

Fluorescence Microscopy

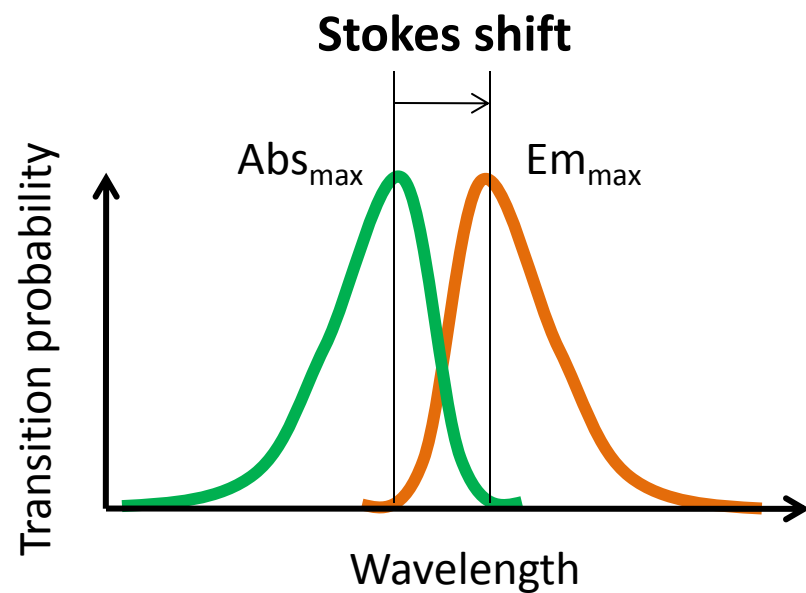
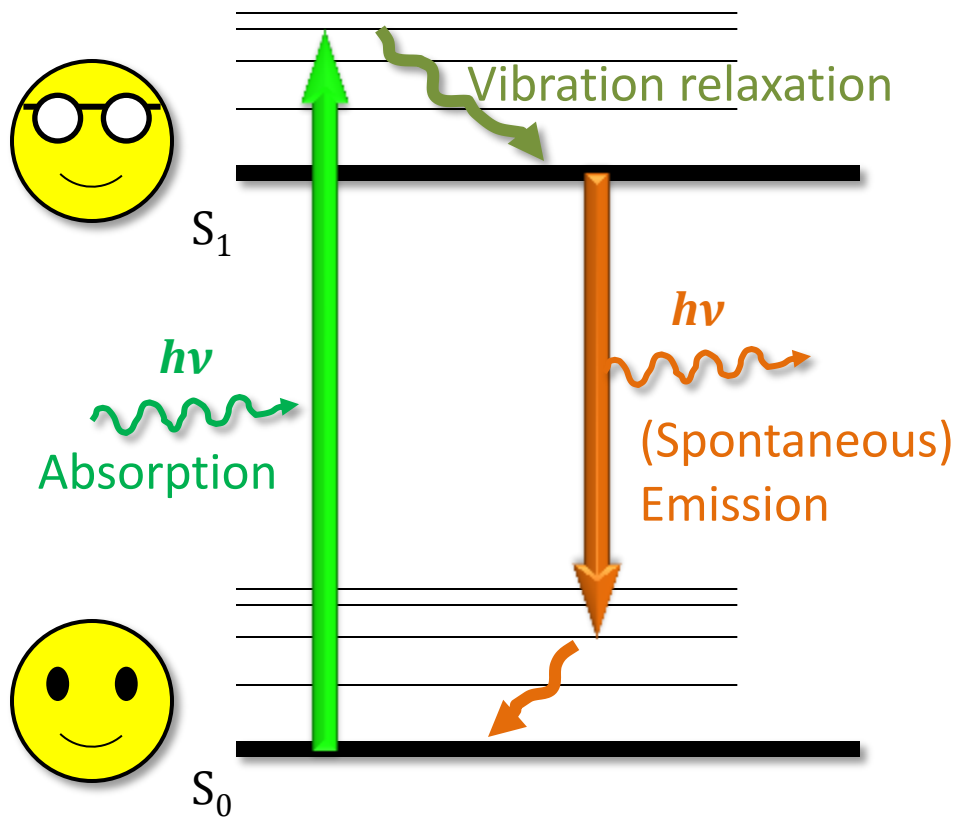
II. Fluorescent dyes



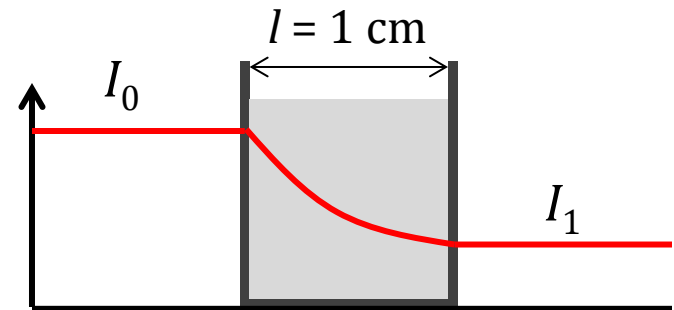
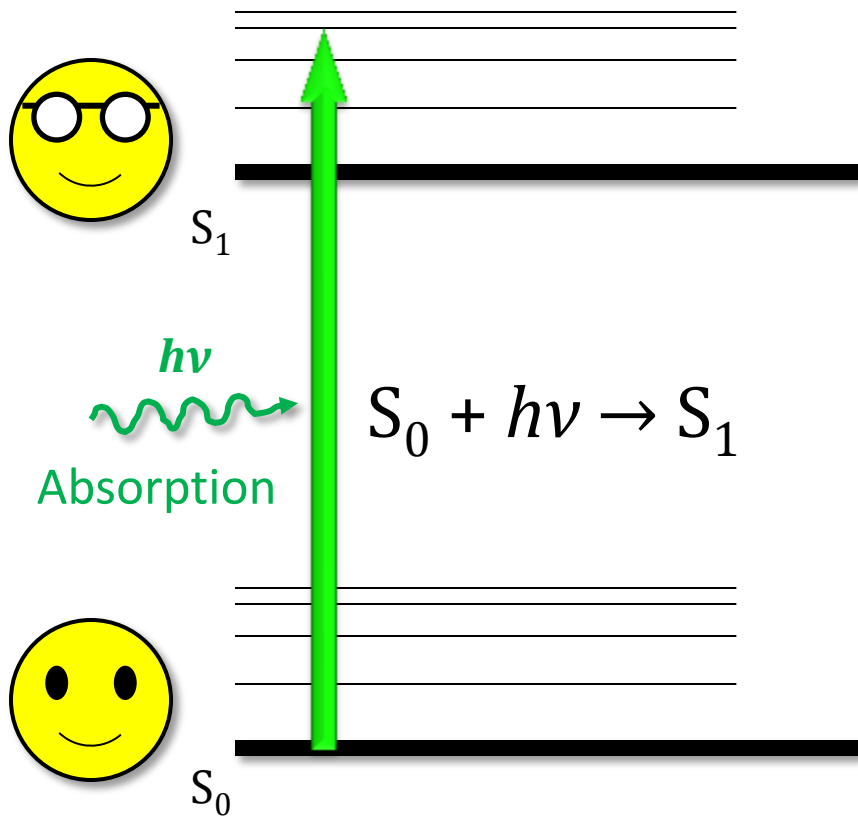
Bo Huang

2013.03.26

The fluorescence process



Extinction coefficient



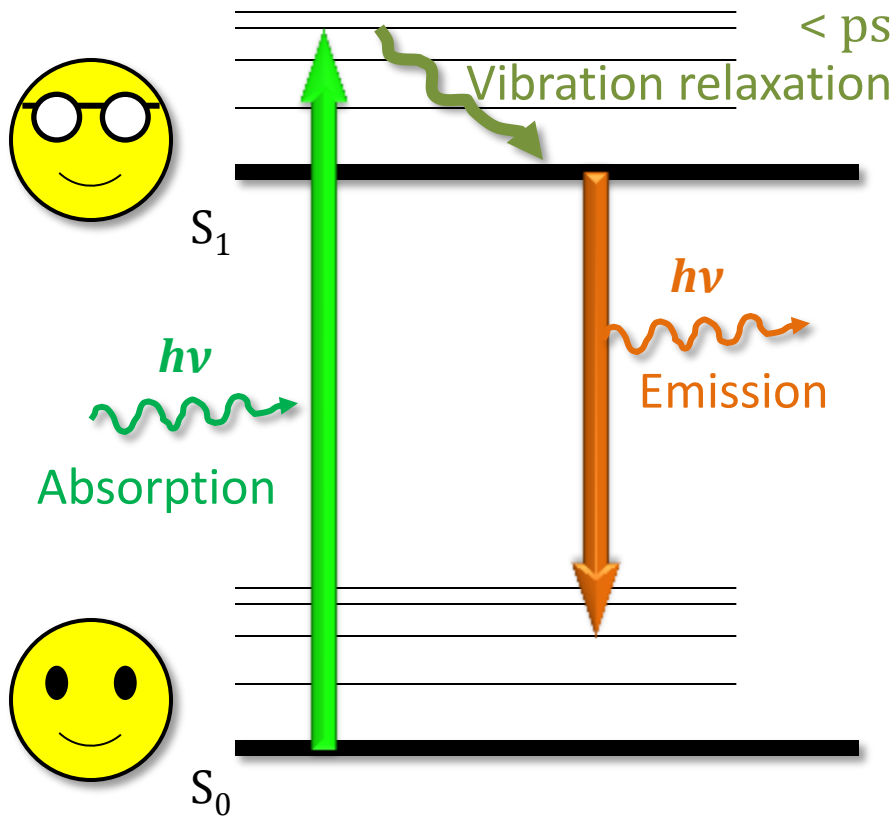
$$\text{Abs} = -\log_{10}(I_1/I_0)$$

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

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

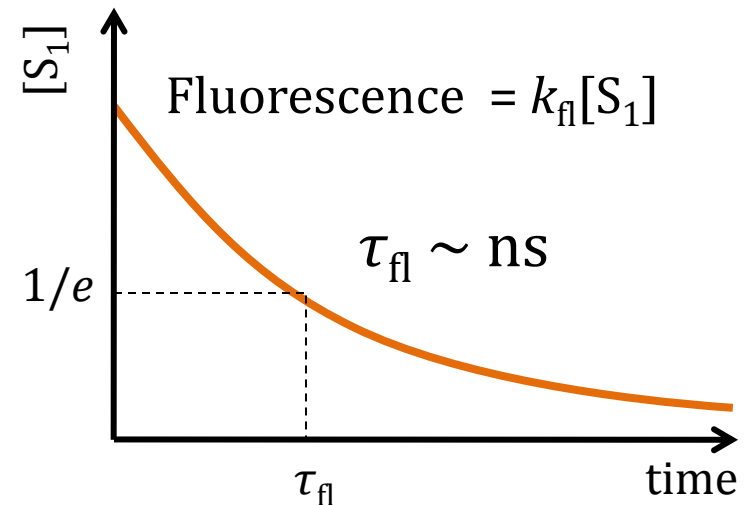
$1 \mu\text{M}, 1 \text{ cm} \rightarrow A \approx 0.1$ (T = 80%)

Fluorescence Lifetime

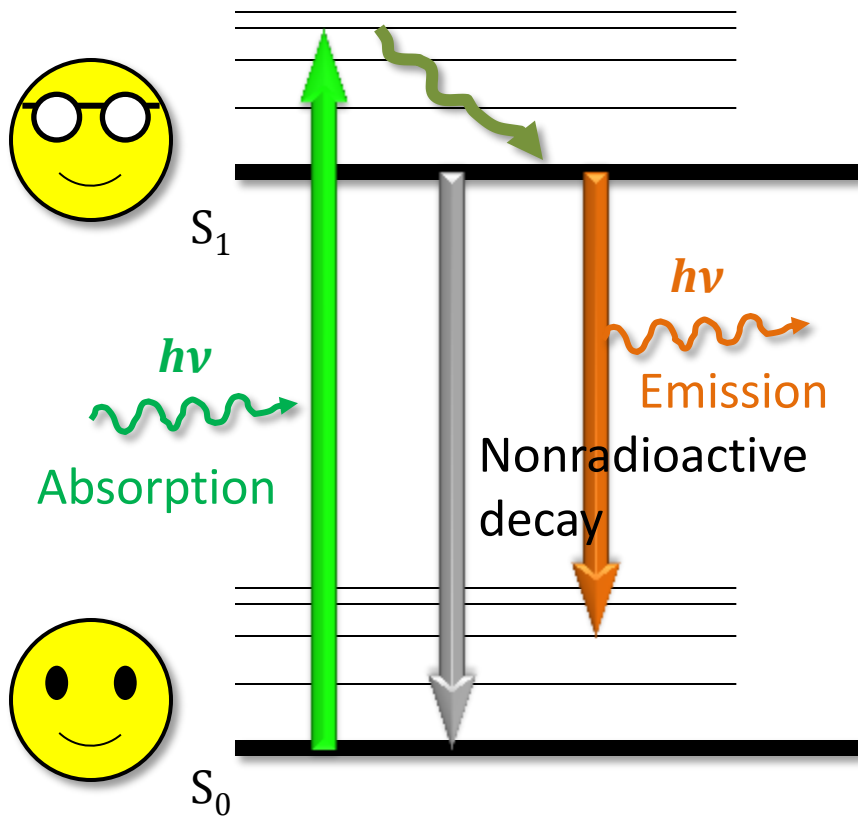


Rate constant = k_{fl}

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



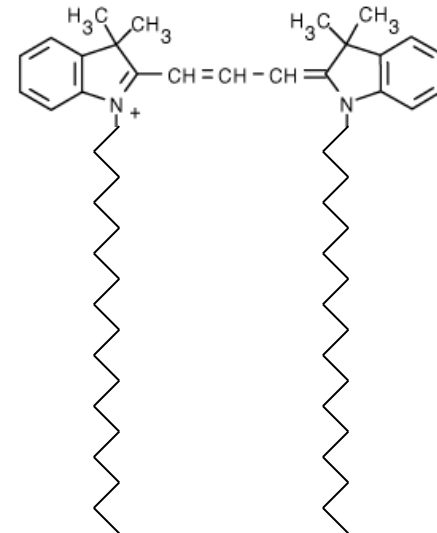
Quenching



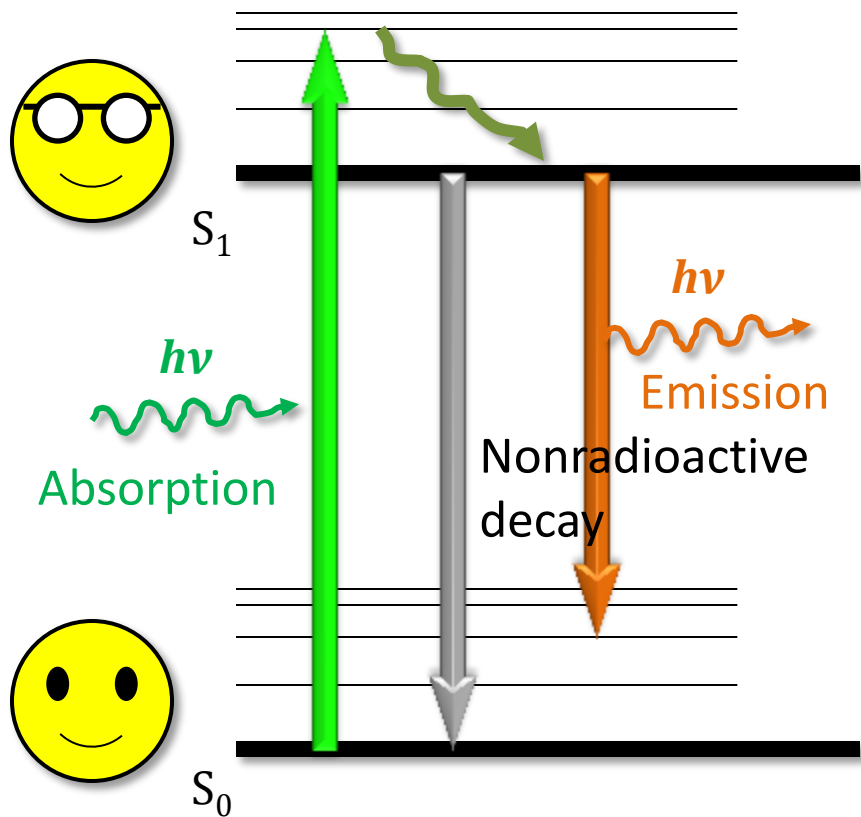
Non-radioactive decay:

Energy dissipation through structural flexibility

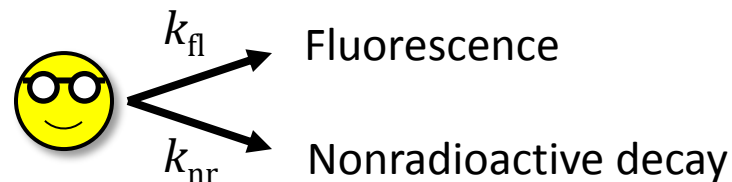
Dil



Quantum Yield



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

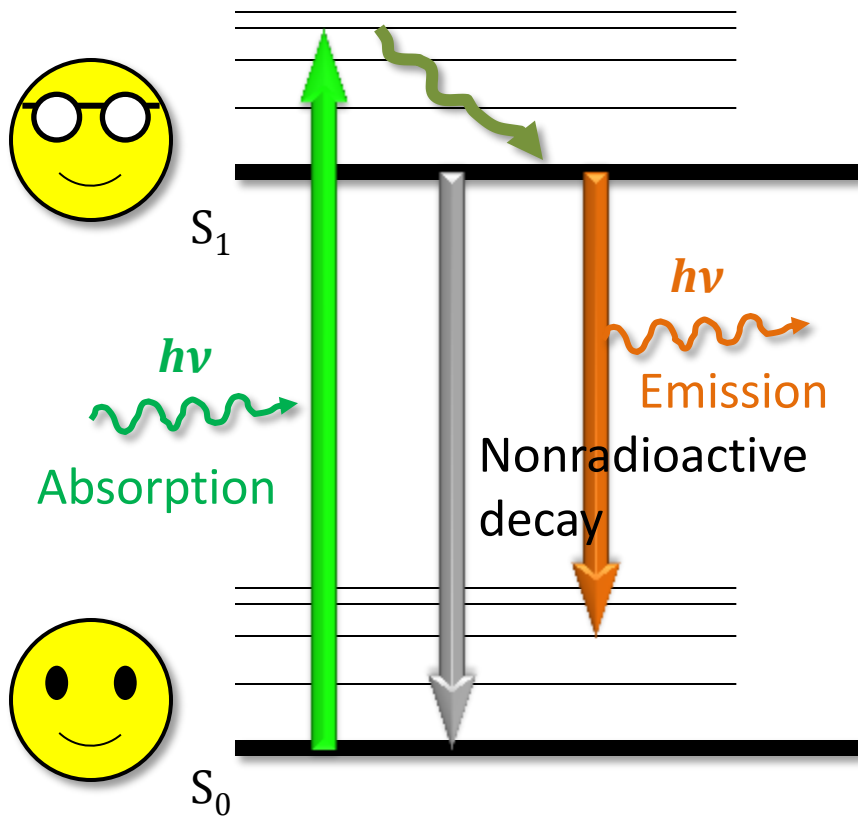


$$QE = k_{fl} / (k_{fl} + k_{nr})$$
$$= 1 / (1 + \tau_{fl} k_{nr})$$

$$\tau_{fl} \uparrow, QE \downarrow$$

$$k_{nr} \uparrow, QE \downarrow$$

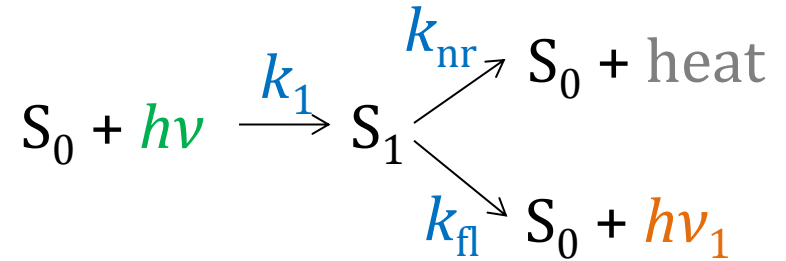
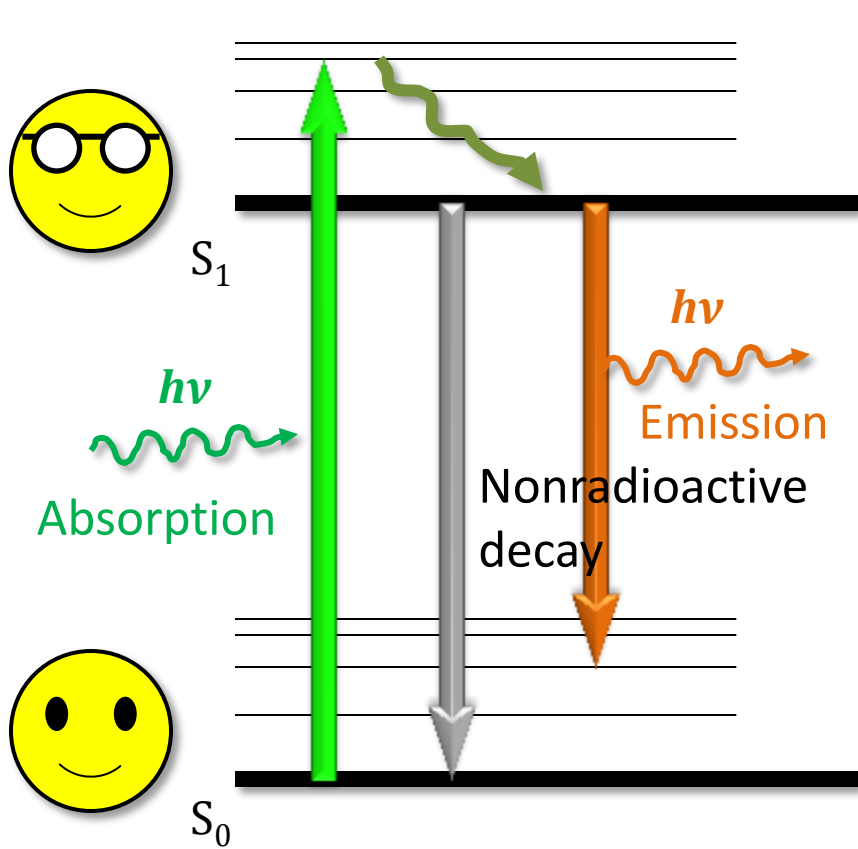
Quantum Yield



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

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 fluorophore



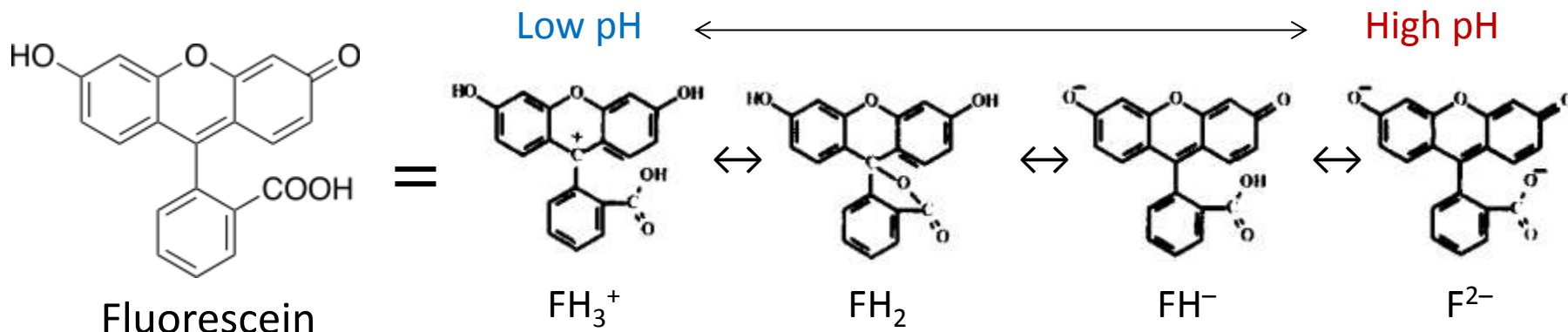
Brightness \approx

EC (wavelength)

\times

QE

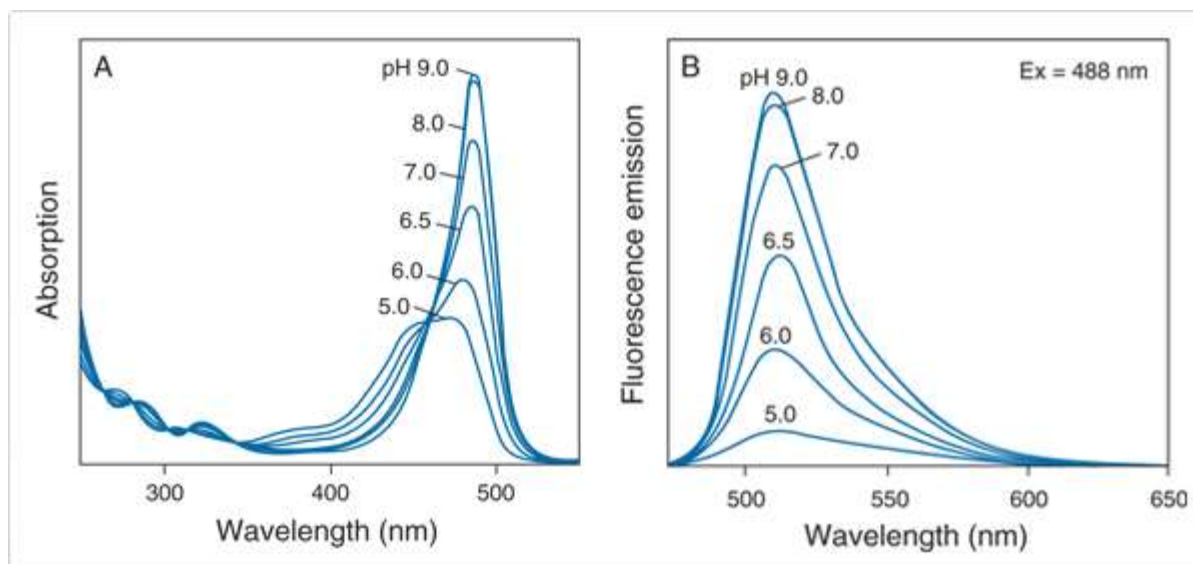
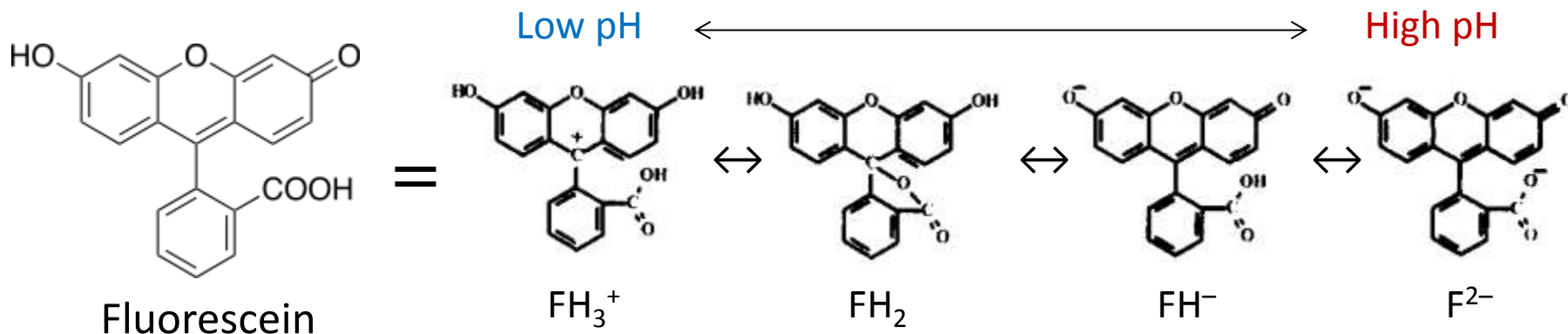
Brightness can be affected by the environment



Summary of absorption, fluorescence and protolytic properties of fluorescein

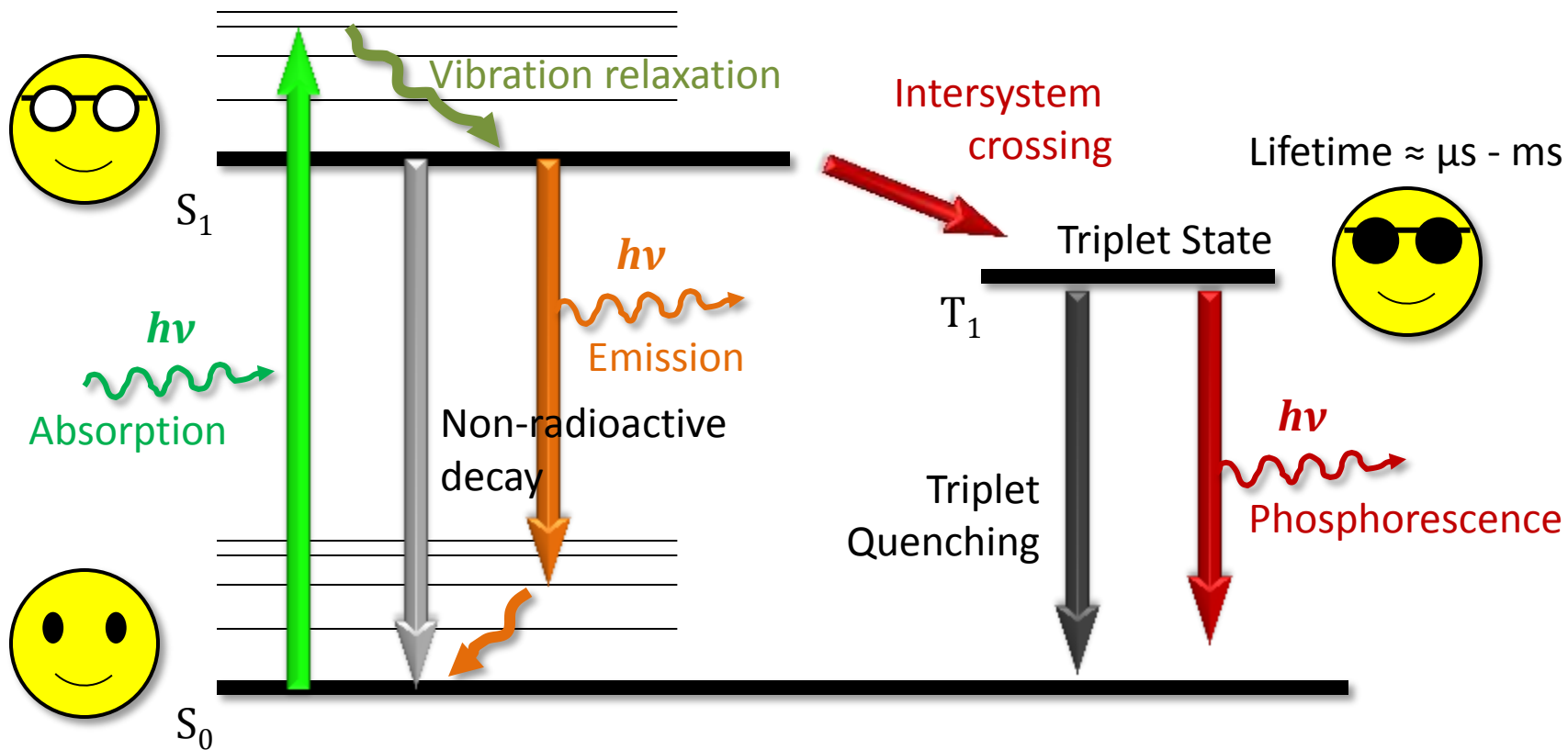
	FH_3^+	FH_2	FH^-	F^{2-}
$\epsilon/\text{M}^{-1} \text{ cm}^{-1} (\lambda/\text{nm})$	53000(437)	3600(475)	29000(472)	76900(490)
$\epsilon/\text{M}^{-1} \text{ cm}^{-1} (\lambda/\text{nm})$	7100(297)	11000(434)	29000(453)	9500(322)
$\epsilon/\text{M}^{-1} \text{ cm}^{-1} (\lambda/\text{nm})$	33000(250)		700(310)	14000(283)
$\epsilon/\text{M}^{-1} \text{ cm}^{-1} (\lambda/\text{nm})$			17000(273)	43000(239)
τ/ns			3.0	4.1
Φ^f	$\sim 0 (\text{pH} > 1.5)$	~ 0	0.37	0.93
Φ^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

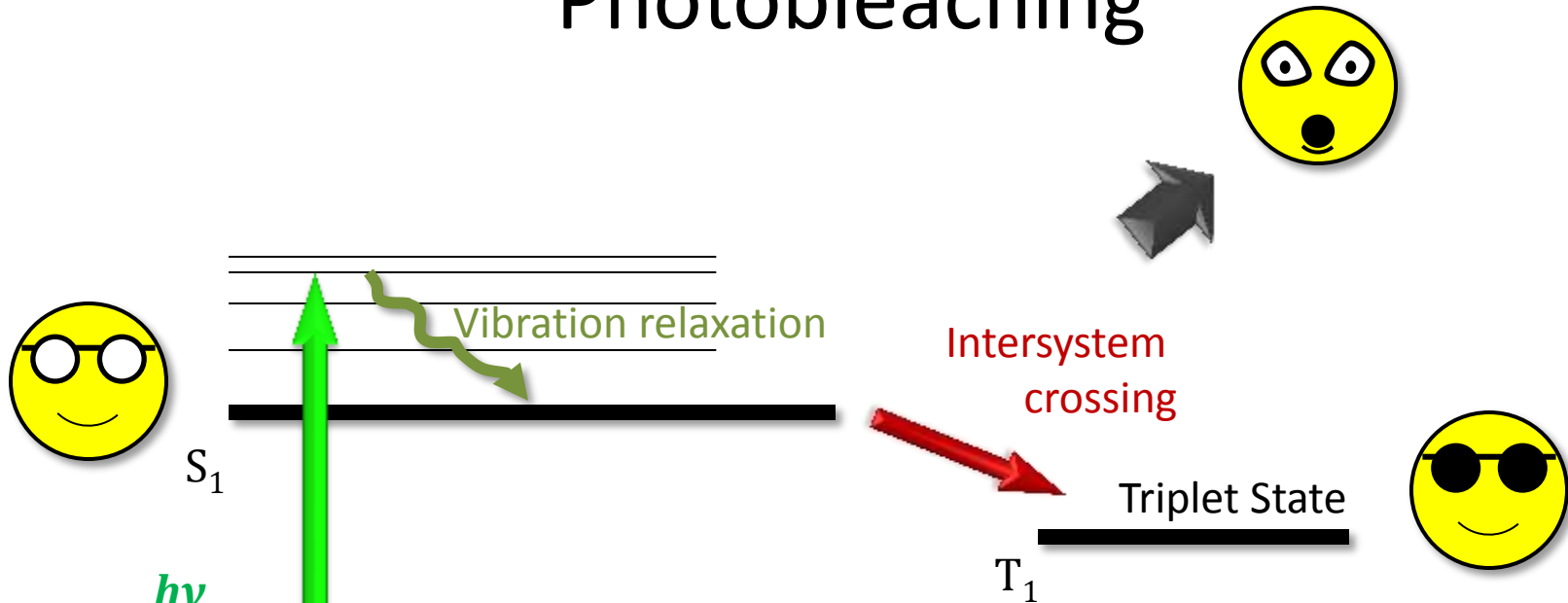


Invitrogen

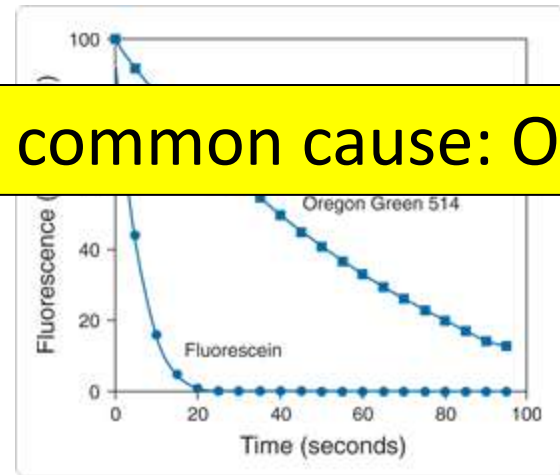
The Full Jabłonski Diagram



Photobleaching

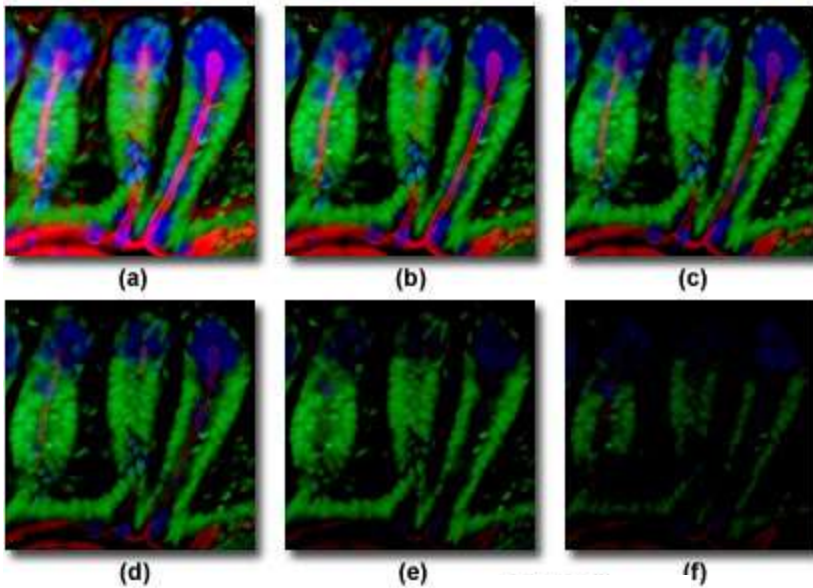


Most common cause: O_2



Photobleaching is our enemy

Differential Photobleaching in Multiply-Stained Tissues



Michael Davidson

For all fluorescence imaging:

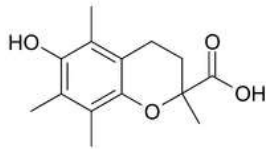
- Kills signal
- Alters the relative contrast

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.



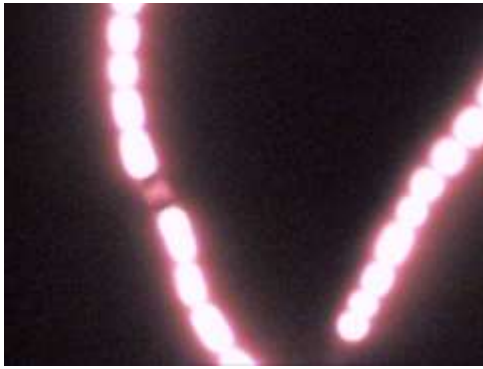
Fight against photobleaching, cont.

$$\begin{array}{ccccccc} \text{Fluorescence} & & \text{Excitation} & & \text{Fluorophore} & & \text{Fluorophore} & & \text{Detection} & & \text{Detector} \\ \text{signal} & = & \text{energy} & \times & \text{brightness} & \times & \text{concentration} & \times & \text{efficiency} & \times & \text{sensitivity} \\ & & \uparrow & & & & & & \uparrow & & \\ & & \text{Intensity} \times \text{Exposure time} & & & & & & \text{Objective, Filters, Light path} & & \end{array}$$

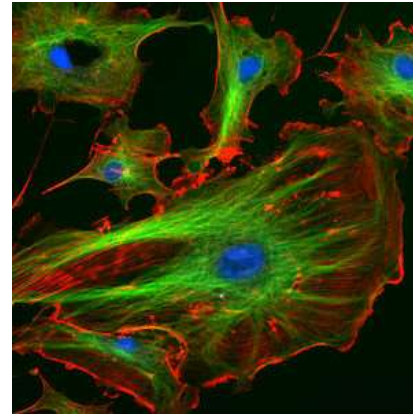
- Labeling as densely as possible
- Budget the photons
 - Only expose when observing
 - Minimize exposure time & excitation power
 - Use efficient filter combinations
 - Use high QE, low noise camera

Choice of fluorophores

Natural fluorescence

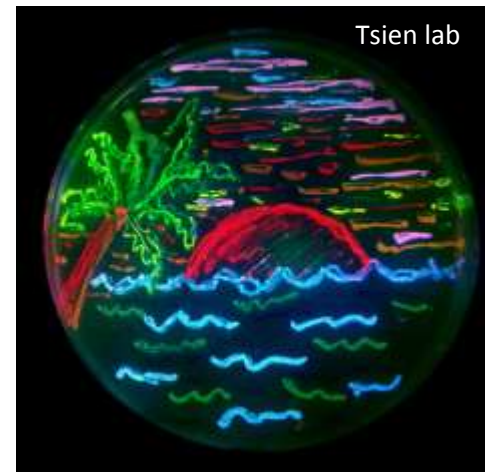


Organic dyes



Fluorescent proteins

Inorganic fluorophores



Inorganic fluorophores

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

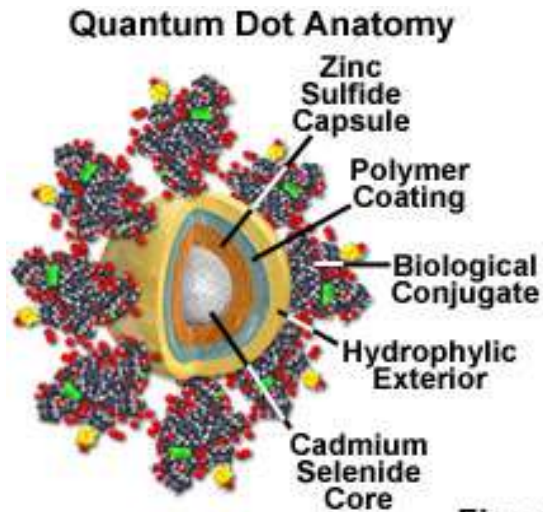
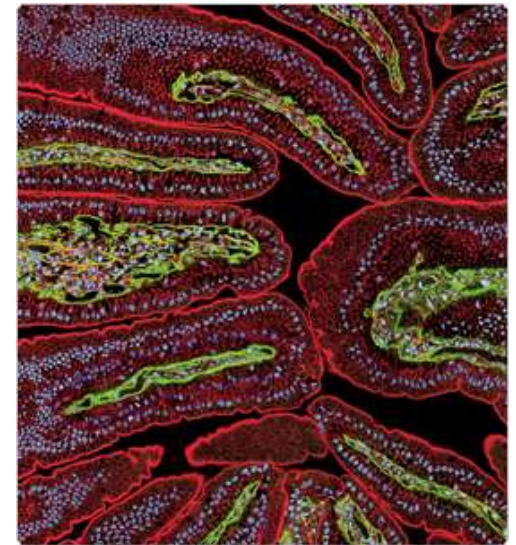
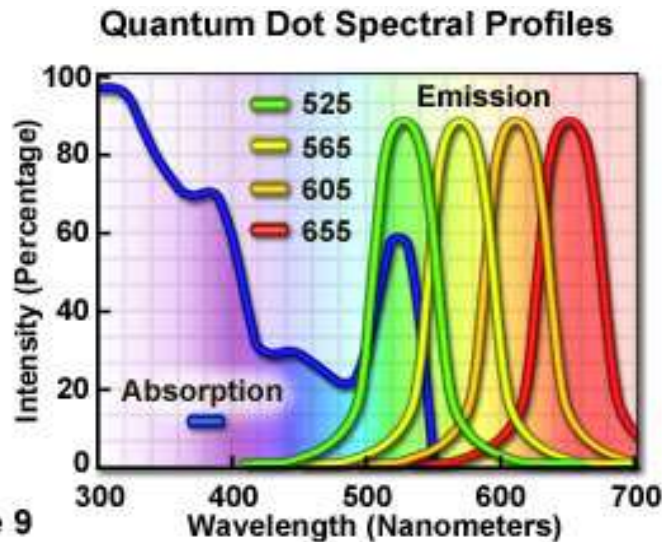
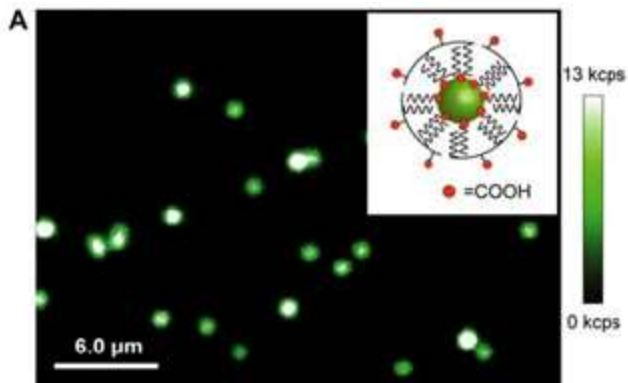


Figure 9

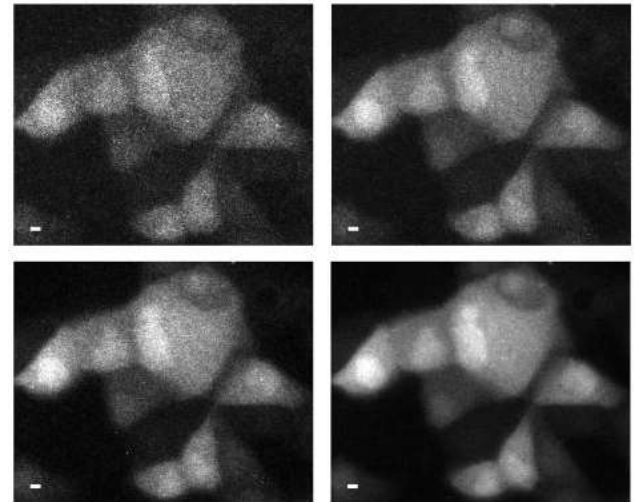


Inorganic fluorophores

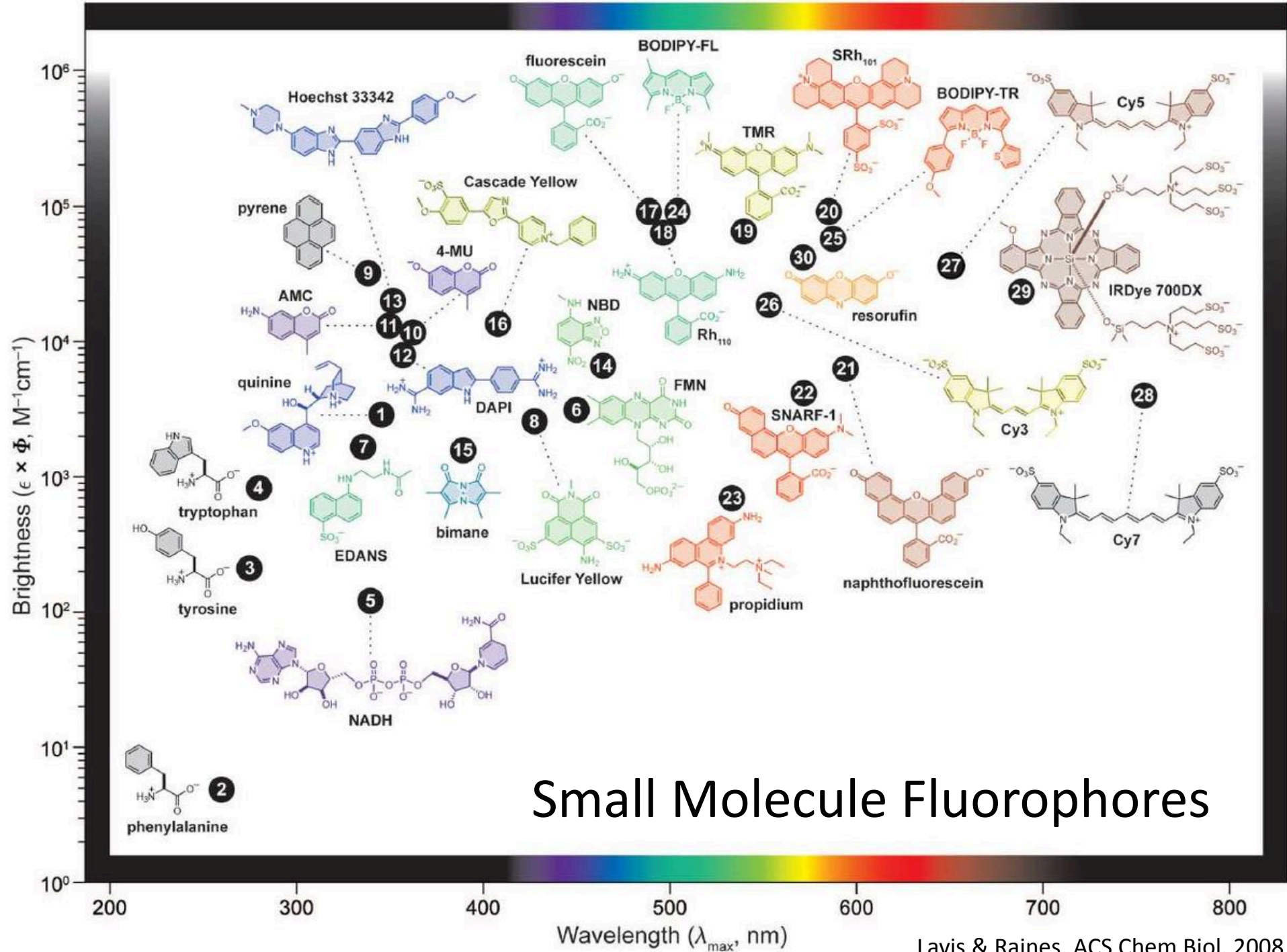
- Quantum dots
- Lanthanides
 - Very large Stokes or anti-Stokes shift (UV \rightarrow Red or Red \rightarrow Green)
 - Sharp emission peaks
 - Extremely long life time (μs - ms)



Bruce Cohen



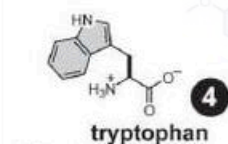
Gahlaut et al., Cytometry A, 2010



Intrinsic Fluorescence from Amino Acids

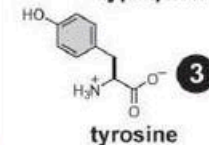
Brightness ($\epsilon \times \Phi$, $M^{-1}cm^{-1}$)

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



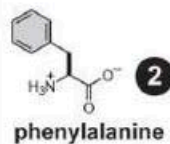
$A_{max} = 280 \text{ nm}$, $Ext = 5,600$

$Em_{max} = 348$



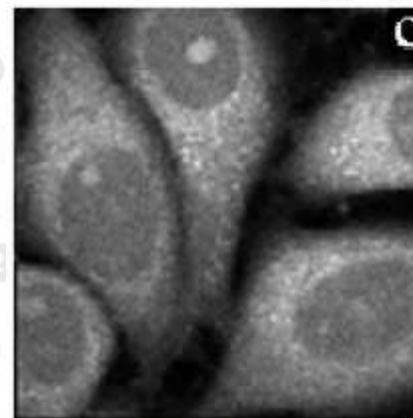
$A_{max} = 274 \text{ nm}$, $Ext = 1,400$

$Em_{max} = 303$



$A_{max} = 257 \text{ nm}$, $Ext = 200$

$Em_{max} = 282$



Tryptophan fluorescence image

Wavelength (λ_{max} , nm)

200 300 400 500 600 700 800

Fluorescent Enzyme Co-factors

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

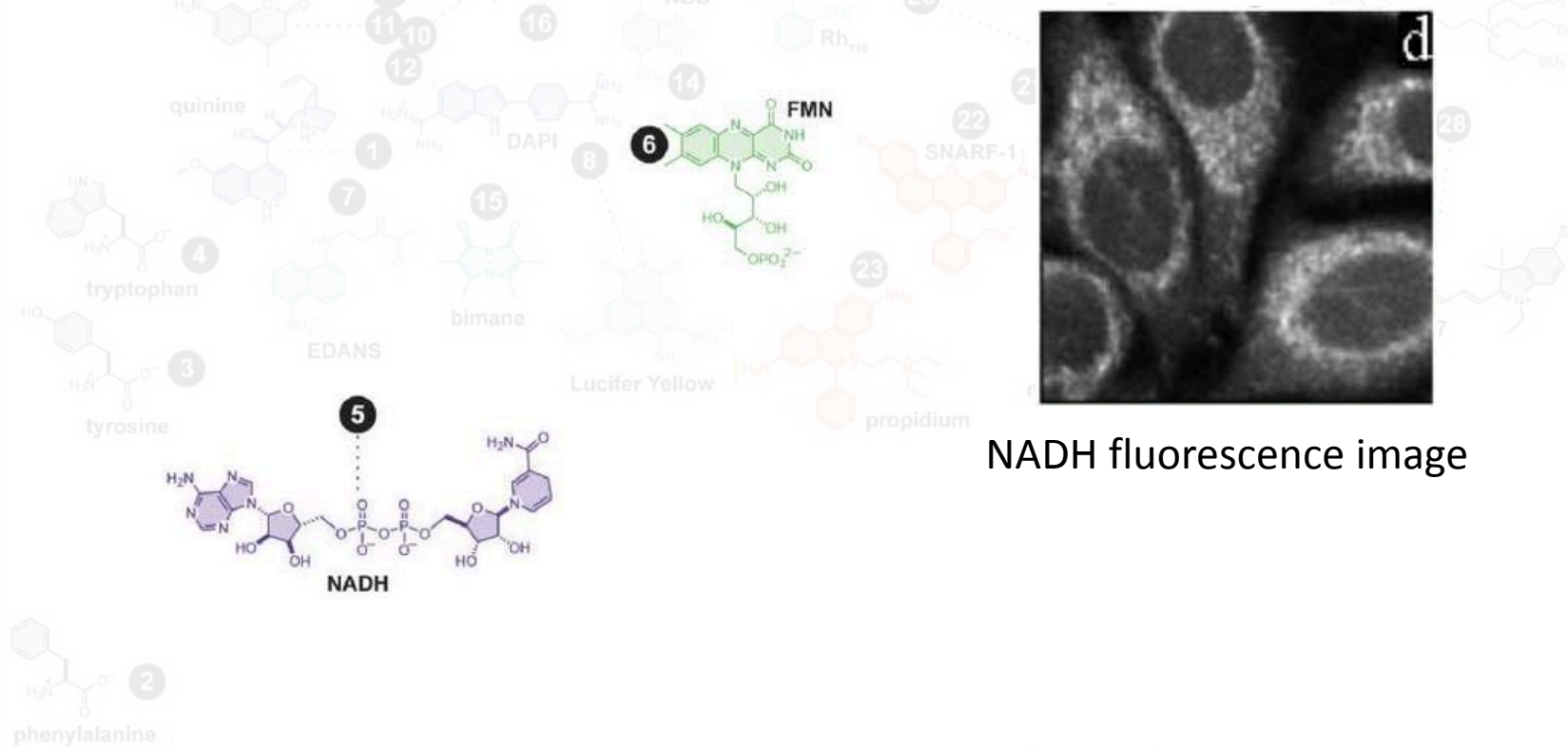
500

600

700

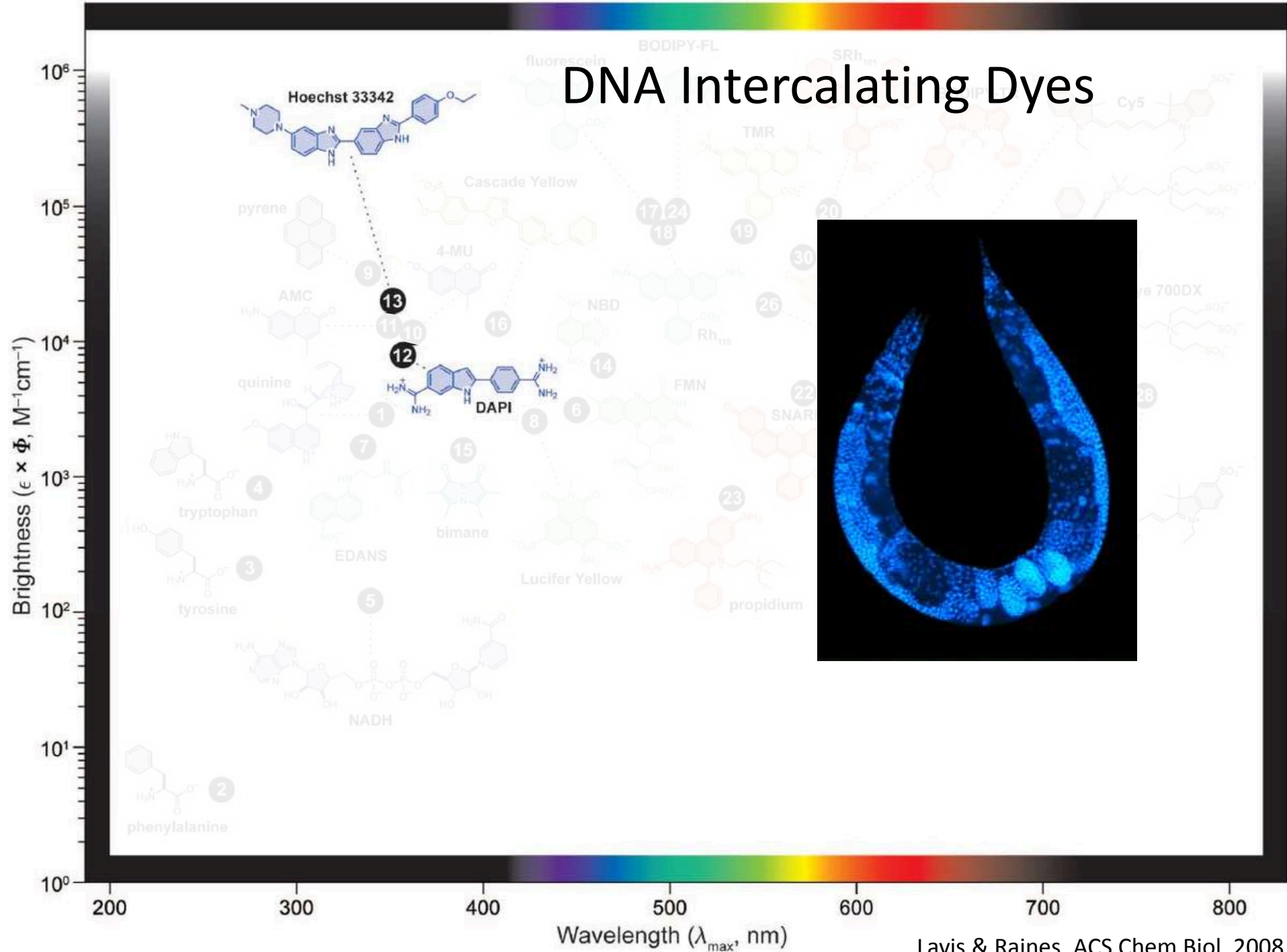
800

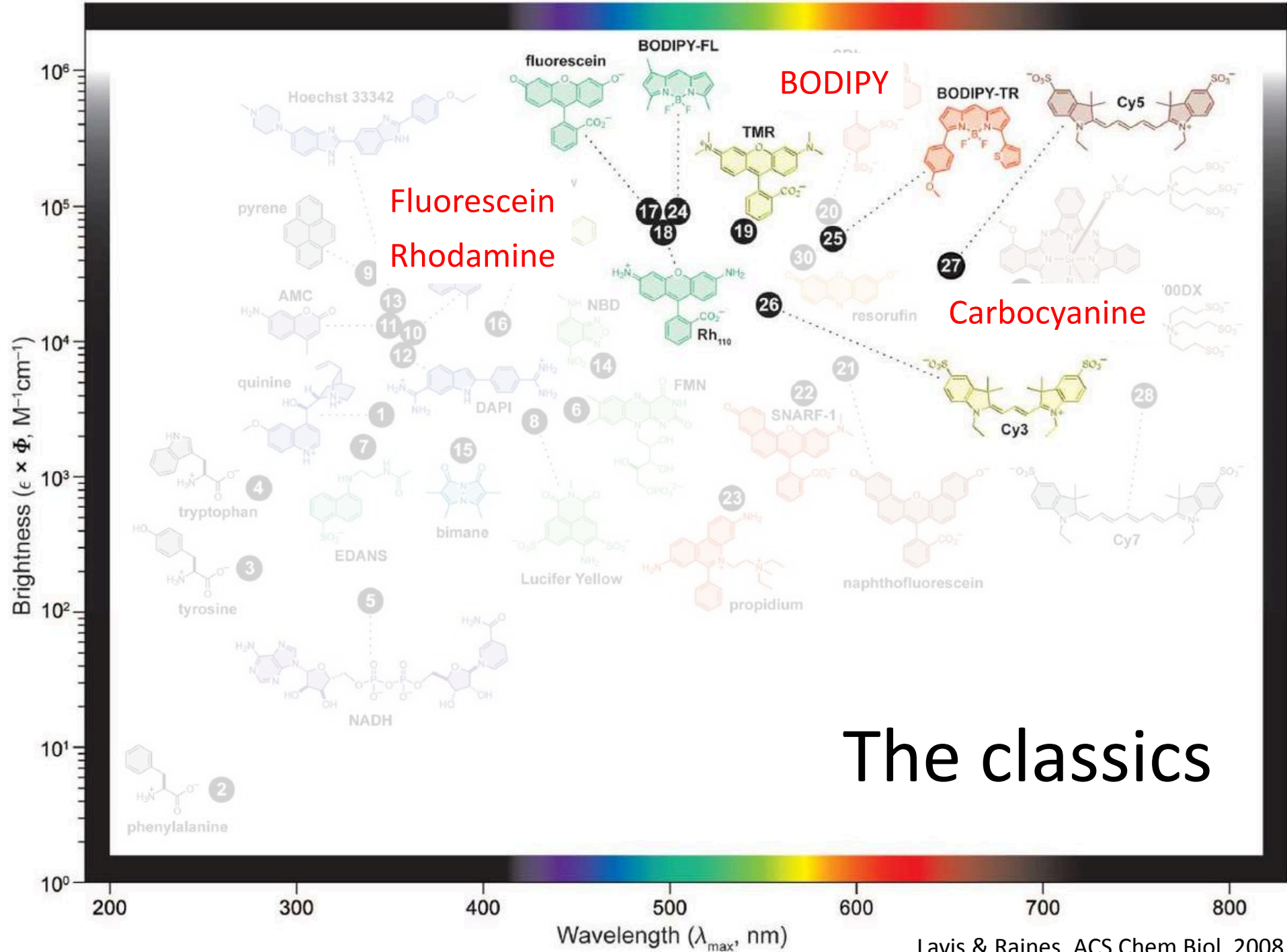
Wavelength (λ_{max} , nm)



NADH fluorescence image

DNA Intercalating Dyes





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

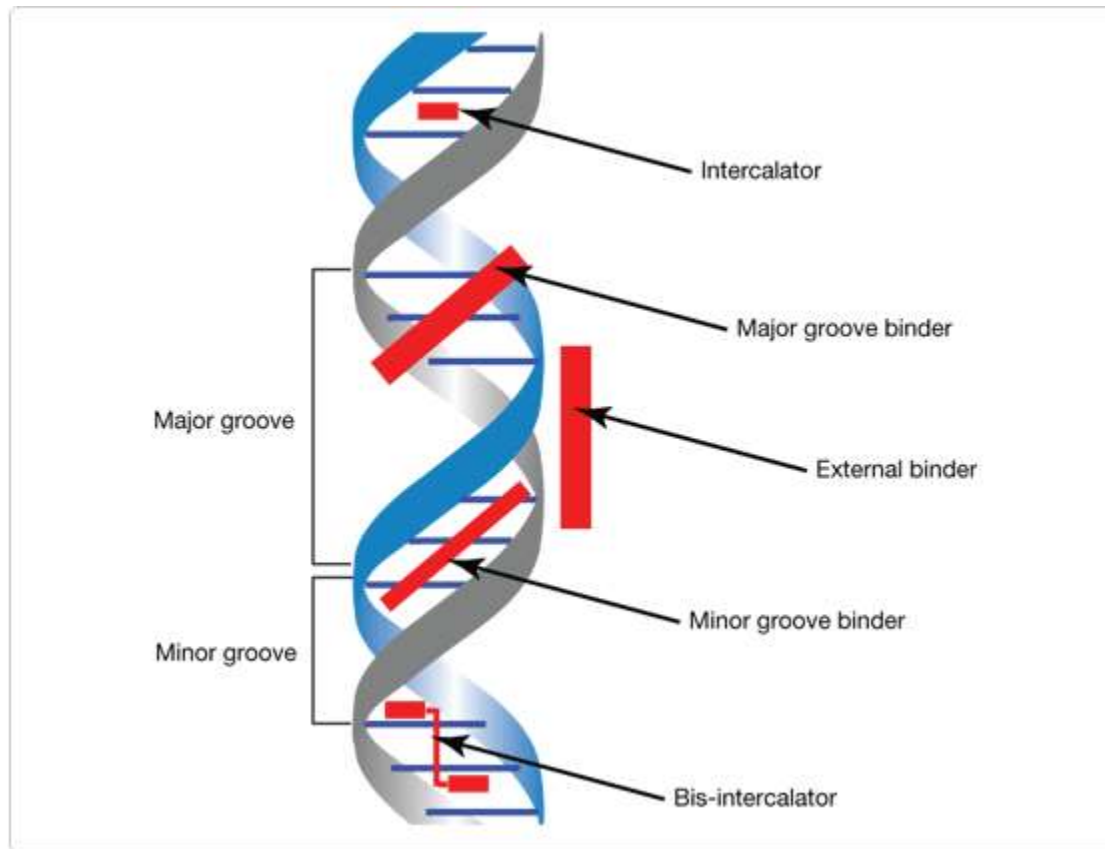
- Small
 - e.g. DNA labeling
- Bright and photostable
- Wide range of wavelengths
 - UV to NIR
- Most of them lack labeling specificity by itself.
 - Specific probes for nucleic acid, plasma membrane, mitochondria
- Almost all “great” ones are unable to cross the membrane.

(Specific) delivery of small molecule dyes



Functional dyes

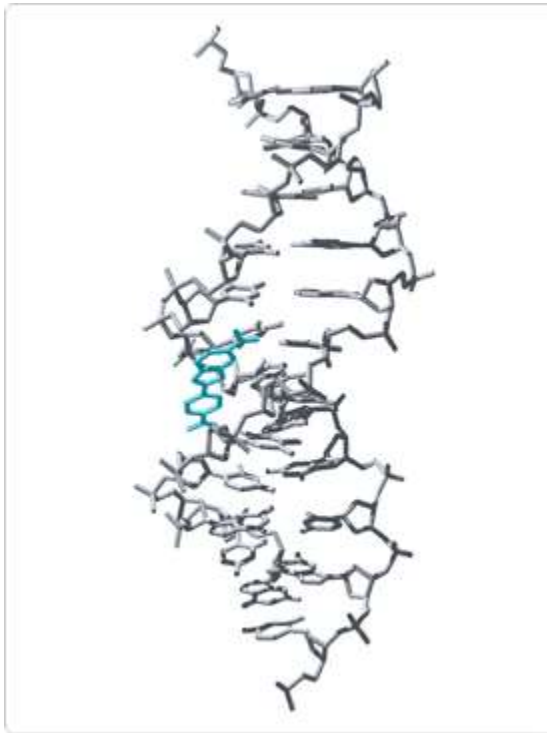
- Nucleic acid intercalating dyes
 - QE increment upon binding to DNA/RNA



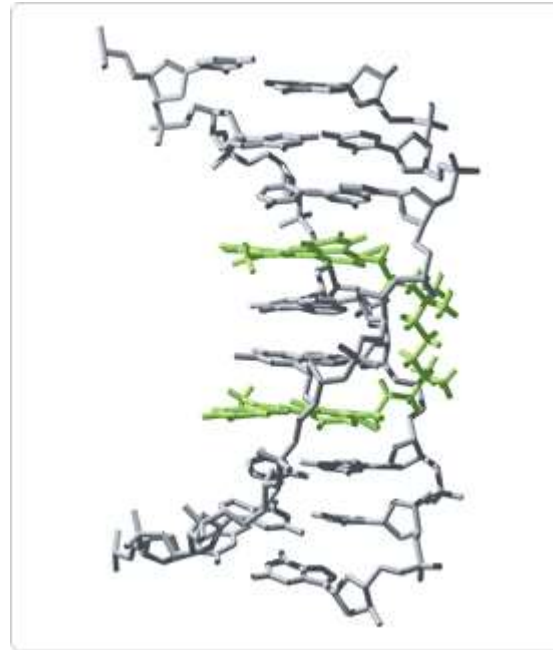
Functional dyes

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

DAPI



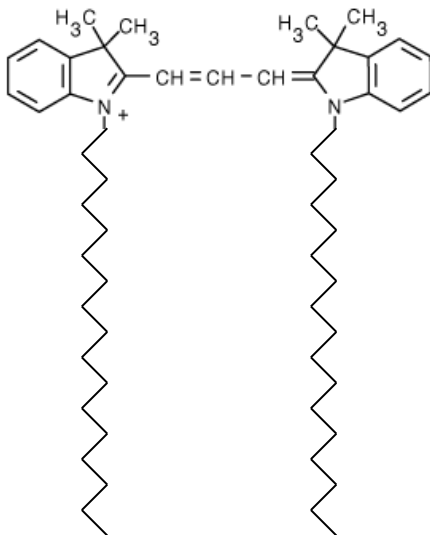
TOTO-1



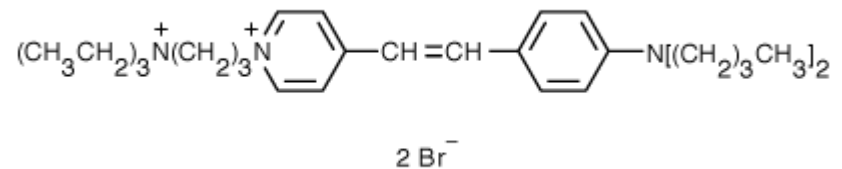
Functional dyes

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

Dil

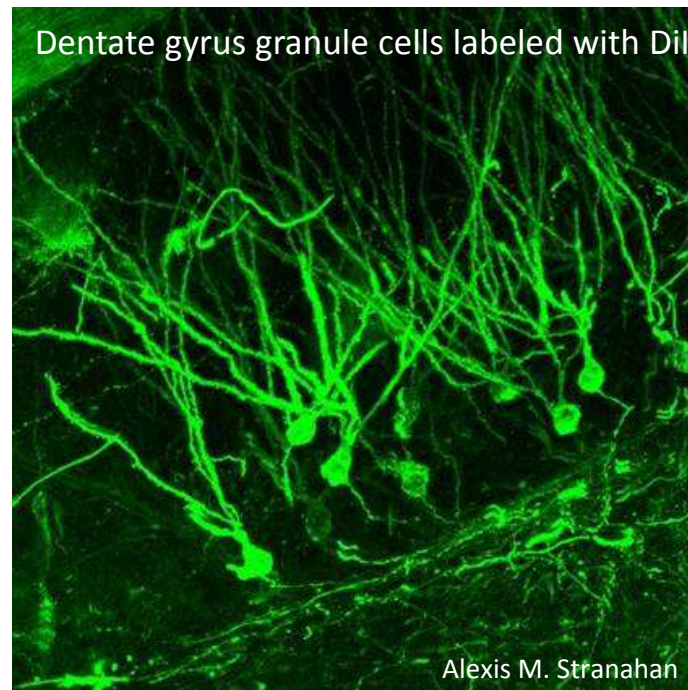


FM 1-43



Functional dyes

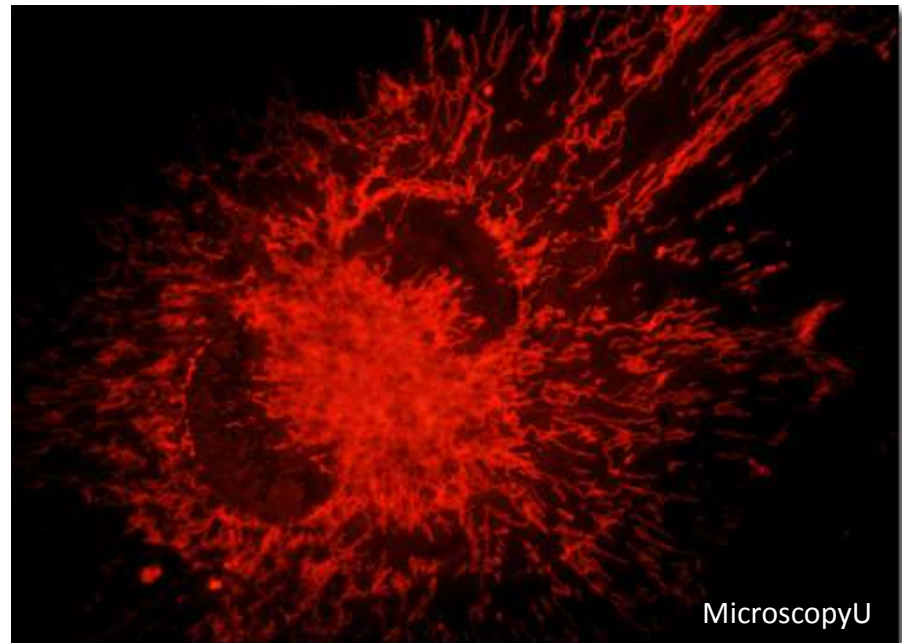
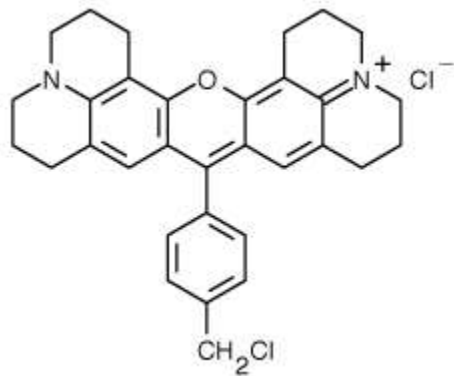
- Nucleic acid intercalating dyes
- Membrane stains
 - Amphiphilic dyes that partition in lipid bilayers



Functional dyes

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

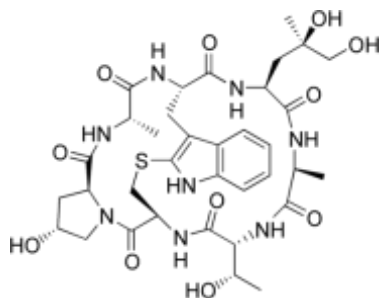
MitoTracker Red CMXRos



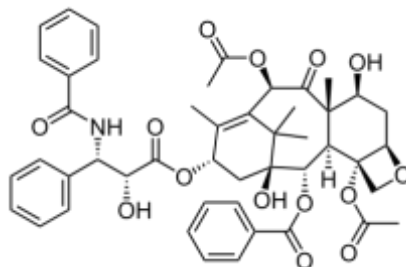
Small molecules probes

- Small molecules that bind proteins

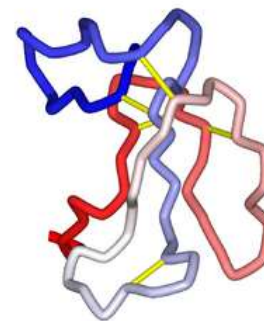
Phalloidin



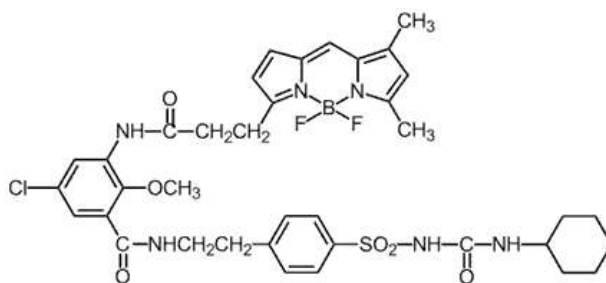
Taxol



α -bungarotoxin



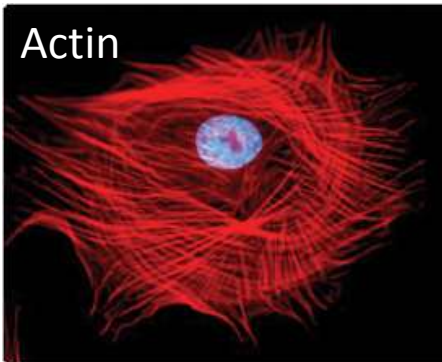
ER Tracker Green



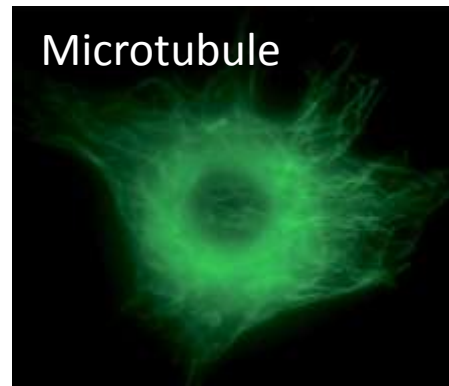
Small molecules probes

- Small molecules that bind proteins

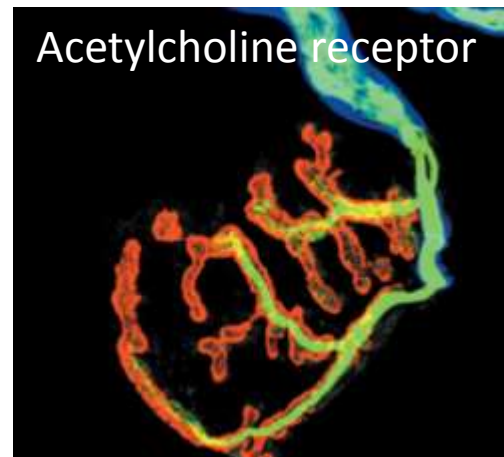
Phalloidin



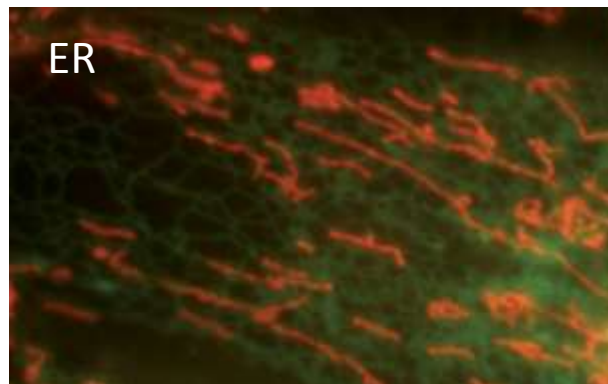
Taxol



α -bungarotoxin



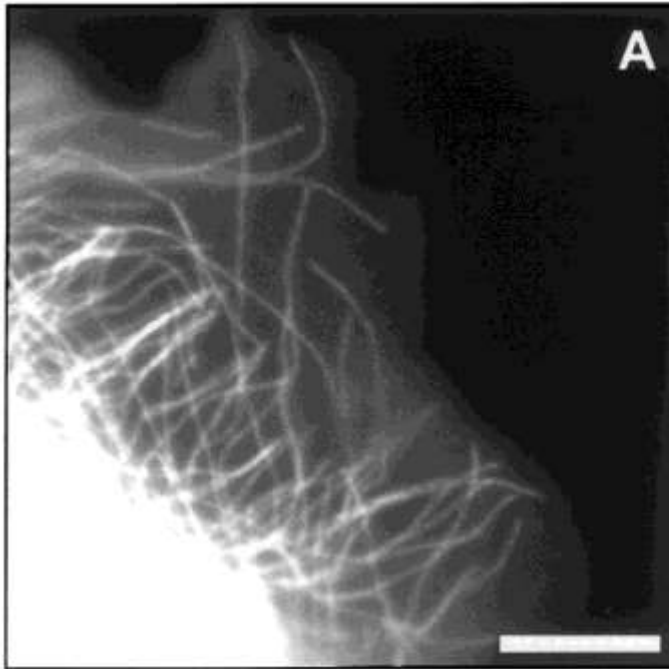
ER Tracker Green



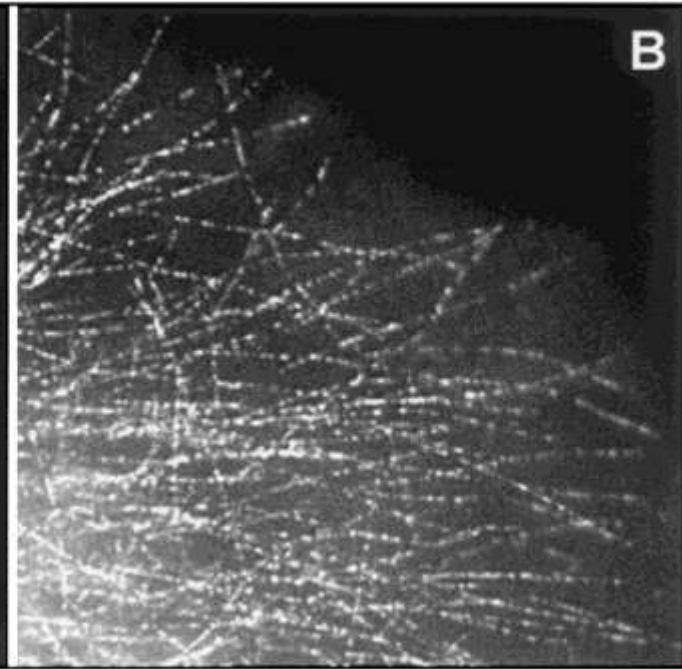
Large molecule labeling

- In vitro reconstituted systems
- Injecting labeled proteins

10% labeled tubulin



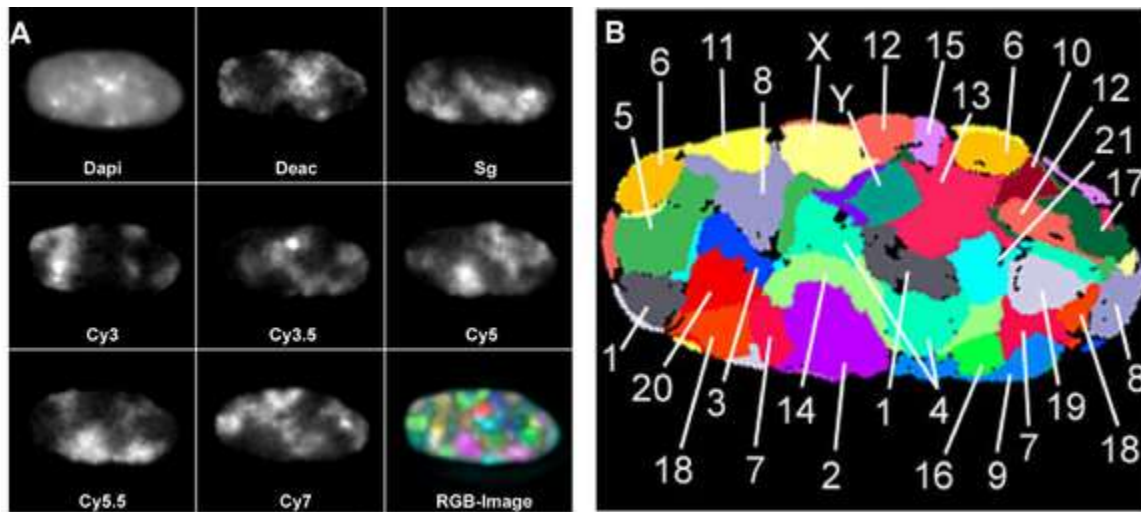
0.25% labeled tubulin



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

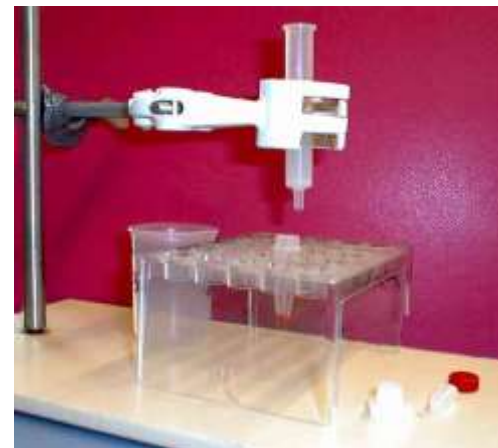
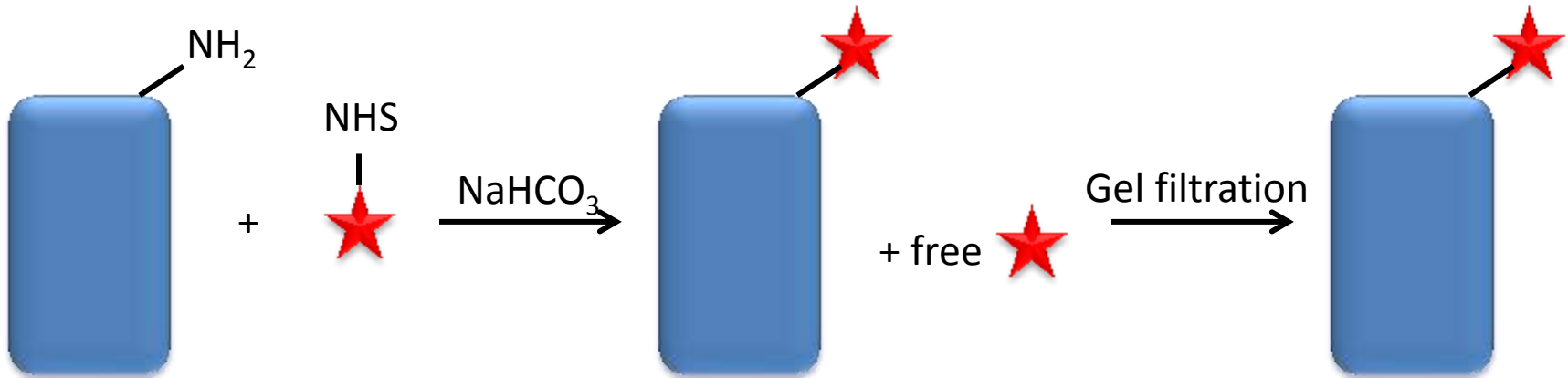
- In vitro reconstituted systems
- Injecting labeled proteins
- Fluorescence in situ hybridization (FISH)
- **Biotin-avidin interaction**
 - Labeled avidin/streptavidin
 - Biotin-dyes

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



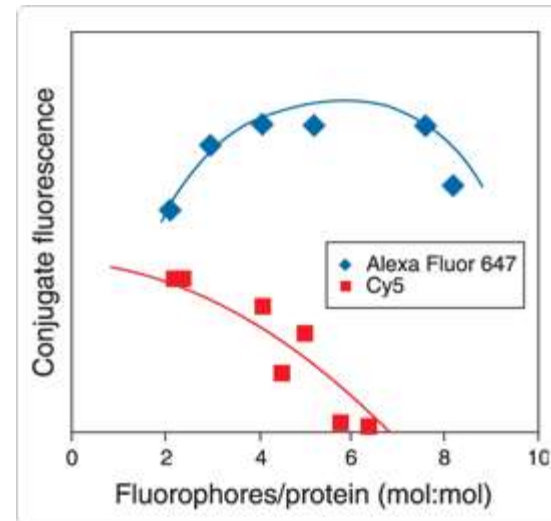
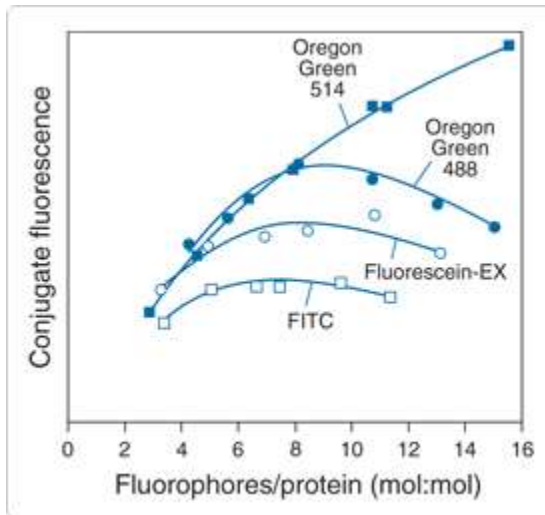
$$\text{Dye concentration} = A_{\text{max}} / \text{E.C.}_{\text{dye@max}}$$

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

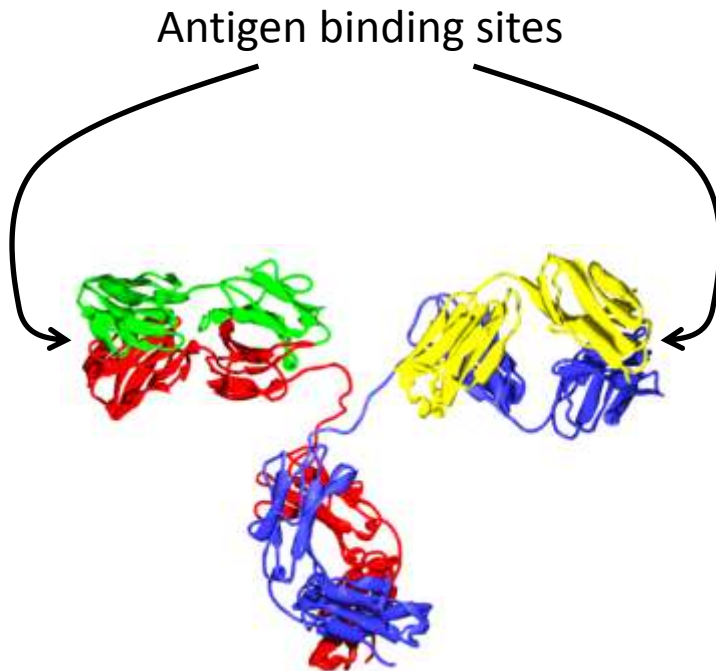
$$\text{Dye per protein} = \text{Dye concentration} / \text{Protein concentration}$$

Too high labeling stoichiometry can make the protein dead...

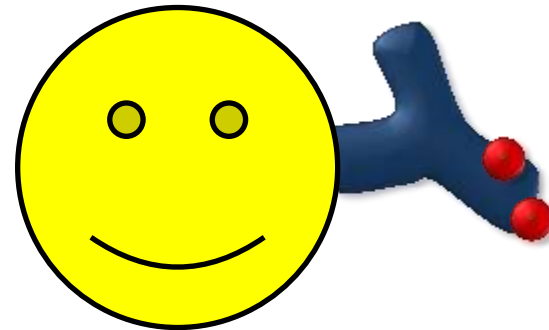
and lead to self-quenching



Immunofluorescence

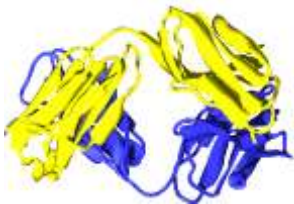


Direct immunofluorescence



Primary antibody binding efficiency can be < 10%

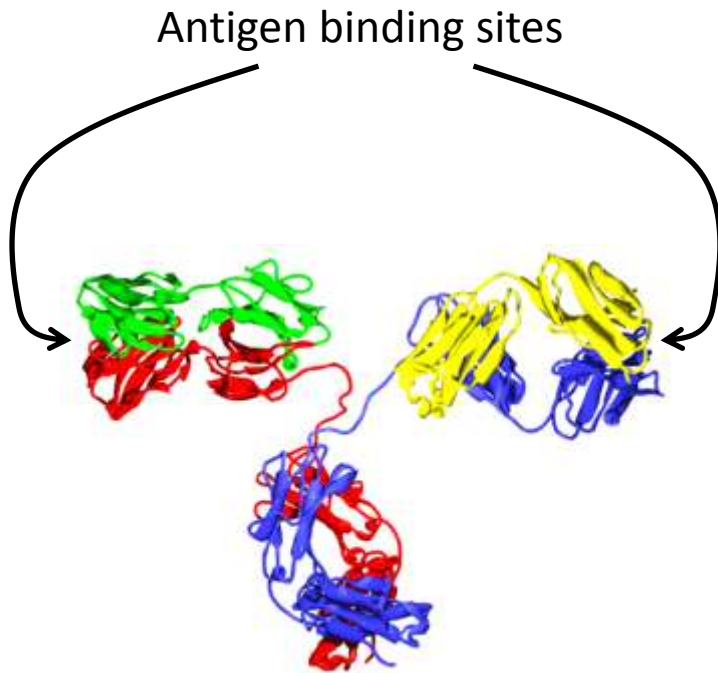
Fab fragment



Nanobody



Immunofluorescence



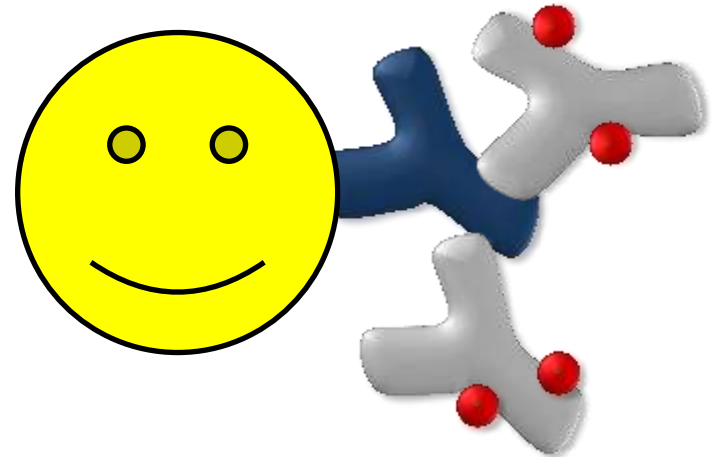
Fab fragment



Nanobody



Indirect immunofluorescence



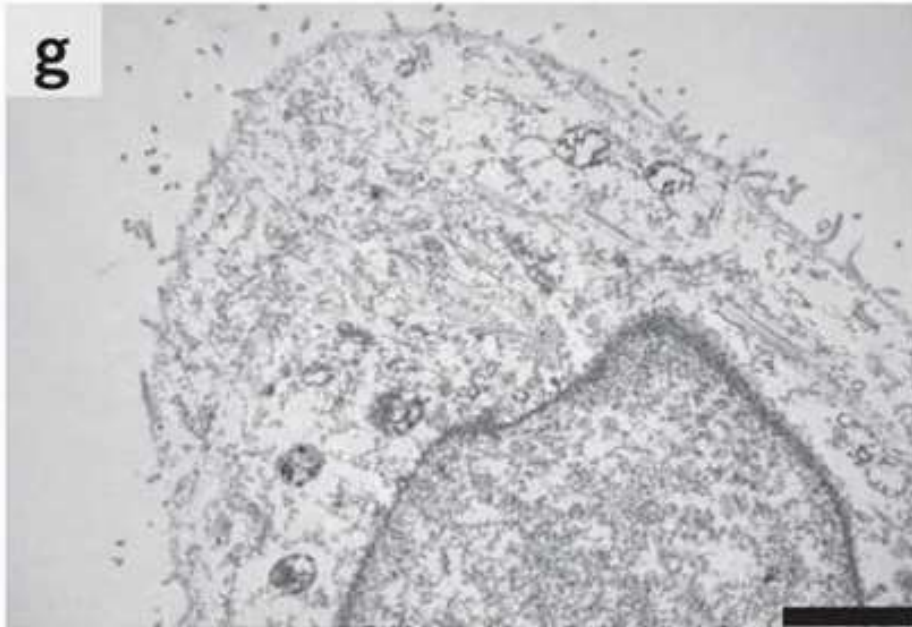
Fixation methods

- 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 (NaBH_4 reduction)
 - Required for electron microscopy

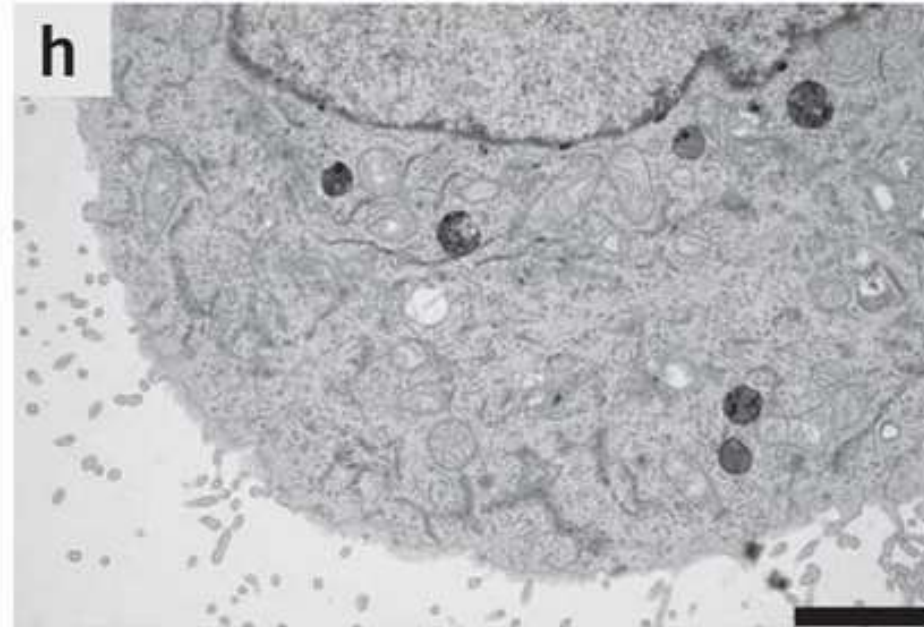
Fixation methods

- Methanol
- Formaldehyde (Paraformaldehyde)
- Glutaraldehyde

Methanol



2% glutaraldehyde



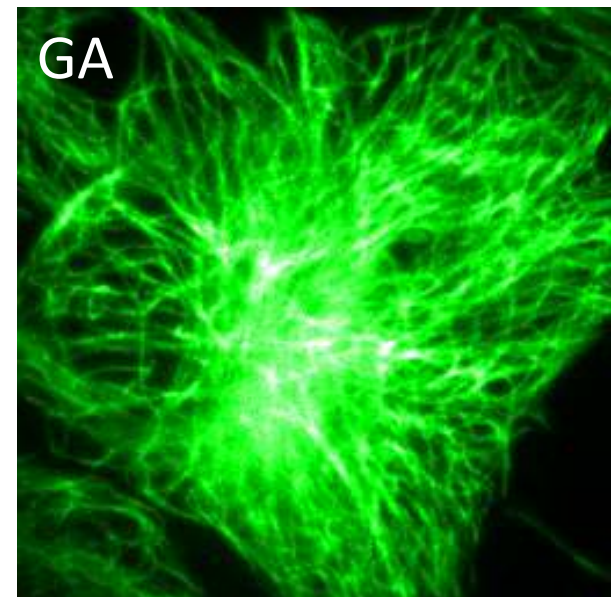
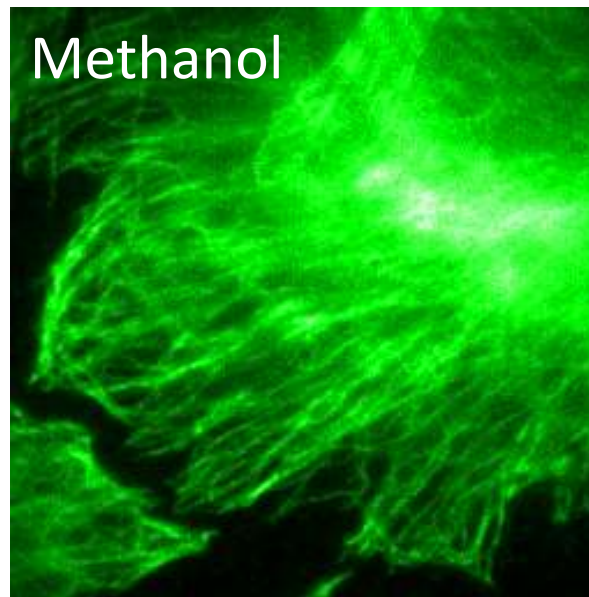
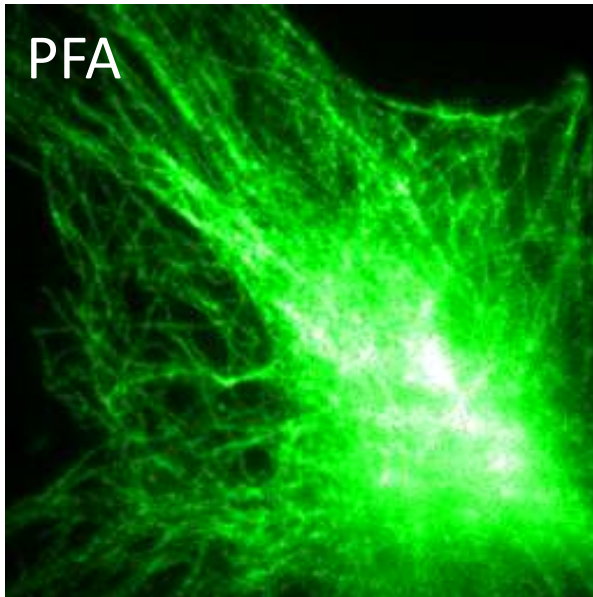
Nature Methods 9, 152–158 (2012)

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 Sjollem & Ben N G Giepmans

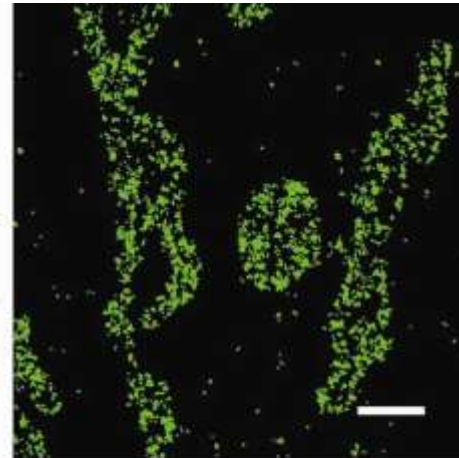
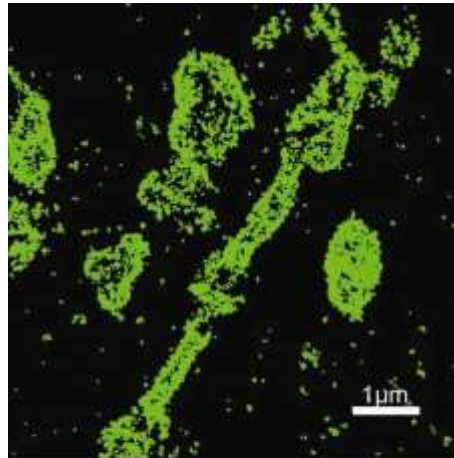
Nature Methods 9, 152–158 (2012) | doi:10.1038/nmeth.1855

Some fixation artifacts become visible in super-resolution microscopy

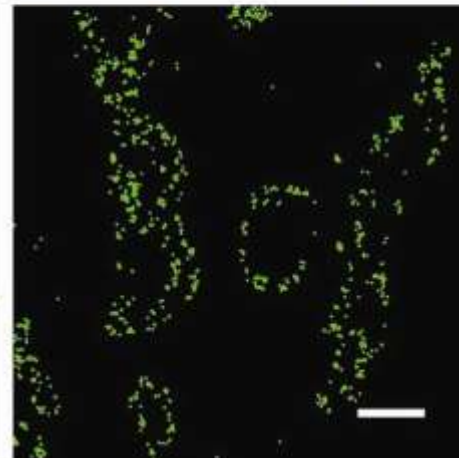
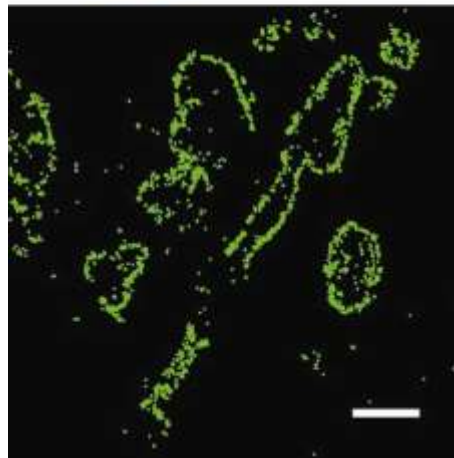
Tom20 –
mitochondria
outer membrane

Glutaraldehyde fixation

Formaldehyde fixation



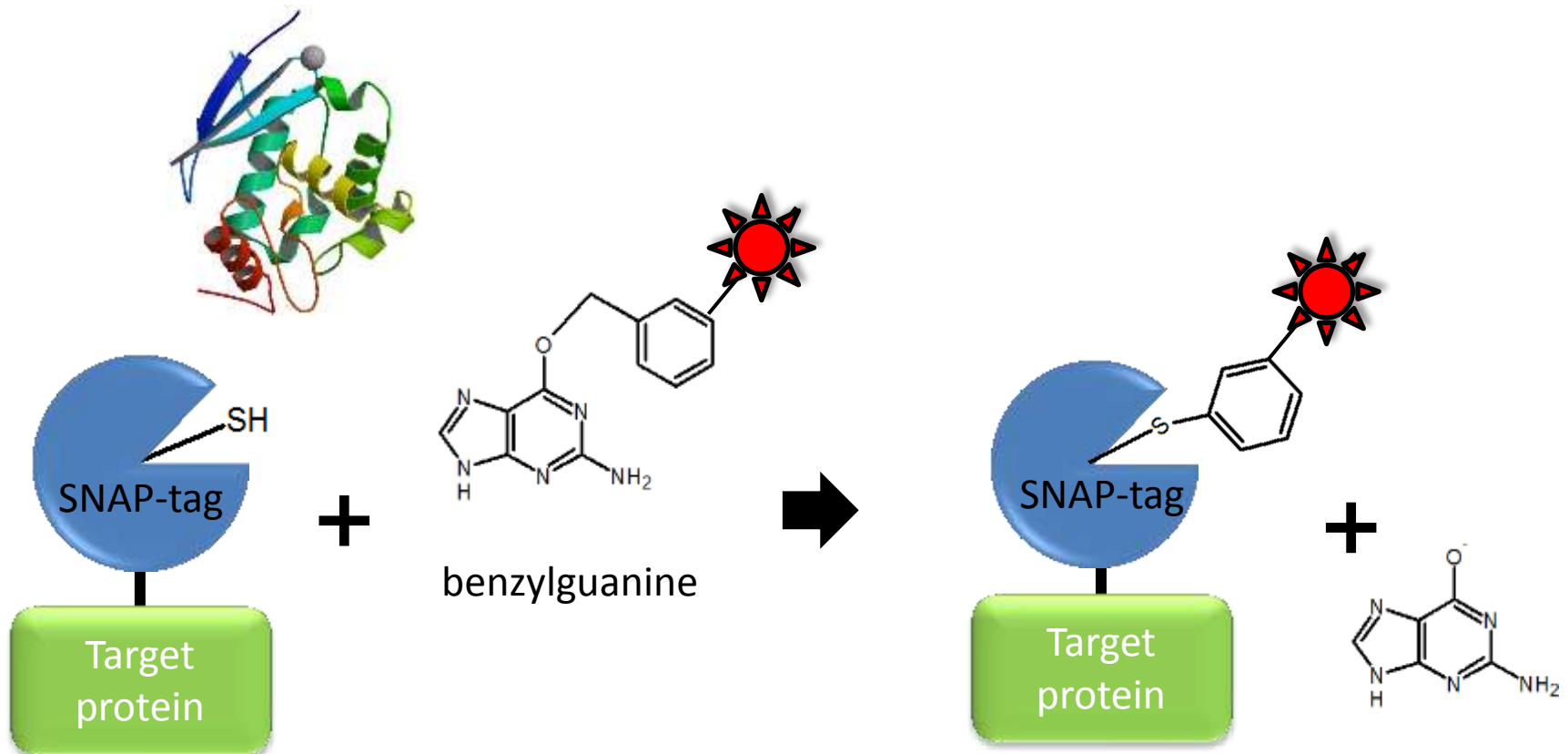
Projection



Section through
the middle

The hybrid approaches

- SNAP-tag, CLIP-tag, HALO-tag, TMP-tag...
 - SNAP-tag: based on human O⁶-alkylguanine-DNA-alkyltransferase (hAGT)



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