Fluorescence Microscopy

I. Fluorescent optics

Bo Huang

2013.03.25

Discovery of Fluorescence

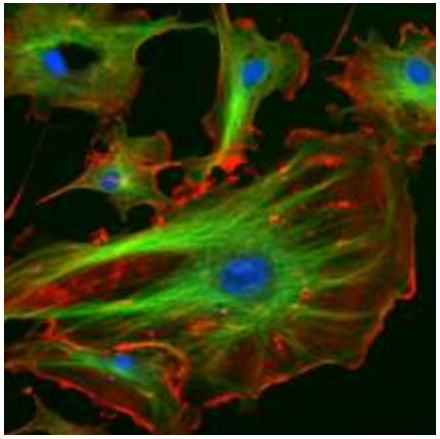
Sir John Herschel

1845

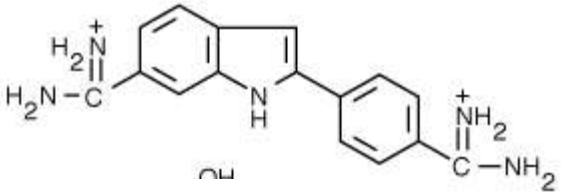
G.G. Stokes

1852

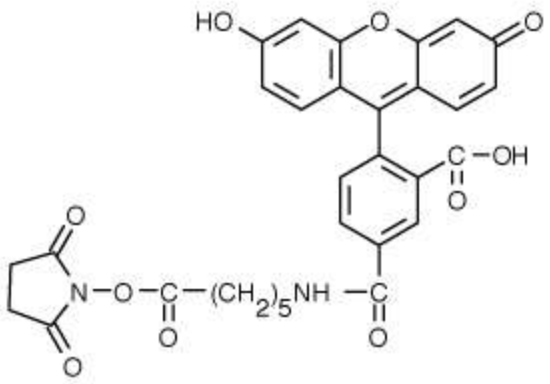
Things that fluoresce



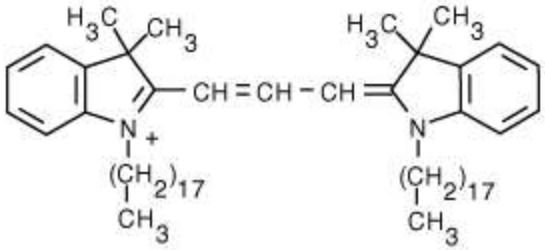
Molecules that fluoresce



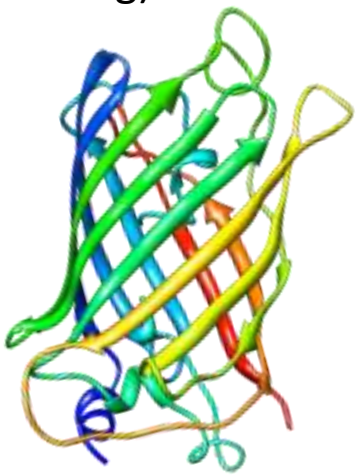
DAPI (DNA stain)



Fluorescein (protein labeling)

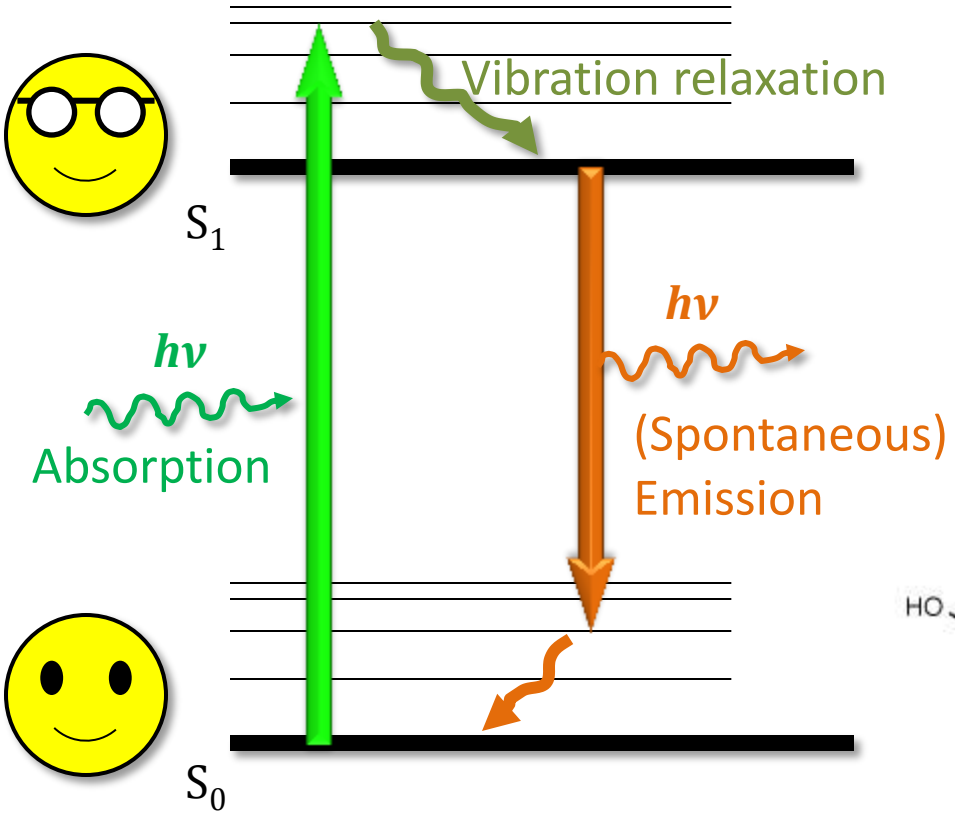


DiI (plasma membrane stain)

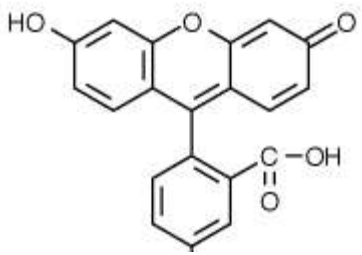


GFP (fluorescent protein)

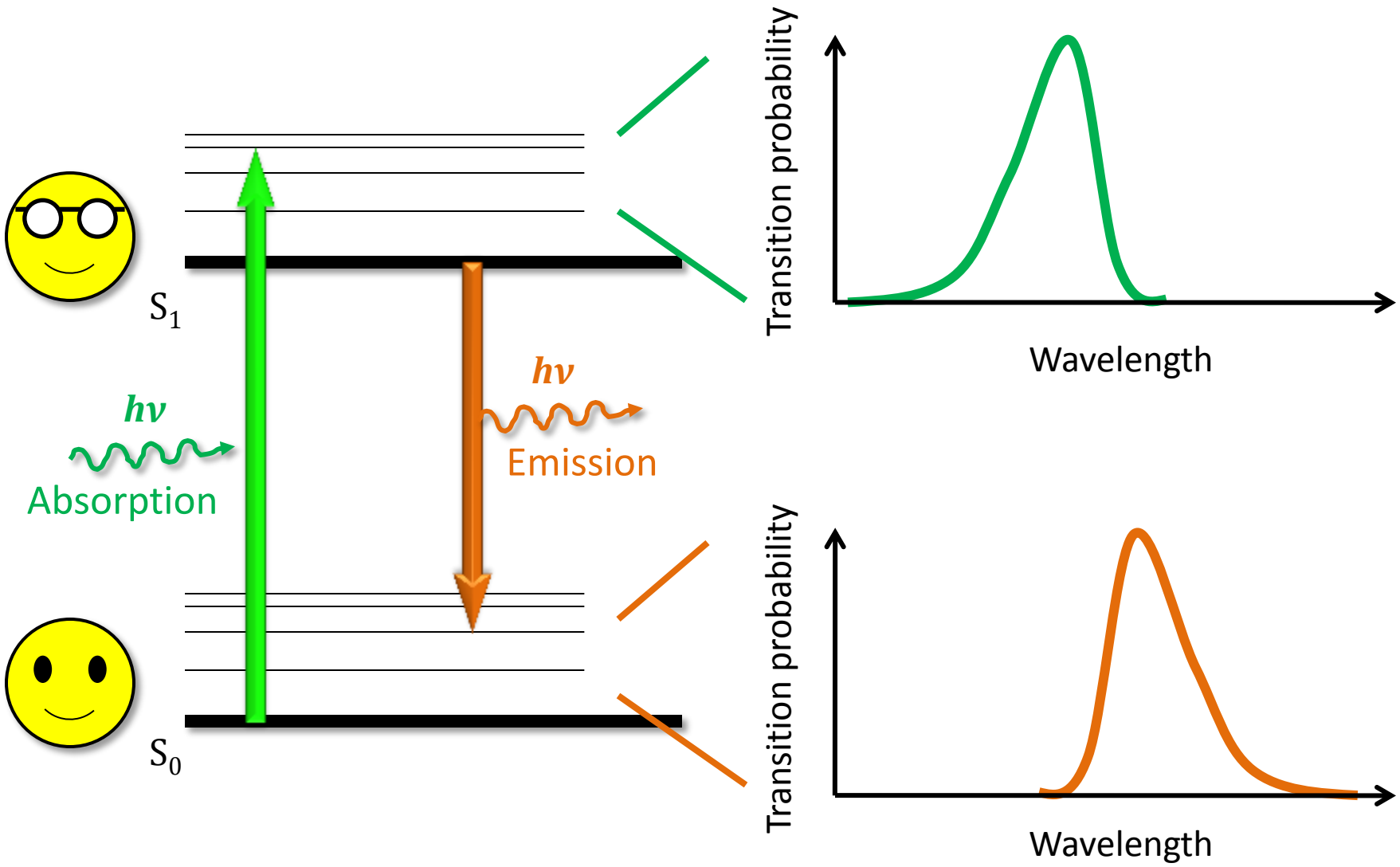
Jabłonski diagram



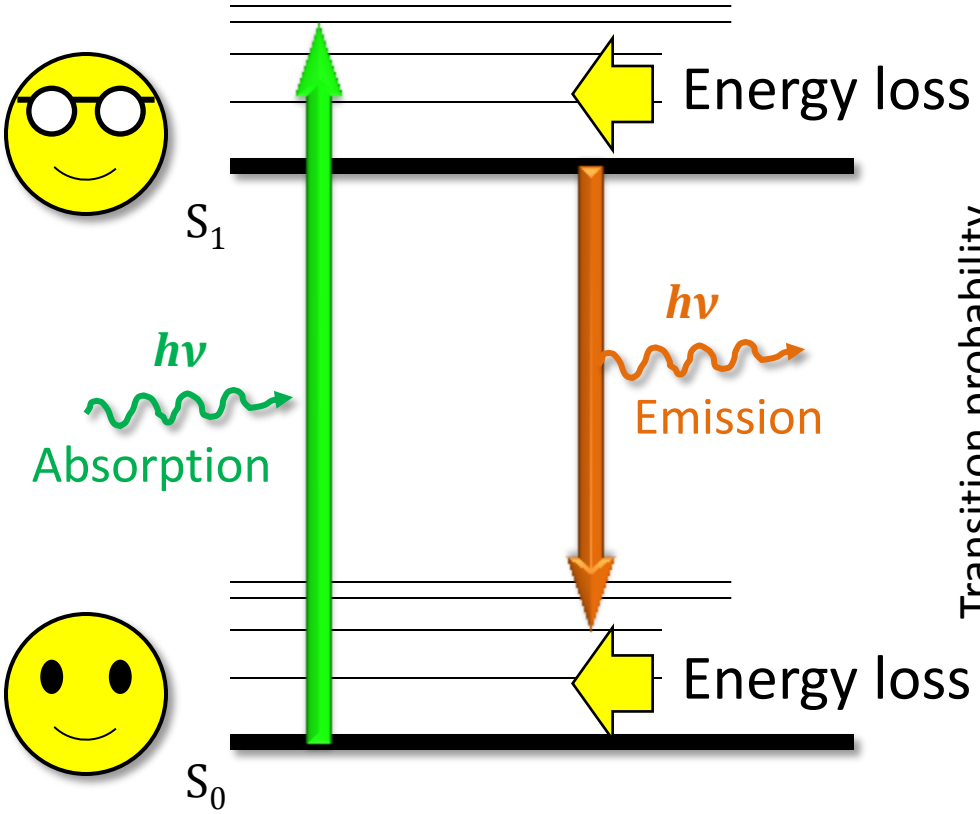
Alexander Jabłonski

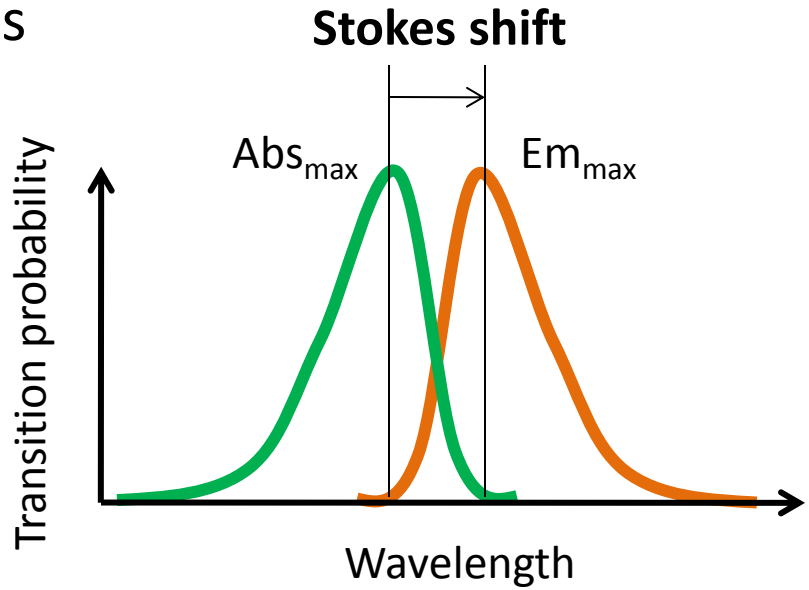


Excitation and Emission Spectra



Stokes Shift

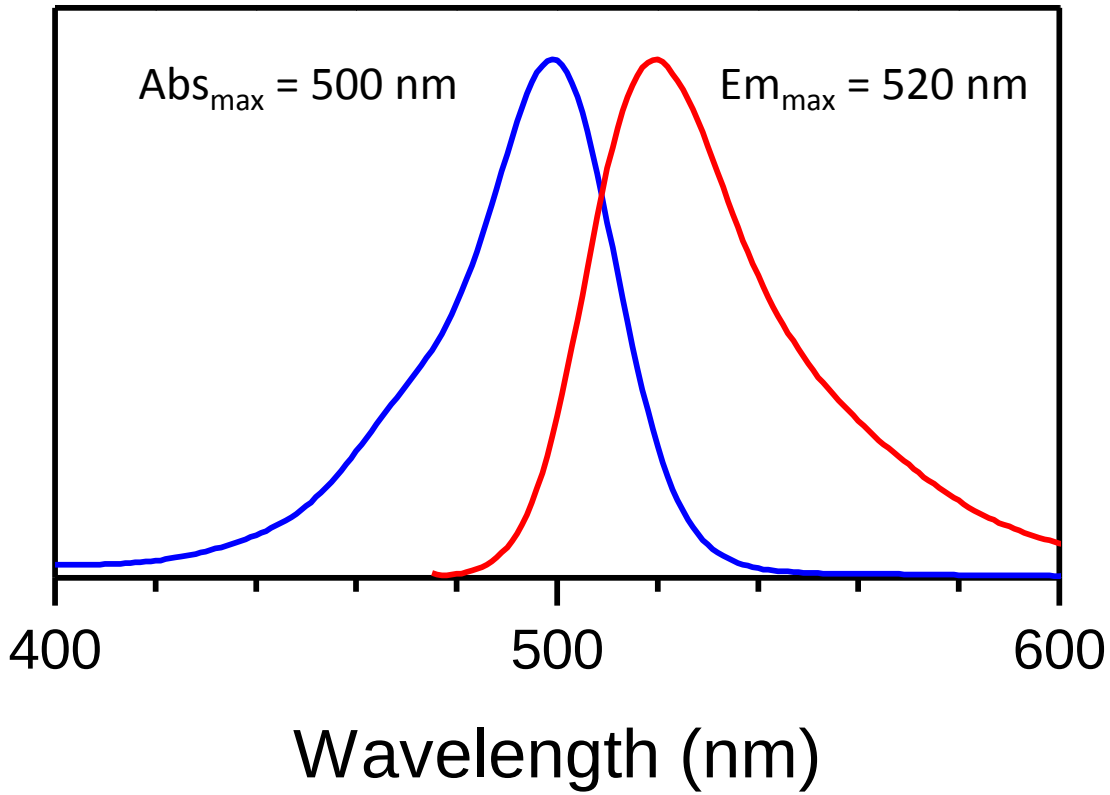


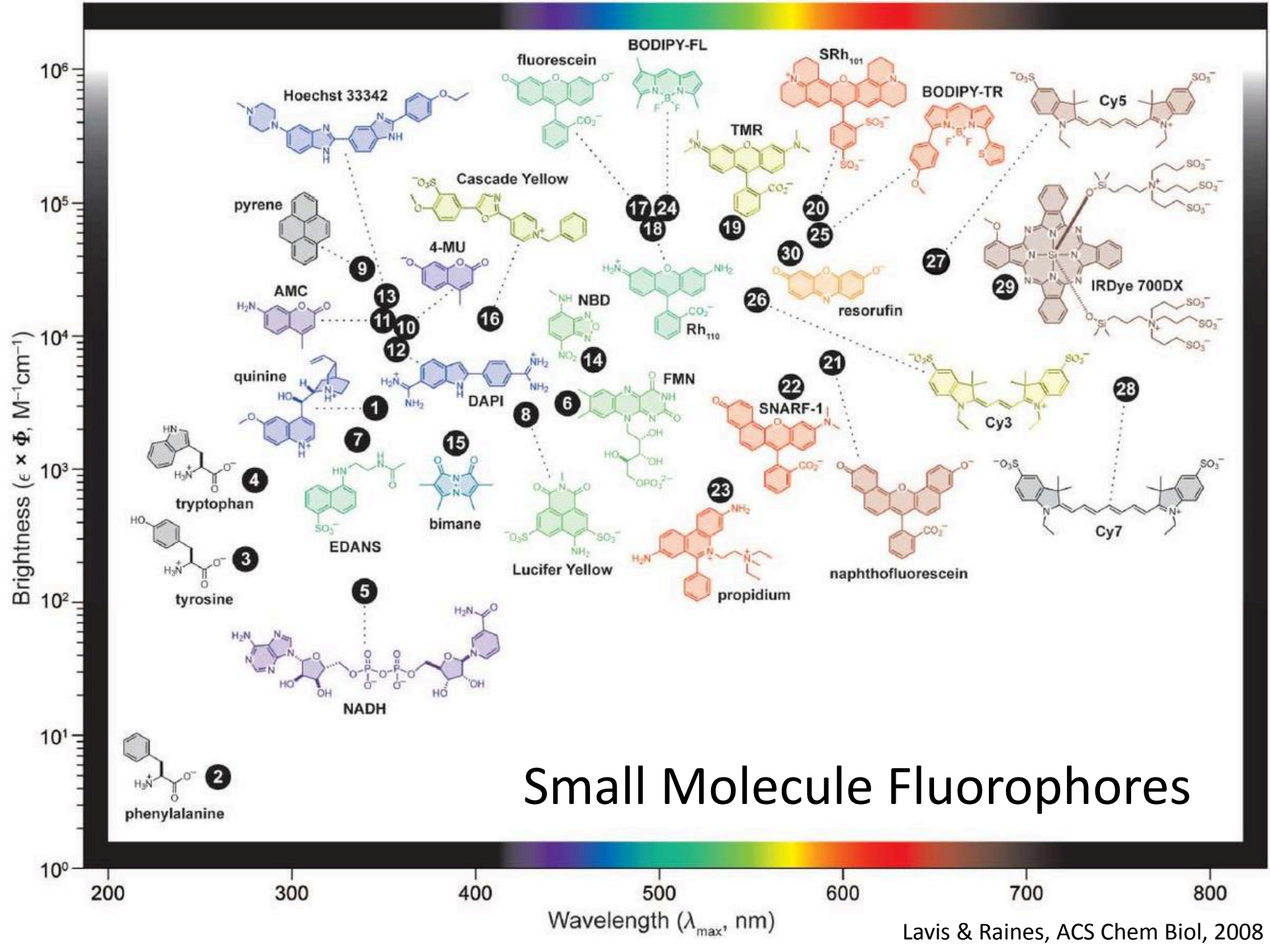


Typically 20-30 nm

Excitation and emission wavelengths

Alexa Fluor 488





Small Molecule Fluorophores

Lavis & Raines, ACS Chem Biol, 2008

Fluorescent proteins

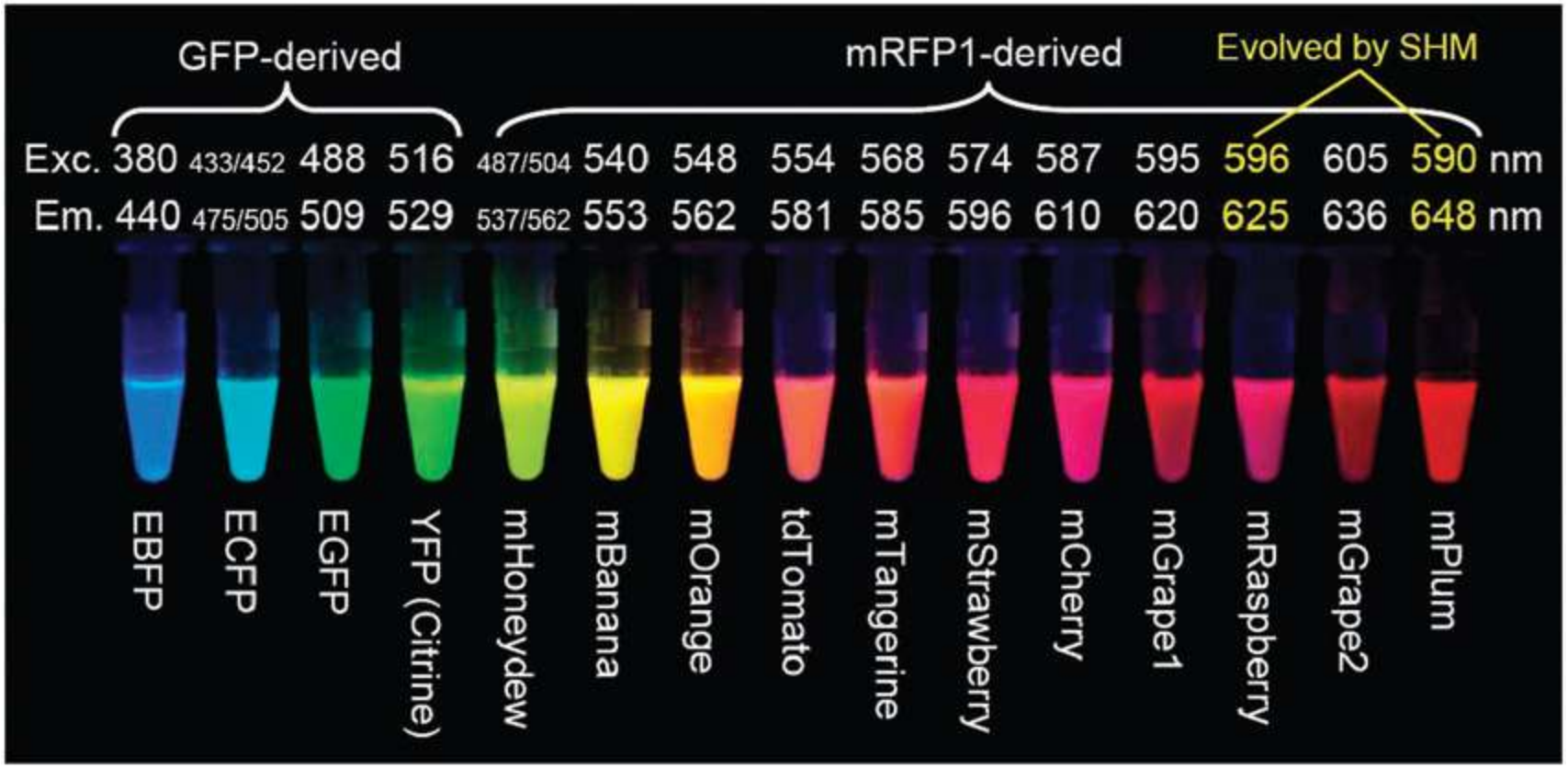


Image from Tsien lab

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

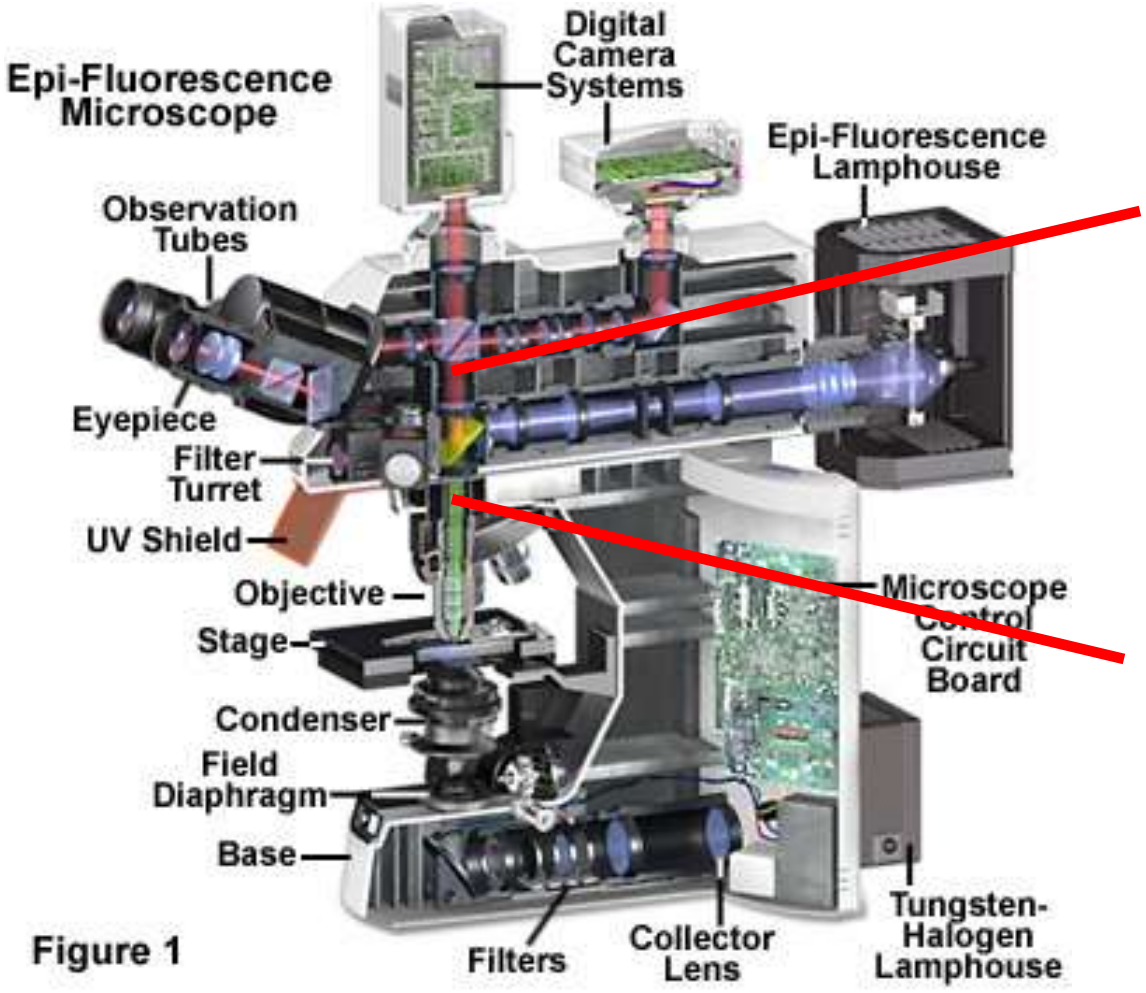


Figure 1

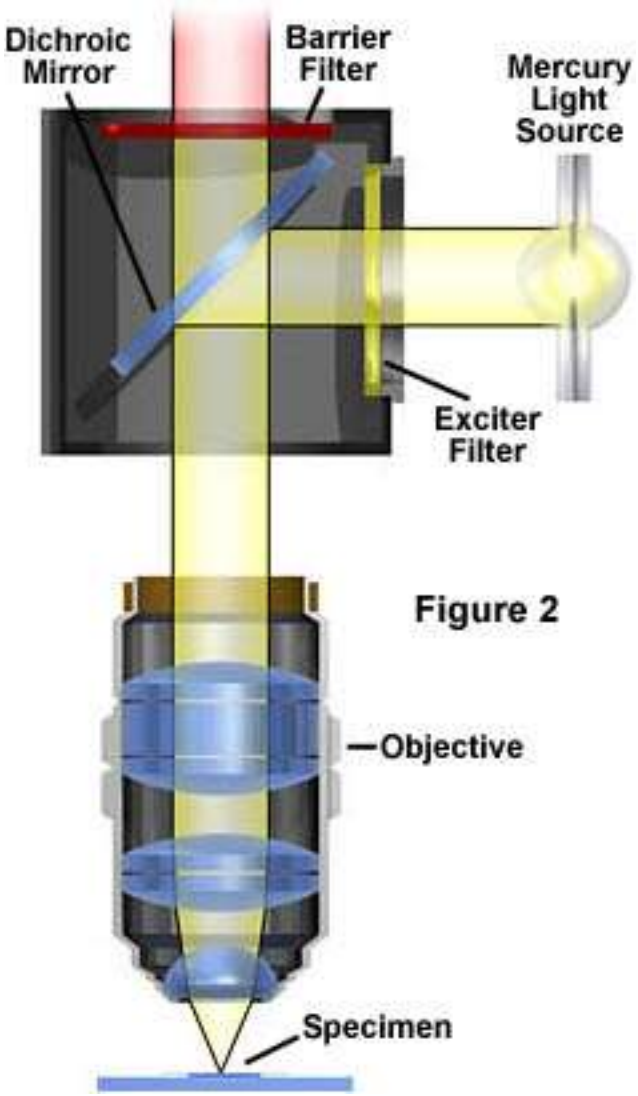
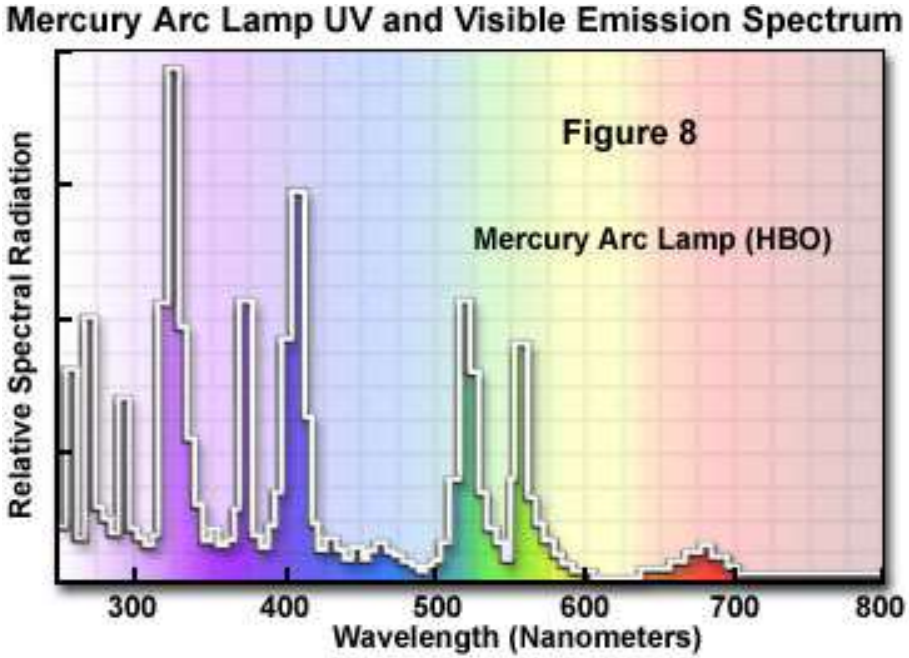
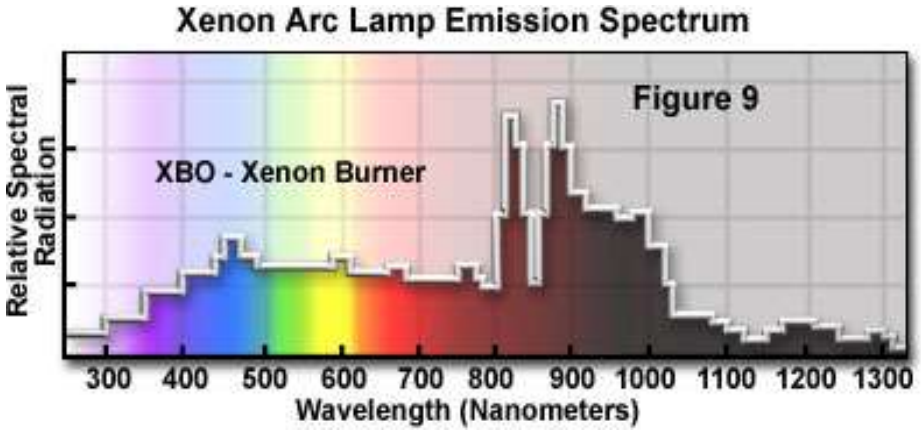


Figure 2

Excitation light sources – Lamps

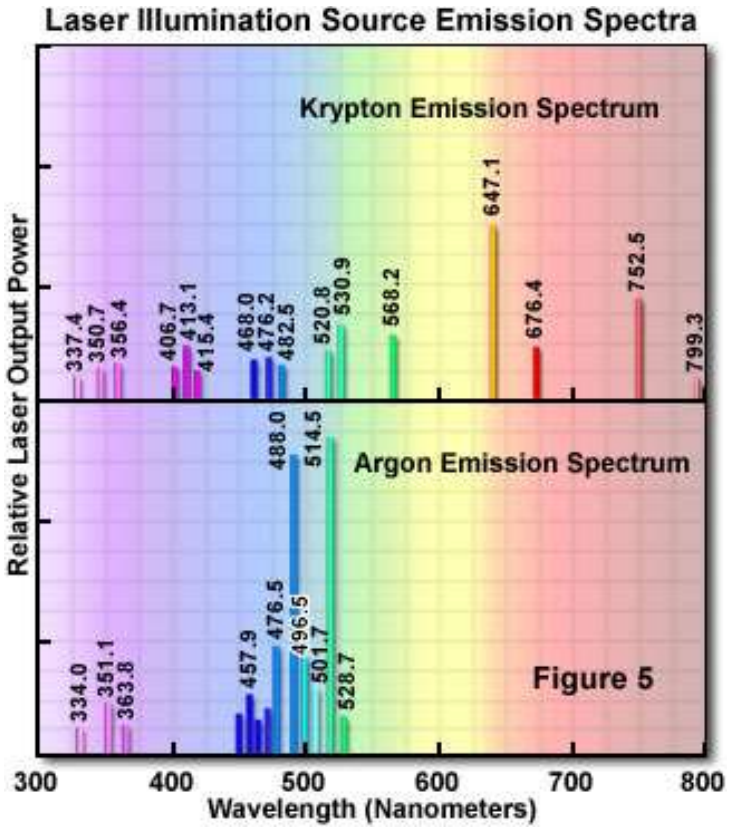


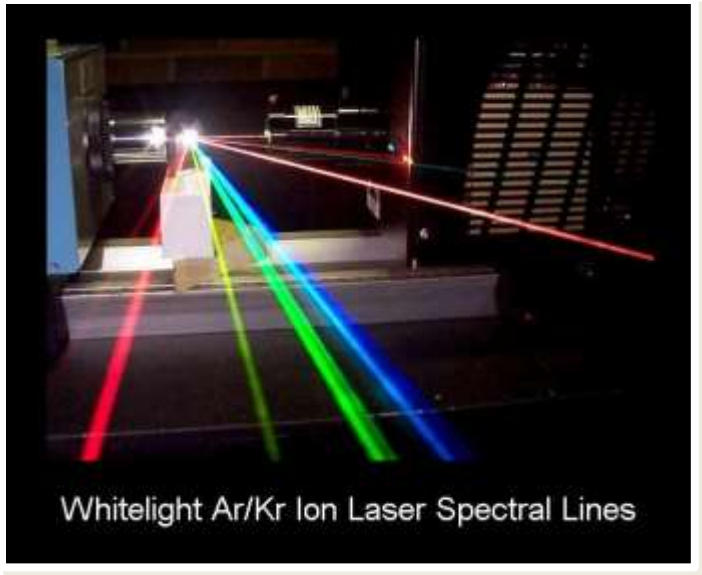


Excitation filter required.

Excitation light sources – Laser

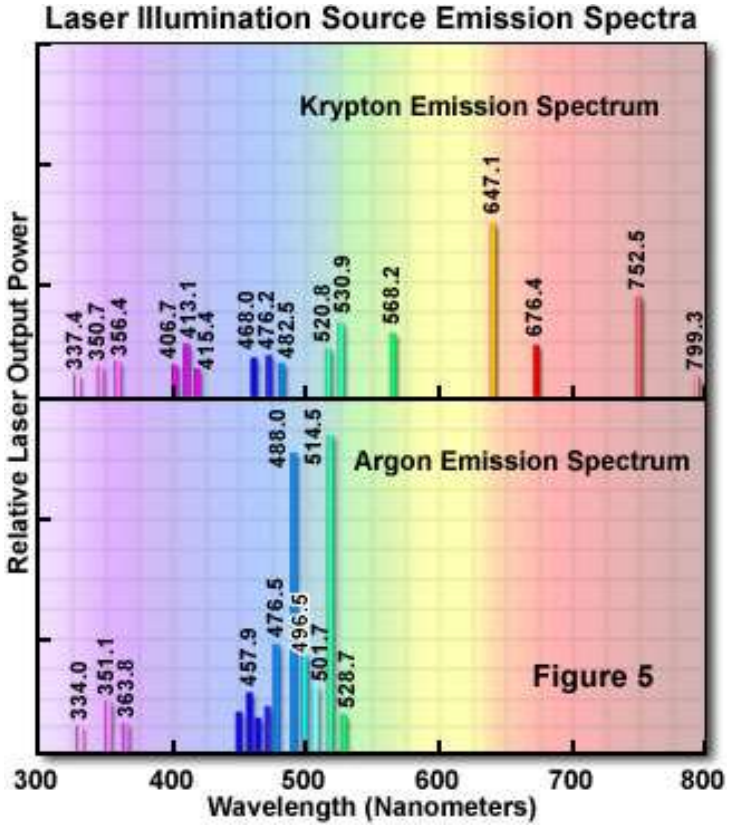
Ar / Kr ion laser: 488, 514, 568, 647





Excitation light sources – Laser

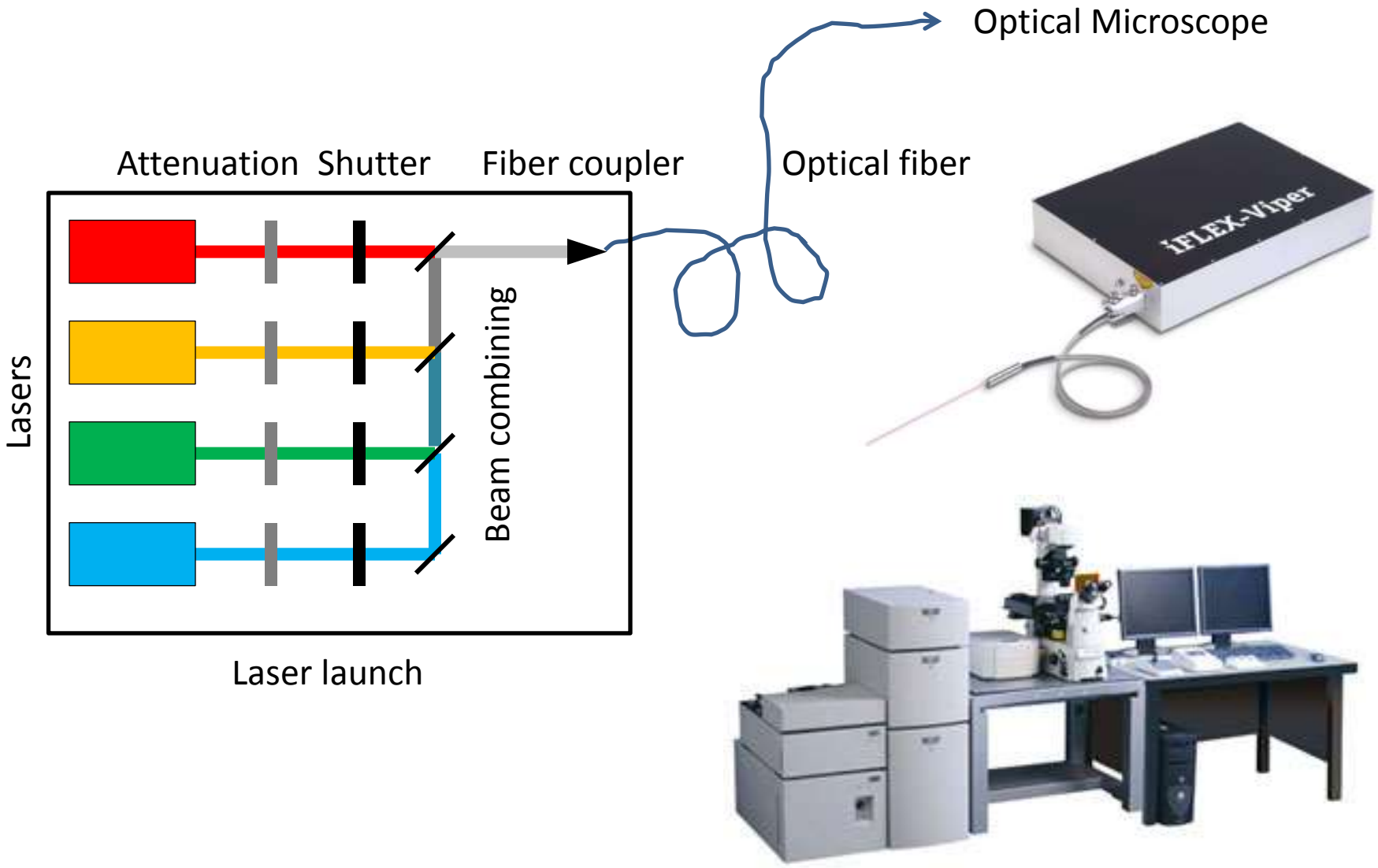
Ar / Kr ion laser: 488, 514, 568, 647



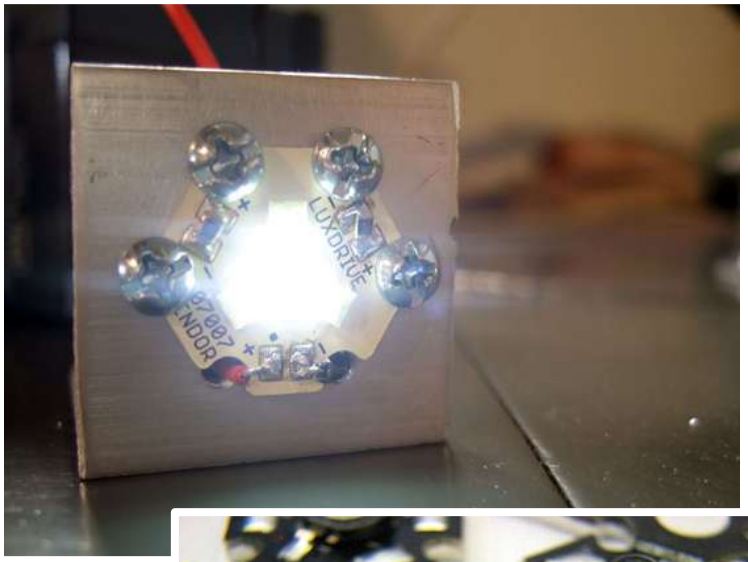
Solid state lasers: 488, 532, 561…

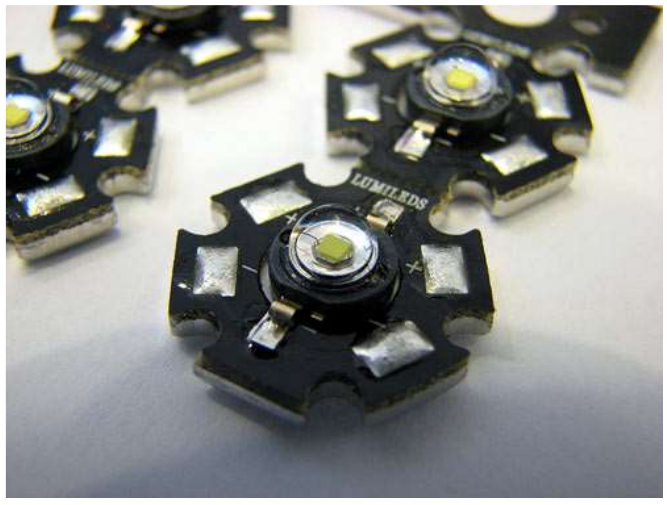
Diode lasers: 375, 405, 488, 635, 660…

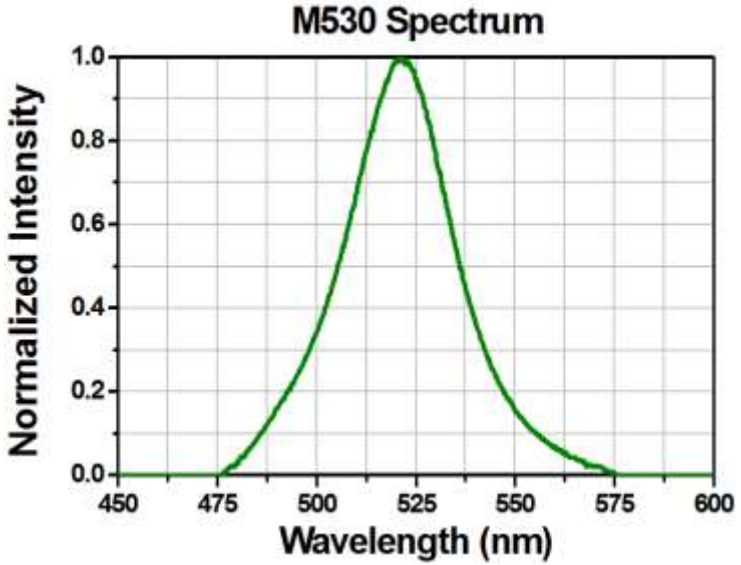
Excitation light sources – Laser

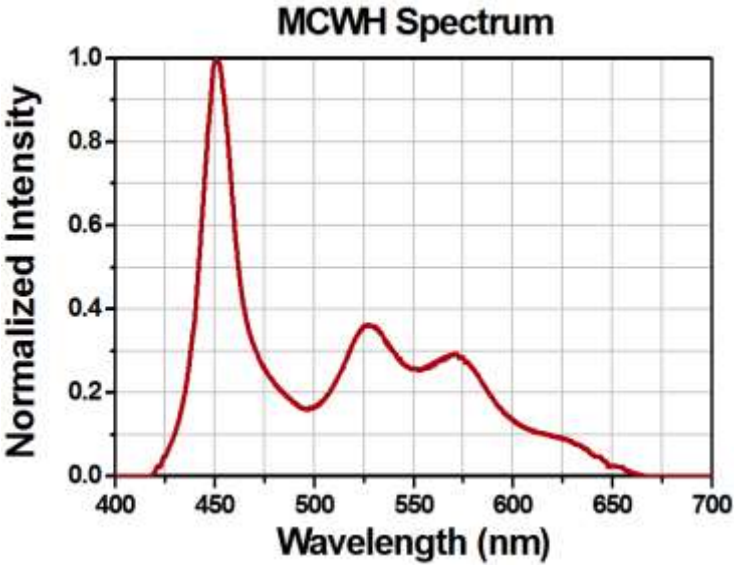


Excitation light sources – LED and others

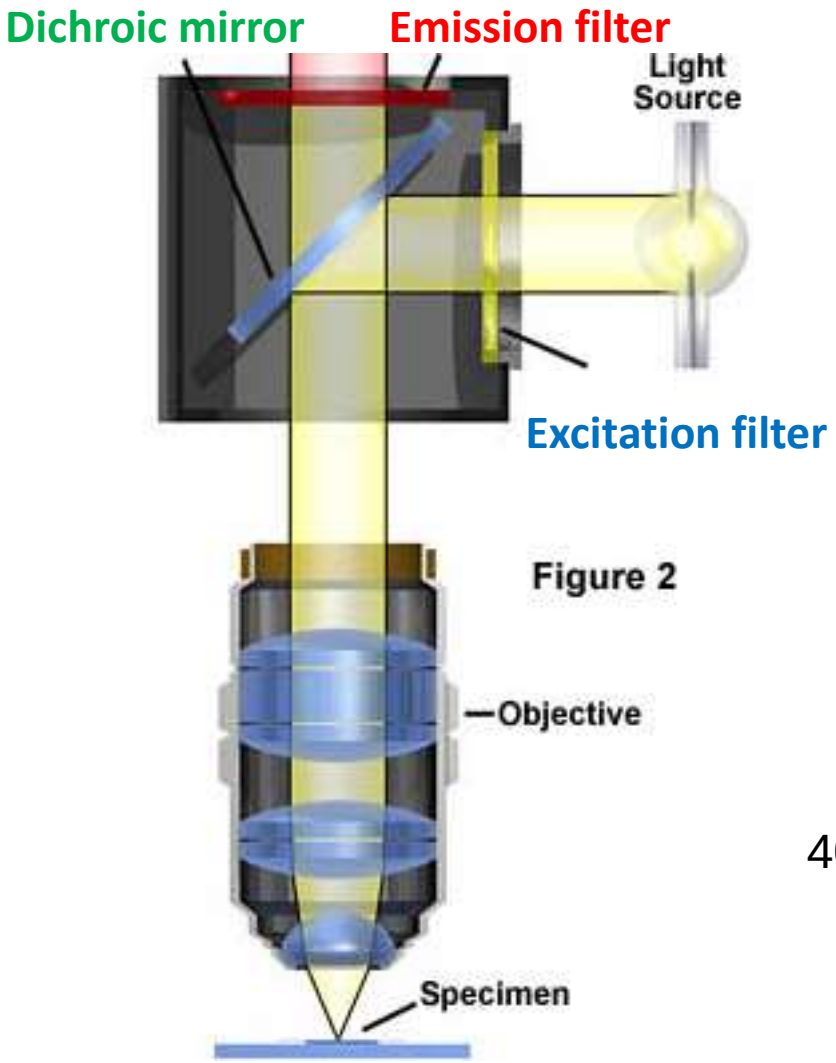


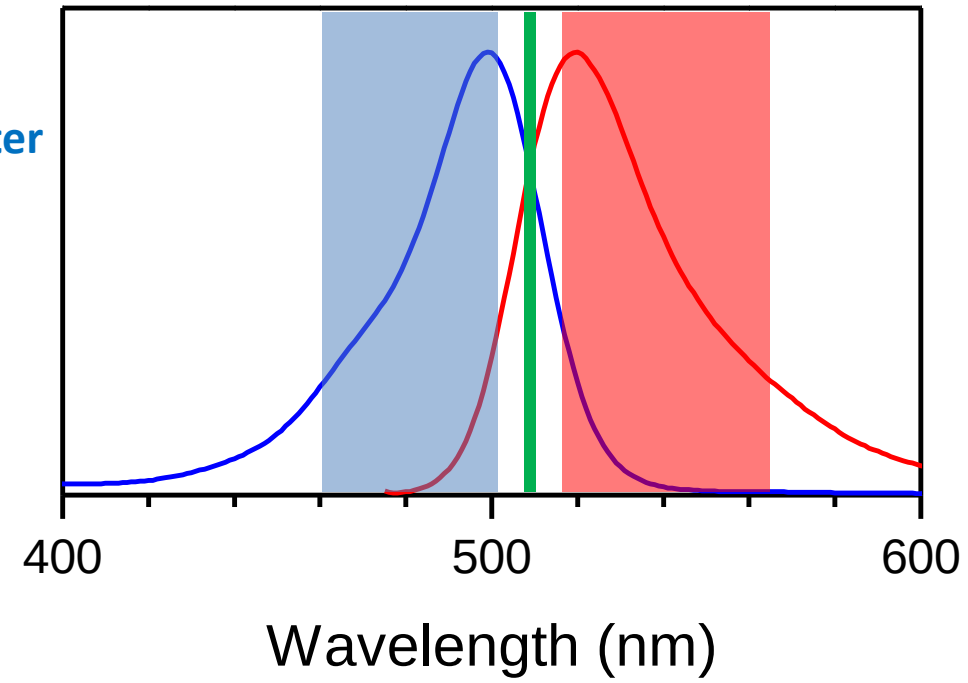




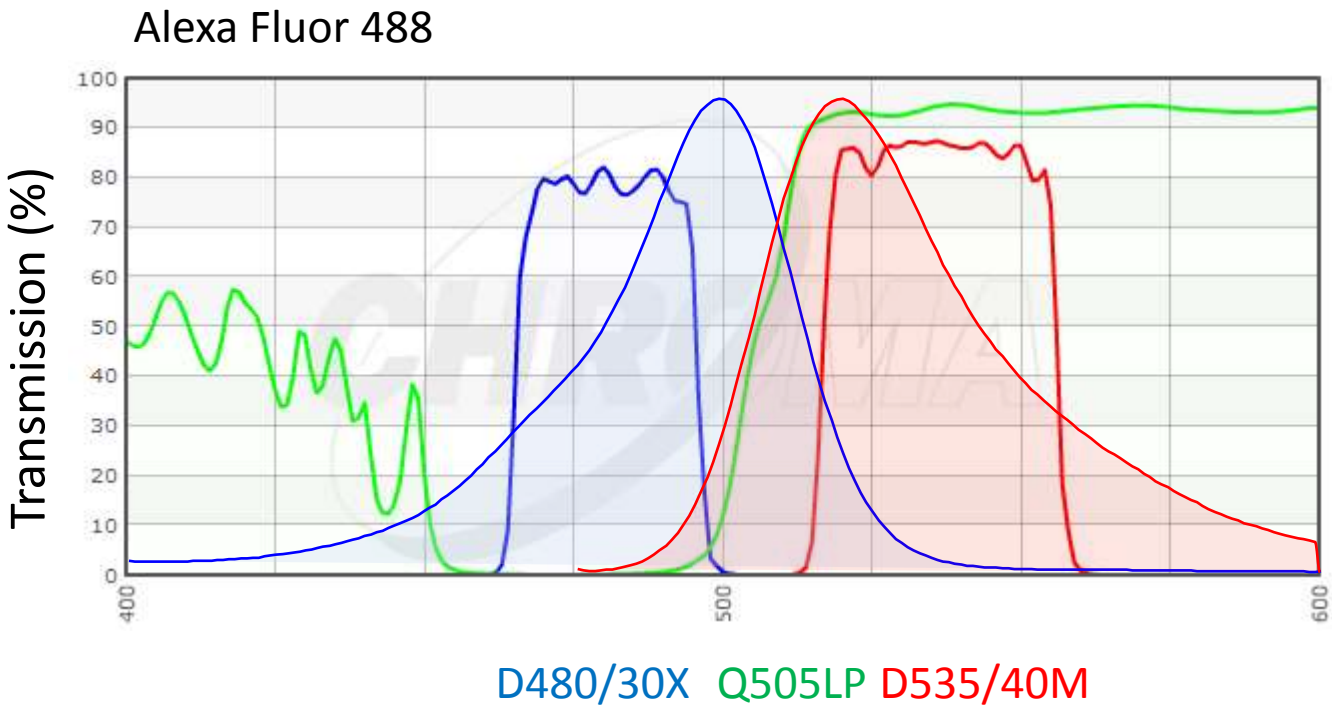


Filter components





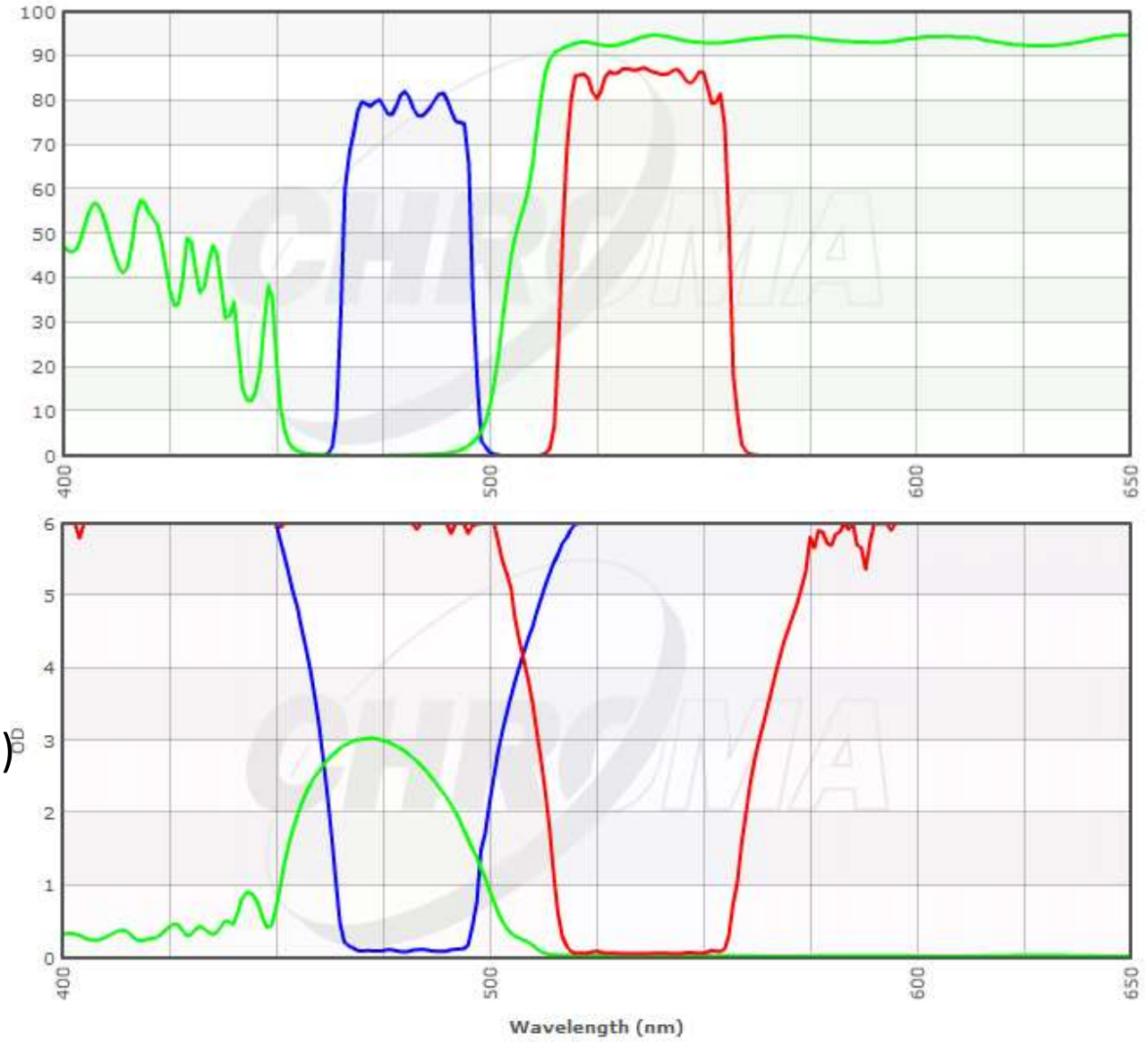
An example of a “filter set”



Transmission vs. Optical density

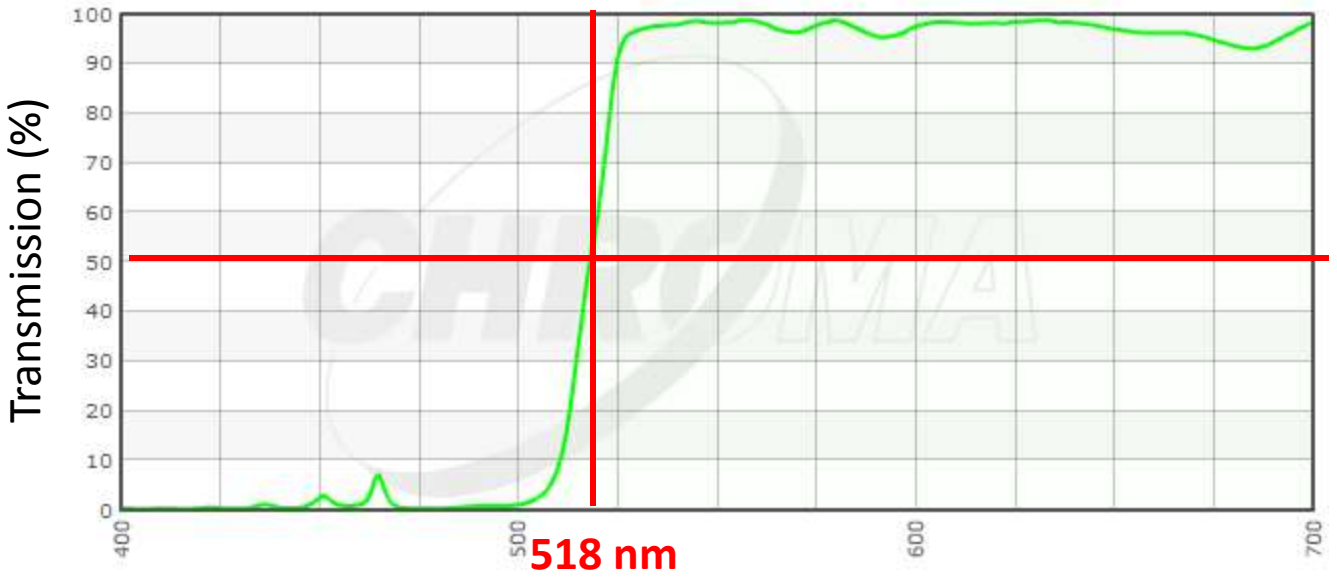
Transmission (%)

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

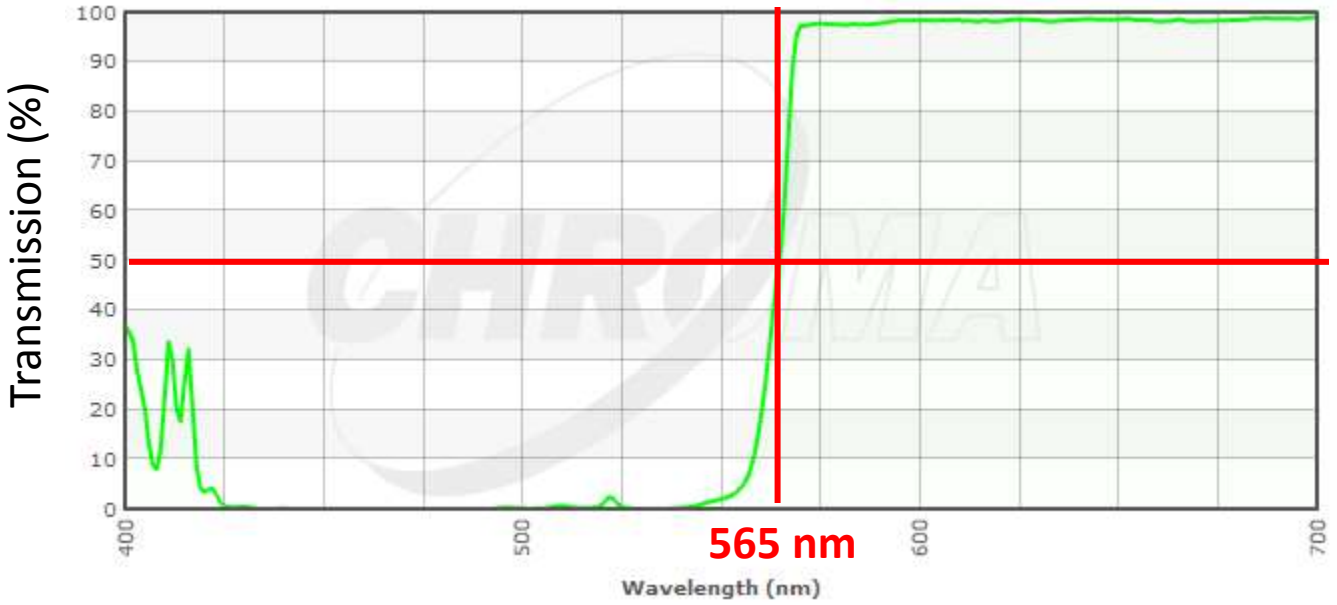


Filter names – Dichroic mirror

515DCLP

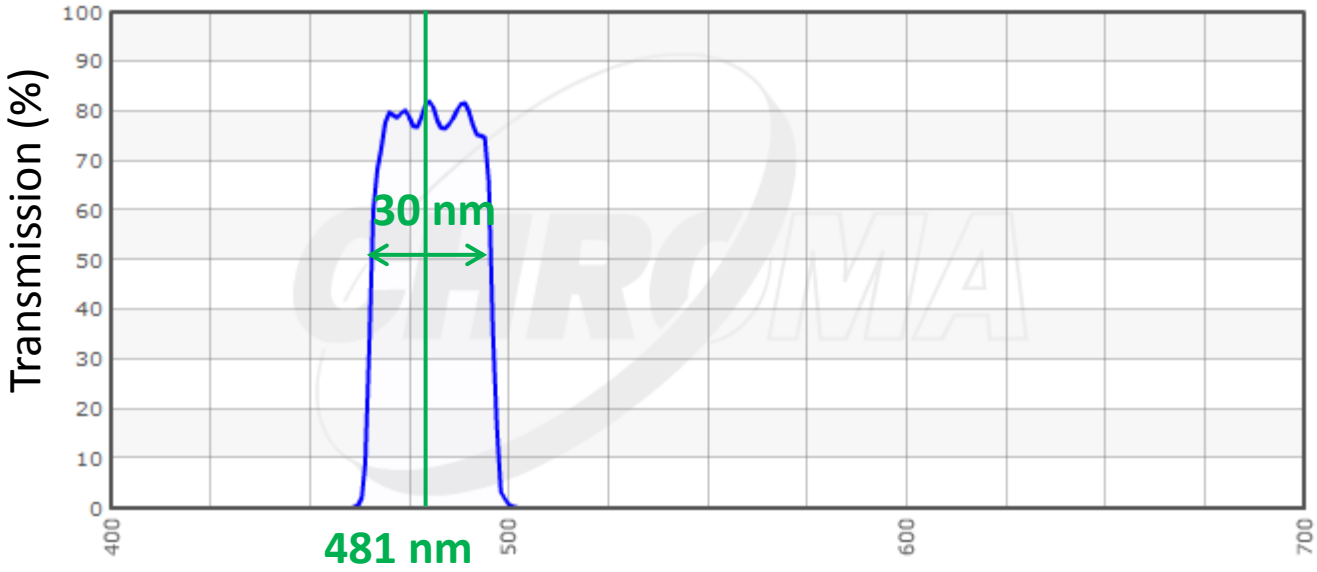


T565LPXR

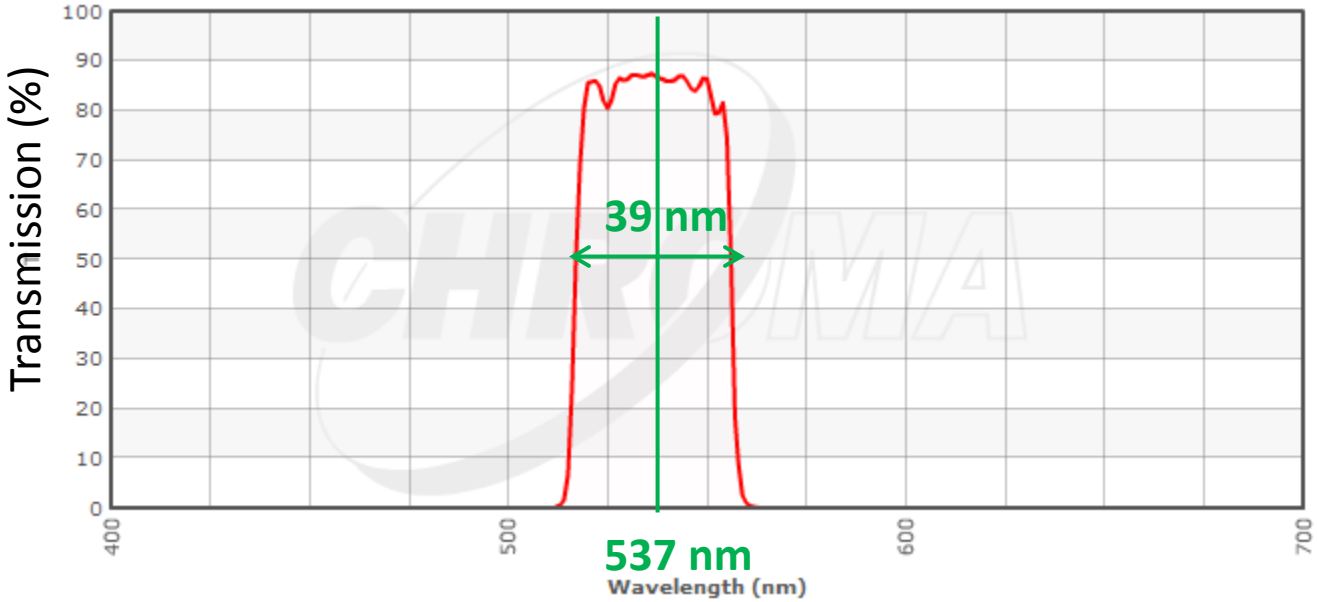


Filter names – Bandpass filters

D480/30X



D535/40M

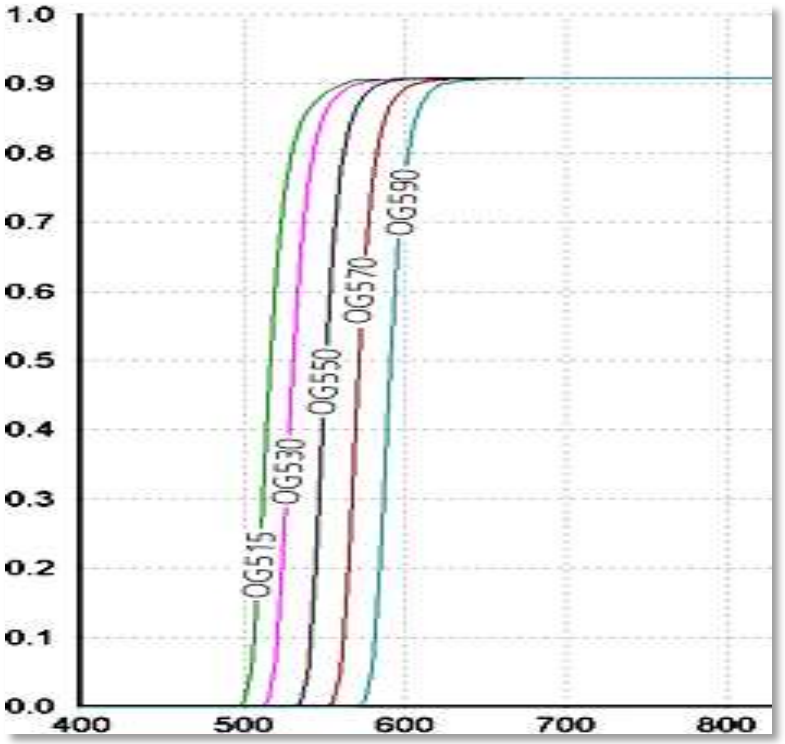


Inexpensive filters: color glasses

Absorptive
(colored glass)

OD ≈ 2

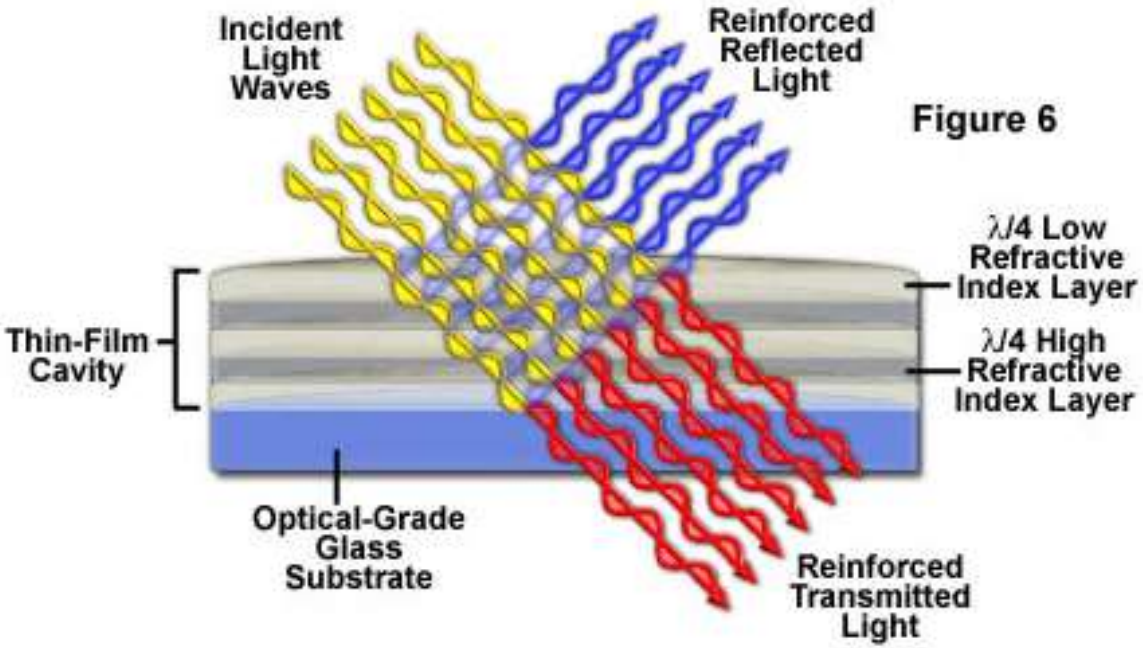
OG550 =
Orange Glass,
50% transmission at 550 nm



Dielectric filters

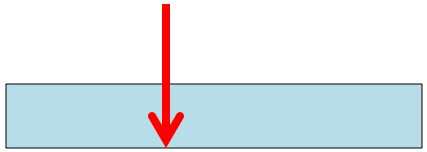
Interference
(dielectric)

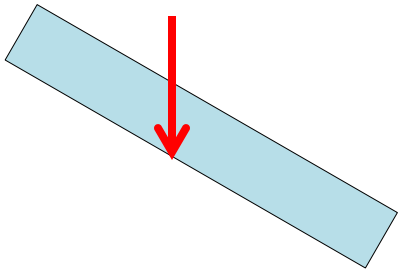
OD > 6
Transmission < 10^{-6}

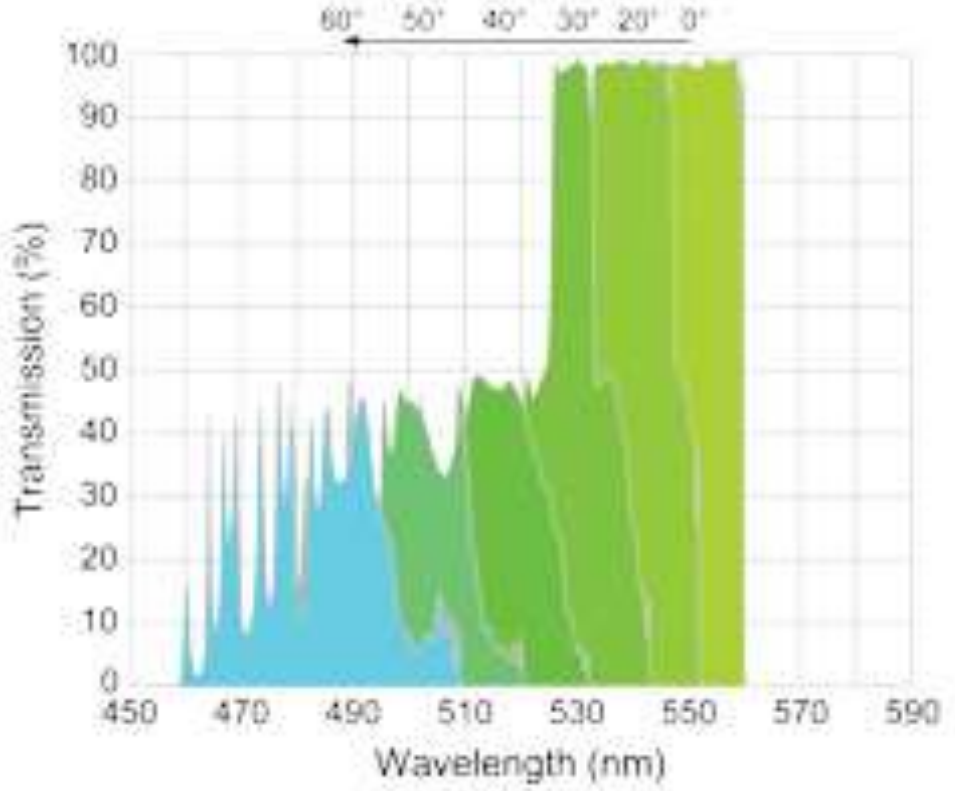


Vendors: Chroma, Semrock, Omega

Interference filter is sensitive to incident angle



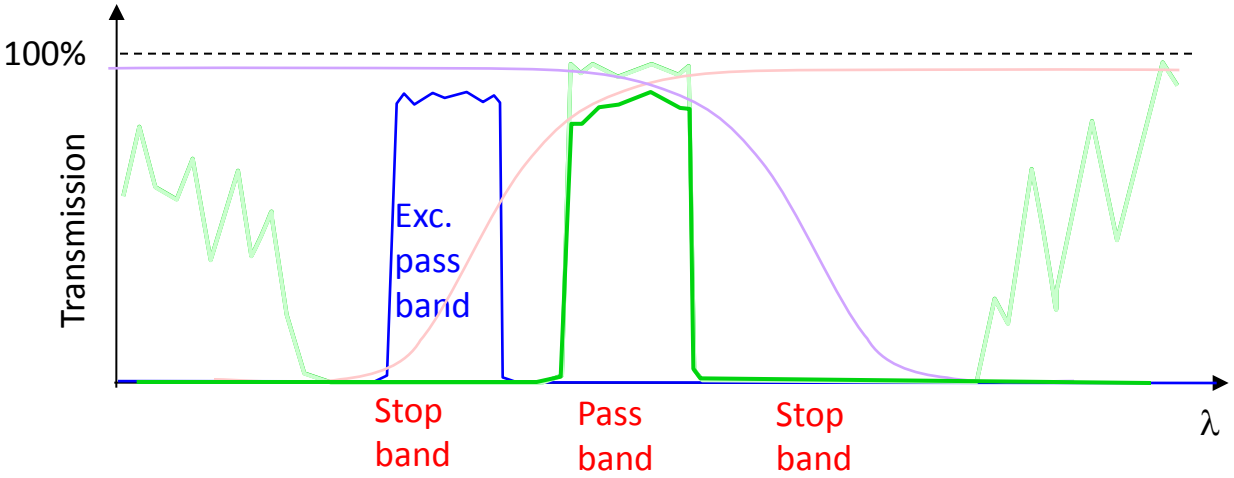


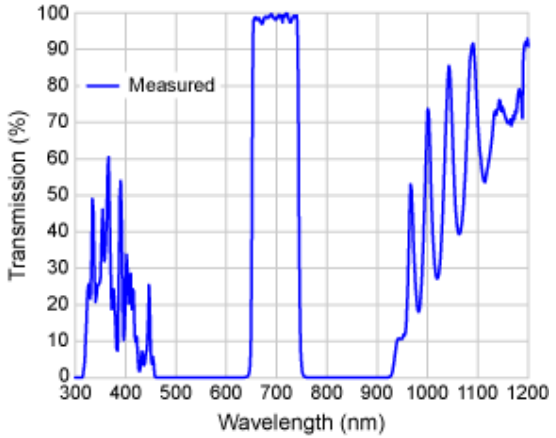


Semrock website

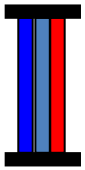
Stop band of interference filters

Unblocked bandpass interference filter





Semrock 697/75



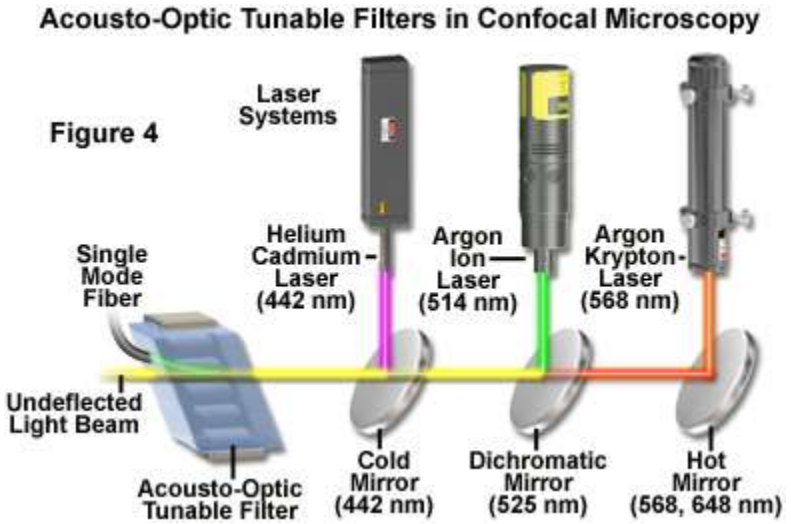
UV absorber
Interference filter
IR absorber

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

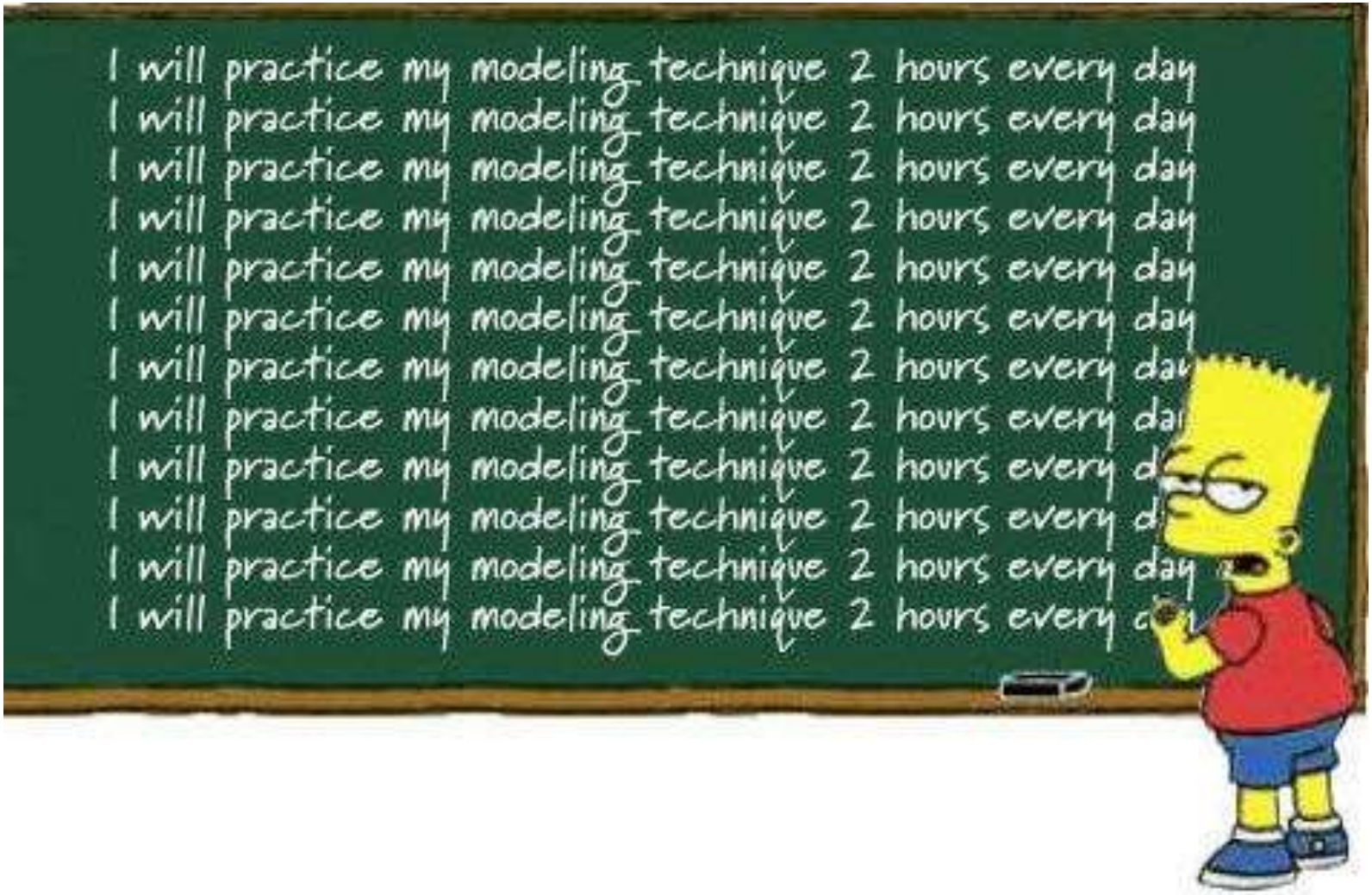
From Nico Stuurman

Tunable filters

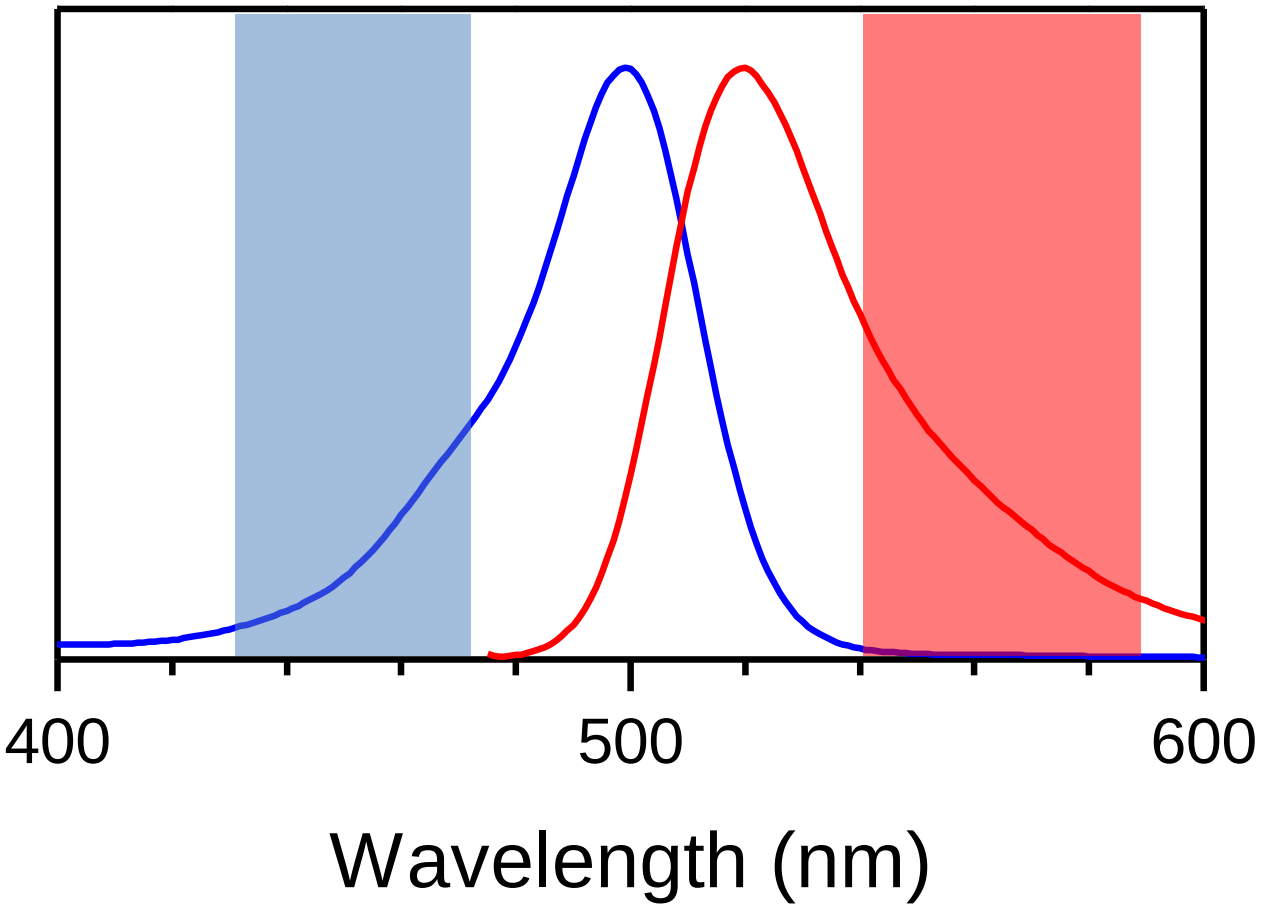
- Liquid crystal filter
- Acoustical optical tunable filter (AOTF)
  - Modulated by ultrasound wave in a crystal
  - Fast switching (µs)
  - Polarization sensitive
  - Mostly for excitation laser



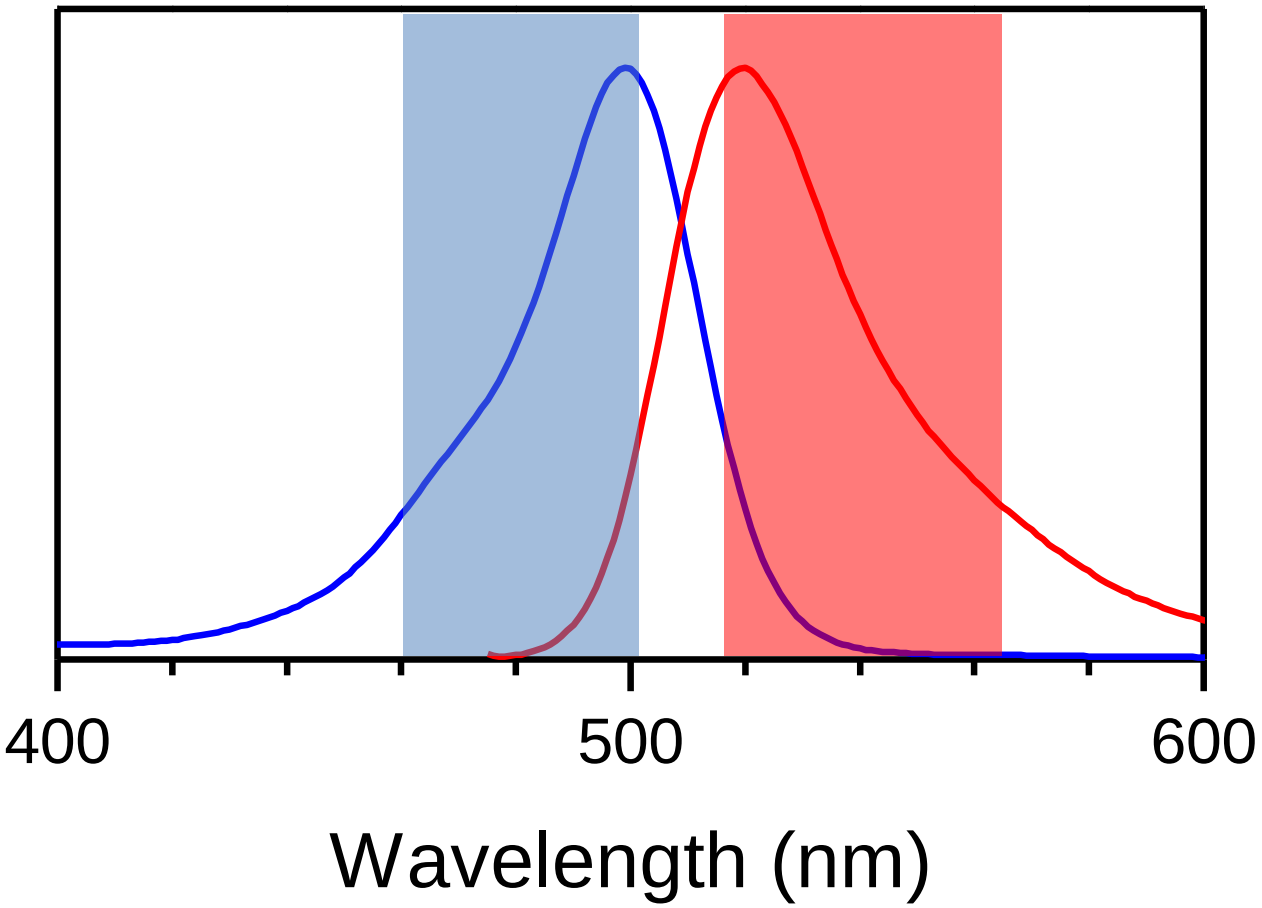
Practical concerns



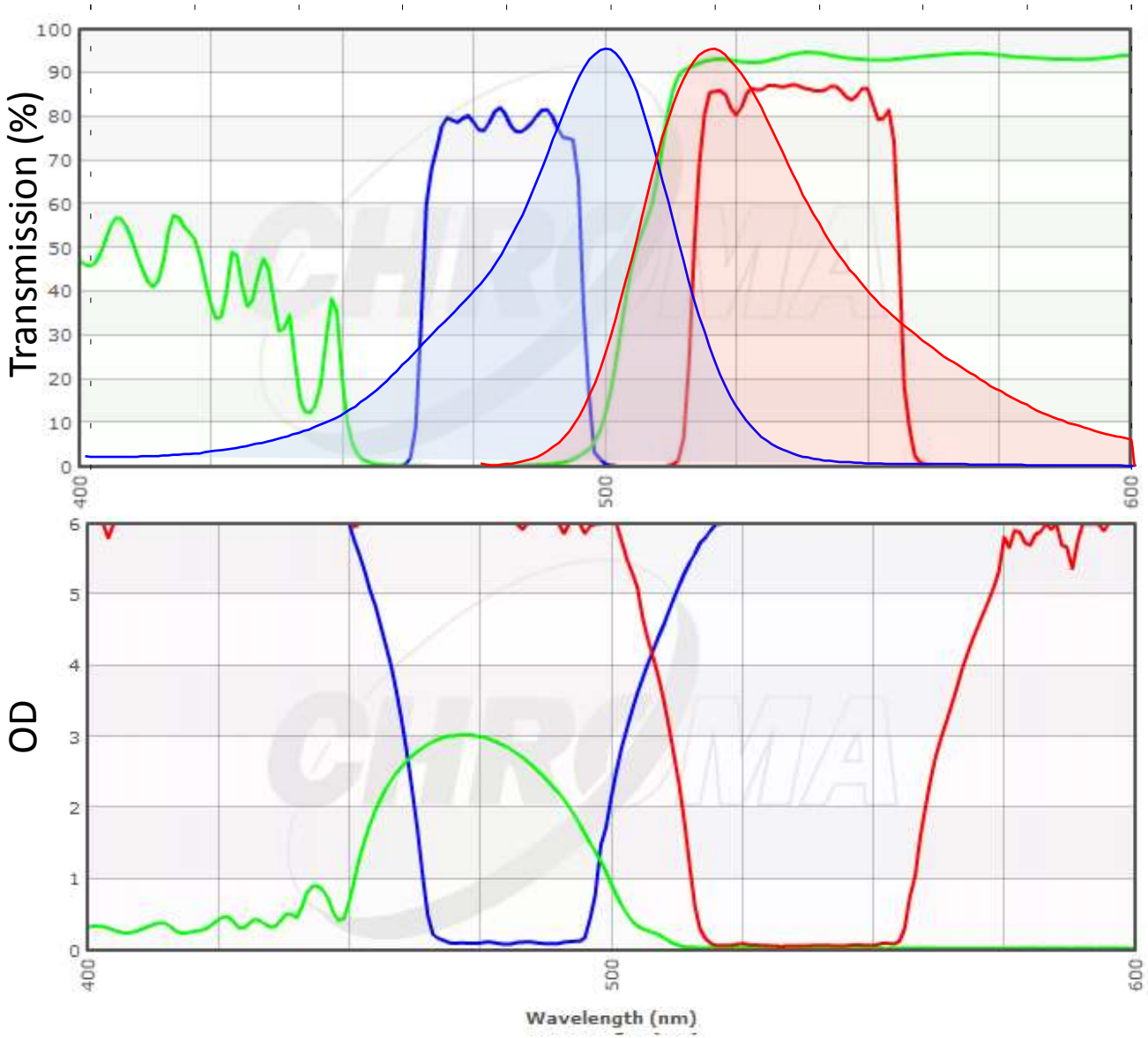
Matching the filters with the spectra



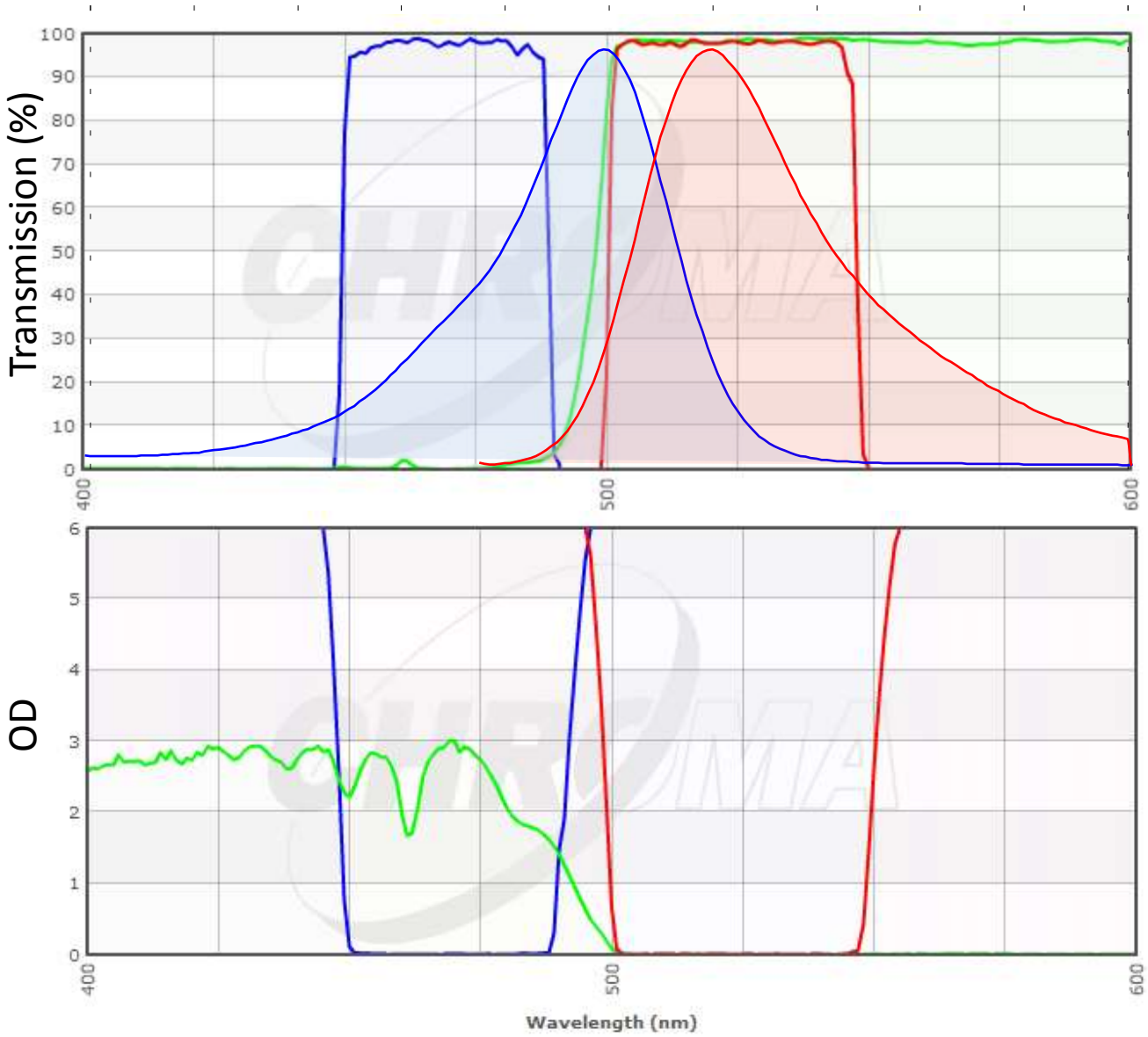
Matching the filters with the spectra



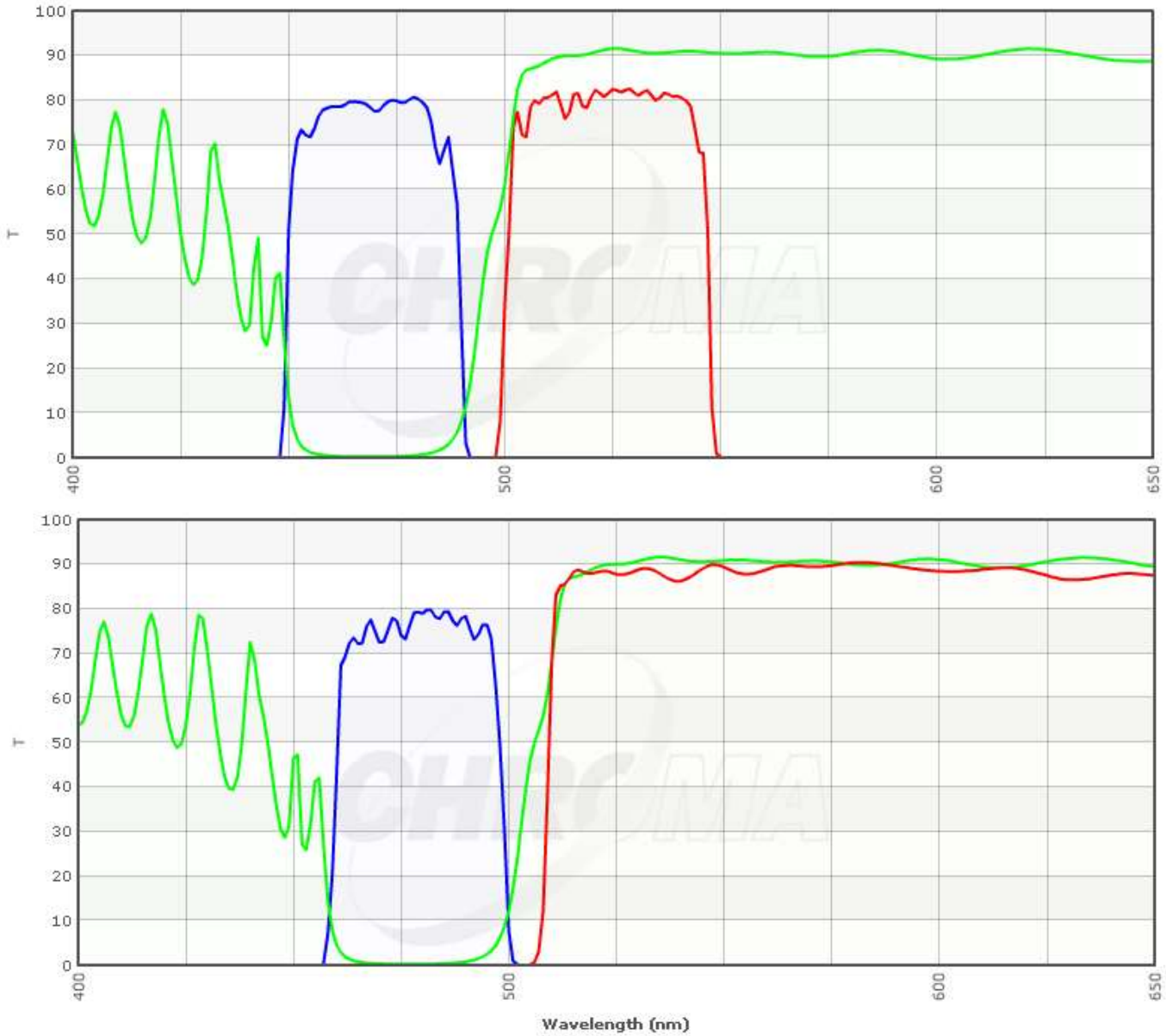
The choice of a “filter set”



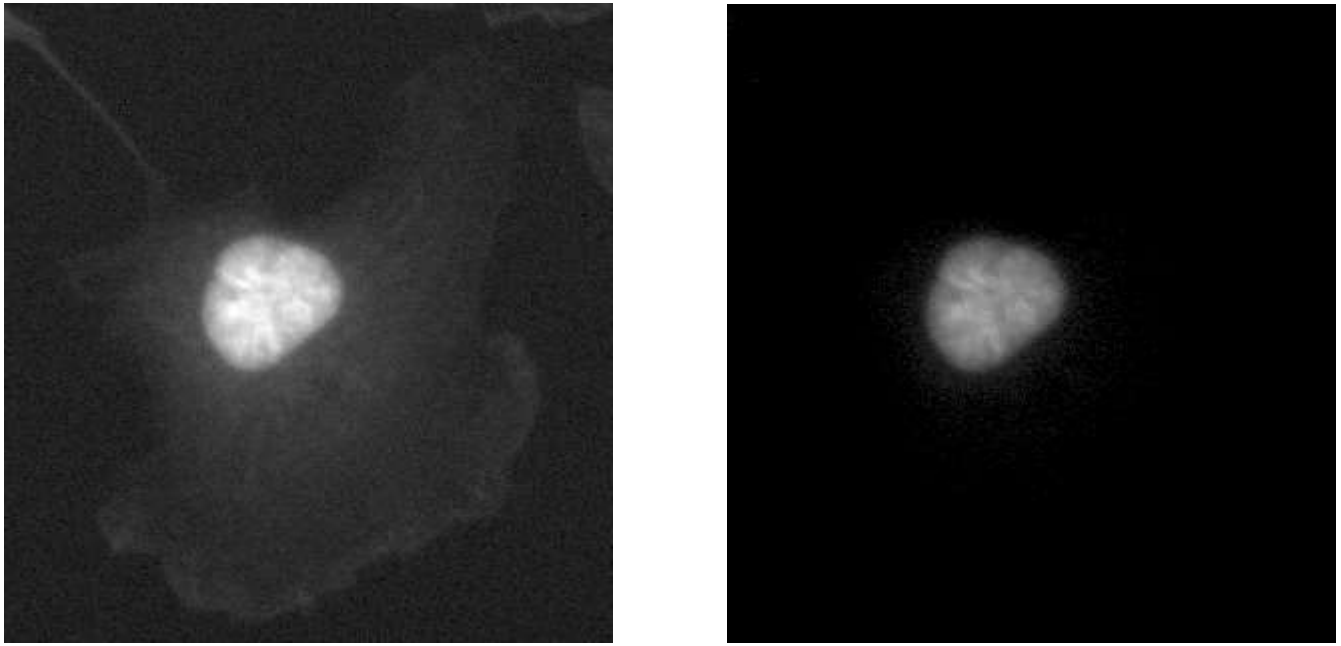
Better ($$$) filters give higher efficiency



Long pass vs. Band pass



Wider is not necessarily wiser

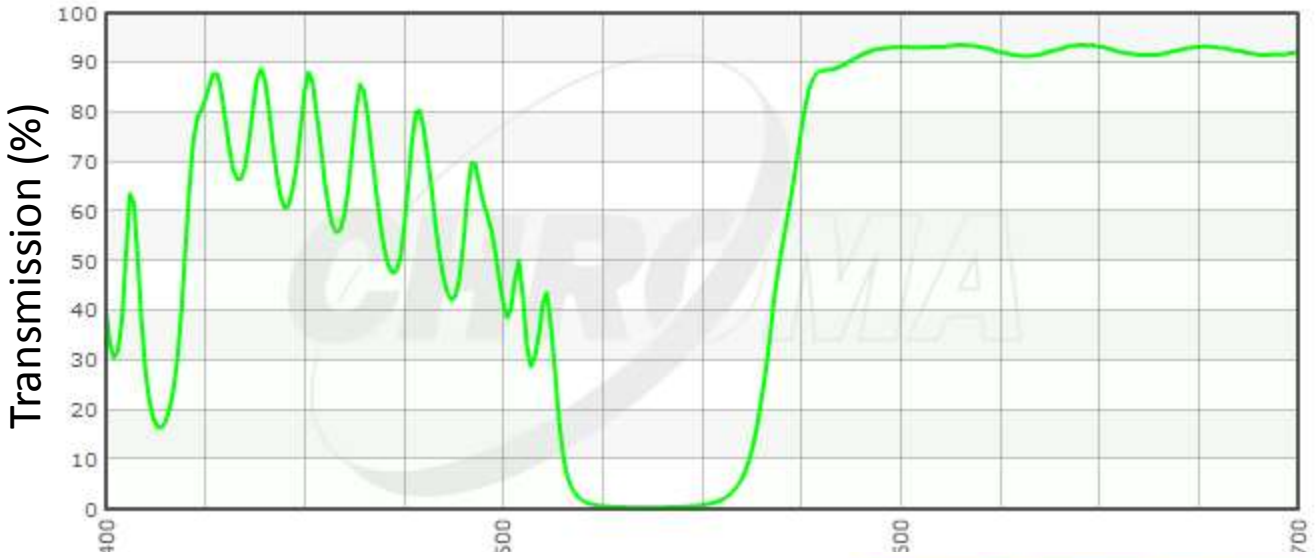


DAPI nucleus staining, long pass vs. bandpass

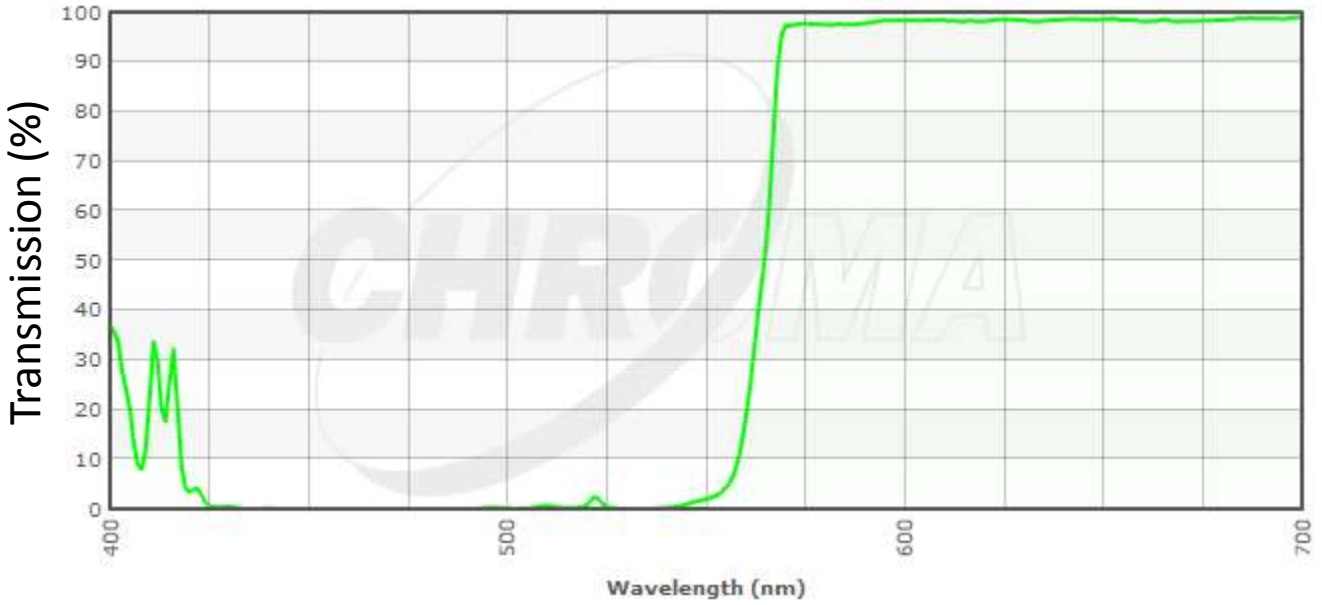
From Nico Stuurman

Dichroic mirrors does not have infinite reflection band

565DCLP
Chroma

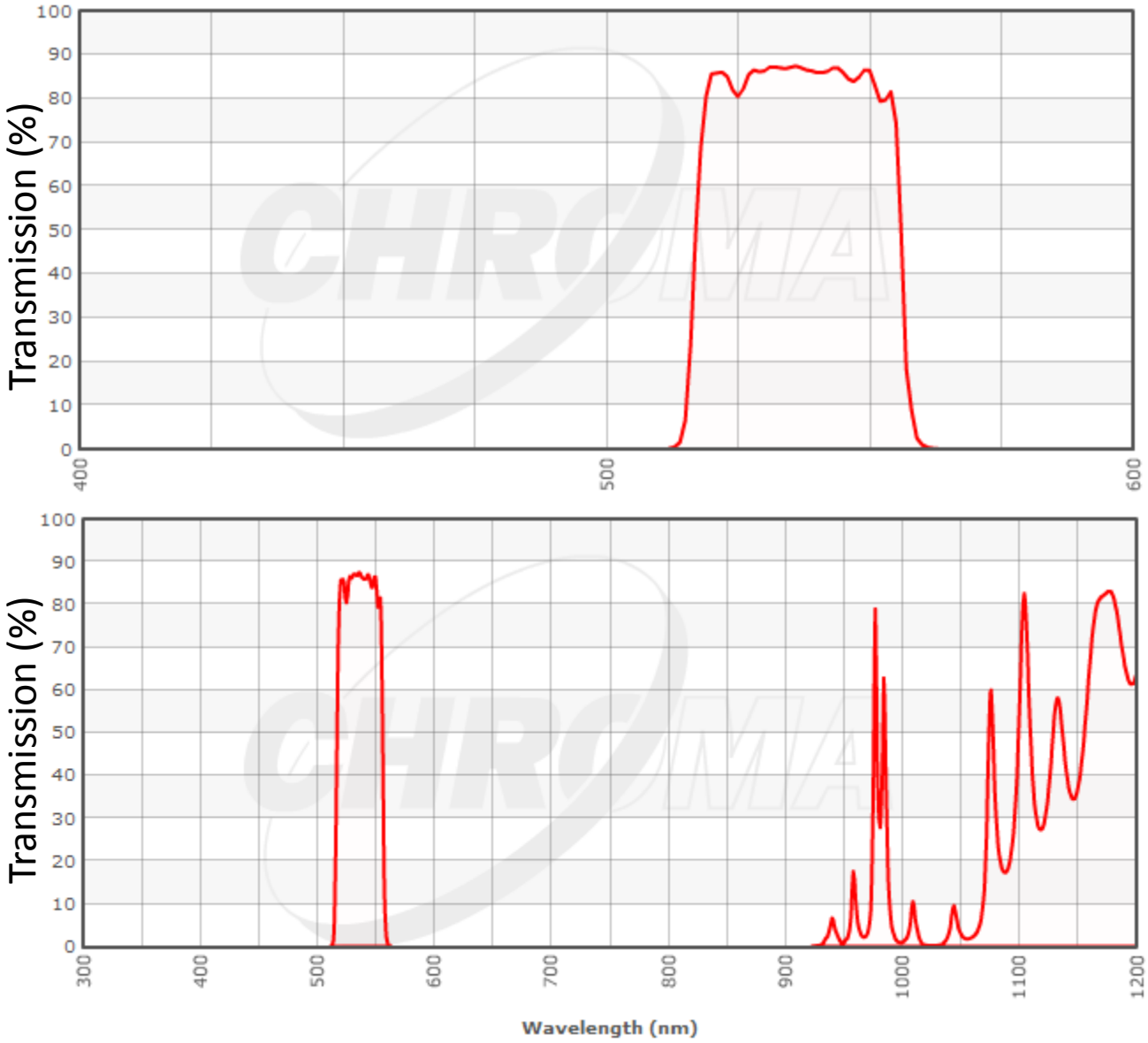


T565LPXR
Chroma



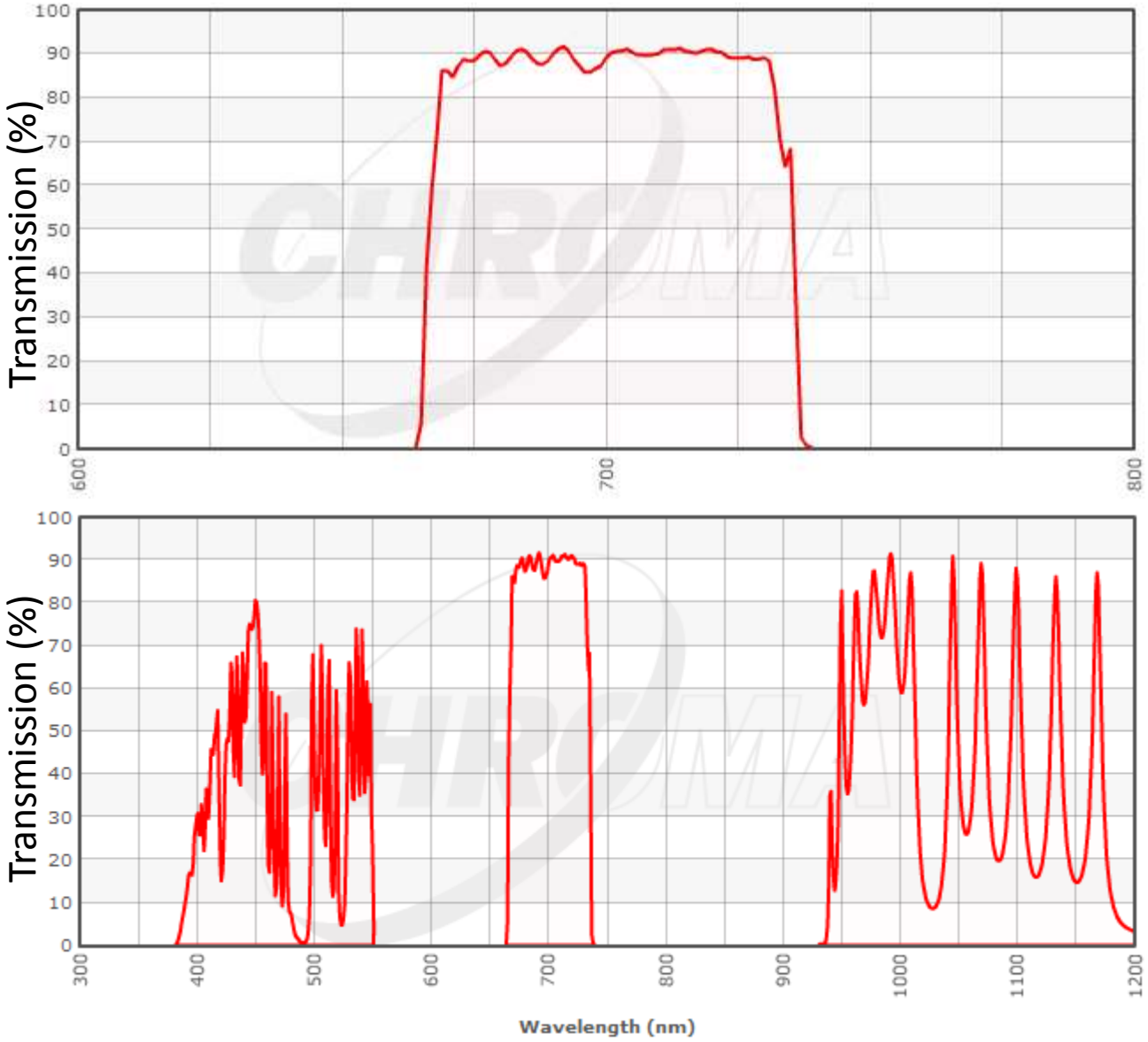
Emission filters might have leaking bands

D535/40M
Chroma

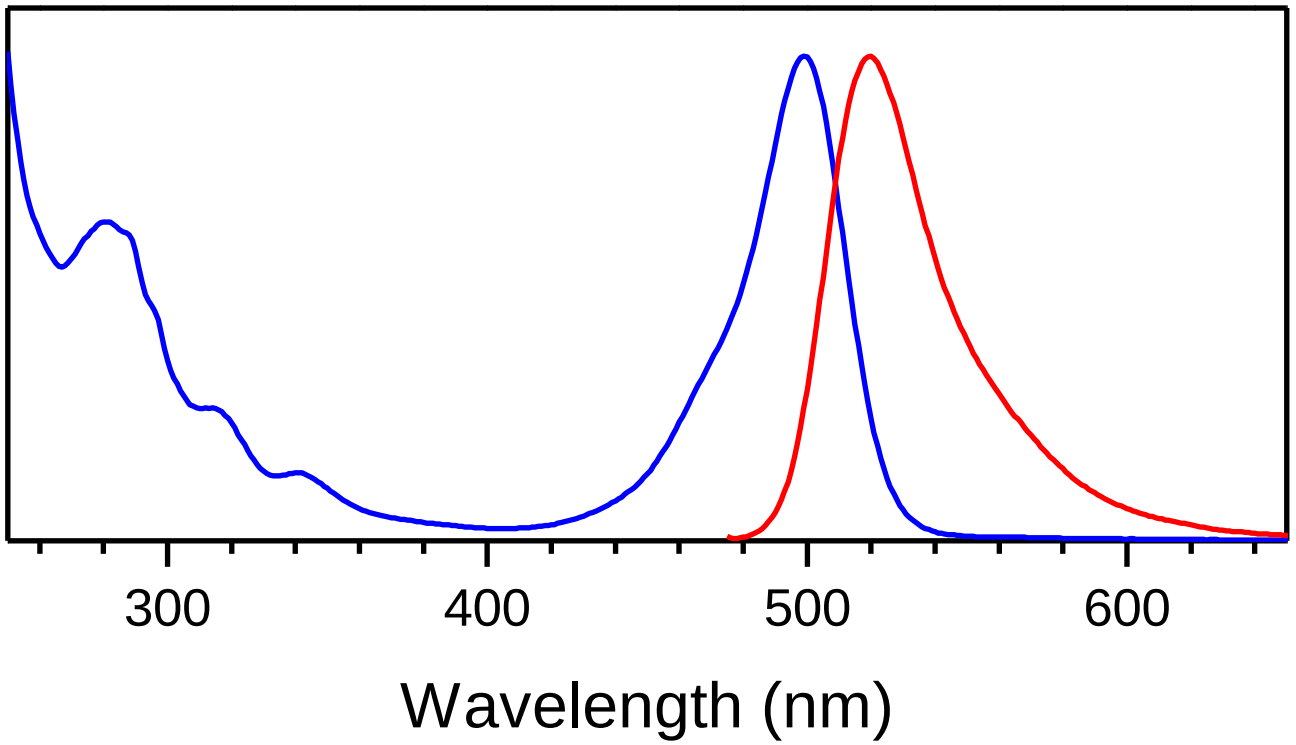


Emission filters might have leaking bands

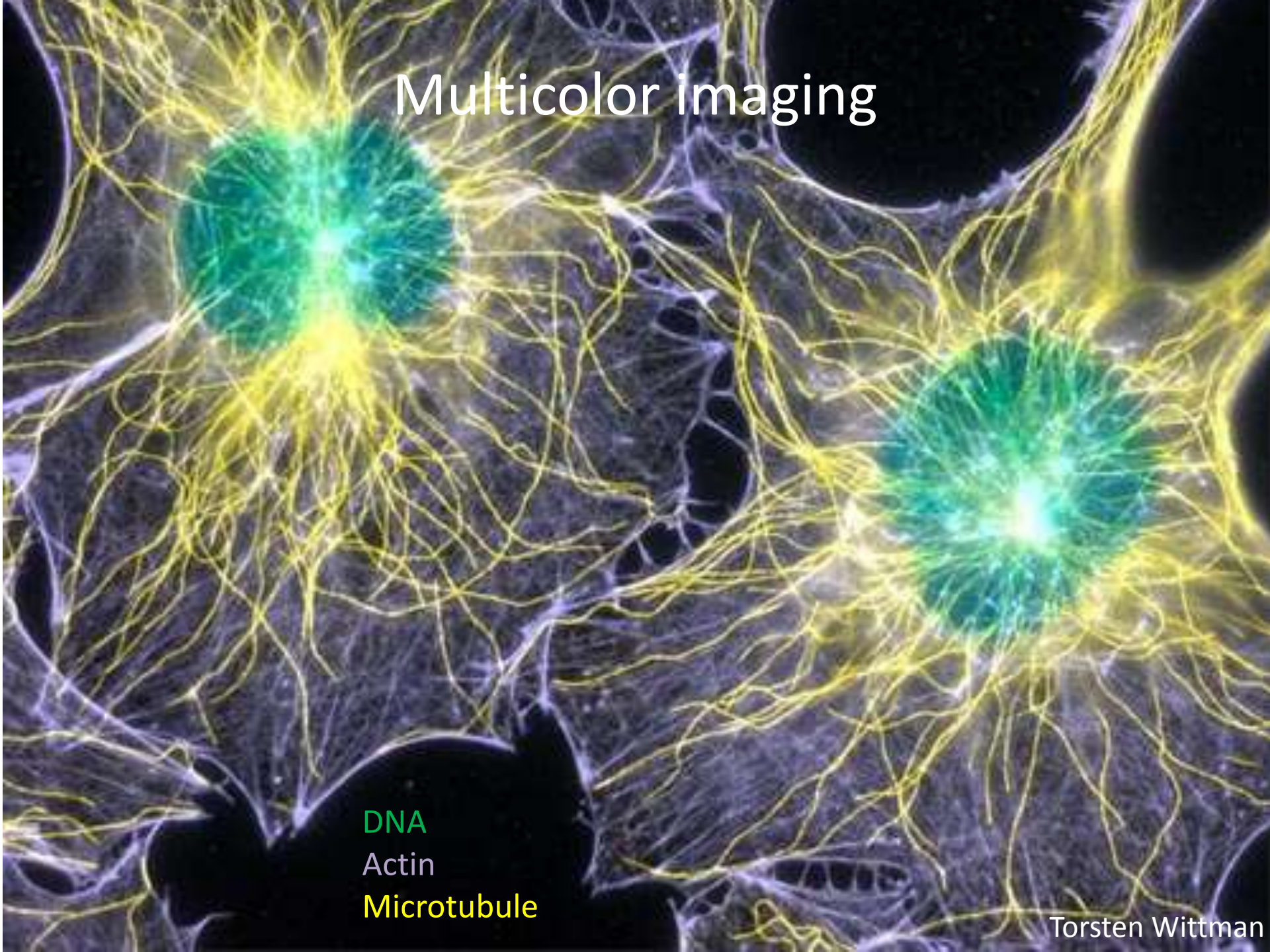
HQ700/75M
Chroma



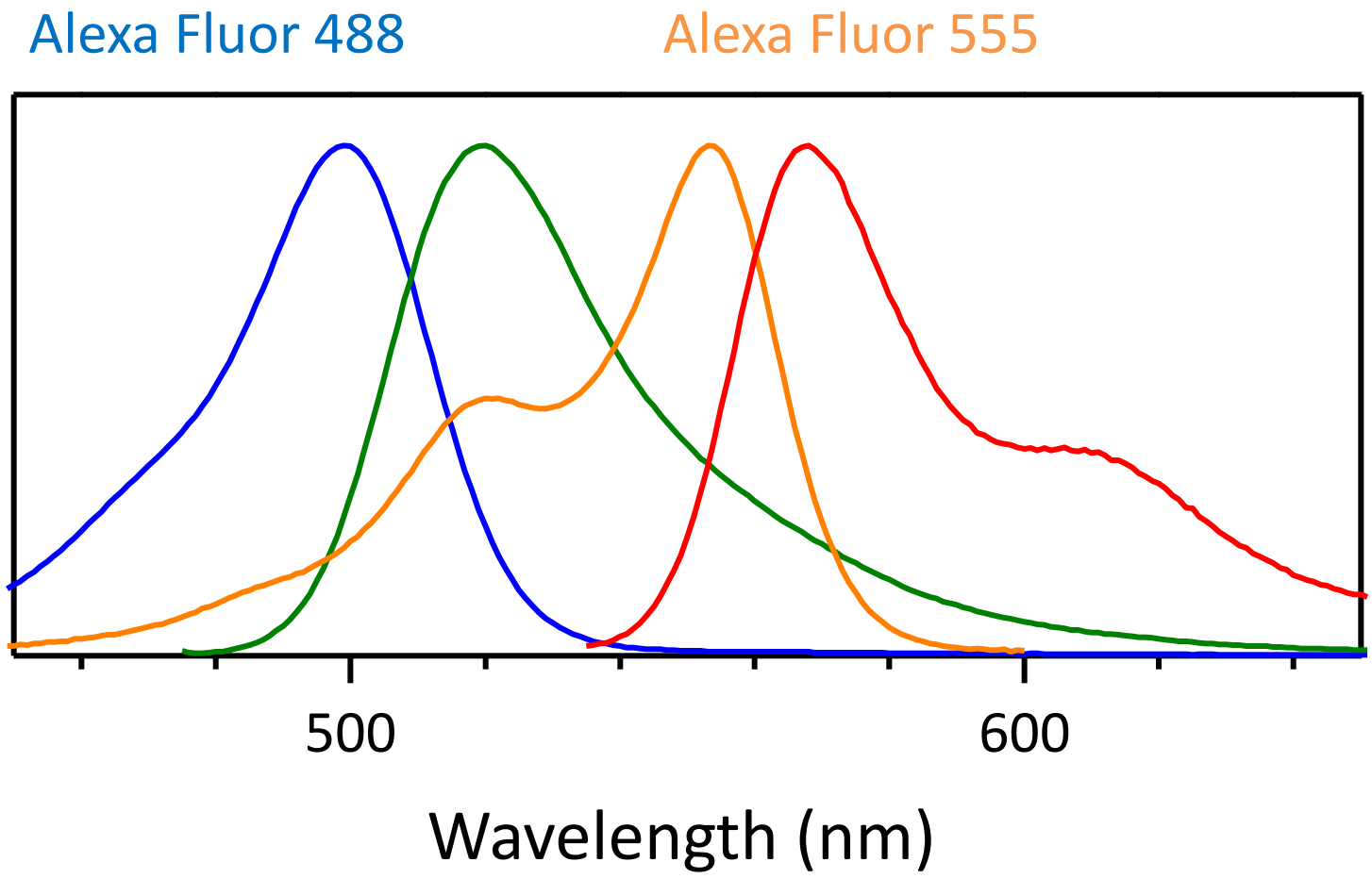
Fluorescence spectra has tails



Multicolor imaging

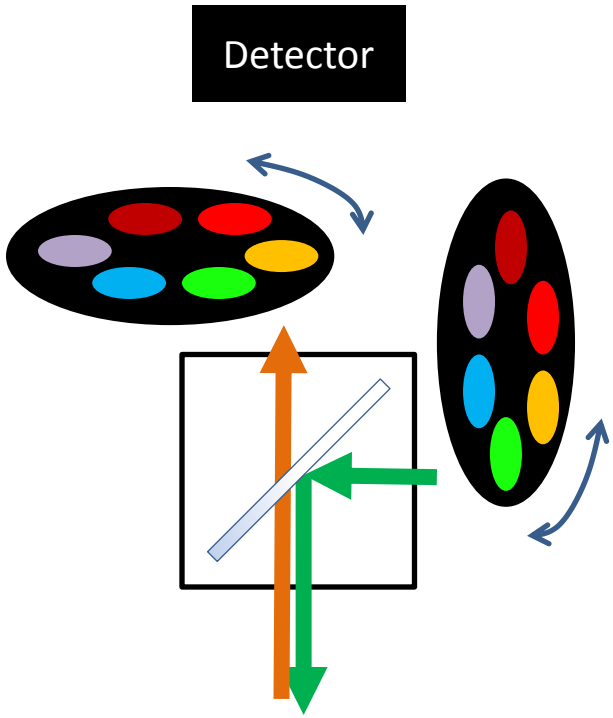


Imaging more than one thing at a time

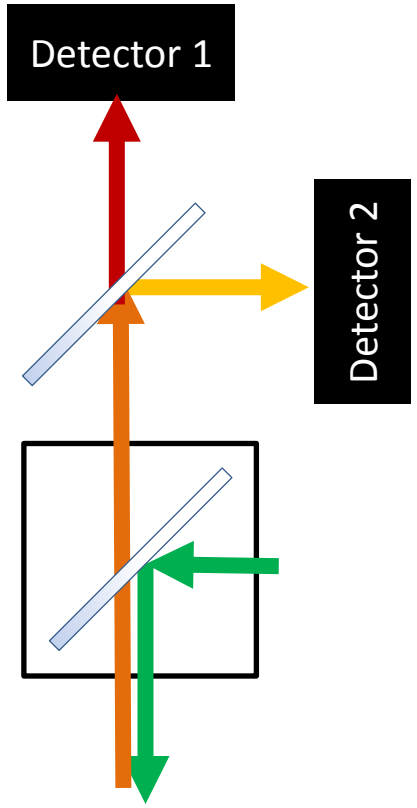


Schemes for multicolor imaging

Cube switching



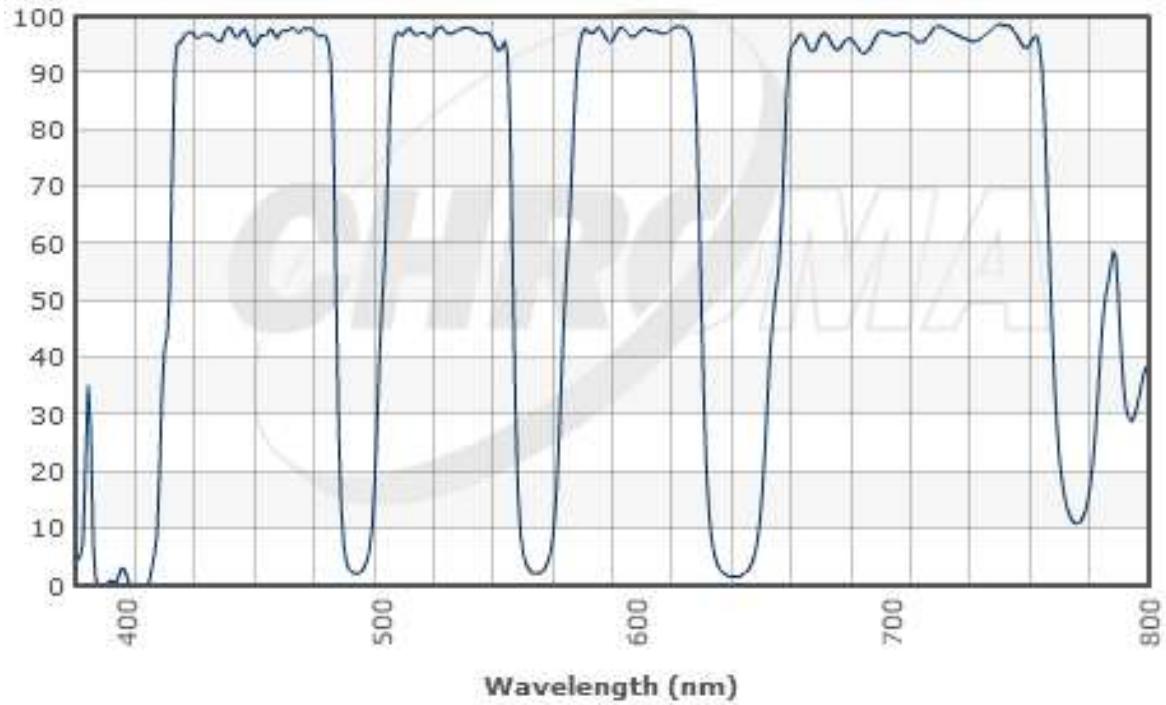
Filter switching



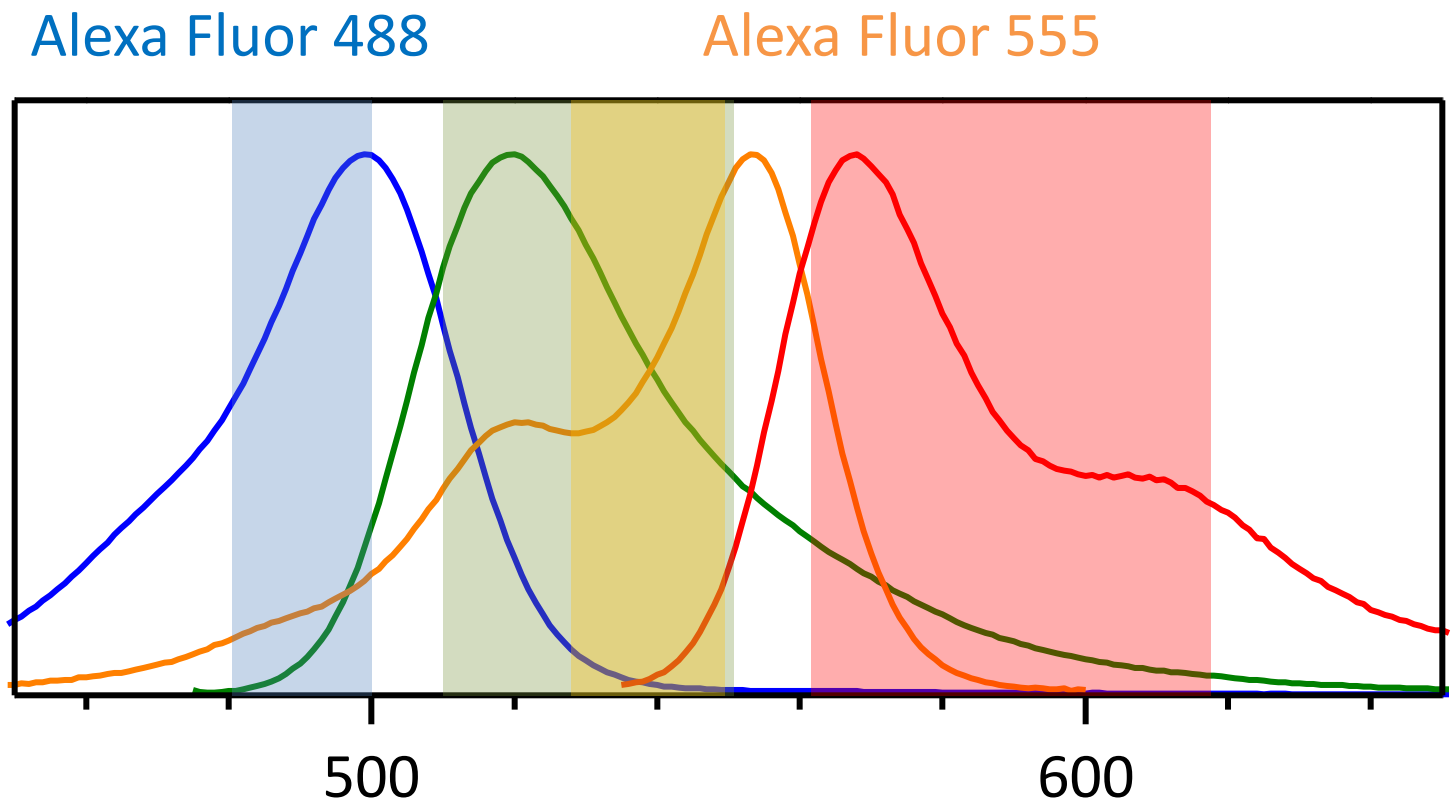
Multiple detectors

Polychroic mirror and multi-bandpass filter

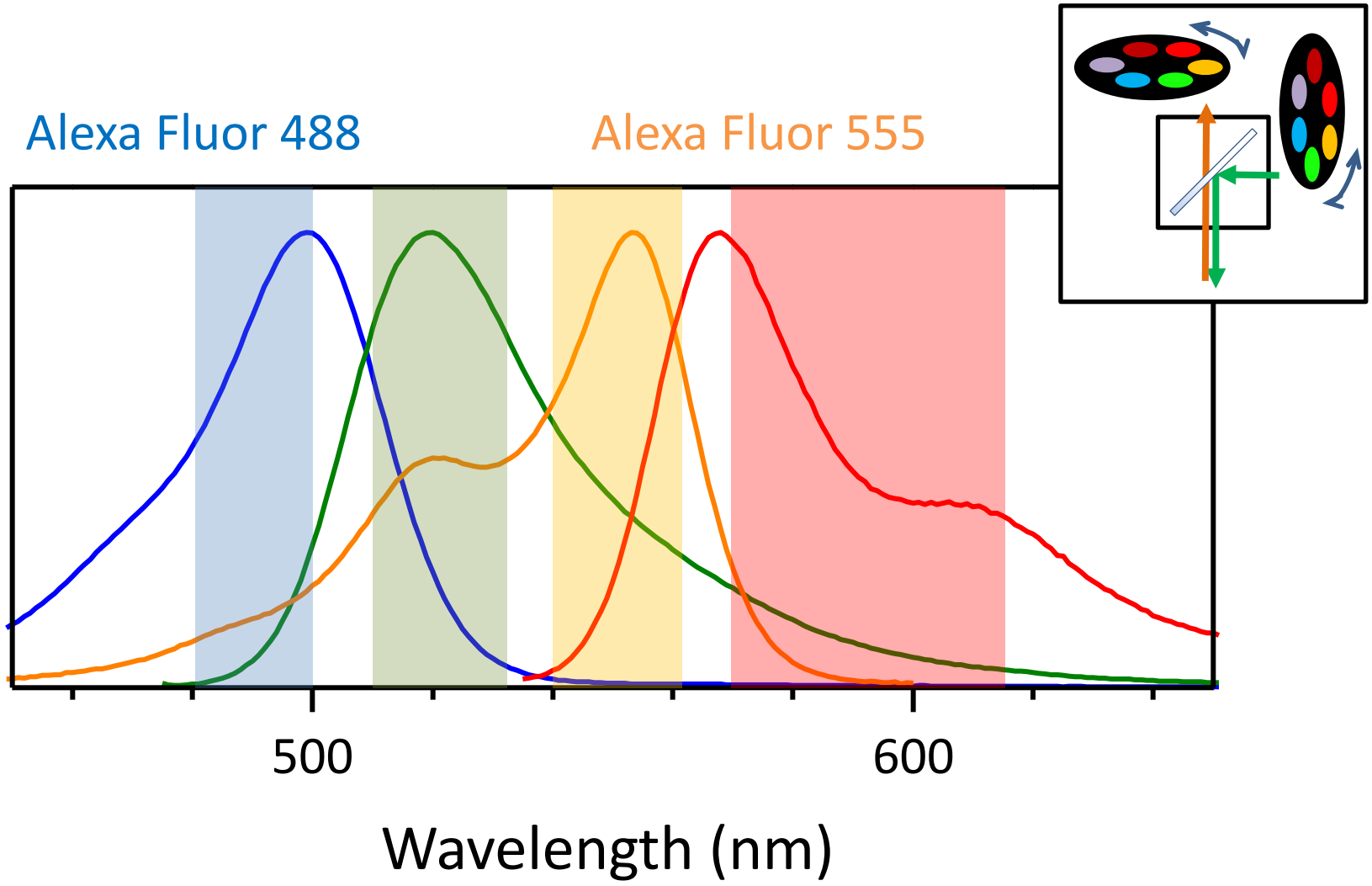
ZT408/488/561/640RPC
Chroma



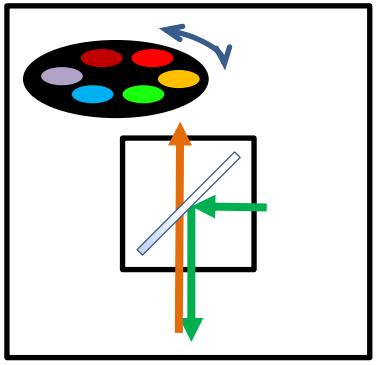
Cube switching



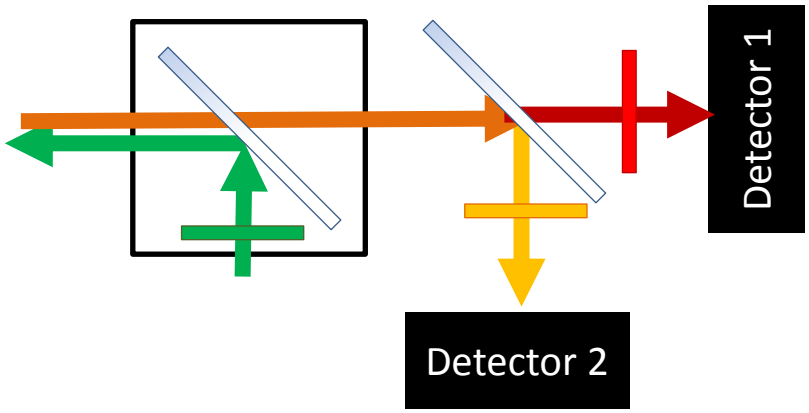
Filter switching – both Ex and Em

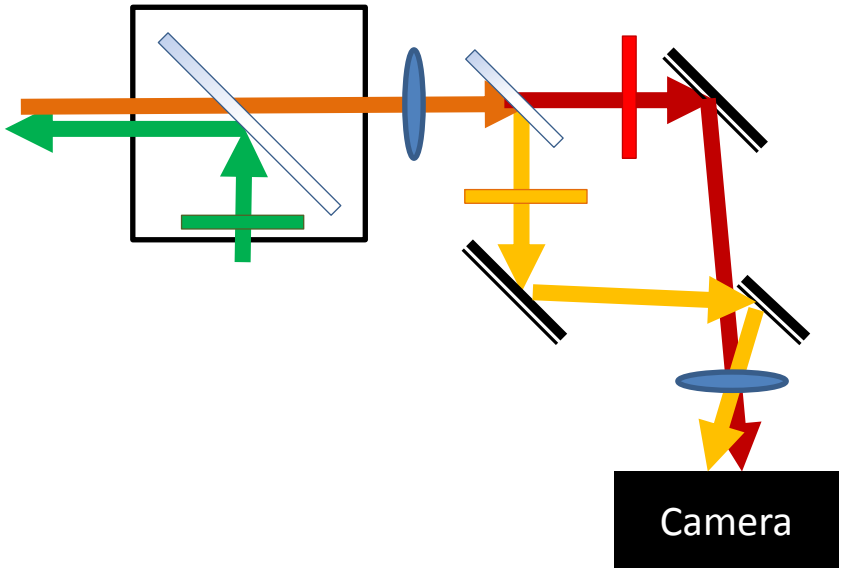


Filter switching – Emission only



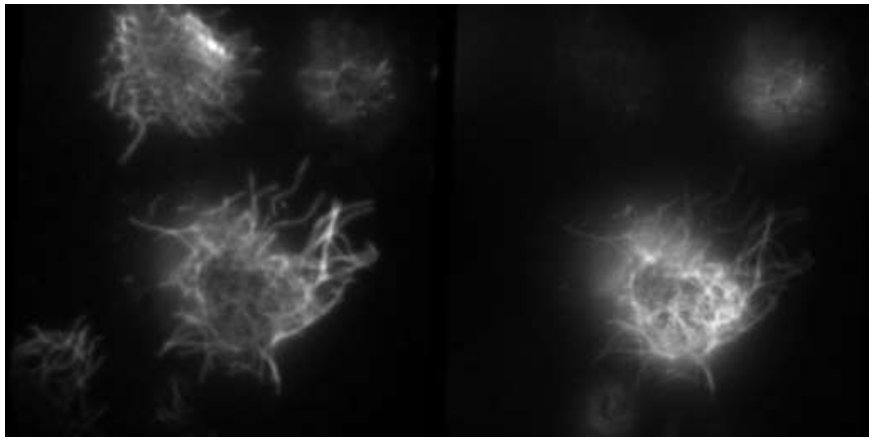
Simultaneous two channel detection



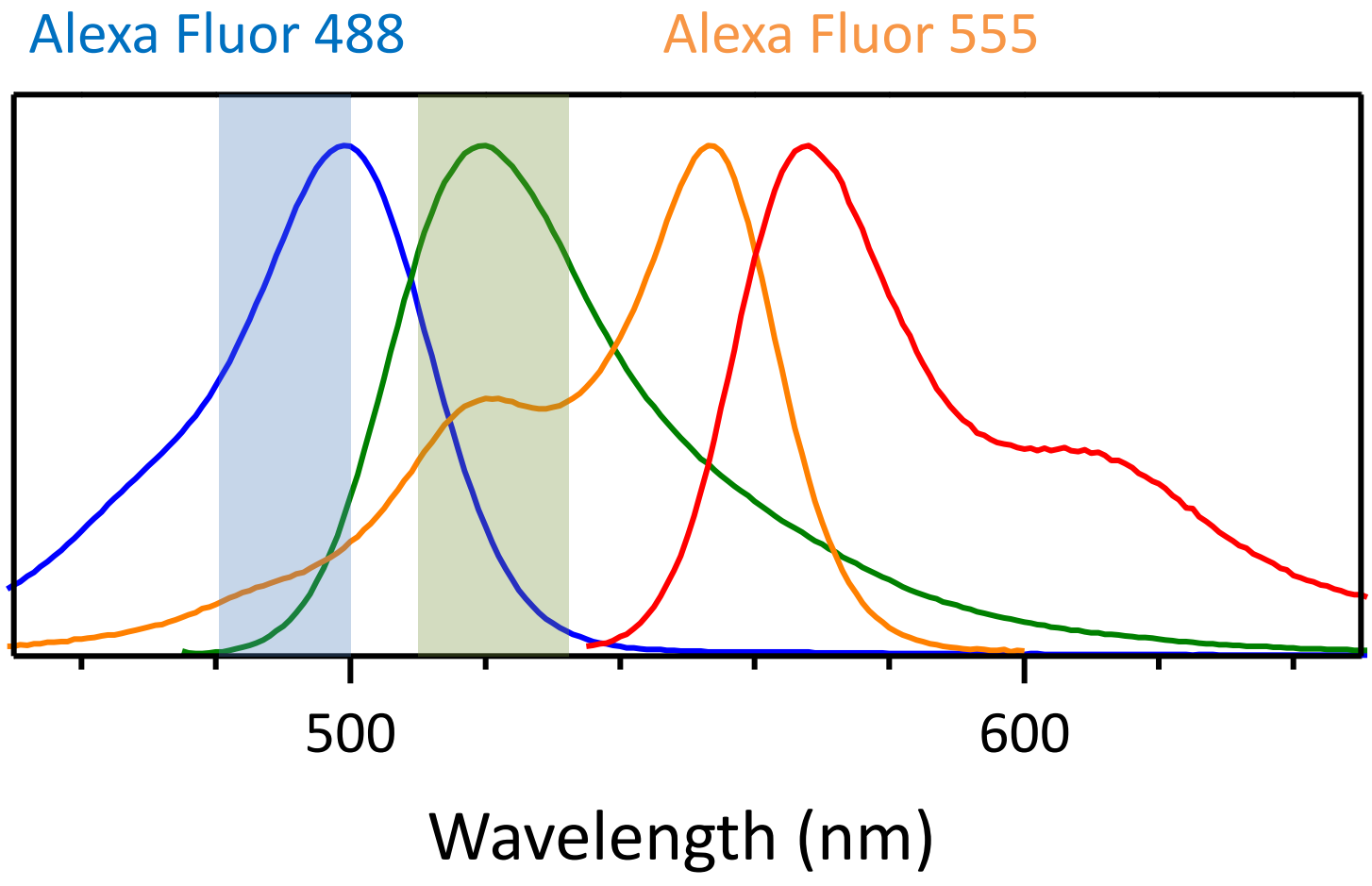


Long wavelength (reflected)

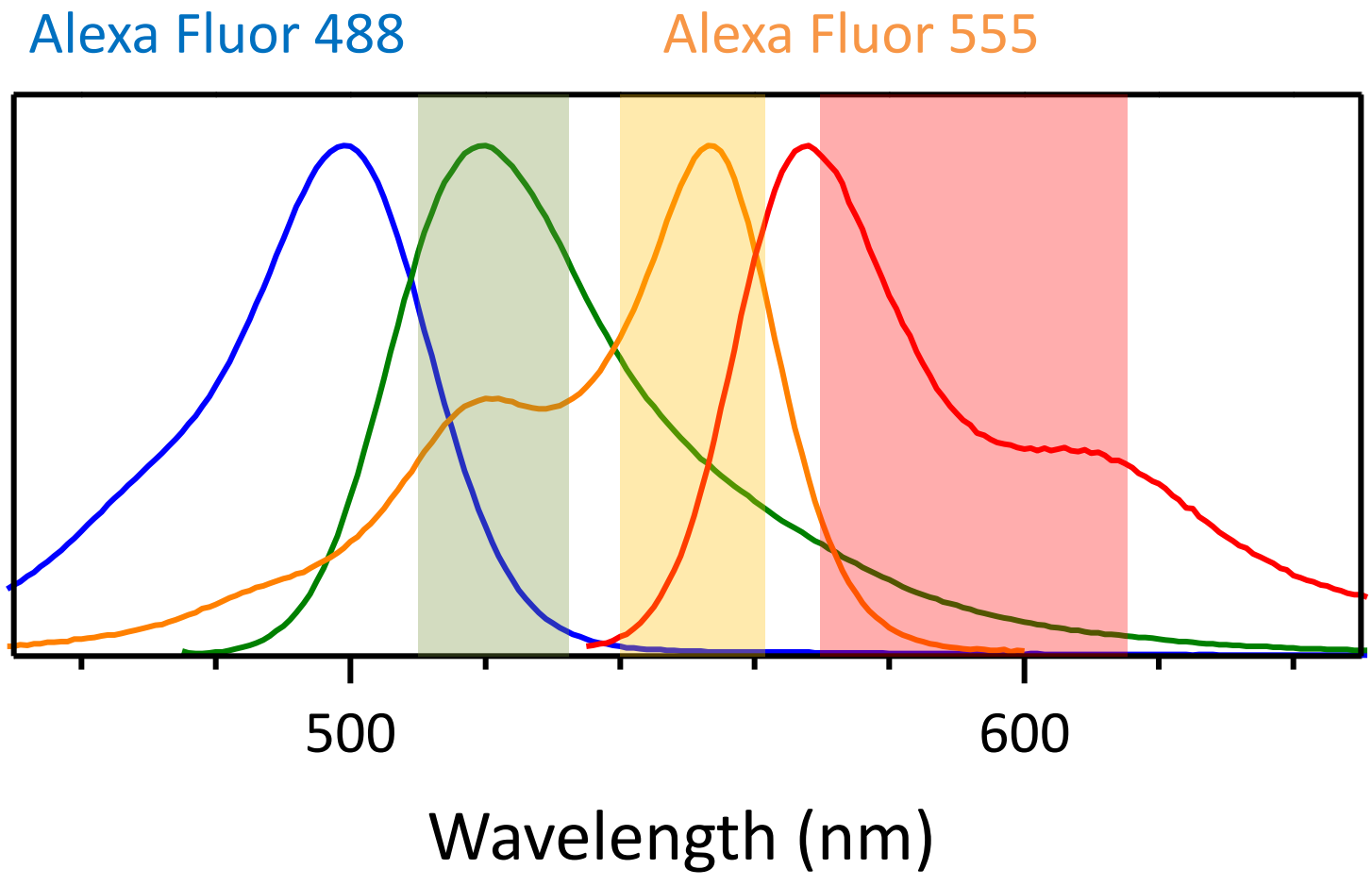
Short wavelength (transmitted)



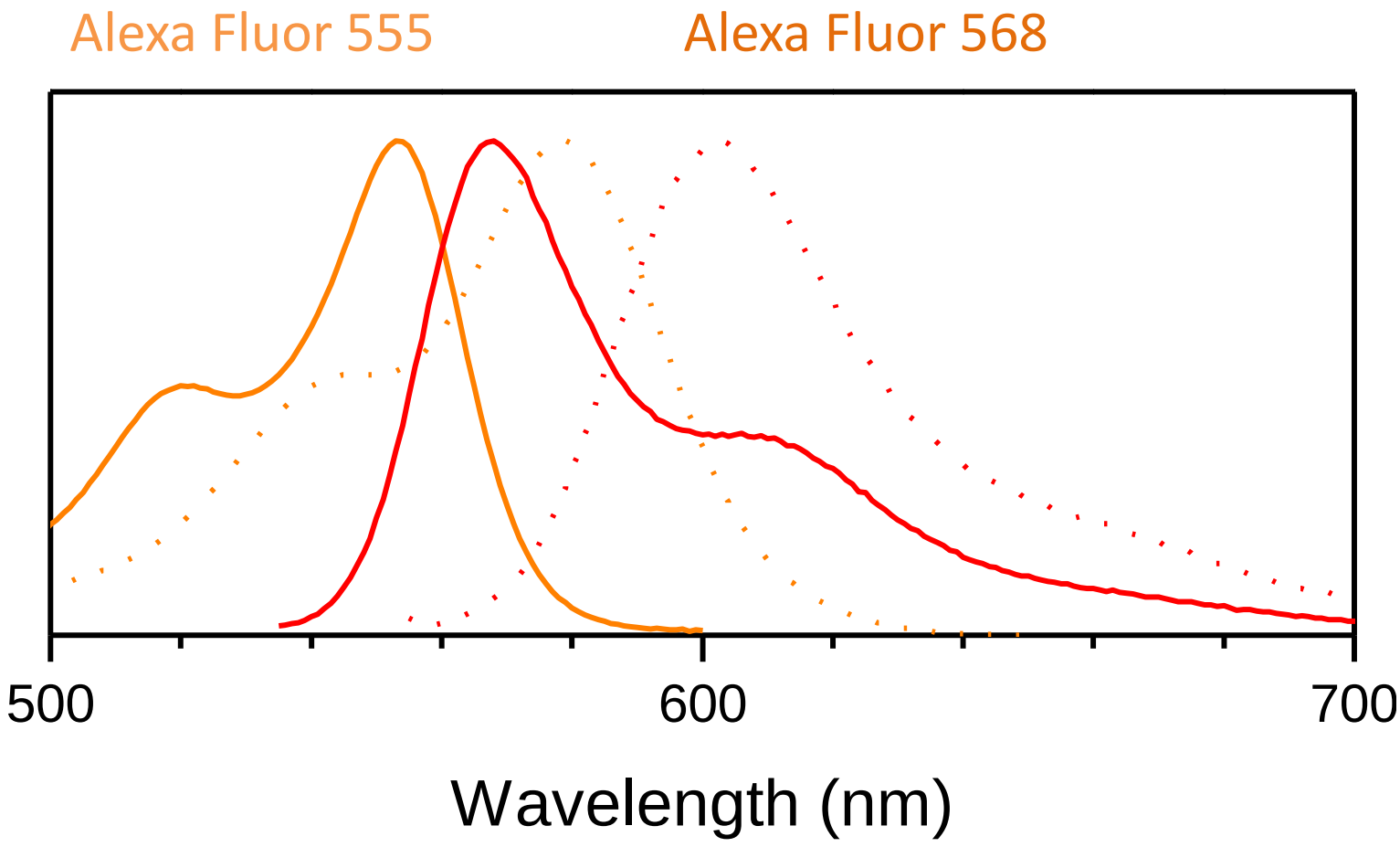
Crosstalk between channels – excitation



Crosstalk between channels – emission



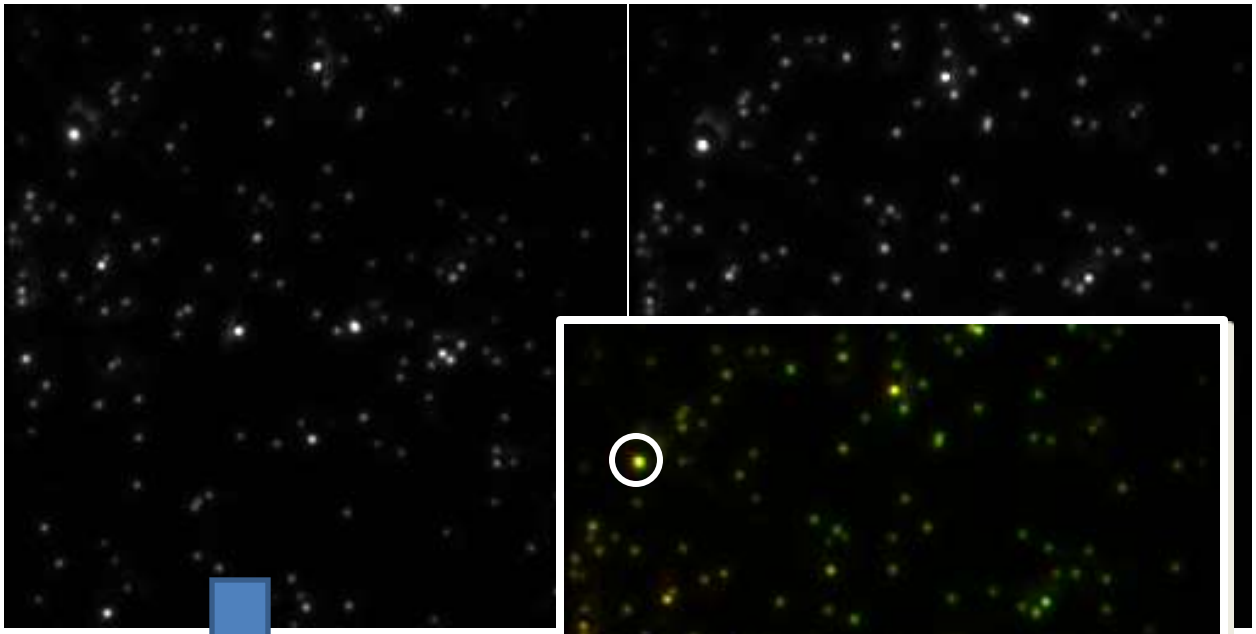
Something too close…



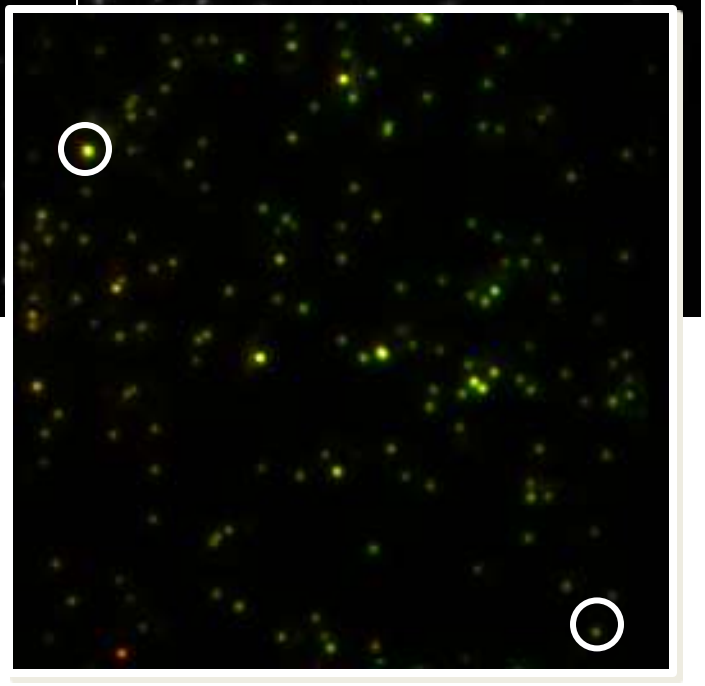
One last concern to address before time is up…

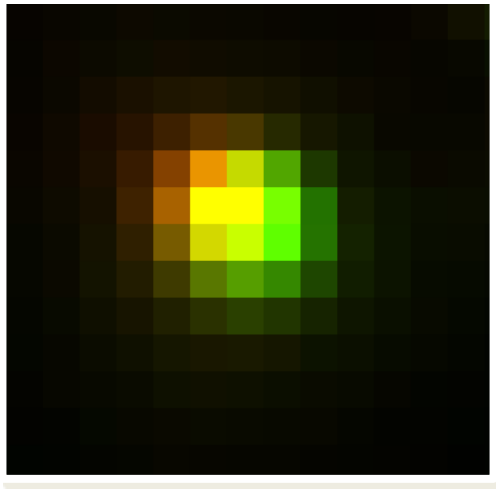
Image registration

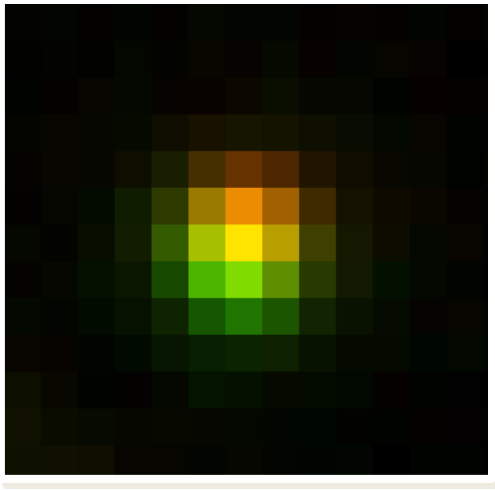
Fluorescent beads with signal in both channels



Translation alignment







Thanks!

- Nico Stuurman
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