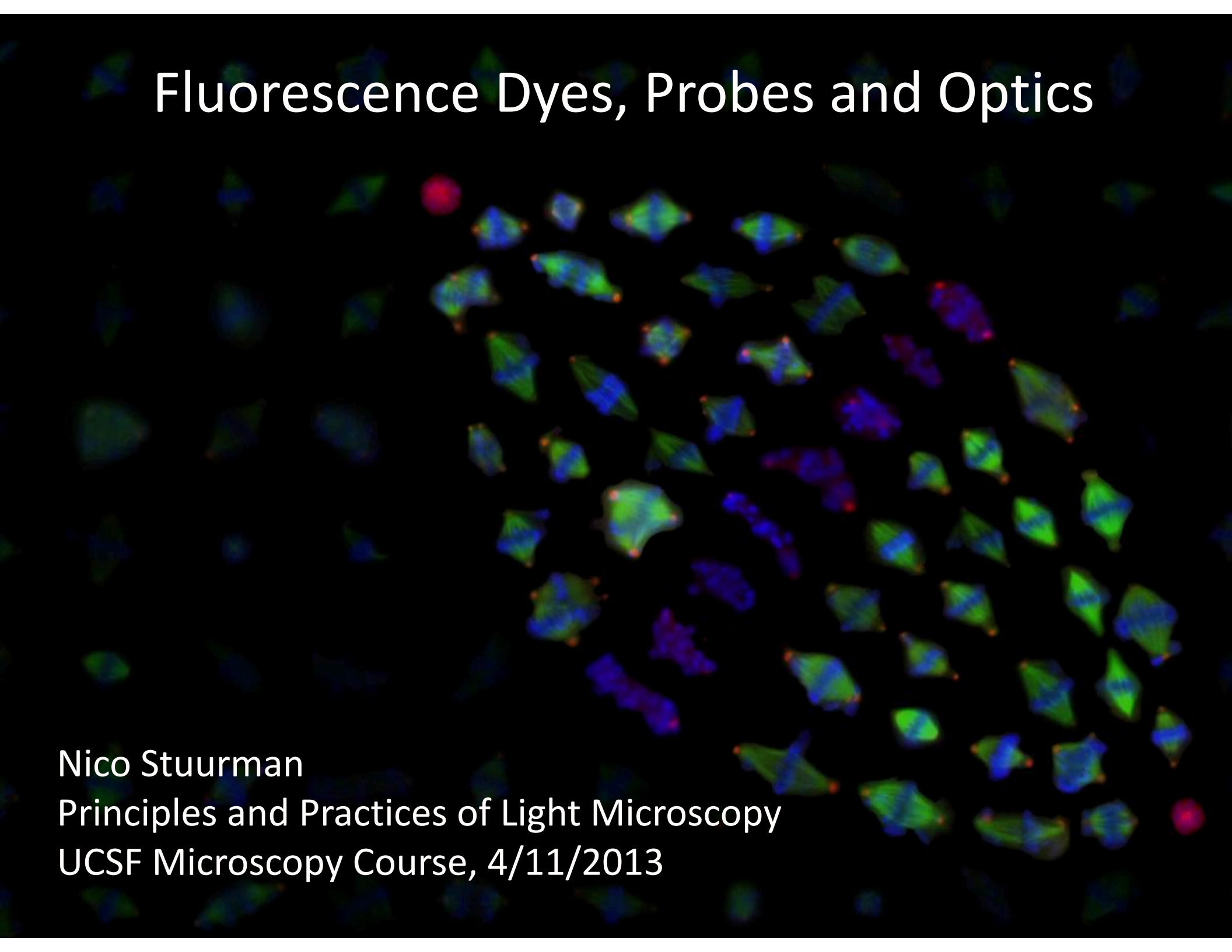
Fluorescence Dyes, Probes and Optics



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

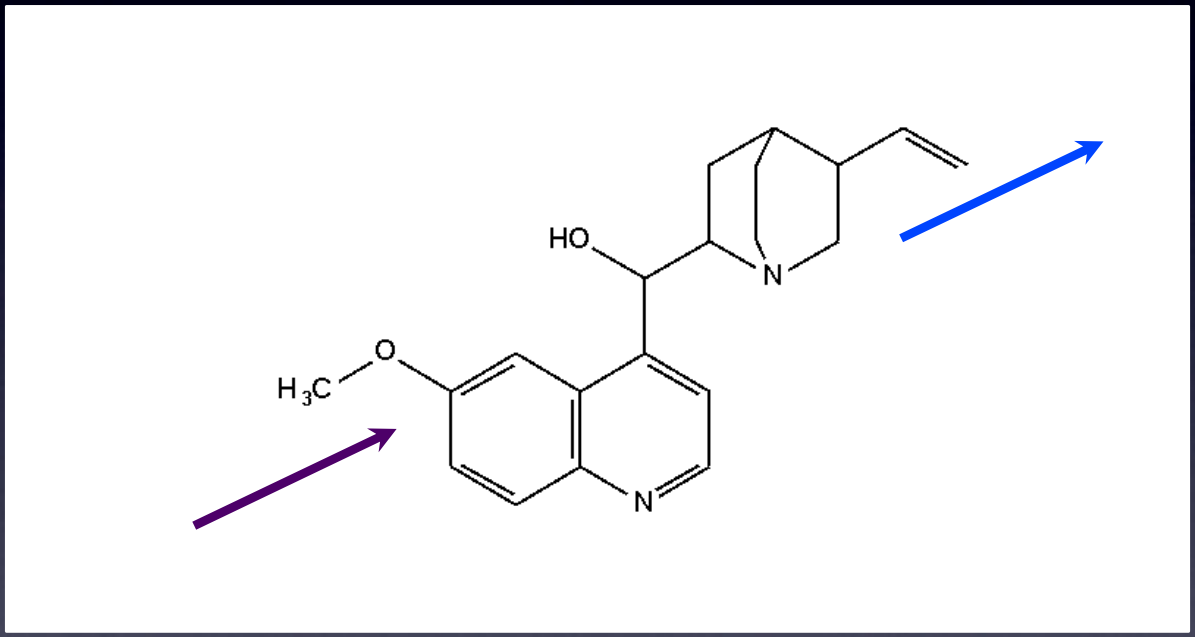
Why Fluorescence?

- 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…..

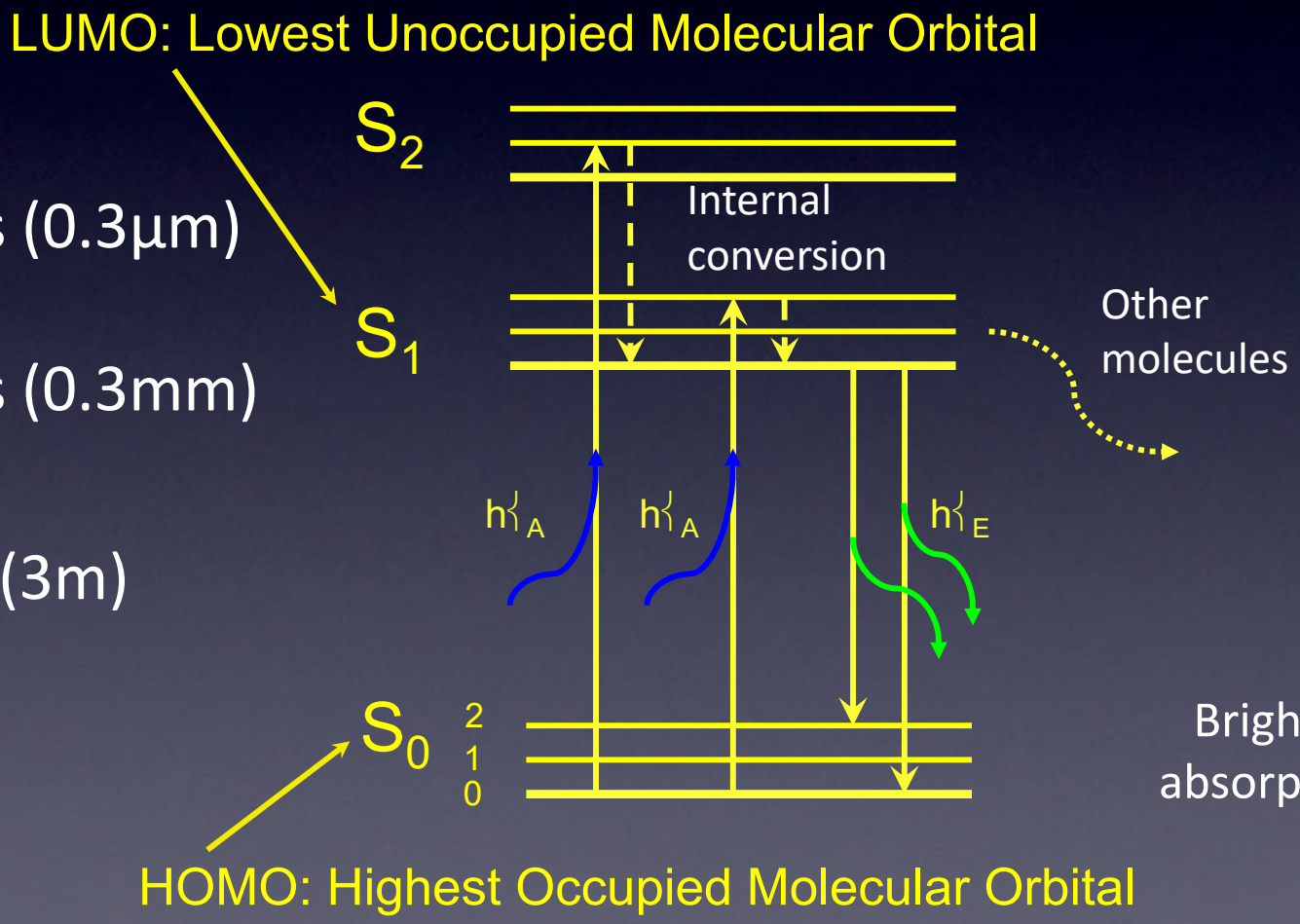
Excitation/Emission



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

Fluorescence

Jablonski diagram



10^{-15}s (0.3μm)

10^{-12}s (0.3mm)

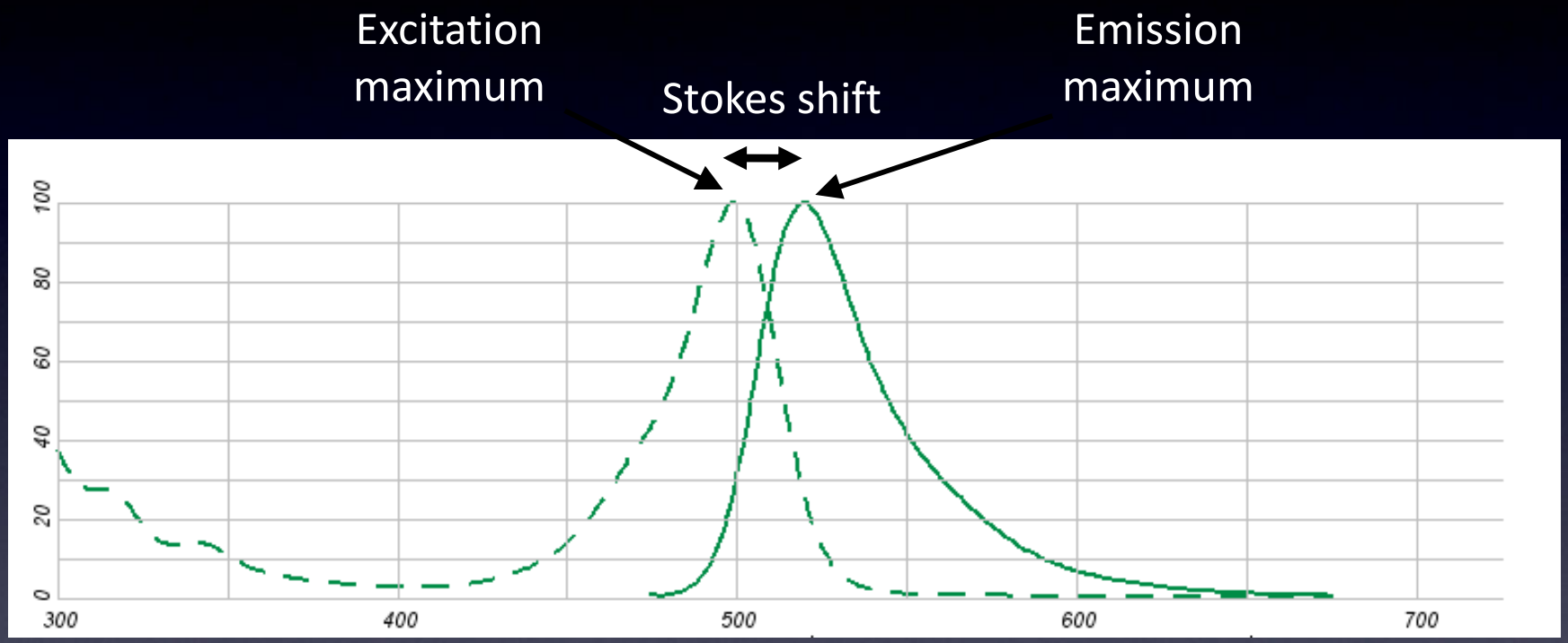
10^{-8}s (3m)

QE: ratio of absorbed and emitted photons

Relation between lifetime and QE

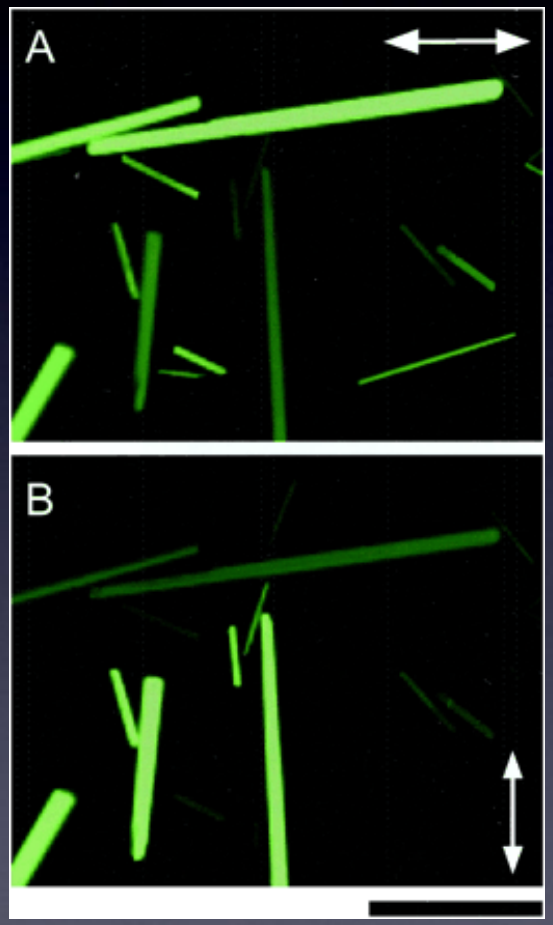
Brightness: determined by absorption coefficient and QE

Fluorescence Spectra



Alexa 488

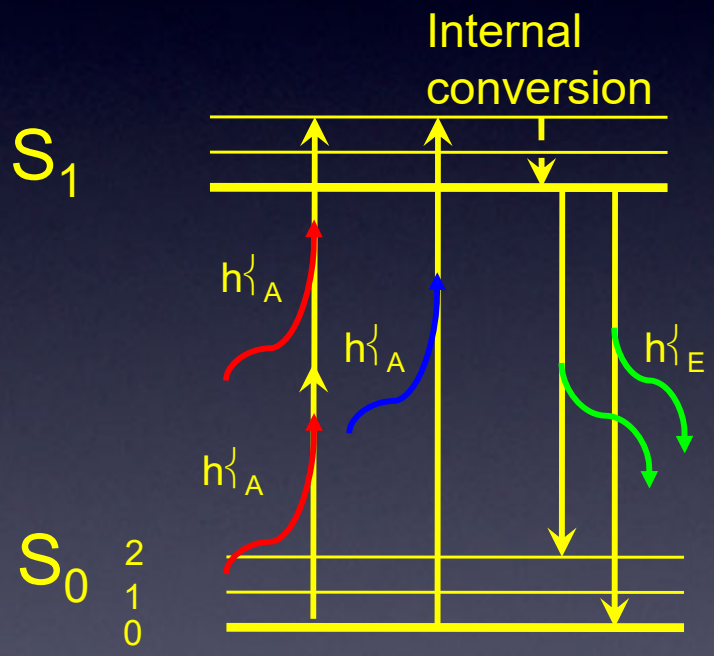
Polarization



Native GFP crystals

Shinya Inoué

Multi-photon excitation



Brad Amos, MRC, Cambridge

Fluorescent Dye Types

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

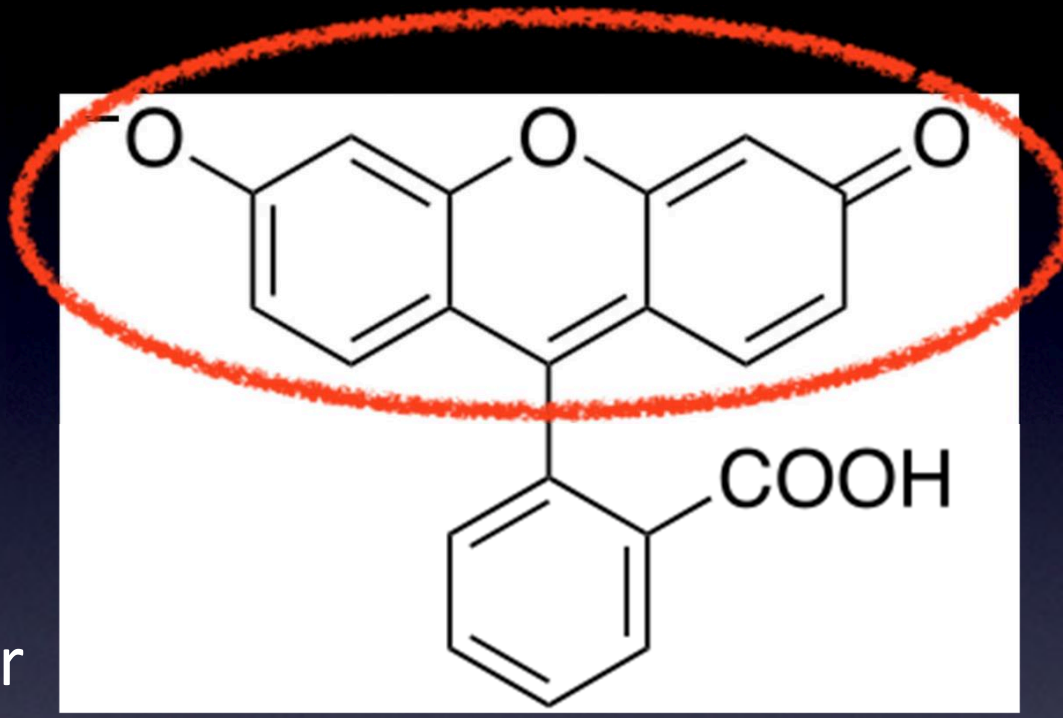
First synthesized fluorescent dye: Fluorescein

Extended conjugate structure

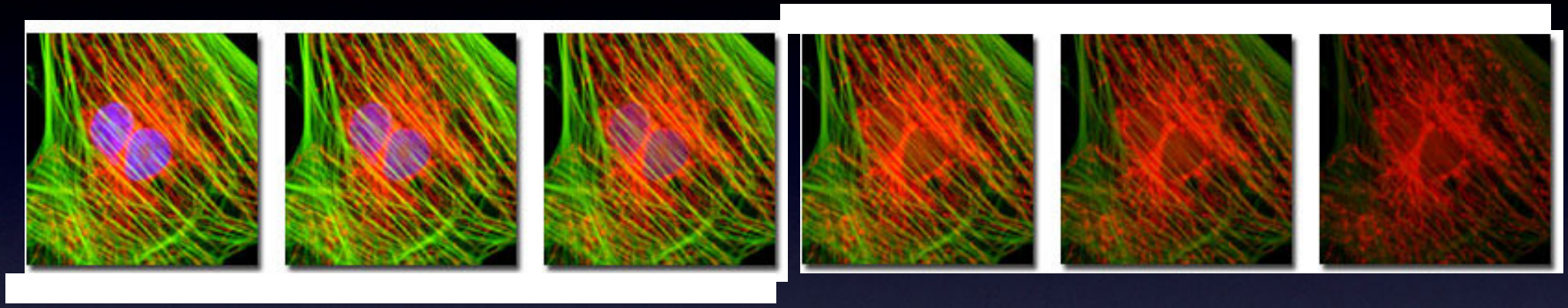
Extended orbital > small energy difference HOMO/LUMO

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

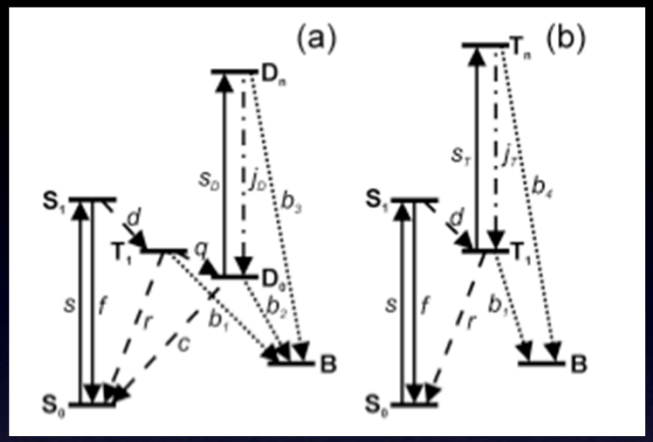


Decrease in emission intensity after exposure

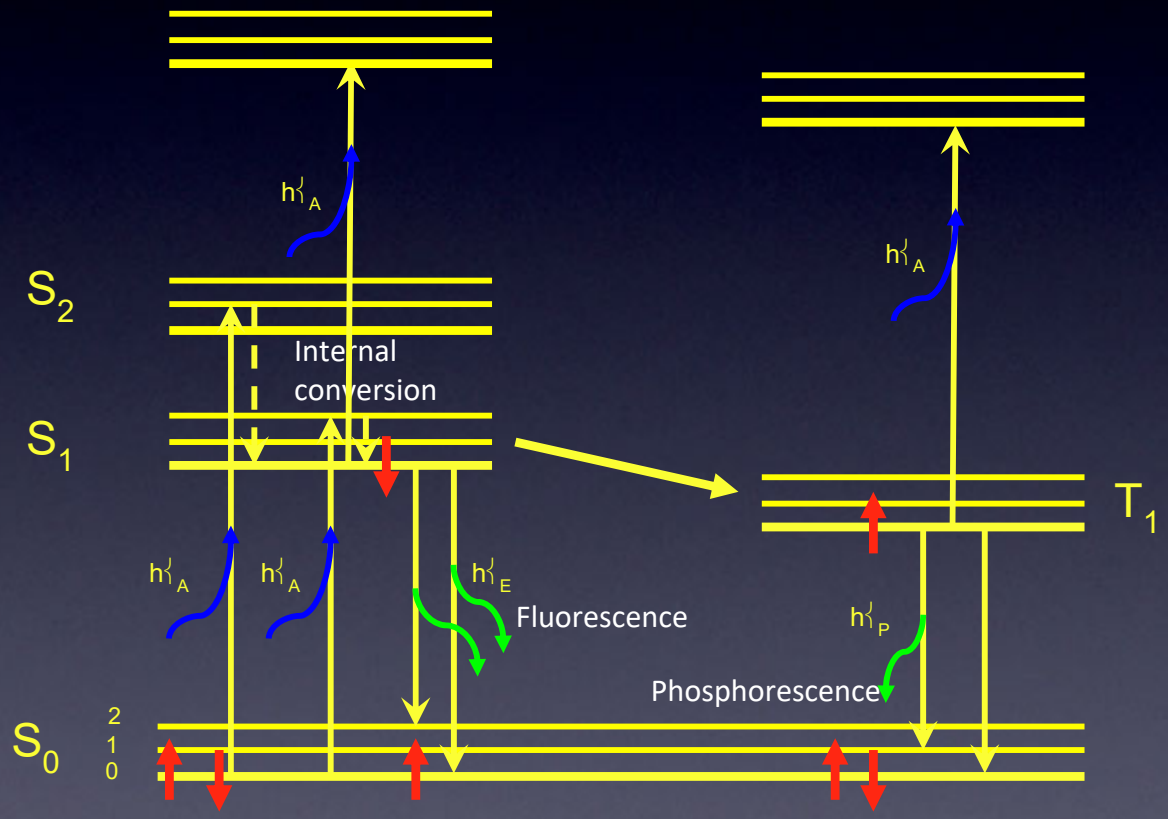
Exciting a molecule once has a probability Q_b of killing it

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

332/456
QY 0.77

Fluorescein

490/520
QY 0.925

Rhodamine

554/573
QY 0.28

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

Organic Dyes

Cyanine dyes

554/568
QY 0.14

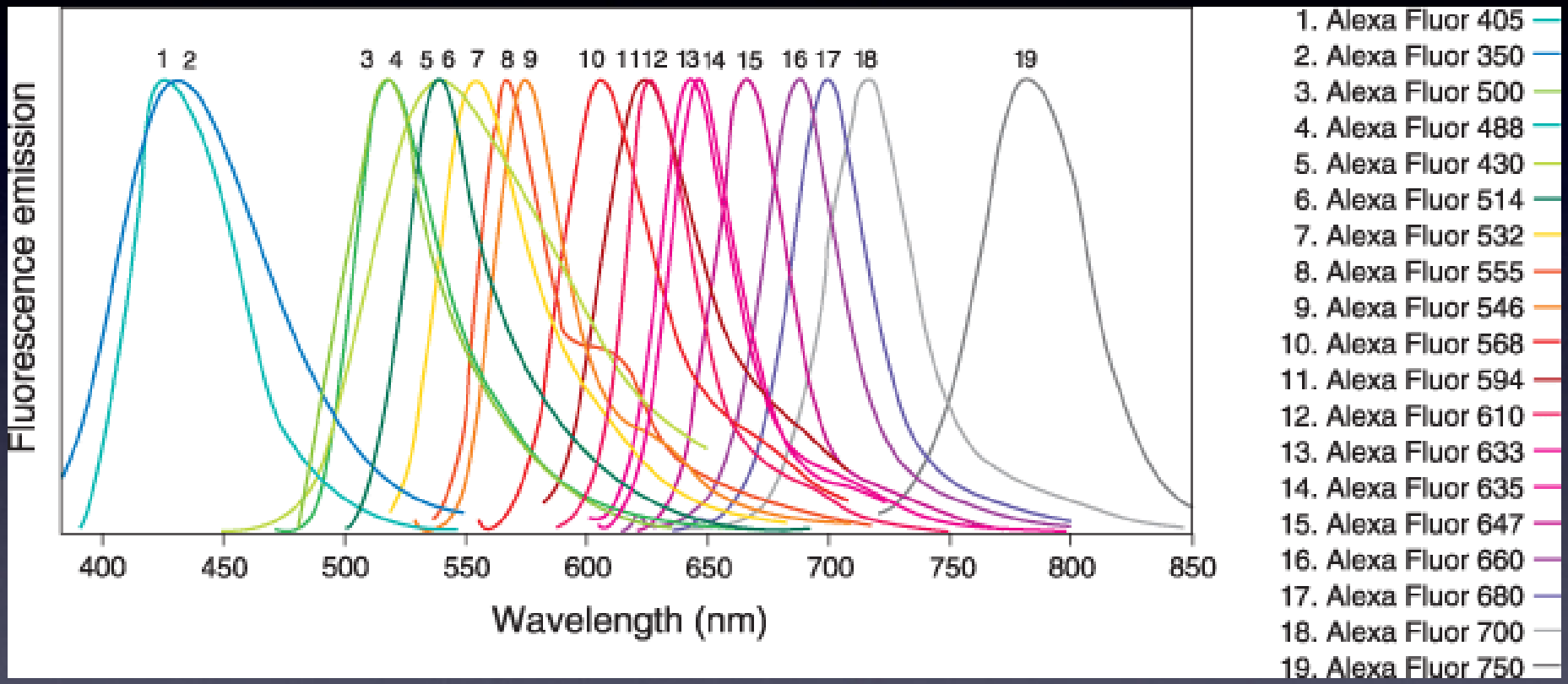
652/672
QY 0.18

Also, Cy2, Cy5.5

Alexa dye series

499/517
QY 0.60

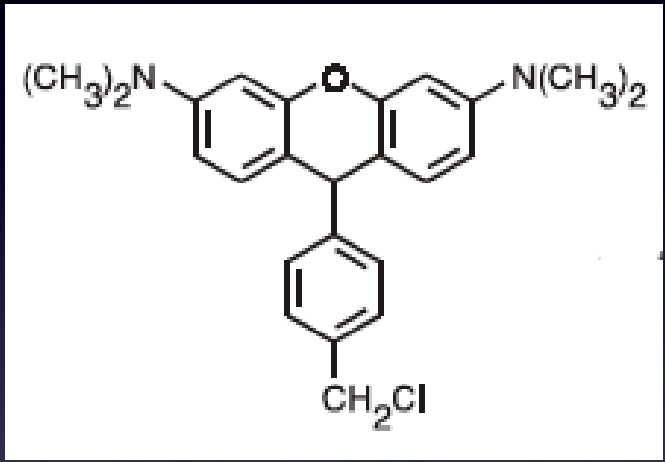
The Alexa Series Emission Spectra



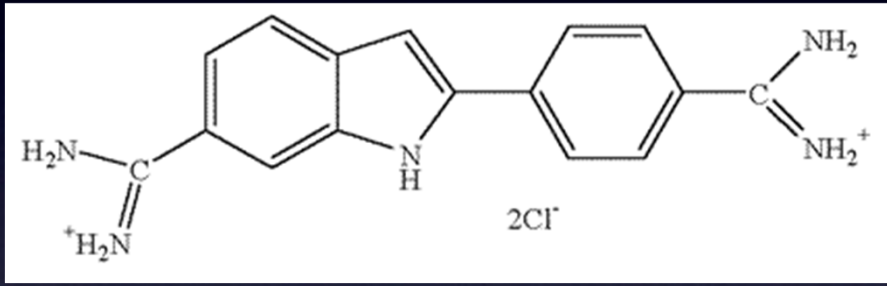
Coumarins Rhodamines Cyanines

Molecular Probes (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

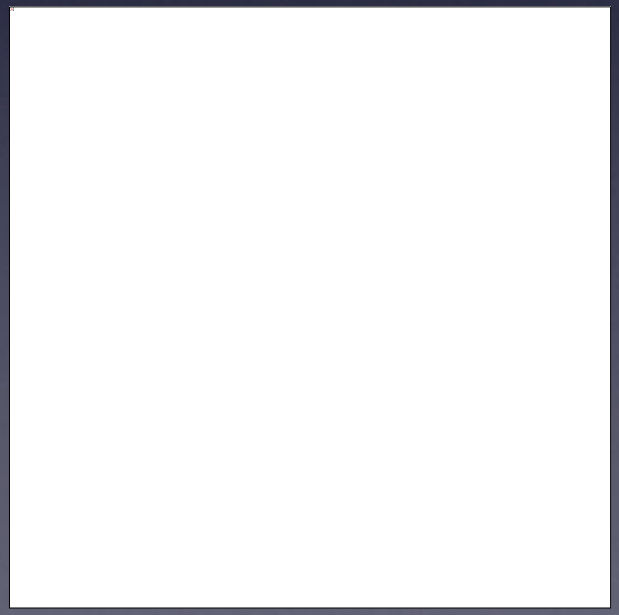
Conjugation of organic dyes

Chemistry/Method

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

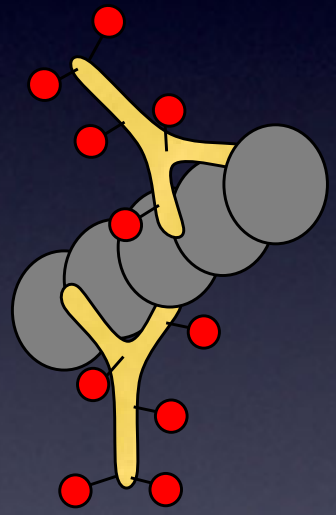
- Small molecules, i.e. phalloidin, taxol
- Proteins: labeling site unspecific
- Antibodies: direct/indirect labeling (Label density)

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

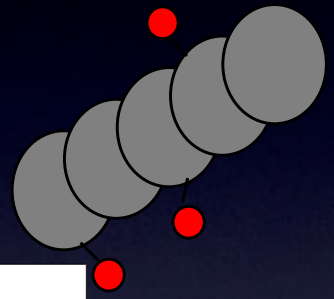


Fluorescent labeling

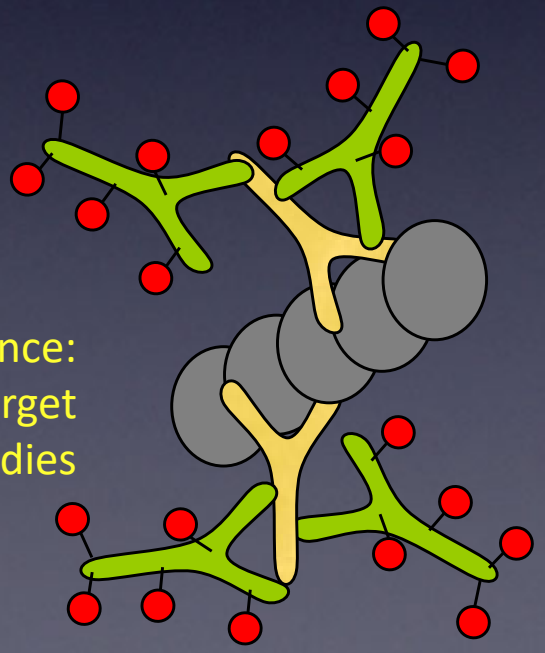
Direct immunofluorescence:
labeled antibodies against target



Direct labeling (& microinjection)
of target molecules



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

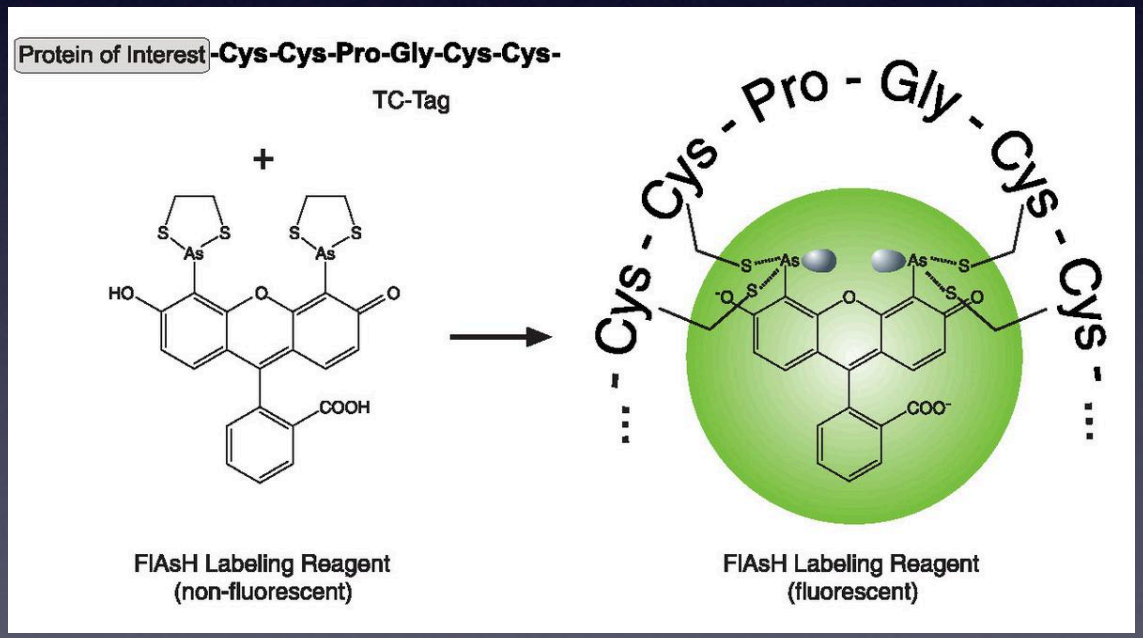


Site-Specific Labeling

Chemistry/Method

Sulfhydryl groups (cystein): maleimide

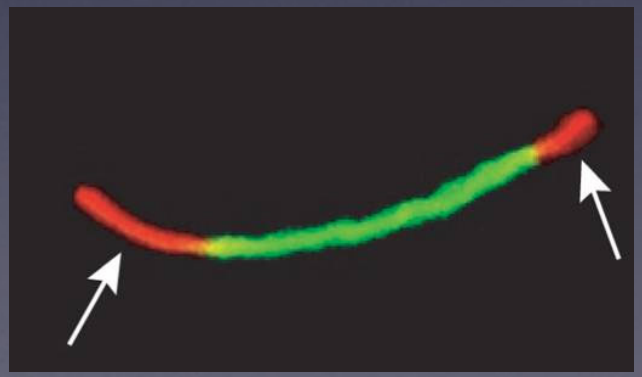
Engineer Cys-light version of target protein



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

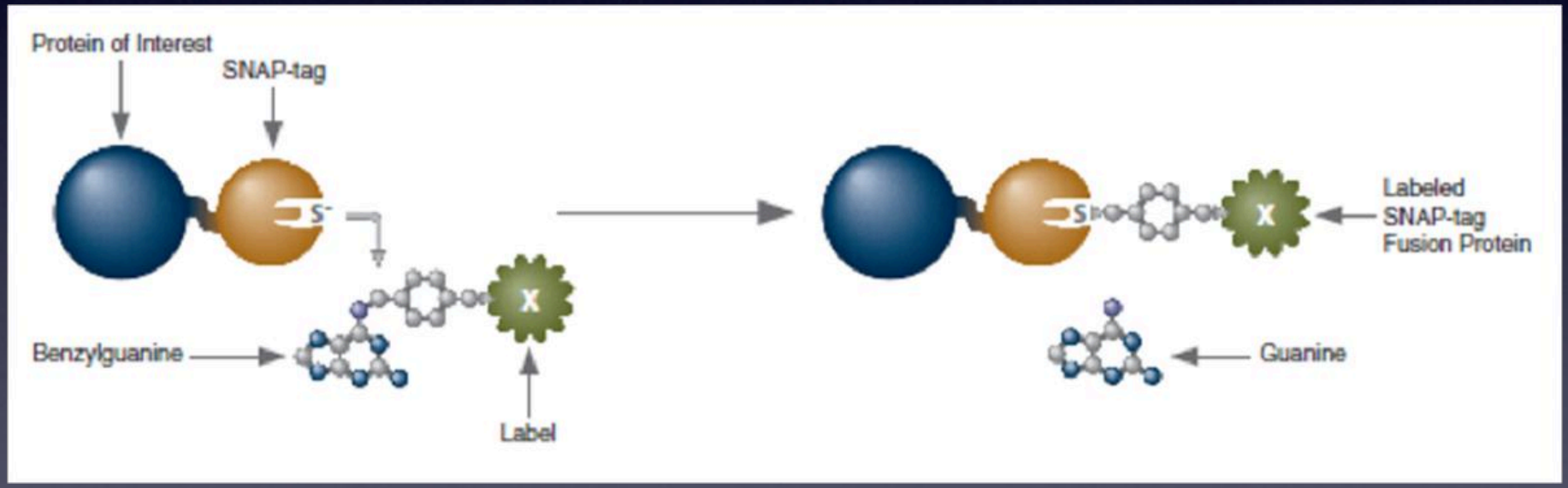
FlAsH/ReAsH

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

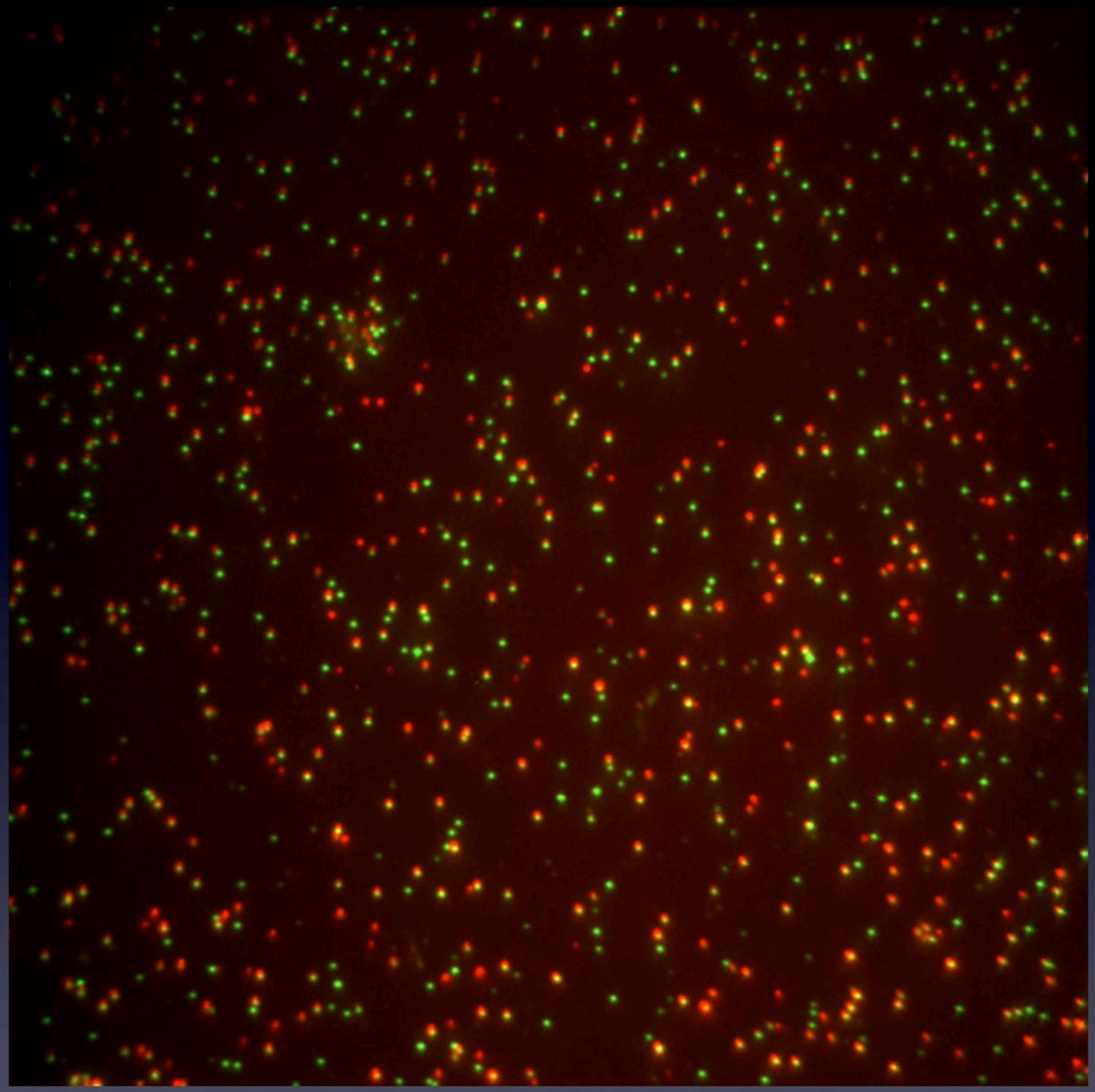


Site-Specific Labeling

SNAP tag
Benzylguanosine reacts with modified DNA repair enzyme (O6-alkylguanine-DNA alkyltransferase (AGT)



Similar strategy: Halo tag, CLIP tag, more?



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

Quantum-dots

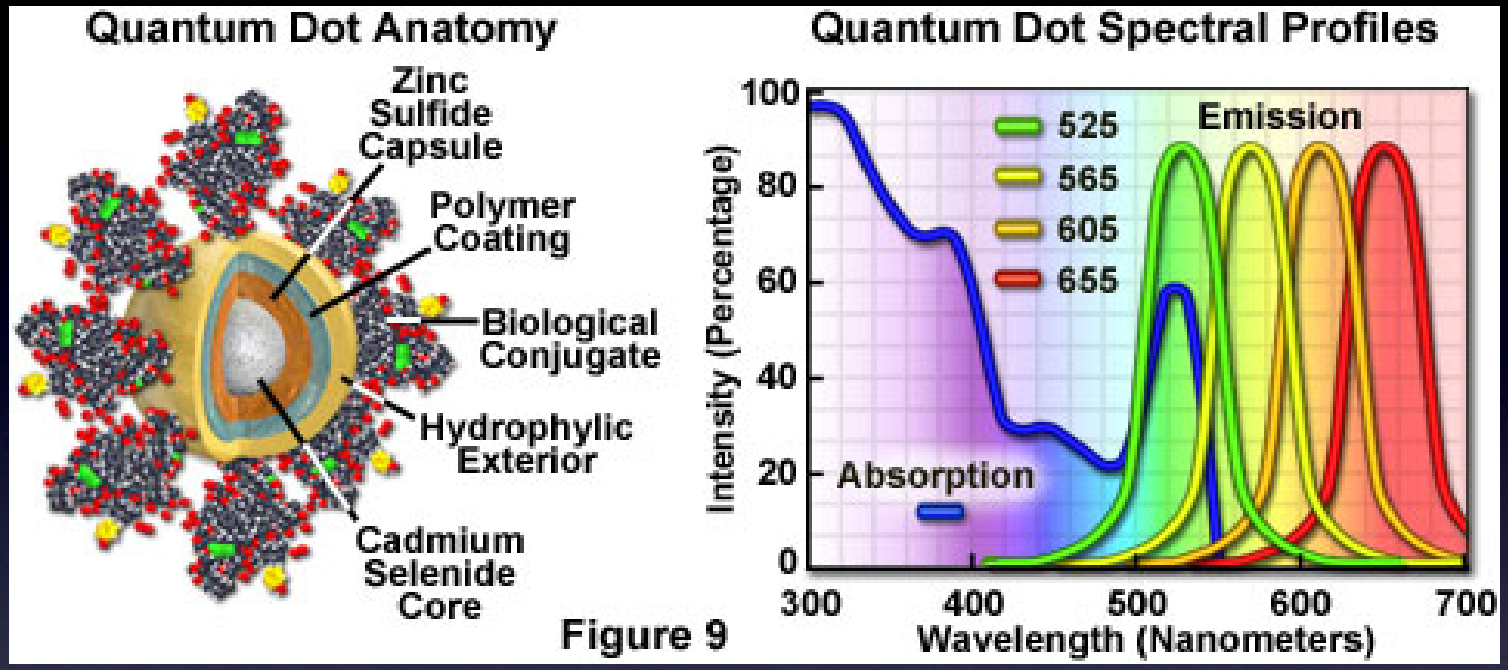


Figure 9

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

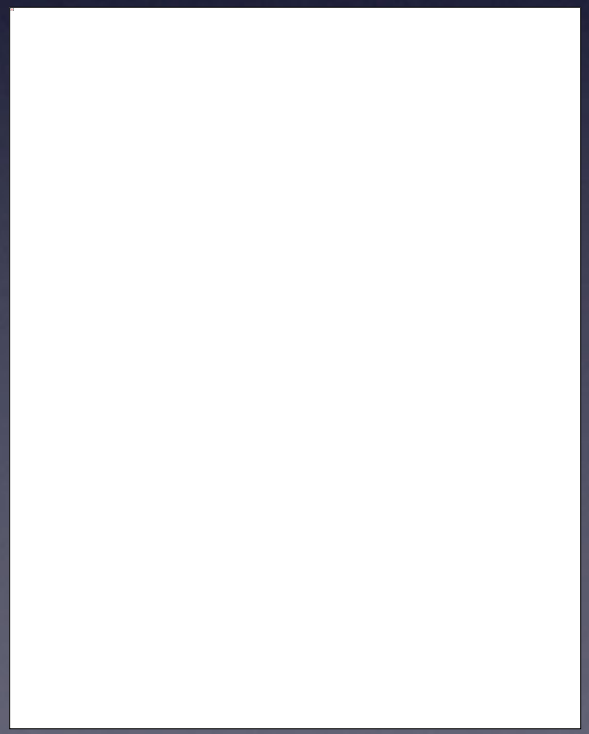
Disadvantages
- Large size
- Multivalent linkage

Quantum dot labeling

- Biotin/Streptavidin Linkage
- Biotin maleimide (in vitro)
- Biotin HaloTag/SNAP
- Biotin carrier protein
- BiotinLigase/AP1

Mono-valent QDots:
Justin Farlow
Zev Gartner Lab

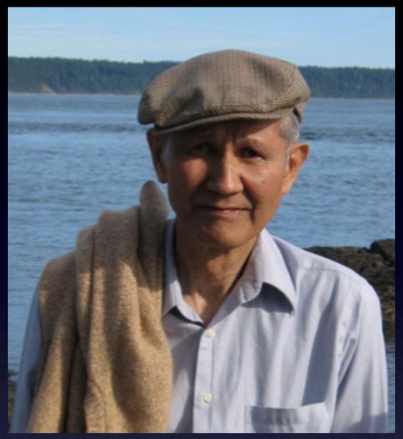
- Antibody Conjugates – immunohistochemistry
- Direct linkage to proteins/peptides – targeting to cell compartments

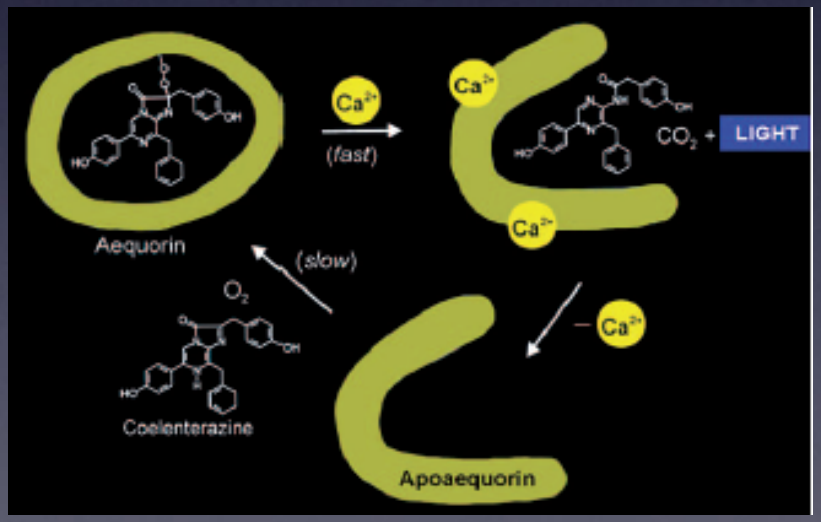


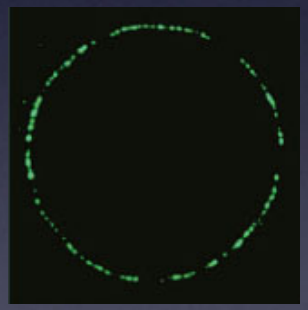
Qdot labelled dynein via HaloTag:Biotin:Streptavidin linkage moving on axonemes

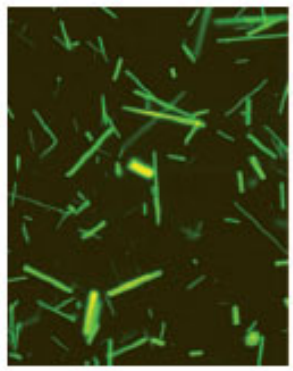
Fluorescent proteins

Discovery









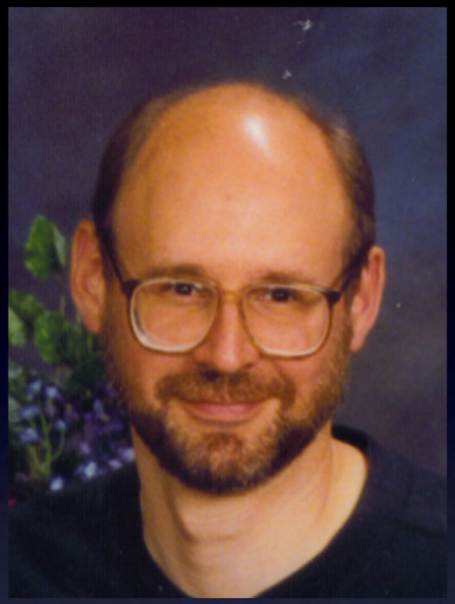
GFP (100 mg)

Denature at 90 °C
Digest with papain
Extraction with butanol at pH 1
TLC purification

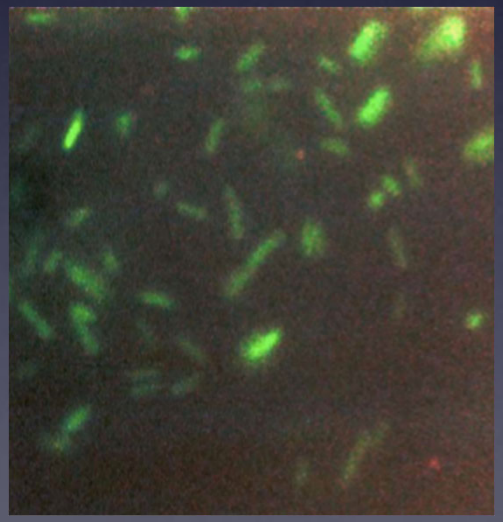
Isolated chromophore (0.1 mg)

Images from Osamu Shimomura

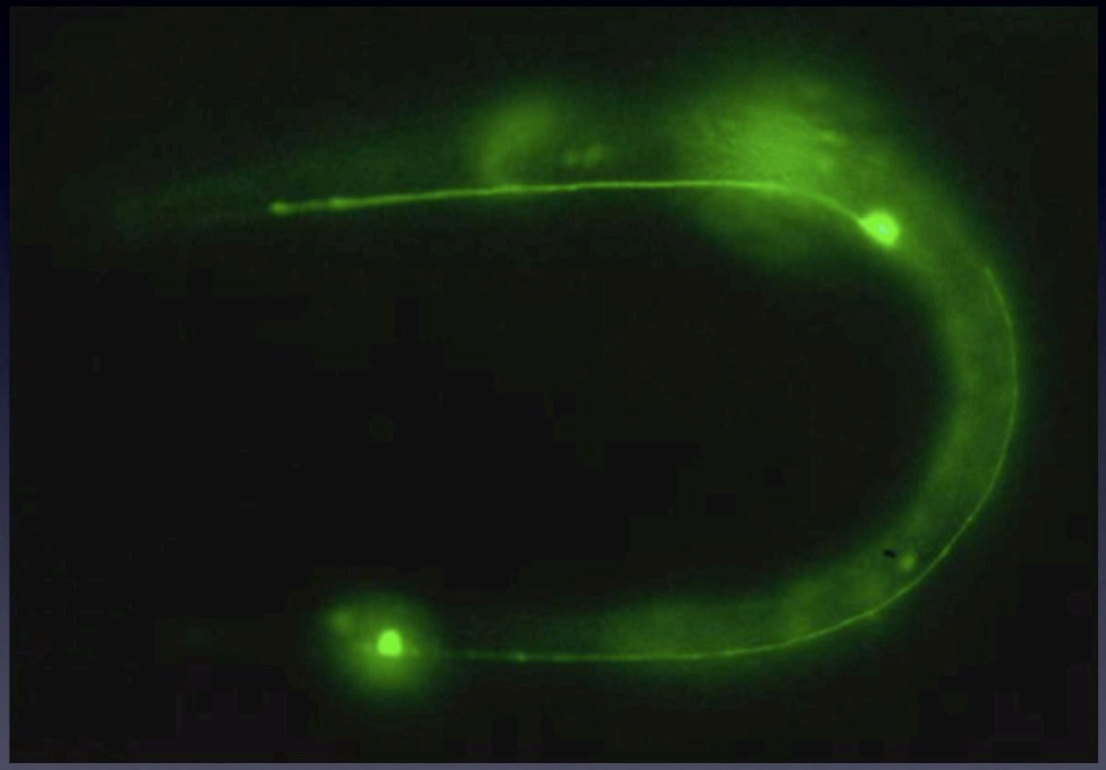
No co-factors needed!



Douglash 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

Lukyanov et al.

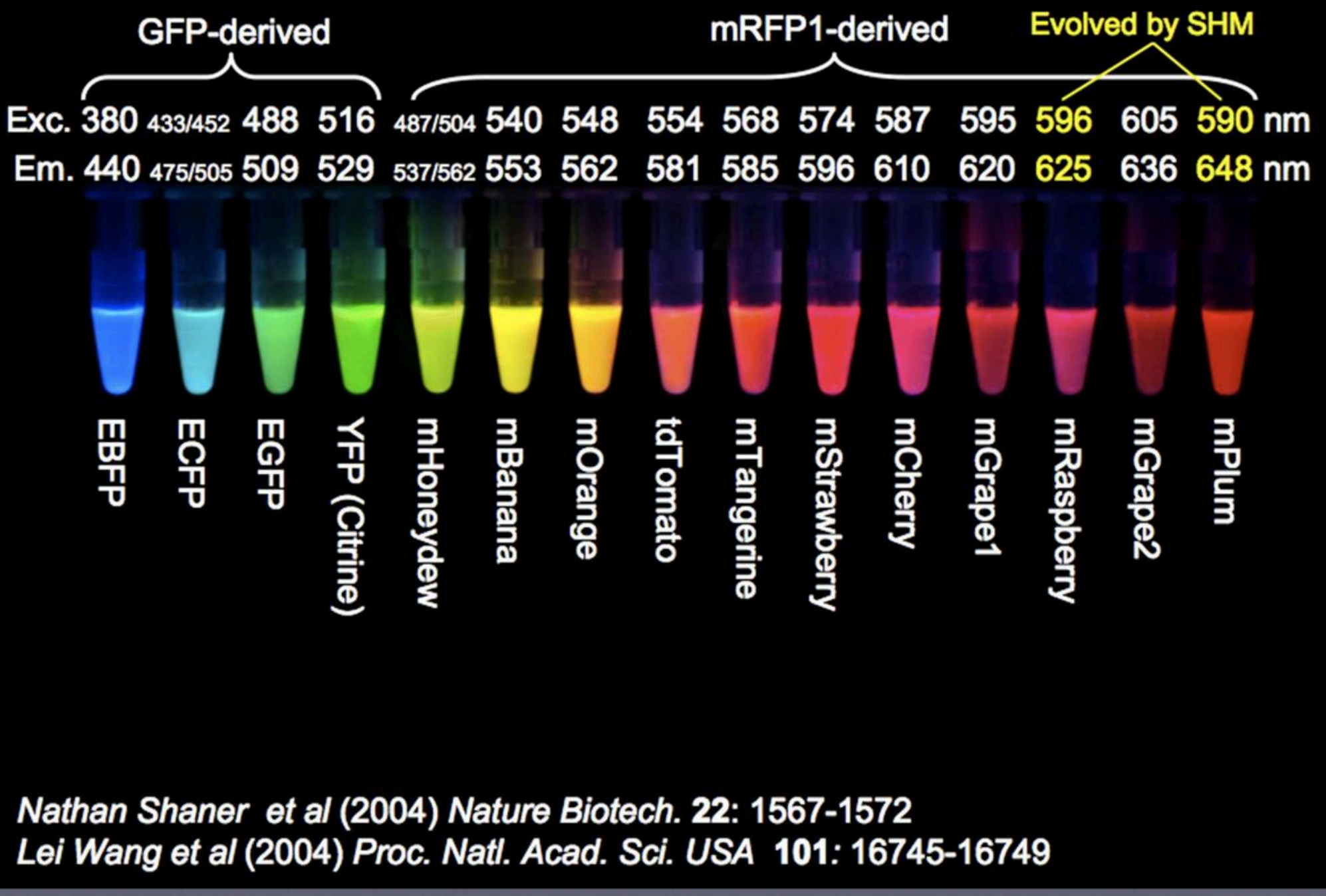
Fished for GFP-like proteins in coral reef obtained from Moscow pet shops.

dsRed: obligate tetramer, slowly maturing from green to red

Coral reef: Discosoma

http://www.scholarpedia.org/article/Talk:Fluorescent_proteins#

The 2004 palette of nonoligomerizing fluorescent proteins



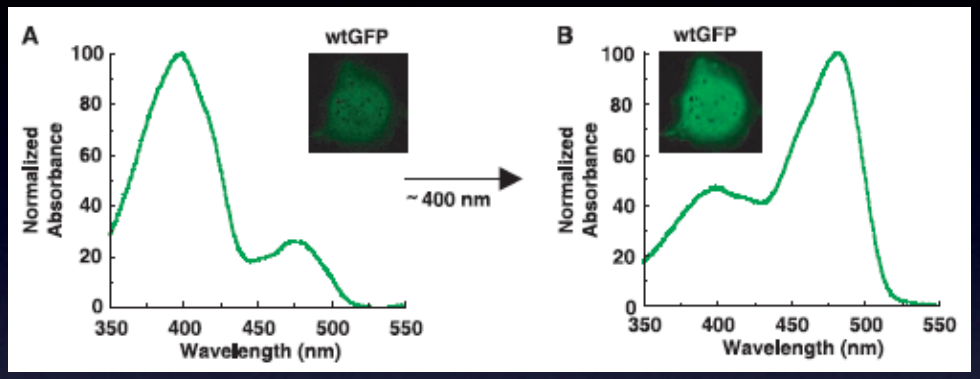
Nathan Shaner et al (2004) *Nature Biotech.* **22**: 1567-1572
Lei Wang et al (2004) *Proc. Natl. Acad. Sci. USA* **101**: 16745-16749

Switchable fluorescent proteins

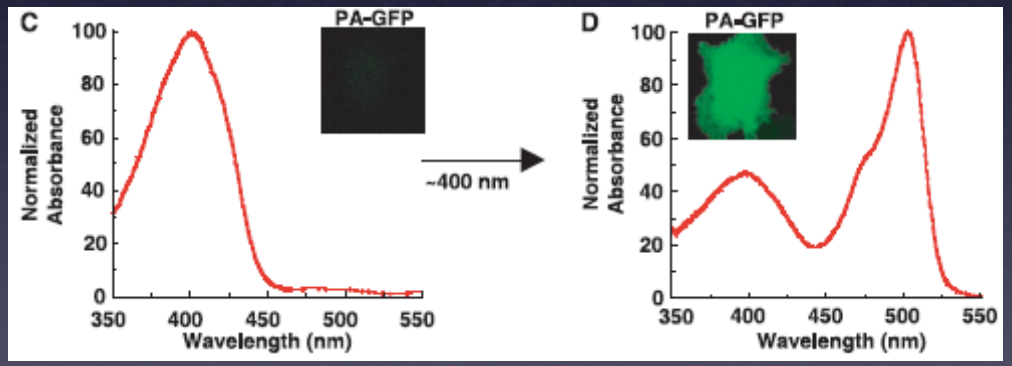
Fluorescence that can be activated or altered by light

Activatable

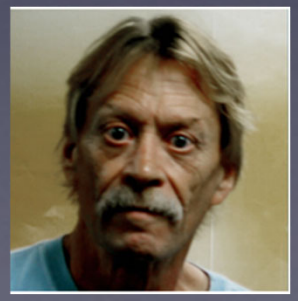
PA-GFP, …



PA-GFP (T203H), 100:1 contrast



George H. Patterson and Jennifer Lippincott-Schwartz, 2002



Michael Davidson

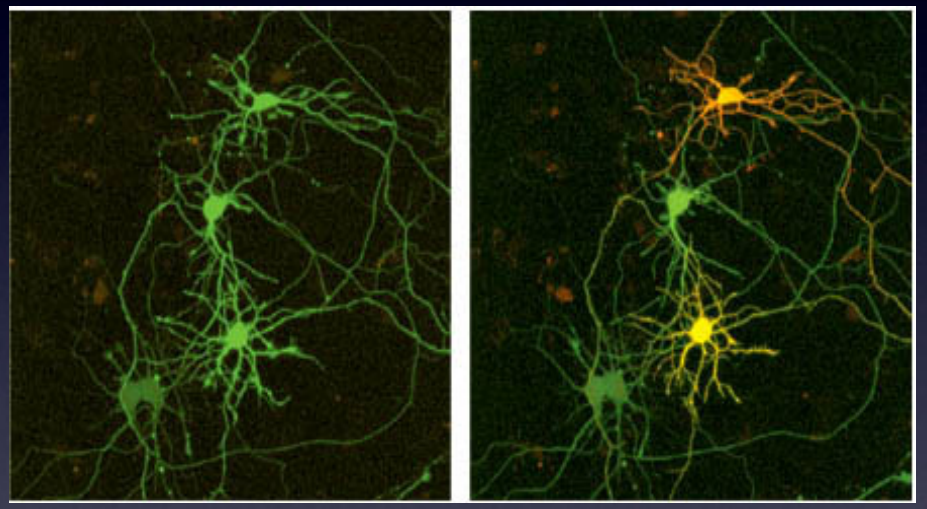
Color-changing

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

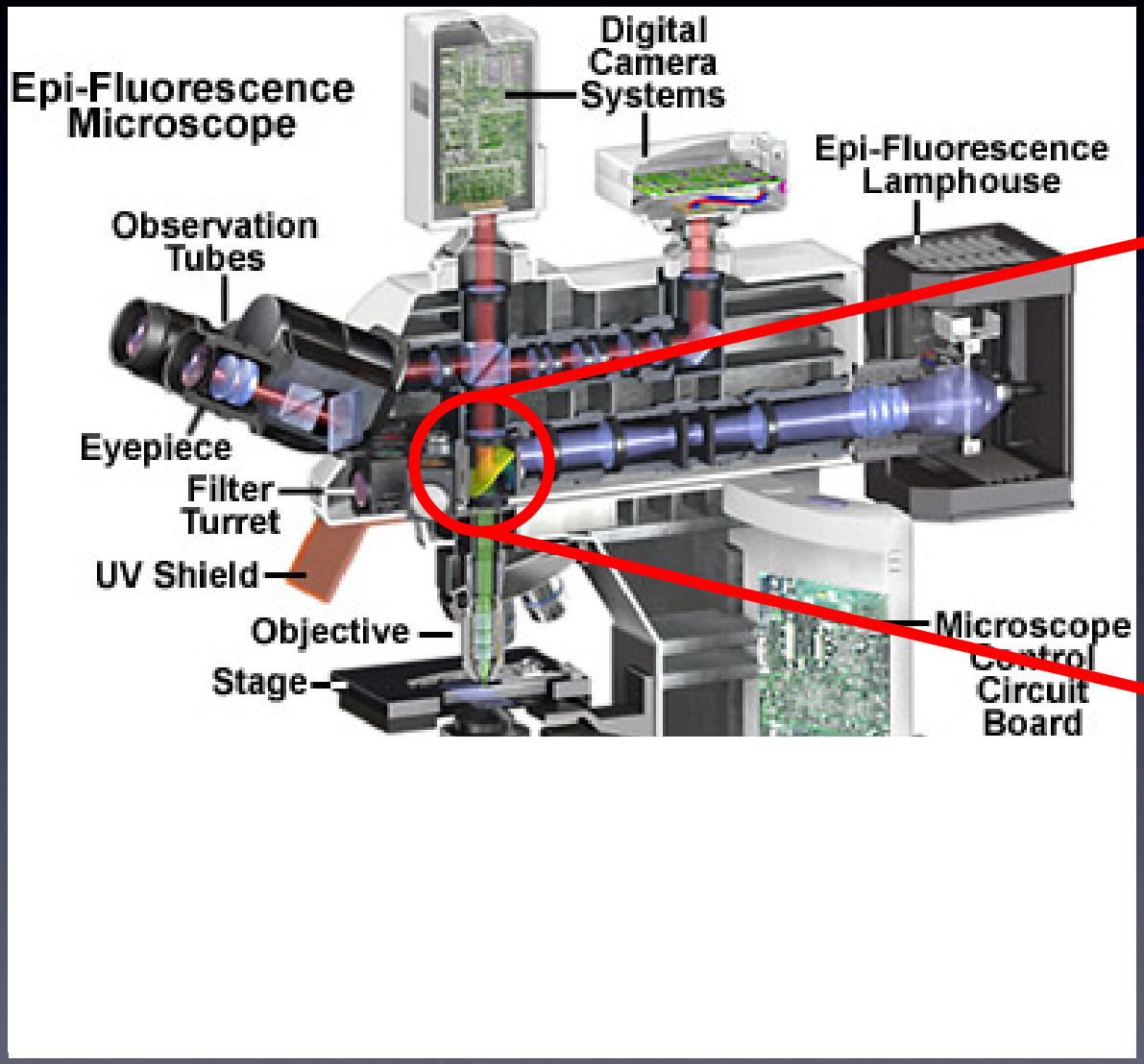
Ryoko Ando*†, Hiroshi Hama*, Miki Yamamoto-Hino*, Hideaki Mizuno*, and Atsushi Miyawaki*‡

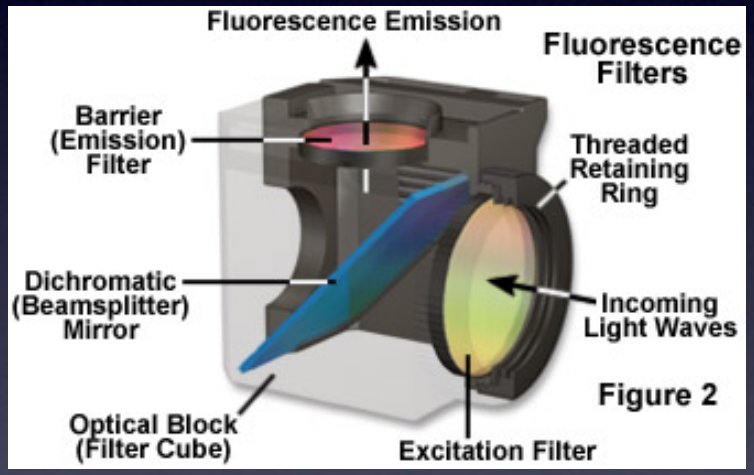
We happened to leave one of the protein aliquots on the laboratory bench overnight. The next day, we found that the protein sample on the bench had turned red, whereas the others that were kept in a paper box remained green. Although the sky had been partly cloudy, the red sample had been exposed to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

Reversibly switchable

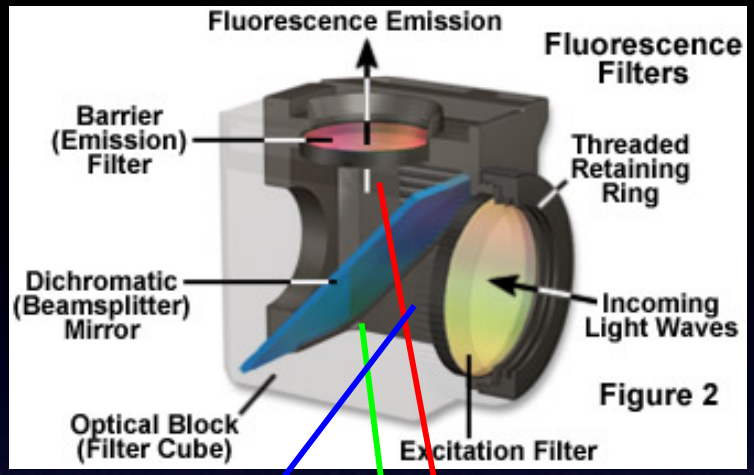
KFP, Dronpa

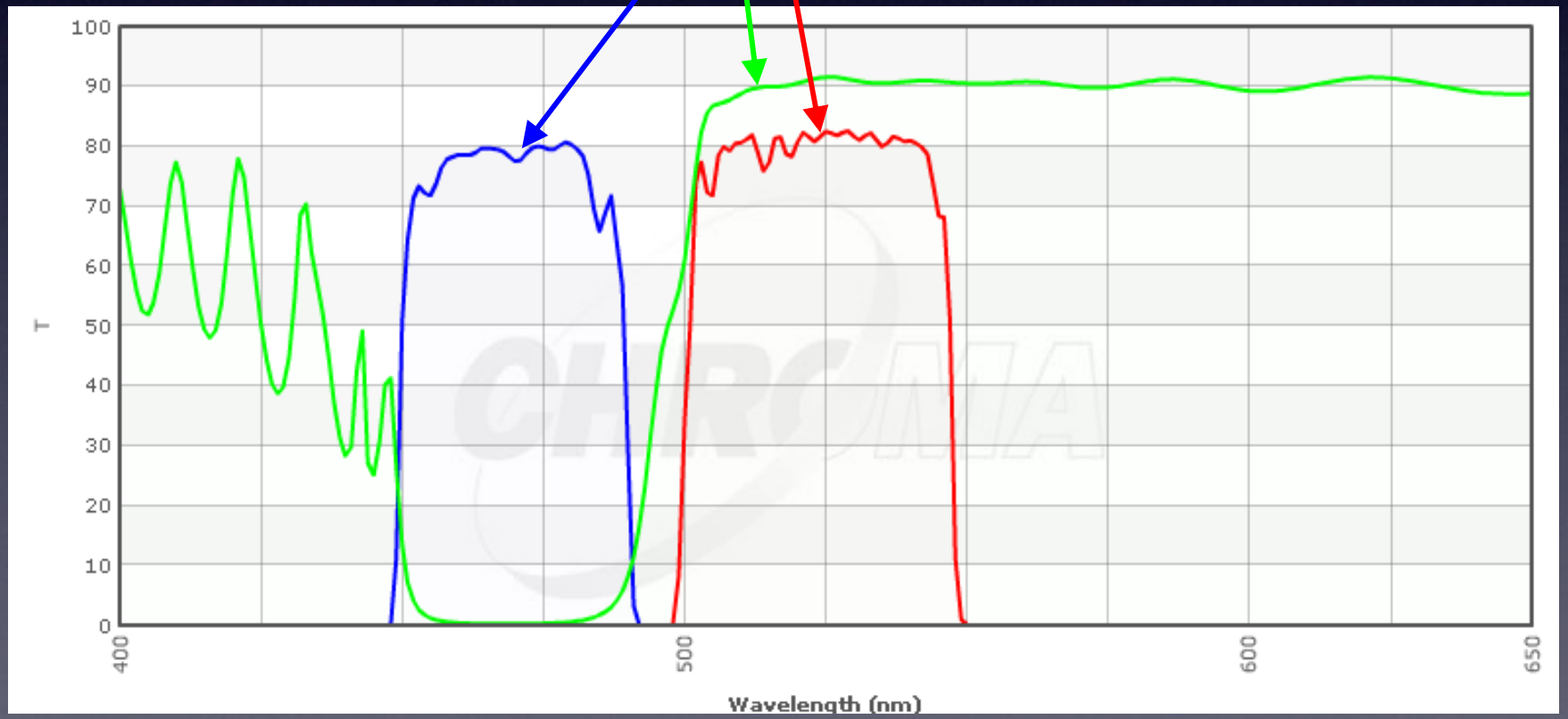
The Epifluorescence Microscope





Ploem


Fluorescence Emission
Fluorescence Filters
Barrier (Emission) Filter
Threaded Retaining Ring
Dichromatic (Beamsplitter) Mirror
Incoming Light Waves
Figure 2
Optical Block (Filter Cube)
Excitation Filter


100
90
80
70
60
50
40
30
20
10
0
T
400
500
600
650
Wavelength (nm)

Types of Filters

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

Colored Glass Filters

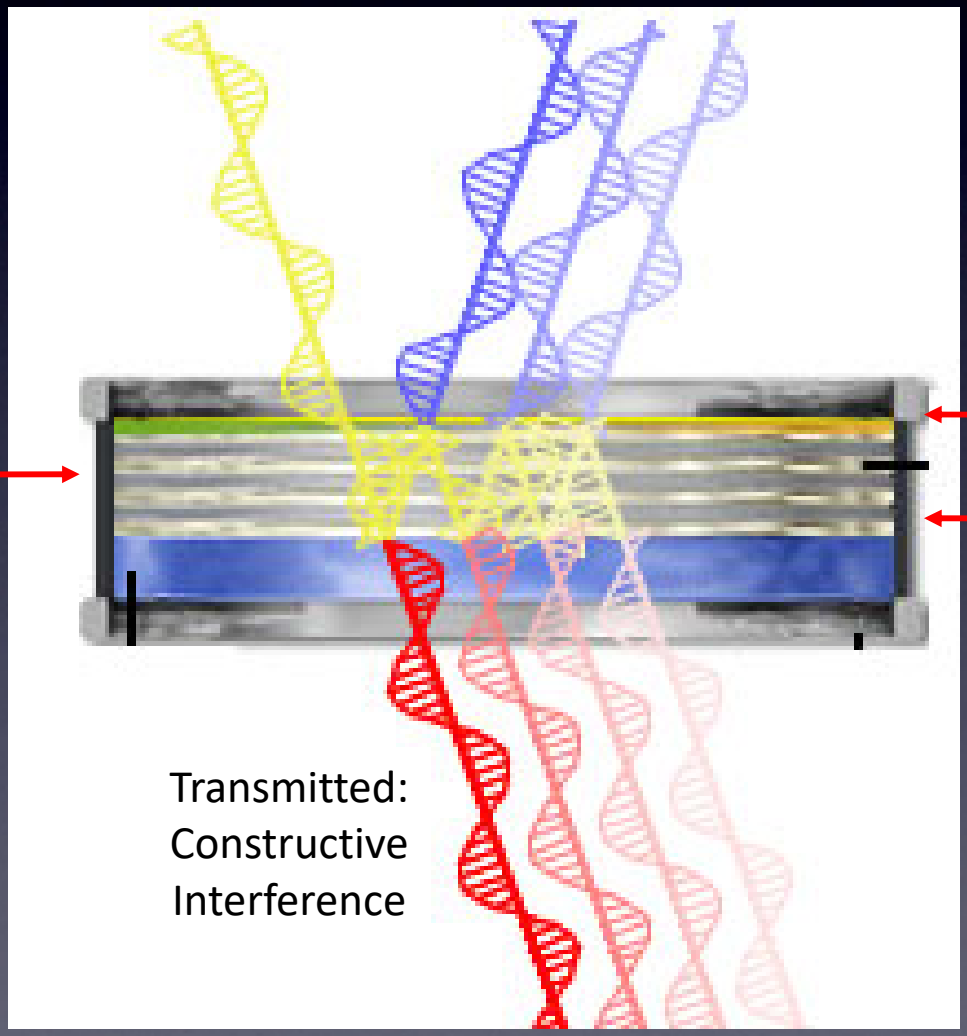
- Cheap
- Sturdy
- Independent of angle of incidence
- Small selection
- Spectra have poor slope and poor peak performance
- Autofluorescence
- Absorb Get Hot

→

Interference Filters

Incident Light

Reflected: Destructive Interference



Transparent layer

Semi-reflective coatings

Transmitted: Constructive Interference

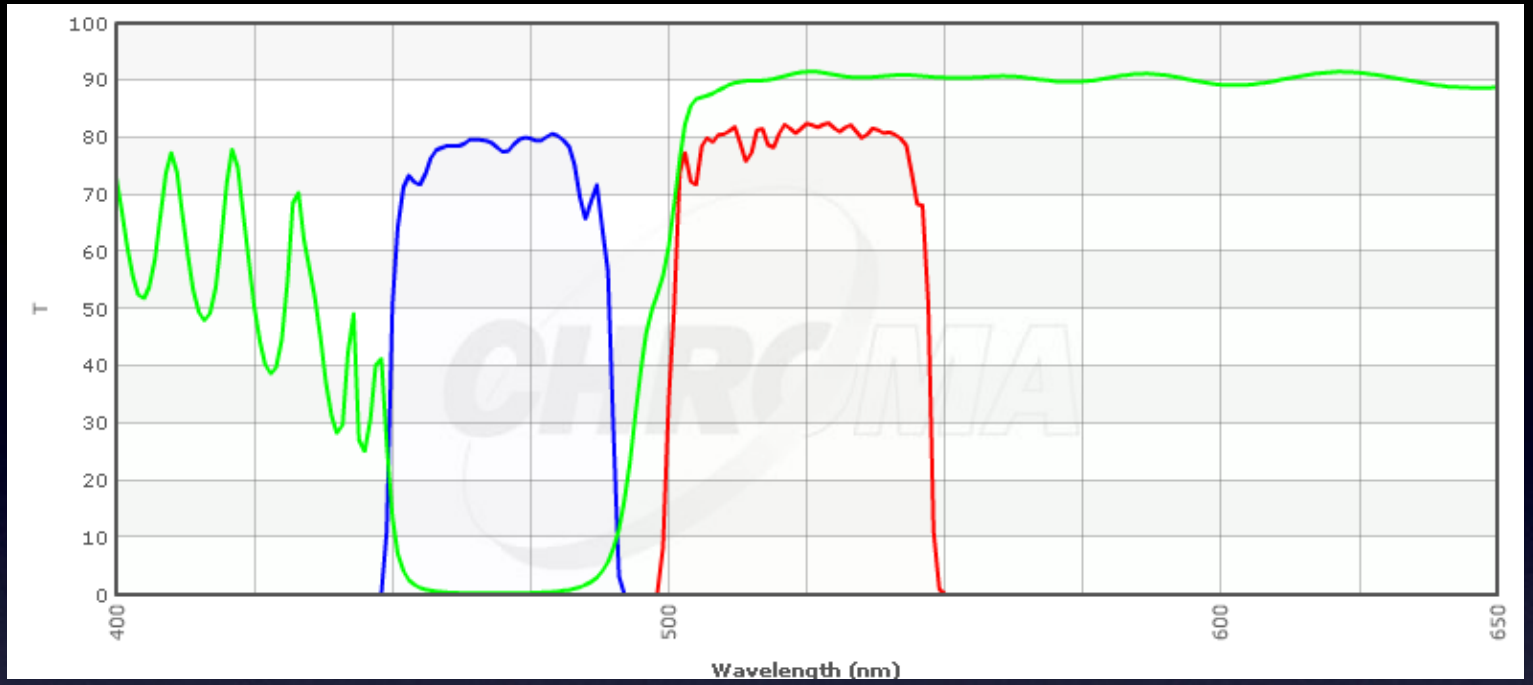
Vendors:

Chroma

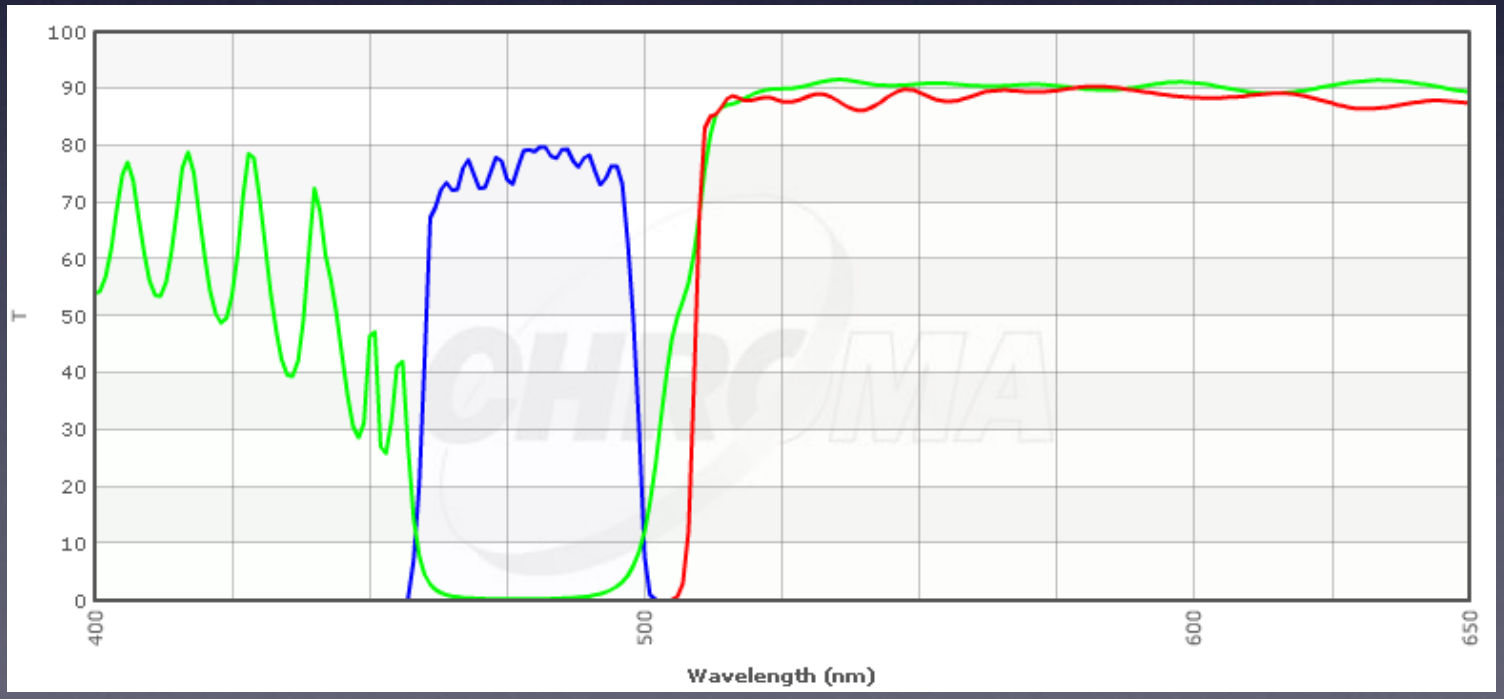
Semrock

Omega

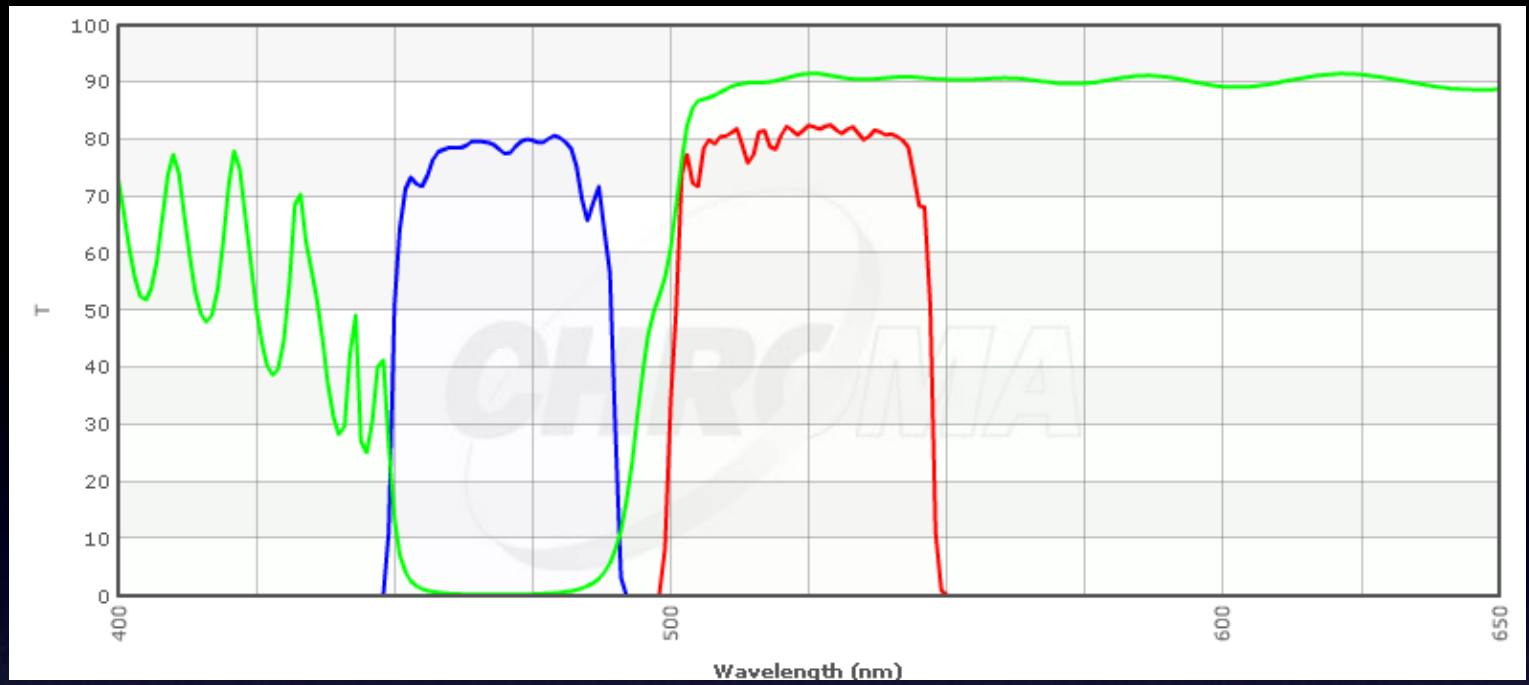
Bandpass



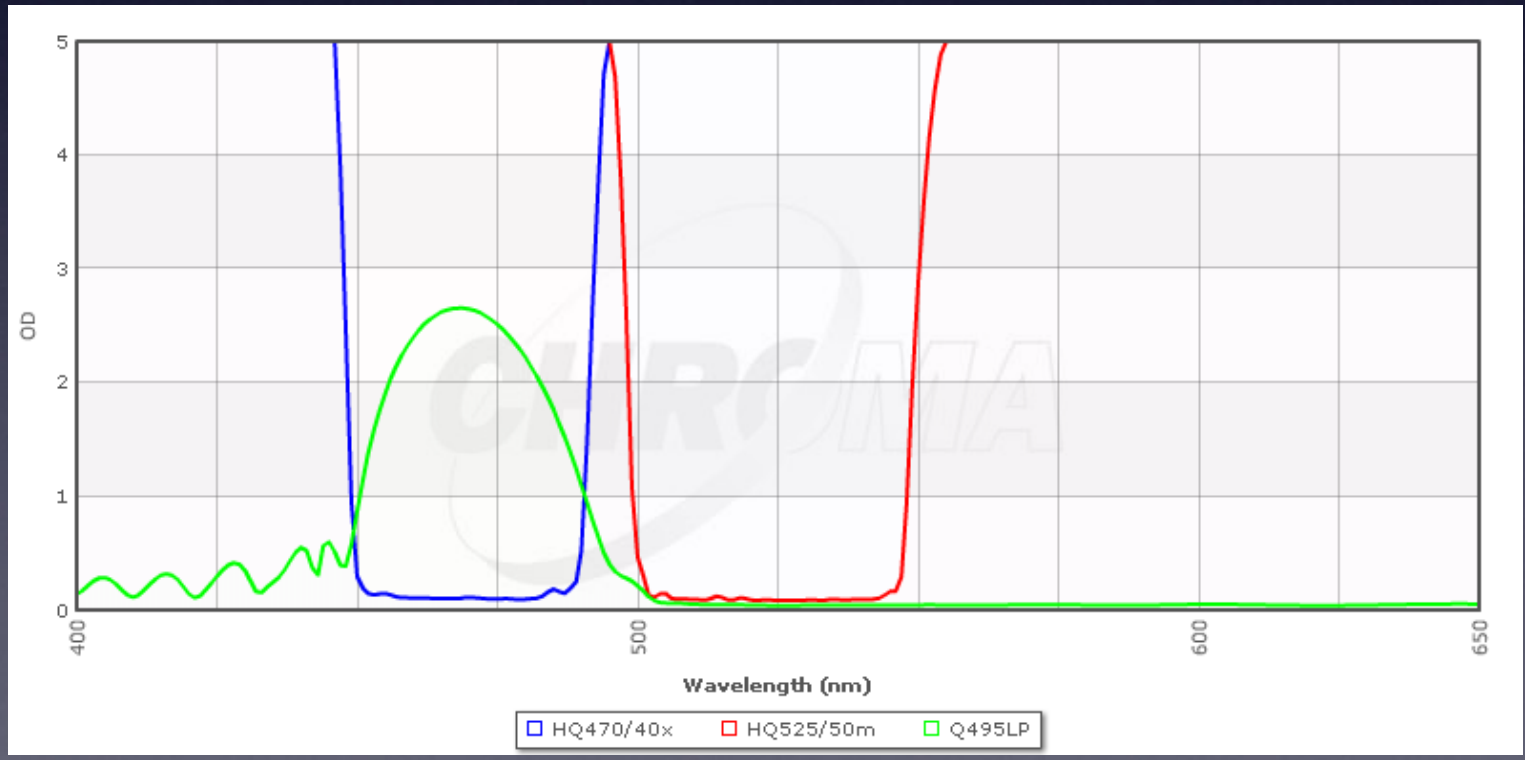
Longpass



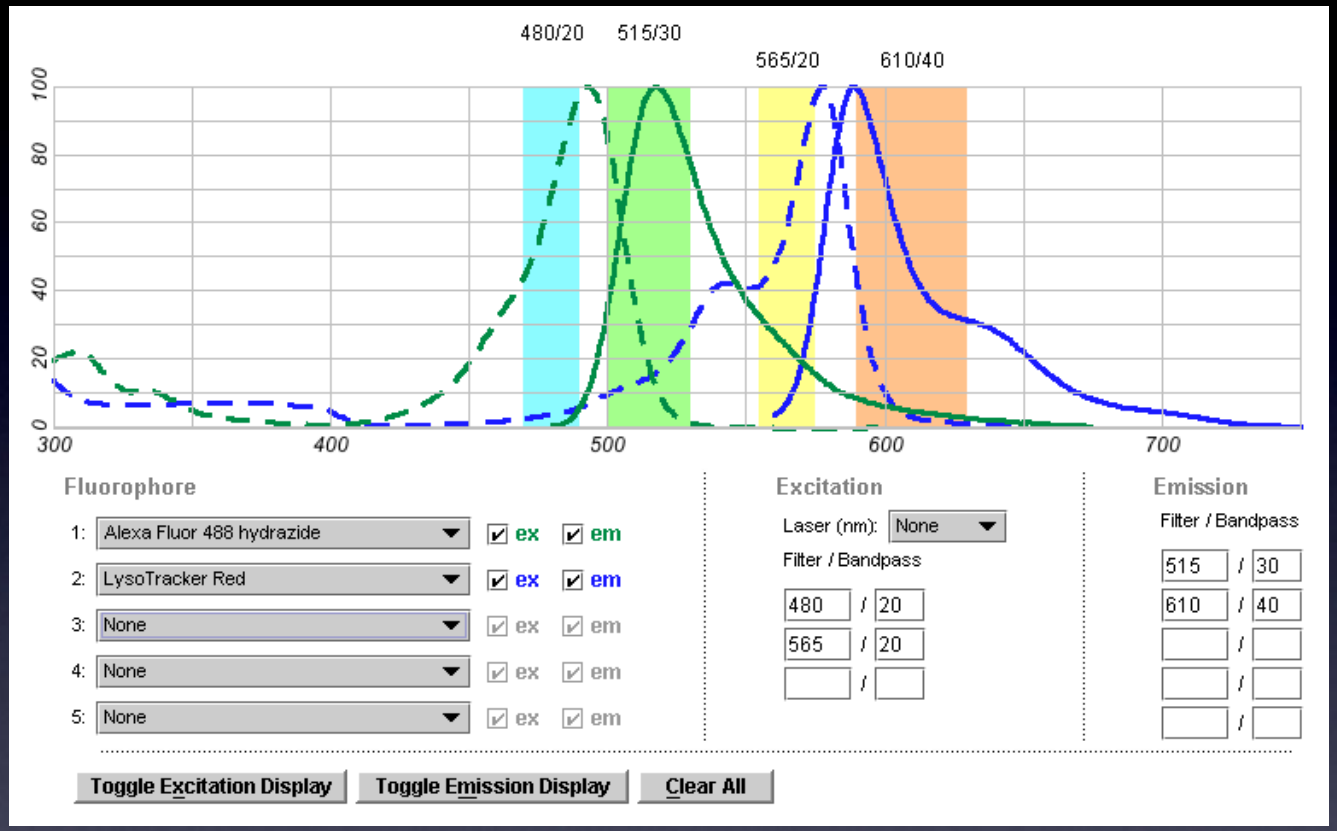
%T


100
90
80
70
60
50
40
30
20
10
0
T
400
500
600
650
Wavelength (nm)

OD


5
4
3
2
1
0
OD
400
500
600
650
Wavelength (nm)
HQ470/40x
HQ525/50m
Q495LP

Matching Filters and Fluorophores

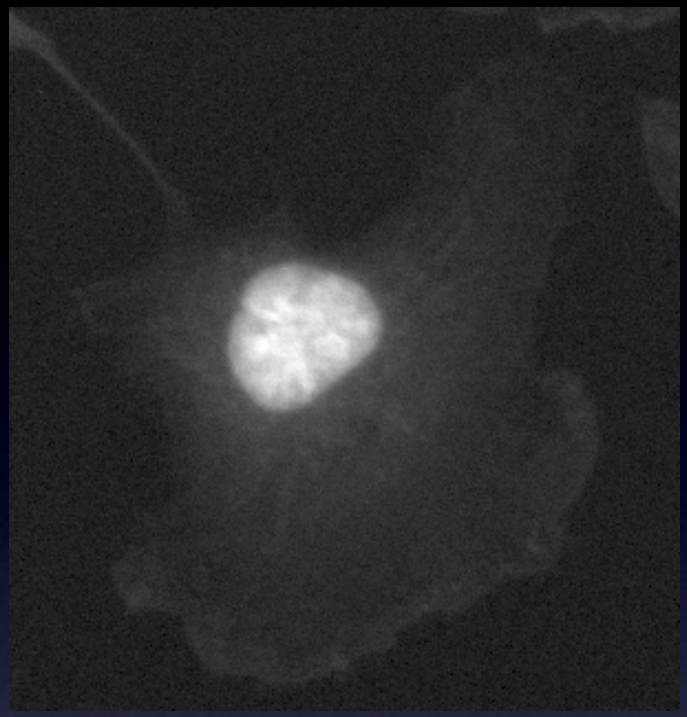


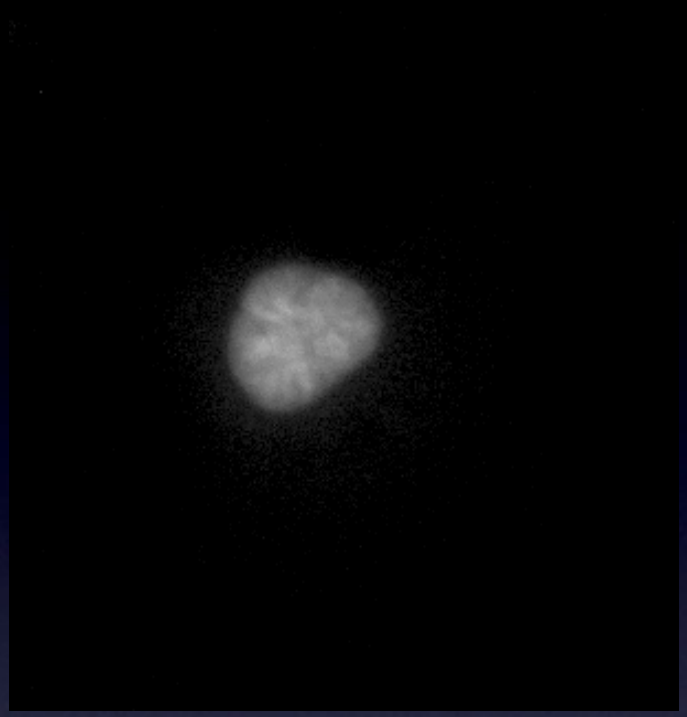
http://probes.invitrogen.com/resources/spectraviewer/

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

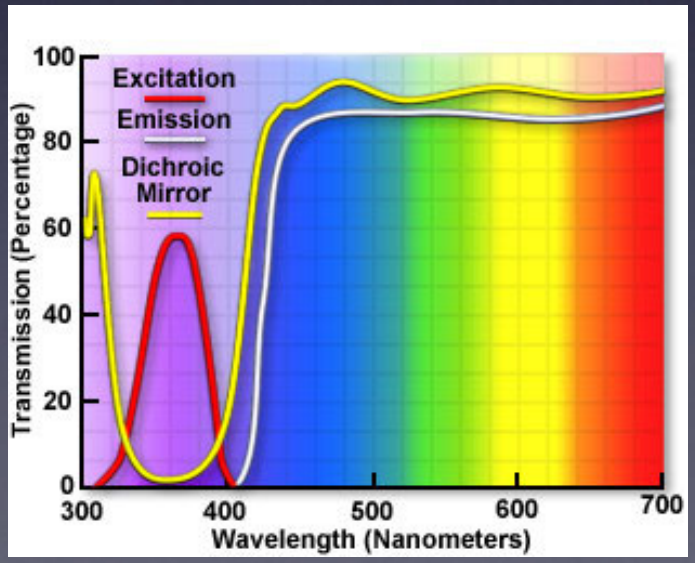
https://www.omegafilters.com/curvo2/index.php

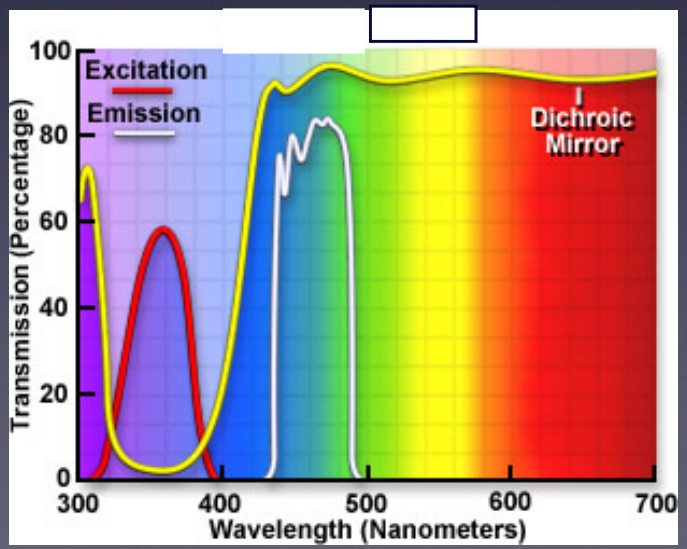
Choose filters that separate fluorophores





Two different UV filter sets





Choose filters that maximize excitation and emission

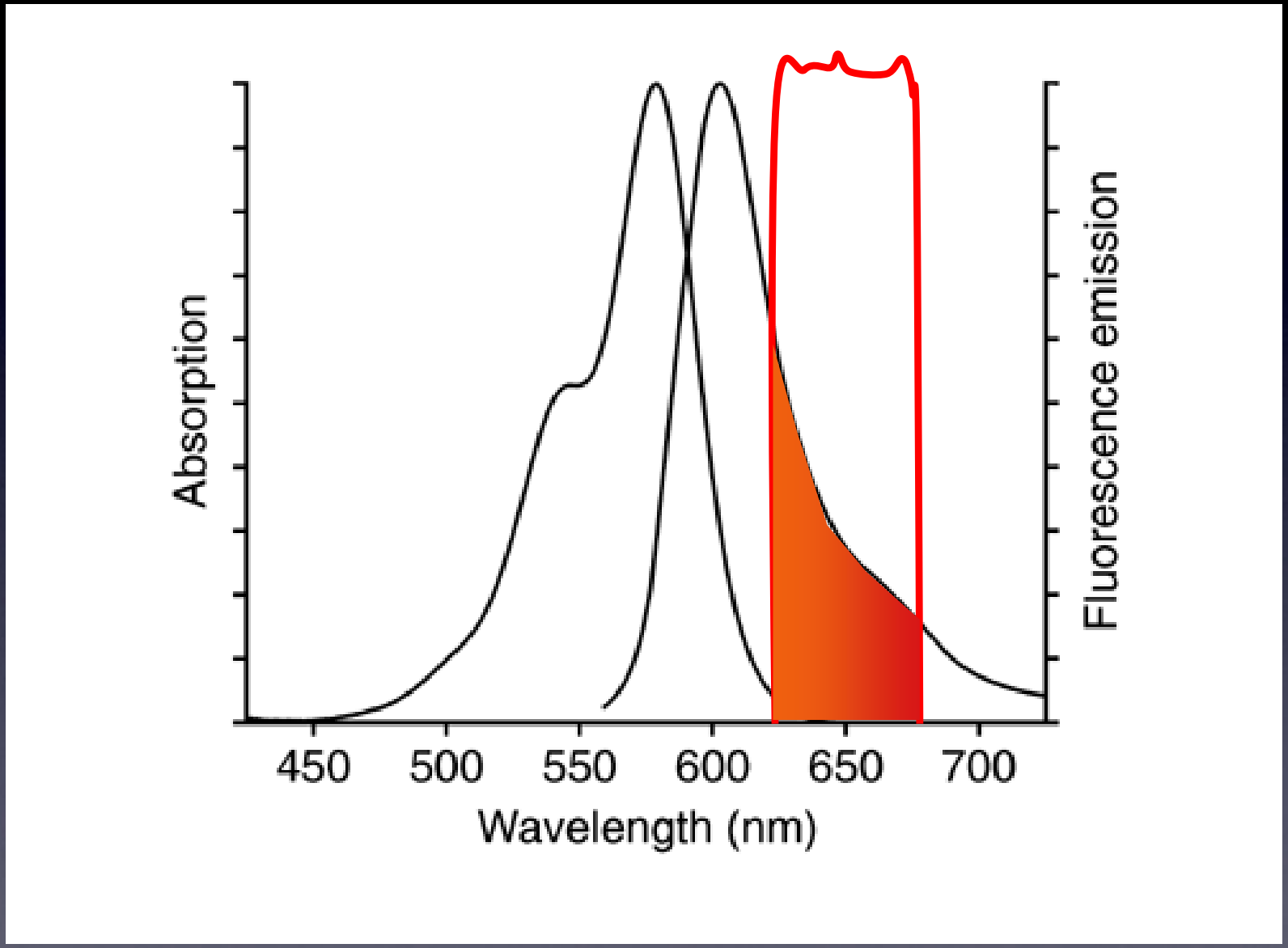


Image from www.probes.com

Choose filters that maximize excitation and emission

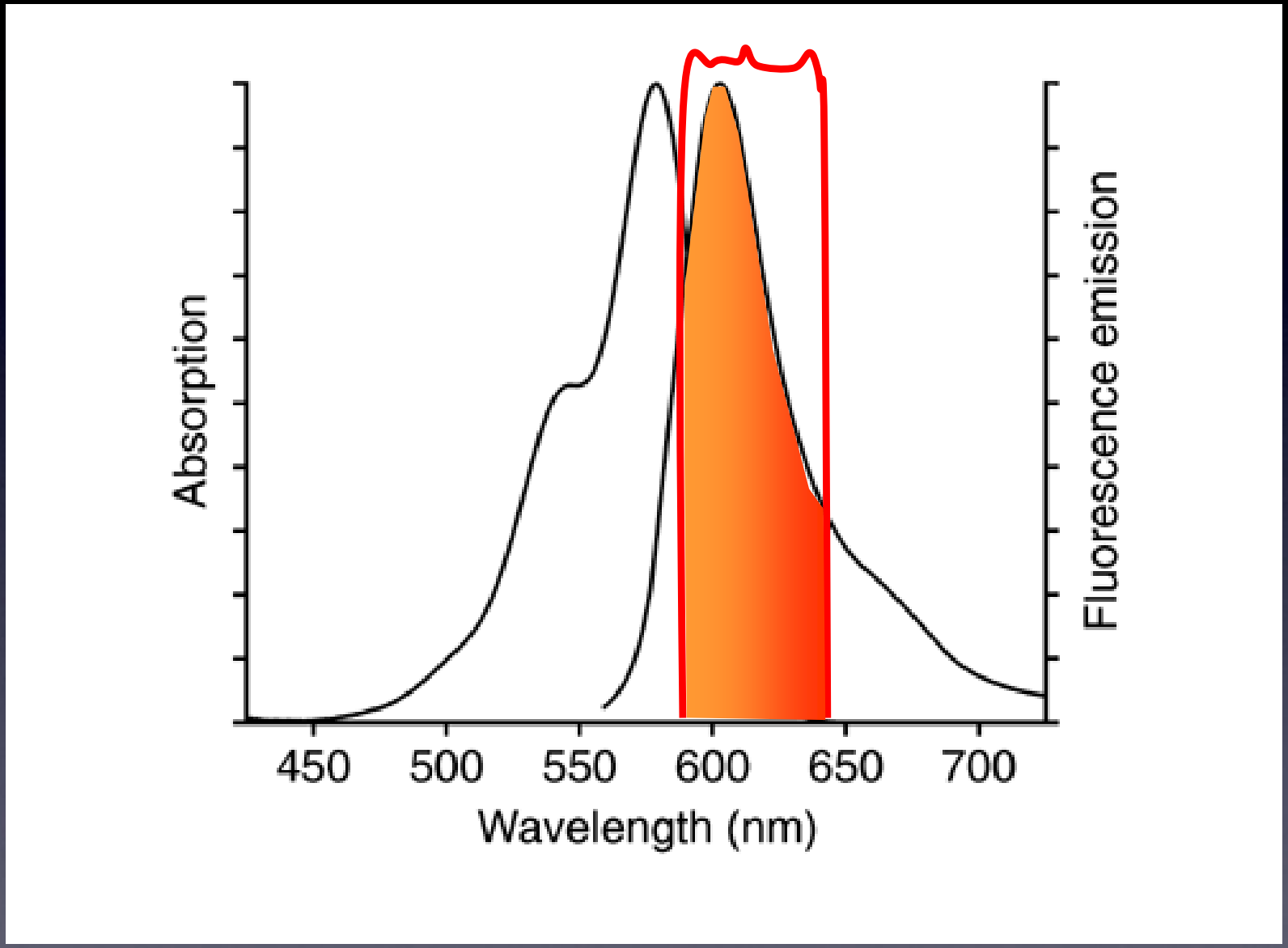
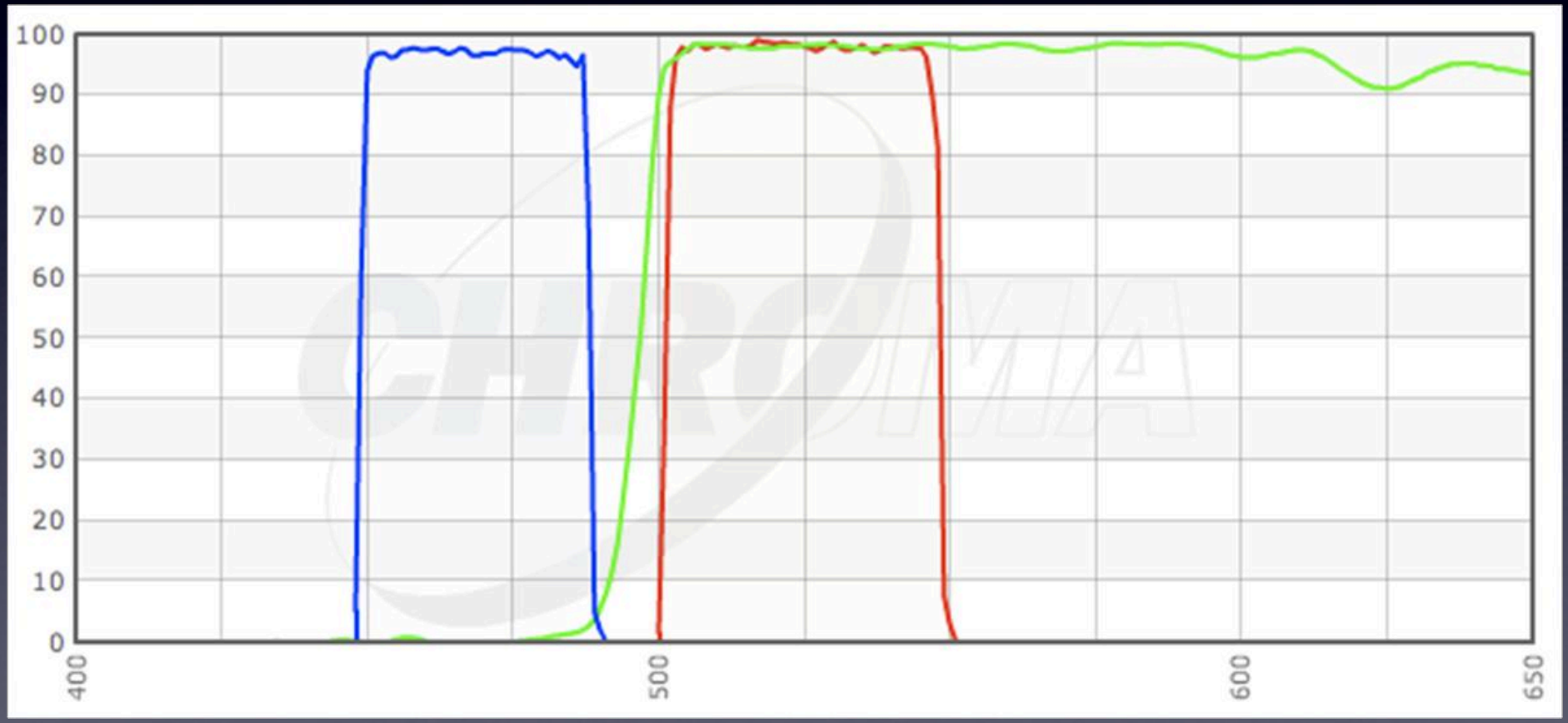


Image from www.probes.com

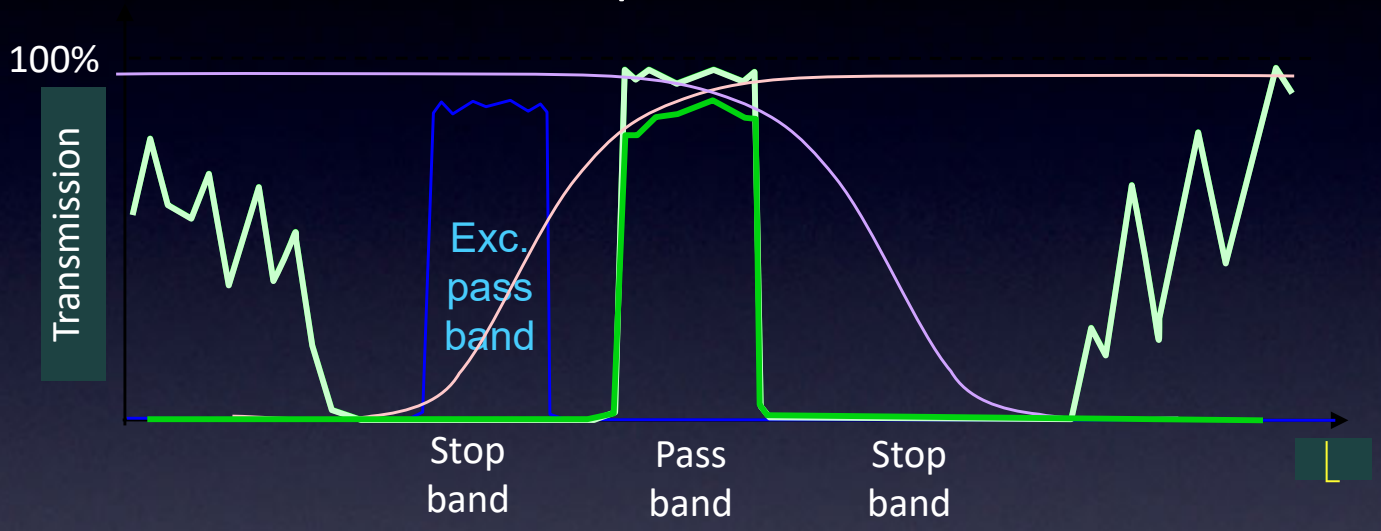
Newer hard-coatings are great!

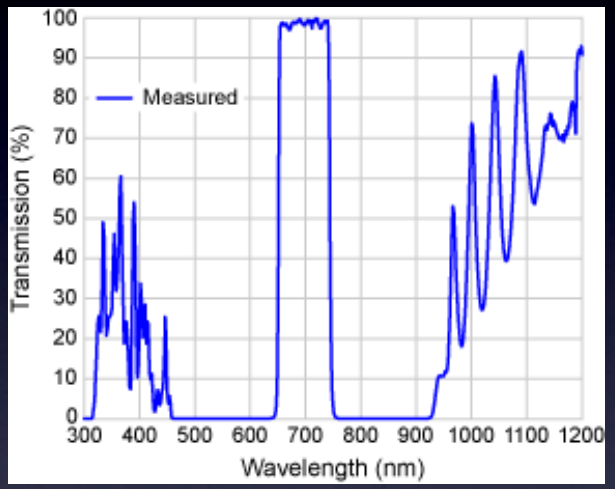


Blocking

Interference filters have finite stop bands

Unblocked bandpass interference filter





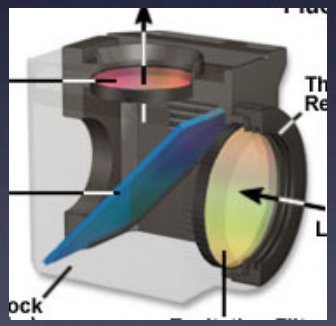
Semrock 697/75

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

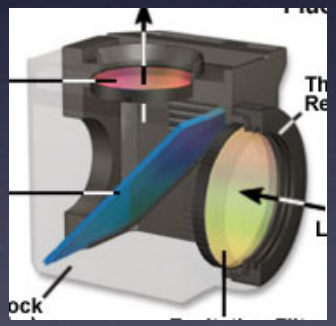
Often excitation filters are blocked,
but emission filters unblocked.
→ Red autofluorescence or room light
may get through your blue emission filter

UV absorber
Interference filter
IR absorber

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

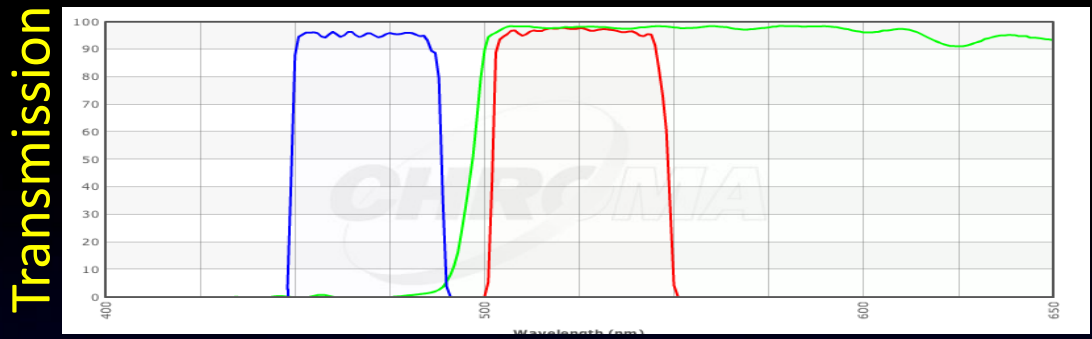


Faster Wavelength Selection: Multiple Band Pass Filters & Filter Wheel(s)

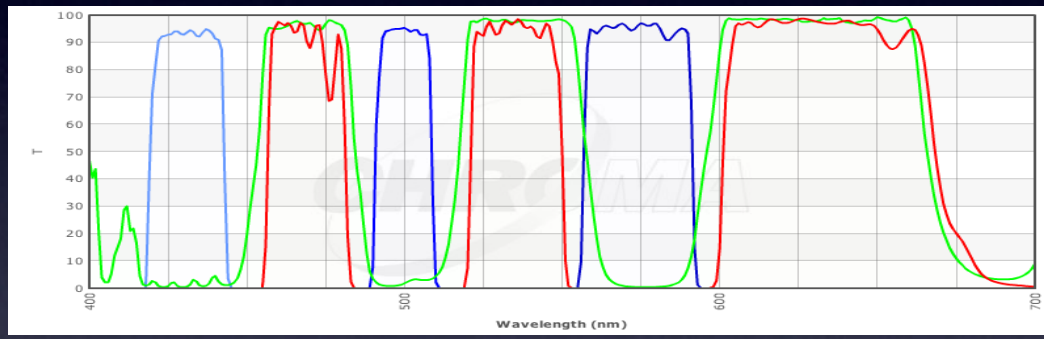


Filter schemes

Single wavelength sets
- Most efficient
- Best separation
- Very slow to change



Multi-band filters
- Multi-band everything
- See all colors at once
- For color cameras
- Bad crosstalk

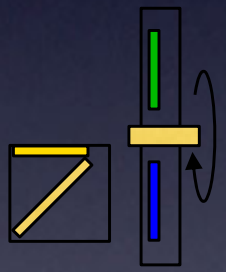


- "Pinkel" scheme

Multi-band dichroic

Multi-band emitter

Single-⌞ exciters



Chroma triple Pinkel set

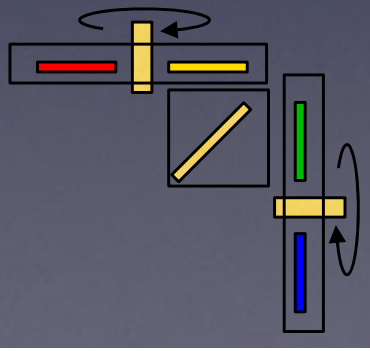
- Excitaton filter wheel
- Separate image at each wavelength
- Better separation

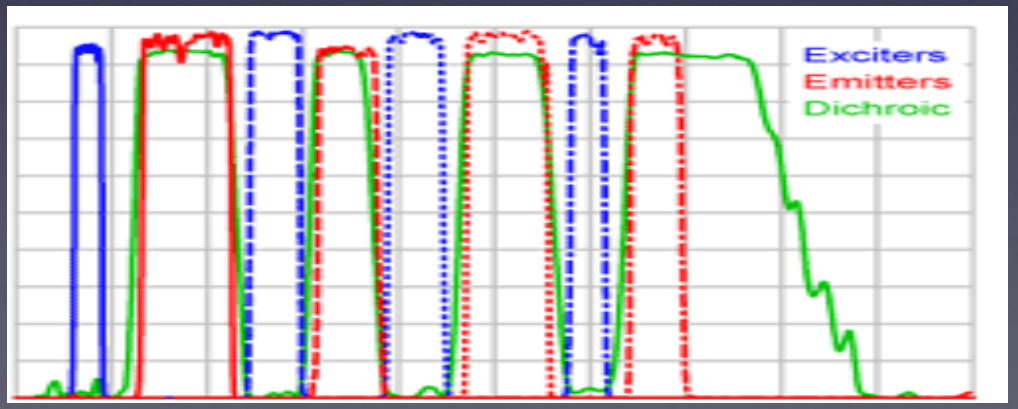
- "Sedat" scheme

Multi-band dichroic

single-band emitters

Single-⌞ exciters





Semrock quad Sedat set

- Two filter wheels
- Even better separation

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!)