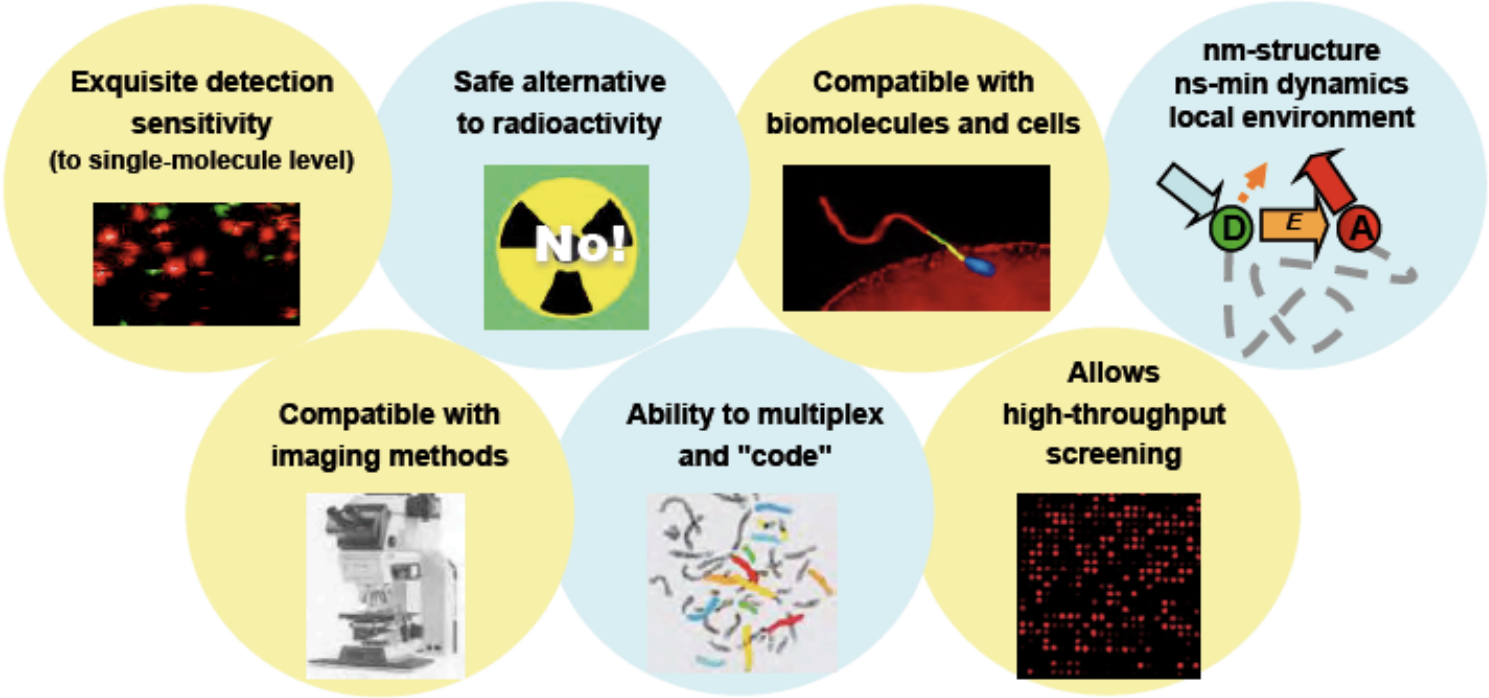
FLUORESCENCE

Introduction to Fluorescence
Fluorochromes
Techniques in fluorescence for live cell imaging

Advantages of fluorescence



1933-2008: 175 years of fluorescence

From ensembles…

1833 First description of fluorescence (Brewster, chlorophyll)

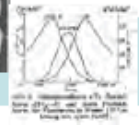
1849 First publication on fluorescence (Herschel, quinine)

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

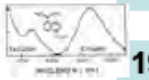
1930's Perrin observes FRET

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

…to single molecules!

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

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

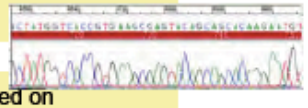
1989 First single-fluorophore observation at cryogenic T (Moerner) (1 fluorophore)

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

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

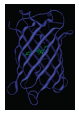
1996 First FRET observation at single-molecule level (Ha *et al.*)

2000 Single-fluorophore in single-cells (Schutz)



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

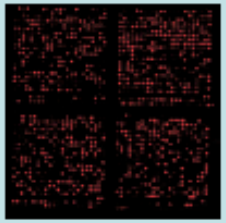
2008 **Nobel prize in chemistry to O.SHIMOMURA, M. CHALFIE, and R Y TSIEN** for the discovery and development of the green fluorescent protein, GFP



Fluorescence applications in biology

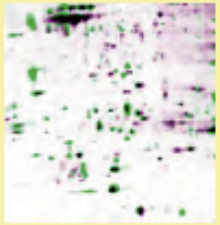
Genomics

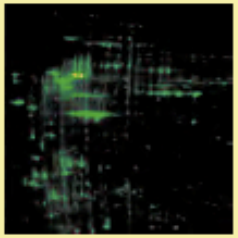
- genome sequencing
- real-time PCR
- DNA arrays



Proteomics

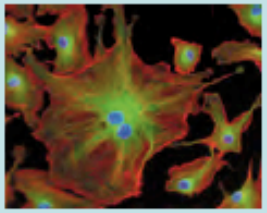
- protein arrays
- protein interactions
- protein expression profile





"Cellulomics"

- in-situ hybridization
- protein localization
- protein movement



Interactions

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

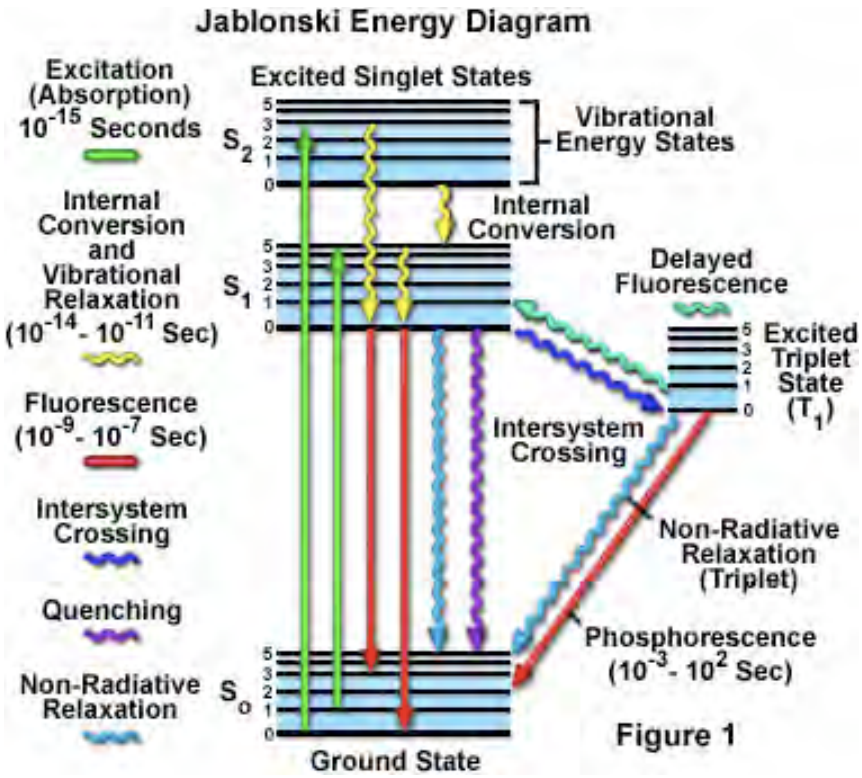
JABLONSKI DIAGRAMS

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

Excitation: 1 photon with energy hvEX excite the electrons of the fluorochrome that from S0 jump to excited singlet S1 o S2. The excited state exist for a definite time (nanoseconds) in which the fluorochrome undergoes into conformational changes and interact with the molecular environment.

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**

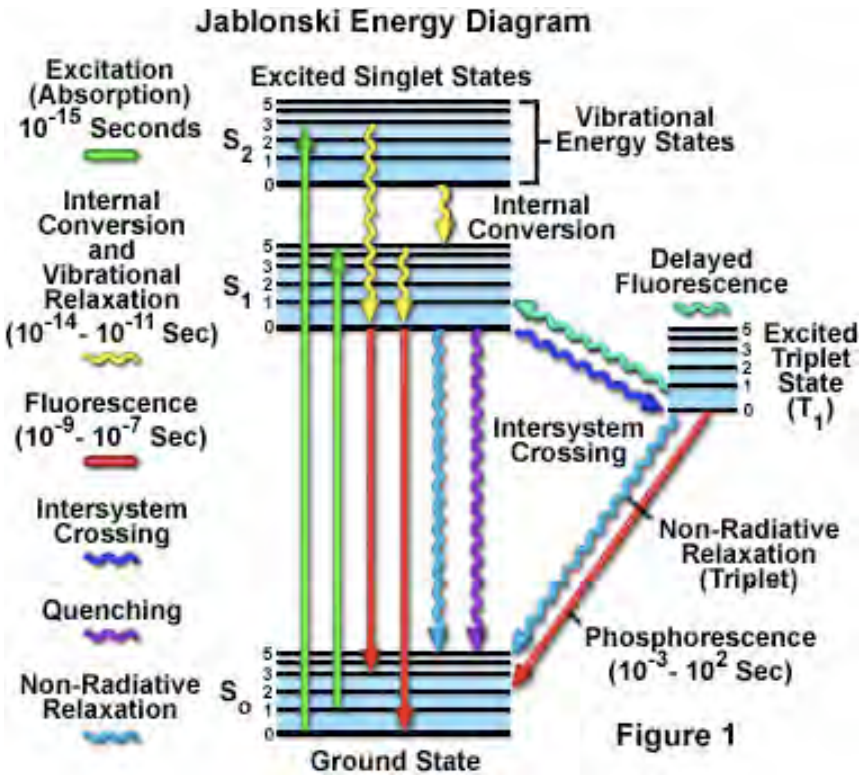
Emission. If the excited fluorochrome return to S0 from S1 emitting photons, the process is called fluorescence. Due to the energy dissipation during the internal conversion, the emission energy hvEM will be lower as compared with hvEX, and therefore λEX < λEM.



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

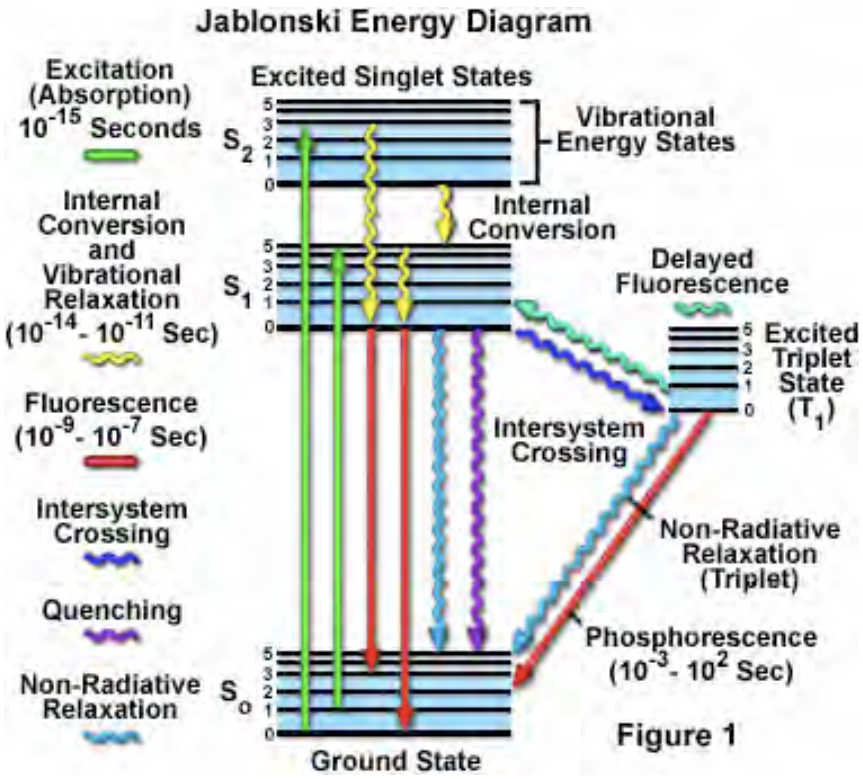
not all the molecules initially excited by absorption return to the ground state (S_0) by fluorescence emission. Other processes such as **intersystem crossing**, **collisional quenching**, **fluorescence resonance energy transfer (FRET)** may also depopulate S_1.

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.



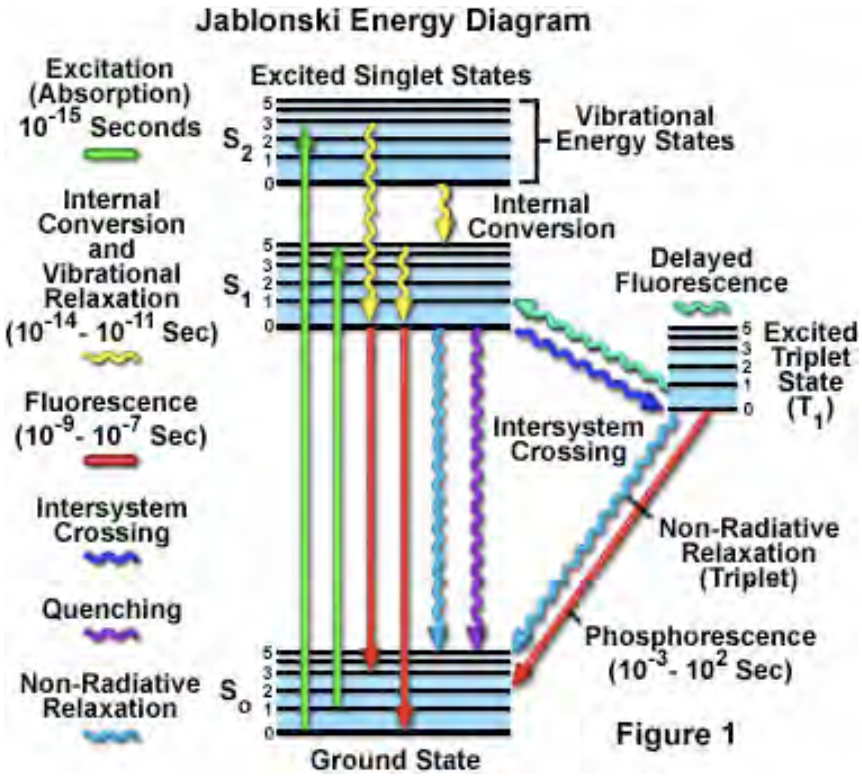
Intersystem crossing: phosphorescence and delayed fluorescence

Molecule relaxation to the lowest triplet state that ultimately results either in emission of a photon through phosphorescence or a transition back to the excited singlet state that yields delayed fluorescence



Collisional quenching

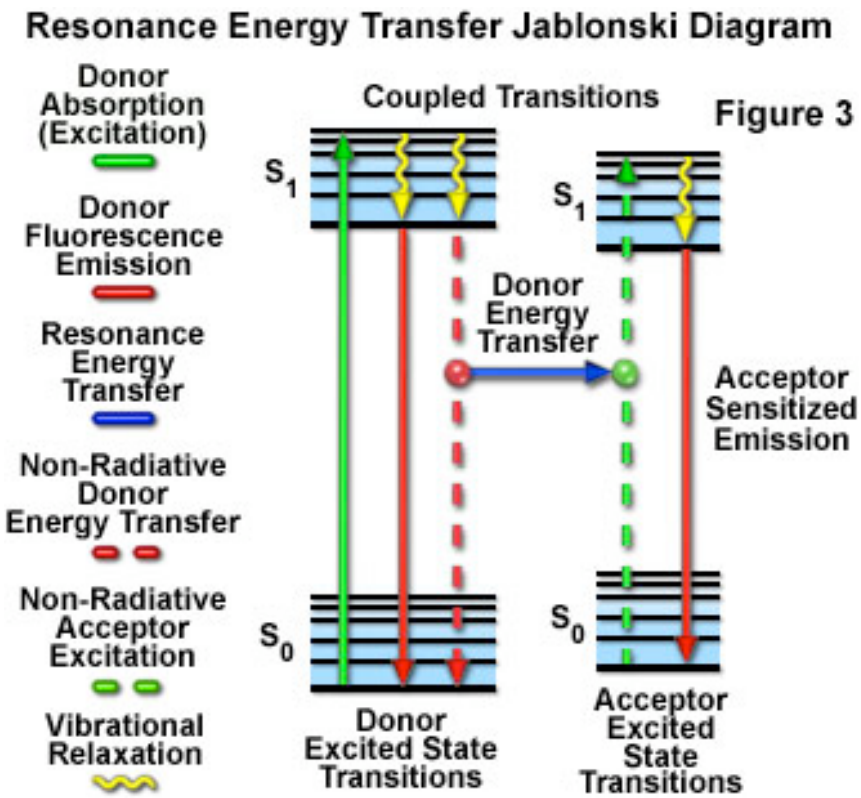
Fluorescence quenching can be defined as a bimolecular process that reduces the fluorescence quantum yield without changing the fluorescence emission spectrum; it can result from transient excited-state interactions (collisional quenching) or from formation of non fluorescent ground-state species. Self-quenching 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.

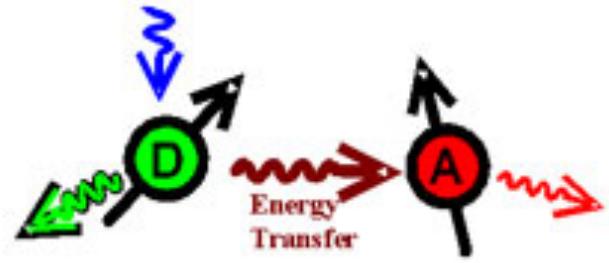
A donor chromophore in its excited state can transfer energy by a non radiative, long-range dipole-dipole coupling mechanism to an acceptor chromophore in close proximity (typically <10nm). This energy transfer mechanism is termed "Förster resonance energy transfer" (FRET), named after the German scientist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

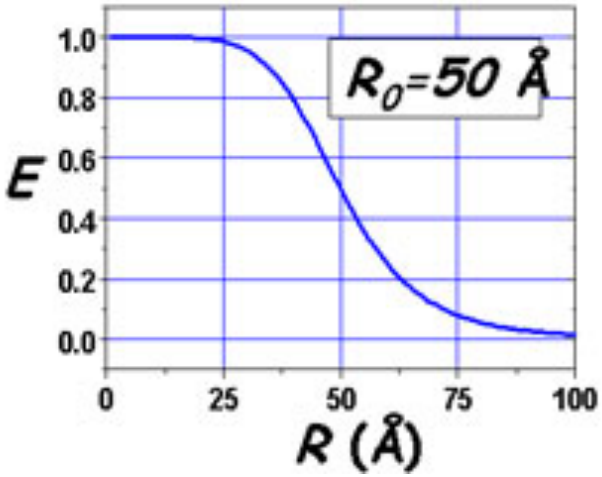


http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Technical-Notes-and-Product-Highlights/Fluorescence-Resonance-Energy-Transfer-FRET.html

FRET: FÖster Resonance Energy Transfer

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



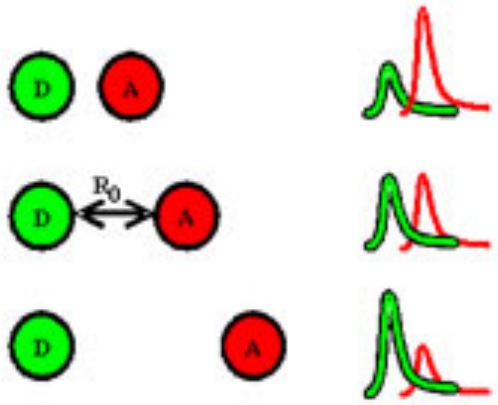


Energy Transfer Efficiency

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

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.

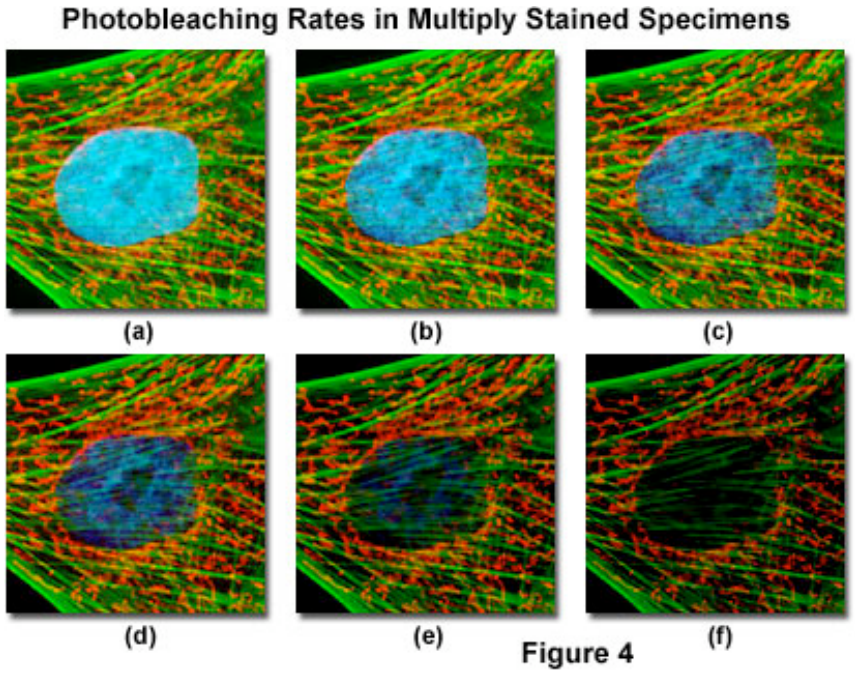
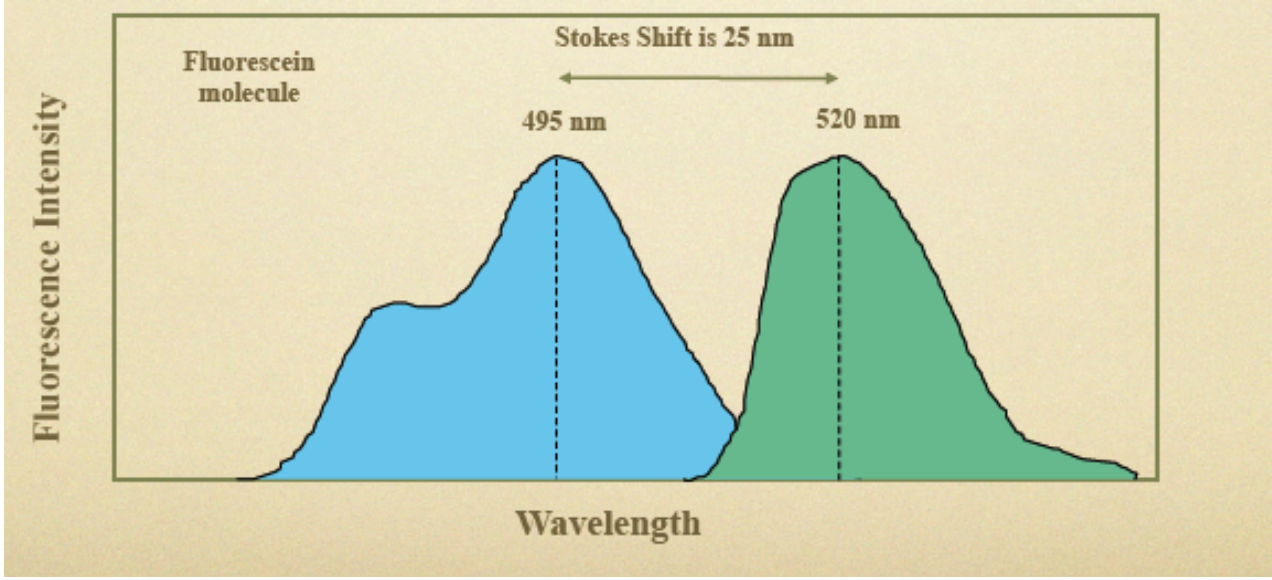


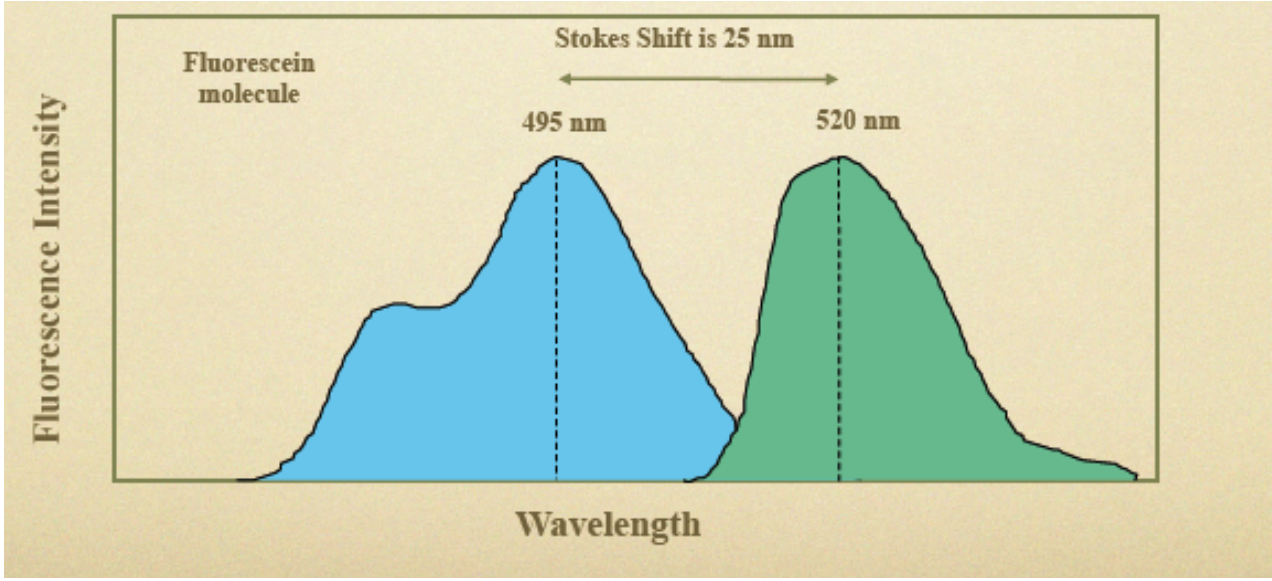
Figure 4

Properties of the fluorescence spectra



- For polyatomic molecules, single electron transition are represented by energy spectra called **excitation** and emission SPECTRA

Properties of the fluorescence spectra



1. **Stokes shift**: Fluorescence emission occurs at longer wavelengths than absorption.

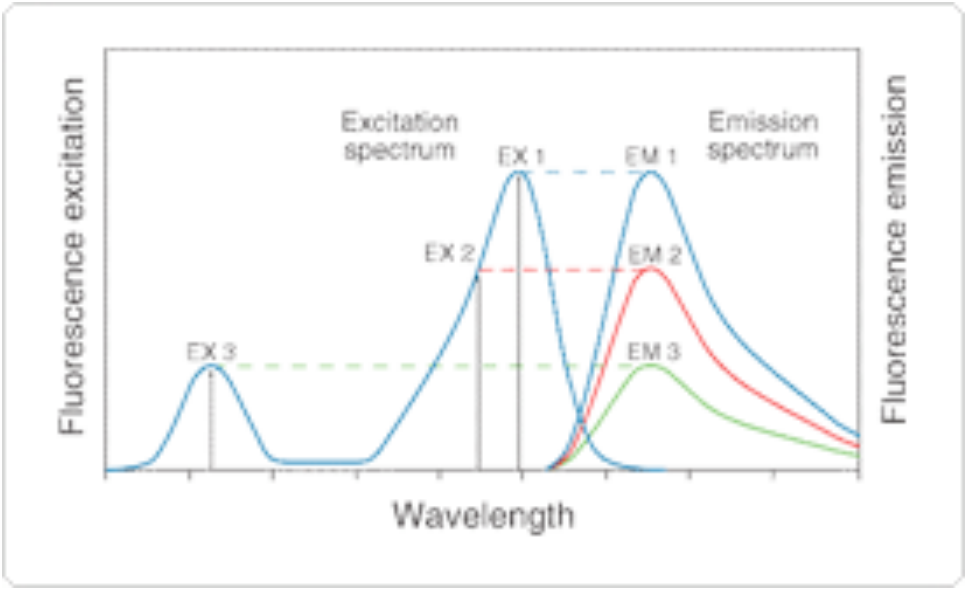
- Due to energy losses during the excitation emission that lead to:
- $E_{EM} < E_{AB}$
- Losses mainly due to internal conversion before and after emission; other factors contribute.
- Stoke shift is expressed as the wavelength difference between the maxima of $S_0 \text{-->} S_1$ and $S_1 \text{—>} S_0$ transitions

2. Kasha's rule: Emission spectra and λ max are independent of excitation wavelength.

On the other end, the intensity of fluorescence emission depends from λ exc

Due to emission occurring solely from the lowest vibrational level of S1

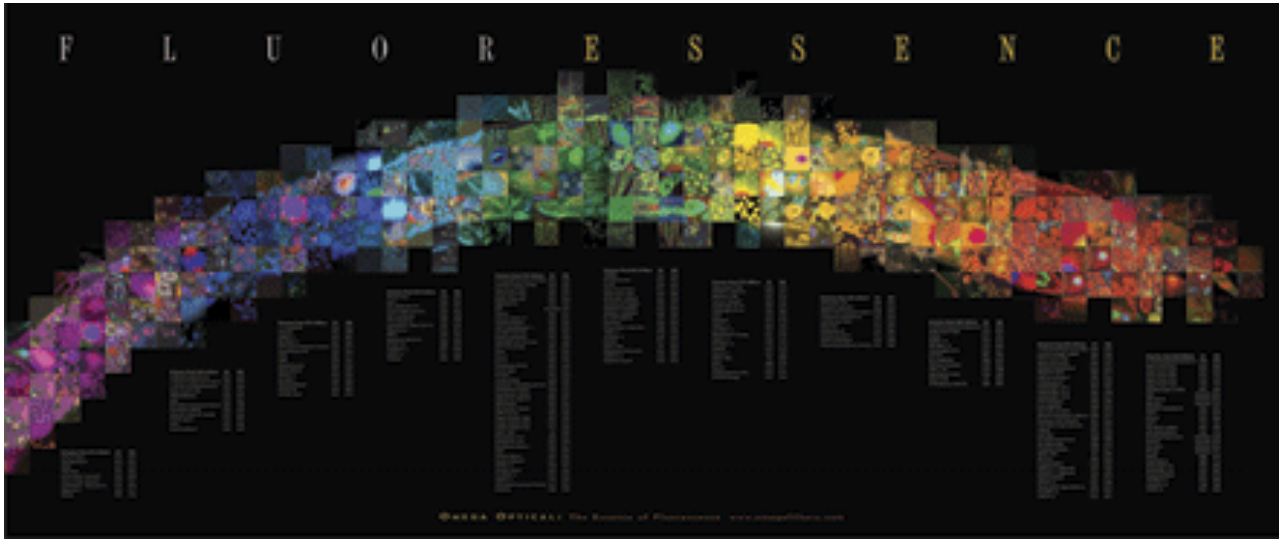
Due to rapid internal conversion from higher electronic and vibrational states (completed much before emission)



Fluorochromes

Organics e inorganics Fluorochromes

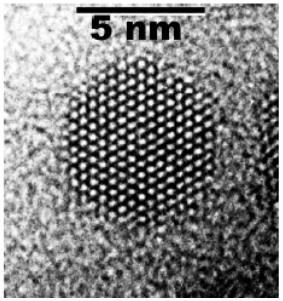
Protein-based fluorochromes

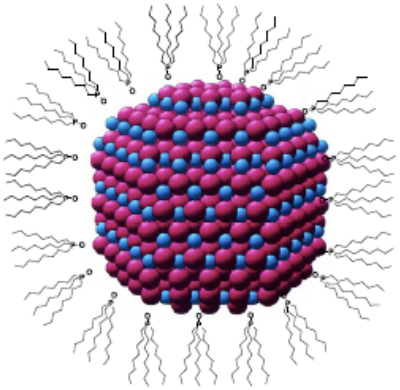


Semiconductors nanocrystals: QDOTS

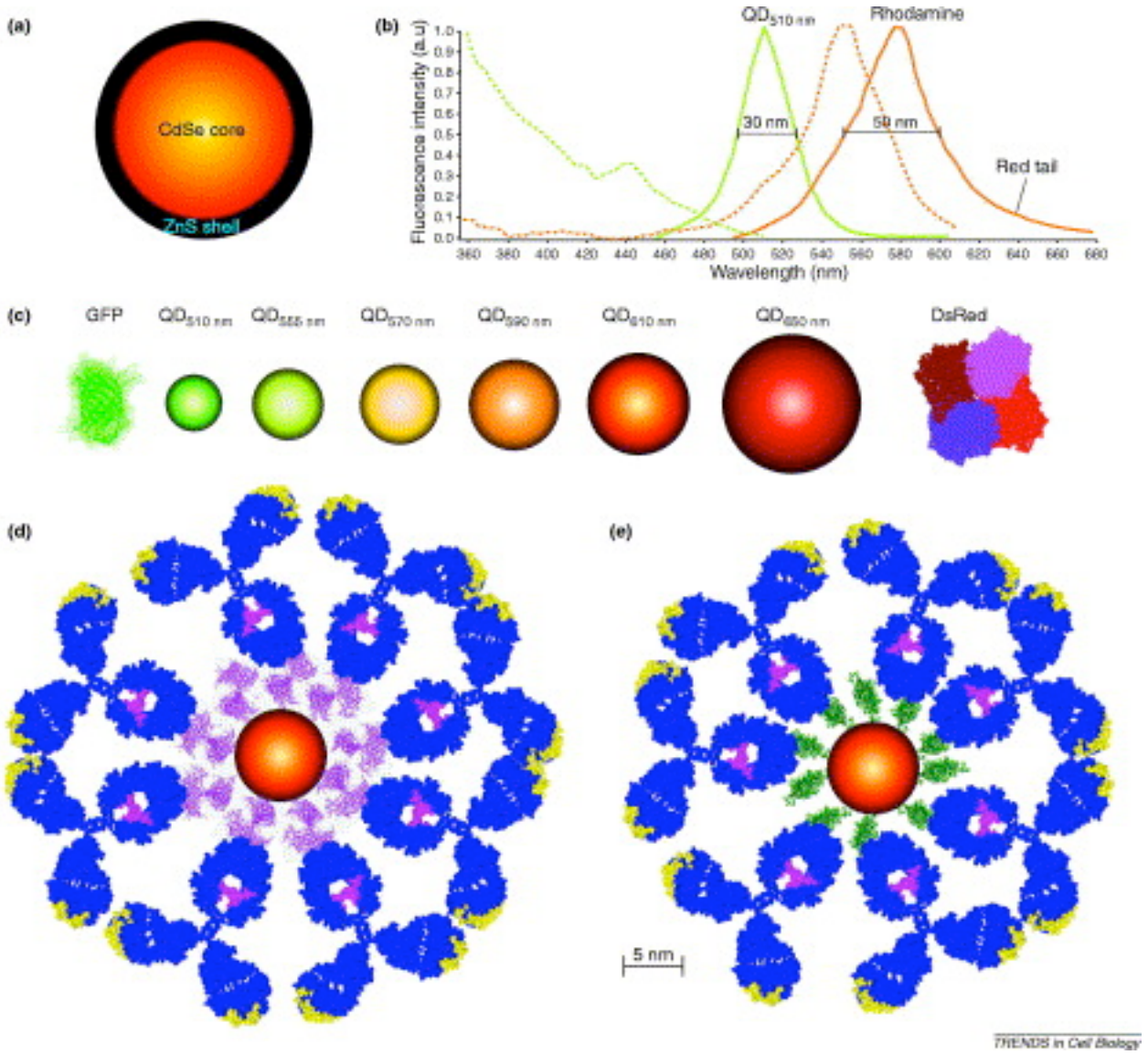
Nanocrystals composed of a core of a semiconductor material (CdSe), enclosed within a shell of another semiconductor (ZnS) that has a larger spectral band gap.

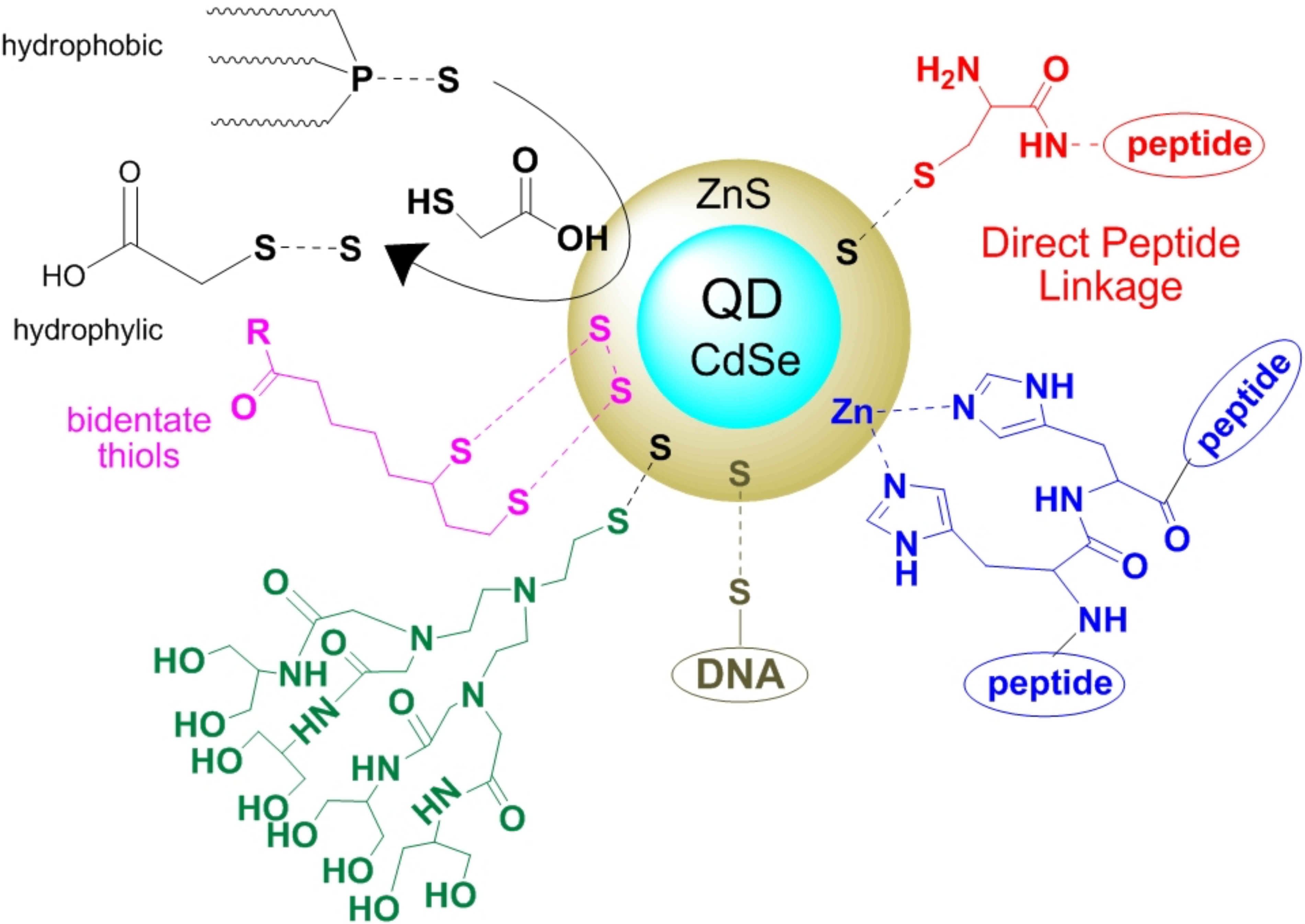
Typical diameter: 2-10 nm





- Size tunable emission = predictable relationship between the size of QD and its emission wavelength
- Strong light absorbance
- Bright fluorescence
- Narrow symmetric emission bands
- High photostability
- Excellent stability of optical properties upon conjugation to biomaterials


(a)
CdSe core
ZnS shell
(b)
$QD_{510\ nm}$
Rhodamine
Fluorescence intensity (a.u)
30 nm
58 nm
Red tail
Wavelength (nm)
(c)
GFP
$QD_{510\ nm}$
$QD_{555\ nm}$
$QD_{570\ nm}$
$QD_{590\ nm}$
$QD_{610\ nm}$
$QD_{650\ nm}$
DsRed
(d)
(e)
5 nm
TRENDS in Cell Biology



hydrophobic
P
S
O
HO
S
S
HS
O
OH
hydrophylic
ZnS
QD
CdSe
H2N
O
HN
peptide
S
S
Direct Peptide
Linkage
R
O
bidentate
thiols
S
S
S
S
S
S
S
DNA
Zn
N
NH
N
N
H
HN
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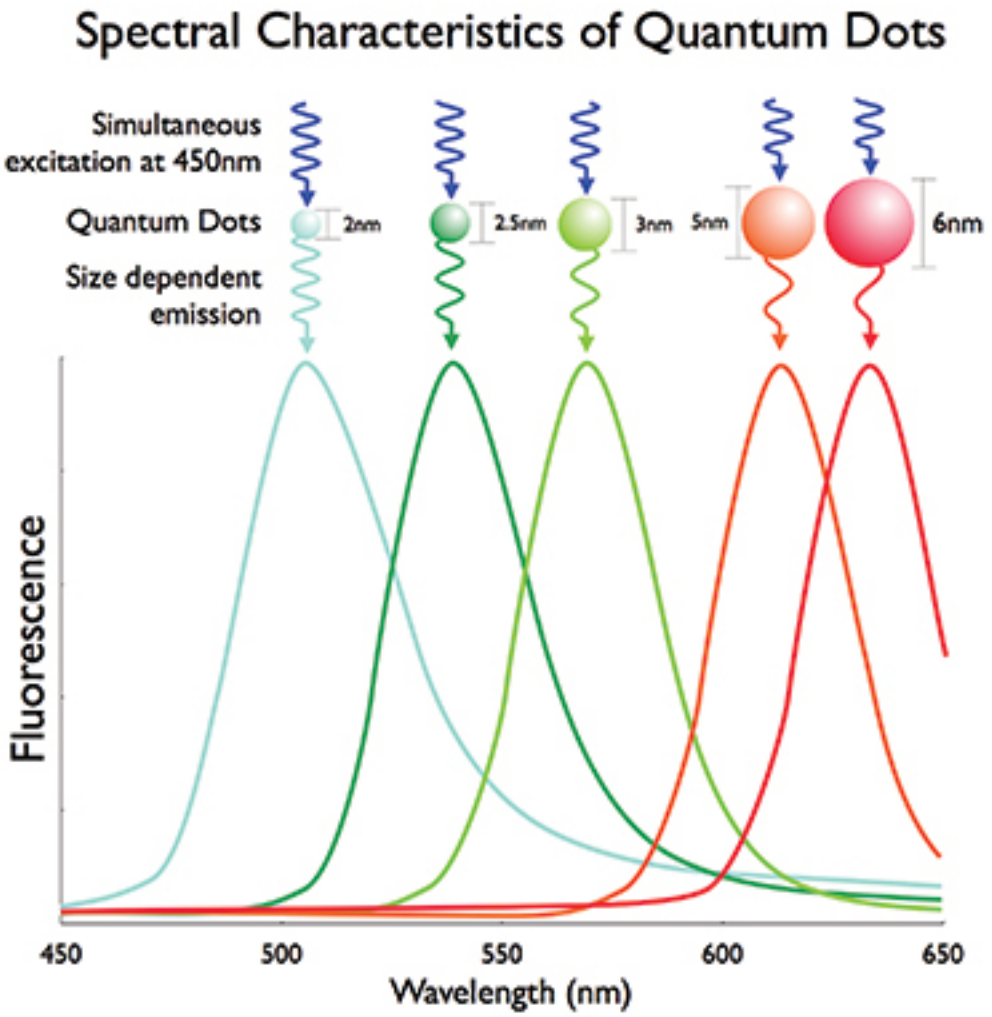
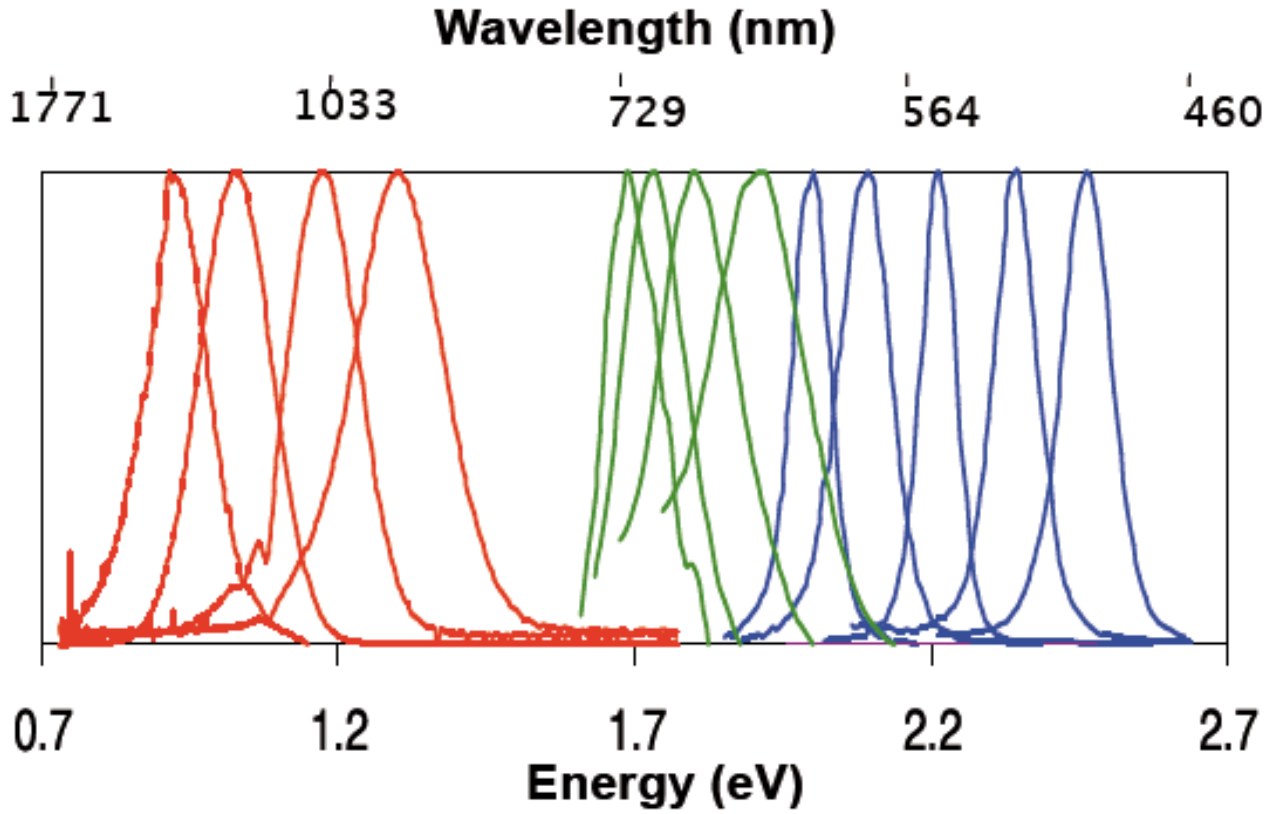
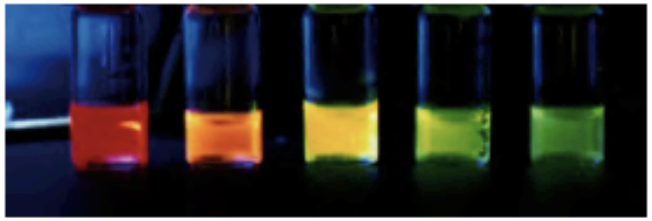

Spectral Characteristics of Quantum Dots
Simultaneous excitation at 450nm
Quantum Dots
Size dependent emission
2nm
2.5nm
3nm
5nm
6nm
Fluorescence
450
500
550
600
650
Wavelength (nm)



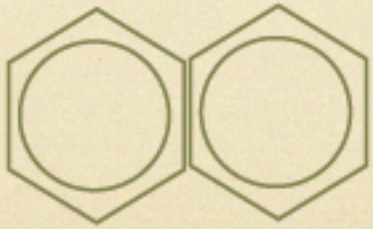
Red--InAs, left to right: 60Å, 46Å, 36Å, 28Å
Green--InP, left to right: 46Å, 40Å, 35Å, 30Å
Blue--CdSe, left to right: 46Å, 36Å, 31Å, 24Å, 21Å

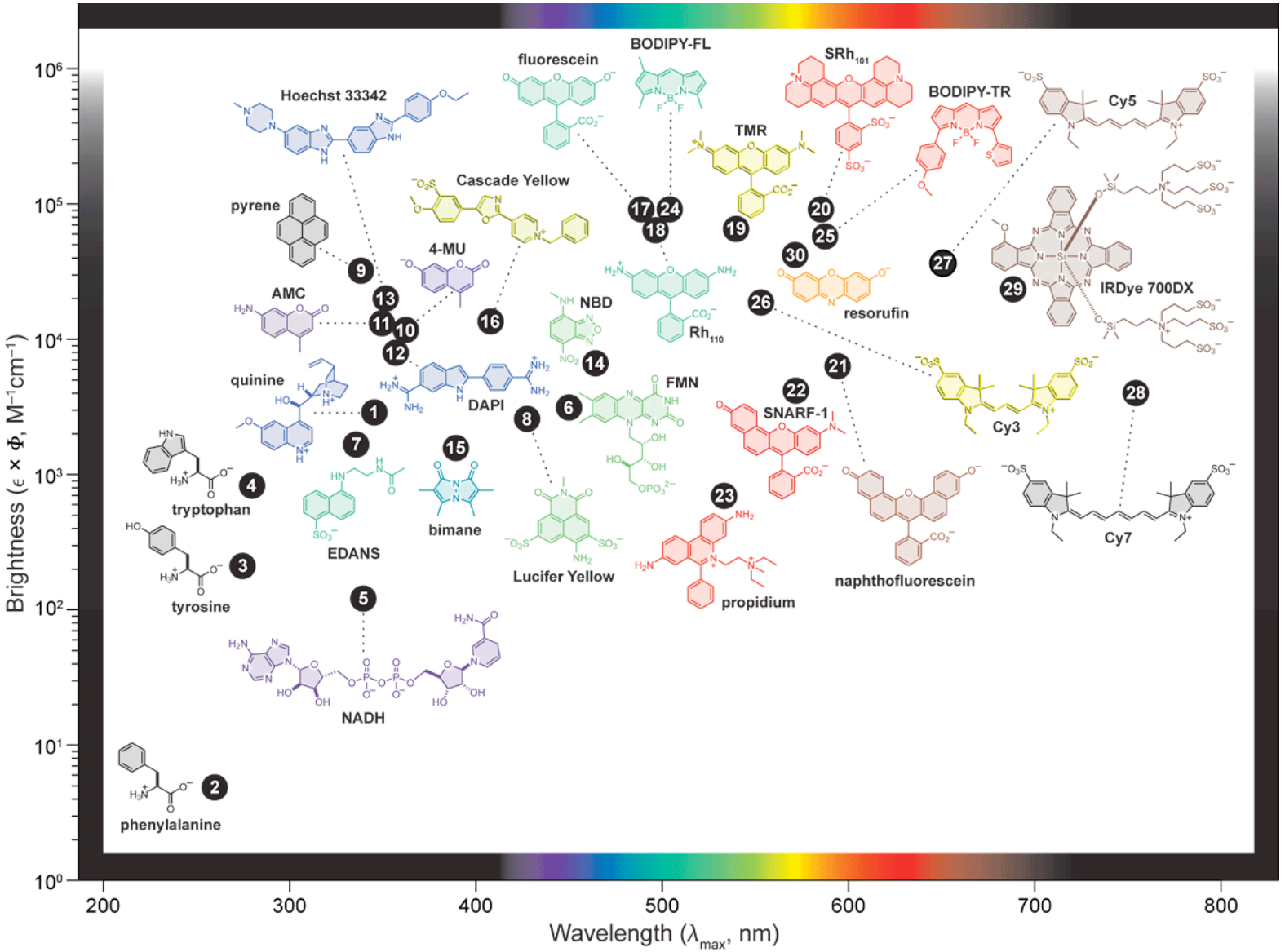


Alivisatos, Bruchez, et al.

Organic and protein based Fluorochromes

- Chromophores are components of molecules which absorb light
  - E.g. from protein most fluorescence results from the indole ring of tryptophan residue
  - They are generally aromatic rings




Brightness (ε × Φ, M⁻¹cm⁻¹)
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
200
300
400
500
600
700
800
Wavelength (λmax, nm)
Hoechst 33342
fluorescein
BODIPY-FL
SRh101
BODIPY-TR
Cy5
TMR
Cascade Yellow
pyrene
4-MU
AMC
NBD
Rh110
resorufin
IRDye 700DX
quinine
DAPI
FMN
SNARF-1
Cy3
tryptophan
EDANS
bimane
Lucifer Yellow
propidium
naphthofluorescein
Cy7
tyrosine
NADH
phenylalanine
1
2
3
4
5
6
7
8
9
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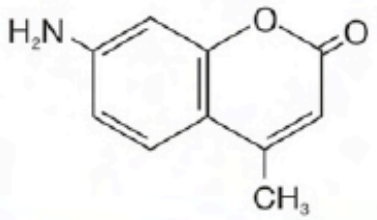
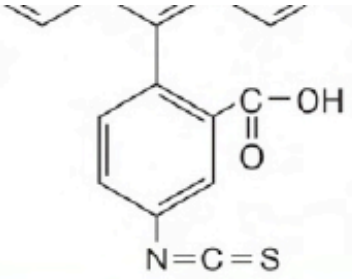
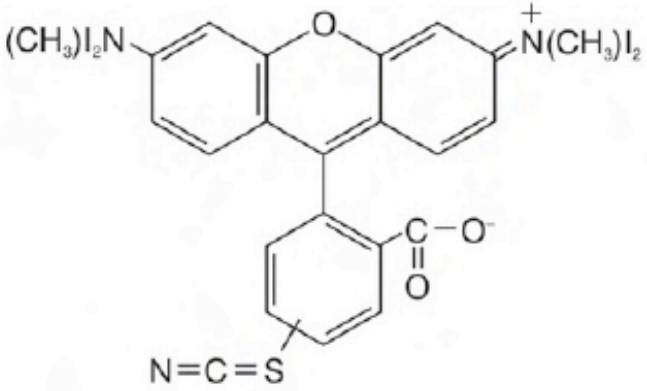


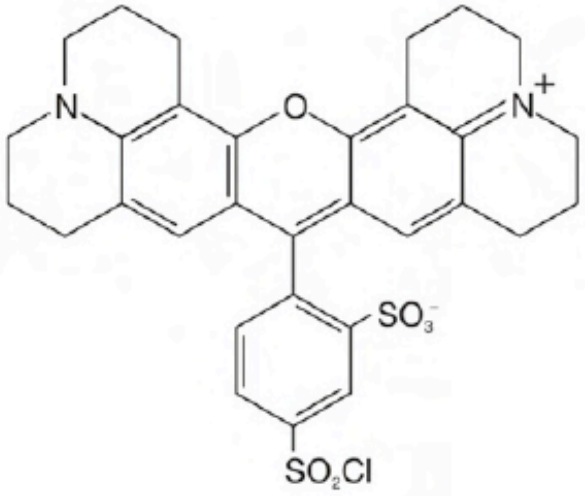
AMCA



FITC

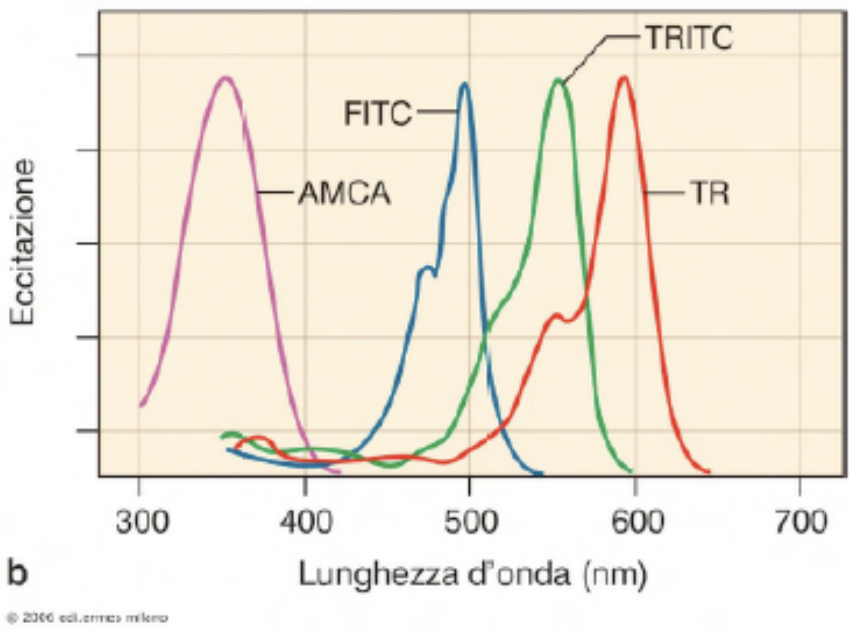


TRITC

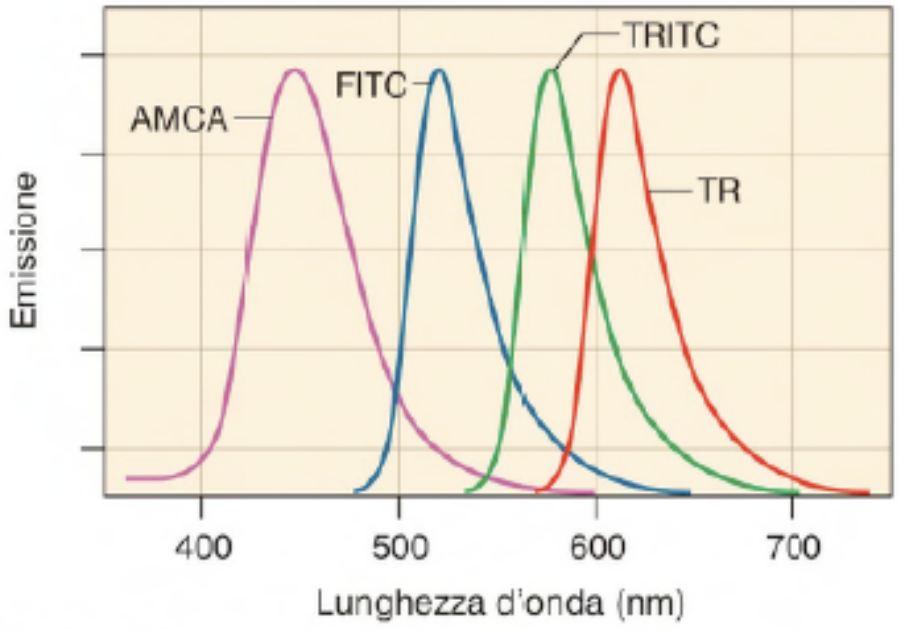


TR

a



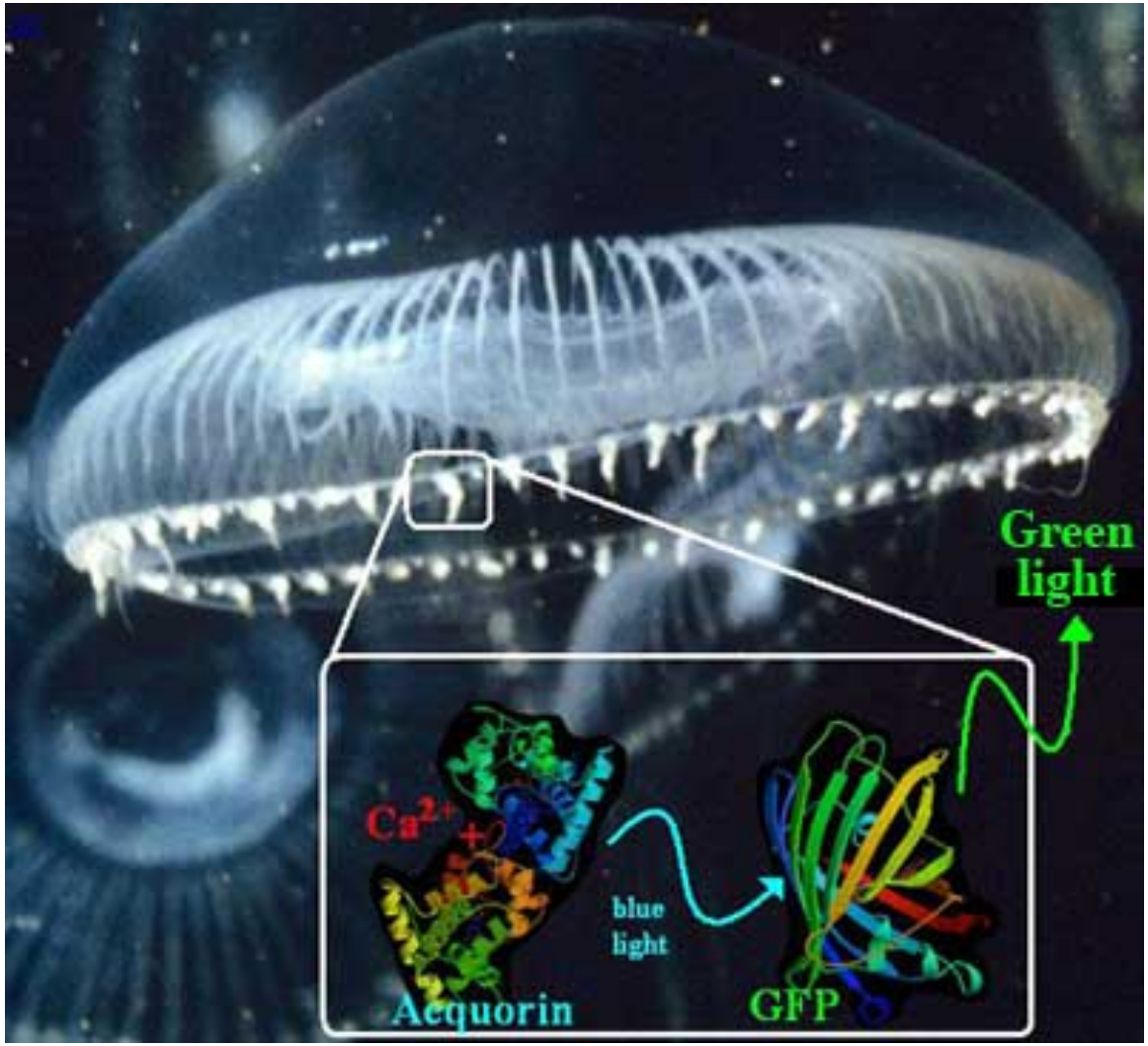
b



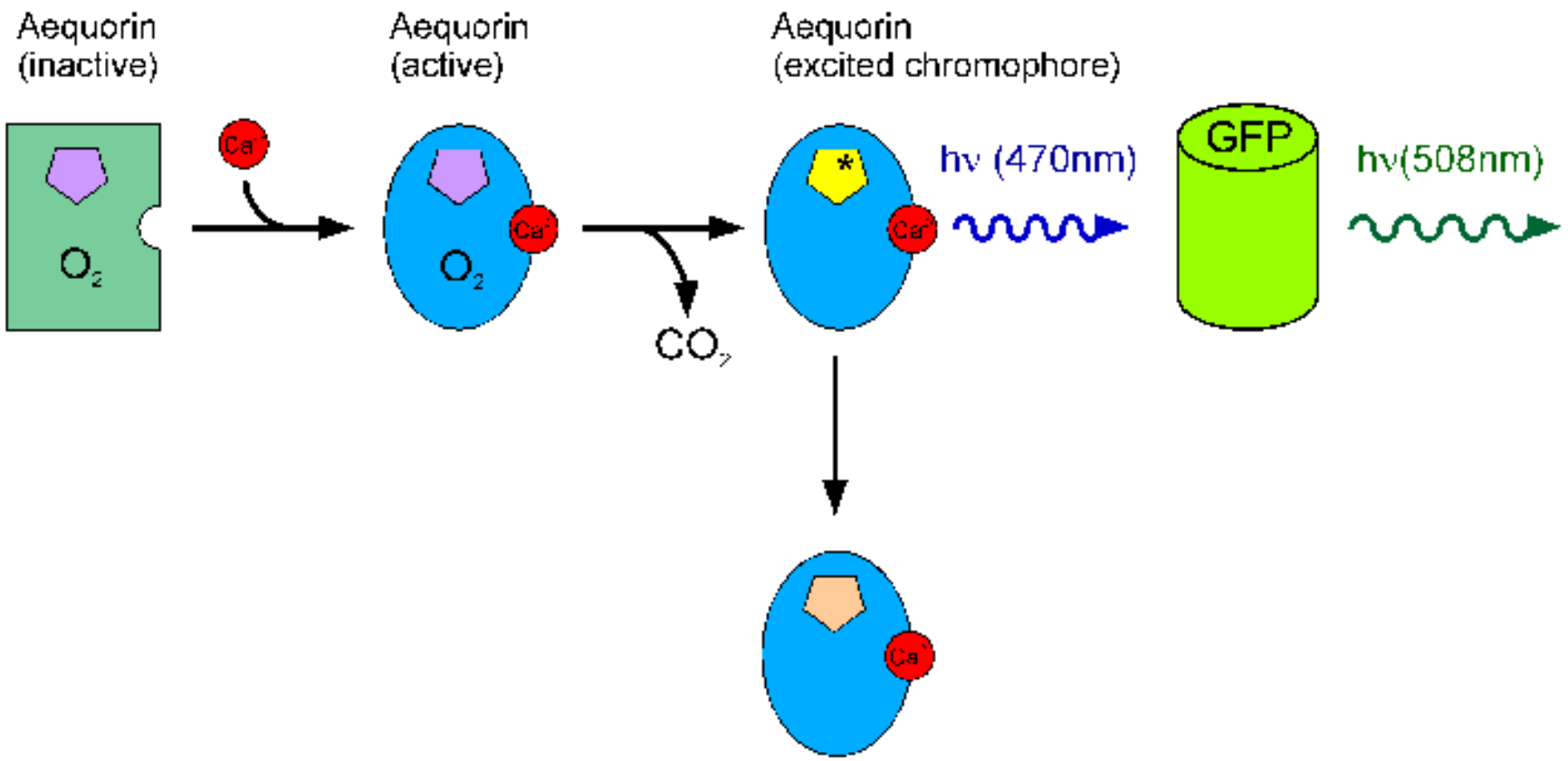
Protein-based indicators

The primary advantages of fluorescent protein-based indicators over simple organic dyes are that they can be designed to respond to a much greater variety of biological events and signals, targeted to subcellular compartments, introduced into a wider variety of tissues and intact organisms, and they very rarely cause photodynamic toxicity.

Aequorin and GFP from Aequoria Victoria



Aequorin activation and GFP excitation



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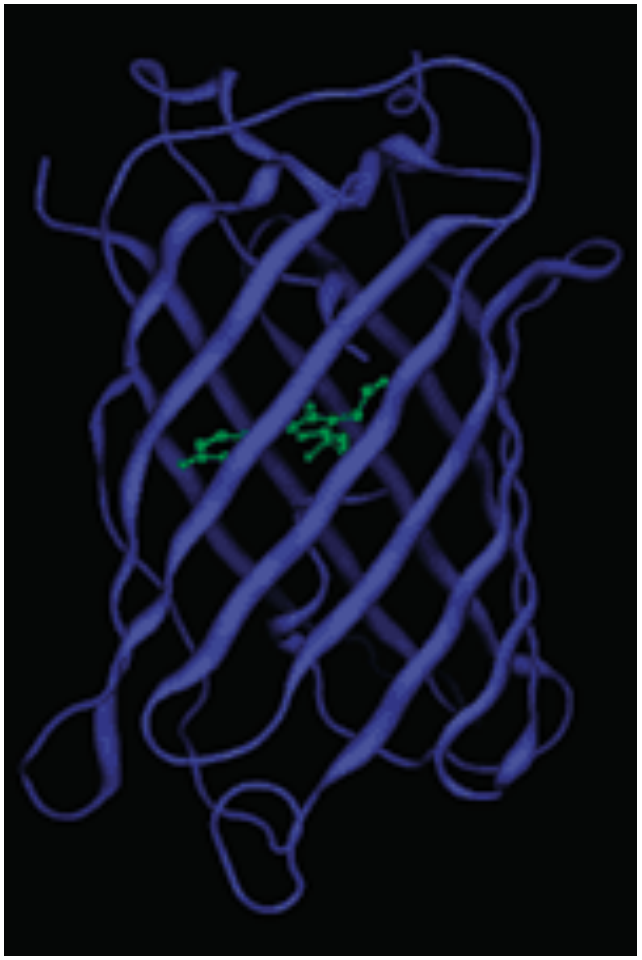
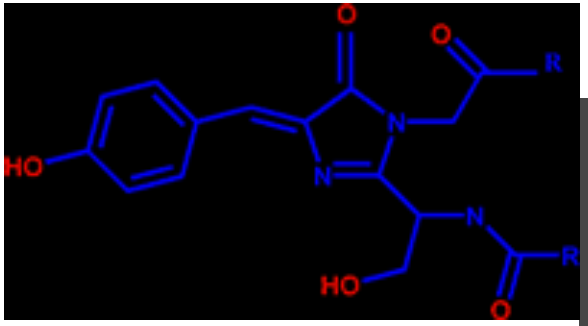
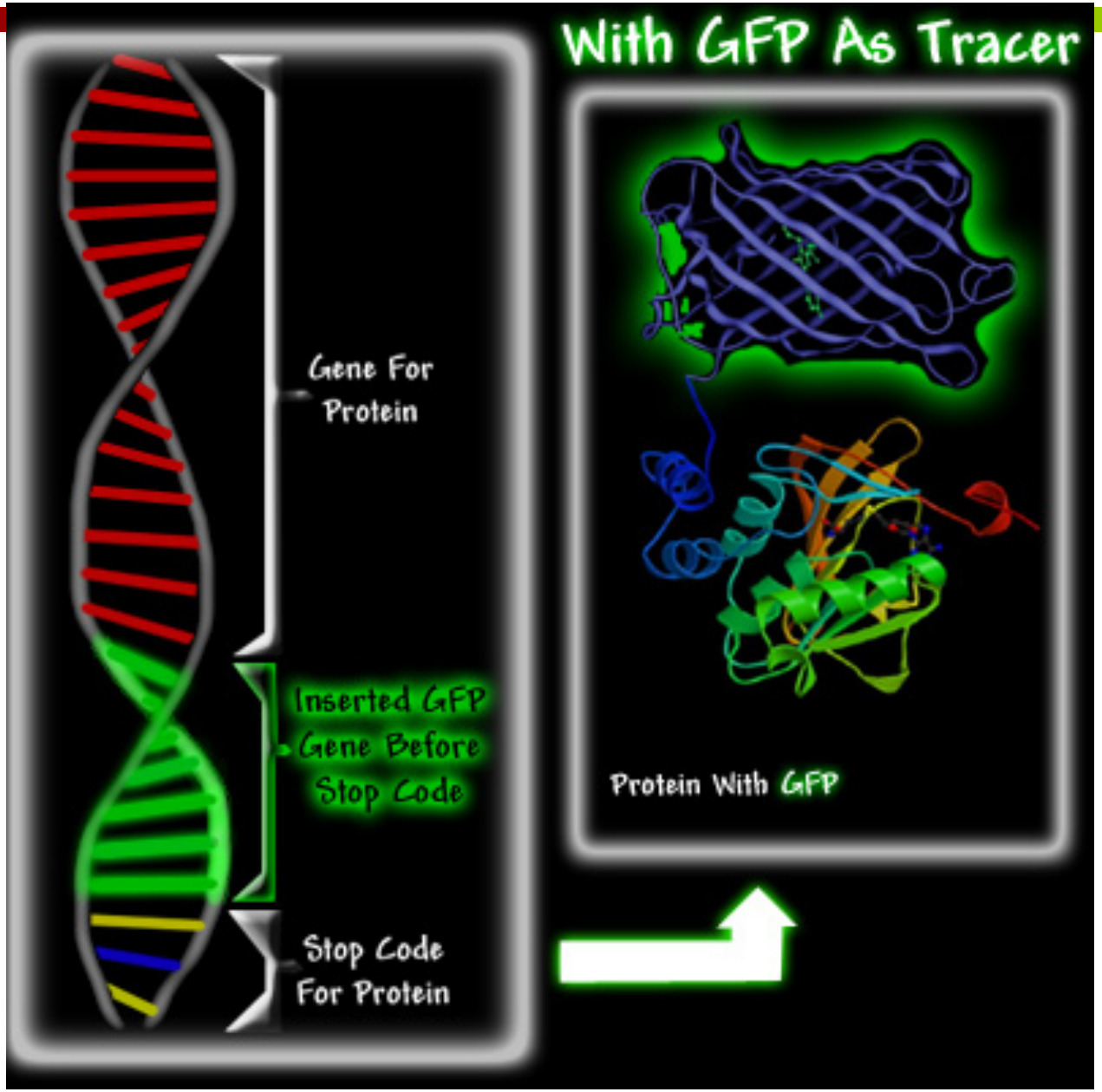




GFP sequence and structure


With GFP As Tracer
Gene For Protein
Inserted GFP Gene Before Stop Code
Stop Code For Protein
Protein With GFP

ADVANTAGES OF GFP AS TRACER AND REPORTER MOLECULE

- If enough protein with attached GFP is made, it should be easy to detect and to trace it as it moved through the cell, because irradiating the cell with ultra violet light would cause the GFP attached to the protein to fluoresce.

ADVANTAGES OF GFP AS TRACER AND REPORTER MOLECULE

- 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 neurons, whereas the diffusion of large proteins would be difficult.

ADVANTAGES OF GFP AS TRACER AND REPORTER MOLECULE

• 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

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

Nobel lecture: constructing and exploiting the fluorescent protein paintbox†

Roger Y. Tsien

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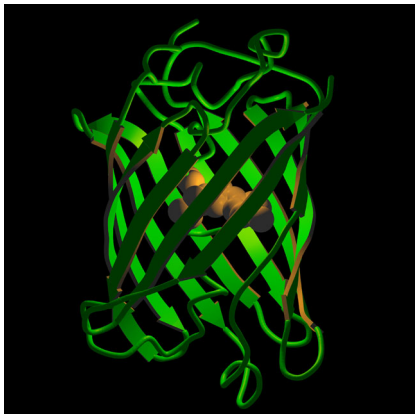
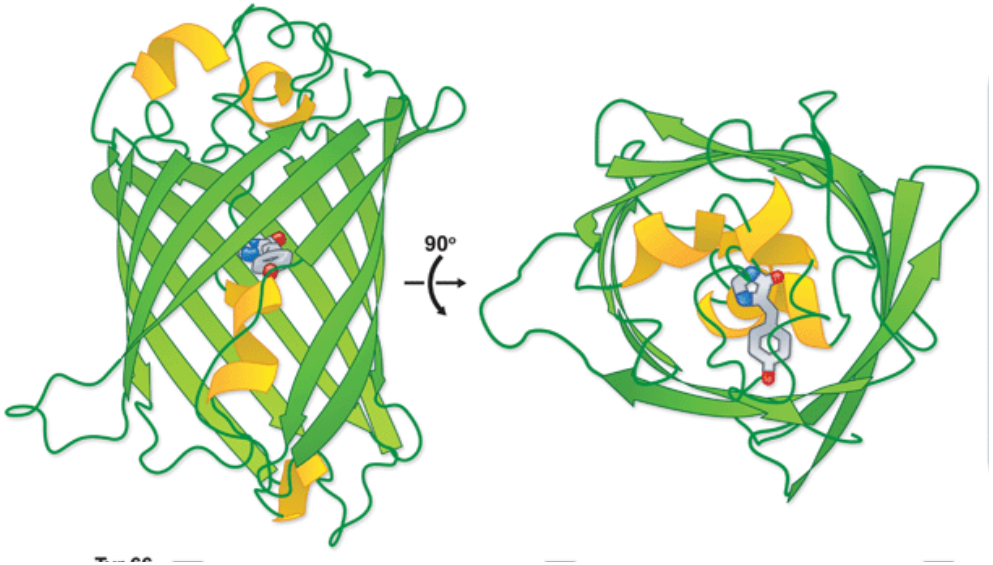
A written version of Professor Roger Tsien's Nobel Lecture.

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

GFP-derived proteins





…….Because the chromophore of GFP was mostly constructed from **a tyrosine at position 66**, I asked Heim (the postdoc) to mutate this amino acid to tryptophan, the other aromatic amino acid most conducive to absorbance and fluorescence. This alteration practically destroyed the fluorescence.

After the failure of my naı¨ve rational idea, Heim decided to mutate GFP randomly.

GFP-derived proteins

To our delight, he soon found a blue-fluorescing mutant (Fig. 4, upper right quadrant), which upon sequencing proved surprisingly to contain a histidine at position 66. Later we discovered that tryptophan at 66 gives an even more useful and bleach-resistant cyan fluorescent protein (CFP), intermediate between blue and green, but only if additional mutations carve out extra room inside the protein to accommodate the bulky tryptophan (Fig. 4, lower right quadrant)

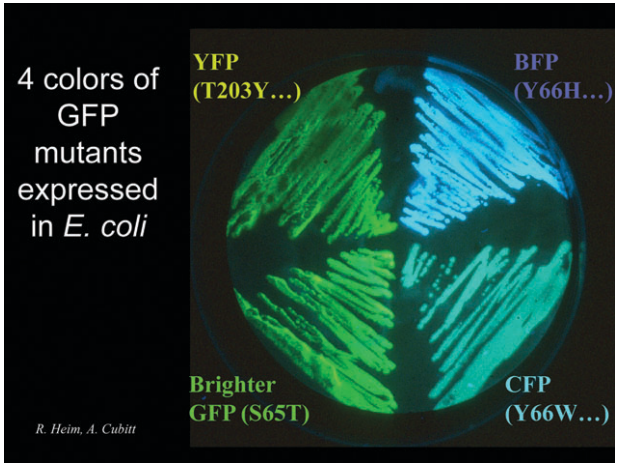


Fig. 4 Fluorescence from streaks of bacteria expressing (clockwise from upper right) improved blue, cyan, green, and yellow fluorescent proteins. Each streak is labeled with the mutation most responsible for its spectral alteration.

GFP-derived proteins

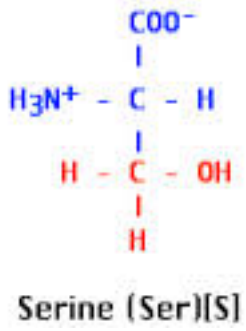
Next goal was for Tsien to create a **GFP excited by blue light**.

….. 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, a big one in the UV and a much smaller one in the blue?**

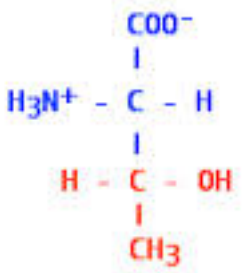
I hypothesized that the major **UV peak** was due to the **chromophore structure** as guessed by Shimomura and Prasher whereas the **minor blue peak was** due to a small fraction of the chromophores undergoing dehydration **of serine 65** to a dehydroalanine.

GFP-derived proteins

…….. Heim then tried replacing serine 65 by threonine. Even though threonine is the amino acid most closely resembling serine, differing only by a CH2 group, the unwanted UV peak disappeared completely, the blue peak became 5–6 fold higher, and it even shifted 10 nm to longer wavelengths (Fig. 6).



Serine (Ser)[S]



Threonine (Thr)[T]

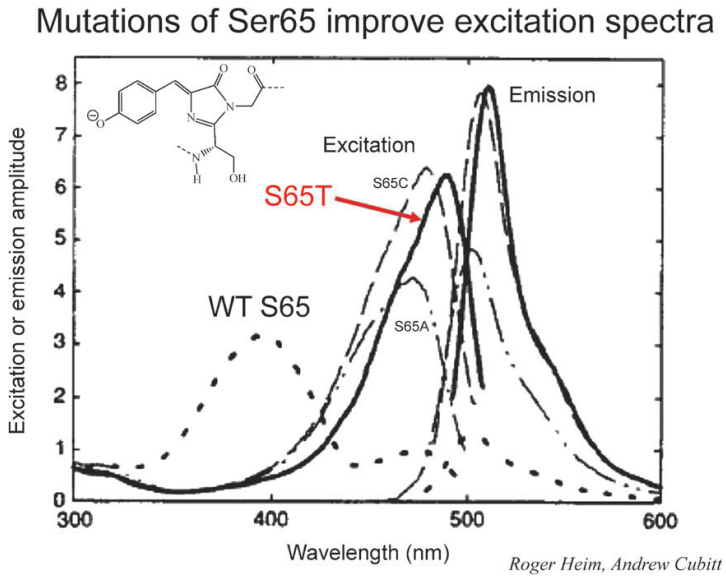


Fig. 6 Excitation and emission spectra of wild-type (WT) GFP and several mutants of Ser 65.[25] The inset shows the structure of the wild-type chromophore.

GFP-derived proteins

We therefore suggested this mutant, **"S65T", as** a general improvement on wild-type GFP (Fig. 4, lower left quadrant).

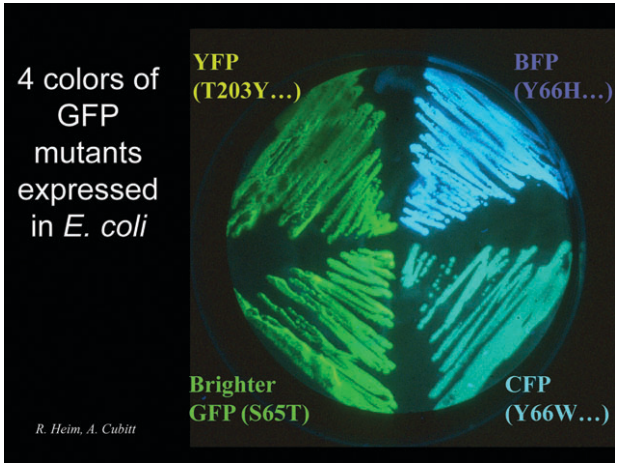


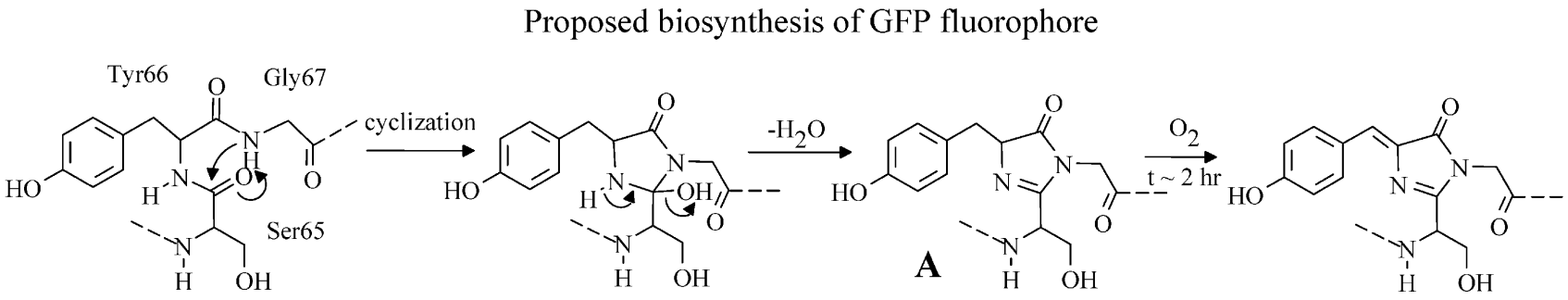
Fig. 4 Fluorescence from streaks of bacteria expressing (clockwise from upper right) improved blue, cyan, green, and yellow fluorescent proteins. Each streak is labeled with the mutation most responsible for its spectral alteration.

GFP-derived proteins

It still bothered me that we did not understand how the chromophore (technically, a p-hydroxybenzylideneimidazolidinone) inside GFP was spontaneously formed from serine 65, tyrosine 66, and glycine 67.

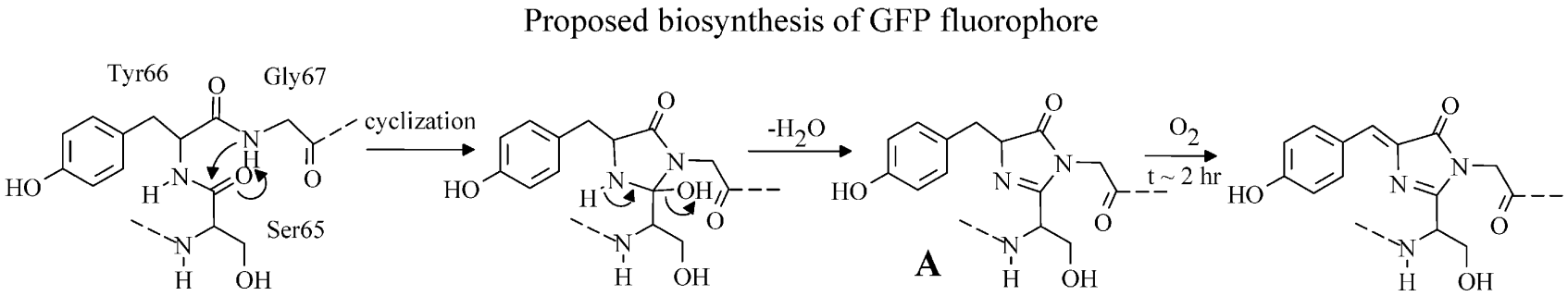
There seemed to be no biochemical precedent for such a post-translational modification, which not only formed a new heterocyclic ring but also dehydrogenated the a-b single bond of tyrosine to a double bond.

Dehydrogenations either evolve hydrogen gas (H2), which I thought most unlikely in this case, or require an oxidant to carry away the two hydrogen atoms. The only oxidant we could directly control within the cells was atmospheric O2.



GFP-derived proteins

Heim therefore grew GFP-expressing bacteria under strictly anaerobic conditions and was pleasantly surprised to find that the protein was made but not yet fluorescent. Upon re-exposure to air, that protein became green fluorescent over a few hours. We were therefore lucky that the requisite oxidant, O2, is available in all organisms except obligate anaerobes, yet can be readily eliminated to demonstrate its necessity. This discovery allowed us to propose a plausible mechanism for chromophore formation



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.

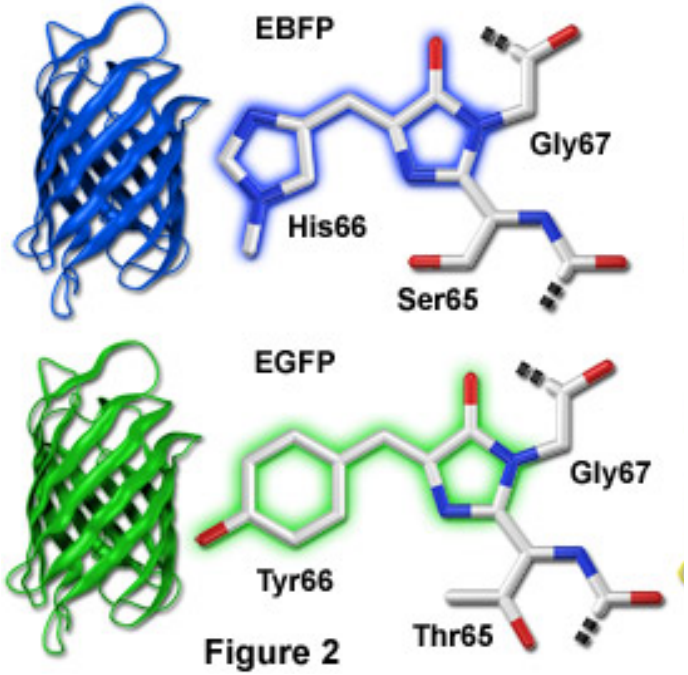
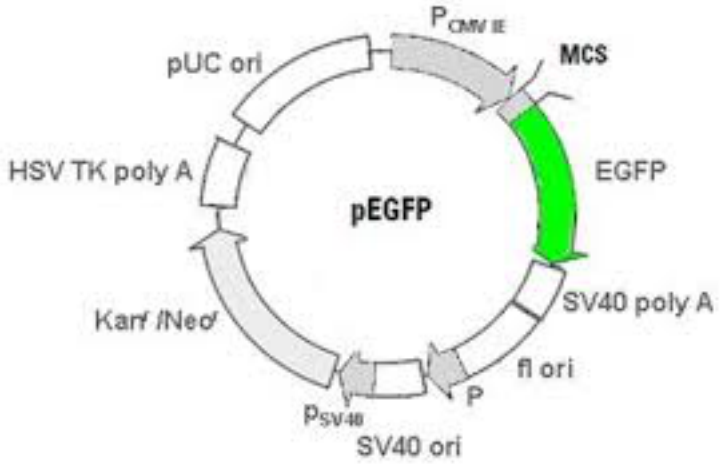


Figure 2



Solving GFP Crystal structure

…….The above improvements were all made without any three dimensional structural information, which clearly would greatly facilitate further engineering……..When Jim Remington at the University of Oregon e-mailed me in May 1995 to get an expression vector for GFP, I suggested that he solve the S65T structure, because that ought to be publishable even if another group got the wild-type structure first. Within a few months, Dr Mats Ormo¨ in Remington's lab had solved the crystal structure (Fig. 8), using selenomethionine substitution for phasing……..

Solving GFP Crystal structure

……The protein was an almost perfect cylinder, 2.4 nm in diameter by 4.2 nm long, composed of eleven beta-strands surrounding a helix running up the central axis, into which the chromophore was inserted. The chromophore was deeply buried at the center of the protein, explaining how it could be shielded from solvent and rigidified to make it fluorescent. (Once the protein is denatured, the exposed chromophore completely loses its fluorescence.)

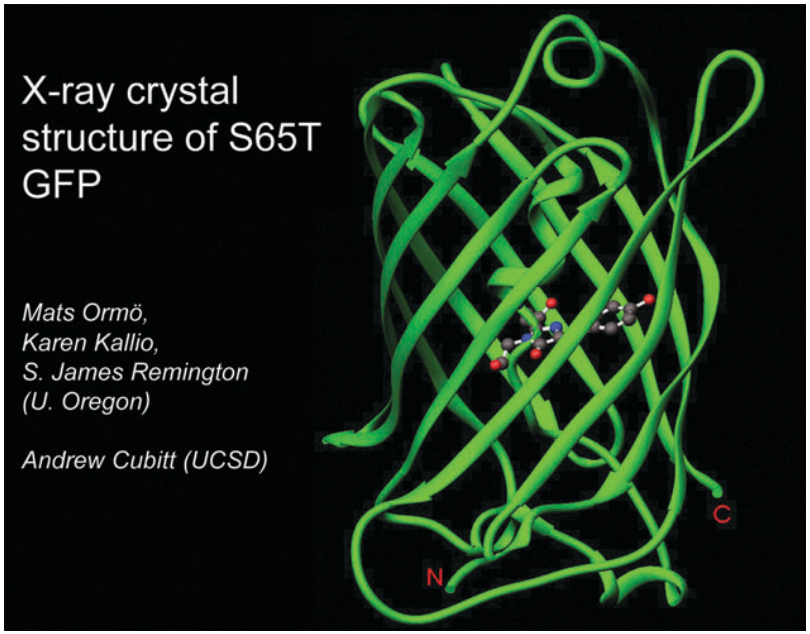
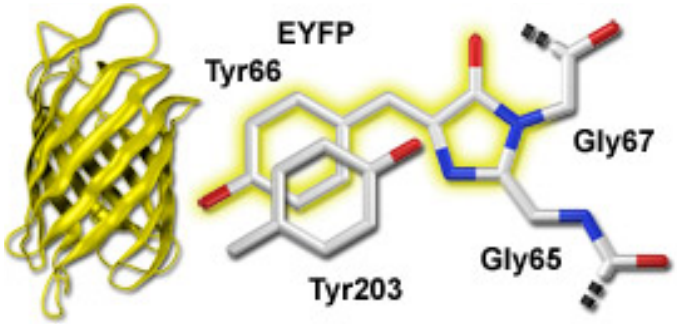


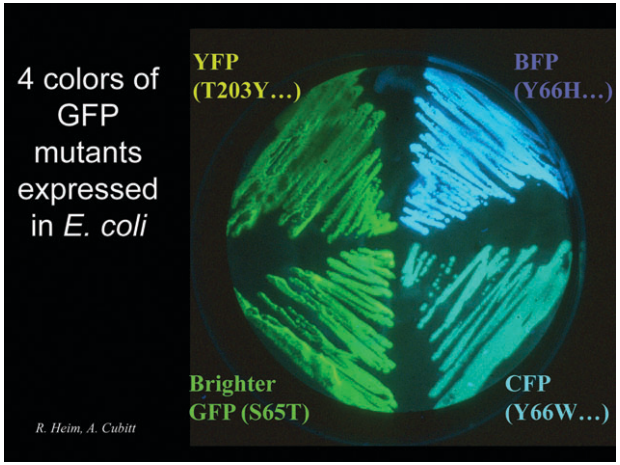
Fig. 8 X-ray crystal structure of the S65T mutant of GFP.[31] Alpha helices and beta strands are shown as ribbons, connecting segments as tubes, and the chromophore in ball-and-stick representation. *N*- and *C*-terminii are marked.

……..We could also rationalize why the chromophore had to be formed spontaneously, because no enzyme could reach through the completely encapsulating shell formed by the rest of GFP……..

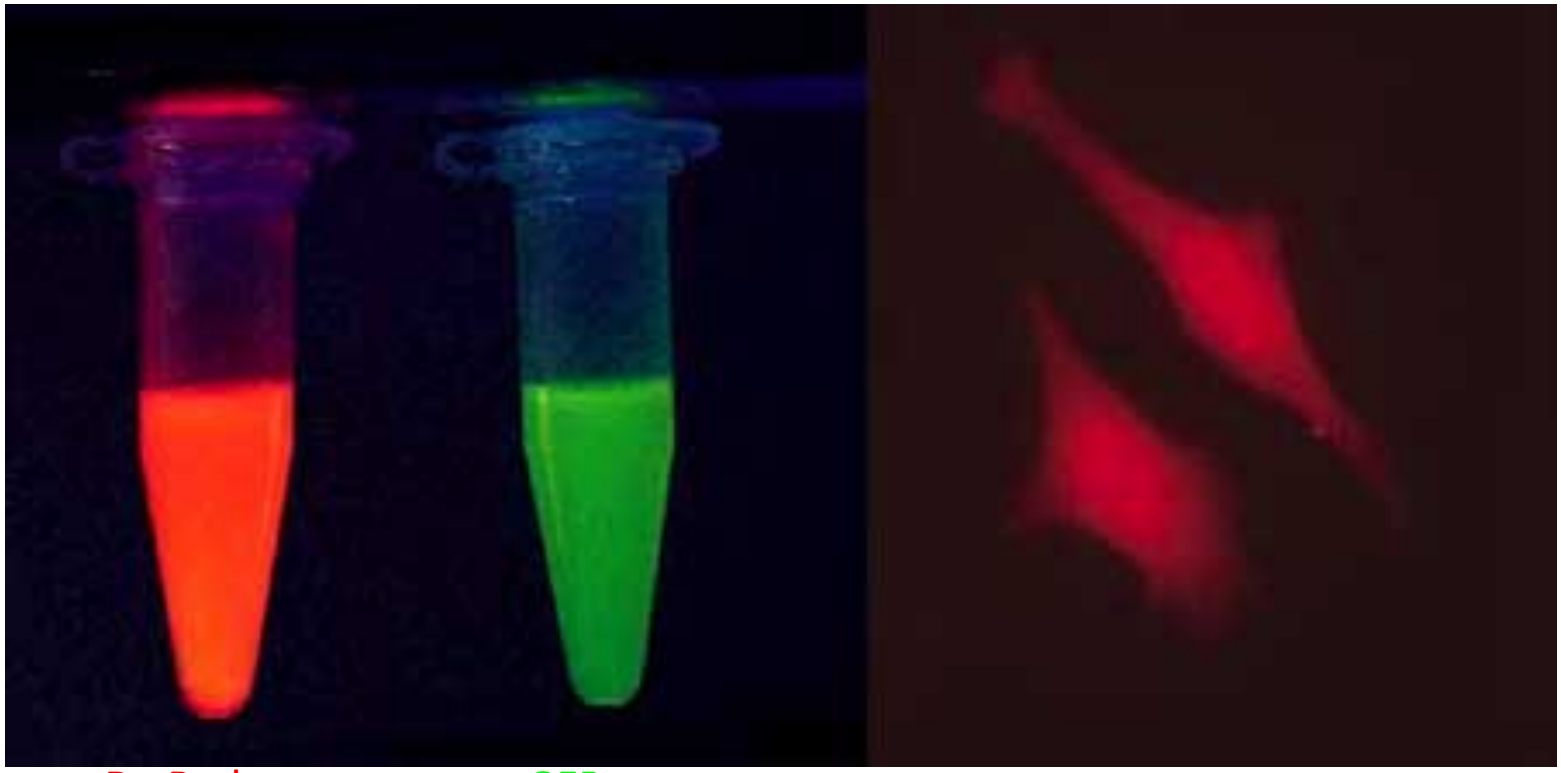
GFP-derived proteins: YFP

Remington noticed a cavity next to the chromophore and suggested that it could accommodate an aromatic ring, which might shift the fluorescence wavelengths.
To introduce this ring, Andrew Cubitt, a new postdoc in my lab**, mutated Thr 203 to various aromatic amino acids, followed by re-annealing**. Indeed, both excitation and emission maxima increased about 20 nm, producing a noticeably more yellowish fluorescent protein, **hence dubbed YFP** (Fig. 4, upper left quadrant). 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.





many new GFP-like proteins in non-bioluminescent and sometimes even non-fluorescent marine organisms.

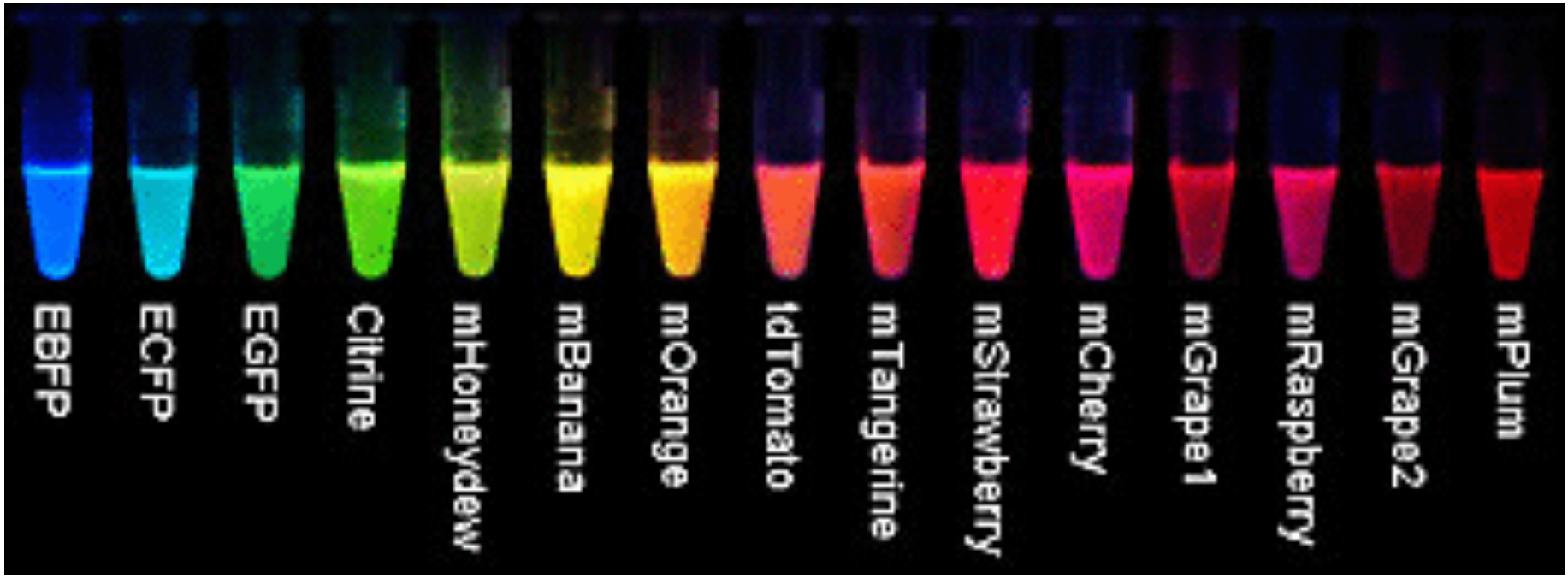


Ds-Red GFP

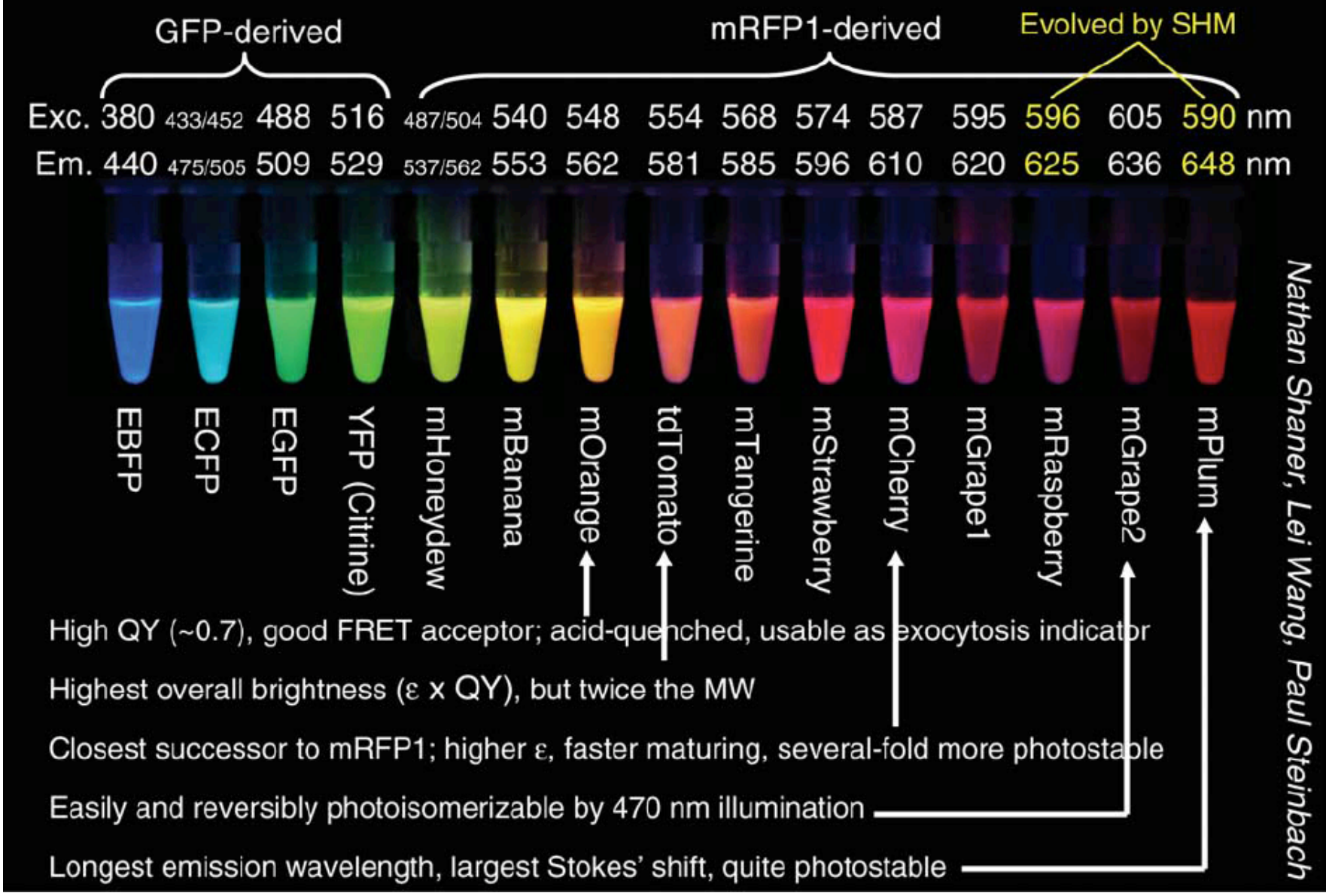
In august 2007 Lukyanov reported a bright, fast folding fluorescent protein that emits light in the far-red. The protein is named Katushka. The monomeric form of the protein is called mKate. It was isolated from a brilliant red sea anemone.

Katushka Ds-Red

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).



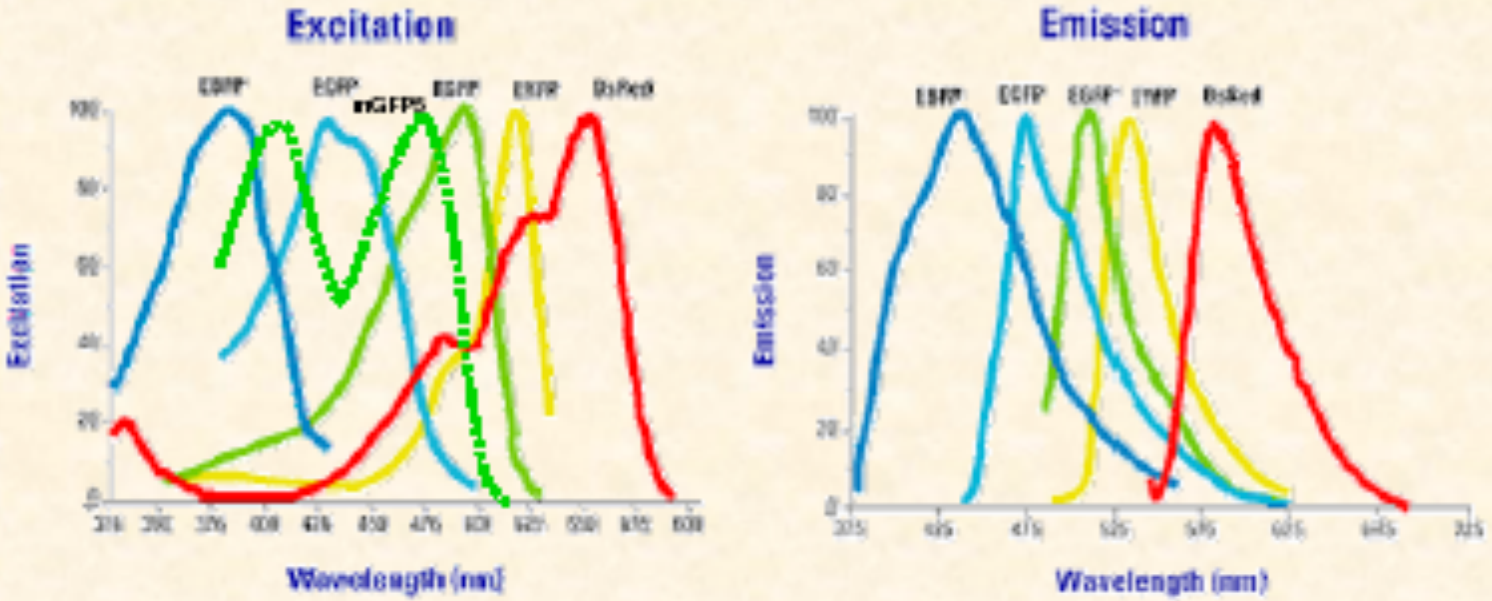
The 2004 palette of nonoligomerizing fluorescent proteins

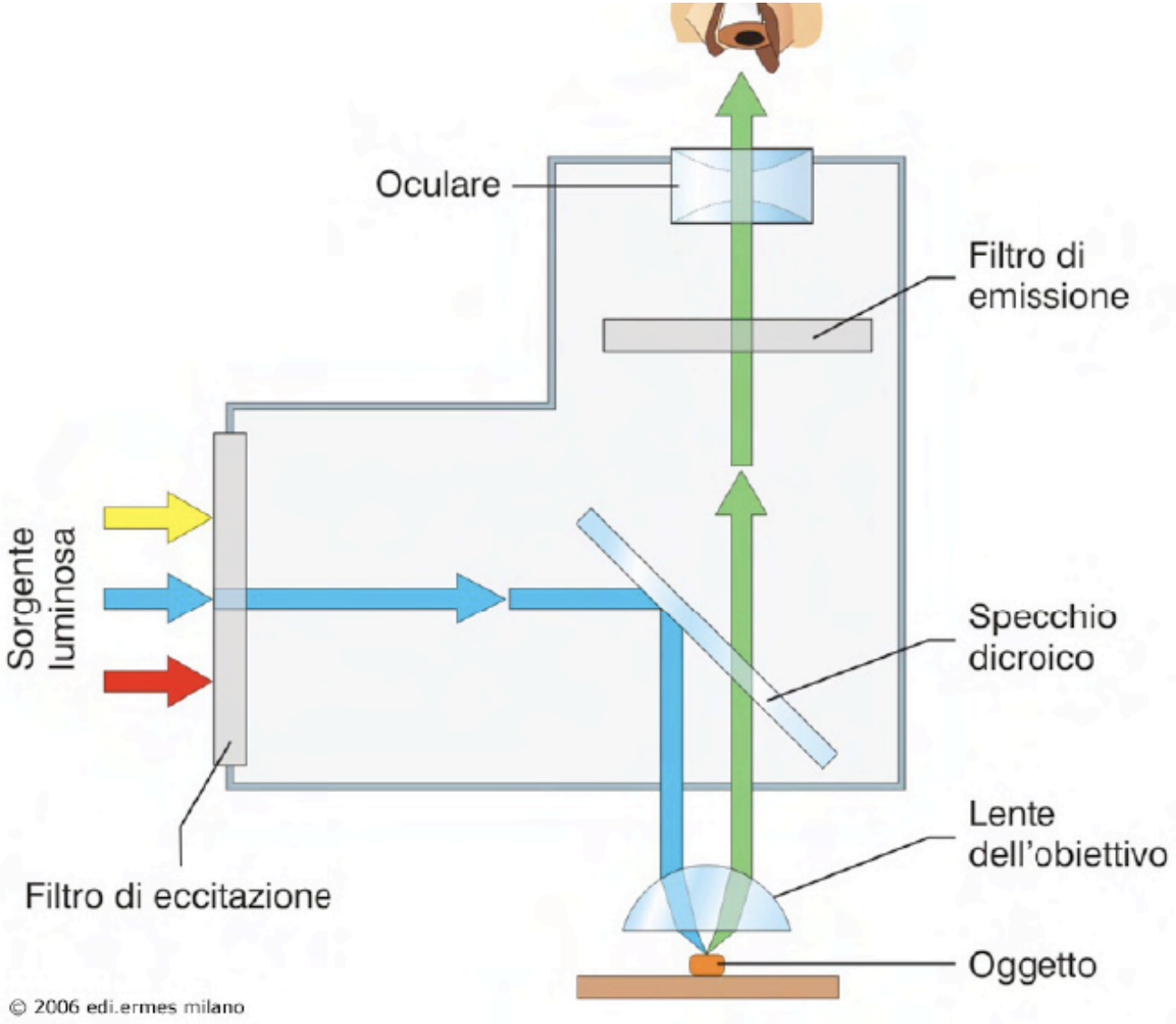


Spectral variants of fluorescent proteins

RFP~CFP < GFP <YFP

3 : 5 : 8 approximate relative brightness




Oculare
Filtro di emissione
Sorgente luminosa
Specchio dicroico
Lente dell'obiettivo
Filtro di eccitazione
Oggetto
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