Fluorophores, INTRINSIC OR NATURAL FLUOROPHORES

Intrinsic protein fluorescence originates with the aromatic amino acids tryptophan (trp), tyrosine (tyr), and phenylalanine (phe) (Figure 1). The indole groups of tryptophan residues are the dominant source of UV absorbance and emission in proteins. Tyrosine has a quantum yield similar to tryptophan, but its emission spectrum is more narrowly distributed on the wavelength scale. This gives the impression of a higher quantum yield for tyrosine. In native proteins the emission of tyrosine is often quenched, which may be due to its interaction with the peptide chain or energy transfer to tryptophan. Denaturation of proteins frequently results in increased tyrosine emission. Emission from phenylalanine is observed only when the sample protein lacks both tyrosine and tryptophan residues, which is a rare occurrence. The emission of tryptophan is highly sensitive to its local environment, and is thus often used as a reporter group for protein conformational changes. Spectral shifts of protein emission have been observed as a result of several phenomena, including binding of ligands, protein–protein association, and protein unfolding. The emission maxima of proteins reflect the average exposure of their tryptophan residues to the aqueous phase. Fluorescence lifetimes of tryptophan residues range from 1 to 6 ns. Tryptophan fluorescence is subject to quenching by iodide, acrylamide, and nearby disulfide groups. Tryptophan residues can be quenched by nearby electron-deficient groups like $–NH_3^+$, $–CO_2H$, and protonated histidine residues.

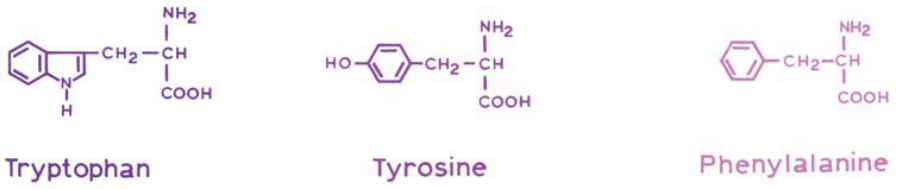
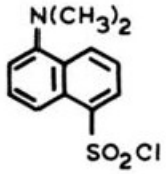


Figure 1. Intrinsic biochemical fluorophores.

Protein-Labeling Reagents



Numerous fluorophores are available for covalent and noncovalent labeling of proteins. The covalent probes can have a variety of reactive groups, for coupling with amines and histidine side chains in proteins. Dansyl chloride (DNS-Cl) was originally described by Weber, Dansyl chloride is widely used to label proteins. Dansyl groups can be excited at 350 nm, where proteins do not absorb. Since dansyl groups absorb near 350 nm they can serve as acceptors of protein fluorescence. The emission spectrum of the dansyl moiety is also highly sensitive to solvent polarity, and the emission maxima are typically near 520 nm.

Fluoresceins and rhodamines are also widely used as extrinsic labels (Figure 2). These dyes have favourably long absorption maxima near 480 and 600 nm and emission wavelengths from 510 to 615 nm, respectively. In contrast to the dansyl group, rhodamines and fluoresceins are not sensitive to solvent polarity. An additional reason for their widespread use is the high molar extinction coefficients near 80,000 M^{-1} cm^{-1}. A wide variety of reactive derivatives are available, including

iodoacetamides, isothiocyanates, and maleimides. Iodoacetamides and maleimides are typically used for labeling sulfhydryl groups, whereas isothiocyanates, N-hydroxysuccinimide, and sulfonyl chlorides are used for labeling amines. Frequently, commercial labeling reagents are a mixture of isomers.

Figure 2. Structures of the dyes.

RED AND NEAR-INFRARED (NIR) DYES

The cyanine dyes were initially used as membrane potential probes and evolved into some of the more commonly used long-wavelength dyes. Long-wavelength probes are of current interest for several reasons. The sensitivity of fluorescence detection is often limited by the autofluorescence of biological samples. As the excitation wavelength becomes longer, the autofluorescence decreases, and hence detectability over background increases. Long-wavelength dyes can be excited with laser diodes. The most familiar long-wavelength dyes are the cyanine dyes. Such dyes have absorption and emission

wavelengths above 550 nm. The cyanine dyes typically display small Stokes shift, with the absorption maxima about 30 nm blue shifted from the emission maxima. A wide variety of conjugatable cyanine dyes are available. Charged side chains are used for improved water solubility or to prevent self association, which is a common cause of self-quenching in these dyes. Lipid side-chains are used to bind these dyes to membranes. Additional long-wavelength dyes are shown in Figure 3. Some rhodamine derivatives display long absorption and emission spectra, as seen for Rhodamine 800. The oxazine dyes display surprising long absorption and emission maxima given their small

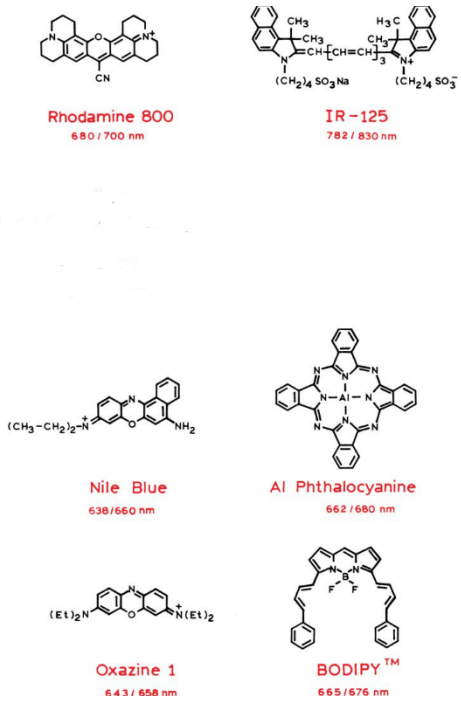


Figure 3.

Representative NIR dyes.

size. Extended conjugated systems result in long absorption and emission wavelengths, as shown for IR-125 and thiazole orange. Dyes of this class have been extensively characterized for use as long-wavelength probes and in DNA sequencing.

Solvent and Environmental Effects

Effects of Solvent Polarity

Emission from fluorophores generally occurs at wavelengths that are longer than those at which absorption occurs. This loss of energy is due to a variety of dynamic processes that occur following light absorption (Figure 4). The fluorophore is typically excited to the first singlet state (S_1), usually to an excited vibrational level within S_1. The excess vibrational energy is rapidly lost to the solvent. If the fluorophore is excited to the second singlet state (S_2), it rapidly decays to the S_1 state in 10^{-12} s due to internal conversion. Solvent effects shift the emission to still lower energy due to stabilization of the excited state by the polar solvent molecules. Typically, the fluorophore has a larger dipole moment in the excited state (μE) than in the ground state (μG). Following excitation the solvent dipoles can reorient or relax around μE, which lowers the energy of the excited state. As the solvent polarity is increased, this effect becomes larger, resulting in emission at lower energies or longer wavelengths. In general, only fluorophores that are themselves polar display a large sensitivity to solvent polarity. Nonpolar molecules, such as unsubstituted aromatic hydrocarbons, are much less sensitive to solvent polarity. Fluorescence lifetimes (1–10 ns) are usually much longer than the time required for solvent relaxation. For fluid solvents at room temperature, solvent relaxation occurs in 10–100 ps. For this reason, the emission spectra of fluorophores are representative of the solvent relaxed state. Examination of Figure 4 reveals why absorption spectra are less sensitive to solvent polarity than emission spectra. Absorption of light occurs in about 10^{-15} s, a time too short for motion of the

fluorophore or solvent. Absorption spectra are less sensitive to solvent polarity because the molecule is exposed to the same local environment in the ground and excited states. In contrast, the emitting fluorophore is exposed to the relaxed environment, which contains solvent molecules oriented around the dipole moment of the excited state. Solvent polarity can have a dramatic effect on emission spectra. Figure 5 shows a photograph of the emission from 4-dimethylamino-4'-nitrostilbene (DNS) in solvents of increasing polarity. The emission spectra are shown in the lower panel. The color shifts from deep blue (λ_{max} = 450 nm) in hexane to orange in ethyl acetate (λ_{max} = 600 nm), and red in n-butanol (λ_{max} = 700 nm). Similar to DNS because of the similar electron-donating and -accepting groups on the fluorophore. The dimethyl amino group is the electron donor. The nitro group and carbonyl groups are both electron acceptors. The high sensitivity to solvent is due to a charge shift away from the amino groups in the excited state, towards the electron acceptor. This results in a large dipole moment in the excited state. This dipole moment interacts with the polar solvent molecules to reduce the energy of the excited state.

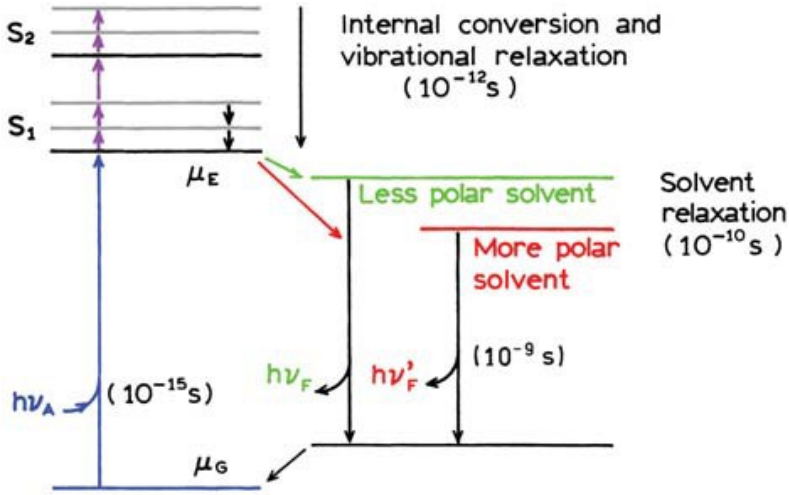


Figure 4. Jablonski diagram for fluorescence with solvent relaxation.

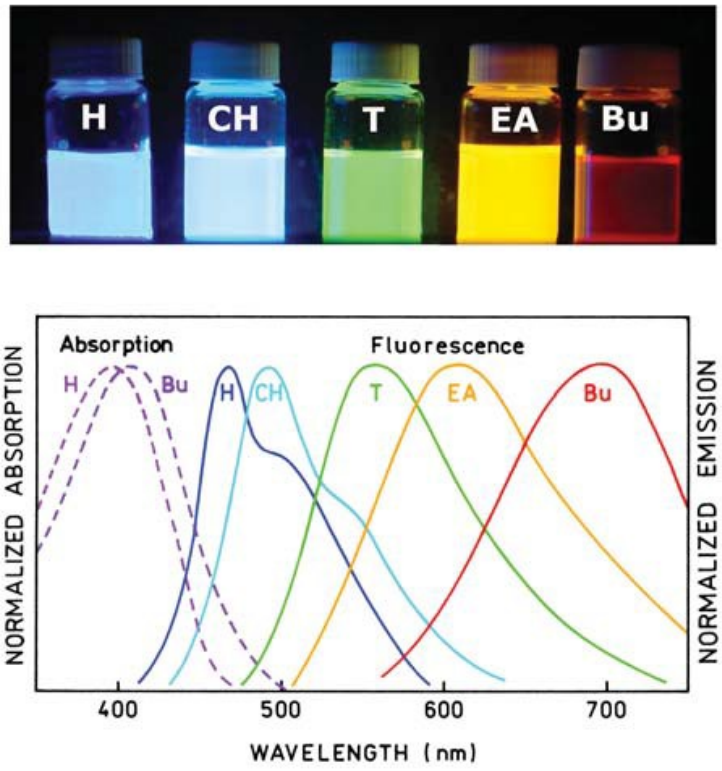


Figure 5. Photograph and emission spectra of DNS in solvents of increasing polarity. H, hexane; CH, cyclohexane; T, toluene; EA, ethyl acetate; Bu, n-butanol.

GENERAL SOLVENT EFFECTS:

THE LIPPERT-MATAGA EQUATION

The theory of general solvent effects provides a useful framework for consideration of solvent-dependent spectral shifts. In the description of general solvent effects the fluorophore is considered to be a dipole in a continuous medium of uniform dielectric constant (Figure 6). This model does not contain any chemical interactions, and hence cannot be used to explain the other interactions which affect the emission. These other interactions, such as hydrogen bonding or formation of charge transfer states, are sometimes detected as deviations from the general theory.

The interactions between the solvent and fluorophore affect the energy difference between the ground and excited states. To a first approximation this energy difference (in cm^{-1}) is a property of the refractive index (*n*) and dielectric

constant (Ɛ) of the solvent, and is described by the Lippert-Mataga equation :

$$\bar{\nu}_A - \bar{\nu}_F = \frac{2}{hc}\left(\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right)\frac{(\mu_E - \mu_G)^2}{a^3} + \text{constant} \qquad \text{.....(1)}$$

In this equation *h* (= 6.6256 x 10^{-27} ergs) is Planck's constant, *c* (= 2.9979 x 10^{10} cm/s) is the speed of light, and *a* is the radius of the cavity in which the fluorophore resides. $\bar{\upsilon}_A$ and $\bar{\upsilon}_F$ are the wavenumbers (cm^{-1}) of the absorption and emission, respectively. Equation1 is only an approximation, but there is reasonable correlation between the observed and calculated energy losses in non-protic solvents. By non-protic solvents we mean those not having hydroxyl groups, or other groups

capable of hydrogen bonding. The Lippert equation is an approximation in which the polarizability of the fluorophore and higher-order terms are neglected. These terms would account for second order effects, such as the dipole moments induced in the solvent molecules resulting by the excited fluorophore, and vice versa.

It is instructive to examine the opposite effects of Ɛ and *n* on the Stokes shift. An increase in *n* will decrease this energy loss, whereas an increase in Ɛ results in a larger difference between $\bar{\upsilon}_A$ and $\bar{\upsilon}_F$. The refractive index (*n*) is a high-frequency response and depends on the motion of electrons within the solvent molecules, which is essentially instantaneous and can occur during light absorption. In contrast, the dielectric constant (Ɛ) is a static property, which depends on both electronic and molecular motions, the latter being solvent reorganization around the excited state. Briefly, an increase in refractive index allows both the ground and excited states to be instantaneously stabilized by movements of electrons within the solvent molecules. This electron redistribution results in a decrease in the energy difference between the ground and excited states (Figure 6). For this reason most chromophores display a red shift of the absorption spectrum in solvents relative to the vapor phase. An increase in Ɛ will also result in stabilization of the ground and excited states. However, the energy decrease of the excited state due to the dielectric constant occurs only after reorientation of the solvent dipoles.

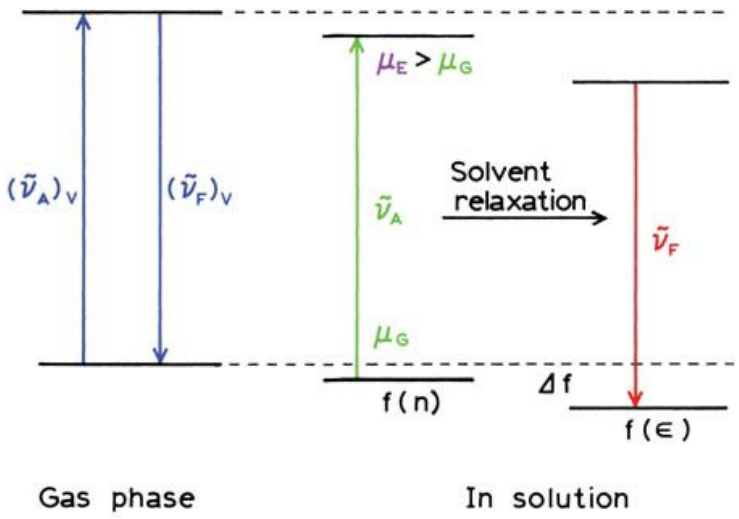


Figure 6. Effects of the refractive index (*n*) and dielectric constant (Ɛ) on the absorption and emission energies.

The term inside the large parentheses in eq. 1 is called the orientation polarizability (Δf). The first term (Ɛ – 1)/(2 Ɛ + 1) accounts for the spectral shifts due to both the reorientation of the solvent dipoles and to the redistribution of the electrons in the solvent molecules . The second term (n^2 – 1)/(2n^2 + 1) accounts for only the redistribution of electrons. The difference of these two terms accounts for the spectral shifts due to reorientation of the solvent molecules (Figure 6, right), and hence the term orientation polarizability. According to this simple model, only solvent reorientation is expected to result in substantial Stokes shifts. The redistribution of electrons occurs instantaneously, and both the ground and excited states are approximately equally stabilized by this process. As a result, the refractive index and electronic redistribution has a comparatively minor effect on the Stokes shift.