Biological Spectroscopy

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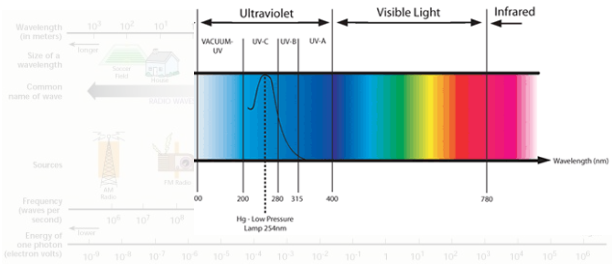
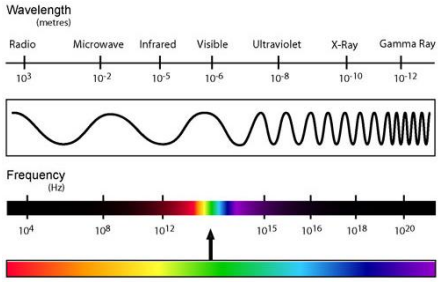
The electromagnetic spectrum



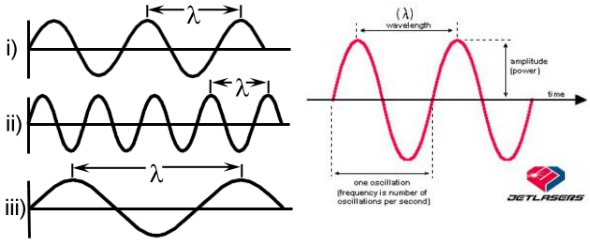
The electromagnetic spectrum



The **longer** the wavelength the **lower** the energy

The **shorter** the wavelength the **higher** the energy eg. UV light from sun causes the sunburn not the red visible light

Frequency and wavelength

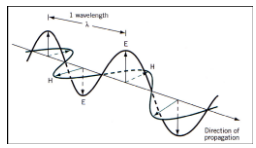


$$\lambda = c / \nu$$

λ - wavelength, meter
C – wave's velocity, m/s
ν - frequency, s^{-1}=Hz

Wave–particle duality

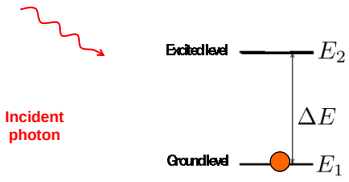
Light is a Wave:



Light is a Particle: - Light also acts as a stream of particles with defined units

Light is made up of particles called photons with defined energy

What is absorption?



$$E_2 - E_1 = \Delta E = h\nu$$

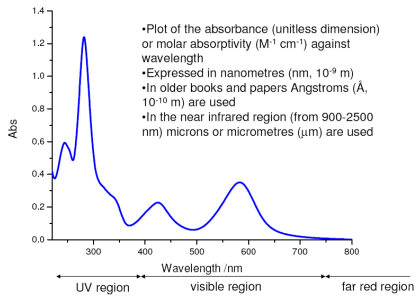
Absorption of electromagnetic radiation is the way by which the energy of a photon is taken up by the electron.

Only photons of certain energy will be absorbed

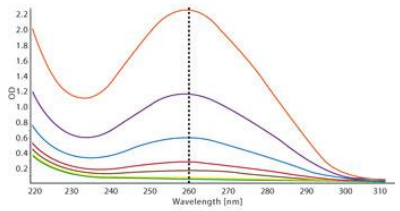
⇓

Only light of certain wavelength will be absorbed

Example of absorbance spectrum

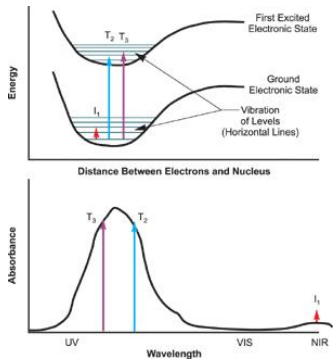


DNA absorbance

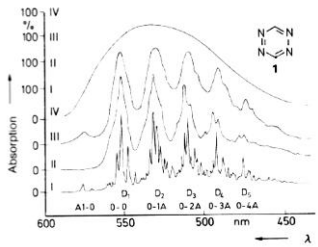


Why not a single peak?

Why not a single peak?

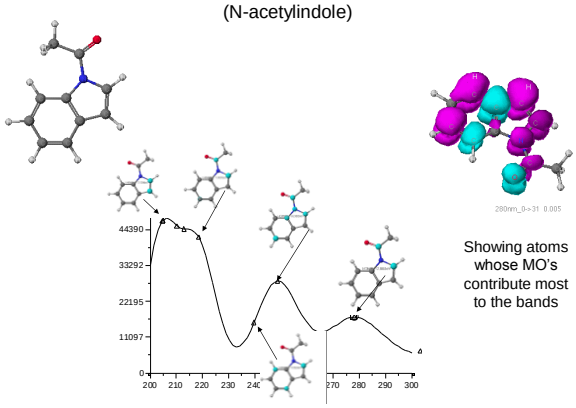


Low temperature – resolving peaks



Tetrazine spectra: Electronic, vibrational and rotational energy levels

The orbitals involved
(N-acetylindole)



Showing atoms whose MO's contribute most to the bands

Different Spectroscopies

- UV-Vis – concentration determination, redox changes, identification
- Fluorescence – emission of UV/Vis by certain molecules
- FT-IR – vibrational transitions of molecules
- FT-NMR – nuclear spin transitions
- X-Ray Spectroscopy – electronic transitions of core electrons

Why should we learn this stuff? After all, nobody solves structures with UV any longer!

Many organic molecules have chromophores that absorb UV

UV absorbance is about 1000 x easier to detect per mole than NMR

Still used in following reactions where the chromophore changes. Useful because timescale is so fast, and sensitivity so high. Kinetics, esp. in biochemistry, enzymology.

Most quantitative Analytical chemistry in organic chemistry is conducted using HPLC or any chromatography with UV detectors

One wavelength may not be the best for all compound in a mixture.

Absorbance spectroscopy

UV/Vis-NIR spectroscopy

What region of the EM spectrum are we interested in?

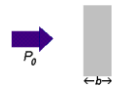
UVC	210-280 nm
UVB	280-320 nm
UVA	320-400 nm
Visible	400-700 nm
Far Red	700-1100 nm
Near-Infra red	1100-2500 nm (9000 cm^{-1}- 4000 cm^{-1}) (increasingly important for materials science)
Mid Infrared	2500-6000 nm (4000 - 60 cm^{-1}) (unusual for electronic absorptions)

Terminology

Light Intensity – *P*, lesser after passing through the sample, $P < P_0$

Transmittance - The light passing through a sample

$T = P / P_0$ *can be expressed in %*

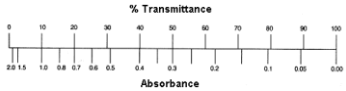


T=100% - no absorbance,
T=0% – no light left after sample

Absorbance - A measure of the extent to which a substance transmits light

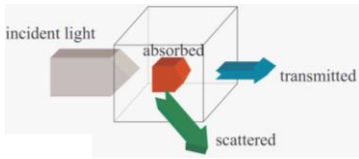
A= log (1/T)

A=0 no absorbance, corresponds to 100% transmittance
A=1 - only 10% light left
A=2 - only 1% light left



Absorption and Transmission

Consider a beam of light on a material – It can be scattered, absorbed, or transmitted



• Transmitted light
– Light emerges propagating in the same direction as the incident light

• Absorbed light
– Energy from light is absorbed in the volume of the material

• Scattered light
– Light emerges in a different direction from the incident light

The Bougier-Beer-Lambert Law

An Empirical law – i.e. based on observation not theoretical derivation

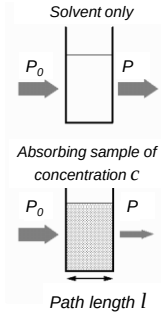
The Bougier-Beer-Lambert Law

$A = \log (P_0/P)$

$A = \varepsilon \times c \times l$

c – *concentration of the light-absorbing matter*
l – *light path length*
ε – *extinction coefficient, or coefficient of proportionality; an intrinsic property of the light absorbing matter*

Usuallty is measured at particular wavelength

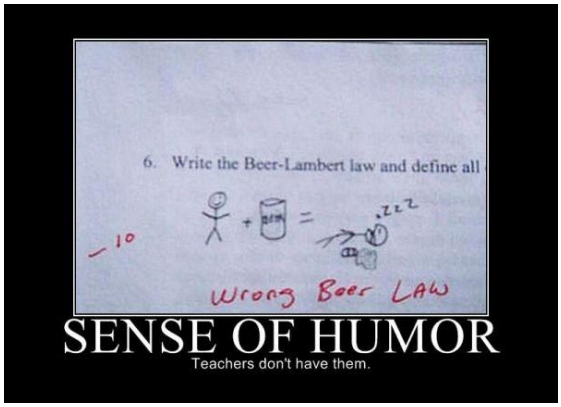


The Bougier-Beer-Lambert Law

- The intensity of light passing through a sample decreases exponentially and the absorbance of light is proportional to the concentration of the chromophore
- The absorbance of light is proportional to the pathlength through which the light travels

$$A = \varepsilon c l$$

A is absorbance, c is concentration (mol L^{-1}), l is pathlength (in cm) and ε is the molar absorptivity (L mol^{-1} cm^{-1})



Beer-Lambert Law

Linear absorbance with increased concentration--directly proportional

Makes UV/VIS spectroscopy useful for quantitative analysis

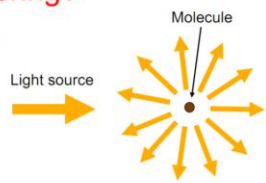
Above a certain concentration the linearity curves down, loses direct proportionality. Due to molecular associations at higher concentrations. Must demonstrate linearity in validating response in an analytical procedure.

A beam of radiation can be attenuated (extinguished) not only by absorption but also by **scattering**

What is scattering?

When light encounters matter, matter not only re-emits light in the forward direction, but it also re-emits light in all other directions.

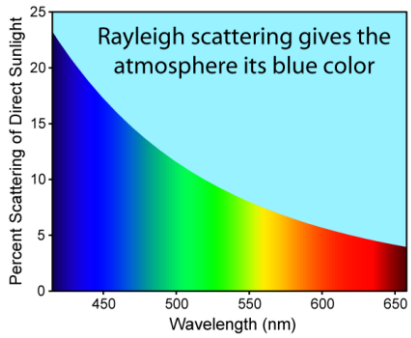
This is called **scattering** – the redirection of radiation out of the original direction of propagation.



Contrast with absorption, which involves conversion of EM energy to heat or chemical energy.

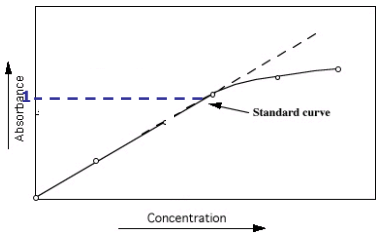
Light scattering is everywhere. All molecules scatter light. Surfaces scatter light. Scattering causes milk and clouds to be white and the daytime sky to be blue. It is the basis of nearly all optical phenomena.

Rayleigh scattering

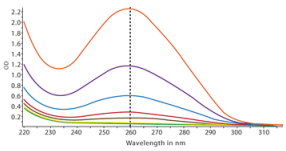


Beer-Lambert Law

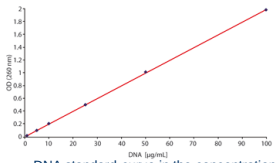
- Beer's law is valid at low concentrations, but breaks down at higher concentrations
- For linearity, **A < 1**



Examples



Absorbance spectrum of different concentrations of calf thymus DNA



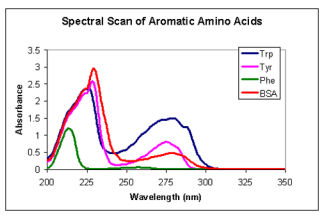
DNA standard curve in the concentration range from 0.1 to 100 µg/mL

UV determination of DNA or RNA

ε = 0.020 µg/ml^{-1} cm^{-1}

It means that 1 µg/ml would give you 0.02 Abs at 260 nm in a cuvette of 1 cm lengths

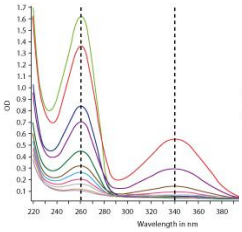
Examples

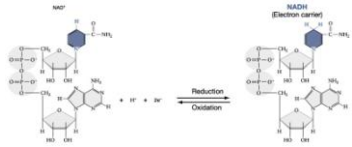


UV determination of protein concentration

If no extinction coefficient information exists for a protein or protein mixture of interest, and a rough estimate of protein concentration is required for a solution that has no other interfering substances, assume εpercent = 10. Most protein extinction coefficients (εpercent) fall in the range 4.0-24.0.3 Therefore, although any given protein can vary significantly from $\varepsilon_{1\%}$ = 10, the average for a mixture of many different proteins will likely be close to 10.

NADH/NAD+





Millimolar redox extinction coefficient of NADH is $^{1cm}Abs_{340nm}$ of 6.22

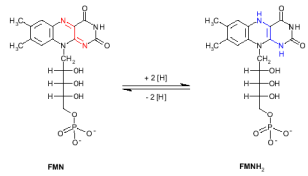
$^{1cm}_{1mM}\varepsilon_{340nm} = 6.22$

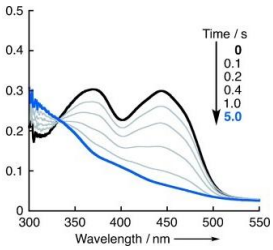
It means that 1mM solution gives absorbance of 6.22 at 340 nm in 1 cm cuvette

Flavins

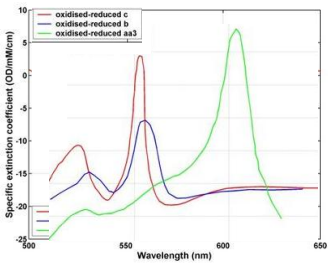
FMN or FAD

Important cofactors for many enzymes

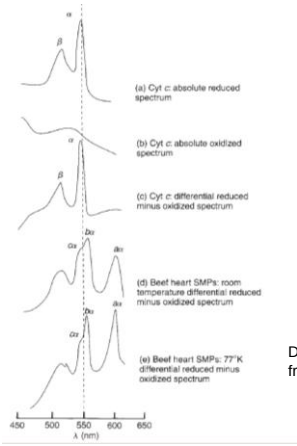


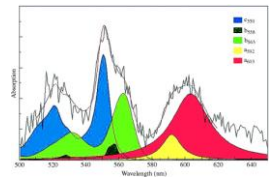


Cytochromes



Cytochromes

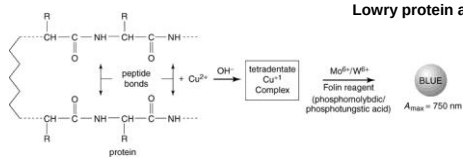


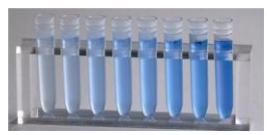


Deriving separate spectra of cytochromes from a suspension of cultured cell

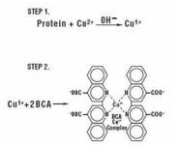
Examples

Lowry protein assay

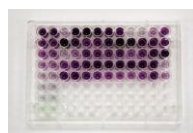


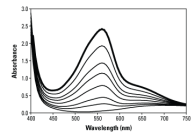


Examples



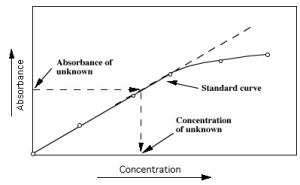
Protein determination: BCA method (Bicinchoninic Acid)



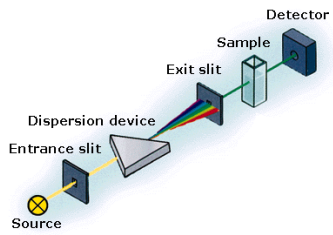


Beer-Lambert Law

- If your unknown has a higher concentration than your highest standard, you have to ASSUME that linearity still holds (NOT GOOD for quantitative analysis)
- Unknowns should ideally fall within the standard range
- Scattering



Scheme of the spectrophotometer

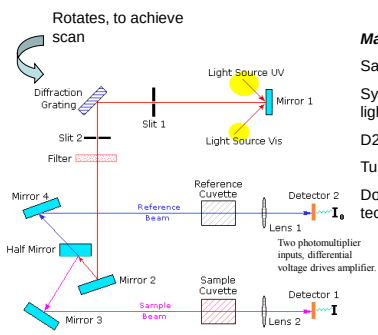


Light source: Mercury-Xenon Arc Lamp, Tungsten-Halogen Lamp, Light Emitting Diodes (LEDs) or Lasers

Monochromator: supplies light within a narrow range of wavelengths

Detector: Photomultiplier or photon counter – measures the light intensity

How Do UV spectrometers work?



Matched quartz cuvettes

Sample in solution at ca. 10^{-5} M.

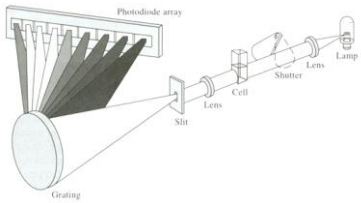
System protects PM tube from stray light

D2 lamp-UV

Tungsten lamp-Vis

Double Beam makes it a difference technique

Diode Array Detectors

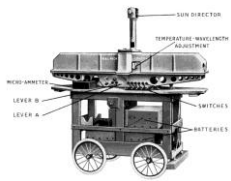


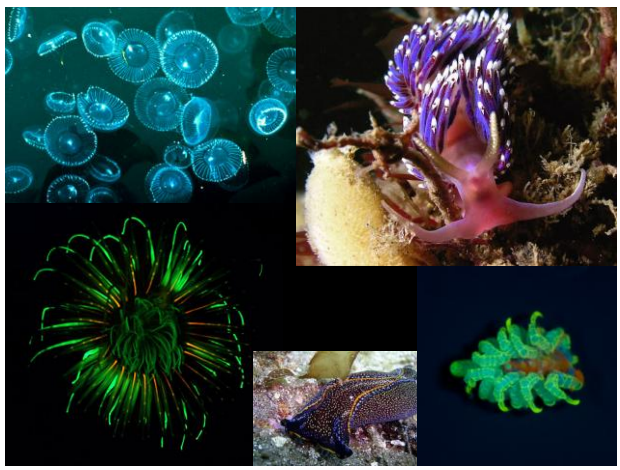
Diode array alternative puts grating, array of photosens. Semiconductors after the light goes through the sample. Advantage, speed, sensitivity,

Resolution is 1 nm, vs 0.1 nm for normal UV (can be solved by including more diodes).

High resolution requires more photodiodes, i.e. higher price.

Various types of spectrophotometers





Principles of Fluorescence

Luminescence

- Emission of photons from electronically excited states
- Two types of luminescence:
  - Relaxation from singlet excited state
  - Relaxation from triplet excited state

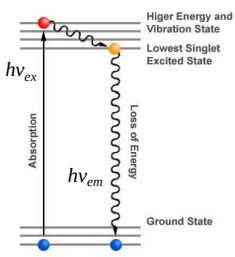
We will consider

Photoluminescence - a process in which a substance absorbs photons (electromagnetic radiation) and then re-radiates photons.

Principles of Fluorescence

3. Types of emission
   - Fluorescence – return from excited singlet state to ground state; does not require change in spin orientation (more common of relaxation)
   - Phosphoresence – return from a triplet excited state to a ground state; electron requires change in spin orientation
   - Emissive rates of fluorescence are several orders of magnitude faster than that of phosphorescence

Principles of Fluorescence

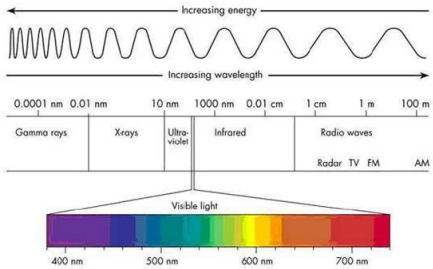


Absorption of light – excitation of the electron and jump from the ground state to the higher vibrational state – femtoseconds 10^{-15}

Decay from the higher vibrational state to the lowest excited state – picoseconds 10^{-12}

Decay to the ground state with emission of a photon – nanoseconds 10^{-9}

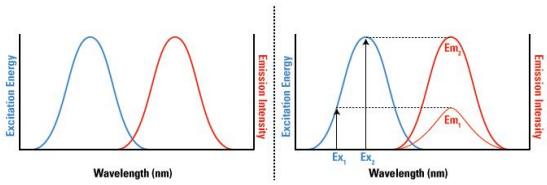
Wavelength and energy



The **longer** the wavelength the **lower** the energy

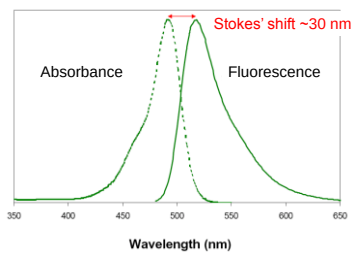
The **shorter** the wavelength the **higher** the energy eg. UV light from sun causes the sunburn not the red visible light

Fluorescence



The excitation and emission spectra of a fluorophore and the correlation between the excitation amplitude and the emission intensity. General diagram of the excitation and emission spectra for a fluorophore (left). The intensity of the emitted light (Em1 and Em2) is directly proportional to the energy required to excite a fluorophore at any excitation wavelength (Ex1 and Ex2, respectively; right).

Fluorescence

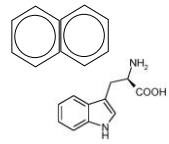


Mirror-image rule
• Emission spectra is typically a mirror-image of the absorption spectra (excitation spectra)

Stokes (guy with tonic water) **Shift**
• The energy of emission is less than energy of absorption
• Emission occurs at longer wavelengths

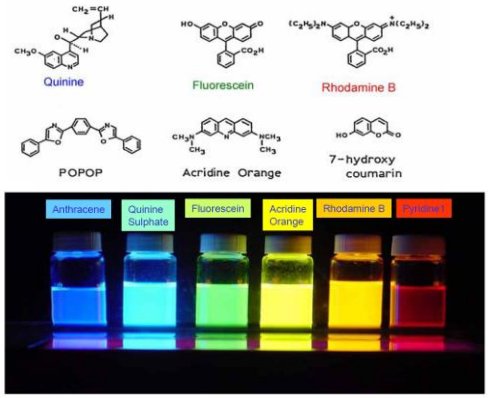
Fluorescence

•Chromophores are components of molecules which absorb light
•They generally have aromatic rings
•If they are able to emit light – they are fluorophores. In protein most fluorescence results from the tryptophan residues



•Quantum yield (Q, Φ) -gives the efficiency of the fluorescence process = the number of emitted photons relative to the number of absorbed photons
•the higher the Q the brighter the emission
•max Q is 1.0 or 100%
•fluorophores with Q ~ 0.15 or 15% are still considered good

Common Fluorophores



Instrumentation

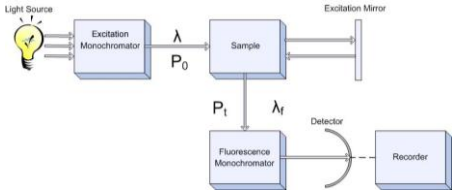
Spectrofluorometers

Fluorescence scanners

Microplate readers

Fluorescence microscopes

Fluorospectrophotomener components



Light source: Mercury-Xenon Arc Lamp, Tungsten-Halogen Lamp, Light Emitting Diodes (LEDs) or Lasers

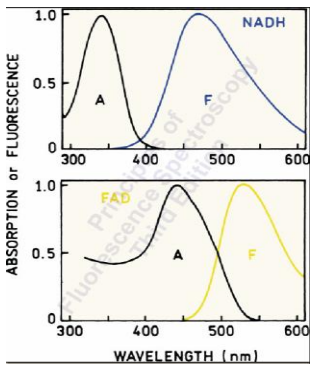
Monochromator: supplies light within a narrow range of wavelengths

Detector: Photomultiplier or photon counter – measures the light intensity

Fluorescence spectroscopy in biochemical applications:

1. **Steady-state** (constant illumination and observation)
2. **Resonance energy transfer (RET)** (Förster distances)
3. **Fluorescence anisotropy** (photoselective excitation by polarized light, information on size and shape; protein-protein associations, membrane fluidity etc)
4. **Time-resolved** (measures intensity or anisotropic decay, pulse of light)
5. **Quenching** (information on the solvent accessibility of the fluorophore)
6. **Fluorescence Correlation Spectroscopy (FCS)** (association reactions in very small volumes)
7. **Single molecule detection (SMD)** (on immobilized fluorophores)
8. **Cellular imaging** (confocal microscopy)

Fluorescence of protein cofactors



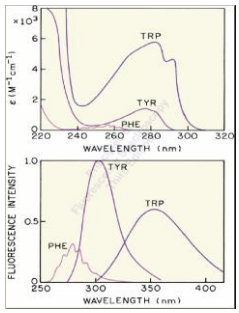
Some of the protein cofactors can be identified in by characteristic spectra:

e.g. NADH or NADPH – maximum emission at 460 nm

FAD or FMN – flavin cofactors maximum emission at 525 nm

Redox-dependent spectra

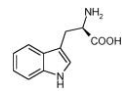
Absorbance and fluorescence of tryptophan and tyrosine



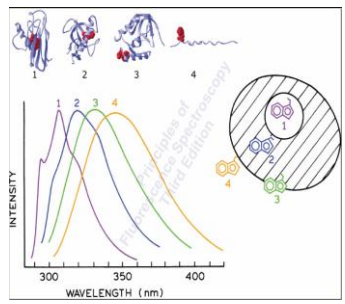
Fluorescence in polypeptides is provided by aromatic amino acids

Trp emission is fingerprint of protein conformation

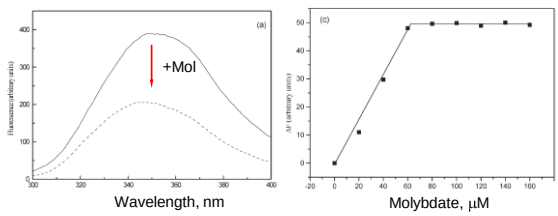
Spectral characteristics of tryptophans
- indole ring fluorescence is dependent on the environment/localization
- from 315 to 355 nm
- could shift due to conformational changes



Spectral classes of Trp
- emission max ~ 315 nm – very hydrophobic environment, usually buried residue
- emission max ~ 330-340 nm – partially exposed residue
- emission max ~ 350 nm – exposed residue, also in denaturated proteins



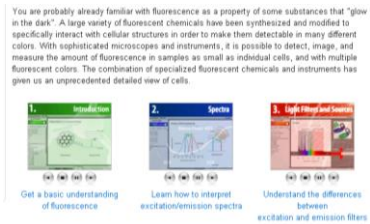
Simple fluorescent measurements (ligand binding)



Change in the fluorescent spectra of molybdate-sensing protein ModE from *E.coli* upon addition of molybdate. Aliquotes of ligand were added to a solution of 60 μM protein and changes in the fluorescence at 350 nm were monitored. Saturation occurres at the concetration of 60 μM of ligand, showing that there is one binding side per protein. Binding of the ligand leads to 50% quenching of the fluorescence. [Boxer et al., 2004].

Extra resources in the web

http://www.invitrogen.com/site/us/en/home/support/Tutorials.html



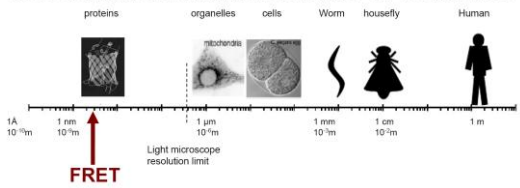
FRET = Förster Resonance Energy Transfer

Non-radiative energy transfer between molecules with overlapping emission-excitation spectra.

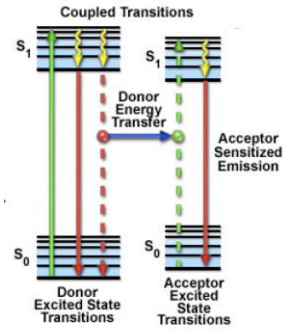
First Identified in 1946 by Theodor Förster

FRET: The Size

Allows for qualitative and quantitative measurement of very close interactions



FRET = Förster Resonance Energy Transfer



Energy is transferred through the resonant coupling of the dipole moments of donor and acceptor

Energy transition between a donor and acceptor with a finite probability based on proximity

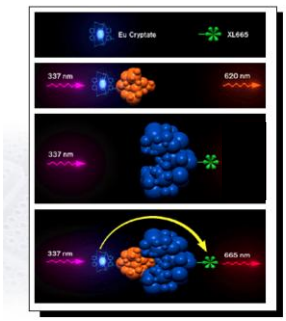
DISTANCE : Donor and acceptor molecules must be in close proximity (10-100 Å)

SPECTRUM OVERLAP: The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor

NO "FLUORESCENCE ENERGY" TRANSFER

FRET = Förster Resonance Energy Transfer

FRET: principle



- 2 fluorescent molecules (here blue and green)
  - One is the « donor » (blue)
  - One is the acceptor (green)
- When both are very close to each other, FRET occurs. The emission of the donor is reduced and the emission of the acceptor is increased.
- Allows to quantitate interactions at a molecular level.

What dyes to use?

Spectral Properties of DyLight Fluorescent Dyes

Emission	Fluor	Ex/Em†	See Spectra	ε††	Spectrally Similar Dyes
Blue	DyLight 350	353/432		15K	Alexa Fluor® 350, AMCA
Blue	DyLight 405	400/420		30K	Alexa Fluor 405, Cascade Blue®
Green	DyLight 488	493/518		70K	Alexa Fluor 488, fluorescein, FITC
Yellow	DyLight 550	562/576		150K	Alexa Fluor 546, Alexa Fluor 555, Cy3®,TRITC
Red	DyLight 594	593/618		80K	Alexa Fluor 594, Texas Red®
Red	DyLight 633	638/658		170K	Alexa Fluor 633
Red	DyLight 650	652/672		250K	Alexa Fluor 647, Cy5®
Near IR	DyLight 680	692/712		140K	Alexa Fluor 680, Cy5.5®
Near IR	DyLight 755	754/776		220K	Alexa Fluor 750
Infrared	DyLight 800	777/794		270K	IRDye® 800

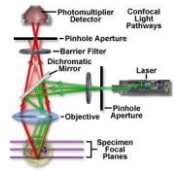
†Excitation and emission maxima in nanometers (+/- 4nm) in phosphate-buffered saline (PBS)
††Molar extinction coefficient (M^{-1} cm^{-1}) at the absorption maximum

Application of FRET

- Receptor / ligand binding
- Detection of nucleic acid hybridization
- Membrane fusion assays
- Distribution and transport of lipids
- Protein folding or conformational changes in proteins
- FRET can also be used for binding assay, as it also detects distance changes at the molecular level

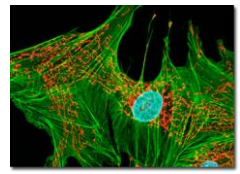
Fluorescent confocal microscopy

Confocal laser scanning microscopy is a technique for obtaining high-resolution optical images with depth selectivity. Excitation lasers with different wavelengths can be used and emission from different fluorophores obtained (it is possible to measure up to 5-6 channels).

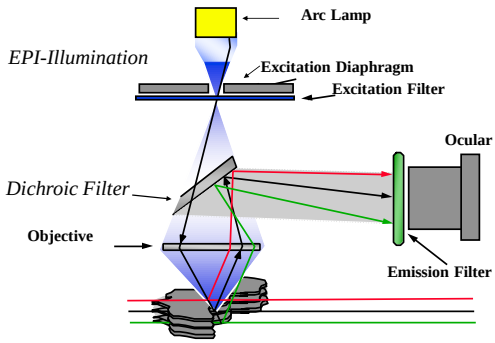


Dermal Fibroblast Cells

GREEN – Actine - Alexa Fluor 488
RED – mitochondria - MitoTracker Red
BLUE - DNA probe - DRAQ5

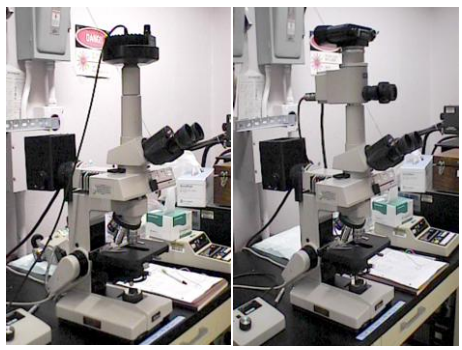


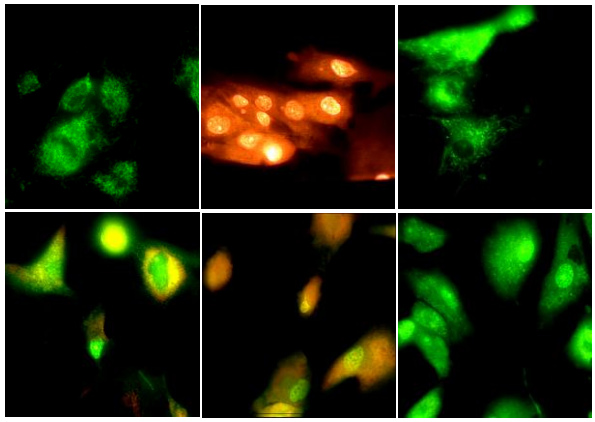
Fluorescent Microscope



Fluorescence Microscope with

Color Video (CCD) 35 mm Camera



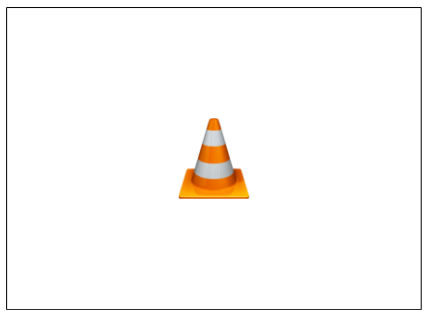


Probes for Proteins

Probe	*Excitation*	*Emission*
FITC	488	525
PE	488	575
APC	630	650
PerCP™	488	680
Cascade Blue	450	
Coumerin-phalloidin	350	450
Texas Red™	610	630
Tetramethylrhodamine-amines	575	
CY3 (indotrimethinecyanines)	540	575
CY5 (indopentamethinecyanines)	640	670

Tracking mitochondria (red fluorescence) and microtubule from cytoskeleton (green fluorescence)

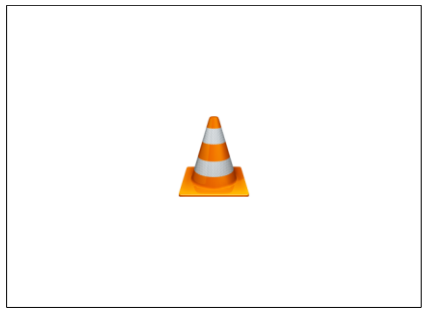
http://www.youtube.com/watch?v=N51QgkRI26I



FRAP - Fluorescence Recovery After Photobleaching

http://www.youtube.com/watch?v=LicQb_SnCSI

Method to monitor mobility of membrane proteins



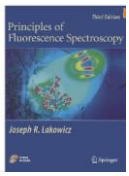
Textbooks and online resources

http://www.chm.davidson.edu/vce/spectrophotometry/index.html

http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

http://www.enzim.hu/~szia/cddemo/edemo0.htm

Physical biochemistry : applications to biochemistry and molecular biology - David Freifelder *(any edition)*



Principles of Fluorescence Spectroscopy
Joseph R. Lakowicz
(Ed. 2006 or earlier)

Green Fluorescent Proteins

Dr Alexander Galkin

The First Human GFP Transgenic?

The Nobel Prize in Chemistry 2008

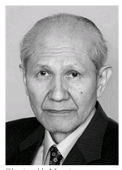


Photo: U. Montan
Osamu Shimomura
Prize share: 1/3

Photo: U. Montan
Martin Chalfie
Prize share: 1/3

Photo: U. Montan
Roger Y. Tsien
Prize share: 1/3

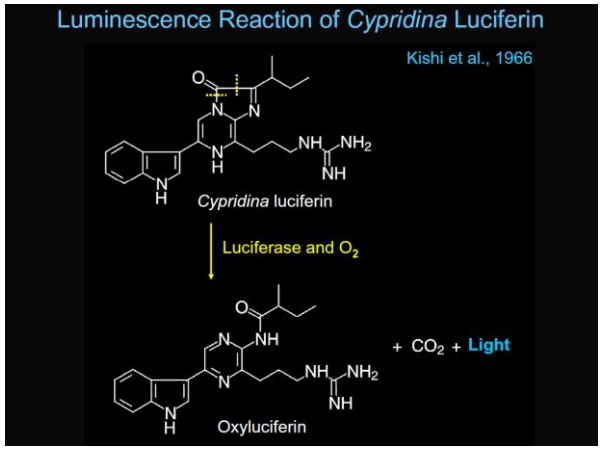
The Nobel Prize in Chemistry 2008 was awarded jointly to Osamu Shimomura, Martin Chalfie and Roger Y. Tsien *"for the discovery and development of the green fluorescent protein, GFP"*.

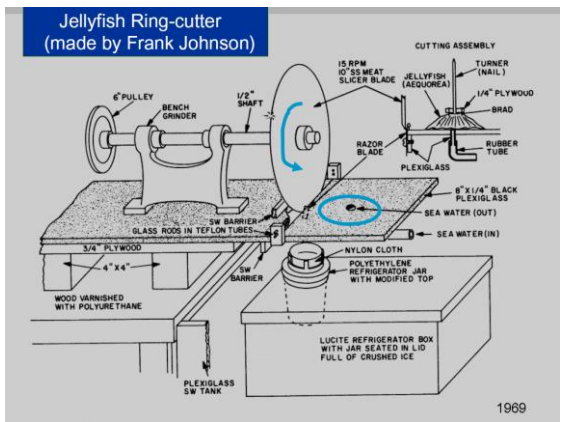
+Douglas Prasher

Jellyfish Collectors (1974)

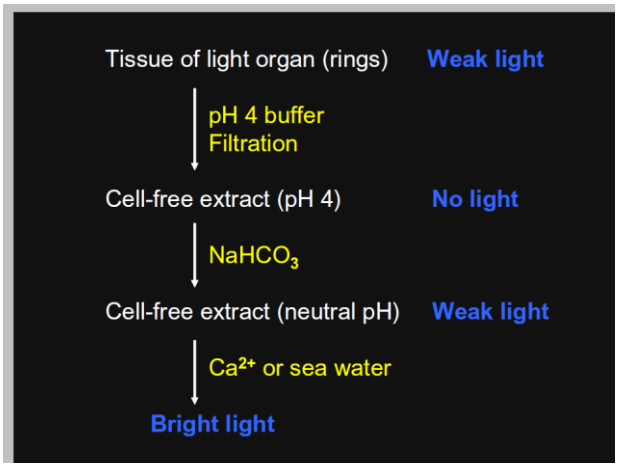
Osamu Shimomura – initially tried to extract luciferase from jellyfish, but failed!
50000 jellyfish were processed to isolate aequorin and GFP

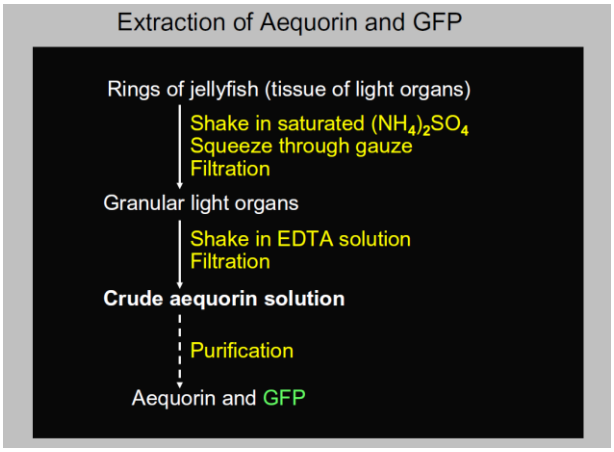
Nobel Prize, 2008

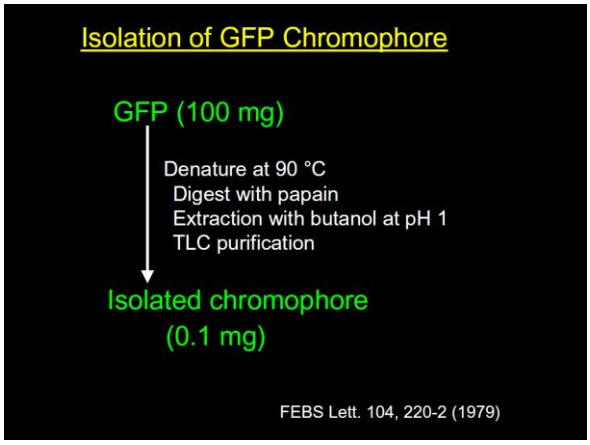




Test-run of ring-cutters by Johnsons,1968


Tissue of light organ (rings)
Weak light
pH 4 buffer
Filtration
Cell-free extract (pH 4)
No light
NaHCO3
Cell-free extract (neutral pH)
Weak light
Ca2+ or sea water
Bright light

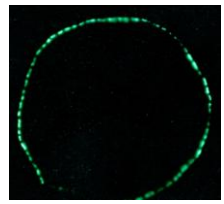

Extraction of Aequorin and GFP
Rings of jellyfish (tissue of light organs)
Shake in saturated (NH4)2SO4
Squeeze through gauze
Filtration
Granular light organs
Shake in EDTA solution
Filtration
Crude aequorin solution
Purification
Aequorin and GFP


Isolation of GFP Chromophore
GFP (100 mg)
Denature at 90 °C
Digest with papain
Extraction with butanol at pH 1
TLC purification
Isolated chromophore
(0.1 mg)
FEBS Lett. 104, 220-2 (1979)

Green Fluorescent Protein (GFP) from hydroid jellyfish *Aequorea victoria*

Discovery – 1962
Cloning – 1992
Application – 1994

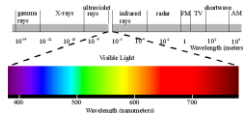
Day light



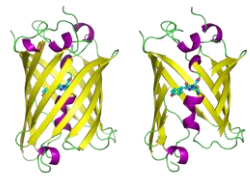
Luminescent in dark

Wild Type GFP

- GFP traditionally refers to the protein first isolated from the jellyfish *Aequorea victoria*.
- GFP is a protein which exhibits bright green fluorescence when exposed to blue light.



- The protein composed of 238 amino acids (26.9kDa).
- The protein sequence and its resultant three-dimensional folding structure into the 11-strand β-barrel, is most likely crucial to the formation of the chromophore and its bioluminscene.



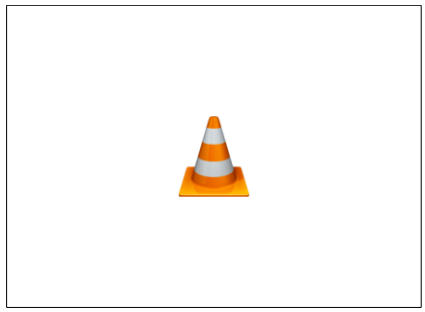
The Fluoropore Active Site

- GFP has the ability to exhibit intrinsic fluorescence thanks to three amino acids that cyclise (Ser65-Tyr66-Gly67) and then undergo an oxidation step during a complex maturation process.

The 3 amino acids forming the chormophore in GFP undergoes oxidation and cyclization.

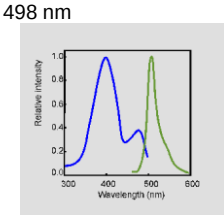
The formed active chromophore contains conjugated double bonds. These double bonds store and release the energy from electrons.

Green Fluorescent Protein

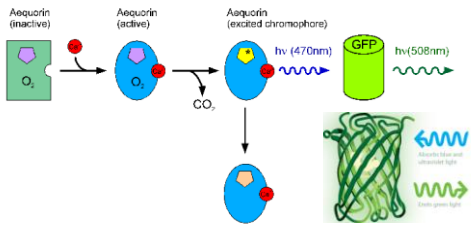


Wild Type GFP

- The GFP from *A. victoria* has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 509 nm which is in the lower green portion of the visible spectrum.
- The GFP from the sea pansy (*Renilla reniformis*) has a single major excitation peak at 498 nm

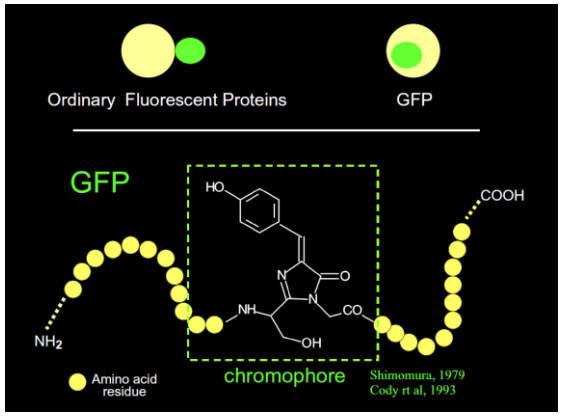


Wild Type GFP



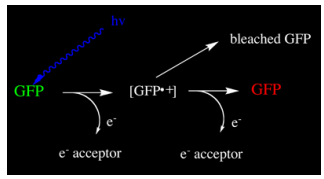
Blue light is given off by another chemical reaction (bioluminescent or chemiluminescence reaction) involving the protein aequorin inside the jellyfish. This blue light is absorbed by the conjugated double bonds of the chromophore resulting in the release of energy in the form of a visible green light (fluorescent reaction).

http://www.lifesci.ucsb.edu/~biolum/chem/index.html

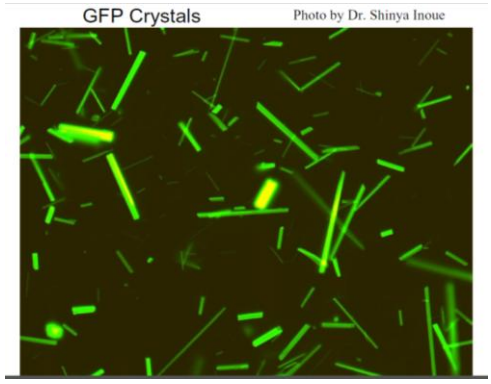


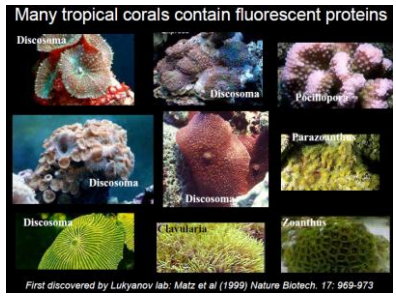
Function in nature?

Lukyanov and his group have found that fluorescent proteins can act as electron donors when excited. According to their proposal published in Nature Chemical Biology the excited green chromophore (GFP) donates an electron to an electron acceptor forming a short lived intermediate. If no electron acceptor is available the intermediate is permanently bleached, however if it reacts with an electron acceptor the GFP reddens (GFP).

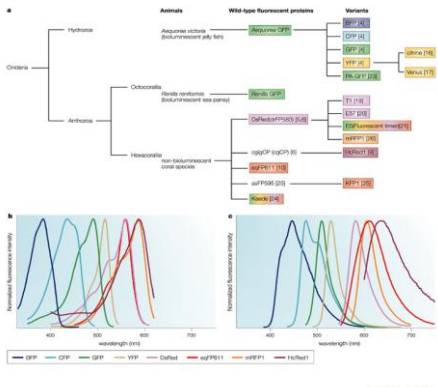


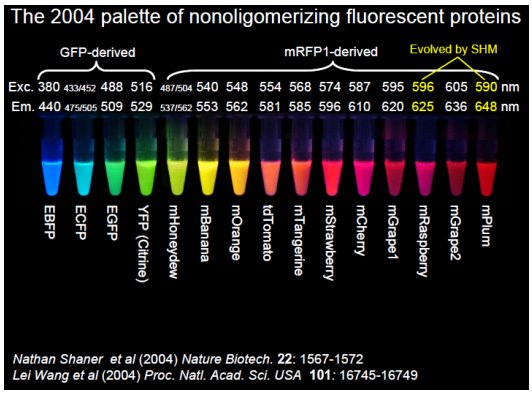
The purpose of both bioluminescence and GFP fluorescence in jellyfish is unknown.





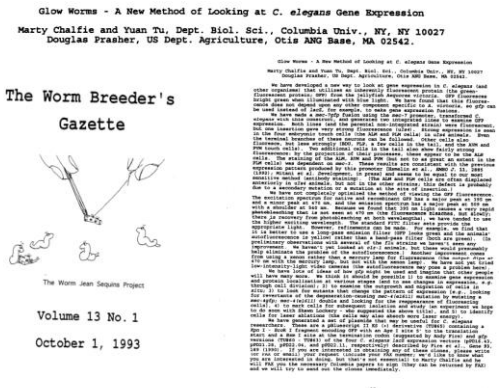
Site-directed mutagenesis





Summarizing Green Fluorescent Protein features for applications:

- **Small**: only 26.9kDa in size.
- **Very stable**: has an unusual barrel structure encompassing the hexapeptide chromophore. GFP is resistant to heat, alkaline pH, detergents, photobleaching , chaotropic salts, organic salts, and many proteases.
- **Fluorescence properties**: absorbs light at 395 and 470nm, emits green fluorescence at 509nm. Now altered spectrum available from mutants.
- **Versatile**: could be cloned and expressed in almost all organisms, and every major cellular compartment.
- **Auto-fluorescence**: no fixation needed, no sacrifice of host, no co-factors needed .
- Non-invasive and nontoxic in most cases.
- **Quantifiable**: its expression is easily detected, and quantifiable.


Glow Worms - A New Method of Looking at *C. elegans* Gene Expression

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The Worm Breeder's Gazette

The Worm Jean Sequins Project

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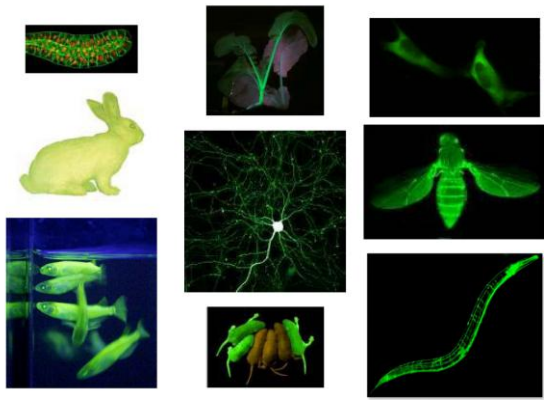


limitations with wild-type GFP

- wild-type GFP can be excited by light at two different absorption peaks, which the optimal excitation one is by UV light. This is problematic if one wants to use GFP in live cells, since UV radiation can damage them.
- Hence, using techniques such as **site-directed mutagenesis**, mutants have been created with strong absorption peaks corresponding to blue light.

Limitations with wild-type GFP

- Wild-type GFP folds poorly at 37°C, so it limits its usefulness for *in vivo* experiments in the lab.

✓ mutants of GFP such as Enhanced Green Fluorescent Protein (EGFP) get around this problem.

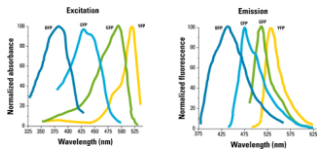
- It can take up to four hours to fold GFP correctly

✓ an engineered version known as Superfolder GFP partly solves this problem.

- A smorgasbord of mutants have been derived from GFP to emit different wavelengths of light; these include :
  - cyan fluorescent protein (CFP)
  - yellow fluorescent protein (YFP)
  - blue fluorescent protein (BFP)
- The palette of fluorescent proteins available to researchers is supplemented by DsRed, a red fluorescent protein derived from the Discosoma coral, and its derivatives.

Changes in the amino acids in the fluorophore region, as well as in amino acids downstream from the fluorophore, result in changes in the color the fluorophore emits.

Color	Amino acids 64 65 66 67 68 69 72 145 146 153 163 203
Green	wtGFP ---Phe-Ser-Tyr-Gly-Val-Gln----Ser------Tyr-Asn-------Met----Val---------Thr----
Green	EGFP ---Leu-Thr----------
Blue	EBFP ---Leu-Thr-His----------Phe----------
Yellow	EYFP ---Phe-Gly----------Leu---------Ala----------Tyr----
Cyan	ECFP ---Leu-Thr-Trp----------Ile-------Thr-----Ala----------

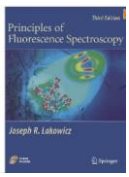


Textbooks and online resources

http://www.chm.davidson.edu/vce/spectrophotometry/index.html

http://www.invitrogen.com/site/us/en/home/support/Tutorials.html

GFP:
http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP-1.htm
http://www.rcsb.org/pdb/101/motm.do?momID=42
http://www.chm.bris.ac.uk/motm/GFP/GFPh.htm



Principles of Fluorescence Spectroscopy
Joseph R. Lakowicz
(Ed. 2006 or earlier)

THE END