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MASTER PRAKTIKUM

Fluorescence Correlation Spectroscopy

Authors:
Nemanja Markesevic, Assegid Flatae, Florian Sledz, Amr Farrag and Mario Agio

Nano-optics group
Department or Physics

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Abstract

Faculty of Physics
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In this document we present a technique called Fluorescence Correlation Spectroscopy (FCS).
In Chapter 1 we briefly introduce absorption and fluorescence. In Chapter 2, without a strict formalism a theoretical background which should be sufficient for understanding the procedures and for data processing is presented. The principles of fluorescence microscopy and confocal setups are explained in Chapter 3. In Chapter 4, students will learn how FCS can be related to the diffusion process and how to extract relevant information about the observed system. The instructions for performing the experiments are given in Chapter 5.
In the last chapter (Chapter 6) we define students' tasks and give general instructions for a written report.
Our goal is to teach students about fluorescence microscopy and to make them familiar with this powerful technique, FCS, widely uses in biology, physics and physical chemistry.

Contents

Chapter 1

Fluorescence

1.1 Optical transitions in molecules

Spectroscopy probes the properties of matter using the interaction between electromagnetic waves and matter. This includes molecules, which are quantum mechanical systems with discrete energy levels (vibrational, rotational and electronic). Molecules can, for example, absorb ultraviolet or visible light undergoing a transition to a higher electronic energy level. Likewise, the absorbed energy can be lost through the emission of a photon. This process can be fast (of the order of 1 ns), when the transition does not involve a change in the electronic spin, and it is called fluorescence. On the other hand, phosphorescence can be a very slow process (up to seconds), because the associated transition requires a change in the electronic spin, which is in principle forbidden.

1.1.1 Jablonski diagram

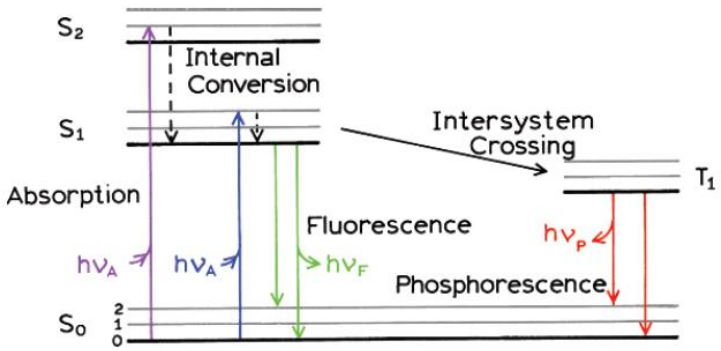

FIGURE 1.1: Jablonski diagram is a simple way to represent processes of absorption, fluorescence, phosphorescence, intersystem crossing (from a singlet to a triplet state), internal conversion (nonradiatively, from a higher to a lower singlet state) [1].

The processes of absorption, fluorescence and phosphorescence can be easily presented on Jablonski diagram (Figure 1.1). Upon excitation, an electron is transferred to the higher electronic state (S_1 or S_2). Each electronic state has its vibronic states (for example S_0 is presented with its vibronic states 0, 1, 2). An electron can either return to the ground state, S_0 and emit a photon (fluorescence), or it can first go

to the triplet state (intersystem crossing), T_1 and after few milliseconds emit a photon (phosphorescence). There is also a possibility that the electron goes back to the ground state non-radiatively [1].

1.1.2 Fluorescence quantum yield

When a fluorescent molecule absorbs a photon, it can either go back to its ground state radiatevely or it can loose its energy in the non-radiative process. Fluorescence quantum yield of a molecules represents the ratio of number of emitted and absorbed photons. This value, depending on the type of molecule can be lower than 1 % or can be close to 100 %.
Another way to define quantum yield is by using the radiative and non-radiative decay rates, γ_r and γ_{nr}, respectively [2]:

$$Q = \frac{\gamma_r}{\gamma_r + \gamma_{nr}} \tag{1.1}$$

Working with molecules with a high quantum yield has a lot of advantages, mostly due to the fact that the larger number of emitted photons enables easier detection.

1.1.3 Saturation intensity

With the increase of the intensity of the excitation light, the emission intensity will also increase. At the beginning, for low excitation powers, this increase will be linear with the excitation power. However, after some point, the linearity will be lost and it will lead to saturation.
To understand a saturation process, it is important to know that once in the excited state, a molecule cannot absorb more photons. Only after it returns to the ground state and a photon is emitted, it can absorb photons again. Therefore, the increased excitation intensity cannot further increase the number of emitted photons.

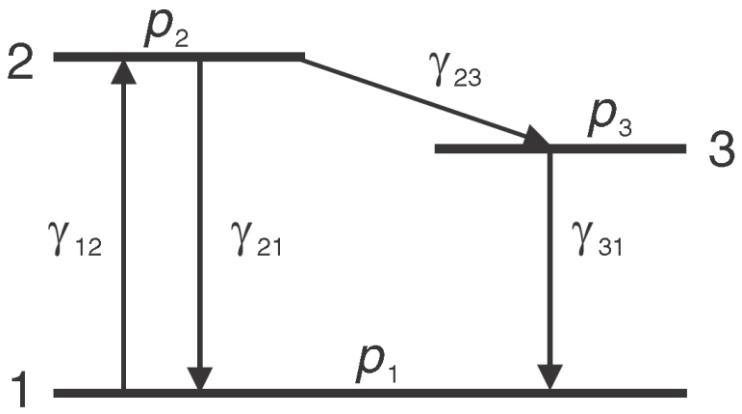

FIGURE 1.2: A quatum emitter approximated by a three-level system. Levels 1 and 2 are singlet states and level 3 is a triplet or a dark state [2].

A simplified version of Jablonski diagram is presented in Figure 1.2. The singlet ground and the singlet excited states are denoted by numbers 1 and 2, respectively. The triplet state is indicated by number 3. These levels are connected by the excitation and decay rates and one can formulate a system of differential equations (1.2, 1.3, 1.4, 1.5) for the change of the populations p_1, p_2 and p_3. The Equation 1.5 states that the emitter has to be in one of the three states at any moment.
The absorption process is described by the rate γ_{12} and the decay from the state 2 to the state 1 is described by $\gamma_{21} = \gamma_r + \gamma_{nr}$. Likewise, the rates γ_{23} and γ_{31} describe transitions from the level 2 to the level 3 and from the level 3 to the level 1, respectively [2].

$$\dot{p}_1 = -\gamma_{12}p_1 + (\gamma_r + \gamma_{nr})p_2 + \gamma_{31}p_3 \tag{1.2}$$

$$\dot{p}_2 = \gamma_{12}p_1 - (\gamma_r + \gamma_{nr} + \gamma_{23})p_2 \tag{1.3}$$

$$\dot{p}_3 = \gamma_{23}p_2 - \gamma_{31}p_3 \tag{1.4}$$

$$1 = p_1 + p_2 + p_3 \tag{1.5}$$

For the steady state, the populations are constant in time and thus their time derivatives are equal to zero ($\dot{p}_1$, $\dot{p}_2$, $\dot{p}_3 = 0$).

The rate at which the system emits photos is given by:

$$R = p_2\gamma_r \tag{1.6}$$

Further calculations brings us to the following relation [2]:

$$R = R_\infty \frac{\frac{I}{I_s}}{1 + \frac{I}{I_s}} \tag{1.7}$$

The constants R_∞ and I_s are defined as:

$$R_\infty = \gamma_r(1 + \frac{\gamma_{23}}{\gamma_{31}})^{-1} \tag{1.8}$$

$$I_S = \frac{\gamma_r + \gamma_{nr} + \gamma_{23}}{\sigma(1 + \frac{\gamma_{23}}{\gamma_{31}})}\hbar\omega \tag{1.9}$$

In Equation 1.9, σ represents the absorption cross-section of a molecule, $\hbar$ is the reduced Planck's constant ($\hbar = 1.05457 \times 10^{-34} Js$) and ω is the angular frequency of the excitation light.

A simple interpretation would be that R_∞ represents an emission intensity for the case in which the excitation intensity is infinite. The excitation intensity at which the emission intensity is 50 % of R_∞ is the saturation intensity, I_s. Measurements should be performed under the I_s value [2].

1.1.4 Examples of fluorescent molecules

Fluorescent molecules typically contain aromatic rings with delocalized π-electrons and some examples are shown in the Figure 1.3a. From a quantum mechanical point of view, a fluorescent molecule can be described as a particle-in-the-box system: the

larger the box, the longer the wavelength.
In the case of molecules with a large π-electron delocalization, the emission is typically in the visible range. Their excitation and emission properties are different, and their spectra span a wide range, from UV to red or IR. Structural formulas of several diffenet organic dyes (Figure 1.3a) and real emission colors of their fluorescent solutions (Figure 1.3b) show only few examples from an enormous world of fluorescent molecules.
For readers who are more interested in the subject, please check Cyanines. Cyanines (for example: Cy3, Cy5 and Cy7) are a family of fluorescent dyes where one can clearly see that the size of a delocalized π-electronic cloud determines their optical properties. The length of the carbon chain which connects two aromatic rings is responsible for the difference of their emission wavelength maxima (200 nm between Cy3 and Cy7).

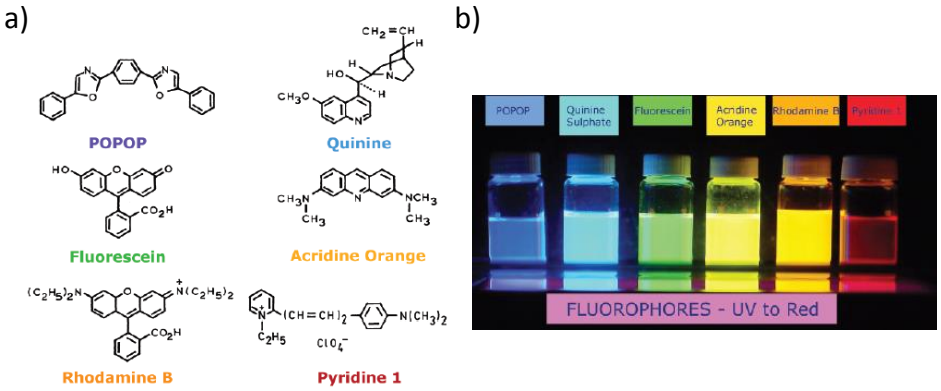

FIGURE 1.3: Examples of fluorescent molecules. a) Structural formulas, b) Their fluorescent solutions (real colors) [1].

1.2 Rhodamine 6G

Rhodamine 6G (Rh6G) is a highly fluorescent molecule, soluble in water, methanol and other organic solvents. Its high quantum yield (around 95 % in water) makes it suitable for different sorts of experiments. In the Figure 1.4a one can see the chemical structure of the molecule, and in the Figure 1.4b absorption and emission spectra of Rh6G in water are presented. In principle, this molecule can be excited with different types of lasers; the only important thing is that the laser wavelength overlaps the absorption spectrum of the molecule. For example, in our laboratory we have a green semiconductor continuous wave (CW) laser (532 nm). One clearly sees from the Figure 1.4b that 532 nm is in the range where this molecule absorbs light. This molecule could be also excited by a laser (CW or pulsed) which emits light at a wavelength of 488 nm.

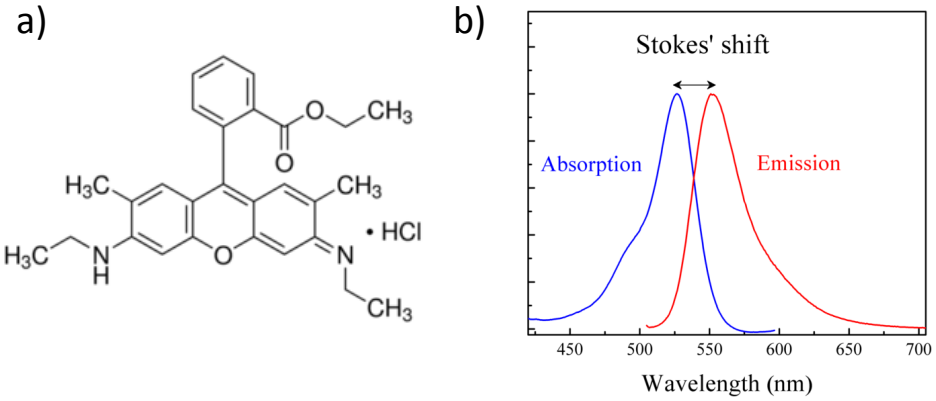

FIGURE 1.4: Rhodamine 6G. a) Structural formula, b) Absorption and emission spectrum [3]. The fluorescence emission occurs at a longer wavelength with respect to the wavelength of the incident light. This difference is called Stokes shift.

Chapter 2

Introduction to Fluorescence Correlation Spectroscopy

2.1 Auto-correlation and Cross-correlation

Before we explain the principles of FCS, it is important to discuss briefly the mathematical apparatus necessary for the interpretation of the data. The auto-correlation of the funcion F is defined as the averaged product of the function value at time t, $F(t)$, with its value after a delay time τ, $F(t+\tau)$ [1]:

$$R(\tau) = \langle F(t)F(t+\tau)\rangle = \frac{1}{T}\int_0^T F(t)F(t+\tau)dt \tag{2.1}$$

One of the interpretations can be that the auto-correlation function discovers the repetitive pattern in the signal. For example, if we look at a sine or a cosine functions, we can immediately notice some regularities (such as periodicity of the functions). For more complex patterns one must use mathematical tools as mentioned above.

Similarly, a cross-correlation of two different functions, F and P, is defined as :

$$S(\tau) = \langle F(t)P(t+\tau)\rangle = \frac{1}{T}\int_0^T F(t)P(t+\tau)dt \tag{2.2}$$

2.1.1 Auto- and Cross-correlation in Fluorescence (Cross-) correlation spectroscopy

Fluorescence Correlation Spectroscopy (FCS) or Fluorescence Cross-Correlation Spectroscopy (FCCS) is a technique that is widely used in different fields in order to monitor processes ranging from simple diffusion in solution to the chemical reactions, diffusion of molecules on the membranes and others [1].
If you understood the concept of of auto- and cross-correlation, it will not be a problem to understand the basics of FC(C)S. More details about experimental setup will follow in Chapter 3. For now, it is important to know that the fluorescence is detected by photon counters. The number of photons received per second is defined as intensity (F). For convenience, the fluorescence signal can also be split with a 50/50 beam splitter and detected by two equal detectors. This is a way to avoid some artifacts, and will also be discussed later (see afterpulsing in Chapter 3).

The intensity fluctuation, δF around its mean value, $\langle F\rangle$, is defined as:

$$\delta F = \langle F\rangle - F(t) \tag{2.3}$$

The important part is that the intensities detected by the two detectors are recorded on the computer. The auto-correlation of the fluorescence intensity, normalized by the average intensity squared, is given by [1]:

$$G'(\tau) = \frac{\langle F(t)F(t+\tau)\rangle}{\langle F\rangle\langle F\rangle} = 1 + \frac{\langle \delta F(0)\delta F(\tau)\rangle}{\langle F\rangle^2} \tag{2.4}$$

In this expression, t is replaced with 0. Some authors consider that working with the auto-correlation of fluorescence fluctuations is more convenient [1]:

$$G(\tau) = \frac{\langle \delta F(0)\delta F(\tau)\rangle}{\langle F\rangle^2} \tag{2.5}$$

Similarly a cross-correlation of the intensities and fluorescence fluctuations can be defined.

Chapter 3

Experimental approach to FCS

3.1 Optical setup

In order to examine the fluorescent properties of molecules, there are several main components that are needed. A light source excites emitters and detectors detect fluorescence. There are many types of light sources, such as different lamps, diodes and lasers. In our experiments we will use a semiconductor CW laser and the wavelength of its laser light is 532 nm.
The set of mirrors to guide the laser light towards the sample or fluorescent light towards detectors, interference filters to reject unwanted scattered or reflected light, objectives to focus light tightly on the sample are only few other optical elements present in the optical setup.

3.1.1 Detectors

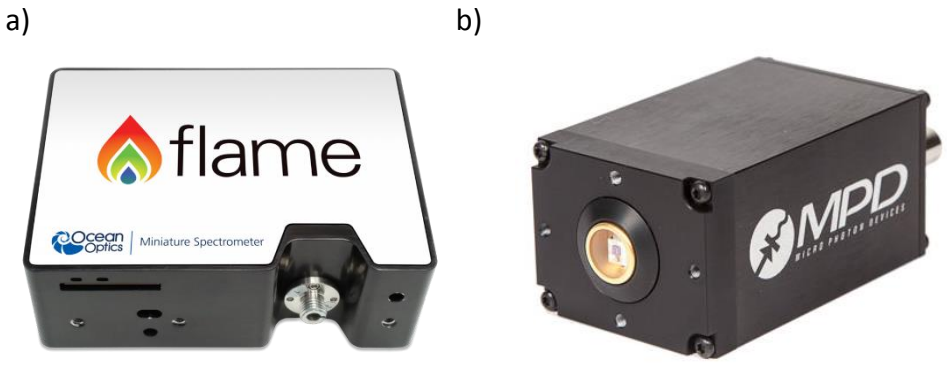

FIGURE 3.1: Photon detectors. a) Spectrometer, b) Single Photon Avalanche Diode (SPAD).

To measure fluorescent spectra, a dispersion element is necessary, for example a prism or a diffraction grating. A photon detector in this case is typically a photomultiplier (PMT) or a charge coupled device (CCD). In our setup, a compact fiber-coupled Ocean Optics spectrometer (Figure 3.1a) contains a diffraction grating and a small CCD array.
On the other hand, if we are only interested in the number of detected photons, then a Single Photon Avalanche Diode (SPAD) is a good choice (Figure 3.1b). The working principle of a CCD and a SPAD is different. While in a CCD the light is projected

on a capacitor array, causing each capacitor to accumulate an electric charge proportional to the light intensity at that location, in the SPADs the absorption of a single photon is sufficient to generate one electron-hole pair and trigger an avalanche multiplication process [4].

3.1.2 Dark counts and afterpulsing

All detectors have their own internal noise, dark counts, mostly caused by thermal effects, which produce pulses even in the absence of illumination. The other big problem is afterpulsing.
It is important to know that these are artifacts and their appearance has nothing to do with the optical properties of the examined emitters.
During the avalanche formation, some trapped carriers can get accelerated by the intense electric field across the p-n junction. They further start another avalanche and these new afterpulses are correlated with a previous avalanche pulse [4]. This is indeed a huge problem in FCS, since only one detector is used. On the other hand, this is exactly why it is more convenient to split the fluorescent signal on two detectors and perform FCCS. In the cross-correlation, these artifacts will not be present, since there is no correlation between afterpulsing of the two different detectors.

3.1.3 Time Tagged Time-Resolved (TTTR) data collection

The principles of TTTR data collection are explained in detail elsewhere [5]. In order to examine fluorescence dynamics, it is important to record the arrival times of all photons with respect to the beginning of the experiment (time tag), and additionally to record their arrival with respect to the excitation pulse (this holds for a pulsed excitation).

However, we will be using continuous wave laser and we record our files in T2 mode (Figure 3.2). The events from the two channels (two detectors) are recorded and treated equally and independently with the highest resolution the hardware supports; in our case, the time tag resolution is 250 ps [5].

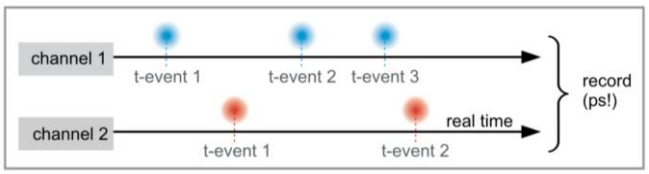

FIGURE 3.2: T2 mode. Photon arrivals are time-tagged with a picosecond resolution by two different independent channels [5].

3.1.4 Confocal setup

The scheme of a typical setup used in FCCS is presented in the Figure 3.3a. Laser light is reflected from a dichroic mirror (DM) and focused by the objective lens onto the sample. Fluorescence light, collected by the same objective, is transmitted through the DM, further split by the 50/50 beamsplitter (BS) and focused on two

SPADs. Since DM is not sufficient to reject all reflected laser light, emission filters in front of the detectors are added to ensure that only fluorescence from the molecules reaches them. Now it is possible to calculate correlation functions.

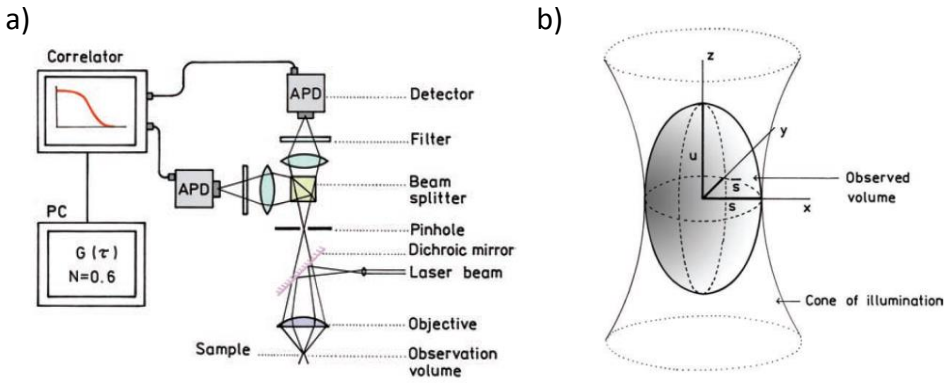

FIGURE 3.3: Confocal detection. a) Schematic representation of a confocal setup, b) Volume defined by the focused laser light and the pinhole [1].

It is important to mention that for the detection of a small number of fluorescent molecules it is essential that the detection volume is small. Otherwise the background signal from different types of scattering (for example Raman scattering) will be comparable (or larger) to the fluorescent signal we want to measure. For this reason the laser light should be tightly focused using an objective lens. However, that is not sufficient, because the out of focus light can also contribute to the signal. By placing a pinhole in the emission path, the out-of-focus light is rejected and the observed volume is defined (Figure 3.3b) [1].

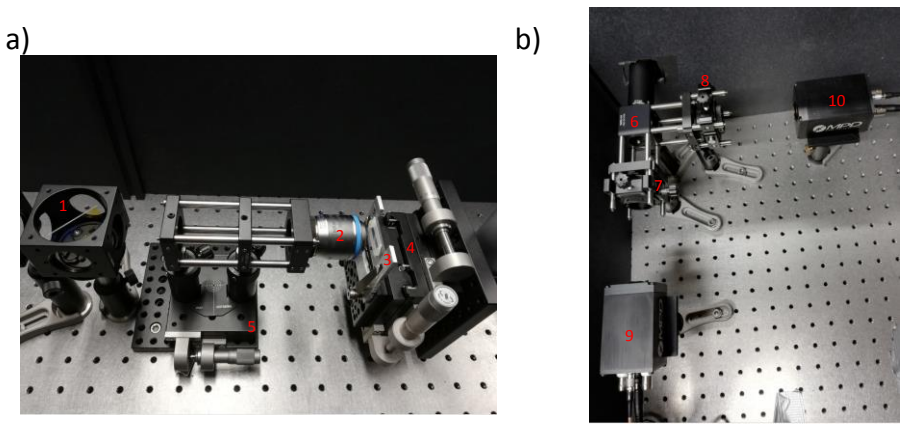

FIGURE 3.4: Parts of the confocal setup in our laboratory. a) Excitation path: 1 - DM, 2 - Objective lens, 3 - sample, 4 - xy-stage, 5 - z-stage. b) Detection path: 6 - BS, 7, 8 - lenses, 9, 10 - SPADs.

The parts of the experimental setup from our lab are presented in Figure 3.4. One part of the excitation path is presented in Figure 3.4a: light reflected by the DM is directed through the objective lens onto the sample. The sample stage can be controlled in the x and y directions. The z-stage is used to focus light transmitted through the objective lens.

A photo of the detection path with SPADs is shown in Figure 3.4b. Light reflected from the BS is further focused by the lenses on two SPADs.

3.1.5 Confocal volume - geometrical interpretation

Confocal volume is defined by the properties of the setup, mainly by the wavelength of the laser and the properties of objective lens. There is a limit up to which one can focus a Gaussian beam. According to scalar diffraction theory, the resolution in the xy-plane is:

$$r_{xy} = \frac{0.61\lambda}{NA} \tag{3.1}$$

The resolution in the z-direction is given by:

$$r_z = \frac{2n\lambda}{NA^2} \tag{3.2}$$

In these equations, λ represents the laser wavelength, NA is a numerical aperture of the objective lens, and n is the index of refraction of the medium in which the emitters are dissolved. The numerical aperture is one of the most important characteristics of a microscope objective and it is given by a dimensionless number that characterizes the range of angles over which the objective lens can accept or emit light.

The pinhole is small enough to reject out-of-focus light, but large enough to pass the light from the illumination spot. A three-dimensional Gaussian can approximate the profile associated with the confocal volume presented in Figure 3.3b [1]:

$$p(r) = I_0 e^{-2\frac{x^2+y^2}{s^2}} e^{-2\frac{z^2}{u^2}} \tag{3.3}$$

In this case, the radius s and half-length u define distances at which the intensity decreases to e^{-2} of its maximal value.
In practice, there are several ways to determine the size and the shape of the confocal volume. One of the best known and widely used is to perform a 3D scan of a single fluorescent bead whose size is much smaller than the diffraction limit [6].

Chapter 4

FCCS and Diffusion

4.1 Diffusion

Molecules and other particles move randomly in solutions. This kind of process is well known as Brownian motion. The diffusion of molecules represents their thermal motion and depends on the molecule size and its interaction with the environment.
To calculate an auto-correlation function for isotropic, three dimensional diffusion, we should start from the basic laws of diffusion. First, we should define the concentration as the number of molecules divided by the product of the observation volume and the Avogrado's number, N_A ($\mathrm{N_A} = 6.022\,141\,29 \times 10^{23}\,\mathrm{mol}^{-1}$).
The local concentration, $C(\vec{r},t)$ can be expressed as a sum of the average concentration, $\langle C\rangle$ and the diffusion-driven stochastic fluctuation of the concentration, $\delta C(\vec{r},t)$:

$$C(\vec{r},t) = \langle C\rangle + \delta C(\vec{r},t) \tag{4.1}$$

Further, the fluctuations change according to Fick's law [7]:

$$\frac{\partial \delta C(\vec{r},t)}{\partial t} = D\nabla^2 \delta C(\vec{r},t) \tag{4.2}$$

The detailed solving of all differential equations is beyond our scope. At this point we will focus on their solutions. For further reading, please refer to Reference [8].

The correlation function for the diffusion in three dimensions has this form [1]:

$$G(\tau) = G(0)(1+\frac{\tau}{\tau_D})^{-1}(1+(\frac{s}{u})^2\frac{\tau}{\tau_D})^{-\frac{1}{2}} \tag{4.3}$$

The diffusion time, τ_D is dependent on the size of the detection volume and the diffusion coefficient D [1]:

$$\tau_D = \frac{s^2}{4D} \tag{4.4}$$

The diffusion coefficient, on the other hand, depends on the temperature T, the viscosity of the medium in which molecules move η, and their hydrodynamic radius, r:

$$D = \frac{kT}{6\pi\eta r} \tag{4.5}$$

where k represents the Boltzmann's constant ($k = 1.381 \times 10^{-23}\,\mathrm{J\,K^{-1}}$).

If we fit the correlation function given by Equation 4.3, one parameter that we obtain is the diffusion time, τ_D. The other parameter is $G(0)$, and it is inversely-proportional to the average number of molecules in the detection volume [1]:

$$G(0) = \frac{1}{N} \tag{4.6}$$

For known sample concentration, one can easily determine the effective volume, V_{eff}:

$$V_{eff} = \frac{N}{N_A \langle C \rangle} \tag{4.7}$$

Here N_A represents the Avogadro's number.

4.1.1 Interpretation of the equations

It is not easy to understand immediately how to interpret the quantities obtained from the correlation curve. Given the fact that the theory of FCS is based on the Poisson statistics, the number of fluorescent molecules in the volume can be described by the Poisson distribution [1]:

$$P(n, N) = \frac{N^n}{n!} e^{-N} \tag{4.8}$$

$P(n, N)$ represents the probability that n molecules are present in the volume, when the average number in the volume is N. For example, if $N = 0.6$, that means that the probability that there are no molecules in the volume is 55%. To have only one molecule present, the probability is 33%. And for two molecules present at the same time in the volume, the probability drops to only 10% [1].

The diffusion time is a quantity that depends on the geometry of the volume, as given in Equation 4.4; the larger the radius, the larger the diffusion time. That simply means that τ_D is not a good quantity to be taken as a reference, since every setup will have a slightly different shape of the detection volume. However, the diffusion coefficient should be constant if the samples are kept under the same experimental conditions (temperature, viscosity).

Chapter 5

Instructions for measurements

5.1 Measurements

In this Chapter we will present many technical details. Namely, the main ideas have already been presented. However, every optical setup is slightly different and also the Software used for the data collection and processing can vary significantly. This Chapter is more like a cookbook; it is here to guide you through the experiments. By following the instructions you can perform measurements, save and process data.

5.1.1 Samples

We will examine Rhodamine 6G molecules in water and water/glycerol mixtures. The movement of molecules depends strongly on the viscosity of the medium. The higher the percentage of glycerol, the more viscous the environment would be and thus the slower the movement. These solutions will be sealed in the home made flow chambers to prevent an evaporation and leakage of the sample. Teaching assistants will take care of the sample preparation before the experiment takes place.
For all measurements, the first step would be clamping the sample to the sample stage. The sample stage can be moved in the x-y plane using micrometer screws. However, in order to focus light, one should move the objective which is on a separate micrometer stage and can translate along the z-axis (Figure 3.4).

5.1.2 Measurements of fluorescence spectra

In the optical setup the fiber-coupled spectrometer is not in the main optical path. There is a flip-mirror which will steer the emitted light in the direction of the optical fiber. Maybe small adjustments of the mirror are needed to improve the coupling with the optical fiber.

To determine the optical spectra of Rh6G it is important to distinguish the fluorescence signal of Rh6G from the background signal (all photons that are not related to the fluorescence of Rh6G). This means that one has to excite the solvent (water in this case, without Rh6G) in which Rh6G will be dissolved and collect all the photons. Despite the fact that water itself is not fluorescent, there is always some stray light that could contribute to the signal. Once we determine the background signal, it is easy to subtract it from the signal obtained in the separate measurement from the Rh6G dissolved in water.
In our experiment, we will use a commercially available program developed for spectroscopy. Open the program OceanView (on the Desktop). Choose the option QuickView. In the Acquisition Group Window (Figure 5.1 #1)change the integration time to 2s. It is convenient during the focusing process. The excitation power should be around 20 μW. In the case of over-saturation, one should decrease the integration

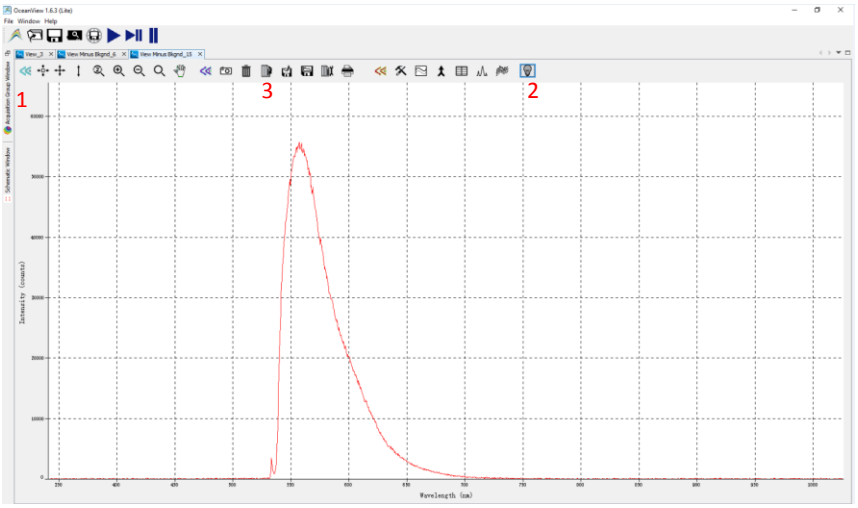

FIGURE 5.1: OceanView software. By clicking on the Acquisition Group Window #1, one can choose the desired integration time. Gray bulb #2, is for a background subtraction. To copy the data on the clipboard, one should click on the icon designated by #3.

time to 0.5 s. In order to perform a measurement, once everything is aligned, the laser should be blocked and the gray light bulb should be pressed (Figure 5.1 #2). After the laser light is unblocked and the sample is illuminated, one obtains a fluorescent signal. Choose the icon to copy the data on the clipboard (Figure 5.1 #3). Open Notepad and paste the data (CTRL+V). It is important to remove the first line of letters not to cause some issues while loading the file in Python for example. Save the data!

5.1.3 Correlation curves

After the SPADs have been switched on, one should approach the sample with the objective, using a z-stage, and focus light on the molecules inside the flow chamber. When the count rate reaches its maximum, that means that everything is well focused. The data collection is performed using the TimeHarp 260N system. For convenience, the data collection and processing steps are explained in the Figure captions (Figure 5.2 and Figure 5.3).
It is important to open a new directory and save the project as .ptu files (Figure 5.2 #2). However, for the fitting procedure and for a program QuickFit 3.0 [9] it is essential to save histograms as .corr files (Figure 5.2 #4).
To obtain a good correlation curve, an acquisition time of approximately 300 s is recommended. Please note that the auto-correlation (red, AA and green, BB) curves in Figure 5.2 #3 are different from the cross-correlation curve (blue, AB). The increase we observe for the short correlation times is a consequence of afterpulsing. As mentioned before, one can solve the problem by performing cross-correlation of the signals from two detectors.

In order to extract raw data from .corr file, one should open QuickFit 3.0 and choose an FCS wizard from the menu. Load the file, press Next (several times) and Finish at the end. Once the curve is shown, choose the fitting model (3D Normal Diffusion) as shown in Figure 5.3 #1 and press the button Fit Current (Figure 5.3 #5). In principle, this program gives all the necessary FCS parameters.

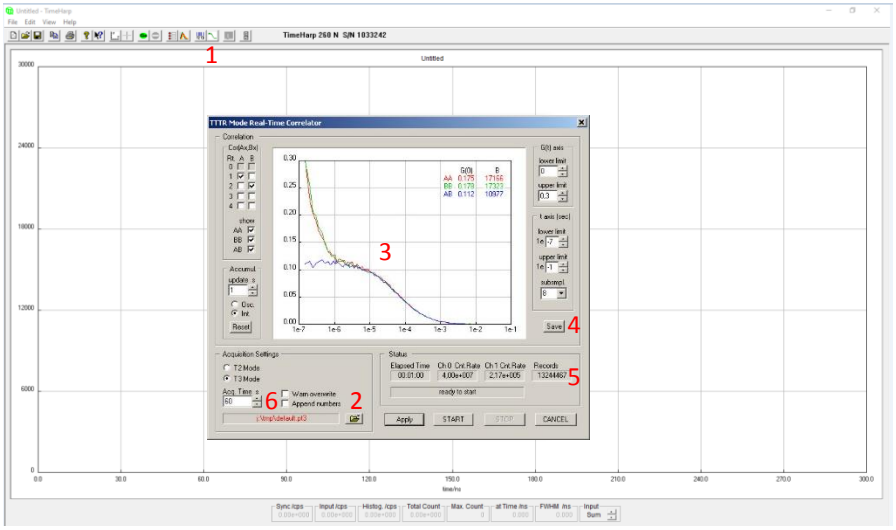

FIGURE 5.2: TimeHarp 260N software. By clicking on the icon #1, the TTTR Real Time Correlator window will appear. It is important to choose the directory where your files will be saved #2. The correlation functions will appear in the window #3. Also the correlation curve should be saved #4. The total number of counts will appear #5. Depending on the type of measurement, the total integration time can be changed #6.

However, we would like you to fit the cross-correlation curve and find the relevant parameters yourself. For this, right click on the graph, choose the option Copy Data and select only the first two boxes as shown in Figure 5.3 #6. Save the data as .txt file. If you use Python, be sure either to delete or skip the first row with letters, otherwise np.loadtxt(...) will report an error!

5.1.4 Saturation curve

In this case we count the number of photons that both detector collect over time. This value will be presented in Figure 5.2 #5. It is important to measure the number of counts for different laser powers: 0 (laser blocked), 1, 2, 5, 10, 20, 40, 50, 80,100 μW. The laser power can be measured with a power meter and tuned by the neutral density filters in front of the laser. Obviously, the intensities with the units of counts per seconds can be obtained after the division of the total number of counts with the total acquisition time. The measurement errors should be taken into consideration. Plotting the emission intensities as a function of laser power gives us a saturation curve, which should be fitted using the model given by Equation 1.7.

To have good correlation curves, it is important to excite the emitters at a power lower than the saturation power!

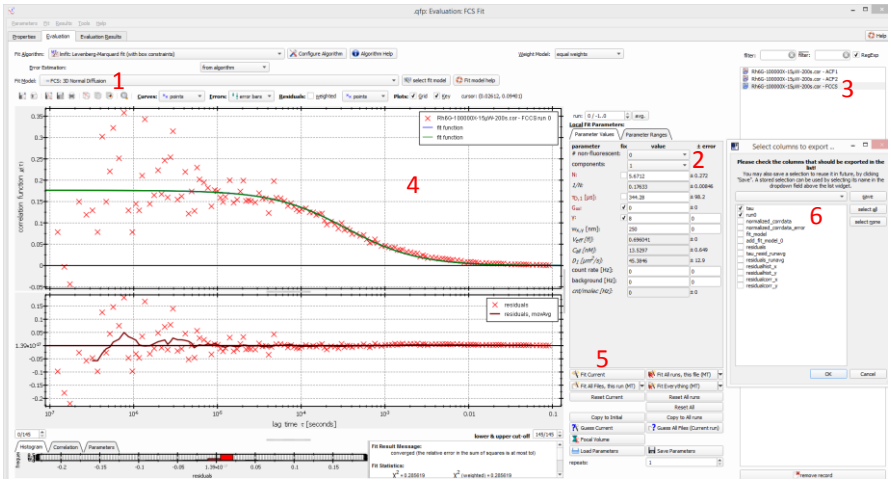

FIGURE 5.3: QuickFit 3.0 for correlation curve fitting. Fit model: 3D Normal Diffusion#1, number of components 1, non-fluorescent 0 #2. Choose only the FCCS curve (the other two are FCS curves which also show undesired afterpulsing) #3. The data points will appear in the window #4. To fit the curve with the chosen model, press Fit Current #5. To save the data, right click on the graph, choose option Copy Data and then select only first two data sets #6.

Chapter 6

Students' tasks

6.1 Tasks

1. Calculation of the confocal volume based on Equations 3.1 and 3.2 for the optical system in the laboratory (the numerical aperture of the objective lens is $NA = 0.6$ and $\lambda = 532nm$).

2. Measurements of the emission spectrum for a high concentration of Rh6G molecules. Plot the emission spectra and make a comment on the curve shape. How is it related to the shape presented in Figure 1.4?

3. Measurement of the saturation curve for a known concentration of Rh6G in water, fitting the data using the model given by Equation1.7 and obtaining R_∞ and I_s from the fit. For the saturation curve fitting, 10 data points would be sufficient, as suggested in Chapter 5. To estimate the average values and measurement errors, it is recommended to collect photons for 30 s and to repeat the same measurement five times. This is a good approach to get an average value with the corresponding error.

4. Estimation of the effective volume based on the results obtained from the FCCS curves for a sample of known concentration. Please compare this value with the calculated value. To calculate the effective volume, you can use Equation 4.7.

5. FCCS measurements of Rh6G dissolved in a mixture of water and glycerol. The goal is to compare the diffusion times and show that they increase with the increase of a glycerol content in the mixture. Five samples will be prepared with different ratios of water and glycerol: 100 % H_2O; 75 % H_2O, 25 % glycerol; 50 % H_2O, 50 % glycerol; 25 % H_2O, 75 % glycerol; 10 % H_2O, 90 % glycerol.

6.2 Output

1. A good report should be clearly structured. Split it into several parts: Abstract, Introduction, Theory, Experimental Section, Data analysis, Discussion and Conclusions.

2. Figures should have figure captions with explanations. All graphs should have labeled axes and visible data points and curves. Curve fits and fitted parameters should be included.

3. All quantities should be followed by the calculated error.

4. It is important to use your own words to explain what you observe in the experiment and to interpret the data. Graphs and numbers without interpretation are not sufficient.

If at any point during your data processing or report writing you have any doubts, you are encouraged to contact your teaching assistants. We are more than happy to help you finish your task and learn as much as possible.

We wish you good luck with experiments!

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