Fluorescence:
- Absorption
- Relaxation to ground vibrational level of \( S_0 \)
- Emission

The lifetime of the excited state (~ 10^-8 sec) makes fluorescence a valuable probe of biological macromolecules.

The emission spectrum is always displaced to lower energy. Vibrational relaxation prior to emission. The displacement of the emission spectrum to longer wavelengths - Stokes' Shift.

Excitation:
The fluorophore exists in some ground state (\( S_0 \)). A photon of energy \( h \nu_{EX} \) is supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore. This creates an excited electronic singlet state (\( S_1' \)).

Excited-state lifetime:
The excited state exists for a finite time (typically 1-10^-10 - 10^-9 seconds). During this time, the fluorophore undergoes conformational changes and is also subject to interactions with its molecular environment.

The energy of \( S_1' \) is partially dissipated, yielding a relaxed singlet excited state (\( S_1 \)) from which fluorescence emission originates. Not all molecules initially excited by absorption (Stage 1) return to the ground state (\( S_0 \)) by fluorescence emission.

Other processes such as collisional quenching, fluorescence energy transfer and intersystem crossing may also depopulate \( S_1 \).

Quantum Yield \( \phi = \frac{\text{Number of emitted photons}}{\text{Number of absorbed photons}} \)

Emission spectra usually mirror images of the absorption spectrum

Properties of a strongly fluorescing molecule
Large molar absorptivity (extinction coefficient, \( \epsilon \)). The excited state must be populated for fluorescence to occur.

Einstein coefficients - probability of spontaneous emission of light from the excited state of a molecule is proportional to the molecule’s ability to absorb light.
Stokes Shift

- is the energy difference between the lowest energy peak of absorbance and the highest energy of emission.

The Stokes shift is fundamental to the sensitivity of fluorescence techniques because it allows emission photons to be detected isolated from excitation photons.

![Stokes Shift Diagram](image)

Quantum Yield

From Einstein's coefficients:

\[ \Phi = \frac{1}{k_r + \sum_k k_i} \]

where \( \Phi \) is the "quantum yield" for the system.

Having absorbed a photon, what is the likelihood of a photon being re-emitted? This probability is given by the "quantum yield" (\( \Phi \)) for the system:

\[ \Phi = \frac{k_i}{k_r + \sum_k k_i} \frac{\tau}{\tau} \]

Biological fluorophores

Intrinsic fluorophores

Proteins

Tryptophan dominates protein fluorescence spectra
- high molar absorptivity
- ability to quench tyrosine and phenylalanine emission by energy transfer.

Aromatic amino acids

The amino acids phenylalanine, tyrosine, and tryptophan have \( \pi-\pi^* \) transitions.

There is a pattern of weak bands from 240 - 300 nm and much more intense bands between 190 - 220 nm.

The weak bands are allowed by vibronic coupling (L).

Tryptophan:

Described as a three level system: the ground state, and two excited states \( L_a \) and \( L_b \), with permanent and transition dipole moments given in the Table. The orientations of the dipole moments in the molecular frame are different.

Since the \( L_a \) and \( L_b \) levels are nearly degenerate, their transition energies are taken to be both at 280 nm

<table>
<thead>
<tr>
<th>state transitions</th>
<th>( \mu_x )</th>
<th>( \mu_y )</th>
<th>( \mu_z )</th>
<th>dipole moment (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 0 \rightarrow L_a )</td>
<td>0.690, 0.723</td>
<td>0.356, 0.394</td>
<td>0.751, 0.640</td>
<td>2.0</td>
</tr>
<tr>
<td>( L_a \rightarrow L_b )</td>
<td>0.707, 0.707</td>
<td>0.707, 0.707</td>
<td>0.707, 0.707</td>
<td>3.5</td>
</tr>
<tr>
<td>( 0 \rightarrow L_b )</td>
<td>0.707, 0.707</td>
<td>0.707, 0.707</td>
<td>0.707, 0.707</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Unit vectors in the direction of the different dipole moments for the lowest transition in tryptophan.

Two dipoles brought side by side, relative orientation (parallel vs. antiparallel) - the energy of individual molecules, \( E \), will split into two new states with the energies \( E' \) and \( E'' \). parallel dipoles repel make up an overall higher dipole moment i.e. stronger absorption - higher energy state - blue shift of absorption antiparallel dipoles attract cancel each other to make a weak absorption - lowers the energy of that state - red shift of absorption
Dipole moments of tryptophan.

Full arrows represent the transition dipole moments, dashed ones represent the permanent dipole moments. G stands for ground state. Dipole moments belonging to excited state La (Lb) are depicted in red (blue).

The two lowest absorption bands in tryptophan are ascribed to the long and short axes of the indole ring.

The weak band at 280 nm is $\nu_a$ and the strong band at 220 nm is $\nu_B$. The weak band has a large orbital angular momentum change.

After a molecule absorbs light, it takes time for it to re-emit light. During this time the molecule can undergo:

1) solvent rearrangement
2) rotation of the molecule
3) loss of energy to neighboring molecule (energy transfer)
4) reaction (quenching)

Ground state dipole moment: 2.1
Excited state dipole moment: 5.4

More polar in the excited state: electron density changed

Protein Fluorescence

Proximity of aromatic groups in a folded protein results in efficient energy transfer between these groups.

Absorbance Max

Phenylalanine 257.4 nm
Tyrosine 274.6 nm
Tryptophan 279.8 nm

Proteins containing all three aromatic residues emit fluorescence light typical of Trp
Proteins containing only Tyr and Phe emit light typical of Tyr.

Trp residues that are exposed to water fluoresce maximally at a wavelength of 350 nm, while totally buried Trp residues emit at about 330 nm.

(Eftink et al., Biochemistry 26: 8338-8346 (1987).)

Fluorescence by a protein is complex when there is more than one aromatic side chain.

The proximity of aromatic groups in a folded protein results in efficient energy transfer between these groups.

Light absorbed by one chromophore is transferred to another that absorbs at a longer wavelength, which can then emit the energy as fluorescence.
Local electric fields cause spectral shifts

Solvent can affect the ground state and excited state molecules causing spectral shift

Example: H bonding to tryptophan. Changes its absorption by about 10 nm

Normalized fluorescence spectra of tryptophan

Residues belonging to five spectral classes A, S, I, II and III
- A: Indole in Cyclohexane
- III: 100% Ethanol

The fluorescence spectra of most proteins are dominated by the tryptophan contribution.

Emission spectrum of tryptophan undergoes a significant red shift upon exposure to a more polar environment.

Protein Denaturation

Unfolding: Trp exposed to water

Trp at position 19 in more stable part of the protein than Trp at position 99
**Fluorescence:**
- Absorption
- Relaxation to ground vibrational level of $S_1$
- Emission

The lifetime of the excited state (~ $10^{-8}$ sec) makes fluorescence a valuable probe of biological macromolecules.

**Nucleic acids** - strongly absorbing molecules are non-fluorescent

Several other processes compete with fluorescent for returning the molecule to the ground state

a) Internal conversion: conversion of electronic energy to vibrational energy. Especially effective if $S_0$ and $S_1$ vibrational levels overlap

b) Intersystem crossing. The "forbidden" (hence, low probability) of the excited state singlet to a triplet state.

Most nucleic acids are nonfluorescent. DNA base analogs, 2-aminopurine (an adenine analog) and isoxanthopterin (IXP, a guanine analog) are fluorescent and quenched by incorporation into double-stranded structures.

**Noncovalent DNA probes.**

Acridine Orange
- 500/526 nm DNA
- 460/650 nm RNA

Ethidium Bromide
- 518/605 nm

**Binding of Ethidium-Bromide to Transfer RNA**

Yeast phenylalanine tRNA

Ethidium Bromide
Ethidium bromide can intercalate into nucleic acid structures. It binds well to both DNA and RNA.

Fluorescence investigations of EB - tRNA interactions have indicated a "strong" binding site and one or more "weak, non-specific" binding sites.

Enzyme cofactors NADH, FAD and pyridoxal/pyridoxamine are fluorescent.

Extrinsic probes: Protein labeling reagents

Table 3.3. Fluorescent Amino Reagents

<table>
<thead>
<tr>
<th>Probe</th>
<th>Use</th>
<th>Absorption</th>
<th>Emission</th>
<th>T (°C)</th>
<th>hν (eV)</th>
<th>eS (eV⁻¹ m² cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH, FAD, pyridoxal</td>
<td>Protein modification</td>
<td>350</td>
<td>6.4</td>
<td>28</td>
<td>4.8</td>
<td>410.4</td>
</tr>
</tbody>
</table>
| Fluorogenic amino reagents provide sensitive detection of amino acids, peptides, and proteins.

Viscosity probes. Fluorescence intensity influenced by viscosity

At high viscosity, internal motions are hindered, and the molecule is strongly fluorescent.

At low viscosity, however, the excited state is rapidly depopulated by charge transfer from the amino group to the vinyl group.
Green Fluorescent Protein (GFP) - bioluminescent jellyfish *Aequorea victoria*.
- Obvious β-barrel structure, with chromophore housed within the barrel.
- Remarkably, the chromophore is formed spontaneously (from Ser-65, Tyr-66, Gly-67) upon folding of the polypeptide chain, without the need for enzymatic synthesis.
- It is possible to insert the gene for GFP into cells and use the resulting protein as a reporter for a variety of applications.

![Diagram of Green Fluorescent Protein](image)

**Binding of ANS (anilinonaphthalene sulfonic acid) by bovine serum albumin (BSA)**

λ_{ex}=280 nm

Emission from ANS alone in aqueous solution is very weak. In the presence of BSA, the fluorescence of ANS is increased, and the wavelength maximum is shifted to lower wavelength ("blue shift"), the result of adsorption onto nonpolar regions of the BSA surface.

Tryptophan emission of serum albumin decreases with the binding of ANS. Example of resonant energy transfer.

![Diagram of ANS binding](image)

**Fluorescence Resonance Energy Transfer (FRET)**

**Primary Conditions for FRET**

Donor and acceptor molecules must be in close proximity (typically 10–100 Å).

The absorption spectrum of the acceptor must overlap fluorescence emission spectrum of the donor.

Donor and acceptor transition dipole orientations must be approximately parallel.

**Förster Radius**

The distance at which energy transfer is 50% efficient (i.e., 50% of excited donors are deactivated by FRET) is defined by the Förster radius (R₀).

The magnitude of R₀ is dependent on the spectral properties of the donor and acceptor dyes.
The efficiency of transfer varies with the inverse sixth power of the distance.

Distance-dependence was demonstrated by Lubert Stryer.

Energy is transferred between the naphthyl group (right) and the dansyl group.

The proline spacers adopt a helical configuration, permitting calculation of the average distance between the donor and acceptor groups for a given length spacer.

The measured efficiencies (in this case the enhancement of the dansyl fluorescence) confirmed the expected $1/r^6$ dependence.

Define $R_0$ as the distance at which energy transfer is 50% efficient.

At $R_0$, $k_1 = 1/\tau$.

The distance dependence of the energy transfer efficiency ($E$)

$$r = \left( \frac{1}{E} \right)^{1/6} R_0$$

Where $r$ is the distance separating the centers of the donor and acceptor fluorophores, $R_0$ is the Förster distance.

The efficiency of transfer varies with the inverse sixth power of the distance.

When the $E$ is 50%, $R=R_0$.

Distances can generally be measured between $0.5R_0$ and $1.5R_0$.

Fluorescence Resonant Energy Transfer (FRET, RET)

FRET is a process by which excitation energy is transferred from one chromophore (the donor chromophore) to another chromophore (the acceptor chromophore). The donor must be fluorescent; the acceptor need not be.

1) The energy transfer process is non-radiative dipole-dipole interaction.

2) The probability of energy transfer depends on the extent of overlap of the donor emission spectrum and the acceptor absorption spectrum.

3) Energy transfer has two manifestations:
   - The intensity of the donor emission spectrum is reduced.
   - Fluorescence emission by the acceptor - called "sensitized emission."

4) Energy does not go back and forth from donor to acceptor.

5) The efficiency of energy transfer is strongly dependent on the distance between the donor and acceptor and the orientation of the donor and acceptor transition dipoles.

$$E \approx 1/r^6$$

The magnitude of $R_0$ depends on the degree of overlap between the donor emission spectrum and the acceptor absorption spectrum, which is given by the overlap integral, $J$. For calculating $J$, the normalized emission spectrum is used

$$J = \frac{n}{\pi} \int_{0}^{\infty} E_{D,v} \cdot E_{A,v} \cdot \lambda \cdot d\lambda$$

$$R_0 = \frac{1}{9000} \int_{0}^{\infty} \frac{S_{D,v} \cdot E_{A,v} \cdot \lambda \cdot d\lambda}{J}$$

If wavelength is expressed in cm and $c$ in M^{-1} cm^{-1},

$$R_0 = 9.78 \times 10^7 \left[ (x^2 - 1)^2 \right]^{1/4}$$

$x$ is the refractive index, and $Q_0$ is the donor quantum yield in the absence of acceptor. $a^s$ is the "orientation factor.

Depending on the geometry of the donor/acceptor transition dipoles, $a^s$ can assume values from 0 to 4. It is usually assumed that $a^s = 2/3$, the value for donors and acceptors that randomize their orientations prior to energy transfer.

Distance-dependence of the energy transfer efficiency ($E$)

$$r = \left( \frac{1}{E} \right)^{1/6} R_0$$

Where $r$ is the distance separating the centers of the donor and acceptor fluorophores, $R_0$ is the Förster distance.

The efficiency of transfer varies with the inverse sixth power of the distance.

When the $E$ is 50%, $R=R_0$.

Distances can generally be measured between $0.5R_0$ and $1.5R_0$.

Theory

In the absence of energy transfer, the lifetime is $\tau$.

In the presence of acceptor, $\frac{1}{1/\tau} = 1 + k_2$, and $k_2 = \frac{1}{\tau} = \frac{1}{\tau}$.

The efficiency ($E_T$) of depopulation by RET is then

$$E_T = \frac{k_2}{1/\tau + k_2} = \frac{1/\tau - 1/\tau}{1/\tau - 1/\tau}$$

So $\frac{1}{\tau} = 1 - E_T$

Since $\phi_0 = \frac{1}{\tau}$ and $\phi_1 = \frac{1}{\tau}$, then $\phi_1 - \phi_0 = \frac{I}{I_D} = 1 - E_T$.
The overlap integral $J$ is defined by:

$$J = \int_{\lambda_1}^{\lambda_2} f_D(\lambda) c_A(\lambda) d\lambda$$

Where $\lambda$ is the wavelength of the light, $c_A(\lambda)$ is the molar extinction coefficient at that wavelength and $f_D(\lambda)$ is the fluorescence spectrum of the donor normalized on the wavelength scale:

$$f_D(\lambda) = \frac{F_D(\lambda)}{\int F_D(\lambda) d\lambda}$$

Where $F_D(\lambda)$ is the donor fluorescence per unit wavelength interval.

**Applications**

- Estimation of inter-chromophore distances in biological macromolecules, using intrinsic and extrinsic probes
- Strategy for assaying molecular proximity
- Biological phenomena that have been examined by FRET include:
  - Protein-ligand interactions
  - Protein-protein interactions
  - Protein folding/unfolding
  - DNA denaturation/renaturation
  - Protein-membrane interactions
  - Membrane diffusion (both protein and lipid)

- Diffusion-enhanced energy transfer from long-lived donors (e.g., lanthanides) allows the distance of a chromophore from the surface of a macromolecule to be determined.

**Quenching**

Certain moieties are particularly efficient at de-exciting the excited state - mostly through interaction with the electron in the excited state orbital.

High-molecular weight ions or triplet species can be effective quenchers. Examples include O$_2$, I$^-$, and acrylamide.

### Combined dynamic and static quenching

$$\frac{F}{F_0} = 1 + K_{s}Q + (1 + K_{d}Q) = 1 + (K_{d} + K_{s}Q)[Q] + K_{d}K_{s}Q^2$$

- $1 + K_{app}[Q]$, where $K_{app} = \left(\frac{F}{F_0} - 1\right)\left[\frac{1}{[Q]}\right]$,
- $K_{s}$ for complex formation

Quenching can also be used to recognize conformational changes that occur with substrate, ligand, or effector binding.

Data for the cAMP receptor protein with two Trp

In the absence of cAMP, only one of the two Trp residues are quenched by acrylamide. Addition of cAMP causes both chromophores to become sensitive to the quencher.
**Dynamic quenching with fractional accessibility**

Downward curvature is observed in the S-V plot when only a fraction of the fluorophores is accessible to quencher. In this case, a modified Stern-Volmer plot of \( \frac{F_0}{|\Delta F|} \) vs \( \frac{1}{[Q]} \) will be linear. The reciprocal of the vertical intercept is the fraction of the fluorophore accessible to quencher. The dashed lines in the figures show the behavior observed when the "inaccessible" population has a \( K_D \) value one-tenth that of the accessible population.

---

**Fluorescence anisotropy**

Since excitation and emission dipoles have a particular orientation, fluorescence is anisotropic (i.e., has a preferred orientation).

If irradiated a perfectly oriented, immobilized sample were irradiated with light polarized parallel to the absorption transition dipole, the resulting fluorescence would have the same polarization as the incident light (provided that the emission transition dipole is parallel to the absorption transition dipole).

If the molecules in the sample were free to tumble prior to emission, the polarization of the emitted light could differ from the incident light.

The extent to which the fluorescence anisotropy is decreased is strongly dependent on the rate at which the molecule rotates or tumbles i.e., its rotational correlation time.

In solution, the fundamental (or intrinsic) anisotropy of a fluorophore is given by the expression below, where \( \beta \) is the angle between the excitation and emission transition dipoles. If the transition dipoles are parallel, then \( r_0 \) has a value of 0.40.

\[
r_0 = A_0 \left[ \frac{2 \cos^2 \beta - 1}{5} \right]
\]

This is the value that is measured in viscous medium at low temperature, conditions under which rotational motions are largely frozen out.

Since rotation of the chromophore prior to emission decreases the observed anisotropy values, anisotropy measurements can be used to measure rotational correlation times. If instrumentation is available for time-resolved measurements, the anisotropy decay for a spherical protein molecule will be a single exponential:

\[
r(t) = r_0 \exp \left[ -\frac{t}{\theta} \right] = r_0 \exp \left[ -\frac{t}{\tau} \right]
\]

In this equation, \( \theta \) is the rotational correlation time, and \( D \) is the rotational diffusion coefficient.

A graph of \( \ln[r(t)] \) vs \( t \) will have a slope of \(-1/\theta\).
The method can be used even if the fluorophore is depolarized by segmental (i.e., local) motion, as well as by the overall tumbling of the protein molecule, provided that the segmental motions are much faster than the rotational diffusion.

Examples:
The figure shows the blue-shift that occurs when the peptide is bound by calmodulin.
The emission maximum shift and the increase in the maximal fluorescence intensity suggest that the tryptophan residue is in a more apolar environment.
The figure to the right shows the changes in the anisotropy of a peptide fragment of myosin light-chain kinase upon titration with calmodulin, which binds the peptide.

- The increase in the anisotropy indicates that the peptide interacts tightly with calmodulin.
- The maximal anisotropy appears at a calmodulin:peptide ratio of 0.93, implying stoichiometry not greater than 1:1.
- The shape of the curve suggests that at a calmodulin concentration of 10^{-8} M (equivalent to the peptide concentration) there is no free peptide.
- The anisotropy of tryptophan emission of the free peptide is 0.033 and of the fully bound peptide 0.170.

Time-resolved fluorescence spectroscopy

Time-resolved data contain more information than is available from steady-state measurements, e.g., consider a protein containing two Trp residues.

- Because of spectral overlap, it is usually not possible to resolve the signals from the two residues.
- If the two Trp residues have different fluorescent lifetimes, their contributions to the overall fluorescence behavior can be determined from time-resolved measurements.

- One can then determine how each is influenced by the interactions of the protein with substrates, effectors, or other macromolecules.

What is the “lifetime” of a fluorophore?

Absorption and emission processes are almost always studied on populations of molecules and the properties of the typical members of the population are obtained from the macroscopic properties of the process.

The behavior of an excited population of fluorophores is described by a rate equation:

\[ \frac{d n^*}{d t} = -n^* \Gamma + f(t) \]

where \( n^* \) is the number of excited elements at time \( t \), \( \Gamma \) is the rate constant of emission and \( f(t) \) is an arbitrary function of the time, describing the time course of the excitation. The dimensions of \( \Gamma \) are sec^{-1} (transitions per molecule per unit time).
If excitation occurs at $t = 0$, the equation is:

$$\frac{d\dot{n}}{dt} = -\dot{n} \Gamma$$

and describes the decrease in excited molecules at all further times. Integration gives:

$$\dot{n}(t) = \dot{n}(0) \exp(-\Gamma t)$$

The lifetime, $\tau$, is equal to $\Gamma^{-1}$.

If a population of fluorophores are excited, the lifetime is the time it takes for the number of excited molecules to decay to 1/e or 36.8% of the original population according to:

$$\frac{\dot{n}(t)}{\dot{n}(0)} = e^{-t/\tau}$$

In pictorial form:

The fluorescence lifetime, $\tau$, is the time at which the intensity has decayed to 1/e of the original value. The decay of the intensity with time is given by the relation:

$$I = I_0 e^{-t/\tau}$$

Excited state lifetimes have traditionally been measured using either the impulse response or the harmonic response method. In principle both methods have the same information content. These methods are also referred to as either the "time domain" method or the "frequency domain" method.

As shown in the intensity decay figure, the fluorescence lifetime, $\tau$, is the time at which the intensity has decayed to 1/e of the original value. The decay of the intensity with time is given by the relation:

$$I = I_0 e^{-t/\tau}$$

Where $I_0$ is the intensity at time $t$, $\alpha$ is a normalization term (the pre-exponential factor) and $\tau$ is the lifetime.

It is more common to plot the fluorescence decay data using a logarithmic scale as shown here.
Quenching phenomena are best studied using time-resolved measurements, because one can readily distinguish static and dynamic quenching.

Formation of static ground-state complexes does not decrease the decay time of the uncomplexed fluorophores because only the unquenched fluorophores are observed in a fluorescence experiment.

Dynamic quenching is a rate process acting on the entire excited-state population and thus decreases the mean decay time of the excited-state population.

RET is also best studied by time-resolved measurements.

e.g. if a protein contains a donor and acceptor, and the steady-state measurements indicate that the donor is 50% quenched by the acceptor.

- the observation of 50% donor quenching can be due to 100% quenching for one-half of the donors or to 50% quenching of all the donors, or some combination of these two limiting cases.

- Steady-state data cannot distinguish between these two cases. However, very different donor intensity decays would be observed for each case.

The down side to time-resolved measurements is that the instrumentation is highly specialized and costly.

Two experimental strategies: time-domain and frequency-domain.
Using the phase shift and relative modulation one can thus determine a phase lifetime ($\tau_p$) and a modulation lifetime ($\tau_m$).

If the fluorescence decay is a single exponential, then $\tau_p$ and $\tau_m$ will be equal at all modulation frequencies.

If, however, the fluorescence decay is multiexponential then $\tau_p < \tau_m$ and, moreover, the values of both $\tau_p$ and $\tau_m$ will depend upon the modulation frequency, i.e.,

$$\tau_p (\omega_1) < \tau_p (\omega_2) \quad \text{if} \quad \omega_1 > \omega_2$$

Typical phase and modulation data

<table>
<thead>
<tr>
<th>Frequency (MHz)</th>
<th>$\tau_p$ (ns)</th>
<th>$\tau_m$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6.76</td>
<td>10.24</td>
</tr>
<tr>
<td>10</td>
<td>6.02</td>
<td>9.70</td>
</tr>
<tr>
<td>30</td>
<td>3.17</td>
<td>6.87</td>
</tr>
<tr>
<td>70</td>
<td>1.93</td>
<td>4.27</td>
</tr>
</tbody>
</table>

Frequency-domain (FD) measurements. Also called phase modulation.

The sample is excited with intensity-modulated light. The intensity is varied at a high frequency comparable to the reciprocal of the decay time. When a fluorescent sample is excited in this way the emission is forced to respond at the same modulation frequency. Since the excited state has a finite lifetime, the emission is delayed relative to the excitation. The delay is measured as a phase shift, $\phi$, which is used to calculate the decay time. The peak-to-peak height of the emission is also decreased relative to that of the modulated excitation beam. This phenomenon, called demodulation, $\omega$, can also be used to calculate the decay time.

Typical phase and modulation data are usually presented as shown below:

A case of multi-exponential decays is shown here for a system of two lifetime species of 8.7 ns and 3.1 ns and a 1 to 1 mixture (in terms of fractional intensities)

Multifrequency phase and modulation data is usually analyzed using a non-linear least squares method in which the actual phase and modulation ratio data (not the lifetime values) are fit to different models such as single or double exponential decays.

The quality of the fit is then judged by the chi-square value ($\chi^2$) which is given by:

$$\chi^2 = \frac{[(Pc - Pm)\tau^2] + (Mc - Mm)\tau^2]}{(2n - f - 1)}$$

where $P$ and $M$ refer to phase and modulation data, respectively, $c$ and $m$ refer to calculated and measured values and $\tau^2$ and $\tau^M$ refer to the standard deviations of each phase and modulation measurement, respectively. $n$ is the number of modulation frequencies and $f$ is the number of free parameters.
Binding of Ethidium-Bromide to Transfer RNA

Ethidium bromide can intercalate into nucleic acid structures
It binds well to both DNA and RNA

Fluorescence investigations of EB - tRNA interactions, carried out for more than 30 years, have indicated a "strong" binding site and one or more "weak, non-specific" binding sites.

What are the lifetimes of the strong and the weak binding sites?

The data were then fit to a 2-component model shown here. In this case the two lifetime components were 22.71 ns with a fractional intensity of 0.911 and 3.99 ns with a fractional intensity of 0.089.

The chi-squared for this fit was 3.06 (note the change in scale for the residual plot compared to the first case shown).

A 3-component model improves the fit still more. In this case:
- $\tau_1 = 24.25$ ns, $f_1 = 0.83$
- $\tau_2 = 8.79$ ns, $f_2 = 0.14$
- $\tau_3 = 2.09$ ns, $f_3 = 0.03$
- $\chi^2 = 0.39$.
Adding a fourth component – with all parameters free to vary - does not lead to a significant improvement in the $\chi^2$. In this case one finds 4 components of 24.80 ns (0.776), 12.13 ns (0.163), 4.17 ns (0.53) and 0.88 ns (0.008).

But we are not using all of our information! We can actually fix some of the components in this case. We know that free EB has a lifetime of 1.84 ns and we also know that the lifetime of EB bound to the "strong" tRNA binding site is 27 ns. So we can fix these in the analysis. The results are four lifetime components of 27 ns (0.612), 18.33 ns (0.311), 5.85 ns (0.061) and 1.84 ns (0.016). The $\chi^2$ improves to 0.16.

We can then go one step better and carry out "Global Analysis". In Global Analysis, multiple data sets are analyzed simultaneously and different parameters (such as lifetimes) can be "linked" across the data sets. The important concept in this particular experiment is that the lifetimes of the components stay the same and only their fractional contributions change as more ethidium bromide binds.

Global Analysis on seven data sets fit best to the 4 component model with two fixed components of 27ns and 1.84ns and two other components of 17.7ns and 5.4ns.

As shown in the plot below, as the EB/tRNA ratio increases the fractional contribution of the 27ns component decreases while the fractional contributions of the 17.7ns and 5.4ns components increase.

"Strong" binding site
Lifetime ~ 27ns

Increase EB conc.

"Weak" binding site
Lifetime ~ 5.4ns

Radiative emission can also occur from the excited triplet state, in which case it is called "phosphorescence."

Phosphorescence is characterized by a very long lifetime (seconds or longer), because emission must be accompanied by the unlikely conversion of the triplet state back to a singlet.

It is difficult to observe in solution at room temperature because of internal conversion and "quenching."
Fourier Series

- Every composite periodic signal can be represented with a series of sine and cosine functions.
- The functions are integral harmonics of the fundamental frequency \( f \) of the composite signal.
- Using the series we can decompose any periodic signal into its harmonics.

Fourier Transform

- Fourier Transform gives the frequency domain of a nonperiodic time domain signal.

Example of a Fourier Transform

Inverse Fourier Transform

\[
S(f) = \mathcal{F}\{s(t)\} = \int_{\infty}^{\infty} s(t) e^{-2\pi ft} dt
\]

\[
s(t) = \mathcal{F}^{-1}\{S(f)\} = \int_{\infty}^{\infty} S(f) e^{2\pi ft} df
\]
A protein uses ATP to convert CO to CO₂. A pair of fluorophores (fluorescein and tetramethylrhodamine) has been chemically attached to the ATP reaction sites. Three labeled protein samples are prepared:

- **Sample I** with fluorescein attached to the ATP binding site
- **Sample II** with tetramethylrhodamine attached to the CO/CO₂ reaction site
- **Sample III** with both fluorophores attached to both the ATP and reaction sites, respectively.

Their quantum yields are measured as follows: $\Phi_I = 0.38$, $\Phi_II = 0.33$, and $\Phi_{III} = 0.22$. $R_0$ for the fluorescein–tetramethylrhodamine pair is 55 Å.

a) Of this pair, which is the donor and which is the acceptor? Why?

b) In **Sample III**, which wavelength is used to excite the fluorophore for a FRET experiment, and which wavelength is used to monitor the fluorescence?

c) What is the energy transfer efficiency ($\eta$)?

d) What is the spatial separation (in Å) between the binding and reaction sites?