Surface patterning to study the interaction between a growing actin network and dynamic microtubules

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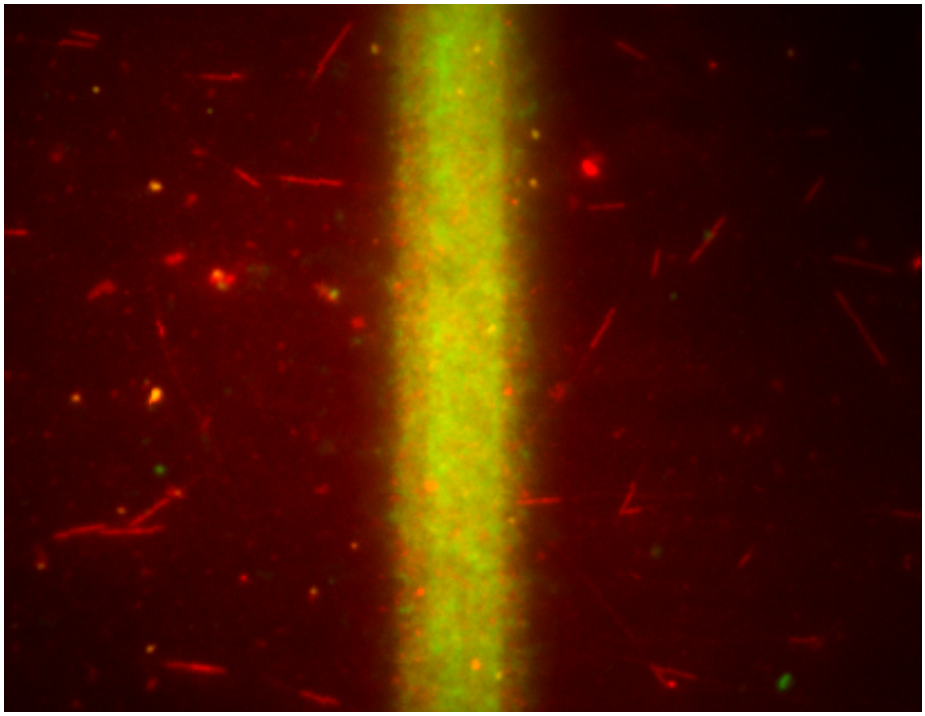

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Abstract

In this thesis we developed an assay to experimentally investigate the dynamic behavior of microtubules in the presence of a growing actin network to gain a better understanding of the interaction between these two cytoskeletal filaments. To visualize the interactions more clearly, we use a surface micropatterning technique that was proposed by *Reymann et. al.* [20]. With this technique an actin network is allowed to grow at a specific place on the surface, while microtubules are attached in-between actin-covered areas. The patterning technique allows easier observation and strongly reduces disturbance of unwanted side interactions of microtubules with actin filaments or networks. First results on the catastrophe time and average growth velocities are presented. We find a slight decrease in catastrophe time for microtubules (from 224.0 to 205.1 seconds) that interact with the actin network and an increase in average growth velocity (from 37.5 to 47.4 nm/s). This new assay makes it able to study interaction between microtubules and actin networks more clearly. It allows to study microtubules and actin network in the exact same condition, such that only their interaction influences the dynamics of microtubules and actin networks. For further research, it would for example be possible to study the changes in microtubule dynamics resulting from changes in the actin network density or from addition of microtubule or actin binding proteins.

Contents

1 Introduction

The simplest living organisms, such as yeast and bacteria consist out of a single cell. In multicellular organisms such as plants and animals, cells form groups to build different structures to perform different functions for the organism. Plant and animal cells are called eukaryotic cells; they typically have a nucleus. The eukaryotic cells have to move, adapt change shape and divide to endure different conditions in the organism. To accomplish this, eukaryotic cells have a cytoskeleton. It was long believed that only eukaryotic cells had a cytoskeleton, but recently it was discovered that also bacteria have a cytoskeleton.[16] The cytoskeleton of a eukaryotic cell consists out of different components, with actin and microtubules being two key elements. These two components by themselves already have interesting properties, but to maintain proper cell function, it has been shown that these polymers have to interact for numerous tasks such as cell division, cell migration, wound healing and the organization of components within the cell. [21]

With developments in microscopy techniques such as confocal microscopy or total internal reflection microscopy, it became easier to observe the cell behavior and individual components such as microtubules. Still, many details in cell mechanisms and protein interactions remain unrevealed. It has for example, been observed that microtubules have a very dynamic behavior, they can polymerize and switch to depolymerization multiple times within minutes, but it is still unclear what causes these catastrophes. Actin filaments can also polymerize and depolymerize, but they treadmill in steady state rather than the dynamic instability that microtubules exhibit. [24]

To study the interaction of these two cytoskeletal polymers, this thesis focuses on building a simple system with a surface micropatterning technique, to be able to study these interactions *in vitro*. We allow an Arp2/3 nucleated actin network to grow in restricted areas and let it interact with dynamic microtubules to mimic the interior of a cell. Patterning of the polymers can make it easier to observe the behavior of the actin network and the dynamic microtubules, because it allows more control over the type of interaction and the location of the growing polymers. It enables to study the interaction of the microtubules and actin when they meet, while the other conditions, such as the temperature, salt concentrations and buffers are exactly the same. Imaging tools enable to measure the average growth velocity of the microtubules, the catastrophe time and the number of pauses. With this patterning technique, we hope to see a difference in these dynamics when the microtubules meet the actin network, to give more insight on the influence of actin-microtubule encounters.

Gaining more fundamental knowledge on the intrinsic behavior of the cytoskeletal filaments and filament-binding proteins can help to understand the functions and the importance of each of them as well as their interactions. With this knowledge it might also be able to understand where and when cellular processes such as cell migration and cell division go wrong and understand how certain diseases such as cancer are caused. Or more importantly how to prevent the disease or cure it.

2 Background

Eukaryotic cells have a cytoskeleton that maintains their structure and plays a major role in the coordination of molecular transport within the cell and motility of the cell throughout its surrounding. The cytoskeleton of the cell is a network of different fibers that are all built from proteins. The cytoskeleton of eukaryotic cells is usually described by dividing it into three major components: microtubules, actin filaments and intermediate filaments. [19]

In this thesis, the focus will be on the interaction between an actin network and microtubules. Actin and microtubules share similar dynamics, they can both polymerize and depolymerize, optionally with an energy source, respectively actin-bound ATP (adenosine triphosphate) and tubular bound GTP (guanosine triphosphate). They both have a polar structure with a fast growing end, which is called the plus end and a slower growing end, which is called the minus end. The dynamics of these polymers are organized by a large repertoire of proteins, which must be highly regulated to maintain proper cell functioning. Several proteins that interact with actin and microtubules also contribute to higher order structures, such as stress fibers, filopodia bundles and contractile rings. [21] This chapter will give an overview on the dynamics of cytoskeletal filaments, in particular actin filaments and microtubules. Considering the wide range of interacting proteins, only the ones used for this project will be discussed in more detail.

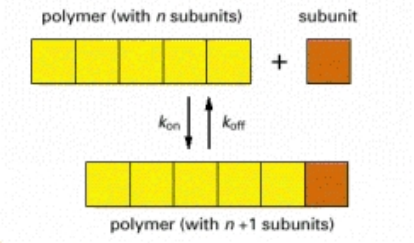
2.1 Polymerization of cytoskeletal filaments

The polymerization process of several cytoskeletal filament shows some similarities. In this section there will be a general explanation of these processes to understand the fundamental properties of filament growth that will be useful for following chapters. A simple model to understand filament polymerization is by describing a filament as a linear polymer where a monomer can be added to the end of the polymer. A monomer is the smallest unit that forms the building block of this polymer. For microtubules this is the $\alpha\beta$-tubulin dimer and for an actin filament these are actin monomers. The rate at which a monomer is added to the polymer is k_{on} and the rate at which the polymer monomer is detached is k_{off}, as is shown in Figure 1a. Where k_{on} is proportional to the concentration of monomers in the solution with the following equation:

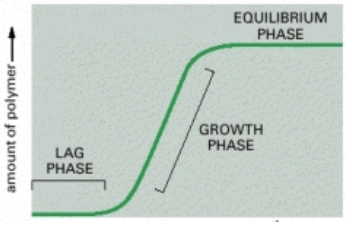
$$\frac{[A_1][A_n]}{[A_{n+1}]} = K = \frac{k_{on}}{k_{off}}$$

With $[A_1]$ the monomer concentration, $[A_n]$ the polymer concentration of the n-mers, $[A_{n+1}]$ the polymer concentration of (n+1)-mers after adding the monomer and K the dissociation constant. As the polymer grows longer, the concentration of the monomers in the solution decreases. This continues until an equilibrium with the critical concentration C_c is reached, with the relation:

$$C_c = \frac{k_{off}}{k_{on}} \left(= K^{-1}\right)$$



(a) A simple model of growth of a polymer by adding monomers at one end with rate k_{on} and detaching with rate k_{off} [2]



(b) Different phases in the growth of polymers. The schematic graph shows the amount of polymers with respect to time. [2]

At this concentration the average elongation rate is zero. Below this critical concentration the polymer on average shrinks until the monomer pool reaches the critical concentration again. Above this critical concentration, polymers will undergo a net growth.

This growth increases very rapidly with increasing monomer concentration. Thus, slight changes in monomer concentration or the binding affinity of monomers, have a large effect on the average length of the polymers in the solution. Actual actin filament and microtubules have a more complex structure than a single string. The monomers of microtubules for example, form a helical and cylindrical structure (see section 2.2). This demands a more complex model such as multi stranded filaments, where more linear filaments are formed next to each other. This differs from the previous model, because now the monomers do not only form longitudinal bonds, but also lateral bonds. This adds more steps because there are more association and dissociation rates involved. Nucleation, which is a stable bond of a few monomers is the rate-limiting step. However, once a nucleus is formed, filament elongation is rapid. The time a polymer needs to form nuclei is called the lag phase. After nuclei have formed, the polymers start to grow rapidly, which is called the growth phase. Polymers continue to grow until the concentration of monomers has decreased enough, such that the growth of the polymers is exactly balanced with the shrinkage of the polymer. A schematic graph of these different phases is shown in Figure 1b. [2, 7]

For now, only passive polymerization at one end of the polymer was considered. The dynamics of the polymer however change if both ends are considered and energy is added to the system. Both, actin and microtubules have a binding side for a nucleoside triphosphate and act as an enzyme to trigger hydrolysis. As long as the nucleoside triphosphate is bound to the monomer, hydrolysis is slow, but once it is bound to a subunit that is part of the filament, hydrolysis happens faster. Without ATP or GTP hydrolysis, the free energy difference for adding monomers is the same at both end, thus the critical concentration is the same at both ends. The ratio of k_{on} and k_{off} must be the same, but the absolute values of these rates can be different. In structurally polar filaments such as actin filaments and microtubules, the rates are much larger at one end than at the other end, but the dissociation constant is still the same. This results in a more dynamic end, called the plus end and a less dynamic end, called the minus end of the polymer. With hydrolysis, two different types of filament structures exist, a part where the ATP (or GTP for tubulin) is bound to the

subunit, or the part where ATP is hydrolyzed to ADP (or GDP). When ATP is hydrolyzed, the free energy released is stored in the polymer lattice. Therefore, the equilibrium constant and the critical concentration are larger for the ADP bound filaments. Thus, in the presence of ATP, at a certain concentration, the ADP bound part will shrink while the ATP bound part will grow. There is a concentration of monomers, at which the subunit addition is faster than the hydrolysis at the plus end, but the hydrolysis is slower at the minus end. The minus end tends more towards disassembly, while the plus end tends towards assembly, this state is called treadmilling.

The longer a certain subunit is part of the filament structure, the is more likely it is to undergo hydrolysis. The middle part of the filament is therefore more likely to have ADP bound subunits. A fast growing end, on the contrary, is more likely to have ATP bound subunits. Thus, an ATP or GTP cap forms at the end of a filament. This depends on the rate of addition and the rate of hydrolysis.[24]

2.2 Microtubule dynamics

Microtubules consist of α- and β- heterodimers of approximately 4 nm thickness and 8 nm in length, that assemble head-to-tail to form a filament as shown in Figure 2. These dimers form the building unit of a microtubule by aligning next to each other, such that around 13 of these form a helical and cylindrical microtubule with a diameter of 25 nm. The β - tubulin drives the polymerization and depolymerization by binding, hydrolyzing and exchanging a guanine triphosphate (GTP). GTP-tubulin binds to an existing microtubule, to form slightly bent protofilament sheets. These sheets close up to form a straight cylinder with a seam where a α-tubulin meets a β-tubulin. The microtubules can alternate between polymerization and depolymerization states, as indicated by the arrows in Figure 2. Although polymerization does not need GTP hydrolysis, switching between polymerization and depolymerization does involve hydrolysis. The GTP-tubulin ends form a natural cap to prevent depolymerization, but this process is not yet entirely understood and a lot of research still needs to be done on this subject. Once the protofilament sheets are closed, the microtubules can either pause and the sheets can open up again, the microtubules can start polymerizing or they can have a catastrophe and depolymerize.[1] Based on electron microscopy images, depolymerized microtubule ends are associated with outward curling protofilaments, illustrated in figure 2.[12] Tubulin dimers that are released during microtubule depolymerization, are mostly GDP-tubulin. The GDP can be exchanged for GTP to make GTP-tubulin, which can be bound to an existing filament again, completing the cycles depicted in Figure 2.

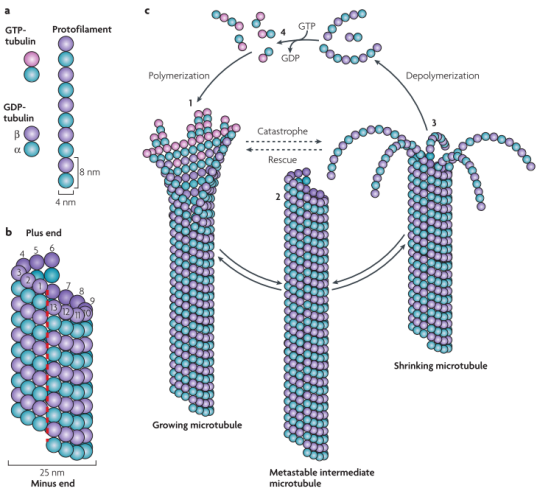


Figure 2: An impression of microtubules and its growth and shrinkage phases. The polymerization-depolymerization cycle it shown, with the role of GTP and GDP- tubulin. (Image obtained from [1])

Microtubule ends change between polymerization and depolymerization dynamically. The microtubule end tends to grow, stop growing (pause) and transits to a rapid depolymerization, which is called a catastrophe. Depolymerization can stop and the microtubule end can start to polymerize again, called a rescue. Figure 3 shown an example of a kymograph of an single dynamic microtubule in red, that has these different events several times. The letters R (rescue) and C (catastrophe), and the different arrows represent the different stages of the microtubule in the kymograph.

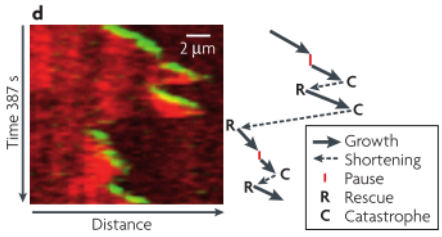


Figure 3: Kymograph of a dynamic microtubule end that shows the different phases. Red is the m-Cherryα- tubulin and green is the EB3-GFP protein that binds to microtubule plus ends. (Image is obtained from [1])

The end of the microtubule has a different conformation than the rest of the microtubule. This and other processes seems to be important in the dynamics of the microtubule, but details of this process are not yet fully understood. Several proteins, called TIP's, can bind to the microtubule ends. Some prefer to bind to a depolymerizing microtubule end, such as XMAP215 and kinesin KLP59C, while Stu2 bind to stable microtubules.[1] Most end-binding proteins however,

bind to the growing end of microtubules. Some of these proteins can have influence on the dynamics of a microtubule. EB1, for example, is known to prefer binding to the polymerizing end of the microtubule and has influence on the growth velocities and number of catastrophes of the microtubule. Interestingly enough, recent data suggest that the EB1 and XMAP215 do not bind to the very end of the microtubule tip, but to a distance of several tubulin dimers behind the end [15]. Other researchers suggest hydrolysis rates and lateral forces between the microtubule dimers have an effect on the initiation of a catastrophe.[13] [17].

2.3 Actin filaments and networks

Similar to microtubules, actin polymerizes and depolymerizes. The monomeric state is usually referred to as G-actin, while polymerized actin is called F-actin or actin filaments. Actin monomers are arranged in a double helical structure, with an increment of 2.7 nm per added monomer [5]. Unlike microtubules, the transition between polymerization and depolymerization is much more gradual and happens less often.[24]

Actin can form several different structures in the cell, depending on the proteins that interact with the actin monomers or the actin filaments. One type of actin networks which is prominent at the leading edge of migrating cells is Arp2/3 nucleated actin networks, which are formed by an interplay of several types of proteins. An impression of these interactions is shown in Figure 4. *In vitro*, actin filaments are formed spontaneously by polymerization of actin monomers in the right buffer conditions. Branched actin filaments are formed by using the Arp2/3 protein complex. The Arp2/3 protein complex binds to the side of an existing 'mother' actin filament and nucleates a 'daughter' actin filament at a 70° angle. The Arp2/3 protein complex is then bound to the pointed end of the 'daughter' filament. [3]. The Arp2/3 complex has an inactive form and needs to be activated before it attaches to a filament and starts a new branch. This activation is done by a nucleation promoting factor, such as WASP or Scar. [3, 11] WASP/Scar is the full length protein, but to activate Arp2/3 complex, only a part of the protein is necessary, such as VCA or pWa. [8, 6] Since actin filaments can also nucleate spontaneously in the absence of activated Arp2/3 complex, spontaneous nucleation has to be suppressed. For the experiments presented in this thesis, profilin is used to prevent spontaneous nucleation. Profilin binds to one side of the actin monomers, such that the actin monomers cannot bind to each other to form a nucleus. [18, 22] The free side of the monomer is however able to bind to other filaments, therefore an existing filament can grow on the barbed end of the filament. Once the actin monomer is bound to an existing filament, the profilin will detach from that particular actin monomer, such that a following actin monomer is able to bind to the filament. [23] Since actin can only grow from existing filaments and the activated Arp2/3 complex, this process promotes nucleation only at the region where VCA is bound.

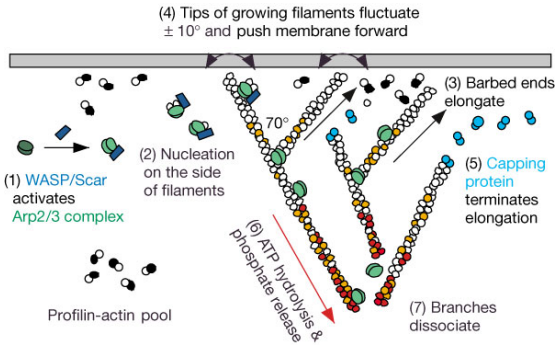


Figure 4: An impression of the proteins involved in the formaion and dissassembly of branched actin networks at the leading edge of migrating animal cells. (Image obtained from [3])

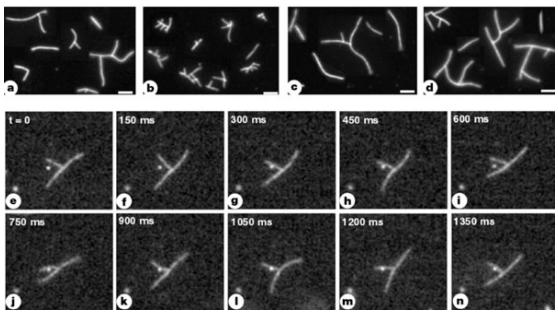


Figure 5: Fluorecent microscopy images on polymerization of actin with the Arp2/3 complex and a capping protein. **(a)** Shows Amoeba actin and profilin and in **(b)** the same, but additionally a capping protein. **(c)** Muscle actin with human profilin and in **(d)** the same, but additionally the Amoeba capping protein. **(e-n)** Shows thermal fluctuations of the branched filaments, in similar conditions as (c). Scale bar is 2.5 μm

3 Method

3.1 Cleaning the coverslip and glass slides

To use total internal reflection (TIRF) microscopy, it is important to have thoroughly cleaned coverslips. Anything that is fluorescent and close to the surface, will be visible and will affect the image quality. The coverslips were cleaned with Base Piranha to remove organic residues. To make Base Piranha, 100 ml of ddH_2O was heated in a beaker in the microwave until it starts to boil, which takes approximately 1,5 minutes. The beaker was placed on a heating plate while stirring and kept at 75 °C. Then 20 ml of 30% NH_4OH was added and after it reheated to 75 °C, 20 ml of 30% H_2O_2. This will result in bubble formation, indicating that there is a reaction. If there are no bubbles, the H_2O_2 is most likely degraded to less than 30%, then more H_2O_2 was added

until bubbles appeared. The magnetic stirrer was removed and the coverslips were placed in the solution for 10 to 15 minutes. The coverslips were rinsed by replacing the Base Piranha with ddH_2O and sonicating for 5 minutes. The ddH_2O was refreshed and sonicated again for 5 minutes. The coverslips were placed in a 100mM KOH and sonicated for 10 minutes and then stored in this solution for no longer than a week. After a week the coverslips need to be cleaned again with the same procedure or new coverslips were cleaned with this procedure. Before using a coverslip for an experiment, it was sonicated in a beaker with ddH_2O for 5 minutes and blown dry with N_2.

An optional method is to clean the coverslips by sonicating them in isopropanol for 20 minutes. Then sonicate them two times with fresh ddH_2O for 5 minutes to remove the isopropanol. Finally, the solution is sonicated in 100 mM KOH in which the coverslips will be stored.

The glass slides do not need to be as thoroughly clean as the coverslips, since this surface is not used for imaging. Cleaning this surface is only necessary to prevent residue in the solution of the flow channels. To clean the glass slide place them in a rack and place the rack in a beaker filled with 1 % Hellmanex. Sonicate for 10 minutes. Replace the Hellmanex with ddH_2O and sonicate for 5 minutes. Empty the beaker of the ddH_2O, fill it with 70 % ethanol and sonicate for 10 minutes. Replace the ethanol with ddH_2O again and sonicate for 5 minutes. Repeat the whole procedure one more time. The glass slides can be stored in ethanol until all the glass slides have been used. To use the glass slides, sonicate them in ddH_2O and blow dry with N_2

3.2 Microtubule seeds

Microtubule seeds are important in these experiments, to construct dynamic microtubules that are immobilized and close to the surface. This is convenient for imaging with a TIRF microscope, because this microscopy technique only allows to image at a close distance to the surface.

To attach the seeds to the surface, the microtubule seeds are grown with biotin labeled tubulin. To make the tubulin visible with the TIRF, a fraction of the tubulin has a fluorophore, such as rhodamine attached to it.

To make the microtubule seeds, mix the components of table 3.2

	Stock concentration	Volume [μl]	Final concentration
Biotin tubulin [1]	50 μM	0.6	3.64 μM
Rhodamine tubulin [1]	50 μM	0.4	2.42 μM
Tubulin [1]	100 μM	1.15	13.94 μM
MRB80		5.27	
GMPCPP	10 mM	0.83	1mM

The tubilin is in a buffer denoted as MRB80, which consists of 80 mM *Pipes*, 4mM $MgCl_2$ and 1 mM $EGTA$, that is brought to a pH of 6.8 with 5 M KOH. The rhodamine tubulin, biotin tubulin and tubulin are ordered from *Cytoskeleton* and GMCPP from *Jena Biosciences*. The solutions were

[1] in MRB80

all aliquoted, flash frozen and stored in the freezer at -80 °C. After mixing the components, the tube containing the mixture was covered with aluminium foil to prevent photobleaching and left at 37 °C for 30 minutes to allow the microtubules to grow. The tube was centrifuged for 5 minutes at 150 000 g in the airfuge from *Beckman-Coulter*. The supernatant was discarded and the pellet was re-suspended with 5.94 μl of MRB80. Then the mixture was left on ice for 20 minutes, to depolymerize the microtubules. 0.66 μl of GMPCPP was added to reach a concentration of 1 mM and than left on ice for 5 minutes. The mixture was left at 37 °C for another 30 minutes. The mixture was centrifuged at 150 000g a second time for 5 minutes and the pellet was again resuspended with MRB80, but now with 10% glycerol. The seeds-mixture was divided in aliquots, flash frozen with liquid nitrogen and stored them at -80 °C. Avoid multiple thawing and freezing when using the seeds, because this will reduce the quality of the seeds. With this procedure it is difficult to control the length of the seeds, therefore the seeds have different lengths. As a consequence, the seed density on the surface of a coverslip can vary per experiment.

3.3 Dynamic microtubules on a coverslip

The microtubule seeds, made in section 3.2, can be used to nucleate dynamic microtubules that can easily be bound to the surface of a coverslip. To bind the seeds to the surface, 0.2 mg/ml polylysine-polyethyleneglycol biotin (PLL-PEG Biotin) was used, diluted in MRB80. A cleaned coverslip was left to incubate with PLL-PEG Biotin, for 20 minutes. Flow channels were made as shown in Figure 6, by sandwiching parafilm between a cleaned glass slide and the coverslip. The parafilm was melted on a hot plate ar 120 °C, such that it sticks the slides together. Streptavidin was flown in and left to incubate for 5 minutes and then flushed out with MRB80. To improve the passivation of the coverslip, the use of κ-casein and pluronic is optional. This was done by leaving a solution of 1 mg/ml of κ-casein, diluted in MRB80 for 10 minutes in the flow channel and then flushing it out with MRB80 again. 1 % Pluronic F-127 was diluted 50-100 times in MRB80 and left for 10 minutes and then again flushed out with MRB80.

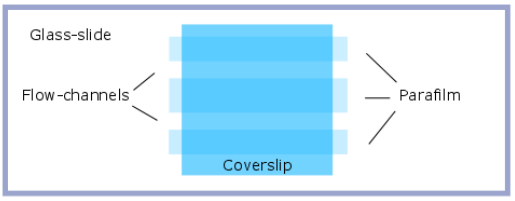


Figure 6: The flow channels, made in between a glass slide and a coverslip. The slide and coverslip are stuck together with parafilm that is cut into channels. The channels have a volume of 7-10 μl, depending on the width of the channel.

Aliquots containing the microtubule seeds were diluted 20 times with MRB80. As was mentioned in section 3.2, it is difficult to control the length and concentration of the seeds in the aliquots. To make sure that enough seeds

attach to the surface it is the most convenient to flush in the seeds into the channel and observe the surface with the TIRF microscope. Usually, a settling time of 5 to 10 minutes is enough to have a sufficient concentration of seeds at the surface. The seeds that did not attach to the surface are flushed out with MRB80. It is possible that flushing the seeds out forces some seeds to come in conact with the surface and attach, resulting in a higher concentration of seeds on the surface. This sometimes aligns some of the seeds with the flow.

After adding the microtubule seeds to the surface, the tubulin mixture is added to allow the formation of dynamic microtubules. For this, the components of tabel 3.3 are mixed. The pre-mix was made to prevent pipetting of small volumes that could cause errors in concentrations.

Pre-mix	Stock concentration	Volume [μl]	Final concentration
κ-casein	5 mg/ml	16.00	1 mg/ml
Methylcellulose[2]	1vol%	8.00	0.1%
KCl	3000 mM	1.32	50 mM
GTP[2]	50 mM	1.60	1 mM
Oxygen scavenger (50x)[2]		1.60	50x diluted
Glucose[2]	1000 mM	4.00	50 mM
Tubulin mix			
Rhodamine tubulin[2]	50 μM	0.80	1.0 μM
Tubulin[2]	100 μM	5.00	25.0 μM
Pre-mix		8.13	
MRB80		6.07	

Methylcellulose and κ-casein, are diluted with MRB80. The oxygen scavenger is in a buffer of 10 mg/ml Catalase, 20 mg/ml Glucose Oxidase and 30 mg/ml DTT. All components of the oxygen scavenger were ordered from *Sigma*. Rhodamine tubulin and tubulin are ordered from *Cytoskeleton* and PLL-PEG Biotin from *Surface Solutions*. Other labeled tubulin, such as Hilyte-488 tubulin or Hilyte-635 tubulin can also be used, these were also ordered from *Cytoskeleton*.

As a last step, the tubulin mixture was added into the flow-cell to obtain dynamic microtubules. The sample is then ready to be observed with the TIRF microscope.

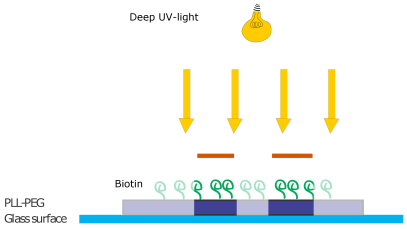
3.4 Microtubule patterning

Figure 7 gives a description of the procedure to pattern the surface of a coverslip. The description only provides the patterning of microtubules, but patterning an actin netowork involves a similar procedure. The first step is to passivate the surface of a coverslip with polylysine-polyethyleneglycol (PLL-PEG) with biotin attached. The next step is to cover the coverslip with a mask and expose the coverslip with the mask on top, with deep UV-light (UV Ozon Cleaner-ProCleaner, *BioForce Nanosciences*, Ames, IA, USA). This is UV-light with wavelengths between 1 and 200 nm. The mask is made of quartz glass, with the desired pattern in chrome on it. The deep UV-light will penetrate the quartz

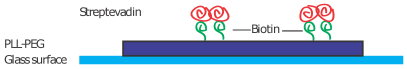
[2]in MRB80

glass, but not the pattern on the mask. The UV-light destroys the PLL-PEG Biotin and should therefore leave the desired pattern on the surface. After the UV-light exposure, a flow chamber is made on the coverslip and the PLL-PEG is flown in, to prevent non-specific sticking to the surface. The streptavidin is flown in, which will only bind to the biotin-attached surface areas and then the seeds are flown in. The seeds contain tubulin with biotin attached to it, thus the tubulin can bind to one of the other binding sites of streptavidin. With these steps the seeds should then only stick to the surface on the desired pattern where PLL-PEG is attached to the surface.

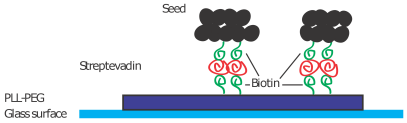
(a) Coat the surface of a coverslip with PLL-PEG Biotin



(b) Illuminate the surface with deep UV-light with a mask on top, to destroy PLL-PEG Biotin in the desired pattern. Passivate the in-between surface with PLL-PEG.



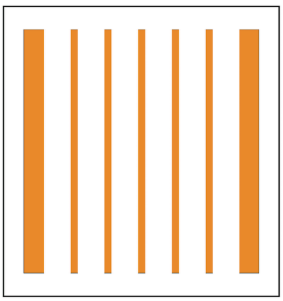
(c) Add streptavidin to the flow channel to bind to the biotin and coat the remaining surface with PLL-PEG



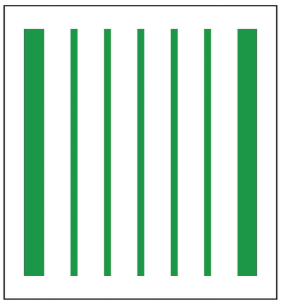
(d) Add seeds to the flow channel, to permanently bind them to the surface

Figure 7: Impression of the patterning process described step by step, shown from the side

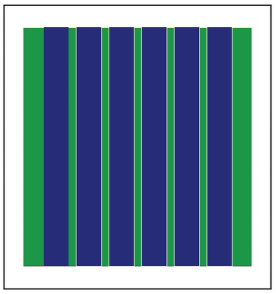
For the mask, different patterns were designed (see the appendix for the designs). The patterns were designed to pattern either Arp2/3 complex based actin networks, microtubule seeds or centrosomes. The different cytoskeletal components, need a different pattern. Actin monomers for example, need VCA and Arp2/3 complex to nucleate actin filament growth. The VCA sticks non-specifically to the surface, thus if the coverslip is passivated with PLL-PEG, the pattern on the mask has to resemble the inverse of desired pattern. For the microtubule seeds, we make use of a PLL-PEG Biotin layer on the coverslip. The chrome pattern on the mask therefore needs to resemble the desired seeds-pattern as is shown in Figure 8a and Figure 8b. Which pattern of the mask is needed, also depends on the order in which the surface is passivated. For example, if the use of PPL-PEG and PLL-PEG Biotin is reversed (PLL-PEG is applied first, the coverslip is illuminated with UV-light and than by PLL-PEG Biotin is applied), the pattern on the mask has to resemble the inverse of the desired pattern on the coverslip. The reverse of the order described in Figure 7



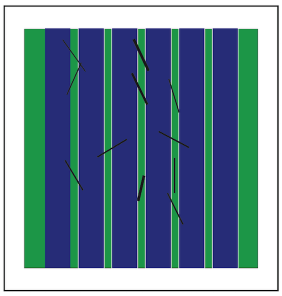
(a) Pattern on the mask, where the orange stripes represents the chrome of the mask



(b) The green stripes are a pattern of PLL-PEG Biotin on the coverslip after illumination.



(c) A pattern of PLL-PEG Biotin in green and coating of PLL-PEG in blue



(d) The coverslip after adding the microtubule seeds

Figure 8: The pattern of the mask and a stepwise impression of the surface of a coverslip while patterning microtubule seeds.

could give a cleaner pattern on the coverslip, but this was not explored for this thesis.

3.5 Actin network patterning

Patterning of the actin network is very similar to the patterning of the microtubules, but there are some small differences. Different proteins are involved that require more in-between spinning steps of the protein mixtures. To passivate the surface, PLL-PEG is used. To control the nucleation of actin filaments the actin nucleation promotor activator VCA is stuck non-specifically to the surface, where the coating is removed from the surface. As explained in previous sections, VCA will activate the nucleator Arp2/3 complex. To pattern the actin network on the surface, a cleaned glasslide and coverslip was used. Both glass surfaces were coated with PLL-PEG, by leaving a droplet of 10 μl of PLL-PEG dissolved in MRB80 at the surface for 20 minutes. The surface was then rinsed with ddH_2O and blown dry with nitrogen gas. The surface is then patterned by placing it on the mask with a droplet of 2.5 μl water in-between and exposing it to a UV-lamp for 6 minutes. Remove the coverslip from the mask by adding more water, this will make sliding it off easier and reduce scratches on the surface.

The surface of the coverslip is now patterned with PLL-PEG and non-PLL-PEG areas. To proceed, the flow channel was made by making channels of parafilm. Then VCA[3] (*Cytoskeleton*) was flown in and left for 5-10 minutes and then flushed out with MRB80. Before using the VCA, it was first spun with an airfuge for 10 minutes at 150 000 g to remove any protein aggregates. To passivate the surface even better, a solution of κ-casein was flow in and left to settle for 5 minutes and then also flown out with MRB80.

The actin was purified in our lab from rabbit skeletal muscle. The batches used were from "July 2014" and "February 2015". Labeled actin monomers were produced by adding the dye Alexa Fluor 647 *Molecular Probes*. To make the protein mixture, the actin first needs to exchange its calcium to magnesium.[4] This was done by adding EGTA with $MgCl_2$ to the labeled and unlabeled actin in the proportions shown in table 3.5. The actin was left in this mixture, for at least 30 minutes, on ice. Then profilin (from *Cytoskeleton*) was added and left for another 5 minutes on ice. All the other proteins were then added to the mixture. The mixture was spun with the airfuge for 10 minutes at 150 000 g, before adding into to the flow channel and observed with the TIRF microscope. Tabel 3.5 shows an example of the protein mixture. Slight changes can be made with the proportions of the used proteins, such as the percentage of the actin labeling, the ratio between profilin and actin and the percentage of methylcellulose. Take care though, that the actin concentration stays below 23 μM when exchanging calcium to magnesium actin monomers, descibed in the table 3.5 at "actin exchange". The actin can already polymerize above this concentration despite the presence profilin, since its concentration exceeds the critical concentration. [14]

[3]VCA was in a buffer of 20 mM Tris (pH 7.5), 20 mM NaCl

Exchange buffer	Stock concentration	Volume [μl]	Final concentration
EGTA	100 mM	0.40	0.4 mM
$MgCl_2$	20 mM	1.00	0.2 mM
Water		98.60	
Actin exchange	Stock concentration	Volume [μl]	Final concentration
Actin (labeled) [4]	47μM	0.51	2μM
Actin (unlabeled) [4]	95μM	1.01	8μM
Exchange buffer		2	
Profilin	47μM	5.11	20μM
G-buffer		3.37	
Protein mix			
Mg-Actin profilin mix	10μM Actin	5.7	2μM Actin
Arp2/3 complex[5]	2.23μM	0.45	50 nM
KCl	3000 mM	0.67	100 mM
ATP [6]	50 mM	0.4	1 mM
Oxygen scavenger (50x)[6]		0.4	50x diluted
Glucose [6]	1000 mM	0.4	50 mM
Methylcellulose [6]	1 vol.%	10.00	0.5 %
G-buffer		1.98	

3.5.1 Adding dynamic microtubules

Adding dynamic microtubules to the system changes the protocol slightly. The surface treatment is similar, but there need to be microtubule seeds flushed in the channels after the surface treatment and there are extra proteins in the solution. To passivate the surface, a droplet of PLL-PEG Biotin was left on the surface for 20 minutes, which was spread out on the coverslip with a piece of parafilm. After washing the surface with demi-water and drying it with nitrogen gas, the flow channel was made. Then VCA, streptavidin and κ-casein where flushed into the channel, following each step with a rinsing step of MRB80, similar to the actin patterning method.

For the protein mixture in tabel 3.5.1, the preparation of actin with profilin was the same. After adding profilin, the actin-profilin mixture was spun in the airfuge for 10 minutes at 150 000 g. The rest of the protein mixture was mixed in a separate tube, because a lot of the proteins in the mixture are stored in solutions containing salts. If these are added to the actin monomer, the monomers could already start polymerizing. After the protein mixture was mixed, it was also spun for 10 minutes in the airfuge at 150 000 g to remove any protein aggregates. It is recomended to prepare three times the volume specified in the tabel for the "actin exchange" to prepare the Mg-actin, prior to adding the Mg-actin to the other proteins. This makes pipeting easier and will reduce deviations in the concentration. Note that in this table, water is used to reach the right concentration, instead of g-buffer that was used in table 3.5.

[4] in G-buffer: 2mM Tris-HCl (pH 7,8), 0.2 mM Na_2ATP 0.2 mM $CaCl_2$, 5mM dithiothreitol (DTT)

[5] In a buffer of 20 mM Tris (pH 7.5), 25 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, *Cytoskeleton*

[6] in MRB80

Also, the concentration of methylcellulose is lower in the final solution and the extra components added are labeled and unlabeled tubulin, GTP, $MgCl_2$ and pipes.

Exchange buffer	Stock concentration	Volume [μl]	Final concentration
EGTA	100 mM	0.40	0.4 mM
$MgCl_2$	20 mM	1.00	0.2 mM
Water		98.60	
Actin exchange	Stock concentration	Volume [μl]	Final concentration
Actin (labeled)[7]	47μM	0.34	1.6μM
Actin (unlabeled) [7]	95μM	0.17	0.8μM
Exchange buffer		0.51	
Water		1.43	
Profilin	47μM	2.55	20μM
Protein mix			
Arp2/3 complex	2.23μM	1.82	200 nM
Tubulin (unlabeled)[8]	100μM	5.00	25μM
Tubulin (labeled) [8]	50μM	0.80	2 μM
KCl	3000 mM	0.67	100 mM
$MgCl_2$	20 mM	0.6	2 mM
ATP [8]	50 mM	0.4	1 mM
GTP [8]	50 mM	0.4	1 mM
Oxygen scavenger (50x)[8]		0.4	50x diluted
Glucose [8]	1000 mM	0.4	50 mM
Methylcellulose	1 vol.%	4.00	0.2 %
Pipes	100 mM	0.48	40 mM

3.6 TIRF microscopy, image processing and analysis

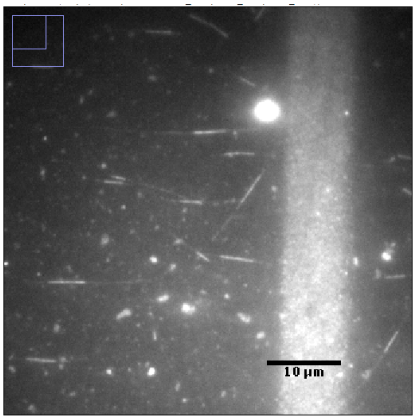
All images shown in the results are made using total internal reflection fluorescence (TIRF) microscopy, which provides a good signal to noise ratio. We used a *Nikon Eclipse* Ti-E inverted microscope, with an Apo TIRF 100 x 1.49 NA objective, a Perfect Focuse System and a Photomerics QuantEM:512SC EMCCD camera. There were three different wavelengths available with this microscope: the 488 nm with a 40 mW Calypso, 561 nm with 50 mW Jive diode-pumped solid state laser and 635 nm with 28 mW Meller Griot laser, that were used depending on the fluorophore that the protein was labeled with. Unless written otherwise, the tubulin was labeled with Rhodamine and actin monomers were labeled with Alexa 647. All the plugins that were used to process the images, were from the program ImageJ and FIJI. With the *Adjust/Brightness* plugin, the images were adjusted to optimal contrast and brightness.

The growth velocities and the catastrophe times were measured from the obtained movies. A line was drawn along the dynamic microtubule as shown in 9b, and a kymograph was made with the *stack/reslice* plugin in ImageJ. This stacks the same line on every image in the time-lapse as is shown in figure

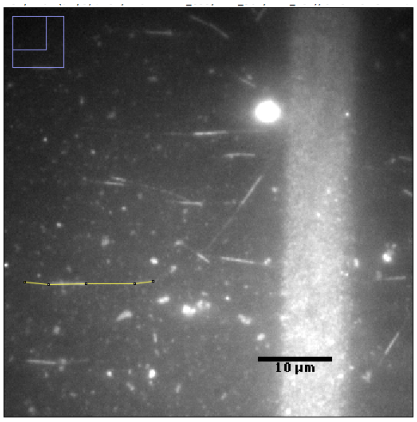
[7] in G-buffer: 2mM Tris-HCl (pH 7,8), 0.2 mM Na_2ATP 0.2 mM $CaCl_2$, 5mM dithiothreitol (DTT)

[8] in MRB80

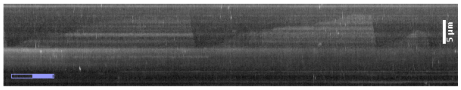
9c. From these kymographs the average growth velocities can be calculated by measuring the difference in length of the microtubule and dividing by the difference in time of the growing dynamic microtubules, which are respectively the height and width of the brighter triangles.



(a) One image of a time-lapse of dynamic microtubules



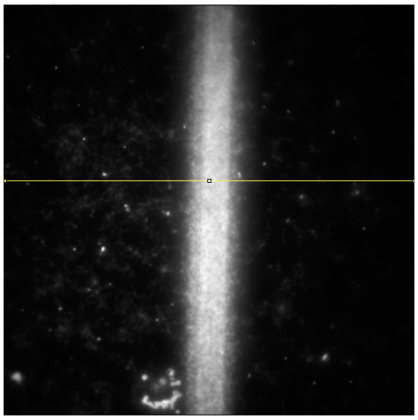
(b) Draw a line along a dynamic microtubule



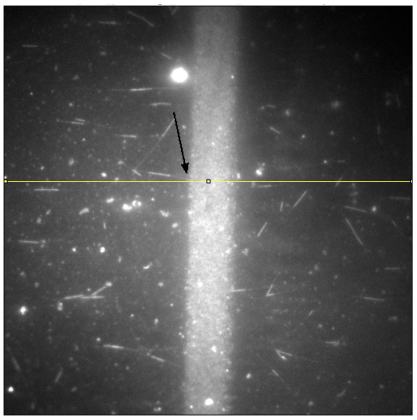
(c) kymograph of one dynamic microtubule with the length along the vertical direction and time in the horizontal direction. The plus-end of the microtubule is repeatedly growing and shrinking.

Figure 9: An example from ImageJ that shows how the microtubule dynamics are measured.

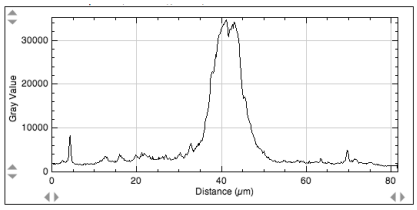
With the patterning technique, it is interesting to see the difference in catastrophe times for microtubules that enter the actin network and the microtubules that do not come near the actin network. To distinguish between the two types, it is neccessary to determine at what distance from the network the microtubules enter. This can be done by looking at the intensity profile of the actin network. A line was drawn perpendiculary to the patterned actin network as is shown in Figure 10a. With the ImageJ plugin *Analyze/ Plot Profile* the intensity profile is plotted this line. Figure 10c shows the intensity profile of the actin network of the line drawn in Figure 9b. The height (y-coordinate) of the drawn line is estimated by the position of the microtubule plus-end near the actin network. From the intensity profile it can then be judged whether the microtuble will enter the actin network. For the profile shown in Figure 10c, the peak increases at 31 μm and finishes at 53 μm (x-coordinates). Dynamic microtubules entering this range of x-coordinates are then counted as interacting.



(a) Image showing the fluorescently labeled actin network in the presence of dynamic microtubules shown in (b). A line was drawn left to right, across the surface at the height a dynamic microtubule is entering the actin network.



(b) Image showing the fluorescently labeled microtubules in the presence of an actin network shown in (a). A line was drawn left to right, across the surface at the corresponding height as in (a).

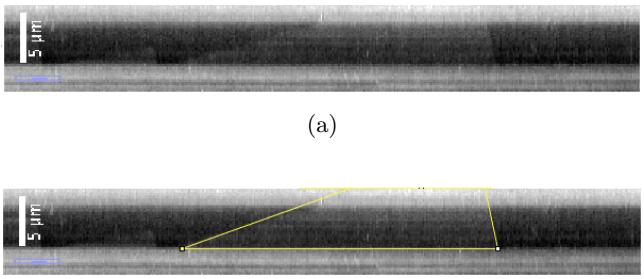


(c) The intensity profile of the surface across the actin network from the line drawn in (a).

Figure 10: To decide if a microtubule was interacting with the actin network, an intensity profile of the actin network was made by drawing a line, at the position where the tip of the dynamic microtubule plus-end is meeting the actin network. The microtubule tip is indicated by the arrow in (b). In this case, if the microtubule-end was between the horizontal distances 31 and 53 μm, the microtubule was considered as interacting with the actin network.

To measure the catastrophe time, the time was measured from where the microtubule starts growing until it starts shrinking. In the kymograph, this is the horizontal length of a saw-tooth from the start until the top of the saw-tooth. The beginning of the kymographs often show saw-teeth that have already started. If the catastrophe time for these initial events was longer that the average catastrophe time, this incomplete saw-tooth was also added to the measurements. For microtubules that interacted with the actin network, the top of the saw-tooth was not visible, possible due to fluorecence signal of the labeled tubulin that is stuck to the actin network pattern . This made it difficult to measure the catastrophe time, because it was not visible when the microtubule started shrinking. Several scenarios are possible in the invisible top. The microtubule could simply keep on growing and then start to shrink. To measure this scenario, the growing and the shrinking of the microtubule

were extrapolated to find the top of the saw-tooth, then the catastrophe time was measured by assuming this top. An alternative case that can be measured is that the microtubule pauses in the invisible part and than starts shrinking. Therefore the time was measured between the start of the saw-tooth and from where the microtubule visible starts shrinking. The average was taken of these two scenarios as the catastrophe time. Other scenarios in this invisible area are multiple pauses and multiple catastrophes and rescues of the microtubule. These scenarios were not taken into account in the measurements.



(a)

(b)

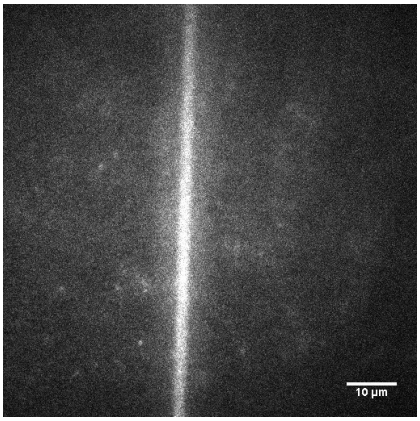
Figure 11: A kymograph of a dynamic microtubule growing into an actin network. At the bottom we see the fluorescent microtubule seed. At the top there is fluorescently labeled tubulin stuck to where the actin network is situated, which makes it difficult to distinguish the tip of the microtubule. (b) Shows how the top of the saw-tooth is found. By extrapolating the growing and shrinking of the microtubule, the triangle is completed. The scale bar is 5 μm and the total width is 96 seconds.

To measure the average growth speed, the height δl and width δt from the start to the top was measured. $\frac{\delta l}{\delta t}$ gives the average growth speed of the microtubule. If the top of the saw-tooth was not visible, as is shown in Figure 11, the growing and shrinking of the visible dynamic part of the microtubule was extrapolated to completed the triangle. Then the height and its corresponding width of this triangle was measured to calculate the average growth speed.

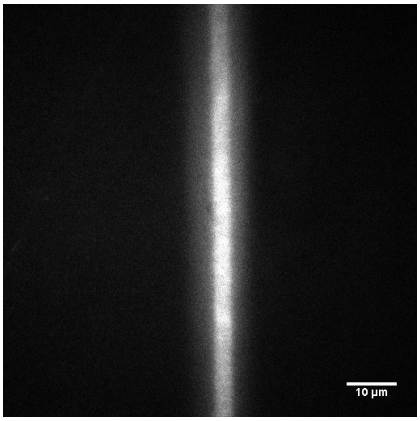
4 Results

4.1 Patterning of labeled streptavidin

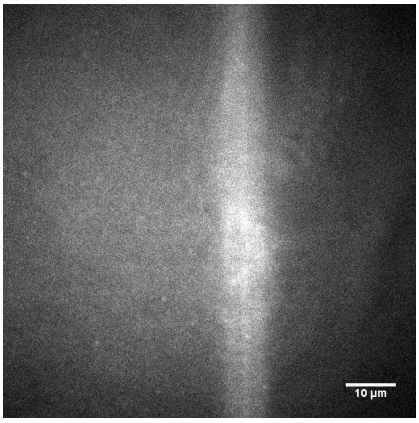
A good start for patterning the surface with dynamic microtubules and an actin network, is by patterning PLL-PEG Biotin on the surface and attaching labeled streptavidin to this. The labeled streptavidin makes the pattern visible with TIRF microscopy and gives an indication of the quality of the pattern.



(a) 1-minutes UV-exposure time



(b) 5-minutes UV-exposure time



(c) 15-minutes UV-exposure time

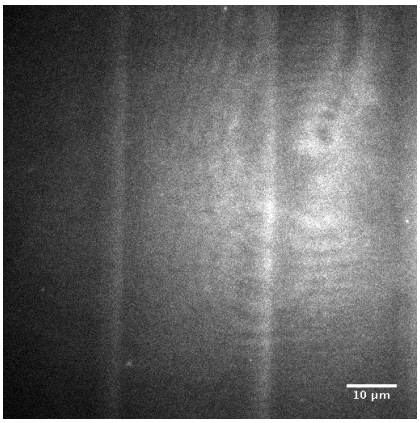
Figure 12: Patterning of streptavidin on the surface with different exposure times to the UV-light of the PLL-PEG Biotin coated coverslip.

For these images, the mask with a stripped pattern was used. The stripes on the mask have a width of 5 μm, the distance between 2 stripes is 60 μm. Figure 12 shows patterned labeled streptavidin made with different deep-UV exposure times. This is the time that the coverslip, which was coated with PLL-PEG Biotin was exposed to deep-UV light, with the mask on top. The exposure times shown are 1, 5 and 15 minutes. The images show different contrasts for the different exposure times. Each sample shows a stripe of labeled streptavidin on the surface, but the sample with the 5 minute exposure time has the clearest

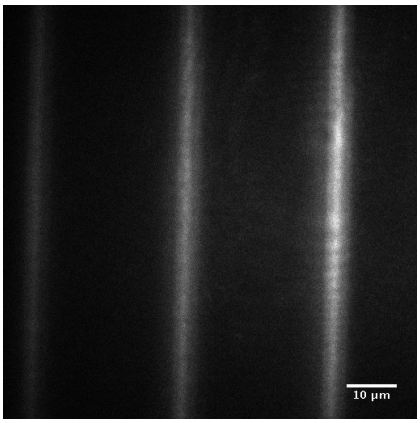
contrast. For the 1 minute exposure time, the sample shows more fluorescent on the whole surface. The sample with 15 minute exposure time has too little fluorescence, that causes more background noise on the image. From these images, it is however not clear if the streptavidin is functional, that is to say if there is enough streptavidin on the pattern to bind the seeds and little enough on the rest of the sample to reject seed-binding. Quantification of the intensities is difficult to obtain, since the concentration of the streptavidin bound to the surface depends on factors that are hard to control. It not only depends on the concentration of streptavidin that is flown into the chambers, but also on the cleanness of the glass used, the freshness and the concentration of all the products, its settle time and the laser intensity. For these experiments, an attempt to control these factors was made by using the same diluted proteins, the same laser intensity and glass from the same cleaning day. The intensity profile also depends on the microscope setup (e.g. the TIRF angle) and the laser intensity varies across the image surface. It is typically highest in the middle and lowest in the corners.

4.1.1 κ-casein

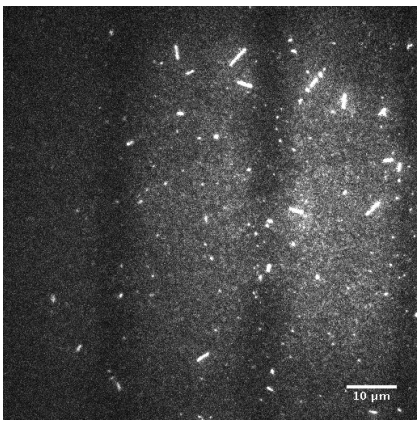
To improve the surface passivation, κ-casein is often used to unspecifically bind to the surface and hence block further protein absorption. The results in figure 13 show the difference in microtubule adhesion to passivated areas of the coverslip when the surface is treated with κ-casein and when it is not treated. For these images, the surface is coated with PLL-PEG Biotin and then exposed to deep UV-light for 5 minutes, the PLL-PEG was left for 40 minutes, labeled streptavidin and for one channel, κ-casein was left in for 5 minutes. Stabilized microtubules were flushed in both channels and the images shown below were made after 20 minutes.



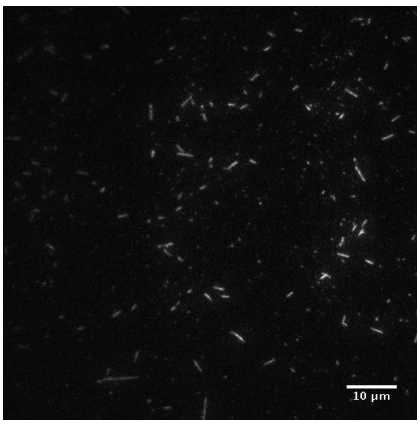
(a) Image showing fluorescent streptavidin on a patterned surface where κ-casein was not used for blocking.



(b) Image showing fluorecent streptavidin on a patterned surface where κ-casein is used for blocking.



(c) Image showing fluorescent microtubule seeds on a patterend surface where no κ-casein was used.



(d) Image showing fluorescent microtubule seeds on a patterned surface where κ-casein was used for passivation.

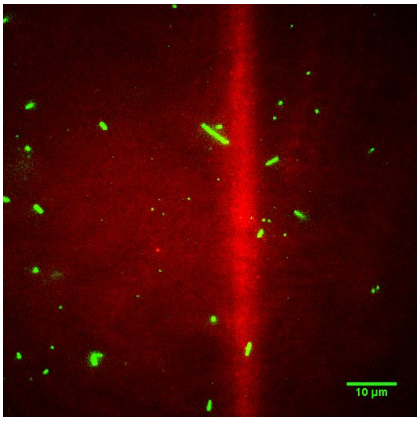
Figure 13: Images of a patterned surface with fluorescently labeled streptavidin and labeled seeds where (a) and (c) is without the use of κ-casein and (b) and (d) are with κ-casein, these images were made 20 minutes after the seeds were added. The surfaces were treated with a 5-minute UV-light exposure time.

The Figures 13a and 13b show a clear difference in contrast with and without the surface treatment of κ-casein. When κ-casein is used on the surface, less labeled substances are on the surface between the stripes. This suggests that the use of κ-casein prevents unwanted labeled particles to stick to the surface. In Figures 13c and 13d show stabilized microtubules, labeled with rhodamine. In Figure 13c, where the surface is not treated with κ-casein, there are a lot of labeled particles stuck to the surface. Surprisingly though, there are not that many seeds sticking to the surface. Since the surface is less passivated, we anticipated to observe more seed sticking to the areas inbetween the streptavidin stripes. A possible explanation is that other smaller particles, such as labeled

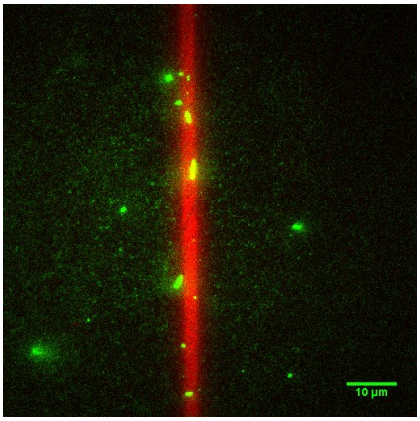
tubulin that are already stuck on the surface, prevent the seeds from sticking. In Figure 13d, where there is κ-casein on the surface, there are a lot more seeds attached to the surface and there is a lot less other unwanted labeled particles attached. The patterning of the seeds is not that clear in this image, even though streptavidin has a higher intensity on the stripes. The results in section 4.2 reveal that the low contrast is a consequence of a too short UV-light exposure time.

4.2 Patterning stabilized microtubules

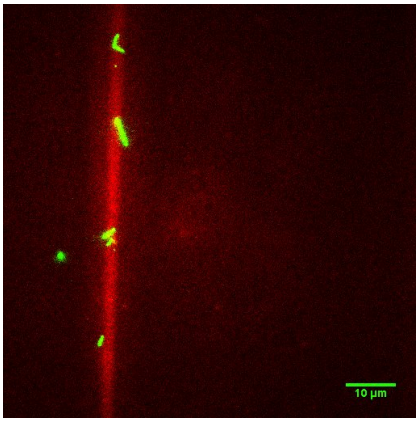
Different exposure times have an effect on the patterning of labeled streptavidin. To observe the effect on stabilized microtubules these were added into flow-channels and attached to the surface. The following results show experiments with different exposure times to pattern the surface which was coated with PLL-PEG Biotin and where seeds were added afterwards.



(a) 1 minutes UV-exposure



(b) 5 minutes UV-exposure



(c) 15 minutes UV-exposure

Figure 14: Patterning of microtubule seeds using different exposure times to the UV-light of the PLL-PEG Biotin coated coverslip, with a mask to create a pattern. Red: fluorescently labeled streptavidin. Green: Stabilized fluorecently labeled microtubule seeds.

In Figure 14 shows labeled streptavidin and seeds attached to the surface of a cleaned coverslip in green. Labeled streptavidin, which binds biotin, is shown as red in the images. The different panels in Figure 14 display patterns created with different UV-exposure times. In all cases, a bright stripe of labeled streptavidin is formed. This indicates notably higher concentrations of streptevidin at non UV-exposed areas compared to the UV-exposed areas, as we already saw in section 4.1. In Figure 14a, the microtubule seeds are bound randomly to the surface. Even though there is a clear red stripe of streptavidin visible on the surface, the concentration of streptavidin on the rest of the surface seems to be high enough to bind microtubule seeds. This results in randomly distributed seeds on the surface. In Figure 14b there is a slight preference of the microtubules that bind to the areas where there is a higher streptavidin concentration. There are however, still a lot of microtubules binding to the surface in between the stripes. Although the intensity, coming from the streptavidin, is lower in between these areas, it could still be that there is some functional streptavidin bound to the surface. Figure 14c, the seeds seem to have a high preference for the areas where the intensity from the labeled streptavidin is the highest. The areas between the pattern have less seeds attached, indicating that the streptavidin concentration is too low to facilitate seed binding surface.

4.3 Patterning of actin filaments

A brief attempt was made to pattern the surface with actin filaments with biotin. For this, the surface treatment was the same as for the patterning of microtubules with microtubule seeds. The PLL-PEG Biotin was patterned with an exposure time of 5 minutes and the rest of the surface was passivated with PLL-PEG, κ-casein and pluronic. The actin filaments shown in Figure 15 were made with actin monomers from the "July 2014" batch, where 10% of the actin was labeled with biotin and another 10% was labeled with Alexa 647 nm. The actin filaments were stabilized with a 1:1 molar ratio of phalloidin, and then flushed into the flow-channel for observation.

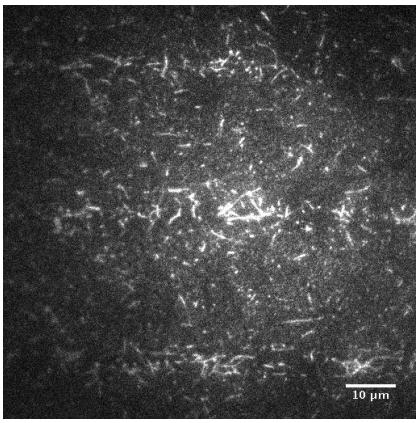


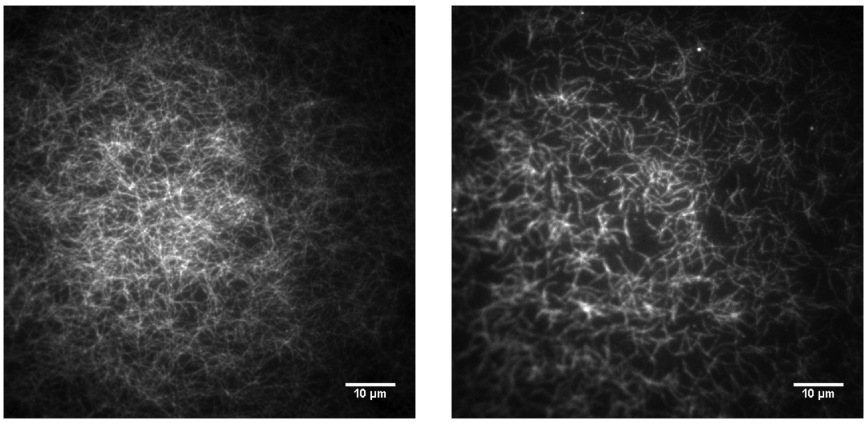
Figure 15: Actin filament patterning using 10% of biotin labeled and 10% Alexa 647 labeled actin monomers. The patterning was made with PLL-PEG Biotin with an UV-exposure time of 5 minutes, the surface passivation with PLL-PEG, κ-casein and pluronic.

Figure 15 shows actin filaments patterned in horizontal stripes. There are however a lot of filaments attached to the surface in between the stripes. This result shows that patterning with the biotin-streptavidin bond is possible, but it does not give the cleanest results. Compared to the microtubules seeds in section 4.2, actin filaments are more difficult to pattern this way. Probably because actin filaments are a lot more sticky and need a better surface passivation. Only the result of a 5 minute UV-exposure time is shown. It could be that different UV-exposure times will give better results though. Another problem with this method is that the filaments are almost fully stuck to the surface. Movement of the actin filament is strongly restricted. For studying the interaction of actin filaments with dynamic microtubules, this approach is not useful. It could be used as an easier substitutional test, to see if PLL-PEG Biotin patterning is sufficient enough to bind filaments.

4.4 Profilin with "old" and "fresh" actin

To properly create an patterned actin network, it is necessary to have control over spontaneous nucleations. From results not shown in this thesis, it was clear that the inhibition did not work well enough to create a patterned actin network. Following results shows some experiments to see what could cause these spontaneous nucleations.

"Old" actin that had been on ice for 31 days has been tested, in comparison with "fresh" actin that had been thawed for 1 day from the freezer.

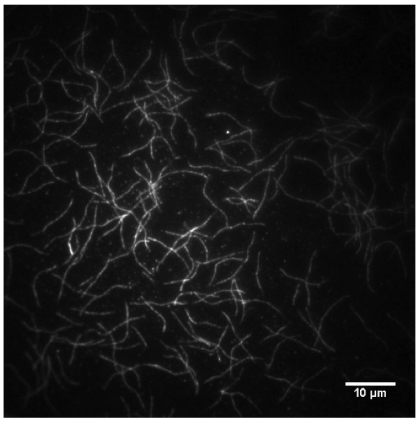


(a) "Old" actin 7 minutes after the polymerization buffer was added.

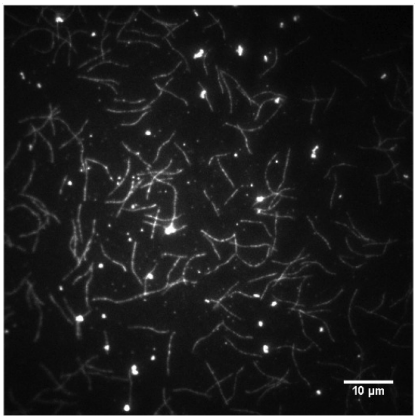
(b) "Old" actin that was premixed with profilin for 45 minutes. Image was taken 7 minutes afer the polymerization buffer was added.

Figure 16: Old actin of 1 uM with 20 % fluorescently labeled actin

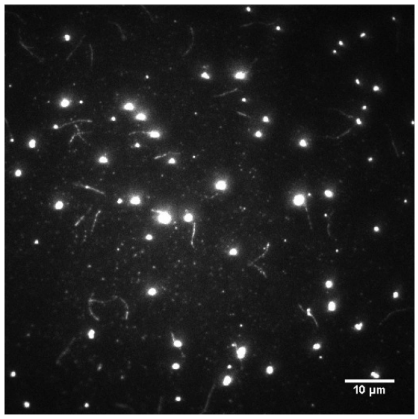
For the old Mg-actin in Figure 16, we made a solution of 1 μM Mg-actin monomers, where 20 % is labeled actin. The images in Figure 16 were taken approximately 7 minutes after the polymerization buffer was added to the actin monomers. Figure 16b has the same concentration of Mg-actin monomers, with the same percentage of labeling, but with 2.5 μM profilin added to the solution. The profilin was first incubated with the actin monomers for at least 30 minutes on ice before adding the polymerization buffer. Both images show a high density of polymerized actin after 7 minutes. Figure 16b shows a slightly lower density of actin filaments, but it is not clear if the density difference is due the profilin in the solution or if it is due to slight changes in concentration.



(a) "Fresh" actin after 18 minutes in the polymerization buffer



(b) "Fresh" actin with profilin, after 7 minutes in the polymerization buffer

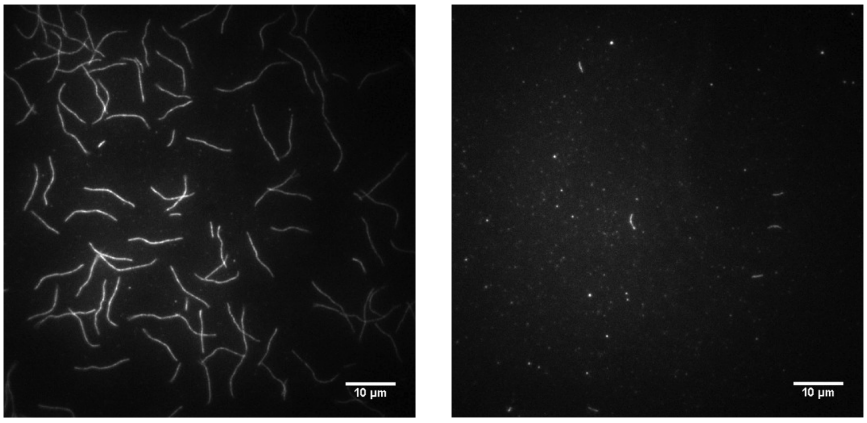


(c) "Fresh" actin, premixed with profilin, after 7 minutes in the polymerization buffer

Figure 17: "Fresh" actin of 1 μM with 20% fluorescently labeled actin

In Figure 17, "fresh" actin was used to polymerize actin filaments. Compared to the "old" actin in Figure 16, the fresh actin also shows actin filaments, but considerably less. In Figure 17, 1 μM of actin monomers with 20% labeled was used again. Figure 17a was made 18 minutes after adding the polymerization buffer and Figure 17b is made 7 minutes after adding the polymerization buffer. Figure 17b shows almost no difference in density of actin filaments compared to Figure 17a, but the time differences between the images, makes it difficult to tell if profilin inhibited polymerization. It is also possible that the surface in Figure 17b has less filaments, simply because it had less time to polymerize. There is however a clear difference between Figure 17b and 17c. For both figures the same concentration of profilin was added, but for Figure 17b, profilin was added at the same time as the polymerization buffer, while for Figure 17c profilin was added to the actin solution approximately 45 minutes before adding the polymerization buffer. Both images were taken 7 minutes after the polymerization buffer was added. The premixed profilin shows a lot less actin filaments than the image

were profilin was not premixed. The images also show a lot of large labeled objects in the solution. From movies made in these solution, it was observed that this were mostly clusters floating around. They were not stuck to the surface of the flow-cell, but were able to freely move around. The objects could be clusters of partly denatured protein that include labeled actin.



(a) "Fresh" and spun actin, after 7 minutes in the polymerization buffer

(b) "Fresh"and spun actin, premixed with profilin, after 7 minutes in the polymerization buffer

Figure 18: "Fresh" and spun actin of 1 uM with 20% labeled

To remove the clusters, the actin and the labeled actin were spun with an airfuge for 10 minutes at 150 000 g, before adding profilin and the polymerization buffers. Figure 18 shows the results of the spun actin, with the same concentrations of Mg-actin monomers at approximately 75 minutes after adding the polymerization buffer. Figure 18a shows actin, without profilin added to the solution. Figure 18b shows the image of actin with profilin in the solution, where the profilin was premixed with the actin monomers, 45 minutes before adding the polymerization buffer. Without profilin added to the solution, a moderate density of long individual filaments is observed. In the presence of profilin, only a few short filaments are visible. These result show that profilin can indeed suppress actin nucleation, provided that it is premixed with actin before the polymerization buffer is added.

4.5 Actin network patterning

In the previous section the working of the proteins involved with the actin network pattering were tested to obtain the best results. Now we have obtained more information on the interaction of profilin and actin, this next section focusses on the patterning of the Arp2/3 nucleated actin network, where the procedure described in section 3.5.1 is used to make the pattern on the surface of a coverslip.

4.5.1 The effect of spinning the protein mixture

In Figure 19 and 24 (see Appendix) the results on actin patterning with a high methylcellulose concentration (0.5%) are shown. For Figure 19, only the VCA was spun in the airfuge at 150 000 g for 10 minutes before attaching this to the surface. The image was taken around 10 minutes after adding actin to the channel, when the actin polymerization was already at a stead-state length. The images in Figure 24 show different area's in the same channel. In this sample, both the VCA and the protein mixture were spun in the airfuge for 10 minutes at 150 000 g, before adding it into the channel. The images where taken around 50 minutes after adding the protein mixture to the channel. For both figures, there was 2 μM of actin monomers of which 20% was labeled and 50 nM Arp2/3 complex. The methylcellulose was diluted in MRB80 to 1% before adding it to the protein mixture, but the oxygen scavenger, glucose and ATP where used the same as described in the protocol.

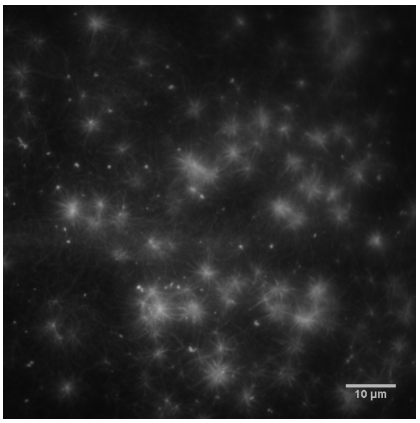
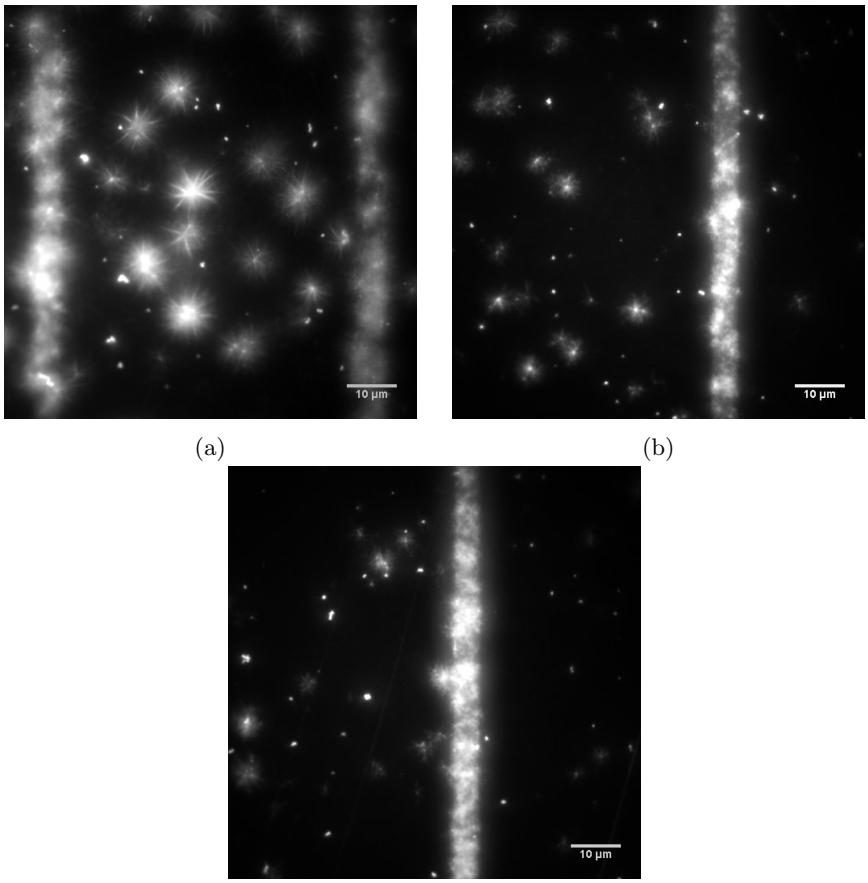


Figure 19: Actin patterning, with spun VCA on the surface and a methylcellulose concentration of 0.5%. The actin was not spun

In Figure 19 there are a lot of actin filaments growing in highly localized, small network clusters. The growth of the actin filaments does not seem to be restricted to certain areas. The image shows actin filaments that seem to bundle at the ends, which is to be expected at the high methylcellulose concentration used.



(a) (b)

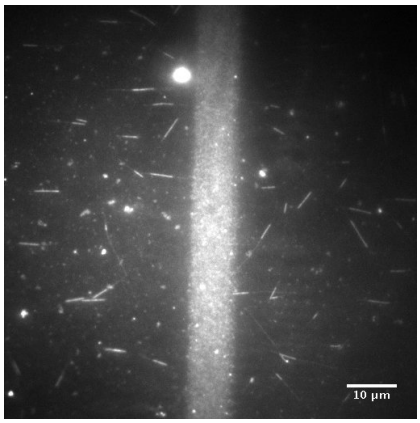
(c)

Figure 20: Different areas on the surface of the same flow channel with an actin pattern. Both the VCA and actin were spun and 0.5% methylcellulose was present.

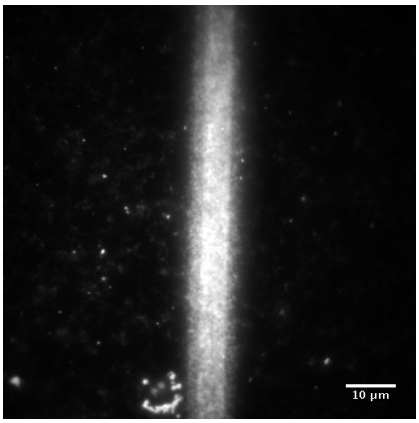
The images in Figure 20 shows a striped pattern on the surface, from where actin can grow. However, different areas in the channel show different results. The coating of the surface does not seem to be uniform throughout the whole sample. For this sample there is also a high methylcellulose level used. The longer actin filaments in the sample show similar bundling effects at their ends as was seen in Figure 19 . There are a few clusters visible at the surface, but the coating seems to be sufficient to be used with this procedure.

4.6 Patterning of actin network with dynamic microtubules

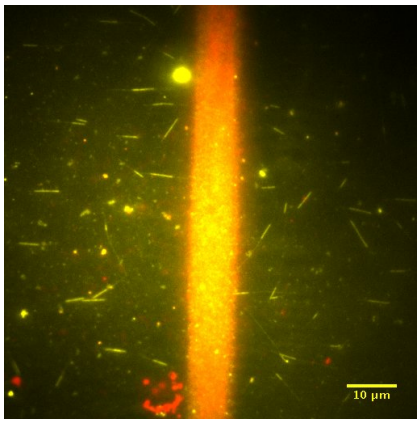
Previous results showed patterning of microtubules and patterning of an actin network. In this section, those two cytoskeletal components are now brought together in one experiment. The actin network is patterned by covering the surface with VCA and passivating the rest of the surface with PLL-PEG Biotin, as is described in section 3.5.1.



(a) Dynamic microtubules labeled with rhodamine



(b) Arp2/3 nucleated actin network growing on top of a VCA-coated stripe, with 20% labeled actin with Alexa Fluor 647



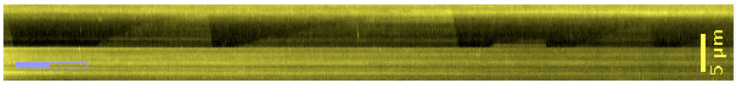
(c) Composition of images, in yellow the microtubules from (a) and in red the actin network from (b)

Figure 21: actin network and dynamic microtubules

Figure 21a shows the dynamic microtubules. The microtubule seeds are made as described in section 3.2, the dynamic part of the microtubules is grown at 27 μM of tubulin, with 7% labeled with Rhodamine. The microtubules were imaged with the TIRF microscope at a wavelength of 561 nm. The actin network at the same position, is visualized with the TIRF microscope at a wavelength of 635 nm in Figure 21b. The actin network was grown at 200 nM Arp2/3 complex, 0.2 % methylcellulose and 2 μM of actin monomers of which 20% were labeled with Alexa Fluor 647. The composition of the two images is shown in Figure 21c.

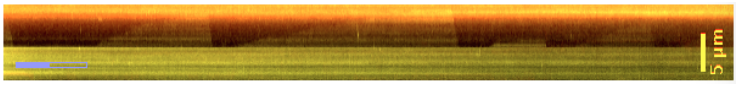
In Figure 21a it is shown that the dynamic microtubules are distributed randomly on the surface. There is a bright stripe of labeled particles at the position of the actin network shown in the other two figures. These could be Rhodamine labeled particles that can stick to this area, because there is no

PLL-PEG Biotin on that part of the surface. The dynamic microtubule consist out of a stabilized seed and dynamic part on both ends of the seed. The seed is brighter because of a higher percentage of labeled tubulin and therefore act as fiducial markers of the dynamic microtubules.



(a) An kymograph of an dynamic microtubule (yellow), growing and shrinking into an actin network. The bottom shows the seeds of the microtubule, the saw-tooth the repeatedly growing and shrinking of the microtubule.

(b) The kymograph of corresponding the Arp2/3 nucleated actin network in red, where the dynamic microtubule is growing into.



(c) The composition of the kymographs (a) and (b)

Figure 22: An kymograph of an dynamic microtubule (yellow), repeatedly growing and shrinking into an actin network (red). Scalebar: 5 μm, Width: 138 seconds

The actin network, shown in Figure 21b is patterned nicely in a stripe-like fashion on the surface. The actin network does not show bundeling, probably because of the low percentage of methylcellulose and high concentration of Arp2/3 complex. the methylcellulose concentration of 0.2% is too low to induce substantial bundeling, while for a concentration of 0.5 % as used in *Reymann et al.* we would expect bundeling of actin filaments to be widespread. [10] The composition of the two images in Figure 21c, shows dynamic microtubules that meet the actin network and other microtubules growing elsewhere and do not meet the actin network. These images show that the buffer conditions are sufficient enough, to grown an Arp2/3 nucleated actin network with dynamic microtubule. It can hence be used to measure the dynamics of microtubules encountering actin networks.

4.6.1 Growth velocities and Catastrophe times of dynamic microtubules

At this point, only one movie has been recorded at the right conditions to measure catastrophe times in the presence of an Arp2/3 nucleated actin network. In this movie there where 8 microtubule measured as not interacting with the network and 9 microtubules as interacting. The movie was 29.25 minutes long, which was sufficiently long to measure multiple catastrophe times of one microtubule. The measurement gave an average catastrophe time of 224.0±28.7 seconds and an average growth velocity of 37.5±3.7 nm/s for the non-interacting microtubules and 205.1±36.9 seconds and average growth velocity of 47.4±4.4

nm/s for the interacting microtubule. The average growth velocities are around the numbers found by *Janson et al.* [9], which reported a growth velocity of 41.6±1.6 nm/s at a tubulin concentration of 28 μM and a temperature of 23 °C.
This data suggest that the interacting microtubules have a slightly higher average growth velocity and a shorter catastrophe time. Careful consideration should be taken with these numbers though, because to find the catastrophe time for the interacting microtubules, the growth and shrinking of the microtubule was extrapolated assuming a constant growth or shrinking velocity. Whenever the microtubule entered the actin network, it was not visible anymore. The catastrophe time could be drastically smaller, if the microtubule had multiple catastrophes and rescues in this area. For the average growth velocities, only the visible area was measured. Since the average growth velocities include pauses, the obtain number could be smaller, if the microtubule had more pauses in the invisible area. Thus, for both quantities the difference between microtubules interacting and not interacting with the actin network, could be larger. Not enough measurements have been made to draw firm conclusion yet. The catastrophe times display a large variability, thus they could change the average catastrophe time drastically, so it is important to average over a sufficiently large munber of events to obtain statistically meaningful averages.Regarding the average growth velocity, it will be important to dertemine the number and duration of pauses for microtubules in contact with actin.

5 Conclusion and Discussion

Patterning microtubules and actin filaments

The simplest experiment that was carried out to test the quality of the surface patterning of a coverslip, was by patterning labeled streptavidin. Streptavidin is a protein that has a high binding affinity for biotin. Thus, by patterning the surface with PLL-PEG Biotin, streptavidin can be patterned on the surface. The images in section 4.1 show that labeled streptavidin is indeed patterned on the surface the way it was intended. The fluorescent intensity pattern observed display a pattern of stripes on the surface of the coverslip that resembles the pattern of the mask. The images show that different deep UV-light exposure times have an influence on the contrast of the stripes. The 1-minute exposure time has a lower contrast than the 5-minute exposure time and the 15 minutes exposure time has such a low intensity of light, that the pattern is barely visible against the background noise. In sum, the 5-minute exposure produced the best visible pattern. The fluorescent patterns are observed as bright and dark areas corresponding to higher and lower streptavidin densities. It is however not clear what the absolute concentration of streptavidin is on the respective parts of the surface. Thus, from this experiment it is not clear if there is a high enough streptavidin concentration on the stripes to bind the microtubule seeds and if there is sufficiently little streptavidin on the rest of the surface not to bind microtubule seeds. To test this, section 4.2 shows microtubule seeds labeled with biotin attached to the surface.

Other than the UV-light exposure times, there are more factors that have an influence on the surface patterning. In section 4.1.1 it is shown that treating the surface with κ-casein, has a large influence on the contrast of the stripes and therefore the quality of the pattern. The surface with no κ-casein, displays a lot more fluorescence on the passivated surface in between the fluorescent streptavidin stripes. The images with the microtubule seeds show that addition of κ-casein reduces other unwanted labeled particles to bind on the surface. These images also show that instead more seeds can bind to the PLL-PEG Biotin stripes on the surface. Presumably because with κ-casein on the PLL-PEG Biotin striped surface, the reduction of the other labeled particles on the surface enabled the seeds to bind to now available spots.

Another factor on the quality of the patterning, which is not shown in this thesis in more detail, is the cleanness of the coverslip, before adding the proteins. The glass used for experiments was cleaned with Base piranha no longer than 5 days prior to the experiments. Longer times showed more dirt particles on the surface of the glass. What drastically improved the contrast of the patterning, was adding 2.5 μl of water between the coverslip and the mask while exposing it to UV-light. The water brings the glass slide and the coverslip close to each other with approximately even distance throughout the whole surface and it reduces movements between the mask and the coverslip during exposure. *Reymann et al.* [20] used a handmade vacuum chamber to achieve this, which could give cleaner results. For our initial testing purposes, adhering the glass by using a droplet of water seemed to be entirely sufficient.

The results in section 4.2 show the surface of patterned labeled streptavidin together with the microtubule seeds. These result show that a short UV-light exposure time, such as the 1-minute exposure time, does not allow to reliably pattern the seeds on the surface. The seeds remain randomly distributed. Longer exposure times, such as the 5 minutes and 15 minutes improved the patterning of the seeds. The microtubule seeds seem more likely to stick where the surface is patterned with PLL-PEG Biotin. Problems with longer exposure time are that the water between the mask and the coverslip dries out during exposure. This will make it more difficult to remove the coverslip without scratching the surface. Exposure times around 7 minutes were preferred with this procedure to pattern the seeds. The next step is to grow dynamic microtubules form the seeds. To go from patterned microtubules seeds to dynamic microtubules, tubulin has to be added in a polymerization buffer to the system.
In section 4.3 an experiment to pattern pre-formed actin filaments is presented. The method of patterning remained the same, but instead of microtubule seeds, stabilized actin filaments tagged with biotin were attached to the surface. We only tested patterning with 5-minute exposure time. Comparing our result with the microtubule seeds, indicated that the patterning of the actin filaments is less well-defined. The higher density suggests a higher affinity of the actin filaments to bind to the PLL-PEG Biotin patterned stripes. However, nonspecific binding may also contribute, since we also observe that many actin filaments attach to the passivated regions in-between the adhesive stripes. Longer exposure times could improve the relative affinity for the stripes. The actin filaments, however appeared to be almost fully attached to the surface and were barely moving. This procedure is less fit to study the interaction with microtubules, because the restricted movement of the actin filaments is less close to a natural situation.

Another approach to study the interaction between microtubules and actin filaments was by inducing growth of an Arp2/3 nucleated actin network in a spatially defined pattern. For this there were several other proteins necessary, such as profilin and VCA. As described in section 2.3, profilin inhibits bulk nucleation. Adding an excess of profilin with actin monomers was hence expected to prevent actin filaments from forming. On the contrary, in-between first results constantly showed actin polymerization, which made it necessary to test the quality of the actin and the profilin. This was done by testing if the time that the actin was stored on ice had influence on the rate of spontaneous nucleation. A possibility is that nuclei are formed if the actin is stored on ice for several weeks.

Experiments to systematically test the interaction of profilin with the actin were carried out as described in section 4.4. These indeed revealed a clear difference between actin that was on ice for a few weeks and actin that was thawed a day before use. When using the old actin, similar filament densities arose in the presence and absence of profilin. The fresh and spun actin showed actin filaments when there was no profilin present, but addition of profilin resulted in a very low filament density. This indicates that the profilin indeed suppresses actin filament nucleation, but it is necessary to use fresh and spun actin. Old actin might have grown nuclei for the actin to polymerize spontaneously. Images of the fresh and unspun actin showed a lot of clusters and some filaments. Spinning was found to be useful to remove clusters and to inhibit spontaneous formation of actin filaments. This is further confirmed by

the difference between unspun "fresh" actin polymerization in Figure 17c and images of spun "fresh" actin in Figure 18b. Another important difference was seen between actin that was premixed and not premixed with profilin. The actin monomers that were premixed with profilin for 30 minutes before adding the polymerization buffer in Figure 17c, showed a lot less actin filaments than the actin monomers that were not premixed in Figure 17b. This indicates that mixing and incubating actin monomers with profilin before adding the polymerization buffer is necessary. The time scale for premixing profilin was not tested systematically. It is possible that times shorter than 30 minutes could be sufficient as well. It was also not tested how long the actin can be kept on ice. To have a good indication of how long the actin can be used after it had been thawed, similar tests should be done every week after it has been thawed.

After gaining sufficient control of the spontaneous nucleation of actin, the actively growing actin network could be patterned on the surface by using VCA, Arp2/3 complex and profilin. The results in section 3.5 show that it is indeed possible to grow an Arp2/3 nucleated actin network in a striped pattern, by using a mask with stripe-thicknesses of 5 μm. The results show that there is a large difference when the protein mixture is spun and or not. When using the spun protein mixture, a much cleaner and more well-defined pattern was obtained. The images also show that it is crucial that the protein mixture is spun to reduce clusters, because these can initiate the growth of an actin network on other areas. Other patterns available on the mask have not yet been tested with this patterning technique, it could be that there is a limit on the thickness of the stripes and the spacing between the stripes to get the desired pattern. The Arp2/3 complex causes branched filaments. In this thesis only one concentration of Arp2/3 complex was tried. Different concentrations of Arp2/3 complex could change the density and the growth of the network upward and perpendicular to the pattern stripes. Methylcellulose is also known to play an important role on the morphology of the network. High percentages of methylcellulose, such as 0.5% have a bundling effect on actin filaments, thus changing the structure of the actin network. [20]

Mimicking actin-microtubule interaction *in vitro*

In this thesis, it has been shown that it is possible to pattern an actin network with the described patterning technique. To study the interaction with dynamic microtubules, these two filaments need to be added together into the same flow chamber. This requires that all the proteins involved need be fully functional at the same buffer conditions. The results shown in section 4.6, show that is it possible to have an Arp2/3 nucleated actin network and dynamic microtubules in the same conditions. While our experimental results show that the microtubules can dynamically interact with an Arp2/3 nucleated actin network, we also encountered some technical difficulties. The images show that there is fluorescently labeled tubulin on the adhesive stripes, where the actin network grows. The dynamic microtubules growing into the network do not have a high enough contrast do be clearly distinguished from the fluorescently labeled tubulin at the surface where the actin network is situated. To increase the contrast, it might be a possibility to use a higher percentage of labeled tubulin to grow dynamic

microtubules. Increasing the labeled tubulin concentration, could however increase the fluorescently labeled tubulin in the adhesive areas and would then not solve this problem. Another possibility is to add labeled EB to the protein mixture and use only unlabeled tubulin. EB is a protein that binds to the growing plus end of the microtubule. Using labeled EB enables to track the plus end of the microtubules and since the necessary concentration of EB is much lower than that of the labeled tubulin, the residual labeling of the striped area might therefore be reduced.
Measurements of the catastrophe time and the average growth velocities indicate that there could be a difference in the microtubule dynamics when the microtubule does or does not meet the actin network. The interacting microtubules have a larger average growth velocity of 47.4±4.4 nm/s compared to 37.5±3.7 nm/s for non-interacting microtubule. They have a shorter catastrophe time of 205.1 ±36.9 seconds compared to the 224.0±28.7 seconds of the non-interacting microtubules, where the error is the standard deviation. It must be noted however that the catastrophe time was measured by extrapolating the growing and shrinking microtubules in the invisible areas. The catastrophe could be drastically smaller if the microtubule has multiple catastrophes and rescues in the invisible areas, which will result in a larger difference between the interacting and non-interacting microtubule. The measured average growth velocity could be lower if the microtubule has more pauses in the invisible area, reducing the difference between the interacting and non-interacting microtubules. The numbers found for the average growth velocities and the catastrophe time are based on a limited number of experiments. With the current errors, the difference in the catastrophe time and growth velocity is not significant. To give a better statistical estimate, more experiments are needed to give evidence of a dynamic interaction of microtubules with an Arp2/3 nucleated actin network.

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Appendix

Mask design

In Figure 23 an impression of pattern designs on the mask (*Delta Mask*, The Netherlands) in chrome on the quartz glass is shown. The quartz glass permits deep UV-light (UV Ozon Cleaner-ProCleaner, *BioForce Nanosciences*, Ames, IA, USA) to penetrate, while the chrome stops the light from passing through. The patterns are designed to be used for actin, microtubule patterning and centrosome patterning. To make the mask designs, the layout editor *Clewin* was used. For patterning microtubules, a pattern mimicking the desired pattern is used, while for patterning an actin network and centrosomes, an inverse of the pattern is necessary. For the centrosomes we designed a dotted pattern with different distances, such that it is for example possible to study the interaction of dynamic microtubules initiated by centrosomes at different distances. There are two different diameters of dots available to test. There is an striped pattern, where the stripes have an angle inbetween, which could force the actin network to bundle. This gives the possibility to study the influence of bundle formation on the dynamics of the microtubules when they interact with the actin network. The pattern is a simple striped pattern and inversed striped patterns with different line thicknesses

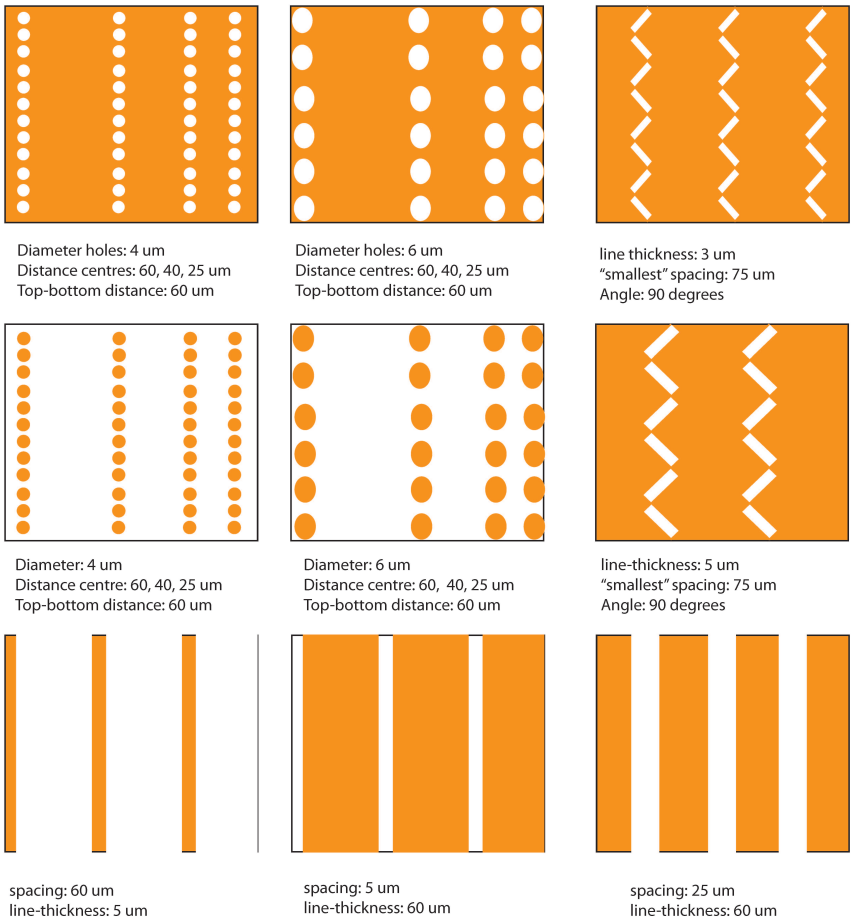
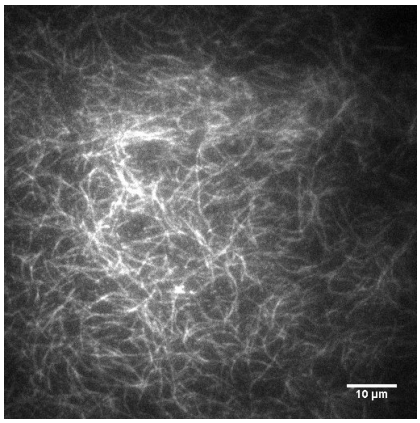


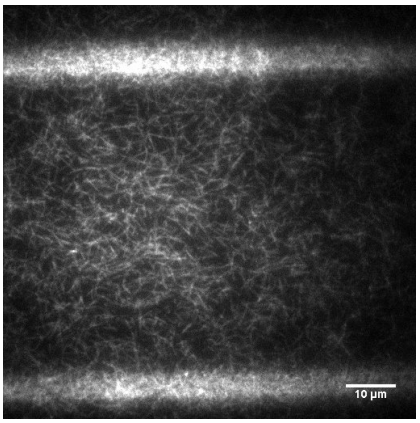
Figure 23: An impression of the mask design. In orange are the patterns in chrome on the quartz glass. The design shown in each pannel is repeated until a surface of one coverslip of 24x24 mm is covered with the same pattern.

Results on an actin network with VCA in solution

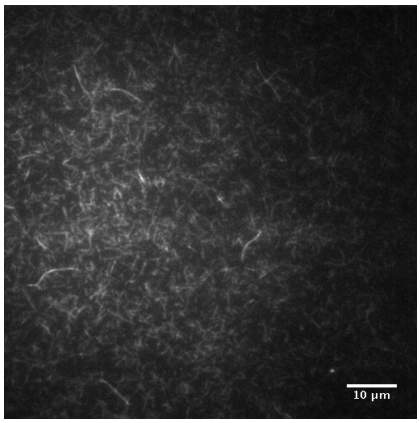
To make sure that the VCA was effective, it was added into the solution with the other proteins. The actin that was used for these experiments was however "Old" actin, that is actin that was thawed from the -80 freezer longer than 4 weeks. The Figures 24a and 24c show 2 μM actin with 20 % labeling, on a surface that was passivated with PLL-PEG and kappa-casein. In Figure 24b and Figure 24d, a pattern of VCA was made on the surface of the coverslip and the rest of the surface was passivated with PLL-PEG and κ-casein. To control the spontaneous nucleation of actin, all samples contained a 3:1 molar ratio of profilin and actin monomers and the profilin was not pre-mixed with the Mg-actin before adding the polymerization buffer. The polymerization buffer includes among others, Arp2/3 complex, which is activated by VCA and should therefore cause a pattern on the surface.



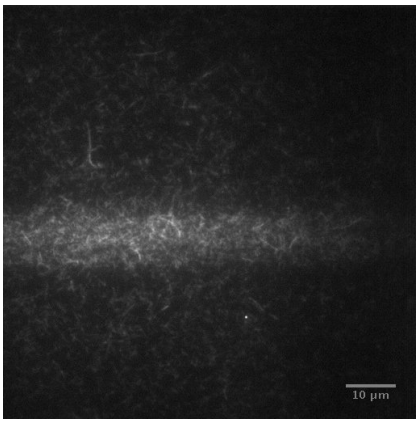
(a) No VCA in the solution or on the surface



(b) No VCA in the solution, but a VCA patterning on the surface



(c) VCA in the solution, without patterning



(d) VCA in the solution and patterning of VCA on the surface

Figure 24: Actin network grown with and without VCA in the solution

Figures 24b and 24d show a striped pattern of actin filaments, but the actin filaments are not only present on the stripes. The images show that the actin

is also allowed to polymerize in the in between areas. For Figure 24d this could be expected, since there is VCA in the solution. There is however no VCA in the solution in Figure 24b, still there are small actin filaments visible in the in between areas.

In the Figures 24a and 24c there is no pattern on the surface. Since there is no VCA in the solution in Figure 24a, actin was not expected to polymerize at all. Clearly this is not the case, there are long filaments visible in the sample indicating that either the actin already contains nuclei or that the profilin is not functional. In Figure 24d, where there is VCA in the solution, there are small filaments visible in the sample. This indicates that the VCA activates Arp2/3 complex and makes smaller branched actin filaments.

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