Prof. Dr. Dieter Braun and Dr. Christof Mast

Brownian motion and single particle tracking

Virtual lab course version

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Supervision:
Juliette Langlais
Maximilian Weingart
Sreekar Wunnava

Please send your request to the following mail address:
praktikum-braun@lists.physik.uni-muenchen.de

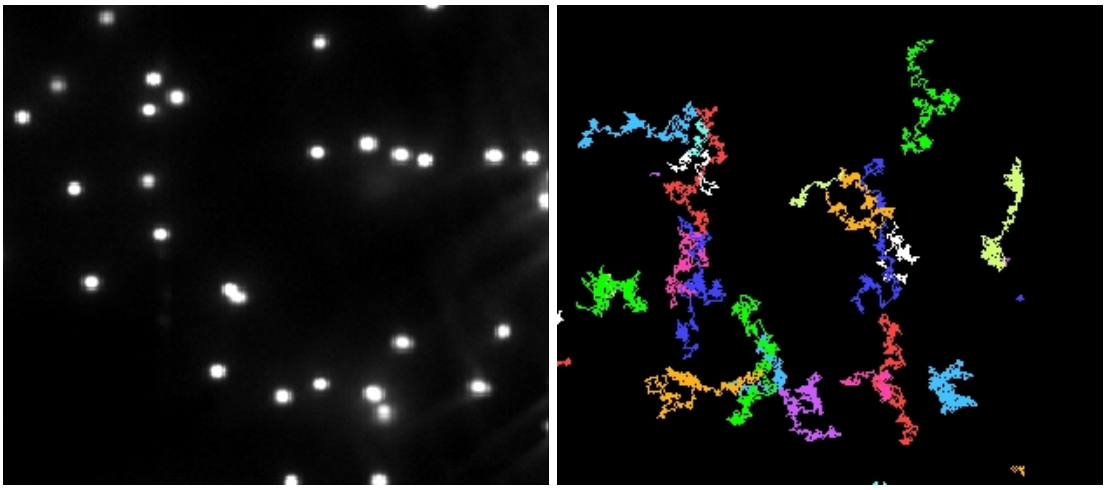

Figure 1: **Left)** Fluorescent particles under a fluorescence microscope. **Right)** color coded particle tracks show the individual beads movements.

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1 Preface

According to the Covid-19 safety regulations at LMU, the lab course will this semester only be possible in virtual form. Nevertheless, we will try to give you an impression of practical work in the lab as far as possible by providing you videos and remote access to one of our computers. For the remote connection, we use the the free client *AnyDesk*. It can be downloaded form `https://anydesk.com/en/downloads/windows`. Please install it on your computer prior to the 1st colloquium.

The lab course will take around 7-8 hours for 1st colloquium and data acquisition. After that you will have a two weeks deadline for data analysis and report preparation. During the 1st colloquium your supervisors will discuss the experiments and the necessary theoretical background with you. Then we will send you a short video about the sample preparation to give you at least a rough impression of the practical work necessary for the experiments. When you are comfortable with the theory and the methods you will be provided with 2–3 prerecorded datasets that you will analyse by connecting to one of our computers via *AnyDesk*. After the basic instructions of the software, your supervisors will be available via mail at any time in case you have any further questions.

Within this practical course you are going to measure the diffusion coefficients of fluorescent polystyrene beads by using a basic version of fluorescence microscopy and statistical data evaluation. Therefore, it will bring you in touch with the following techniques and concepts:

- Fluorescence and fluorescence microscopy
- Motion of particles in fluids (Brownian motion, Diffusion and Drift)
- Simulation of a random walker
- Statistical data evaluation

The following manual will provide you with some theoretical background 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 you attend the colloquium. You will notice that the manual contains a couple of questions, marked in blue. Please be prepared to answer those and other questions in a brief discussion with your supervisors showing your comprehension of the necessary background. You will not need to hand in the questions in written form. Besides that, we would also encourage you to look up other sources on your own if any of the theory remains unclear.

Feel free to ask questions at any time: During the discussion session, about the video of the experiment or while you are working on the analysis. The supervisor's job is to teach you, while your responsibility is to ask questions.

Have fun!

2 Fluorescence

Fluorescence is a phenomenon where a molecule (fluorophore) absorbs electromagnetic radiation and re-emits it at a longer wavelength. This molecular phenomenon finds applications in various fields of science and also in daily use (e.g. fluorescent lamps).

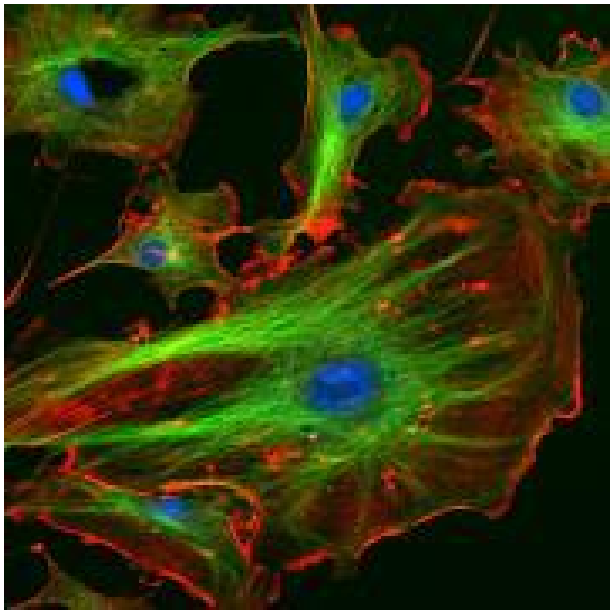

Figure 2: Fluorescence picture of living cells, with microtubuli stained in green, actin filaments in red and cell nuclei in blue.

Especially in biological and biophysical research fluorescence microscopy is highly advantageous over bright-field microscopy. Most cellular features are in the range of nanometers (proteins) to micrometers (nucleus) and are not intrinsically coloured or fluorescent. Thus, observing them requires staining or marking regions/molecules of interest with specific fluorescent dyes. This not only allows us to enhance the contrast of the samples but, in some fluorescence microscopy techniques, allows us to monitor the dynamics of the system as well. In the above Figure 2, actin filaments, microtubuli and the nuclei of cells are stained with different fluorescent dyes and can easily be distinguished. An informative tutorial about fluorescence can be found at: `http://www.invitrogen.com/site/us/en/home/support/Tutorials.html`.
In the following sections, we look at the principle of fluorescence and its application in fluorescence microscopy.

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 (Fluorophores) are organic or inorganic molecules that can absorb a photon of specific energy, get exited and re-emit a photon of lower energy. The wavelengths of the light that is absorbed must be within the characteristic absorption/excitation spectrum of the molecule and similarly, the emitted light is within the emission spectrum. Maximum Absorbance can be obtained at the centre of the peak, at the so-call absorption wavelength.

After absorbing a photon of right energy, 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 and loses some energy in rotations or oscillations. This new state still has a higher energy than the ground state, so the system emits a photon corresponding to the energy gap between this new state and the ground state, and returns to the ground state. Thus, the emitted light has higher wavelength than the absorption wavelength. In Figure 3 an energy diagram and the typical spectrum of the fluorescent dye Cy5 are shown. We recommend checking out the *invitrogen spectra viewer* at `http://www.lifetechnologies.com/order/spectra-viewer`. It is a very useful tool provided by a supplier of dyes to find the right dye-filter combination (for the use of filters see section 2).

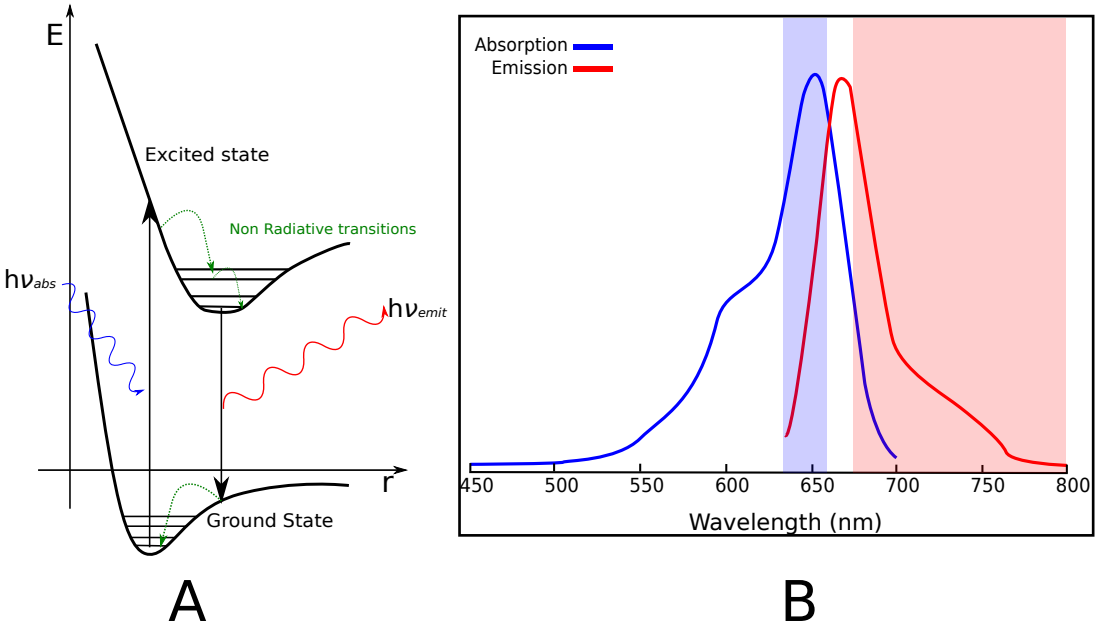

Figure 3: **A)** Excitation and emission of a fluorophore. The x-coordinate is the molecule conformation and the y-coordinate the energy. **B)** Excitation and emission spectra of Cy5. The shaded regions show possible ranges for absorption and emission filters.

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

Upon long exposures with high intensity light, some of the dye molecules get irreversibly destroyed and 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. Even though it seems like a disadvantage, some techniques specifically use this property of fluorophores.

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 and optical resolution

The Numerical Aperture (NA), given as a dimensionless number, defines the ability of an optical element (e.g. lens objective) to focus light and can be calculated as $\mathrm{NA} = n \cdot \sin\phi$, where n

is the refractive index of the medium between the object and the optical system, ϕ is half of the maximum opening-angle for which light can enter the optical system. To have a short focus distance and be able to see small objects, a high NA is wanted. A common trick in microscopy to increase the value 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

$$d_{\min} = \frac{0.61 \cdot \lambda}{\mathrm{NA}}, \tag{1}$$

where $d_{\min}$ is the minimal distance between two points to be discriminated and λ is the wavelength of the light.

Question: Which visible light's color gives the highest resolution? Can you think of a difference between DVD and BluRay based on equation 1? What do you need to take into account if using shorter wavelengths?

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.

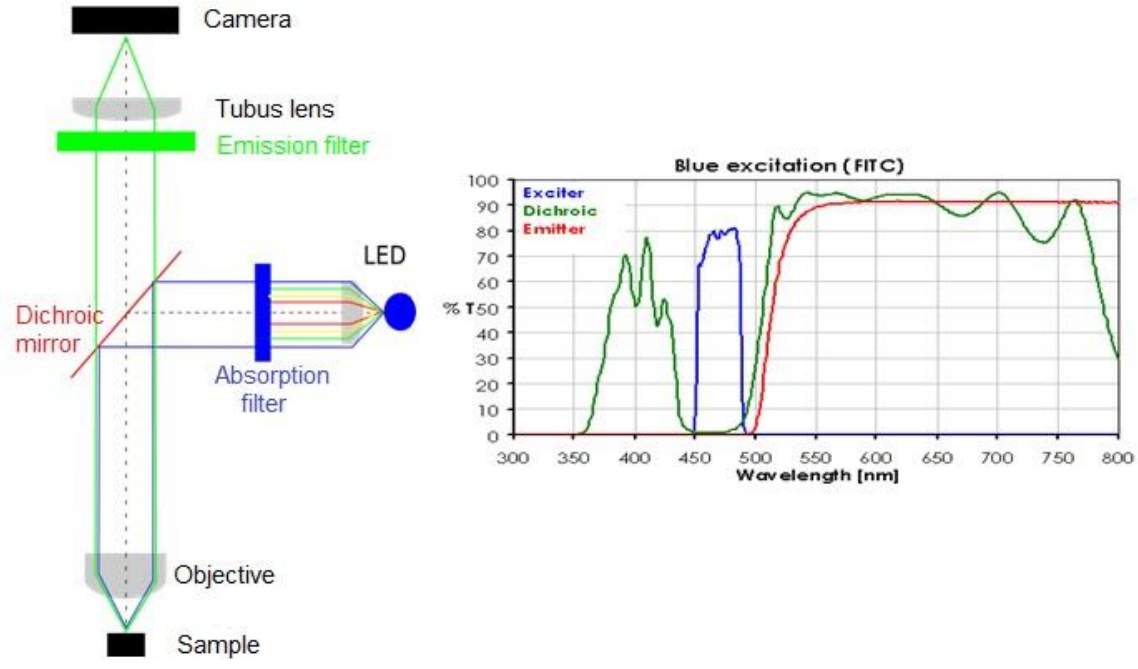

Figure 4: **left)** Optical path of a typical fluorescence microscope. **right)** Characteristics of a filter set.

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 fluorophores at the same time. Which additional components have to be added to the setup shown in Figure 4?

3 Motion of Particles in Fluids

If molecules of a certain species are diluted in a liquid or gas, i.e. are surrounded by bulk molecules of another species, they are subject to different kinds of motion. Which type of motion they experience depends on the exact setting and the boundary conditions of the system. In this experiment, we will mainly deal with the three most common types: Brownian motion, diffusion and drift.

3.1 Brownian Motion

In the experiment fluorescence labeled beads are diluted in water. All particles will show a random jiggle motion, which is called Brownian motion. It originates from collisions with solvent molecules (i.e. water molecules in our case), or generally speaking, other molecules in solution. The intensity of the movement depends on the temperature and rises with increasing temperature.

3.2 Diffusion

Diffusion is an important mechanism in the microscopic world and describes the movement of particles in a solution along a concentration gradient. Especially in biological systems many transport processes are driven by diffusion. 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, the particles move from regions of higher concentration to regions of lower concentration, driven by the thermal motion of the bulk molecules. Due to statistical (entropical) reasons, this leads to the equilibration of the 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

$$\mu(\vec{r}, t) = \mu_0(\vec{r}, t) + k_\mathrm{B} T \ln c(\vec{r}, t). \tag{2}$$

with the Boltzmann constant $\mathrm{k_B}$ and the temperature T. 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

$$\vec{F_1}(\vec{r}, t) = -\nabla \mu(\vec{r}, t). \tag{3}$$

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' law:

$$\vec{F_2}(\vec{r},t) = -6\pi\eta a\vec{v}(\vec{r},t) \tag{4}$$

with η being the dynamic viscosity of the solvent, a being the radius of the particle and $\vec{v}$ being the flow velocity. Combining now equations 2 – 4 this gives us

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

Even though the collision forces due to Brownian motion are much larger than the forces from the gradient in chemical potential, they cancel out on average since they are equally likely in each direction. So, for the average propagation velocity of the species, only the chemical potential is important.

We can now rearrange equation 5 to obtain the net particle flow

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

Using the Stokes-Einstein equation

$$D = \frac{k_\mathrm{B}T}{6\pi\eta a} \tag{7}$$

we can rewrite it to get Fick's first law

$$\vec{j}(\vec{r},t) = -D\nabla c(\vec{r},t). \tag{8}$$

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

$$\frac{\partial c(\vec{r},t)}{\partial t} + \nabla\vec{j}(\vec{r},t) = 0, \tag{9}$$

we get Fick's second law:

$$\frac{\partial c(\vec{r},t)}{\partial t} = D\triangle c(\vec{r},t). \tag{10}$$

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

$$c(x,t) = \frac{1}{\sqrt{4\pi Dt}}\exp\left(-\frac{x^2}{4Dt}\right). \tag{11}$$

From this partial differential equation, we can calculate that the expectation value for the average traveled distance of a particle is zero

$$\langle\ \vec{r}\ \rangle = \int \vec{r}\ c(\vec{r},t)\ d\vec{r} = 0, \tag{12}$$

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:

$$\langle\ \vec{r}^2\ \rangle = \int \vec{r}^2\ c(\vec{r})\ d\vec{r} \tag{13}$$

gives

$$\langle\ \vec{r}^2\ \rangle = \alpha Dt. \tag{14}$$

Where $\vec{r}$ is the traveled distance in time t and $\alpha = 2d$, where d is the dimension of the motion (or rather the dimension of observation).

With equation 14 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.

3.3 Drift

Drift is a directed motion that is caused by external forces to the system. Since we should not have any concentration gradients in our samples, drift would occur as a net movement in a specific direction.

Question: How could you adapt equation 14 to account for possible drift with drift velocity $\vec{v}_D$? What could cause drift in our experimental setup?

4 Setup

The following section describes the setup that is used to record the image sequences of the beads in solution. The self-built microscope (as sketched in Figure 4) applies 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 dicroic mirror. 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.

To keep the beads in place, a $100 \times 1000\,\mu m^2$ (inside geometry) sized rectangular capillary of borosilicate glass is used as probe chamber and sealed with modeling clay to prevent evaporation.

A peltier element (PC-128-10-05, Telemeter Electronic GmbH, Donauwörth, Germany) is used to set the temperature and to create a temperature difference.[1] On one side is a silicon sample holder (high heat conductivity).

5 Measurements

The aim of the experiments is to measure 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 is recorded for further analysis.

The adapted version of the equation 14,

$$\langle\, \Delta\vec{r}^2 \,\rangle = \alpha D \Delta t, \tag{15}$$

[1] 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.

and the exercise during the 1st colloquium will help you understand how the diffusion coefficient D is measured by tracing the tracks of single particles, starting at an arbitrary initial time. Therefore, equation 15 only considers time intervals Δt. For the experiment itself, you will not have to calculate D for every timestep manually, one of the *LabView* programs will do it for you.

To get more statistics, we compare all position differences of one trajectory. 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 and get a slope of αD (cf. equation 15). How exactly the statistical data evaluation in the applied algorithm works will be discussed with your supervisors during the 1st colloquium.

The following part will explain good experimental practice - even though you will not do the experiment yourself, you should read it carefully and compare how it is done in the video.

1. Inspect the setup that you will be working with (try to do it for the picture in the video). Can you recognize the main elements of the fluorescence microscope on the physical set up? 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. Sample preparation: In this experiment, you are supposed to measure the diffusion coefficients of beads of different diameters. The provided stock solutions are at a too high concentration, so they need to be diluted in order to be able to track single beads: Overlapping and high bead densities should be avoided so that the software can differentiate them. Still, there should be enough visible beads to get decent statistics. The solution are prepared using the micro-pipettes of our lab and mixed by vortexing and centrifugation. There are a few general rules for working with them:

   - Always use them with latex-gloves. This has mainly two reasons: On the one hand, your skin should be protected when working with chemicals. On the other hand, also your samples should be protected form contamination with organic material, which is especially important, if you are working with RNA or DNA.
   - Before pipetting, clean the working area with a 70 % isopropanol/water mix to remove dirt and micro-organisms.
   - 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 (in the video) 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.

   If you need water in your probes, always use MilliQ H_2O. This is a deionised water for laboratory use treated to contain the minimal possible amount of ions, just H^+ and OH^-.

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

   Question: What is the molarity of water? Is it possible to dilute that?

   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. Important remark: Avoid doing a single dilution of a factor higher than 100, it is better to do several ones successively if necessary.

   Question: Propose a dilution step to prepare a solution of 2.5 µM from a stock of 150 µM.

3. After preparing the solution in an Eppendorf sample tube, it can be filled into the capillaries by simply dipping the in the solution. Capillary forces will do the rest. After that the capillaries are sealed on both ends using modeling clay (this prevents drift and evaporation).

4. The capillaries are positioned under the steel plate on the Peltier element in front of the microscope. The steel plate is hold in place by two magnets on the side of the copper block. By focusing inside the capillary (changing the slide in the axial direction) the beads will appear on the screen. The objective must not touch the capillary, this might destroy the lens.

5. Find the best values for the parameters in the image acquisition and tracking program (or check which values have been used in the video). Note the used binning, exposure time, frame rate, objective (magnification and NA) and the analysis threshold for your report!

6. The provided *LabView* program (Tracker) will record an image sequence of 300 hundred frames, exported as a series of single tiff files into a folder of the computer. 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.

   Sometimes, if you use temperatures too low, you get problems with condensing water. In this case, you should turn up the temperature a bit. A too high temperature might cause problems with leaking capillaries. Whenever you change the temperature, wait a short period of time until the temperature is equilibrated. The ITC4005 is configured with a temperature and voltage protection. If you set your new temperature too high/low from the current one 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 again.

6 Analysis

You will be provided with a particle tracking program written in *LabView*. This particle tracker gives the positions of every particle over time as output together with the diffusion coefficients obtained through simple and advanced fit. The principle of the algorithm implemented in that program will be explained during the 1st colloquium.

Use that VI to obtain the diffusion coefficients. 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. During the lab course, you will have access to a computer in the lab via AnyDesk, which has the *LabView* programs as well as all the prerecorded data sets you need.

7 *LabView* Introduction

In this practical course, you will use programs coded in the programming language *LabView* to evaluate your data. In contrast to most other programming languages *LabView* is a graphical language. Instead of writing lines of code, there are various graphical objects for all types of data and operations that are connected by colored wires. *LabView* makes it quite easy to generate graphical user interfaces and follow the flow of data once the program is running.

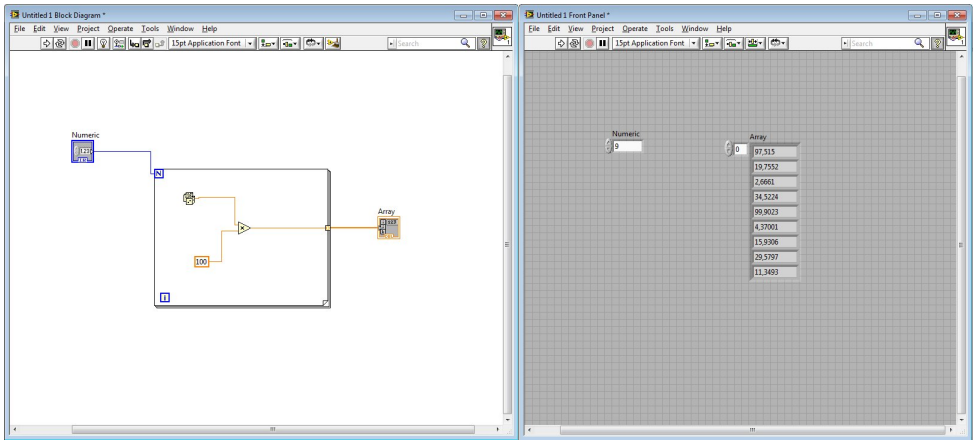

Figure 5: *LabView* example program. On the left you can see the Block Diagram, in which the actual 'code 'is written. The right side shows the front panel with the simultaneously generated user interface.

In *LabView*, all 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 stops 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.

8 Report

Please prepare a report (max. 20 pages) of the experiments in English and send it to us within the next two weeks. A well written report should be an independent scientific document. Remember to give sufficient information so that even the readers who have not read the lab manual should be able to understand the aim, methodology and the results of the experiments described in the report. Make sure that the report answers the following questions:

- **Introduction and Background**: What was the goal of the experiment? Briefly summarize the relevant theoretical background. Among others, shortly explain the difference between random motion, diffusion and drift in your own words. `DO NOT` copy from this lab manual.
- **Experiment and Results**: Briefly explain the setup and the experimental procedure. `DO NOT` copy from this lab manual.
  What are your results? Explain them using figures and **plots**. Also think about what else you could see/plot from your data. Try to get as much information out of it as possible (maybe you have even new ideas).
- **Simulation**: Simulate a random walk in 3D (in the language of your choice e.g. Python). Project those results onto a 2-dimensional plane. Implement the same algorithm that the *LabView* program is using (as explained during the 1st colloquium) and calculate the diffusion coefficient.
  Also, project the 2D trajectories of 1–2 of your best measurements onto 1D.
- **Discussion**: Make a logical connection of all the results from your experiment and simulations and discuss your findings. Are your findings reliable? What are potential error sources? What did you learn from the simulation and projection of your 2D tracks onto 1D? How does the change in dimension affect the diffusion coefficient?

8.1 Presentation of Results

For the 2nd colloquium we would ask you to prepare a short presentation of your results (max. 10–15 min). Just briefly explain the basic concept of the experiment, show and discuss your findings (especially plots). Prepare a few slides (e.g. PowerPoint) or use any other suitable presenting technique you like. Be prepared to answer some questions based on experiment, presentation and report.