Molecular Motors

Enzyme kinetics using the example of muscle protein myosin II

Carina Pelzl (carina.pelzl@tum.de)
Philip Bleicher (p.bleicher@tum.de)
Etienne Loiseau (etienne.loiseau@tum.de)

October 3, 2014

Biophysics lab course for physicists

Contents

1 Introduction

This experiment gives insights into the kinetics of enzymatic reactions using the example of an enzyme, that can be found in the muscle muscle, that can hydrolyze ATP (adenosine triphosphate): the muscle protein myosin II.
The goal of the experiment is to verify the correlation between the production rate and the substrate concentration of an enzymatic reaction, which is described in our case by the "Michaelis-Menten-Equation".

Therefore, in our experimental set-up, the so-called *motility assay*, molecular motors are attached to a functionalized glass surface and by addition of ATP the fixed motors can move fluorescently labeled actin filaments. This movement can be observed by the means of fluorescent microscopy. By diluting the ATP concentration in the assay, you will observe and quantify the change of velocity of the actin filaments.

2 Muscles and their components

2.1 Actin

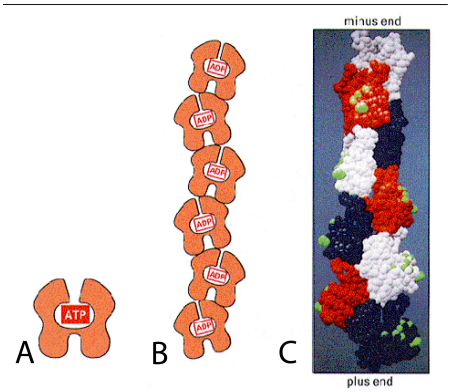

Figure 1: Schematic illustration of G-Actin (**a**, single monomer) and F-Actin (**b**, filamentous actin, polymer) and molecular depiction of F-Actin (**c**).

Actin is a globular protein with a binding pocket for ATP, that is in the center of the protein. Actin in its monomeric form is called G-actin for that reason (Fig. 1a). If actin exceeds a critical concentration, G-actin can polymerize into long actin filaments which form helical polymers and is thus called F-actin (Fig. 1b, c). Vice versa, if the actin concentration is lower than the critical concentration, filaments will depolymerize.
Actin is a polar filament: the monomers are all aligned and thus we have two

different ends, the (+)- or barbed end and the (–)- or pointed end. Both ends differ in their polymerization and depolymerization rate. While the (+)-end is characterized by a fast kinetic, the (–)-end (de)polymerizes a lot slower.

2.2 Myosin

Myosin II plays an important role during contractile activities in both muscle and non-muscle cells. The smallest functional unit of myosin II is a dimer that consist of two heavy chains, to which four lights chains are attached (Fig. 2). The two heavy chains dimerize with their long domains by helically twisting around each other and thus form the so-called coiled-coils.

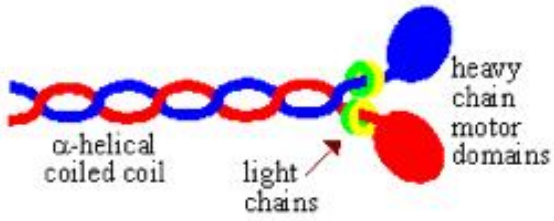

Figure 2: Structure of myosin II dimers

Myosin II not only forms dimers, but also polymers, the mini filaments. In muscles, about 200 myosin molecules self assemble in a parallel manner into bipolar mini filaments (Fig. 3). The globular head domains of the heavy chains are bent towards the side and hence are flexibel.

Here, we use HMM (Heavy Mero Myosin) which is a shortened form of myosin II and thus cannot form mini filaments. HMM exists only as a dimer.

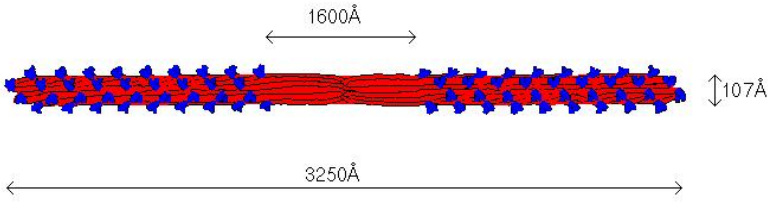

Figure 3: Structure of myosin filaments

2.3 Structure of muscles

Already in the 19th century microscopic examinations of muscles were conducted. Thereby, it was discovered that skeletal muscles, also called striped or striated

muscles, are composed of alternating zones that are optically dense and less dense (Fig. 4). The dense zones are called A-band and the less dense zones I-band. The A-bands are characterized by a central bright zone (H-zone), in which a thin line runs down the middle, called the M-line. The I-band is divided by the Z-line (from the German "Zwischenscheibe"). Between two Z-lines or Z-discs the fundamental unit of a muscle is defined as the so-called sarcomere with a length of about 2.5µm. The human biceps for example consists of at least ten million sarcomeres.

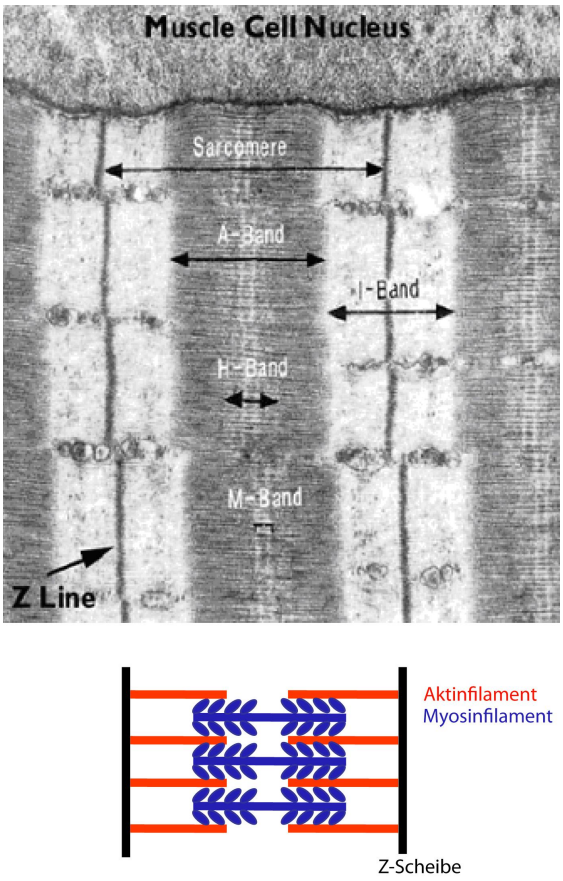

Figure 4: Structure of a skeletal muscle.

How can the stripes be explained?
The bright I-band consists mainly of actin filaments and the A-band mainly of myosin filaments. The Z-line defines individual sarcomeres. The actin filaments of the bright I-band range far into the optical dense A-bands, i.e. at the outer edge of the A-band, the actin filaments overlap with the myosin filaments. The inner zone of the A-band mainly consists of only myosin filaments (H-zone) and is separated by the M-line.

2.4 Actin, Myosin II and their interplay in muscles

Chemomechanical cycle of Myosin II

The energy for this mode of transport within the muscle is provided in the form of adenosine triphosphate (ATP). Chemical energy is gained through hydrolysis of the high-energy phosphate bond, which is then transformed into mechanical energy by the myosin. This is called the chemomechanical cycle.

In combination with actin filaments the chemomechanical cycle of myosin can be described like as below (Fig. 5). In the absence of ATP the myosin is cocked at 45 degrees and in its presence at 90 degrees. This cyclic alteration of orientation causes the necessary translocational force, which is executed by many myosins along the filaments and hence is responsible for the muscle contraction.

- During the initial state, the myosin head is bound to the actin filament (Fig. 5a).
- By binding of ATP the myosin can detach from the actin (Fig. 5b).
- The myosin head hydrolyzes the bound ATP to ADP and phosphate, but keeps both products. The hydrolyzation of ATP causes a conformational change and thus tension of the myosin head. The myosin head rotates by 45 degrees to 90 degrees (Fig. 5c).
- Now the myosin head forms a new cross bridge to the neighboring actin molecule. The actin causes the release of the phosphate and shortly after the release of the ADP (Fig. 5d).
- This leads to an opposed conformational change of the myosin head, that operates as a power stroke. After this movement the myosin head detaches and moves back to its original position. There a new active site at the actin is available, since through the relative movement of the myosin compared to the actin, the old attachment site is translocated (Fig. 5e).

As long as ATP is available, the cycle can start again, so that the myosin filament is continuously walking along the actin filament towards the Z-disk. Every stroke of the roughly 400 heads along a thick filament causes a translocation of about 10 nm and repeats 50 times per second during a strong contraction of the muscle.

Sliding filament model

As mentioned, muscles consist of many parallel sarcomeres arranged in series. If the muscle needs to contract, every single sarcomere shortens and thus leads to an

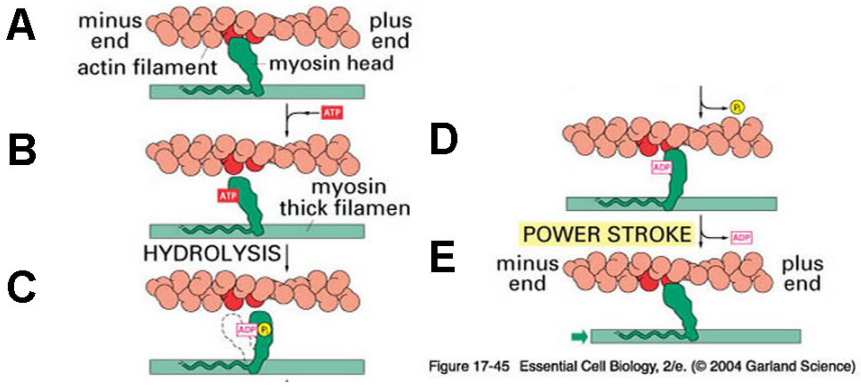

Figure 5: Chemomechanical cycle of Myosin II

overall contraction of the whole muscle.
These contractions were observed in living frog muscles using an interference microscope in the 1950's. The optically thinnest I-band becomes the optically thickest band during contraction.

Also for this reason the *sliding filament theory* is postulated. This theory implies that the shortening of the muscle fibre is based on a gliding mechanism where the actin filaments slide telescopically into the myosin filaments, and hence the Z-disks approach and the actin and myosin filaments generate a large overlap (Fig. 6).

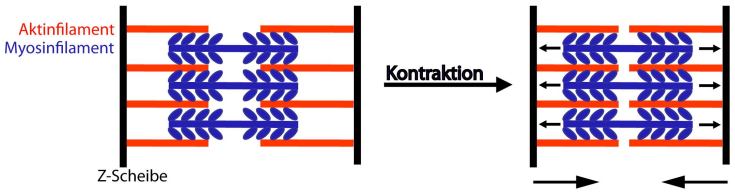

Figure 6: Model for the sliding filament theory

Question 1.

- *What is a protein? What are the proteins in our experiment?*
- *What is an enzyme? What is the enzyme in our experiment?*
- *Is ATP a protein? If so or if not so, why?*
- *What is a buffer? Why do we need it in our experiment?*
- *Explain the chemomechanical cycle.*
- *Can you now explain, why the rigor mortis (German: "Totenstarre") happens?*

3 Michaelis-Menten kinetics

Kinetics of the enzyme reaction: The Michaelis-Menten equation

$$\nu = \frac{\nu_{\max} \cdot [S]}{K_{\min} + [S]} \tag{1}$$

Here, ν is the production rate of the enzyme, which in this case is directly proportional to the gliding velocity of the actin filaments in the experiment.

Below we use the following abbreviations:
$[E]$ = concentration of the enzyme (myosin II)
$[S]$ = concentration of the substrate (ATP)
$[ES]$ = concentration of the enzyme-substrate complex (enzyme + ATP)
$[P]$ = concentration of the reaction product (ADP, phosphate)

For reactions, which are catalyzed by enzymes, we start with the assumption, that enzyme and substrate form a complex, which again can dissociate into the product and a free enzyme. If you additionally take into account that all reactions are reversible, we get the following formula:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P + E$$

Since $[S] \gg [P]$, the backward reaction of the product P can be neglected. Hence, the formula changes to:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E$$

The efficiency of the enzymatic reaction is determined by the production rate:

$$\nu = \frac{d[P]}{dt} = k_2[ES] \tag{2}$$

To calculate ν we need $[ES]$. For changes of $[ES]$ we also need to consider, that the enzyme-substrate complex can be formed as well as dissociated.

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_2)[ES] \tag{3}$$

A high substrate concentration results in a stationary state of the complex concentration $[ES]$, in which then this concentration is the determining factor. Even though after addition of the substrate the concentration of the enzym-substrate complex rises, after a while the slope slows down due to the backward reaction until it reaches a stationary state.

$$\frac{d[ES]}{dt} = 0 \tag{4}$$

From eq. (3) and eq. (4) follows:

$$k_1[E][S] = (k_{-1} + k_2)[ES] \tag{5}$$

Dissolving eq. (5) to $[ES]$:

$$[ES] = \frac{k_1}{k_{-1} + k_2}[E][S] = \frac{[E][S]}{(k_{-1} + k_2)/k_1} = \frac{[E][S]}{K_M} \tag{6}$$

where $K_M = (k_(-1)+k_2)/k_1$ is the Michaelis constant, and $[E]$ is the concentration of free enzyme, which is the total amount of enzyme ($[E_T]$) minus the amount of enzyme bound in the ES complex:

$$[E] = [E_T] - [ES] \tag{7}$$

Applying eq. (6) and eq. (7) leads to

$$[ES] = \frac{([E_T] - [ES])[S]}{K_M}$$

This equation can be transformed:

$$[ES] + [ES]\frac{[S]}{K_M} = \frac{[E_T][S]}{K_M} \rightarrow [ES](1 + \frac{[S]}{K_M}) = \frac{[E_T][S]}{K_M}$$

With eq. 2 we get:

$$[ES] = \frac{[E_T][S]}{K_M}/(1+\frac{[S]}{K_M}) = \frac{[E_T][S]}{K_M} \cdot \frac{K_M}{K_M + [S]} = [E_T] \cdot \frac{[S]}{K_M + [S]} = [E_T] \cdot \frac{[S]}{K_M + [S]}$$

With eq. (2), we can then write the following:

$$\nu = \frac{d[P]}{dt} = k_2[ES] = k_2[E_T] \cdot \frac{K_M}{K_M + [S]}$$

$$\nu = \frac{\nu_{\max} \cdot [S]}{K_{\min} + [S]}$$

The constants K_M and $\nu_{\max}$ are two parameters, that completely define the kinetic behavior of an enzyme depending on the substrate $[S]$, if $[S] \gg [E]$. We can determine both constants experimentally with a series of reaction, where we

keep $[E_T]$ constant, while varying $[S]$. The parameter, we will measure is the production rate ν, which is here the gliding velocity of the actin filaments. By plotting ν against $[S]$, we can extract K_M and $\nu_{\max}$ from the graph. Due to its hyperbolical form of the graph it is difficult to determine $\nu_{\max}$.

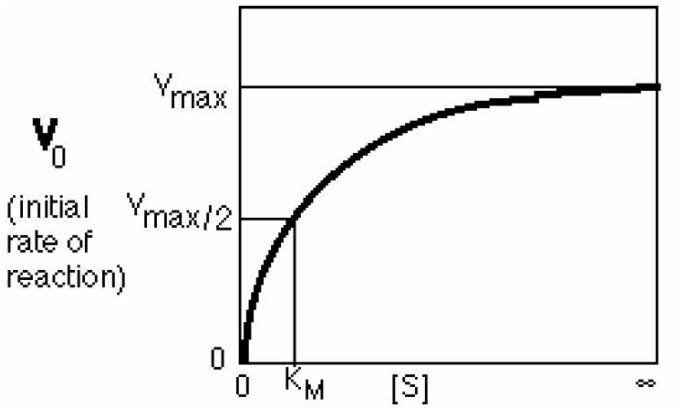

Figure 7: Example plot of the Michaelis-Menten equation.

For this reason, we chose a linear transformation of the Michaelis-Menten equation, where the inverse production rate $1/\nu$ depends linearly on the inverse substrate concentration $1/[S]$.

$$\frac{1}{\nu} = \frac{1}{\nu_{\max}} + \frac{K_M}{\nu_{\max}} \cdot \frac{1}{[S]}$$

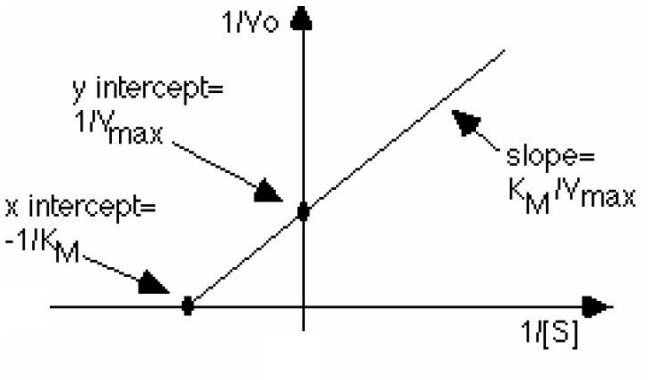

Figure 8: Example plot of the linear Michaelis-Menten equation.

K_M and $\nu_{\max}$ are determined from the intersection with the two axes and the slope of the graph.

For the application of the Michaelis-Menten theory in this assay, we assume ATP to be the substrate to analyze the enzymatic activity of myosin. The reaction product will be phosphate and adenosine diphosphate (ADT). Since chemical energy is converted into mechanical energy while moving the actin filaments, the velocity of the actin filaments is proportional to the production rate. The movements of the actin filaments can be visualized by means of fluorescent microscopy.

4 Fluorescence microscopy

Generally, radiation of a certain wavelength can not resolve any structures which are much smaller than this wavelength. The maximum resolution limit is determined by the wavelength of the light, e.g. in the visible range 400nm (violet) up to 700nm (red). The resolution limit of a light microscope, where we can still distinguish two objects, depends both on the wavelength of the light and the numerical aperture (N.A.) of the used objective.

Figure 9: Objective used in the lab course.

To visualize single molecules, we attach fluorescent dyes to them. This dye then absorbs light with a certain wavelength and emits the light with a longer wavelength. This mechanism is applied in a fluorescence microscope by sending white light through a filter of the appropriate excitation wavelength and the fluorescent light is collected, again filtered and imaged onto a CCD camera, where the molecules are observed on a dark background. With the help of fluorescence microscopy we can detect particle positions to an accuracy of 0.2µm, which would not be possible with a normal bright field microscope due to its bright background.

The advantage of fluorescence microscopy in biology is the possibility to work on/with living objects, which is not possible with e.g. an electron microscope

due to the necessity of vacuum. A typical picture of fluorescently labeled actin filaments is shown in Fig. 11.

Here, we use a dye which has an excitation wavelength of 488 nm (blue) and an emission wavelength of 550 nm (green).

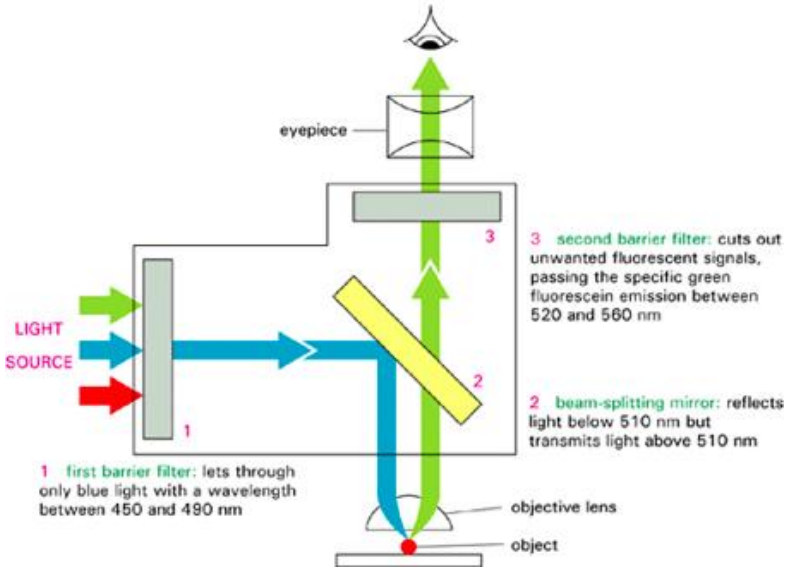

Figure 10: Schematic principle of a fluorescence microscope: A set of filters consists of two band elimination filters and one dichroic mirror, which splits the beam depending on its color. Picture taken from Alberts et al. "Molecular biology of the cell"

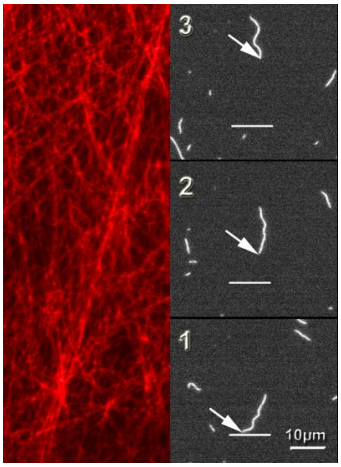

Figure 11: Fluorescence picture of actin filaments (left, image colored in red) and single actin filament in a motility assay (right, time series).

Question 2.

- What is written on the objective? Explain.

- What is the formula to calculate the resolution limit? What is the resolution limit in our case?* What are the dimensions of an actin filament? Is it comparable to the resolution limit? Why do we see it? Is the resolution limit better for fluorescence microscopes compared to bright field microscopes?*

*- What are the so-called super-resolution techniques?***

*- Explain fluorescence briefly and include a scheme with the energy levels. Why is the emitting wavelength longer?**

*- What is the difference to phosphorescence? How do the energy levels differ to those from fluorescence? Where do you encounter phosphorescence in everyday life?***
(Answer in the final report.)*
*(** Answer, if interested.)*

5 Experimental procedure

This part will not be questioned during the preliminary discussion, but you still need to read it in advance!

With the use of a fluorescence microscope, interactions between muscle proteins myosin II and actin in the presence of ATP are investigated by several *in vitro* assays.
To verify the above mentioned Michaelis-Menten equation, the velocity of the actin filaments, which is measured for the production rate of the enzymatic reaction, needs to be determined for different ATP concentrations.

5.1 Preparations

Coating of cover slips with nitrocellulose

In order to assure binding of myosin to the cover slips, they need to be covered with nitrocellulose. For this, they are immersed in a nitrocellulose solution (0.1% in isoamyl alcohol) and dried in the fume hood. This will be already prepared for you.

Assembly of the flow cell

First, you need to assemble a flow cell, which we will put on the microscope. Therefore, we take a glass slide, place two stripes of Parafilm® on it as distance

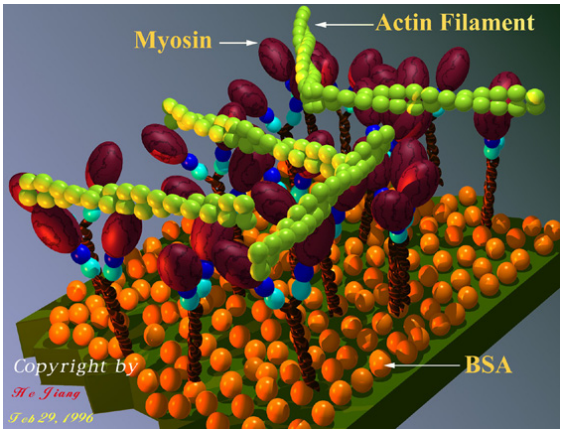

Figure 12: Schematic view of the motility assay

stripes and on top a cover slip, that is coated with nitrocellulose. This device is heated up until the parafilm turns clear. Then, press the cover slip with a pair of tweezers towards the glass slide to ensure tight binding. Now we have a hollow space with our desired chamber volume. Here, which we can fill the chamber with our proteins and buffers, by flowing them in on the one side and removing the outflow on the other side.

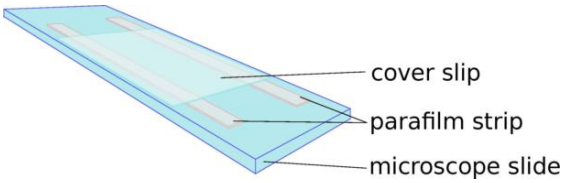

Figure 13: Schematic view of the flow cell

The following, you need to prepare in advance:
We use Myosin II with just a short tail instead of its actual long tail domaine, which is called HMM. HMM cannot form mini filaments and hence is well suited for the motility assay. All proteins must be stored on ice at all times to ensure their functionality. As a solvent we use the so-called AB buffer, which keeps the myosin in an environment similar to the body.

AB (Assay buffer)

25 mM	Imidazol hydrochlorid pH 7.4
25 mM	KCl
4 mM	$MgCl_2$
1 mM	EGTA
1 mM	DTT

This buffer is premixed at a 10-times concentration, which you need to dilute. The DTT must be freshly added.

1x AB
4,4 mL dist. Wasser
0.5 mL 10x AB
100 µL DTT
Store the buffer on ice at all times!

To allow interaction of actin only with the myosin and not with the cover slip, we need to cover all the remaining sticky nitrocellulose, which is not covered with myosin. This is done by the protein BSA and by that we prevent the actin from sticking to the cover slip as well.
Therefore, we need to prepare the following solution:

1x ABSA
3,9 mL dist. Wasser
0.5 mL 10x AB
0.5 mL BSA (10mg/ml in H_20)
100 µL DTT
Store the buffer on ice at all times!

The actin filaments are already labeled with a fluorescent dye (Atto488) by the means of the mushroom poison phalloidin, which binds to the filaments. Here, the dye is attached to the phalloidin. Therefore, the actin solution must be stored in the dark, e.g. wrapped in aluminum foil.

To keep the fluorescent dyes stable and capture any free radicals like oxygen, we need an enzyme mixture, which does exactly that:

GoC enzyme mixture
10 µL Glucose oxidase (Gox or X)
10 µL Catalase (Cat)
30 µL ABSA
Store the enzyme mixture on ice at all times!

Since we have a stock solution of actin filaments and HMM, we need to dilute these protein solutions.

HMM (5mg/ml)/200
398 µL AB
2 µL HMM

Actin (5µM)/200

398 µL	AB
2 µL	Actin

For the inititation of the reaction, we still need the ATP, the "fuel", which is finally included in the motility buffer. This motility buffer has to be prepared shortly before every experiment:

Motility-Buffer

43 µL	ABSA
5 µL	GoC
1 µL	20 % Glucose in H_2O
1 µL	ATP (100 mM)

5.2 Assembly of the motility assay

30 µL	Myosin	WAIT 3 min!! (keep myosin time to bind)
100 µL	ABSA	rinse
30 µL	Actin	WAIT 2 min!! (keep actin time to bind)
50 µL	Motility buffer	add

Add a little drop of immersion oil on the cover slip and with this side facing the objective, put the sample on the microscope. Adjust the focus, so that you see the actin filaments running on the screen.

5.3 Measurements

You need to prepare eight samples with different concentrations of ATP. Take movies with the program "Micro Manager" (Acquisition – Multi-D Acquisition – Acquire!) with about 200 time points for each concentration at a frame rate of 100 msec (Interval). Within the same program ("ImageJ" - Freeware: imagej.nih.gov/ij/) you can extract the distance which a filament has covered, by first make a overlay in time (Stacks – Z-project), select the tracks with the freehand line tool and check the time the filament needed to cover the track from the movie. Make sure, that the filament is not stopping in between, since that will change your calculated velocity.

The experiments should be conducted at varying ATP concentrations. Therefore, pre-dilutions of ATP are added to the motility buffer. For the first sample we use 1 µl of the 100mM ATP stock concentrations, which is diluted 50-fold and thus results in a final ATP concentration of 2000 µM in the motility buffer. For the 2nd sample, pre-dilute some of the ATP stock concentration 1:1 and from that

we use again 1 µl for the motility buffer. For the 3rd sample further dilute the pre-diluted ATP concentration, etc.

- 2000 µM
- 1000 µM
- 400 µM
- 100 µM
- 50 µM
- 20 µM
- 10 µM
- 5 µM

Negative controls. You can do them in between or at the beginning.

- As a negative control build a sample without motors.
- As another negative control build a sample with motors, but do not add ATP to the motiliy buffer.

Question 3. *What do you expect for each of these controls? Does this match with your experiment? Explain this behavior.**
(Answer also in the final report.)*

5.4 Analysis and Discussion

Your report should of course include a short introduction, a rough sketch of the experiment, your results and a discussion. The main focus of your report should be the discussion of your data and the answering of the questions below. This list serves as a guidance and is not exclusive.

Question 4. *What is the formula to calculate the resolution limit? What is the resolution limit in our case? What are the dimensions of an actin filament? Is it comparable to the resolution limit? Why do we see it? Is the resolution limit better for fluorescence microscopes compared to bright field microscopes?*

Question 5. *Explain fluorescence briefly and include a scheme with the energy levels. Why is the emitting wavelength longer?*

Question 6. *What do you expect for each of the negative controls? Does this match with your experiment? Explain this behavior.*

Plot the velocities of the actin filaments as a function of the ATP concentrations. Determine the Michaelis-Menten constant and $\nu_{\max}$ from both the conventional and the inverse representation and insert both fits into the plot.
If some data points are far off, you can exclude them from your evaluation, but you need to discuss, why. Please plot and fit the data both with and without the values, that are off. Does the data points influence the values of K and $\nu_{\max}$? Why or why not?

Question 7. *Why do the values of both K and ν_{max} differ from the two fits? Discuss!*

Question 8. *Which fit is better? Why? Which values do you trust more? Why? Discuss!*

Question 9. *What is the difference between the two representations? What do you learn from it as a future scientist?*

Please hand in your report within four weeks to your lab adviser.