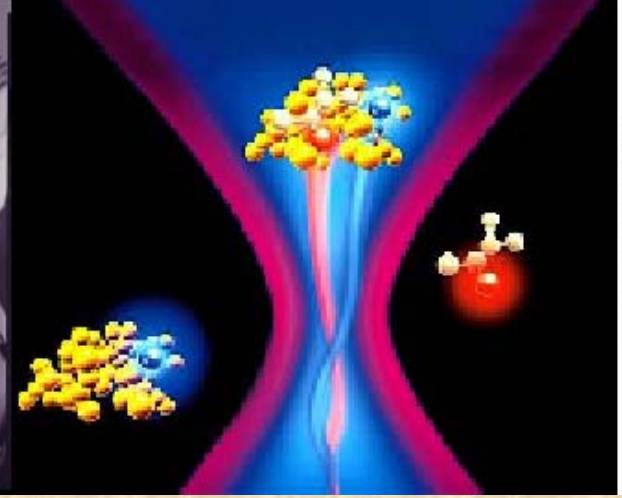
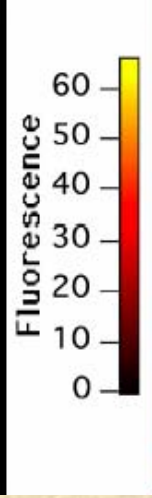
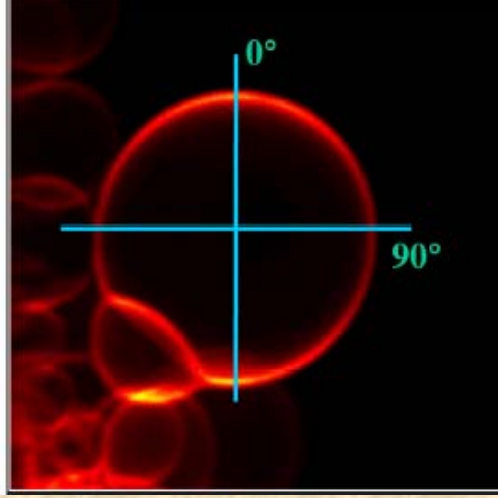






Fluorescence Workshop
UMN Physics
June 8-10, 2006

Basic Spectroscopic Principles
Joachim Mueller

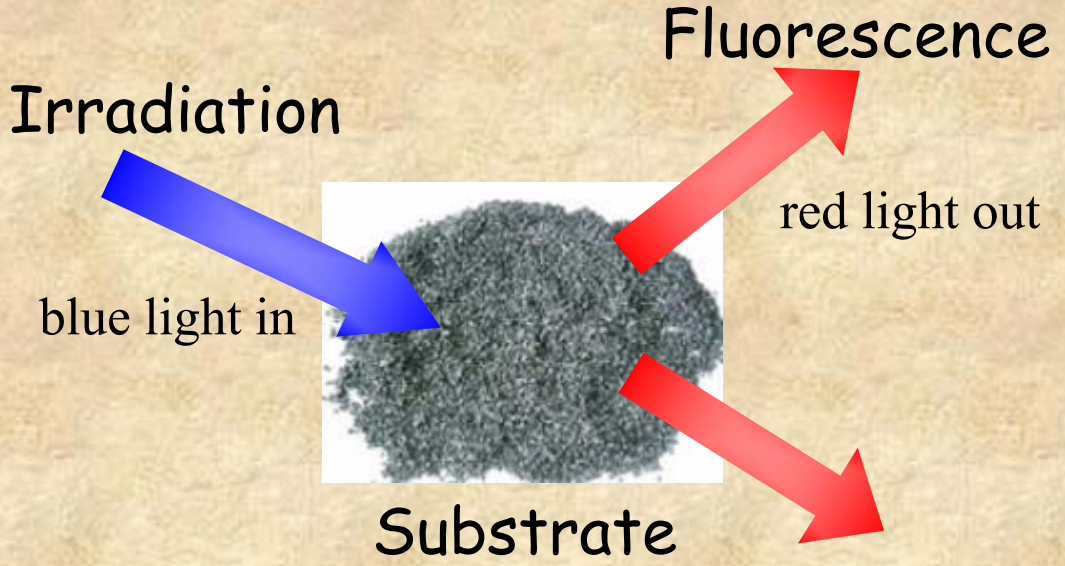
Fluorescence, Light, Absorption, Jablonski Diagram, and Beer-Law

What is fluorescence?

First stab at a definition:

Fluorescence describes the emission of light from a substance being irradiated by light of a different color

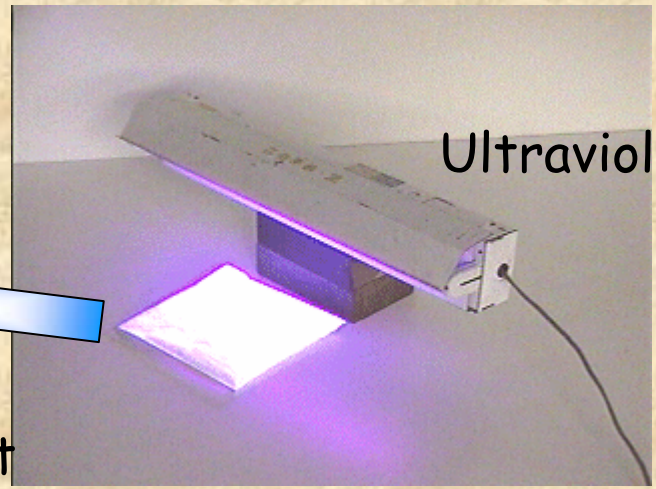
Example:



A fluorescent substance is also called a fluorophore

Example of a fluorescent substance

Whiter than white:



Fluorescent chemicals are typically added to laundry soap to make white clothing appear "whiter and brighter." In the presence of even a small amount of UV, the fluorescence due to the residual chemical remaining after the clothes are rinsed is sufficient to cause white shirts, socks, or T-shirts to have a blue/white glow. Soap manufacturers would like consumers to believe that their soaps are actually making the whites "whiter."

More examples

Tonic water contains quinine which fluoresces blue when exposed to ultraviolet light from a black light.

A $20 dollar bill has fluorescent strips that fluoresce when illuminated by the ultraviolet light

A $20 bill under normal room light illumination

A $20 bill illuminated with UV light.

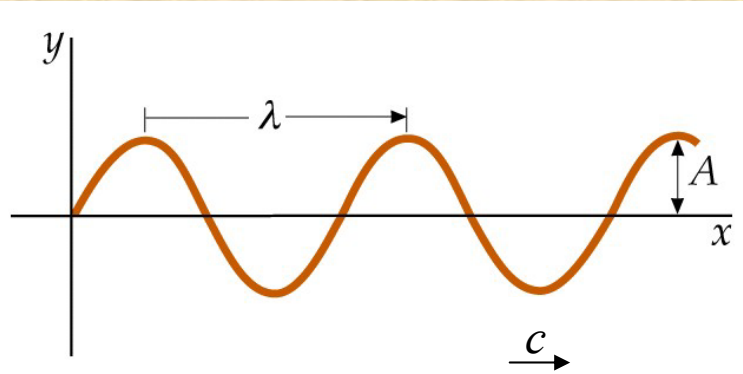
Fluorescein is a widely used fluorophore that emits in the green

Basic Properties of Light (1)

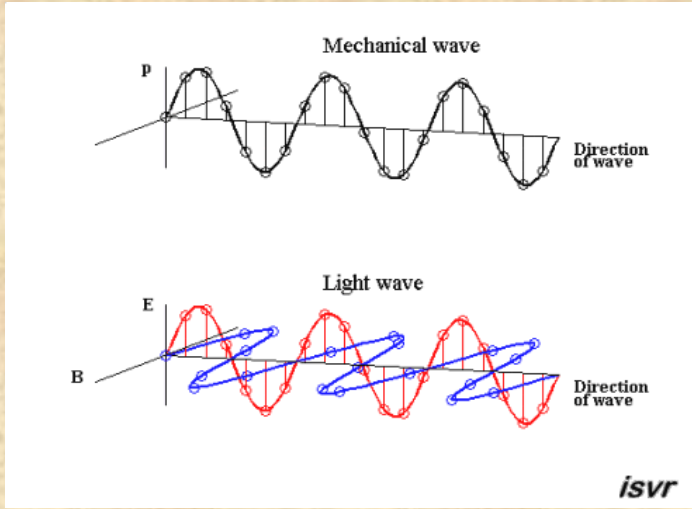
Light is form of electromagnetic radiation:

Two useful ways to look at light: as a wave or as a particle

- wave-picture



Amplitude A
wavelength λ
frequency f
velocity c



E: electric field
B: magnetic field

Tip: you can in almost all cases ignore the presence of the B-field in fluorescence applications.

speed of light: $c = \lambda f = 3.00 \times 10^8 \,\mathrm{m/s}$

Basic Properties of Light (2)

- particle-picture: Photons
  - Think of photons as the smallest 'unit' of waves
  - most phenomena related to light or electromagnetic radiation is explained by 'radiation-waves'
  - Fluorescence is usually explained by interaction of single photons and single molecules
  - The energy of a photon is proportional to the frequency

$$E = hf$$

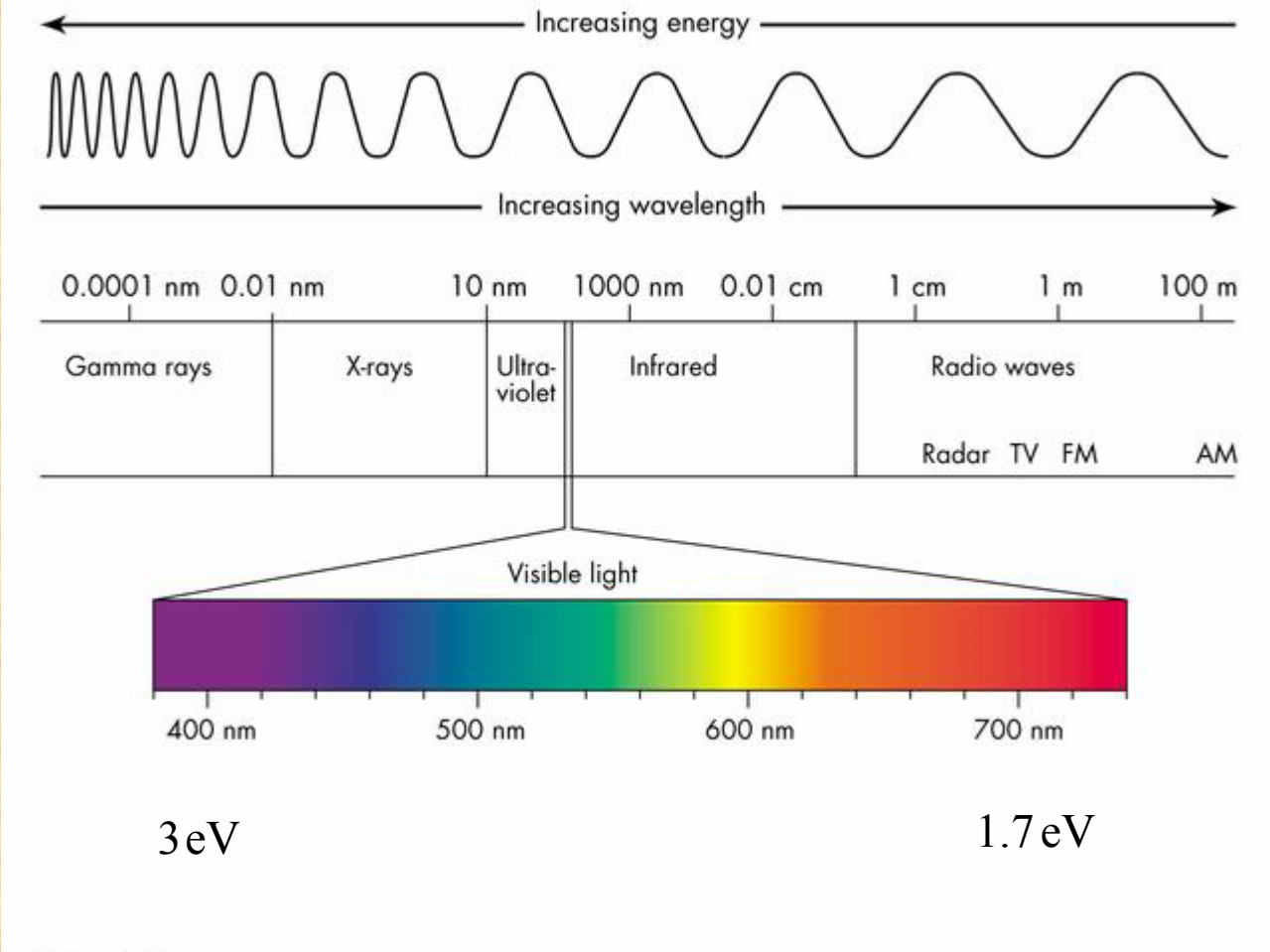
Planck's constant $h = 6.6 \times 10^{-34}\,\text{J}\times\text{s}$

$= 4.1 \times 10^{-15}\,\text{eV}\times\text{s}$

Remember: $c = \lambda f$, thus $E = hf = \dfrac{hc}{\lambda}$

Basic Properties of Light (3)

- Electromagnetic Spectrum



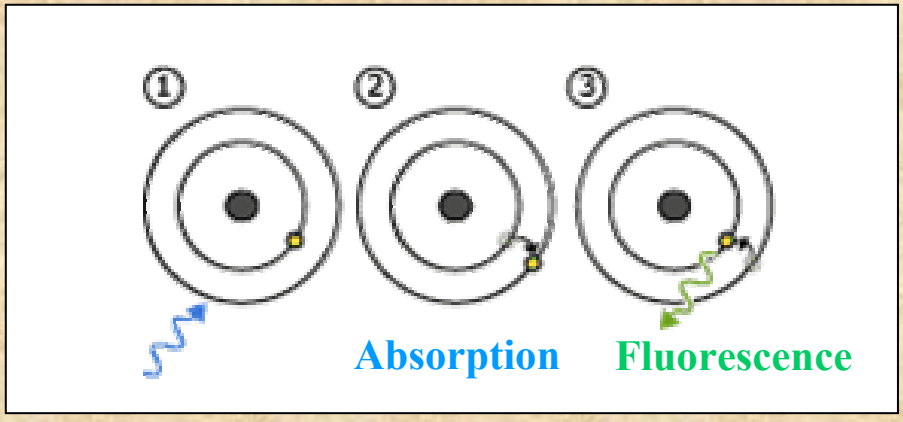
$$E = hf = \frac{hc}{\lambda}$$

3 eV

1.7 eV

Light Interaction with Matter

1) Blue photon hits atom
2) Electron (yellow) absorbs the blue photon and transitions to a previously empty orbital of higher energy
3) Electron (yellow) looses energy by emitting a green photon and falls back to its original orbital

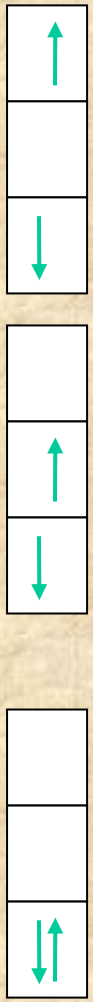


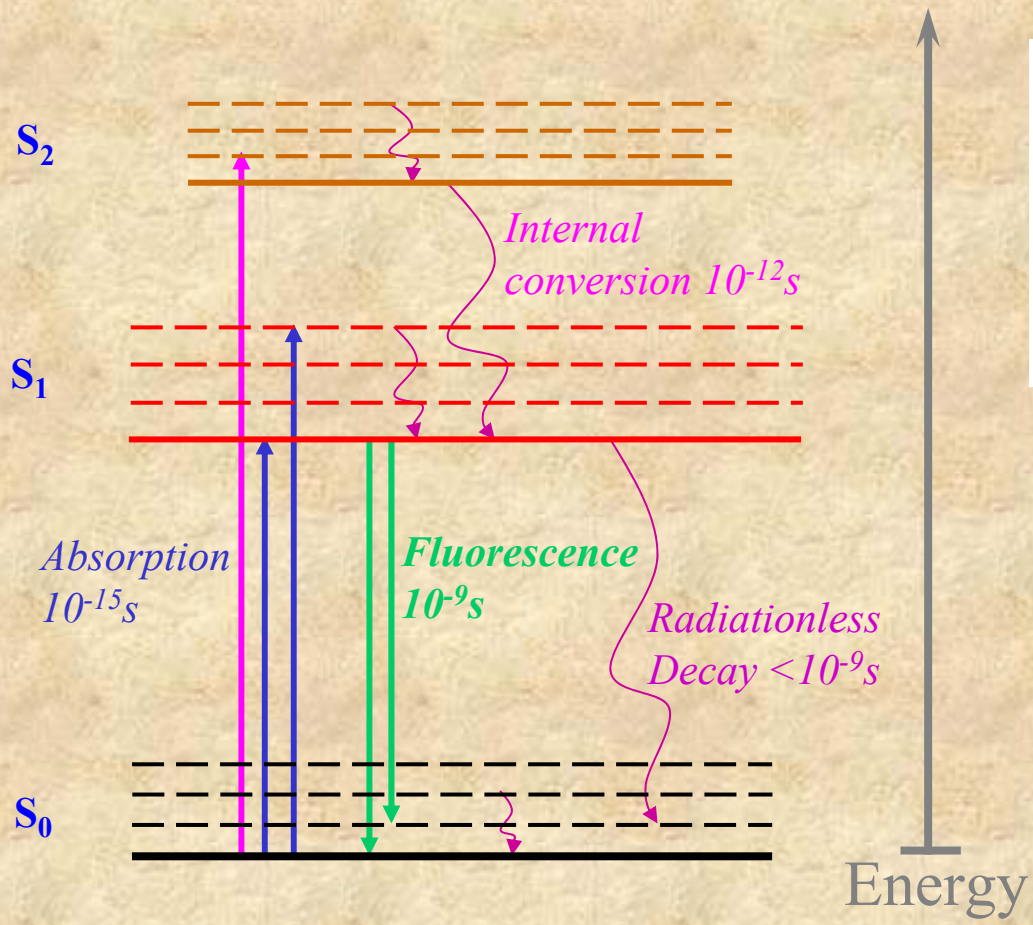
this process (illustrated for atoms) is also the same for molecules.

Note that the fluorescent light (green photon) has a lower energy than the absorbed light (blue photon). This was first observed by George Stokes (1852) and is known as **Stokes shift**.

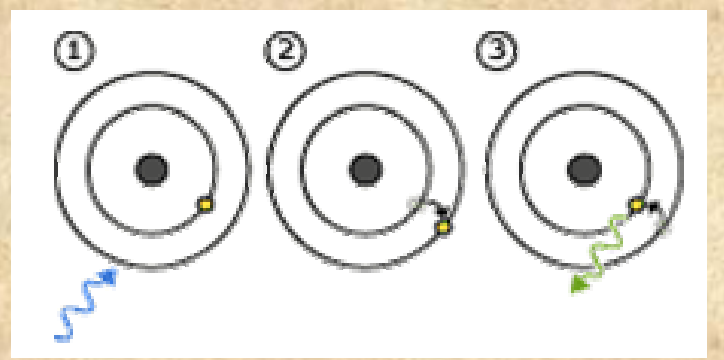
Simplified Jablonski Diagram

The life history of an excited state electron in a luminescent probe





Relate to picture from previous slide::



S : singlet state (all electrons in the molecule are spin-paired)

S_i : *i*-th electronic state

S_0 : electronic ground state

vibrational level

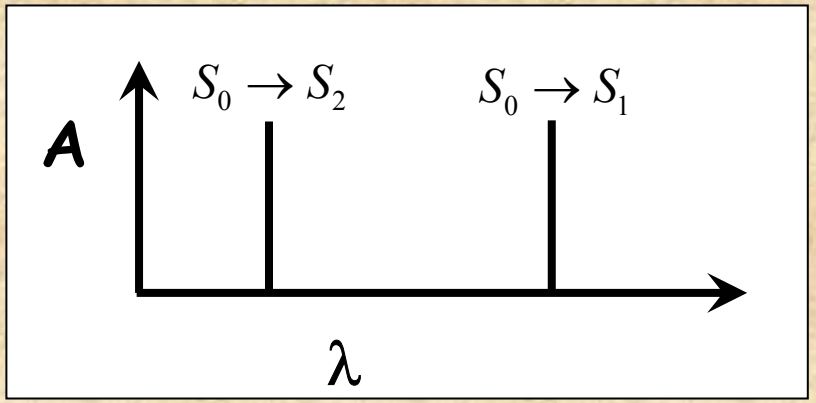
electronic level

Note:

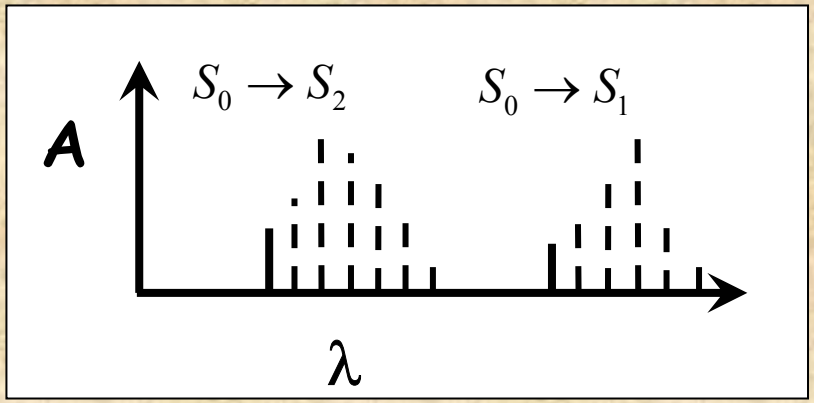
- ***Jablonski Diagram explains Stokes shift (emission has lower energy compared to absorption)***
- ***Most emission (fluorescence) occurs from the lowest vibrational level of S_1***

A Quick Note: Solvent Broadening

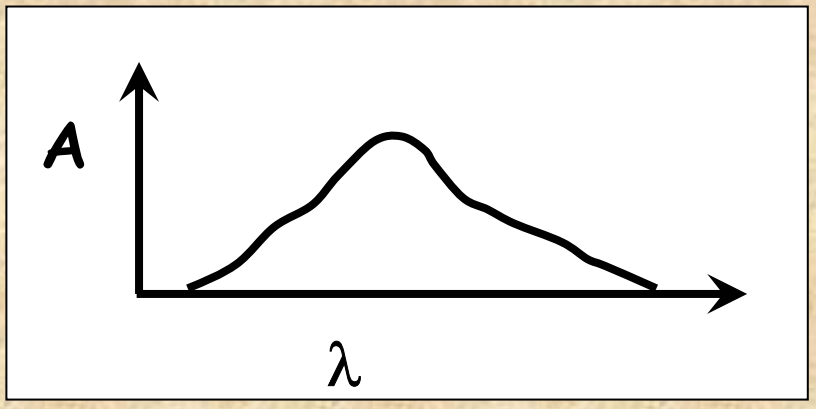
- electronic transitions give rise to sharp line spectra



- including vibrational states broadens the spectrum, but still a line spectrum



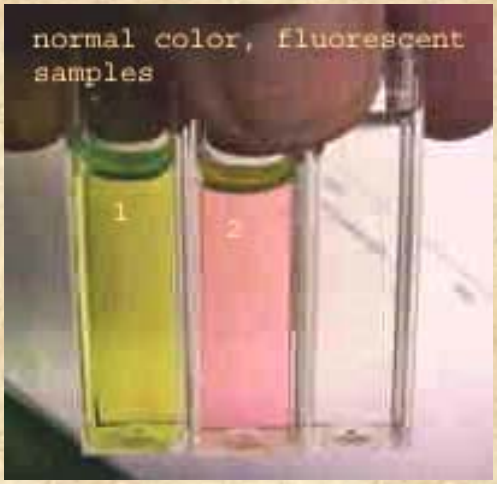
- but real spectra in solution look like this



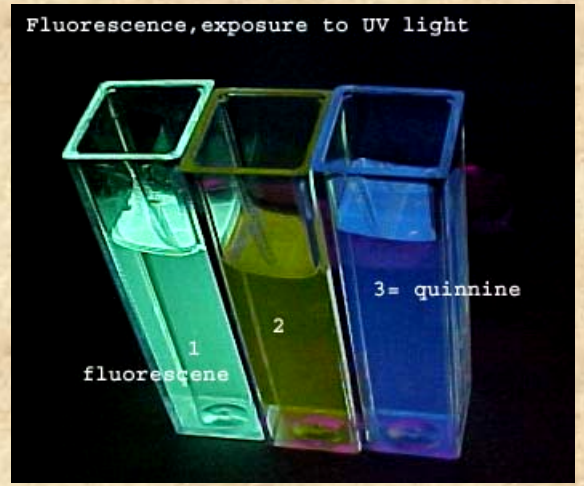
Exercise

Three samples:
(1) Fluorescein solution (2) Rhodamine (3) Quinine

room light



exposure to UV light



exposure to blue light

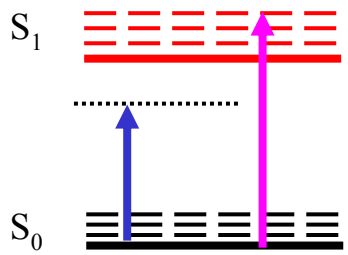
Why is there no fluorescence of the quinine solution when exposed to blue light?

(1) Fluorescein is yellow (because it absorbs in the blue)
(2) Rhodamine is pink (because it absorbs green light)
(3) Quinine is colorless (because it absorbs no visible light)

When exposed to UV light all three species fluoresce

- remember that quinine is colorless
- absorbs no visible light (including blue)
- therefore it is not excited and can't fluoresce

insufficient energy of blue photon to reach the first excited state of the quinine molecule; thus no absorption and therefore no fluorescence.



energy of UV photon matches the energy needed to reach the first excited state; thus absorption and subsequent fluorescence.

Stokes Historic Experiment (1852)

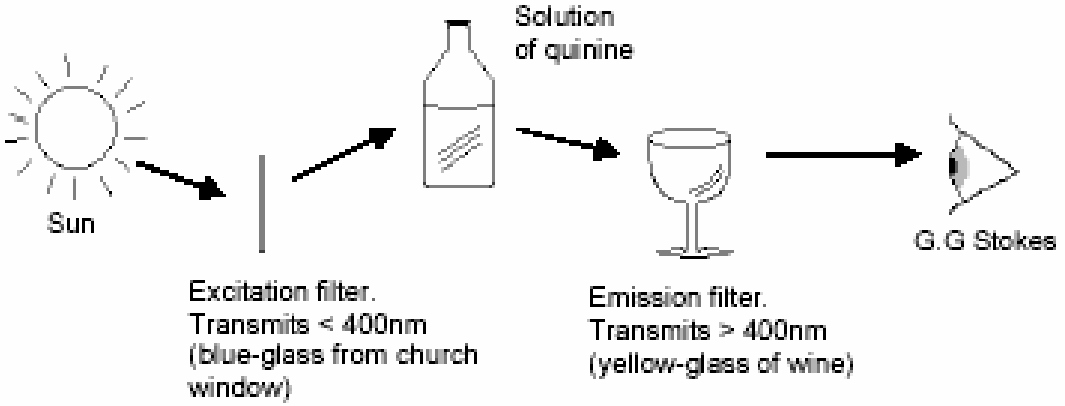
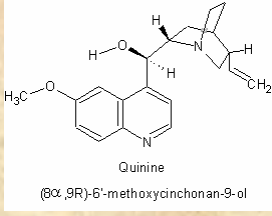
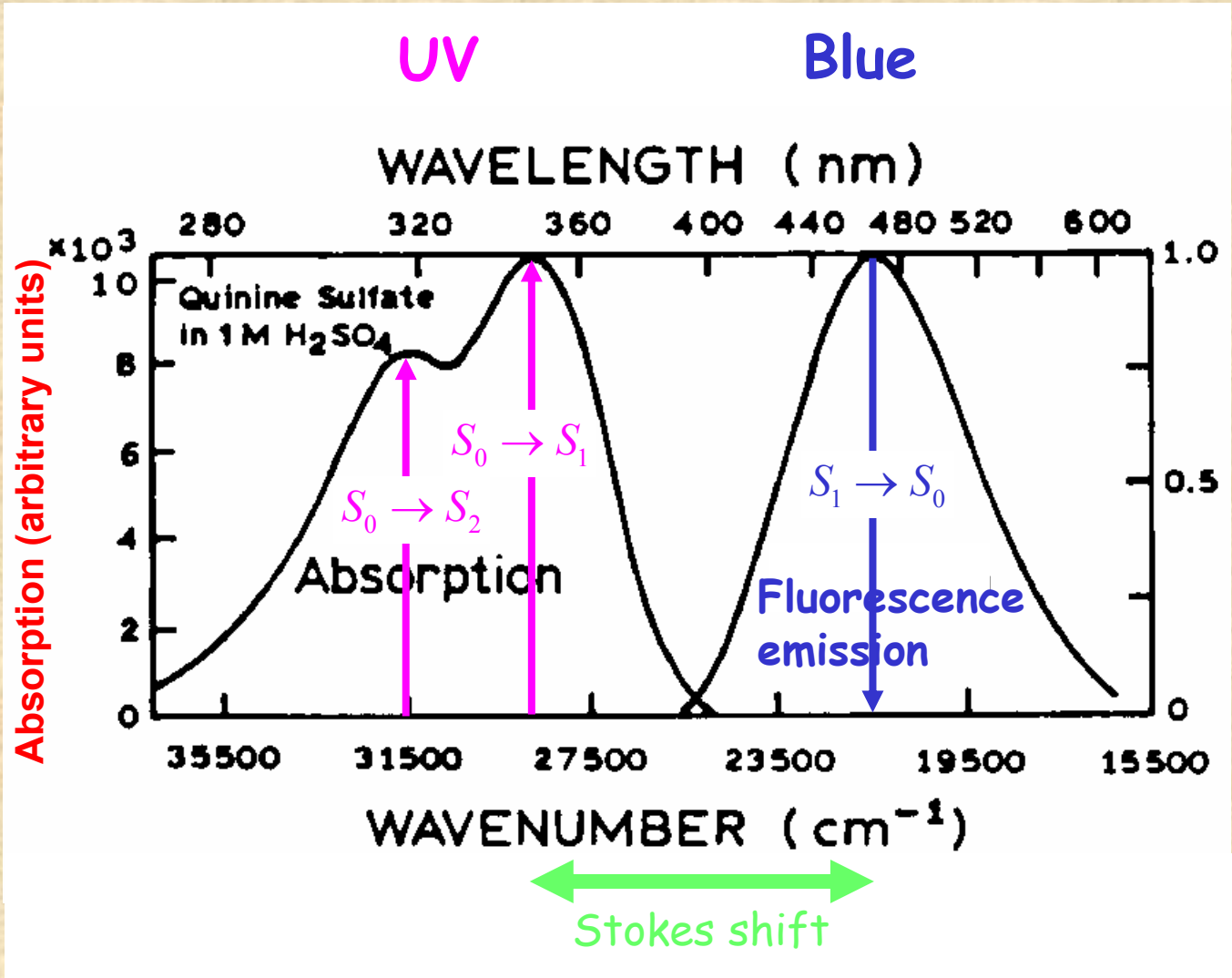


Figure 1-2. Experimental set-up used by G.G. Stokes.

In the experiment outlined, the blue glass transmits light below 400nm that is absorbed by quinine. The light emitted from quinine has a longer wavelength (450nm) and is allowed to pass through the glass of wine and reach the observer. If however the filters (blue glass and glass of wine) switched place the UV radiation could not reach the quinine solution, and the emission could not penetrate the wineglass.

Absorption spectrum of quinine

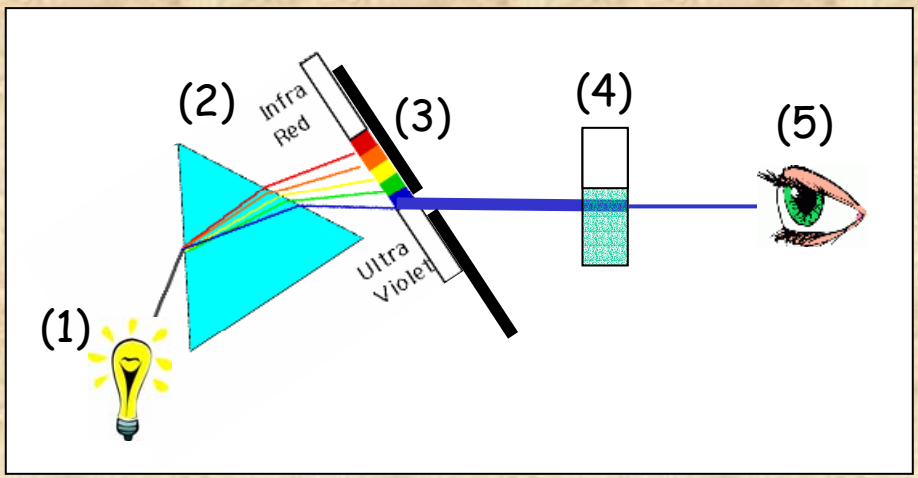




Note that the spectra are broad, because of **solvent broadening**

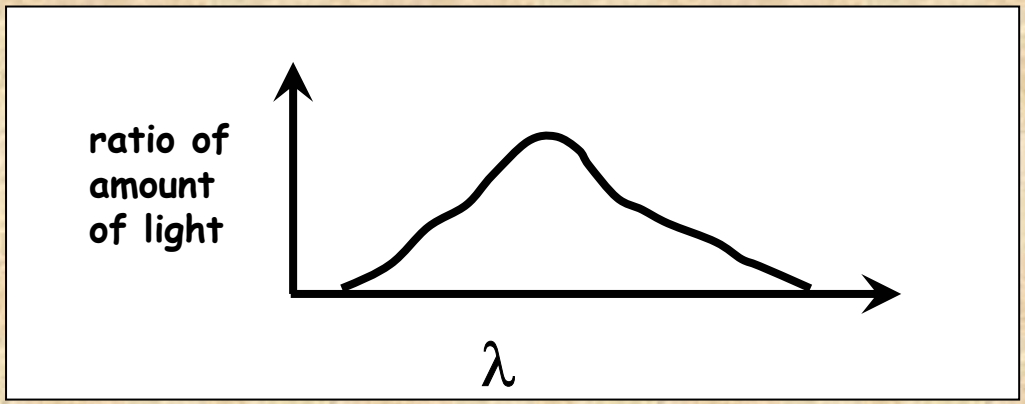
Absorption Spectrometer: Principle

Idea: Measure how much light is absorbed by substrate



(1) white light source
(2) separate into colors (wavelengths)
(3) select color with a slit
(4) Light passes through sample
(5) Transmitted light is measured with a photo detector
(6) Compare the amount of light received with and without sample

Repeat this measurement at all wavelengths (colors) of interest and plot the ratio of light with and without sample



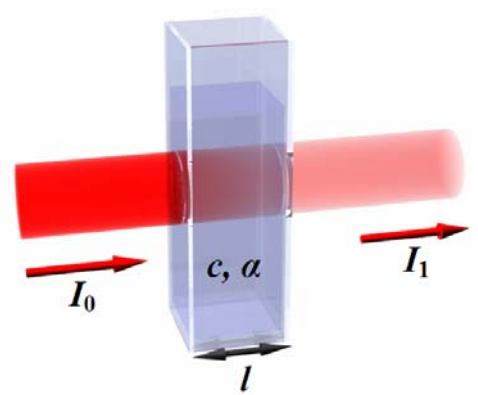
Absorbance A and Transmittance T

$$\text{Intensity } I = \frac{\text{Energy}}{\text{time} \times \text{Area}} = \frac{\text{Power}}{\text{Area}}$$

$$\text{Energy} = (\#\text{ of photons}) \times \left(\frac{hc}{\lambda}\right)$$

energy per photon

Intensity of light decreases as it passes through the sample because of absorption of photons



I_0 : intensity of incident light
I_1 : intensity of transmitted light

$$\text{Transmittance } T \equiv \frac{I_1}{I_0}$$

$$\text{Absorbance } A \equiv -\log T = -\log \frac{I_1}{I_0}$$

Interpretation of Transmittance:

$$T(\lambda) = \frac{I_1}{I_0} = \frac{\frac{\text{Energy}_1}{\text{time} \times \text{Area}}}{\frac{\text{Energy}_0}{\text{time} \times \text{Area}}} = \frac{(\#\text{ of transmitted photons per second})}{(\#\text{ of incident photons per second})} \frac{hc/\lambda}{hc/\lambda}$$

$$T(\lambda) = \frac{\#\text{ of transmitted photons per second}}{\#\text{ of incident photons per second}}$$

Absorbance *A* and Transmittance *T*

Absorbance $A \equiv -\log T$

Transmittance $T \equiv \frac{I_1}{I_0} = \frac{(\#\text{ of transmitted photons per second})}{(\#\text{ of incident photons per second})}$

Note:
Although Absorbance has no physical units it is custom to add Optical Density (OD) to the absorbance value.

T	A	
1	0 OD	for every 100 photons entering the sample, 100 leave
0.1	1 OD	for every 100 photons entering the sample, 10 leave
0.01	2 OD	for every 100 photons entering the sample, 1 leaves
0.001	3 OD	for every 1000 photons entering the sample, 1 leaves

The useful range of most absorption spectrometers is 0.01-2.0 OD units

A = 3 OD requires to distinguish between 999 and 1000 detected photons!
That is difficult to achieve.

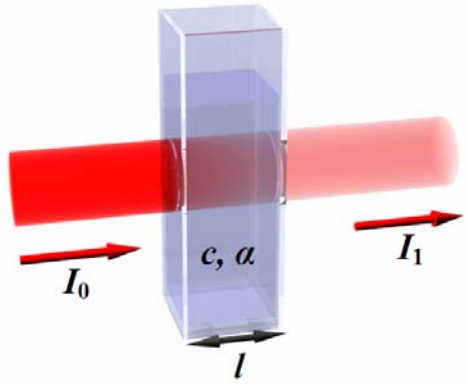
The Beer-Lambert Law

the Beer-Lambert law (also known as Beer's law) relates the absorption of light to the properties of the material through which the light is traveling.

$A \propto c$ *Absorbance is proportional to concentration*

$A \propto l$ *Absorbance is proportional to length of optical path through sample*

Beer-Lambert law $A = \varepsilon c l$



The proportionality constant ε is called the molar extinction coefficient

Example:

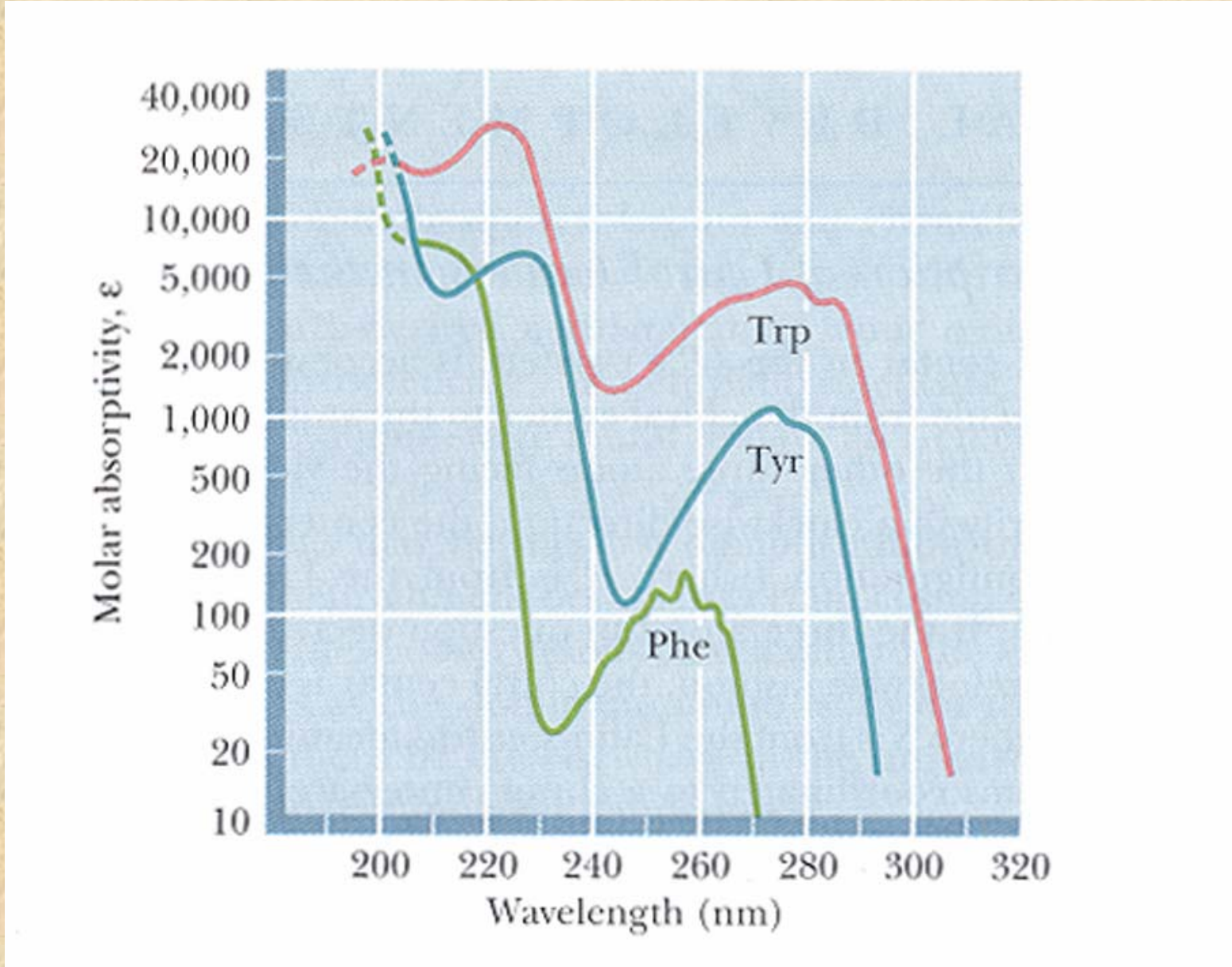
The extinction coefficient of fluorescein (pH 9.5) is ~93,000 $M^{-1}cm^{-1}$ at 490nm

The length of the cuvette is 1 cm.

An absorption of 0.019 corresponds to a concentration of ~2 x 10^{-7} M

An absorption of 1.86 corresponds to a concentration of 2 x 10^{-5} M

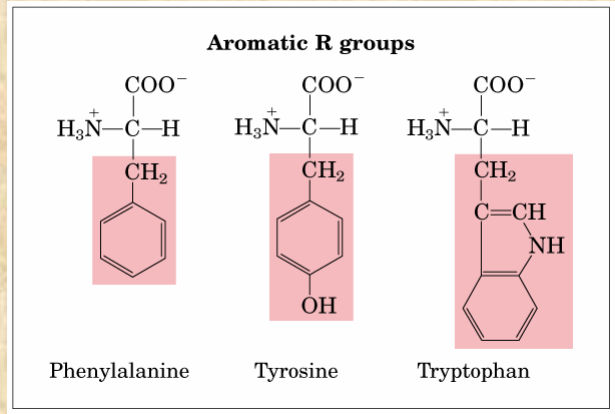
Absorption Properties of Proteins



Intrinsic Protein Fluorescence

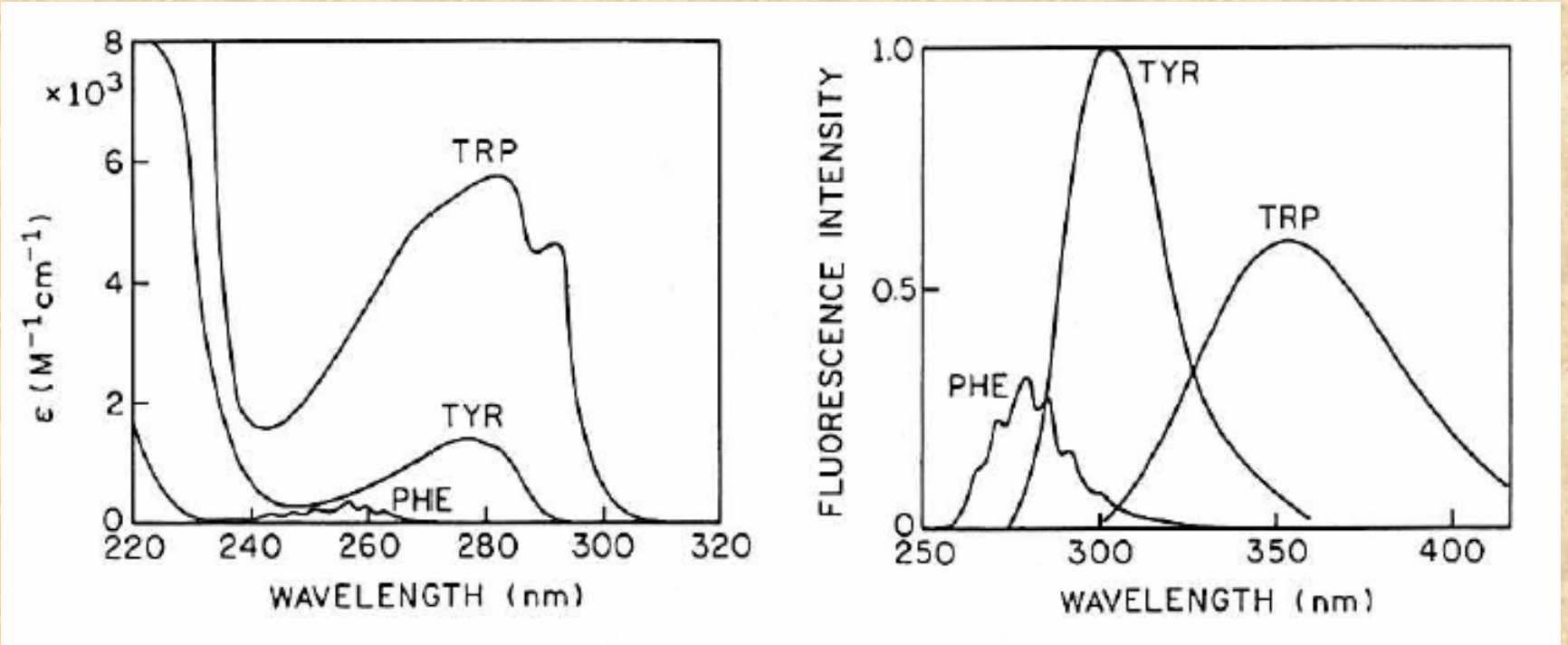
Fluorescent Amino Acids:

tryptophan (trp)
tyrosine (tyr)
phenylalanine (phe)



Absorption

Emission

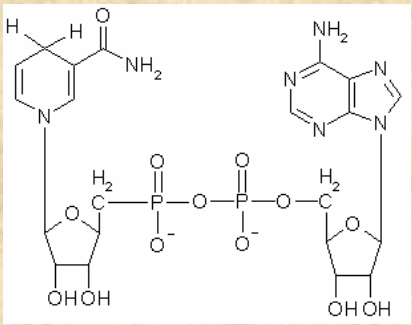


Intrinsic Protein Fluorescence

Fluorescent enzyme cofactors that bind to proteins:

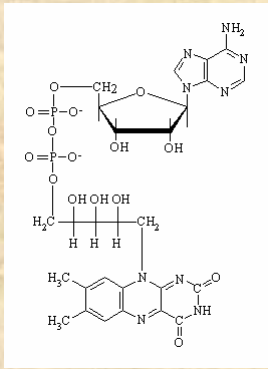
Examples: NADH
Riboflavins

NADH

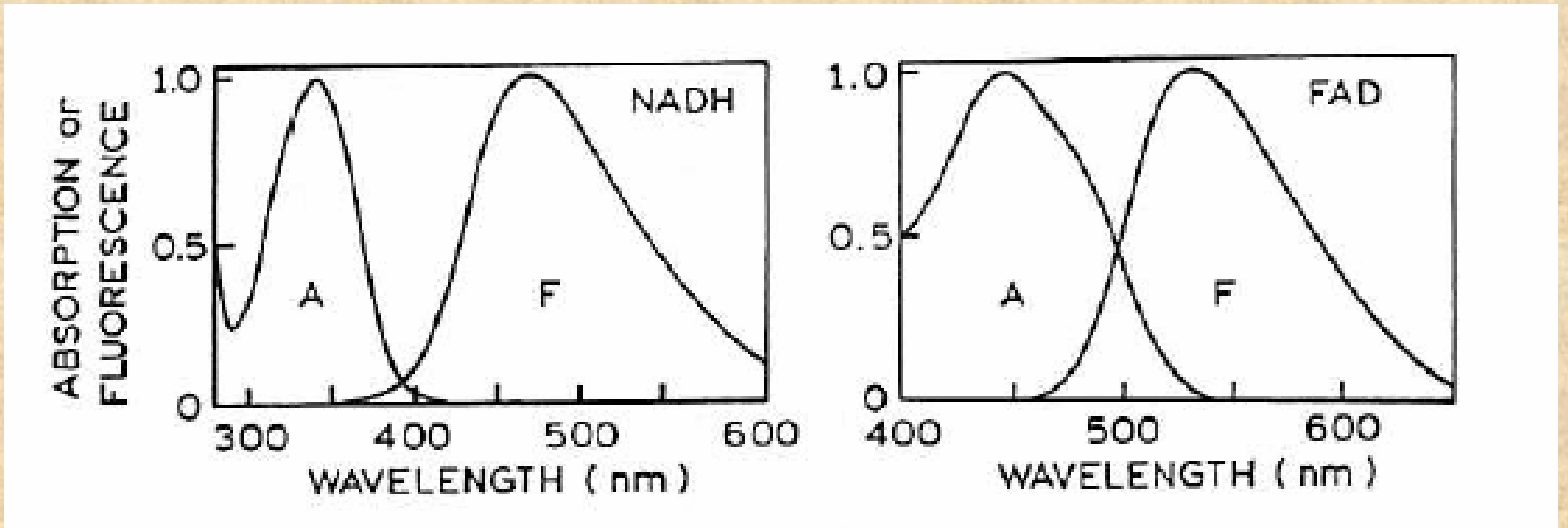


Nicotinamide Adenine Dinucleotide

FAD

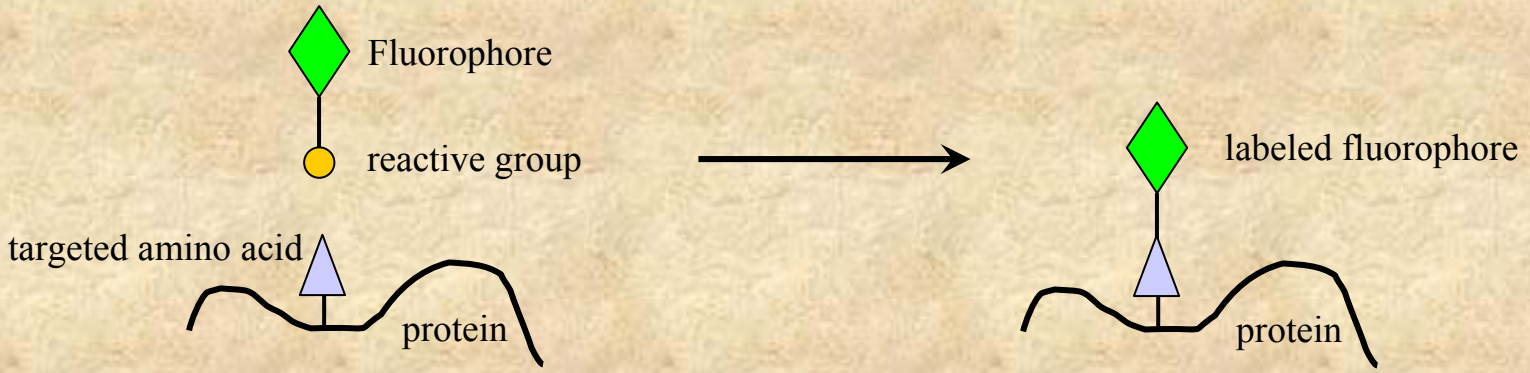


flavin adenine dinucleotide (FAD)



Extrinsic Protein Fluorescence

Covalent labeling of proteins with fluorophores

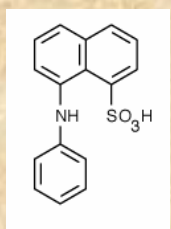


Example: amine-specific reagents (targets lysine)
thiol-specific reagents (targets cysteine)

Non-covalent labeling of proteins with fluorophores

Example: ANS

1,8-ANS (1-anilinonaphthalene-8-sulfonic acid)



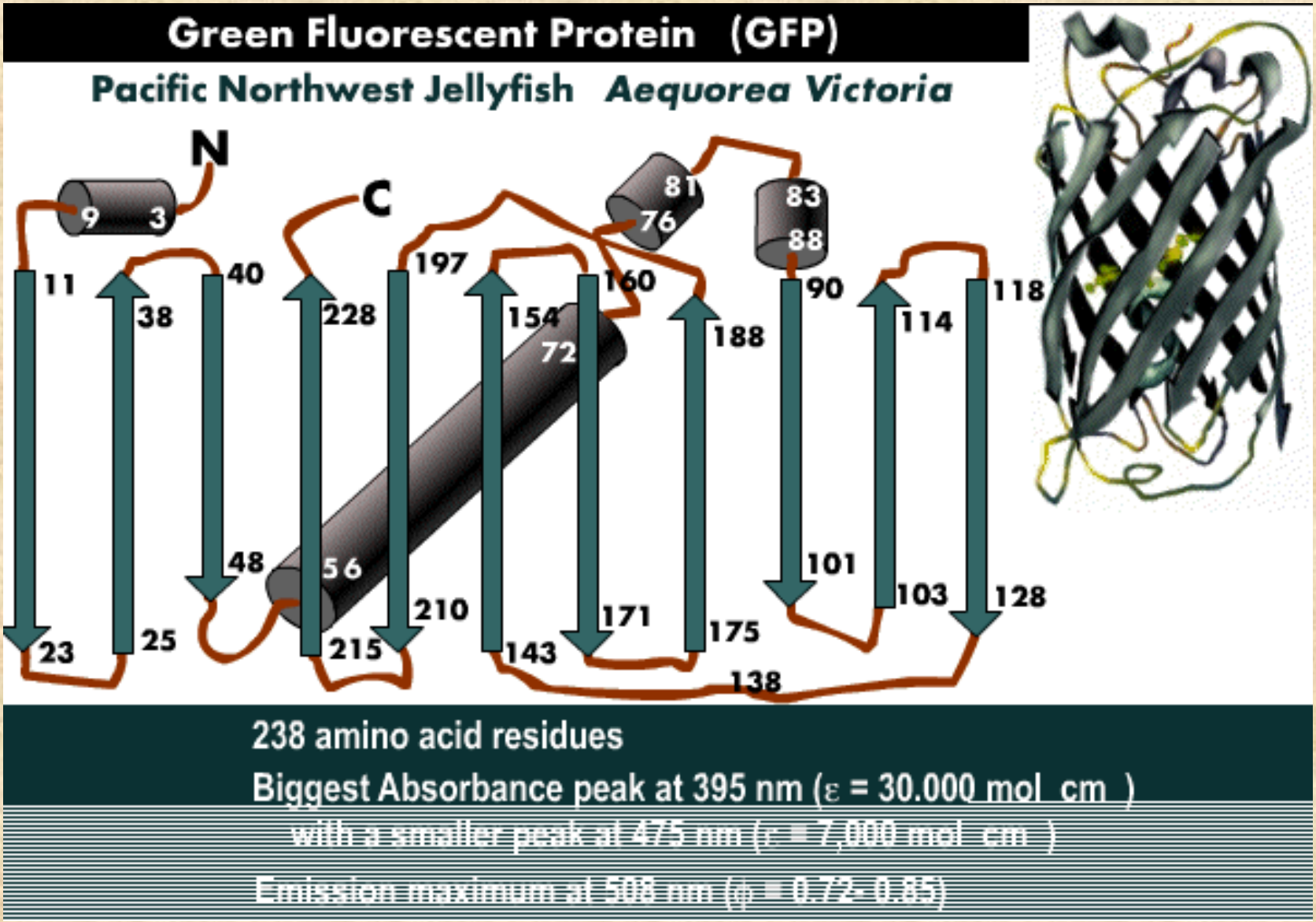
This dye binds to proteins that have hydrophobic pockets (bovine serum albumin) Dyes of this class are typically weakly (or nonfluorescent) in aqueous solution, but acquire strong fluorescence when bound to proteins.

Note fluorescent labels for membranes and DNA (RNA) exist, but won't be discussed here.

Green Fluorescent Protein

The past decade has witnessed an explosion in the use of the family of naturally fluorescent proteins known as Green Fluorescent Proteins or GFPs. GFP, a protein containing 128 amino acid residues, was originally isolated from the pacific northwest jellyfish *Aequorea Victoria*.

Secondary/3-D structure of the GFP

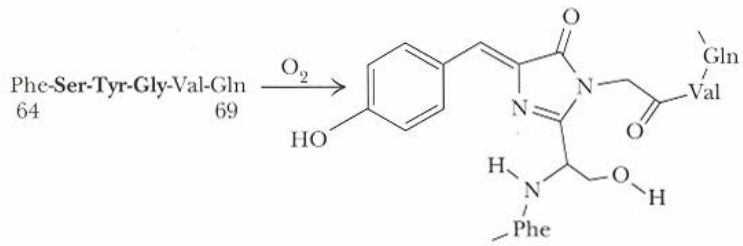


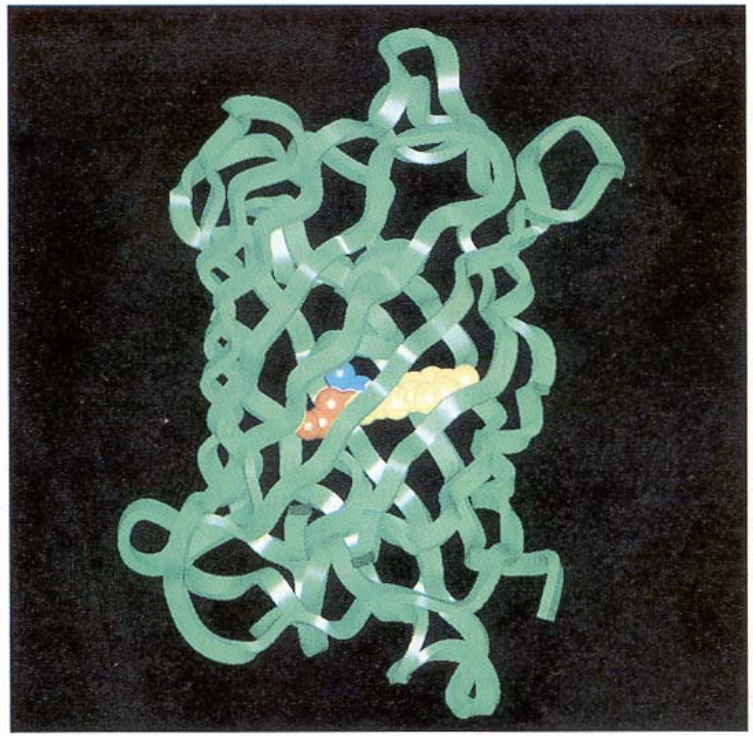
GM

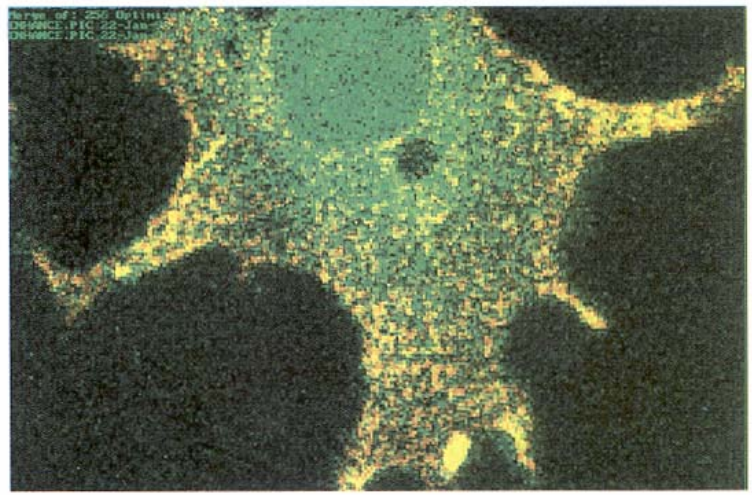
Green Fluorescent Protein—The "Light Fantastic" from Jellyfish to Gene Expression

Aquorea victoria, a species of jellyfish found in the northwest Pacific Ocean, contains a **green fluorescent protein (GFP)** that works together with another protein, **aequorin,** to provide a defense mechanism for the jellyfish. When the jellyfish is attacked or shaken, aequorin produces a blue light. This light energy is captured by GFP, which then emits a bright green flash that presumably blinds or startles the attacker. Remarkably, the fluorescence of GFP occurs without the assistance of a **prosthetic group** —a "helper molecule" that would mediate GFP's fluorescence. Instead, the light-transducing capability of GFP is the result of a reaction between three amino acids in the protein itself. As shown below, adjacent **serine, tyrosine,** and **glycine** in the sequence of the protein react to form the pigment complex—termed a **chromophore.** No enzymes are required; the reaction is autocatalytic.

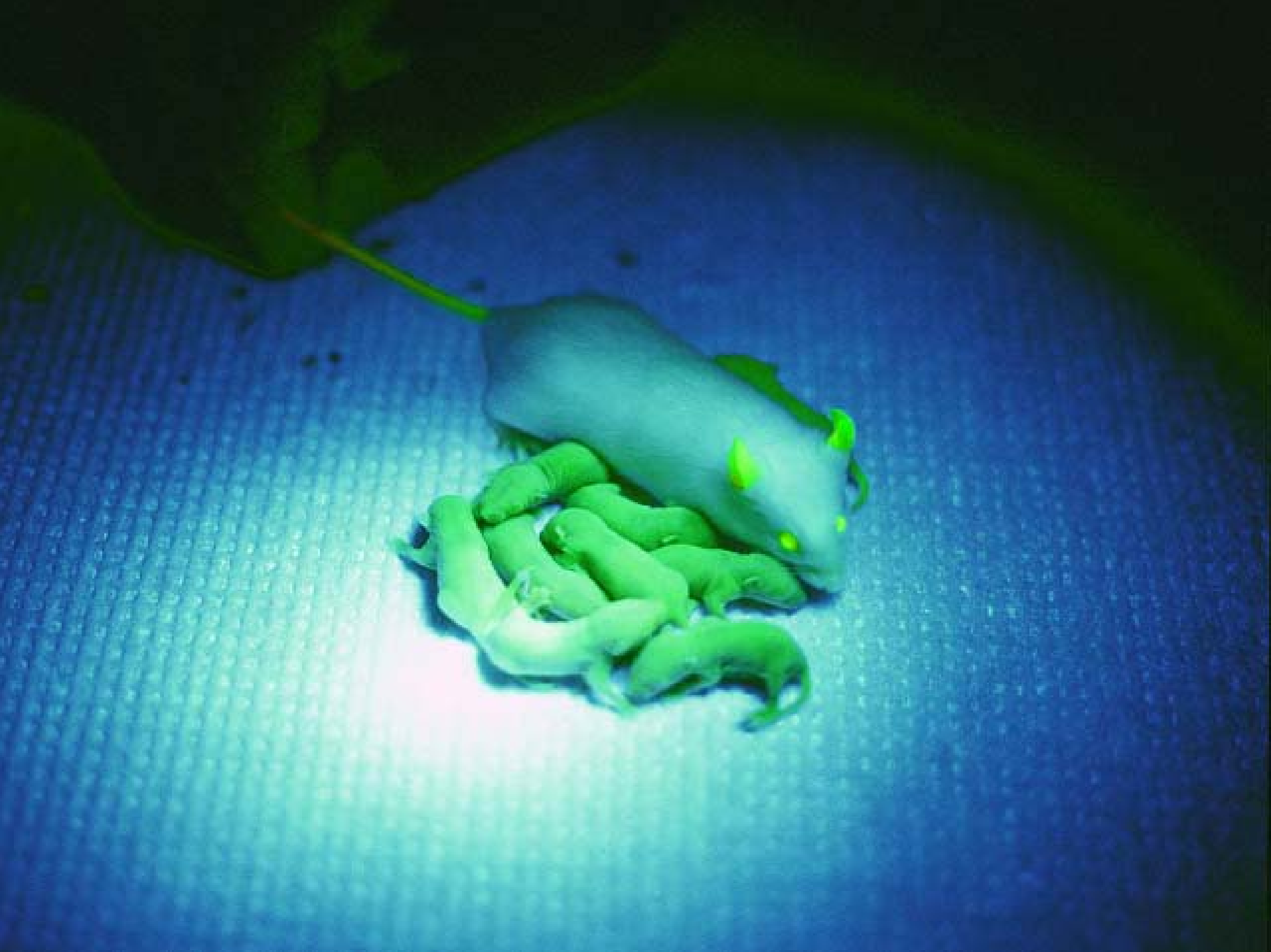
Because the light-transducing talents of GFP depend only on the protein itself (upper photo, chromophore highlighted), GFP has quickly become a darling of genetic engineering laboratories. The promoter of any gene whose cellular expression is of interest can be fused to the DNA sequence coding for GFP. Telltale green fluorescence tells the researcher when this fused gene has been expressed







Autocatalytic oxidation of GFP amino acids leads to the chromophore shown on the left. The green fluorescence requires further interactions of the chromophore with other parts of the protein.



GFP and variants

Other colors available as well

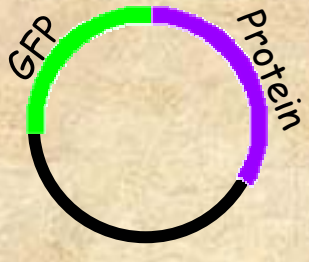
use Fluorescent Proteins as optical labels:

GFP: CFP:

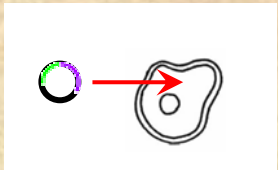
YFP: RFP:

Genetic Labeling:

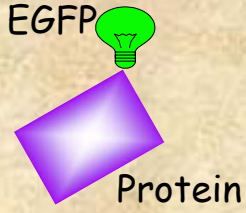
(A) Construct plasmid



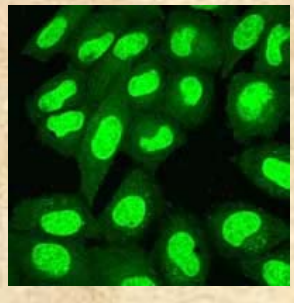
(B) Transfect cells



(C) Cell makes protein



(D) Measure cells



Excitation and Emission Spectra of Fluorescent Proteins

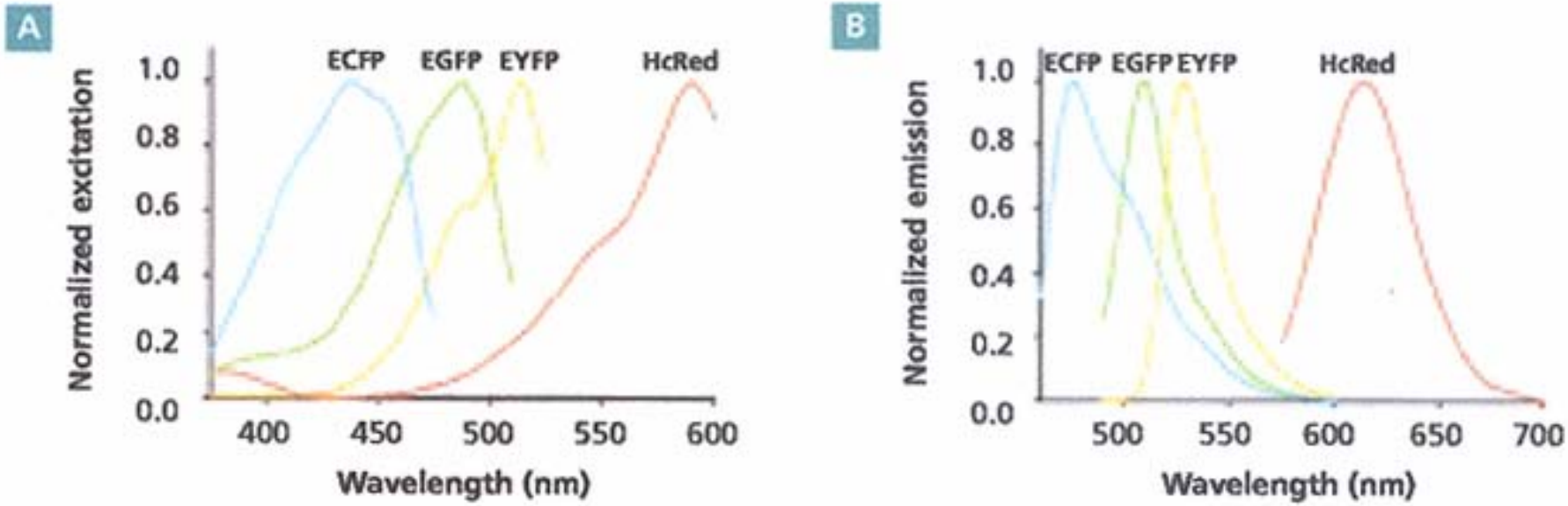


Figure 1. Excitation and emission spectra of BD Living Colors™ ECFP, EGFP, EYFP, and HcRed. HcRed's excitation maximum = 588 nm; emission maximum = 618 nm. EGFP's excitation maximum = 489 nm; emission maximum = 508 nm. EYFP's excitation maximum = 514 nm; emission maximum = 527 nm. ECFP's excitation maximum= 434 nm; emission maximum = 477 nm.