LMU Munich
Physics Department
Chair for Applied Physics
Gaub lab

Lab course G3

Motility Assay

Enzyme Kinetics and Molecular Motors

July 23, 2013

1 Introduction

How do muscles work and which components cause their contraction? This question was an object of numerous investigations in fields of medicine, biology, and recently also physics. Already in the 19th century many microscopic studies on the structure of muscles have been conducted, focusing mainly on the stripe-like pattern - typical feature of the muscle tissue (fig. 1).

Stripes in muscle consist of alternating optically dense and less dense regions. The dense regions are known as A-stripes, the less dense areas as I-stripes. The A-stripes have a characteristic bright zone in the center called H-zone. Along the middle of the H-zone runs another dark line. The I-strips are separated by the dark Z-line. Area between two Z-lines is a basic unit of the muscle - sarcomere. A human biceps for example, contains at least ten million sarcomeres (fig. 3).

In the 40s it was found that adenosine-5'-triphosphate (ATP) is an energy source for muscle contraction. For the first time it was shown that muscles are molecular machines that convert chemical energy into mechanical energy. However, the role of ATP in living organisms is much wider. It does not only provide the energy for muscles contraction but also serves as an important energy storage molecule in living cells. Chemical energy becomes available when ATP is enzymatically converted (hydrolyzed) into ADP and phosphate ion (fig. 3). This energy can be used to drive biosynthesis, transport processes and perform mechanical work. Every day about 65 kg ATP are repetitively synthesized and hydrolysed in human body.

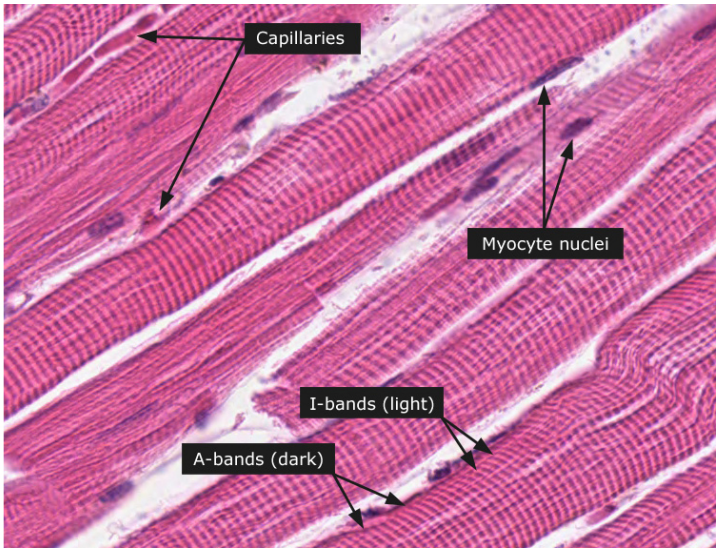


Figure 1: Microscpic image of muscle tissue with typical stripe-like pattern visible.

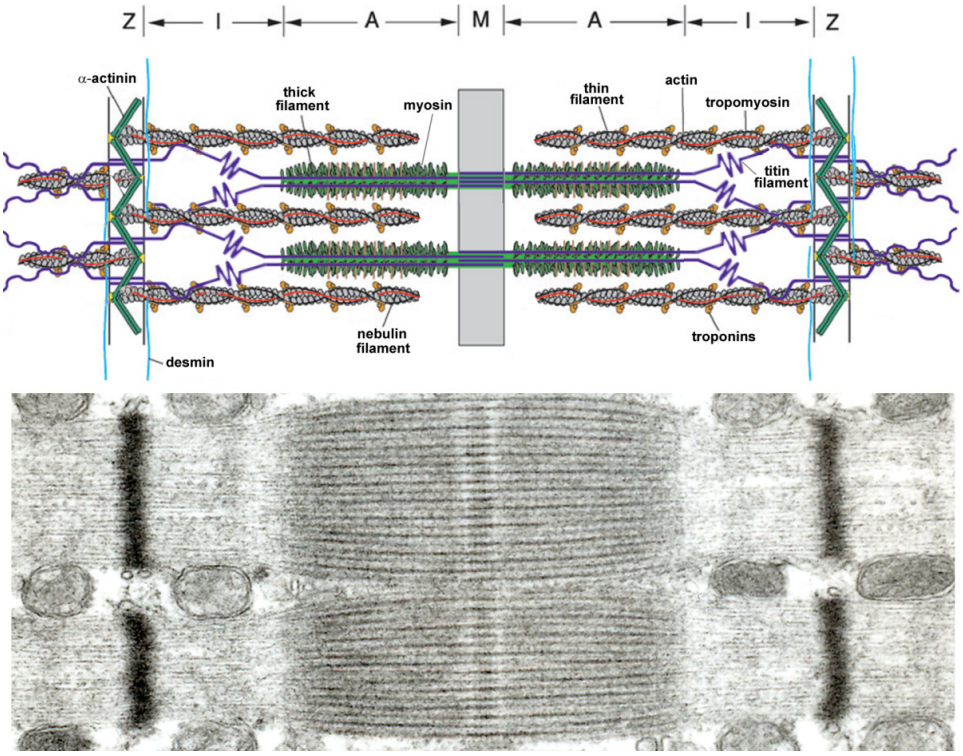


Figure 2: *Top:* Simplified model of two muscle sarcomeres in parallel. The sarcomere is comprised of the thin (mostly actin) filaments, the thick (mostly myosin) filaments, and the giant filamentous molecule titin. The thin filaments are anchored in the Z-line, where they are cross-linked by α-actinin. The thick filament is centrally located in the sarcomere and constitutes the sarcomeric A-band. The myosin heads, or cross-bridges, on the thick filament interact with actin during activation. Titin spans the half-sarcomeric distance from the Z-line to the M-line, thus forming a third sarcomeric filament. In the I-band region, titin is extensible and functions as a molecular spring that develops passive tension upon stretch. In the A-band titin is inextensible due to its strong interaction with the thick filament.
Bottom: Electron micrograph of the ultrastructural organization of sarcomeres. Bright areas consist of thin filaments (actin) and are called I-strips. Dark areas are called A-strips and consist mainly of thick myosin filaments. Figure from: Ottenheijm, C. A., Heunks, L. M. & Dekhuijzen, R. P. Diaphragm adaptations in patients with COPD. Respir Res 9, 12 (2008).

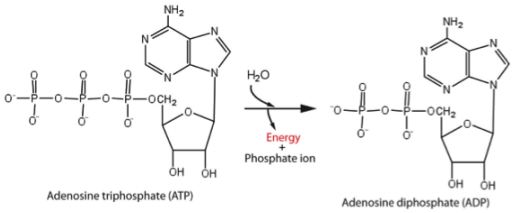
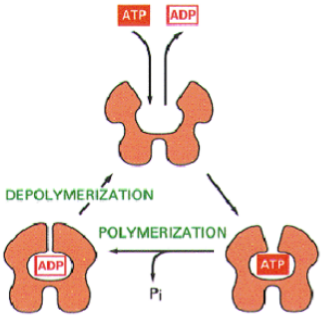


Figure 3: Hydrolysis of the ATP to ADP releases chemical energy stored in the pyrophosphate bond. 7.4 kcal are released per mole of ATP.

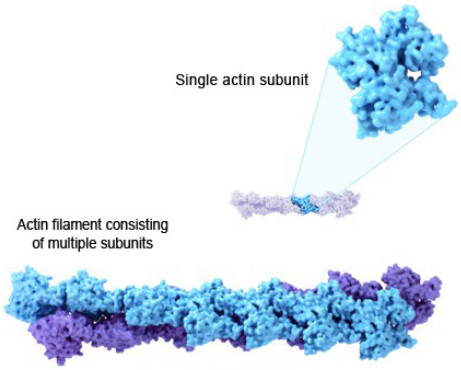
The next major scientific advance in in understanding how muscles work was the identification of the three proteins: **actin**, **myosin** and **titin**. These are the three main components of the muscle that build the sacromere and allow muscle contraction.

1.1 Actin Filaments

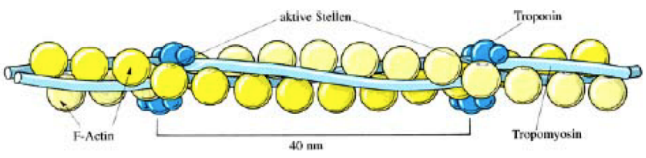
G-actin is a globular protein with a centrally located ATP binding site. If the binding site is occupied by an ATP, multimerization of G-actin monomers occurs and an F-actin polymer is formed (fig. 4a, 4b). Critical G-actin concentration and presence of ATP is is necessary to trigger the polymerization. At a lower G-actin concentration depolymerization of the filament starts.



(a) Polymerization cycle of actin.



(b) Formation of an actin polymer.



(c) The I-strip (fig. 2) consists mostly of actin filaments.

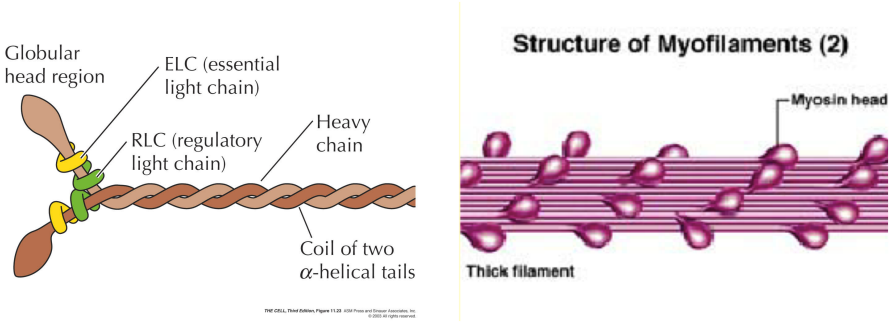
Figure 4: Actin and actin filaments.

A functional actin filament (fig. 4c) consists of two coiled F-actin fibers and two tropomyosin fibers. The tropomyosin fibers are located in the grooves between the F-actin fibers and connected by troponin complexes. Actin filaments are the main component of sarcomere I-stripes.

1.2 Myosin Filaments

Myosin molecules are generally composed of a head, neck, and tail domain. The head domain is a motor domain that binds the filamentous actin and uses ATP hydrolysis to generate force and to "walk" along the filament. The neck domain acts as a linker and as a lever arm for transducing force generated by the catalytic motor domain. The neck domain can also serve as a binding site for myosin light chains which are regulatory proteins. The tail domain mediates interaction with other myosin subunits.

A myosin filament consists of about 200 single myosin II molecules, each one having a molecular mass of about 490 kDa. The myosin II molecule is composed of two heavy motor chains (HMM) and four light chains (LMM). Heads of the heavy chains form a globular motor head domain while the tails two chains interact to form an α helical coiled coil (fig. 5a). Coiled tails of single myosin molecules are winding in parallel and make up a backbone of the myosin filament. Myosin molecules attach to each other in such a way that the myosin motor heads show up in in a regular manner on the filament surface (fig. 5b).



(a) Structure of the myosin II. (b) Structure of a myosin filament.

Figure 5: Myosin and Myosin Filaments.

1.3 Titin

Beside myosin and actin, titin plays a fundamental role in the ensemble of muscle proteins. Titin is a giant protein with a molecular weight of about 3.6 MDa. It forms its own filament structure which is stretched through half the sarcomer. One titin molecule has a length of about one micrometer and is built from up to 300 globular protein domains. Most of the titin domains

bins to actin and myosin filaments, giving muscle its stability. The part of titin which is not bound to actin and myosin provides muscle elasticity.

1.4 Sliding filament theory

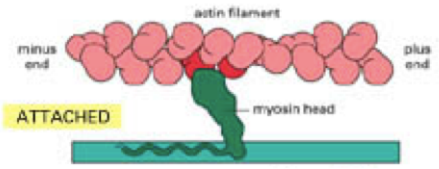
At the beginning of 1950s in vivo observations on frog muscle fibers with the interference microscope showed a change from low to high optical density of the I-band during contraction of the muscle. As explanation to this observation the sliding filament theory was postulated: the contraction of the muscle fiber is based on a telescopic sliding of the myosin into the actin filaments while the z-discs approach each other. This leads to an increased overlap of the myosin and actin filaments and therefore to an increased optical density. The sliding movement is caused by the forces between actin and myosin chains.

The electron microscope images show that actin and myosin filaments are bond to each other via heads of the myosin molecules. The finding that these cross bridges are responsible for the ATPase activity of the myosin proved the sliding filament model. In absence of ATP the myosin binds to the actin filament and adding ATP loosens these strong bonds. Combined, of myosin and actin show high ATPase activity, while myosin alone has rather low activity on ATP. This is consistent with the idea of muscles acting as machines converting chemical into mechanical energy.

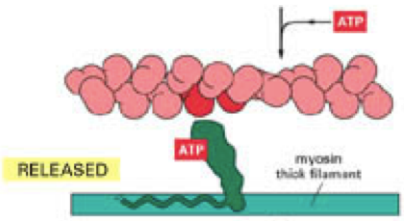
The mechanical activity of myosin, especially of the head domain, could first be observed via X-ray diffraction on contracting muscles. The addition of ATP changes the angle between the head domains and the actin filament from 45° to 90°. This periodic change in orientation of single myosin heads produces the forces which sum up and cause the sliding of the filaments and consequently contraction of the muscle. A detailed description of the reaction cycle of myosin is given below.

1.5 Myosin reaction cycle

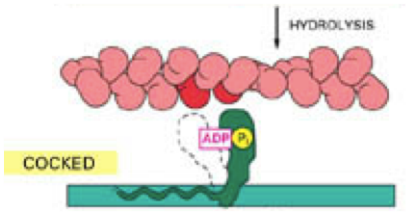
1. In the initial state myosin is bond via his head domain to actin.



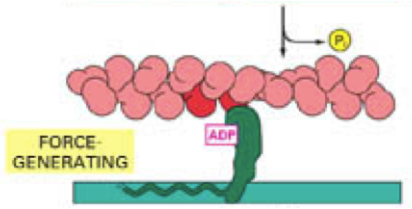
2. The myosin head hydrolyses ATP to ADP and an inorganic phosphate ion (Pi) and keeps the two products. The ATP dissociation leads to a tilt of the myosin head, the angle between the head domain and the actin filament changes from 45° to 90°C.



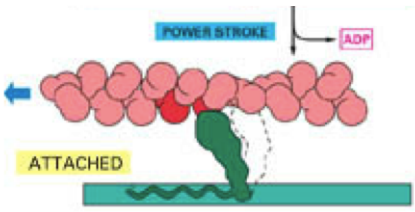
3. Binding of ATP loosens this bond.



4. The myosin head forms a new bridge to the adjacent actin molecule. The actin causes the release of the Pi ...



5. ... and subsequent release of the ADP. This causes a conformational change of the myosin head which acts like a power stroke. Now the next, adjacent active actin is available for the next cycle.



This cycle can be repeated as long as ATP is present and the thick filament moves steadily along the thin filament towards the z-disc. Every single stroke of the approximately 500 heads of a thick filament contributes

about 10 nm to the sliding with a repetition rate of about 5 Hz during strong contraction.

1.6 Gliding assay of actin filaments

Goal of this lab course is to perform an in vitro observation of the interaction between actin and myosin filaments. For this purpose myosin molecules are bond to a functionalized glass surface. After addition of ATP free actin filaments are moved by these myosins over the surface (fig. 6). The analysis of the enzymatic activity of myosin is done using the Michaelis-Menten kinetics. KInetic datawill be extracted from a video analysis of the free moving actin filaments. The optical acquisition of the data will be done by fluoresence microscopy.

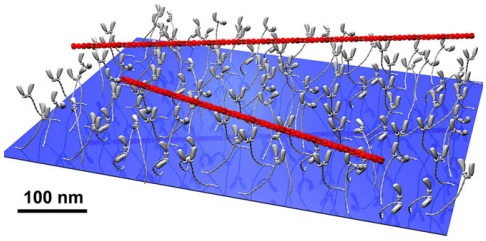


Figure 6: Schematics of the experimental concept. Fluorescently labelled actin filaments slide on the surface being pushed by immobilized myosin molecules.

2 Fundamental Theory

2.1 Kinetics of enzymatic reactions: The Michaelis- Menten equation

For simple enzymatically catalysed reaction one can assume that enzyme (E) and substrate (S) form a complex (ES) that after the processing decays again into free enzyme and product (P). If all reactions are reversible, then the following scheme holds:

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

Often the back reaction of the product P is so slow that it can be neglected:

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

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

$$v = \frac{d[\mathrm{P}]}{dt} = k_2[\mathrm{ES}] \tag{1}$$

The of formation of the ES complex depends on concentration of the enzyme and the substrate (2nd order kinetics). The ES complex can dissociate to form a product or it can fall back to free enzyme and substrate:

$$\frac{d[\mathrm{ES}]}{dt} = k_1[\mathrm{E}][\mathrm{S}] - (k_{-1} + k_2)[\mathrm{ES}] \tag{2}$$

In the case of the very high substrate concentration a stationary state regarding the concentration of the complex is reached, because the enzyme concentration is the limiting factor in the reaction kinetics. After addition of substrate the number of enzyme-substrate complexes would increase, but after a while the increasing decay rate slows down this increase until an equilibrium of the complex concentration is reached. This is so called stationary state approximation:

$$\frac{d[\mathrm{ES}]}{dt} = 0 \tag{3}$$

It follows that:

$$k_1[\mathrm{E}][\mathrm{S}] = (k_{-1} + k_2)[\mathrm{ES}] \tag{4}$$

$$[\mathrm{ES}] = \frac{k_1}{k_{-1} + k_2}[\mathrm{E}][\mathrm{S}] = \frac{[\mathrm{E}][\mathrm{S}]}{K_M} \tag{5}$$

where the Michaelis-Menten constant is $K_M = \frac{k-1+k2}{k1}$.

The free enzyme concentration [E] equals the total enzyme concentration [E_0] minus the enzyme concentration bound in [ES] complexes:

$$[\mathrm{E}] = [\mathrm{E}_0] - [\mathrm{ES}] \tag{6}$$

Thus:

$$[\mathrm{ES}] = \frac{([\mathrm{E}_0] - [\mathrm{ES}])\,[\mathrm{S}]}{K_M} \tag{7}$$

Which can be simplified to:

$$[\mathrm{ES}]\left(1 + \frac{[\mathrm{S}]}{K_M}\right) = \frac{[\mathrm{E}_0][\mathrm{S}]}{K_M} \tag{8}$$

$$[\mathrm{ES}] = \frac{[\mathrm{E}_0][\mathrm{S}]}{K_M}\frac{K_M}{K_M + [\mathrm{S}]} = [\mathrm{E}_0]\frac{[\mathrm{S}]}{K_M + [\mathrm{S}]} \tag{9}$$

From equation 2 we know that:

$$v = \frac{d[\mathrm{P}]}{dt} = k_2[\mathrm{ES}] = k_2[\mathrm{E}_0]\frac{[\mathrm{S}]}{K_M + [\mathrm{S}]} \tag{10}$$

Finally we obtain the equation describing the speed of enzymatic reaction depending on the substrate concentration, called Michaelis-Menten equation:

$$v = \frac{\nu_{max}[\mathrm{S}]}{K_M + [\mathrm{S}]} \tag{11}$$

where K_M and ν_{max} are parameters that define the kinetic behaviour of an enzyme as a function of the substrate concentration [S], if [S]$\gg$[E]. Those parameters can be determined in series of experiments in which enzyme concentration is kept constant while substrate concentration is varied. Initial reaction rate ν_0 is measured and plotted against [S] (fig. 7).

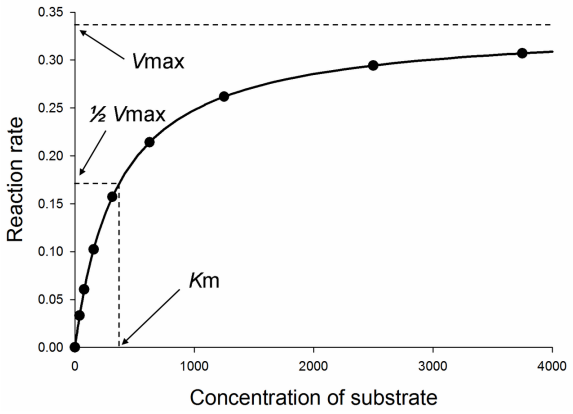


Figure 7: Plot representing enzymatic reaction rate for different substrate concentrations for Michaelis-Menten kinetics.

Due to the hyperbolic shape of this plot it is hard to extrapolate data to infinity for the determination ν_{max}. For that reason a linear transformation of the Michaelis-Menten equation is used, where the inverse production rate $\frac{1}{\nu_0}$ is a linear function of the inverse substrate concentration $\frac{1}{[\mathrm{S}]}$:

$$\frac{1}{\nu_0} = \frac{1}{\nu_{max}} + \frac{K_M}{\nu_{max}}\frac{1}{[\mathrm{S}]} \tag{12}$$

Kinetic parameters of enzymatic reaction can be easily extracted by plotting experimentally determined value of $\frac{1}{\nu_0}$ against $\frac{1}{[\mathrm{S}]}$ (fig. 8).

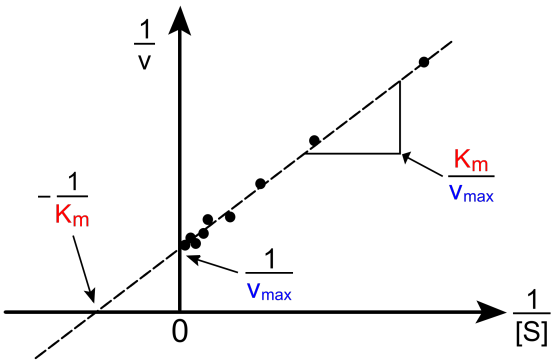


Figure 8: Illustration of the linear transformation of the Michaelis-Menten equation to so called Lineweaver-Burke plot.

2.2 Fluoresence microscopy

Radiation of a certain wavelength cannot be used to observe structural details that are much smaller than its wavelength. The theoretical resolution limit of optical microscopy is given by the wavelength of the used visible light and the numerical aperture of the microscope lens system and is described by Abbe diffraction limit:

$$d = \frac{\lambda}{2nsin\theta} \tag{13}$$

where d is the resolvable feature size, λ is light wavelength, n is a refractive index of the medium that light travels in and $\theta$is a half-angle subtended by the optical objective lens. $nsin\theta$ is called the numerical aperture (NA) and can reach about 1.4 in modern optics. That means that for a wavelength of 546 nm used in this experiment and a numerical aperture of 1.4 the resolution limit is approximately 200 nm.

To detect single molecules with a size smaller than diffraction limit they can be labelled with fluorescent dyes. Those dyes absorb photons of certain energy and emit ones with other, lower energy. Such labelled molecule can be observed by illuminating it with with light of the absorption wavelength and looking at it through a filter which let pass only the emitted wavelength. This leads to an image of the fluorescently labelled molecule on a dark background. This technique is called fluorescence microscopy. In figure 9 a typical fluoresence microscope is presented.

With help of the fluoresence microscopy particles which cannot be detected due to an bright background can be located. Advantage of this technique is high resolution and the ability to work biologically relevant conditions. However, it is important to notice that fluorescence microscopy is also subject to diffraction limit. Due to the close to zero background single molecules of sizes of few nanometers can be detected using fluorescence

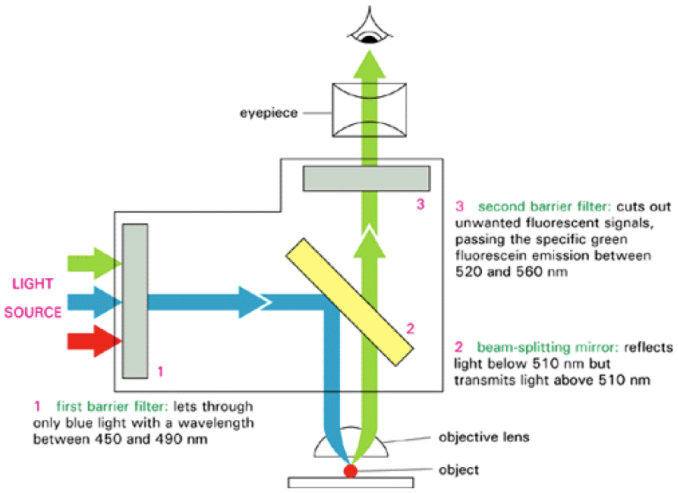


Figure 9: Schematics of the fluorescent microscope. A filter set consists of two blocking filters and one dichroic mirror. In this example the filter set is chosen for the detection of fluorescein.

but if they are closer to each other that diffraction limit, they can not be individually resolved.

The main limitation of fluorescent microscopy is photobleaching - a process in which fluorophores lose their ability to fluoresce as they are illuminated. Photobleaching occurs as the fluorescent molecules are damaged by the electrons excited during fluorescence. Photobleaching can severely limit the time over which a sample can be observed by fluorescent microscopy. Several techniques exist to reduce photobleaching such as the use of more robust fluorophores, minimizing illumination and use of photoprotective scavenger chemicals.

To image actin filaments in this experiment orange fluorescent dye conjugated with phalloidin group is used. Phalloidin is a small bicyclic peptide that selectively binds to polymeric F-actin. The spectra of the dye and of the mercury lap used as an illumination source are presented in figure 10.

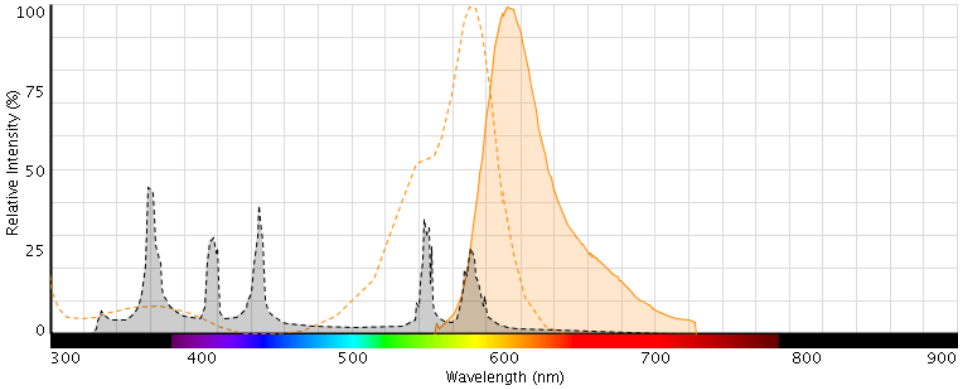


Figure 10: Absorbance and fluorescence spectra of the dye used in this experiment (orange) and emission spectra of the mercury lamp used as a light source (grey).

3 Experimental procedure

By means of a fluorescence microscopy we will perform in vitro investigations on the interaction between the muscle proteins myosin II and actin in presence of ATP. The Michalis-Menten equation can be used to find out the parameters describing the enzymatic behaviour of actin-myosin system. To acheive this, the speed of the actin filaments, which is a measure for the myosin-ATP reaction rate, will be determined at different ATP concentrations.

3.1 Necessary equipment and chemicals

The following solutions should be ready before the start of the measurements.

- **AB buffer, pH 7.4**:
  - 25 mM Imidazole hydrochloride
  - 25 mM KCl
  - 25 mM $MgCl_2$
  - 25 mM EGTA (ethylene glycol tetraacetic acid)
  - 25 mM DTT (dithiothreitol)

  This is a basic protein buffer used in the experiment. It is provided as a 10x concentrate. DTT has to be added on the same day.
- **ABSA buffer**:
  - 0.5 ml AB buffer
  - 0.5 ml BSA (10 mg/ml)
  - 4 ml H_2O

  This buffer contains BSA, a protein used to block and shield the active cover slip surface before addition of actin.

- **Actin** labelled with the phalloidine functionalized with a dye molecule. We recognize the dye by its pink color. This solution has to be stored on ice.
- **Glucose oxidase**, 0.12 mg/ml
- **ATP**, 0.1 M. This solution has to be stored on ice.
- **Myosin**, 0,51 mg/ml. In our experiment only the heavy motor chains (HMM) of the myosin II are used. This solution has to be stored on ice.

3.2 Assembly of the fluid cell

Fluid cell for fluorescence microscopy observations has to be fabricated. Firstly, the cover glass slips are modified with thin layer of nitrocellulose. Nitrocellilose has a non-specific affinity for amino acids and can be used to immobilize myosin molecules on the surface.

As a second step, glass slide and a cover glass slip are fixed on top of each other with double sided adhesive tape, as shown in fig. 11. The space between glass plates has a volume few tenths of micrometers and can be filled up with the liquid sample due to capillary forces.

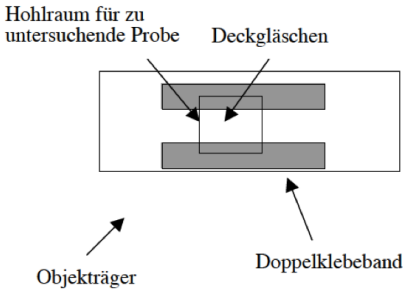


Figure 11: Scheme of the fluid cell used in the experiment.

3.3 Preparation of protein solutions

The following solutions have to be prepared shortly before each experiment.

- **HMM solution**:
  - 2 μl of myosin stock
  - 98 μl of AB buffer
- **Actin solution**:
  - 1 μl of actin stock
  - 299 μl of AB buffer
- **Motility buffer**:
  - 88 μl AB buffer
  - 5 μl Glucose oxidase

– 5 μl 20% glucose
– variable amount of ATP

This buffer is used to observe actin filaments movement in presence of ATP. Glucose oxidase and glucose are added slow down bleaching of dye molecules. Buffer has to be prepared fresh before every single measurement.

3.4 Sample preparation

1. Add ∼40 μl of HMM solution to fluid chamber. Wait 3 minutes.
2. Rinse with 100 μl of ABSA buffer
3. Add ∼40 μl of actin solution to fluid chamber. Wait ∼10s.
4. Add ∼40 μl of motility buffer.

3.5 Measurements

Add a droplet of immersion oil to the coverglass slip of and put the fluid chamber with the oil side on the objective. Adjust the objective such that actin filaments running over myosin become visible.

Perform the following experiments:

- Determine the kinetic of the enzymatic hydrolysis of ATP by myosin. Record the videos of the moving actins in liquid chambers filled with different ATP-concentration (2000, 400, 100, 50, 20, 10 and 5 μM).
- Determine how the temperature influences the kinetics of gliding assay. Perform the measurement of actin speed in room temperature and after cooling the flow chamber on ice.
- Estimate the usage of ATP in the measured system. After measuring the first concentration of ATP keep the fluid chamber in the fridge and measure it again at the end of the day. How did the speed of the actin filaments change?

3.6 Evaluation of the results

- With help of the ImageJ software (short introduction during the lab course) measure the speed of the actin filaments in recorded videos.
- Draw a graph of the actin filamets speed versus the ATP concentration. Interpret the outcome with help of the in paragraph 2.1 describing Michaelis-Menten equation. Determine the K_M and ν_{max}. What assumptions have to be met in order to use Michaelis-Menten kinetics?
- Interpret the effect of the temperature on the reaction.
- Estimate changes in ATP concentration during the reaction. How much ATP is used?