FLUORESCENCE

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. Uppsala University . Platform . Biological Visualization .

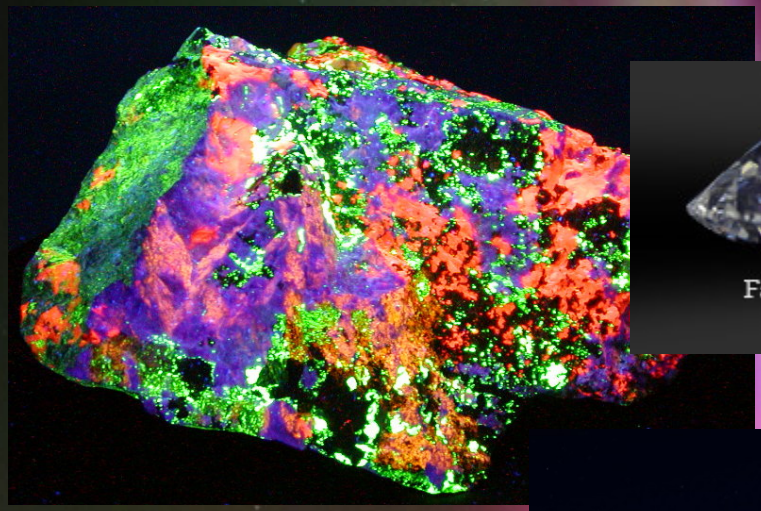
BIOVIS

Light & Electron Microscopy : Flow Cytometry : Image Analysis

Information

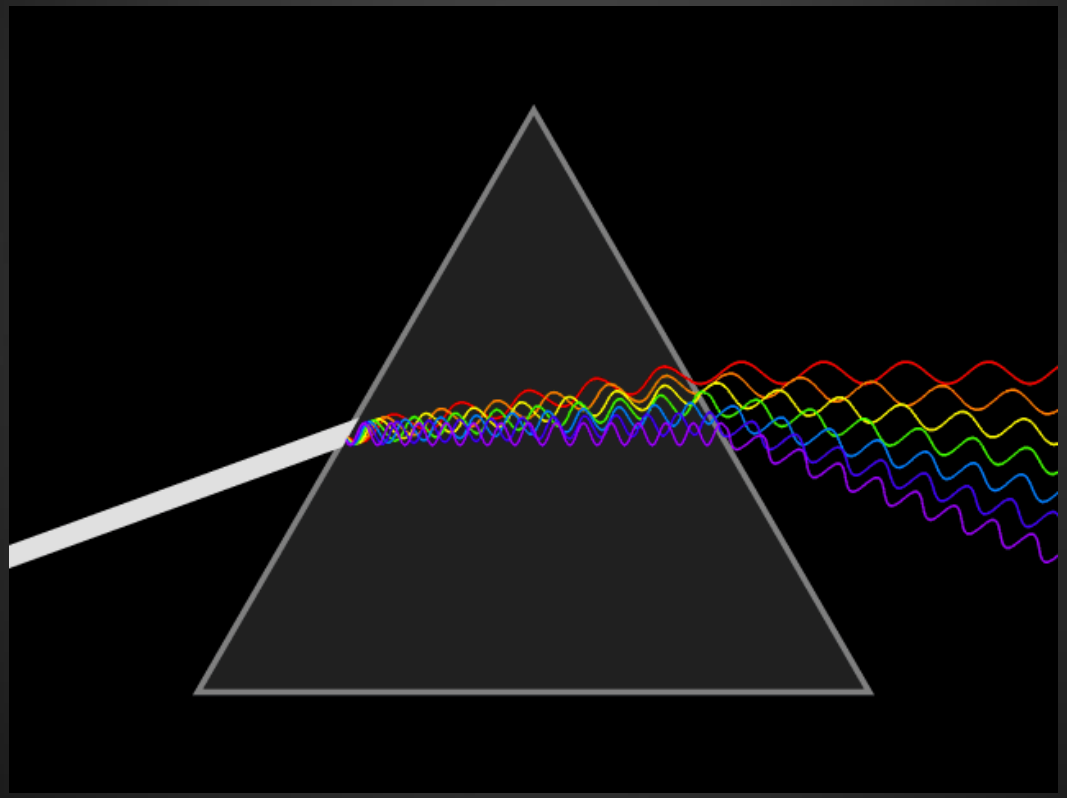
This lecture contains images and information
from the following internet homepages

http://micro.magnet.fsu.edu/primer/index.html
http://www.microscopyu.com/
http://www.olympusmicro.com/primer/lightandcolor/index.html

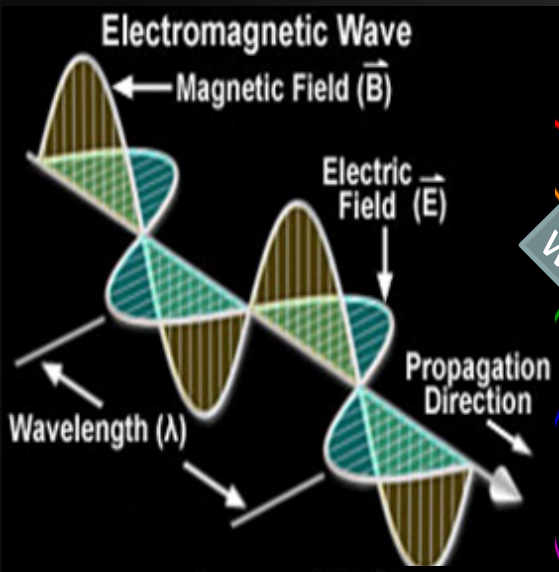


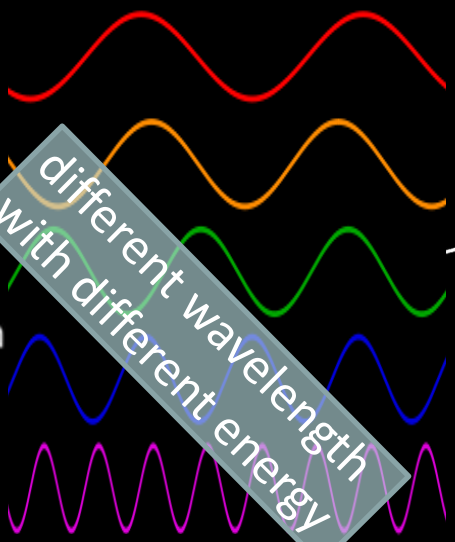

Faint
Medium
Strong
Very Strong

Light phenomenon



Wave or particle? … Wave!





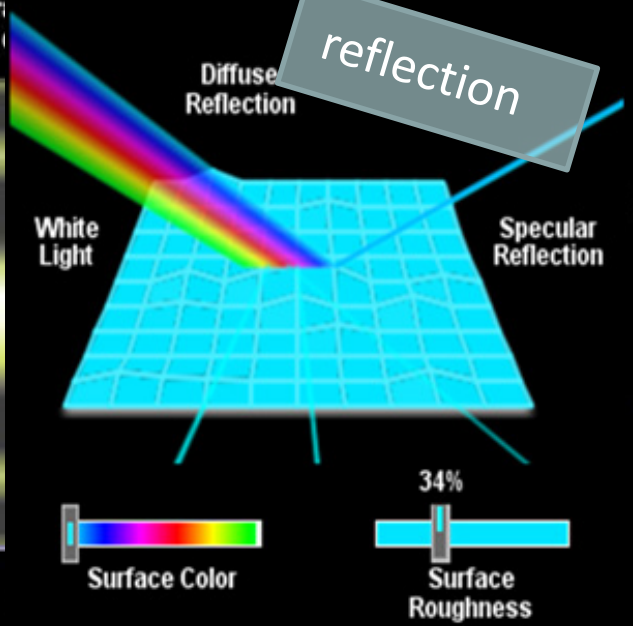
different wavelength with different energy

Dispersion due to refraction

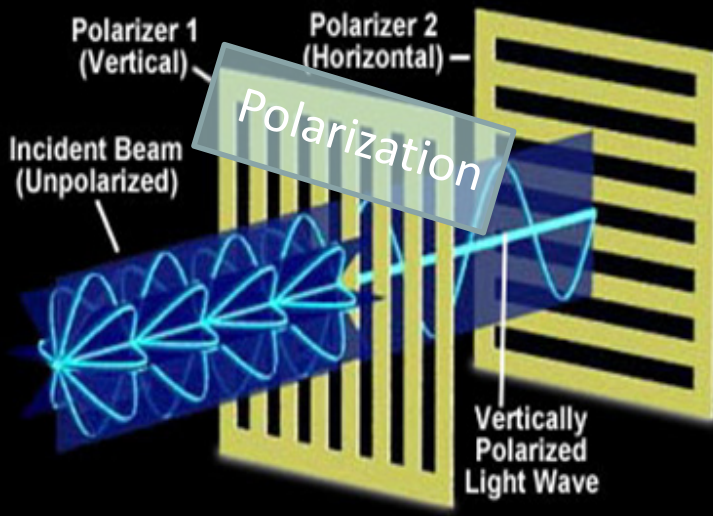
absorption filtering

Light Passing Through Crossed Polarizers

diffraction & interference

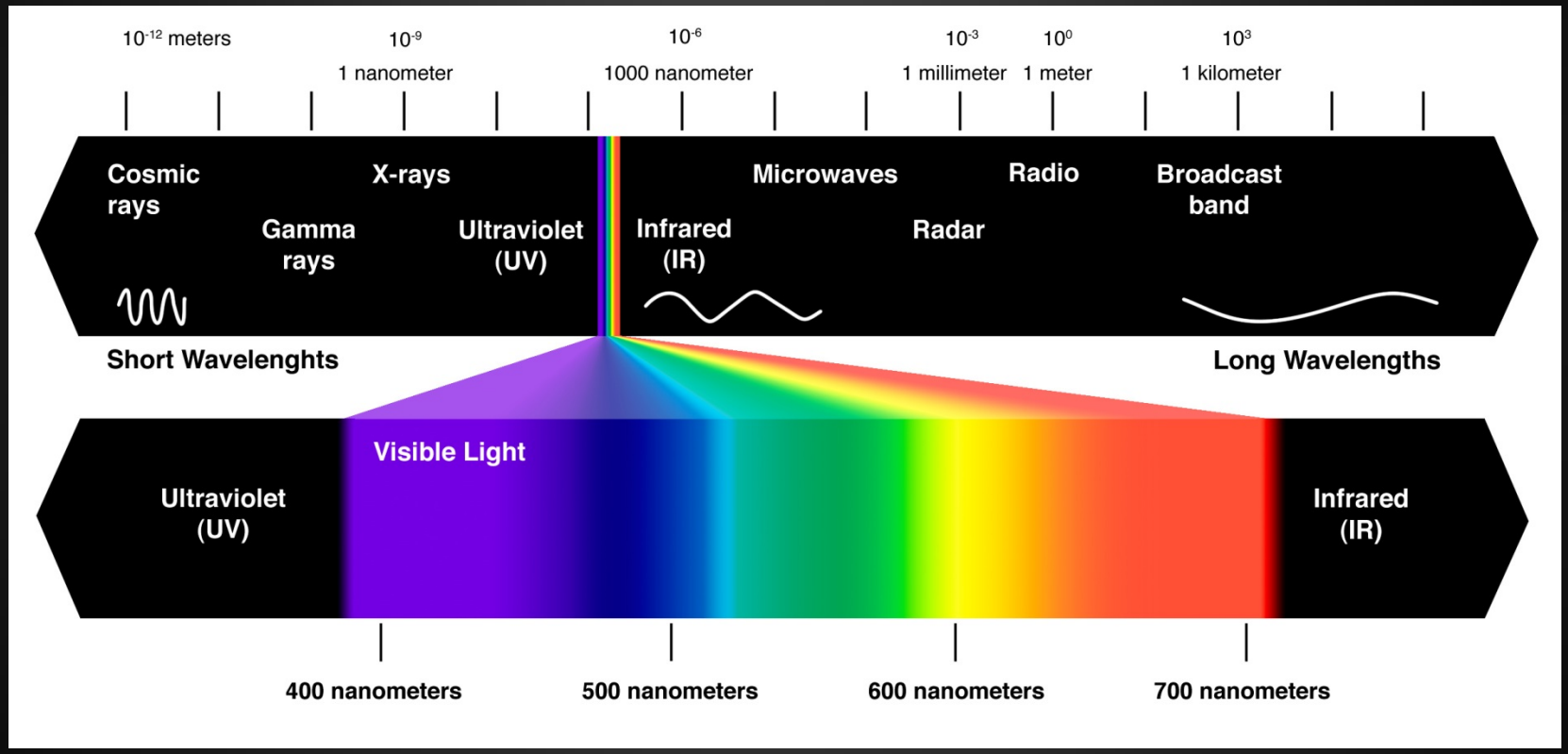


reflection

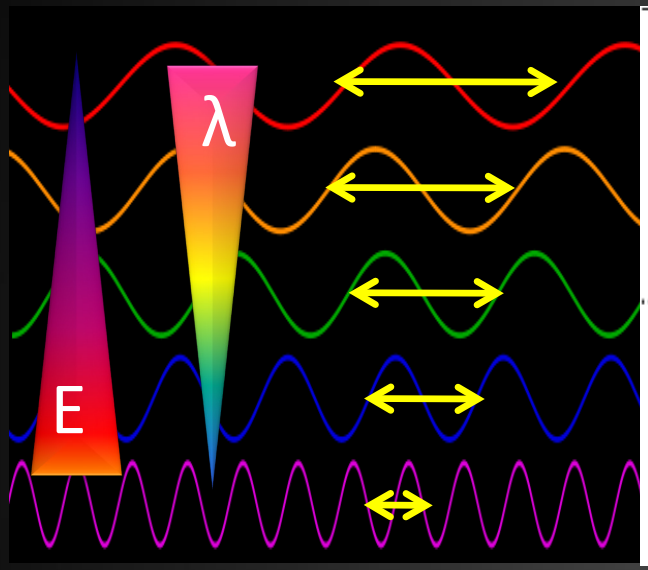


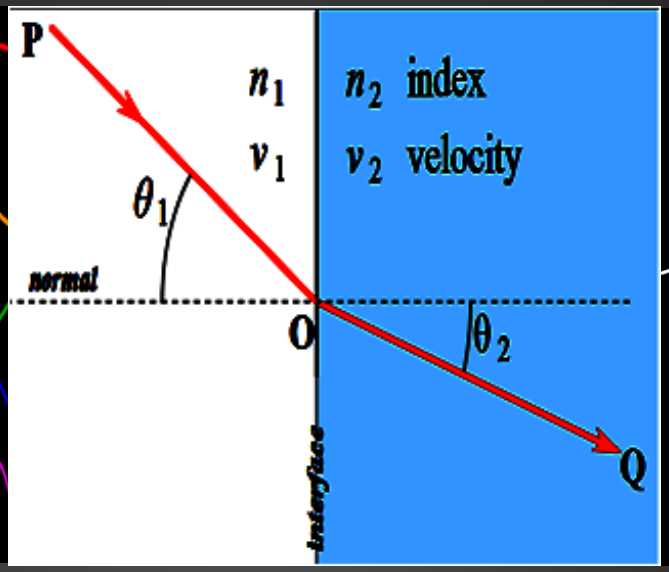
Polarization

Wave or particle? ... Wave!



Wave or particle? … Wave!





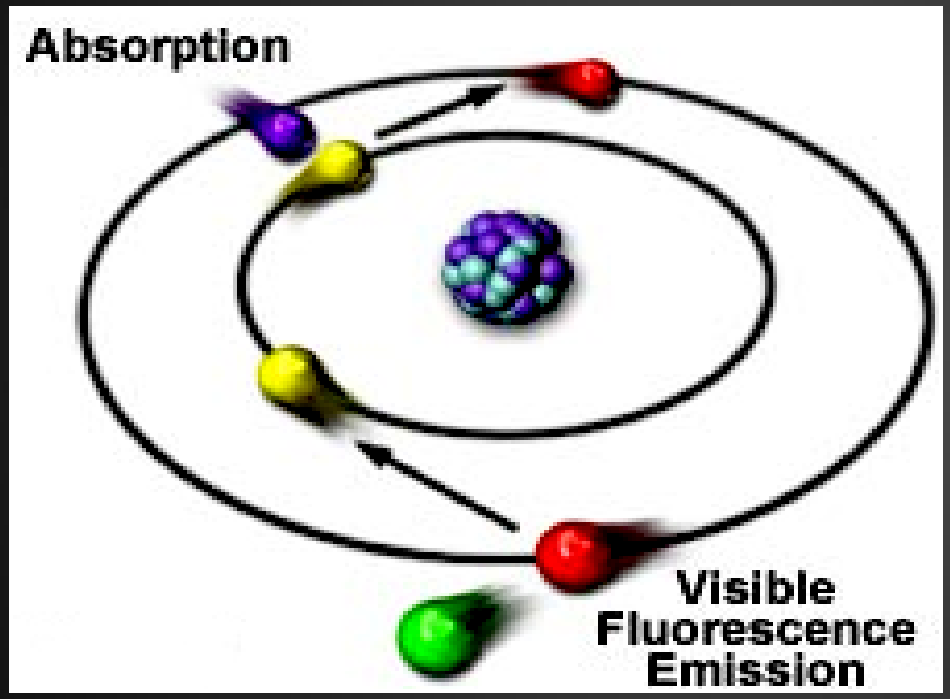
- Light travels in vacuum C = 300.000 km/sec
- Seems to slows down in denser matter.
- → Refractive index n = C/v (v= speed in matter)
- → light is 1.5 x slower at n=1.5
- →Light changes direction between different dense materials : shorter λ refract more than longer λ

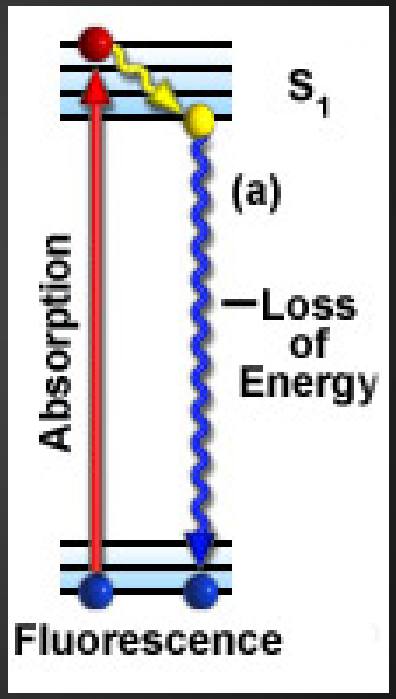
→ Optic lenses, effects in sample...

→ shorter wavelength (blue) has higher energy

Wave or particle? … Particle!

Principle of fluorescence





Light behaves sometimes as quantized energy pockets, light has particle behavior.

Fluorescence basics

Fluorescence basics

Definition:

Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation.

Usually the emitted light has a longer wavelength, and therefore lower energy than the absorbed radiation.

Emission of light happens in time scale of nano second – so to speak immediately

Compared to **Phosphorescence**:

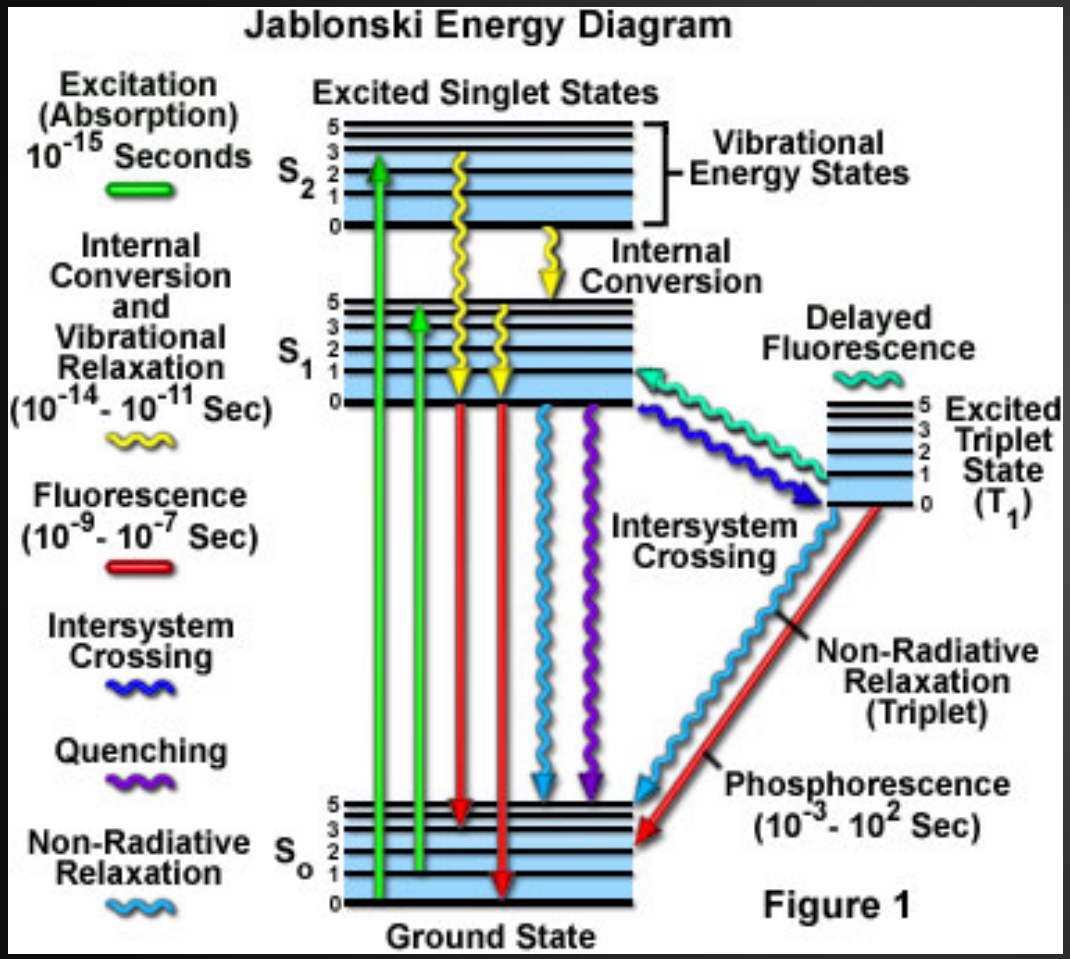
- specific type of photoluminescence related to fluorescence.

Unlike fluorescence, a phosphorescent material does not immediately emit light.

Absorbed radiation may be re-emitted for up to several hours after original excitation.

(wikipedia ;))

Fluorescence basics

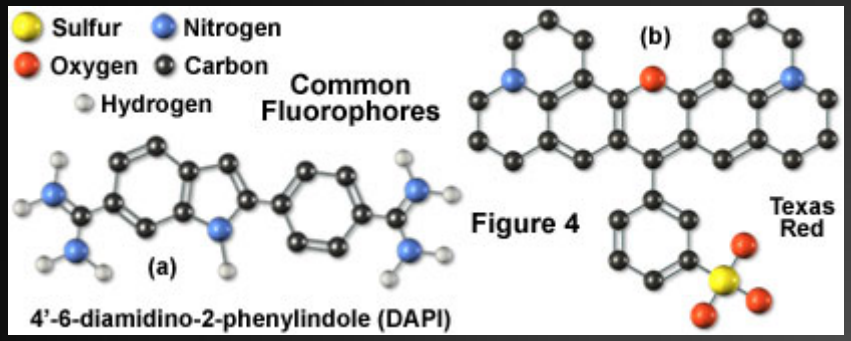


http://www.olympusmicro.com/primer/java/jablonski/jabintro/

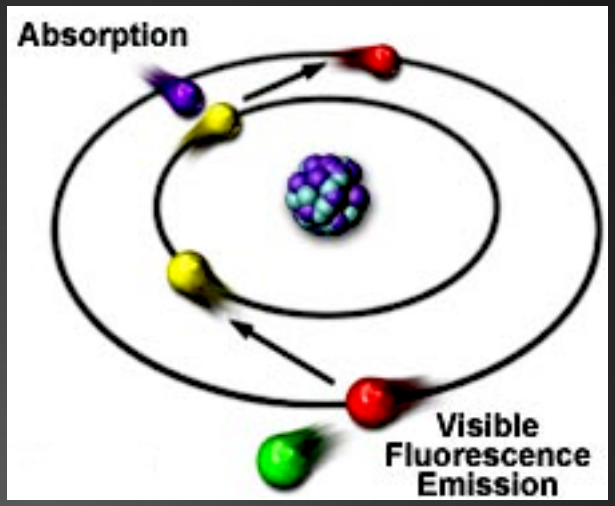
10^{-15} s	: 1 femto sec
10^{-14} s	: 10 fs
10^{-13} s	: 100 fs
10^{-12} s	: 1 pico sec
10^{-11} s	: 10 ps
10^{-10} s	: 100 ps
10^{-9} s	: 1 nano sec
10^{-8} s	: 10 ns
10^{-7} s	: 100 ns
10^{-6} s	: 1 micro sec
10^{-5} s	: 10 µs
10^{-4} s	:100 µs
10^{-3} s	: 1 milli sec
10^{-2} s	: 10 ms
10^{-1} s	:100 ms
10^{0} s	: 1 second

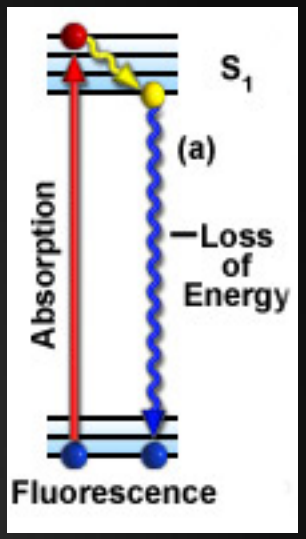
Fluorescence basics

Examples of fluorescent probes

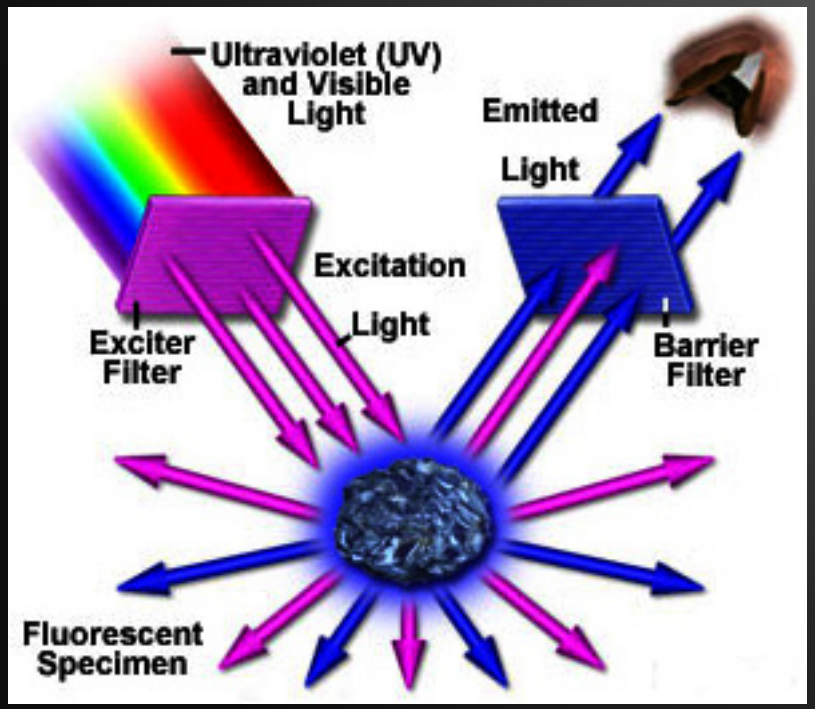


Principle of fluorescence

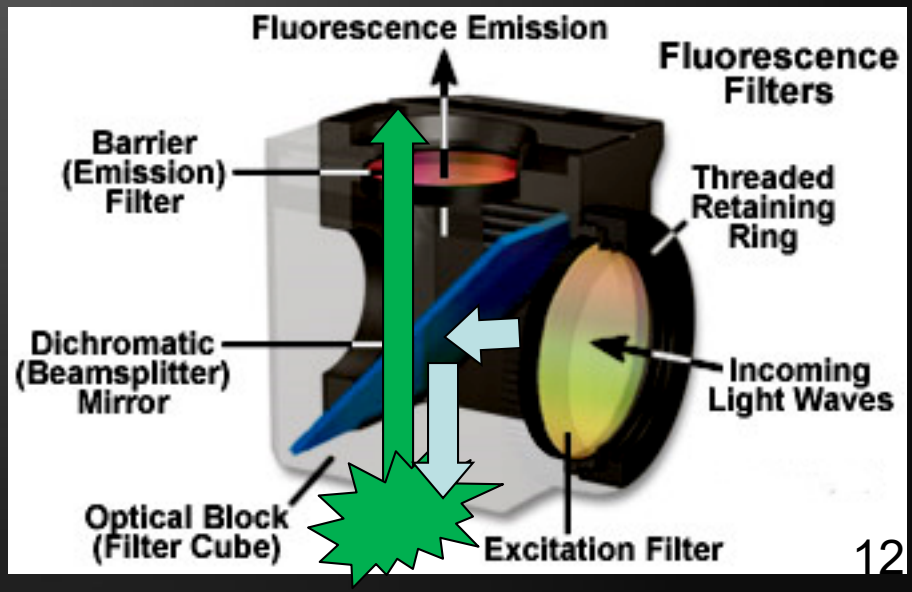




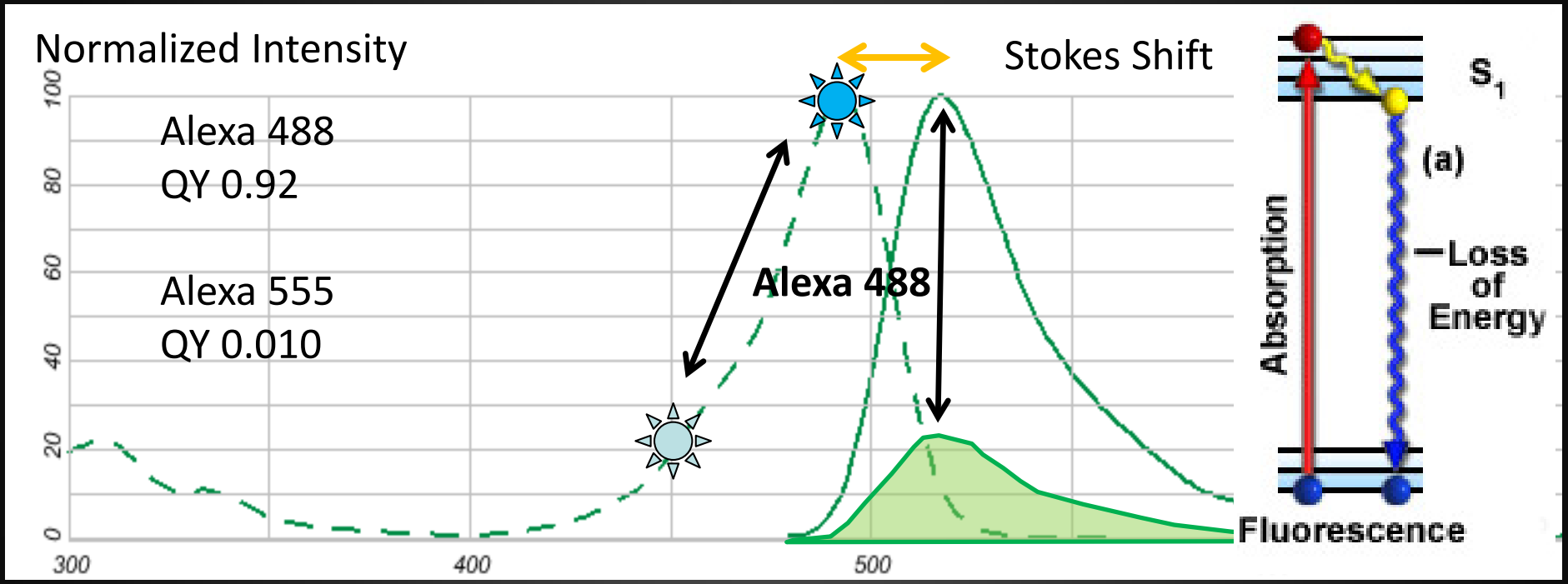
Principle of fluorescent microscope



Excitation-Emission filter cube



Fluorescence: The spectra



X axis: λ in nm

Y-axis: Intensity or probability of event that A) fluorophore absorbs the light for excitation (dashed line) and B) Fluorophore emits ligth (full line)

- Ex peak at 100% → em peak at 100 %, ex 20% → em 20 %, same range of emission
- Stokes shift: gap between ex-peak and em peak = (loss of energy, dissipation)
  →important for separation of excitation and emission light in microscope etc

<u>Other important features of fluorophores:</u>

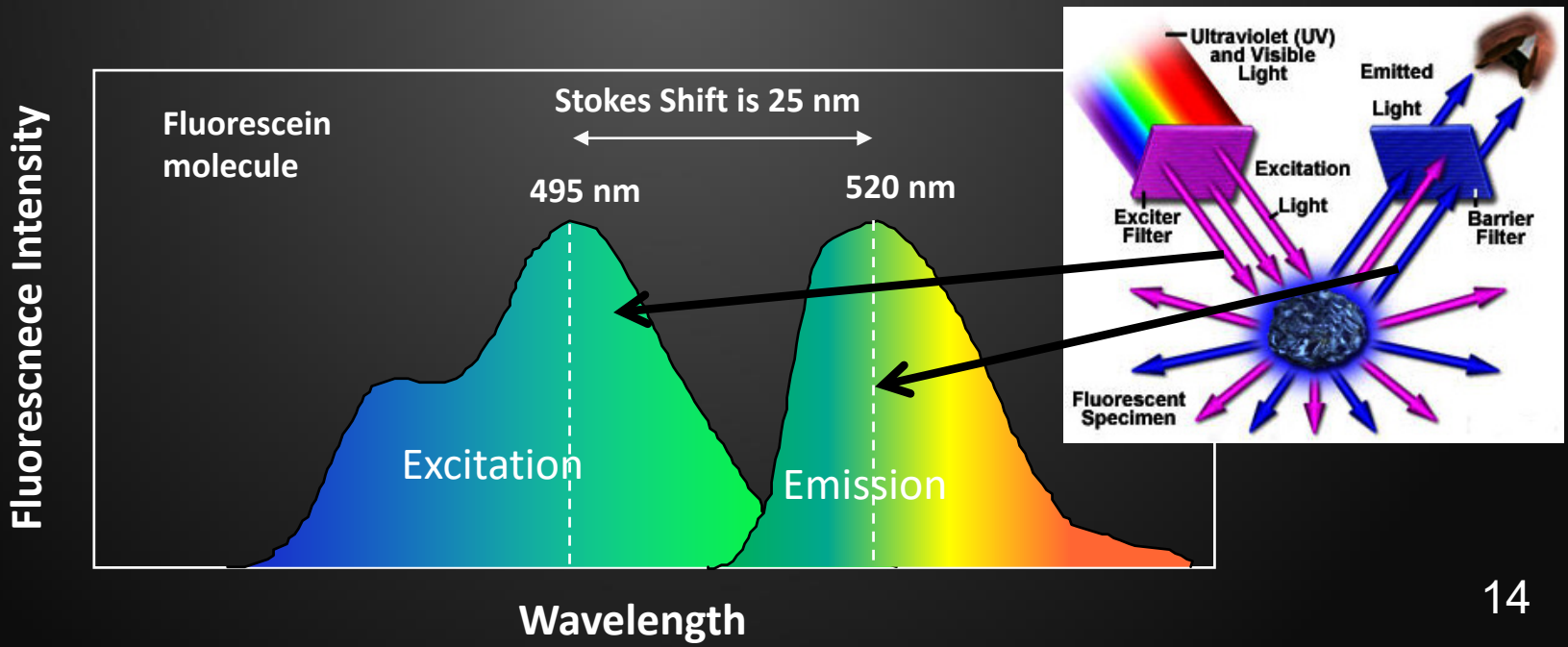
- Extinction coefficient: absorbtion efficiency of a photon at particular wavelength
- Quantum yield: proportion of photons emitted at λ em to those absorbed at λ em

Stokes shift

Stokes shift is the energy difference between the lowest energy peak of absorbance and the highest energy of emission.

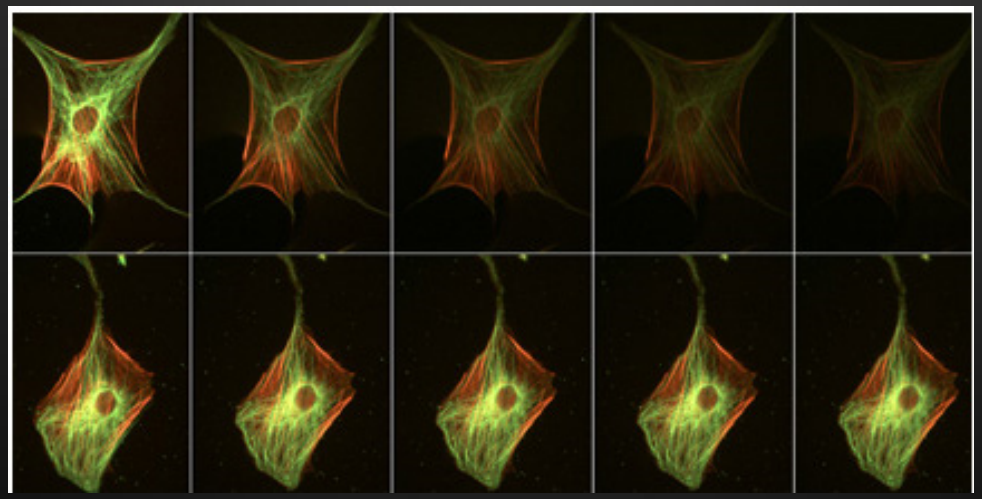
The Stokes shift is an extra for observing fluorescence; without it there would be (almost) no way to distinguish between excitation and emitted light

Probes with varying Stokes shifts are very useful for multicolor applications

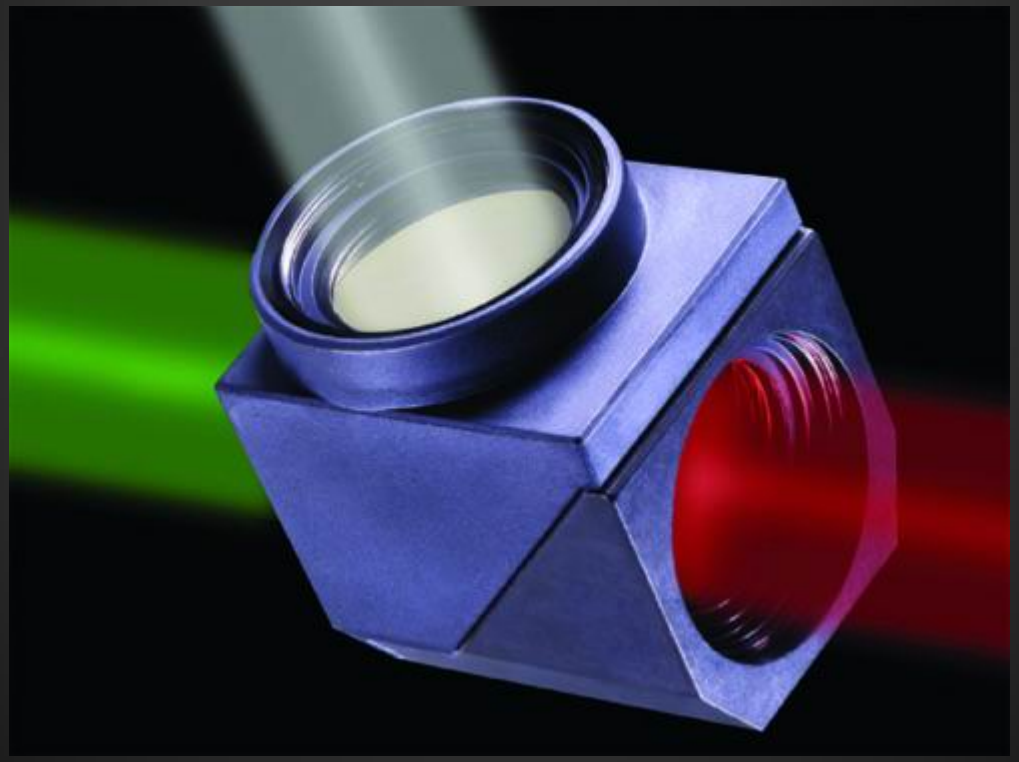


Photobleaching, quenching

- Defined as the **irreversible destruction** of an excited fluorophore
- Methods for countering photobleaching
  - Illuminate for shorter times
  - Use high magnification, high NA objective
  - Use wide emission filters – more signal to capture (may create problems with multiple probes)
  - Reduce excitation intensity
  - Use "**antifade**" reagents (not compatible with viable cells)



Filter and dichroic mirror



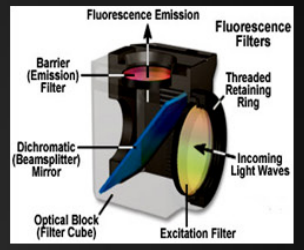
Filter and dichroic mirror

Longpass
LP filter

Shortpass
SP filter

Bandpass
BP filter

Dichroic mirror, Beamsplitter
FT 550



LP 550
All light with
λ> 550 passes

SP 550
All light with
Λ< 550 passes

BP 505-550
All light bw
505 550 passes

All light with
Λ < 550 passes
All light with
λ > 550 gets
reflected

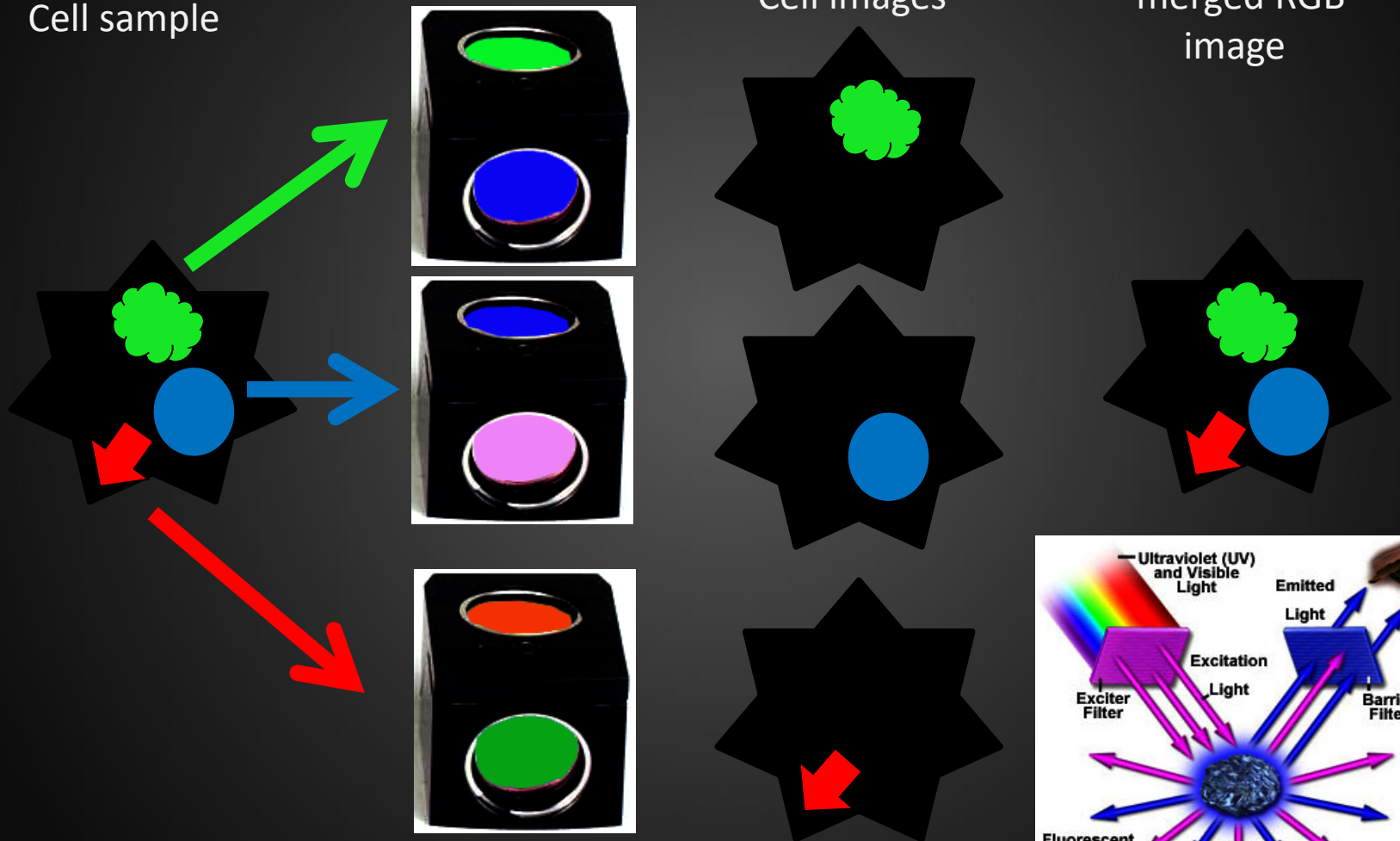
Light which is not passing might be absorbed
OR reflected (to other filter)

Dealing with fluorescence

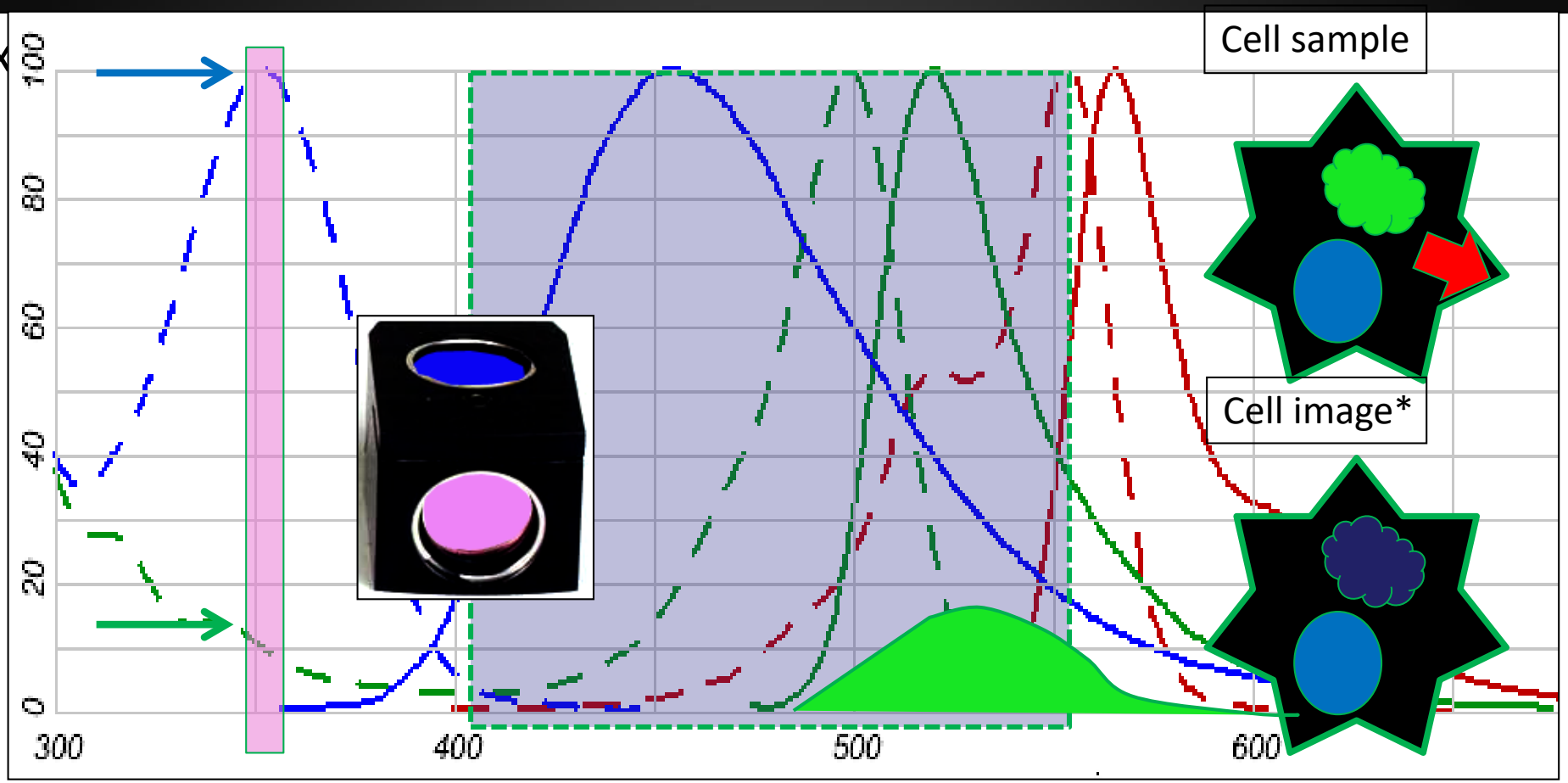
Cell sample

Cell images

merged RGB image

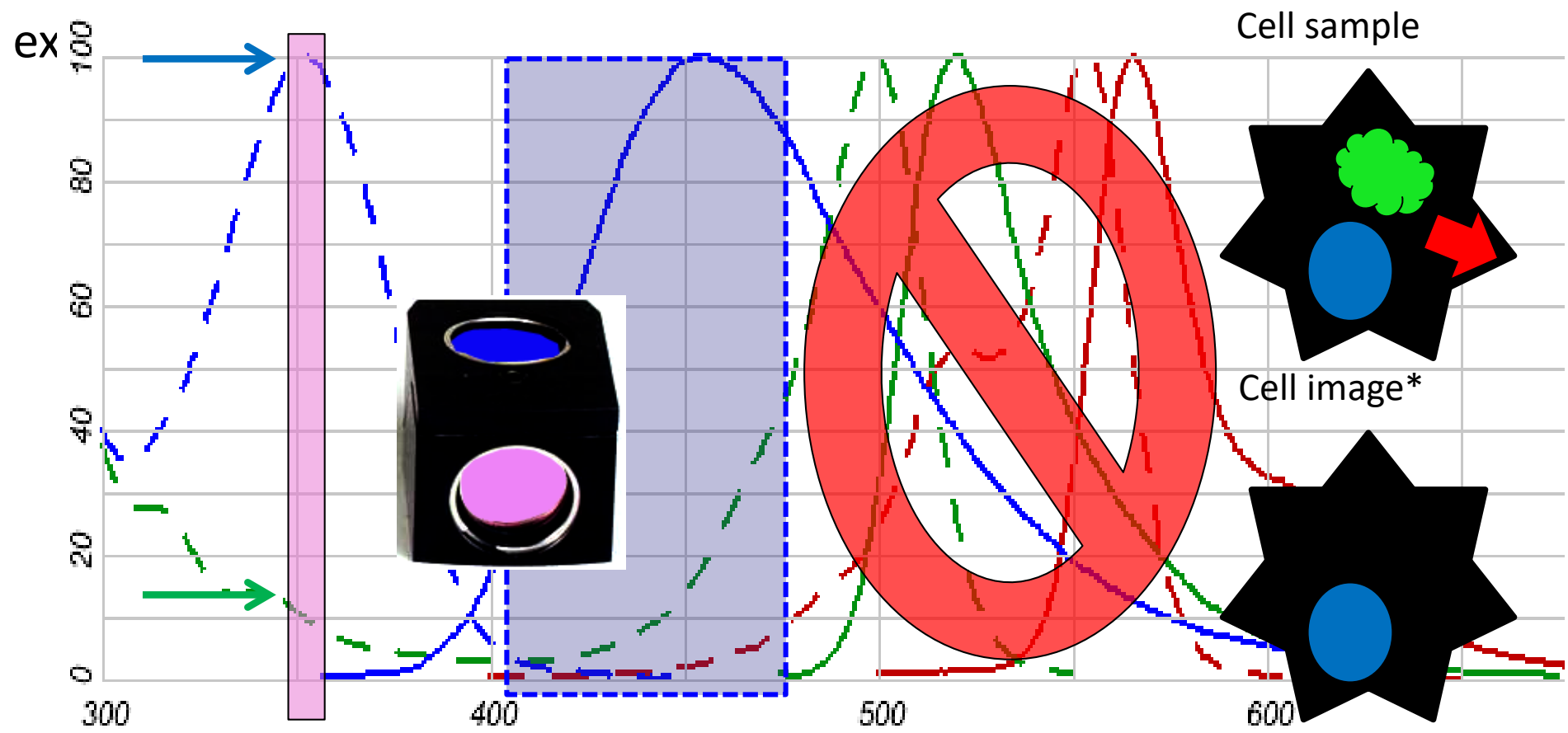


Dealing with fluorescence



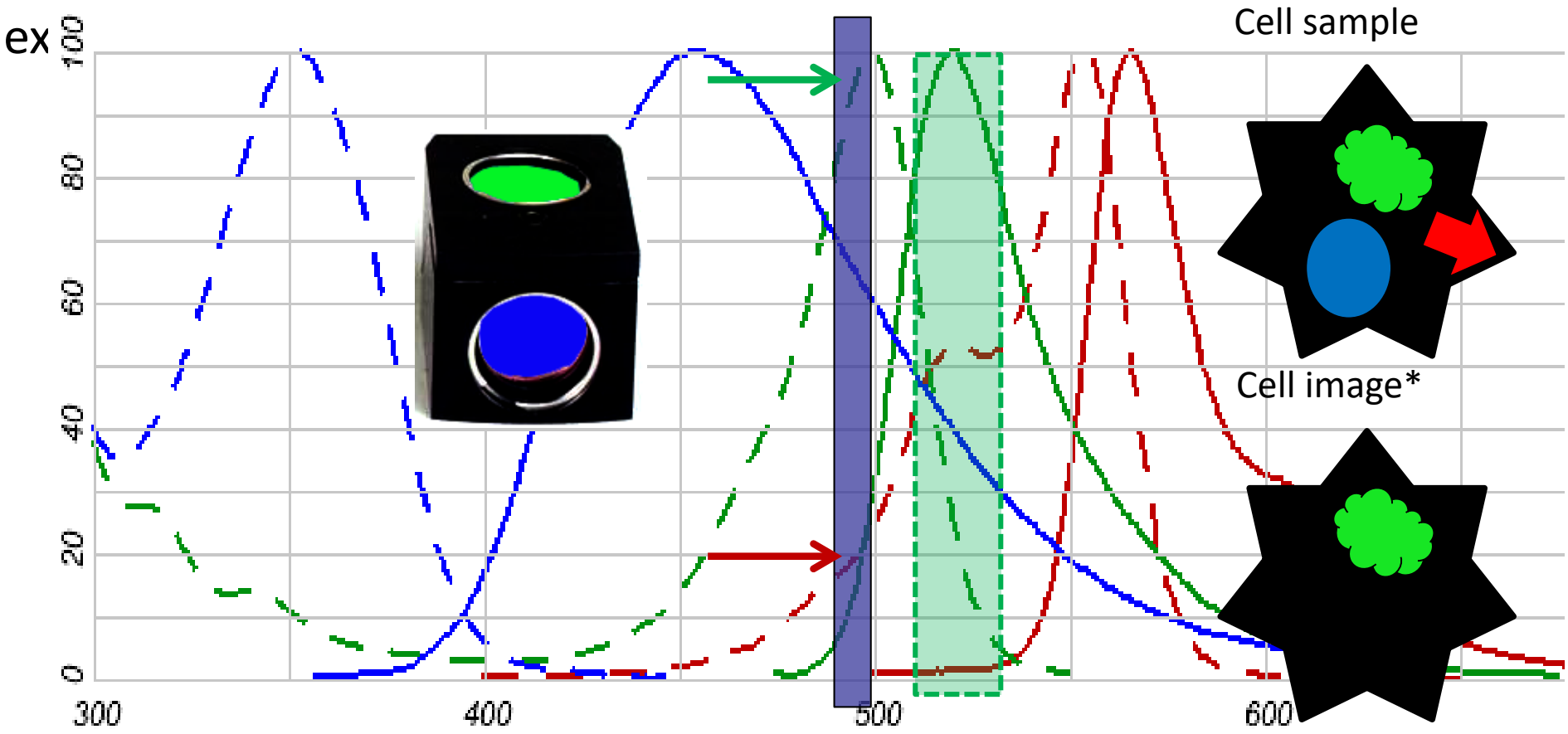
Excitation 350 nm excitates Blue and Green, using BP filter 400-550 collects them both. *Remember: the camera is color blind.
You decide with your choice of filter what it will see.

Dealing with fluorescence



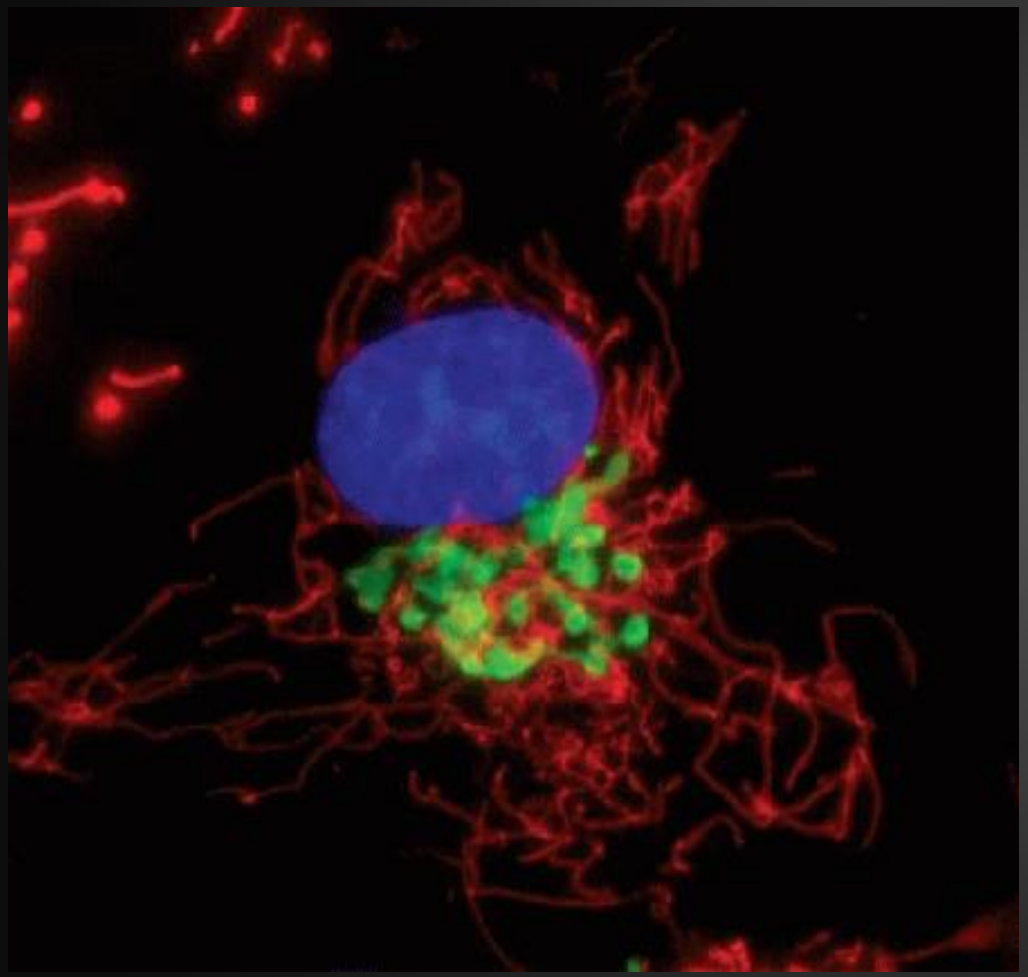
Excitation 350 nm excitates Blue and Green, using BP filter 400-480 collects only the blue. *Remember: the camera is color blind. You decide with your choice of filter what it will see.

Dealing with fluorescence



Excitation 480 nm excitates Green and Red, using BP filter 510-530 collects only the green. *Remember: the camera is color blind. You decide with your choice of filter what it will see.

Specific Organelle Probes



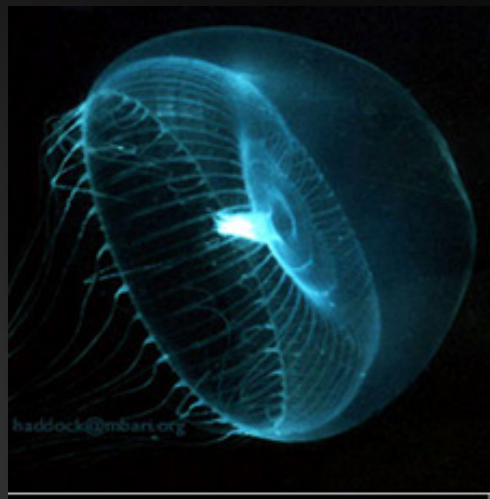
Gibco® human aortic smooth muscle cells (HASMC, Cat. No. C0075C) were transduced with
CellLight™ Golgi-GFP ,
CellLight™ Mitochondria-RFP
Hoechst 33342
Imaging was performed on live cells using a DeltaVision® Core microscope + standard DAPI/FITC/TRITC filter sets.

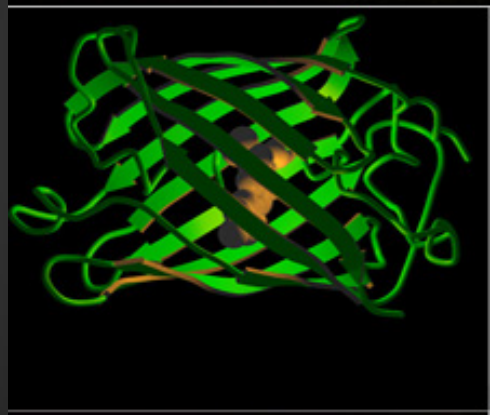
http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Image-Gallery/Image-Detail.10239.html

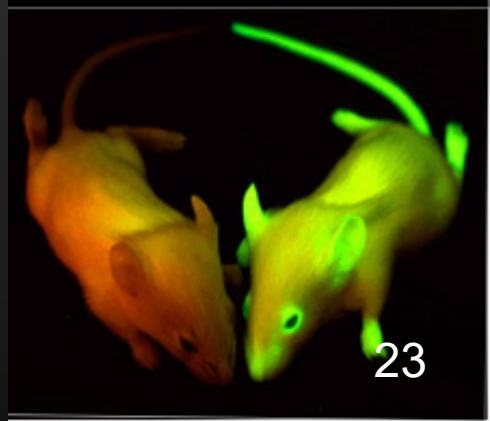
Fluorescent protein

- GFP - *Green Fluorescent Protein*
  - GFP is from the chemiluminescent jellyfish *Aequorea victoria*
  - excitation maxima at 395 and 470 nm (quantum efficiency is 0.8) Peak emission at 509 nm
  - contains a p-hydroxybenzylidene-imidazolone chromophore generated by oxidation of the Ser-Tyr-Gly at positions 65-67 of the primary sequence
  - Very stable
  - Major application is as a reporter gene for assay of promoter activity
  - requires no added substrates
  - Now in the enhanced form of eGFP , eYFP, eCFP

http://gfp.conncoll.edu/
http://brainwindows.wordpress.com/category/gfp
http://www.biojobblog.net/2008/10/08/gfp-finally-gets-its-due/







Important points

- Fluorescence is the primary information source for confocal microscopes and flow cytometry equipment
- Fluorescence emission is longer than the exciting wavelength
- Dye molecules must be close to, but below saturation levels for optimum emission
- Fluorescence probes must be appropriate for the excitation source and the sample of interest
- Correct optical filters must be used for multiple color fluorescence emission

THANKS FOR YOUR ATTENTION!