Introduction to Spectroscopy and Fluorescence

For students of HI 6001-125

"Computational Structural Biology"

Willy Wriggers, Ph.D.

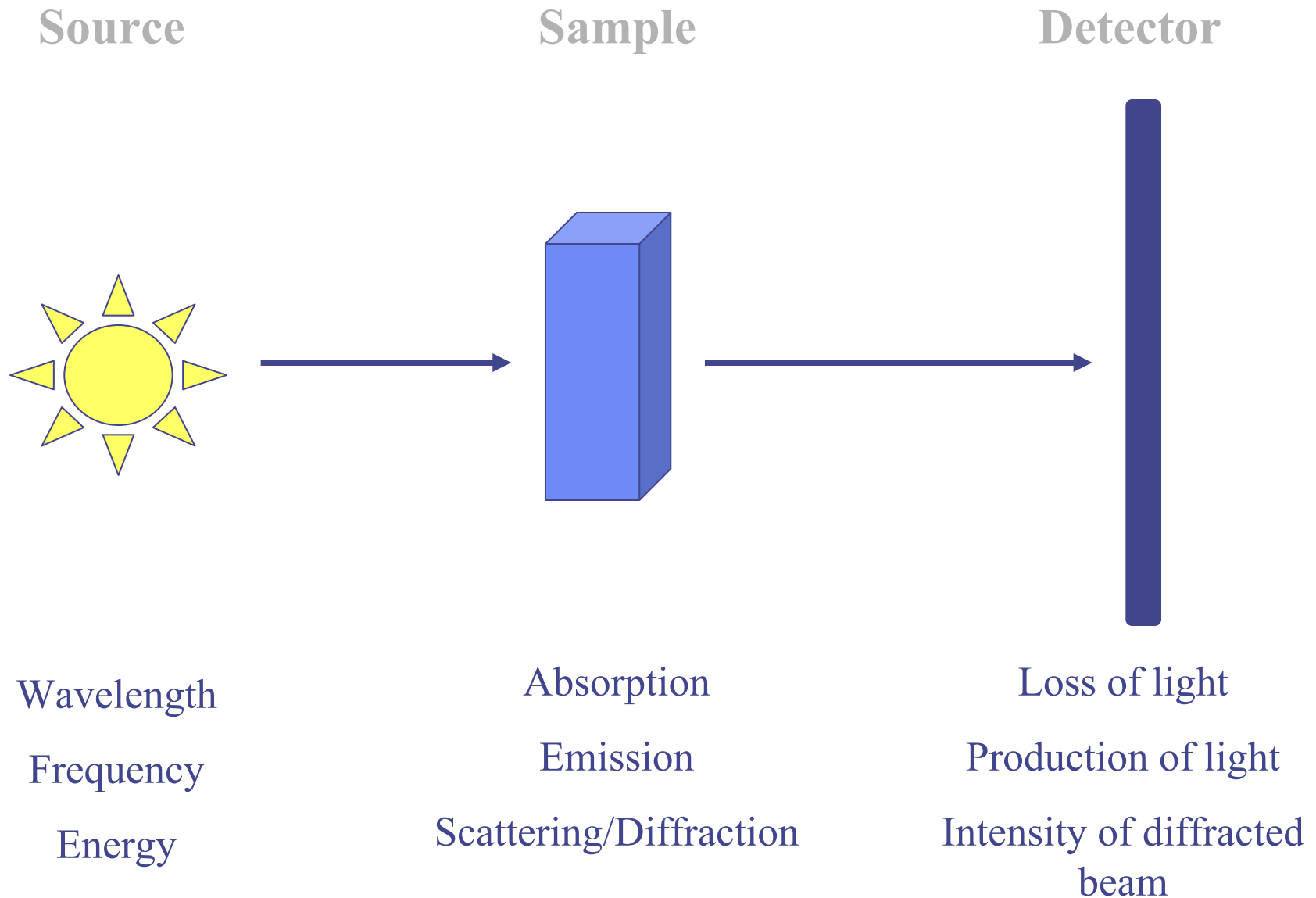Adopted from material by Mathew Baker, Univ. of Bath

http://staff.bath.ac.uk/bssmdb/

http://biomachina.org/courses/structures/05.html

What is Spectroscopy?

•By using different parts of the electromagnetic spectrum (different types of light) we can "see" separate details.

•The "size" and energy of the light waves restrict their uses, as does our ability to measure & interpret them.

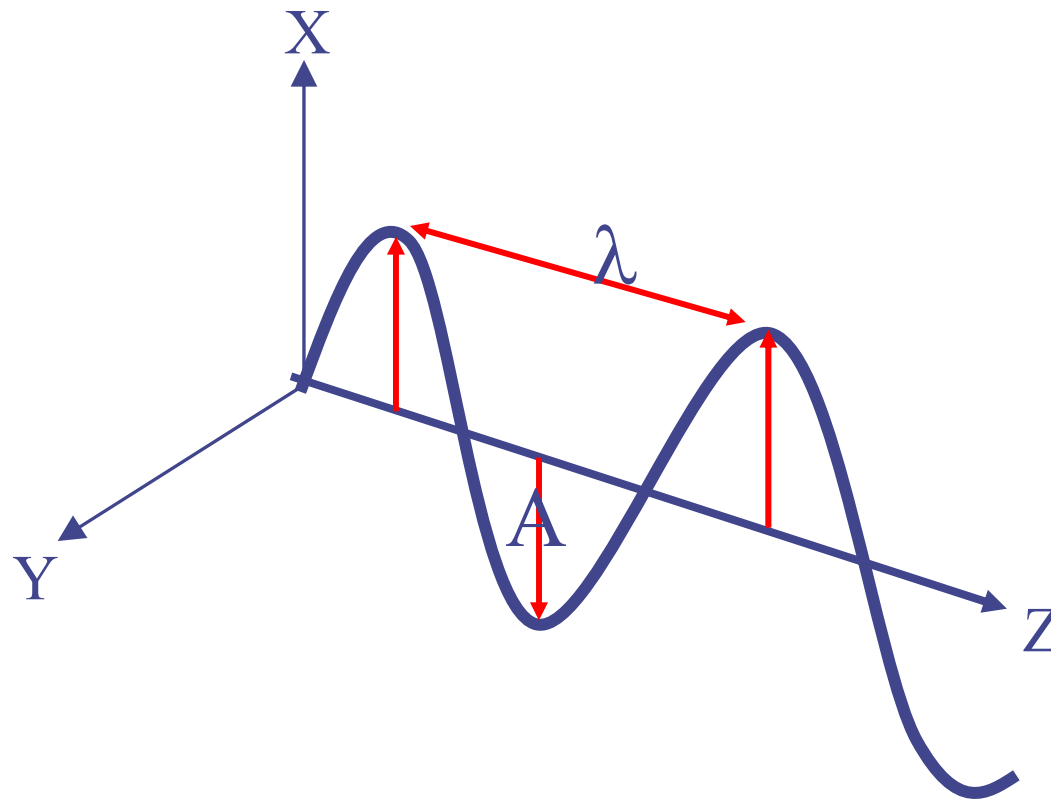•Redefine how you think of "seeing".

Interactions with Sample

Source   **Sample**   **Detector**

Wavelength   Absorption   Loss of light

Frequency   Emission   Production of light

Energy   Scattering/Diffraction   Intensity of diffracted beam

Electro-Magnetic Radiation

A =Amplitude ν = frequency

λ = Wavelength   Phase

X

λ

A

Y

Z

Electro-Magnetic Radiation

- The Frequency (ν) & wavelength (λ) of a wave are related by:

$$\nu = c/\lambda$$

($c = 3 \times 10^8 \, m.s^{-1}$)

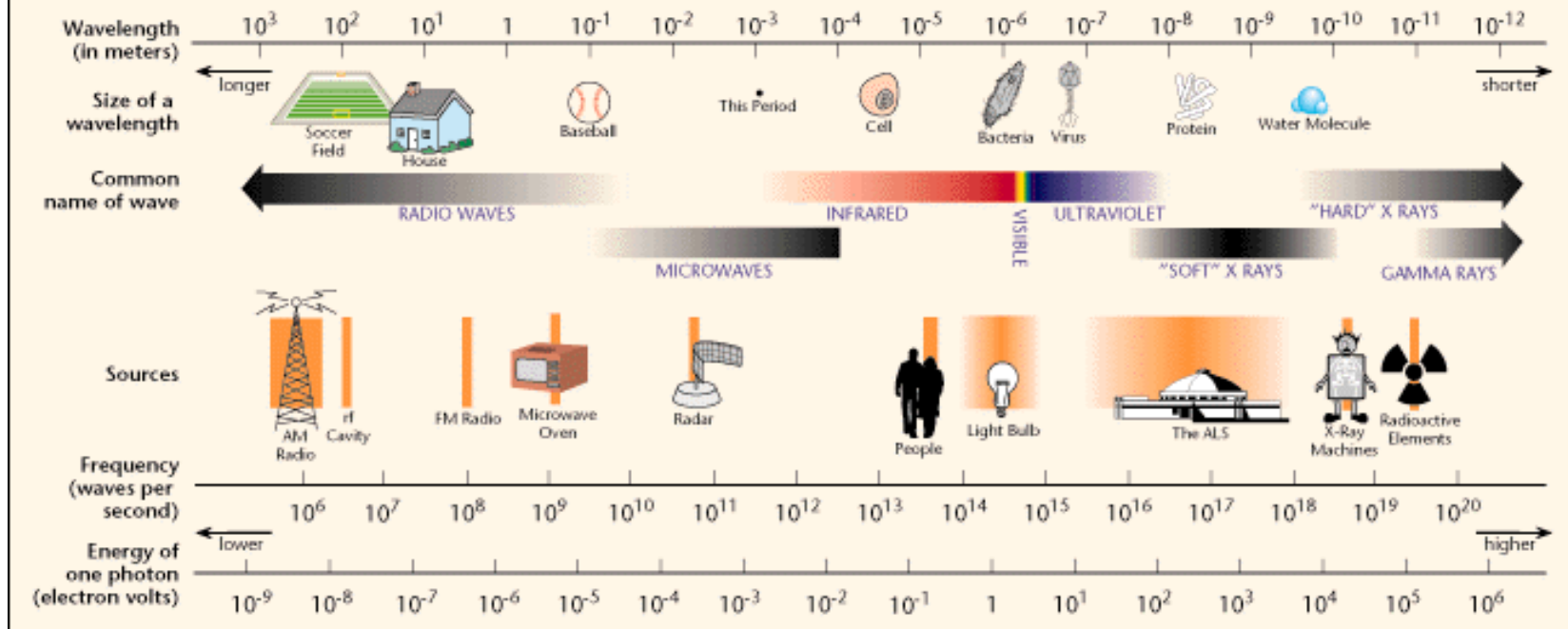- Frequency can be converted to energy (e) by:

$$e = h\nu$$

($h = 6.63 \times 10^{-24} \, J.s$)

- Scales: $1 \, nm = 10^{-7} \, cm = 10 \, \text{Å}$

THE ELECTROMAGNETIC SPECTRUM

Wavelength (in meters)	10^3	10^2	10^1	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}

Size of a wavelength

← longer   shorter →

Soccer Field   House   Baseball   This Period   Cell   Bacteria   Virus   Protein   Water Molecule

Common name of wave

RADIO WAVES   INFRARED   VISIBLE   ULTRAVIOLET   "HARD" X RAYS

MICROWAVES   "SOFT" X RAYS   GAMMA RAYS

Sources

AM Radio   rf Cavity   FM Radio   Microwave Oven   Radar   People   Light Bulb   The ALS   X-Ray Machines   Radioactive Elements

Frequency (waves per second)	10^6	10^7	10^8	10^9	10^{10}	10^{11}	10^{12}	10^{13}	10^{14}	10^{15}	10^{16}	10^{17}	10^{18}	10^{19}	10^{20}

← lower   higher →

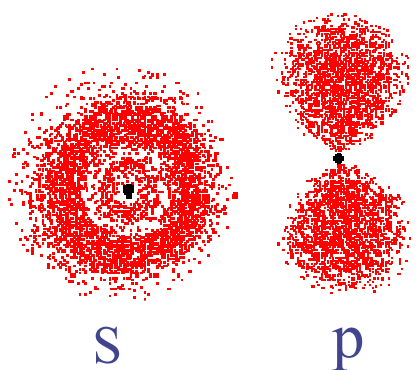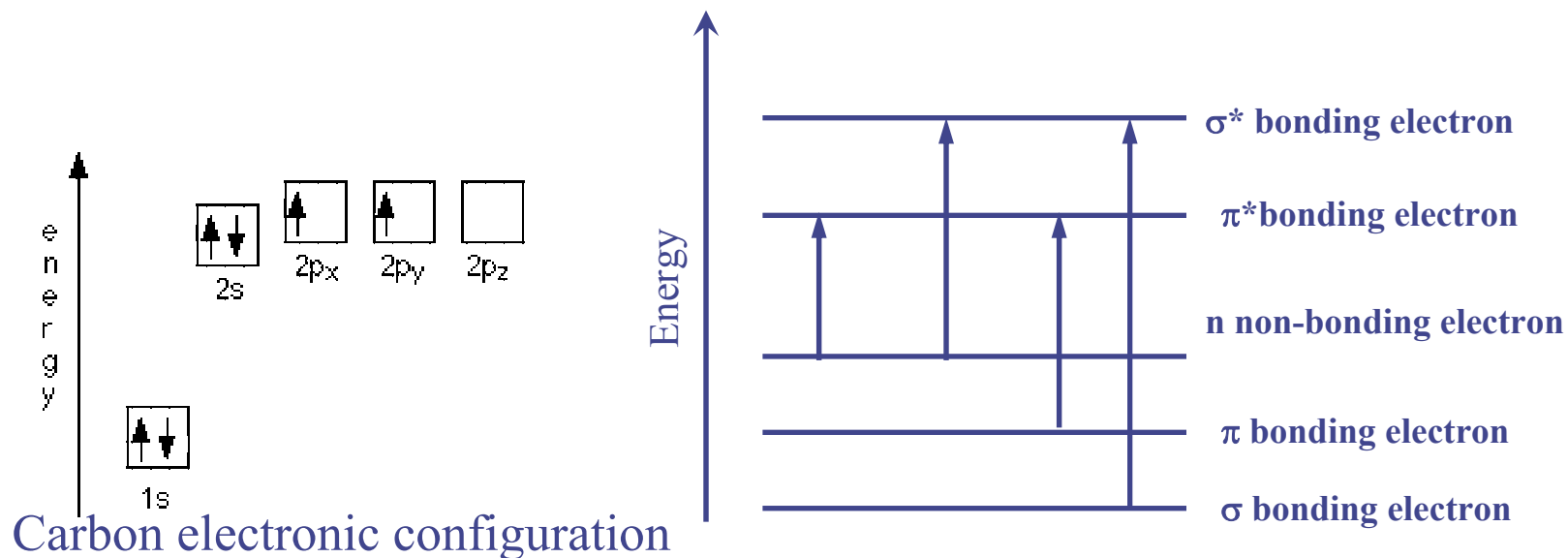| Energy of one photon (electron volts)	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	1	10^1	10^2	10^3	10^4	10^5	10^6

$$\text{Energy} = \frac{hc}{\lambda}$$

Electron Energy Levels

•The circles represent energy levels - representing increasing distances from the nucleus. The further out the energy level the higher its energy.

s   p

•In organic molecules, the energies of orbitals increase in the order

$$\sigma < \pi < n < \pi^* < \sigma^*$$

Carbon electronic configuration

σ^* bonding electron

π^* bonding electron

n non-bonding electron

π bonding electron

σ bonding electron

When EM Radiation Hits a Molecule

• Energy from a light source interacts with protein molecules in several ways.

•At an atomic bonding level we see promotion of electrons to higher energy levels.
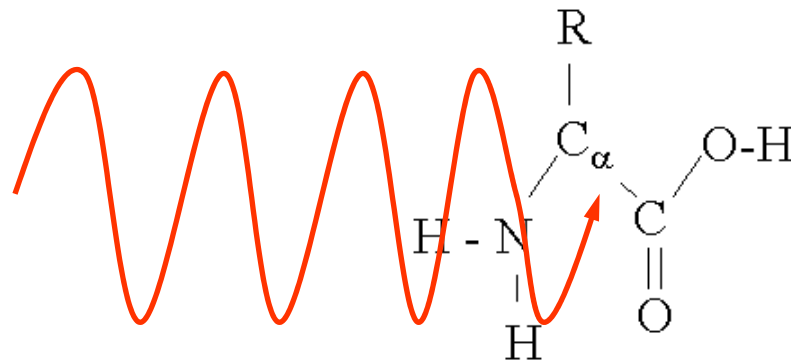
•On a molecular level we see absorption and emission.

•Transition between energy levels is not just confined to electrons; chemical bonds can have a variety of vibrational energy levels & atoms connected together by covalent bonds can rotate relative to each other.

•ΔE (elec) approx 20 kJmol^{-1} ΔE (vib) approx 2.5 kJmol^{-1} ΔE (rot) approx 0.25 kJmol^{-1}

Transitions

• A transition occurs when the energy of a molecule changes from one state to another - e.g. from ground to first excited state. Such transitions can be shown on a potential energy diagram

e ➡ **e** →
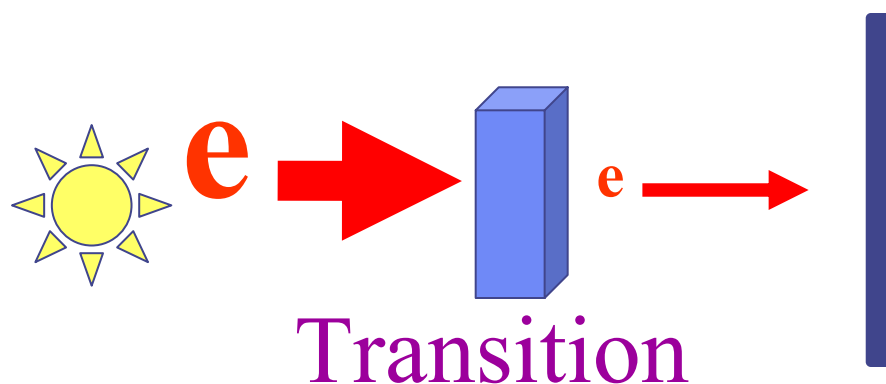
Transition

• Absorption occurs when radiation causes an increase in energy of the system with which it interacts.

e ➡ **e** →
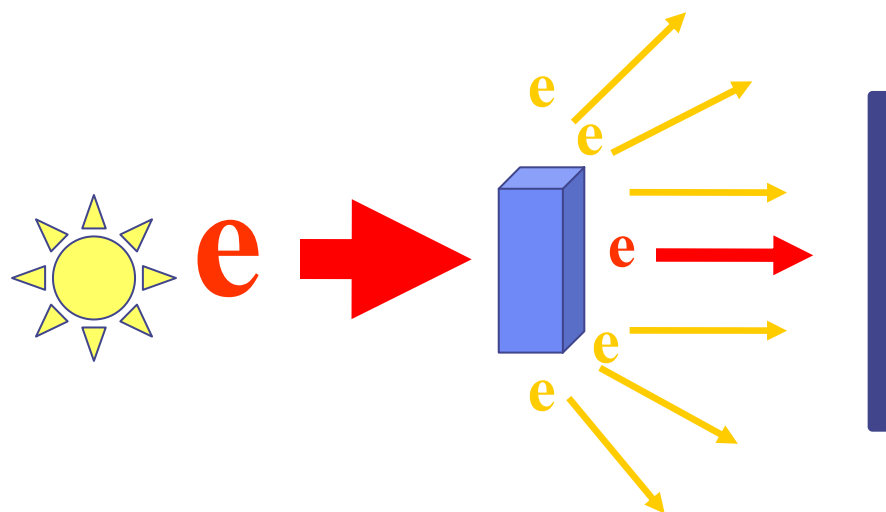
• Emission occurs when radiation is produced by a system during a transition from a higher energy level back to a lower one.

Transitions

- The frequency of radiation required to promote a transition between 2 energy states (E1 to E2)

$$\Delta E = (E2 - E1) = h\nu$$
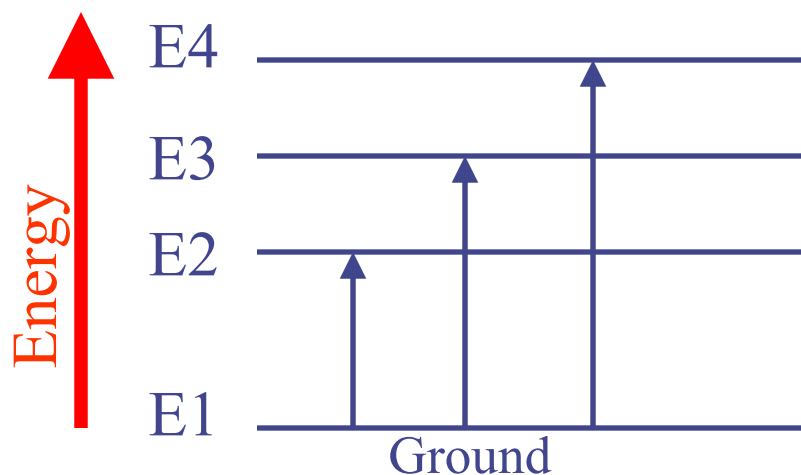
- The total energy of a molecule is the sum of distinct reservoirs of energy: Translational, vibrational, rotational, electron/nuclear spin orientation states

- Ground state = lowest energy, becomes more 'occupied' as molecules are cooled to absolute zero. E1, E2,…etc are excited states

- The type of radiation used causes different types of transitions

Transitions
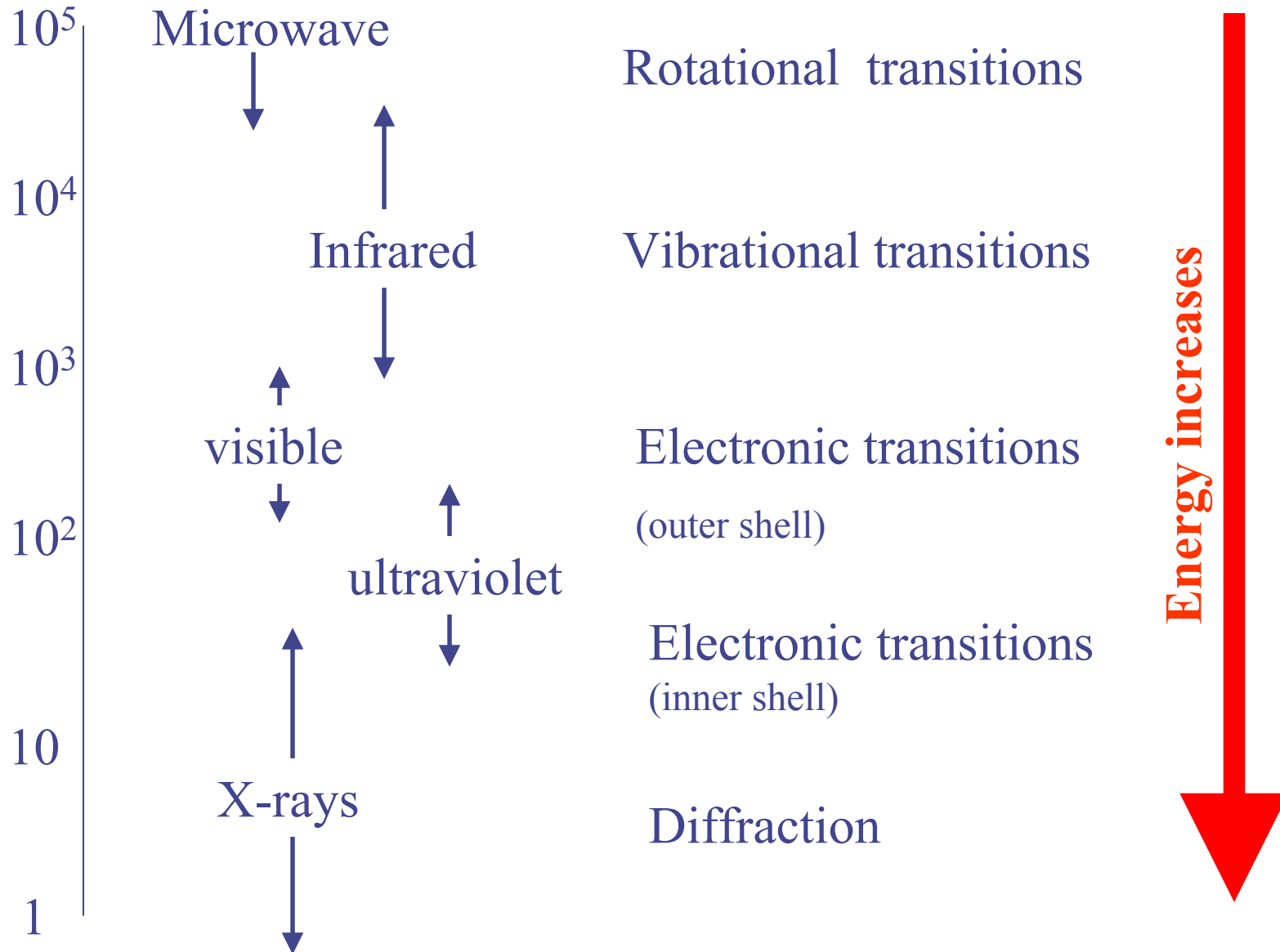
Fluorescence Spectroscopy

•Some chromophores are quite rigid & inflexible thus have a limited range of vibrational energy levels.

•Often the vibrational energies of the excited & ground states do not overlap so it is not possible for them to return to the ground state by losing energy as heat.

•In fluorescence spectroscopy, energy is lost by radiative transistion.
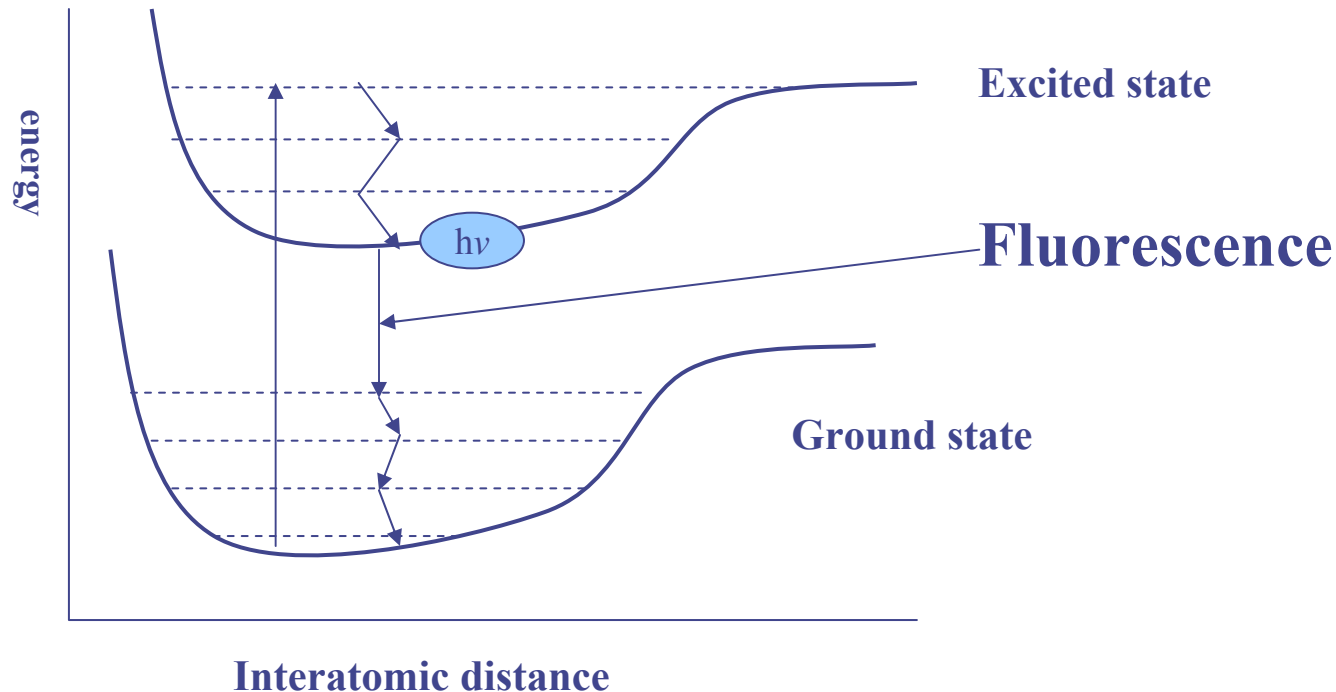
Fluorescence Spectroscopy

• A portion of the energy absorbed is re-emitted as light.

• The emitted light is always of longer wavelength (lower energy).

• We can quantify fluorescence by the quantum yield, Q

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

Under a given set of conditions, Q will have a fixed value for a particular fluorophore, with a maximum possible value of 1.

• This gives fluorophores a characteristic fluorescence spectrum as well as a characteristic absorption spectrum.
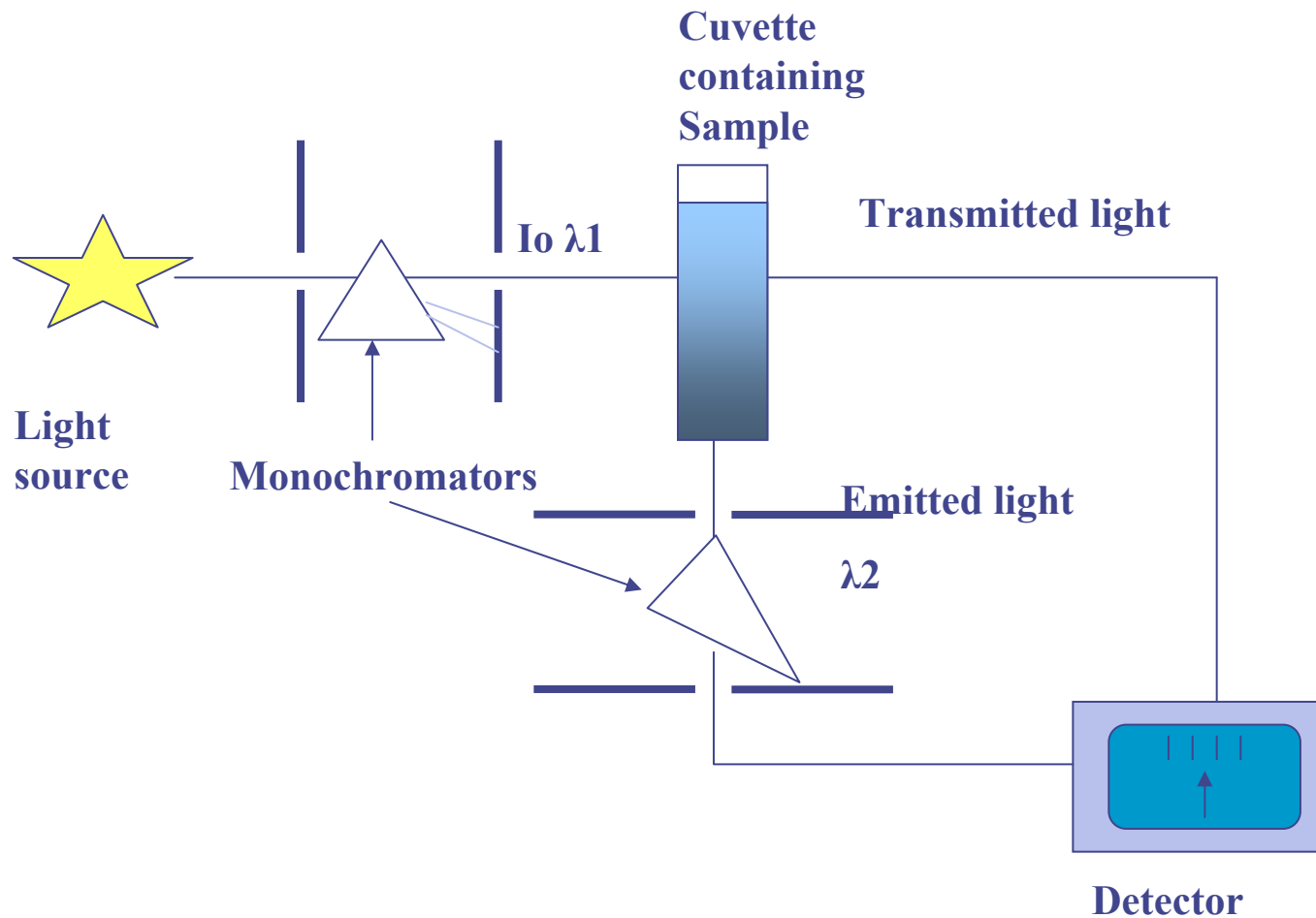
- Intrinsic fluorophores in proteins include Trp, Tyr & Phe. Trp & Tyr give stronger spectra than Phe, & Tyr is frequently quenched as a result of proton transfer in the excited state.

- The bases of DNA nucleotides & of some co-factors (e.g. NAD) are also intrinsic fluorophores, although they produce weak spectra.

Importance of Aromatic Amino-Acids

Tyrosine

Role in structure: Partially hydrophobic, Tyrosine prefers to be buried in protein hydrophobic cores. Tyr involved in *stacking* with other *aromatic* side-chains.

Role in function: Tyr contains a reactive hydroxyl group, thus making it much more likely to be involved in interactions with non protein atoms.

Tryptophan

Role in structure: Being hydrophobic, Tryptophan prefers to be buried in protein hydrophobic cores. The aromatic side chain can also mean that Tryptophan is involved in *stacking* interactions with other *aromatic* side-chains.

Role in function: As it contains a non-carbon atom (nitrogen) in the aromatic ring system, Tryptophan is more reactive than Phenylalanine though it is less reactive than Tyrosine. The Tryptophan nitrogens can play a role in binding to non-protein atoms, but such instances are rare.

Importance of Aromatic Amino-Acids

Phenylalanine

Role in structure: Being hydrophobic, Phenylalanine prefers to be buried in protein hydrophobic cores. The aromatic side chain can also mean that Phenyalanine is involved in *stacking* interactions with other *aromatic* side-chains.
Role in function: The Phenylalanine side chain is fairly non-reactive, and is thus rarely directly involved in protein function, though it can play a role in substrate recognition.

In general
Hydrophobic amino acids can be involved in binding/recognition of hydrophobic ligands such as lipids. Aromatic residues can also be involved in interactions with non-protein ligands that themselves contain aromatic groups via *stacking* interactions.

A common role for Tyr (Thr) within intracellular proteins is phosphorylation. Protein kinases frequently attach phosphates to Tyrosines in order to facilitate the signal transduction process. Protein kinases are highly specific (i.e. Tyrosine kinases generally do not work on Serines/Threonines and *vice versa*).

Example: Tryptophan

Shows characteristic absorption & fluorescence spectra.
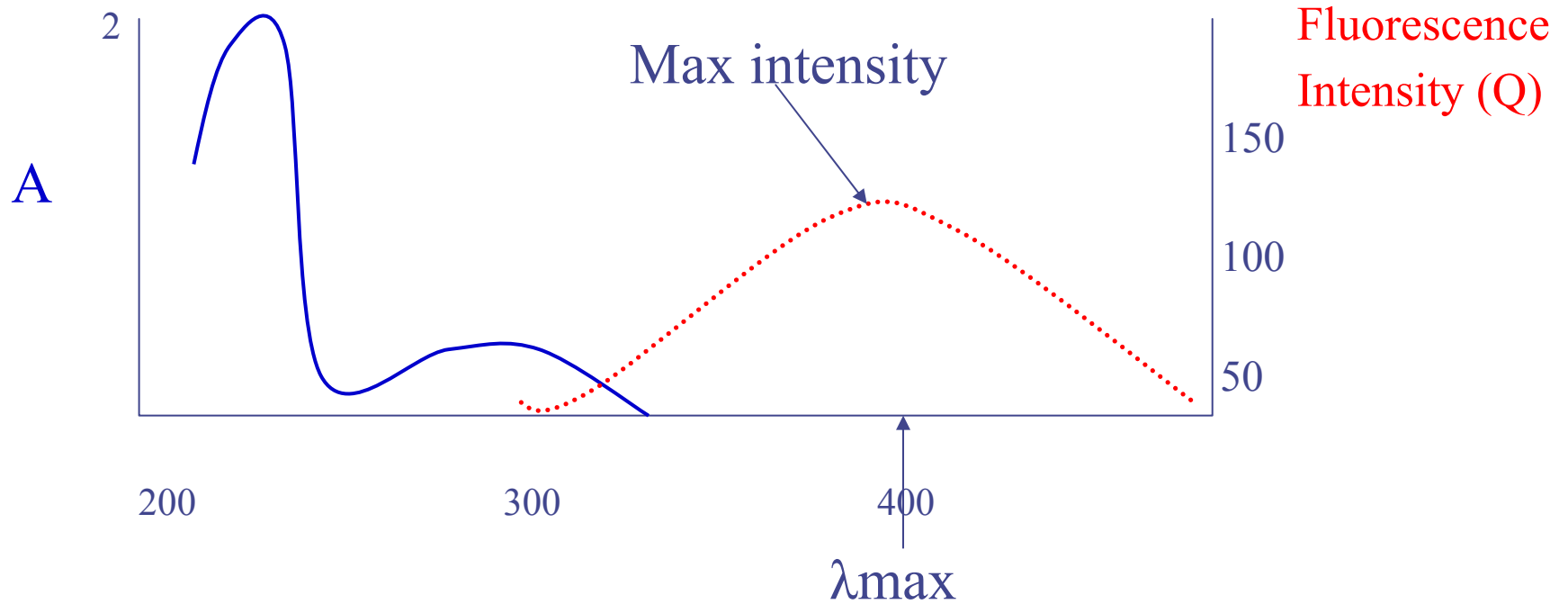
Uses of Fluorescence

•Analytical assays for organic compounds, eg. metabolic pathways.

•Structural – local conformation of aromatic amino acids, tertiary structure, denaturation transitions.

•Binding studies.

•Chemiluminescence.

•Fluorescence microscopy.

•Fluorescence is more sensitive to fluorophore environment than UV/visible spectroscopy due to the increased time the molecule stays in the excited state.

Fluorophores

•Substances which display fluorescence should have:

     •Rigid structures

     •Delocalised electrons (alternate single & double bonds, aromatic rings).

     •Intense U.V absorption bands (π to $\pi*$ transition, e.g. Trp Em 5700 m^{-1} cm^{-1}

     •Short excited state lifetimes ($<10^{-9}$ sec).

     •Good overlap between electron orbitals of ground & first excited states.

•Two ways of measuring fluorescence:

     •Emission spectrum- excitation λ constant, measure fluorescence intensity of emission against λ, I.e. spectrum of emitted light.

     •Excitation spectrum – measure fluorescence intensity at different excitation λ, similar to absorption spectra.

Physical Origin

S₁' _____

2

S₁

1

3

S₀ _____
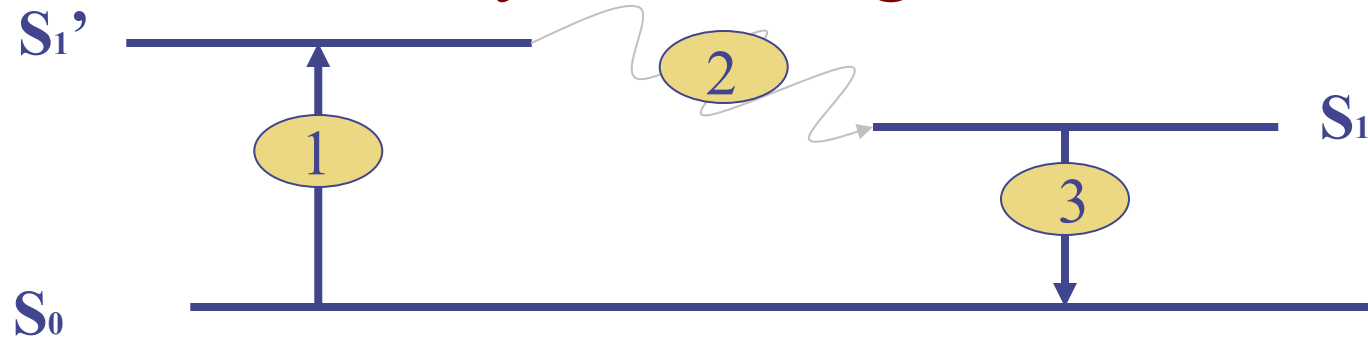
1 **Excitation:** A photon of energy $h\nu_{(EX)}$ is absorbed by the flourophore, creating an excited electronic singlet state (S1').

2 The fluorophore undergoes conformational change & interacts with it's molecular environment. S1' energy is partially dissipated, to give a relaxed singlet state (S1) from which fluorescence emission originates. Not all molecules excited by stage 1 return to the ground state (S0) by flourescence emission: quenching, energy transfer and intersystem crossing can also lead to depopulation of S1.

3 A photon of energy $h\nu_{(EM)}$ is emitted, returning the fluorophore to the ground state S0. The wavelength of emission is longer and the energy is lower

Energy Loss & Quenching

•Only a proportion of the light absorbed is emitted as radiation, energy may be lost in vibrational transitions & heat transfer with solvent as well.

•Two further processes can diminish amount of light energy emitted from the sample.

•**Internal quenching** due to intrinsic structural feature e.g. structural rearrangement.

•**External quenching** interaction of the excited molecule with another molecule in the sample or absorption of exciting or emitted light by another chromophore in sample.

•All forms of quenching result in non-radiative loss of energy.

•**Intersystem crossing** from excited singlet to excited triplet state. Transition occurs between the singlet ground state (electrons are anti-parallel & paired) to an excited triplet state (electrons are parallel and unpaired).

•Return to ground state much slower process than fluorescence, = Phosphorescence. Emitted radiation is of an even longer wavelength because the energy difference between the two is small.

Singlet excited state S_1

Intersystem crossing

Triplet state

Phosphorescence

Singlet ground S_0
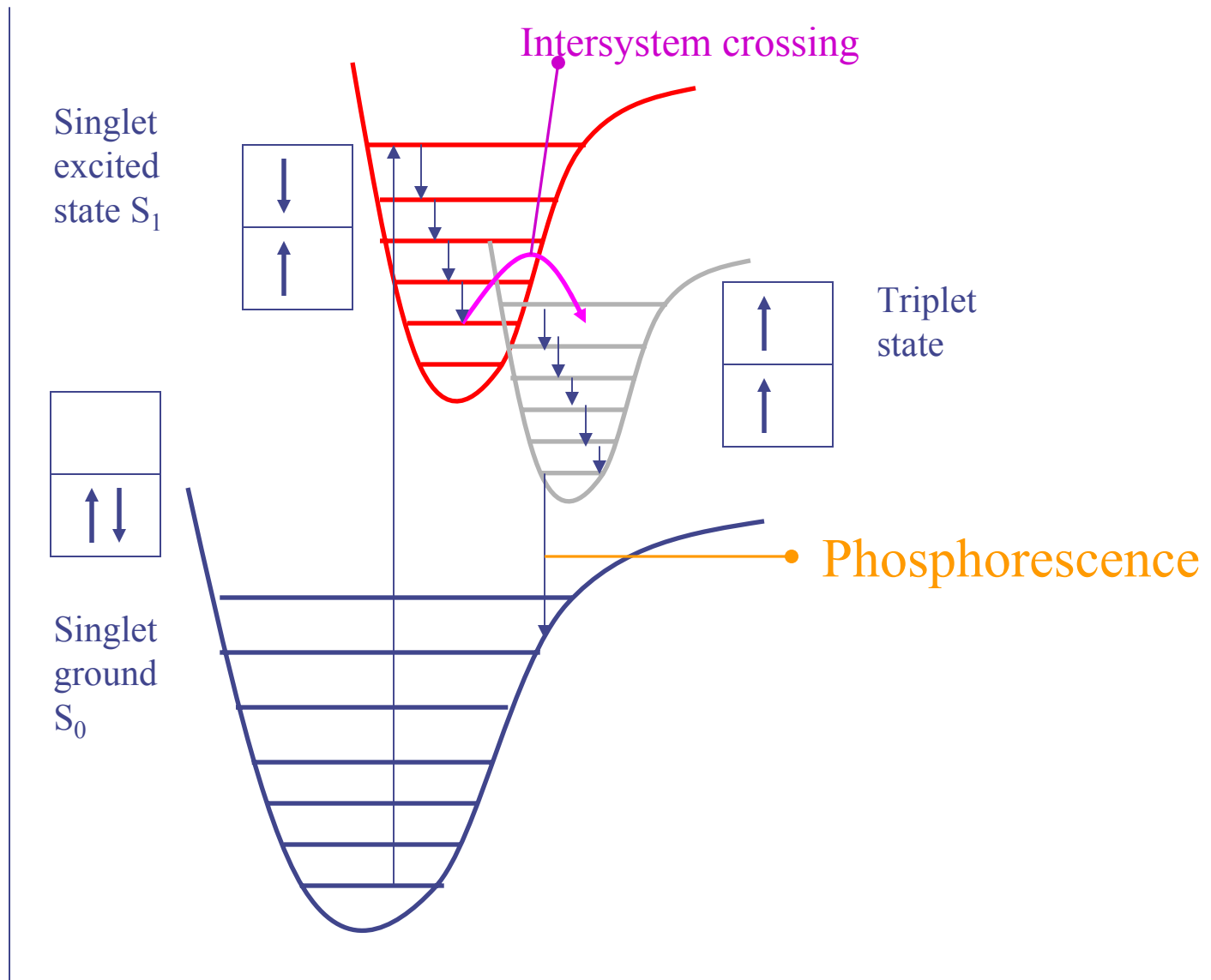
Interpreting Fluorescence Spectra

• All fluorescence of a protein is due to Trp, Tyr or Phe, unless it contains an extrinsic fluor or fluorescent co-factor.

•The λmax of Trp shifts to shorter wavelengths & the intensity of λmax increases as the polarity of the solvent decreases.

If the λmax is shifted to shorter wavelengths when the protein is in a polar solvent, the Trp must be internal.

if λmax is shifted to shorter wavelengths when the protein is in a non-polar solvent the Trp is on the surface of the protein or conformation changes such that it is brought to the surface.

•If a quencher (iodide, nitrate ions) quenches Trp fluorescence it must be on the surface of the protein. Failure to do so means its either internal, in a small crevice, or in a highly charged region.

•Trp fluorescence is quenched by neighboring protonated acid groups. If the pK measured by monitoring Trp fluorescence is the same as the pK for a known ionisable group (e.g. a carboxyl) then the group must be near the Trp.

•If a substance that effects the Q of the free amino acid doesn't effect the fluorescence of the protein it must do so by producing a conformational change.

•If a substance binds to a protein & Trp fluorescence is quenched, either there is a conformational change as a result of binding or Trp is in or near the binding site.

What Can be Learned from Intrinsic Fluorescence?

- The 3 Trp of a hypothetical protein have a much greater fluorescence intensity and shorter λmax than does free Trp

A-without co-factor

B-with cofactor

C- free Trp
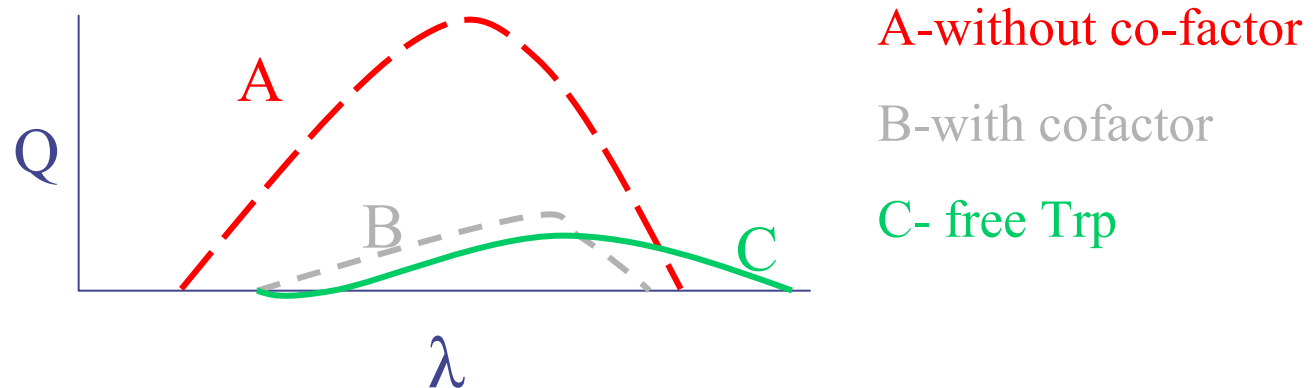
- When the cofactor is added, intensity decreases & λmax gets longer. Conformation of the protein must have changed either to make one or more of the Trp available to polar solvent or bring them near charged groups.

- Addition of iodide ions has no effect on fluorescence spectrum before or after cofactor binds. Hence no Trp on surface (or in binding site) before or after addition of co-factor; they have probably been moved to a more polar, internal region.

Probing the Active Site of an Enzyme

•A hypothetical enzyme is known to contain a single Trp and its λmax is nearly the same as that for free tryptophan suggesting that it is in a polar environment. The Q is low, suggesting that a quencher might be nearby:
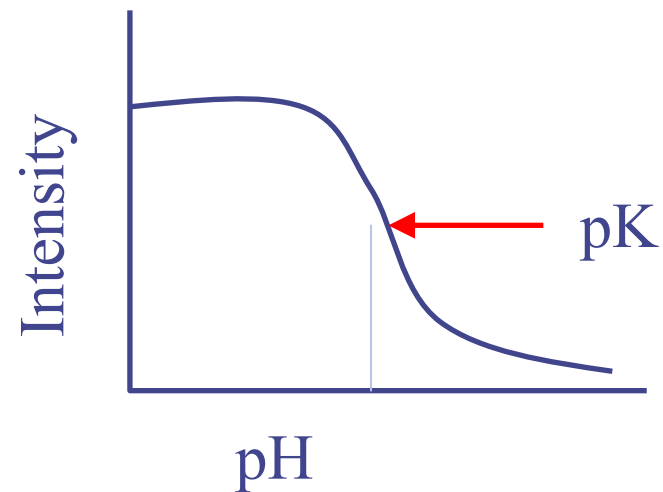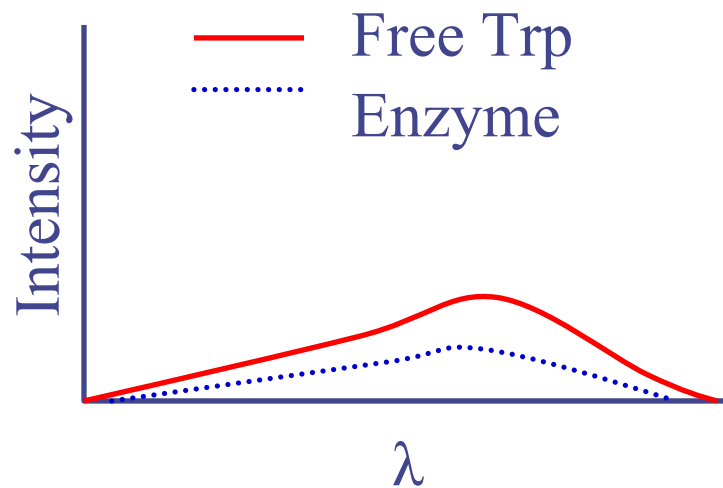
•Acid titration decreases Q and the pK of the transition is that of a carboxyl a.a. side chain (3-4), so the low Q is caused by the proximity to a carboxyl.

Probing the Active Site of an Enzyme
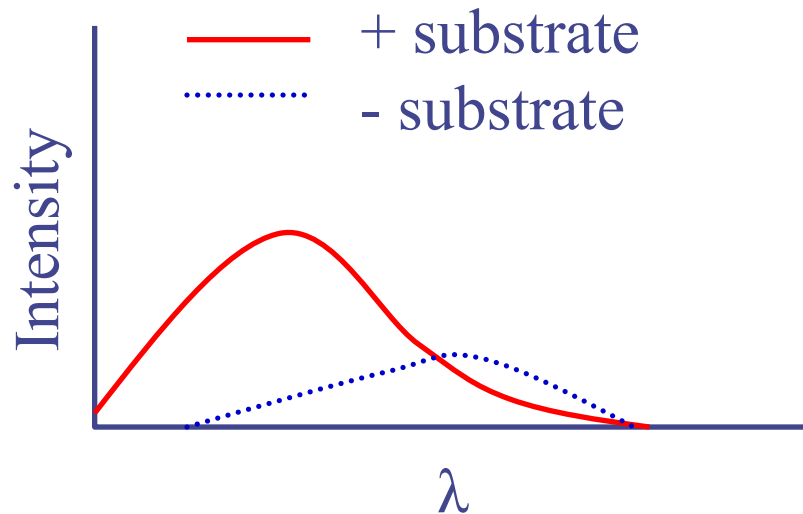
The addition of the substrate shifts λmax to shorter wavelengths & increases Q.
The shift in λmax indicates that the Trp is in a less polar environment.
This could mean that there has been a conformational change moving the Trp to a non-polar region or that Trp is in or near the binding site & that substrate binding excludes water. The increase in Q suggests that carboxyl quenching is eliminated.

The likely answer is that the Trp is in the active site & the substrate binds either to the Trp or the carboxyl.
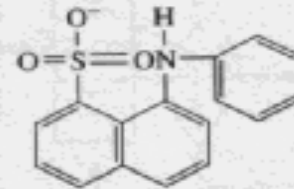
Fluorophores

Tyrosine
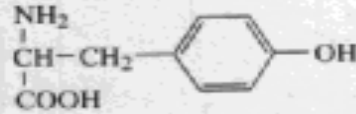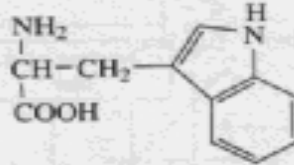
Tryptophan

ANS (1-Anilino-8-napthalene sulphonate
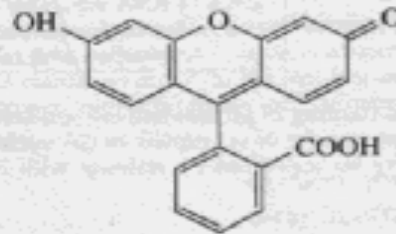
Fluorescin

Ethidium bromide
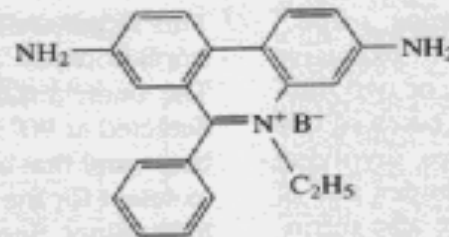
Acridine orange
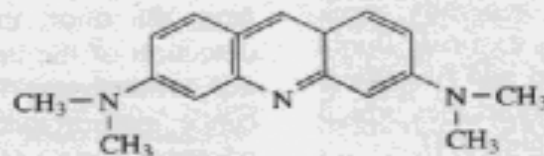
Extrinsic Fluorophores

- Fluorophores such as ANS are only strongly fluorescent when bound to a protein.

- ANS will bind to mildly hydrophobic pockets on the proteins surface (e.g. active site).

- Kd can be calculated by carrying out competition experiments with ANS and ligand.

Ligand Binding

- Binding of ligands to proteins often causes changes to 3-D structure.

- If this causes a change in the environment of the fluorophore (intrinsic or extrinsic), can result in a measurable change in the fluorescence spectrum.

- Provided the fluorophore has a unique location in the protein, such changes of fluorescence at a particular wavelength (ΔF) can be used to calculate Kd the dissociation constant (good for active siteTrp). $P + L \rightarrow PL$

$$Kd = \frac{[P]\,[L]}{[PL]}$$
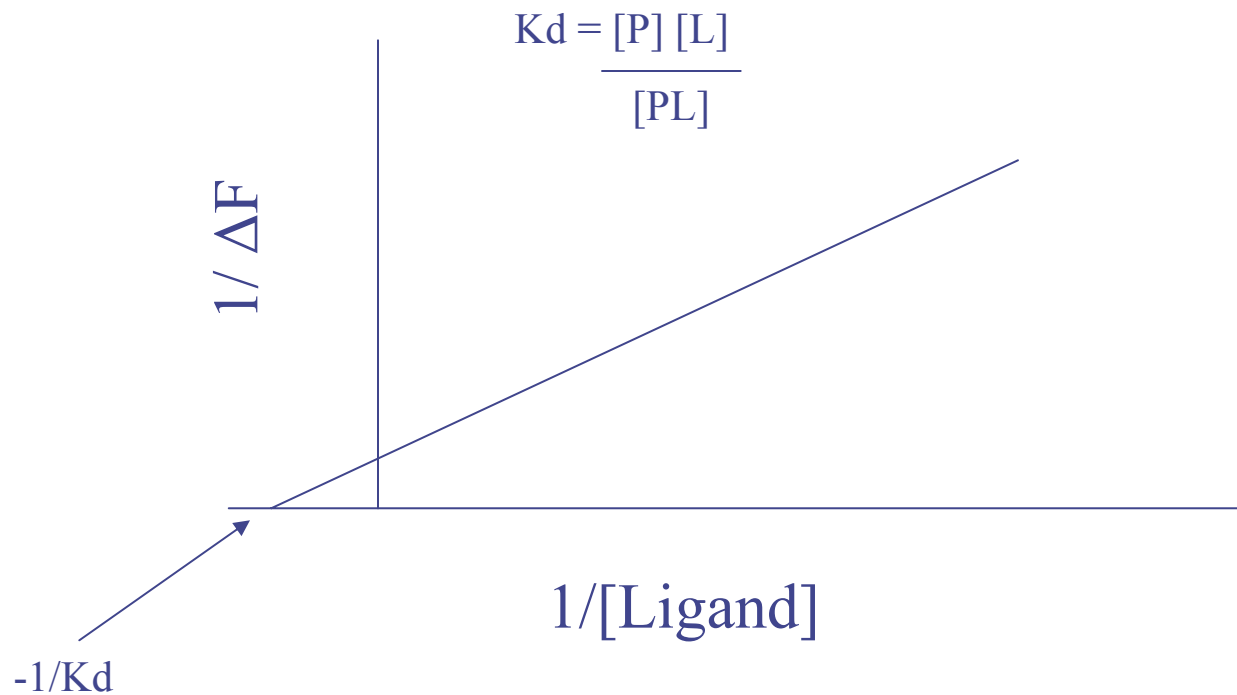
Lineweaver-Burke Plot
See e.g. http://www.dentistry.leeds.ac.uk/biochem/thcme/enzymekinetics.pdf

Extrinsic Fluorophore Example

- Hemoglobin is a complex of a small prosthetic group with the protein apohemoglobin. The extrinsic fluor ANS fluoresces when added to solutions of apohemoglobin but not with hemoglobin.

- The addition of heme to the apohemoglobin-ANS complex eliminates the fluorescence by displacing ANS.

- This tells us that ANS and the heme group bind to the same site. Since ANS is only fluorescent in highly non-polar environments, the heme must bind to a highly non-polar site.

- This would give valuable clues in the interpretation of X-ray diffraction patterns. E.g. you would know that a certain configuration of amino acids could be the point of interaction between heme and protein when building the structure.

Strandedness of Polynucleotides

• The extrinsic fluor acridine orange shows an increase in Q & a shift in λmax when bound to polynucleotides.
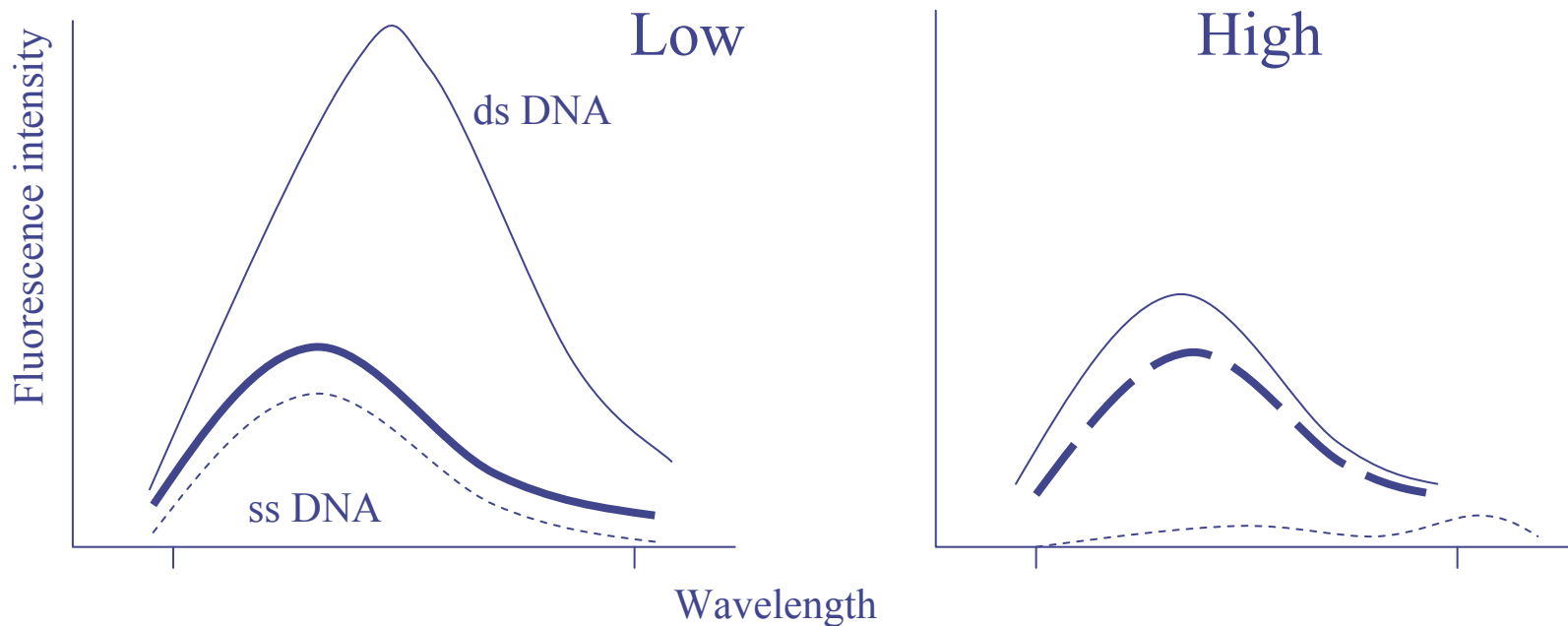
• When saturating amounts of acridine orange are added the values of λmax are significantly different for double stranded & single stranded polynucleotides. Hence, the structures can be distinguished in this simple way. If a sample contains both ds & ss polynucleotides, it will have 2 peaks, one for each value of λmax.

Fluorescence Resonance Energy Transfer (FRET)

• A fluorophore has a characteristic λmax in both its absorbance & emission spectra.

• FRET can occur when the emission spectra of one fluorophore (A) overlaps with the absorbance λmax of a second fluorophore (B).

• If the fluorophores (extrinsic or intrinsic) have unique locations within the protein or complex, it is possible for emission light energy from A to be absorbed by B and to be emitted as part of B's emission spectrum

Fluor A

A/Q

Absorbance   emission

λ1   λ2

Fluor B

A/Q

Absorbance   emission

λ2   λ3

λ (nm)
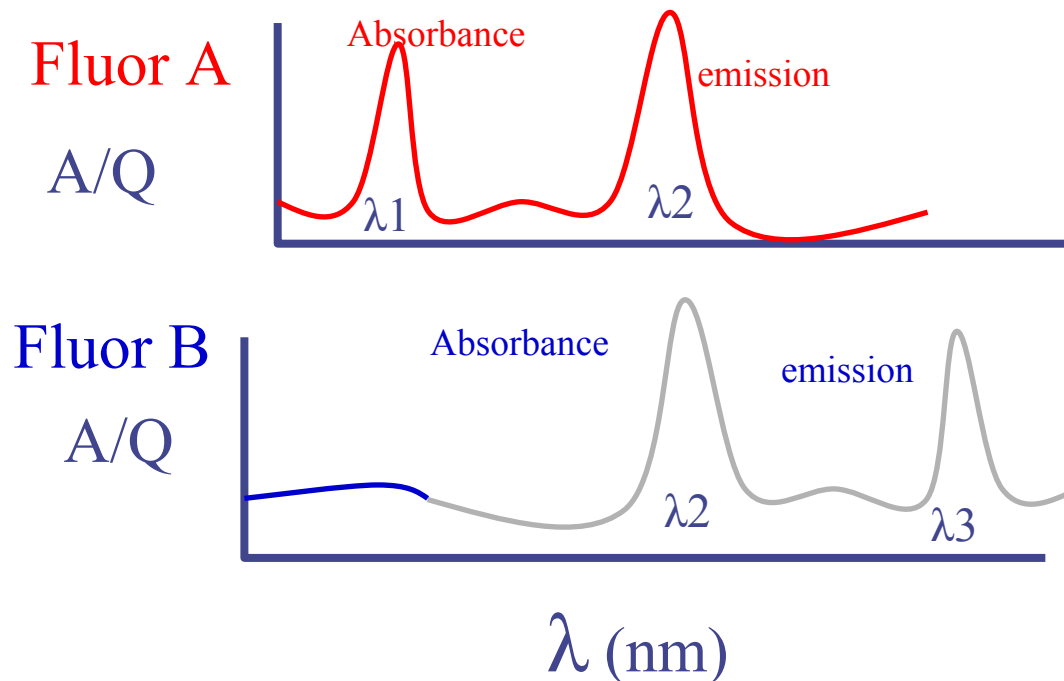
• FRET is dependent on the distance, R, between the two fluors. Used to measure distances in proteins, membranes, & macromolecular assemblies 10 to 80 Å apart.

• Efficiency of the energy transfer (E) from donor to acceptor (A to B) defined by

$$E = \frac{R_0^6}{R_0^6 + R^6}$$

Fluor A   Fluor B

R
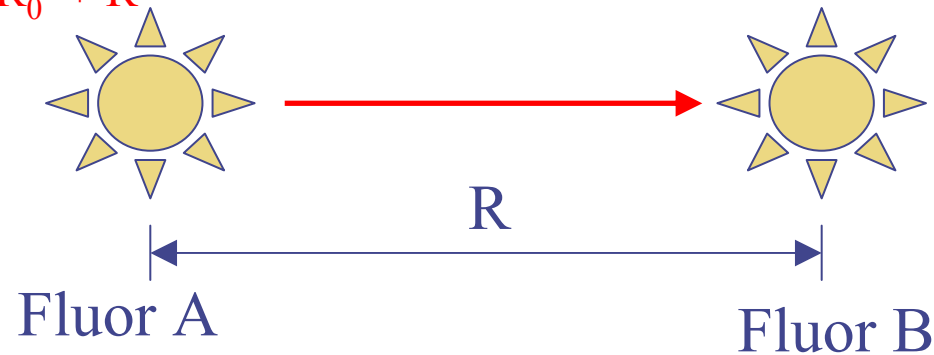
• R = distance between A & B, R_0 is a constant calculated from absorption and emission spectra.

• E can be calculated from fluorescence intensity (F)

$$E = Fda / Fd$$

Fda = F in the presence of acceptor, Fd = F in the absence of acceptor.

• Once E is known, R can be calculated from the first equation if R_0 is known.

FRET Uses

• Can probe the topology of membrane proteins which can't be done by X-ray or NMR.

•Used to study movement in contractile proteins e.g. actin & myosin.

•Can determine changes upon receptor ligand binding, monitoring the formation of tetraplex & duplex DNA formation & mapping of active sites.

Limitations

•Limited range (10 – 80 Å) if R is > 80Å. E is very small

•Need 2 fluorophores, a donor & an acceptor. Trp is a good donor.

•Assumptions are made in the calculation of R_0 such as the orientation of the donor & the acceptor.

Importance

•Tyr to Trp transfer explains why we don't see Tyr fluorescence in proteins.

•Spectroscopic ruler.

•Quite fast & simple compared to 3-D structure determination.

FRET Application Example

Rhodopsin is the light absorber in the discs of rod cells in eye. It is a complex between a protein, opsin & a fluor cis-retinal, which absorbs given light. The Mr of rhodopsin (40 kDa) implies that if the molecule were spherical, its diameter would be 45 Angstrom.

A fluor, which served as an acceptor, was bound to a particular part of opsin. FRET experiments showed that this area if 75 Angstrom away from the cis-retinal group

This suggests that rhodopsin is an elongated protein long enough to traverse membrane of the retinal disc. Proposed that rhodopsin might serve as a light sensitive gate that controls the ion flow across the membrane.

Membrane

Retinal

George Eade
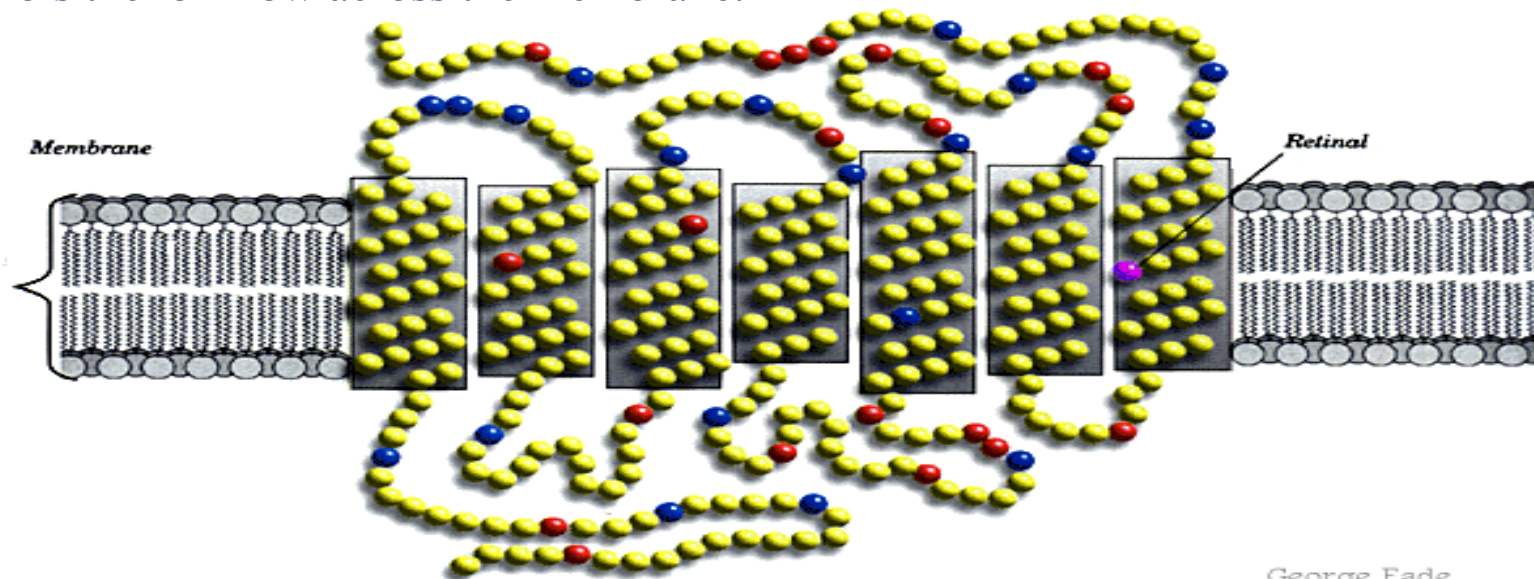
Fluorescence & Protein Folding

- One of the main techniques used to study protein folding.

- Folded proteins compact structures - residues adopt highly defined conformations.

- When a protein unfolds from active (N) to unfolded (U) sidechains become free to rotate around $C\alpha$ & $C\beta$ bonds & move freely in solution. Trp is normally buried in the hydrophobic core of the protein.

- When a protein unfolds Trp comes in contact with solvent & may also be able to interact with quenching agents.

N ⇄ U
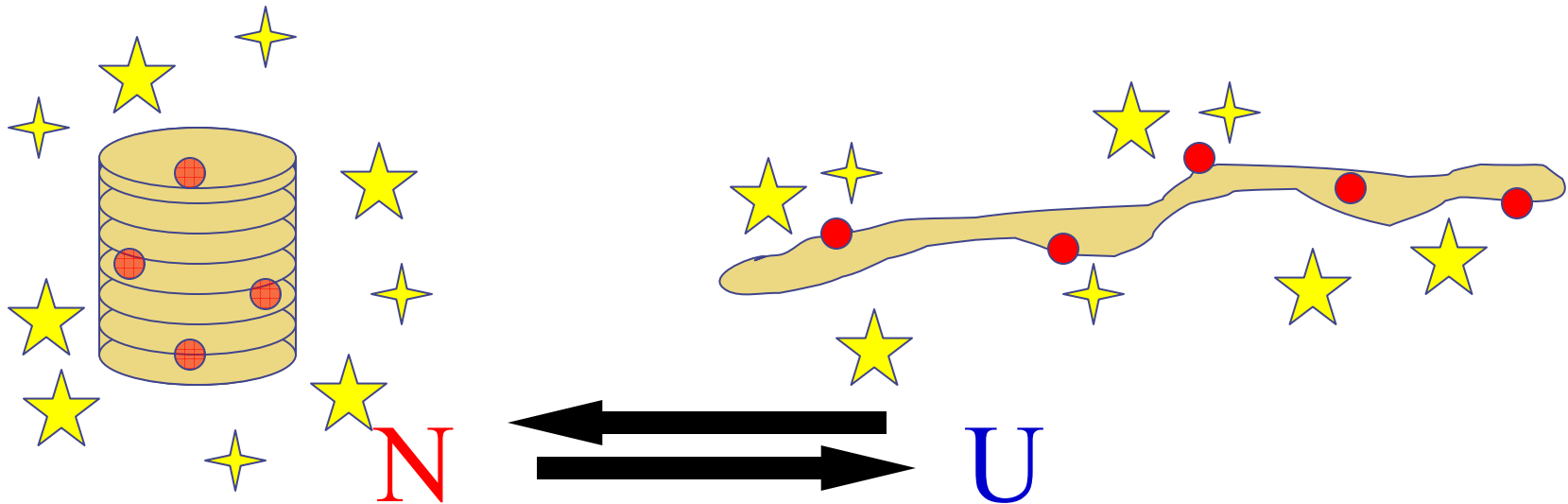
Fluorescence & Protein Folding

•Four main experimental parameters are usually observed

1.Intensity may increase or decrease causing an increase or decrease of Q

2.The fluorescence lifetime (T) may decrease as a result of quenching; (e.g. encounters with disulphide bonds or amines)

3.Radiation emitted from an unfolded protein is usually of lower energy & longer wavelength (red shift)

4.Quenching of fluorescence by an external quencher such as potassium iodide. KI has a strong negative charge precluding it to diffuse into the hydrophobic interior of folded & partially folded proteins. Quenching of fluorescence by KI is therefore good evidence for the exposure of Trp to solvent during protein folding.

Recap

- Flourescence is a cyclical process & only a portion of the energy absorbed is re-emitted as light.

- Emitted light is of a lower energy & longer wavelength than absorbed energy.

- Quenching.

- Difference between fluorescence & phosphorescence.

- Emission spectrum = monitor fluorescence intensity at a constant excitation wavelength.

- Excitation spectrum = monitor fluorescence intensity at different excitation wavelengths holding emission wavelength constant.

- In proteins, 3 amino-acids are fluorescent: Trp, Tyr, Phe = intrinsic fluorophores.

- Extrinsic fluorophores e.g. chemical dyes.

- FRET provides distance measurements.

Resources

Textbook:

Cantor and Schimmel, Chapters 7 and 8.

WWW:

•Fluorescence Spectroscopy:

http://chemistry.rutgers.edu/grad/chem585/lecture2.html

http://www.probes.com/handbook/sections/0001.html

•FRET:

http://www.probes.com/handbook/boxes/0422.html

•Enzyme Catalysis:

http://www.dentistry.leeds.ac.uk/biochem/thcme/enzymekinetics.pdf

Figure and Text Credits

Text and figures for this lecture were adapted from the following sources:

- http://staff.bath.ac.uk/bssmdb/biol0020intro_to_spectroscopy.ppt
- http://staff.bath.ac.uk/bssmdb/Fluorescence2.ppt

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