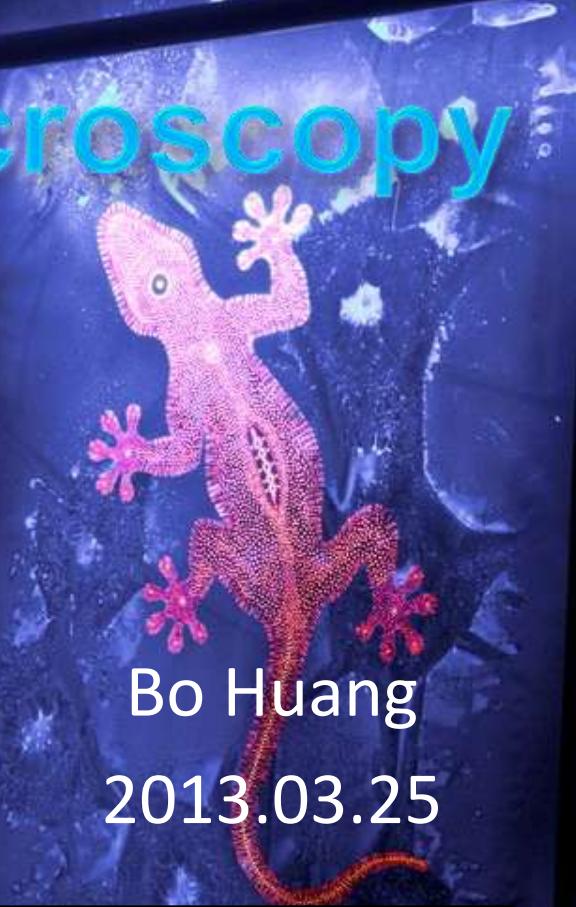


Fluorescence Microscopy

I. Fluorescent optics



Bo Huang

2013.03.25



THORLABS



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probes | invitrogen
by life technologies™

SUTTER INSTRUMENT

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SCOPELED™

HAMAMATSU

89 NORTH



Discovery of Fluorescence

Sir John Herschel



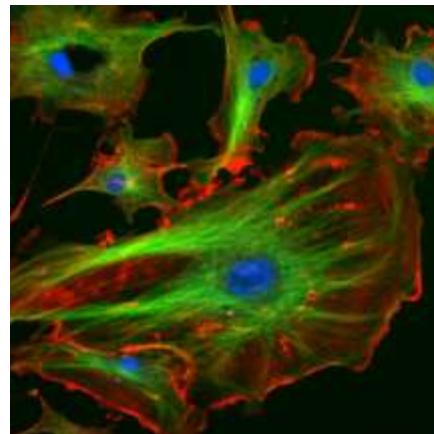
1845

G.G. Stokes

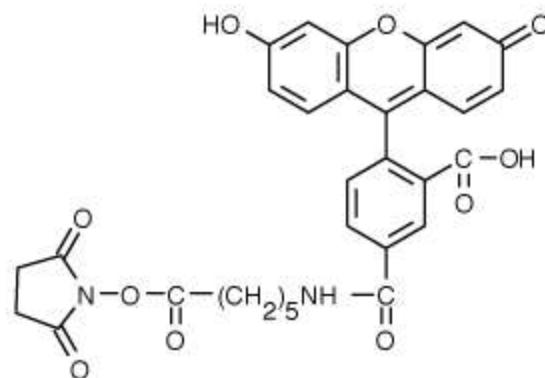
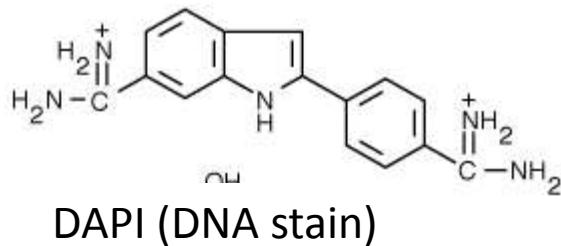


1852

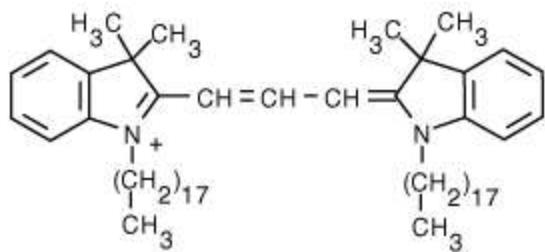
Things that fluoresce



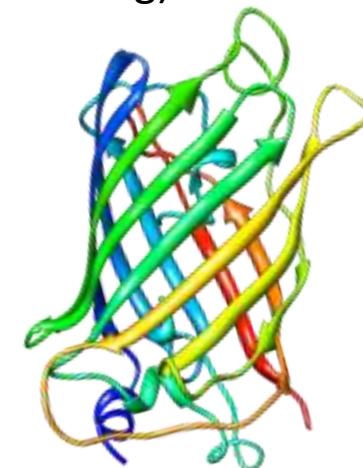
Molecules that fluoresce



Fluorescein (protein labeling)

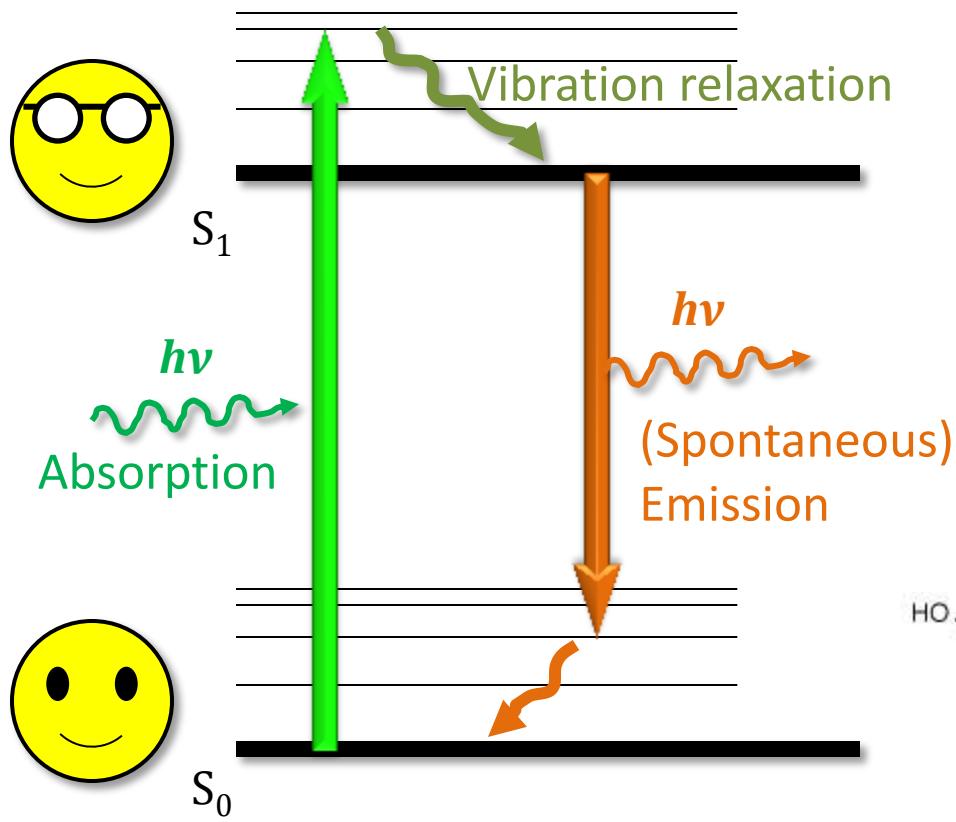


Dil (plasma membrane stain)

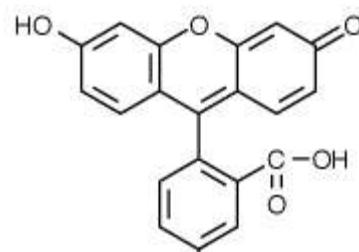


GFP (fluorescent protein)

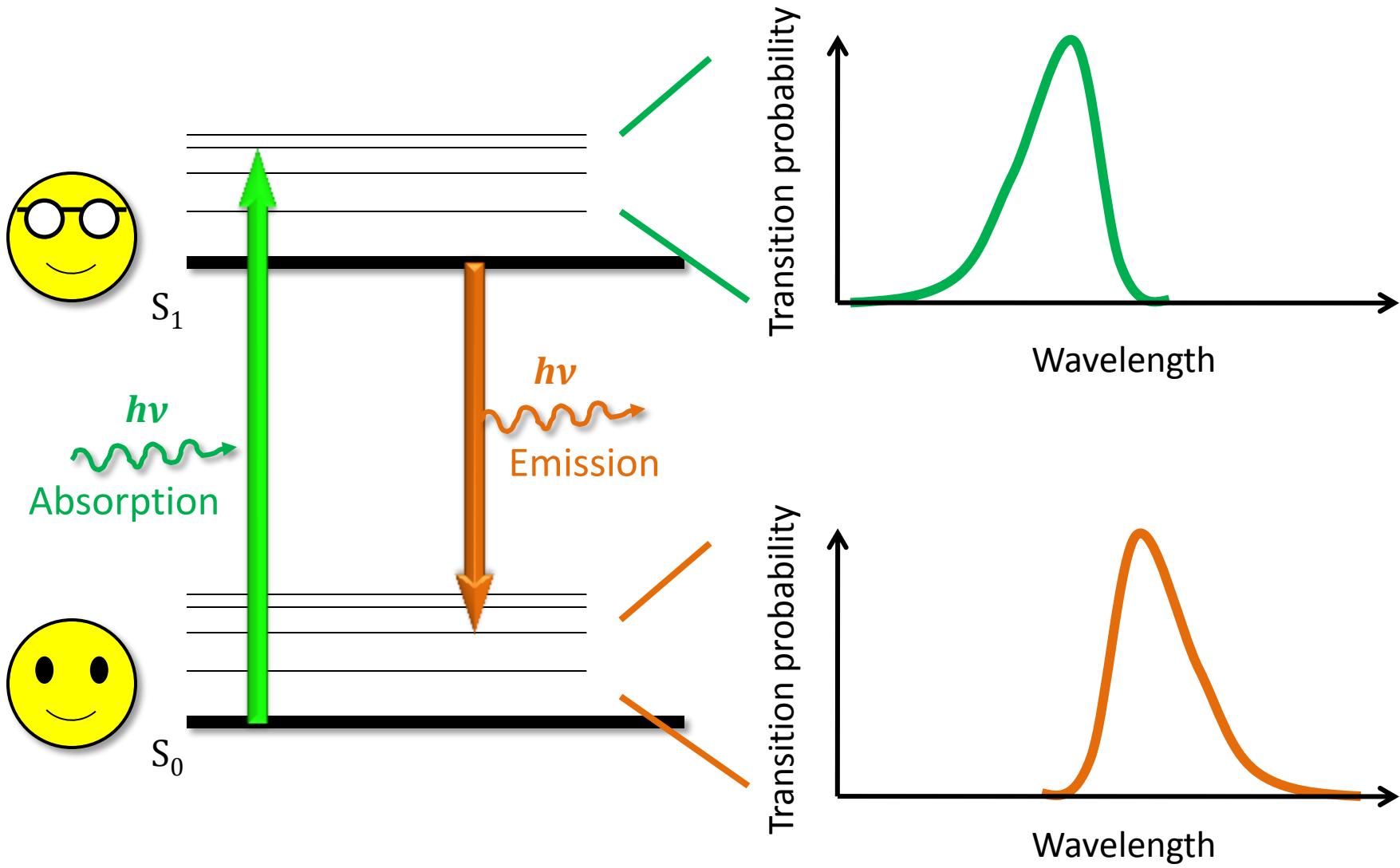
Jabłonski diagram



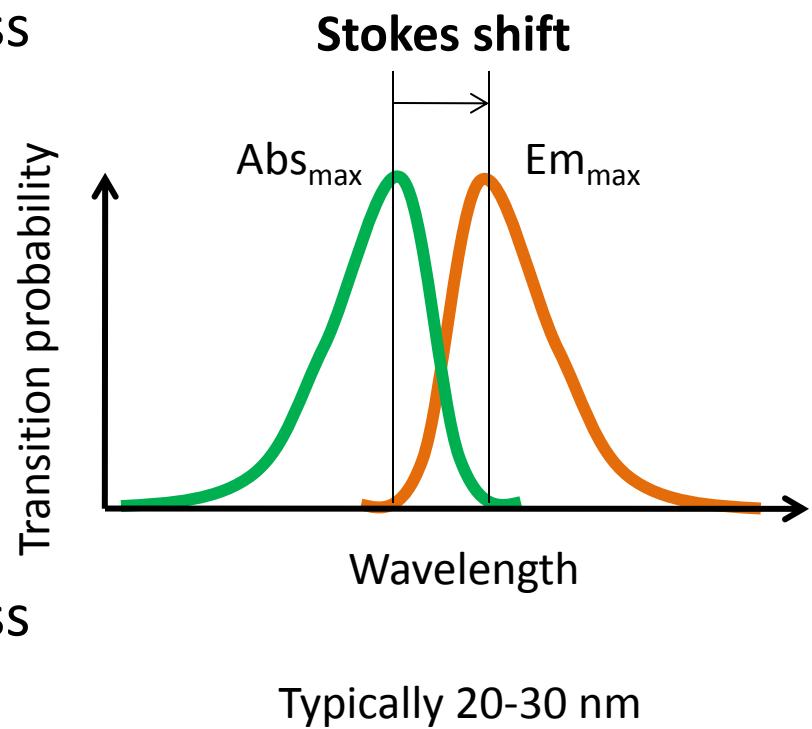
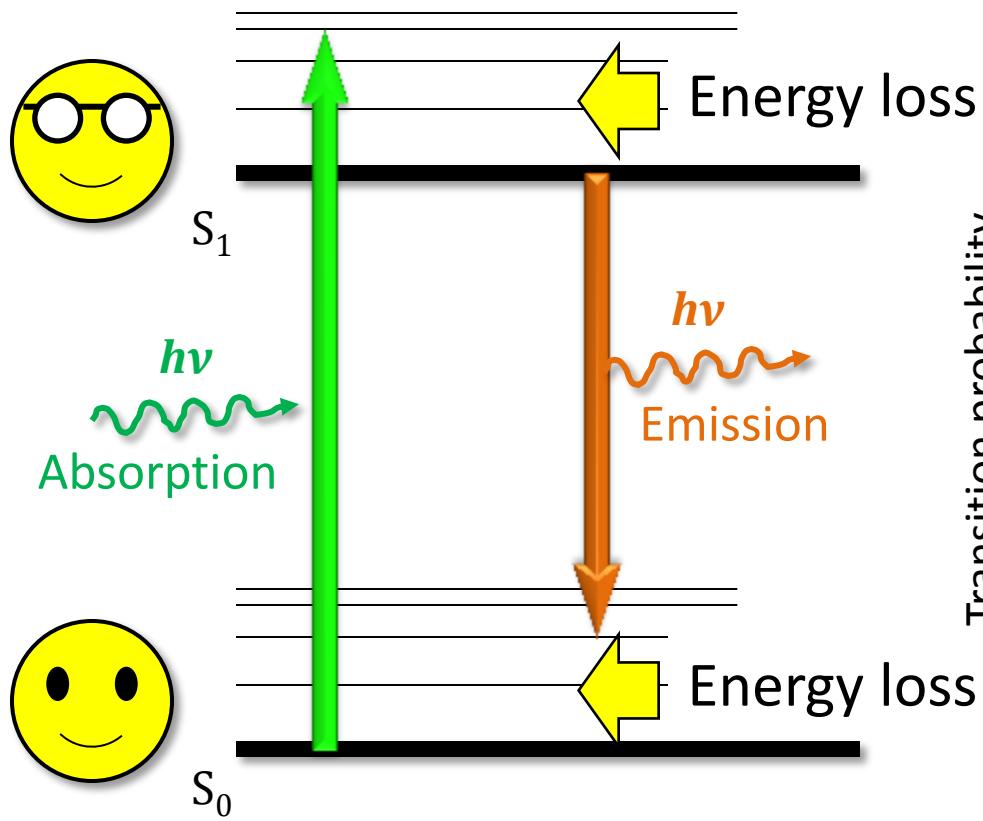
Alexander Jabłonski



Excitation and Emission Spectra

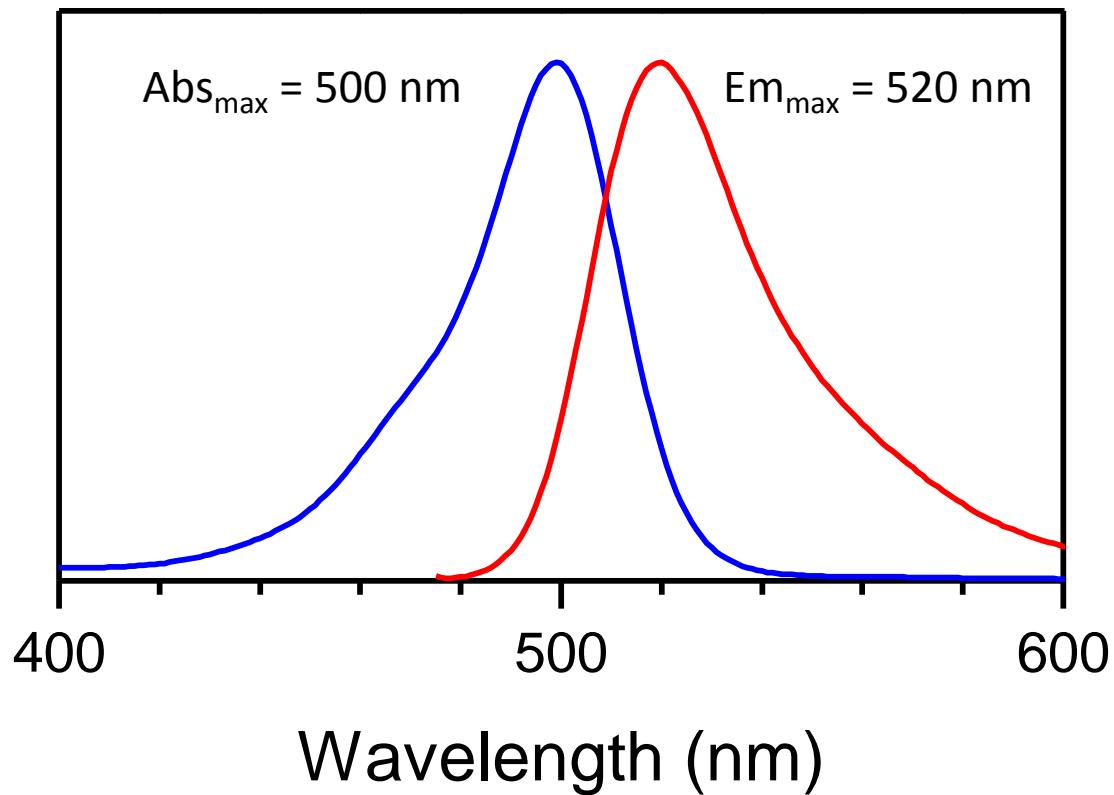


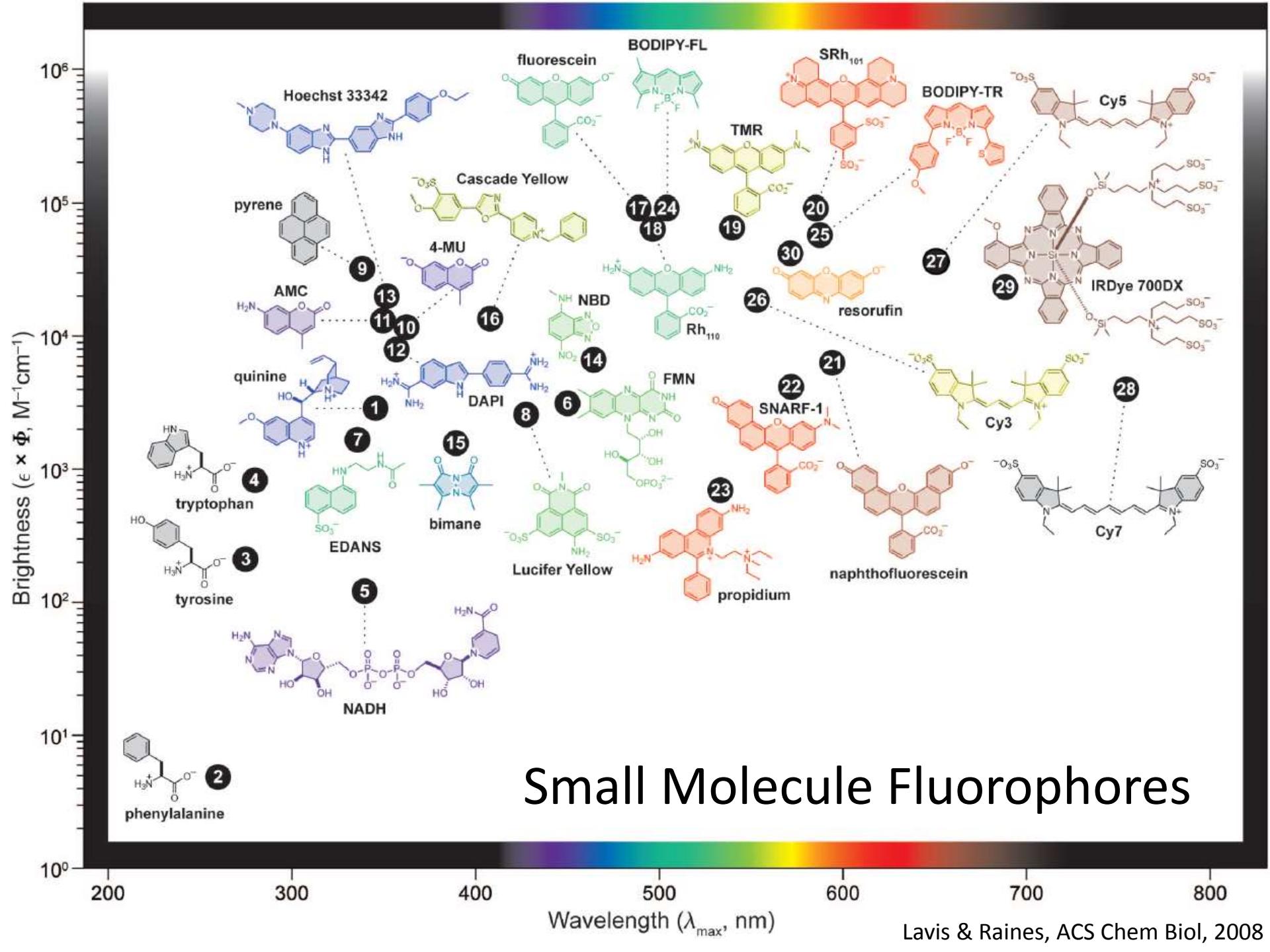
Stokes Shift



Excitation and emission wavelengths

Alexa Fluor 488





Fluorescent proteins

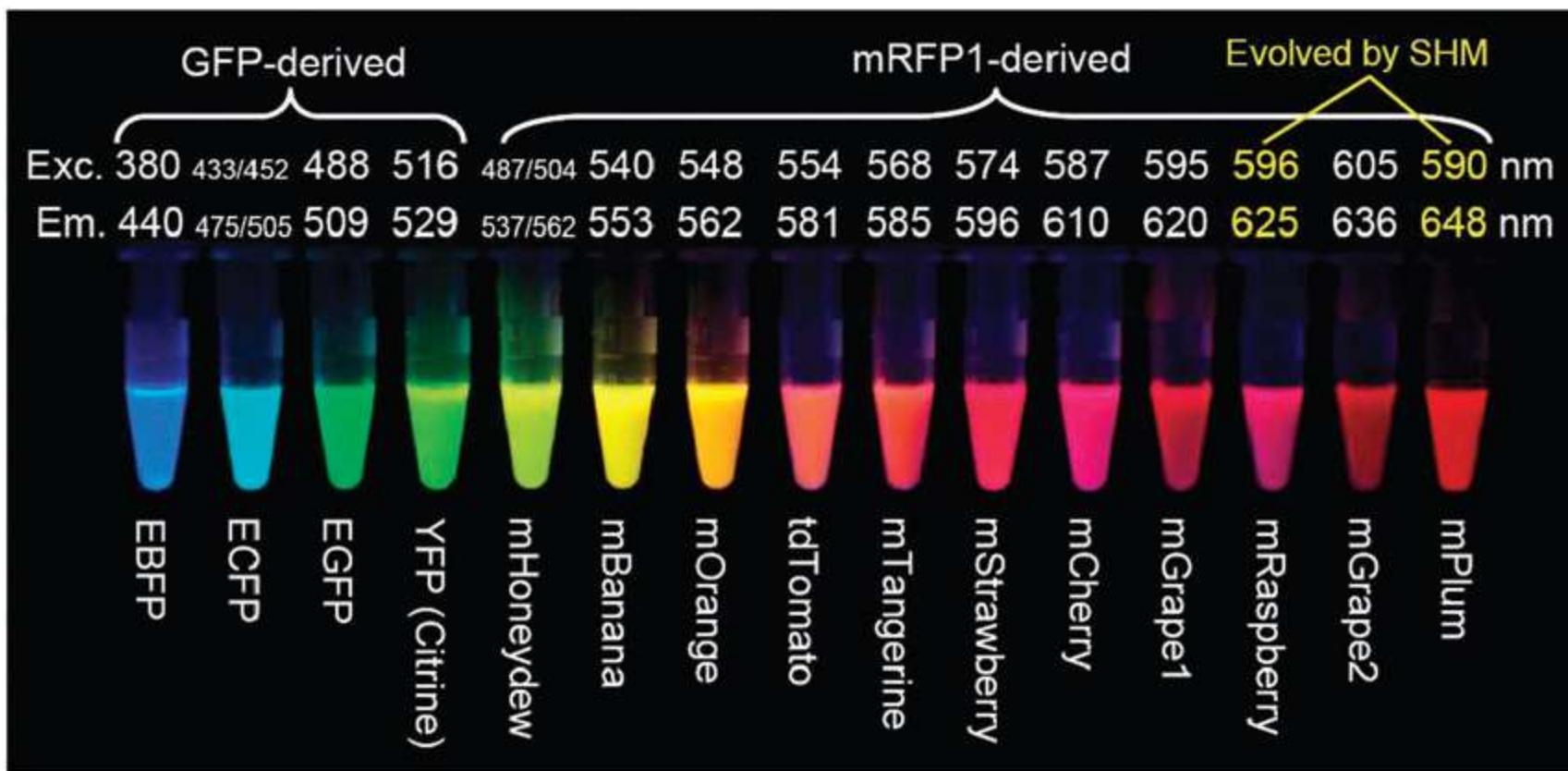


Image from Tsien lab

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

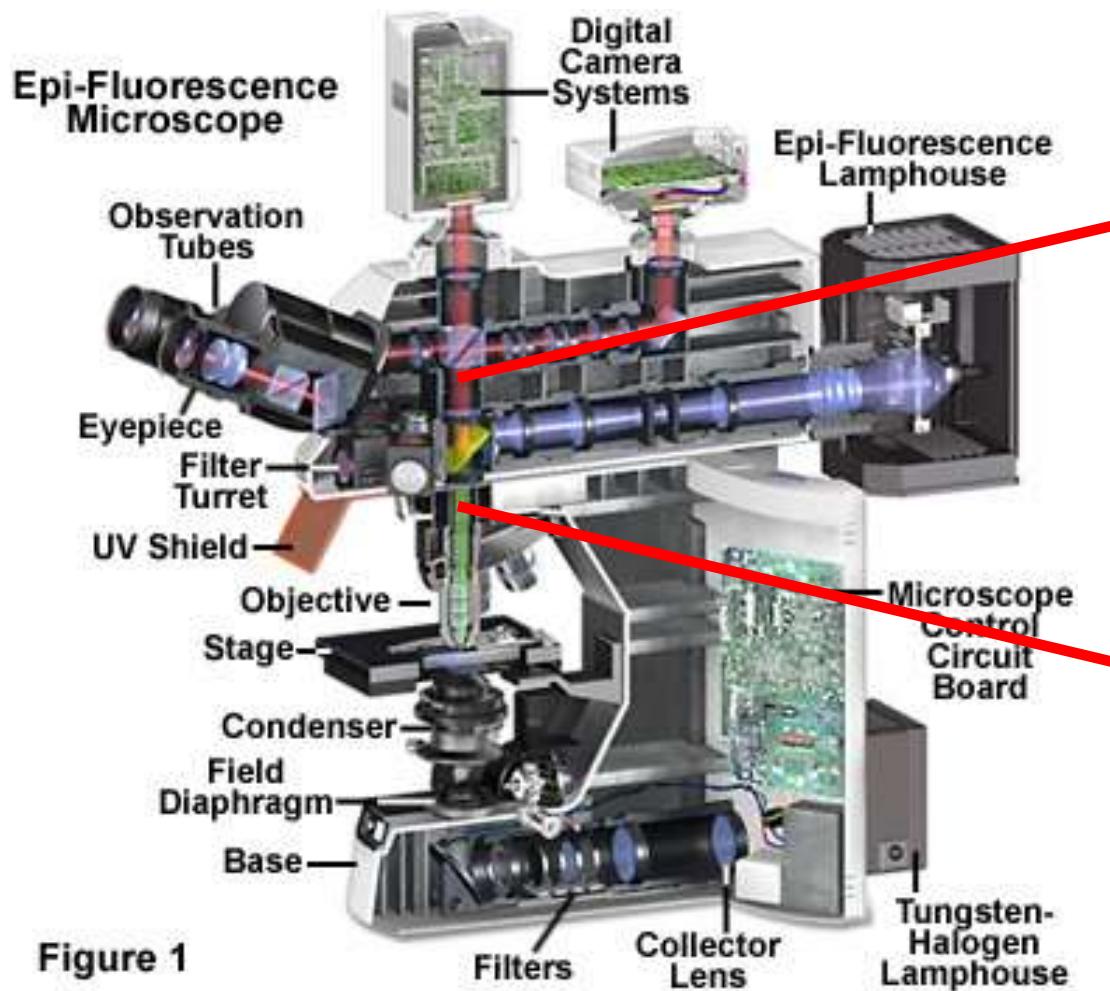


Figure 1

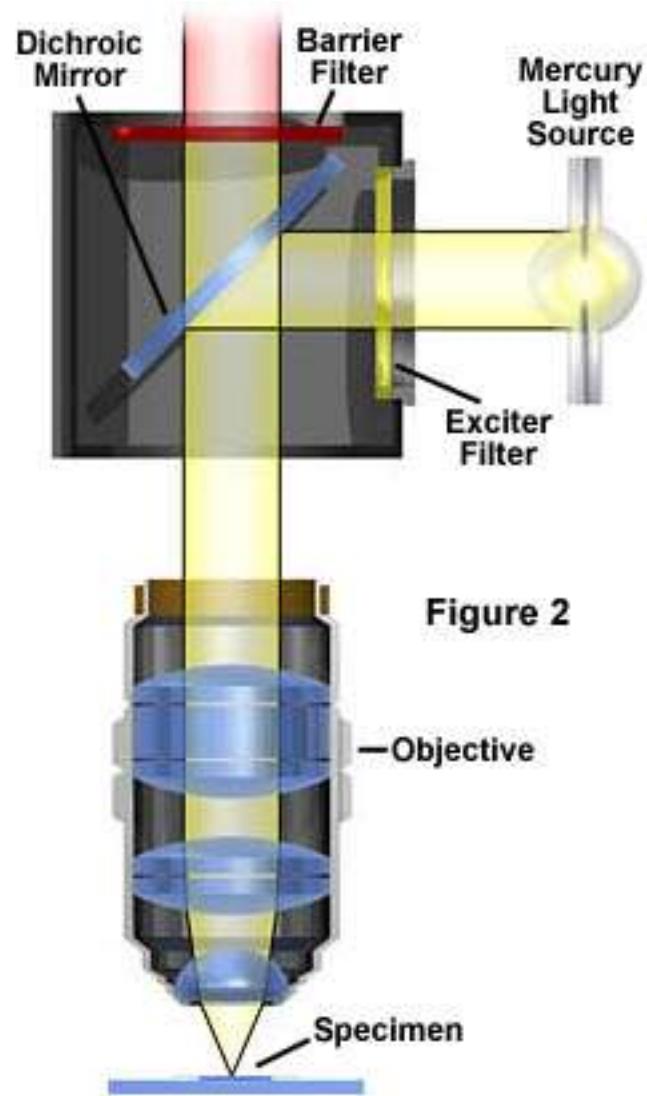
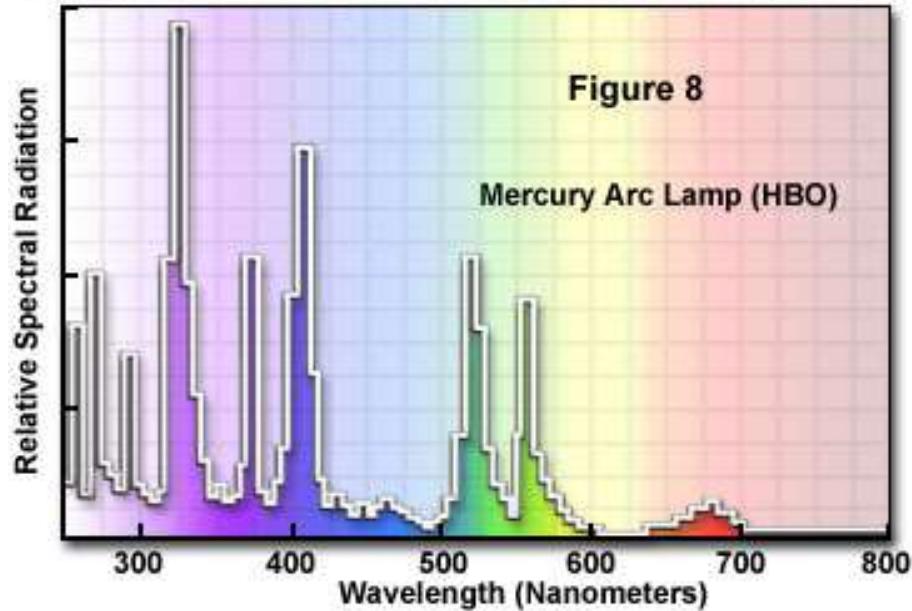


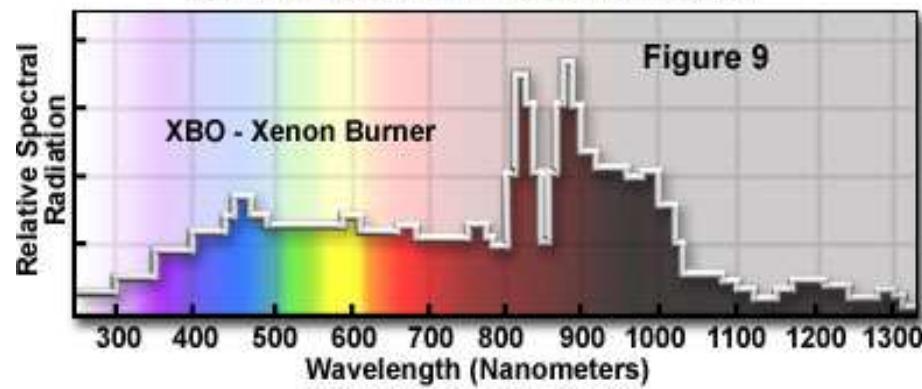
Figure 2

Excitation light sources – Lamps

Mercury Arc Lamp UV and Visible Emission Spectrum



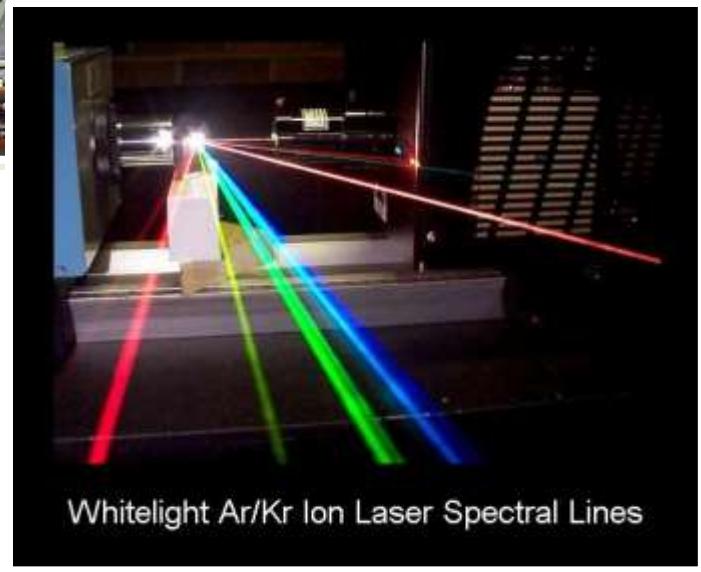
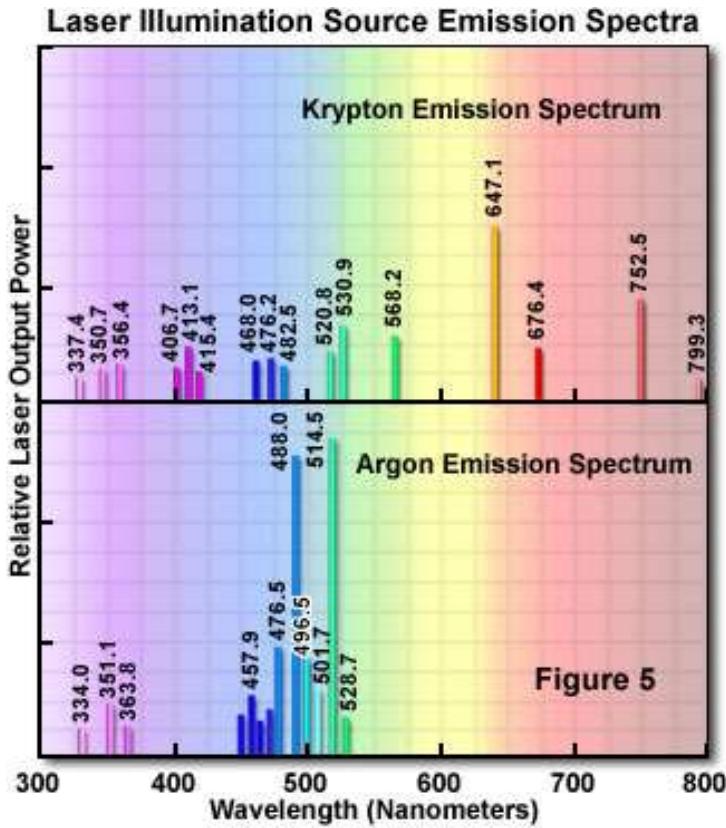
Xenon Arc Lamp Emission Spectrum



Excitation filter required.

Excitation light sources – Laser

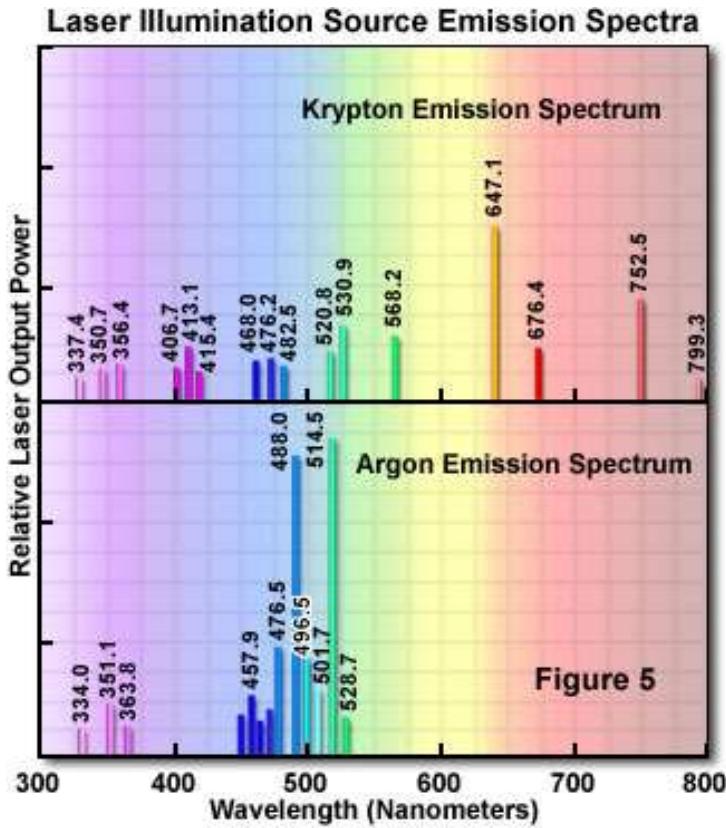
Ar / Kr ion laser: 488, 514, 568, 647



Excitation light sources – Laser

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

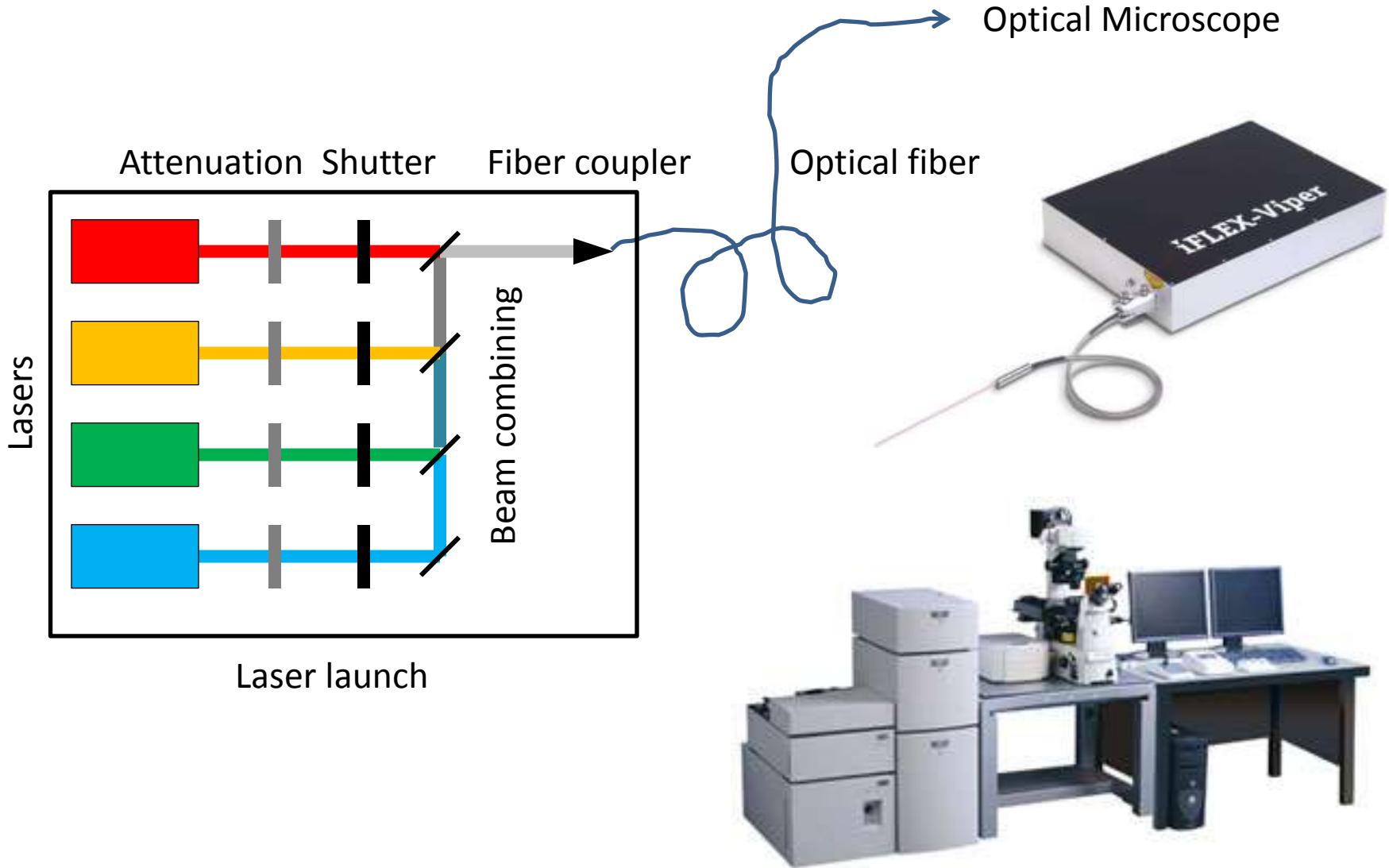
Solid state lasers: 488, 532, 561...



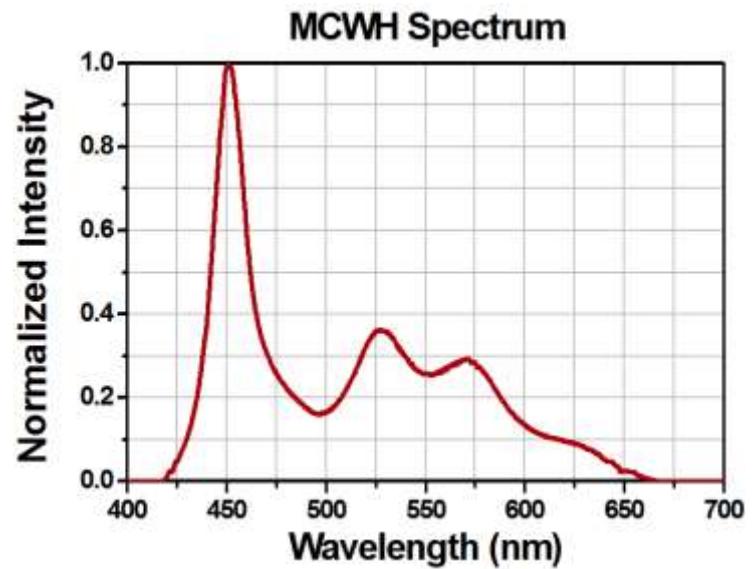
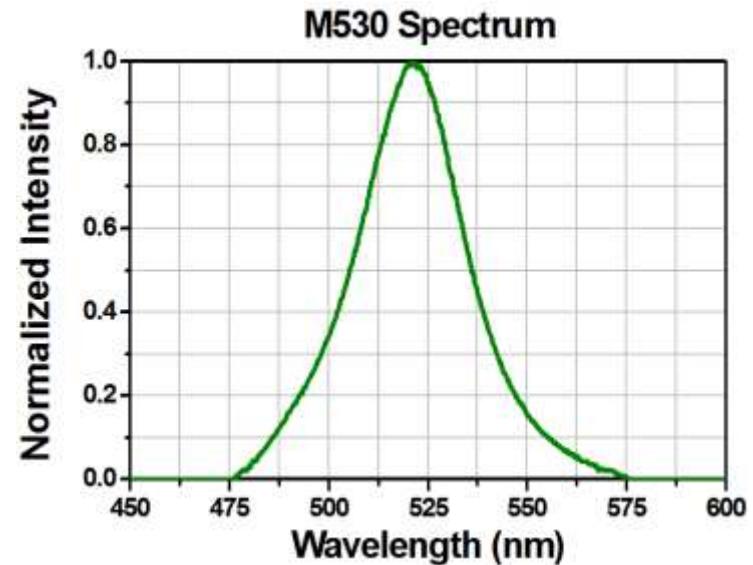
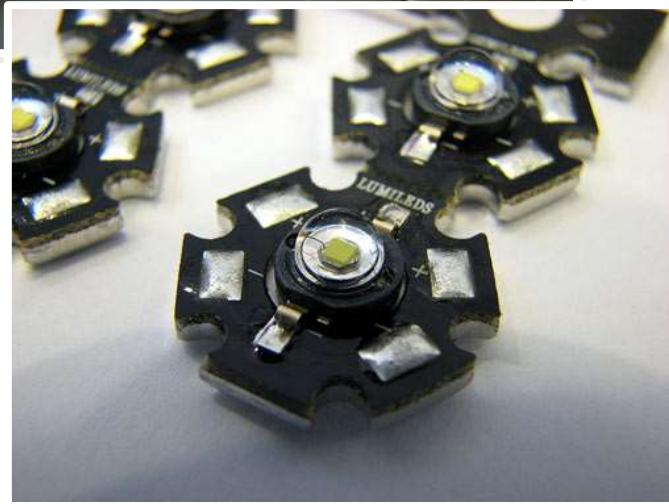
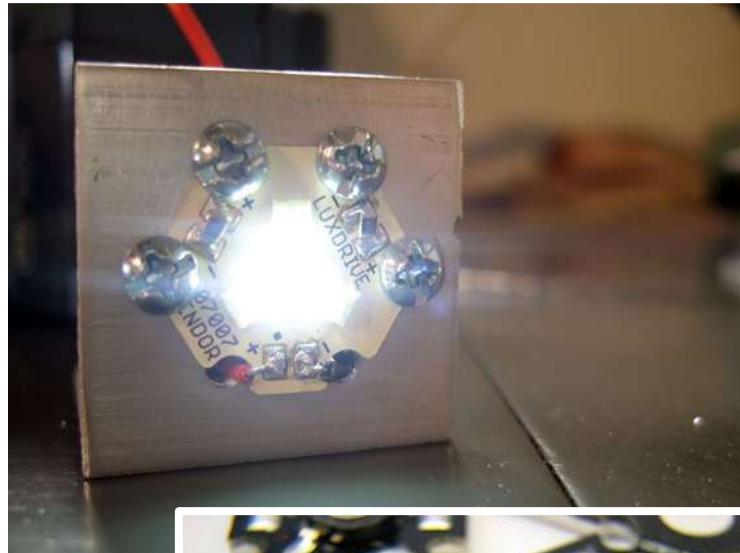
Diode lasers: 375, 405, 488, 635, 660...



Excitation light sources – Laser

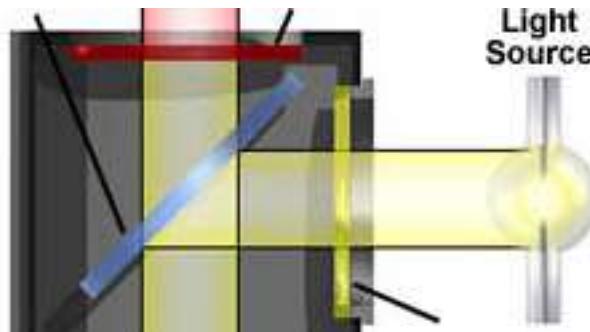


Excitation light sources – LED and others



Filter components

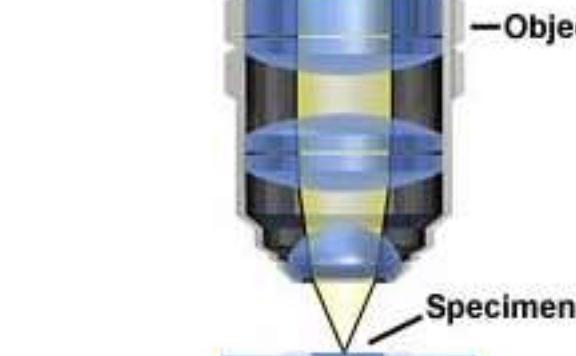
Dichroic mirror Emission filter



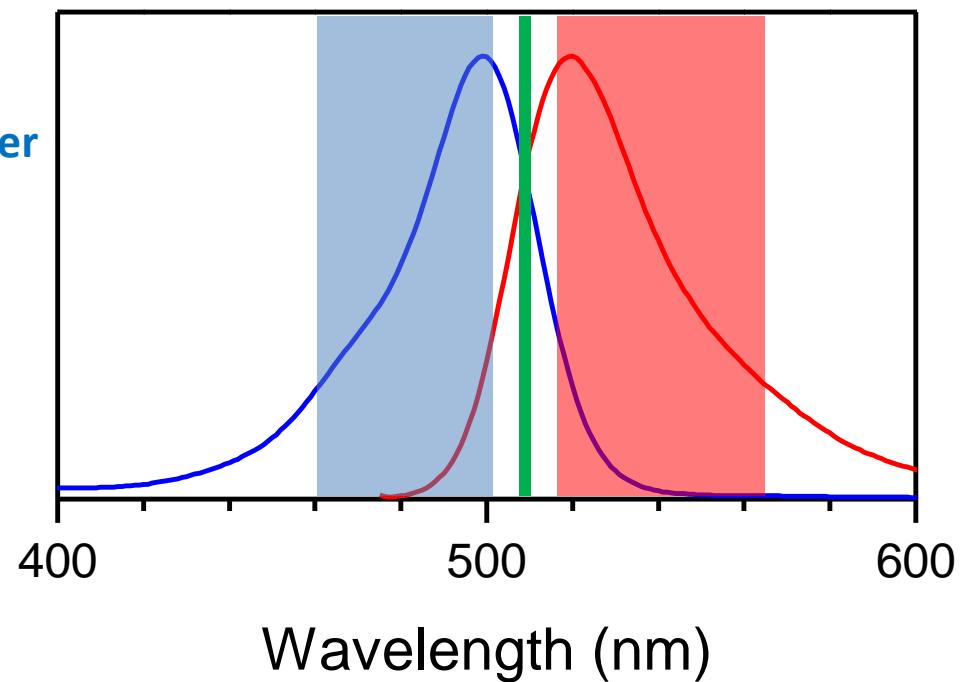
Excitation filter

Figure 2

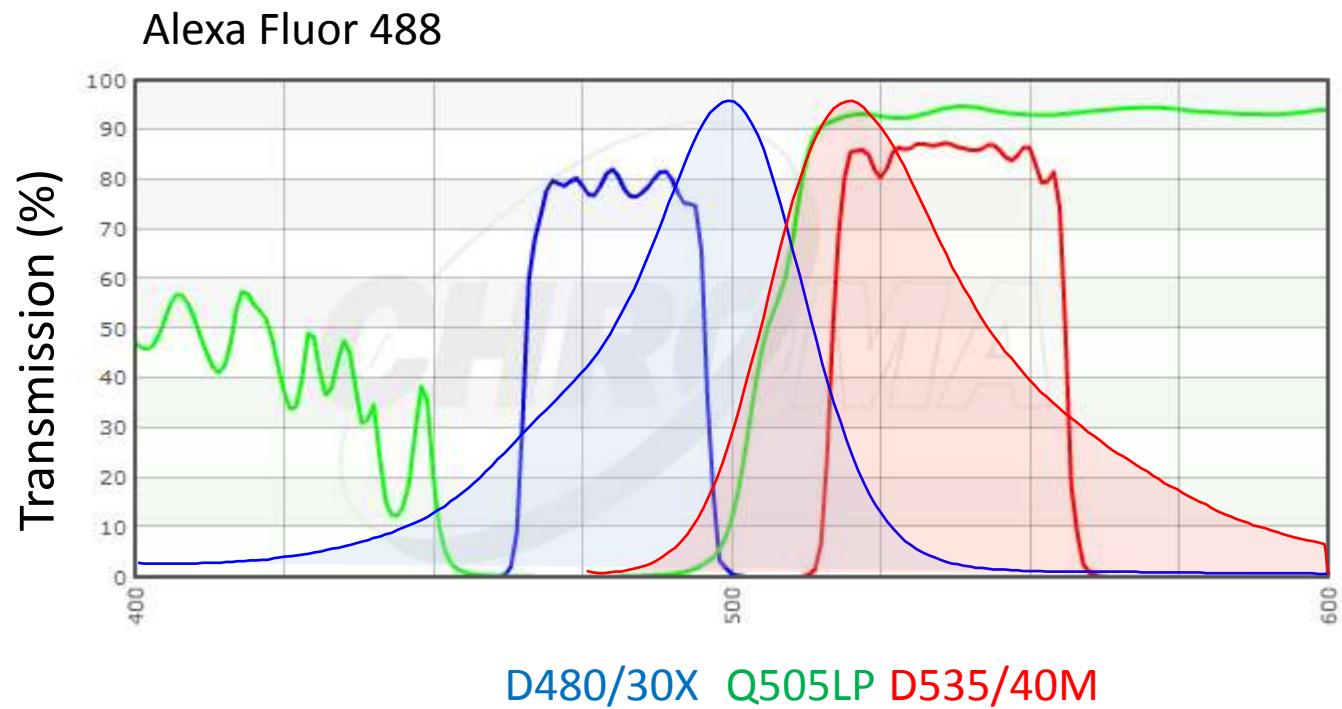
—Objective



Specimen

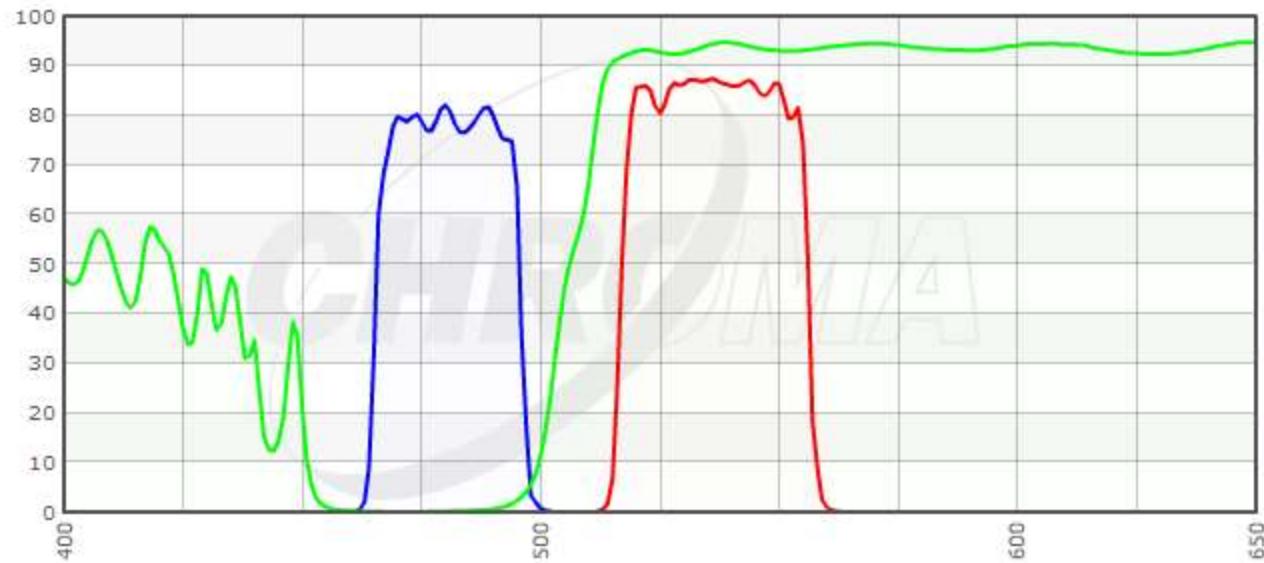


An example of a “filter set”

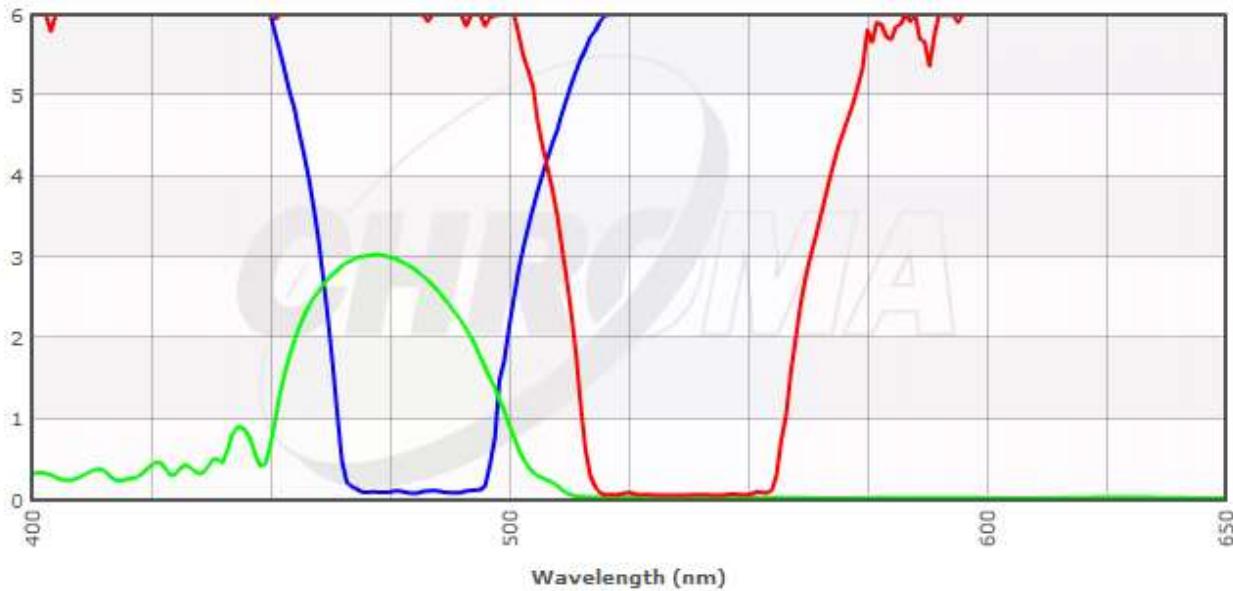


Transmission vs. Optical density

Transmission (%)

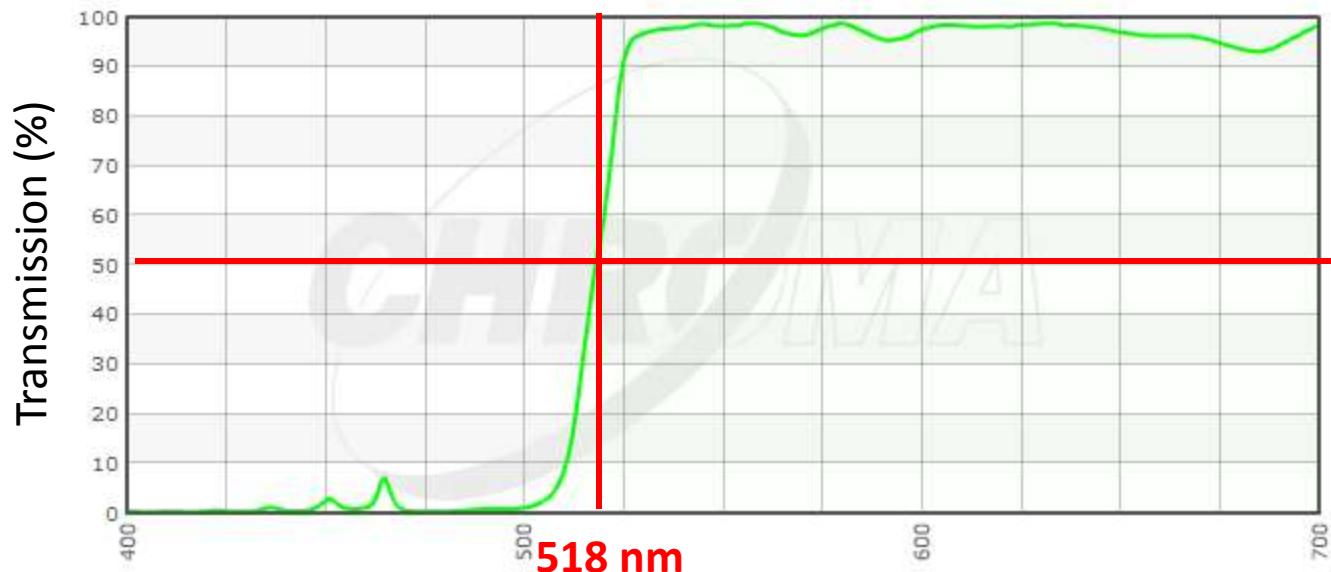


$$\text{OD} = -\log_{10}(\text{Transmission})$$

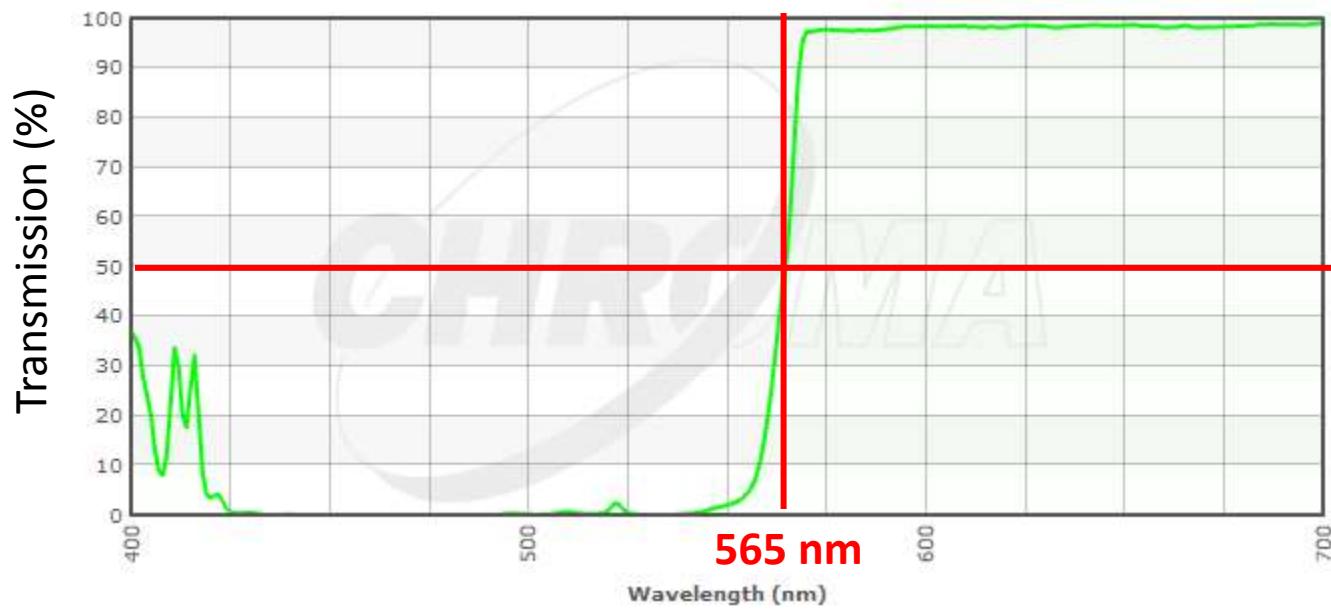


Filter names – Dichroic mirror

515DCLP

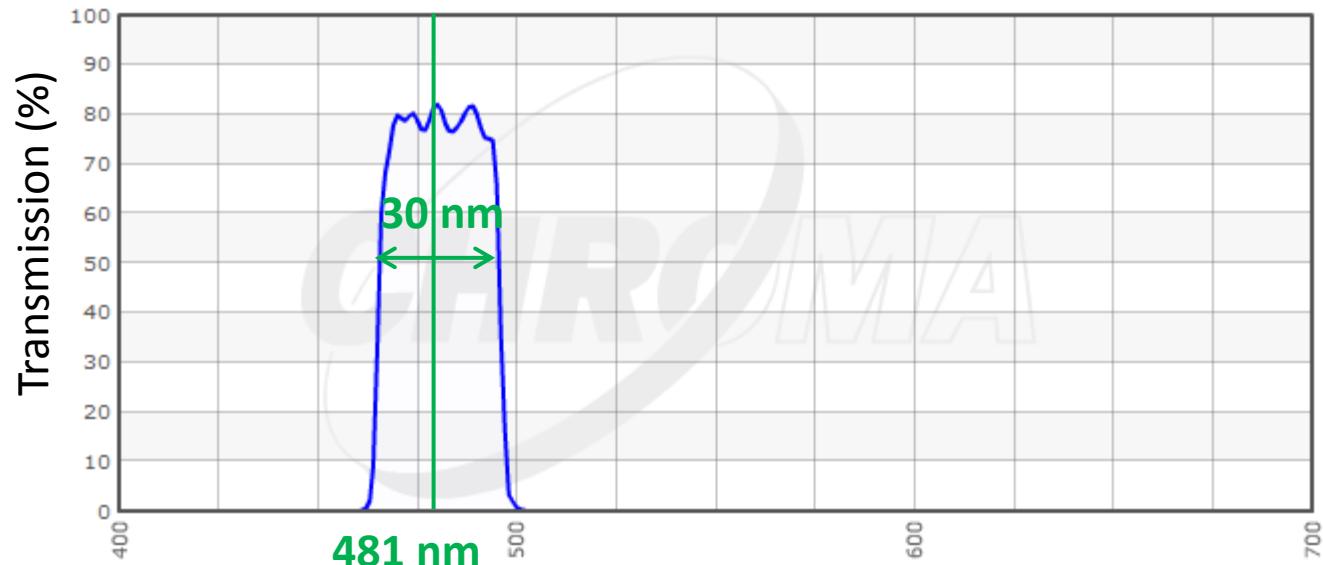


T565LPXR

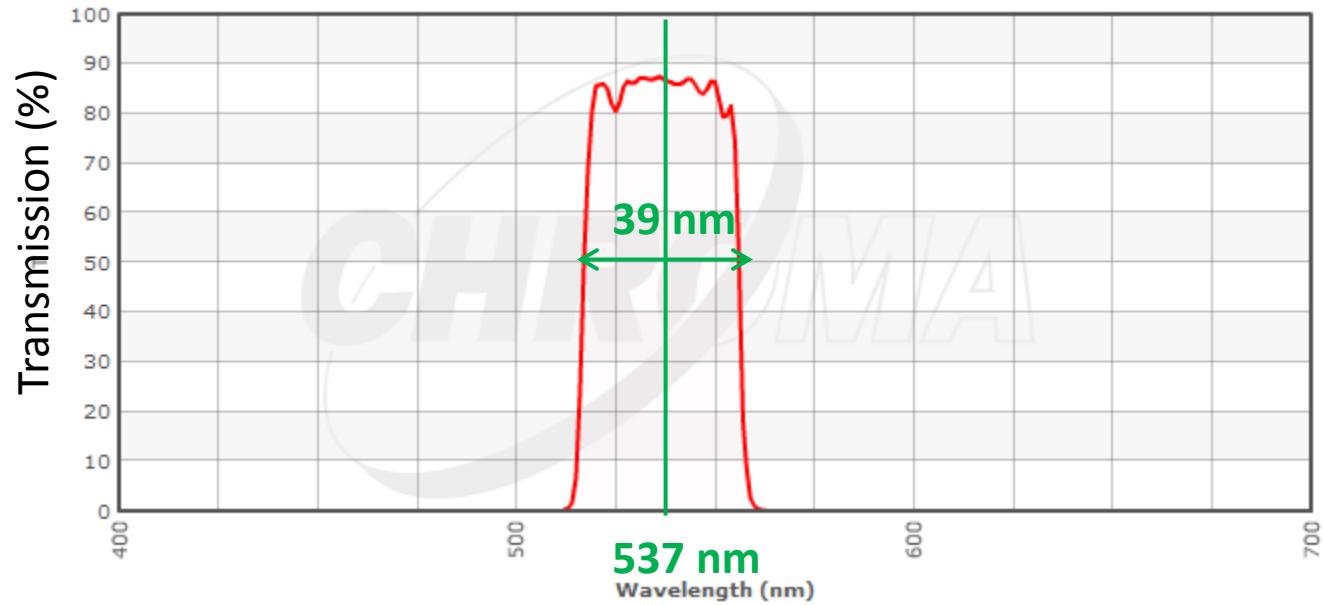


Filter names – Bandpass filters

D480/30X



D535/40M



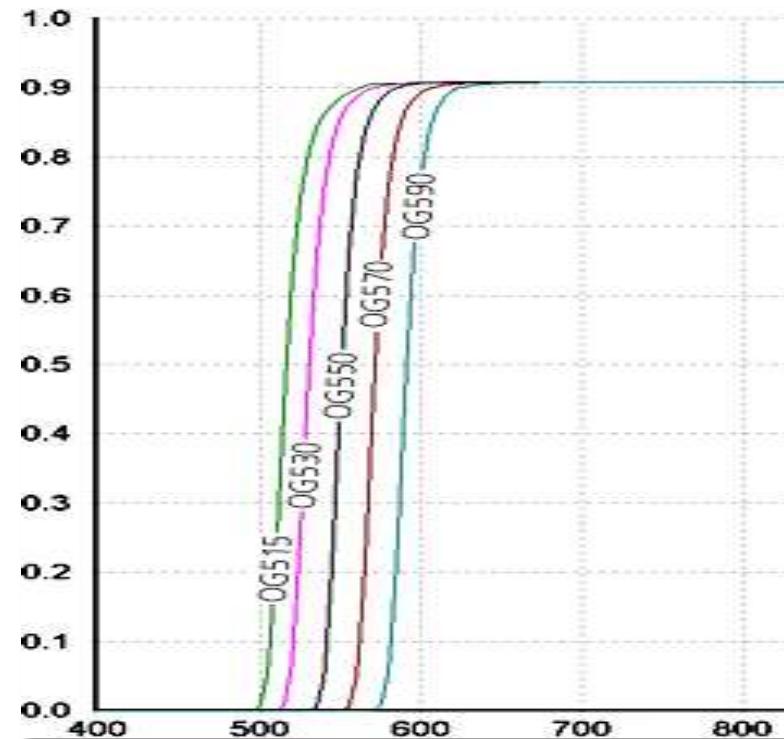
Inexpensive filters: color glasses



Absorptive
(colored glass)

OD \approx 2

OG550 =
Orange Glass,
50% transmission at 550 nm

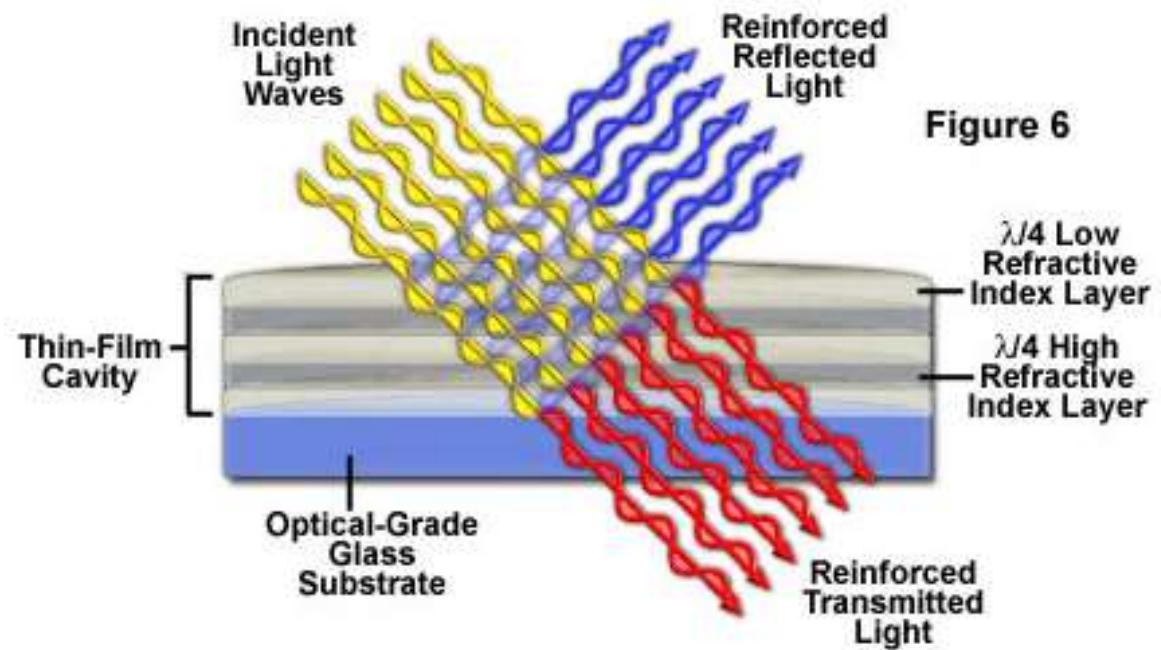


Dielectric filters



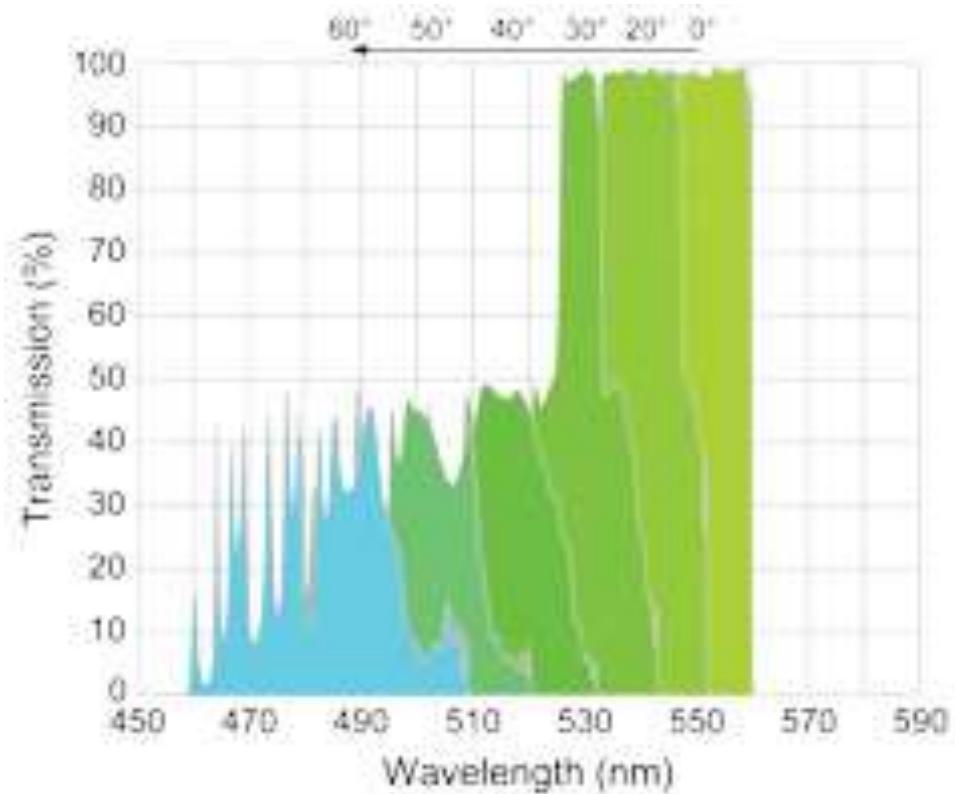
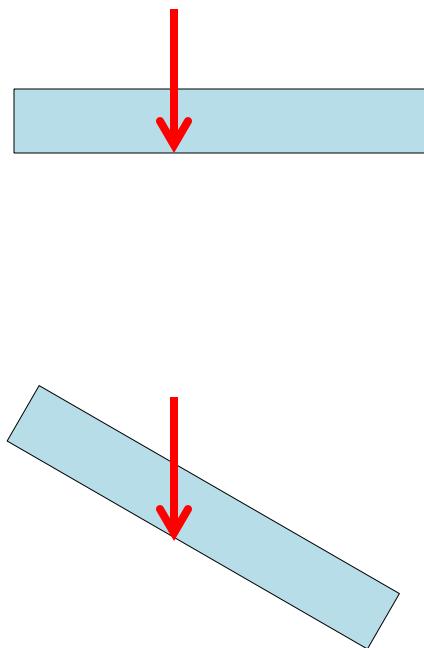
Interference
(dielectric)

OD > 6
Transmission < 10^{-6}



Vendors: Chroma, Semrock, Omega

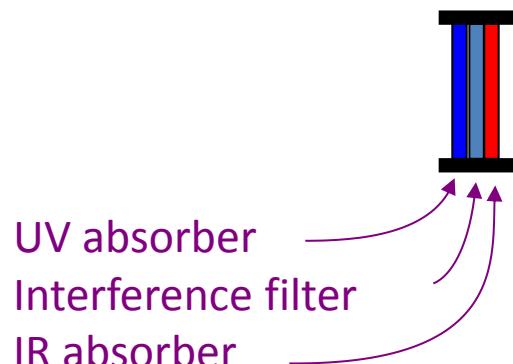
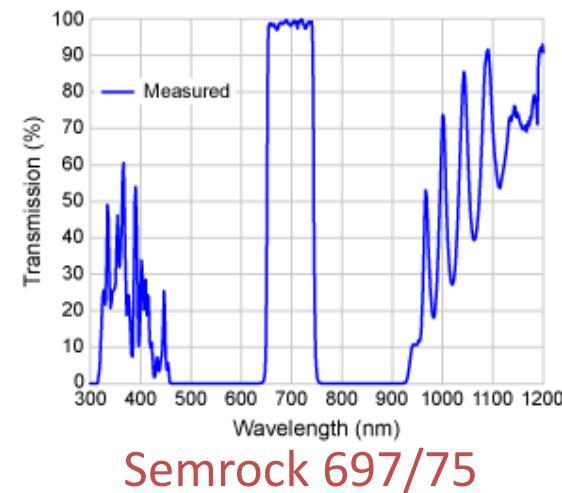
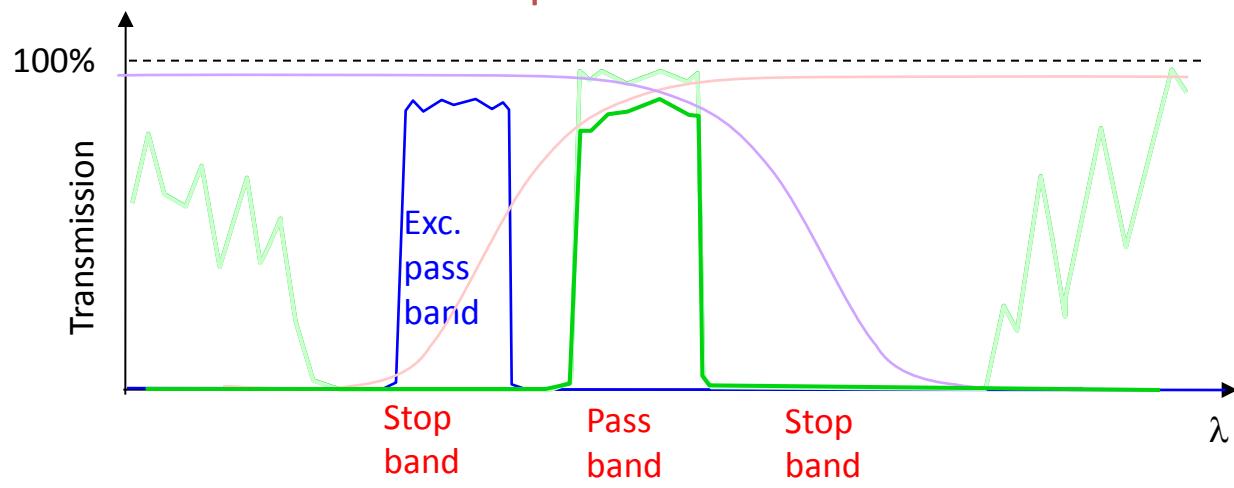
Interference filter is sensitive to incident angle



Semrock website

Stop band of interference filters

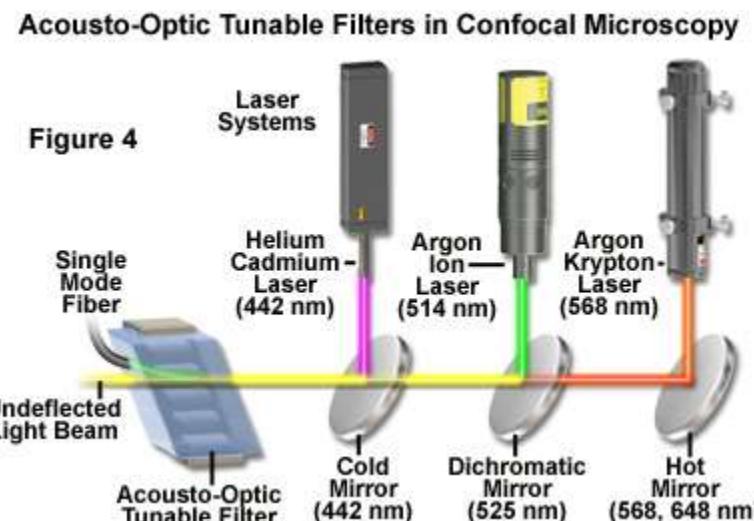
Unblocked bandpass interference filter



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

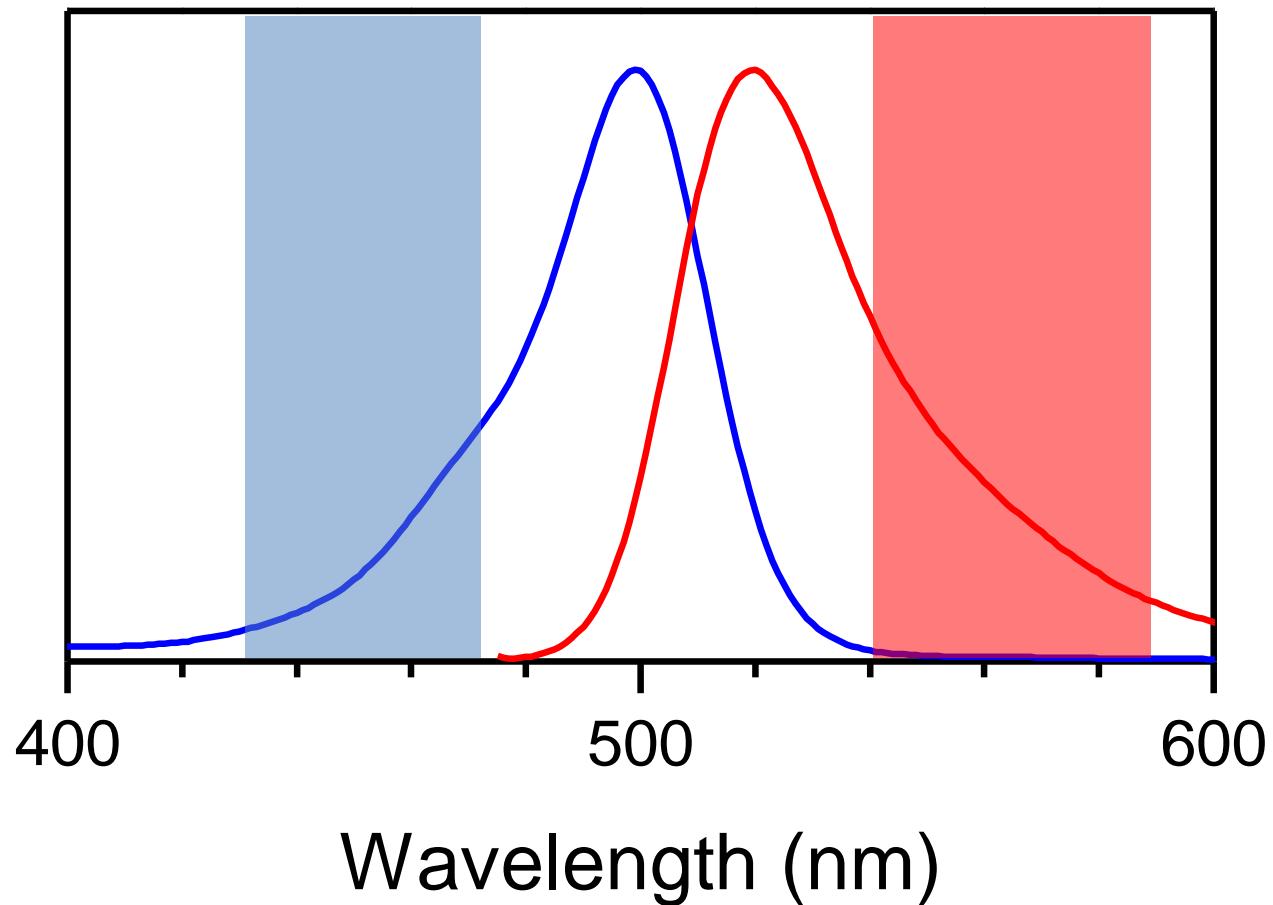


Practical concerns

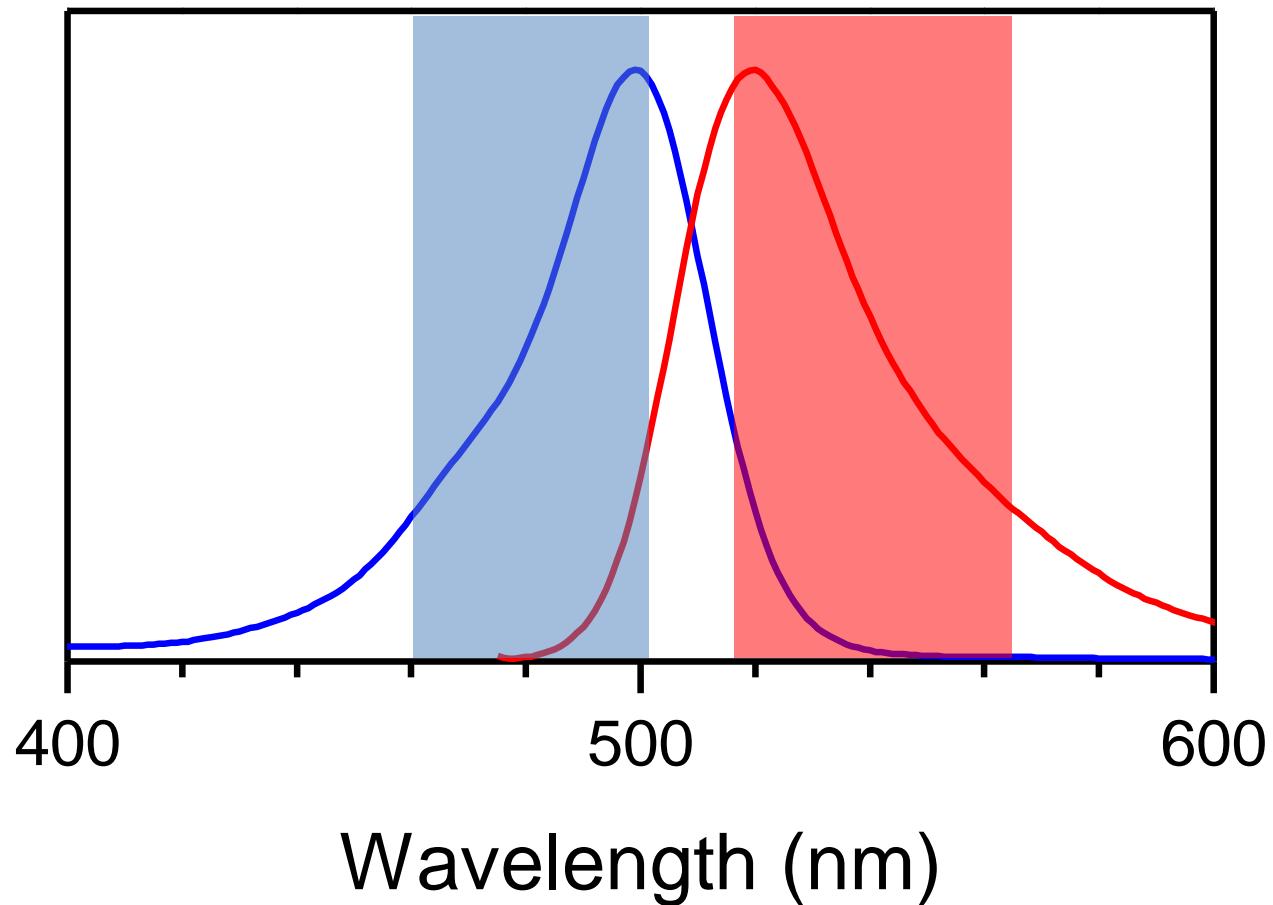
I will practice my modeling technique 2 hours every day
I will practice my modeling technique 2 hours every day
I will practice my modeling technique 2 hours every day
I will practice my modeling technique 2 hours every day
I will practice my modeling technique 2 hours every day
I will practice my modeling technique 2 hours every day
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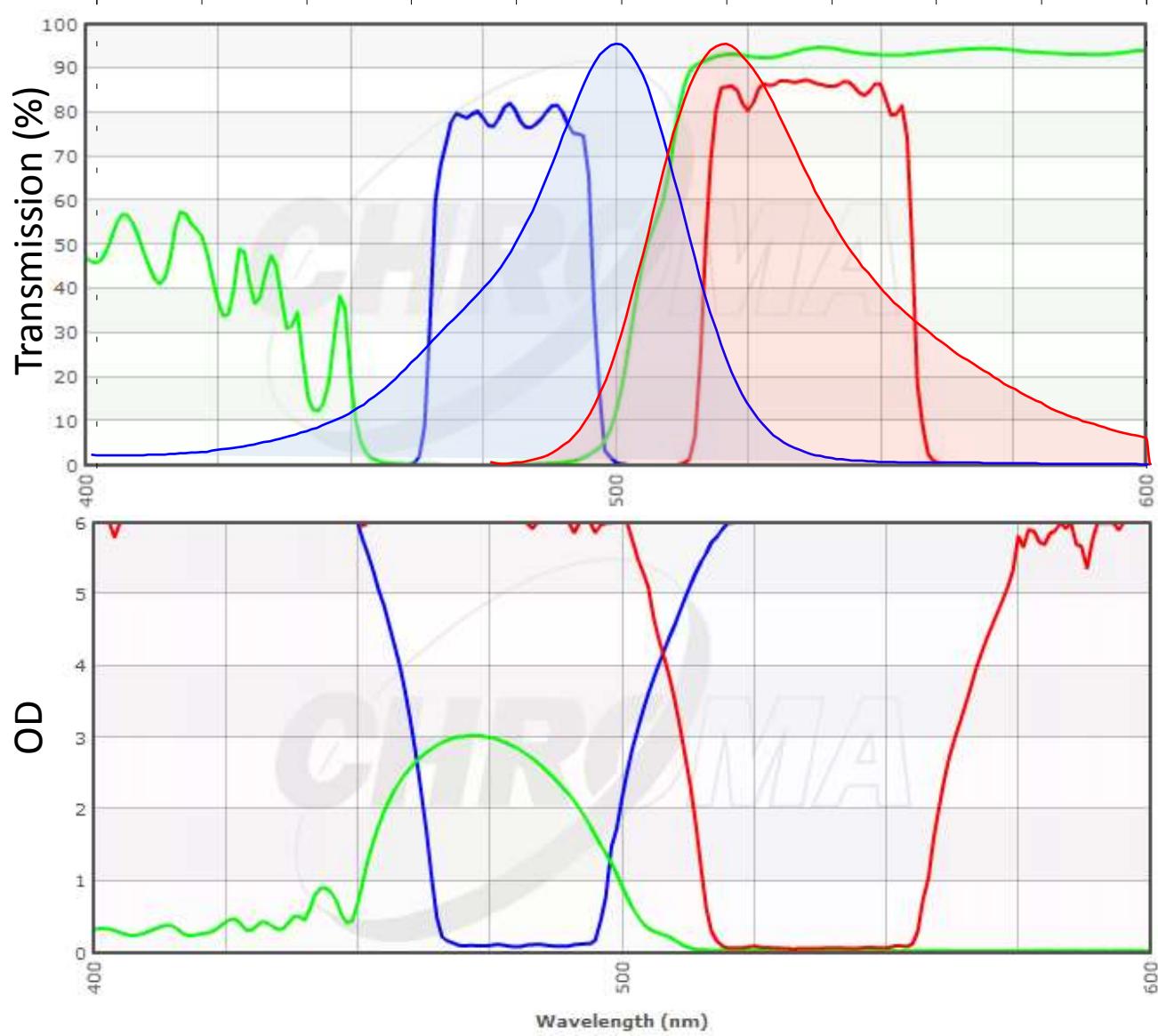
Matching the filters with the spectra



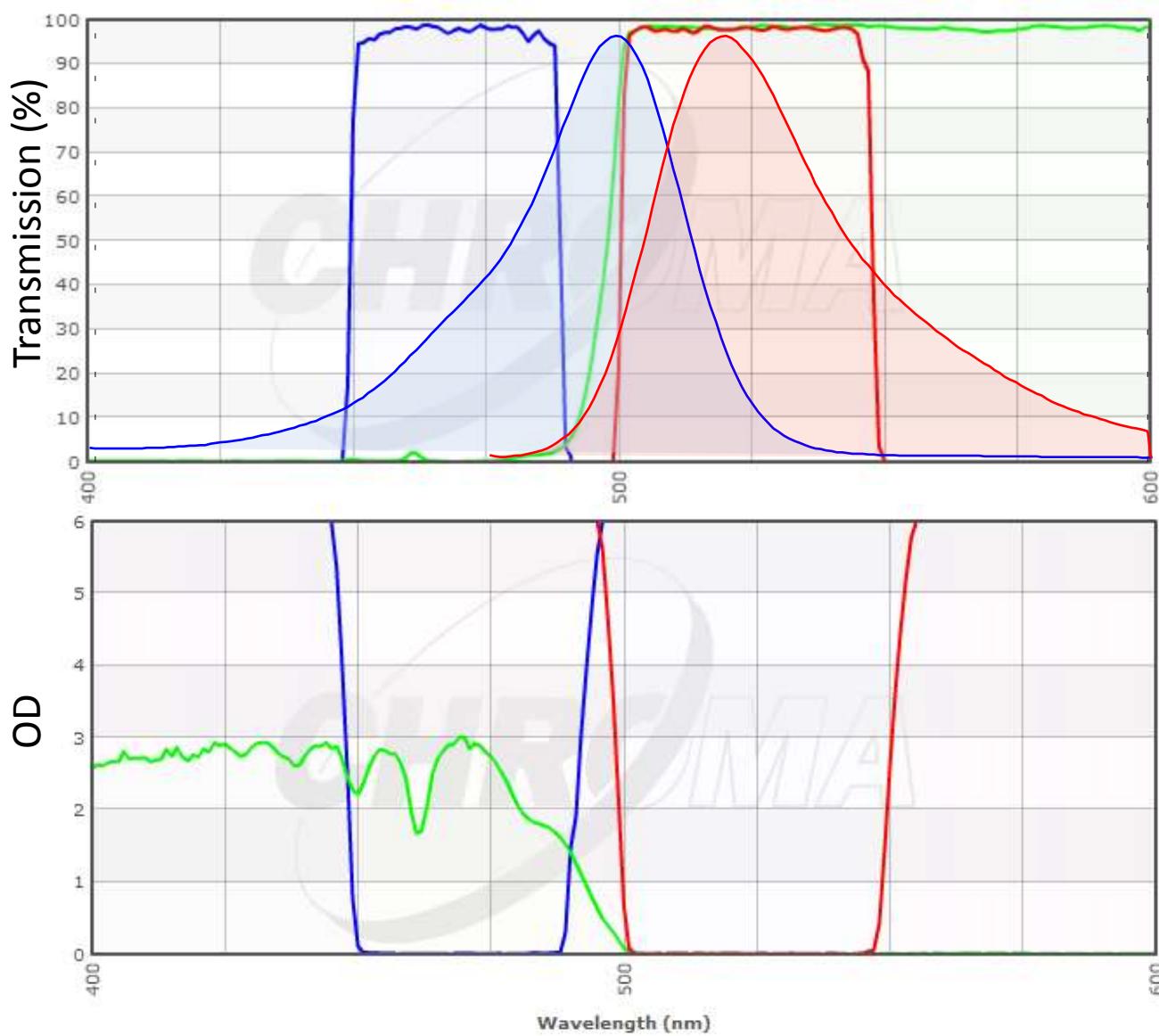
Matching the filters with the spectra



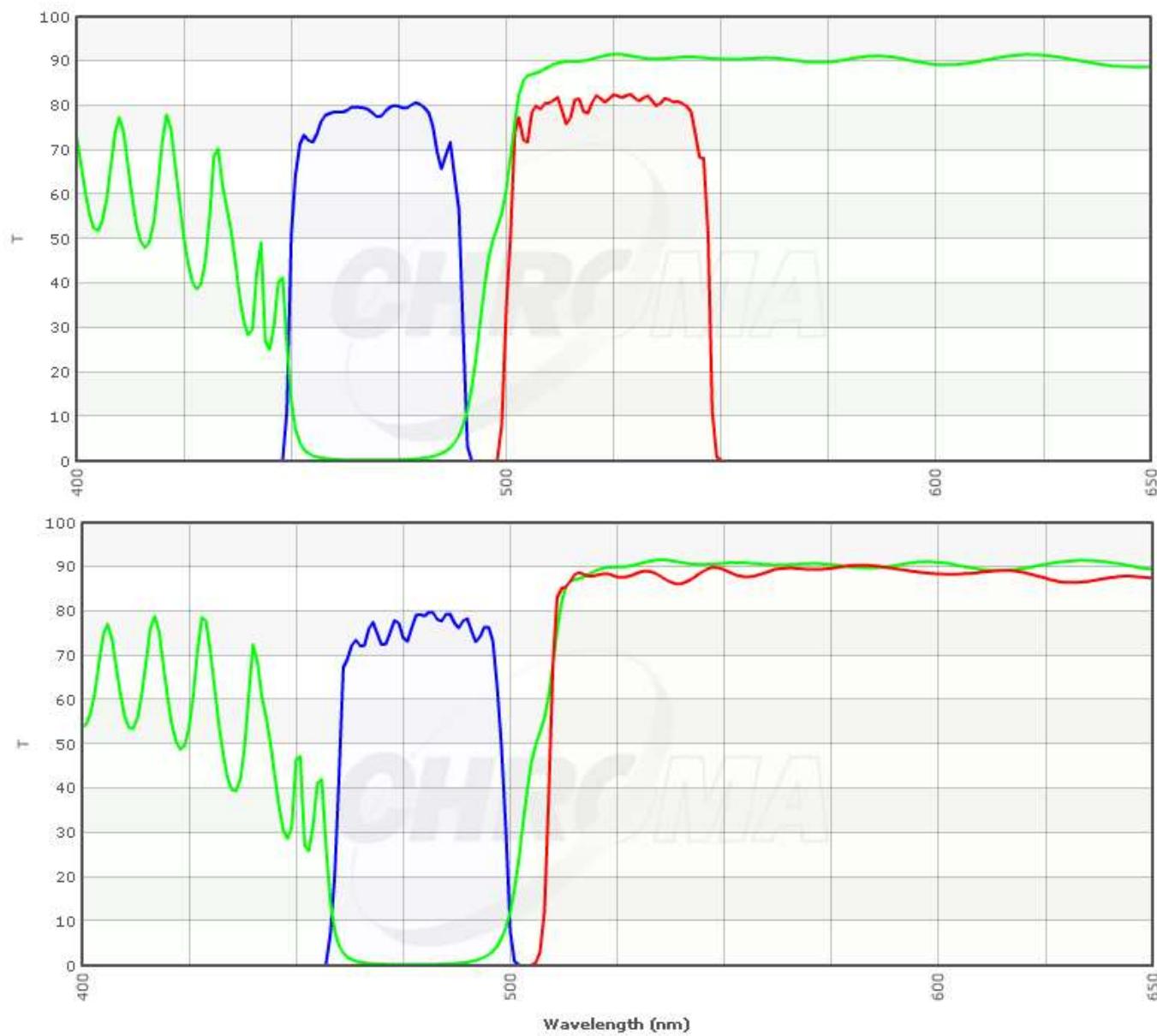
The choice of a “filter set”



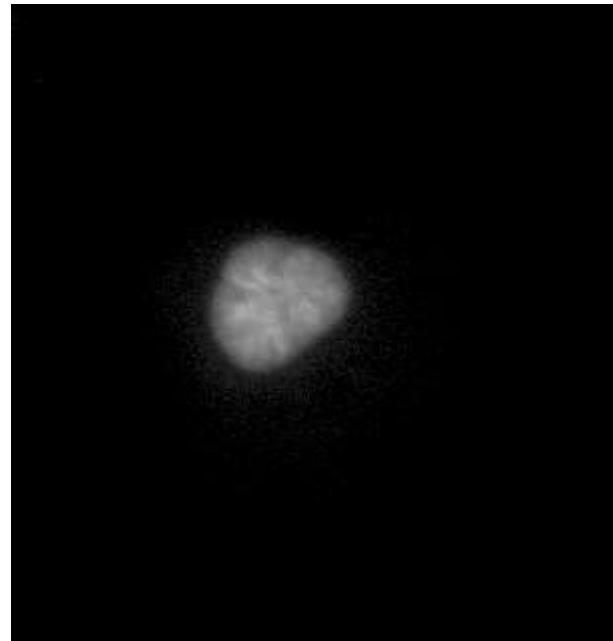
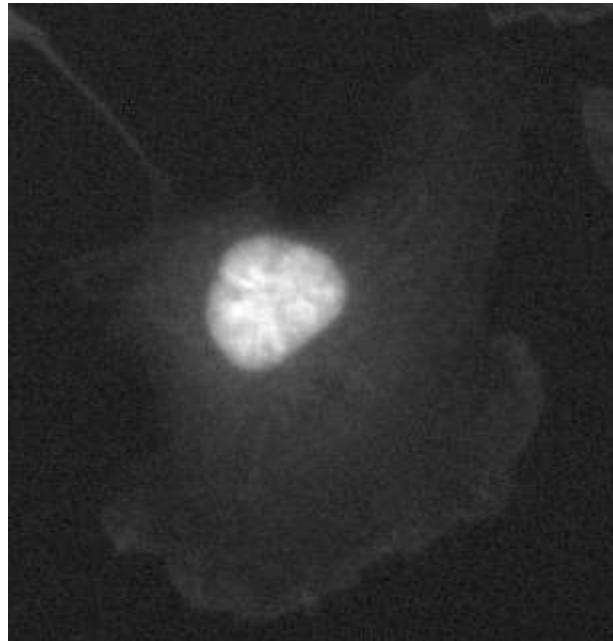
Better (\$\$\$) filters give higher efficiency



Long pass vs. Band pass



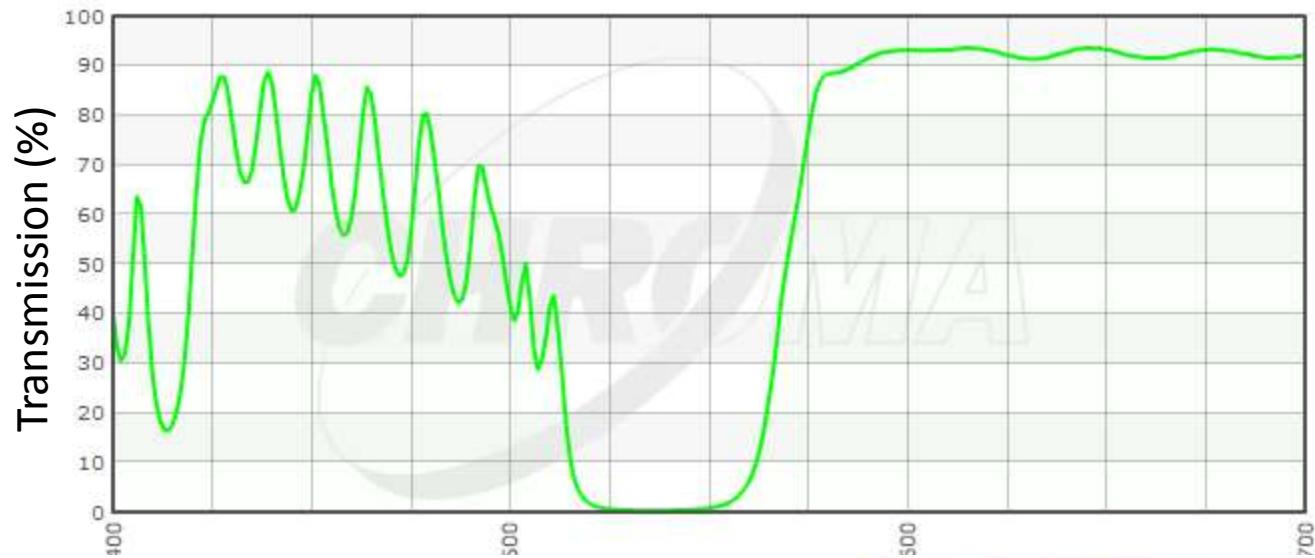
Wider is not necessarily wiser



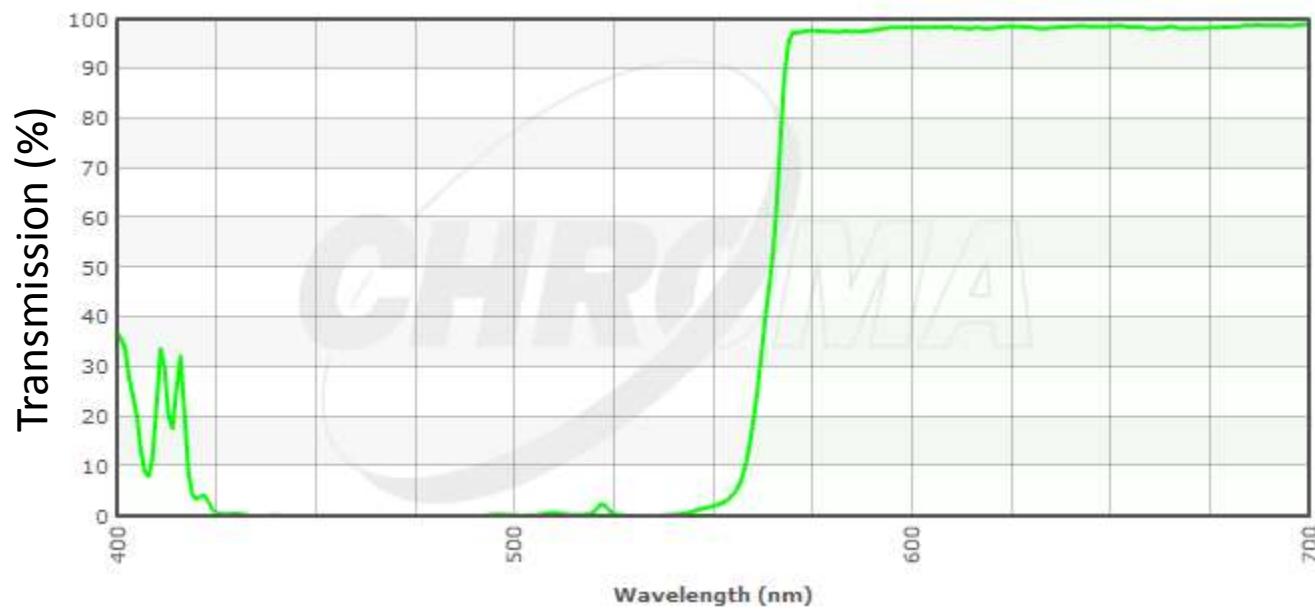
DAPI nucleus staining, long pass vs. bandpass

Dichroic mirrors does not have infinite reflection band

565DCLP
Chroma

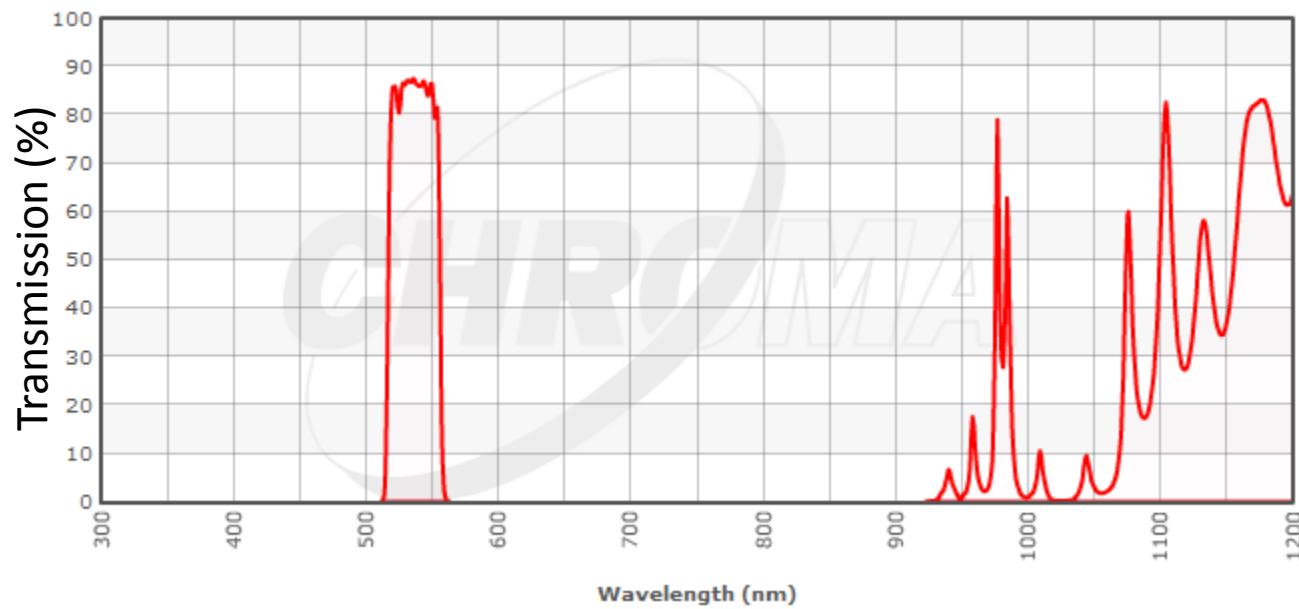
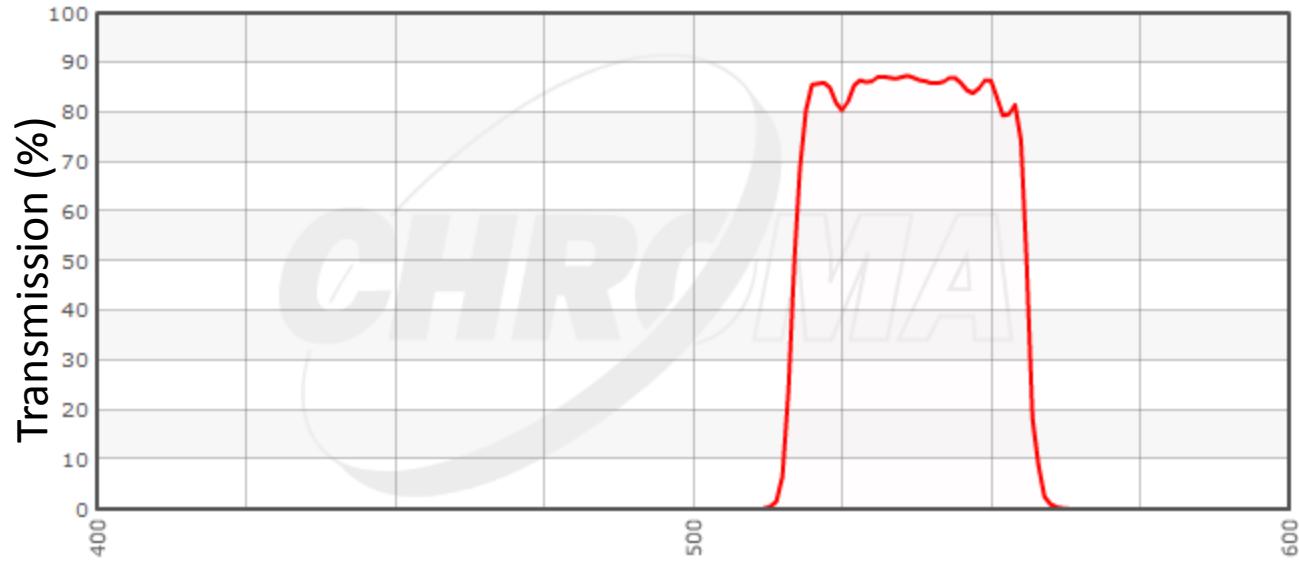


T565LPXR
Chroma



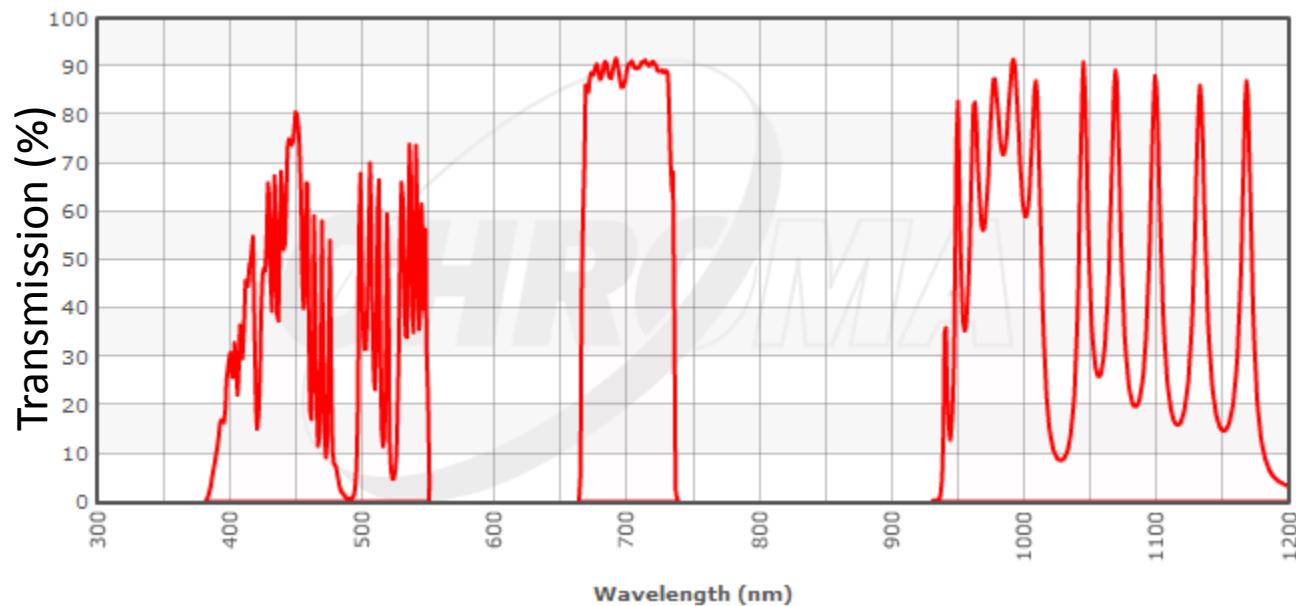
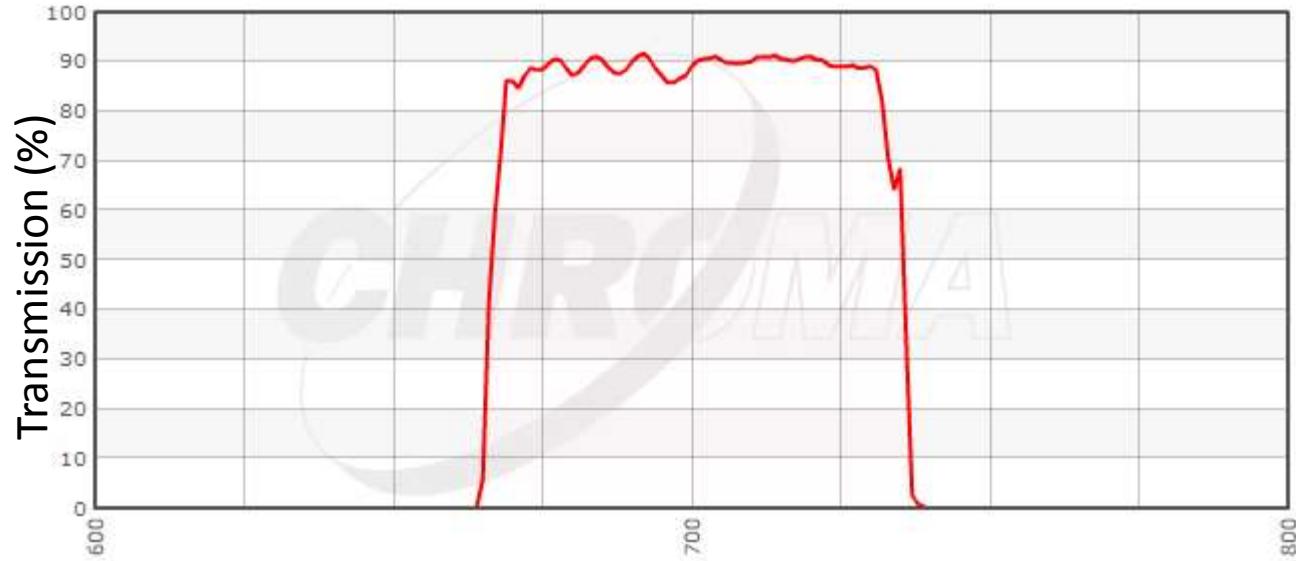
Emission filters might have leaking bands

D535/40M
Chroma

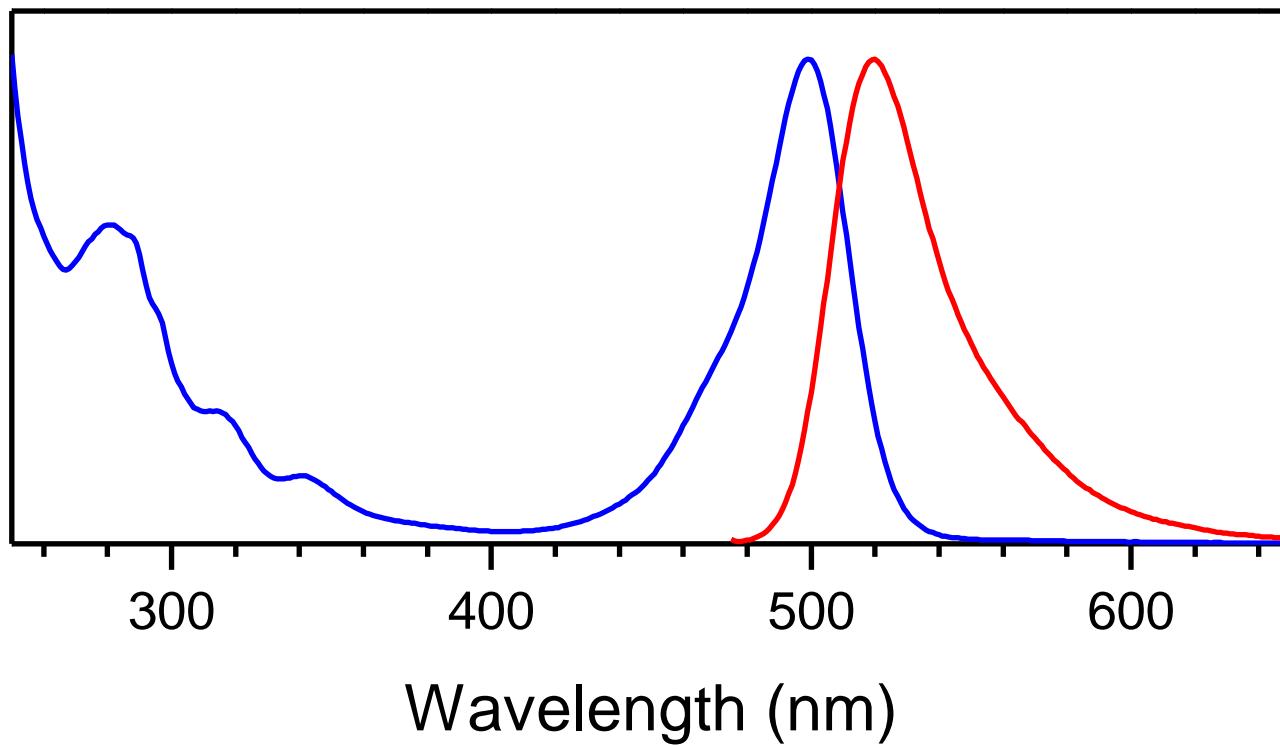


Emission filters might have leaking bands

HQ700/75M
Chroma



Fluorescence spectra has tails



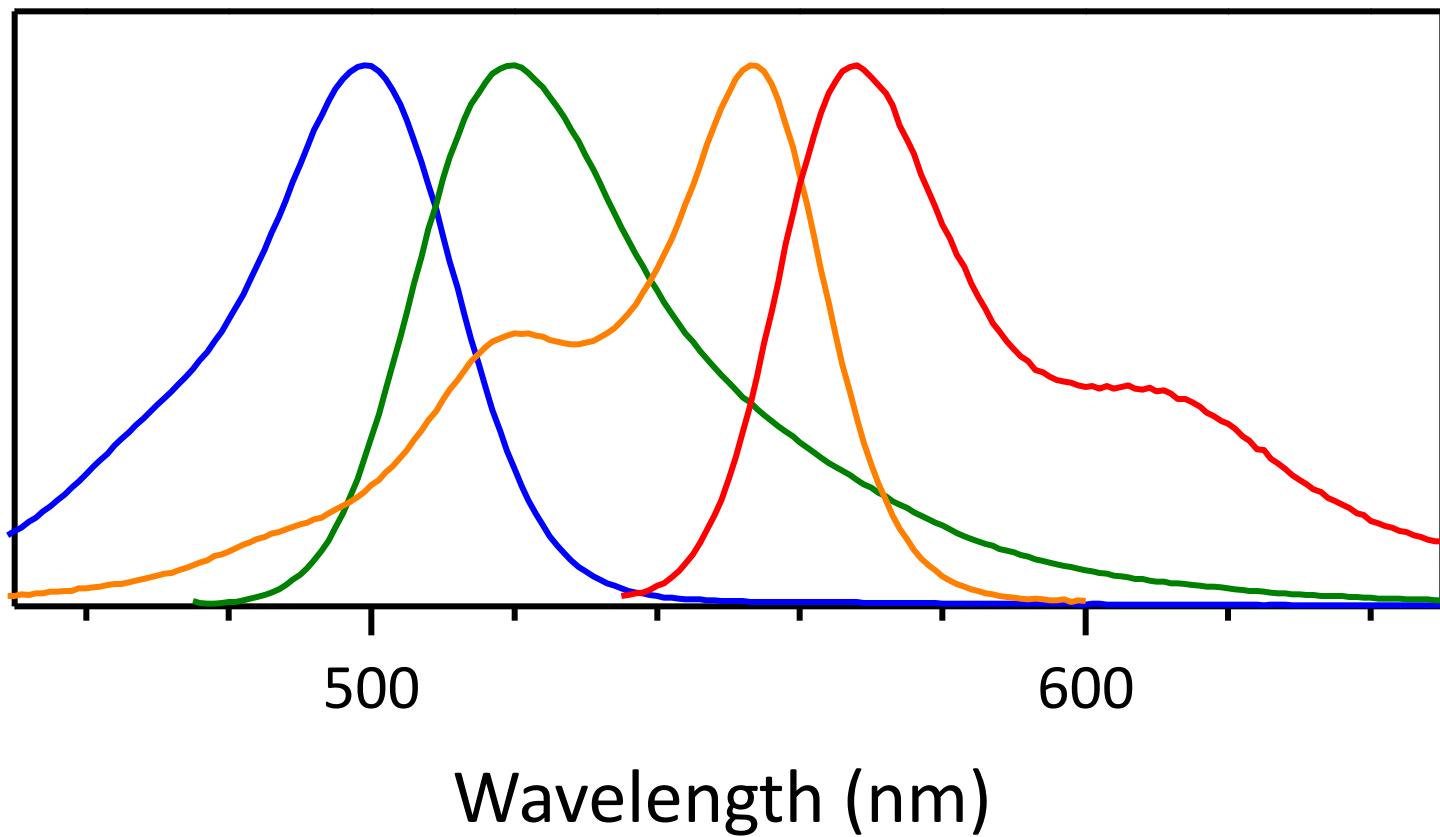
Multicolor imaging

DNA
Actin
Microtubule

Imaging more than one thing at a time

Alexa Fluor 488

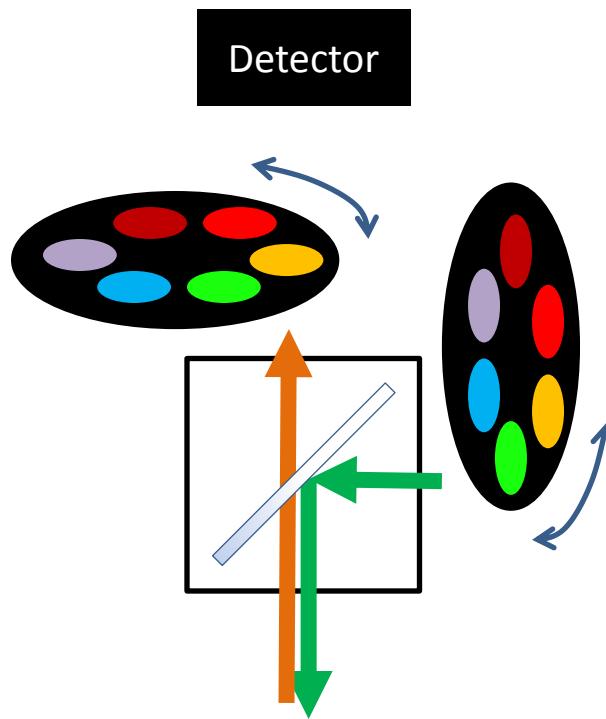
Alexa Fluor 555



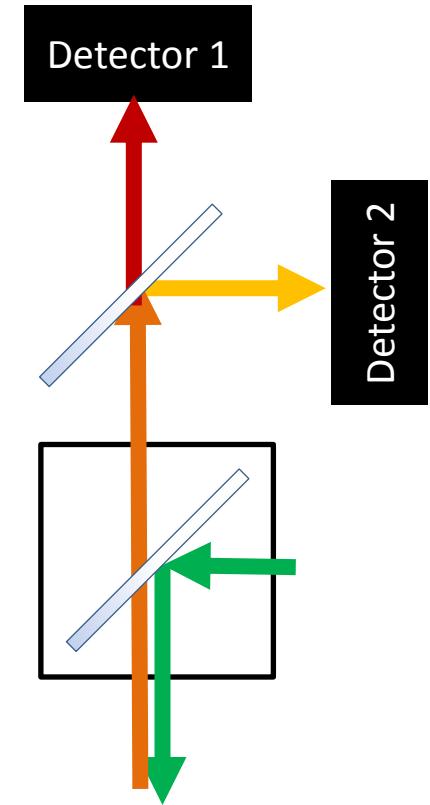
Schemes for multicolor imaging



Cube switching



Filter switching

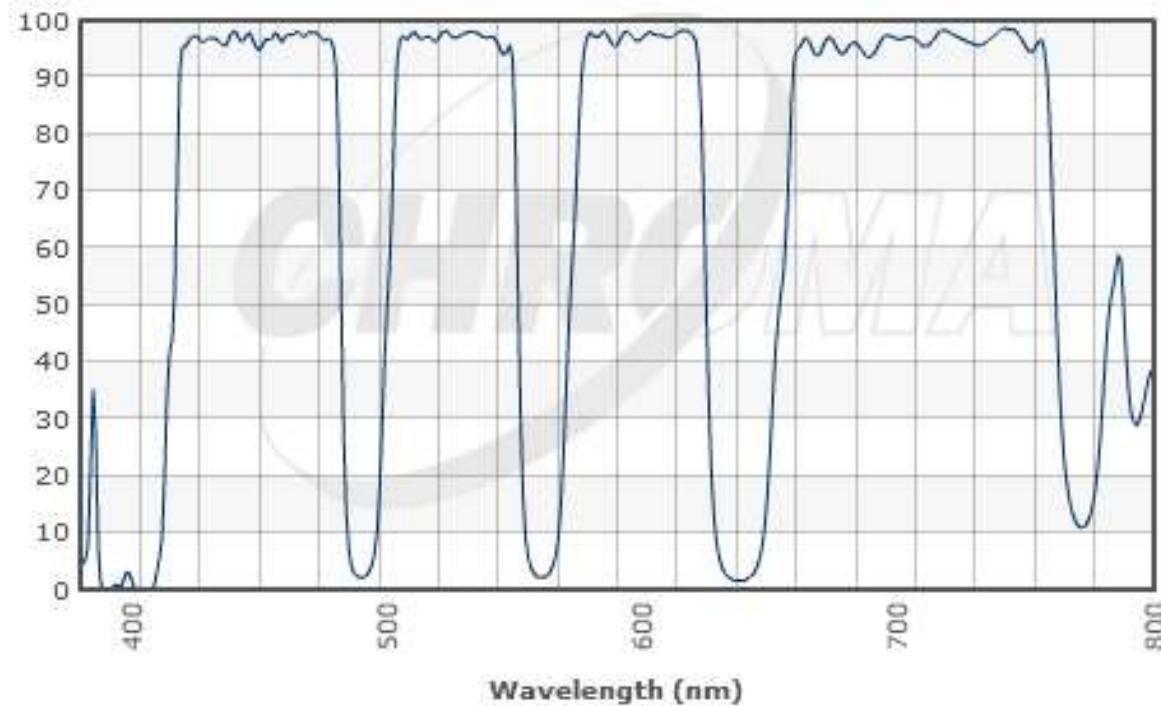


Multiple detectors

Polychroic mirror and multi-bandpass filter

ZT408/488/561/640RPC

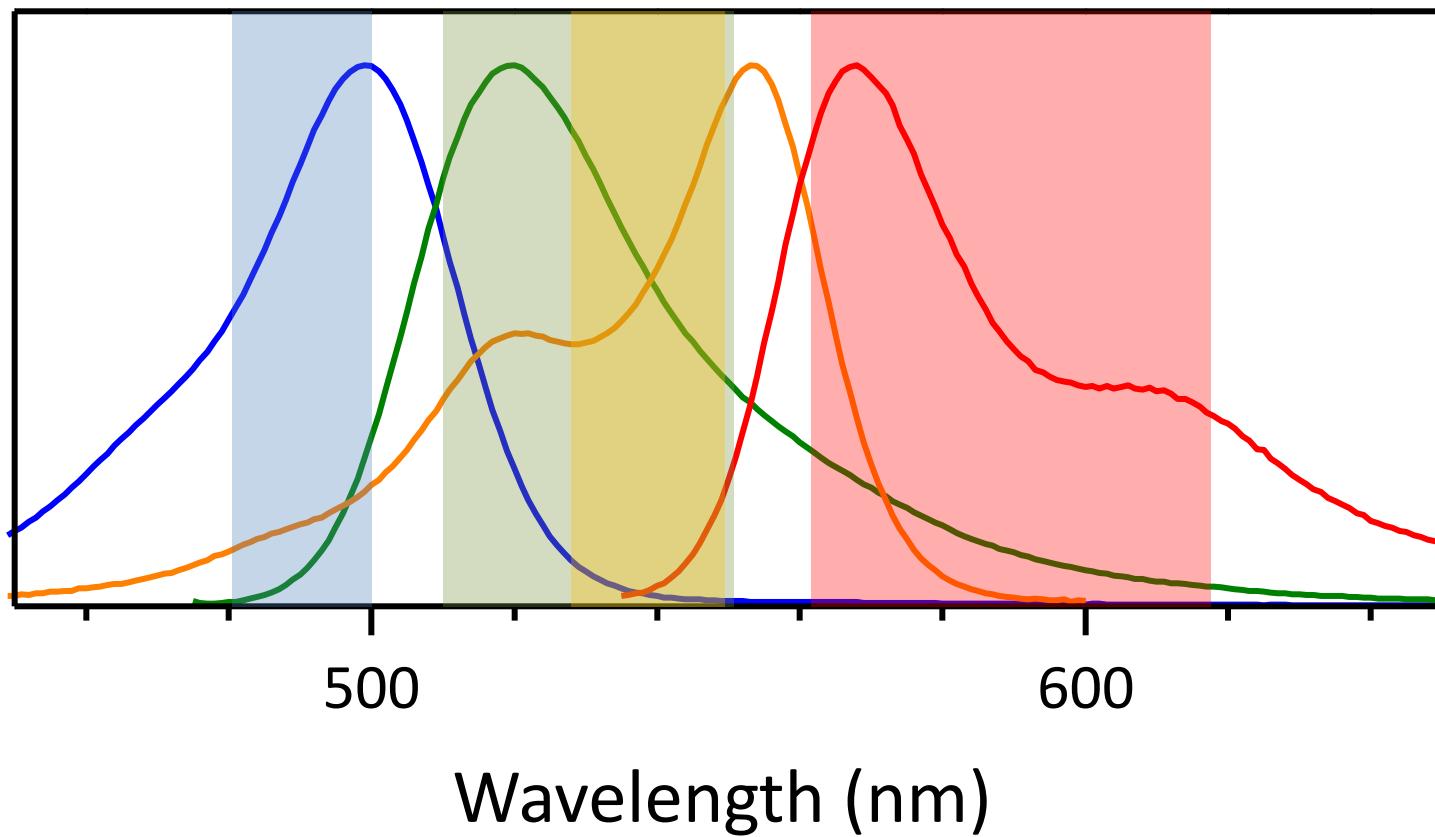
Chroma



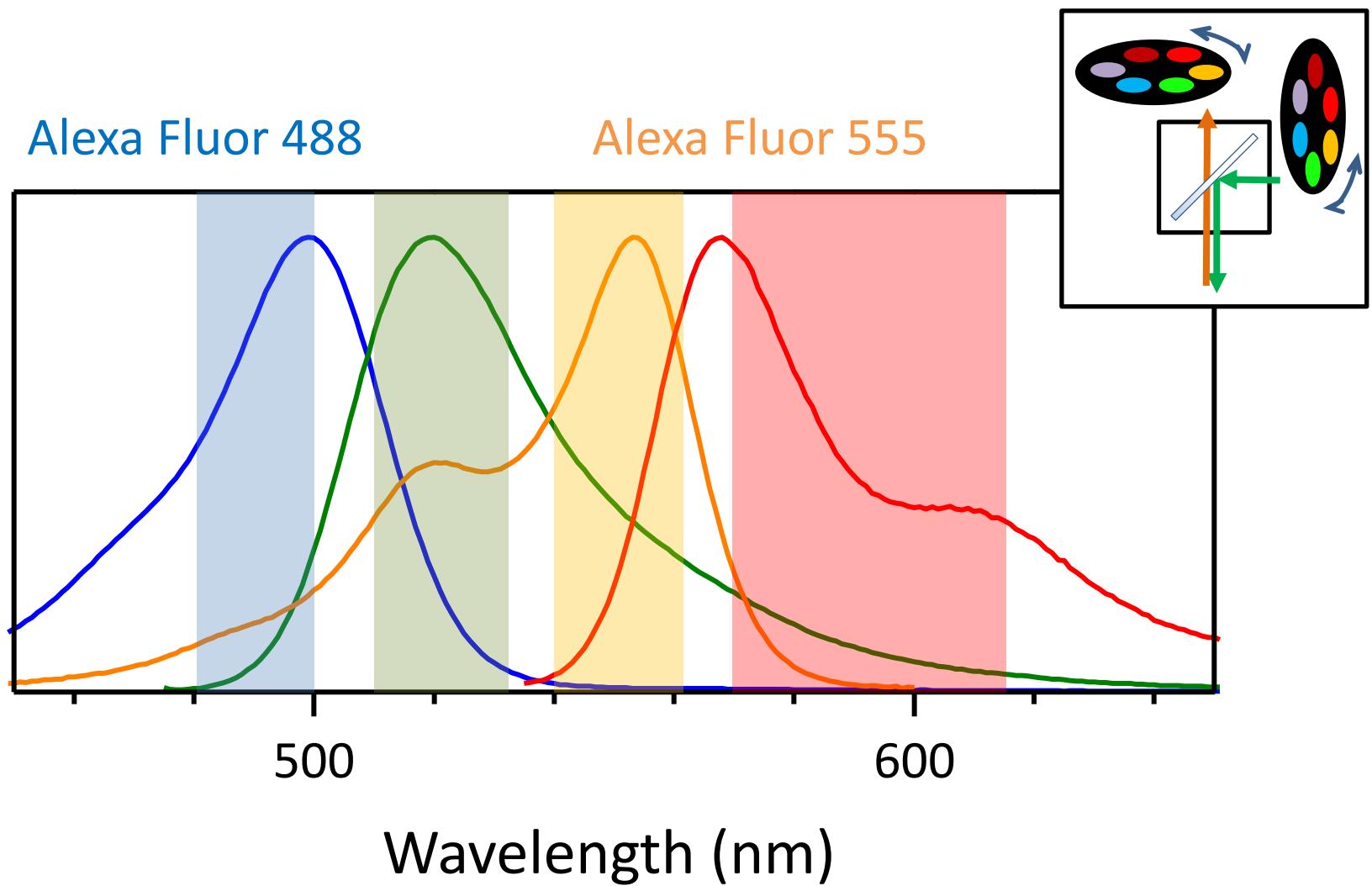
Cube switching

Alexa Fluor 488

Alexa Fluor 555

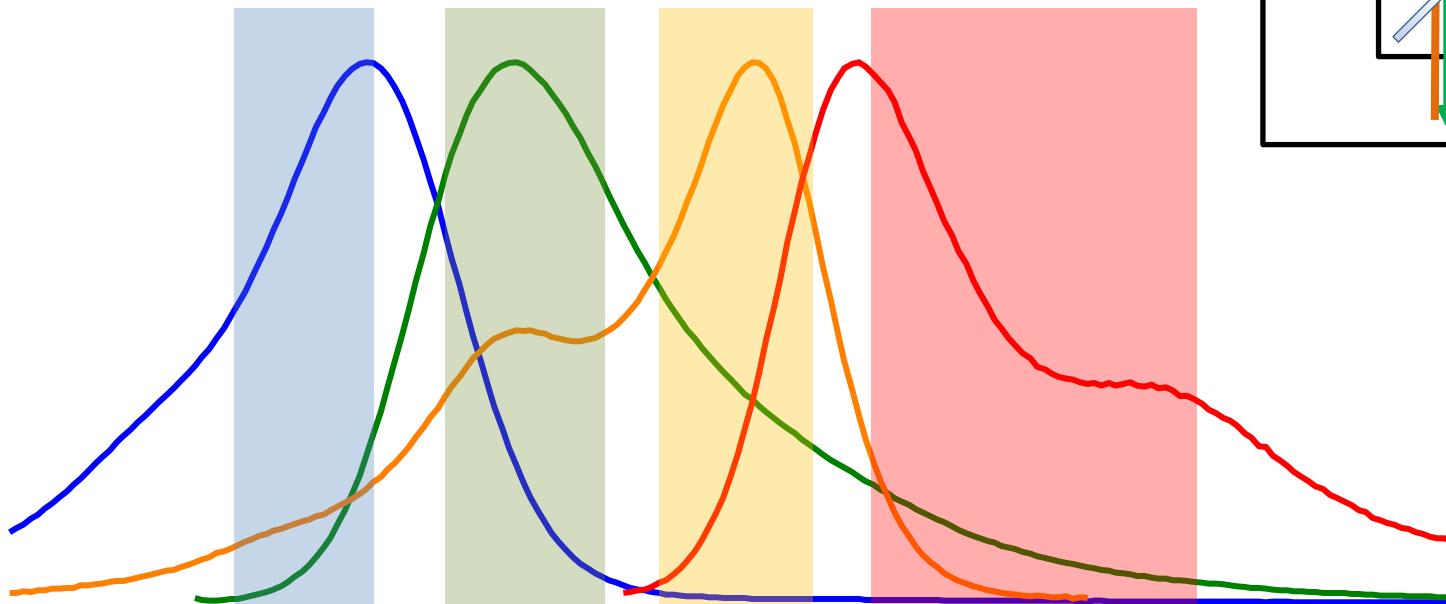


Filter switching – both Ex and Em

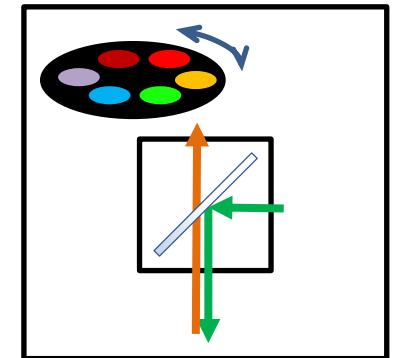


Filter switching – Emission only

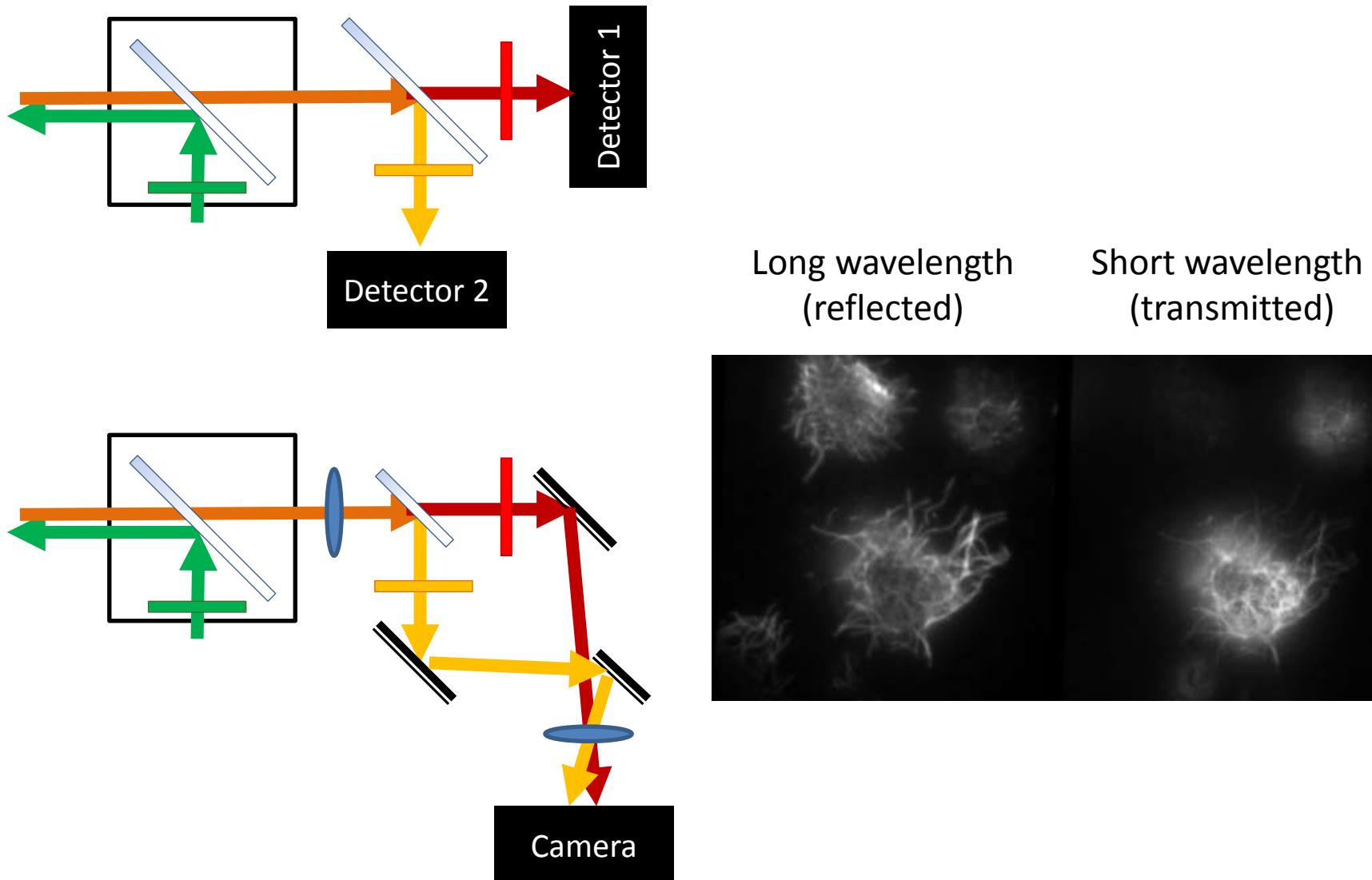
Alexa Fluor 488



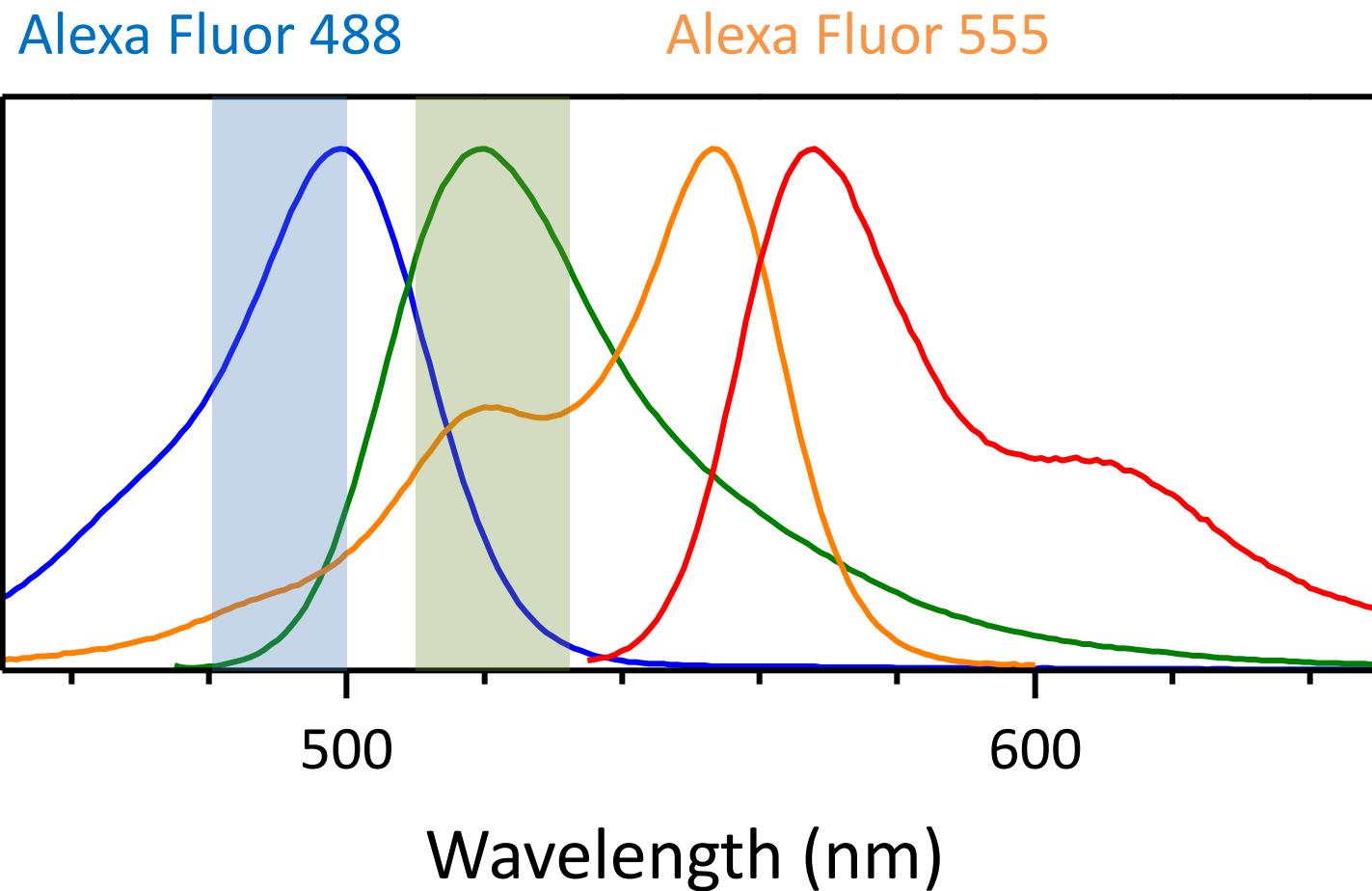
Alexa Fluor 555



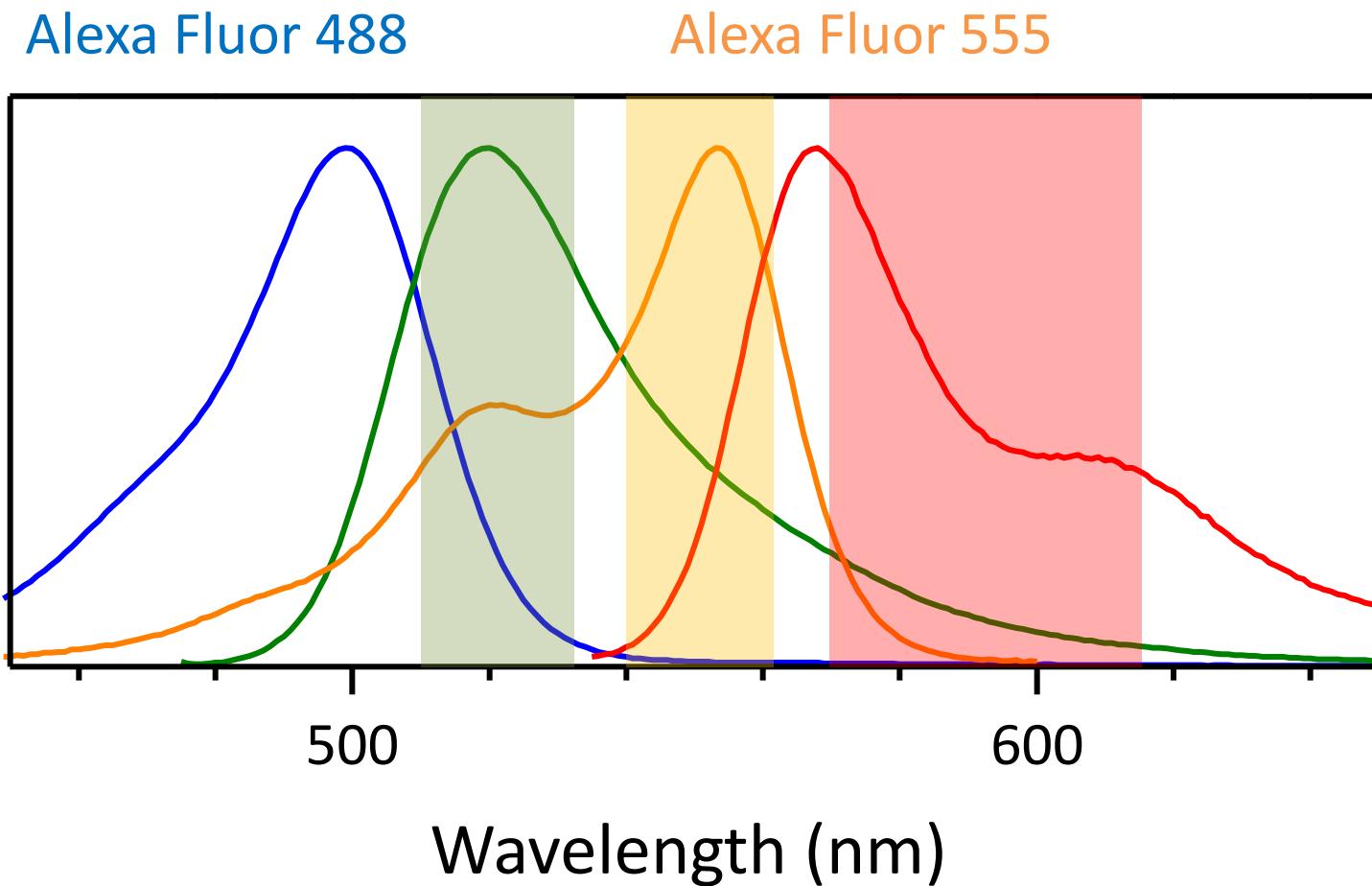
Simultaneous two channel detection



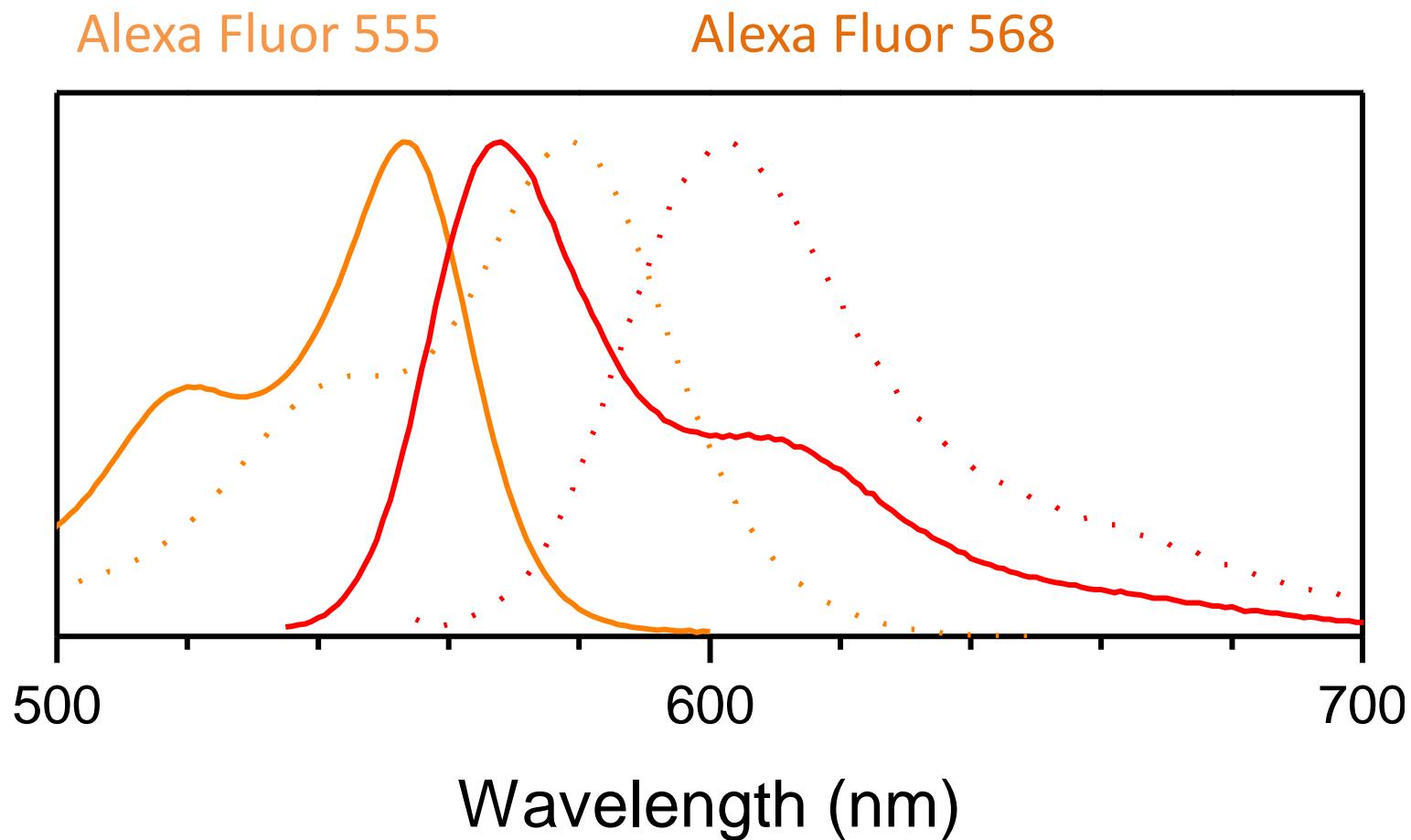
Crosstalk between channels – excitation



Crosstalk between channels – emission



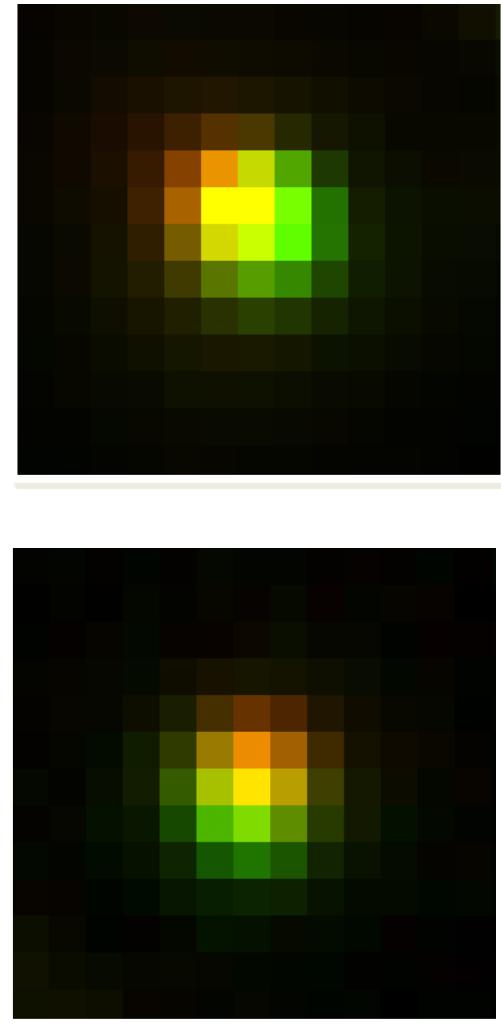
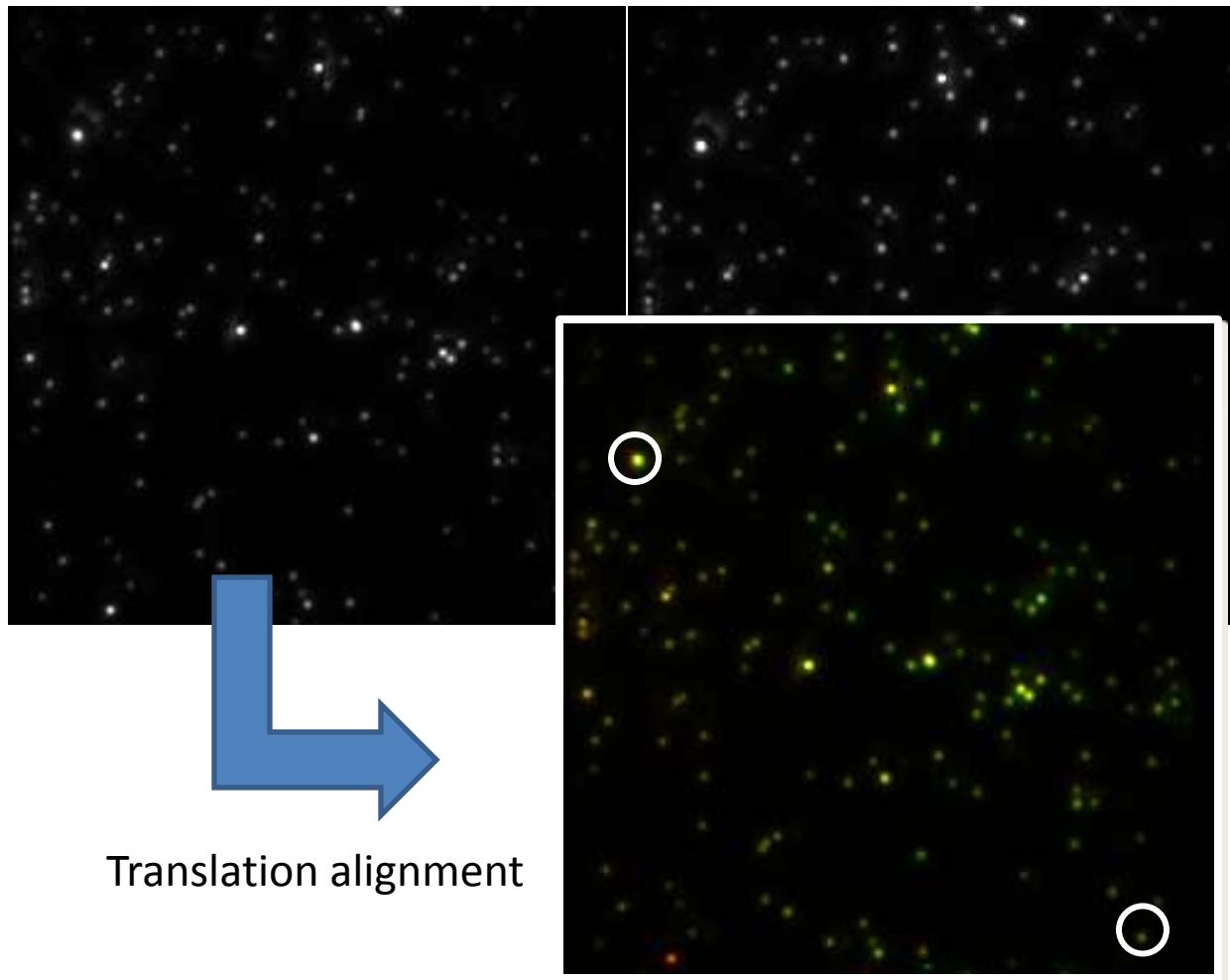
Something too close...



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

Image registration

Fluorescent beads with signal in both channels



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>