LABORATORY OF ELEMENTARY BIOPHYSICS

Experimental exercises for III year of the First cycle studies

Field: „Applications of physics in biology and medicine"

Specialization: „Molecular Biophysics"

Fluorescence decay kinetics measurements of typical fluorescence dyes (PB16)

Projekt *Fizyka wobec wyzwań XXI wieku* współfinansowany ze środków Unii Europejskiej w ramach Europejskiego Funduszu Społecznego

I. Introduction

The purpose of the exercise is to familiarize the student with the fundamentals of fluorescence decay measurements. You will measure the fluorescence lifetime of typical fluorescence dyes or mixtures thereof using the method of Time-Correlated Single Photon Counting (TCSPC).

I.1. Phenomena of fluorescence

The compounds having carbon rings with a conjugated π-bond system can absorb and emit ultraviolet and visible radiation (200-800 nm). Such bond arrangement reduces the energy difference between the π and π* levels and increases the probability of $\pi \rightarrow \pi^*$ and $\pi^* \rightarrow \pi$ transitions. The distribution of electronic states and intensity of absorbed or emitted radiation depends on the molecule structure and microenvironment.

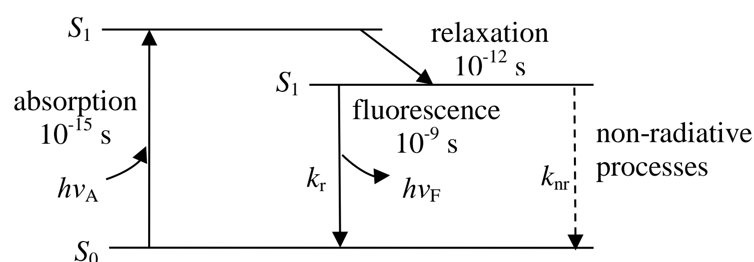


Figure 1. Jabłonski diagram showing the processes of absorption, fluorescence and solvent relaxation. For simplicity only the first singlet excited state S_1 is indicated and neither vibrational nor triplet levels are shown. k_r and k_{nr} are the rate constants for fluorescence emission and non-radiative processes, respectively (for convenience all possible non-radiative decay paths are characterized by the single rate constant k_{nr}).

The processes that occur following light absorption are usually illustrated by the Jablonski diagram (Fig. 1). Excited molecule tends to return to the lowest vibrational level of the singlet ground electronic state S_0. From higher vibrational level of either S_1 or S_2 molecule rapidly relaxes to the lowest vibrational level of S_1. At the same time, due to a different charge distribution in the excited state relative to the ground state, solvent molecules reorient around the excited molecule (solvent relaxation). It leads to lower the energy of the excited state. The time scale of this non-radiative processes is about 10^{-13}-10^{-12} seconds.

There are three possible ways to return to the ground state from the lowest vibrational level of S_1: (a) radiative transitions to various vibrational levels of S_o i.e. **fluorescence** emission (time scale 10^{-10}-10^{-8} s); (b) the non-radiative transitions to high vibrational levels of S_0 and fast relaxation to the lowest vibrational level; (c) non-radiative transitions to the first triplet state T_1 (intersystem crossing), which, despite the small difference in S_1 and T_1 energies

are very slow, as they are accompanied by forbidden spin conversion. Emission from T_1 is termed **phosphorescence** (the process not shown in Fig.1). Examination of the Figure 1 reveals that fluorescence occurs at longer wavelengths than that of absorption. This phenomenon is called the Stokes shift.

Not every absorption act leads to the emission of a photon. This fact is described by quantity called fluorescence quantum yield (Q). The quantum yield is defined as the ratio of the number of photons emitted to the number of photons absorbed. Both emissive and non-radiative processes depopulate the excited state. The fraction of fluorophores that decay through emission, and hence the quantum yield, is given by:

$$Q = \frac{n_F}{n_A} = \frac{k_r}{k_r + k_{nr}} \tag{1}$$

where k_r and k_{nr} are the rate constants for fluorescence emission and non-radiative processes, respectively.

After excitation with short puls of light, the initial population of fluorophores in the excited (N_0) state decays with a rate $k_r + k_{nr}$ according to:

$$\frac{dN(t)}{dt} = -(k_r + k_{nr})N(t) \tag{2}$$

where $N(t)$ is the number of excited molecules at time t following excitation.
The equation (2) implicates an exponential decay of the excited state population:

$$N(t) = N_0 \exp(-(k_r + k_{nr})t) = N_0 \exp(-t/\tau) \tag{3}$$

where τ equal the inverse of the total decay rate, $\tau = (k_r + k_{nr})^{-1}$, is called the lifetime of the excited state. It is the average time the molecule spends in the excited state prior to return to the ground state.

In a fluorescence experiment we do not observe the number of excited molecules, but rather fluorescence intensity, which is proportional to $N(t)$. Hence, the time-dependent intensity $I(t)$ is given by:

$$I(t) = I_0 \exp(-t/\tau) \tag{4}$$

The equation (4) follows that at t = τ the fluorescence intensity decreases to 1/e of its initial value.

Samples very often display more than one decay time. Among the reasons of multi-exponential or even non-exponential decays are: heterogeneity of fluorophore, the excited state reactions or fluorescence resonance energy transfer (FRET).

The efficiency of radiative and non-radiative deactivation processes strongly depends on various environmental factors (pH, viscosity, polarity, temperature, the presence of

ligands). It makes the time-resolved fluorescence measurements a valuable tool for study of fluorophore microenvironment and the excited state reactions.

I.2. Methods of fluorescence decay measurements

Two methods of measuring time-resolved fluorescence are in widespread use: the time-domain and frequency-domain (also called phase-modulation) methods. In time-domain or pulse fluorometry, the sample is excited with a pulse of light and then the decay of fluorescence signal is observed. In frequency-domain methods sample is excited with intensity-modulated light at a high frequency (typically near 100 MHz). The emission is forced to respond at the same modulation frequency. The lifetime of the fluorophore causes the emission to be delayed in time relative to the excitation. This delay is measured as a phase shift (φ), which can be used to calculate the decay time.

I.3. Time-correlated single photon counting

We will focus on the pulse method, because it will be used during this exercise. At present most of the time-domain measurements are performed using time-correlated single photon counting (TCSPC). The method is based on the precise, repeated registration of time between the excitation pulse and the observed photon. The conditions are adjusted so that less than one photon is detected per laser pulse (typically 1 photon per 100 excitation pulses) (Fig. 2A). The measurements generate a histogram of the number of photons counted at each time interval (Fig. 2B). The histogram represents the time decay, that would be observed when many fluorophores are excited and numerous photons are observed.

Specialized electronics, that act like a stopwatch with picosecond resolution, are used for measuring the time delay between the excitation and emission. A diagram of time-correlated single photon counting system is shown in Figure 3. The excitation pulse that excites the samples, at the same time sends an electrical START signal to the time-to-amplitude-converter (TAC). TAC generates a voltage ramp that is a voltage that increases linearly with time. Photon emitted by the sample, after passing through the monochromator, goes to the photomultiplier tube (PMT). PMT sends a STOP signal to TAC, which stops the voltage increase. In this way, the TAC registers a voltage proportional to the time difference (Δt) between the excitation and emission signals. The voltage is converted to a digital value by the analog-to-digital converter (ADC), that is stored as a single event with the measured time delay. Repeating this procedure time and again times leads to the histogram shown in Figure 2B.

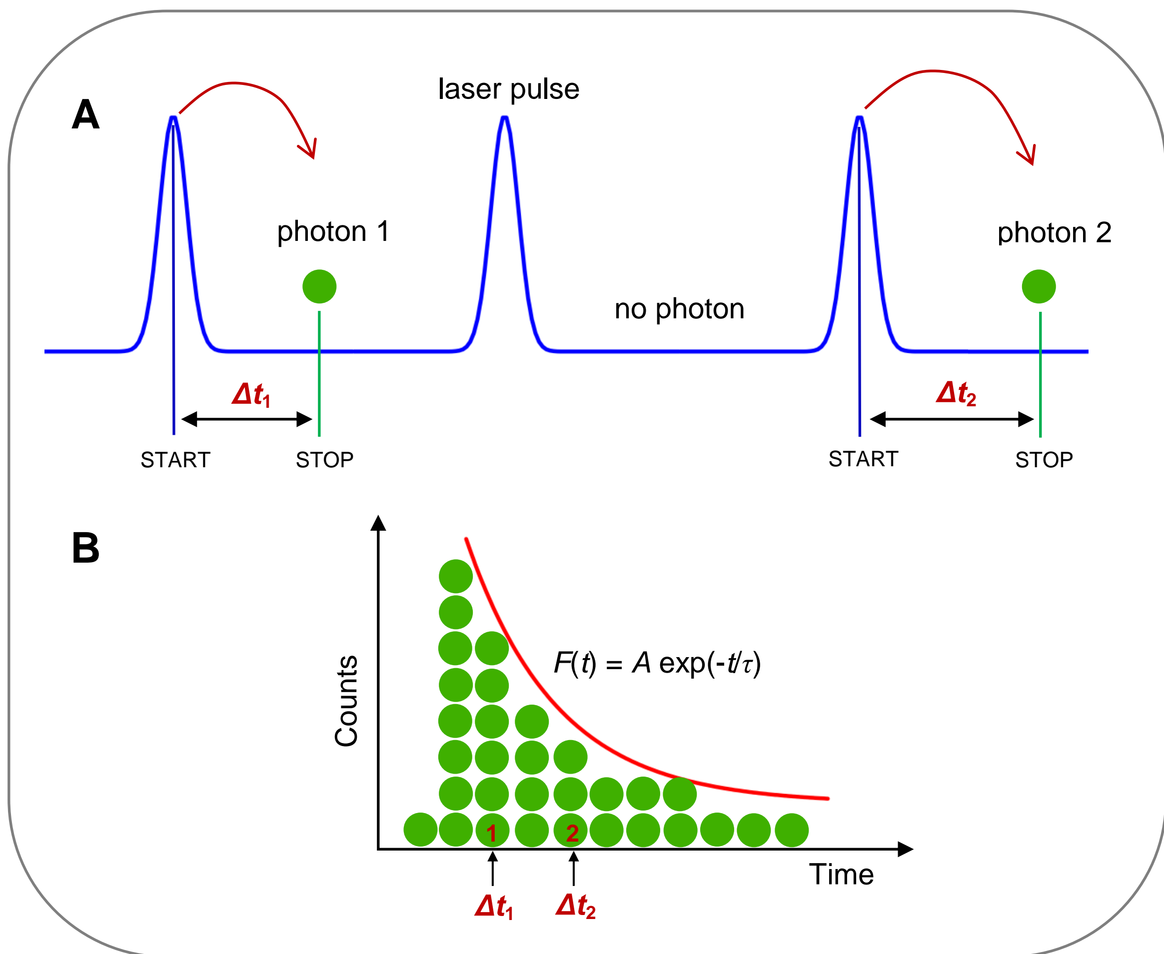


Figure 2. Principle of time-resolved fluorescence measurements with time-correlated single photon counting. (A) Measurement of time between the excitation pulse and photon emission and (B) the histogram of number of photons corresponding to each start-stop time.

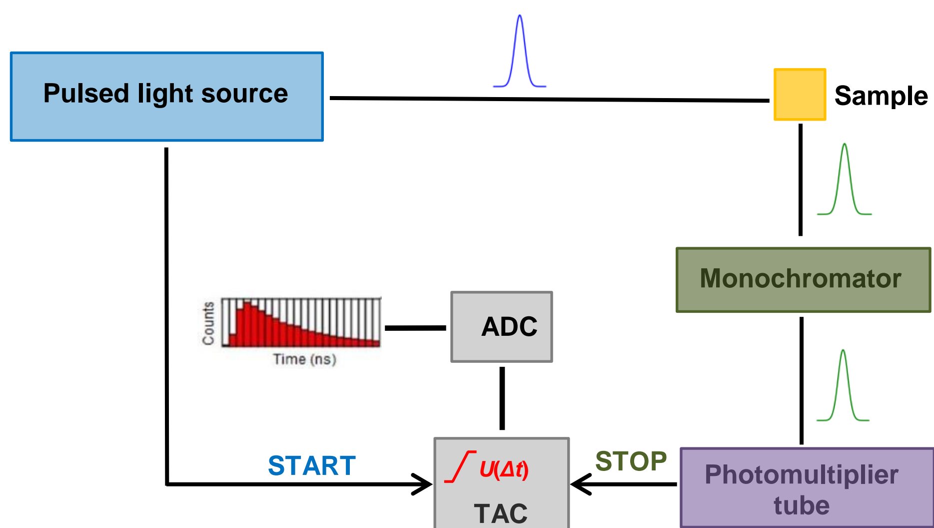


Figure 3. Electronic schematic for TCSPC

For TCPSC measurements it is necessary to maintain a low probability of registering more than one photon per cycle. At present the electronics are not fast enough to measure multiple photons per pulse when the lifetimes are in the nanosecond range. If more than one photon is emitted per excitation pulse, this leads to overestimation of early photons in the histogram and to faster decay (pile-up distortion). To obtain the true decay one has to keep count rate $< 2\ \%$ with respect to the excitation rate. The precondition is achieved by simply attenuating the light level.

I.4. Analysis of a fluorescence decay data

It is important to understand that only the ideal system with an infinitely sharp excitation pulse and infinitely accurate detectors and electronics would measure the true decay curve $I(t)$. In fact, the experimentally measured intensity decay $F(t)$ is a convolution of the instrument response function (IRF) with decay function $I(t)$:

$$F(t) = \int_0^t IRF(t) I(t - \hat{t}) d\hat{t} \tag{5}$$

The way to determine the actual intensity decay curve $I(t)$ that best matches the experimental data is an iterative reconvolution. This numerical analysis requires measuring of IRF. The typical approach is to place a scattering medium in the sample compartment (for example silica solution – Ludox). In Figure 4 are shown the exemplary experimental decay data, the instrument response function IRF and the fitted exponential model. The IRF width depends on the laser pulse width as well as the timing uncertainty of the detector and the TCSPC electronics.

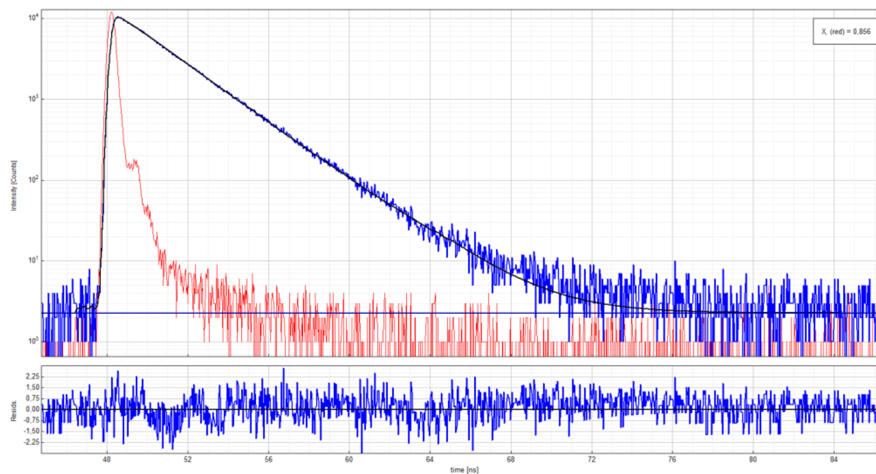


Figure 4. Analysis of a decay data. Top panel shows experimental decay data (blue), IRF (red) and the fitted exponential model (black). Bottom panel displays the weighted residuals.

II. The requirements for entrance test

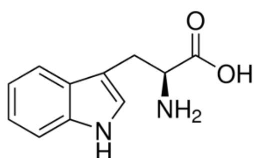
1. The phenomenon of absorption and emission of electromagnetic radiation
2. Fluorescence, fluorescence emission spectrum, fluorescence decay, fluorescence quantum yield and lifetime
3. Methods of fluorescence decay measurements
4. Time-correlated single photon counting, pile-up effect
5. Analysis of a fluorescence decay data

III. Experimental part

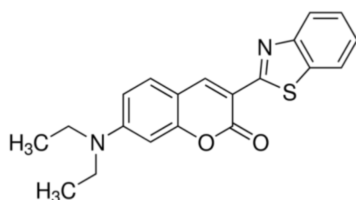
During the exercise you will measure the fluorescence lifetimes of typical fluorescence dyes or mixtures thereof using fluorescence lifetime spectrometer FT300 (PicoQuant) and time-correlated single photon counting method. The fluorimeter is equipped with two types of pulsed light sources with selectable repetition frequencies (up to 40 MHz): (i) laser diode heads with pulse width of ~ 50 ps (available wavelengths 405 and 485 nm) and (ii) the pulsed LEDs with pulse width of ~ 1 ns (wavelengths: 270, 280, 300, 340 and 450 nm).

You will be provided with:

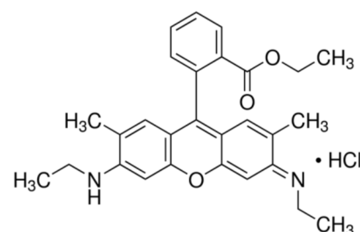
– Ludox scattering solution
– tryptophan in water
– coumarin 6 in ethanol
– rhodamine 6G in ethanol
– mixture of coumarin 6 and rhodamine 6G in ethanol
– a pair of absorption quartz cuvettes of 1 cm optical path length
– fluorescence quartz cuvette of 1 cm optical path length



tryptophan



coumarin 6



rhodamine 6G

1. Register absorption spectra of dyes using a spectrophotometer: for tryptophan in the range of 230-350 nm, and for coumarin 6 and rhodamine 6G in the range of 300-600 nm. Do you see any correlation between the structure of the compound and the maximum position of the absorption spectrum? On the basis of the measured spectra, select appropriate excitation sources for given sample.

2. Measure the fluorescence decays for each sample for several emission wavelengths. Use more than one excitation source if possible. Register the instrument response function (IRF) using Ludox scattering solution. What excitation source and emission wavelength should be selected for IRF measurements?

3. Do an analysis of the acquired data using FluoFit software to determine lifetimes for each sample. Perform IRF reconvolution with exponential decay model (1, 2 or 3 components). Always start from the monoexponential model and check whether adding another component will reduce the $\chi 2$ value and improve the residuals distribution.

IV. Final report

Final report should include all elements typical for the experiment description (abstract, introduction, description of the experimental system, results and discussion).

The results should be shown in tables and figures. The fluorescence decay data should be analysed using FluoFit program.

The following issues should be taken into account:

1. Justification of excitation sources selection on the basis of the measured absorbance spectra
2. Determination of lifetimes for each sample
3. Analysis and interpretation of lifetime dependence on excitation source and/or emission wavelength

V. Bibliography

1. „Principles of Fluorescence Spectroscopy” Joseph R. Lakowicz
2. „Time-Correlated Single Photon Counting”, Michael Wahl, PicoQuant Technical Note (http://www.picoquant.com/images/uploads/page/files/7253/technote_tcspc.pdf)