

# Fluorescence Dyes, Probes and Optics

Nico Stuurman  
Principles and Practices of Light Microscopy  
UCSF Microscopy Course, 4/11/2013

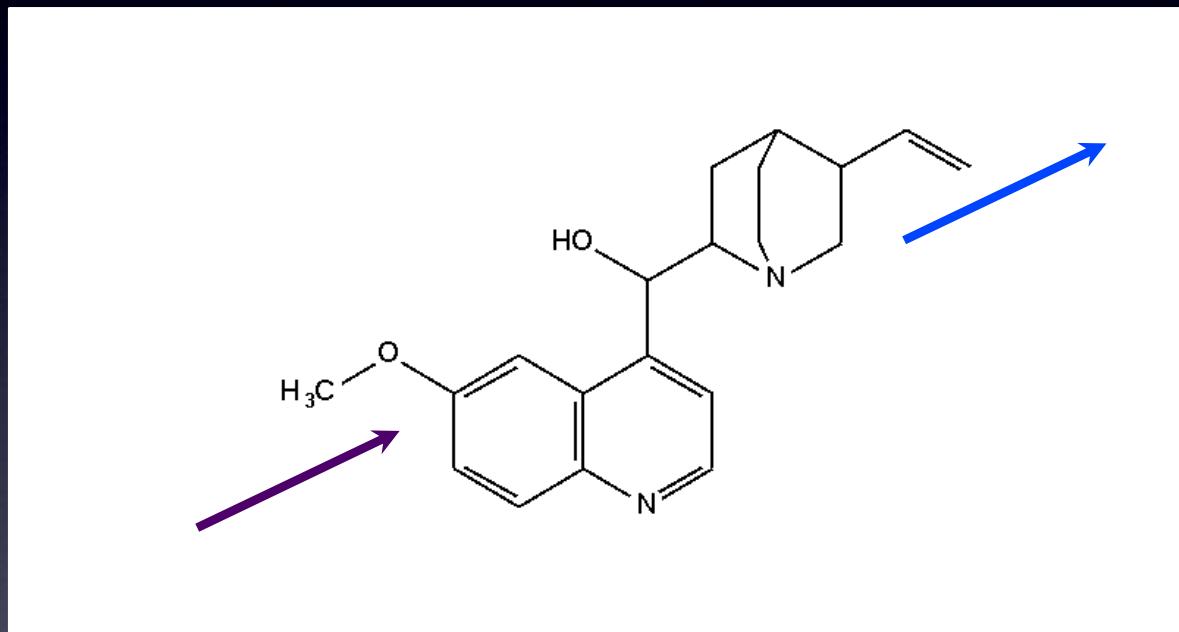
# Why Fluorescence?

- High Contrast
- High Specificity
- Quantitative
- Live Cell Imaging

# What is it?

Sir John Frederick William Herschel, 1854: Though perfectly transparent and colorless when held between the eye and the light, or a white object, it yet exhibits in certain aspects, and under certain incidences of the light, an extremely vivid and beautiful celestial blue colour, which, from the circumstances of its occurrence, would seem to originate in those strata which the light first penetrates the liquid.....

# Excitation/Emission

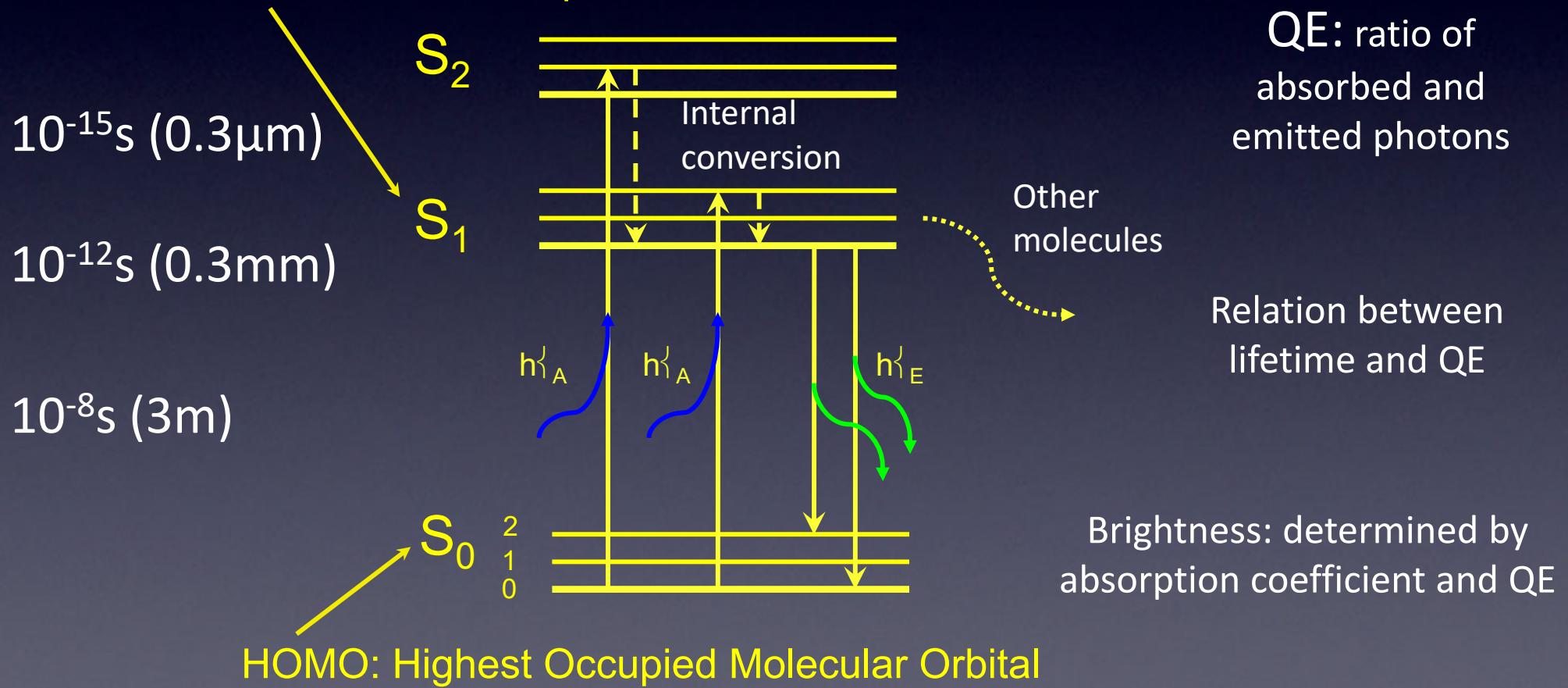


Emission light is longer wavelength (lower energy)  
than excitation light

# Fluorescence

## Jablonski diagram

LUMO: Lowest Unoccupied Molecular Orbital

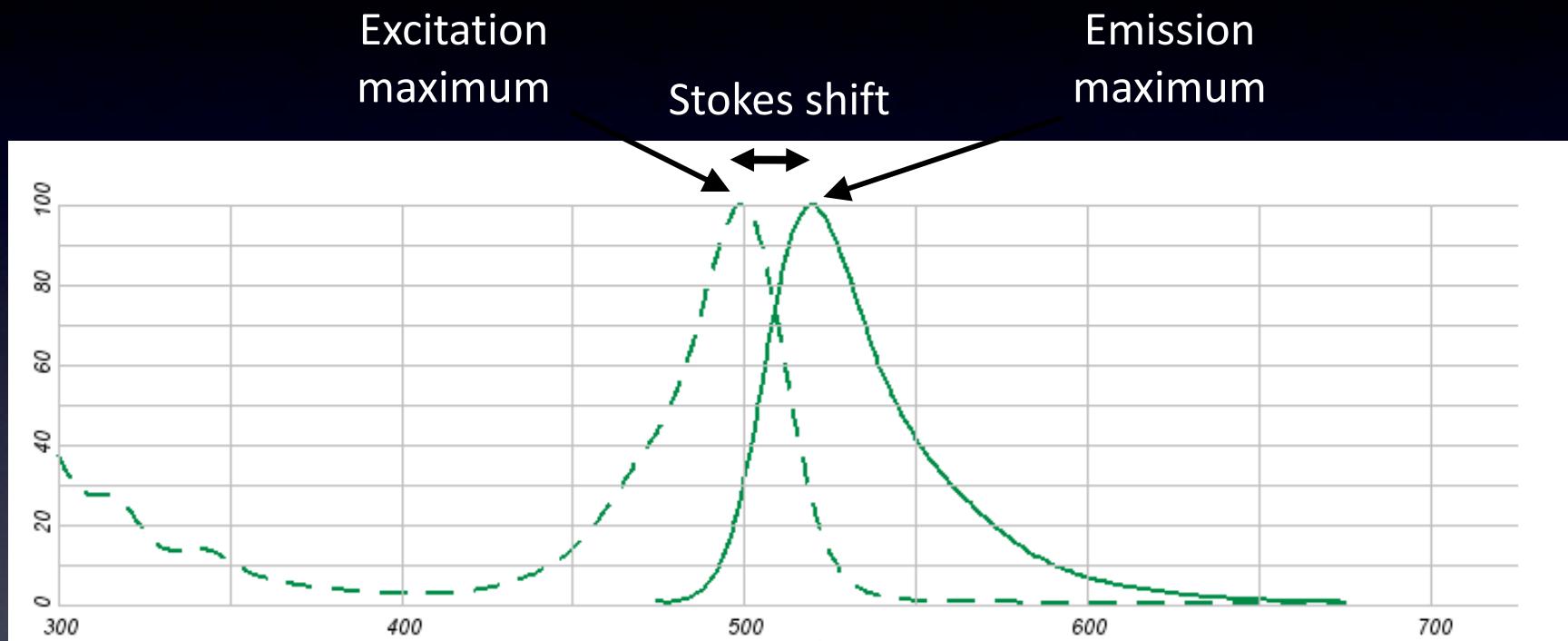


QE: ratio of absorbed and emitted photons

Relation between lifetime and QE

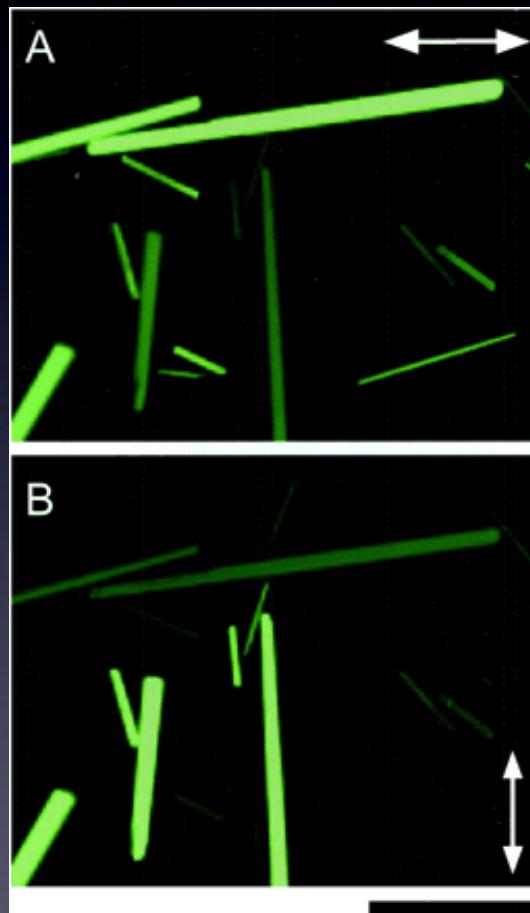
Brightness: determined by absorption coefficient and QE

# Fluorescence Spectra



Alexa 488

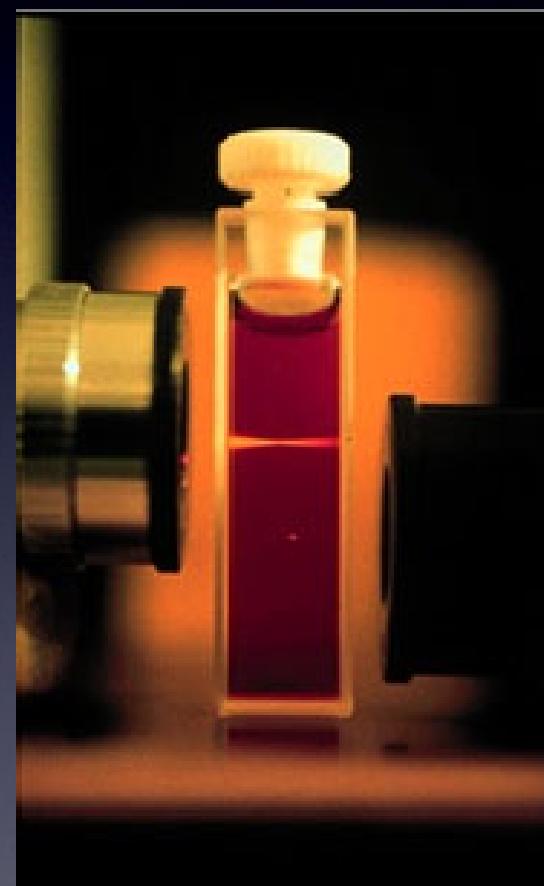
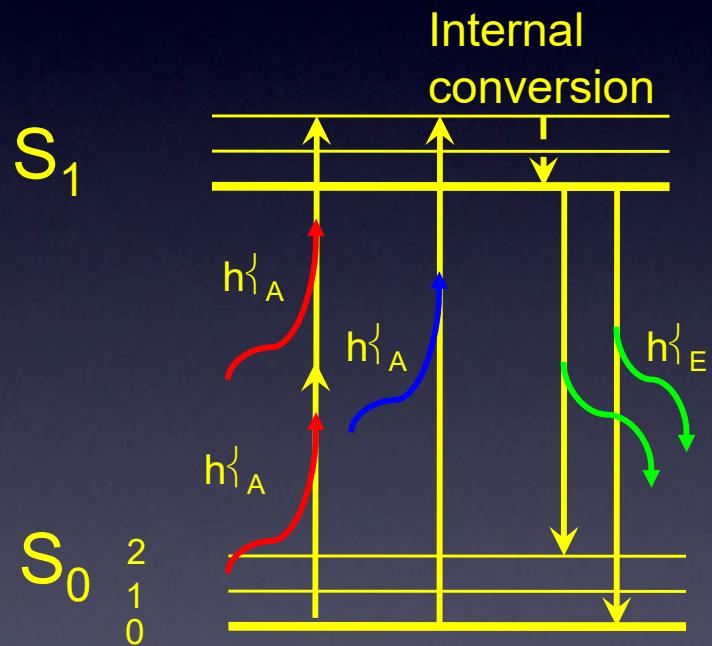
# Polarization



Native GFP crystals

Shinya Inoué

# Multi-photon excitation



Brad Amos, MRC, Cambridge

# Fluorescent Dye Types

- Organic dyes
- (Phycobiliproteins)
- (Lanthanide Chelates)
- Fluorescent Nanocrystals
- Fluorescent Proteins

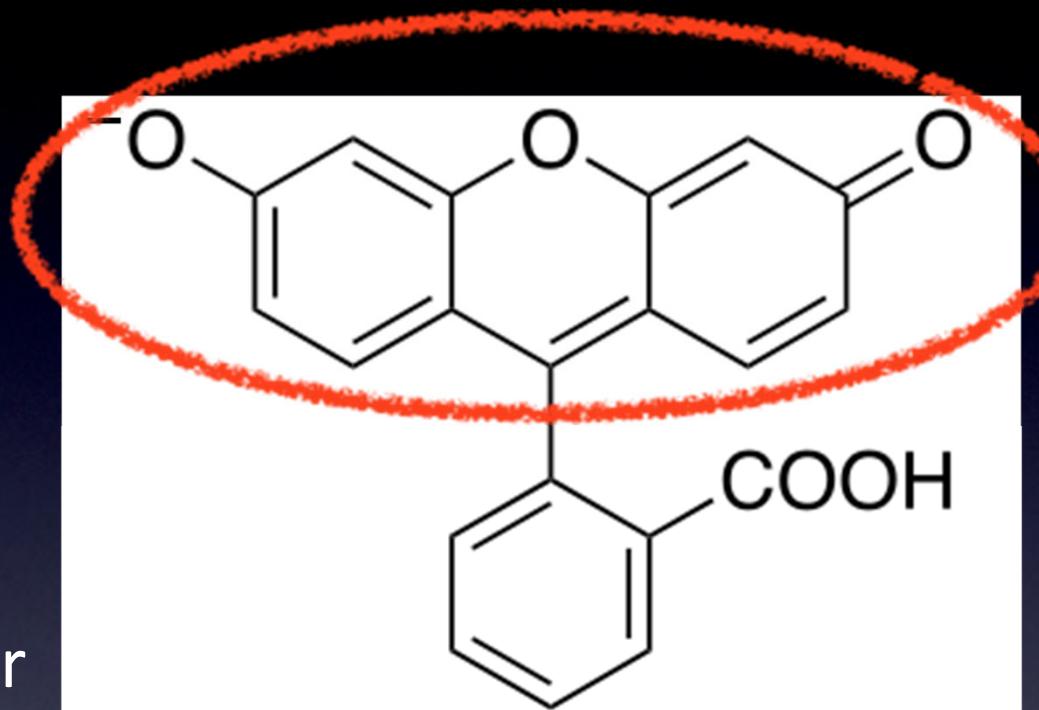
# First synthesized fluorescent dye: Fluorescein

Extended conjugate structure

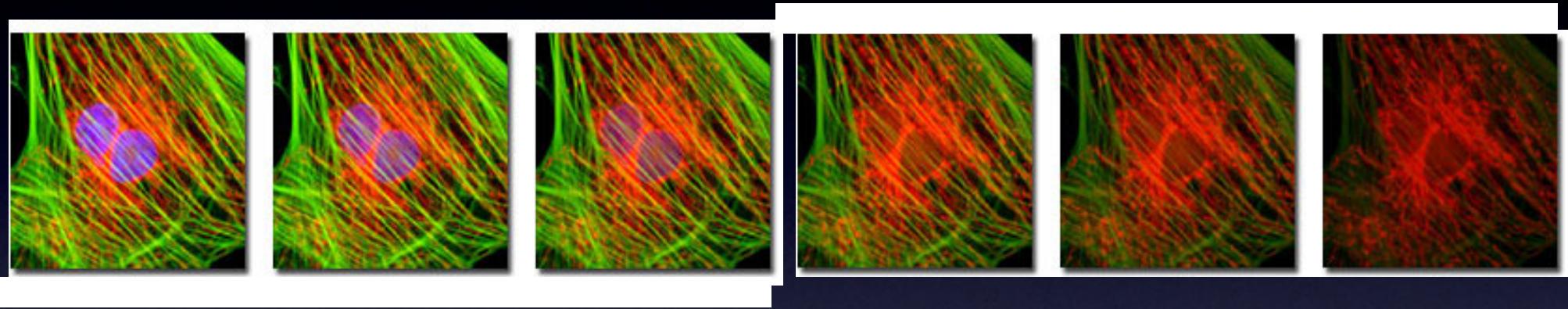
Extended orbital > small energy difference HOMO/LUMO

Electron donor ( $O^-$ ) and acceptor (=O) at the two ends

Rigid (no rotatable bonds in conjugated structure > Prevents energy loss from LUMO by bond rotation



# The Enemy: Photo-bleaching

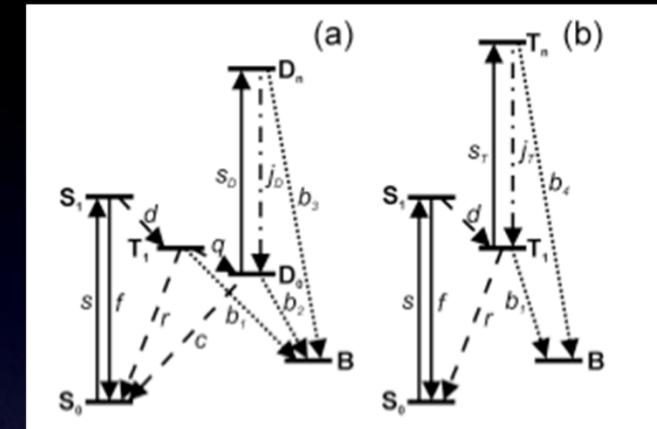
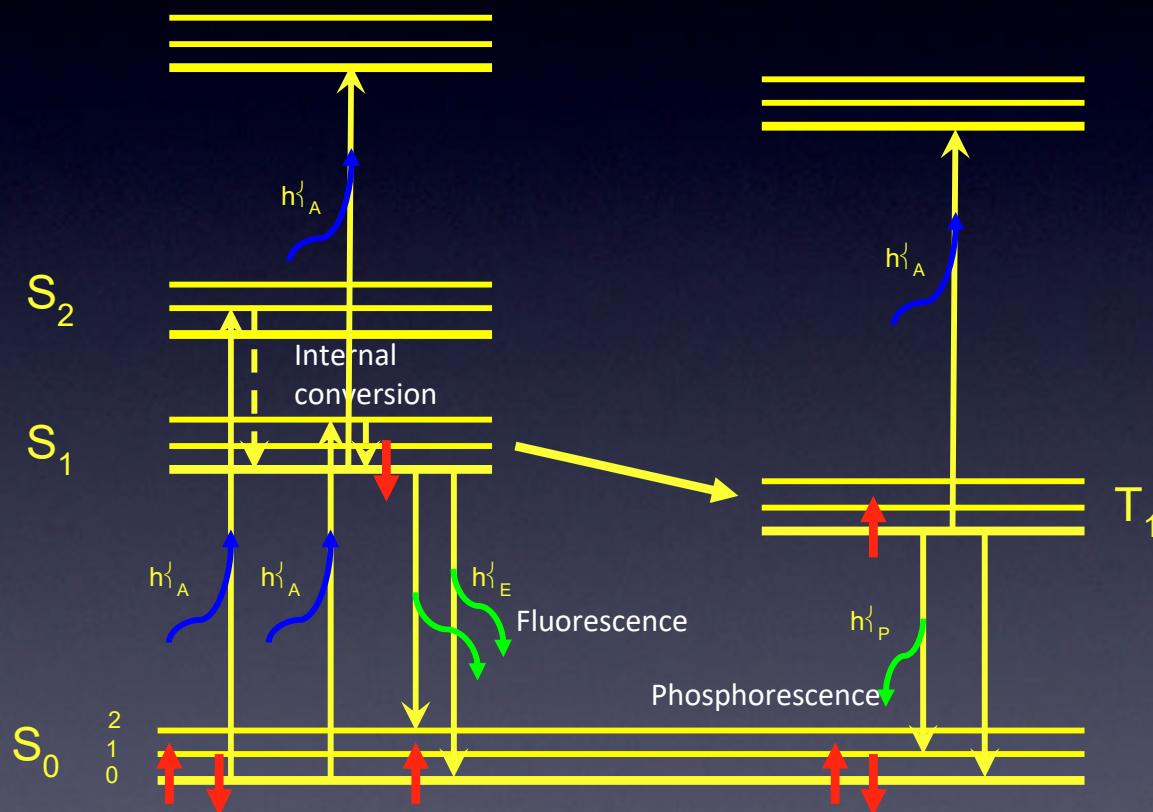


Decrease in emission intensity after exposure

Exciting a molecule once has a probability  $Q_b$  of killing it

Each molecule will emit only a finite number of photons

# Photo-Bleaching - Mechanisms



Zondervan et al., J. Phys. Chem. A , 2004, 108:1657–1665.

$O_2$   
 $\bullet O_2$  Singlet Oxygen  
**Bad!**

Except when used in CALI (Chromophore-assisted light inactivation)

# What to do about photo-bleaching?

- Select fade-resistant dyes
- Label densely
- Decrease bleaching by anti-fade mounting media
- Glycerol
- Oxygen scavengers
- Free-radical scavengers
- Triplet state quenchers

Note: some anti-fade agents quench some dyes.

- Budget the photons you have
- Only expose when observing
- Minimize exposure time & excitation power
- Use efficient filter combinations
- Use high QE, low noise camera
- Use simple light path

# Organic Dyes

## *The Classics*

Coumarin

Fluorescein

Rhodamine

332/456  
QY 0.77

490/520  
QY 0.925

554/573  
QY 0.28

- Systems of conjugated bonds that share electrons
- Larger system  $\square$  longer wavelength

# Organic Dyes

Cyanine dyes

Alexa dye series

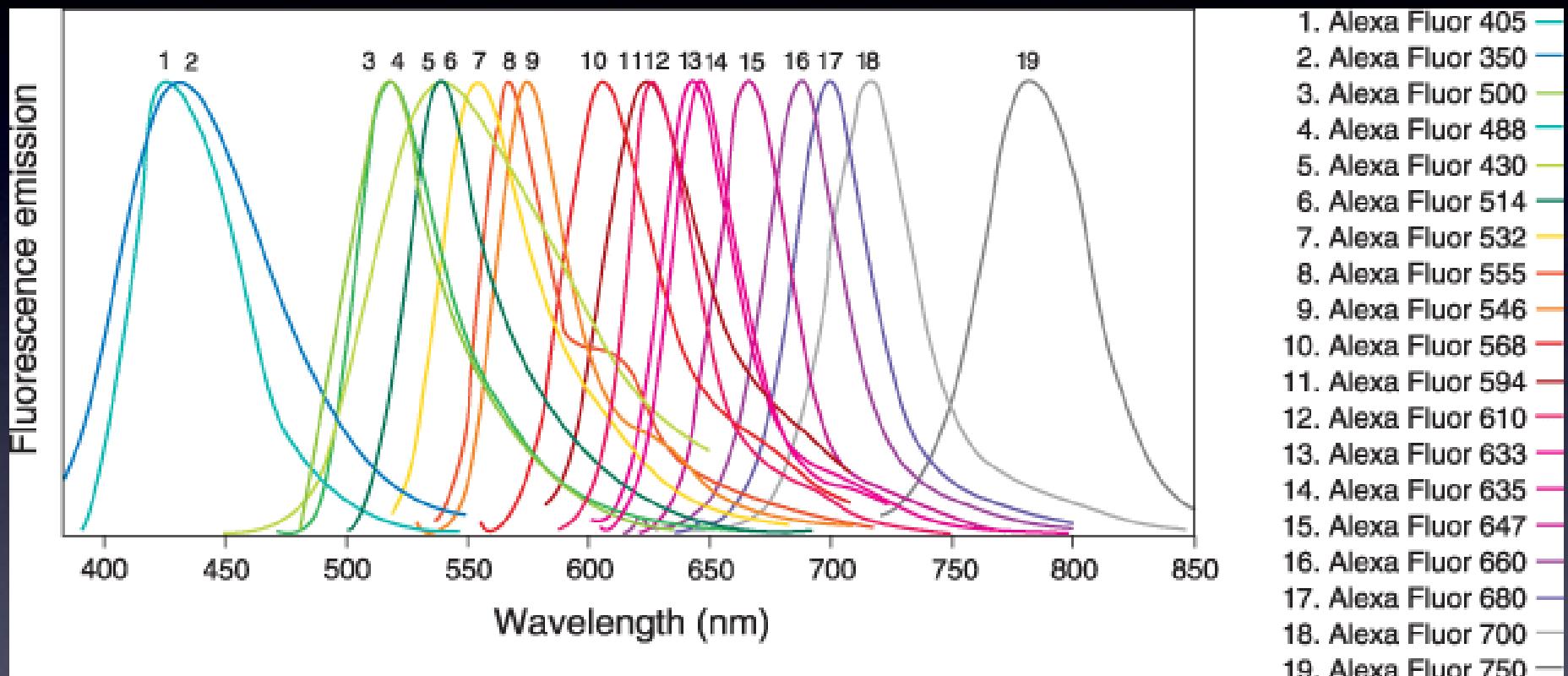
554/568  
QY 0.14

652/672  
QY 0.18

Also, Cy2, Cy5.5

499/517  
QY 0.60

# The Alexa Series Emission Spectra



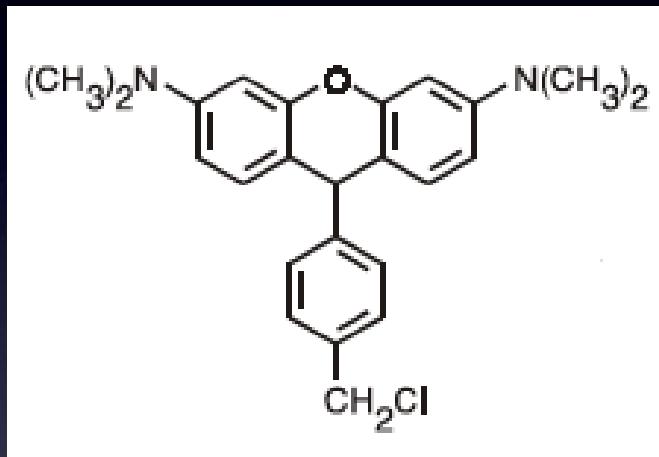
Coumarins

Rhodamines

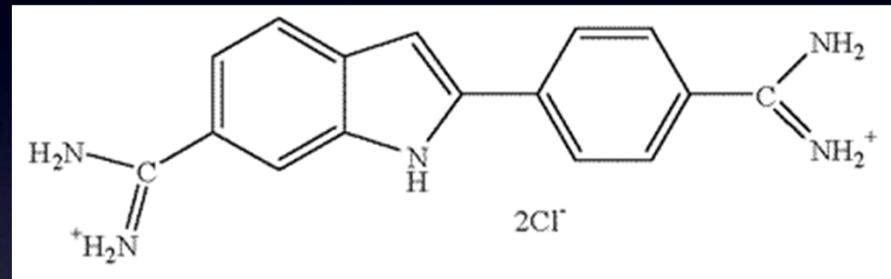
Cyanines

Molecular Probes ([www.probes.com](http://www.probes.com)) - now In Vitrogen

# From Dye to Probe: Small dyes that are Probes



Mitotracker  
Oxidized in mitochondria in  
fluorescent compound



DAPI  
Hoechst33258  
Hoechst 33342  
~20 fold enhancement  
TOTO, YOYO

# Conjugation of organic dyes

Chemistry/Method

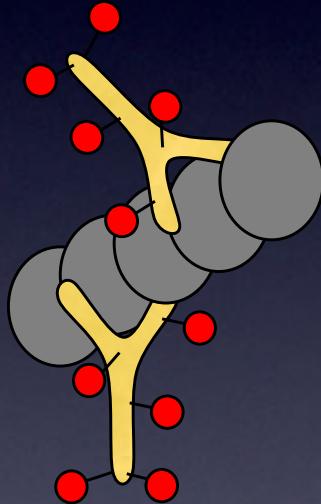
Amino groups (lysine, K): succinimidyl ester or isothiocyanate

- Small molecules, i.e. phalloidin, taxol
- Proteins: labeling site unspecific
- Antibodies: direct/indirect labeling (Label density)

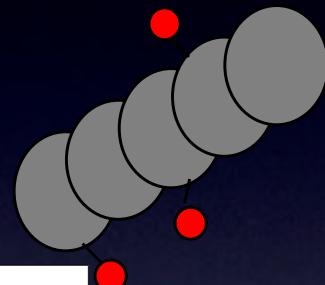
Example:  
Dynein driven gliding of microtubules  
labelled with TMR on lysine residues.

# Fluorescent labeling

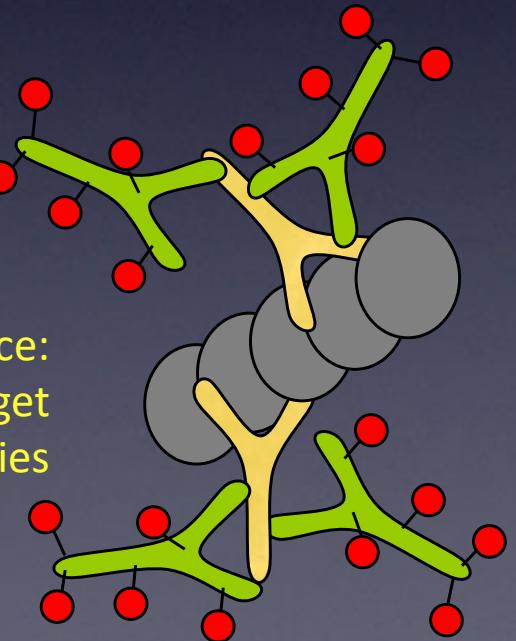
Direct immunofluorescence:  
labeled antibodies against target



Direct labeling (& microinjection)  
of target molecules



Indirect immunofluorescence:  
Unlabeled antibodies against target  
Labeled antibodies against those antibodies

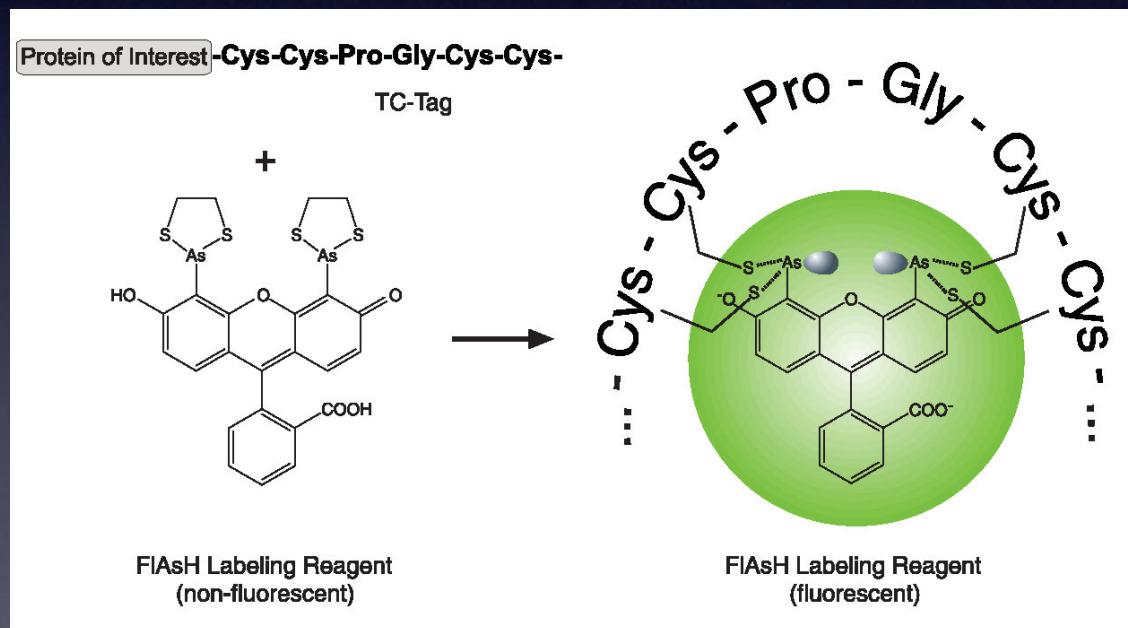


# Site-Specific Labeling

## Chemistry/Method

Sulfhydryl groups (cystein): maleimide

Engineer Cys-light version of target protein

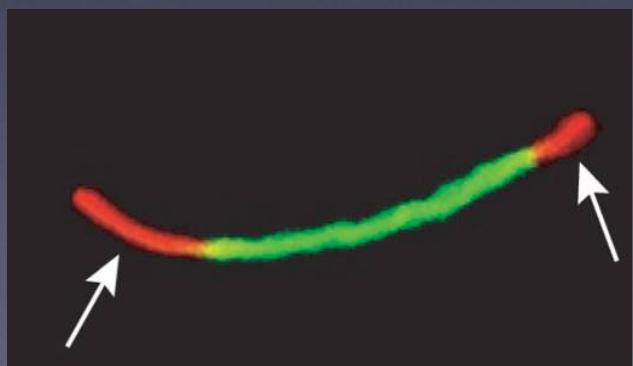


Example:

Newly synthesised connexins (ReAsH:Red) are added to the outer edges of existing gap junctions (FlAsH:Green). Gaietta et al 2002

## FlAsH/ReAsH

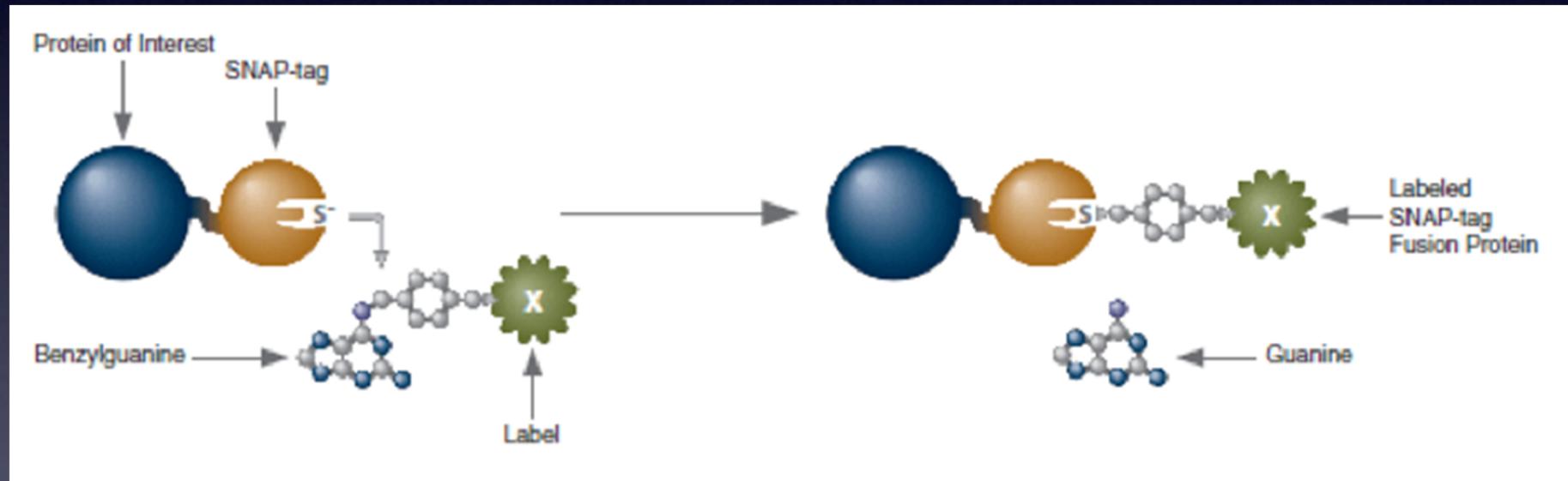
Labeling protein with tetra-cysteine motifs  
(Tsien lab/Invitrogen):



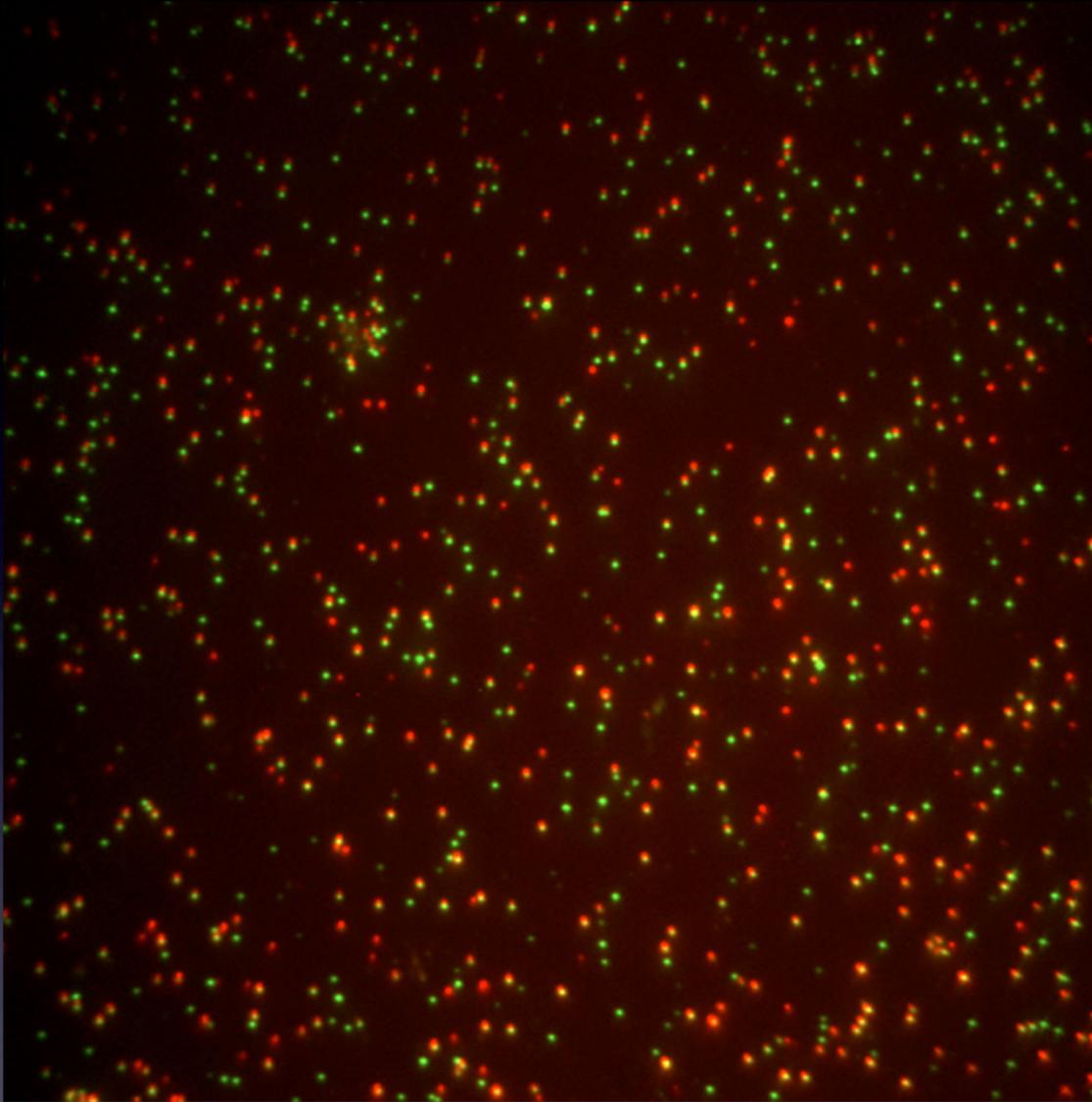
# Site-Specific Labeling

SNAP tag

Benzylguanosine reacts with modified DNA repair enzyme (O<sub>6</sub>-alkylguanine-DNA alkyltransferase (AGT))

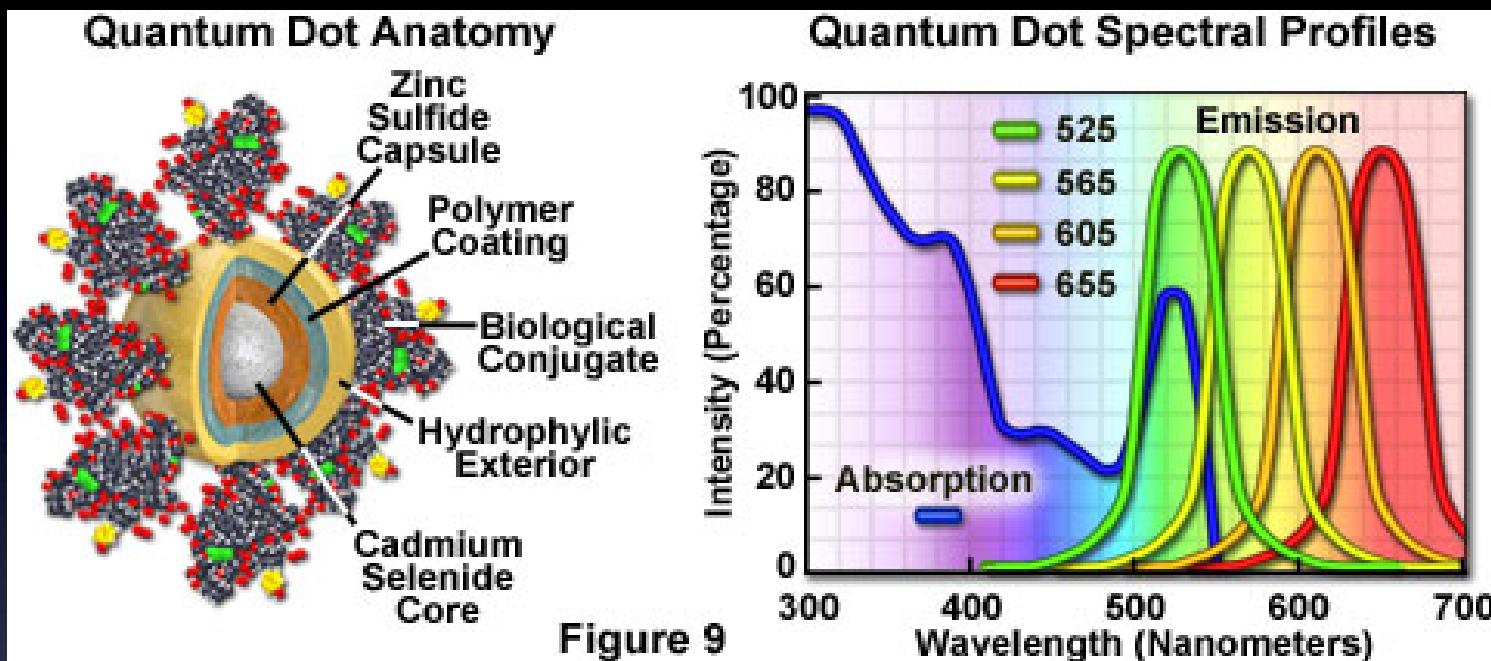


Similar strategy: Halo tag, CLIP tag, more?



Single molecules of Dynein labeled with  
SNAP Surface 647 and CLIP-cell TMR Star  
(Gira Bhabha)

# Quantum-dots



nanometre-scale crystals composed of atoms of an inorganic semiconductor material

## Advantages

- Very bright
- Very photostable
- Excitation possible at a single wavelength
- Visible in electron microscope

## Disadvantages

- Large size
- Multivalent linkage

# Quantum dot labeling

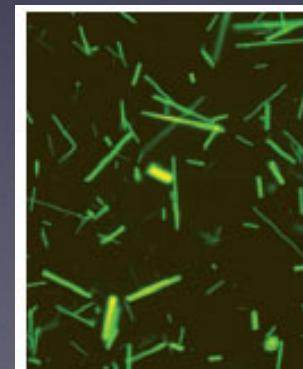
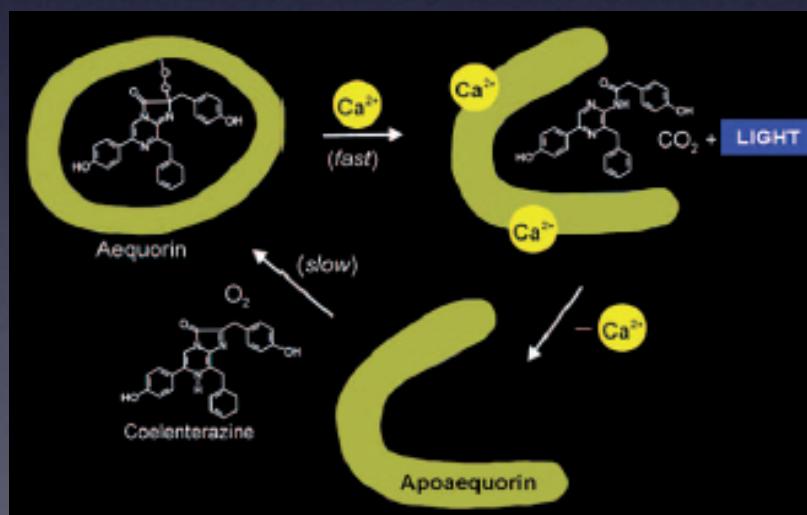
- Biotin/Streptavidin Linkage
- Biotin maleimide (in vitro)
- Biotin HaloTag/SNAP
- Biotin carrier protein
- BiotinLigase/AP1
- Antibody Conjugates – immunohistochemistry
- Direct linkage to proteins/peptides – targeting to cell compartments

Mono-valent QDots:  
Justin Farlow  
Zev Gartner Lab

Qdot labelled dynein via  
HaloTag:Biotin:Streptavidin  
linkage moving on axonemes

# Fluorescent proteins

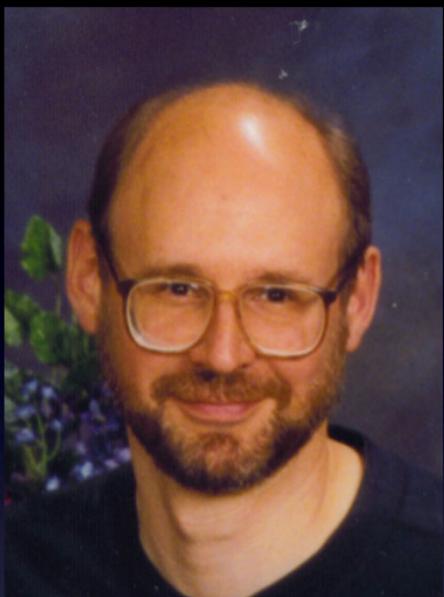
## Discovery



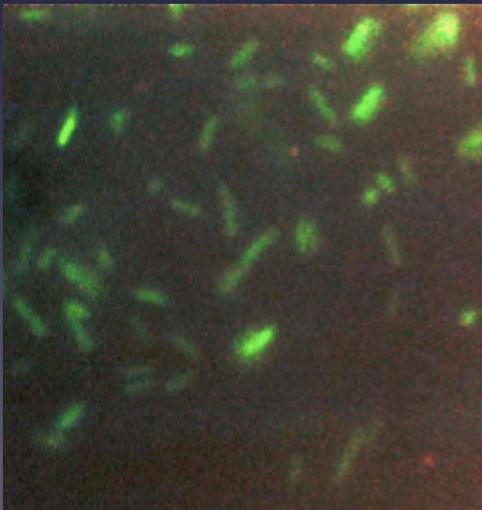
GFP (100 mg)  
↓  
Denature at 90 °C  
Digest with papain  
Extraction with butanol at pH 1  
TLC purification  
↓  
Isolated chromophore (0.1 mg)

Images from Osamu Shimomura

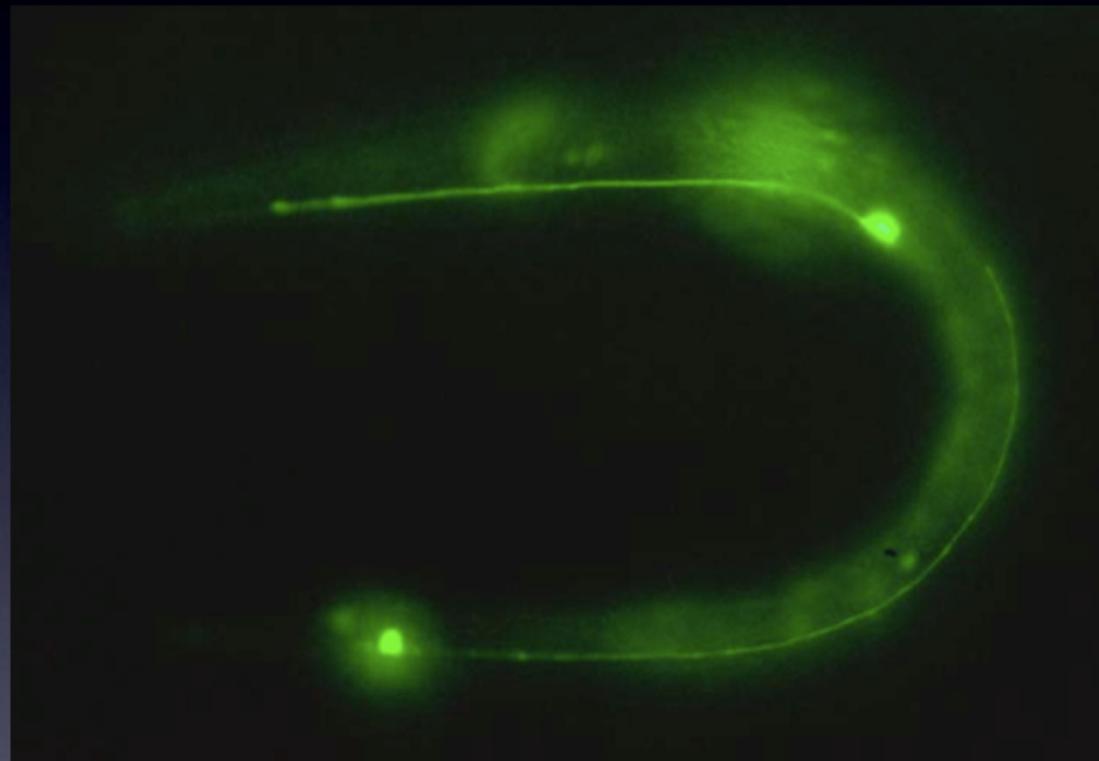
# No co-factors needed!



Douglass Prasher



First GFP expression in E.  
coli



and *C. elegans*

Images from Martin Chalfie

# GFP Optimization

## First Round

- Shift excitation peak from ~390 to ~480 (S65)
- Improved folding at higher temperatures
- Prevent dimerization (A206K), important!
- Created color mutants (BFP, CFP, YFP)

# Red fluorescent proteins



Coral reef: Discosoma

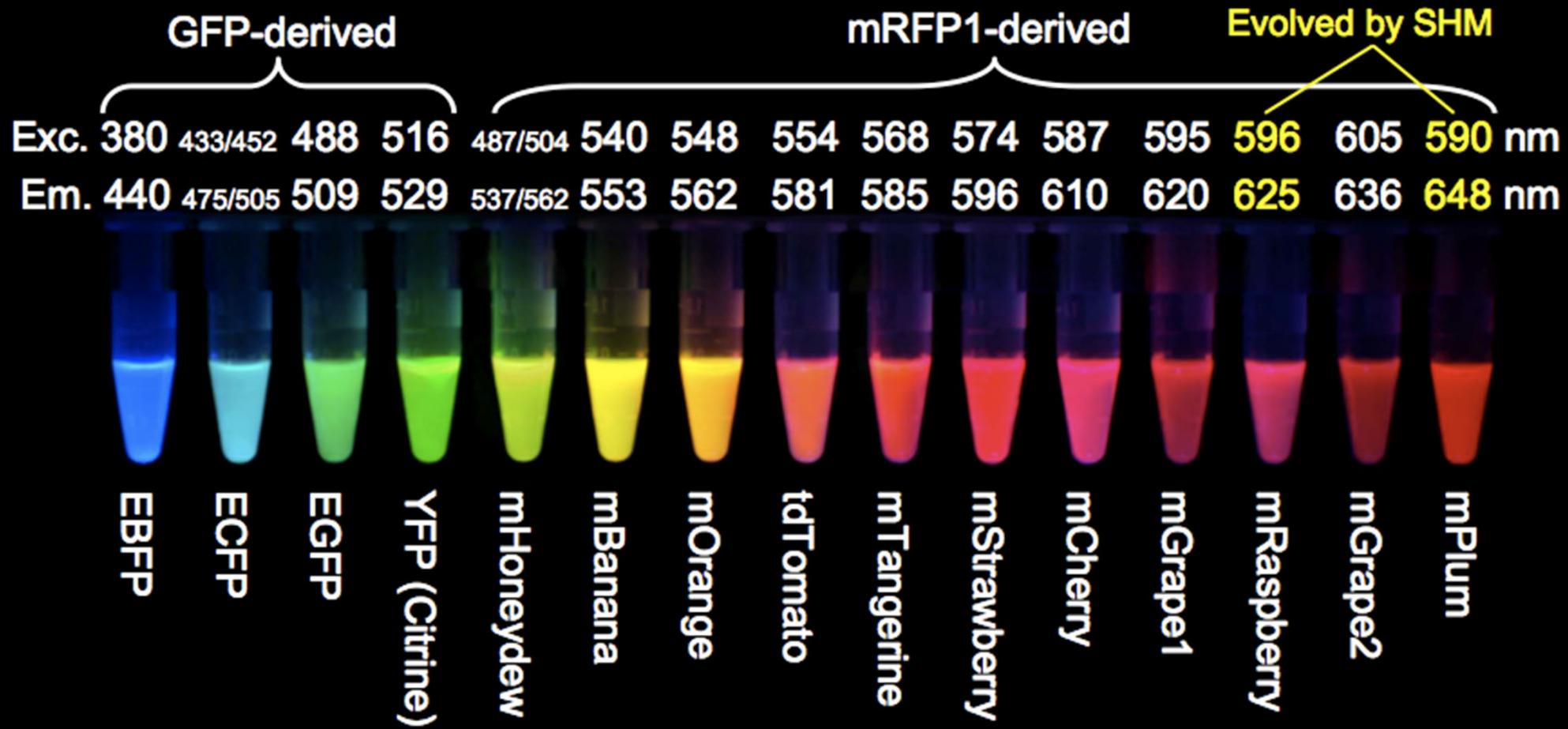
Lukyanov et al.

Fished for GFP-like proteins in coral reef obtained from Moscow pet shops.

dsRed: obligate tetramer, slowly maturing from green to red

[http://www.scholarpedia.org/article/Talk:Fluorescent\\_proteins#](http://www.scholarpedia.org/article/Talk:Fluorescent_proteins#)

# The 2004 palette of nonoligomerizing fluorescent proteins



Nathan Shaner et al (2004) *Nature Biotech.* **22:** 1567-1572

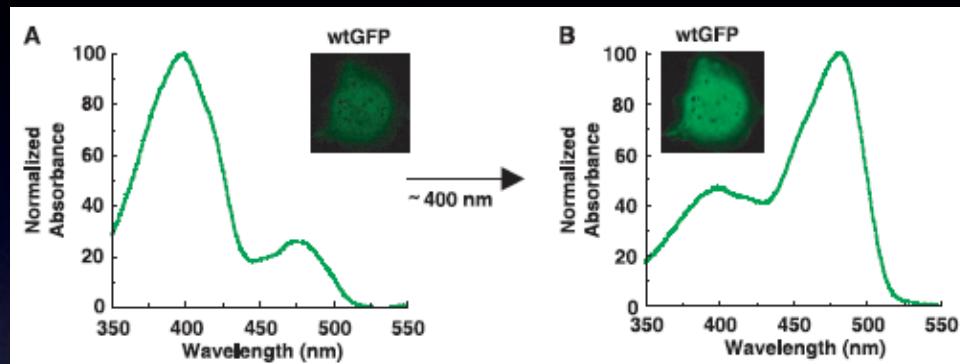
Lei Wang et al (2004) *Proc. Natl. Acad. Sci. USA* **101:** 16745-16749

# Switchable fluorescent proteins

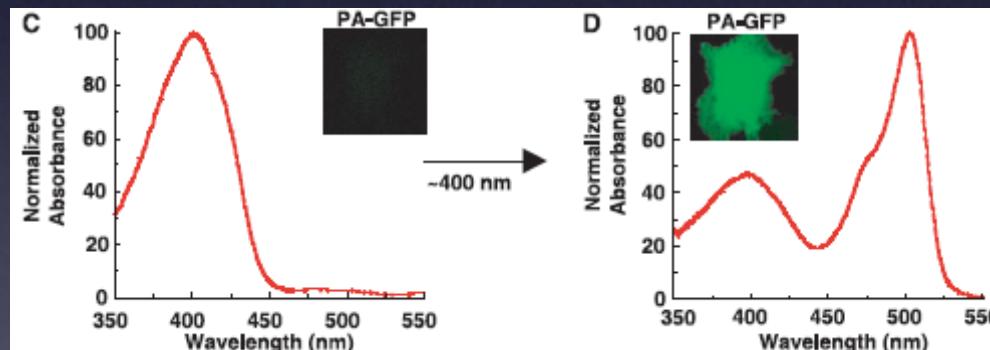
Fluorescence that can be activated or altered by light

Activatable

PA-GFP, ...



PA-GFP (T203H), 100:1 contrast



George H. Patterson and Jennifer Lippincott-Schwartz, 2002

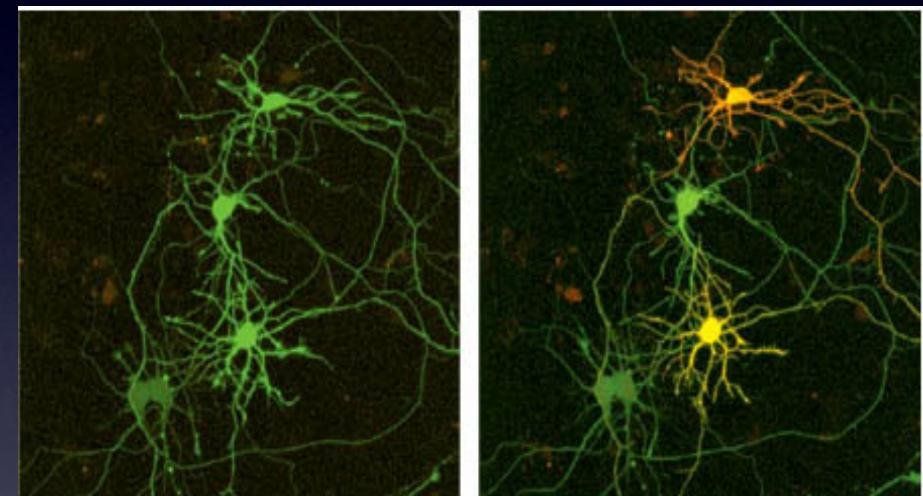
Color-changing

Green-red:

Kaede, EosFP, KikGR,...

Cyan-green:

PS-CFP



An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein

Ryoko Ando\*,†, Hiroshi Hama\*, Miki Yamamoto-Hino\*, Hideaki Mizuno\*, and Atsushi Miyawaki\*\*

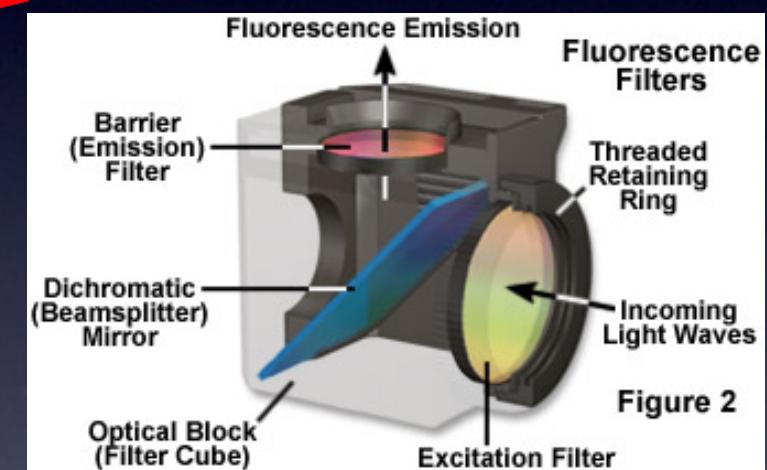
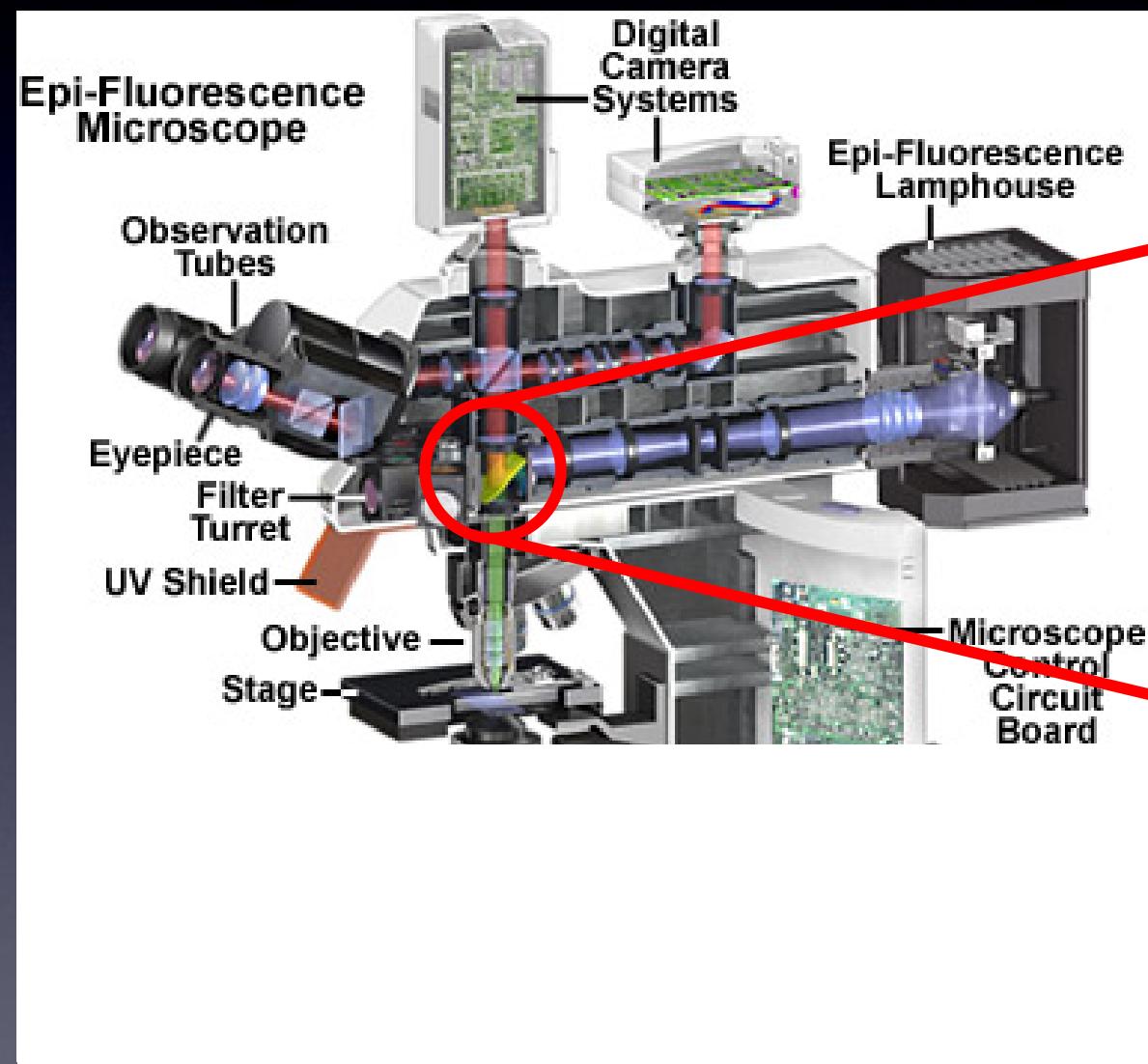
We happened to leave one of the protein aliquots on the laboratory bench overnight. The next day, we found that the protein sample on the bench had turned red, whereas the others that were kept in a paper box remained green. Although the sky had been partly cloudy, the red sample had been exposed to sunlight through the south-facing windows.



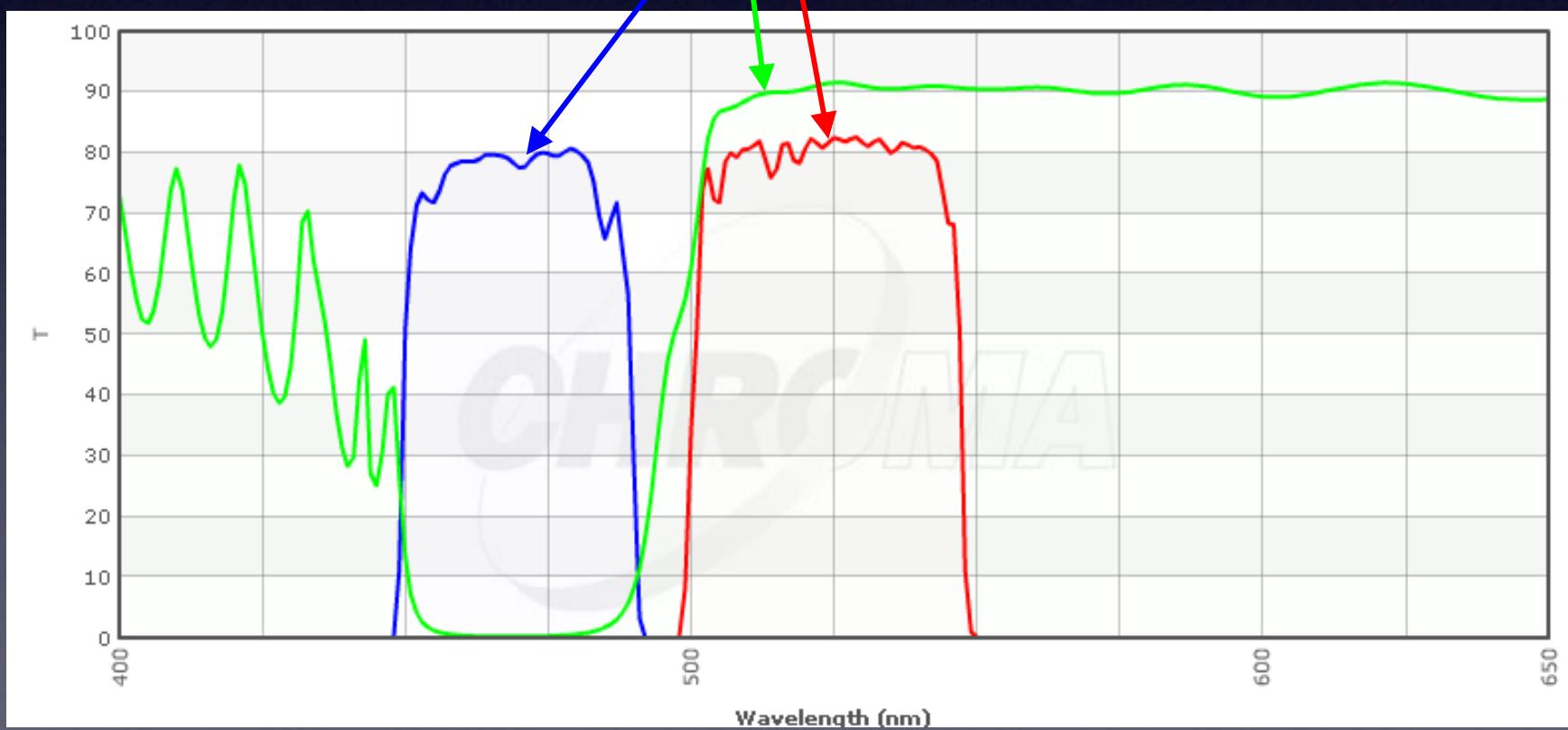
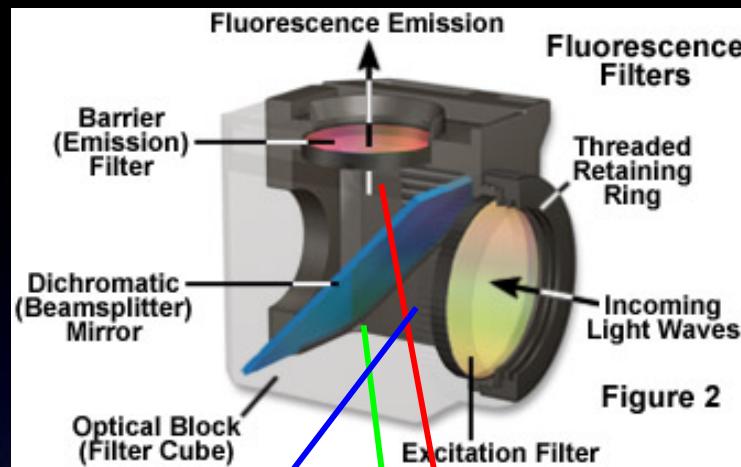
Michael Davidson

Reversibly switchable  
KFP, Dronpa

# The Epifluorescence Microscope



Ploem



# Types of Filters

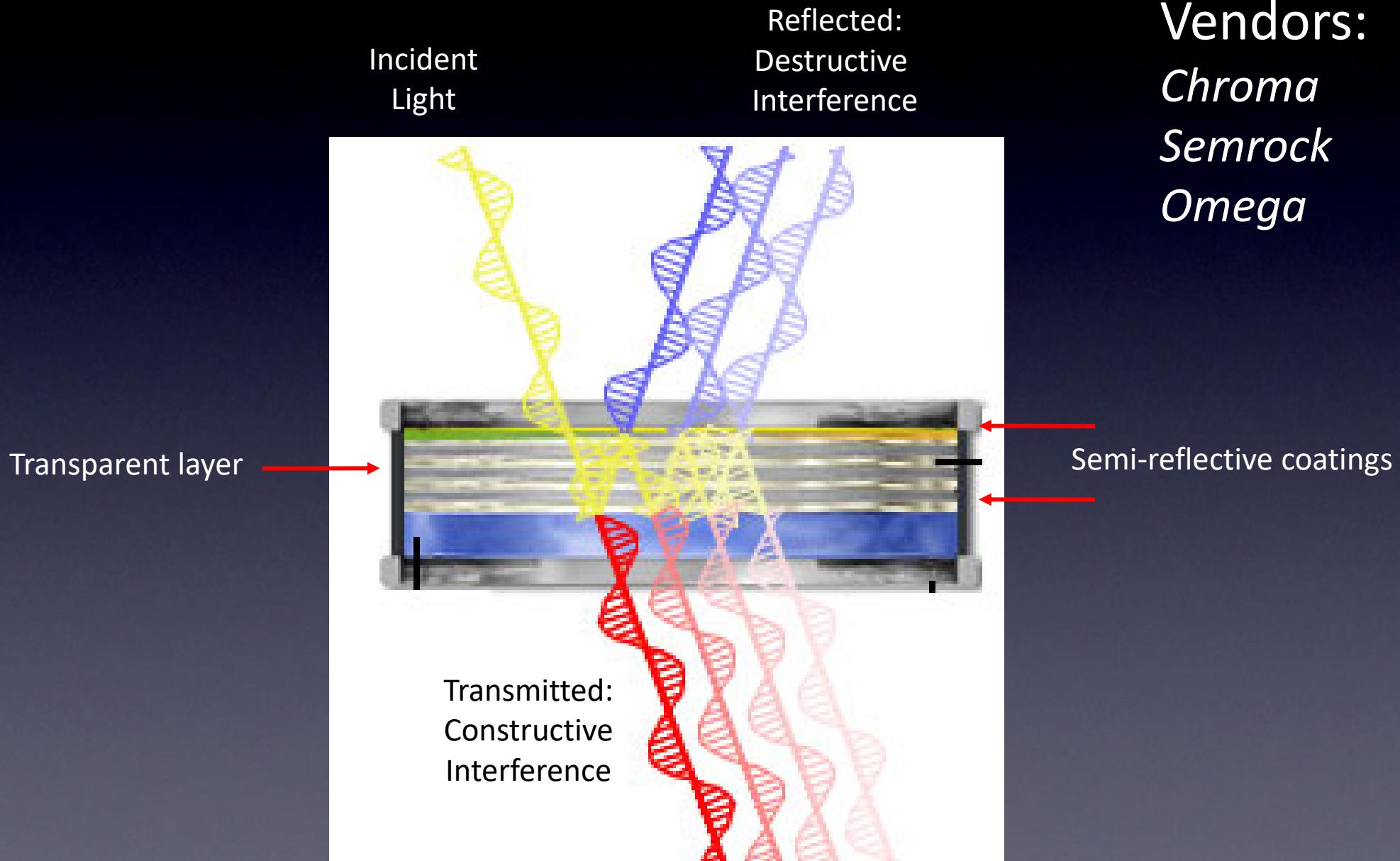
- Absorption (“colored”) glass
- Interference (thin-film coatings) Filters
- Acousto Optical Filters
- Liquid Crystal Filters

# Colored Glass Filters

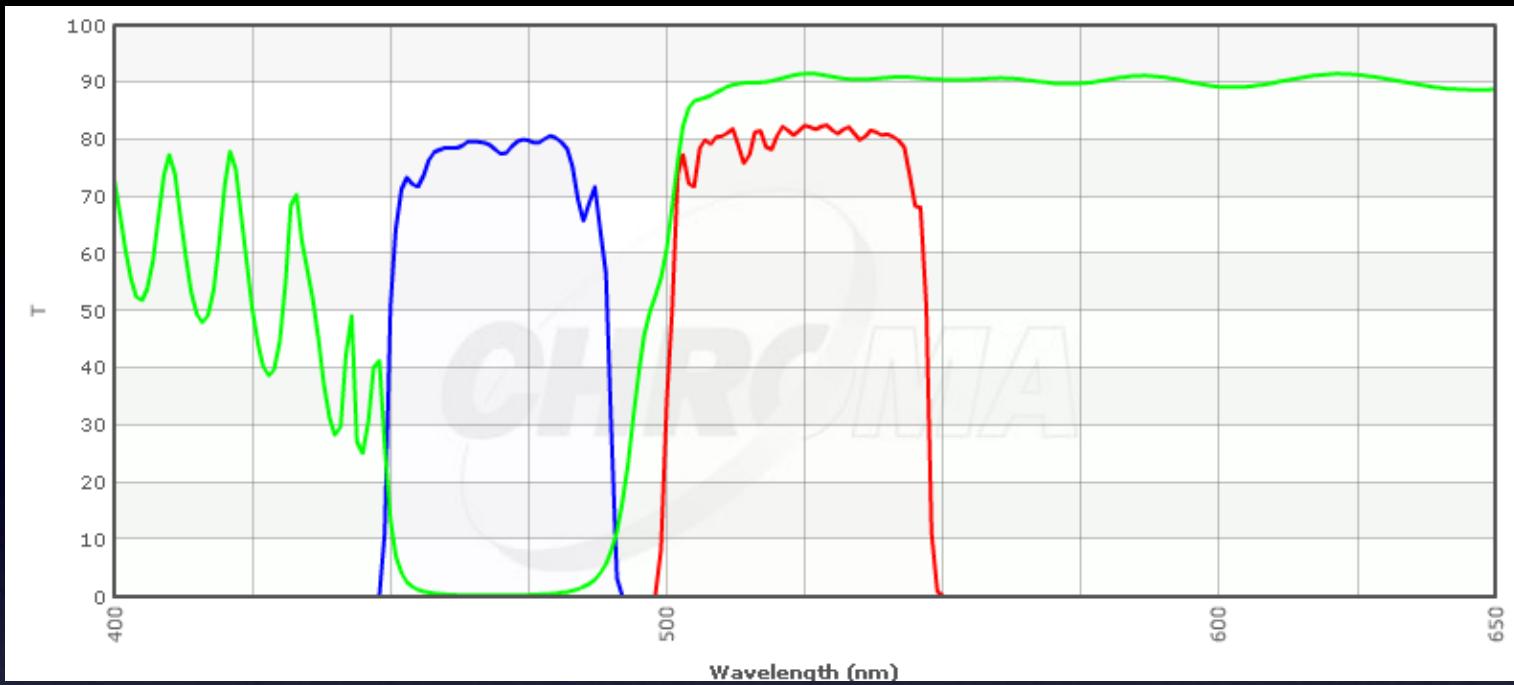
- Cheap
- Sturdy
- Independent of angle of incidence
- Small selection
- Spectra have poor slope and poor peak performance
- Autofluorescence
- Absorb    Get Hot



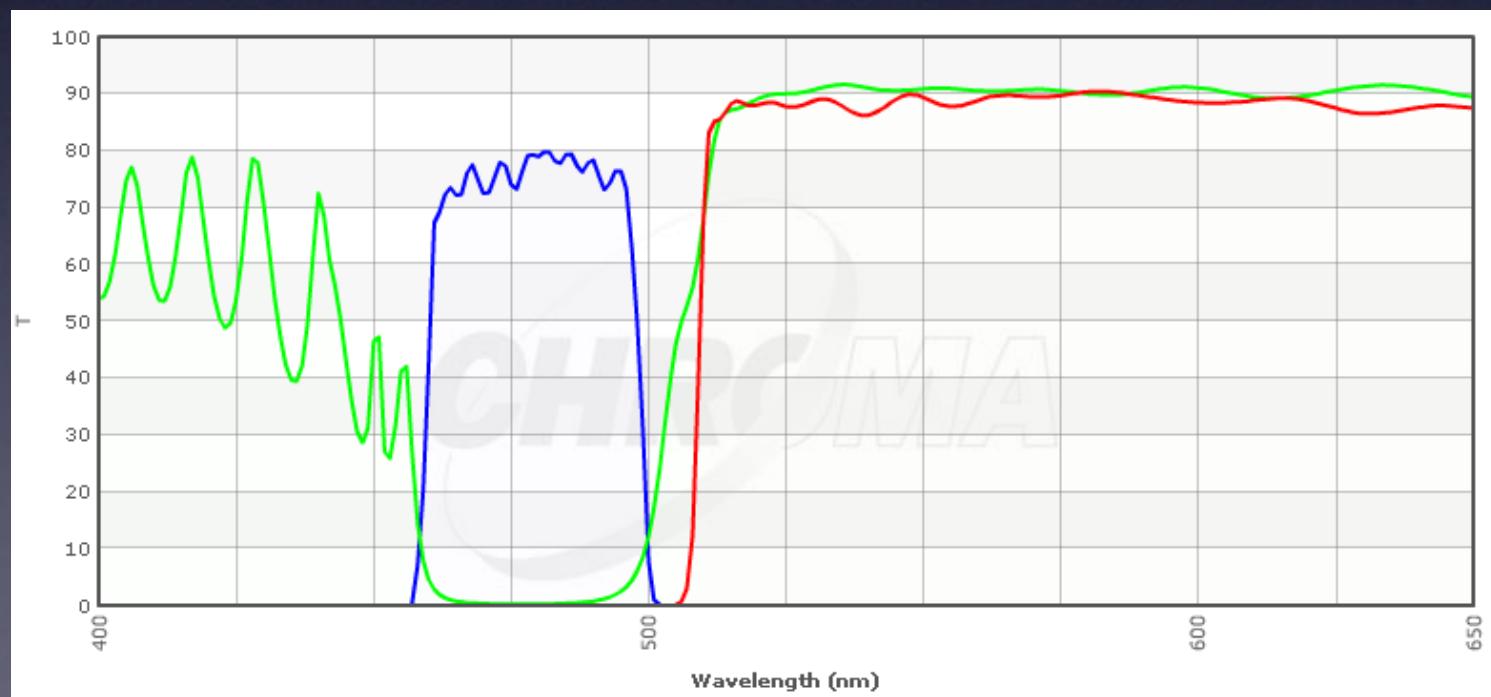
# Interference Filters



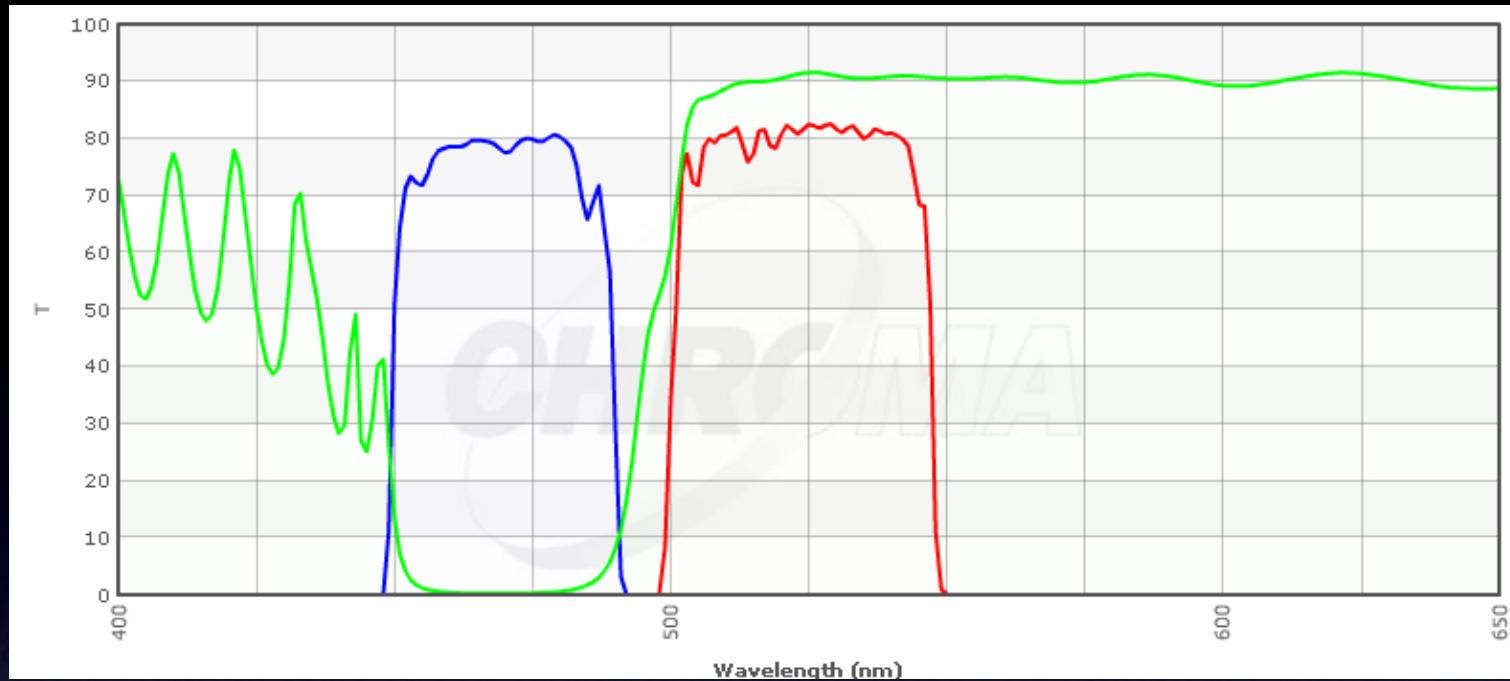
Bandpass



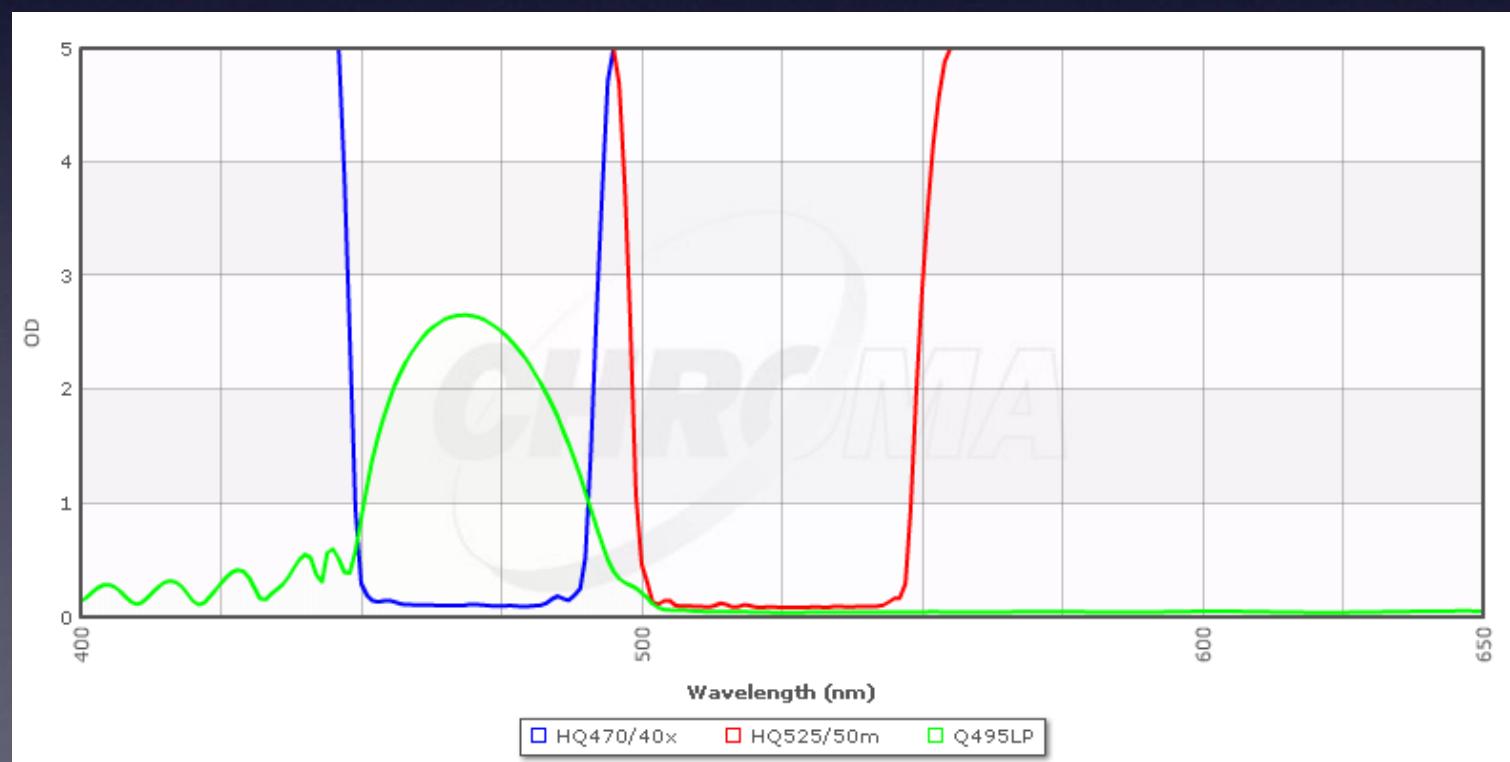
Longpass



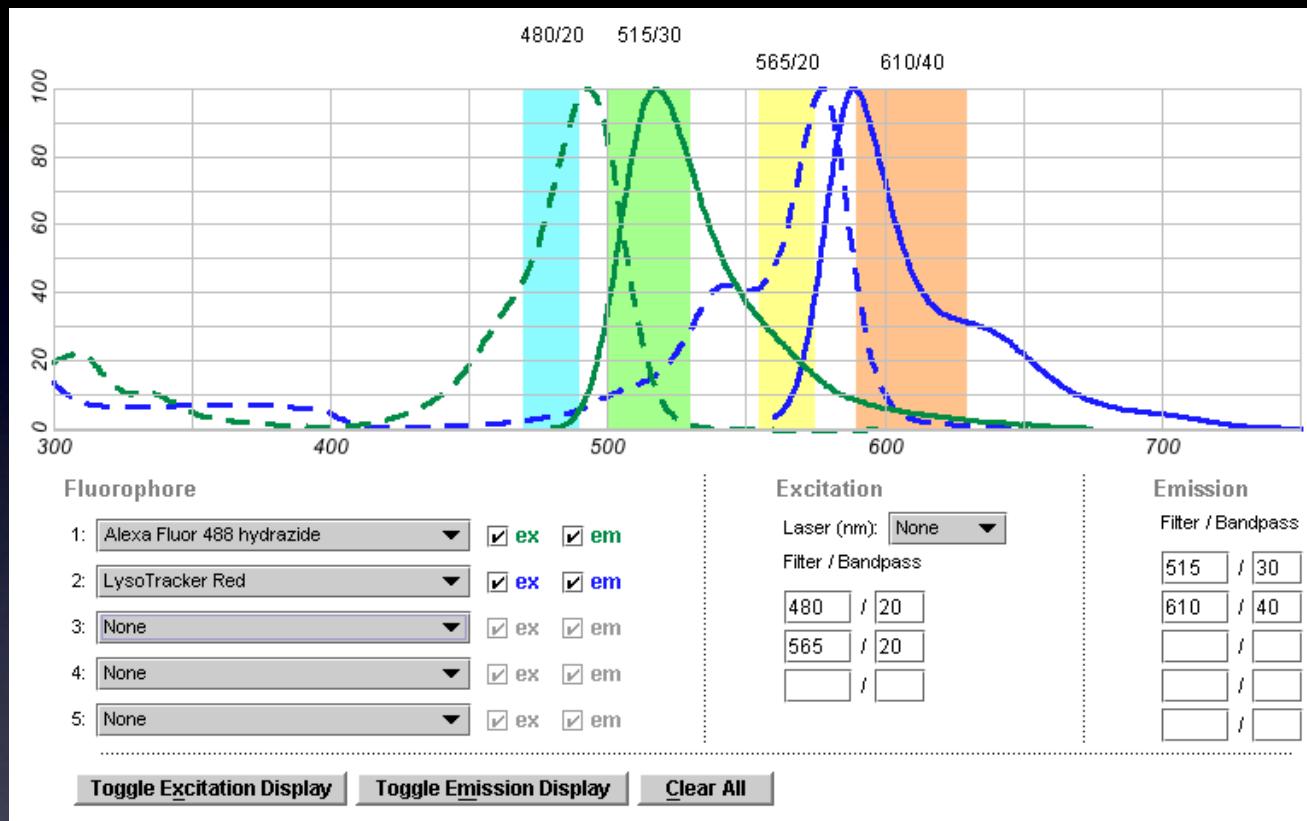
%T



OD



# Matching Filters and Fluorophores

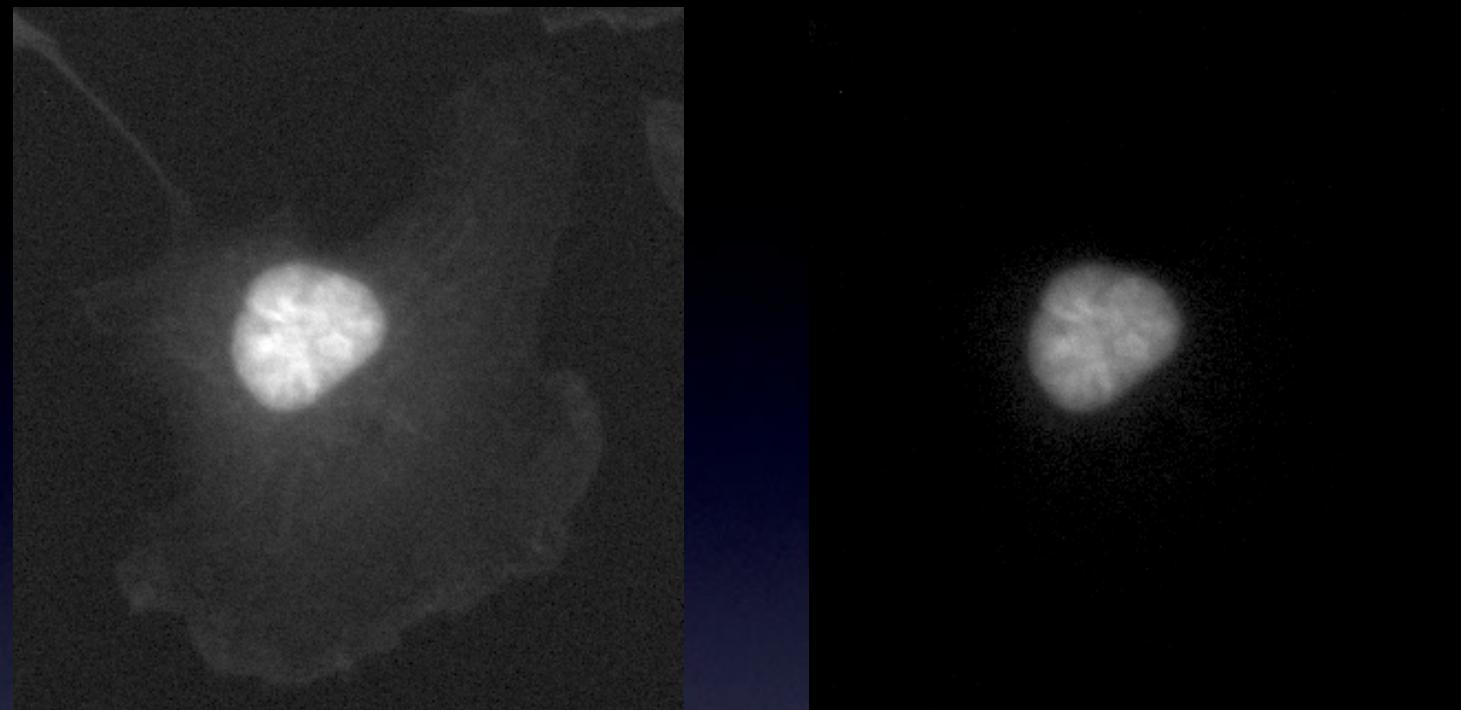


<http://probes.invitrogen.com/resources/spectraviewer/>

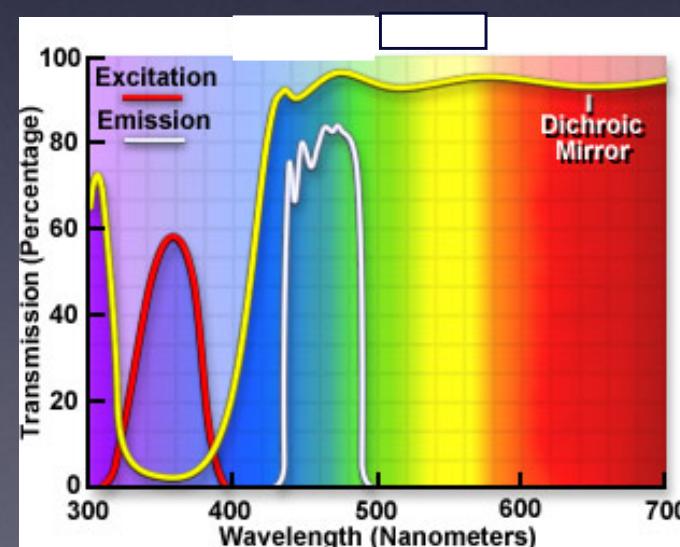
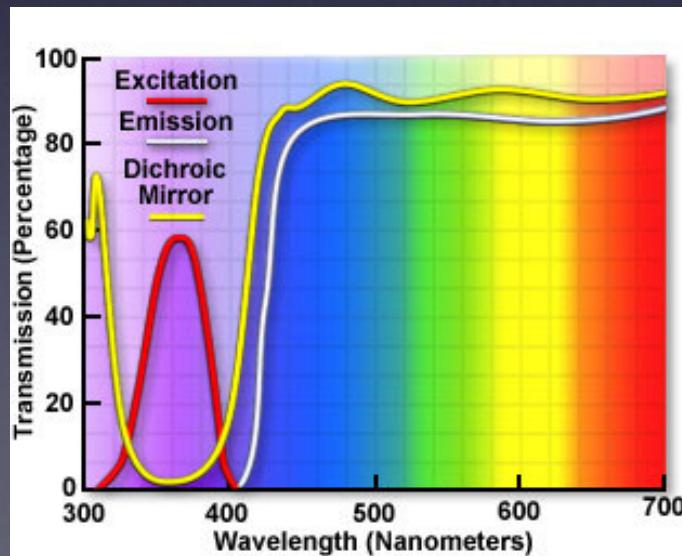
<http://fluorescence.nexus-solutions.net/frames6.htm>

<https://www.omegafilters.com/curvo2/index.php>

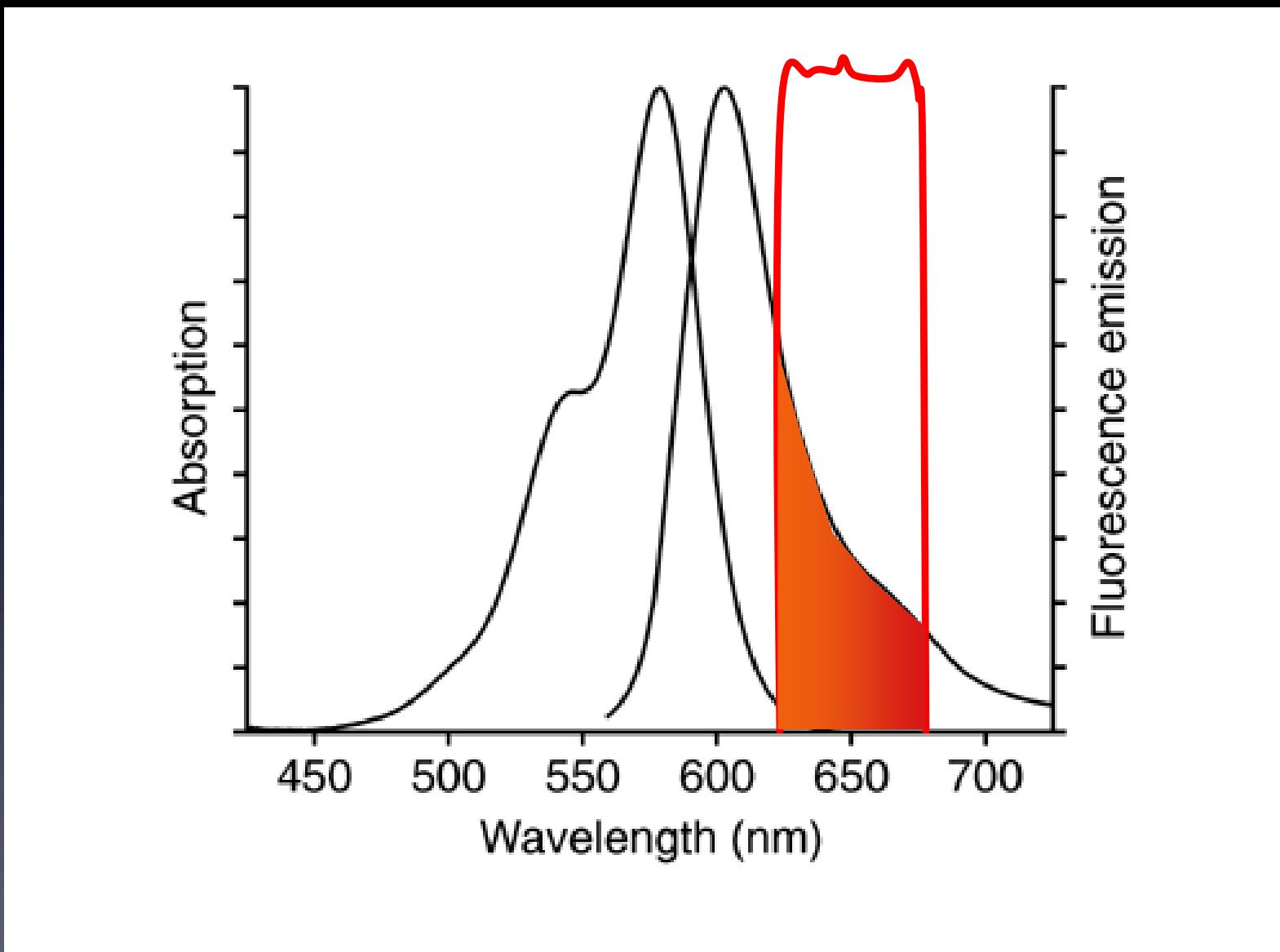
Choose filters that separate fluorophores



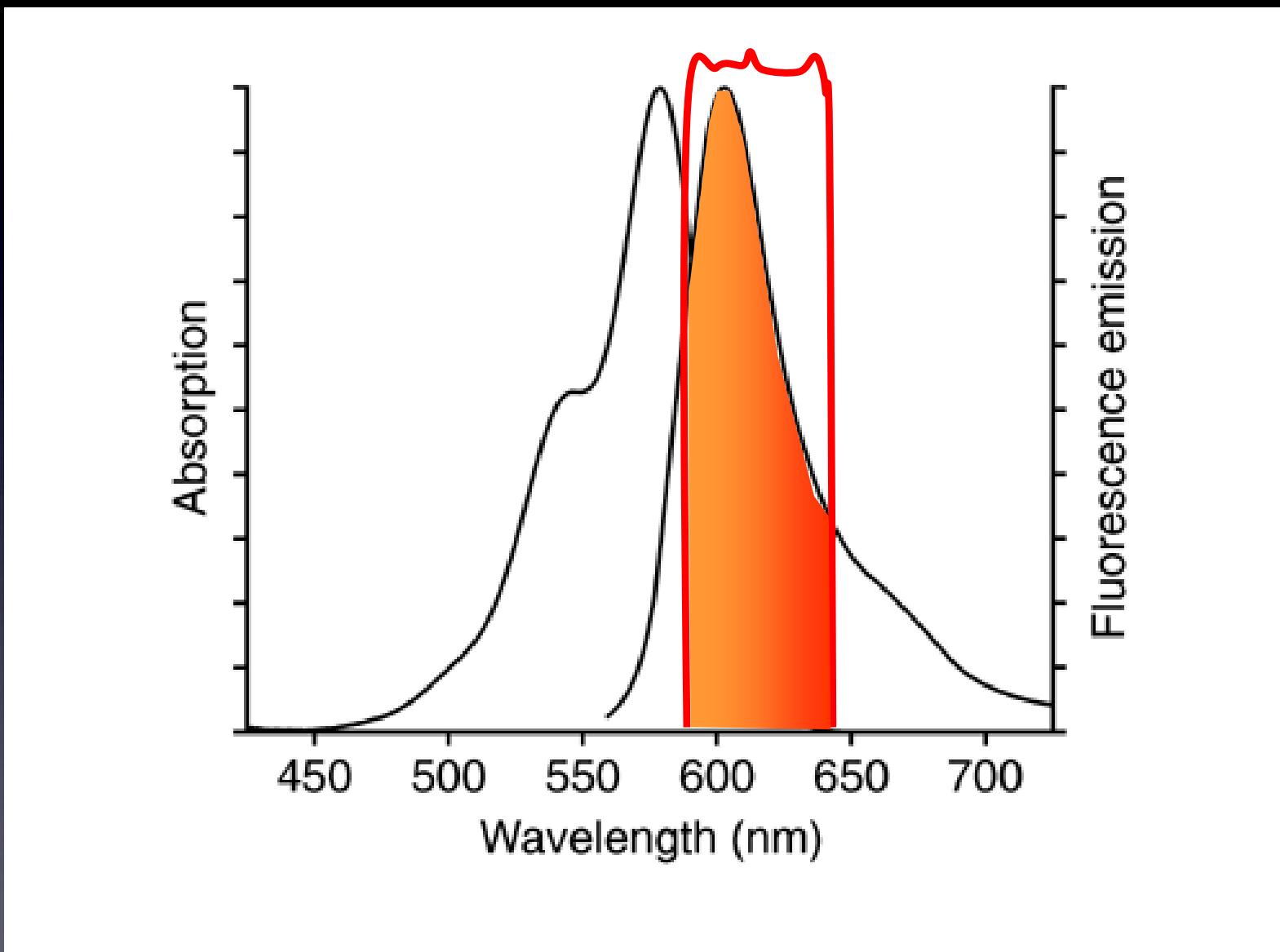
Two different UV filter sets



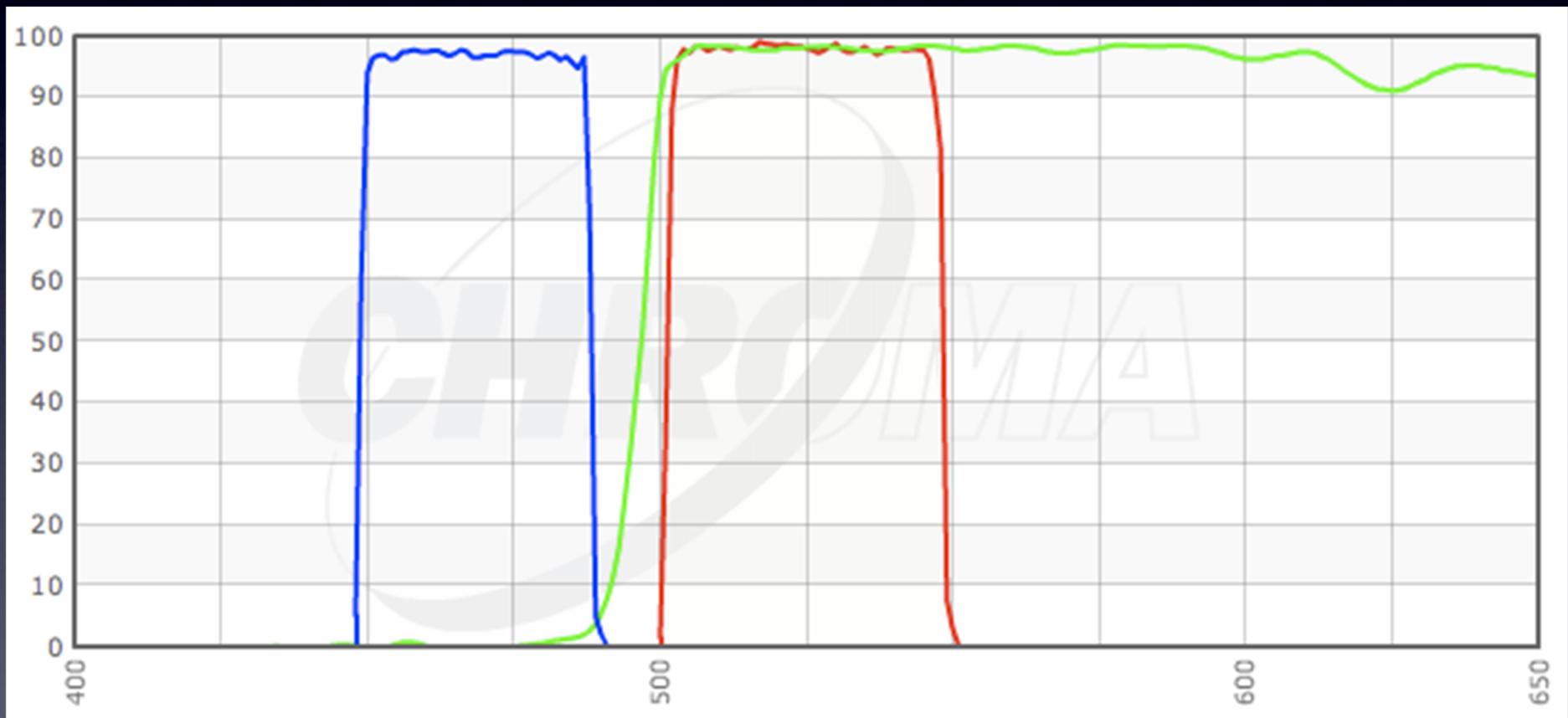
**Choose filters that maximize excitation and emission**



**Choose filters that maximize excitation and emission**



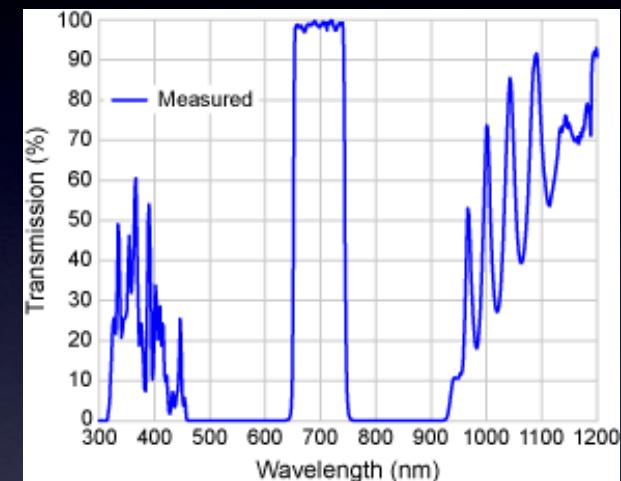
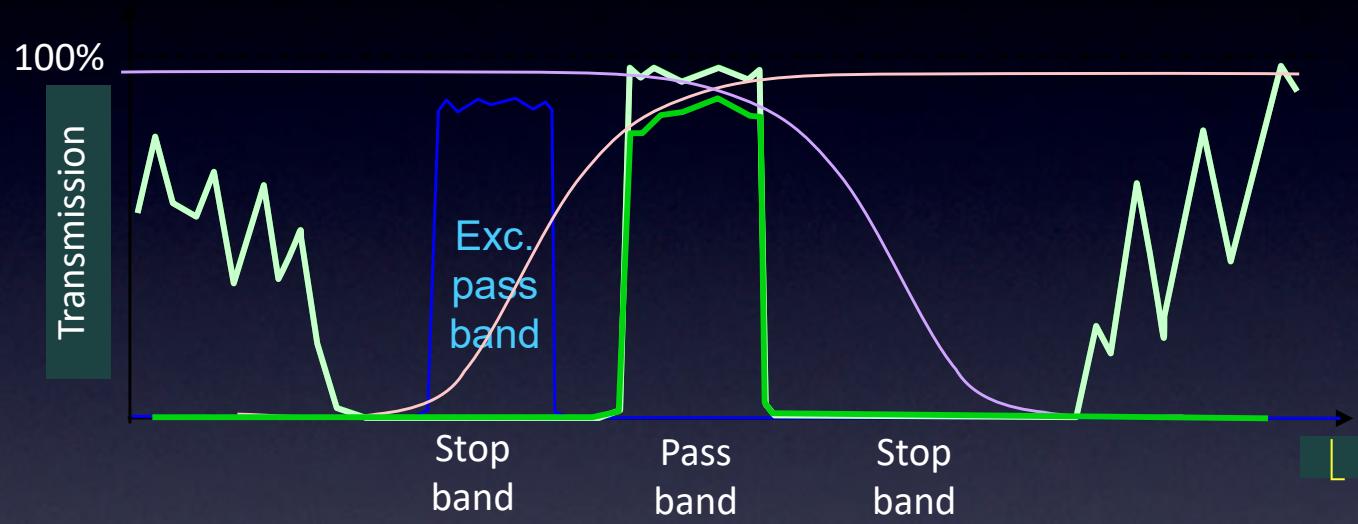
# Newer hard-coatings are great!



# Blocking

Interference filters have finite stop bands

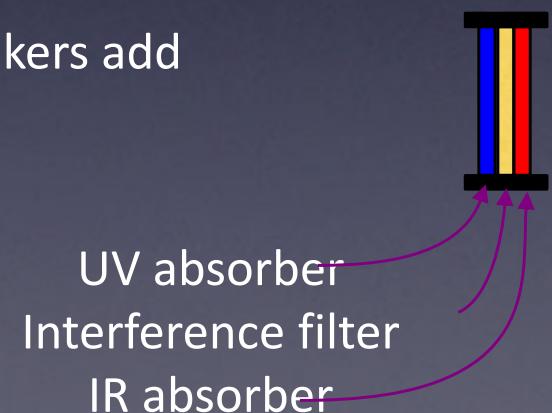
Unblocked bandpass interference filter



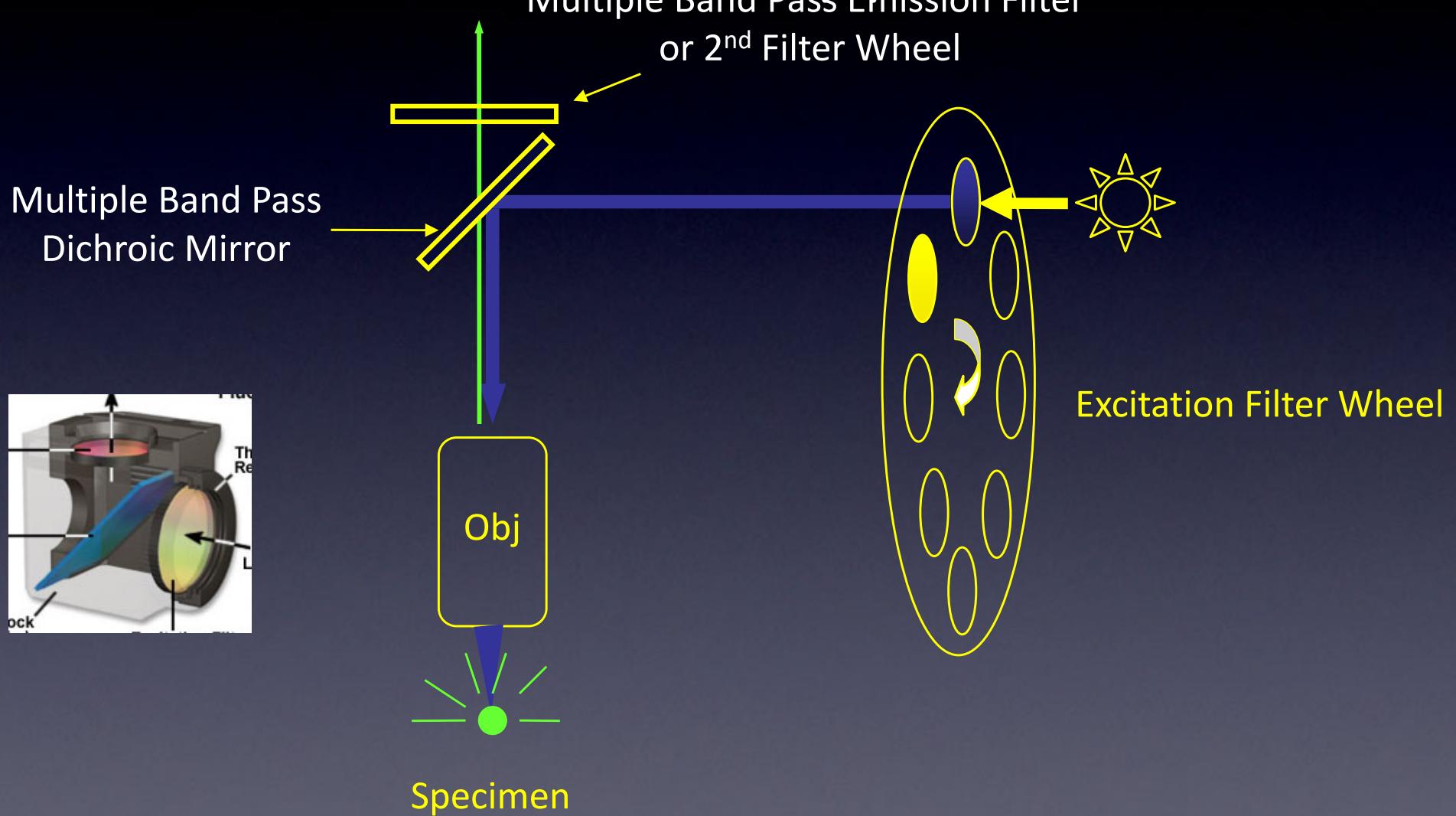
Semrock 697/75

To block unwanted transmission from UV to IR, filter makers add absorption glass to the filter.

Often excitation filters are blocked,  
but emission filters unblocked.  
→ Red autofluorescence or room light  
may get through your blue emission filter

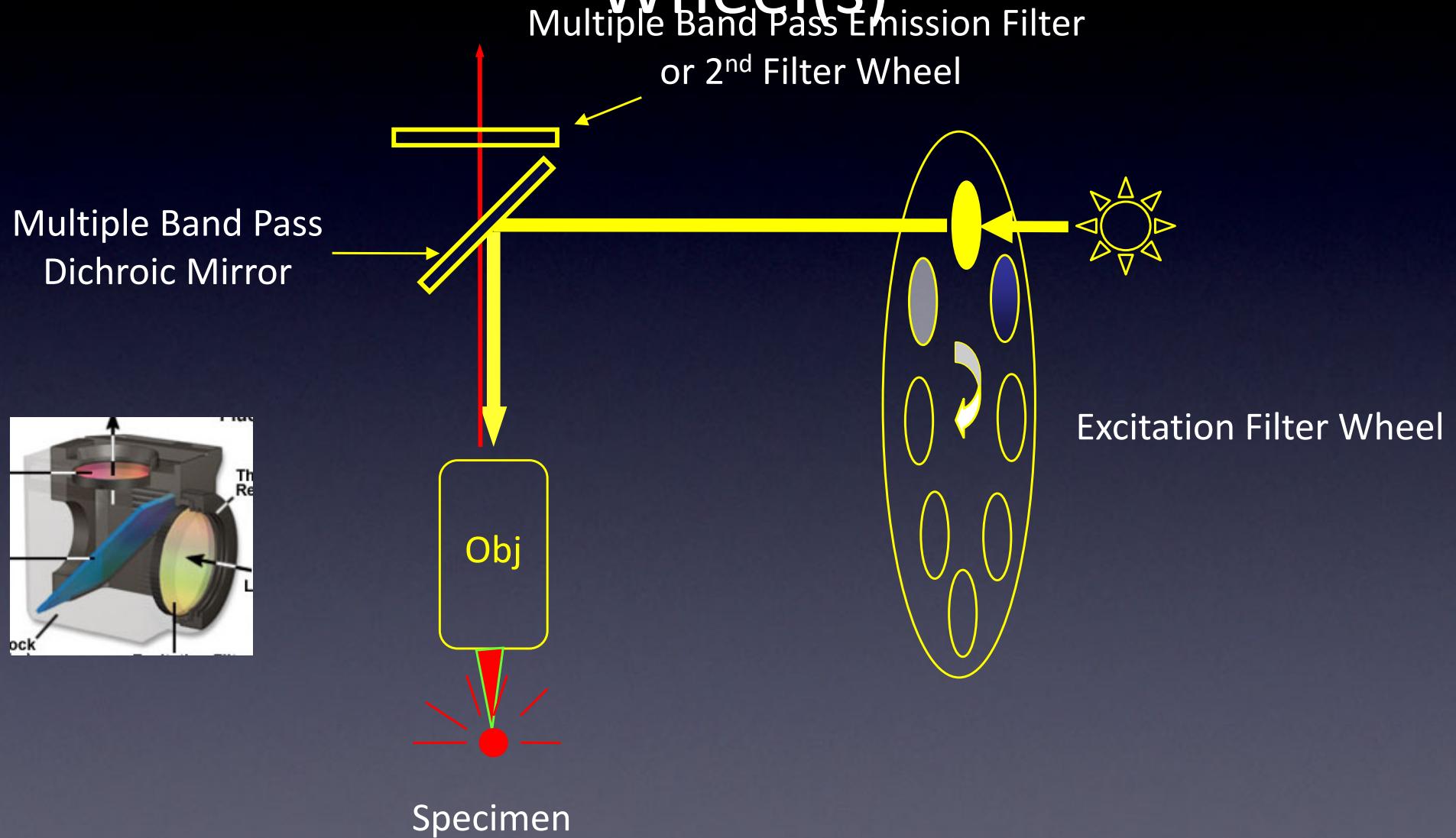


# Faster Wavelength Selection: Multiple Band Pass Filters & Filter Wheel(s)



# Faster Wavelength Selection: Multiple Band Pass Filters & Filter

## Wheel(s)



# Filter schemes

## Single wavelength sets

- Most efficient
- Best separation
- Very slow to change



Transmission



## Multi-band filters

- Multi-band everything
- See all colors at once
- For color cameras
- Bad crosstalk
- “Pinkel” scheme

Multi-band dichroic

Multi-band emitter

Single-exciters

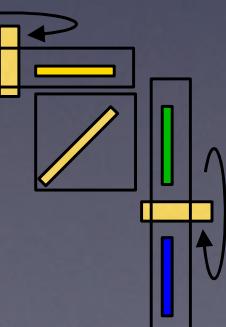
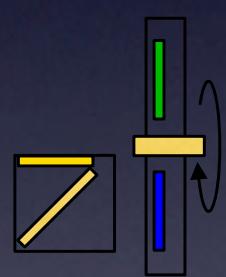
- Exciton filter wheel
- Separate image at each wavelength
- Better separation

“Sedat” scheme

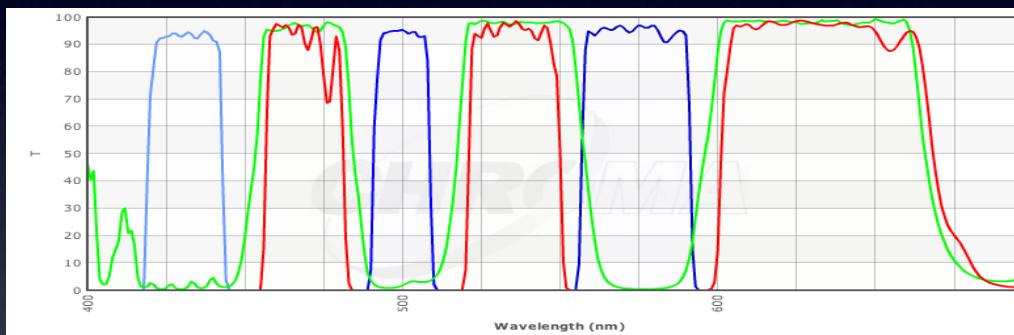
Multi-band dichroic  
single-band emitters

Single-exciters

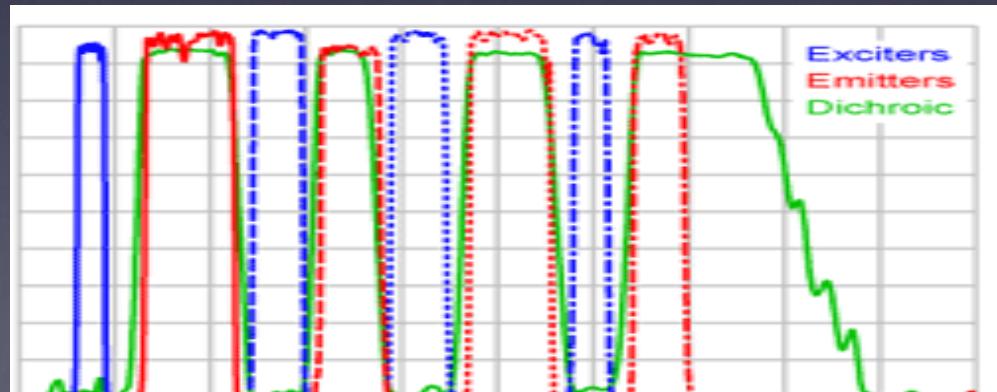
- Two filter wheels
- Even better separation



Wavelength

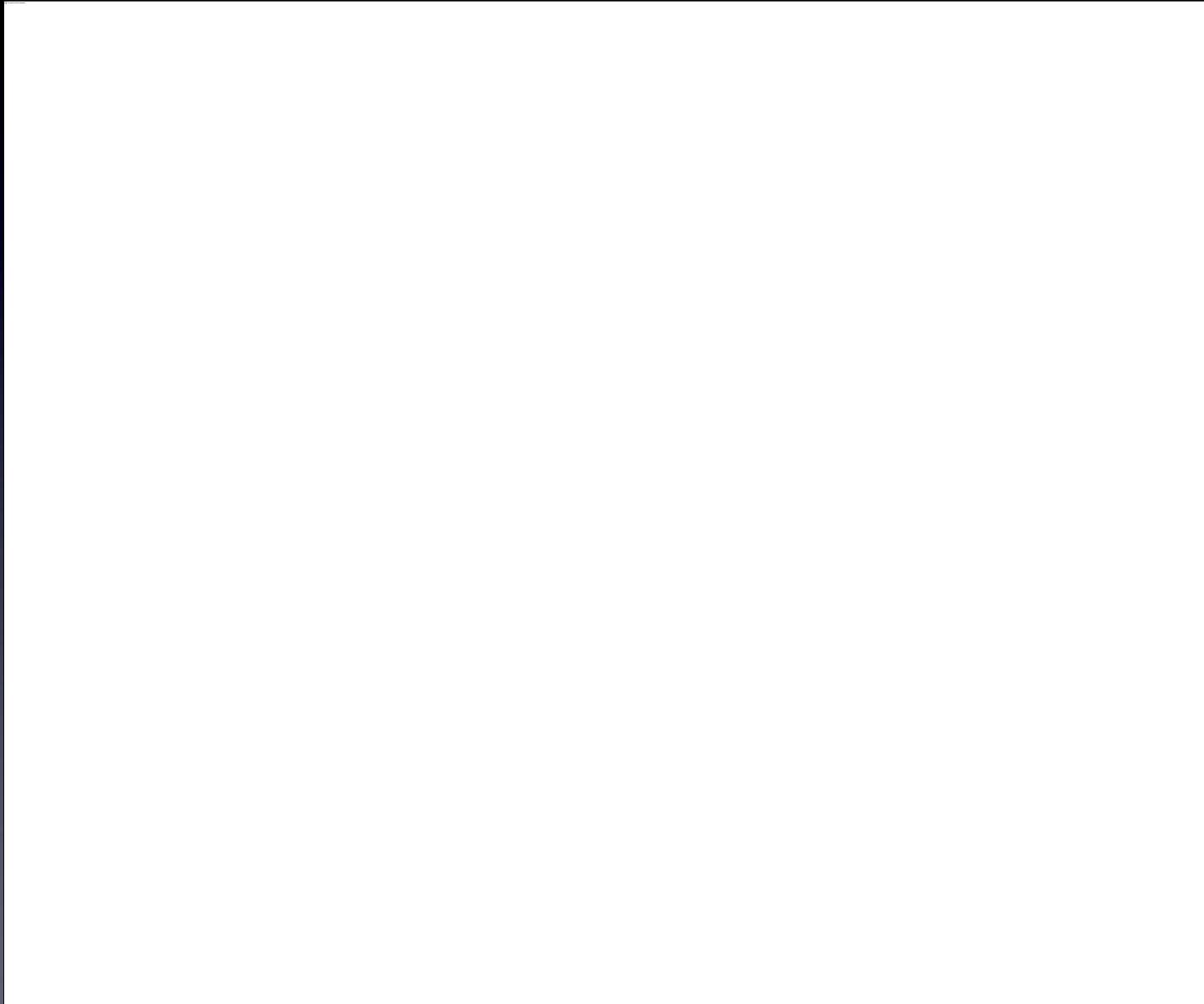


Chroma triple Pinkel set



Semrock quad Sedat set

# Koehler illumination



# Acknowledgements and Resources

- Kurt Thorn
- Bo Huang
- Mats Gustafsson
- Jennifer Waters

Lakowicz - Principles of Fluorescence Spectroscopy

Goldman et al. - Live Cell Imaging: A Laboratory Manual

Day and Davidson, Chem Soc Rev, 2009(38) 2887

<http://www.microscopyu.com>

<http://www.chroma.com> (Filter Handbook!)