Micro-assisted fabrication of collagen matrix to study cell mechanical behaviour under micro-confinement

Master thesis in measurement science and instrumentation

By

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Abstract

The aim of this work has been to optimize a microenvironment for study of the behaviour of living cells. This type of microenvironment has previously been used to compare adhesion ability for different cell types and those experiments have indicated a potential application in diagnostic of oral cancer [1]. Bovine serum albumin (BSA) has been used to generate a protein repellent surface which is extra repellent to extra cellular matrix (ECM)-proteins. When adsorbed on a substrate ECM proteins (such as collagen type I used in this project) have the ability to support cell adhesion. Samples with series of parallel BSA stripes with micrometric width and millimetric length have been fabricated using the technique of micro-contact printing. An elastic material (PDMS) containing parallel ridges with the dimensions described were inked with a solution containing BSA and used to deposit stripes of BSA at a cover slip. The micro gaps between the stripes have been used to depose collagen I. We then have a substrate presenting strong variations for the adhesive interactions between cells and substrate. The patterned surface is large enough to study simultaneously a population of single cells. A vital part of this project has been to evaluate effects of variations in deposition of both BSA and collagen I. Collagen I have the ability to polymerize into fibers under suitable conditions. The polymerization of collagen depends on pH, and this parameter has been varied for different samples. Polymerization has been induced within micro-channels taking advantage of the material properties of PDMS to control the pH changes. We document interesting fabrication and control of the collagen polymerization, even if it was not possible to achieve a complete study of this complex micro-system. Atomic force microscope (AFM) has been used for imaging and quantification of characteristics of different samples. The AFMs ability to detect variations at the nanoscale has revealed the high degree of accuracy needed for this purpose. Finally some of the prepared samples were tested in cell experiments. BSA deposition of was tried using both hydrophobic and hydrophilic PDMS. Those experiments revealed a significant thicker BSA layer on the substrate using hydrophobic than hydrophilic PDMS. The concentration of glutaraldehyde used was also varied. Samples made using concentrations of 0,625%, 1,25% and 2,5% glutaraldehyde (diluted in PBS) did not give any

significant differences. Having two layers of BSA on top of each other were found to give a denser layer. Experimentation with different methods of deposing collagen resulted in samples covered with collagen fibers of varying sizes and quantities and potensial for development is demonstrated. The cell experiments indicated only slightly different cell spreading dynamic on the samples chosen for those experiments and for the cell type used.

Contents

Acknowledgements

1.0 Theory

In this work living cells has been studied in a microenvironment made of BSA and ECM. For a cell to be able to adhere in the microenvironments prepared it must change and adapt to the new surroundings. Some knowledge about how the cytoskeleton of a cell is built up is required to get an understanding of how the cell interacts with a surface consisting of ECM proteins which favours cell adhesion. This chapter also provide some information about BSA which is used to for its ability to block interactions with other proteins, and in particular ECM proteins. This section ends with a presentation of relevant previous work which has been an inspiration to this project.

1.1 The cell

We have two types of cells. Those are the prokaryotic cells which are typically independent organisms, and eukaryotic cells which are usually found in multicellular organisms and the type we focus on here. The eukaryotic cell is an extremely complex structure that supports microbiologists with a tremendous amount of different challenges. The subject is huge, but this introduction will only implement a certain level of detail on a few components that are most relevant to the project. Important components of the eukaryotic cell includes the nucleus were DNA of the cell is stored and mitochondria which produce most of the cells adenosine triphosphate which give the cell power. The cell gets most of its mechanical strength and flexibility from the cytoskeleton. The cytoskeleton is considered very relevant to the project, and is covered in more detail in the next section [2].

microtubule

centrosome with
pair of centrioles

chromatin (DNA)

nuclear pore

nuclear envelope

extracellular matrix

vesicles

5 μm

lysosome

actin
filaments

peroxisome

ribosomes
in cytosol

Golgi
apparatus

intermediate
filaments

plasma
membrane

nucleolus

nucleus

endoplasmic
reticulum

mitochondrion
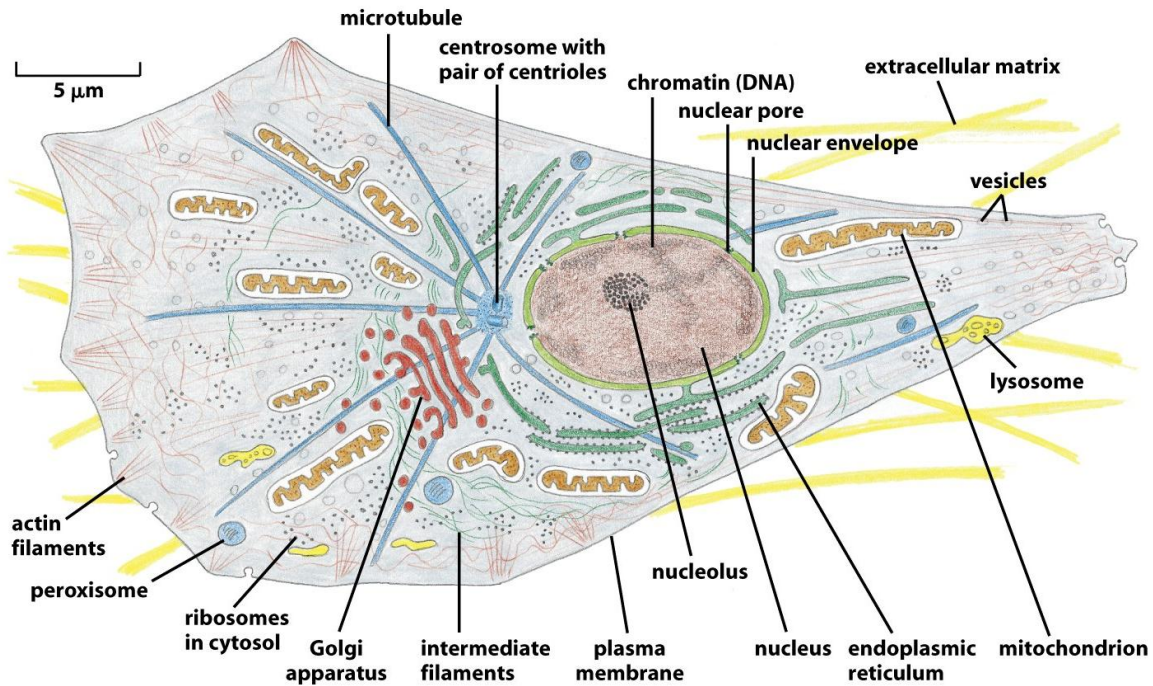
Figure 1: The eukaryotic cell. *The drawing shows the inside of a eukaryotic cell, and the most important components are labeled.*

1.2 The cytoskeleton

When studying cells spreading, it is essential to know something about how the cytoskeleton of the cell functions. The cytoskeleton of a eukaryotic cell is a system of filaments that support the cell with many important functions. Among those are maintaining mechanical strength of the cell, help it to be correctly shaped, and rearrange internal components as the cell grow, divide and adapt to changing circumstances. To manage this, the cytoskeleton depends on three different filaments which need to work together. Those filaments are formed by three different families of protein molecules, also called subunits of the filament. Each of those filaments has distinct mechanical properties, dynamics, and biological roles. But they all share the fundamental principles that provide the basis of understanding how the cytoskeleton works and how the different elements cooperate. Although the individual protein molecules of the cytoskeleton are

only a couple of nanometers in size, the cytoskeleton is spanning all over the cell from one end to the other. Many polymers such as RNA and DNA are hold together by covalent bounds. For the different filaments of the cytoskeleton, this is not the case. Here it is the much weaker non covalent interactions which are working in holding the different filaments subunits together. This provides the subunits to quickly join together and separate without any bounds being formed or removed [3]

Actin filaments

The actin filament is made of helical polymers of the actin protein. Two rows of subunits laced together to make a right handed helix of the actin protein is called an actin filament. (*The evolution of the cytoskeleton*) The diameter of this filament is about 5-9 nm. The actin filament is flexible, and builds up a network which covers the whole cell, but the highest concentration is found in the part of the cell called the cortex.

The actin filaments determine the shape of the cells surface, and are also responsible for movement of the cell on a surface. In order to manage this, they depends on hundreds of accessory proteins which both regulate the spatial distribution and the dynamic behaviour of the cytoskeleton. They are also converting information signals into physical cytoskeletal movements, and connect the cytoskeleton to other cell components. The actin filament is dynamic, meaning that they normally are remodelling and replaced every 48 hours in average. There are two distinct ends on an actin filament. They are called the plus end, and the minus end. The plus end is the more dynamic of the two, meaning that growth and shrinkage are fast compared to the other [3].

Microtubules

Microtubules are made up of the protein tubulin. There are two different tubulin proteins called α-tubulin, and β-tubulin. Microtubules are shaped as long hollow cylinders with an outer diameter of 25 nm, and this causes them to be less flexible than the actin filaments. They appear

long and straight and usually have one end attached to a centrosome. This is an organizing centre for microtubules. As the actin filament, microtubules also have a plus and a minus end. α-tubulin are exposed at the minus end, and β-tubulin at the plus end. The minus end is always the end attached to the centrosome, and the plus end points outwards. Because of this the centrosome also functions as a cytoskeletal organizer which holds the microtubules together in a defined geometry. Microtubules control intracellular transport, and positions of membrane-enclosed organelles. The microtubules are also dynamic structures [3]

Intermediate filaments

Those are straight fibers with a 10 nm diameter. Many of those fibers are twisted together in a way that make them very strong and supply the cell with mechanical strength. Accumulation of many intermediate filaments is what makes up tough appendages as nails and hair. Intermediate filaments have the ability to stay at a constant location, and with the same length and diameter throughout the entire lifetime of the cell. This is being stable, and is opposite to the dynamic behaviour of actin filaments and microtubules [3].
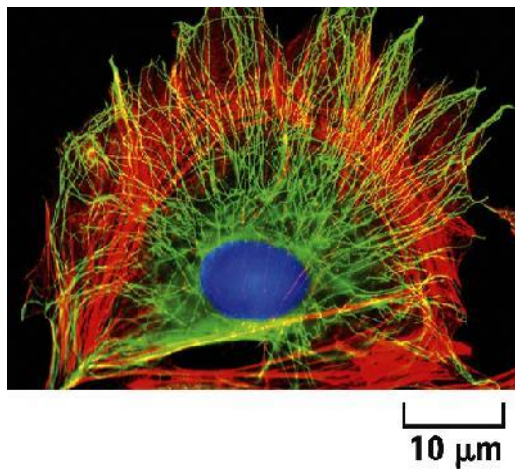

Figure 2: Fixed and labeled cell. *Microtubules are shown in green, and actin filaments in red [3].*

1.3 The extracellular matrix

In this section the extracellular matrix protein (ECM) which favours cell adhesion is described. The cells cytoskeleton interacts with the ECM through the use of integrin receptors. Finally the structure of collagen type I which is the specific type of ECM used in this project is presented.

In a multicellular organism the cells need to be able to interact with each other. The most fundamental interactions are those which hold cells together. This can happen either as cell-cell junctions were two cells bound directly together, or as a cell interacting with extracellular matrix (ECM), which will soon be explained in more detail. These attachments control the orientation of the cells internal structure. Which attachments are made are essential for the way cells move within the organism, and obviously good communication between cells are necessary as the body grows, changes shape and heals. Lacking this communication causes a lot of different diseases.

Tissues in mammals are not solely made up of cells, and a major part of the tissues is the extracellular matrix (ECM). This is a complex network of polysaccharides and proteins that are secreted by the cells. Various concentrations of ECM are found in different types of tissues. In bulky tissues such as bone, tendon and inner layer of the skin big amounts of ECM is present. In those tissues we usually have more ECM than cells, and here it determines the physical properties of the tissue.

Cytoskeleton binds to the ECM through integrin receptors on the cell membrane. This interaction is very important in cell adhesion on ECM. Integrins are characteristic for multicellular animals, and are used to perform various tasks. The integrin connection between ECM and the cytoskeleton is not only a physical connection, but can also be used to send information forward and backward between the interior of the cell and the binding with the ECM. The integrins are flexible, and if tension is applied to it this can cause it to tighten its grip both on the intracellular and extracellular structures. If tension is released this can cause release of this tension. So the integrins can transmit both mechanical, and molecular signals. It can also convert one of these signal types to the other. Those properties are used by the cell to control the orientation of the ECM [Ref (2)]. There are a large amount of different integrins. In humans at least 24 different types are known. An integrin is composed of two glycoprotein subunits. For all 24 except one of

the integrins, it is connected to the actin filament of the cytoskeleton. Talin protein is used as an important component in the linkage between the integrin and the actin filament [4].
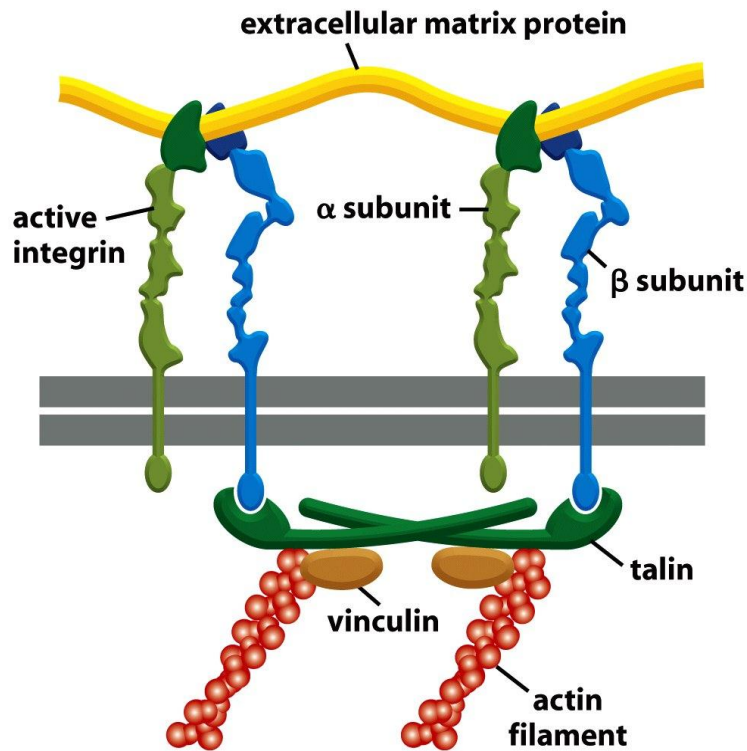
Figure 19-45 Molecular Biology of the Cell 5/e (© Garland Science 2008)

Figure 3: Integrin connecting to extra cellular matrix (ECM). This picture shows an integrin subunits connecting to an ECM protein [4].

The most important of the proteins in the ECM for mammals are collagen. Collagen is secreted in very large quantities by connective tissue cells, but also by other cell types. In mammals collagen alone represent 25% of the total protein mass. Collagen is build up of α - chains. The α - chain has a helical structure consisting of different types of amino acids. The structure of the helix is glycine-x-y, where often times x is proline and y hydroxproline. Glycine is the smallest of the amino acids, and is necessary in every third position for the helix to fold correctly. As seen in the figure three of those chains joining together form a collagen molecule. Glycine is the only protein small enough to be in the interior part of this triple helix. Usually many collagen proteins assemble into higher-order polymers called collagen fibrils. Those are structures with about 10nm-300nm with, and a hundreds of micrometers long. Often collagen fibrils again aggregate

into larger bundles which are several micrometers in diameters and visible in light microscope as collagen fibers. The making of those polymers are called polymerization. Collagen can also form gels when it appears in relatively high concentration [5]. Today 28 different types of collagen have been identified. These collagen types can again be categorized by their macromolecular structures. Type I which is the type used in this project is a fibril type, and is by far the most common collagen protein. Col I is found in bone, skin, tendons, ligaments, cornea and internal organs [4].
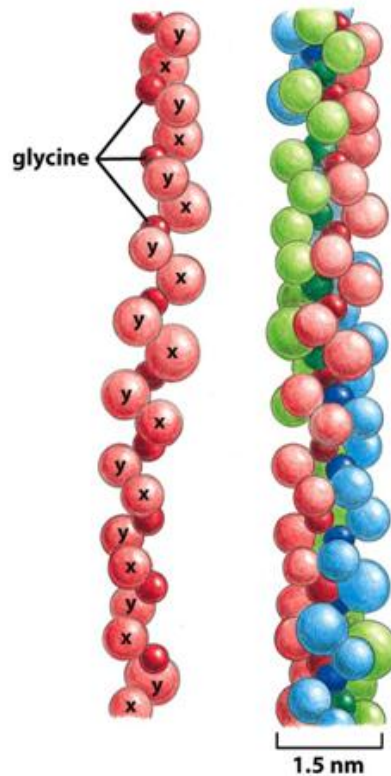
Figure 4: Structure of the collagen molecule. To the left is a model of a single collagen α – chain. To the right three of those chains, each represented in different colours are wrapped around each other to form a triple helix.

1.4 Polymerization of collagen at different pH values

A study of collagen assembly onto a flat surface and at different pH values revealed that the pH of the collagen solution plays an important role in collagen polymerization [7]. A mica disc of 6,5mm in diameter was used as supporting surface. The collagen used in with study was composed at 97% collagen type I and 3 % collagen type III . The mica surface was flushed with a buffer solution containing collagen at a concentration of 0,1mg/ml. After 10 minutes adsorption time the same buffer solution without collagen was used to rinse of the molecules only loosely bound to the surface. Imaging was performed using atomic force microscopy in tapping mode. The results show that polymerization of collagen into fibers does not appear for pH 4,5 and lower. This changed when the pH is increased to values between 5,5 and 9,5. The mica surface then ended up completely covered with fibrillar structures. The flow (caused by the flushing of the buffer solution containing collagen) induced alignment of the collagen fibers, and more than 95% of the fibers were aligned parallel to each other in the direction of the flow. Increasing the pH to values between 5,5 and 9,5 also made the fibers bigger. When the pH increased to 10,5 only a few fragments (similar to the ones obtained at pH between 5,5 and 9,5) were obtained. The results obtained for the different pH values are shown in Figure 5 [7].
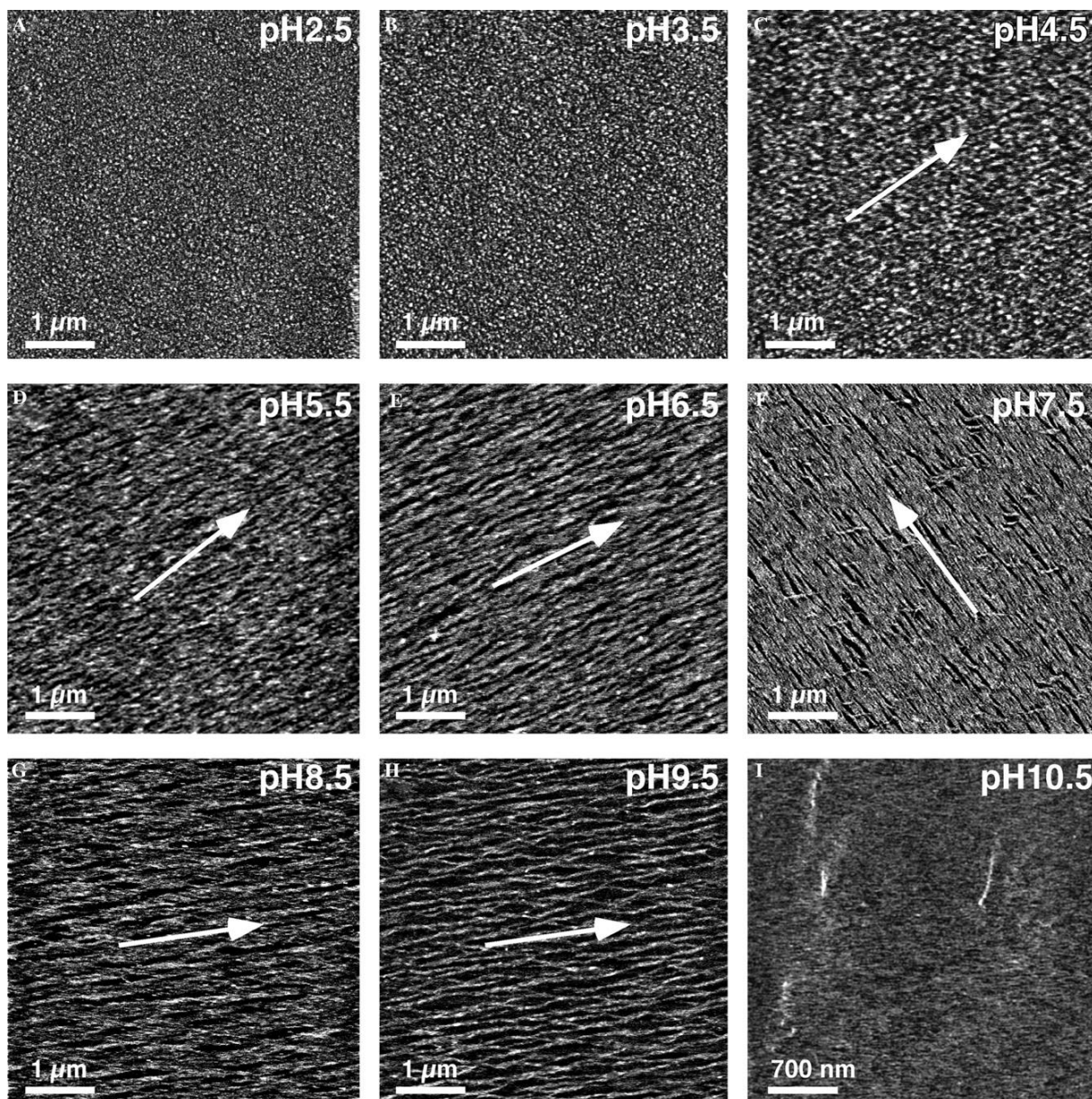
Figure 5: Collagen assembled at different pH values. *Arrows show the direction for which the buffer solution with collagen was guided over the surface (flow direction). Collagen assembled at pH values ranging from 2,5 to 10,5. At pH 2,5 and 3,5 the collagen assembled to the surface as globular protrusions. Increasing the pH to 4,5 resulted in elongated globules. For pH values between 5,5 and 9,5 a surface covered with fibrillar structure were obtained. At pH 10,5 only a few fibers were found at the surface [7].*

1.5 Dynamic phase transition in cell spreading

A study of cells spreading (adhering) on ECM covered substrates showed that cells go through three different phases during this process. In each of the phases the cells were spreading with different speed. A power law describing the growth area in the different phases was invented. As the cell is spreading in phase i the area growth is described:

(1) $\ln(A(t)) = a_i \ln(t)$

Here t is the time of spreading and ai is the growth exponent in phase i. The first phase was denoted the basal phase. In this phase the cells are testing the suitability of the surface, and the spreading is rather slow ($a_1 = 0,4 \pm 0,2$). The basal phase is followed by the continuous phase were the spreading is significantly faster($a_2 = 1,6 \pm 0,9$). The final phase is named the contractile phase and here the cell spreading slows down again $a_3 = 0,3 \pm 0,2$ [9].

1.6 Bovine Serum Albumin (BSA)

Bovine serum albumin (BSA) is a protein present in blood. It can adsorb on hydrophilic and hydrophobic surfaces (eventually with conformation changes). It is commonly used in immuno-stain protocols to prevent unspecific interactions between anti-bodies and proteins and in general between proteins. When working with a suspension of a specific and purified protein, it can be added in the suspension in order to prevent and minimize adsorption (and consequently loss) of the purified protein onto tube and pipette-tip surface. The primary structure of BSA is a sequence of 580 amino acids and its secondary structure is made at 54% of α-helixes and 40% β-sheets. The overall shape of a BSA molecule is globular with the following dimensions: 14 x 4 x 4 nm^3. BSA belongs to the class of soft proteins, which can easily change their structure and conformation [10]. It can be used to prevent cell adhesion to surface by preventing the adsorption of other proteins that could mediate cell adhesion to surface [11].
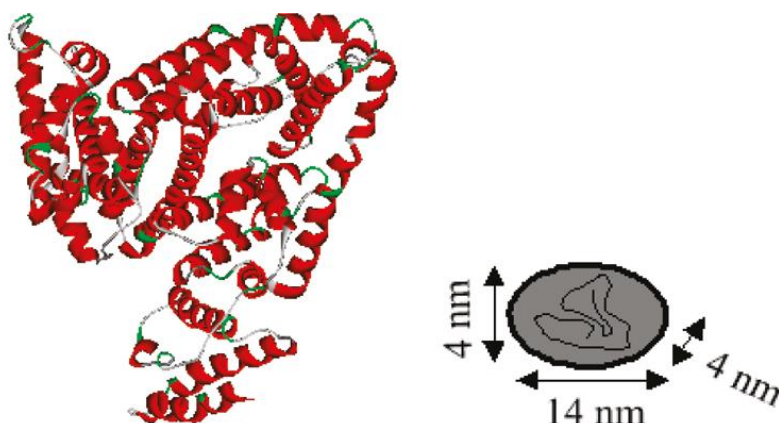

Figure 6: Structure of bovine serum albumin (BSA). *The BSA molecule is built up of α-helixes (left) fold in a globular tertiary structure (right) [10].*

2.0 Techniques

2.1. Atomic force microscopy (AFM)

The last 30 years, the development of various scanning-probe microscopy techniques and especially atomic force microscopy (AFM), has revolutionized the world of microscopy and the surface characterization at nanoscale. In comparison to electron microscopy, AFM allows imaging of non-conducting samples in air or in solution. AFM gives pieces of information about surface topography as well as local characterization of mechanical properties. In this project, one takes advantage of the molecular resolution imaging to characterize artificial matrices made of biological proteins such as BSA and collagen I. This section explains the working principle of the AFM and the repellent and attractive forces described by the Lenard-Jones potential. Further a step by step description of how imaging was performed in this project is provided.

A nanoscopic sharp tip attached to the end of a microscopic cantilever is moved across the surface to image. A typical diameter of the sharp tip is ~ 10nm. The cantilever is 150μm long, 35μm wide and a few micrometers thick. Through a system integrating a laser diode, a photodiode, a mirror, a piezoelectric actuator and an electronic controller for signal acquisition and a feedback signal generation, it is possible to detect cantilever deflection and to adjust the cantilever deflection by varying the distance between the surface and the nanoscopic tip interacting with the sample surface. The short-distance interactions between the AFM tip and the nanoscopic structure of the close-by area induce cantilever deflection. From this controlled nanoscopic interaction and by scanning the sample surface, it is possible to get topographical information about the sample. The laser beam hits the cantilever over the tip, and is reflected on a mirror and further on to the photodiode. This photodiode works as a position sensitive detector:

nanoscopic position changes are detected. According to the imaging mode chosen, the controller adjusts voltage applied on the piezoelectric crystal to move the cantilever up and down depending on the measured cantilever deflection. A piezoelectric crystal dilates and contracts under variation of applied voltage.
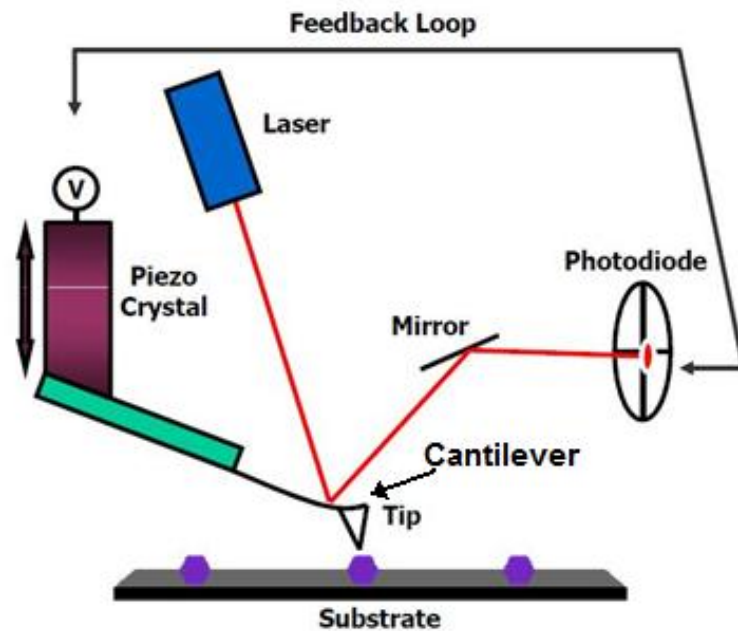

Figure 7: Schematic of the atomic force microscope system. The lasers position on the photodiode is translated into a feedback signal which change the voltage which is put on the piezo crystal and give us information about how the substrate look like. [12].

The Lennard - Jones potential

To understand the interaction between the tip and the surface it is crucial to understand which forces are present. When the AFM tip is interacting with the surface, it is essentially only one atom at the tip that is interacting with the surface. The Lennard- Jones potential is describing how the energy between two atoms is varying with the distance between them. Knowledge of this potential is a nice tool for understanding how the AFM works. The Lennard-Jones potential is given:

$$(2) \quad V(r) = -4\epsilon \left[\left(\frac{\sigma}{r}\right)^6 - \left(\frac{\sigma}{r}\right)^{12} \right]$$

Here ϵ is the depth of the potential wall, σ is the finite distance with no potential energy between the two particles and r is the distance between the particles. As seen by the formula we have two terms here. The first one r^{-12} is representing the repulsive effect, or Pauli repulsion which occurs when the atoms are very close. The r^{-6} term is caused by the longer ranged, weaker and attractive van der Waals force [13]. Note that the Lennard-Jones potential is only a model describing the energy between two atoms and not totally accurate. The Lennard-Jones potential is plotted against corresponding empirical values in Figure 8. This potential is very similar to the curve we get by plotting the interaction forces between the AFM tip and the surface. The interaction force as a function of distance between tip and sample is plotted in Figure 10 below.
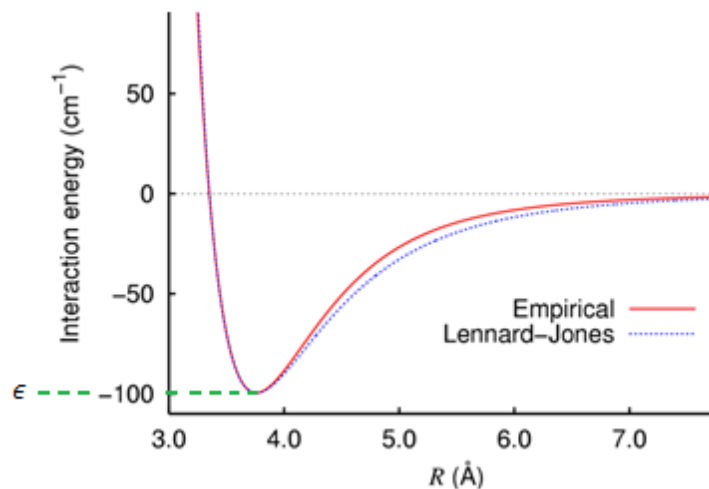

Figure 8: The Lennard - Jones potential plotted against corresponding empirical values [12].

Different modes can be used to image a surface. In **contact mode** which is the mode were the tip is closest to the surface, and the repulsive force is the domineering between the tip and the sample. There are several ways to measure topography in this mode. In constant height mode the cantilever deflection is measured as the sample is scanned horizontally (at constant height). If the sample surface investigated contains abrupt steps this could cause large deflections and thereby a large force which could damage the sample. This problem is avoided in the constant deflection mode. Here the height of the sample is adjusted to keep the deflection of the cantilever constant using the feedback loop. [14] Other modes used are based on a principle with a vibrating cantilever (AC modes). The cantilever is set to vibrate with a fixed frequency. As the tip distance to the sample surface change, so will the forces between the tip and the sample. This induces a change in the cantilever-frequency which can be recorded to obtain information about the sample. The mode with a vibrating cantilever was originally used with a relatively large tip-surface distance (**non contact mode**). Later vibrating tips were also used successfully closer to the surface. In **tapping mode** the vibrating tip is actually touching the surface [15]. In tapping mode the tips amplitude is heavily increased compared to non contact mode. Tapping mode is the mode used in this project. Very recently a technique is developed using both the first and second order resonance frequency of the cantilever. This technique is called **bimodal dual AC** imaging. The developers claim that this allows higher contrast and apply less force on the sample than the tapping mode.
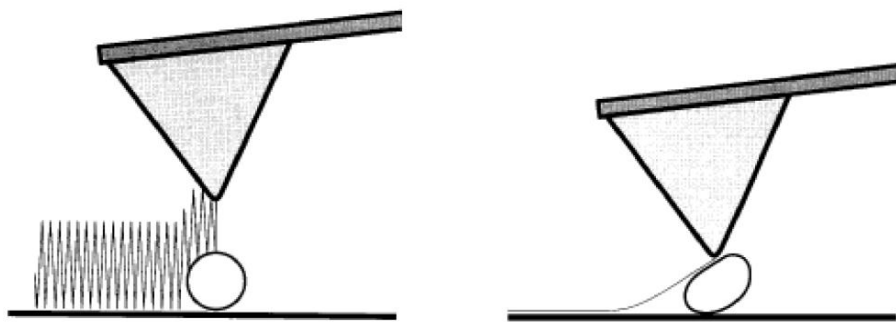
Figure 9: Schematic of tapping mode and contact mode. In tapping mode (left) a vibrating cantilever is used. In contact mode (right) the tips contact with the surface induces a deflection of the cantilever that provides information about the surface [16].
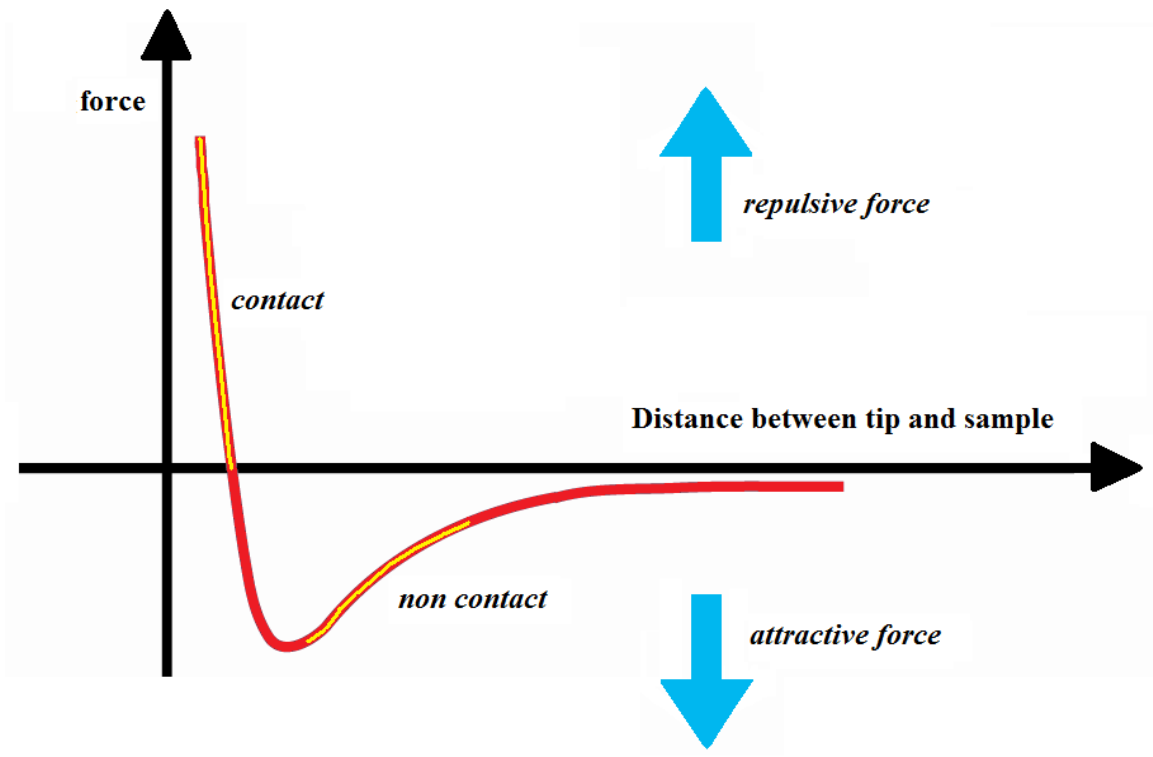
Figure 10: Force between tip and sample as a function of distance. *The areas of operation with contact mode and non-contact mode are marked on the graph. With AC mode we can work within both areas.*

The AFM used for this work is the Molecular Force Probe, MFP-3D from Asylum Research, California, USA . It consists of two different parts. First, the **head,** on which the cantilever is mounted. The head also contains the piezo element that moves the cantilever against the surface or away from it, manual mechanical adjustments of the laser-spot position on the cantilever and also to zero the deflection signal of the position sensitive detector (photodiode in Figure 7). The second part is the **base** on which the head is placed when imaging. The base contains the sample stage, which includes the piezo-electric scanner to move the sample during imaging and micrometric screws to manually move the sample stage in order to image at different locations at the sample. The base is placed on the top of an inverted microscope (Nikon).
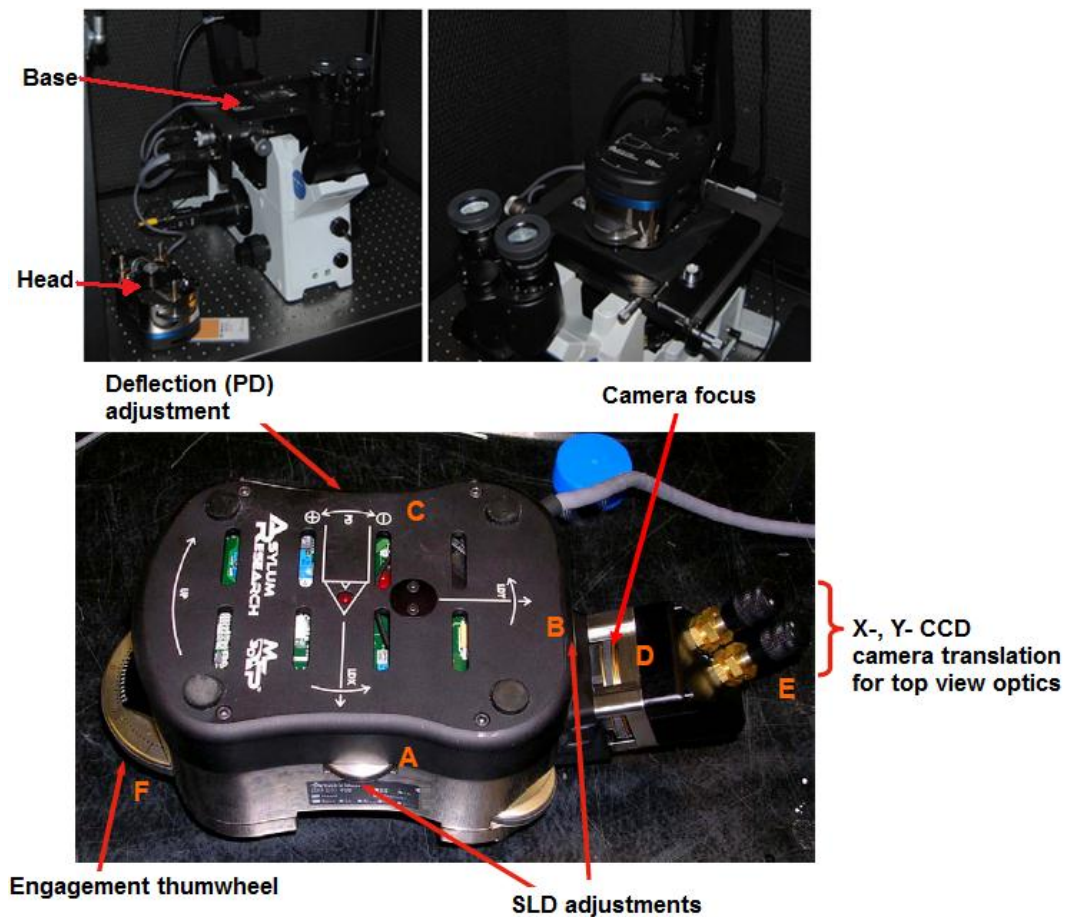
Figure 11: MFP-3D AFM from Asylum Research. The main parts of the AFM are the head and the base. As seen at the upper left picture those can be separated from each other. On the upper right picture the head are placed on the top of the base. This is the imaging position. At the bottom picture the different parts of the head are shown.

The cantilever needs to be calibrated before imaging. First, the MFP-3D software is started. Also the digital controller and the light for the optical microscope are turned on .The type of AFM tip used in this project is the Tap 300-G (Innovative solutions Bulgaria Ltd, Sofia, Bulgaria), with a resonance frequency about 300(±100) kHz , a force constant of 40N/m (range 20-75N/m), and a tip radius of less than 10nm [18]. The AFM tip is located at the extremity of a micro-cantilever, which has been fabricated at the border of millimetric chip. Tip, cantilever and chip together form the probe. The dimensions of the probes different parts are shown in Figure 12.
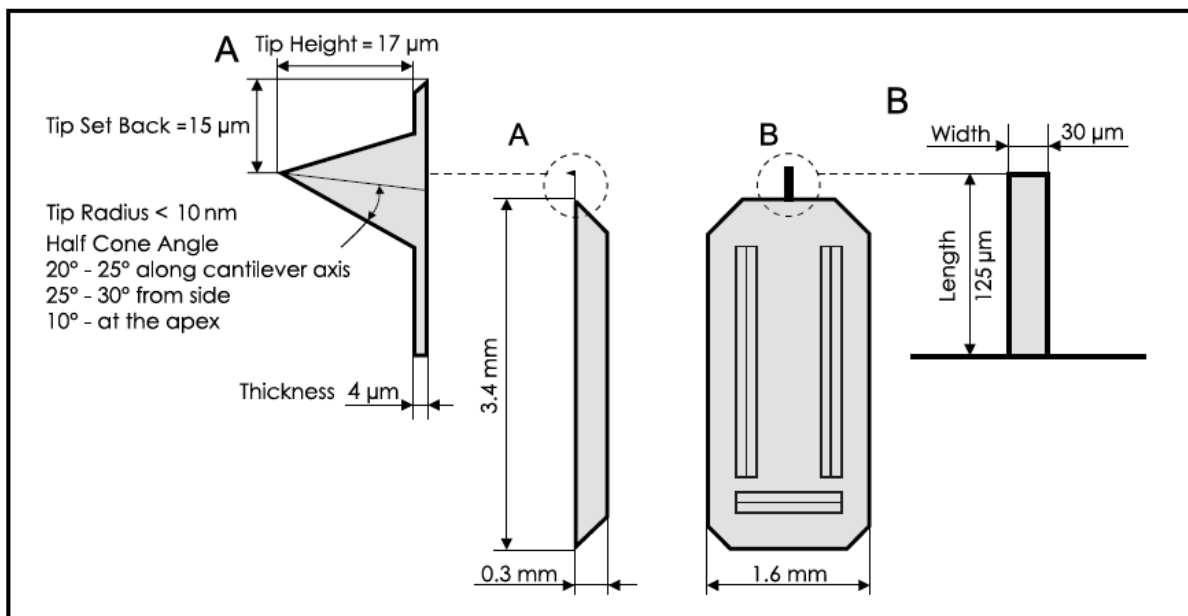
Figure 12: Dimensions of the tip cantilever and chip forming the probe. The schematic provides a view from the side (A) and the top (B)[18].

The probe is mounted on the probe holder. The probe holder is removed from the head and put on a probe holder stand to mount a new tip. This process of mounting/changing the tip is shown in Figure 13.
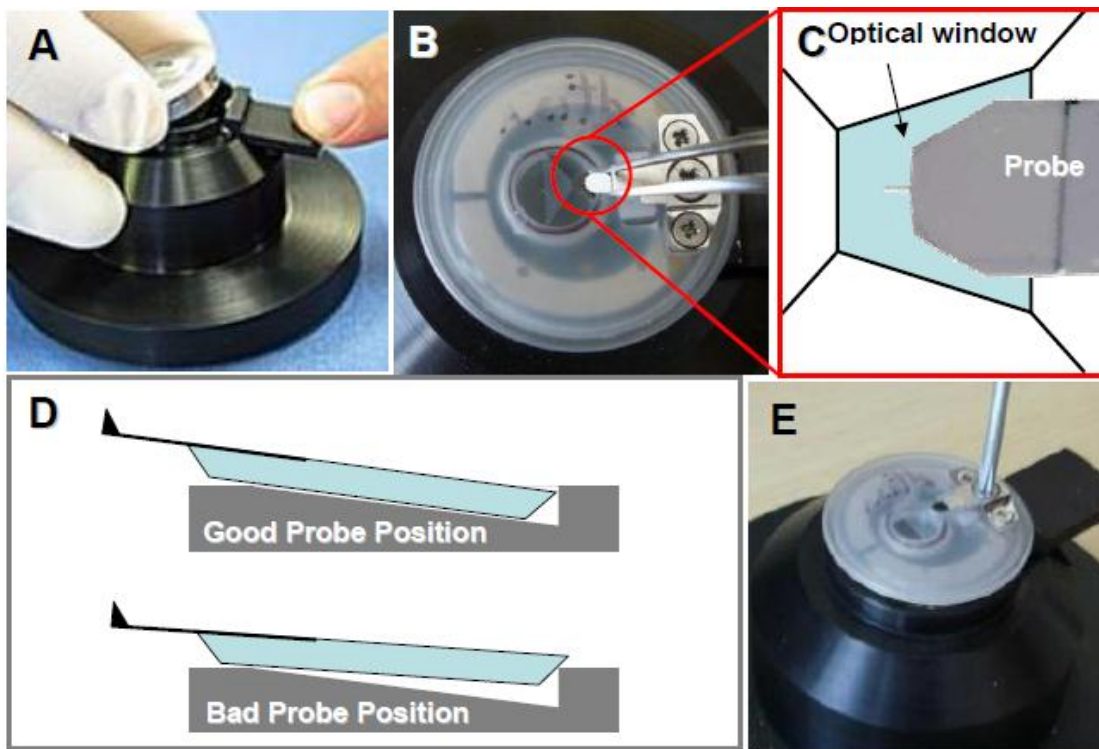
Figure 13: Mounting AFM tip. (A) Cantilever holder is put into the cantilever holder stand. (B)The tip is put on the right position using a tweezer. (C) Good probe position shown from above. (D) Good tip position from the side. (E) Screw is tightened. [AFM manual]

The sample is attached on a microscope slide using tape (see Figure 14) and the slide is placed on the sample stage. After, the AFM head is placed on the base as shown in Figure 11.
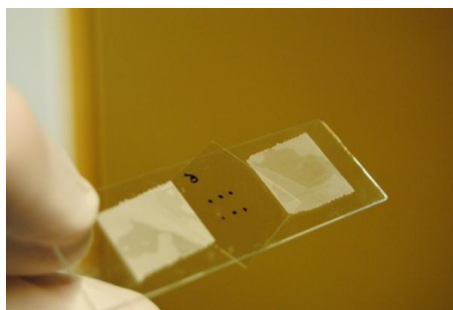
Figure 14: Glass cover slip (typical sample) taped on a microscope slide.

The next step is to align the laser beam on the cantilever. The orientation of a mirror located at the backside of the AFM head is adjusted in order to get an image of the cantilever on the camera chip. This image is displayed onto in computer screen. As the laser is turned on, one sees either the laser spot somewhere on the cantilever, a reflection of the laser spot and nothing. The XY adjustment screws enable to direct the laser beam onto the cantilever as shown in Figure 15. The "Sum" value in the "Sum and Deflection Meter" panel is maximized (usually around 3,5- 5V depending on the probes). Finally, the position of the photodiode (PD) is adjusted to set the deflection signal to zero. This is made with the adjustment screw located at the left side of the AFM head (see Figure 11).

Figure 15: **Laser spot on the cantilever.** *The picture is showing the optimized position of the laser spot on the cantilever [AFM manual]*

The AC mode is used for imaging sample in air during this project. The Auto Tune function of the software is first used to find the resonance frequency and to set the drive frequency and amplitude (it can be found in the Tune tab in the Master panel). The parameter "Target percent" is set to -5%, meaning that the drive frequency will be set at a frequency at the left side of the resonant peak and corresponding to a 5% lower amplitude than the resonance amplitude . Consequently, the cantilever should be excited in a regime where the repulsive forces dominate (Figure 10). It is important that the tip is relatively far from the sample surface when setting the working frequency so that the forces between tip and sample can be neglected. When the auto tuning is completed the "cantilever tune" window displays the results (Figure 17). The drive amplitude was set so that the target amplitude equal 1,0V. The next step is to engage the tip on the oscillating cantilever with the sample.

When approaching the sample the amplitude will start to be damped by the forces form the surface. A set point is selected before approaching. This is the amplitude for which the feedback – loop will kick in, and make sure that this particular amplitude is maintained. The set point is shown in the main tab of the master panel and was normally set to 800-950 mV during approach. When this amplitude is reached a sound is made by the software. Now you need to be very careful with further approach since this could cause the tip to crash into the sample. Before starting the imaging the set point was typically changed to around 600 mV. Another important parameter that should be mentioned is the integral gain shown in the main tab of the master panel. This parameter changes the responsiveness of the feedback loop. If this value is too low, the feedback can't track the surface very well, in which case an abrupt step could cause the tip to crash. If it on the other hand is too high, unwanted oscillations could appear and make distortion to our picture. Typically a value around 5 worked fine. The pixel density and the scan rate are also chosen in the same window. Pixel density is set to either 256 or 512 scan points and scan lines. Which density is chosen depends on the resolution needed. Higher numbers give higher resolution on the pictures, but it also takes longer time to image pictures with higher resolution. The Scan Rate is normally put at 1Hz. An increase of this number would make the picture faster to image, but also typically give less detail.
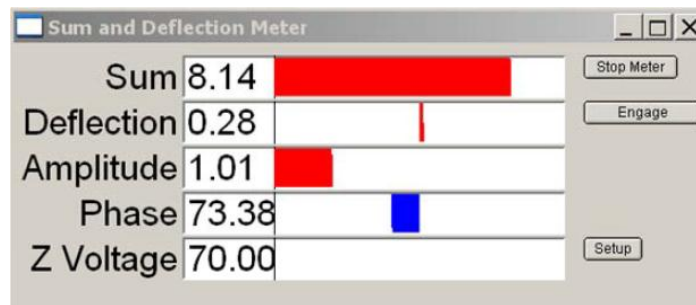
Figure 16 : *Sum and deflection meter from MFP-3D software.*
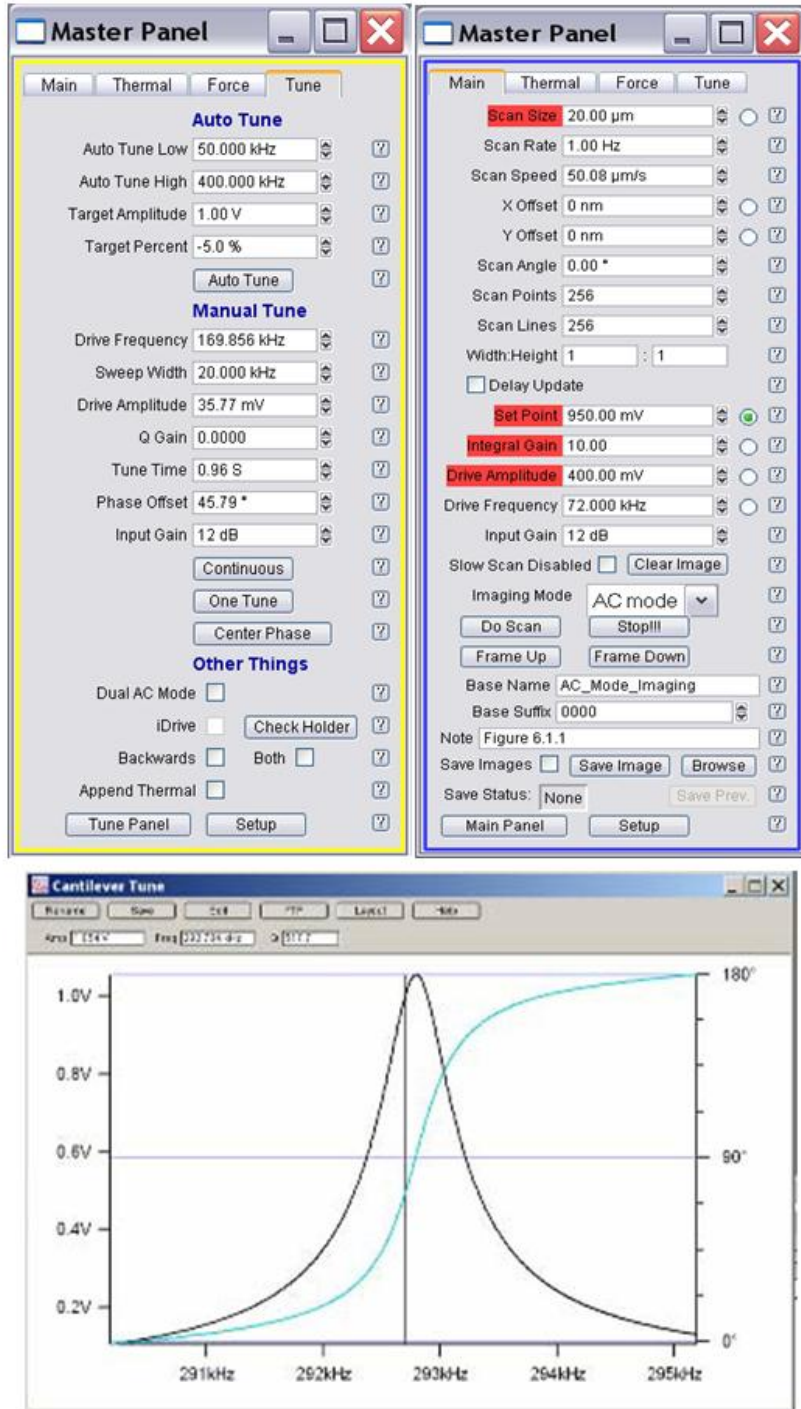
Figure 17: MFP-3D software. *Top left: Master panel of the software with Tune tab selected.*
Here we can select the working frequency of the tip. Top right: Master panel with the main tab
selected. This is where most important parameters are selected. Bottom: Tuning diagram for the
cantilever after frequency is selected.

When taking a picture with the AFM, automatically three different pictures show up on the screen. Those are height, phase and amplitude pictures. It is only the height pictures that have been used in this project. Range and offset are parameters that do not affect the imaging, but only the grey scale on the picture. They can be changed after the picture is taken. It is very important to choose those parameters so they are suitable for the details you want to point out. In Figure 18 is an example of how it is sometimes not possible to get all the information needed with one picture. You then either need more than one picture or decide which details are priorities.
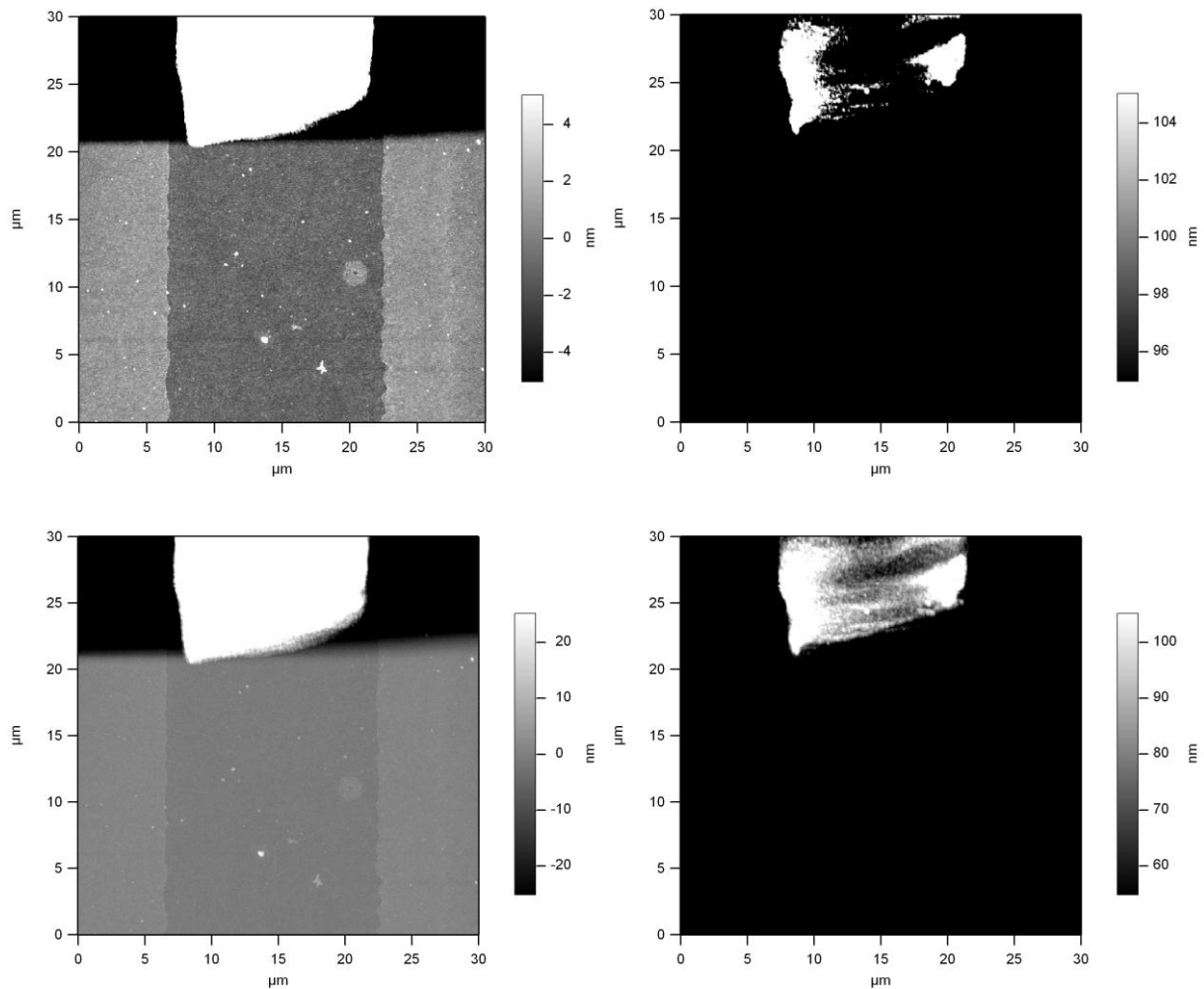
Figure 18: The same AFM picture with different range and offset. Upper left: offset is 0 and range is 10nm. Upper right: offset is 100nm and range is 10nm. Lower left: Offset is 0 and range is 50nm. Lower right: Offset is 100nm and range is 50nm.

2.2 Deposition of BSA by micro contact printing documented by AFM

In this work, surface coating with bovine serum albumin (BSA) is used to generate a protein-repellent surface and especially repellent for proteins of the extracellular matrix (ECM-proteins), which mediate cell adhesion to substrate. Collagen I is the ECM-protein used here to fabricate micro-areas on which cells adhere and spread. Series of parallel BSA micro-stripes (microscopic width and millimetric length) on a surface are fabricated and the micro-gaps between them are used to depose collagen I assembly in different manner. We have then a substrate presenting strong variations of adhesive interactions between cells and substrate at the microscopic scale. The patterned surface is large enough ($\sim 25\ mm^2$) to study simultaneously a population of single cells. The fabrication technique used to fabricate BSA micro-stripes is based on the so-called "micro-contact printing" technique and is described in this section. Efforts have been placed in optimizing the technique.

2.2.1 Fabrication of stamp for micro-contact printing

Micro contact printing belongs to the soft lithography techniques. Most of these techniques use polydimethylsiloxane (PDMS) or other polymers with similar characteristics as material to fabricate microstructures. PDMS is often chosen to fabricate microstructures to be used with living cells. PDMS is an elastomeric material with biocompatible properties, chemically rather non reactive with aqueous solutions, transparent, permeable to gases and only moderately permeable to water [20]. For micro-contact printing applications, PDMS is used to fabricate stamps with microstructures. These stamps can be inked with molecules (adsorption of molecules on PDMS surface). During stamping, micro-patterns of molecules are transferred on the substrate. PMDS stamps are negative replicate of a micro-structured master made by photolithography. The fabrication process of the master is illustrated in Figure 19.
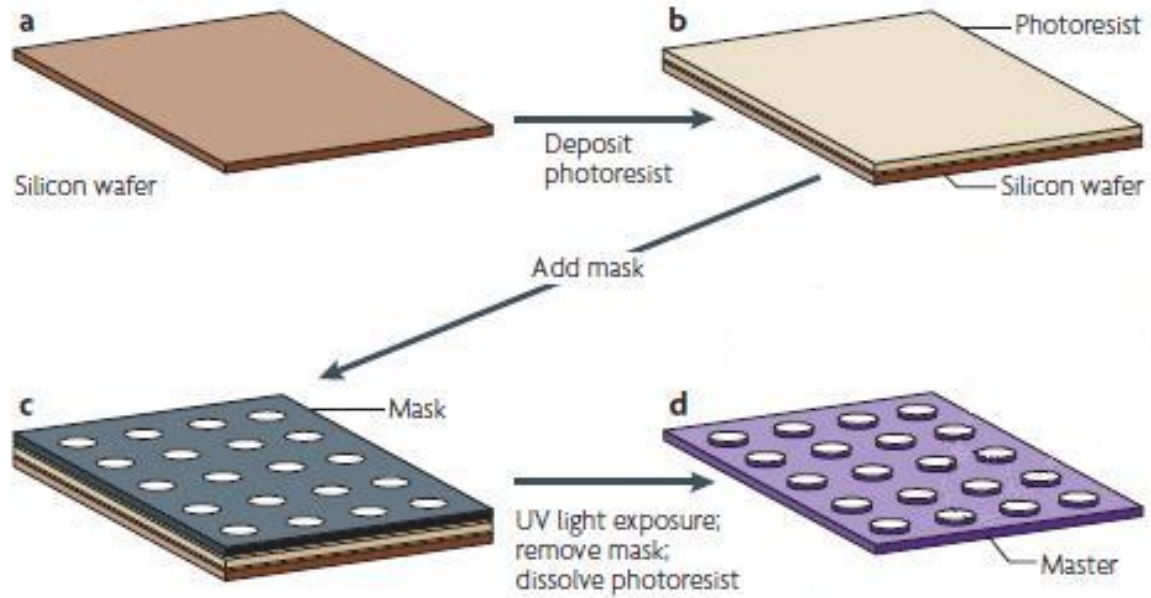
Figure 19: Principle in the making of the master used to replicate structures in PDMS. Note that the pattern here are different from the one we use in this work [20].

The micro-pattern is drawn using a CAD (computer-aided-design) software and a mask is ordered to a producer. The mask is usually a fin chrome layer with holes corresponding to the micro-pattern. With such a mask, the photolithography process can be implemented as briefly described here. First a photoresist is spin coated on a silicon wafer to form a microscopic layer. Then the mask is placed on top of the photoresist layer and UV light is used to expose the photoresist layer through the mask, i.e. the photoresist is exposed only on micro-area corresponding to the designed pattern. Then the sample is baked to induce local crosslink within the UV exposed areas in the case of a negative photoresist. The appropriated organic solvent dissolves the photoresist that is not cross-linked and as a result, the designed micro-pattern has been transferred in 3D-microstructres. The piece of silicon wafer with photoresist micro-structures is called master or mold.

The PDMS oil is mixed with a curing agent at a ratio 1:10 (curing agent:PDMS). Crosslinking occurs in 2 hours at 65°C and occasionally samples have been overnight at 65°C. After curing, the resulting material is transparent and elastic. The elastomeric surface is hydrophobic. PDMS stamps used in this project have a thickness of either 1mm or 3mm.

To reproduce the micro-pattern of the master in a PDMS layer, the PDMS mixture is added in a petri dish. This petri dish is put in vacuum for 30 minutes to be degassed. Before the master is put in contact with the PDMS mixture, spacers are placed on the boarders of the master as seen in Figure 20. The spacers are cured PDMS pieces of thickness adjusted to 1 or 3mm. The master is then placed on the PDMS mixture and gently pressed against the petri-dish bottom. First contact of the master with PDMS mixture is done with a slight angle to minimize the air-bubble trapping and then the petri dish is placed in vacuum again to remove air bubbles that may still have formed. Finally, the petri dish is placed at 65°C. After curing, the PDMS layer is peeled off from the master and micro-structures have been replicated at the PDMS-layer surface.
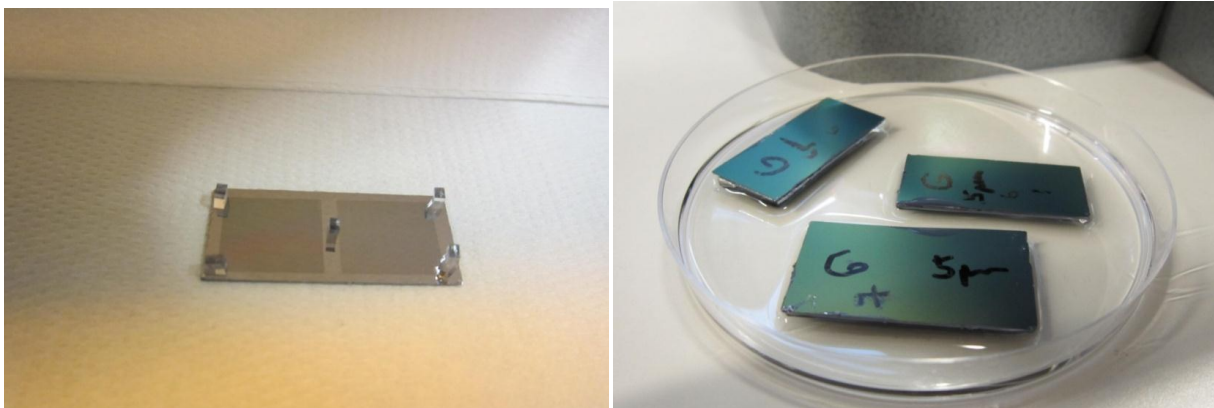
Figure 20: Photograph of master during fabrication of PDMS stamps. Left: Master with 3mm spacers. Right: Master in contact with freshly mixed PDMS oil and curing agent.
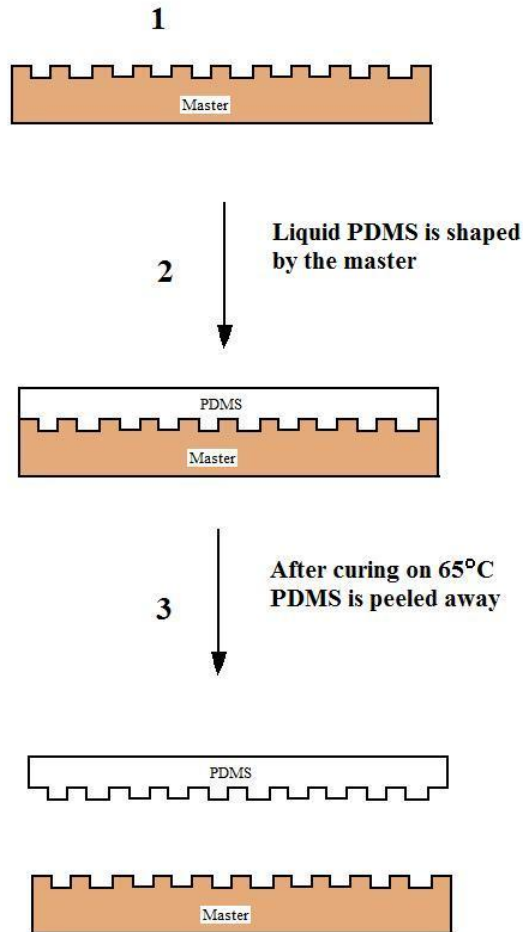
Figure 21: Fabrication process of polydimethylsiloxane (PDMS) stamps. *PDMS silicon oil is shaped by the master. After curing at 65℃ for two hours the PDMS is peeled of, and we have a PDMS-stamp with the replicated structure of the master.*

This micro-structured PDMS layer is now ready to be used as a stamp. First, the stamp has to be inked with a solution containing molecules, such as for example proteins, and dried quickly with nitrogen gas. When placed in contact with a surface, molecules at the upper parts of the stamp are transferred onto the surface. Figure 22 illustrates the fabrication process.
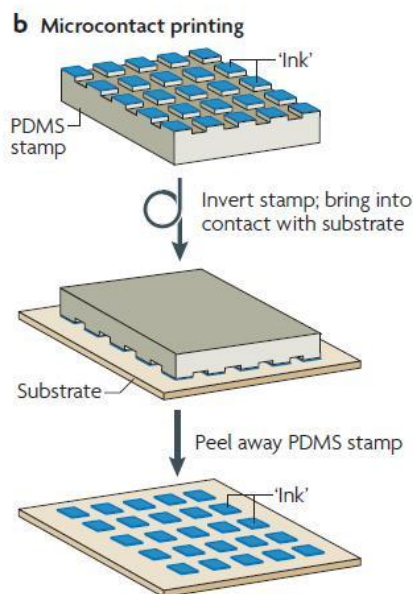
Figure 22: Micro-contact-printing process. *The stamp is inked (typically with molecules) and the pattern from the stamp is transferred to the substrate [20].*

2.2.2 Micro-contact printing of BSA stripes

Bovine Serum Albumin (BSA) coating by micro-contact printing is used to make a protein-repellent surface. BSA is known to minimize protein adsorption and consequently prevent cell spreading [11]. Parallel BSA micro-stripes are micro-contact printed and afterwards the whole sample surface is incubated collagen I. Other ECM proteins could also have been used. Cells will attach and spread on the micro-area, for instance micro-stripes, coated with collagen I. The geometry of a micro-stripe was chosen because it mimics to some extend in vivo cell confinement, but also the cell confinement on the stripe shapes the cell body and the quantification of the cell spreading is more straightforward. A typical sample surface on which cells will be platted is depicted in Figure 23.
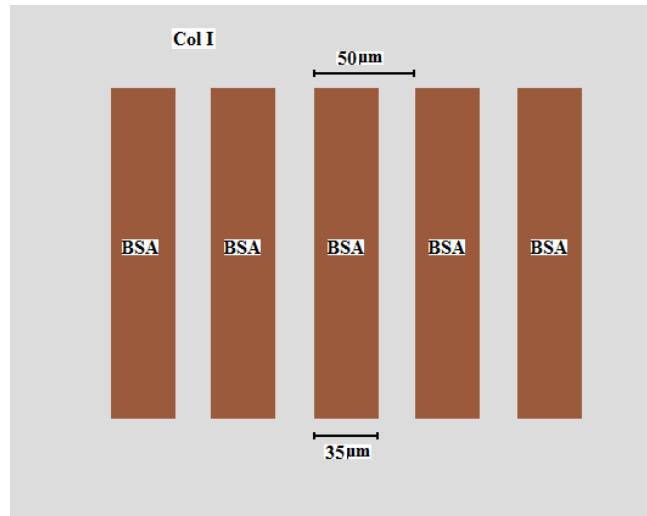
Figure 23: Schematic of the sample surface on which cells will be plated. *Grey area represents the area coated with collagen and the brown area represents area coated with BSA.*

Sample preparation

The PDMS stamp is made of parallel ridges with a width of 35μm. The distance between the center of one ridge to the next one is 50μm. The substrate is a 24mm x 24mm glass cover slip. First the substrate has to be aminosilanated to fix covalently amino-groups on the glass surface. A protocol describing this can be found in appendix. Aminosilanated cover slips were stored up to two weeks during this work. Aminosilanated cover slips are incubated with glutaraldehyde resulting in glass surface presenting aldehyde groups (glutaraldehyde contains two aldehyde groups: one reacts and binds one amino group at the surface and the second is available for further reactions, but likely not with the surface). This incubation is done by injecting 200μl of glutaraldehyde solution between two cover slips, and let those incubate for 15 minutes in the solution. They are rinsed 3 times with 1ml distilled water (again by injection between both cover slips) and finally they are separated and dried with nitrogen gas. Figure 24 illustrates this process.
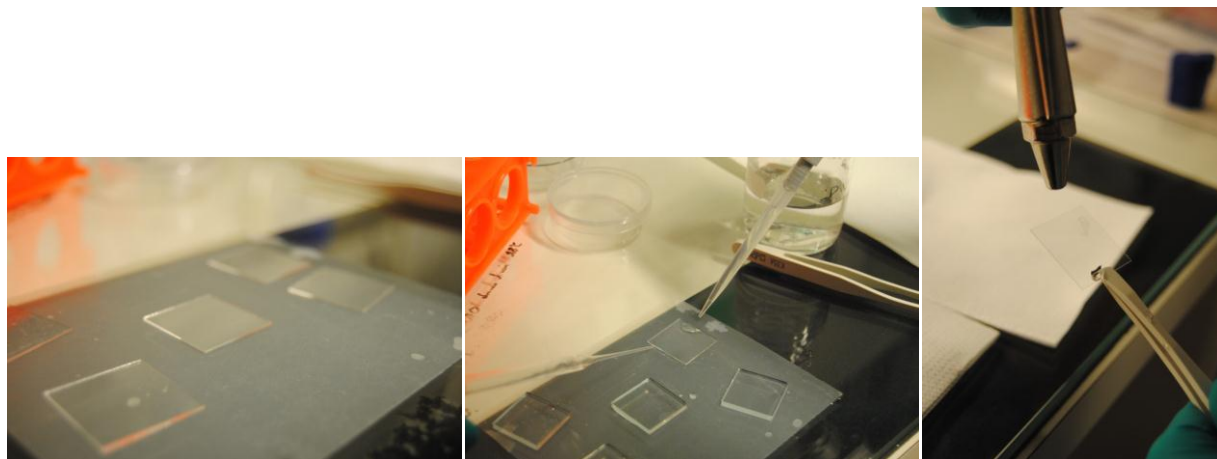
Figure 24: Photographs of incubation, rinsing and drying steps during sample preparation.
Left: Pairs of cover slips are incubated with glutaraldehyde. Middle: Cover slips are rinsed with
distilled water. Distilled water is injected at one side and aspirated away on the other. This is
repeated six times for each pair. Bottom: A cover slip is dried using nitrogen gas. The best is to
direct the flow towards the tweezer as in the picture. This is because the tweezer potentially can
contain some dust that could contaminate the sample.

Preparation of PMDS stamps and micro-contact printing of BSA

The PDMS stamps are cut into rectangular pieces, and then place under vacuum for 30 minutes.
Afterwards each of them is incubated on the top of a 200µl BSA solution droplet laying on
parafilm®. Since the stamp have been degassed, if air bubbles form at the stamp surface in the
solution, it is likely that the air will diffuse in the PDMS and the bubbles will disappear, leaving
access to the surface for the solution and consequently the BSA. This favours uniform BSA
coating. Then, stamps are rinsed with distilled water and finally dried. The PDMS stamps can
now carefully be stamped on the prepared cover slips (see previous section). The stamps are left
in contact with the cover slips for 30 minutes. During this time, BSA molecules from stamp
ridges covalently bind to the cover-slip surface. When the stamp is removed, BSA micro-stripes
(with a width equal to the ridge width) remain on the cover slip. The samples are first rinsed with

PBS and then distilled water before to be dried Figure 26 shows an AFM micrograph of BSA stripes fabricated as described above.

Figure 25*: Top: PDMS - stamps incubating on top of droplets with BSA. Bottom: PDMS - stamps incubating on cover slips covered with glutaraldehyde. Black dots are drawn on the back side of the slips so the position can be found after the stamps are removed*
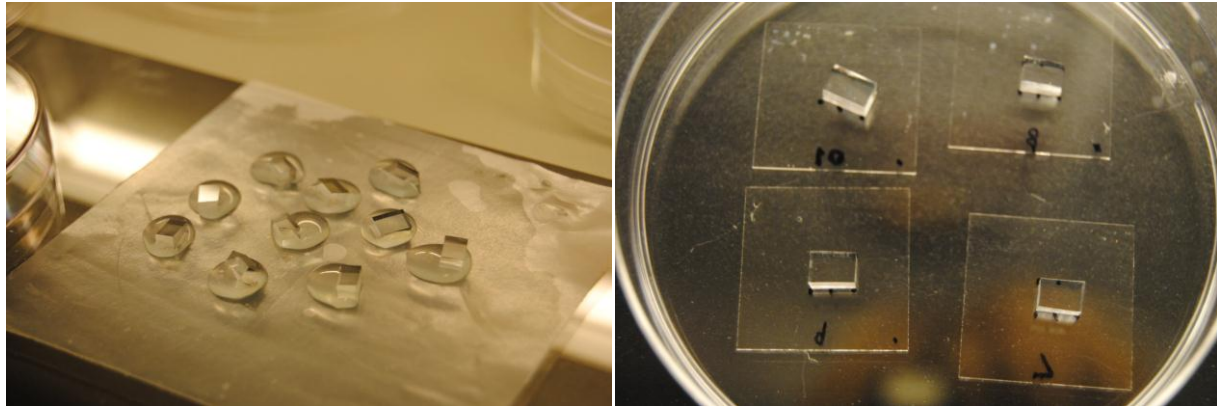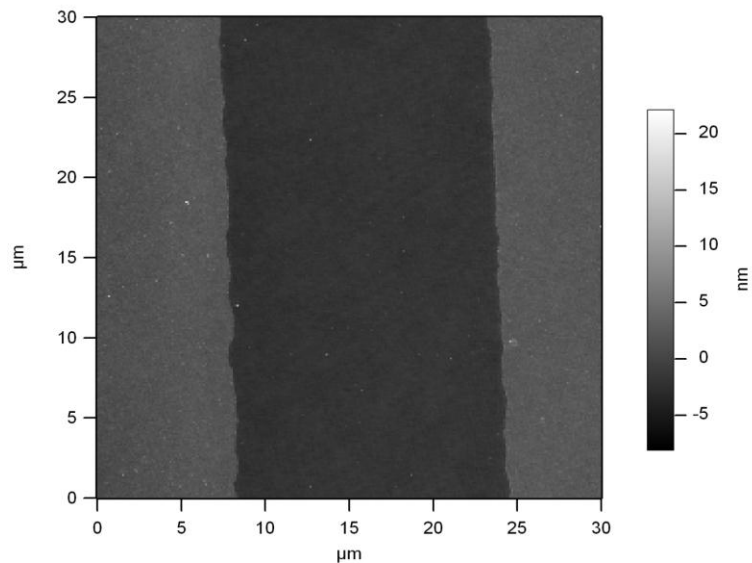

Figure 26: AFM micrograph of micro-contact printed BSA micro-areas. *. The15μm dark stripe in the middle is without BSA and the boarders are coated with BSA.*

2.2.3 Optimization of micro-contact printing

An important point for this thesis was to characterize with AFM imaging and based on the knowledge to optimize the deposition of BSA on the substrate for applications to the study of cell spreading. Obviously, a dense and uniform covering is wished. Different variations of the micro-contact printing protocol described above have been implemented: first the hydrophilicity and hydrophobicity of the stamp has been considered, second the rinsing time of stamps, and third the concentration of glutaraldehyde used to crosslink the BSA to the surface.

Hydrophilicity and hydrophobicity of the PDMS stamp surface

Jeyachandran et al. have published a study of BSA molecules adsorption from phosphate-buffered saline (PBS) to hydrophilic and hydrophobic surfaces. They have concluded that BSA adsorbed with stronger interactions on the hydrophilic surface. The same study shows that BSA adsorption saturates at around 50% of surface coverage on the hydrophobic surface, whereas for the hydrophilic surface the BSA covered 95% [10]. In light of this, it is interesting to investigate how the BSA transfer during stamping could be affected The PDMS surface is originally hydrophobic, but an oxygen-plasma treatment makes the PDMS surface hydrophobic. A set of samples, made using both hydrophobic and hydrophilic stamps was prepared to compare the deposition of BSA. Pictures were taken with the AFM. The height of the BSA layer was measured using the MFP-3D software (see "uncertainty section"). AFM micrographs of the both kind of samples are shown in Figure 27. Since the grey scale representing the height is the same for both pictures, it is clear that the BSA layer made using hydrophobic PDMS is higher than the one made with hydrophobic PDMS.
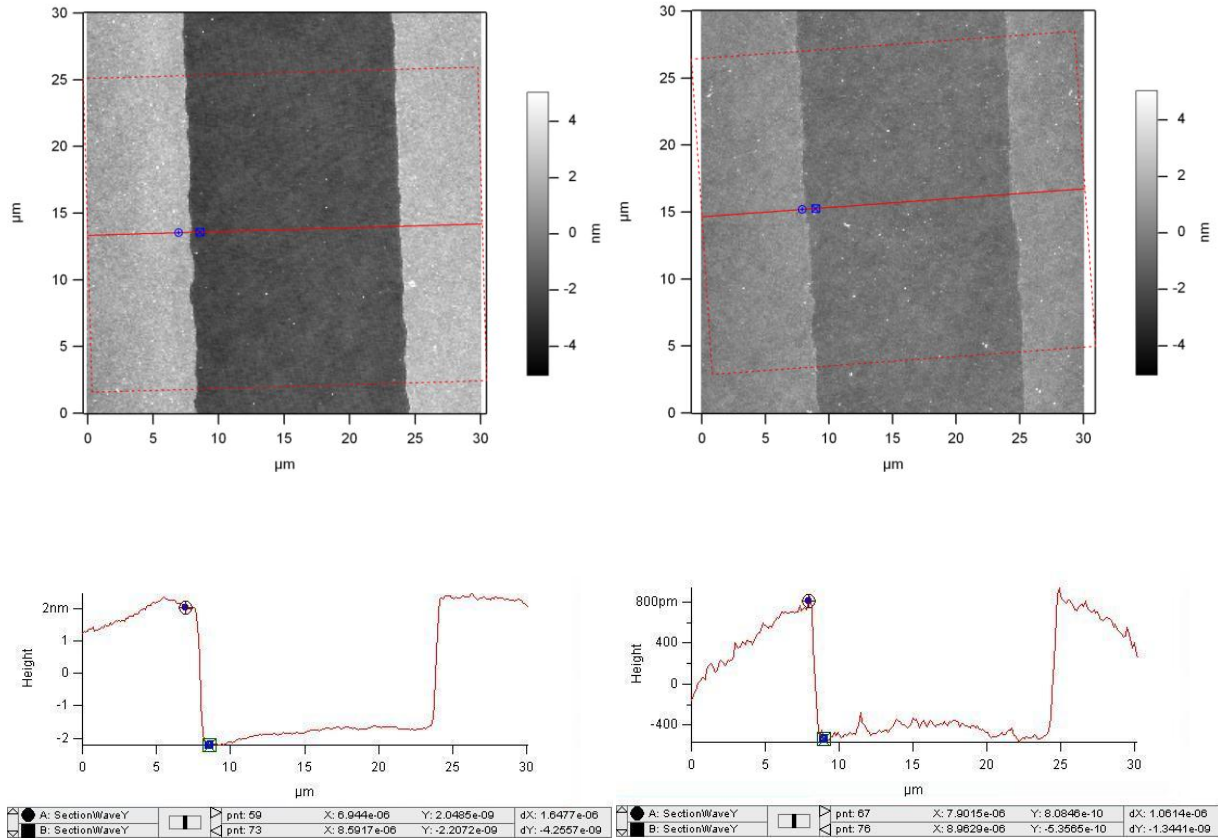
Figure 27 AFM pictures of BSA stripes stamped with hydrophobic and hydrophilic PDMS stamps. Right: The stripe in the middle is without BSA and the boarders contain BSA. The stamp was hydrophilic. Left: The stripe in the middle is without BSA and the boarders contain BSA. The stamp was hydrophobic. Both left and right: The difference in height between the BSA layer and the part without BSA are measured with the analyse panel in the MFP-3D software (graphs below each picture). The vertical span between the cursors in each of the graphs indicates the corresponding height of the BSA layer.

Rinsing of stamps

During the rinsing of the PDMS stamps on which BSA is adsorbed, BSA could possibly desorb. Different periods of time of rinsing have been tried to investigate the influence on BSA transfer. The first protocol is as follow. After incubation   for 15 minutes on the BSA droplets, stamps are put in a small petri dish with the patterned side facing up. Immediately, a large volume (5-10 ml) of distilled water is poured over the stamp to cover it.   And immediately again, the water is poured away, resting droplet aspirated and the stamp is dried with nitrogen gas and placed in contact with the sample. All this occurs within approx 30-60s, meaning that the sample was a few seconds immersed in water.   To investigate the effect of the rinsing step on the BSA transfer the following durations of rinsing have been used: 0s, a few seconds and 30min. This was done both for the hydrophilic and hydrophobic PDMS stamps. As it can be seen in figure 10a, when the hydrophobic stamp is not rinsed, the BSA deposition is not homogeneous.   With hydrophilic stamps, the absence of rinsing does not seem to impact strongly the BSA transfer compare to the stamp rinsed for a few seconds. The transfer of BSA was similar after a few seconds or 30 min rinsing. The rinsing step does not seem to play a significant role, but it is needed especially in the case of hydrophobic PMDS stamps. A short rinsing time of a few seconds was chosen as a standard.
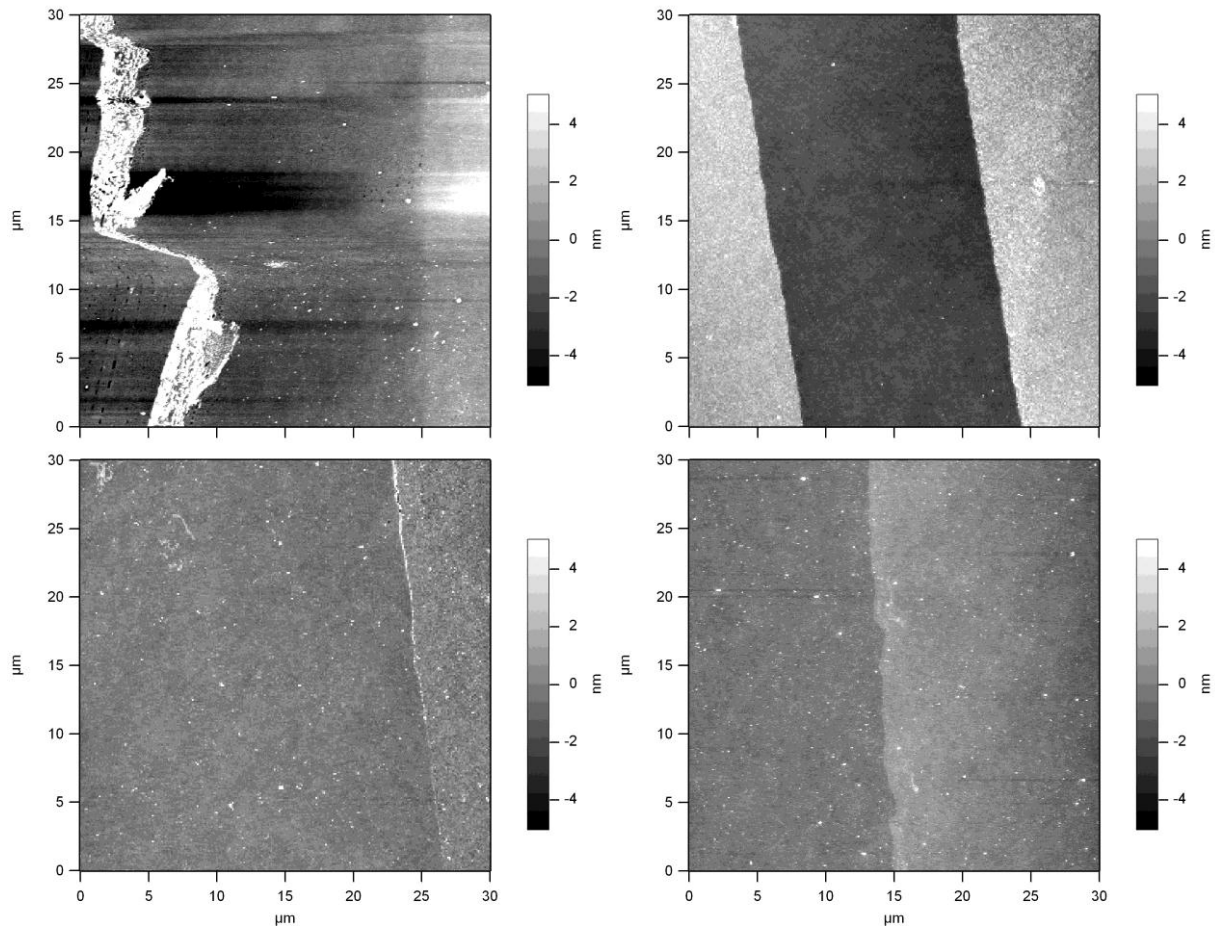
Figure 28: AFM micrographs of micro-contact-printed BSA by PDMS stamps prepared differently: effect of rinsing. *Top left: Cover-slips stamped with hydrophilic PDMS. The PDMS - stamp was rinsed 30 minutes in distilled water before stamping. Top right: Cover-slip surface stamped with hydrophilic PDMS. No rinsing with water after incubation with BSA. Bottom left: Cover-slip surface stamped with hydrophilic PDMS. No rinsing with water after incubation with BSA. Bottom right: Cover-slips stamped with hydrophilic PDMS. The PDMS - stamp was rinsed 30 minutes in distilled water before stamping.*

Glutaraldehyde concentration

In this work, glutaraldehyde is used as cross-linking agent to bind BSA to aminosilanated cover-slips. Even if glutaraldehyde is commonly used in biotechnologies to crosslink or to immobilize proteins, its reaction with primary amines of proteins is complex. It depends on the solution pH, protein structure, temperature and reagent concentrations. In 2004, in a review article, Migneault et al. highlighted that there is no single mechanism responsible for interaction of glutaraldehyde with proteins [21]. Potentially the concentration of glutaraldehyde could play an important role in the way the BSA molecules bind to the surface. The viscous solution of glutaraldehyde at 50% is diluted in phosphate buffer saline (PBS) and these diluted solutions are used to incubate samples. The following concentrations have been used: 0,625%, 1,25% and 2,5% glutaraldehyde in the PBS. The samples were then imaged with AFM to evaluate to quality of BSA transfer.

2.2.4 BSA layer underlying the micro-contact printed BSA layer.

In general, the transfer of proteins during micro-contact printing is more efficient, if the single proteins or the dense protein layer on the stamp are still hydrated. It limits also protein denaturation, which occurs anyway during interaction between a protein and a surface, but at different degrees depending on the protein structure. To increase water contents at the interface during micro-contact printing, BSA was homogeneously adsorbed on glass cover slips. The adsorption is made from BSA in PBS solution onto an aminosilanated glass cover slip previously incubated with glutaraldehyde. A layer of BSA, called "underlying BSA layer", is consequently covalently linked to the glass. Then, samples with and without underlying BSA layer are incubated with glutaraldehyde and BSA is micro-contact printed on them.

 AFM pictures of the micro-contact printed BSA layer are taken. As it can be seen in figure 14, the BSA coverage appears to be more homogeneous with the underlying BSA layer. Without it, nanometric holes can be seen in the BSA layer (black spots in Figure 14, left).
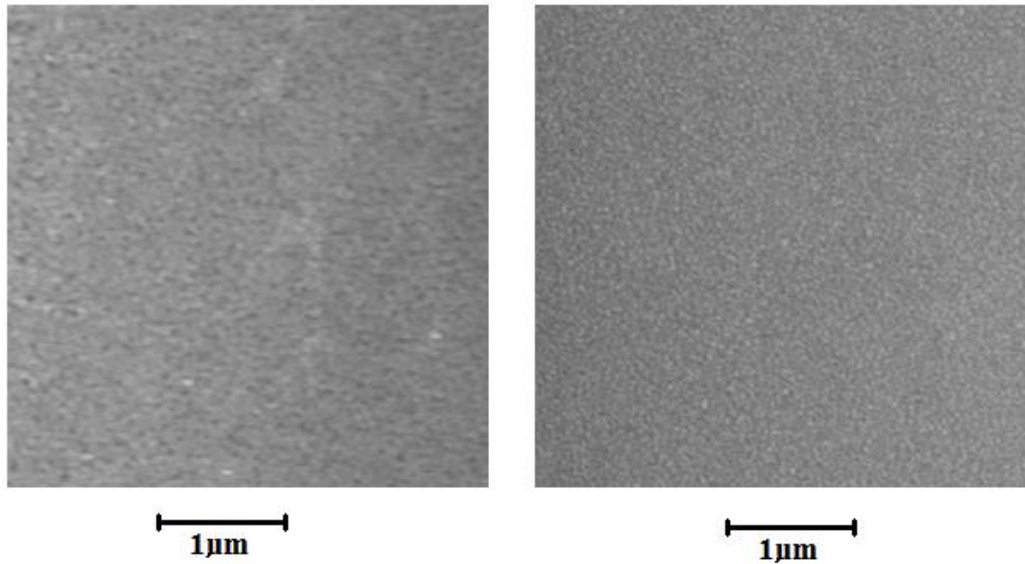
Figure 14: AFM pictures of BSA micro-contact printed areas. Left: micro-contact printing on aminosilanated glass cover slip pre-incubated with glutaraldehyde Right: micro-contact printing on underlying BSA layer pre-incubated with glutaraldehyde.

In conclusion, the concentration of glutaraldehyde did not affect the sample fabrication significantly. The conditions leading to an apparently denser and homogeneous BSA layer after micro-contact printing were chosen: use of hydrophobic PDMS stamp and of an underlying BSA layer. The glutaraldehyde was used at 2,5% in PBS solution.

2.3 Collagen deposition

In order to obtain samples that alternate micro-stripes coated with BSA (micro-contact printed) and collagen I as described in Figure 23, it was necessary to incubate samples with collagen I solutions after or during micro-contact printing. The PMDS stamp can be removed and the sample can be incubated with a collagen I solution. But there is a second alternative: when the PDMS stamp is still in contact with the sample (a BSA layer linking the stamp and the sample during printing), the stamp forms micro-channels on the top of the micro-areas that needs to be covered with collagen I assembly. Collagen I solution can be injected in these micro-channels to incubate the surface. Various approaches can be developed to fabricate collagen I assembly with different structures. Of importance is that the collagen I monomers can polymerize under appropriated conditions. The stock solution of collagen type I is a solution at pH 2 and is stored at 4°C. Both pH and temperature influence collagen polymerization. Polymerization occurs in the following pH range: 6,5 - 8,5 and at temperatures between 20°C and 37°C [22]. To induce collagen I polymerization and assembly, the pH of the stock solution needs to be increased to reach a value within the appropriated pH range. The increase of pH was achieved in different ways: by dilution or assisted by the PMDS stamp playing the role of a micro-system to regulate and control pH.

2.3.1 Preparing samples with collagen from a PBS solution

The stock solution of collagen was diluted in a buffer solution, phosphate buffered saline (PBS), which has a pH of 7,4. The pH of the collagen I solutions was adjusted to be 7,4. In order to incubate a defined sample surface with a defined solution volume, PMDS chambers are used. The chamber is removed before AFM imaging. A circular punch was used to make a hole in a PDMS layer large enough to contain approximately 300µl of solution. Micro-contact printing is

made at the center of this chamber (see figure 29). The stamps can be removed after a solution has been added in the chamber. The PDMS stamp was removed carefully with a scalpel by lifting one side of the stamp progressively and without return to previous position. The PBS in the chamber was aspirated away and replaced by new PBS a couple of times to rinse. After rinsing, 200µl of PBS was left in the chamber. Then a 1:10 solution of collagen solution and PBS was prepared in a small tube, and quickly after preparation 100µl of this solution was added to the chamber by falling droplets at the center of the chamber (see Figure 30).
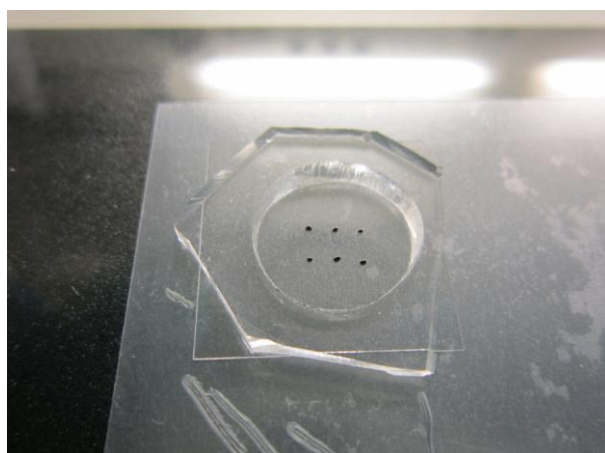
Figure 29: A circular PDMS chamber surrounding the sample surface. The black dots are used as guidelines for imaging.
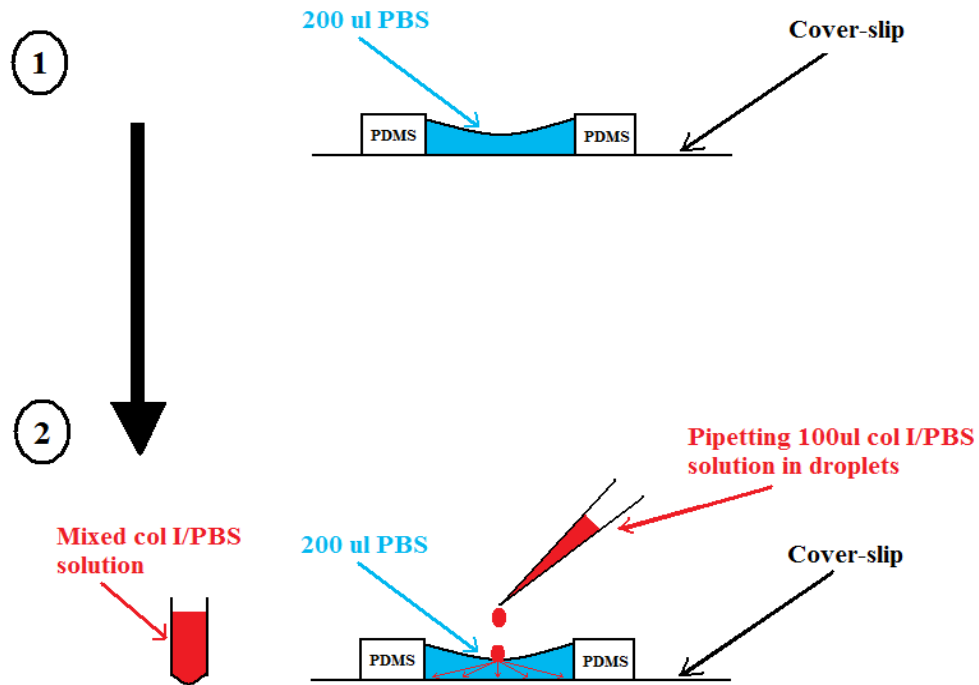
Figure 30: Illustration describing deposition of collagen using a dilution of the protein in PBS. 1: The chamber of PDMS surrounding the sample surface (as in Figure 29) is filled with 200μl of PBS. 2: Droplets of collagen-solution are pipette onto the sample surface.

Mixing vs. no mixing of the solution in the PDMS chamber

After addition of the collagen solution into the chamber (droplet by droplet), samples were incubated for 15min or the solution in the chamber was first thoroughly mixed by pipetting 100μl in and out several times and finally let to incubate for 15min. During the incubation, collagen I fibers that have already form in solution as the pH was increased, interacted with the aldehyde groups at the surface. The purpose of the mixing was to obtain a better distribution of the collagen on the sample, but from another hand, Fengzhi Jiang et al. have used a hydrodynamic flow to direct collagen fibers. Consequently, mixing can induce orientation of fibers at the surface introducing non-controllable heterogeneities in collagen deposition [7]. PDMS chambers

were put on aminosilated cover-slip pre-incubated with glutaraldehyde. The PDMS chambers are circular with a diameter of 15mm (see Figure 29).
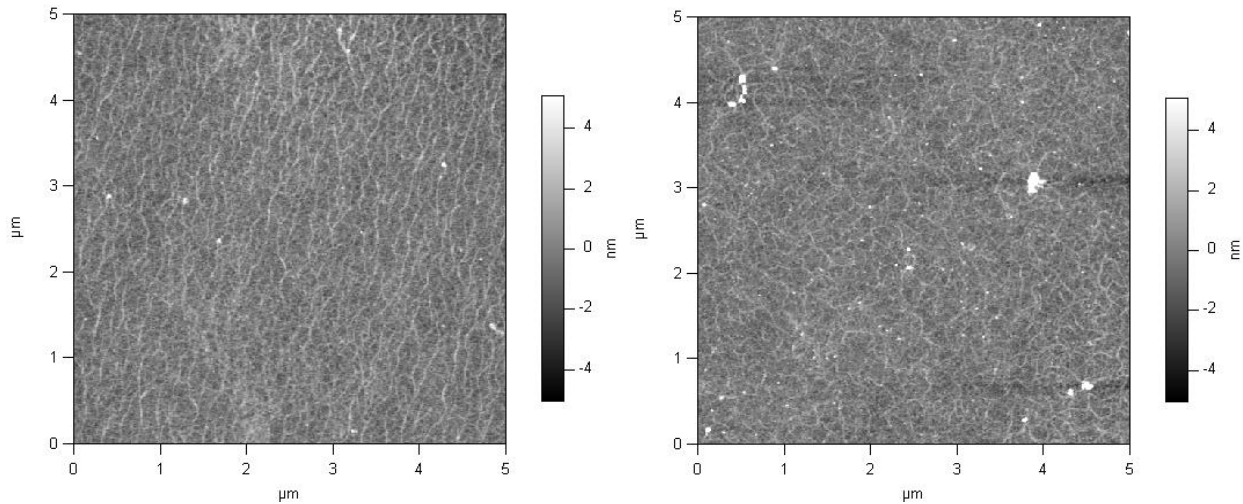
Figure 31: AFM pictures showing the difference between mixing and no mixing of the solution in the chamber. To the left the collagen-PBS solution is mixed with a pipette causing the fibers to be more aligned compared to the picture to the right were no mixing of the solution in the chamber is done.

After trying this a couple of times it was clear that mixing in many cases but not always did affect the fibers by directing them (Figure 31). This is something that could be interesting to investigate further, but a way to control this process more precisely would be needed. The distributions of the fibers on the samples were not that different. In both cases the central parts of the chamber contained a rather uniform layer of collagen. But closer to the boarders of the chamber, parts with less or without collagen were found. The stamped area is typically about $4 \times 6mm$. If the PDMS-chamber is placed on the sample in such a way that the stamped area ends up at the boarder of the chamber, we risk not getting an uniform layer of collagen at the sample. However if we make sure the stamped area is centered as much as possible in the chamber, experiments indicate a nice distribution of collagen.

Deposition of collagen onto underlying BSA layer

When samples for micro-contact printing are prepared with an underlying BSA layer, collagen will adsorb onto this layer that has been pre-incubated with glutaraldehyde. Such a sample has been prepared. The result is shown to the right in Figure 32. Collagen fibers adsorbing with a higher density onto the aminosilanated cover slips pre-incubated with glutaraldehyde (see Figure 31) than onto the cover slip with underlying BSA layer.
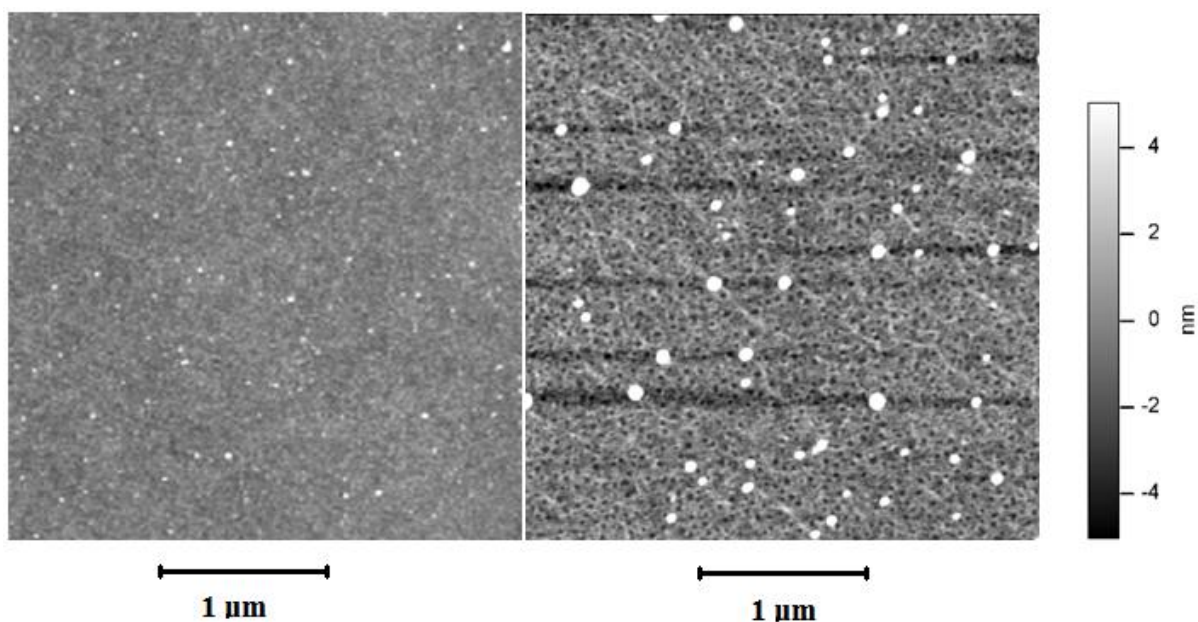

Figure 32: AFM pictures of BSA layer (not micro contact printed) with and without collagen. Left: BSA surface without collagen used as a control. Right: BSA surface with collagen. We see that some collagen fibers are sticking to the surface to the right.

2.3.2 Deposition of collagen assisted by micro-channels

During micro-contact printing (see section 2.2.2), the PMDS stamp forms parallel micro-channels at the sample surfaces. This offers the possibility to incubate the sample with collagen by using these channels before to remove the stamp. The pH of the acidic collagen I solution has to be increased and this has been done in two different ways: first acidic collagen solution is injected at one entrance of the micro-channels, when a basic ammonia solution is injected at the second entrance. Second, micro-channels are filled with collagen and an ammonia atmosphere is created around the PDMS stamp. Ammonia molecules diffuse in PDMS and increase the pH of the collagen solution.

Glass cover slips are prepared with the underlying BSA layer (see section 2.2.4), incubated with glutaraldehyde and rinsed. Then the micro-structured and hydrophobic PDMS stamps are cut to make micro-channels of approximately 4mm in length. The thickness of the PMDS layer is chosen to be 3mm. The same 1mm-thick piece of glass is used as a standard reference to make sure that the channel length is constant for all experiments. After incubation with BSA, the stamps were placed in contact with the glass cover slip as described above (see section 2.2.2).

Interaction of collagen and ammonia solutions in micro-channel

The PMDS stamps (incubated with BSA) are let in contact with the sample (underlying BSA layer incubated with glutaraldehyde) from 15min and then they are placed in the vaccum chamber for 30min. After, samples are removed from the chamber one after the other to continue the fabrication process (the rest of the samples remain under vacuum). A piece of glass is put on the top of the PDMS stamp: by limiting the surface of contact between PDMS and air, the diffusion of air into the degassed PDMS stamp is slower and the PDMS block aspirate air for a longer time. Solution drops (approx 20µl) are placed at the both entrances of channels trapping air in the channel. This trapped air diffuses progressively in the PDMS block and the air pressure in the micro-channel decreases. Being slightly higher than the channel pressure, the atmospheric pressure pushes the solutions into the micro-channels. If the PDMS-stamp volume is big enough

solutions will fill the channels completely.   The stock collagen solution is put on one side of the stamp (in contact with the entrances of the channels), and an ammonia solution is put on the opposite side (also in contact with the entrances of the channels) as shown in Figure 33. With a light microscope, one observes that the solutions are flowing closer to each other and finally meet inside the channels. The solutions are flowing faster in the centred channels than in the channels closer to the boarder of the stamp (where more air can be 'pumped' from the atmosphere). As the solutions have met (no air in the channel) in nearly all of the channels in the central part of the stamp, the incubation time starts. 15minutes was used. Finally, the stamps are removed and the samples were rinsed three times with 1ml PBS, followed by three times with 1ml distilled water. Finally the samples were dried with nitrogen for AFM imaging.
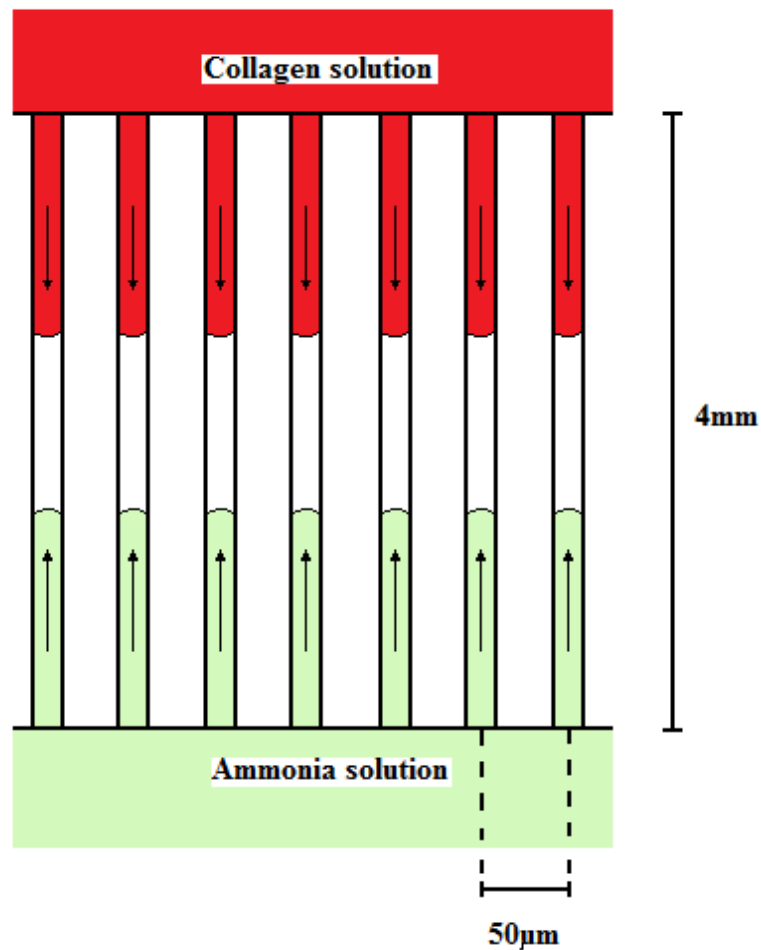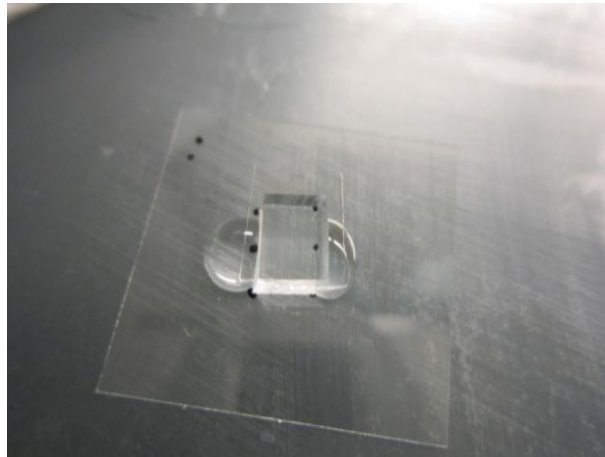
Figure 33: PDMS- stamp with a collagen droplet at one side, and an ammonia droplet at the other. *Top: Photo of a PDMS stamp with a collagen droplet at one side, and ammonia droplet at the other. Bottom: Schematic showing the two solutions flowing inside the channels. The solutions will eventually meet inside the channels.*

When the collagen solution having a very low pH and the ammonia solution with a very high pH meet in the micro-channel in the other, the pH in the collagen solution will increase and induce polymerisation. It could be that a gradient of pH occurs through the length of the channel. This should cause the collagen to polymerize differently at different locations inside the channels. A gradient of fiber diameter and/or density could be generated in the collagen assembly (see Figure 34).
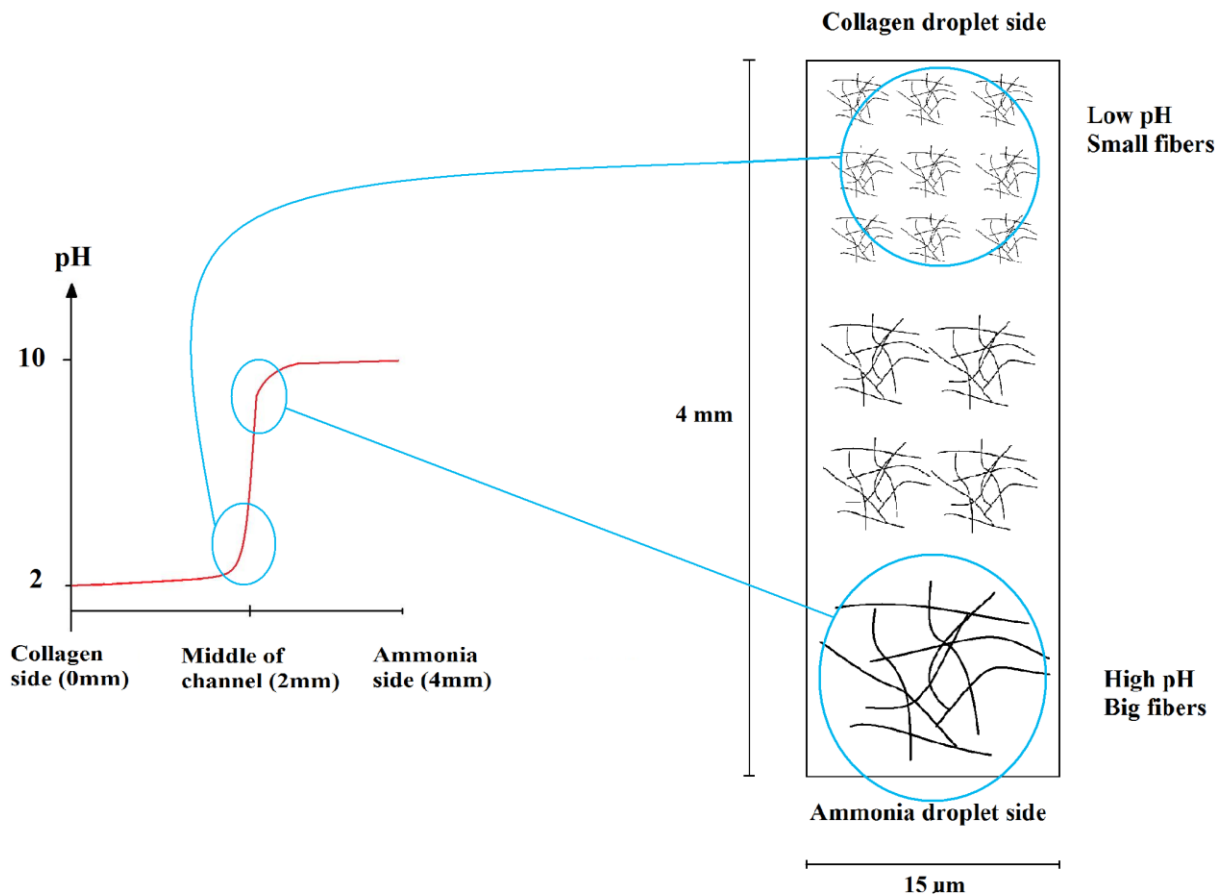
Figure 34: Schematic showing the working hypothesis of the experiment. Big fibers of collagen are polymerizing at one side of the channel and small fibers at the other because of the differences in pH between side starting with an ammonia droplet next to the channels entrances and the opposite side starting with a collagen droplet.

In order to generate a pH gradient, the ammonia solution was used at different dilutions in distilled water. Samples made using concentrations of 1% and 0,025% ammonia are documented in the results section. 15 minutes incubation time was used for all the samples.

Interaction of collagen solution in micro-channel with ammonia vapor

As shown in Figure 35, samples (a PDMS stamp incubated with BSA on a cover slip with underlying BSA layer pre-incubated in glutaraldehyde) was placed with a small container of ammonia (approx 5-6ml at a concentration of 25%) on a piece of parafilm on the bench, and a large petri dish was put over both of them to create an ammonia atmosphere. Previously, the micro-channels have been filled with collagen stock solution. The large petri dish was pressed down in the parafilm and slightly rotated to seal the chamber. The ammonia in the container evaporates and the ammonia molecule diffuses in the PDMS block. This diffusion is favoured by the previous degassing of the PDMS just before addition of collagen in the micro-channels. The pH value increase inside the micro-channels and polymerization starts. Incubation times of 2 minutes, 30 minutes and incubation over night were used. After incubation the stamp was removed and the sample was washed three times with 1ml PBS and three times with 1ml distilled water. Then it was dried for AFM imaging.

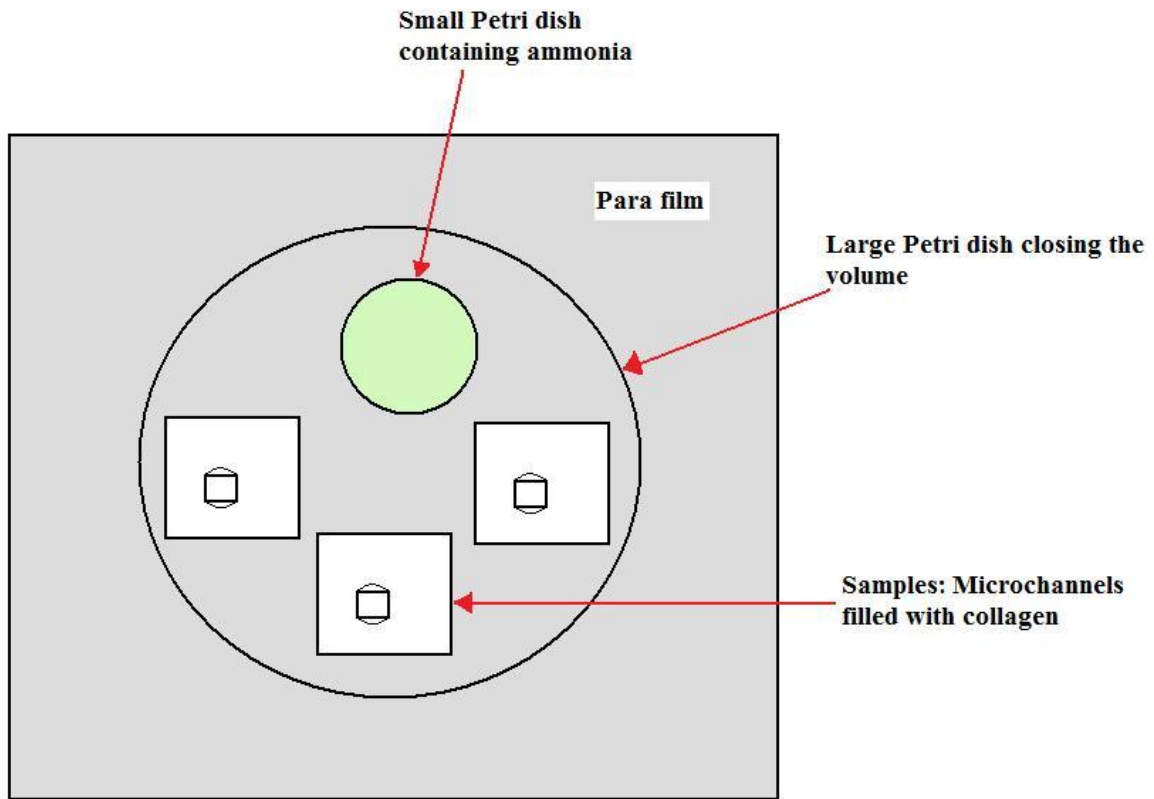*Figure 35 Schematic of samples with collagen filled channels exposed to evaporating ammonia inside a closed chamber.* The cover-slips are covered with BSA, and the stamps are inked in a BSA solution, making a double BSA layer in the parts where the stamp touches the cover-slip.

2.4 Experiments with cells

After fabrication and characterization of several different types of matrixes, some of them were chosen to use to study cell adhesion and spreading.

2.4.1 Preparation of sample with cells and imaging

The cells, which will be added on the sample are in suspension in cell culture medium. Cover slips are mounted with the help of vacuum grease on the back of a petri dish with a circular hole in it using grease. Cell suspension can be then poured on to sample to allow cells to interact with the surface. At the opposite side of the circular hole in the petri dish, a glass piece is mounted to form a chamber ensuring homogeneous distribution of cells on the sample surface. A cover slip mounted this way is shown in Figure 36.

Figure 36: Cover slip mounted on a petri dish using grease. [1] This was done to be able to add an aqueous solution on the coverslip.

After mounting the sample to the petri dish, Dulbecco's modified eagle medium (DMEM) containing 2% Pen-Strep was added to the sample and let to incubate. Pen-Strep is used to prevent bacteria development. Then to pipettes were used to add a medium (DMEM, 2%Pen-

Strep, 10% FBS) containing cells. Each pipette was adjusted to the same volume, one was used to add cell suspension at one side and the other to remove medium from the other side. This was repeated until the sample surface had a nice distribution of cells. The sample was then put in the incubator at 37°C and 5% CO2. The sample was then take to the microscope stage for imaging at defined times, and put back in the incubator again after each round of imaging. Pictures were taken with phase contrast using 10 x objectives. Preparation of samples for imaging is described step by step in PROTOCOL 4, and adding of cells to the sample is described in PROTOCOL 5. A picture of cells spreading with and without confinement can be seen in Figure 37.
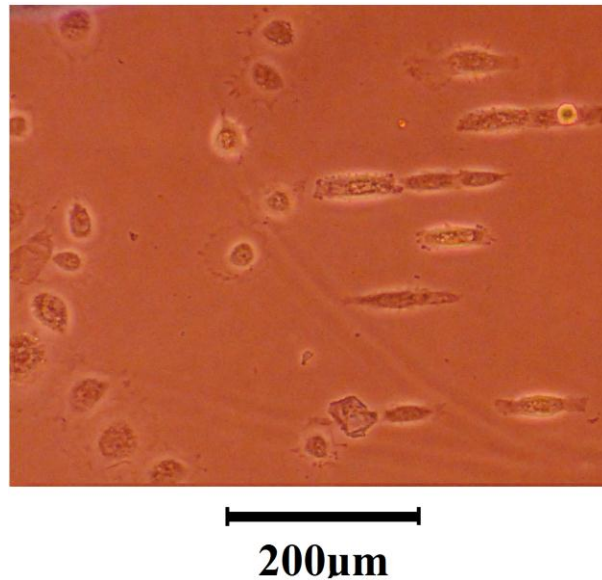

200µm

Figure 37: Picture of cells taken using phase contrast 10x objective. The picture is taken at the boarder of the sample, so the cells right in the picture are spreading with confinement and the cells to the left without.

2.4.2 Image processing

The measurement of the cells spreading is carried out using the software ImageJ. This is free software, and can be downloaded from their website. It contains several different tools, but in this project it is only the function of measuring the length of a straight line has been used.
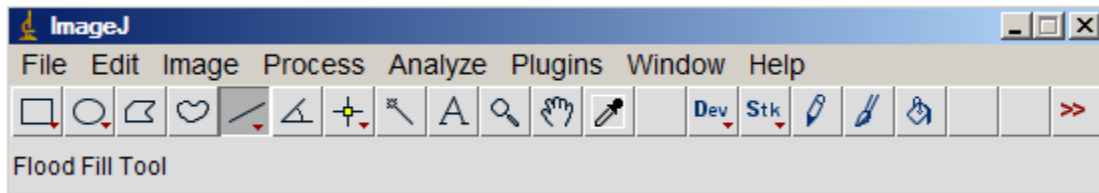
Figure 38: The ImageJ program window

Before measurements of length of cells can start, the scale has to be set. For this a reference picture is taken of a micrometer scale using the same objective as the one used to take pictures of cells. This picture can be seen in Figure 39. The distance between the objective and the micrometer scale is the same as the distance between objective and sample surfaces analyzed. So the distance between to point at the reference picture corresponds to the same distance at the pictures of the cells.
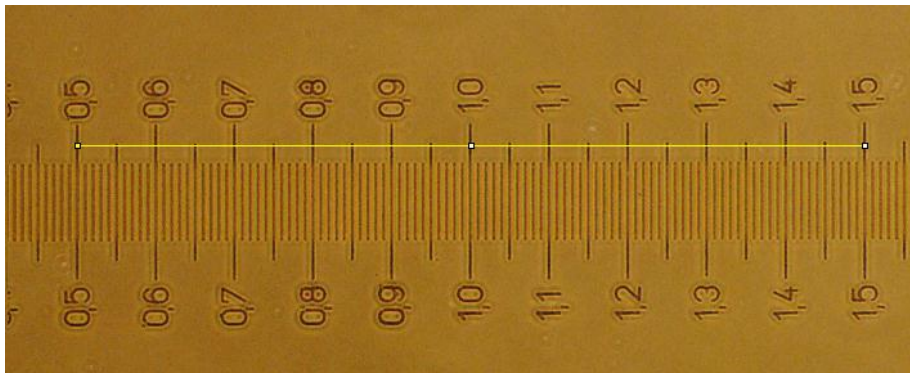
Figure 39: Reference picture taken to set the scale in ImageJ. *A distance known to be 1mm is selected, and this distance is compared to the number of pixels this length corresponds to. The distance to pixel ratio is the same for this picture as for the pictures of cells, so it can be used as a reference when measuring length of cells.*

When the scale is set measurements of cells can be taken. Images of cells are then imported to the software and the same tool for measuring the length of a straight line is now used on cells. This is shown in Figure 74.

3.0 Results and analysis

3.1 Evaluation in documentation with AFM

The AFM pictures shown in the results section come together with additional information in form of graphs and statistics. For the AFM pictures presented to investigate adsorption of BSA under various conditions the heights of the BSA layers are given. For the samples imaged containing collagen the height of the collagen layer are given, and for most of those also the thickness of the collagen fibers are presented. Both size of collagen fibers and height of the collagen layer are presented as a function of position on the sample. The thickness of the collagen fibers are measured five times following the guidelines described below, and the standard deviations of those measurements are given together with the value. The evaluation of the uncertainties in measurement of the height of collagen layer leads to Table 2.

Evaluation of uncertainty in height measurements

When evaluating the height of the collagen layer we use a tool in the MFP-3D software. When measuring height of collagen layer it is important to average over a certain length of BSA-collagen boarder. The tool used provides this opportunity, and the importance of this is illustrated in Figure 40. It can be seen that the graph representing the height in the picture get smoother as the number of pixels averaged over increases.
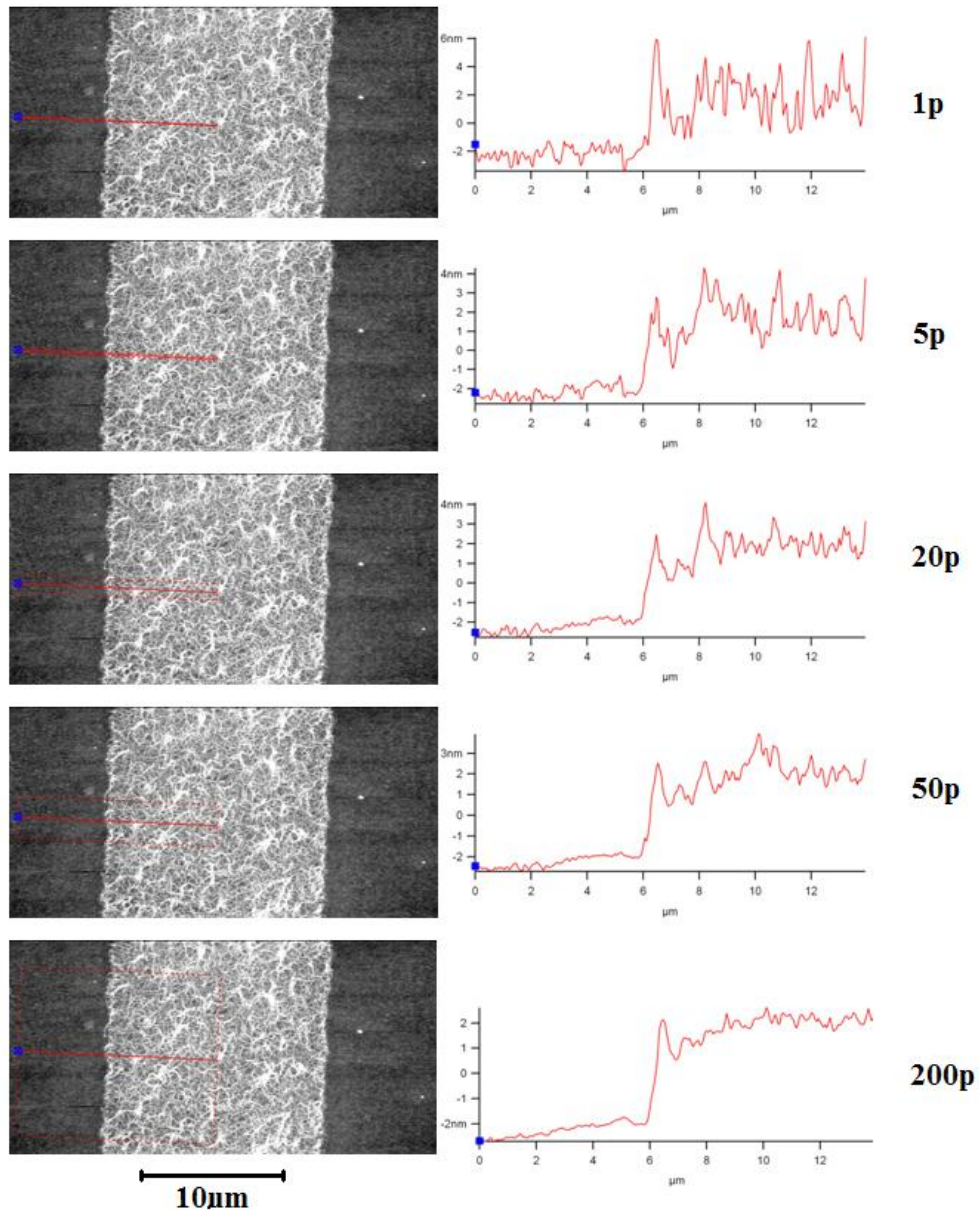
Figure 40: Increasing number of pixels when making calculation of height of collagen layer.
It is seen that the graph representing the height of collagen layer gets smoother as the number of pixels increase from 1 (top) to 200 (bottom).

A measurement of height of collagen is carried out at 5µm of border (between collagen and BSA) or more. 5µm corresponds to 84 pixels in Figure 40 (this depends on the scale of the AFM picture). After selecting the square used to measure height, you can get help reading the values by

adding cursors to the graph. Then information of the cursors position becomes visible. There is an uncertainty in this step which is illustrated in Figure 41. In this figure the cursors are placed with maximum (upper) and minimum span (lower) showing the largest error that could realistically be done at this measurement. When trying to hit a value that is closer to the average height of the layer, better accuracy is obtained.

Figure 41: Measurement of height of collagen layer. *Top: AFM picture the measurement was carried out on. Middle: Section graph showing height differences in the picture. The coursers are put in position to have maximum span in height (4,24nm). Bottom: Section graph showing height differences in the picture. The coursers are put in position to have minimum span in height (2,35nm).*
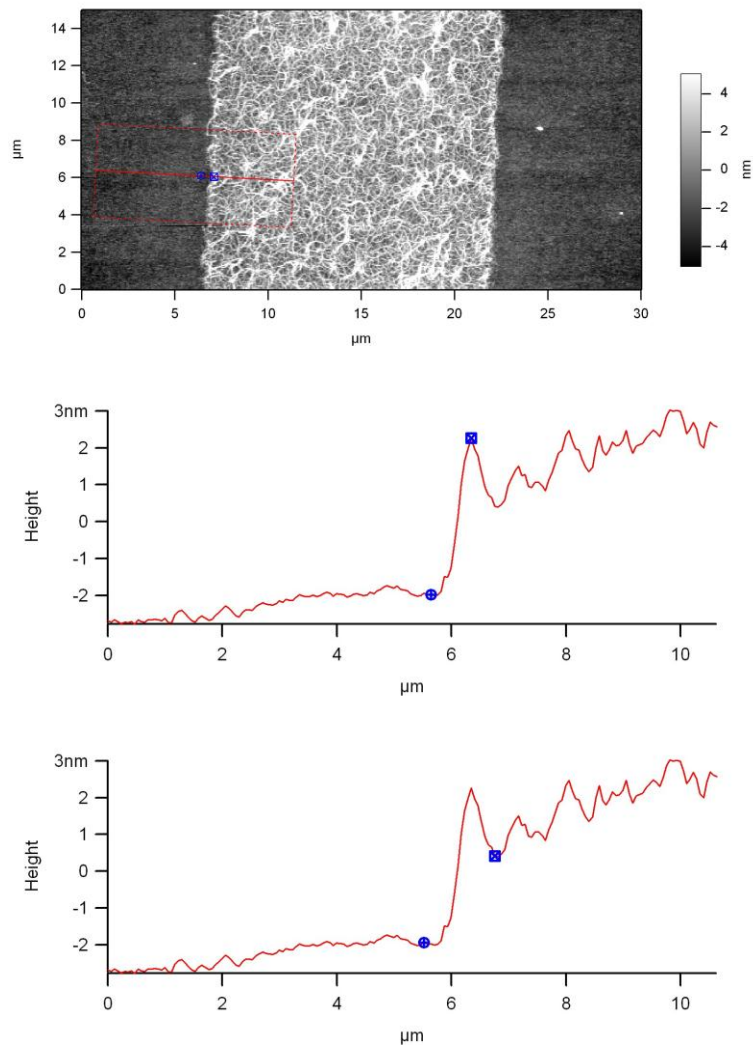
Another thing to be aware of is that the AFM pictures comes with a certain shift in the height depending on distance between positions. So points relatively far away from each other tend to be shown in the picture as having a larger difference in height than what is actually true. This is illustrated in Figure 42.
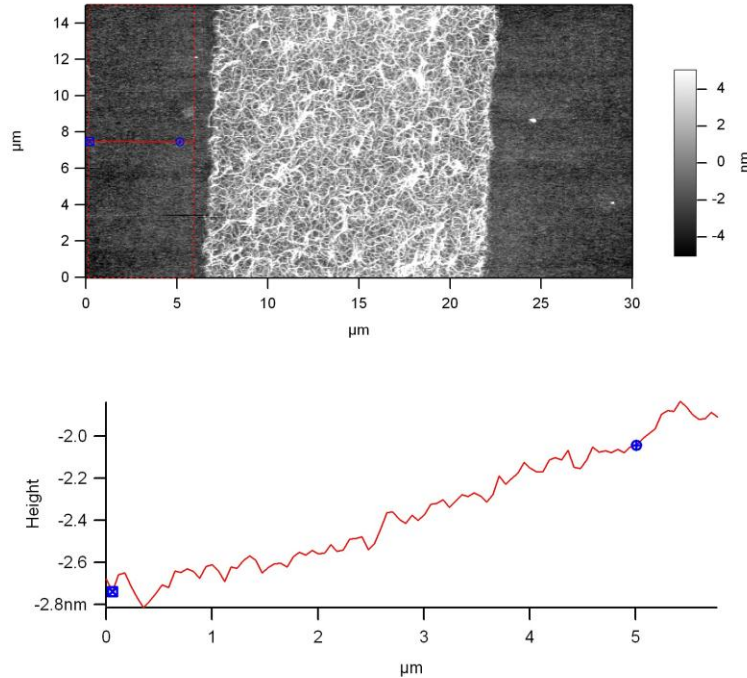
Figure 42: Graphics explaining shift in the AFM picture. The two points are shown in the graph to have about 0,7nm difference in height even though the difference is probably less.

To limit the uncertainty of this shift we want to hold the cursors relatively close to each other. Following the guidelines described above 10 measurements were done at the picture presented in this discussion. This gave a mean value of 3,6nm and a standard deviation (SD) of 0,3nm (measurements in appendix).

We also have to keep in mind that we now are calculating the height of collagen with the height of the BSA layer as reference. This could result in negative numbers which means the collagen layer is lower than the BSA layer. It also means that we have to take the uncertainty of the height of the collagen layer into consideration when calculating the total height of the collagen layer.

63
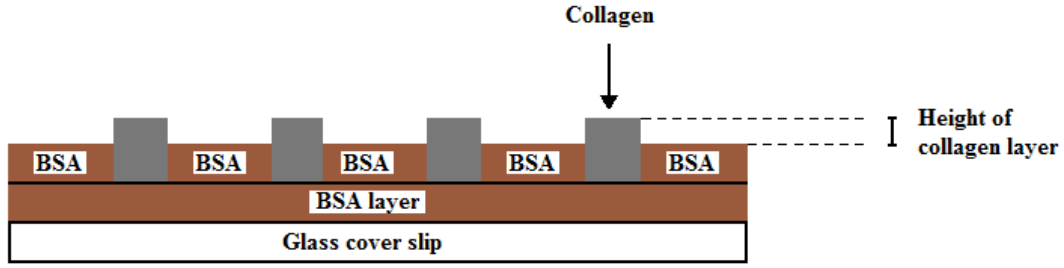
Figure 43: Schematic of a cover slip with collagen and BSA. The figure shows what is meant by height of collagen layer. The top of the micro contact printed BSA is used as reference when measuring the height of the collagen layer.

In addition to the standard deviation calculated here we also have to take into consideration that the BSA layer also has an uncertainty. For this we use the value found in Table 2 for the standard deviation of the micro contact printing with an extra BSA layer. Now the formula to relate the standard variation of two random variables (X and Y) to the individual standard deviations is used:

$$(3) \quad SD(X + Y) = \sqrt{Var(X) + Var(Y) + 2Cov(X,Y)}$$

Here we have that $Var(X) = \left(SD(X)\right)^2$ and $Cov(X,Y)$ is the covariance between the two variables

Assuming that the covariance between the variables is 0 gives:

$$(4) \quad SD(X + Y) = \sqrt{Var(X) + Var(Y)}$$

Using this formula with height of collagen layer as one variable and height of BSA layer as another (seen in Figure 40, Figure 41 and Figure 42) gives a total standard deviation of 0,7nm.

This is an uncertainty useful for most of the calculations of height of collagen layer. However there are some cases where the uncertainty is obviously bigger. An example of this is shown in Figure 44. In this case the difference between the maximum and minimum span (described in Figure 41) will become very big (about 20nm) and we get a larger uncertainty.
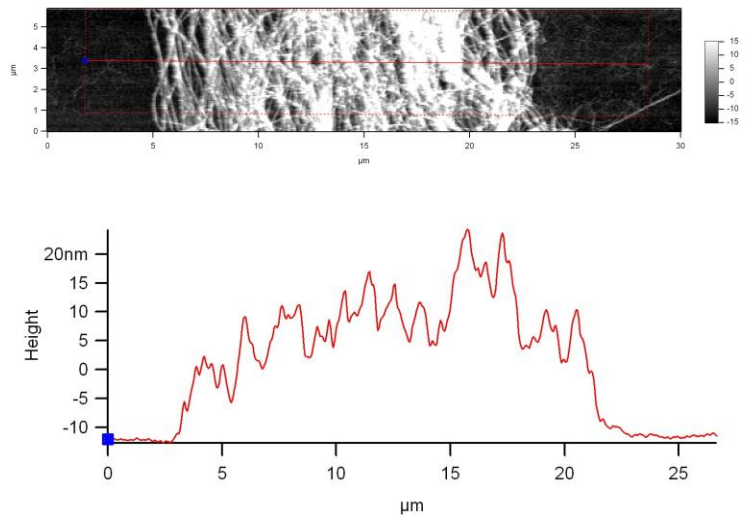
Figure 44: AFM picture and measurement of height of collagen layer using the MFP-3D tool.
The uncertainty in the height of the collagen layer (middle of the picture) is obviously very big.

To get an idea what the actual uncertainty were in those cases, the height of the collagen layer seen in Figure 44 was measured 5 times. This gave an average height of 10nm (SD = 2nm). We see that the domineering uncertainty is now the height of the collagen layer and not the height of the BSA layer as we found for the measurements based on Figure 41. Using formula 4 with height of collagen layer in Figure 44 as one variable, and the height of the BSA layer for hydrophobic stamping of BSA on another BSA layer (value found in Table 2 at page 72) gives SD(X+Y)=2nm.

Type of sample	Standard deviation
Height of BSA layer is the domineering uncertainty (Based on measurements form sample shown in *Figure 41*)	0,7nm
Measurement collagen layer is the domineering uncertainty. (Based on measurements from sample shown in *Figure 44*)	2nm

Table 1: Standard deviations used as uncertainties when height of collagen. *The smallest value is used when the height of the BSA layer is the domineering uncertainty and the largest is used when the measurement of the height of the collagen layer is the domineering uncertainty.*

Evaluation of uncertainty in thickness of collagen fibers

When calculating thickness of collagen fibers it is preferable to have a smaller scale on the AFM picture. The problem with larger pictures is that the fibers are so small that it becomes hard to select single fibers. After selection it also leaves us with a low resolution which makes it difficult to perform accurate measurements. So to make better estimations of the thickness of the fibers some zoom in pictures are made at different positions. AFM pictures illustrate the benefit of the smaller scale in Figure 45.
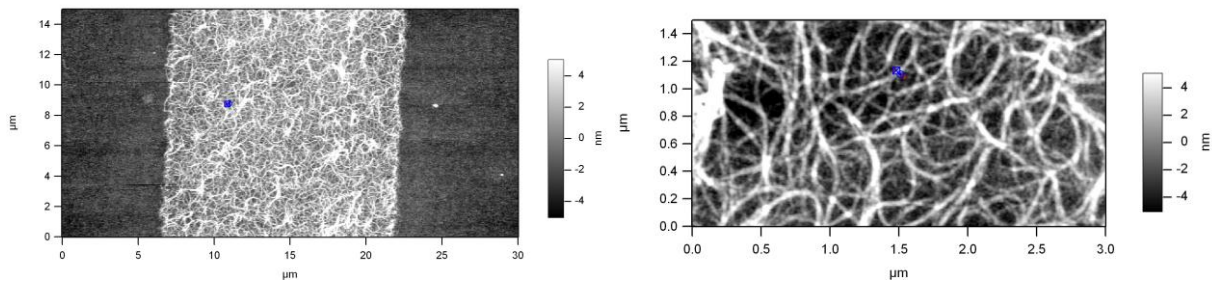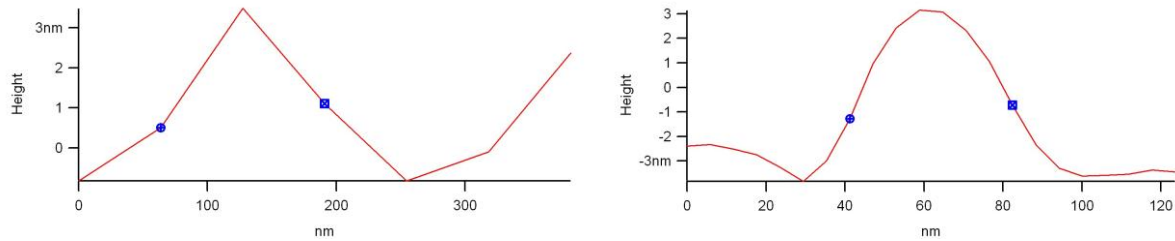
Figure 45: AFM pictures and corresponding graphs illustrating the problem with large pictures when estimating the thickness of the collagen fibers. *The pictures are presented with the corresponding graph below. Left: There is in fact only one value between the two cursors which is the top point of the graph. In between the cursors and the top the software use line interpolation. Obviously this makes accurate measurements difficult. It is also hard to say if there is one fiber or more fibers which are selected. Right: This is a zoom in picture from the same position as the other picture. Here there are several values between the cursors so the interpolation is smoother, and we can perform measurement with larger accuracy.*

The problem illustrated in Figure 45 gets smaller as the fibers get bigger. This is because the peak in the graph consists of more pixels when the fibers are thicker. A measurement of a relatively thick fiber is shown in Figure 46.
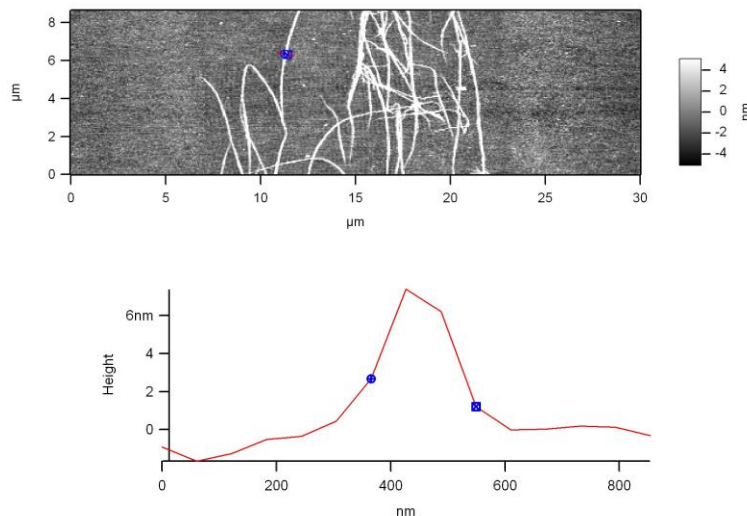
Figure 46: AFM picture and corresponding graph showing the profile of a relatively large collagen fiber. *As we can see the graph still is not as smooth as for the smaller scale picture to*

the left in Figure 45, but it is better than the picture to the right in the same figure which has the same picture scale as this but with smaller fibers.

Measurements are taken as full with of the peak at ⅓ of the maximum height for all pictures. The peak height is estimated as the height-span between the highest minimum of the two minimums next to the peak and the peak top. Following the guidelines presented here, several values are calculated for each picture, and the standard deviation is given for each measurement.

3.2 Results: characterization of matrices by AFM

The AFM pictures shown in this section are presented together with additional information in form of graphs and statistics. For the type of samples presented to investigate adsorption of BSA under various conditions, the corresponding heights of the BSA layers are given. For the samples imaged containing collagen the height of the collagen layer are given. For many of the pictures also the thickness of the collagen fibers is measured. Both size of collagen fibers and height of the collagen layer are presented as a function of position in the microchannel.

3.2.1 Micro-contact printing of BSA
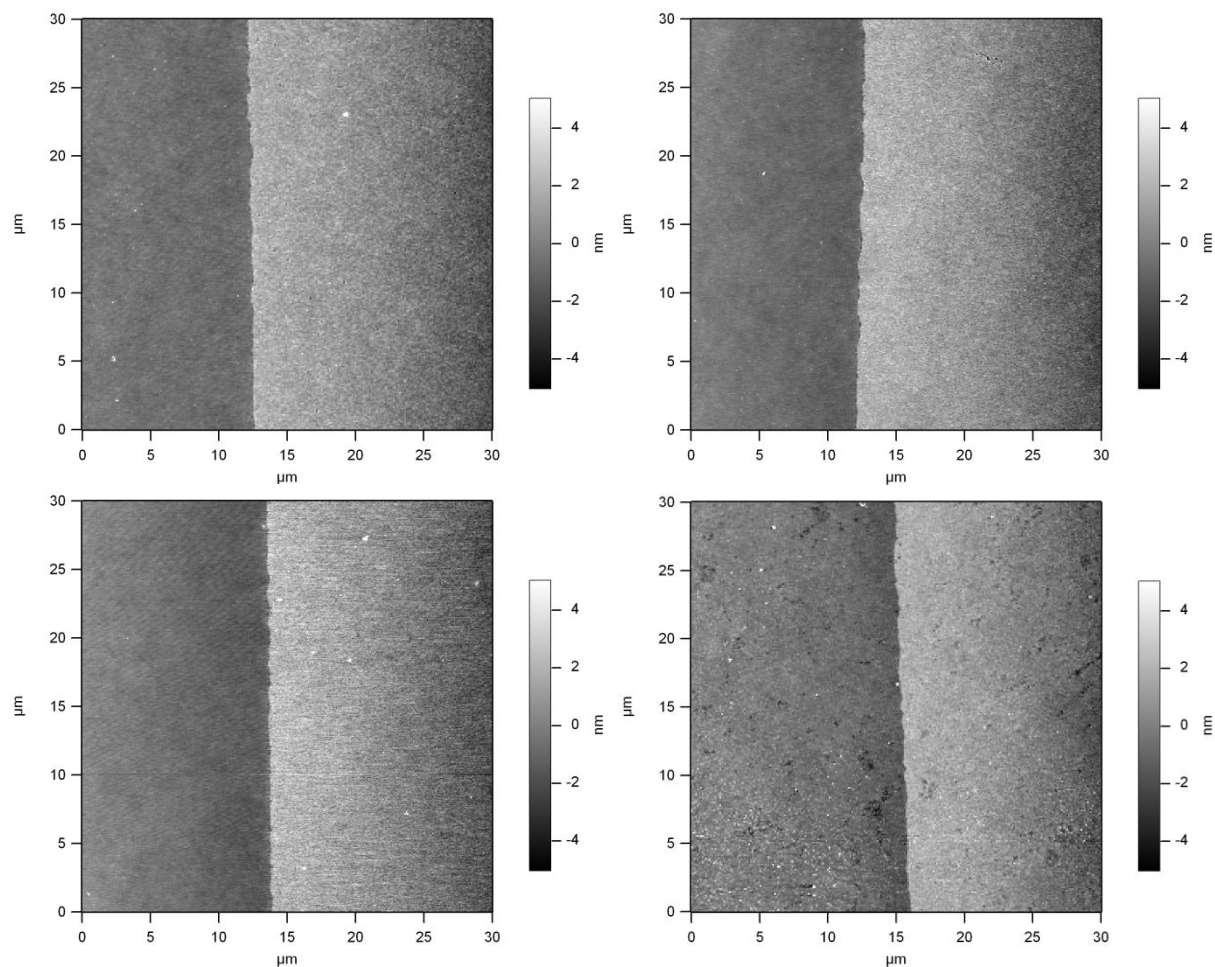
Figure 47: AFM pictures taken of samples made by micro contact printing of BSA using hydrophobic PDMS. *Top left: Glutaraldehyde diluted 0,625% in PBS used as crosslinker when making a micro contact print of BSA on an aminosilanated coverslip. Top right: Glutaraldehyde diluted 1,25% in PBS used as crosslinker when making a micro contact print of BSA on an aminosilanated coverslip. Bottom left: Glutaraldehyde diluted 2,5% in PBS used as crosslinker when making a micro contact print of BSA on an aminosilanated coverslip. Bottom right: Glutaraldehyde diluted 1,25% in PBS used as crosslinker when making a micro contact print of BSA on top of a BSA layer. The underlayer of BSA was deposited using glutaraldehyde with the same concentration.*
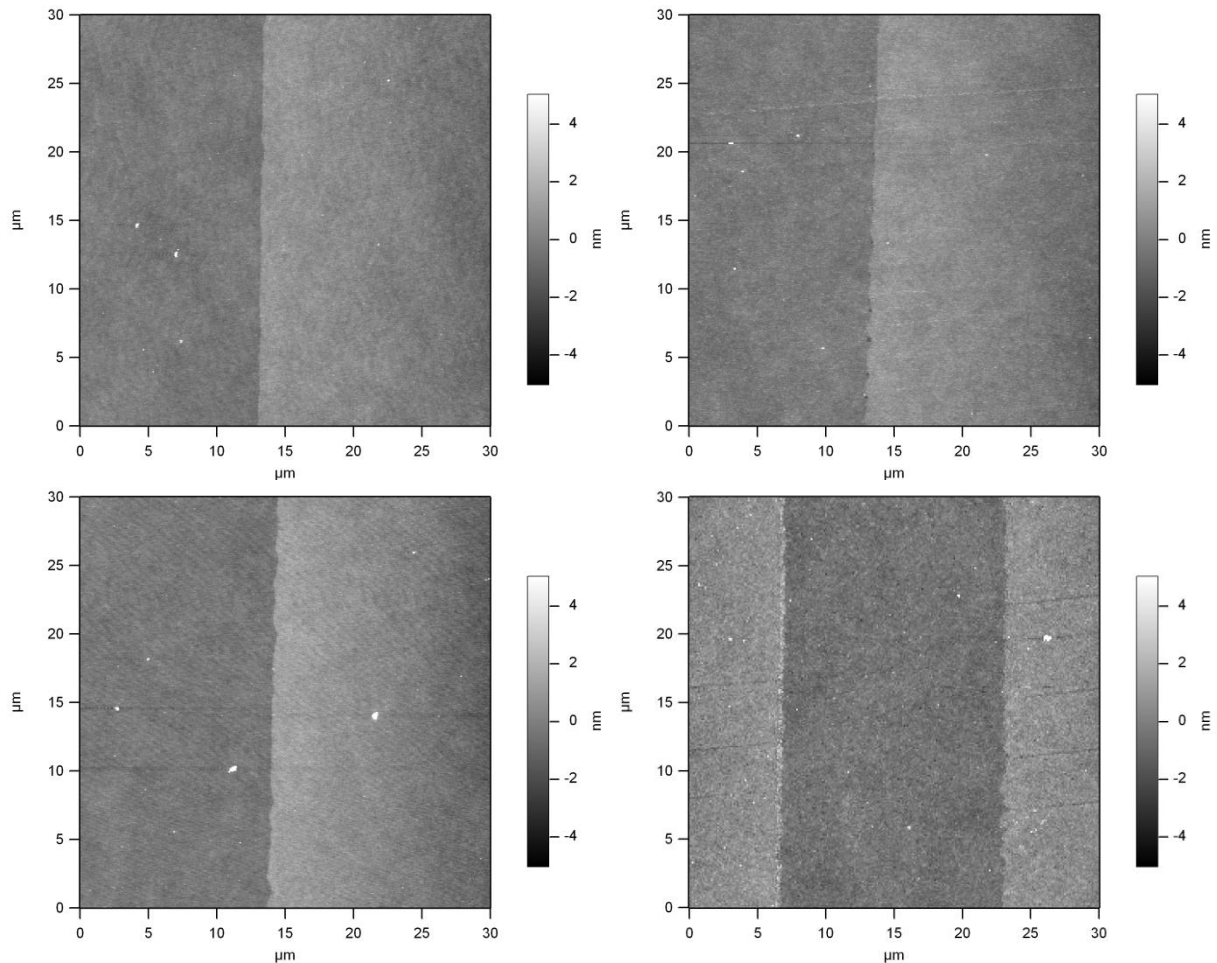
Figure 48 **AFM pictures taken of samples made by micro contact printing of BSA using hydrophilic PDMS.** *Top left: Glutaraldehyde diluted 0,625% in PBS used as crosslinker when making a micro contact print of BSA on an aminosilanated coverslip. Top right: Glutaraldehyde diluted 1,25% in PBS used as crosslinker when making a micro contact print of BSA on an aminosilanated coverslip. Bottom left: Glutaraldehyde diluted 2,5% in PBS used as crosslinker when making a micro contact print of BSA on an aminosilanated coverslip. Bottom right: Glutaraldehyde diluted 1,25% in PBS used as crosslinker when making a micro contact print of BSA on top of a BSA layer. The underlayer of BSA was deposited using glutaraldehyde with the same concentration.*

The types of samples seen in Figure 47 and Figure 48 were fabricated for optimization of BSA deposition by micro-contact printing. Concentrations of 0,625% , 1,25% and 2,5% glutaraldehyde (diluted in PBS) were tried with both hydrophilic and hydrophobic PDMS stamps. The effect of an underlying BSA layer covering the coverslip before micro contact printing was also investigated. AFM pictures of the samples were taken at the scale 30µm×30µm (for the most part) and at least 0,5mm distance in between each picture. Most of the samples were also reproduced to get stronger statistics and confirm reproducibility. A summary of the measurement of the height of the BSA layers described is provided in Table 2. In the appendix all the measurements can be found.

	0,625% glutaraldehyde in PBS	1,25% glutaraldehyde in PBS	2,5% glutaraldehyde in PBS	2,5% glutaraldehyde BSA on BSA
Hydrophilic stamps	$1,3 \pm 0,2$nm (7 pictures from 2 samples)	$1,1 \pm 0,3$nm (7 pictures from 2 samples)	$1,1 \pm 0,4$nm (6 pictures from 2 samples)	$1,3 \pm 0,2$nm (3 pictures from 1 sample)
Hydrophobic stamps	$3,8 \pm 0,3$nm (7 pictures from 2 samples)	$3,6 \pm 0,6$nm (7 pictures from 2 samples)	$3,4 \pm 0,6$nm (7 pictures from 2 samples)	$4,0 \pm 0,7$nm (5 pictures from 2 samples)

Table 2: Table presenting measurements of height of BSA layers deposited in different ways. Each picture used to obtain these results contributed with a stripe - length of at least 10µm.

From these results it seems clear that micro-contact printing with hydrophobic PDMS give us a higher BSA layer than printing with hydrophilic PDMS. Glutaraldehyde concentrations tried does not seem to influence the outcome significantly. When averaging over all the height found for BSA layer stamped with hydrophobic PDMS on glass (BSA on BSA excluded) we get 3,6nm (SD=0,5 and n=21). Corresponding for hydrophilic stamps is 1,2nm (SD=0,3 and n=20).

3.2.2 Deposition of collagen from PBS solution

No underlaying BSA layer
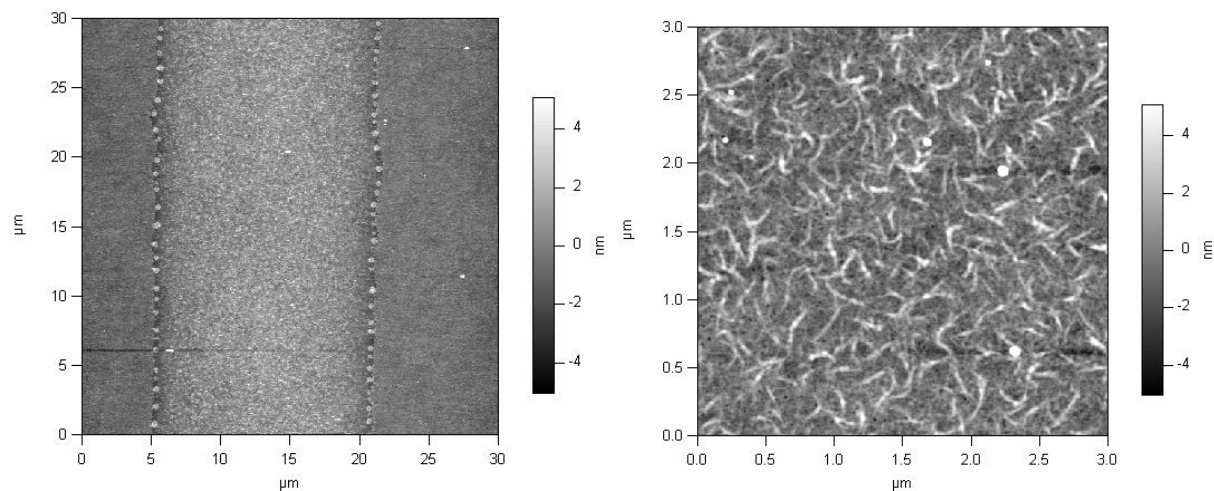
Figure 49: AFM pictures taken of a sample made by using protocol 2 a) without an extra BSA layer. Left: 30μm×30μm scan size used at the AFM. Collagen stripe in the middle of the picture, BSA stripes at the boarders. Right: 3μm×3μm scan size used at the AFM to zoom in at the collagen stripe seen in the middle of the top picture.

This type of samples was widely investigated and found to be uniform. The height of the collagen layer was found to be 1,5nm (SD=0,7nm) and the size of the collagen fibers 59nm (SD= 6nm, n=5). The small circles seen along the borders between the collagen and BSA is observed also at other types of samples. They are most probably a defect on the master used to replicate the PDMS that the BSA was printed with.

With underlaying BSA layer
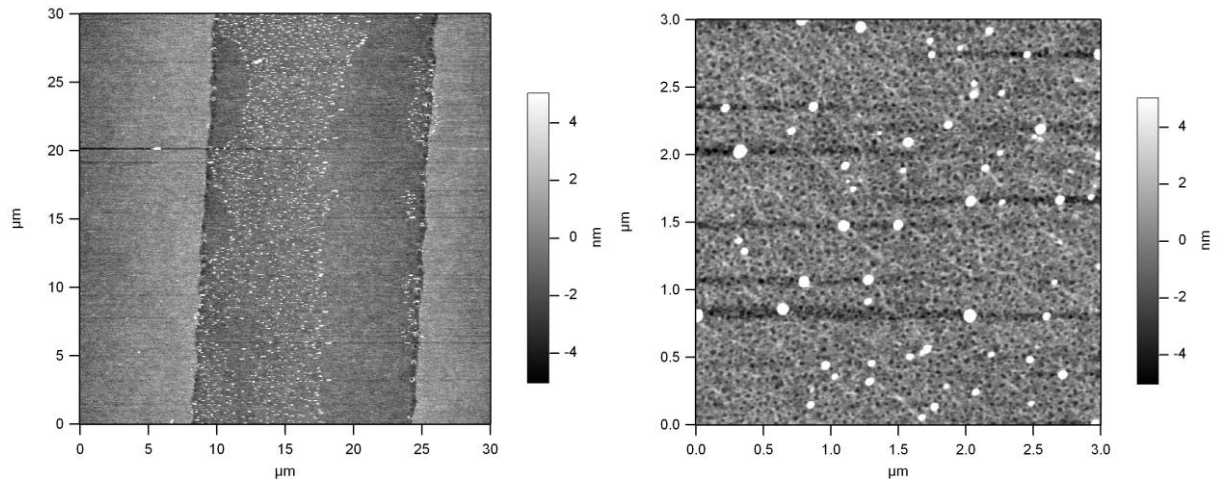
Figure 50: AFM pictures taken of a sample made by using protocol 2 a) with an extra BSA layer. *Left: 30μm×30μm scan size used at the AFM. Collagen stripe in the middle of the picture, BSA stripes at the boarders. Right: 3μm×3μm scan size used at the AFM to zoom in at the collagen stripe seen in the middle of the top picture. The zoom was done at the part were white spots can be seen.*

The pictures taken of this type of sample indicates that it is covered uniformly with a thin layer of collagen. The height of the collagen layer was found to be -0,8nm (SD=0,7nm is given in *Table 1*), and the size of the collagen fibers 36nm (SD=8nm, n=5).

3.2.3 Interaction of collagen and ammonia solutions in micro-channels

Ammonia 0,025% and collagen in micro channels

Figure 51: Montage consisting of AFM pictures taken of a sample made by using protocol 2 c) with ammonia diluted 0,025% in distilled water. The numbers to the right of each picture indicate position in the channel with the upper pictures position as reference.

Only a few fibers were found on this sample. Those were mostly found close to the entrance of the channel at the side were the collagen droplet was put. At 2mm (about middle) and further no fibers were found. The thickness of the fibers measured at the top picture was 59nm (SD=9, n=5). The height of the collagen layer is provided in Figure 52.

Figure 52: Height of collagen layer-- sample made by using protocol 2 b) – 0,025% ammonia.

Standard deviation used for error bars are taken from Table 1.
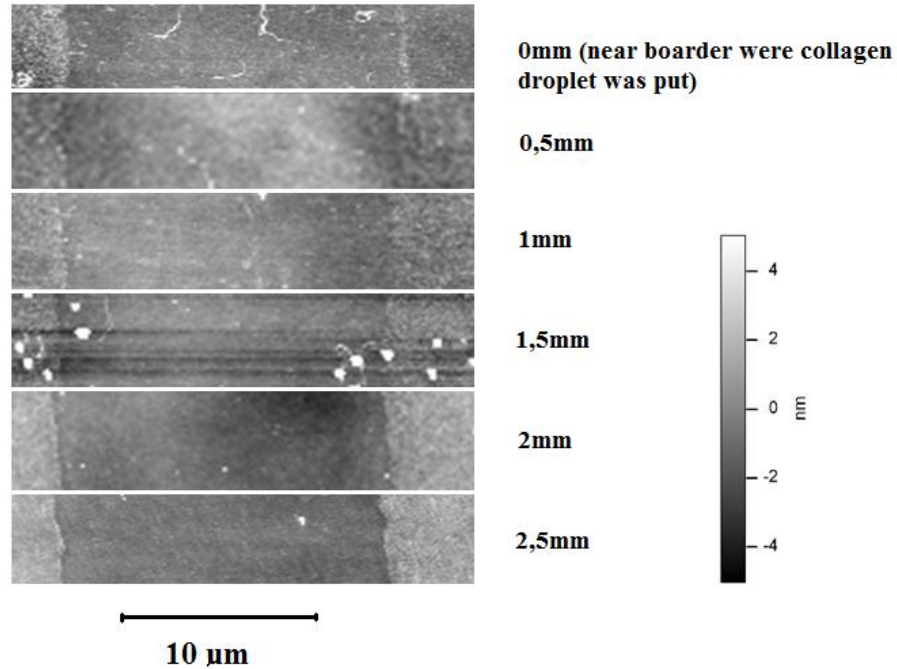
Ammonia 1% and collagen in micro channels (type 1)

Figure 53: Montage consisting of AFM pictures taken of a sample made by using protocol 2 c) with ammonia diluted 1% in distilled water. The numbers to the right of each picture indicate corresponding position in the channel with the upper pictures position as reference.

This sample has a thin layer of small collagen fibers at the top. As we move from the picture close to the entrance where the collagen droplet was put, toward the side where the ammonia droplet was put, we see that the size of the fibers increases. Between 1mm and 1,5mm huge amounts of large collagen fibers are found, both at the glutaraldehyde, but also at the micro contacted BSA layer. The latter is not wanted because it will allow the cells to spread on the BSA. For higher values than 1,70mm no collagen was found. The height of the collagen layer is provided in Figure 54, and the thickness of the fibers in Figure 55.
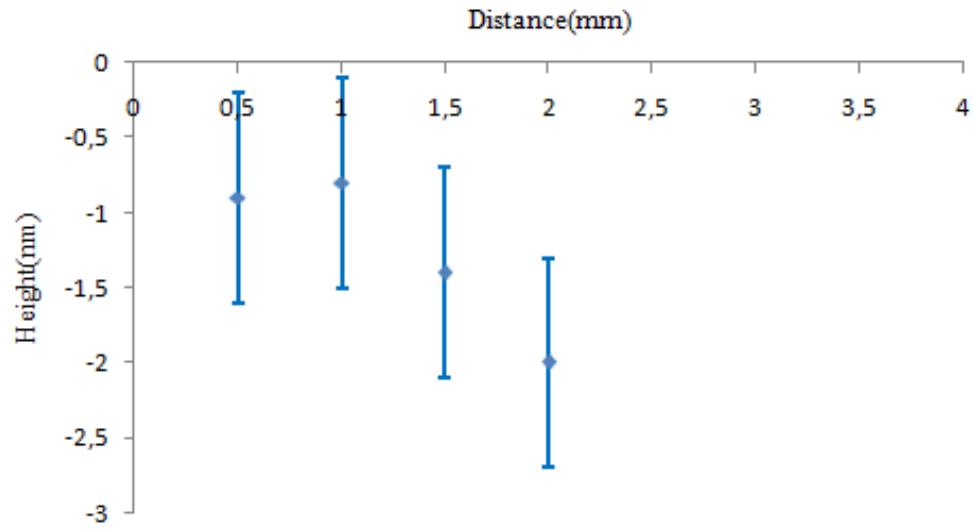
Figure 54 : Height of collagen layer-- sample made by using protocol 2 c) – 1% ammonia (Type 2). Standard deviations used for error bars are taken from Table 1.At 0,5mm, 1mm and 1,5mm the uncertainty in the measurement of collagen is domineering, and for the other values, the uncertainty of the BSA layer is the domineering.

Figure 55 Thickness type 2: Thickness of collagen fibers-- sample made by using protocol 2 b) – 1% ammonia (Type 2). Standard deviations used as error bars are based on 5 measurements each.

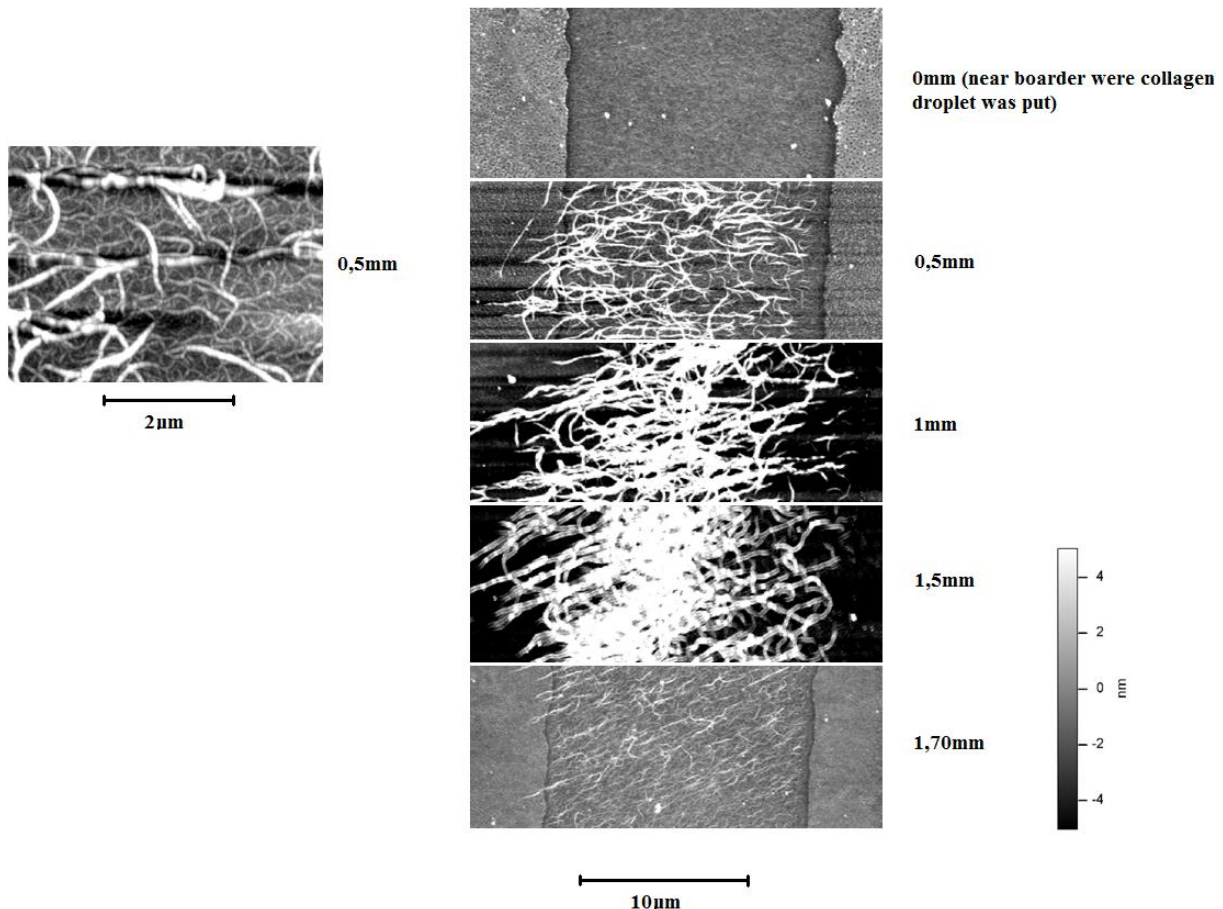# Ammonia 1% and collagen in micro channels (type 2)
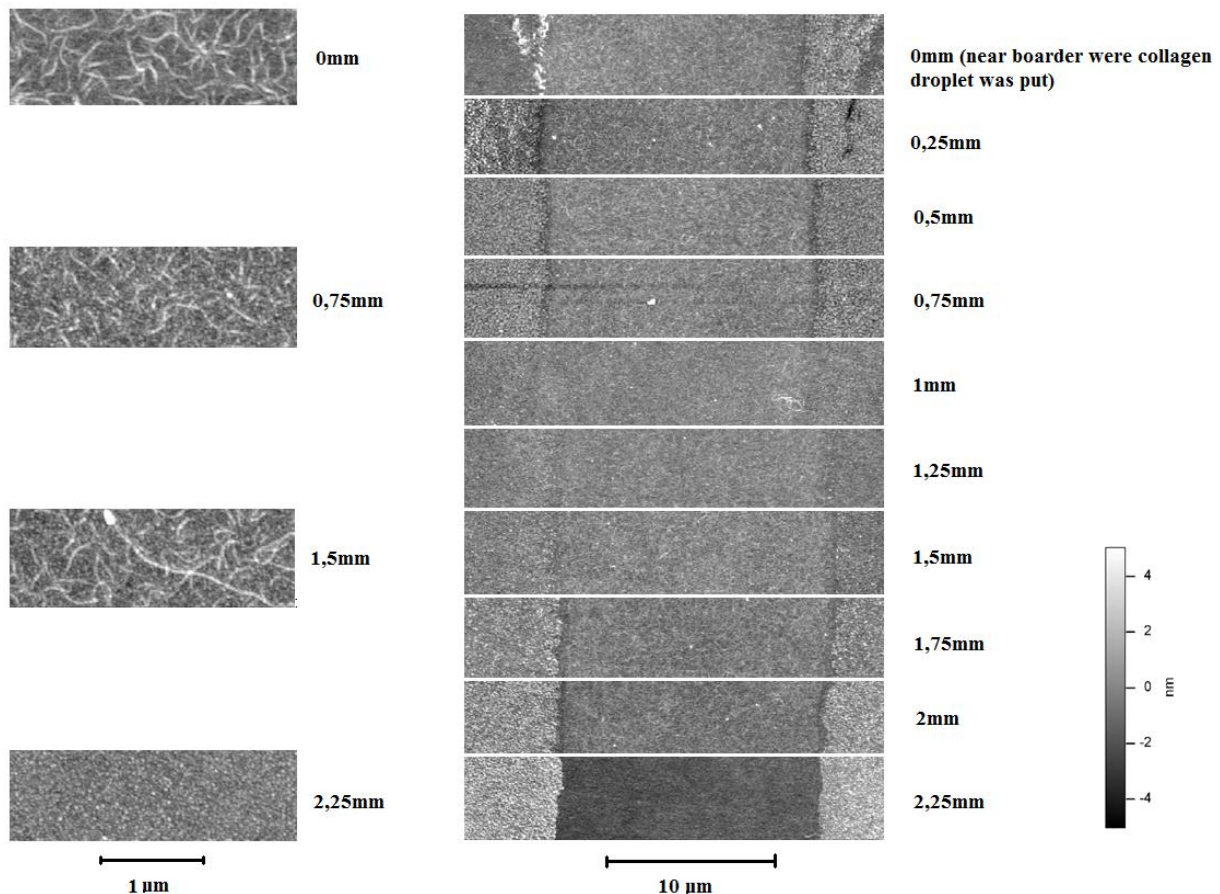
Figure 56: Montage consisting of AFM pictures taken of a sample made by using protocol 2 c) with ammonia diluted 1% in distilled water. The numbers to the right of each picture indicate corresponding position in the channel with the upper pictures position as reference.

This sample was made the same way as the previous, but when comparing Figure 53 and Figure 56 it is clear that the result was different. A significant amount of time has been spent in a struggle to reproduce this type of sample. The height of the collagen layer for this sample is found in Figure 57 and thickness of the fibers is found in Figure 58.
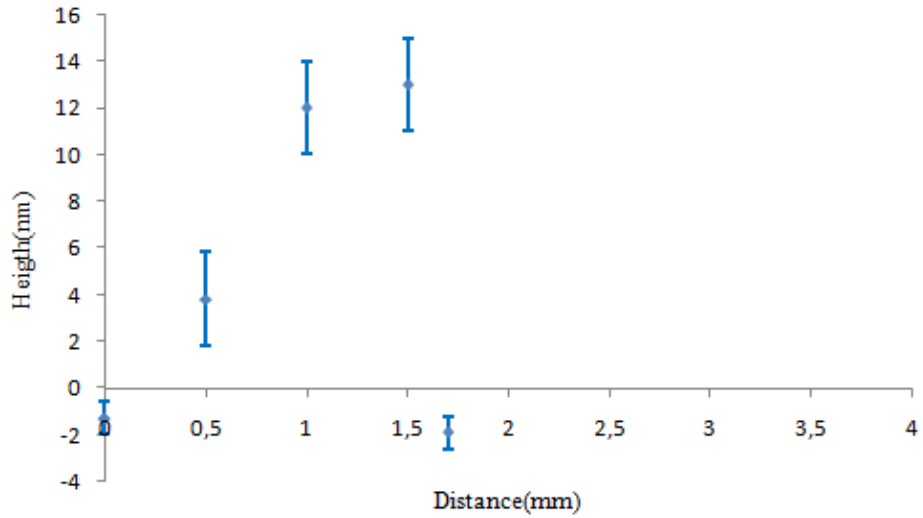
Figure 57:   Height of collagen layer-- sample made by using protocol 2 b) – 1% ammonia (Type 1). Standard deviation used for error bars are taken from Table 1.
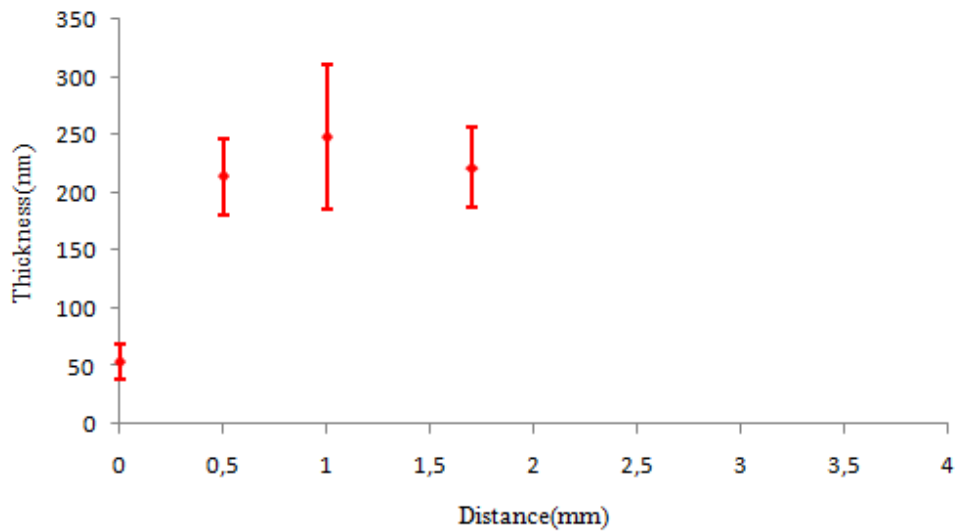

Figure 58 : Thickness of collagen fibers-- sample made by using protocol 2 b) – 1% ammonia (Type 1). Standard deviations used as error bars are based on 5 measurements each.

3.2.4 Interaction of collagen solution and ammonia vapour in micro-channels

This type of samples was expected to be symmetric about the middle of the channels. This is best indicated in Figure 64, Figure 68, and Figure 70 (the middle of the channels will be around 1,75mm for those).

Incubation time 2 minutes

Figure 59: Montage consisting of AFM pictures taken of a sample made by using protocol 2 b) with an incubation time of 2 minutes in chamber with evaporating ammonia. The numbers to the right of each picture indicate position in the channel with the upper pictures position as reference.

This type of sample gave a small amount of fibers at 0,5mm and 1mm. At 1,5mm no fibers were found. Since the sample is expected to be symmetric about the middle (1,75mm), one would think that there are also some fibers present at about the same distance from center, at the opposite side of the sample.

Figure 60: Height of collagen layer-- sample made by using protocol 2 b) - 2 min incubation time. Standard deviation used for error bars are taken from Table 1.

Figure 61: Thickness of collagen fibers-- sample made by using protocol 2 c) – 2 min incubation time. Standard deviations used as error bars are based on 5 measurements each.

Incubation time 30 minutes

Figure 62: Montage consisting of AFM pictures taken of a sample made by using protocol 2 c) with an incubation time of 30 minutes in chamber with evaporating ammonia. The numbers to the right of each picture indicate position in the channel with the upper pictures position as reference.
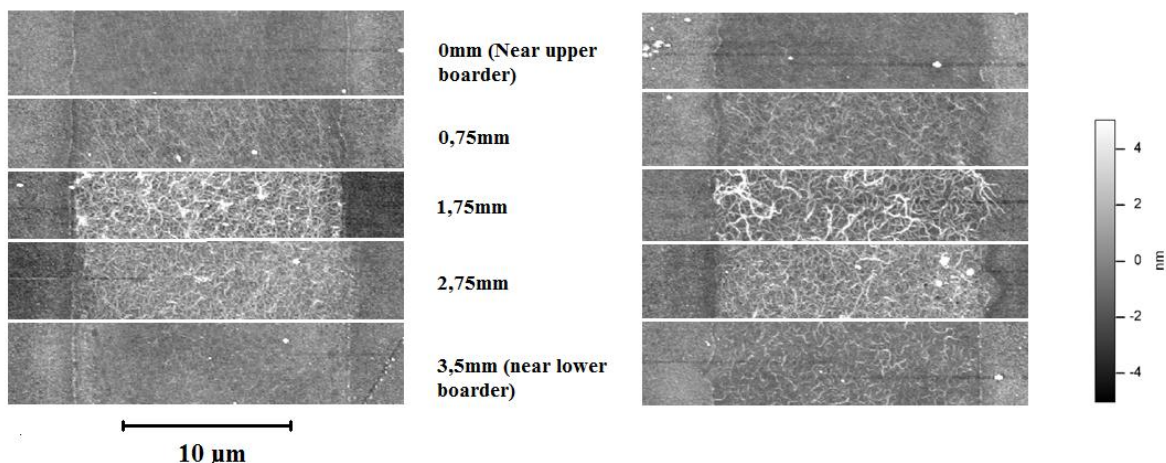
Figure 63 : *Montage consisting of AFM pictures taken of a sample made by using protocol 2 c) with an incubation time of 30 minutes in chamber with evaporating ammonia.* The numbers to the right of each picture indicate position in the channel with the upper pictures position as reference.

This sample was made several times, and was found to have a nice reproducibility. It was found to have a thin collagen layer at the boarders of the channels. These layers were growing in height towards the center of the channel were the peak of the height was found (1,75mm). The height of the collagen layer is shown in Figure 64. However there were only small differences found in the thickness of the collagen fibers as we can see in Figure 65.
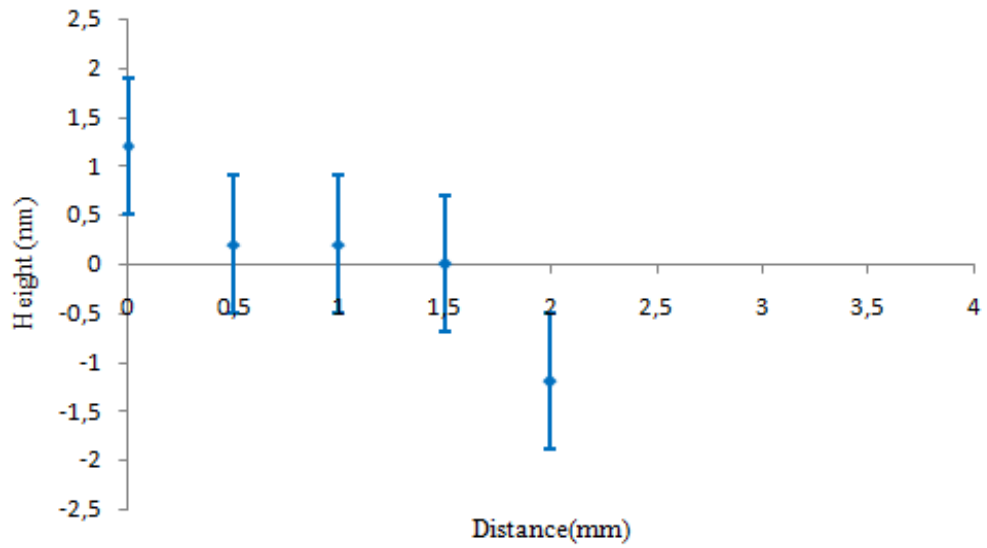
Figure 64: Height of collagen layer-- sample made by using protocol 2 b) – 30 min incubation time. Standard deviation used for error bars are taken from Table 1.
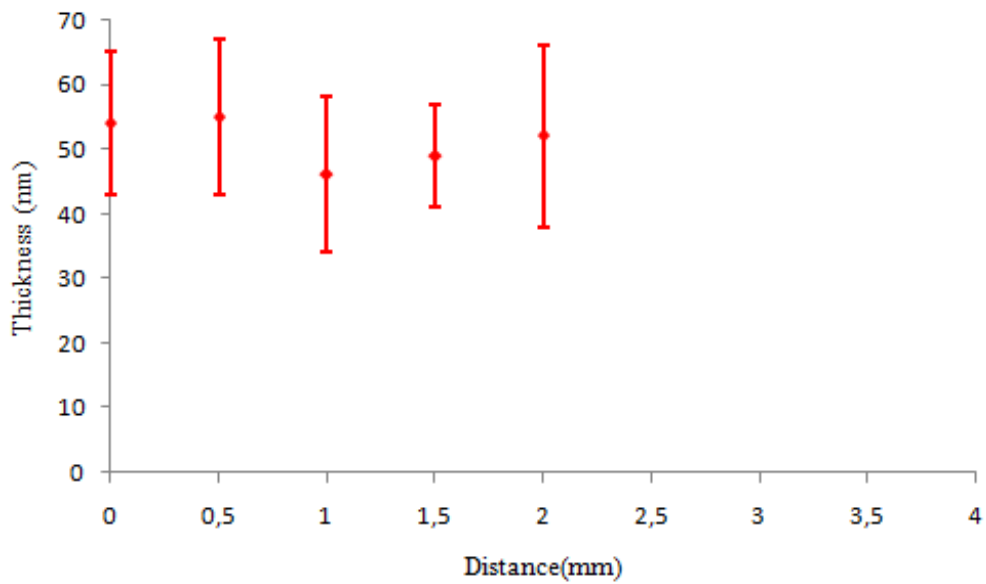

Figure 65: Thickness of collagen fibers-- sample made by using protocol 2 c) – 30 min incubation time. Standard deviations used as error bars are based on 10 measurements each.

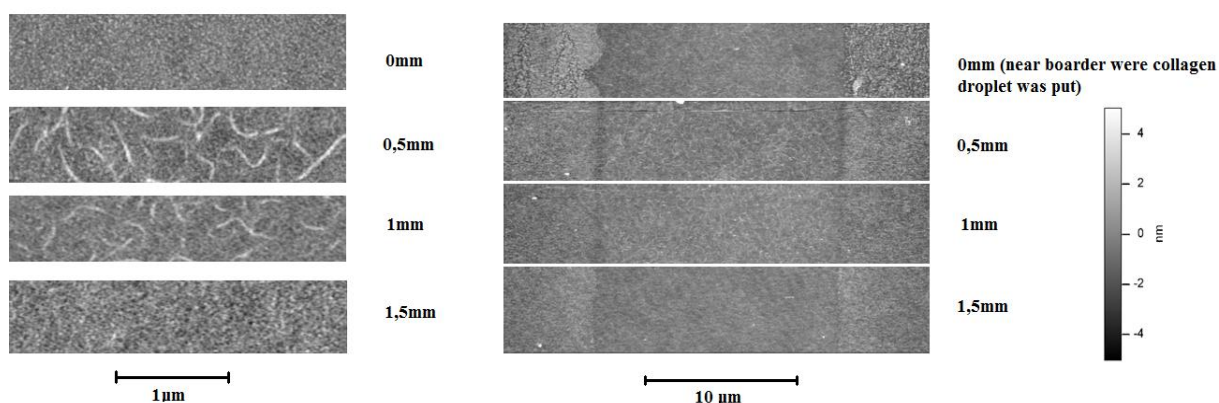# Incubation time 60 minutes

Figure 66: Montage consisting of AFM pictures taken of a sample made by using protocol 2 c) with an incubation time of 60 minutes in chamber with evaporating ammonia. The numbers to the right of each picture indicate position in the channel with the upper pictures position as reference. Note that the collagen is located to the left in the picture at position 0,5mm and not in the middle as it is for the other pictures.
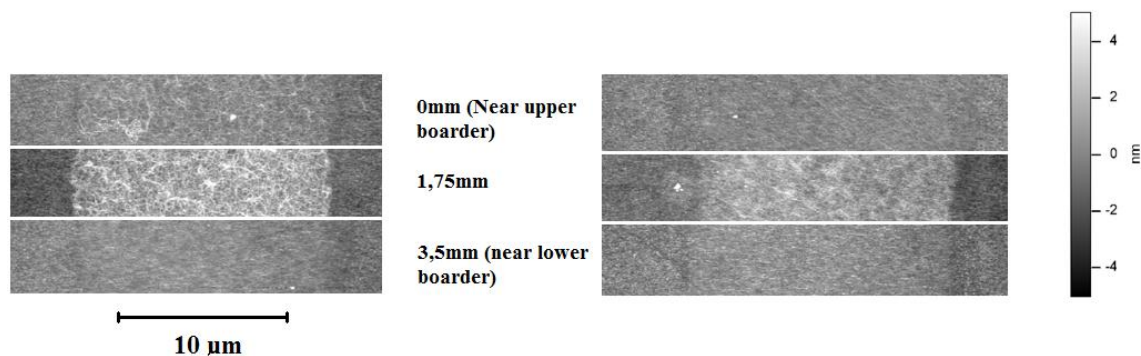
Figure 67: Additional pictures. Montage consisting of AFM pictures taken of a sample made by using protocol 2 c) with an incubation time of 60 minutes in chamber with evaporating ammonia. The numbers in between the pictures indicate position in the channel with the upper pictures positions as reference.

This sample had very similar characteristics with the corresponding with a 30 minutes incubation time. A thin layer of collagen was found at the boarders of the stripes, increasing in heights towards the middle of the channels. The sample had certain symmetry about the center (1,75mm), but the peak of the height of the layer was found at 1,5mm. The height of the collagen layer can be seen in Figure 68. Zoom in pictures to measure the thickness of the fibers were not taken, but it is very likely that we would find a characteristic similar to what is obtained in Figure 65.
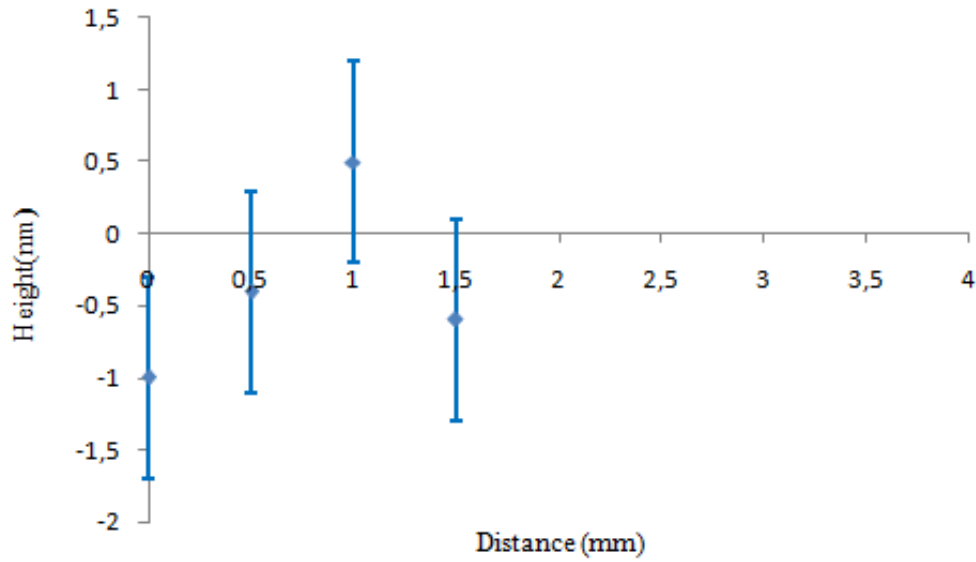
Figure 68: Height of collagen layer-- sample made by using protocol 2 b) - 60 min incubation time. *Standard deviation used for error bars are taken from Table 1.*
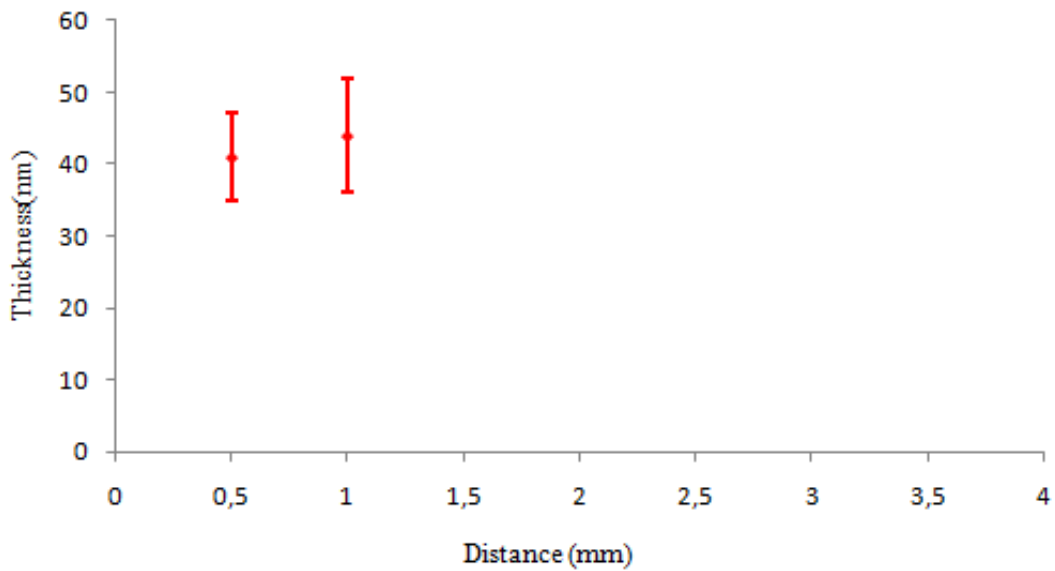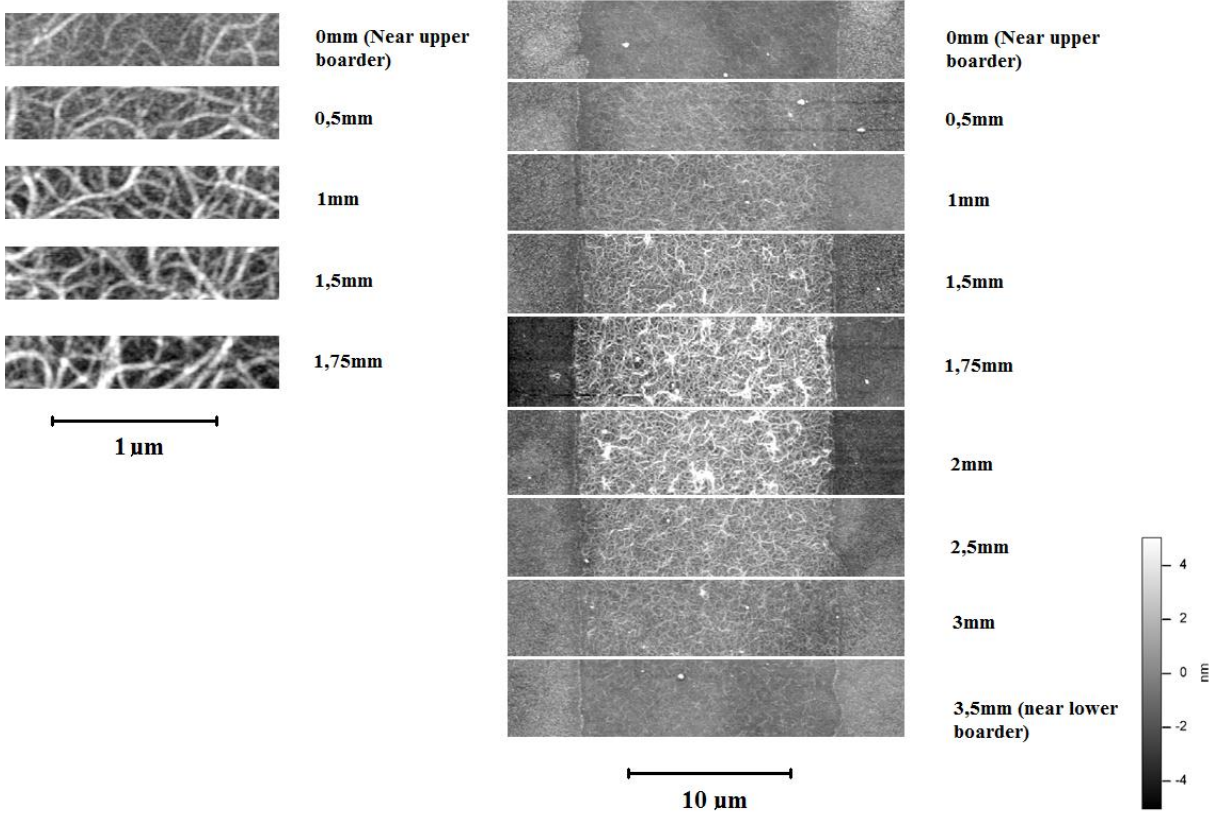
Incubation time 120 minutes
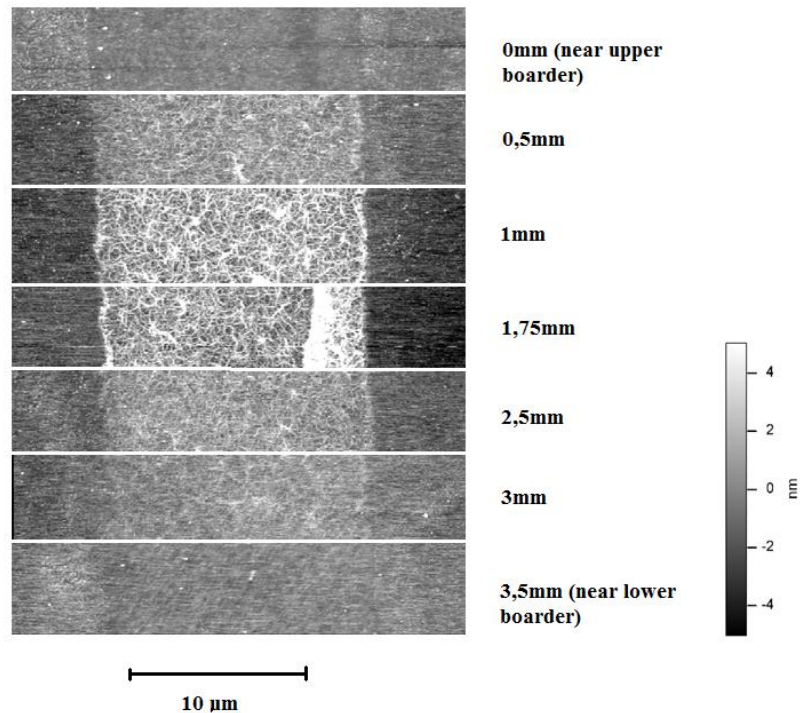
Figure 69: Montage consisting of AFM pictures taken of a sample made by using protocol 2 c) with an incubation time of 120 minutes in chamber with evaporating ammonia. The numbers to the right of each picture indicate position in the channel with the upper pictures position as reference.

This sample had very similar characteristics with the corresponding with a 30 minutes incubation time. A thin layer of collagen was found at the boarder of the stripe, increasing in height towards the middle of the channel. The sample had certain symmetry about the center (1,75mm), with the highest layer of collagen documented at this point. However it seems like a higher layer of collagen could exist between 1mm and 1,75mm. The height of the collagen layer can be seen in Figure 70. Zoom in pictures to measure the thickness of the fibers were not taken, but it is very likely that we would find a characteristic similar to what is obtained in Figure 65.
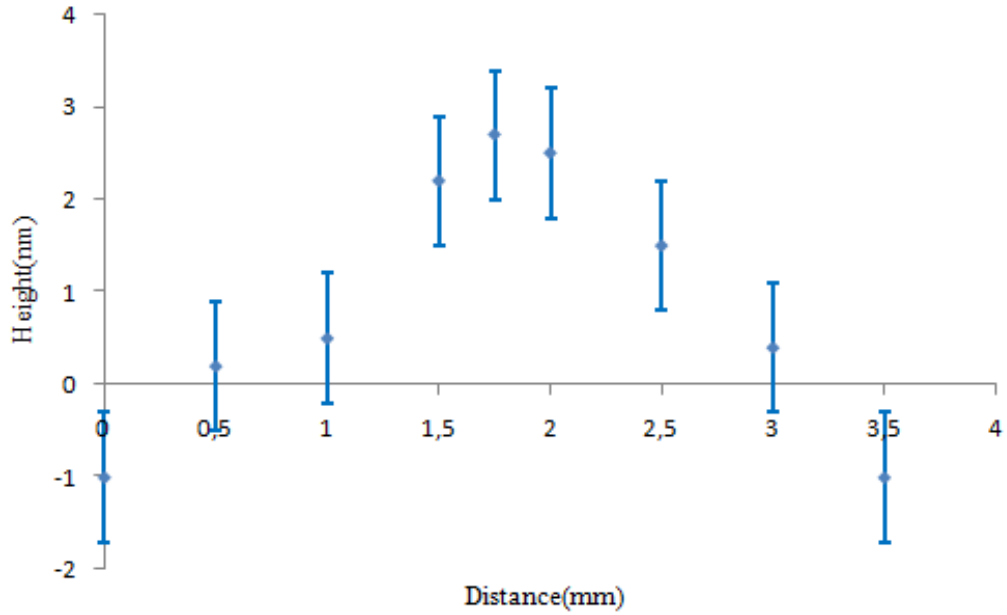
89
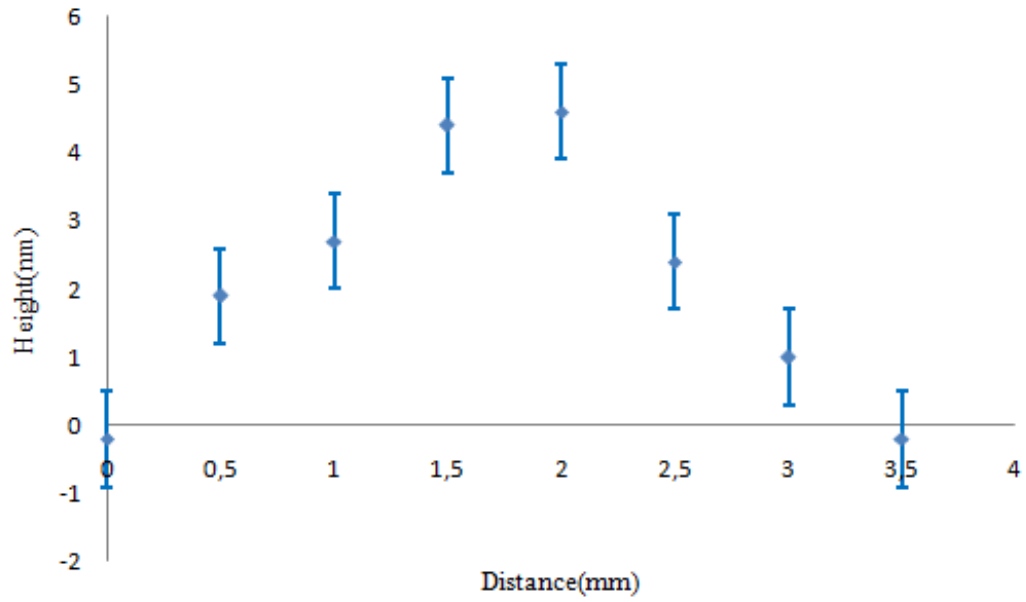
Figure 70: Height of collagen layer-- sample made by using protocol 2 b) - 120min incubation time. *Standard deviation used for error bars are taken from Table 1.*
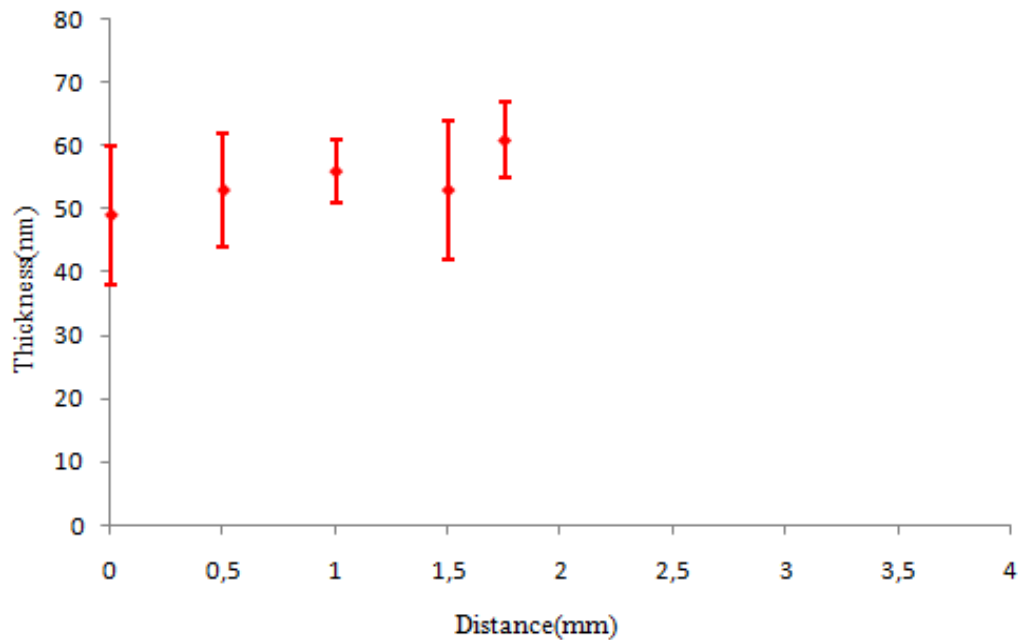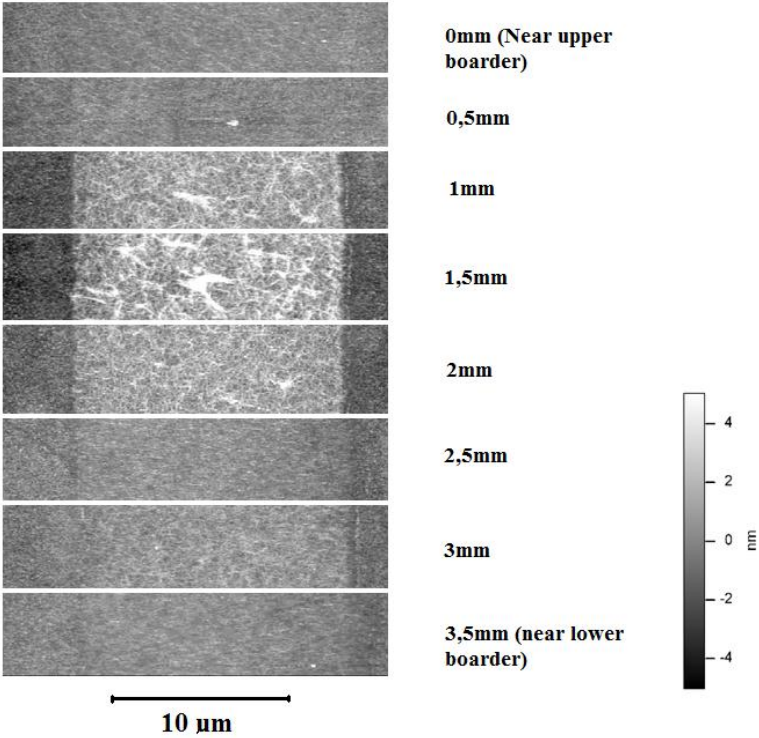
Incubation over night
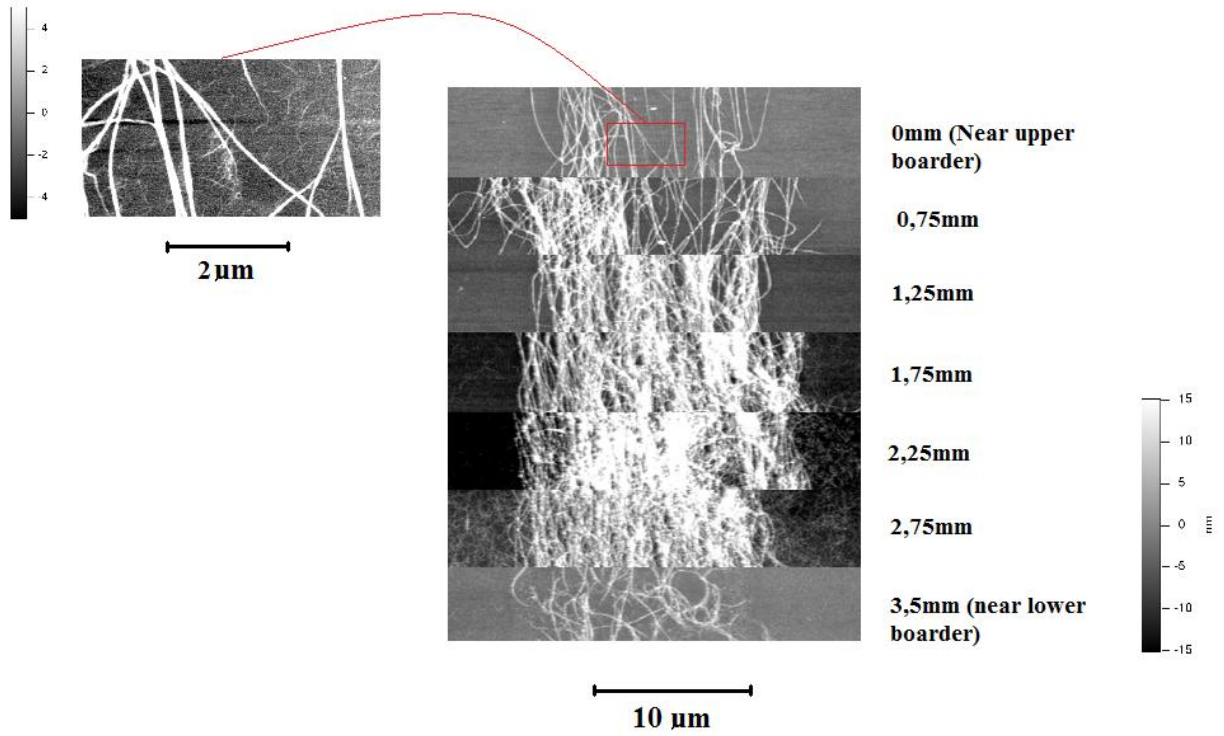
Figure 71: Montage consisting of AFM pictures taken of a sample made by using protocol 2 c) with incubation over night in chamber with evaporating ammonia. The numbers to the right of each picture indicate position in the channel with the upper pictures position as reference.

This sample had a very long incubation time, and the outcome was also completely different from what were observed at short incubation times (less than 2 hours). A relatively small amount of fibers were found at the boarders of the sample. Towards the middle of the channels the height of the collagen layer was increasing, giving certain symmetry in height about the center (1,75mm). Height of the collagen layers are shown in Figure 72. The zoom in picture done at 0mm shows that there is a layer of small collagen fibers which are covered by bigger fibers. Probably this is the case throughout the channel. The measurements of the thickness of collagen fibers (Figure 73) indicate a slight increase in the thickness of the fibers from the boarder towards the middle of the sample. The underlayer of small fibers is not considered in the measurements shown in Figure 72 and Figure 73.
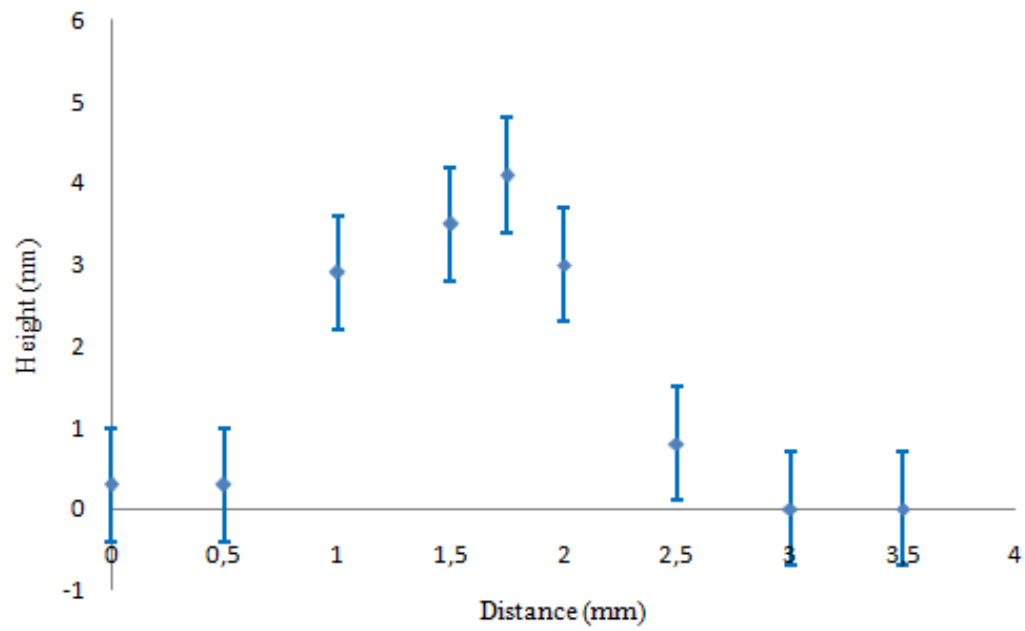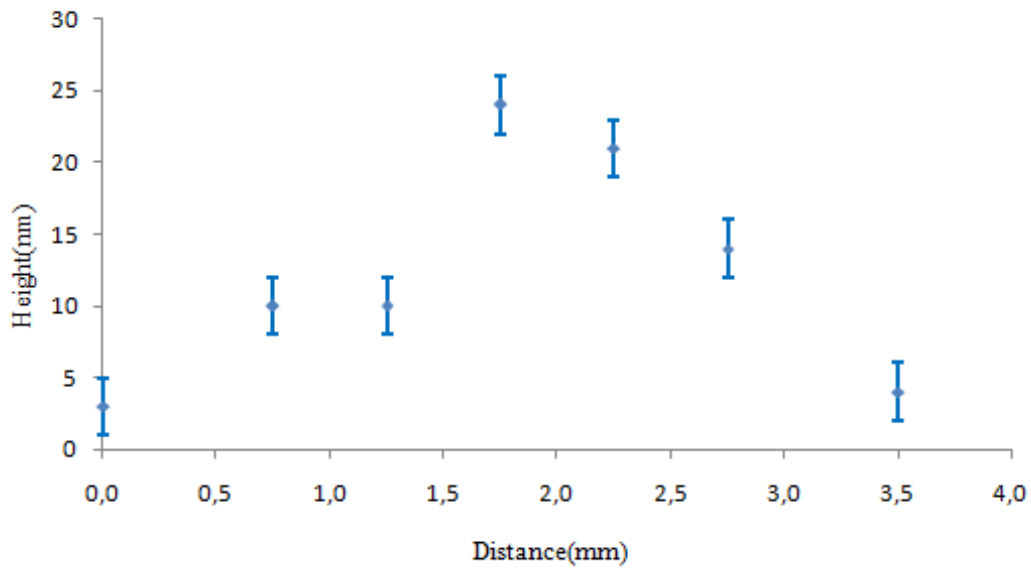
***Figure 72 : Height of collagen layer-- sample made by using protocol 2 b) – Incubated over
night.*** *Standard deviation used for error bars are taken from Table 1.*
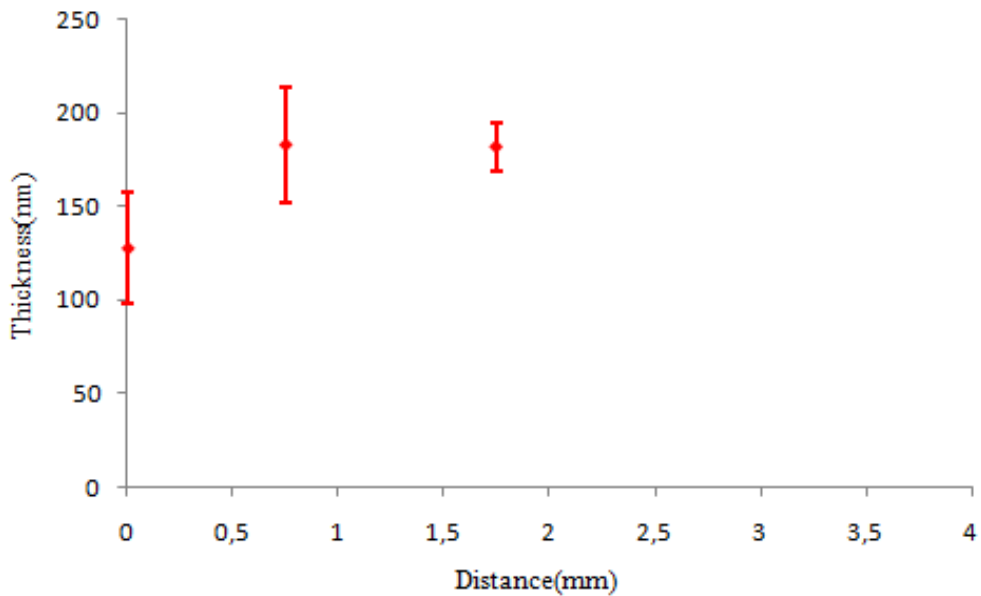
***Figure 73: Thickness of collagen fibers-- sample made by using protocol 2 b) – Incubated over
night.*** *Standard deviations used as error bars are based on 5 measurements each.*

3.3 Uncertainties in cell measurements

To investigate the uncertainty caused by ImageJ measurements, the length of the same single cell were measured 20 times. This gave an average length of 61,5 µm and a standard deviation of 1,7 µm. This uncertainty do not influence the graphs presented significantly. Therefore it was excluded from further analysis. All measurements of this cell (seen in Figure 74) are found in the Appendix F.

200 µm
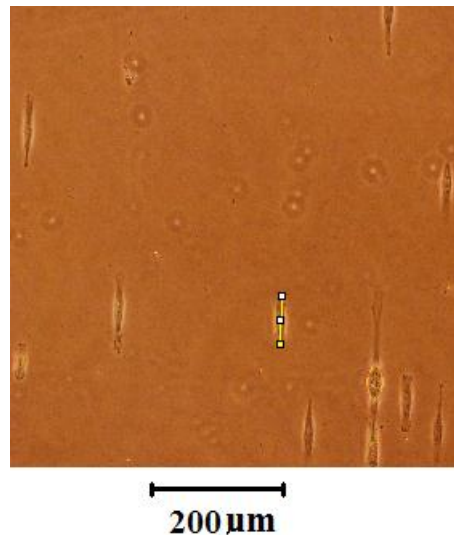
Figure 74: ImageJ used to measure the length of a cell. *This cells length was measured 20 times, and this data were used to calculate a standard deviation.*

Normalizing of cell graphs

The speed of the cell adhesion and the size of the cell before are found to be correlated. Bigger cells typically spread faster. Ideally we would like all the cells used in these experiments to have the same size, but this is not the case. To compensate normalization has been done for some of the graphs presenting cell spreading. The unit of length is in those cases the average of the cells size (before spreading). Improvement using normalization is shown in Figure 76, which is a normalized version of Figure 75.

3.4 Results: cells spreading on collagen matrices

3.4.1 Collagen from PBS buffer

No underlaying BSA

Figure 75: Extra BSA layer - Length of cells as function of time - 11 cells. *The black curve shows average length of the cells measured at that particular time and the corresponding standard deviation. Error bars for the individual cells were too small to show up on the graph. Height of collagen layer is -0,8nm (SD=0,7nm) and thickness of fibers are 36nm (SD=8).*

This graph is based on length measurements of 11 cells, were 6 cells are tracked during 120 minutes of spreading and 5 cells for 135 minutes. All the marked points on the black curve except at 0 minutes consist of an average of either of those two groups of cells. The black curve has a few peaks located at 30, 60 and 120 minutes. Those are for the most part caused by the cell

marked with dark red in the graph. This cell spreads significantly faster than the other cells, and all the peaks are at times were this cells average are included in the measurement. The cell marked with a purple line also spreads relatively fast. When looking at the cells size before spreading (at time 0), we see that those two cells were the two biggest. This trend is the reason why it was decided to normalize the graphs when comparing cells spreading at different substrates. Sample cells are put on are seen in Figure 50.

No underlaying BSA normalized
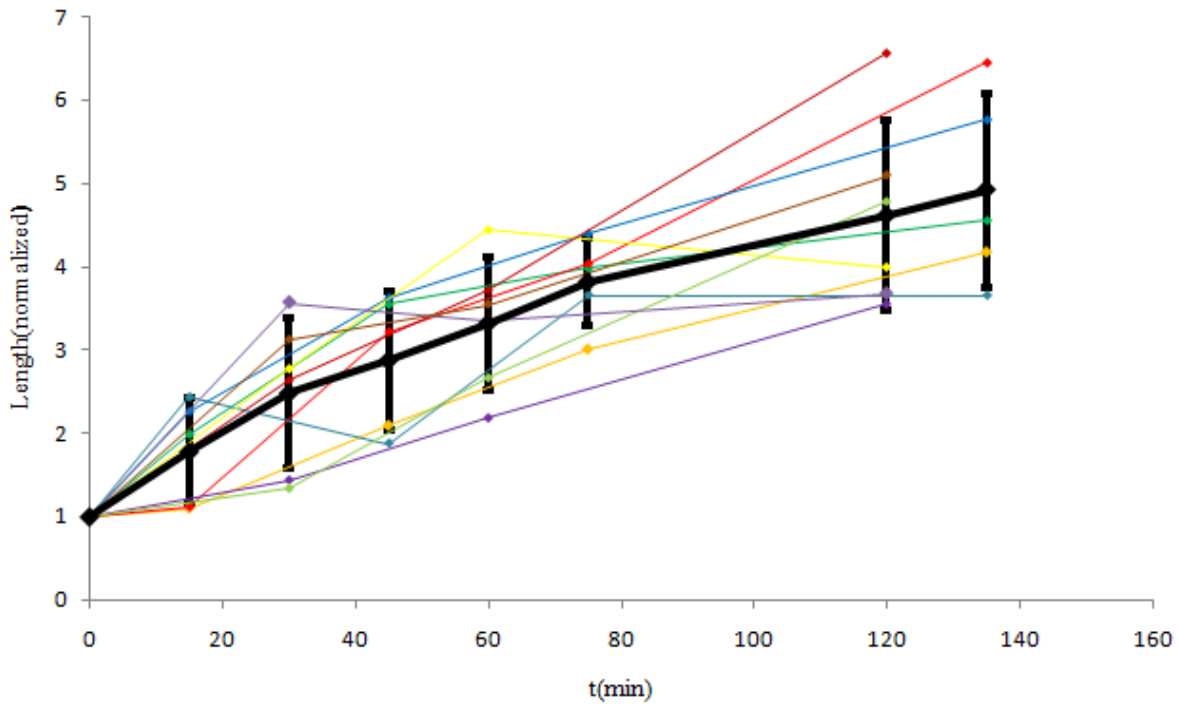
Figure 76 : Extra BSA layer – Normalized length of cells as function of time - 11 cells. *The black curve shows average of the normalized lengths of the cells measured at that particular time and the corresponding standard deviation. Error bars for the individual cells were too small to show up on the graph. Height of collagen layer was -0,8nm (SD=0,7nm) and thickness of fibers where 36nm (SD=8nm).*

This normalized graph is based on length measurements of 11 cells, were 6 cells are tracked during 120 minutes of spreading and 5 cells for 135 minutes. All the marked points on the black curve except at 0 minutes consist of an average of either of those two groups of cells. Figure 76 are based on the same measurement as Figure 75. We see that in this normalized version has a little less dispersion between the cells. This is especially clear when comparing the two figures at 120 minutes. At this particular time Figure 75 had measurements ranging from 81µm to 225µm. The normalized graph had values ranging from 3,6 to 6,6. With an average cell size before spreading of 24µm the values for the normalized graph corresponds to a ranging from 86µm to 158µm. Cells used at this sample had initial sizes ranging from 17µm to 39µm.

With underlaying BSA
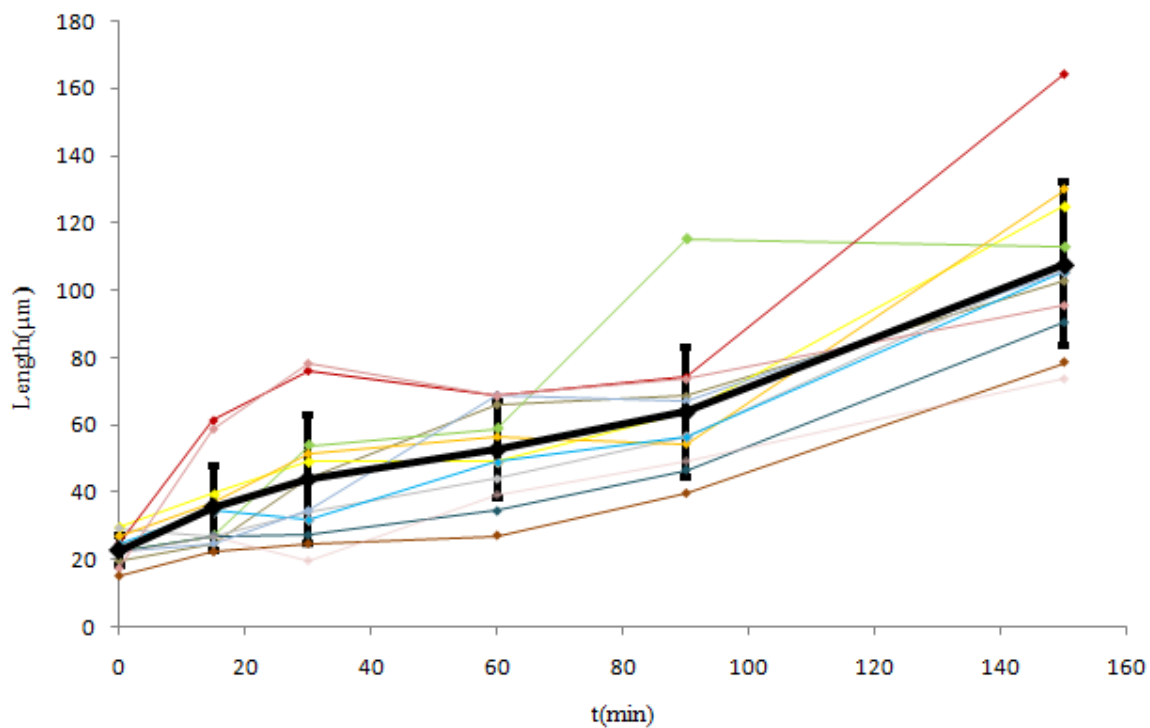
Figure 77: No extra BSA layer - Length of cells as function of time - 12 cells. *The black curve shows average length of all cells and the corresponding standard deviations. Error bars for the individual cells were too small to show up on the graph. Height of collagen layer was 1,5nm (SD=0,7nm) and thickness of fibers where 59nm (SD=6nm).*

The sample used for those cells are shown in Figure 49. The 12 cells considered on this sample had an initial size ranging from 15µm to 29µm and after 150 minutes of spreading the cells sizes were in between 74µm and 164µm. So slightly less dispersion is seen for those cells than the ones shown in Figure 75.

3.4.2 Deposition of collagen assisted by microchannels
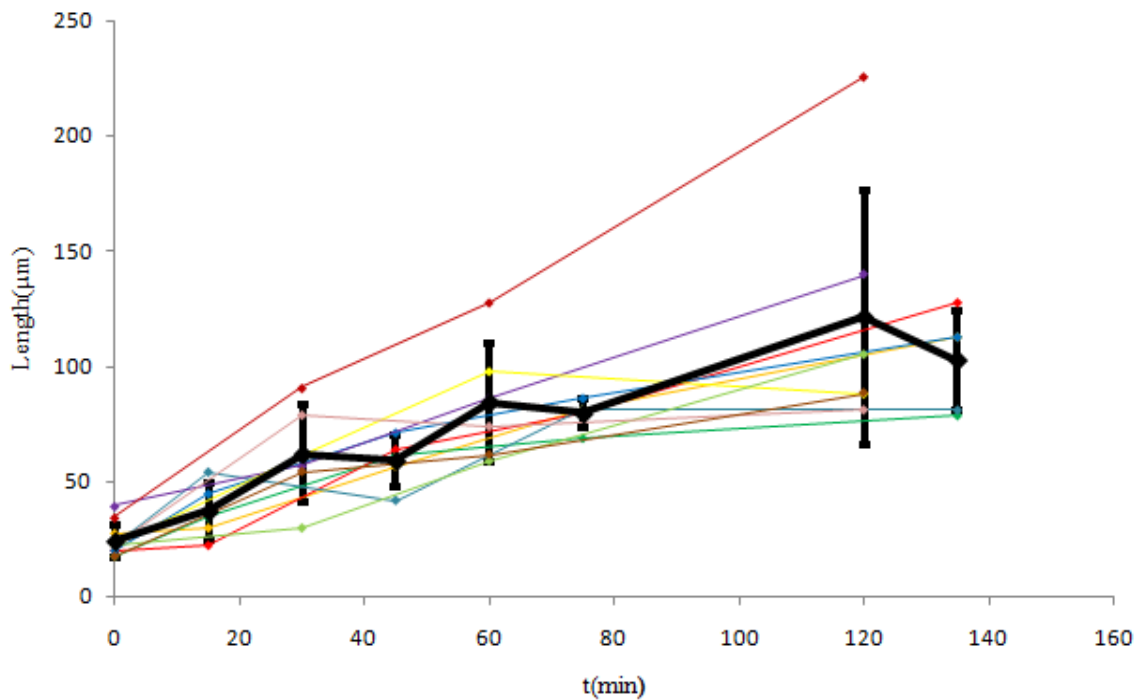
Sample made using chamber with evaporating ammonia - boarder
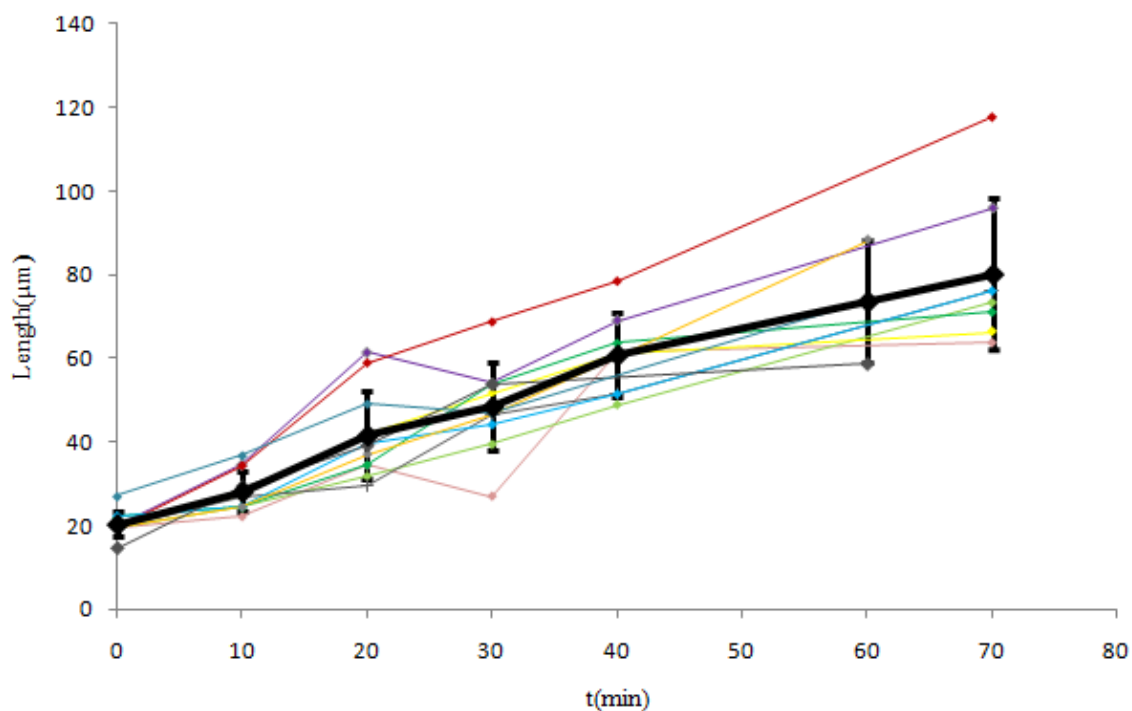
Figure 78: Length of cells as function of time - 11 cells. *The black curve shows average length of the cells measured at that particular time and the corresponding standard deviation. Error bars for the individual cells were too small to show up on the graph. Height of collagen layer is between -1nm (SD=0,7nm) and 0,4nm (SD=0,7nm).Thickness of fibers are between 49nm (SD=11nm) and 53nm (SD=9nm).*

Cells spreading on this sample had an initial size ranging between 15µm and 27µm. Of the 11 cells that were investigated, 8 cells were followed for 70 minutes, and 3 cells for 60 minutes. After 70 minutes the lengths of the cells were found to be between 64µm and 118µm.

Sample made using chamber with evaporating ammonia - center
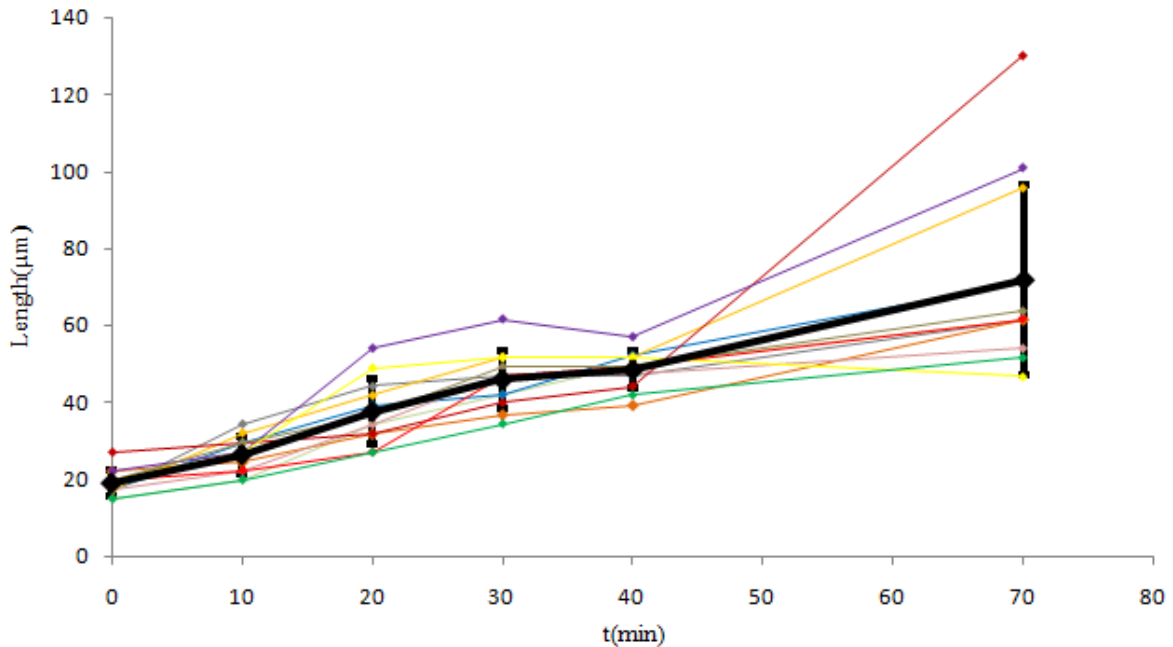
Figure 79 : Length of cells as function of time - 12 cells. *The black curve shows average length of all cells and the corresponding standard deviation. Error bars for the individual cells were too small to show up on the graph. Height of collagen layer is between 2,2 nm (SD=0,7nm) and 2,7nm (SD=0,7nm). Thickness of fibers is between 53nm (SD=11nm) and 61nm (SD=6nm.)*

Cells spreading on this sample had an initial size ranging between 15µm and 27µm. The 11 cells that were investigated were followed for 70minutes. After 70 minutes the lengths of the cells were found to be between 47µm and 130µm.

3.4.3 Comparison of cells spreading on collagen layers with different height
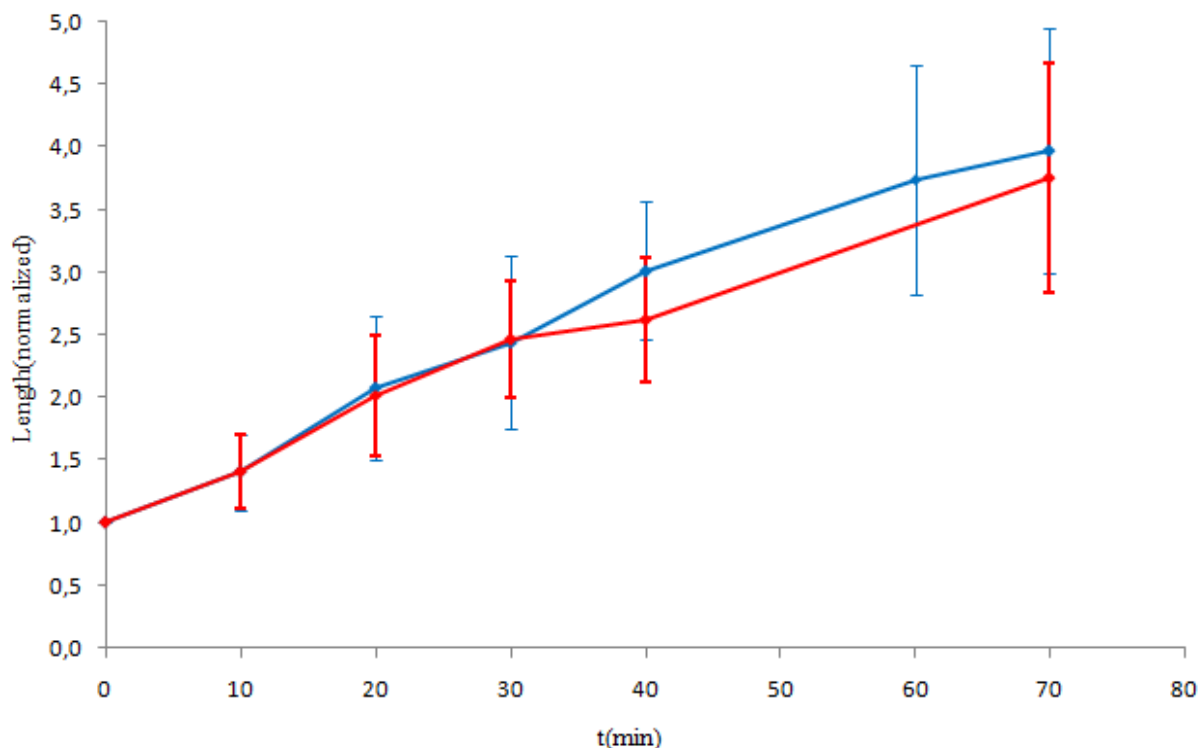
Figure 80 : Normalized length of cells as function of time – comparison 1. Sample surfaces made using protocol 2b) Red curve (average 12 cells): Height of collagen layer is between 2,2 nm and 2,7nm. Thickness of fibers is between 53nm and 61nm. Blue curve (average 11 cells): Height of collagen layer is between -1 nm and 0,4nm. Thickness of fibers is between 49nm and 53nm.

Normalized graphs for cells spreading at different height of collagen are provided in Figure 80. The red line indicate average spreading of 12 cells spreading at collagen were height of the collagen layer is between 2,2 and 2,7nm and the size of the collagen fibers are varying between53nm and 61nm. The blue line indicate the average spreading of 11 cells spreading at collagen were height of the collagen layer is between -1nm and 0,4nm and the size of the collagen fibers are varying between 49nm and 53nm. The two curves show little difference between cells spreading at the two different substrates. The statistics are relatively weak, and the difference in heights of the collagen layers are not as large as preferred.

3.4.3 Comparison of cells spreading with and without underlaying BSA
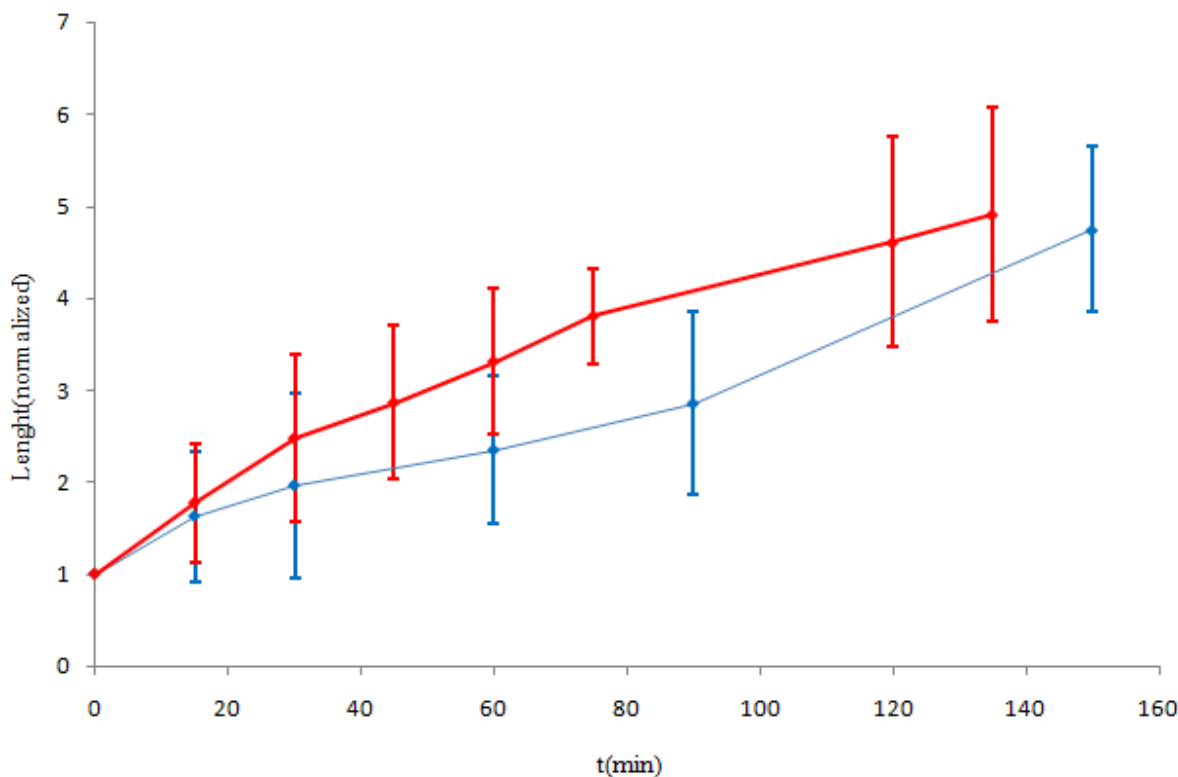
Figure 81: Normalized length of cells as function of time – comparison 2. Sample surfaces made using protocol 2a). Red curve (11 cells): Collagen on layer of BSA – Height of collagen layer is -0,8nm and thickness of fibers are 36nm (SD=8). Blue curve (12 cells): Collagen not on a layer of BSA-Height of collagen layer is 1,5nm.Thickness of fibers are 57nm (SD=6).

Normalized graphs for cells spreading on collagen with and without an underlayer of BSA are provided in Figure 81. The red line indicates 11 cells spreading with an underlayer of BSA. The collagen fibers found at this sample (see sample in Figure 49) had an average thickness of 36nm and a height of collagen layer of -0,8nm. The blue line indicates 12 cells spreading without an underlayer of BSA. The thickness of the fibers found at this sample (see sample in Figure 50) were 57nm and the average height of the collagen layer was 1,5nm.

We see that the cells spreading on the thinnest fibers (and underlayer of BSA) indicated by the red line spread faster than the cells spreading on the thicker fibers (no BSA underlayer) indicated by the blue line. The difference between the two is especially significant between 30 and 75 minutes. After 75minutes the blue line starts to spread a little faster than the red. It is possible that it is a trend found, but the statistics are relatively weak so no strong conclusion can be drawn.

Conclusion and outlook

In this work new protocols have been developed, to fabricate micro-structured collagen matrices that can be used to study cell spreading as function of the matrix structure. The fabrication of matrices assisted by micro-contact printing and by in situ polymerisation of collagen in micro-channels leads to a better control on matrix structure and consequently a control of the communication between cell and matrix. Relatively strong statistics on deposition of BSA from hydrophilic and hydrophobic stamps using micro contact printing were obtained. These results indicated a significantly higher layer of BSA using hydrophobic stamps (3,6nm) than hydrophilic stamps (1,2nm). The concentrations of glutaraldehyde used to crosslink BSA to the substrate did not seem to influence the results significantly. Collagen was deposited on the samples using several different techniques, and this resulted in collagen fibers ranging in thickness from 36 to 248nm. The heights of the layers of collagen at different samples varied from lower that the deposited BSA (hydrophobic stamps used) to 24nm. This is an interesting achievement to be able to control the thickness of the collagen matrix: fibers adsorbed directly on the glass surface mimic a stiff matrix. On the other hand, a thin layer of collagen, i.e. a collagen gel, will present a mechanical compliance to the pulling force applied by cells. In this study, it no effect is seen on cell-spreading dynamic, but for other cell types sensitive to another range of mechanical forces, theese samples could be a useful tool. If the fabrication of some samples was robust, the reproducibility of some of the others has been a challenge, and especially so for those of the samples with large collagen fibers. First, this means that there is room to develop various protocols and various matrix structures. For the samples for largest fibers, but also other it has occurred that collagen fibers were not well confined on micro-stripes after removing the stamp, i.e. fibers were on the top of the printed BSA layer. This enables the cells to adhere outside the gaps between the BSA stripes and this make them more difficult to analyze. For those reasons the samples with the largest fibers were not included in the experiments with cells. The experimentation with cells was then performed on samples only presenting small differences in size and amount of fibers. Only tiny differences in cell adhesion speed were found. A way to develop reproducible samples with larger fibers exclusively located in between the BSA stripes would be interesting for investigating if the cells behaviour changed using this type of

microenvironment. A possible way to do this could be to this could be more experimentation with the use of collagen polymerization in PBS buffers of varying pH values and temperature. A little bit of experimentation has already been done here during this project, so far without any significant results. Exchanging collagen with another type of ECM as for instance fibronectin could also reveal a different cell behaviour. The attempts made in this project to bind collagen from a PBS buffer solution on top of a BSA-layer using glutaraldehyde, has indicated that the fibers do not bind as well to this surface as they do without the BSA layer. Improvement here would be preferable, because printing on top of the BSA underlayer tends to give denser BSA print. A possible solution to this could be to exchange glutaraldehyde with EDC/NHS. EDC/NHS is a crosslinker which is used together with collagen in other studies [23]. Higher resolution in the AFM imaging could also be helpful, especially when dealing with tiny collagen fibers and for analyzing the fibers internal structure. Asylum Research California, USA has developed bimodal imaging. This is a new imaging technique which includes a frequency near the second order resonance frequency (in addition to near the first order resonance which is the case in traditional tapping mode) when setting the drive frequency for the piezoelement. This technique provides increased contrast and unlike normal tapping mode, high resolution at extremely gentle pico-Newton level forces. The technique is still being improved, and it could be a really helpful tool for analyzing small fibers with less error, and also to characterize the internal structure of the collagen fibers.

If the type of microenvironment presented in this thesis is improved sufficiently it could possibly be a tool for diagnostics of oral cancer, since cell spreading could be a marker of cancerous state of cells. Much due to the use of tobacco and alcohol [25] this is large a large problem worldwide. Oral cancer counts for 3% of all diagnosed cancer, and more than 300 000 cases of oral cancer are being diagnosed every year [26]. Some reports have also indicated lower survival rates for younger patients than older, which makes the problem even more disturbing [27]. Experiments using this type of environment to compare cancer associated cells with normal oral fibroblasts have already revealed some differences between the behaviors of the two cell types [1]. A further development of the environment based on some of the suggestions in this section could increase those differences and contribute to develop faster detection and a better treatment of the affected patients.

Appendix A

PROTOCOL 1: Aminosilanation of glass coverslips

1. Take the number of coverslips you want to prepare and place them in a special designed Teflon holder. Each holder can contain a maximum of 10 coverslips. Place the holder(s) in a glass container and add distilled water until the entire Teflon holder(s) is covered. Place the container in an ultrasonic device for 20 minutes at 50 ℃ and 100% power.

2. Replace distilled water with an Extran solution. Extran is diluted 1-10 with distilled water, and this solution is used to clean the cover slips. Put the container back in the ultrasonic device for 20 minutes at 50 ℃ and 100% power.

3. Pour the extran solution back. This can be reused the same day. Rinse the cover slips 6 times in distilled water, and then leave the slips in distilled water for 30 minutes. During this time you prepare the aminosilylation solution in a beaker.

4. Aminosilylation solution consist of:
   - 5 ml MilliQ water
   - 6 µl acetic acid
   - 94 ml methanol
   -1 ml (3-Aminopropyl)triethoxysilane

5. Incubate the slips in this solution for 15 minutes.

6. Turn the incubator on at 120℃, so it is ready for step 7.

7. Discard the solution in special waste, and the add methanol in the beaker to rinse. Wait 2 minutes, and discard the methanol in special waste. Repeat one time.

8. Place the slips in the incubator at 120°C for 10 minutes.

Appendix B

PROTOCOL 2: Making samples with micro contact printing

1. Remove the master from the cured PDMS, and cut out the parts with patterns. Use 3mm thick stamps if making micro channel sample and 1mm if adding collagen solution diluted in PBS.

2. Place the PDMS with striped pattern in a petri dish with the striped side up. Look at it in the microscope and make sure you have the desired stripe with. Keep track of the direction of the stripes.

3. Put a special designed rectangular glass piece on the top of the PDMS. The glass pieces long sides should be perpendicular to the stripes in the PDMS. Now cut along each long side of the glasspiece to make channels with constant length (4mm long channels is used here). The lengths of the channels are only important for the samples with ammonia and collagen solution inside the channels because those samples aren't uniform. When making uniform samples the exact lengths of the channels are not important and you can cut rectangular pieces without using the glass piece as reference.

4. Place the prepared PDMS stamps in a petri dish. And put them into vacuum for at least 30 minutes.

5. While waiting: Place half of the aminosilanated cover slips you are going to prepare on a parafilm in front of you. Add 200µl of glutaraldehyde solution with desired consentration (2,5% in PBS is the standard) on each of them. Place the rest of the aminosilanated cover slips on top of them so they form pairs of cover slips with glutaraldehyde solution in between (sandwiches). Let them stay like this for 15 minutes to incubate.

6. After 15 minutes in glutaraldehyde: Rinse the samples with distilled water. Add 1 ml water in between the cover slips and aspirate away. Repeat 5 more times. Leave them as sandwiches with water in between.

7. If you don't want a BSA layer on your cover slips you skip step 8 and 9.

8. Dry the samples using nitrogen under pressure and repeat step 5 and 6 exchanging the glutaraldehyde solution with BSA(1% in PBS). Make sure that it is the same side that is incubated with glutaraldehyde that is incubated with BSA . This results in coverslips with a BSA layer.

9. Repeat step 5 again. Make sure that it is the same side as before that is incubated with glutaraldehyde this time.

10. While waiting for coverslips: Prepare a parafilm with 200µl droplets of BSA (1% in distilled water) equal to the number of stamps prepared. Put one stamp on top of each droplet with the patterned side in contact with the droplet. Make sure that the whole patterned area is in contact with the BSA solution. Leave the stamps here for 15 minutes to incubate.

11. After 15 minutes in glutaraldehyde: Do step 6.

12. Dry one cover slip and place it on a parafilm with the BSA (aldehyde groups) surface up.

13. After stamps have incubated for 15minutes on droplets: Take one piece of PDMS and place it in a petri dish with patterned side up. Make sure that it sticks to the petri dish by pressing gently on it at the side. Pour distilled water in the petri dish and aspirate right away. Dry the stamp quickly and gently stamp it on the cover slip prepared in step 12. Make center the PDMS stamp on the cover slip. Repeat step 12 and 13 until all the cover slips are stamped with BSA pattern.

14. Leave the stamps on the cover slips to incubate. Draw black dots on the back to indicate the stamps position.

a) Only for samples with collagen in a PBS solution

1. After 30 minutes incubation: Add a circular PDMS chamber with diameter 15mm around the stamp. Pour 200μl PBS in the hole and gently remove the stamp with a scalpel. Repeat this for all samples.

2. Make a mixture of collagen solution and PBS (1:10). Add 100μl of this solution as droplets in the center of the chamber, and incubate for 15 minutes.

3. After 15 minutes: Remove the PDMS chamber, and rinse first 3 times with 1ml PBS and then 3 times with water aspirating away each time.

4. Dry the samples and store them in small petri dishes for further use either with cells or for AFM imaging.

b) Only for samples in ammonia chamber

1. After 15 minutes of incubation: Put the samples in petri dishes and put them in the vacuum chamber for 30 minutes.

2. After 30 minutes in vacuum: Fill a small petri dish with 4ml fresh ammonia solution (25% in water) and place it on a parafilm. Take the stamps out of vacuum one at the time and put them on the light microscope stage. Add 15 μl collagen solution from stock as a droplet in contact with all the channels entrances at one side. Do the same on the opposite side, and watch in the light microscope as the solutions flow from both sides and meet in the middle. Repeat this for all the samples.

3. Place the samples next to the small petri dish prepared with ammonia and close the chamber with a large petri dish that you hold upside down and press down to cover the

samples and the small petri dish. At the same time as you press the petri dish you slightly turn it. This prevent the area inside the chamber from contact with surrounding air, and the samples are now exposed to evaporating ammonia.

4. After desired time, open the chamber and remove the small petri dish. Add 1ml PBS so it surrounds the sample and remove the stamp. Rinse with three times with 1ml PBS and three times with 1ml distilled water and aspirate away each time. Do this for all samples.

5. Dry the samples and store them in small petri dishes for further use either with cells or for AFM imaging.

c) Only for samples with ammonia and collagen in channels

1. After 15 minutes of incubation: Put the samples in petri dishes and put the petri dishes in the vacuum chamber for 30 minutes.

2. After 30 minutes in vacuum: Take the stamps out of vacuum one at the time, place a small piece of glass so it cover the top part of the PDMS stamp, and put the sample on the light microscope stage. Add 15 µl collagen solution from stock as a droplet in contact with all the channels entrances at one side. Do the same on the opposite side with ammonia solution with desired concentration, and watch in the light microscope as the solutions flow from both sides and meet inside the stamps. After solutions have met in most of the central channels (it flows faster in the central channels) start to take the time. Repeat this for all the samples.

3. After 15 minutes: Add 1ml PBS so it surrounds the sample and remove the stamp. Rinse three times with 1ml PBS and three times with 1ml distilled water and aspirate away each time. Do this for all samples.

4. Dry the samples and store them in small petri dishes for further use either with cells or for AFM imaging.

Appendix C

PROTOCOL 3: Splitting of cells

1. Aspirate the medium around the cells in the Petri dish.

2. Add 10ml PBS to rinse. Aspirate right away.

3. 3. Add 3ml trypsin, and put the petri dish in the incubator for 1-2 minutes. This is done for the cells to detach the surface.

4. Tilt the petri dish a little without spilling. Aspirate at the bottom and release at the top five times. Make sure that the cells detach. If they don't, incubate for one more minute.

5. Add the cell suspension to a tube, and add 12 ml medium (DMEM with 10% FBS) Centrifuge this tube at 1000rpm for 2 minutes. After centrifuging the cells should be seen at the bottom of the tube.

6. Aspirate away the liquid above the cells without aspirating any cells. Add 5ml new medium (DMEM with 10%FBS) in the tube. Aspirate at the bottom and release at the top a couple of times to get a homogeneous distribution. Bubbles should be avoided as much as possible.

7. Prepare two new petri dishes with 10ml medium (DMEM with 10% FBS) in each. Add 1/5 (1ml) of the cell suspension to one petri dish and 1/3 (1,7ml) of the cell suspension to the other. Write on the top of the new petri dishes your name, date and the number of splittings the new cell cultures has been through.

8. Check in the microscope that the new petri dishes contain a nice distribution of cells, and place them in the incubator for storage. The tube with cell suspension can be stored in the

refrigerator for a couple of hours. Make sure to write the time and date on it before putting it in the refrigerator.

9. This procedure should typically be repeated after 2 days for the 1/3 solution and 3 days for the 1/5 solution.

Appendix D

PROTOCOL 4: Preparing a sample for cell experiment

1. Take the same number of petri dishes with circular holes (diameter=15mm) as samples you want to add cells to.

2. Make a mixture of 0,5ml blue and 0,5ml yellow liquid (this forms a glue) and add this at the bottom side of the petri dishes as close to the holes as possible using a pipette tip. Because the glue will stiffen within five minutes a maximum of 2 petri dishes should be added glue to before step 3 is done.

3. Add the cover slips with micro pattern on the prepared petri dishes. Make sure that it is the side of the cover slip with micro pattern that is in contact with the glue. Center the patterned area in the hole, and make sure no glue comes here. Gently press at the back side of the cover slip so that the glue covers the whole area around the circle. This is important to avoid leakage.

4. If you have more than two samples repeat step 2 and 3until all your coverslips are mounted.

5. Take a number the special designed glass pieces stored in 70% ethanol equal to the number of samples you have. Wipe the glass pieces with some paper to dry them before use.

6. Distribute some paper to working surface in front of you. Make a new porsion of glue. Place a petri dish (with cover slip mounted on the back) on the working space with the back of the cover slip in contact with the paper. Add two small droplets of glue on the petri dish at the boarder of the circular hole, and add a glasspiece on the top. The

glasspiece should be glued so that you have access to the area under the glasspiece with a pipette from two sides. Do this for all your petri dishes and glass pieces. If the glue dries out, make new.

7. Put the prepared samples in larger petri dishes for protection until they are used with cells.

Appendix E

PROTOCOL 5: Adding cells to sample and imaging

1. Mount the camera on the microscope and make sure that the setup is ready so that pictures can be taken. It is the 20× phase contrast objective that should be used for this.

2. Take the samples prepared for cell experiments and add 200 µl of medium (DMEM with 2% Pen Strep) below the glass piece in the hole were the slip is mounted. Pen Strep is added in the medium to prevent bacterial contamination on the sample.

3. Make a 1/10 dilution of cells with medium (DMEM with 10% FBS and 2% Pen Strep).

4. Take two pipettes and adjust them both to 100µl. One is used to pipette cell suspension in under the glass plate at one side, the other is used to simultaneously remove DMEM at the opposite side. Repeat this 3 times.

5. Place the samples in the incubator (37°C , 5% CO2).

6. After 15 minutes in the incubator you can start to take set of pictures of the sample surface with the desired intervals. Put the samples back in the incubator after each set of pictures.

Appendix F

Measurements used for uncertainty calculations

Height of BSA is the domineering uncertainty

Measurements (nm):

1. 3,2
2. 3,4
3. 3,4
4. 3,5
5. 3,3
6. 3,9
7. 3,9
8. 3,8
9. 3,5
10. 3,8

Mean: 3.6nm

SD: 0.3nm

Height of BSA is the collagen is the domineering uncertainty

Measurements (nm):

1. 9
2. 11
3. 12,5
4. 8
5. 11,5

Mean: 10,5nm

SD: 2nm

Measurements of length of one cell in using ImageJ

Measurent nr.	Length (µm)
1	61.3
2	61.5
3	61.3
4	61.3
5	64.1
6	63.9
7	63.9
8	61.3
9	59.0
10	59.0
11	63.9
12	59.0
13	61.3
14	61.3
15	61.5
16	61.3
17	61.3
18	61.5
19	63.8
20	58.8
Mean	**61.5µm**
SD	**1.7µm**

Appendix G

All measurements of heights of BSA layers

Here all the measurements used to create Table 2 on page 72 follows. In the type description it is seen if the PDMS used were hydrophobic (phob) or hydrophilic (phil), and the glutaraldehyde concentration (diluted in PBS) used is given as a number afterwards. Types marked BSA on BSA indicates stamping of BSA on another BSA layer. In these cases the glutaraldehyde concentration given is used both to bind the first layer to the cover slip, and the second BSA layer to the first (through micro-contact printing). Average (AVG) is given for each type. The sample row indicate how many samples were used to measure this particular type of sample, and also how many pictures were used from each of the samples. All measurements uses are based on at least 10µm of boarder of the printed BSA.

type	height (nm)	sample		type	height (nm)	sample
phob 0,625	4,3	a		phil 0,625	1,5	a
phob 0,625	4,1	a		phil 0,625	1,2	a
phob 0,625	3,7	a		phil 0,625	1,0	a
phob 0,625	3,4	a		phil 0,625	1,4	a
phob 0,625	3,8	b		phil 0,625	1,2	b
phob 0,625	3,5	b		phil 0,625	1,4	b
phob 0,625	3,7	b		phil 0,625	1,3	b
AVG phob 0,625	**3,8**	**2 samp**		**AVG phil 0,625**	**1,3**	**2 samp**
phob 1,25	3,2	a		phil 1,25	1,5	a
phob 1,25	3,2	a		phil 1,25	1,1	a
phob 1,25	3,1	a		phil 1,25	1,2	a
phob 1,25	3,0	a		phil 1,25	1,5	a
phob 1,25	4,1	b		phil 1,25	0,9	b
phob 1,25	4,2	b		phil 1,25	0,7	b
phob 1,25	4,4	b		phil 1,25	0,9	b
AVG phob 1,25	**3,6**	**2 samp**		**AVG phil 1,25**	**1,1**	**2 samp**

phob 2,5	4,4	a		phil 2,5	1,6	a
phob 2,5	3,2	a		phil 2,5	1,3	a
phob 2,5	3,8	a		phil 2,5	1,4	a
phob 2,5	3,7	a		phil 2,5	0,9	b
phob 2,5	2,5	b		phil 2,5	0,9	b
phob 2,5	2,9	b		phil 2,5	0,6	b
phob 2,5	3,3	b		**AVG phil 2,5**	**1,1**	**2 samp**
AVG 2,5 phob 2,5	**3,4**	**2 samp**				
phob 2,5 BSA on BSA	4,6	a		phil 2,5 BSA on BSA	1,5	a
phob 2,5BSA on BSA	3,9	a		phil 2,5 BSA on BSA	1,4	a
phob 2,5 BSA on BSA	4,8	a		phil 2,5 BSA on BSA	1,1	a
phob 2,5 BSA on BSA	3,3	b		**AVG phil 2,5 BSA on BSA**	**1,3**	**1 samp**
phob 2,5BSA on BSA	3,6	b				
AVG phob 2,5 BSA on BSA	**4,0**	**2 samp**				

Table A.1: All measurements of heights of BSA layers. *See Table 2 on page 72 for a summary of those measurements.*

List of references

1. C. F. Marthinussen, *Quantification of cell spreading in a confined microenvironment: A tool for cancer diagnostics?*, Master thesis in measurement science and instrumentation at university of Bergen, 2010.

2. B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, *Molecular biology of the cell*, 2008, 5th edition, Garland science, New York, 1-27.

3. B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, *Molecular biology of the cell, 5th edition*, 2008 Garland science, New York, 965-1005.

4. B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, *Molecular biology of the cell, 5th edition*, 2008, Garland science, New York, 1031-1085.

5. M. T. Sheu, J. C. Huang, G. C. Yeh, H. O. Ho, *Characterization of collagen gel solutions and collagen matrices for cell culture*, 2008, Biomaterials, vol 22, 1713-1719.

6. D. E. Przybyla, J. Chmielewski, *Higher order assembly of collagen peptides into nano- and microscale materials,* 2010, Biochemistry vol 49, 4411-4419.

7. F. Jiang, H. Hörber, J. Howard, D. J. Müller, *Assembly of collagen into microribbons: effects of pH and electrolyte,* 2004, Journal of structural biology, vol 148, 268-278.

8. B. Wickestad, K. Gull, T*he evolution of the cytoskeleton,* 2011, The journal of cell biology, vol 194, 513-525.

9. H. G. Döbereiner, B. D. Thaler, G. Giannone, H. S. Xenias, M. P. Sheetz, *Dynamic phase transistions in cell spreading, 2004,* Physical review letters, vol 93, 108105-08.

10. Y. L. Jeyachandran, E. Mielczarski, B. Rai, and J. A. Mielczaski, *Quantitative and qualitative evaluation of adsorption/desorption of bovine serum albumin on hydrophilic and hydrophobic surfaces, 2009,* American chemical society, vol 19, 11614-20.

11. Y. Yu, P. Q. Ying, G. Jin, *Competitive Adsorption between Bovine Serum Albumin and Collagen Observed by Atomic Force Microscope,2004,* Chinese Chemical Letters Vol. 15, 259-263.

12. Nano 160-Lecture20, B. Holst, 2010.

13. T. Stifter, O. Marti, B. Bhushan, *Theoretical investigation of the distance dependence of capillary and van der waals forces in scanning force microscopy*, 2000, Physical review B, vol 62, 13667-73.

14. Y. F. Dufrêne, *Atomic force microscopy, a powerful tool in microbiology*, 2002, Journal of Bacteriology, vol 19, 5205-13.

15. F. J. Giessib, *Advances in atomic force microscopy, 2003,* Reviews of modern physics, vol 75, 943-983.

16. N. C. Santos, M. A. R. B. Castanho, *An overview of the biological applications of atomic force microscopy,* 2003, Biophysical chemistry, 133-149.

17. E. T. Herruzo, R. Garcia, R. B. Proksch, I. Revenko, and M. G. Heaton, *Bimodal dual AC AFM imaging of collagen fiber ultrastructure,* 2011 Asylum research, Calefornia, USA, no journal.

18. Tap300 Datasheet, Innovative solutions Bulgaria Ltd, Sofia, Bulgaria.

19. R. Fuierer, *Asylum research Procedural operation manualette*, 2008, (AFM manual).

20. D. B. Weibel, W. R. DiLucio and G. M. Whitesides, *Microfabrication meets microbiology,* 2007, Nature reviews microbiology, vol 5, 209-218.

21. I. Migneault, C. Dartiguenave, M. L. Bertrand, and K. C. Waldron, *Glutaraldehyde: behaviour in aqueous solution, reaction with proteins, and application to enzyme cross-linking, 2004,* Biotechniques, vol 5, 798-802.

22. K. E. Sung, G. Su, C. Pehlke, S. M. Trier, K. W. Eliceiri, P. J. Keely, A. Friedl, D J. Beebe, *Control of 3-dimentional collagen matrix polymerization for reproducible human mammary fibroblast cell culture in microfluidic devices,* 2009 Biomaterials, vol 30, 4833-4841.

23. N. E. Vrana, N. Builles, H. Kocak, P. Gulay, V. Justin, M. Malbouyres, F. Ruggiero, O. Damour and V. Hasirci, *EDC/NHS cross-linked collagen foams as scaffolds for artificial corneal stroma*, 2007, J Biomater Sci Polym Ed, 1527-1545.

24. C. D. W. Wilkinson, M. Wood, J. Gallagher, A. S. G. Curtis, *The use of materials patterned on a nano-and micrometric scale in cellular engineering*, 2002, vol 19, 263-269.

25. A. Mashberg, P. Boffetta, R. Winkelman and L. Garfinkel, *Tobacco smoking, alcohol drinking, and cancer of the oral cavity and oropharynx among U.S. veterans*, 1993, Cancer, 1369-1375.

26. T. Tanaka and R. Ishigamori, *Understanding Carcinogenesis for Fighting Oral Cancer*, 2011, Journal of Oncology, Article ID 603740, 10 pages.

27. K. Annertz, H. Anderson, A. Björklund, T. Möller, S. Kantola, J. Mork, J. H. Olsen and J. Wennerberg, *Incidence and survival of squamous cell carcinoma of the tongue in Scandinavia, with special reference to young adults*, 2002, Int. J Cancer, vol 101, 95-99.