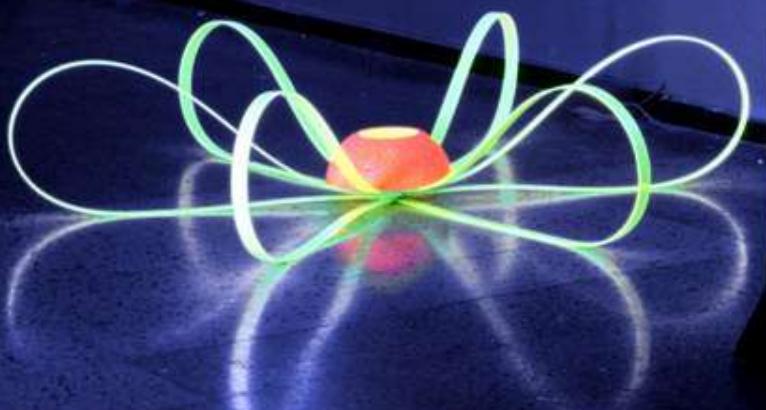


# Fluorescence Microscopy

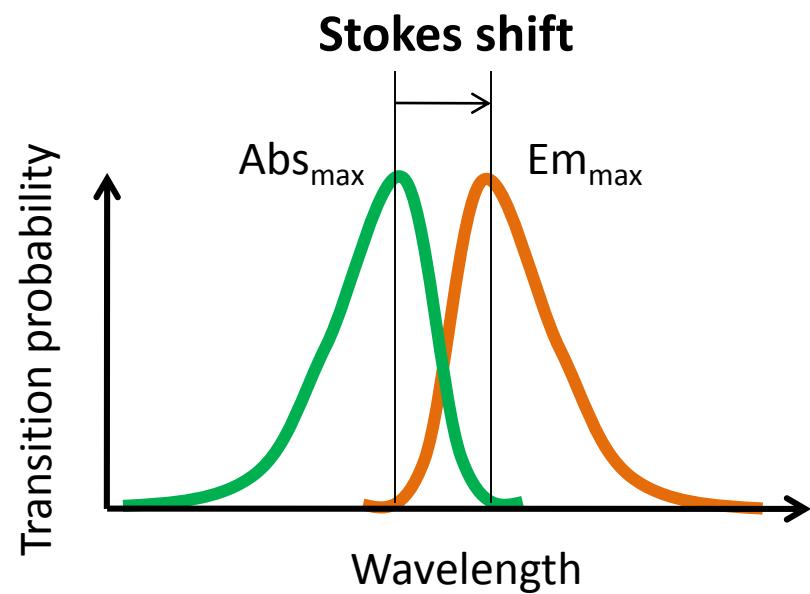
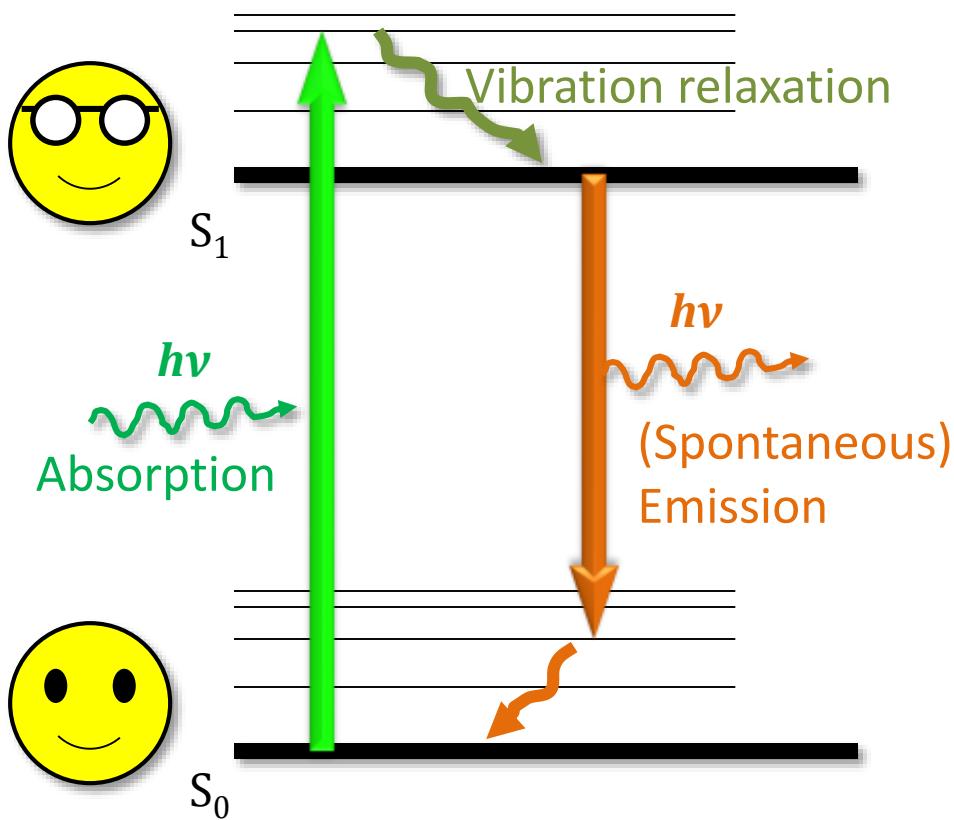
## II. Fluorescent dyes



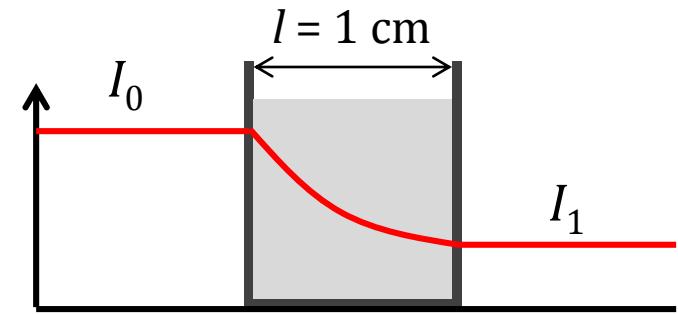
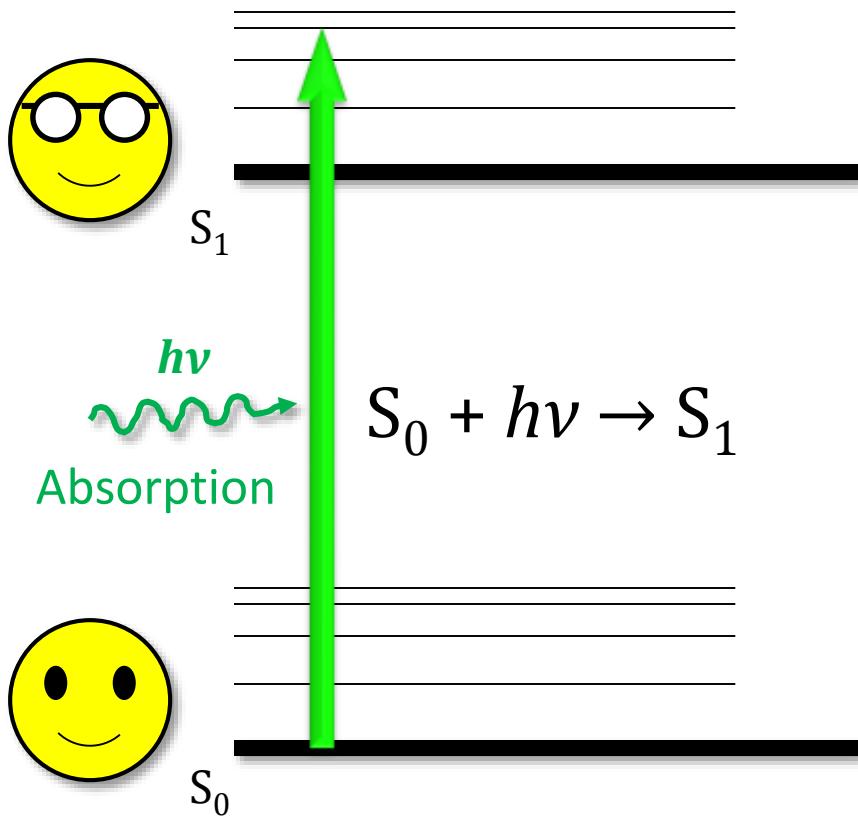
Bo Huang

2014.07.30

# The fluorescence process



# Extinction coefficient



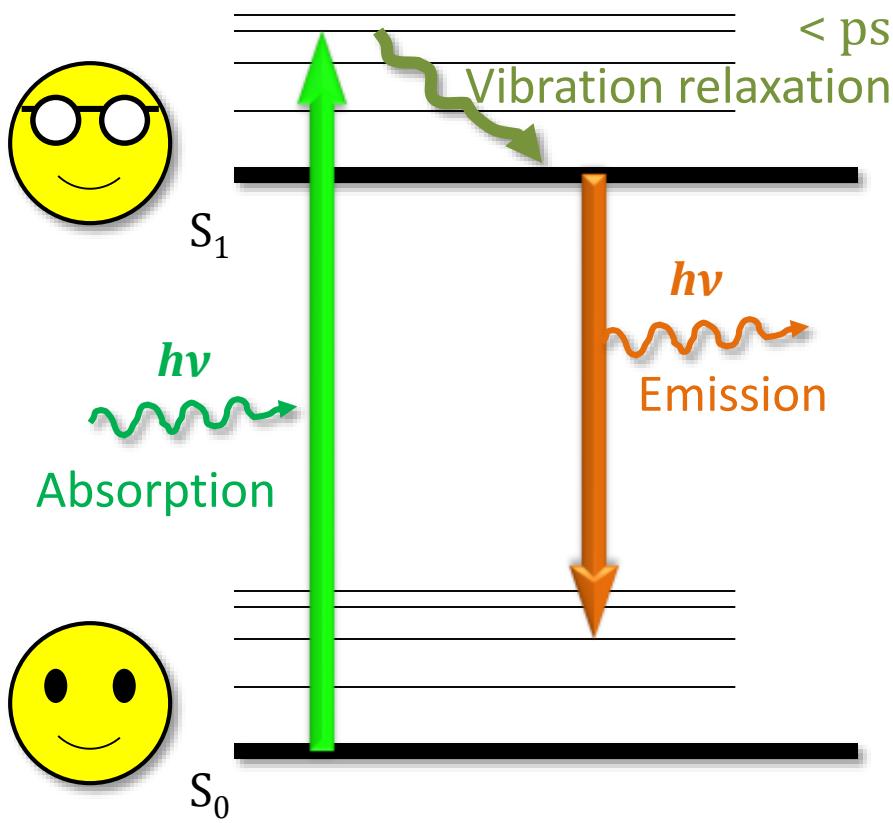
$$\text{Abs} = -\log_{10}(I_1/I_0)$$

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

Typically  $50,000 \sim 200,000 \text{ M}^{-1}\text{cm}^{-1}$

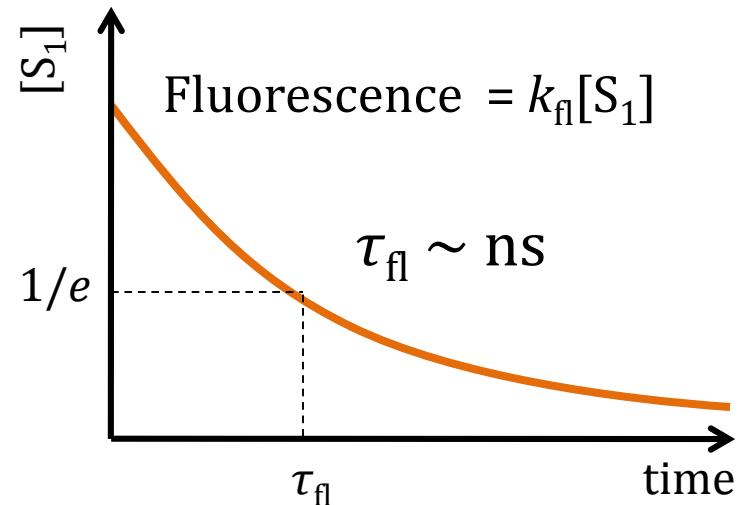
$1 \mu\text{M}, 1 \text{ cm} \rightarrow A \approx 0.1 \text{ (T = 80%)}$

# Fluorescence Lifetime

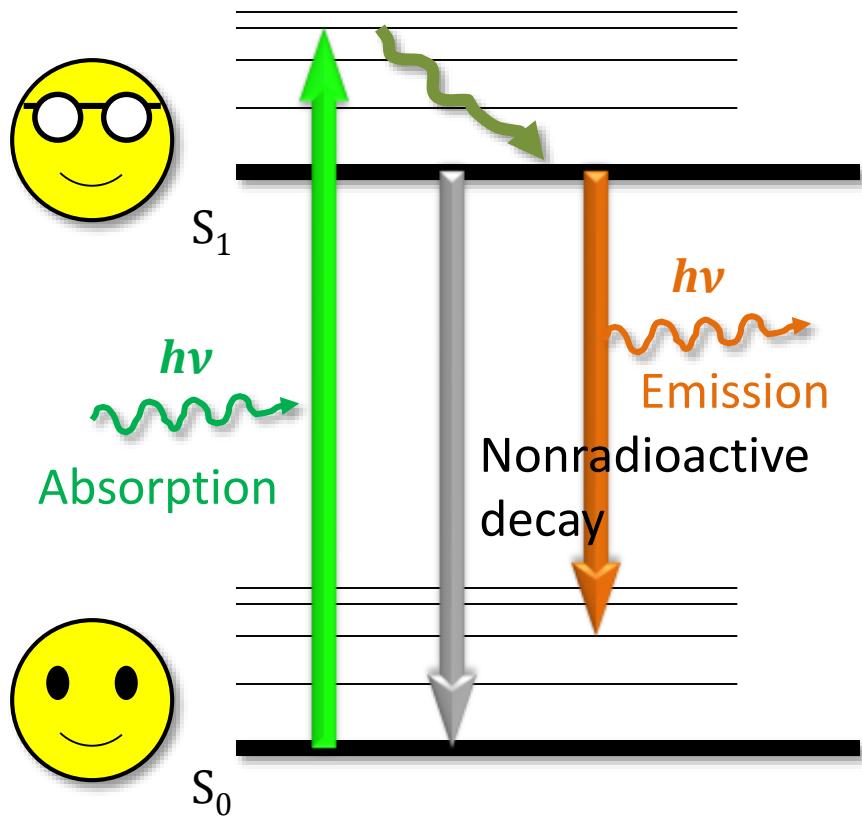


Rate constant =  $k_{\text{fl}}$

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



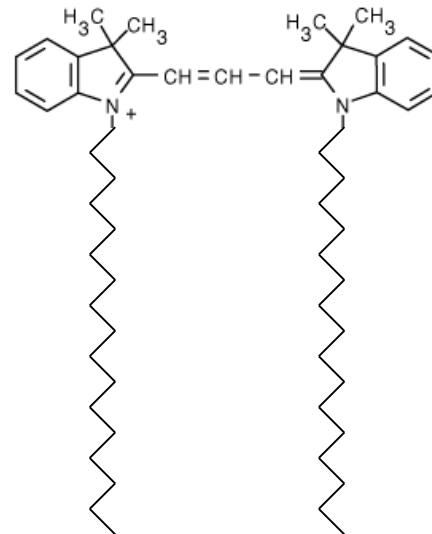
# Quenching



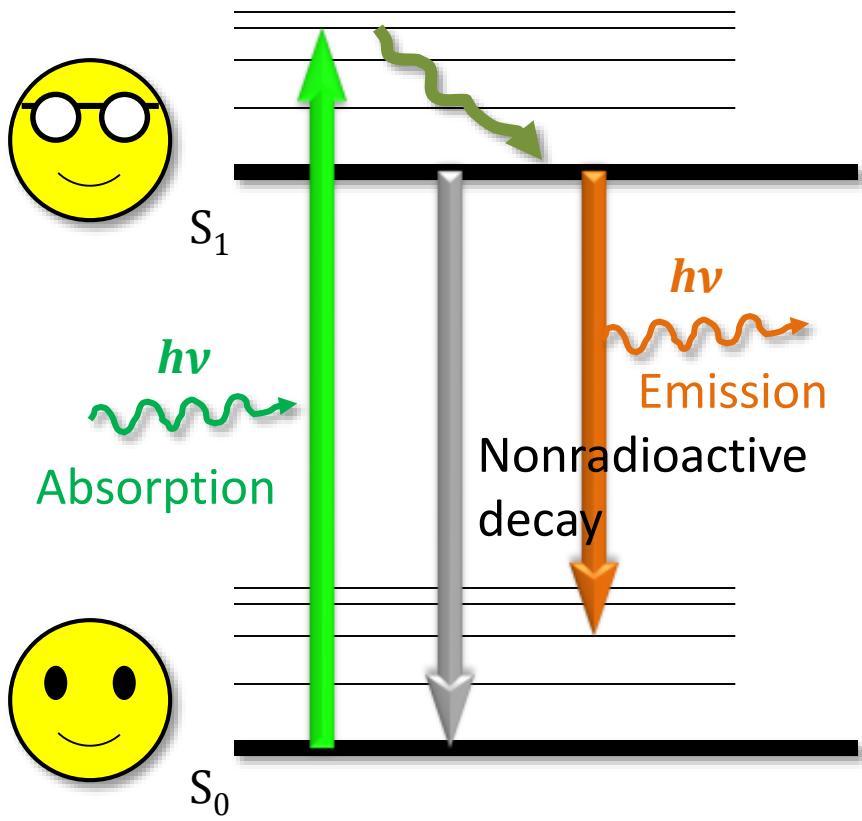
Non-radioactive decay:

Energy dissipation through  
structural flexibility

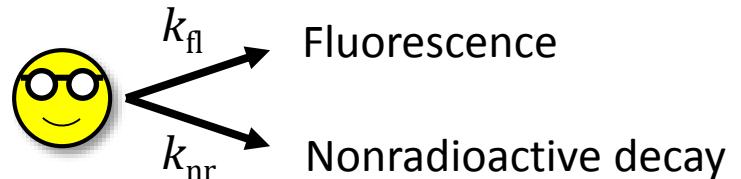
Dil



# Quantum Yield



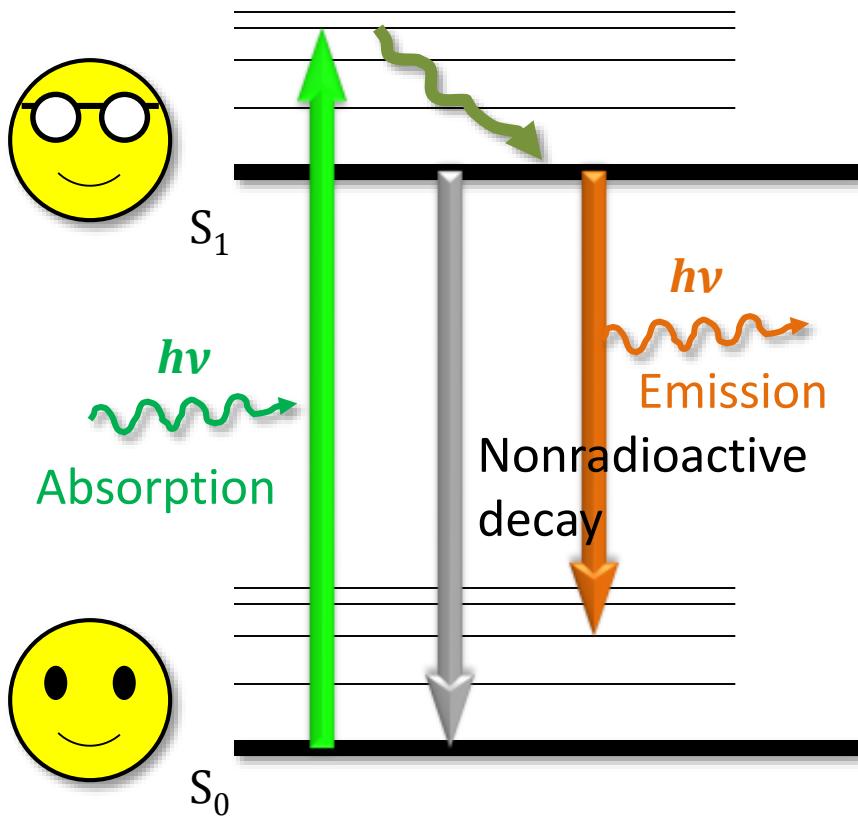
$$QE = \frac{\text{emitted photon}}{\text{absorbed photon}}$$



$$\begin{aligned} QE &= k_{fl} / (k_{fl} + k_{nr}) \\ &= 1 / (1 + \tau_{fl} k_{nr}) \end{aligned}$$

$$\begin{aligned} \tau_{fl} \uparrow, \quad QE \downarrow \\ k_{nf} \uparrow, \quad QE \downarrow \end{aligned}$$

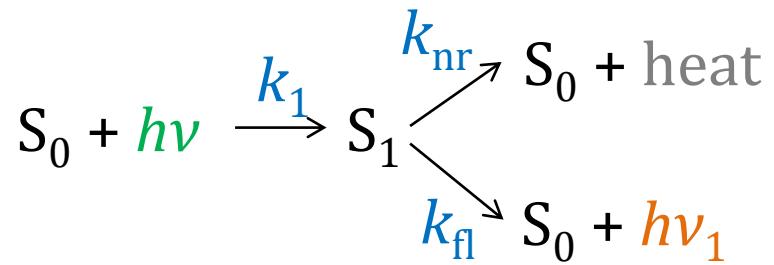
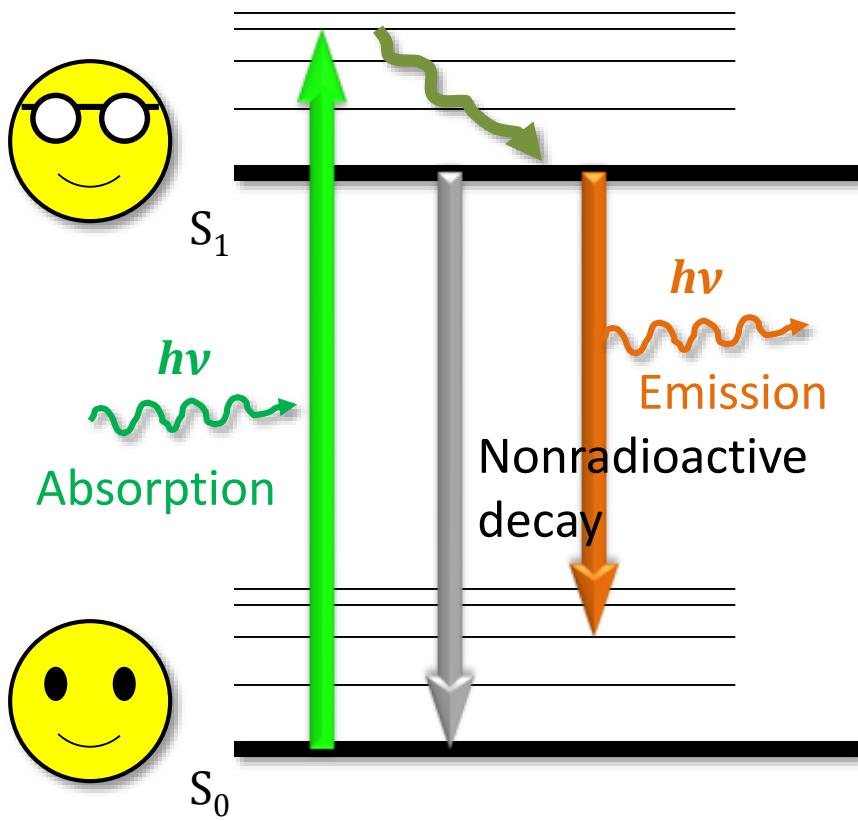
# Quantum Yield



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

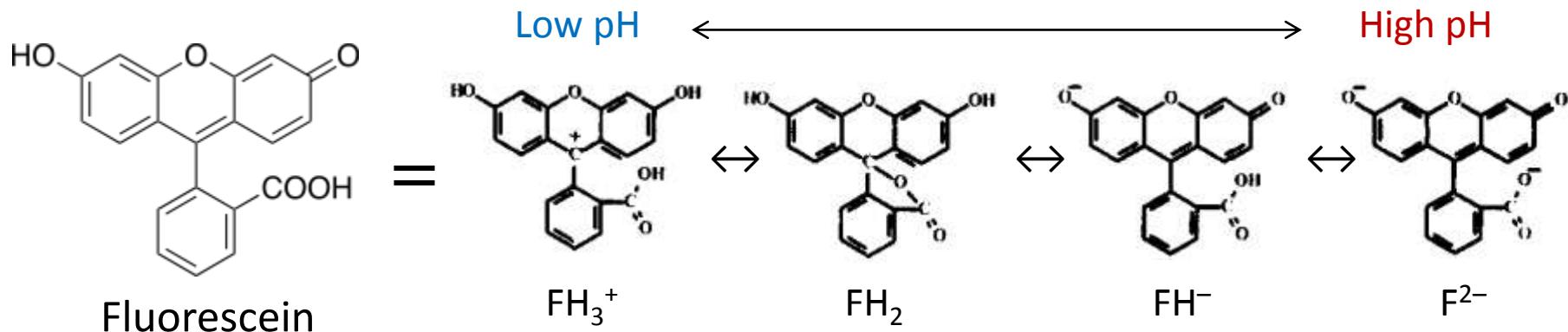
Fluorophore	QE
Fluorescein in ethanol	0.97
Tryptophan, pH 7.2	0.14
EGFP	0.60
EGFP chromophore by itself	0.0005

# The brightness of a fluoropore



Brightness  $\approx$   
EC (wavelength)  
 $\times$   
QE

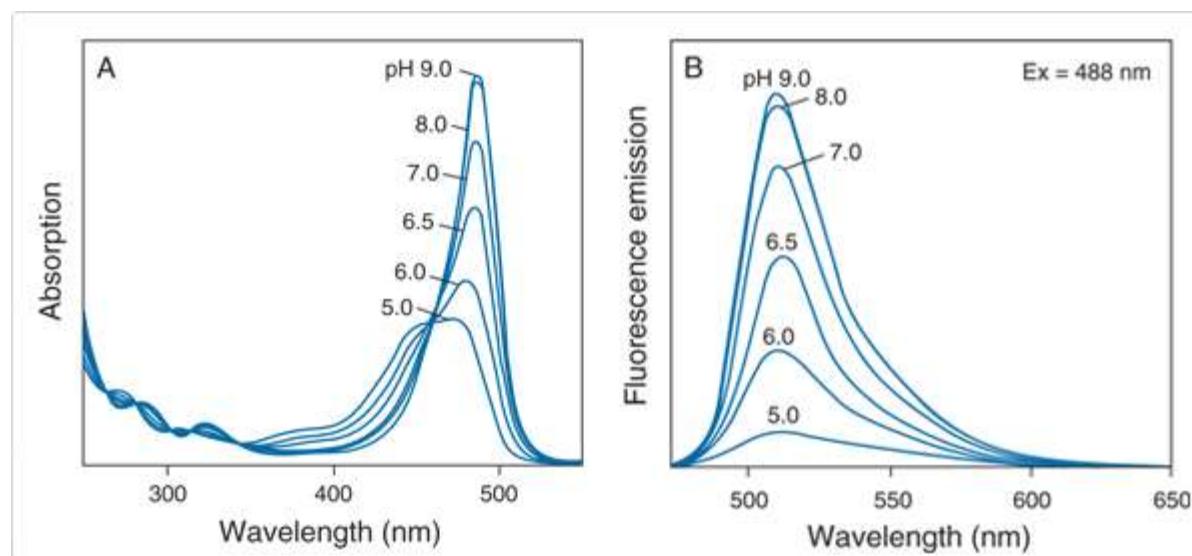
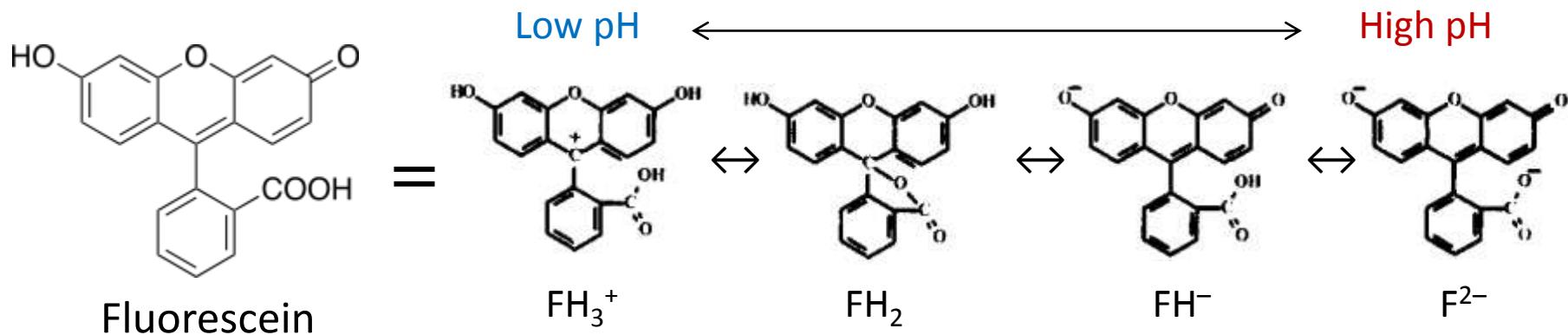
# Brightness can be affected by the environment



Summary of absorption, fluorescence and protolytic properties of fluorescein

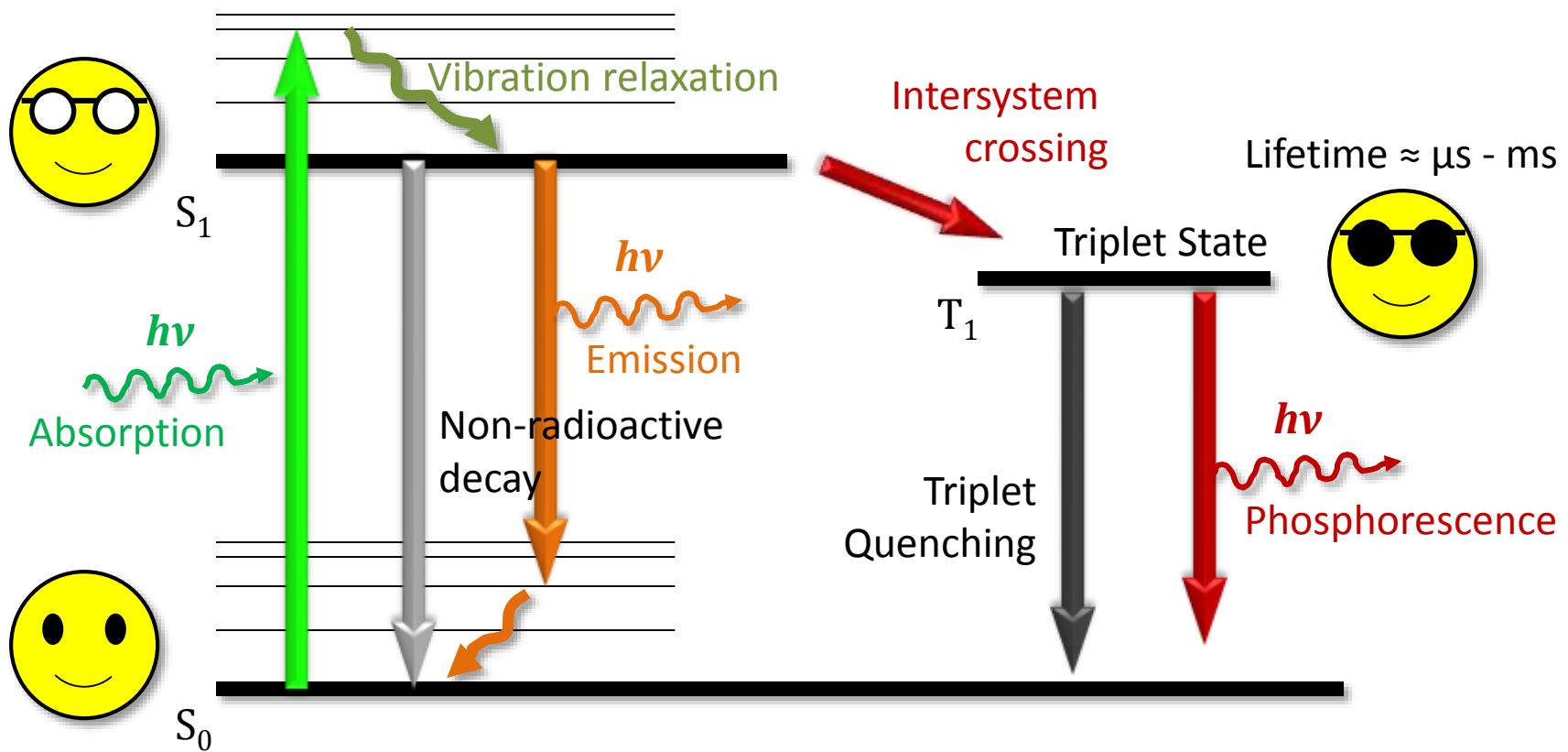
	$\text{FH}_3^+$	$\text{FH}_2$	$\text{FH}^-$	$\text{F}^{2-}$
$\epsilon/\text{M}^{-1} \text{cm}^{-1} (\lambda/\text{nm})$	53000(437)	3600(475)	29000(472)	76900(490)
$\epsilon/\text{M}^{-1} \text{cm}^{-1} (\lambda/\text{nm})$	7100(297)	11000(434)	29000(453)	9500(322)
$\epsilon/\text{M}^{-1} \text{cm}^{-1} (\lambda/\text{nm})$	33000(250)		700(310)	14000(283)
$\epsilon/\text{M}^{-1} \text{cm}^{-1} (\lambda/\text{nm})$			17000(273)	43000(239)
$\tau/\text{ns}$			3.0	4.1
$\Phi^f$	$\sim 0(\text{pH} > 1.5)$	$\sim 0$	0.37	0.93
$\Phi^c$	0.6	0.8	—	
$\text{p}K_a'(1 \text{ M})$	2.14	4.20	6.0	
$\text{p}K_a'(50 \text{ mM})$	2.09	4.30	6.41	
$\text{p}K_a$	2.08	4.31	6.43	

Brightness can be affected by the environment

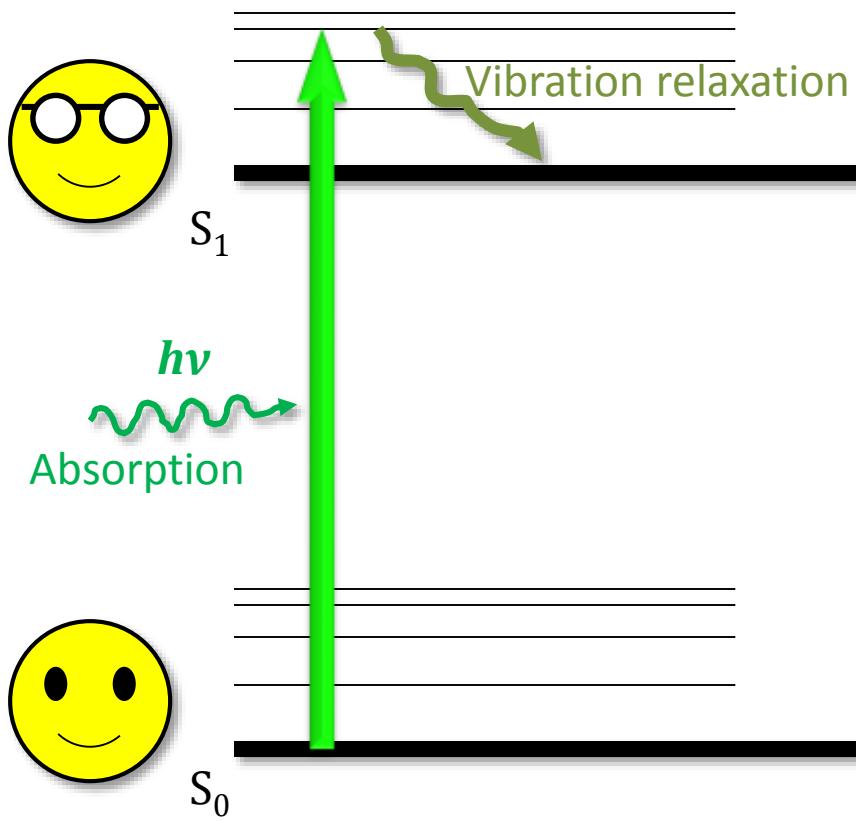


Invitrogen

# The Full Jabłonski Diagram



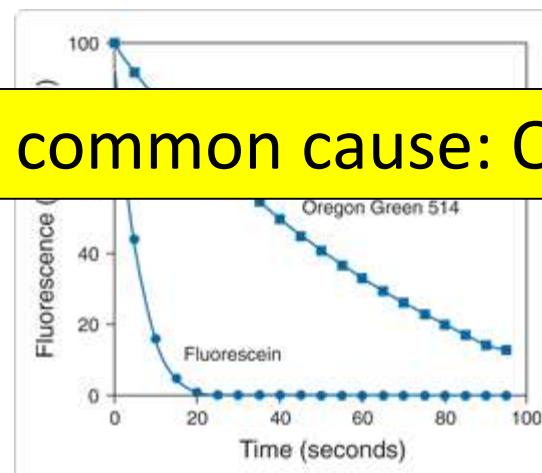
# Photobleaching



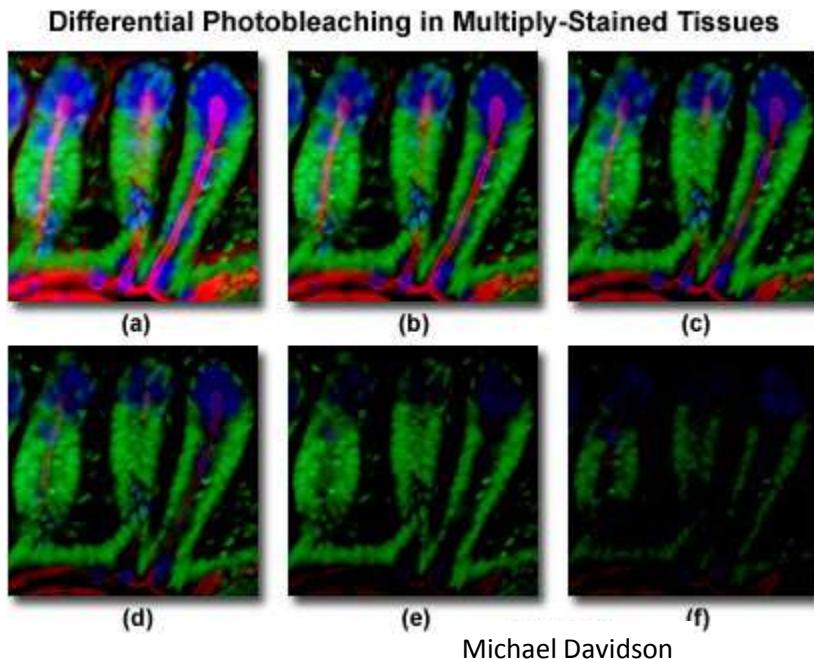
Intersystem  
crossing

Triplet State

Most common cause:  $O_2$



# Photobleaching is our enemy



For all fluorescence imaging:

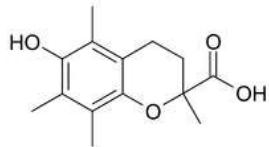
- Kills signal
- Alters the relative contrast

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.

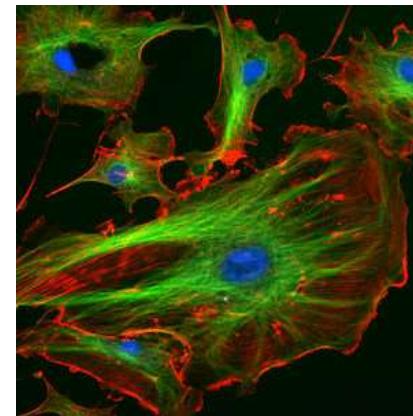
- Labeling as densely as possible
  - Budget the photons
    - Only expose when observing
    - Minimize exposure time & excitation power
    - Use efficient filter combinations
    - Use high QE, low noise camera

# Choice of fluorophores

Natural fluorescence



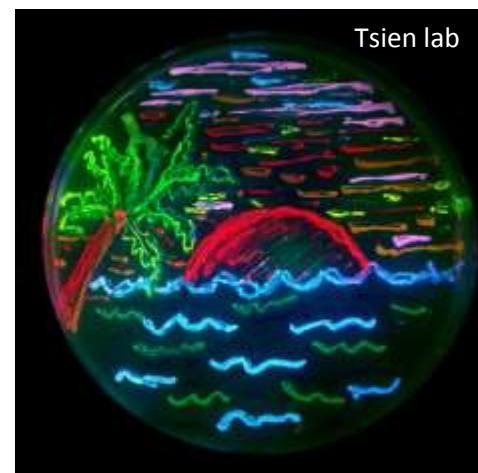
Organic dyes



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)

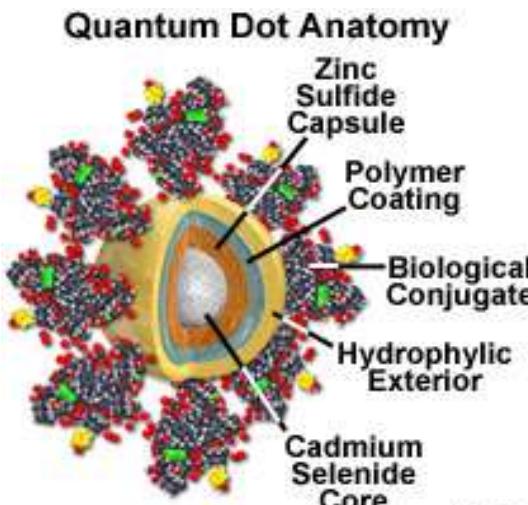
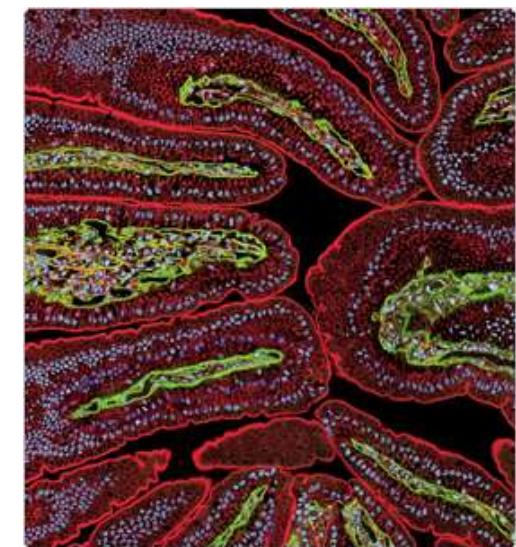
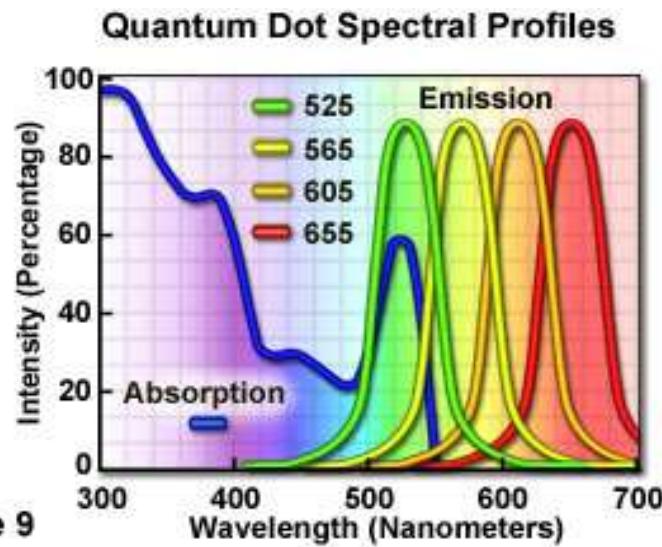
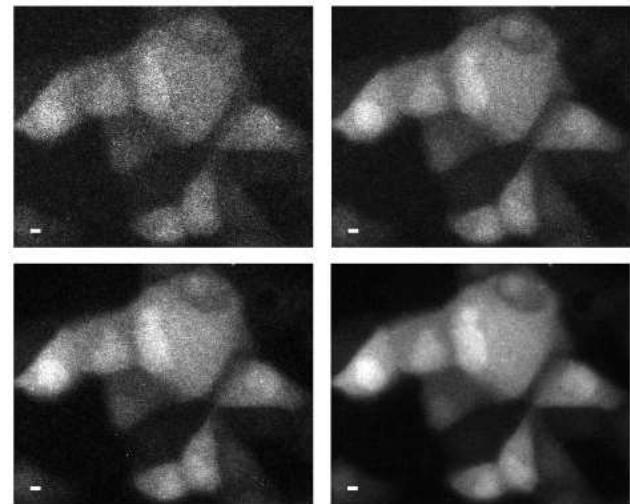
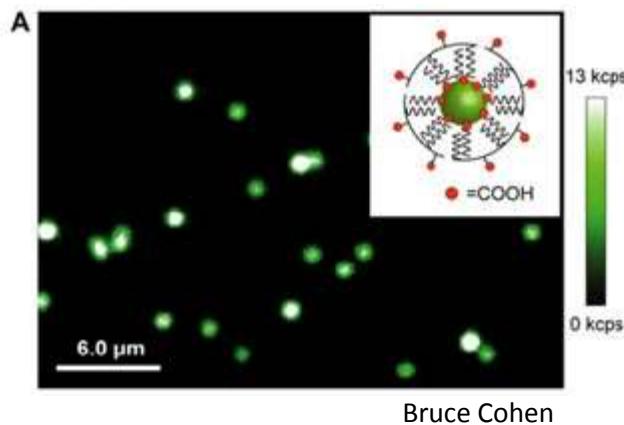


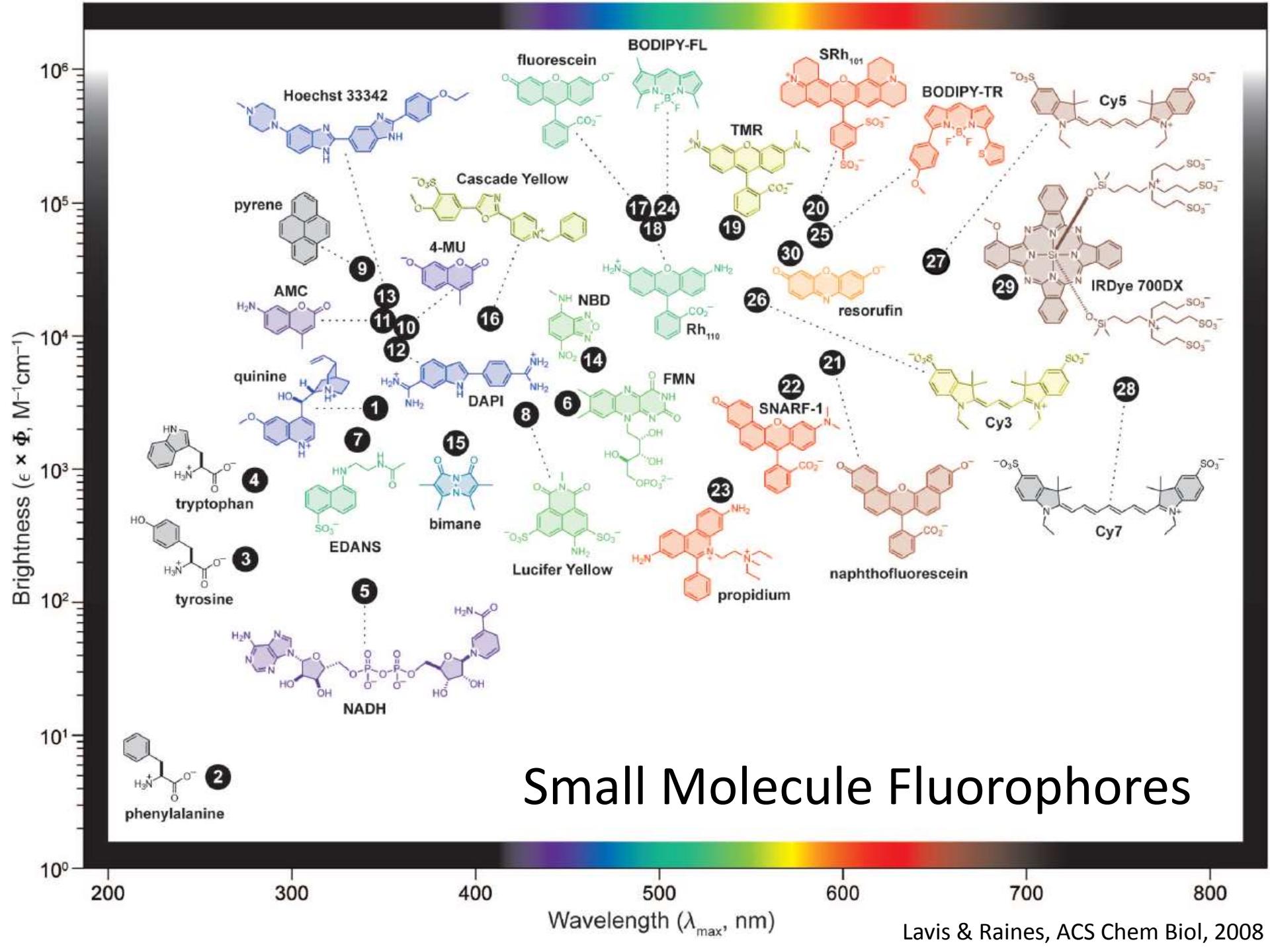
Figure 9



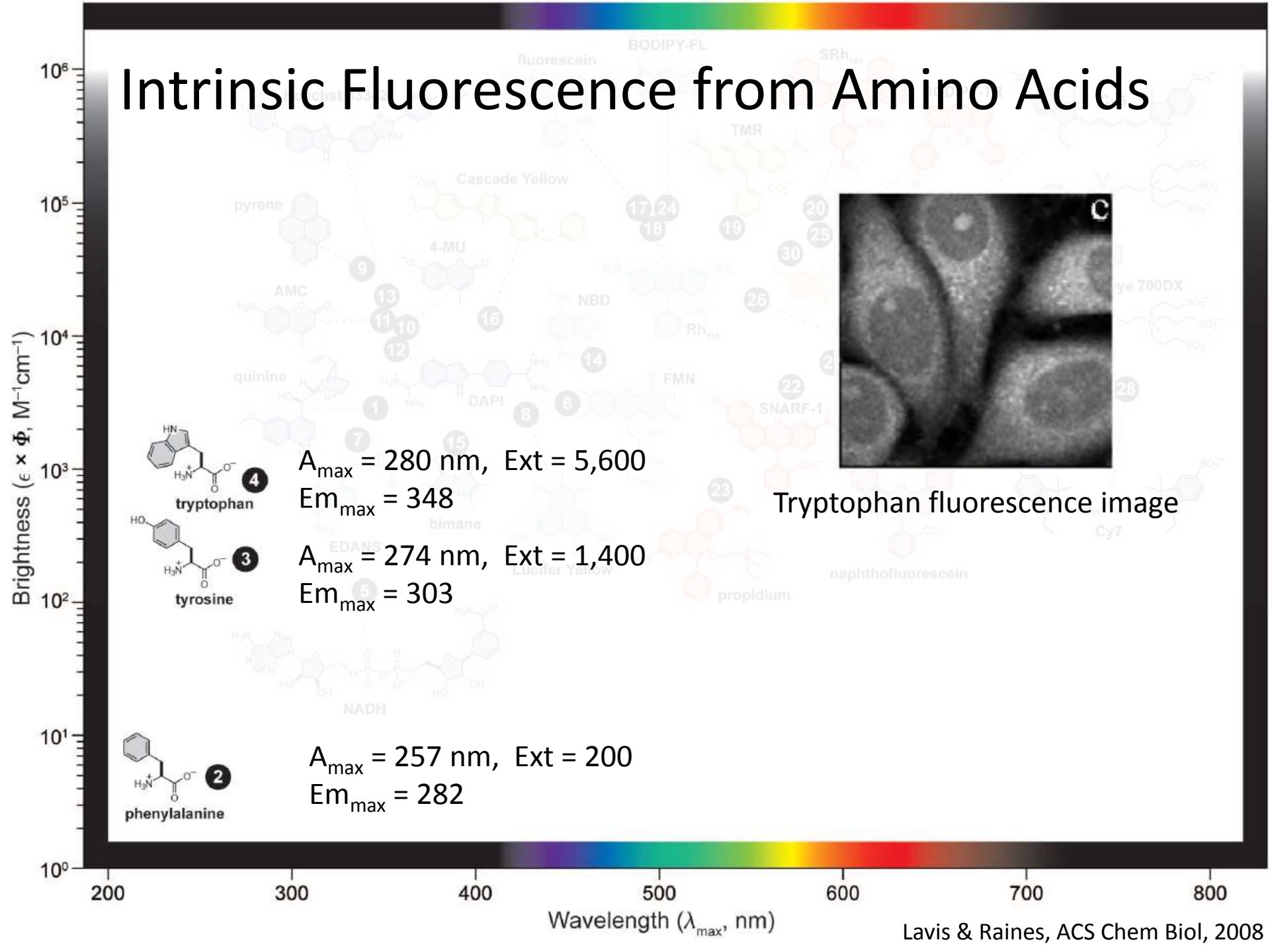
# Inorganic fluorophores

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

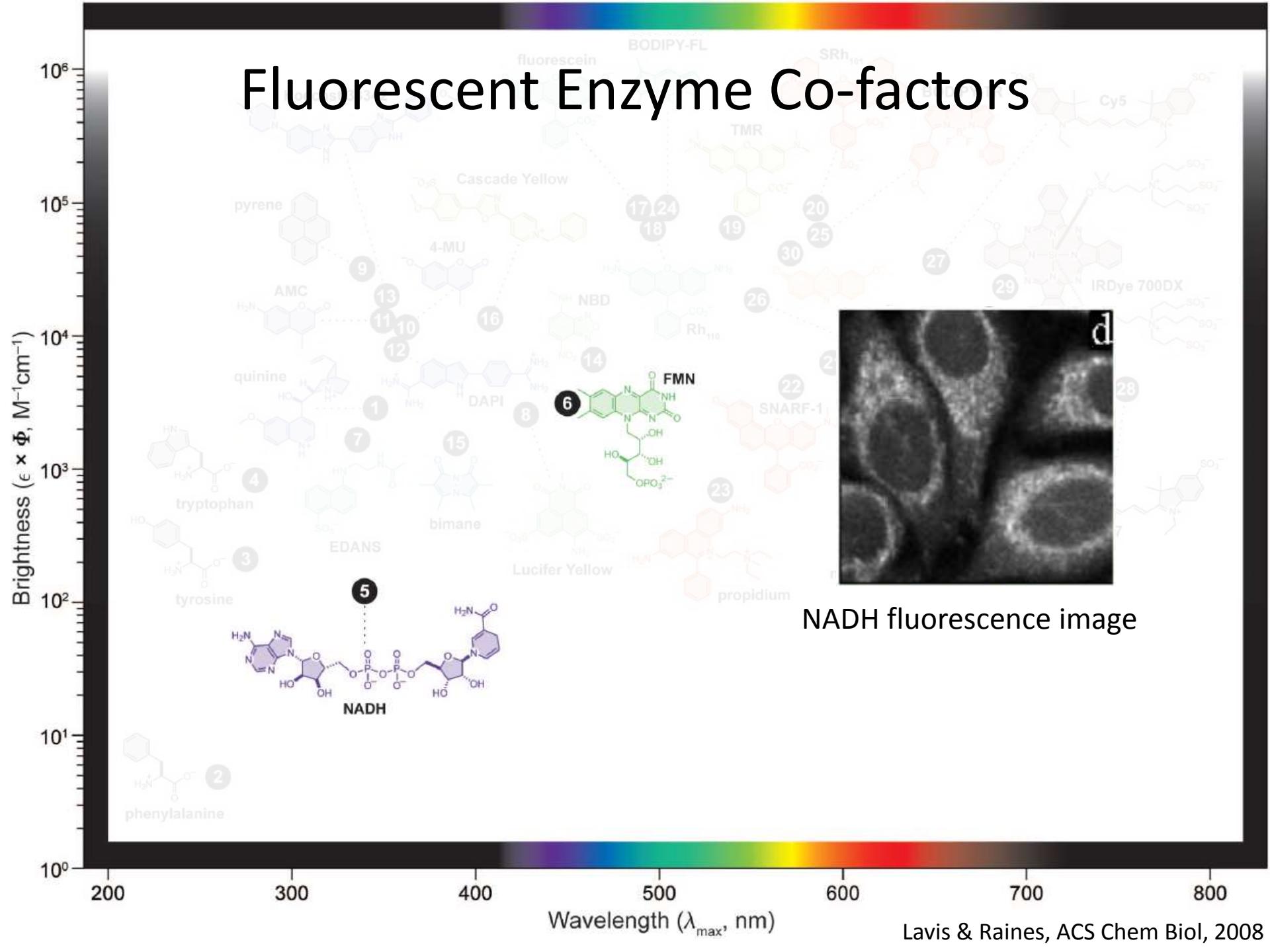


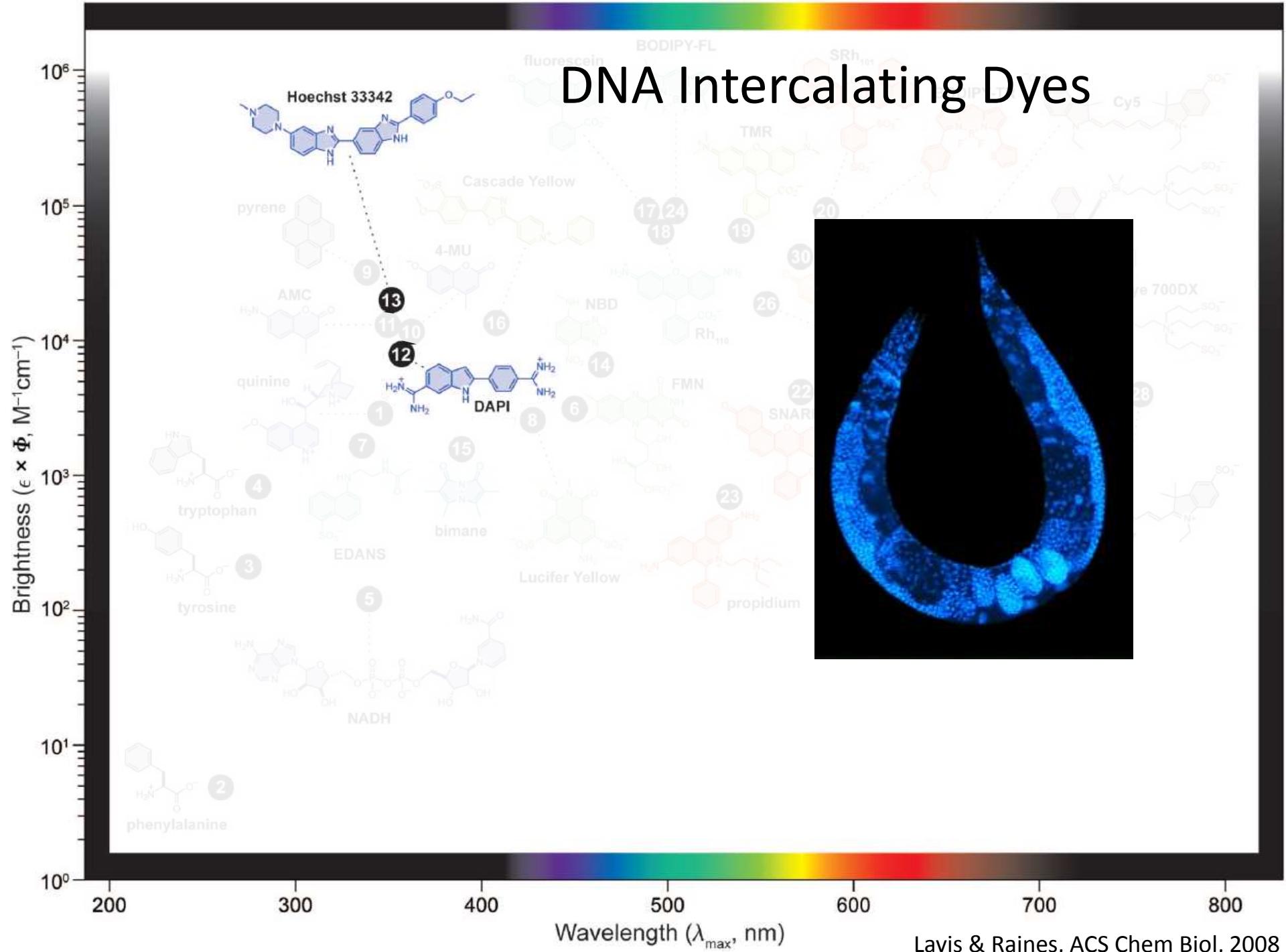


# Intrinsic Fluorescence from Amino Acids



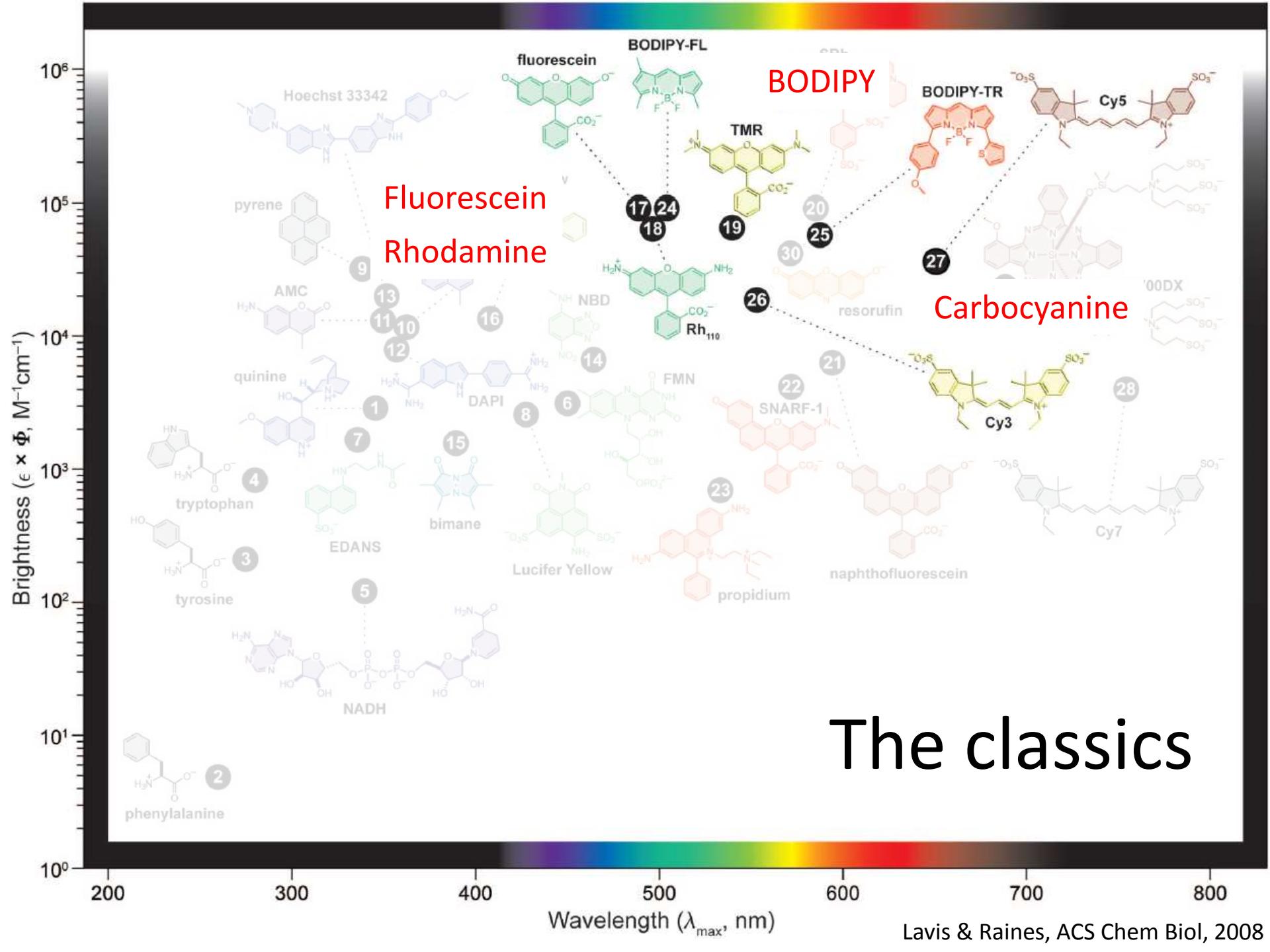
# Fluorescent Enzyme Co-factors





# DNA Intercalating Dyes

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

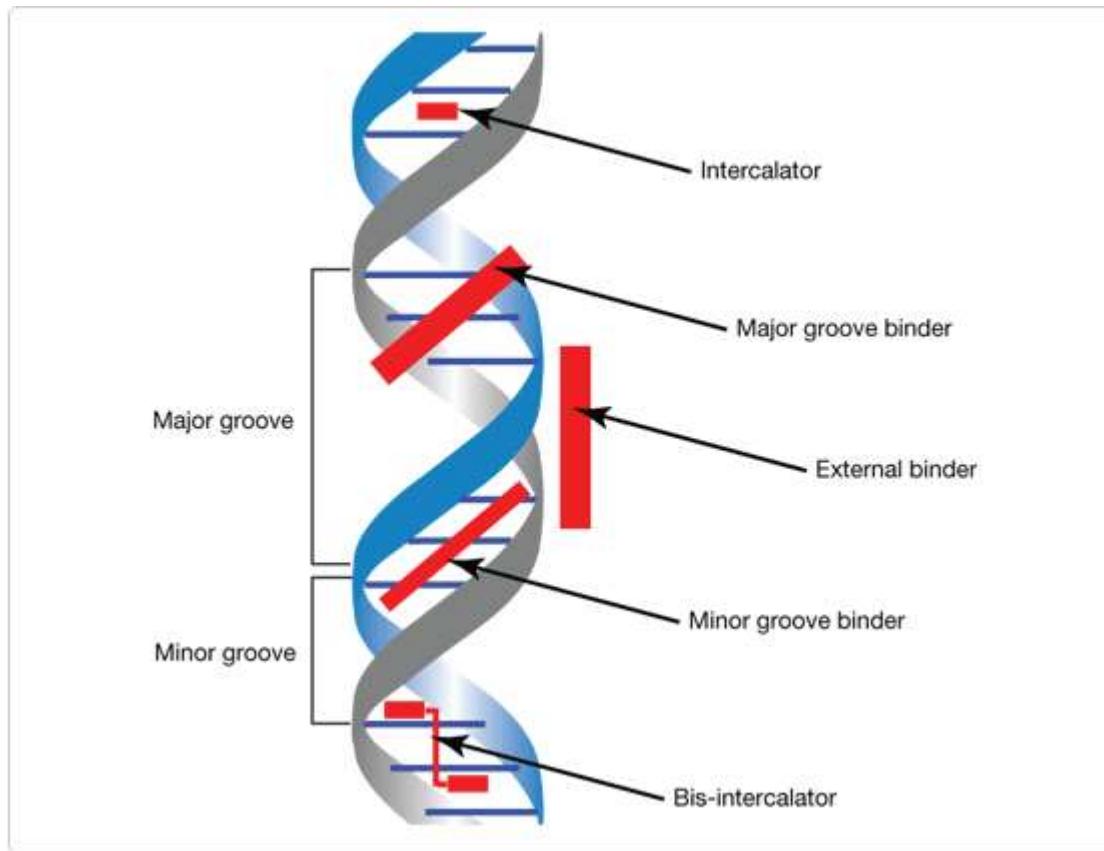
- Small
  - e.g. DNA labeling
- Bright and photostable
- Wide range of wavelengths
  - UV to NIR
- Most of them lack labeling specificity by itself.
  - Specific probes for nucleic acid, plasma membrane, mitochondria
- Almost all “great” ones are unable to cross the membrane.

# (Specific) delivery of small molecule dyes



# Functional dyes

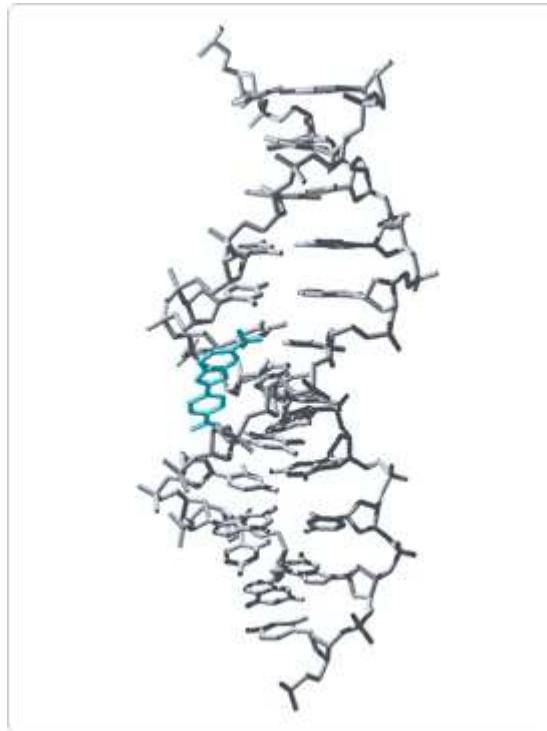
- Nucleic acid intercalating dyes
  - QE increment upon binding to DNA/RNA



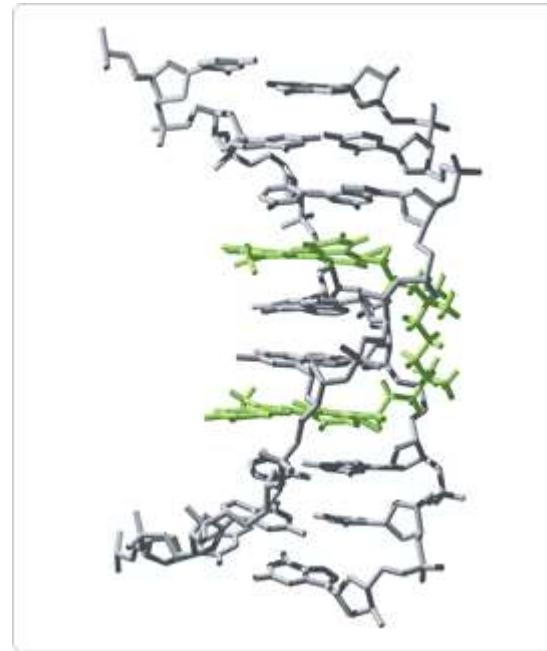
# Functional dyes

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

DAPI



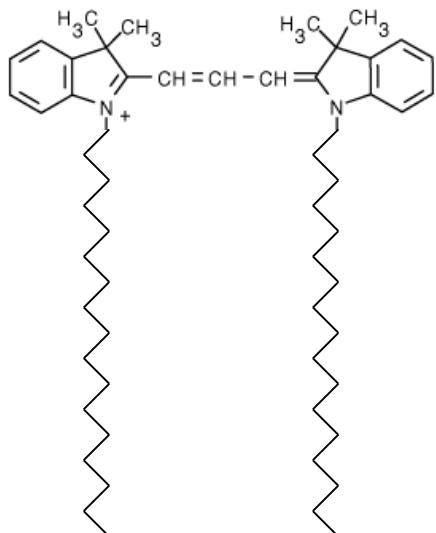
TOTO-1



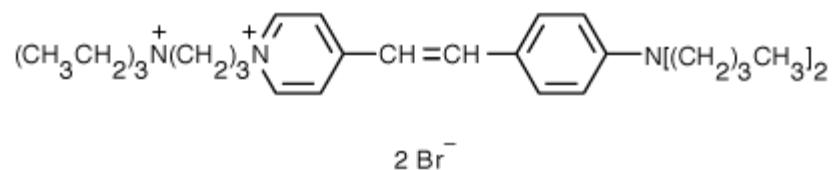
# Functional dyes

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

Dil



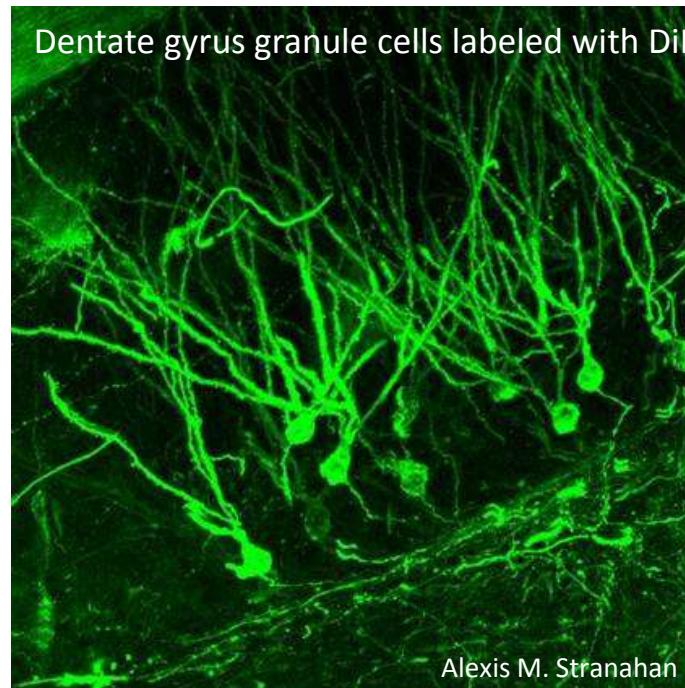
FM 1-43



$2 \text{Br}^-$

# Functional dyes

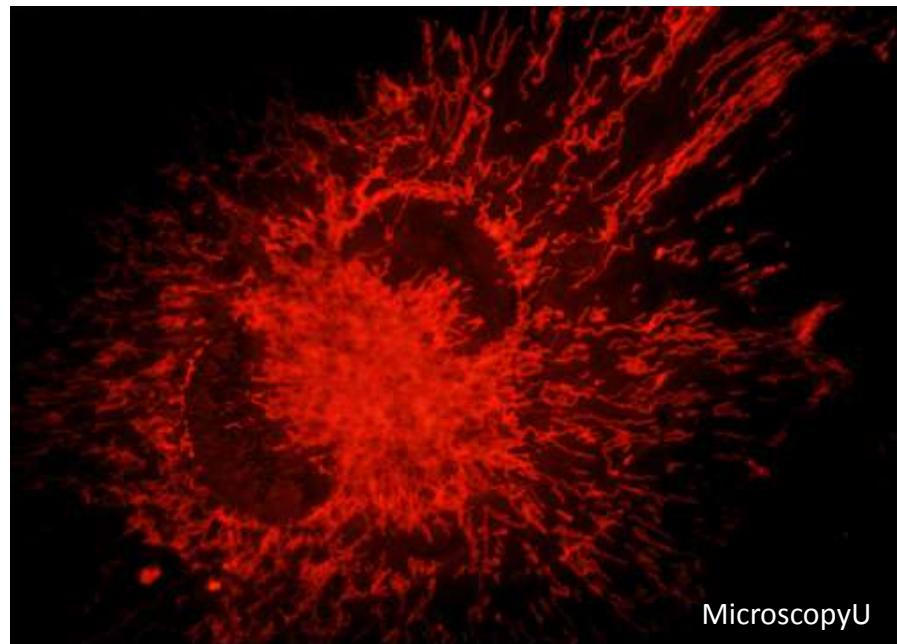
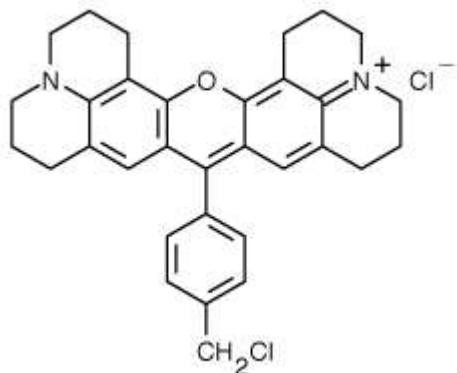
- Nucleic acid intercalating dyes
- Membrane stains
  - Amphiphilic dyes that partition in lipid bilayers



# Functional dyes

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

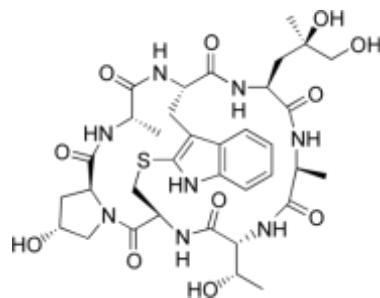
MitoTracker Red CMXRos



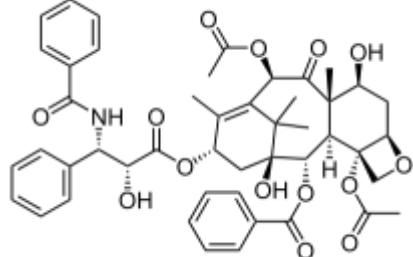
# Small molecules probes

- Small molecules that bind proteins

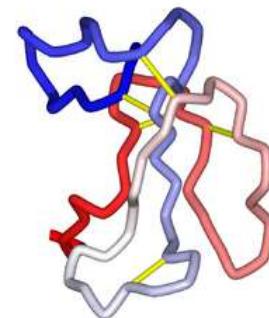
Phalloidin



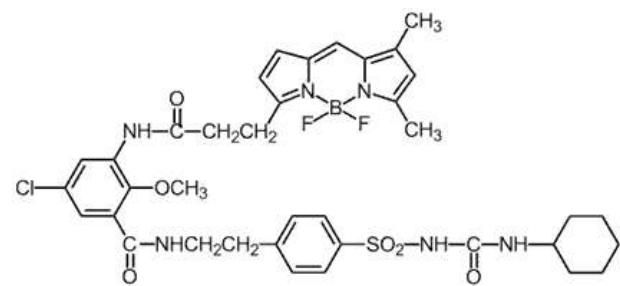
Taxol



$\alpha$ -bungarotoxin



ER Tracker Green



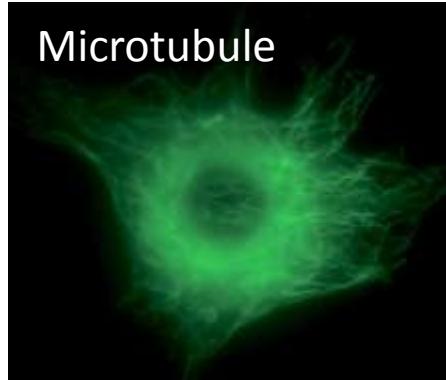
# Small molecules probes

- Small molecules that bind proteins

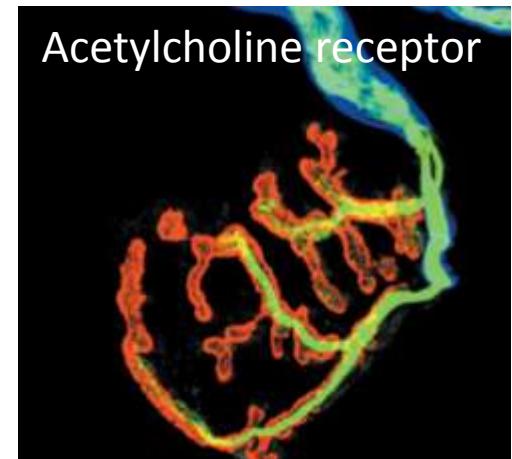
Phalloidin



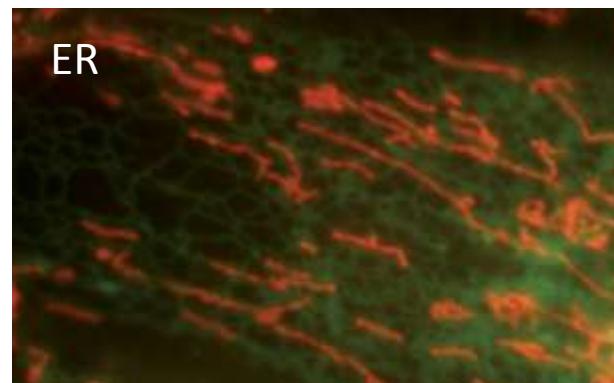
Taxol



$\alpha$ -bungarotoxin



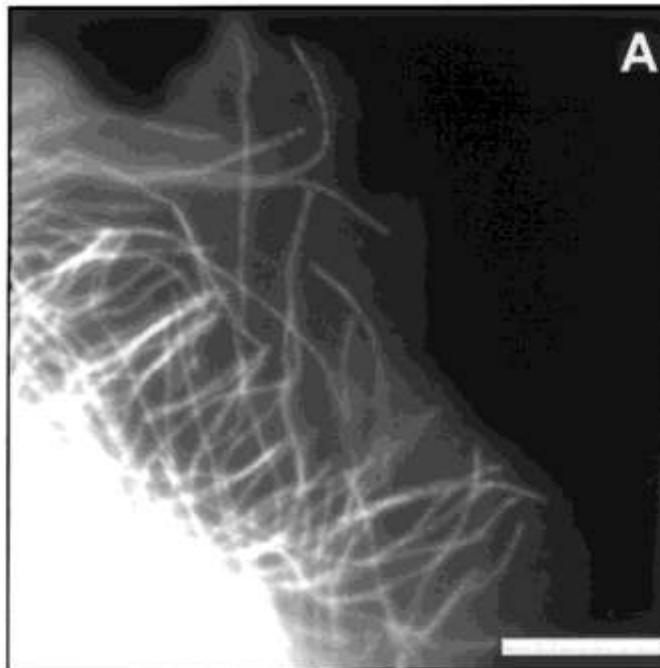
ER Tracker Green



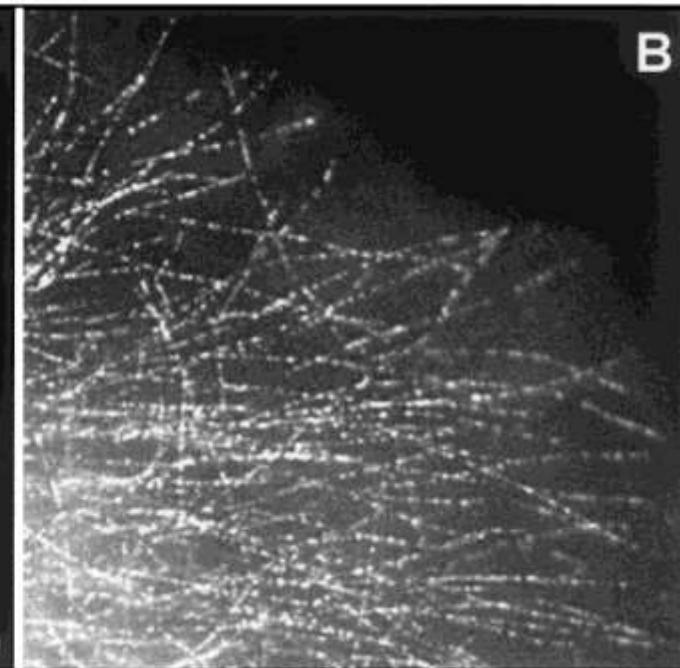
# Large molecule labeling

- In vitro reconstituted systems
- Injecting labeled proteins

10% labeled tubulin



0.25% labeled tubulin

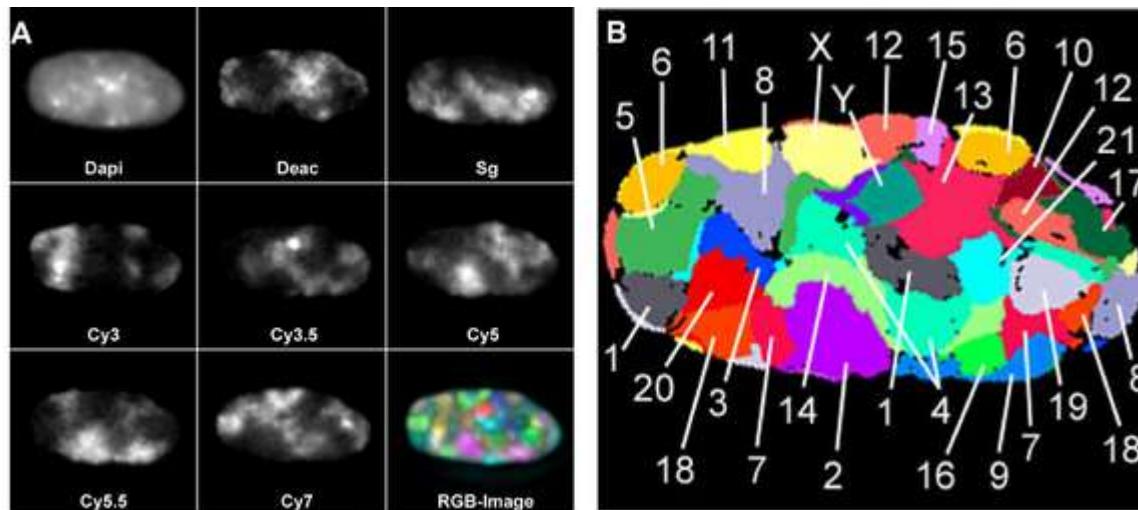


Waterman & Salmon, FASEB J, 1999

# 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

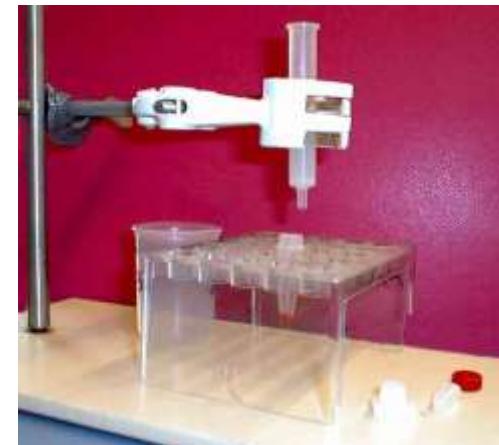
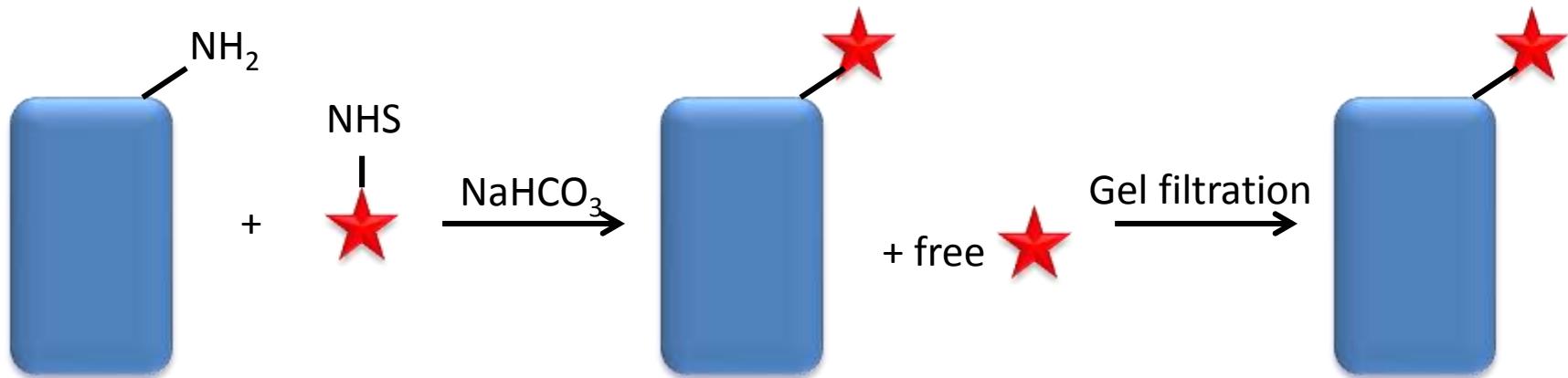
- In vitro reconstituted systems
- Injecting labeled proteins
- Fluorescence in situ hybridization (FISH)
- Biotin-avidin interaction
  - Labeled avidin/streptavidin
  - Biotin-dyes

# 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

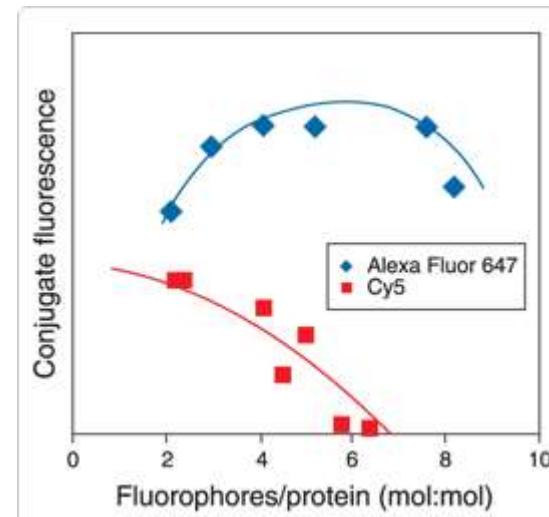
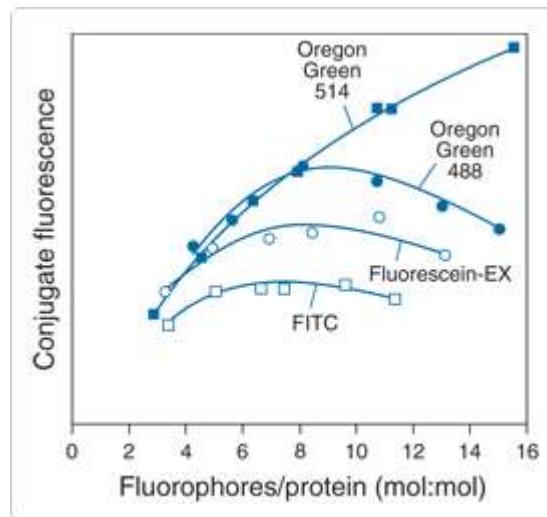


$$\text{Dye concentration} = A_{\max} / \text{E.C.}_{\text{dye}@\max}$$

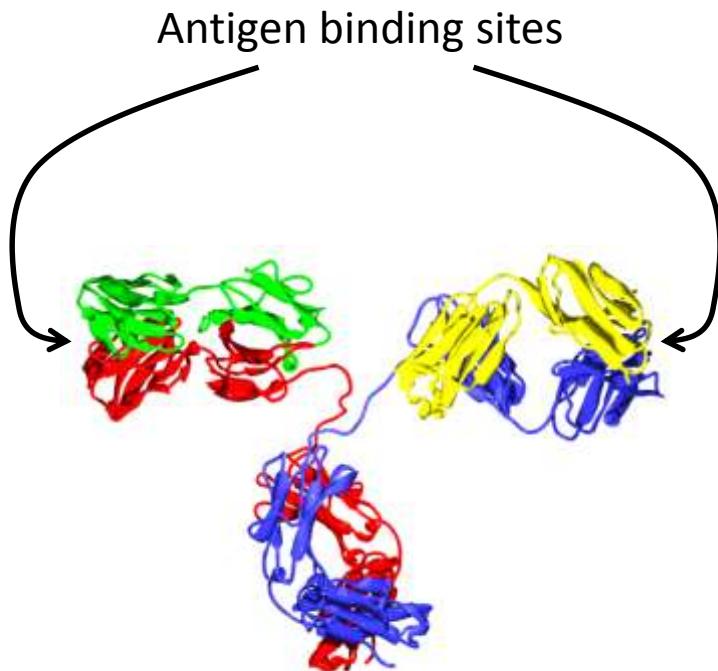
$$\text{Protein concentration} = (A_{280} - \text{dye contribution}) / \text{E.C.}_{\text{protein}@280}$$

$$\text{Dye per protein} = \text{Dye concentration} / \text{Protein concentration}$$

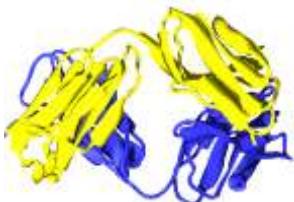
Too high labeling stoichiometry can make the protein dead...  
and lead to self-quenching



# Immunofluorescence



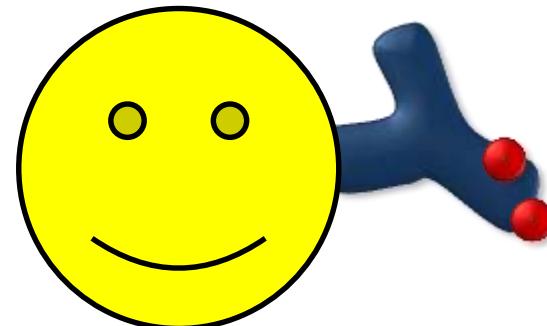
Fab fragment



Nanobody

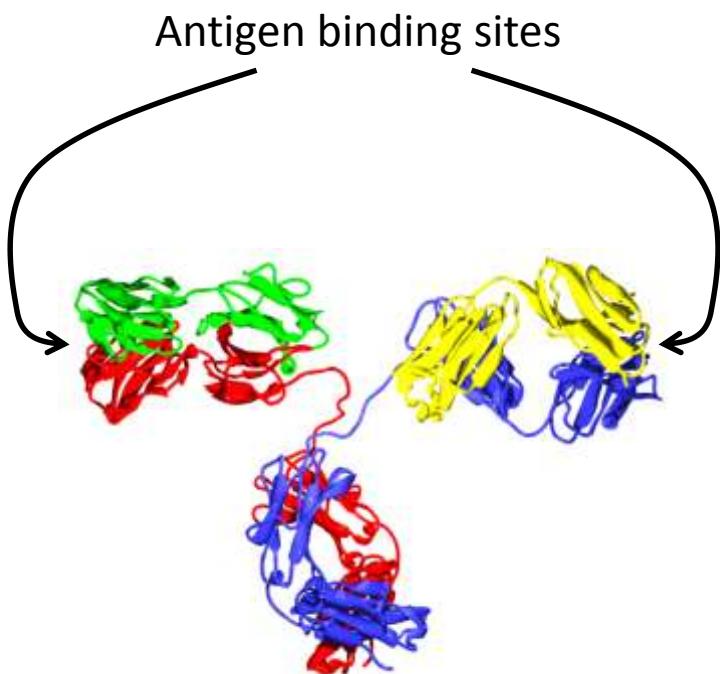


Direct immunofluorescence

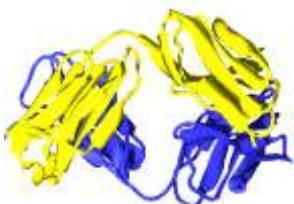


Primary antibody binding efficiency can be < 10%

# Immunofluorescence



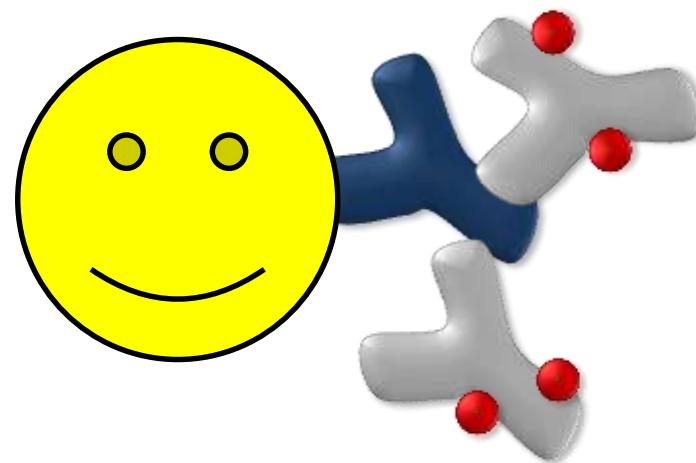
Fab fragment



Nanobody



Indirect immunofluorescence



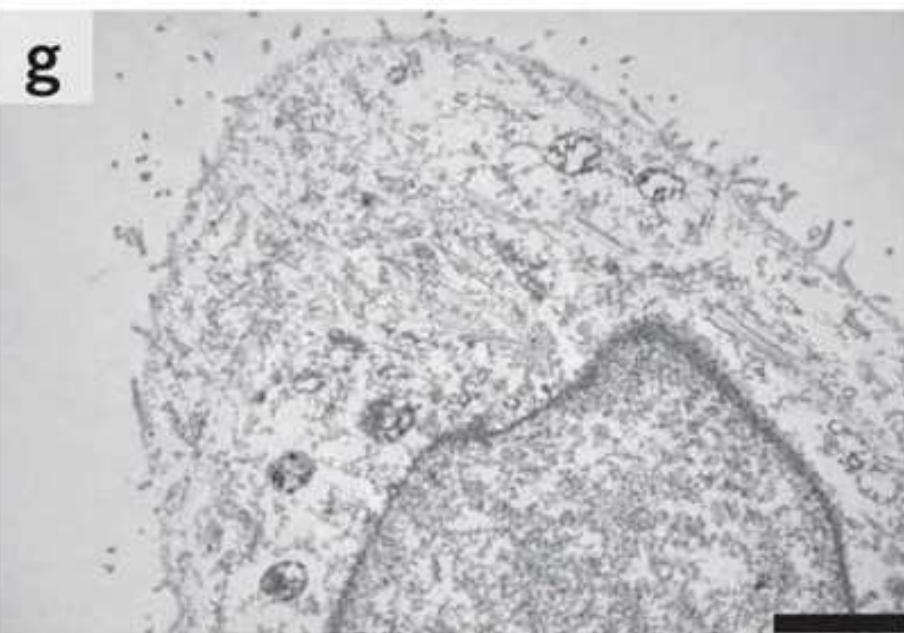
# Fixation methods

- 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 ( $\text{NaBH}_4$  reduction)
  - Required for electron microscopy

# Fixation methods

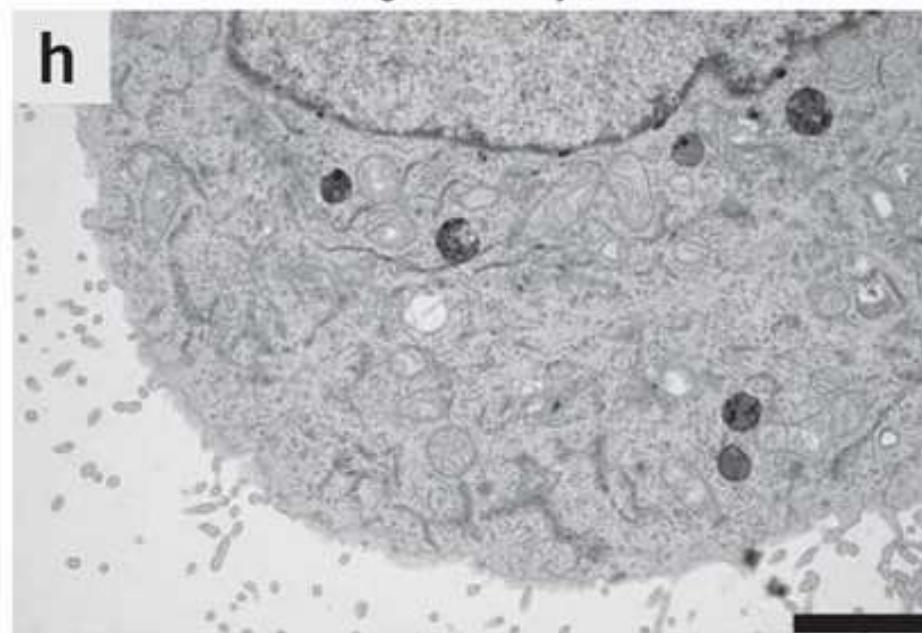
- Methanol
- Formaldehyde (Paraformaldehyde)
- Glutaraldehyde

Methanol



**g**

2% glutaraldehyde



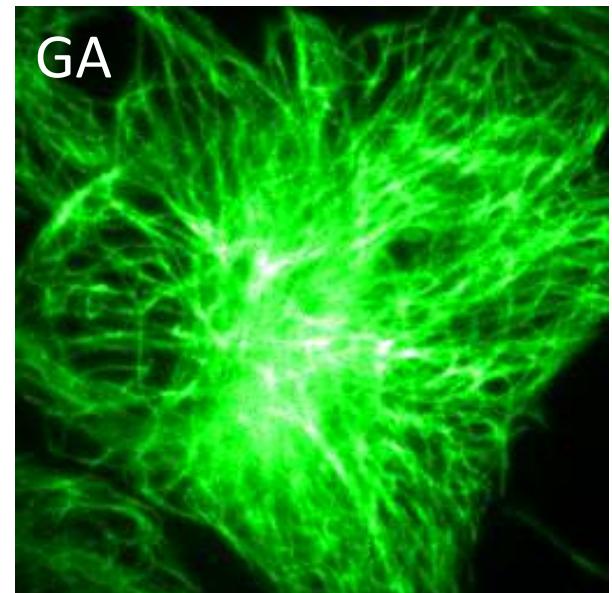
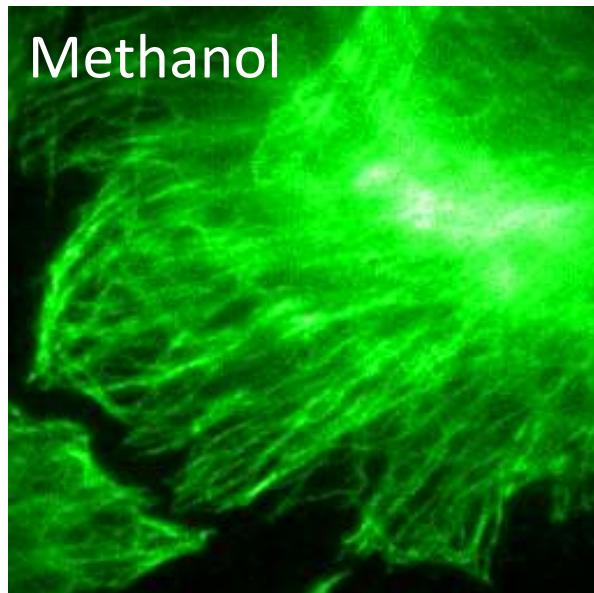
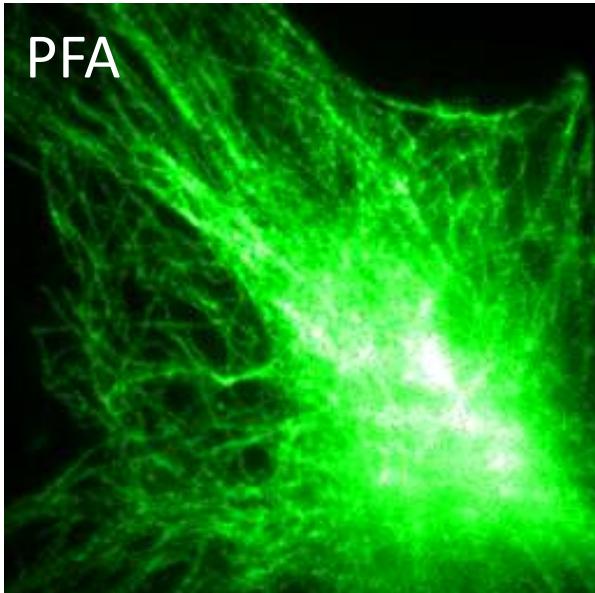
**h**

# 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

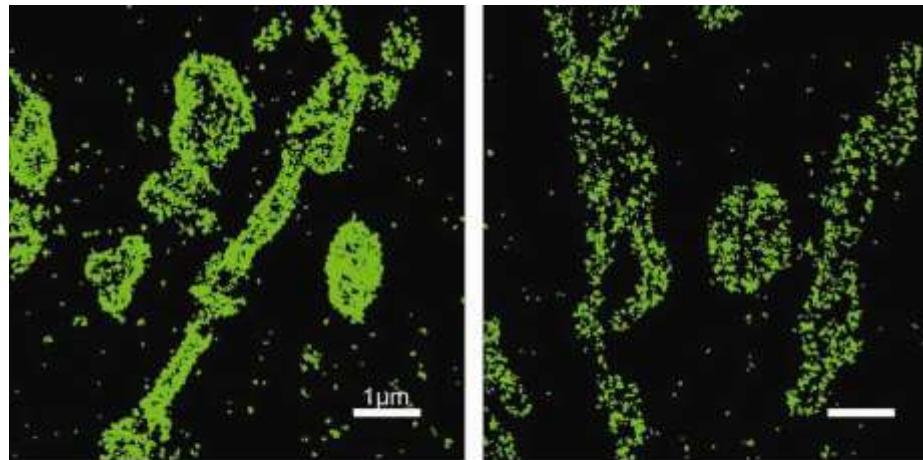
Ulrike Schnell, Freark Dijk, Klaas A Sjollema & Ben N G Giepmans

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

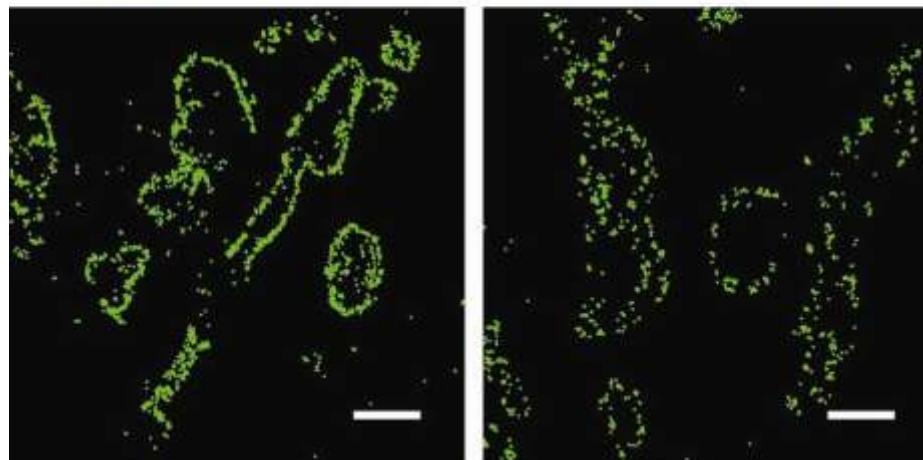
# Some fixation artifacts become visible in super-resolution microscopy

Tom20 –  
mitochondria  
outer membrane

Glutaraldehyde fixation      Formaldehyde fixation



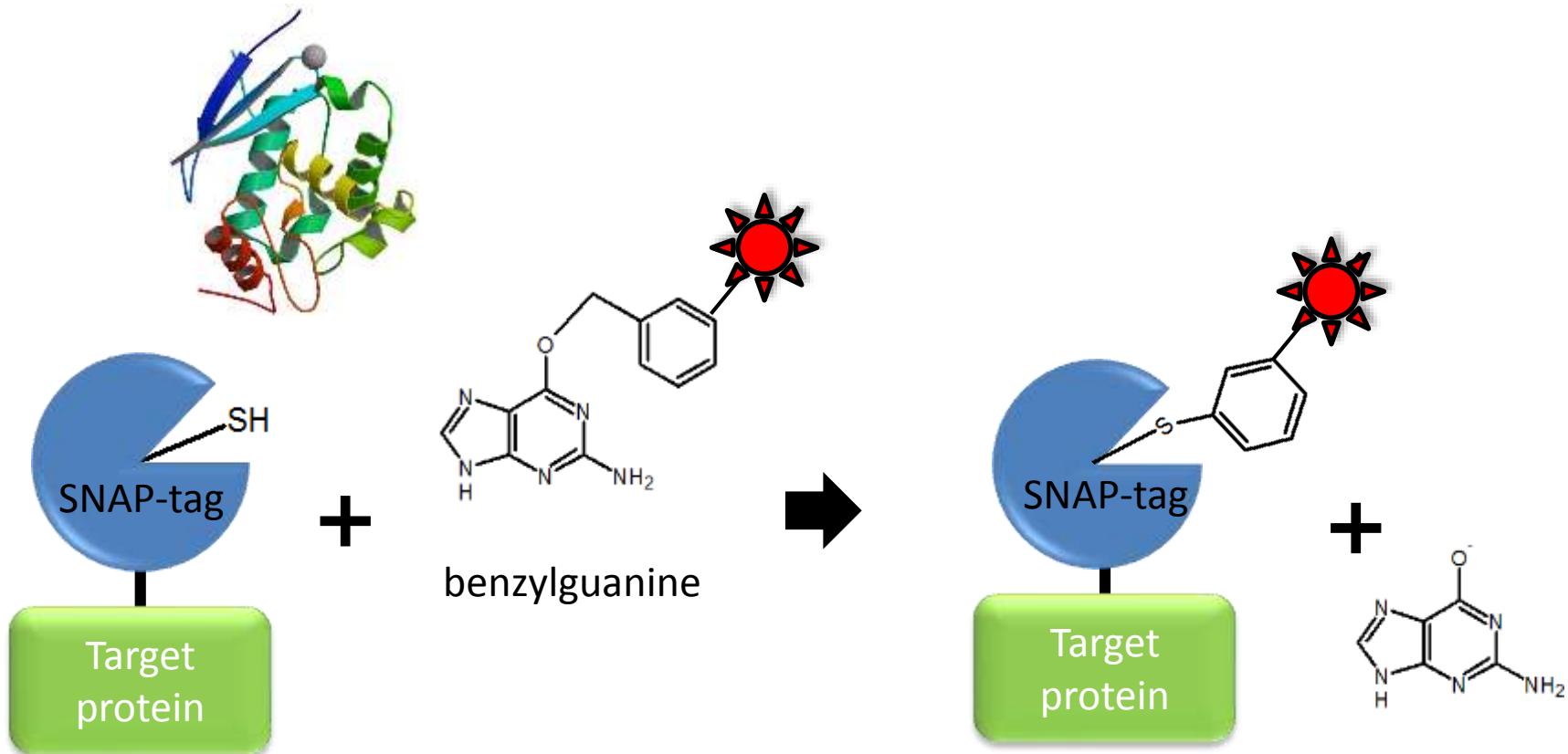
Projection



Section through  
the middle

# The hybrid approaches

- SNAP-tag, CLIP-tag, HALO-tag, TMP-tag...
  - SNAP-tag: based on human O<sup>6</sup>-alkylguanine-DNA-alkyltransferase (hAGT)



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