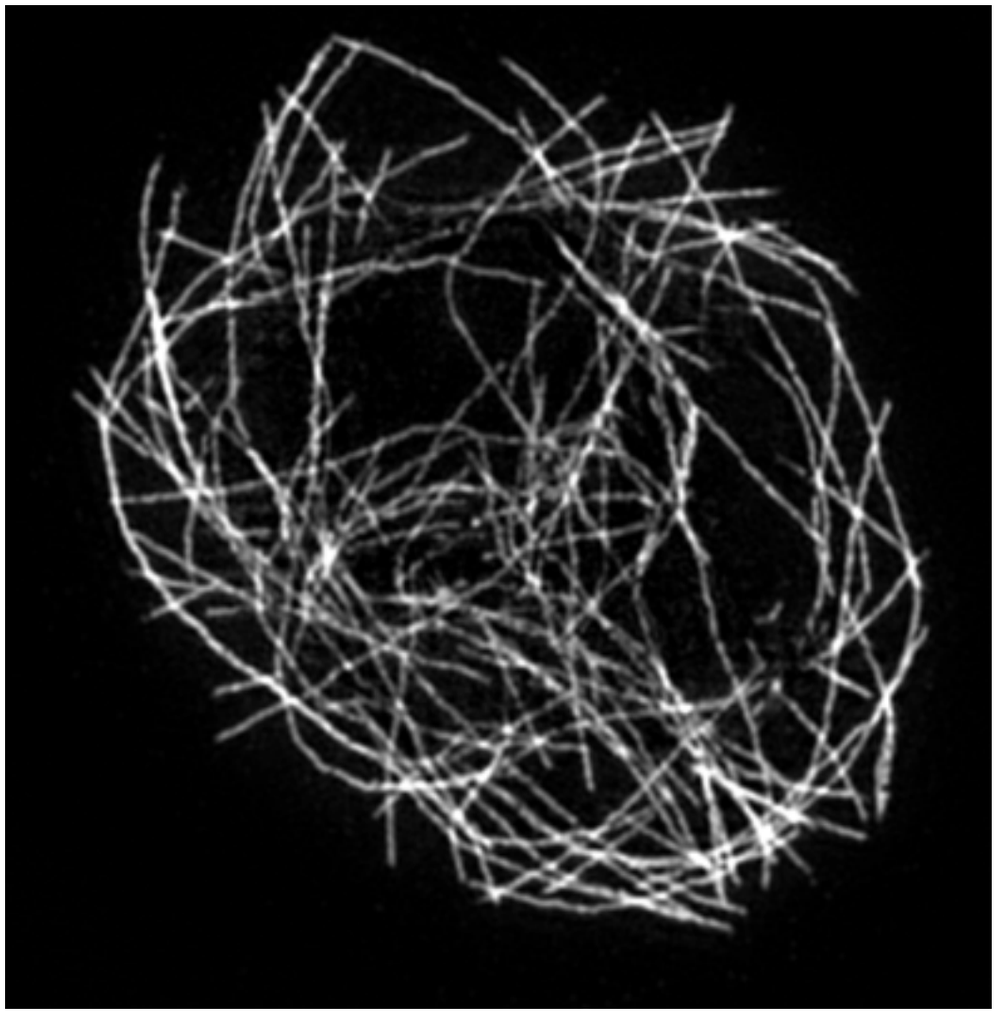
Fixation procedures - important considerations

1. Preserve structural features

….As life!



Jupiter::GFP live (MT)



PFA fixed / Anti-Tubulin

Fixation procedures - important considerations

2. Preserve antigenicity (for immuno)

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….Trial and error
Ab specific!

Fixation procedures - important considerations

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….Trial and error
Ab specific!

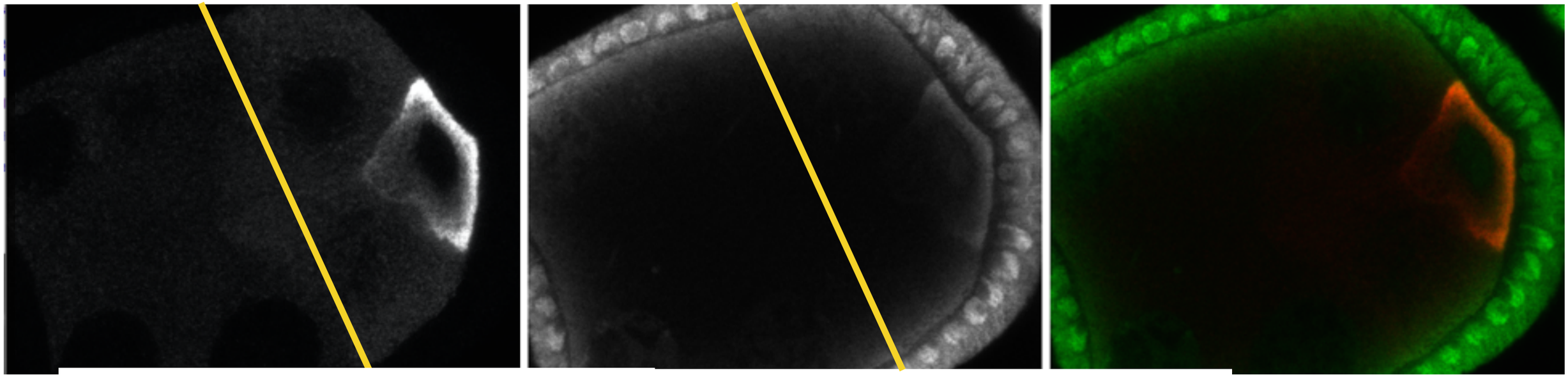
Concentrations of PFA / Glutaraldehyde

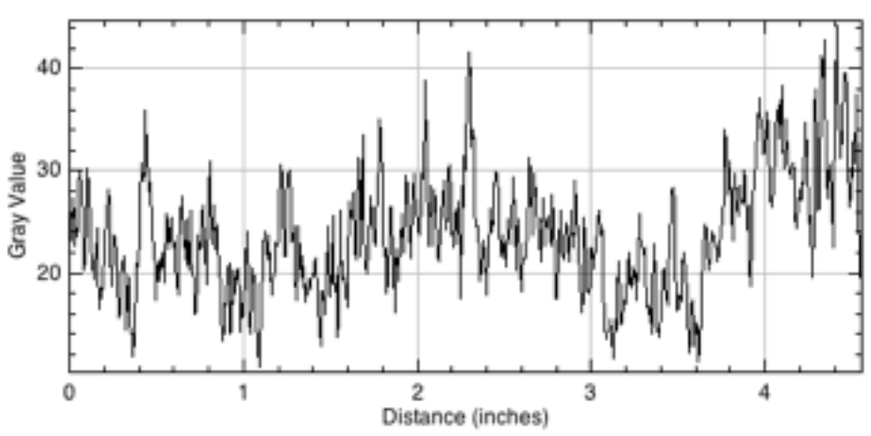
Fixation procedures - important considerations

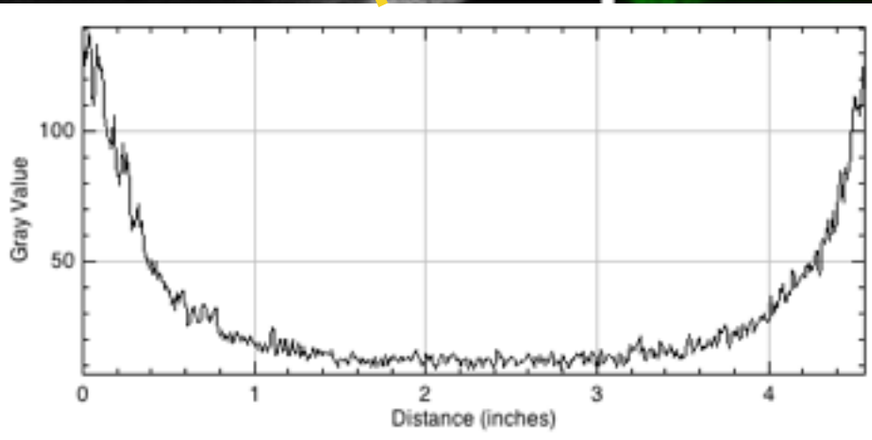
3. Enable Penetration

Fixation procedures - important considerations

3. Enable Penetration







Detergents / Protease treatment
Small probes

Fixation procedures - important considerations

4. Reduce Background

Fixation procedures - important considerations

4. Reduce Background

reduce Glutaraldehyde

thorough rinses and washes

blocking steps

Widely applicable labeling: immunlabelling

probe for proteins of interest

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1^0 antibody e.g. mouse anti-tubulin

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probe for proteins of interest

1^0 antibody e.g. mouse anti-tubulin

2^0 antibody - dye conjugated

e.g. goat anti-mouse, Alexa 546

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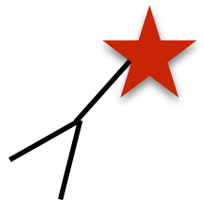


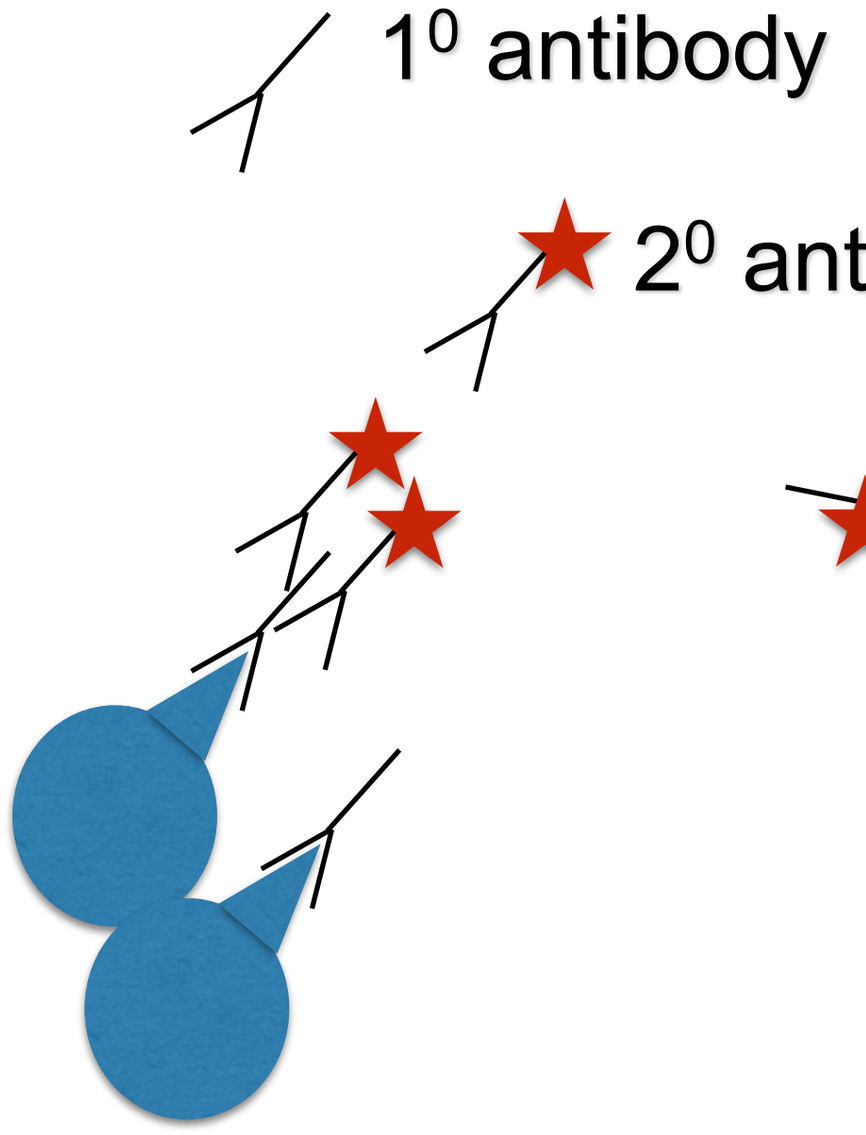
2^0 antibody - dye conjugated

e.g. goat anti-mouse, Alexa 546

2^0 antibody (Camelid or FAB fragment)

Widely applicable labeling: immunlabelling

probe for proteins of interest



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Widely applicable labeling: smFISH

probe for DNA or RNA

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probe for DNA or RNA

1. Fixation
2. Permeabilisation
3. Unfolding (Hybe/Temp)
4. Annealing
5. Other labelling (e.g. immuno)
6. Mounting e.g. Vectashield

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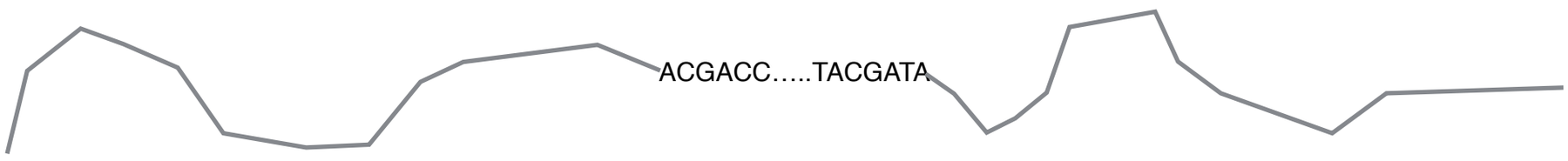

(unfolded) DNA or RNA target

Widely applicable labeling: smFISH

probe for DNA or RNA

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2. Permeabilisation
3. Unfolding (Hybe/Temp)
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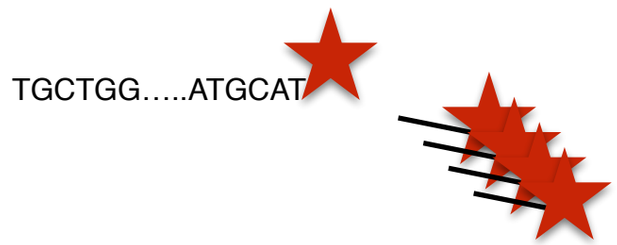
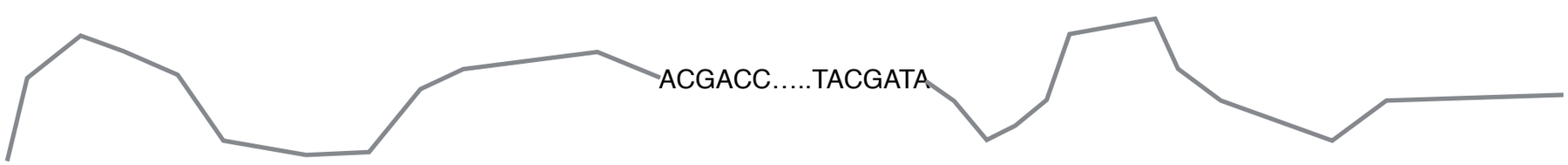
nx distinct nucleic acid

20-mer probes





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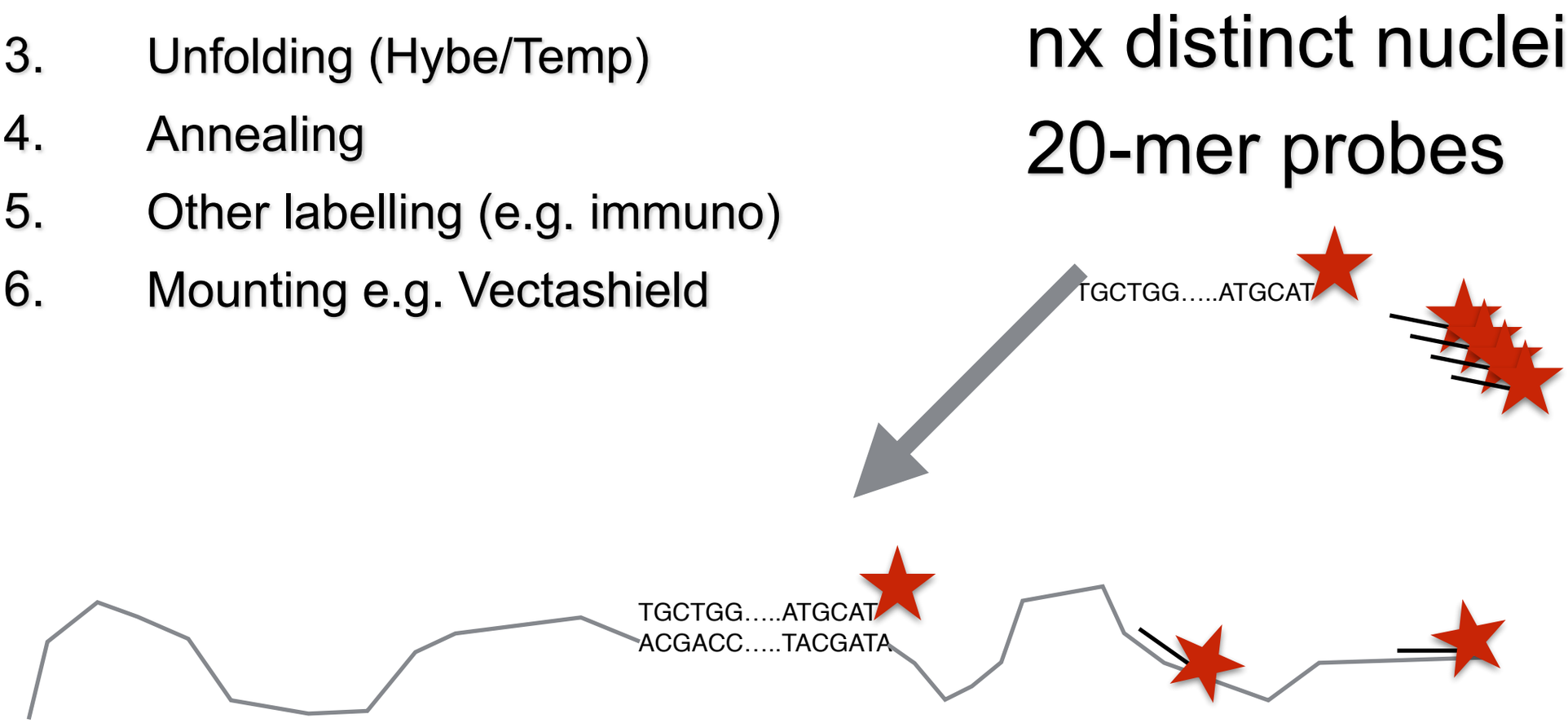

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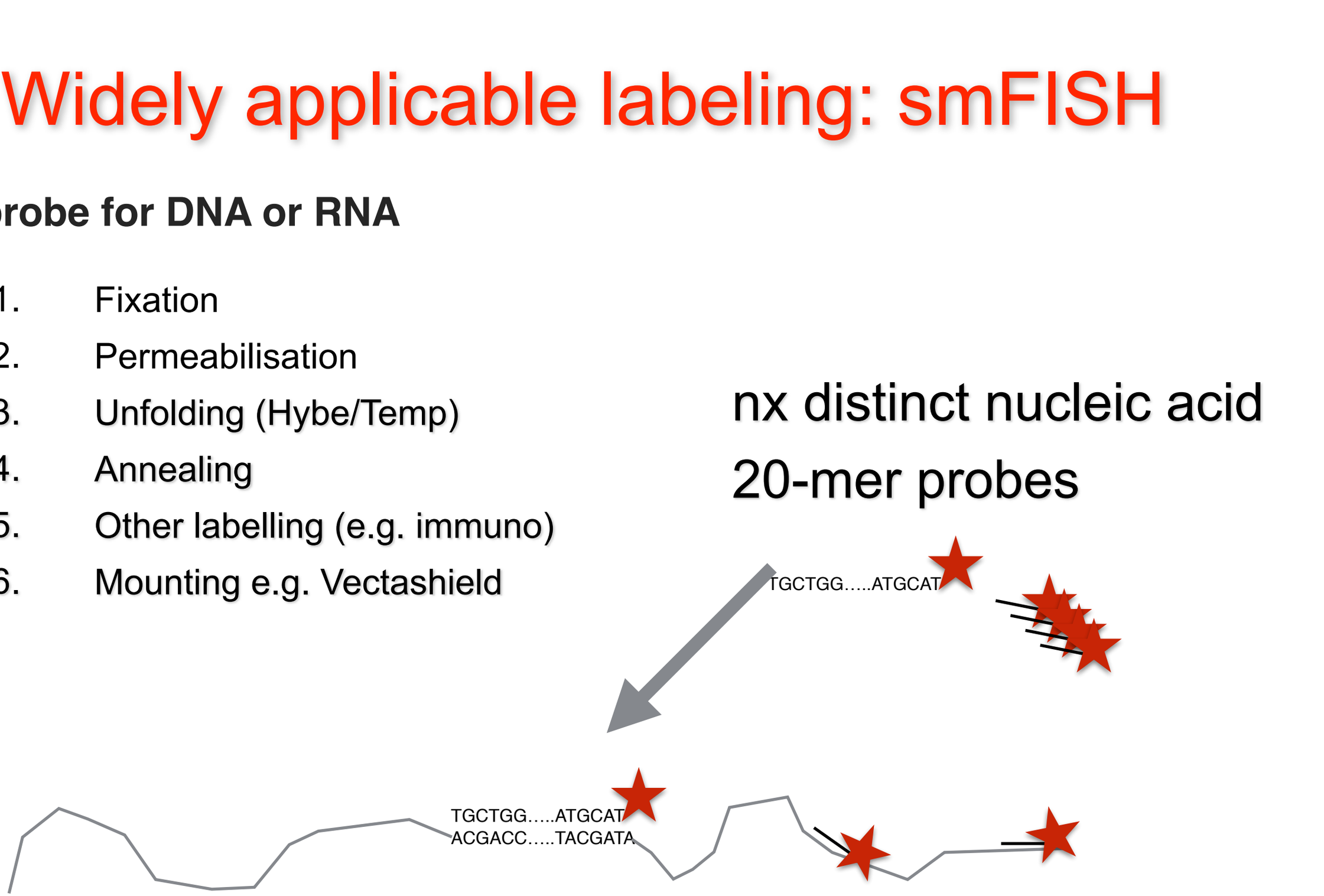


Famine et al., 1998- *Science*
Raj et al., 2008- *Nature Methods*
Little et al., 2011- *PLoS Biology*
Trcek et al., 2015- *Nature Comm*
Abbaszadeh & Gavis, 2016- *Methods*

END

Technical Tips

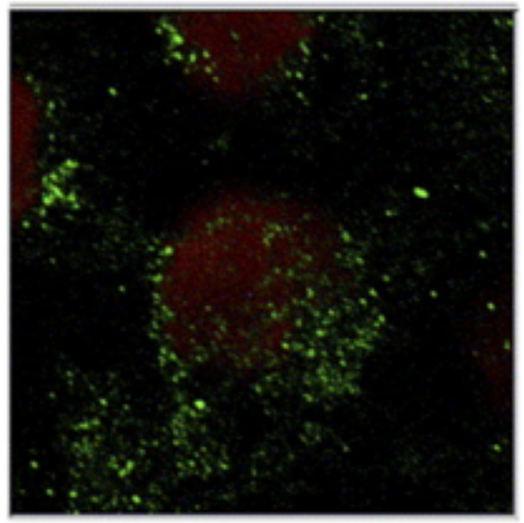
Two Types of Fixation

Denaturing fixation:

Cold methanol or cold acetone stored at -20 °C, samples submerged at -20 °C for 5 to 10 min

- destroys 3D protein structure
- dissolves lipids into micelles
- poor morphological preservation and poor protein retention
- makes some epitopes accessible
- best used after cross-linking fixation

MeOH

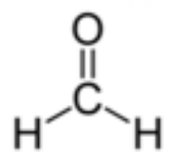


Cross-linking fixation:

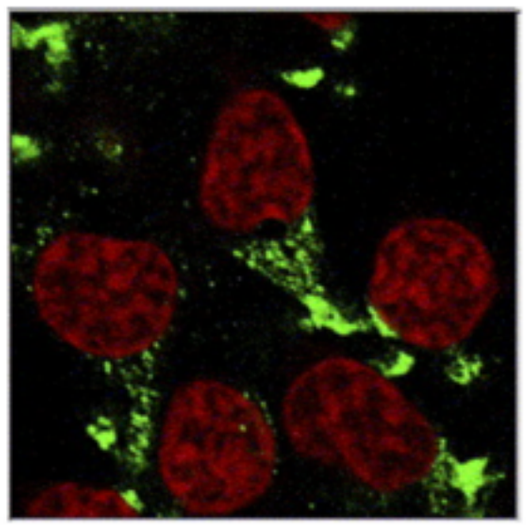
- aldehyde groups cross-link molecules in cells and tissues
- extensive cross-linking prevents antibody penetration

Formaldehyde used for immunocytochemistry in light microscopy

- cross-links 1° amines of Lys and Arg, sulfhydryl groups of Cys, OH groups, double bonds
- binds to amino acids, peptides, proteins and some lipids, but not RNA, DNA or most sugars
- retention of DNA and RNA due to protein cross-linking
- for cultured cells fixation usually for 20 min in 2 - 4% formaldehyde



PFA



Buffers for fixation

- pK range must be 7.0-7.3
- Maintain stable pH and have to have the same tonicity as the cells (same conc. of solutes)
- Usually phosphate buffer but specialist buffers possible: MOPS, TES, HEPES, PIPES

How to prepare cells for fixation

- Grow adherent cells on coverslips for fixation in multiwell plates
- Fix non-adherent cells in suspension after pelleting and resuspending or fix on poly-lysine coated coverslips (0.1mg/mL)

Permeabilisation

Aim: to allow fixative to enter the cells/tissue more quickly if necessary
to allow antibodies to penetrate fixed cells/tissue
done by removing lipids with detergents

Detergents:

- polar lipids with a hydrophilic (water soluble) end and a hydrophobic end that binds the hydrophobic moieties of water insoluble compounds and renders them hydrophilic

Nonionic detergents:

- contain methyl groups that participate in hydrogen bonds and are able to solubilise membranes but do not destroy protein-protein interactions

Triton X-100: used to permeabilise unfixed or lightly fixed eukaryotic cell membranes (0.1% in PBS)
Tween 20: milder than Triton X-100, used to reduce surface tension in blocking, antibody incubation and wash steps (0.1%)
Nonidet P-40 (Igepal Ca-630 from Sigma-Aldrich): used to permeabilise unfixed cells (0.1% in PBS for 5-10s)

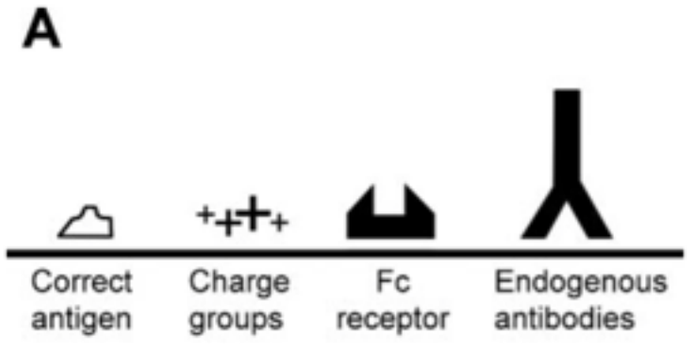
Ionic detergents:

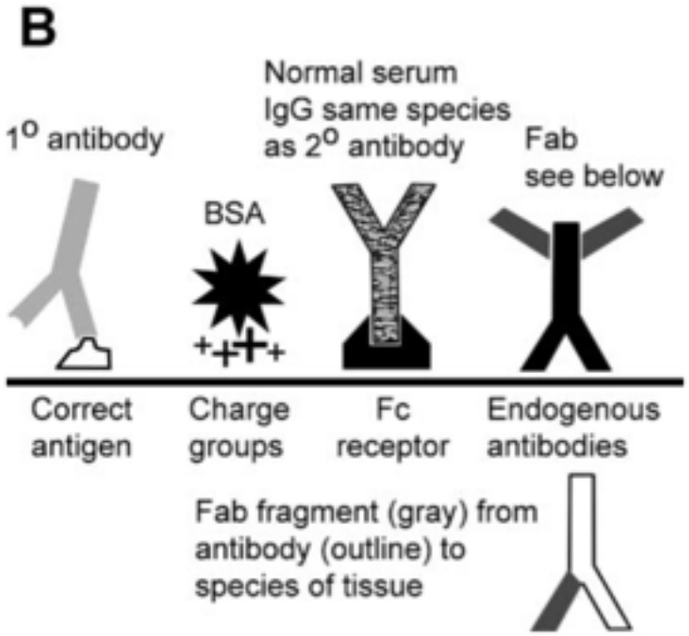
- have highly charged hydrophilic groups and are very effective at solubilising membranes, but also destroy native three dimensional protein structures

SDS, deoxycholate, CHAPS
Not used for immunocytochemistry

Blocking

Aim: to allow binding of antibodies only to appropriate sites





Sources of nonspecific binding:

Charged groups

Occur on proteins (esp. histones) or lipids

Also generated by fixation in formalin or glutaraldehyde

To block use bovine serum albumin at 10-30mg/mL (fraction V)

Fc receptors

On macrophages and other immune cells, which bind any antibody

To block whole IgG 1° and 2° antibodies from binding to Fc receptors, incubate cells in buffer containing 5-10% normal serum from the host species of the 2° antibody

Endogenous antibodies

Only a problem for 2° antibodies recognising the same species as your tissue/ cells and only at inflammation sites or in cell cultures of immune system cell types

To block use Fab fragments raised in the same species as the 2° antibody that recognise the species of your tissue/cells as part of the blocking procedure

For general blocking can also try MAXblock (Active Motif): protein based, non-mammalian blocking agent, no cross-reactivity with 2° antibodies

How to choose primary antibodies:

1. Published literature recommendation
2. Product recommended for immunocytochemistry
3. High specificity for the antigen of interest in your species
4. Species the Ab was raised in compatible with other Abs in your experiment

How to store antibodies:

10 µL aliquots in -70 freezer, after defrosting: in fridge for short-term

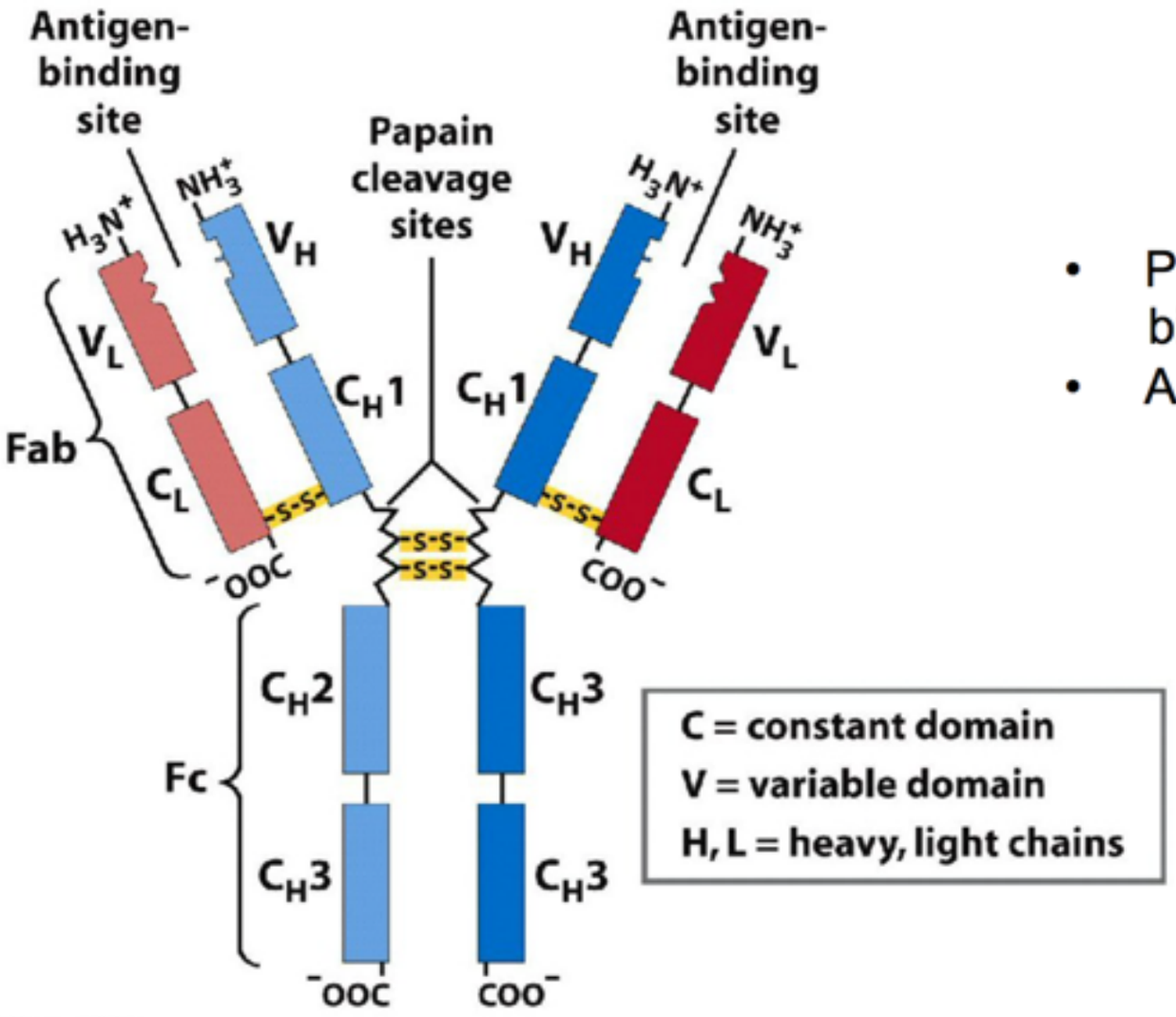


Figure 5-21a
Lehninger Principles of Biochemistry, Fifth Edition

- Preferred: IgG isotype, more consistent generation and binding
- All constant domains are recognised by 2° Abs

Polycolonal antibodies contain multiple clones of antibodies produced to different epitopes of the antigen

Monoclonal antibodies, originally from one mouse, contain a single antibody from one clone of B-cells to a single epitope on the antigen

Affinity-purified Abs best in theory because they have bound to the antigen, but some of the strongest binding Abs cannot be eluted from the affinity columns and are lost.

Polyclonal antibodies

Advantage:

- High levels of labelling because they bind several epitopes on the same protein

Disadvantages:

- Can label multiple proteins that share epitopes
- Different batches have different antibodies

Monoclonal antibodies

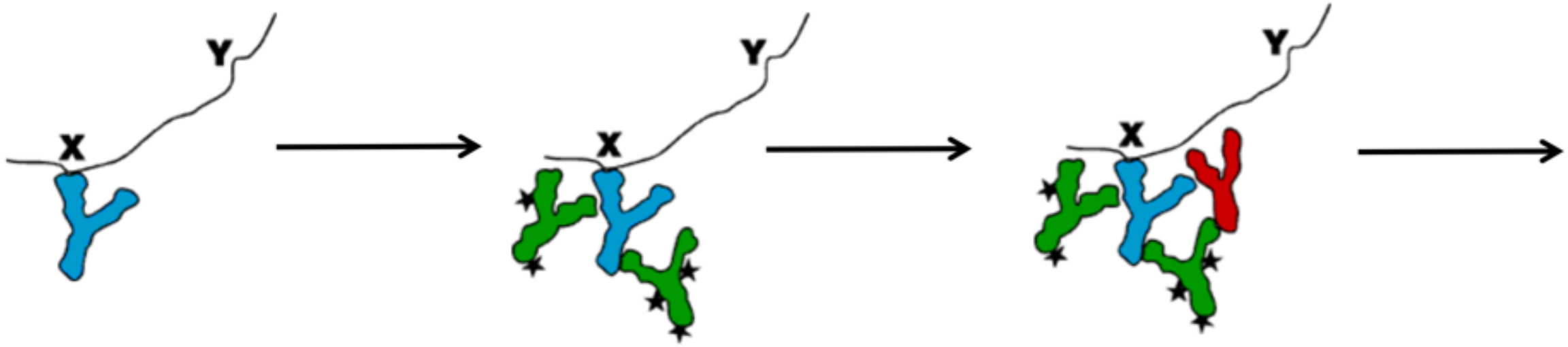
Advantages:

- Single epitope selected for high specificity
- Different clones can be generated to different epitopes on the same antigen
- Single clone can recognise post-transcriptionally modified protein (e.g. phosphorylation)
- Same clone can be generated indefinitely

Disadvantages:

- Low levels of labelling possible
- Mostly from mice

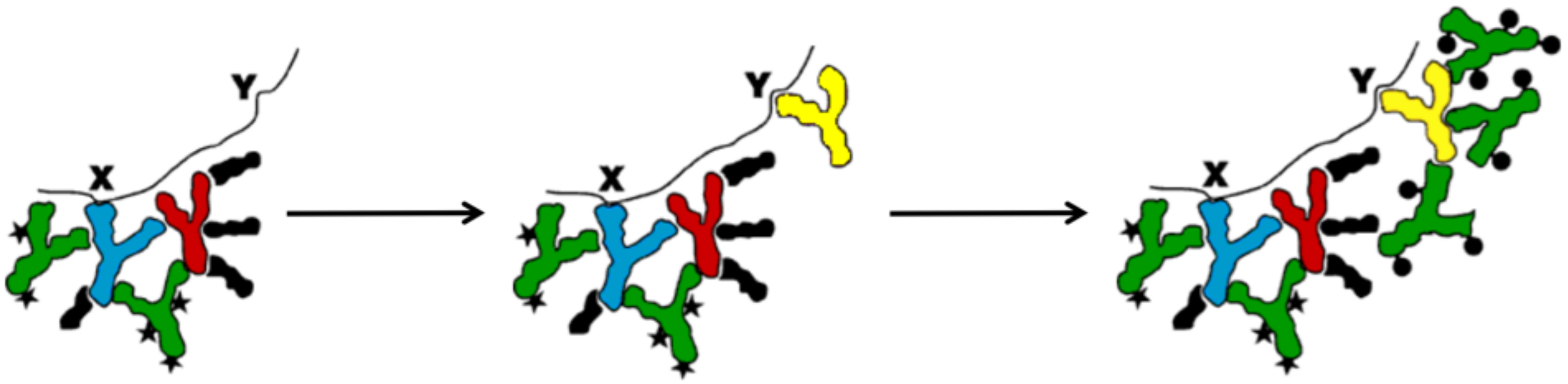
Two 1° antibodies from the same species



rabbit anti-X

goat anti-rabbit IgG Alexa 488

IgG from normal rabbit serum



Fab fragment goat anti-rabbit IgG

rabbit anti-Y

goat anti-rabbit IgG Alexa 568

1° Antibody Controls

- check localisation of fluorescent fusion proteins in live imaging
- compare tissue sections from a normal animal/cells and a knockout animal/cells – not often possible, knockout might not be complete
- single band on western blot or better immunoprecipitation followed by gel and silver staining
- immunocytochemical comparison with known antibody against same target or fluorescent fusion protein

2° Antibody Controls

- omit the 1° antibody and block with normal serum if you see background
- purchase 2° antibodies from reliable manufacturers
- when choosing a 2° antibody for a 1° mouse antibody the 2° frequently needs to be able to bind to the subclass of the IgG used as the 1° antibody

Washes

- Wash with agitation (unless your cells dislodge easily) for 5-10 min for each wash step
- Wash 7 times leaving 10-20% of the buffer each time to prevent drying of your cells/tissue
- Or wash 3 times removing all buffer and replacing it immediately
- If cells/tissue dry out in between washes background is increased and cannot be removed

Washes after the 1° antibody

- Incomplete removal of the 1° antibody does not increase background but lowers the amount o specific labelling because the 2° antibody reacts with the 1° in solution decreasing its conc.

Washes after the 2° antibody

- Incomplete removal of the 2° antibody increases background

Reference Material

http://www.olympusmicro.com/
Very comprehensive and well written

http://micro.magnet.fsu.edu/primer/anatomy/anatomy.html
Very comprehensive

Immunocytochemistry a practical guide for biomedical research
Richard W. Burry, Springer 2010
http://www.jacksonimmuno.com/technical

Molecular Biology of the Cell, fifth edition.
Alberts et al. Chapter 9: Visualizing cells, page 579-616