MICROSCOPIA OTTICA IN BIOLOGIA CELLULARE [675SM]

aa 2023/2024, 2nd semester

Lesson 3

Aula exCLA, edificio C1, 15:00-18:00

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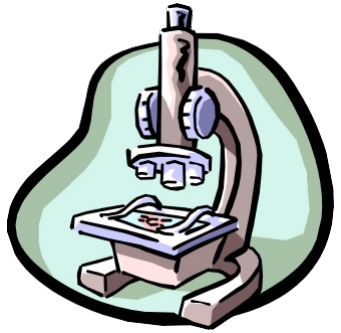
UNIVERSITÀ DEGLI STUDI DI TRIESTE
Dipartimento di Scienze della Vita

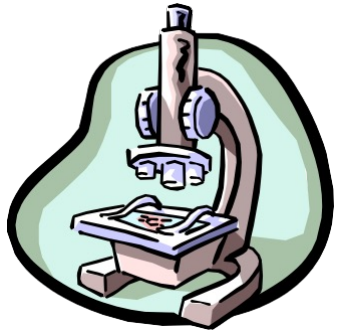
CONTRASTING TECHNIQUES – A REMINDER…



- Brightfield: absorption
- Darkfield: scattering
- Phase Contrast: phase interference
- Differential Interference Contrast (DIC): polarization + phase interference

➡ Fluorescence Contrast

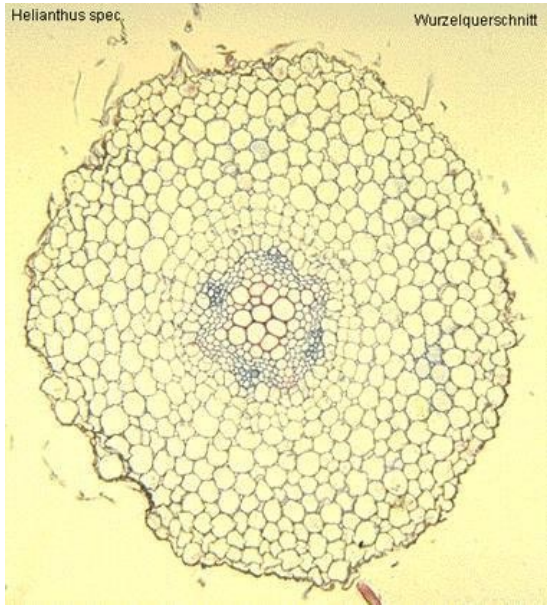
BRIGHTFIELD



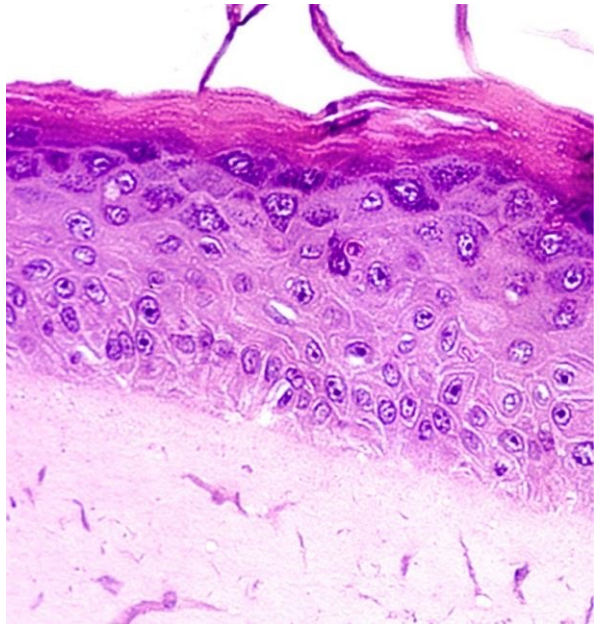
Principle:
Light is transmitted through the sample and absorbed by it.

Application:

- Only useful for specimens that can be contrasted via dyes
- Very little contrast in unstained specimens
- With a bright background, the human eye requires local intensity fluctuations of at least 10 to 20% to be able to recognize objects.

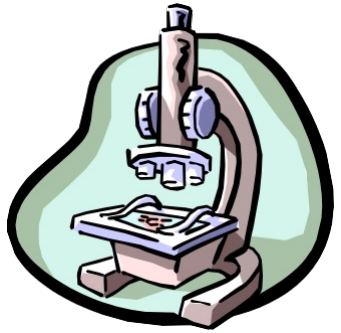


Cross section of sunflower root
(http: /www.zum.de/Faecher/Materialien/beck/12/bs12-5.htm)



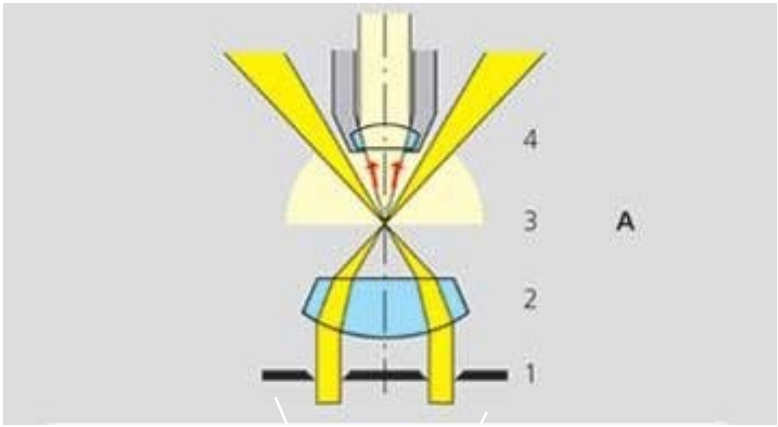
Piece of artificially grown skin
www.igb.fhg.de/.../dt/PI_BioTechnica2001.dt.html

DARKFIELD



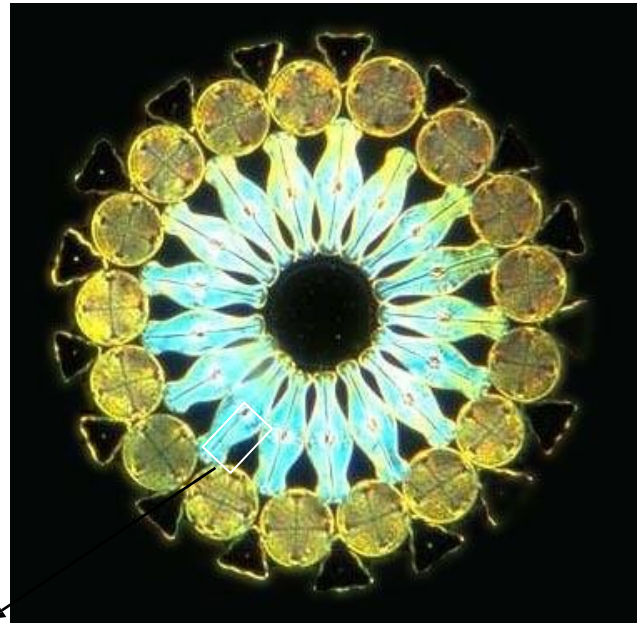
Principle:

The illuminating rays of light are directed through the sample from the side by putting a dark disk into the condenser that hinders the main light beam to enter the objective. Only light that is scattered by structures in the sample enters the objective.

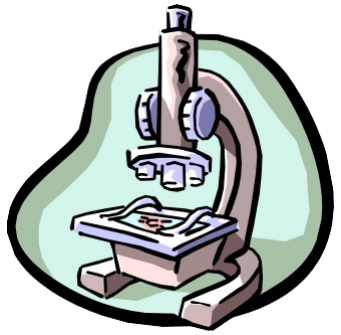


Application:

- Diatoms and other unstained or colourless specimens

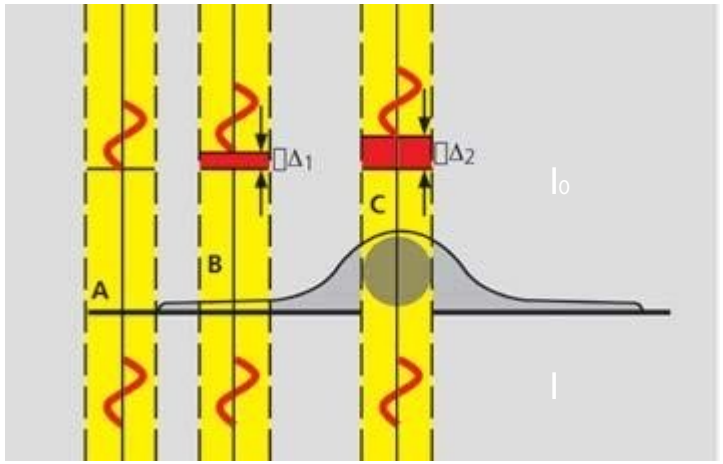


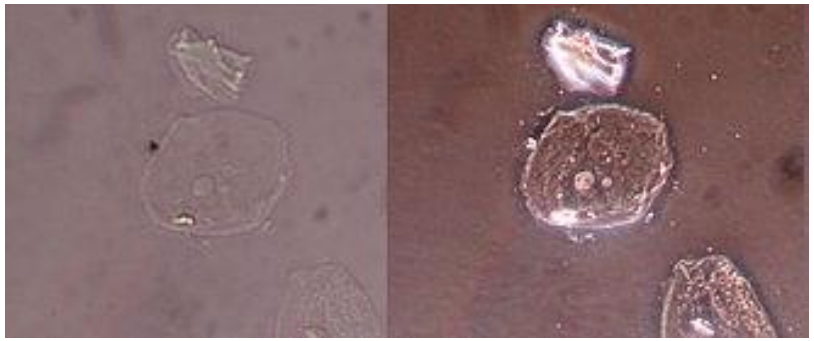
PHASE CONTRAST

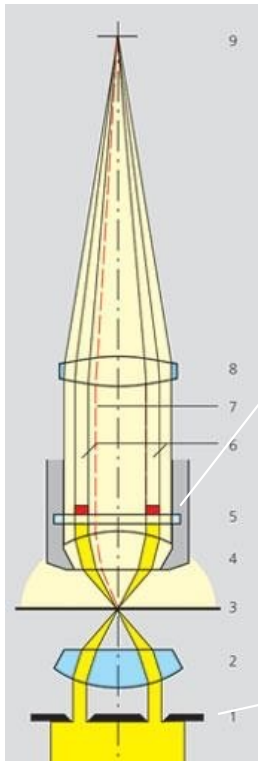


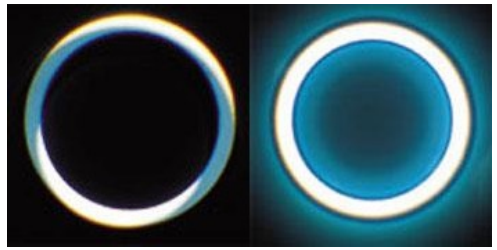
Principle:

Incident light is out of phase with transmitted light as it was slowed down while passing through different parts of the sample. when the phases of the light are synchronized by an interference lens, a new image with greater contrast is seen.

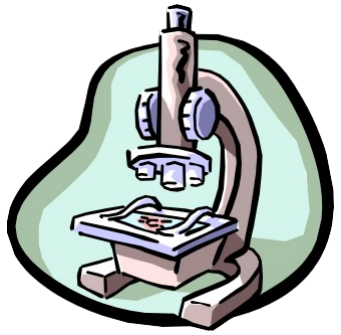








POLARISATION CONTRAST



Principle:

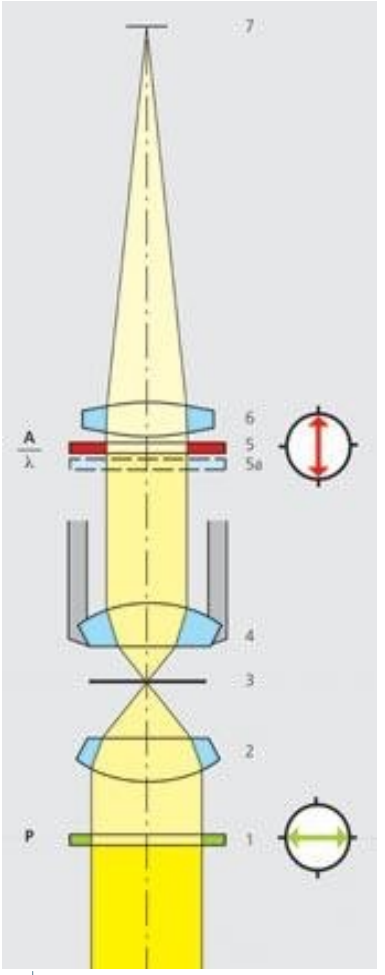
Polarized light is used for illumination. Only when the vibration direction of the polarized light is altered by a sample placed into the light path, light can pass through the analyzer. The sample appears light against a black background. A lambda plate can be used to convert this contrast into colours.

Application:

Polarization contrast is used to look at materials with birefringent properties, in which the refractive index depends on the vibration direction of the incident light, e.g. crystals or polymers.

Analyzer

Lambda plate



Polarizer

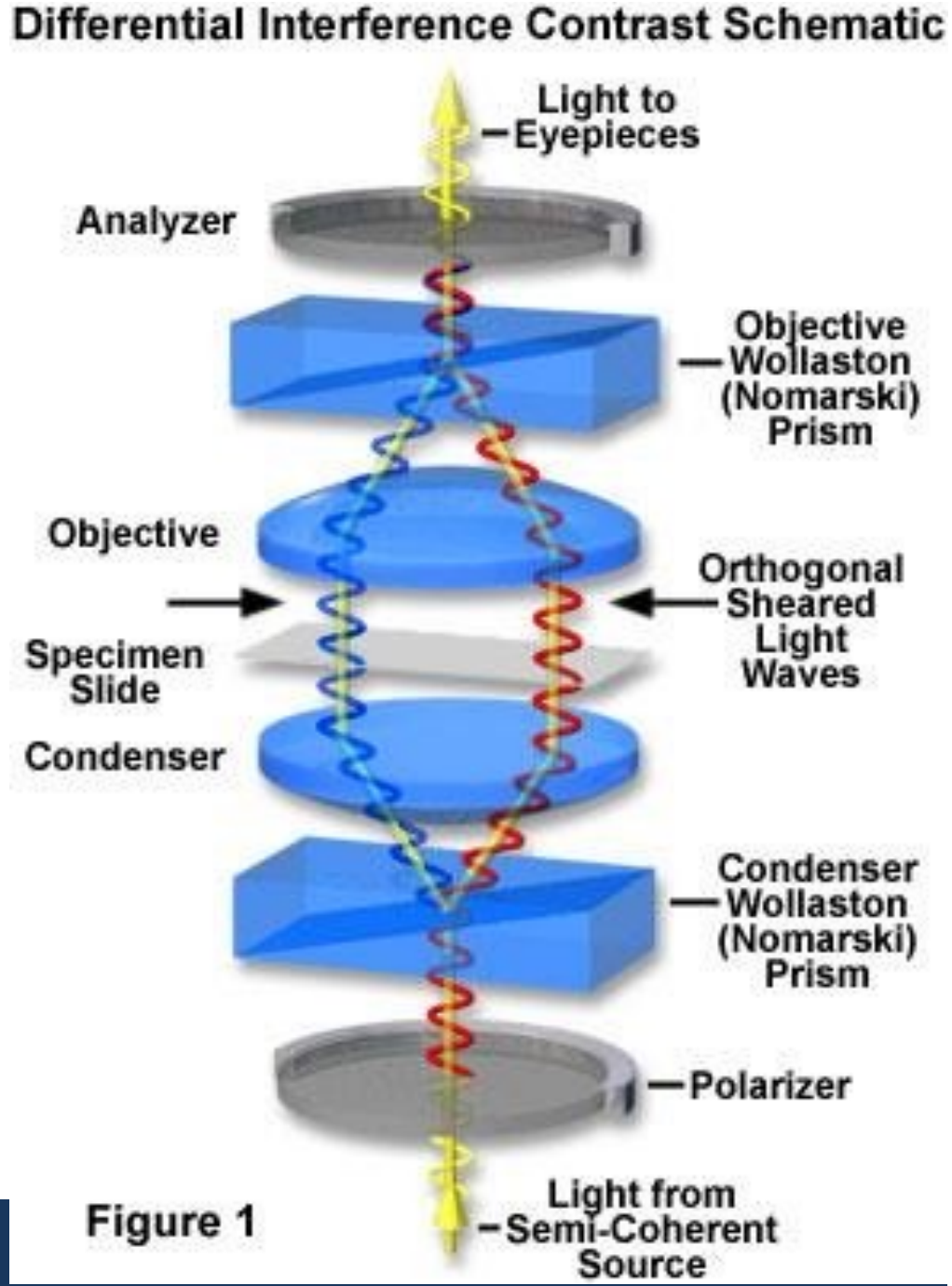
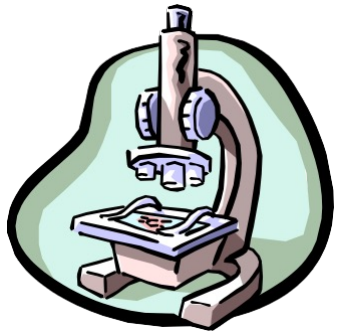
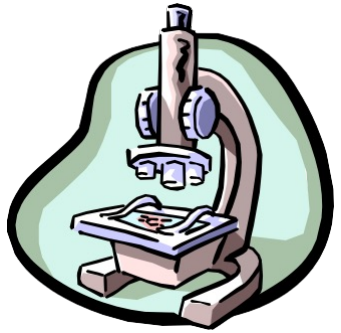

Differential Interference Contrast Schematic
Light to Eyepieces
Analyzer
Objective Wollaston (Nomarski) Prism
Objective
Orthogonal Sheared Light Waves
Specimen Slide
Condenser
Condenser Wollaston (Nomarski) Prism
Polarizer
Light from Semi-Coherent Source

Figure 1




Shear Axis

CONTRASTING TECHNIQUES – A REMINDER…



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- Darkfield: scattering
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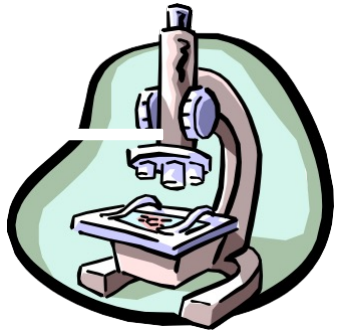
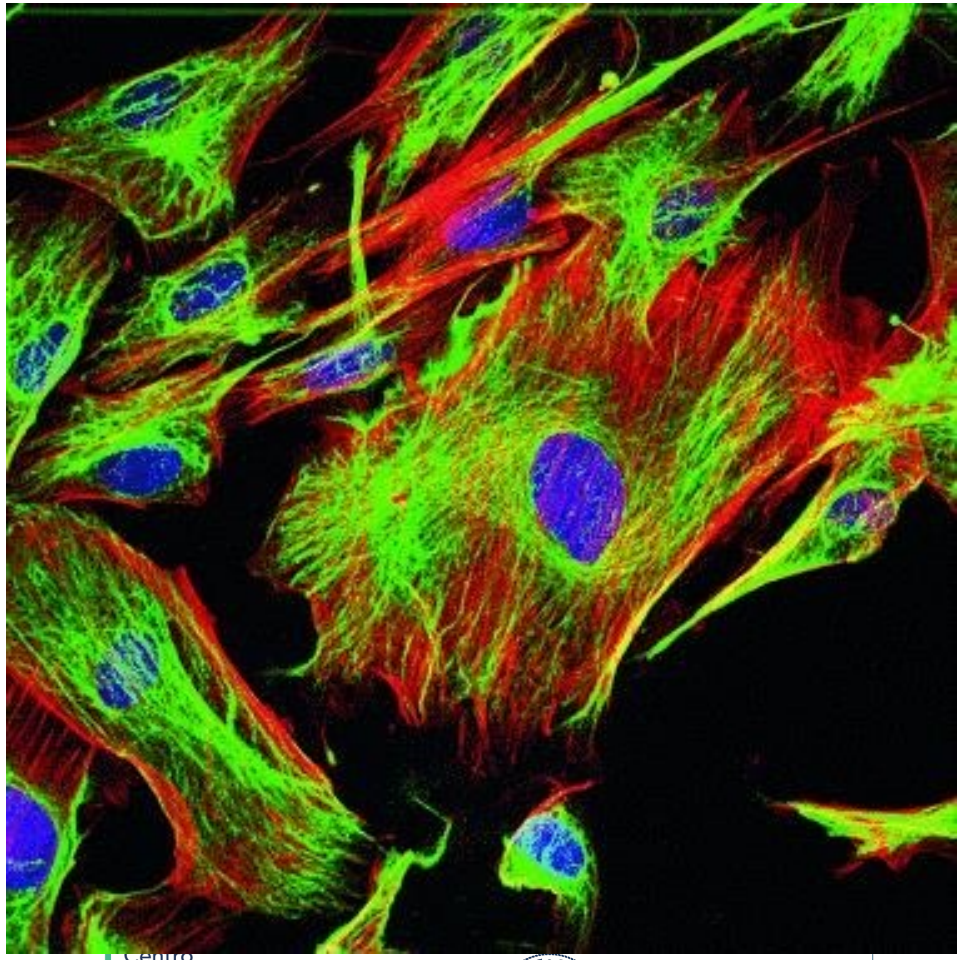
➡ Fluorescence Contrast

WHY FLUORESCENCE MICROSCOPY?

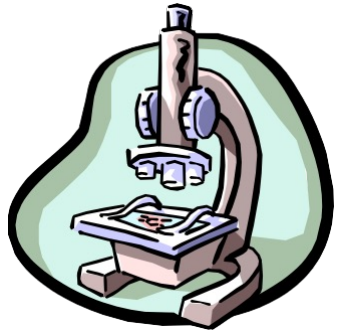


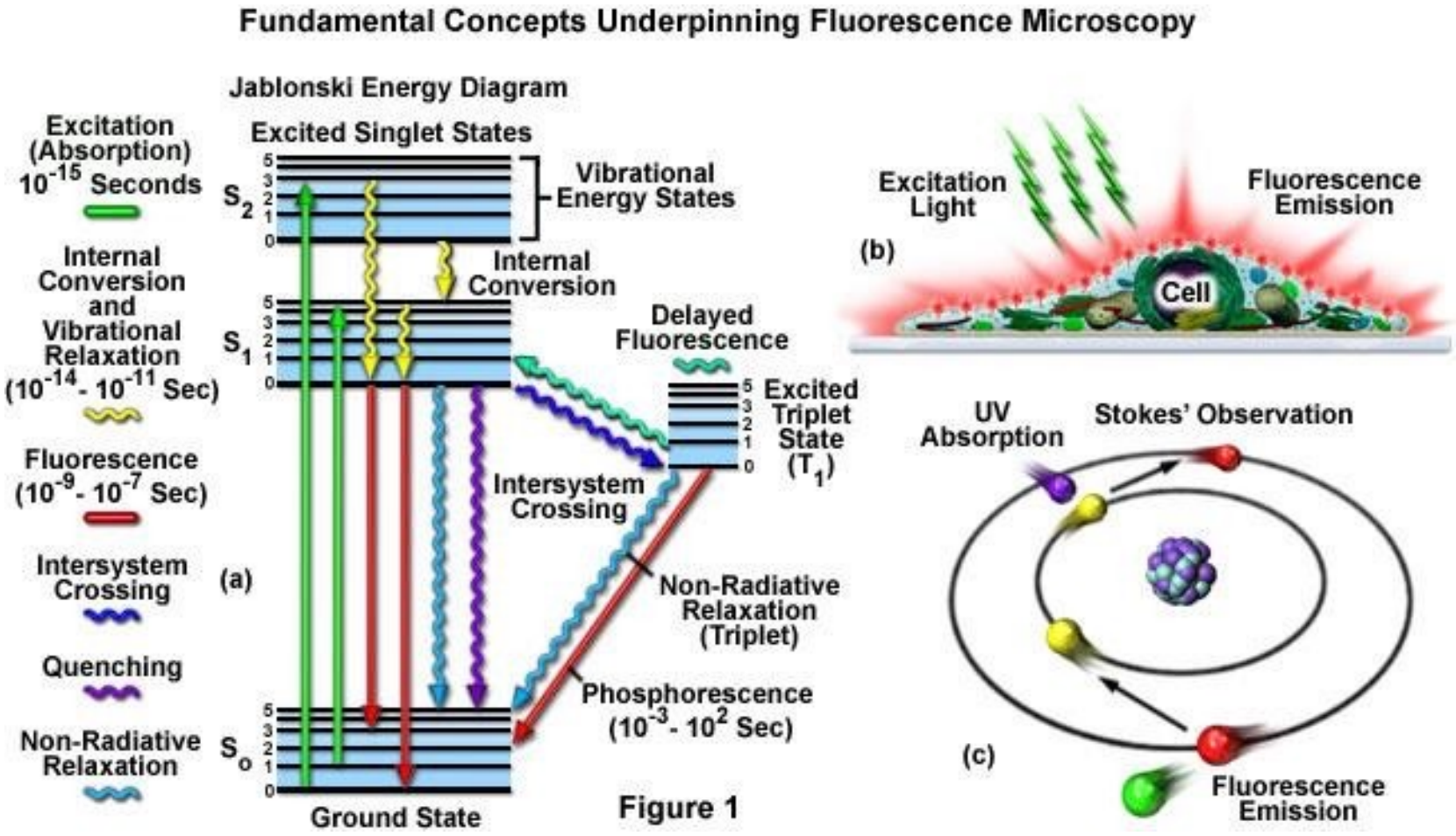
High resolution

High contrast
High specificity
Quantitative
Live Cell Imaging

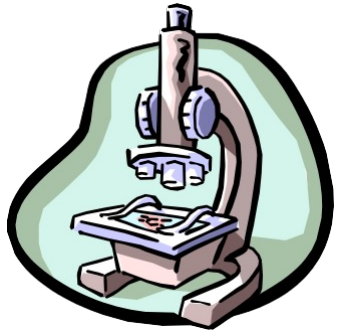


FLUORESCENCE PRINCIPLE



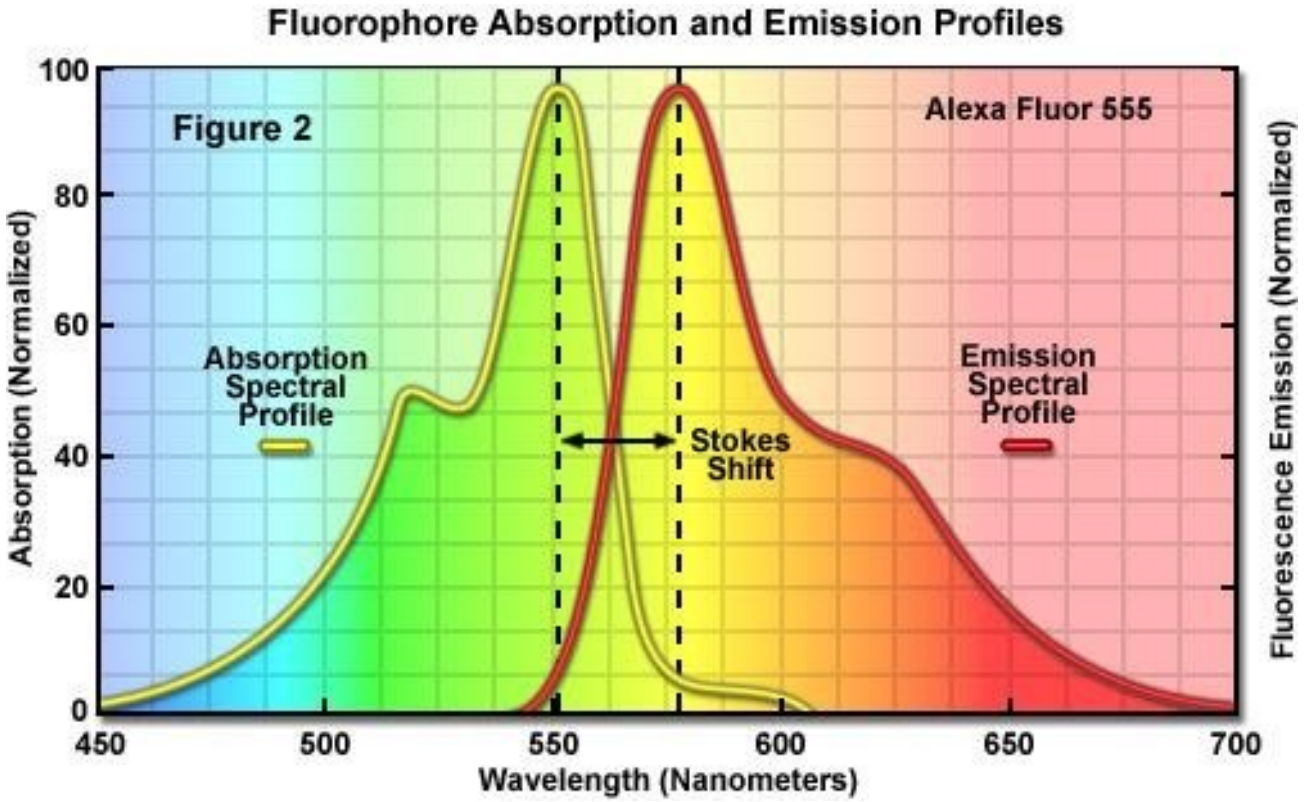


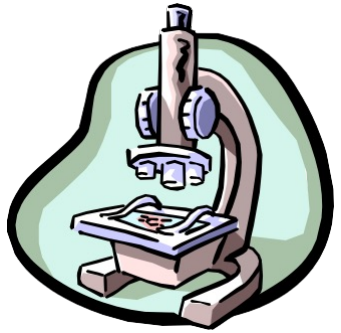
FUNCTIONING



- A component of interest in the specimen is specifically labeled with a fluorescent molecule called a **fluorophore.**

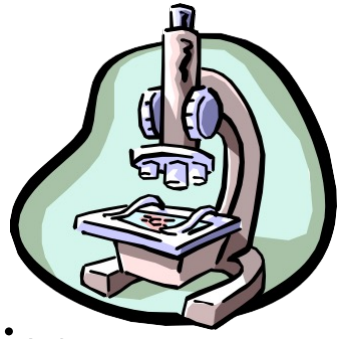
- The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit longer wavelengths of light (of a different color than the absorbed light).



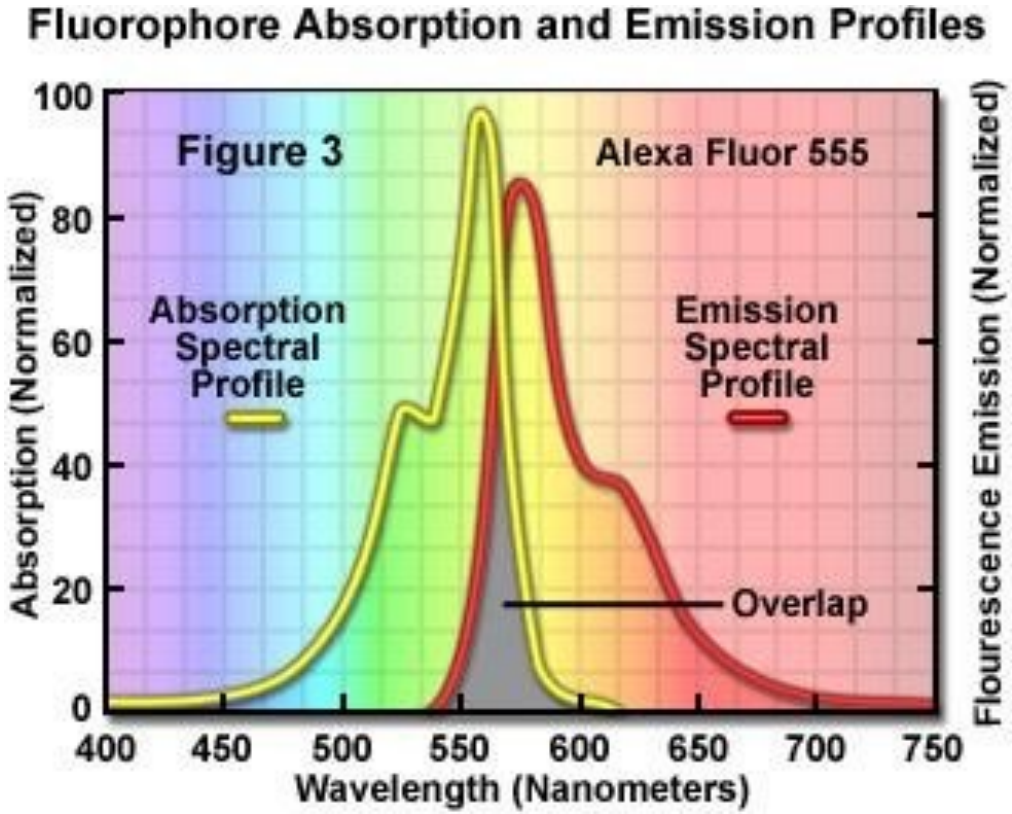


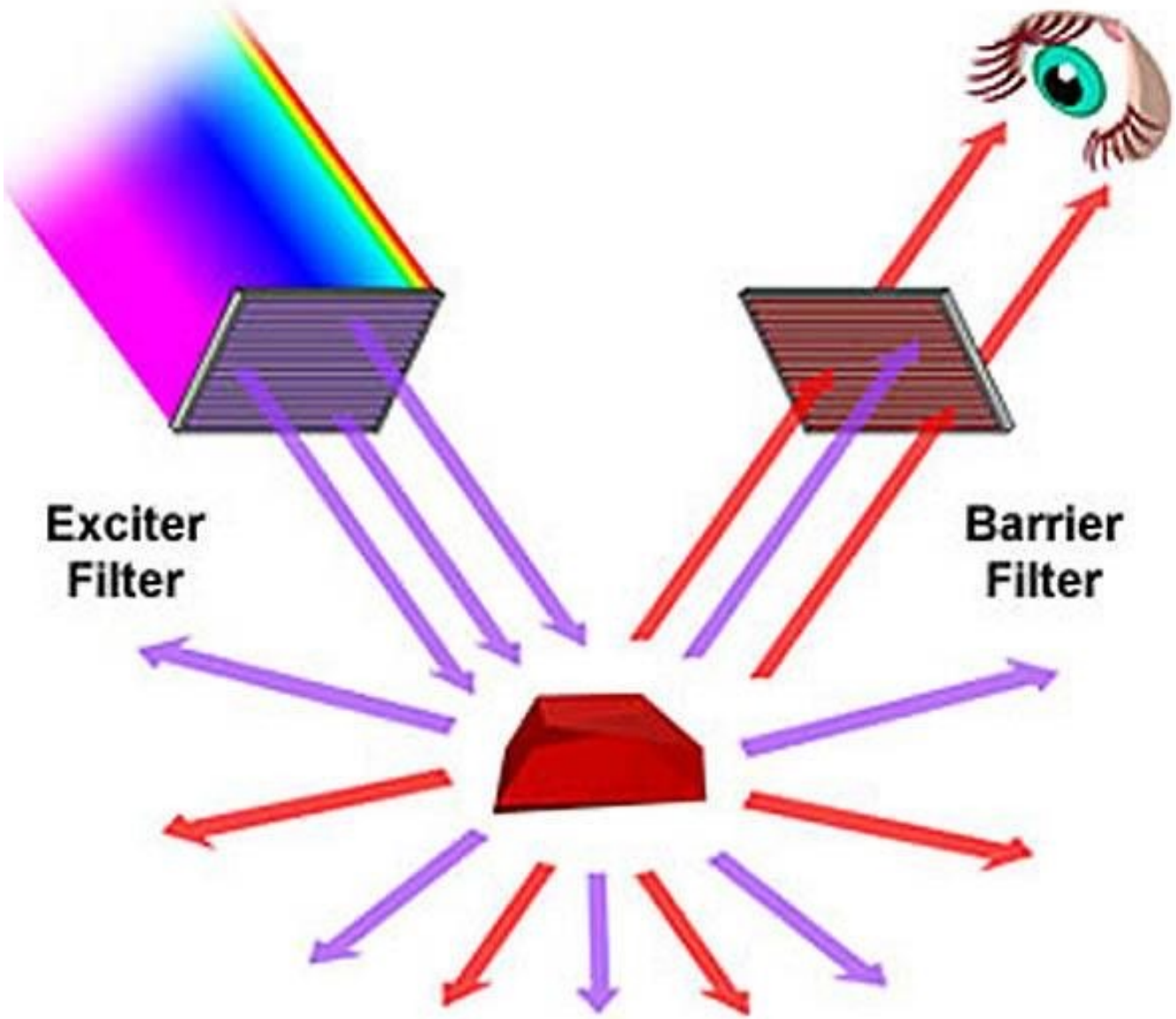
Molecules absorbing the energy of electromagnetic radiation will jump to a higher energy level. When certain excited molecules return to the ground state they emit radiation. This phenomenon is known as fluorescence. Fluorescent molecules are known as fluorochromes or fluorophores.

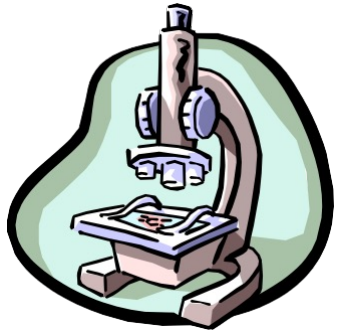
STOKE'S SHIFT



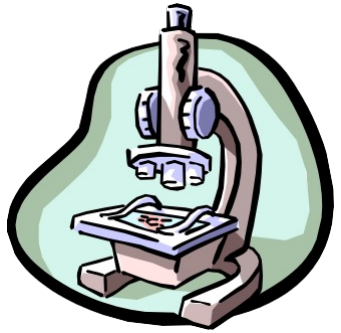
✓As Stokes' shift values increase, it becomes easier to separate excitation from emission light through the use of fluorescence filter combinations.




Exciter
Filter
Barrier
Filter



COMPONENTS OF A FLUORESCENT MICROSCOPE



- light source
  - (xenon arc lamp / mercury-vapor lamp / metal *halide* lamp / LED)
- excitation filter,
- dichroic mirror
- emission filter

A fluorescence microscope is basically a conventional light microscope with added features and components that extend its capabilities.

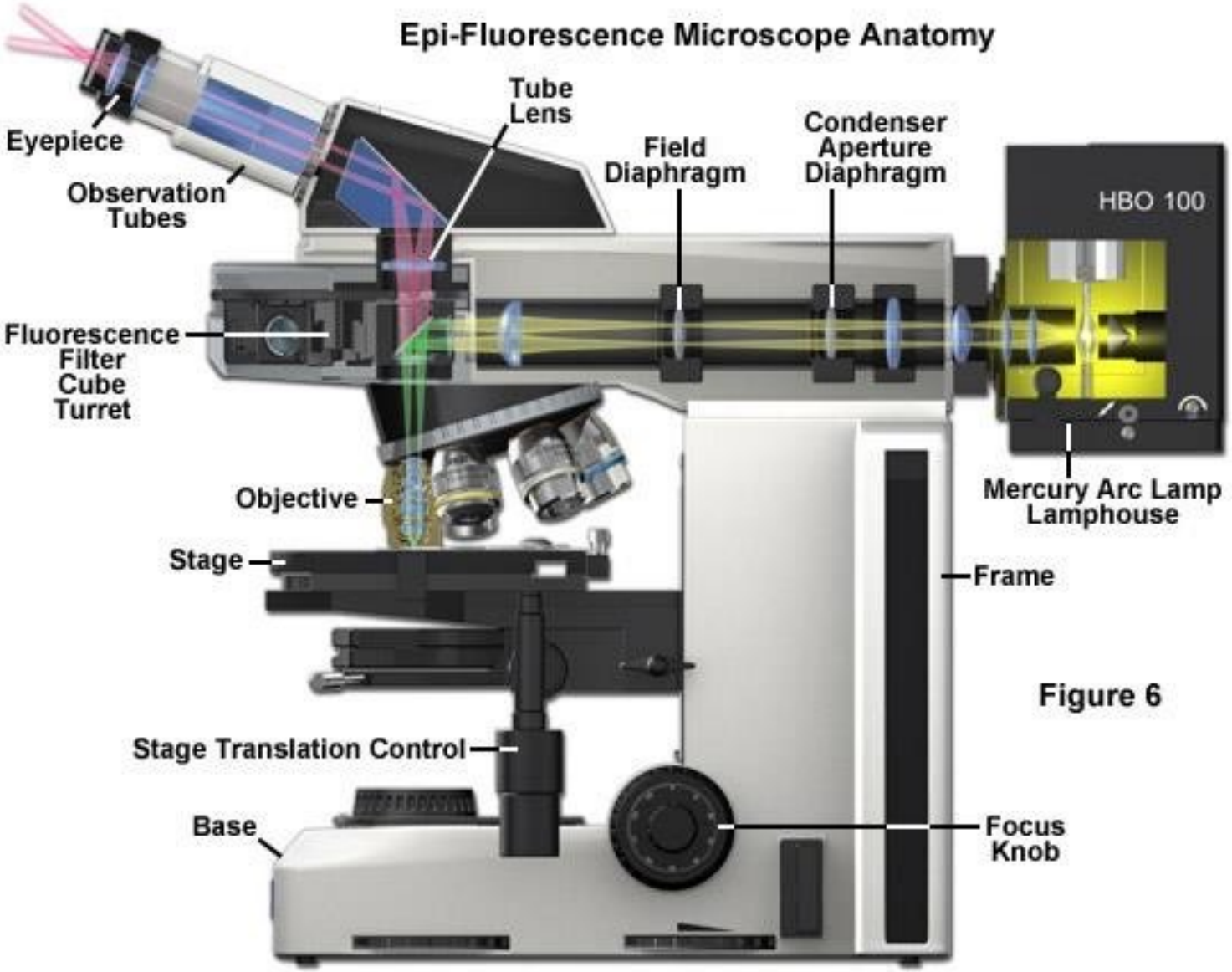
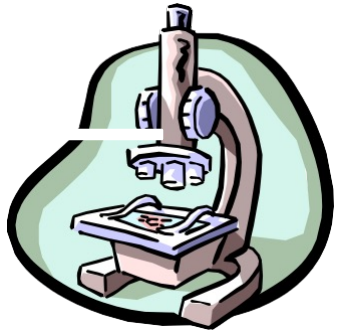
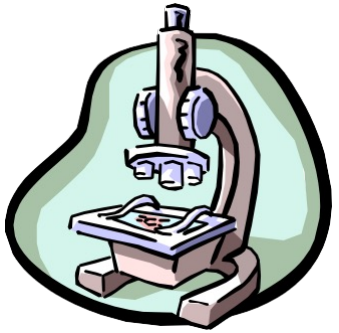

Epi-Fluorescence Microscope Anatomy
Eyepiece
Observation Tubes
Tube Lens
Field Diaphragm
Condenser Aperture Diaphragm
HBO 100
Fluorescence Filter Cube Turret
Objective
Stage
Mercury Arc Lamp Lamphouse
Frame
Stage Translation Control
Base
Focus Knob

Figure 6



LIGHT SOURCES



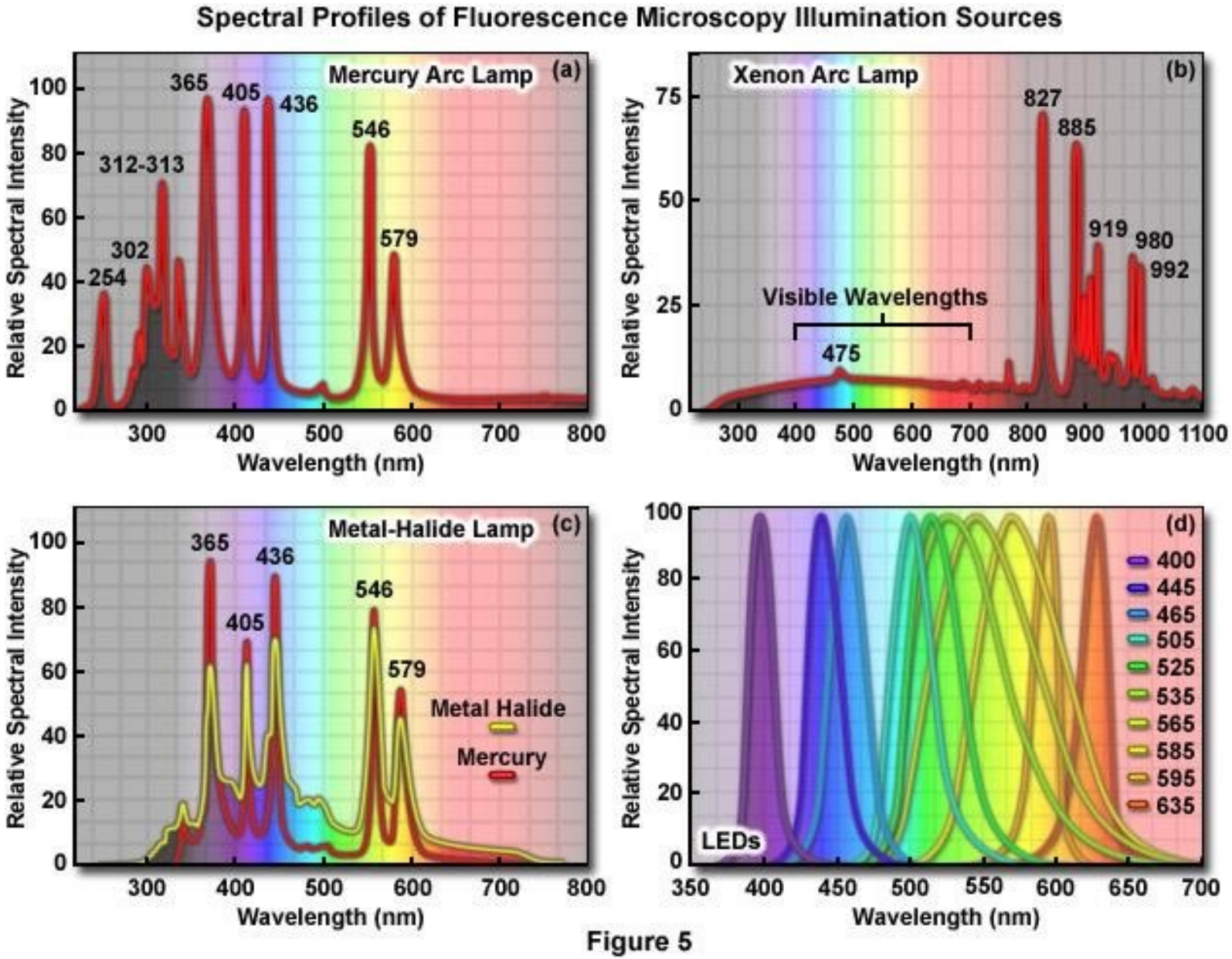


Figure 5

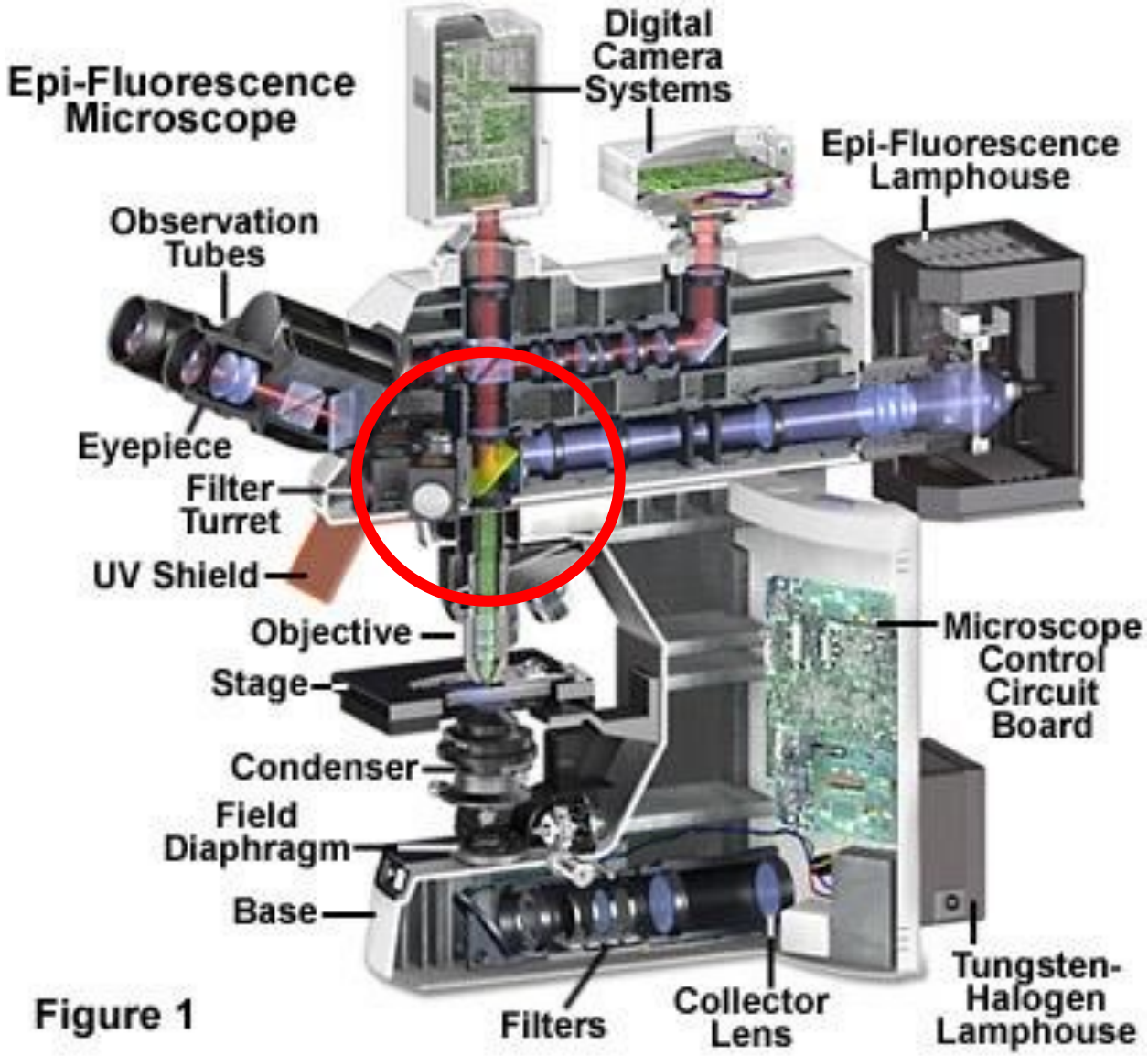
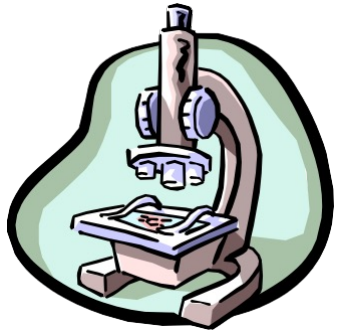


Figure 1



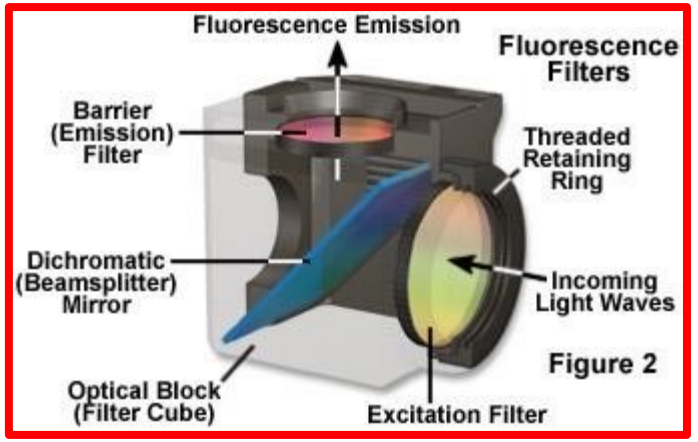
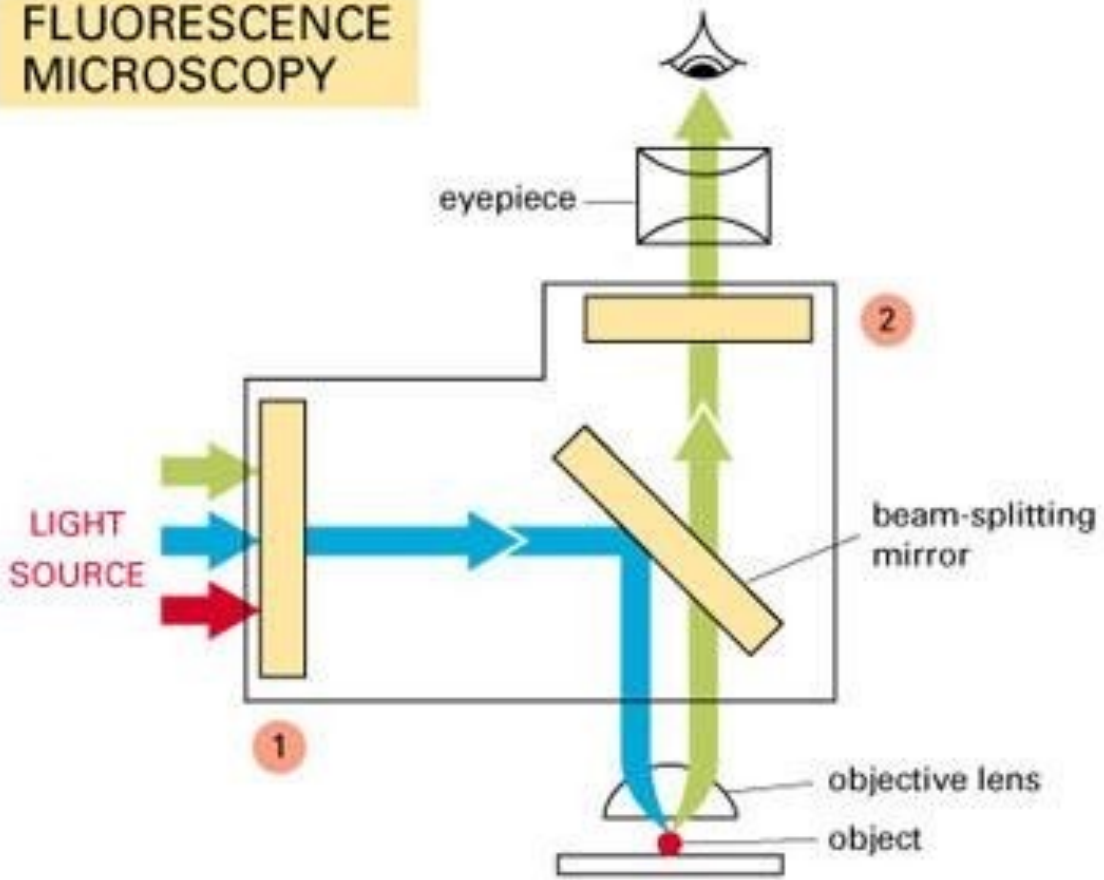
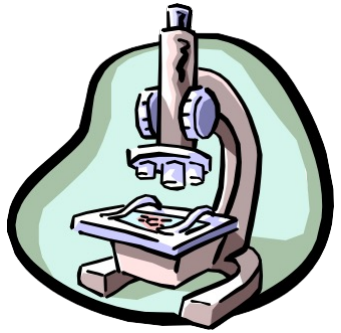


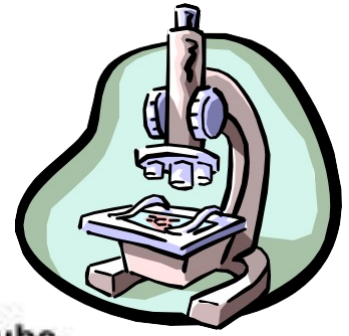
Figure 2

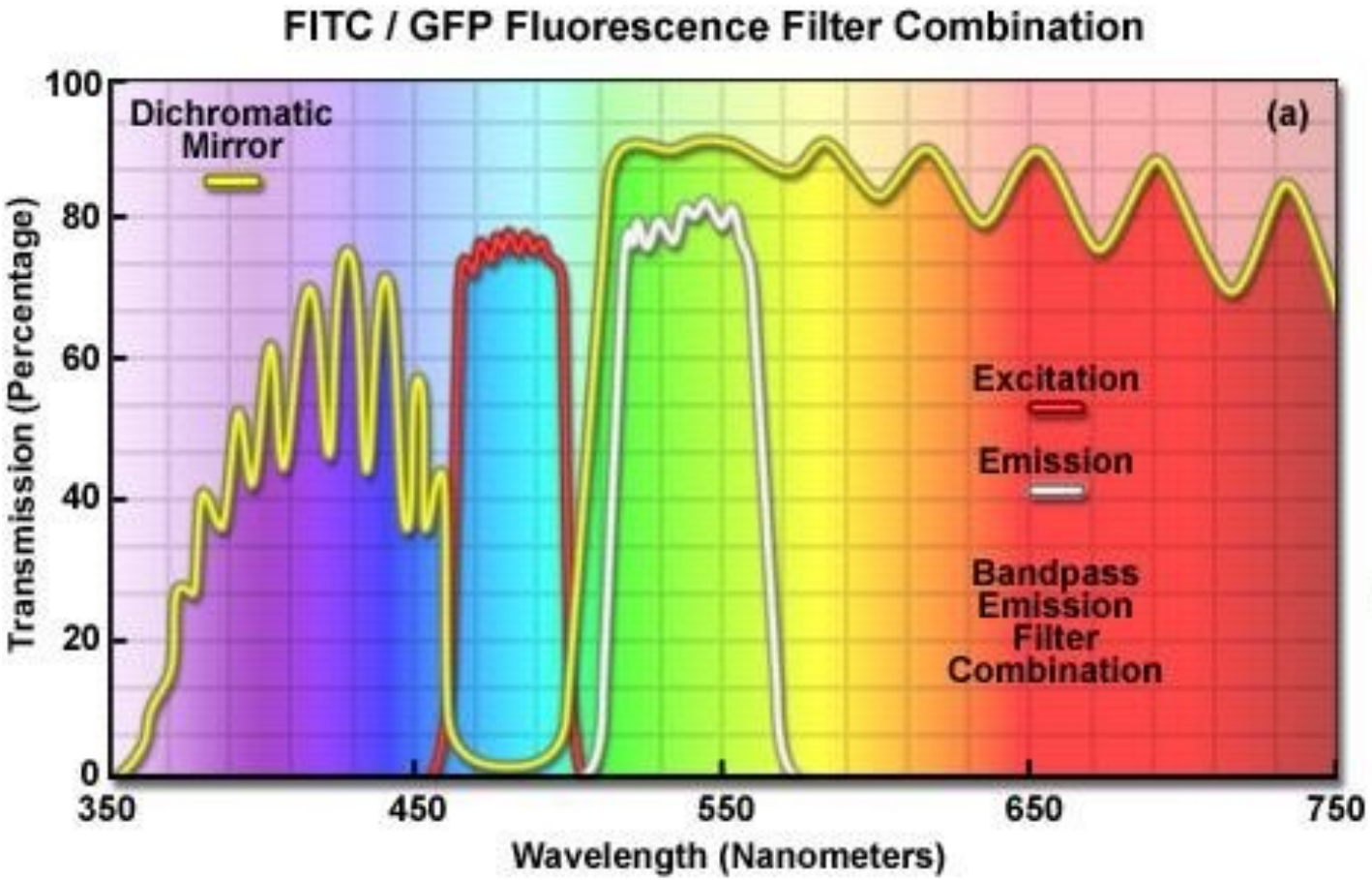
FLUORESCENCE MICROSCOPY



Fluorescent dyes used for staining cells are detected with the aid of a *fluorescence microscope*. This is similar to an ordinary light microscope except that the illuminating light is passed through two sets of filters. The first (1) filters the light before it reaches the specimen, passing only those wavelengths that excite the particular fluorescent dye. The second (2) blocks out this light and passes only those






FITC / GFP Fluorescence Filter Combination
Dichromatic Mirror
(a)
Excitation
Emission
Bandpass Emission Filter Combination
Transmission (Percentage)
100
80
60
40
20
0
350
450
550
650
750
Wavelength (Nanometers)

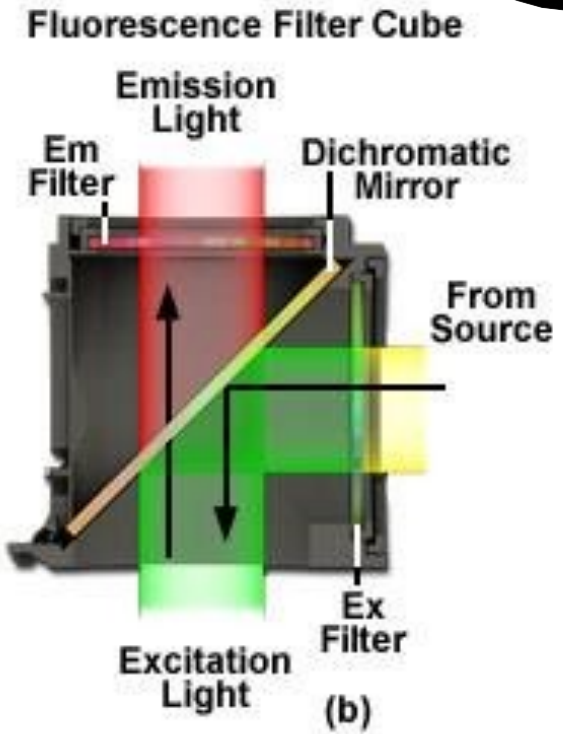
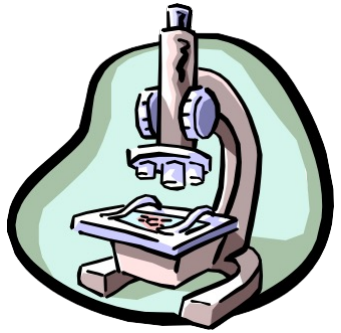

Fluorescence Filter Cube
Emission Light
Em Filter
Dichromatic Mirror
From Source
Ex Filter
Excitation Light
(b)

Figure 4

THE DICROIC MIRROR – dichroic: two color



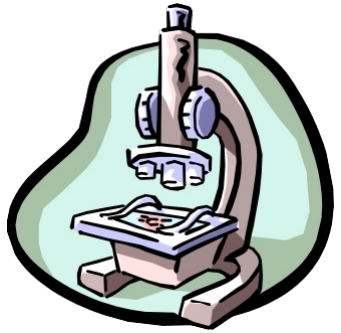
- Each dichroic mirror has a set wavelength value - the **transition wavelength value** - which is the wavelength of 50% transmission.

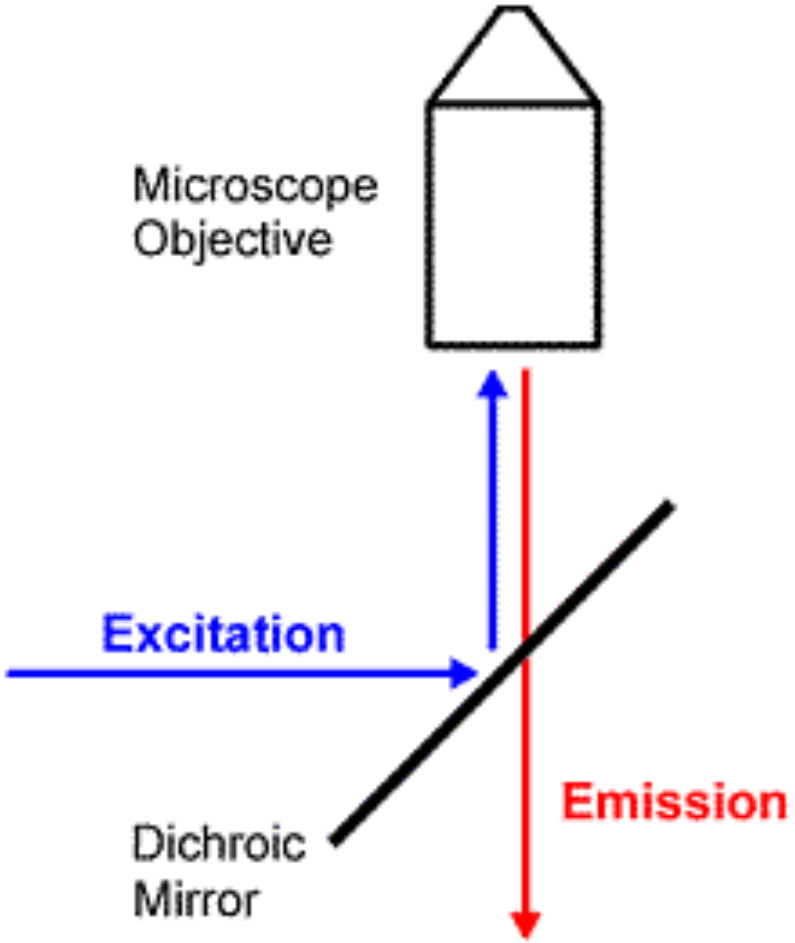
- wavelengths below the transition wavelength value are reflected (90%)

- wavelengths above this value are transmitted (90%)

- Ideally, the wavelength of the dichroic mirror is chosen to be between the wavelengths used for excitation and emission.

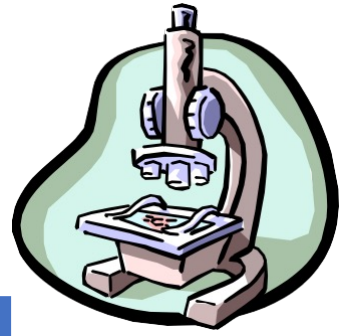
THE DICROIC MIRROR – dichroic: two color





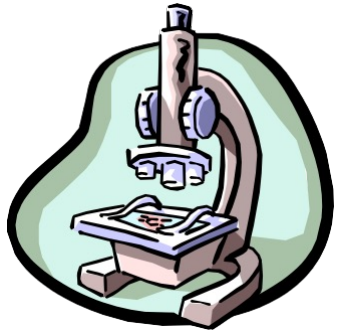
- The **excitation** light reflects off the surface of the dichroic mirror into the objective.
- The fluorescence **emission** passes through the dichroic to the eyepiece or detection system.

THE FILTERS

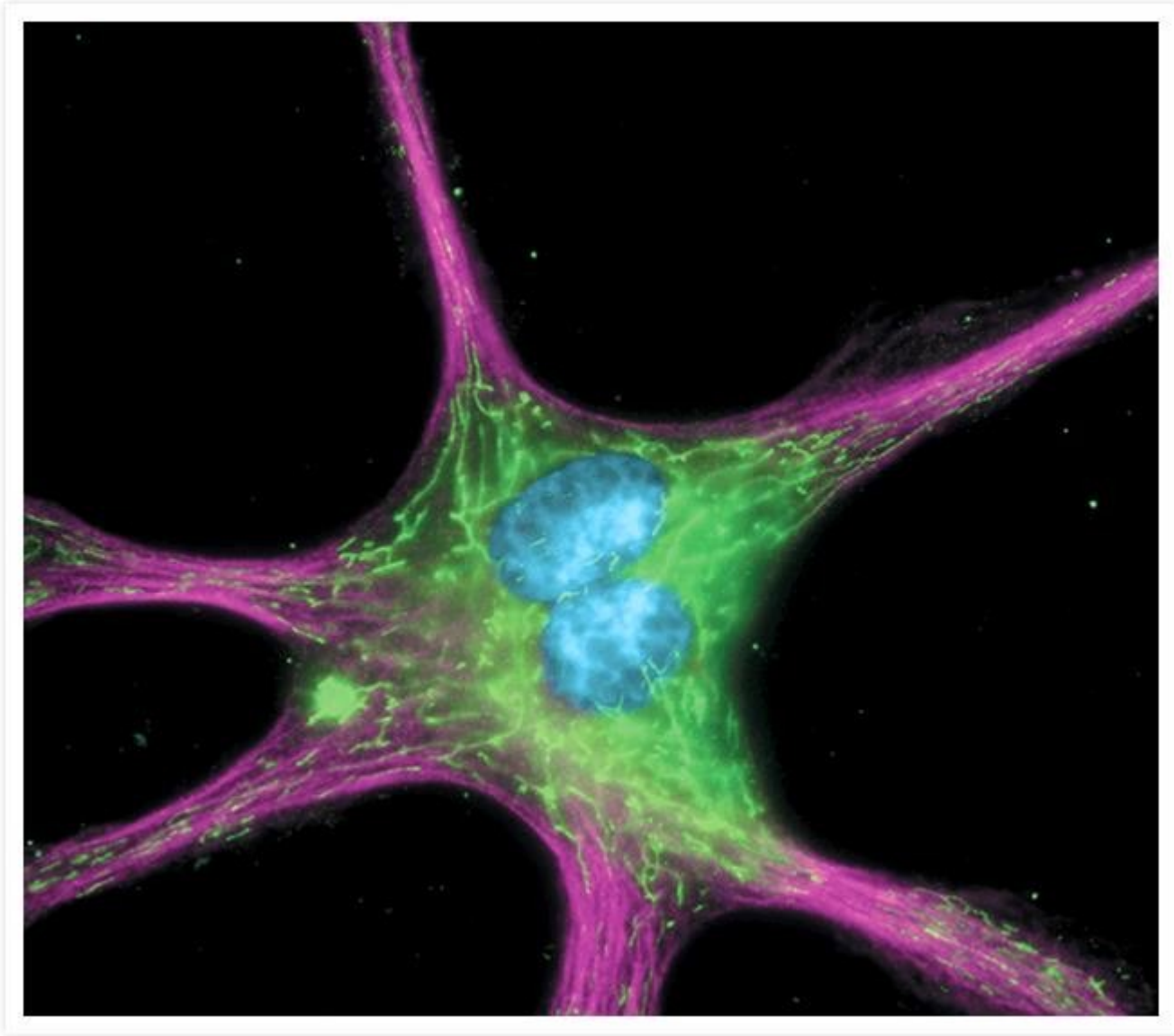


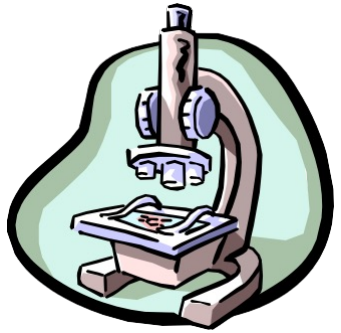
Excitation Filter	Emission Filters
to select excitation wavelength	to select emission wavelength
	to remove traces of excitation light
placed in the excitation path just prior to the dichroic mirror	placed between sample and ocular/camera/ detector

BASIC CONCEPTS

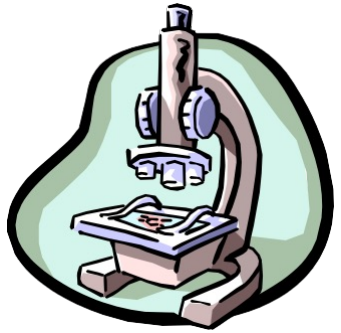


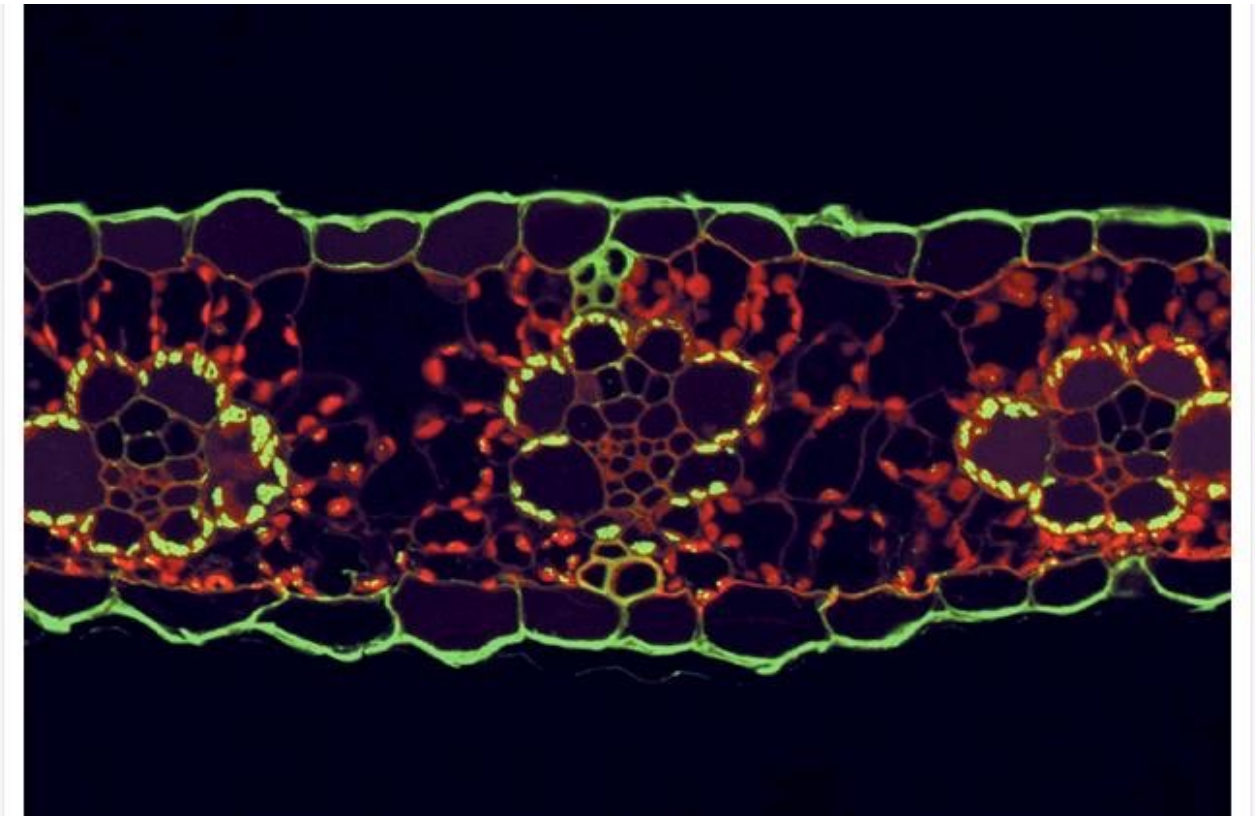
- excitation light radiates specimen
- weaker emitted light to make up the image is separated
- use fact that the emitted light is of lower energy and has a longer wavelength
- The fluorescent areas can be observed in the microscope and shine out against a dark background with high contrast



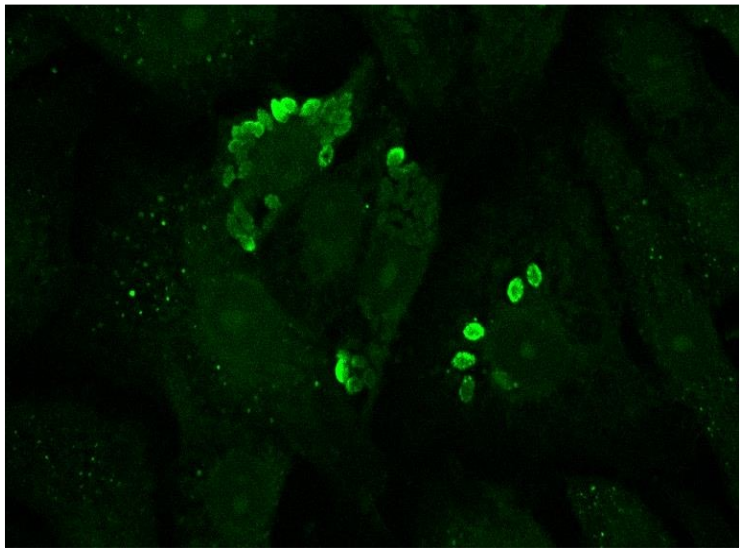


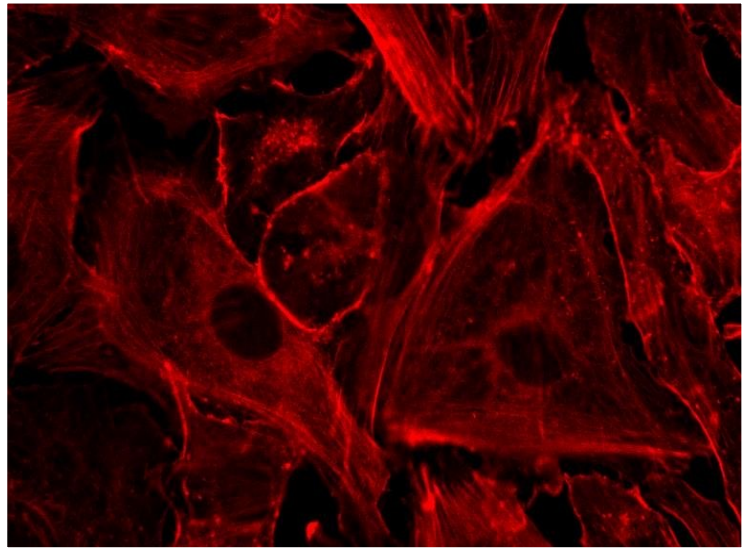
The cytoskeleton of a fixed and permeabilized bovine pulmonary artery endothelial cell detected using mouse monoclonal anti– -tubulin antibody (A11126), visualized with Alexa Fluor 647 goat anti–mouse IgG antibody (A21235) and pseudocolored magenta. Endogenous biotin in the mitochondria was labeled with green-fluorescent Alexa Fluor 488 streptavidin (S11223) and DNA was stained with blue-fluorescent DAPI (D1306, D3571, D21490).

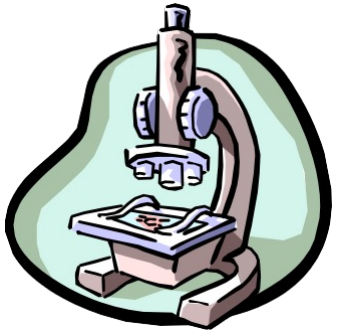


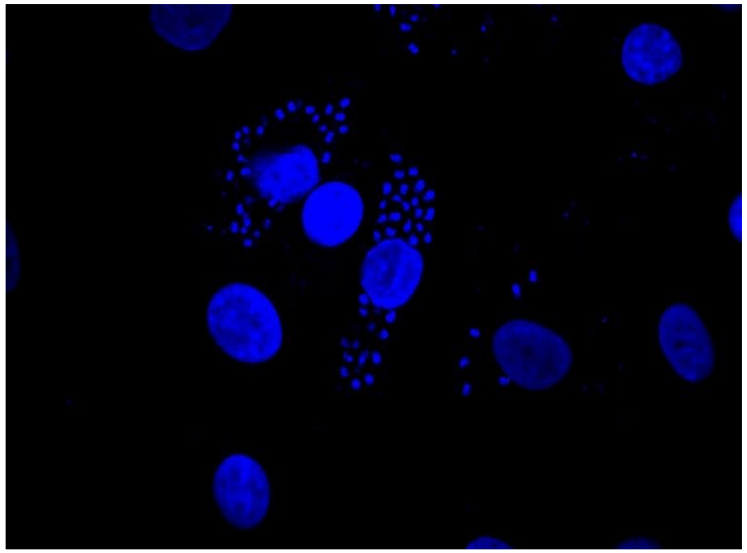


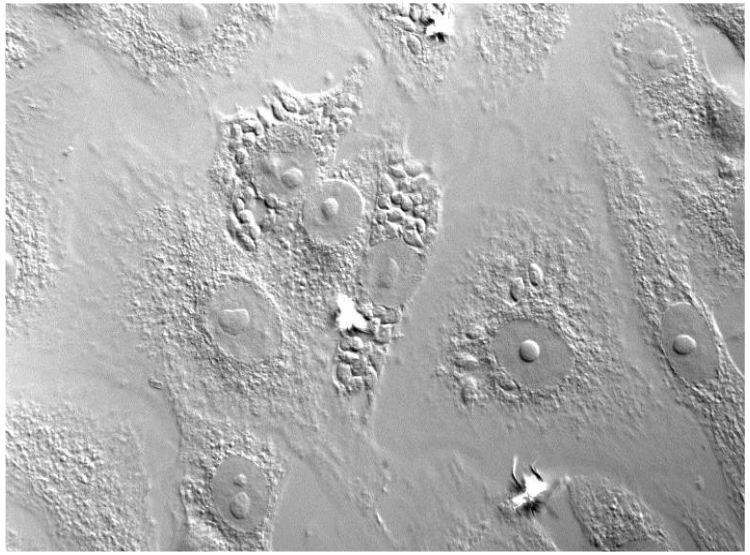
A 2.0 µm maize leaf section illustrating the immunolocalization of the enzyme ribulose bisphosphate carboxylase (rubisco) in the chloroplasts of the bundle sheath cells surrounding the vascular bundles. Maize is a C4 plant and, as a result, spatially segregates components of the photosynthetic process between the leaf mesophyll and the bundle sheath. Rubisco was localized using a rabbit anti-rubisco antibody and visualized using the highly cross-adsorbed Alexa Fluor 488 goat anti–rabbit IgG antibody (A11034). The remaining fluorescence is due to the autofluorescence of chlorophyll, which appears red and is localized to the mesophyll plastids; lignin, which appears dull green and is localized to the xylem of the vascular bundle; and cutin, which appears bright green and is localized to the cuticle outside the epidermis. Image contributed by Todd Jones, DuPont.



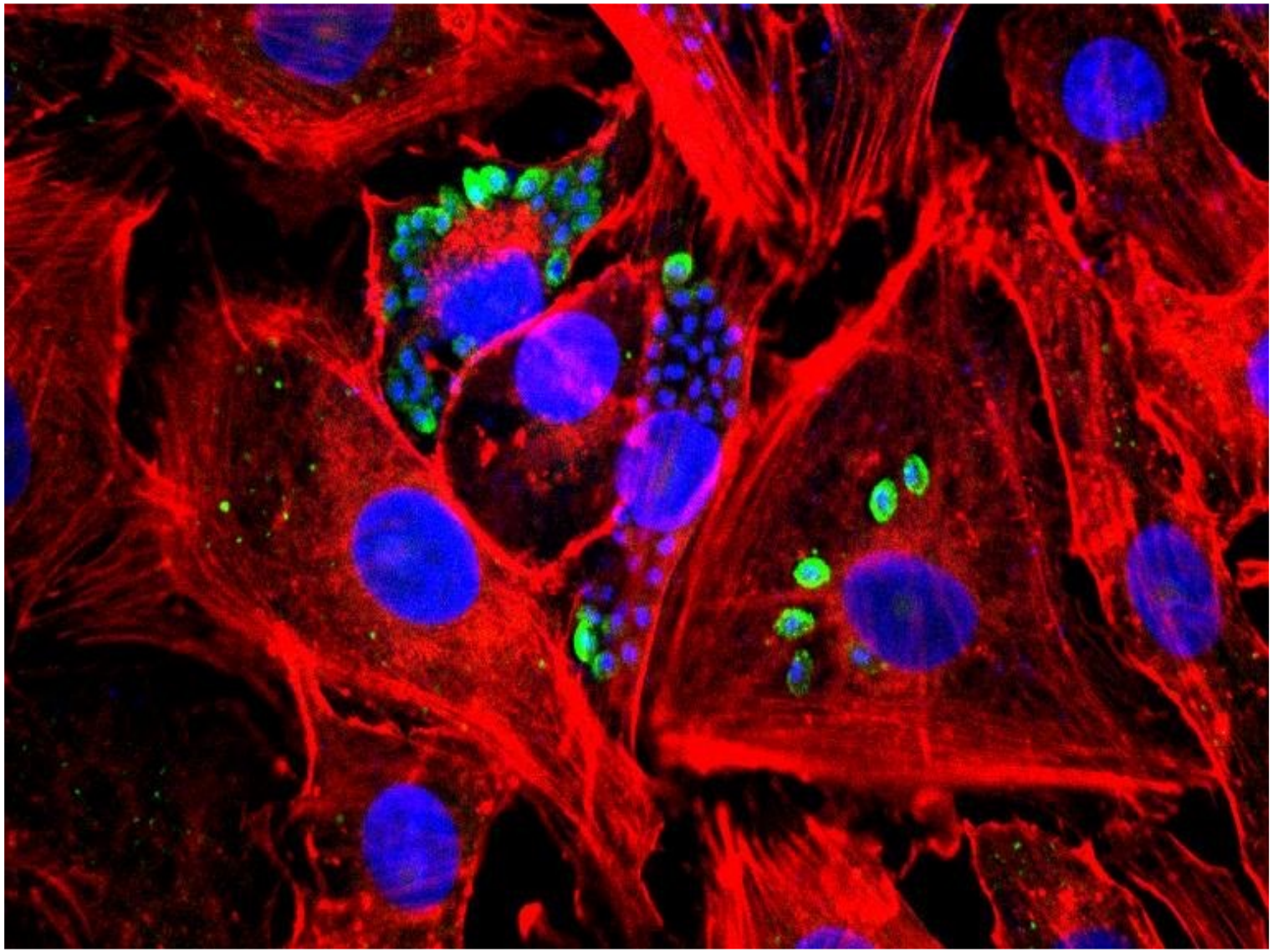


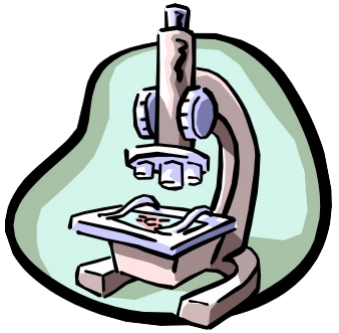






Actin - Rhodamine-phalloidin (red), Antibody to *T.cruzi* – FITC (green),
DNA – Dapi (blue)





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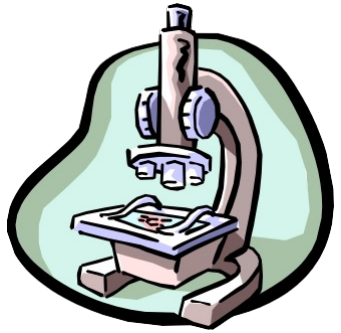
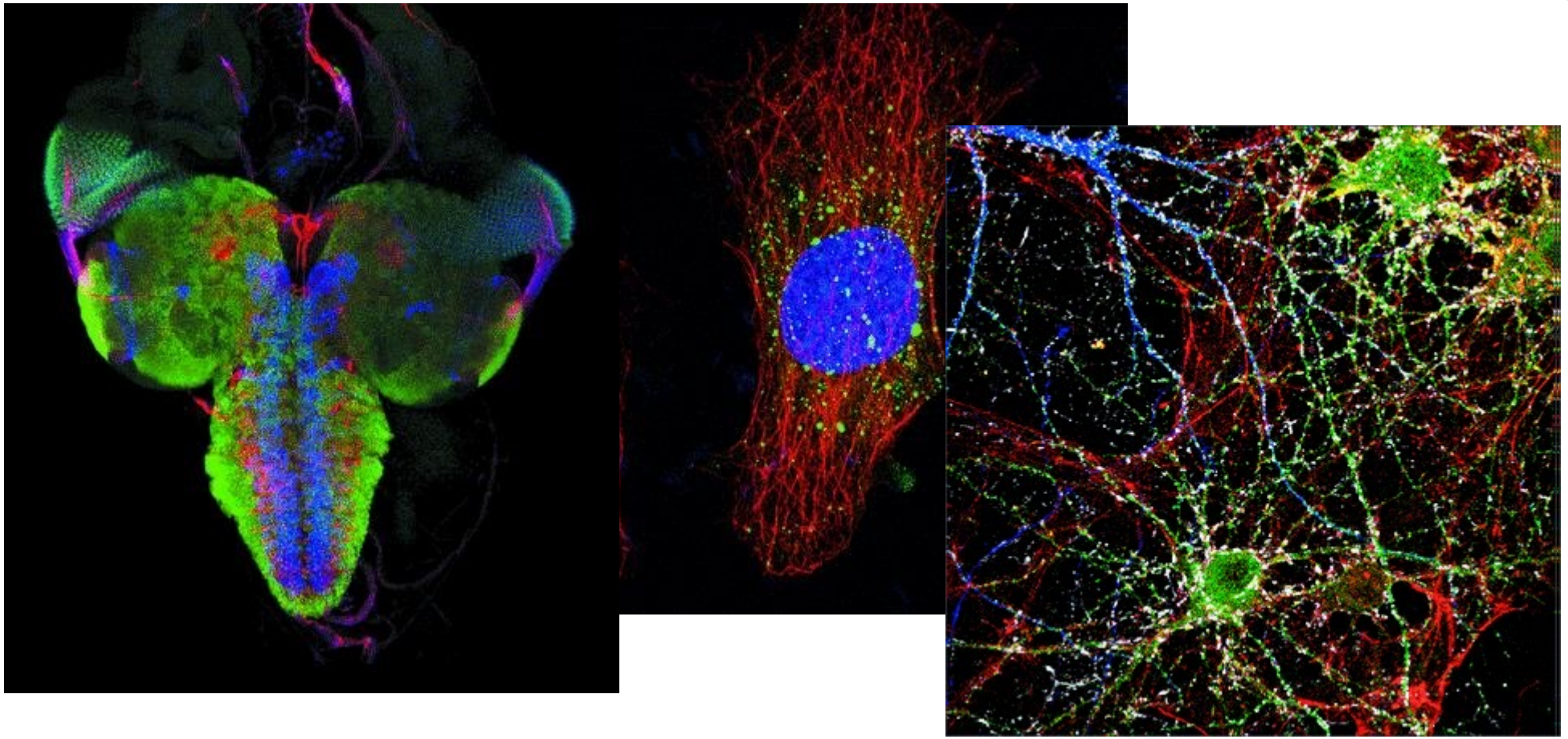
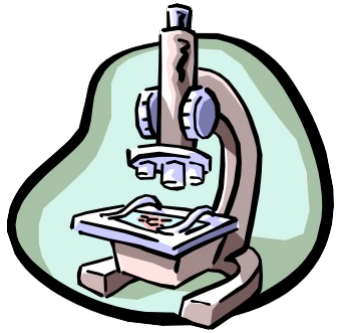


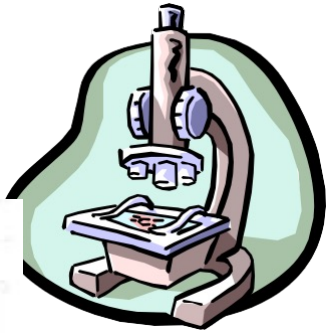


CHROMOPHORES



- Chromophores are components of molecules which absorb light
- e.g. from protein most fluorescence results from the indole ring of tryptophan residue
- They are generally combined aromatic groups, planar or cyclic molecules with several π-bonds
- Used alone as dyes or conjugated to macromolecules (antibodies!!)

FLUOROPHORES (Fluorochromes, chromophores)



Probe	Ex (nm)	Em (nm)	MW	Notes
Reactive and conjugated probes				
Hydroxycoumarin	325	386	331	Succinimidyl ester
Aminocoumarin	350	445	330	Succinimidyl ester
Methoxycoumarin	360	410	317	Succinimidyl ester
Cascade Blue	(375);401	423	596	Hydrazide
Pacific Blue	403	455	406	Maleimide
Pacific Orange	403	551		
Lucifer yellow	425	528		
NBD	466	539	294	NBD-X
R-Phycoerythrin (PE)	480;565	578	240 k	
PE-Cy5 conjugates	480;565;650	670		*aka* Cychrome, R670, Tri-Color, Quantum Red
PE-Cy7 conjugates	480;565;743	767		
Red 613	480;565	613		PE-Texas Red
PerCP	490	675		Peridinin chlorphyll protein
TruRed	490,675	695		PerCP-Cy5.5 conjugate
FluorX	494	520	587	(GE Healthcare)
Fluorescein	495	519	389	FITC; pH sensitive
BODIPY-FL	503	512		
TRITC	547	572	444	TRITC
X-Rhodamine	570	576	548	XRITC
Lissamine Rhodamine B	570	590		
Texas Red	589	615	625	Sulfonyl chloride
Allophycocyanin (APC)	650	660	104 k	
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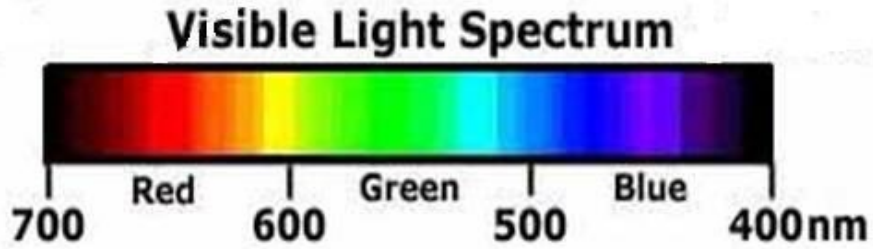
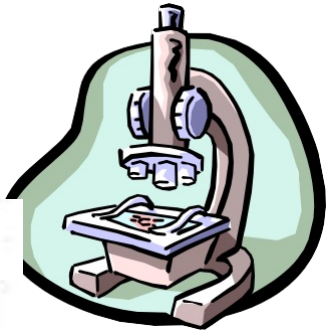


Alexa Fluor dyes [antibody conjugates] *(Molecular Probes)*				
Alexa Fluor 350	343	442	410	
Alexa Fluor 405	401	421	1028	
Alexa Fluor 430	434	540	702	
Alexa Fluor 488	499	519	643	QY 0.92
Alexa Fluor 500	503	525	700	
Alexa Fluor 514	517	542	714	
Alexa Fluor 532	530	555	724	QY 0.61
Alexa Fluor 546	561	572	1079	QY 0.79
Alexa Fluor 555	553	568	1250	QY 0.1
Alexa Fluor 568	579	603	792	QY 0.69
Alexa Fluor 594	591	618	820	QY 0.66
Alexa Fluor 610	610	629	1285	
Alexa Fluor 633	632	648	1200	
Alexa Fluor 647	652	668	1300	QY 0.33
Alexa Fluor 660	663	691	1100	

FLUOROPHORES (Fluorochromes, chromophores)



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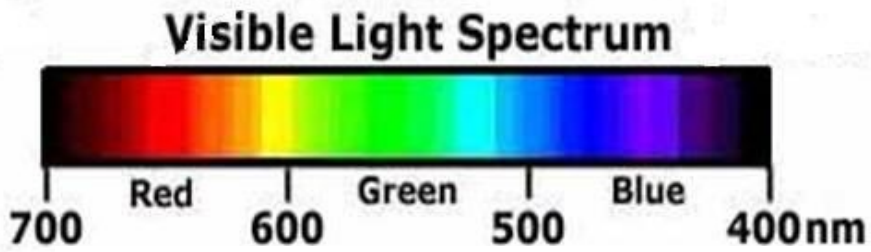
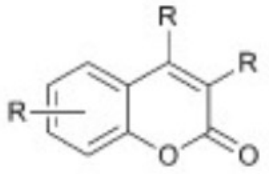
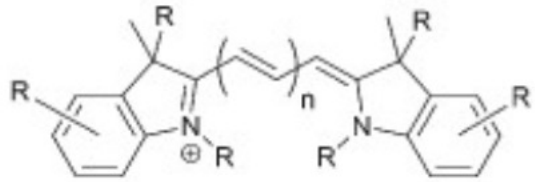


Coumarin

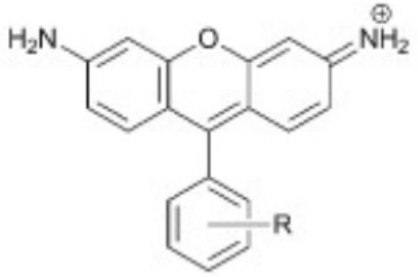


Cyanine

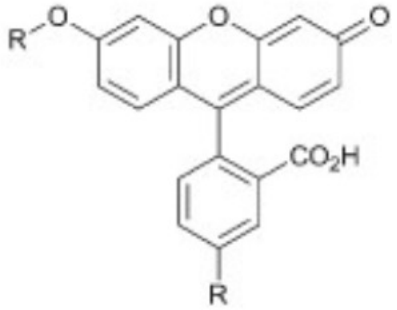


n = 1-3

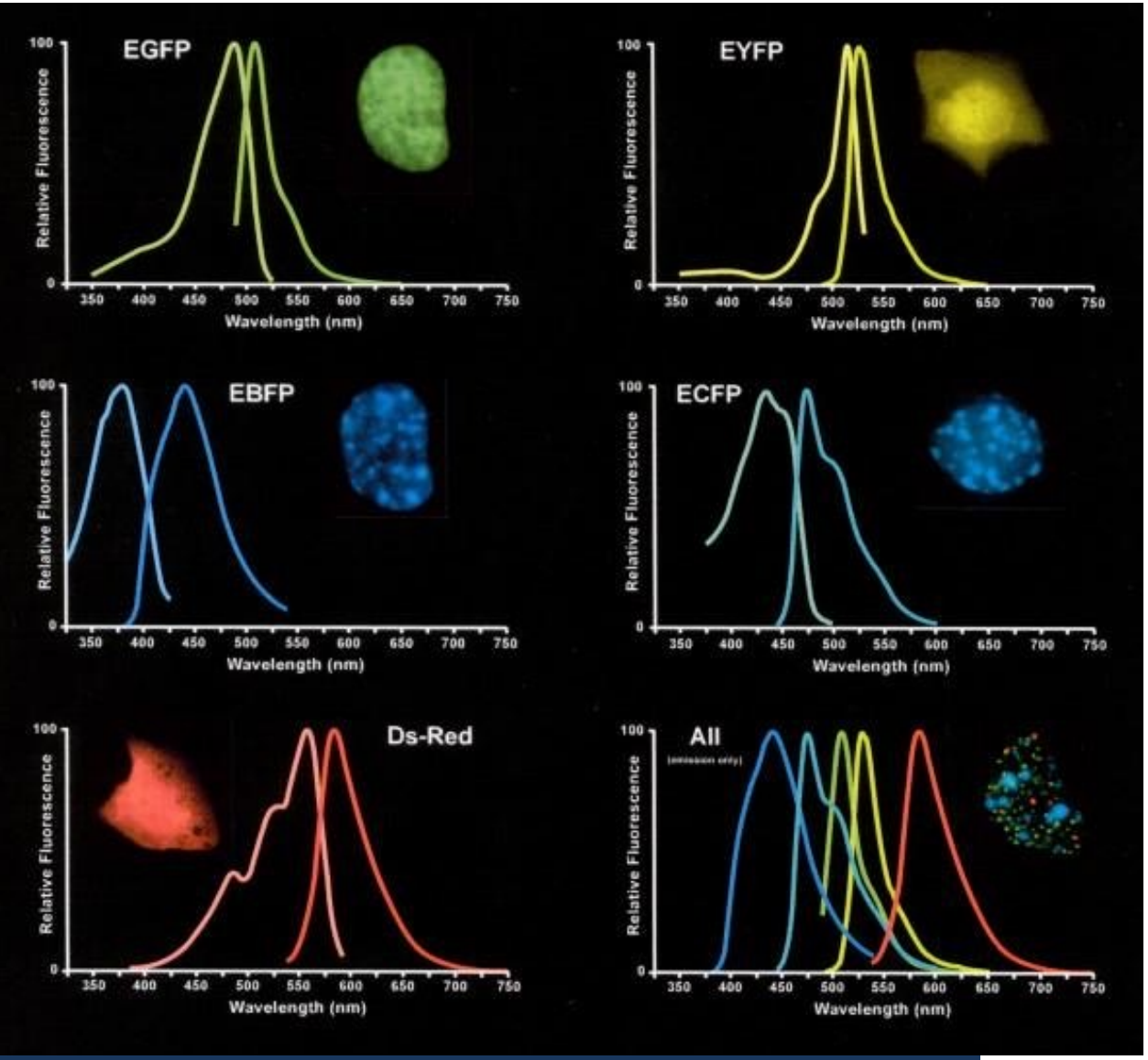
Rhodamine

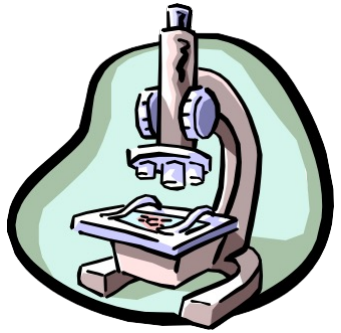


Fluorescein



FLUORESCENT PROTEINS





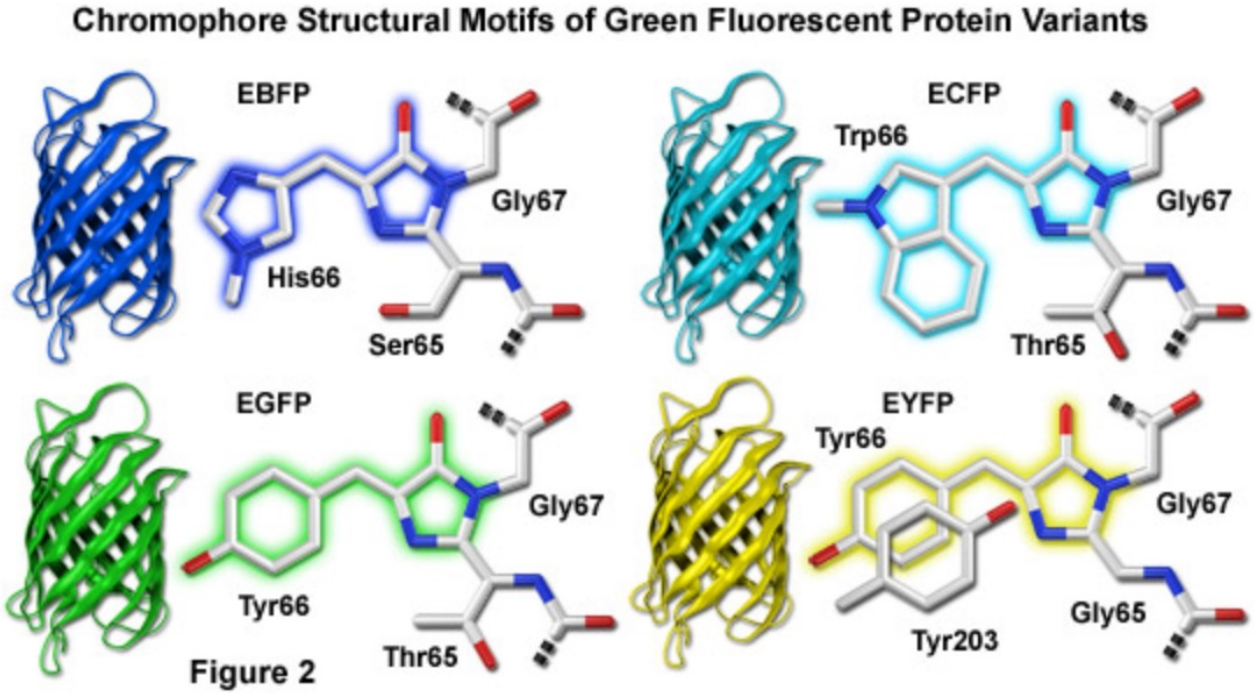
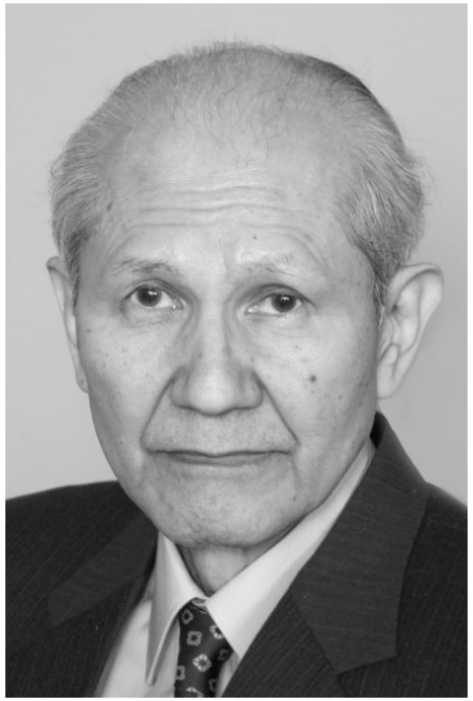


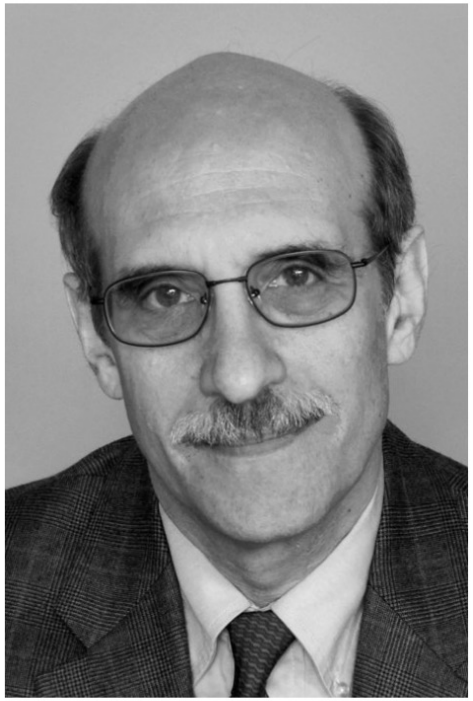
Figure 2



© The Nobel Foundation. Photo: U. Montan

Osamu Shimomura

Prize share: 1/3



© The Nobel Foundation. Photo: U. Montan

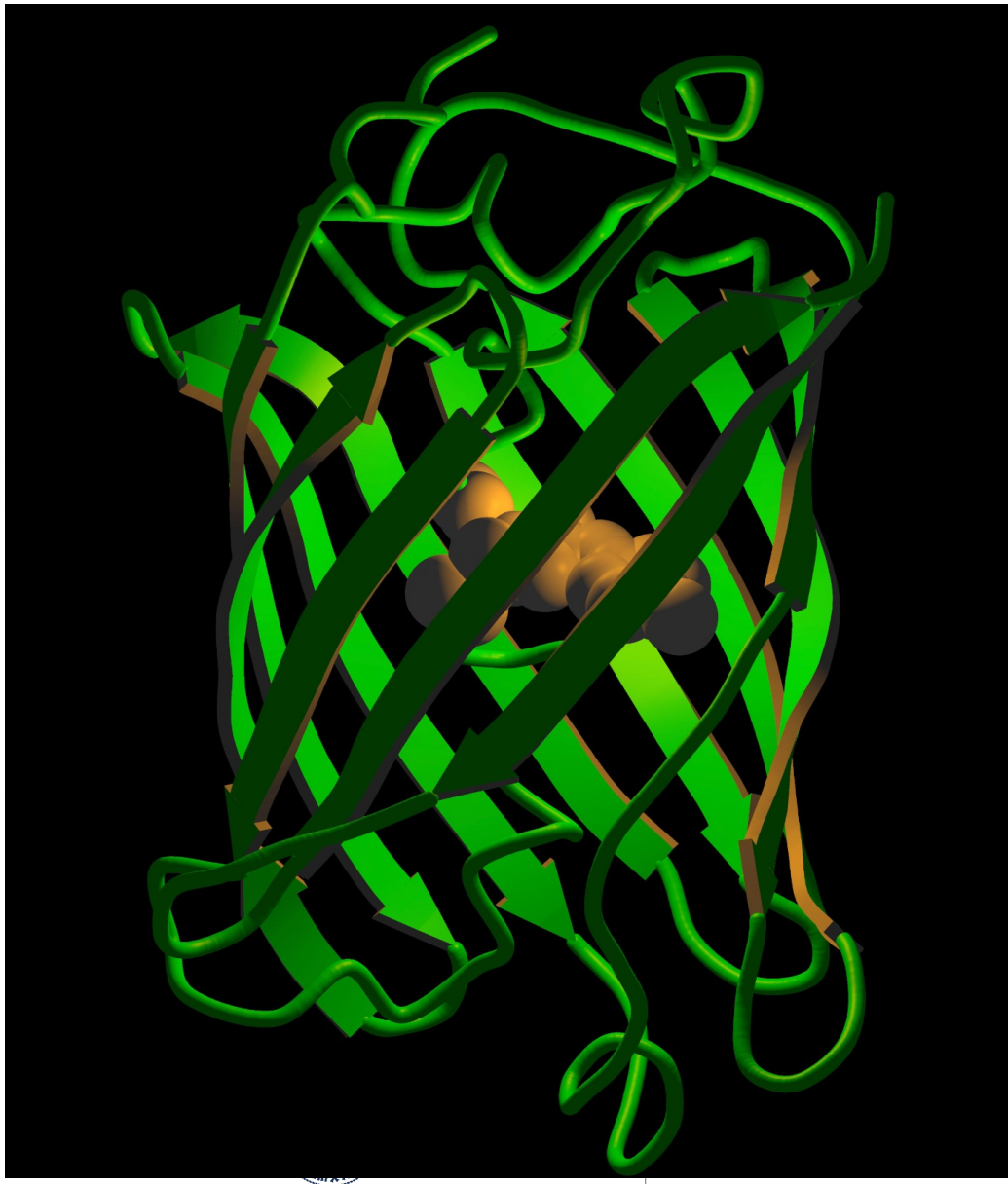
Martin Chalfie

Prize share: 1/3

© The Nobel Foundation. Photo: U. Montan

Roger Y. Tsien

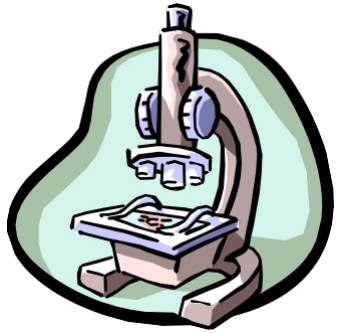
Prize share: 1/3



The Nobel Prize in Chemistry 2008 was awarded jointly to Osamu Shimomura, Martin Chalfie and Roger Y. Tsien "for the discovery and development of the green fluorescent protein, GFP"

Mitosis in Rat Kangaroo Epithelial Kidney Cells

CONSIDERATIONS



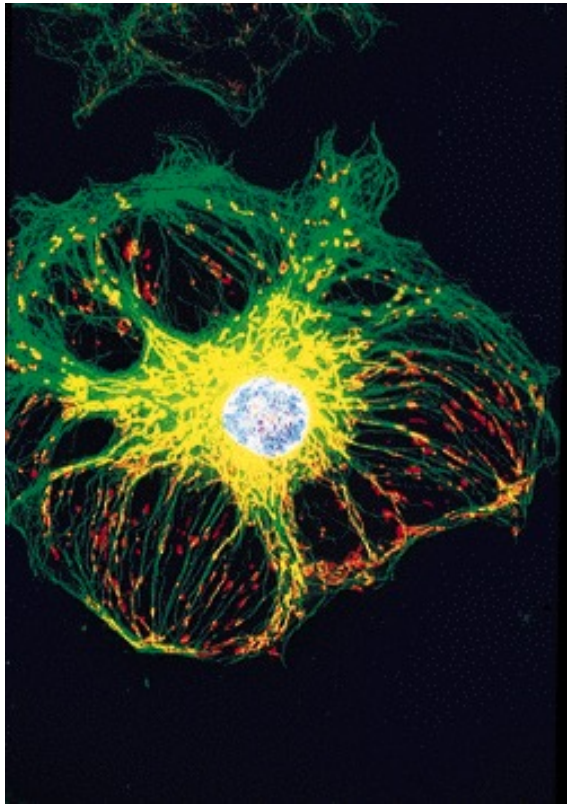
The **intensity** is related to the **probability** of the event

Wavelength relates to the **energy** of the light absorbed or emitted

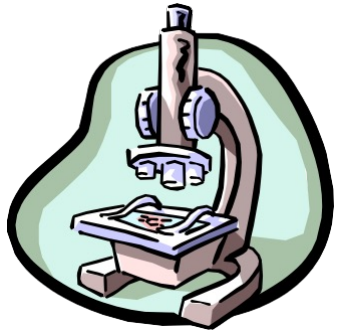
the **longer** the wavelength the **lower** the energy

the **shorter** the wavelength the **higher** the energy

MULTICHANNEL FLUORESCENCE

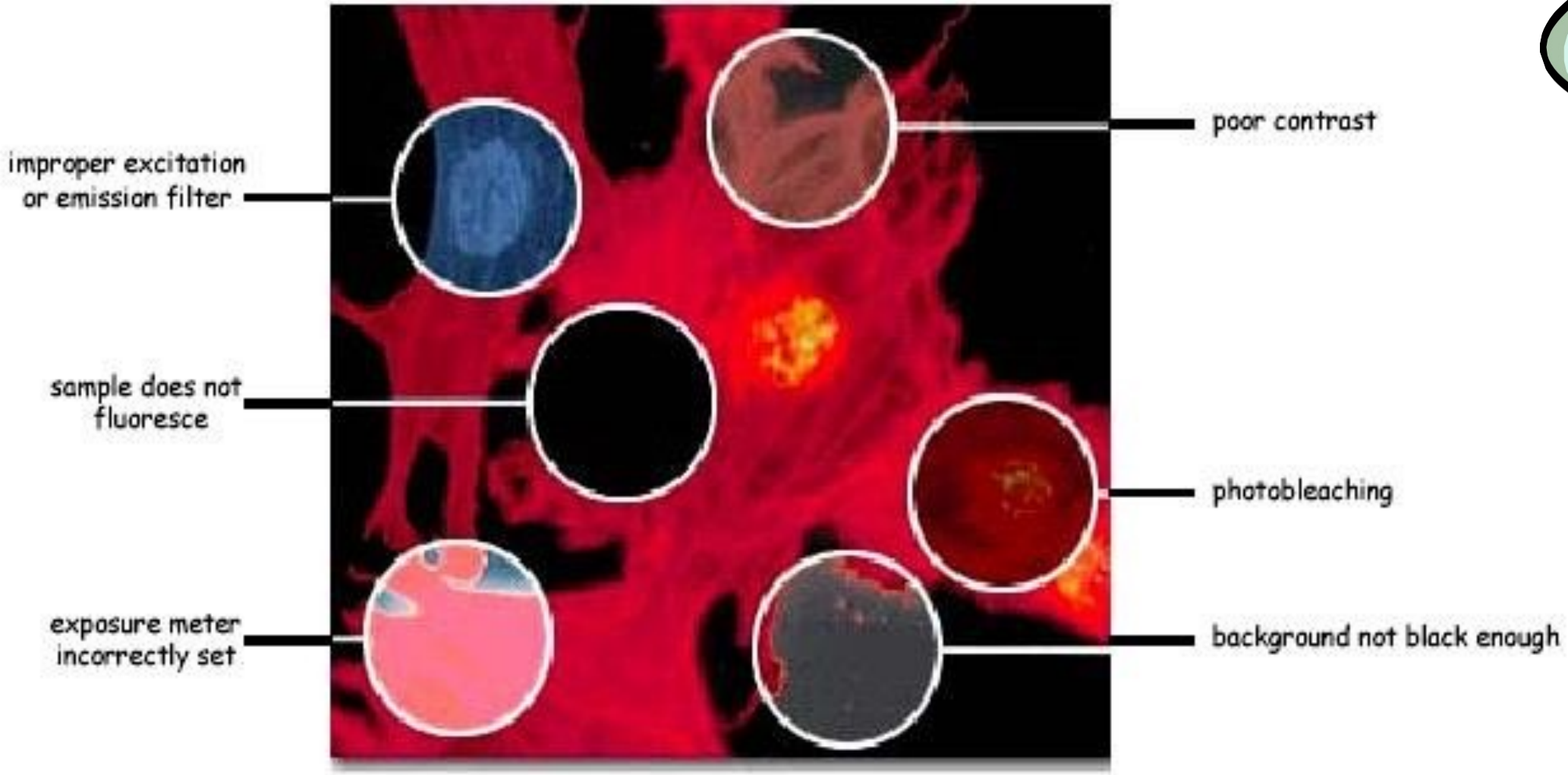


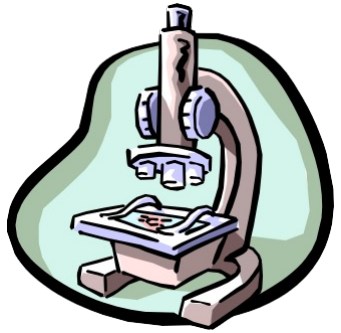
Arterial edothelial cell
Ch1(Green) FITC Tubulin
Ch2(Red) mitotracker
Ch3(Blue) DAPI



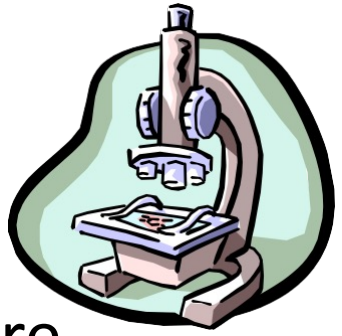
- Direct coupling to macromolecules
- Fluorescent dyes and substrates
- Fluorescent fusion proteins
- Fluorescent Antibodies

Problems with Fluorescence Microscopy



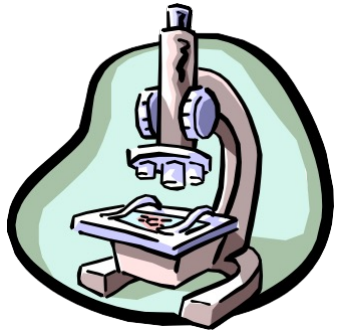


PHOTOBLEACHING



- Defined as the irreversible destruction of an excited fluorophore (discussed in later lecture)
- Methods for countering photobleaching
  - Scan for shorter times
  - Use high magnification, high NA objective
  - Use wide emission filters
  - Reduce excitation intensity
  - Use “antifade” reagents (not compatible with viable cells)

QUENCHING



Not a chemical process

Dynamic quenching:

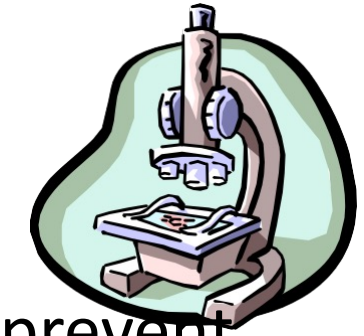
Collisional process usually controlled by mutual diffusion

Typical quenchers: oxygen, aliphatic and aromatic amines (IK, NO2, CHCl3)

Static Quenching:

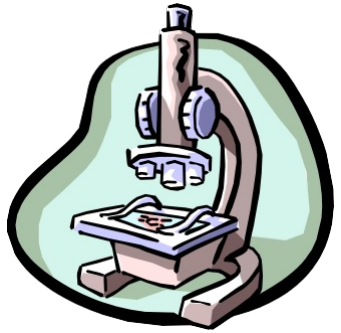
Formation of ground state complex between the fluorophores and quencher with a non-fluorescent complex (temperature dependent – if you have higher quencher ground state complex is less likely and therefore less quenching

ANTIFADE AGENTS



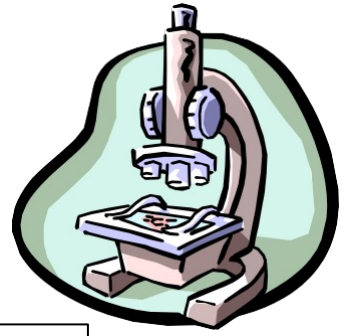
- Many antifade agents act by reducing oxygen concentration to prevent formation of singlet oxygen
- Satisfactory for fixed samples but not live cells!
- Antioxidants such as propyl gallate, hydroquinone, p-phenylenediamine are used
- Reduce O_2 concentration or use singlet oxygen quenchers such as carotenoids (50 mM crocetin or etretinate in cell cultures); ascorbate, imidazole, histidine, cysteamine, reduced glutathione, uric acid, trolox (vitamin E analogue)

STAINING

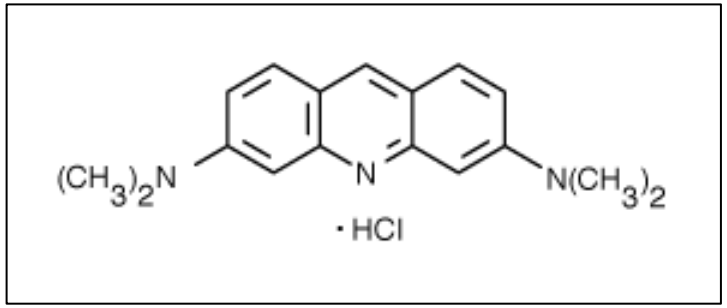


	Fluorescent dyes	Coupled fluorophore
Fixed sample	Most DNA dyes	to proteins: Antibodies, lectins, streptavidin to other molecules (nucleic acids,..) Fluorescent protein coupling (usually genetically encoded)
Live sample	Some DNA stains Organelle stains (mitotracker, lysotracker, ER tracker)	Ready access only for surface targeting coupled fluorophores Main application for genetically encoded fluorescent proteins

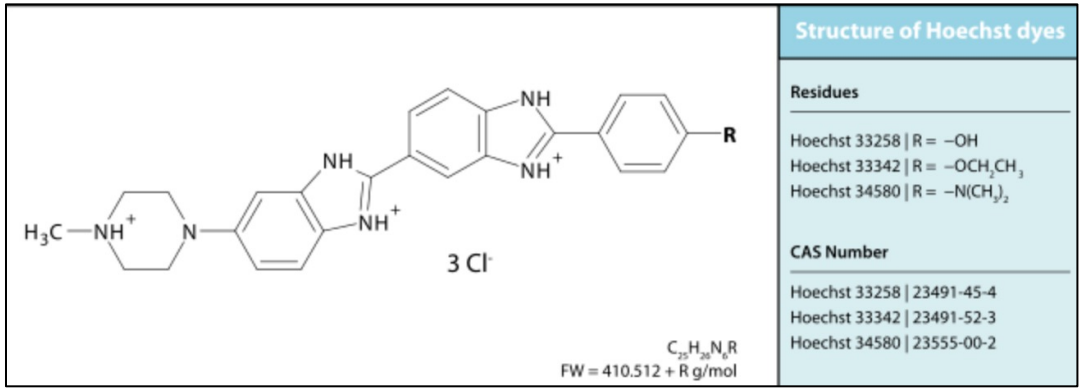
DNA PROBES



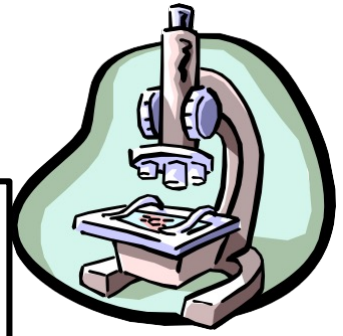
- Acridine Orange
  - Metachromatic dye
    - concentration dependent emission
    - double stranded NA - Green
    - single stranded NA - Red



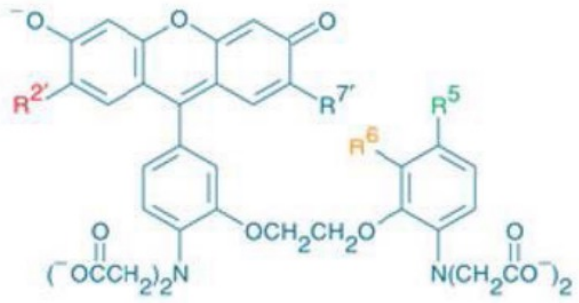
- AT/GC binding dyes
  - AT rich: DAPI, Hoechst, quinacrine
  - GC rich: antibiotics bleomycin, chromamycin olivomycin, rhodamine 800



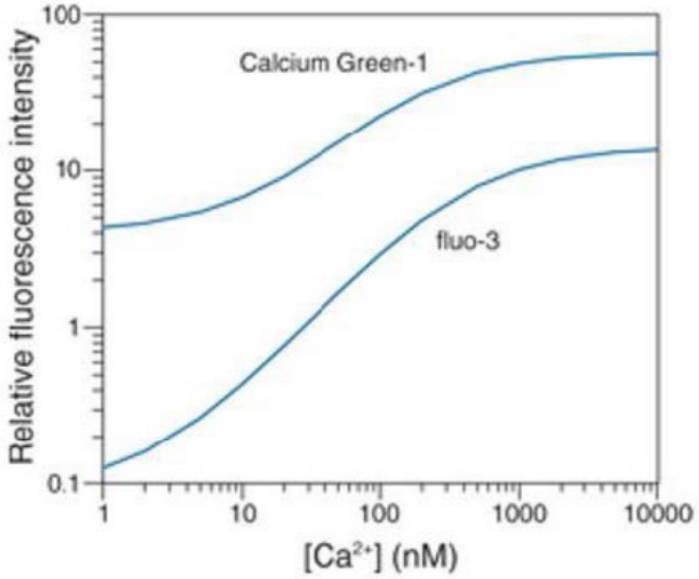
PROBES FOR IONS



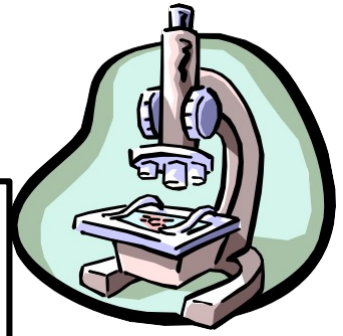
• INDO-1	E_x350		E_m405/480
• QUIN-2	E_x350		E_m490
• Fluo-3	E_x488		E_m525
• Fura -2	E_x330/360		E_m510



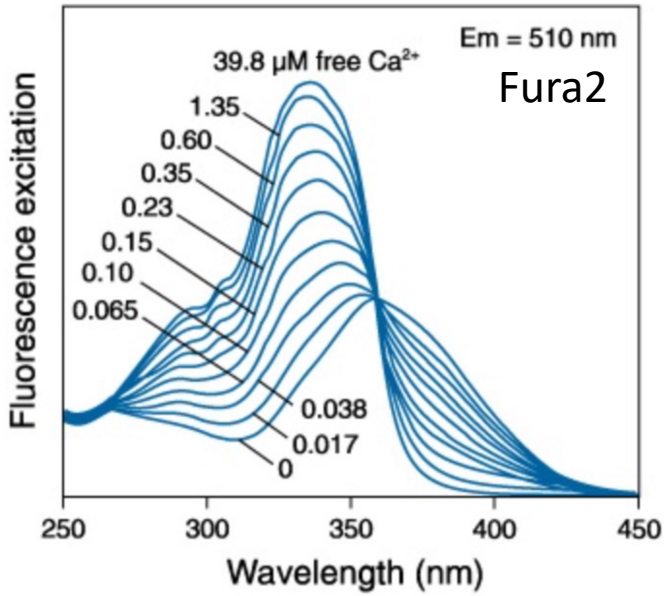
Indicator	$K_d(Ca^{2+})$	$R^{2'}$	$R^{7'}$	R^5	R^6
Fluo-3	0.39 µM	Cl	Cl	CH_3	H
Fluo-4	0.35 µM	F	F	CH_3	H
Fluo-5F	2.3 µM	F	F	F	H
Fluo-5N	90 µM	F	F	NO_2	H
Fluo-4FF	9.7 µM	F	F	F	F

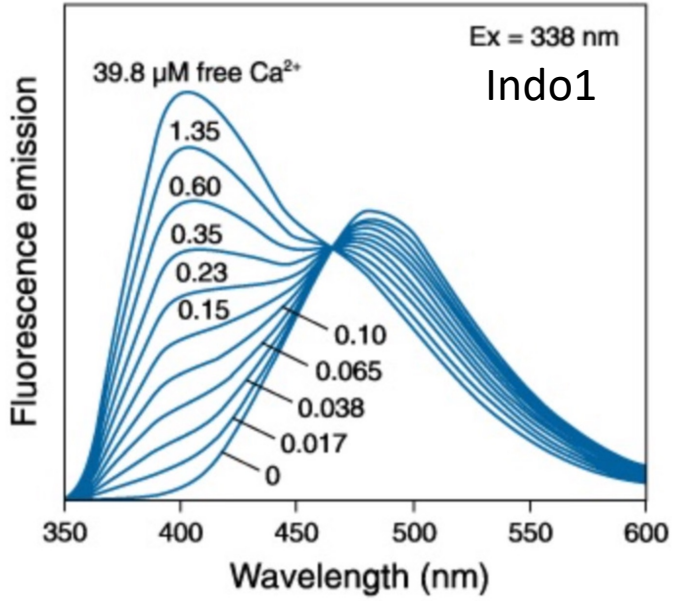


PROBES FOR IONS

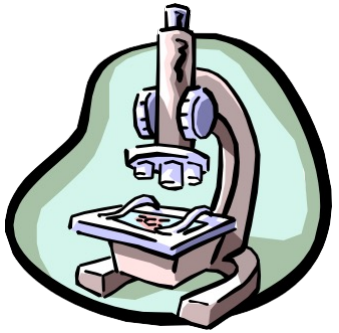


• INDO-1	E_x350	E_m405/480
• QUIN-2	E_x350	E_m490
• Fluo-3	E_x488	E_m525
• Fura -2	E_x330/360	E_m510

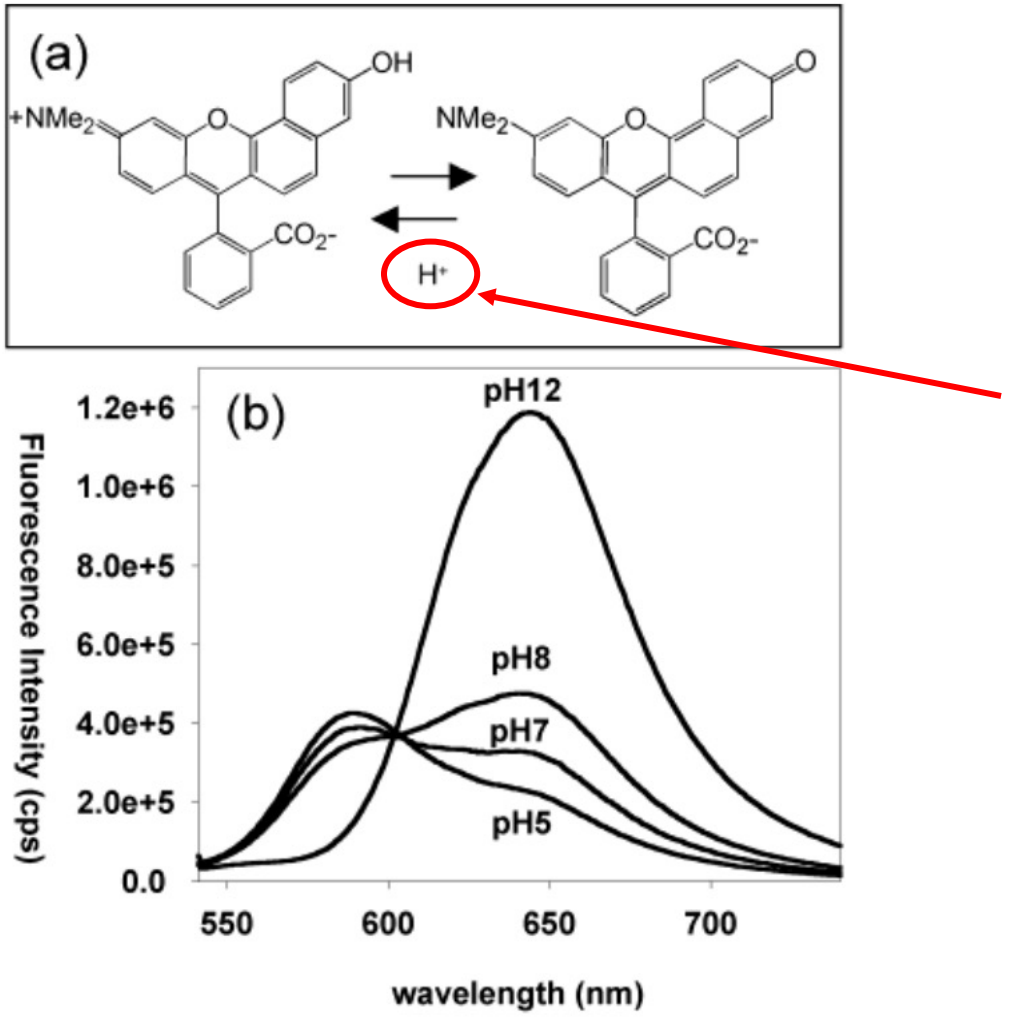




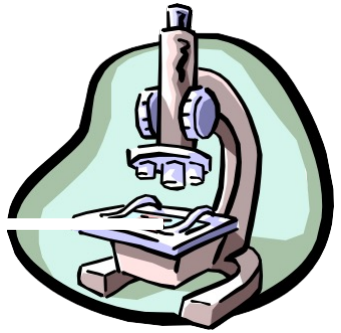
PH SENSITIVE INDICATORS



SNARF-1



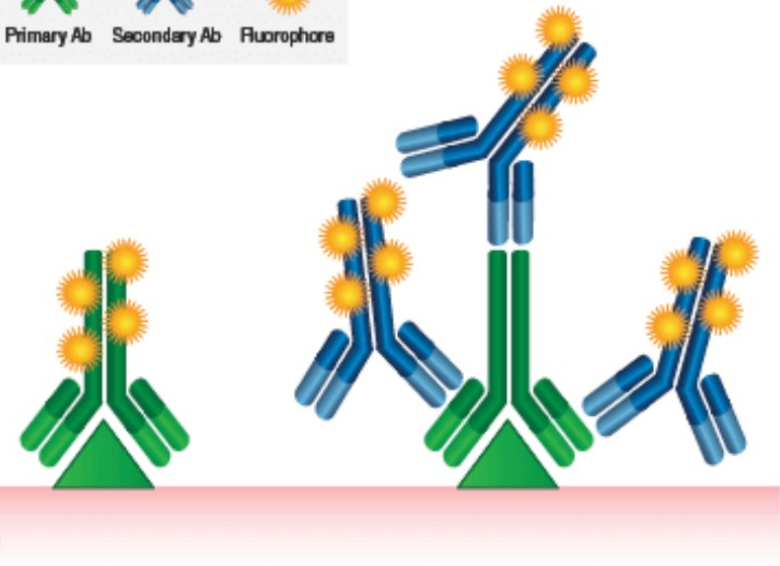
SPECIFIC ORGANELLE PROBES



Probe	Site	Excitation	Emission
BODIPY	GOLGI	505	511
NBD	GOLGI	488	525
DPH	LIPID	350	420
TMA-DPH	LIPID	350	420
RHODAMINE 123	MITOCHONDRIA	488	525
DIO	LIPID	488	500
DII-CN-(5)	LIPID	550	565
DIO-CN-(3)	LIPID	488	500

BODIPY - borate-dipyrromethene complexes
NBD - nitrobenzoxadiazole
DPH – diphenylhexatriene
TMA - trimethylammonium

Fluorophore-conjugated ANTIBODIES



① Fixation

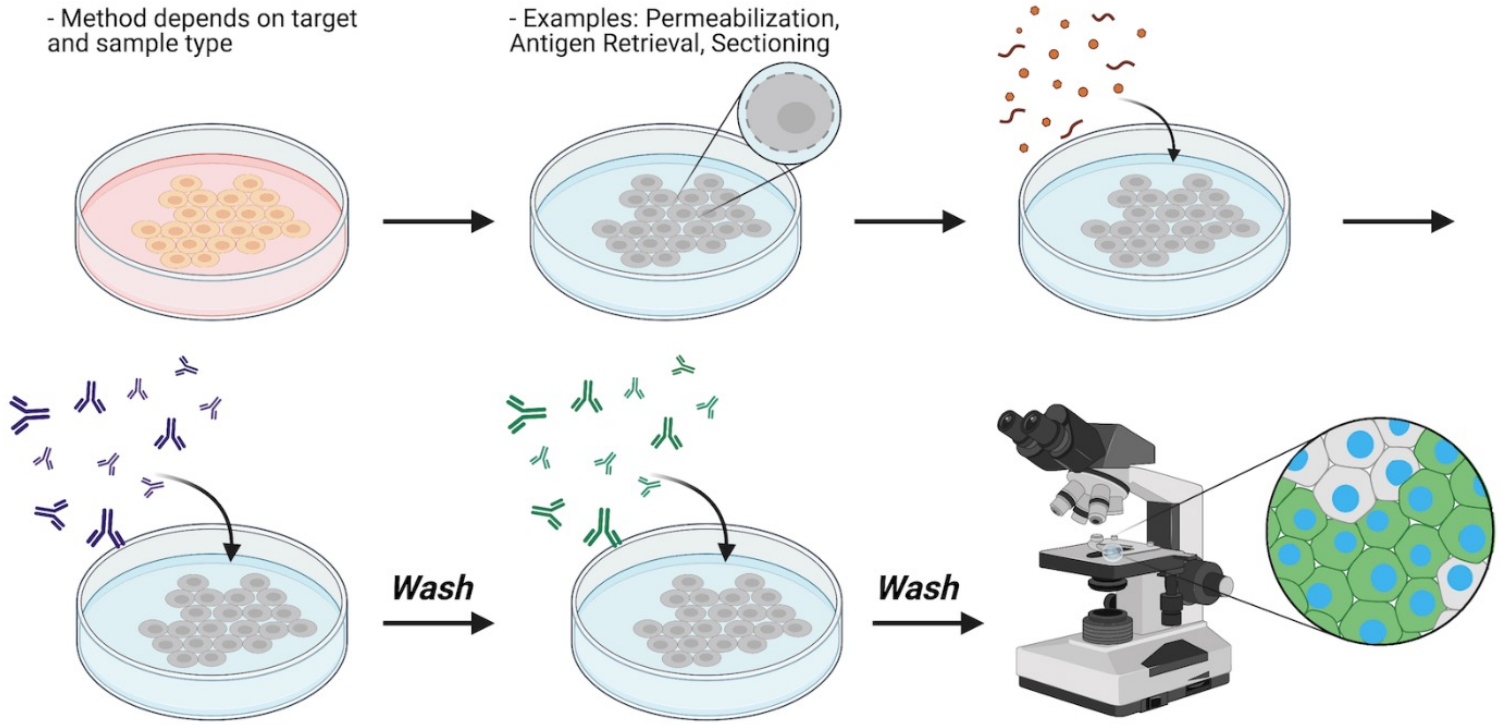
- Maintain sample morphology with minimal impact to target epitope
- No "1 size fits all" method
- Method depends on target and sample type

② Sample Prep

- Ensure that antibodies can access target
- Methods depend on target and sample type
- Examples: Permeabilization, Antigen Retrieval, Sectioning

③ Blocking

- Reduce non-specific binding of antibodies
- May need to optimize type of block, concentration, incubation time



④ Primary Antibody

- 1st antibody binds to your target
- May need to optimize concentration and incubation time

⑤ Secondary Antibody

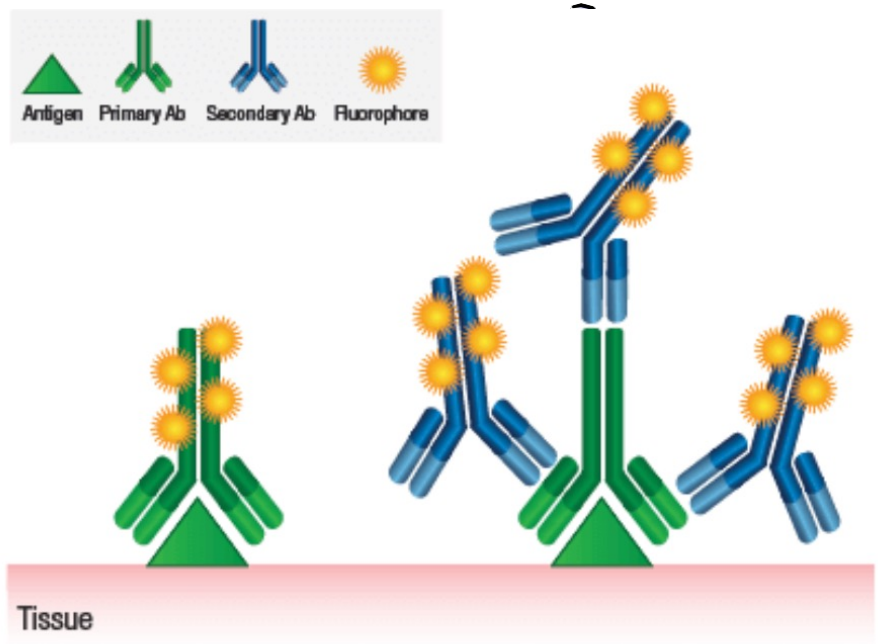
- 2nd antibody conjugated to fluorophore binds to the 1st
- May need to optimize the concentration and incubation time as well

⑥ Preservation & Imaging

- Maintain sample integrity and fluorescence
- Methods depend on sample type, microscopy tools available, and experimental goals
- Examples: Counterstaining, Mounting sample on slides

Antigen Detection	Direct immunofluorescence with primary antibody conjugated to a fluorophore	Indirect immunofluorescence with secondary antibody conjugated to a fluorophore
Protocol	Parallel staining	Parallel staining
Primary Antibody	Same host species can be used for multiple targets	Different host species or isotype for each target
Seconday Antibody	No	Yes
Signal Amplification	None	Moderate

Fluorophore-conjugated ANTIBODIES



- Which target protein? -> Selection for specificity
- Accessability of target (cell surface, intracellular)
    - Permeabilisation after fixation
- Blocking of unspecific sites
- Controls for specificity of antibody
- Mounting

Antigen Detection	Direct immunofluorescence with primary antibody conjugated to a fluorophore	Indirect immunofluorescence with secondary antibody conjugated to a fluorophore
Protocol	Parallel staining	Parallel staining
Primary Antibody	Same host species can be used for multiple targets	Different host species or isotype for each target
Seconday Antibody	No	Yes
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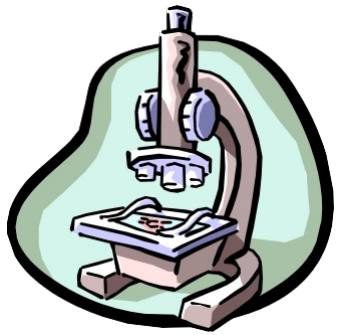
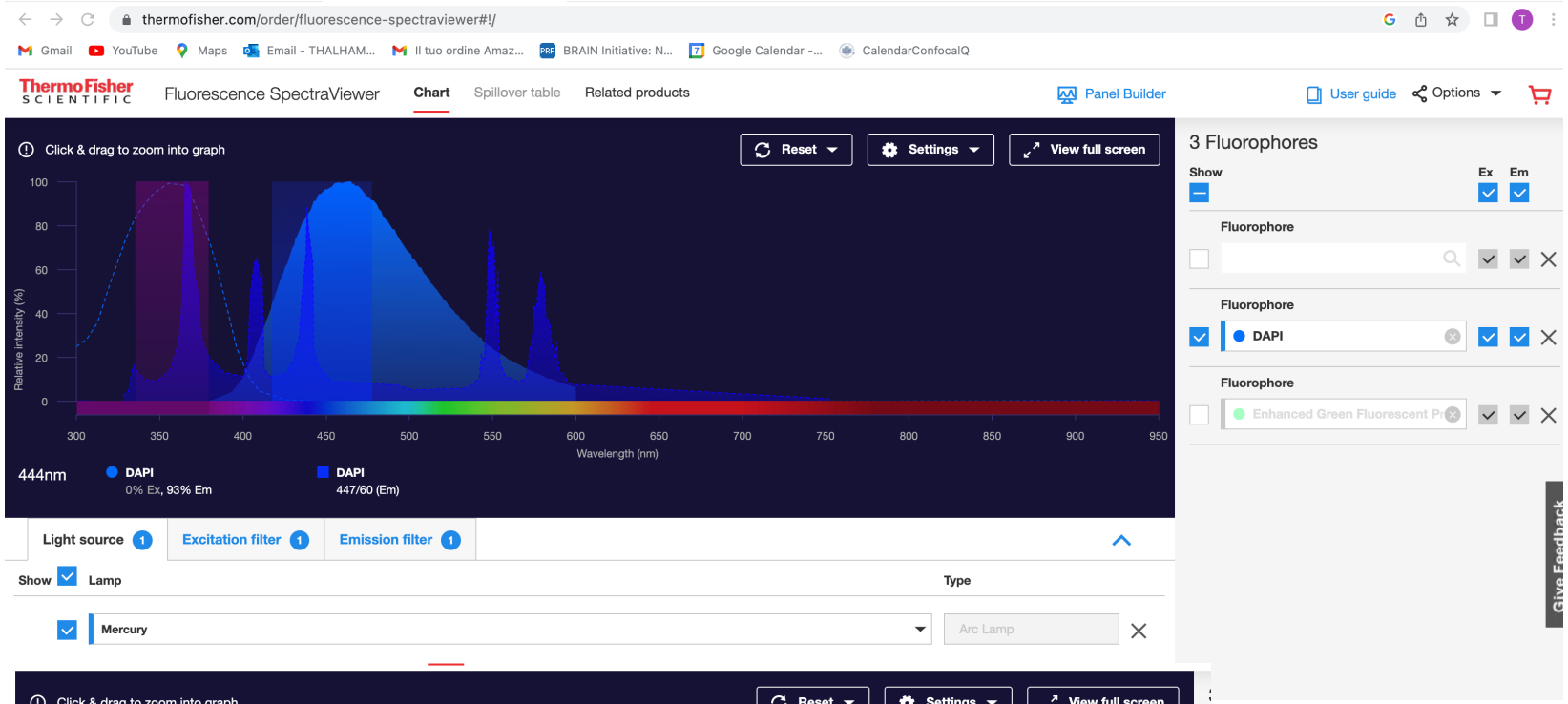
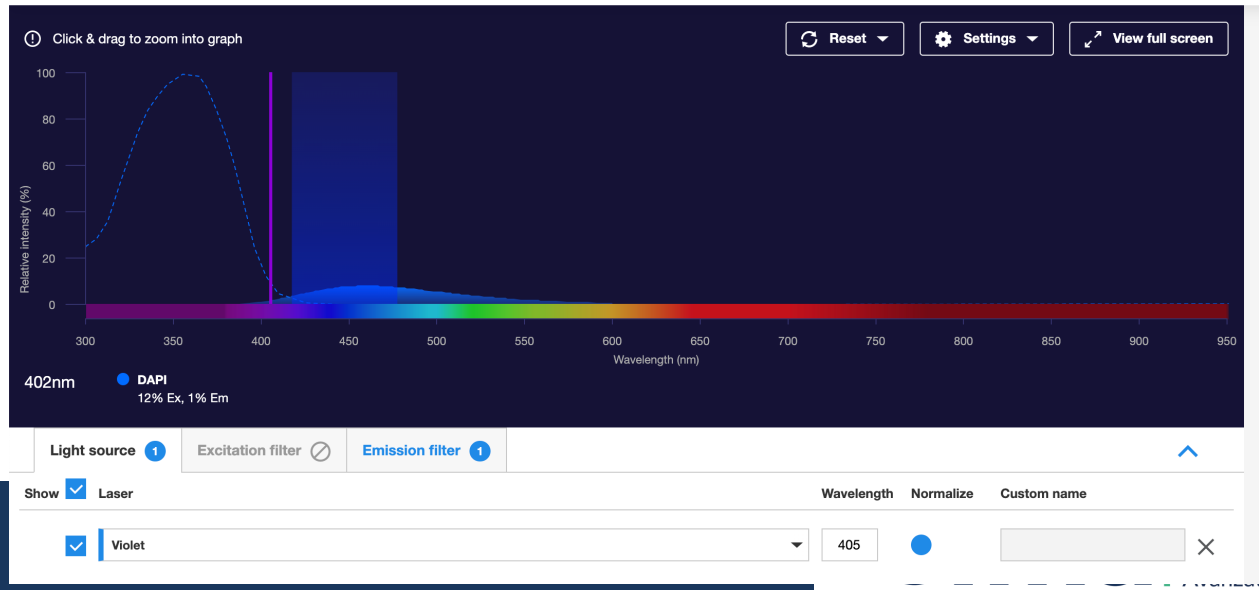
FILTER COMBINATIONS



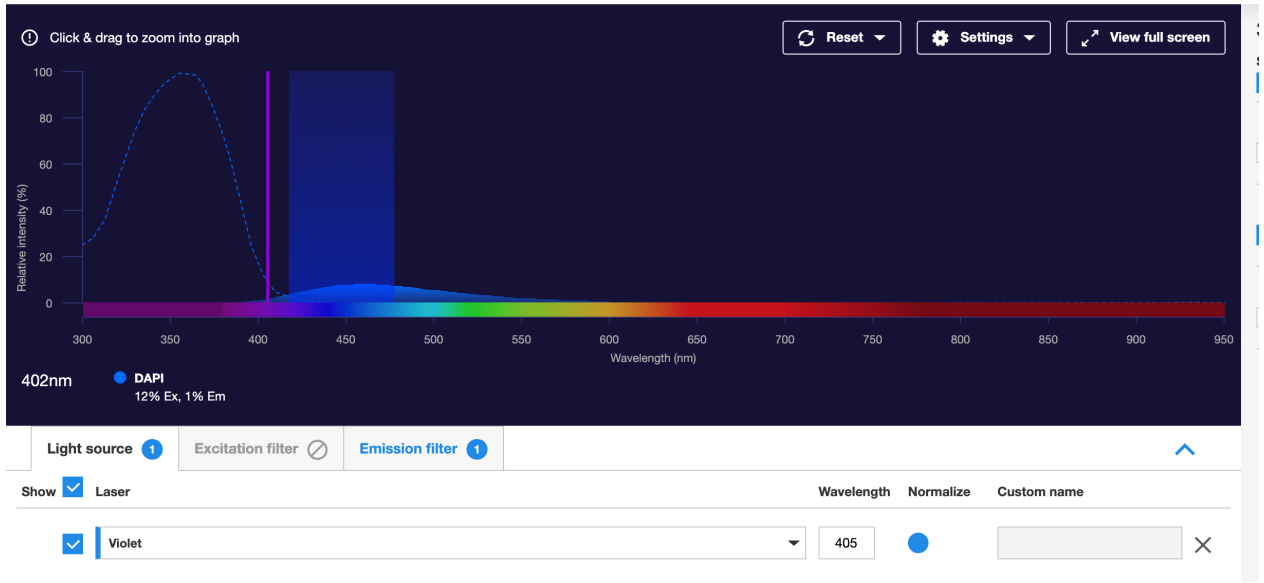


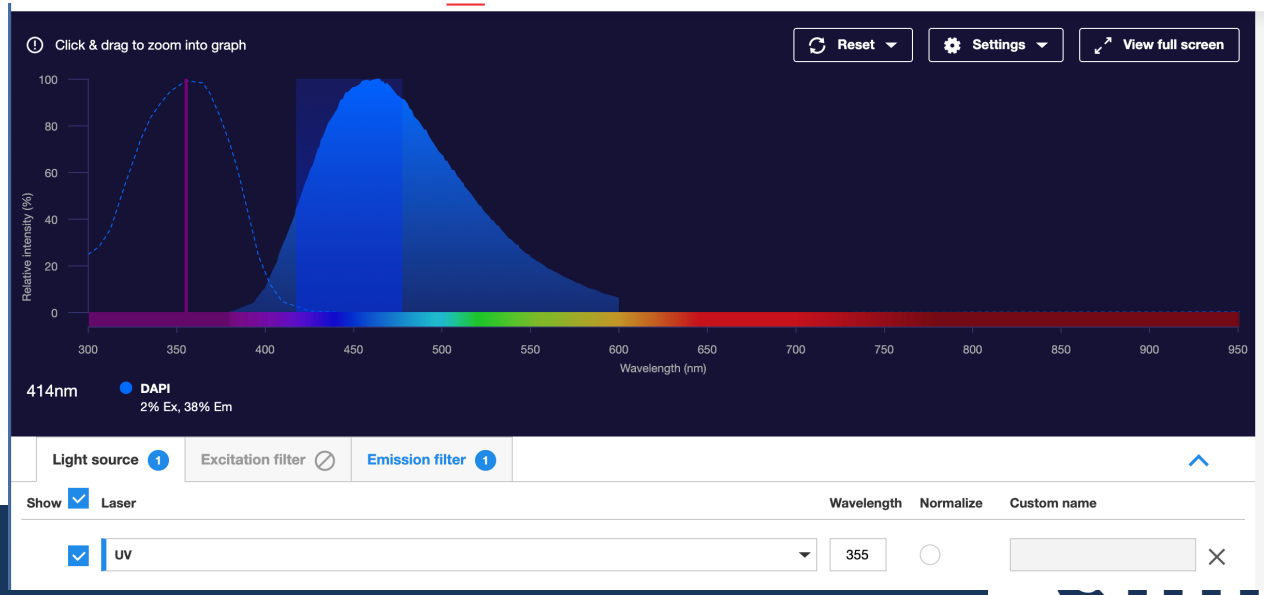


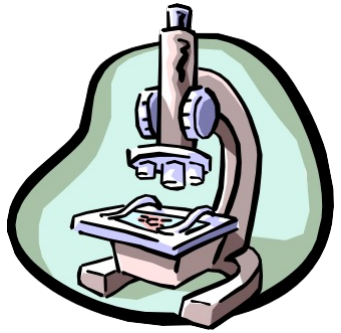
https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of light source!!

FILTER COMBINATIONS







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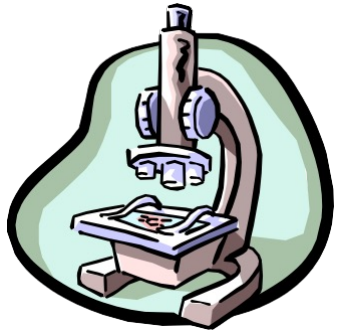
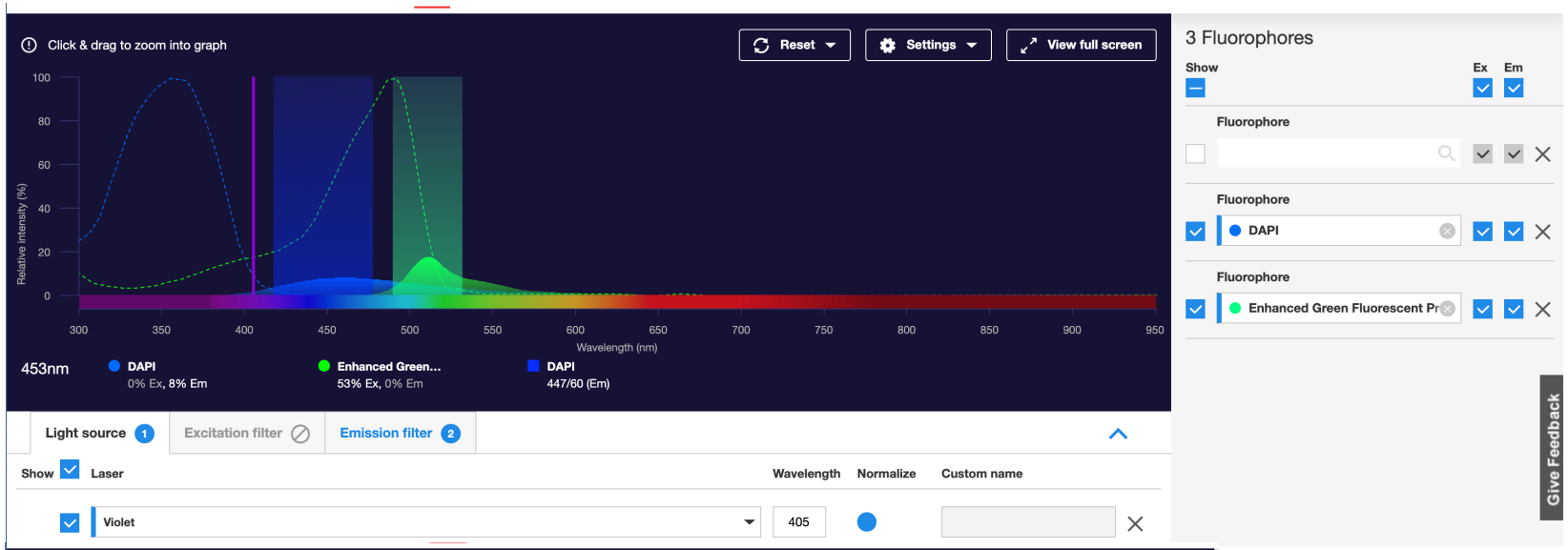
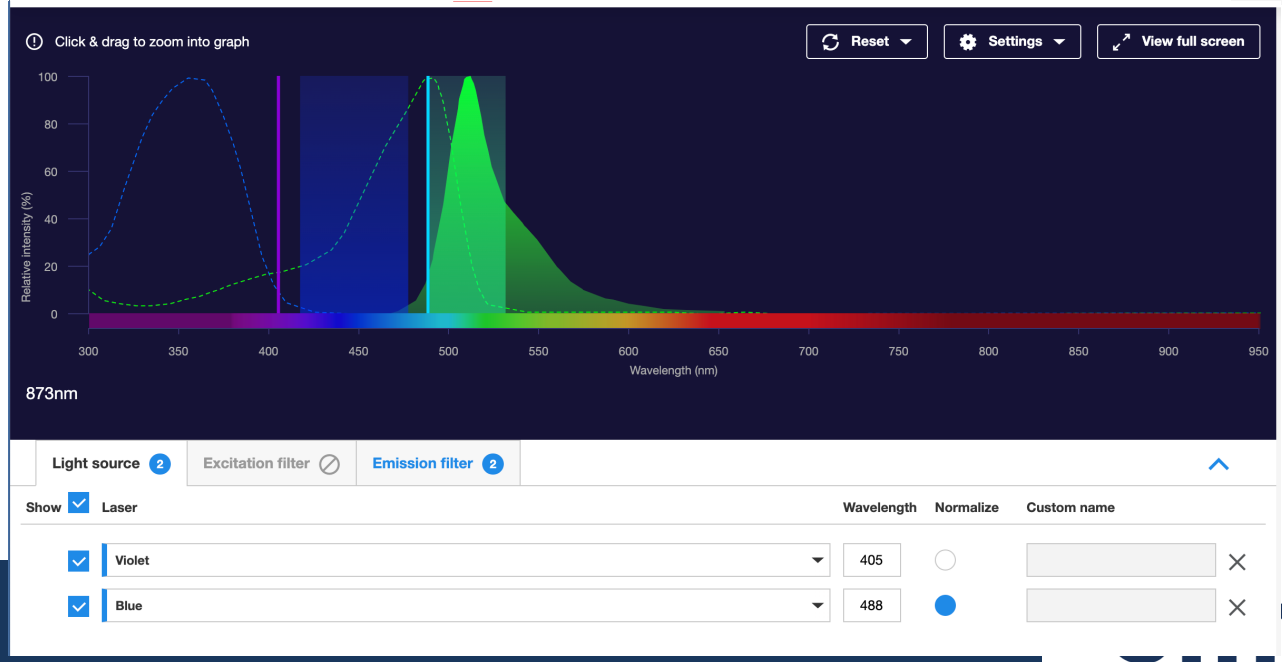
FILTER COMBINATIONS







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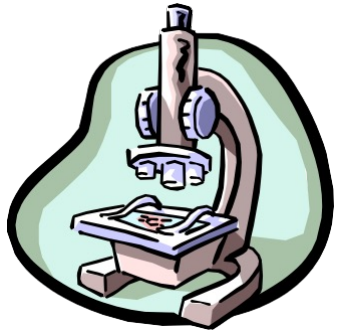
Beware of light source!!

FILTER COMBINATIONS



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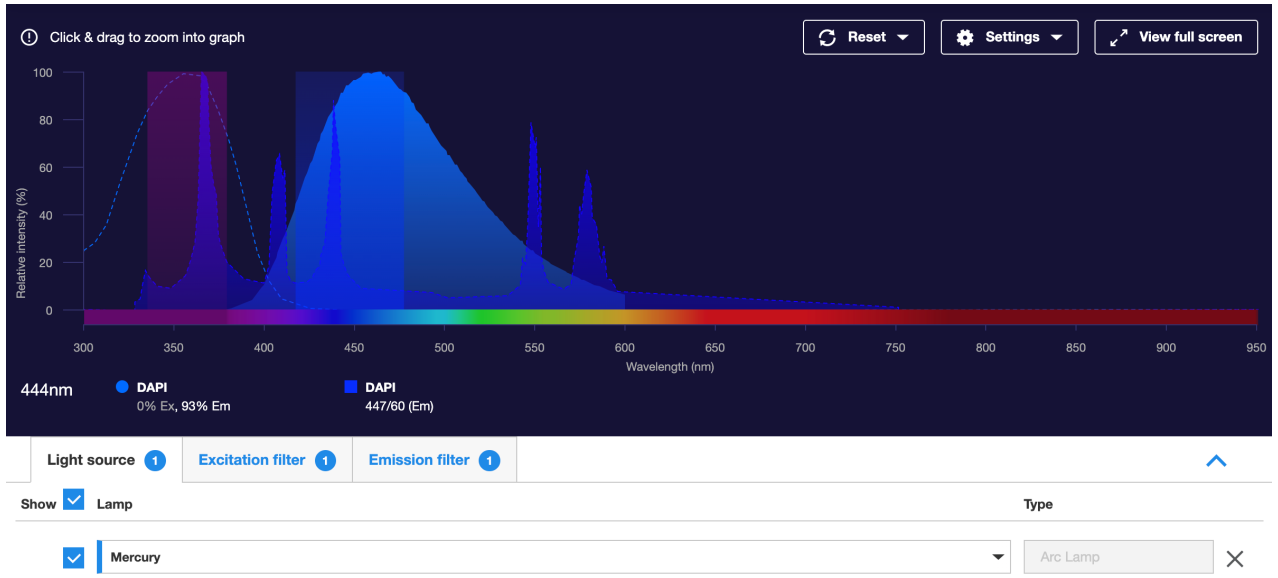
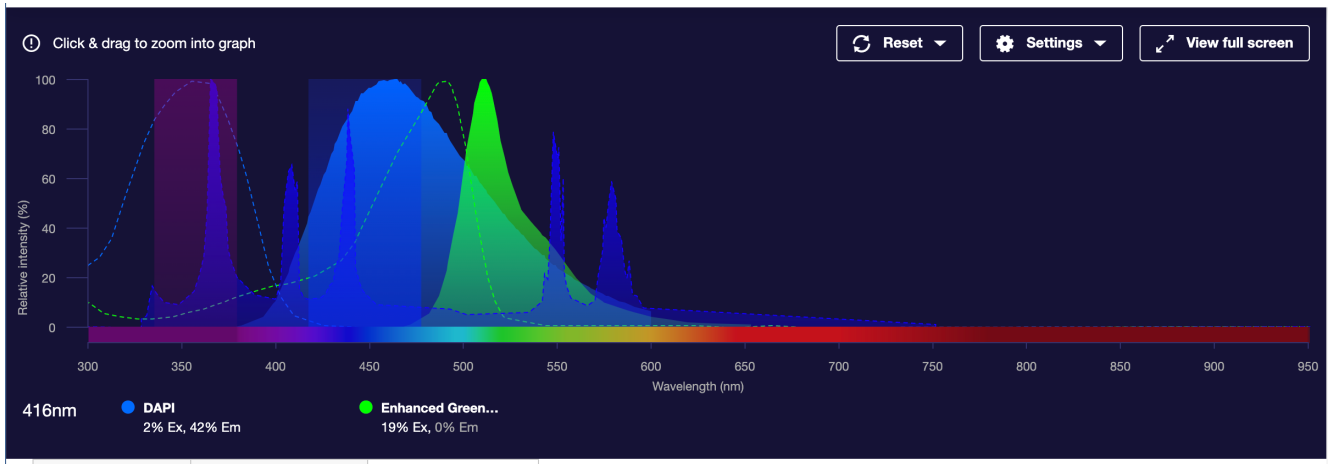
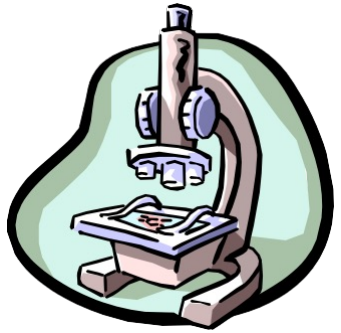
FILTER COMBINATIONS



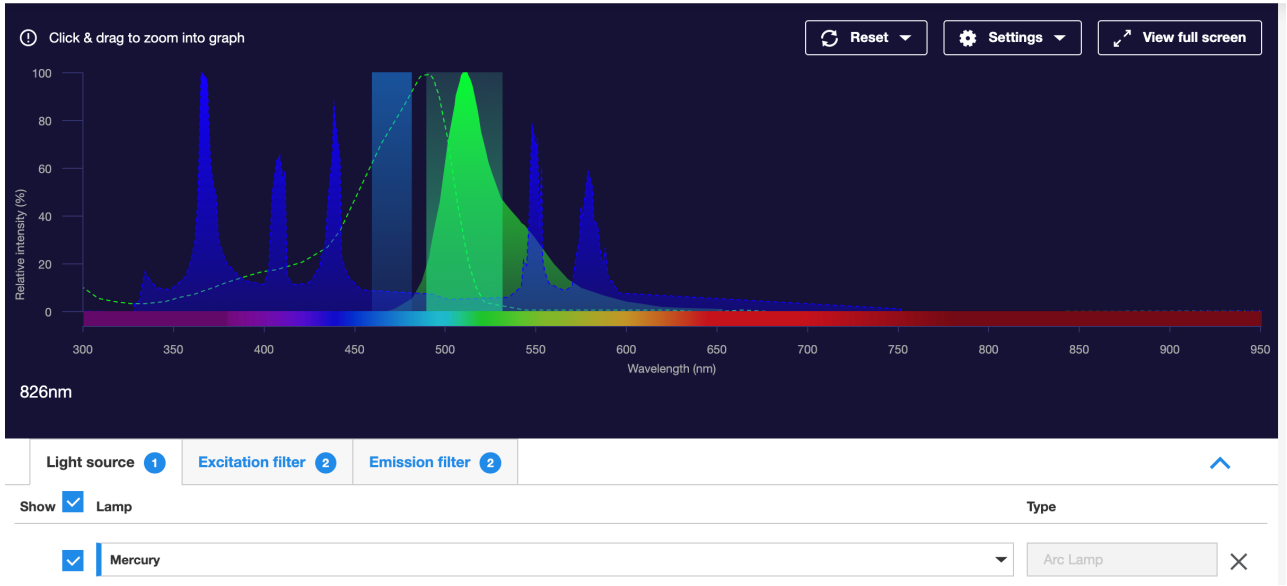


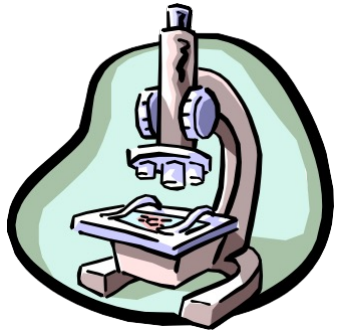


https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Check for crosstalk!!

FILTER COMBINATIONS

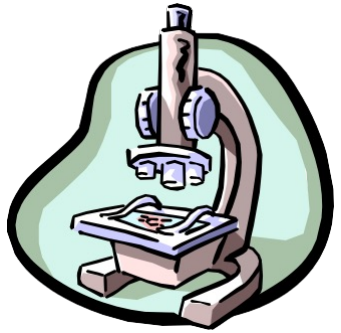


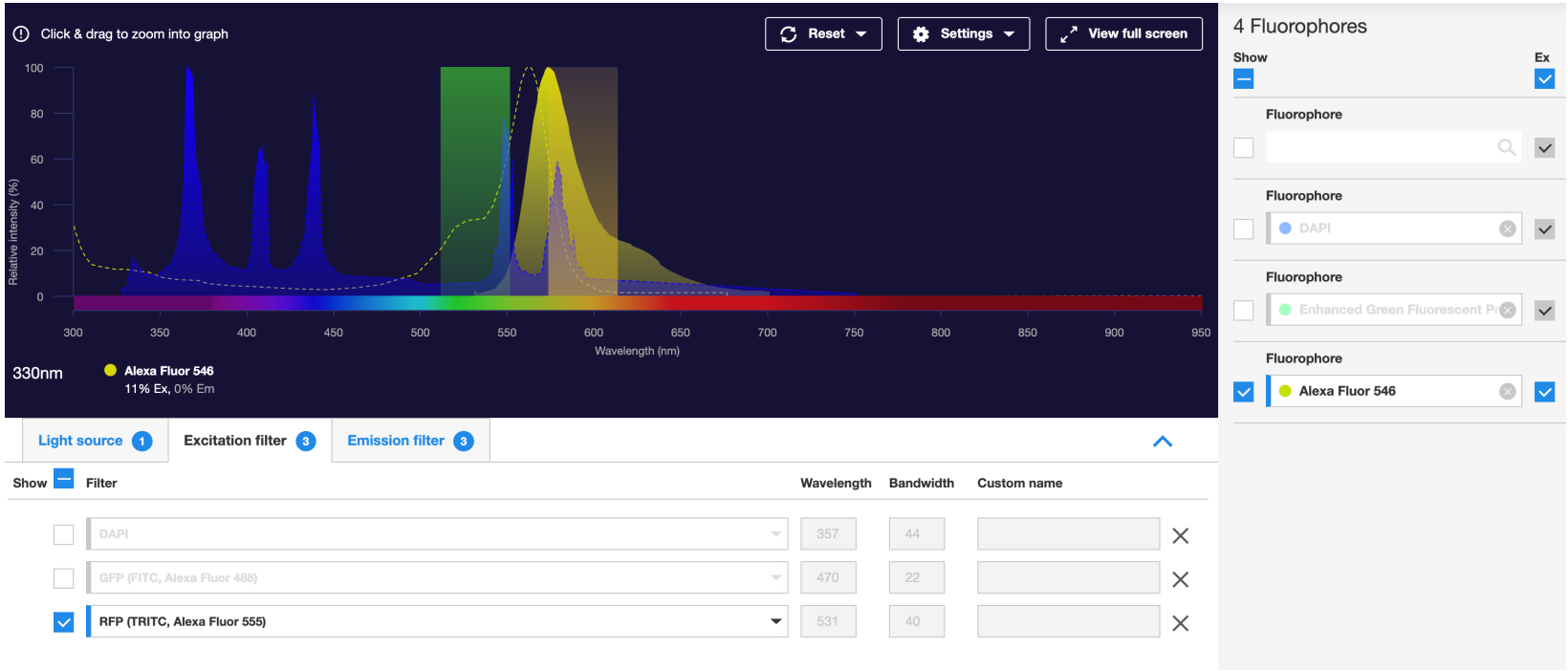


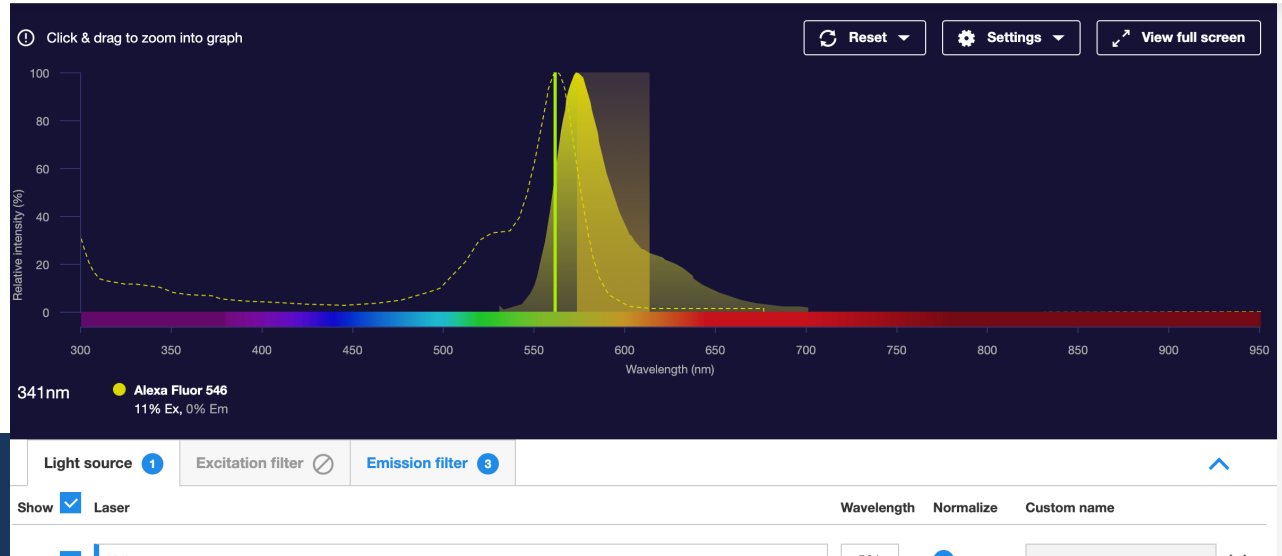
https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Check for cross-talk!!

FILTER COMBINATIONS



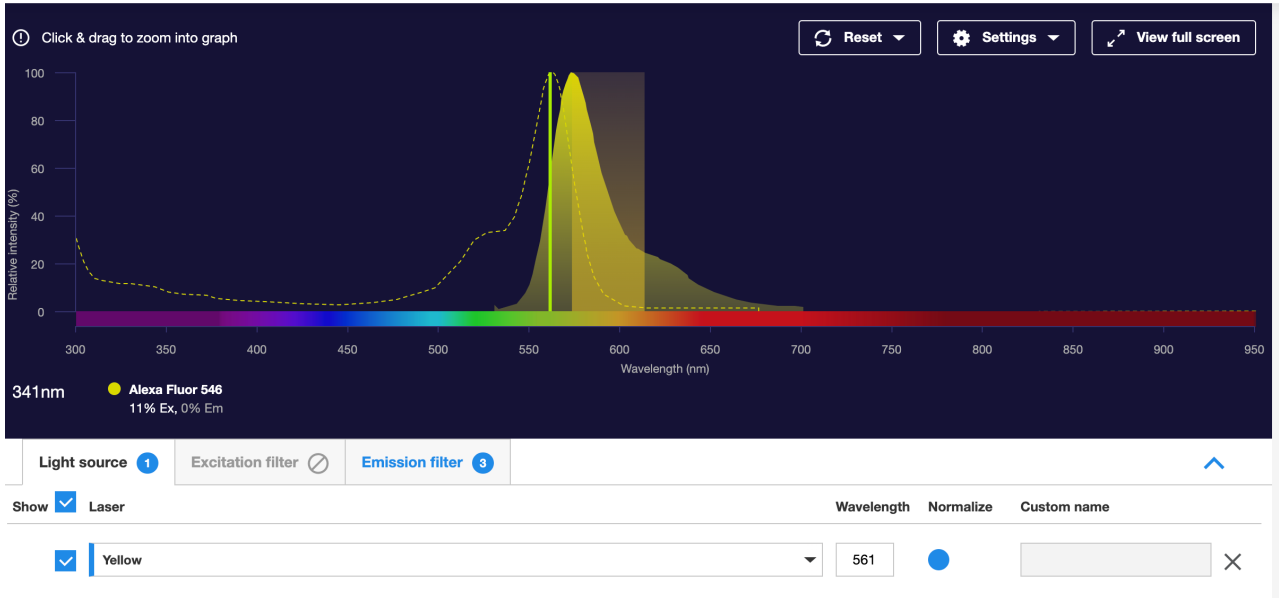


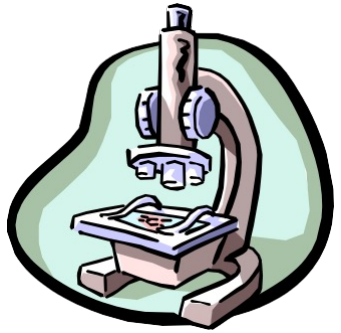


https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of filter!!

FILTER COMBINATIONS

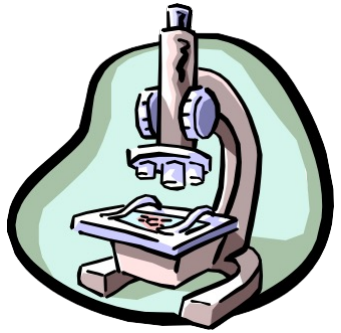


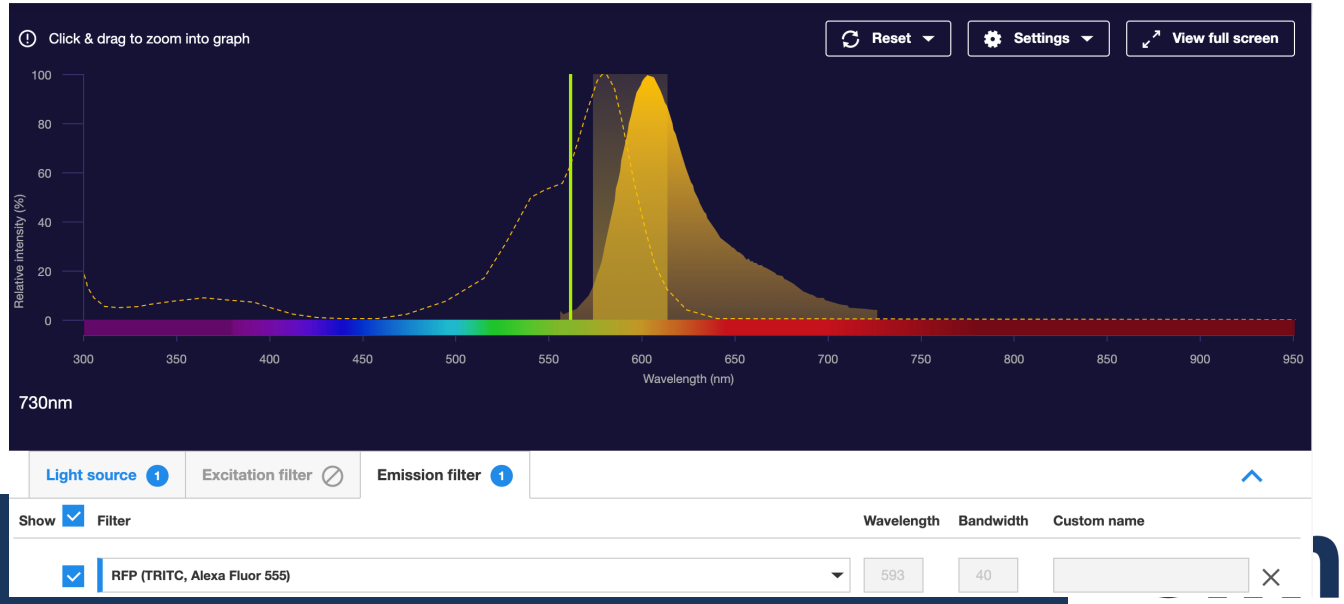


https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of crosstalk!!

FILTER COMBINATIONS

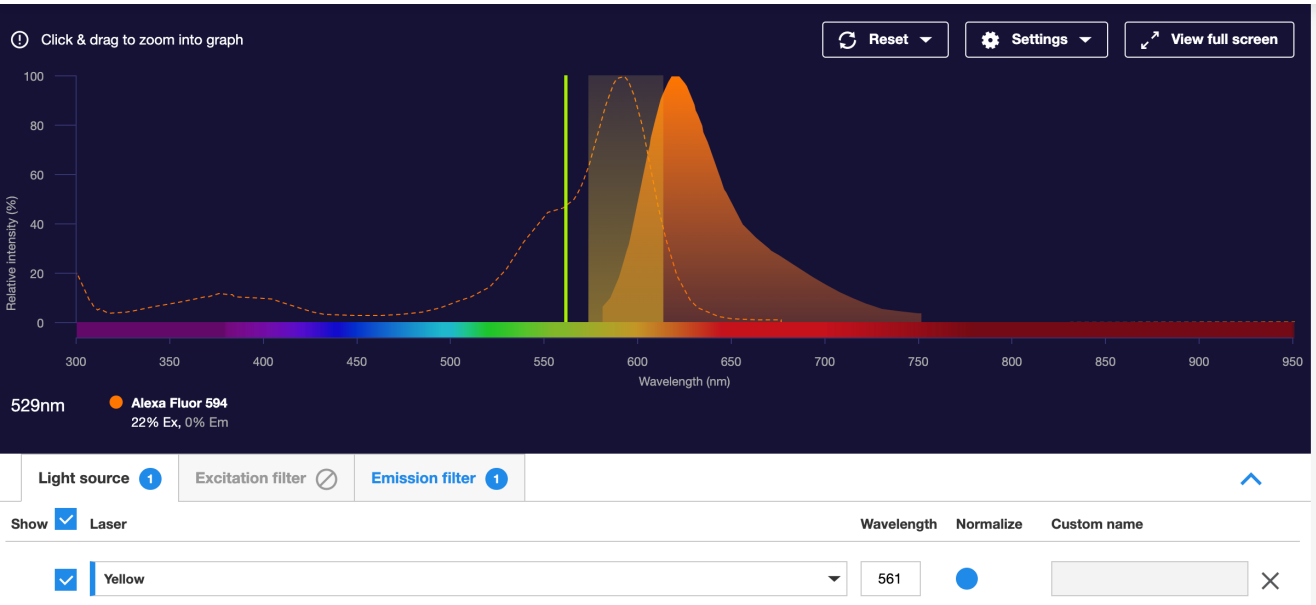


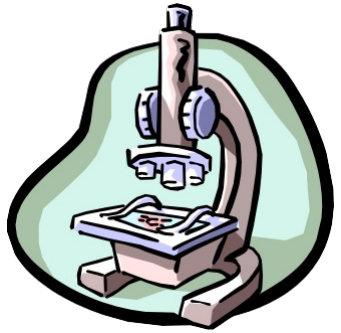


https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of fluorophore!!

FILTER COMBINATIONS

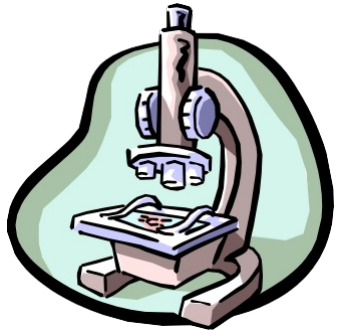


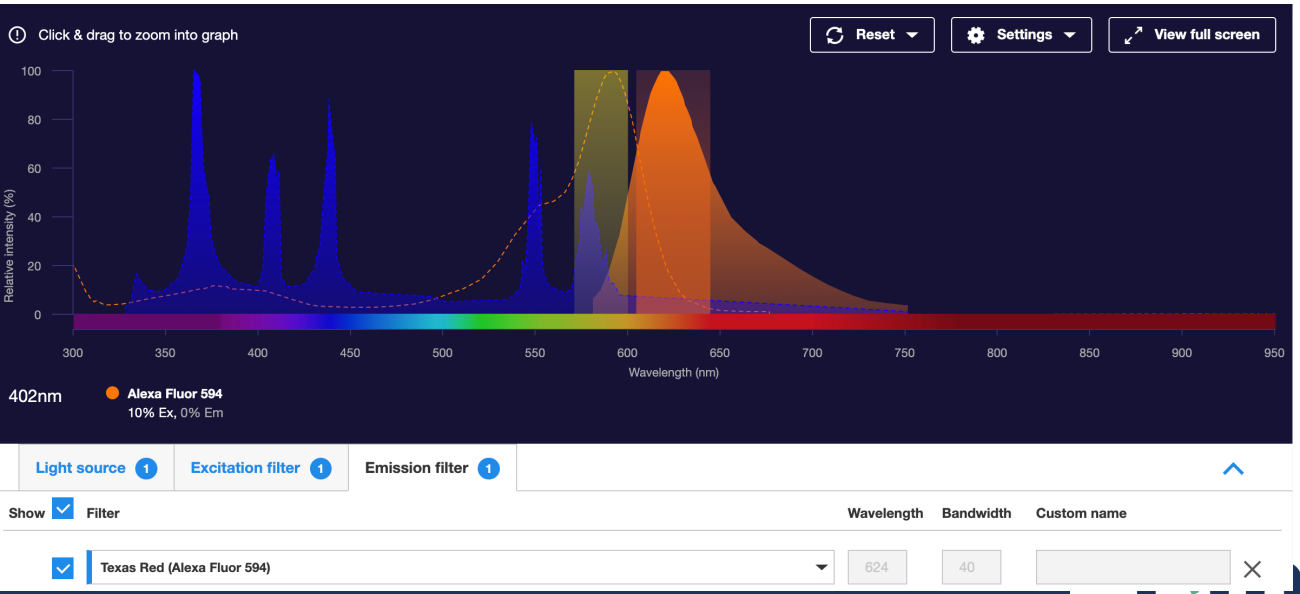


https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of fluorophore!!

FILTER COMBINATIONS

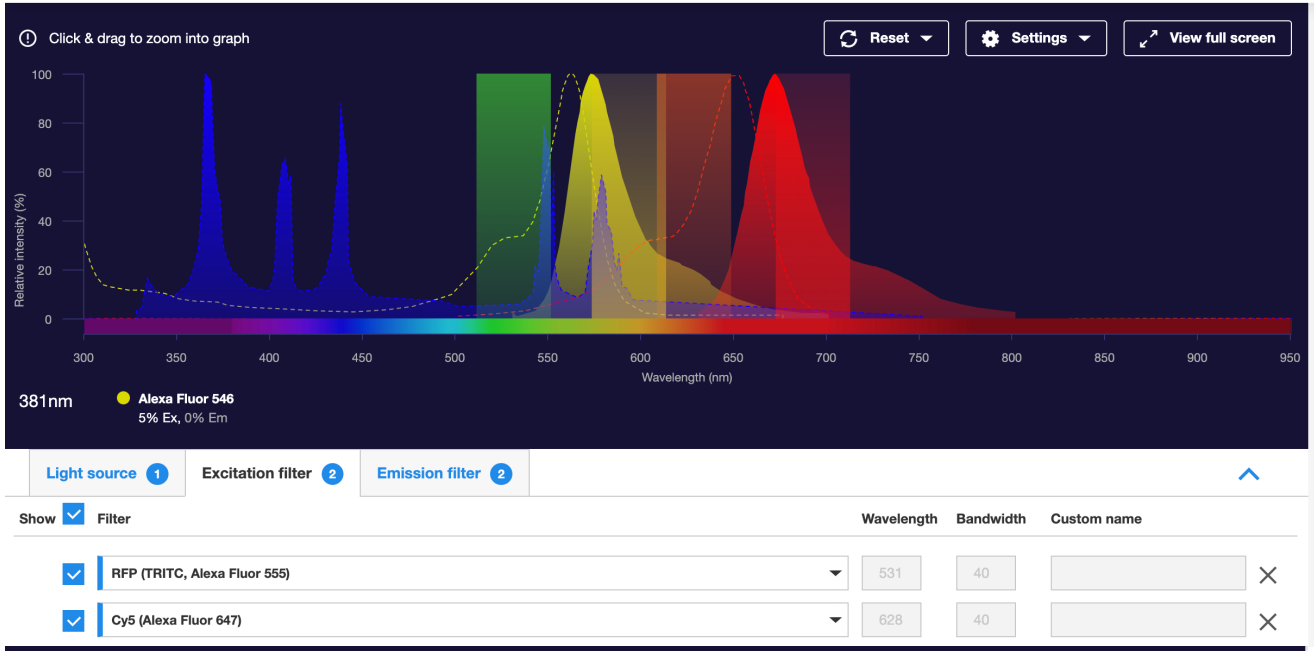


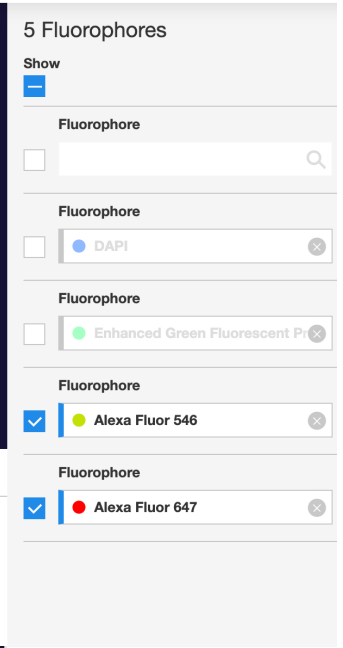


https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Beware of filter cube!!

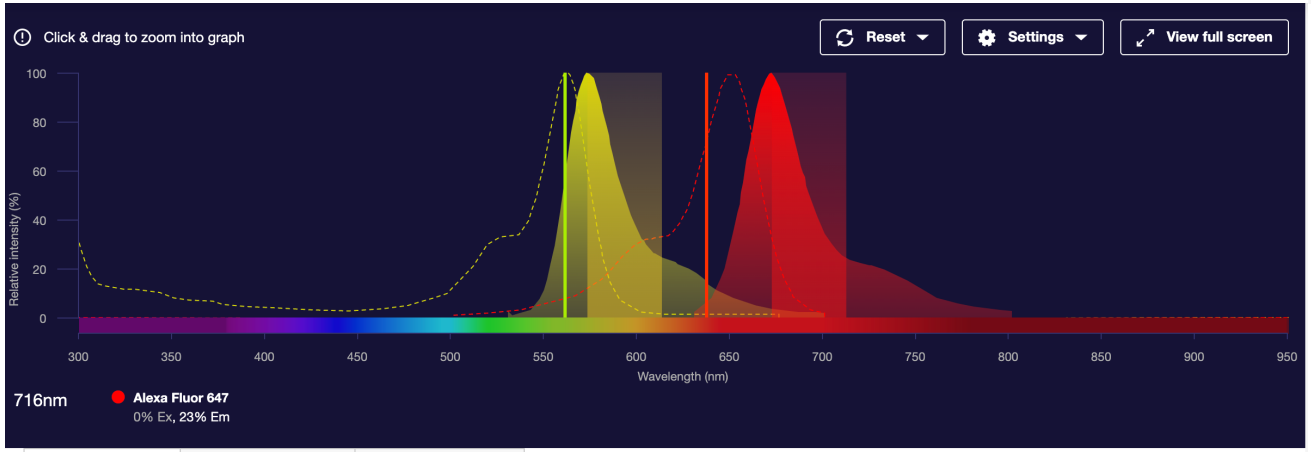
FILTER COMBINATIONS





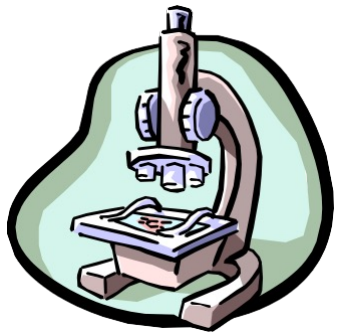
Light source 1 | Excitation filter 2 | Emission filter 2

Show	Filter	Wavelength	Bandwidth	Custom name
☑	RFP (TRITC, Alexa Fluor 555)	531	40	
☑	Cy5 (Alexa Fluor 647)	628	40	



Light source 2 | Excitation filter | Emission filter 2

Show	Laser	Wavelength	Normalize	Custom name
☑	Yellow	561	●	
☑	Red	637	○	



https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Farred is not visible to eye!!

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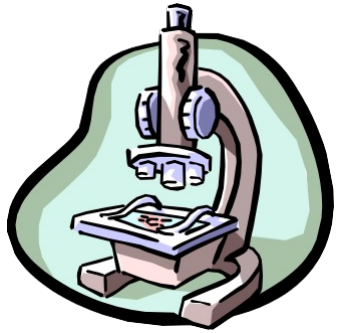
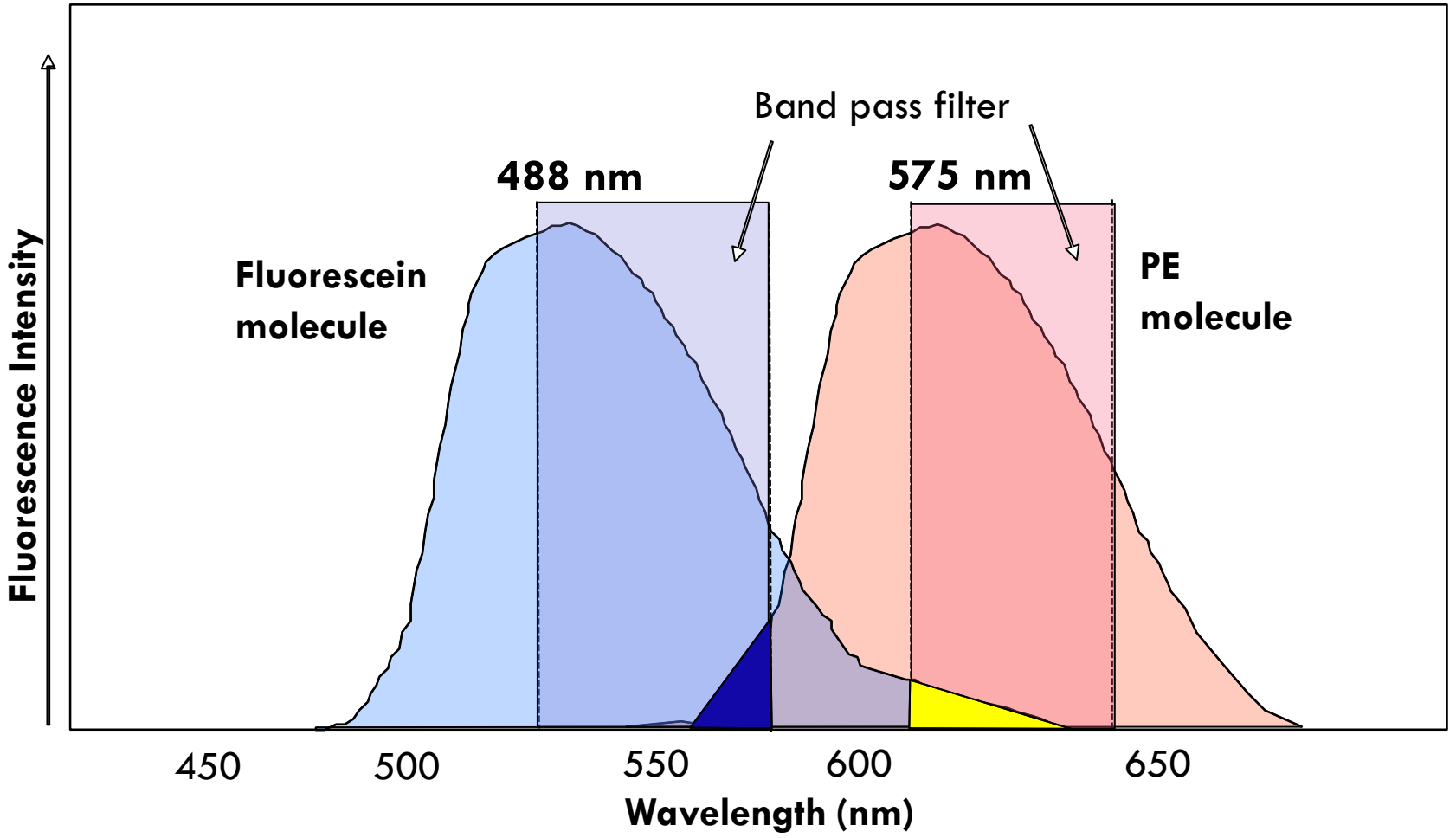
Dipartimento di Scienze della Vita

FLUORESCENCE OVERLAP

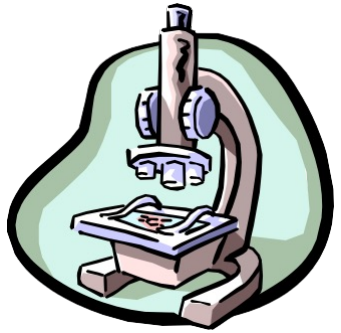




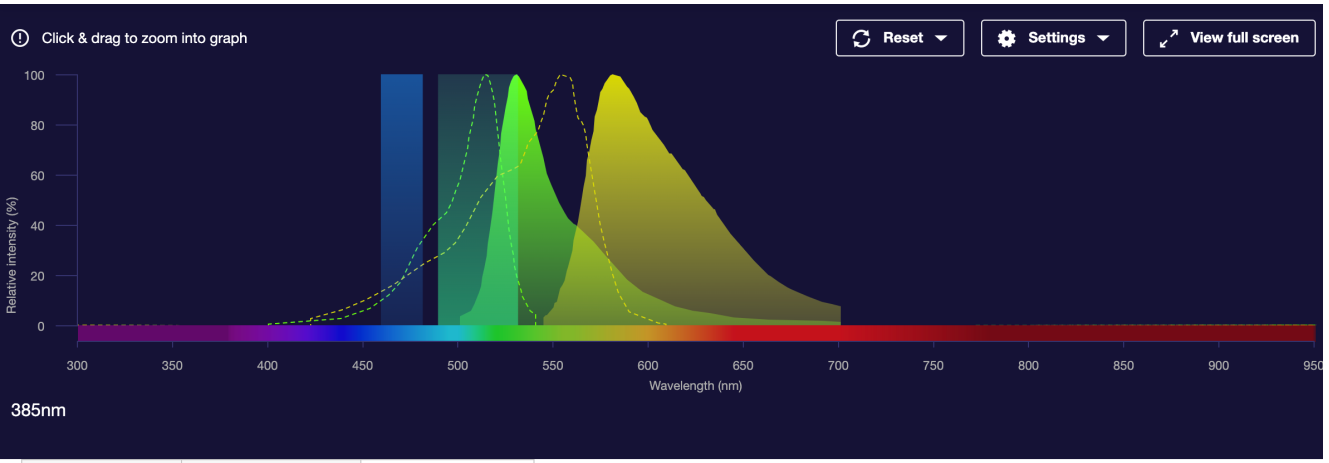
Overlap of FITC fluorescence in PE PMT

Overlap of PE fluorescence in FITC PMT

FLUORESCENCE OVERLAP

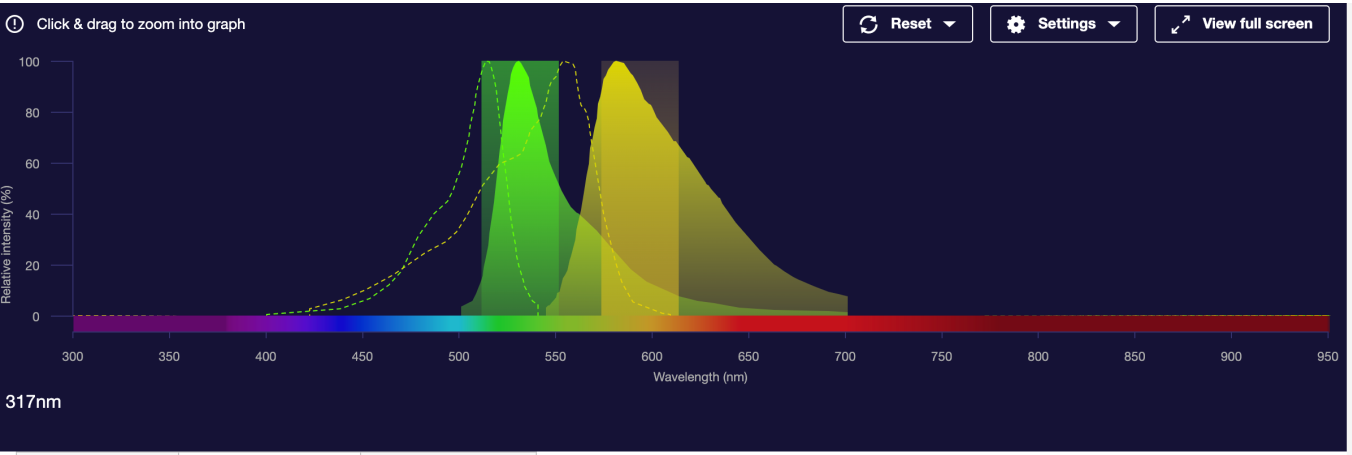


FLUORESCENCE OVERLAP



Light source 1 | Excitation filter 2 | Emission filter 2

Show	Filter	Wavelength	Bandwidth	Custom name
☑	GFP (FITC, Alexa Fluor 488)	510	42	



Light source 1 | Excitation filter 2 | Emission filter 2

Show	Filter	Wavelength	Bandwidth	Custom name
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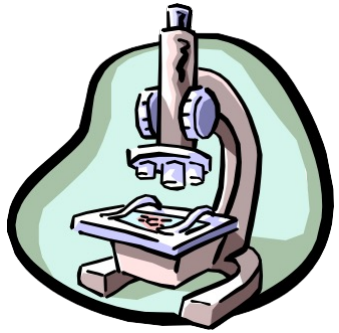
☑	RFP (TRITC, Alexa Fluor 555)	531	40	



Overlap!!

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