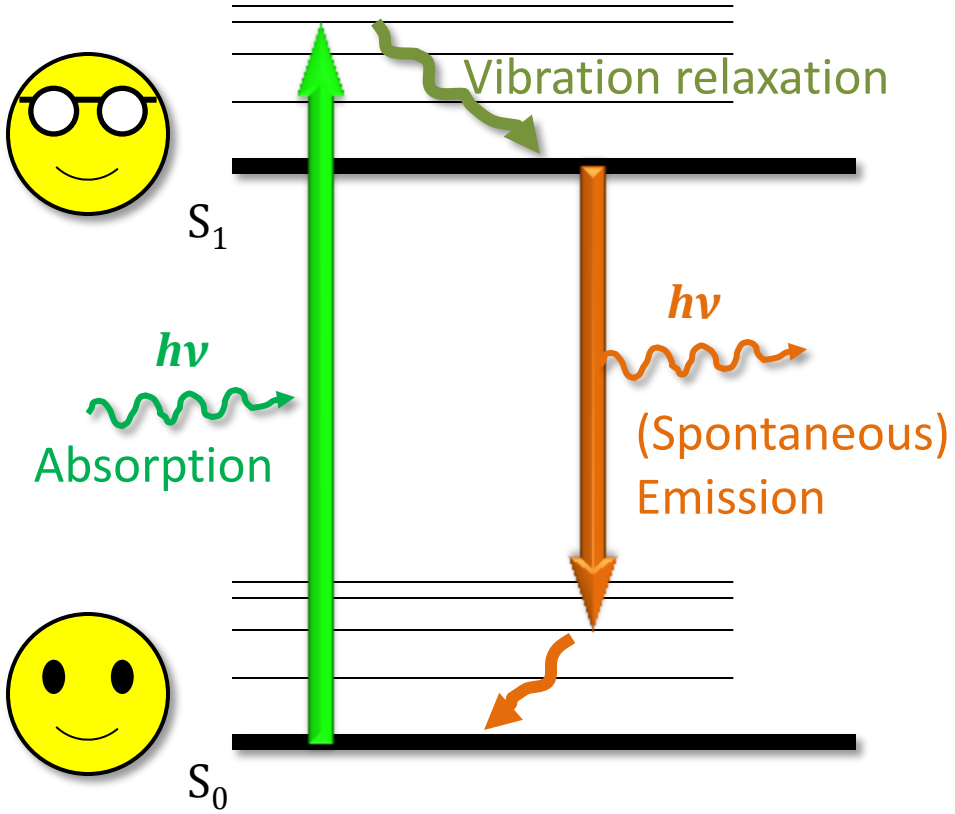
Fluorescence Microscopy

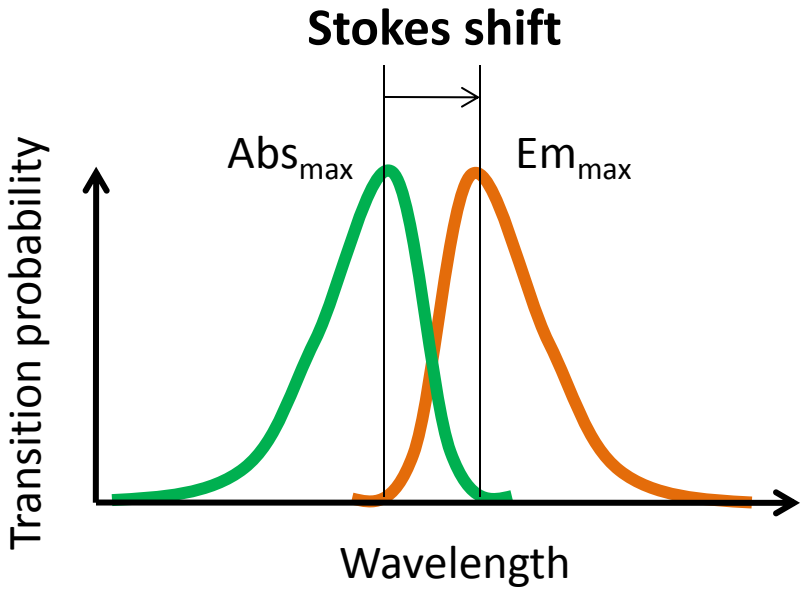
II. Fluorescent dyes

Bo Huang

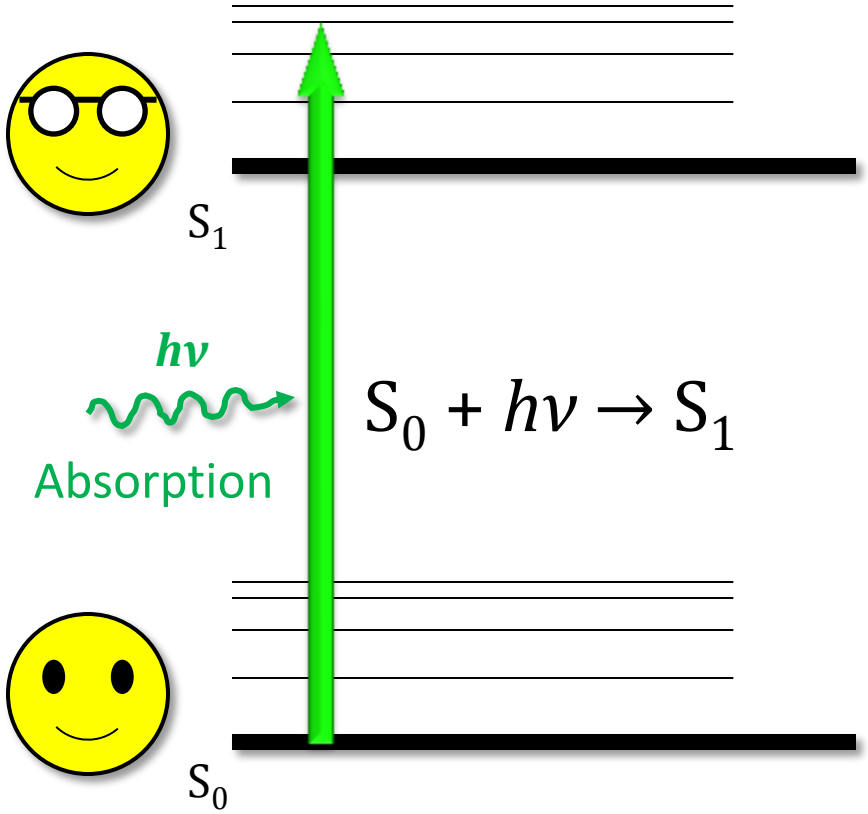
2013.03.26

The fluorescence process

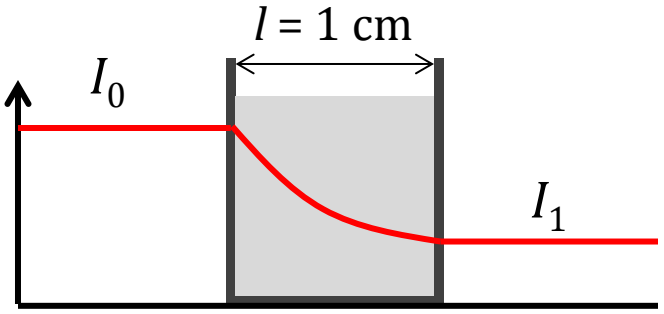




Extinction coefficient



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



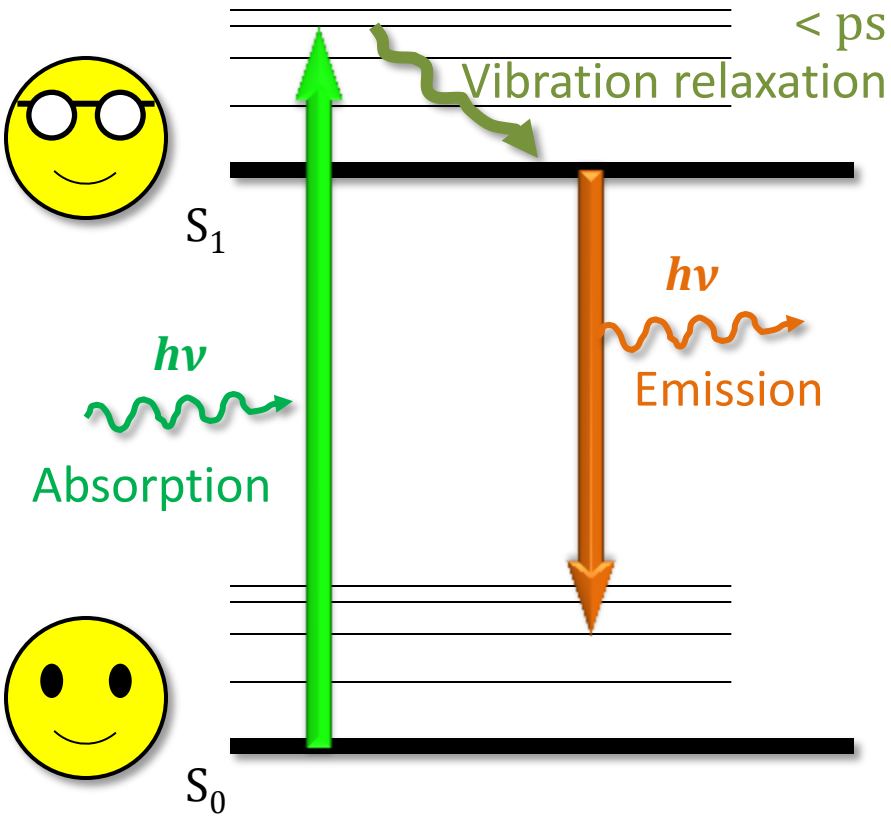
$$\text{Abs} = -\log_{10}(I_1/I_0)$$

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

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

1 μM, 1 cm ➔ A ≈ 0.1 (T = 80%)

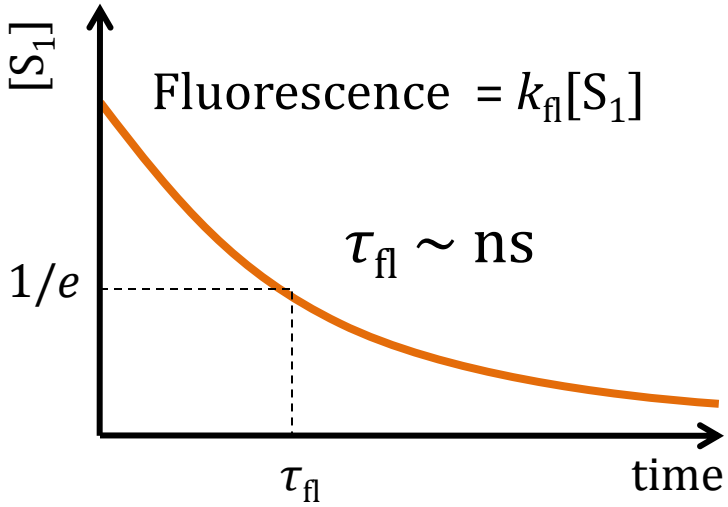
Fluorescence Lifetime



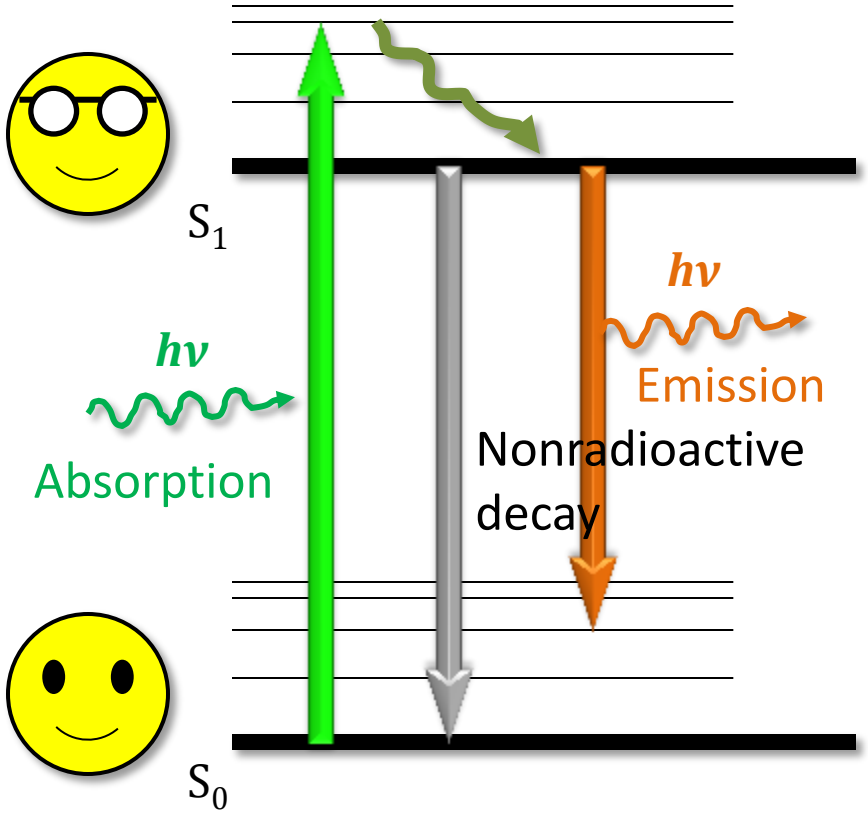
$$S_1 \rightarrow S_0 + h\nu$$

Rate constant = k_{fl}

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



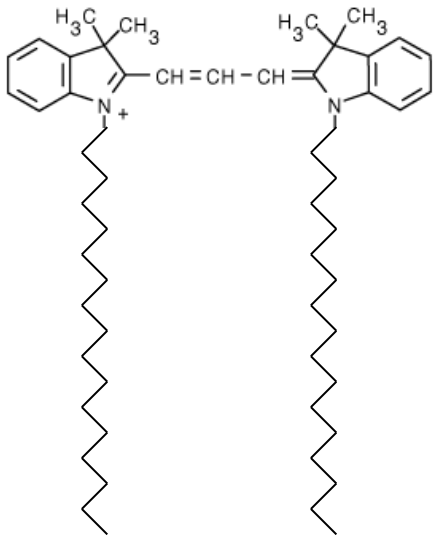
Quenching



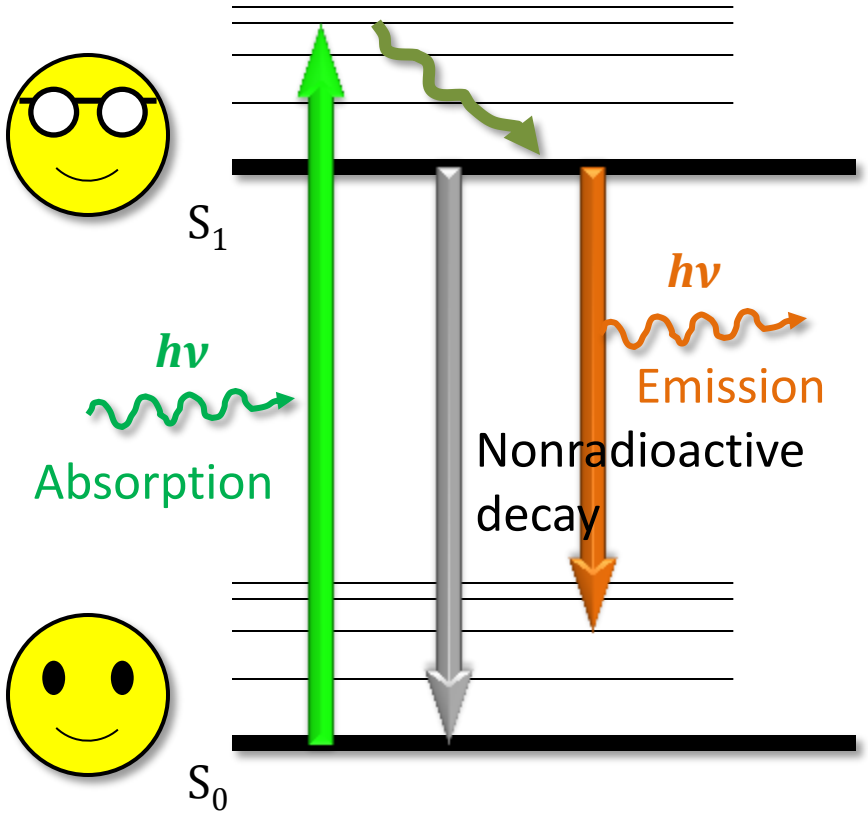
Non-radioactive decay:

Energy dissipation through structural flexibility

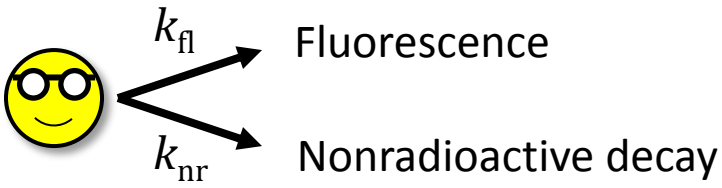
DiI



Quantum Yield



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

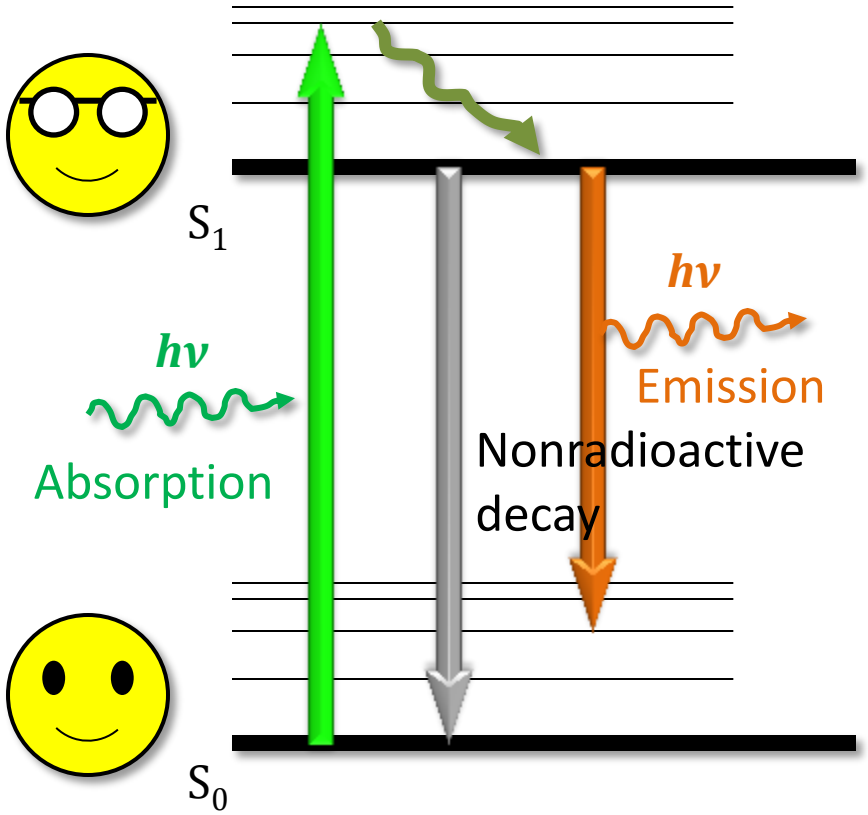


$$\mathrm{QE} = k_{\mathrm{fl}} / (k_{\mathrm{fl}} + k_{\mathrm{nr}})$$
$$= 1 / (1 + \tau_{\mathrm{fl}}\, k_{\mathrm{nr}})$$

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

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

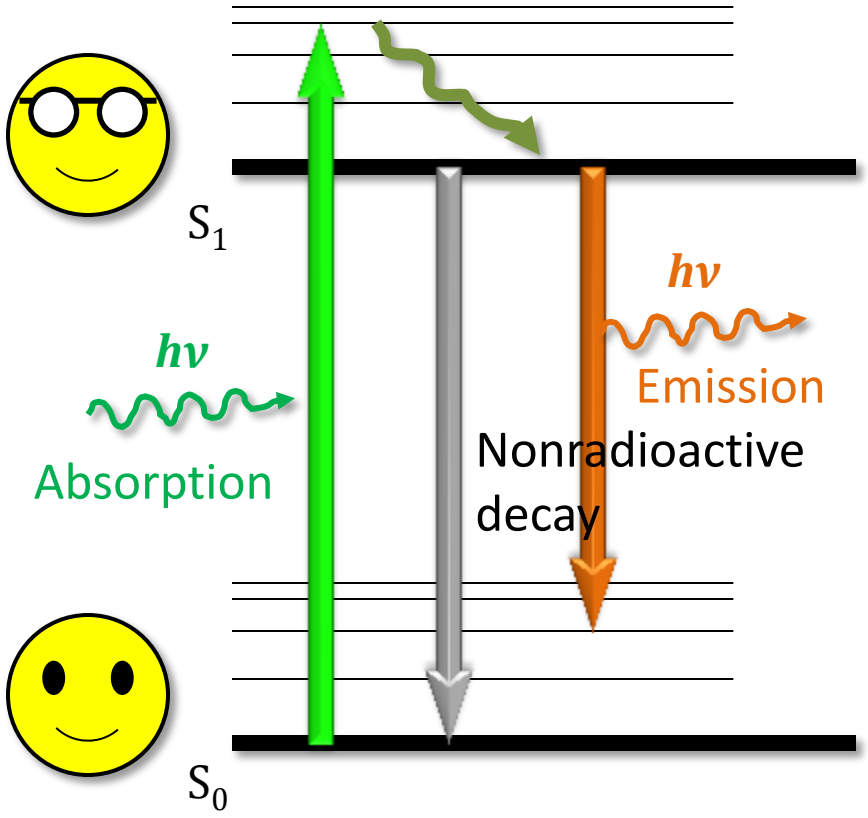
Quantum Yield



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

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



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

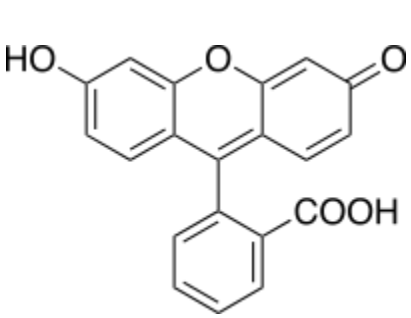
Brightness ≈

EC (wavelength)

×

QE

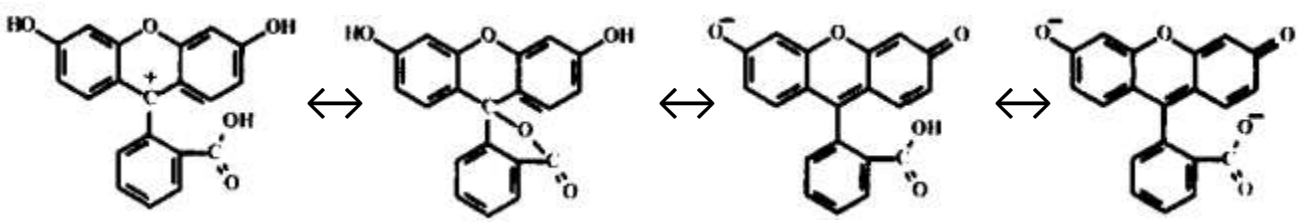
Brightness can be affected by the environment



Fluorescein

=

Low pH ⟷ High pH



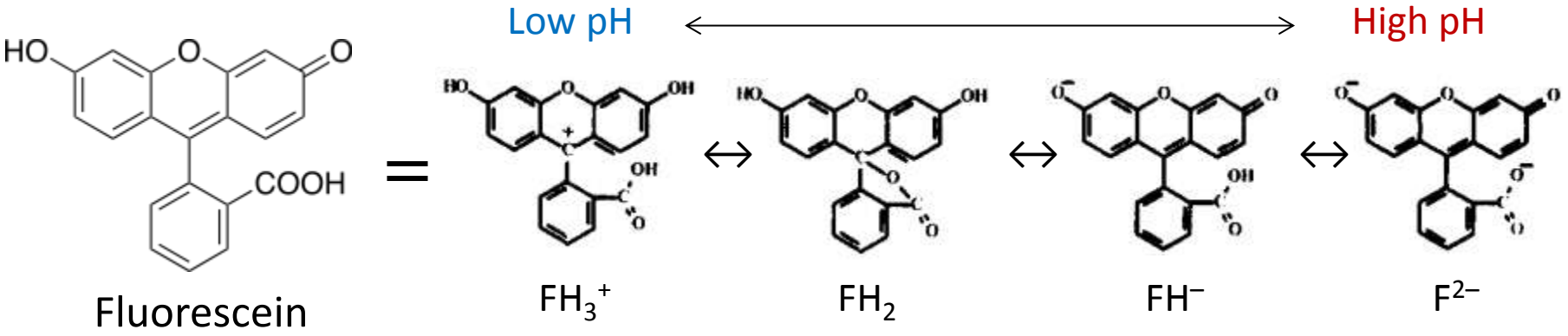
FH_3^+ ↔ FH_2 ↔ FH^- ↔ F^{2-}

Summary of absorption, fluorescence and protolytic properties of fluorescein

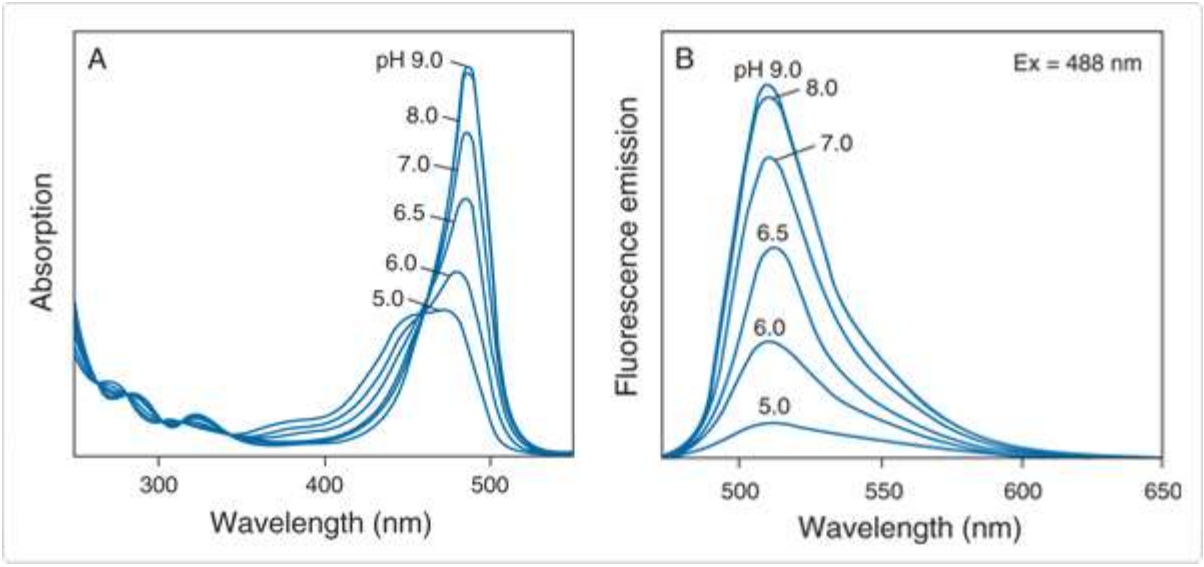
	FH_3^+	FH_2	FH^-	F^{2-}
ε/M^{-1} cm^{-1} (λ/nm)	53000(437)	3600(475)	29000(472)	76900(490)
ε/M^{-1} cm^{-1} (λ/nm)	7100(297)	11000(434)	29000(453)	9500(322)
ε/M^{-1} cm^{-1} (λ/nm)	33000(250)		700(310)	14000(283)
ε/M^{-1} cm^{-1} (λ/nm)			17000(273)	43000(239)
τ/ns			3.0	4.1
Φ^f	~0(pH > 1.5)	~0	0.37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

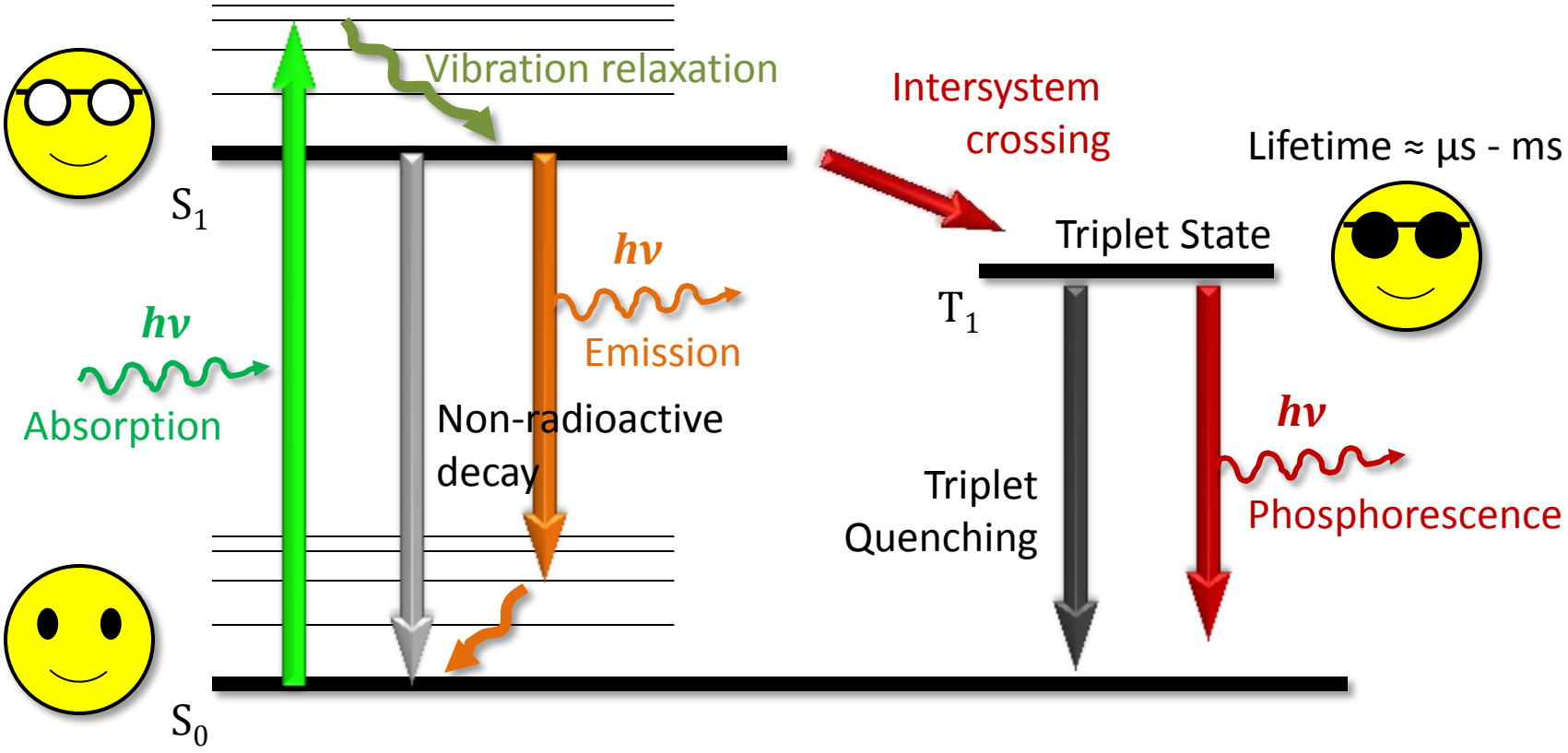


Fluorescein

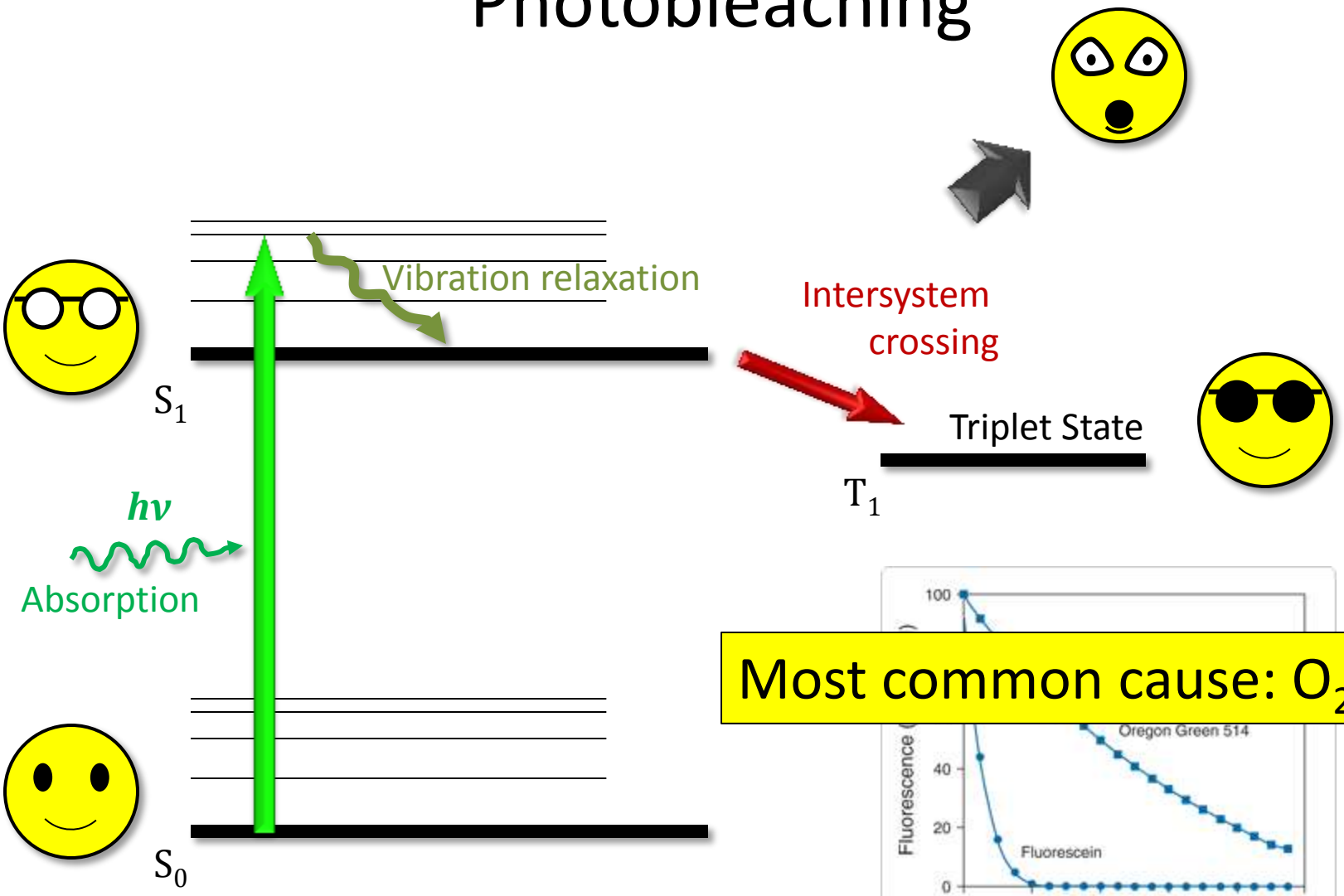


Invitrogen

The Full Jabłonski Diagram

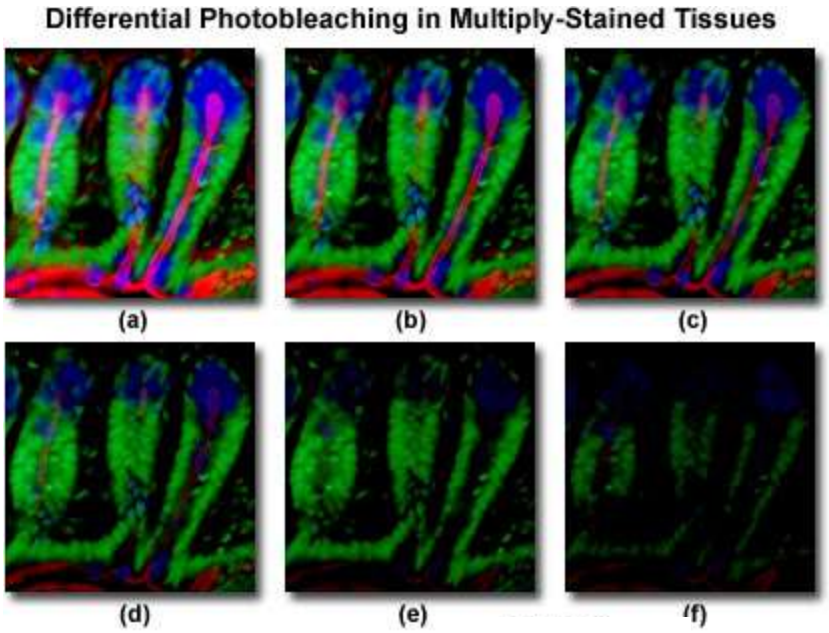


Photobleaching



Most common cause: O_2

Photobleaching is our enemy



Michael Davidson

For all fluorescence imaging:

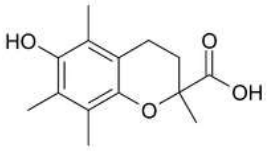
- Kills signal
- Alters the relative contrast

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.

Fluorescence signal = Excitation energy × Fluorophore brightness × Fluorophore concentration × Detection efficiency × Detector sensitivity

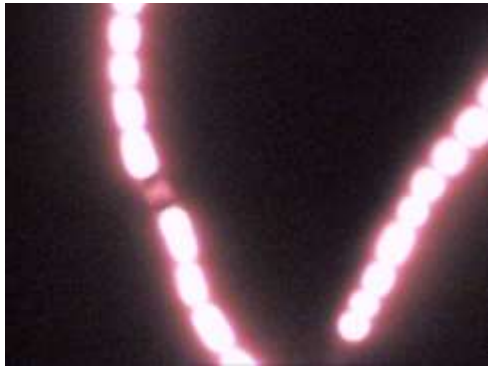
↑ Intensity × Exposure time

↑ Objective, Filters, Light path

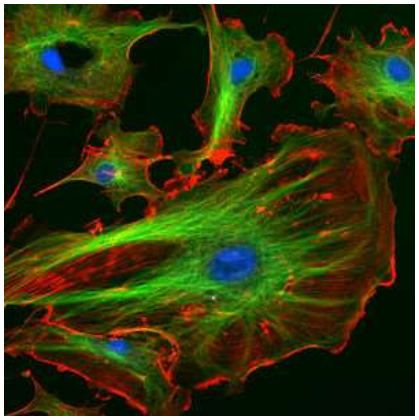
- Labeling as densely as possible
- Budget the photons
  - Only expose when observing
  - Minimize exposure time & excitation power
  - Use efficient filter combinations
  - Use high QE, low noise camera

Choice of fluorophores

Natural fluorescence

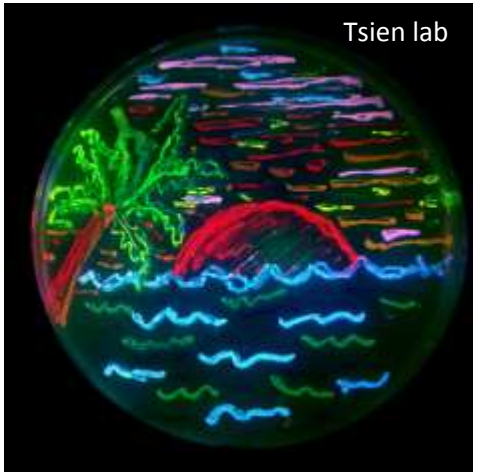


Organic dyes



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)

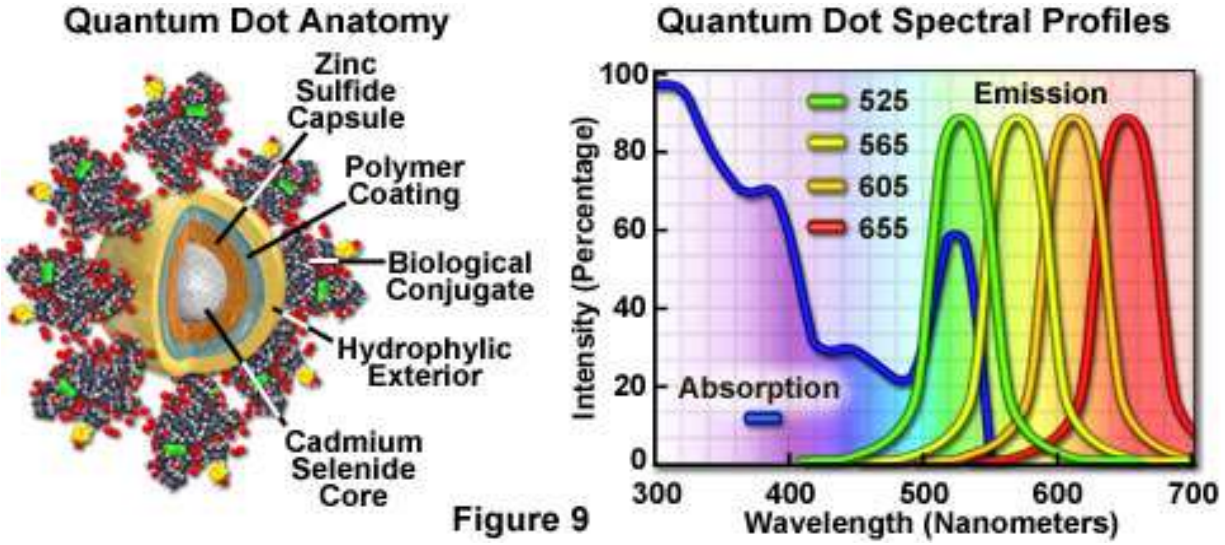
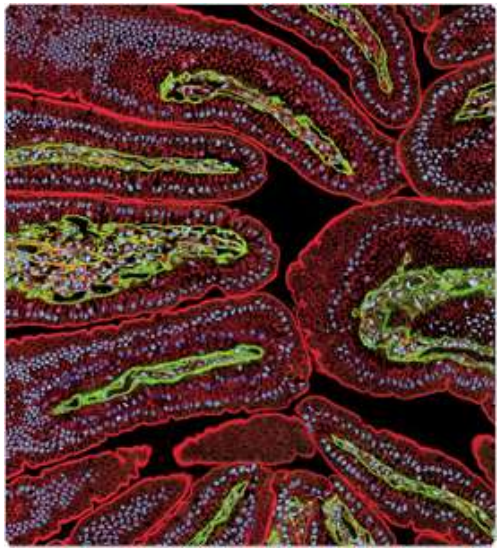
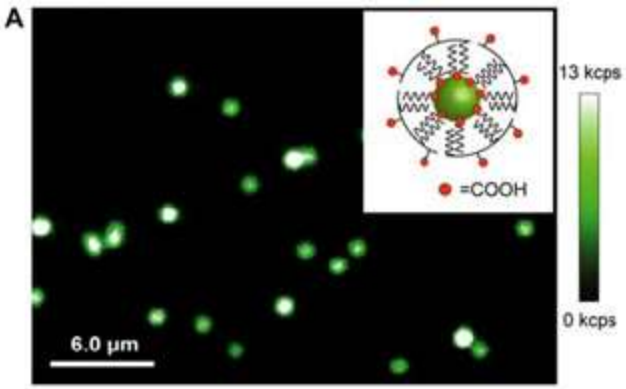


Figure 9

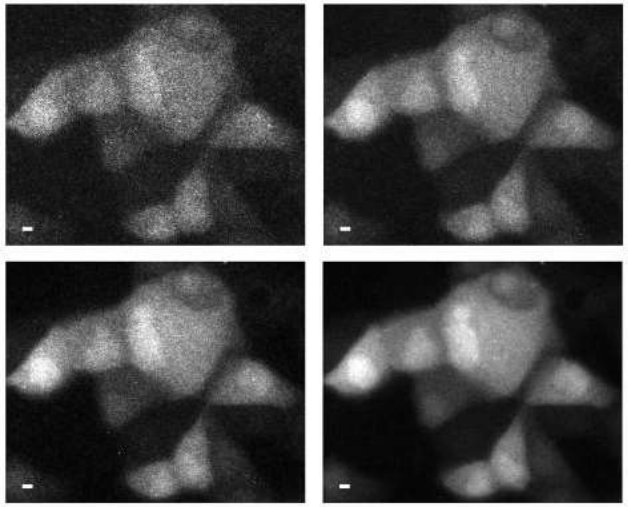


Inorganic fluorophores

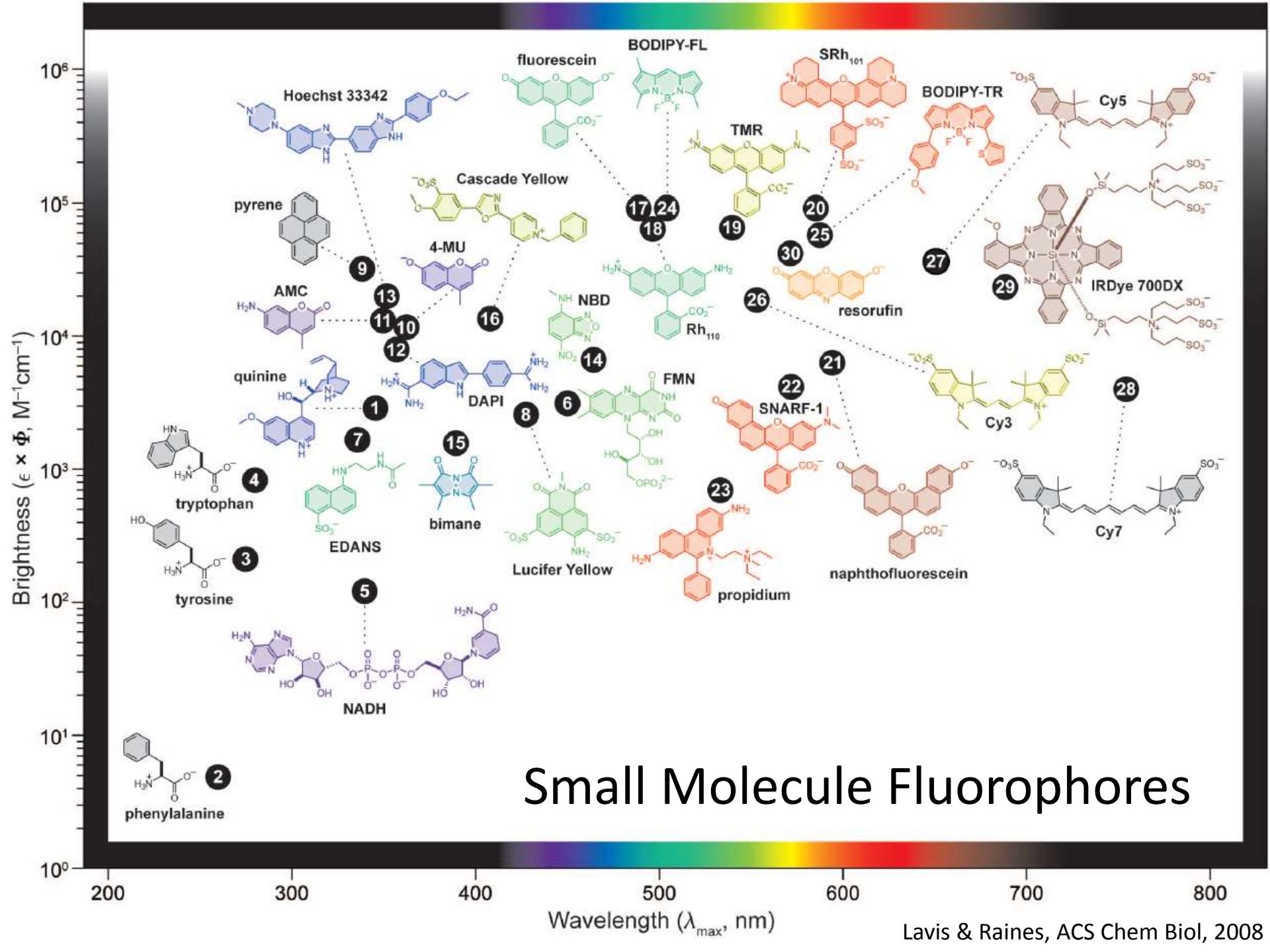
- Quantum dots
- Lanthanides
  - Very large Stokes or anti-Stokes shift (UV → Red or Red→Green)
  - Sharp emission peaks
  - Extremely long life time (µs - ms)



Bruce Cohen



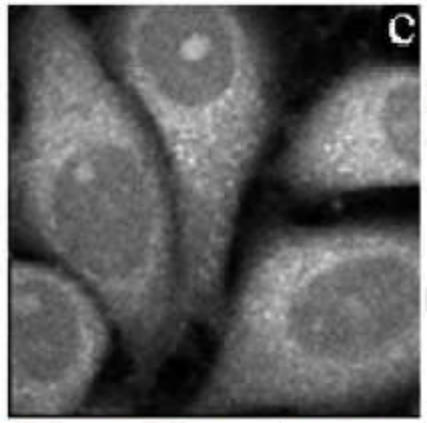
Gahlaut et al., Cytometry A, 2010



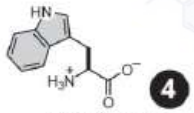
Small Molecule Fluorophores

Lavis & Raines, ACS Chem Biol, 2008

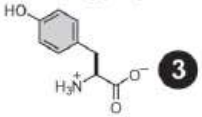
Intrinsic Fluorescence from Amino Acids



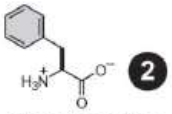
Tryptophan fluorescence image



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



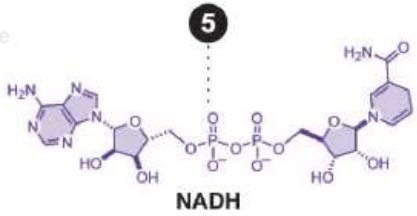
A_{max} = 274 nm, Ext = 1,400
Em_{max} = 303

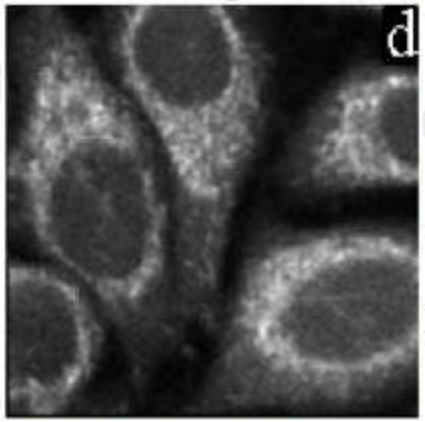


A_{max} = 257 nm, Ext = 200
Em_{max} = 282

Lavis & Raines, ACS Chem Biol, 2008

Fluorescent Enzyme Co-factors





NADH fluorescence image

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

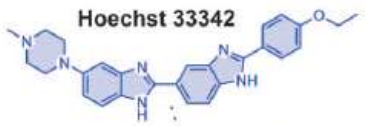
10^6 10^5 10^4 10^3 10^2 10^1 10^0

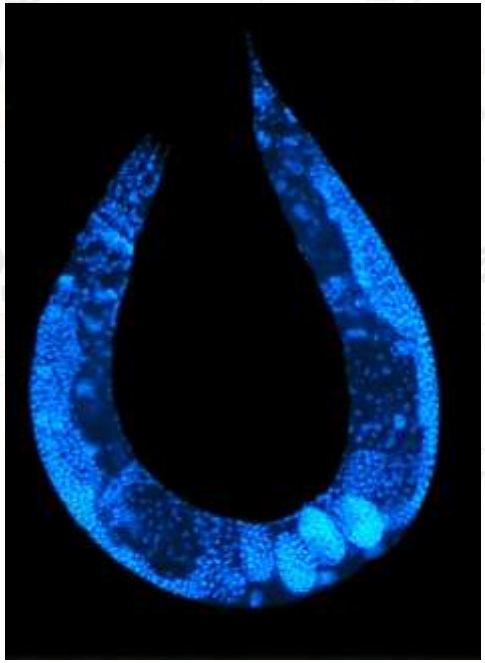
200 300 400 500 600 700 800

Wavelength (λ_{max}, nm)

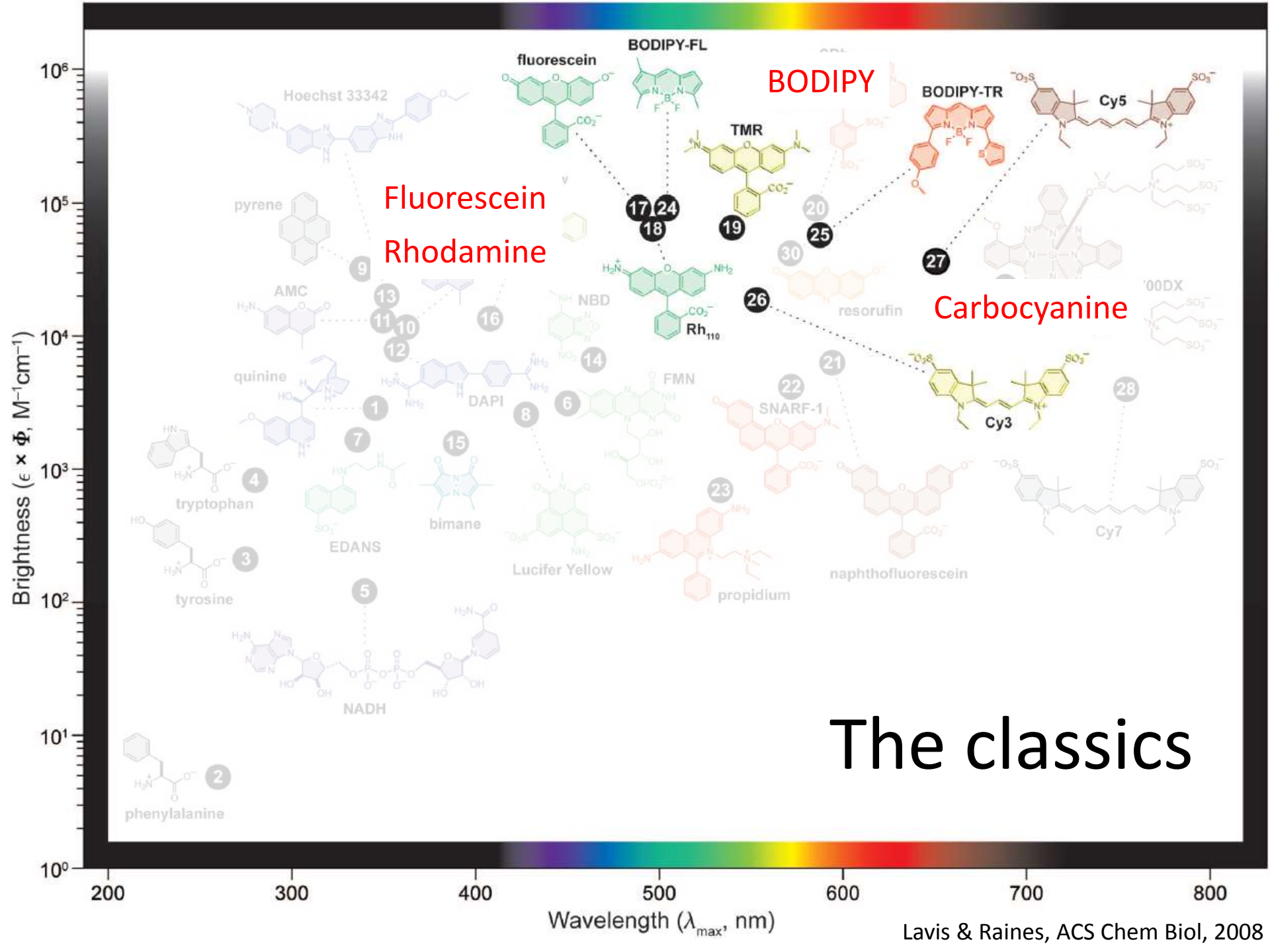
Lavis & Raines, ACS Chem Biol, 2008

DNA Intercalating Dyes





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.

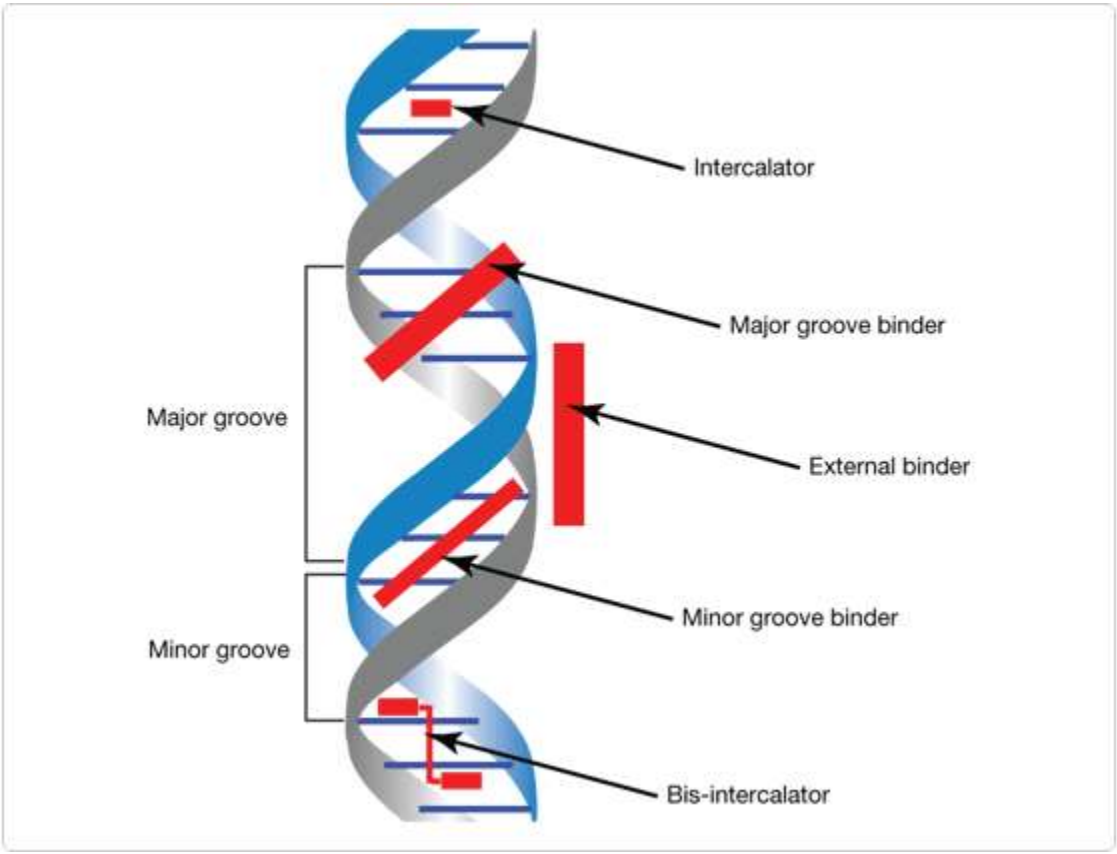
Small molecule dyes

- 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

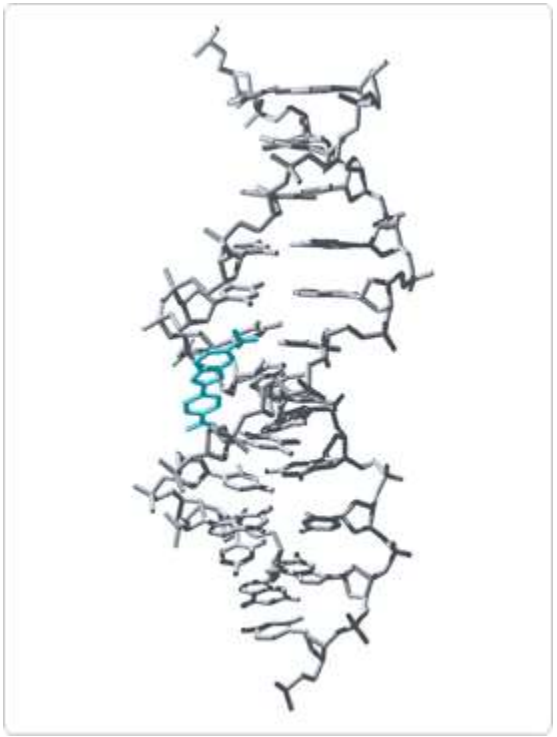
- Nucleic acid intercalating dyes
  - QE increment upon binding to DNA/RNA



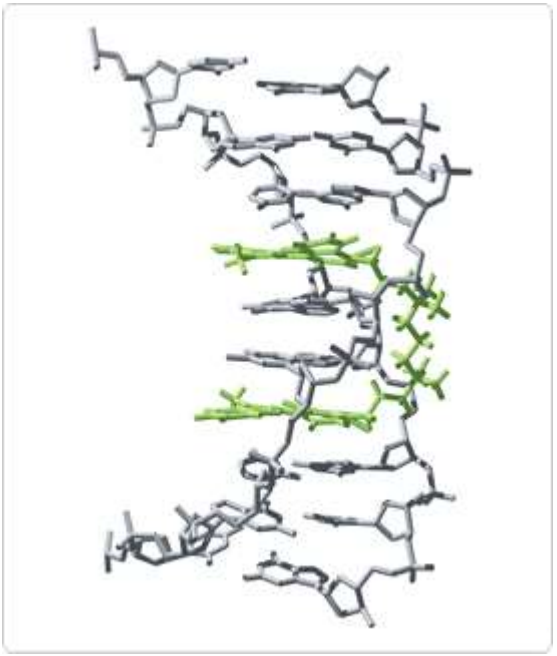
Functional dyes

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

DAPI



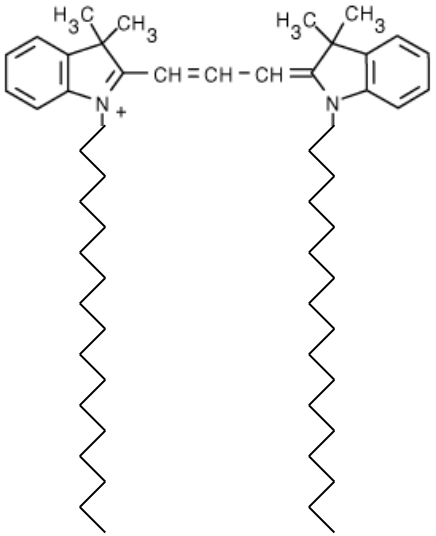
TOTO-1



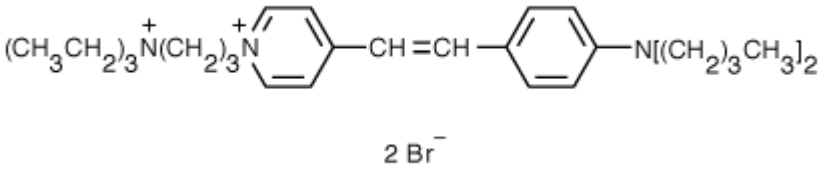
Functional dyes

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

DiI



FM 1-43



Functional dyes

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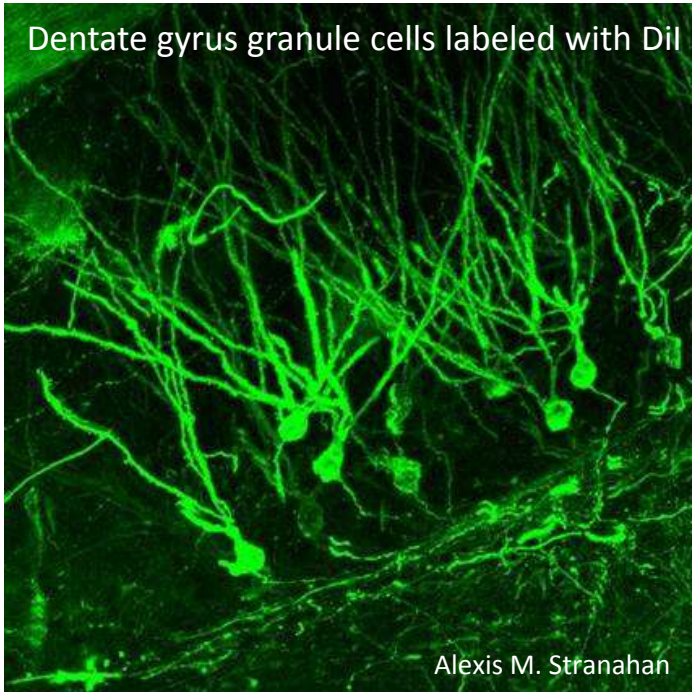
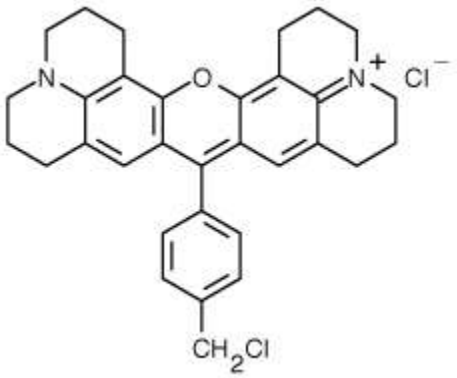
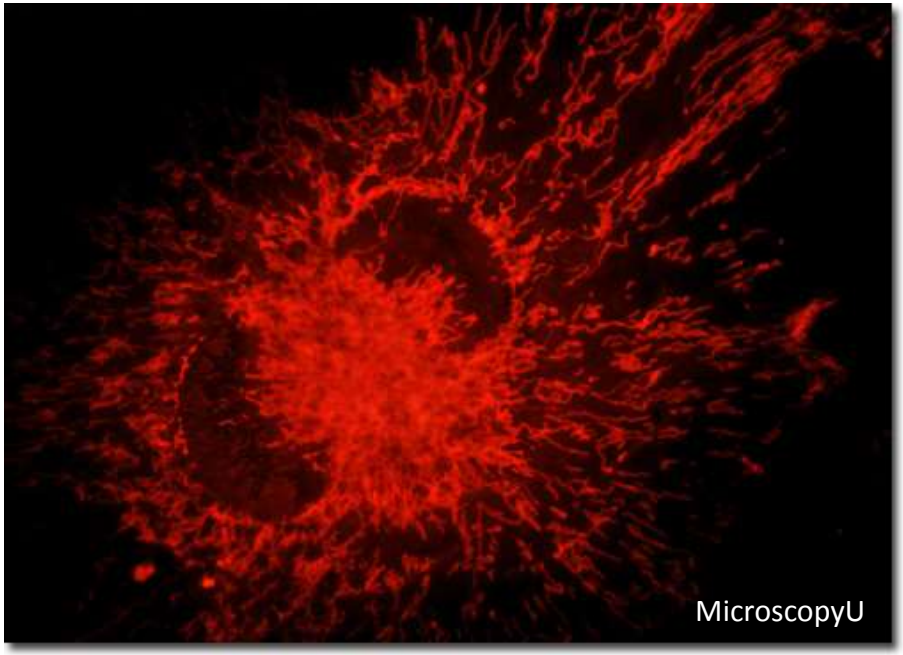


Functional dyes

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

MitoTracker Red CMXRos

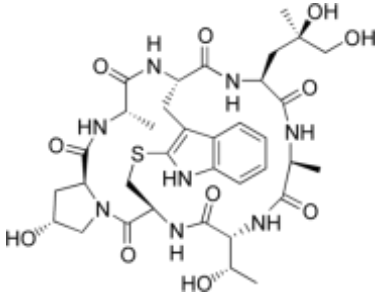




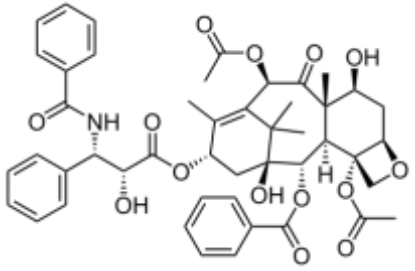
Small molecules probes

- Small molecules that bind proteins

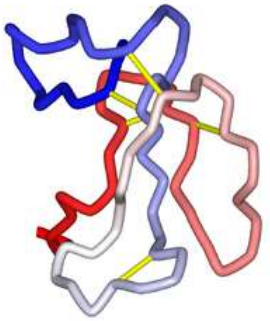
Phalloidin



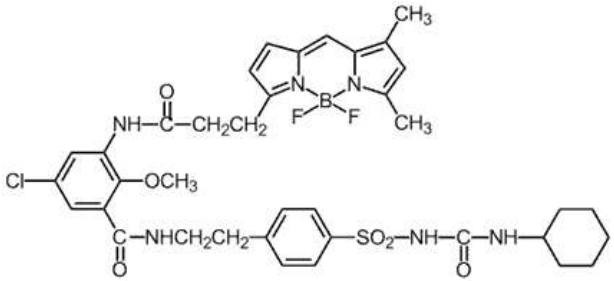
Taxol



α-bungarotoxin



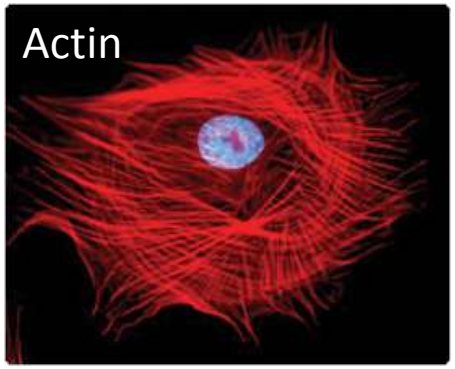
ER Tracker Green



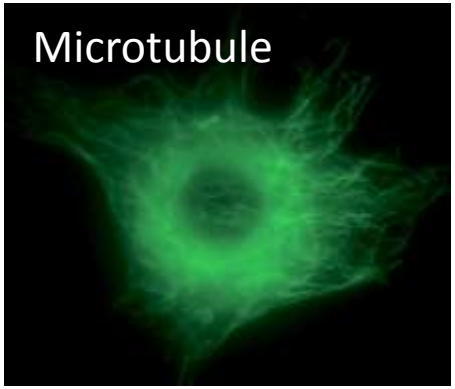
Small molecules probes

- Small molecules that bind proteins

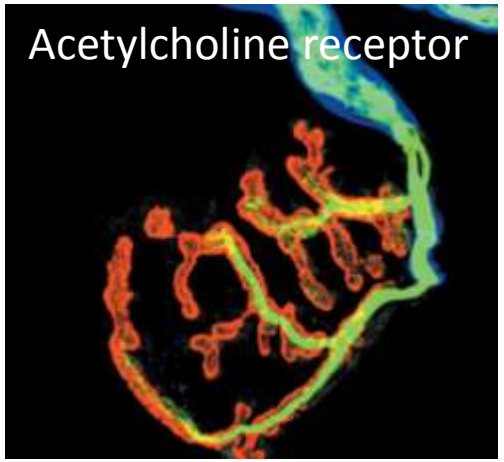
Phalloidin



Taxol



α-bungarotoxin



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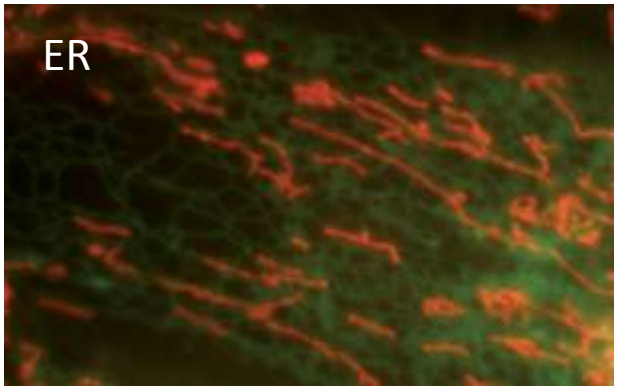
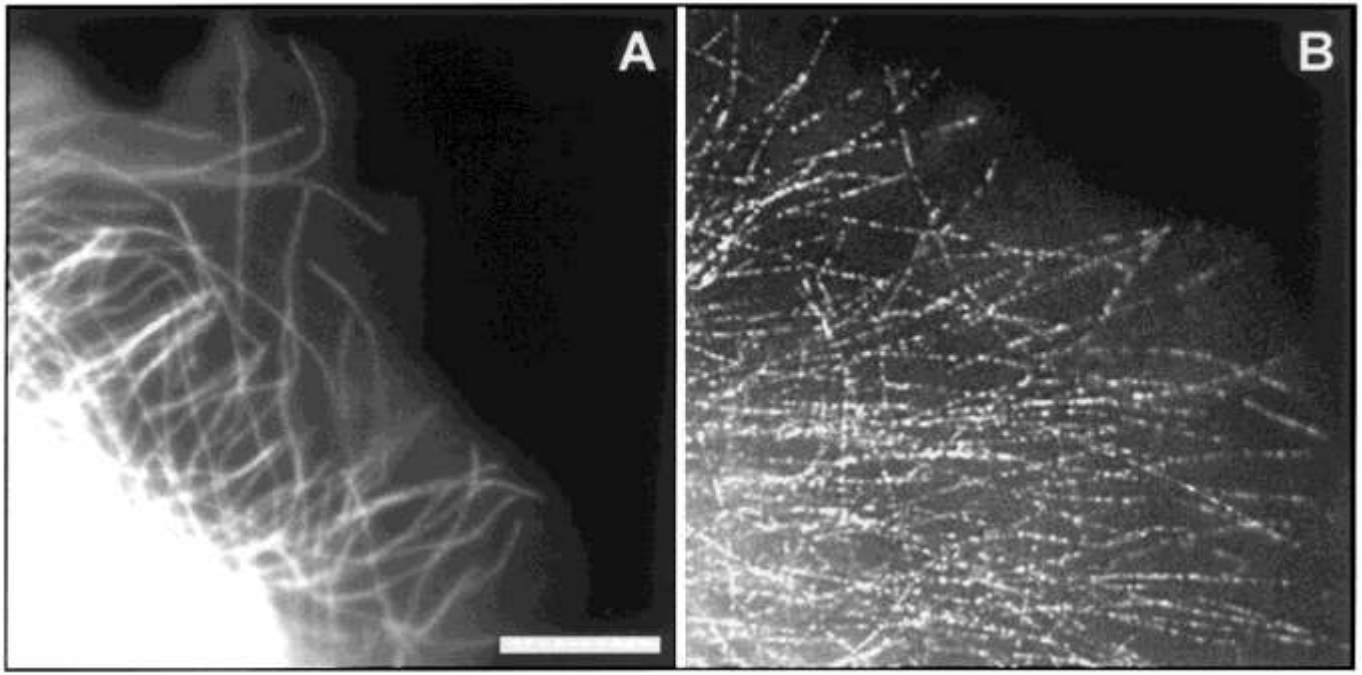


Large molecule labeling

- In vitro reconstituted systems
- Injecting labeled proteins

10% labeled tubulin

0.25% labeled tubulin

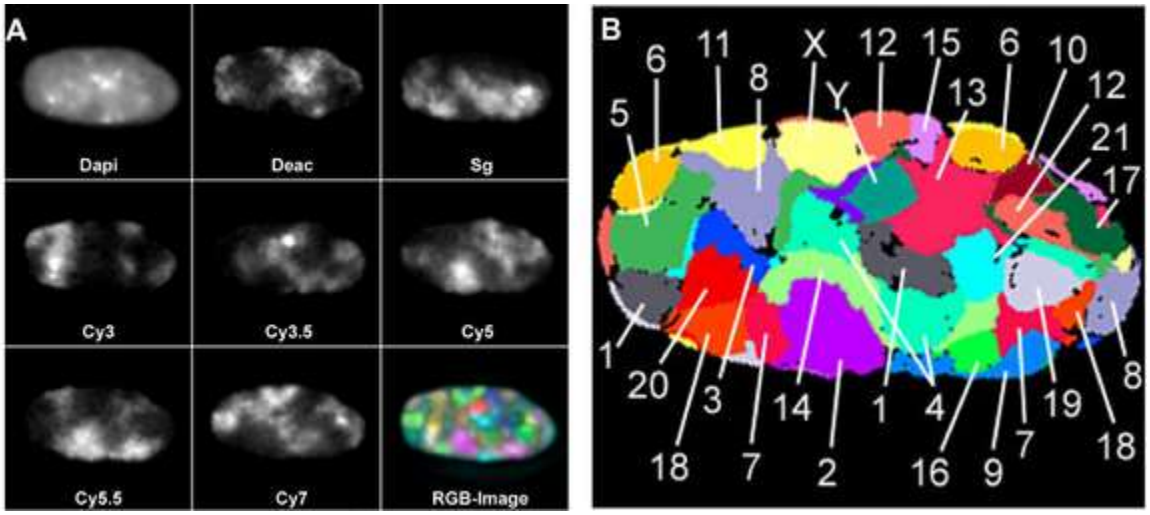


Waterman & Salmon, FASEB J, 1999

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

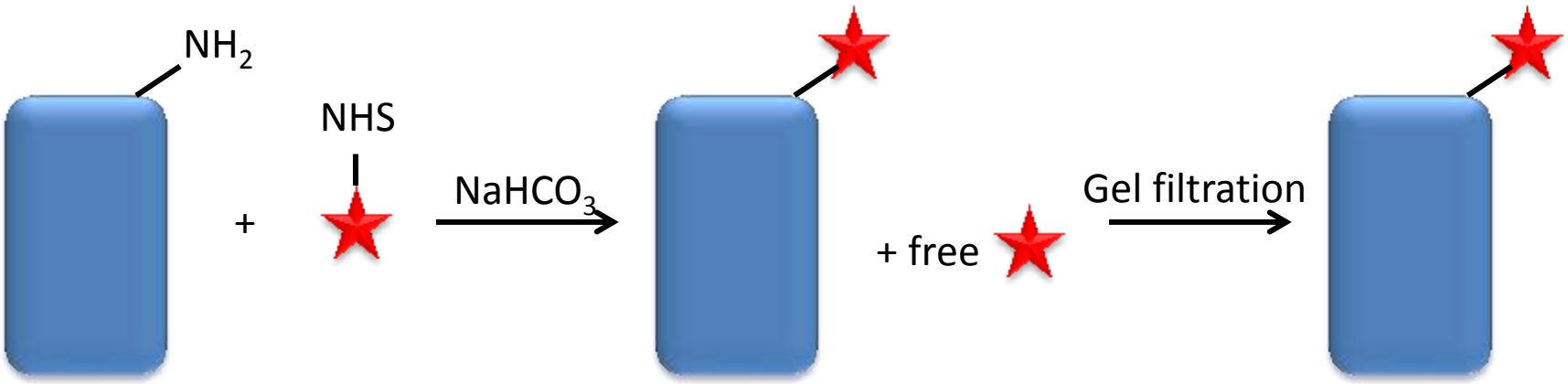
- In vitro reconstituted systems
- Injecting labeled proteins
- Fluorescence in situ hybridization (FISH)
- Biotin-avidin interaction
  - Labeled avidin/streptavidin
  - Biotin-dyes

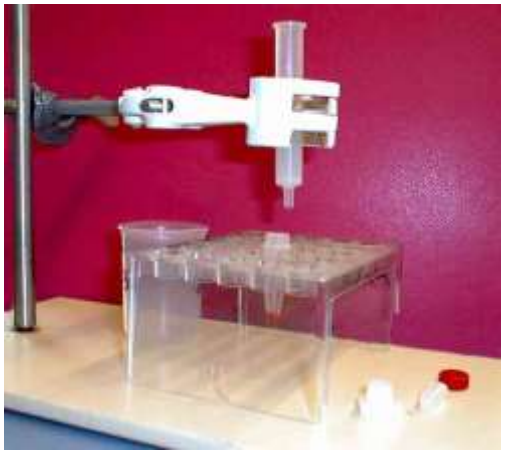
Large molecule labeling

- 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

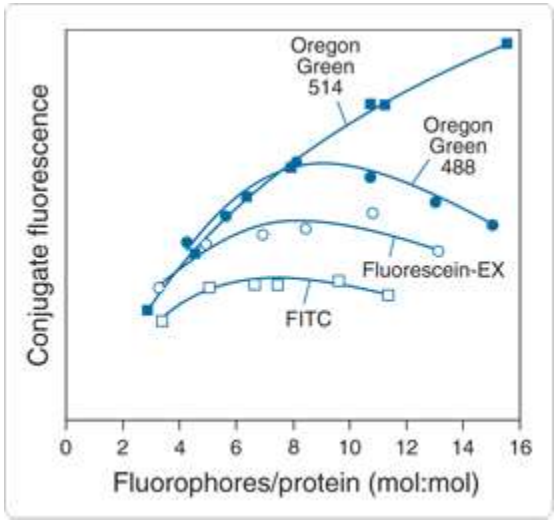
Dye concentration = A_{max} / E.C. $_{dye@max}$

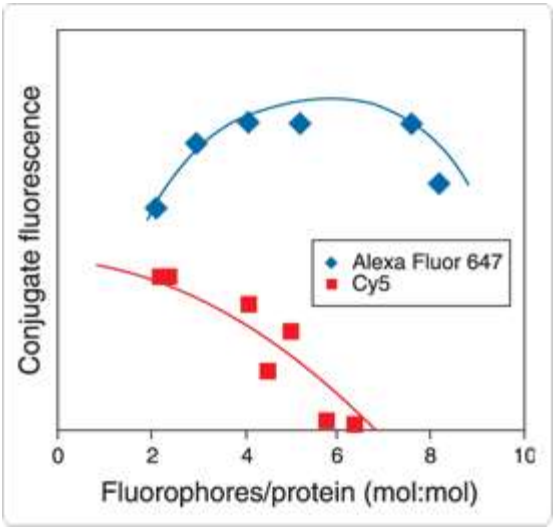
Protein concentration = (A_{280} – dye contribution) / E.C. $_{protein@280}$

Dye per protein = Dye concentration / Protein concentration

Too high labeling stoichiometry can make the protein dead…

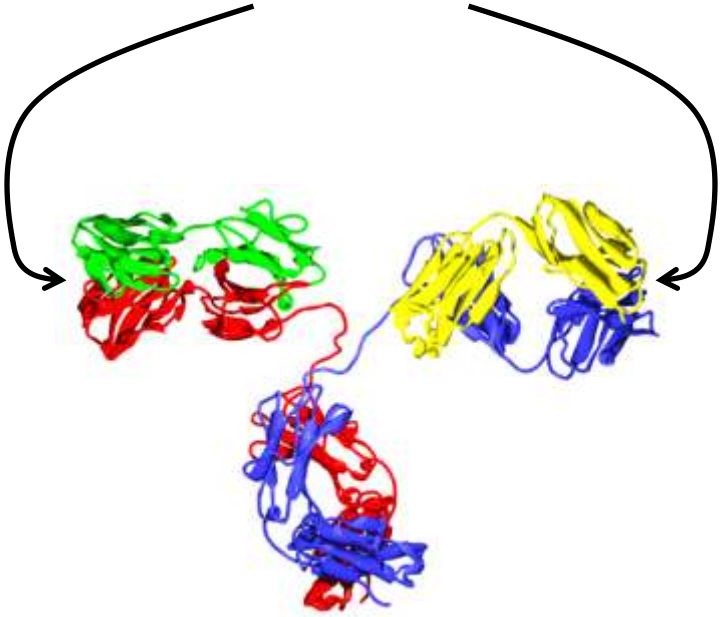
and lead to self-quenching



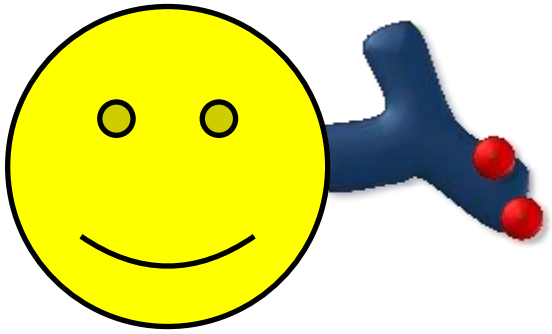


Immunofluorescence

Antigen binding sites

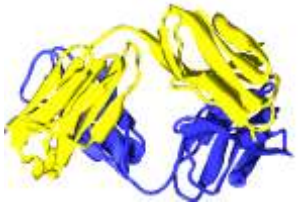


Direct immunofluorescence



Primary antibody binding efficiency can be < 10%

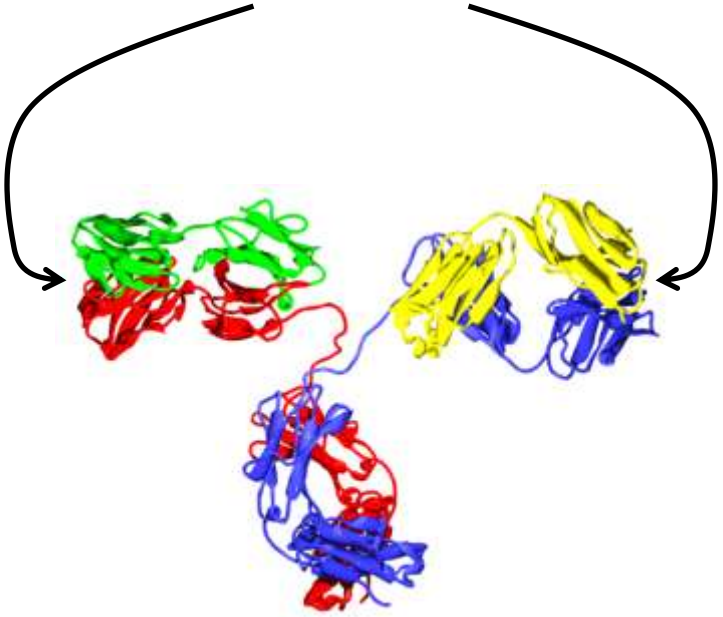
Fab fragment



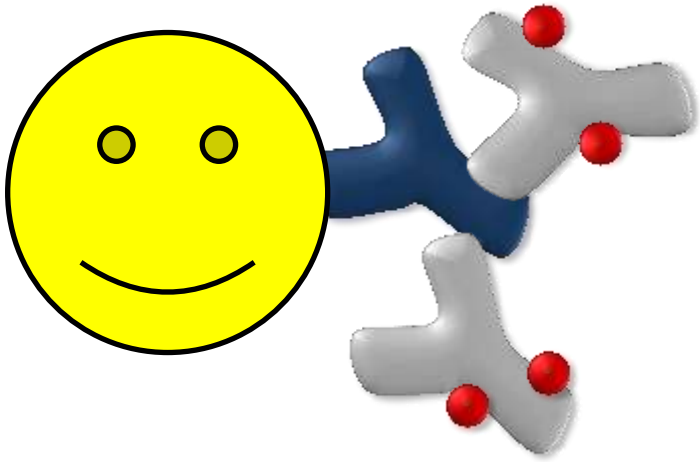
Nanobody

Immunofluorescence

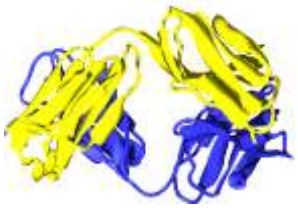
Antigen binding sites



Indirect immunofluorescence



Fab fragment



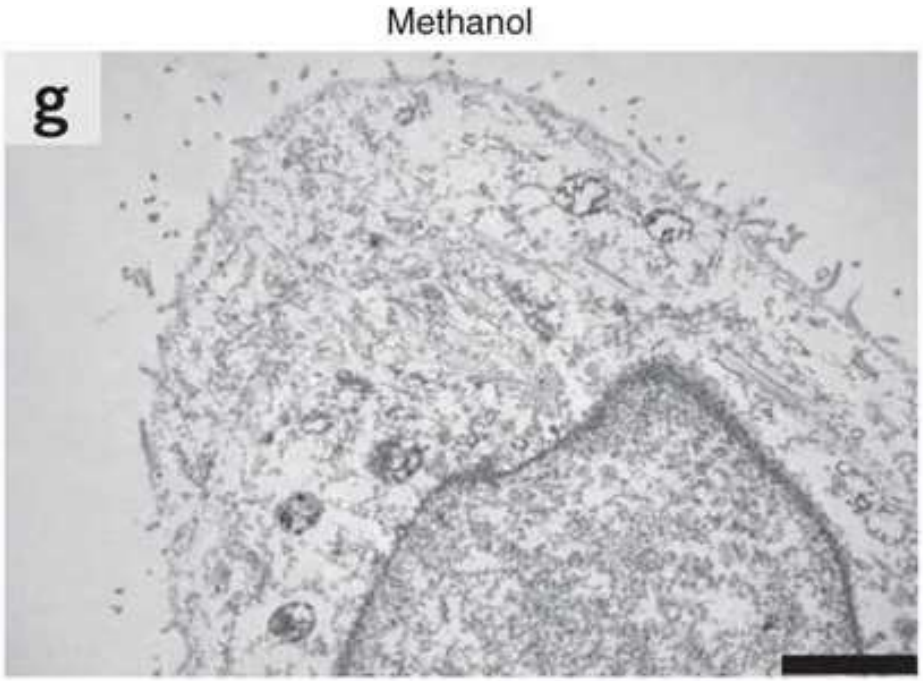
Nanobody

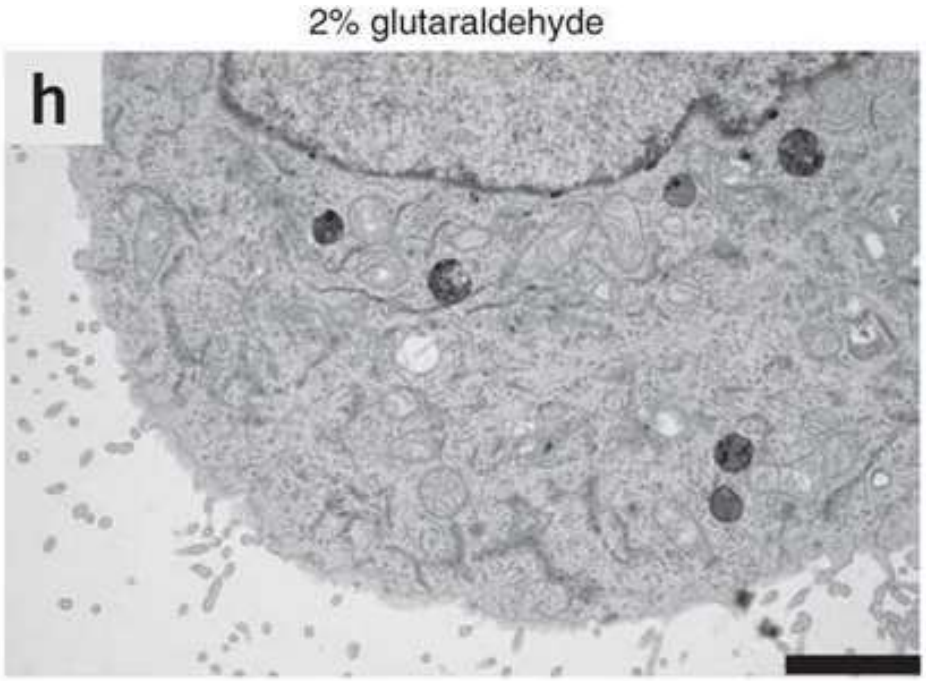
Fixation methods

- 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





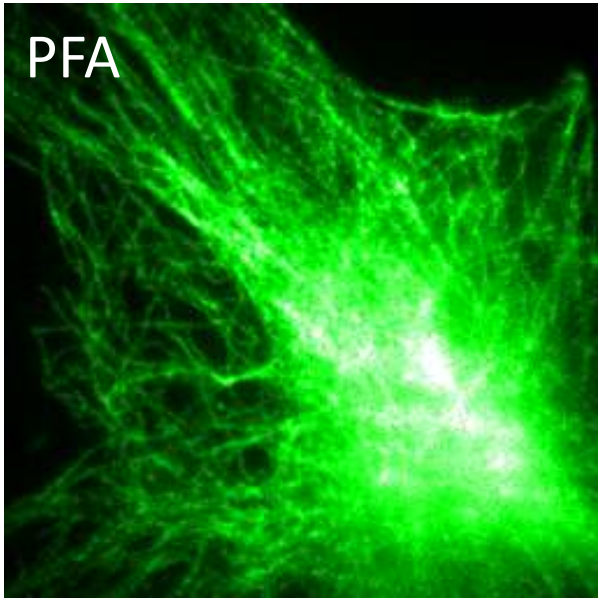
Nature Methods **9**, 152–158 (2012)

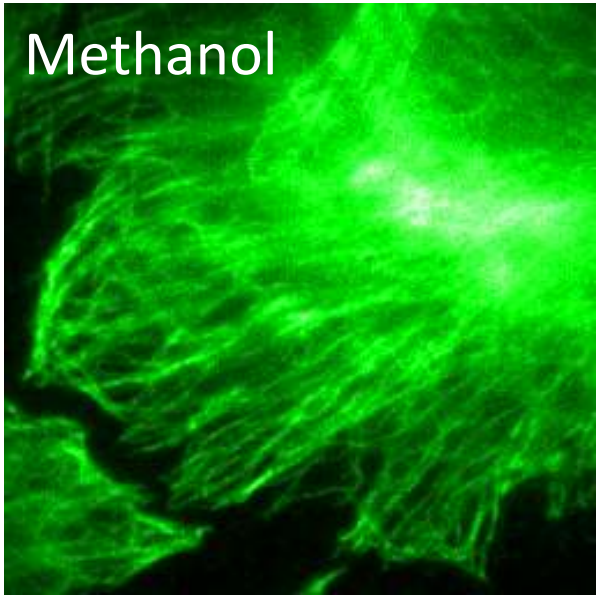
Membrane permeabilization

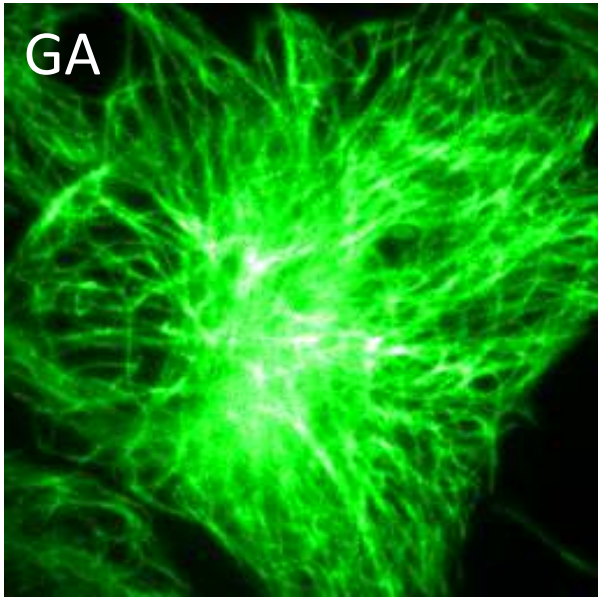
- Acetone
- Non-ionic detergents
  - Triton X-100, Tween-20
    - Extracting lipids, making holes on the membrane
- “Mild” detergents
  - Saponin
    - Extracting cholesterol only, permeabilizing the plasma membrane while saving the organelle membranes

Fixation artifacts

Microtubules







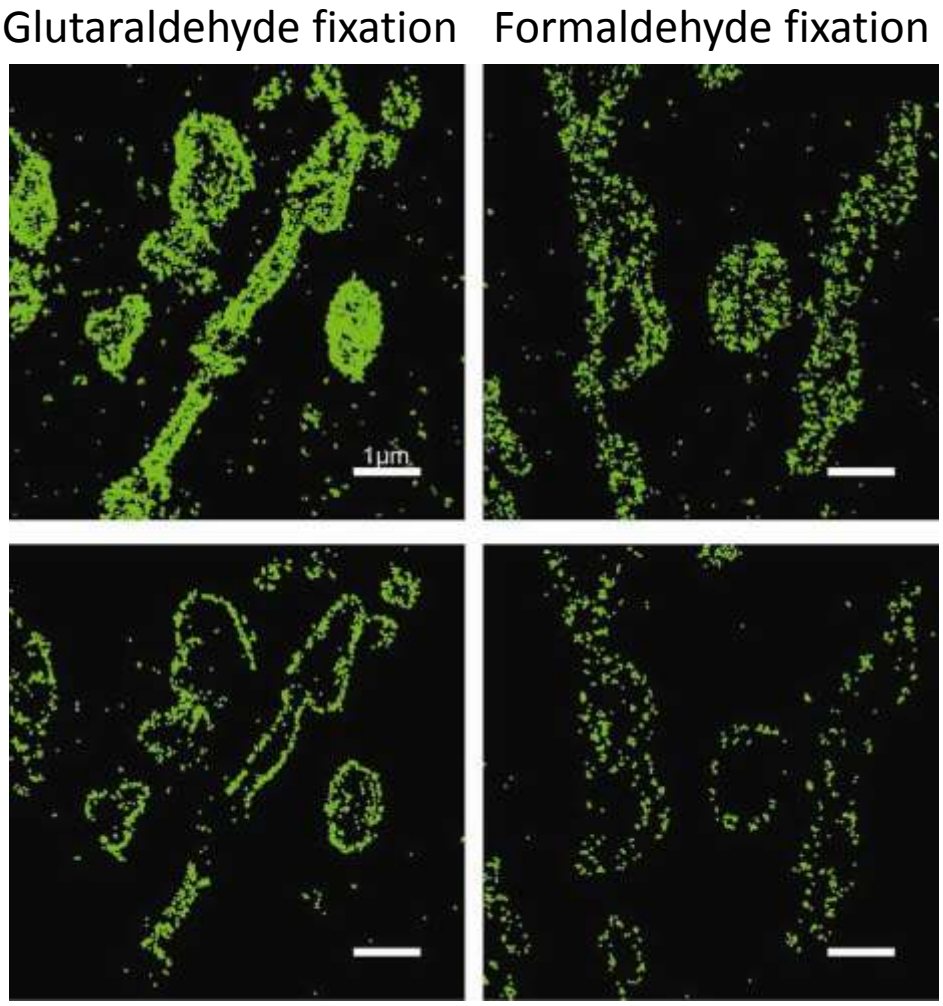
Immunolabeling artifacts and the need for live-cell imaging

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

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

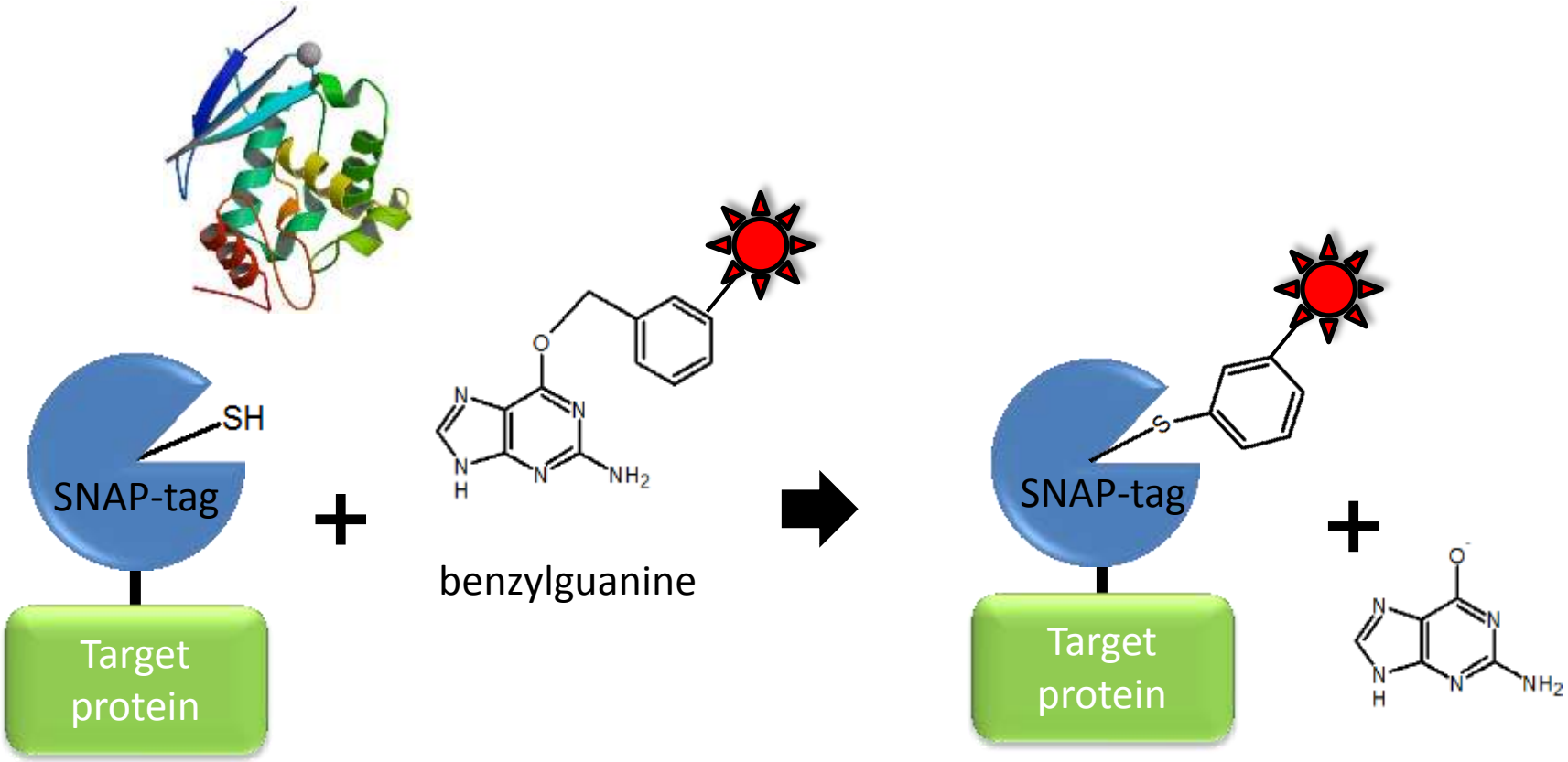
Some fixation artifacts become visible in super-resolution microscopy

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)



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