Tutorial

Thermophoresis of DNA

This experiment is thought to bring you in touch with the use of fluorescent dyes, which are needed for some of the most important tools in physics in general and in biophysics especially. Here we use two different fluorescent molecules, to give you a little insight into the possibilities.

Furthermore we use the thermodynamic principle of thermophoresis. This effect describes the movement of particles in a temperature gradient, which causes a concentration gradient. This effect is known for more than 150 years phenomenologically. However, the theory, as well as the application as an analysis method for liquid solutions is still subject of contemporary research.

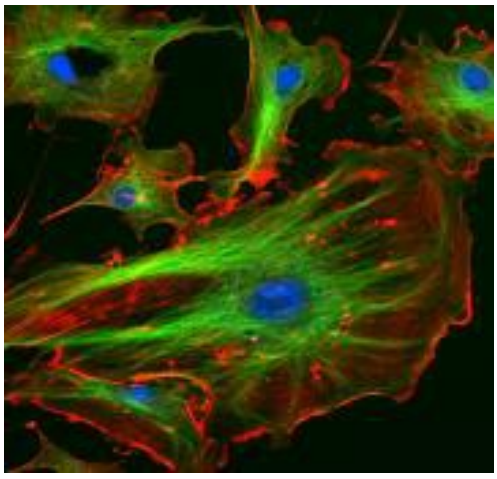


Figure 1: Fluorescence picture of living cells, with microtubuli in green, actine filaments in red and cell nuclei in blue

Munich, 07/27/16

Preamble

One of the most important abilities of a physicist is to ask questions and find good answers. Within this text, you will find questions now and then. The questions are marked with stars.

*) One star means, you should definitely be able to answer this question with the knowledge of previous lectures. In the improbable case you cannot answer one of these questions, follow the hints or check any standard physics book.

**) Two stars mean, the question is interesting but advanced. Let's have some fun!

Hint: Don´t annoy your supervisor by appearing unprepared. In the end, he has the power to grade you.

Your own questions: Please feel very very encouraged to ask all the questions that come to your mind, during your preparation, during the colloquium, during the experiment and afterwards. The supervisor's job is to teach you, while your responsibility is to ask questions.

Have fun with the experiments!

Theory

In the theory part you learn some basics about fluorescence microscopy and the background of thermophoresis.

1.1 Fluorescence

Introduction

In many applications in science it is the major task to observe **only one or some** components of complex systems such as cells or ensembles of molecules. Biology and biophysics research is nowadays concentrated on the size scale of micrometers to nanometers, which means on length scales of cells down to single proteins.

A very common way to fulfill this task is to mark molecules with fluorescent dyes and to observe the emitted light of these molecules. In FIGURE 1 on the front page, actin filaments, microtubuli and the nuclei of cells are dyed with different fluorescent dyes and can be distinguished easily.

Very nice 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 in consideration: The fluorescent dye and the fluorescence microscope.

***) Question 1: Why is it not possible, to observe Proteins, mRNA and so on directly with an ordinary microscope?** Hint: What is the size of a normal Protein like actin or a mRNA in orders of magnitude? What does the Abbé Criteria tell you? What is the wavelength of visible light?

How does a fluorescent dye work?

Fluorescent dyes are organic or inorganic molecules with a metastable energy level. They absorb light of a certain wavelength, the so called absorption wavelength, relax to a lower, metastable energy level (which means it has a longer residue time than statistically expected) from which they further relax to the ground level by emitting a photon of a slightly longer wavelength. In FIGURE 2 an energy diagram and the typical spectrum of the fluorescent dye Cy5 are shown. The right side is a screenshot from the *'invitrogen spectra viewer'*, which is a very useful online tool provided by a supplier of dyes to find the right dye-filter combination. Its free, you find it here: http://probes.invitrogen.com/servlets/spectraviewer

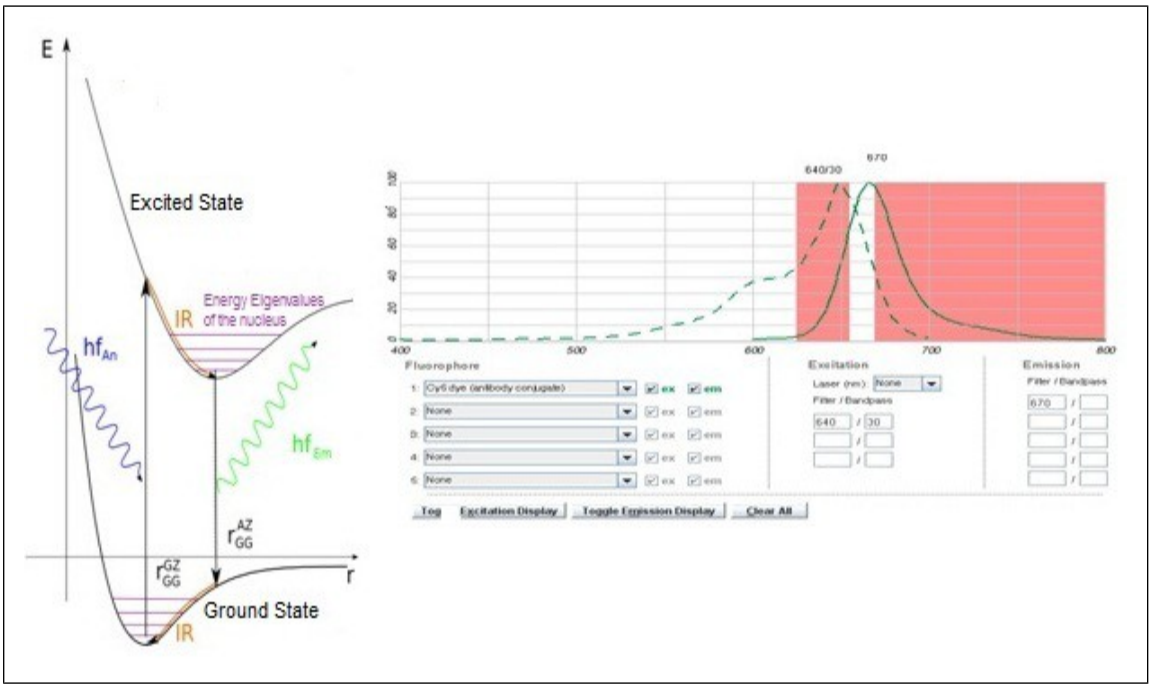


**Figure 2: Left: Excitation and Emission of a fluorophore;
Right: Excitation- and Emissionspectra of Cy5 (Red areas mark the transmission wavelengths of the filter)**

To understand the process in more detail a quantum mechanical description is required. The Schrödinger equation for the electron wave functions of a molecule provides the potential for the movement of the nucleus which tends to the minimum in such an energy landscape.

If energy in form of photons with the adequate wavelength is applied to the electronic system it is lifted into the excited state. Subsequently it loses energy due to rotations or oscillations in order to reach the local energy minimum. 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 longer than the absorption wavelength.

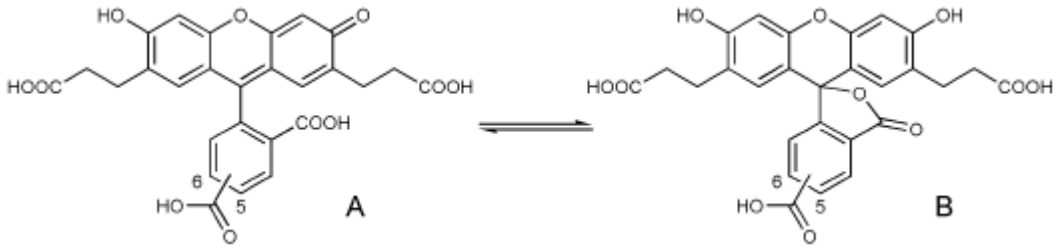
In the course of time the ability of fluorescence of the dyes decreases. This is caused by an effect called photo bleaching. In this process the photon energy changes the potential energy of the electrons (e.g. isomeric transitions) instead of inducing the emission of another photon. Thus the energy eigenvalues of the system change and it is no longer excitable with the applied absorption light. Subsequently the fluorophores are afflicted with permanent bleaching.

****) Question 2: There are a couple of techniques to investigate molecules that are based on Fluorescence. Could you name one or two of them?**

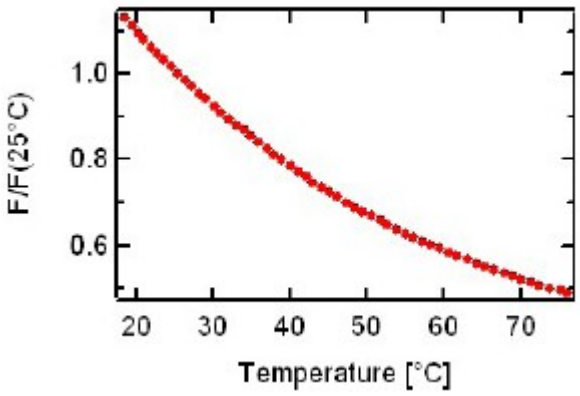
****) Question 3: We don't use it here but it is interesting: What does FRET mean? How does it work?**

Temperature dependence of fluorescent dyes

The temperature dependence of the fluorescence dye is determined in independent measurements with 2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), that serves as a pH-sensitive indicator in classical microscopy.



The structural isomerism of A and B are keto enol tautomerizations, that are present in physiological (pH = 7.4) conditions. The thermal dependence of BCECF is illustrated below, whereas the relative fluorescence is normalized to 1 at 25° C.



An increase in the temperature of 1K displaces the pH-value of the TRIS-buffer instantly about approximately -0.03 pH units. The BCECF fluorescence decreases due to fast proton transitions linearly with moderate thermal enhancements of about -0.95%/K. By measuring the percentage drop of the relative fluorescence in the laser heat spot, we extract its caused temperature elevation in the solution.

As soon as one knows the temperature dependence of the dye, one can use it as a thermometer. With this technique, we will measure the temperature of the heat spot.

Fluorescence Microscope

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.

***) Question 4: How does a normal light microscope work? What components do you need at least? Please be able to make a small drawing of all lenses and light path.**

A typical setup is depicted in the left part of FIGURE 3: The excitation light is filtered from a LED light source and directed onto the sample via a dichroic mirror. The special characteristic of this mirror is to split up absorption and emission light of fluorescent dyes. It allows the transmission of the emission light, while it reflects the excitation light. This feature is displayed in the spectrum in FIGURE 3. An additional emission filter in front of the tubus lens finally absorbs or reflects all remaining light with unwanted wavelengths to ensure that only the emission light gets to the camera. The insertion of so many filters reduces the noise, which is immensely important for biophysical applications.

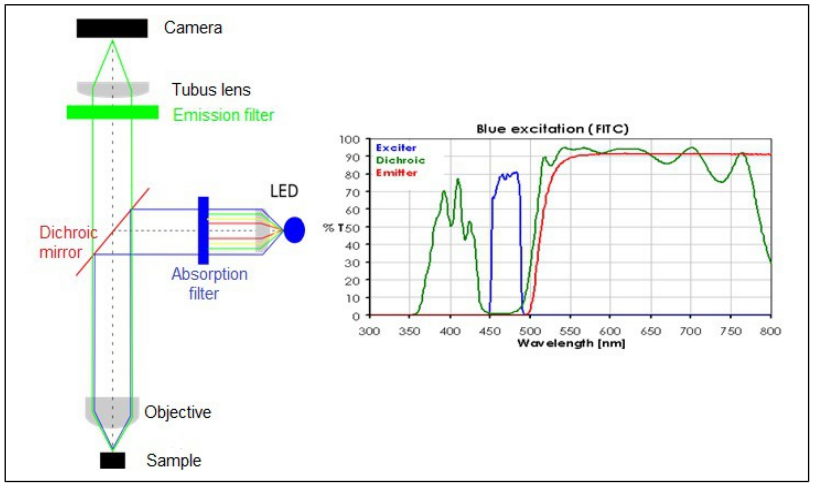


Figure 3: Left: Optical path of a typical fluorescence microscope; Right: Characteristics of a dichroic mirror

****) Question 5: In our lab, we use fluorescence microscopes which allow to observe two different colors at the same time. What additional components do you need for that?**

Light sources and lasers

For fluorescence microscopy, light sources with high intensities and a well defined color spectrum are highly needed. Specialized light sources are used such as halogen lamps, steam lamps with several metal steams (Hg, Na) LED´s in different colors and intensities and lasers. In our setup, we use a LED for illumination and an infrared laser to establish the temperature gradient.

Therefore it is necessary to lead your attention to the dangers the work with lasers in general and invisible laser beams in special brings with it.

- Never look into the laser directly or indirectly. Use a laser card to make the IR Laser visible.
- Before you switch on anything, consider the full way of the laser beam. Make clear where you probably could get in contact with the laser.
- Make sure that there are no reflective parts around the laser beam (like aluminum, adornment, rings ect.)
- The laser is controlled by a controlling voltage between 0-0.5V NEVER apply more; otherwise the laser is gone immediately.
- The laser is guided into the instrument via a light fiber that looks like a yellow cable. The fiber is intrinsic part of the laser, as soon it gets folded or damaged, the laser is gone too.

In general: the laser beam of the instrument you use is completely closed, as long as you don´t disassemble something, you are pretty save. Nevertheless it is important that you are aware of the laser.

***) Question 6: Describe the basic principle of a laser. Hint: Use the term "stimulated emission, the description is oral and should not last longer than three minutes.**

1.2 Thermophoresis

Introduction

The motion of particles along a temperature gradient is called thermophoresis, thermodiffusion or Ludwig-Soret-effect. A demonstrative example for this phenomenon is the black fume above some heaters. A temperature gradient forms between the hot heater and the cold wall. Thus the black smoke moves towards the wall. For gases an easy explanation can be found: A particle is hit by several other particles from all sides; but those from the hot side carry a larger momentum than those from the cold side. Therefore a net momentum towards the cold side builds up and the particle is effectively pushed into this direction. However, for fluids the phenomenon is much more complex and even the reverse effect has been observed.

Diffusion

Thermophoresis creates a gradient in concentration and therefore is always accompanied by a counteracting diffusion process. The most important facts about diffusion are presented in the following paragraph.

Diffusion describes the net flow of particles due to a concentration difference. An example from daily life is a drop of ink in a water glass. After sufficiently long time it will have been diluted all-over the glass. This phenomenon is driven by entropy. A highly concentrated ink drop involves a much smaller phase space volume that the equally diluted solution. From thermodynamics it should be known that the entropy is defined as

$$S = k_B \ln\left(\frac{\Omega}{\Omega_0}\right) \quad (1),$$

where Ω is the phase space volume and Ω_0 the unit volume. This treatment is strongly simplified since in fact enthalpic contributions due to solvation have to be taken into account, but are neglected here. Fick's law of diffusion and the continuity equation lead to a differential equation, which is just in some special cases analytically solvable:

$$j = -D\frac{\partial c}{\partial x} \text{ and } \frac{\partial c}{\partial t} = -\frac{\partial j}{\partial x} \text{ hence } \frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2} \quad (2)$$

Theoretical Description of Thermophoresis

***) Question 7: Summarize the most important formulas you need to describe a spherical capacity.**

It is necessary to find a model which explains all findings and gives quantitative information about the concentration changes due to thermophoresis. In principle two approaches can be made: The first one assumes a stringent non-equilibrium thermodynamic; the second one a global non-equilibrium with local equilibriums around each particle for moderate temperature gradients, for which the equilibrium thermodynamic can be applied. Until now no inconsistency with the second hypothesis could be found, which is also approved by several experiments. Therefore a short summary is given here:

The movement of particles in temperature gradients suggests the analysis of the flow densities. The total flow density j consists of two parts – the flow due to normal diffusion j_D and the flow caused by thermodiffusion j_T:

$$j = j_D + j_t = -D\nabla c - D_T c \nabla T \quad (3)$$

with the diffusion constant D and the thermophoretic mobility D_T. In a local equilibrium the total flow density is zero, which leads to a differential equation for the concentration c:

$$\frac{dc}{c} = -S_T\, dT \quad (4)$$

with the Soret-coefficient $S_T = \frac{D_T}{D}$. The solution of this differential equation is:

$$\frac{c(x)}{c(x_0)} = \mathrm{e}^{-S_T \Delta T} \quad (5)$$

where the concentration of an arbitrary point x_0 has been normalized to c_0. From thermodynamics the relation between the concentration and the Gibb's free energy is known as:

$$G = G_0 + k_B T \ln\left(\frac{c}{c_0}\right) \quad (6)$$

Rearrangement provides:

$$\frac{c}{c_0} = \mathrm{e}^{\frac{\Delta G}{k_B T}} \quad (7)$$

For small concentration and temperature gradients (viz. near the local equilibrium) equations (5) and (7) can be linearized, which gives a descriptive explanation of the quite abstract Soret-coefficient:

$$-S_T\, dT = \frac{dG}{k_B T} \quad (8)$$

Since for the entropy the equation $S = -\frac{dG}{dT}$ is valid, the Soret-coefficient can be determined as:

$$S_T = -\frac{S}{k_B T} \quad (9)$$

Thus the Soret-coefficient is proportional to the entropy of the system. It is important to keep in mind that only the local entropy difference can be considered here. This value can also be negative, as long as the global entropy change is not negative.

To find tangible expressions for the entropy and hence for the Soret-coefficients it is necessary to determine the Gibb's free energy and to differentiate with respect to the temperature.

At first the ideal gas contribution which is discussed above is considered:

$$dG = \frac{1}{\rho}\, d\,\Pi \quad (10)$$

with the osmotic pressure $\Pi = \rho\, k_B T$ and the ion density ρ

This leads to $S_T^{ideal\,gas} = \frac{1}{T}$ (11)

Secondly the contribution of the ionic shielding of the effective, molecular charge due to the dissolved salts, has to be taken into account. This system can be regarded as a spherical capacitor: The molecular surface is taken as the inner plate and the cloud of counter ions as the outer sphere. The Debye length λ_{DH} describes the distance at which the induced field of the molecule's charge has dropped to a fraction of 1/e (see Figure 4) and can be calculated with:

$$\lambda_{DH} = \sqrt{\frac{\varepsilon k_B T}{N_A e^2 \sum c_i z_i^2}} \quad (11)$$

with the permitivity $\varepsilon = \varepsilon_r \varepsilon_0$

***) Question 8: What is the Debye length of a 10 mM KCl Solution?**

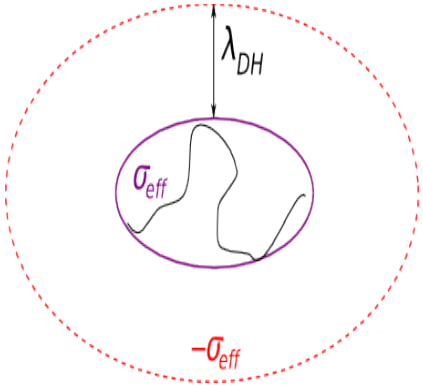


FIGURE 4: SHIELDING OF THE SURFACE CHARGE IN WATER

The field energy of a capacitor is known as:

$$E = \frac{Q_{eff}^2}{2C} \quad \text{with the capacity} \quad C = 4\pi\varepsilon \frac{R(R+\lambda_{DH})}{\lambda_{DH}} \quad (12)$$

When the energy is derived by the temperature, the Soret coefficient S_T can be calculated.

All contributions (also those not discussed here) combined lead to the following equation for the Soret-coefficient:

$$S_T(\lambda_{DH}) = \frac{(e * q_{per\ base} * bases)^2}{16 * \pi * \epsilon_r * \epsilon_0 * k_B T^2 * \lambda_{DH} * (1 + R/\lambda_{DH})^2} + S_T^0 \quad (13)$$

1.3 Binding affinities

***) Question 9: What does the mass-action-law tell you, under which constraints is it valid?**

The reaction of a simple bimolecular binding process of a single ligand L to a binder B leading to the formation of a complex LB, is characterized by the stochiometric equation:

$$[L]^{free} + [B]^{free} \Leftrightarrow [LB] \quad (14)$$

The dissociation constant K_D is an indicator for the affinity between the ligand and the binder. It is defined as the equilibrium constant that describes the equilibrium achieved between the velocity of association and dissociation.

We get the following rate equation:

$$\frac{d[B]^{free}}{dt} = k_{off}[LB] - k_{on}[B]^{free}[L]^{free} \quad (15)$$

with the rate constants k_{off} and k_{on}. If the reaction is in equilibrium we get our dissociation constant:

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[B]^{free}[L]^{free}}{[LB]} \quad (16)$$

With total ligand concentration $[L] = [L]^{free} + [LB]$ and total binder concentration $[B] = [B]^{free} + [LB]$ we get:

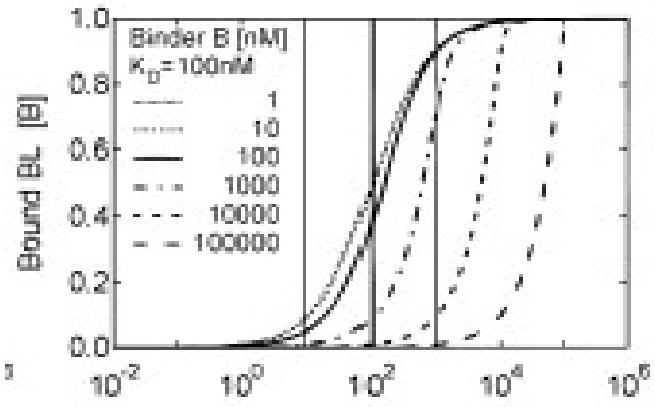
$$K_D = \frac{([L] - [LB])([B] - [LB])}{[LB]} \quad (17)$$

which is an application of the law of mass action.

To get K_D out of our data, we use above equation in a rearranged form:

$$\frac{[LB]}{[B]} = \frac{[L] + [B] + K_D - \sqrt{(([L] + [B] + K_D)^2 - 4[L][B])}}{2[B]} \quad (18)$$

with [LB]/[B] as the fraction of bound binders. A measurement of the fraction of bound binder as a function of the concentration of **total ligands** yields the so-called binding isotherm. It is a S-shaped curve, when plotted on a semi-log scale and can be used to extract the binding constant with a fit according to above equation for known concentration of ligand and binder.



Hint: To analyze your data, you normalize the fit so that the upper plateau is on one and the lower one is on zero.

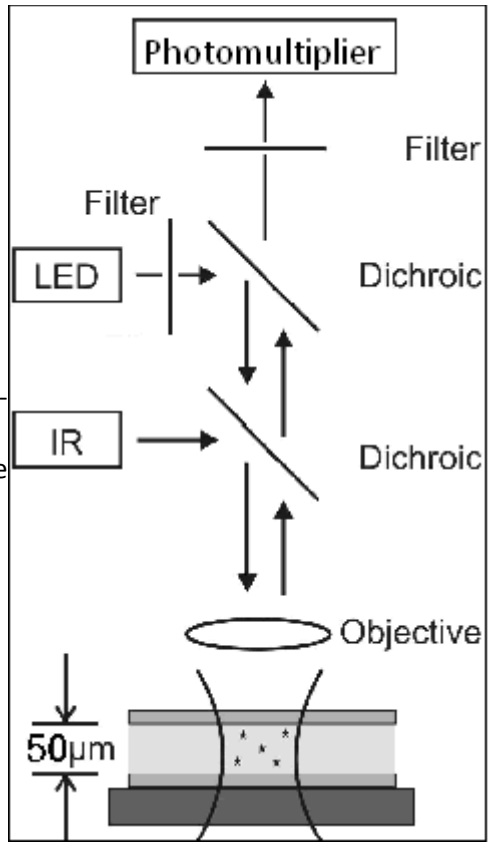
****) Question 10: What we use here is a 2-state model. Can you think of other analysis methods, where two-state models are used?**

2 Setup and Measurement

Combining fluorescence microscopy and optical implemented heating by the use of an infrared laser (IR-laser), it is possible to analyze the thermophoretic mass transport with high accuracy. In biosciences, fluorescence methods are well established and a broad choice of model systems is available, ranging from spherical polystyrene-particles to short fluorescent-labeled DNA-molecules to nanocrystals. In order to gain a clearer understanding of micro fluidic processes, the experiments are conducted using low volume samples well below 10µL and small sample concentrations (1nM – 1µM). Intramolecular interactions are negligible. The fluorescence detection of the molecules and the optical manipulation by a thermal gradient occur on a length-scale of only a few tens micrometers. Hence, the whole measurement range can be displayed to a CCD-camera with high resolution. This kind of setup allows detecting causes for artifacts, e.g. impurities and fluid drifts instantaneously. Using thin micro fluidic measurement chambers, undesired side effects of the temperature distribution are inhibited, such as convection. In addition, the small capillaries permit a two dimensional description [8]. In the following the setup is presented.

2.1 Setup

The setup is shown schematically in the figure on the rigth. As excitation light, we use a collimated contiunuous Light Emitting Diode with a centric wavelength at 530nm. The LED is attached on the back side of the microscope, whose optical path initiates parallel above the infrared laser (IR-laser). The fiber coupled IR-laser (Fibotech, Meiningen, Germany) exhibits a central wavelength of 1480nm and a power of < 200mW and enters the setup laterally between microscope body and the 40x air objective (Partec, Görlitz, Germany) with a numerical aperture of 0.8. The actual magnifcation factor was put to a test by measuring a microscale, resulting in 35.4-fold. The IR beam is then coupled into the path of fluorescence light with a heat-rejecting "hot mirror" (NT46-386; Edmund Optics, Barrington, USA) and is focused in the sample level by the objective, where it partially excites the fluorophores of the probe. The composite metal capillaries (CMS, Shipley, UK) are made of chemically unreactive borosilicate glass, possessing a rectangular geometry of a height-width dimension of 50x500µm. Since diffusion causes convection, plane measurement chambers are utilized.



A fraction of the emitted light of the sample goes straightly up into the CCD. On its way, the beam crosses both dichroids and a emission filter, that ensures that no excitation light reaches the camera. The emission light is monitored by a Luca S 658M CCD camera (Andor Technology, Belfast, Northern Ireland) with a quadratic pixel size of 10x10µm, a maximum frame rate of 37.2, a quantum efficiency of 52% between 500 and 600nm and a digitization of 14bit.

2.2 Thermophoresis Curves

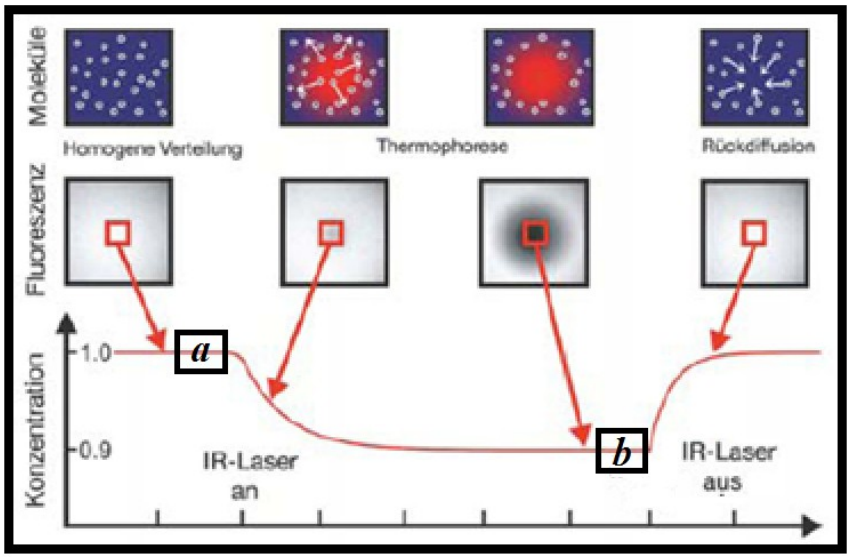


Figure 5: Typical Thermophoresis Curve

FIGURE 5 depicts the typical course of the fluorescence signal during a thermophoresis measurement. To visualize the occurring processes a fluorescence image and the molecule distribution for four different points are inserted. The impact of the laser illumination on the fluorescence signal is clearly visible. However, thermophoresis is always superimposed by other effects. There is usually a jump in the graph at the moment the laser is turned on or off . This is mainly caused by the temperature dependence of the fluorophores intensity *I'(T)*. When the thermophoretic signal finally exceeds this effect a slight bend of the curves is noticeable. The further course of the graphs shows the decrease of intensity due to thermodiffusion and the approach to steady state where the effect compensates with backdiffusion. After the laser is turned off the molecules diffuse back. This part of the graph gives information about the diffusion coefficient *D*.
All named processes are further superimposed by the exponential bleaching of the fluorophores which occurs during illumination. To obtain precise Soret-coefficients the curve has to be fitted to EQUATION (12). However, the following linear extension is valid for small temperature gradients, where $\Delta T * D_T / D \ll 1$ which can be assumed in the experiments for low laser intensities.

$$S_T = F'(T) + \frac{1 - b/a}{\Delta T} \quad (19)$$

where a and b are the mean intensities of the areas indicated in FIGURE 5 , ΔT the temperature difference induced by the laser and F'(T) is the temperature dependence of the dye intensity. Both ΔT and I(T) are read from calibration curves. Alternatively you can choose an area shortly after the laser is switched on (after the temperature jump) as area (a) and set F'(T) as zero.

3. Experiments

You will perform three types of experiments.

- Temperature dependence of BCECF dye.
- Binding affinity between a DNA aptamer and its ligand using NanoTemper Pico Instrument.
- Binding of DNA aptamer with High-Throughput MST setup

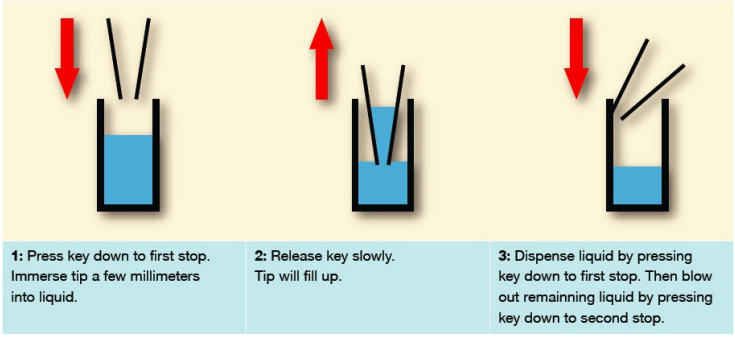
**) What is an aptamer and what can it be used for?

You will get detailed information about the experiments from your supervisor when you are here.

All the preparation you need is to read and understand this document, bring some pens and a lab book to write down parameters, and a big portion of good mood and curiosity.

Good Pipetting:

Figure 1: Forward pipetting



1: Press key down to first stop. Immerse tip a few millimeters into liquid.	**2:** Release key slowly. Tip will fill up.	**3:** Dispense liquid by pressing key down to first stop. Then blow out remainning liquid by pressing key down to second stop.

- Immerse the pipette tips only a few millimeters into the medium.
- Prewet the pipette tips a few times before aspiration and mix your sample properly using the pipette (vortexing is not enough at low volumes)
- Hold the pipette vertically during aspiration. Move the filled tip up against the wall of the vessel to avoid residues of liquid on the outside of the tip.
- Look at your sample and the pipette tip. There should not be any bubbles in it.

3.1 Temperature Dependence of BCECF dye

- Prepare a solution of 20 nM BCECF (stock-solution @ 50 µM) in 10 mM TRIS (pH=7.4).
- Fill a capillary (MST premium) with the solution. Place the capillary onto the holder and into the NanoTemper machine.
- The NanoTemper machine should detect more than 5000 counts at the capillary positions. At 25000 counts saturation is reached. The LED power should be adjusted accordingly, as a compromise between bleaching and a sufficient signal-to-noise ratio.
- You will repeat the measurement for temperatures between 22 °C and 38 °C with a step of 2 °C. First, let the temperature to equilibrate, then perform a short measurement (3 seconds). Change the temperature.
- Analyse how fluorescence of BCECF depends on temperature.
- Now cool the stage back to 22 °C. Perform short thermophoresis measurements (3 seconds IR laser on-time to focus only on the temperature jump) with MST powers of 20%, 40%, and 80%. To what temperature changes do these percentages of MST power correspond?

3.2 Binding Curve of ATP-aptamer and AMP

Here we want to measure the binding constant K_D between an ATP-aptamer and its ligand AMP. Then, we want to perform a control measurement between a mutant ATP-aptamer and AMP. Therefore, we use a Cy5-labeled ATP-aptamer/mutant ATP-aptamer at constant concentration and a 16-step titration series of an unlabeled AMP that binds ATP-aptamer but not mutant ATP-aptamer.

Experiment

1. Heat ATP-aptamer for 1 minute at 85 °C and let it cool at RT. This will help the aptamer to adopt the working conformation.
2. Prepare 200µl of 10nM ATP-aptamer in ATP-aptamer selection buffer (SB). The stock-solution has a concentration of 85µM.
3. Prepare a 16-step titration series of AMP in SB starting at 5mM. The stock-solution has a concentration of 200mM. Each titration step should have a volume of 10µl.
4. Now add 10µl of the ATP-aptamer solution from step 1 to 10µl of the diluted AMP. In this way you always obtain 1x SB with 5nM ATP-aptamer and varying AMP concentration starting at 2.5mM. It is advisory to incubate the mixed samples for 1-2 hours at RT before the next step.
5. Load the samples into capillaries (MST premium). Place the capillaries onto the holder. The capillary with 2.5mM AMP is at the front.
6. Adjust the LED power as before.
7. The IR laser power (MST power) should be set to 40%.
8. Program three replicates.
9. The laser on-time (thermophoresis) should be 30s and the off-time (back diffusion) 20s.
10. Repeat the measurement with the mutant ATP-aptamer (ATPm-aptamer; stock-solution @ 200 µM) and AMP.

Analysis

The text-file you get from the Nanotemper analysis gives you a table of concentrations and relative fluorescence values. Plot the binding curve and normalize it, so that the upper level has the value 1 and the lower value the level 0. Explain in your protocol, why this is reasonable! What is the correct axis label?

Now fit a curve to your data using formula (18) to obtain the equilibrium constant K_D. What are your fit parameters and do they seem reasonable?

3.3 Binding of DNA Aptamer with High-Throughput MST setup

In this experiment, we want to validate what we measured with the commercial NT setup. We are going to use the same solutions, but will enjoy the rapid transfer of multiple 2.5 nl droplets by the Labcyte Echo 550 liquid handler. To generate the entire transfer automatically, we need to have 3 different dilutions for the ligand.

Experiment

1. Heat ATP-aptamer for 1 minute at 85 °C and let it cool at RT. This will help the aptamer to adopt the working conformation.
2. Prepare 20 µl of 20 µM ATP-aptamer (ATPm-aptamer) in selection buffer (SB).
3. Prepare a 3-step titration series (2x 1:64) of AMP in SB starting at 200mM. Each titration step should have a minimal volume of 20µl.
4. Transfer solutions into well plate
5. Add 1 µl mineral oil in used wells of 1536 well plate
6. Centrifuge both plates @1500g for 1 min
7. Start transfer; As soon as the liquid handler is done, extract the well plate and quickly centrifuge @1500g for 15 min.
8. Adjust the LED power (~200 mA) and set IR laser power to 65 mV.
9. Import the transfer protocol you used at the liquid handler ('*.csv')
10. The laser on-time (thermophoresis) should be 30s and the off-time (back diffusion) 20s.
11. Click YES for XY and Z Focus
12. Choose folder for your acquired images and metadata
13. Start measurements

Analysis

The text-file will give you a table of concentrations and relative fluorescence values. Plot the binding curves and normalize it, so that the upper level has the value 1 and the lower value the level 0. Do not forget to add error bars!

Now plot and fit a curve to your data using formula (18) to obtain the equilibrium constant $K_{D.}$ What are your fit parameters and compare them to the NT results.

Bibliography

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