Lab Course B2 in Lab NU115

Brownian motion and single particle tracking

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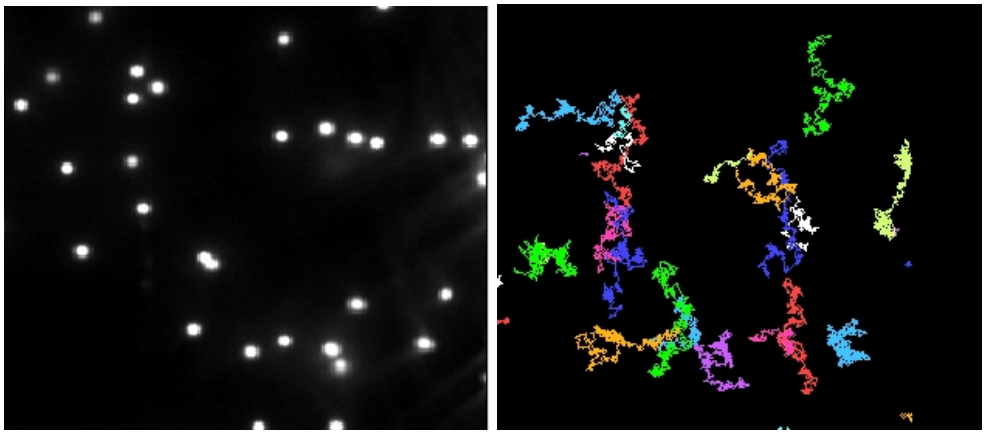

Figure 1: **Left)** Fluorescent particles under a fluorescence microscope. **Right)** color coded particle tracks show the individual beads movements.

Contents

1 Aim of this course

This lab course will get you in touch with two of the most essential concepts in biophysics: fluorescence microscopy and brownian motion. You will use a basic version of fluorescence microscopy to determine the diffusion coefficient of fluorescent beads. We chose a basic version of fluorescence microscopy for you, such that you can easily learn the basics and appreciate the power and elegance of the technique. At the same time you should get a feeling for how it is to work in the lab and what kind of problems you encounter when trying to obtain good results.

The lab course will take a full day. In the morning you will discuss the experiment and its background with your supervisor and get to know the tools. In the afternoon you will have the opportunity to work mostly on your own and carry out the measurements. Note that you can contact your supervisor at any time. Please bring a USB stick with you to save some experimental data for your report.

The following manual will provide you with some background information on fluorescence microscopy and diffusion, which will help you to better understand the experiments and subsequent analysis. We ask you to thoroughly read through the manual before coming to the lab course. While reading, you will notice that the manual also contains a couple of questions, marked in blue. Please be prepared to answer those and other questions in a brief discussion with your supervisor showing that you comprehend the content of this manual before starting with the experiments. You will not need to hand in the questions in written form.

We also encourage you to ask questions at any time: during the discussion session, the experiment or while you are working on the analysis. The supervisor's job is to teach you, while your responsibility is to ask the questions.

Have fun with the experiments!

2 Fluorescence

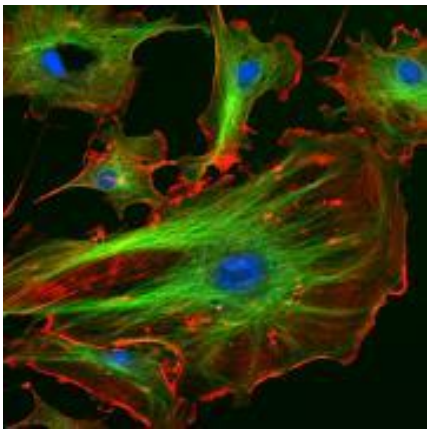

Figure 2: Fluorescence picture of living cells, with microtubuli stained in green, actin filaments in red and cell nuclei in blue.

In many applications in science, the major task is to observe one or more components of complex systems such as cells or ensembles of molecules. Research in biology and biophysics nowadays concentrates on the length scale of micrometers to nanometers, which corresponds to the size of cells down to single proteins. A very common way to fulfil this task is to mark the molecules of interest with fluorescent dyes and to observe the emitted light of these molecules.

In Figure 2, actin filaments, microtubuli and the nuclei of cells are stained with different fluorescent dyes and can easily be distinguished. Recommendable tutorials about fluorescence can be found on: `http://www.invitrogen.com/site/us/en/home/support/Tutorials.html`. To understand how fluorescence works, two aspects have to be taken into consideration: the fluorescent dye and the fluorescence microscope.

Question: Why is it not possible to observe proteins, mRNA and other biomolecules with an ordinary microscope?

Question: Fluorescence microscopy can help overcome this problem. What other techniques are used to investigate macromolecules?

Principle of Fluorescence

Fluorescent dyes are organic or inorganic molecules with a metastable energy level. They absorb light within their characteristic spectrum around the so-called absorption wavelength. If energy in form of a photon with the adequate wavelength hits an electronic system (i.e. a molecule), the system is excited to a higher quantum state. It instantly relaxes to a lower, metastable energy level (which means it has a longer residue time than statistically expected). Subsequently, it relaxes to a lower energy state. In this process, it loses energy due to rotations or oscillations. This new state has still a higher energy than the ground state, but also a slightly lower energy than directly after the excitation. To reach the ground state again, the system emits a photon with the wavelength corresponding to the remaining energy gap. Therefore the emission wavelength is slightly higher than the absorption wavelength. In Figure 3 an energy diagram and the typical spectrum of the fluorescent dye Cy5 are shown. The latter one is a screen-shot from the *invitrogen spectra viewer*, which is a very useful tool provided by a supplier of dyes to find the right dye-filter combination. It is free, you find it here (for the use of filters see section 2): `http://www.lifetechnologies.com/order/spectra-viewer`.

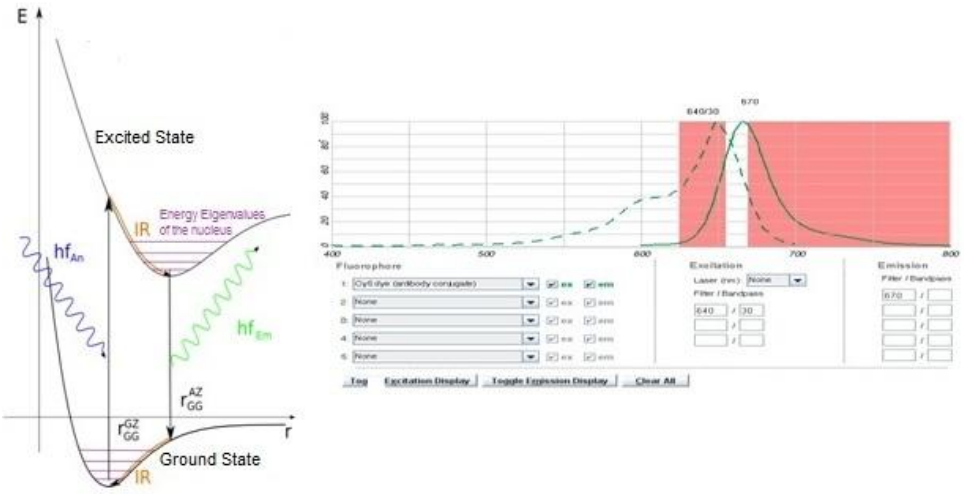

Figure 3: **Left)** excitation and emission of a fluorophore. The x-coordinate is the molecule conformation and the y-coordinate the energy. **Right)** excitation and emission spectra of Cy5 (red areas mark the transmission wavelengths of the filter).

Question: How do energy and wavelength of a photon relate?

In the course of illumination, some of the dye molecules get destroyed, so the overall fluorescence intensity decreases. This is also called photo-bleaching. In this process, the photon energy changes the shape of a molecule (e.g. isomeric transitions) instead of inducing the emission

of another photon. Thus the energy levels of the system change and it is no longer excitable with the applied absorption light. Consequently, the fluorophores are afflicted with permanent bleaching.

Question: There are a couple of techniques to investigate molecules that are based on fluorescence. Explain three of the following: TIRF, STED, PALM/STORM, FCS, FRAP, Light-sheet microscopy.

Question: We do not use it here but it is an interesting technique: What does the abbreviation FRET stand for? How does it work?

Numerical Aperture

The following paragraph should enable you to choose the right objective. The numerical aperture is defined as $\mathrm{NA} = n \cdot \sin\phi$, where n is the refractive index whilst ϕ is half the opening angle. The NA defines the ability of an optical element (e.g. lens, objective) to focus light. The higher the NA, the smaller the focus. A common trick is to add immersion oil between the objective and the coverslip of the sample.

Question: Why does adding immersion oil lead to a larger numerical aperture?

Abbé found the optical resolution of a microscope to be $d_{\min} = \frac{0.61 \cdot \lambda}{\mathrm{NA}}$, where $d_{\min}$ is the minimal distance between two points to be discriminated and λ is the wavelength of the light.

Question: How does the depth resolution change with NA? Explain why.

Fluorescence Microscope

Question: How does a normal light microscope work (two lenses)? What is a virtual image? Be able to answer this question and the lensmaker's equation $\frac{1}{f} = \frac{1}{b} + \frac{1}{g}$ with a drawing.
If you are interested, more complex microscopes are shown at `http://micro.magnet.fsu.edu/primer/anatomy/kohler.html`.

The setup of a fluorescence microscope resembles a normal light microscope. However, the objective is infinity corrected, which means that the light is parallelized between the tubus lens and the objective. This facilitates the insertion of optical devices into the optical path without influencing the imaging.

A typical setup is depicted in the left part of Figure 4: The excitation light is filtered from an LED light source. The filter cleans up the light and allows only a narrower band of wavelengths to pass. The light is then directed onto the sample via a dichroic mirror. The special characteristic of this mirror is to split up absorption and emission light of fluorophores. It allows the transmission of the emission light, while it reflects the excitation light. The transmission characteristics for the optical elements are displayed in Figure 4.

An additional emission filter in front of the tubus lens finally reflects or absorbs all remaining diffuse light to ensure that only the emission light gets to the camera. The insertion of filters reduces the noise, which is immensely important for biophysical applications.

Question: In our lab, we use fluorescence microscopes that allow observation of two different colors at the same time. Which additional components have to be added to the setup shown in Figure 4?

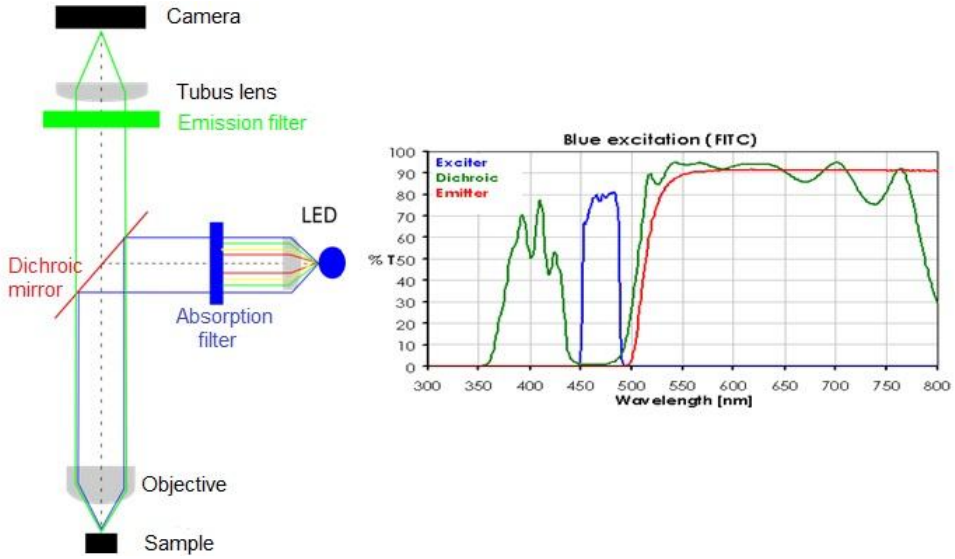

Figure 4: **left)** optical path of a typical fluorescence microscope. **right)** characteristics of a filter set.

3 Diffusion

Diffusion is a very important mechanism in the microscopic world. For example, in living cells it is responsible for some transport processes. An example from daily life is a drop of ink in a glass of water. After sufficiently long time it will have spread throughout the glass. In general, diffusion describes the movement of particles in a solution due to the thermal motion of the bulk molecules. Due to statistical (entropical) reasons, this leads to the equilibration of concentration gradients.

Let us take a look at a diluted solution of particles with concentration c. The chemical potential in such a solution is given by

$$\mu(\vec{r},t) = \mu_0(\vec{r},t) + k_\mathrm{B}T \ln c(\vec{r},t). \tag{1}$$

with k_B being the Boltzmann constant and T being the temperature. Therefore, the potential is position dependent and varies with the concentration. The thermodynamic force resulting from the gradient of a chemical potential is given by

$$\vec{F_1}(\vec{r},t) = -\nabla\mu(\vec{r},t). \tag{2}$$

It is counterbalanced ($\vec{F_1}+\vec{F_2}=0$) by the friction in the solution. For laminar flow and spherical particles, this counter force is given by Stokes:

$$\vec{F_2}(\vec{r},t) = -6\pi\eta a\vec{v}(\vec{r},t) \tag{3}$$

with η being the dynamic viscosity of the solvent, a being the radius of the particle and $\vec{v}$ being the flow velocity. From this we can get

$$\vec{v}(\vec{r},t) = -\frac{k_\mathrm{B}T}{6\pi\eta a}\frac{\nabla c(\vec{r},t)}{c(\vec{r},t)}. \tag{4}$$

In addition, all particles will show a random jiggle motion, which is called Brownian motion. It originates from collisions with solvent molecules, or generally speaking, other molecules in solution. The intensity of the movement depends on the temperature. Although the collision forces are much larger than the forces from the gradient of the chemical potential, they cancel

out on average as they point in all directions. So, for the average propagation velocity of the species, only the chemical potential is important.

The net particle flow is given by

$$\vec{j}(\vec{r},t) = c(\vec{r},t) \cdot \vec{v,t}(\vec{r}) = -\frac{k_\mathrm{B}T}{6\pi\eta a}\nabla c(\vec{r},t). \tag{5}$$

Using the Stokes-Einstein equation

$$D = \frac{k_\mathrm{B}T}{6\pi\eta a} \tag{6}$$

we can rewrite it to get Fick's first law

$$\vec{j}(\vec{r},t) = -D\nabla c(\vec{r},t). \tag{7}$$

If we take into account that the particle number is constant (continuity equation),

$$\frac{\partial c(\vec{r},t)}{\partial t} + \nabla\vec{j}(\vec{r},t) = 0, \tag{8}$$

we get Fick's second law:

$$\frac{\partial c(\vec{r},t)}{\partial t} = D\triangle c(\vec{r},t). \tag{9}$$

This partial differential equation can be analytically resolved in some cases (special initial conditions). For example in one dimension, a delta function $c(t=0) = \delta(x)$ will diffuse like

$$c(x,t) = \frac{1}{\sqrt{4\pi Dt}}\exp\left(-\frac{x^2}{4Dt}\right). \tag{10}$$

From this partial differential equation, we can calculate that the expectation value of how far a particle diffuses on average, is zero

$$\langle\ \vec{r}\ \rangle = \int \vec{r}\ c(\vec{r},t)\ d\vec{r} = 0, \tag{11}$$

because the probability is the same in all directions. In this equation the probability to find a particle at position $\vec{r}$ at the time t can be substituted with the concentration. We therefore look at the second moment:

$$\langle\ \vec{r}^2\ \rangle = \int \vec{r}^2\ c(\vec{r})\ d\vec{r} \tag{12}$$

gives

$$\langle\ (\vec{r})^2\ \rangle = \alpha D t. \tag{13}$$

Where $\vec{r}$ is how far the particle diffused in t time and $\alpha = 2d$, where d is the dimension of the motion (or the dimension of the observation).

With equation 13 we can calculate D from the tracks of single particles. In other words: If we can track single particles, we do not need to measure concentration changes, but we can measure the diffusion coefficient in a homogeneous particle solution.

4 Setup

A self-built microscope is used to observe bead positions in solution. For excitation we use a collimated continuous Light Emitting Diode (M505L2-C4; Thorlabs, Newton, New Jersey, USA) with a center wavelength at 505 nm, an optical beam power between $335 - 620$ mW (depending on LED wavelength). A maximum drive current of 1000 mA should **not** be exceeded! The LED

light passes an excitation filter and is coupled in from the side of the microscope via a dichroic mirror (see Figure 4). To keep the beads in place, a $100 \times 1000\,\mu m^2$ (inside geometry) sized rectangular capillary of borosilicate glass will be used as probe chamber. It will be sealed with modeling clay to prevent evaporation. The magnification is performed by a 20x Zeiss objective with an NA of 0.5. The emitted light crosses the dichroic mirror and the emission filter, which ensures that no excitation light reaches the camera. The emission light is monitored by an AVT-Stingray F145B camera (Allied Vision Technologies) with a quadratic pixel size of 6.45 µm. In the camera head, some pixels can be combined to make the camera faster and increase the sensitivity. This is called binning. The drawback is the decreased spacial resolution.

Question: Compare the pixel size with the optical resolution.

A peltier element (PC-128-10-05, Telemeter Electronic GmbH, Donauwörth, Germany) is used to set the temperature and to create a temperature difference. On one side is a silicon sample holder (high heat conductivity). Do not drive the peltier element above 15.8 V or 9 A! The LED and Peltier element are operated by a diode and temperature controller (ITC4005; Thorlabs, Newton, New Jersey, USA). The ITC4005 keeps the Peltier element at a certain temperature by adjusting the current.

5 Measurements

The main task in this experiment will be the measurement of the diffusion coefficient for beads of different sizes in aqueous solution at multiple temperatures. To do so, we use polystyrene beads (Invitrogen F8888) with a fluorescine-dye label (the properties are equivalent to BCECF; use the spectra-viewer to obtain the excitation and emission wavelengths). A fluorescence microscope with the matching filters and illuminating LED allows us to visualize these beads. Via a CCD camera, the movement over time will be recorded for further analysis.

Equation 13 will help you to practically measure the diffusion coefficient D. The diffusion process does not have a designated beginning. To get more statistics, we can compare all position differences of one trajectory. How far has the particle moved? Then we sort these values for the timesteps Δt and average over all values for each Δt. Plotting these averages in a diagram $\langle\, \vec{r}^2 \,\rangle$ over t, we can fit a line through origin (in 0 time it cannot diffuse anywhere) and get a slope of αD (cf. equation 13). With equation 6 you can calculate the theoretical values and compare them to the measured ones.

Performing the following steps should lead you to a successful experiment:

1. Make yourself comfortable with the surrounding setup: It is important that you know the stuff you are working with. Make sure the filters are correctly placed! Otherwise the intensity of light will drop dramatically, the light path will be misaligned and therefore no image will be shown. In case of questions, ask your tutor.

2. Prepare the samples: In this experiment, you are supposed to measure the diffusion coefficients of beads of different diameters. Use the stock solutions and dilute them to a level, where you are able to track single beads along their way. The visible ones should not overlap too often, but you should see some beads to get a decent statistic. For the preparation of your samples, you will use the micro-pipettes of our lab. There are a few rules for working with them:

    - Always use them with latex-gloves.

- Do not touch anything else than the upper part of the pipette. Do not touch the pipette tips with your fingers, your tutor will show you how to attach them to the pipette. Try to avoid getting chemicals/sample on your pipettes.
- Change the pipette tip for every mixing process. Dispose the old tip in the tip litter! In case you do not, stock solutions could be contaminated (and therefore become useless) with whatever was in and on the pipette tip.
- Before pipetting, clean the working area with a 70 % isopropanol/water mix to remove dirt and micro-organisms.

If you need water in your probes, always use MilliQ. This is a special cleaned water for laboratory use. It has the minimal possible amount of ions, just H^+ and OH^-.

Question: How many H^+ and OH^- ions do you expect?

Question: What is the molarity of water? Is it possible to dilute that? How and under which circumstances?

To find the best concentration, use the data from the manufacturer, or simply give it a try. A good starting point should be: 1 µl of stock solution, 199 µl of water.

Question: Propose a dilution step to prepare a solution of 2.5 µM from a stock of 150 µM. General nota bene (not necessary for the calculation): One dilution should never exceed a factor of 100.

3. After preparing your solution in an Eppendorf sample tube, fill the capillary simply by using capillary forces. After that seal the capillary using modeling clay (this prevents drift and evaporation).

4. Position the capillary underneath the copper plate right above the microscope and focus inside the chamber until you see the beads. Do not touch the sample with the objective, this might destroy the objective. The positioning is done by placing the capillary onto a steel plate and then clicked underneath the copper plate which is connected to the peltier element. The Steel plate will be hold by the magnetic field which is generated by two magnets.

5. Find the best values for the parameters LED illumination. Note the used binning, exposure time, frame rate, objective (magnification and NA) and the analysis threshold for your report!

6. Record a movie using the provided LabView software. It will export the frames of the movie to a series of single tiff files into a folder of the computer. Take about 300 pictures. Be careful to always specify a new folder, as it will overwrite older frames without asking. Record movies for all particle sizes each at varying temperatures between 20 °C and 55 °C. You should not freeze the probe and you should not boil it. Sometimes if you use temperatures too low, you get problems with condensing water. In this case, you should turn up the temperature a bit. Wait a short period of time until the temperature is equilibrated to the set temperature. The ITC4005 is configured with a temperature and voltage protection. If you set your new temperature to high/low from your current temperature the voltage protection will turn your TEC off. In this case turn it on and slowly in-/decrease the temperature to your demanded set-point.

6 Analysis

We prepared a particle tracker program in LabView that gives the positions of every particle over time as output. Use that VI to program a routine to obtain the diffusion coefficient. Ask

your tutor for help if needed. If you have time during the preparation for this course, you can get familiar with the LabView programming language.

7 Lab View Introduction

In this practical course, you will use the visual programming language *LabView* to evaluate your data. To make you more familiar with this program, you will have to complete the analysis software used for this experiment. Below, you will find information on how to complete this task. The rest will be explained by your tutor.

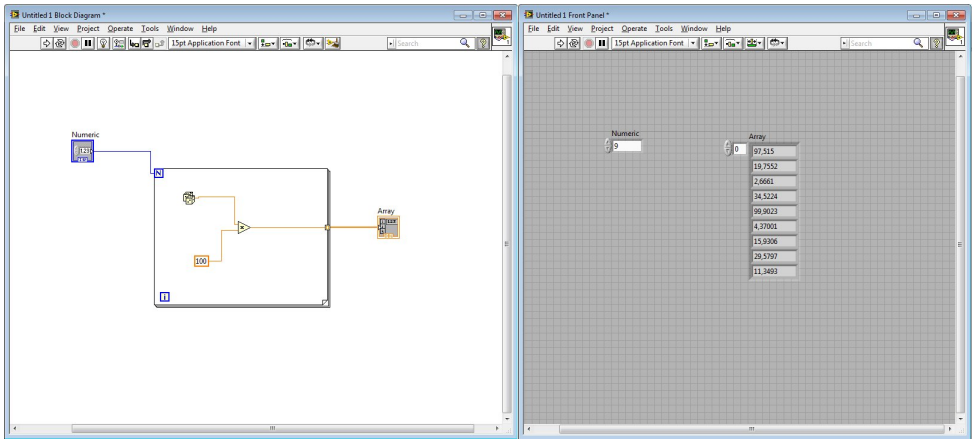

Figure 5: LabView example program.

In LabView, all the structures you know from other programming languages (loops, operations, arrays etc.) are visible objects. In Figure 5, left side (Block Diagram), is a short example showing a random number generator (two dices) inside a for-loop. The loop starts at $i = 0$ and stop after $i = 8$, completing nine rounds. Each round, the number generator creates a random number between 0 and 1, which is then multiplied by a constant factor of 100. The results are fed into an array. On the right hand side is the front panel, where you can read out your data and in this case specify how many rounds the for-loop should complete. This then also determines the length of your array. In general you do your programming in the Block Diagram and control it from you front panel. The latter shows e.g. graphs, control buttons, arrays etc. and allows you to save your data.

In your experiment, you will track the position of diffusing beads, from which you can calculate the distance covered per time step for every bead. You will only have to complete a small part of the tracking and evaluation software that contains the calculation of the bead displacement.

8 Report

During the next week please write a protocol of the experiment in English and not exceeding 20 pages, answering the following question:

- Overview and preparation: What did you do? You do not need to reproduce this tutorial, but please give a **really short** overview of the important parts of the theoretical part. Among others, shortly explain the difference between random motion, diffusion and drift in your own words.
- Simulation: Simulate a random walk in 3D (in the language of your choice e.g. python). Project those results onto a 2-dimensional plane. How does this change the diffusion constant?
- Experiment and discussion: What were your results? Discuss them using **plots**. Did you encounter difficulties? What are potential error sources? Are your findings trustworthy? How do they compare to the theoretical predictions?
- Further understanding: Project the 2D trajectories of 1 to 2 of your best measurements onto 1D. Compare your findings to the simulation. What do you find? How do the diffusion coefficients relate?