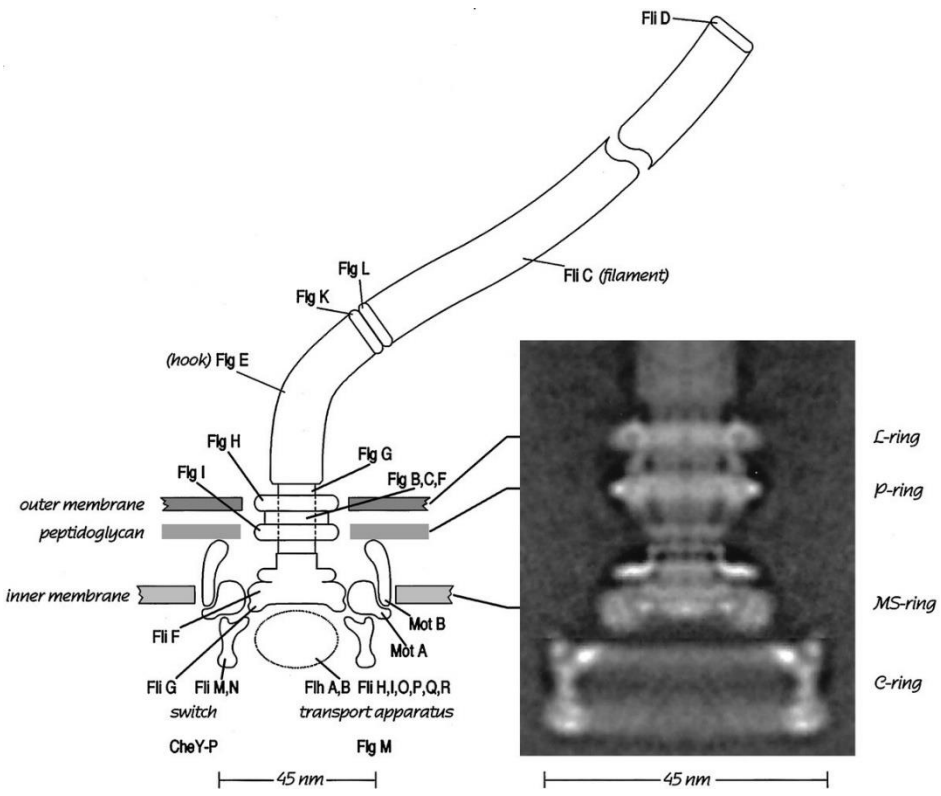
Biophysics Lab Course

ulm university universität ulm

Optical tweezers

Investigation of the E.coli Flagellar Motor using Optical Tweezers

Subjects: optical tweezers, torque of the flagellar motor



Experiments:

The principal functionality of optical tweezers is demonstrated. The torque generated by the flagellar motor of E.coli is calculated in two ways. Their angular velocity is measured and they get investigated with an optical trap.

1 Introduction

Diffusion is not sufficient to perform the various tasks necessary for living processes. To accomplish directed movements, each cell contains an immense number of various molecular motors. On the one hand there is the group of linear motors. These motors hydrolyze ATP (adenosine triphosphate) and couple the release of energy to a directed movement along filaments in the cell. An example for these motors are myosin II and myosin V, which "walk" along actin filaments similar to a tightrope artist.

On the other hand there are rotary motors like the ATP synthase and the bacterial flagellar motor. Both of them use a proton gradient to generate energy. While the ATP synthase produces ATP in the respiratory chain, the flagellar motor propels bacteria during their search for the best living conditions. The force and conformational change produced by a single molecular motor is in the range of a few piconewton ($1\text{pN} = 10^{-12}\text{N}$) and nanometers ($1\text{nm} = 10^{-9}\text{m}$), respectively.

A technique to investigate these extremely small motions and the functioning of molecular motors is the optical tweezers: it consists of a focused laser beam, which can trap and handle dielectric particles with a high spatial resolution. The focused laser beam works as a light-based hookean spring with a stiffness in the range of 0.1 $\frac{\text{pN}}{\text{nm}}$. The aim of this practical course experiment is getting familiar with the theoretical principle of optical tweezers and to use this method for a mechanical characterization of the bacterial flagellar motor of Escherichia coli. In the next section (1.1) we will introduce the flagellar motor and its functioning, while in the second one (1.2) we offer a description of physical principles that optical tweezers underlie.

Range: pN, nm.

1.1 The bacterial flagellar motor

Locomotion and taxes

Most bacteria are able to move to search for optimal living conditions. While cyanobacteria and several other bacteria crawl along surfaces, most other bacteria can swim using thread-like extensions called flagella. These flagella are located on the surface of the cell body and are connected to a molecular rotational motor anchored in the membrane. For example, Escherichia coli possesses about four flagella which are stochastically distributed on the cell surface. The direction of the motor rotation determines the movement of the bacterium: during counterclockwise rotation the flagella form a bundle and the bacterium moves forward; clockwise rotation makes the bundle fall apart so that the directional movement stops and the bacterium starts tumbling. External stimuli

Counterclockwise → Clockwise
(Move) (detect the enviro & select direction)

influence the rotation, e.g. receptors on the surface of the bacterium check the concentration of nutritions and harmful substances and feedback the rotational direction. If the direction of the actual movement of the bacterium seems favourable, the motor continues rotating counterclockwise. As soon as the environment is getting less favourable, the motor switches to clockwise rotation and the bacterium starts tumbling. By this the bacterium gets statistically reoriented and "tries out" whether directional movement in the new direction is advantageous. If this is not the case, the bacterium starts tumbling again. The response of the bacterium to a concentrational gradient of an attractant/alarm substance is called positive/negative chemotaxis.

chemotaxis
Response to concentration gradient-

Structure and functioning of the flagellar motor

Bacterial flagella are polymers (diameter 15 nm, length 15 µm) composed of the protein flagellin. The motor rotating a flagellum has a diameter of approximately 45 nm and comprises about 20 different proteins. It can roughly be divided into two parts (see figure 1):

- The stator (static part of the motor) is anchored to the plasma membrane. It is mainly composed of the proteins MotA and MotB which form a ring consisting of eight subunits (four MotA and two MotB per subunit). Each subunit contains at least one proton channel.
- The rotor (rotating part of the motor) is a set of protein rings up to 45 nm in diameter, comprising proteins like FliG, FliM and FliN. The rotor is connected to the helical flagellum.

An inward-directed electrochemical gradient of ions across the membrane provides the free-energy source for the motor rotation. In case of a proton-fuelled motor this driving force is called protonmotive force. Due to the proton flux through the MotA/MotB complex this anchored complex changes its conformation and interacts with FliG from the rotating part of the motor so that a torque is generated.

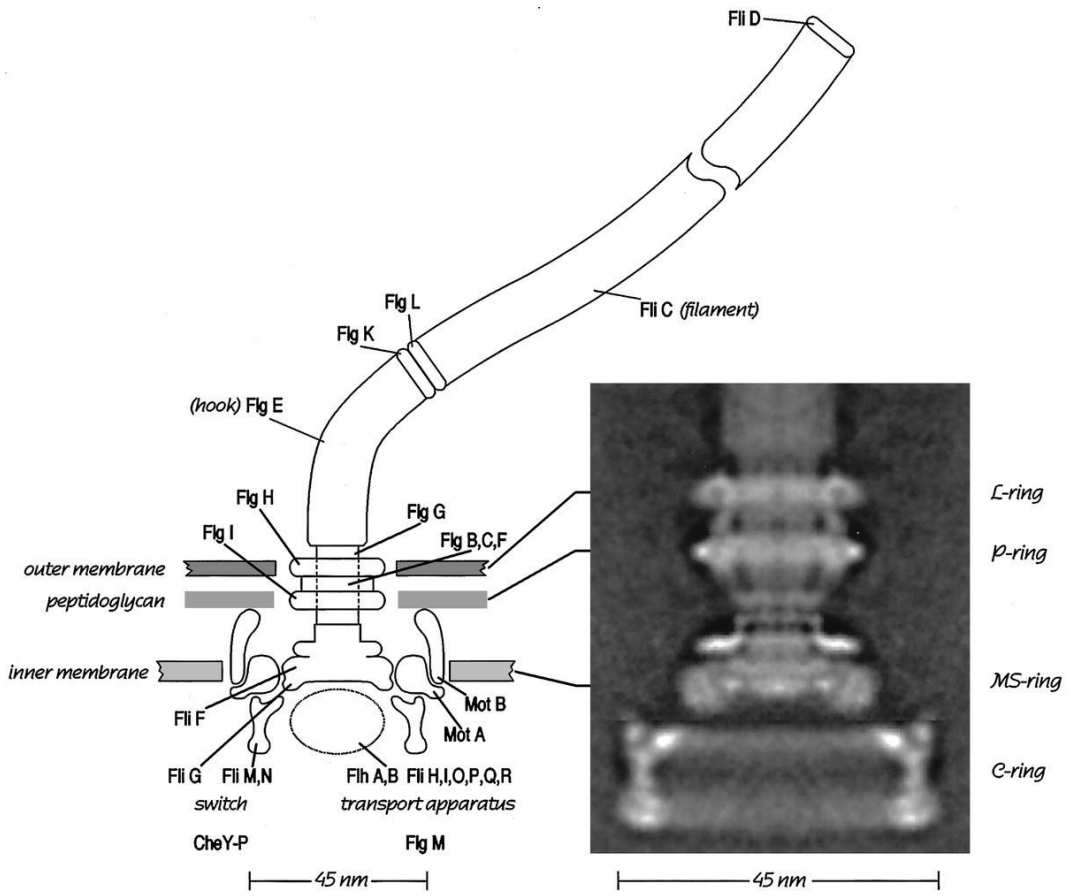


Figure 2: Structure of the flagellar motor.

Left: Proton-flux through this complex causes an interaction of the complex with FliG from the rotor part of the motor, generating a torque.

Right: Electron-microscopical reconstruction [H.C. Berg, The Rotary Motor of Bacterial Flagella, Annu. Rev. Biochem. 2003.]

1.2 physical principle of the optical tweezers

Light carries energy as well as momentum; therefore it can exert force on matter. However, this effect called *radiation pressure* is very weak. In the mid-eighties Arthur Ashkin demonstrated that, given appropriate circumstances, this phenomenon can be used to trap and manipulate microscopic particles by using light.

Physical Theory

In case of objects with a dimension larger than the wavelength of the light ($d \gg \lambda$), simple geometric optics are sufficient to illustrate the active principle of optical tweezers. Let us have a look at a light ray, colliding with a bead with a refractive index n_b (e.g. glass, n = 1.5) higher than the medium n_m (e.g. water, n = 1.33; figure 2). As a consequence of scattering at the water/bead surface and the bead/water surface, the direction of the light ray is changed. This effect is coupled with a corresponding momentum change of light and a momentum transfer to the bead.

Now imagine a parallel pencil of rays with a gaussian intensity profile (e.g. a laser) instead of a light ray. In this case the sum of all momentum transfers causes the bead to be dragged to areas of higher light intensity (figure 2A). The component of the force which drags objects in the direction of the intensity gradient is called *gradient force*. Perpendicular to the gradient force due to the gaussian shape of the beam, another force component is acting, pulling the bead in the same direction of the light propagation. This force component is referred to as *scattering force*, and it is due to the fraction of the beam that is reflected by the bead. To trap the bead also along the beam axis, one can focus the pencil of rays by a lens, like an objective with a high numerical aperture, obtaining a gradient force opposing the scattering force (figure 2B). If gradient force and scattering force are balanced, the bead - e.g. polystyrene beads, cell organelles or whole cells - can be trapped steady and held near the laser focus.

Geometric optics do not apply to objects with a dimension much smaller than the wavelength of the light ($d \ll \lambda$), here the dielectric nature of the trapped object (e.g. polystyrene bead, but also cell organelles or whole cells) is a strict requirement for the functioning of the trap, and electrodynamics have to be applied to theoretically describe the active principle of optical tweezers. This case will not be exemplified in this instruction.

For most biophysical applications is $d \approx \lambda \approx 1\mu m$, so that neither particle optics nor electrodynamics apply here. In this regime no approximations can be made and the theoretical description becomes very complex.

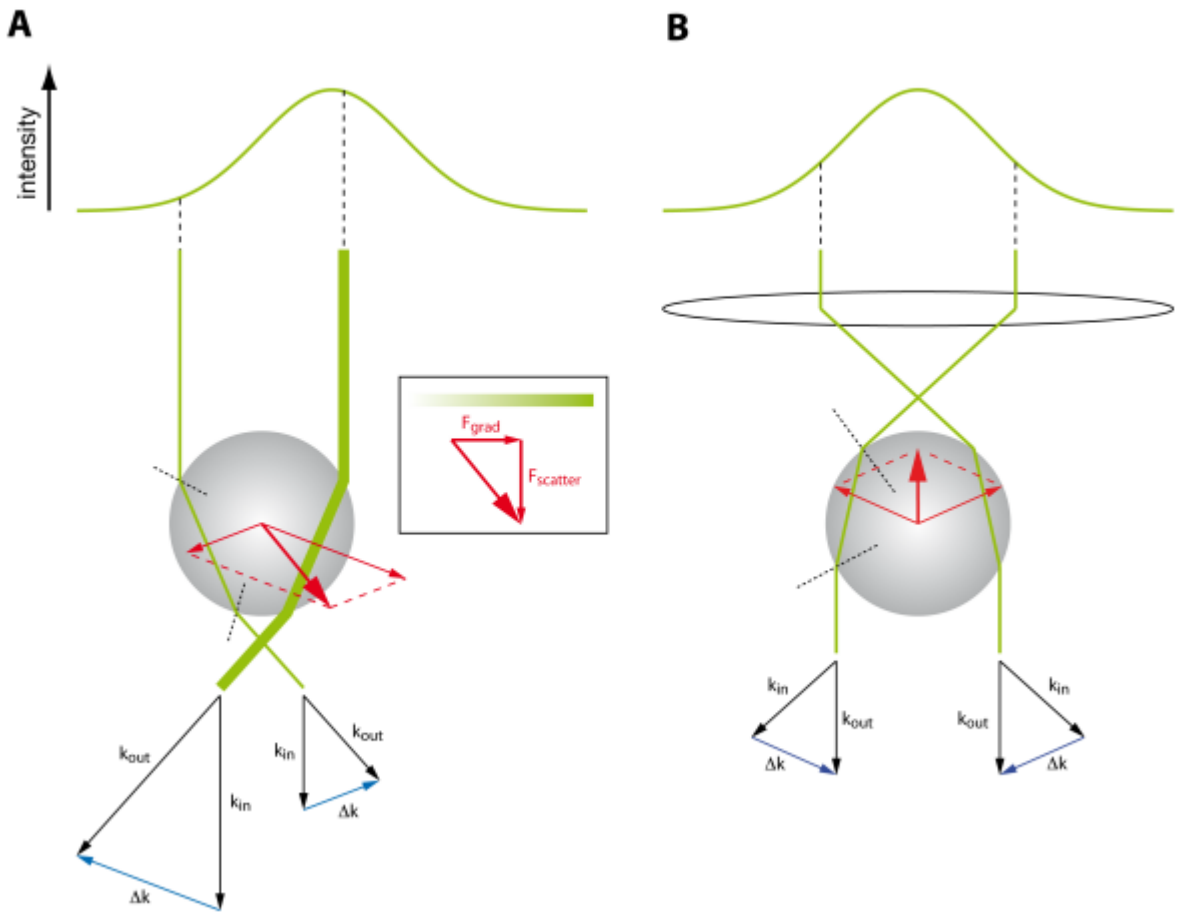


Figure 2: The principle of optical tweezers in the context of geometric optics.

A If a dielectric bead resides in a two-dimensional light intensity gradient like a Gaussian beam profile of a laser (green), the momentum changes of the single light rays (blue) cause a resulting force (red), which drags the bead in the direction of maximal intensity (F_{grad}) as well as accelerates the bead in the direction of light propagation ($F_{scatter}$).

B Focussing the light generates a component of the gradient force facilitating the trapping of a bead near the focus.

Functioning

Optical tweezers operate as a Hookean spring in a certain range, characterized by a spring constant: if a moderate force acts on a particle trapped by the tweezers, the laser focus exerts a restoring force proportional to the deflection on the particle. With increasing force the linearity between deflection and restoring force is lost. After a critical force - called *escape force* - has been reached, the laser focus cannot keep a hold on the particle and the particle escapes from the trap.

2 Experiment

In this section we show how to investigate the mechanics of the flagellar motors by using optical tweezers. In more detail, we want to measure the torque generated by a single flagellum: to do that we work with bacteria which are bound to the surface of a cover glass by a single flagellum (they can be easily recognised, as they are the ones which freely turn around a fixed point). The rotational mechanics of such a bacterium can be described in a simple way, in first approximation assuming only three forces acting on it:

The torque generated by the motors M, the viscous drag D of the medium and the optical trapping torque T.

After a short paragraph on the experimental setup (section 2.1), we calibrate the microscope's image and determine its magnification (section 2.2). Then we measure the escape force of the trap (section 2.3), a useful calibration to determine optical trapping torque. In section 2.4 we finally discuss how to measure the motor-generated torque in two different ways: firstly without any trapping, just using the balance between the motor torque and the viscous drag, when the system reaches a state of constant velocity (ω = const; T = 0; M = D). Then, activating the trap and regulating the trapping force until the bacterium stops rotating (ω = D = 0; M = T).

2.1 Experimental setup

In order to perform the experiment a simple optical tweezers instrument is used, consisting of a laser (532 nm) coupled in into a strongly focussing objective lens (100x, 1.3 NA, oil immersion), which is combined with a bright-field microscope. The laser has a peak output power of 450 mW (wear the provided laser safety goggles to protect your eyes!), the electrical current in A (Ampere) is displayed on the controller. The provided power of the laser is too high, so there is a neutral absorber of an optical density OD = 0.6 mounted directly after the laser.

A sketch of the optical components is shown in figure 3. The laser is collimated by two lenses and coupled in into the objective via a beamsplitter. The beamsplitter provides a possibility to use the same objective for focussing the laser and for the microscope. The red light of the LED is transmitted by the beamsplitter and detected with the camera. In between the beamsplitter and camera is a colour filter to block any remaining laser light transmitted by the beamsplitter. The colour filter may be dismounted, to determine the position of the laser trap (only do that if the laser is operated at low power).

The fluid cells are mounted onto a motorized stage which can - controlled by a joystick - move the

fluid cells in x- and y- direction. With a screw the objective can be adjusted in z-direction.

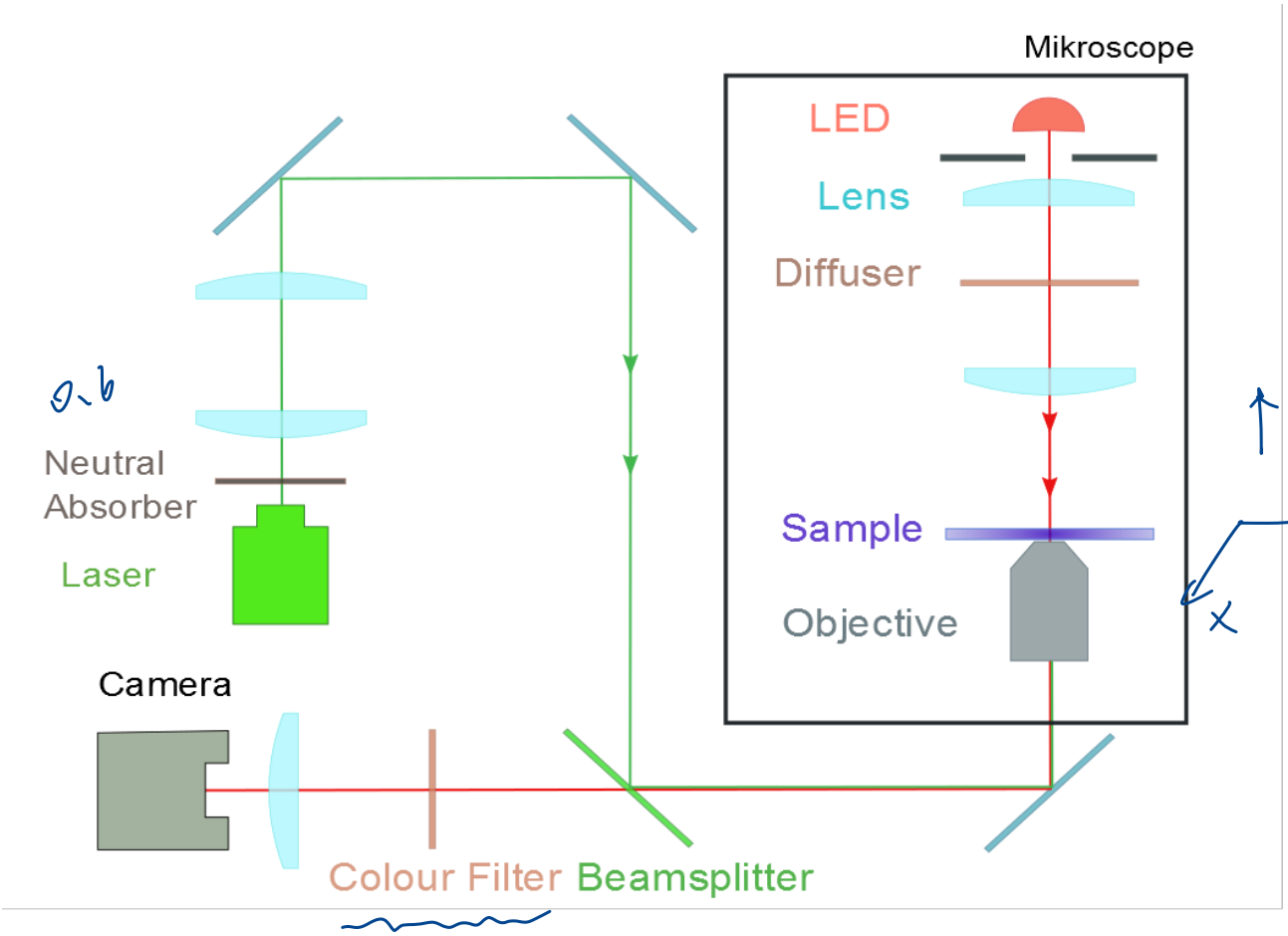


Figure 3: sketch of the optical components in the setup. On the right side is the bright-field imaging microscope. On the left side is the camera and the laser, coupled in via two mirrors and a beamsplitter.

2.2 Calibrating the bright-field image

First the magnification of the microscope on the camera has to be determined. For that a *Stage Micrometer* is used. It is a ruler with a graved-in scale of 1mm length, divided in sections of 10µm.

Experiment 1: put a droplet of immersion oil on the objective lens and mount the ruler on the x-, y-stage. Move the objective upwards until it is in contact with the ruler. Look at the camera's image and generate a focussed image of the ruler's scale. Take a picture and save it for later analyzation. Do at least five measurements on this picture to determine a conversion formula for pixels to µm. To do so you can use for example the free software IMAGEJ. You should end up with a formula similar to: 1 px = 0.07 µm. To dismount the ruler, first draw back the objective again!

2.3 Calibrating the optical tweezer

One method to measure the escape force of optical tweezers is based on viscous friction in a flow, see figure 4. The frictional force of a stationary object in a fluid is

$$F_f = \beta v,$$

with β being the friction coefficient and v the velocity of the fluid. In case of a bead with a radius r in a medium with the viscosity η the Stokes friction coefficient is defined by

$$\beta = 6\pi\eta r.$$

This equation describes a bead located at infinite distance from a surface. For measuring the friction coefficient of a bead at finite distance from a surface, a correction term is added to the Stokes friction coefficient which considers the distance between bead and surface h (Faxen's law).

$$\beta = \frac{6\pi\eta r}{1 - \frac{9}{16}\frac{r}{h} + \frac{1}{8}\left(\frac{r}{h}\right)^3 - \frac{45}{256}\left(\frac{r}{h}\right)^4 - \frac{1}{16}\left(\frac{r}{h}\right)^5}$$

Experiment 2 The escape force of optical tweezers depends linearly on the intensity of the laser. As a measurement of the laser's intensity we use the current value displayed on the controller. This relation needs to be known for the analysis of the flagellar motor's torque.

Preparation of simple fluid cells: Fix two small strips of double-adhesive tape parallel on an object slide, leaving a small space in between them. Stick a cover slip to the strips.

Determination of the escape force in dependence of the laser intensity: Use the provided 1:2000 dilution of polystyrene beads (diameter 2µm) and fill about 8 µL of the dilution into the fluid cell und seal it with nail polish. Mount the fluid cell on the motorized stage. Then trap a bead with the optical tweezers, move the motorized stage with the fluid cell on top and regulate down the laser intensity until the bead escapes from the trap. Assume at this point $F_f = F_O$. To achieve a force-intensity calibration, perform this measurement at four velocities $(0{,}1; 0{,}2; 0{,}3; 0{,}5\,\frac{\text{mm}}{\text{s}})$. Use five different beads per velocity to be able to give an estimation of the error. Write down the velocity and the laser intensity at which the bead gets lost. Think about a way to measure the height of the bead above the glass surface. (Use $h = 0.03\text{mm}$, if you don't find your own value.)

For data evaluation, do a linear regression with your measured data. You should end up with a conversion formula which connects the displayed laser current I with the acting optical force

$$F_O = c_1 * I + c_2.$$

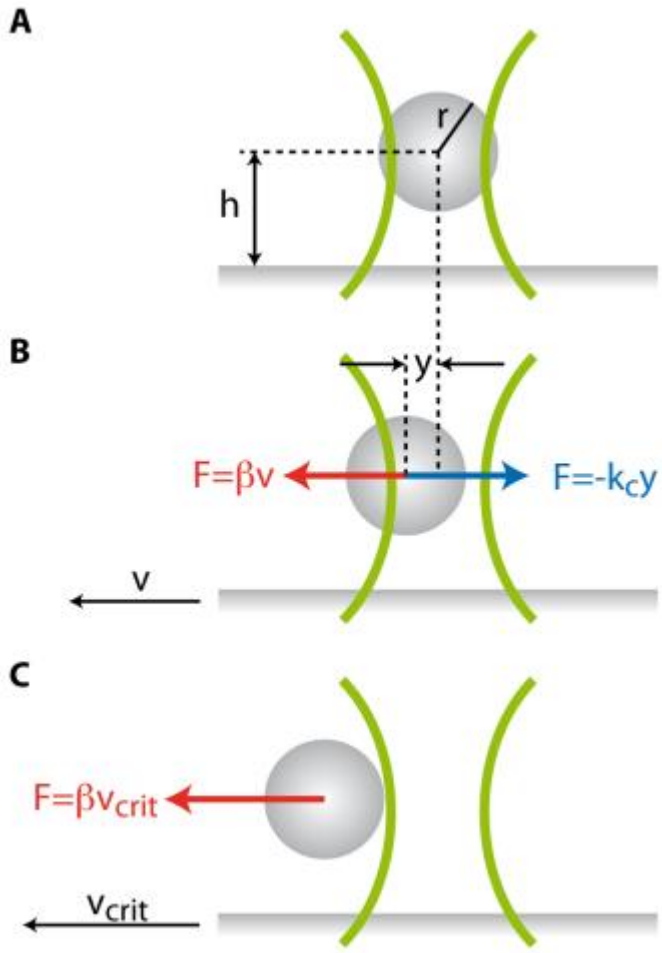


Figure 4: **A** The bead is trapped in a laser focus (green) and held at distance h from the inner surface of a fluid cell.
B When the fluid cell is moved at velocity v, the frictional force $F = \beta v$ as well as the restoring force exerted by the optical tweezers $F = -k\,y$ act on the bead.
C If a critical velocity is reached, the force exerted by the optical tweezers can no longer keep the bead trapped. Thus the proximity to the surface has an important influence on the frictional force if the distance between bead and surface is similar to the size of the bead. Herein lays the major disadvantage of this calibration method, as the distance between bead and surface can be only roughly estimated.

2.4 Investigation of the flagellar motor's torque

In this experiment optical tweezers in combination with a bright-field microscope are used to investigate the torque of the flagellar motor of the bacterium Escherichia coli. The bacterial flagella are adhered to the inner glass surface of the fluid cell - this works in an unspecific way due to a mutation in the flagella protein flagellin of the used bacterial strains KF95 and KF84. If the bacteria adhere to the surface by only one flagellum, the cell body rotates around this anchoring point, when the flagellar motor is switched on.

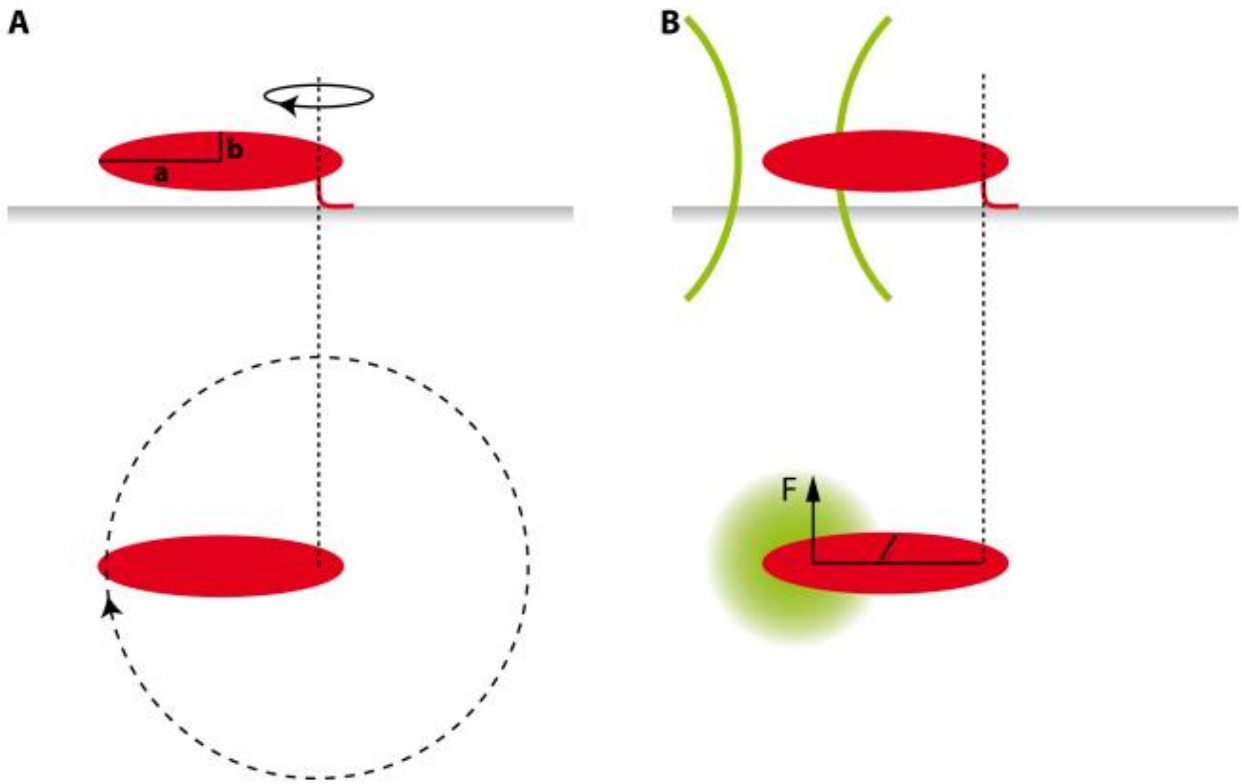


Figure 5: Experimental geometry. Shown are lateral views (top) and top views (bottom) of the geometry with and without optical tweezers. **A** The bacterium (red) is adhered to a glass substrate via its flagellum. It rotates around the anchoring point with the frequency ω. **B** Optical tweezers (green) can hamper the rotation.

Experiment preparation Resuspend bacteria in PBS buffer. Shear them carefully with a pipet to shorten the flagella so that some of the bacteria might adhere with only one flagellum. Fill the bacteria suspension into a fluid cell (this time do not use double-adhesive tape and nail polish, but use vacuum grease instead). Do not seal! After two minutes flush the fluid cell with 200 µL PBS to remove the bacteria not fixed to the glass. Now seal the fluid cell.

2.4.1 Torque estimation using the rotational frequency

A method to estimate the torque of the flagellar motor is measuring the rotational frequency. While rotating the bacterium has to generate a torque against the friction with the surrounding media. To estimate this torque one can approximate the bacterium as a rotating ellipsoid with the half-axes *a* and *b* (see figure 5 **A**). The drag D (and so the torque M at steady-state) is proportional to the angular velocity ω, with a prefactor that depends on the geometry.

$$M = \beta_r \omega$$

The term for the rotational friction coefficient β_r of an ellipsoid rotating around its short half-axis reads as (this equation only applies if the axis is located in the centre of the ellipsoid)

$$\beta_r = \beta_{sphere}\alpha$$

$$\beta_{sphere} = 6\eta V_E = 6\eta \frac{4}{3}\pi a b^2.$$

β_{sphere} is the rotational friction coefficient of a sphere with the same volume as the ellipsoid V_E. The correction factor α depends on the relation $p = \frac{b}{a}$ of the two half-axes:

$$\alpha = \frac{4}{3}\frac{1-p^4}{p^2(-2-p^2S+2S)}$$

$$S = \frac{2}{\sqrt{1-p^2}} \ln\left[\frac{1}{p}\left(1+\sqrt{1-p^2}\right)\right]$$

Experiment 3 Make movies of ten appropriately rotating bacteria - consider, what 'appropriately rotating' means in this context. Analyse the movies to determine the values needed to calculate the torque (You can use IMAGEJ again). Estimate from the average torque, what force a single torque-generating unit (not a whole flagellar motor!) can exert. Assume that the force is created on the edge of the motor (see figure 2).

2.4.2 Torque determination using optical tweezers

Regarding optical characteristics, E. coli are similar to the previously used polystyrene beads. They are about the same size and consist of a material which has a higher refractive index than water. So one can 'catch' bacteria with the optical tweezers, though the wavelength of the previously described experimental setup (a laser of 532 nm) is harmful to them and will kill them after relatively short exposure times. A bacterium which is attached to a surface by one of its flagella and the cell body rotating around this anchoring point, can be caught at the free end with optical tweezers (figure 5 B). If the force exerted by the trap is known, one can easily calculate the torque of the flagellar motor, assuming that the escape force F_{trap} of the trap approximately equals the

force, which is required to just 'catch' the bacterium. So the torque can be calculated as the product of the escape force F_{trap} and the lever arm *l*.

$$M = F_{trap} * l$$

Experiment 5 Find the position of the trap on the camera. To do so, dismount the colour filter in front of the camera and turn on the laser to a low power. Memorize this position and put the filter in its place again.

Place the fluid cell such that a rotating bacterium moves through the laser spot. Now increase the laser intensity quickly until the bacterium is fixed by the force exerted by the optical tweezers. Note the value shown on the power supply and decrease the laser intensity rapidly. The bacterium should start rotating again - otherwise it is unclear whether the bacterium was still alive at the measured value. Take a short movie to specify the length of the lever arm (You can use IMAGEJ again.). Assume the bacterium is trapped at $^2/_3$ of its body length. Measure the flagellar motor's torque of ten individuals.

2.5 Annotations to the experimental procedure

The laser used in this practical course experiment is a class 3B laser. Laser safety glasses are provided and should be worn during the experiment. The single steps of the experimental procedure are listed here again:

- Calibration of the bright-field image
- Determination of the escape force of the optical tweezers in dependence of the laser intensity
- Preparation of the bacteria sample
- Estimation of the torque via the rotational frequency
- Investigation of the torque of the flagellar motor using optical tweezers

3 Data evaluation

The written record should comprise a short description of the physical principles of optical tweezers, a brief summary of the experiment and the measured values. The results as well as potential sources of errors are to be discussed, e.g. why the torques determined with different methods might differ from each other. Consider which method seems more reliable and give reasons for your decision. Calculations of the data analysis should be retraceable.

Please bring an external hard drive with approx. 20 GB of free space.

1. Calibrating the bright field. 10 μm

	pixels	micrometers
1		90.464
2		90.731
3		91.440
4		
5		118.268 nm/pix

8.4 pix/μm

2. Calibrating the optical tweezer (escape force)

$F_f = \beta v$ bead 2.07 μm – d.

Velocity (mm/s) / Attempts	1	2	3	4	5
0.1	1.18	1.18	1.20	1.23	1.28
0.2	1.17	1.17	1.13	1.12	1.15
0.3	1.19	1.28	1.23	1.26	1.23
0.5	1.43	1.48	1.47	1.40	1.34

3 Torque Estimation 1. $D = M$. $T = 0$

Movies:

4. Torque measurement 2.

$L = \frac{2}{3} a$

2 page intro

Data & Analysis

V 6 1.2

V 14

15 1.55

16

17

18

19 1.08

20 1.08

21 1.07 die-

22

~~23~~

24 1.13 1.18

25 1.05 1.0x 1.05

26

27 1.08

28 1.06

29 1.10

1.10

1.06

both