

Fluorescence Microscopy

I. Fluorescent optics



Bo Huang
2013.03.25



Discovery of Fluorescence

Sir John Herschel



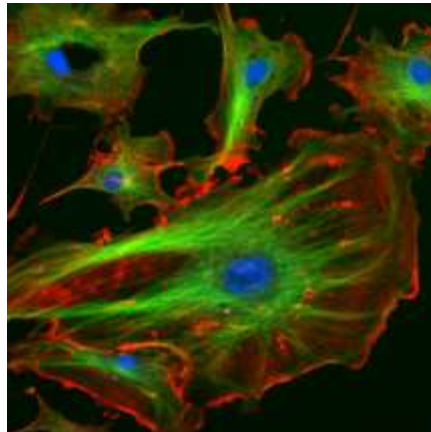
1845

G.G. Stokes

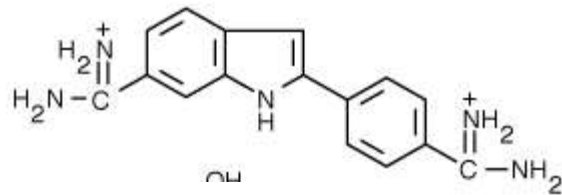


1852

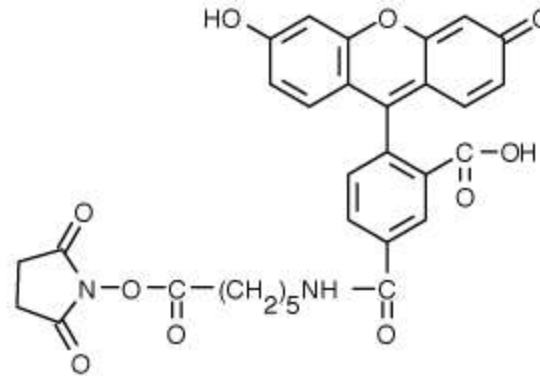
Things that fluoresce



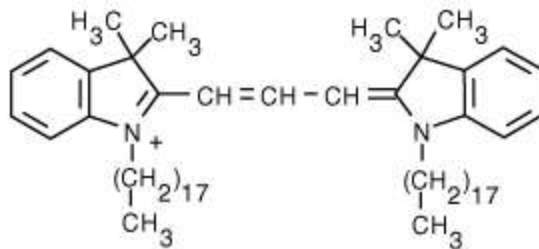
Molecules that fluoresce



DAPI (DNA stain)



Fluorescein (protein labeling)

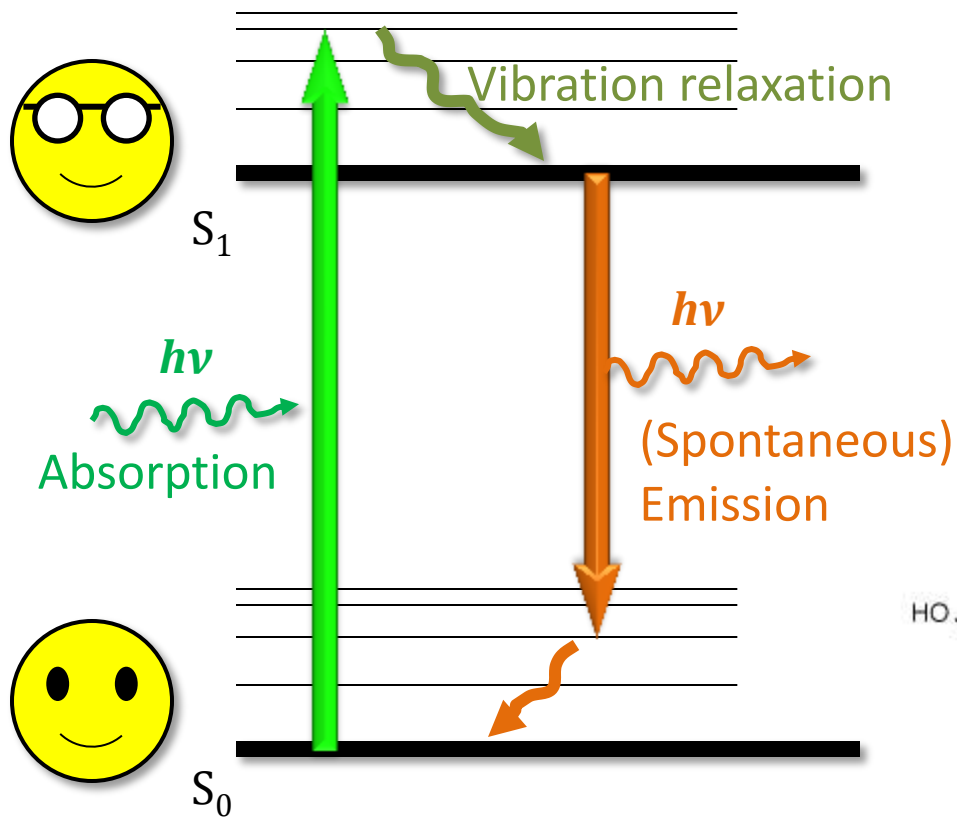


Dil (plasma membrane stain)

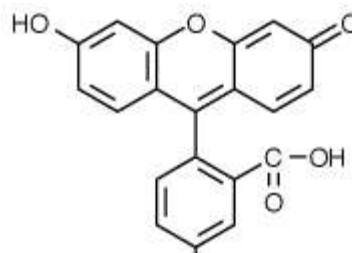


GFP (fluorescent protein)

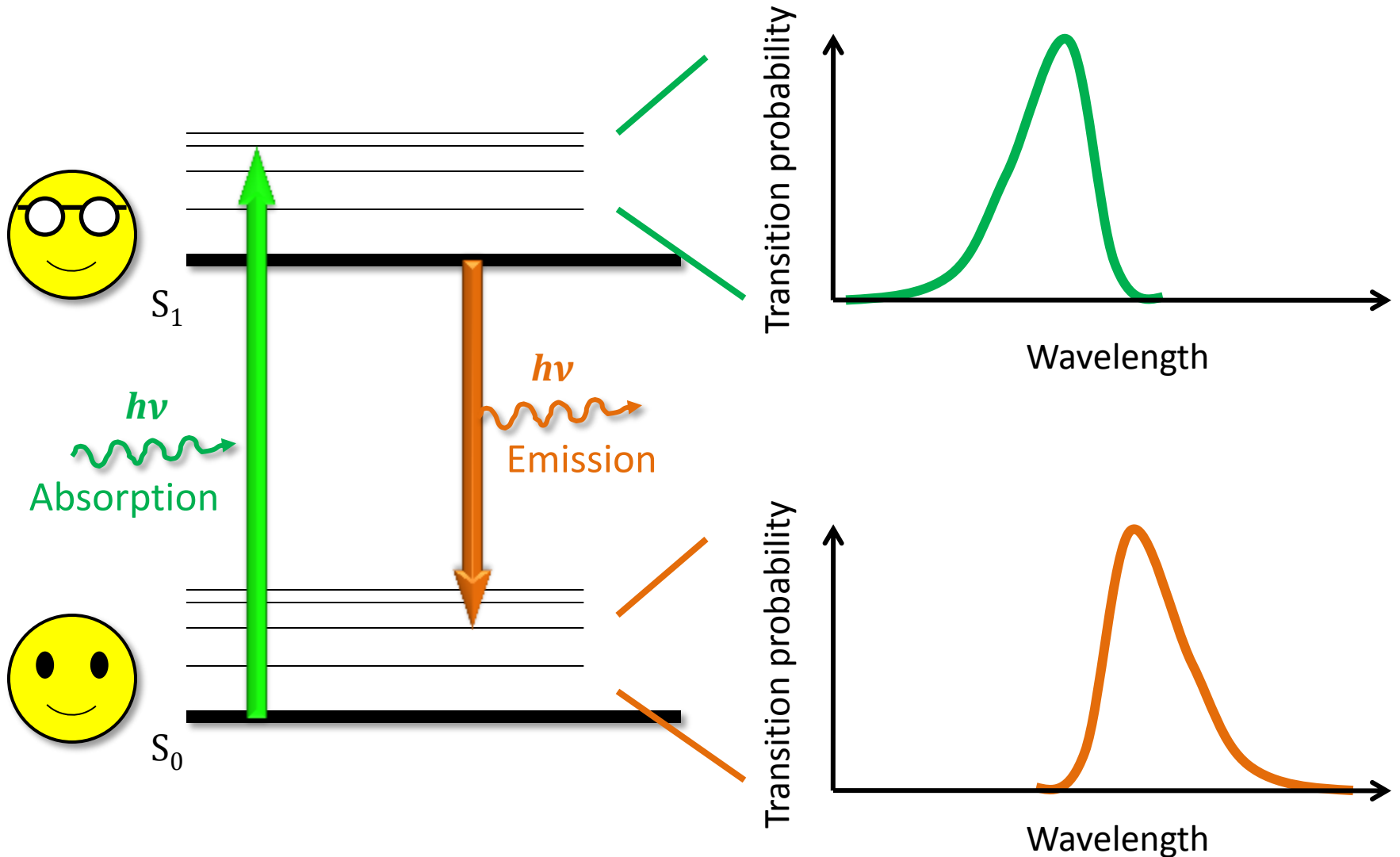
Jabłonski diagram



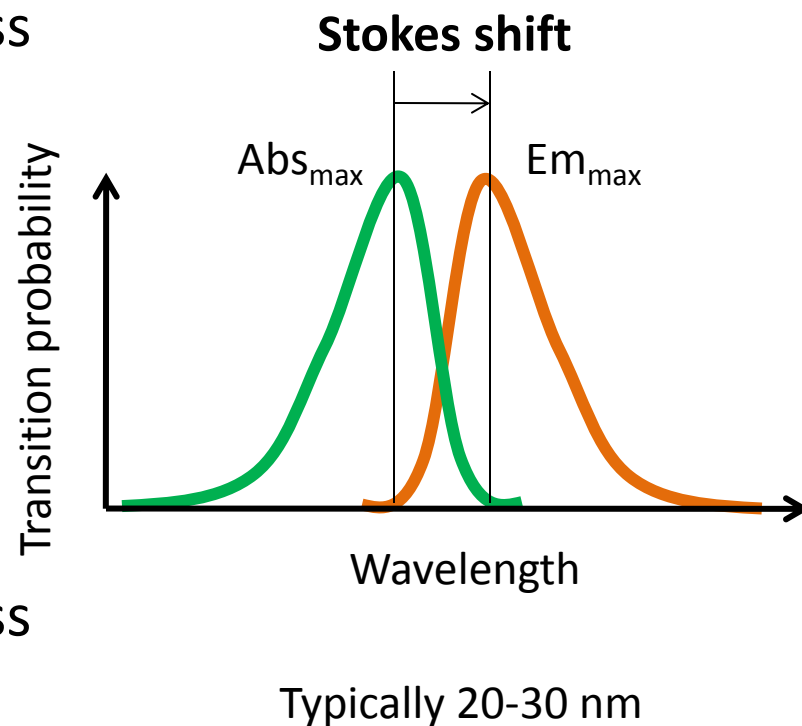
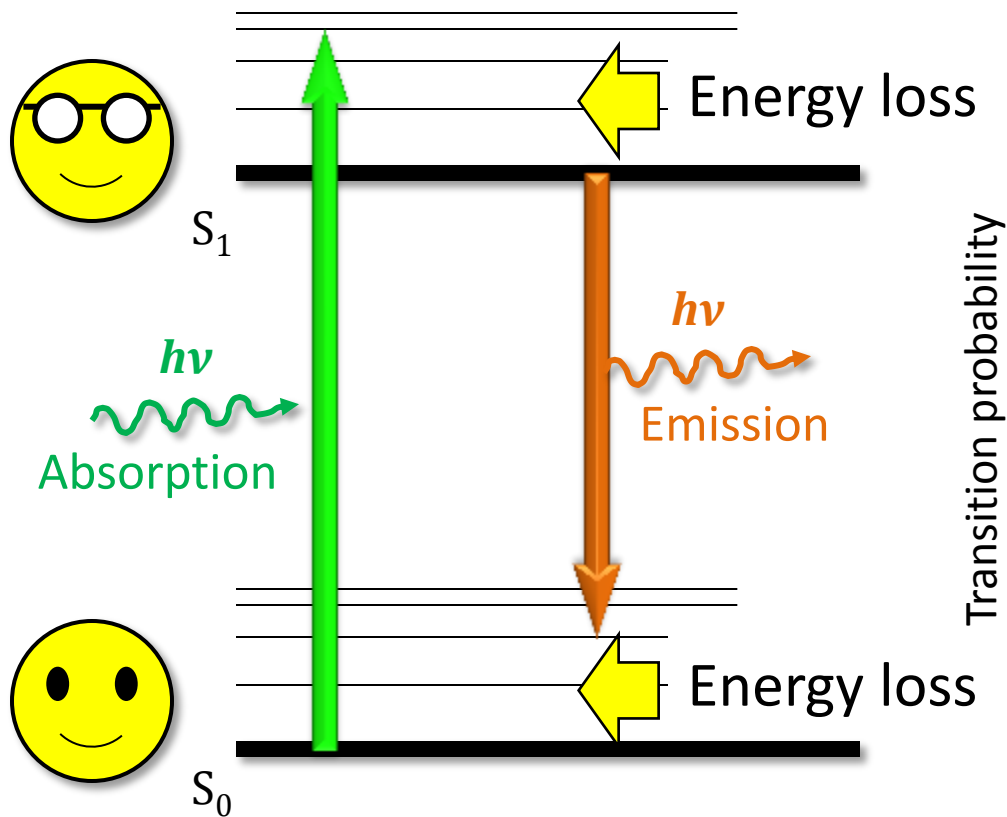
Alexander Jabłonski



Excitation and Emission Spectra

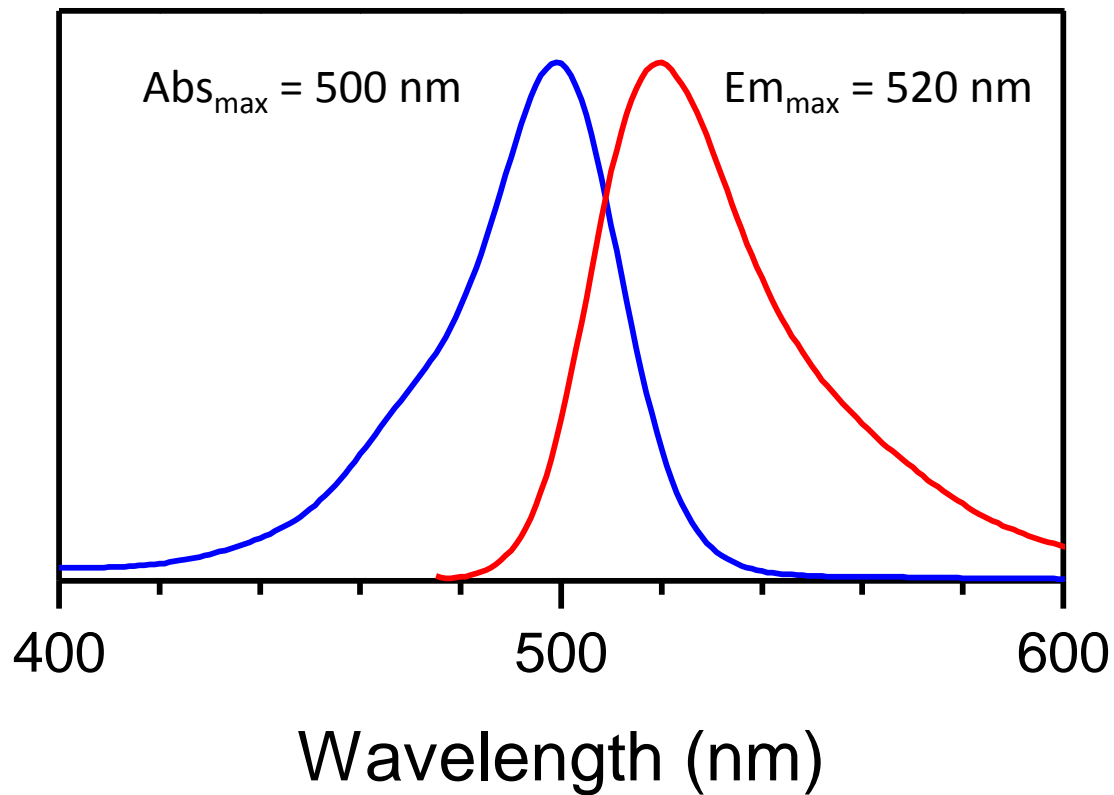


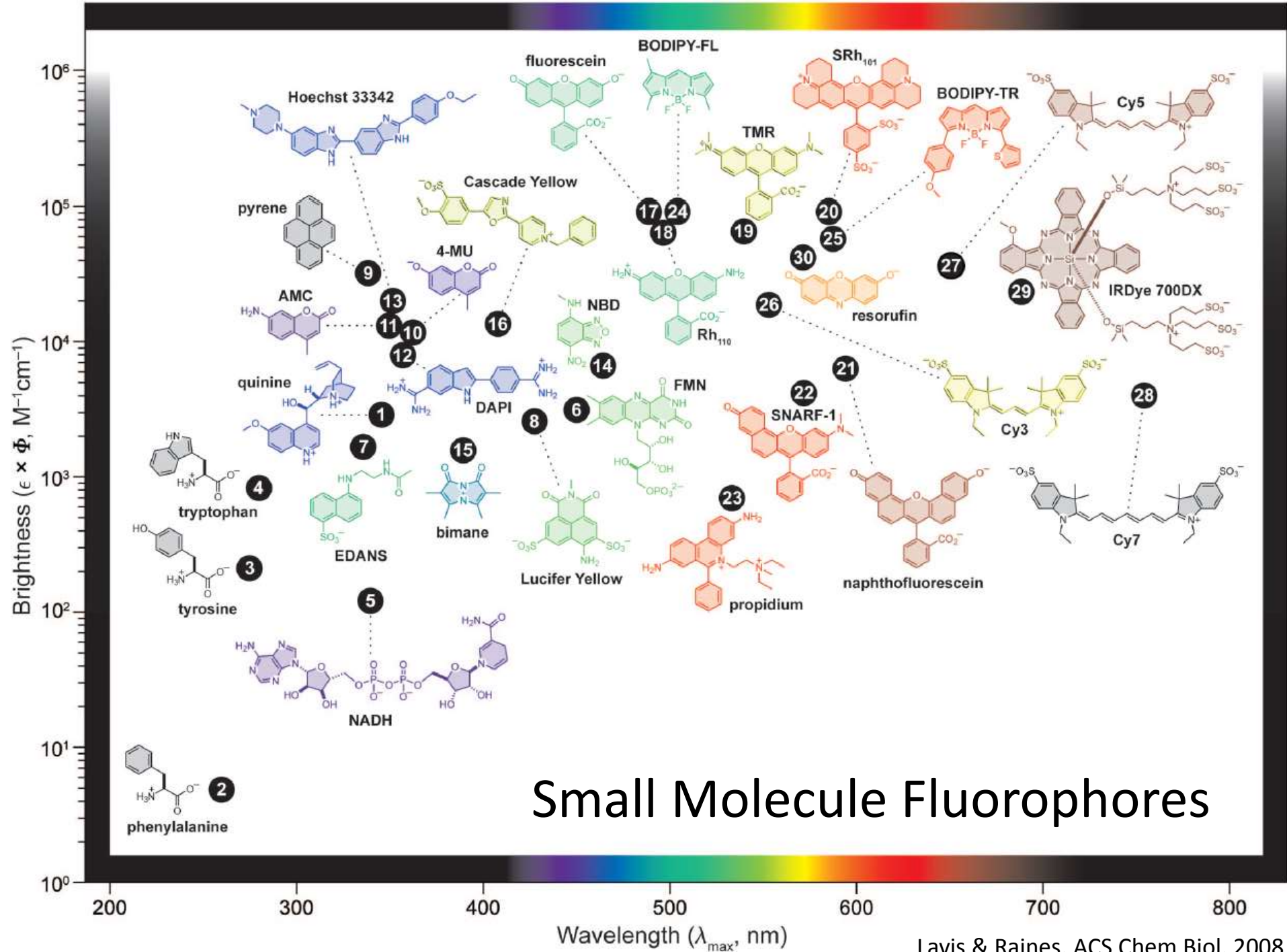
Stokes Shift



Excitation and emission wavelengths

Alexa Fluor 488





Fluorescent proteins

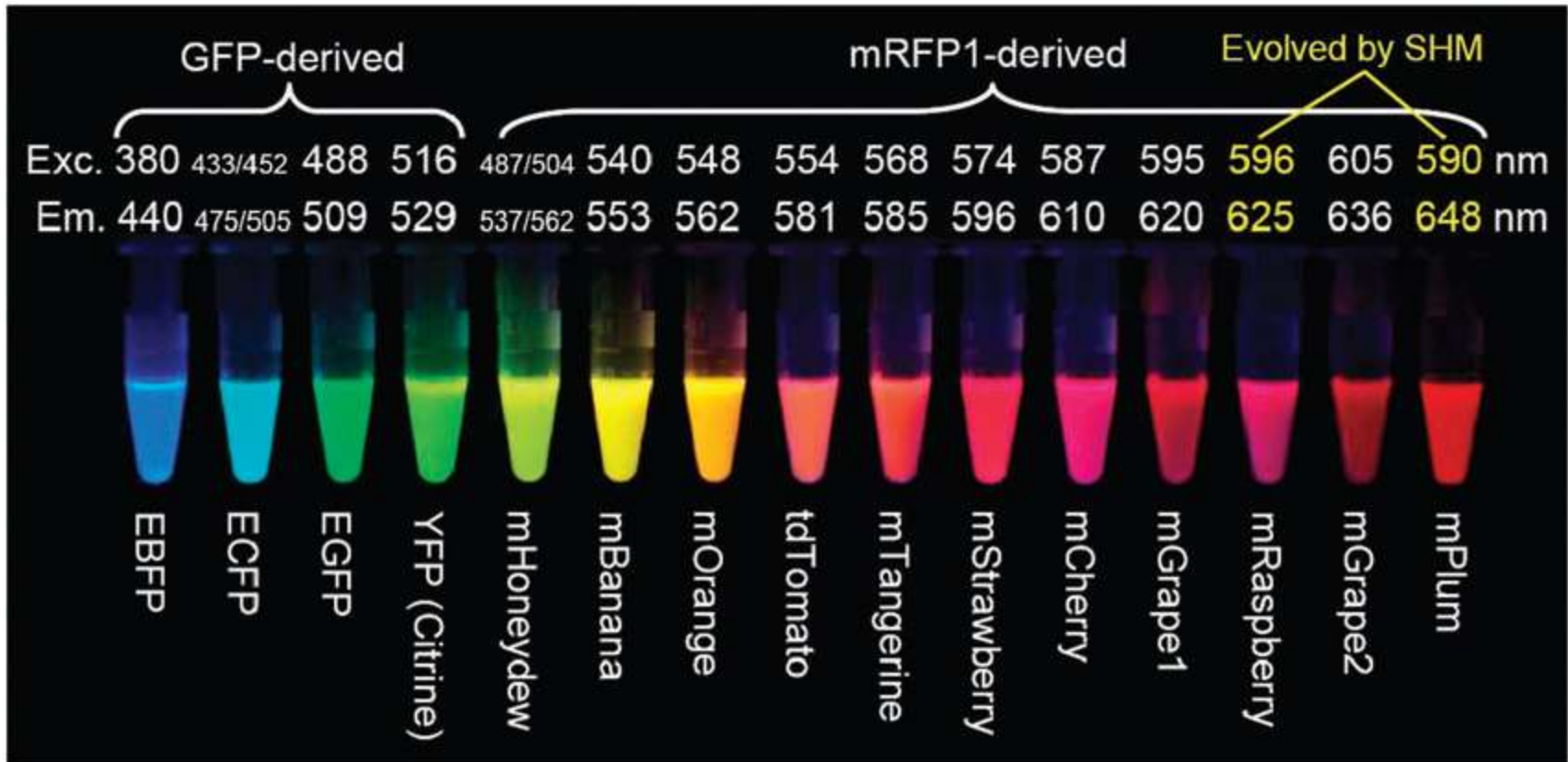
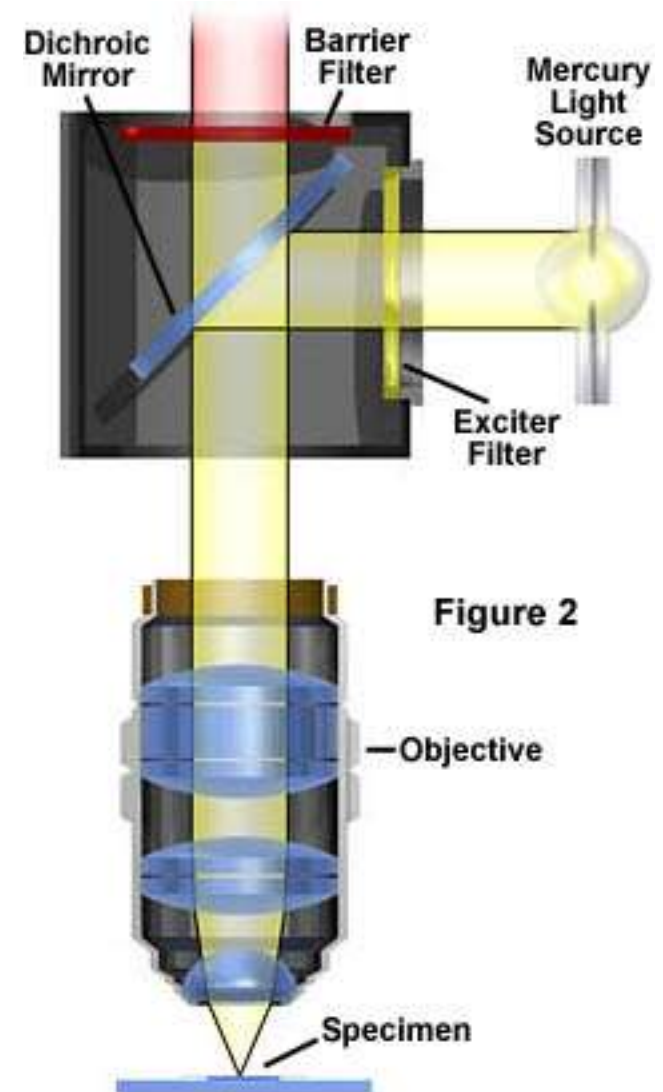
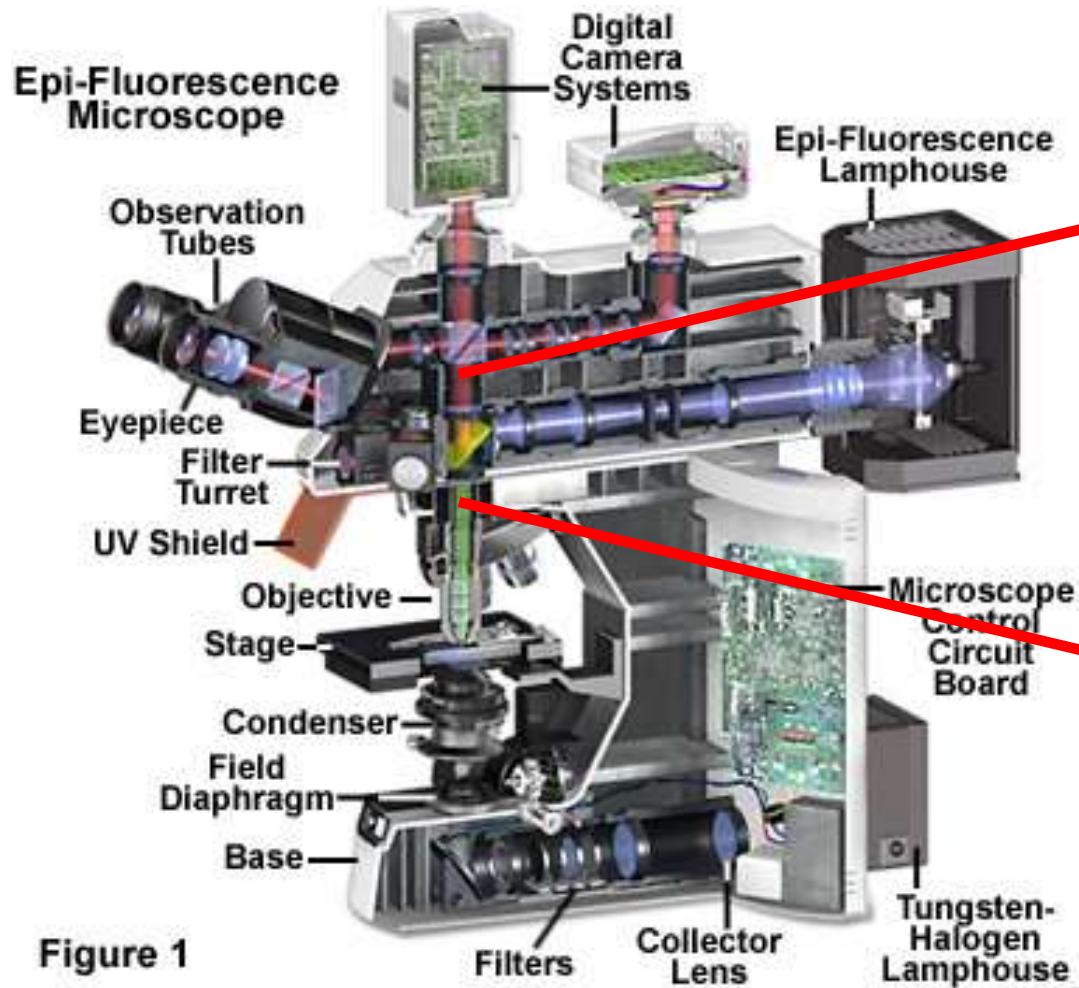


Image from Tsien lab

Fluorophore spectra viewers

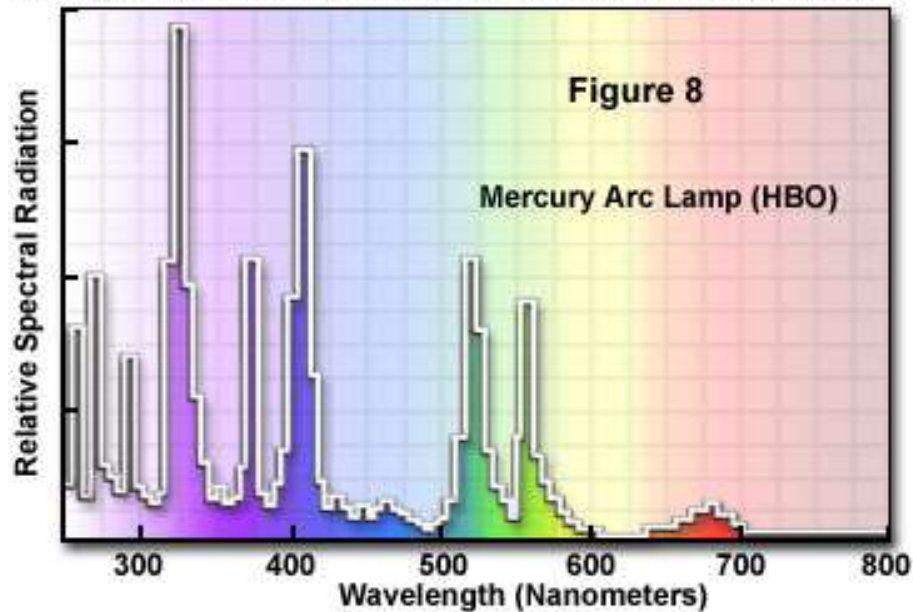
- **Invitrogen**
<http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html>
- **Omega**
<http://www.omegafilters.com/Products/Curvomatic>
- **Zeiss**
https://www.micro-shop.zeiss.com/us/us_en/spektral.php?cp_sid=&f=db
- **U Arizona MCB**
<http://www.mcb.arizona.edu/ipc/fret/index.html>

The Epifluorescence Microscope

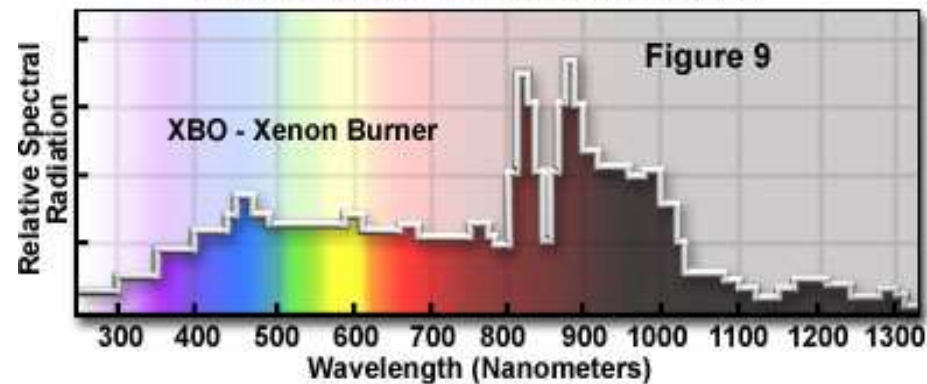


Excitation light sources – Lamps

Mercury Arc Lamp UV and Visible Emission Spectrum



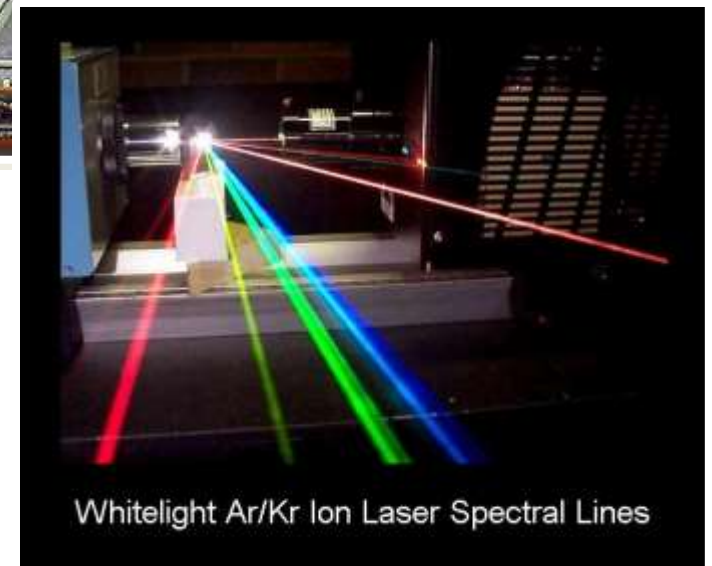
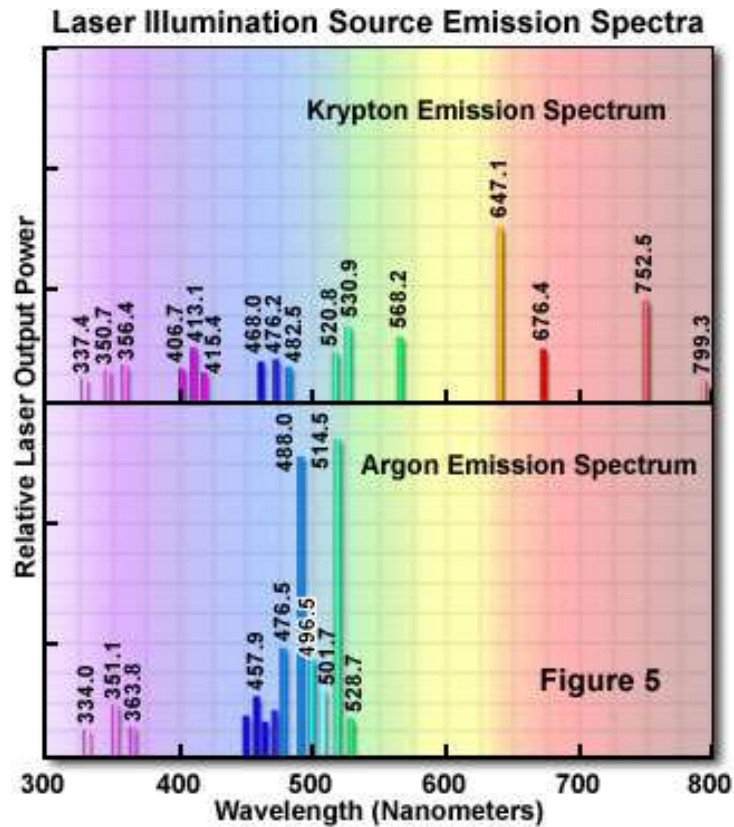
Xenon Arc Lamp Emission Spectrum



Excitation filter required.

Excitation light sources – Laser

Ar / Kr ion laser: 488, 514, 568, 647

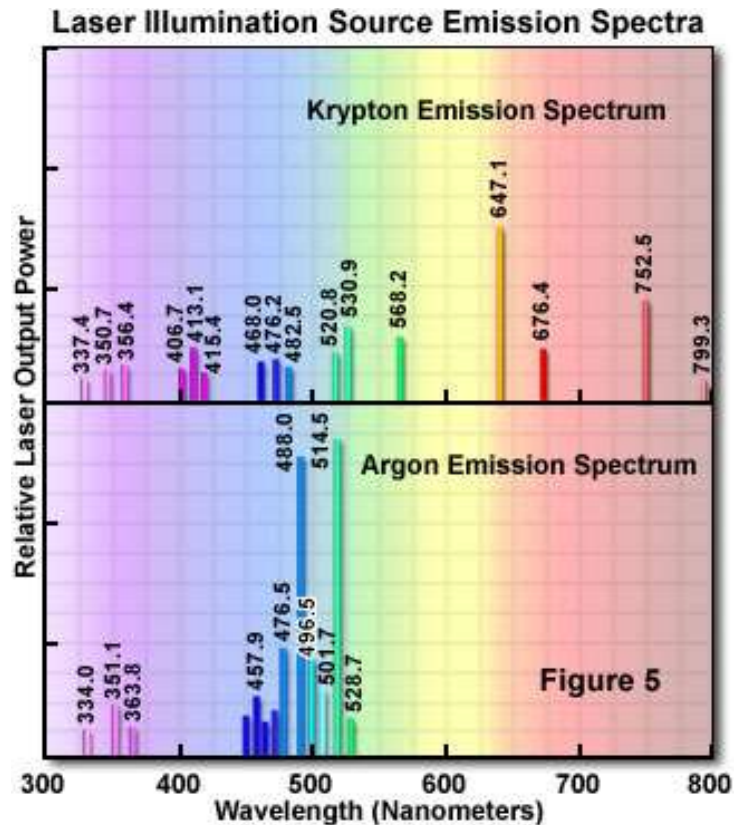


Whitelight Ar/Kr Ion Laser Spectral Lines

Excitation light sources – Laser

Ar / Kr ion laser: 488, 514, 568, 647

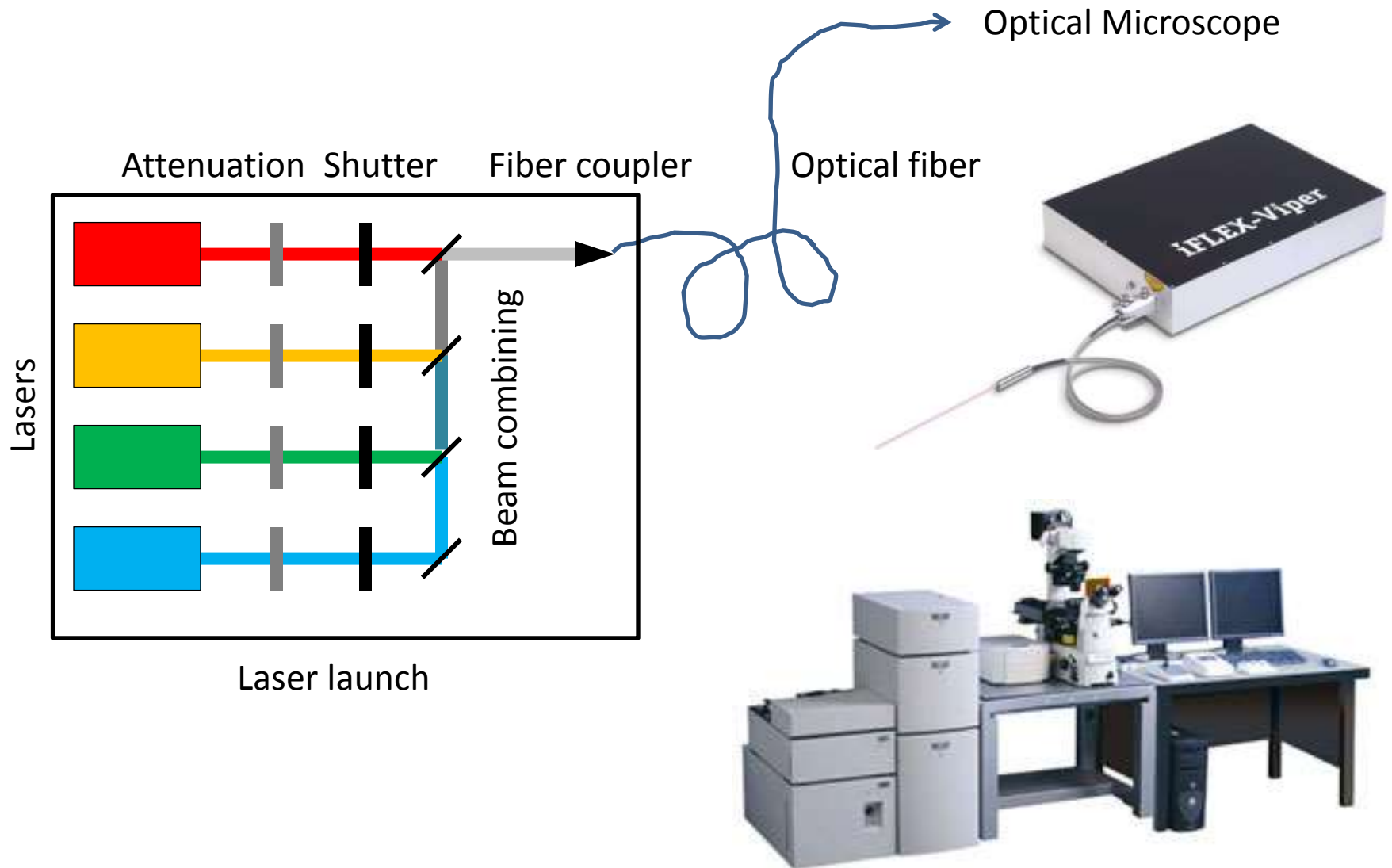
Solid state lasers: 488, 532, 561...



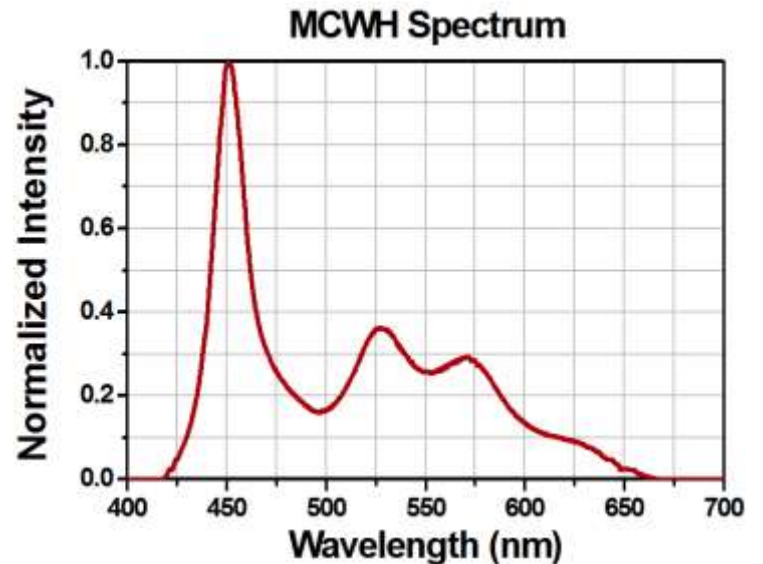
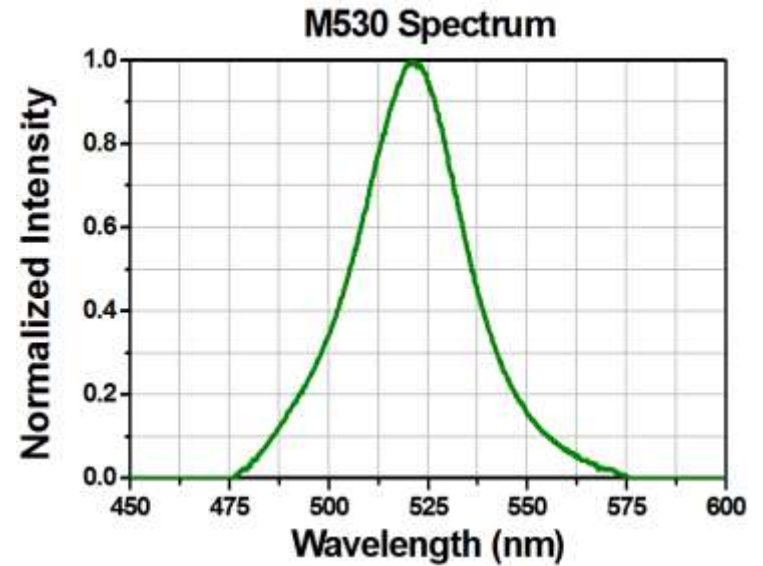
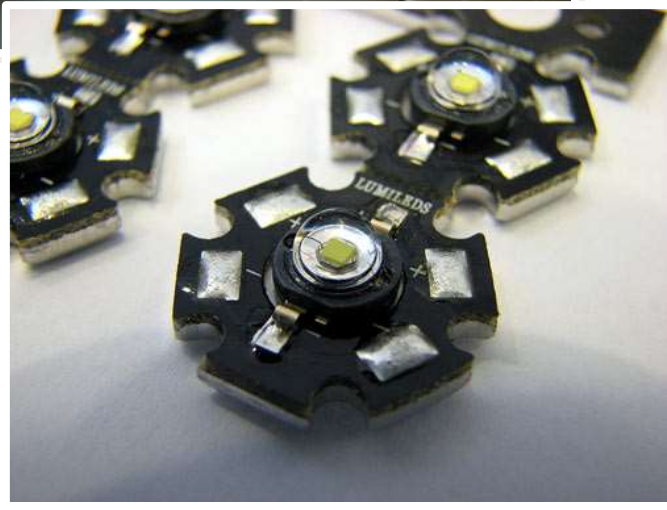
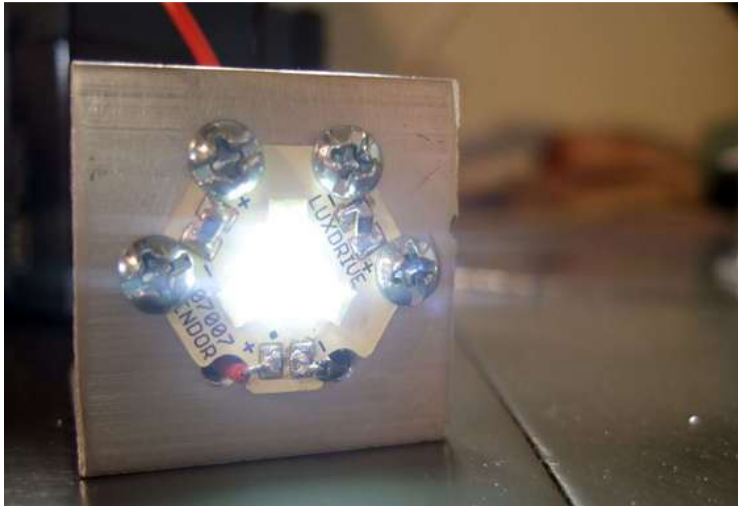
Diode lasers: 375, 405, 488, 635, 660...



Excitation light sources – Laser



Excitation light sources – LED and others



Filter components

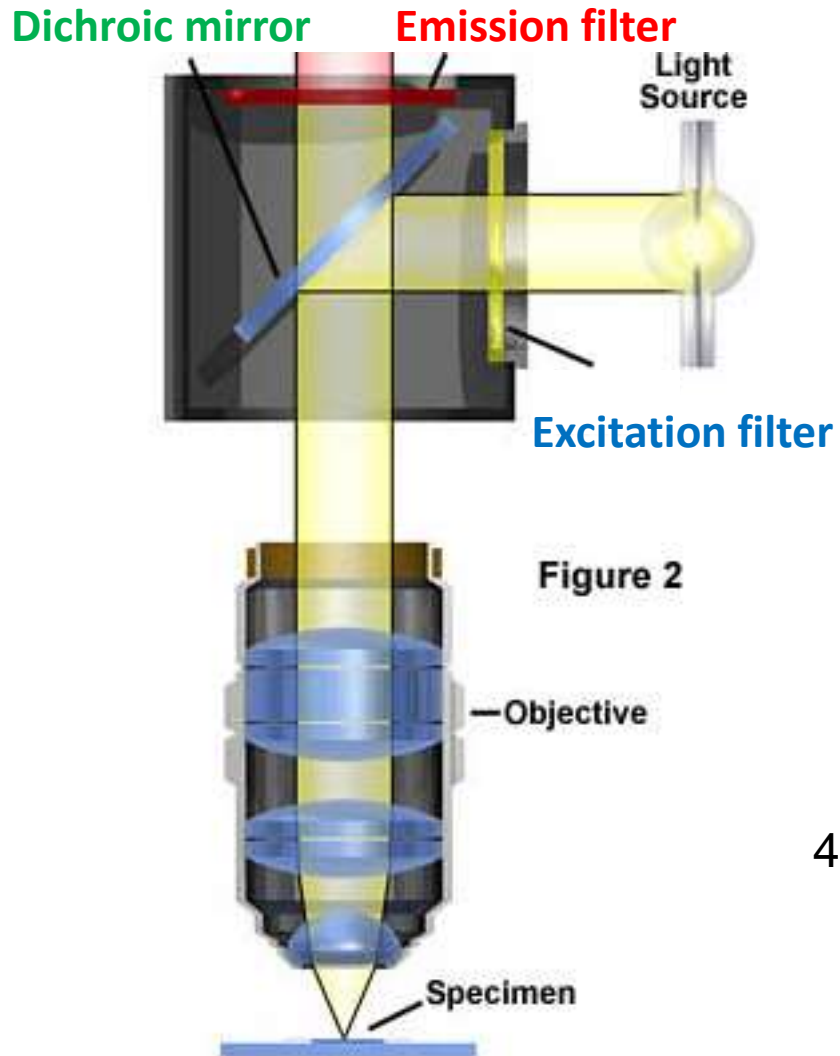
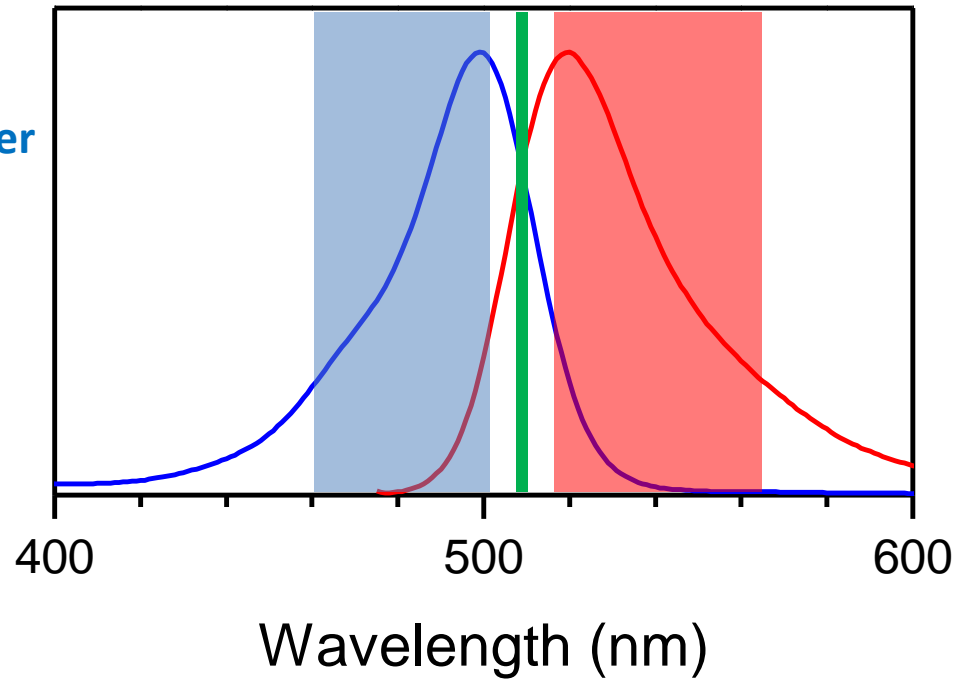
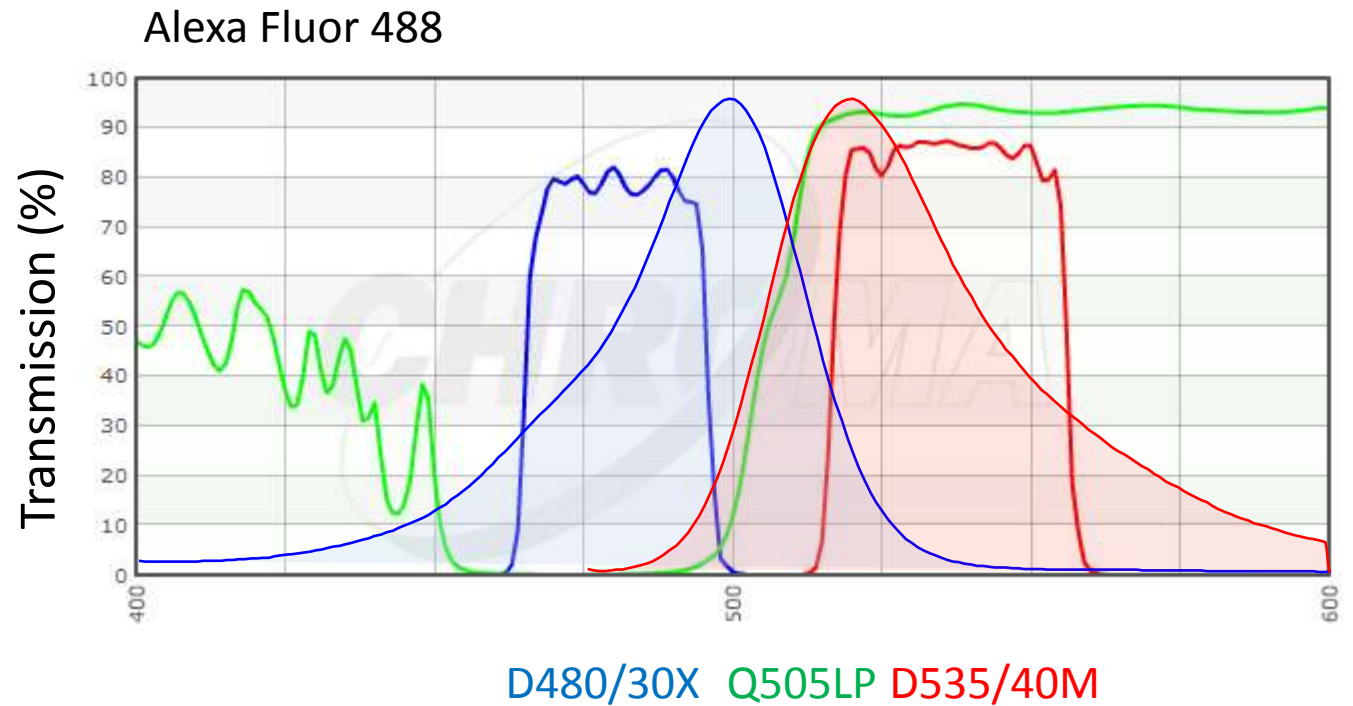


Figure 2

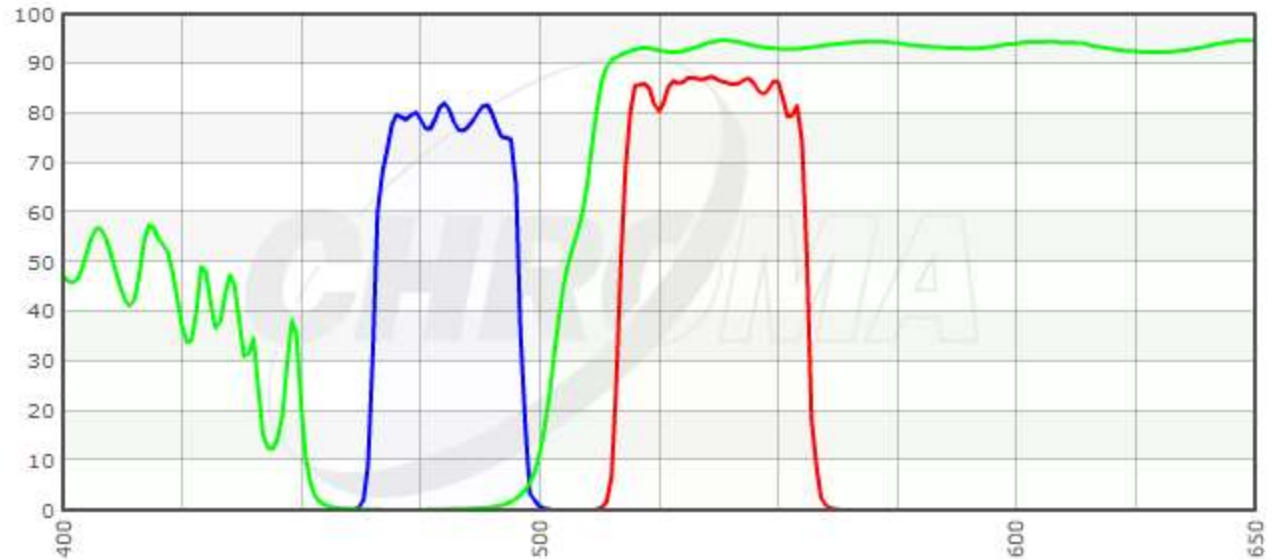


An example of a “filter set”

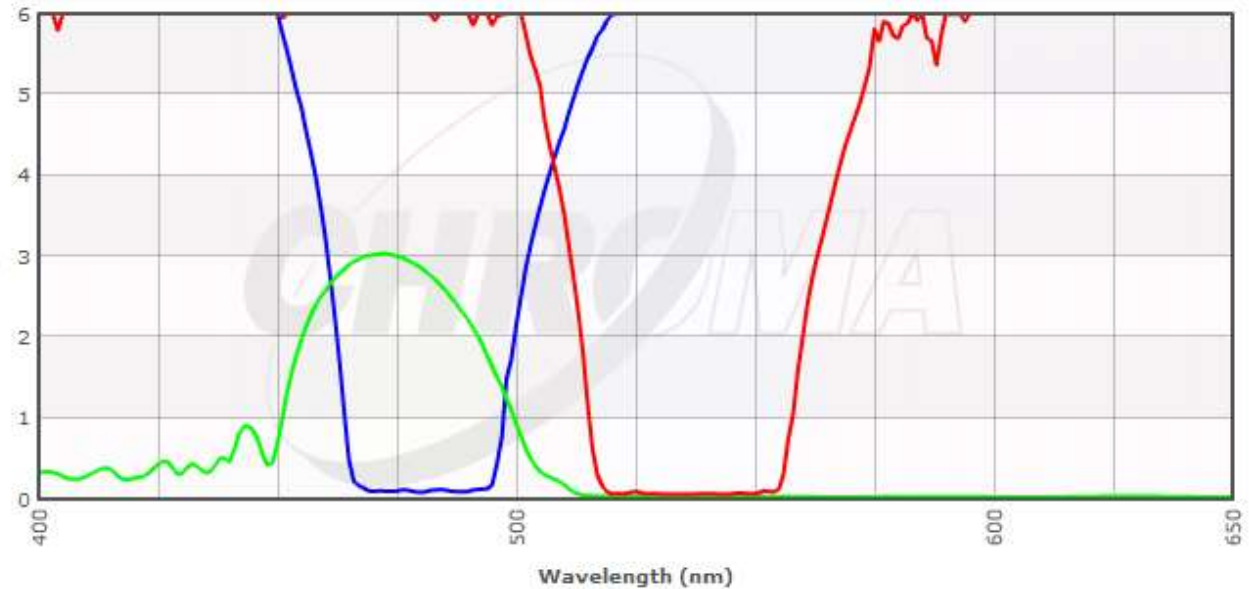


Transmission vs. Optical density

Transmission (%)

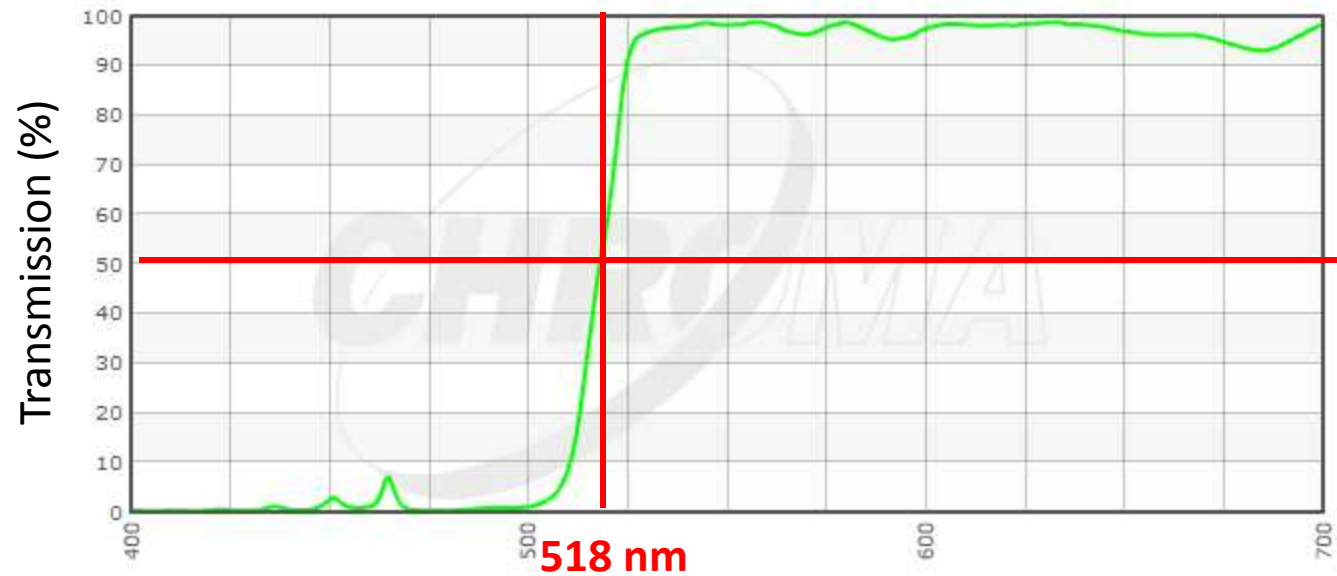


OD =
 $-\log_{10}(\text{Transmission})$

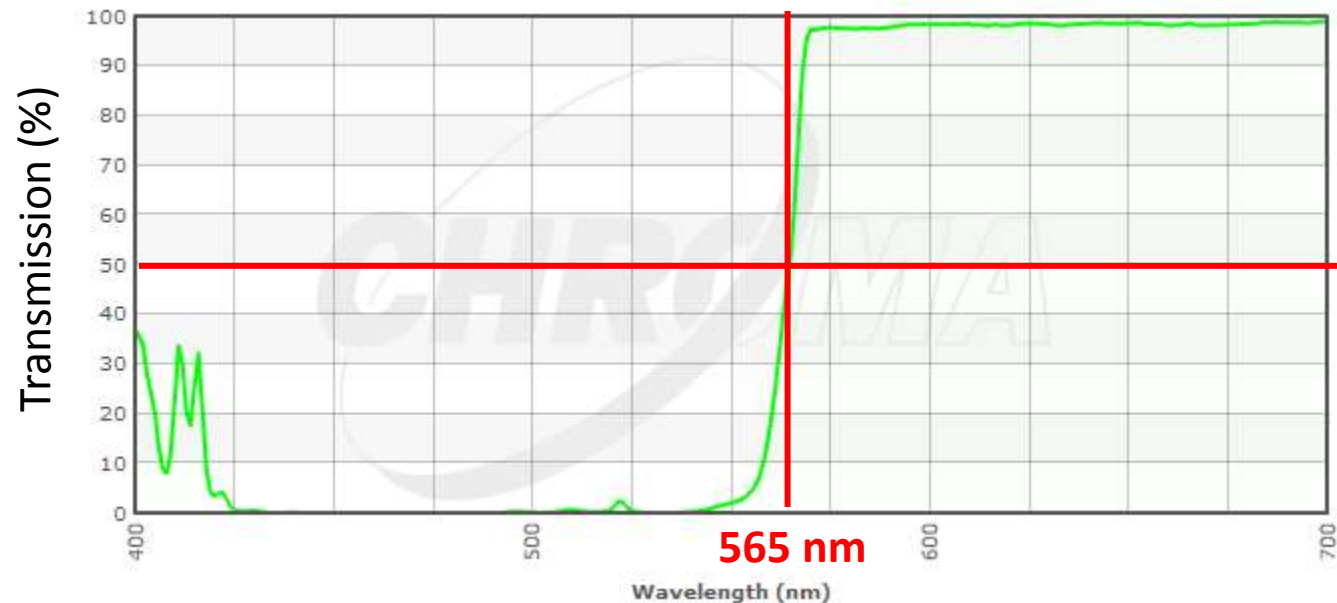


Filter names – Dichroic mirror

515DCLP

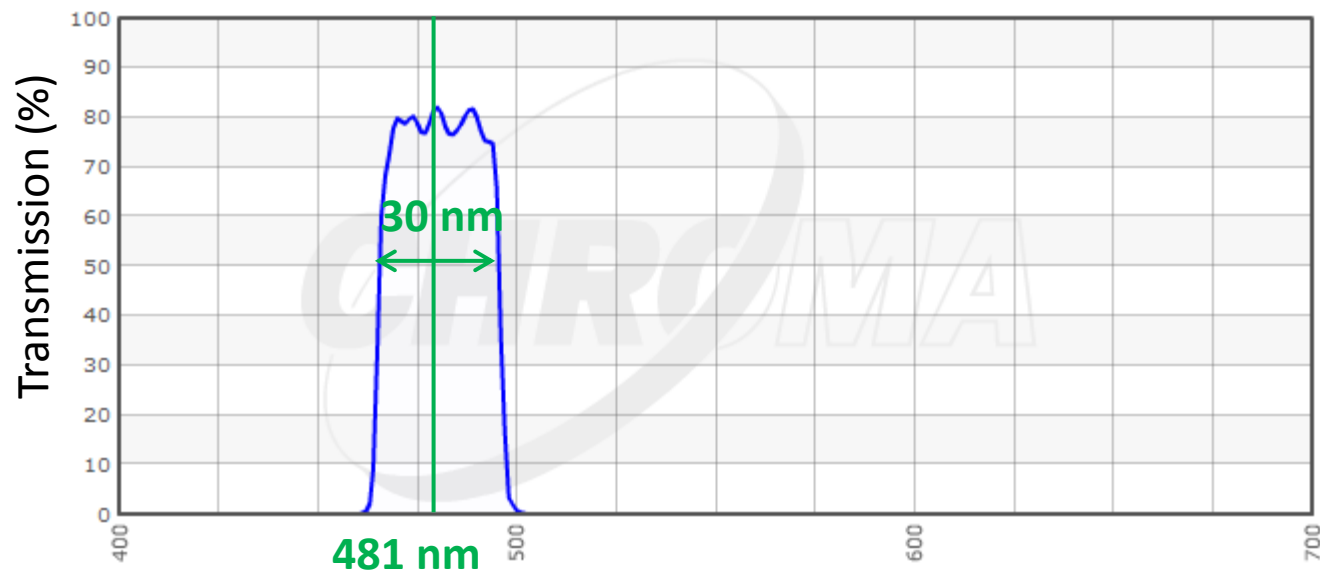


T565LPXR

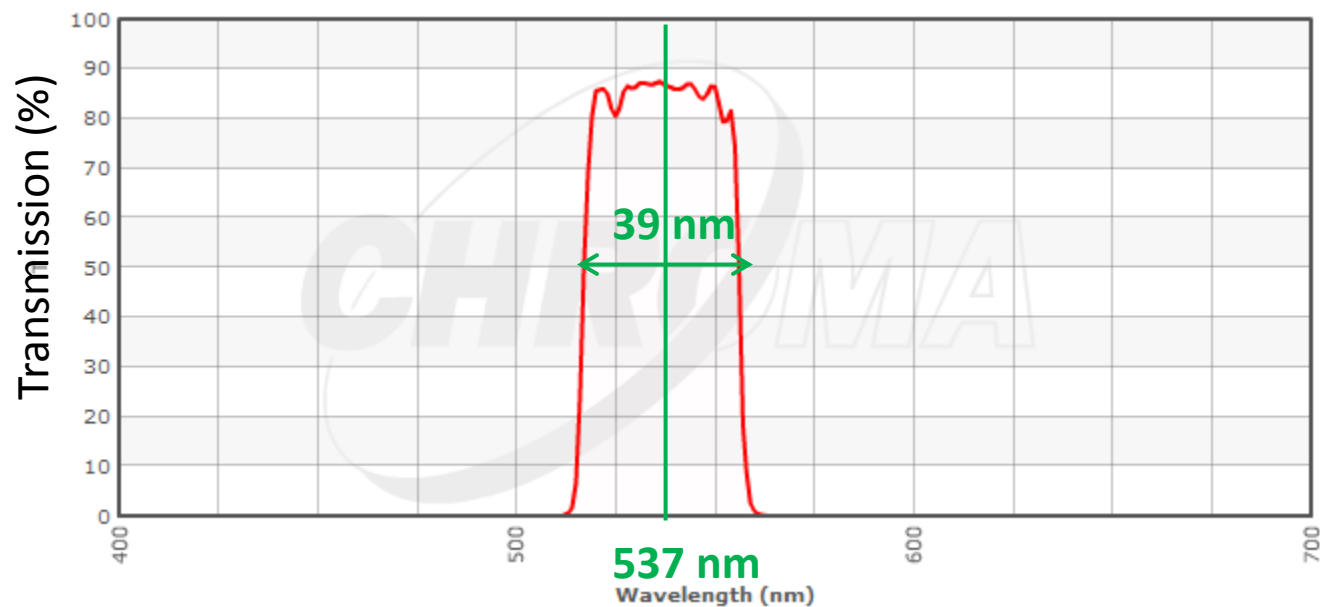


Filter names – Bandpass filters

D480/30X



D535/40M



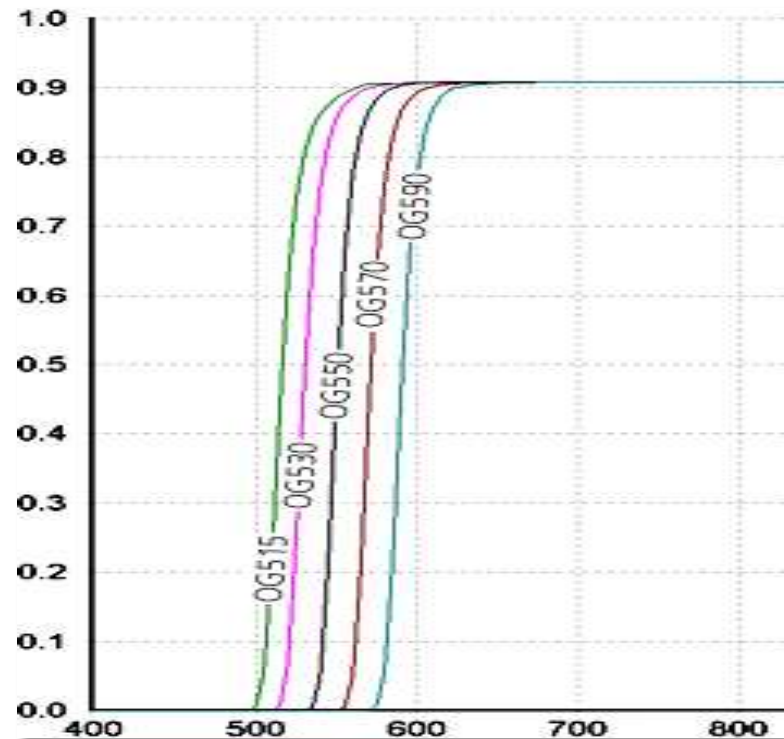
Inexpensive filters: color glasses



Absorptive
(colored glass)

OD \approx 2

OG550 =
Orange Glass,
50% transmission at 550 nm



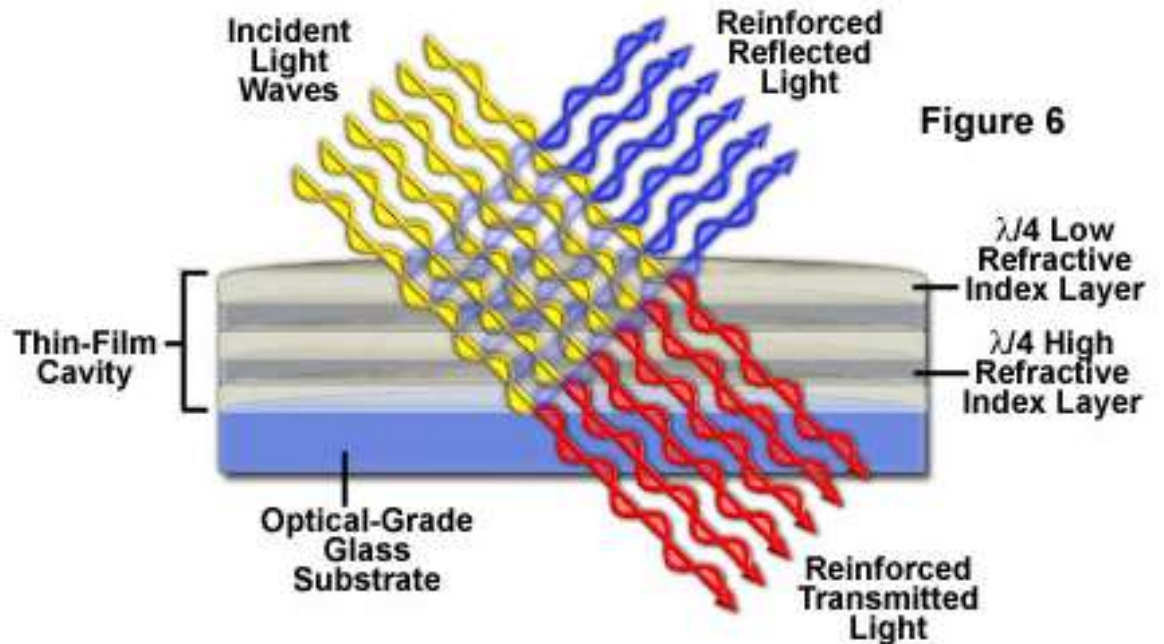
Dielectric filters



Interference
(dielectric)

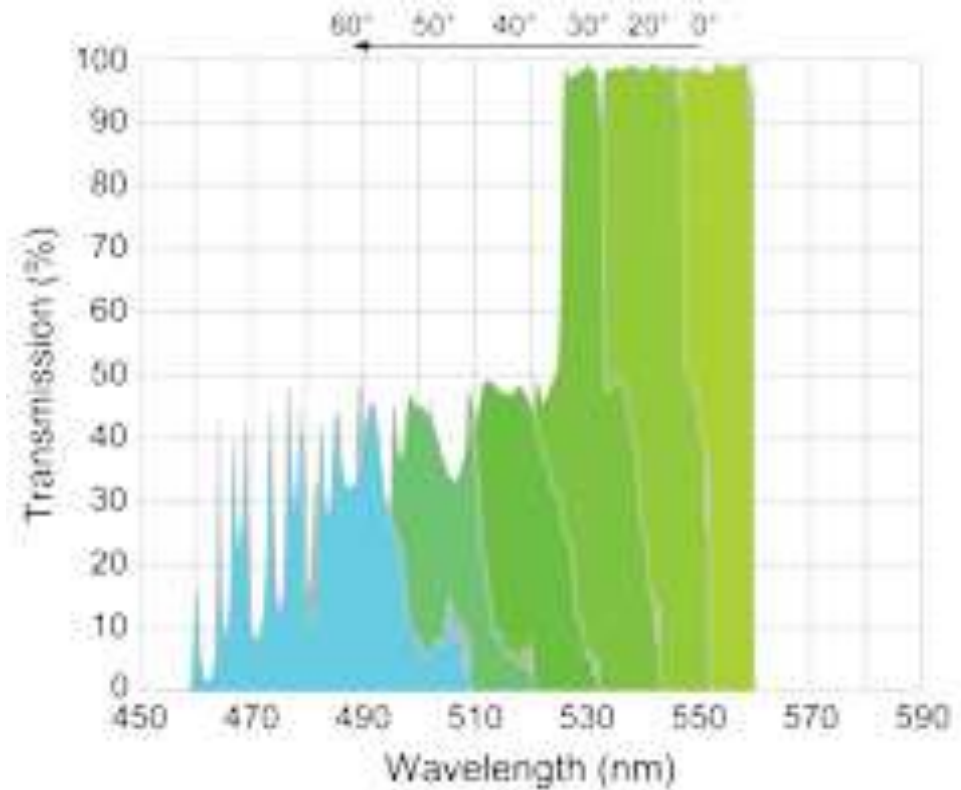
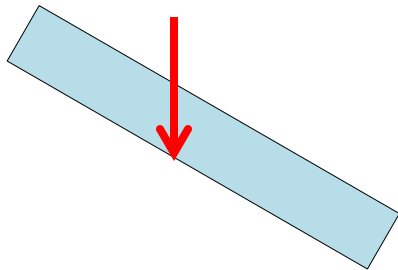
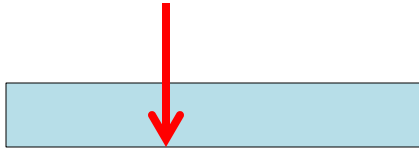
$OD > 6$

Transmission $< 10^{-6}$



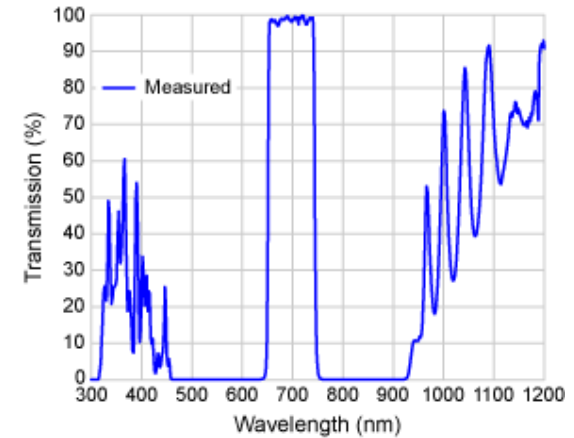
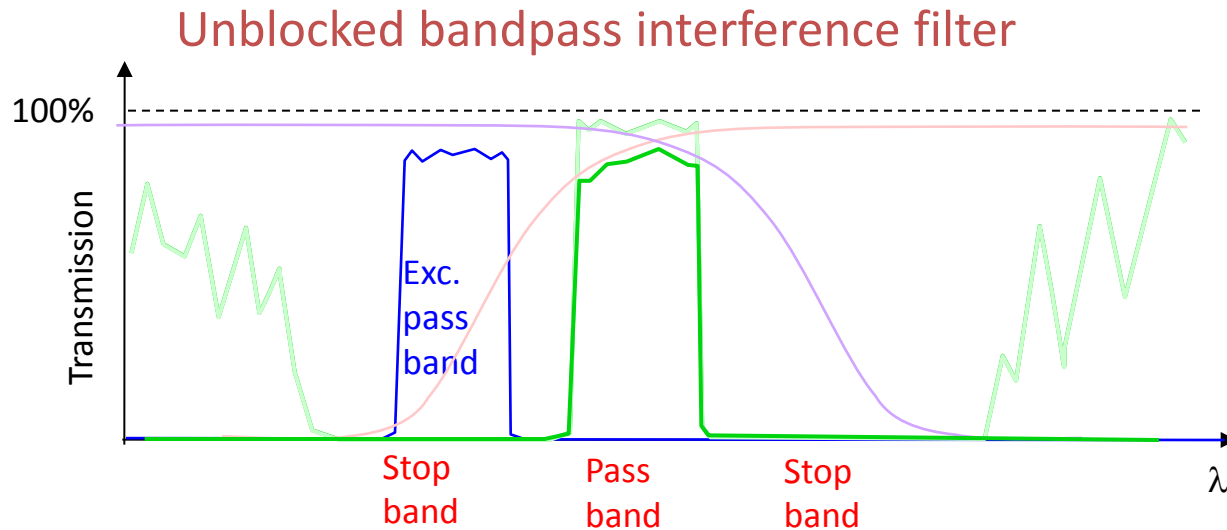
Vendors: Chroma, Semrock, Omega

Interference filter is sensitive to incident angle

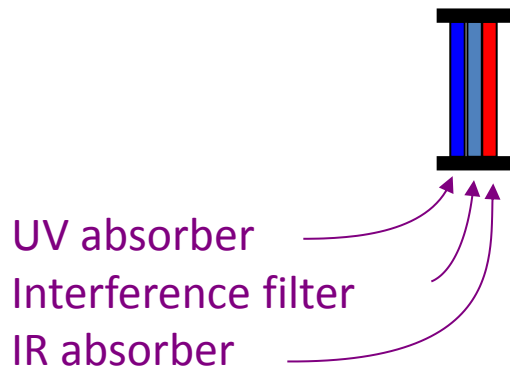


Semrock website

Stop band of interference filters



Semrock 697/75



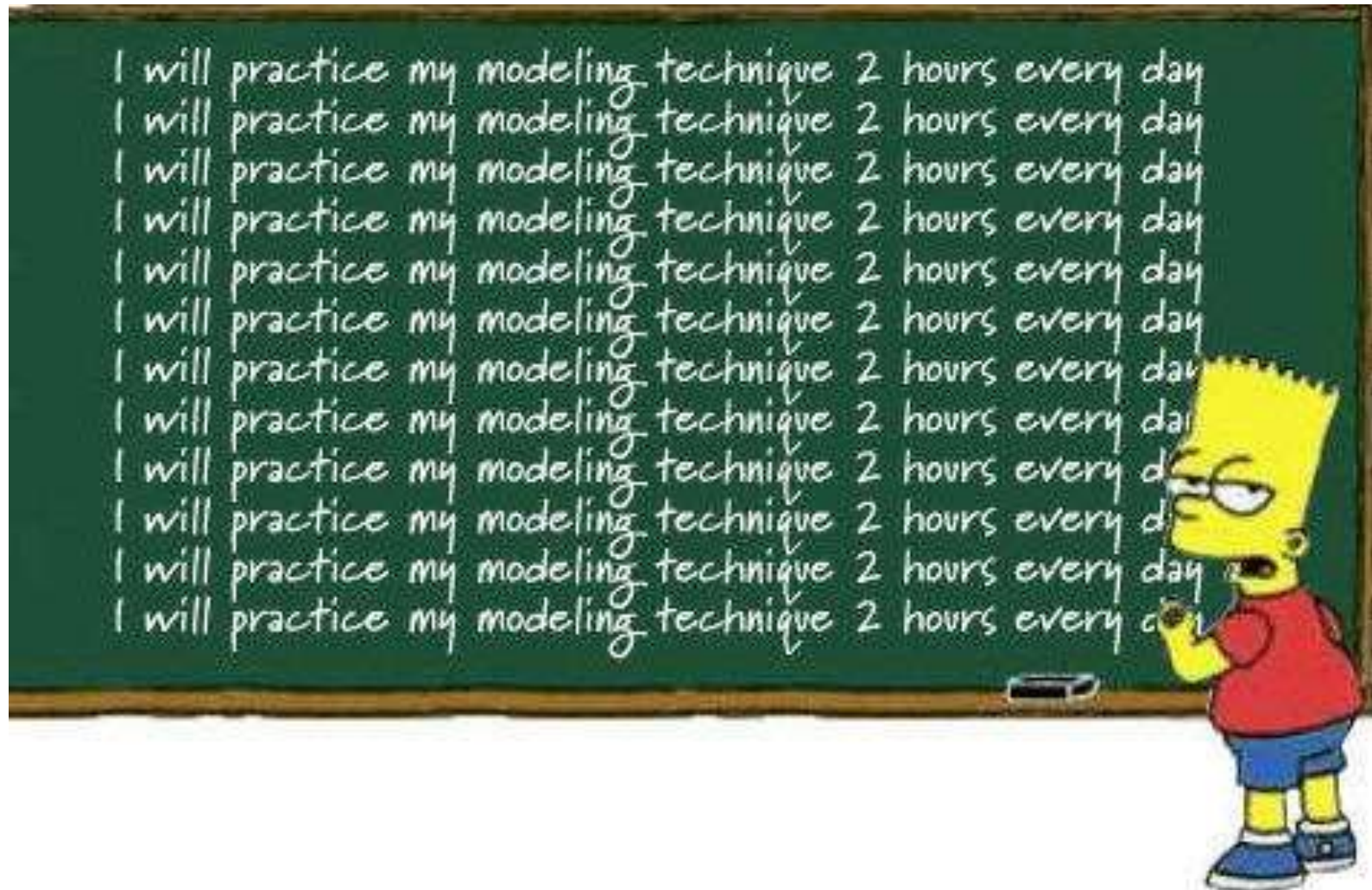
Often excitation filters are blocked,
but emission filters *unblocked*.

Tunable filters

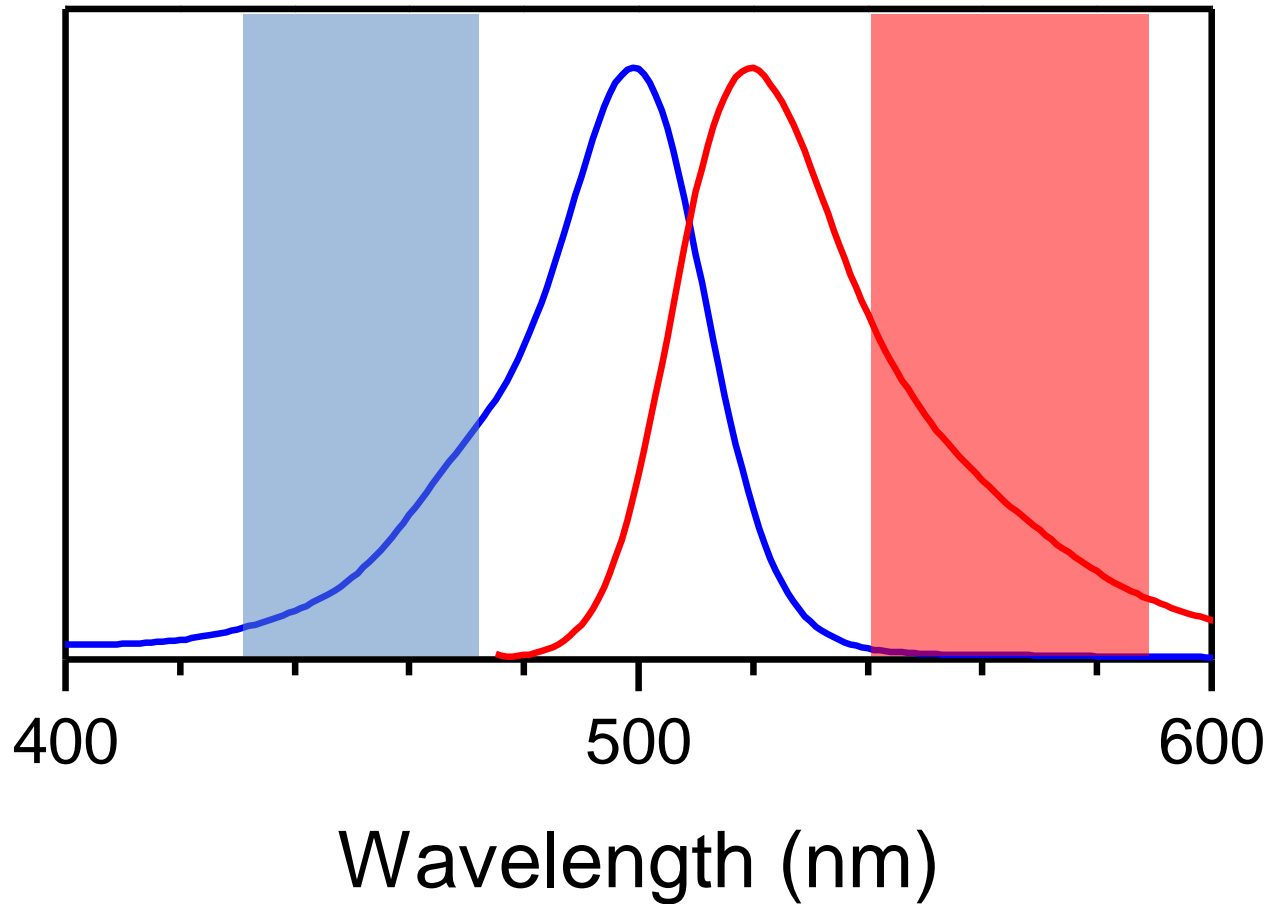
- Liquid crystal filter
- Acoustical optical tunable filter (AOTF)
 - Modulated by ultrasound wave in a crystal
 - Fast switching (μs)
 - Polarization sensitive
 - Mostly for excitation laser



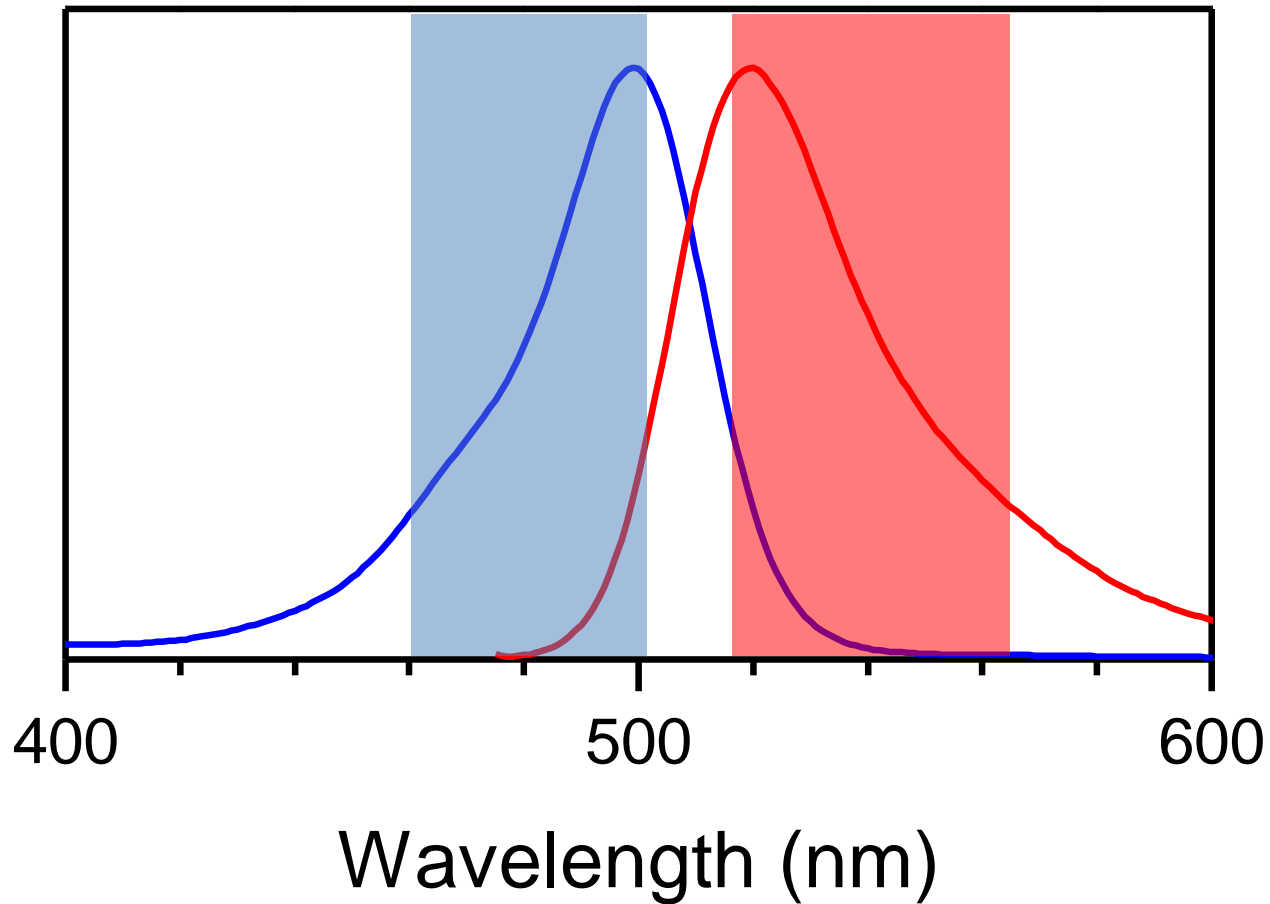
Practical concerns



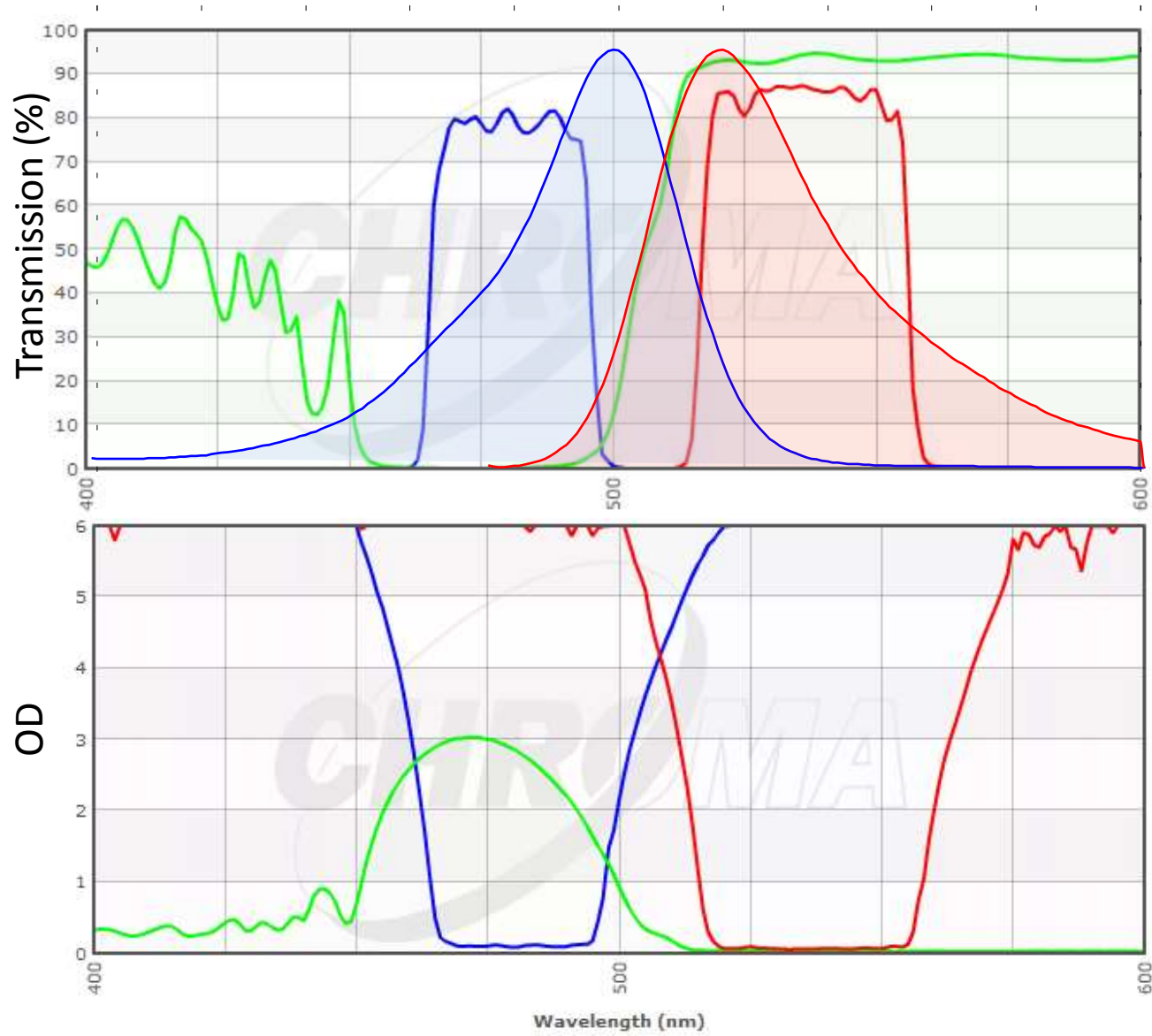
Matching the filters with the spectra



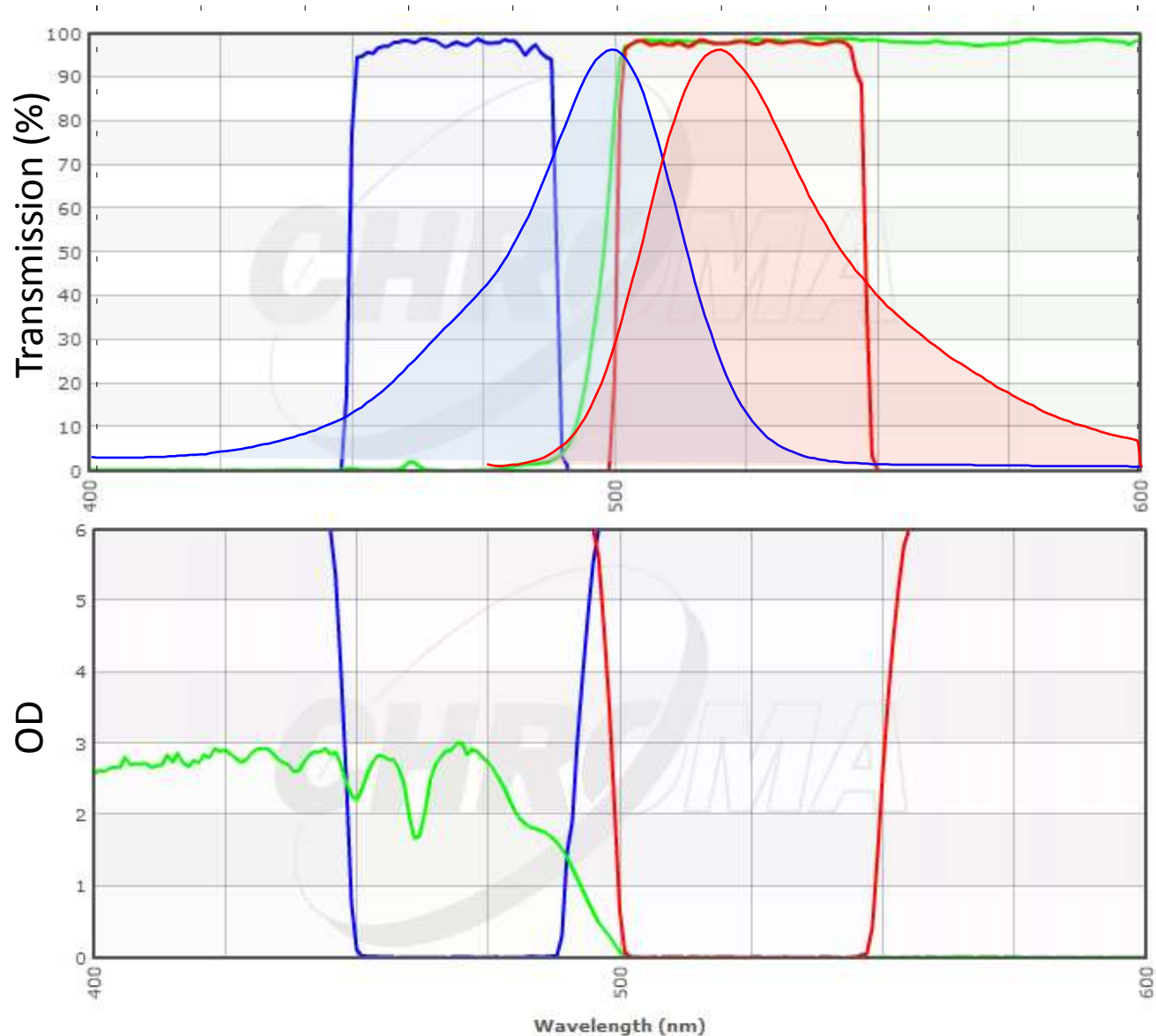
Matching the filters with the spectra



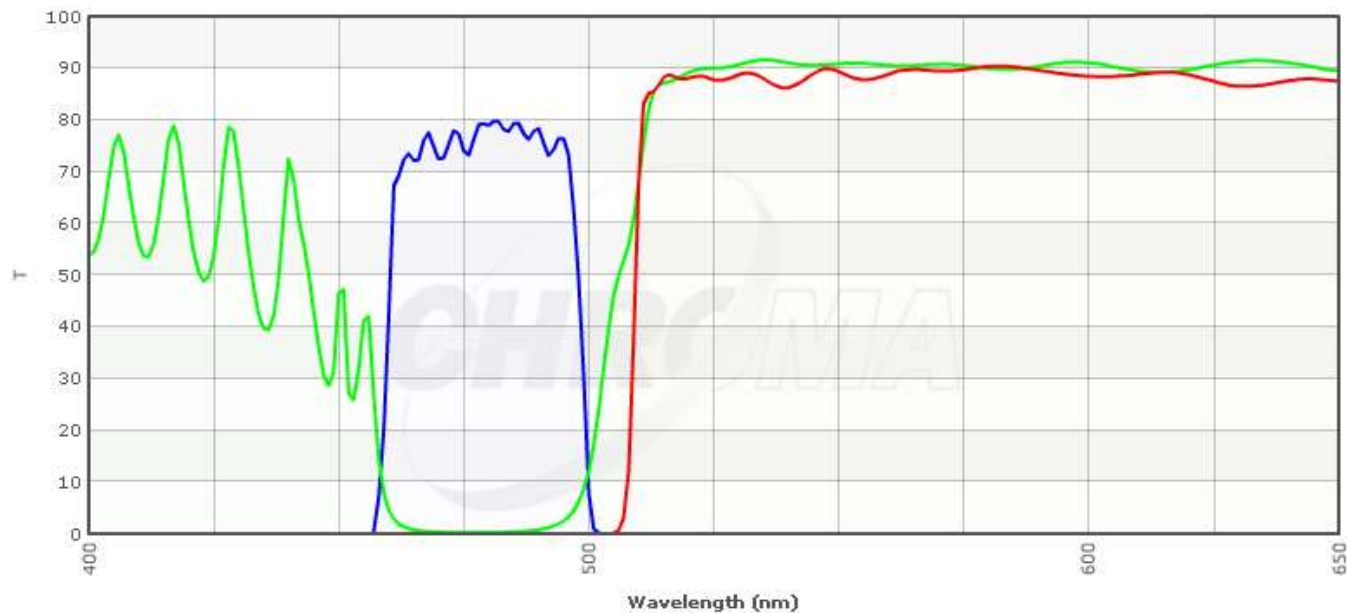
The choice of a “filter set”



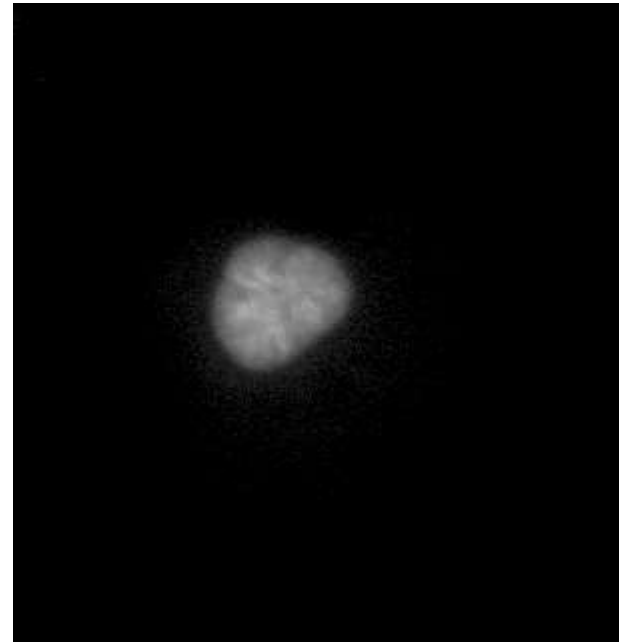
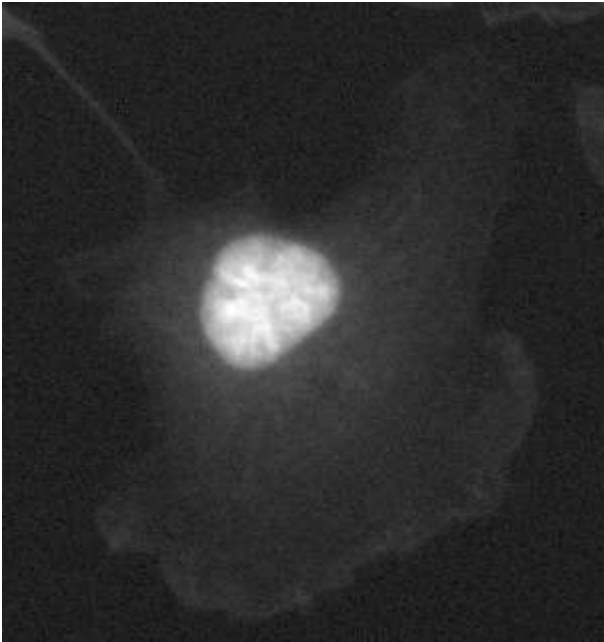
Better (\$\$\$) filters give higher efficiency



Long pass vs. Band pass



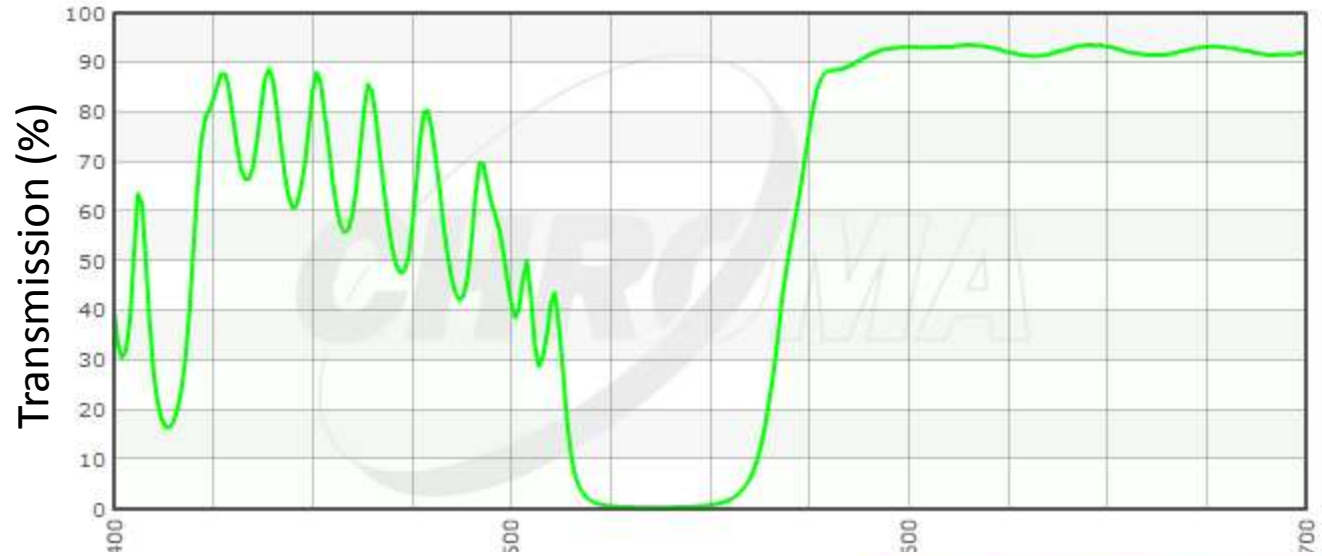
Wider is not necessarily wiser



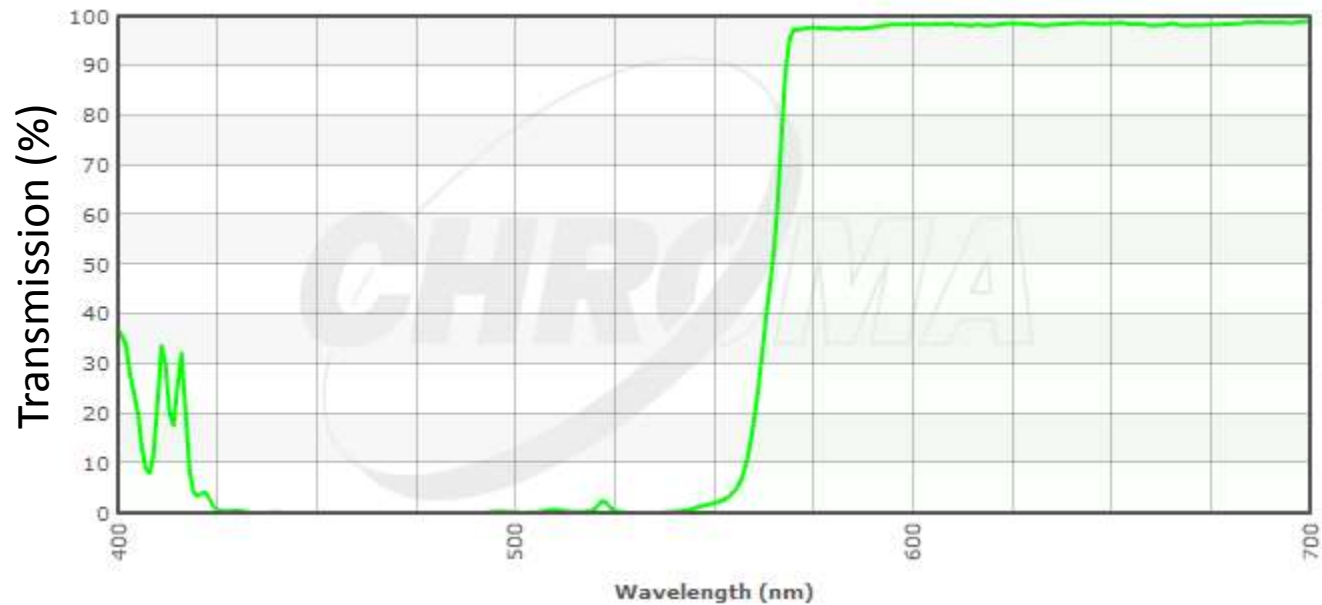
DAPI nucleus staining, long pass vs. bandpass

Dichroic mirrors does not have infinite reflection band

565DCLP
Chroma

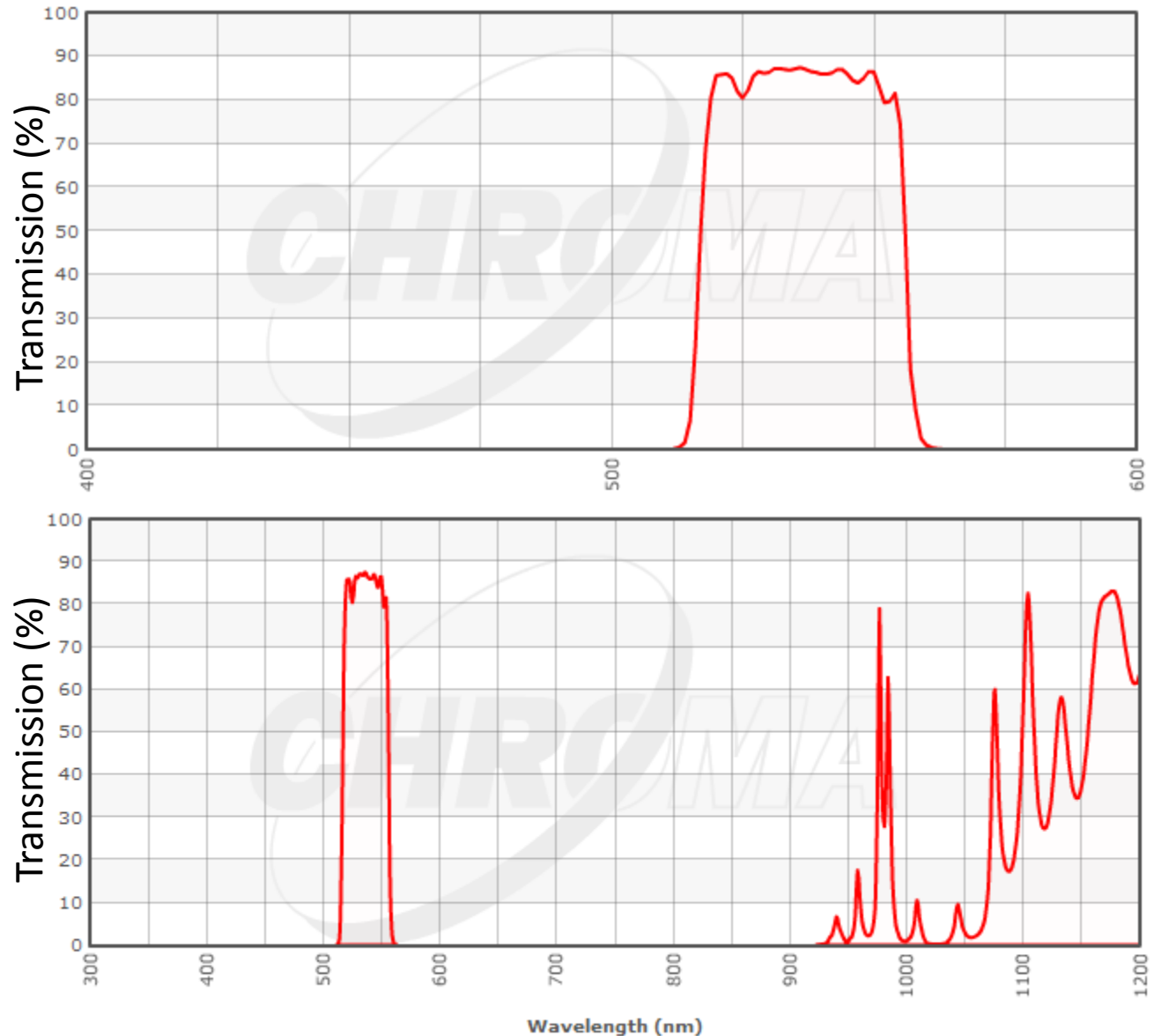


T565LPXR
Chroma



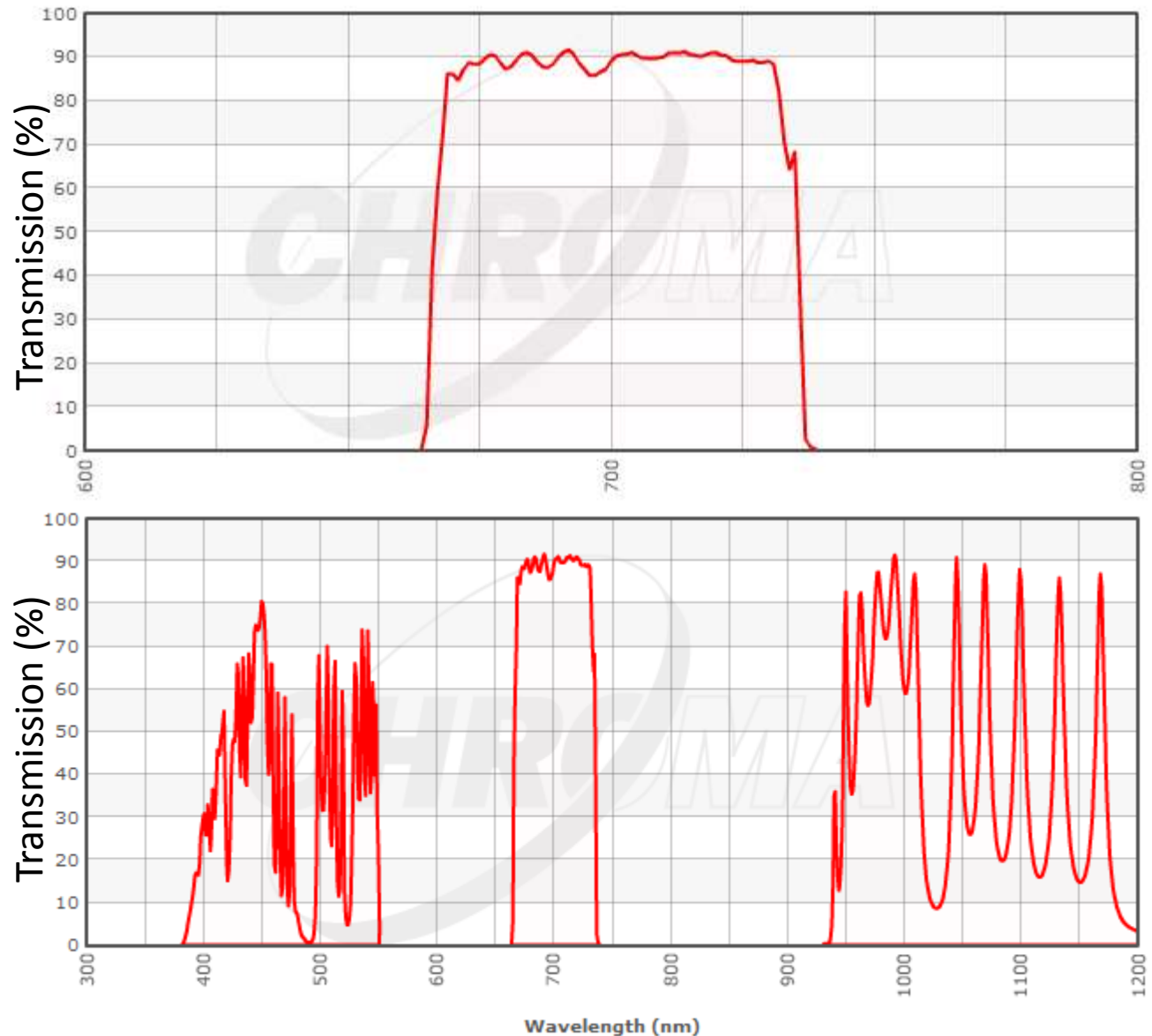
Emission filters might have leaking bands

D535/40M
Chroma

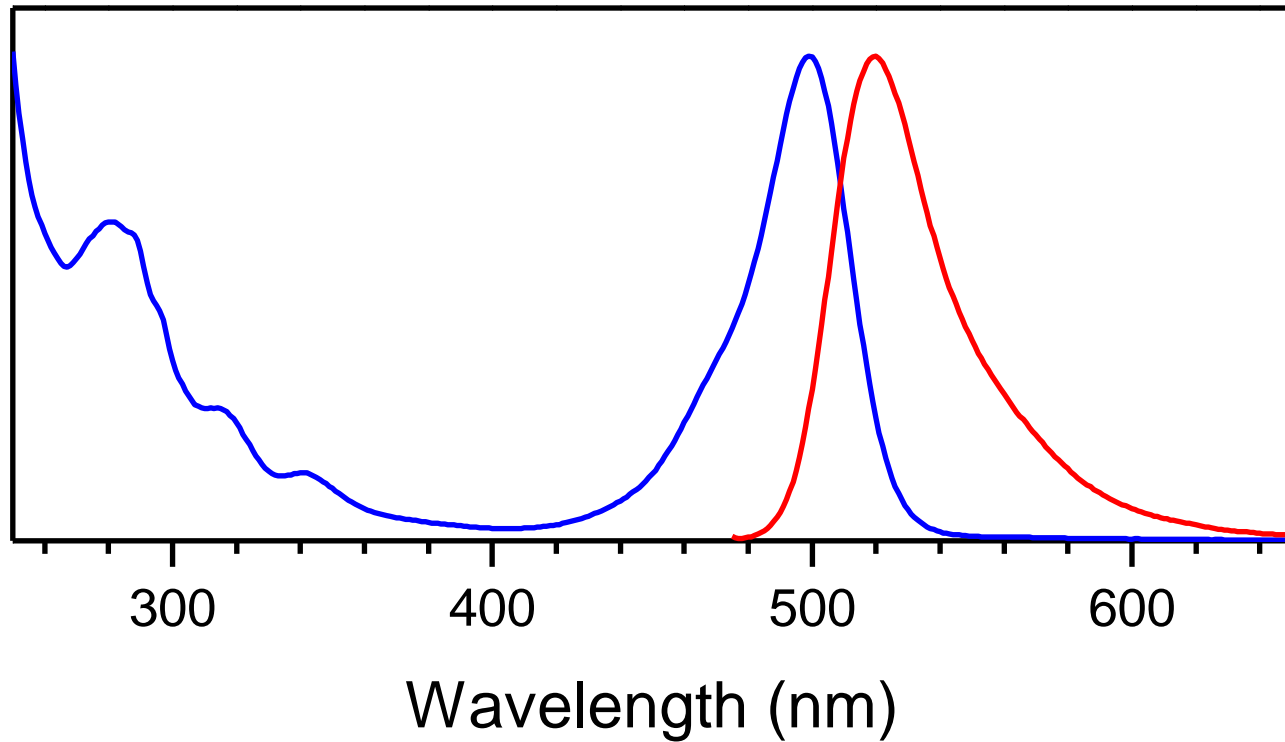


Emission filters might have leaking bands

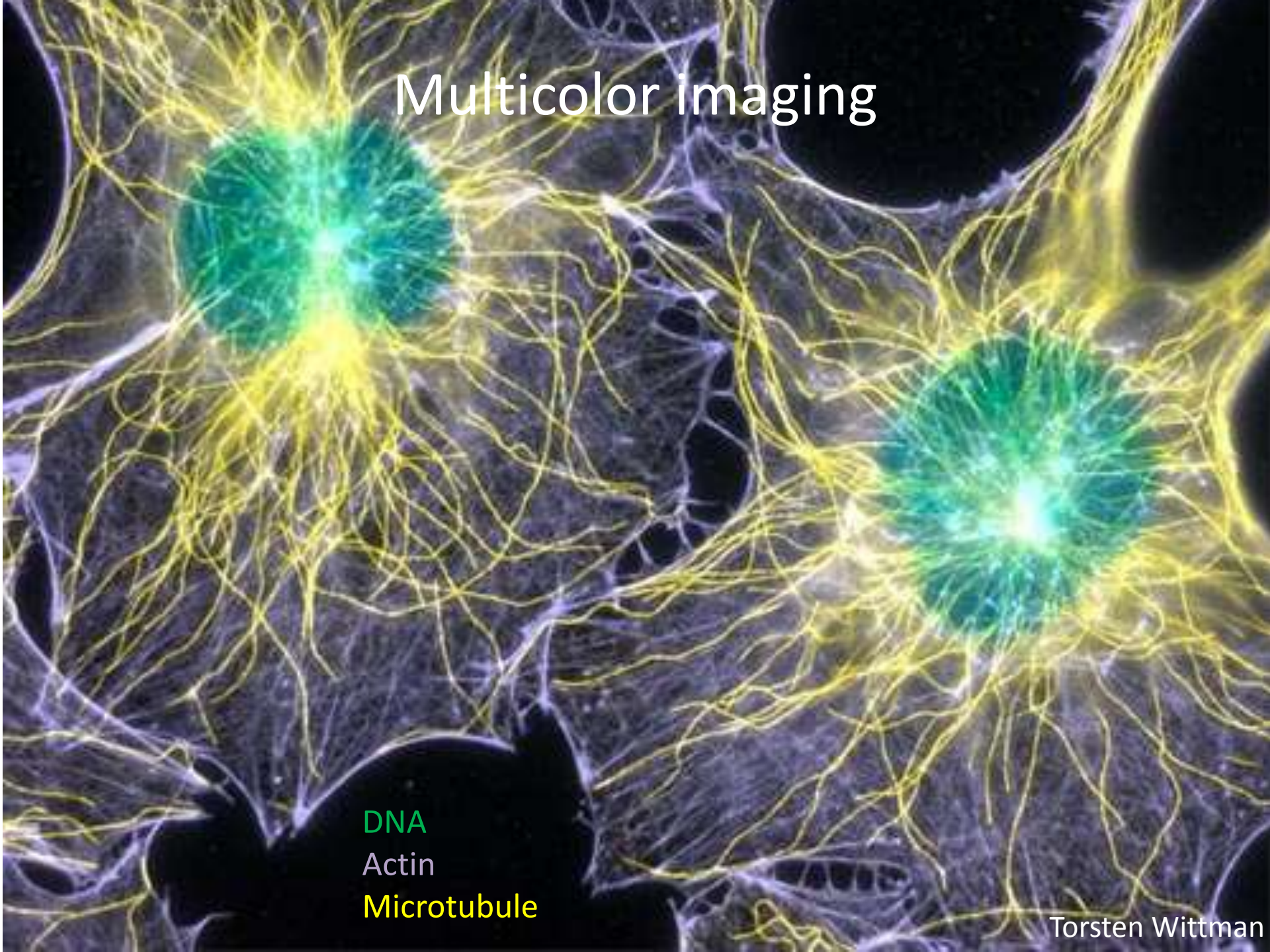
HQ700/75M
Chroma



Fluorescence spectra has tails



Multicolor imaging

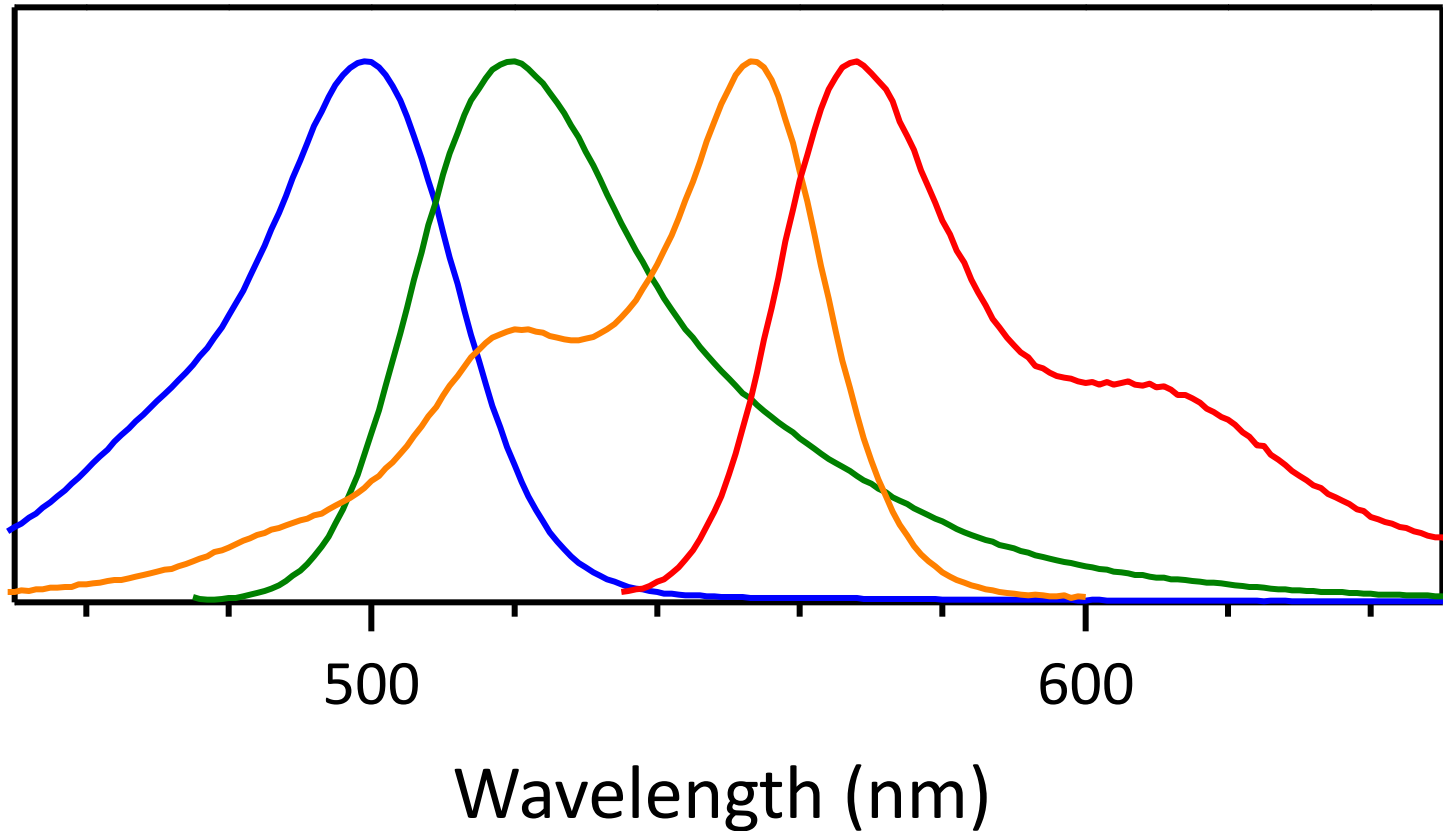


DNA
Actin
Microtubule

Imaging more than one thing at a time

Alexa Fluor 488

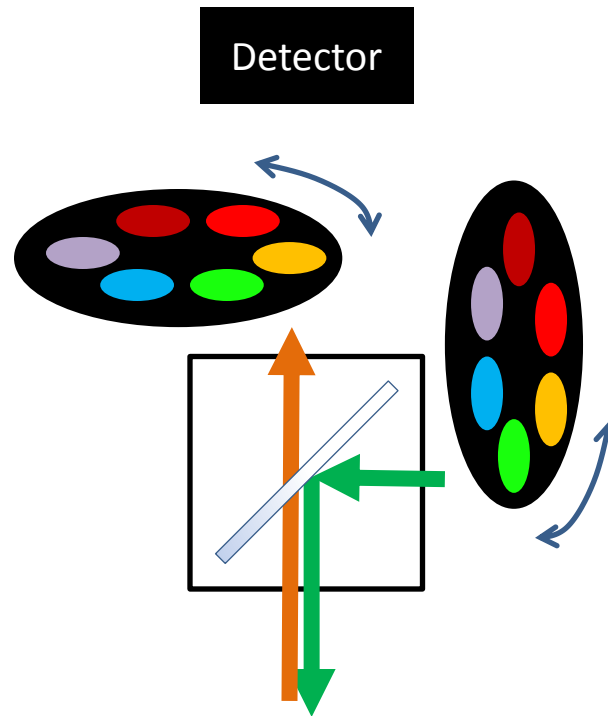
Alexa Fluor 555



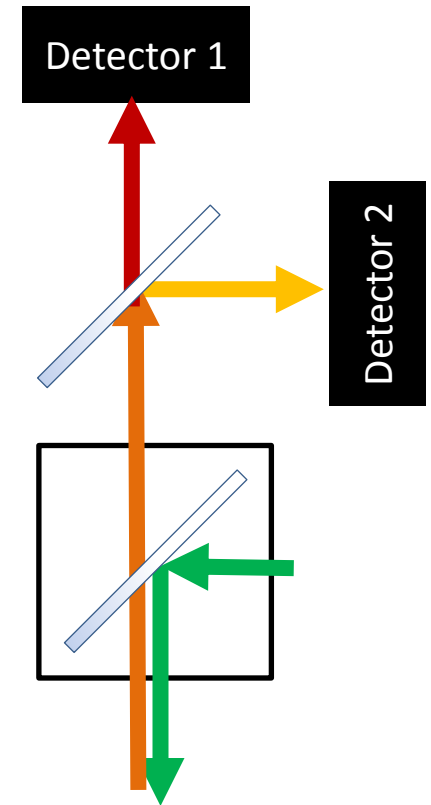
Schemes for multicolor imaging



Cube switching



Filter switching

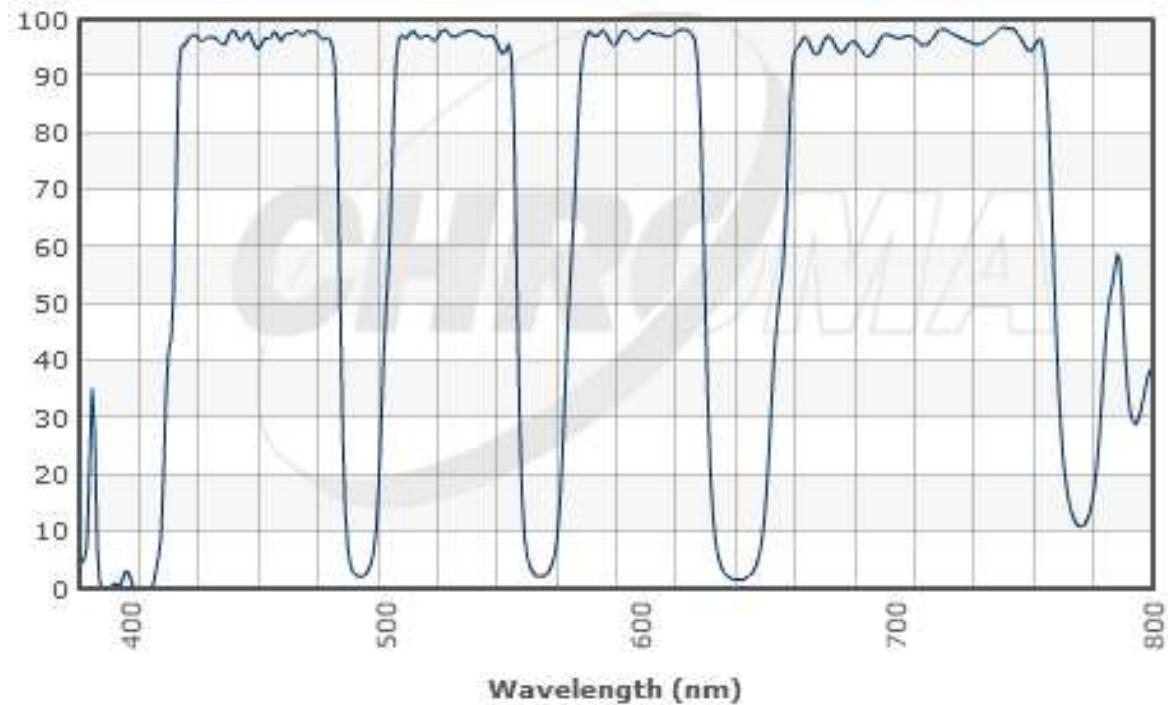


Multiple detectors

Polychroic mirror and multi-bandpass filter

ZT408/488/561/640RPC

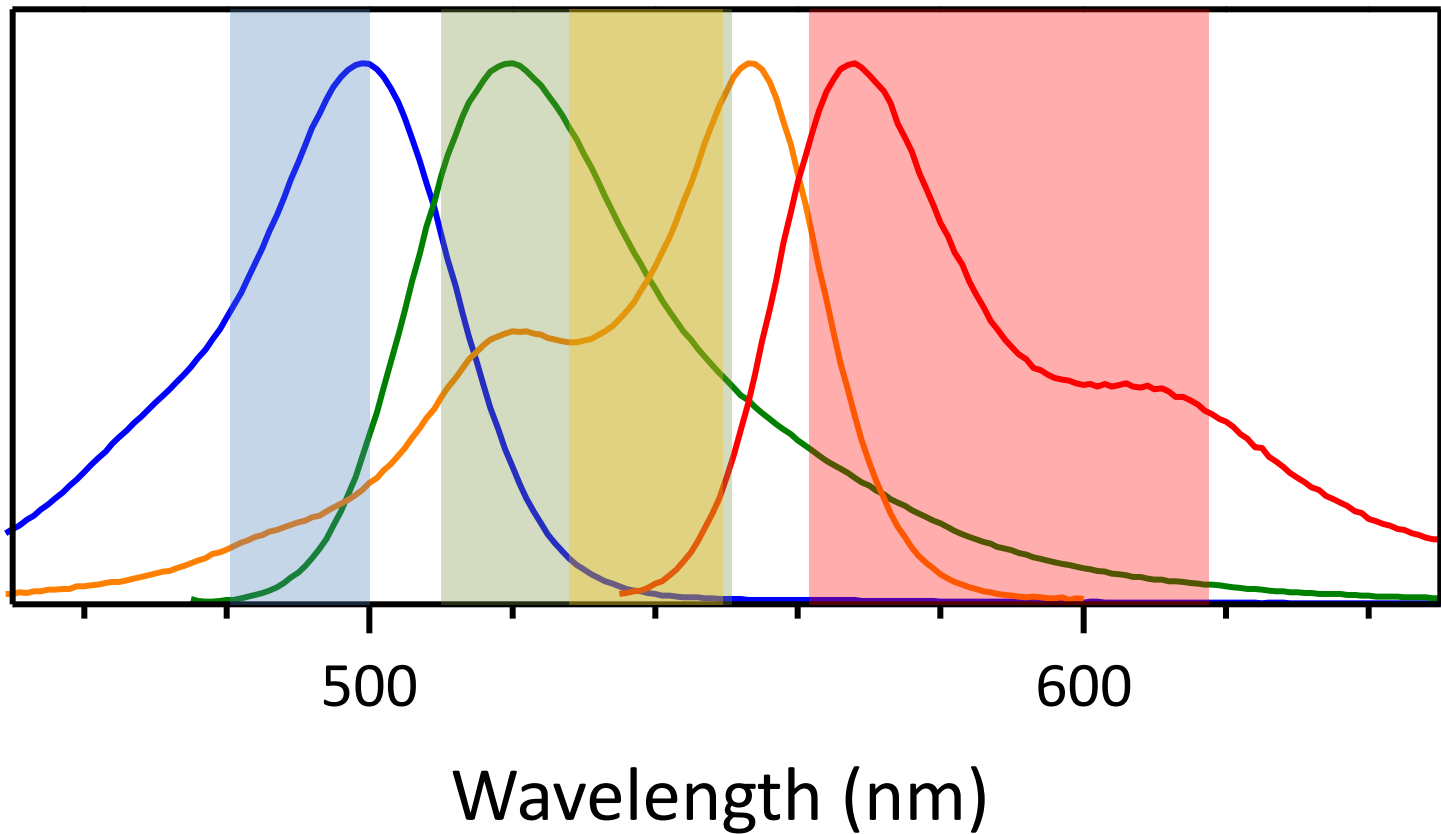
Chroma



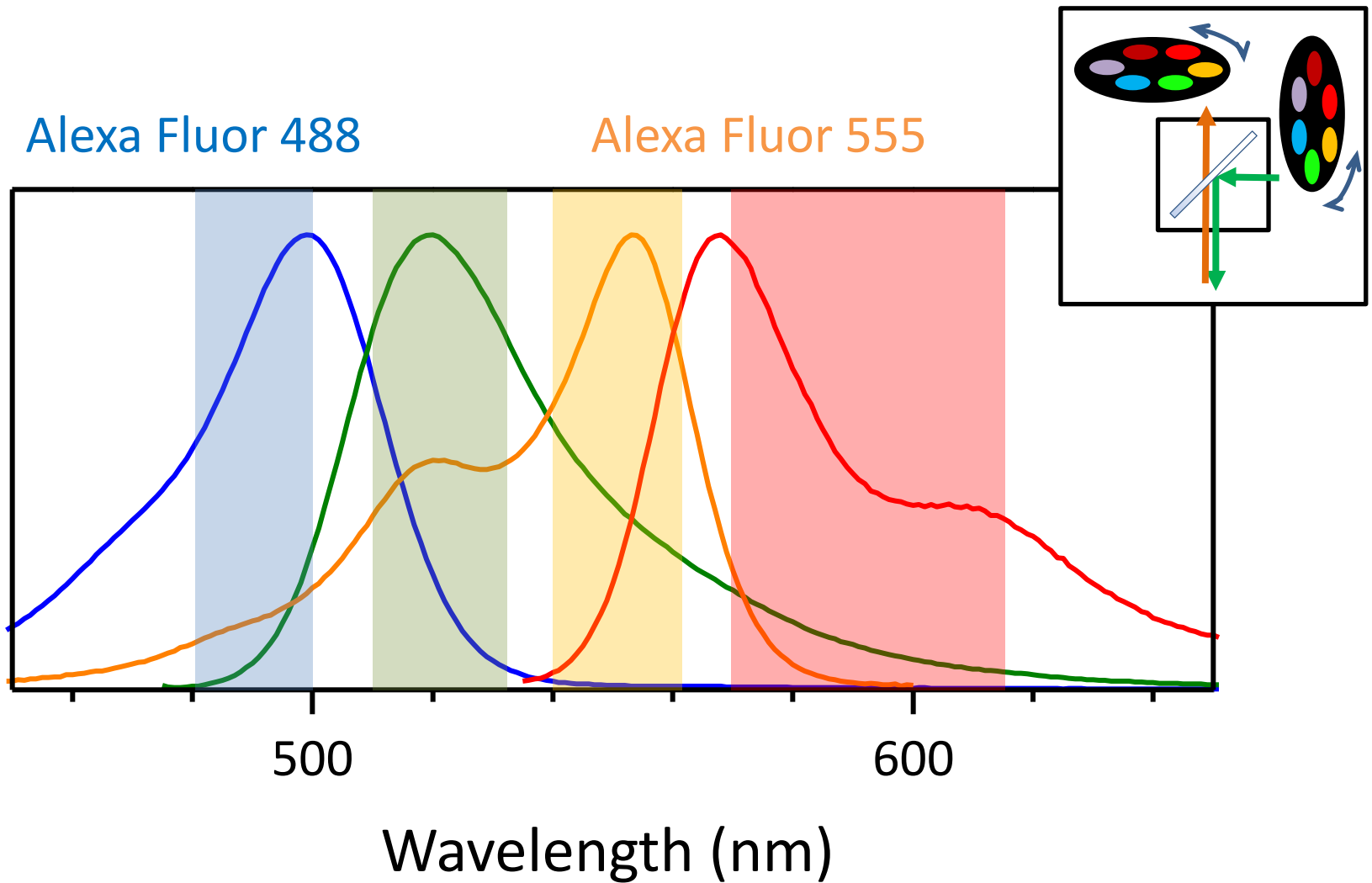
Cube switching

Alexa Fluor 488

Alexa Fluor 555



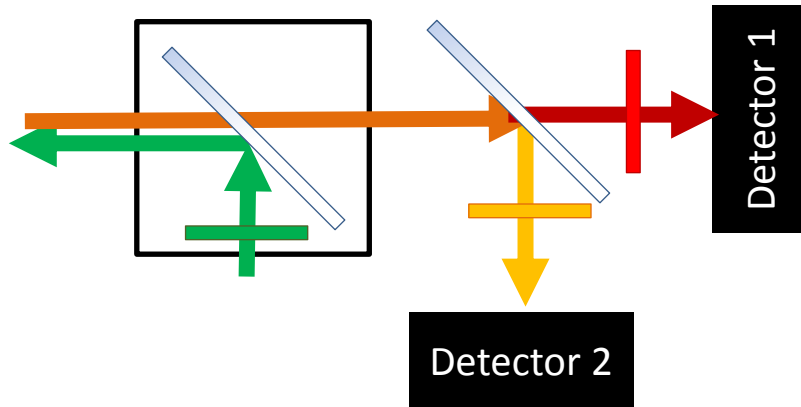
Filter switching – both Ex and Em



Filter switching – Emission only

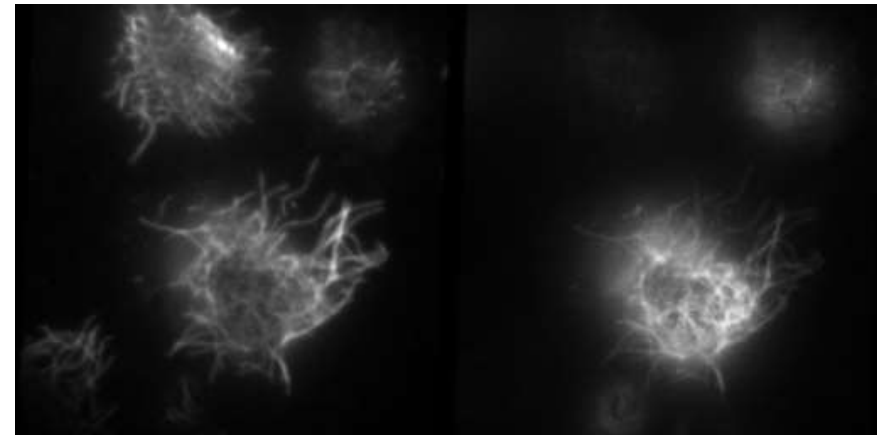
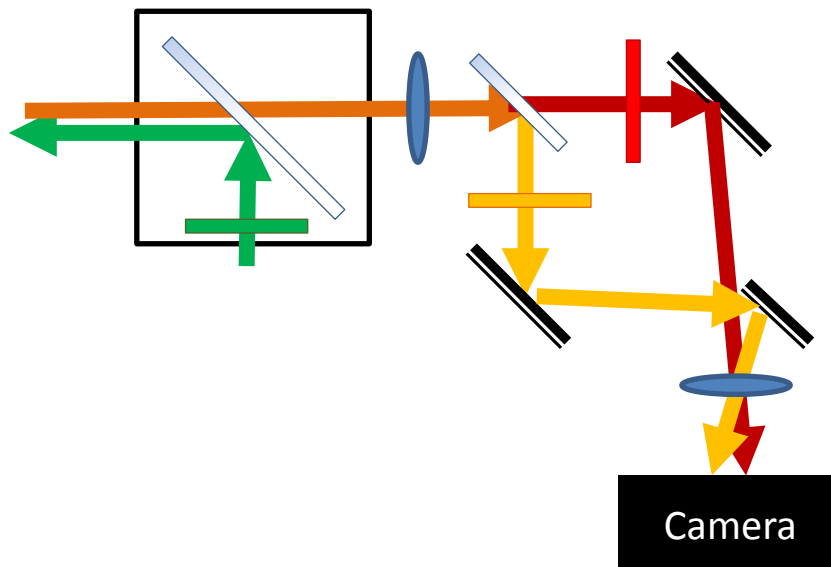


Simultaneous two channel detection



Long wavelength
(reflected)

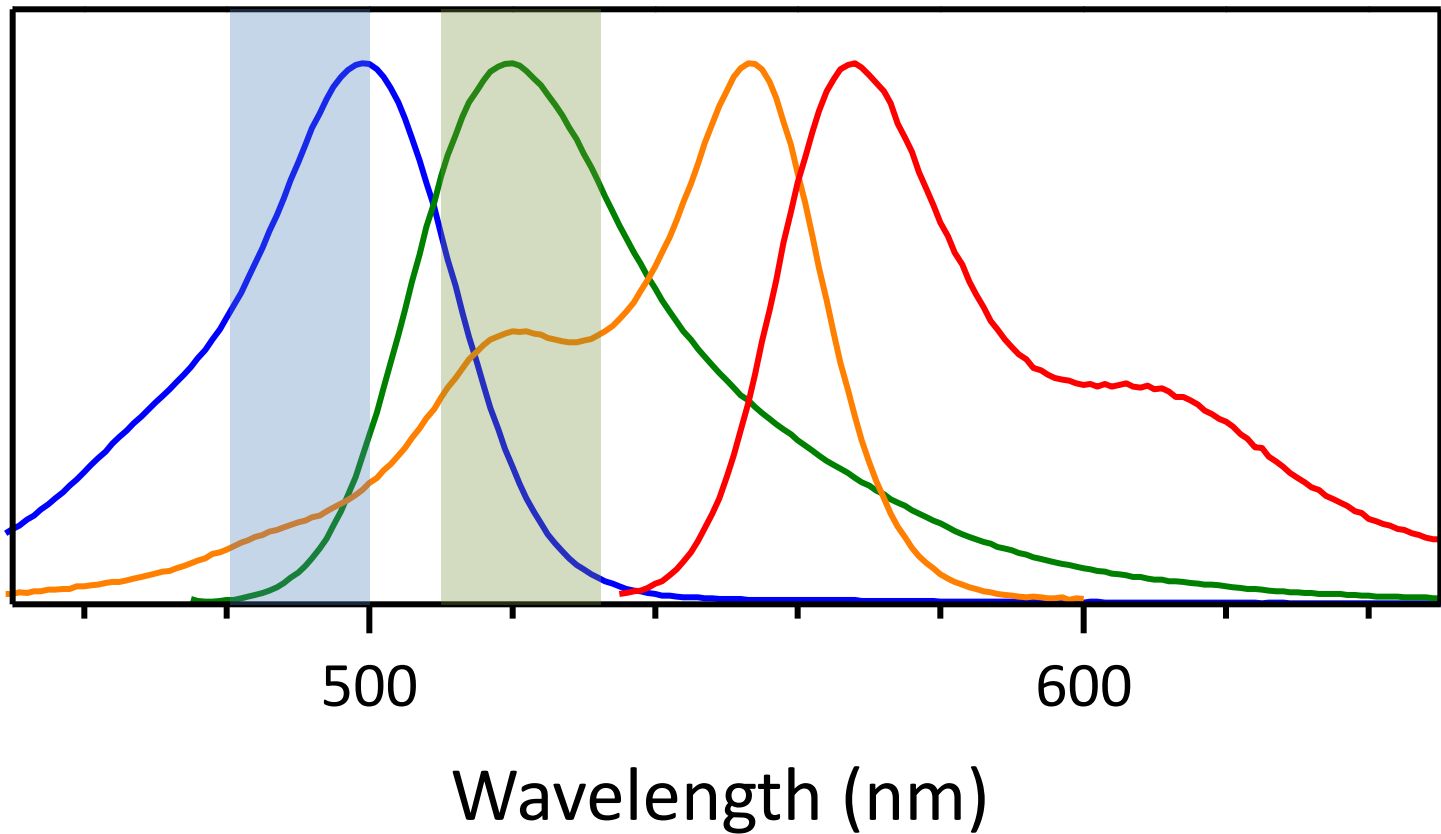
Short wavelength
(transmitted)



Crosstalk between channels – excitation

Alexa Fluor 488

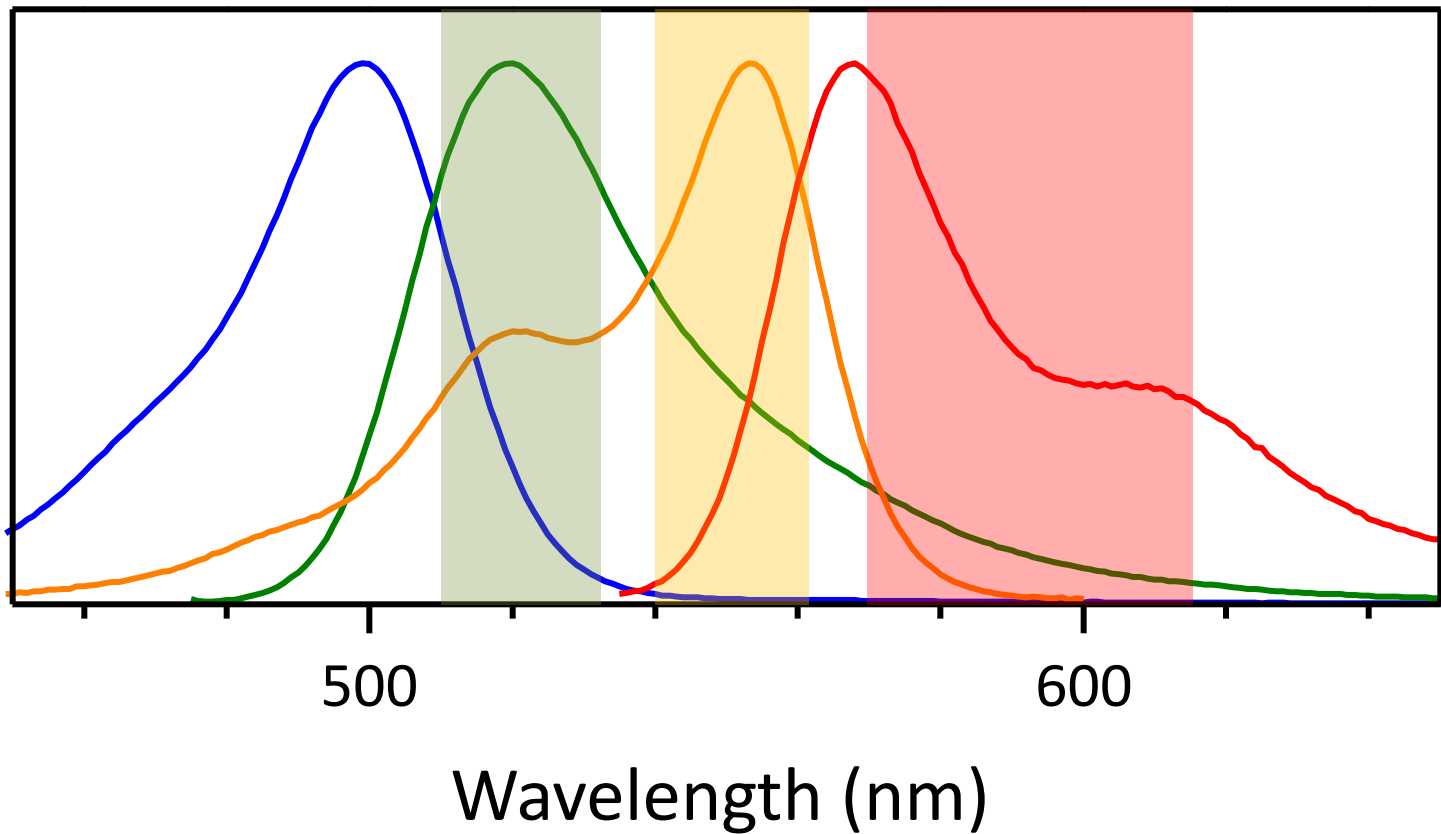
Alexa Fluor 555



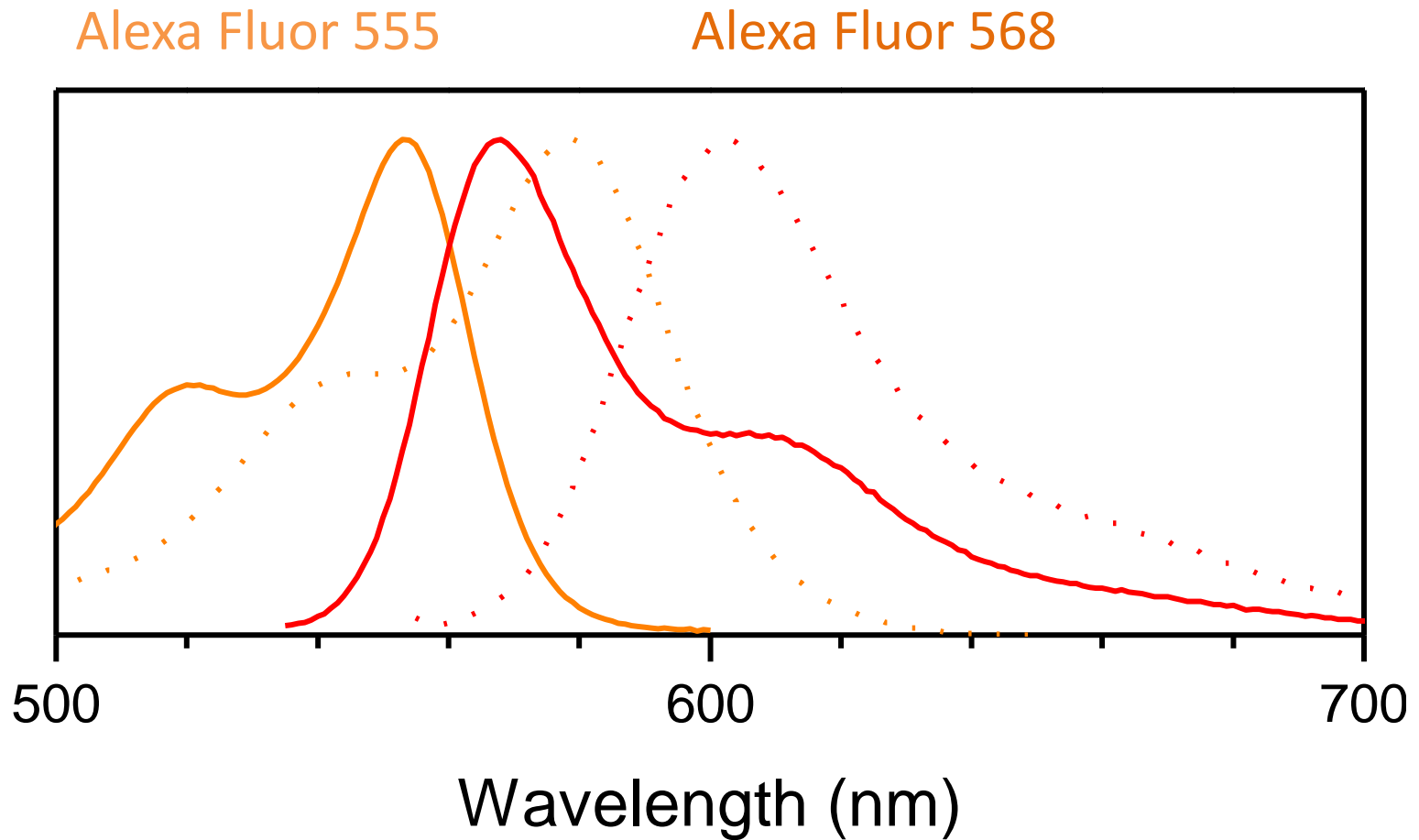
Crosstalk between channels – emission

Alexa Fluor 488

Alexa Fluor 555



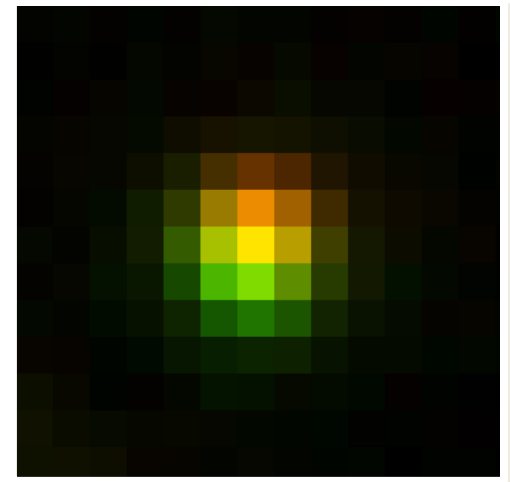
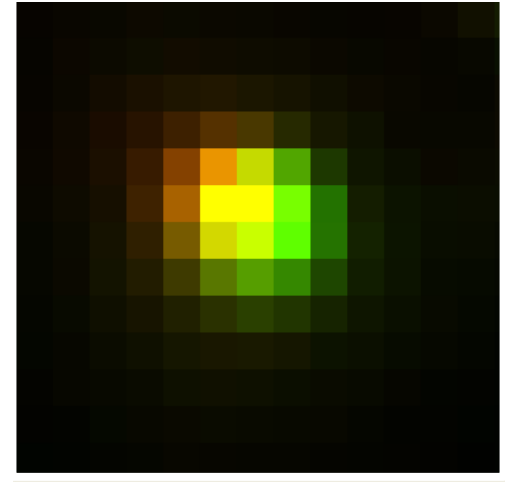
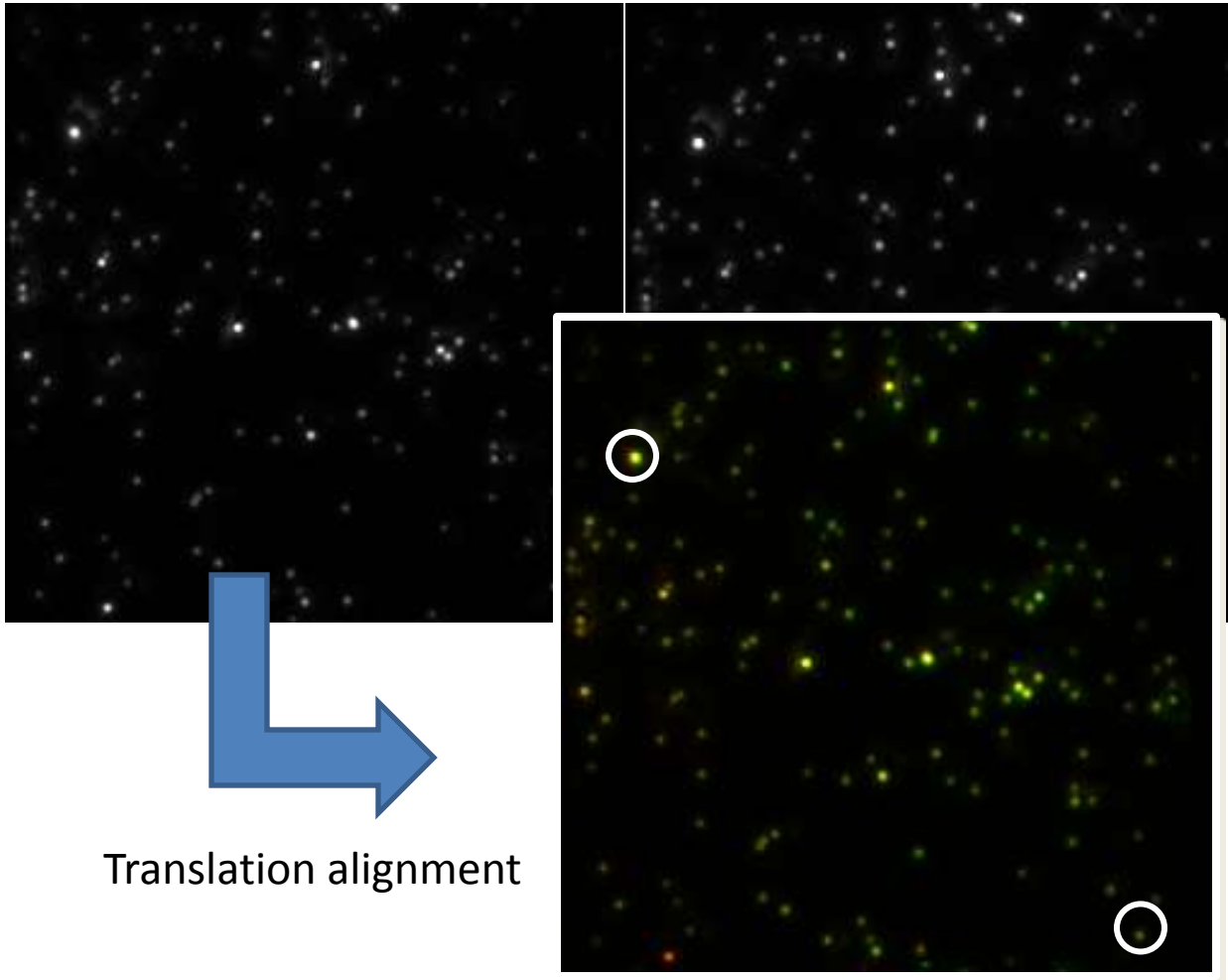
Something too close...



One last concern to address
before time is up...

Image registration

Fluorescent beads with signal in both channels



Thanks!

- Nico Stuurman
- <http://micro.magnet.fsu.edu/>
- <http://www.microscopyu.com>
- <http://olympusmicro.com>
- <http://zeiss-campus.magnet.fsu.edu/>
- <http://www.chroma.com>