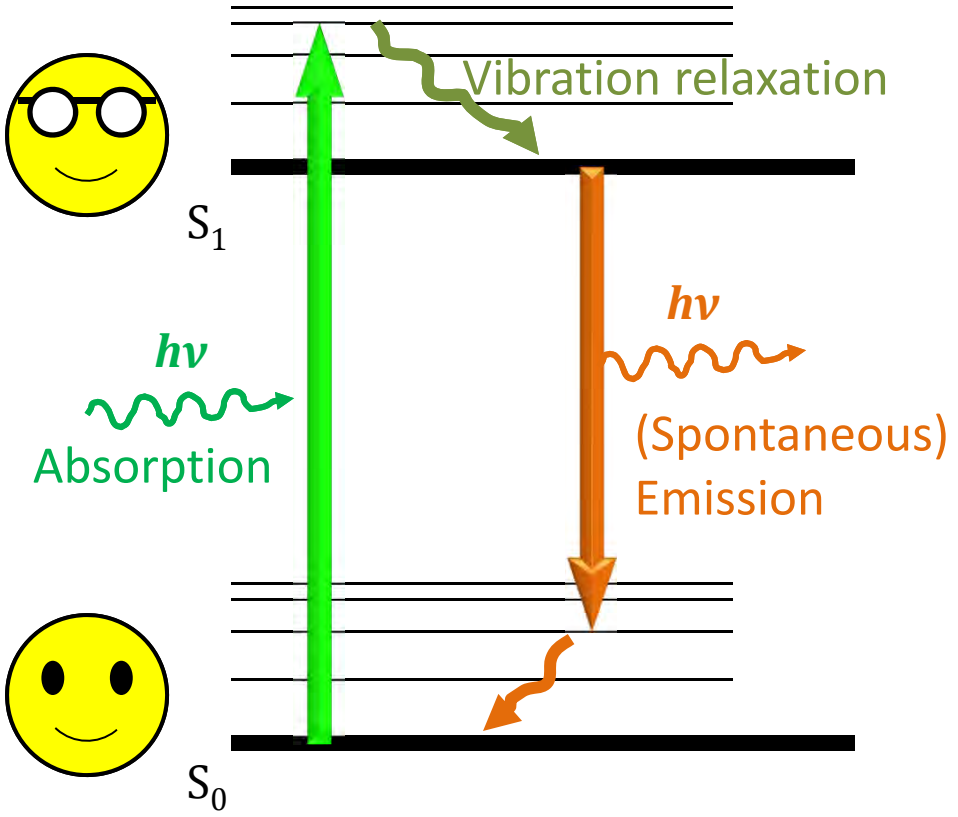
Fluorescence Microscopy

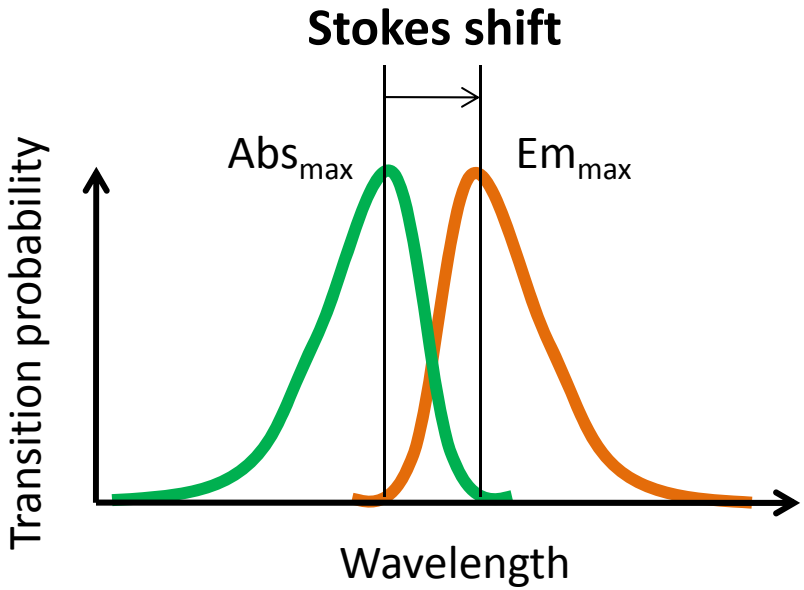
II. Fluorescent dyes

Bo Huang

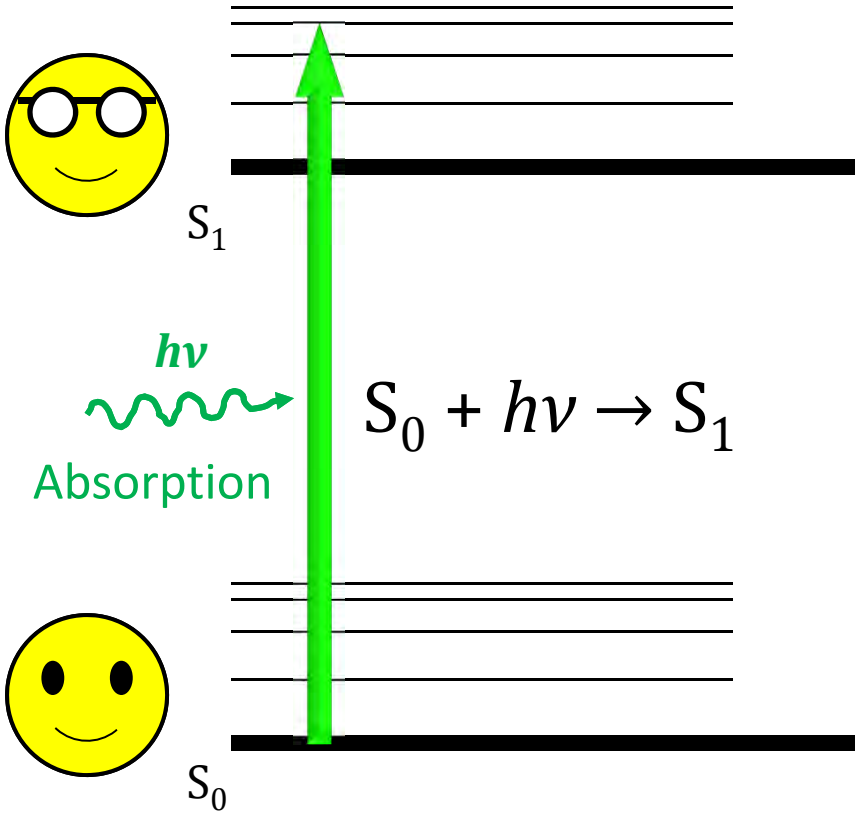
2012.03.27

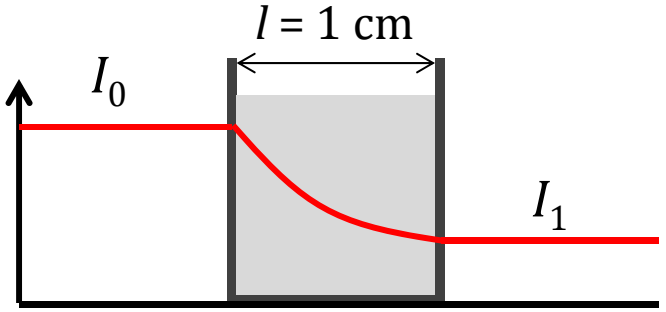
The fluorescence process





Extinction coefficient





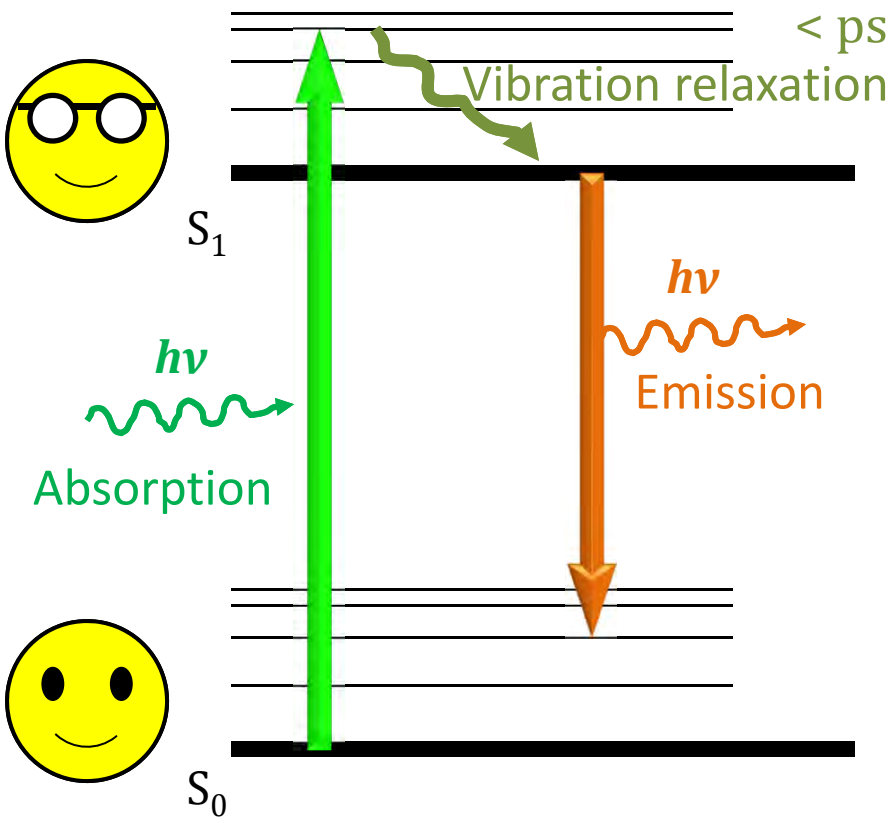
$$\text{Abs} = -\log_{10}(I_1/I_0)$$

$$\varepsilon = \text{Abs} / (l \times c)$$

Typically 50,000 ~ 200,000 $M^{-1}cm^{-1}$

1 μM, 1 cm ➔ A ≈ 0.1 (T = 80%)

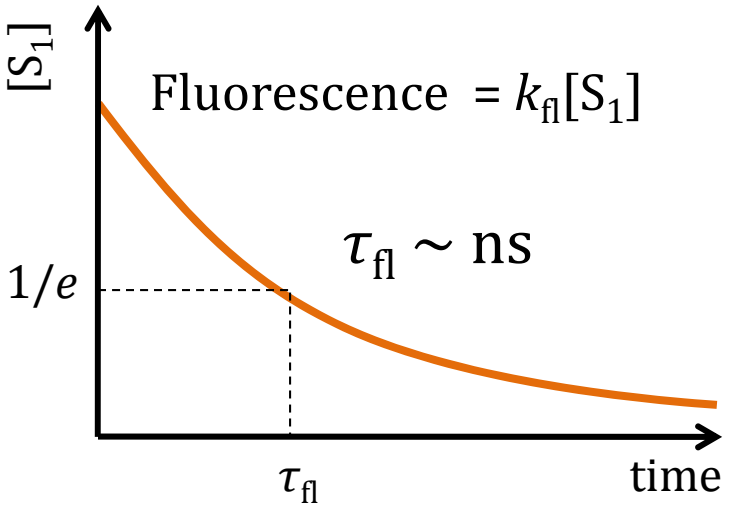
Fluorescence Lifetime



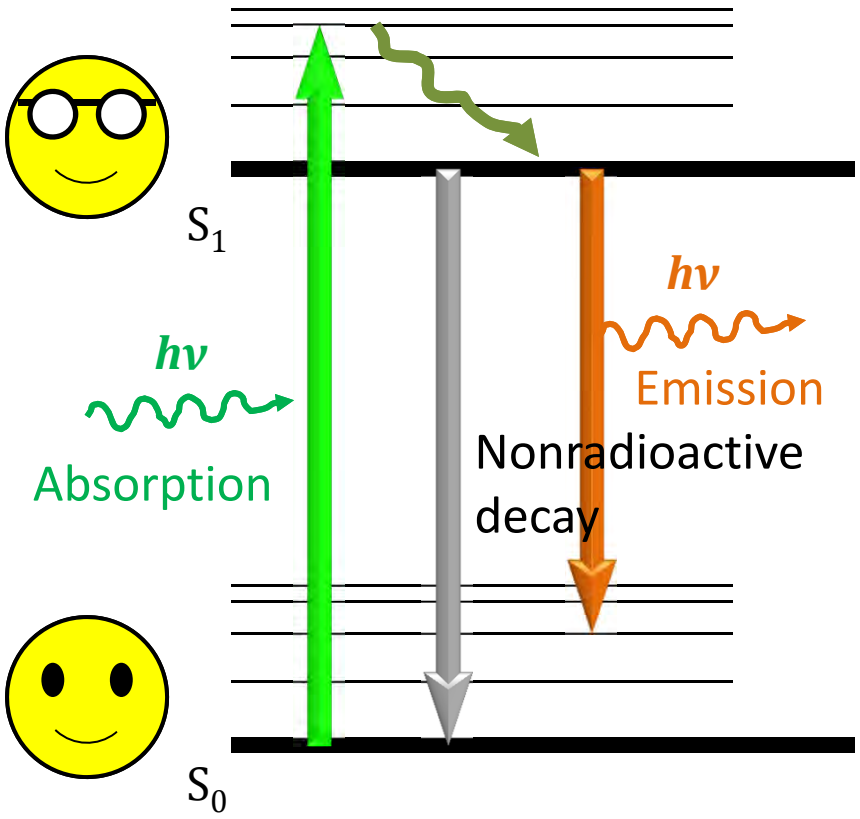
$$S_1 \rightarrow S_0 + h\nu$$

Rate constant = k_{fl}

Lifetime $\tau_{fl} = 1/k_{fl}$



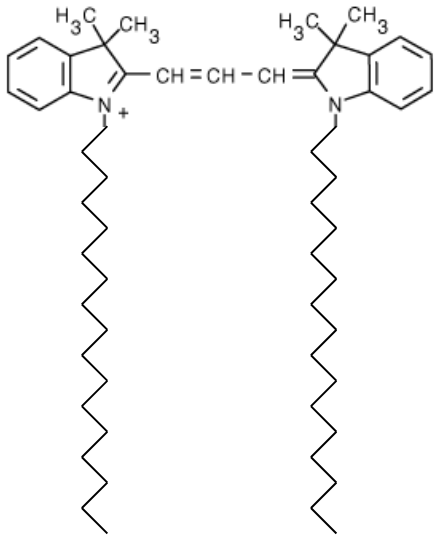
Quenching



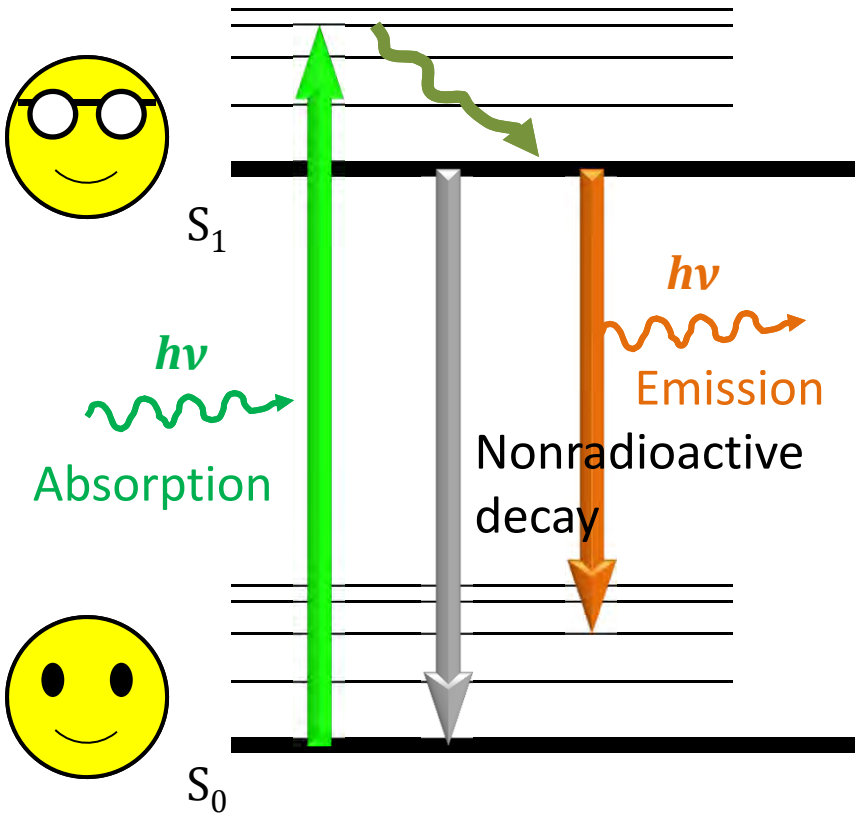
Non-radioactive decay:

Energy dissipation through structural flexibility

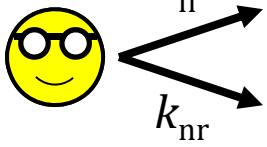
DiI



Quantum Yield



$$\mathrm{QE} = \frac{\text{emitted photon}}{\text{absorbed photon}}$$



k_{fl} Fluorescence

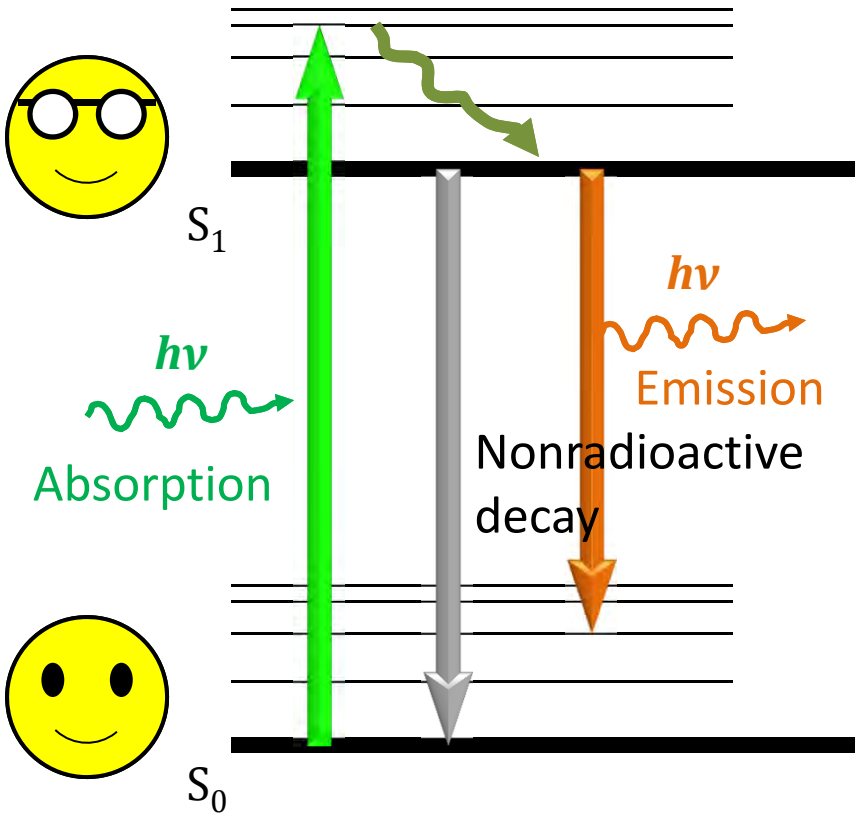
k_{nr} Nonradioactive decay

$$\mathrm{QE} = k_{\mathrm{fl}} / (k_{\mathrm{fl}} + k_{\mathrm{nr}})$$
$$= 1 / (1 + \tau_{\mathrm{fl}}\, k_{\mathrm{nr}})$$

$\tau_{\mathrm{fl}} \Uparrow,\ \mathrm{QE} \Downarrow$

$k_{\mathrm{nf}} \Uparrow,\ \mathrm{QE} \Downarrow$

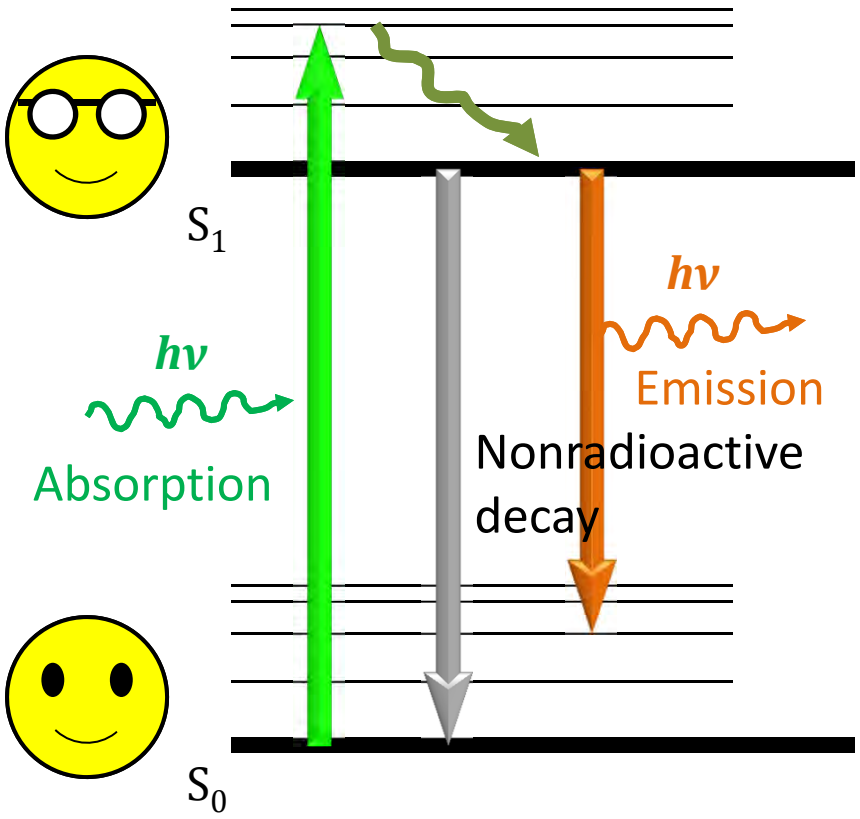
Quantum Yield



$$QE = \frac{\text{emitted photon}}{\text{absorbed photon}}$$

Fluorophore	QE
Fluorescein in ethanol	0.97
Tryptophan, pH 7.2	0.14
EGFP	0.60
EGFP chromophore by itself	0.0005

The brightness of a fluoropore



$$S_0 + h\nu \xrightarrow{k_1} S_1 \begin{cases} \xrightarrow{k_{nr}} S_0 + \text{heat} \\ \xrightarrow{k_{fl}} S_0 + h\nu_1 \end{cases}$$

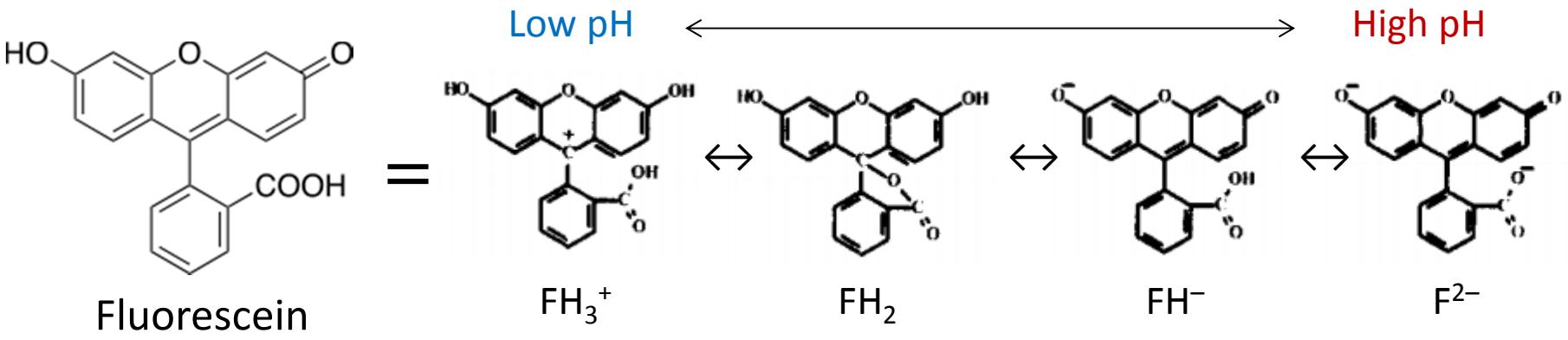
Brightness ≈

EC (wavelength)

×

QE

Brightness can be affected by the environment



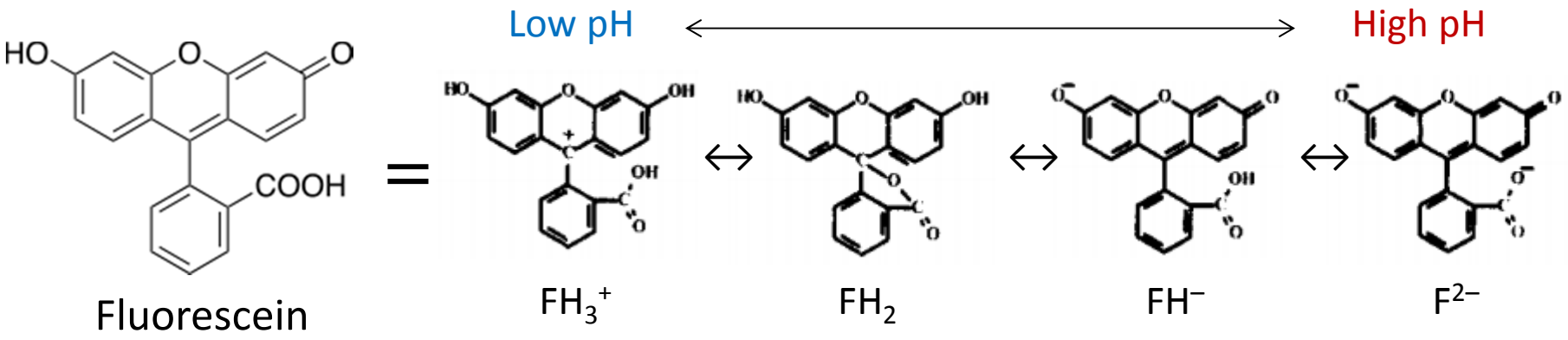
Fluorescein

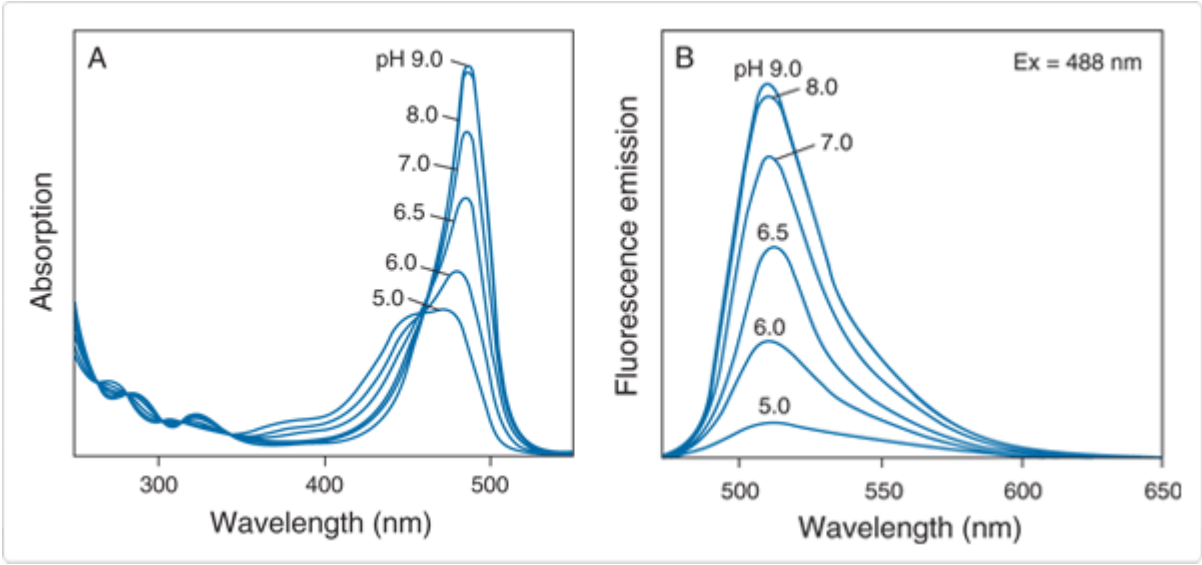
Summary of absorption, fluorescence and protolytic properties of fluorescein

	FH_3^+	FH_2	FH^-	F^{2-}
$\varepsilon/M^{-1}\,cm^{-1}$ (λ/nm)	53000(437)	3600(475)	29000(472)	76900(490)
$\varepsilon/M^{-1}\,cm^{-1}$ (λ/nm)	7100(297)	11000(434)	29000(453)	9500(322)
$\varepsilon/M^{-1}\,cm^{-1}$ (λ/nm)	33000(250)		700(310)	14000(283)
$\varepsilon/M^{-1}\,cm^{-1}$ (λ/nm)			17000(273)	43000(239)
τ/ns			3.0	4.1
Φ^f	~0(pH > 1.5)	~0	0.37	0.93
Φ^c	0.6	0.8	–	
pK'_a(1 M)	2.14	4.20	6.0	
pK'_a(50 mM)	2.09	4.30	6.41	
pK_a	2.08	4.31	6.43	

Sjoeback et al., Spectrochemica Acta Part A (1995)

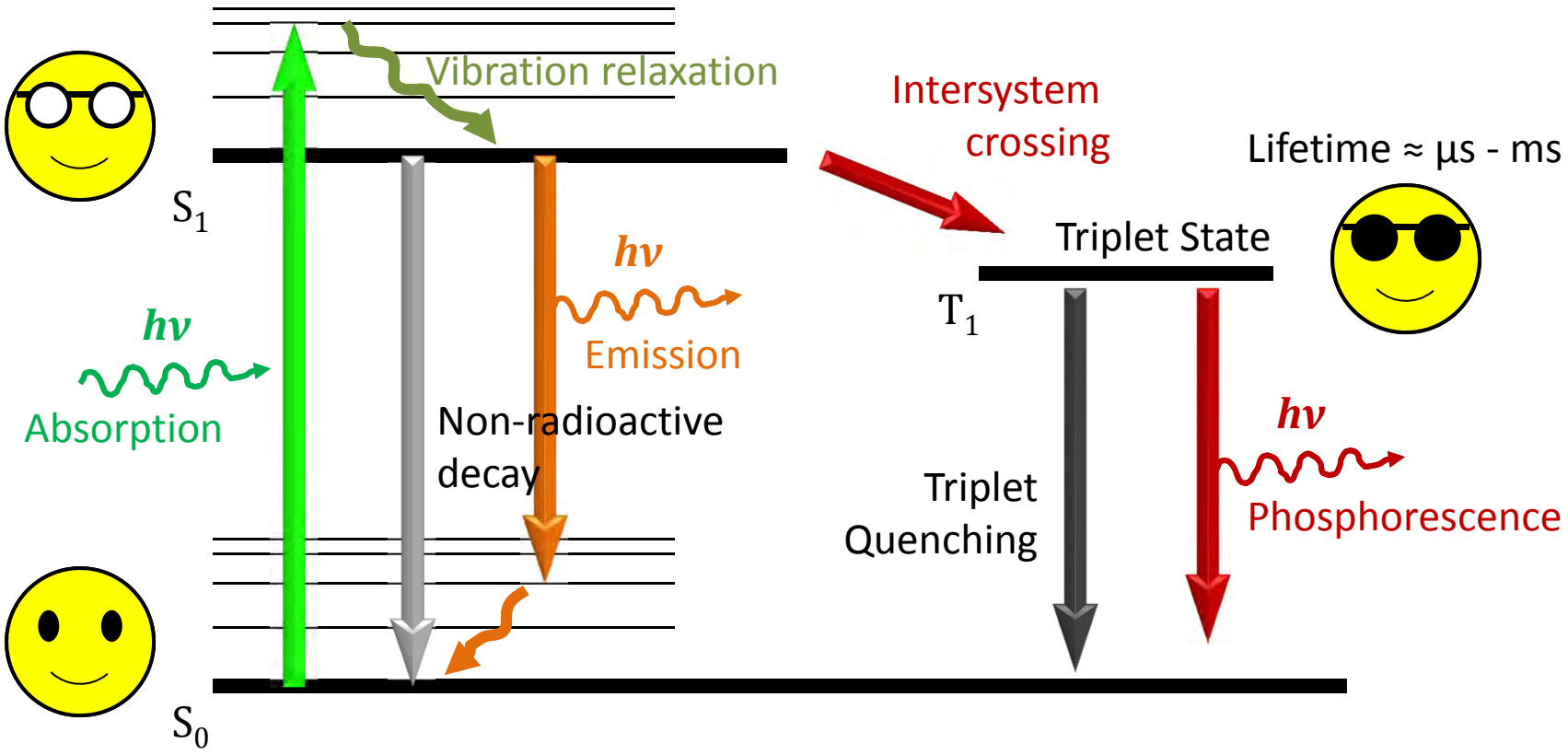
Brightness can be affected by the environment



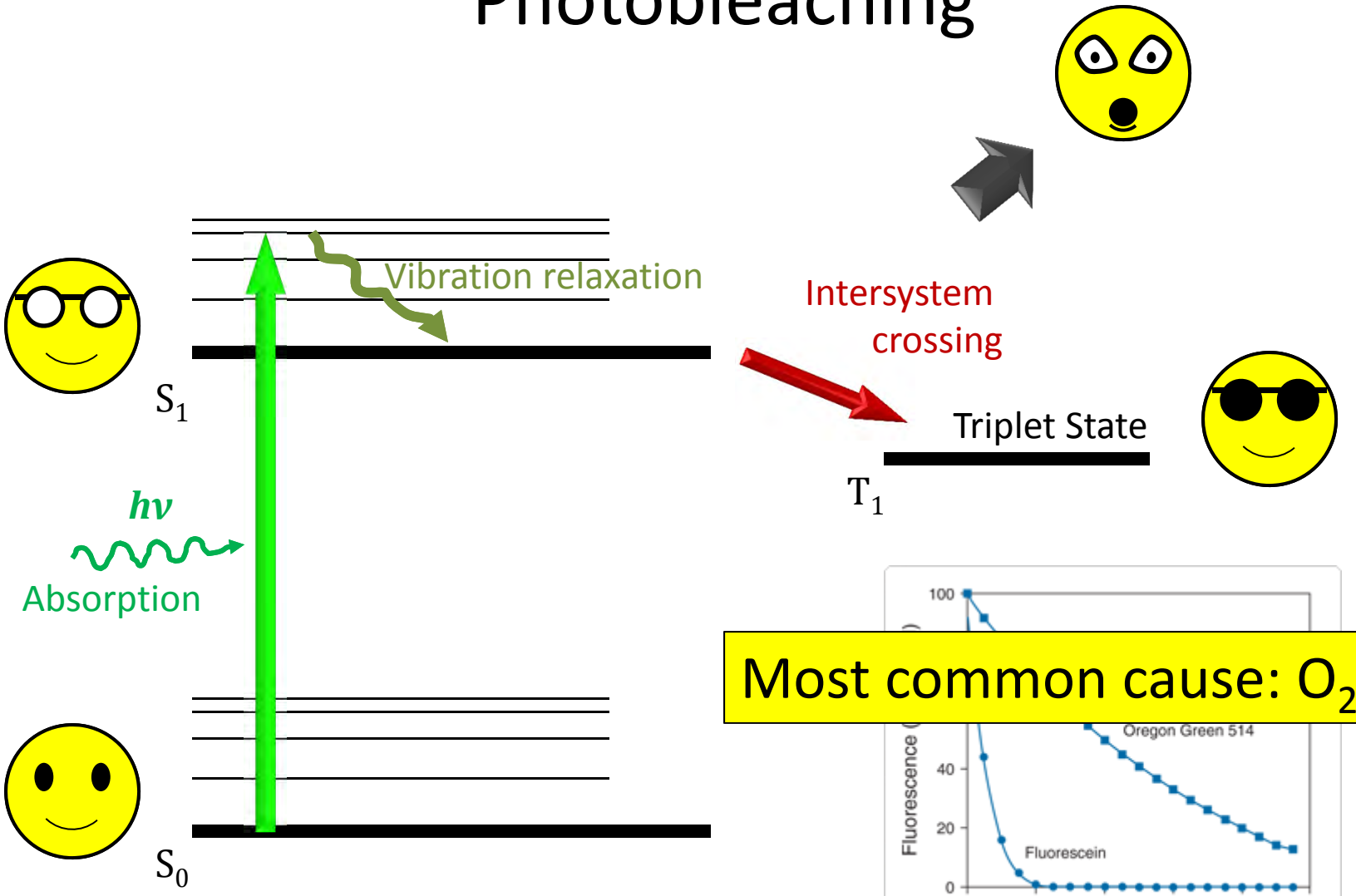


Invitrogen

The Full Jabłonski Diagram

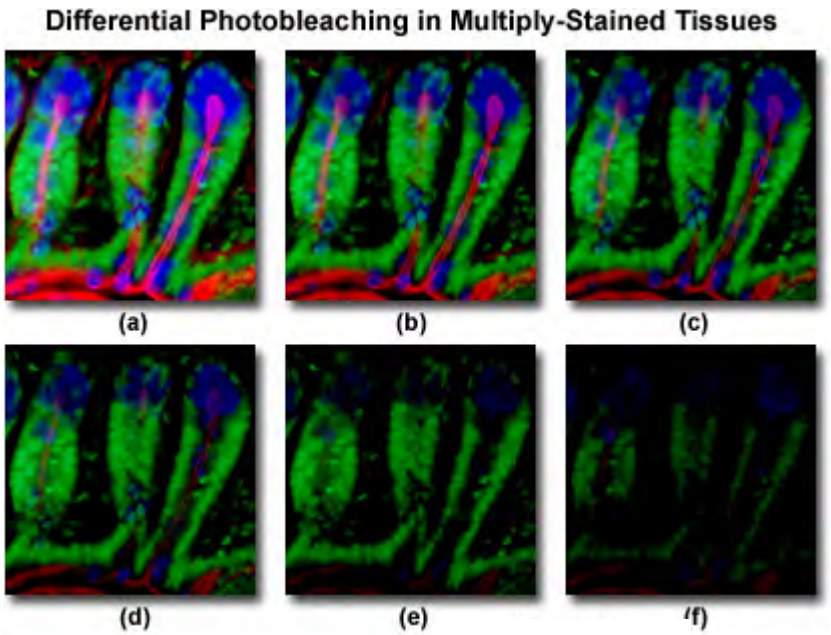


Photobleaching



Most common cause: O_2

Photobleaching is our enemy



Michael Davidson

For all fluorescence imaging:

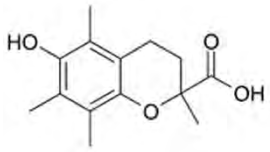
- Kills signal
- Alters the relative contrast

Live imaging:

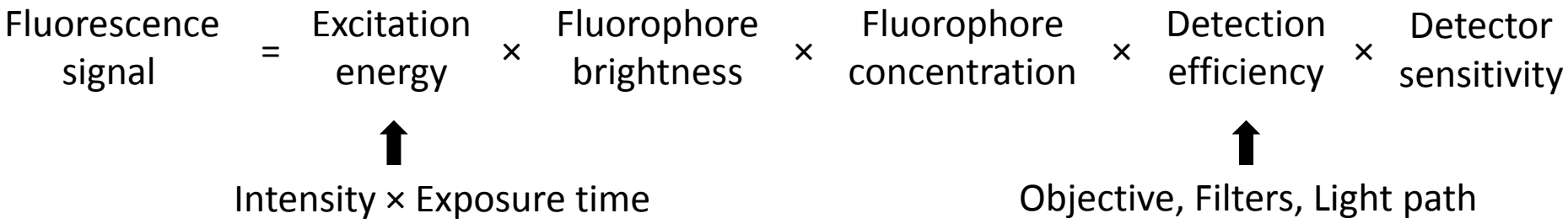
- Limits observation duration
- Phototoxicity from reactive oxygen species

Fight against photobleaching

- Choose “great” dyes
- “Anti-fade” mounting media
  - Glycerol
  - Oxygen scavengers
  - Free-radical scavengers and triplet quenchers
    - Trolox, mercaptoethanol, etc.



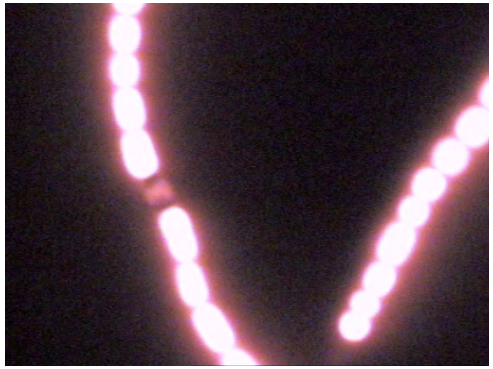
Fight against photobleaching, cont.



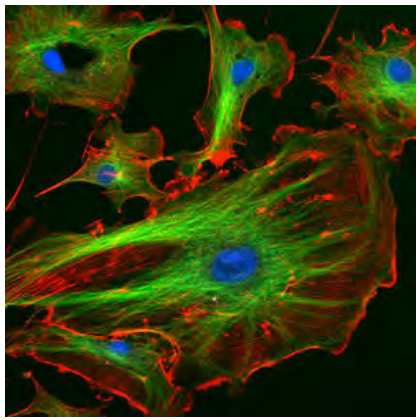
- Labeling as densely as possible
- Budget the photons
  - Only expose when observing
  - Minimize exposure time & excitation power
  - Use efficient filter combinations
  - Use high QE, low noise camera

Choice of fluorophores

Natural fluorescence

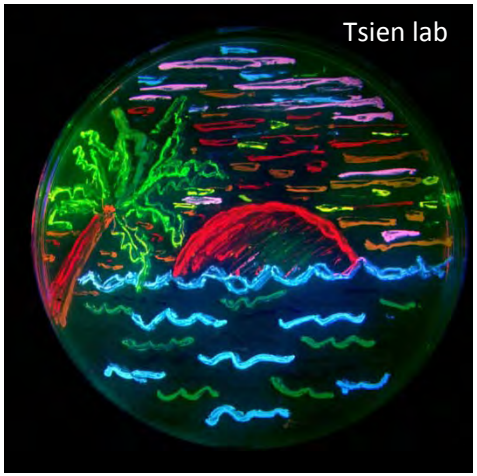


Organic dyes



Inorganic fluorophores

Fluorescent proteins



Inorganic fluorophores

- Quantum dots
  - Extremely bright and photostable
  - Broad excitation, narrow emission spectra
  - Large, difficult for specific labeling (10 – 20 nm)

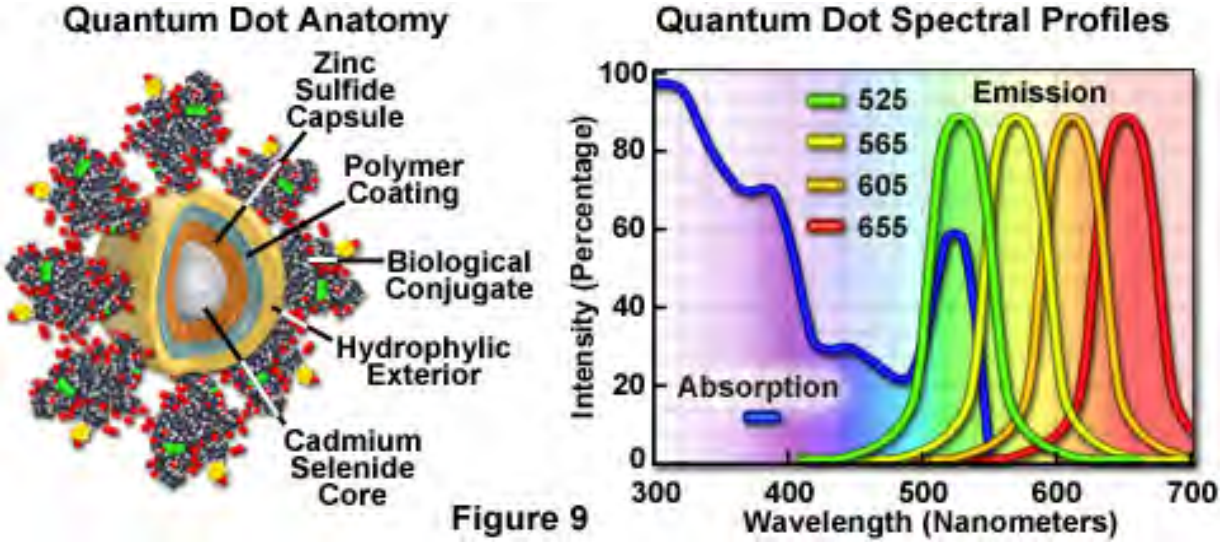
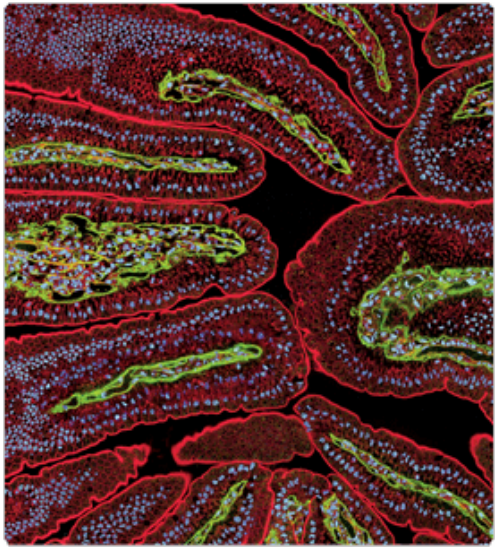
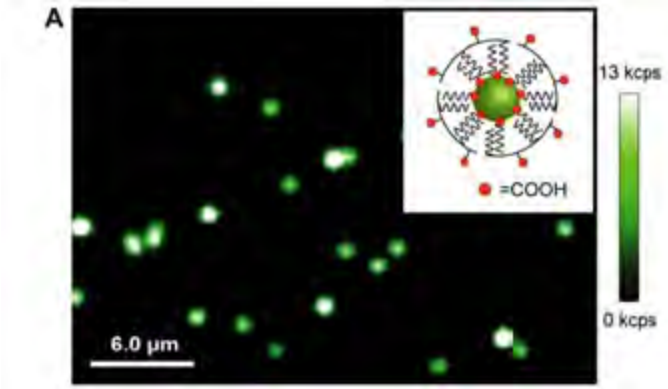


Figure 9

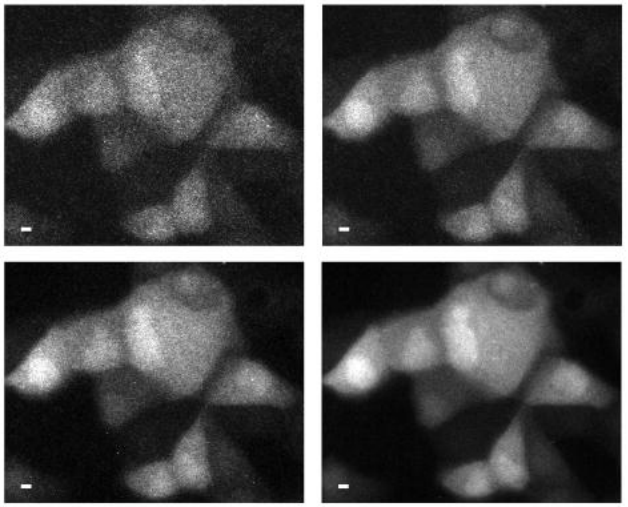


Inorganic fluorophores

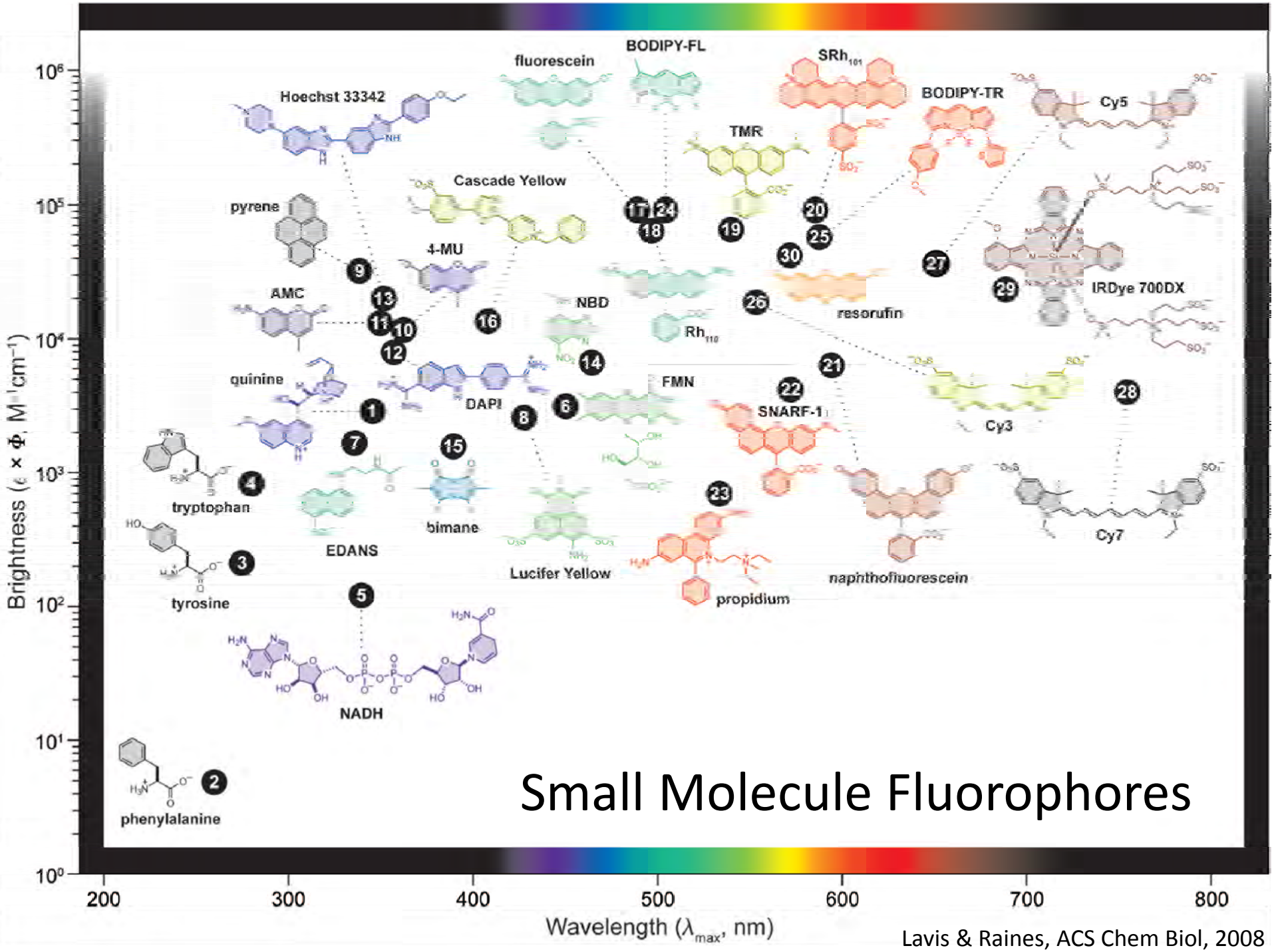
- Quantum dots
- Lanthanides
  - Very large Stokes or anti-Stokes shift (UV → Red or Red→Green)
  - Sharp emission peaks
  - Extremely long life time (µs - ms)



Bruce Cohen

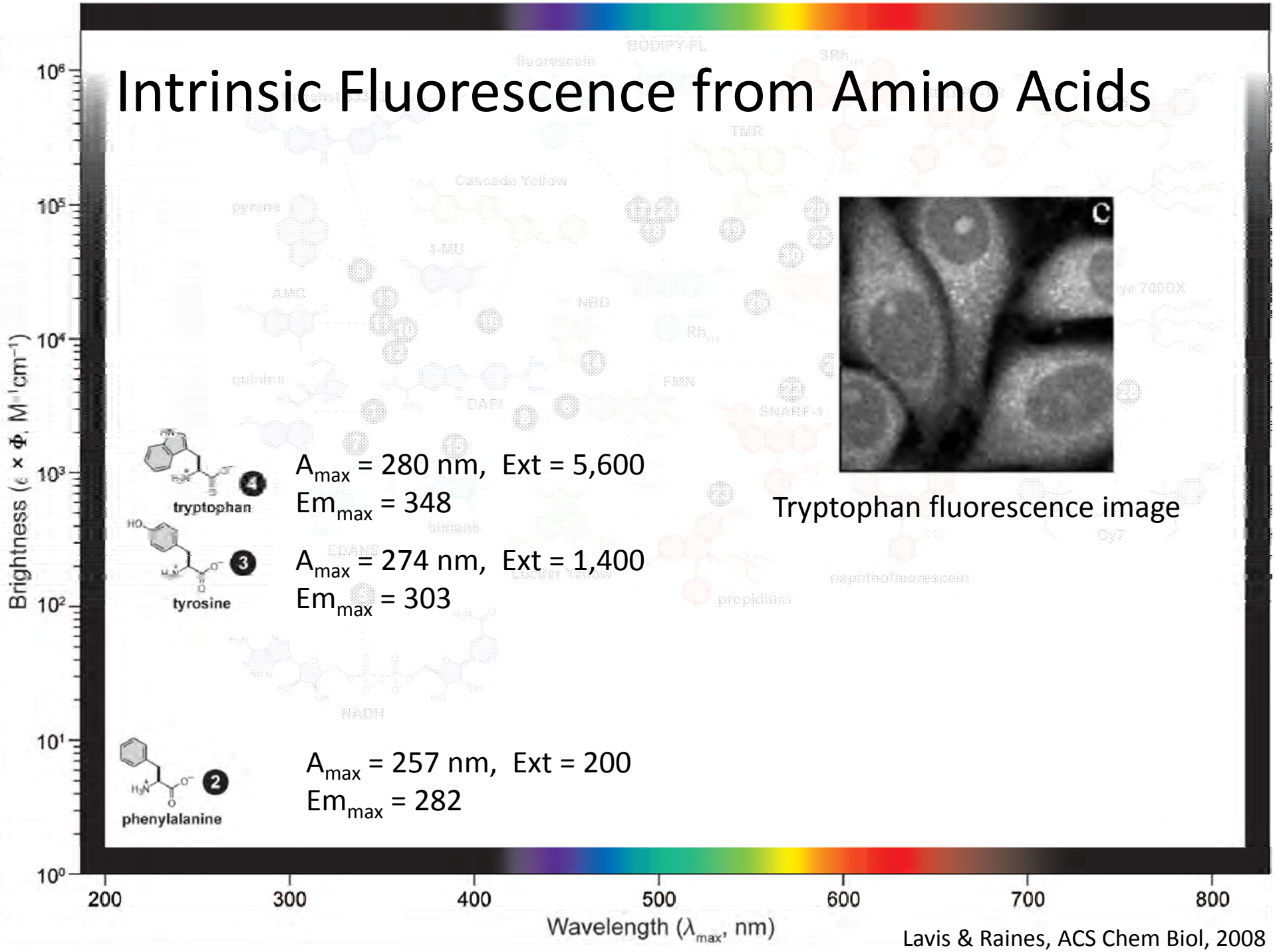


Gahlaut et al., Cytometry A, 2010



Lavis & Raines, ACS Chem Biol, 2008

Intrinsic Fluorescence from Amino Acids



A_{max} = 280 nm, Ext = 5,600
Em_{max} = 348

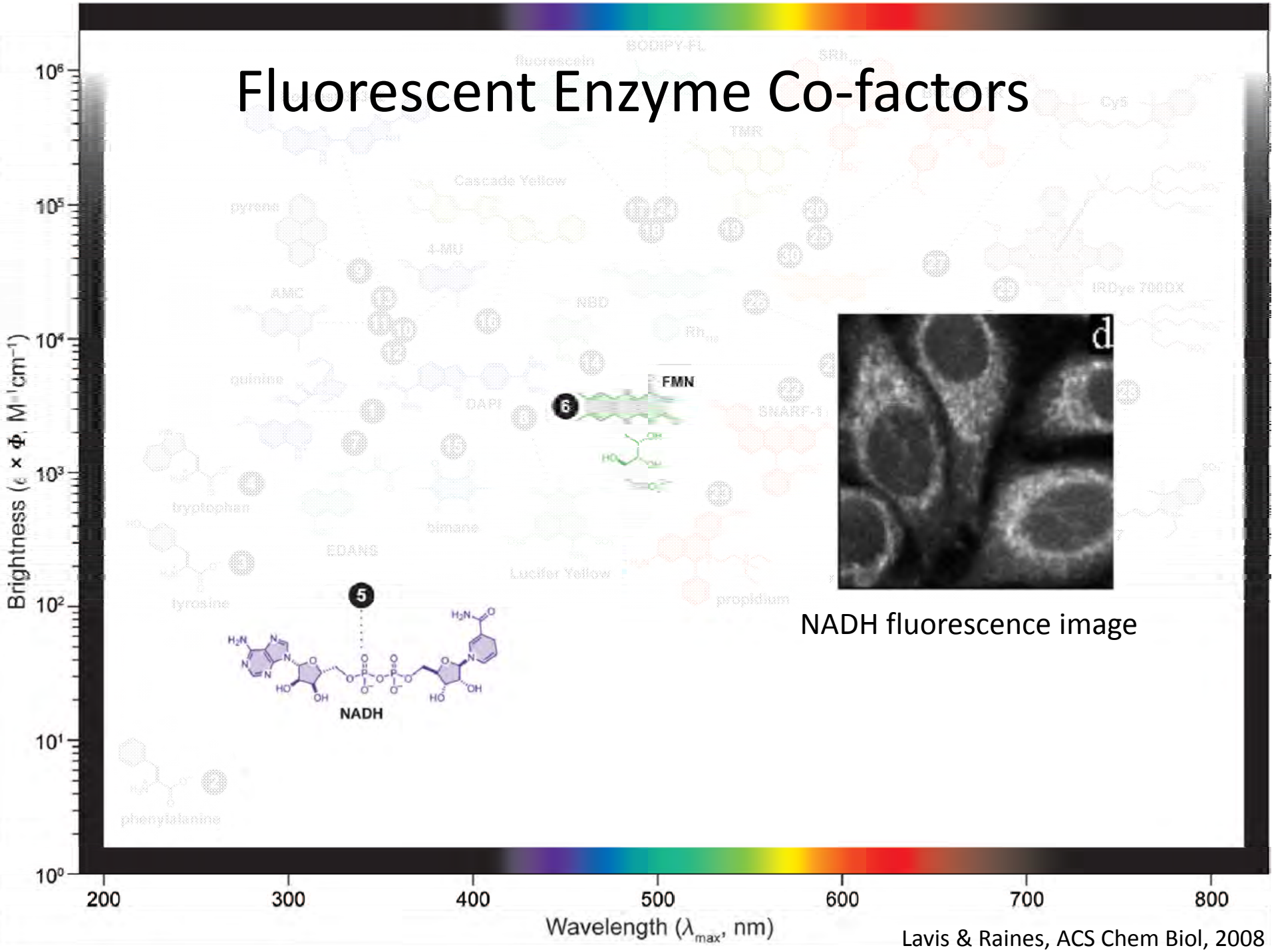
A_{max} = 274 nm, Ext = 1,400
Em_{max} = 303

A_{max} = 257 nm, Ext = 200
Em_{max} = 282

Tryptophan fluorescence image

Lavis & Raines, ACS Chem Biol, 2008

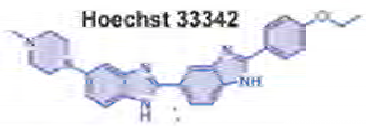
Fluorescent Enzyme Co-factors

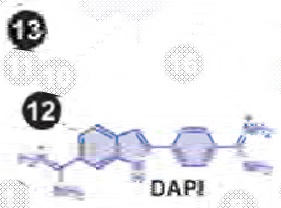


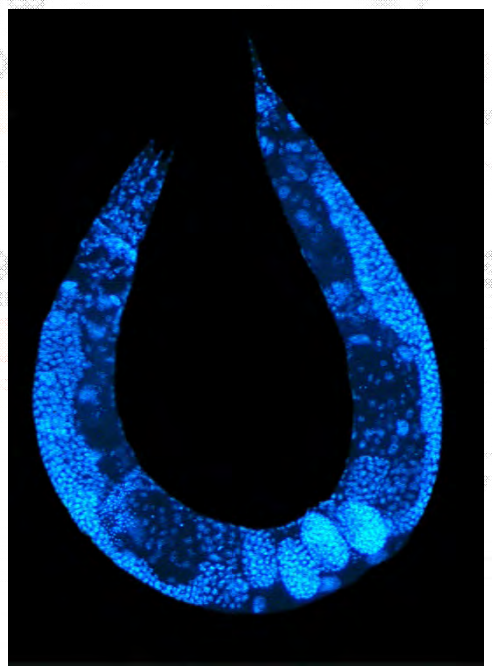
NADH fluorescence image

Lavis & Raines, ACS Chem Biol, 2008

DNA Intercalating Dyes







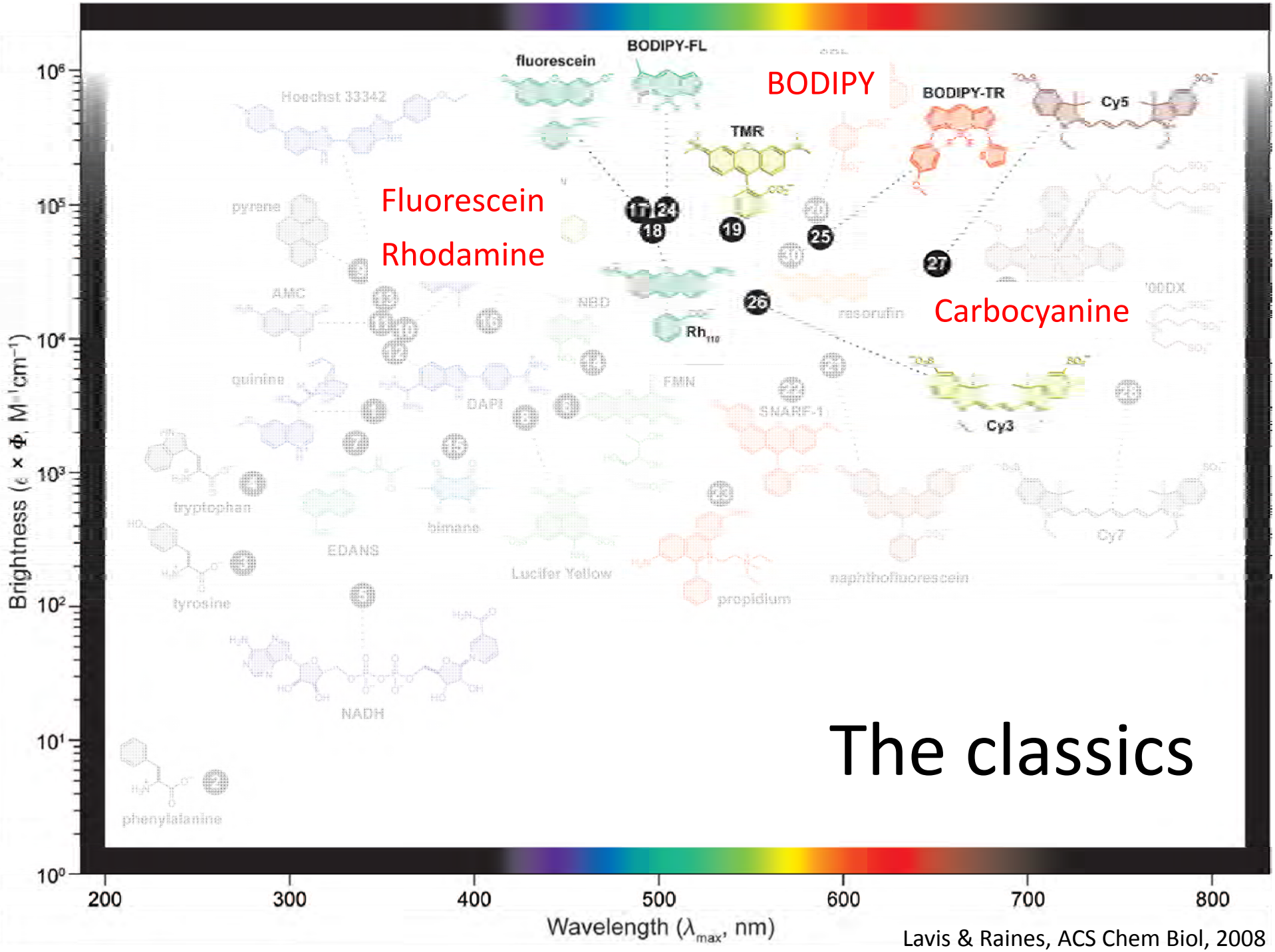
Brightness ($\epsilon \times \Phi$, $M^{-1}cm^{-1}$)

10^6 10^5 10^4 10^3 10^2 10^1 10^0

200 300 400 500 600 700 800

Wavelength (λ_{max}, nm)

Lavis & Raines, ACS Chem Biol, 2008



The classics

Lavis & Raines, ACS Chem Biol, 2008

The new generations

- Alexa Fluor series (Molecular Probes)
- Atto series (ATTO TECH)
- DyLight (Dyomics)
- Many more…

- Check the experimental conditions of the claims.
- Try them out.

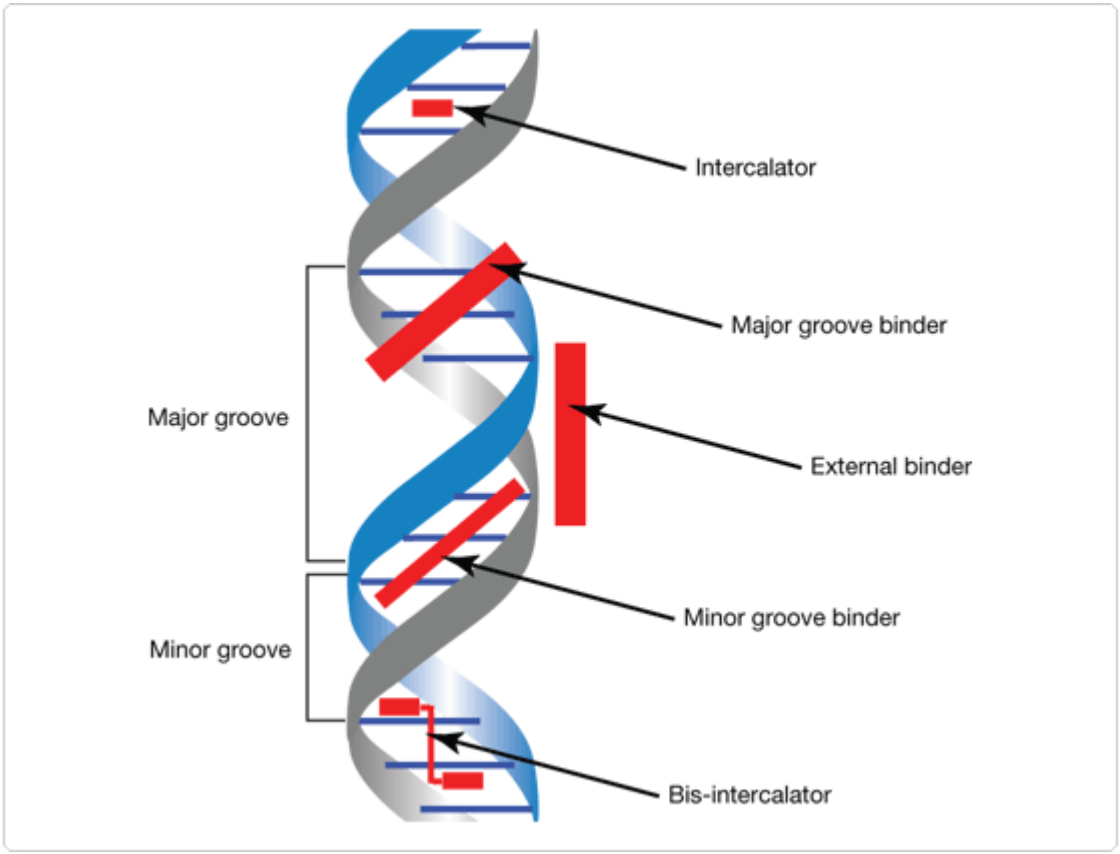
Small molecule dyes

- Small
  - e.g. DNA labeling
- Bright and photostable
- Wide range of wavelengths
  - UV to NIR
- Most of them lack labeling specificity by itself.
  - Specific probes for nucleic acid, plasma membrane, mitochondria
- Almost all “great” ones are unable to cross the membrane.

(Specific) delivery of small molecule dyes

Functional dyes

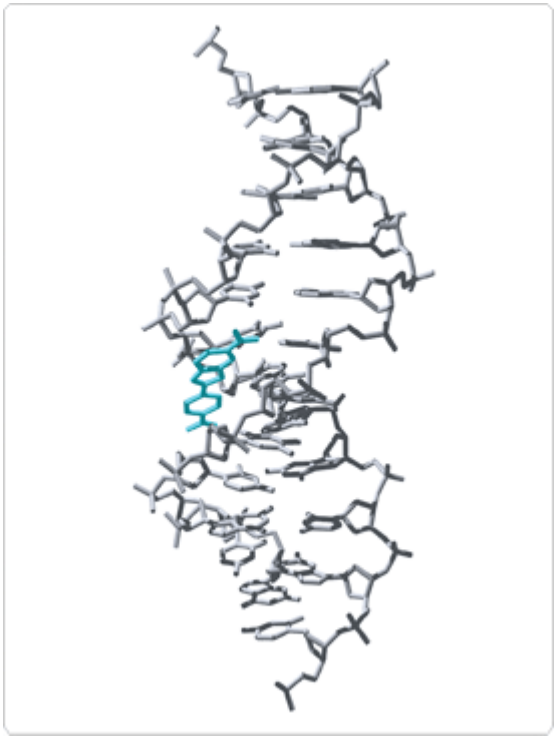
- Nucleic acid intercalating dyes
  - QE increment upon binding to DNA/RNA



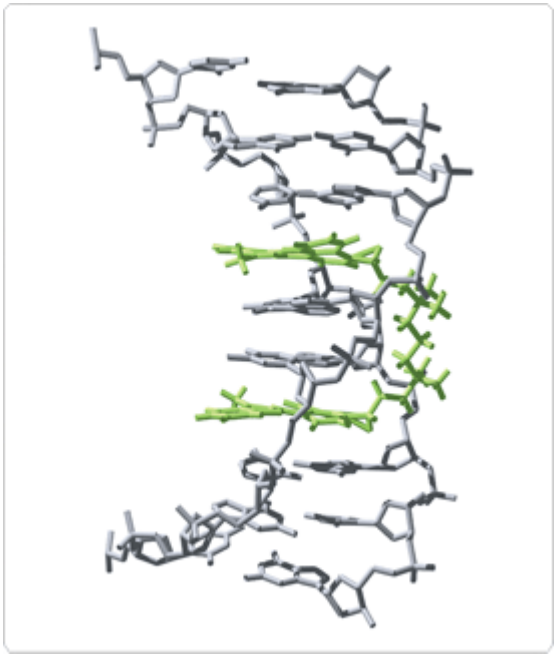
Functional dyes

- Nucleic acid intercalating dyes
  - QE increment upon binding to DNA/RNA

DAPI



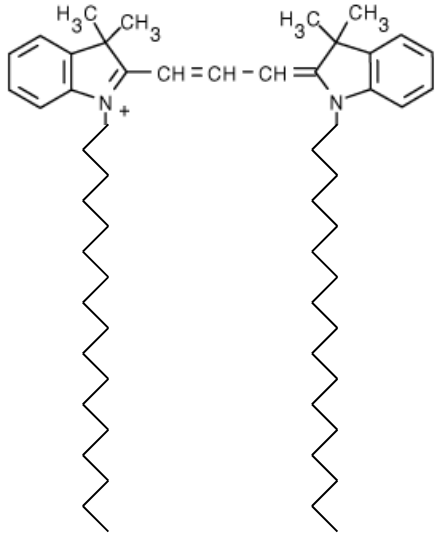
TOTO-1



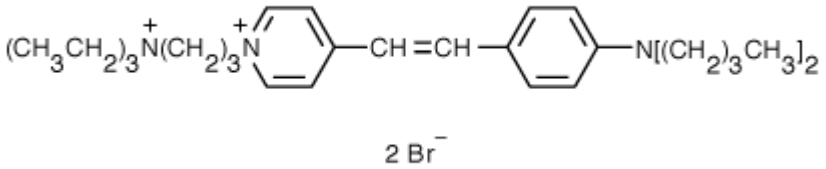
Functional dyes

- Nucleic acid intercalating dyes
- Membrane stains
  - Amphiphilic dyes that partition in lipid bilayers

DiI

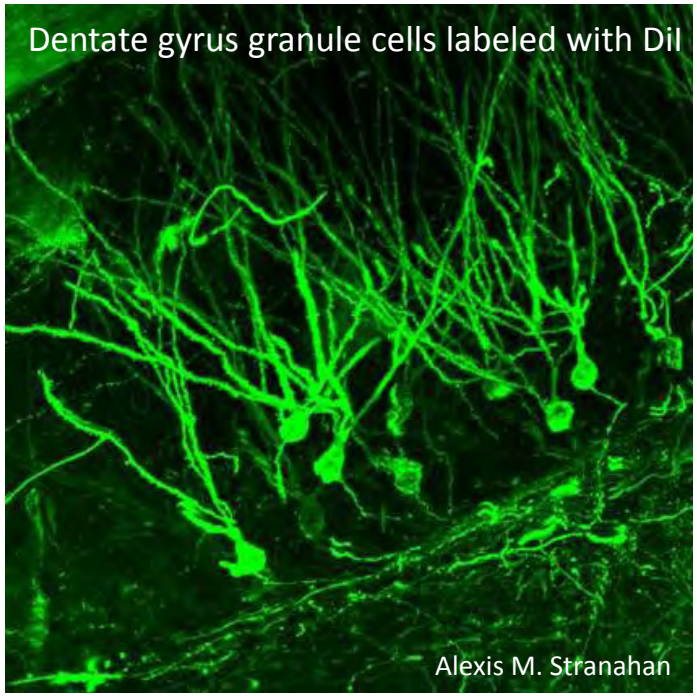


FM 1-43



Functional dyes

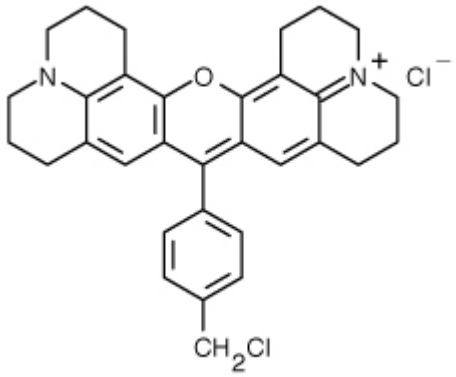
- Nucleic acid intercalating dyes
- Membrane stains
  - Amphiphilic dyes that partition in lipid bilayers

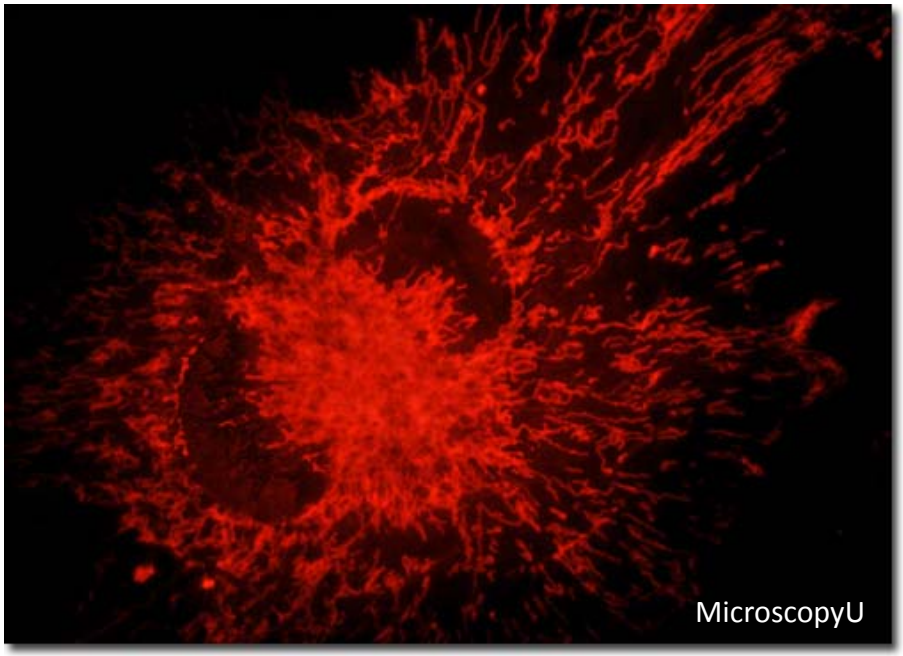


Functional dyes

- Nucleic acid intercalating dyes
- Membrane stains
- Organelle (mitochondria, ER, Golgi, etc.) stains
  - Based on charge and redox properties

MitoTracker Red CMXRos

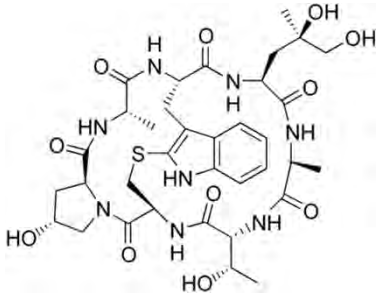




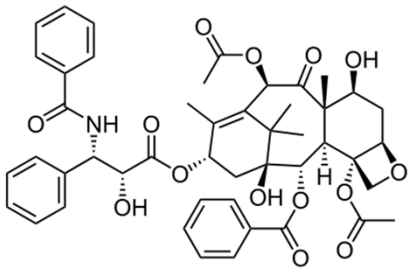
Small molecules probes

- Small molecules that bind proteins

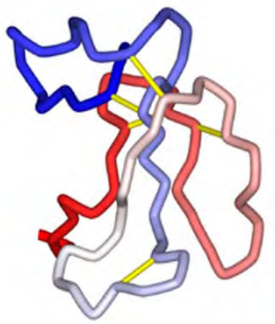
Phalloidin



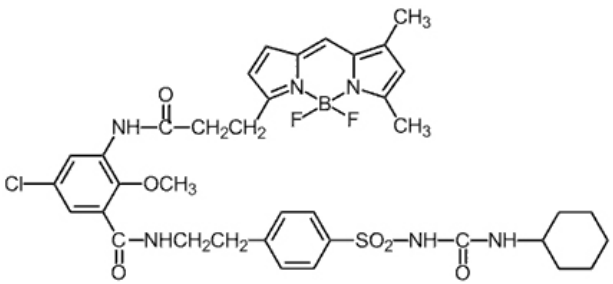
Taxol



α-bungarotoxin



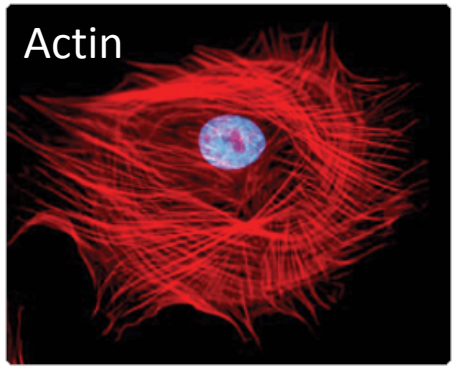
ER Tracker Green



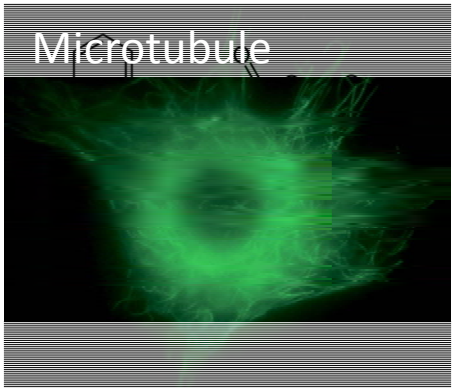
Small molecules probes

- Small molecules that bind proteins

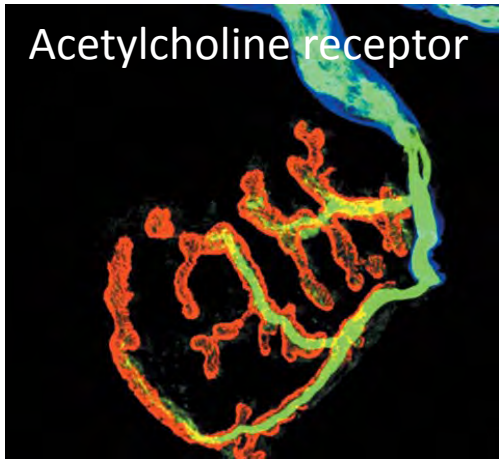
Phalloidin



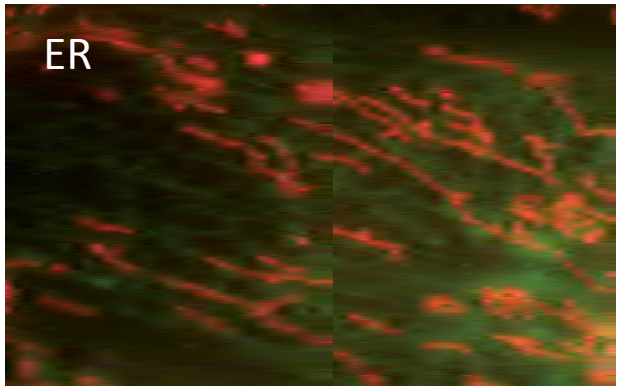
Taxol



α-bungarotoxin



ER Tracker Green

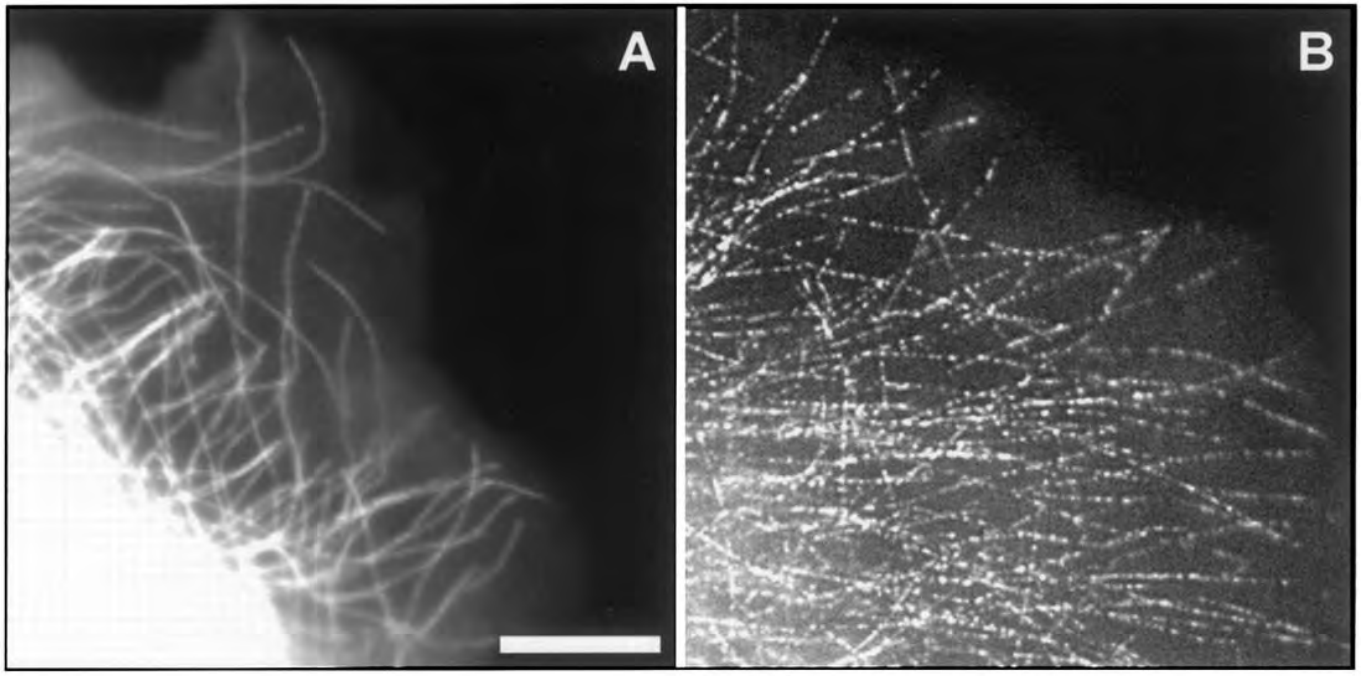


Large molecule labeling

- In vitro reconstituted systems
- Injecting labeled proteins

10% labeled tubulin

0.25% labeled tubulin

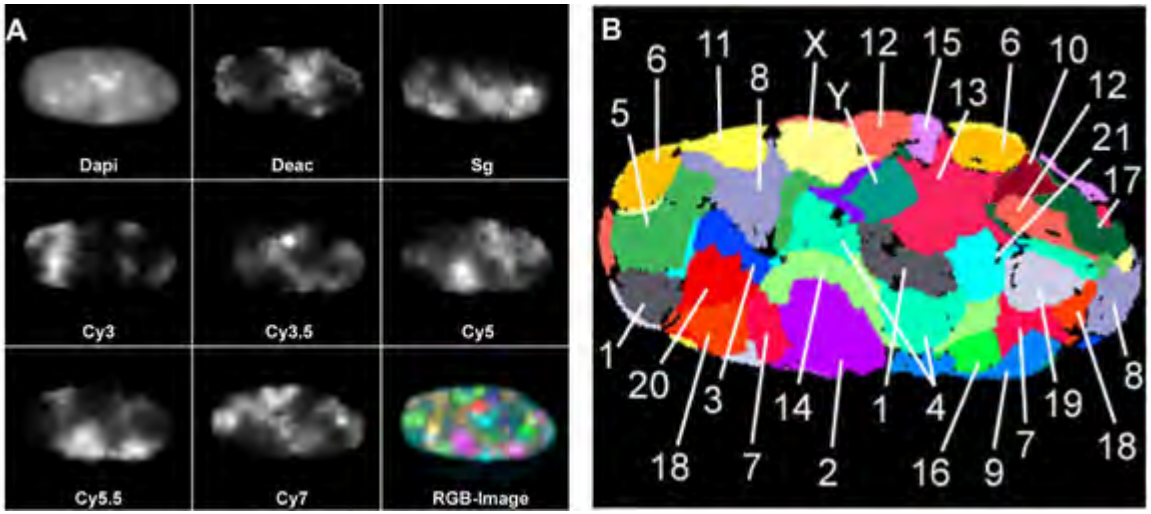


Waterman & Salmon, FASEB J, 1999

Large molecule labeling

- In vitro reconstituted systems
- Injecting labeled proteins
- Fluorescence in situ hybridization (FISH)

8 color 3D FISH to show chromosome territories in human G0 fibroblast nucleus



Bolzer et al., PloS Biology (2005)

Large molecule labeling

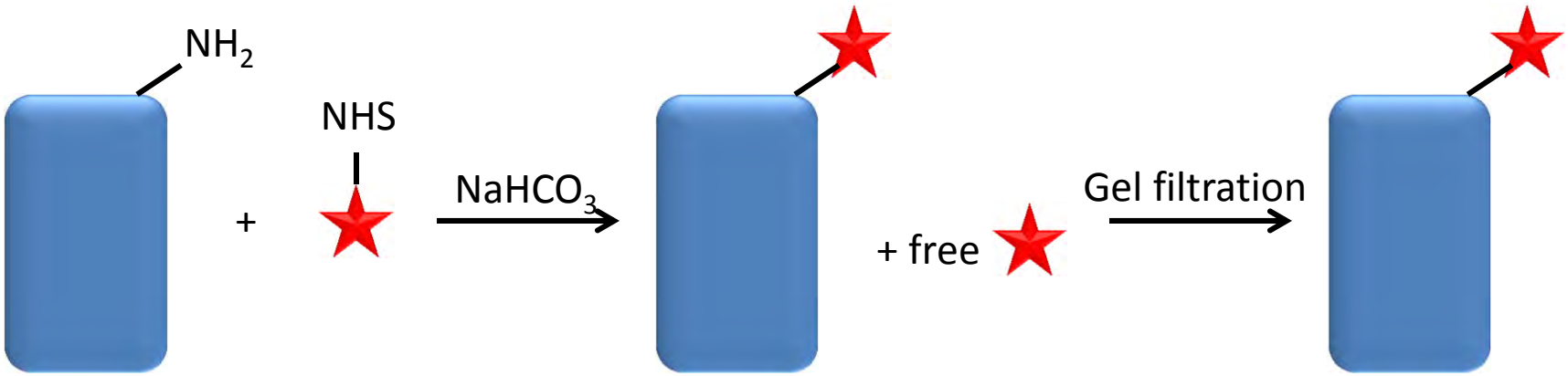
- In vitro reconstituted systems
- Injecting labeled proteins
- Fluorescence in situ hybridization (FISH)
- Biotin-avidin interaction
  - Labeled avidin/streptavidin
  - Biotin-dyes

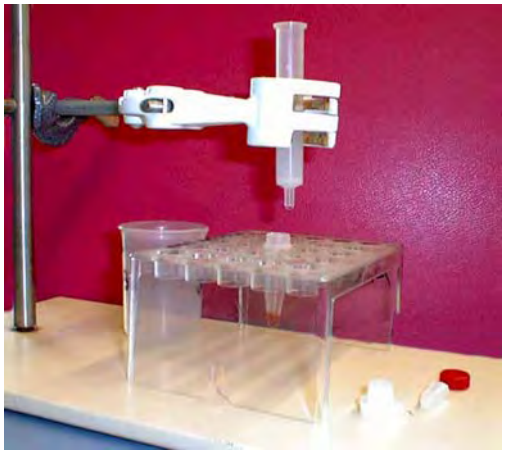
Large molecule labeling

- In vitro reconstituted systems
- Injecting labeled proteins
- Fluorescence in situ hybridization (FISH)
- Biotin-avidin interaction
- Antibody (immunofluorescence)

Labeling reactions

- Amine – succinimidyl ester chemistry (Lys and N-term)
- Thiol – maleimide chemistry (Cys)





Labeling stoichiometry

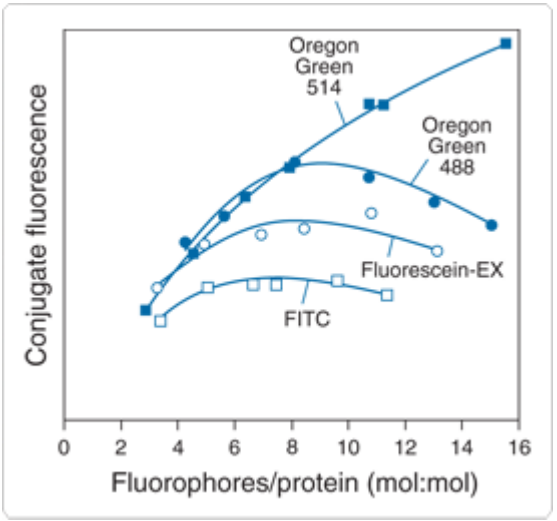
Dye concentration = A_{max} / E.C. $_{dye@max}$

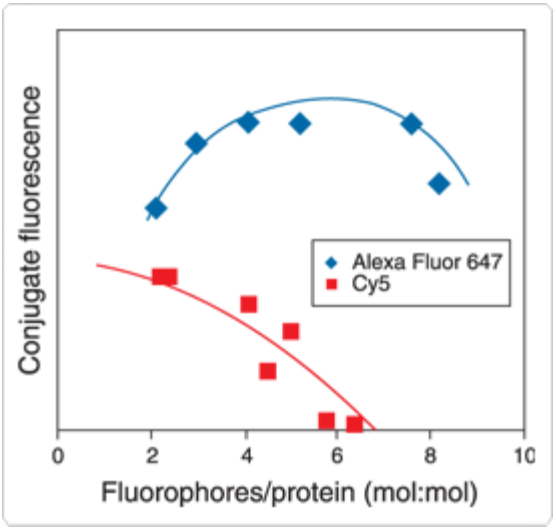
Protein concentration = (A_{280} – dye contribution) / E.C. $_{protein@280}$

Dye per protein = Dye concentration / Protein concentration

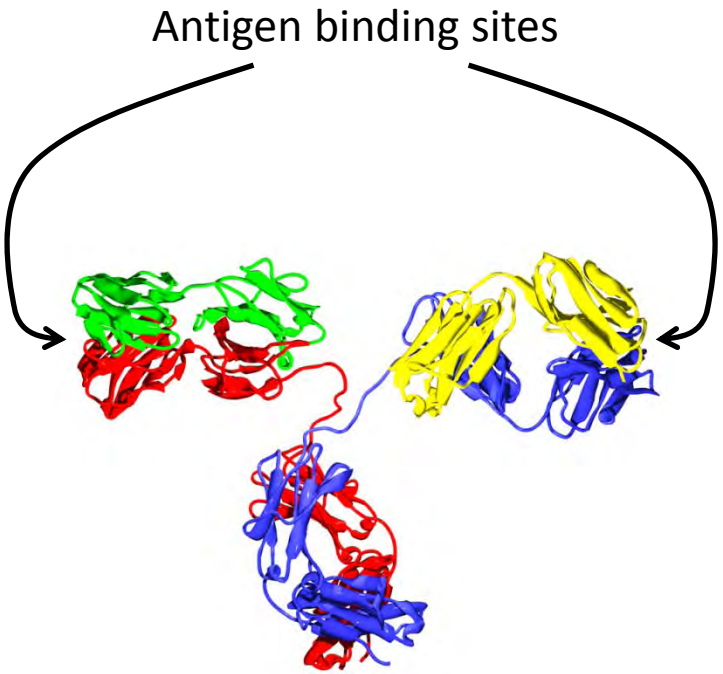
Too high labeling stoichiometry can make the protein dead…

and lead to self-quenching

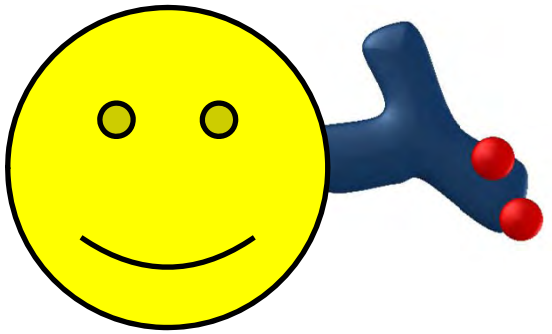




Immunofluorescence

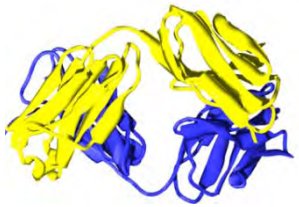


Direct immunofluorescence

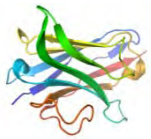


Primary antibody binding efficiency can be < 10%

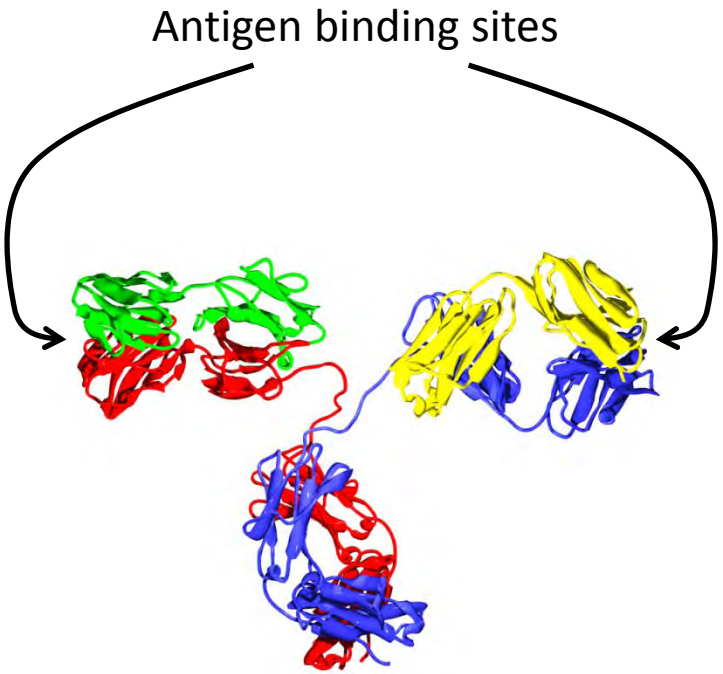
Fab fragment



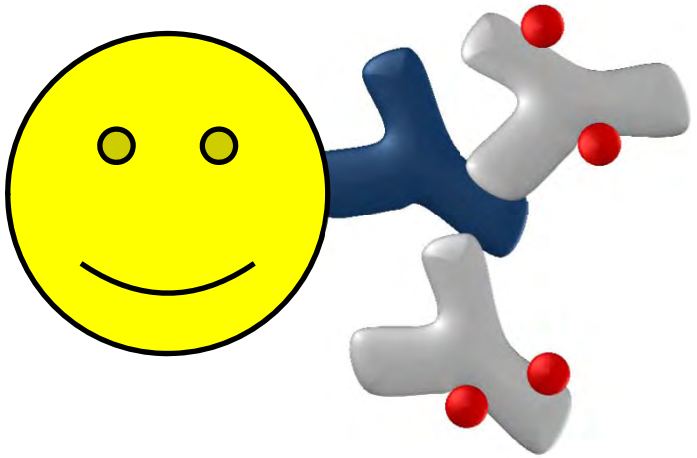
Nanobody



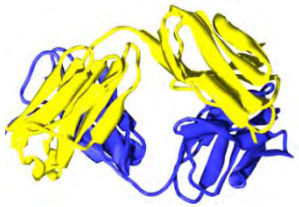
Immunofluorescence



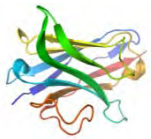
Indirect immunofluorescence



Fab fragment



Nanobody

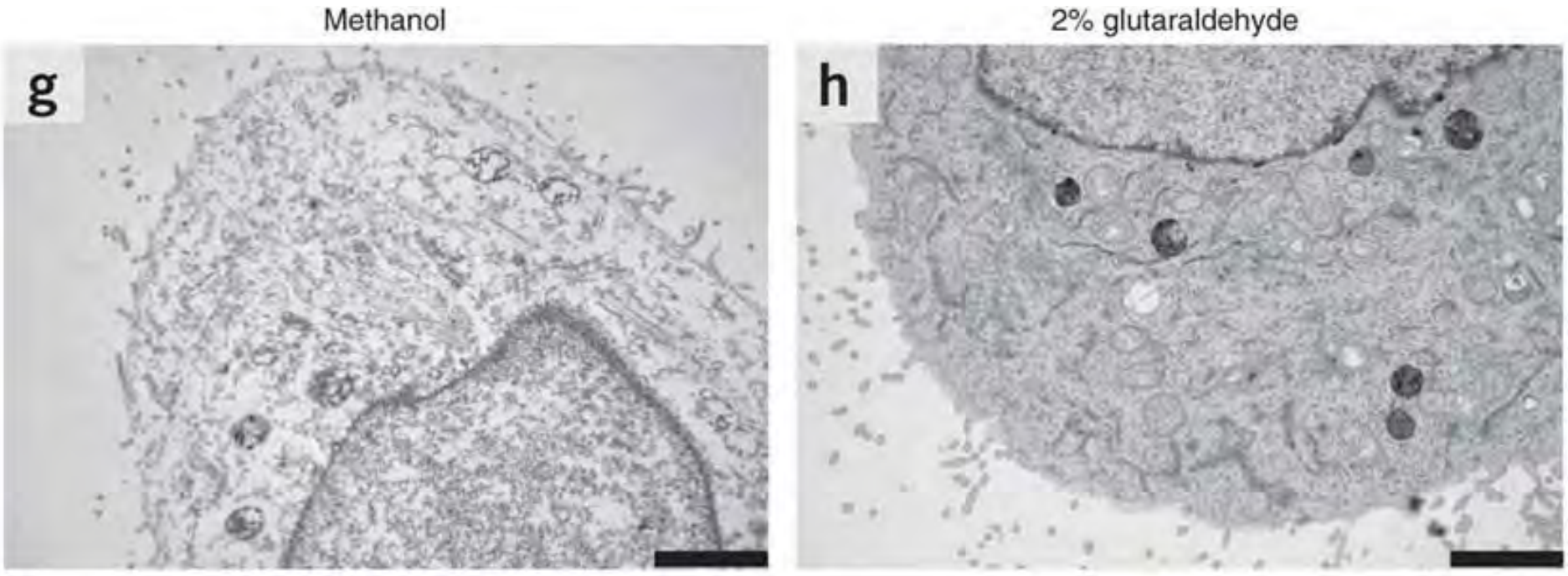


Fixation methods

- Methanol
  - Precipitates proteins in situ, dissolve membrane lipids
  - Good for protein structures, can destroy organelles and extract cytoplasmic proteins
- Formaldehyde (Paraformaldehyde)
  - Mild crosslinking of proteins
  - Most widely used, may not be strong enough crosslinking
  - Common for tissue fixation
- Glutaraldehyde
  - Strong crosslinking, preserving the ultrastructure
  - May mask some epitopes
  - May create fluorescence background ($NaBH_4$ reduction)
  - Required for electron microscopy

Fixation methods

- Methanol
- Formaldehyde (Paraformaldehyde)
- Glutaraldehyde



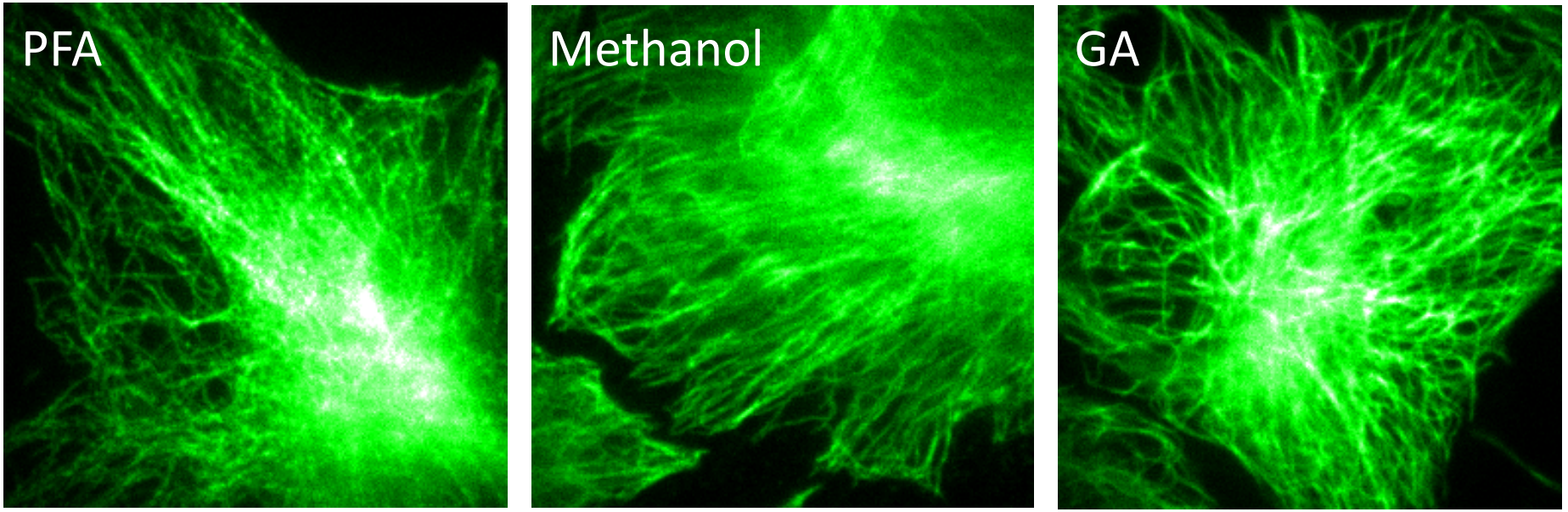
Nature Methods **9**, 152–158 (2012)

Membrane permeabilization

- Acetone
- Non-ionic detergents
  - Triton X-100, Tween-20
    - Extracting lipids, making holes on the membrane
- “Mild” detergents
  - Saponin
    - Extracting cholesterol only, permeabilizing the plasma membrane while saving the organelle membranes

Fixation artifacts

Microtubules



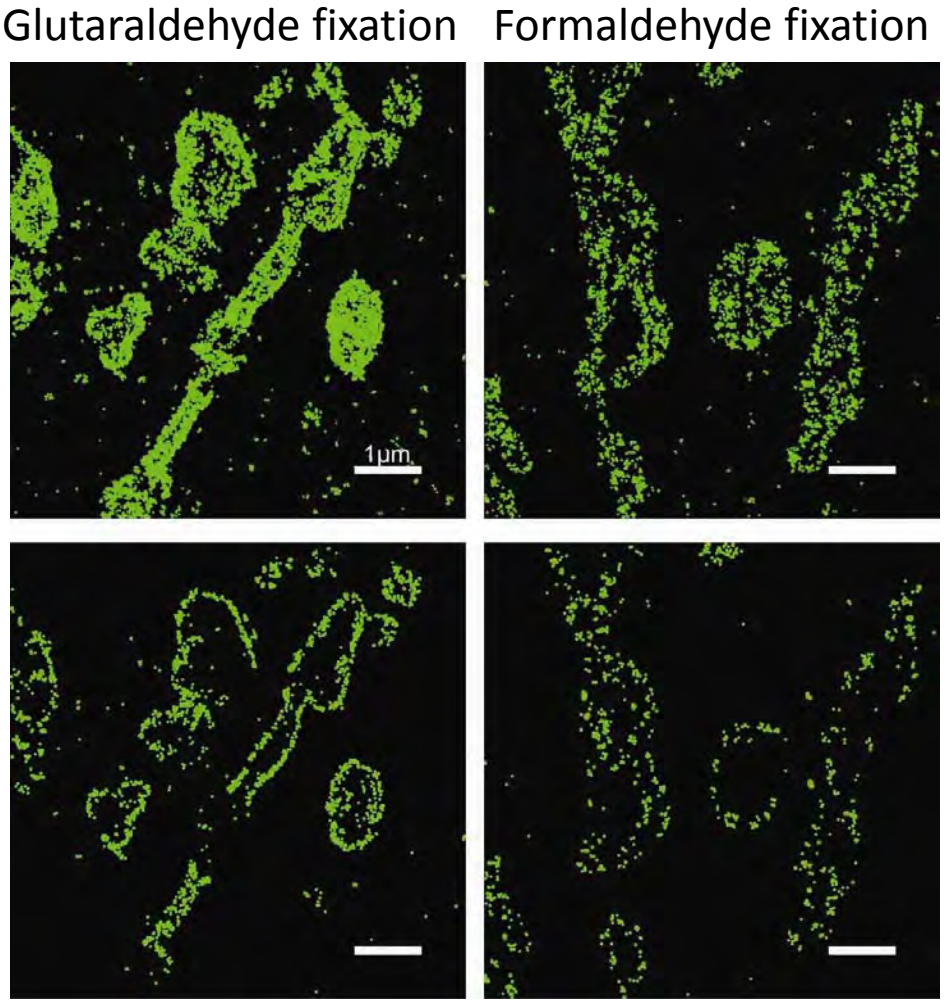
Immunolabeling artifacts and the need for live-cell imaging

Ulrike Schnell, Freark Dijk, Klaas A Sjollema & Ben N G Giepmans

Nature Methods **9**, 152–158 (2012) | doi:10.1038/nmeth.1855

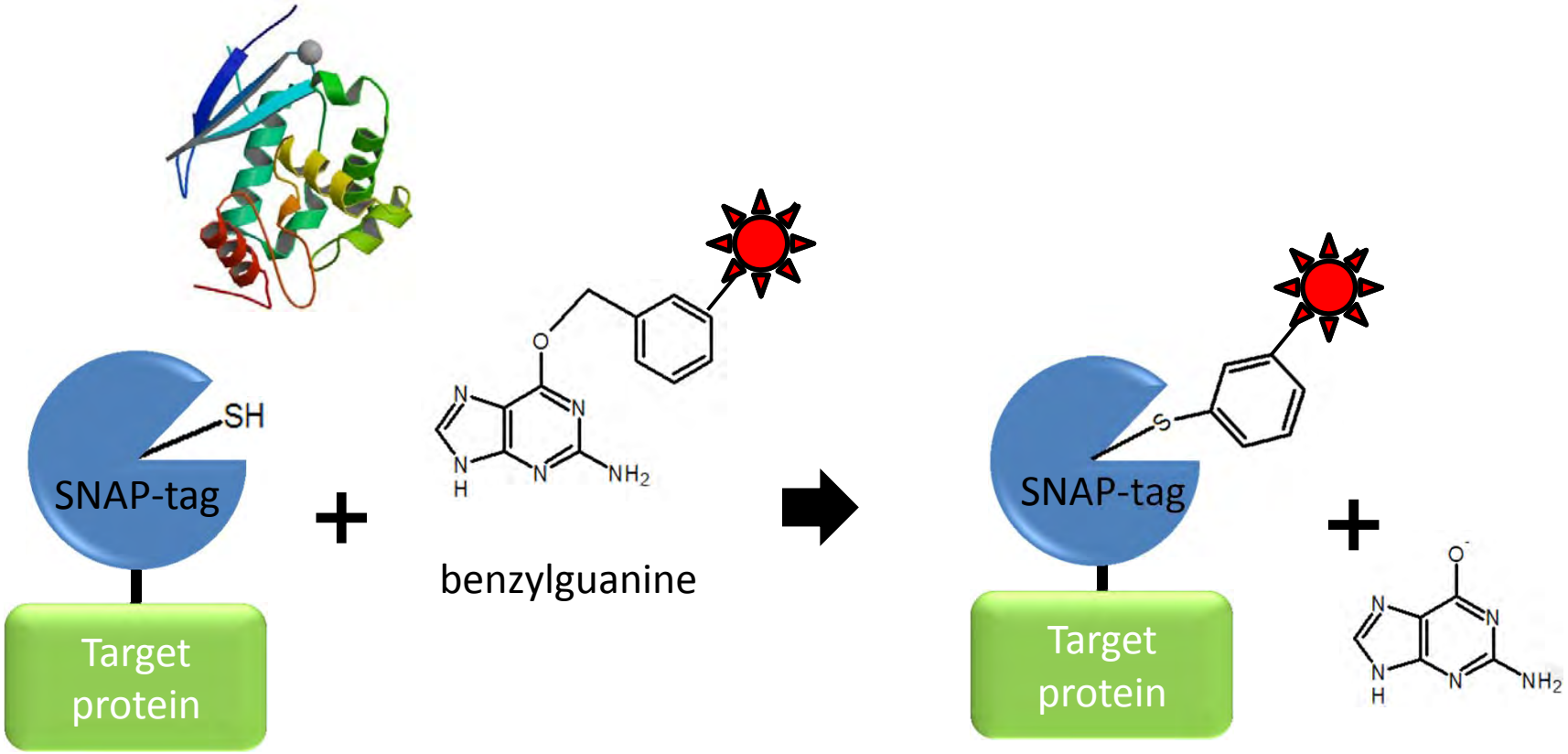
Some fixation artifacts become visible in super-resolution microscopy

Tom20 – mitochondria outer membrane



The hybrid approaches

- SNAP-tag, CLIP-tag, HALO-tag, TMP-tag…
  - SNAP-tag: based on human O^6-alkylguanine-DNA-alkyltransferase (hAGT)



Technical Reference Library

Newsletters & Journals

The Molecular Probes® Handbook

- Fluorescence Fundamentals
- Fluorophores and Their Amine-Reactive Derivatives—Chapter 1
- Thiol-Reactive Probes—Chapter 2
- Click Chemistry and Other Functional Group Modifications—Chapter 3
- Biotin and Hapten Derivatives—Chapter 4
- Crosslinking and Photoactivatable Reagents—Chapter 5
- Ultrasensitive Detection Technology—Chapter 6
- Antibodies, Avidins and Lectins—Chapter 7

The Molecular Probes® Handbook

11th Edition

The most complete fluorescent labeling and detection reference available, *The Molecular Probes® Handbook—A Guide to Fluorescent Probes and Labeling Technologies* contains over 3,000 technology solutions representing a wide range of biomolecular labeling and detection reagents. The significantly revised 11th Edition features extensive references, reorganized content, and new technical notes and product highlights.

Order Your Handbook Today

Order on Amazon.com

Search The Handbook

Enter your search term to search across the entire online handbook.

Search

Table of Contents

Fluorescence Fundamentals