TECHNICAL NOTE

Fluorescence Spectroscopy

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The number of fluorescence techniques applications has been continuously growing over the last 20 years. While initially intended as an analytical tool for the determination of the presence of specific molecules in solutions, fluorescence is now routinely used in biochemistry and biophysics for studying molecular interactions and dynamics, both in solutions and in cells; in clinical immunoassays for the determination of the presence of specific antibodies and antigens; in drug discovery; in life sciences for DNA sequencing; in nanotechnology and material sciences for identification and characterization of new materials.

The reasons of the continuing increase in popularity are multiple: on one hand, it is due to the improvements in the sensitivity of the instrumentation that allows now for the observation of single molecules events on a routine basis; on the other hand, the interface of the instrumentation with the personal computer has increased the automation of the data collection and the sophistication of the data analysis. A third reason for its increased success is due to the introduction in the past thirty years of innumerable and specific chemical probes used as markers for compounds that either do not display fluorescence or only emit a low level of it. The extent of the applications has benefited from the development of the Green Fluorescence Protein (GFP) family that allows for the expression of fluorescent proteins in cells and tissues; a feature that allows the experimenter to follow the whereabouts of proteins in live cells and even tissues in live animals.

Paradoxically, the capabilities of the instrumentation coupled to the computation power of the computer brings new challenges to the field, as novel practitioners are not always aware of the potential pitfalls that lie behind an experiment. In the past few years several articles and books have been published on the subject describing in details the applications of the fluorescence techniques to the chemical and life sciences. A brief article cannot cover such details; our goal is rather to reiterate the fundamental principles of the technique and to mention some of the common pitfall that a user of the technique may encounter.

1. Observables measured in fluorescence

Fluorescence is generally referred to as the emission of photons from a sample following the absorption of photons. There are other means for producing fluorescence in a sample (bioluminescence, sonoluminescence, and electroluminescence) but in the following we will refer exclusively to the phenomenon originated by the absorption of light.

Fluorescence is part of a general class of phenomena named *luminescence; it* is distinguished by the *phosphorescence* as the latter takes, typically, a time of the order of one microsecond (10^{-6} s) or longer while the former takes a time of the order of one nanosecond (10^{-9} s). As we will see in the following, the distinction between the two is described using the more precise terminology of quantum mechanics.

The main five parameters measured in fluorescence spectroscopy are:

- Excitation spectrum
- Emission spectrum
- Decay times (fluorescence lifetimes)
- Quantum yield
- Anisotropy (or polarization)

Recent advancements in fluorescence microscopy have introduced the measurement of additional parameters (diffusion correlation times, brightness) but we will limit our discussion in this chapter to the five parameters listed above and measurable using a spectrofluorometer.

The description of the fluorescence measurable parameters is best understood with the introduction of the Perrin-Jablońsky diagram that is a quantum mechanics representation of the energy levels of a molecular structure.

2. The Perrin-Jablońsky diagram

Figure 1 is a classic representation of the electronic levels of a molecule in solution or in gas phase (in solid phase the energy levels collapse into "bands" although the basic concepts are still valid).

The energy levels occupied by an electron are named "singlet states" and the letters S_0, S_1, S_2, ..., indicate the ground state, the first excited state, etc.; upon absorption of a photon, an electron moves from the ground state S_0 to the excited states. Associated with each electronic level, there are several vibrational and rotational levels, which differ in energy by a smaller amount than the corresponding electronic levels.

Moreover, there are energy transitions that are not directly allowed (forbidden transitions). They are identified as "triplet states" and indicated by T_1, T_2, …, etc.; they also feature associated vibrational and rotational levels.

The absorption probability of a photon in each electronic level is described within the framework of quantum mechanics (energy separation between the levels, momentum and spin of the various levels).

The molecules interact when in presence of photons of the appropriate photon energy E, where

$$E = h\nu = h\frac{c}{\lambda} \qquad [68.1]$$

In the relation, h is the Planck constant (6.626 x 10^{-34} J s), c is the speed of light (2.9979 10^{8} m s^{-1}) while ν and λ are the frequency and wavelength of the electromagnetic wave describing the photon.

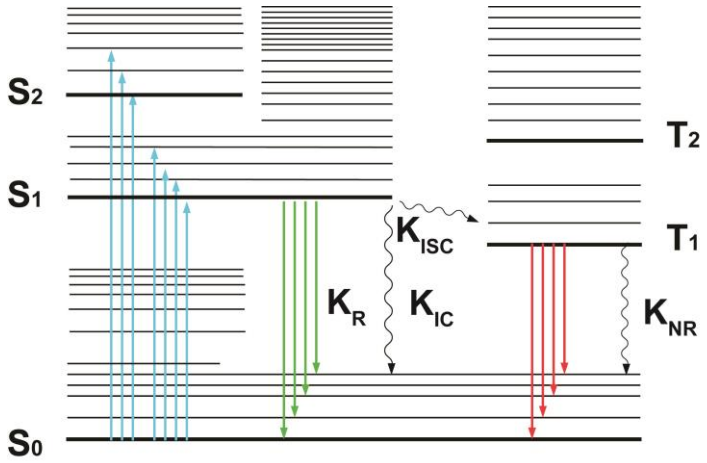

Figure 1. Perrin-Jablonsky energy diagram for a molecular structure. Singlet states are indicated by S_0, S_1, ..; and triplet states by T_1, T_2, ..Internal conversion rate is K_{IC}; intercrossing conversion rate between singlet and triplet states is K_{ISC}; the fluorescence decay rate is K_R, while the non-fluorescence rate is K_{NR}.

For absorption to occur, E has to be of the order of magnitude of the separation between the excited level and the ground state; that is,

$$E \approx E_{S_1} - E_{S_0} \qquad [68.2]$$

Let us consider a population of N molecules in a solution. Upon absorption of photons (blue lines in the figure), a fraction of the molecules undergo a transition from the ground state S_0 to the upper electronic states, S_1, S_2; the final state depending ultimately by the energy of the absorbed photon. The absorption process takes an amount of time of the order of the femtosecond (10^{-15} s) or shorter.

Once in the excited electronic level, the molecules relax fairly rapidly (about 10^{-12} s) to the lowest level of the first excited state S_1; hence, they decay with rate k_R to emit fluorescence (green line in the figure). The characteristic time of the fluorescence is of the order of one nanosecond (10^{-9} s).

There are additional decay routes that are not necessarily associated with the emission of photons; they are indicated by k_{IC} (internal conversion between two electronic states of the same spin multiplicity) and k_{ISC} (intersystem crossing conversion between the S levels and T levels). It is noteworthy to note that the excited level T_1 (triplet state) emit photons; this process is usually termed "phosphorescence" and its characteristic time, as mentioned above, is of the order of one microsecond (10^{-6} s) and longer.

The Perrin-Jabloński diagram (Figure 1) is instrumental to determine the law describing the decay time of fluorescence. If N_1 is the population of the excited level S_1, upon absorption of photons the population of the level changes are described by the relation:

$$\frac{dN_1}{dt} = -(k_R + k_{NR})\, N_1 + f_1 \qquad [68.3]$$

where f_1 is a function that describes the process of the excitation photons (pulsed source, continuous wave source, etc.). By solving the equation (and disregarding f_1), we find the

$$N_1 = N_1(0)\, e^{-t/\tau_S} \qquad [68.4]$$

where τ_S, the decay time of the excited state S_1 is defined as:

$$\tau_S = \frac{1}{k_R + k_{NR}} \qquad [68.5]$$

The fluorescence quantum yield is the fraction of excited molecules that return to the ground state with the emission of fluorescence. From direct examination of the Perrin-Jabłońsky diagram, one simply divides the rate of radiative emission k_R by the total rates of deactivation, which includes both the radiative and non-radiative contributions:

$$\Phi = \frac{k_R}{k_R + k_{NR}} \qquad [68.6]$$

By using the definition of decay times, the quantum yield can also be expressed in terms of lifetimes:

$$\Phi = \frac{\tau_S}{\tau_R} \qquad [68.7]$$

One can say that the quantum yield is the ratio of the number of emitted photons over the total number of absorbed photons.

The five measurable parameters of fluorescence are usually used to describe these processes, namely: the range in wavelengths of the absorption and emission of photons (excitation and emission spectra), the orientation changes during the time the molecules are in the excited states between absorption and emission of the photons (anisotropy or polarization), the fraction of photons emitted over the number of photons absorbed (quantum yield) and the emission rate (decay times). After a brief overview of the instrumentation we will examine in detail the measurement of the five parameters.

3. Instrumentation

The peculiar parameters that characterize fluorescence are measured using "spectrofluorometers"; sometimes, instruments for the measurement of excitation and emission spectra are termed "spectrofluorimeters", while the ones for the measurements of the decay times termed "spectrofluorometers". Yet, the distinction is not anymore as clearly demarked as several instruments allow, in the same unit, to measure both the steady-state (excitation and emission spectra) and the dynamic (decay times and rotational correlation times) properties of the fluorescence.

Usually, in all of the instruments, the fluorescence is collected at an angle of 90 degrees with respect to the optical axis set by the excitation light beam. This geometry maximizes the efficiency of the emission collection and reduces the background due to the excitation light.

It is worthy to mention that absorption spectra can be measured using a spectrophotometer. In this type of instrument the light detector is placed on the same optical axis of the excitation light beam and the instrument detects the amount of light that is being transmitted (that is, not absorbed) through the sample. A spectrophotometer measures the difference in the intensity of two signals (typically, sample transmittance is compared to 100% transmittance); instead, a spectrofluorometer measures a signal (the fluorescence) over a zero background.

The key elements of a spectrofluorometer are the light source, the monochromator and the light detector.

5.1 Light Source

The typical light source utilized in a spectrofluorometer is a high-pressure xenon arc lamp. The bulb of this lamp includes xenon at high pressure that is excited to higher level by the electrical arc established by the current running through the electrodes. The emitted light is a continuous spectrum from (depending

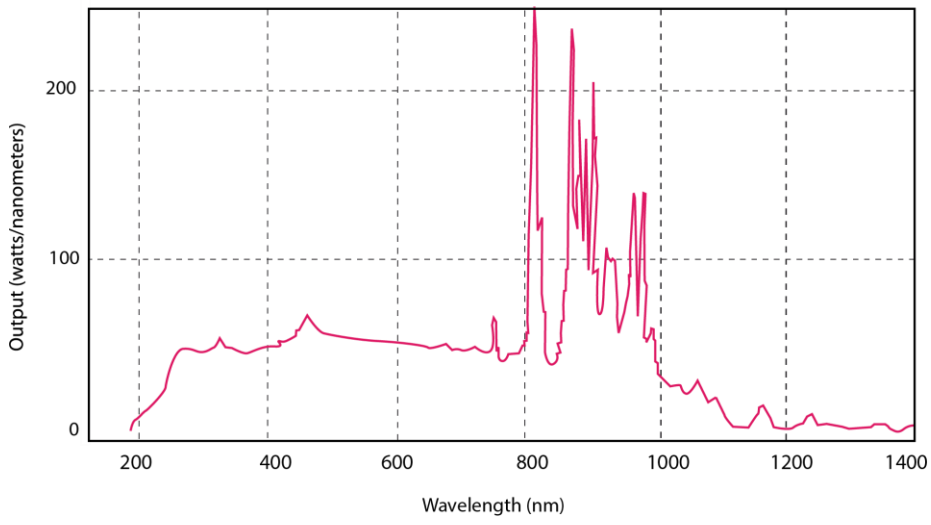

Figure 2. Spectral distribution for the 300W xenon arc lamp (courtesy of ISS).

upon the models and geometries) about 250 nm up to 1100 nm. Figure 2 displays the spectrum of the lamp utilized by ISS. Although the spectrum is relatively flat up to about 800 nm, several sharp resonances are present above that wavelength.

It is worth noting that a variation of this lamp is the Hg-Xe lamp, which contains traces of mercury; this element displays resonances at around 295 nm and this feature allowed for its use as an excitation source for the proteins containing tryptophan.

In the past several years lasers have replaced the xenon arc lamp, specifically for time-resolved applications. Although they emit radiation only at specific wavelengths, their brightness is order of magnitude higher than that of the lamp. In addition, they can be pulsed with fairly narrow pulse widths (about 50 ps for the laser diodes). A recent advancement is the supercontinuum laser (or white laser) that delivers any wavelength in the range from 390 nm up to 2000 nm, featuring 5 ps pulsewidth and (in the model made by Fianium Ltd) the option of selecting the repetition rate up to 40 MHz.

Light emitting diodes (LEDs) are also utilized as light sources especially in the region from 240 nm to 350 nm, where lasers are not available (with exceptions at 266 nm, 315 nm, 325 nm). They are compact, relatively inexpensive and the source of choice when building an instrument dedicated to a specific application.

5.2 Monochromator

Monochromators are utilized to select the wavelength used for irradiating the sample when using a xenon arc lamp; in the collection channel of a spectrofluorometer they are utilized to record the range of wavelengths emitted by a fluorophore (emission spectrum, see below). The simplest monochromator includes a diffraction grating and slits at the entrance and at the output. Light impinging at an angle on the grating is diffracted at a series of angles; usually, the first angle (or first order) is selected for the measurement.

It is important to realize that the transmission of the light traversing a monochromator is affected by two parameters:

1. the wavelength; the grating has a specific transmission curve and some wavelengths are transmitted with a higher efficiency than other wavelengths, a feature to remember when collecting excitation and emission spectra.
2. the polarization status of the radiation; the grating of the monochromator transmits differently radiation with different planes of polarization.

Moreover, it is important to remember that when a monochromator is set to deliver radiation at wavelength λ, it also delivers radiation at 2λ (second order); as an example, if the excitation monochromator is set at 300nm, it delivers radiation ad 600nm too. Typically the intensity of the second

order is about 1/10 the intensity of the first order; still this amount is sufficient to contaminate the emission spectrum. The second order can be eliminated with a judicial selection of filters.

A characterization of every monochromator is the amount of stray light, that is radiation present at any wavelength other than the specific wavelength the monochromator is set at. The stray light is usually measured as the amount of light that is transmitted outside the band pass of the 632.8 nm HeNe laser line. For typical holographic gratings it is 10^{-5} the intensity of the line. While this amount is not typically important for the study of fluorophores in thin solutions, it becomes important when the sample is in a turbid solution or even a solid state. Different strategies are available for the minimization of the stray light, the first being a judicial selection of the grating. Gratings are classified depending upon their fabrication process: the ruled gratings and holographic gratings, with the latter displaying less stray light inhomogeneity as the grooves are formed through the interference process of two laser beam in a photosensitive material, while in the former the grooves are formed mechanically.

Gratings can be arranged in different designs to build a monochromator, the two more popular being the Czerny-Turner and the Seya-Namioka.

5.3 Light Detectors

In all the instruments the fluorescence signal is converted into current by a photomultiplier tube (PMT), or photodiode (instruments for lifetime measurements may utilize other types of detectors too, such as hybrid PMTs, microchannel plate detectors or streak cameras).

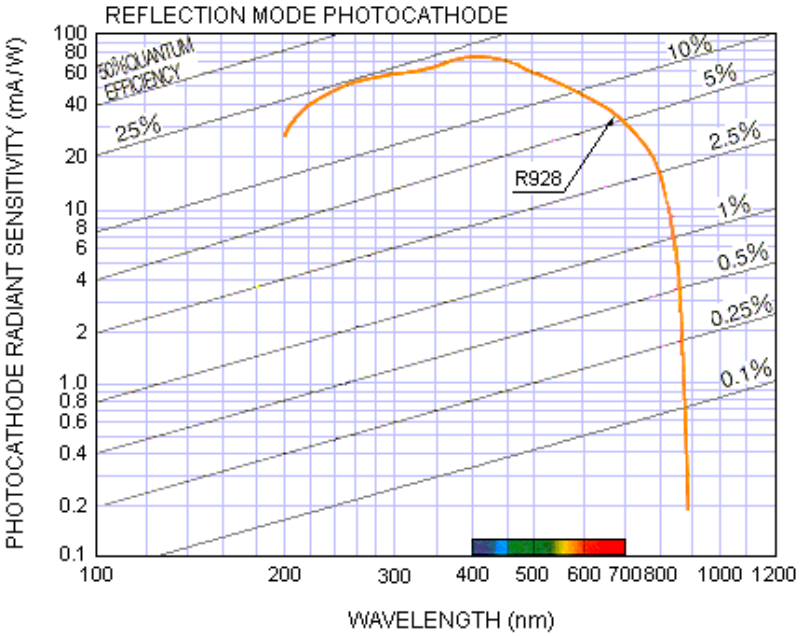

Figure 3 Wavelength range for a photomultiplier tube model R928 (*courtesy of Hamamatsu*)

Photomultiplier tubes are sensitive within a set wavelength range that is determined by the material used in the photocathode. Figure 3 displays the region of sensitivity for the PMT Model R928 by Hamamatsu. The PMT can be utilized in the region from about 230 nm to about 830 nm. It is apparent that even within the operational wavelength region, the sensitivity is not the same; the non-linearity of the sensitivity introduces an artifact in the data such that a correction to the data has to be introduced.

A spectrofluorometer includes other optical elements such as lenses and mirrors; moreover polarizers are utilized for anisotropy measurements. The operational region of the instrument is given by the superposition of the wavelength transmission of the various elements of the instruments. Even within this region, the variation in transmission has to be taken into account when measuring the fluorescence parameters. The procedures will be outlined in the measurements sections below.
Figure 4 displays the technical diagram of the K2 Multifrequency Phase Fluorometer made by ISS, an instrument capable of measuring all of the relevant fluorescence parameters.
The standard light source is a 300 W xenon arc lamp. Continuous wave (cw) lasers, pulsed lasers (including the multi-photon laser) and light emitting diodes (LEDs) can be coupled to the K2 as well; typically these sources are utilized for the measurement of the decay times of fluorescence.

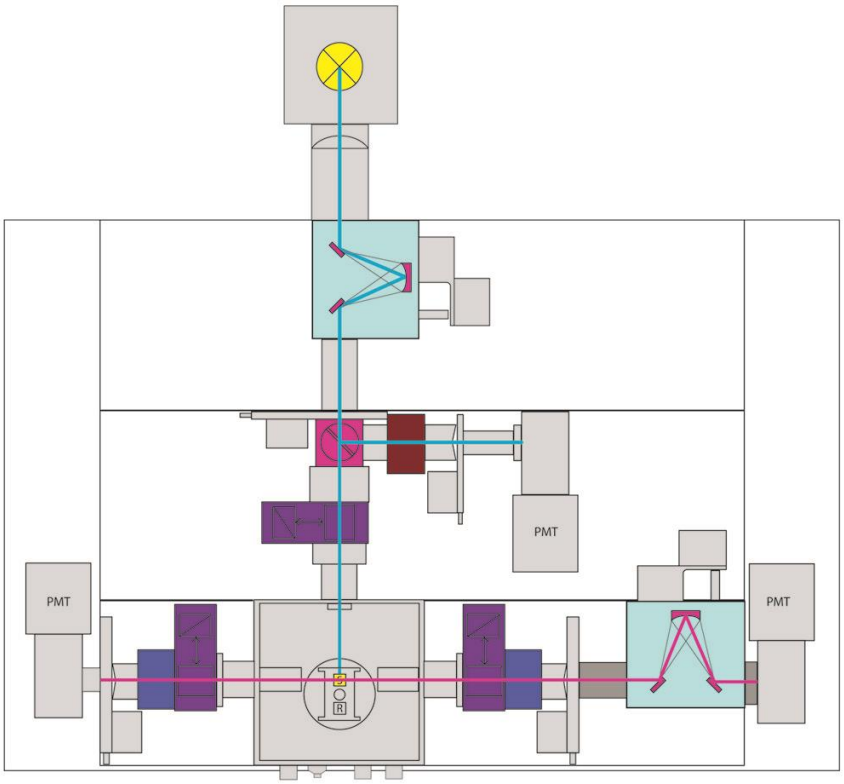

Figure 4. PC1 Photon Counting Spectrofluorometer *(courtesy of ISS)*

The light emitted by the source travels through the excitation channel that comprises the monochromator, a filter holder and the polarizer holder; the monochromator selects the wavelength of the light that excites the sample. The fluorescence emitted by the sample is collected through the left or the right channels; the right channel includes the emission monochromator.

The instrument includes polarizers' holders, filters holders, shutters for blocking the light from reaching the sample and the detectors. All of these components are required for automated measurement acquisition.

5.4 Instrumentation for steady-state fluorescence: analog and photon counting

Two general schemes are utilized to process the signal collected by the PMT: in one scheme, named *analog detection*, the signal from the PMT goes through a current-to-voltage converter, an amplifier and, finally, it is digitized by and analog-to-digital converter. The signal is then displayed on, and/or stored in, the computer.

In another scheme, named *photon counting detection*, the signal from the PMT goes through an amplifier discriminator that allows for the selection of pulses over a set threshold. A counter in the processing unit counts the number of photons collected per seconds by the detector. This parameter is then displayed by the software on, or stored in, the computer.

Although the advantage of analog detection is in the capability of processing signals within a high dynamic range and fast response, its overall sensitivity is lower than the sensitivity of photon counting detection. Ultimately the choice of one scheme over the other depends upon the specific application.

5.5 The Measurement of decay times: Frequency-domain and time-domain techniques

The instrumentation for the measurement of fluorescence decays times is broadly classified as belonging to one of two groups, time-domain and frequency-domain techniques.

The time-domain technique includes the single photon counting, the multiscaler and the time correlated single photon counting (TCSPC); the TCSPC is usually the technique utilized more often. The frequency domain technique comes in an *analog* version (AFD) and a *digital* version (DFD) that has just been introduced.

In TCSPC, a photon is counted within a set time period with a high precision. The time period is defined by the intervals between the pulses of the excitation light (repetition rate of the light source) and the precision is given by the acquisition electronics (mainly the time-to-amplitude converter (TAC) and the analogue-to-digital converter (ADC) components). For instance, when using an excitation light, emitting pulses at 80 MHz, the time period is the distance between two such pulses (12.5 ns). Typically, the repetition rate of some light sources can be set by the user.

At the arrival of each pulse on the light detector, a high precision timer is triggered which records how much time has passed between the arrival of the excitation pulse and the emitted photon.
The TAC unit produces a signal, proportional to the arrival time of the photon, different arrival times records are grouped in different memory locations (bins) of computer memory.
To interpret the lifetime time information obtained by a TCSPC instrument a histogram of the arrival times records is built. For a single exponential decay, a curve similar to the one of Equation [5] is collected and the decay time τ is determined using a minimization technique to fit the experimental data to the theoretical decay model.

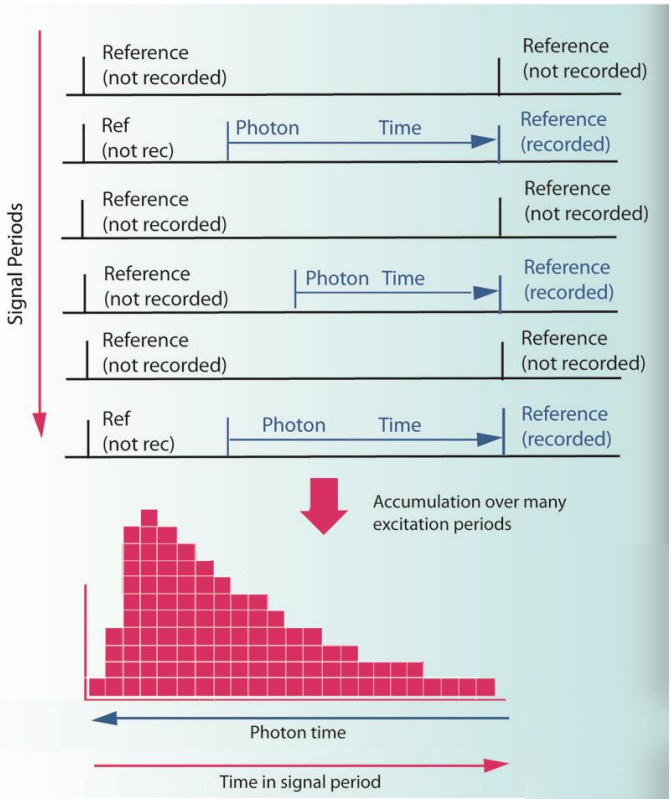

Figure 5. Principle of Start-Stop mechanism utilized in TCSPC data acquisition.

The frequency domain technique is more versatile as it can perform either with pulsed sources used for TCSPC or with the modulation of the excitation light source: the modulated excitation results in a modulated fluorescence with a phase and modulation which is dependent on the lifetime of the excited fluorophores.
The instruments utilized in frequency domain technique are called multifrequency phase fluorometers (MPF) or, simply, frequency domain fluorometers. The underlying operational principle of a MPF is illustrated by Figure 6 for a continuous wave source. The excitation light $E(t)$ is modulated at a frequency ω; its modulation is characterized by an alternating component AC_{EX} and an average

component DC_{EX}. The fluorescence light is modulated at the same frequency ω, but its phase is delayed by the quantity ϕ and the overall modulation $\left(AC/DC\right)_{EM}$ is less than the original modulation of the excitation light. A frequency-domain instrument measures the phase shift ϕ and the demodulation m of the fluorescence; both quantities are related to the decay time (see equations [7, 8]. For a single exponential decay, the decay time is related to the phase angle and to the modulation by the following relations:

$$\tau_P = \frac{1}{\omega}\tan\phi \qquad [68.8]$$

$$\tau_M = \frac{1}{\omega}\sqrt{\frac{1}{m^2}-1} \qquad [68.9]$$

Such measurements are repeated at several different values of the modulation frequency, ω ranging typically from two or three for a single exponential decay, to up to twenty-twenty five for multiple exponential decays. The decay times τ_i are determined using a minimization technique to fit the experimental data.

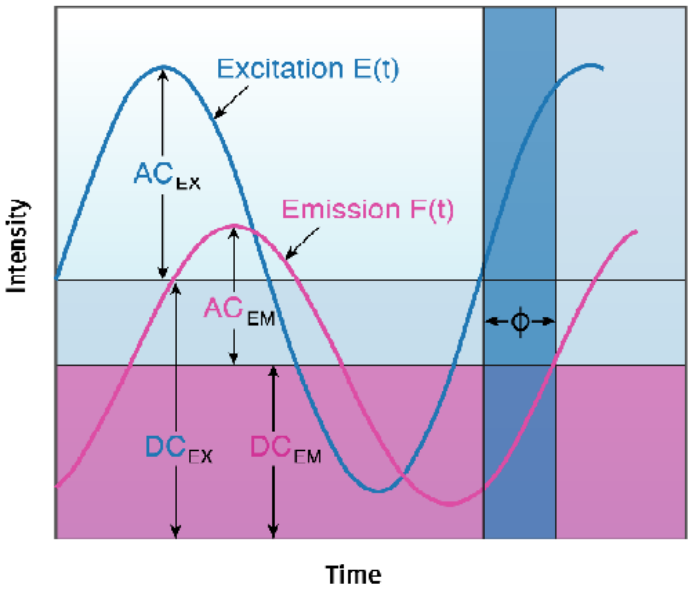

Figure 6. Schematics of the excitation and emission light in frequency-domain spectroscopy; the emission light is phase-shifted and demodulated with respect to the excitation light.

The first modern frequency-domain instrument has been introduced by Spencer and Weber in 1969. In this instrument the light source is modulated at a frequency ω and the light detector is modulated at a frequency $(\omega+\Delta\omega)$; the two frequencies being provided by phase-locked frequency synthesizers. The approach is also known as "heterodyning". The output signal includes components at the sum (2ω) and the difference ($\Delta\omega$) frequency; the low signal component $\Delta\omega$, called the "cross-correlation frequency", which is typically in the range from 1 Hz to 20 KHz, is utilized to determine the phase shift and the demodulation of the fluorescence. From the phase and modulation of the $\Delta\omega$ frequency, the phase and the modulation of the fluorescence can be determined relative to that of a reference lifetime.

6 Fluorophores

Generally fluorophores are divided into *intrinsic* and *extrinsic*. Intrinsic fluorophores are the natural components of a system (typically biological macromolecule) that exhibit fluorescence that can be measured; for instance the aromatic amino acids tyrosine, tryptophan and phenylalanine of the proteins, NADH, the flavins, the porphyrins-based compounds such as chlorophylls. Extrinsic probes include all those molecules that are foreign to the system or were added to it artificially (fluorescent probes and labels – organic dyes, quantum dots or biological fluorophores), such as fluorescein, ANS (1,8-anilinonaphthalene sulfonic acid), which are introduced by the experimenter. Such molecules can be covalently linked to the molecule under study or non-covalently as is the case for DPH (diphenylexatriene), used to study membranes.

7 Measurements

7.1 Excitation spectrum

The excitation spectrum displays the emission intensity distribution at one wavelength while scanning the excitation wavelength over a range. Practically, for the acquisition of the excitation spectrum, the emission monochromator of the spectrofluorometer is set at a fixed wavelength (in the sample emission range) and the excitation monochromator is scanned over a range of wavelengths (the range that corresponds to the sample absorption range). Referring to the Jablońsky-Perrin diagram of Figure 1, when acquiring the excitation spectrum one detects photons emitted by the molecules at a set wavelength (represented by one of the green lines), while scanning the wavelength of the radiation (energy of photons) sent to the sample from high energy to low energy (blue lines).

If there are no changes occur to the molecule in the excited state, then the excitation spectrum closely resembles the absorption spectrum acquired with a spectrophotometer, yet, in most instances, it does not: in order for the two to match, a suitable correction of the instrumental factors has to be applied. The main

culprit of the differences is due to the lamp; it features a peculiar emission spectrum of its own, that is, the intensity of the emitted radiation is not constant at all the wavelengths. In order to correct for this effect, a small fraction of the excitation light is diverted in the Reference channel of the spectrofluorometer (Figure 2) where it passes through the quantum counter and it is collected by the reference detector. The quantum counter, usually a stable fluorophore at a high concentration in solution, delivers a number of photons proportional to the absorbed signal; therefore, at each wavelength, we have a signal proportional to the signal emitted by the lamp; this signal is utilized to correct the fluorescence signal collected in the emission channel. Although this correction addresses most of the concerns, it does not completely correct the excitation spectrum as the beam splitter utilized to divert part of the excitation light into the reference channel reflects differently the two planes of polarization. For a full correction to be implemented, one should place a cuvette with a scattering solution in the sample compartment and acquire an emission spectrum over the wavelength range of interest; then acquire the emission spectrum of the fluorophore and divide it by the spectrum of the scatterer. In this way, the excitation spectrum is fully corrected. Practically, the correction introduced by using the quantum counter and the reference channel is sufficient; one should nonetheless specify the experimental conditions when publishing the spectrum.

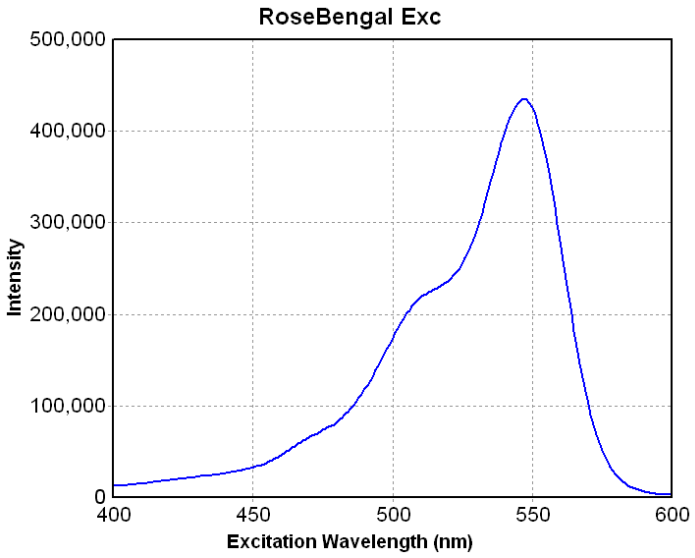

Figure 7. Excitation spectrum of Rose Bengal in a water solution, acquired using the PC1 Photon Counting Spectrofluorometer (*courtesy of ISS*). The spectrum was acquired by scanning the excitation monochromator from 400 nm to 600 nm in steps of 1 nm; at each position data were acquired for 1 second. The fluorescence was observed at 610 nm.

7.2 Emission spectrum

The emission spectrum of a fluorophore is most likely the most popular experimental measurement carried out in fluorescence. The spectrum is acquired by setting the excitation wavelength at a fixed value (one of the blue lines of Figure 1) and then by scanning the emission monochromator over a range of emission wavelengths (the green lines of Figure 4).

There are a few general rules that apply to emission spectra:

1. The emission of fluorescence occurs at wavelengths longer than the excitation wavelength (Stokes shift).
2. The shape of the emission spectrum does not change by changing the excitation wavelength.
3. The emission spectrum is a mirror image of the excitation spectrum of lower energy.

An examination of Figure 1 explains as to why the first rule holds. When the molecules are excited, they relax to the lowest vibrational level of the excited states and, from there, they emit fluorescence. Fluorescence photons have a lower energy than excitation photons (that is the fluorescence occurs at longer wavelengths than the excitation). Hence, we also gather that the shape of the emission spectrum does not change by changing the excitation wavelength. Finally, rule 3 establishes that the emission spectrum ($S_1 \rightarrow S_0$ transition) is a mirror image of the absorption transition involving the same levels ($S_0 \rightarrow S_1$ transition). If the excitation spectrum includes transitions to higher levels, the emission spectrum will not be a mirror image of the excitation. There are exceptions to the mirror image rule: for instance when *p*-terphenyl is excited the nuclei undergo a geometric rearrangement upon absorption and the emission spectrum shows the additional vibrational structure. Excited-states reactions can also result in emission spectra that mark a departure from the mirror rule; and so the formation of complexes (for instance Pyrene).

As for the excitation spectrum, the emission spectrum is affected by experimental artifacts, namely, the transmission of the emission monochromator and the sensitivity of the light detector: The transmission of the monochromator varies with the wavelengths and, moreover, it features different transmission for the two planes of polarization of the light (see below for the definition of light polarization); the sensitivity of the light detector varies with the wavelength. All these variation have to be accounted for in order to acquire a "true' emission spectrum. To this respect, one distinguishes between technical spectrum (the spectrum acquired by an instrument) and the corrected spectrum (the technical spectrum that has been corrected for the experimental artifacts). Manufacturers typically provide correction files for an instrument; these factors are embedded in the software and corrected spectra can be acquired on line; or spectra can be corrected afterwards. Practically, one does not need to correct a spectrum unless it is meant for publications; even in that event, it is completely acceptable to specify that the spectrum is a *technical* spectrum rather than a *corrected* one. There are some instances when corrected spectra are required; when calculating the quantum yield of a fluorophore one has to calculate the area under the spectrum; the spectrum has to be corrected for providing the proper value. Another instance occurs when using the Förster Resonance Energy Transfer (FRET), a useful tool for estimating the distances between two interacting and close fluorophores.

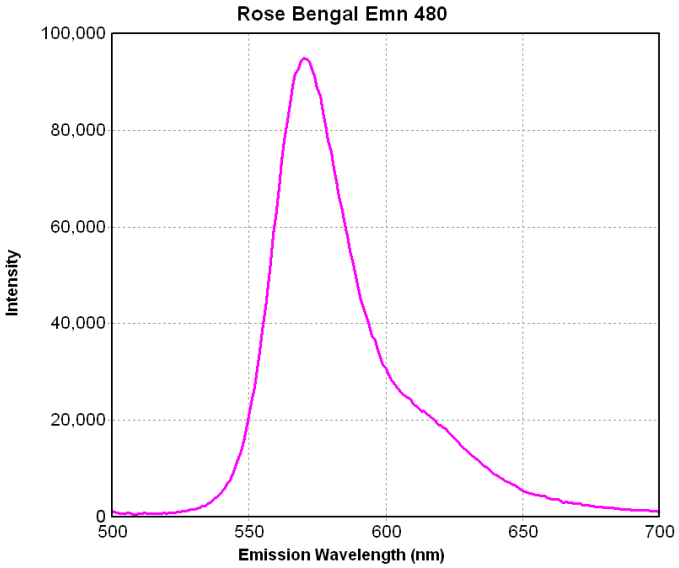

Figure 8. Emission spectrum of Rose Bengal water solution, acquired using the PC1 Photon Counting Spectrofluorometer (*courtesy of ISS*). The excitation monochromator was set at 490 nm. The emission spectrum was acquired by scanning the emission monochromator from 500 nm to 700 nm in steps of 1 nm; at each position data were acquired for 1 second.

Besides the instrumental artifacts, the emission spectra are sometimes distorted by experimental artifacts that a practitioner of the field needs to be aware of, namely:

1. Background fluorescence
2. The second order of the monochromator
3. The Raman spectrum of water

Background fluorescence occurs when the fluorophore is diluted in a solution and the solvent (for example, buffer) emits some fluorescence of its own at the emission wavelength utilized in the experiment; the resulting emission spectrum is the superposition of the individual spectra of the solvent and the fluorophore. In this case, one can acquire the emission spectrum of the solvent alone and subtract it from the emission spectrum of the solution in order to obtain the emission spectrum of the fluorophore. We mentioned about the second order in the paragraph covering the monochromators: when a monochromator is set to deliver radiation at wavelength λ, it also delivers radiation at 2λ (second order); although the intensity is about 1/10 of the intensity of the first order, it is sufficient to introduce distortions when measuring turbid solutions and solid samples. The second order can be eliminated with a judicial selection of filters.

Finally, when working with water as a solution, the Raman peaks are present at a wavelength that is 3,400 cm^{-1} longer than the excitation wavelength:

$$\lambda_{Ex}^{-1} - \lambda_{R}^{-1} = 3,400\ cm^{-1} \qquad [68.10]$$

As an example, when exciting at 300 nm an emission peak appears at 334 nm; when exciting at 350 nm, an emission peak appears at 397 nm. Note that, while the position of the peak is fixed in unit of wavenumbers ($1/\lambda$), the position varies when dealing in wavelengths (λ); the change in the peak position with the change of the excitation wavelength allows for the user to discern the peak from other peaks or artifacts. The intensity of the Raman peak provides a simple tool to verify the status of the light source of the spectrofluorometer; measured periodically, one can have a pretty good idea of the derating of the xenon arc lamp and make a decision as to when replace the lamp.

7.3 Decay times of fluorescence

The fact that the decay times of many fluorophores are in the range of 1 -30 ns is truly amazing as this time scale is typical of molecular interactions in biological systems (enzyme conformational shifts, rotational motions in proteins, photosynthetic reactions, etc.) in physiologically active systems.

The decay time is affected by many parameters of the microenvironment (temperature, ions, polarity, viscosity, electric fields) and this is the reason it is widely utilized for studying molecular interactions. For instance, the decay time of ANS in water is about 100 ps; when ANS is bound to a protein the lifetime is 8-10 ns. The lifetime of ethidium bromide is 1.8ns in water; it is 22 ns when bound to DNA and 37 ns when bound to tRNA.

Finally, the lifetimes can be used an analytical tool as well for the characterization of the presence of specific dyes or simply for the quantitation of complex fluorescent mixtures (the type of crude oil provided by a well, the dye in a hair spray or a soap, the production process of paper, counterfeiting of banknotes and of drugs, etc.).

Back in 1962, Strickler and Berg published a relation to estimate *a priori* the excited state lifetime of a fluorescent molecule. Yet, its usefulness is limited because of the variation of lifetimes due to the experimental conditions. That is, the best way to know the lifetime of a fluorophores if to measure it directly.

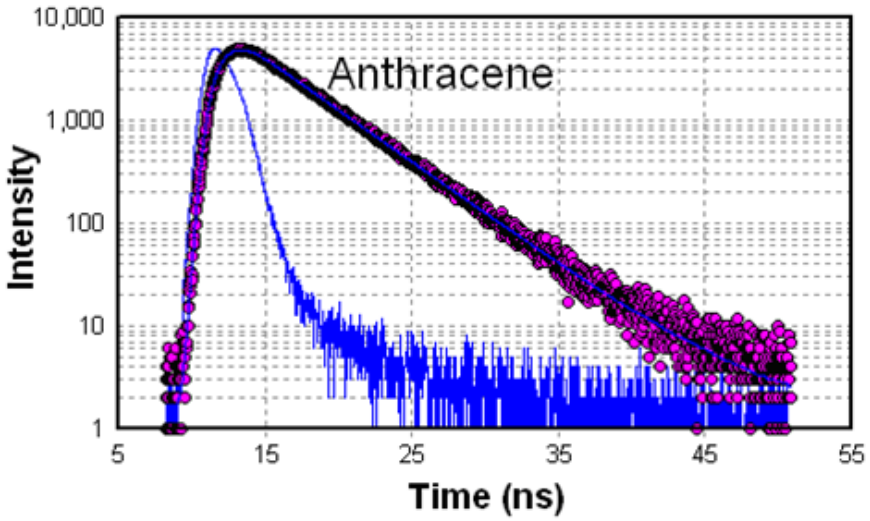

Figure 9. Decay curve of Anthracene in ETOH using a TCSPC instrument (ChronosBH, by ISS).

Figure 9 displays the decay time of Anthracene in ETOH using the ChronosBH, a TCSPC instrument, by ISS. The light source is a pulsed LED emitting at 335 nm. A high pass filter (WG 385, 50% transmission at 385 nm) was used to separate the fluorescence. A single lifetime of 4.2 ns was determined using the fitting routine of the software.

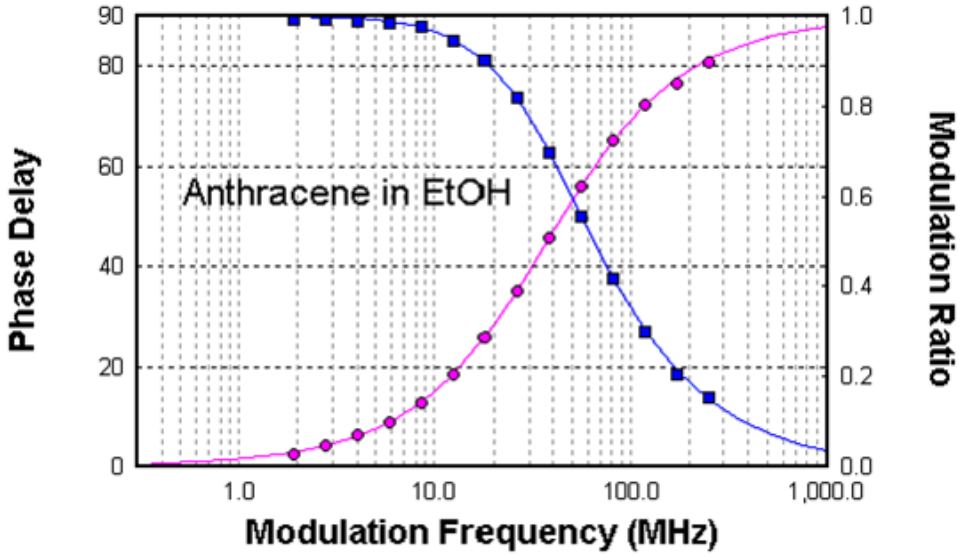

Figure 10. Decay curve of Anthracene in ETOH using a frequency-domain instrument (ChronosFD, by ISS).

Figure 10 displays the decay time of Anthracene in ETOH using the ChronosFD, a frequency-domain instrument. Phase and modulation data were acquired at fourteen different modulation frequencies ranging from 2 MHz to about 250 MHz. The light source is a pulsed LED emitting at 370 nm. A high pass filter (WG 389, 50% transmission at 385 nm) was used to separate the fluorescence. A single

lifetime of 4.2 ns was determined using the fitting routine of the software. In both techniques the decay times are recovered by using a fitting algorithm (least square analysis); the algorithm the theoretical functions that best minimize the differences with the experimental points. Other approaches are available for the data analysis, such as the maximum entropy method (MEM) and the phasor analysis.

7.4 Quantum yield

The quantum yield is a parameter that varies widely from molecule to molecule. A few examples are reported in Table I below. Clearly, when looking for a fluorescent probes there are advantages in selecting one featuring a high quantum yield!

molecule	wavelength range (nm)	Temperature (ºC)	solvent	Quantum yield
Benzene	270-300	20	ethanol	0.04
Anthracene	360-480	20	ethanol	0.27
Tryptophan	300-380	25	H_2O	0.14
Rhodamine 101	600-650	20	ethanol	1.0

Table I. Quantum yield values of selected molecules

We refer the reader to the literature listed in Further References for the measurement of the quantum yield. We only recollect that there is a direct mode and a relative mode. The direct mode encompasses the use of the integrating sphere, an accessory of the spectrofluorometer that allows for the determination of the number of photons emitted by a sample. The relative mode allows for the determination of the quantum yield of a sample by comparison to a reference of known quantum yield. Both measurements require particular attention to the details.

7.5 Anisotropy and polarization

Anisotropy (or polarization) is a popular application of fluorescence spectroscopy as it allows for the measurement of the rotation of molecules as well as of their shape and size and the rigidity of molecular structures.

A light beam is described as an electromagnetic wave with an electric vector $\vec{E}$ and a magnetic vector $\vec{B}$ perpendicular between them; both are also perpendicular to the direction of propagation of the light beam $\vec{k}$. Natural light can be described as the superposition of innumerable such single wave representations.

When working with natural light a particular direction of the electric vector $\vec{E}$ can be selected by using a polarizer; such wave is said to be "polarized" (Figure 11.).

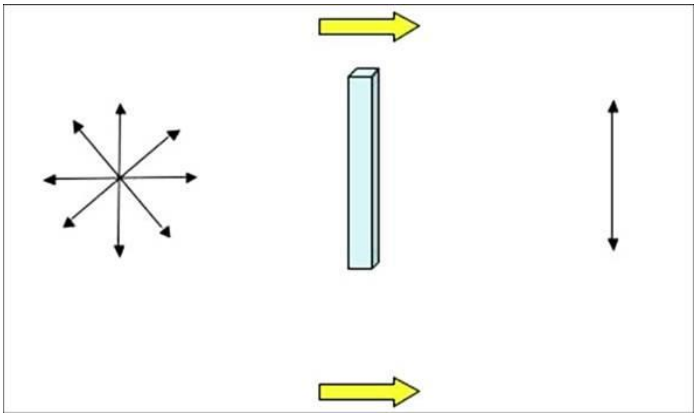

Figure 11. An unpolarized light beam traverses a polarizer; a plane of polarization is selected.

Polarized light can be utilized for interesting experiments and applications. When polarized light with the proper energy illuminates an ensemble of molecules (Figure 12) only molecules with the excited state dipole moment $\vec{M}_A$ (or transition moment) oriented in the same direction of the electrical field (polarization) can absorb the photons.

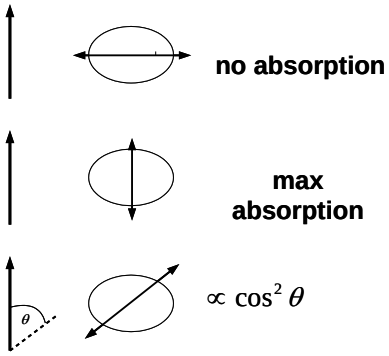

Figure 12. Molecules with the electric dipole featuring a component parallel to the direction of the electric filed of the excitation light have a probability for absorption of a photon.

If the direction of polarization of the excited beam and the direction of the dipole moment of the molecule are perpendicular to each other, no absorption takes place. In intermediate cases, the probability of the absorption is proportional to $\cos^2\theta$, where θ is the angle between the vector $\vec{E}$ of the exciting light and the vector $\vec{M}$ of the transition moment dipole (Figure 12).

Because of the preferential absorption rules of the molecules, a polarized light introduces a *photoselection* of the molecules. As the distribution of the excited fluorophores is anisotropic, the fluorescence is anisotropic too. Any change in the direction of the transition moment $\vec{M}_A$ during the time the molecule spend in the excited level will result in a decrease of the anisotropy, that is the overall polarization of the fluorophores solution will decrease. The decrease in the anisotropy can be due to several reasons:

- Difference in direction between the absorption and emission transition moments. This happens as the transitions moments of the excited states S_1 and S_2 may not be the same; yet, molecules emit from the lowest vibrational level of S_1.

- Brownian motion. Molecules in the excited state enter into collisions with the molecules of the solvent or with molecules of the same species and, as a result, the direction of the emission transition moment changes.

- Energy transfer to another molecule featuring a different orientation.

Anisotropy is measured using a spectrofluorometer equipped with polarizers; one polarizer is mounted in the excitation beam (Figure 2) and a second polarizer is inserted in the emission channel. The anisotropy is defined as:

$$r = \frac{I_{VV} - gI_{VH}}{I_{VV} + 2gI_{VH}} \qquad [68.11]$$

And the polarization is

$$P = \frac{I_{VV} - gI_{VH}}{I_{VV} + gI_{VH}} \qquad [68.12]$$

The two parameters, anisotropy and polarization, describe the same phenomenon; they are related to each other by

$$P = \frac{3r}{r + 2} \qquad [68.13]$$

(In the following description we will refer to anisotropy only). In the relations above, I_{VV} is the measured fluorescence intensity with the polarizer in the excitation channel in the (V)ertical position and the polarizer in the emission channel in the (V)ertical position; I_{VH} is the measured fluorescence intensity with the polarizer in the excitation channel in the (V)ertical position and the polarizer in the emission channel in the (H)orizontal position.

The number *g*, called the *g-factor*, is given by $g = \frac{I_{HV}}{I_{HH}}$, where the letters *V* and *H* refer to the positions of the polarizers in the excitation and emission channel, respectively. The *g-factor* corrects the anisotropy values for the artifact introduced by the instrument; as is the case for emission spectra, the instrument has different transmission properties for the two planes of polarization.

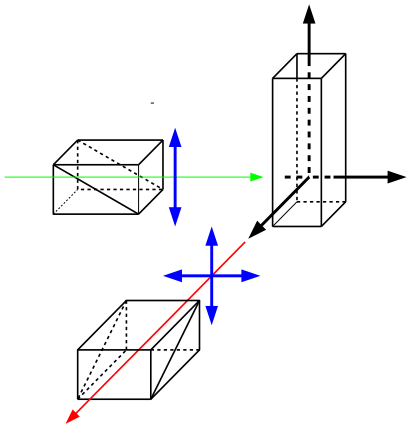

Figure 13. Experimental setup for anisotropy measurements. The spectrofluorometer has a polarizer in the excitation channel and a second polarizer in the emission channel. The intensity of the fluorescence reaching the light detector is measured for different orientation of the polarizers (see relation [9]).

Figure 14 displays the polarization values for Erythrosine in water along with the excitation spectrum; the fluorescence is collected at 550nm. The polarization is negative for wavelengths below 360 nm and then rises sharply up to 400 nm and stays almost constant above 400 nm. The reason for this behavior is due to the fact that the excitation at the short wavelengths favors the transition $S_0 \rightarrow S_2$, while at the longer wavelengths the transition $S_0 \rightarrow S_1$ is the one excited: as the fluorescence is always emitted by the lowest vibrational level of S_1, it is an indication of the different orientation of the transition moments of the excited levels S_1 and S_2. Practically, when using anisotropy measurements one has to select and specify the excitation wavelength (and chose a wavelength displaying a high value of polarization).

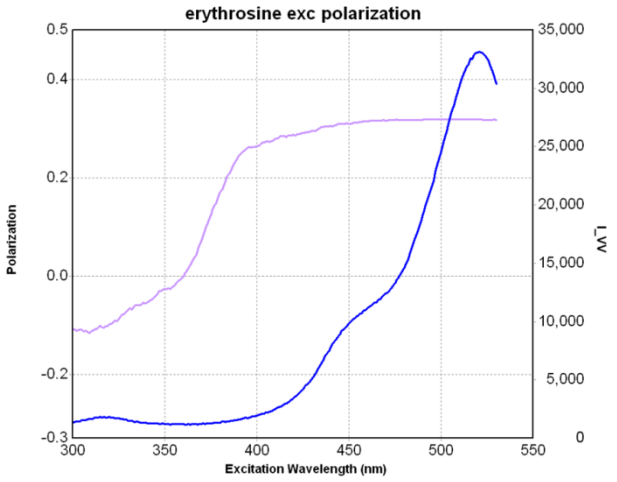

Figure 14. Excitation polarization spectrum for erythosine (purple line) in water; the excitation spectrum is represented (blue line). Fluorescence is collected at 550nm.

What are the values that the anisotropy can assume? In order to answer this question one has to introduce the emission transition moment $\vec{M}_E$ and distinguish the two cases:

(a.) $\vec{M}_E$ and $\vec{M}_A$ are parallel; and

(b.) $\vec{M}_E$ and $\vec{M}_A$ are not parallel.

Without going into the details of the calculations (the interested reader can consult one of book by Valeur cited in the References), we note that for the case of the two moments being parallel and in absence of any motion, it is $r_0 = 0.4$; this value is called the *fundamental anisotropy*. When the two moments are not parallel the values are confined in the range:

$$-0.2 \le r_0 \le 0.4 \qquad [68.14]$$

The case of the decrease of anisotropy due to Brownian motion collisions is very interesting one for its practical applications. This is the case when molecules in the excited state rotate due to collisions with the solvent. The amount of the depolarization depends upon the value of the decay time of the molecule, the size of the molecule, the viscosity and temperature of the solvent. In fact, let us suppose that the decay time is of the same order of the rotational time; it is found that the anisotropy decays, for a spherical molecule, according to the following relation:

$$r(t) = r_0 \exp(-6D_r t) \qquad [68.15]$$

where D_r is the rotation diffusion coefficient. From the Stokes-Einstein relation $D_r = \frac{RT}{6V\eta}$, where V is the hydrodynamic volume of the molecule, η is the solvent viscosity, R is the gas constant and T the absolute temperature. D_r can be determined by resolving equation [13] using time-resolved fluorescence techniques. Alternatively, if the decay is a single exponential decay, it can be solved using steady-state technique. As:

$$\bar{r} = \frac{1}{\tau}\int_0^\infty r(t) \exp(-t/\tau)\, dt \qquad [68.16]$$

By direct substitution one finds

$$\frac{1}{\bar{r}} = \frac{1}{r_0}(1 + 6D_r\tau) \qquad [68.17]$$

This is the Perrin equation; it allows for the evaluation of the decay times by measurements of the steady-state polarization! In some literature, the quantity $\tau_C = 1/6D_r$, called the rotational correlation time, is used. This case is strictly valid for a spherical molecule. When the more complex shape of a general ellipsoid is considered, the motion is described by three rotational diffusion coefficients associated with each of the rotational axis. The relation between the rotational correlation times and the rotational diffusion coefficients is no longer simple. The anisotropy decay is described by:

$$r(t) = \beta_1 e^{-t(4D_1+2D_2)} + \beta_2 e^{-t(D_1+5D_2)} + \beta_3 e^{-t(6D_2)} \qquad [68.18]$$

where

$$\tau_1 = \frac{1}{(4D_1 + 2D_2)}$$
$$\tau_2 = \frac{1}{(D_1 + 5D_2)} \qquad [68.19]$$
$$\tau_3 = \frac{1}{(6D_2)}$$

In this expression the quantities β_1, β_2, β_3 represent expressions for the angles between the absorption and emission dipoles and the axes of the ellipsoid; D_1 and D_2 are the diffusion coefficients around the axis of symmetry and equatorial axes respectively.

There are physical conditions where a probe is restricted to motion within an angle; for instance the case of a probe in a membrane. In these cases, the anisotropy does not decay to zero. A hindered rotator is described by the following expression

$$r(t) = \left(r_0 - r_\infty\right)\exp\left(-t/\tau_c\right) + r_\infty \qquad [68.20]$$

Table II below lists a few applications of the technique that spans from the physical-chemistry research all the way to clinical applications.

spectroscopy	Separation of excited states
polymers	Local viscosity Molecular orientation Chain dynamics
immunology	Antigen-antibody reactions Immunoassays
molecular biology	Proteins interactions Nucleic acids-proteins interactions Biological membranes Micellar systems

Table II. Selected applications of anisotropy measurements

8 Conclusions

Fluorescence is a sensitive technique that, although started as an analytical tool, is used more and more for the study of molecular interactions *in-vitro* and in cells; in fact, it is nowadays capable of detection of single molecules on a routine basis. The fluorescence decay time of typical fluorophores falls in a window (1 -20 ns) suitable for the observation of several molecular processes of biological relevance. The spectral properties of fluorophores are changed by several processes including collisions with other molecules, rotational diffusion, and formation of complexes; moreover, the fluorescence properties are sensitive to changes of the environment such as pH, electrical fields, concentration, temperature, polarity. These features have expanded the applications of fluorescence to fields as diverse as the development of sensors for monitoring the presence of specific analytes (O2, ions) *in-vitro* and *in-situ*; to the development of sensors for the measure of physical parameters (materials under high pressure, mechanical properties of materials). A variety of research instruments is available for the measurement of the general and specific parameters of the fluorescence. Dedicated instruments are utilized for the measurements in specific immunoassays (polarimeters), in drug discovery (microwell plates and microarrays), cell sorting (cytofluorometers), genome sequencing.

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