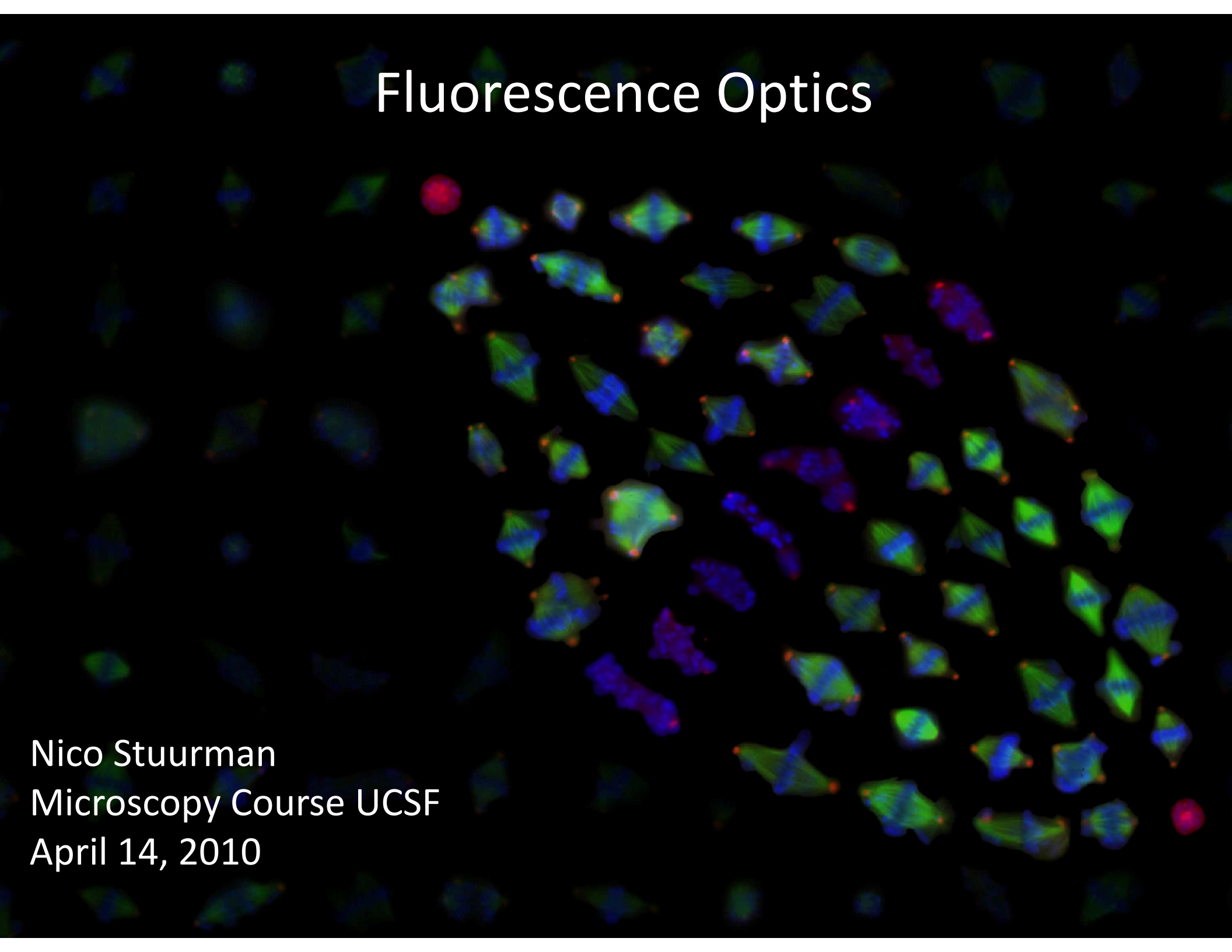
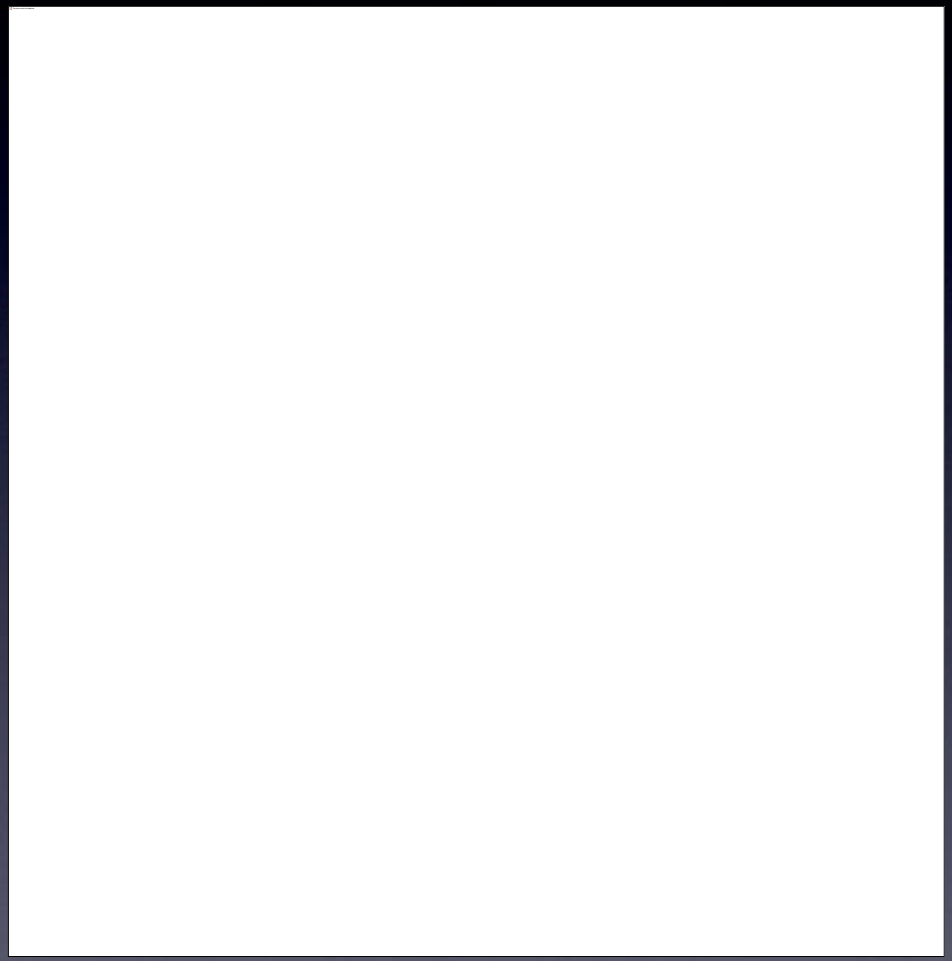
Fluorescence Optics



Nico Stuurman
Microscopy Course UCSF
April 14, 2010

Why fluorescence?

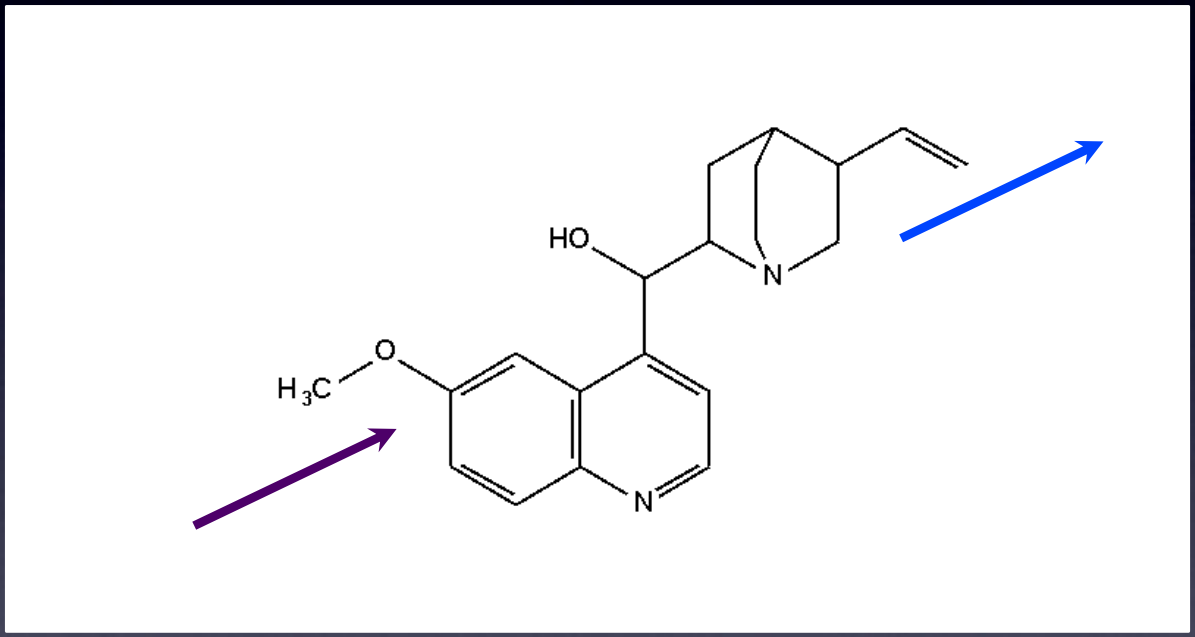


- High contrast
  - Signal against dark background
- Highly specific, multi-color labeling
  - GFP etc.
  - Antibodies
- Live imaging
  - GFP etc.
- Quantitative
- Sensors for [Ca], pH, …

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

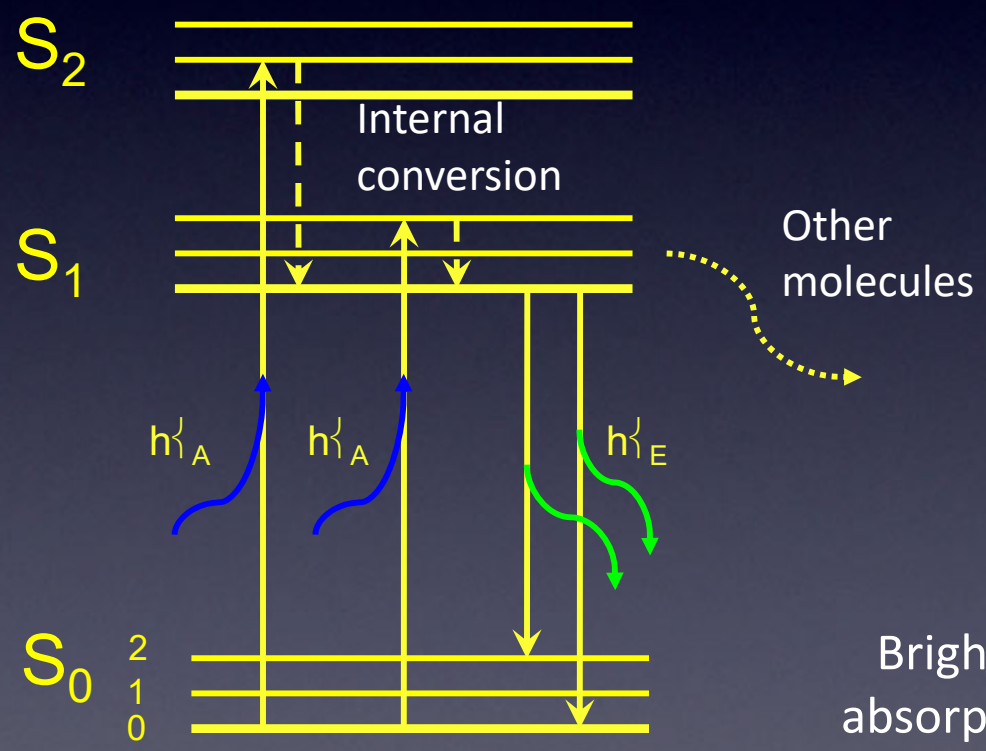
Fluorescence

Jablonski diagram

10^{-15}s (0.3µm)

10^{-12}s (0.3mm)

10^{-8}s (3m)



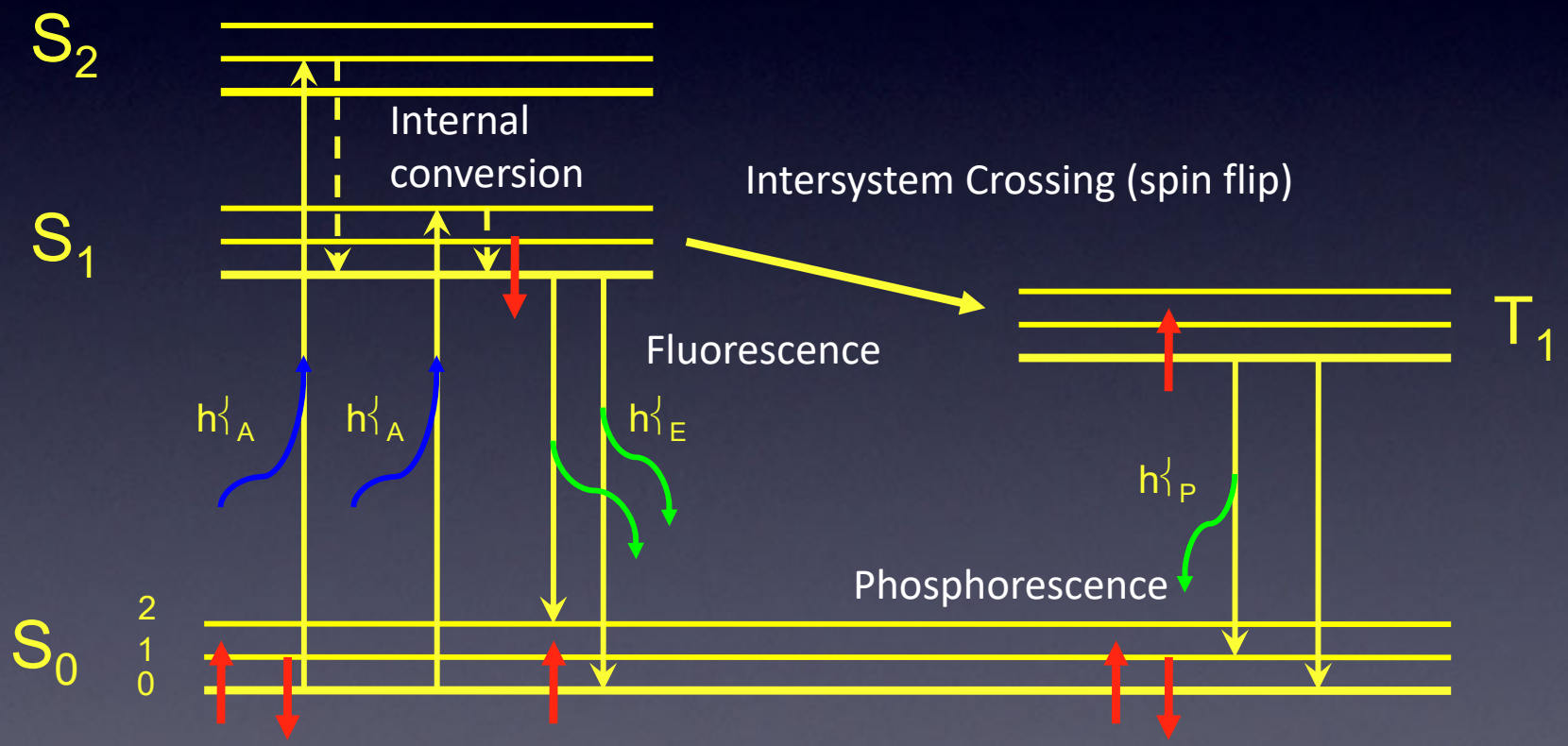
QE: ratio of absorbed and emitted photons

Relation between lifetime and QE

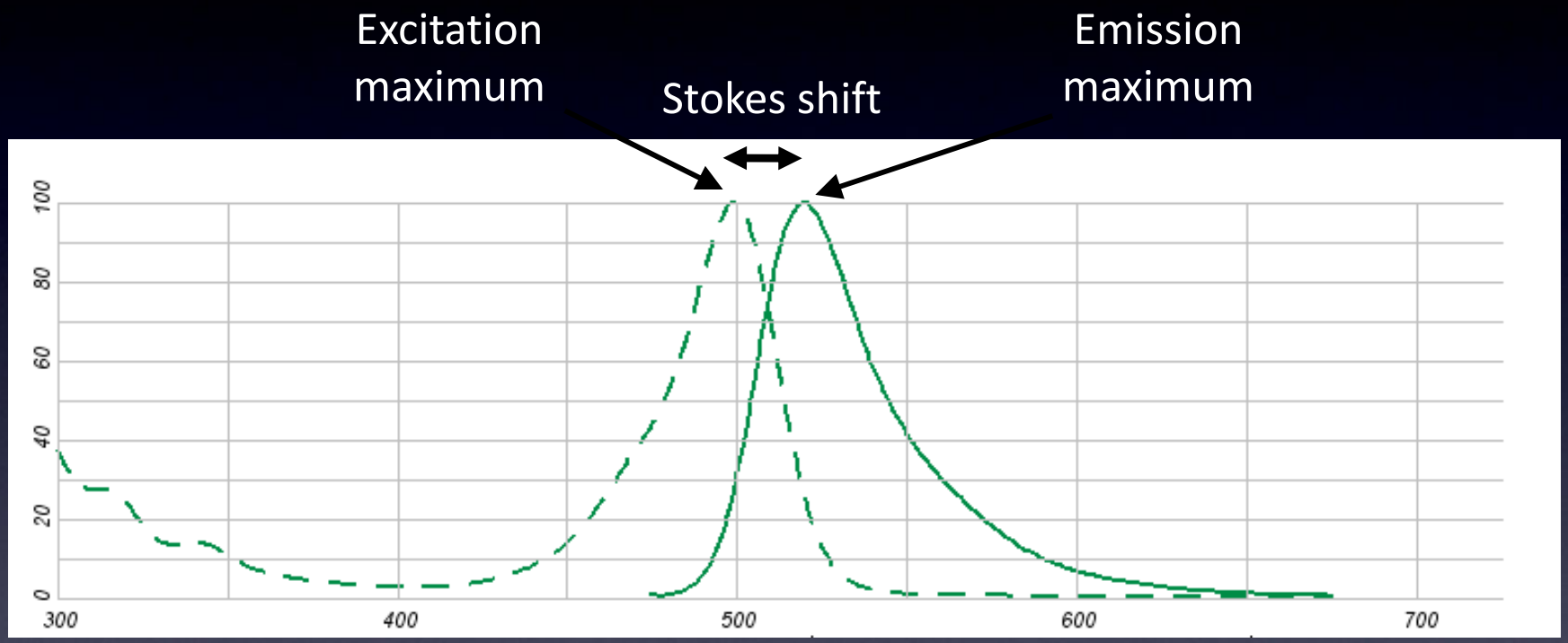
Brightness: determined by absorption coefficient and QE

Fluorescence

Jablonski diagram

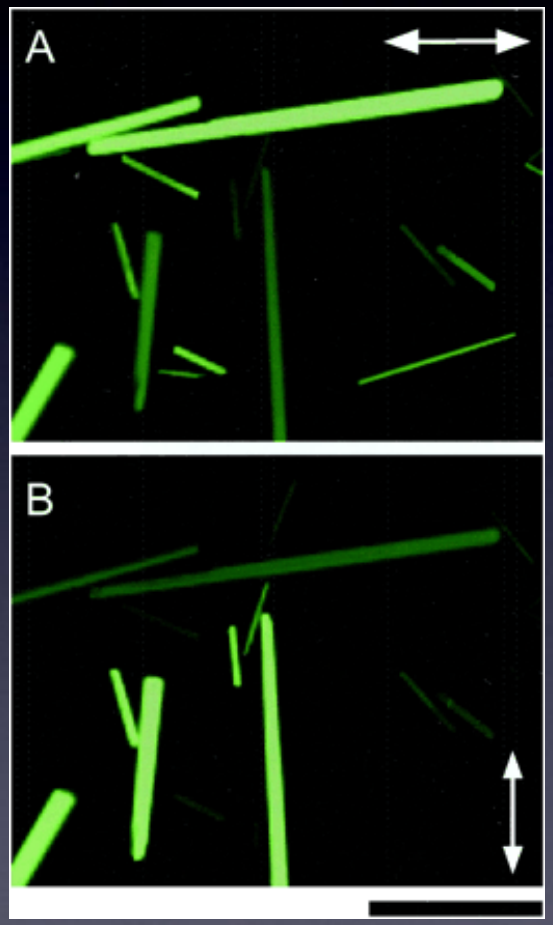


Fluorescence Spectra



Alexa 488

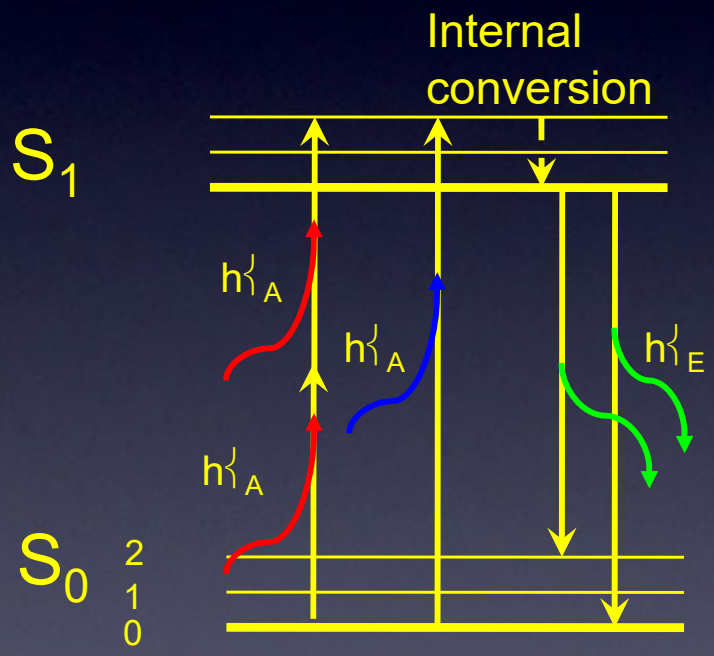
Polarization

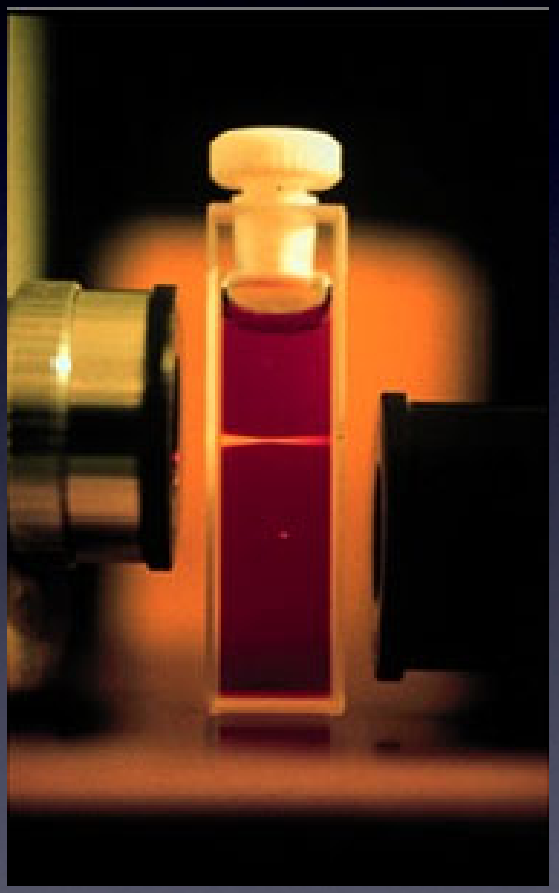


Native GFP crystals

Shinya Inoué

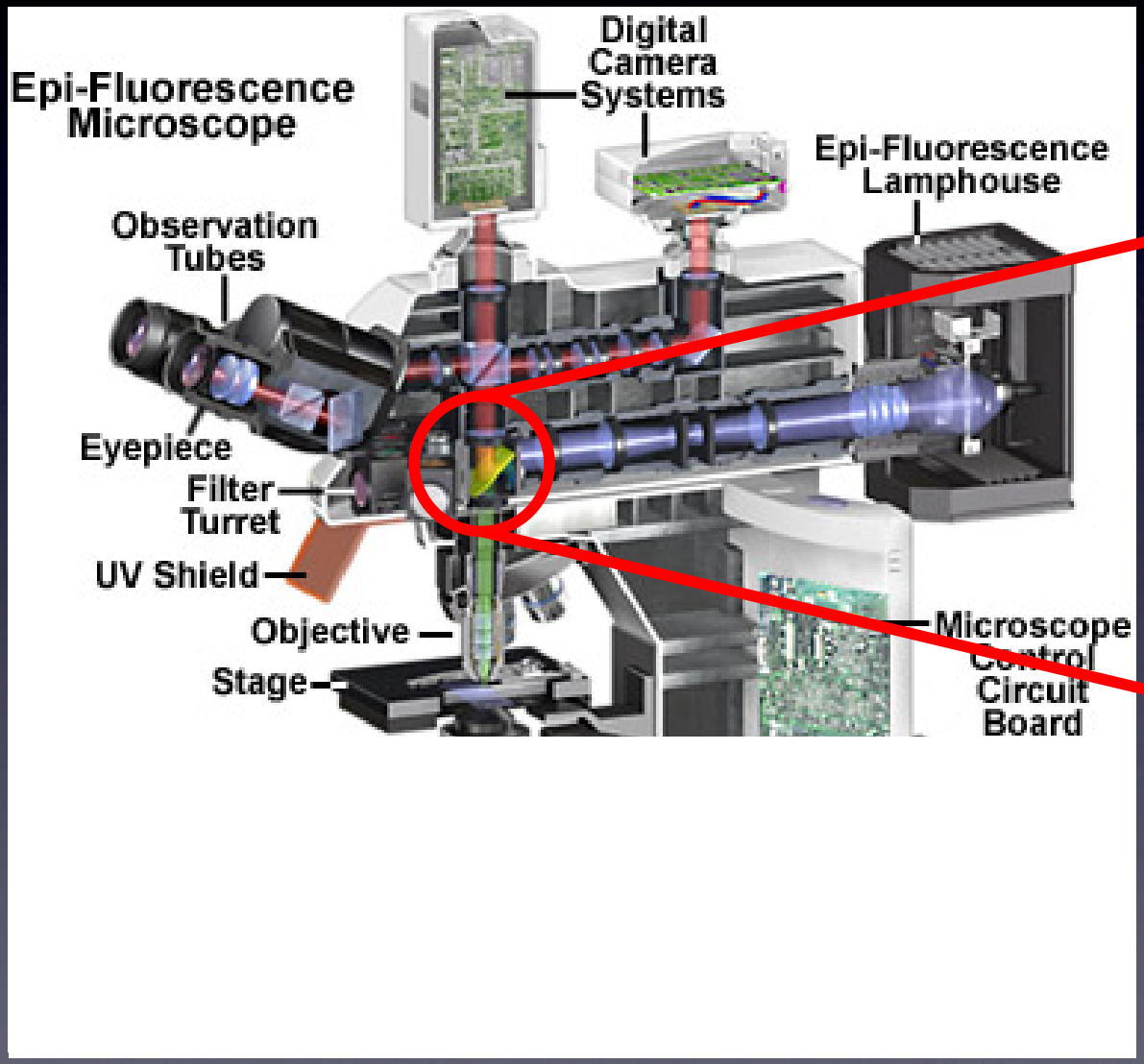
Multi-photon excitation

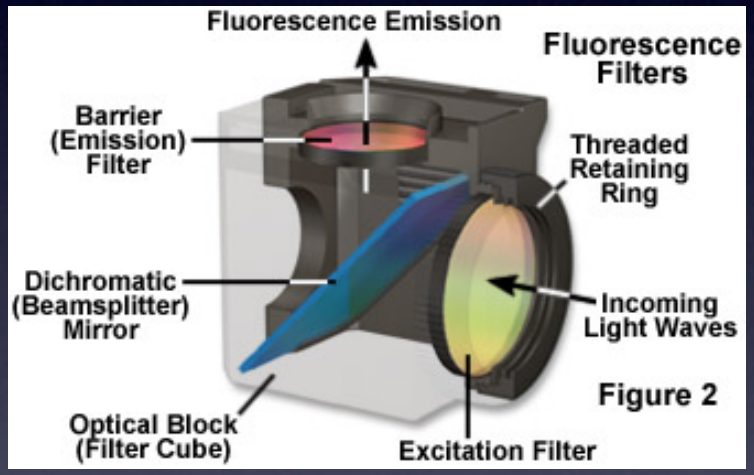




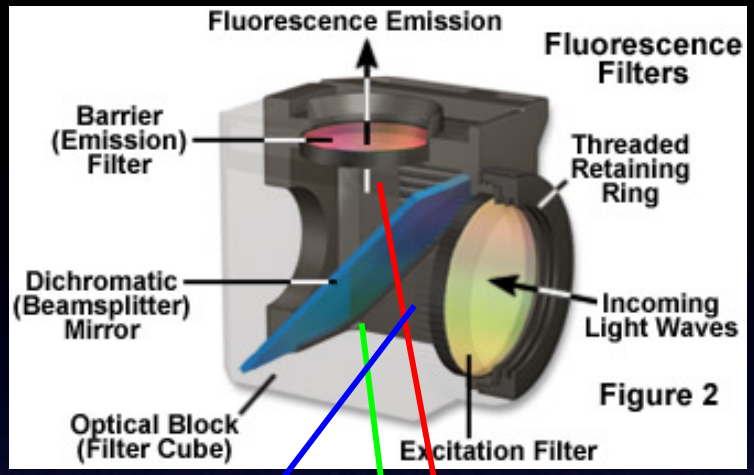
Brad Amos, MRC, Cambridge

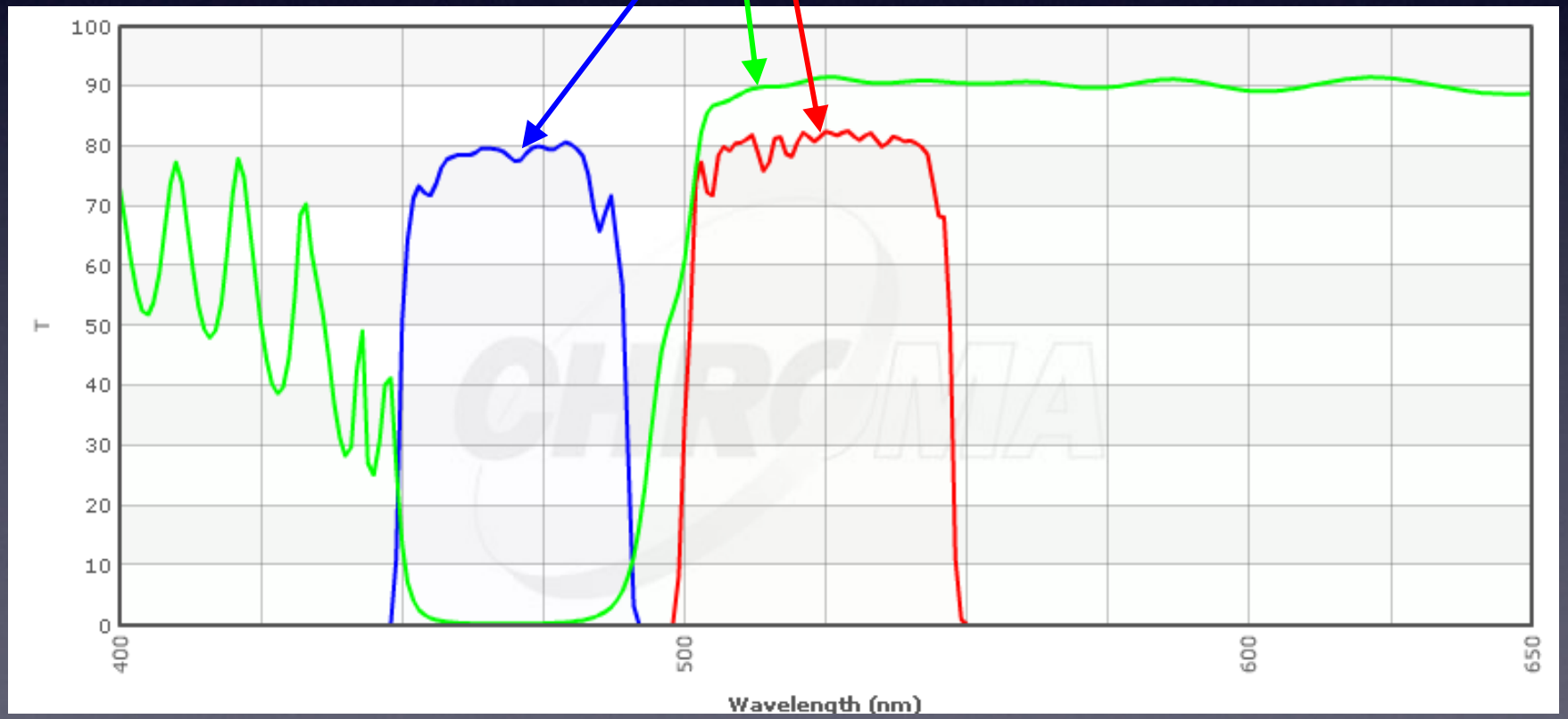
The Epifluorescence Microscope





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
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40
30
20
10
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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

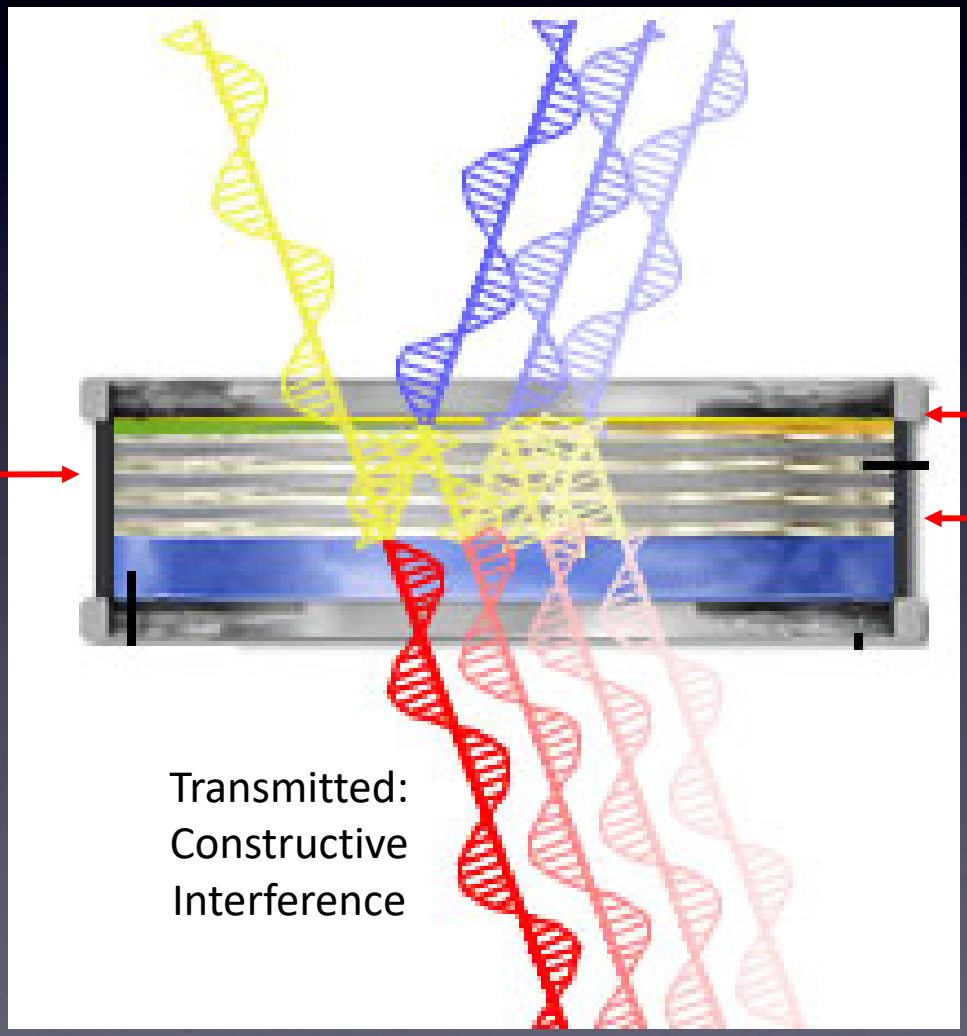
- Cheap
- Sturdy
- Independent of angle of incidence
- Small selection
- Spectra have poor slope and poor peak performance
- Autofluorescence
- Absorb Get Hot

→

Interference Filters

Incident Light

Reflected: Destructive Interference



Transparent layer

Semi-reflective coatings

Transmitted: Constructive Interference

Vendors:

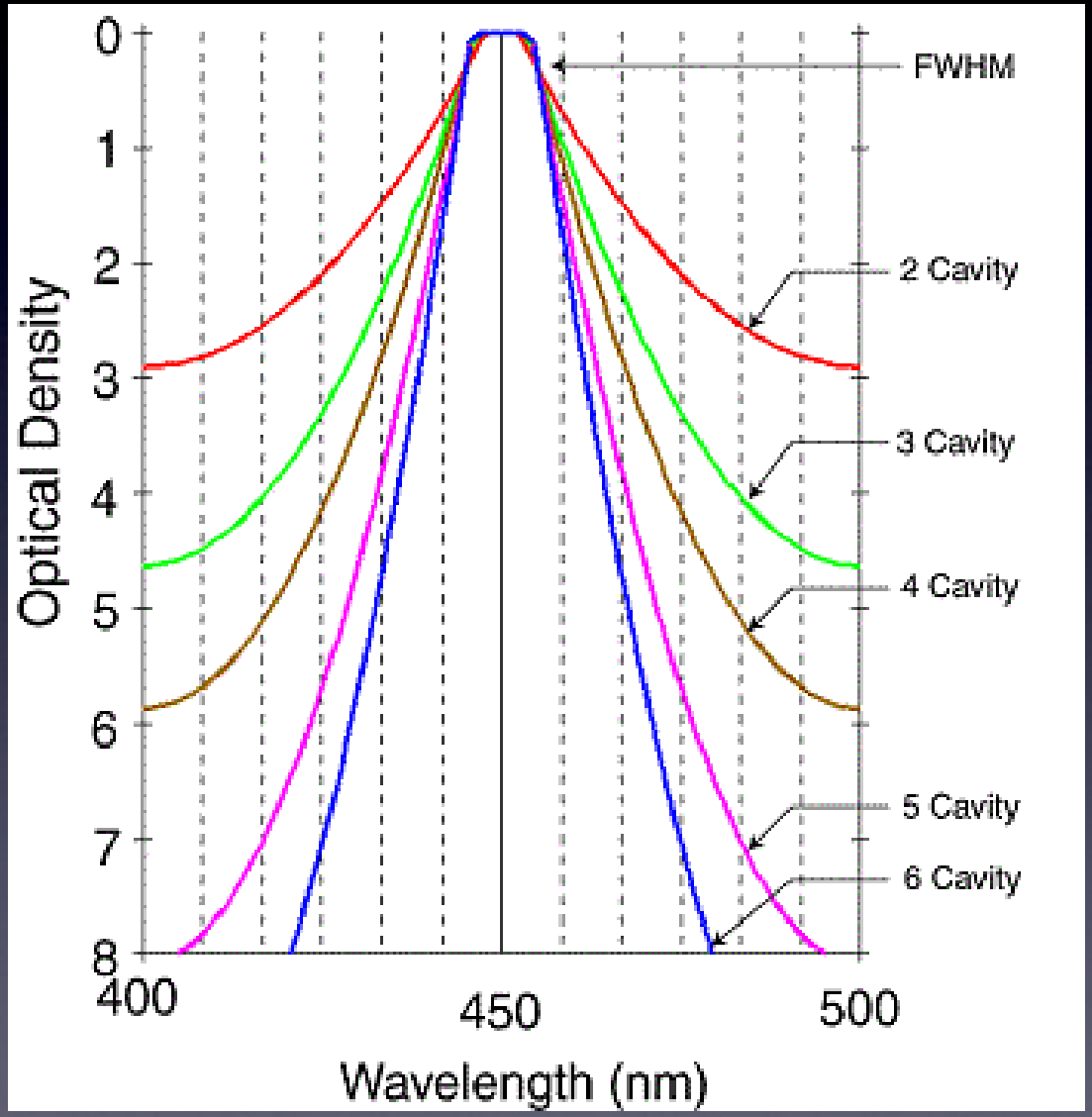
Chroma

Semrock

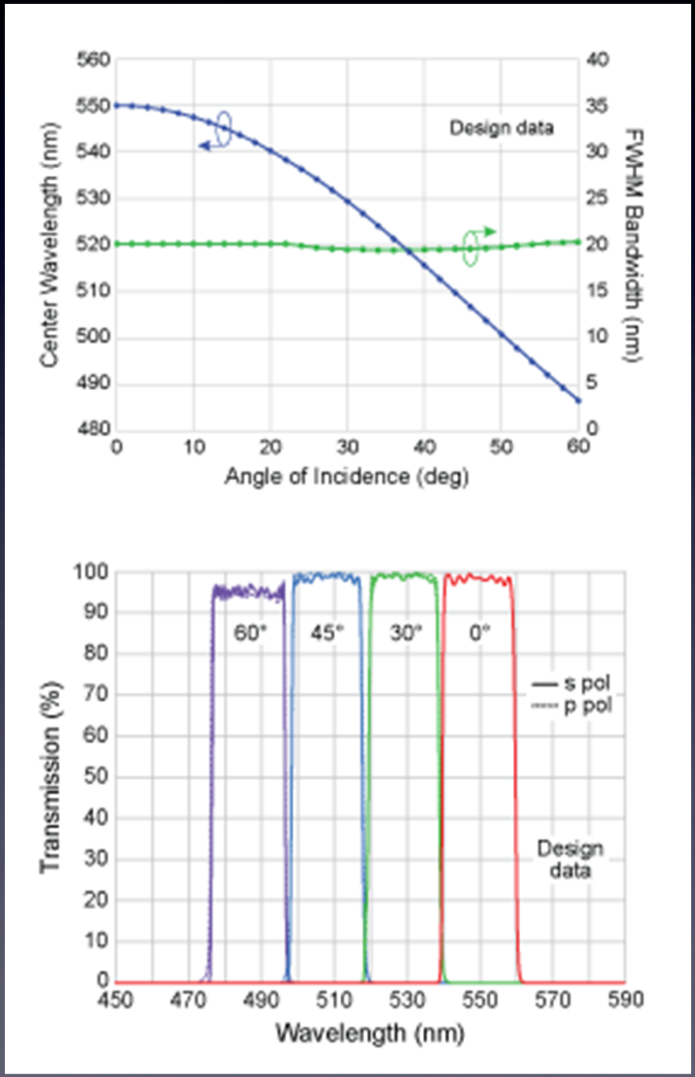
Omega

Interference Filter Design

(multiple cavities)



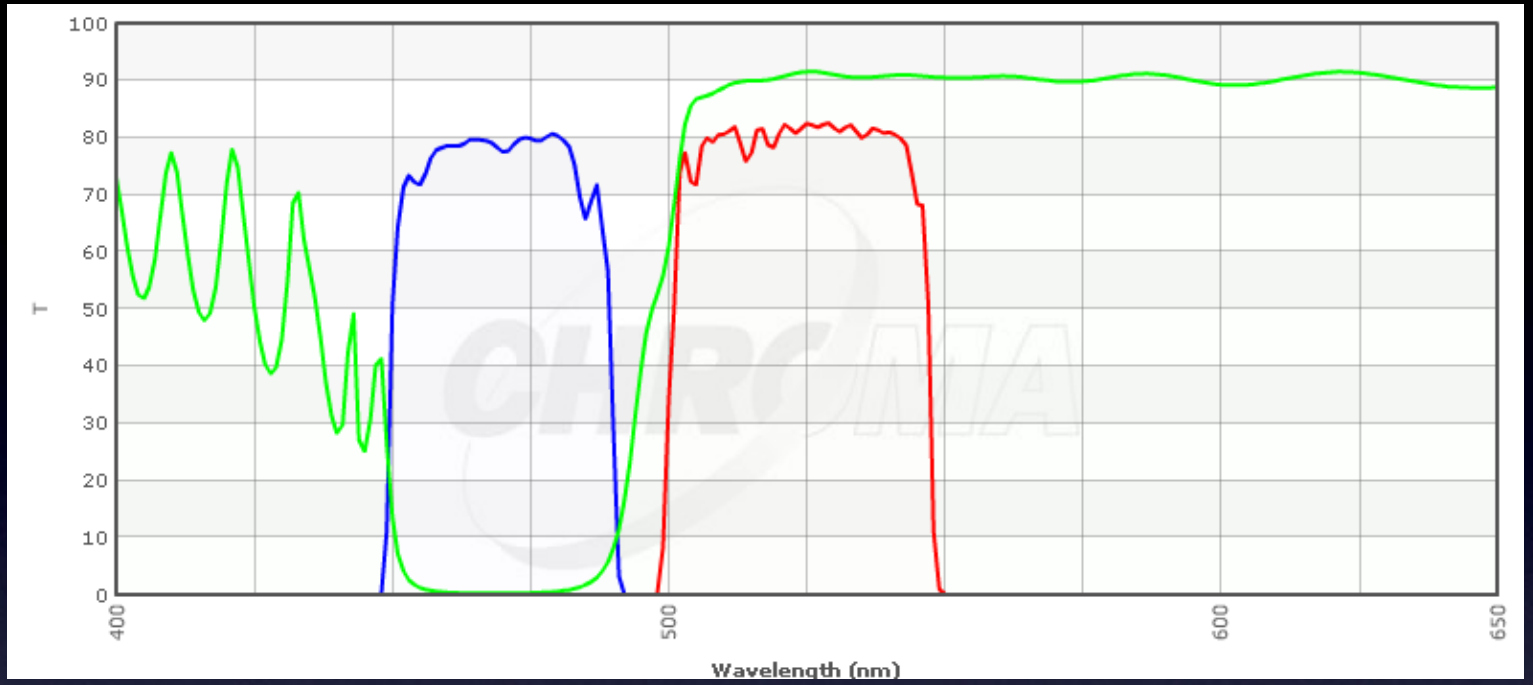
Highly sensitive to angle



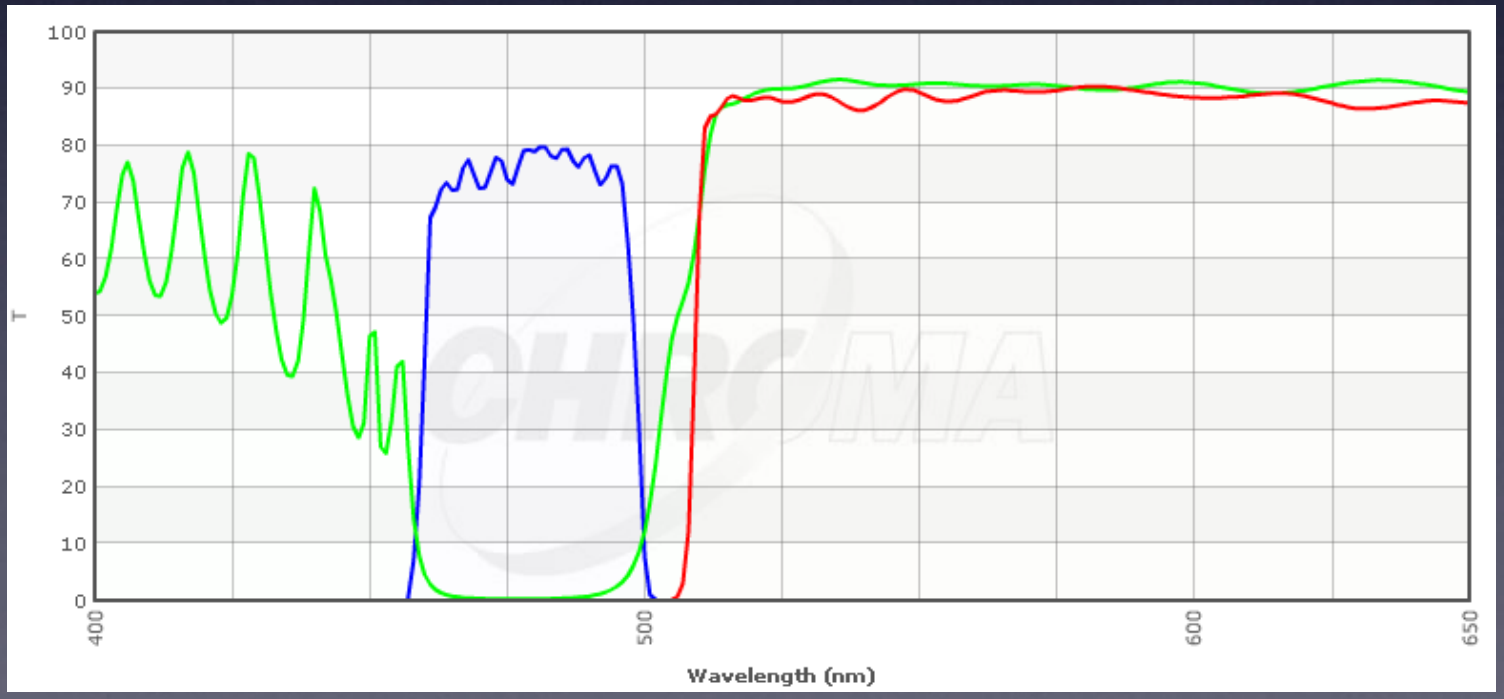
Positive spin on this pitfall:
Tunable Bandpass Filters!

(Semrock *VersaChrome*)

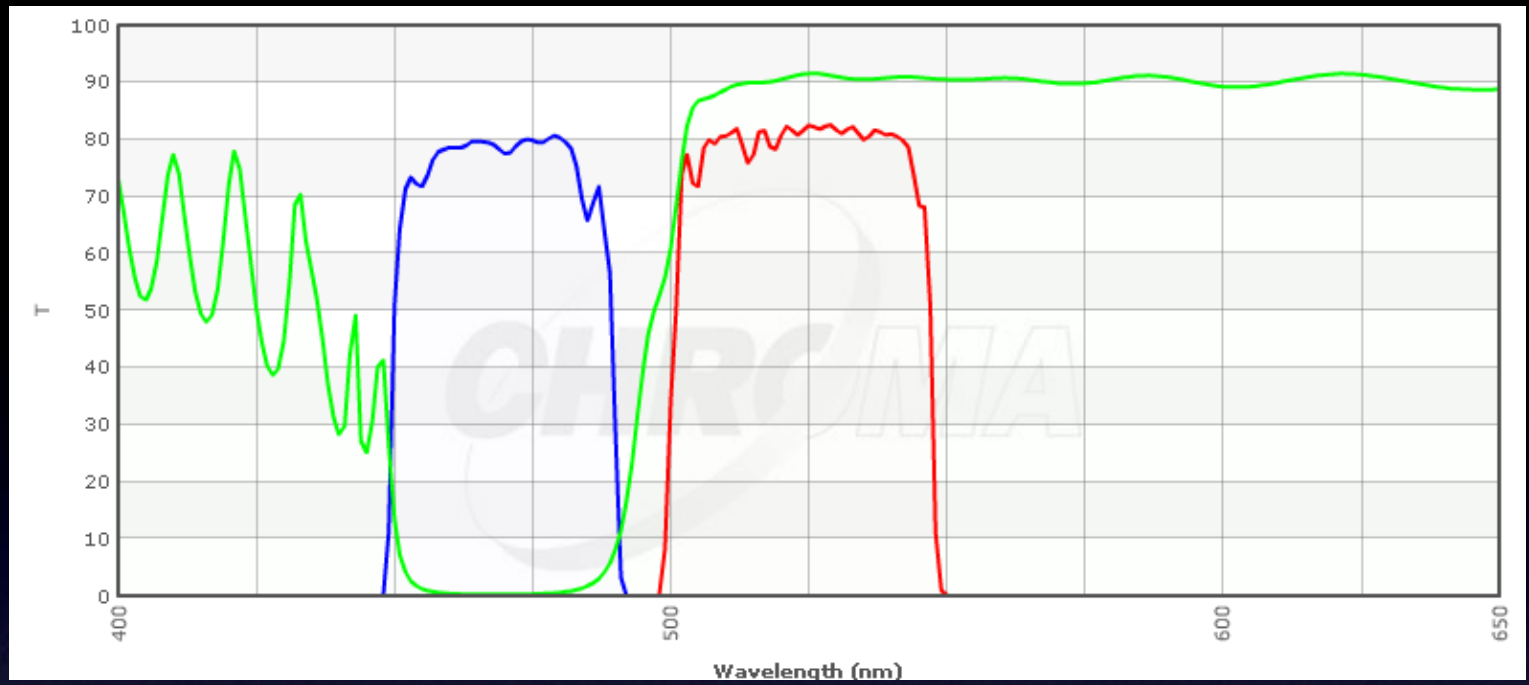
Bandpass



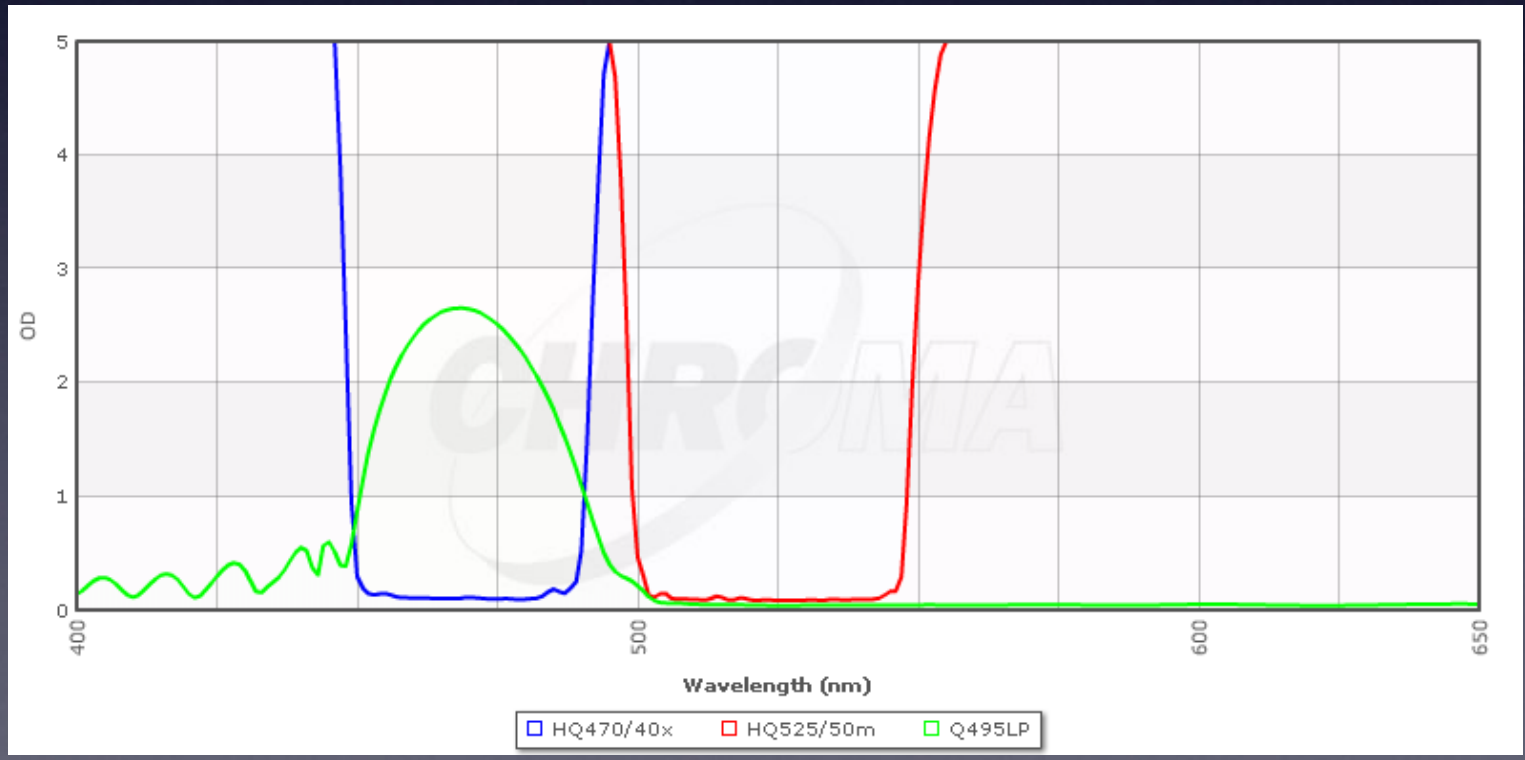
Longpass



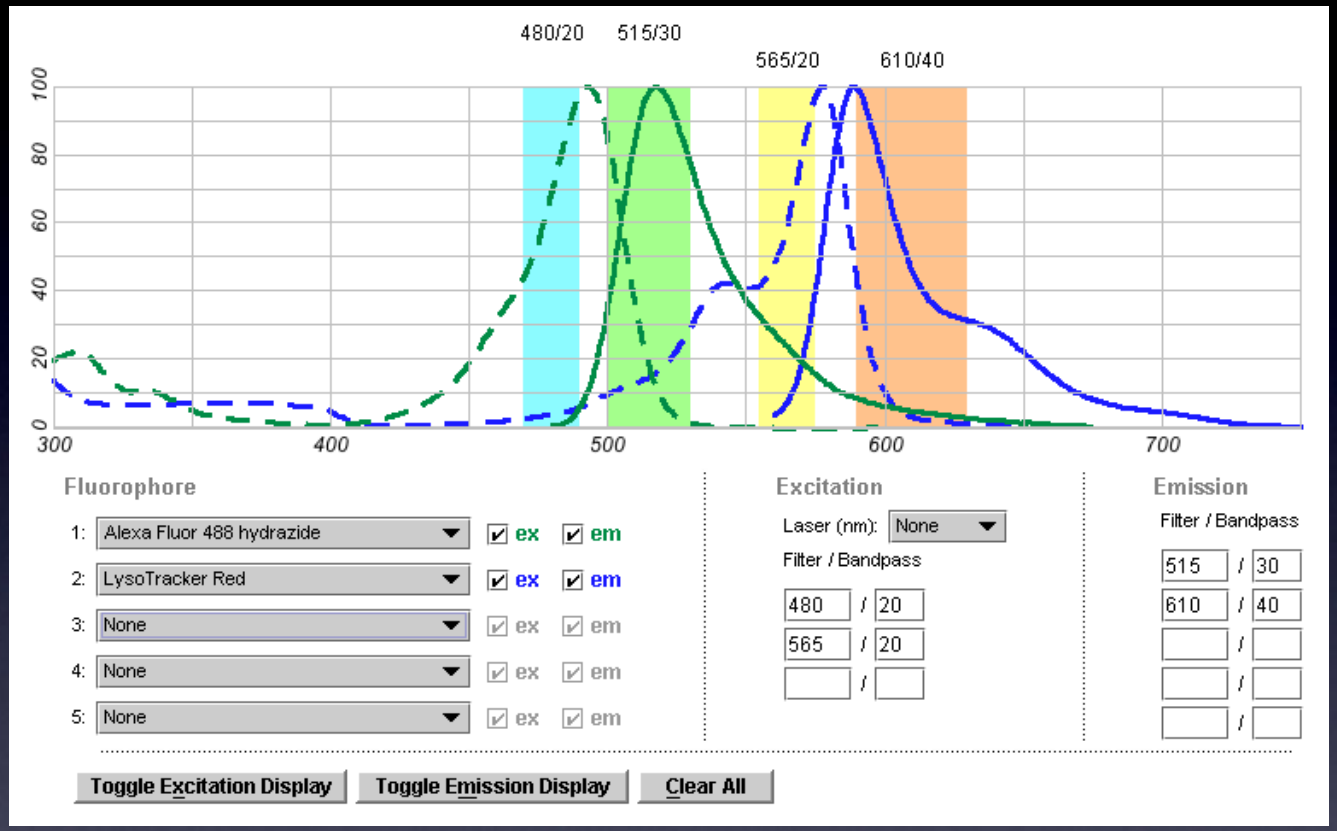
%T


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

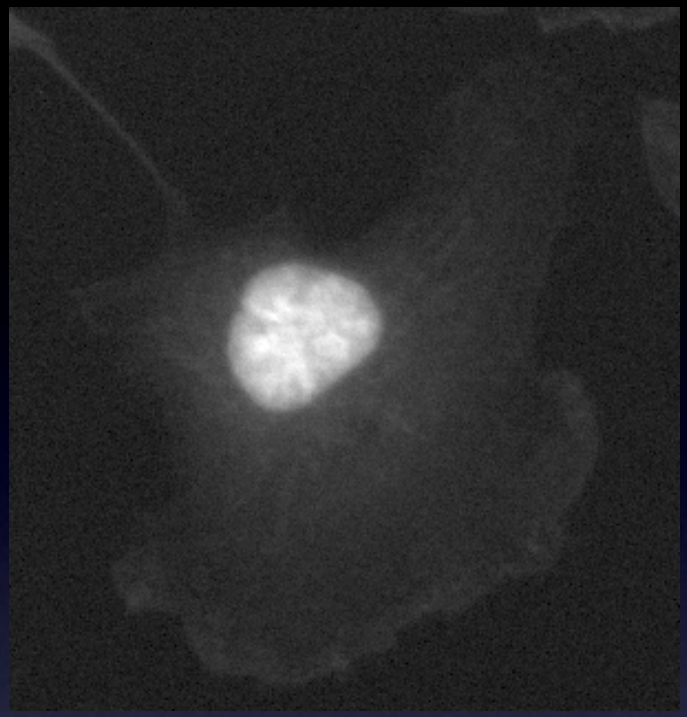


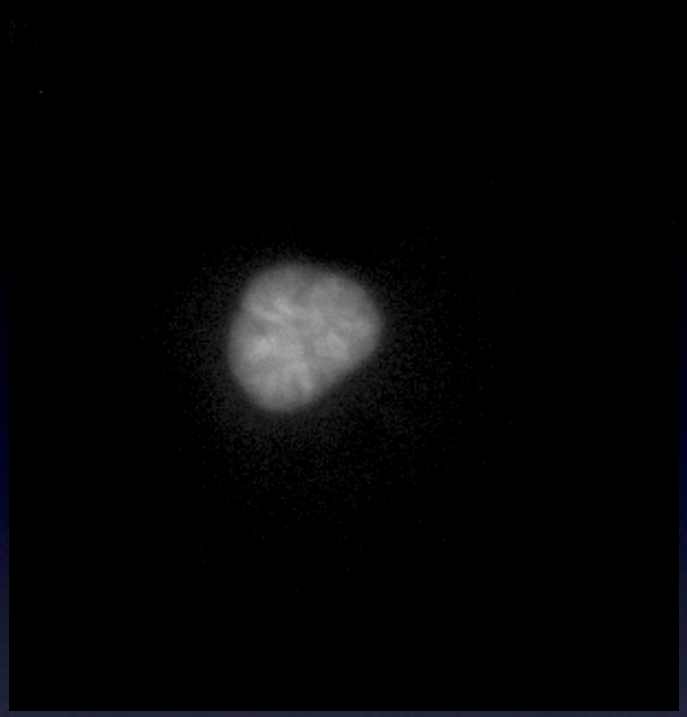
http://probes.invitrogen.com/resources/spectraviewer/

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

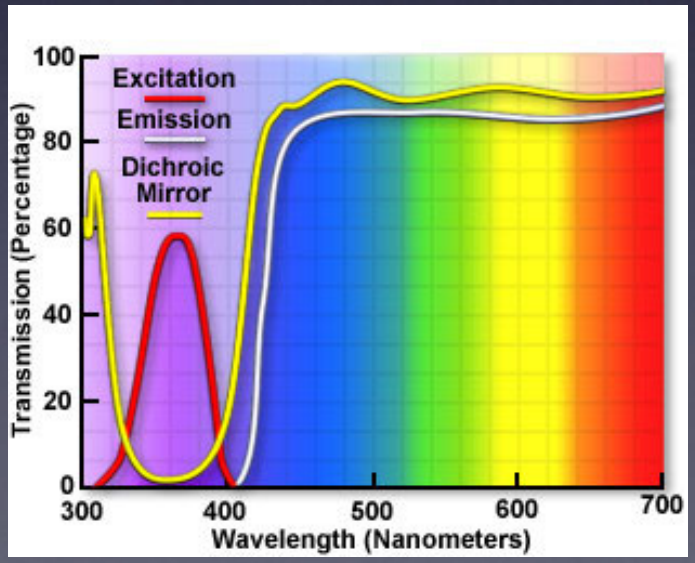
https://www.omegafilters.com/curvo2/index.php

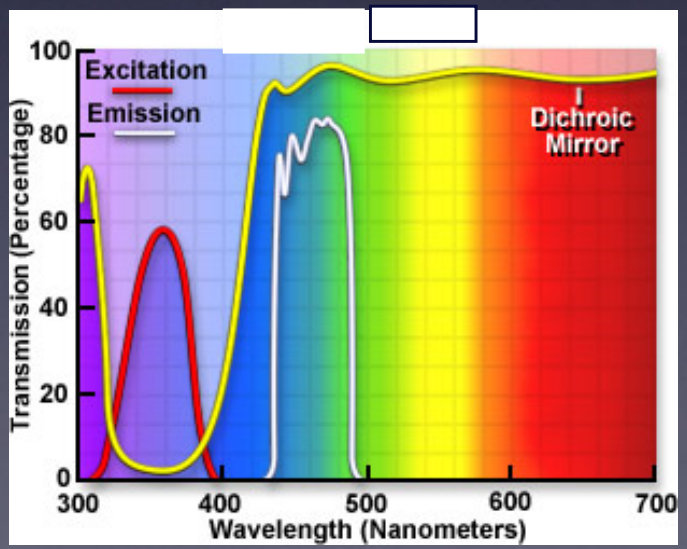
Choose filters that separate fluorophores





Two different UV filter sets





Choose filters that maximize excitation and emission

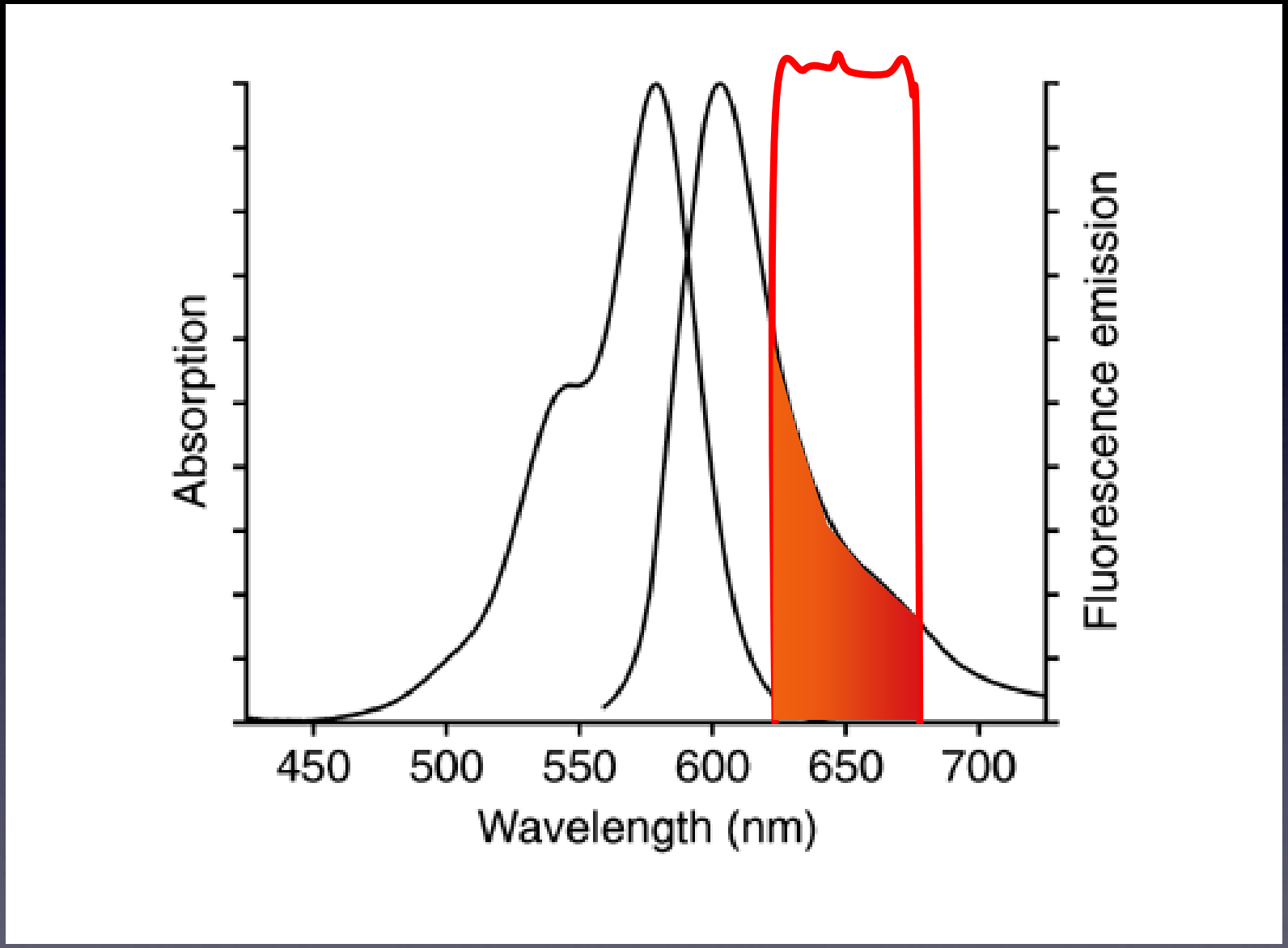


Image from www.probes.com

Choose filters that maximize excitation and emission

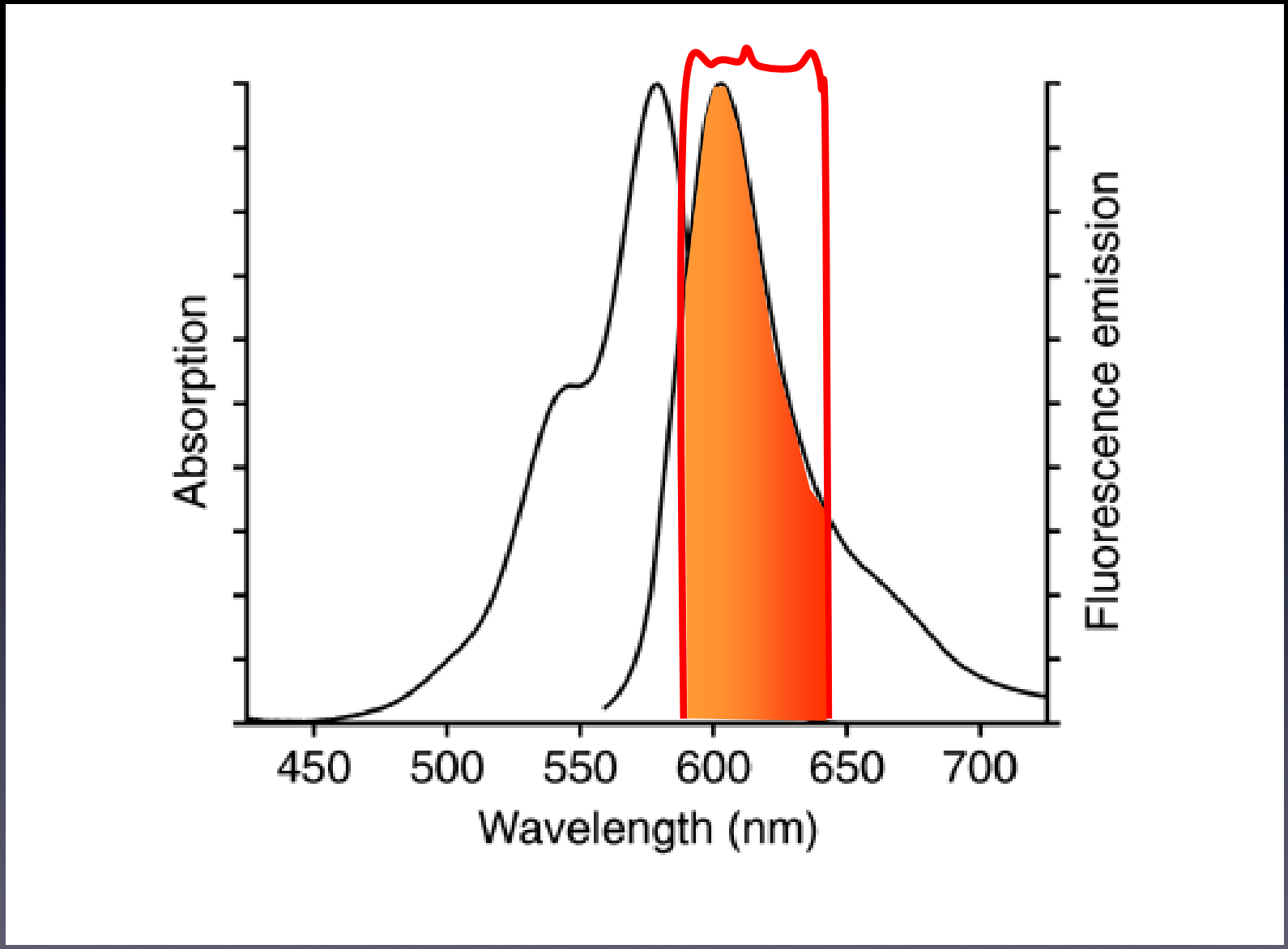
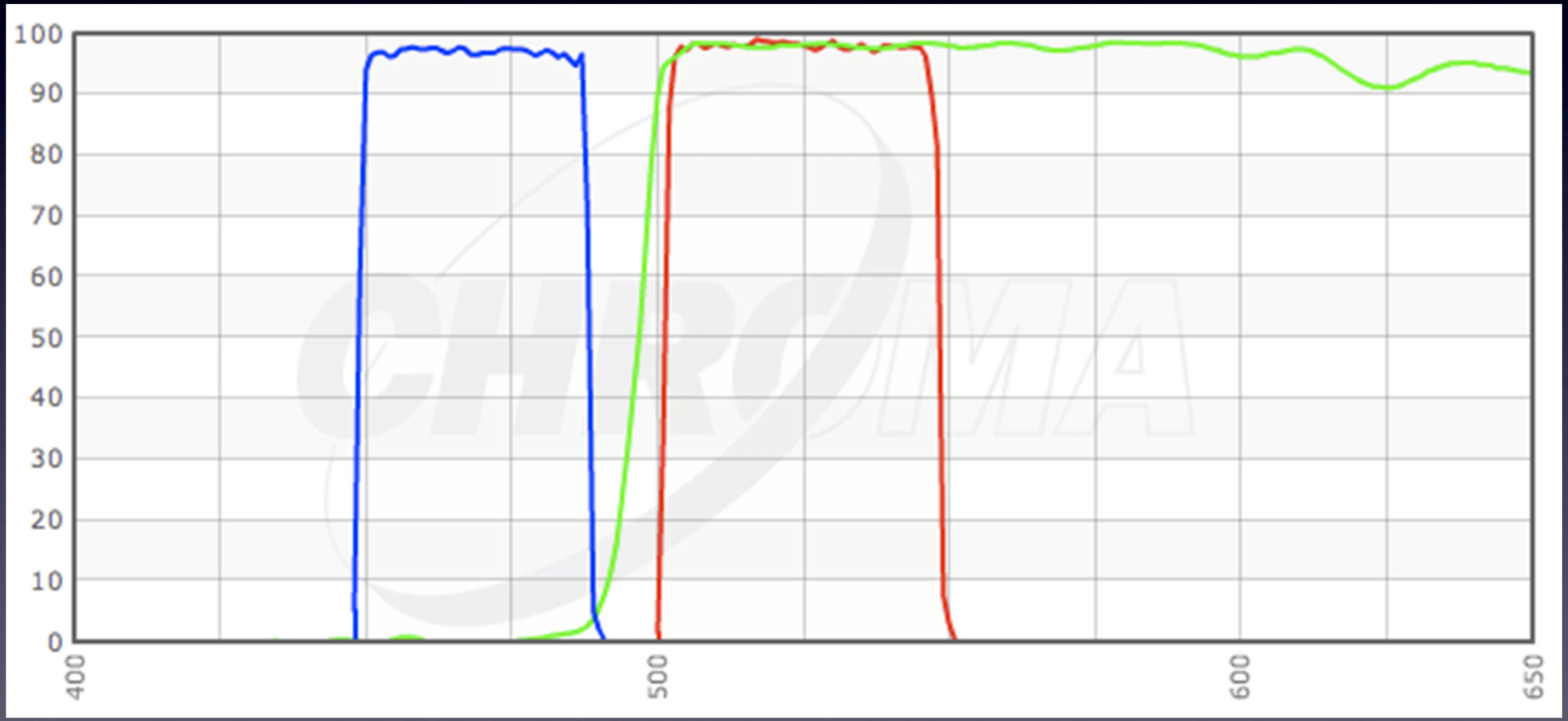


Image from www.probes.com

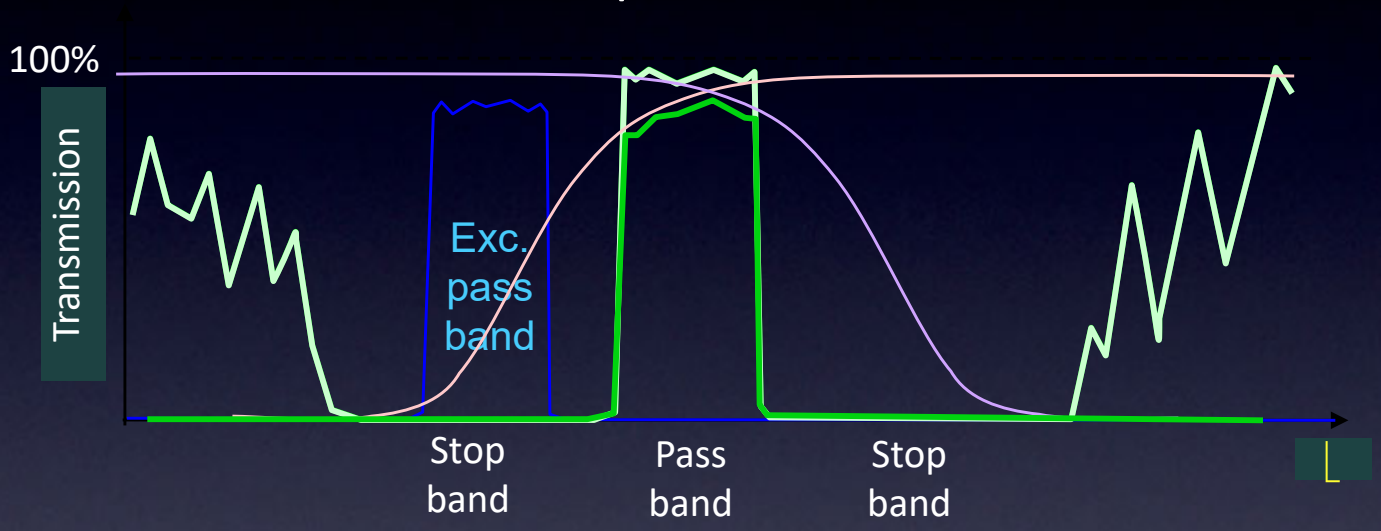
Newer hard-coatings are great!

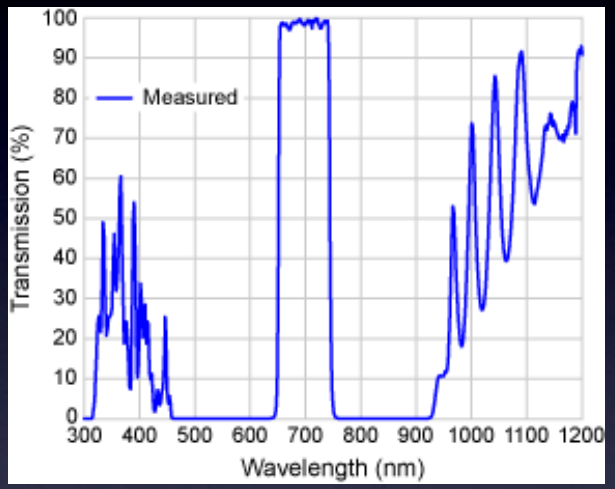


Blocking

Interference filters have finite stop bands

Unblocked bandpass interference filter





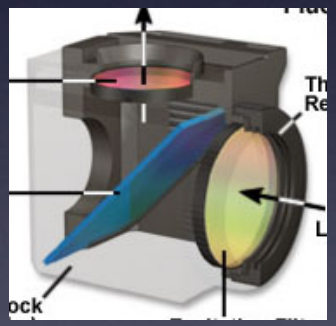
Semrock 697/75

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

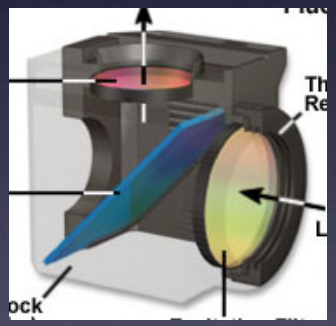
Often excitation filters are blocked,
but emission filters unblocked.
→ Red autofluorescence or room light
may get through your blue emission filter

UV absorber
Interference filter
IR absorber

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

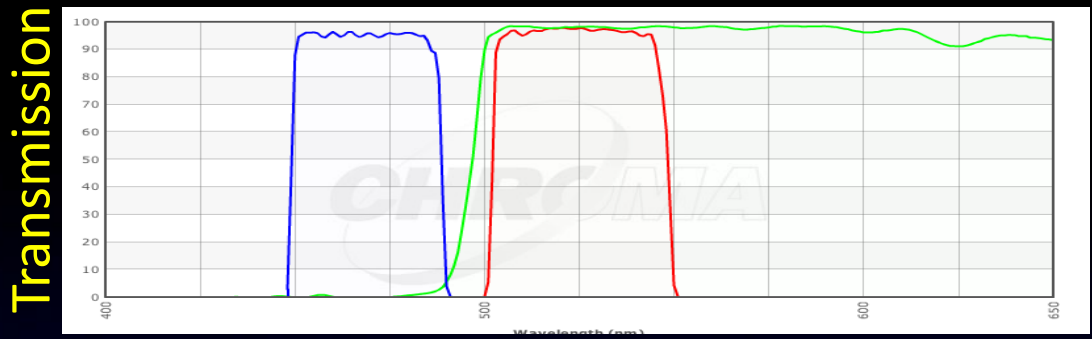


Faster Wavelength Selection: Multiple Band Pass Filters & Filter Wheel(s)

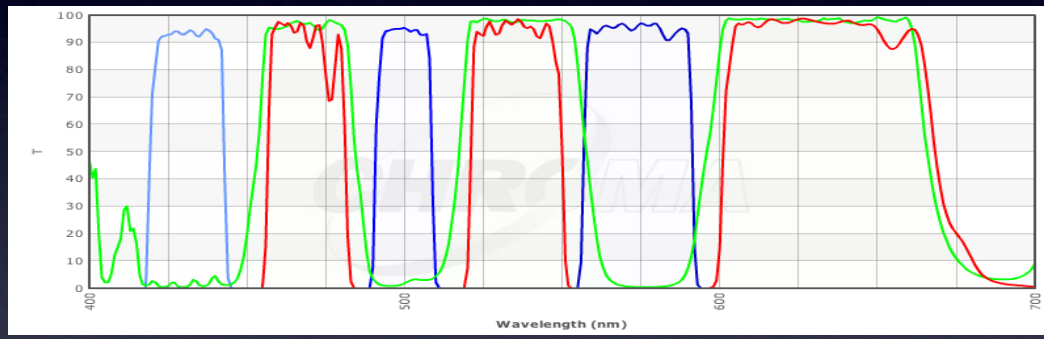


Filter schemes

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

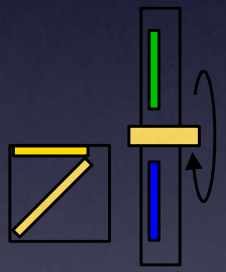


- "Pinkel" scheme

Multi-band dichroic

Multi-band emitter

Single-⌞ exciters



Chroma triple Pinkel set

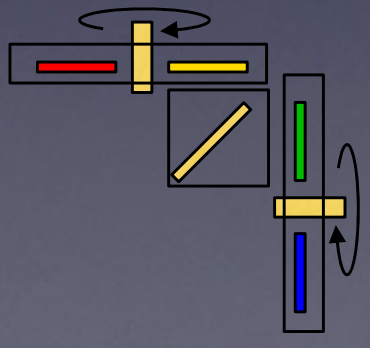
- Excitaton filter wheel
- Separate image at each wavelength
- Better separation

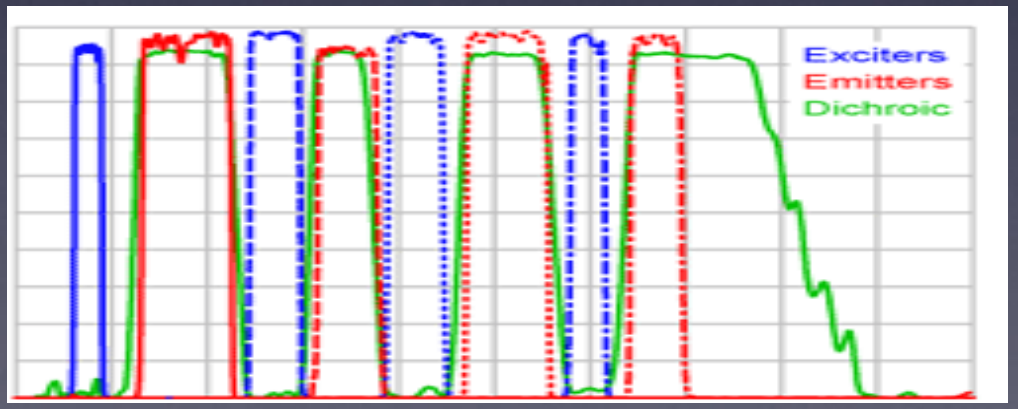
- "Sedat" scheme

Multi-band dichroic

single-band emitters

Single-⌞ exciters

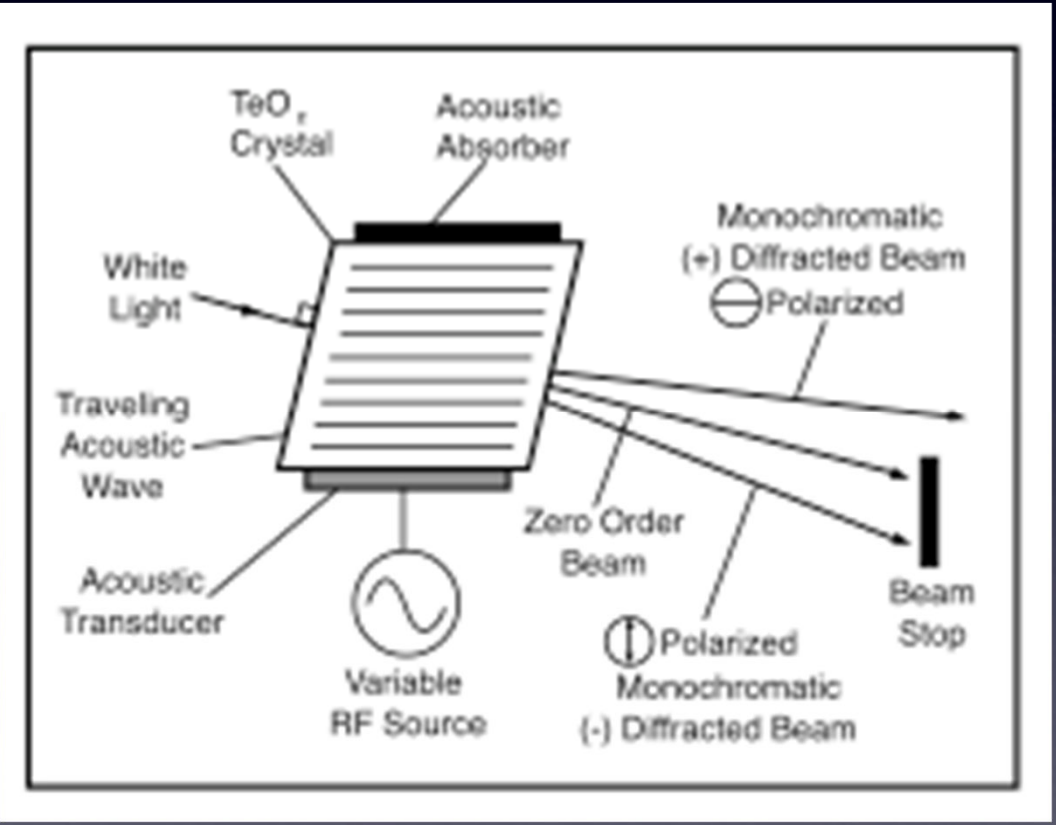




Semrock quad Sedat set

- Two filter wheels
- Even better separation

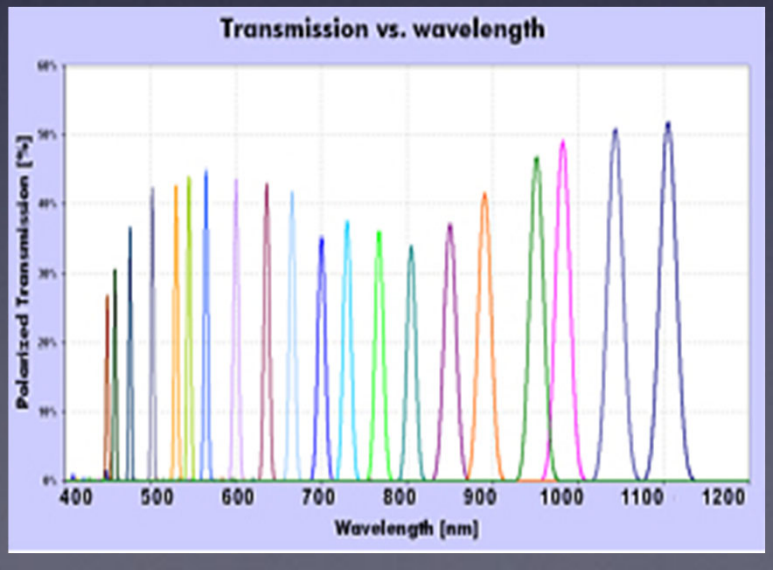
Acoustical Optical Tunable Filter



- Switches and modulates intensity
- Fast! (sub-microseconds)
- Mainly used for excitation laser light
- Polarization dependent

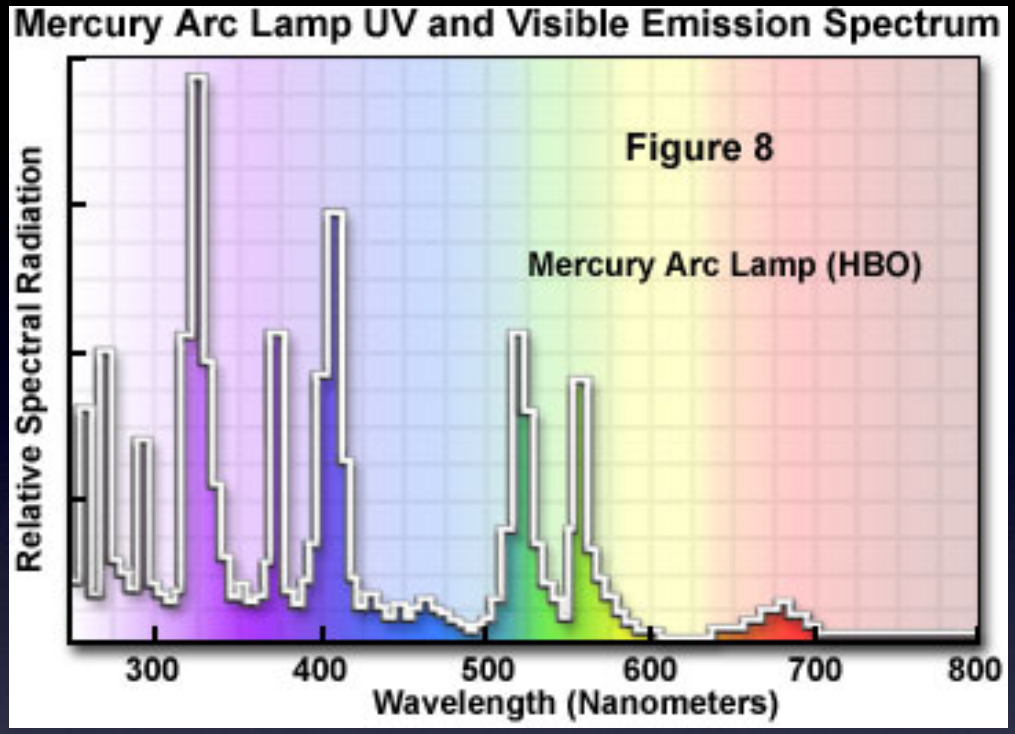
Also: AOM or Bragg cell

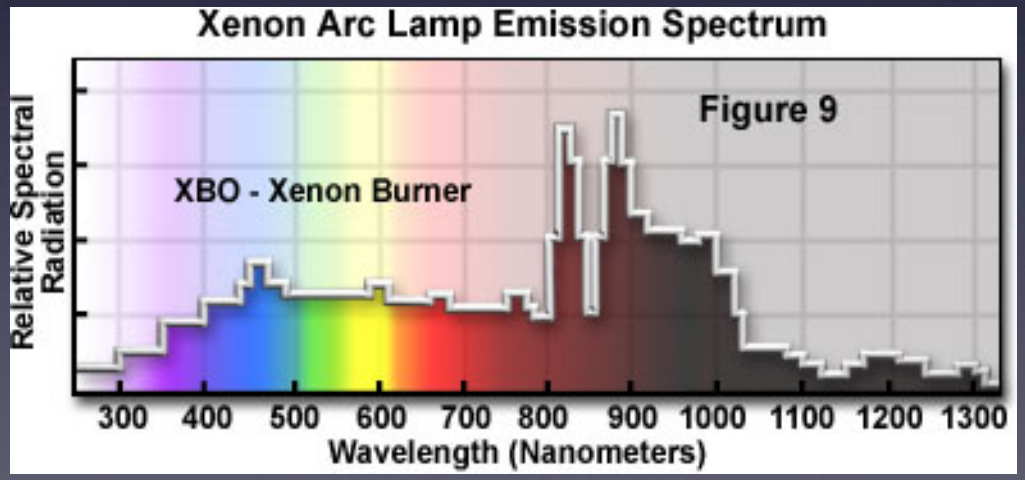
Liquid Crystal Filters



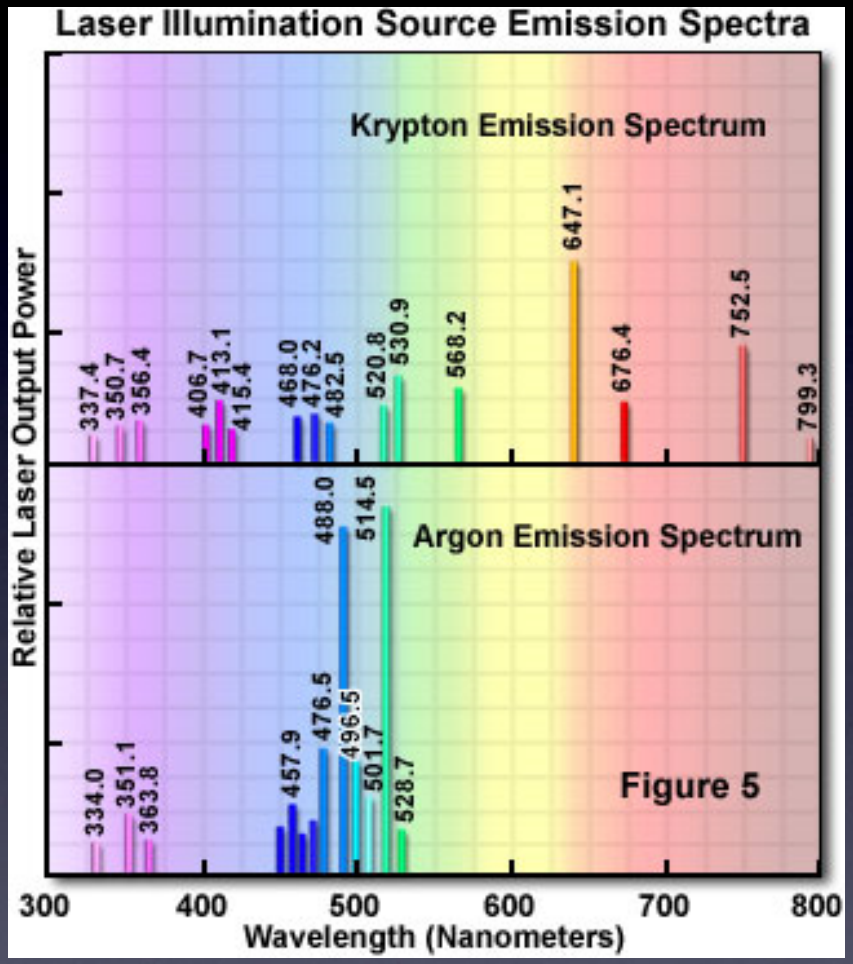
- Example: Lyot filter: Uses Birefringence and polarizers
- Shifts in (100) ms time range
- Maximum transmission is 50%, blocking max 10^{-5}

Light source spectra





LEDs are here!



Solid-state lasers: many, many lines available

Koehler illumination

Thanks!

- Mats Gustafsson
- Kurt Thorn
- Jennifer Waters
- http://micro.magnet.fsu.edu/
- http://www.microscopyu.com
- http://olympusmicro.com
- http://zeiss-campus.magnet.fsu.edu/
- http://www.chroma.com (Filter Handbook!)