Fluorescence spectroscopy in biochemistry: teaching basic principles with visual demonstrations

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Abstract

Although most biochemistry curricula include some treatment of light absorption and spectrophotometry, discussion of fluorescence spectroscopy is generally omitted. This omission is unfortunate given the increasing use of fluorescence in many fields of biochemical research. In this paper we briefly review the principles and applications of fluorescence in biochemical systems, from the viewpoint of teaching fluorescence in undergraduate curricula. Simple practical demonstrations are presented, which clearly demonstrate important concepts yet require minimal specialized equipment. © 2001 IUBMB. Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

The interaction of light with matter has been widely utilized in the physical, chemical and biological sciences. Within the field of biochemistry, spectroscopic methods have assumed an important role and have helped to put much of the discipline on a quantitative basis. Examples of the current use of fluorescence in biochemistry range from fluorimetric assays for biomolecules (e.g., proteins, lipids, nucleic acids, carbohydrates), fluorimetric enzyme assays, DNA sequencing analysis, and fluorescence in situ hybridization (FISH) to identify specific DNA and/or RNA sequences in tissues. More recently, an exciting new development has been the use of molecular genetics methods to fuse the gene for the intrinsically fluorescent green fluorescent protein (GFP), originally isolated from coelenterates such as the Pacific jellyfish *Aequoria victoria*, to other target genes for subsequent expression in living cells (for a vivid discussion of GFP and for many exciting pictures visit the website: http://www.biochemtech.uni-halle.de/PPS2/projects/jonda/intro.htm). In addition, much of the current research into protein and membrane dynamics has involved fluorescence spectroscopy (either in cuvettes or through the microscope) of intrinsic biomolecular fluorescence, for example from proteins (specifically from tyrosine or tryptophan residues) as well as exogenous fluorescent probes (either protein or membrane specific).

Most, if not all, biochemistry curricula include detailed discussion of light absorption by biomolecules such as porphyrins, flavins, chlorophylls, carotenoids, proteins and nucleic acids. These lectures include discussion of the wavelengths absorbed and concepts such as the Beer–Lambert Law. Molecules that absorb light typically lose the excess energy by non-radiative processes, for example, as heat to the solvent. In a few cases, however, the excited state energy may be re-emitted as light, a phenomenon known as fluorescence.

However, while biochemistry curricula include a detailed discussion of light absorption and spectrophotometry, a description of the theory and application of fluorescence spectroscopy is not commonly presented. Thus biochemistry curricula may be lagging behind current practice wherein knowledge of fluorescence principles is increasingly pertinent to biochemists and molecular biologists.

Here we present a brief, basic coverage of principles and applications of fluorescence, perhaps suitable as the basic outline for a module of undergraduate teaching. The discussion is supported by simple and generally inexpensive examples for practical demonstrations,

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which require a minimum of specialized equipment. For more detailed coverage of fluorescence we refer readers to several books and articles therein [1–6] . Internet web sites provide another useful resource for information on fluorescence, such as that of Molecular Probes Inc. (http://www.probes.com), which presents a very large collection of fluorescence microscopy images. Another interesting fluorescence related website is that of the Laboratory for Fluorescence Dynamics (http://lfd.uiuc.edu/). Finally, a very good discussion of some fluorescence concepts which can be simply illustrated using common yellow and pink highlighter pens has been given by Fery-Forgues and Lavabre [7] and a simple, lost-cost fluorometer has been described by Delorenzi et al. [8].

2. Overview of fluorescence

In general terms, a fluorescent molecule is one that absorbs light at a given wavelength and then, after a finite duration (on the order of nanoseconds), emits light of a different (generally longer) wavelength. Light absorption is characterized by the absorption spectrum and the extinction coefficient (a measure of the extent of light absorption). The information content of fluorescence emission is somewhat different, and encompasses several parameters including the relative fluorescence intensity or quantum yield, excitation and emission spectra, polarization, and lifetime of the excited state. Our discussion will include a brief introduction to each of these parameters.

A good starting point for a discussion of fluorescence principles is a simplified Jablonski diagram (Fig. 1). A Jablonski diagram is used to represent the energy levels of a molecule. These energy levels include the ground electronic state (S_0), which typifies a molecule "at rest" and also higher energy electronic states (e.g., S_1, S_2), reached upon the absorption of light. Each electronic state of a molecule also contains numerous vibrational and rotation energy levels that fully describe the energetics of the system. A molecule can absorb a photon which contains energy equal to the difference between its ground state and one of its excited states (recall that the energy of a photon is given by $E = hv$ where h is Planck's constant (6.27×10^{-27} erg-s) and v is the frequency of the light). In Fig. 1, the thick lines represent the lowest energy levels of the electronic states while the thin lines represent the vibrational modes. The rotational energy levels (not depicted here) would be superimposed on each vibrational level. Transitions from the ground state to an excited energy level, represented by arrows, thus occur upon the absorption of light. At room temperature, most molecules would be in the lowest vibrational level of the ground electronic state (the distribution of molecules among the various energy levels is given by Boltzmann's
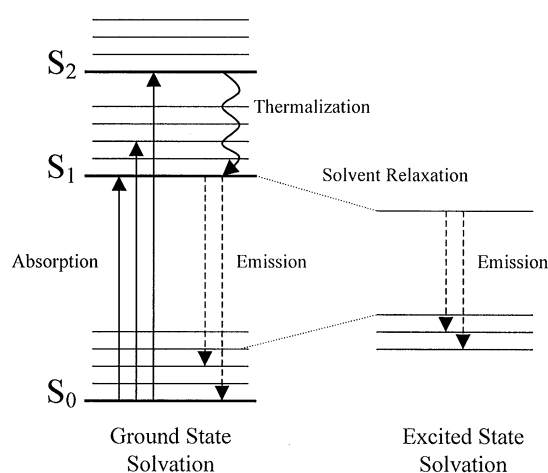

Fig. 1. A simplified Jablonski energy level diagram depicting absorption (—) and emission (- - - -) processes as well as thermalization and solvent relaxation.

law [4]). Absorption of a photon by a molecule occurs essentially instantaneously relative to nuclear motion ($\sim 10^{-15}$ s), a fact known as the Franck–Condon Principle. The energy of the photon leads to a redistribution of a molecule's electron cloud, which results in an altered dipole moment, and the excited molecule will ordinarily momentarily be in an upper vibrational level of an excited electronic state. The exact vibrational and electronic level reached will depend upon the energy content, or color, of the light absorbed. Students should be reminded that the energy of the electromagnetic radiation is higher at shorter wavelengths (since energy is directly proportional to v, the frequency of the light, and $v = c/\lambda$, where c is the velocity of light).

Regardless of the excited energy reached, however, the molecule will rapidly ($\sim 10^{-12}$ s) lose energy to its environment through non-radiative modes involving nuclear motions, and will revert to the lowest vibrational level of the lowest electronic excited state. This process is depicted in Fig. 1 and is termed thermalization. The molecule may persist in this lowest level of the S_1 state for a period of time known as the fluorescence lifetime (technically the fluorescence lifetime is the reciprocal of the rate constant for returning to the ground state). This excited state lifetime may last for a period of picoseconds to hundreds of nanoseconds. For most fluorophores of interest and utility in biochemistry, however, the fluorescence lifetimes are in the range of several nanoseconds to a few tens of nanoseconds. The Jablonski diagram illustrates why the absorption and emission spectra are typically broad bands, namely because of the distribution of the higher vibrational levels that can be reached from transitions from the ground state (absorption) and the distribution of vibrational levels in the ground state that can be reached from transitions from the lowest excited state (fluorescence). The Jablonski diagram also clearly
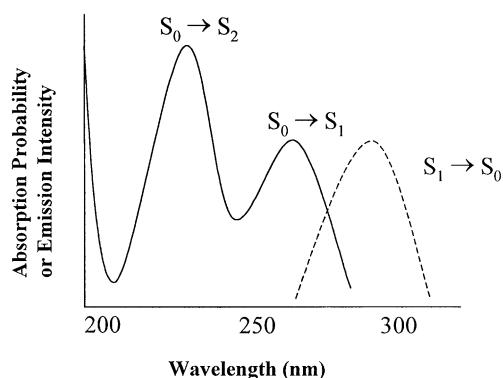
Fig. 2. Representative absorption (—) and emission (- - - -) spectra for a simple organic molecule, such as phenol.

indicates why the wavelength (or color) of the fluorescence is independent of the wavelength absorbed: regardless of the wavelength absorbed and subsequent upper energy level reached, the fluorescence will always occur from the lowest vibrational level of the first excited state (S_1). This independence of the emission from the excitation is known as Vavilov's law. A further consequence of these facts is that the emitted light is usually at longer wavelengths (lower energy) than the absorbed light; this difference is called the Stokes Shift (named after Sir George G. Stokes who first introduced the term "fluorescence"). In some cases, further reduction of energy occurs during the fluorescence lifetime by a phenomenon known as solvent relaxation. This involves re-orientation of solvent dipoles around the new excited state dipole. The reorientation favors new dipole–dipole interactions and lowers the overall energy of the system. Since this new, more stabilized S_1 level is at lower energy, any subsequent fluorescence will also be at lower energy (longer wavelength). Other molecular phenomena, such as non-radiative energy transfer to an appropriate acceptor molecule or quenching due to formation of a stable or transient complex with molecules such as oxygen or heavy metals (to name but a few) can also lead to deactivation of the excited state without the emission of light.

Fig. 2 shows representative absorption and emission spectra for a simple organic molecule (such as phenol) which might result from transitions between the energy levels depicted in Fig. 1. Fig. 2 illustrates two principles described above: the two absorption maxima represent incident photon energy levels matching electronic transitions in the absorbing molecule; and the broad bands reflect the distributions of vibrational levels in the excited and ground states.

Fig. 3 shows a simple, visual demonstration of fluorescence. In this figure, a simple UV handlamp or "blacklight" (such as often used in geology demonstrations) is used to illuminate various solutions of fluorophores placed in test tubes. In a darkened room the emitted light is clear and dramatic.

We want to emphasize that the concentrations given in the figure legends for all fluorophore solutions are very approximate. In practice, the instructor may simply insert the tip of a pasteur pipette into the solid fluorophore, giving enough in the tip to make a concentrated stock solution. Then, through trial and error, the instructor can determine the final concentrations which work best for the demonstrations, given the type of handlamp, ambient lighting, *et cetera*.

If the handlamp being utilized has both 366 and 254 nm available (the usual lines from a mercury hand lamp), one can illuminate the samples alternatively with both wavelengths and demonstrate that the fluorescence is invariant (except for intensity) with the exciting wavelength (one should note that the 254 nm illumination will not penetrate deeply into the glass test tubes but enough light will reach the sample to convey the effect; if quartz tubes or cuvettes are available the effect is much more dramatic). It should be noted that there are some occupational health and safety issues in handling the fluorescent probes and UV light. However, by using the recommended procedures as detailed in materials safety data sheets (MSDS) from the suppliers, the compounds and handlamp may be handled safely. The visual demonstrations presented in this article can be extended by obtaining excitation and emission spectra using a spectrofluorometer, if it is available. The excitation spectrum should in most cases correspond to the absorbance spectrum taken using a spectrophotometer (some care must be taken here, however, and the reader should consult the references for a discussion of instrument correction factors). The actual emission spectra corresponding to the emissions shown in Fig. 3B are given in Fig. 3C.

3. Quenching of fluorescence

Emission of light is not the only way in which a molecule can lose the excited state energy and return to the ground state. Other processes which can lead to a "non-radiative" transition include deactivating collisions with solvent molecules, transfer of the excited state energy to another "acceptor" molecule (this phenomenon is known as fluorescence resonance energy transfer (FRET)), and interactions with a "quencher"—a molecule which can deactivate the excited state either by formation of a non-fluorescent complex or by a collisional process. Quenchers that work via complex formation tend to be very selective in the type of fluorophores with which they interact. Many aromatic molecules can interact with one another via weak van der Waals forces; since most fluorophores are based on aromatic ring structures one finds that they can often interact with non-fluorescent aromatic molecules and form a weak, non-fluorescent complex. An example of this type of interaction is the quenching of ethidium bromide by caffeine (see Ref. [9]
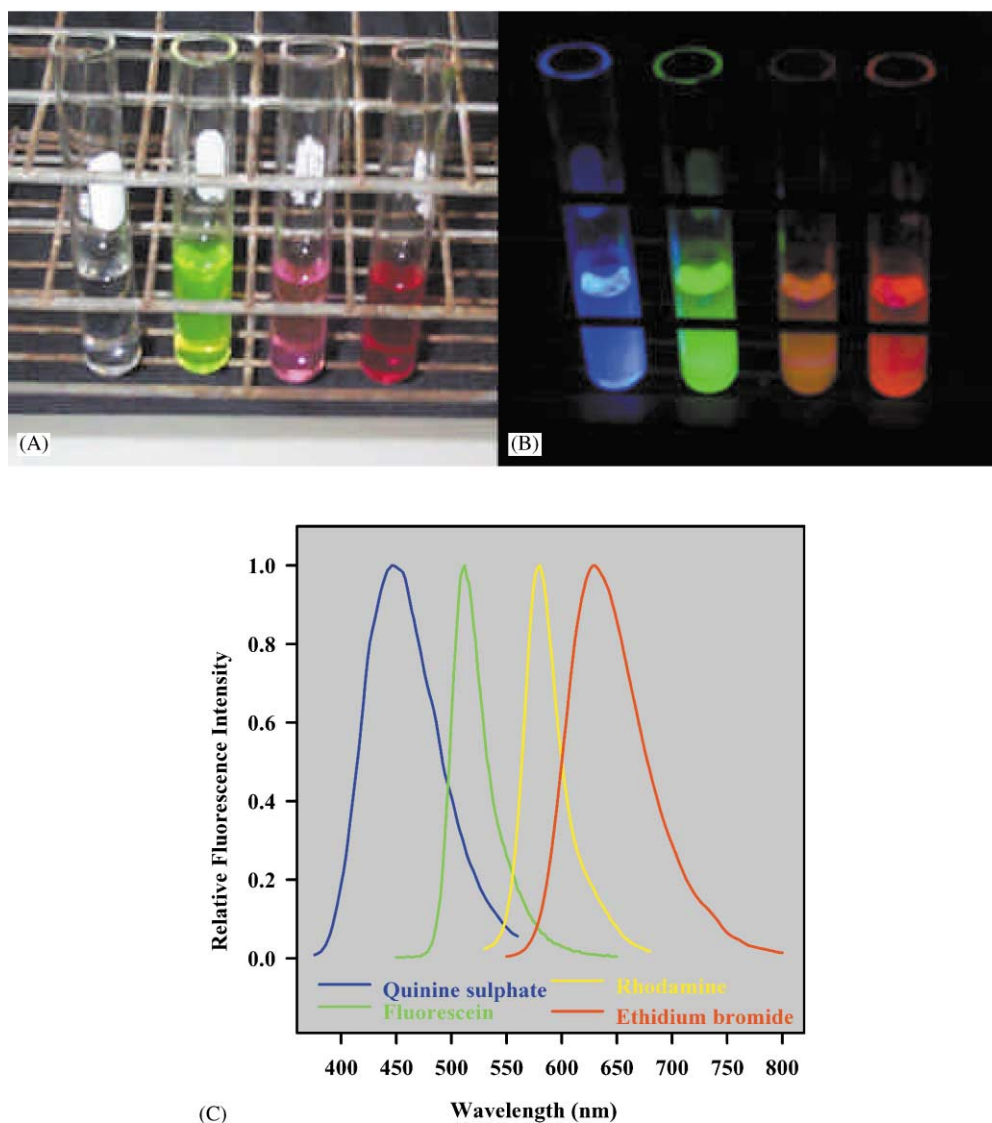
Fig. 3. A visual demonstration of fluorescence emission from varying compounds at approximate concentrations of $0.01\,mg\,mL^{-1}$. In each of the photos, the compounds are from left to right; quinine sulphate in water ($0.1\,M\,H_2SO_4$ preferable), fluorescein in water (slightly alkaline pH preferable), rhodamine B in water and ethidium bromide in ethanol. Panel A shows the test tubes in room light. The edge of the hand lamp can be seen at the bottom of the picture. Panel B shows the test tubes illuminated with a UV hand lamp (excitation 366 nm) in a darkened room. Panel C shows normalized emission spectra obtained from a spectrofluorometer (ISS PC1; ISS Inc, Champaign, IL USA), using excitation at 366 nm.

for a very detailed discussion of this phenomenon). Collisional quenchers, however, tend to be small molecules, the most common being oxygen (O_2), iodide (I^-) and heavy metals such as mercury (Hg^{2+}). The demonstration shown in Fig. 4 illustrates the principle of fluorescence quenching. In this example the fluorescence intensity of a solution of fluorescein is shown to be decreased by the addition of potassium iodide (KI). This demonstration may be extended to include sequential additions of KI and a rough estimation of the extent of quenching. The relationship between the initial fluorescence before addition of quencher (F_0), the fluorescence in the presence of quencher (F), the so-called "bimolecu-

lar quenching constant" (k_+^*), the quencher concentration [Q], and the fluorescence lifetime in the absence of quencher (τ_0) is given by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_+^*[Q]\tau_0.$$

The plus sign in the bimolecular quenching constant is traditional and simply refers to the fact that the process is an association between two molecules—in normal kinetic usage, for example, k_+^* usually means association and k_-^* usually means dissociation. A Stern–Volmer plot of the quenching of fluorescein with KI is shown in Fig. 5.
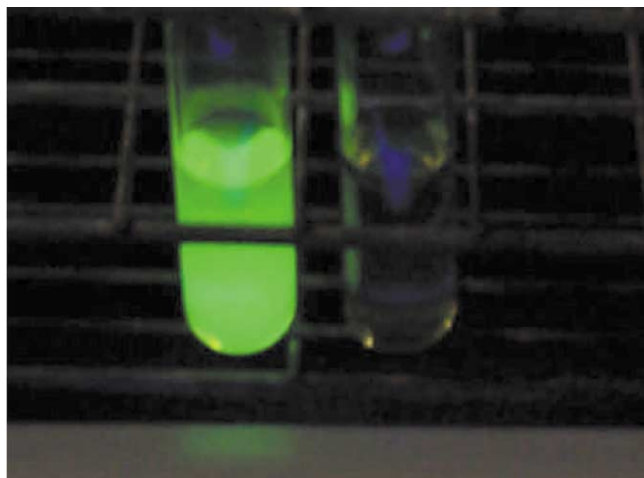
Fig. 4. Two test tubes of fluorescein (approximately $0.01\,mg\,mL^{-1}$) in water in the absence (left) and presence of potassium iodide. A few crystals of KI were added directly and dissolved in the tube to quench the fluorescence. Samples were illuminated by UV hand lamp (excitation 366 nm).
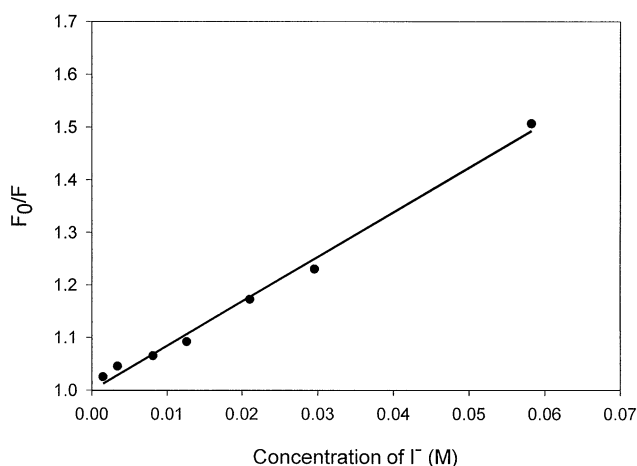

Fig. 6. Fluorescence from tubes containing approximately 10 mL of $0.01\,mg\,mL^{-1}$ aminonaphthalene sulphonate (ANS) in water before (right) and after addition of 2 mL of 1% (wt/vol) bovine serum albumin (BSA). Samples were illuminated by UV hand lamp (excitation 366 nm).

Fig. 5. Stern–Volmer plot of quenching of fluorescein fluorescence by KI. Fluorescein, $1\,\mu M$ in $50\,mM\ KPO_4$ buffer, pH 8.0, was excited at 488 nm, the emission was collected through a yellow filter (Corning 3–69) that blocks light below $\sim 500\,nm$ but passes all longer wavelengths. We should note that at higher iodide ion concentrations the curve deviates from linearity, specifically curving upwards somewhat. The reason for this effect is beyond the scope of the present discussion (see Ref. [4] for more discussion).

4. Applications to proteins

In the next example, shown in Fig. 6, the effect of environment on fluorescence is dramatically demonstrated. When aminonaphthalene sulphonate (ANS) is dissolved in water, it emits only a very weak, yellow fluorescence. Upon addition of a solution of protein (in this case 1% bovine serum albumin (BSA), although human serum albumin (HSA) will work as well), an immediate, dramatic increase in fluorescence is noted along with a color change (from yellow to blue–green). The underlying basis for the increase of emission is movement of ANS from water to a hydrophobic environment on the surface of BSA. Water molecules effectively lower the energy of ANS's excited state (hence the lower energy, yellow emission) and also quench the fluorescence. The binding of ANS to BSA is consistent with the biological role of albumin, which circulates in the bloodstream and carries non-polar molecules, such as fatty acids, through this aqueous environment.

5. Solvent relaxation/viscosity

As discussed earlier, the excited fluorophore usually has a dipole moment (charge separation) which differs from that of the ground state molecule. Hence, during the excited state lifetime there will be a tendency for the solvent molecules, which usually have a small dipole moment, to reorient around the newly created excited

The slope of the quenching curve is $\sim 8\,L\,mol^{-1}$ and equals $k_+^* \tau_0$. The unquenched fluorescence lifetime of fluorescein in this solution is 4.0 ns and hence the bimolecular quenching constant is $\sim 2 \times 10^9\,M^{-1}\,s^{-1}$. Such quenching studies are important since they can provide direct information on the diffusion of small molecules in solution and in cellular interiors and hence give insights into solvent viscosities and solvent accessibilities.
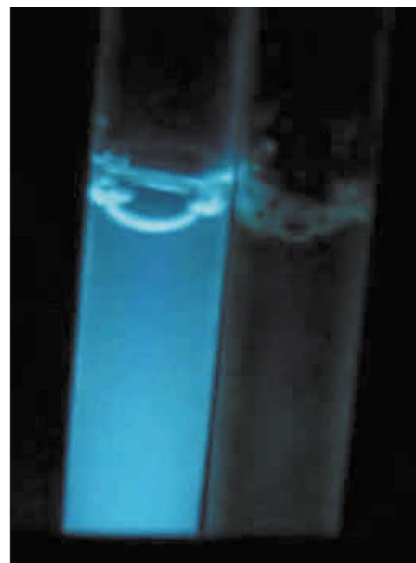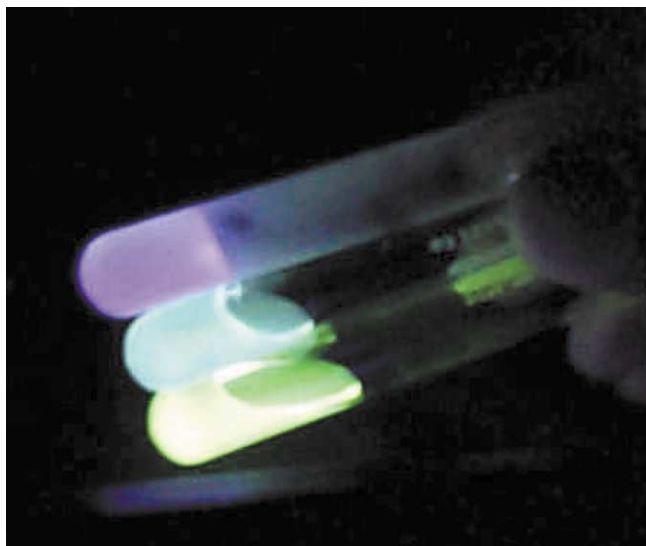
Fig. 7. Laurdan in glycerol, cooled to $-70°C$ by immersing the tube in dry ice/propanol (top), at room temperature (25°C) (middle) and heated to $\sim 80°C$ by immersing the tube in hot water (bottom). The laurdan (approximately $1\,mg\,mL^{-1}$) was dissolved in $\sim 0.25\,mL$ of dimethyl formamide which was then added to $\sim 15\,mL$ of glycerol and the resulting mixture was briefly mixed, followed by heating to $\sim 80°C$, vortexing for complete mixing, and distribution into three tubes. Samples were illuminated by UV hand lamp (excitation 366 nm).

incline both tubes (the room temperature and the cold tube) and see that the glycerol flows in the green tube but not in the blue tube. One can also compare simultaneously tubes which are cold, room temperature and hot as shown in Fig. 7 and easily see the difference in the color of the fluorescence. Prodan and Laurdan are, in fact, extensively used in studies of biological membranes to investigate the "fluidity" (which is the inverse of the viscosity) of membranes. The demonstration may also be made using water ice, however the viscosity change and hence the blue shift is not nearly as dramatic as that seen with dry ice/acetone.

6. Summary

In this paper we provide a simple introduction to the principles of fluorescence, coupled with simple visual demonstrations, suitable for presentation to undergraduate students. The presentation is necessarily incomplete, and simply mentions some important principles without expanding on their nature. Readers with a sustaining interest should consult the references cited which provide a much more rigorous treatment of theory and application of fluorescence in biochemical systems.

state dipole. This reorientation process usually occurs very rapidly in fluid solvents, such as water, and invariably leads to a red-shift in the emission since the stabilized excited state dipole emits from a lower energy level (see Fig. 1). In very viscous solvents, however, one can effectively demonstrate the solvent relaxation phenomenon as shown in Fig. 7. In this figure one sees fluorescence from the probe Laurdan (6-dodecanoyl-2-dimethylamino naphthalene) (although the analogous probe Prodan (6-ethanoyl-2-dimethylamino naphthalene gives very similar results). The probes emits with a greenish fluorescence in glycerol at room temperature or above, but at lower temperatures (and hence greatly increased viscosity) result in a bluish fluorescence. The demonstration is most effectively done by holding two tubes of the probe dissolved in glycerol (it is best to first dissolve some probe in ethanol or dimethyl formamide and then to add a few drops to the glycerol tubes and invert them repeatedly while holding them under hot water). Then, while illuminating both tubes with the handlamp, one can be immersed in a dry ice/acetone mixture, which rapidly vitrifies the glycerol. As the solution cools one will notice that the green fluorescence begins to turn to blue. Specifically, the blue emission will begin to appear near the outside of the solution and, as the cold penetrates into the test tube, the blue color will penetrate more towards the center of the tube. When the tube emits a uniform blue color one can then gently

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