

XXXI. OTDK Biológia Szekció
EXAMINATION OF MYOSIN THICK FILAMENT
MORPHOLOGY USING ATOMIC FORCE
MICROSCOPY

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Abstract

One of the most basic processes in the healthy operation of an organism is muscle contraction which is based on the interaction between actin and myosin. Myosin molecules under physiological conditions are often organized into thick filaments, the detailed structure of which is still unknown. Synthetic myosin thick filaments provide us a great opportunity to shed light on the assembly of these polymers in vitro. Using atomic force microscopy allows us to observe filaments through high-quality images in a close to native environment.

During this project, we performed experiments in order to derive a detailed description of thick filament structure and filament assembly. Thick filaments were synthesized from rabbit back muscle myosin using a dialysis protocol. To investigate the process through transient states in the assembly of myosin dimers into filaments, we prepared synthetic filaments using buffers ranging from 0 mM to 120 mM potassium-chloride (KCl) concentration.

While imaged on mica, thick filaments show an opened-up nature, thereby enabling us to study the internal organization of synthetic filaments. We observed a difference in width depending on ionic strength, as the average was 103 ± 23 , 179 ± 34 , 167 ± 38 , 162 ± 43 , 159 ± 41 nm at 0, 30, 60, 90 and 120 mM KCl, respectively. To further investigate the difference induced by ionic strength, additional experiments were performed when the buffer was changed at different steps, suggesting an irreversible yet strongly buffer-affected surface-filament interaction. Results on mica were confirmed with experiments using nitrocellulose-covered surface, which revealed the same ionic strength dependency.

On high resolution images free myosin molecules as well as fine structural details were observed, for example a core structure specific to 0 mM KCl filaments which varied in length and width according to overall filament dimensions. Furthermore, at higher ionic strength, we were able to resolve subfilaments constituting the trunk and branching out by the ends of the filament into individual myosin tails. An average filament is consisted of about 3-10 subfilaments and seems to be organized into a twisted, helical structure.

Our results on morphological differences might lead to a better understanding of the underlying physicochemical mechanism behind filament assembly, also providing a good starting point to investigate polymerization processes as well as actomyosin interactions.

1 Introduction

In our project, we aimed to shed light on the assembly of thick filaments from myosin monomers and how this process is influenced by the environment. The in vitro model system used to study the native assembly consisted of synthetic thick filaments. Information about the sample was collected by imaging with atomic force microscopy, the technique most suitable for single-molecule examination of biological samples.

1.1 Myosin Thick Filament

Myosin is an example - probably the most important one - for **motor proteins**, meaning that in the presence of 'molecular fuel' adenosine triphosphate (ATP) it has the ability to exert force on other cellular elements, and change their or its own position within the cell. A single myosin has a long, rope-like **tail** domain and a globular **head**. Two myosins can form a dimer by wrapping around each other with their tails. This dimer - often named monomer - is a fully functioning unit, but dimers of type II myosin can interact by their tails domains and form a structure called **thick filament**. Under physiological conditions the filament has an approximately cylindrical shape, with the paired heads of the consisting dimers on the surface. (See figure 1.)

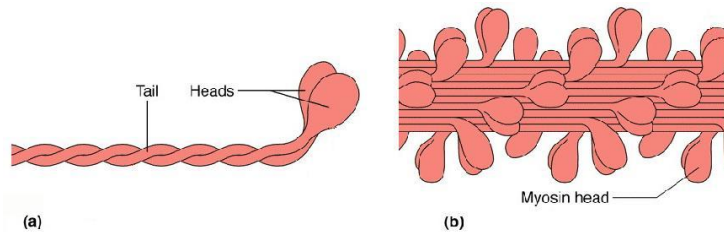


Figure 1: (a) Schematic image of myosin dimer. (b) Portion of myosin thick filament.

Myosin's main role in our system is the motor activity underlying most contraction based processes, for example traction. Understanding the mechanisms of muscle contraction and myosin's role in it required a number of significant discoveries through the 20th century. Until the 1930's, muscle had been thought to consist one type of molecule, and only since then we know that contraction is caused by the adenosine triphosphate dependent interaction between two different polymers. The so-called Sliding Filament Model - proposed in 1969 [1] - is based on the relative movement of actin and myosin, induced by the motor activity of the myosin heads. Myosins bound into a thick filament are moving along the actin thin filaments, pulling the Z-discs (boundaries) of the sarcomere, shortening the muscle cell. This activity is automatic, the presence or lack of ATP can switch the mechanism on or off, but it is also affected by many other factors like Ca^{2+} influx and the activity of additional muscle proteins.

The model was refined during the last decades thanks to the high resolution results from crystallization and AFM experiments, and the introduction of using **synthetic thick filaments** as a great in vitro model system for native myosin and myosin-like molecules. [2] As the structure of both thin and thick filaments become more detailed we are closer to reveal the details of their function as well. However, still many questions are unresolved about the components and exact mechanism of muscle contraction.

1.2 Atomic Force Microscopy

From the beginning of the 20th century a series of inventions in microscopic techniques helped us to a better understanding of the structure of organic and inorganic materials. **Light microscopy** in itself was and is a powerful tool in the examination of different biological samples, from leaf-sections as we all remember from Biology classes to cell nuclei and chromosomes. Despite its limitations in resolution induced by the relatively large wavelength of the illuminating light, the very diversified family of light microscopes is still the most common for in vitro measurements.

The first breakthrough expanding our measuring capacity was the introduction of **Electron Microscopy** (EM), which lowered the limit of resolution from 200-300 *nm* to a few Angstroms (0.2-0.8 *nm*). This gave the opportunity to image molecular level structures never seen before and shed light on many structures in the cell governing processes not understood up to that point. However, the operating conditions (samples must be viewed in vacuum and require conductive coating) makes imaging quite roundabout and in vivo systems cannot be studied in an electron-microscopy experiment.

The solution for this problem was provided by the family of Scanning Probe Microscopes invented in the 1980's, for example **Atomic Force Microscope** (AFM). [3] An advantage of this microscope family is that the achievable magnification is not diffraction-limited like the aforementioned ones.

The AFM is a surprisingly small-size equipment, the heart of which consists of a **cantilever** with a sharp, nanometre scale **tip** (probe) at its end which actually performs the scanning (**fig. 2**). When it is in the proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever. This deflection is measured indirectly by photodiodes, that detect the shift of a laser spot aimed onto the top of the cantilever and reflected from it. The vertical deflection data tells us about the interaction between the sample and the tip, and fed back to the piezoelectric scanner which can adjust the height to keep distance.

Further improvements in biological experiments were caused by the introduction of new modes in which the AFM can be operated, starting from contact to non-contact and tapping mode.

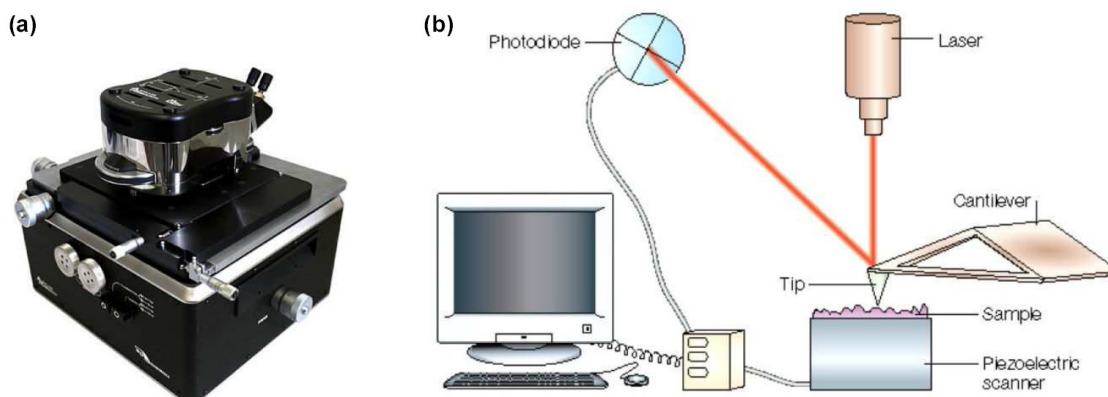


Figure 2: (a) Atomic force microscope. (*Molecular Force Probe 3D*, Asylum Research) (b) Schematic representation of the components of the microscope.

In **contact mode** the tip is in very close proximity to the sample (touching it), so it literally corresponds to the case when someone 'maps' an object by slowly stroking it. In the case of many biological samples this method is too rough and possibly damages the sample, so scanning is usually carried out differently. However, its advantage is that in such proximity the studied molecules can even bind to the tip, therefore we are able to apply force on the structures - for example proteins - attached to the surface. Once the examined structure is bound to the tip, moving the cantilever away from the surface means a pulling force on the sample. In this mode, cantilever bending - measured from the reflected laser spot - is translated into exerted force value. Therefore the force needed to reach a given distance holds information about our sample's inner structure.

In the other two modes, the controller oscillates the cantilever at a very high rate - up to a few hundred kHz - and the tip interacts with (but not through mechanical contact) the sample on the down stroke of each cycle, resulting in a change in the amplitude or the resonant frequency of the oscillation. In **non-contact mode**, the tip is relatively far from the surface, the forces between the two are very low, so high resolution is hard to achieve and no imaging in liquid is possible.

On the other hand, in tapping or **intermittent contact mode** the tip is closer to and eventually tapping (hence the name) the surface. The modulation in amplitude induced by repulsive or attractive forces is measured as cantilever deflection. The primary advantage of intermittent contact mode is that the lateral forces between the tip and the sample can be eliminated, which greatly improves the image resolution. Moreover, high resolution images can be obtained in air and in liquid as well, and as the obtained height values are always fed back to the controller, the tip does not cause harm to the structures. It is the most widely used operating mode, especially suitable for soft and poorly fixed samples like biological macromolecules.

The information gained from the deflected laser spot's position is translated into a height value at the cantilever's current xy position. To map the sample, the cantilever is moved over subnanometre wide lines back and forth, and the height data assigned to the points of a rectangular area can be combined into a topographical map of the surface. Since the background surface - for example mica - is usually atomically flat, every structure attached to it has distinctive height value, and therefore noted on the AFM micrograph by an outstanding colour.

The data collected in tapping mode is separated into three output images which altogether describe the sample. **Height data** are the representation of the cantilever position used in the feed-back system, hence a good measure of the height of surface structures but does not show distinct edges of these features. **Amplitude images** show edges of samples indeed, since they are actually the difference between trace and retrace (back and forth) scanning data. **Phase images** as their name suggest obtained from the phase shift introduced by interactions with the scanned object. They contain information on varying elastic properties as well as chemical interactions between tip and sample over the scanned area, hence useful to tell apart structures low compared to irregularities on the surface.

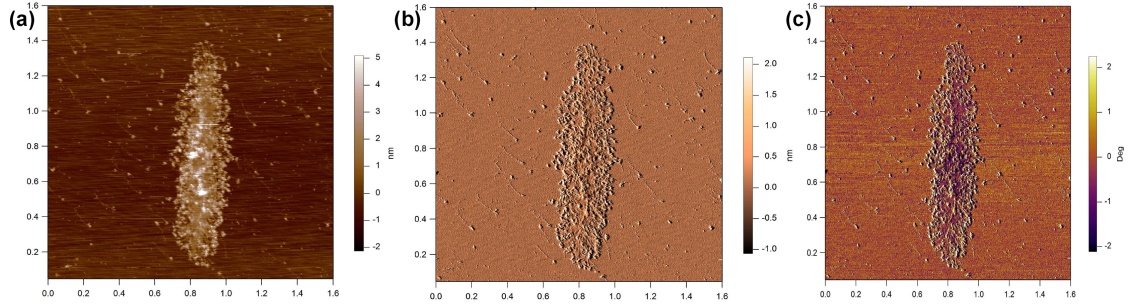


Figure 3: Height (a), amplitude (b) and phase (c) outputs imaged by MFP3D. The imaged structure is a synthetic myosin thick filament, but free myosin molecules (both head and tail domains) are visible as well, especially in (b). Image dimensions are in μm .

2 Materials and methods

The thick filaments we imaged were polymerized from free myosin by slowly decreasing the solution ionic strength. The myosin was purified from rabbit and stored in glycerine on -20°C . The synthetic filaments were polymerized using **dialysis**, proposed in [4] to maintain a higher number of formed filaments compared to controlled dilution. In different experiments the myosin was dialysed on 4°C against dialysis buffers of various **potassium-chloride** concentrations ($[\text{KCl}]$).

The imaging was carried out with Atomic Force Microscope in aqueous conditions. A 10-nm tip cantilever was used suitable for the observation of nanometre-scale structures in buffer. The ideal setting of scanning parameters depended on the actual sample (method of preparation, dilution factor, incubation time), so for each imaging these parameters needed refinement. The microscope was controlled with the dedicated **IgorPro 6.04** software’s MFP3D module, which provided real-time image of the scanned area.

In each experiment a desired number of imaged filaments were defined. This goal was usually surpassed by about 10% to overcome occasional not analysable TFs. In many cases the data used to describe a given population were collected from several experiments supporting generality. The populations were characterized by their average **length** and **width**, the latter measured at three perpendicular planes. The measurements were carried out within the IgorPro framework, and measured data sets were compared using statistical tests.

2.1 Thick filament preparation

Thick filaments were prepared by dialysis from rabbit back muscle myosin, purified based on the method of Margossian and Lowey [5] and stored in a glycerinated solution. The dialysis buffer consisted of 10 mM imidazole (set to pH 7.0 by adding HCl), 2 mM MgCl_2 and 0 to 120 mM KCl (varying over experiments) in a final volume of 1 l. Adenosine triphosphate (ATP) was added to the buffer right before dialysis, in concentration of 1 mM. For some experiments investigating the difference induced by the absence of ATP, this last step was skipped.

Synthetic thick filament polymerization by dialysis was carried out in a specific microcassette, constructed from an Eppendorf tube, for experiments involving such small volumes. The lid was separated from the tube’s body, and the glycerinated myosin ($\sim 150\ \mu\text{l}$ to $150\ \mu\text{l}$ buffer) was pipetted into it. The tube was cut about 4-5 mm from the top, and this ‘ring’ was used to keep the membrane secured. The previously hydrated dialysis tubing was attached to a glass mixing rod by one corner (using parafilm or a cotton yarn). This prevented the microcassette from floating on the surface of the buffer, which could have dried out the sample. Finally, the

myosin was dialysed at 4°C against 300 *ml* of the buffer with slow stirring for more than 1 hour. Then the buffer was changed and the process repeated with finally leaving the sample to dialyse overnight.

The thick filament solution was pipetted out from the microcassette after cutting the dialysis membrane with a scalpel. The solution was in every step moved carefully using a **cut pipette tip** instead of a normal one, to minimize sheer forces. Therefore the final volume of the thick filaments is imprecise, as well as the dilution factor which was about 1:3 in the sample. The thick filaments solution if stored on ice could be used for AFM imaging for 3-4 days.

In addition to thick filament synthesis, for some experiments **nitrocellulose-coated coverslips** had to be prepared. Rectangular coverslips were used, but first they had to be cleaned to avoid irregularities of the surface caused by contaminating particles. Cleaning involved sonication in acetone for >20 minutes. Then 0.25 M nitrocellulose was applied to the surface. Since spin-coating was found to create an erratic cover on the glass, I decided to prepare coverslips by **dipping**. Still, nitrocellulose surfaces were rough compared to atomically-flat mica, so after at least 12 hours of drying the coverslips were tested with AFM and used only if meeting certain conditions.

2.2 Imaging by AFM

The sample prepared by dialysis was further diluted with different factors. Usually we started out with a 100x dilution obtained in two 10x diluting steps (20 μ l sample/prior dilution to 180 μ l buffer), and further changed according to the image quality. The optimal dilution (calculated from the original microcassette solution) depending on the ionic strength was between 1:50 and 1:4000.

The solution was deposited on **freshly cleaved mica** or nitrocellulose-covered surface and incubated for 10 minutes. After incubation the sample was **washed** 5 times to remove molecules in the solution not attached to the surface (which otherwise could cause distortions if binding to the scanning tip). Cut pipette tips were used not only for the steps moving TF solution, but also for washing, to avoid high fluid velocities caused by the very small tip diameter.

The imaging was done with a **Molecular Force Probe 3D** atomic force microscope (Asylum Research, Santa Barbara, CA). Previous experiments suggested that using a **Biolever mini** (BL-AC40TS-C2) cantilever with a 10-nm-radius tip not only improves quality as expected, but sometimes crucial to image thick filaments, because the sample (especially at lower ionic strengths) interacts more with BL-RC150VB-C1. For dry experiments (nitrocellulose surface check, dried samples) BL-AC160TS was used.

Starting the AFM experiment consisted of the following steps:

1. First, we set up the AFM without the sample (typically until the incubation period) by placing the cantilever within the holder in the microscope and locating the laser on the tip of the cantilever.
2. The cantilever was tuned to check laser positioning and determine its **resonant frequency** in air. Sometimes the automatic tuning was modified manually if another peak at a more characteristic frequency seemed suitable.
3. After incubation and washing, the sample was placed on the microscope table. In dry scanning step 4 followed, while experiments with BL-AC40TS continued by lowering the AFM head till the cantilever holder submerged into the liquid layer. Then step 2 was repeated after adjustment of the laser spot to determine the in-buffer resonant frequency.
4. Using the optical part of the microscope the cantilever tip was checked, as well as the surface, and the sample was moved if any larger structures (e.g. step in the mica) were visible in the targeted scanning area. In liquid occasional bubbles were avoided by moving the AFM head carefully back and forth or adding more buffer.
5. The cantilever was slowly descended close to the surface (using the positioning wheels on the head), until a decrease in amplitude caused by attractive interactions with the sample was detected.

After setting up the microscope and finding the targeted surface, scanning was initiated and the output image followed **real-time** in IgorPro. Initial parameters were set to values obtained from literature and prior knowledge (see in table 1), and refined mainly according to the height images, although all three outputs held some information about measurement quality and were taken into consideration. Most undesired effects could be eliminated by adjusting the parameters, for example during the first 2-3 scans the set point often had to be changed to follow the drift in free amplitude resulting from temperature equalization between the sample and the head. Further noises required turning the not necessary electrical equipments off or scanning a neighbouring area to avoid tip damage.

2.3 Data acquisition and analysis

The filaments were examined at 0 mM, 30 mM, 60 mM, 90 mM and 120 mM KCl concentrations (abbreviated in the following as 0 mM TFs, etc.). We found that images of **12 μm** by both dimensions with 512×512 pixels are optimal for the study of the whole population, because the achieved resolution ($\sim 23 \text{ nm}$ pixel

Table 1: *Typical settings for myosin imaging in liquid (BL-AC40TS) and dry sample imaging (BL-AC160TS).*

Scanning parameter	BL-AC40TS	BL-AC160TS
Resonant frequency (in air)	110 kHz	335 kHz
Resonant frequency (in buffer)	20 kHz	-
Set point	420 mV	400 mV
Free amplitude	500 mV	550 mV
Integral gain	8.8	15

size) is high enough for statistical measurements (length, width), but the scanning-time/captured-filament ratio is still relatively good. For each ionic strength, first an ideal dilution was determined taking into consideration the following aspects: minimal filament overlapping, minimal free myosin molecules between the filaments producing noise, maximal number of TFs per area. This dilution depended on the current sample, so even at same KCl concentrations had to be recalculated for each experiment. Typically a dilution factor of 200-1000 was the most suitable.

Our aim was to image enough filaments for later statistical analysis ($n \approx 100$) at each ionic strength, so about **100-120 TFs** were collected at each ionic strength condition, counting only those completely in the scanned area and not overlapping with other filaments. In average 4-5 recordings were needed to have sufficient data. Similarly, ~ 60 TFs were captured for the study of filament morphology on nitrocellulose-covered surface, from samples prepared in 0 mM, 60 mM and 120 mM KCl buffer.

In buffer change experiments 25-30 filaments were imaged, since the question addressed was whether they compose a distinct population. However, sometimes more snapshots could reveal information about the way of transition if it happened on a time-scale comparable to scanning as discussed later.

For high-resolution study of internal structure about 10 filaments (selected from large-scales images) were imaged at each ionic strength with slow scanning speed. Due to the technique of scanning the area (along horizontal lines), vertical structures can be imaged with the best quality, hence we modulated the angle of the scan according to the chosen filament. If the population had elements with different types of morphology each were selected to zoom on, in the ratio they appeared within the population.

All the image analyses were carried out in the framework provided by **IgorPro** capable of handling the multiple layers of AFM output data (height, amplitude and phase 'intensity' values). Height images were used for morphological characterization, but for some features phase images can hold interesting information, despite their sometimes poor quality resulting from the imaging optimization method. Measurements for statistical purposes were performed on lower resolution images.

After comparing the images obtained at different ionic strengths, we assumed that analysing length and width was sufficient to point out the significant differences between filaments. To eliminate possible fluctuations caused by smaller features like individual myosin heads, all measurements were carried out by averaging filament data along a **5 pixel wide line** instead of taking a single pixel cross-section.

Length was measured along the main axis of the filament, by obtaining a **base height** value (typically not zero) from both the image and the cross-section graph and calculating the x distance between the two cursors located at delimiting base points. (See figure 5.) Because of the occasional presence of not fully-formed TFs or not organized myosin aggregations, a length-criterion of $l \geq 500 \mu m$ was stated to discriminate these structures from real synthetic filaments, based on previous findings of average native and synthetic filament length. [6]

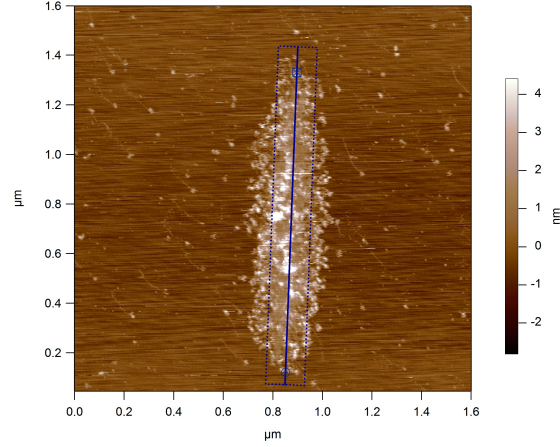


Figure 4: *The method of length measurement on a filament. The central blue line indicates the centre of the cross-section, dashed lines the area in which filament values are averaged.*

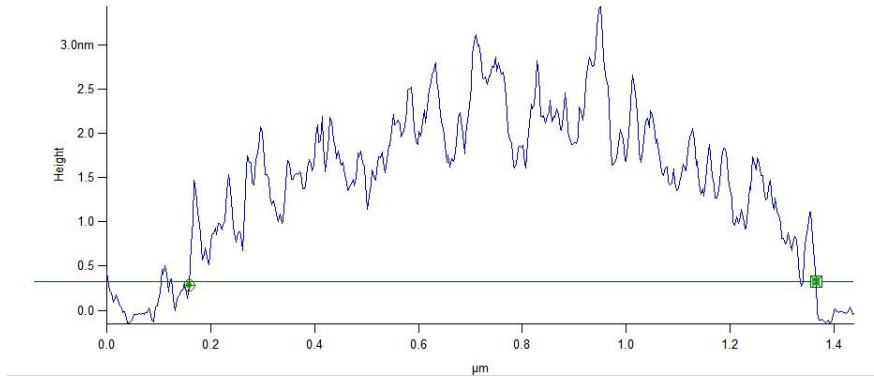


Figure 5: *Cross-section graph taken along the line in fig. 4. Baseline is in green, the length measured as the distance between green cursors.*

The width of the filament was measured at three different points: in the **centre** of the filament and at **1/4** and **3/4** of its length, 1/4 denoting the point belonging to the left/upper side of the TF (figure 6). At each point, first a base height was defined and the **base width** calculated similarly to length measurements. Then the **width at half-max** was determined by taking the distance of the two points of the cross-section graph being at $(\text{maximum-height} - \text{base-height})/2$, as shown in figure 7.

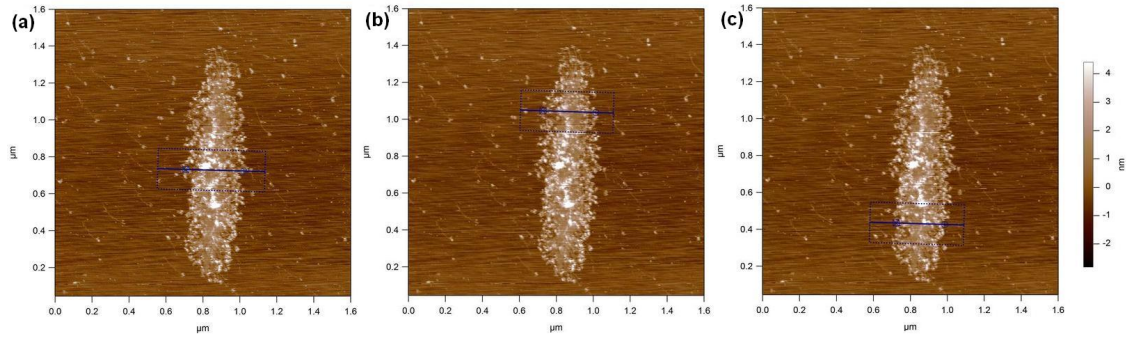


Figure 6: *The method of width measurement on a filament. The central line and dashed lines indicated the cross-section where height values are studied in the centre of the filament (a), at 1/4 (b) or at 3/4 (c).*

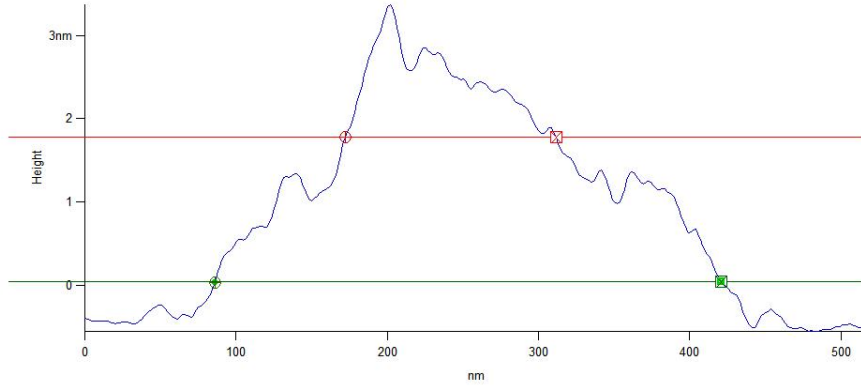


Figure 7: *Cross-section graph of a filament in its centre, as shown in figure 6a. Green line indicates the baseline, the distance between the green cursors being the width at base. Red line is at the half of the maximal height, width at half-max is measured between red cursors.*

3 Results

The outcome of an AFM sample imaging experiment is affected by numerous factors. Many features of the images depended on whether the ideal imaging parameters were found, and did not hold any information about the sample’s quality. For the first time we had to determine an ideal parameter set applicable for further measurements to obtain the best quality on zoomed and lower resolution images as well. Except for some inevitable external noise sources (like the 50 Hz mains hum) varying the parameters helped us to images where as small features as individual myosin tails were perceptible. These images suggest periodicity and helical organization of a tail bundle within the synthetic filament. However, giving quantitative measures on such characteristics is beyond the aim of this study. On lower resolution scans, statistical differences between TFs prepared against different buffers could be observed and quantified.

3.1 Ionic strength dependence

Filament morphology showed clear **[KCl] dependence** as illustrated by the height images in figure 9. First of all, TFs dialysed in a no KCl buffer showed many distinguishing features, corresponding to previous results. [4] Their average width was significantly smaller than other filaments’, while in many cases a compact and high structure - called **core** - was observable in the centre of the filament. The dimensions of the core differed according to the dimensions of the filaments, sometimes incomplete cores were visible present only in a fracture of a TF. On close-up images, further details of this structure were seen, but its components are not well-recognizable - it can be that the middle of the filament was not as fixed to the mica as the sides, and therefore the tip was not able to resolve the molecules consisting this part.

The outstanding characteristics of 0 mM filaments can be further demonstrated by comparing their population to TFs prepared at another KCl concentration. As later discussed, average width appears to be in inverse ratio to ionic strength, therefore 120 mM filaments were used for comparison as the ones closest in morphology. Width values calculated for all filaments as the average of the three measuring points were tested for difference of their means when variances are unequal. The two populations ($n_{0mM} = 106$, $n_{120mM} = 109$) were found to be significantly **different** with $p \ll 10^{-6}$ in both their half-max and base width, which is clearly visible if the two width-distributions are plotted simultaneously. (Fig. 8.)

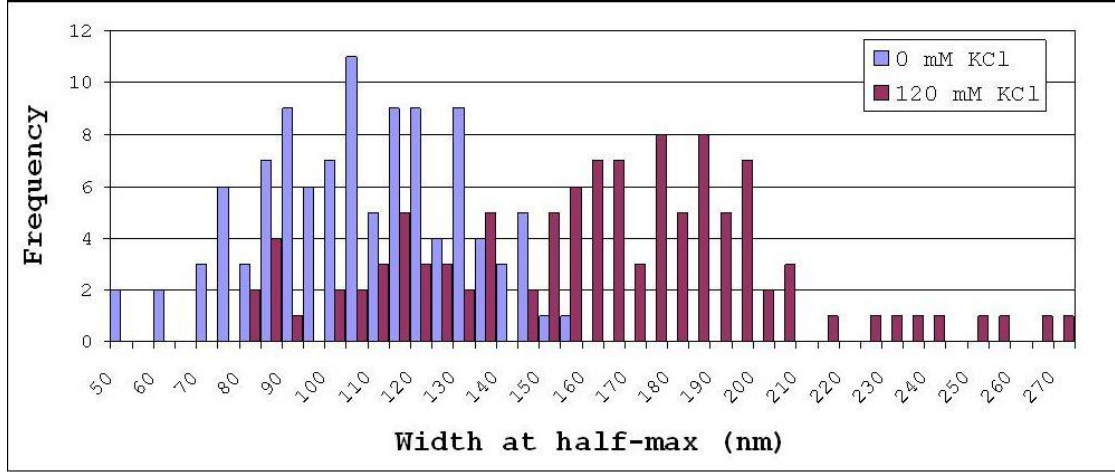


Figure 8: Comparison of width value distribution of 0 mM and 120 mM KCl TF populations.

Leaving the 0 mM TFs out and examining only the other four populations, some trends in length and width could be observed. The average width slightly decreased from 30 mM to 120 mM KCl filaments, while the length increased, resulting in a decrease in the width-to-length ratio. Since the increase of length was more dominant, the rough estimation of area as base-width $\times$ length also increased with KCl concentration. Interestingly, 0 mM filaments did not stand out in this case. (See figure 10 and table 2.)

It is important to note that the above data are affected by many factors due to the nature of our measurement (e.g. the 10-nm-width of the scanning tip), hence width and length values can be inaccurate and different from what could be observed in a similar set-up using electron microscopy. Nevertheless, these factors are identical for all measurements, therefore the trends and features distinguishing the different ionic strength populations - which we focus on in order to understand filament formation - are not by-products of our method.

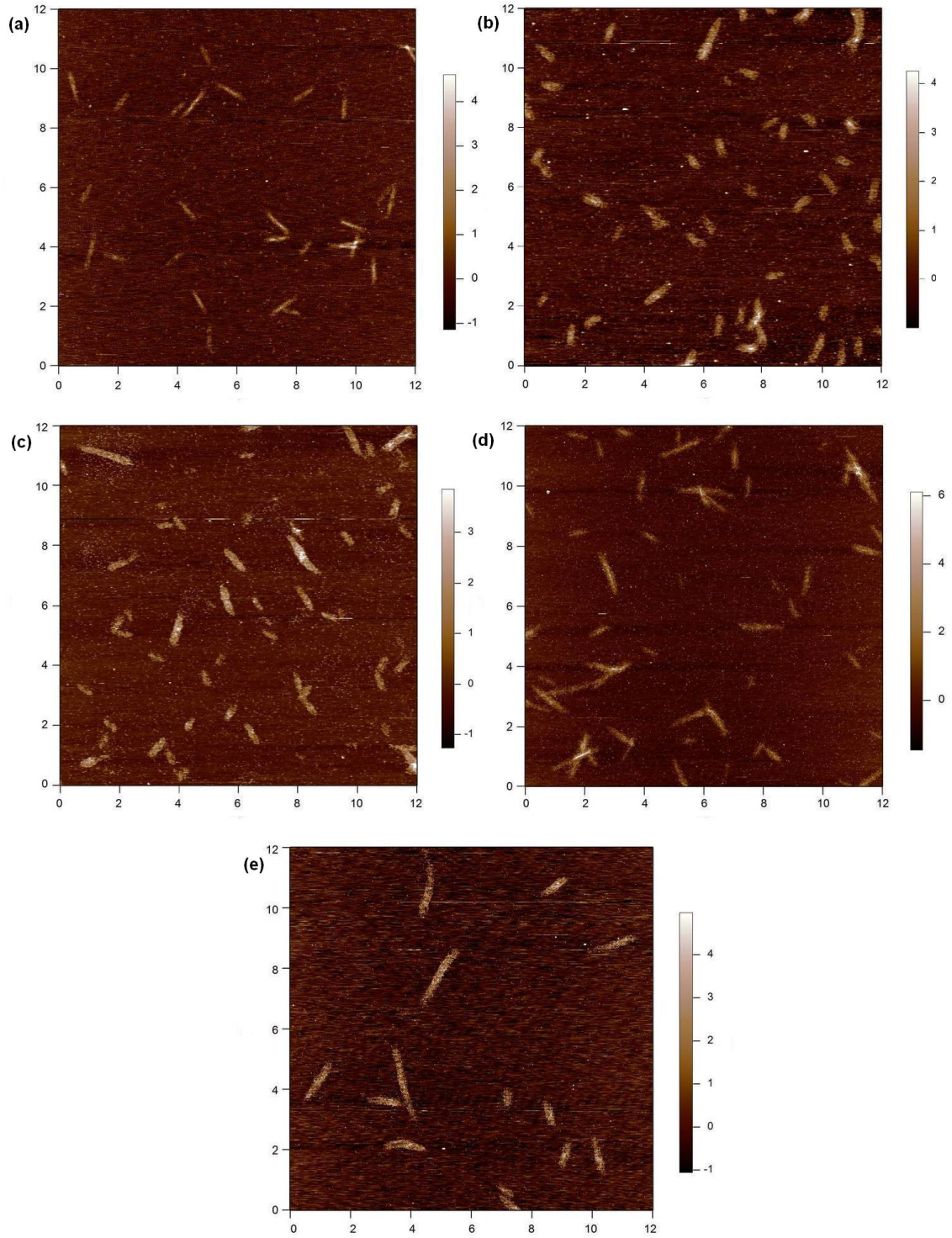


Figure 9: Large-scale height images of filament populations prepared at 0 mM (a), 30 mM (b), 60 mM (c), 90 mM (d) and 120 mM (e) KCl concentration. Image dimensions are in μm . Colour scales indicate the height values in nm.

Table 2: Summarizing table of average values and standard deviations of the different measures describing filament morphology for each KCl concentration.

KCl concentration	0 mM	30 mM	60 mM	90 mM	120 mM
Length [nm]	908	716	753	1079	1382
Standard dev. [nm]	231	161	262	394	536
Width (at half-max) [nm]	104	179	167	162	159
Standard dev. [nm]	23	34	38	43	41
Width (at base) [nm]	182	271	250	249	242
Standard dev. [nm]	24	36	41.04	43	39
Width/length	0.12	0.25	0.22	0.16	0.13
Standard dev.	0.048	0.06	0.053	0.049	0.057
Area [μm^2]	0.16	0.20	0.21	0.28	0.34
Standard dev. [μm^2]	0.045	0.061	0.083	0.119	0.145
Δwidth [nm]	29	41	43	47	49
Standard dev. [nm]	23	34	35	35	38

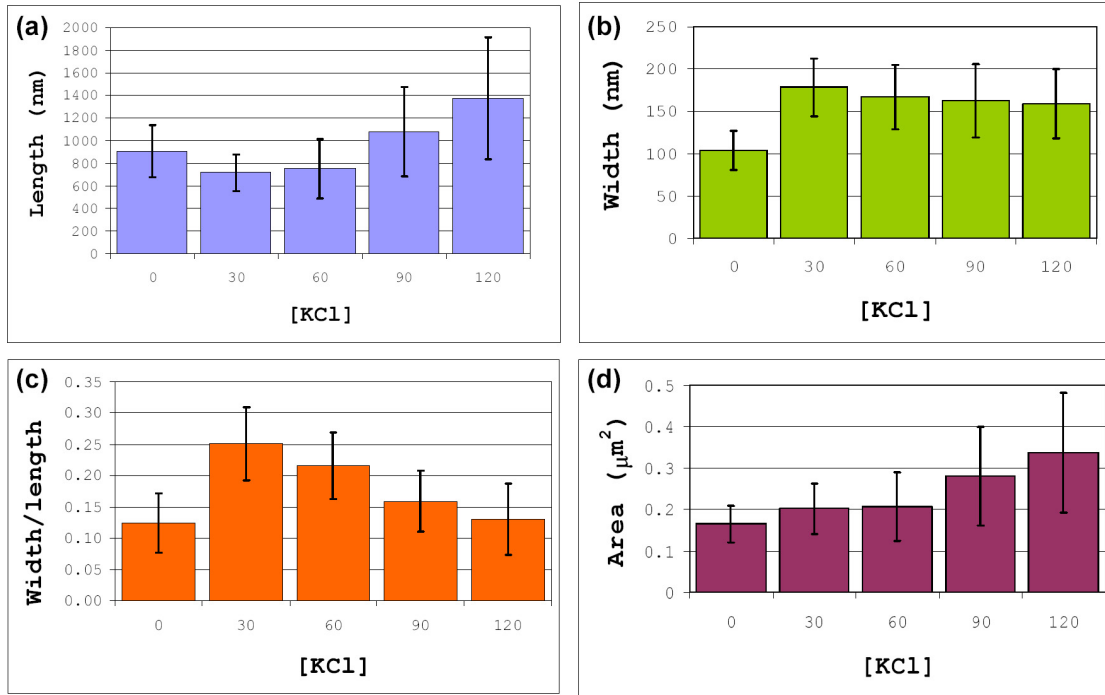


Figure 10: Average length (a), width (b), width/length ratio (c) and area (width-length, d) of TF populations at different ionic strengths. Columns show the averages, standard deviations are indicated as error bars. Width is measured at half of the maximal height. For detailed data see table 2.

In addition, based on the idea of previous studies using different techniques [6] [7], we analysed how the three widths measured by the opposing ends and the centre are related to each other. Taking the average of the two end-widths and comparing it to the central width shows that synthetic filaments do not have a narrower part in the centre - called a bare zone - observable in native TFs. Comparing the ends to each other and plotting the $|\text{width}_{1/4} - \text{width}_{3/4}| = \Delta(\text{width})$ value of all the imaged filaments shows that although this difference is usually small, there are several TFs when it is comparable to the filament width. (Fig. 11.)

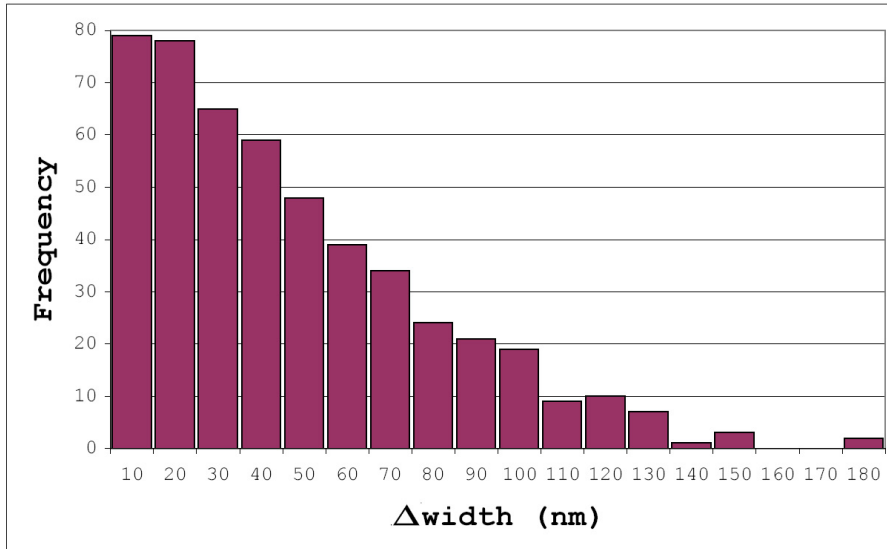


Figure 11: *Distribution of $|\text{width}_{1/4} - \text{width}_{3/4}|$ values examined using all filament populations from the study.*

3.2 Buffer change experiments

In the aim of understanding the mechanisms behind the aforementioned differences, we studied what effect shifting the ionic strength can indicate in filament morphology. As different ionic strengths resulted in varying TFs, we were interested in whether these variations can be directly induced by us changing the environment. This experiment also seemed promising as a possibility to analyse on what time-scale the [KCl]-dependent parts of filament formation happen.

The two directions of buffer change ($\Delta[KCl] < 0$ or $\Delta[KCl] > 0$) were tested by changing the concentration from 120 mM to 0 mM and from 0 mM to 30 mM, respectively. The new buffer was introduced to the system at different points: either already **adsorbed** filaments were washed with a different KCl-concentration buffer; or the **dilution** prior adsorption was carried out at an already changed ionic strength.

When ionic strength decreased, in the first case no difference was observable, despite of the 0 mM buffer environment, the TF population had the characteristics of a 120 mM one. On the other hand, if the filaments were introduced to the new buffer before adsorption, in some measures they became similar to 0 mM TFs. Apparently, the average length was not affected by the buffer change, while the width significantly decreased and a core structure was visible on many filaments. (Fig. 12, 14.) Statistical tests also support the conclusion that the population created by the second method significantly differs from the original 120 mM TFs ($p \ll 10^{-6}$), while method-one population is similar.

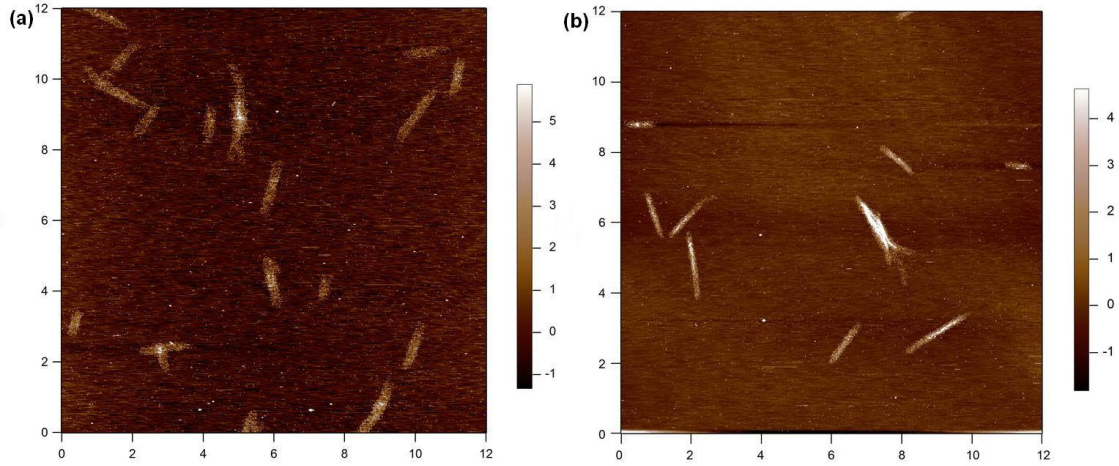


Figure 12: Large-scale images of filaments prepared in 120 mM KCl buffer. Buffer was changed to no KCl buffer after (a) and before (b) adsorption. Image dimension are in μm , height intensities in nm.

When originally 0 mM filaments were used and we increased the ionic strength during the experiment, the results showed no difference according to the time point of buffer change, but another parameter, **temperature** proved to play an important role. In both 'adsorption' and 'dilution' case, the changed buffer induced a spreading-out process, making the filaments wider and lower, while core structures disappeared. The new filament population was approximately homogeneous, similar to 0 mM filaments in length and to 30 mM filaments in width, significantly different from the original population.

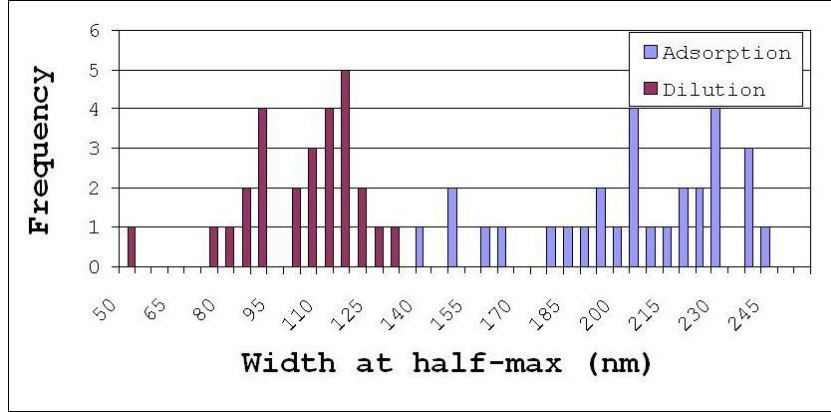


Figure 13: Comparison of width value distribution over TF populations in buffer change experiments (0 mM buffer added to 120 mM filaments). Buffer was changed on already surface-bound filaments (adsorption) or during the dilution prior to incubation (dilution).

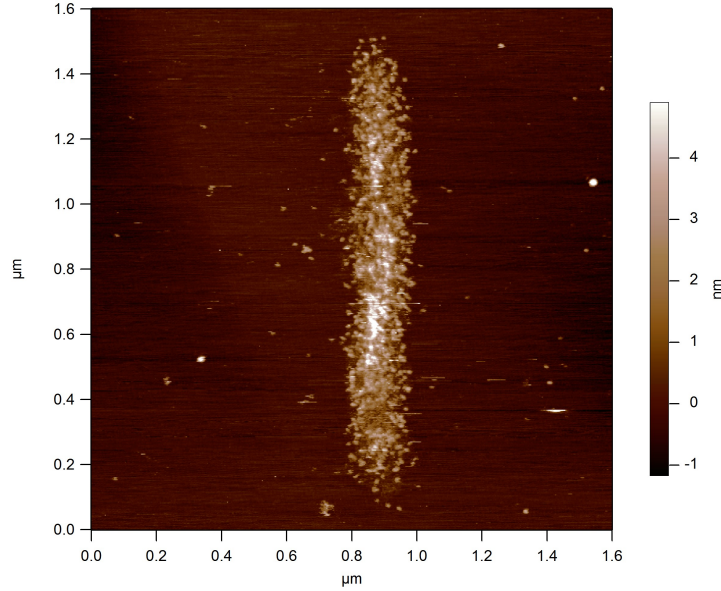


Figure 14: Zoomed high-resolution height image of a filament prepared in 120 mM, diluted and adsorbed in 0 mM KCl buffer. A core structure is clearly visible along the main axis of symmetry.

However, earlier results suggested that appropriate experimental set-up can allow us to capture a transient state of mixed population consisted of narrow 0 mM and wide 30 mM-like TFs. (Fig. 15.) The only notable change in our experiment was that - to minimize bubble formation - room-temperature buffer was used, while in earlier experiments the filaments were washed with ice-cold buffer. Therefore the adsorption experiment was repeated with **ice-cold** 30 mM KCl buffer, and similarly to previous results, narrower filaments with a core were observable as well as already spread-out ones. (Fig. 17.)

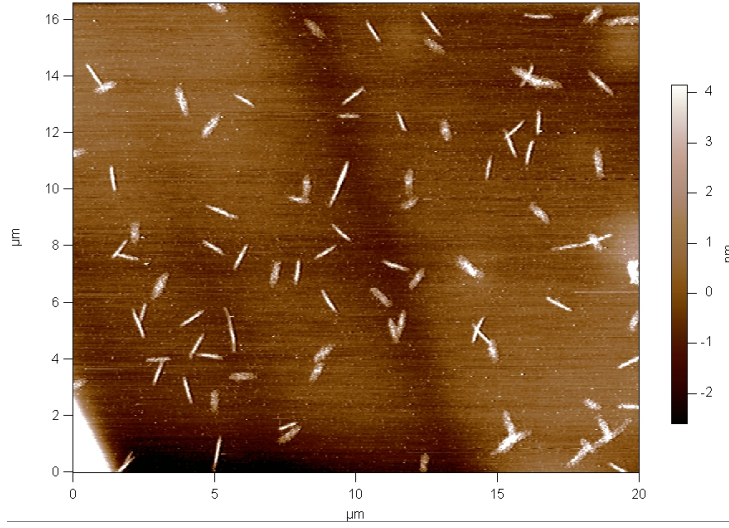


Figure 15: Large-scale images of 0 mM TFs in 30 mM KCl buffer recorded during a previous experiment series. The population is a mixture of two dissimilar filament types. Image dimensions are in μm , height values in nm.

Additionally, the mixture of the two populations moved towards the more spread ones on newer scans (fig. 16), as the time needed for scanning was enough for more and more transitions to occur, revealing some qualitative information about the kinetics of the process. Probably the warming-up of the buffer also contributed to this effect.

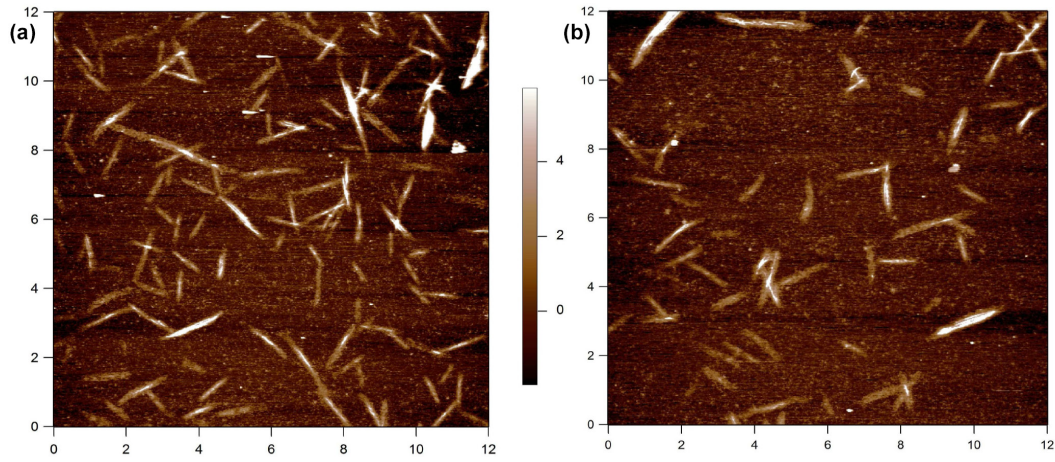


Figure 16: Large-scale images of 0 mM filaments washed with ice-cold 30 mM KCl buffer. (a) First scan after washing. (b) Fourth scan after washing. Narrow-type filaments are still visible but the majority of TFs is spread-out.

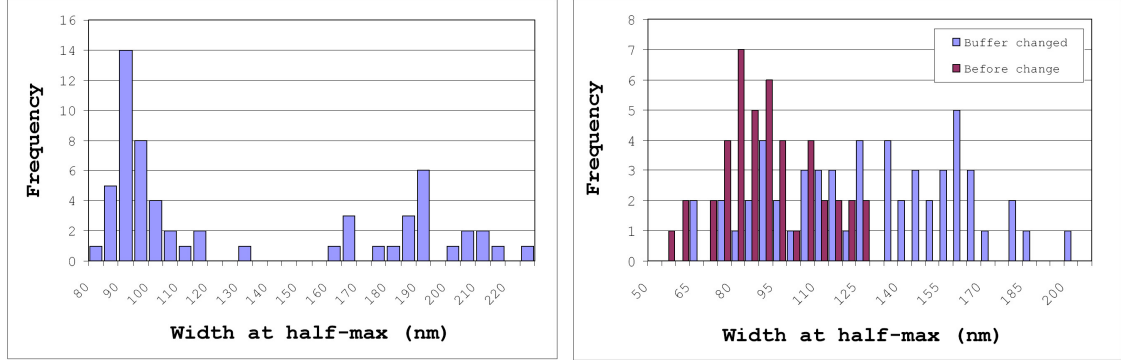


Figure 17: Width-distribution of filaments in buffer change experiment introducing 30 mM KCl ice-cold buffer to no KCl TFs. (a) Data from 2007. Two distinct populations are observable. (b) Recently acquired data. After change a new population appears between the narrow one still there. Image and intensity units as before.

To sum up, we found that filaments already adsorbed to the surface had a lower limit for their width, presumably resulting from irreversible surface-filament interactions. They could widen and interact on a larger area with the mica, but not the other way around. However, if TFs were still in solution, their buffer affected the way they bound to the surface and consequently their width. Moreover, the buffer-induced interaction was slower and less complete at low temperature.

3.3 Filaments on nitrocellulose

Comparing our results to other measurements on synthetic thick filaments [7] [8] [9] raises the question how much of the observed morphology is intrinsic for the filament structure, and how much of it is induced by specific interaction with the mica surface. Hence I designed a control experiment to examine the very same structures bound to a surface with other characteristics. Additionally, future experiments involving the TIRF microscope - built together with our AFM - were also in mind, because thin coverslips and **nitrocellulose** are better for fluorescent imaging. Synthetic thick filaments were able to bind to the nitrocellulose surface, and although it was not as flat as freshly cleaved mica, the sample was easily observable and distinguishable from the other structures on the surface.

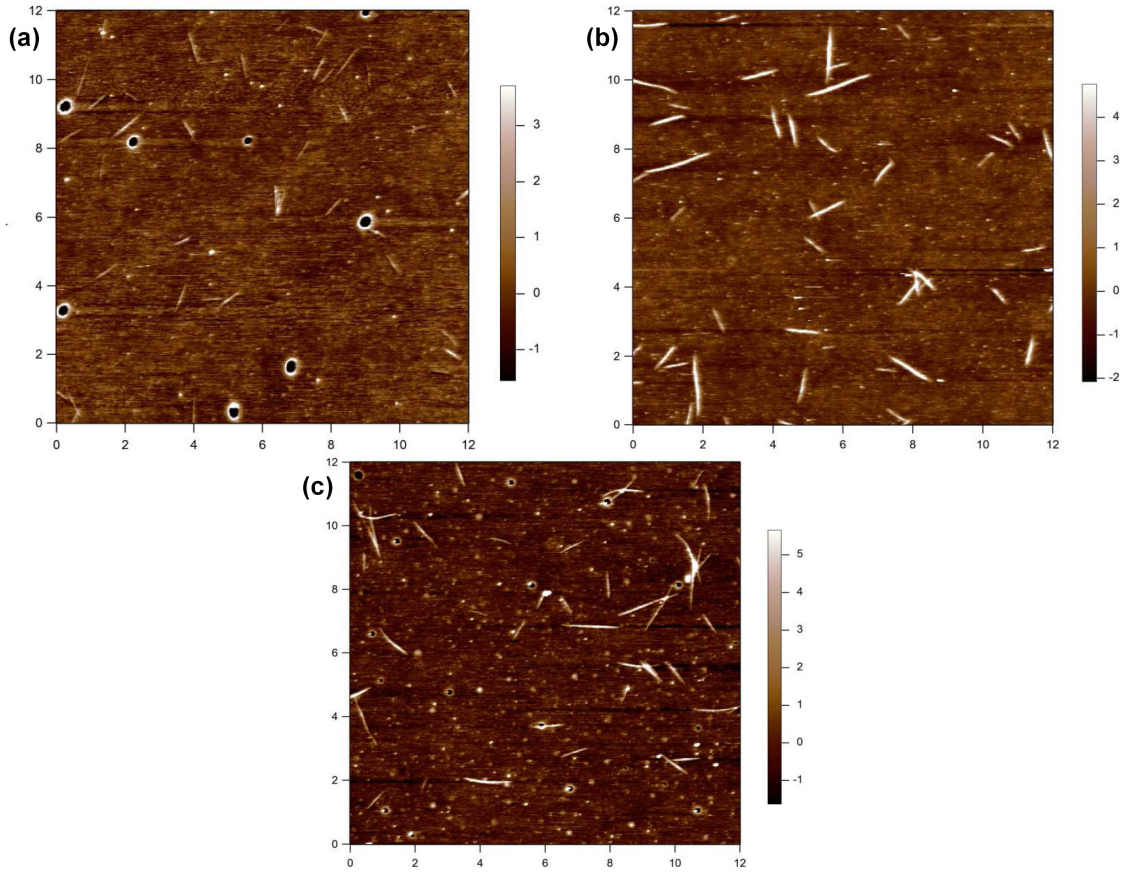


Figure 18: Large-scale height images of filament populations prepared at 0 mM (a), 60 mM (b) or 120 mM (c) KCl concentration and adsorbed to nitrocellulose surface. Image dimensions are in μm . Colour scales indicate the height values in nm.

In general, filaments looked very different on nitrocellulose, being way much narrower on this type of surface, closer to the appearance published before [8] [6].

However, several similarities could be observed between the two sets of results, for example the average length of TFs at a given ionic strength was on the same scale. More importantly, the width showed the same ionic strength dependence, although the absolute value of width-differences dropped, compared to the average width they were still significant, and the trend observed in figure 10. held for these measurements. (Fig. 19.)

Since 120 mM images were obtained from a more noisy experiment (due to objects on the nitrocellulose), the 60 mM KCl population is used to illustrate that 0 mM filaments again form a distinct cluster. (See figure 20.) The difference of widths was statistically tested and found significant with $p \ll 10^{-6}$ for both 60 mM and 120 mM populations.

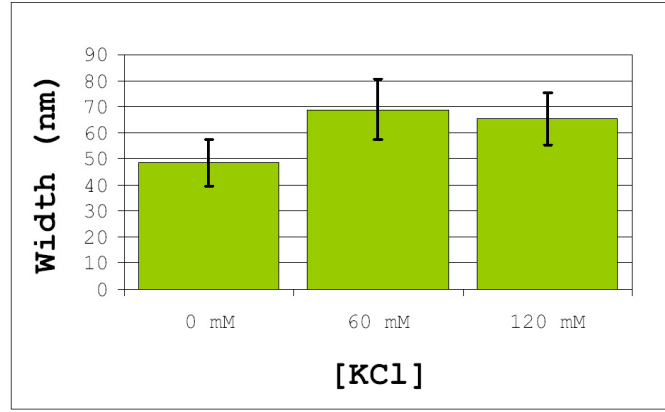


Figure 19: Average width values of 0 mM, 60 mM and 120 mM KCl filament populations on nitrocellulose. Standard deviations are indicated with error bars.

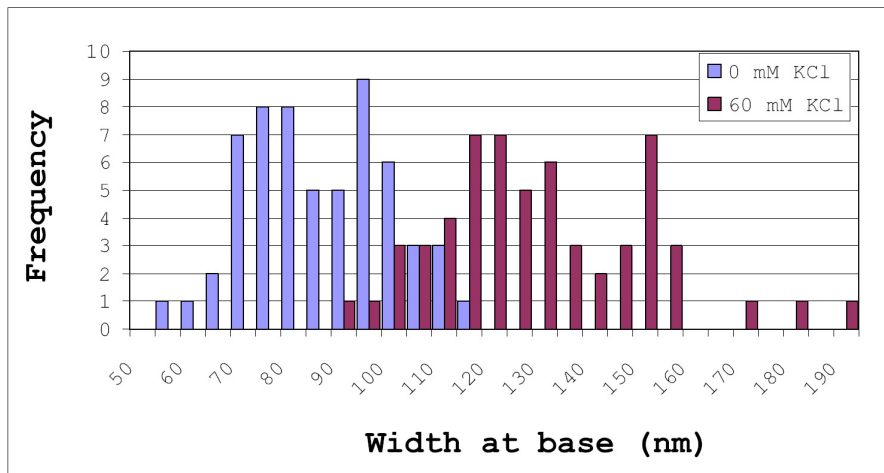


Figure 20: Histogram of width values measured on nitrocellulose-bound 0 mM and 60 mM KCl filaments. (cf. figure 8)

Apparently, the nitrocellulose did not induce much interaction with the TFs, so they put on a more compact form instead of opening up. The assumption that some characteristics like filament volume are independent of the adsorbing surface was supported by the - not quantified - observation that not only the width was reduced on nitrocellulose, but the height values were several times greater than in previous experiments (fig. 21). In some way filaments on mica versus nitrocellulose could be understood as a book on its joint opened or closed. Unfortunately, the metaphor proved to be right in the case when attempts were made to further investigate the filament structure on high-resolution images. Imaging was complicated by surface irregularities, and because of the looser interaction with the surface, most myosin heads were not fixed, hence impossible to precisely capture with the tip which rather pushed them.

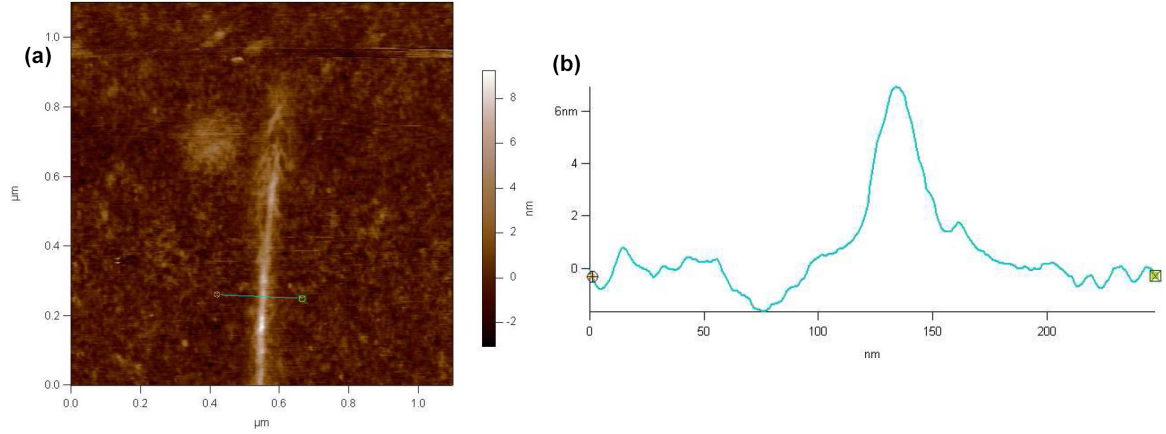


Figure 21: (a) High-resolution image of 60 mM thick filament adsorbed to nitrocellulose-covered surface. (b) Cross-section of the filament taken along the line indicated on the height image. The TF is unambiguously thinner and higher than mica-bound specimen.

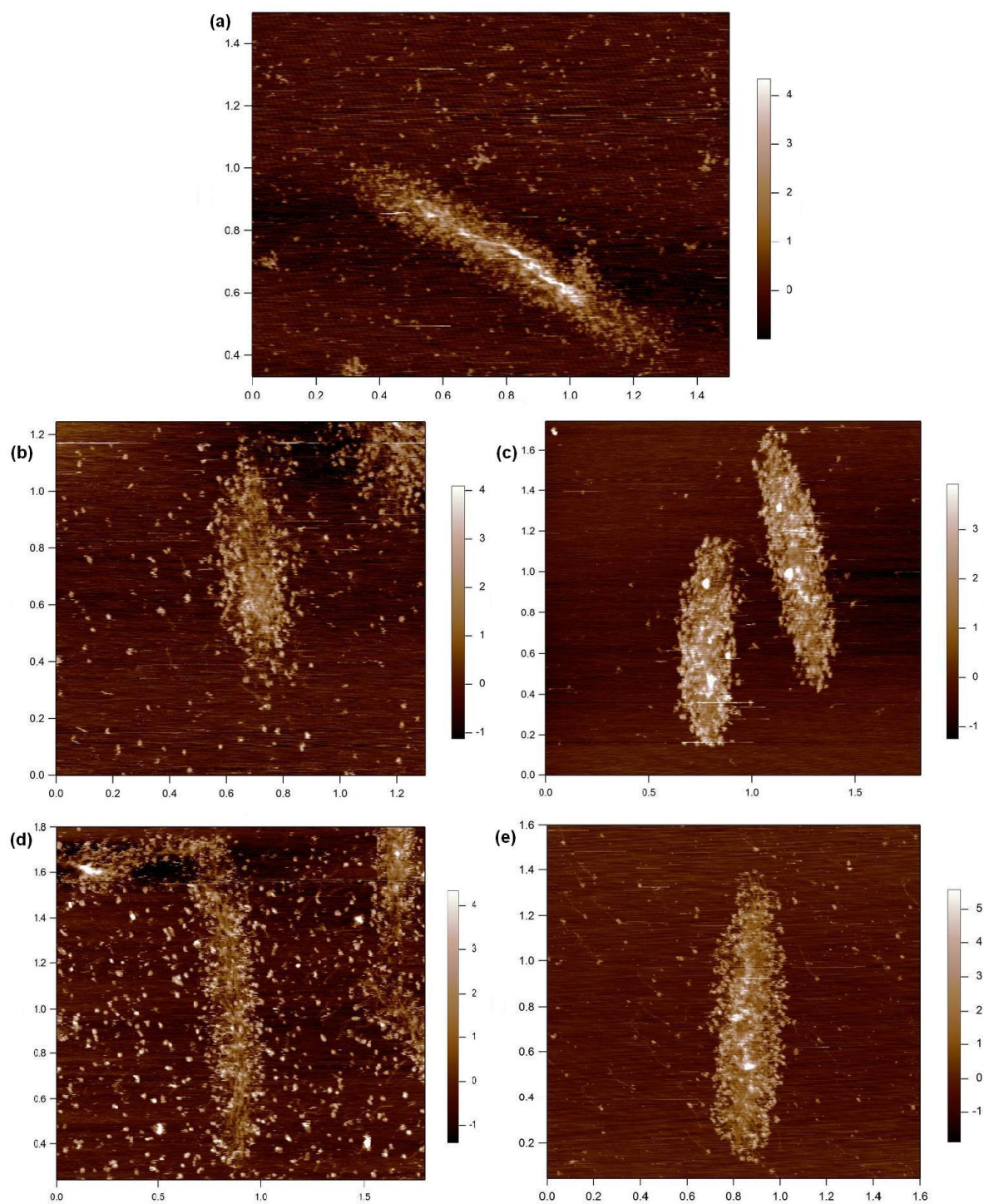


Figure 22: High-resolution height images of filaments prepared at 0 mM (a), 30 mM (b), 60 mM (c), 90 mM (d) and 120 mM (e) KCl concentration. Image dimensions are in μm . Colour scales indicate the height values in nm.

3.4 High-resolution images

Beside the aim to conclude in general statements concerning thick filament morphology and underlying interactions, we were also interested in the structural details of filaments and the capabilities of our experimental set-up to reveal internal organization. Therefore the special opened-up nature of TFs applied on mica proved to be very useful in the process of tapping subfilament structures mainly formed of myosin tails.

As the compactness of the filaments differed with ionic strength ([10] and see table 2), the zoomed images were of different quality and could highlight varying features of synthetic TFs. Filaments prepared in no KCl buffer were much less spread, therefore the organization of tails linking the monomers was not observable, but the core structure was resolved more than on large-scale shots. Although a portion of the filament could not be imaged in details because of the high number of loosely fix myosin heads, on the sides individual myosin heads can be distinguished. 30 mM and 60 mM filaments could reveal more details on structure, but the features often were a bit blurred, not completely distinct, probably due to a quite high affinity to interact with the tip induced by the ion concentration. Finally, in buffer with KCl concentration of 90 mM and 120 mM the combination of spread-out morphology and less sample-tip interactions resulted in detailed images, where subfilamental structures as well as free myosin heads and tails could be observed. (Figure 22.)

In the end regions of the TFs, **branches** unambiguously containing many myosin tails - since they had the width of several tens of free myosin tails - were visible as if they were loosened parts of a braid forming the filament's trunk. An average of 3-10 **subfilaments** were observable in this region, which at the very end of the TF were branching out into individual myosin tails connected to distinct heads. In some filaments the structure was disturbed around the middle and one or two thicker branch were seen leaving the trunk, causing a measurable drop in width.

Furthermore, the filament structure along the main axis of symmetry appeared to be **periodic**, as cross-section graphs revealed (figure 5.) Since the graphs were always obtained by averaging the intensity values over a wide band, this periodicity could not arise from the accidental arrangement of myosin heads. Although detailed analysis of periodicity was not the aim of this study, a period of ~ 25 nm (and its multiples) could be observed in several imaged filaments, besides the shorter period corresponding to myosin head dimensions. [8] [11] [12] This finding requires further investigation and spectral analysis, though.

Another feature observable thanks to the branching around the edges of the filament was **polarity**. [13] In the upper 1/3 myosins pointed upwards (head domain upper than tail), similarly in the lower 1/3 downwards, while in the middle they were mostly horizontal or a mixture of slightly up- or down-directed molecules. In some TFs the point where polarity switches - not necessarily in the centre of the filament - could be precisely determined.

In addition to the organization of synthetic filaments, **free myosin molecules** were also measurable on the close-up images. Since the site for interaction with the mica is the neck part of the molecule, heads were fixed enough to be imaged. Moreover, at high [KCl] both heads and tails bound to the surface stronger, so heads could be scanned precisely, looked approximately globular - sometimes even the two domains were visible - and also tails appeared on the output images. The parameters of the molecule (contour length of the tail, dimensions of the head domains) were consistent with literature data. [14]

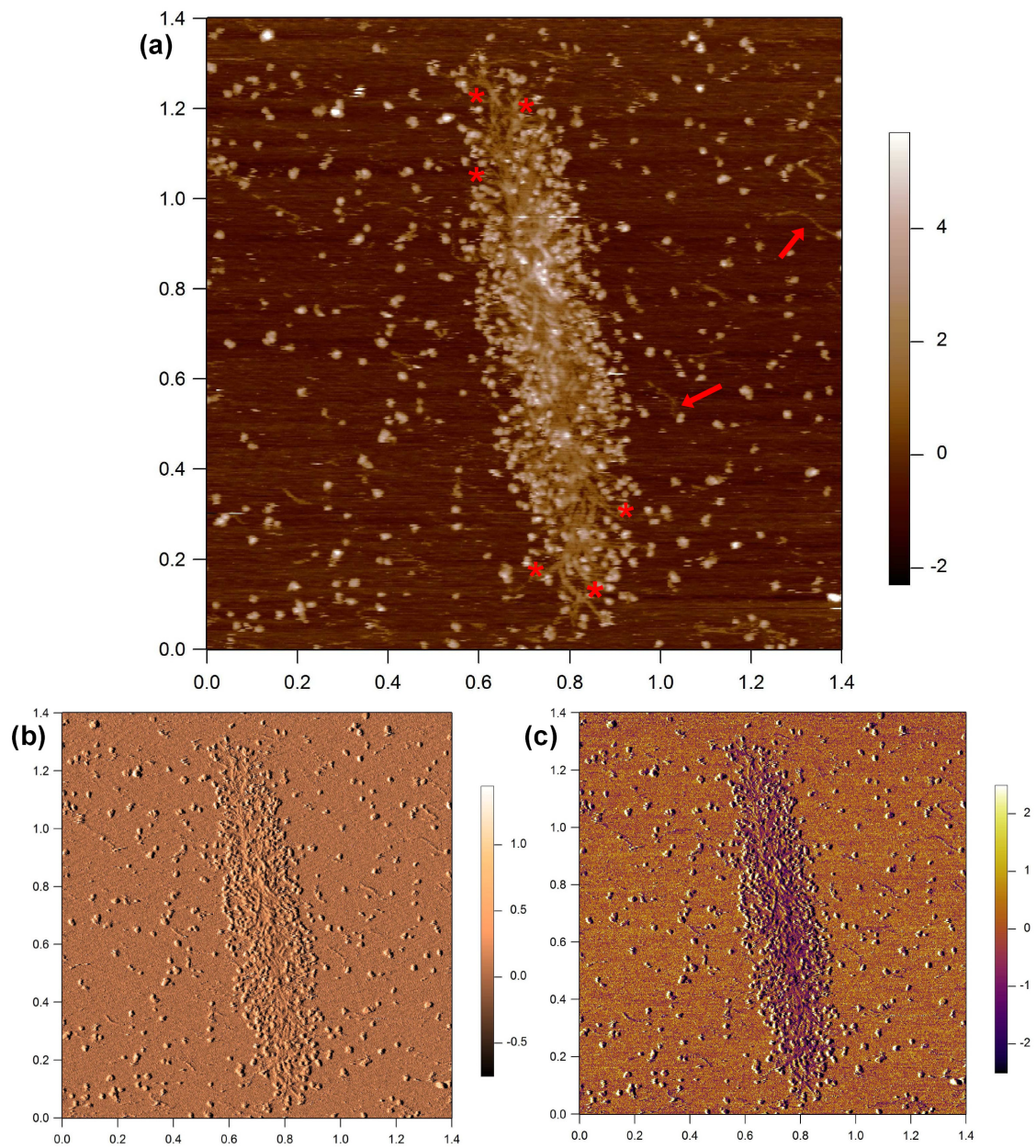


Figure 23: *High-resolution images of a typical thick filament dialysed in 90 mM KCl buffer. Beside the main filament, free myosin heads and sometimes also tails are visible. (a) Height image. Subfilaments are indicated with asterisk, free monomers (head and tail domain together) with arrow. (b) Amplitude image. (c) Phase image. Image dimensions are in μm , unit of colour scale is nm (a, b) and degree (c).*

3.5 Other experiments

Higher ionic strength

As the maximal KCl concentration used for sample preparation in the previous experiments was 120 mM, the question naturally arises whether higher [KCl] can hold extra information about filament morphology. It is known that thick filaments do not form at $[\text{KCl}] > 0.2 \text{ M}$, which is why myosin is usually stored in a high-salt solution containing 0.5 M KCl. In addition, previous experiments with a sample dialysed against a buffer of 150 mM KCl concentration were unsuccessful, suggesting that it can be an upper limit for this preparation method. Therefore starting with 120 mM KCl filaments and slowly raising the ionic strength in a buffer change experiment seemed the best way to study this regime.

The 120 mM KCl filaments before buffer replacement looked the same as described and illustrated in previous paragraphs. After the addition of 100 μl 1 M KCl solution to 10 ml buffer, the sample was washed with this new buffer and imaged immediately. At **130 mM KCl** less TFs filaments were found on an average imaged area, while the number of free myosins increased - similar to the effect of pH raising in [15] - suggesting that some filaments depolymerized and washed out or bound to the surface as free molecules. Although some filaments still had the 120 mM characteristics, many TFs were more spread and loose. Moreover, high-resolution images revealed that their structure were much less organized, sometimes as if captured in the middle of decomposition, like in figure 24.

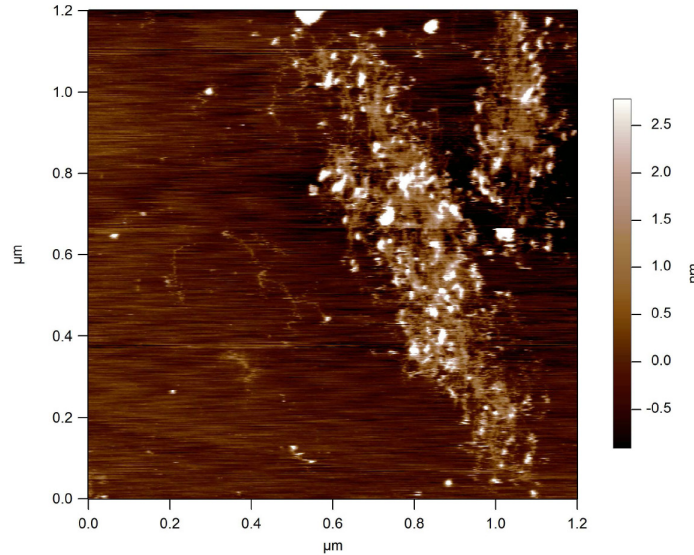


Figure 24: *Synthetic thick filament prepared and adsorbed at 120 mM KCl, then washed with 130 mM KCl buffer. The structure is loosened with subfilaments still holding together but farther from each other.*

Afterwards the ionic strength was further increased by raising $[\text{KCl}]$ to 150 mM. The 130 mM buffer was replaced in 5 steps - to avoid a lower ionic strength mixture and wash out released particles - and approximately the same area scanned. In this case no thick filaments were observed but an increased free myosin density on the mica surface. In addition, image quality became worse as in a higher ionic strength buffer the mica itself has some interaction with the tip producing artefacts.

ATP-free thick filaments

In the aim of investigating not only the structure but also the function of myosin thick filaments, in future experiments actin is planned to be added to the system. Since imaging of this more complex system is only possible in the absence of ATP, first a completely ATP-free sample had to be examined (but still without adding actin).

Filaments prepared in no ATP 60 mM KCl buffer imaged with the same parameters as before, showed similar form on large-scale (fig. 25) and close-up images. Hence in a system containing actin studying the structural changes induced in either thin or thick filaments, our prior results describing synthetic myosin filaments can be used. So if these future experiments reveal interesting arrangement of actin (for example on fluorescent images) it can be compared to the known properties of myosin, also any change observed in TF morphology on AFM micrographs is an effect of actin in the system, not the lack of ATP.

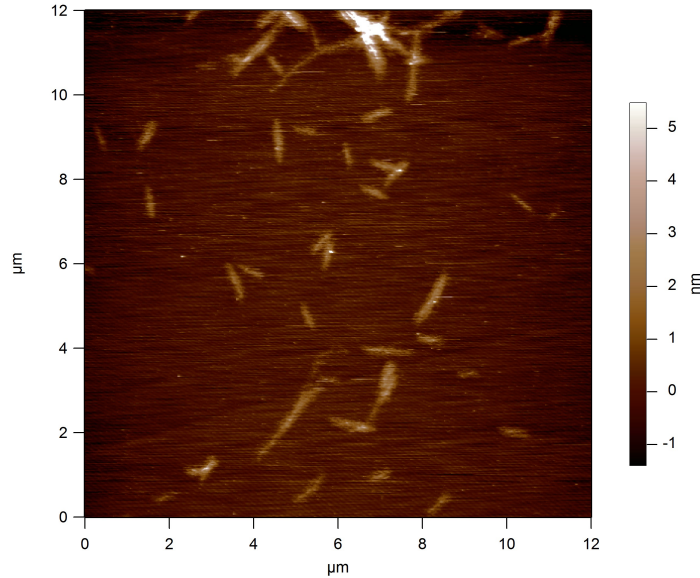


Figure 25: Height image of synthetic thick filaments prepared and diluted in a standard myosin dialysis buffer containing 60 mM KCl and no ATP. (cf. figure 9 c.)

4 Discussion

During this project, we managed to image synthetic myosin thick filaments under aqueous condition and confirmed that TF **morphology** is highly **ionic strength dependent** (at constant pH) and that filaments dialysed against a 0 mM KCl buffer form a distinct population with a unique core structure in many filaments. Closer images on this core suggests that it can be a dense volume of myosin heads not in interaction with the surface and slightly swinging as the tip moves close.

Statistical analysis considering the other 4 populations suggests some trends for the [KCl]-dependence of width and length, quantifying the striking difference observed on scans. Moreover, comparing the width values measured at three points of a given filament revealed some interesting characteristics of synthetic filaments as the lack of a bare-zone and **asymmetry**.

We also performed buffer change experiments to further investigate the effect of ionic strength on filament morphology. [KCl] of the buffer was lowered or raised on filaments either already adsorbed to the surface or still in solution with the aim to induce change in TF width.

Replacement of 120 mM with 0 mM KCl buffer - assumed to result in decreased width - was ineffective on surface bound molecules, while changed filament morphology if applied before adsorption. This means that the interaction between myosin heads and mica is **irreversible** but the heads' affinity to bind the surface is affected by KCl concentration, hence the less spread-out morphology of TFs diluted in 0 mM buffer.

On the other hand, changing [KCl] from 0 mM to 30 mM affected the filaments regardless of the state when we applied the higher ionic strength buffer. Moreover, if ice-cold instead of room-temperature buffer was used the transition did not happen instantaneously, and we could study **intermediate states** as well as the time-development of the process. These observations support a conformational change, the speed of which is determined by the energy contained in the system.

The next step of the project was to study how mica-specific are the above described discoveries, via experiments carried out using nitrocellulose-coated coverslips instead. Although filaments were much more compact and closer to the morphology obtained by electron microscopy, their features depended on the [KCl] of the dialysis buffer. Therefore the ionic strength dependence of average width and length of TF populations is caused by not only an enhanced specific interaction between mica surface and sample. Indeed, the **intrinsic** structural features of the filaments are also affected by the amount of ions in the buffer, and this variety contributes to the observed morphological differences.

We could also obtain high-resolution images of the synthetic myosin thick filaments, revealing structural details of TFs as well as free myosin dimers. Using mica-surface for fixation and high ionic strength buffers resulted in an opened-up form of filaments, individual myosin heads and tails recognizable along the side and end regions of the TF. Therefore many molecules could be tracked and their polarity determined. The images confirmed that myosin thick filaments are **bipolar**, with a centre not necessarily aligned with the centre of the filament. At the end regions of the filament, due to that most molecules in the region were strongly bound to the surface, the internal structure became clearly visible. Myosin **bundles** radiating from the core of the filament were scanned as they further branched out to individual tails. In addition, we observed **periodicity** in height along the main axis of symmetry, with a definitely higher period than that resulting of the spatial arrangement of head domains. These results suggest that a central trunk consisted of myosin tails is built up from several subfilaments in a **helical** fashion, a way shown for thick filaments from tarantula but not described before for vertebrate myosin.

To sum up, we described how the formation of synthetic myosin thick filaments depends on the environment's ionic strength - controlled by potassium-chloride - by binding the filaments to mica surface and imaging them in buffer with atomic force microscope. We studied the morphology as well as the internal structure of filaments utilizing the high resolution limit of AFM. Through different control experiments we could confirm that filament morphology observed by us is mostly the result of internal organization rather than specific interactions with the surface. We could also obtain high-resolution images of synthetic thick filaments and reveal fine details of internal structure as subfilaments. Hopefully, our results will contribute to our knowledge on thick filament assembly and this experimental set-up capable of capturing structural details will be of use in future studies investigating the interactions of muscle components essential for contraction.

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