# Fluorescence Spectroscopy

Molecular Energy Levels and Absorption Spectra

The phenomenon of fluorescence is intimately linked with that of **absorption** by molecules of energy in the form of photons of visible (or ultraviolet) light. To understand the phenomenon of fluorescence, we must thus first understand the process of light absorption.

In general a molecule can absorb a photon only if the energy provided by the photon (E_{photon}) equals the energy (which we'll call $\Delta E_{transition}$) that is needed to promote a molecule from the **ground state**, in which the molecule is found immediately before absorbing the photon, to some higher-energy **excited state**. The energies of visible and ultraviolet photons are sufficient to promote an electron in a molecule from its normal ground-state orbital to a higher-energy orbital (typically from a bonding or nonbonding orbital to an antibonding orbital). From this fact in isolation we might expect the UV/visible absorption spectrum of a molecule to be fairly simple, with one symmetrical and relatively sharp peak centered at $\lambda = c/\nu = hc/(\Delta E_{transition})$ for each possible electronic transition (recall that the energy of a photon, E_{photon}, equals $h\nu = hc/\lambda$). In fact, however, peaks in the UV/visible spectra of real molecules are typically broad, may be asymmetric and/or contain 'fine structure' such as multiple peaks, shoulders, etc.. To understand the basis for these features of absorption spectra, we need to consider in more detail the possible energy levels of molecules.
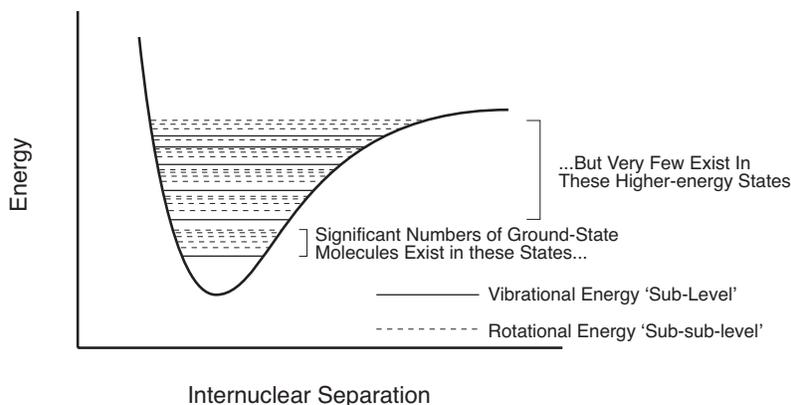
An important result from quantum mechanics is that molecules can exist only in specific electronic states (this you know already, of course) and as well only **in specific rotational and vibrational states**. Associated with each electronic state of a molecule is a **family** of different vibrational and rotational energy states. This is most easily shown graphically for a diatomic molecule (e.g., Cl_2), where for each electronic state we plot the separation-energy curve and then show the associated vibrational/rotational states. A plot of this type for one electronic state (e.g., the ground state) is shown below.

Vibrational Energy 'Sub-Level'

---------- Rotational Energy 'Sub-sub-level'

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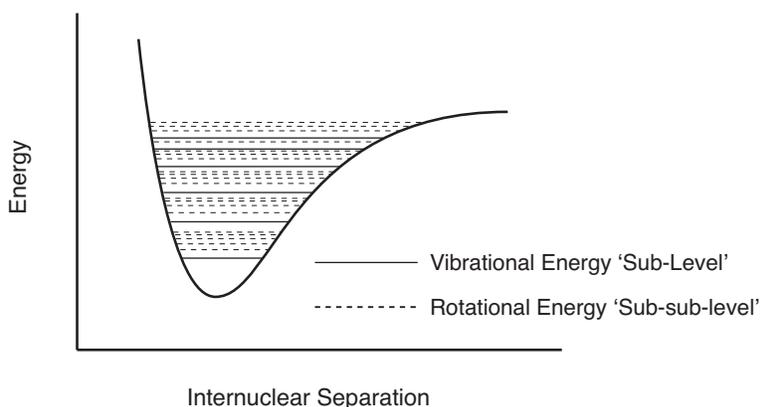
We will use diagrams of this nature, which are strictly appropriate only for diatomic molecules, to represent *schematically* the energy levels for larger molecules as well; for the latter the same principles hold, but the complete energy diagrams would be rather complex to represent graphically.

What does this have to do with molecular UV/visible absorption spectra? Consider a transition in which a particular electron is promoted from its ground-state orbital to a particular higher-energy orbital. You might at first expect that this would correspond to a unique transition between a unique ground state and a unique excited state. However, because the molecule in both the ground and the excited state can have different vibrational

and rotational energy levels, this is not the case. On one hand, in the **'equilibrium' ground state** from which the molecules are excited, the molecules exist in *several* **rotational** 'sub-sub-levels':

...But Very Few Exist In These Higher-energy States

Significant Numbers of Ground-State Molecules Exist in these States...

—————— Vibrational Energy 'Sub-Level'

- - - - - - - - - Rotational Energy 'Sub-sub-level'

Internuclear Separation

On the other hand, photon absorption can promote the molecule to *any* of the vibrational./rotational substates of the excited electronic state. Below are shown the possible transitions (arrows) from 'the' ground to 'the' excited state (for clarity the rotational 'sub-sub-levels' for each electronic/vibrational state are omitted):

Excited State

Ground State

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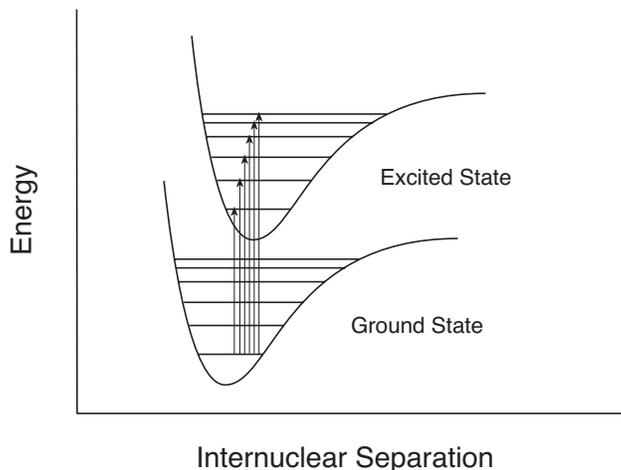
Note that each of the arrows shown above represents a different possible transition, even though all transitions are between the same two *electronic* states. The energy change in each of these transitions is different, indicating that each will correspond to a different peak wavelength $\lambda = hc/(\Delta E_{transition})$. There are in fact many more individual transitions (arrows) than are shown in the above diagram, since I have omitted all the further complexity introduced by the different *rotational* 'sub-sub-states.' The overall effect of these various energy sub- (and sub-sub-) levels is to split the spectral peak for a given electronic transition into a *family* of overlapping peaks. In solution the band for each individual transition is itself somewhat broadened due to solvent interactions of the molecule, and the overall absorption is often 'smeared out' to one very broad, fairly smooth peak:

Effect of Vibrational and Rotational Splitting

Idealized Spectrum for
a Single Transition

Effect of Vibrational Splitting
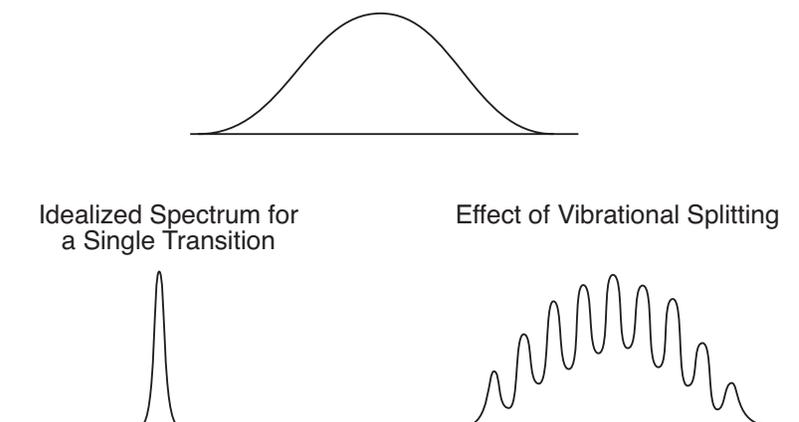
Thus a typical peak in the UV/visible spectrum for a molecule of interest is in fact a smeared summation of many different transitions between different vibrational and rotational substates. In some cases the smearing is incomplete, and we can see at least hints of the individual component peaks due to individual vibronic bands (as in the second of the three spectra above). In this case we say that 'vibrational fine structure' can be seen in the spectrum.

It is clear from the above discussion that the overall form of an absorbance spectrum depends on two properties of the individual transition peaks that comprise it:
    (a) the **energies** $\Delta E_{transition}$ of each individual transition in the energy-level diagram, which determine the **positions** (peak wavelength $\lambda = hc / \Delta E_{transition}$) of the individual (overlapping) transition peaks; and
    (b) the relative **intensities** (roughly, the peak area) of each individual transition.

What we have **not** discussed so far is exactly what factors determine properties (a) and (b). Without going into excessive detail, we can identify two major factors of importance. The first of these is the nature of the molecular orbital wavefunctions for the ground and excited states of the molecule, which we can simply summarize as 'details of the structure of the molecule' (if you want more grisly details, see pp. 356-360 of Cantor and Schimmel). The second is the **nature of the solvent environment**, a topic that we will return to in a later section.

From Absorption to Fluorescence

As noted above, the actual absorption of light by a molecule occurs very quickly, on a time scale of roughly 10^{-15} sec. This is too fast to permit any significant motions of the atoms (more specifically, of the *nuclei*) in the molecule itself or in the solvent molecules surrounding it. This is known as the **Franck-Condon principle**, and the excited state that the molecule is 'promoted' to immediately upon absorption of the photon is called the **Franck-Condon excited state**.

As we will see, in order for a molecule (fluorescent or not) to return from the Franck-Condon excited state to the equilibrium ground state, the molecule must undergo three distinct processes sequentially:

<u>Step 1</u> - On a time scale in the picosecond range, the newly excited molecule adjusts to adopt a **minimum free energy** while remaining in the excited electronic state. After these adjustments are complete, the molecule will exist in a new, **lower-energy excited state** known as the **equilibrium excited state**.

<u>Step 2</u>- The electron in the higher-energy orbital returns to the lower-energy orbital. This step may entail the emission of a photon as fluorescence, but there are also other, competing mechanisms to return the electron to the lower-energy orbital *without* photon emission. The return of the electron to the ground-state orbital is very rapid (time scale of the order of 10^{-15} sec). As a result, the molecule initially returns to a **Franck-Condon ground state**, in which it may have **excess vibrational energy** and a **nonoptimal interaction with the surrounding solvent molecules**.

<u>Step 3</u> - The molecule in the Franck-Condon ground state will undergo **relaxation** to the **equilibrium ground state,** in much the same way that the Franck-Condon excited state earlier relaxed to the equilibrium excited state.

In the two following sections we will discuss in more detail these three processes and their consequences for the overall phenomenon of fluorescence.

<u>'Relaxation' of the Franck-Condon Excited State</u>
The energy of the Franck-Condon excited state is typically higher than that of the equilibrium excited state. This is true for two reasons. First, in the Franck-Condon excited state the molecule may exist in a vibrational substate other than the lowest-energy vibrational level. One part of 'relaxation' of the Franck-Condon excited state is thus the conversion of the molecule to the lowest-energy vibrational level of the excited state:
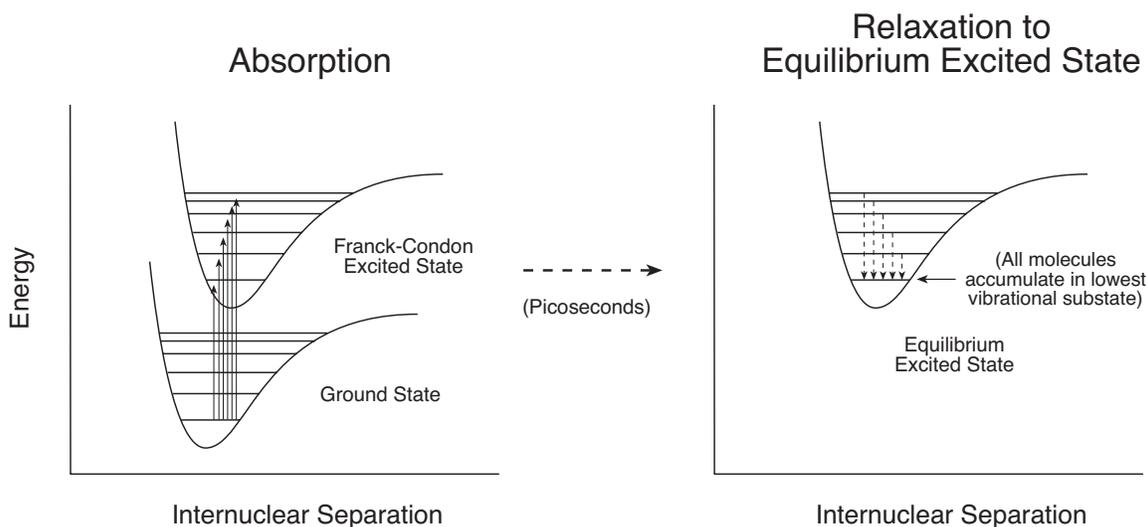

Second, the electronic distribution, and hence various other properties such as the dipole moment, are different for a molecule in the excited state compared to the ground state. As a result, immediately after absorbing a photon the excited-state molecule will typically *not* exhibit an optimal interaction with the solvent molecules around it. The second aspect of 'relaxation' of the Franck-Condon excited state is thus that an excited-state molecule and the solvent molecules around it will adjust their positions and orientations to achieve a new arrangement of solvent molecules, which is optimally adjusted to the properties of the *excited-state* molecule. This is frequently a different, and lower-energy, arrangement of solvent and solute molecules than the one that characterized the Franck-Condon excited state. For these two reasons the equilibrium excited state attained after excited-state relaxation is of significantly *lower energy* than the Franck-Condon excited state.

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‘Deexcitation’ of the Excited State, and Relaxation Back to the Equilibrium Ground State
In the equilibrium excited state just described, the molecule still has one electron in the excited-state orbital. The average delay between absorption of a photon and emission of fluorescence is of the order of nanoseconds (nsec) or tens of nsec for a typical fluorescent species. This means that after photon absorption a molecule typically will have time to reach the *equilibrium* excited state before emitting a photon. However, once a molecule ‘decides’ to return to the electronic ground state, the actual transition itself happens extremely rapidly (on a time scale of the order of 10^{-15} sec.), too fast to permit any motions of the nuclei within the molecule or in the surrounding solvent molecules. We thus say that the newly ‘deexcited’ molecule returns initially to a **Franck-Condon ground state**.

In two important respects the transition just noted is similar to the earlier process of ‘promotion’ of the molecule to the Franck-Condon *excited* state. First, a given excited-state molecule may return to *any* of the vibrational (and rotational) substates of the electronic ground state, giving the possible transitions shown below:

Photon Emission (Fluorescence)

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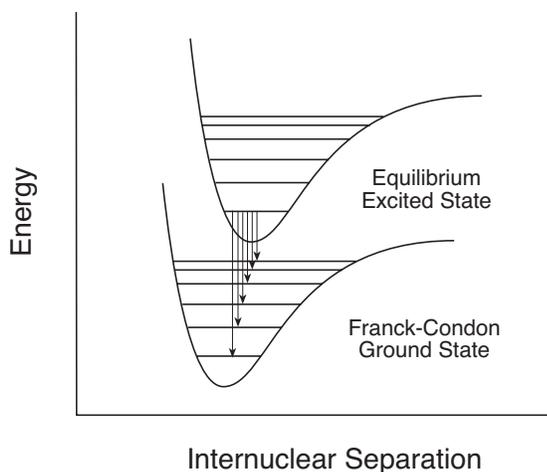
‘The’ transition to the ground state is thus again a **family** of possible transitions to individual sublevels of the Franck-Condon ground state. Second, in the Franck-Condon ground state the newly ‘deexcited’ molecule may find itself initially surrounded by solvent molecules in an arrangement which was optimal to solvate the *excited*-state molecule but which is *not* optimal to solvate the *ground*-state molecule. As a result, the Franck-Condon ground state will typically dissipate some of its energy (both by returning to the lowest-energy vibrational level and by ‘reoptimizing’ its interactions with the solvent) in order to return to the *equilibrium* ground state (in which the molecule existed before photon absorption). These processes (and the reasons for them) are similar to those which drive the relaxation of the Franck-Condon excited state to the equilibrium excited state.

Translating Molecular Behavior into Observable Fluorescence Properties
We will now consider two questions of critical importance in understanding fluorescence spectroscopy: first, at what **wavelengths** will the fluorescence be emitted (i.e., what will be the **shape and the position** of the fluorescence spectrum), and second, what will be the

intensity of the emitted fluorescence? To answer the first question, recall that 'the' transition of a molecule from the equilibrium excited state to the Franck-Condon ground state is not a unique transition but rather a family of possible transitions to various vibrational (and rotational) substates of the electronic ground state. This means that the **shape** of a fluorescence spectrum will exhibit the same general features as does the absorbance spectrum; it will in generally be fairly **broad**, may be **asymmetric** and may reveal **'vibrational fine structure'** (shoulders, partially resolved component peaks, etc.).

What about the **position** of a fluorescence spectrum, and more precisely, what will be the relationship between the fluorescence and the absorption spectrum? The general answer to this question can be understood by considering the energy diagram below:
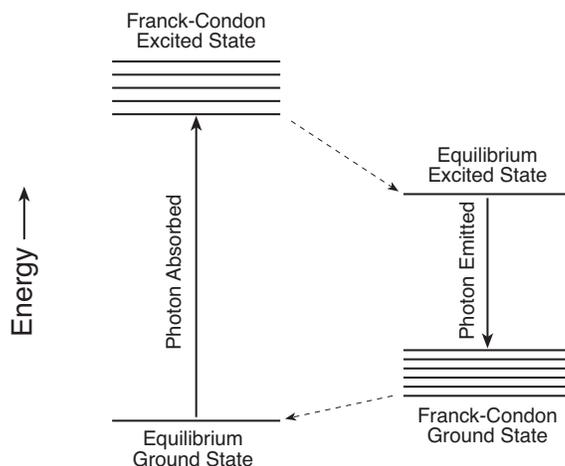
Note that as shown here and as discussed above, the energy of the equilibrium excited state must be *less than or equal to* that of the Franck-Condon excited state. Likewise, the energy of the equilibrium ground state must be *less than or equal to* that of the Franck-Condon ground state. Recall as well that the energy of the photon *absorbed* equals the energy difference between the equilibrium excited state and the Franck-Condon excited state, and that the energy of the photon *emitted* equals the energy difference between the equilibrium ground state and the Franck-Condon ground state (arrow lengths in the above diagram).

From the above four statements we can conclude that **the energy of the photon emitted as fluorescence must be less than or equal to (typically less than) the energy of the photon initially absorbed**. This means in turn that **the wavelength of light emitted as fluorescence will be greater than or equal to (and typically greater than) the wavelength of the light absorbed**, since $\lambda = hc/E_{photon}$.

If we extrapolate this last statement from the behavior of a single molecule to that of a whole population of fluorescent molecules, we conclude that **the emission (fluorescence) spectrum for a fluorescent molecule will be shifted to longer wavelengths with respect to the excitation (absorption) spectrum**.

We now turn to the question of the **intensity** of fluorescence emitted by a sample, which can also be a useful source of information about the behavior and environment of a molecule. For this discussion we will have to introduce some additional information about how molecules can return from an excited state to the ground state.

Determinants of Fluorescence Intensity: Competition among Energy-Dissipating Pathways
You may have wondered whether every molecule that absorbs a photon must emit fluorescence to return to the ground state. The answer is 'no,' because emission of a photon is not the only way that a molecule in an excited electronic state may return to the ground state. There are typically other, competing pathways to achieve this end as well, and for many molecules these alternative, **radiationless** (or **nonradiative)** processes are so efficient that the molecules emit little if any fluorescence at all.

There are a number of detailed pathways by which a molecule that has absorbed a photon can return to the ground state <u>without</u> emitting a photon; these are known collectively as pathways of **radiationless** (or **noradiative**) **decay** of the excited state. For our purposes it is useful to group these processes into three classes:
(1) The molecule may be able to return the electron to the ground-state orbital while converting its excess *electronic* energy to excess *vibrational* or *rotational* energy, which can then be transferred to the surrounding medium. The transformation of energy within the molecule in this manner is known as **internal conversion**. For many molecules this is the major or exclusive means of dissipation of the energy provided by photon absorption.
(2) The molecule may transfer its excess electronic energy to a special type of molecule called a **quencher** and thereby return to the electronic ground state without emitting a photon. Quenchers such as acrylamide, Br^- and I^- ions, etc. act by transiently colliding or complexing with excited-state molecules. One special class of quenchers, however, can 'accept energy at a distance' by a phenomenon known as **resonance energy transfer**, which we will discuss in a later section.
(3) The molecule may undergo **intersystem crossing** from the excited state discussed above, which is technically known as a **singlet state** (one in which all electron spins are paired), to a **triplet state** (which has two unpaired spins). It is much 'harder,' in quantum-mechanical terms, for a triplet-state molecule to convert to the ground state than for a singlet-state molecule to do so. As a result, molecules that convert to the triplet state return to the ground state either by eventual internal conversion or by a distinct, relatively slow process of photon emission called **phosphorescence**.
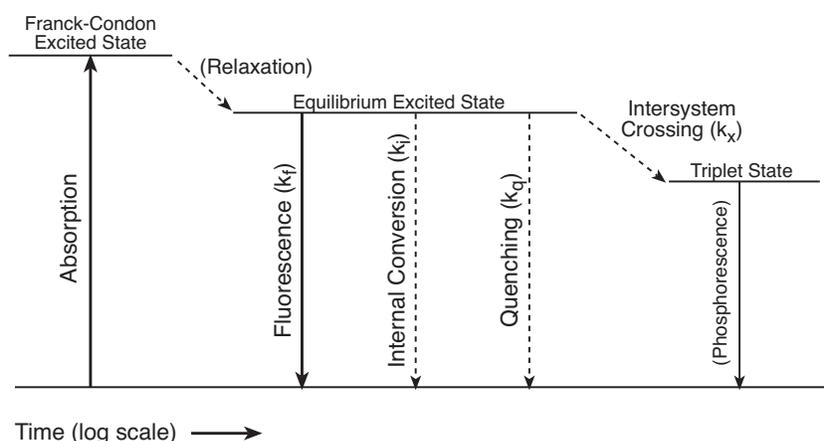
The above diagram summarizes the alternative possible 'fates' of a molecule in the excited state; here k_f, k_{ic}, k_q and k_{ix} are respectively the first-order rate constants for emission of fluorescence, internal conversion, quenching and intersystem crossing (the latter three processes, all of which lead to outcomes **other** than fluorescence, are those described in (1)-(3) above). If (as is usually the case) the rate of relaxation of the Franck-Condon excited state to the equilibrium excited state is much faster than is the overall rate of decay of the

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equilibrium excited state, we can relate the above rate constants to two important characteristics of a molecule's fluorescence. The first is the overall rate of decay of the excited state, which is more often reported as the excited state **lifetime**, τ_f:

$$\tau_f = 1 \Big/ \sum k_i$$

[1]

where $\sum k_i$ is the sum of all the rate constants for processes by which the excited state can decay (k_f, k_{ic}, k_q, k_{ix}, etc.). The lifetime of the excited state is most simply determined (at least in conceptual terms) by simultaneously exciting many fluorescent molecules in a sample with an, ultrafast (psec or sub-psec) laser pulse, then monitoring the time course of subsequent fluorescence emission. For a homogeneous population of fluorescent molecules, the resulting time course of fluorescence emission will be exponential and obey the equation

$$F(t) = F_o \exp\left(-t/\tau_f\right)$$

[2]

Note that 'the' lifetime of the excited state is thus really analogous to an *average* lifetime for the whole population of fluorescent molecules.

A second important quantity, the **quantum yield Φ**, can also be readily calculated by solving the differential equation appropriate for the above kinetic scheme. The quantum yield is the ratio of photons emitted to photons absorbed; it has limiting values of 1.0 if fluorescence is 'perfectly efficient' and 0 if the molecule absorbs light but is entirely *non*fluorescent. The quantum yield is given by the formula

$$\Phi = k_f \Big/ \sum k_i$$

[3]

The quantum yield is thus a measure of how efficiently fluorescence 'competes' with other, radiationless pathways for decay of the excited state. We can thus understand why a molecule like thymidine absorbs light energy efficiently around 260 nm but shows negligible fluorescence: for this molecule the rate constant for fluorescence, k_f, is so small with respect to the other rate constants for decay of the excited state that the quantum yield is negligible. On the other hand, a molecule like fluorescein has a *high* quantum yield because the rate constant for emission of fluorescence is large compared to the rate constants for competing processes of radiationless decay of the excited state.

Environmental Sensitivity of Absorbance and Fluorescence Spectra
We have noted already that a fluorescent molecule in its various states interacts in important ways with its solvent environment. In fact, as we will see in this section, we can often monitor the characteristics of the molecular fluorescence to learn something about the nature of its environment. As we discuss below, two important examples of this approach are to determine experimentally the **polarity** of a fluorescent molecule's environment and to determine the degree of exposure of fluorescent protein residues (e.g., tryptophan residues) to the surrounding solvent.
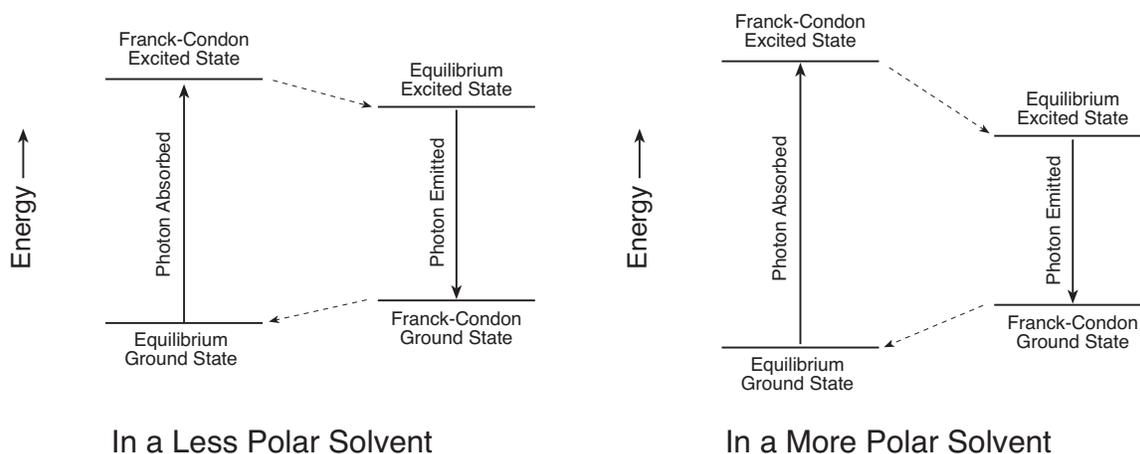
When we speak generically of the 'polarity' of the environment of a fluorescent molecule, we are really referring to two distinct characteristics of the molecule's environment. The first is the local dielectric constant, which is a measure of the magnitude of the **static (i.e.,**

permanent) **dipoles** present in the immediate environment of the fluorescent species. For example, methanol and water have relatively high dielectric constants, reflecting the fact that each of these types of solvent molecules has a rather high dipole moment. A second aspect of the 'polarity' of a molecule's environment is its **polarizability**, i.e., the ability of the environment to generate **induced dipoles** in response to the presence of charges or dipoles. In general, a molecule with a substantial dipole moment will be 'happier' (i.e., have a lower free energy) in a **more polar environment**. (note for example how polar sugar molecules dissolve much more readily in polar solvents like water than in apolar ones like hexane).

Fluorescent molecules typically exhibit a **greater** dipole moment in the excited than in the ground state. Solvents of different polarities may therefore *differentially* affect the energies of the ground and excited states for a given molecule. Moreover, since molecules of a given species in the *Franck-Condon* excited and ground states may interact with the surrounding environment differently than they do in the corresponding *equilibrium* states, there exists a rich variety of ways in which solvent polarity can affect the fluorescence spectrum of a given molecule. Consider again the energy–level diagram shown below for a complete 'fluorescence cycle':
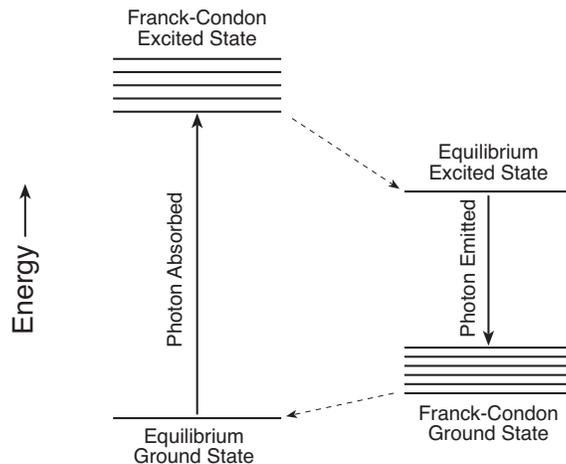
The relative energies of all these states can be altered (depending on the specific structural properties of the molecule) by changes in the molecular environment. Consider for example the hypothetical example illustrated on the next page (I show only one vibrational level for each state). Here the solvent indicated as 'more polar' is assumed to have a higher permanent dipole moment. Since an excited state usually has a higher dipole moment than the ground state, a solvent of this type will *raise* the energy of the Franck-Condon excited state but can *lower* the energy of the equilibrium excited state. (The larger dipole moments of more polar solvent molecules will impose a greater energetic 'penalty' when the arrangement of solvent molecules vis-à-vis the solute is *non*-optimal, as in the Franck-Condon excited state. At the same time, solvent molecules with a larger dipole moment can offer a greater energetic 'reward' when the arrangement *becomes* optimal (as in the equilibrium excited state). As a result, and as shown by the lengths of the transition arrows in the above figure, in the more polar solvent the molecule will show a **higher-energy ('blue-shifted') excitation transition**, and a **lower-energy ('red-shifted') emission transition.**

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Franck-Condon Excited State

Equilibrium Excited State

Energy →

Photon Absorbed

Photon Emitted

Equilibrium Ground State

Franck-Condon Ground State

Franck-Condon Excited State

Equilibrium Excited State

Energy →

Photon Absorbed

Photon Emitted

Equilibrium Ground State

Franck-Condon Ground State

In a Less Polar Solvent

In a More Polar Solvent

It can often be very complicated to predict quantitatively the effects of environmental polarity on fluorescence. For example, the 'polarity' of a solvent actually has two aspects, which can affect quite differently the fluorescence of a given molecule. The 'polarity' of the solvents discussed in the above example was a result of the *permanent* dipoles of the solvent molecules. However, a solvent may also be more or less **polarizable**, meaning that it can give rise to smaller or larger *induced* dipoles when the solvent molecules interact with a charged or dipolar solute. Dipole induction is essentially instantaneous (as fast as the excitation and emission transitions themselves) while reorientation of permanent solvent dipoles is much slower. The effects of the two aspects of solvent 'polarity' on the energy levels in the 'fluorescence cycle' (and hence on the excitation and emission spectra) are typically different and can even be opposite in sign.

To simplify dealing with the above complexities, it is common to determine the fluorescence of a given species in a series of solvents of varying polarities, thereby 'calibrating' how that probe's fluorescence varies with the polarity of the environment. We can then examine the fluorescence spectra for the probe in some novel environment (e.g., a membrane or a protein binding site) and estimate the polarity of the novel environment from these 'calibration data.' We can also monitor **changes** in the polarity of the probe environment through shifts in the fluorescence emission or excitation spectra. This latter approach can be used for example to follow conformational changes in a protein by monitoring changes in the fluorescence of intrinsic tryptophan residues or extrinsic (covalently or noncovalently bound) fluorescent probes.

Application: Monitoring Environments of Fluorescent Molecules or Groups

To illustrate some of the applications of the above results, we will consider two examples using fluorescence to probe the environment of a specific molecule or group. Both of these will reflect a fairly common application, namely monitoring the state of a protein or peptide tryptophan residue.

In the first example, suppose that we have a globular protein containing a single trypophan residue, and that we wish to determine whether that residue is buried within the protein core or exposed on the surface. At least two approaches can be used to achieve this end. First, we can estimate the polarity of the tryptophan's environment by measuring its fluorescence excitation and emission spectra and comparing these to reference spectrra obtained for simpler compounds (e.g., N-acetyltryptophanamide) in solvents of varying polarity.
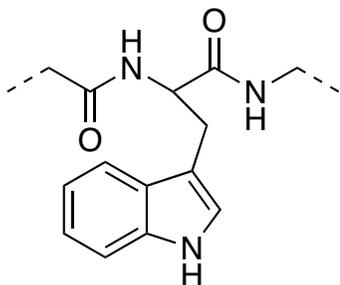
Tryptophan residue
in protein
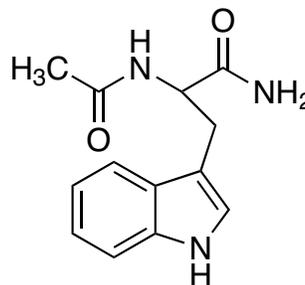
N-acetyltryptophanamide

The data might show for example that in an aqueous buffer, the fluorescence emission spectrum for the tryptophan residue in the protein peaks at a much shorter wavelength (and has a much higher peak intensity) than is observed for N-acetyltryptophanamide in water. We would also find in control experiments that the peak of the emission spectrum maximum for the latter model compound shifts to progressively lower wavelengths (i.e., exhibits a blue shift) as the solvent polarity decreases. We would then conclude that the tryptophan residue in the protein is in an environment significantly more hydrophobic (less polar) than water, and thus that the tryptophan residue was presumably buried in the protein interior. This conclusion could be further tested by measuring the susceptibility of the tryptophan fluorescence residue to quenching by water-soluble quenchers like **acrylamide or iodide (I⁻) ion**.

The usual method to determine quenching efficiency is to measure the fluorescence intensity (or quantum yield) for the species of interest as a function of the concentration of quencher. Starting from equation [3] above (we'll skip the details), it is possible to derive the expression

$$F_o/F = 1 + K_q \cdot c_Q$$

[4]

where K_q is a constant representing the efficiency of quenching, F_o is the intensity of fluorescence measured in the absence of quencher and F is the intensity of fluorescence measured in the presence of quencher at a concentration c_Q. In this equation K_q has a different physical interpretation depending on whether quenching is **collisional** (due to transient interactions between the excited-state molecule and the quencher species) or is due to complex formation between the fluorescent species and the quencher. In practice this distinction is not always critical (e.g., in the present example). If the protein-incorporated tryptophan residue is quenched with markedly lower efficiency (gives a much smaller value of K_q) than is N-acetyltryptophanamide, we can again conclude that the tryptophan residue is buried within the protein interior.

In a second hypothetical example we will consider how the disposition of a tryptophan residue, incorporated into an antibacterial peptide, could be determined when the peptide is bound to lipid bilayers (as a simple model for the bacterial membrane). We could first measure the excitation and emission spectra for the tryptophanyl residue in the presence and absence of lipid vesicles. Suppose that we observed a marked **blue shift** for the peptide fluorescence emission spectrum in the presence of lipid vesicles, indicating that the peptide tryptophan residue was incorporating into a more hydrophobic (less polar) environment upon binding to the vesicles. We might then hypothesize that the trytophan residue was

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exposed to the aqueous solvent in the soluble form of the peptide but was inserting into the hydrophobic region of the bilayer upon binding to lipids. This hypothesis could be tested further in at least two ways. First, we could examine whether the vesicle-bound peptide was less accessible than the soluble peptide to quenching by a hydrophilic species such as iodide ion. This could be tested in much the same manner that was described above for the example of tryptophan in a soluble protein. Second, we could determine whether the vesicle-bound peptide shows a lower fluorescence (indicating some degree of quenching) when the normal lipids in the vesicles are replaced by brominated *quencher* lipids:

Normal Phospholipid

Brominated Analogue
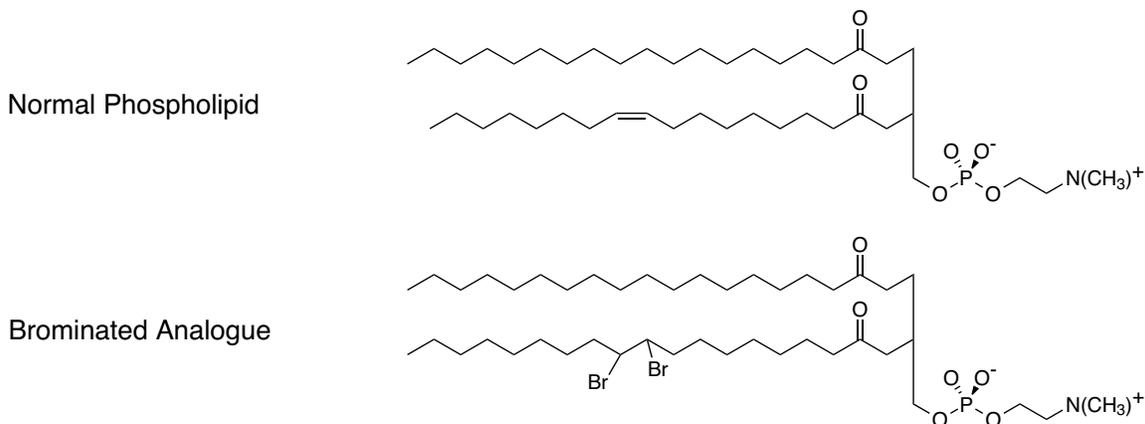
If the peptide-incorporated tryptophan penetrates into the acyl-chain (hydrophobic) region of the lipid bilayer, it will show some degree of fluorescence quenching (reduction in quantum yield/fluorescence intensity) when bound to the *brominated* lipid vesicles compared to the normal lipid vesicles. This is now a common method to investigate the localization of tryptophan residues in membrane-bound proteins or peptides.

The environmental sensitivity of the tryptophan fluorescence in the second example has another interesting application. Tryptophan residues typically give stronger (more intense) fluorescence when present in more hydrophobic environments. If we thus measure the intensity of fluorescence of a fixed amount of peptide in the presence of increasing concentrations of lipid vesicles, we can determine the affinity constant K_a (or the dissociation constant $K_d = 1/K_a$) for peptide-lipid binding:

$$F = F_o + (F_{max} - F_o) \cdot [\text{Lipid}]/(K_d + [\text{Lipid}])$$
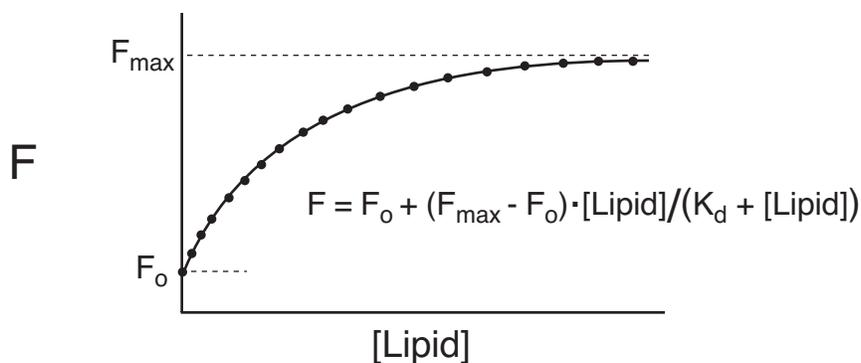
where the value of the dissociation constant K_d is determined by fitting the data to the standard binding equation shown in the figure.

Resonance Energy Transfer and Measurement of Intermolecular Distances

We have noted that a molecule in an excited electronic state can interact with a quencher to return to the ground state without emitting fluorescence. Many quenching interactions are contact-dependent and thus require that the excited-state molecule and the quencher approach to within a few Angstroms. However, some combinations of molecules can participate in **resonance energy transfer**, an interaction through which an excited-state molecule of one type, the **donor**, can transfer its excess energy to an **acceptor** molecule, usually of a second type, *even when the acceptor molecule is up to several tens of Angstroms away*. In this manner the donor molecule (**D**) can return from the excited to the ground state while the acceptor molecule (**A**) is promoted from the ground to the excited state. The acceptor species then can return to the ground state by the usual mechanisms, including potentially by emitting a photon if the acceptor species is itself fluorescent.

Resonance energy transfer (often abbreviated as RET, or as FRET for 'fluorescence resonance energy transfer') can thus often be monitored in at least two different ways:
(1) As a quenching of the donor fluorescence. This occurs because in the presence of the acceptor species, the excited state of the donor species acquires an additional means to return to the ground state without fluorescing, namely transferring its excess energy to the acceptor species. In the limit of 100% energy transfer, all of the excited-state donor molecules would transfer their excess energy to the acceptor, and no fluorescence would be observed from the donor species – all the energy absorbed by the donor species as photons would be 'siphoned off' by the acceptor.
(2) As fluorescence emitted by the acceptor species when the donor species absorbs photons. As already noted, when an acceptor molecule 'siphons off' energy from an excited-state donor molecule, it is itself promoted to an excited state. The return of the acceptor molecule to the ground state can occur by several mechanisms, including photon emission if the acceptor species is itself fluorescent. In this case, if donor and acceptor molecules within a sample exhibit RET, we can excite the sample at the *donor* excitation maximum and observe some fluorescence emitted at the *acceptor* emission maximum.

The efficiency of energy transfer (ε) can be quantitated by measuring the first effect discussed above as follows:

$$\varepsilon \equiv RET\ efficiency = \frac{\Phi_D(A\ absent) - \Phi_D(A\ present)}{\Phi_D(A\ absent)}$$

[5]

where Φ_D is the quantum yield of the donor. If we carry out an RET measurement for two samples, one with the acceptor present and the other with no acceptor present, under otherwise identical conditions (same amount of donor molecules in each sample, same intensity of the exciting light, etc.) we can replace the above equation by the equivalent form

$$\varepsilon = \frac{F_D(A\ absent) - F_D(A\ present)}{F_D(A\ absent)}$$

[6]

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where F is the measured fluorescence of the donor species **D** under each indicated condition. From basic physical theory, for any given donor-acceptor pair (or for a population of donor-acceptor pairs, all of which have the same spacing), we can calculate that

$$\varepsilon = \frac{1}{1 + \left(R / R_o\right)^6}$$

[7]

where R is the distance between the donor and acceptor species and R_O is called the **Förster length**. For a fixed value of R_O this function has the following appearance:
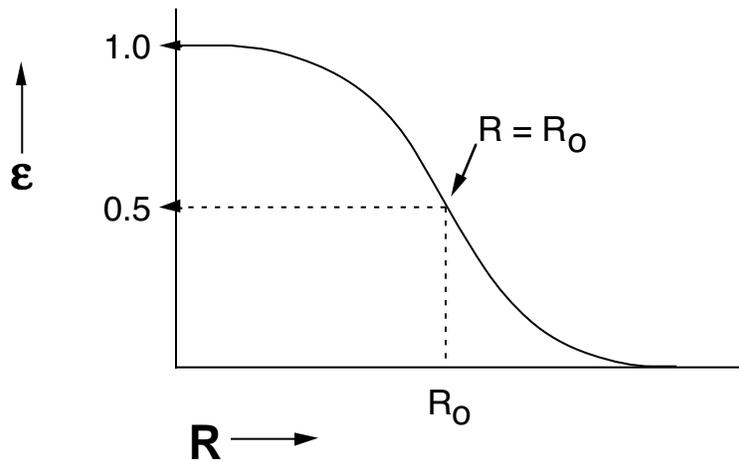
The Fôrster length R_O is a function of the mutual orientation of **D** and **A** (a factor we won't concern ourselves with here) and of the degree to which the excitation spectrum of **A** overlaps the emission spectrum of **D**:
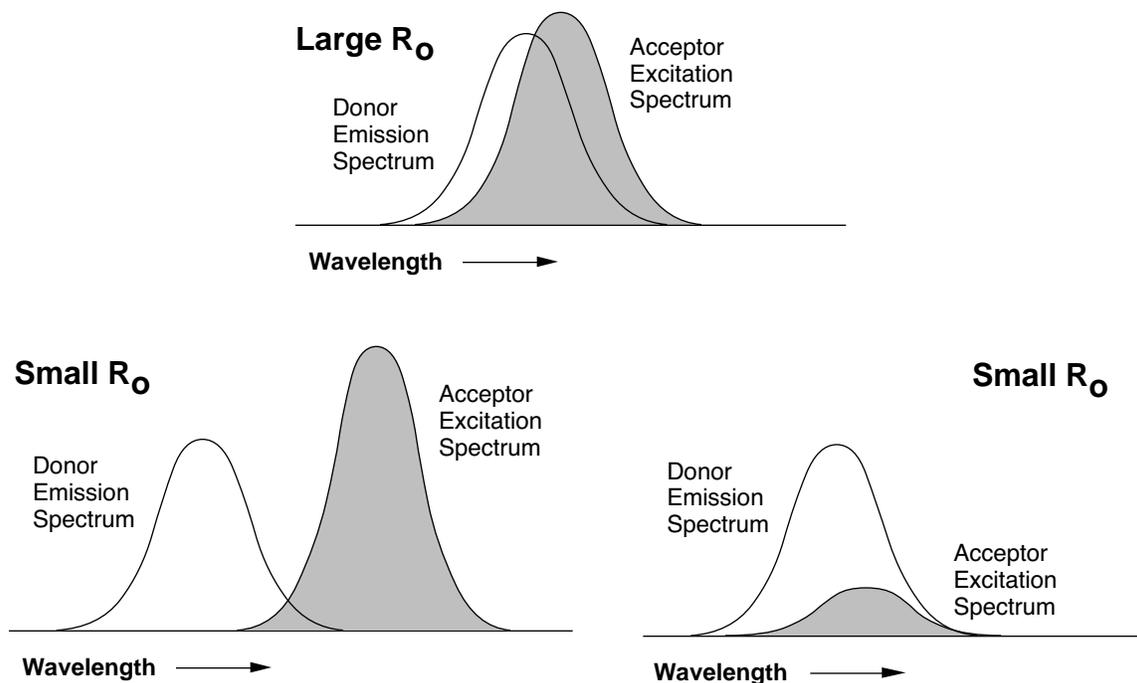
For typical donor-acceptor pairs the R_O-value is of the order of 20-60 Å. As the graph above shows, the energy-transfer efficiency is most sensitive to the donor-acceptor distance R when R is in the range $R_O/2$ to $2R_O$, i.e., about 10-120 Å for typical donor and acceptor species. These distances are of the order of macromolecular dimensions, making RET a very useful tool for mapping distances within macromolecules or molecular complexes. As well, since RET can act over distances of the order of typical protein dimensions, it can be used to monitor processes such as the oligomerization of proteins within membranes and the binding of fluorescent ligands (serving as donor or acceptor species) to macromolecules (which contain an endogenous or exogenous acceptor or donor, respectively).

Fluorescence Anisotropy (Polarization) Measurements

The Transition Dipole
The wavelength of visible or UV light is much longer than the dimensions of a typical molecular orbital, so the molecule 'feels' a passing wave of light simply as a **time-dependent oscillation in the local electronic field**. This can have the effect of allowing 'mixing' between the ground and higher electronic (/vibrational/rotational) states, which in turn can allow the molecule to undergo a transition from the ground state to one of these excited states. The efficiency of this process for a particular molecule depends on the spatial distribution of the relevant ground- and excited-state orbitals and on their relationship to the axis of polarization of the light wave. Evaluation of the mixing of states promoted by these factors leads to the following important result:

> Associated with every possible transition of a molecule between ground and excited electronic states, there exists an **excitation transition dipole** which consists of both a magnitude and a direction and which is a function of the relevant molecular-orbital wavefunctions. **The efficiency of absorption of light by molecules undergoing that transition is proportional to (a) the magnitude of the transition dipole, and (b) $\cos^2\theta$, where θ is the angle between the absorption transition dipole and the axis of polarization of the light beam.**

This is a fundamental result, which shows us that the absorption of light is **directional** - it depends on the orientation of the molecule's transition dipole with respect to the direction of polarization of the light, with maximal absorption taking place when the two are co-parallel $(\cos^2(0^o) = 1)$ and minimal absorption when the two axes are perpendicular $(\cos^2(90^o) = 0)$. This is the phenomenon of **linear dichroism**.

The phenomenon of polarized absorption has a parallel in the phenomenon of fluorescence emission. Just as for a given absorption (excitation) transition there exists an excitation transition dipole, for a transition in which light is emitted as fluorescence there exists an **emission transition dipole**. The consequence of this latter fact is that **light emitted as fluorescence by a given molecule tends to be polarized along the axis of the emission transition dipole for that molecule**. I say 'tends to be' because the axis of polarization of the emitted photon is a probabilistic quantity; it has the highest probability to be oriented parallel to the emission dipole axis and the lowest probability to be aligned perpendicular to this axis.

To make these directional factors a bit more concrete, let us consider the special (but not uncommon) case of a fluorescent molecule whose excitation and absorption transition dipoles are co-parallel. Suppose that we had a single, uniform crystal in which all of the fluorescent molecules were oriented in the same direction. Then we would notice two things if we excited this ordered, rigid sample with polarized light:

(a) **The extent of light absorption would vary** as we changed to orientation of the crystal (and hence of the fluorescent molecules) with respect to the axis of polarization of the light. This phenomenon is known as (linear) **dichroism**..

(b) The light emitted as fluorescence would itself be **preferentially polarized** (though not perfectly polarized) along the direction of the emission dipoles of the fluorescent molecules (which in this crystalline sample all have the same orientation).

These directional aspects of light absorption/emission have important implications when we consider the phenomenon of **fluorescence polarization**, which allows us to gain information about how molecules change their orientation (through rotational motions) as a function of time.

Remember that the time intervening between the excitation of a fluorescent molecule and the subsequent emission of fluorescence is of the order of 10^{-9} to 10^{-8} sec. This time is comparable to the time required for many molecules in biologically interesting systems to rotate randomly ('tumble') in space. We can estimate the rates at which the molecules tumble by measurement of **fluorescence anisotropy**. In the following discussion, for simplicity we will consider molecules with elongated fluorescent groups whose excitation and emission transition dipoles are co-parallel. We will thus refer simply to 'the' transition dipole for such molecules, remembering however that there are in reality distinct dipoles for excitation and emission.

Suppose that we excite a sample of randomly oriented fluorophores with a beam of plane-polarized light (polarized along, say, the **z**-axis). Then as noted above, the probability of light absorption is proportional to $\cos^2\theta$, where θ is the angle between the axis of polarization of the excitation beam (**z**) and the excitation (absorption) transition dipole of the fluorophore. Molecules whose (absorption) transition dipoles are **parallel** to the polarization direction **z** have the **highest** probability of absorbing light from the exciting beam, while molecules whose transition dipoles are exactly perpendicular to the polarization axis have a negligible probability of excitation. Molecules with intermediate orientations will have intermediate probabilities of absorbing light over any given interval:

Plane of Light Polarization

| Highest Excitation Probability | Negligible Excitation Probability | Intermediate Excitation Probability |
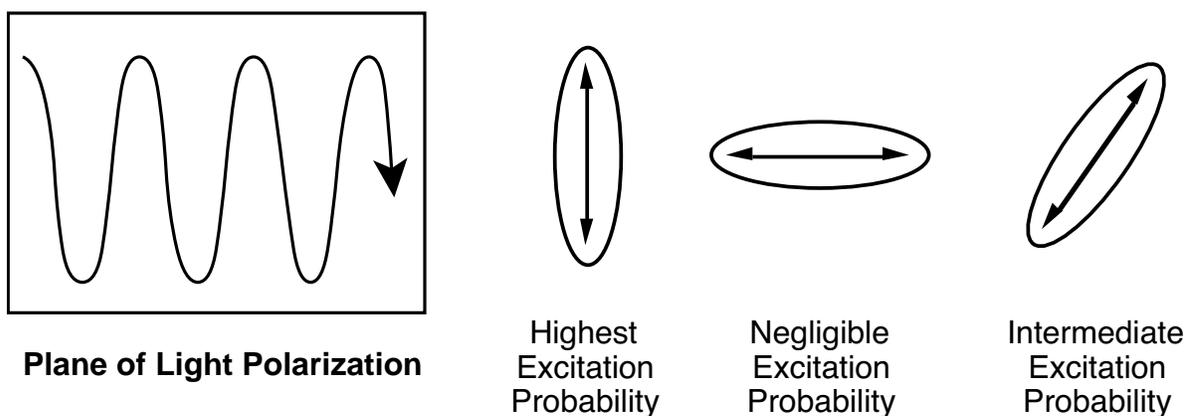
Suppose now that we 'zap' a sample of fluorescent molecules with a **very brief** (psec.) pulse of **z-polarized** light, which is too fast for any molecular rotation to occur during the pulse itself. If we could examine the distribution of molecules that were excited by the pulse, just after the pulse ended we would observe that the distribution of excited-state species is not random. Instead, this distribution is biased toward species whose transition dipoles are oriented toward the **z**-axis, since molecules with their transition dipoles oriented more closely parallel to the **z**-axis are those with higher probabilities of absorption:

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Orientations of Excited-
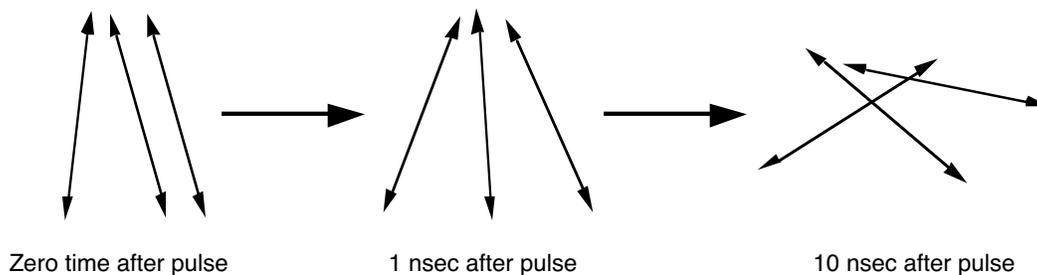State Molecules
Immediately after Pulse
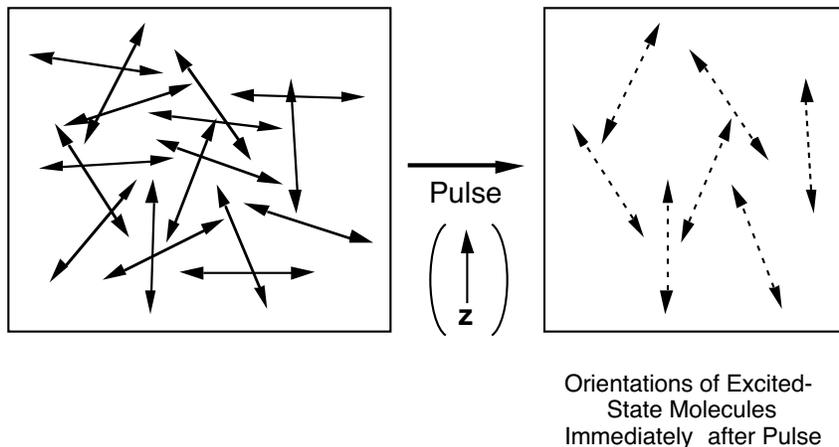
What will happen over the next few nanoseconds after the pulse is turned off? Two things. First, the excited molecules will gradually release their excess energy as fluorescence. As discussed previously, in the simplest case (where all the molecules are in essentially the same environment), the fluorescence intensity emitted by the sample will obey the simple exponential relationship

$$F(t) = F_o \cdot e^{-t/\tau_F}$$

[2]

where τ_F is the fluorescence lifetime. Note that (also as discussed above) the fluorescence emitted by any given molecule will be preferentially polarized along the molecule's transition dipole at the moment the (fluorescence) photon is emitted. This means that **if there were no molecular motion between the time of the exciting pulse and the emission of fluorescence, the emitted fluorescence would show a large degree of polarization in the z-direction**.

The second thing that can happen after the pulse is turned off is that the molecules may rotate ('tumble' is a better word, since the process is a random one rather than a smooth, steady propellor-like motion). As they do so, the average orientation of the transition dipoles for the excited-state molecules, which right after the pulse is biased toward the **z**-axis, will gradually show **less** preferential orientation along this axis (since the molecular rotations are random):

Zero time after pulse   1 nsec after pulse   10 nsec after pulse

So, molecules that emit fluorescence at very early times after the pulse ends will still have a large degree of preferential alignment along the **z**-axis, and the fluorescence emitted at early times will thus be significantly **polarized** along the **z**-axis. However, if (random) molecular

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motions are occurring on a time scale comparable to the fluorescence lifetime, the fluorescence emitted at **later** times will be **less** polarized along the **z**-axis.

How do we quantitate the degree of polarization of a beam of emitted fluorescence? The most useful measure is a quantity called the **anisotropy** of the light, which is determined as follows. We split our beam of light into two (equal) parts and pass one through a polarizing filter set with its axis parallel to the **z**-axis; we call the intensity of the light that gets through this filter $I_{||}$. The second half of the original light beam we pass through a polarizing filter with its axis oriented perpendicular to the **z**-axis; we call the intensity of the light that passes through this filter $I_{\perp}$. We then calculate the **fluorescence anisotropy** as

$$A = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$$

[8]

The factor of 2 in the denominator of the above equation represents the fact that there are actually two perpendicular axes of space that are also perpendicular to **z**, and in the above experiment we only measure the light polarized along one of these **z**-perpendicular axes.

The **maximum** possible anisotropy of the fluorescence emitted from a macroscopically unoriented sample is 0.4, observed when there is **no molecular motion** between the times of photon absorption and fluorescence emission. If we allow the molecules to tumble (randomly) to a *limited* extent between the times of photon absorption and emission, we expect that the emitted light will be less (but still somewhat) polarized, i.e., it will have a reduced but nonzero anisotropy. This fact is the key to analyzing molecular motions from **time-dependent fluorescence anisotropy** measurements.

Remember that after a fast excitation pulse different excited-state molecules will emit fluorescence at different times (typically ranging up to several nsec) after the time of the pulse. If we measure the anisotropy of the fluorescence emitted as a function of time after the pulse, we expect that the earliest fluorescence will be highly anisotropic (since it arises from molecules that have had essentially no time to 'tumble' or 'wobble' after excitation). By contrast, the fluorescence measured a few nsec later may be less anisotropic (since the molecules emitting this fluorescence have had more time undergo random rotations). If we have a solution of fluorescent molecules in a simple solvent (one that does not impose any restrictions on the molecular orientation), we will in fact observe a simple exponential decay of the fluorescence anisotropy toward zero (for as long as we can still collect enough photons to measure the anisotropy!):


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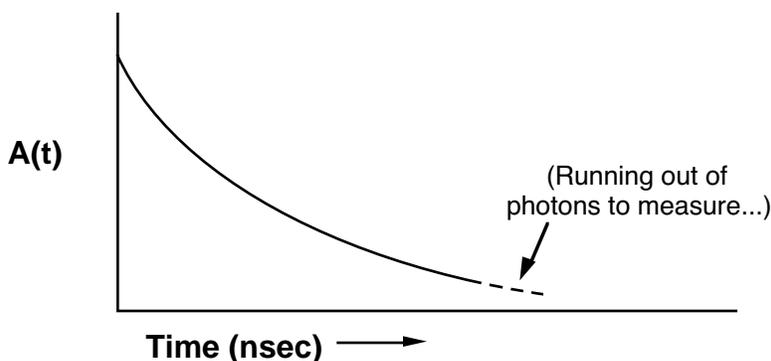
where the time parameter in A(t) represents time elapsed since the pulse (which was so fast that we can consider it 'instantaneous').

The above anisotropy trace, for a molecule in a simple 'isotropic' solvent (where there is no special constraint to tumbling in any particular direction), provides two key pieces of information. First, the **rate** of decay of A(t) reflects the **rate** of molecular tumbling, and it is quantitated by a parameter called the **rotational correlation time** τ_c, which crudely represents the average time for the molecule to tumble (randomly) through about 63% of the maximal amplitude of the motion(s) it is 'allowed' in its environment. Second, the amplitude of the decay tells us something about the **freedom** (or **amplitude**) of the molecular tumbling: the lower the plateau value to which A(t) is tending, the greater is the freedom of motion. (Note the important distinction between the **rate** and the **freedom** (**amplitude**) of molecular motion).

For a spherical molecule (say, a protein) in solution, the rotational correlation time τ_c is given by the expression

$$\tau_c = V_h \eta / kT$$

[9]

where V_h is the hydrated volume of the molecule, η is the viscosity, $k = 1.38 \times 10^{-16}$ erg mol^{-1} ($^\circ$K)$^{-1}$ and T is the absolute temperature (in $^\circ$K). If the orientation of a fluorophore is rigidly fixed within the molecule, the fluorophore will move (tumble) time in the same manner as the whole protein and therefore with the same rotational correlation time. If however the fluorophore can exhibit additional motions within the protein (for example, limited rotation around a partially flexible side chain), it will exhibit a partial loss of fluorescence anisotropy at a rate faster than the rotation of the entire protein. The resulting anisotropy-decay profile will then be biphasic:
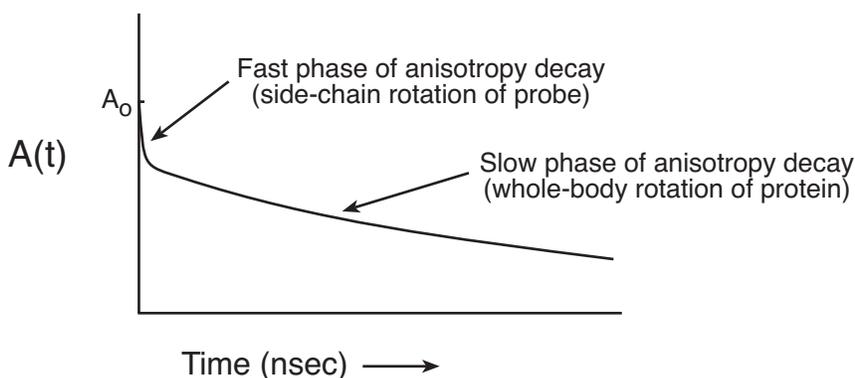
Time-dependent fluorescence anisotropy measurements thus can offer a rich (though sometimes complex) source of information about the motions of fluorescent residues within macromolecules, membranes and other biologically interesting environments.