Biological Fluorescence

Dr Alexander Galkin
Level 3
BIOMOLECULAR STRUCTURES

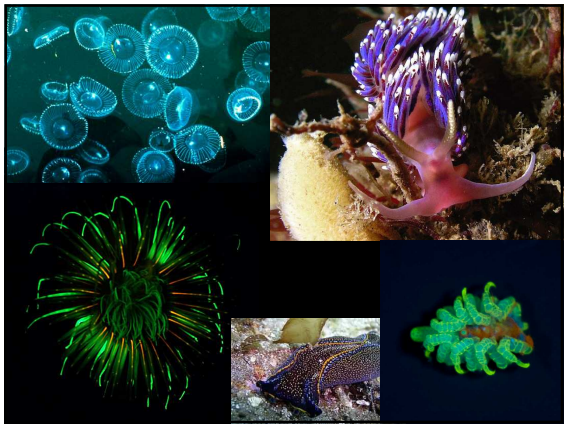
Contact Details

Dr Alexander Galkin

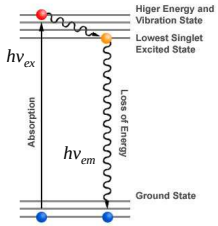
Email: a.galkin@qub.ac.uk

Office: MBC Room 186

Tel: (028) 90972166



Fluorescence

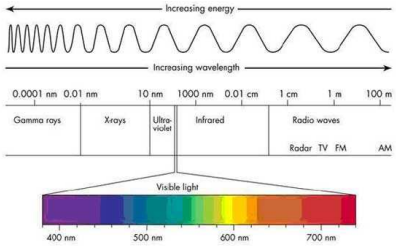


Absorbtion of light – excitation of the electron and jump from the ground state to the higher vibrational state – femtoseconds 10^{-15}

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

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

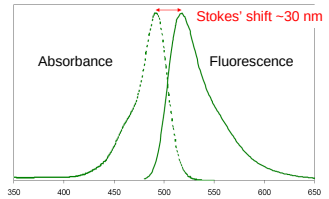
Wavelength and energy



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

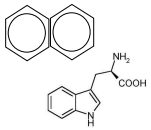
Fluorescence



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

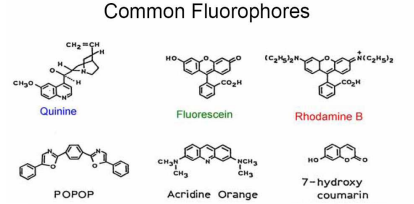
Fluorescence

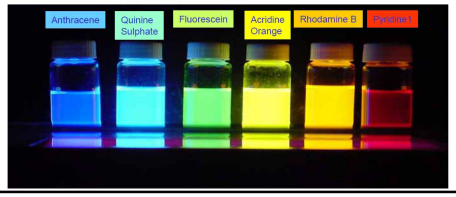


•Chromophores are components of molecules which absorb light
•They generally have aromatic rings
•If they are able to emit light – they are fluorophores. In protein most fluorescence results from the tryptophan residues

•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

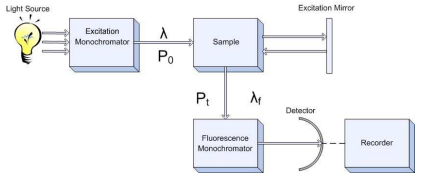
Spectrofluorometers

Fluorescence scanners

Microplate readers

Fluorescence microscopes

Fluorospectrophotomener components



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

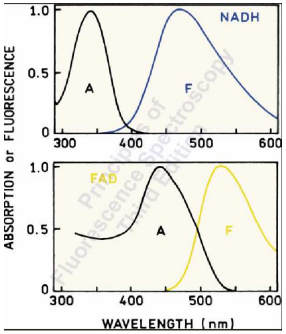
Monochromator: supplies light within a narrow range of wavelengths

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



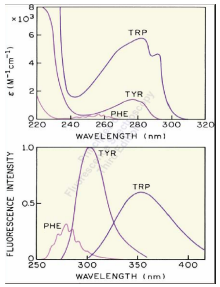
Some of the protein cofactors can be identified in by characteristic spectra:

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

FAD or FMN – flavin cofactors maximum emission at 525 nm

Redox-dependent spectra

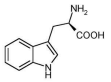
Absorbance and fluorescence of tryptophan and tyrosine



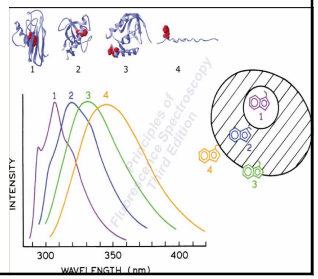
Fluorescence in polypeptides is provided by aromatic amino acids

Trp emission is fingerprint of protein conformation

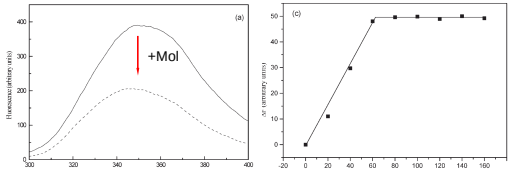
Spectral characteristics of tryptophans
- indole ring fluorescence is dependent on the environment/localization
- from 315 to 355 nm
- could shift due to conformational changes



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].

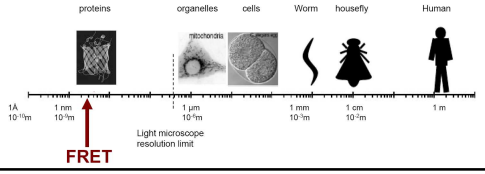
FRET = Förster Resonance Energy Transfer

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



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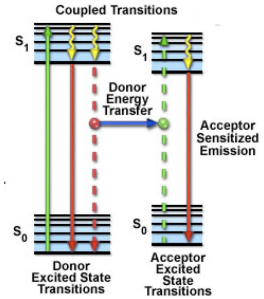


Energy is transferred through the resonant coupling of the dipole moments of donor and acceptor

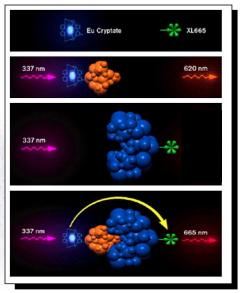
Energy transition between a donor and acceptor with a finite probability based on proximity

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

FRET = Förster Resonance Energy Transfer

FRET: principle



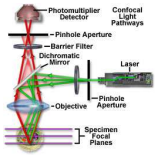
- 2 fluorescent molecules (here blue and green)
  - One is the « donor » (blue)
  - One is the acceptor (green)
- When both are very close to each other, FRET occurs. The emission of the donor is reduced and the emission of the acceptor is increased.
- Allows to quantitate interactions at a molecular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

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

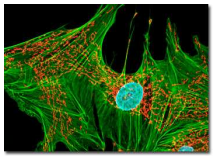
Fluorescent confocal microscopy

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



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

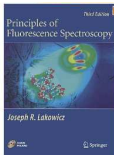
http://www.youtube.com/watch?v=N51QgkRI26I

FRAP - Fluorescence Recovery After Photobleaching

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

Method to monitor mobility of membrane proteins

Textbook



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

THE END