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Investigation of the *E. coli* Flagellar Motor using Optical Tweezers

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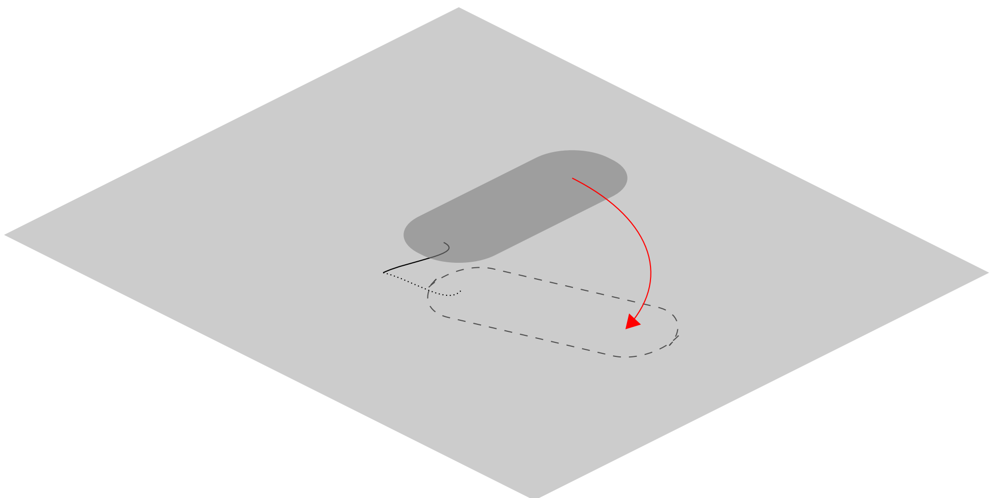

Contents

Things to note before the practical session

- Please read the following introduction and experimental notes carefully, and try the exercises.
- Please, if possible, have one member of your group bring a laptop for recording the data from the experiments.
- Either write in your lab books on on a laptop as much as possible of what the lab demonstrator is telling you - many of these things will be important for writing up your report.

1 Introduction

Diffusion is not sufficient for performing the various tasks necessary for living processes. To accomplish directed movements, each cell contains a large number of different molecular motors.

These can be divided into two types: linear and rotary. Linear motors hydrolyse ATP (adenosine triphosphate) and couple the release of energy to a directed movement along filaments in the cell. Examples of these motors include myosin II and myosin V, which 'walk' along actin filaments, in a way that is similar to that of a tightrope artist.

Rotary motors, such as ATP synthase and the bacterial flagellar motor, use a proton gradient to generate energy. ATP synthase produces ATP in the respiratory chain and 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 piconewtons ($1\,\mathrm{pN} = 10^{-12}\,\mathrm{N}$) and nanometers ($1\,\mathrm{nm} = 10^{-9}\,\mathrm{m}$), respectively. A technique that can be used to investigate these extremely small motions and the functioning of molecular motors, is *optical tweezers*: these consist of a focused laser beam which can trap and handle dielectric particles with a high spatial resolution, usually on the order of nanometers. The focused laser beam works as a light-based hookean spring with a stiffness that is usually in the range of 0.1 pN/nm (see figure 1).

The aim of this practical course experiment is to become familiar with the theoretical principle of optical tweezers and to use this method for the mechanical characterization of the bacterial flagellar motor of Escherichia coli.

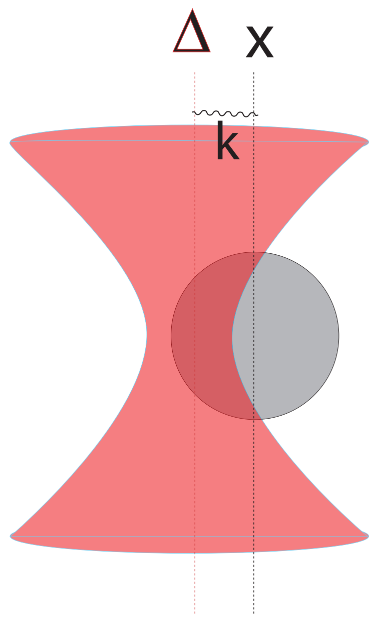

Figure 1: Schematic of optical tweezers: a dielectric bead is trapped in a focused laser beam. As the center of the bead x moves relative to the trap center Δ, it feels an approximately linear restoring force: $F = k(\Delta - X)$.

1.1 The bacterial flagellar motor

Most bacteria are able to move to search for optimal living conditions. While cyanobacteria (formerly called blue-green algae) 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 influence the rotation, e.g. receptors on the surface of the bacterium check the concentration of nutrients and harmful substances and feedback the rotational direction.

If the direction of the movement of the bacterium seems favorable, the motor continues rotating counterclockwise. As soon as the environment becomes less favorable, 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 concentration gradient of an attractant/alarm substance is called positive/negative chemotaxis.

Exercise 1. *If* E. coli *did not possess flagella and relied exclusively on diffusive processes for locomotion: How long would it take for the bacterium to get* 1 cm *closer to its 'destination'? Use the viscosity of water (*$\eta = 10^{-3}\,\mathrm{Pas}$*), equation 2 and the Einstein relation* $D\beta = k_B T$ *to calculate the diffusion coefficient for a one-dimensional diffusion of the bacterium (approximately round-shaped with a diameter of* $2\,\mu\mathrm{m}$*). The temperature of the surrounding environment is* 293 K.

Structure and function 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 2):

- 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 eleven 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.

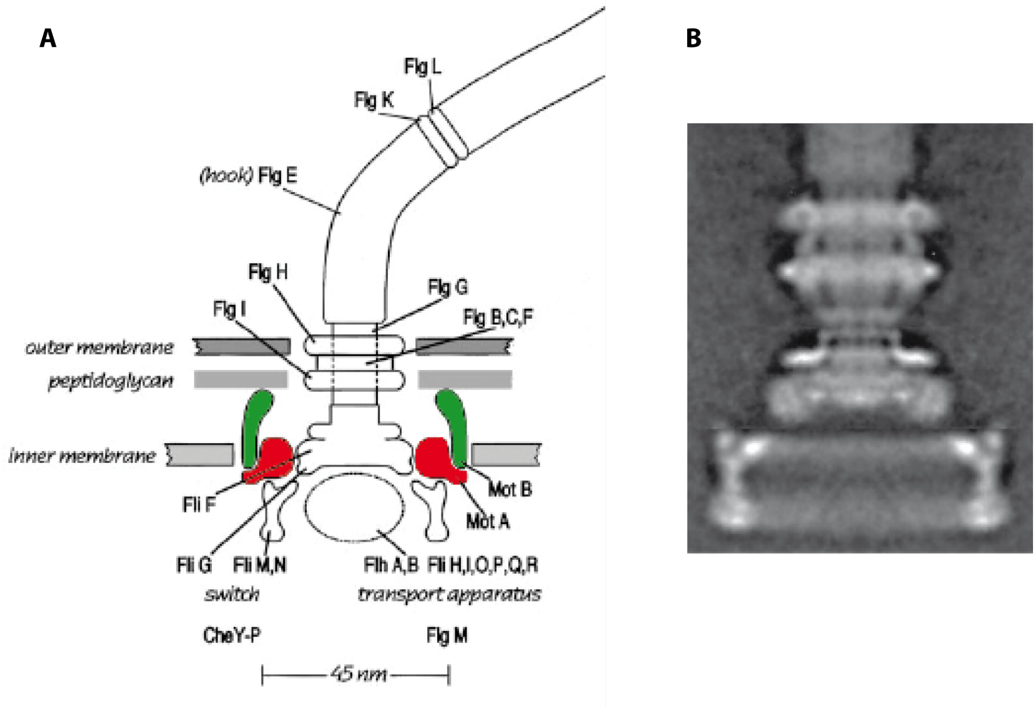

Figure 2: Structure of the flagellar motor. **A** Schematic view. Eleven MotA/MotB subunits (red and green) form a ring anchored to the plasma membrane. Proton-flux through this complex causes an interaction of the complex with FliG from the rotor part of the motor, generating a torque. **B** Electron-microscopical reconstruction [H.C. Berg, The Rotary Motor of Bacterial Flagella, Annu. Rev. Biochem. 2003.]

An inward-directed electrochemical gradient of ions across the membrane provides the free-energy source for the motor rotation. In case of a proton-'fueled' 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.

1.2 Active principle of the optical tweezers

Being a electromagnetic wave, light carries energy as well as momentum; therefore it can exert force on matter. However, this effect called *radiation pressure* is very weak.

Exercise 2. *Calculate the force of a laser beam ($P = 1\,\mathrm{mW}$) exerts on a perfectly reflecting surface (perpendicular incidence of light)?*

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.

Theory

In case of objects with a dimension larger than the wavelength of the light ($d \gg \lambda$), simple particle optics are sufficient to illustrate the active principle of optical tweezers.

Let us have a look at a light ray, incident upon with a bead with a refractive index n_b (e.g. glass, $n = 1.5$) higher than that of the surrounding medium n_m (e.g. water, $n = 1.33$; figure 3). 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 the light and a momentum transfer to the bead.

Now imagine a parallel beam of light 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 3A). The component of the force which drags objects in the direction of the intensity gradient is called the *gradient force.*

Another force component acts perpendicular to the gradient force due to the Gaussian shape of the beam, 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 beam of rays using a lens, such as an objective with a *high* numerical aperture, obtaining a gradient force opposing the scattering force (figure 3B).

If the gradient force and scattering force are balanced, the bead — e.g. silica beads, cell organelles or whole cells — can be stably trapped and held near the laser focus.

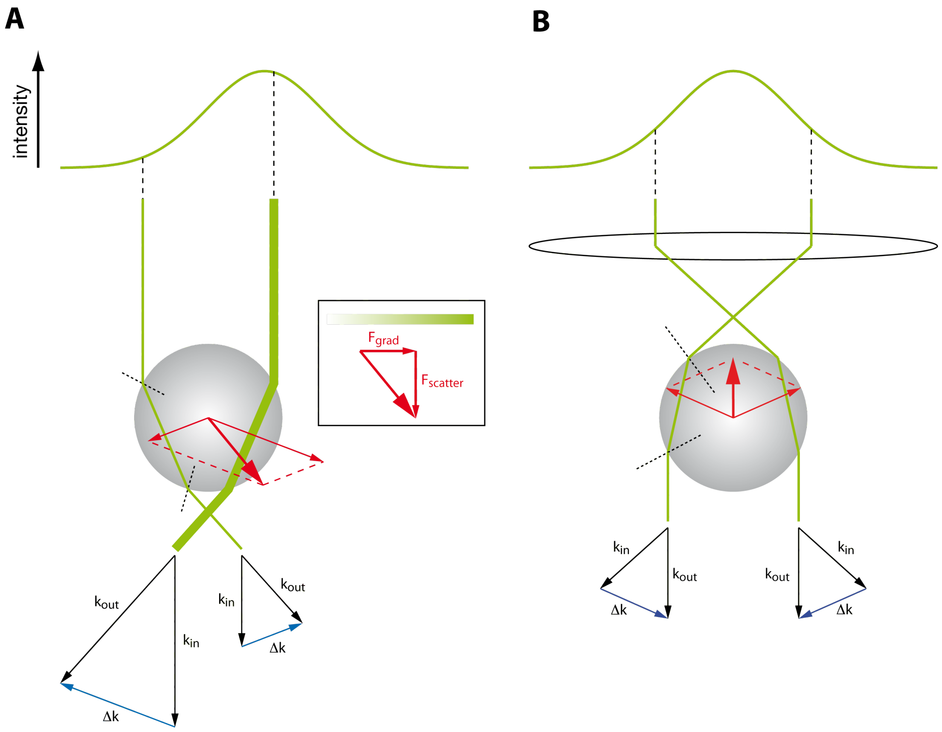

Figure 3: The active principle of optical tweezers in the context of particle optics. **A** If a dielectric bead is in a two-dimensional light intensity gradient such as the Gaussian beam profile of a laser (green), the momentum changes of the single light rays (blue) cause a resulting force (red), which drags the bead in the direction of the greatest intensity (F_{grad}) as well as accelerating the bead in the direction of light propagation ($F_{scatter}$). **B** Focusing the light ray generates a component of the gradient force facilitating the trapping of a bead near the focus.

Function

Optical tweezers operate in a certain range as an Hookean spring 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. With increasing force the linearity between deflection and restoring force is lost. After a critical force — called the *escape force* — has been reached, the laser focus can no longer hold on the particle and the particle escapes from the trap.

2 Experimental procedure

In this section we explain how to investigate the mechanics of flagellar motors by using optical tweezers. Specifically, we want to measure the torque generated by a single flagellum: this requires bacteria which are bound to the surface of a cover glass by a single flagellum (they can be easily recognized, as they are the ones who freely turn around a fixed point). The rotational mechanics of such a bacterium can be described in a simple way, in a first approximation assuming only three forces acting on it: the torque generated by the motors M, the viscous drag F_d of the medium and the optical trapping force F_t.

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:

- Determination of the escape force of the optical tweezers in dependence of the laser intensity
- Investigation of the torque of the flagellar motor using optical tweezers

2.1 Experimental setup

A simple optical tweezers instrument is used, consisting of a laser (532 nm) coupled in into a strongly focusing objective lens (100×, 1.3 NA, oil immersion), which is combined with a bright-field microscope. The fluid cells are mounted onto a motorized stage which can — controlled by a joystick — move the fluid cells in x and y direction. The stage cab be manually adjusted in the z direction using a screw.

2.2 Determination of the optical tweezers' escape force

One method to measure the escape force of optical tweezers is based on viscous friction in a flow. The frictional force of a stationary object (or viscous drag acting on a stationary object) in a fluid is:

$$F_d = \beta v, \tag{1}$$

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:

$$\beta = 6\pi\eta r. \tag{2}$$

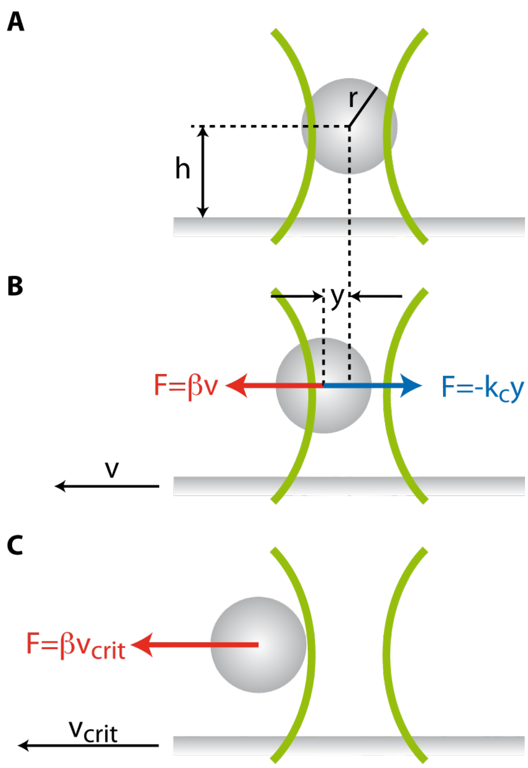

Figure 4: **A** A bead is trapped in a laser focus (green) and held at distance h from the inner surface of a fluid cell. **B** When the fluid cell is moved at velocity v, the frictional force $F_d = \beta v$ as well as the restoring force exerted by the optical tweezers $F_t = -k_c y$ act on the bead. **C** If a critical velocity is reached, the force exerted by the optical tweezers can no longer keep the bead trapped.

Experiment 1. *The escape force of optical tweezers depends linearly on the intensity of the laser. This relation needs to be known for the analysis of the flagellar motor's torque.*

Preparation of simple fluid cells: Fix two small strips (about $2.5\,\mathrm{cm} \times 0.5\,\mathrm{cm}$*) of double-adhesive tape parallel on an object slide, leaving a space of approximately* $2.5\,\mathrm{cm} \times 1\,\mathrm{cm}$ *in-between them. Peel off the protective foil and stick a cover slip to the strips.*

Determination of the escape force in dependence of the laser intensity: Prepare a 1:3000 dilution of silica beads (diameter $2\,\mu\mathrm{m}$*,* 5% *(w/v)) in water, pipette about* $30\,\mu\mathrm{L}$ *of the dilution into a fluid cell and seal it with vacuum grease. Put a droplet of immersion oil onto the cover slip and mount the fluid cell on the motorized stage — oil droplet on the bottom, thinner glass, side touching the objective lens.*

Check the focus of the camera by varying the z distance manually, until *beads appear small and dark.*

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. To achieve a force-intensity calibration, perform this measurement at four velocities ($0.08, 0.17, 0.21$ *and* $0.25\,\frac{mm}{s}$*). Use enough different beads per velocity and modify the direction (up, down, left, right; no diagonal movements) to be able to give a statistically significant result and an estimate of the error (suggested number: 20 beads per velocity, 5 in each direction).*

2.3 Investigation of the bacterial flagellar motors' 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 through a nonspecific interaction, made possible by a mutation in the flagella protein flagellin of the bacterial strains KF95 and KF84. If the bacteria adhere to the surface by only one flagellum, the cell body rotates around this anchoring point.

Experiment 2. *Starting with bacterial strain KF95, gently pipette the bacterial suspension into a fluid chamber. Do not seal! Watch the* E. coli *move in the bright-field of the setup and monitor them adhering to the surface. After about two minutes flush the fluid cell with* $200\,\mu$L *PBS to remove the bacteria that are not fixed to the glass. Now seal the fluid cell.*

2.3.1 Torque determination using optical tweezers

Regarding optical characteristics, *E. coli* are similar to the previously used silica beads. They are about the same size and consist of a material which has a higher refractive index than water. As a result, one can "catch" bacteria with the optical tweezers, though the wavelength of the laser (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_c of the tweezers approximately equals the force, which is required to just "catch" the bacterium. Thus, the torque can be calculated as the product of the escape force F_c times the length of the lever arm, l:

$$M = F_c \cdot l. \tag{3}$$

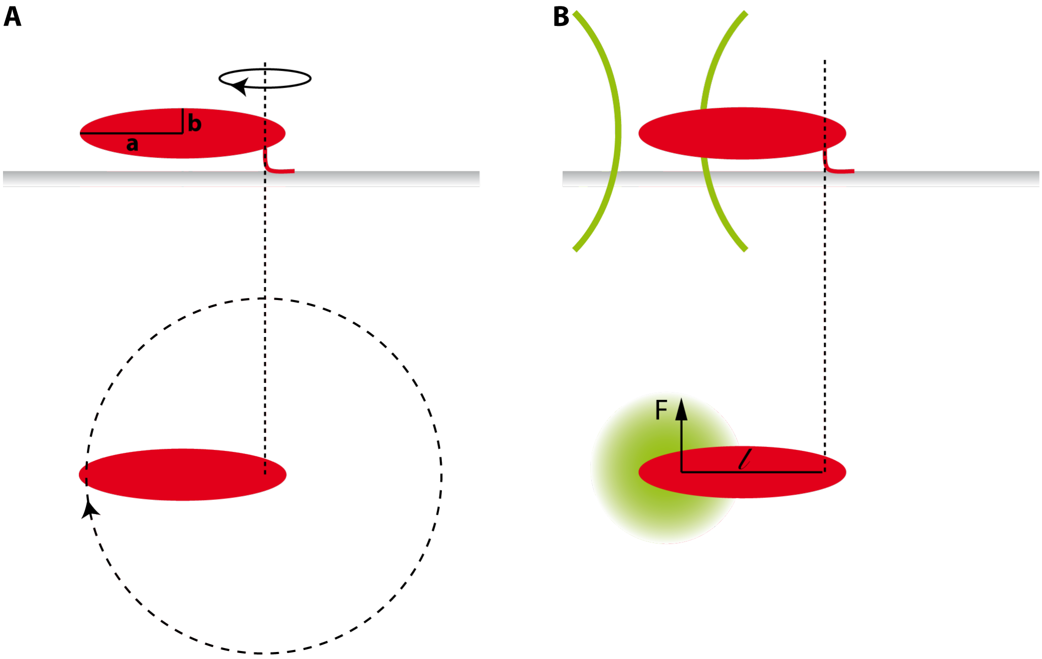

Figure 5: Experimental geometry. Shown are side views (top) and top views (bottom) of the geometry with and without optical tweezers. **A** A bacterium (red) is adhered to a glass substrate via its flagellum. It rotates around the anchoring point with a frequency, f. **B** Optical tweezers (green) can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

Experiment 3. *Mark the position of the trap in* OpenBox *and write down the coordinates. 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. Write down 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, meaning that this data point should be discarded.*

Take a short movie (10 - 20 frames) to obtain the length of the lever arm. Measure the flagellar motor's torque of ten individual bacteria. You will need to know the conversion from pixels into μm.

Exercise 3. *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 at the edge of the motor (see figure 2).*

Appendix: Torque estimation using the rotational frequency

Another method to estimate the torque of the flagellar motor is by measuring the frequency at which bacteria rotate. If we assume that the bacteria are rotating at a constant rate, then the torque generated by the flagellar motor must be equal and opposite to the torque resulting from the viscosity of the fluid.

To estimate this torque one can observe a bacterium rotating about its short axis, and approximate it as a rotating prolate ellipsoid with the half-axes a and b (see figure 5 A).

Similarly to what was shown in equation 1, the viscous torque M_{vis} at equilibrium is proportional to the angular velocity ω, with two prefactors: β_{rot} (that is the rotational equivalent of the Stokes coefficient β), and the *Perrin factor* α that accounts for the fact that the rotating object is not a sphere but an ellipsoid. Therefore

$$M_{\text{vis}} = \alpha\beta_{\text{rot}}\omega. \tag{4}$$

Note again that this equation applies only to an ellipsoid rotating about its short axis!

The explicit expressions of α and β_{rot} are as follows:

$$\alpha = 8\pi\eta a b^2 \tag{5}$$

$$\beta_{\text{rot}} = \frac{4}{3} \cdot \frac{(1/p)^2 - p^2}{2 - S[2 - (1/p)^2]} \tag{6}$$

where $p = a/b$. The factor S is given by

$$S = 2\frac{\text{atanh}\xi}{\xi} = \frac{1}{\xi}\ln\left(\frac{1+\xi}{1-\xi}\right)$$

where

$$\xi = \frac{\sqrt{|p^2 - 1|}}{p}.$$

Therefore by measuring the rotational frequency one can calculate the angular velocity ω, and after having measured a and b, calculate the viscous torque exerted on a rotating bacterium.

Experiment 4. *Take movies of about ten appropriately rotating bacteria — consider, what 'appropriately rotating' means in this context. Analyze the movies to determine the values needed to calculate the torque.*

3 Solutions to the exercises

3.1 Exercise 1

For one-dimensional diffusion applies:

$$< x^2 >= 2Dt \tag{7}$$

with the diffusion coefficient

$$D = \frac{k_B T}{\beta}. \tag{8}$$

Using a friction coefficient for a bead according to Stokes (equation 2) $\beta = 6\pi\eta r$ one obtains

$$t = \frac{< x^2 >}{2D} = \frac{6\pi\eta r < x^2 >}{2k_B T} \tag{9}$$

Applying these equations the result is:

$$\mathrm{t} = \frac{6\pi \cdot 10^{-3}\,\frac{\mathrm{kg}}{\mathrm{m\cdot s}} \cdot 10^{-6}\,\mathrm{m} \cdot 10^{-4}\,\mathrm{m}^2}{2 \cdot 1,381 \cdot 10^{-23}\,\frac{\mathrm{kg\cdot m^2}}{\mathrm{s^2\cdot K}} \cdot 293\,\mathrm{K}} = 2,3 \cdot 10^8\,\mathrm{s}\ (\textit{about 7 years}) \tag{10}$$

3.2 Exercise 2

The change of momentum Δp during a collision with a perfectly reflecting surface is

$$\Delta p = 2p. \tag{11}$$

Furthermore the following relation between energy and momentum of a photon applies

$$p = \frac{E}{c} \tag{12}$$

with the velocity of light c. The force acting on the surface after momentum conservation is

$$F = \frac{\Delta p}{\Delta t} = \frac{2p}{\Delta t} = \frac{2E}{c\Delta t} = \frac{2P}{c}, \tag{13}$$

where P denotes the incident light power.
With $P = 1\,\mathrm{mW}$ and $c = 3 \cdot 10^8\,\mathrm{m/s}$ one obtains a force of about 7 pN.