Cellular and Molecular Biophysics

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CFU 5 LM Biotecnologie Industriali- 6 LM Fisica - A.A. 2024/25

Corso di laurea in LM Biotecnologie Industriali- LM Fisica

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and Systems Biology

FLUORESCENCE

I. Introduction to Fluorescence

II. Fluorochromes

III. Biological and biomedical applications

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I. Introduction to Fluorescence

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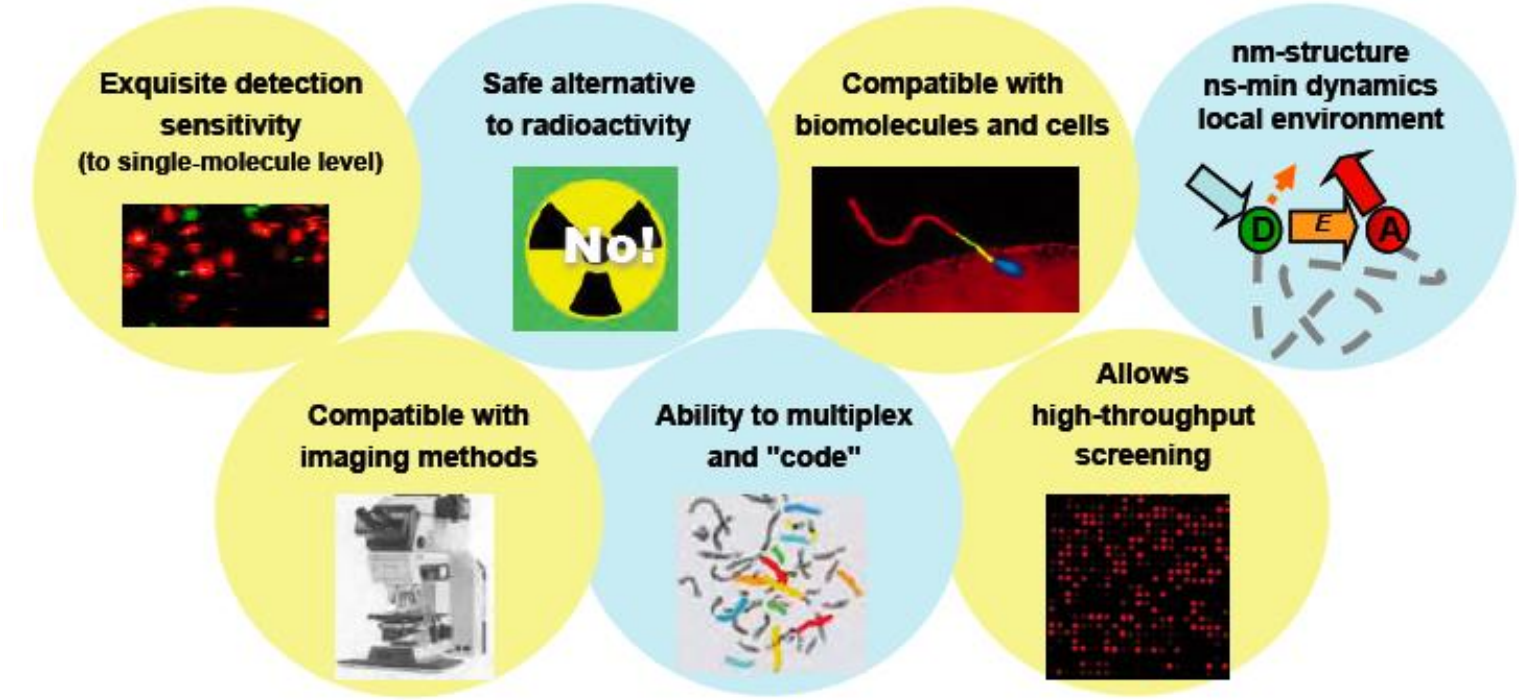
What is fluorescence?

It’s the **property of some compounds**, once hit by a light radiation,

to emit the absorbed light (in the form of photons)

at a lower frequency and, consequently, at a longer wavelength

Advantages of fluorescence



1933-2008: 175 years of fluorescence

From ensembles…

…to single molecules!

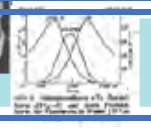
1833 First description of fluorescence (Brewster, chlorophyll)

1849 First publication on fluorescence (Herschel, quinine)

1852 Sir Stokes coins the term "fluorescence" (from fluorspar crystals, CaF_2; "fluor", to flow)

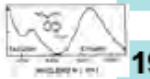
1930's Perrin observes FRET

1935 Jablonski diagram



1948 Förster describes FRET

1950's NIH funds development of a research fluorometer



1951 Weber describes Fluorescence Anisotropy, introduces the first designer probe: dansyl

1976 First fluorescence-based single-molecule observation (Hirschfeld) (~100 fluorophores)

1987 First multi-fluorophore observation at RT in solution (Keller) (phycoerythrin, 34 fluorophores)

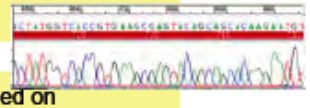
1989 First single-fluorophore observation at cryogenic T (Moerner) (1 fluorophore)

1990 First single-fluorophore observation at RT in solution (Keller) (1 fluorophore)

1993 First single immobilized fluorophore observation at RT (Betzig) (1 fluorophore)

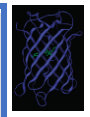
1996 First FRET observation at single-molecule level (Ha *et al.*)

2000 Single-fluorophore in single-cells (Schutz)



2003 Complete Human Genome based on fluorescence DNA sequencing; several groups on quest for single-molecule sequencing

2008 Nobel prize in chemistry to O.SHIMOMURA, M. CHALFIE, and R.Y. TSIEN for the discovery and development of the green fluorescent protein, GFP



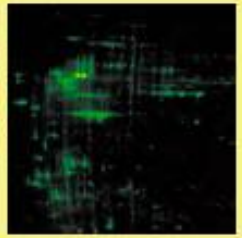
Fluorescence applications in biology

Genomics

- genome sequencing
- real-time PCR
- DNA arrays

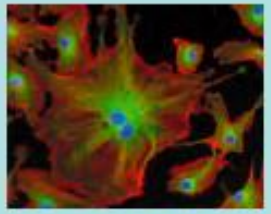
Proteomics

- protein arrays
- protein interactions
- protein expression profile



"Cellulomics"

- in-situ hybridization
- protein localization
- protein movement



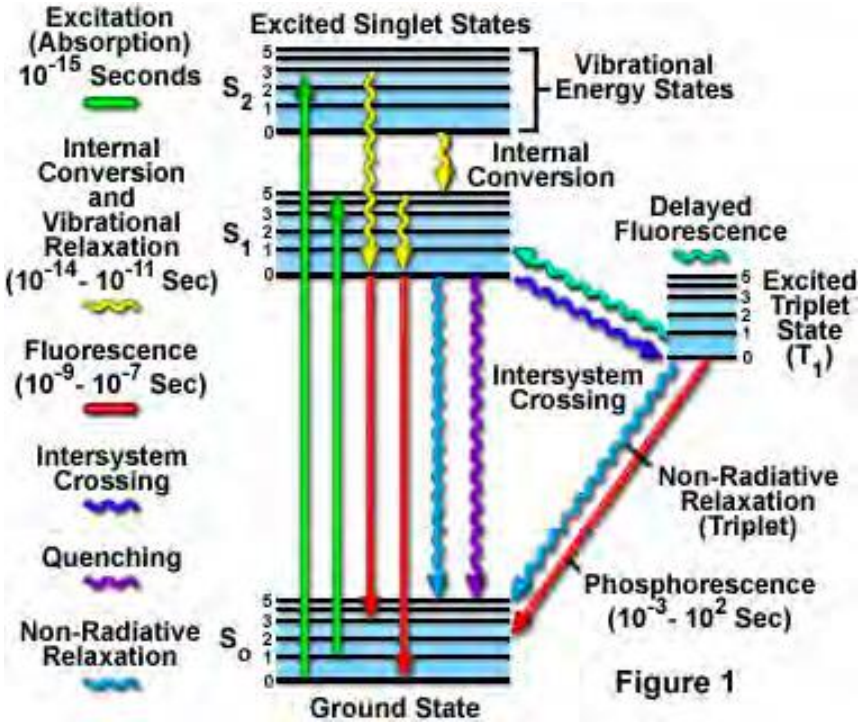
Interactions

- protein-DNA
- protein-drug
- DNA-drug

Jablonski energy diagram

Fluorescence is a property of particular compounds to emit light absorbed at lower frequency as compared with absorption radiation

Excitation: 1 photon with energy hv_{EX} excite the electrons of the fluorochrome that from S_0 jump to excited singlet S_1 o S_2. The excited state exist for a definite time (nanoseconds) in which the fluorochrome undergoes into conformational changes and interact with the molecular environment.



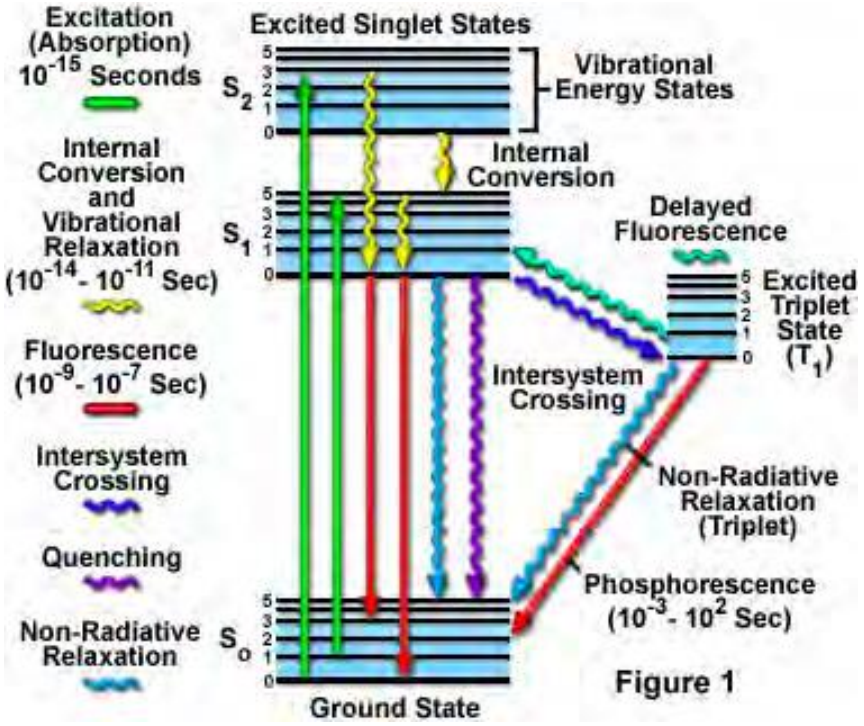
http://www.olympus-lifescience.com/en/microscope-resource/primer/java/jablonski/lightandcolor/

$$hv_{EM} < hv_{EX}$$

Jablonski energy diagram

Fluorescence is a property of particular compounds to emit light absorbed at lower frequency as compared with absorption radiation

The excited electron in a high state if vibrational energy has the tendency rapidly go back to the lower vibrational energy level of the excited state S1=0 dissipating part of the energy in heating **Internal conversion**



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$$hv_{EM} < hv_{EX}$$

Jablonski energy diagram

Fluorescence is a property of particular compounds to emit light absorbed at lower frequency as compared with absorption radiation

Emission. If the excited fluorochrome return to S_0 from S_1 emitting photons, the process is called fluorescence. Due to the energy dissipation during the internal conversion, the emission energy hv_{EM} will be lower as compared with hv_{EX}.

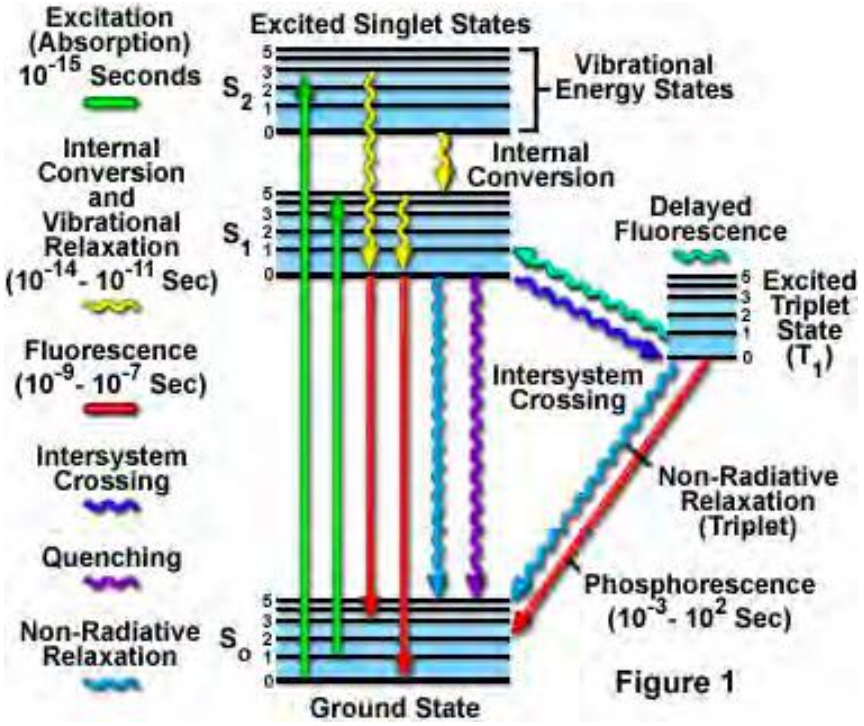


Figure 1

http://www.olympus-lifescience.com/en/microscope-resource/primer/java/jablonski/lightandcolor/

$$hv_{EM} < hv_{EX}$$

Not all the molecules initially excited by absorption return to the ground state (S_0) by fluorescence emission.

Other processes such as intersystem crossing, collisional quenching, fluorescence resonance energy transfer (FRET) may also depopulate S_1.

The **fluorescence quantum yield**, which is the ratio of the number of fluorescence photons emitted to the number of photons absorbed, is a measure of the relative extent to which these processes occur.

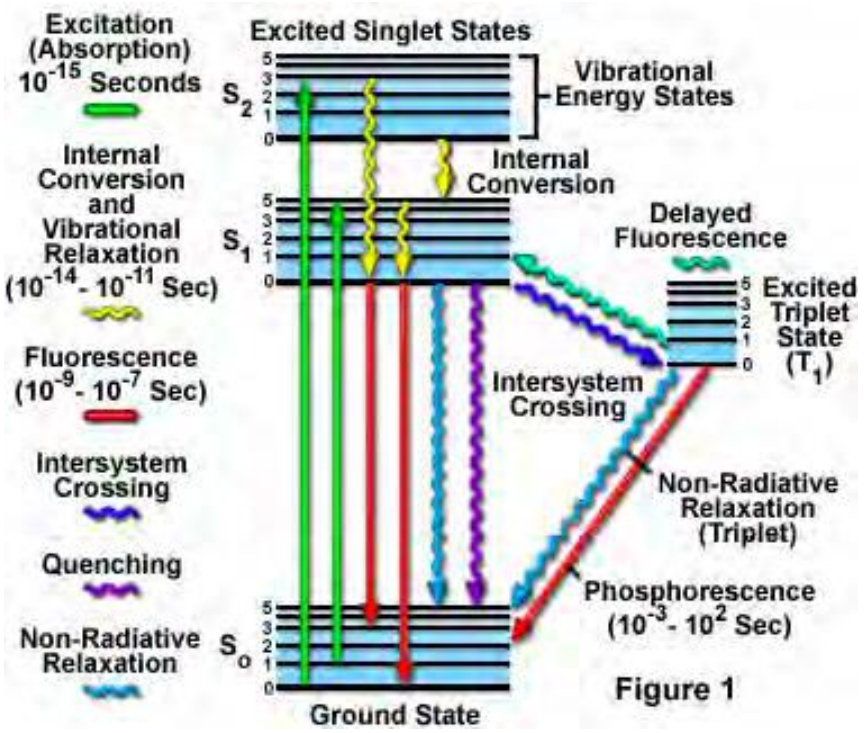


Figure 1

$$\Phi_f = \frac{\text{Number of photons emitted}}{\text{Number of photons absorbed}}$$

$$0 \leq \Phi_f \leq 1$$

Intersystem crossing: phosphorescence and delayed fluorescence

Intersystem crossing (ISC): molecule relaxation to the lowest triplet state that ultimately results either in emission of a photon through phosphorescence, a transition back to the excited singlet state that yields delayed fluorescence or in photochemical reaction with molecular oxygen.

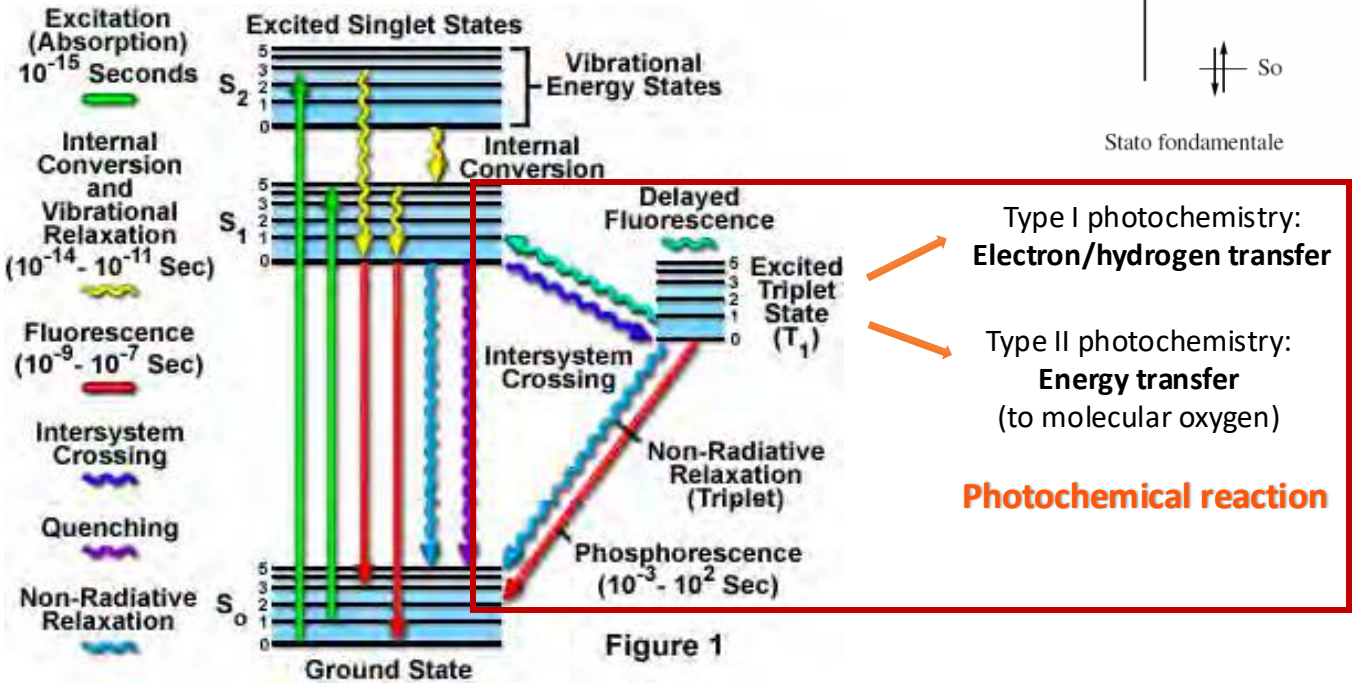
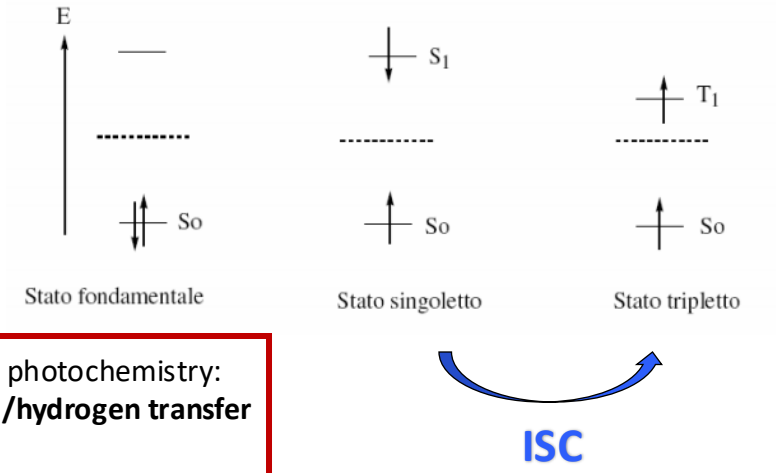


Figure 1

Type I photochemistry:
Electron/hydrogen transfer

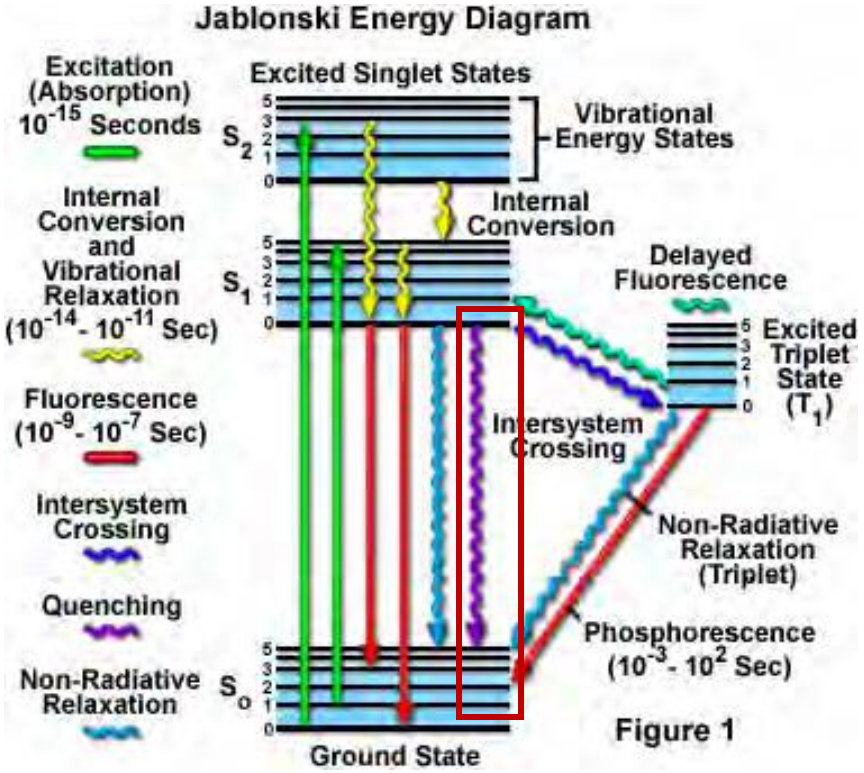
Type II photochemistry:
Energy transfer
(to molecular oxygen)

Photochemical reaction



Collisional quenching

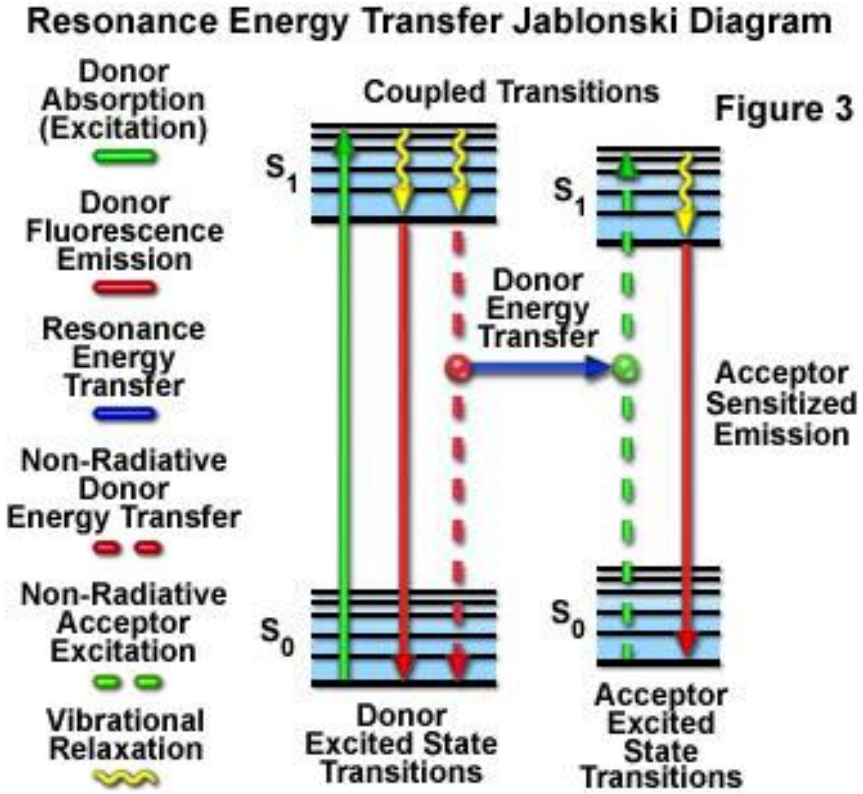
Fluorescence quenching can be defined as a bimolecular process that reduces the fluorescence quantum yield without changing the fluorescence emission spectrum; it can result from transient excited-state interactions (collisional quenching) or from formation of non fluorescent ground-state species. Self-quenching is the quenching of one fluorophore by another; it therefore tends to occur when high loading concentrations or labeling densities are used



FRET: FÖster Resonance Energy Transfer

FRET is a quantum-mechanical phenomenon that occurs when two fluorochromes are in molecular proximity. It describes an energy transfer mechanism between two chromophores.

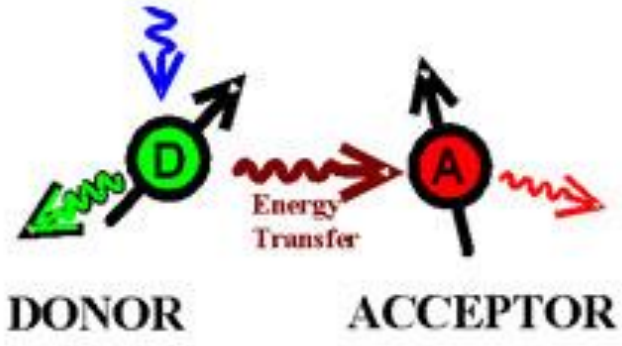
A **donor chromophore** in its **excited state** can **transfer energy** by a non radiative, long-range dipole-dipole coupling mechanism **to an acceptor chromophore in close proximity** (typically < **10 nm**). This energy transfer mechanism is termed "Föster resonance energy transfer" (FRET), named after the German scientist: Theodor Föster.



http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Technical-Notes-and-Product-Highlights/Fluorescence-Resonance-Energy-Transfer-FRET.html

FRET: Föster Resonance Energy Transfer

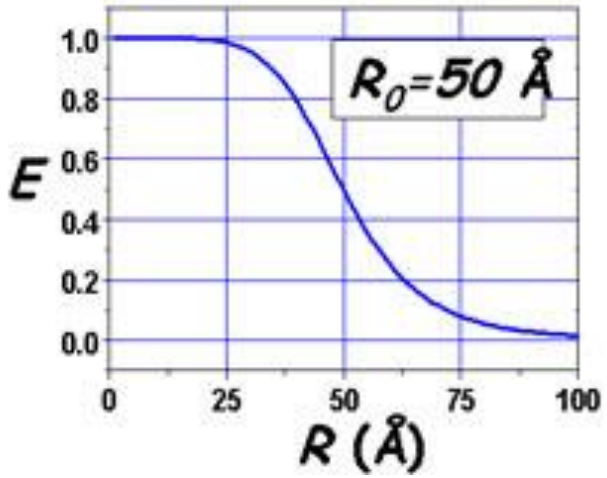
FRET is well-suited to the study of protein-protein interactions, which occur on a similar spatial scale.



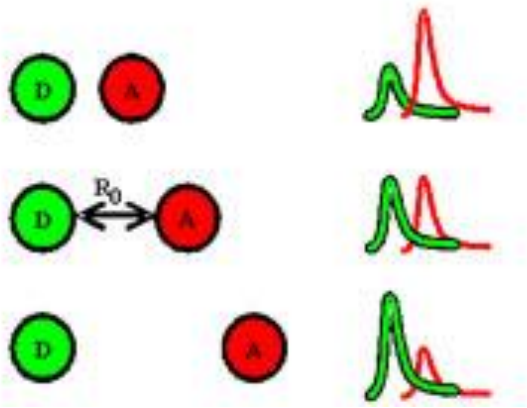
Energy Transfer Efficiency

$$E = \frac{1}{1+(R/R_0)^6}$$

R_0=50% transfer efficiency distance
3nm~7nm



"Spectroscopic Ruler"



Photobleaching

Photobleaching is the photochemical destruction of a fluorophore.

Photobleaching occurs when a fluorophore permanently loses its ability to fluoresce due to photon-induced chemical damage and covalent modification.

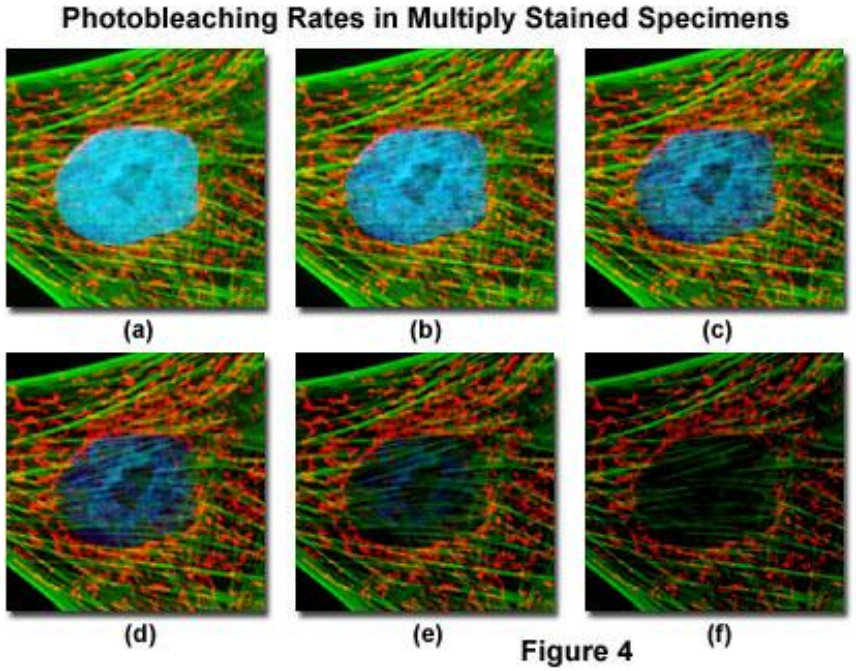
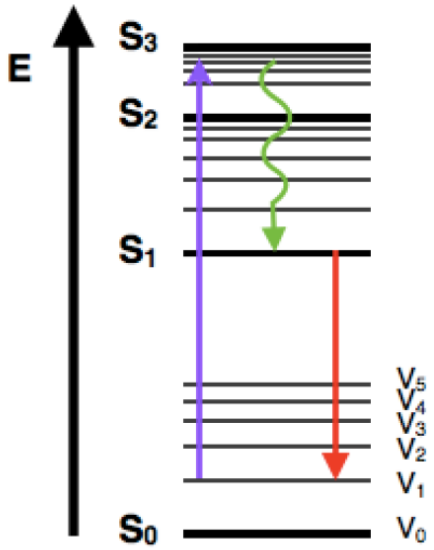
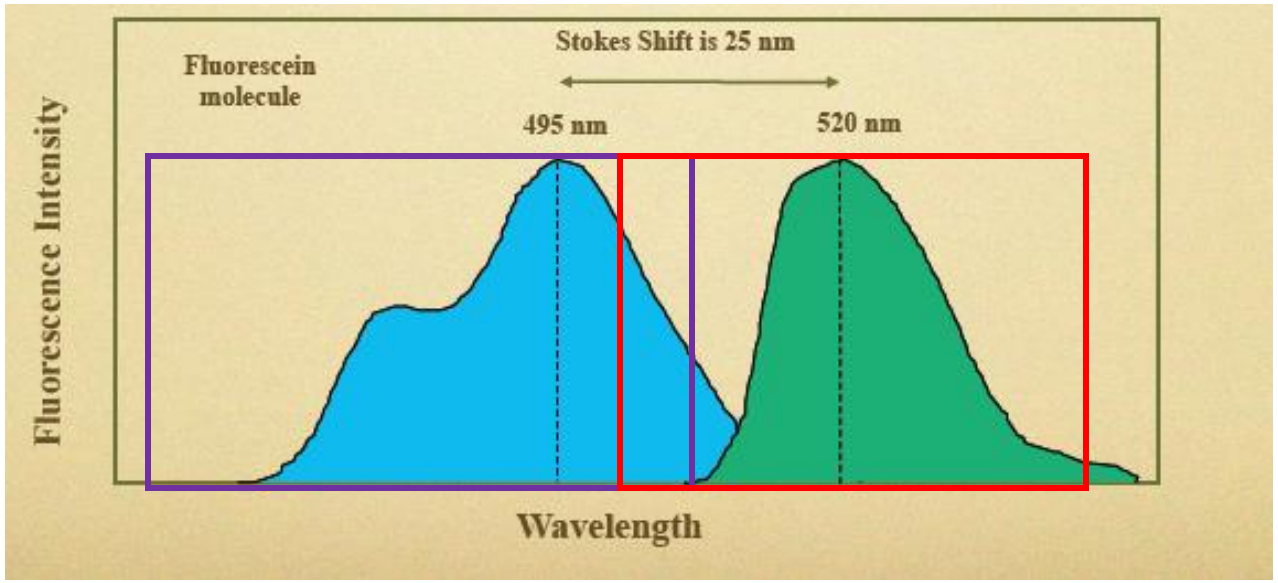


Figure 4

Properties of the fluorescence spectra

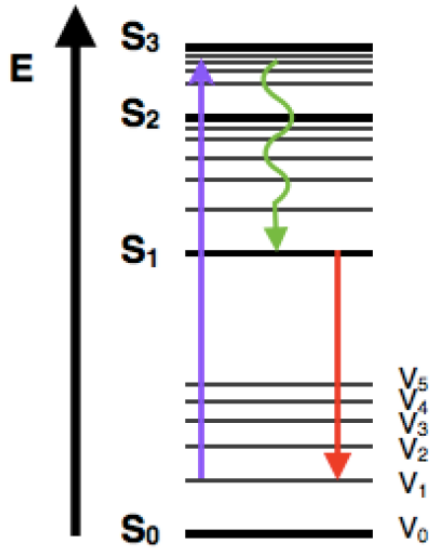


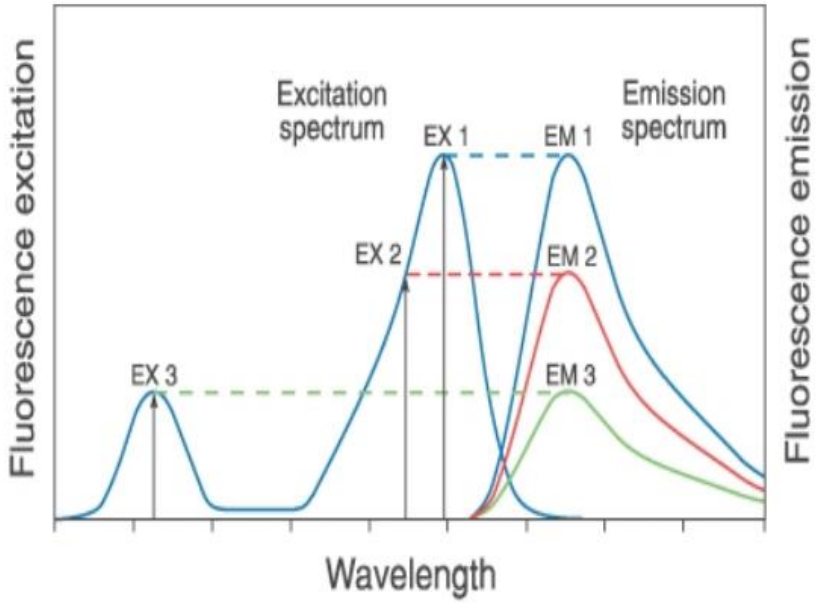


- For polyatomic molecules, single electron transition are represented by energy spectra called **excitation** and **emission** SPECTRA
- Due mainly to internal conversion: $E_{EM} < E_{EX}$
- **Stoke shift** is expressed as the wavelength difference between the maxima of S_0-->S_1 and S_1—>S_0 transitions

$$\lambda_{EM} - \lambda_{EX}$$

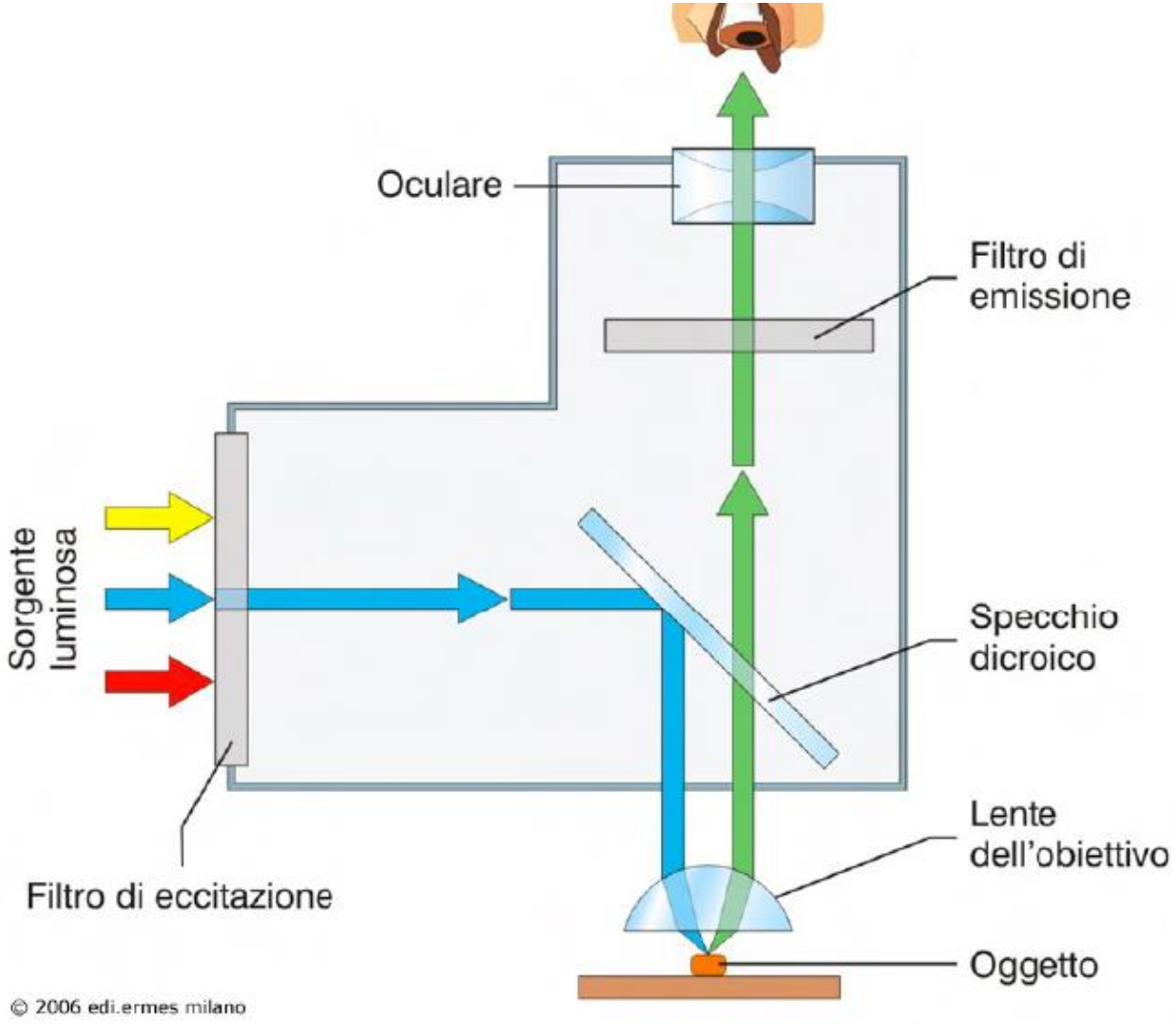
Properties of the fluorescence spectra





- Due to rapid internal conversion from higher electronic and vibrational states (completed much before emission), emission occurs solely from the lowest vibrational level of S_1, so….
- **Kasha's rule:** emission spectra and λ_{em} max are independent of excitation wavelength. The intensity of fluorescence emission depends from λ_{ex}
- **$Q = I_0 (2.3 \varepsilon bc) \Phi$**

Fluorescence microscope



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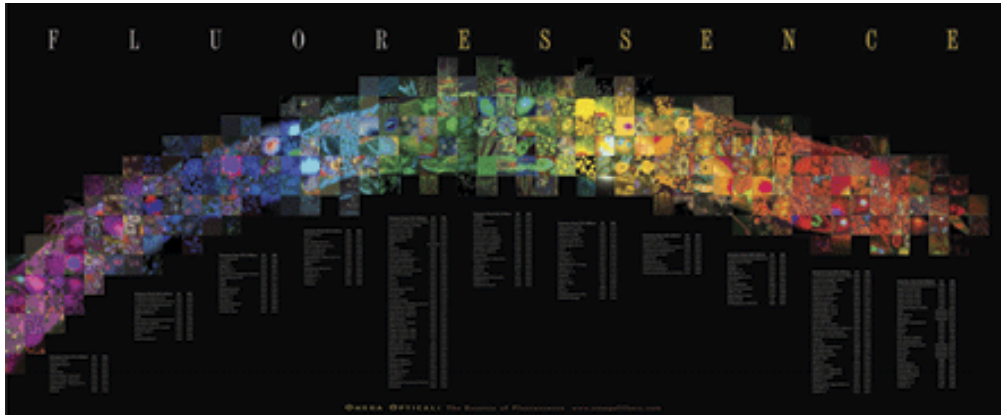
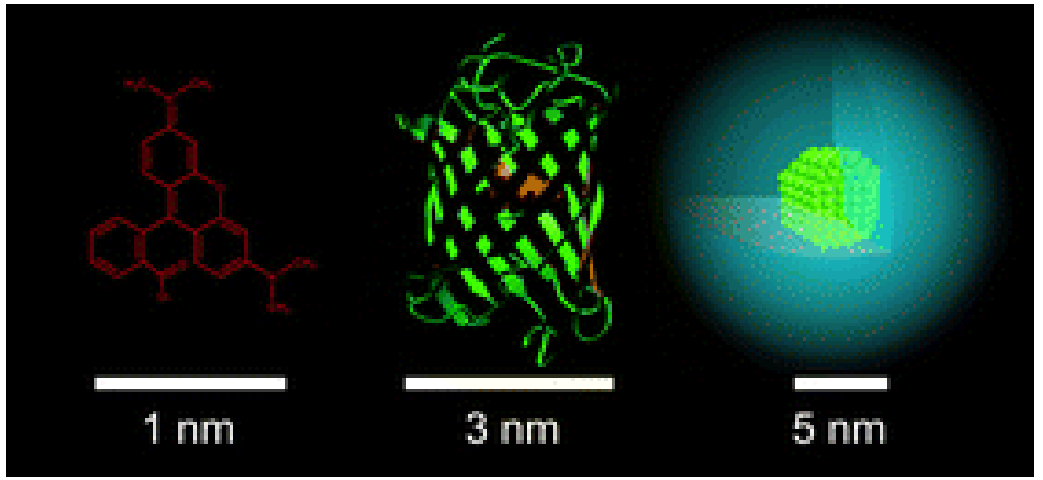
Fluorochromes

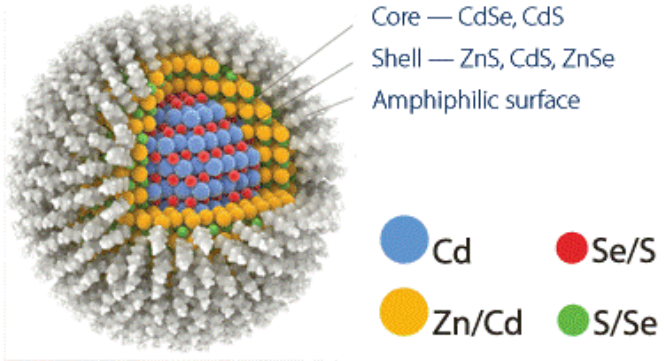


- **INORGANIC**
- **ORGANIC**
- **PROTEIN-BASED**



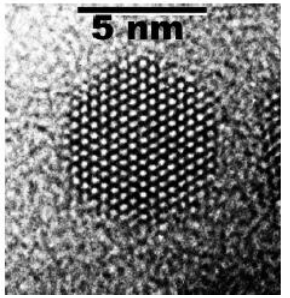
A. Burns et al., Chem. Rev. Soc., 35 (2006) 1028-1042

Semiconductors nanocrystals: QDOTS

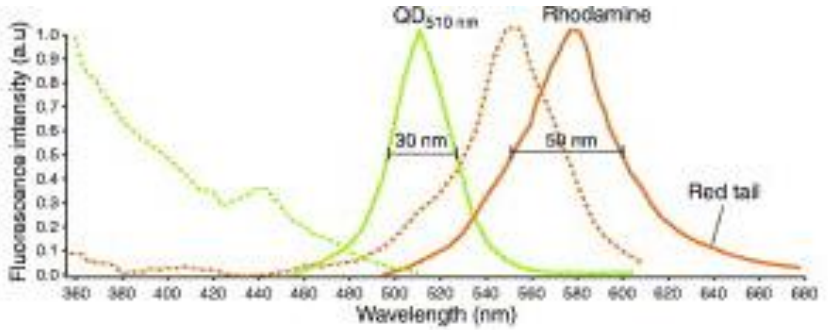


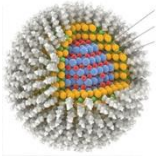
Nanocrystals composed of a core of a semiconductor material (CdSe), enclosed within a shell of another semiconductor (ZnS) that has a larger spectral band gap.

Typical diameter: 2-10 nm



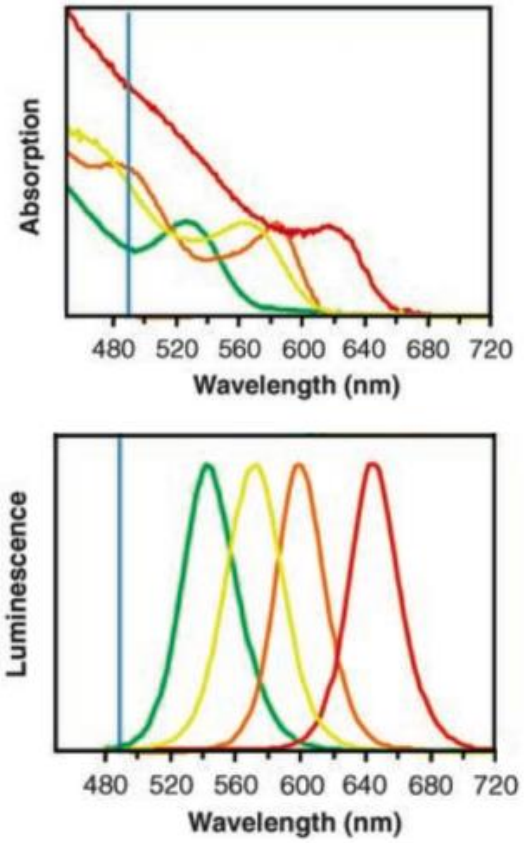
- Strong light absorbance
- Bright fluorescence
- Narrow symmetric emission bands
- High photostability

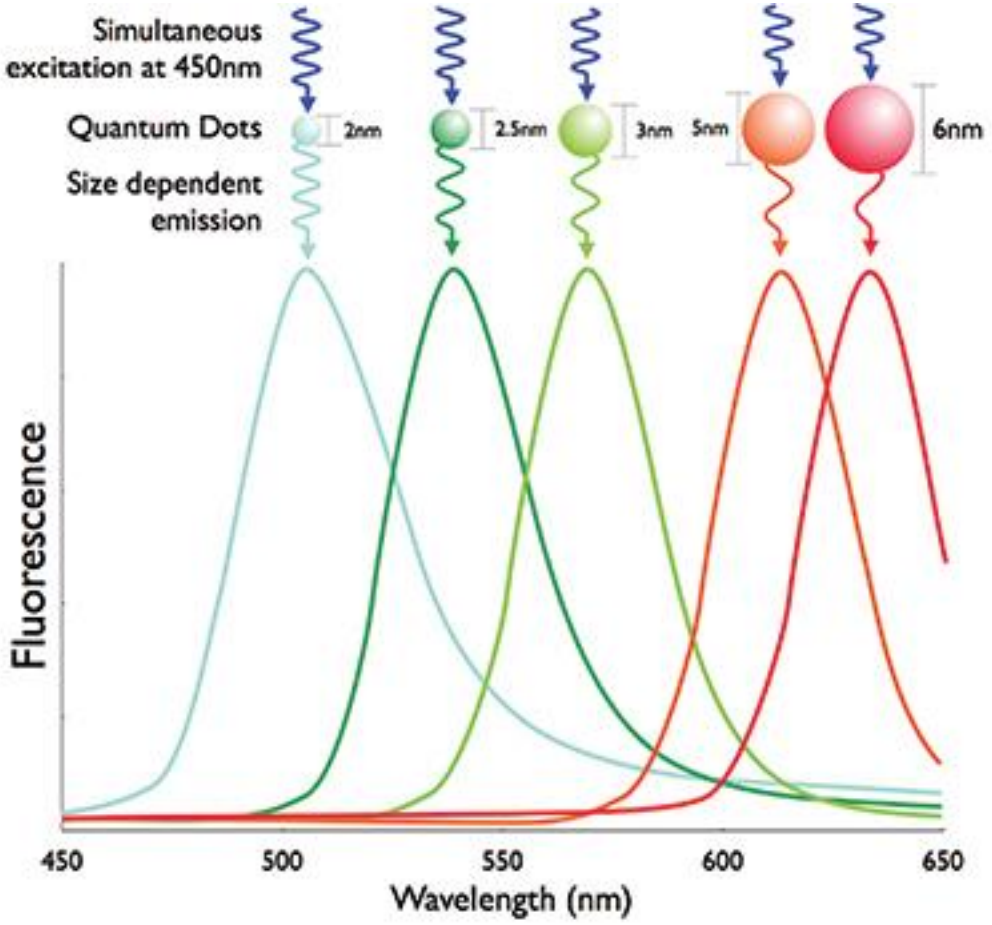


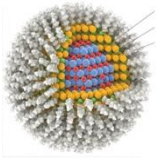


Semiconductors nanocrystals: QDOTS

- **<u>Size tunable emission</u>** = predictable relationship between the size of QD and its emission wavelength

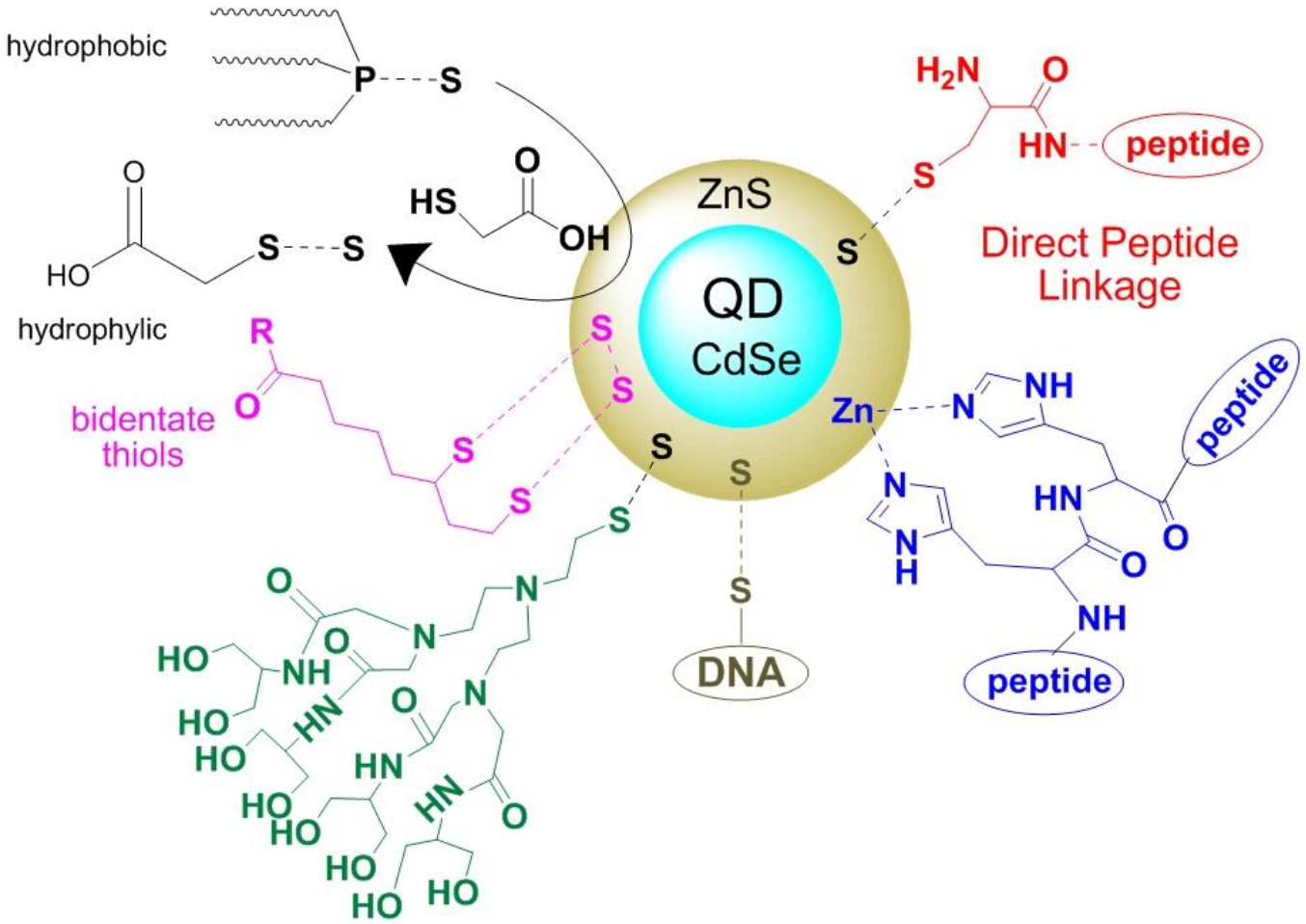


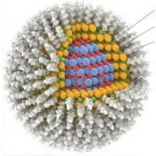




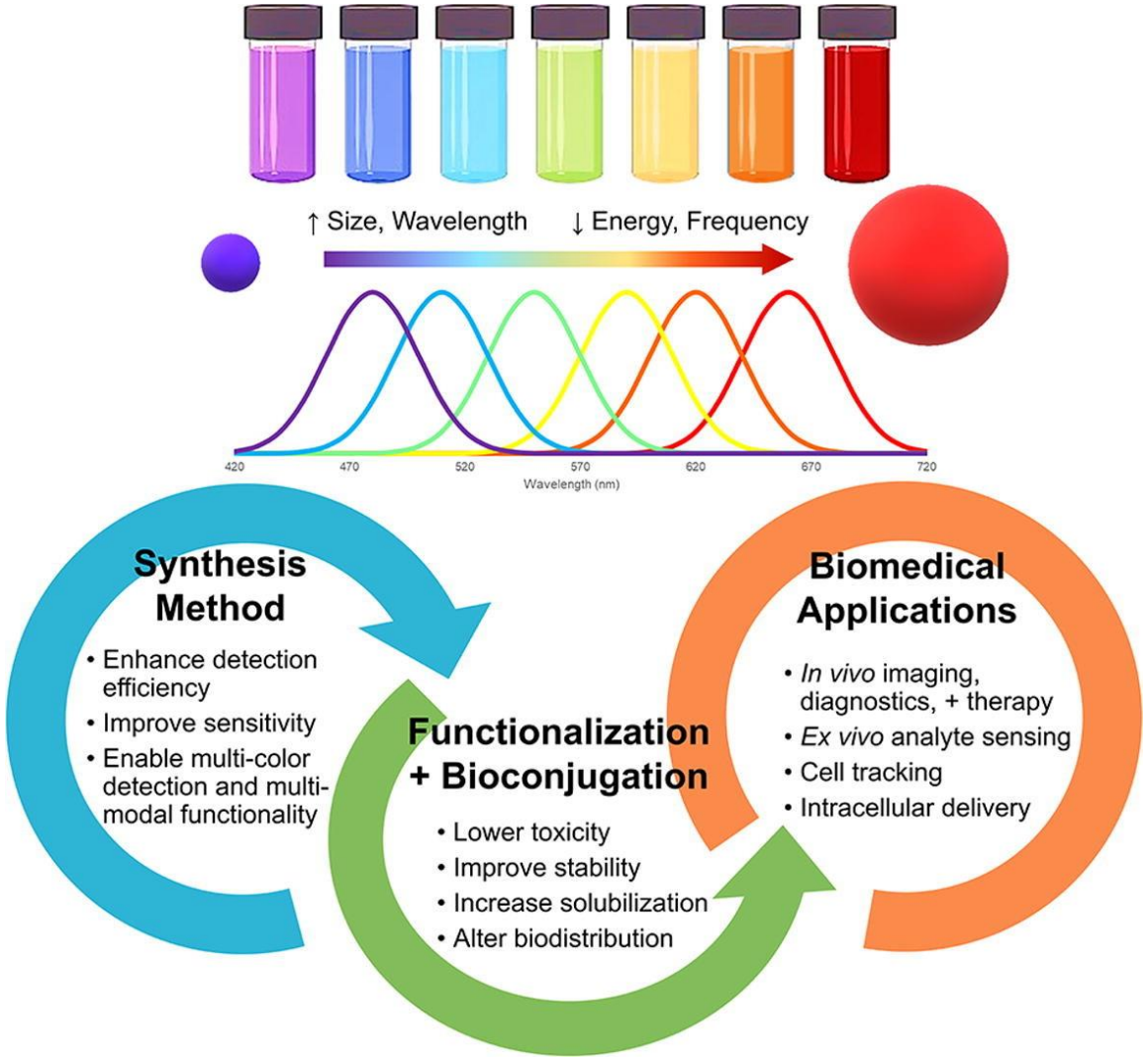
Semiconductors nanocrystals: QDOTS

- Excellent stability of optical properties upon conjugation to biomaterials



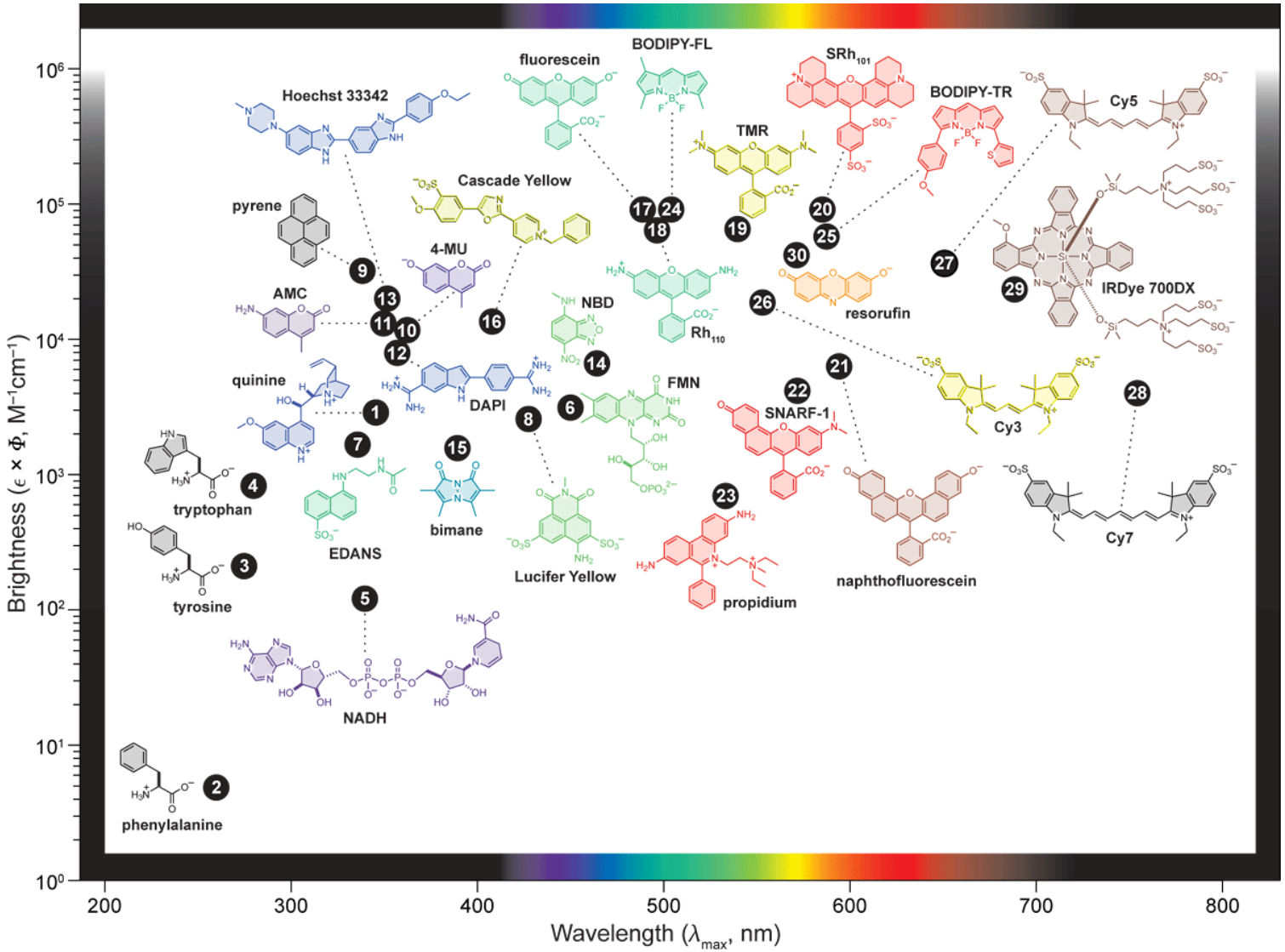


Semiconductors nanocrystals: QDOTS



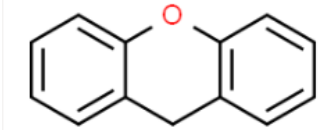
Wagner *et al.*, Acta Biomaterialia 94 (2019) 44–63

Organic fluorescent dyes

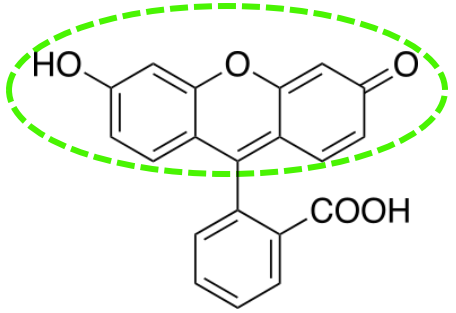


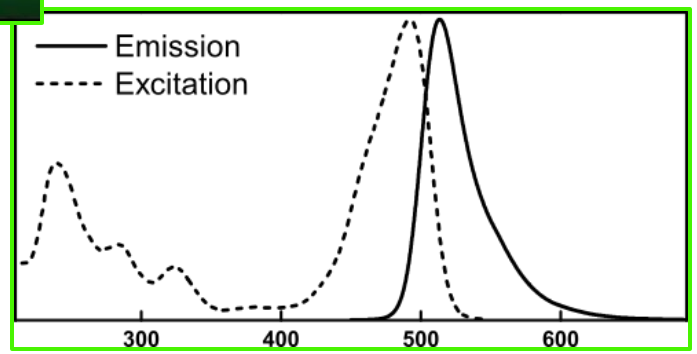
Organic fluorescent dyes

XANTHENE-BASED

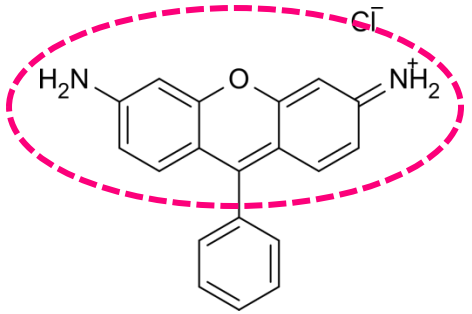


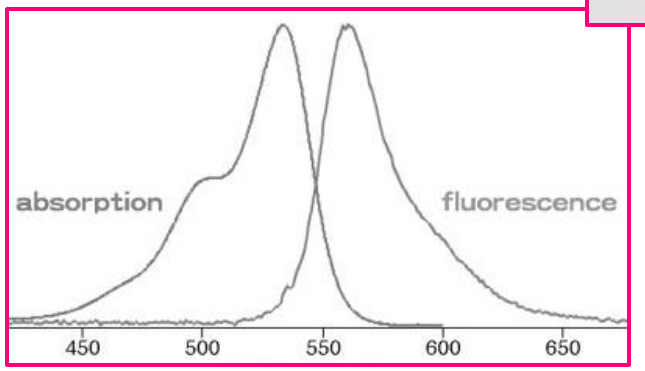
fluoresceine family





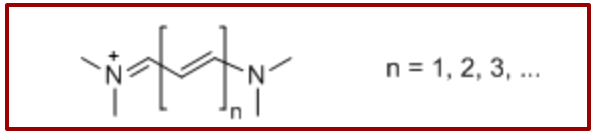
rhodamine family

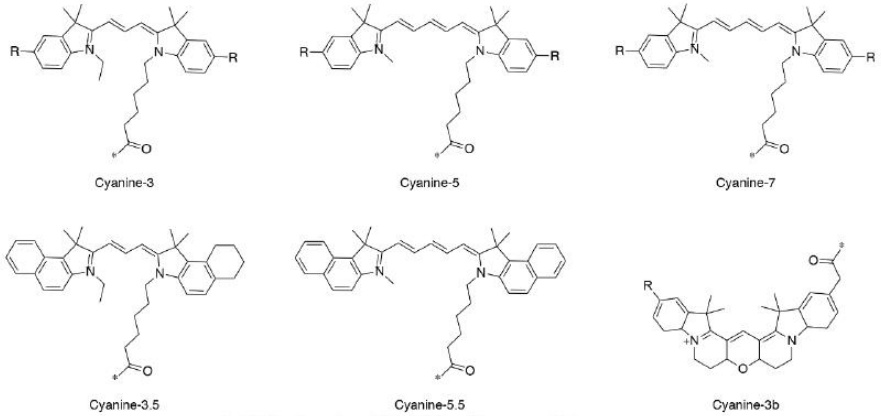


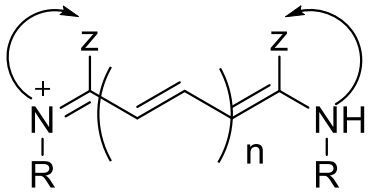


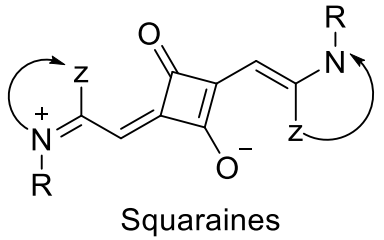
Organic fluorescent dyes

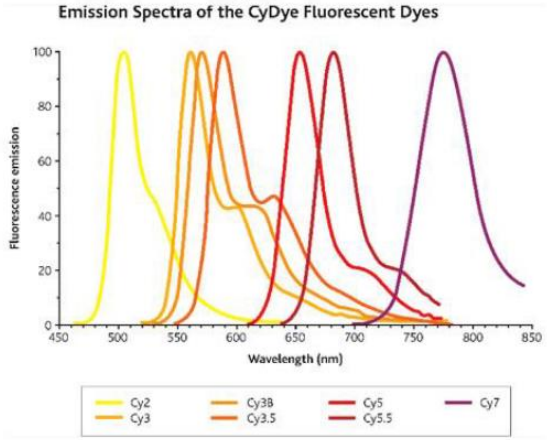
POLYMETHINIC BRIDGE-BASED





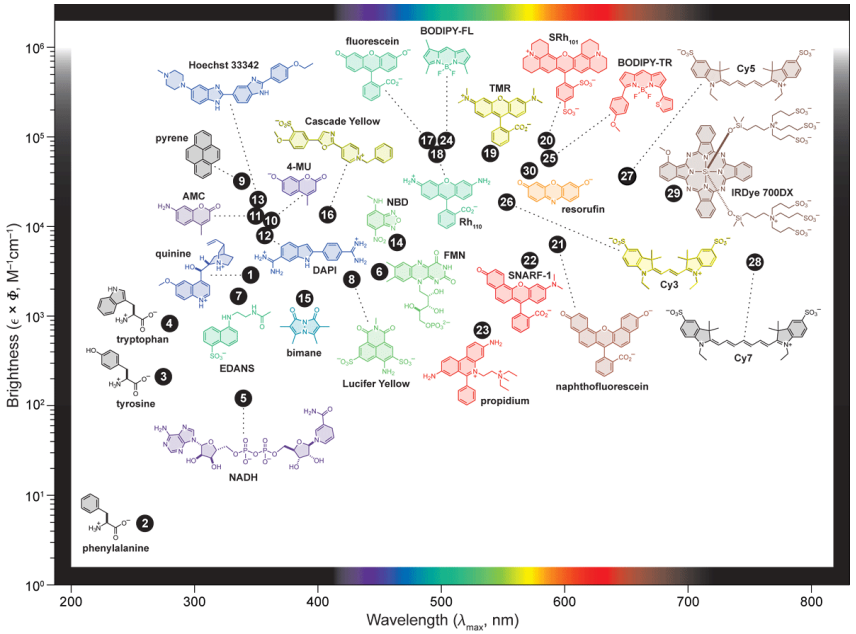






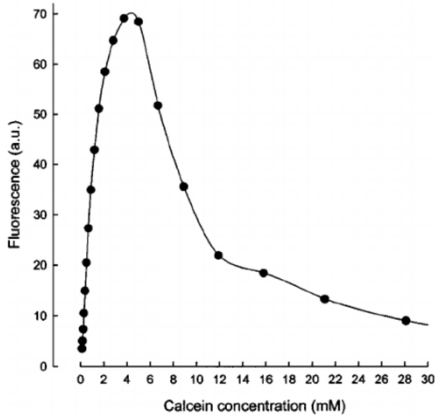
- 1. Excellent optical properties (high ε and ϕ)
- 2. High structural versatility
- 3. **Tunable spectral properties**
- 4. Wide application fields

Organic fluorescent dyes

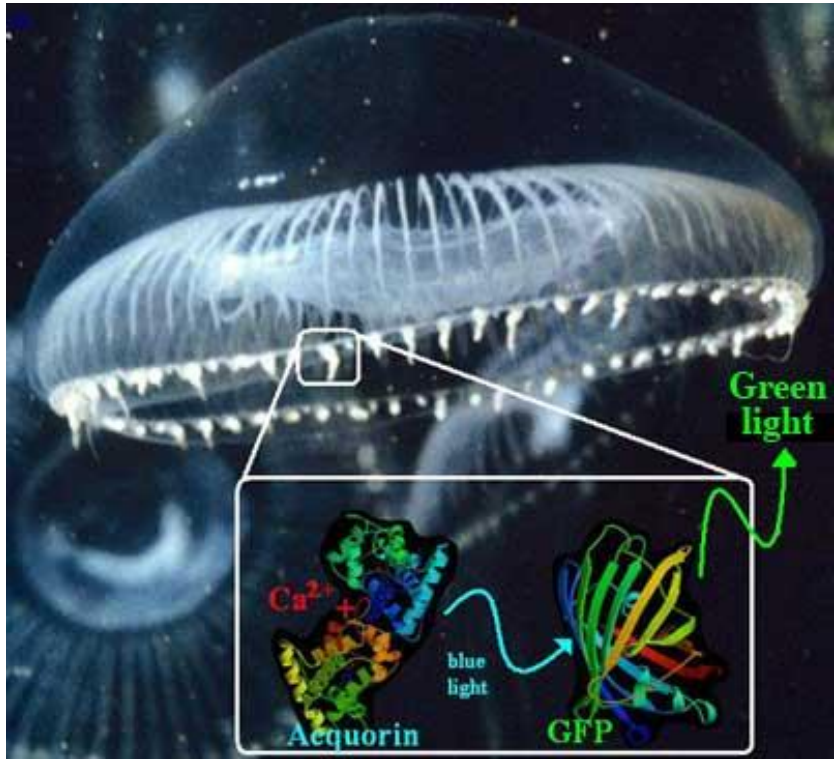


- Wide pool of molecules
- Huge amount of reference data in literature on their photophysical behaviour
- Versatility for bioconjugation

- Concentration quenching
- Limited photostability
- Limited control in cell targeting



Protein-based indicators



REVIEW ARTICLE www.rsc.org/ibiology | Integrative Biology

Nobel lecture: constructing and exploiting the fluorescent protein paintbox†

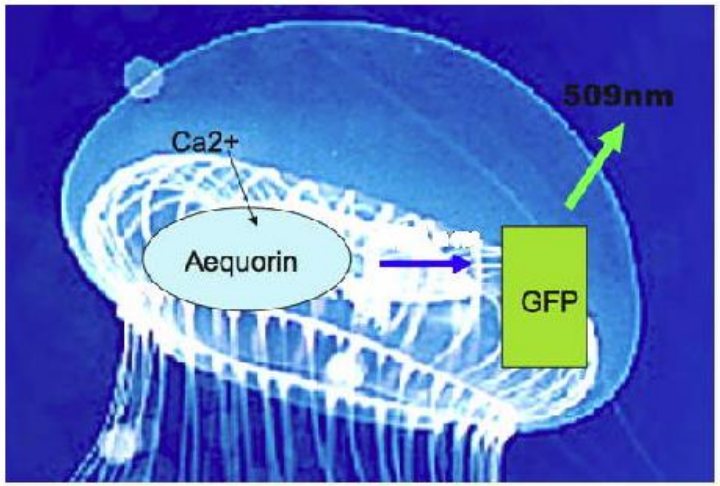
Roger Y. Tsien

Received 15th December 2009, Accepted 22nd December 2009
First published as an Advance Article on the web 22nd February 2010
DOI: 10.1039/b926500g

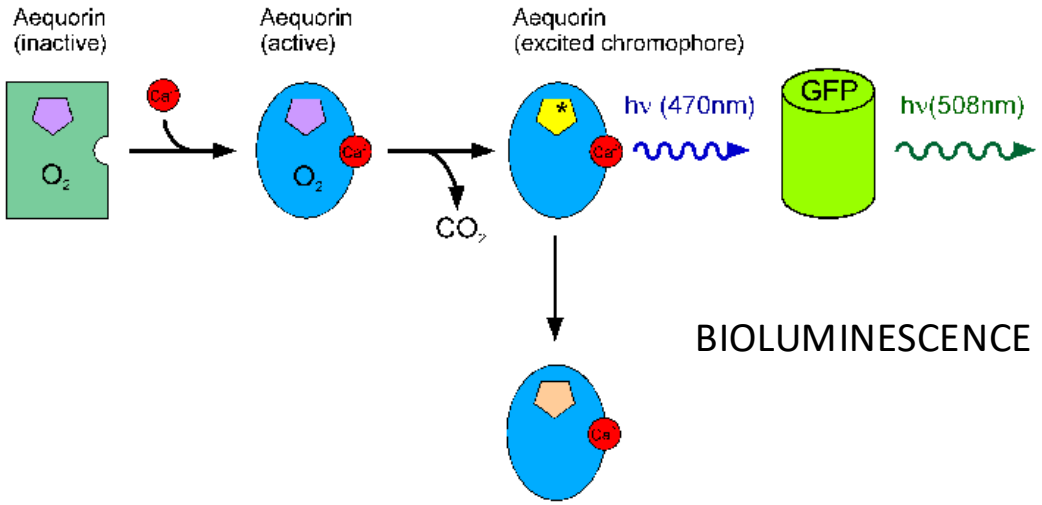
A written version of Professor Roger Tsien's Nobel Lecture.

- Synthetized from cell itself
- They can be designed to respond to a great variety of biological events and signals
- Allow to target different subcellular compartments and to easily detect and trace protein
- They can be introduced into a wider variety of tissues and intact organisms
- They very rarely cause photodynamic toxicity

Aequorin and GFP from *Aequoria Victoria*



Upon binding calcium, aequorin generates an electronically excited product that undergoes radiationless energy transfer (blue arrow) to the GFP fluorescent state, which emits the green light (509 nm)



GFP-derived proteins

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Motivation

Meanwhile fluorescence imaging of the second messenger cAMP (cyclic adenosine 3′,5′-monophosphate) had become one of my main research goals by 1988. I reasoned that the best way to create a fluorescent sensor to detect cAMP with the necessary affinity and selectivity inside cells would be to hijack a natural cAMP-binding protein. After much consideration of the various candidates known at the time, I chose cAMP-dependent protein kinase, now more commonly abbreviated PKA. PKA contains two types of subunits, regulatory and catalytic. In the absence of cAMP, the regulatory subunits tightly bind and inhibit the catalytic subunits. When cAMP becomes available, it binds to the regulatory subunits, which then let go of the catalytic subunits, which in turn start transferring phosphate groups from ATP onto selected proteins.[5–7] But how could activation of PKA by cAMP be made directly visible inside a single living cell? From my graduate student days I had been fascinated by a biophysical phenomenon called fluorescence resonance energy transfer (FRET), in which one excited dye molecule can transfer its energy to a close neighbor, much as a football or basketball player can pass the ball to a teammate with diminishing probability of success the greater the distance between the players. If we could attach one type of dye molecule to the regulatory subunits and the other type of dye molecule to the catalytic subunits, FRET would be possible in intact PKA, because the subunits are in intimate contact. But once cAMP had broken up the PKA complex and allowed the subunits to drift apart, FRET would be disrupted and a change in fluorescence color should be observable.

GFP-derived proteins

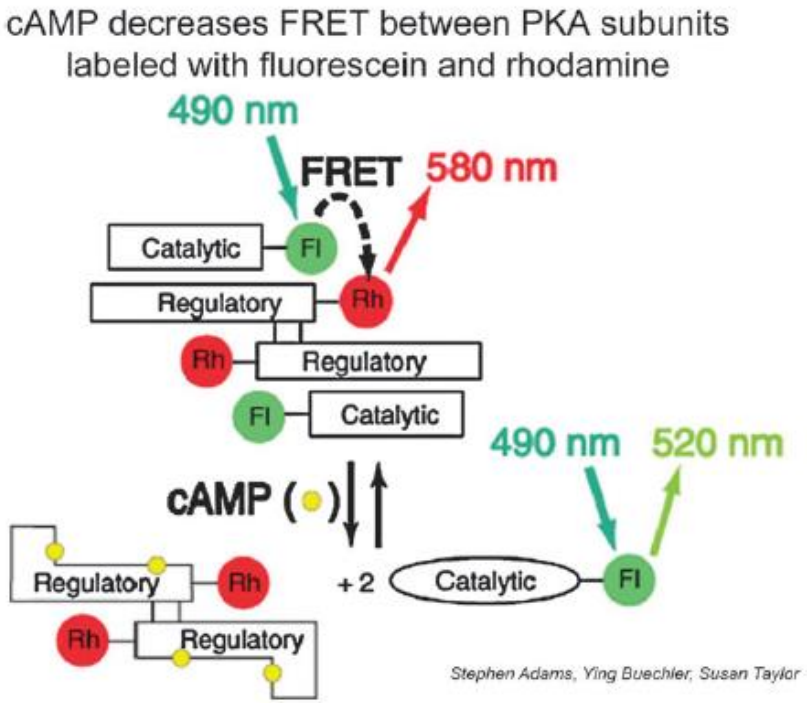

Fig. 1 Schematic cartoon showing how cAMP-induced dissociation of regulatory from catalytic subunits of protein kinase A (PKA) can be reported by loss of FRET from fluorescein to rhodamine labels.

Tsien, Integr. Biol., 2 2010 77-93

Although the cAMP sensor was moderately successful, the general approach would have been very difficult to extend to other proteins because it required high level expression and purification of soluble proteins or subunits, controlled attachment of two different dyes *in vitro* to distinct domains or subunits without destroying the function of the protein, repurification, and microinjection back into living cells. Such cells had to be large and robust enough to tolerate poking with a hollow glass needle, and the experimenter had to be patient and dexterous, unlike me. All of the above obstacles could be circumvented if we had genes encoding two fluorescent proteins of the appropriate colors. These genes could be fused to the genes for the protein(s) of interest. One would still have to get the fusion genes into the cell(s) to be studied, but standard methodology has been worked out for most cells of interest. Introducing genes into cells (transfection) is generally much easier than introducing proteins, because each cell needs only one or a few copies of DNA (compared to billions of molecules of protein), the cell has plenty of time to recover from any membrane damage, and one can selectively propagate those cells that have successfully assimilated the DNA. Once in the cells, these genes would hopefully make composite proteins *in situ* that would both fluoresce and preserve native biological function.

GFP-derived proteins

Prasher *et al* (1992) clone GFP

Gene, 111 (1992) 229–233

GENE 06296

Primary structure of the *Aequorea victoria* green-fluorescent protein

(Bioluminescence; Cnidaria; aequorin; energy transfer; chromophore; cloning)

Douglas C. Prasher[a], Virginia K. Eckenrode[b], William W. Ward[c], Frank G. Prendergast[d] and Milton J. Cormier[b]

SUMMARY

Many cnidarians utilize green-fluorescent proteins (GFPs) as energy-transfer acceptors in bioluminescence. GFPs fluoresce in vivo upon receiving energy from either a luciferase-oxyluciferin excited-state complex or a Ca^{2+}-activated photoprotein. These highly fluorescent proteins are unique due to the chemical nature of their chromophore, which is comprised of modified amino acid (aa) residues within the polypeptide. This report describes the cloning and sequencing of both cDNA and genomic clones of GFP from the cnidarian, *Aequorea victoria*. The *gfp10* cDNA encodes a 238-aa-residue polypeptide with a calculated M_r of 26888. Comparison of *A. victoria* GFP genomic clones shows three different restriction enzyme patterns which suggests that at least three different genes are present in the *A. victoria* population at Friday Harbor, Washington. The *gfp* gene encoded by the λGFP2 genomic clone is comprised of at least three exons spread over 2.6 kb. The nucleotide sequences of the cDNA and the gene will aid in the elucidation of structure-function relationships in this unique class of proteins.

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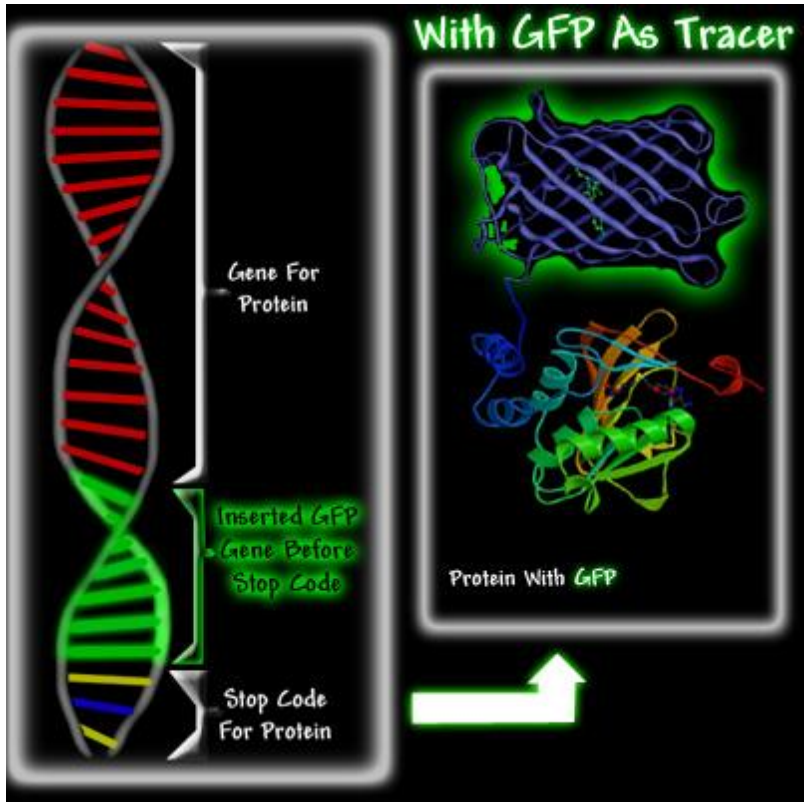
Supported in part by a Mellon Award from the Woods Hole Oceanographic Institution (27/50.44) and a grant from the American Cancer Society (NP640) to D.C.P.



GFP-derived proteins

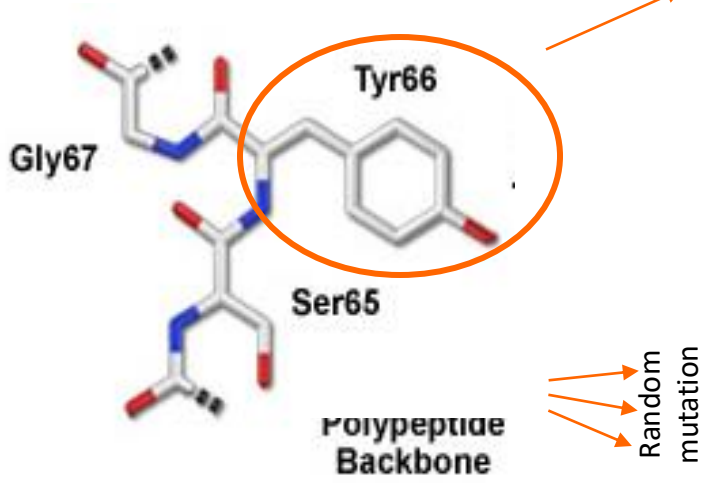
Why GFP?

- GFP is a fairly **small protein**. This is important because a small protein attached to the protein of interest is less likely to hinder its proper function. Its small size would also allow it to follow the fused protein, especially in organelles like the nucleus, whereas the diffusion of large proteins would be difficult.
- **Once GFP is made in the jellyfish, it is fluorescent**. Most other bioluminescent molecules require the addition of other substances before they glow. For example, aequorin will glow only if calcium ions and coelenterazine have been added, and firefly luciferase requires ATP, magnesium, and luciferin before it luminesces. This would make GFP a much more versatile tracer than either aequorin or firefly luciferase, which were being used as tracers.

GFP-derived proteins

Initial experiments

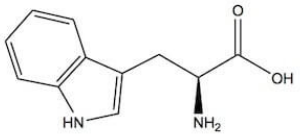
Unfortunately, the GFP fluorescence signals were very weak and highly variable from cell to cell. This confirmed that wild-type GFP was too unreliable,



Random mutation

+ additional mutations

Trp66



No fluorescence

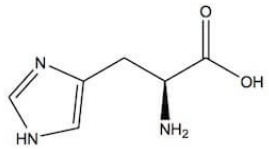
CFP

↑ useful

↑ bleach-resistant

+ additional mutations

His66



BFP

↑ greater performance

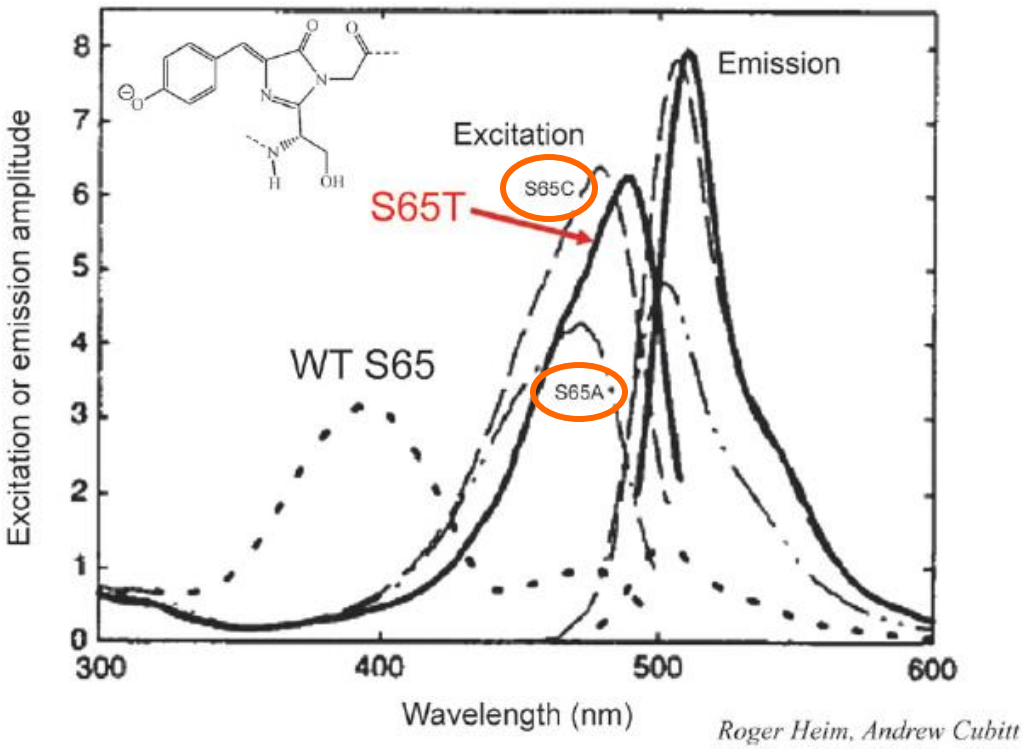
GFP-derived proteins

Next goal was for Tsien to create a **GFP excited by blue light**.

We still had one other big problem before we could use FRET from the blue mutant ("BFP") to GFP to sense protein conformational changes or protein-protein interactions. Ideally, GFP should be excitable only by the same blue wavelengths as BFP emits. Then irradiation with ultraviolet (UV) light to selectively excite BFP would either give blue emission in the absence of FRET, or transfer the energy to GFP to glow green. However, the original GFP was more strongly excited by UV than by blue, so that GFP was a very poor acceptor of FRET from BFP. Why did GFP have two excitation peaks, one big one in the UV and a much smaller one in the blue? I hypothesized that the major UV peak was

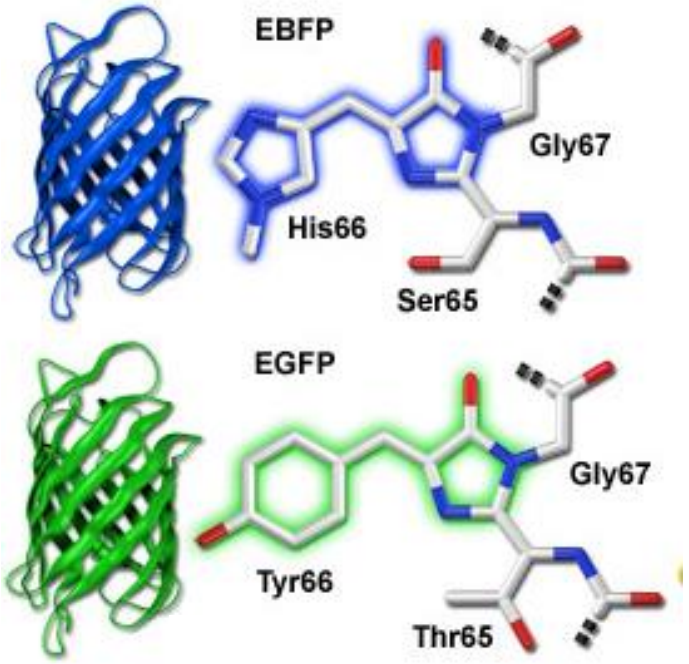
Mutation of **Ser65** improve excitation spectra

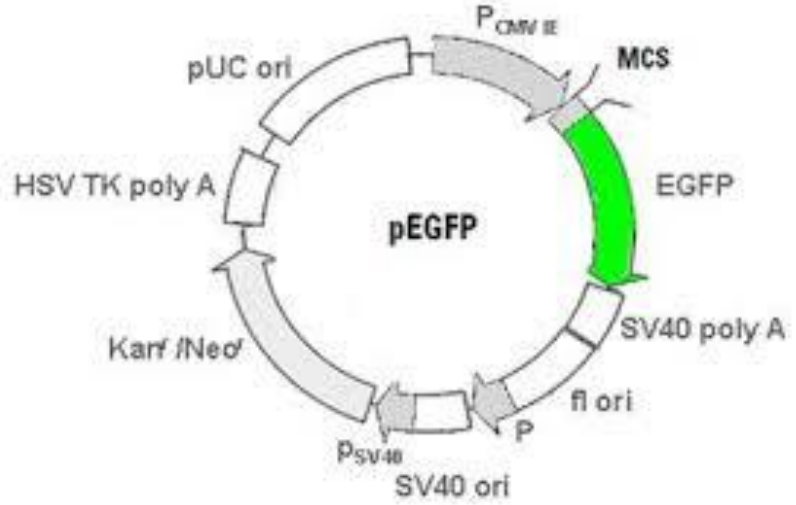
We therefore suggested this mutant, "S65T", as a general improvement on wild-type GFP



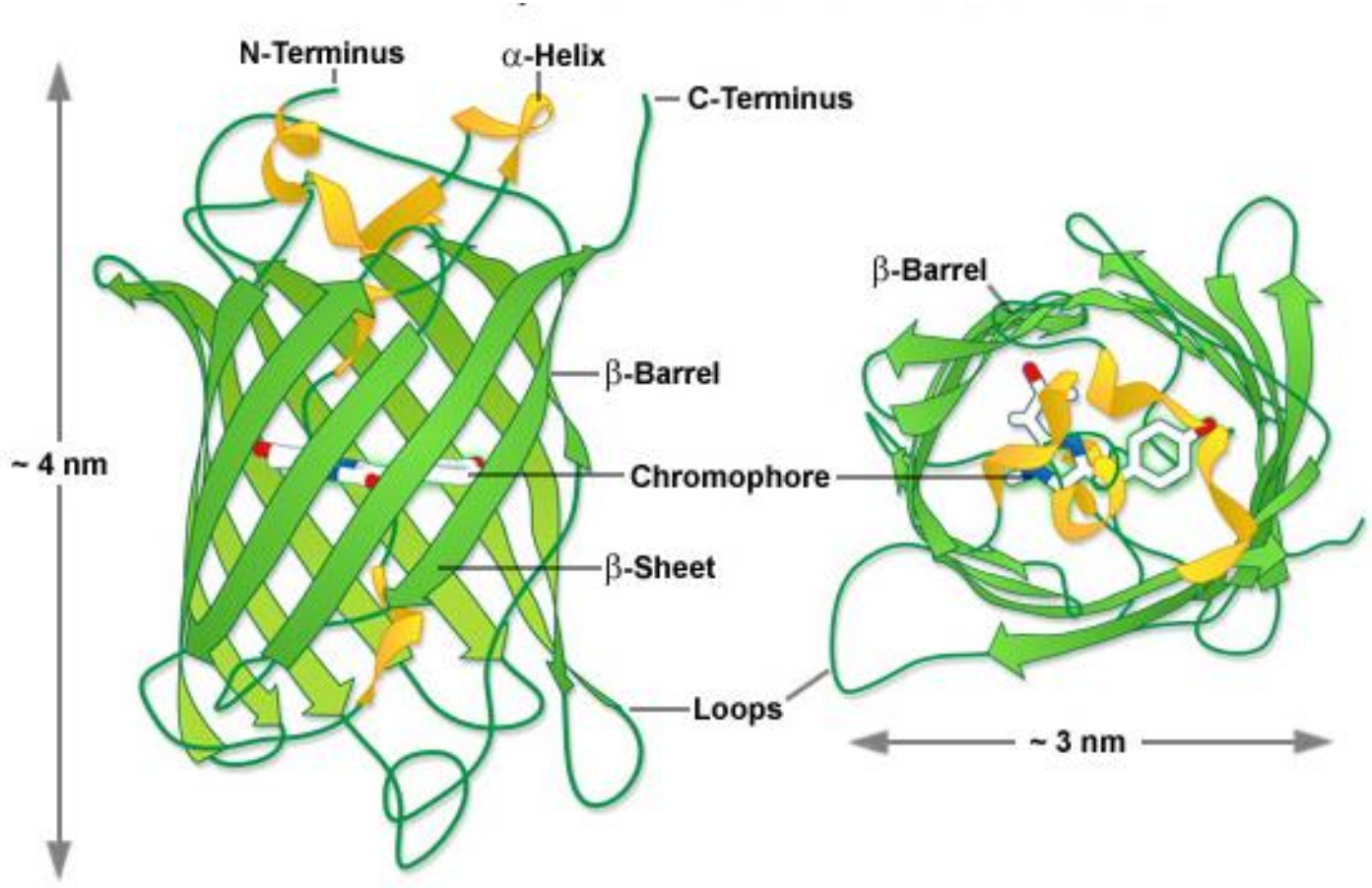
GFP-derived proteins

In a random screen for optimal mutations of residues 55 to 74, Cormack *et al*. confirmed the value of S65T and added another mutation, **F64L, which permits folding at warmer temperatures**. The resulting double mutant, ‘‘**enhanced GFP**’’, was aggressively marketed by Clontech, a molecular biology supply company, and became the basis for most subsequent applications of GFP, even when these two mutations are not explicitly acknowledged.





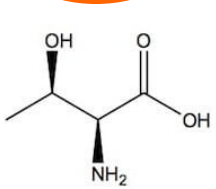
GFP S65T structure

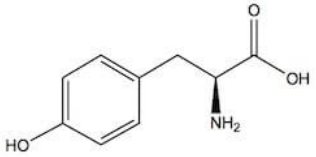


1996

GFP-derived proteins

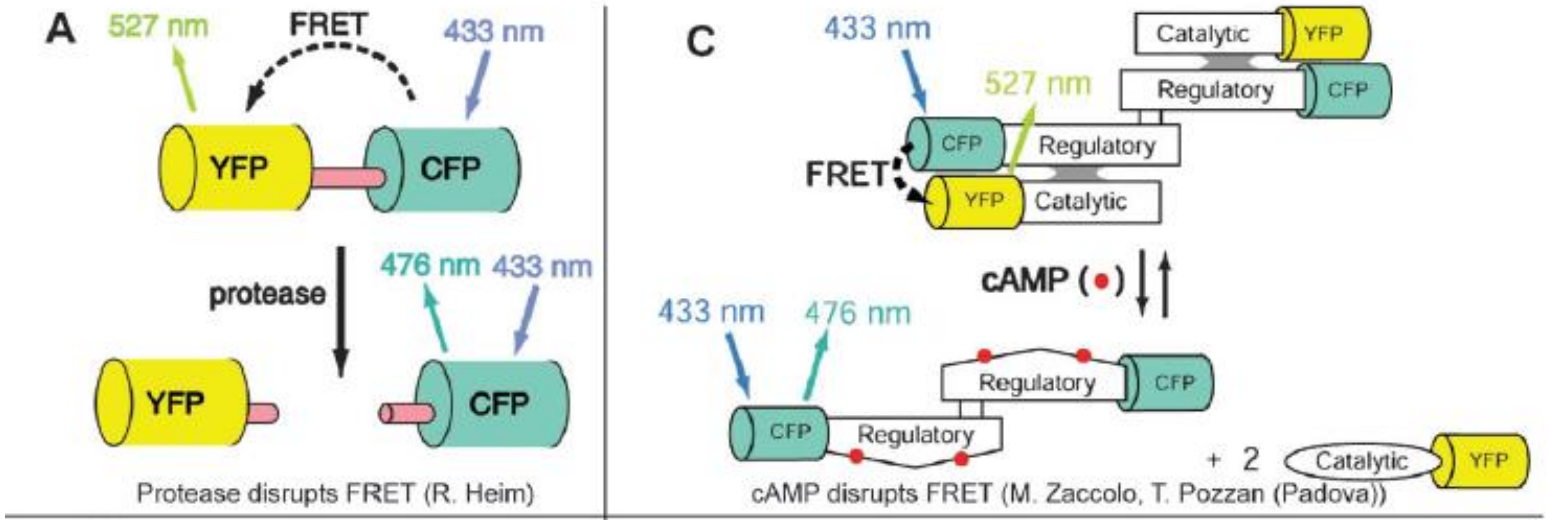
Thr203 → **Tyr203** ⇒ **YFP**



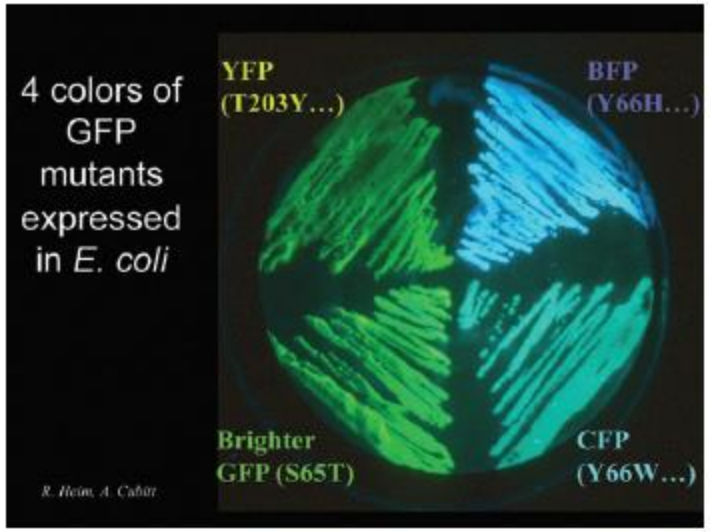


These shifts made YFP a good FRET acceptor from CFP. We were glad to have the CFP/YFP pair to replace our previous BFP/GFP combination, because BFP was too easy to bleach and required potentially injurious UV excitation.

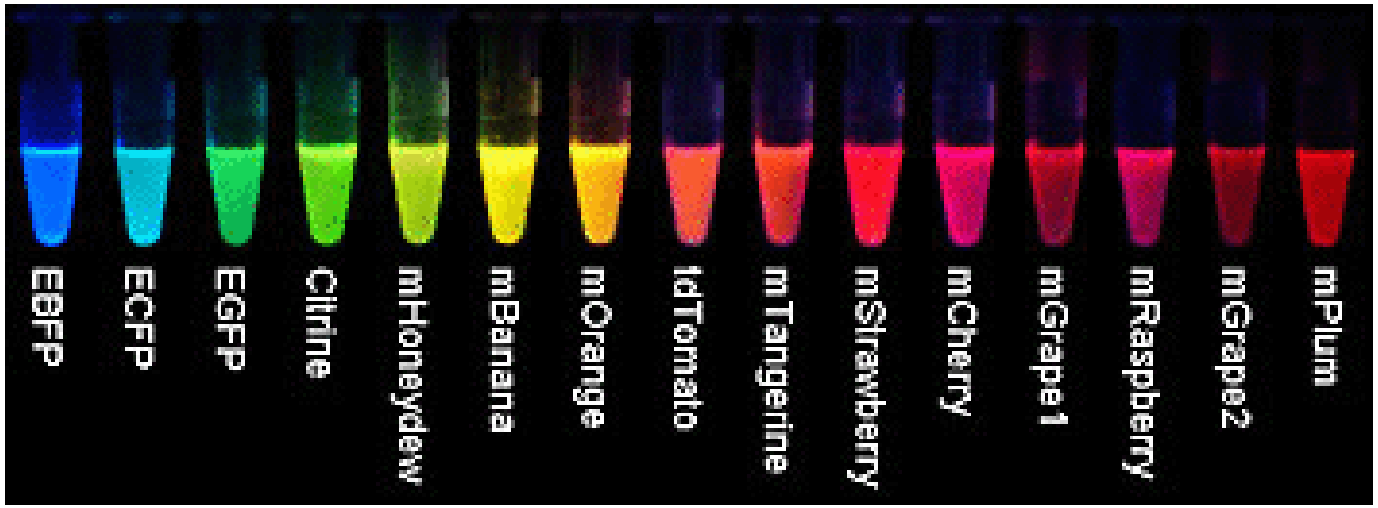
Examples of genetically encoded FRET sensors

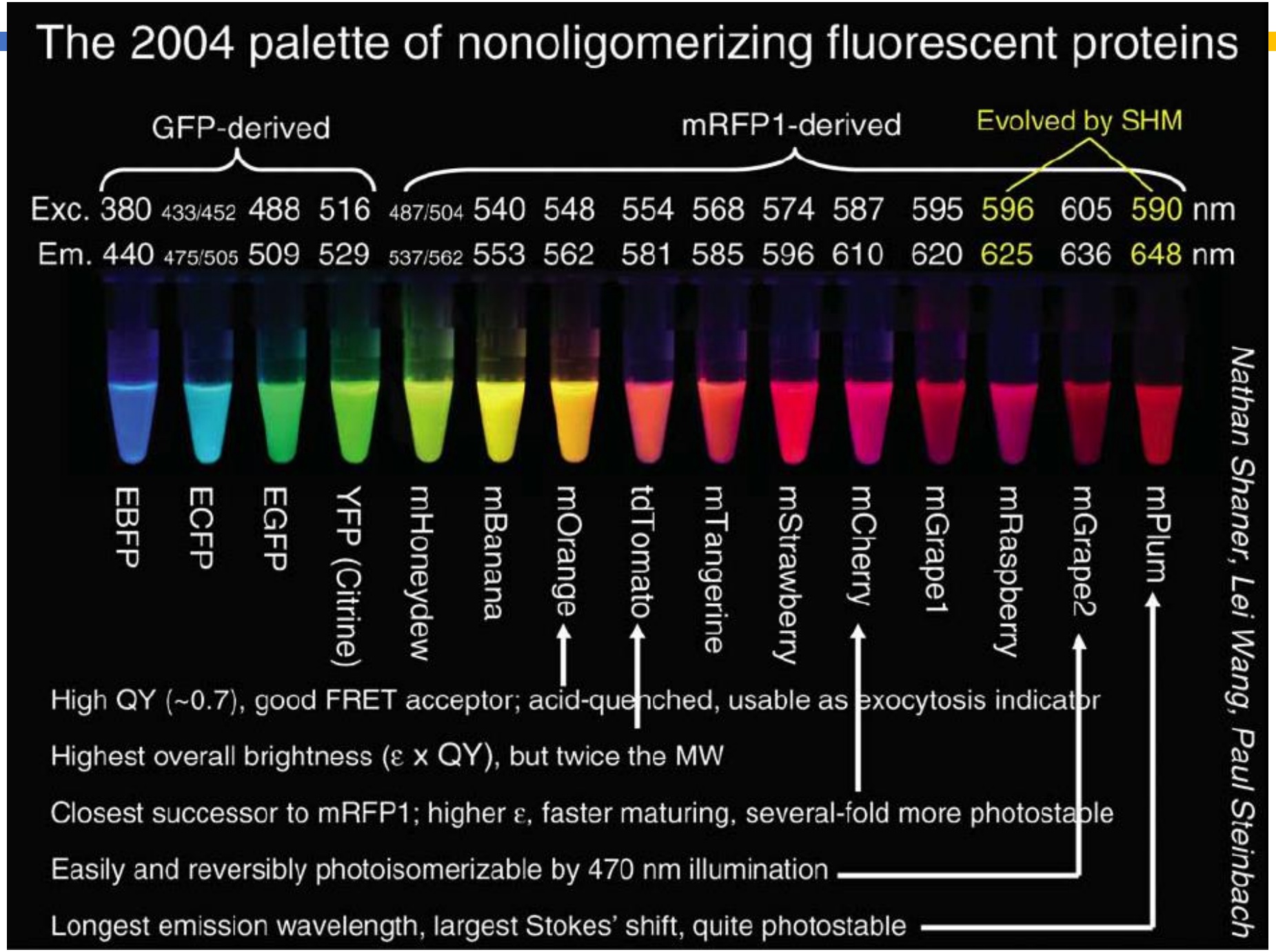


GFP-derived proteins



Roger Tsien is responsible for much of our understanding of how GFP works and for developing new techniques and mutants of GFP. His group has developed mutants that start fluorescing fast than wild type GFP, that are brighter and have different colors (see below, the E stands for enhanced versions of GFP, m are monomeric proteins and tdTomato is a head-to-tail dimer).




The 2004 palette of nonoligomerizing fluorescent proteins
GFP-derived
mRFP1-derived
Evolved by SHM
Exc. 380 433/452 488 516 487/504 540 548 554 568 574 587 595 596 605 590 nm
Em. 440 475/505 509 529 537/562 553 562 581 585 596 610 620 625 636 648 nm
EBFP
ECFP
EGFP
YFP (Citrine)
mHoneydew
mBanana
mOrange
tdTomato
mTangerine
mStrawberry
mCherry
mGrape1
mRaspberry
mGrape2
mPlum
High QY (~0.7), good FRET acceptor; acid-quenched, usable as exocytosis indicator
Highest overall brightness (ε x QY), but twice the MW
Closest successor to mRFP1; higher ε, faster maturing, several-fold more photostable
Easily and reversibly photoisomerizable by 470 nm illumination
Longest emission wavelength, largest Stokes' shift, quite photostable
Nathan Shaner, Lei Wang, Paul Steinbach

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Thank you