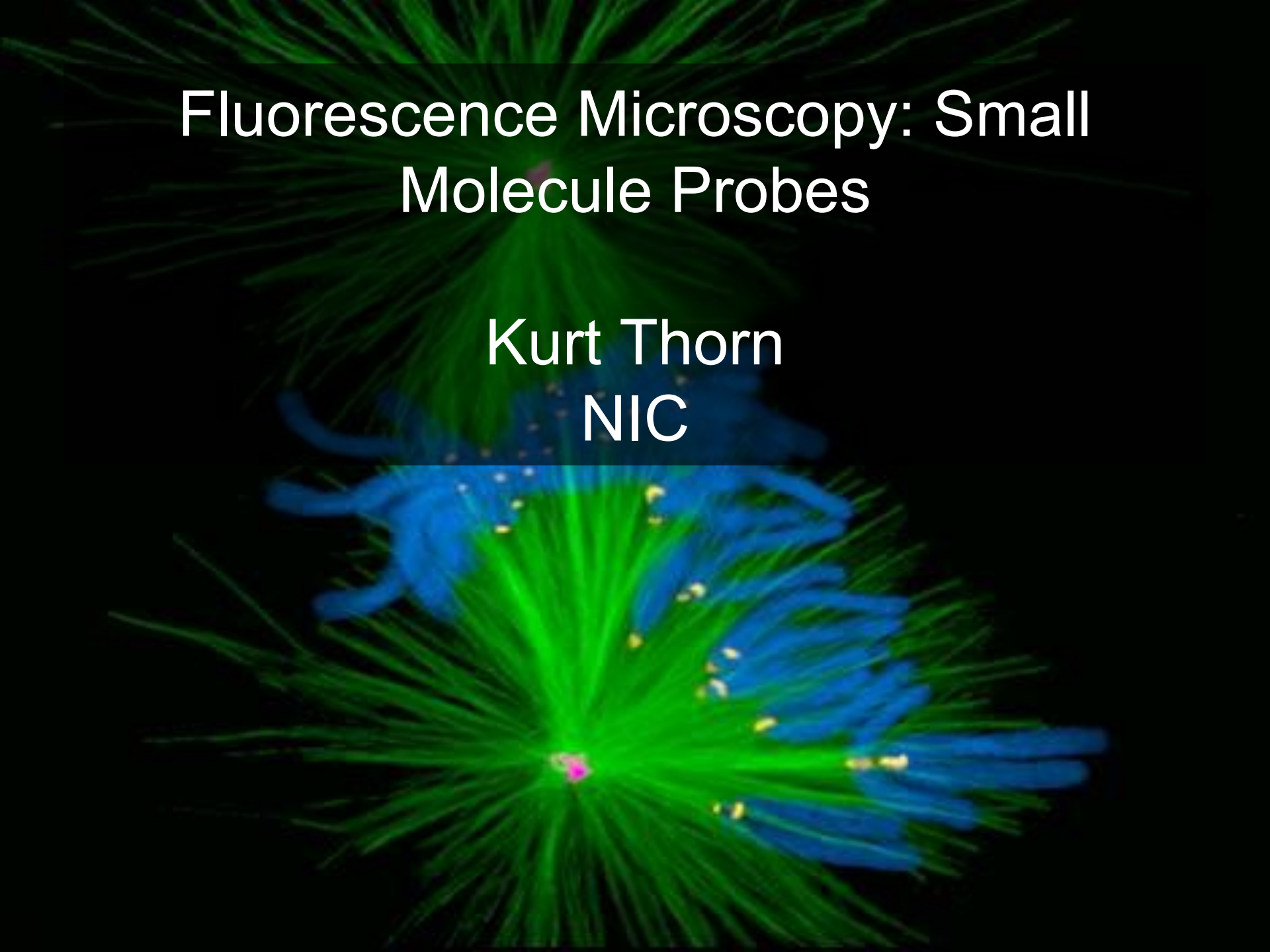
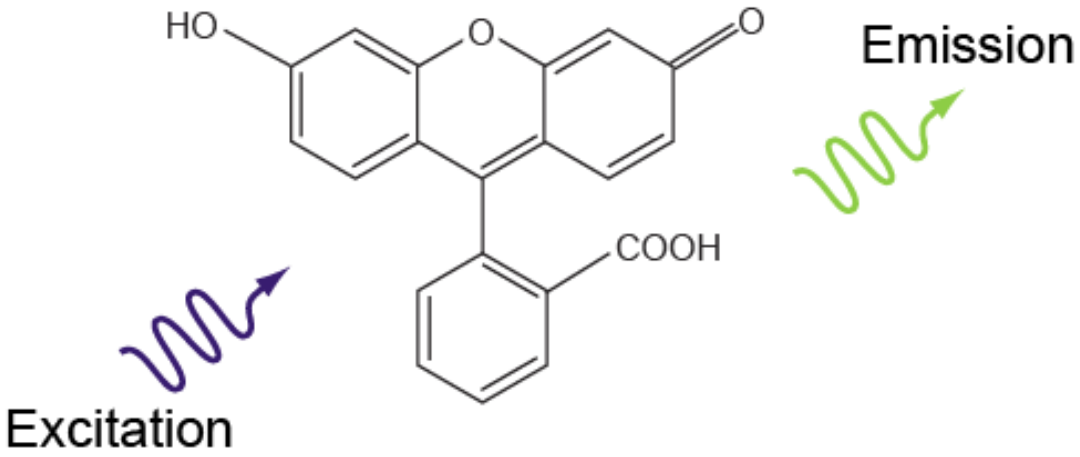
Fluorescence Microscopy: Small Molecule Probes

Kurt Thorn
NIC

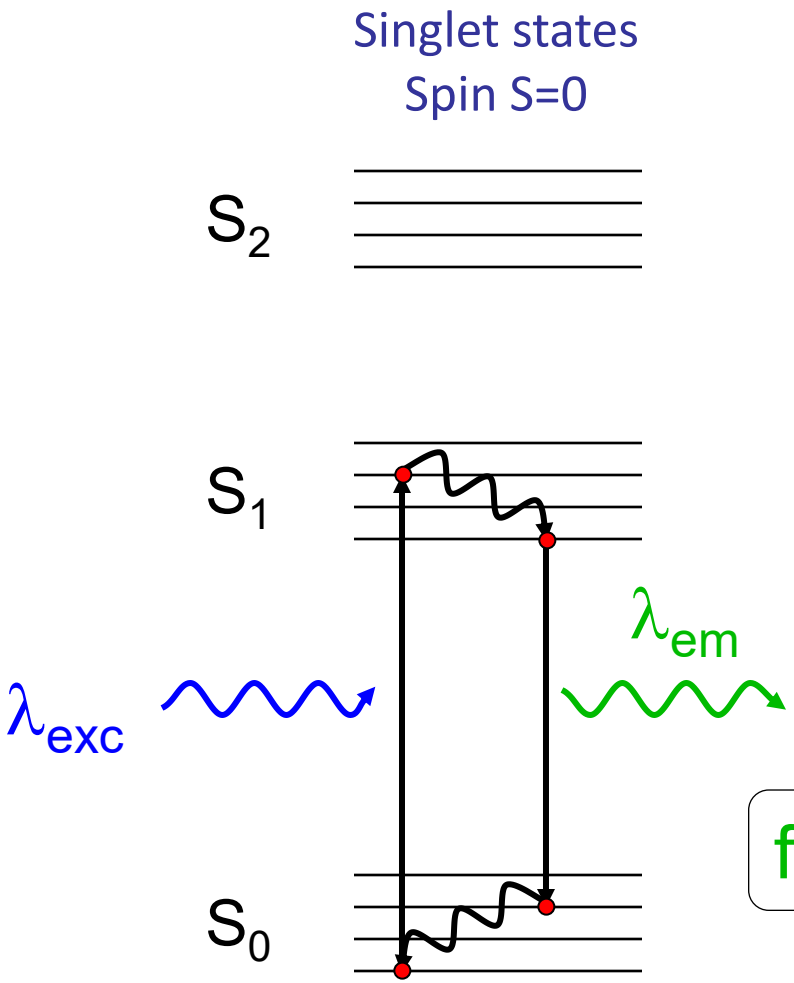


What is fluorescence?



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

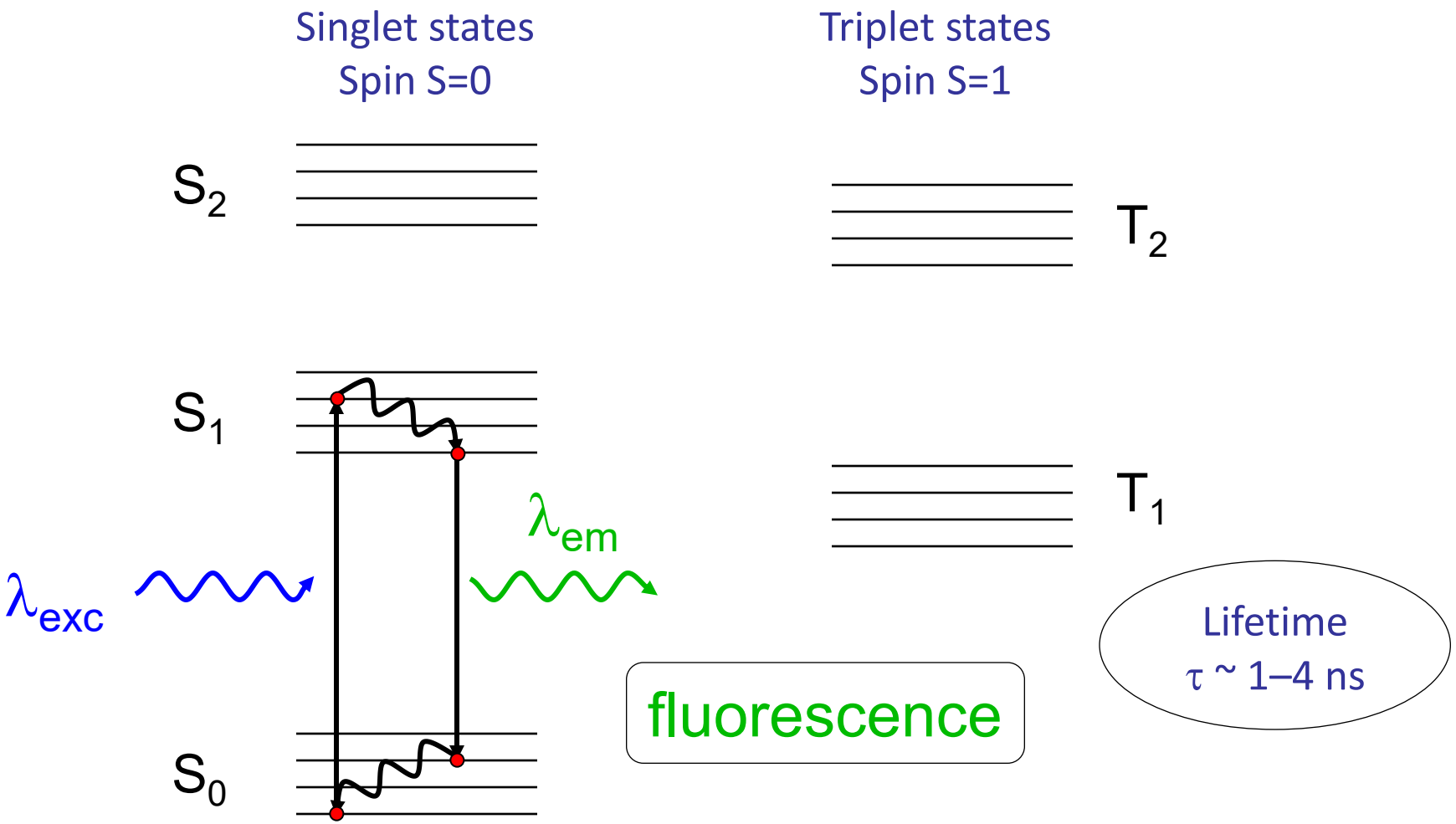
Jabłonski diagram
(Molecular energy diagram)



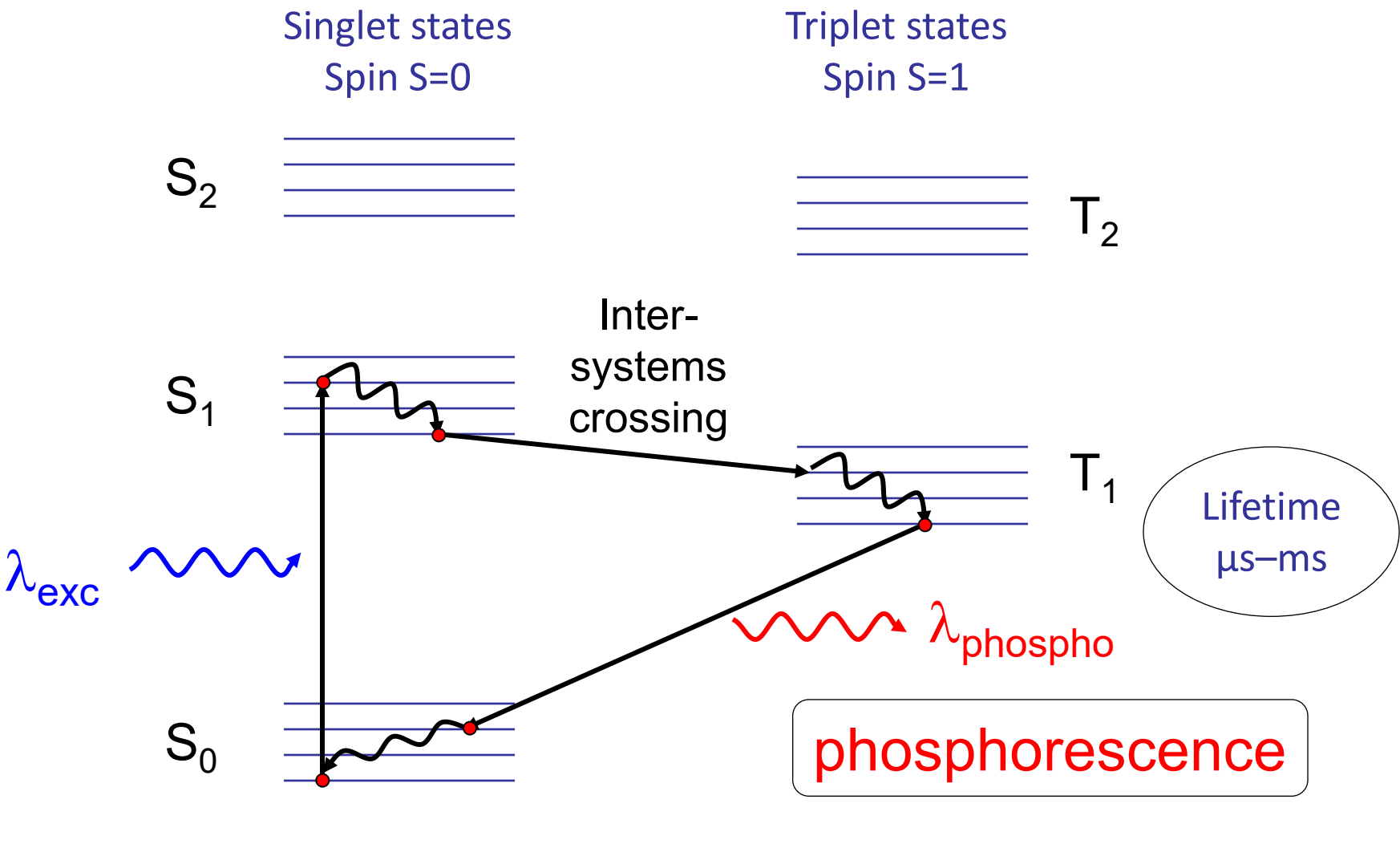
fluorescence

Lifetime
τ ~ 1–4 ns

Jabłonski diagram
(Molecular energy diagram)

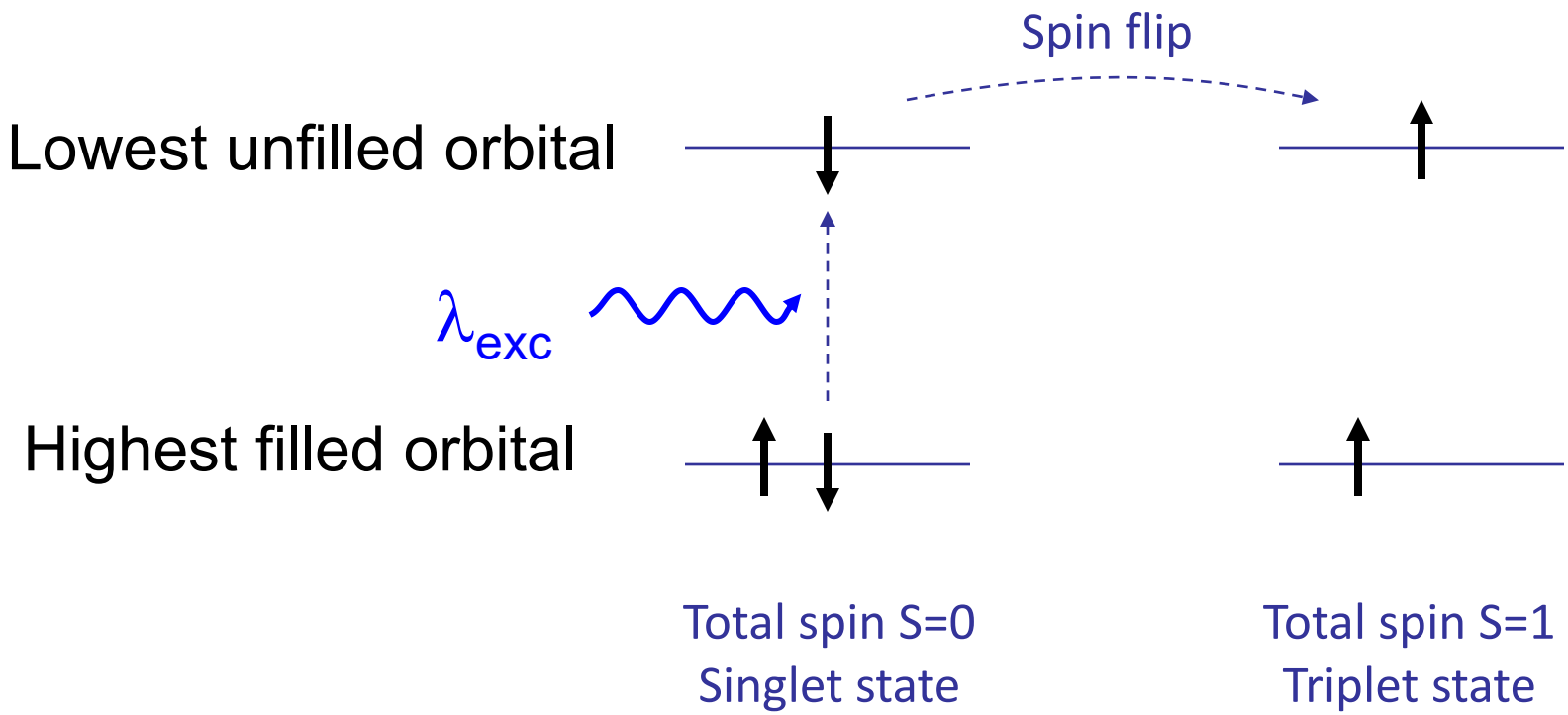


Jabłonski diagram
(Molecular energy diagram)



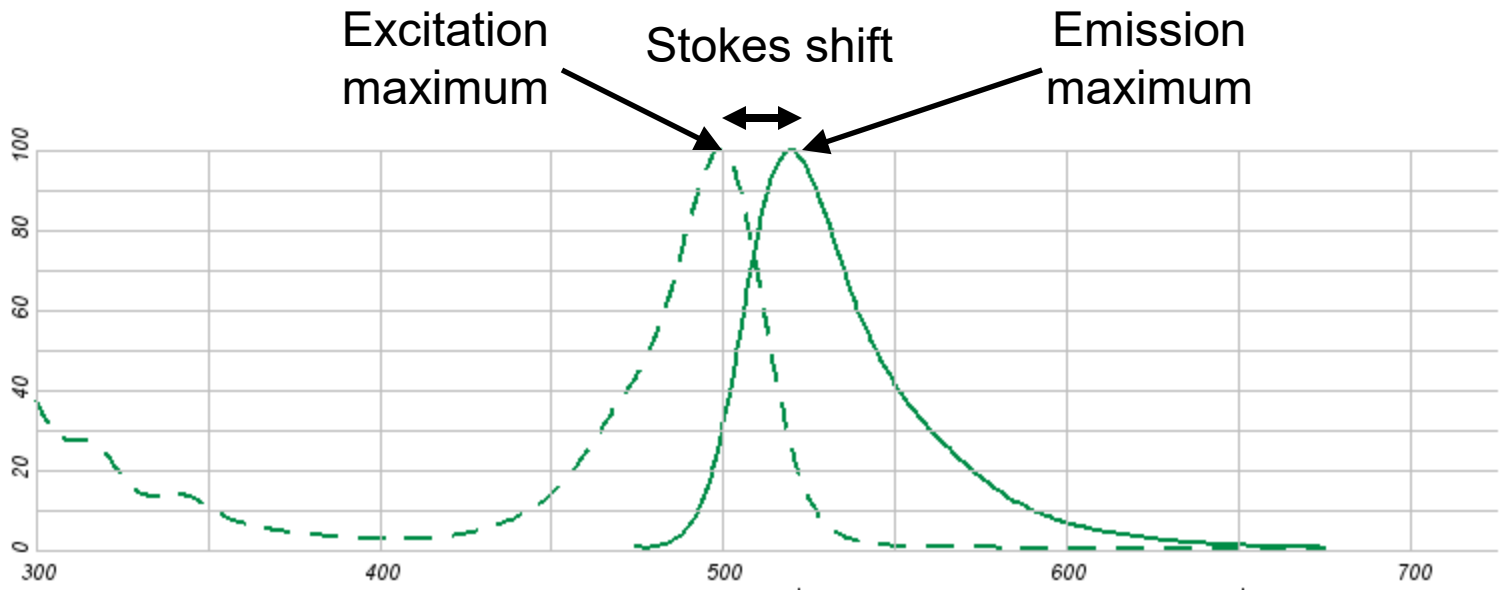
Singlet and Triplet States

Orbital states of *each electron*



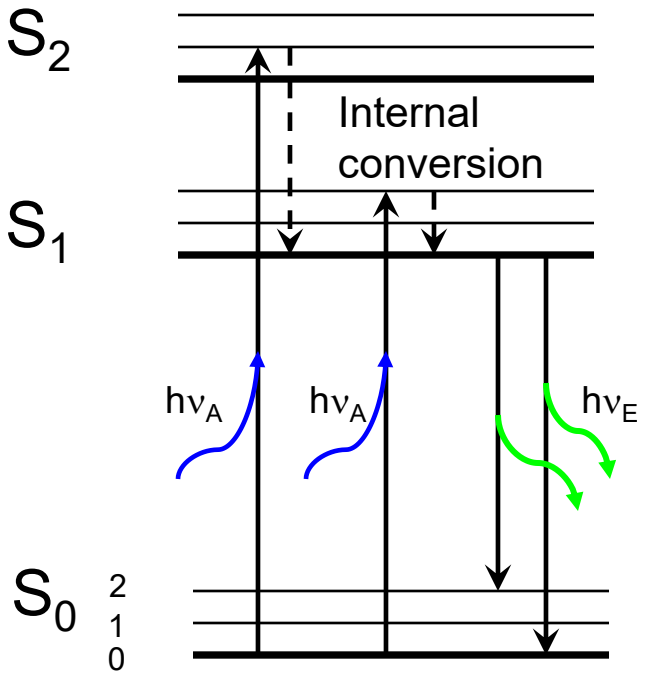
Spin flips are “dipole forbidden” → unlikely → long triplet lifetime

Fluorescence Spectra



Alexa 488

Fluorophore saturation

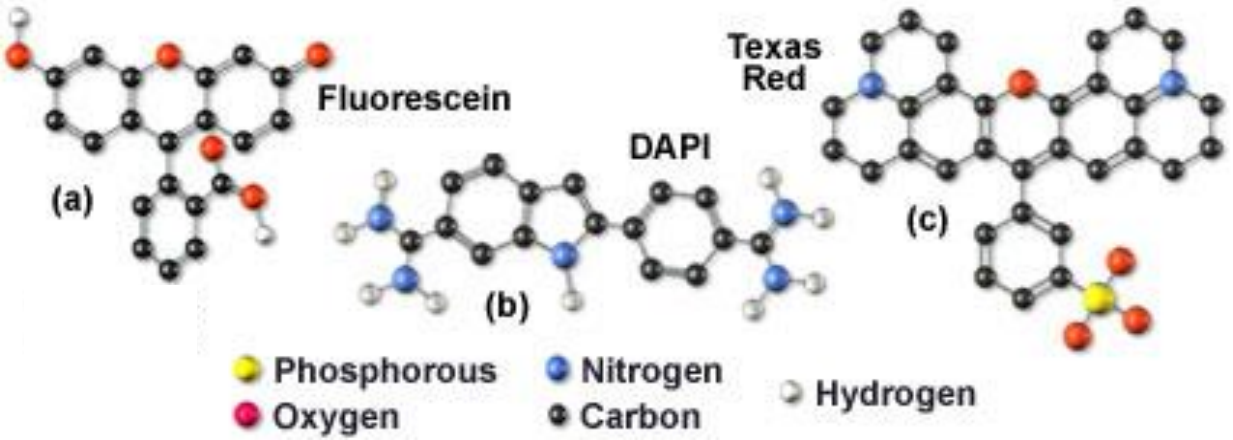


Fluorescence lifetime is ~1-5ns

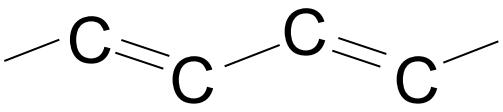
Once illumination intensity is high enough to excite the fluorophore as soon as it deexcites, further intensity increases will not increase brightness

Usually only a problem for confocal

Fluorescent molecules



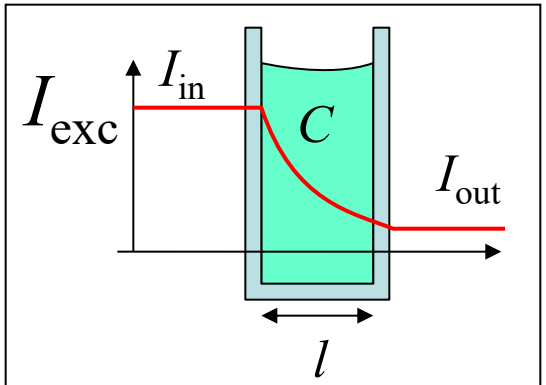
Systems of conjugated bonds
that share electrons



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.
  $\propto Q_f / Q_b$



$$I_{out} = I_{in} 10^{-\varepsilon l C}$$

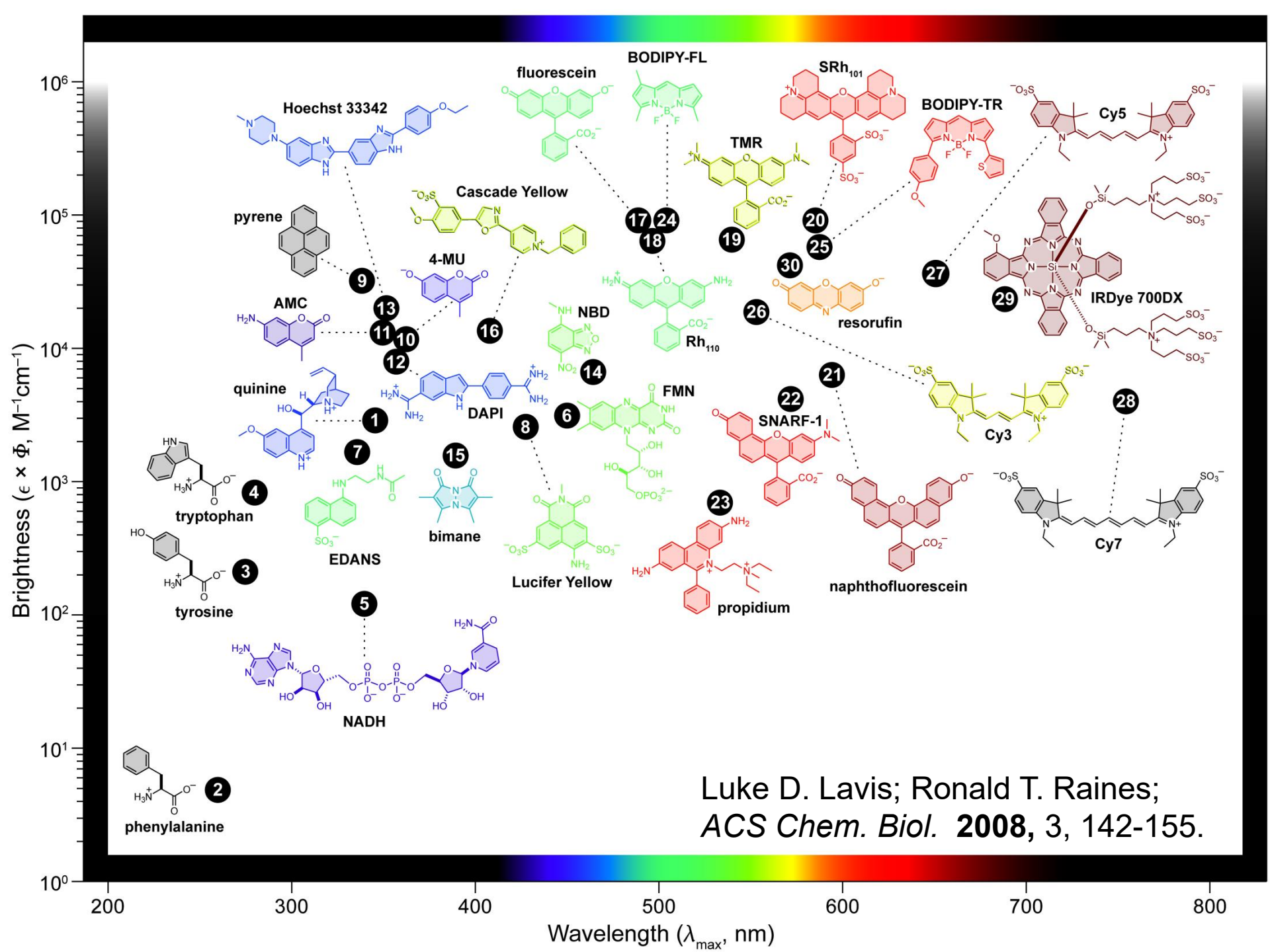
ε: extinction coefficent ($M^{-1}cm^{-1}$)

l: length (cm)

C: concentration (M)

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



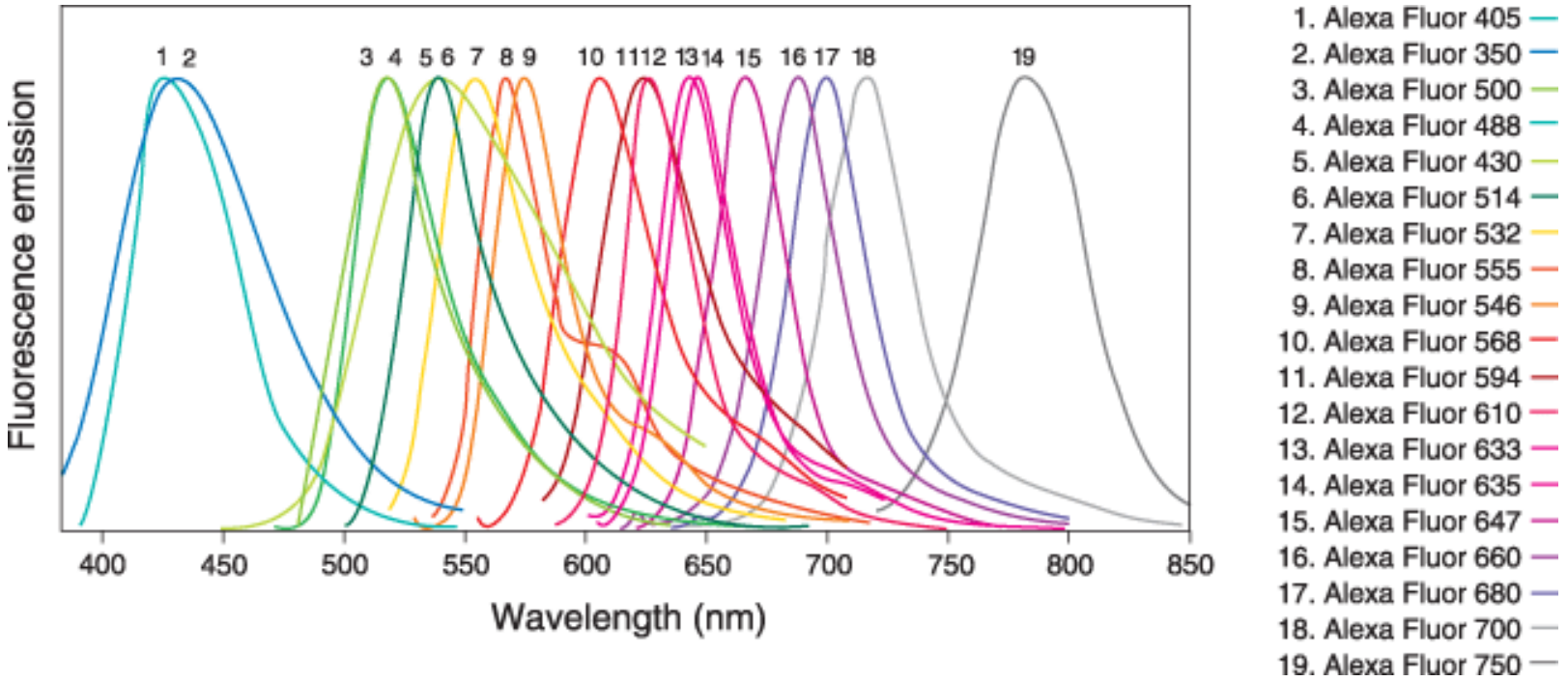
Luke D. Lavis; Ronald T. Raines;
ACS Chem. Biol. **2008,** 3, 142-155.

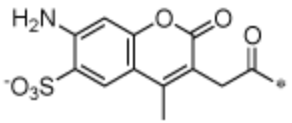
Commercial Dye Series

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

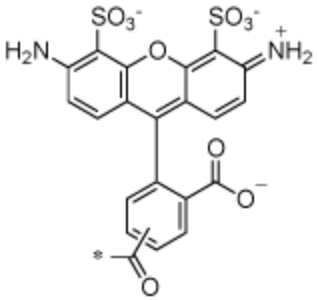
Not chemical families – marketing families

Alexa Fluors





Alexa Fluor® 350



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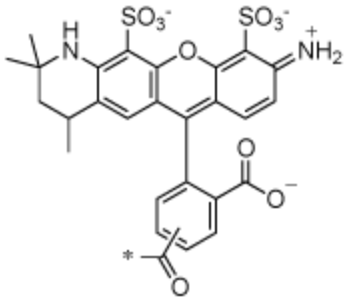

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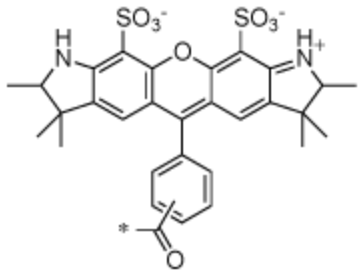
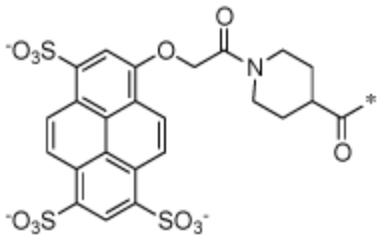
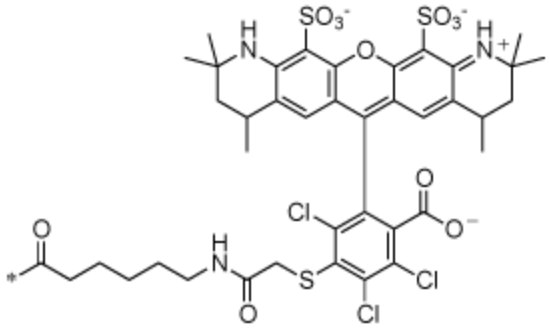


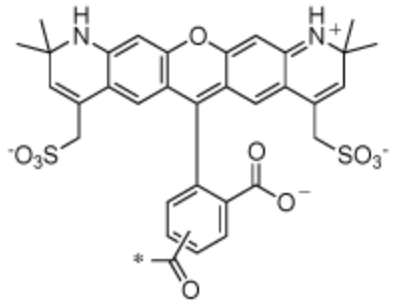
Alexa Fluor® 532



Alexa Fluor® 405



Alexa Fluor® 546



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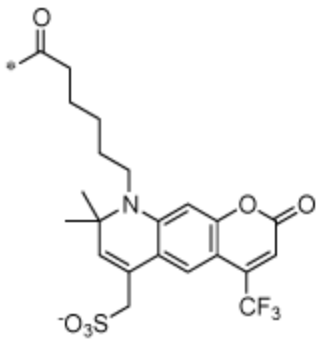

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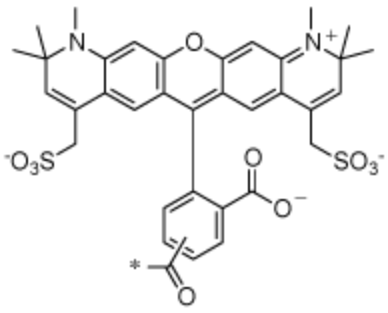

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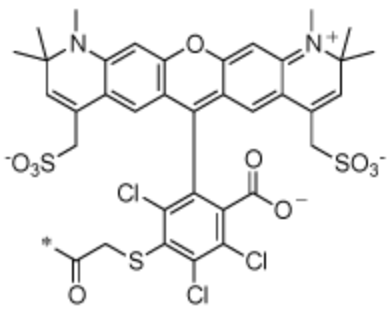
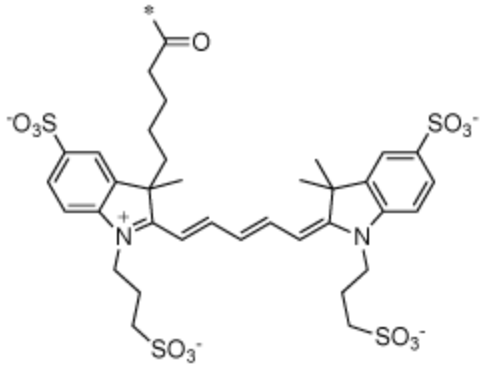


Alexa Fluor® 610



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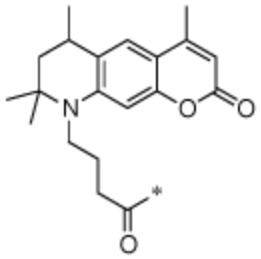

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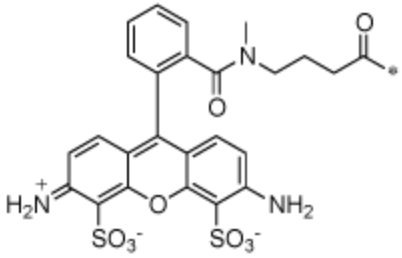

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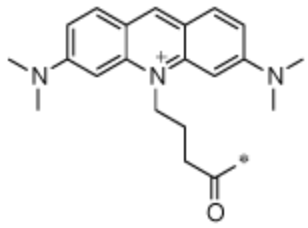

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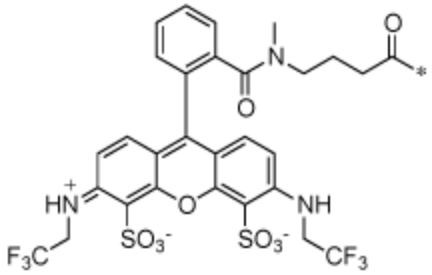

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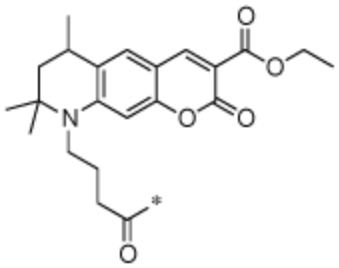

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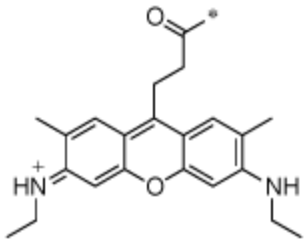

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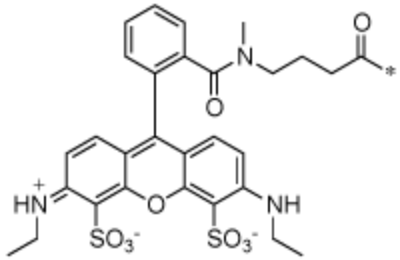

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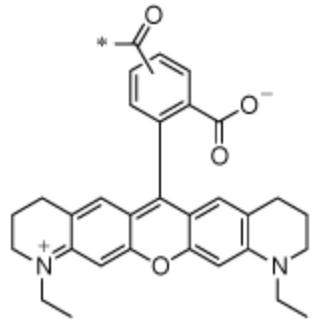

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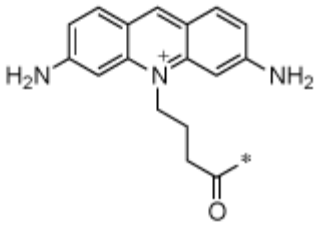

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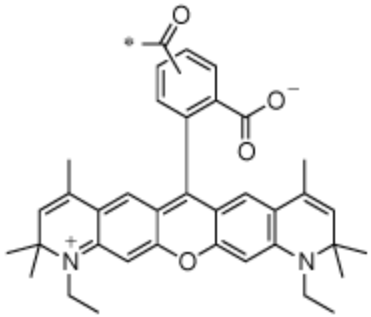

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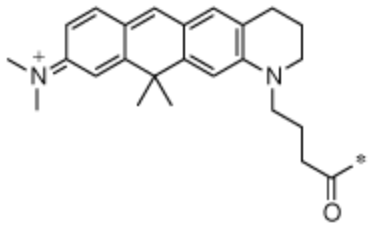
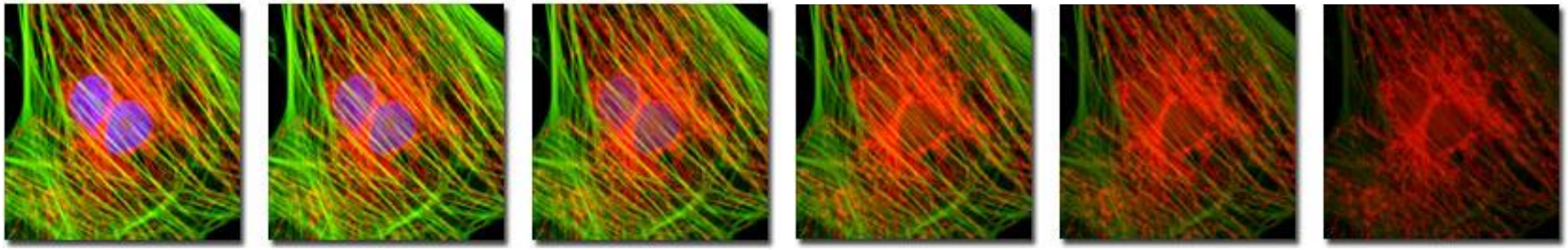


ATTO 610

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

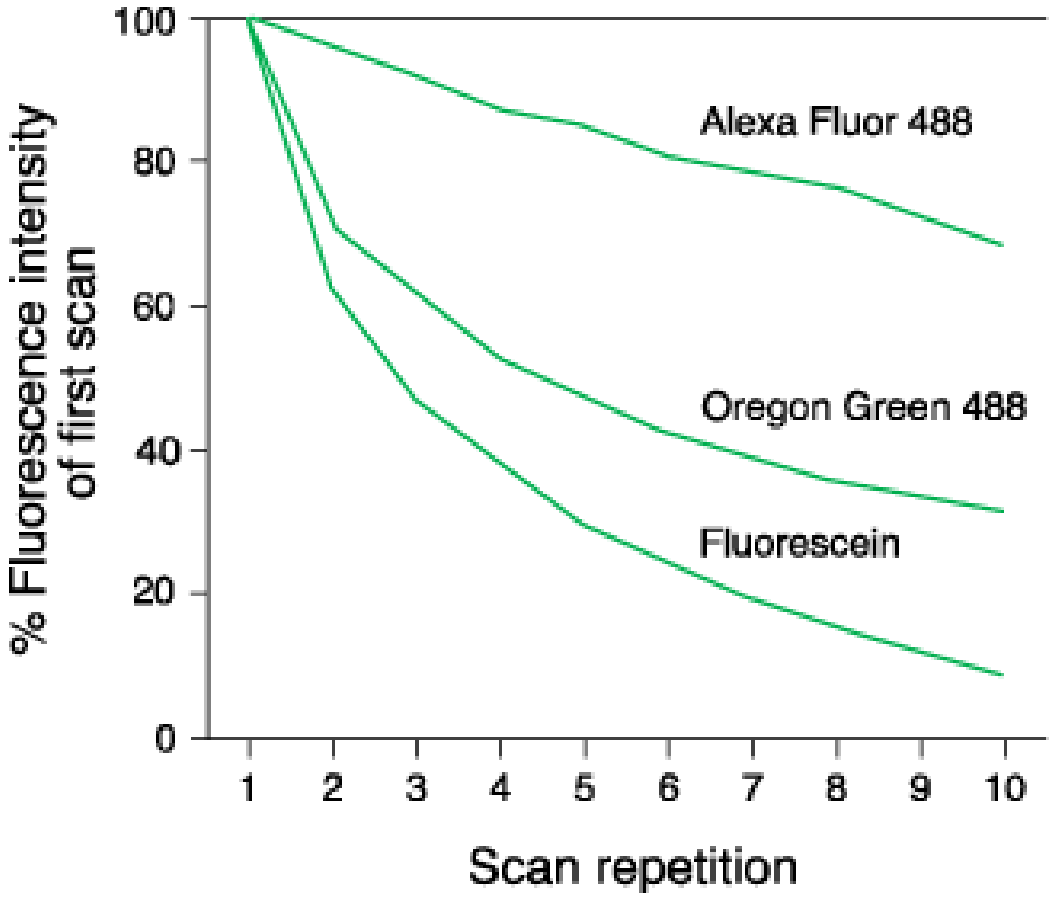
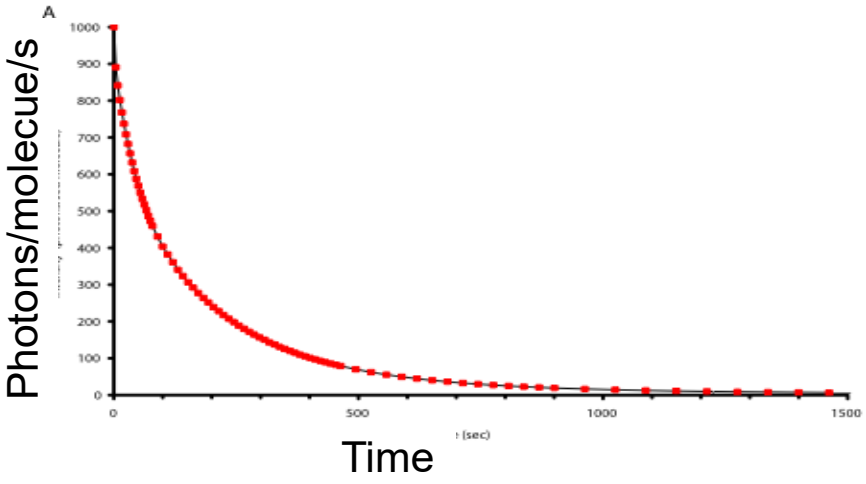
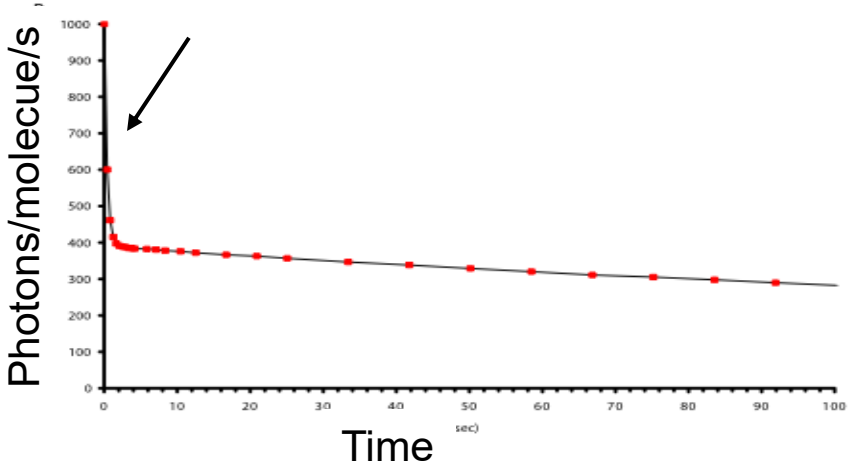


Photo-bleaching of fluorescent proteins



mCherry

Single-exponential bleaching



Emerald

Double-exponential bleaching

Fast- and slow-bleaching populations?

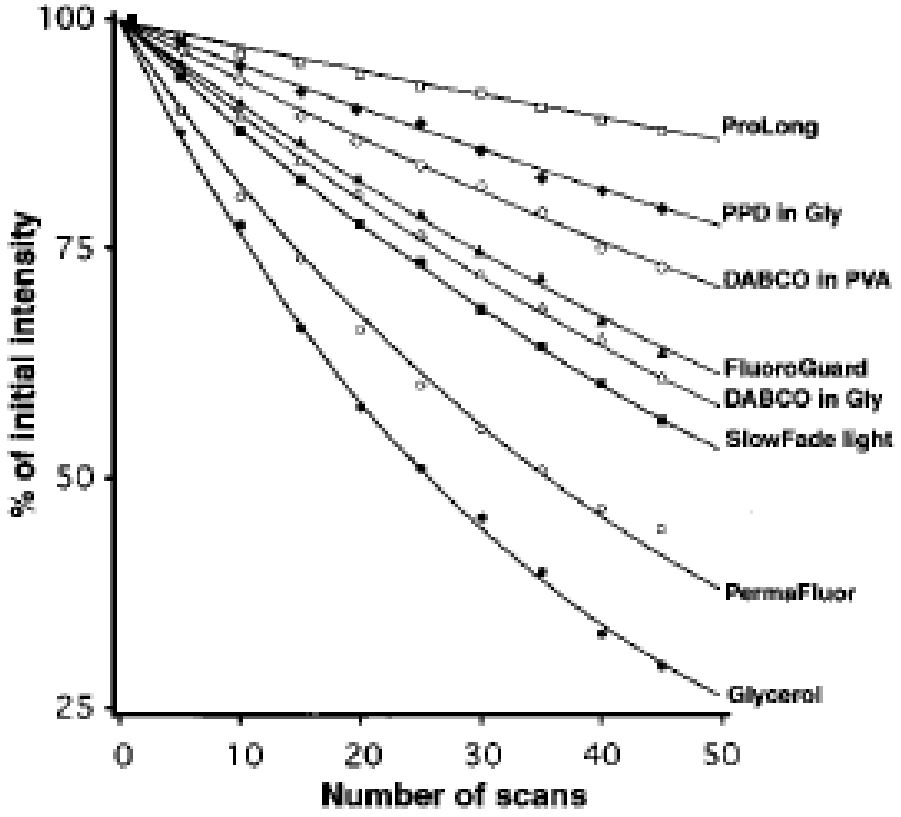
What to do about photo-bleaching?

- 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.

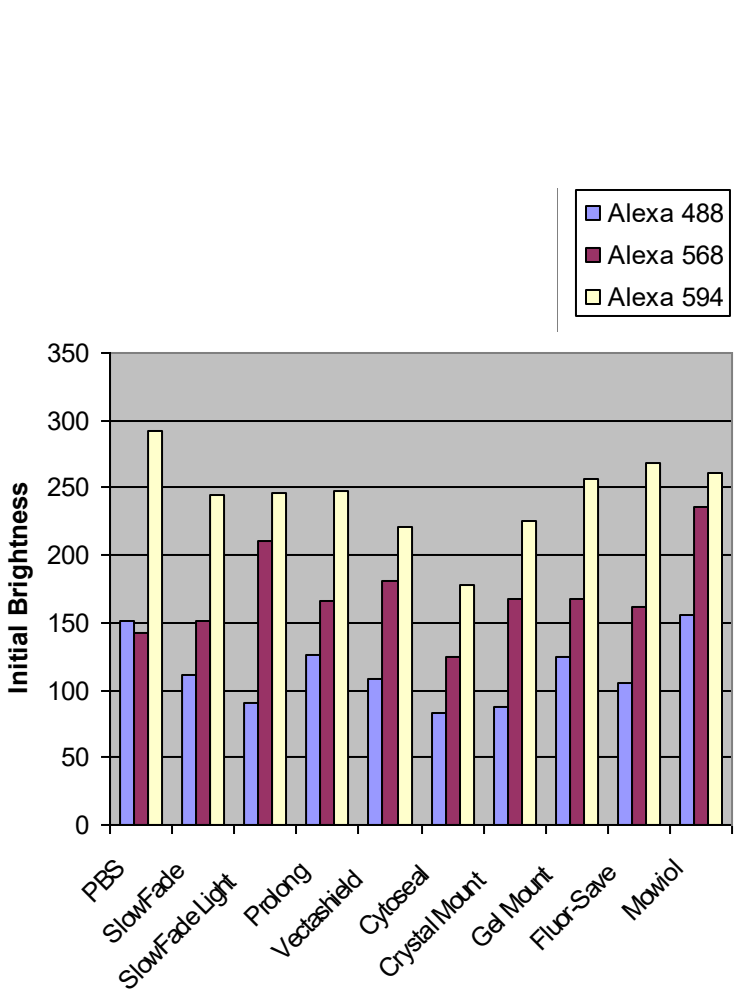
- Budget the photons you have
  - Only expose when observing
  - Minimize exposure time & excitation power
  - Use efficient filter combinations
  - Use highly QE, low noise camera
  - Use simple light path

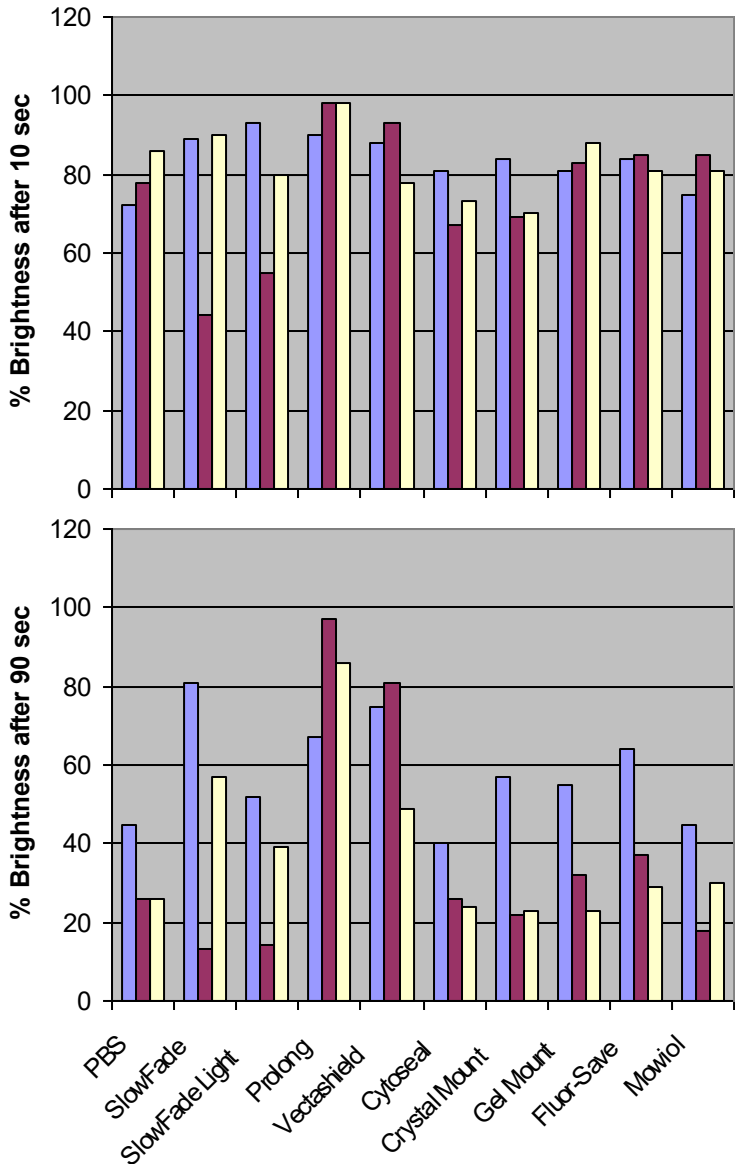
Effect of mounting medium on FITC bleaching



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

Effect of mounting media on Alexa bleaching



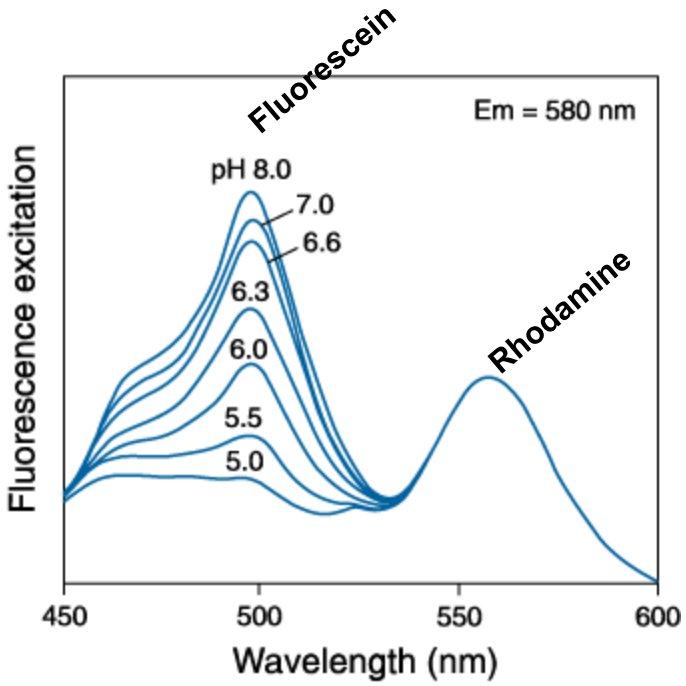


Factors affecting overall brightness

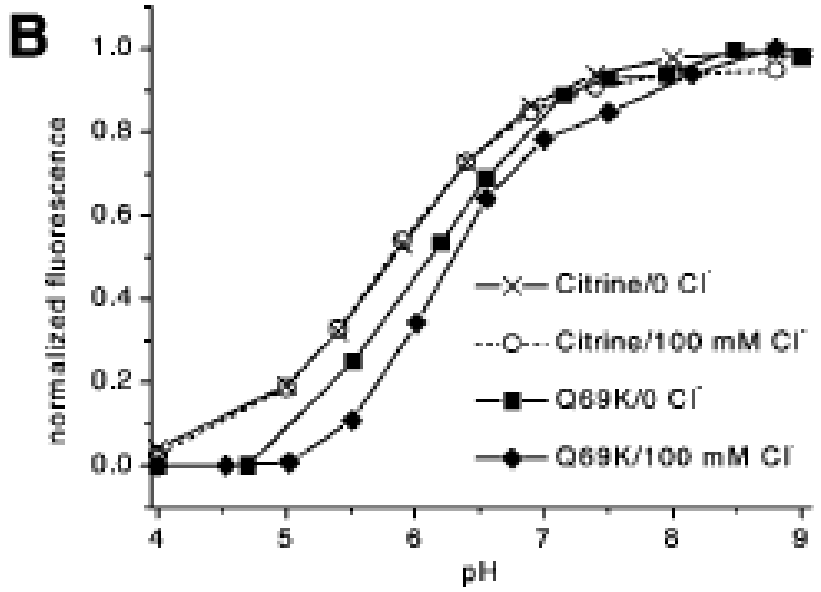
- Intrinisic brightness
- Spectrum of arc lamp/lasers
- Lamp/laser power
- Filter set transmission
- Quantum efficiency of detector
- Photobleaching
- Quenching / maturation / other dye-specific effects

pH dependence of dyes

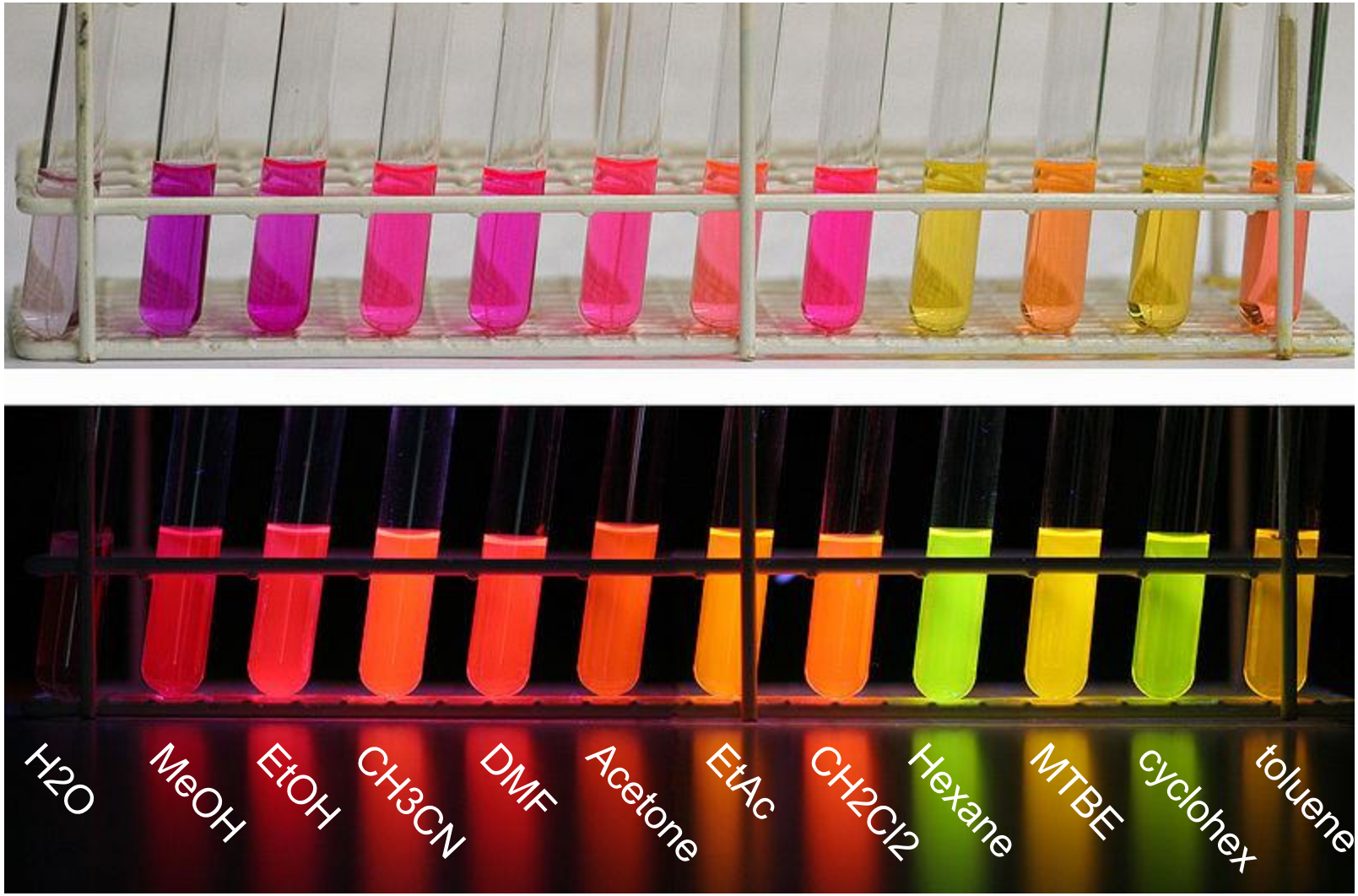
Mixed Fluorescein and Rhodamine



YFP variants



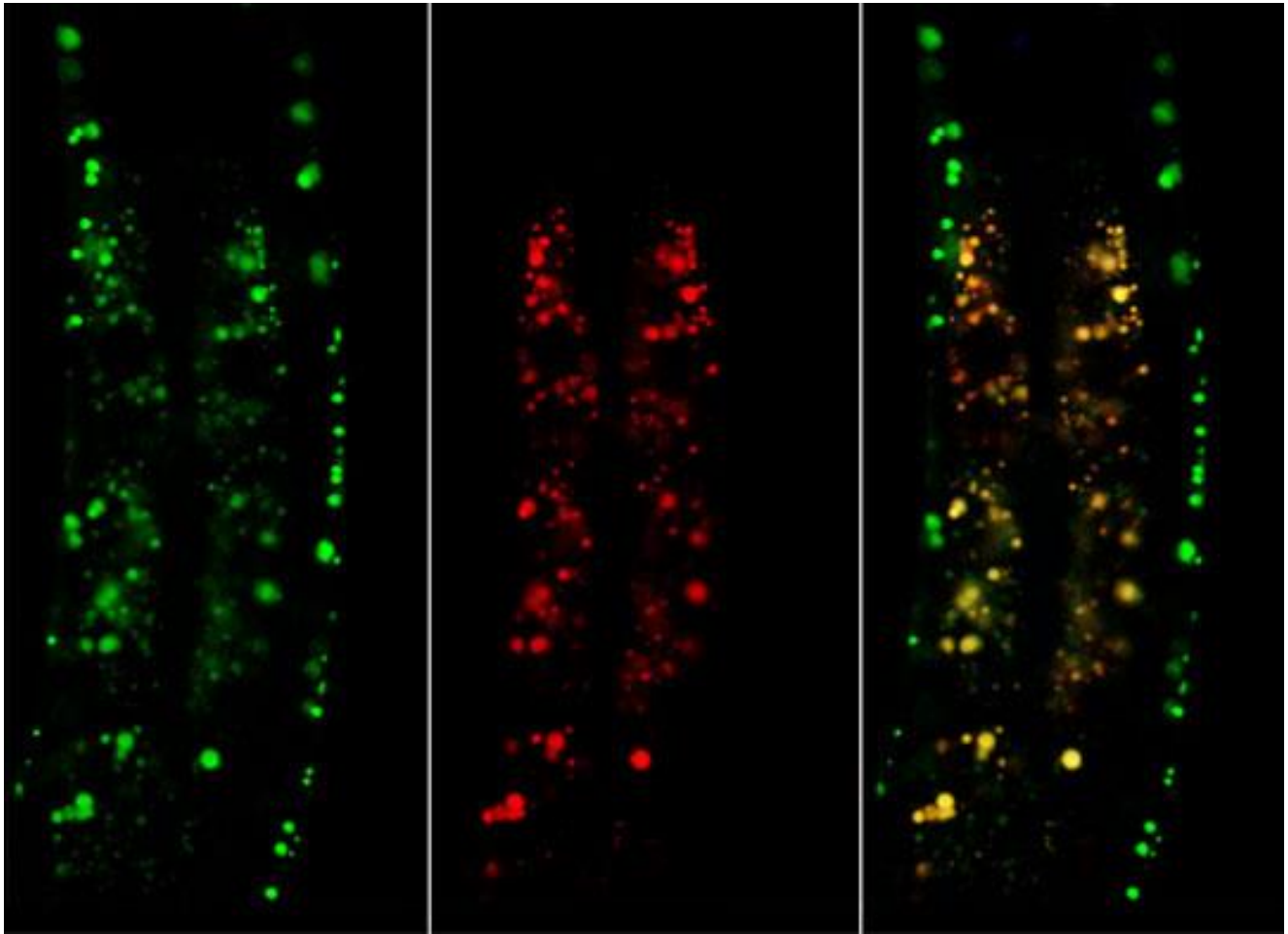
Environmental Dependence: Nile red



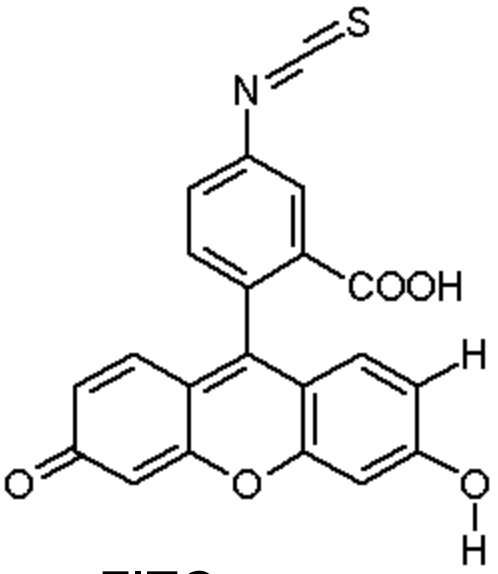
http://en.wikipedia.org/wiki/File:Nile_red_01.jpg

Environmental Dependence: Nile Red

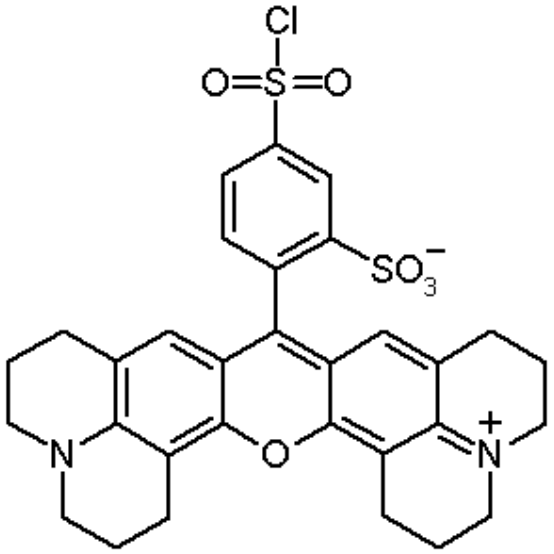
C. elegans staining with Nile Red and BODIPY-fatty acid
(Kevin Jones, Ashrafi lab)



Fluorescent dyes in Biology



FITC

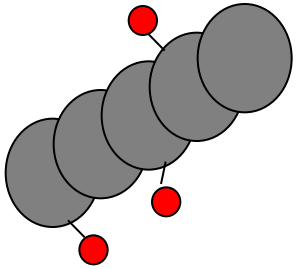


Texas Red

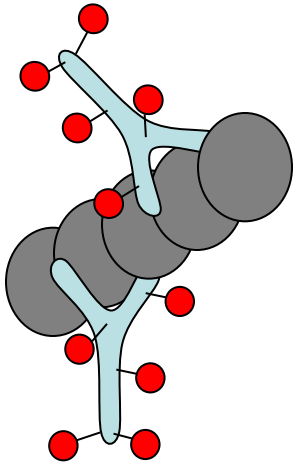
- Protein labeling: couple to amino- or sulfhydryl groups
- Direct and indirect (immuno-) fluorescence

Fluorescent labeling

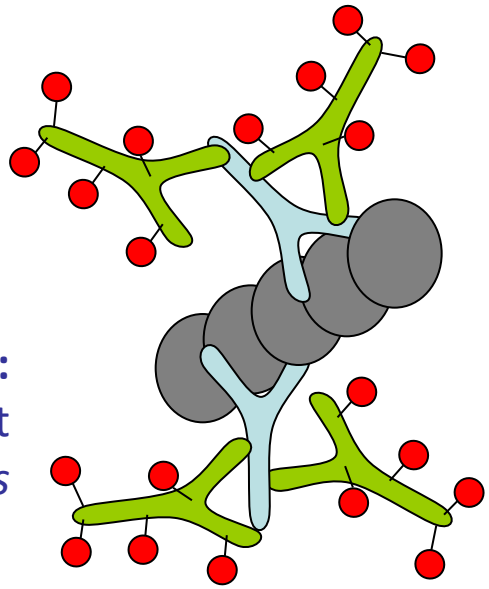
Direct labeling (& microinjection)
of target molecules



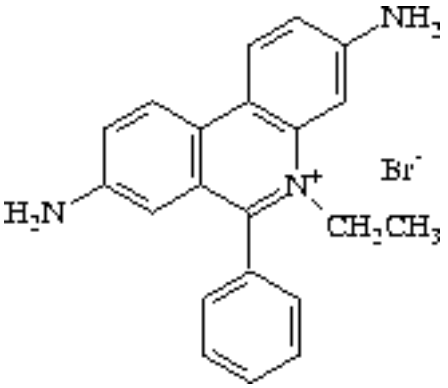
Direct immunofluorescence:
labeled antibodies against target



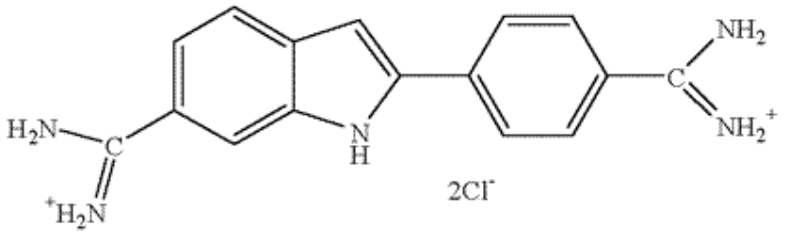
Indirect immunofluorescence:
Unlabeled antibodies against target
Labeled antibodies *against those antibodies*



DNA Probes



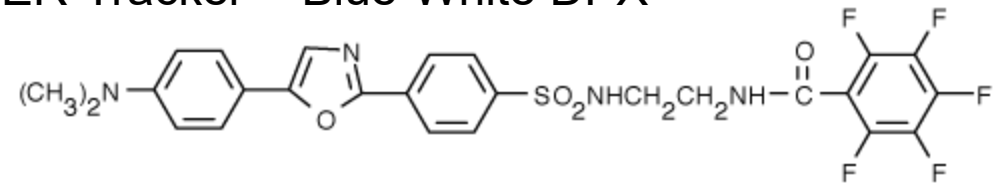
Ethidium Bromide
~30 fold enhancement



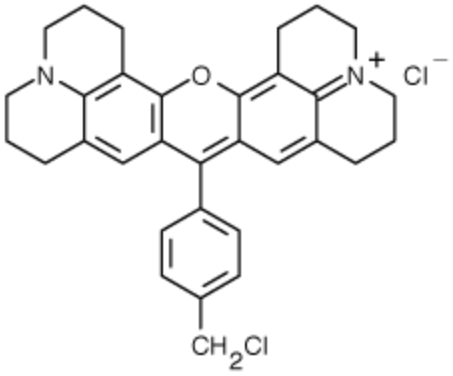
DAPI
Hoechst 33258
Hoechst 33342
~20 fold enhancement

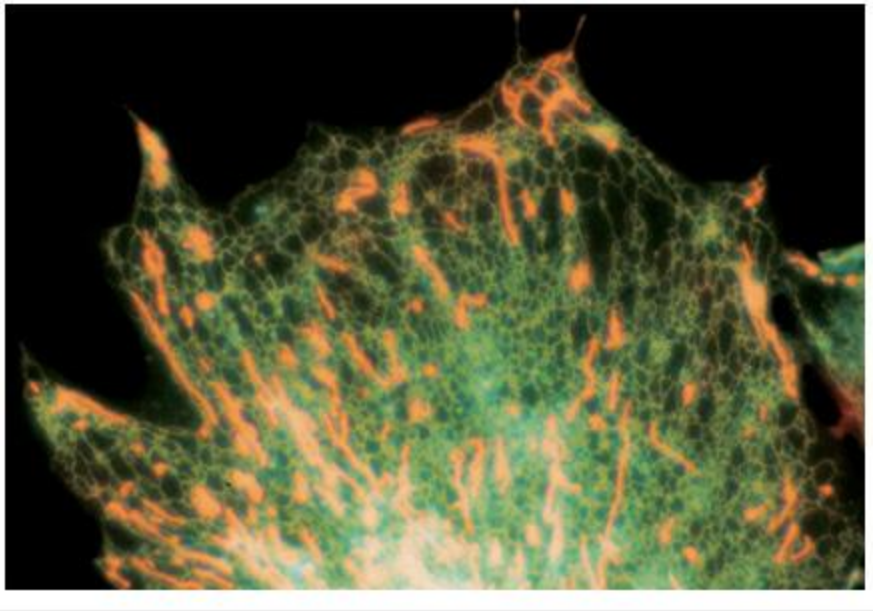
Other probes

ER-Tracker™ Blue-White DPX



MitoTracker Red CMXRos





Probes for Golgi, lysosomes, and peroxisomes are also available

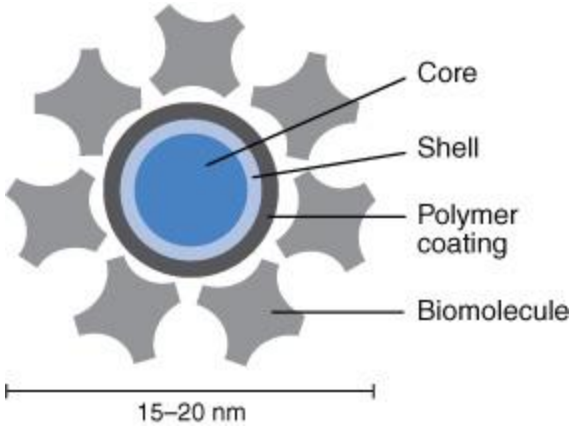
Small molecules – pros / cons

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

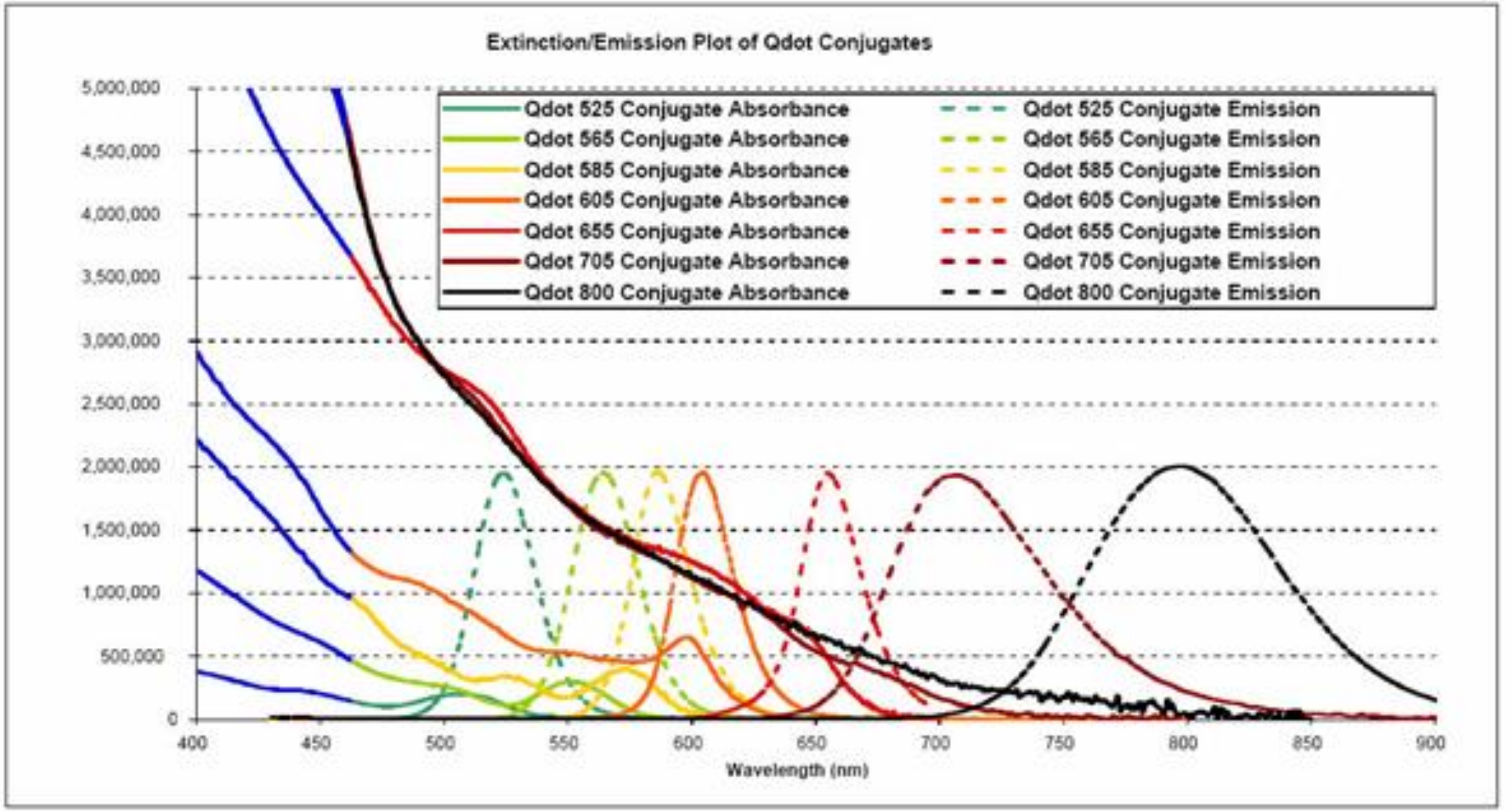
- Require fixing and staining, which can lead to artifacts
- Potential self-quenching and environmental sensitivity

Quantum dots

- “Artificial atoms” composed of small semiconductior nanocrystals



Quantum dots - spectra



Quantum dots – pros / cons

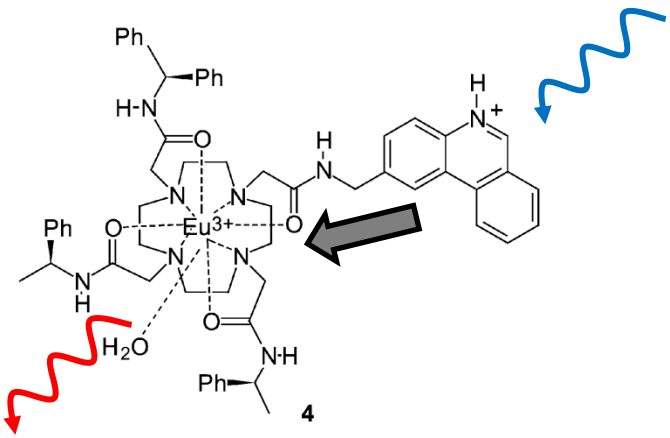
- Little to no photobleaching
- Very bright
- Can use single excitation wavelength for multiple dyes
- Narrow emission spectra

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

Lanthanides: Atomic phosphors

Phosphorescence from atomic 4f transitions

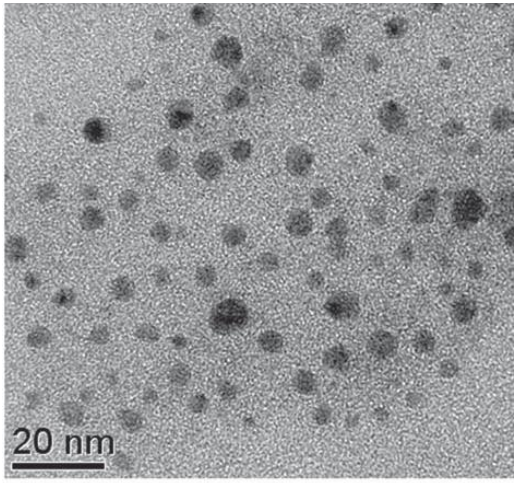
Organic dye antennas

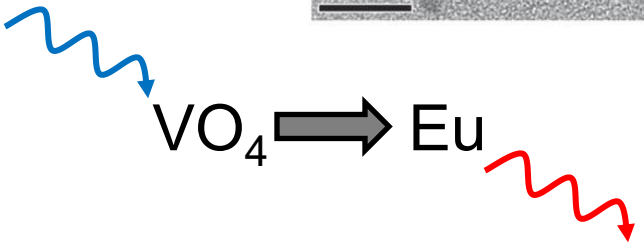


Inorganic (nano)crystals

$Eu:YVO_4$

$Eu:Y_2O_3$



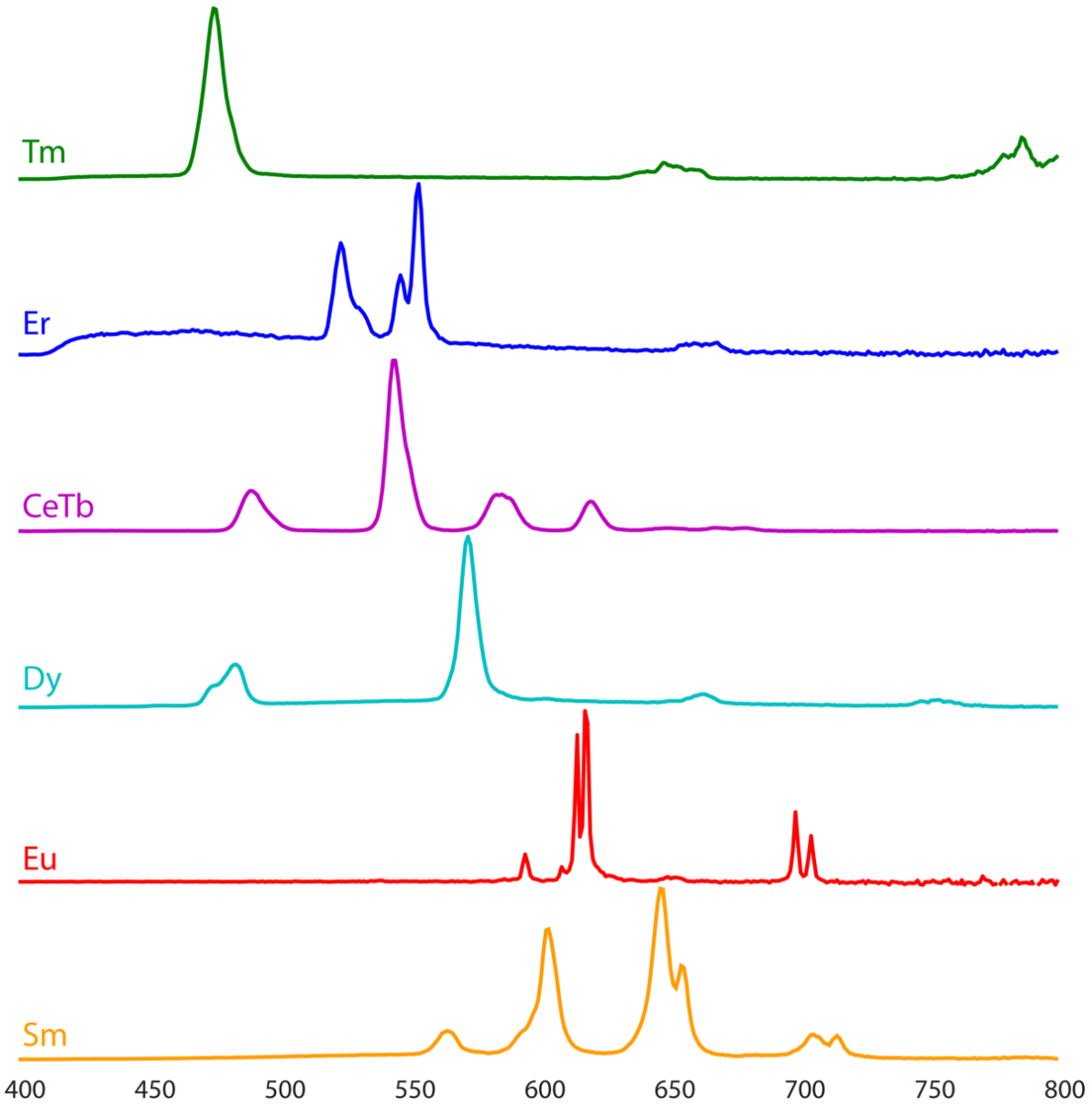


Unique lanthanide properties

- Very long lifetime: 100 μs – 1 ms
- Very narrow emission lines
- Can change wavelength by changing lanthanide
- No photobleaching

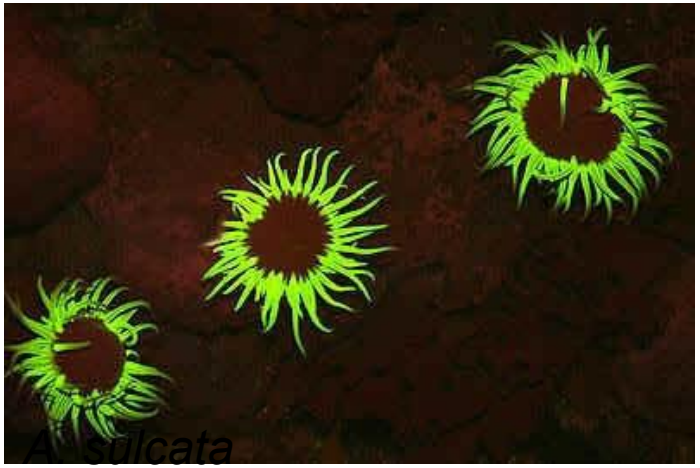
- Uses:
- Time resolved luminescence
- Lanthanide phosphors

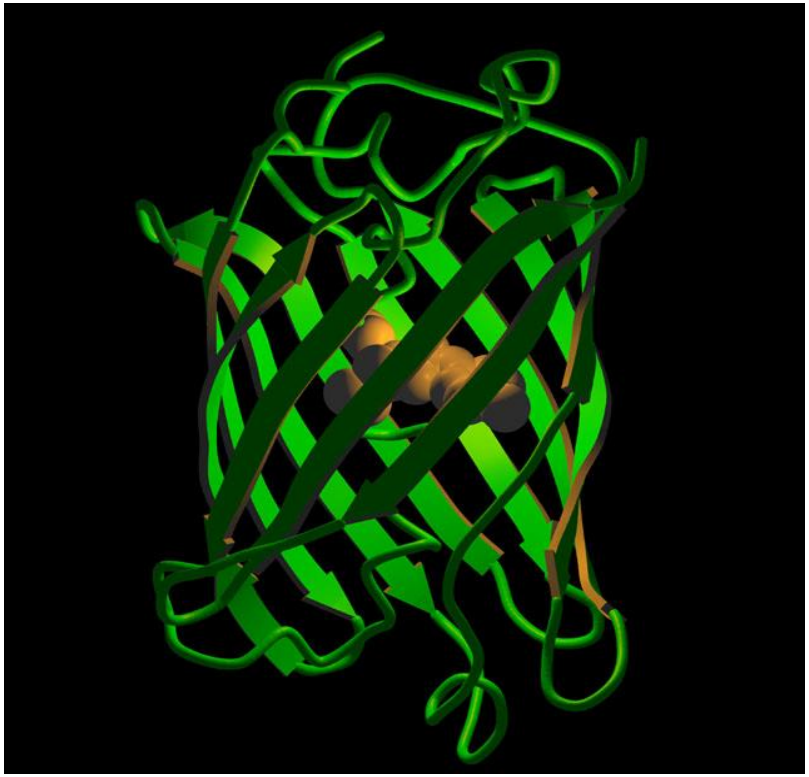
Lanthanide Nanophosphors



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