Humboldt-Universität zu Berlin - Institut für Physik
Advanced Practical Course

Time-correlated single photon counting - script
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The time-correlated single photon count (TCSPC) is a statistical measurement method used to investigate radiating optical transitions. It will be the focus of this experiment as an example for event-based measurement methods. The solubility behaviour of pheophorbide a in ethanol-water mixtures and its embedding in micelles will be investigated. Pheophorbide a is a dye that belongs to the group of porphyrins. The parameter investigated is time-resolved fluorescence and its polarization.
The measuring method itself, as well as its optimization, is to be learned in order to achieve reliable results on the object of investigation.

Imagine, your boss gives you the order to carry out a measurement for which you have to set up a TCSPC measuring station.
Then there are several steps for you to perform:

- **Accumulation of information about the desired technique and the object of investigation -** this work step should take place BEFORE the date of the experiment. The bibliography on the website of the experiment is only an introduction and does not claim to be complete. So also use the library/the Internet - you should be informed about all red colored terms as well as the mentioned questions!
- **Building the setup** - This task will be taken care of and is not part of your practical training. All necessary components are available and positioned.
- **Adjustment of the setup** - This is where you come into play.
- **Adjustment of the operating parameters** of the components used - A maximum signal-to-noise ratio is almost always aimed at. The signal strength is also aspired, since stronger signals allow shorter measurements, but usually subordinate to the SNR. Here the PMT detector voltage and the detection threshold of the TCSPC electronics are of particular interest - on the day of the test you should be able to explain why.
- **Sample conditioning and optimization of measurement parameters** - In addition to the detector, there are other devices and materials involved in the experiment that need to be well selected. An obvious example of this is the damage threshold of detectors, always start new measurements at low laser power. In addition to the laser power, the optical density of the sample to be examined is of particular interest in this experiment - Why?
- **The actual measurement** - usually the lightest part of the workload, but also the most interesting - prepare your samples and record them well.
- **Evaluation** - Analyze your measurements and present your results clearly (in PRESENTATIVE tables and graphs) - this should ALWAYS be done promptly (also in your later research work, because at some point you should present your results and not always the subject is as small and well-defined as in the practical course)

- **The next step of iteration** - (you can only realize this part to a limited extent during the internship, but you can write down what you would do) Option 1: you could bring your results into agreement with the theory, fine - reproduce this! Option 2: It didn't work (several times) - try to find the cause and expand your theory or improve the experiment!

Let's get started:

The research group Photobiophysics deals with carrier systems for photosensitizers (PS) in photodynamic therapy (PDT).

Pheophorbide a (Pheo) is a good PS, but very poorly soluble in water and the applications of PDT are all in aqueous environments. So the PS has to be transported somehow controlled to the target area. A simple possible solution is to embed the PS in micelles. Your task now is to prove that the embedding in the micelle works and that the PS in the micelle remains photophysically active. The fluorescence intensity and decay time should be taken as a measure.

As just described, however, the actual measurement is only at the end of a series of necessary tasks to ensure that you actually measure what you intend to.

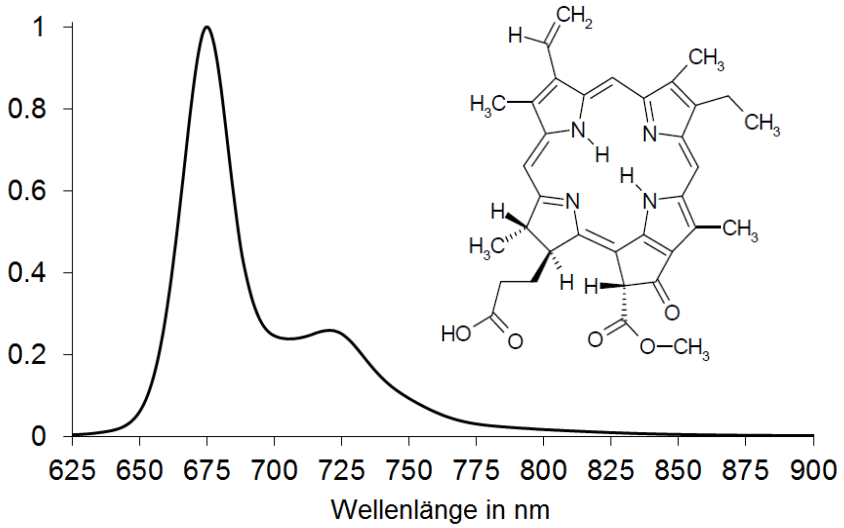

Abbildung 1 *Structural formula and normalized fluorescence spectrum of Pheophorbide a*

Why single photon counting?

The desired time resolution of a fluorescence measurement is in the picosecond range (10^{-12} s). The energy of a photon in the visible range is about 2 eV, i.e. in the range of 10^{-19} J. Thus, although one is still three orders of magnitude away from the absolute limit according to the uncertainty relation, the low-pass effect of all electronical components involved can already be clearly felt, which is why analog measuring procedures fail. The TCSPC avoids this by decoupling the temporal measurement from the intensity measurement - why is this possible?

Working tasks:

1. Preparation

In preparation for the test, familiarize yourself with the devices used (their design and function): (pulsed) laser, time-amplitude converter, lambda⁄2 plate, constant fraction discriminator, ...
Think about the terms highlighted in red in the text, like: Laser, laser modes, modelocking, Q-switching, Einstein coefficients, Huygens-Fresnell principle, Fourier transformation.
Get familiar with the basics of luminescent materials: fluorescence, phosphorescence, Jablonski diagram, as well as the terms solubility, micelles, ...

2. optical design and control program

Prepare the setup for the measurements. First get an overview of the test setup, align the devices and check the light paths. SYNC OK appears in the program if the reference signal is set correctly. Sinusoidal interference in the measurement signal is generally also due to unfavorable illumination of the reference diode.
Produce a sample with a low pheophorbide a concentration (about OD 0.1) in ethanol. Now insert the cuvette into the holder and set the operating voltage of the detector to approx. 0.8V first. Familiarize yourself with the settings of the program (e.g. through test measurements).
Two filters can be positioned in front of the PMT for wavelength selection. For all scattered light measurements (apparatus function) please use the grey (neutral) filter and for all measurements on Pheo the (red) interference filter 665-675 nm (the slider positions for this are clearly marked).

3. optimization of the TCSPC setup regarding PMT voltage and detection threshold value

Insert the scattering cuvette (strongly diluted Ludox) and set the detector voltage to 800V and the laser intensity to very low so that no more than 2*10^5 photons are detected at the 20 mV threshold. Now carry out measurements of the response function at CFD threshold values of 5 - 50 mV without changing the other parameters.
Compare signal (the range of the response peak) and noise (the range between the peaks and select a meaningful threshold based on it).
With this threshold you repeat the procedure for detector voltages between 600 and 1000 V and select a meaningful voltage.
Determine the parameters where the ratio between the noise signal and the relevant signal is lowest. For this purpose, it is advisable to calculate the quotient from the number of all noise signals to all relevant signals and to display it in tabular form.
For all subsequent measurements, use the optimum operating voltage of the detector determined here and the threshold value of the CFD in order to be able to compare the results better.

4. response function

Record a response function using the scattering cuvette and display the selected curve graphically.
Carry out this measurement for several different count rates and compare the half-widths of the apparatus curves as well as the falling flank. You can recognize a meaningful countrate by the fact that a further reduction of the countrate does not narrow the response function.
In each data file you need a measurement block that contains the response function (ideally BLOCK 1) for later evaluation of the fluorescence data.
Once you have recorded the response function in block 1 at the beginning, you can leave it there and always save it. Normally you should always redefine it, but here the fluctuations are very small.

5. test parameters

Optimize the measurement parameters (excitation intensity/count rate, OD of the sample) to avoid measurement errors (peak pile-up or reabsorption).

5.1. Peak Pile-Up Effect

Insert a sample of Pheo into the setup (OD < 0.2) and carry out measurements with different intensities of the laser pulse/count rate (the CFD count rate may go up to 10^7 for this partial task). Display your result graphically and in tabular form and select a meaningful setting for further measurement. Discuss the Chi^2 values depending on the intensity and choose a suitable intensity for the following measurements. Discuss the distribution of residuals with regard to the pile-up effect!

5.2. Reabsorption

Prepare at least 5 samples with different concentrations of Pheo so that the optical density is between 0,1 and 1,5.
Measure the fluorescence lifetime for these samples. Display your results graphically and in tabular form and draw conclusions for sample conditioning.

6. fluorescence lifetime (base value)

Measure the fluorescence lifetime of Pheo at optimal settings and graphically display your result as well as the residuals of the fit. Evaluate the measurement.

7. pheophorbide a in ethanol-water mixtures

Prepare at least 5 samples of the same concentration of pheophorbide a, but with different ethanol to water ratios. The strongest effects are to be expected for water contents above 50%. One of the samples should contain as much water as possible. Compare the results with a suitable ethanol sample.
Perform a measurement for each of these samples and display it graphically. Discuss and interpret the changes in the measurement results as the water content increases.

8. Triton X-100

Triton X-100 is a detergent that embeds pheophorbide in micelles to make them water soluble. Add 3-4 drops of the 10 % stock solution of Triton X-100 to the sample in 7. with the highest possible water content and repeat the measurements. Display your results graphically and compare them with those from 7.
In the evaluation, describe how Triton X-100 works and explain why pheophorbide a fluoresces again.

9. Prove the embedding of the pheo into the micelles

Compare the decay of the anisotropy of Pheo in ethanol with Pheo in water/triton. - Turn the polarization filter behind the cuvette for both samples first to the vertical position (i.e. the detected photons are polarized parallel to the incoming ones) and to the horizontal position (i.e. the detected photons are polarized perpendicular to the incoming ones) and perform the measurement.
Calculate the time-resolved anisotropy and display all results graphically.
Compare the temporal behavior for pheophorbide a in ethanol and in ethanol/water with Triton X-100. Discuss and interpret the differences between the two kinetics.
To help you in your search for information, here follows a short introduction, also to name the terms to be explained in context (in red). Further information on PS, laser, TCSPC and photomultipliers can be found on the experiment's website (user: fpr, pw: laser50).

Setup and Principle of the TCSPC

The TCSPC is intended to investigate radiating optical transitions in the range of many ps to many ns. However, because of the low pass functionality of most electronical components, the problem arises that it is not possible to measure quickly and accurately at the same time. TCSPC avoids this problem by decoupling detection and time measurement. The basic idea here is that a large number of identical, non-interacting particles (or molecules) behave statistically in exactly the same way as a single particle (or molecule).

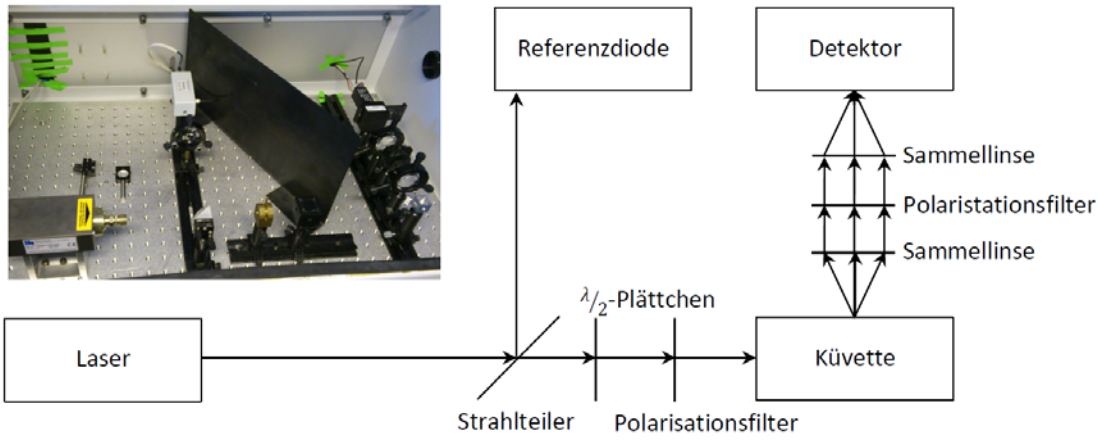

Abbildung 2 *setup*

The laser is operating in pulsed mode. The laser hits a semi-transparent mirror. This divides the beam path into two components. One meets a reference diode. The other is attenuated using a lamda/2 plate and a vertically oriented polarization filter and then hits the sample.
If a pulse is emitted by the laser, a part of this pulse hits the reference diode, which starts the time measurement. The other part of the pulse, attenuated, hits the cuvette containing the fluorescent substance. If a photon of the pulse interacts with a fluorophore, the latter is excited. When returning to the ground state, this fluorophore then emits a photon with a certain probability for reaching the detector to terminate the measurement with a trigger signal. The detector is a photomultiplier whose pulses must pass through a constant fraction discriminator in order to be measured reliably.

For the electronics it is better to start the measurement with the detector pulse (inverse TCSPC), because to avoid the pileup effect only about every 100 pulses a photon can be detected at all. If the signal reference diode would be used as start signal, the electronics would have to start a new measurement with each pulse. But many of them would be aborted because no photon could be detected. Starting the measurement at the detector, on the other hand, ensures that every measurement started is completed. The reference diode then ends the measurement and together with the pulse frequency of the laser provides the start time of the laser pulse belonging to the detected photon. In addition, each measurement always causes a certain dead time, a period in which the detector is virtually "blind", which would unnecessarily lengthen the measurement.

Fluorescence

Luminescence generally refers to the emission of photons from an electrically excited state. Depending on the type of excited state, a distinction is made between fluorescence and phosphorescence. In order to understand the difference, two electrons of a system (e.g. an atom) should be considered.
If two electrons are present in the same electrical state, they must differ in their spin state according to the Pauli principle. One of the electrons is therefore in the spin-up state the other in the spin-down state. The sum of the spin quantum numbers of the two electrons is thus zero and the electrons are called paired. If there are also unpaired electrons, which in principle can be in

the spin-up or spin-down state, the sum of the spin quantum numbers need no longer be zero. A measure for the sum of all spin quantum numbers of a system is the multiplicity M=2|S|+1. If, for example, only paired electrons are present, then the multiplicity results to 1 and one speaks of the so-called singlet state. If the sum of the spin quantum numbers is 1, the multiplicity is 3 and we speak of the triplet state. The difference between singlet and triplet state can be seen, for example, in spectroscopy, when the degeneracy is eliminated (e.g. in the magnetic field). Here the number of visible spectral lines corresponds exactly to the multiplicity, i.e. in a singlet state one sees one line, in a triplet state three lines.
Fluorescence is a transition without change of multiplicity, while phosphorescence is a transition with change of multiplicity.

The Jablonski Diagram

Each electron of the system occupies a certain energy level in a certain state of vibration. For an energy level scheme, it should be noted that the energy differences between different energy levels are significantly greater than the energy differences between the vibrational states. If an electron is excited from its ground state to a higher energy level in a certain vibrational state, it will change into the lowest energy vibrational state of the energy level within a few picoseconds. The probability of transition between two states of equal multiplicity is antiproportional to $(E)^3$.
The energetic distance of the vibrational levels of an excited state decreases with increasing energy (the parabolic approximation of the molecule potential curves above the generalized core coordinate - applies only to small values). Thus the higher vibration levels of the next lower state show a clearly higher density of states than the lowest vibration levels of the currently occupied state. According to Fermi's Golden Rule a transition is therefore very probable.
These just described radiation-free transitions are called Internal Conversion.
In general, the energetic distances of both the energy levels and the corresponding vibration levels become larger with decreasing excitation energy, so that the transitions also become slower. The slowest transition is consequently the one from the vibration-free S_1 state to S_0. The lifetime of this state can be several nanoseconds. Thus other processes than the internal conversion are in principle only possible from this state (rule of Kasha). These include fluorescence, intermolecular energy and electron transfer and intersystem crossing.
The lifetime of this state is therefore of highest importance for the description of the properties of a dye, as it is the starting point for all downstream processes. Fluorescence is particularly suitable for its measurement as it is a natural process and its observation does not influence the molecules involved. At this point, however, it should be noted that the decay time of fluorescence (and thus of S1 occupation) is influenced by various processes. Fluorescence with its "natural lifetime" is only one of a number of draining channels. However, the actual experimental lifetime is the reciprocal of the sum of all rate constants involved.
Although basically forbidden, under certain conditions (strong spin orbit coupling in the case of cyclic conjugated pi-electron systems or heavy atom effect) the spin state of the electron can change during the radiation-free transition. It thus changes from a singlet state to a triplet state (intersystem crossing). The electron can no longer simply change to the ground state, because there is already an electron in the same spin state. In addition to the release of a photon, the spin state of the electron must also change again. This process is then called phosphorescence. Compared to fluorescence, the transition rate is much lower and the lifetime of the excited states much higher.

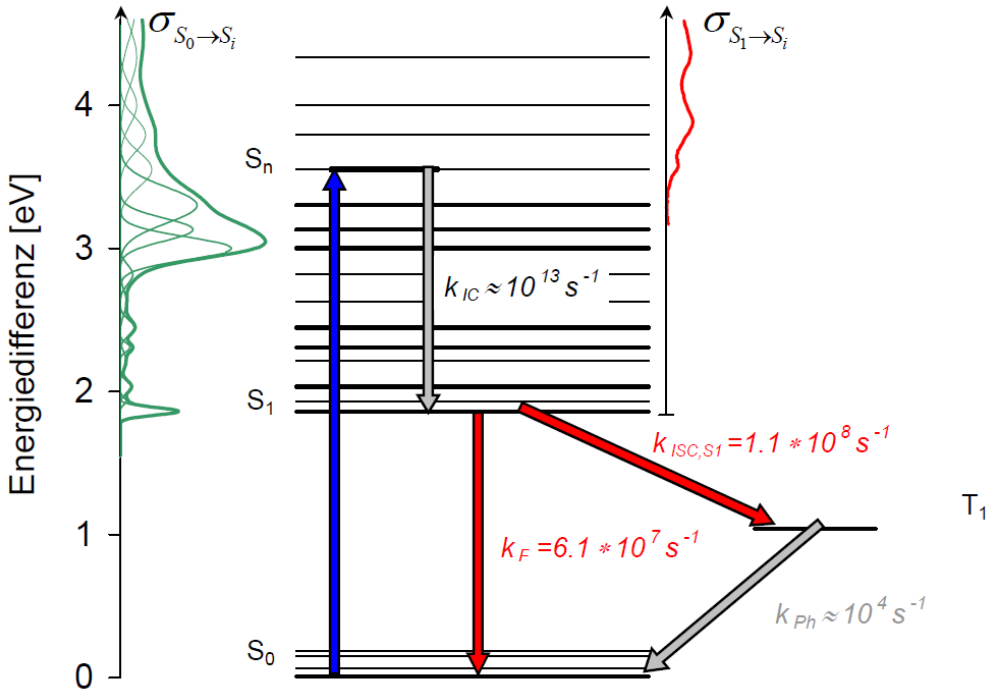

Abbildung 3 *Jablonski-Diagram of Pheophorbide a in Ethanol. – A onedimensional Energy scheme to illustrateall involved states and transitions between them. For better understanding, also the corresponding absorption is show on the left.*

Quantum yield

In the context shown, the so-called fluorescence quantum yield Φ_{Fl} is an interesting parameter. It describes the proportion of photons emitted by a substance to the photons absorbed by it. Due to radiation-free transitions, the number of photons emitted is smaller than the number of photons absorbed and the quantum yield is therefore smaller than one. If k_{Fl} is the transition rate of radiating transitions and the transition rate of radiation-free transitions is free, then the following applies:

$$\Phi_{Fl} = \frac{k_{Fl}}{k_{Fl} + k_{sonst}} \tag{1.1}$$

Consequently, if the fluorescence quantum yield is known, the natural lifetime can be calculated from the measured fluorescence decay time.

Anisotropy of a fluorophore

Fluorophores have the property of absorbing or emitting electromagnetic radiation according to their transition dipole moment. The transition dipole moment of the fluorophore is a vectorial quantity whose direction describes the polarization direction of the light at which the substance preferentially absorbs or emits.

The fluorescent substance is now usually in the form of an isotropic solution, the transition dipole moments are randomly oriented according to the Boltzmann distribution. The anisotropy is only created by excitation with a linearly polarized laser pulse. Preference is given to molecules where the angle between the polarization of the excitation light and the transition dipole moment is small. Since the absorption and emission dipole moments of a molecule have a fixed angle, this generally leads to polarized fluorescence. The fluorescence anisotropy r is calculated:

$$r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \tag{1.2}$$

Where in general, it has to be considered that the detection sensitivity can be polarization-dependent (in our case, however, this dependency is negligible - consider why?).
Since the fluorophores now move in the solution, in particular rotate, the sample gradually approaches an isotropic one again. The temporal behaviour depends strongly on the shape of the investigated dye and its environment. In the case of a spherical molecule, the anisotrope would simply drop exponentially.

$$r = (r_o - r_\infty) \cdot e^{-t/\tau_{Rot}} + r_\infty \tag{1.3}$$

where the rotational decay time red is significantly determined by the molecule size and the environment. A residual anisotropy always occurs when the rotation of the molecule is hindered by coupling or embedding in a very large carrier.

$$\tau_{Rot} = \frac{\eta V}{k_B T} \tag{1.4}$$

Here η is the viscosity, V the volume of the molecule, k_B the Bolzmann constant and T the temperature.
An important conclusion is that measurements where the influence of the molecular rotation should not influence the measurement results (e.g. fluorescence decay times) require a reasonable positioning of the observation polarization filter. What does this look like and why (keyword: "magic angle")?