Topic Overview

Fluorescence

Erkin Kuru

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1. Spectrum and Light
2. Color
   - Kelvin
3. Chromophores vs Flurophores
4. Fluorescence
   - Important terminology revealed
   - What makes a molecule a fluorophore?
5. Important fluorescent dyes
6. Conclusion: Consider following when creating a novel fluorophore

1. Spectrum and Light

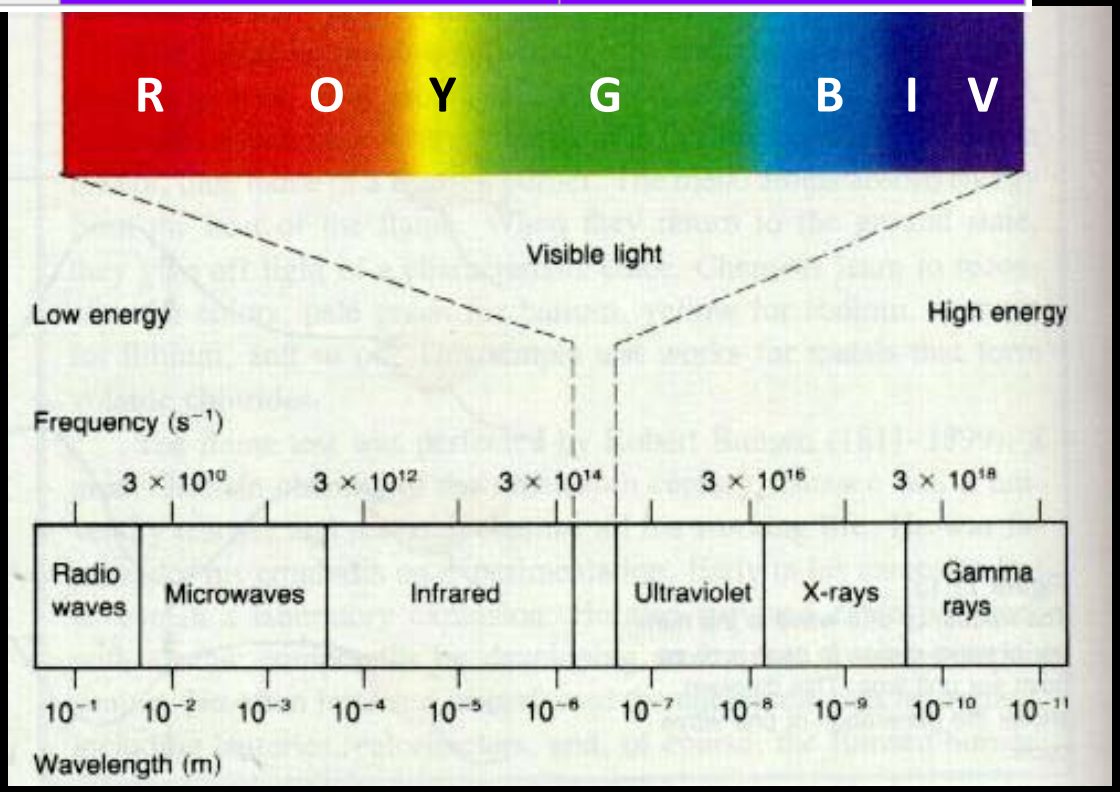
color	wavelength interval	frequency interval
red	~ 700–635 nm	~ 430–480 THz
orange	~ 635–590 nm	~ 480–510 THz
yellow	~ 590–560 nm	~ 510–540 THz
green	~ 560–490 nm	~ 540–610 THz
blue	~ 490–450 nm	~ 610–670 THz
violet	~ 450–400 nm	~ 670–750 THz

LIGHT = ENERGY

Frequency X Planck's = **ENERGY**

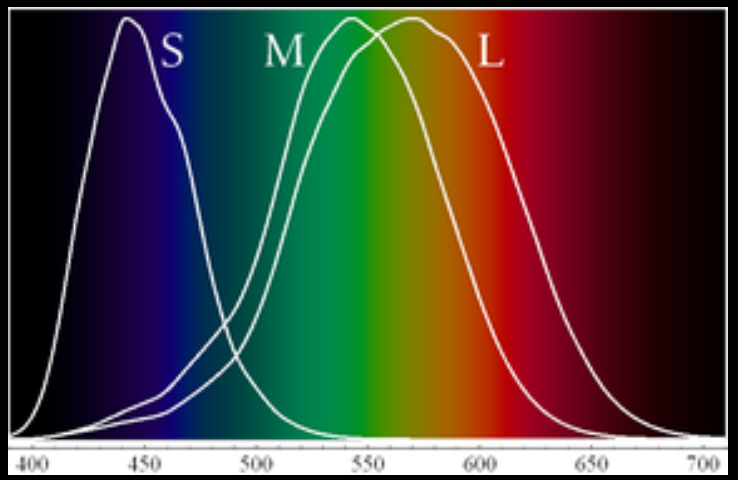
v X h = **ENERGY**

1/Wavelenght X Planck's = **ENERGY**



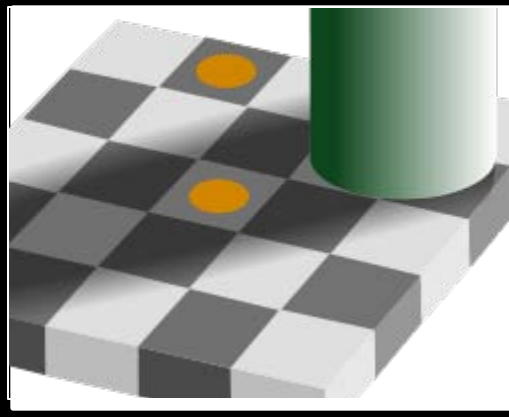
KNOW what color of a given wave length is

- Universe is colorless...
- Color is all about perception
  - Short (**S**) a.k.a. blue, Medium (**M**) a.k.a. green , and Long (**L**) a.k.a. red cone types



Cone type	Name	Range	Peak wavelength
S	β	400–500 nm	420–440 nm
M	γ	450–630 nm	534–545 nm
L	ρ	500–700 nm	564–580 nm

  - We will see the following object having the same color:
    - An object emitting/reflecting **green**
    - An object emitting/reflecting in **yellow** and blue together
  - Color is a function of physics of the object and perception
  - The Range means that different people might have different perception of colors…

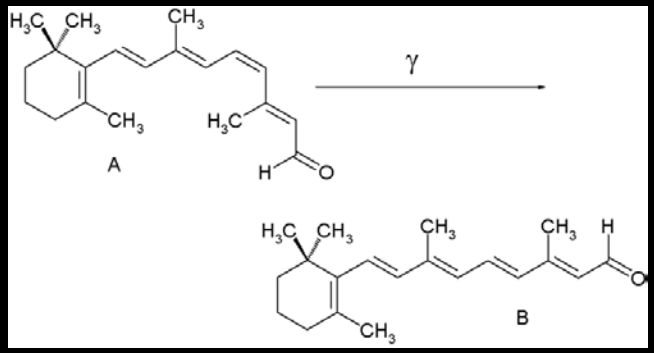


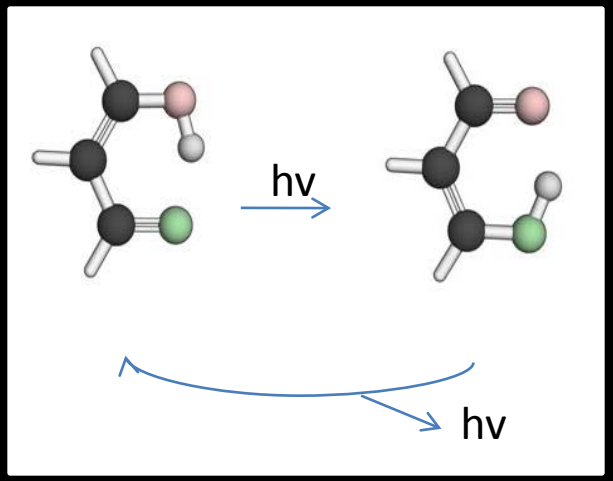
- Color can additionally be expressed in Kelvin
- Different photon energies that a metal emits at different Kelvins
- Very relevant for photography

Degrees Kelvin	Type of Light Source	Indoor (3200k) Color Balance	Outdoor (5500k Color Balance
1700-1800K	Match Flame		
1850-1930K	Candle Flame		
2000-3000K	Sun: At Sunrise or Sunset		
2500-2900K	Household Tungsten Bulbs		
3000K	Tungsten lamp 500W-1k		
3200-3500K	Quartz Lights		
3200-7500K	Fluorescent Lights		
3275K	Tungsten Lamp 2k		
3380K	Tungsten Lamp 5k, 10k		
5000-5400K	Sun: Direct at Noon		
5500-6500K	Daylight (Sun + Sky)		
5500-6500K	Sun: through clouds/haze		
6000-7500K	Sky: Overcast		
6500K	RGB Monitor (White Pt.)		
7000-8000K	Outdoor Shade Areas		
8000-10000K	Sky: Partly Cloudy		

Based on information from the book [digital] Lighting & Rendering
Chart and colors (c)2003 Jeremy Birn for www.3dRender.com

- Chromophores
  - A.k.a. Pigments
  - Will absorb certain wavelengths
  - Will reflect (or transmits) rest
  - Color of a chromophore is the collection of the **reflected** wavelenghts
  - It wont emit!
  - Color depends on the incident light!
  - Objects have the color of the light leaving their surface
- Fluorophores
  - Will absorb certain wavelengths
  - Reflected or transmitted light is not important, it will rather emit!
  - Color of fluorophore is the collection of **emitted** wavelenghts
  - Color is independent of the incident light!





Important similarity:

Things that make a molecule a chromophore or fluorophore is almost identical, I think…

-Conjugated molecules (e.g. aromatic groups)

-Metal complexes

4. Fluorescence: Important Terminology Revealed

- Chemoluminescence
  - Emission of light as the result of a chemical reaction (with limited emission of heat).

- Bioluminiscence
  - Production and emission of light by a living organism.
  - Is actually Bio-Chemoluminescence

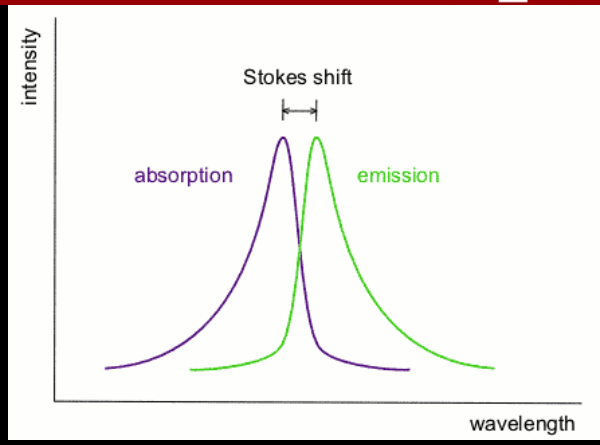
- Photoluminescence
  - absorption of photons causing re-radiation of photons

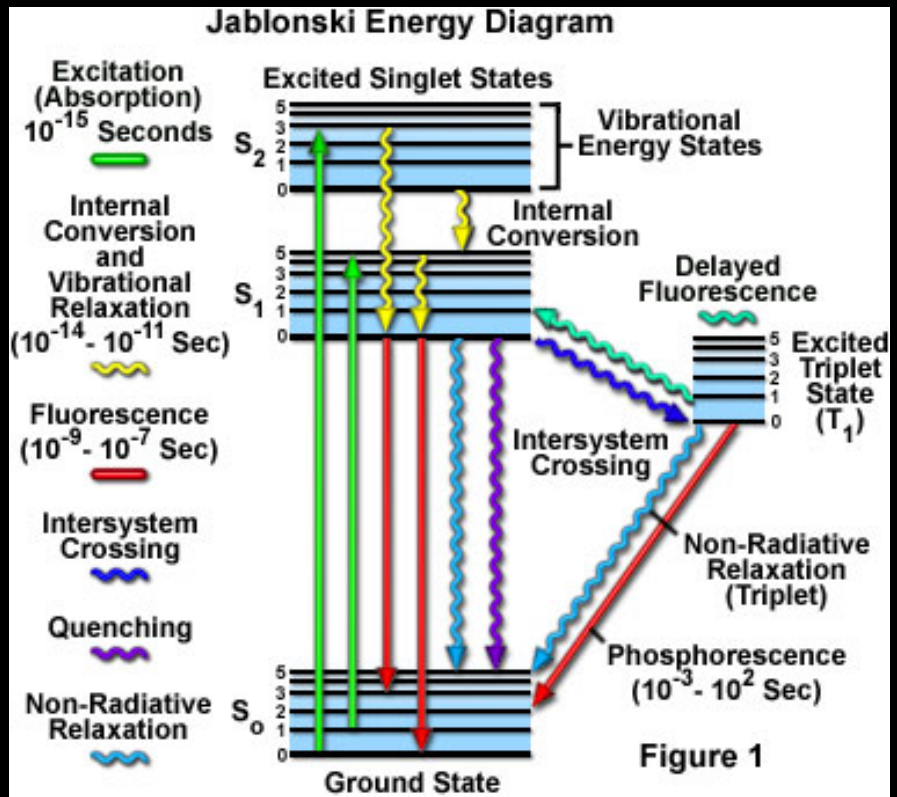
1. Photophosphorescence; delayed re-radiation
   - Transformation to triplet state
   - Energy in triplet state is trapped
   - Thus slow process of radiative transition to singlet state
2. Fluorescence
   - Instant radiative transition to singlet state.
   - Fast Emission

4. Fluorescence: Important Fluorescence Terminology Continued

- **Strokes shift:** The energy difference between the lowest energy peak of absorbance and the highest energy of emission
  - Kasha's rule:
    - Emission occurs only from S1
    - Emission wavelength is independent of excitation wavelength
    - With changing energy of illumination;

      only **intensity** will change you'll get the same emission spectrum





- Jablonski Energy Diagram

Tells it all…

- Extinction coefficient, ε
  - The molar extinction coefficient is a direct measure of a dye's ability to absorb light.
  - Larger extinction coefficients indicate that the absorption of a photon (or quantum) in a given wavelength region is more likely.
  - Varies between 5000 and 200,000 $cm^{-1}M^{-1}$ (at absorption maxima)
  - In general, dyes with large Stokes shifts have smaller extinction coefficients
- Quantum Yield, ϕ
  - Amount of absorbed energy which is released as energy (i.e fluorescence)
  - Ratio of the number of quanta emitted compared to those absorbed (and is usually a value between 0.1 and 1.0).
  - Quantum yield values below 1 are the result of the loss of energy through nonradiative pathways, such as heat or a photochemical reaction

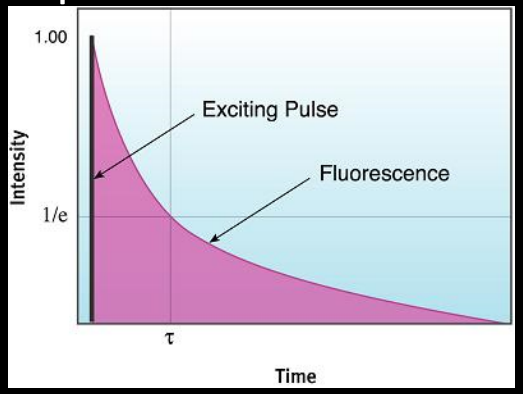
$$\phi = \frac{\text{Number of emitted photons}}{\text{Number of absorbed photons}}$$

  - Decrease in quantum yield upon coupling to proteins and nucleic acids
  - Very sensitive to the environment, pH, temperature, and the structure of the fluorophore itself.
  - A fluorophore might have a high extinction coefficient but low quantum yield and verse versa.
- Brightness = Quantum Yield x Extinction coefficient

4. Fluorescence: Important Fluorescence Terminology Continued

- Intensity: How many molecules at a given wavelenght is;
  - in excited state (in the case of excitation)

  OR
  - In ground state (in the case of emission)
  - Best way to increase signal is increasing fluorophore concentration
- Fluorescence lifetime



- Photo stability
  - Ability of a dye to undergo repeated cycles without being destroyed while in the excited state.
  - **Photobleaching** = Destruction of the excited state dye
  - Each fluorescein molecule can be excited on average about 30,000 times before it is destroyed. (an average stability)

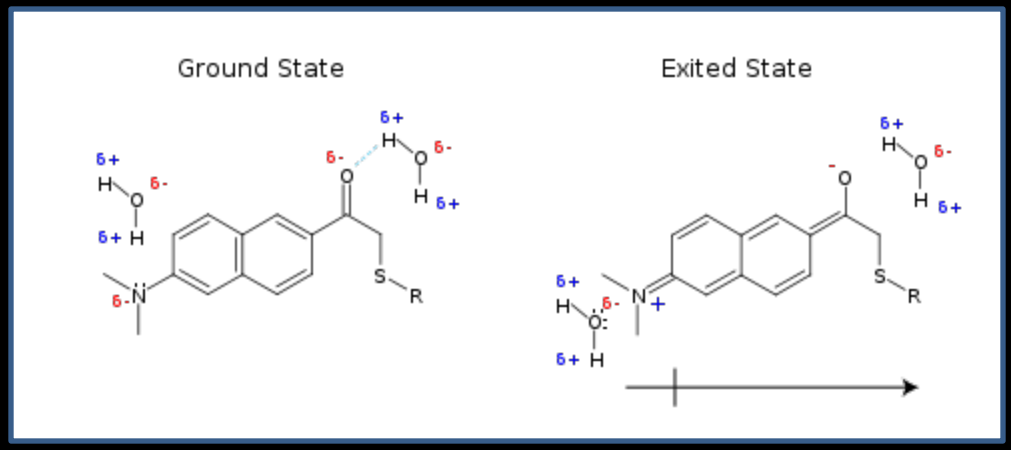
A newbie's naive attempt to understand…

1. Conjugated system

- Usually give strong colors
- Absorbtion of these compounds excites pi electrons (π to π*)
- If fewer than 8 conjugated double bonds = emission in UV and colorless
- Every additional double bond shifts emission spectra toward infrared. So the system will be mostly responsive to lower energy illumination.
- When excited, delocalized electrons stabilize the energy absorbed

2. Effect of the functional groups and environment

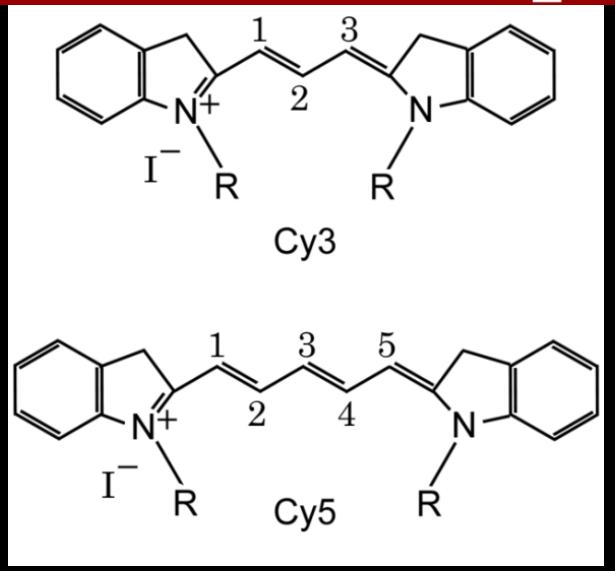
- When the electron donating and withdrawing groups are places on the opposite sides of an aromatic system and if it is a polar environment :
  - When Excited usually a larger dipole moment builds up
  - Before emission the polar solvent molecules reorrient stabilizing this otherwise unstable excited state
  - Energy of the excited state is lowered!!
  - Fluorophores with high dipole moment change has larger Stroke shift



5- Important Fluorescent dyes: **Cy3 , Cy5 and FITC**

Cy3 and Cy5

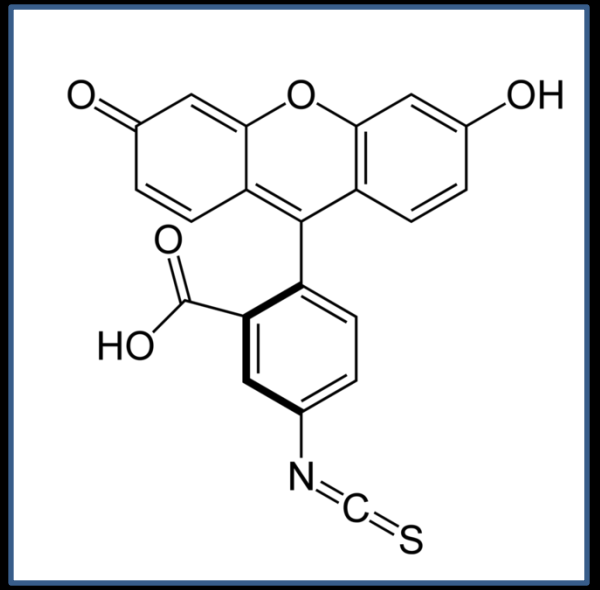
- Cyanine is name of the synthetic dye family
- Cy3 and Cy5 are water-soluble cyanines.
- Wide applications



	Absorption max. (nm)	Emission max (nm)	Emission color	Extinction coefficient	Quantum Yield
Cy3	~550	570	Green	70.000	0.15
Cy5	650	670	Red	69,000	0.28
FITC	495	521	Green	70,000	0.80

FITC

- Widely used
- reactive towards nucleophiles including amine and sulfhydryl groups on proteins
- Not very photostable
- A derivative is Alexa 488



5. Important Fluorescent dyes: **Seth's** & **BODIPY**

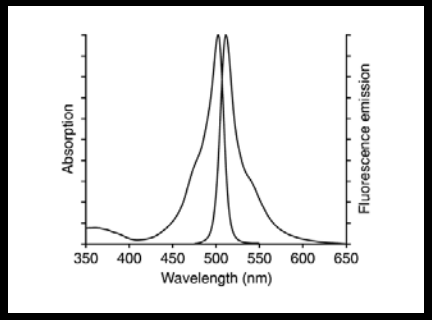
SETH'S

- Produced by Seth
- To be characterized.
- Cheap
- Water soluble
- Small
- Important Question: Is it photostable?

	Absorption max. (nm)	Emission max (nm)	Emission color	Extinction coefficient	Quantum Yield
Seth's	~375-400	~420-450	Blue	unknown	Unknown
BODIPY FL	505	513	Green	80,000	0.9

Boron-dipyrromethene (BODIPY)

- Small Stroke shift. (i.e excitation and emission maxima is very close)

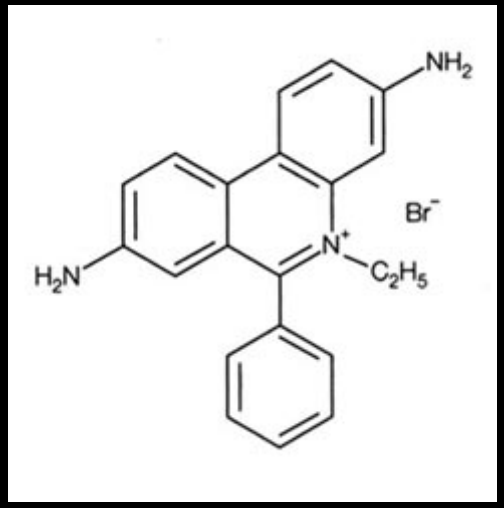


- Very high Quantum Yield even in water
- Usually environmental independent fluorescence

5. Important Fluorescent dyes: **Ethidium Bromide** & **DAPI**

ETHIDIUM BROMIDE (aka EtBr)

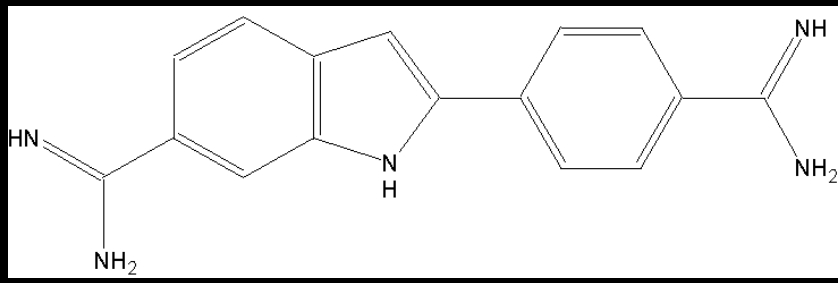
- Bind between base pairs in DNA
- When bound to DNA its fluorescence is enhanced 25 fold!
- Enhancement due to striped water molecules upon binding
- Cheap



	Absorption max. (nm)	Emission max (nm)	Emission color	Extinction coefficient	Quantum Yield
EtBr	302	602	Orange	5,000	0.3
DAPI	359	461	Violet	27,000	0.58

DAPI

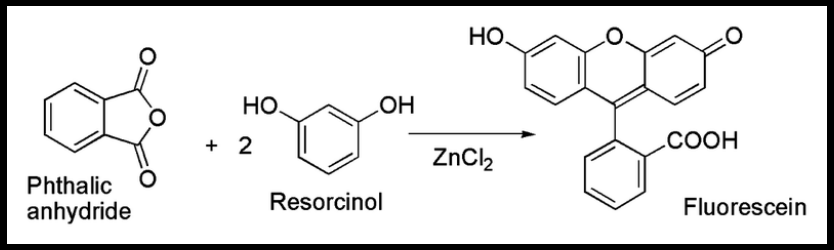
- Strongly binds to AT pairs of DNA
- Cell permeable!
- When bound its fluorescence increases significantly!

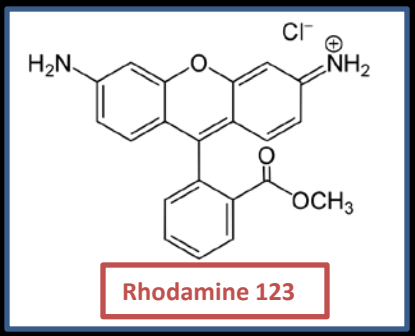


5. Important Fluorescent dyes: **Alexa Dyes**

Alkynyl Alexa 488

- Produced by Invitrogen
- Numbered according to their excitation maxima.
- Spans 350nm-750nm (with dozens of dyes)
- Synthesized through sulfonation of **coumarin**, **rhodamine**, **xanthene**, and **cyanine dyes**.
- Sulfonation makes Alexa Fluor dyes negatively charged and hydrophilic.
- Alexa Fluor dyes are generally more stable, brighter, and less pH-sensitive
- But much more expensive



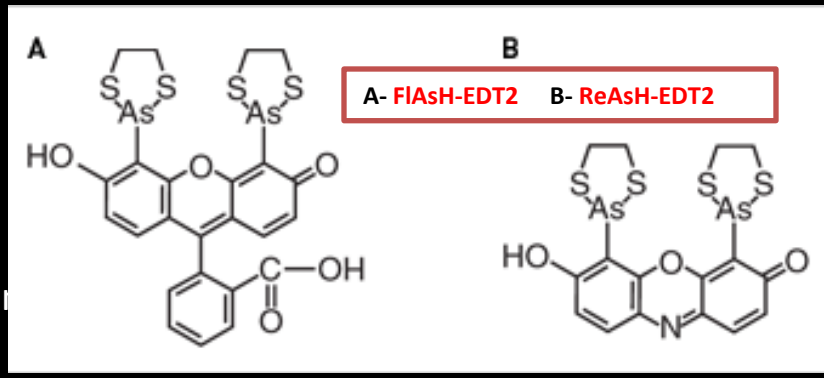


	Absorption max. (nm)	Emission max (nm)	Emission color	Extinction coefficient	Quantum Yield
Alexa Fluor® 350	346	442	Blue	19,000	Unknown
Alexa Fluor® 405	401	421	Blue	34,000	Unknown
Alexa Fluor® 430	433	541	Green/Yellow	16,000	unknown
Alexa Fluor® 488	496	519	Green	71,000	0.92

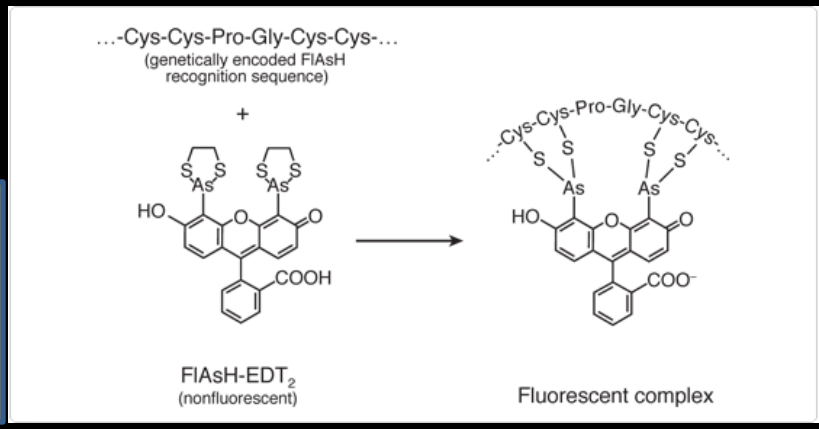
5. Important Fluorescent dyes: **FlAsH** & **ReAsH vs GFP**

FlAsH &ReAsH

- Developed by Roger Tsien
- Both biarsenical dyes
- Smallest expression tag based labelling
- Alternative top GFP? Comparable emission and extinctio
- Arsenic binds to a Tetracysteine tag.
- Fluorescent enhancement upon binding!
- We're considering to utilize this approach in the future
- It seems like it has big issues with nonspecific binding.

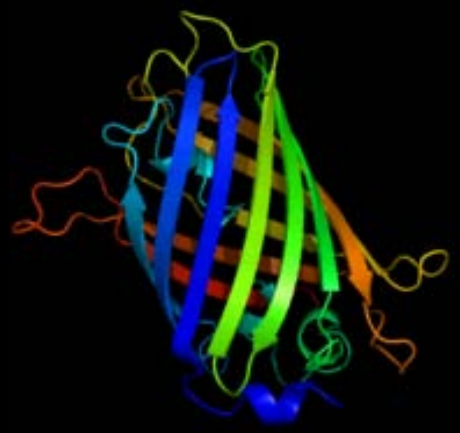


	Absorption max. (nm)	Emission max (nm)	Emission color	Extinction coefficient	Quantum Yield
FlAsH	~508	528	Green	70.000	0.85
ReAsh	593	608	Red	69,000	0.48
GFP	395	509	Green	55,000	0.60



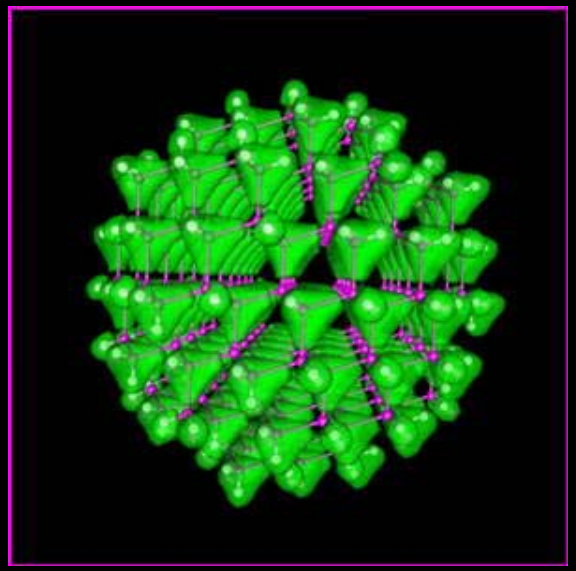
GFP

- Isolated by Roger Tsien
- Genetically encoded
- Huge 238 aa 30kDa



5. Important Fluorescent dyes(!): **Quantum Dots**

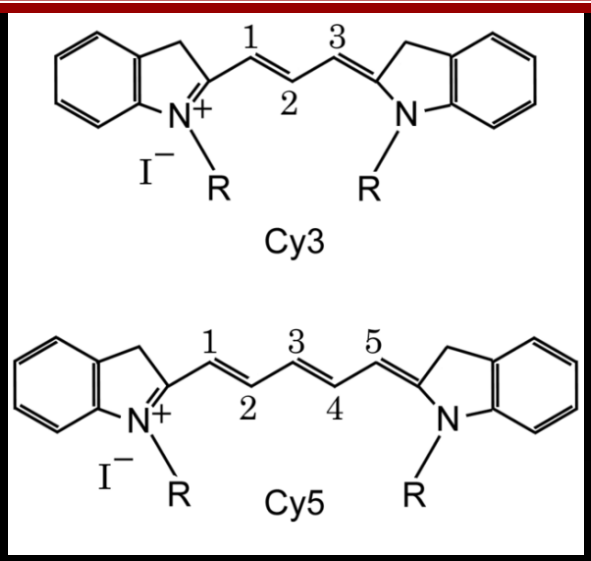
- Semiconductor nanocrystals. (100 – 100,000 atoms)
- Because they are semiconductors they can be excited and can emit light
- The smaller the crystal;
  - Higher gets the position of excited state
  - Higher gets the energy required to excite
  - Higher gets the energy released
  - Shorter gets the light emitted

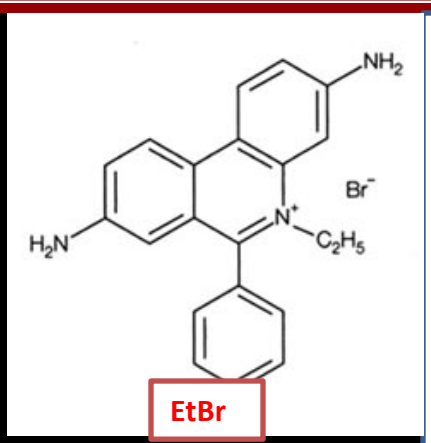


- High extinction coefficient (20 times)
- Comparable Quantum Yield
- Incredibly high photostability! (100 times)
- We should consider…

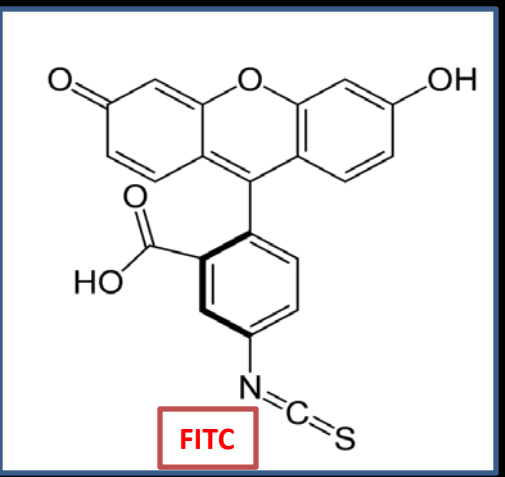
5. Important Fluorescent Dyes: Summary

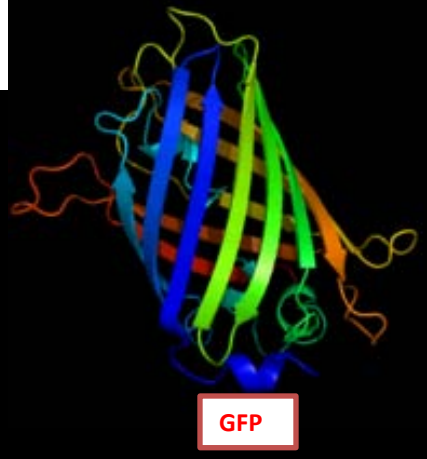
Alkynyl Alexa 488



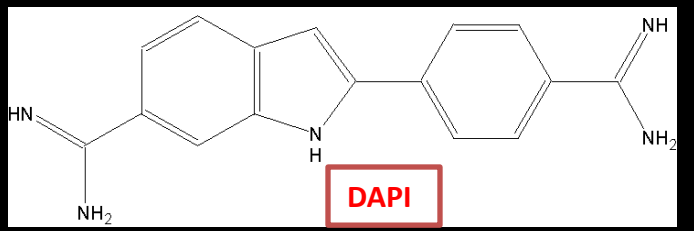


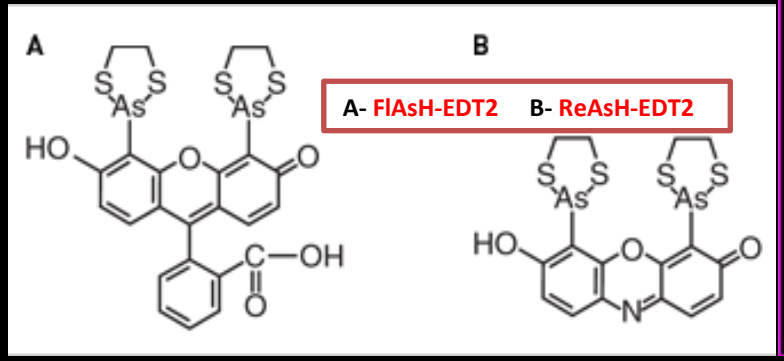
Seth's

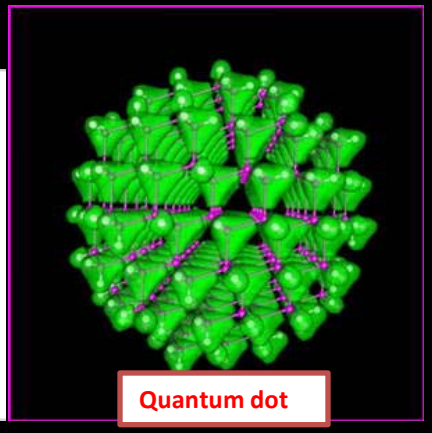




Alkynyl BODIPY







6. Conclusion: Consider following when creating a novel fluorophore

- Cheap
- Preferably small
- Conjugated system
- Modular
  - Easily add different reactive groups
  - Change Solubility
- Photostable
- pH stable
- Close to 1 Quantum Yield
- Brighter
- High absorbance (i.e. high Extinction coefficient)