Resonance Energy Transfer

Resonance energy transfer (RET), sometimes called fluorescence resonance energy transfer (FRET), provides an opportunity to measure the distances between sites on macromolecules. Förster distances are typically in the range of 15 to 60 Å, which is comparable to the diameter of many proteins and to the thickness of membranes, the distance between a donor and acceptor can be calculated from the transfer efficiency.

The use of RET to measure protein association and distance is shown in Figure 1 for two monomers that associate to form a dimer. Suppose one monomer contains a tryptophan residue, and the other a dansyl group. The Förster distance is determined by the spectral overlap of the trp donor emission with the dansyl acceptor absorption. Upon association RET will occur, which decreases the intensity of the donor emission (Figure 1). The extent of donor quenching can be used to calculate the donor-to acceptor distance in the dimer. It is also important to notice that RET provides a method to measure protein association because it occurs whenever the donor and acceptor are within the Förster distance.

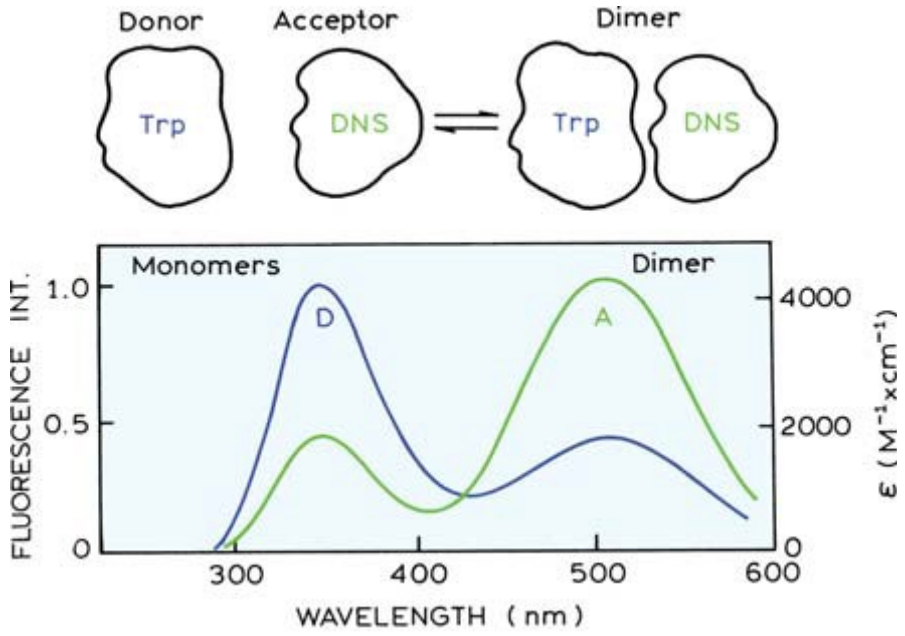


Figure 1. Energy transfer between donor- (D) and acceptor- (A) labeled monomers, which associate to form a dimer. In this case the donor is tryptophan and the acceptor DNS.

Single-Molecule Detection

Observations on single molecules represent the highest obtainable sensitivity. Single-molecule detection (SMD) is now being performed in many laboratories. At present most single-molecule experiments are performed on immobilized fluorophores, with fluorophores chosen for their high quantum yields and photostability . A typical instrument for SMD consists of laser excitation through microscope objective, a scanning stage to move the sample and confocal optics to reject unwanted signals. SMD is now being extended to include UV-absorbing fluorophores, which was considered unlikely just a short time ago. The probe 2,2'-dimethyl-p-quaterphenyl (DMQ) has an absorption maximum of 275 nm and an emission maximum of 350 nm. Figure 2 (left) shows intensity images of

DMQ on a quartz cover slip. The spots represent the individual DMQ molecules, which can yield signals as high as 70,000 photons per second. The technology for SMD has advanced so rapidly that the lifetimes of single molecules can also be measured at the same time the intensity images are being collected (right). The individual DMQ molecules all display lifetimes near 1.1 ns.

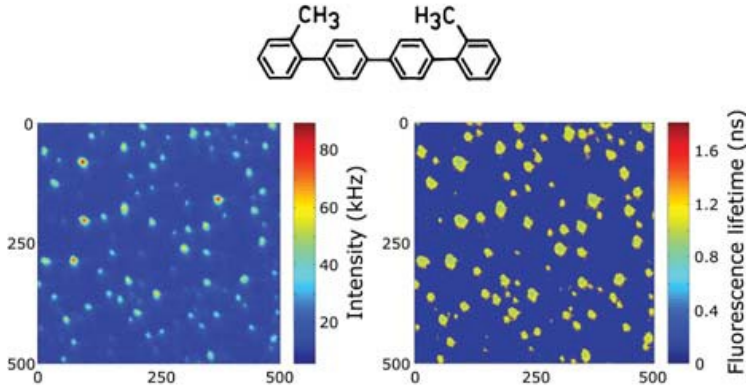
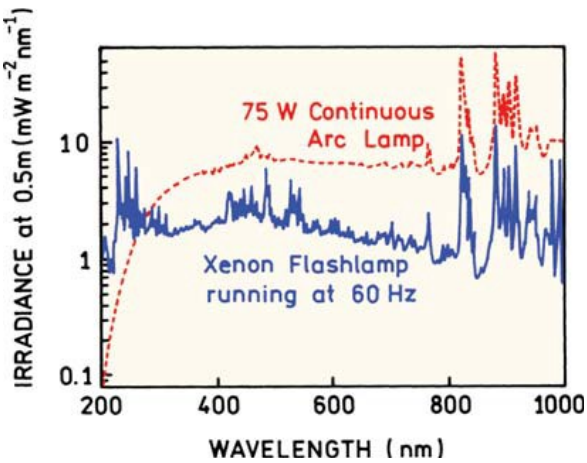


Figure 2. Single-molecule intensities and lifetimes of DMQ in a quartz slide. Excitation at 266 nm.

Instrumentation for Fluorescence Spectroscopy

1. LIGHT SOURCES:-

At present the most versatile light source for a steady-state spectrofluorometer is a high-pressure xenon (Xe) arc lamp. These lamps provide a relatively continuous light output from 250 to 700 nm (Figure 3), with a



number of sharp lines occurring near 450 nm and above 800 nm. Xenon

Figure 3. Spectral output of a continuous xenon arc lamp and a xenon flash lamp.

arc lamps emit a continuum of light as a result of recombination of electrons with ionized Xe atoms. These ions are generated by collisions of Xe atoms with the electrons that flow across the arc. Complete separation of the electrons from the atoms yields the continuous emission. Xe atoms that are in excited states but not ionized yield lines rather than broad emission bands. The peaks near 450 nm are due to these excited states. The output intensity drops rapidly below 280 nm.

2. MONOCHROMATORS

Monochromators are used to disperse polychromatic or white light into the various colors or wavelengths. This dispersion can be accomplished using prisms or diffraction gratings. The monochromators in most spectrofluorometers use diffraction gratings rather than prisms. The performance specifications of a monochromator include dispersion, efficiency, and stray light levels. Dispersion is usually given in nm/mm. The slit width is sometimes expressed in mm, which requires knowledge of the dispersion. A monochromator for fluorescence spectroscopy should have low stray light levels to avoid problems due to scattered or stray light. By stray light we mean light transmitted by the monochromator at wavelengths outside the chosen wavelength and bandpass.

Monochromators are also chosen for high efficiency to maximize the ability to detect low light levels. The slit widths are generally variable, and a typical monochromator will have both an entrance and exit slit. The light intensity that passes through a monochromator is approximately proportional to the square of the slit width. Larger slit widths yield increased signal levels, and therefore higher signal-to-noise ratios. Smaller slit widths yield higher resolution, but at the expense of light intensity. If the entrance slit of the excitation monochromator is already wide enough to accept the focused image of the arc, then the intensity will not be increased significantly with a wider slit width. If photobleaching of the sample is a problem, this factor can sometimes be minimized by decreasing the excitation intensity. Gentle stirring of the sample can also minimize photobleaching. This is because only a fraction of the sample is illuminated and the bleached portion of the sample is continuously replaced by fresh solution.

3. OPTICAL FILTERS

3.1. Colored Filters

While spectrofluorometers have monochromators for wavelength selection, it is often important to use optical filters in addition to monochromators. Optical filters are used to compensate for the less-than-ideal behavior of monochromators. Also, when the spectral properties of a fluorophore are known, maximum sensitivity is often obtained using filters rather than monochromators. A large

range of filters are available. The manufacturers typically provide the transmission spectra of the filters. Before the advances in thin film technology, most filters were colored-glass filters. Colored- glass filters can transmit a range of wavelengths (Figure 4, top). Some color filters are called long-pass filters and transmit all wavelengths above some particular wavelength (bottom). The names

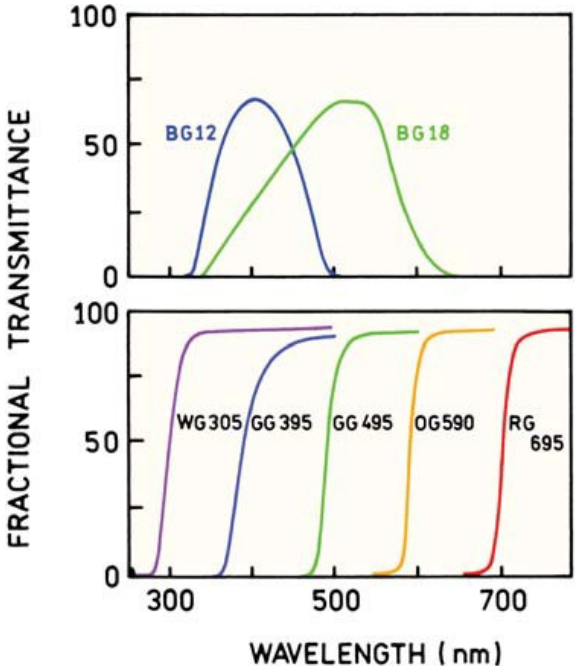


Figure 4.
Transmission spectra of some typical colored glass filters.

of the filters divide them into classes according to their colors (BG, blue glass, GG, green glass, etc.).

In turbid or dilute samples the scattered light can be orders of magnitude more intense than the fluorescence. This is also true for two-photon excitation when the excitation wavelength is longer than the emission wavelength. For these reasons transmission curves are often presented on a logarithm scale, which makes it possible to determine if the filter transmits 1% or much less than 1% of the light at any given wavelength.

4. PHOTOMULTIPLIER TUBES

Almost all fluorometers use photomultiplier tubes (PMTs) as detectors, and it is important to understand their capabilities

and limitations. A PMT is best regarded as a current source. The current is proportional to the light intensity. A PMT responds to individual photons, and the pulses can be detected as an average signal or counted as individual photons. A PMT vacuum tube consists of a photocathode and a series of dynode (dynode is an electrode in a vacuum tube that serves as an electron multiplier through secondary emission) which are the amplification stages. The photocathode is a thin film of metal on the inside of the window. Incident photons cause electrons to be ejected from this surface. The generation efficiency of photoelectrons is dependent upon the incident wavelength. The photocathode is held at a high negative potential, typically –1000 to –2000 volts. The dynodes are also held at negative potentials, but these potentials decrease toward zero along the dynode chain. The potential difference between the photocathode and the first dynode potential is generally fixed at a constant voltage by a Zener diode, at values ranging from –50 to –200 volts. This potential difference causes an ejected photoelectron to be accelerated toward the first dynode. Upon collision with the first dynode the photoelectron causes 5 to 20 additional electrons to be ejected, depending on the voltage difference to this dynode. This process continues down the dynode chain until a current pulse arrives at the anode. The size of this pulse depends upon the overall voltage applied to the PMT. Higher voltages result in an increased number of electrons ejected from each dynode, and hence higher amplification. PMTs are useful for low level light detection because they are low-noise amplifiers. Little additional noise is

created as the electrons pass through the PMT. Amplification outside of the PMT generally results in more noise being added to the signal.

5. CCD Detectors

There is a growing usefulness of charge-coupled devices (CCDs) in fluorescence spectroscopy. CCDs are imaging detectors with remarkable sensitivity and linear dynamic range. CCDs typically contain 10^6 or more pixels (a minute area of illumination on a display screen). Each pixel acts as an accumulating detector where charge accumulates in proportion to total light exposure. The charge at each pixel point can be read out when desired, to obtain a two-dimensional image. CCDs are used widely in fluorescence microscopy. Small spectrofluorometers using CCDs are commercially available. The sensitivity can be rather good, as seen from the fluorescein emission spectra . When combined with an LED light source the entire instrument becomes a solid state device.

QUANTUM YIELD STANDARDS

The easiest way to estimate the quantum yield of a fluorophore is by comparison with standards of known quantum yield. Some of the most used standards are listed in Table 1. The quantum yields of these compounds are mostly independent of excitation wavelength, so the standards can be used wherever they display useful absorption. Determination of the quantum yield is generally accomplished by comparison of the wavelength

integrated intensity of the unknown to that of the standard. The optical density is kept below 0.05 to avoid inner filter effects, or the optical densities of the sample and reference (*r*) are matched at the excitation wavelength. The quantum yield of the unknown is calculated using

$$Q = Q_R \frac{I}{I_R} \frac{OD_R}{OD} \frac{n^2}{n_R^2}$$

where Q is the quantum yield, I is the integrated intensity, OD is the optical density, and n is the refractive index. The subscript R refers to the reference fluorophore of known quantum yield. In this expression it is assumed that the sample and reference are excited at the same wavelength, so that it is not necessary to correct for the different excitation intensities of different wavelengths.

Table 1 Quantum Yield Standards

Compound	Solvent	λ_{ex} (nm)	°C	Q	Reference
Quinine sulfate	0.1 M H_2SO_4	350	22	0.577	45
		366	–	0.53 ± 0.023	46
β-Carboline[a]	1 N H_2SO_4	350	25	0.60	40
Fluorescein	0.1 M NaOH	496	22	0.95 ± 0.03	47
9,10-DPA[b]	cyclohexane	–	–	0.95	48
9,10-DPA	"	366	–	1.00 ± 0.05	49–50
POPOP[c]	cyclohexane	–	–	0.97	48
2-Aminopyridine	0.1 N H_2SO_4	285	–	0.60 ± 0.05	50–51
Tryptophan	water	280	–	0.13 ± 0.01	52
Tyrosine	water	275	23	0.14 ± 0.01	52
Phenylalanine	water	260	23	0.024	52
Phenol	water	275	23	0.14 ± 0.01	52
Rhodamine 6G	ethanol	488	–	0.94	53
Rhodamine 101	ethanol	450–465	25	1.0	54
Cresyl Violet	methanol	540–640	22	0.54	55

[a]β-carboline is 9H-pyrido[3,4-β]-indole.
[b]9,10-DPA, 9,10-diphenylanthracene.
[c]POPOP, 2,2'-(1,4-phenylene)bis[5-phenyloxazole].

ABSORPTION OF LIGHT AND DEVIATION, FROM THE BEER-LAMBERT LAW

A fundamental aspect of fluorescence spectroscopy is the measurement of light absorption. While the theory of light absorption is well known, a number of factors can result in misleading

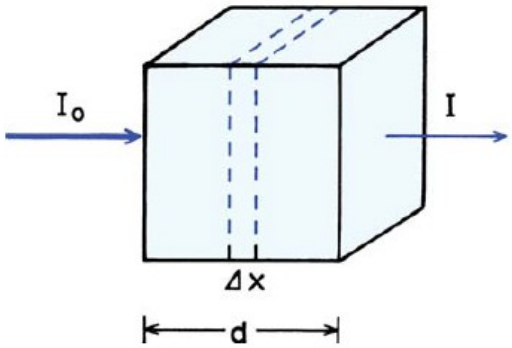


Figure 5. Light absorption.

measurements of light absorption. We will first derive the Beer-Lambert Law, and then describe reasons for deviations from this law. Consider a thin slab of solution of thickness *dx* that contains *n* light-absorbing molecules/cm^3 (Figure 5). Let σ be the effective cross-section for absorption in cm^2. The light intensity *dI* absorbed per thickness *dx* is proportional to the intensity of the incident light *I* and to both σ and *n*, where *n* is the number of molecules per cm^3:

$$\frac{dI}{dx} = -I\sigma n$$

This is the Beer- Lambert equation, which is generally used in an alternative form:

$$\log \frac{I_0}{I} = \varepsilon cd = \text{optical density}$$

where Ɛ is the molar extinction coefficient (in M^{-1} cm^{-1}) and *c* is the concentration in moles/liter.

Deviations from Beer's Law

Beer's Law predicts that the optical density is directly proportional to the concentration of the absorbing species. Deviations from Beer's law can result from both instrumental and intrinsic causes. Biological samples are frequently turbid because of macromolecules or other large aggregates that scatter light. The optical density resulting from scatter will be proportional to $1/\lambda^4$ (Rayleigh scattering), and may thus be easily recognized as a background absorption that increases rapidly with decreasing wavelength.

If the optical density of the sample is high, and if the absorbing species is fluorescent, the emitted light cannot reach the detector. This effect yields deviations from Beer's law. Hence, this effect can be minimized by keeping the detector distant from the sample, and thereby decreasing the efficiency with which the fluorescence emission is collected.

If the absorbing species is only partially soluble, it may aggregate in solutions at high concentrations. The absorption spectra of the aggregates may be distinct from the monomers. An example is the common dye bromophenol blue. At concentrations around 10 mg/ml it appears as a red solution, whereas at lower concentrations it appears blue. Depending

upon the wavelength chosen for observation, the deviations from Beer's law may be positive or negative. The factors described above were due to intrinsic properties of the sample. Instrumental artifacts can also yield optical densities that are nonlinear with concentration. This is particularly true at high optical densities. For example, consider a solution of indole with an optical density of 5 at 280 nm. In order to accurately measure this optical density, the spectrophotometer needs to accurately quantify the intensity of I_0 and I, the latter of which is 10^{-5} less intense than the incident light I_0. Generally, the stray light passed by the monochromator, at wavelengths where the compound does not absorb, are larger than this value.