A new Touch to Atomic Force Microscopy
Smart probing of biological and biomedical systems at the nanoscale

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Chapter 1

Introduction

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Atomic Force Microscopy (AFM) is a microscopy technique which works on the principle of feeling surface topography with a sharp probe. With the right choice of parameters it has enough resolving power to visualize single atoms, hence its name. The AFM was invented in 1986 by Binnig et al. \cite{1} and its usefulness in biological research was quickly realized: it allows high resolution imaging in a physiological environment as was shown conclusively by the first AFM images of living cells in 1991 \cite{2}. The AFM has also been used to image many other biological objects, including single DNA molecules, purified proteins and membrane proteins in reconstituted or native membrane. Besides imaging, AFM enables mechanical manipulation and measurement of samples. The probe which is used to feel the surface can also be used to push into or pull away from the surface. This has been used to investigate the role of forces for everything from tissues down to single proteins. For all these reasons, AFM is finding its way from physics labs not only into biological research, but into medical research as well.

Despite its spreading use in many different types of research, AFM still has a lot of potential for optimization. This thesis describes advances in AFM
in a few areas that we identified as important for biological and biomedical research as well as the application of these advancements in such research. This chapter will first provide a quick introduction to AFM and will then focus on the particular advances and applications described in this thesis.

1.1 Atomic Force Microscopy

AFM is a member of the family of scanning probe microscopes. With these techniques, a probe is raster-scanned over a surface to measure local properties. Usually the surface topography is mapped by keeping the interaction between probe and surface at a predefined level. The first such technique was Scanning Tunneling Microscopy where an electrical current across a tunneling junction was measured. Binnig et al. subsequently developed the AFM to add the ability to measure non-conductive surfaces. With AFM, a force on the probe is measured using the deflection of a cantilever on which the probe is situated.

Several different variants have been developed to measure the force between tip and surface, based either on measuring the static deflection of the cantilever ("contact mode") or on driving the cantilever harmonically and measuring its interaction with the surface through amplitude or frequency-shift ("dynamic mode"). Contact mode has the benefit that the applied force is directly related to the deflection and easily calculated. It has as drawback that the deflection might drift over time with temperature, so that the applied force is not known anymore, and the probe can easily damage fragile structures on the surface as it scans them laterally. Dynamic mode is more gentle as most of the time the probe is not in contact with the surface and it hardly exerts lateral force. The instantaneous perpendicular force can be higher though, and the applied force is not easily calculated. Dynamic mode is used in this thesis for imaging, while contact mode is used for force mapping, which is described below.

Obviously the probe is an important factor in determining the lateral resolution achievable with this technique. Many different types of probes can be bought commercially; most are manufactured using silicon etching techniques. The probe itself usually has a pyramidal shape with a tip with a radius from
a few nanometer to tens of nanometers. The tip end may also be modified in a variety of ways to achieve sharper tips, higher aspect ratios, hardness, durability or specific surface chemistry. The probe is mounted on a cantilever, the spring constant of which can range from \(1 \text{ mNm}^{-1}\) to \(>100 \text{ Nm}^{-1}\). Softer probes are often used in contact mode and in biological research to minimize the applied force; stiffer probes are often used in dynamic mode because it is easier to excite them in liquid and their higher resonance frequency allows for higher scanning speeds.

Dynamic mode AFM can be done in a variety of ways, all of which excite the cantilever mechanically, usually harmonically and close to the cantilever resonance frequency, and measure the response of the cantilever to an interaction between the tip and the surface. Most commonly, and for all dynamic mode measurements in this thesis, the amplitude is measured, although other possibilities include measuring the resonance frequency shift or the phase shift. The amplitude is reduced by the tip-surface interaction; a feedback mechanism detects the changes in the amplitude and adjusts the height of the tip above the surface to keep a constant amplitude while the tip is scanned over the surface. This is used to generate a topographical height map of the surface at the nanoscale. At the same time, other properties can be measured as well, such as the phase difference between drive signal and cantilever response. This can be used for example to map material properties, although a quantitative interpretation is often difficult.

Contact mode is used in this thesis for force mapping. With force mapping, the probe is not scanned laterally over the surface, but moved vertically into and out of contact on each point of a raster pattern, while recording deflection, or force, versus height at all positions. From this data it is possible to calculate mechanical properties of the sample at all positions visited, as well as a topographical image similar to what can be obtained using normal AFM imaging. As the tip is continuously moved into and out of contact, the disadvantages of contact mode mentioned above — drift in the applied force and disruptive lateral forces — do not apply.
1.2 Probes

As mentioned above, the tip is an important factor determining the performance of an AFM. Much of the work in this thesis is therefore concerned with the tip and possible modifications and enhancements to the tip.

Chapter 2 describes modification of tips with carbon nanotubes (CNTs) for higher resolution, gentler imaging and smarter tip chemistry. CNTs are exactly what their name implies: tubes with a diameter down to 1 nm made of carbon. With their extremely high aspect ratio and small diameter, nanotubes are ideal candidates for AFM tips. However, when using thin nanotubes, the length and angle of the nanotube have to be carefully controlled to get a usable tip. Chapter 2 describes in detail the mechanics of nanotubes that are needed to understand how to make reliable tips and subsequently our protocol for the production of such tips.

Chapter 3 shows the use of nanotubes on a biological sample: the Nuclear Pore Complex (NPC) and explains the advantages of using CNT tips. The most important advantage is that on soft samples, the extremely high aspect ratio allows gentler imaging as less of the sample is deformed. This allowed us to image fragile fibers in the NPC. Also, CNT AFM measurements show some quite different phase images with similar imaging parameters. Unambiguous assignment of differences in these phase images to material properties is not possible, but we hypothesize that hydrophobic interactions play a part here. Force mapping, perhaps in dynamic mode, might be a good way to investigate this further.

In chapter 4 we take a close look at bio-chemically sensitive AFM imaging. Our findings indicate that it is possible to obtain single molecule binding and unbinding rates from such measurements, but that these rates are influenced by even the most careful imaging parameters. The high aspect ratio of CNTs and the possibility of directed modification of the tip end of CNTs should provide a more flexible and a better defined system than the currently used modification protocols.

In chapter 5 we investigate the mechanical properties of tissue of the aortic wall and the effect of two diseases, Marfan syndrome and aneurysms, on the
strength of the tissue. For this we used both normal silicon pyramidal tips and, quite contrary to the use of nanotube tips in the other chapters, tips consisting of a sphere with a diameter of 5 µm. The aortic wall is made of network structures with the primary components being collagen and elastin and using differently sized tips in force mapping allowed to probe both individual fibers of the network and the space in between them with the sharp tips and the overall network response using the blunt tips. The analysis shows clearly that one must understand the mechanical response of the tissue at both these levels to understand how and why the tissue sometimes fails and ruptures under the mechanical stress caused by the heartbeat.

1.3 Data from AFM

While AFM originated as a microscopy technique and is still most often used as such, the technique also allows for quantitative measurements of various properties of samples, based on the measurement of forces. Mostly contact mode is used for this, as it is straightforward to calculate the actual applied force from the deflection of the cantilever. When testing mechanical properties of samples, often the Hertz model is used to fit force versus indentation curves and to find thus the Young's modulus of the sample, even though it can be difficult to automate fitting with this model and the mechanics of non-homogeneous and non-isotropic samples like tissues or cells are not adequately described by a single Young's modulus.

In chapter 5 we use contact mode force mapping to evaluate the mechanical strength of healthy tissue from the aortic wall versus diseased tissue which is more likely to fail mechanically and rupture. From the force curves, the Young’s modulus is calculated using the Hertz model, using a smart fitting procedure to circumvent having to locate the first point of contact as has to be done normally. This approach allows completely automated analysis, which is important when working with large numbers of force curves. Even though strictly speaking the Hertz model is not applicable, we can still the mechanical construction of the tissues from these measurements. Also, we see some clear non-hertzian behaviour in the force curves, which we can relate to the specific qualities of
CHAPTER 1. INTRODUCTION

the tissue. This study would also clearly benefit from a tight integration of AFM with optical techniques, notably confocal microscopy, to correlate the results of probing with the AFM tip with 3-dimensional and large-scale structural changes in the tissue. Here, we performed separate confocal and AFM measurements.

Dynamic mode can also be used for quantitative measurements of interactions with the right data analysis techniques. For example, recently algorithms have been developed to extract force and energy versus distance relationships from dynamic mode force distance curves. Another special dynamic imaging mode is simultaneous topography and recognition imaging (TREC), where the cantilever motion is filtered specifically for adhesion between a molecule attached to the tip and one attached to the surface under investigation. This mode is used to identify proteins on a surface. In chapter 4 we analyse such a measurement on a model system in great detail for the first time. From this analysis it is in principle possible to calculate both binding and unbinding rates between the two molecules on a single molecule level. Comparison of these results with calculations from theory is difficult at the moment, but with some advances, mostly achievable through the use of smarter tips and tip chemistry, this should become possible, as we also argue in this chapter.
Bibliography


Chapter 2

Atomic Force Microscopy with Nanotube Tips

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After a general introduction into nanotubes we will outline the benefits that nanotubes bring for Atomic Force Microscopy (AFM) in section 2.1. We address the restrictions to nanotube AFM probes, posed by nanotube properties, in section 2.2. Next, in section 2.3 we will describe our production method and how we have succeeded to produce reliable nanotube probes.

The use of Carbon Nanotubes (CNTs) as probes for AFM was proposed by Dai et al. [9] as early as 1996, five years after CNT’s had been brought to the general attention of the scientific population. More than ten years later, very few people are using CNT AFM probes, despite the advantages that nanotube are expected to offer compared to standard probes: they have an extremely high aspect ratio and the nanotube end interacting with the surface is made up of a closed sheet of carbon, which is chemically quite inert and suffers little from wear. Apparently, there are some issues which offset these advantages.
2.1 Carbon nanotubes

Carbon nanotubes have already for a long time fascinated researchers. The first mentioning of CNTs can be traced to 1889 [10]. The first conclusive evidence for the existence of multi-walled CNT’s (MWCNTs) came in 1952 [16], while the first experimental evidence of single walled CNTs (SWCNTs) was presented in 1993 [6], [20]. For most people, however, the field was started with a paper by Ijima in 1991 [19]. In the 90’s nanotubes became a popular topic as the ongoing development of the technology for growing them provided for the first time opportunities to work with nanotubes and measure and use their special properties.

One of the special properties of CNT’s which make them interesting for scanning probe applications is their mechanical strength. CNT’s have an extremely high tensile strength and elastic modulus of 0.1 TPa respectively 1.0 TPa [4], [7]. As the covalent bonds between the carbon atoms are very strong, nanotubes should exhibit reduced wear due to scanning compared to conventional silicon or silicon-nitride tips, especially when scanning on harder surfaces such as mica or other crystalline surfaces. In addition, buckling — bending of the cantilever under compressive stress which is further elaborated on in section 2.2 — could possibly be used to limit the applied force to fragile samples as suggested in [21].

Nanotubes can also be good electrical conductors, depending on their precise atomic structure and therefore nanotube tips can be useful for STM of current sensing AFM. Moreover, as it is also possible to electrically isolate everything apart from the very end of the nanotube, very sharp tips can be made for measurements in conducting electrolytes without getting problems due to leakage currents [3].

As their name suggests, nanotubes have a small diameter, down to just one nanometer, and a high aspect ratio, both of which are ideal for obtaining high resolution with AFM. However, if resolution is the only motivation, it has been convincingly shown that standard pyramidal AFM probes are capable of obtaining ultra-high resolution — to the subatomic scale [8]. We propose here that the main benefits of CNT tips are other than this. Especially biological
2.1. CARBON NANOTUBES

Figure 2.1: Schematic depiction of a standard AFM tip (at the left) and a nanotube AFM tip (at the right) in contact with a soft surface. While the standard tip could achieve similar resolution as the nanotube tip on hard surfaces through its final asperity, on soft surfaces it interacts with a much larger surface area.

samples like cells or the nuclear membrane which will be discussed in chapter 3, are often compliant and will deform under the applied force of the AFM tip. As depicted in figure 2.1, this results in a large contact area for normal tips, much larger than the final asperity which would interact with a hard surface. The nanotube, with a radius similar to the final asperity on the normal tip, interacts with a much smaller area of the sample. Moreover, when imaging in liquid, the water molecules between tip and sample have to be pushed out on every approach in tapping mode. With the reduced interaction area of the nanotube tip, less water has to be pushed away. Both effects reduce the energy loss through the interaction, so that imaging is more sensitive and requires less force. This is an important aspect for biological AFM.

The main reason why CNT SPM tips are not yet ubiquitous is their difficult production process. Many people use a manual production method where a nanotube is put on a tip using a nanomanipulator under an optical microscope [9] or inside a SEM [18]. Other production methods involve picking up nanotubes by electric field [17] or mechanically in an AFM [15] and direct growth of the nanotube on the tip [11], [14]. Combinations of the different methods for substeps are also sometimes used, as described in section 2.3. While manual manipulation is a time consuming and expensive process, direct growth of the nanotube on a tip so far doesn’t give good enough control over length and orientation to make useful tips. The limits to the nanotube length and orientation for use as AFM probes will be discussed in the next section.
2.2 Mechanics of nanotubes

Nanotube AFM tips have many advantages over conventional AFM tips, but care needs to be taken to achieve these advantages. Nanotubes that are not of the correct dimensions or that are not aligned well enough will cause unstable imaging. This section will describe all mechanical properties that have to be understood and optimized in order to achieve robust and high performance nanotube AFM tips. The essential conclusion is that CNTs between 3 and 6 nm diameter should be shortened to a length below 80 nm.

2.2.1 Lateral adhesion

Unlike traditional AFM tips, nanotubes are flexible laterally and can deform when sticking to sidewalls. Thus the Van-der-Waals attraction between a nanotube and a sidewall can become fairly large, with measurements suggesting that a force of around 8 nN is required to pull a nanotube off of a SiO$_2$ sidewall in vacuum, independent of contact length [13]. This is considerably more than typical forces in tapping mode with soft (2 N/m) cantilevers and may cause unstable imaging as the feedback overreacts to pull the tip loose again. Moreover, the combination of a low lateral spring constant and long range forces make that the nanotube jumps into contact with the sidewall from quite a distance. From figure 2.2, it can be clearly seen that adhesion is a large problem. It can be alleviated by making the nanotube as short as possible, effectively increas-
2.2. MECHANICS OF NANOTUBES

ing the lateral spring-constant, and by choosing conditions to avoid long range interactions. In liquid buffers, forces usually have a limited range and we did not observe lateral instabilities.

2.2.2 Thermal motion

When using thin nanotubes for sharp AFM probes without wear, it is important to realize the end of the nanotube exhibits thermal motion. The amplitude of the thermal motion can be determined by equating the thermal energy with the potential energy of lateral bending. $1/2kx^2 = 1/2k_BT$, with $x$ the displacement, $k_B$ the Boltzman constant, $T$ the absolute temperature and $k$ the spring constant. The springconstant of a nanotube of radius $r$ and length $L$ can be approximated using the continuum theory: $k = \frac{3\pi r^4 E}{4L^3}$, with $E$ the Young’s Modulus which is around 1 TPa, according to Akita et al [18]. For a nanotube of given radius and a desired resolution, this imposes a maximum length on the nanotube for high resolution imaging. For the measurements described in this thesis with a resolution down to a few nanometer and using the restrictions in length imposed to prevent buckling, as discussed in the next section, we are not limited by thermal motion.

2.2.3 Buckling

Buckling is the mechanical failure of a column under compressive stress along its long axis, as sketched in figure 2.3. When the force on a column exceeds a critical force, it will develop a local deformation such that it can bend and reduce the load. This critical force is given by the Euler formula

$$F_{cr} = \frac{K\pi^2 EI}{L^2}$$

where $K$ is a constant whose value depends on the conditions of end support of the column. For a column with one end fixed and the other end free to move laterally, which is the (worst) case for a nanotube AFM tip, $K = \frac{1}{4}$. $I$, the area moment of inertia, is $I = \frac{\pi r^4}{4}$ for a column with a circular cross-section.

Nanotubes will buckle under a compressive stress and, according to simulations [5], Eulers formula is still valid on this small scale. Nanotubes are special
CHAPTER 2. ATOMIC FORCE MICROSCOPY WITH NANOTUBE TIPS

Figure 2.3: Cartoon of buckling. When a force applied to a column exceeds a threshold force, the column will deform as sketched for the right hand one here. The threshold force depends on geometry and the boundary conditions on the endpoints of the column. While for classical objects the deformation is irreversible, for CNTs it is reversible.

Figure 2.4: Curves recorded with a nanotube AFM tip on a nuclear membrane in air. On the left amplitude (black) and phase (red) versus distance; on the right dissipation (black) and force (red) versus distance as calculated from the amplitude and phase curves according to [1]. The nanotube was about 70 nm long and 8 nm diameter, as measured from SEM images. Cantilever parameters were: $k = 2 \text{ N m}^{-1}$ (nominal value), $f_0 = 78 \text{ kHz}$, $f_d = 82 \text{ kHz}$, $Q = 40$. In these graphs zero is arbitrarily defined as the start of the force distance curve at the far point away from the surface.

At $z = 60$ nm the nanotube first touches the surface and the amplitude is reduced, until the nanotube buckles around $z = 5$ nm and the amplitude is restored to a value even larger than the free amplitude. The amplitude can increase when the cantilever resonance frequency shifts closer to the driving frequency through the interaction with the surface. From here on, the phase doesn’t change much anymore until the pyramid touches the sample surface, indicating dissipation is independent of the amount of buckling. The calculated force increases linearly from this point on. Around $z = 40$ nm the amplitude drops a bit without any apparent change in the phase, corresponding with an increase in the dissipation. Supposedly the configuration of the bundle of single-walled nanotubes changes here.
however in that the deformation is reversible and, when the force is lifted, they will return to their original shape [5]. This is one of the properties which makes AFM nanotube tips extremely reliable. For stable imaging however, buckling should be prevented. If buckling occurs, it leads to a discontinuous jump down in force going into the surface, which in turn leads to a non-monotonic amplitude-distance dependence. Figure 2.4 shows an example of an amplitude distance curve where a nanotube is buckling. In this case, when the setpoint is chosen just before the nanotube buckles, a sufficiently large error in z can cause the feedback to drive the tip into the surface until the silicon pyramid touches, as the amplitude gets larger when getting closer to the surface.

The critical buckling force depends strongly on the dimensions of the nanotube. Figure 2.5 shows the critical force as a function of nanotube length for two nanotubes of different diameters. The Young’s modulus $E$ for the nanotubes is assumed to be 1 TPa, according to Akita et al [18]. Simulations show that nanotubes which are under an angle with the sample surface and thus experience a force component normal to their long axis, buckle already at 3x lower forces for a 20 degrees tilt angle[2, 5, 21].

In tapping mode AFM, forces on the order of a nanonewton are normal.
Moreover, with our production process (section 2.3) it is impossible to control the alignment of the nanotube very precisely in the viewing direction. Thus, especially when using thin nanotubes, we should take care to prevent buckling by making short nanotube tips. Generally, using bundles of single-walled nanotubes or single multi-walled nanotubes which are between 3 and 8 nm diameter and up to 80 nm long, we experience no imaging instabilities.

### 2.3 AFM tip production

For making CNT AFM tips we used the method of direct manipulation inside a SEM, because we found that the precise control over the process that this method allows combined with the constant monitoring of the nanotubes state is crucial for reliably making robust probes. In this section I will first describe the nanomanipulator that we used for the mounting procedure and subsequently the different steps involved to create robust CNT AFM probes: alignment, cutting and gluing of the nanotubes.

#### 2.3.1 Nanomanipulator

All nanomanipulation described in this thesis is done inside a 30 kV FEI NovaSEM with a field emission source and a magnetic immersion lens system, with a measured resolution of 1.3 nm. This microscope allows a good view on the manipulation process even for very thin nanotubes (< 3 nm). In order to fit inside the SEM, the manipulator needs to be high-vacuum compatible, built of non-magnetic materials and fit in the limited space under the final lens. Figure 2.6 shows the manipulator.

For manipulation, we need a manipulator with a travel range in all three spatial directions of 5 mm and a precision better than one nanometer. To achieve this, the manipulator was built with a coarse and a fine stage. The fine stage consists of a piezo element with 15 μm scan range and has the (AFM) tip holder on top. The coarse stage is built using three Attocube piezo stepper motors and has the nanotube sample on top. These motors have a range of 4 or 5 mm and a specified minimum step size of 25 nm. They are non-magnetic,
2.3. **AFM TIP PRODUCTION**

Figure 2.6: nanomanipulator design. The technical drawing on the right shows the various elements of the design. On the left is the piezo element for fine movement. On top of this is the tip holder. On the right the three attocube stages can be discerned, with on top of them the holder for nanotube samples. The two holders can be removed easily to replace tip or sample. The photograph shows the finished manipulator, also with the piezo stage at the left and the coarse stage on the right. The holders are mounted without samples.

high vacuum compatible and a stack with three motors — for three spatial directions — can be made to fit under the SEM lens. While the specifications for the coarse motor seem almost good enough for mounting by themselves, the stack with all three motors is not very robust and when taking a single step the whole assembly vibrates with an amplitude much larger than the stepsize. This necessitates the use of a piezo stack for fine movement. The motors are also very fragile and will break or not reach specifications when not handled very carefully. Figure 2.6 shows the manipulator.

### 2.3.2 Alignment

We usually mount an AFM tip on the fine stage while we mount a sample with nanotubes protruding from an edge or sticking out from a piece of soot on the coarse stage. After bringing the AFM probe and nanotube sample in each other’s vicinity in the SEM and selecting a suitable nanotube, the fine stage is used to approach this nanotube with the tip from below. Van-der-Waals forces make the nanotube stick along its length to the tip. Figure 2.7 shows a sequence of images demonstrating how the nanotube is brought into
CHAPTER 2. ATOMIC FORCE MICROSCOPY WITH NANOTUBE TIPS

Figure 2.7: Picking up of a nanotube. Please note that the nanotube tip in this sequence was not made for imaging, so less care was taken with respect to alignment and thickness of the nanotube. The sequence starts in the top left with the nanotube attached to the tip. The next images show how the nanotube is pulled away and how its orientation is defined by its original orientation on the tip and the pulling process. The continuous high resolution imaging deposited enough carbon to fix the nanotube during the process. The final images show how the nanotube is detached from its original support. Images courtesy of A.F. Beker.
contact with the AFM tip, aligned and pulled away somewhat from the raw material. When aligning the nanotube in this stage, it is important to make it stick out from the very end of the tip, to make sure that the AFM imaging is only performed by the nanotube. The nanotube needs to be somewhat free from its surrounding material in order to be able to cut it as otherwise the large electric field gradient imposed during cutting (as described in section 2.3.3 will cause nearby nanotubes to jump into contact with the tip as well.

As discussed in section 2.2, the nanotube has to be aligned well to the sample surface normal in the AFM to prevent problems with buckling. It is important to have a good alignment already in the first step in the manipulation process. Later on, the direction of the nanotube can be adjusted by pushing the nanotube against a support and fixing its induced alignment by depositing amorphous carbon locally by zooming in with the SEM onto the place where the nanotube extends from the tip apex. Depositing too much material in this way is however undesirable, because this will also deposit material on the nanotube itself.

In the direction parallel to the electron beam, it is not possible to control the alignment, because the depth of view in a SEM is quite large. By using a tip which is sharp and has a high aspect ratio to start with, we find however that the alignment is good enough in this direction. Figure 2.8 shows an AFM
tip with a nanotube viewed from two different directions from which we can see that the nanotube is aligned well.

2.3.3 Cutting

After alignment of the nanotube on the tip, it has to be broken off from its original support and it has to be cut to the right length. For the nanotube tips used throughout this thesis, we have chosen to cut the nanotubes by sending an electrical current through the nanotube while inside the SEM. Other possibilities proposed for cutting nanotubes include sending a current through the nanotube while imaging in an AFM on a conductive surface [22], using a high intensity electron beam [12] or using a focused ion beam. We have chosen the first method because, in contrast to cutting in the AFM, it allows an in situ view of the result during the cutting process and easy further processing in the SEM, e.g. for gluing. While cutting by a high intensity electron beam allows the best control over the cutting process, the SEM chamber and the cantilever need to be very clean to prevent deposition of amorphous carbon while cutting.

Cutting is performed in a few steps. First, the nanotube is cut from its original support by applying a voltage between the AFM tip and the nanotube sample. Typically, a voltage of 20-30 V is needed when the nanotubes are supported on carbon tape. The position of breaking is ill controlled in this step, but usually the nanotubes break where they are bent or buckled or where they touch other nanotubes. After this initial cut, resulting nanotubes are usually too long to be used as an AFM probe. For the next cutting step, the nanotube is approached to a metal support until electrical contact is made, taking care that the distance between the end of the original silicon AFM tip apex and the metal support corresponds to the desired nanotube length. The approach can be checked visually with the SEM or by monitoring the current while applying a small voltage. After a contact has been established, the voltage is increased slowly until the current suddenly drops to zero, indicating that the contact is broken. Normally, the nanotubes break where they touch the metal support, possibly because the contact resistance is higher than the resistance across the nanotube and the heat developed in the contact breaks the nanotube. This
allows good control over the length of the nanotube.

### 2.3.4 Improving fixation

The final step in the production of CNT AFM tips is to increase the reliability by applying extra material over the contact area of nanotube and AFM tip. When scanning on rough surfaces or when inserting the tip into water, the Van-der-Waals force between the nanotube and the silicon of the tip may otherwise not be enough to prevent loss of the nanotube. The SEM we used for nanotube tip production is also equipped with a gas injection system, which can be used to perform electron beam induced deposition (EBID). In this process, a gaseous precursor consisting of a metal atom (platinum in this case) attached to organic ligands is let into the vacuum chamber through a hollow needle at 100 \( \mu m \) distance from the AFM tip. The precursor gas molecules adsorb on the tip and are broken down by the electron beam. The organic wreckage desorbs, leaving the platinum behind. In this way, material can be grown on selected places on the sample. This was used to apply extra material over the contact between nanotube and silicon tip as close to the end of the tip as possible. We
found that this greatly increases the reliability and longevity of CNT AFM tips under rough circumstances. Figure 2.9 shows a nanotube tip before and after applying EBID.

We have found nanotube tips prepared according to the procedures described here to be reliable and to generate good, reproducible, high-resolution and artifact-free AFM images. Figure 2.10 shows a nanotube tip before and after extensive measurements on nuclear membrane in air, as described in section 3.4.

However, working with very thin nanotubes takes a lot of time. One crucial aspect of the montage is carbon deposition in the SEM during imaging. To minimize deposition it is good practice to work at the lowest magnification which allows to image the nanotubes. This does make the manipulation cumbersome and more time consuming. Combining all steps described before is time consuming in itself. Also, some of the steps are liable to damage the nanotube or render it unusable in other ways, especially the processes of aligning and cutting. All in all, it generally took about 4 hours to produce one nanotube, if no accidents happened. In contrast, for some other applications using thicker nanotubes where the alignment and length were less critical, the manipulation process could be performed as quickly as 15 minutes. We found out that storage and transport of the nanotube tips need special precautions as well. Nanotube AFM tips are best stored in metal containers to prevent build-up of
static charge, to prevent discharges due to high gradients of electric fields near the nanotube tip end.
Bibliography


Chapter 3

Imaging Nuclear Pores with Nanotube AFM Tips

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The Nuclear Pore Complex (NPC) forms a complex gateway organizing all traffic into and out of the cell nucleus. As such, it has an important role in regulation of transcription and translation processes in the cell. For many new medicines it is important to understand how they interact with the NPC to optimize their uptake into the nucleus. However, like all membrane proteins, NPCs are hard to study using biochemical methods and not much is known yet about the way they function. Much structural information has been obtained using various methods of electron microscopy (EM). AFM has also been used to obtain structural and functional information [15], but, especially in liquid, the nuclear membrane is difficult to image as it is very soft owing to its double bilayer architecture. The nuclear pores themselves are tall structures, about 100 nm in diameter and protruding about 40 nm above the membrane at the cytoplasmic face with a deep, hydrophobic channel in the middle.

In this chapter we will image NPCs with Carbon nanotube AFM tips. We
CHAPTER 3. IMAGING NUCLEAR PORES WITH NANOTUBE AFM TIPS

expect the high aspect ratio of the nanotube tips to localize the interaction with
the sample and therefore to image the sample gentler and more reliably. At the
same time, this sample puts the robustness of nanotube tips to the test: it is
a rough surface with both hydrophilic and hydrophobic patches. Imaging this
stably shows that the nanotube tips are indeed reliable.

3.1 Nuclear Pore Complexes

Specialized cellular compartments have enabled eukaryotes to become very
complex. In particular, the spatial separation of transcription and translation
of DNA provides eukaryotes with powerful mechanisms for controlling gene
expression. This also requires selective transport between the nucleus, where
transcription takes place, and the cytoplasm, where translation takes place. All
eukaryotes have highly conserved Nuclear Pore Complexes (NPC’s) which are in-
serted in the double membrane around the nucleus and which are responsible
for all transport into and out of the nucleus. Small molecules like ions and small
proteins can diffuse through these complexes unhindered. Larger molecules
have to be transported actively through the channel after being tagged by nu-
clear import factors. The limiting size for free diffusion is generally believed to
be around 49 kDa \([5]\), or 4–5 nm.

The structure of NPC’s has been established to 6 nm resolution using elec-
tron microscopy. Together with biochemical data this gives a good picture of
the way NPC’s are built up \([7]\). Figure 3.1b shows the tentative structure of
NPC’s as determined from many different analysis methods. NPC’s consist of
multiple copies of at least 30 different proteins, called nucleoporins \([13, 6]\).
These assemble into subunits of the NPC, 8 of which together form the final
structure \([9]\). The NPC is one of the largest protein complexes in cells, with
a mass of 120 MDa. It has a donut-like shape with an outer diameter of
100 – 120 nm. Both on the nucleoplasmic and the cytoplasmic side it has
filaments of several tens of nanometers long. At the nucleoplasmic side these
form into a basket-like structure. At the cytoplasmic side the filaments are too
flexible to be imaged by high resolution SEM. Since the images in figure 3.1 are
produced averaging many individual electron microscopy images of NPC’s, the
3.1. NUCLEAR PORE COMPLEXES

Figure 3.1: a. Schematic showing the different structural elements of the nuclear pore complex (cf. [10]). Note that some of the elements are controversial. The blue mass designated 'macromolecule' represents a vague mass often seen in electron microscopy images. This could be a molecule in transport or a deformable or less organized part of the NPC, such as FG repeat filaments. The side channels which are depicted here to provide transport routes for inorganic ions are also controversial. While it is clear that ions and small proteins can pass unhindered, it is not clear whether this happens via side-channels or via a small open volume in the center of the pore.

b. (cf. [8]) NPC structure from an integrated analysis approach (EM, SPM and biochemical methods). This analysis has mapped out the location of many of the constituent proteins of the NPC, but still cannot answer the question on how the pore functions.
flexible filaments average out and do not appear. These cytoplasmic filaments are called FG repeats as they are mostly made up of repeats of phenylalanine and glycine.

While mechanistic descriptions of the transport processes in NPC’s are not very detailed and there is a lot of controversy about them, there is quite some biochemical data available on the processes which occur in NPC’s. Many proteins have been identified that are involved in specific import to or export from the nucleus. A comprehensive overview of proteins involved is given in [1]. There are families of proteins which have a binding domain for FG repeats and often directly, but sometimes through adaptor proteins specifically recognize cargo. These proteins adopt different configurations, with different binding affinities to their cargo, depending on the presence of one of the two forms of the small RAN protein: RAN-GTP or RAN-GDP. The cell maintains a steep gradient of these different forms across the nuclear membrane, thus giving directionality to the transport.

Several studies [11, 14] using EM and immuno-gold labelling have indicated that the cytoplasmic filaments are involved not only in recognition but also in transport of cargo through the NPC. In the model proposed in these papers the cargo binds to the filaments, which then bring the cargo to the central pore or even direct the cargo all the way through the pore to the nuclear side. Some studies show that specific transport from the cytoplasm to the nucleus for at least some biochemical pathways is possible even without these filaments [16].

It is likely that FG rich filaments extend not only into the cytoplasm and nucleoplasm but also mostly fill the channel of the pore [8]. They leave open a small central channel of about 10 nm diameter through which small proteins and particles can diffuse freely, while blocking larger cargo, unless this cargo contains binding sites for the hydrophobic FG repeats. The binding sites for the FG repeats are usually provided by the transport factors which lead molecules through the NPC.

While electron microscopy has provided much data on the structure and function of NPC’s, it always only provides snapshots of the state of the pores. AFM has the promising ability to image the pores in their native environment and ultimately to provide direct imaging of import of cargo and the associated
structural changes. One of the requirements is a tip which can probe the tentative structures inside the NPC. Nanotube tips seem well suited for this task for several reasons. They have a high aspect ratio, which is needed to image deep inside the pore. Also, as hydrophobicity seems an important attribute for uptake through the pore, the hydrophobic nature of nanotubes might help to enter the pore with the tip. Here we used CNT tips prepared according to the methods laid out in chapter 2 to image NPCs in native nuclear membrane in air and in liquid.

3.2 Methods

Preparation of Cell Nuclei. Xenopus laevis (African clawed frog) females were anesthetized with 0.1% ethyl m-aminobenzoate methanesulfonate (Serva, Heidelberg, Germany), and their ovaries were removed. Oocytes were dissected from ovary clusters and stored in modified Ringer's solution (87 mM NaCl, 6.3 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, 10 mM HEPES, 100 units/100 µg penicillin/streptomycin, pH 7.4) until used. For isolation of the cell nuclei, the oocytes were transferred into nuclear isolation medium (NIM) composed of 90 mM KCl, 26 mM NaCl, 5.6 mM MgCl2 (corresponding to a free Mg²⁺ concentration of 2 mM), 1.1 mM EGTA, and 10 mM HEPES and titrated to pH 7.4. Additionally, we added 1.5% polyvinylpyrrolidone (M₄ 40,000; Sigma) to compensate for the lack of macromolecules in NIM, mimicking the intact cytosol. The presence of polyvinylpyrrolidone is crucial to prevent the swelling (>100% in the absence of polyvinylpyrrolidone) of total nuclear volume that occurs instantaneously after isolation in pure electrolyte solution. We used X. laevis oocytes because of their size. They provide the possibility to manually prepare the nucleoplasmic and cytoplasmic faces of a nuclear envelope for AFM investigations.

Cargo blocking was achieved by incubating the nucleus with a mutant of one of the import factors for nuclear pores: importin beta 45–462. This mutant binds irreversibly to the fg-repeats in the central channel and thus accumulates and blocks transport.

Preparation of Nuclear Envelopes. After the oocytes were placed in NIM,
nuclei were isolated manually by piercing the oocyte with two pincers. Individual intact nuclei were picked up with a Pasteur pipette and transferred to a glass coverslip placed under a stereomicroscope. The chromatin was then carefully removed using sharp needles, and the nuclear envelope was spread on BD Biosciences Cell-Tak™-coated glass, with the nucleoplasmic side facing downwards or upwards. Finally, for measurements in liquid the samples were washed and kept in PBS. For measurements in air the specimens were washed with deionized water and dried.

**Atomic Force Microscopy.** AFM was mostly performed on a Veeco Multimode AFM using a Nanoscope V controller, while some measurements were performed on a Scientec PicoAFM. Nanotubes were mounted on Olympus AC240TS cantilevers (nominally 2 N m$^{-1}$; resonance frequency 70 kHz in air.

**Phase imaging.** As we will present images showing a diverse response in the phase channel of amplitude modulation dynamic mode AFM (AM-AFM) later in this chapter, we discuss here the most important elements influencing (phase-)imaging in AFM. The phase channel is often used to visualize the positional dependence of mechanical sample properties as explained in [4], but as the cantilever response in dynamic mode AFM is complicated and the amplitude is not in effect constant during imaging of rough samples, it is not straightforward to do so quantitatively.

In AM-AFM the cantilever is driven at its resonance frequency and feedback is performed at the amplitude, which changes when the tip gets into contact with the surface. Now consider the simple case where tip surface interactions are zero when the tip is not in contact with the surface, and when the tip does touch the surface it acts as a linear spring. The interaction with the surface changes the cantilevers’ mechanical behaviour, in particular its effective resonance frequency and its effective quality factor, the latter of which is determined by cantilever damping and the energy dissipation in the tip sample interaction. As sketched in figure 3.2 a change in the effective resonance frequency and quality factor influences the measured amplitude and phase.

When the tip is only slightly indenting the surface, a change in the average distance between tip and surface, as happens when scanning topographical features on a surface, hardly influences the damping but does influence the
3.2. METHODS

Figure 3.2: Change in cantilever spectrum amplitude and phase when the resonance frequency shifts to a higher frequency.

effective stiffness of the cantilever. Thus both amplitude and phase shift by an appreciable amount. As the changes in stiffness are small there is a linear relationship between amplitude and phase in this case.

On the other hand, when the amplitude setpoint is chosen such that the sample is indented somewhat more on each tap, a change in material properties of the surface can change the damping of the tip and lead to contrast in the phase channel. This has been shown convincingly on mixtures of polymer where there are no appreciable topographical features [4] and thus the amplitude response is flat but the phase shows marked contrast. In general, it can be said that if the phase image shows features that are not related to features in the amplitude image, sample properties contributed a large part to the contrast in the phase channel.

The situation gets more complicated when we consider that the equation of motion for the tip is very nonlinear. This may cause there to be several stable solutions to the equation of motion for the cantilever, between which it can switch randomly and which have different relations between amplitude and phase. Also, these solutions may appear or disappear depending on surface properties or even on topography if the feedback is too slow and the tip moves closer and further from the sample [12]. Jumping between different solutions will show up through large changes in the phase and small hiccups in
the amplitude as the feedback attempts to regulate the amplitude back to its setpoint.

When amplitude and phase are measured as a function of distance from the sample surface, the interaction profile can be reconstructed [2], but in normal imaging where amplitude and phase are measured at a single height there is not enough information to deconvolute the different contributions to the amplitude and phase and sample properties cannot be determined from first principles. However, by comparing images and using knowledge about the sample it is possible to draw conclusions for which interactions are important.

3.3 NPC’s studied in liquid using CNT tips

3.3.1 Nucleoplasmic side

Figure 3.3a shows an image of the nucleoplasmic side of the nuclear membrane recorded with a normal tip. This area contains several tens of NPCs. The nucleoplasmic side of the nuclear pores is difficult to image with AFM as the nuclear basket, the structure of filaments extending into the nucleus, is soft and fragile. The image quality depends sensitively on the quality of the sample preparation and the tip: from many repeated sample preparations, typically only a small number gives images where the nuclear basket is clearly visible and not destroyed during imaging using standard pyramidal tips. The long tails pointing to the right show that the setpoint was set so closely to the free amplitude that the feedback could not follow the surface well. This minimizes the force and is necessary to get images of this sample. In this image one can even recognize the individual filaments which extend from the ring at the membrane to the top of the basket.

Figure 3.3b shows the membrane imaged with a nanotube tip. The image presented shows the nuclear baskets and individual filaments. The quality of the image is about as good as for the best images with a normal tip, although we did not test many preparations for the nucleoplasmic side with nanotube tips, as we mainly focussed on imaging the cytoplasmic side. There are some ‘jumps’ in the topography, showing up as stripes which suddenly end and which we
3.3. NPC’S STUDIED IN LIQUID USING CNT TIPS

Figure 3.3: The nucleoplasmic side imaged with a normal tip (left) and a nanotube tip (right). The normal tip produces a very detailed image, however the imaging force is set so low that the tip has trouble following the surface on the downward slopes.
contribute to the cantilever switching between two solutions of the equation of motion. With the nanotube tips, we could decrease the setpoint enough to follow the surface accurately also downhill. In contrast to normal tips we could do this without increasing the interaction strength so much that we damaged the surface. As we discuss also in section 2.1, we think this is due to the reduced contact area between nanotube and sample compared to a normal tip. As the sample is compliant, it deforms under the load applied by the AFM tip. A normal tip, which might have a small final diameter but widens away from the surface, forms a larger contact area than a nanotube tip. This will increase the sample deformation as well as the amount of liquid that has to be removed on each tip oscillation. Both effects increase the energy dissipation per cycle, which causes enhanced sample damage.

### 3.3.2 Cytoplasmic side

When imaging the cytoplasmic side of the nuclear membrane in liquid with nanotube tips, we see a number of qualitative differences as compared to pyramidal tips. During measuring we often changed parameters like driving frequency and setpoint amplitude, resulting in different types of images. However, also without changing parameters, we saw changes in the image quality and type, most likely because of changes in temperature or changes in liquid composition — e.g. because of evaporation of water — or tip contamination.

Some images show a topography with clear correlation between amplitude and phase images, while in other images this is not the case. We will present here an overview of the types of images we observe, with examples all collected from the same sample using the same tip during one day. While the data set presented here is the most diverse and with the highest quality data that we obtained using nanotube tips, unfortunately we do not have a SEM image of this particular tip after the experiment. The images of single pores shown in the figures 3.5 – 3.8 are cut-outs of larger images, showing pores that are representative for all pores in those images.

Imaging parameters were varied between images; especially changes to drive amplitude and amplitude setpoint, and drive frequency resulted in qualitatively
Figure 3.4: Cytoplasmic side of NPC with normal tip in liquid.

different images. The amplitude setpoint was varied between 60% – 95% of the free amplitude, which was varied from an estimated 5 nm – 20 nm. Drive frequency was varied above and below the resonance peak in the acoustic drive spectrum. The original images range in size from 0.8 µm – 2.5 µm with tip speeds ranging from 1.6 µm s\(^{-1}\) – 5.0 µm s\(^{-1}\). Different tip speeds did not result in qualitatively different images.

The sample used for the experiments in this section was prepared with pores plugged with cargo, as described in section 3.2. For reference, we first present one image taken with a normal AFM tip (of TYPE ) in figure 3.4. Clearly visible in this image are the ring structure of the pores and a large plug in the center, consisting of cargo trapped in the pore. Whether substructure is visible in the ring depends sensitively on the quality of the preparation and the AFM tip. In this image there is no substructure easily discernable, although in other similarly prepared samples sometimes subunits are visible. We do not have images of high enough quality to show substructure with pores plugged with cargo.

Figures 3.5, 3.6, 3.8 show cut-outs of different AFM images of NPCs taken with CNT tips, showing different contrast mechanisms in AC mode. All NPCs have the same lateral dimensions with an outer diameter of slightly above 100 nm, although the dimensions of the pictures can be slightly different. The figures all show height, amplitude and phase in the left, middle and right col-
Figure 3.5: AFM images of the cytoplasmic side of NPCs with a CNT tip showing a large phase response. The height scale in the topographical images in this set is 70 nm; the colour-scale in the amplitude images is 50 mV (sensitivity was not calibrated for these measurements, but this corresponds with 1-2 nm); the colour-scale in the phase images is 50°.
3.3. NPC’S STUDIED IN LIQUID USING CNT TIPS

Figure 3.6: AFM image of the cytoplasmic side of NPCs with a CNT tip showing different imaging mechanisms on one pore. The height scale in the topographical images is 50 nm; the colour-scale in the amplitude images is 60 mV (sensitivity was not calibrated for these measurements, but this corresponds with 1–2 nm), the colour-scale in the phase images in the upper two rows is 10°, in the lower two rows it is 30°.

Different rows within one figure show one and the same pore from different, subsequent images. Please note that lateral thermal drift can cause distortion to the images, so that, even though features can be recognized between NPCs in one set, they can look distorted with respect to each other. The pore shown is always representative of all pores in the particular series of images (usually there are between 10–20 pores in any one image). Differences in contrast mechanism were induced by varying imaging parameters, in particular drive amplitude and amplitude setpoint, and drive frequency.

Figure 3.5 shows a series of AFM images where the phase image is markedly different from the amplitude image. The ring as well as cargo in the center show a larger phase lag than the material directly surrounding the pore. This signifies that the tip is sensitive to surface properties in the phase channel.

Figure 3.6 shows a series of images, taken of one and the same pore. The top two images are different from the bottom two images, presumably because there
are two different imaging mechanisms that result in two qualitatively different types of images. The images show a number of common topographical features and the images of the pores can still be recognized as being from the same pore. The upper images more clearly show subunits in the ring. The lower images more clearly show filaments reaching from the outer rim to the cargo in the center. All images are slightly distorted because of drift. Figure 3.7 shows the NPC of fig 3.6 with two neighbouring NPCs and a crosssection through it showing its steep features with slopes up to 60° both uphill and downhill.

Figure 3.8 shows successive images of one pore with substructure visible at the bottom inside the pore. This might be the FG repeat mesh which is thought to be inside the pore [8]. With high quality images we always see something inside pores. Interestingly, the measured height from the top of the ring to the bottom in these images, 15–18 nm is slightly less than the maximum depth measured with normal, hydrophilic tips, where heights up to 25 nm are reported [3].
3.3. NPC’S STUDIED IN LIQUID USING CNT TIPS

Figure 3.8: AFM image of the cytoplasmic side of NPCs with CNT tip showing structure inside the pore. This series shows three different pores, one in the upper two rows, one in the middle two rows and one in the last row. The height scale of the topography images is 40 nm, the colour scale in the amplitude is 60 mV (sensitivity was not calibrated for these measurements, but this corresponds with 1–2 nm), the colour-scale in the phase images is 10°. All these pores show structure at a depth of around 15 nm in the pore. This structure is clearly different from the filaments in figure 3.6 in that the filaments do not start at the height of the outer rim and they seem less organized.
3.4 NPC's studied in air using CNT tips

While one of the strong points of AFM is that the technique allows to investigate biological samples under real-life conditions at high resolution, it can still be useful to perform experiments in air as well. In air, the cantilever has much less damping and therefore a higher quality factor. As the resonance peak is sharper, the response of the cantilever to changes on the surface becomes larger and changes in sample properties show up more clearly. While whole cells do not survive in air, isolated proteins or membranes may be preserved quite well, because there is always a thin waterfilm covering the sample, originating from the humidity of the air. We expect that the tip will be especially sensitive to hydrophobicity of the sample as this will influence the presence and properties of the liquid film on the surface.

Figure 3.9 shows clearly how sensitive AFM imaging can be to the imaging conditions. Even though these images contain a lot of interesting data and details, we have not been able to link this to known structures of the NPC or to deduce the sample properties responsible for these striking images.

3.5 Discussion

We have shown that nanotube AFM tips are useful for imaging complex real-life samples, such as Nuclear Pore Complexes (NPCs). From these measurements we can see that the benefit that these tips bring does not predominantly lie in higher resolution, but rather in other properties of the nanotubes, such as reduced tip-sample interaction and their hydrophobicity.

On the cytoplasmic side of NPCs, the use of nanotube tips has allowed us to image filament-like structures which extend from the rim of the pore to cargo trapped in the center. While such filaments have been observed before by electron microscopy [11], they have not yet been imaged with AFM, despite many measurements on similarly prepared sample. These filaments have been postulated to be the cytoplasmic filaments bringing cargo captured in the cytoplasm towards the pore. These are likely delicate structures and can easily be deformed or destroyed by AFM imaging. We believe that the reduced interaction
3.5. DISCUSSION

Mode switching: the phase changes by a large amount; the topography shows a jump to the new where the amplitude equals the setpoint.

Figure 3.9: Topography and phase images of the cytoplasmic side of NPCs with a CNT tip in air. These images were measured using a PicoPlus AFM (Agilent). Both images were recorded on the same sample, with the same tip, the lower image about 30 minutes after the upper one. Imaging conditions were almost equal, although slight adjustments had to be made to keep the tip in contact with the surface. Image size for the top row is 600 nm, for the bottom row 700 nm. The height scale in the top topographical image is 125 nm, in the bottom image only 60 nm. The phase images are uncalibrated, but as they have been taken without changing tip, they can be compared. the top image has a colour scale ranging 6 V, the bottom image 1 V.

We hypothesize that the tip picked up some material while scanning, altering its interaction with the sample and rendering two such different phase images. Even after after extensively testing different imaging parameters, we could not again capture images like the upper one, suggesting a permanent change in the tip-end. Also noteworthy is the clear signatures of mode switching of the cantilever in the upper image.
between nanotube tip and sample has been crucial to be able to image these filaments.

We have some counterintuitive results when comparing tip convolution in images from nanotube tips and normal tips. Contrary to what one might expect, the images made using nanotube tips do not exhibit steeper profiles. However, we can not rule out that this is because the nuclear pores do not have steeper features than those imaged with nanotube tips. Also, when pores do not have trapped cargo, images made with normal AFM tips show pores that are a bit deeper than in images made with nanotube tips. While this seems to suggest that normal tips are better able to probe inside the pore than nanotube tips, the images with nanotube tips consistently show structure on the bottom of the pore where normal tips never show this. We suggest that the structure that we see with nanotube tips is the FG repeat mesh, which prevents larger proteins from diffusing through the pore. We also suggest that the difference in measured depth is because the rim of the pore, or perhaps the filaments on top of the ring, are sensed differently with normal, hydrophilic tips and hydrophobic nanotube tips.

On the nucleoplasmic side of the membrane we could image the soft and fragile nuclear baskets without destroying them. Using normal tips, people have been able to image the same features with perhaps even slightly better contrast, but needed to keep the imaging force very low, to the point that the feedback had trouble following the surface downhill. With the nanotube tips we found the preparation to be less critical to obtain good resolution and we could use lower setpoint amplitude, making the measurement easier and less susceptible to drift. This can be explained by the nanotubes high aspect ratio: less surface contributes to the interaction between tip and sample, making the tip more sensitive to the local topography.

The large differences between observed images on the cytoplasmic side shows that the AFM is sensitive to surface properties of the sample. Unfortunately, it is not straightforward to separate topographical effects from contributions from different tip-sample interactions as the details of the interaction and its influence on the tip motion depend sensitively on the settings and environment. The observed variety of images does not stem from a variety of
properties in NPCs: within one larger-scale image, all NPCs always show the same features and contrast in the different channels.

When amplitude and phase are uncorrelated, the phase image certainly has a large contribution from local variations in surface properties. For example in figure 3.5 this is the case. The ring and some cargo in the center show a large phase offset, while there is no corresponding feature in the amplitude image. The hydrophobic nature of the protein we used to block the central channel suggests that the phase contrast is due to hydrophobicity, although we cannot exclude other material properties such as elasticity. Using similar arguments, Kramer et al [3] propose that the inside of the pore is hydrophobic, but not the outer rim.

To be able to determine the nature of the interaction it would be necessary to perform amplitude- and phase-distance curves at each point in the image. This would allow to determine force and dissipation profiles as a function of distance between tip and surface, giving quantitative information on the type and strength of the interaction [2]. Also for this experiment the higher aspect ratio of nanotubes would ensure that the measured interaction is between the tip and only a small area on the sample surface and thus ensure the most sensitive measurement.

In conclusion, the use of nanotube tips has allowed us to observe for the first time the FG repeat filaments of the Nuclear Pore Complex with AFM. The use of nanotube tips allows more reliable and easier imaging of fragile structures. AFM imaging with nanotube tips results in a rich variety of images, which we interpret as the probing of a rich variety of tip sample interactions. Understanding of these interactions would likely help to understand the functioning of NPCs.

Bibliography


CHAPTER 3. IMAGING NUCLEAR PORES WITH NANOTUBE AFM TIPS
Chapter 4

Recognition Imaging

One of the possible future applications for CNT AFM tips is their use in high resolution recognition imaging. In simultaneous topography and recognition imaging (TREC), explained in detail in section 4.1, the AFM tip is functionalized via a flexible linker with a ligand at its end. The specific binding of this ligand to a receptor on the sample surface is recognized by its characteristic influence on the cantilever motion. With standard tips, the length of the linker, about 7 nm, limits both the resolution and the maximum scanning speed [1]. The linker cannot be shorter for geometrical reasons: from its random anchoring point on the tip we need one and only one linker be able to reach the surface. Nanotubes with an open end can be functionalized specifically at the -COOH groups present only at the end of the nanotube, giving the opportunity to select any linker length. This length is however also important in other aspects: it sets the optimum amplitude for scanning, it sets the maximum volume the ligand can diffuse through and therewith the effective concentration of the ligand and, finally, it determines the forces the ligand feels while bound to its binding partner on the surface as the linker stretches during the cantilever oscillation. Before functionalizing nanotubes it is important to understand how these factors influence the statistics for binding and the signal to noise ratio for the detection of binding. In this chapter we investigate these various issues by comparing measurements with a standard AFM tip with numerical computations. In section 4.1 we will first introduce recognition imaging. In section 4.2
we will then introduce the theory we used to model trec imaging and some considerations on the validity of the approximations for this particular experiment. Next, in section 4.3, we will introduce the model system used for the experiments described in this chapter. In section 4.4 we will show the experimental results and describe the analysis we performed and its results. In section 4.5 we will discuss the implications of our findings for recognition imaging.

We find that trec imaging is currently limited by a combination of factors, including linker length limiting lateral resolution, cantilever properties limiting the signal to noise ratio and receptor-ligand interactions limiting scanning speed. Moreover, these factors are linked in non-trivial ways. One solution to be better able to tune individual parameters and make recognition imaging more effective is to use nanotube tips. Incidentally, this would also allow more quantitative results to be extracted from our analysis and might make this method useful to study single molecule binding kinetics, as discussed in sections 4.4 and 4.5. The measurements described in this chapter have, however, been performed with standard AFM tips.

4.1 Simultaneous topography and recognition imaging

While AFM has very high spatial resolution, allowing to image single proteins, because of tip convolution it is almost always impossible to recognize proteins in a natural membrane by their geometry alone. To overcome this limitation simultaneous topography and recognition imaging (TREC) has recently been developed by P. Hinterdorfer et al. \[1\], \[2\] to extend AFM with the capability to specifically recognize single proteins on surfaces. This method utilizes specific biochemical binding of a ligand to the protein of interest in the sample.

For TREC imaging, a standard AFM tip is chemically modified according to the methods developed by P. Hinterdorfer et al. \[6\]. This method binds protein to the AFM cantilever through a flexible polymer linker, where the density of linkers is tuned such that on average only one protein can reach the sample surface. This technique is popular for force spectroscopy measurements \[7\]
4.1. SIMULTANEOUS TOPOGRAPHY AND RECOGNITION IMAGING

Figure 4.1: Timetraces of a cantilever scanning a surface with proteins with feedback turned off (cf. [1]). While the authors labeled the vertical axes here ‘Amplitude’, ‘Deflection’ is what is actually plotted. On the left specific binding is blocked: on the right the ligand can bind specifically to proteins on the surface. While on the left the top half of the cantilever oscillation is unaffected by topography, on the right there is a clear affect, which can be attributed to specific single molecular binding.

because the linker separates non-specific interactions, between AFM tip and surface, spatially from protein-ligand dissociation in force distance curves. This is also what allows TREC imaging to separate topographical information from binding in the amplitude signal.

With TREC imaging, the deflection signal of the cantilever is split in a bottom and a top half, see figure 4.1. The bottom half of the oscillation signal corresponds to the downward swing of the cantilever, when it touches the sample surface. It provides topographical information and is used to perform feedback on. Thus it is analogous to the amplitude signal in Magnetic AC (MAC) mode AFM. The top half of the signal provides the recognition signal, as this is where the linker is stretched. The top and bottom half of the oscillation can be treated independently because of the low quality factor, $Q$, of the cantilever oscillation, which for soft cantilevers is often below unity in liquid near a surface. On a conceptual level, this is similar to performing two contact mode (dc) measurements at the same time: one to probe the topography and one to probe the binding events.

Figure 4.2 shows a typical recognition image. Unlike one might expect at first thought, unbinding is a stochastic process, with a finite possibility to happen even directly above the binding site. On second thought, however, this is what should be expected: to get a detectable signal, the cantilever must be
Figure 4.2: Typical recognition image at high magnification. This image will be discussed in detail in section 4.4. The lateral dimensions are 200x200 nm, height scale is 2.5 nm in the topography image and 0.35 V in the recognition image. It was recorded on a PicoPlus AFM equipped with the optional TREC module. The left image shows the topography of S-layer, a bacterial protein which forms 2D crystals and the right image shows the corresponding recognition image. Some of the proteins making up the lattice have been modified to bind to the protein tethered to the tip so isolated recognition sites show in this image, as darker patches. Binding and unbinding are stochastic and both can happen anytime the tip is within reach of a binding site as shown by light areas inside the darker patches.

pulling on the bond. This force, however, causes the bond to break every now and then. Binding and unbinding under force are important to understand when attempting to optimize TREC imaging and the analysis of these processes is an important part of this chapter.

4.2 Theory for recognition imaging

4.2.1 unbinding rates

Unbinding of single proteins is well understood theoretically and there is independent experimental data available for the interaction we will be investigating in section 4.4. The specific interaction between two bio-molecules is described well by a potential well with an energy barrier to the unbound state [8]. Without applying any external force, the bond will break eventually because of thermal
activation. The rate of unbinding in this limit is

$$\nu = \nu_0 \exp\left(\frac{-E_B}{k_B T}\right) \quad (4.1)$$

Here $E_B$ is the height of the barrier, $\nu_0$ the natural attempt frequency, $k_B$ the Boltzmann constant and $T$ the temperature. Applying a force $F$ tilts the energy landscape, lowering the barrier and quickening the unbinding. The unbinding rate changes to

$$\nu = \nu_0 \exp\left(\frac{-E_B - F x_\beta}{k_B T}\right) \quad (4.2)$$

assuming that the barrier has a sharp maximum at a distance $x_\beta$ from the binding site [8].

Usually in force spectroscopy experiments, the force is increased over time and the force at the moment of unbinding is registered. Because of the stochastic nature of unbinding a distribution of unbinding forces will be found. For the case where the force is linearly increased in time with a rate $r_F$, the most likely force of unbinding $F^*$, which is the peak in the distribution, can be found to be

$$F^* = F_\beta \ln(r_F) \quad (4.3)$$

when the loading rate $r_F$ is sufficiently large so that the bond is not broken spontaneously. Here the definition of the dimensionless loading rate was used as given in [3]: $r_F = (\Delta f/\Delta t)/\nu_0 f_a$, where $\nu_0 = (1/t_D)\exp(-E_B)$, which contains the Arrhenius dependence on barrier energy, scaled by a characteristic time constant $t_D$ for diffusive escape. $f_a = k_B T/x_\beta$. $F_\beta$ is then the threshold where the applied force becomes more important than thermal activation. Measuring $F^*$ as a function of $r_F$ allows to extract $E_B$ and $x_\beta$ characterizing the bond.

For TREC experiments however, the loading rate is not constant over time. As the tip is oscillated, so is the force. Moreover, the flexible linker binding the protein to the tip is a nonlinear spring, turning the often sinusoidal tipmotion into a sharply peaked oscillation of the force. This makes it impossible to solve equation 4.2 analytically to find the unbinding rate for this experiment. Therefore we integrated the equation of motion of the tip numerically to find the force on the bond and consequently the unbinding rate. To be able to calculate the tip motion we used a simplified model of the experiment, as discussed in more detail in section 4.2.3.
4.2.2 Binding rates

It is difficult to quantitatively describe the binding rate during a TREC experiment. In bulk, the binding rate is dependent on the concentration of both binding partners and their mutual affinity. In TREC experiments, one of the binding partners is bound to the sample surface and its structure and orientation is not known in atomic detail. The binding rate is partly determined by the concentration of the protein on the tip, which can diffuse through a small volume limited by the linker but also by the combined geometry of tip and sample.

As the extension of the linker is governed by entropy according to the worm-like-chain model, there is a gradient in effective concentration of the protein around the tip. The exact dependence of the concentration with distance is difficult to calculate, due to effect of excluded volume from the nearby surfaces and the protein itself on the statistics of the linker conformations. Therefore we will approximate the space available to the protein here as a cylinder with a radius defined as the distance where the energy for stretching the linker equals $k_B T$. Vertically, the space is defined by the oscillation amplitude of the cantilever.

4.2.3 Model

Figure 4.3 shows a schematic picture of the model used for calculations. The tip oscillates with a peak-to-peak amplitude close to but smaller than the linker length and moves lateraly to scan the surface. At the same time, the linker molecule allows the ligand to diffuse through a small volume around the tip. Owing to the worm-like-chain characteristics of the linker [5], the effective volume available to the ligand to explore by thermal energy has a radius of about half the linker length. The timescale to cross the effective volume by diffusion is much less than the period of oscillation of the cantilever. When the tip comes closer to the receptor on the surface there is an increasing chance that the ligand will bind. Once the ligand is bound, the tip feels a force everytime it stretches the linker at the top of its oscillation cycle. This reduces the cantilever amplitude and produces the TREC signal. At the same time, the ligand might
be pulled of the receptor by this force. The settings when doing TREC measurements will have to be optimized so that, when the tip is above the receptor, enough force is generated to detect binding but not too much, so that usually the ligand and receptor will remain bound. When the tip passes the receptor and starts to move away, the linker is stretched more on every oscillation cycle until the force will get too large and the ligand and receptor unbind.

We analyze the measurements in this chapter in terms of binding and unbinding rates and compare these experimental rates with calculations. To do calculations based on the seemingly simple picture outlined above, we have to make a number of far-reaching simplifications. For calculating binding rates, we have to calculate the effective concentration of ligand at the position of the receptor. Ideally, we would calculate the partition function for the ligand and the flexible linker and use that to find the chance for the ligand to be near the binding site. While this is possible for an unconstrained worm-like-chain, this is not possible at the moment for a worm-like-chain confined between two surfaces as is the case here. Instead, to calculate an effective concentration, we consider the molecule to move freely through a cylinder with a height set by the oscillation amplitude and a radius determined by the distance past where
it takes more than the thermal energy to stretch the linker.

For calculating unbinding rates we do numerical calculations based on the equation of motion of the tip. We include all forces depicted on the schematic in figure 4.3: the driving force on the cantilever, a repulsive tip-sample interaction, a force from the linker based on the worm-like-chain and a thermal driving force. Unbinding is modeled as a randomly distributed process based on equation 4.2. Here, we also made a few simplifications: the linker is regarded as a worm-like-chain without geometrical constraints due to the nearby surfaces, as is usually done in analyzing force spectroscopy data from experiments with similar geometries, and the sizes of the receptor and ligand are not taken into account. In section 4.5 we will discuss the various issues that arise from these simplifications.

4.3 Experimental Model system

To analyze TREC imaging to the detail as we will do in section 4.4, it helps if the topography shows little corrugation. Although the quality factor of the cantilever is low, it is not so low that crosstalk between topography and recognition is completely suppressed, especially if the error signal becomes large.

As a model system we used S-layer protein fused with strep-tag on the
4.3. EXPERIMENTAL MODEL SYSTEM

sample-surface and streptavidin on the tip. S-layer is the most common cell surface layer found on prokaryotes. It has a crystalline protein structure, which, after purification, reassembles spontaneously on solid supports such as silicon or lipid bilayers. We used S-layer protein SbpA of Bacillus Sphaericus CCM 2177. Four copies of one protein combine to form the basic square structure of the lattice. The lattice has a low corrugation of only a few Angstrom over large distances. Besides being a regular and flat template for binding sides, the lattice also serves as an internal lateral size calibration in our experiment with a known lattice constant of 13.9 nm.

Some of the S-layer proteins were fused with strep-tag through genetic engineering. This small poly-peptide is an artificial ligand with extremely high affinity for streptavidin, even higher than the affinity of biotin to streptavidin. Activity of the fused construct was tested by force spectroscopy measurements on a lattice fully consisting of modified proteins, as described in [4]. The probability of finding binding events in the force curves, and more importantly the reduction of this probability after biochemically blocking the interaction, is a good test for functionality of the tip. We found a binding probability of 13%, which reduced to 3% after blocking the specific binding by adding free biotin to the solution. From force spectroscopy data we found the dissociation constant $k_{off} = 2.38 \text{ s}^{-1}$, which is the dissociation rate at zero applied force, $x_9 = 0.352 \text{ nm}$. For the TREC measurements described here we used a preparation with a 1:7 ratio of modified and unmodified protein.

The cantilever was modified according to the protocol developed in [6], attaching a streptavidin molecule covalently to the AFM tip through a flexible Poly-Ethylene-Glycol (PEG) linker with a length of 7 nm. We used Agilent MAC mode Type IV cantilevers E with a nominal spring constant of 0.1 Nm$^{-1}$. The AFM was an Agilent 5500 AFM. We did all measurements in Magnetic AC (MAC) mode, with the drive frequency set at about 75% of the free cantilever resonance frequency in liquid far away from the surface. TREC signals were recorded in ‘full amplitude’ mode using Agilent electronics. Measurements were performed at different drive and setpoint amplitude, recording both left-to-right and right-to-left (‘trace’ and ‘retrace’) of topography, amplitude and recognition signals.
4.4 Results and analysis

4.4.1 Localization of the binding site

As a first step in the quantitative analysis of TREC imaging, it is necessary to locate the binding site exactly, so that subsequently we can find the distances from the site where the ligand binds and unbinds. Finding this location is also an interesting goal in itself (private communication with Jilin Tang, JKU Linz).

Before we can do this there is a practical issue to solve with the AFM images. We used a small scanning area of 200 nm on a side to get many datapoints for each recognition spot. As a result of the small imaging size and moderate scanning speed, there is considerable drift in the images. As we need to calculate distances accurately, we have to correct for the resulting image deformation. The topographical image, which shows the square lattice of S-layer with known lattice constant, is well suited to base this correction on. We assume here that the drift is constant over the acquisition time of a single image and therefore we use a linear correction. Knowing where the maxima in a FFT image should be for a square lattice, it is straightforward to calculate the coordinate transformation which generates the observed position of the maxima in the measured data. Applying the inverse transformation compensates for the drift during imaging. After this correction we find that the trace and retrace topography images are still shifted by a small amount with respect to each other. We quantify this shift by performing a cross correlation between both topography channels and compensate for this as well. We are sure this shift is not caused by lagging feedback as the error image only shows noise and the shift is several pixels large. As we need to perform interpolation to get the corrected image as in figure 4.5, we do all image analysis on raw data and then perform the coordinate transformation on the location of features that we find in the raw data, before we continue processing them further.

TREC images show recognition spots with a size of about 15 nm. This size is set mainly by the contour length of the linker which binds the ligand to the AFM tip. To single out the recognition spots in the recognition images, we threshold the images. For thresholding we use an automatic procedure known
4.4. RESULTS AND ANALYSIS

Figure 4.5: Drift correction. Thermal drift causes distortion of the images as in effect the sample moves relative to the tip. The left images show raw data before drift correction. Although the S-layer is known to form a square lattice, the FFT image does not show a square lattice. The right images are the same data after drift correction as described in the text. All figures in the rest of this chapter show data from this image, which is also shown with a recognition image in figure 4.2. The lateral size is 200x200 nm, as set in the AFM software. The height scale is 1 nm.
Figure 4.6: Drift-corrected topography and recognition image next to each other. The topography trace and retrace overlay well, as shown by the split view (upper triangle: trace, or left to right, lower triangle: retrace, or right to left). The Recognition has been thresholded and different gray-scale levels indicate the following: dark gray: trace; light-gray: retrace. White spots indicate an overlap between trace and retrace. White rectangles indicate the spots used for analysis in the rest of the chapter. The recognition image is thresholded as described below. Please note that the trace and retrace of the recognition image, aligned using the exact same parameters as the topography, do not completely overlay.

Figure 4.7: Thresholded recognition image with a two enlarged single spots to the right. This image has not been processed for drift correction to avoid interpolation in the image and shows the raw data as analyzed in this section. Clearly visible in the enlarged spots is the stochastic behaviour of binding and unbinding.
as Otsu thresholding. We found that using somewhat different thresholds did not change the outcome of the analysis significantly. As can be seen in figure 4.6 trace and retrace of the recognition images do not overlap, while the topography images overlap well: binding takes place systematically closer to the recognition site than unbinding. This is because when unbinding the linker may be stretched to a length close to its contour length, which takes a lot more than the thermal energy which is available for it on average to stretch to reach the recognition site for binding. We can locate the recognition site now as the center of mass of the combined spot of trace and retrace images after thresholding, see figure 4.6. For this procedure and all analysis presented in the following sections, we used only spots that we could unambiguously separate from neighbouring spots. For the analysis a total of eleven spots from one image, taken without changing imaging parameters, was used. These spots are indicated in figure 4.6 with a blue rectangle. Figure 4.7 shows the same image as it was used for data analysis, i.e. without drift correction.

4.4.2 Binding and unbinding rates

Now that we know the exact location of the binding site, we can check how the interaction between the molecule on the tip and on the sample surface depends on the distance between the tip and the binding site. To this end we will use the data from multiple recognition sites from one image to calculate the effective binding and unbinding rates as a function of distance between the tip and the binding site. We start by calculating the probability that the molecules are bound as a function of the distance to the binding site. We do this by counting the number of pixels below the threshold in the recognition image relative to the total number of pixels at each distance. We find the probability distribution in figure 4.8. Negative distances, in this graph as well as in all subsequent graphs in this chapter, are data from before the point of closest approach of the AFM tip to the binding site on any particular scanline. In other words, negative distances indicate the tip is approaching the binding site, while positive distances are from after the closest approach, where the tip moves away from the binding site.
**Figure 4.8:** Probability that the ligand on the tip is bound specifically to a protein on the surface as a function of distance from the binding site, as determined from TREC images. The asymmetry in probability between when the tip approaches the binding site (negative distances) and when it moves away (positive distances) can be attributed to the fact that, once the ligand has bound, it will take some time to dissociate again, during which time the tip moves further.

**Figure 4.9:** Distributions of the distance of binding events to the binding site. The peak at negative distances is larger than at positive distances because at negative distances there is less chance that the ligand and receptor are bound already, so that there is a larger possibility for a binding event to occur. The dip at zero distance is because the tip spends very little time so close to the binding site. The trace and retrace distributions show the same profile when aligned such that both show approaching the binding site on the left and retracting on the right.
4.4. RESULTS AND ANALYSIS

Next, we look at the occurrence of binding and unbinding events. These can be found as the edges of the thresholded area in the recognition image. Traversing the image in the scanning direction, entering an area where the recognition image is below the threshold corresponds to a binding event, while leaving such an area corresponds to an unbinding event. From this we can create a distribution of binding and unbinding events as a function of distance to the binding site, see figure 4.9. Because we find that the distributions are not significantly different between trace and retrace, we will combine them further on. Again we have made sure that the thresholding is of little importance. A total of about 1300 binding and unbinding events were found in the eleven recognition spots analysed.

The binding and unbinding rates can be calculated from the distributions of distances of binding and unbinding events by taking into account the measured probability of a molecule being bound at a particular position and the time the tip spends at a pixel:

$$r_{\text{binding}}(d) = \frac{P_{\text{binding event}}(d)}{P_{\text{unbound}}(d) T_{\text{pixel}}}$$

Figure 4.10 shows the binding and unbinding rates that we find in this way.
CHAPTER 4. RECOGNITION IMAGING

Unbinding

$0 \quad 2 \quad 4 \quad 6 \quad 8$

Time averaged linker energy (k$_B$T)

Binding

-10 -5 0 5 10

Distance from center (nm)

Figure 4.11: Energy stored in the linker at the moment of binding or unbinding as a function of distance, calculated from the rates in figure 4.10.
4.5 DISCUSSION

It is instructive to convert the binding and unbinding rates into an energy based on Boltzmann statistics:

\[ \text{rate}(E) = \text{rate}(0) \exp \frac{E(0) - E}{k_B T} \]  \hspace{1cm} (4.5)

which allows to find the difference in energy between two states based on the measured difference in dis-association rate from or association rate into that state. Figure 4.11 shows that the energy associated with binding goes up quickly further away from the binding site, especially for the case of association.

4.5 Discussion

It should be noted that the unbinding rate at a position right above the binding site is larger than initially expected from the model. We will discuss here first in more detail the model and where we think it fails. This has relevance to (topographic) imaging quality in TREC imaging in general as we will discuss next. Then we go on to discuss the signal to noise ratio and finish with discussing speed and resolution limitations.

4.5.1 Model

While the calculations give forces and distances that are close to the values measured, it doesn’t capture the experimental results completely. The most obvious difference between model and results is that in the situation sketched in figure 4.3, the tip will image the protein attached to it when this is bound to the recognition site and the tip is over the site as well. In the measurements we never see any trace of the protein on the tip in the topography. This might make sense once we realize that the protein diffuses through the volume available to it on a timescale of 0.01 ms when it is not bound to a recognition site, much faster than the tip oscillation. Effectively the protein generates a cloud below the tip with which the surface is probed. The radius of this cloud is defined by the linker length of around 7 nm, much like a normal average AFM tip. This is in line with the experimental observation that it is virtually impossible to get high resolution topography — requiring a tip radius of about 2 nm — at the same
time as clear recognition (private communication with J. Tang, JKU Linz). This
does not explain what happens when the ligand is bound to the receptor. If the
tip itself is blunt, it then probes the protein sitting on the surface which could
have the same height as the effective cloud that the ligand generates when it is
free.

Related to this is the fact that in the calculations the force on the linker
and therefore the recognition signal is zero, when the tip is above the binding
site, while it gets larger further away from the recognition site. Because of the
before-mentioned effect we calculate too small a distance between AFM tip and
recognition site. This is further aggravated because the model is too simple.
For example, we do not take into account compliance of the protein and we
allow the protein to rotate freely around its binding site on the surface. These
assumptions affect the force on the linker. The unbinding rate that we calculate
is therefore a lower bound.

It is worth noting that if the above issues can be solved it is possible to
investigate single molecule binding kinetics with this experiment. Nanotube
tips would help in this regard: because of the high aspect ratio of the nanotube
the excluded-volume effects will be smaller. It will also be advantageous to
know the anchoring location of the linker on the tip more precisely.

4.5.2 Signal to noise

TREC imaging requires a very precise setting of the amplitude. It only takes
a small change in amplitude to go from an amplitude that is too large, caus-
ing continuous unbinding to an amplitude that is too small, not stretching
the linker enough to generate enough of a TREC signal. For the experiments
described in this chapter the TREC signal is mostly lost if we decrease the am-
plitude by about 10% from the maximum amplitude where we see a recognition
signal. This is because of the very non-linear behaviour of the linker molecule.
TREC imaging would be much more stable in its operation if the amplitude
were not such a critical value. Using miniature cantilevers, which have a much
higher sensitivity in the same bandwidth compared to normal cantilevers, ex-
tends the useably amplitude to about 20% in the numerical simulations. It is
important however to consider that the loading rate during each upward swing is also much higher with miniature cantilevers with higher resonance frequencies, and it is still an open question whether the assumptions used here for the calculations are still valid.

4.5.3 High speed / resolution

The binding and unbinding rates that we find experimentally are much lower respectively much higher than the rates for free molecules in solution at corresponding concentrations and the binding rate is only slightly larger than the unbinding rate. To get a signal in the recognition channel, the force on the bond has to be large enough to have a significant influence on the cantilever motion, and a high force also results in a high unbinding rate [8]. On the other hand, to see recognition events, the binding rate has to be larger than the unbinding rate for a long enough period. The situation as in figure 4.10 where binding and unbinding rates are not too disparate but with the binding rate larger when the tip is over the recognition site, satisfies these requirements. Indeed this data was acquired after lowering the cantilever amplitude just enough to see recognition spots and thus we should indeed expect this to have the optimal situation.

In order to be able to take reliable TREC image, the time averaged binding rate near the binding site must be of similar magnitude as the the pixel rate of the imaging system. This is currently indeed the case for the fastest TREC images we have been able to obtain at a pixel rate of about 1 kHz. It also indicates how further speed increases might become possible in the future.

In order to further increase speed one needs to increase the binding rate. This may be done by reducing the linker length, which would confine the ligand to a smaller volume, increasing the effective concentration, which determines the binding rate. Although the binding rate will ultimately be limited by the diffusion rate of the ligand, for small ligands it should be possible to reduce the linker length to approximately 3 nm, increasing the binding rate by up to a factor 100. Using nanotubes with an open, reactive end will be helpful to be able to choose shorter linker lengths, as these can be modified specifically at
the end and one does not have to worry about whether a linker can reach the sample surface from its random anchoring point on the tip.

This however is not the whole story. Obviously TREC will only work as long as the unbinding rates stay well below the binding rates. The success of further improvements of TREC speed will depend very strongly on whether for such short linkers it will be possible to distinguish the force due to the stretching of these shorter linkers from other forces acting on the cantilever as it scans over the surface.

**Bibliography**


Chapter 5

Mechanical Strength of Tissue probed by AFM

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Contact mode Atomic Force Microscopy (CM AFM) has established itself as a powerful tool to research the role of forces in biology from single proteins to cells and even tissues. This chapter describes AFM force measurements on tissue from the human aortic wall. The AFM was operated in Force-Volume mode (FV mode) where force curves are taken in a regular grid pattern, to obtain spatially resolved maps of the mechanical properties of the tissue, which allows correlation of the local mechanical properties with features observed through other techniques, including normal AFM imaging and optical microscopy. We used both sharp and blunt tips to test the material properties on different lengthscales, as the tissue, like many other types of tissues and cells, shows distinct organization at different lengthscales which we expect to influence the mechanical properties at those lengthscales. One of the major challenges with FV mode is data analysis. The figures presented in this chapter represent tens
of thousands of force curves each.

Tissue from the aortic wall has extraordinary mechanical properties: the aorta experiences large pressure pulses each heartbeat and dilates and contracts with the pressure to ease the pulse for the rest of the vascular system. Sometimes the aorta is permanently dilated, which is called an aneurysm and can eventually lead to failure of the aortic wall, which in turn usually results in death. Aneurysms generally occur in elderly individuals and result from many effects such as inflammation of the aortic wall and weakening of the aortic wall caused by impaired collagen metabolism. However, no biochemical explanation has been identified. Not only acquired inflammation but also inherited disorders, such as the Marfan syndrome, may cause aortic wall weakening [16]. This chapter demonstrates that the biochemical data on collagen quantity and quality of the aortic wall cannot explain the observed differences strength in the aortic wall. 3D confocal imaging and atomic force microscopy show a striking difference of the collagen network in atherosclerotic abdominal aneurysms as compared to normal aortic wall. It is shown that this is accompanied by loss of vessel wall resilience. It is also shown that, independently of their biochemical properties, the physical qualities of tissue are influenced by the degree of network organization. This implies that the structural organisation is a key component in the development of anurismal disease and other extracellular matrix disorders.

For the research described in this chapter we have combined AFM measurements with other techniques, notably confocal microscopy to correlate 3-dimensional and large scale structural changes with the results from the probing by the AFM tip.

### 5.1 Biochemics of Aneurysms

Aortic aneurysms are localized dilatations of the aortic wall. Although aneurysms are generally without clinical symptoms, most aneurysms will ultimately rupture, and bleeding from an aneurysm is now responsible for more than 15000 annual deaths in the US alone. A minority of the aortic aneurysms relate to a monogenetic disorder such as Ehlers Danlos type 4 (collagen type
Marfan Syndrome (a gene defect in the fibrillin gene) and the Loetz Dietz syndrome (TGFbeta), suggesting a link between these defects and aneurysm formation. Yet, the aetiology of the abdominal aortic aneurysm (AAA), by far the most common form of aortic aneurysms, is still unclear.

Although extensive loss of medial elastin is generally considered the hallmark of AAA formation [10], it is now acknowledged that aneurysmal growth, and ultimate rupture relate to impaired collagen homeostasis [15]. Remarkably, while numerous studies sought for putative quantitative changes in aortic collagen, results are controversial with the available studies [17, 11]. And, with the notable exception of Ehlers-Danlos type 4, no clear association between impaired collagen homeostasis and aneurysm growth and/or rupture has been found. In search of a putative collagen disorder as the underlying cause of aneurysm formation in AAA and Marfan syndrome, by far the most common forms of abdominal aneurysm formation, we evaluated possible quantitative and qualitative differences in aortic wall collagen in AAA, Marfan syndrome and normal aortic wall.

5.2 Methods and Materials

Tissue was harvested and frozen as quickly as possible. The tissue was cryo-sliced to 8 micron thickness, perpendicular to the flow of the blood, and stored in a minus 80 refrigerator. The day of the experiment, the tissue was placed in the liquid cell of the AFM and immediately immersed in PBS buffer at pH 7.5. The tissue and slide were allowed to warm to room temperature and imaged immediately.

For optical imaging the tissue was stained after slicing with pico sirus red collagen staining. In confocal microscopy, elastin was observed using its autofluorescence.

AFM AFM nanoindentation offers a number of advantages over traditional indention experiments. First of all, as biological tissue is organized at the nanoscale, a larger indenter averages out all the individual variations in the tissue and is only able to find bulk values. For some measurements, this is good and even desired, but for many biological samples, the interesting length
scales are in the micrometer to nanometer range instead of millimetres. With
the AFM and a variety of tips, the tissue can be probed at a variety of length
scales and it can be determined at what range the disease states appear.

For these measurements we used a Molecular Imaging (Tempe, AZ) Pico
Scan Atomic Force Microscope (AFM) controlled with a custom scripting pro-
gram written in Labview (National Instruments) and Visual Basic 6 (Microsoft).
Data was recorded with a National Instruments card at 100 kSs$^{-1}$ and then
processed into force volume images with higher resolution than can be taken
with the control software supplied with the AFM. All measurements were done
in the Molecular Imaging liquid cell in Phosphate Buffered Saline (PBS) buffer
adjusted to pH 7.5. Care was taken to prevent the tissue from drying as drying
causes large changes in the structure and mechanical properties of the tissue.

We used both sharp and blunt tips to test the mechanical properties of the
tissue at different length scales. For sharp tips, Park Cantilevers (Veeco) with
force constants of 0.12 and 0.58 Nm$^{-1}$ were used. For blunt tips, specially mod-
ified tips from Novascan (Ames, IA) were purchased. These are Park cantilevers
with a force constant 0.58 Nm$^{-1}$ that have a ball attached to them and then
are coated with a layer of poly ethylene glycol which helps prevent the fouling
of these tips. We calibrated the cantilevers using the thermal method. The tips
were stored in PBS between experiments and were reused when possible.

The cantilever comes in a variety of stiffnesses and has been selected for
these experiments to be near the estimated stiffness of the tissue in order to
maximize the force resolution. This is done by estimating the spring constant
of the tissue at the measured level. The conversion from Young's modulus $Y$
to spring constant $k$ is performed with the formula.

$$k = \frac{YS}{L} \quad (5.1)$$

Where $S$ is the cross sectional area and $L$ is the length of the tissue. Assuming
the ball tip as the indentor for the cross sectional area and a sample that is
200 $\mu$m thick and using cartilage’s young’s modulus of 0.6 MPa gives a spring
constant of 3 Nm$^{-1}$. Another check can be done using the forumula for young’s
modulus for an indentor derived from Sneddon [7].

$$k = \frac{2Y^*}{\pi d \tan \frac{a}{2}} \quad (5.2)$$
Where $d$ is the indentation depth, $Y^*$ is the effective stiffness of the tissue and tip combination, and $\alpha$ is the angle of the indentors sharp edge. Once again using the young’s modulus of cartilage as an estimate, the estimated spring constant was $9 \text{ Nm}^{-1}$.

Data analysis Measuring the stiffness of biological samples at the nanoscale is a difficult process. Most measurements of material stiffness have been interpreted with the Hertz model [5]. However, as Stolz et al have pointed out [14], this model is difficult to apply to the force extension curves that are collected with an AFM on biological samples. The first, and greatest, difficulty of fitting the model to the data is that it is difficult if not impossible to determine where the point of contact is with a biological sample. The samples are not homogeneous and the high sensitivity of the AFM tip will cause it to interact with anything on the surface and at the interface. This can have profound impact on the fittings to the Hertz model. Another difficulty is that the Hertz model assumes that the surface of the tested sample is flat and even. This is difficult if not impossible with biological samples. The Hertz model also assumes that the material is homogeneous, which is not the case for biological samples. Also, the Hertz model does not account for friction or adhesion between the sample and the indenter. A final problem is that fitting is nonlinear and subject to many false solutions.

As the Hertz model provides such an error prone method to model the interaction between the AFM cantilever and the tissue, we have decided to follow A-Hassan et al [2] with some modifications to overcome the difficulties in determining the effective Young’s Modulus of the tissue. This technique is more robust to the errors that are inherently present in fitting the Hertz model to data collected from AFM force distance curves. It involves determining the work performed by the cantilever on the tissue sample and then comparing this work to a known standard. By integrating the total area under the force distance curve, local variations are minimized and the curve fitting reduces in difficulty. Most importantly, the exact contact point between the surface and AFM cantilever is no longer needed, as the area under the curve at the contact point is small compared to the area under the total curve.

In our case we did not measure the standard curve with the tips, but instead
used Sneddon’s equation [7] to simulate the curve that we would expect for these particular tips. While this negates a lot of the advantages in this method, it still produces curves that are more consistent with the correct value than fitting Sneddon’s equation to the curves produce. This method also easily allows for the comparison of the data from many tips.

It was also desirable to check the softer components of the tissue. For this, Park Cantilevers were used, which are softer than the cartilage or collagen components of the tissue. These cantilevers have force constants of $0.58 \text{ Nm}^{-1}$ and $0.12 \text{ Nm}^{-1}$. They were calibrated using the thermal calibration method [8]. Both tips were used to measure the behaviour of the tissue and both measured similar stiffnesses. This shows that the force sensitivity of these tips is appropriate for the tissue and does not present an obstacle for measurement. Another effect to check for is the effect of pushing speed on the measurements. We found that in our measurements, there is a negligible effect from the pushing speed on the tissue. Furthermore, care was taken not to indent the tissue by more than a few percent of its thickness to prevent an effect of the hard, supporting substrate on the measurements.

### 5.3 Results

Tissues were first evaluated by conventional biochemical methods. Vascular load-bearing collagen is essentially composed of highly stable type I and III fibrillar collagens that are further stabilized by intra-molecular cross-linking. Biochemical as well as morphometric evaluation showed similar collagen concentrations in aneurysm wall from patients with Marfan syndrome and healthy control aorta, and elevated collagen concentrations in AAA. Type I/III collagen mRNA ratios were similar in Marfan syndrome, AAA and control aorta (Fig. 5.1H, I). Evaluation of collagen cross-linking through quantification of non-reducible, lysyl oxidase initiated collagen cross-links (hydroxylsyl-pyridinoline / lysyl-pyridinoline cross links) indicated increased intra-molecular collagen cross-linking in aneurysm wall of patients with Marfan syndrome as well as in AAA. The findings for AAA are all in line with available literature [18], and do not support a quantitative or qualitative defect in vascular collagen at the
5.3. RESULTS

Figure 5.1: (A,B,C) 3D Confocal images of the human aortic wall, stained with Sirrus Red to show the collagen. (A) Marfan media [top] and adventitia [bottom] (B) Healthy tissue [arranged the same way] (C) AAA tissue [arranged the same way, (E, F, G) Fibrillin-1 staining in the abdominal aorta. adventitia/media (100x). Observe the predominant staining of the adventitia. This was also observed in the Marfan thoracic aorta. (F) Healthy (G) AAA. (H) Histochemical measurements done on healthy aortic tissue, white, aneurismatic tissue, light gray, and Marfan aortic tissue, dark gray. Hydroxyproline / proline is a measure for the amount of collagen in the tissue. HP+LP/TM is a measure for the number of crosslinks per triple helix. HP/LP is the ratio between hydroxylysyl pyridinoline and lysyl pyridinoline. (I) The mRNA expression relative to GAPDH of collagen I, collagen III and three different crosslinks (LOX, LOX-LP and PLOD-2)
chemical level in AAA or aneurysms in Marfan syndrome.

In the absence of a putative defect at the biochemical level, we sought for possible defects in collagen network formation in Marfan syndrome and AAA. Histological evaluation (pico-sirus red collagen staining) showed the distinct collagen organization in the medial and adventitial layers of the normal aortic wall (Fig. 5.1E). A similar pattern but with minor fibrotic changes is seen in Marfan syndrome (Fig. 5.1D). Collagen deposition in AAA, on the other hand, is characterized by complete loss of architecture and deposition of disorganized and condensed collagen (Fig. 5.1F), which is consistent with fibrosis. As two dimensional imaging masks defects in the structural relationships of collagen fibers in the third dimension, we also performed three dimensional visualization of medial and adventitial collagen networks through confocal microscopy (Fig. 5.1A, B, C). This evaluation showed the distinct collagen organization in the medial and adventitial layers of the normal aortic wall even more clearly. Collagen networks in the medial layers are characterized by small interdispersed collagen fibrils that run between circumferential elastic sheets. Adventitial collagen on the other hand is arranged in a highly organized, loose knitting work of ribbon-like collagen bands that provide a high resilience and flexibility. These observations are consistent with experimental and clinical observations showing that the elasticity of the arterial wall essentially resides in medial layer whereas the adventitial layer is essentially responsible for the resilience of the arterial wall.

Evaluation of collagen networks in thoracic aneurysms of patients with Marfan syndrome revealed minor changes in the medial layers of the aortic wall. Conversely, evaluation of adventitial collagen in these patients revealed a dramatically disturbed architecture with deposition of parallel thin collagen fibrils and complete absence of collagen aggregation as seen in normal aortic adventitia (Fig. 5.1D).

Collagen networks in AAA were clearly distinct from aneurysms of Marfan patients or normal aortic wall. Visualization of collagen structures in AAA (Fig. 5.1F) showed extensive arterial wall remodeling with complete loss of architecture and disappearance of the distinction between medial and adventitial collagen organization. Collagen deposition in AAA is best characterized by de-
position of aggregated, parallel collagen sheets that appear to have lost their flexibility. The disordered properties of the collagen networks in Marfan syndrome and AAA suggest that differences in the collagen network structure much more than its composition may underlie the weakening of the aortic wall.

Atomic Force Microscopy (AFM) is an established means to test the mechanical properties of cells, muscle fibers and individual proteins [4, 12, 19, 13, 1, 3, 6, 9]. AFM experiments are performed by pushing a sharp needle into the tissue at multiple points on a surface of the tissue and testing the mechanical response. The tissue response to the indentation is used to calculate the elasticity (Young’s modulus) for the given point as described in section 5.2. Individual elasticity measurements were integrated to create stiffness maps of the vessel wall. The high force resolution of the AFM (piconewtons) as well as its high lateral resolution (several elasticity measurements each square micrometer) makes it ideal for precise mapping of the mechanical properties of tissue at the sub-millimetre level. The elasticity maps can be correlated with high resolution confocal images. Moreover, the individual elasticity measurements can be compiled into a histogram which can be used express the elasticity of the arterial wall.

Tissue was probed at two different levels: a sharp tip (25 nm end radius) was used to probe the tissue at the individual fiber level, while an AFM cantilever with a 5 µm radius ball was used to probe the behaviour of the network at a tissue level. The two tips allow a comparison of the tissue at different length scales. The sharp tip is designed to only react with individual molecules in the tissue, while the larger ball tip is designed to probe the local network as a whole. In both cases we calculate the Young’s modulus as this is independent of the size of the indentor, allowing a direct comparison of the stiffness of the tissue only. This method completes the picture of the mechanical properties of the tissue from the macroscopic level which has already been published down to the level of individual fibers and molecules.

The normal tissue provides a nice starting point for the analysis. Stiffness histograms for the normal aorta (adventitia) show that the tissue response is independent of the size of the tip that is used. This indicates that the normal adventitial layer behaves as a highly coherent network. The stresses encoun-
Figure 5.2: Topography of the various tissues as determined from AFM force volume spectroscopy at 11 nN of constant force. Marfan Tissue probed with the ball tip (A) and a pyramidal tip (B). The small inset in (A) shows the Marfan tissue at 40nN. Healthy Tissue probed with ball tip (C) and a pyramidal tip (D). AAA Tissue probed with ball tip (E) and with pyramidal tip (F). To the right shows comparison of the effective Young’s modulus for different tissue types. Values are plotted on a log scale to improve the comparison of the modulus.
5.3. RESULTS

tered by the tissue are spread over the whole network equally and the scale of the challenger does not matter. The fact that the sharp tip is not able to penetrate the network also indicates that the components of the network are densely crosslinked, as the fibers are not able to slide out of the way easily, and all fibers are pulled when any one fiber is moved. These results have been checked for healthy tissue from multiple donors, with AFM tips having different force constants, and with different tissue thicknesses and we have found the results to be consistent.

The differences with the other tissues are apparent immediately. The stiffness of aneurysmal tissue is wildly different when probed with both the sharp and ball tip. The ball tip, which interacts with the larger extracellular structures, senses a very stiff piece of tissue, which is in line with other observations that the AAA tissue becomes stiffer, but the sharp tip finds that the interconnections between the principle fibers are much weaker than was shown in the healthy tissue. Biochemical experiments have shown that there is an increase the amount of collagen (a very stiff fiber) at the expense of elastin in the aortas of those suffering from aneurysms. Our analysis shows that the smaller tip pushes between the fibers of the extracellular matrix with little resistance, while the larger ball tip is completely unable to push through the network, and only encounters the resistance of the stiff collagen network.

The Marfan tissue shows an intermediate result. The larger ball tip senses a sample that is similar to the healthy tissue. This would seem to indicate that the amount of proteins is normal, as is also shown by the biochemical data, but the sharp tip plunges through the tissue just as it did with the aneurysmatic tissue. This would indicate that the crosslinking of the tissue is deficient and unable to prevent the fibers from sliding out of the path of the sharp tip. Marfan tissue is known for being extremely soft, with this being a confirmation that the density of links in the tissue determine the strength of the whole tissue.

Reconstructions of the stiffness into two dimensional images allows some extra information to be gained, that can be compared to the confocal images. These images show how stress would be distributed across the tissue. It is clear from the images in figure 5.2 that the distribution of the stiffness is very different from one type of tissue to another. Both the healthy and aneurysm
Figure 5.3: Individual approach curves of tip-tissue interactions. Left graph shows the nonlinear interactions common on the AAA tissue with the ball tip. We think that the nonlinearity is a result of one collagen fiber being pushed into a second fiber resulting in markedly stronger forces. Right graph shows the collapses that are common with the Marfan tissue. These could be the result of the collagen fibers slipping to the side of the tip, or could indicate structural weaknesses in the tissue.

Tissues are fairly homogeneous in their stiffness, while Marfan tissue is the most unevenly distributed of the three tissues. This would indicate that the processes that control the homogeneity in these tissues is severely disturbed in the Marfan syndrome where the control that is being exerted is only working at a local level.

Another remarkable and unique finding to the Marfan tissue is that sections of the tissue collapse as the force is applied. These sections appear to be small voids in the collagen network which are also visible in the confocal images. These voids would support the conjecture that the control and repair of Marfan tissue is not controlled through a global process.

5.4 Discussion

The disease mechanism for aneurysma’s is apparent in the results from the combination of 3-dimensional confocal imaging and AFM mechanical measurements. The confocal imaging shows the highly organized network structure within the tissue and how the organization differs in pathological tissues. The AFM measurements show that the mechanical strength of the tissue is defined on different levels in the organizational hierarchy in the tissue. Measurements with blunt tips show the resilience of the overall network of the tissue. At this
level, Marfan tissue is soft while AAA tissue is stiff and noncompliant, with healthy tissue similar to Marfan tissue. Measurements with sharp tips show that both types of pathological tissue are unusually soft, suggesting a common route to failure for the networks in these tissues. Healthy tissue has very similar mechanical properties at the macro- and micro-level, suggesting that load is evenly distributed over the components of the tissue. Confocal images are in agreement with this picture: Both healthy and Marfan tissue show large scale collagen networks in the confocal images, but in Marfan, and especially in the adventitia, the network is less well architected and has less directionality. AAA tissue does not show much organization at all and on a large scale the random collagen organization appears very stiff, while on a small scale faults are easily found.

The observed co-localization of fibrillin and collagen aggregates suggests fibrillin plays a vital role in the organization and the resulting mechanical properties of the collagen networks of the aorta. We expect that this could well explain some of the other symptoms of Marfan syndrome such as herniae and aortic valve malfunctions. Collagen has a major structural role in many (connective) tissues in the body and it appears the organization of collagen in such tissues is also affected or caused by fibrillin.
Bibliography


Chapter 6

Summary

This thesis is about the use of a somewhat uncommon type of microscopy in biology: “Atomic Force Microscopy” (AFM). “Atomic” indicates here the resolution of the microscope: in ideal circumstances individual atoms can be resolved. “Force” indicates the working principle of the microscope: by measuring the force between a tip and a surface. Unlike a conventional microscope the AFM images tiny structures on surfaces by scanning the surface, somewhat like how a grammophone needle scans a l. p.

The AFM images in this thesis are however quite a different size from l. p.'s. The images with the least magnification can be found in chapter 5. These images show tissue from the aortic wall of a human. The aorta is the large artery running down from the heart. The images of the tissue are about 100 $\mu$m x 100 $\mu$m, which is 0.1 mm x 0.1 mm, or about the thickness of a hair. The images with the most magnification can be found in chapter 4. These images show the regular coating of protein that some kinds of bacteria have on the outside. These images are about 0.2 $\mu$m x 0.2 $\mu$m and thus have sides that are 500x smaller than those of the images of the tissue. The regular structure depicted in those images consists of 4 proteins per unit cell of the regular structure so that here we almost see single proteins.

Proteins are large, complex molecules and the machinerie performing (almost) all tasks that have to be performed in our bodies to live: we have proteins digesting food as an energy-source; we have proteins...
CHAPTER 6. SUMMARY

...teins according to the blue-prints in the DNA; we have motor-proteins to move
muscles or to constitute a transport mechanism within cells; etc, etc.

AFM is a very suitable technique for biological research. In contrast to
many other high-resolution techniques, AFM can be used in liquid and at room
temperature or at 37°C, so that living cells can be studied. It is not surprising
then that quite soon after the development of the first AFM for typical physics
research in vacuum in 1986 it was adapted for research on living cells in 1991.

AFM is especially suited for research of membranes. Membranes are the
envelopes of cells and cellular compartments. They are hard to study with
conventional biological and biochemical techniques, but are important: not
only do they keep cells and compartments together, but many cellular activities
take place on membranes: food has to enter the cell and waste has to expelled;
cells keep contact with their neighbours about their health and environmental
factors; nerve impulses are transmitted along membranes; energy production
takes place at membranes; etc. AFM makes it possible to image the proteins
in membranes which perform all these tasks. Chapter 3 is about research on
the membrane around the nucleus of cells and the complex proteins regulating
and performing transport into and out of the nucleus.

AFM gives more possibilities besides imaging of tiny structures: the AFM
can be used in a mode to specifically measure forces and mechanical properties.
The research in chapter 5 is performed using this mode. The tip is pushed
vertically into the surface in a regular grid pattern. The measured force and
the indentation of the surface depend on the properties of the material under
investigation.

There are still more possibilities: In 2003 a special mode was developed to
gain biochemical specificity to recognize proteins on surfaces. While in ideal
circumstances individual atoms can be resolved, with 10x–100x more magnification
than the highest-resolution images in this theses, it is difficult to resolve
single proteins on biological surfaces, and it is certainly difficult to recognize
single proteins in membranes crowded with many different types of proteins.
In this special mode, called TREC for simultaneous Topographical and RECo-
nition imaging, the tip is oscillated above the surface and the topography is
measured each time the tip touches the surface at its lowest swing. A long,
flexible molecule with a protein or ligand at its end has been attached to the end of the tip. The ligand has been chosen to bind specifically to one of the proteins or receptors on the surface. Such specificity is very common in biology as proteins need to cooperate closely with other proteins and recognize them to do so. If the ligand on the tip binds to its receptor on the surface, the tip feels this when it is at its highest point in its swing, where the flexible molecule is stretched completely. Chapter 4 describes such measurement on model systems.

The needle is an important element in AFM. Its sharpness and robustness are the most important factors for the lateral resolution. Carbon nanotubes are theoretically ideal tips. Many measurements in this thesis have been made with this kind of tips. Chapter 2 describes pros and cons of using this kind of tips and important considerations when making them. Carbon nanotubes are a special form of carbon, besides the more common forms of graphite and diamond. A nanotube is a layer of graphite, rolled into a tube. The wall of the tube can be made of as few as one layers of graphite, in which case the tube is very thin, down to less than 1 \( \text{nm} \) or 0.001 \( \mu \text{m} \). Importantly, the diameter of the tube stays constant until relatively far above the surface. Standard AFM tips have a pyramidal shape and broaden quickly away from the surface. One of the most important improvements of nanotube tips in biology is probably not that these tips are a bit sharper at the end compared to standard tips, but that with soft materials, which deform under the pressure of the tip, a much smaller area will get in contact with the tip, due to its high aspect ratio. The small contact area minimizes damage to fragile biological structures. Conversely, sometimes it is beneficial to use much blunter tips than usual. Chapter 5 describes measurements on the mechanical properties of tissues and the role of networks of protein fibers for the strength of the tissue. Here normal tips and extra blunt tips, with a diameter of 5000 \( \text{nm} \) were used to probe the tissue both at the nanoscale and at the microscale, or at the scale of the individual fibrils and the scale of the total network of fibers.

I have used the nanotube tips as developed in chapter 2 for measurements on the membrane around the nucleus. This is a very soft membrane with complex proteins, called “Nuclear Pore Complexes” (NPC’s). These proteins
regulate all traffic into and out of the nucleus, an important process. Signal-
molecules have to enter the nucleus to turn genes on or off; subsequently RNA
(a copy of DNA synthesised especially for making proteins) has to leave the
nucleus. For viral diseases this is also an important point: most viruses have
to enter the nucleus to be able to reproduce. It is not yet clear how NPCs
perform all transport. We show here that nanotube tips are sensitive to surface
properties of NPCs and that these tips image new structures, probably because
they do not destroy these structures, in contrast to standard tips.

Chapter 4 describes research to gain a better understanding of TREC mea-
urements. A good understanding of TREC is complex because of the dynamics
of the different elements involved, like the movement of the tip and the flexible
molecule and the interaction between ligand and receptor. I describe here an
analysis of TREC measurements which produces a relation between the dis-
tance between tip and receptor and how quickly, or how eagerly, the ligand
binds its receptor. I also try to deduce this relation from theory, but this prob-
lem turns out to be very difficult mathematically. From the analysis we can
deduce that the use of nanotube tips would give important benefits: a higher
resolution and a higher speed or sensitivity. Using nanotubes would also allow
easier tuning of the parameters for the specific experiment and make it easier
to perform.

Chapter 5 describes research into the mechanical properties of tissue. The
tissue used here is from the aortic wall, the large artery running down from
the heart. The aorta experiences continuously varying pressure because of the
heartbeat. It has to be flexible to absorb the pressure pulses from the heart,
but strong enough not to tear apart. Sometimes it does tear apart; this is called
a aneurysma. Here we study healthy tissue, tissue after rupture and tissue
from people with a genetic disease which enhances the chance of rupture: the
Marfan syndrome. We use both AFM and light microscopy to find out the
mechanical properties and to find out influence of the structure of the tissue
on the mechanical properties. The complex network of fibers in the tissue gives
the tissue both its flexibility and its strength. The measurements indicate that
the construction of the structure can be faulty and that this causes a weakening
in the aortic wall.
Dit proefschrift gaat over de toepassing van een wat ongewone soort van microscopie in de biologie: “atomaire kracht microscopie”. “Atomair” geeft hier aan wat het oplossend vermogen van de microscoop is: in ideale omstandigheden kunnen individuele atomen zichtbaar worden gemaakt. “Kracht” geeft aan hoe de microscoop werkt: door het meten van de kracht tussen een naald en het oppervlak onder bestudering. Deze techniek lijkt dus in het geheel niet op de bekende optische microscoop, maar beeldt zeer kleine structuren op een oppervlak af door dat oppervlak af te tasten, enigszins vergelijkbaar met hoe een grammofonnaald een plaat aftast.

De AFM (van het Engelse Atomic Force Microscope) afbeeldingen in dit proefschrift zijn echter van een heel ander formaat dan een grammofonplaat. De afbeeldingen met de minste vergroting zijn te vinden in hoofdstuk 5. Deze afbeeldingen tonen weefsel uit de wand van de aorta van een mens, de grote slagader die van het hart naar beneden loopt. De afbeeldingen van het weefsel in dit hoofdstuk zijn ongeveer 100 µm bij 100 µm, dat is 0,1 mm bij 0,1 mm, of ongeveer de dikte van een haar. De afbeeldingen met de meeste vergroting zijn te vinden in hoofdstuk 4. Deze afbeeldingen tonen de regelmatige coating van eiwitten die sommige soorten bacteriën aan de buitenkant hebben. Deze afbeeldingen zijn ongeveer 0,2 µm bij 0,2 µm. De zijden zijn dus 500x kleiner dan van de afbeeldingen van het weefsel. De regelmatige structuur die hier is afgebeeld bestaat uit 4 eiwitten per eenheidscel van het regelmatige patroon en
we kunnen nu dus bijna enkele eiwitten zien.

Eiwitten zijn grote, complexe moleculen en de machinerie die (bijna) alle taken verricht die moeten plaatsvinden om te leven: er zijn eiwitten die voedsel verteren als energiebron; er zijn eiwitten die andere eiwitten maken naar het bouwplan in het dna; er zijn eiwitten als motors om spieren te bewegen of transport in een cel te verzorgen; etc, etc.

Atomaire Kracht Microscopie is een techniek die zeer geschikt is voor biologisch onderzoek. In tegenstelling tot veel andere technieken met een hoog oplossend vermogen, kan AFM toegepast worden in vloeistof en bij kamertemperatuur of bij 37°C, zodat levende cellen kunnen worden bestudeerd. Vrij snel na de ontwikkeling van de eerste AFM voor typisch natuurkundig onderzoek in vacuum in 1986, werd deze techniek dan ook in 1991 al toegepast op levende cellen.

AFM is uitermate geschikt om membranen te bestuderen. Membranen vormen het omhullende oppervlak van cellen en van compartimenten in cellen. Membranen zijn moeilijk te bestuderen met conventionele biologische en biochemische technieken, maar zijn uitermate belangrijk: niet alleen houden ze cellen bij elkaar, er vindt ook veel activiteit plaats op membranen: er moet voedsel de cellen in en afvalstoffen moeten geloosd: cellen houden contact met buurcellen over hun gezondheid en omgevingsinvloeden; zenuwprikkels geleiden langs celmembranen; energieproductie vindt plaats aan membranen; etc, etc. AFM maakt het mogelijk om de eiwitten in de membranen af te beelden die verantwoordelijk zijn voor al deze taken. Hoofdstuk 3 beschrijft onderzoek aan het membraan rond de kern van een cel en de complexe eiwitten in dat membraan die het transport van alle stoffen in en uit de celkern regelen.

De meetmethode met AFM geeft echter ook nog extra mogelijkheden behalve het afbeelden van kleine structuren: de AFM kan gebruikt worden in een modus om specifiek krachten en mechanische eigenschappen te bestuderen. Het onderzoek beschreven in hoofdstuk 5 is in deze modus uitgevoerd. De naald wordt voor deze modus verticaal op en neer bewogen en het oppervlak ingeduwd op een regelmatig raster. De gemeten kracht op de naald en de indentatie, ofwel de mate van indrukking, van het oppervlak hangen af van de eigenschappen van het onderzochte materiaal.
De mogelijkheden zijn nog gevarieerder: In 2003 is een modus ontwikkeld die biochemische specificiteit geeft om eiwitten te herkennen op oppervlakken. Hoewel in ideale omstandigheden individuele atomen zichtbaar kunnen worden gemaakt, met ongeveer 10–100x meer vergroting dan de meest vergrootte afbeeldingen in dit proefschrift, is het op biologische oppervlakken moeilijk om enkele eiwitten af te beelden, en zeker om eiwitten te herkennen in membranen met veel verschillende eiwitten. In deze speciale modus, TREC genaamd voor het Engelse Topography and RE cognition imaging, wordt de naald snel op en neer bewogen boven het oppervlak en wordt de topografie elke keer gemeten op het moment dat de naald in zijn laagste punt is en het oppervlak raakt. Aan het uiteinde van de naald is een lang flexibel molecuul gemaakt met aan het einde een eiwit of ligand dat specifiek bindt aan een bepaald soort eiwit op het bestudeerde oppervlak. Dit soort specifieke bindingen komen veel voor in de biologie omdat eiwitten over het algemeen nauw samenwerken met andere eiwitten en deze herkennen. Als dit ligand aan de naald bindt met zijn specifieke receptor op het oppervlak, voelt de naald dit op het hoogste punt in zijn beweging, als het flexibele molecuul tussen ligand en naald helemaal uitgerekt is. Hoofdstuk 4 beschrijft dit soort metingen aan een modelsysteem.

De naald is een belangrijk element in AFM. De scherpte en robuustheid van de naald bepalen voor een belangrijk deel het oplossend vermogen en de gevoeligheid voor krachten. Een van de in theorie meest ideale types naalden bestaat uit een koolstof nanobuis. Veel metingen in dit proefschrift zijn uitgevoerd met dit soort naalden, die echter niet standaard zijn of eenvoudig te maken. Hoofdstuk 2 beschrijft de voor- en nadelen van dit soort naalden en belangrijke punten om op te letten bij het maken. Koolstof nanobuizen zijn een speciale vorm van pure koolstof, naast de meer bekende verschijningsvormen van grafiet en diamant. Een nanobuis bestaat uit een laag grafiet die opgerold is tot een buis. De wand van de buis kan tot één atoomlaag dun zijn, in welk geval de buis ook zeer dun is, tot minder dan 1 nm of 0,001 µm. Minstens zo belangrijk is dat de diameter van de buis hetzelfde blijft tot relatief ver boven het oppervlak. Standaard AFM naalden hebben de vorm van een piramide en worden dus snel breder weg van het oppervlak. Het meest belangrijke voordeel van nanobuis naalden in biologisch onderzoek is waarschijnlijk niet dat ze aan het einde iets
scherper zijn dan de piramides, maar dat bij zachte materialen, die vervormen onder de druk van de naald, een veel kleiner oppervlak uiteindelijk in aanraking komt met de naald. Het kleinere interactie oppervlak minimaliseert schade aan de fragiele biologische structuren. In tegenstelling hiermee is het soms voordelig om botte naalden te gebruiken. Hoofdstuk 5 beschrijft onderzoek aan de mechanische eigenschappen van weefsel en de rol van netwerken van eiwit fibers in de mechanische sterkte van dit weefsel. Hier hebben we zowel normale naalden en naalden met een diameter van 5000 nm gebruikt om het weefsel af te tasten op zowel de nano- als de micro-schaal, oftewel de schaal van enkele eiwit fibers en de schaal van het gehele netwerk van fibers.

De nanobuis naalden zoals die ontwikkeld zijn in hoofdstuk 2 heb ik in hoofdstuk 3 gebruikt voor metingen aan het membraan rondom de celkern. Dit is een zeer zacht membraan met complexe eiwitten, genaamd “Nuclear Pore Complexes” (NPC). Deze eiwitten regelen al het verkeer de celkern in en uit, een belangrijk proces. Er moeten signaal-moleculen de celkern in om te regelen welke genen aan- of uitgezet moeten worden in reactie op signalen van binnen of buiten de cel; vervolgens moet RNA (een kopie van DNA speciaal voor het maken van eiwitten) de kern uit. Ook voor virale ziektes is dit een belangrijk punt: de meeste virussen moeten de celkern binnendringen om zich voort te planten. Het is nog niet precies bekend hoe de NPCs alle transport uitvoeren. Wij laten hier zien dat nanobuis naalden gevoelig zijn voor oppervlakte eigenschappen van de NPCs en dat de nanobuis naalden nieuwe structuren afbeelden, waarschijnlijk omdat ze, in tegenstelling tot standaard naalden, deze fragiele structuren niet kapotwalsen.

Hoofdstuk 4 beschrijft onderzoek voor een beter begrip van TREC metingen. Een goed begrip van de metingen is complex vanwege de dynamica van de verschillende elementen, zoals de beweging van de naald en het flexibele molecuul en de interactie tussen ligand en receptor. Ik beschrijf hier een analyse van TREC metingen die uitmondt in een relatie tussen de afstand tussen naald en receptor en hoe snel, oftewel hoe graag, de ligand aan de receptor bindt. Ook probeer ik zo goed mogelijk vanuit de theorie deze relatie te vinden, maar dit probleem blijkt wiskundig moeilijk op te lossen. Uit de analyse kunnen we wel afleiden dat het gebruik van nanobuis naalden in dit geval verschillende vo-
ordelen zou opleveren: een hoger oplossend vermogen en hogere meetnauwkeurigheid of hogere gevoeligheid. Gebruik van nanotube naalden zou het ook gemakkelijker maken om de juiste parameters te kiezen voor het specifieke experiment en dit eenvoudiger maken.

Hoofdstuk 5 beschrijft onderzoek naar de mechanische eigenschappen van weefsel. Het onderzochte weefsel is afkomstig van de aorta, de grote slagader die van het hart naar beneden loopt. Deze slagader staat onder continue variërende druk door de hartslag. De aorta moet flexibel zijn om de drukpulsen op te vangen maar sterk genoeg om nooit te scheuren. Dit scheuren gebeurt soms wel, dit heet een aneurysma. Hier bestuderen we gezond weefsel, weefsel na een scheuring en weefsel van mensen met een erfelijke ziekte waardoor de kans op scheuren groter wordt: het Marfan syndroom. We gebruiken hier zowel AFM als licht microscopie om de mechanische eigenschappen te leren en uit te vinden hoe de structuur van het weefsel van invloed is op deze mechanische eigenschappen. Het complexe netwerk van fibers in het weefsel zorgt er uiteindelijk voor dat het weefsel zowel flexibel als sterk is. De metingen geven aanwijzingen dat de opbouw van deze netwerkstructuur mis kan gaan en dat dit kan leiden tot een verzwakte vaatwand.
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Curriculum Vitae

