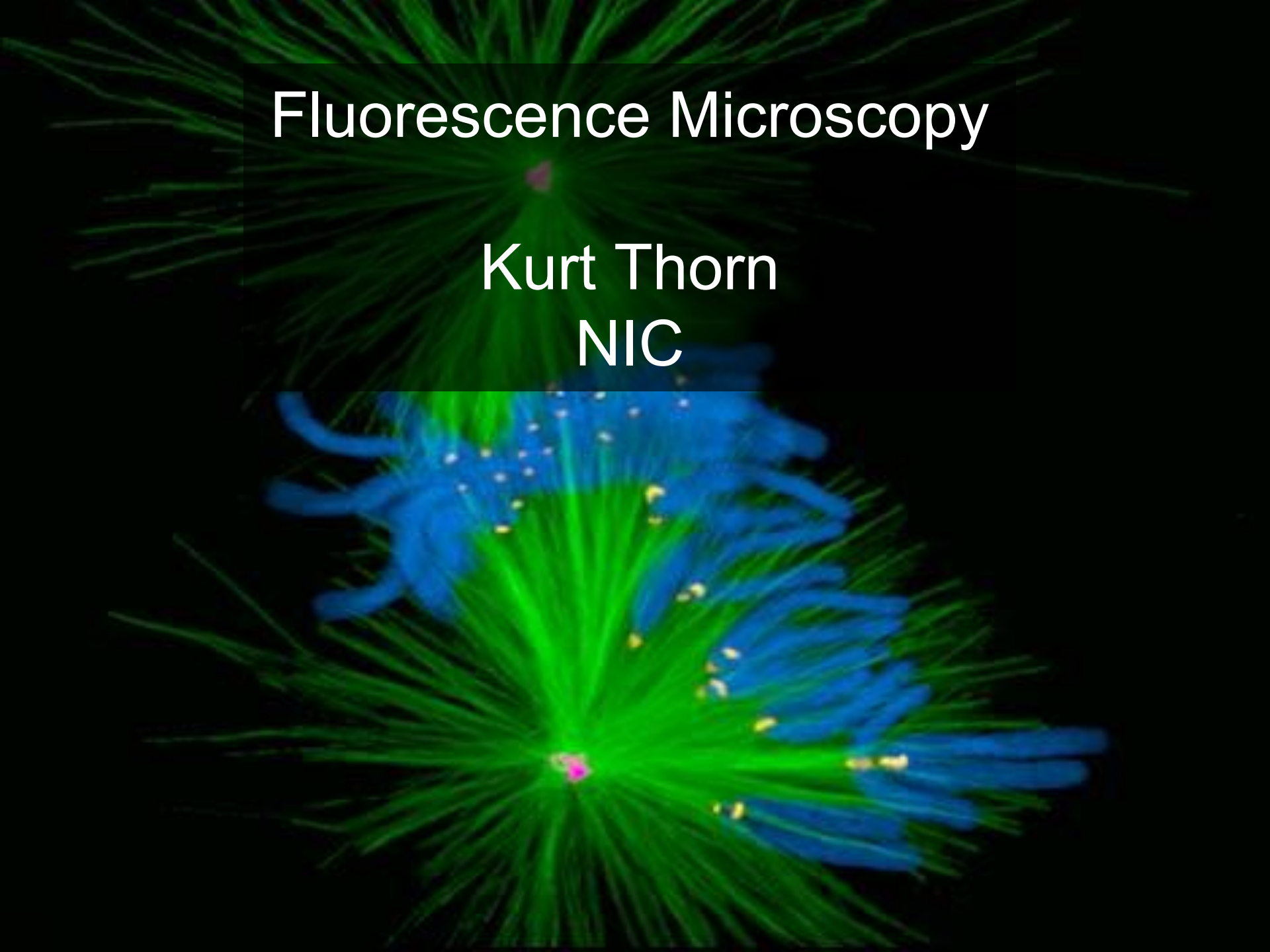
Fluorescence Microscopy

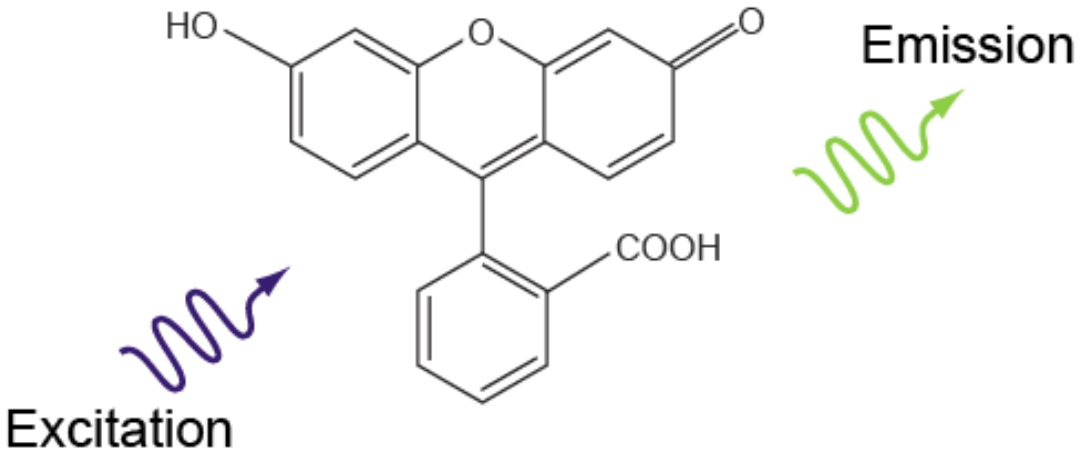
Kurt Thorn
NIC



Why fluorescence?

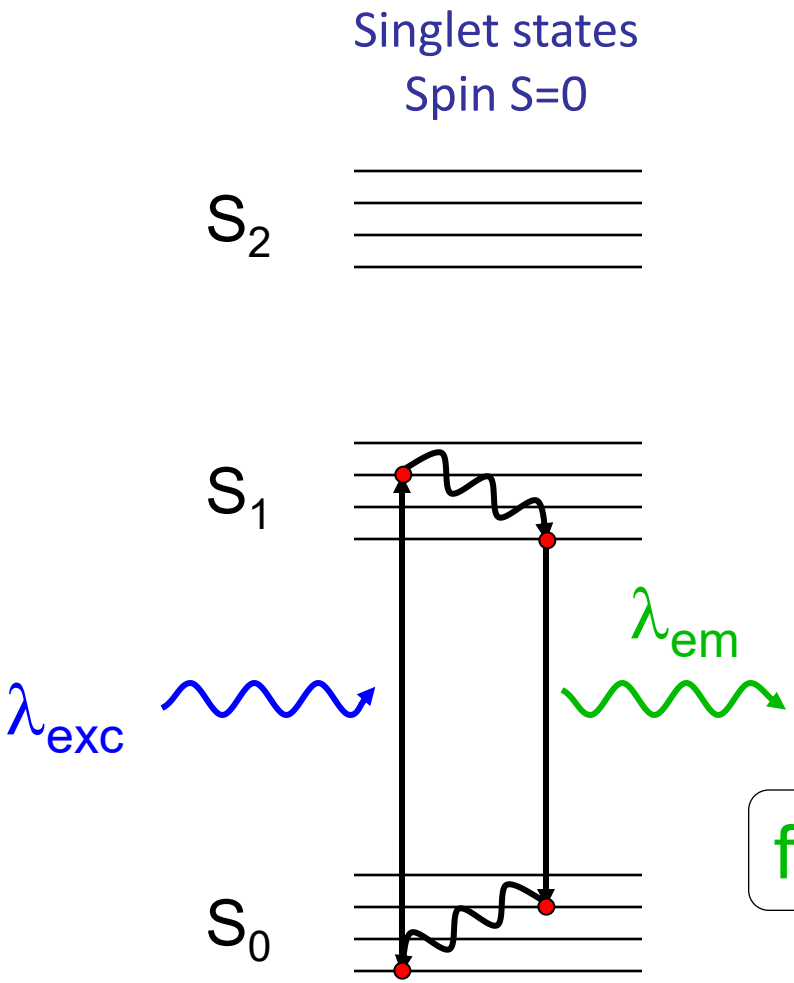
- High contrast
  - Signal against dark background
- Highly specific, multi-color labeling
  - GFP etc.
  - Antibodies
- Live imaging
  - GFP etc.
- Quantitative
- Sensors for [Ca], pH, …

What is fluorescence?



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

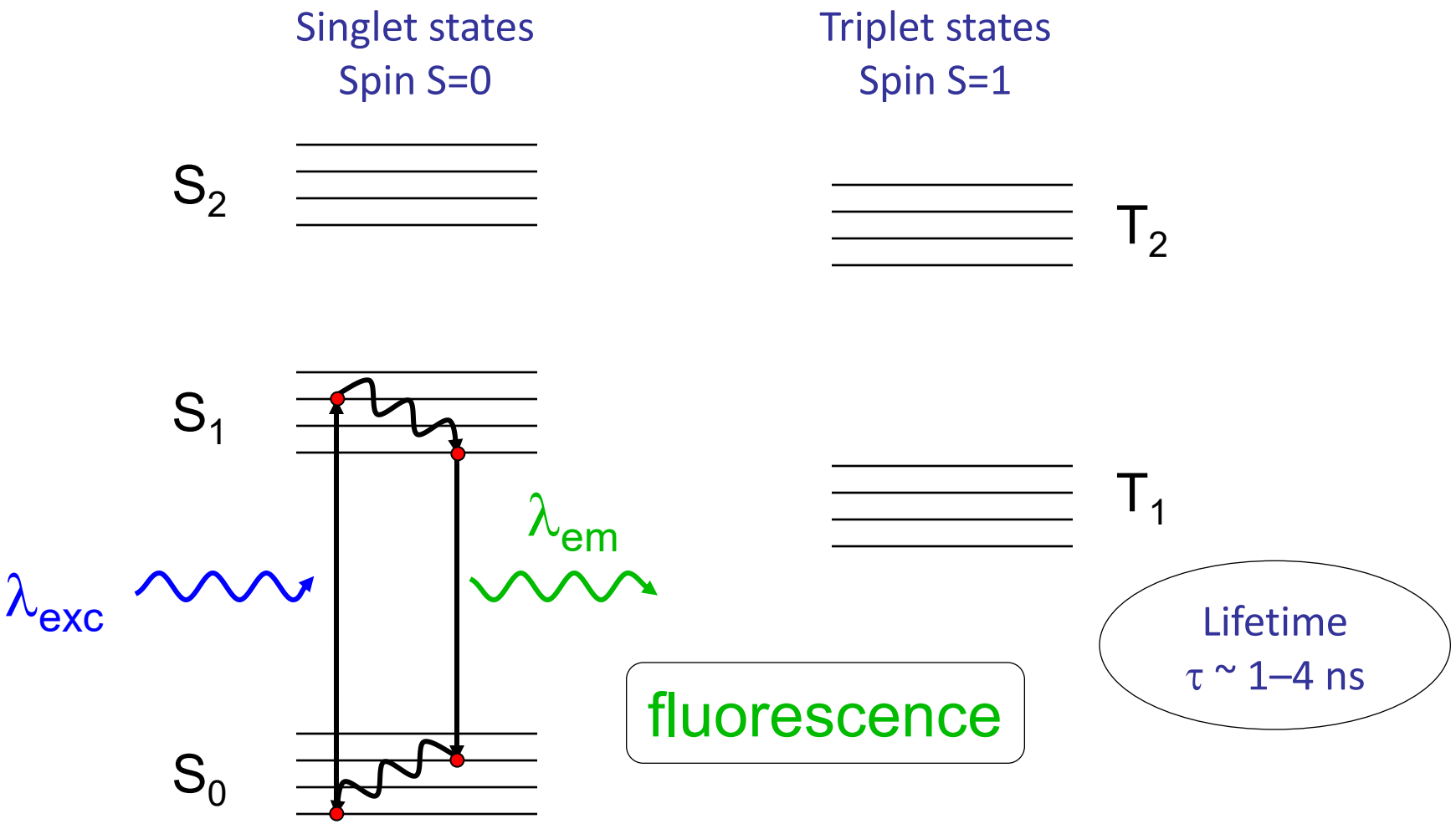
Jabłonski diagram
(Molecular energy diagram)



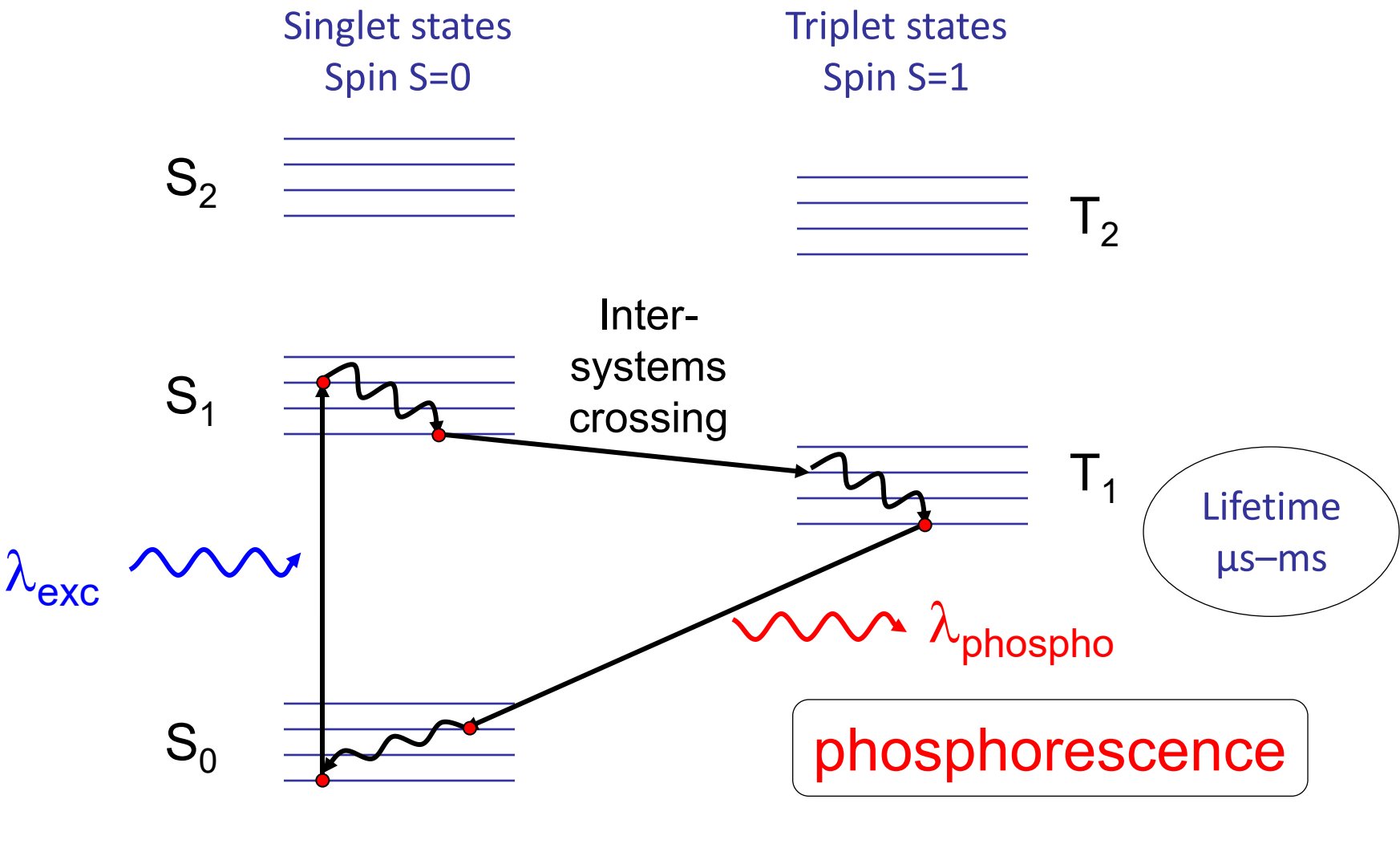
fluorescence

Lifetime
τ ~ 1–4 ns

Jabłonski diagram
(Molecular energy diagram)

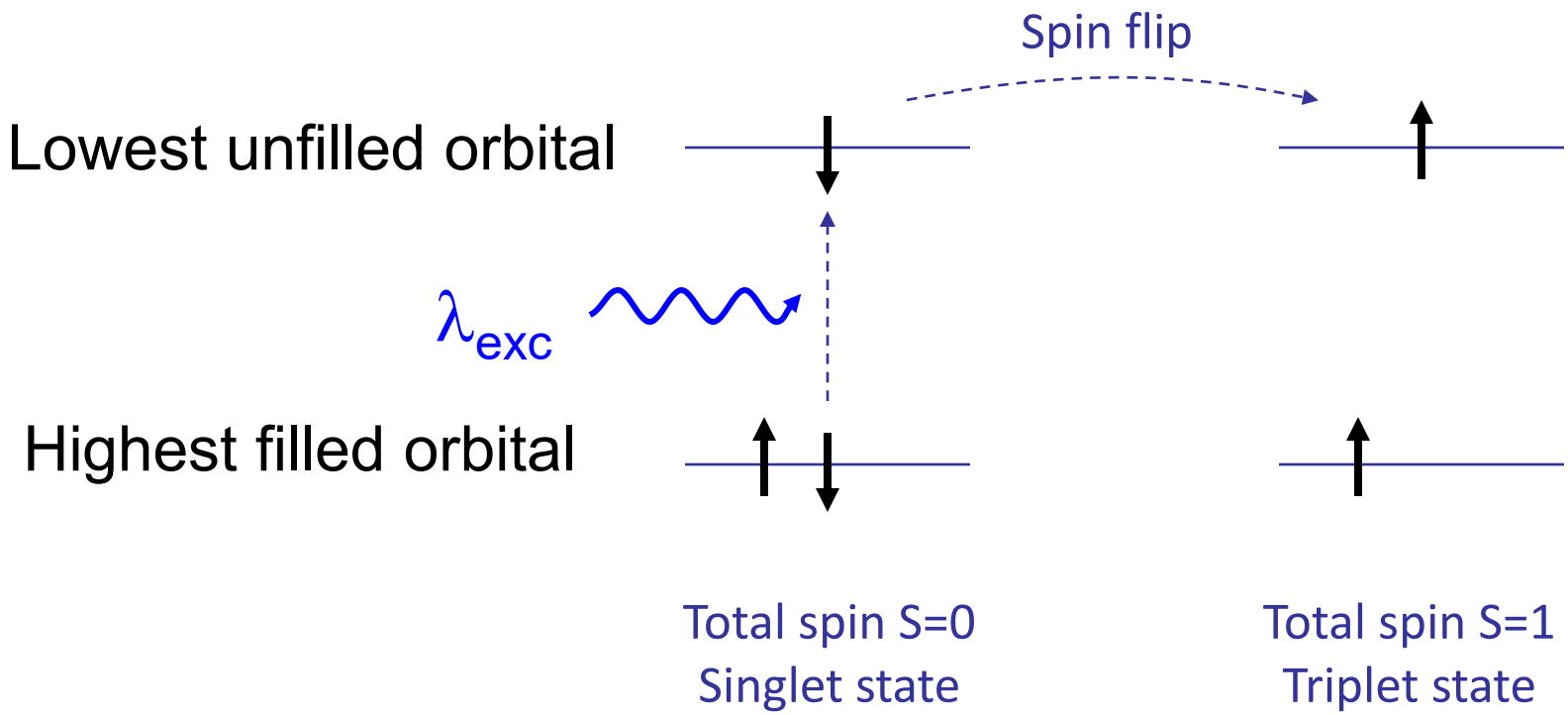


Jabłonski diagram
(Molecular energy diagram)



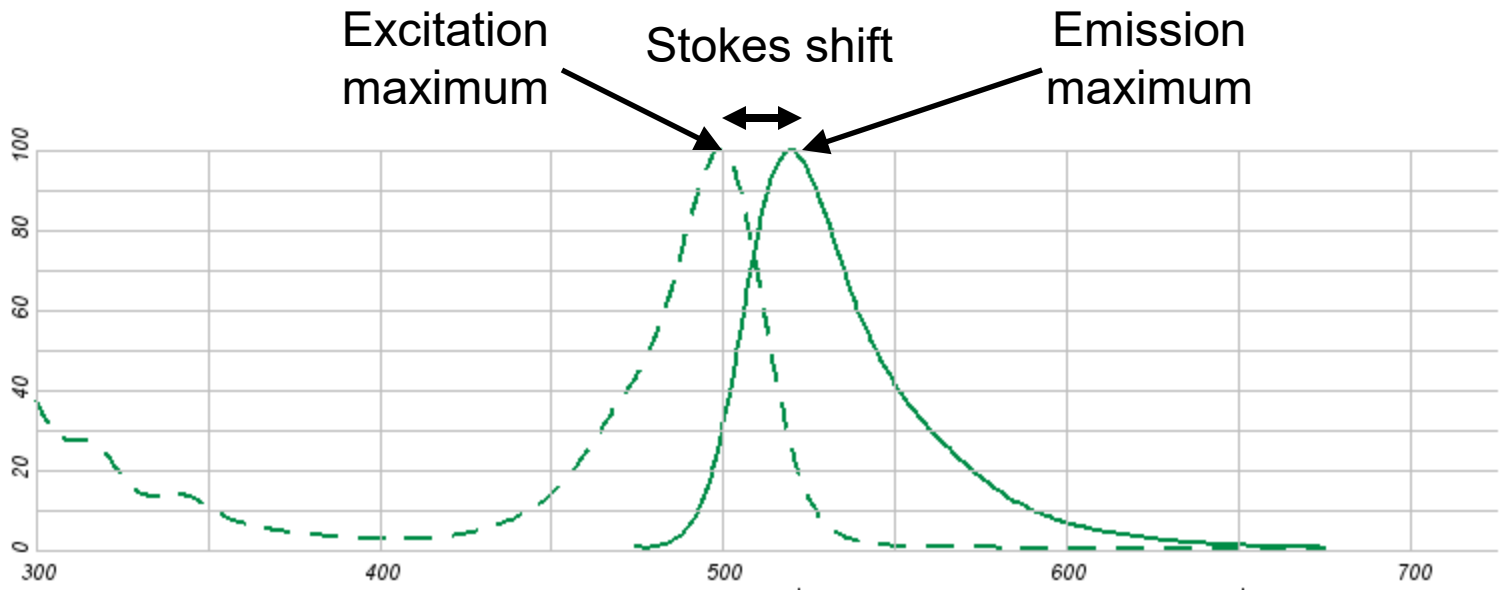
Singlet and Triplet States

Orbital states of *each electron*



Spin flips are “dipole forbidden” → unlikely → long triplet lifetime

Fluorescence Spectra



Alexa 488

The Epifluorescence Microscope

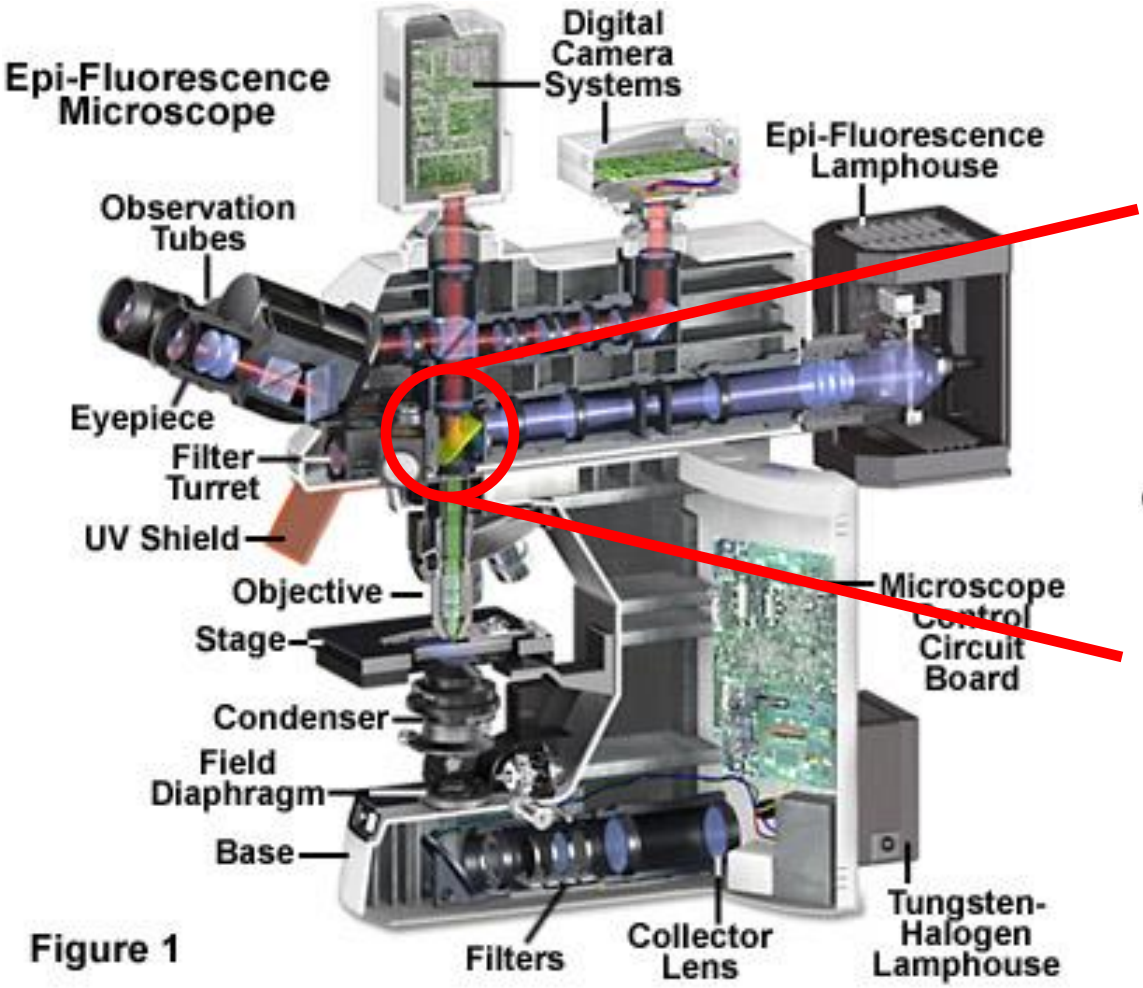


Figure 1

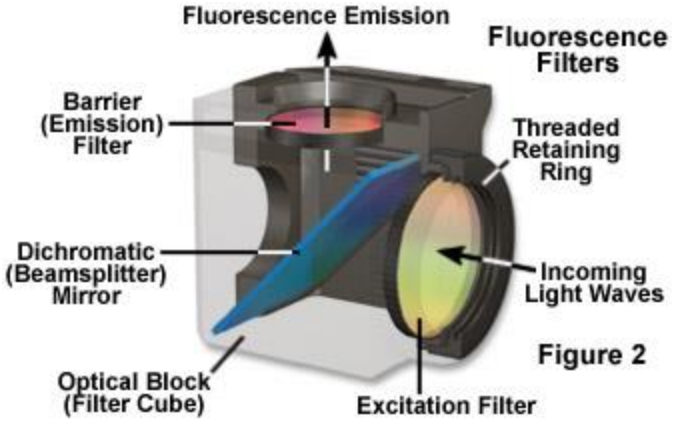


Figure 2

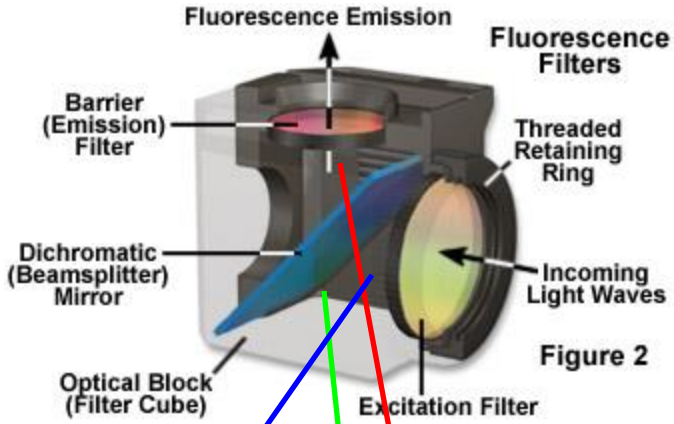
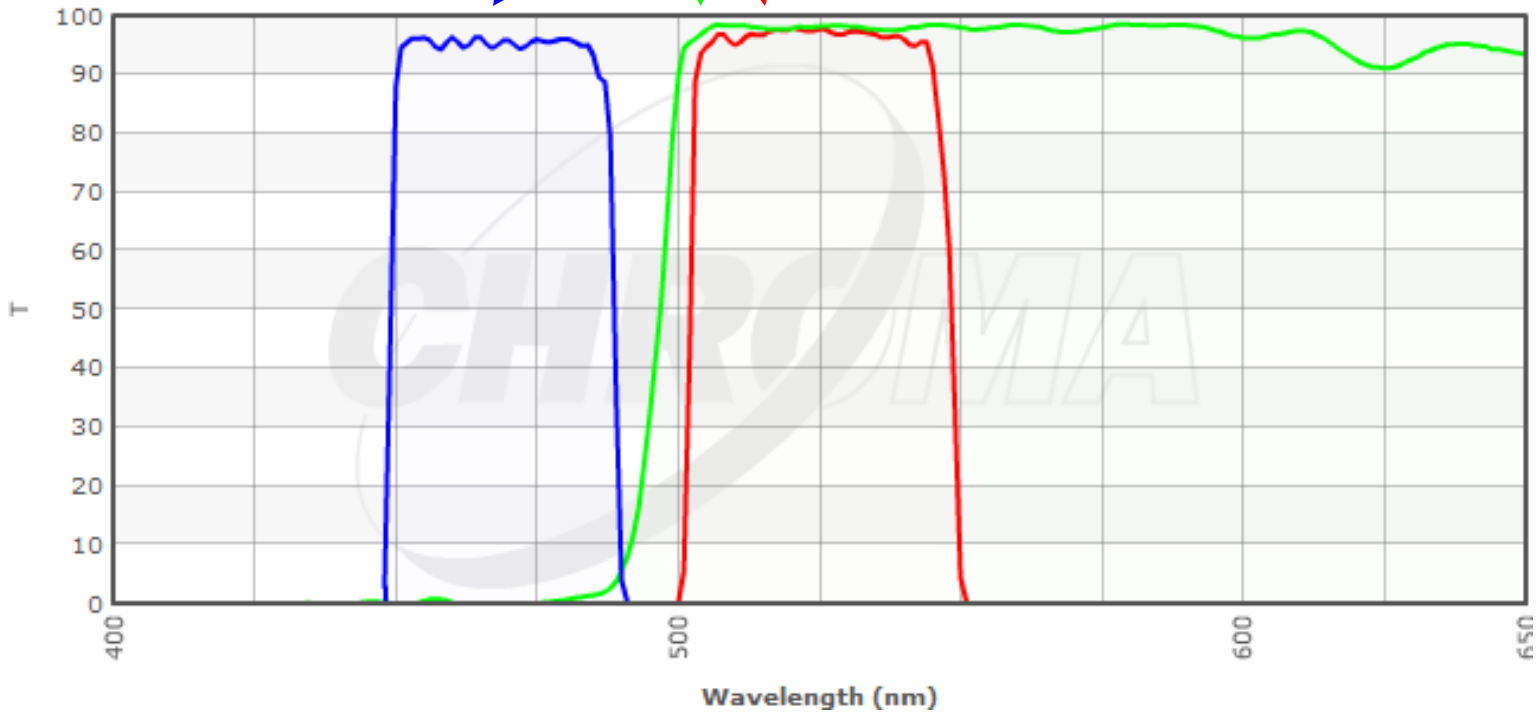
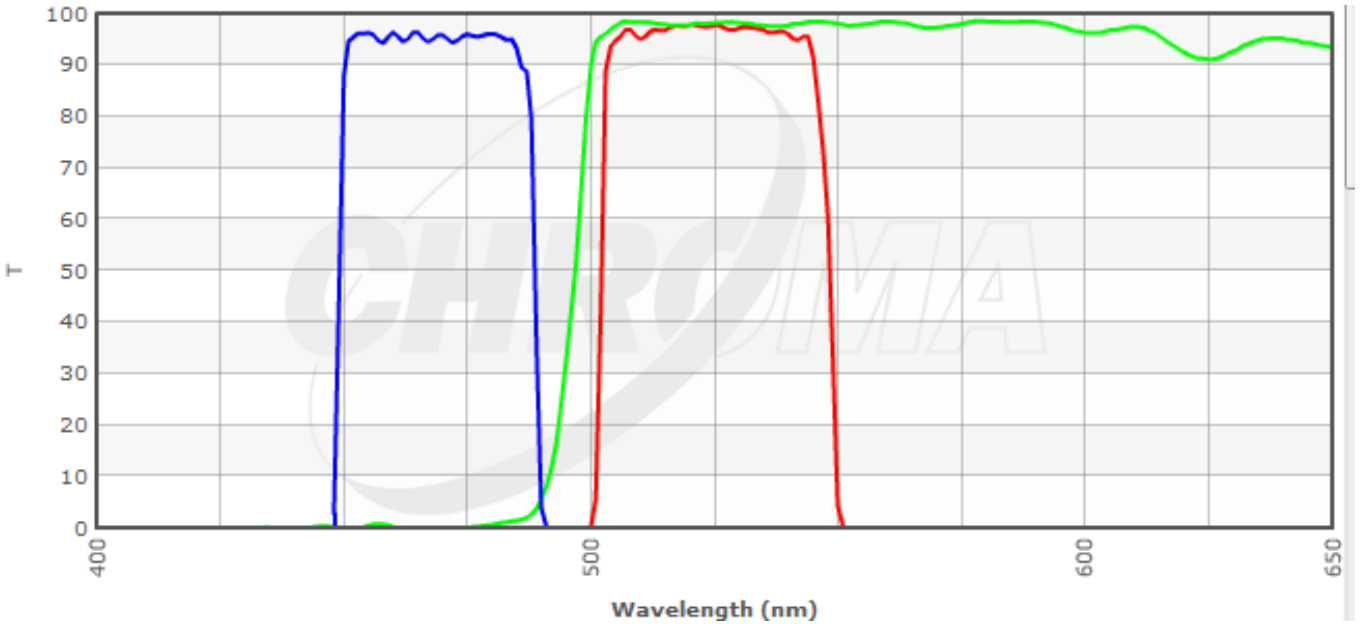

Fluorescence Emission
Fluorescence Filters
Barrier (Emission) Filter
Threaded Retaining Ring
Dichromatic (Beamsplitter) Mirror
Incoming Light Waves
Optical Block (Filter Cube)
Excitation Filter

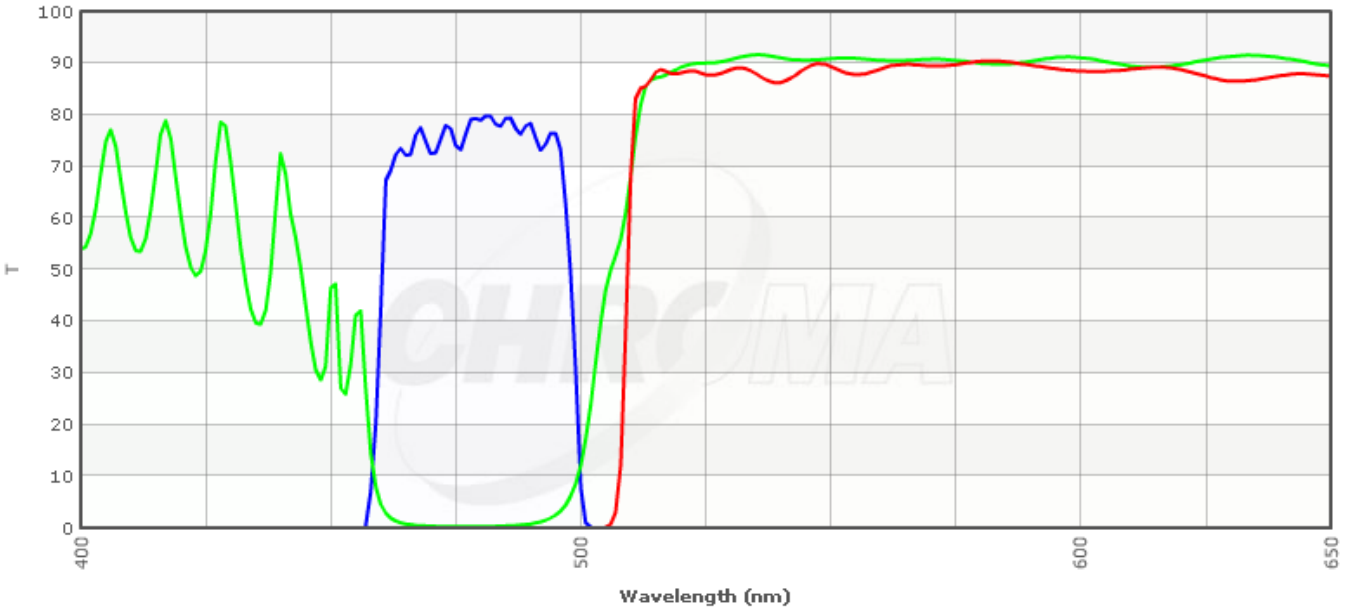
Figure 2


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

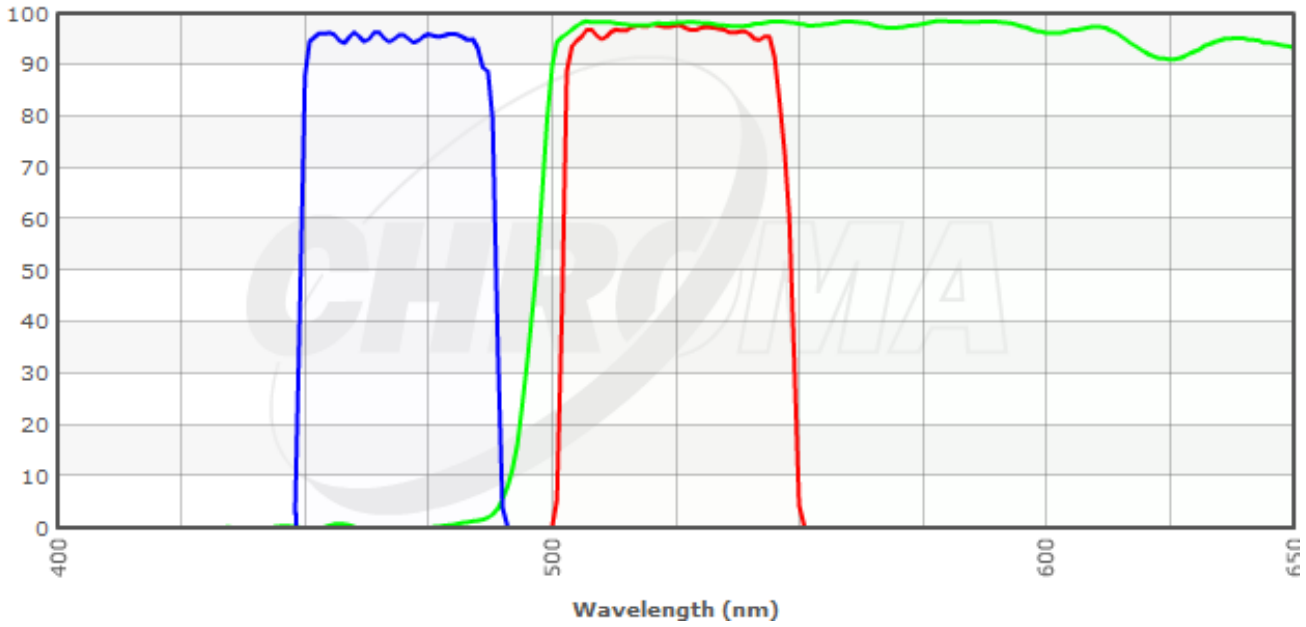
Bandpass



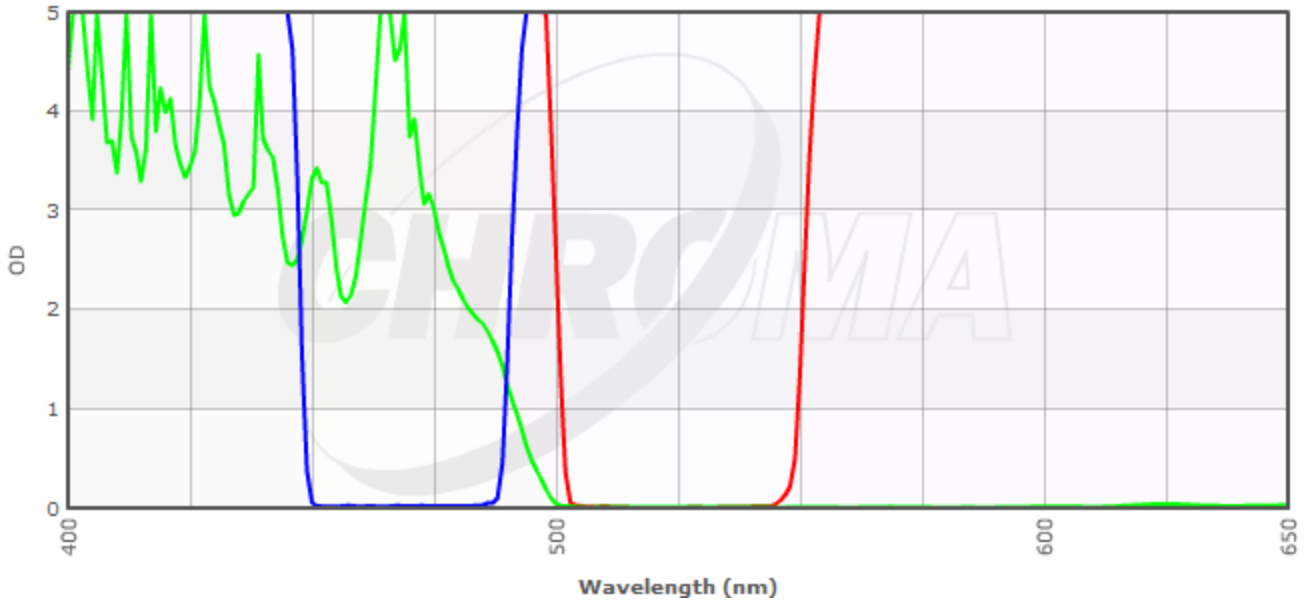
Longpass



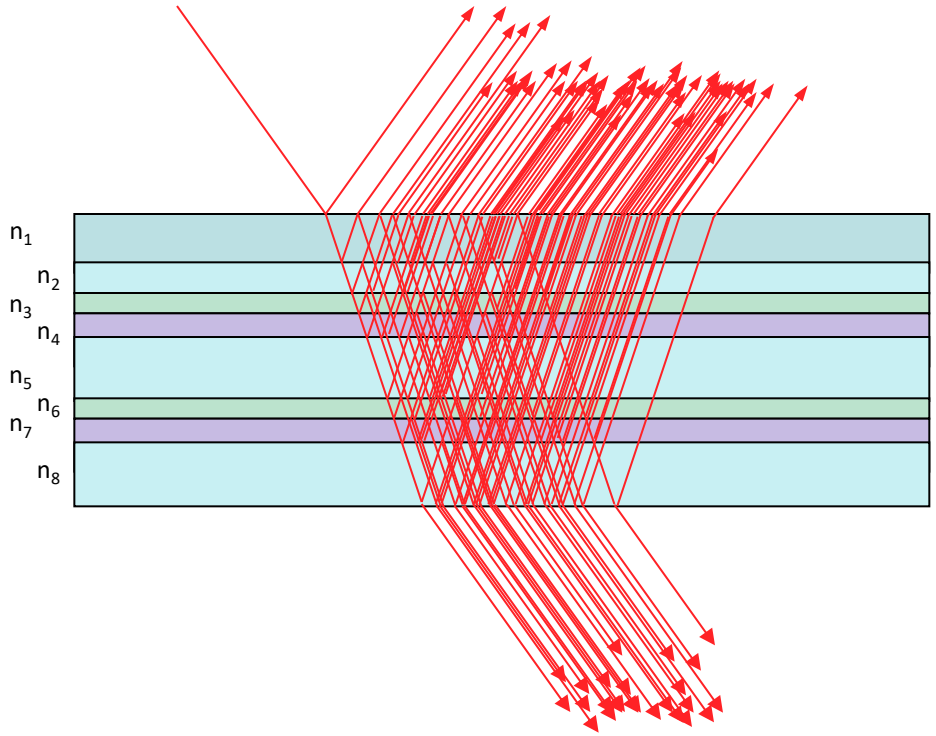
%T


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

OD
$= -\log_{10}(\%T)$


5
4
3
2
1
0
OD
400
500
600
650
Wavelength (nm)

Interference filters



Interference

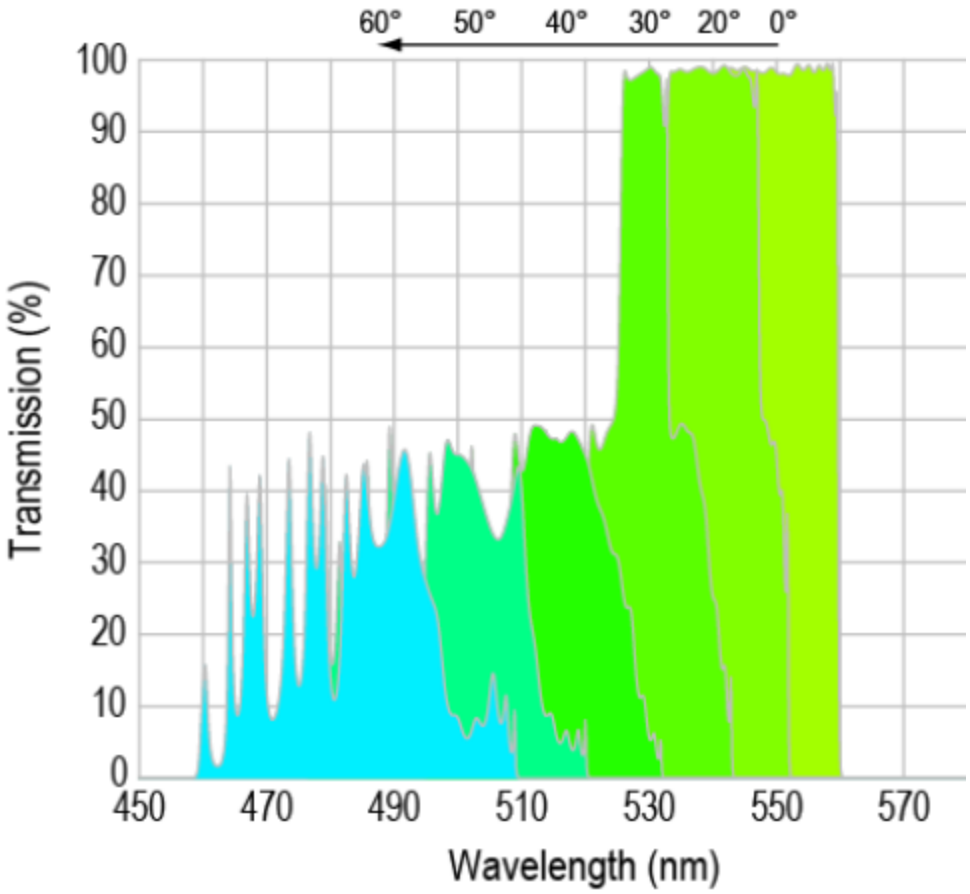
→ Wavelength-Dependent transmission & reflection

→ filter

Filter makers : Chroma
Semrock
Omega

Filter spectrum is angle dependent

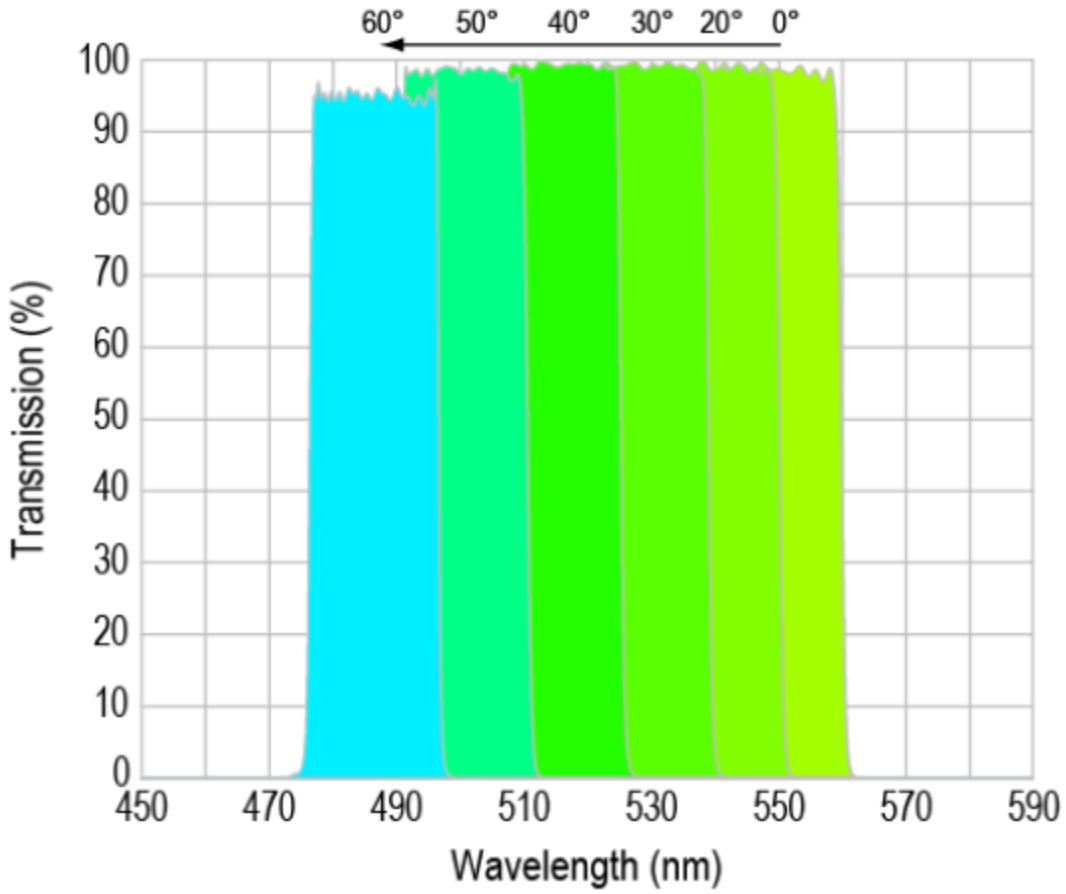
Generally bad



Semrock

Filter spectrum is angle dependent

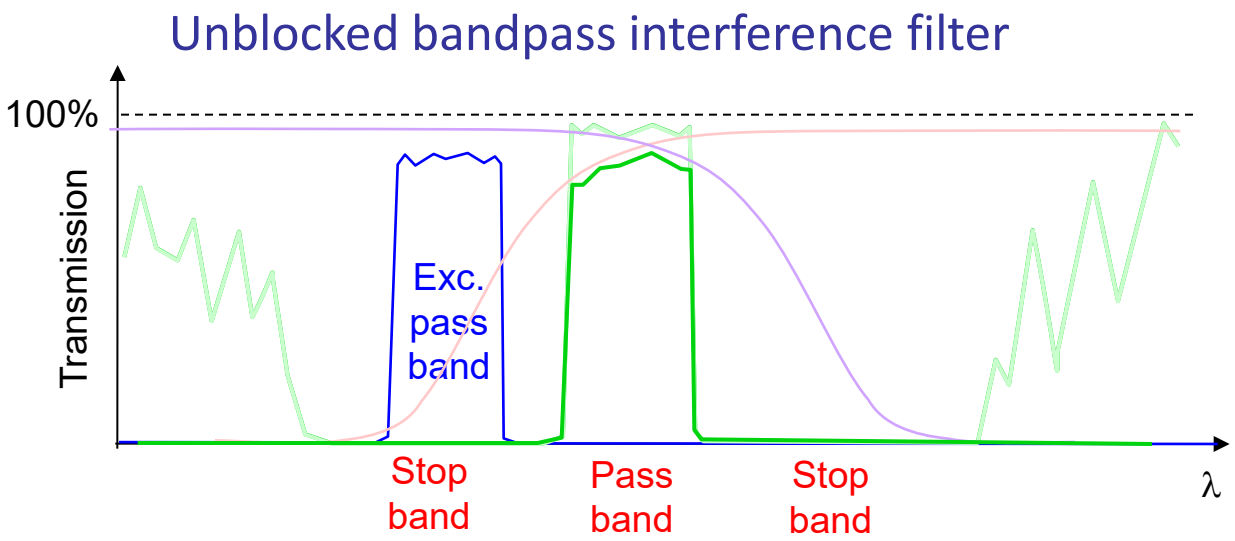
But can be good – Semrock VersaChrome

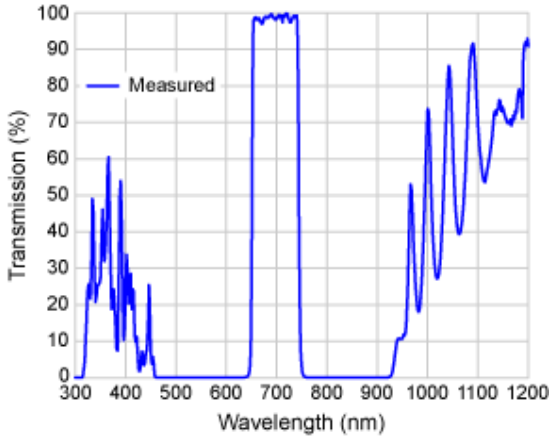


Semrock

Blocking

Interference filters have finite stop bands

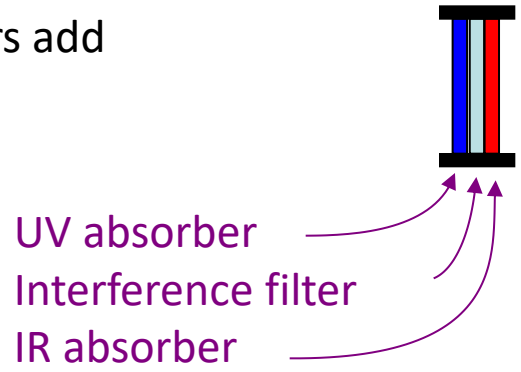




Semrock 697/75

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

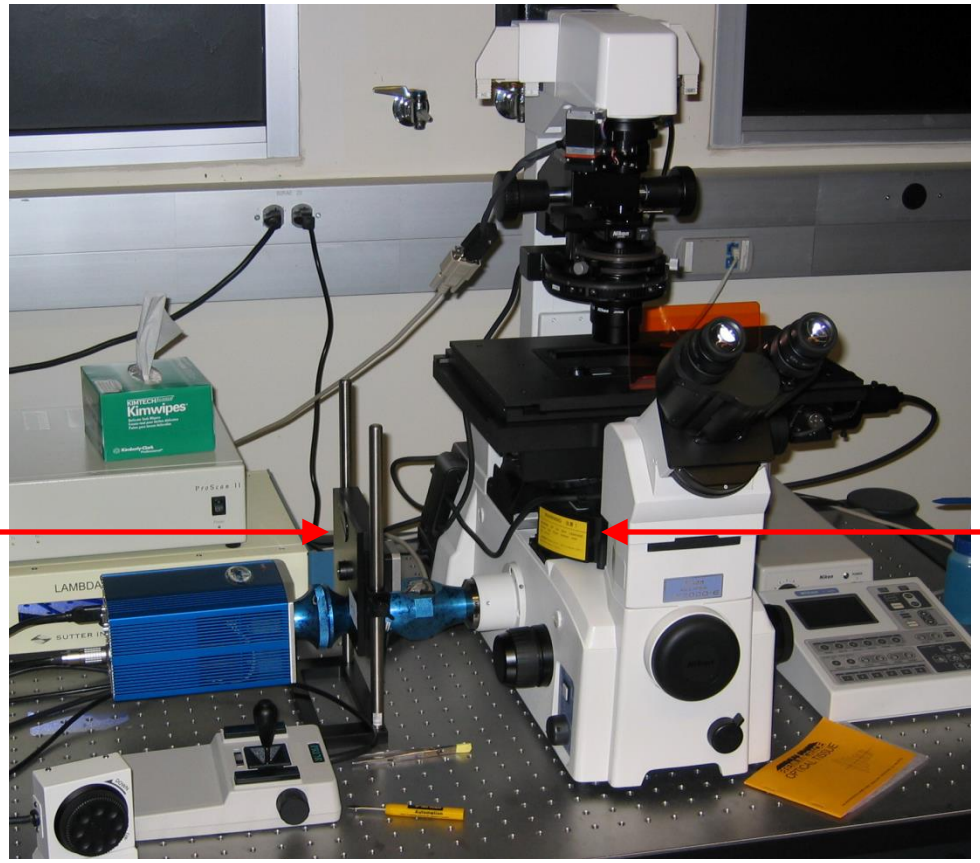


Filter cube nomenclature

- Chroma labels filters as center wavelength / passband (e.g. D350/50x)
- Dichroics are labeled by cut-on wavelength (e.g. 505DCLP)

- Nikon filters use a letter to specify illumination wavelength – e.g. UV, B, G, R
- Letters afterward specify emission profile – e.g. UV-2A vs UV-2E/C

Exploding the filter cube



Emission Filter

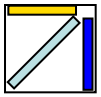
Dichroic Mirror

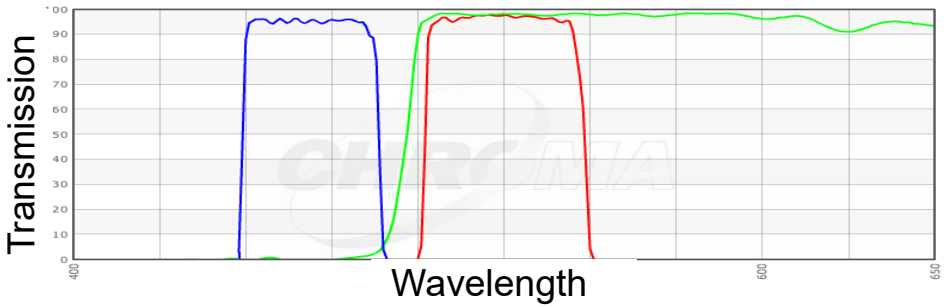
Excitation Filter (off slide)

Filter schemes

Single wavelength sets

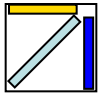
- Most efficient
- Best separation
- Very slow to change λ

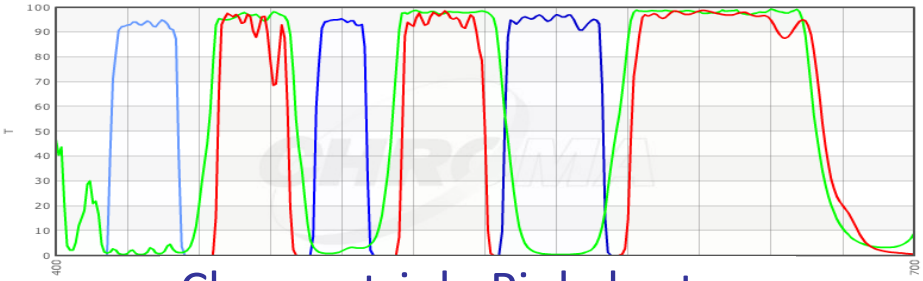




Multi-band filters

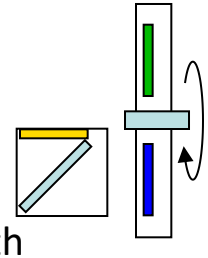
- Multi-band everything
  - See all colors at once
  - For color cameras
  - Bad crosstalk

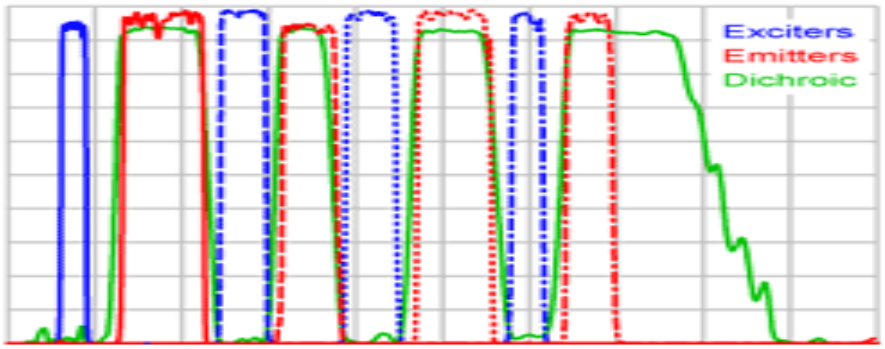




Chroma triple Pinkel set

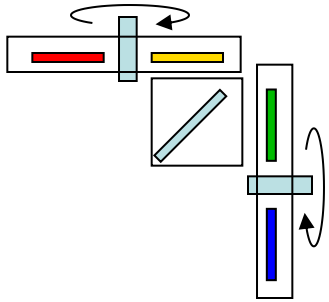
- "Pinkel" scheme
  Multi-band dichroic
  Multi-band emitter
  Single-λ exciters
  - Excitaton filter wheel
  - Separate image at each wavelength
  - Better separation





Semrock quad Sedat set

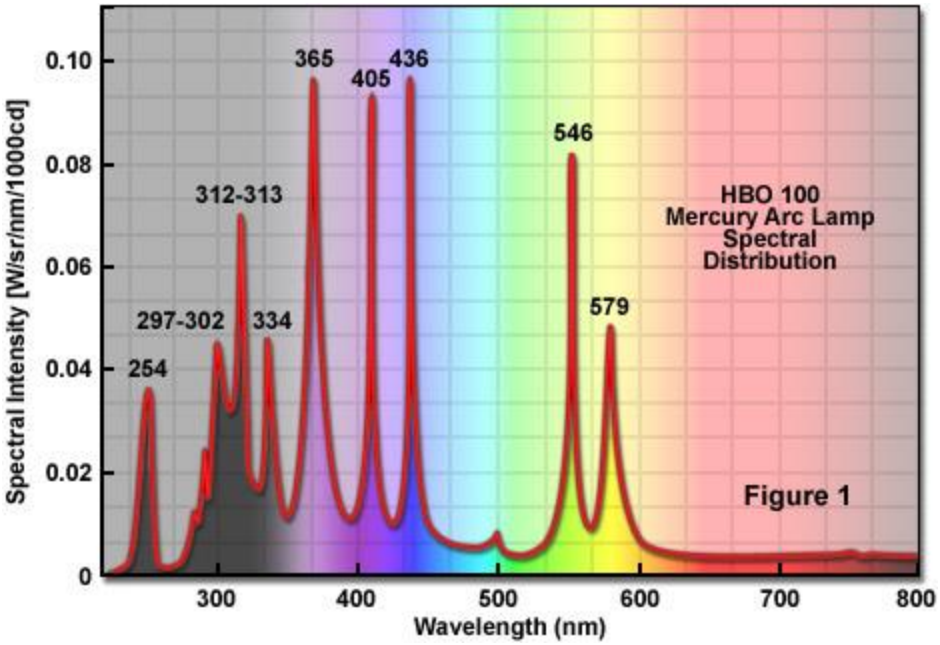
- "Sedat" scheme
  Multi-band dichroic
  single-band emitters
  Single-λ exciters
  - Two filter wheels
  - Even better separation

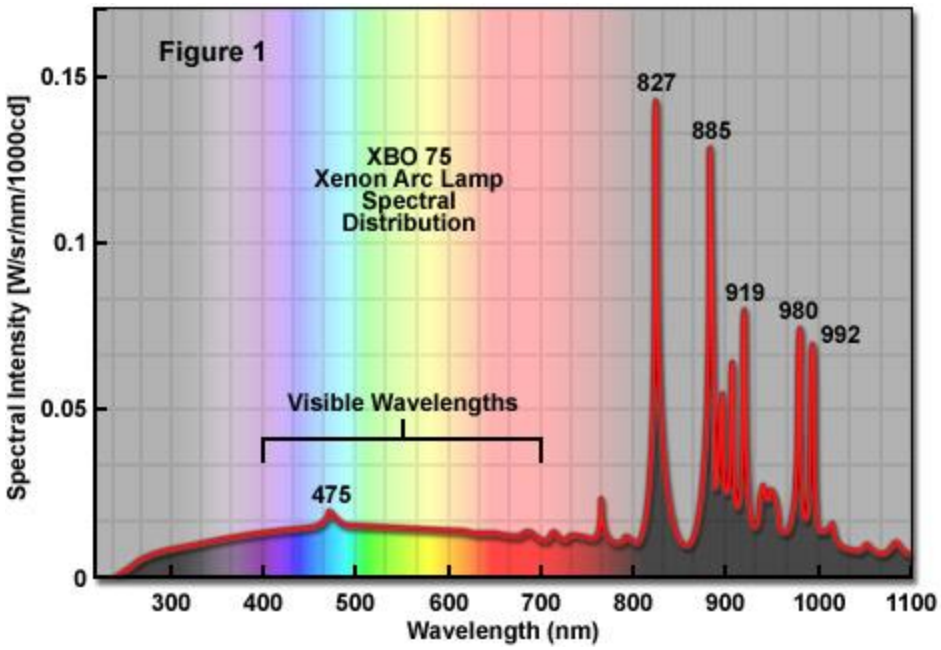


Light Sources

- Arc Lamps
  - Hg and Xe
  - Metal Halide
- LEDs
- Plasma
- Lasers
  - Generally only for collimated illumination (Confocal, TIRF)

Arc Lamp Spectra



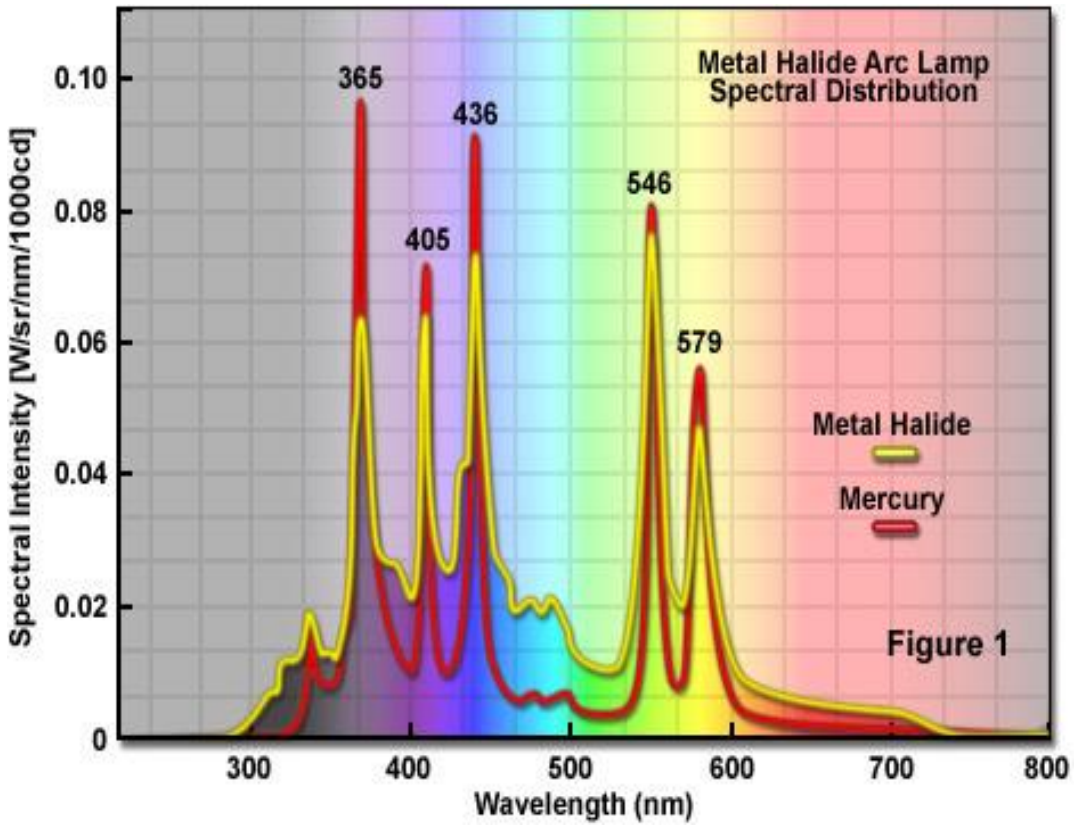


Hg: Brighter if your excitation spectrum matches one of the lines

Xe: More stable, longer lifetime, flat emission in visible is sometimes beneficial

Metal Halide Arc Lamp

Exfo, Intensilight, etc.



Metal Halide Arc Lamp

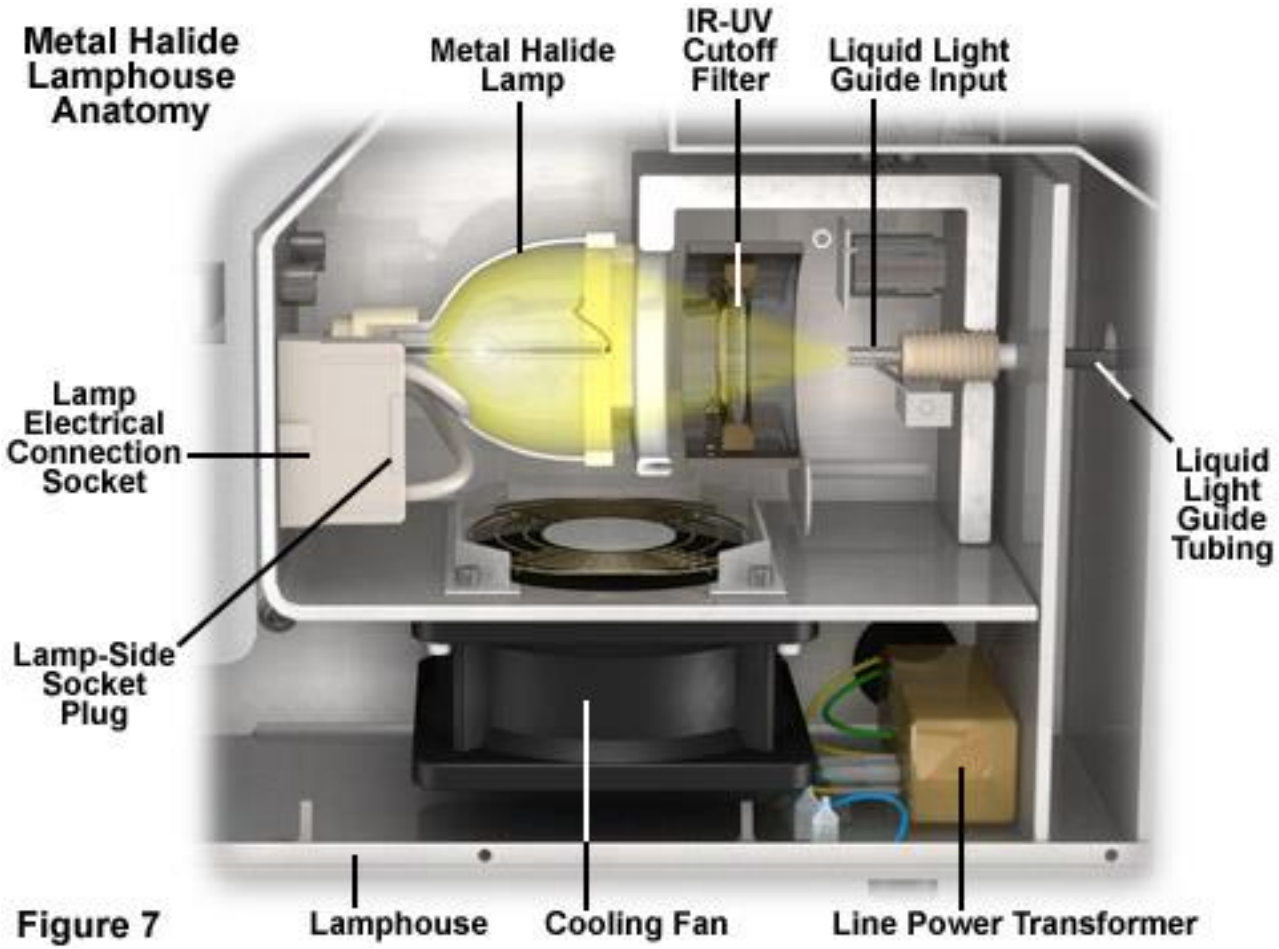
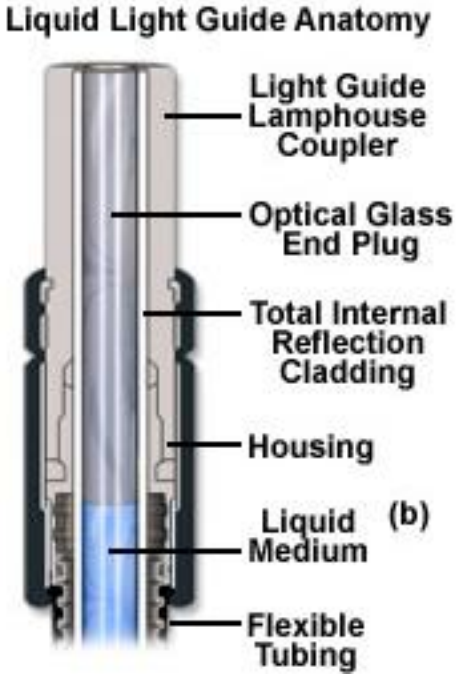
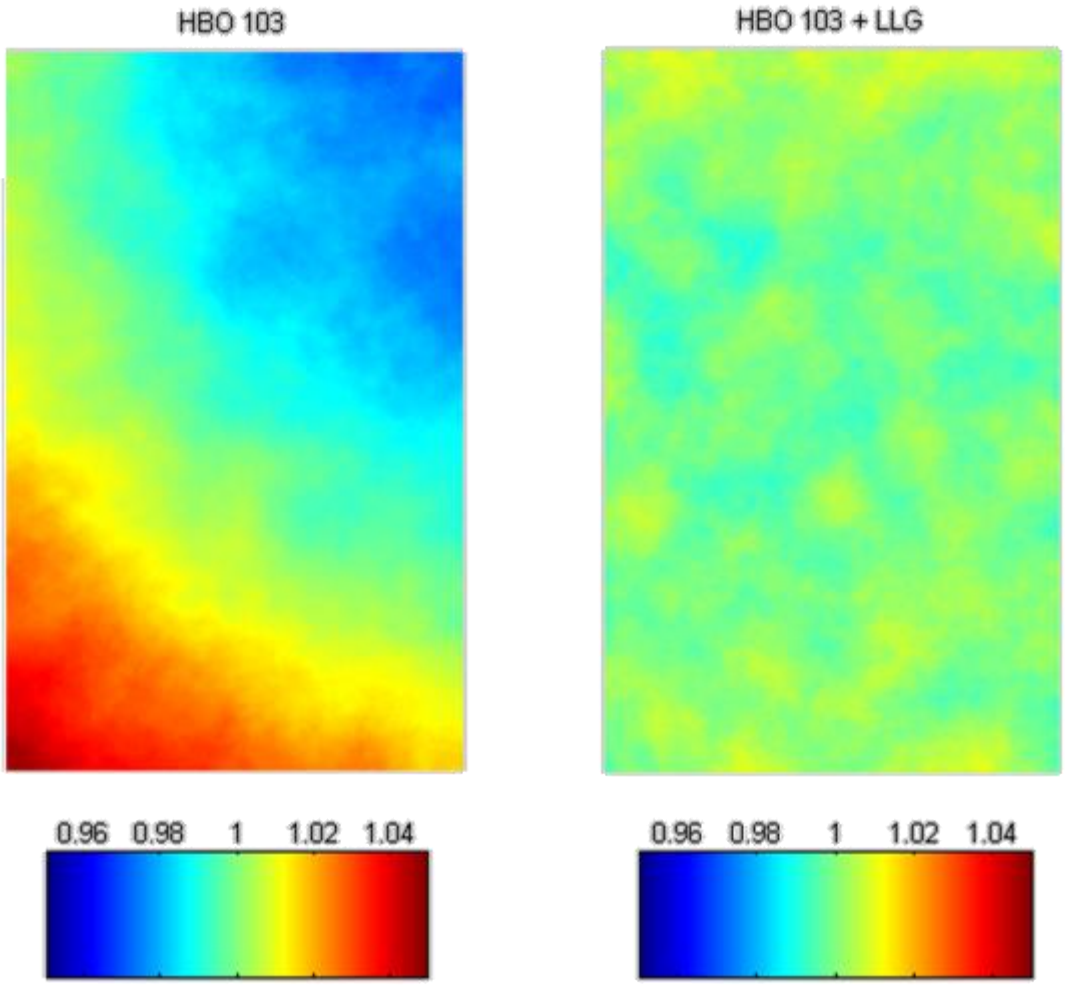


Figure 7



Benefit of Liquid Light Guide

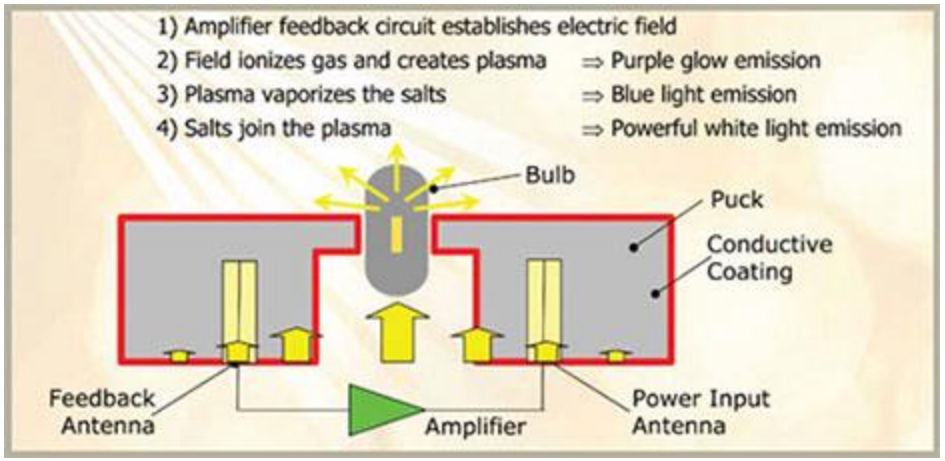
Improved Illumination Uniformity



Plasma Lamp

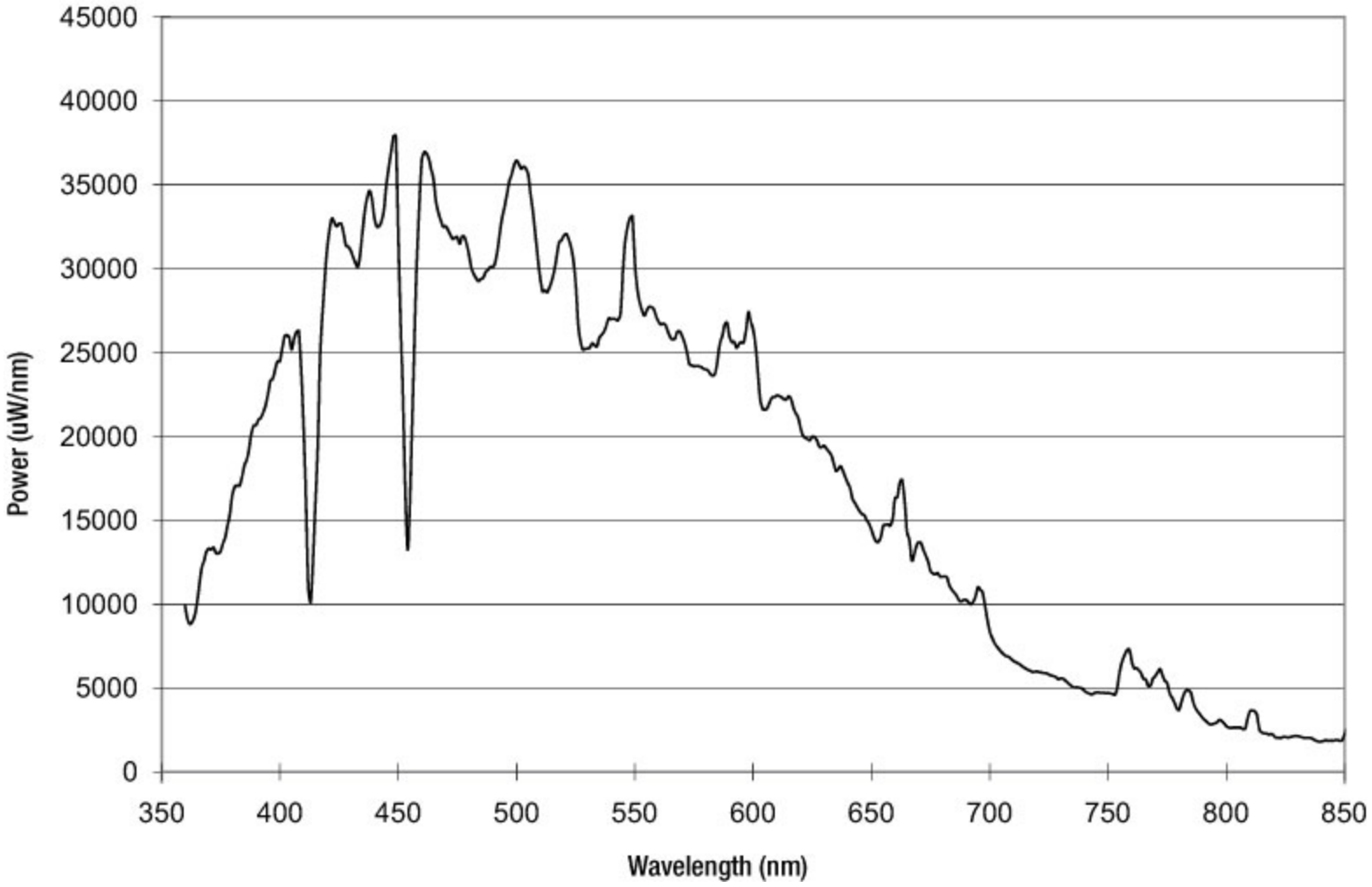
LIFI; Sutter XL

- Electrodeless ‘arc’ source
- Very bright
- 10,000 hr life

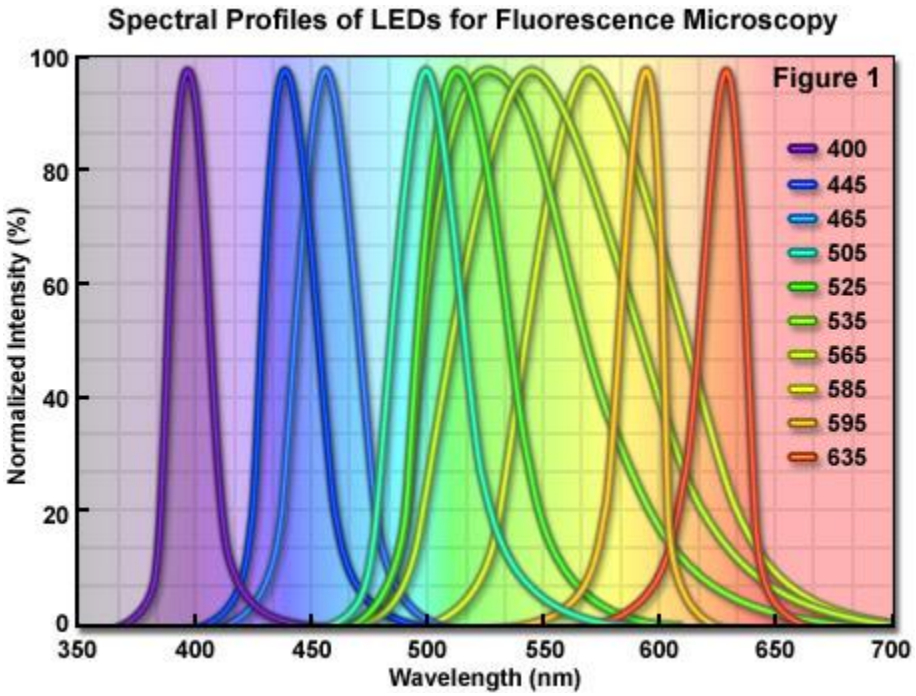


Plasma Lamp

LIFI; Sutter XL



LEDs



Good in the blue / red

Not so good in the green / yellow

Long lifetime

Fast switching

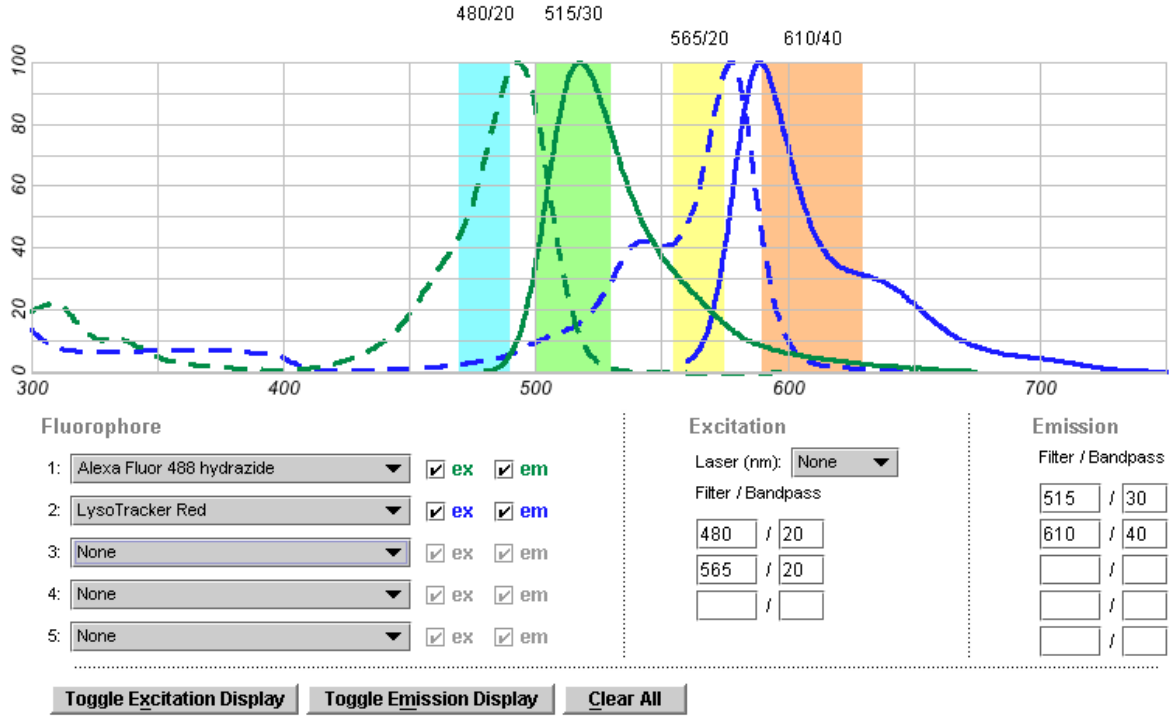
Relative lamp power

Filter Set	Excitation Filter (nm)	Dichromatic Mirror (nm)	Mercury HBO Power mW/Cm²	Xenon XBO Power mW/Cm²	Metal Halide Power mW/Cm²	LED Power mW/Cm²	Tungsten HAL Power mW/Cm²
DAPI (49)[1]	365/10	395 LP	23.0	5.6	14.5	0.70 (365)[3]	0.06[4]
CFP (47)[1]	436/25	455 LP	79.8	25.0	76.0	26.5 (445)[3]	1.0
GFP/FITC (38)[1]	470/40	495 LP	32.8	52.8	57.5	39.2 (465)[3]	2.8
YFP (S-2427A)[2]	500/24	520 LP	20.0	35.4	26.5	10.9 (505)[3]	2.7
TRITC (20)[1]	546/12	560 LP	43.1	12.2	33.5	2.7 (535)[3]	1.4
TRITC (S-A-OMF)[2]	543/22	562 LP	76.0	31.9	67.5	6.6 (535)[3]	3.6
Texas Red (4040B)[2]	562/40	595 LP	153.7	54.4	119.5	7.9 (585)[3]	6.9
mCherry (64HE)[1]	587/25	605 LP	80.9	29.7	54.5	7.2 (585)[3]	4.3
Cy5 (50)[1]	640/30	660 LP	9.1	22.1	13.5	14.9 (635)[3]	4.5

[1]Zeiss Filters [2]Semrock Filters [3]LED Peak Wavelength [4]Tungsten-Halogen Lamp Voltage = 12.2 V

Summary: Metal Halide best; LEDs still not quite bright enough.

Matching Filters and Fluorophores

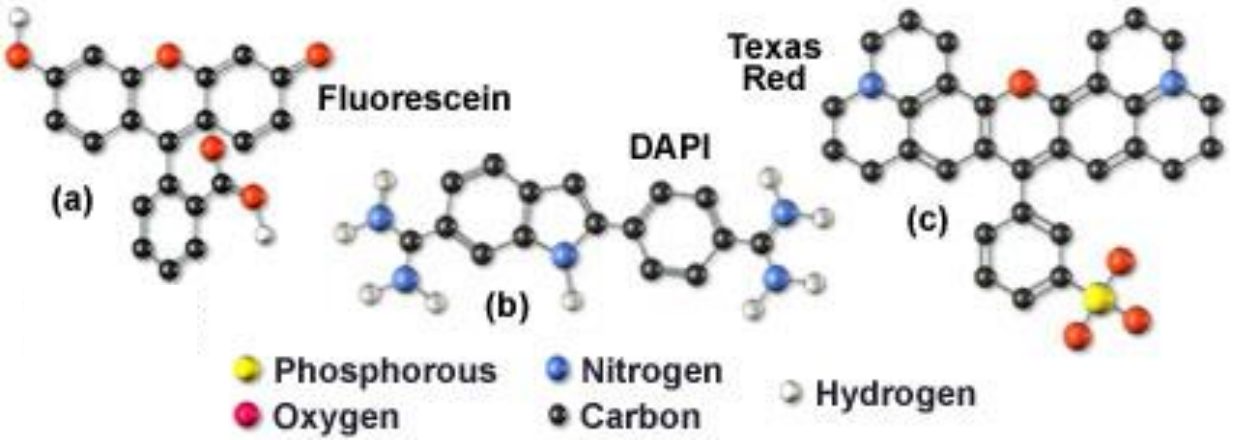


http://probes.invitrogen.com/resources/spectraviewer/

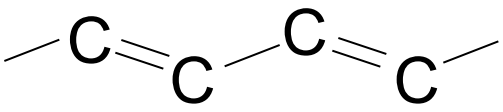
http://fluorescence.nexus-solutions.net/frames6.htm

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

Fluorescent molecules



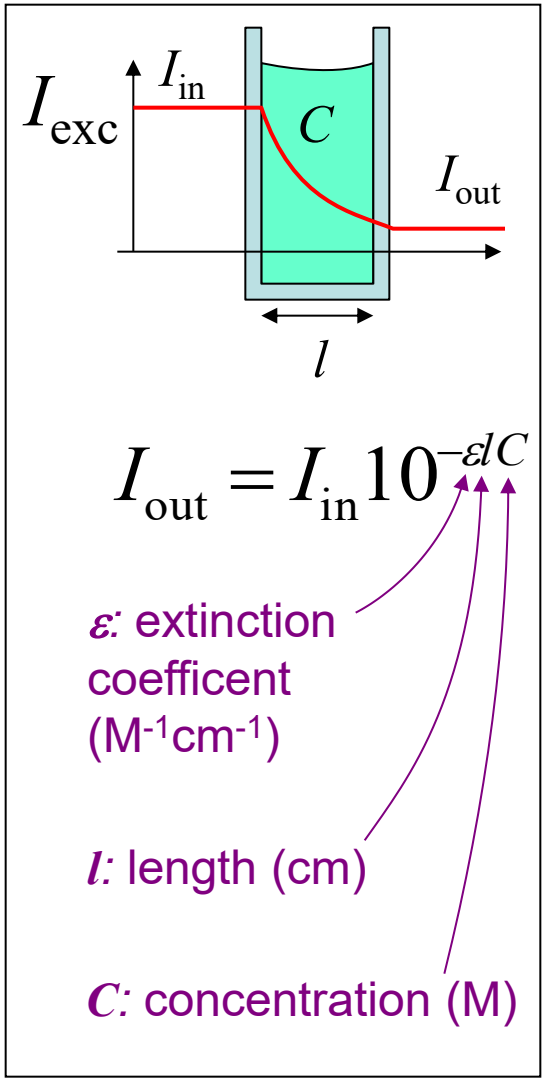
Systems of conjugated bonds
that share electrons



Larger system $\rightarrow$ longer wavelength

Parameters of fluorescent molecules

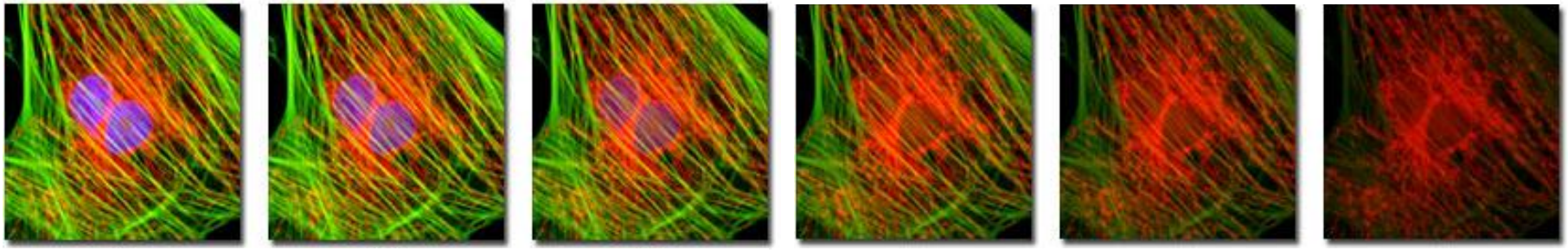
- Excitation & emission maxima
- Extinction coefficient ε
  $\propto$ *absorption cross section*
  $\varepsilon \approx 50{,}000$–$100{,}000$ $M^{-1}cm^{-1}$
- Fluorescence quantum yield Q_f
  = # *Photons emitted / # photons absorbed*
  $Q_f \approx 25$–90%
  Brightness $\propto \varepsilon Q_f$
- Photo-bleaching quantum yield Q_b
  = *average # of photons emitted per molecule before bleaching.*
  Depends on environment.
  $\propto Q_f / Q_b$



Parameters for some common fluorophores

Dye	λ_{ex}	λ_{em}	ε	QY	brightness
DAPI	350	470	27000	0.58	15.7
Fluorescein	490	520	67000	0.71	47.6
Alexa 488	494	517	73000	0.6	43.8
Rhodamine	554	573	85000	0.28	23.8
Cy3	554	568	130000	0.14	18.2
Cy5	652	672	200000	0.18	36
GFP	488	507	56000	0.6	33.6
mCherry	587	610	72000	0.22	15.8
CFP	433	475	32500	0.4	13
YFP	516	529	77000	0.76	58.5

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

Photostability varies between dyes

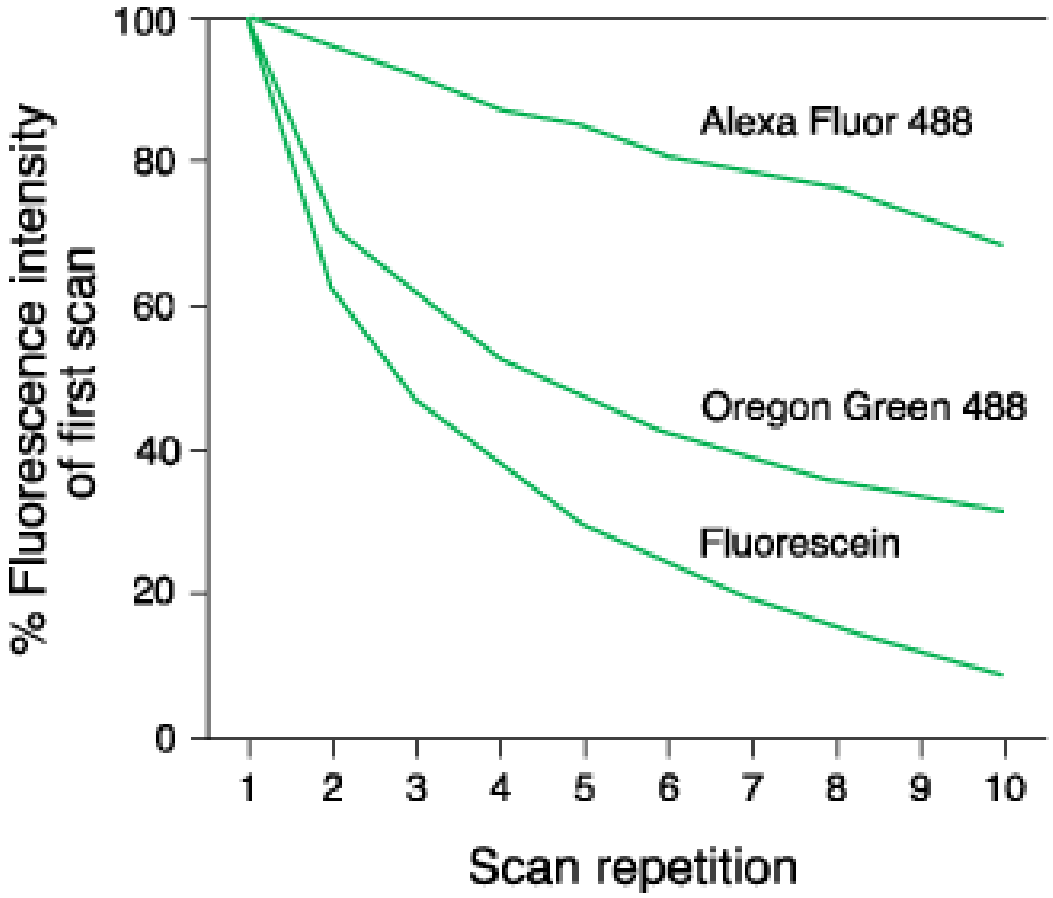
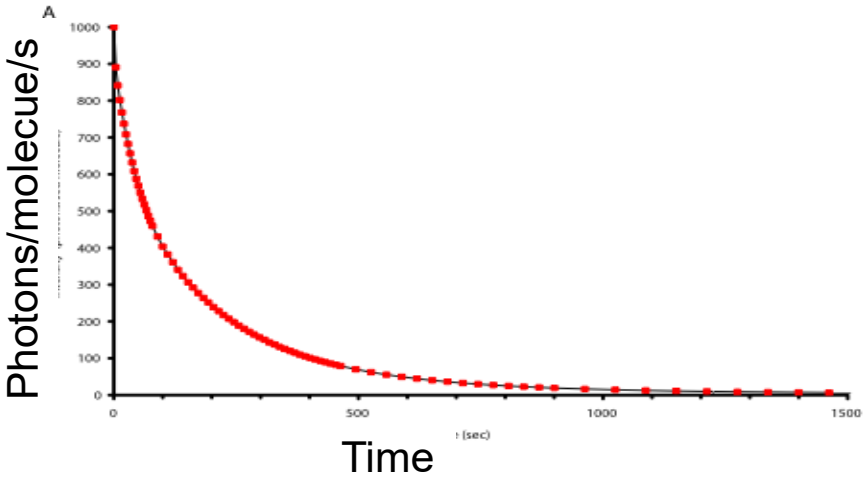
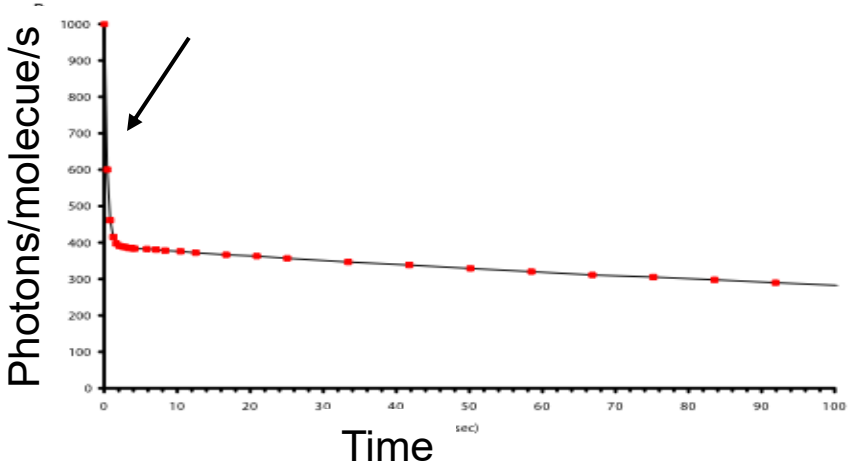


Photo-bleaching of fluorescent proteins



mCherry

Single-exponential bleaching



Emerald

Double-exponential bleaching

Fast- and slow-bleaching populations?

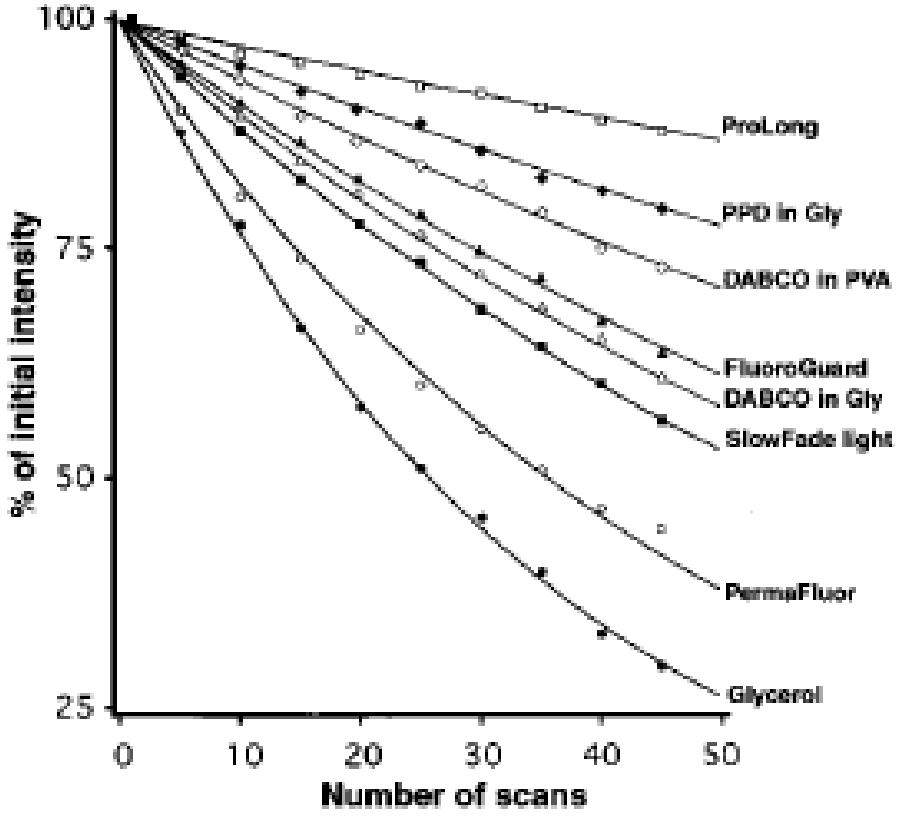
What to do about photo-bleaching?

- 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.

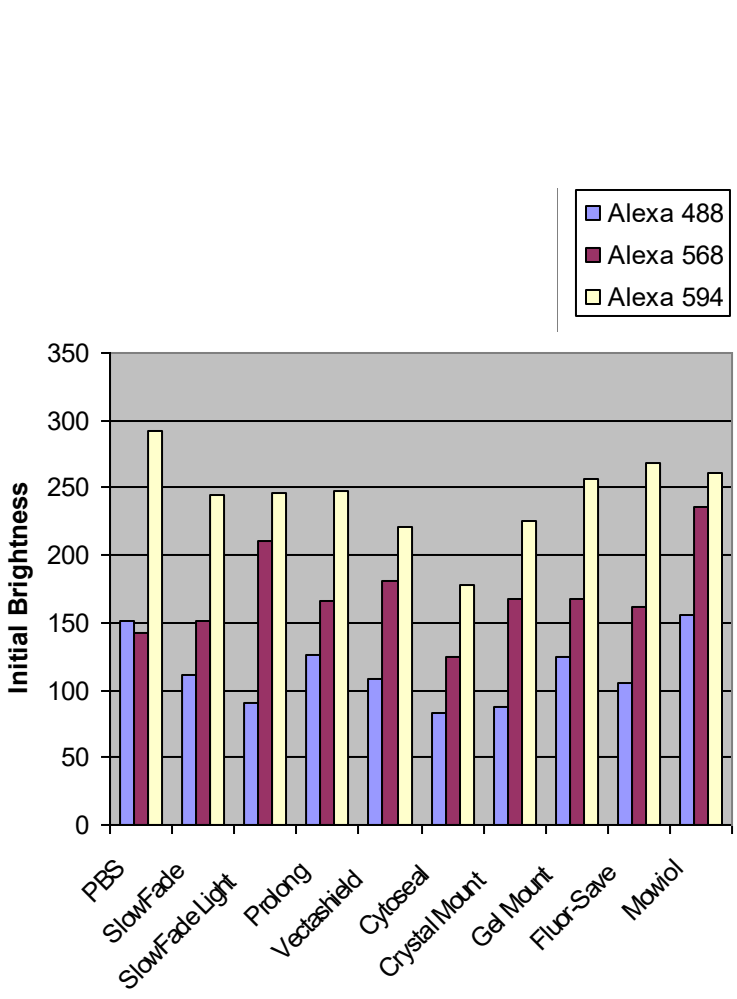
- Budget the photons you have
  - Only expose when observing
  - Minimize exposure time & excitation power
  - Use efficient filter combinations
  - Use highly QE, low noise camera
  - Use simple light path

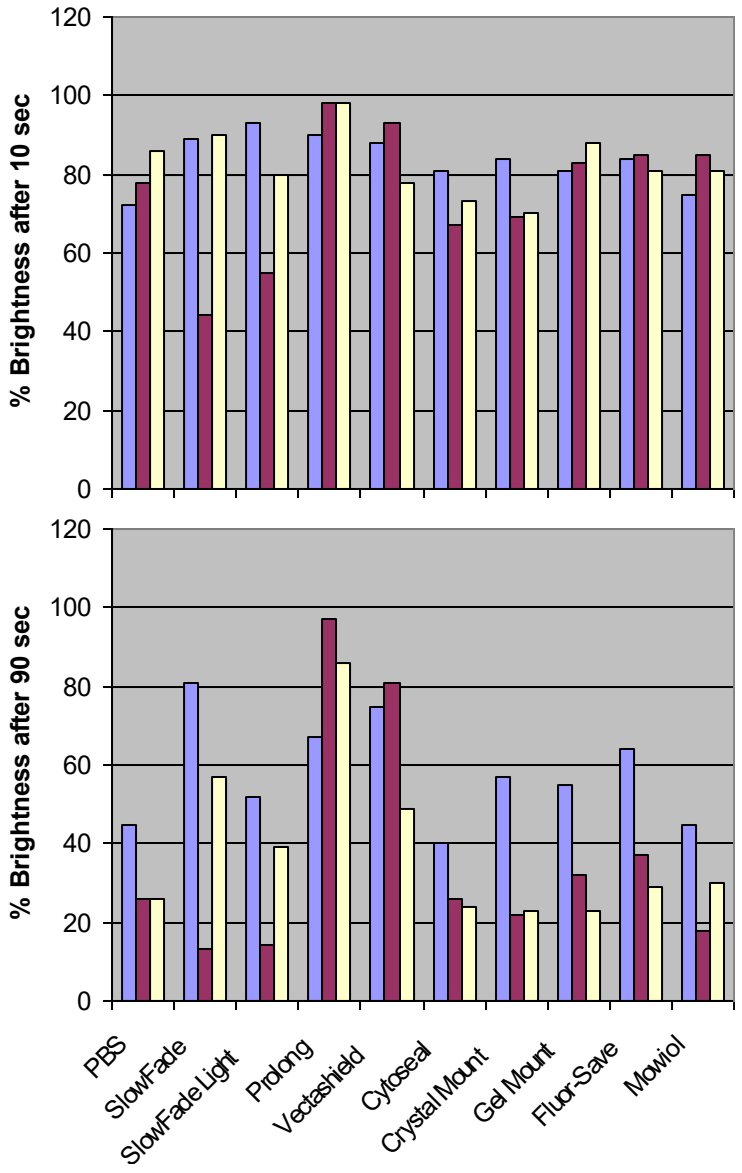
Effect of mounting medium on FITC bleaching



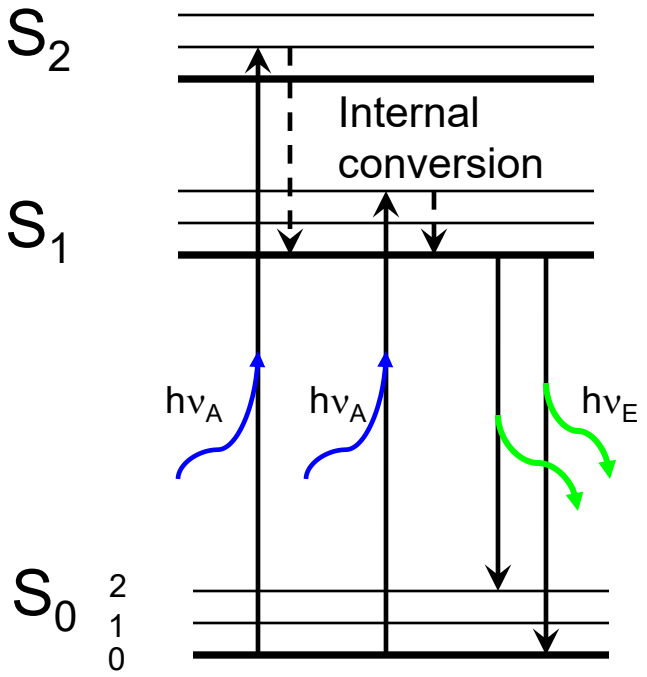
Ono et al. 2001, *J. Histochem Cytochem.* **49**: 305-311

Effect of mounting media on Alexa bleaching





Fluorophore saturation



Fluorescence lifetime is ~1-5ns

Once illumination intensity is high enough to excite the fluorophore as soon as it deexcites, further intensity increases will not increase brightness

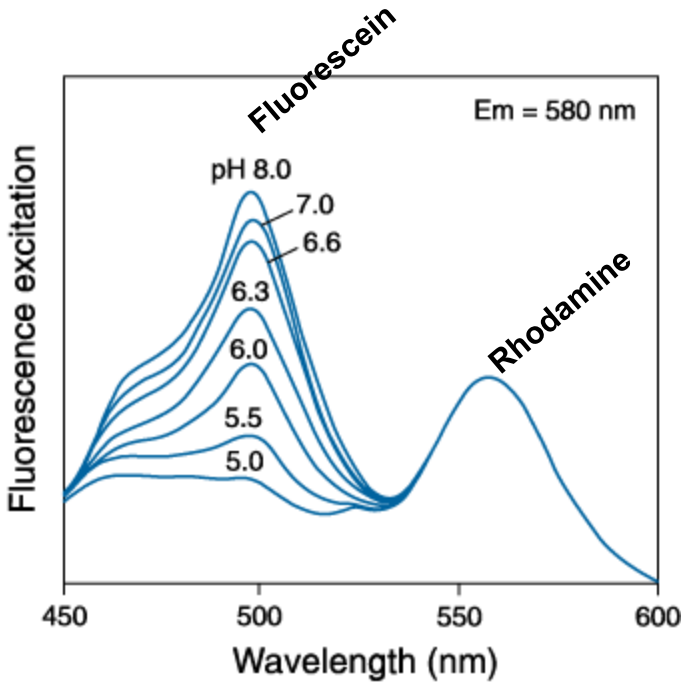
Usually only a problem for confocal

Factors affecting overall brightness

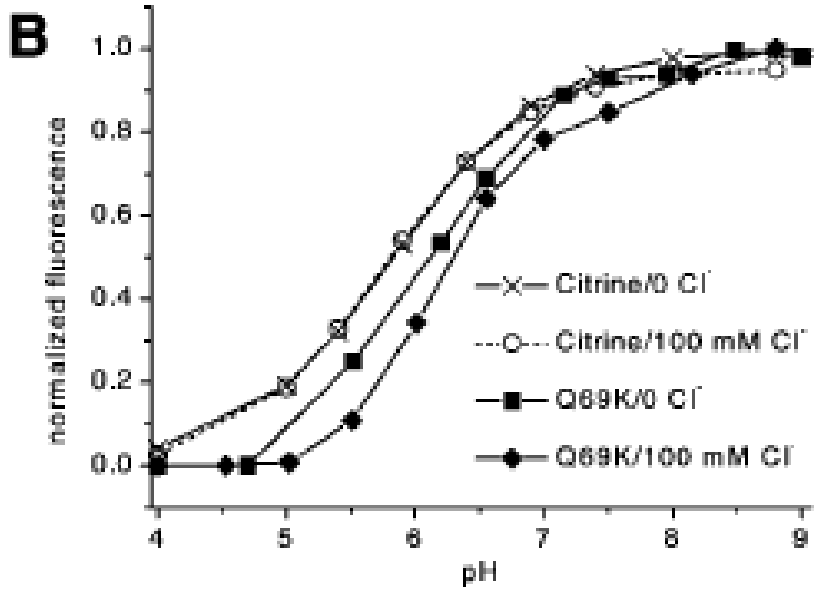
- Intrinisic brightness
- Spectrum of arc lamp/lasers
- Lamp/laser power
- Filter set transmission
- Quantum efficiency of detector
- Photobleaching
- Quenching / maturation / other dye-specific effects

pH dependence of dyes

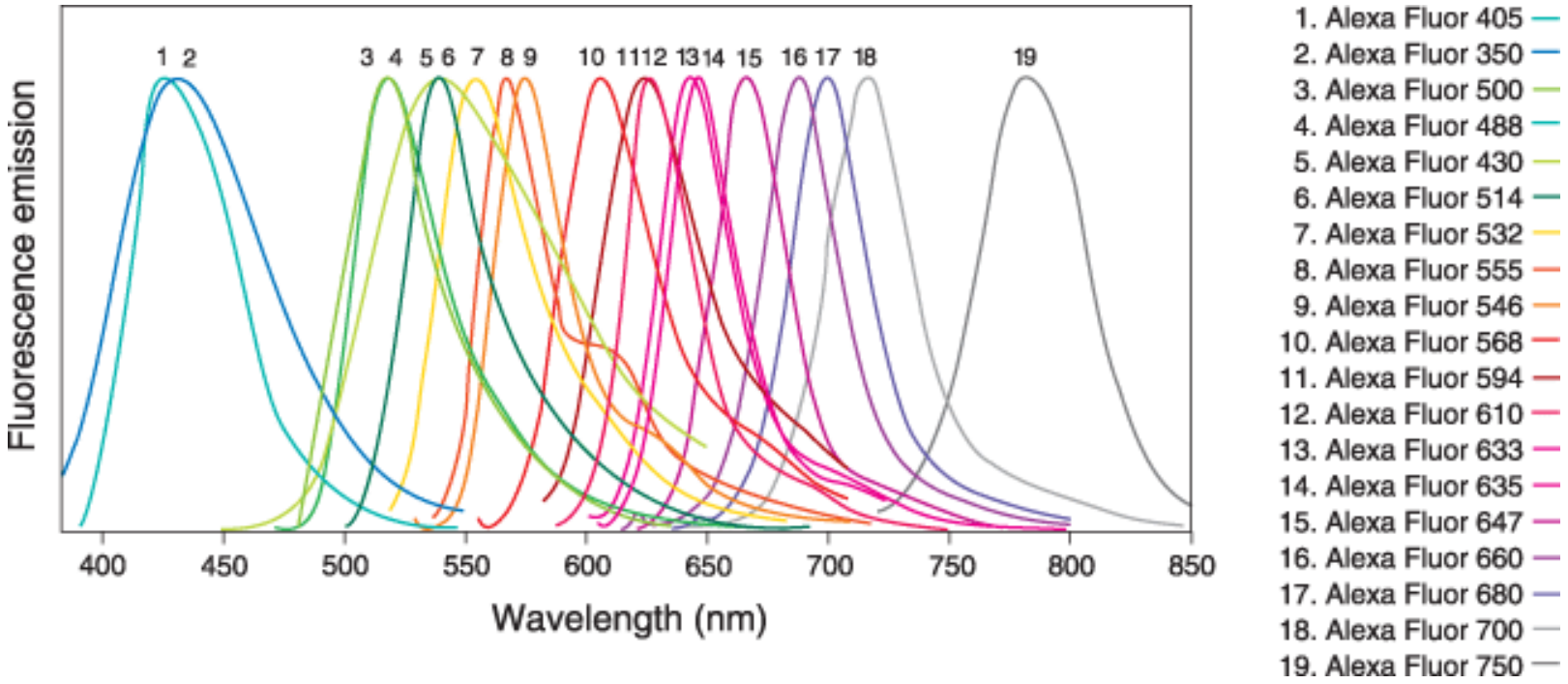
Mixed Fluorescein and Rhodamine



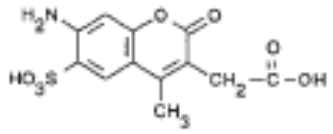
YFP variants



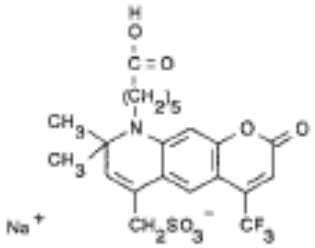
Brief discussions of various fluors



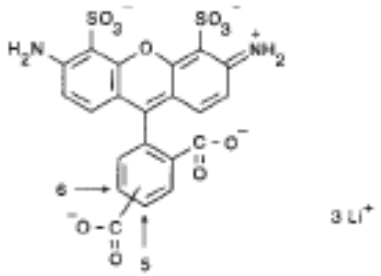
Traditional small molecule dyes



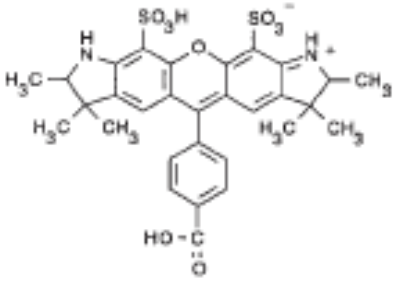
Alexa 350



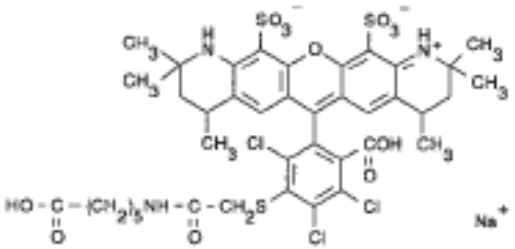
Alexa 430



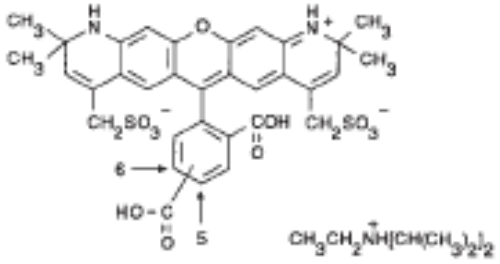
Alexa 488



Alexa 532

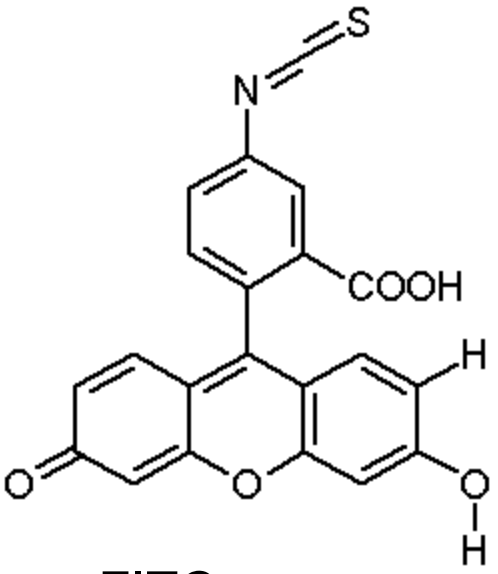


Alexa 546

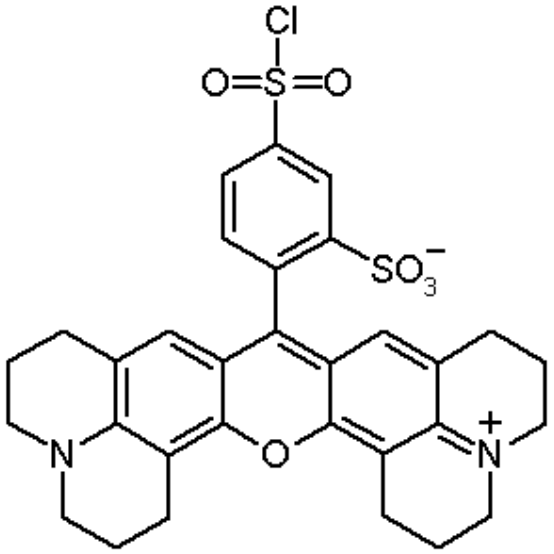


Alexa 568

Fluorescent dyes in Biology



FITC

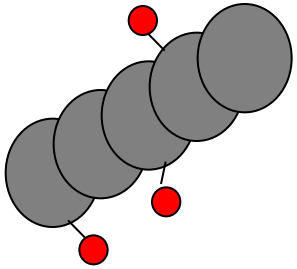


Texas Red

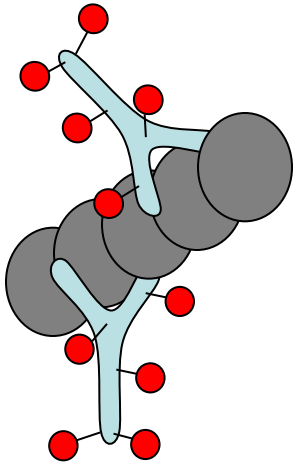
- Protein labeling: couple to amino- or sulfhydryl groups
- Direct and indirect (immuno-) fluorescence

Fluorescent labeling

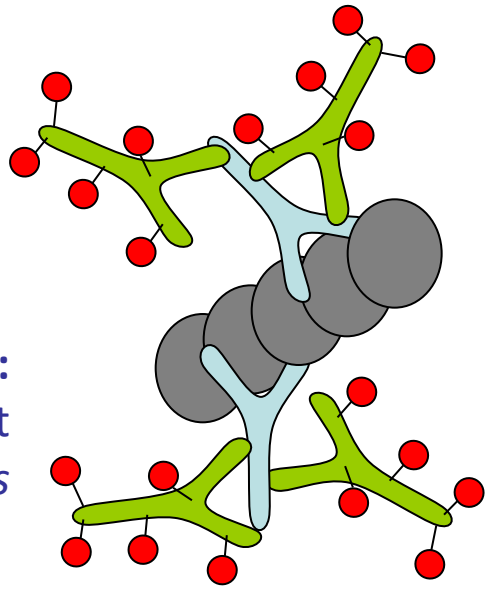
Direct labeling (& microinjection)
of target molecules



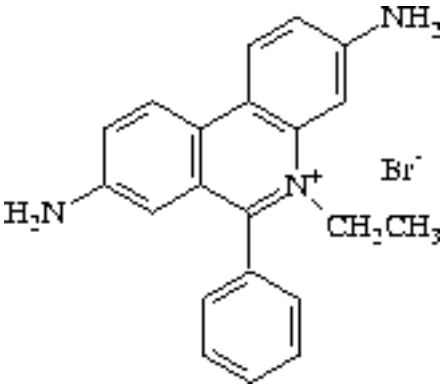
Direct immunofluorescence:
labeled antibodies against target



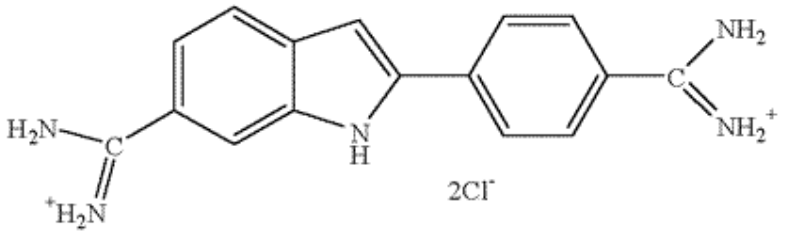
Indirect immunofluorescence:
Unlabeled antibodies against target
Labeled antibodies *against those antibodies*



DNA Probes



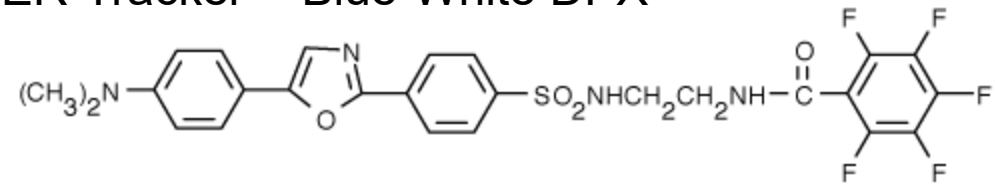
Ethidium Bromide
~30 fold enhancement



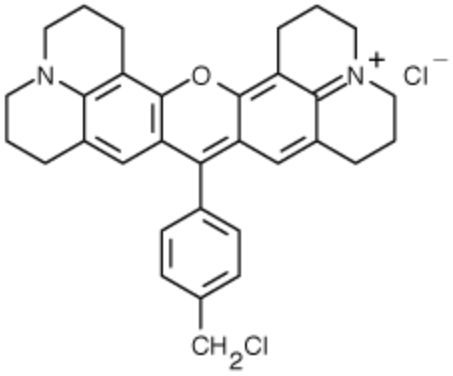
DAPI
Hoechst 33258
Hoechst 33342
~20 fold enhancement

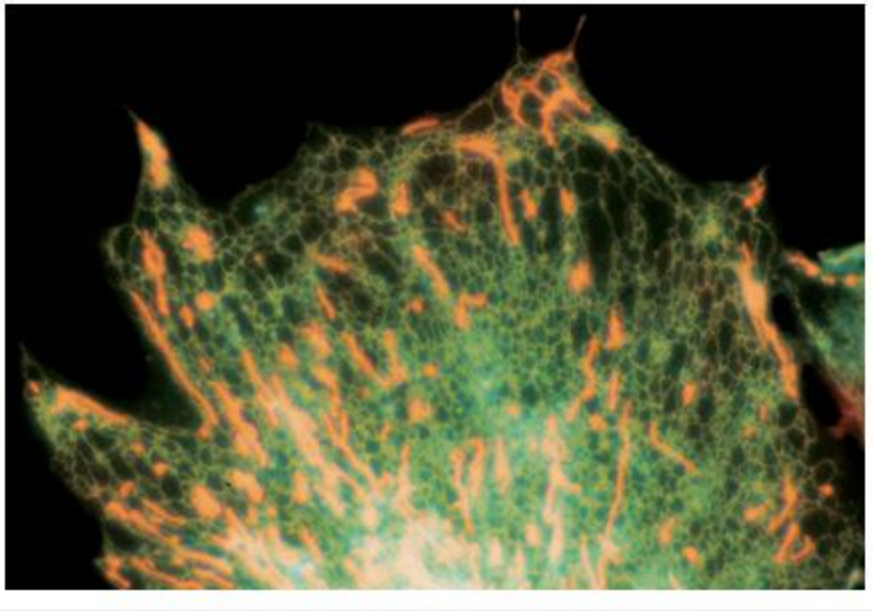
Other probes

ER-Tracker™ Blue-White DPX



MitoTracker Red CMXRos





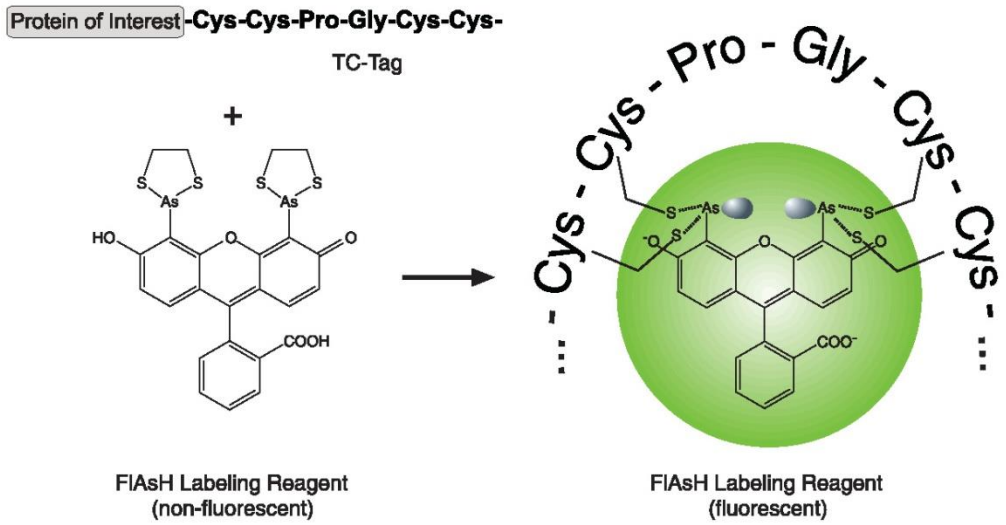
Probes for Golgi, lysosomes, and peroxisomes are also available

Small molecules – pros / cons

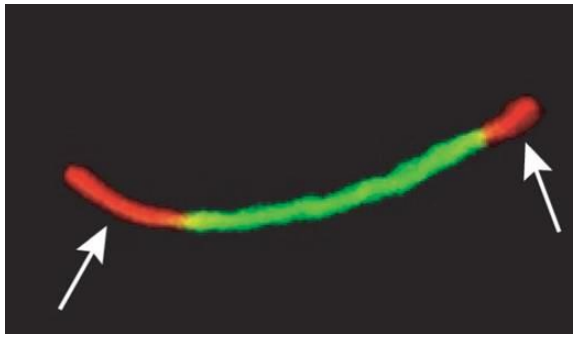
- 1000s available – huge spectral range
- Easy to acquire
- Precisely tailored properties, including environmental sensitivity

- Require fixing and staining, which can lead to artifacts
- Potential self-quenching and environmental sensitivity

FlAsH/ReAsH

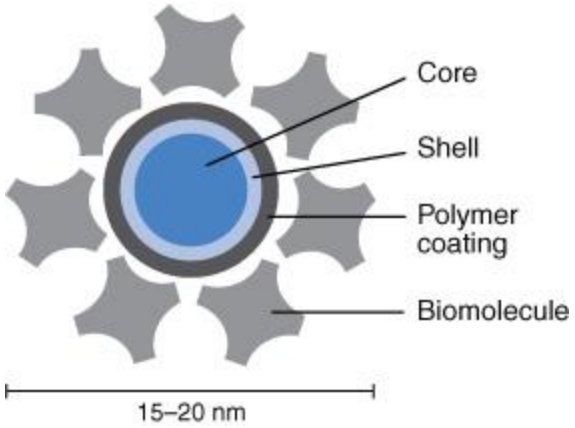


Example:
Newly synthesised connexins (ReAsH:Red) are added to the outer edges of existing gap junctions (FlAsH:Green). Gaietta et al 2002

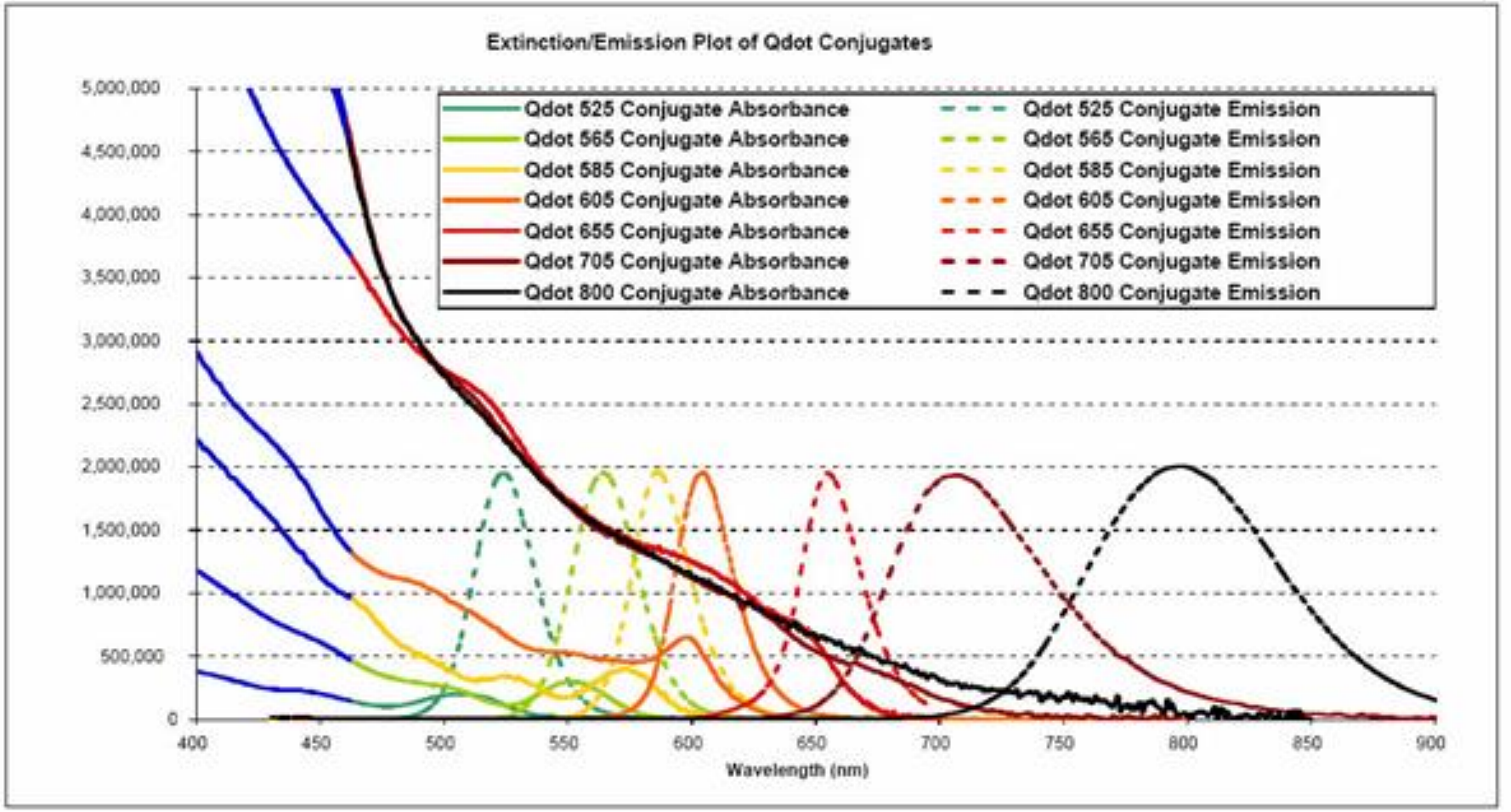


Quantum dots

- “Artificial atoms” composed of small semiconductior nanocrystals



Quantum dots - spectra



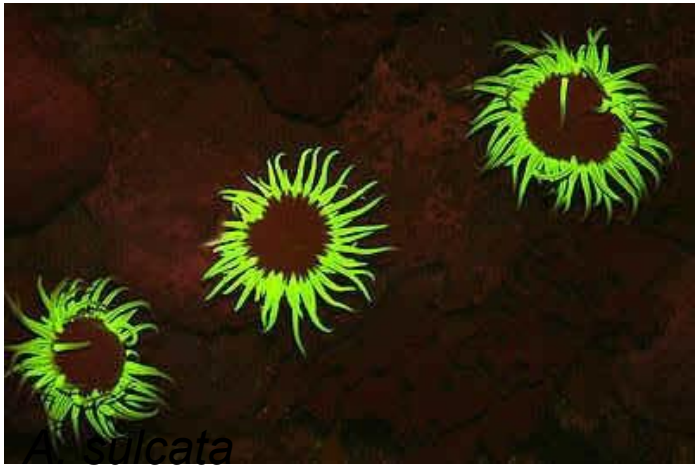
Quantum dots – pros / cons

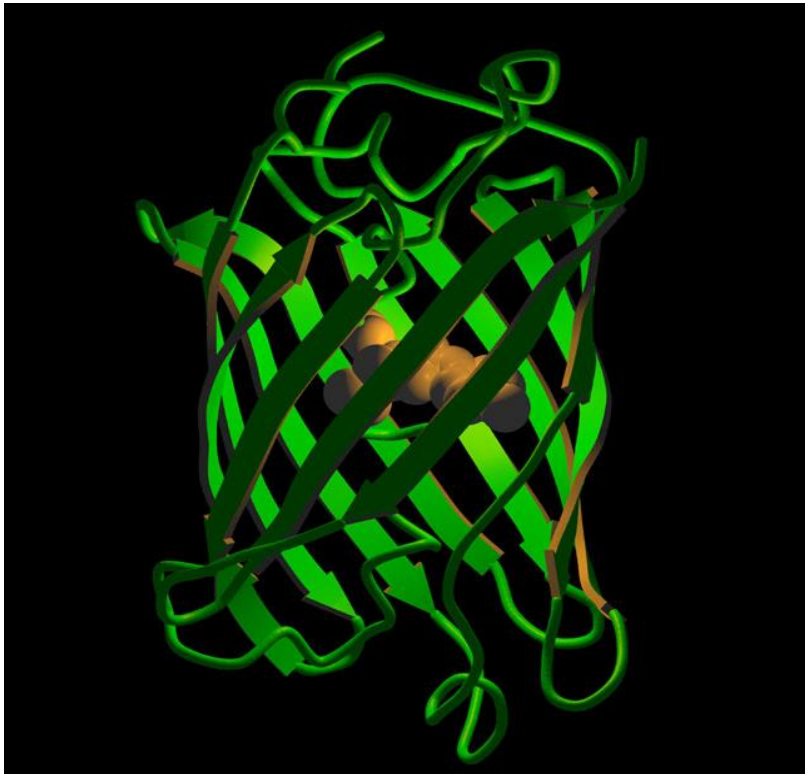
- Little to no photobleaching
- Very bright
- Can use single excitation wavelength for multiple dyes
- Narrow emission spectra

- Large compared to small molecule dyes
- Single quantum dots blink
- Problems with non-specific binding

Fluorescent Proteins and Genetically Encoded Tags

See future lecture!





Resources

www.microscopyu.com

micro.magnet.fsu.edu

www.chroma.com (esp. their handbook on filter design)

www.probes.com (esp. their handbook/catalog)

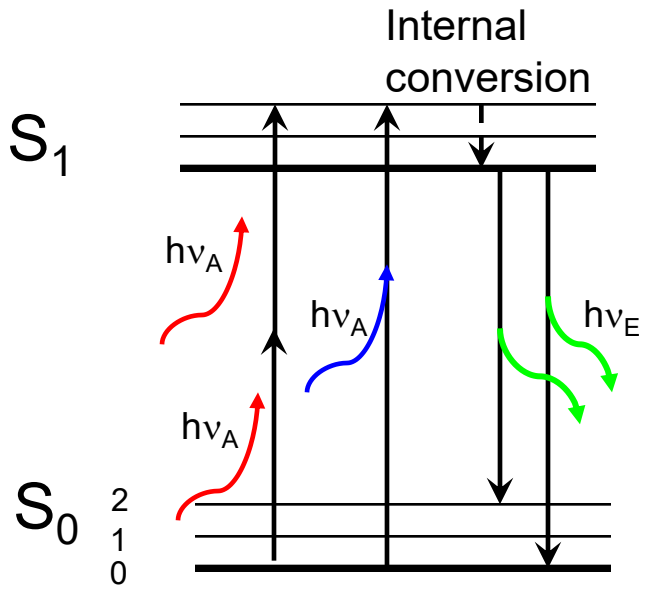
Douglas B. Murphy “Fundamentals of Light Microscopy and Electronic Imaging”

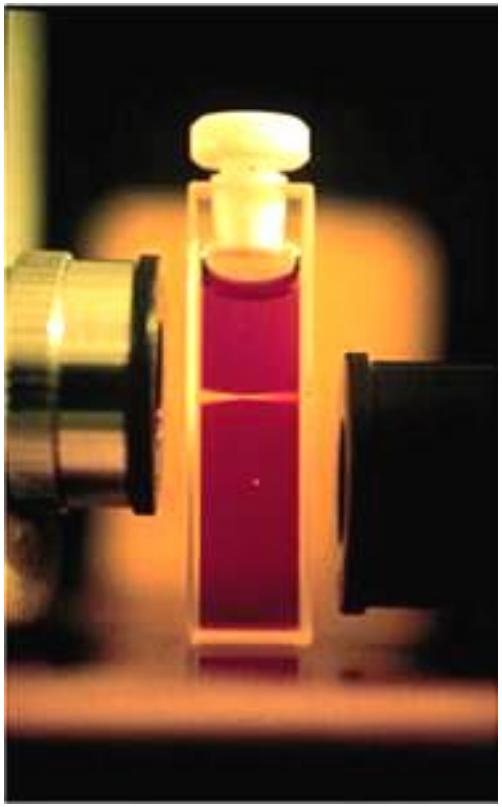
James Pawley, Ed. “Handbook of Biological Confocal Microscopy, 3rd ed.”

Acknowledgements

Nico Stuurman / Mats Gustafsson / Mike Davidson

Multi-photon excitation





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