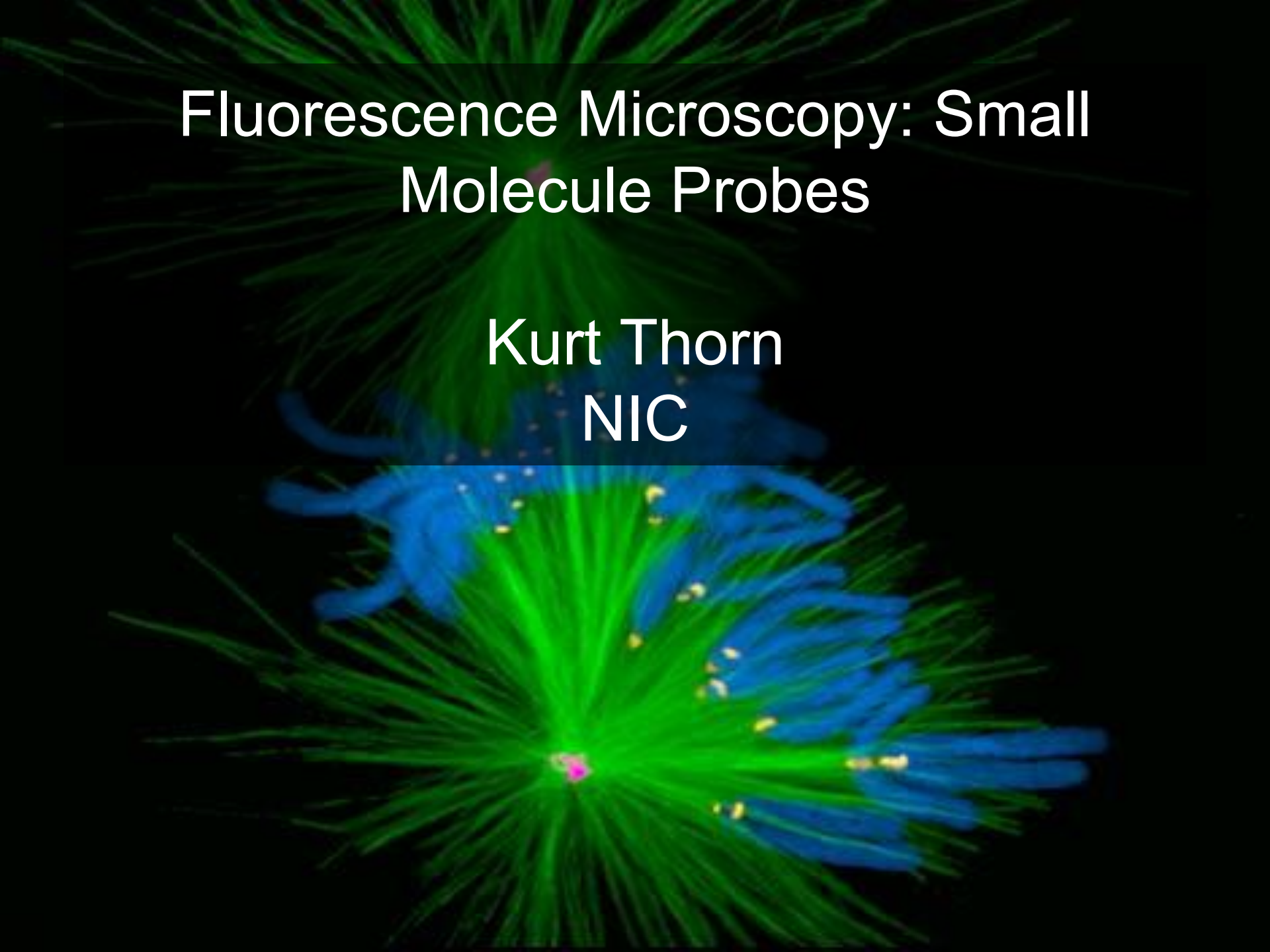
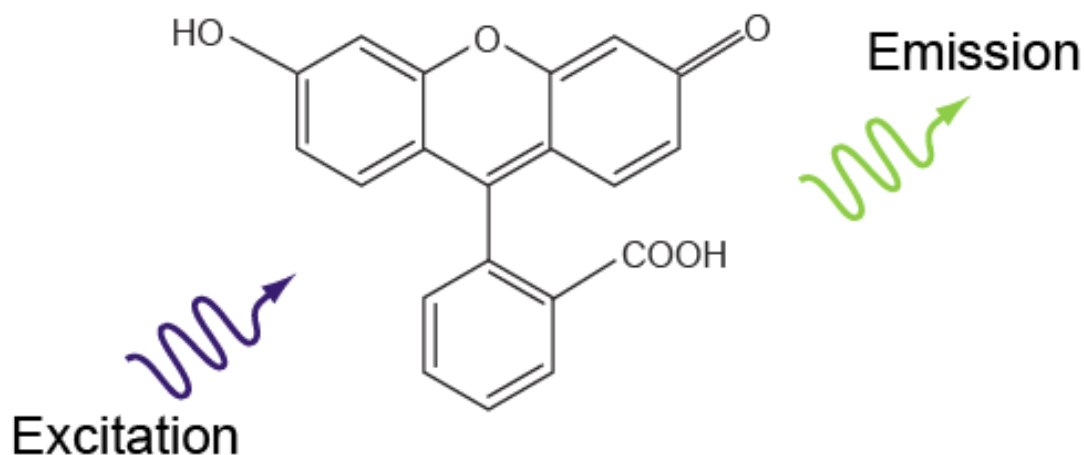


Fluorescence Microscopy: Small Molecule Probes

Kurt Thorn
NIC



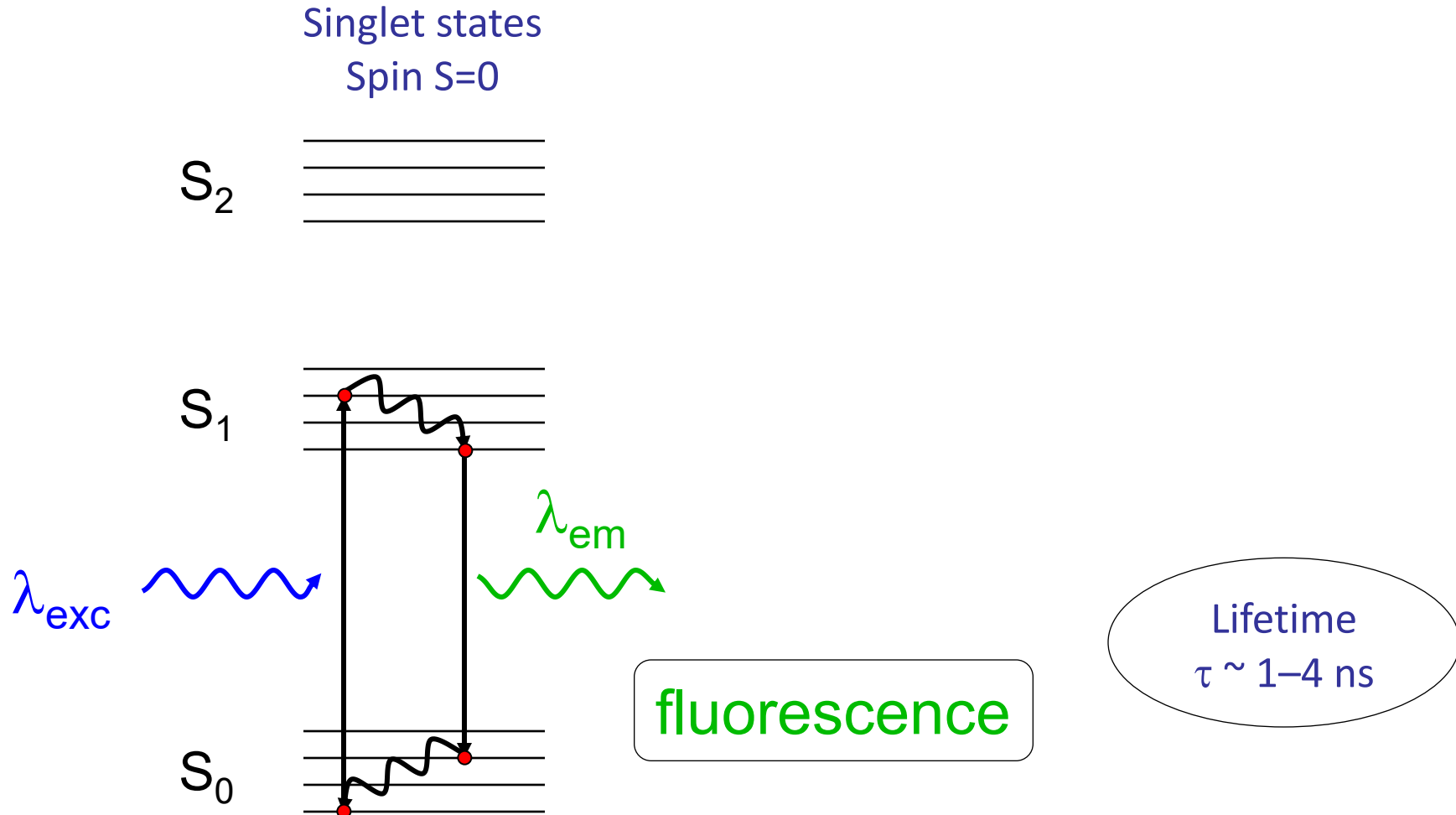
What is fluorescence?



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

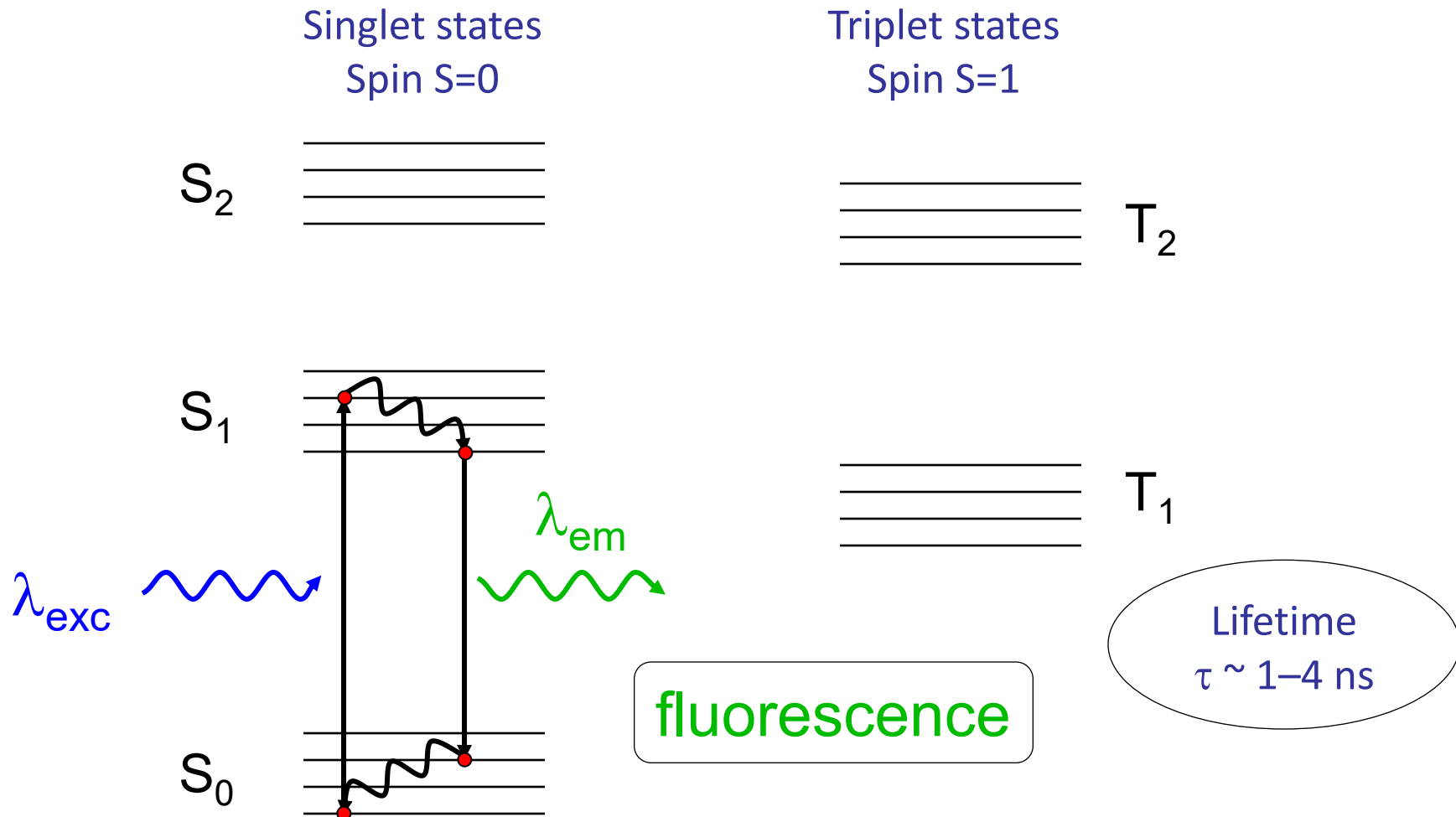
Jablonski diagram

(Molecular energy diagram)



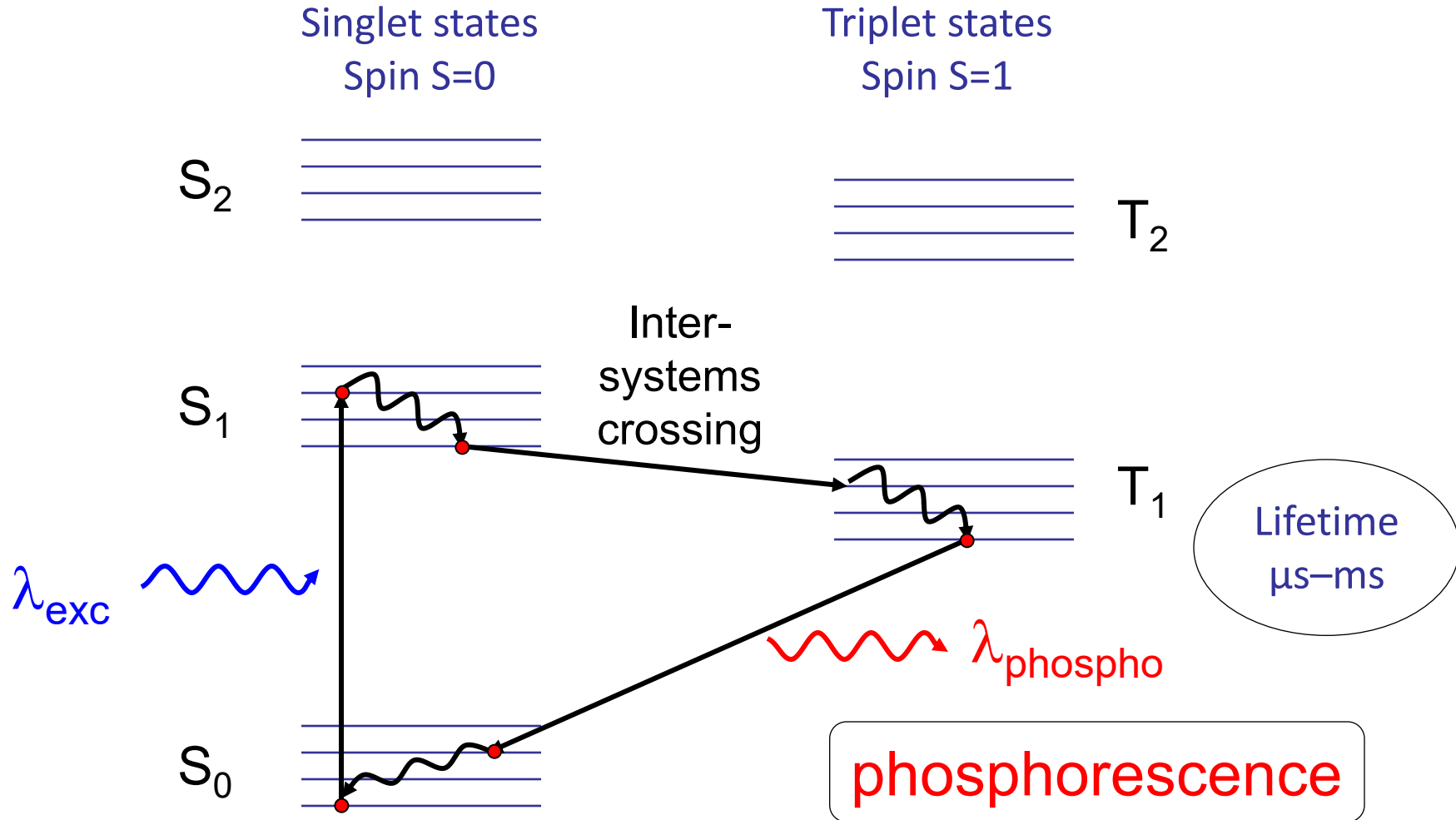
Jablonski diagram

(Molecular energy diagram)

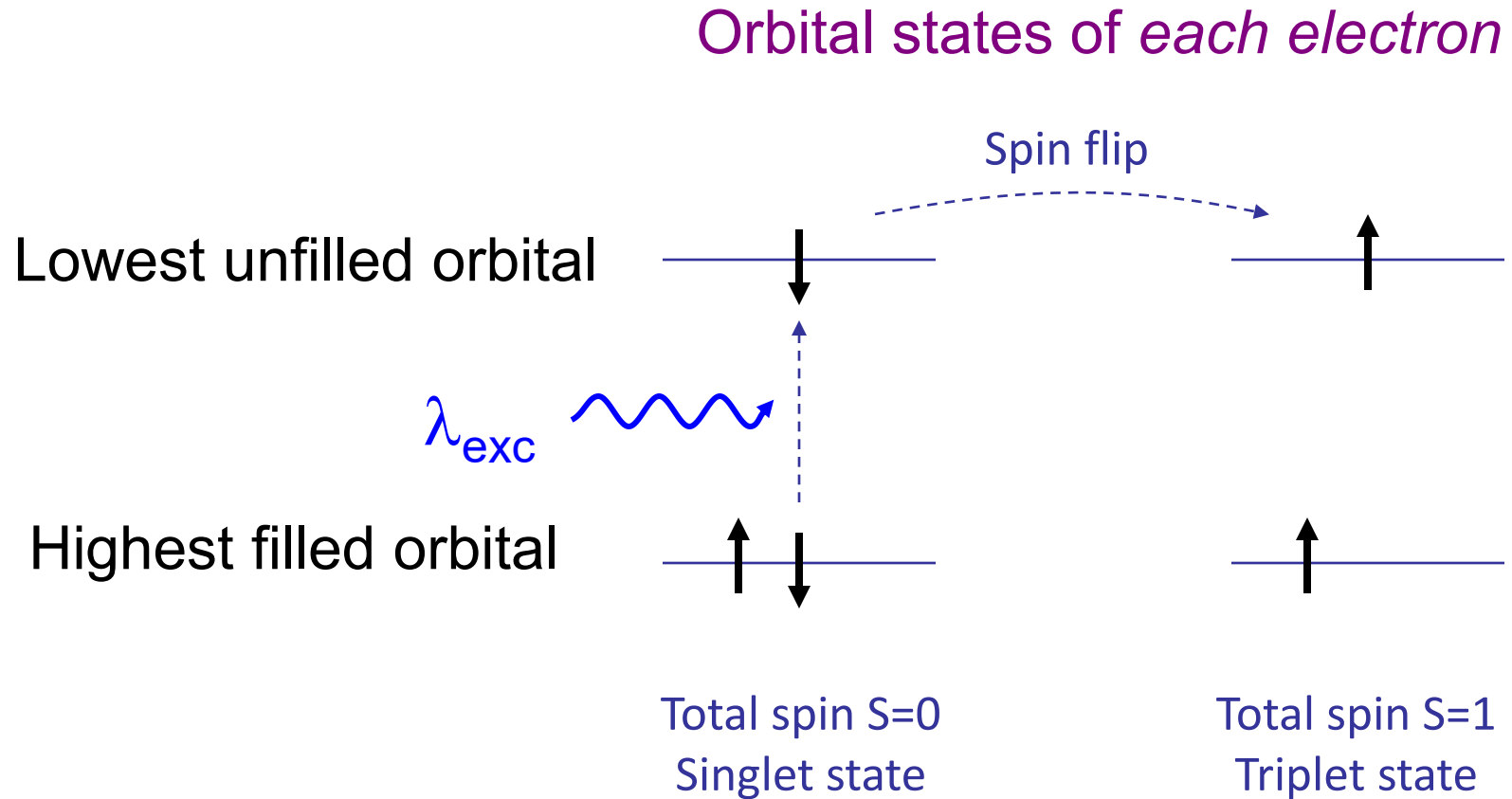


Jablonski diagram

(Molecular energy diagram)

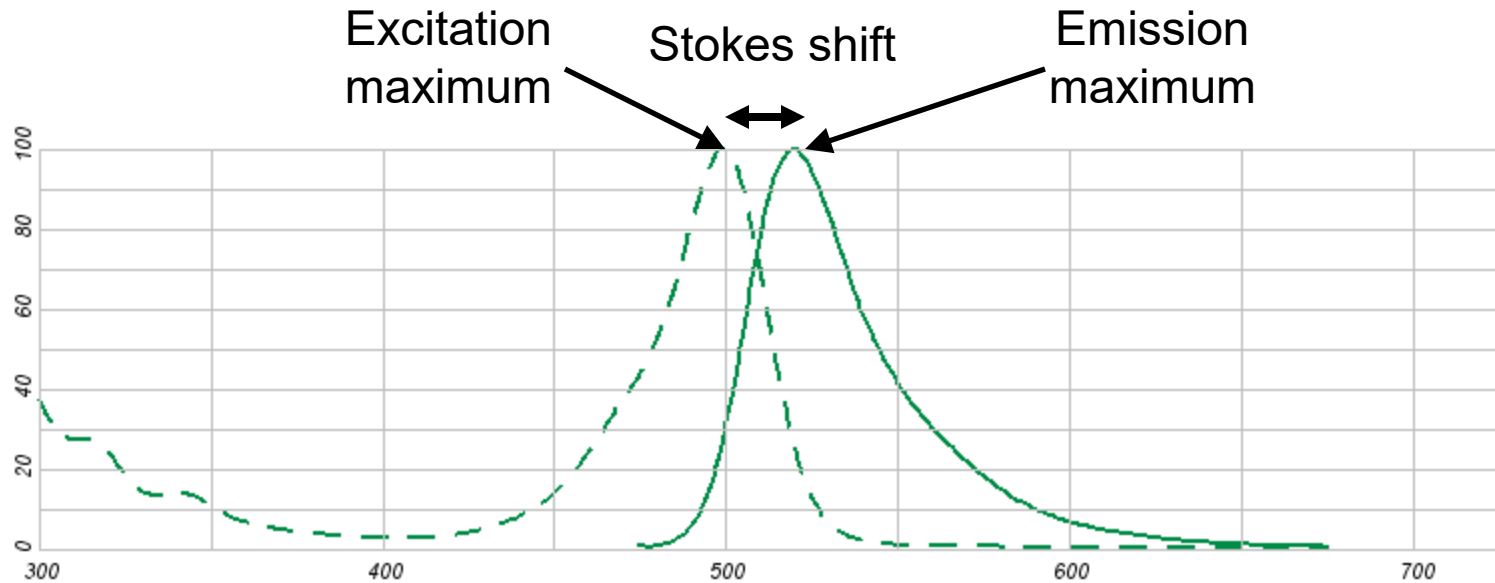


Singlet and Triplet States



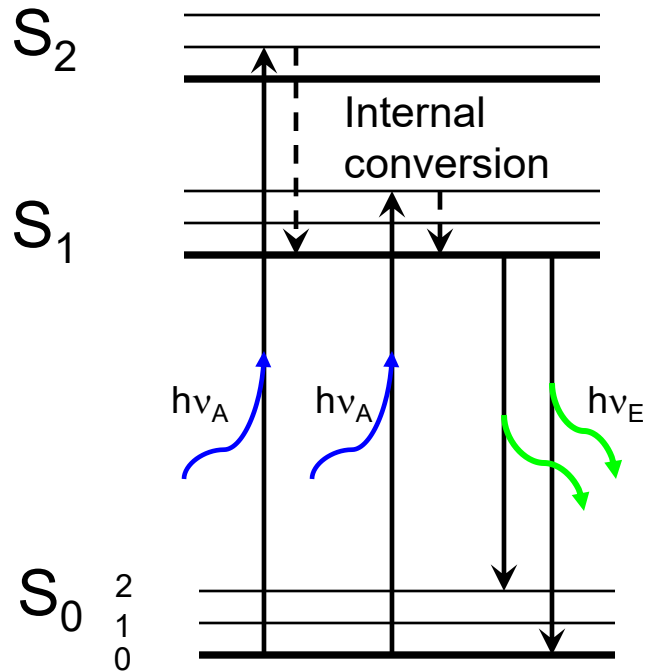
Spin flips are “dipole forbidden” → unlikely → long triplet lifetime

Fluorescence Spectra



Alexa 488

Fluorophore saturation

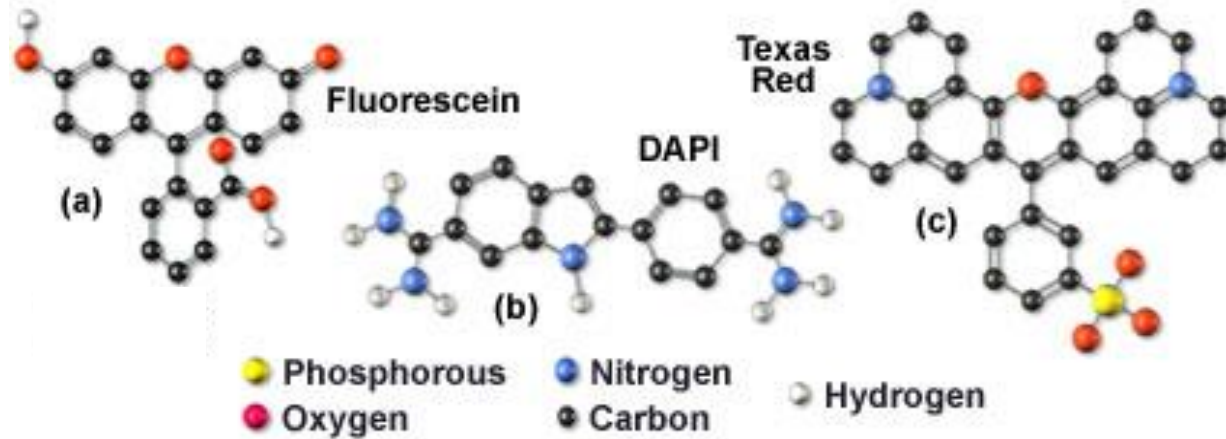


Fluorescence lifetime is $\sim 1-5\text{ns}$

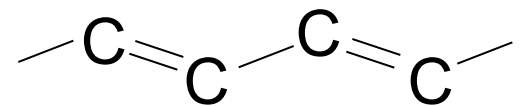
Once illumination intensity is high enough to excite the fluorophore as soon as it deexcites, further intensity increases will not increase brightness

Usually only a problem for confocal

Fluorescent molecules



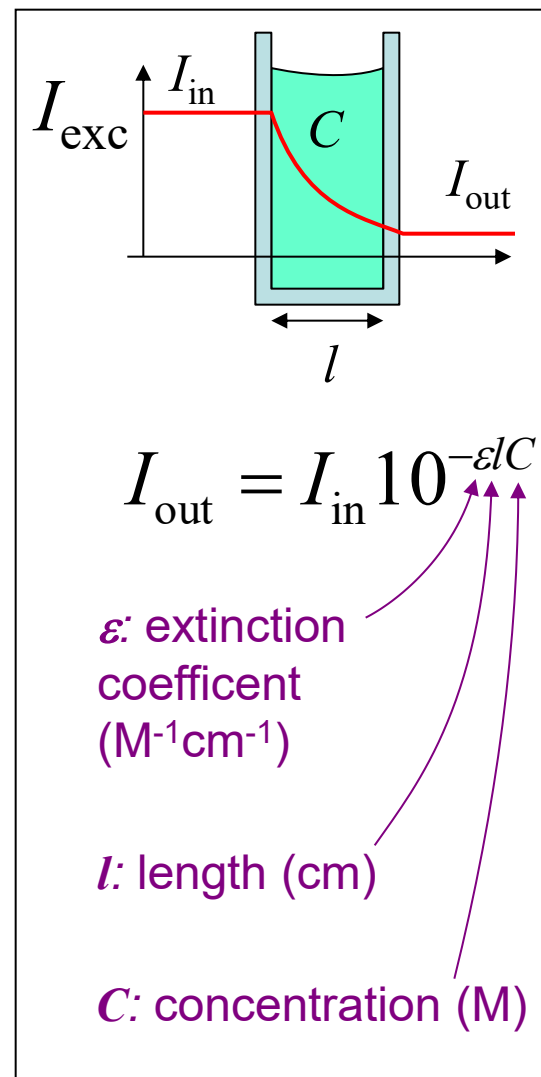
Systems of conjugated bonds
that share electrons



Larger system → longer wavelength

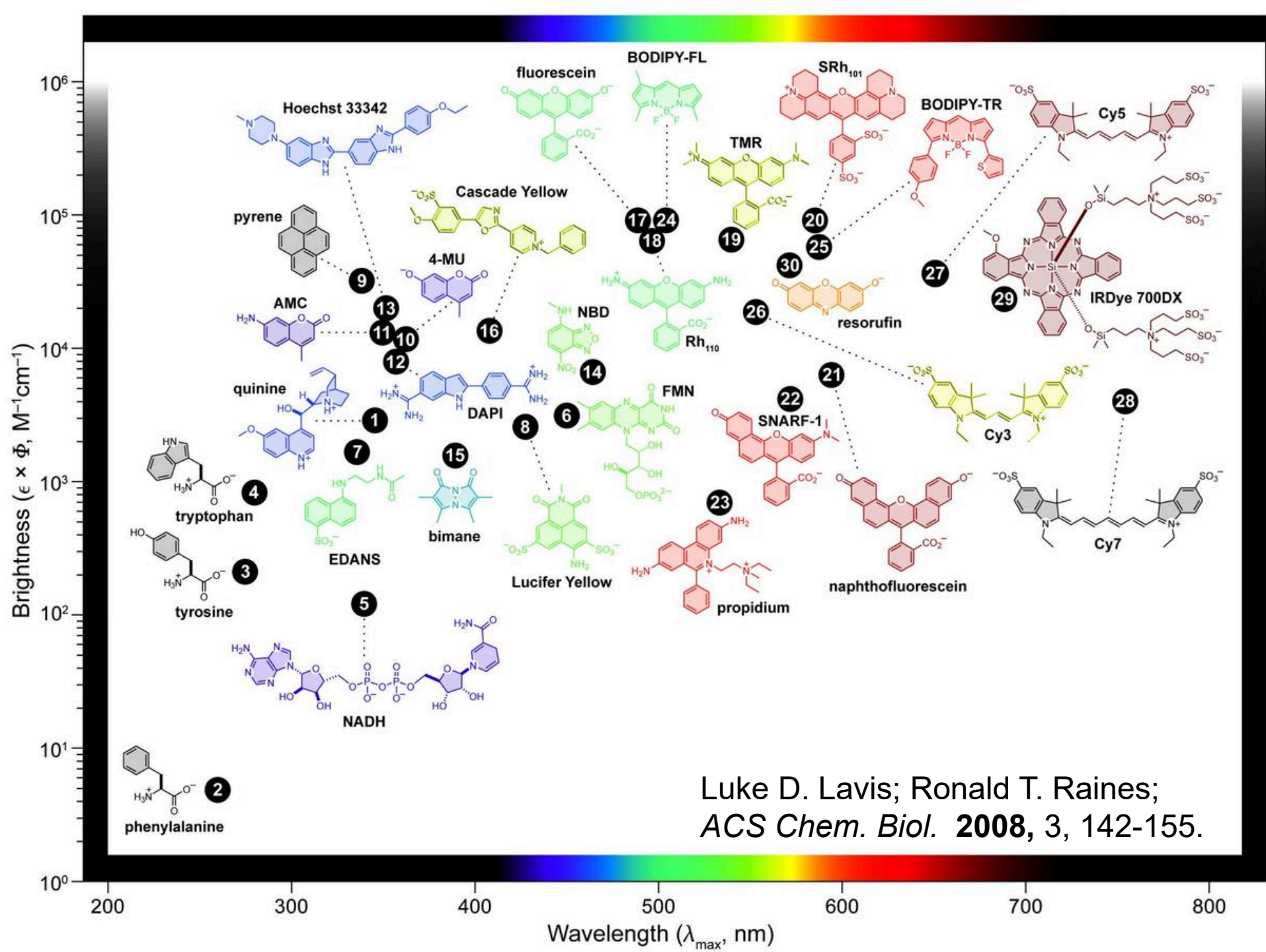
Parameters of fluorescent molecules

- Excitation & emission maxima
- Extinction coefficient ϵ
 \propto absorption cross section
 $\epsilon \approx 50,000\text{--}100,000 \text{ M}^{-1}\text{cm}^{-1}$
- Fluorescence quantum yield Q_f
= # Photons emitted / # photons absorbed
 $Q_f \approx 25\text{--}90\%$
Brightness $\propto \epsilon Q_f$
- 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

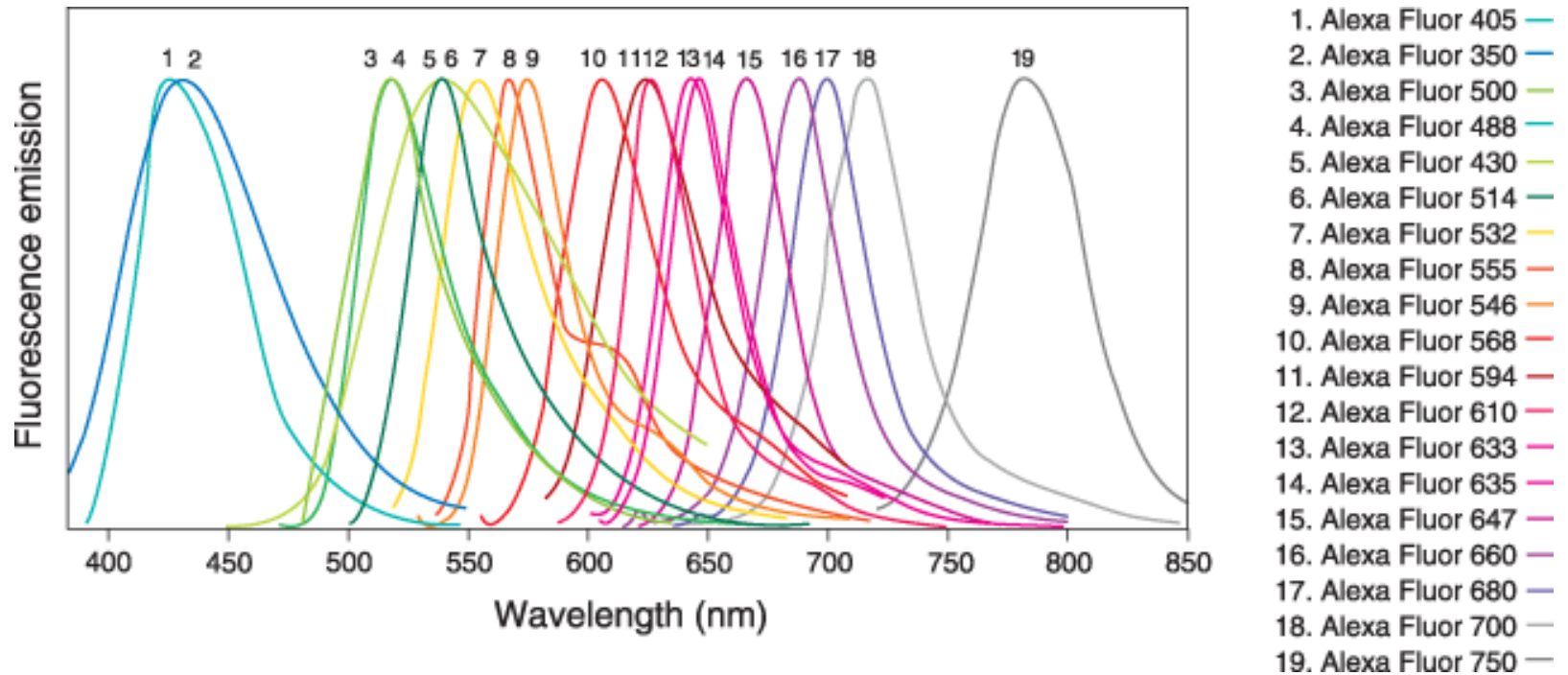


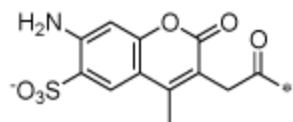
Commercial Dye Series

- Alexa Dyes – Molecular Probes / Life Technologies
- Atto Dyes – Atto-Tec GmbH
- Etc...

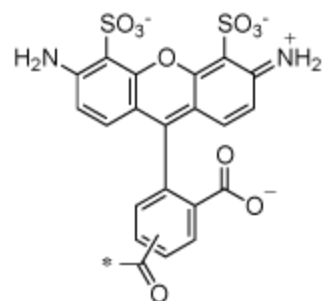
Not chemical families – marketing families

Alexa Fluors

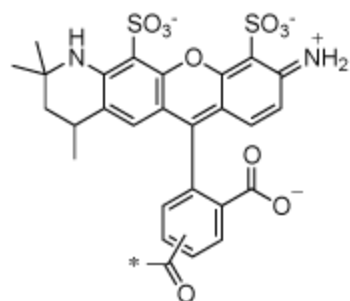




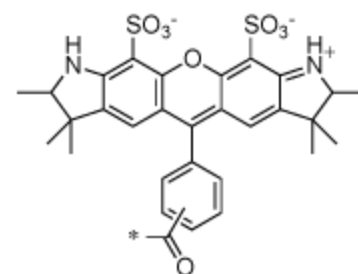
Alexa Fluor® 350



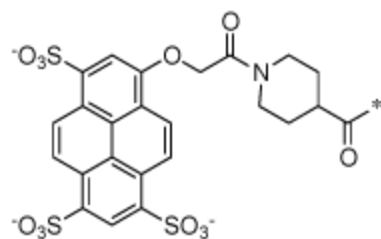
Alexa Fluor® 488



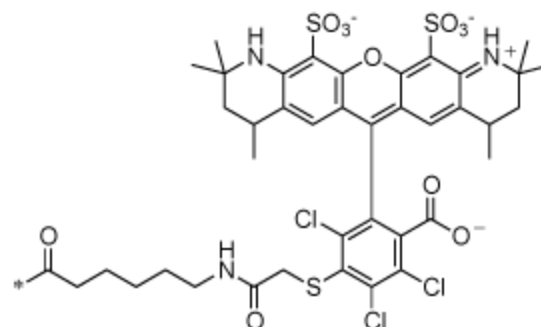
Alexa Fluor® 514



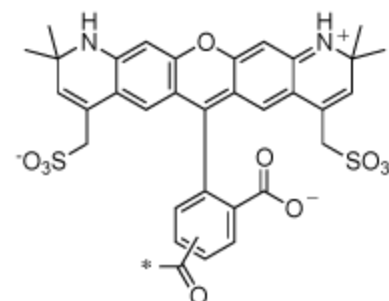
Alexa Fluor® 532



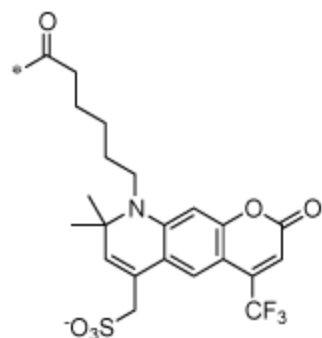
Alexa Fluor® 405



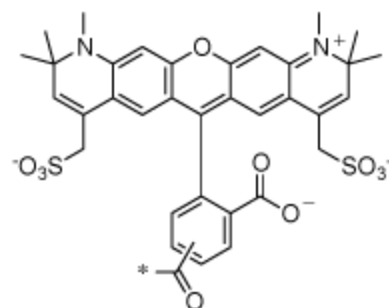
Alexa Fluor® 546



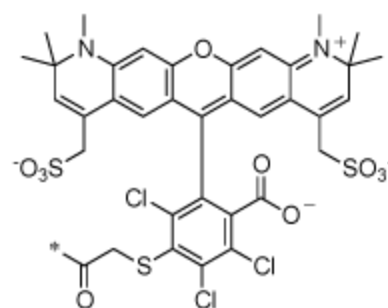
Alexa Fluor® 568



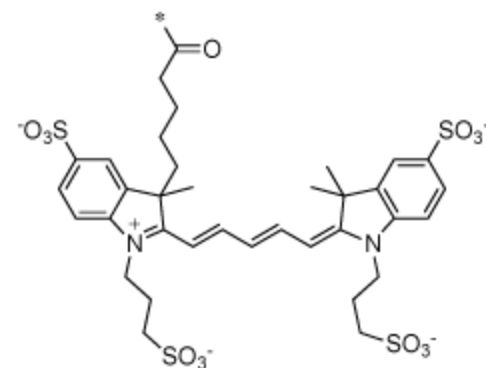
Alexa Fluor® 430



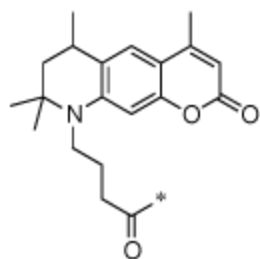
Alexa Fluor® 594



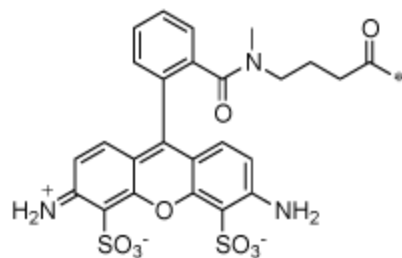
Alexa Fluor® 610



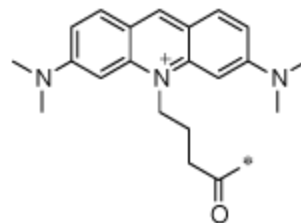
Alexa Fluor® 647



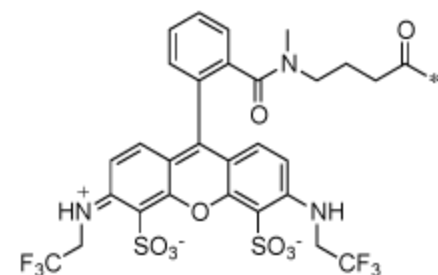
ATTO 390



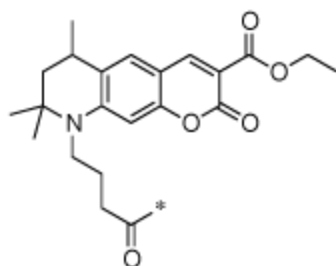
ATTO 488



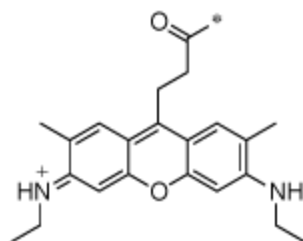
ATTO 495



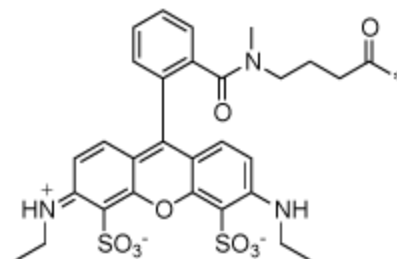
ATTO 514



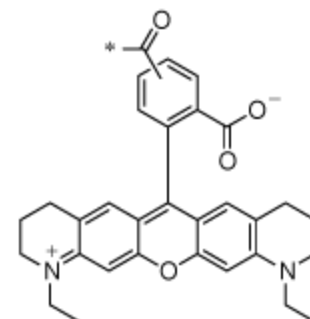
ATTO 425



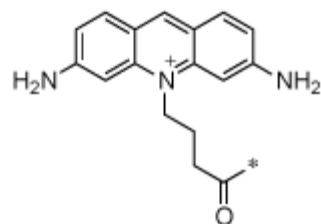
ATTO 520



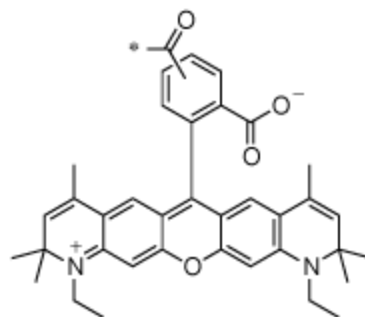
ATTO 532



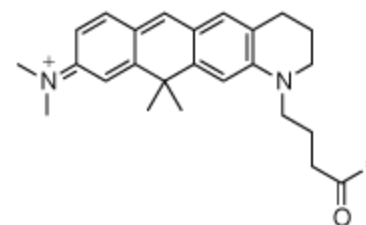
ATTO 565



ATTO 465

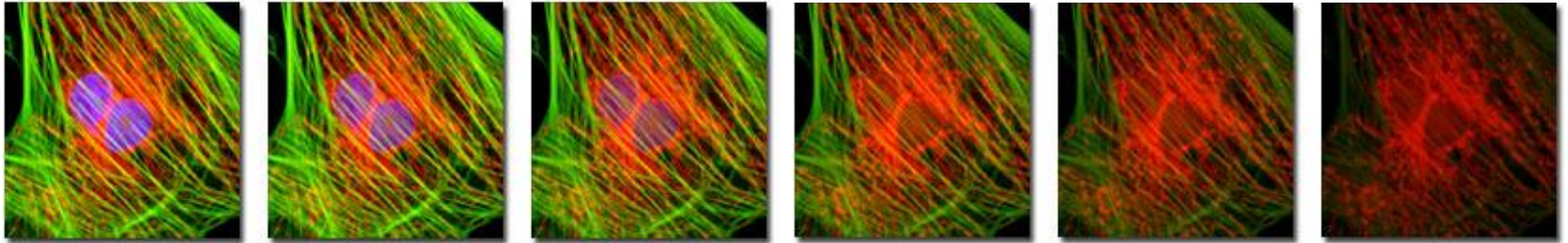


ATTO 590



ATTO 610

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

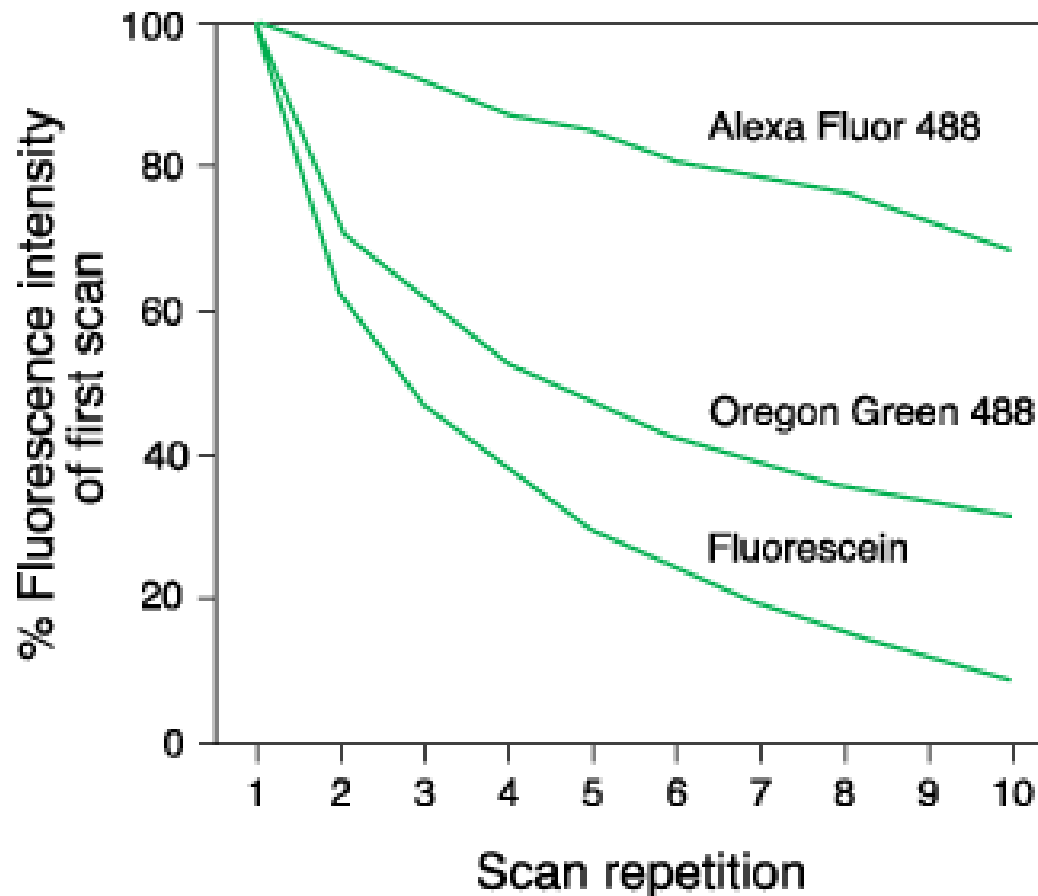
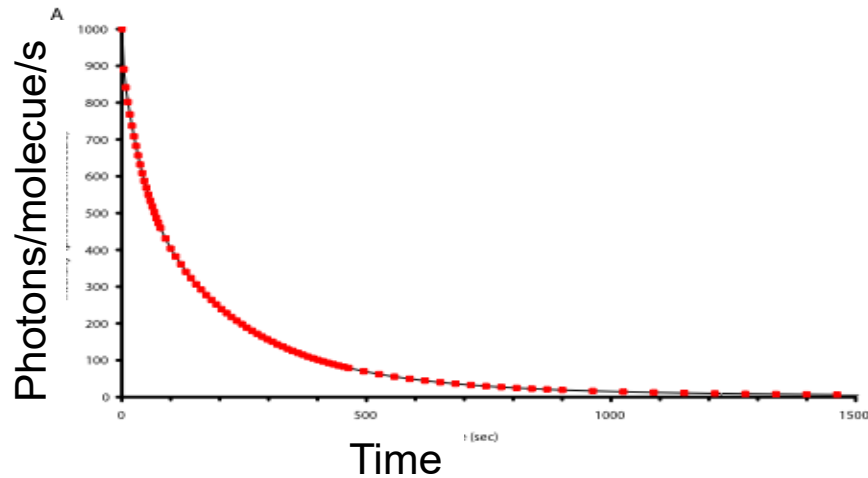
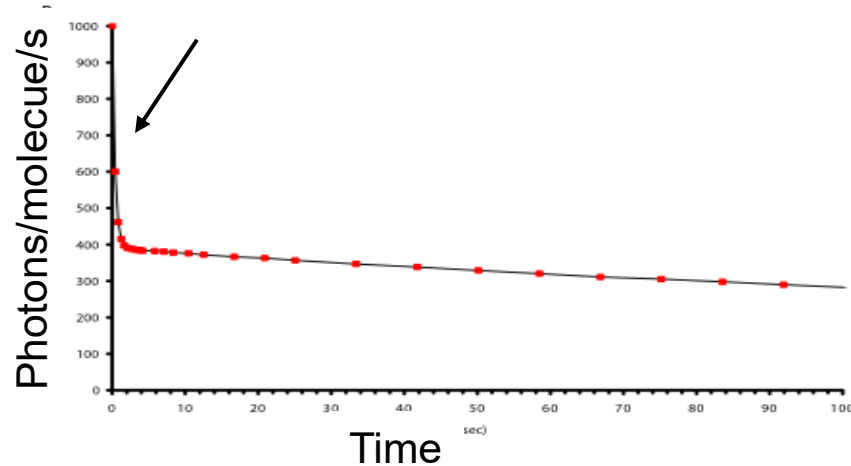


Photo-bleaching of fluorescent proteins



mCherry
Single-exponential bleaching



Emerald
Double-exponential bleaching
Fast- and slow-bleaching populations?

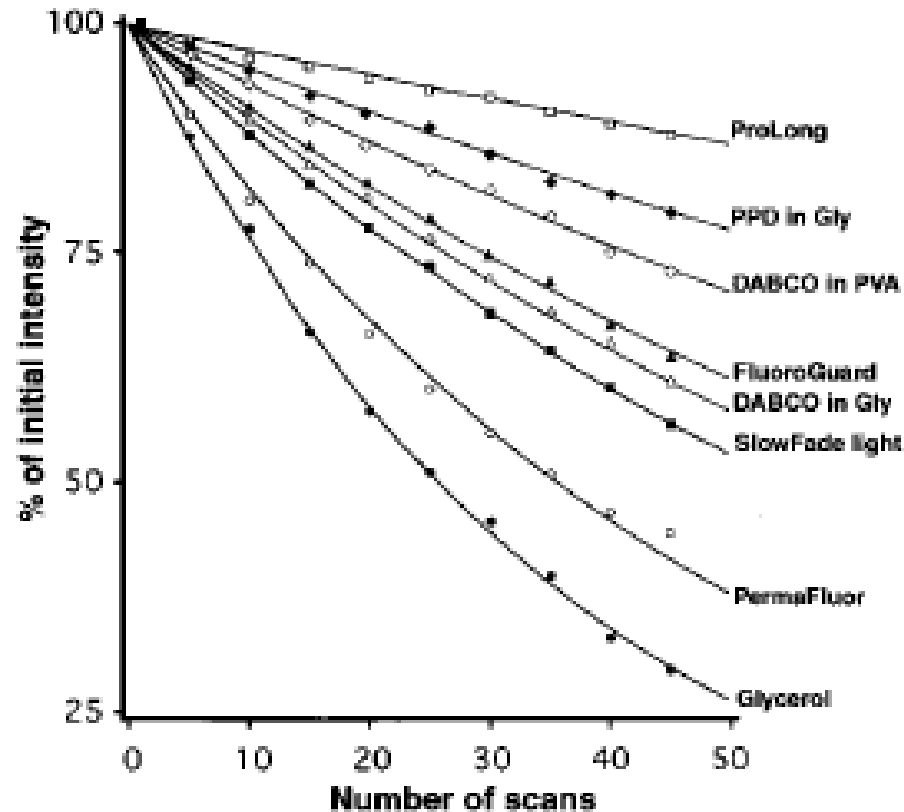
What to do about photo-bleaching?

- 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.

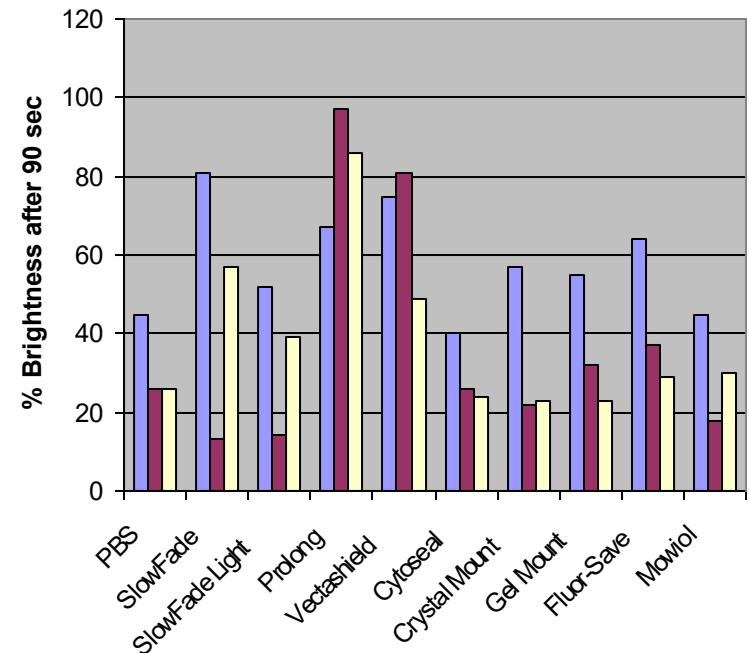
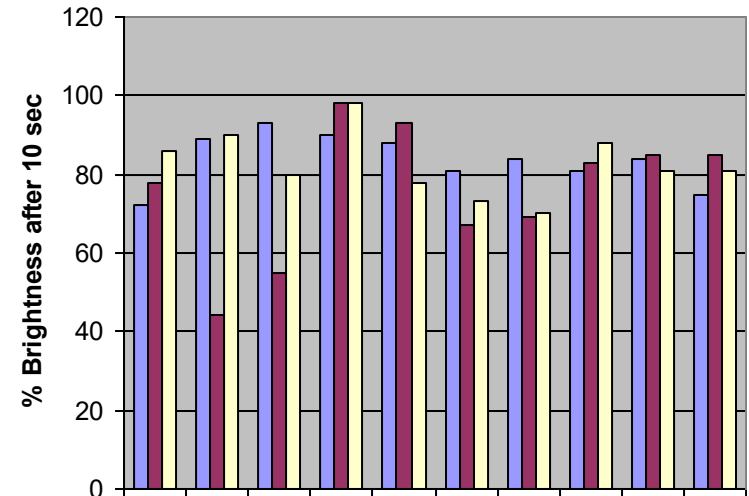
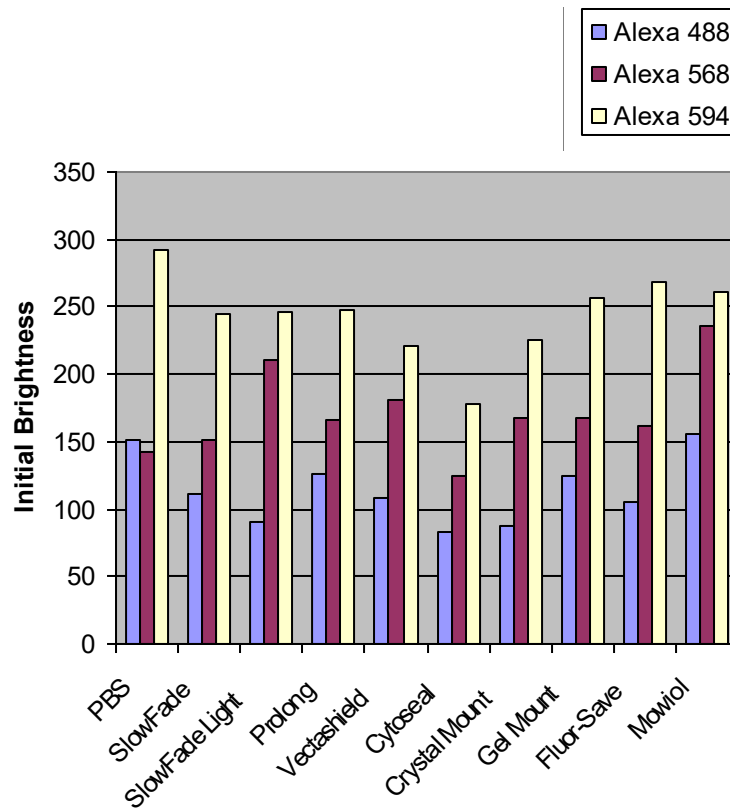
- Budget the photons you have
 - Only expose when observing
 - Minimize exposure time & excitation power
 - Use efficient filter combinations
 - Use highly QE, low noise camera
 - Use simple light path

Effect of mounting medium on FITC bleaching



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

Effect of mounting media on Alexa bleaching

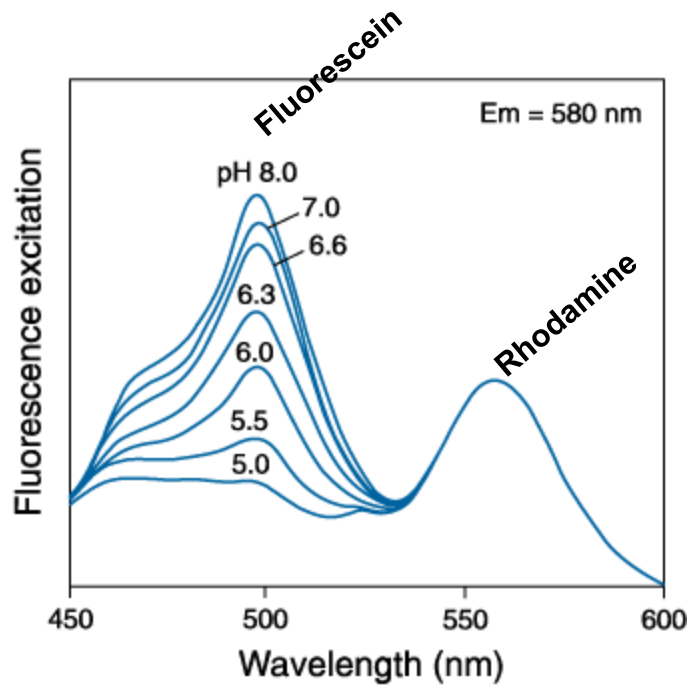


Factors affecting overall brightness

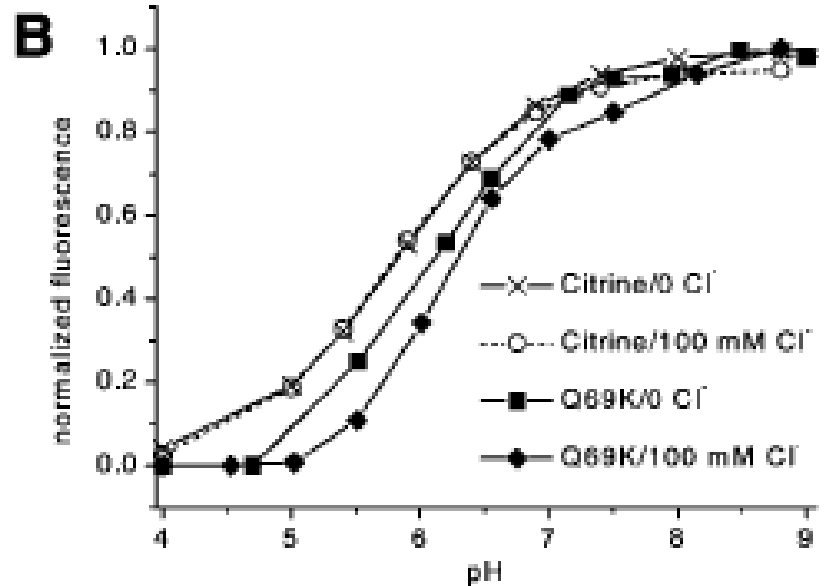
- Intrinsic brightness
- Spectrum of arc lamp/lasers
- Lamp/laser power
- Filter set transmission
- Quantum efficiency of detector
- Photobleaching
- Quenching / maturation / other dye-specific effects

pH dependence of dyes

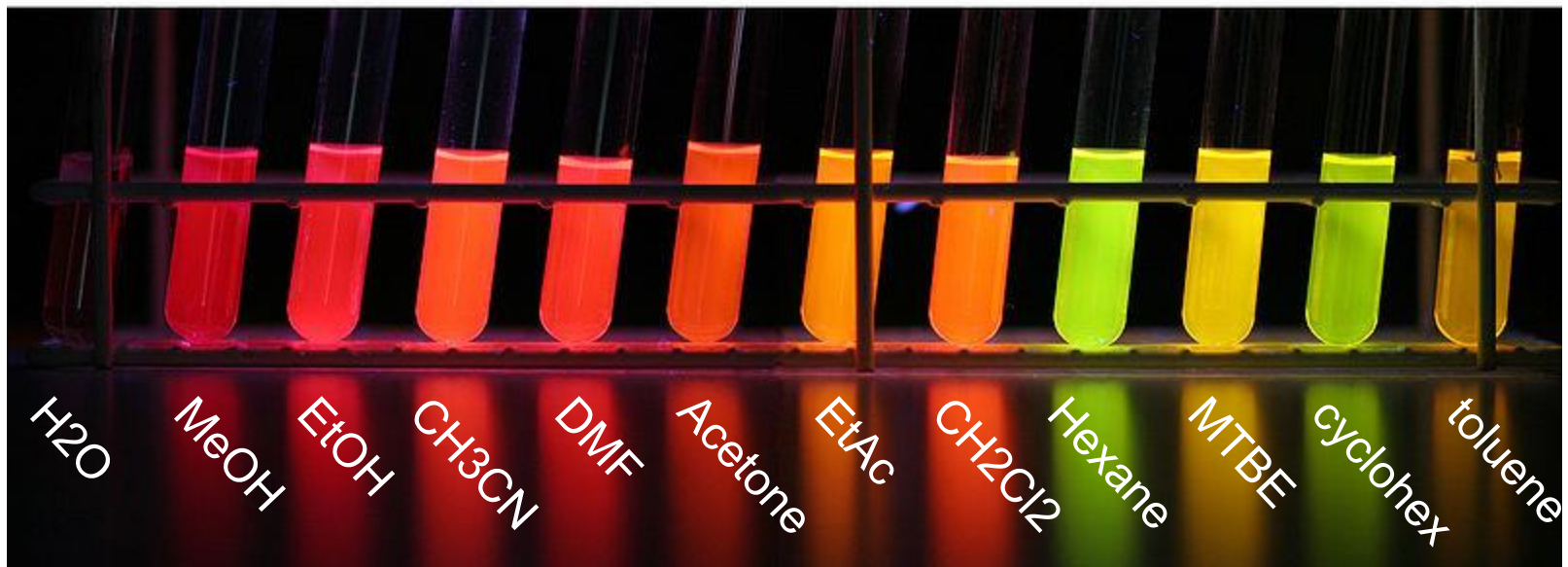
Mixed Fluorescein and Rhodamine



YFP variants



Environmental Dependence: Nile red



H₂O

MeOH

EtOH

CH₃CN

DMF

Acetone

EtAc

CH₂Cl₂

Hexane

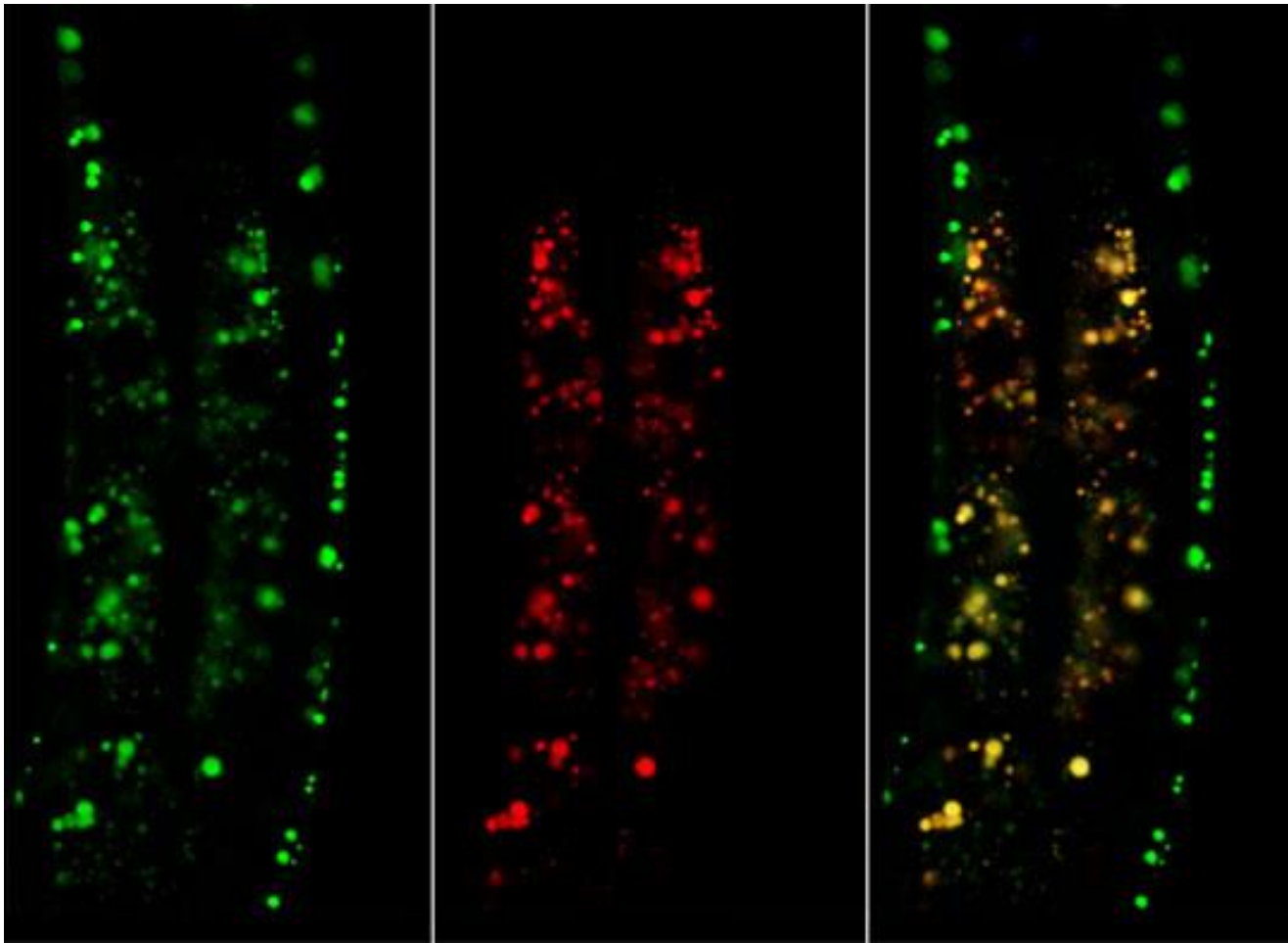
MTBE

cyclohex

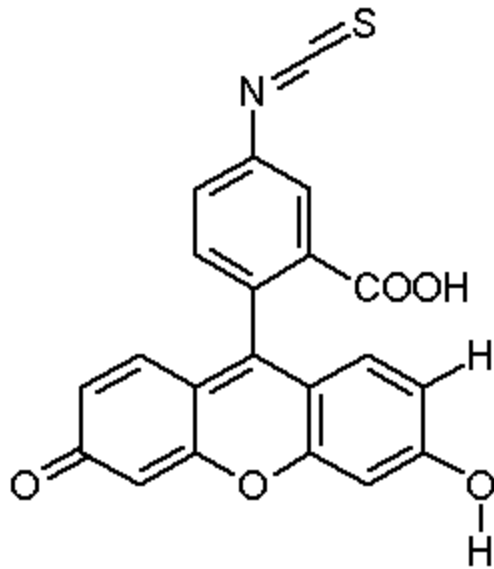
toluene

Environmental Dependence: Nile Red

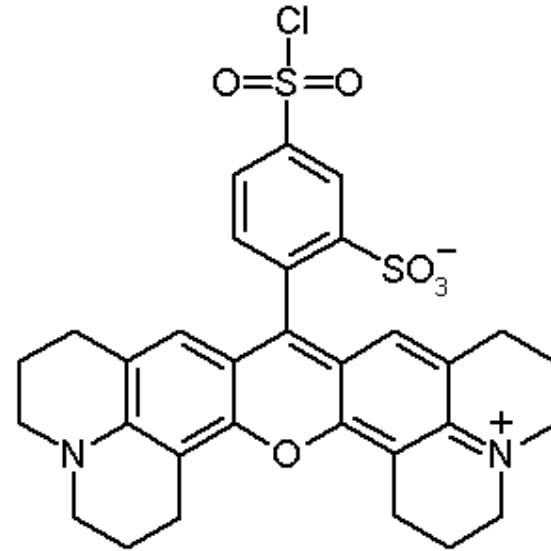
C. elegans staining with Nile Red and BODIPY-fatty acid
(Kevin Jones, Ashrafi lab)



Fluorescent dyes in Biology



FITC

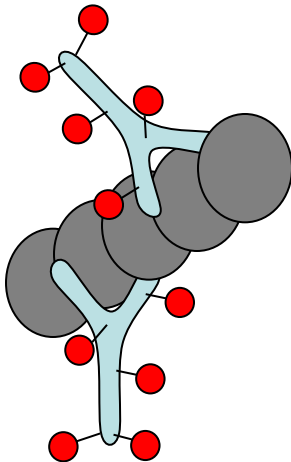


Texas Red

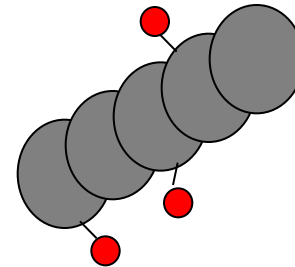
- Protein labeling: couple to amino- or sulfhydryl groups
- Direct and indirect (immuno-) fluorescence

Fluorescent labeling

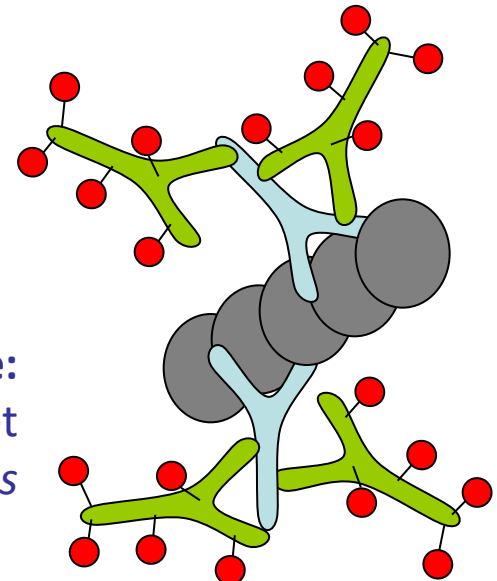
Direct immunofluorescence:
labeled antibodies against target



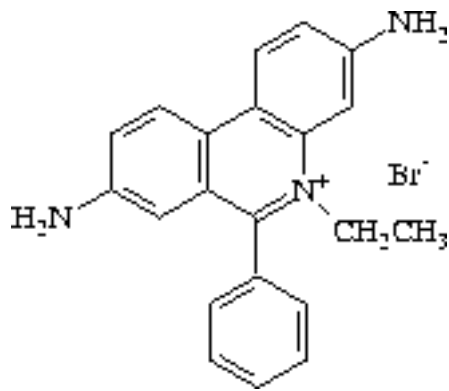
Direct labeling (& microinjection)
of target molecules



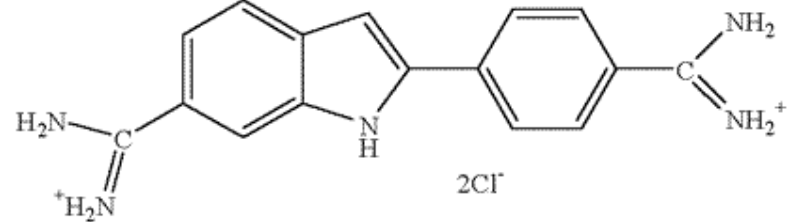
Indirect immunofluorescence:
Unlabeled antibodies against target
Labeled antibodies against those antibodies



DNA Probes



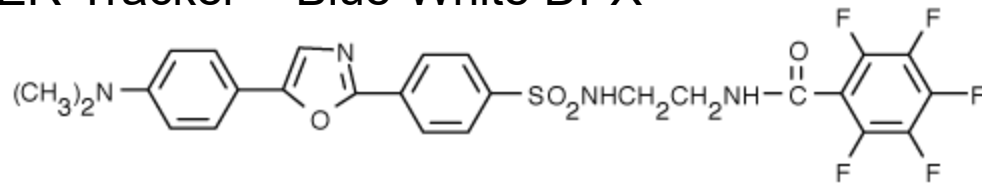
Ethidium Bromide
~30 fold enhancement



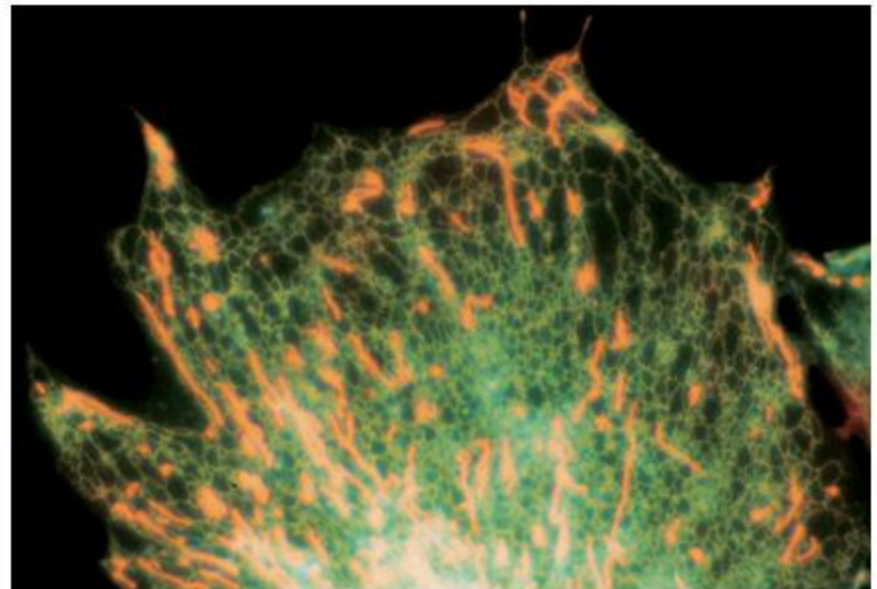
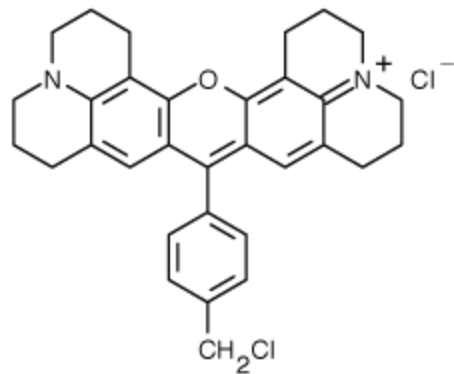
DAPI
Hoechst 33258
Hoechst 33342
~20 fold enhancement

Other probes

ER-Tracker™ Blue-White DPX



MitoTracker Red CMXRos



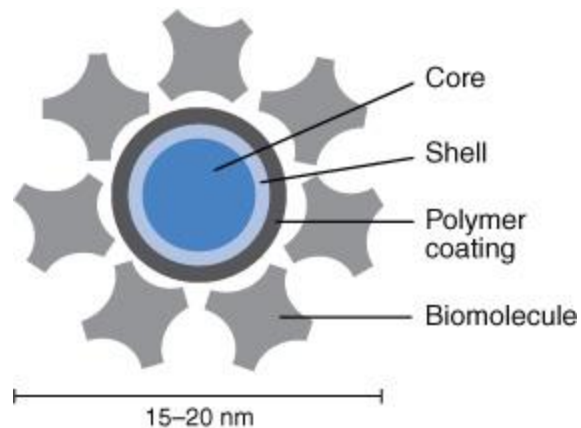
Probes for Golgi, lysosomes, and peroxisomes are also available

Small molecules – pros / cons

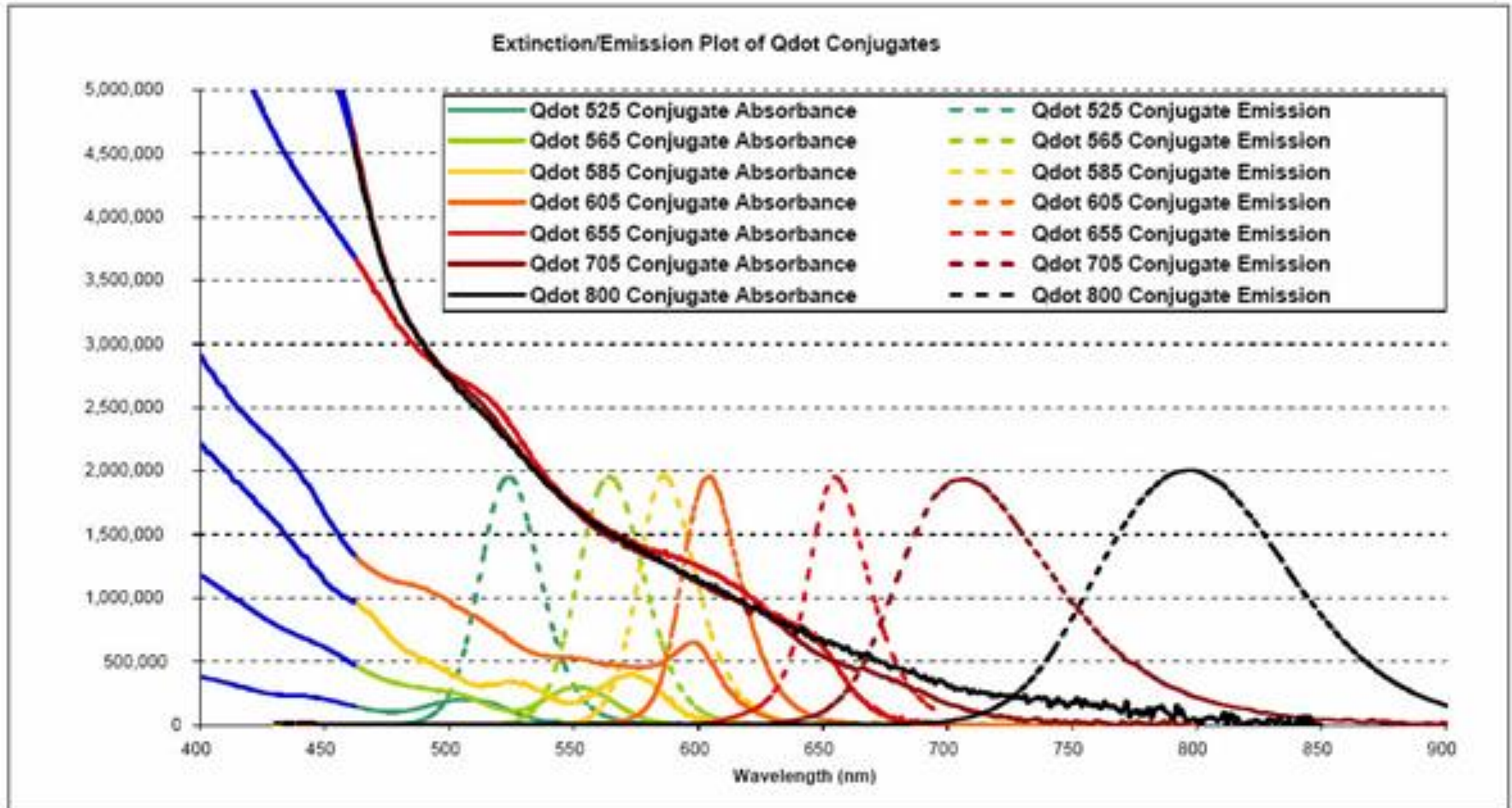
- 1000s available – huge spectral range
- Easy to acquire
- Precisely tailored properties, including environmental sensitivity
- Require fixing and staining, which can lead to artifacts
- Potential self-quenching and environmental sensitivity

Quantum dots

- “Artificial atoms” composed of small semiconductior nanocrystals



Quantum dots - spectra



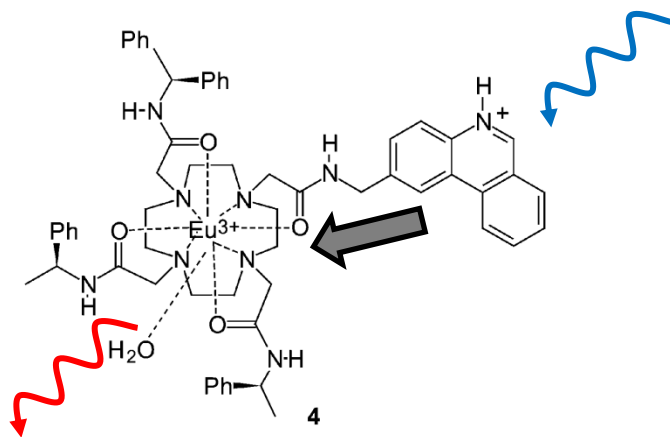
Quantum dots – pros / cons

- 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

Lanthanides: Atomic phosphors

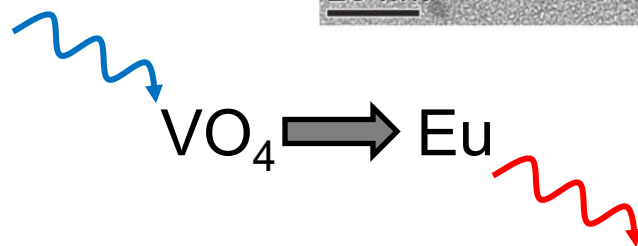
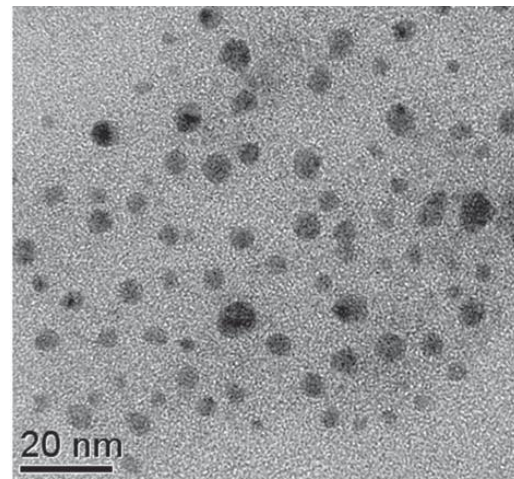
Phosphorescence from atomic 4f transitions

Organic dye antennas



Inorganic (nano)crystals

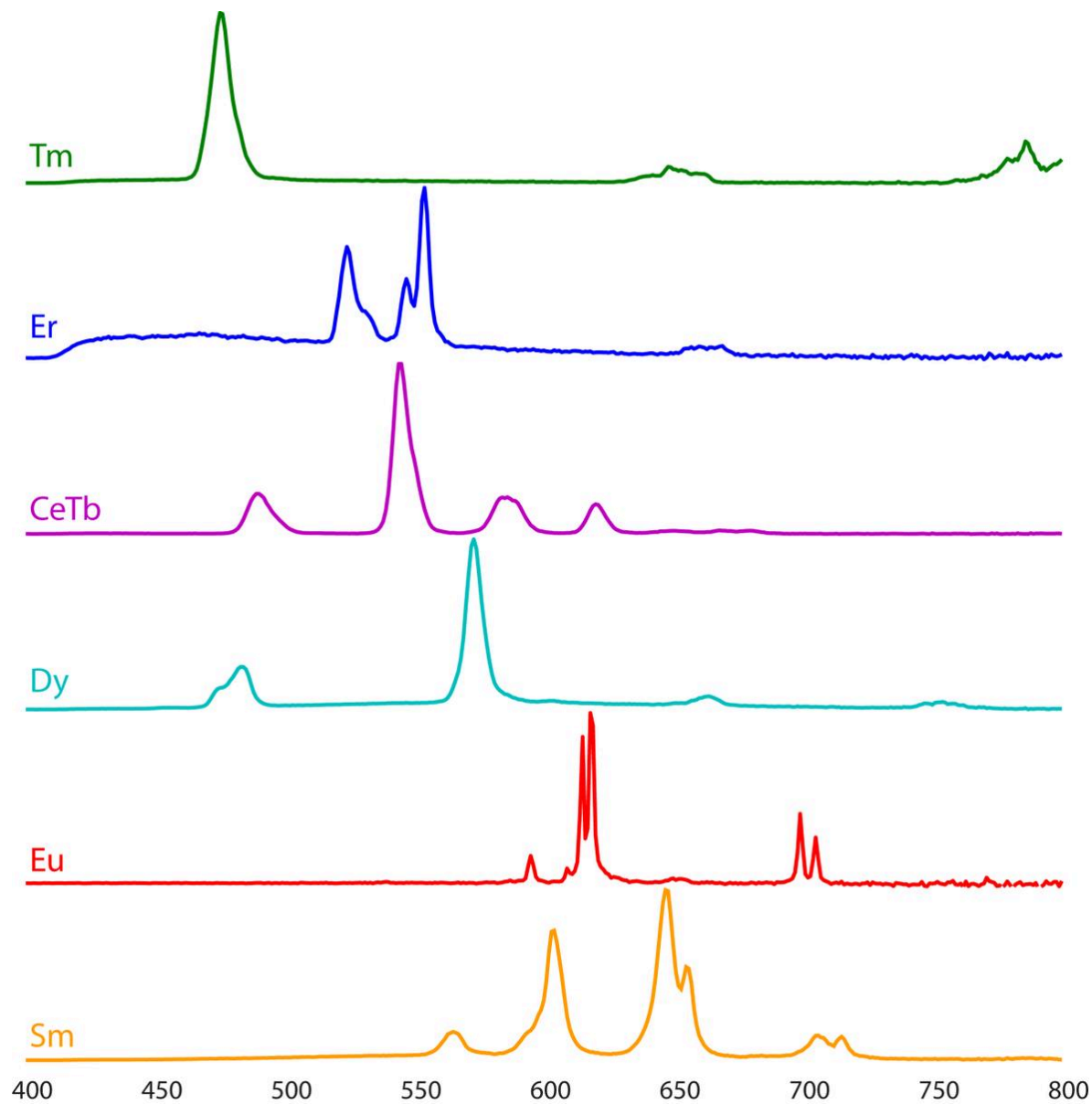
Eu:YVO_4
 $\text{Eu:Y}_2\text{O}_3$



Unique lanthanide properties

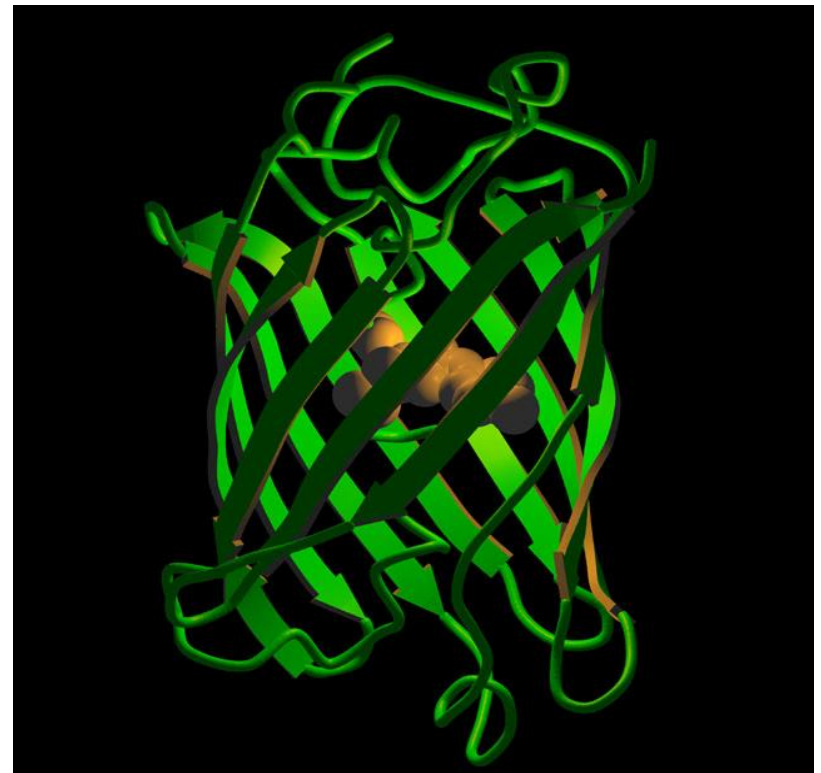
- Very long lifetime: 100 μs – 1 ms
- Very narrow emission lines
- Can change wavelength by changing lanthanide
- No photobleaching
- Uses:
 - Time resolved luminescence
 - Lanthanide phosphors

Lanthanide Nanophosphors



Fluorescent Proteins and Genetically Encoded Tags

See next 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