

Atomic Force Microscopy

Abstract:

The Atomic Force Microscope is a versatile, high resolution microscope for imaging conducting and non conducting materials with nanometer resolution. In this lab course, you will learn the basic principles of AFM, learn how to operate an AFM and how to perform AFM specific image processing.

Specific learning objectives:

After this lab module you will know how to:

- **perform imaging of biological samples using a state-of-the-art AFM system**
- **recognize and remove artifacts in the AFM images (a lot of AFM images in the research papers are poorly processed!)**
- **process and display obtained AFM image to present measured data in the best way**
- **extract quantitative information about the sample from the AFM images**

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1 1. THEORY

1.1 History: from STM to AFM

The atomic force microscope (AFM) was developed in 1986, by Gerd Binnig, Calvin Quate and Christoph Gerber¹ in an attempt to image **non-conducting** samples with atomic resolution. Four years earlier, Binnig, Gerber, Rohrer and Wiebel had invented the scanning tunneling microscope (STM)² that had already succeeded in imaging **conducting** samples with atomic resolution. This was done by applying a potential difference (V_t) between the conducting sample surface and an atomically flat tip, while measuring the quantum mechanical tunneling current between the tip and the sample. If $|V_t|$ is small compared to the workfunction Φ , the tunneling current is given by:

$$I_t(z) = I_0 e^{(-2\kappa_t z)}$$

where z is the gap I_0 is a function of the applied voltage, the density of states in the tip and the sample, and $\kappa_t = 2m\Phi/\hbar$. Because of the exponential dependency of I_t on the distance z , tunneling is a very sensitive method to detect the surface topography. For the same reason, the most current is carried by “front atom” of the tip, so atomic resolution is possible even with relatively blunt tips. The tip is then raster scanned relative to the surface of the sample and the tunneling current is recorded for each point, which than can be plotted as a 2D-image. Since the tunneling current falls off so rapidly with distance, STMs are generally operated in a closed loop,

where the tip-sample distance is held constant using a feedback loop that moves the tip in the out of plane (z) direction. This allows the imaging of samples higher than the decay length of the tunneling probability.

The approach that Binnig, Quate and Gerber used to also image non conducting samples, was to add a conductive “mediator” between the sample and the STM tip in the form of a cantilever. The cantilever was a very thin gold foil with a shard of diamond glued to the back. The thin foil acts as a spring that can push the diamond tip onto the sample surface (see the figure from the original publication in Figure 1). On top of the aluminum foil, the STM is mounted, which is now used to measure the displacements of the cantilever as it is scanned over the surface. Figure 1C shows the first AFM image traces ever recorded.

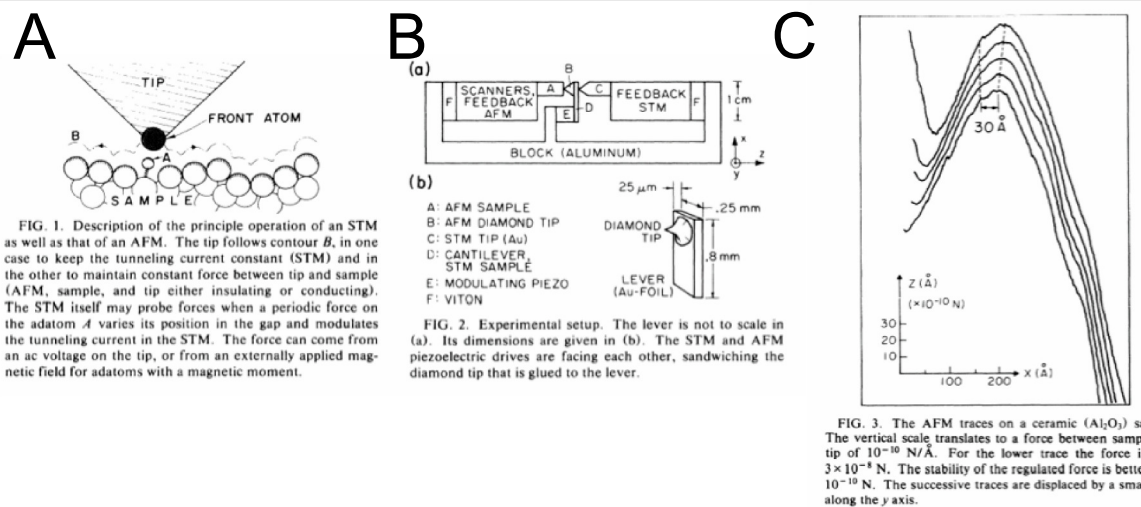


Figure 1: figures from first AFM paper

1.1 Current AFM technology and Instrumentation

AFMs are now widely used in many areas such as materials science, nanotechnology, biophysics, and industrial process control. Modern AFMs are very versatile instruments that can be used to image nanoparticles, single molecules, semiconductor devices and even living cells. Since the first AFM prototypes, the technique has changed significantly and many improvements have made the AFM such a broadly used tool. Two of the main improvements were the development of microfabricated cantilevers and the development of the *optical lever detection*, which has replaced the STM as the cantilever deflection readout technique.

1.1.1 AFM cantilevers as sensitive displacement and force sensors

In order to record high-resolution AFM images even on soft (biological-) samples, it is essential to have cantilevers with a sharp tip, high displacement sensitivity and high force sensitivity. These days AFM cantilevers are fabricated using MEMS fabrication techniques, most often out of silicon or silicon nitride. Figure 2 shows some example of commercial AFM cantilevers. Many different varieties are available to suite the specific applications such as imaging of hard samples in air, or imaging soft samples in aqueous solution.

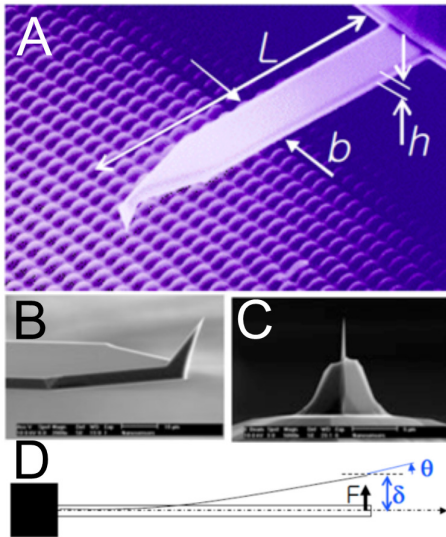


Figure 2: Commercially available cantilevers

In order to use the cantilever as a sensor, we need the relationship between a force (F) acting on the cantilever, and its displacement at the tip (Δz). From elementary beam theory we know that:

$$\Delta z = w(L) = F \cdot L^3 / (3 \cdot E \cdot I)$$

Where $w(x)$ is the z displacement of the neutral axis as a function of the position along the cantilever, L is the cantilever length, E is the elastic module and I is the moment of inertia ($I = b \cdot h^3 / 12$).

For the deflection angle (θ) of the end of the cantilever we can calculate:

$$\theta = dw(L)/dx = F \cdot L^2 / 2 \cdot E \cdot I$$

It is important to note, that both the deflection of the end, as well as the slope at the end of the cantilever is proportional to the force applied to the tip. We can therefore assign the cantilever a spring constant k given as:

$$k = 3 \cdot E \cdot I / L^3$$

1.1.2 Optical lever detection method

While the detection of the cantilever deflection with an STM is a very high resolution method, it is quite cumbersome and impractical in many applications, especially when imaging biological samples in fluid. Therefore, most AFMs these days use a laser to detect the cantilever deflection. This is done by shining a laser onto the back of the reflecting cantilever and measure the angle of the reflected laser beam using a four-quadrant laser diode (see Figure 3).

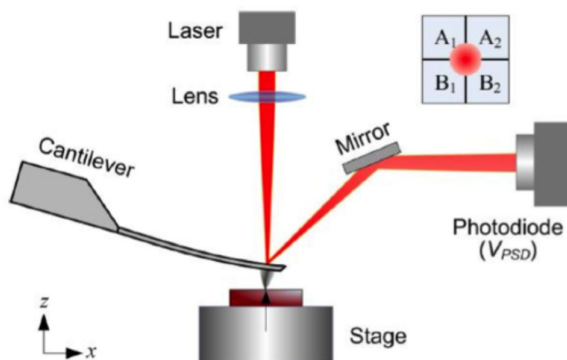


Figure 3: Optical lever deflection method for the detection of the cantilever bending

When the cantilever is deflected, the reflected laser beam hits the four-quadrant photo diode at a different position. From the total amount of light that hits on the four quadrants, the deflection as well as the torsion of the cantilever can be measured.

$$\text{Vertical deflection} = (A_1 + A_2) - (B_1 + B_2)$$

$$\text{Horizontal deflection (torsion)} = (A_1 + B_1) - (A_2 + B_2)$$

Strictly speaking, we are not measuring the cantilever deflection in z-direction, but rather the angle of the neutral axis of the deflected cantilever. But since both the angle as well as the cantilever deflection are proportional to the force (which is proportional to the displacement), we can use the angle change as a measure for the cantilever deflection.

1.1.3 Feedback mode operation

Feedback control is used in AFM for maintaining a fixed relationship, or force, between the probe and the surface. The feedback control operates by measuring the force between the surface and probe, then controlling a piezoelectric ceramic that establishes the relative position of the probe and surface thereby keeping forces between them at a user-specified **setpoint** level. Let us use contact mode as an example. When the control signal (in this case the cantilever deflection) is above the setpoint, the feedback loop will try to reduce the force between the cantilever and the sample by contracting the Z-piezo thereby moving the tip a little further away from the surface. When the deflection is less than the specified setpoint, the feedback loop will expand the piezo to move the tip closer to the sample.

Although signal processing varies according to the image mode used (contact mode, tapping mode, etc), the feedback loop always performs essentially the same function.

The feedback system used to control tip-sample interactions and render images must be optimized for each new sample. This is accomplished by adjusting various gains in the SPM's feedback circuit. This section discusses gains and how they are used to obtain images.

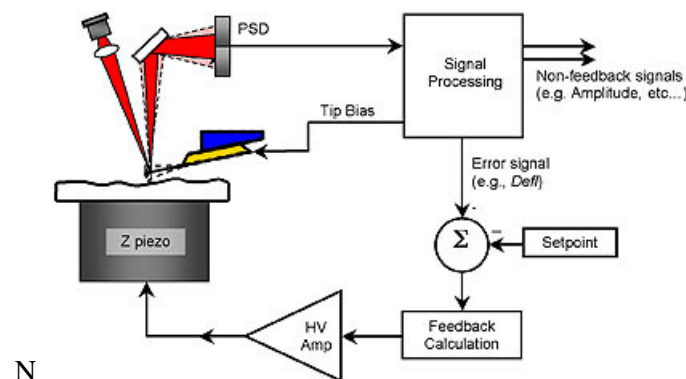


Figure 4: Schematic depiction of AFM feedback loop

In the AFM, the feedback control electronics take an input from the force sensor and compare the signal to a set-point value; the error signal is then sent through a feedback controller. The output of the feedback controller then drives the Z piezoelectric ceramic. The most common

form of feedback control for AFM is the PID controller (**Proportional, Integral and Derivative controller**). The proportional, integral, derivative controller takes the error signal and processes it as follows:

$$Z_v = P \times Z_{err} + I \times \int Z_{err} dt + D \times \frac{dZ_{err}}{dt}$$

where Z_v is the output signal of the controller that goes to the high voltage amplifier for the z-piezo, E_{err} is the error signal, and **P**, **I**, and **D** are the gain settings. By selecting the appropriate P, I and D gain setting, the probe will “track” the surface as it is scanned. The integral term facilitates the probe moving over large surface features and the P and D terms allow the probe to follow the smaller, high frequency features on a surface. When the PID parameters are optimized, the error signal image will be minimal. As a rule of thumb: the higher the feedback gains, the faster the feedback loop can react to changes in topography while scanning. Therefore, in an ideal world, one would set the gains as high as possible. However, since the AFM is not infinitely fast in responding to the output of the PID controller, one can only increase the feedback gains to a certain point. This also limits the maximum achievable scan speed. Establishing where this point is requires practice and some intuition. In the exercise you will learn a simple method to get close to these optimal gain settings.

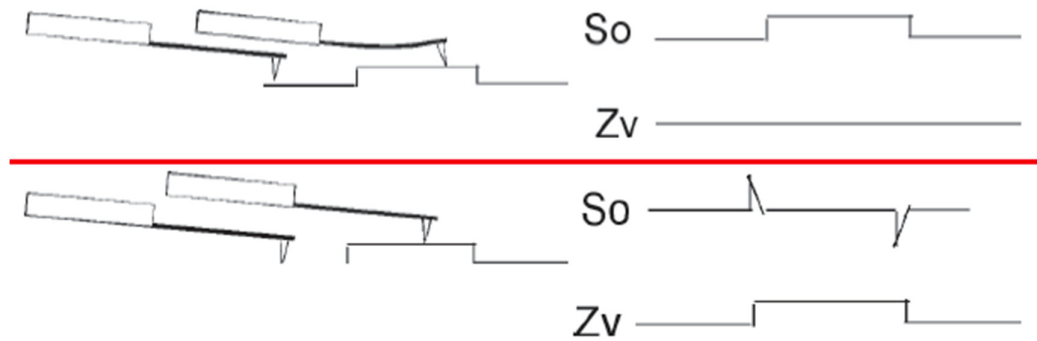


Figure 5: Top: If the PID parameters are all zero, the cantilever will bend as it moves across the surface features. Bottom: If the PID parameters are optimized, the cantilever deflection remains constant while scanning.

1.2 AFM Modes

A force sensor in an AFM can only work if the probe interacts with the force field associated with a surface. In ambient air, the potential energy between the probe and surface is shown in Figure 6. There are three basic regions of interaction between the probe and surface:

- free space
- attractive region
- repulsive region

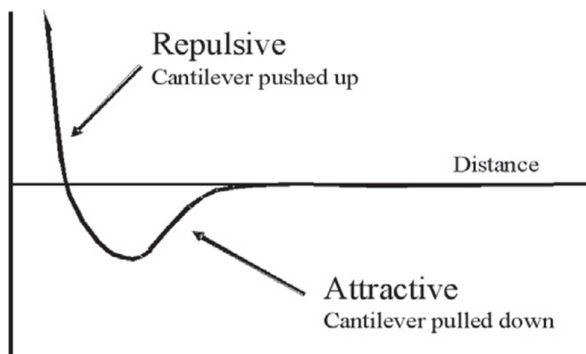


Figure 6: Potential energy diagram of a probe and sample. The attractive potential is caused by the capillary forces from surface contamination.

Attractive forces near the surface are caused by a nanoscopic layer of contamination that is present on all surfaces in ambient air. The contamination is typically an aerosol composed of water vapor and hydrocarbons. The amount of contamination depends on the environment in which the microscope is being operated. Repulsive forces increase as the probe begins to “contact” the surface. The repulsive forces in the AFM tend to cause the cantilever to bend up.

There are two primary methods for establishing the forces between a probe and a sample when an AFM is operated. In contact mode the deflection of the cantilever is measured, and in vibrating mode the changes in frequency and/or amplitude are used to measure the force interaction. As a rule of thumb, the forces between the probe and surface are greater with contact modes than with vibrating modes.

1.2.1 Contact mode

In contact mode, the cantilever is scanned over a surface at a fixed deflection, Figure 7. Provided that the PID feedback loop is optimized, a constant force is applied to the surface while scanning. If the PID feedback parameters are not optimized, a variable force is exerted on the surface by a probe during a scan.

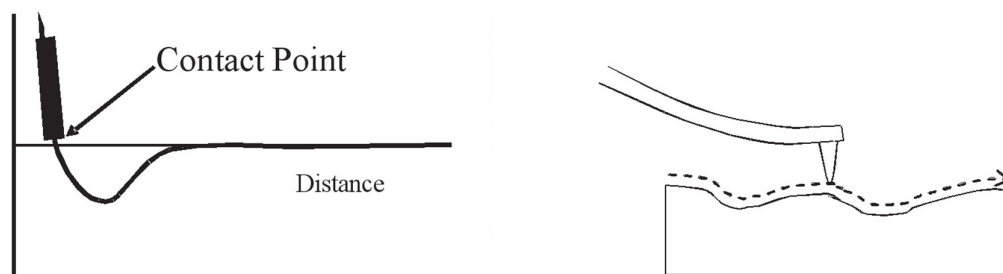


Figure 7: Left: Potential diagram showing the region of the probe while scanning in contact mode. Right: In contact mode the probe glides over the surface.

Contact mode is typically used for scanning hard samples and when a resolution of greater than 50 nanometers is required. The cantilevers used for contact mode may be constructed from silicon or silicon nitride. Resonant frequencies of contact mode cantilevers are typically around 50 KHz and the force constants are below 1 N/m.

1.2.2 Tapping Mode

The constant force that the cantilever exerts on the sample in contact mode is often too large when imaging biological samples. In these cases, it is more advantageous to image in the dynamic mode, often also called tapping mode or intermittent contact mode. In this mode, the cantilever is excited with an external piezo to vibrate close to its resonance frequency (see Figure 8). When the oscillating cantilever approaches the surface, the amplitude of the oscillation decreases, see Figure 8A. One can explain this by looking at the resonance behavior of the cantilever far away from the surface (black curve in Figure 8B), and close to the surface (red curve in Figure 8B). When the cantilever gets close to the surface, an additional restoring force works on the cantilever (the tip sample interaction pushes the cantilever back), and this can be seen as an increase in the spring constant, which manifests itself in a shift of the cantilever resonance frequency to higher values (red curve). In tapping mode, the cantilever is always excited at a fixed frequency that is chosen to be just below the free resonance frequency f_0 . Far away from the surface, the cantilever will then oscillate with amplitude of A_0 . When the cantilever comes closer to the surface, the resonance curve shifts, but the cantilever is still excited with frequency f_0 . The resulting amplitude is the A_1 . It is important to note, that the cantilever still does touch the surface, but only at the bottom swing of the cantilever vibration.

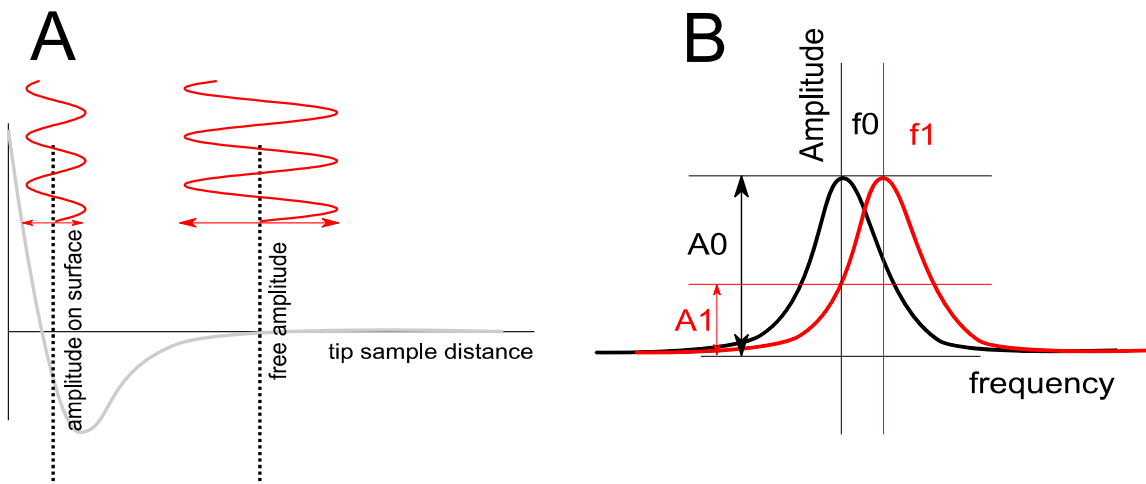


Figure 8: Amplitude dependence in tapping mode.

This drop in amplitude can be used as the feedback parameter for AFM imaging, just like the cantilever deflection in contact mode (only that in tapping mode, decreasing the setpoint value increases the force on the sample, while in contact mode, decreasing the setpoint decreases the force on the sample). Using the change in amplitude as a feedback parameter is called operating the AFM in *amplitude modulation (AM) mode*. One can also track the shift of the resonance frequency of the cantilever when it approaches the surface. That is called *frequency modulated (FM) mode*. AM mode (or tapping mode) is the most common way to operate the AFM. Cantilever for tapping mode are stiffer than cantilever for contact mode to allow for a higher resonance frequency. Typical values for k are 40N/m and f_0 300-400kHz.

1.3 Piezo scanners

SPM scanners are made from piezoelectric material, which expands and contracts proportionally to an applied voltage. Whether they elongate or contract depends upon the polarity of the voltage applied.

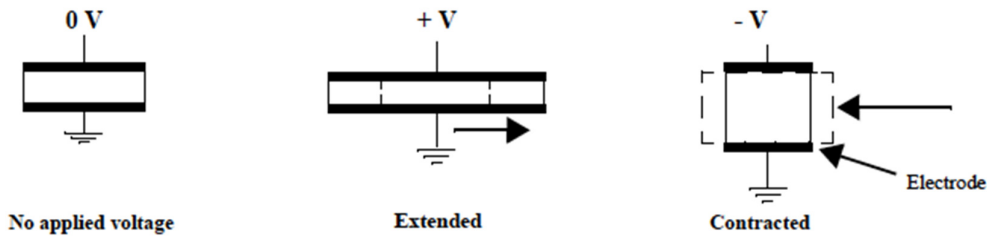


Figure 9: Piezoelectric effect. When a positive voltage is applied to the piezo, it extends, when a negative voltage is applied it contracts.

The scanner is constructed by combining independently operated piezo electrodes for X, Y, & Z into a single tube or flexure scanner, forming a scanner which can manipulate samples and probes with extreme precision in 3 dimensions.

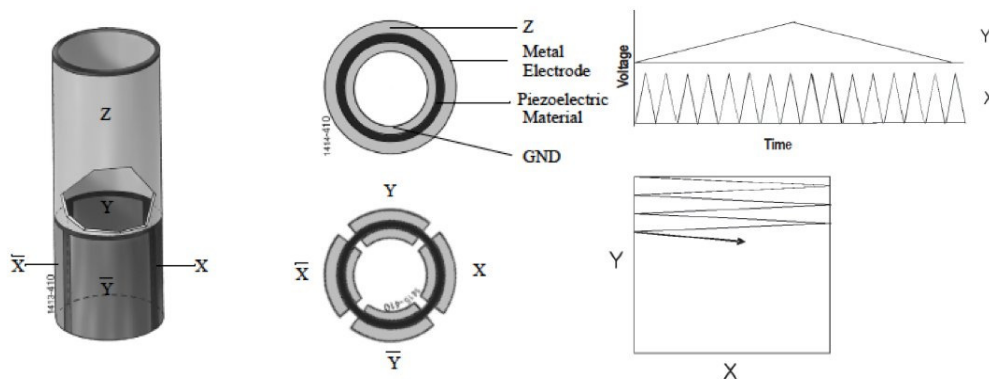


Figure 10: Piezo tube scanner.

1.3.1 Raster scanning

The X-Y signal generators create a series of voltage ramps that drive the x and y piezoelectric ceramics in the AFM, as illustrated in Figure 10. The scan range is established by adjusting the min and max voltage. The position of the scan is established by off setting the voltages to the ceramic. Finally, the scan orientation is rotated by changing the phase between the signals.

1.3.2 Hysteresis

Because of differences in the material properties and dimensions of each piezoelectric element, each scanner responds differently to an applied voltage. This response is conveniently measured in terms of sensitivity, a ratio of piezo movement-to-piezo voltage, i.e., how far the piezo extends or contracts per applied volt. Sensitivity is not a linear relationship with respect to scan size.

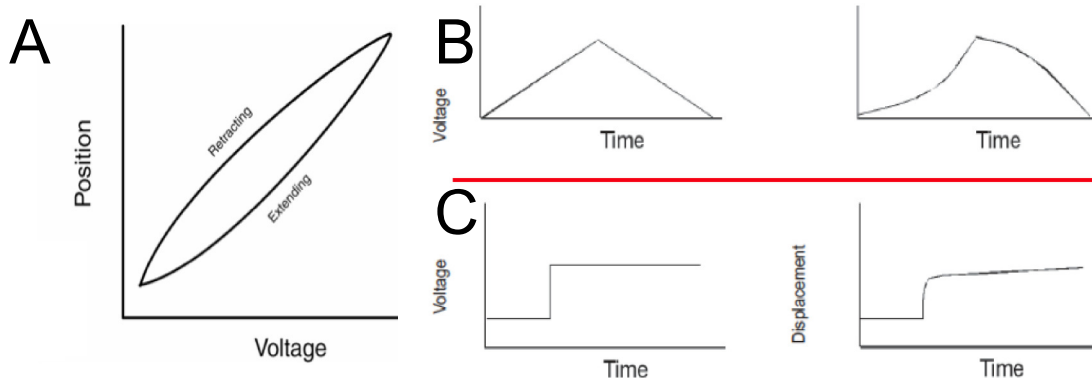


Figure 11: PZT materials have hysteresis. When a voltage ramp is placed on the ceramic, the motion is nonlinear: A) due to the hysteresis, the piezo behaves differently when it expands than when it contracts. B) A triangular command signal (left) results in a distorted output signal (right). C) Creep occurs when a voltage pulse on a PZT causes initial motion followed by drift.

Piezo materials have inherent nonlinearities and hysteresis. The effect of nonlinearity and hysteresis can be seen from the curves in Figure 11. As the piezo extends and retracts throughout its full range, it moves less per applied volt at the beginning of the extension than near the end. The same is true when the piezo is retracting. This causes the forward and reverse scan directions to behave differently and display hysteresis between the two scan directions. Nonlinearity and hysteresis can cause feature distortion in SPM images if not properly corrected (see Figure 12).

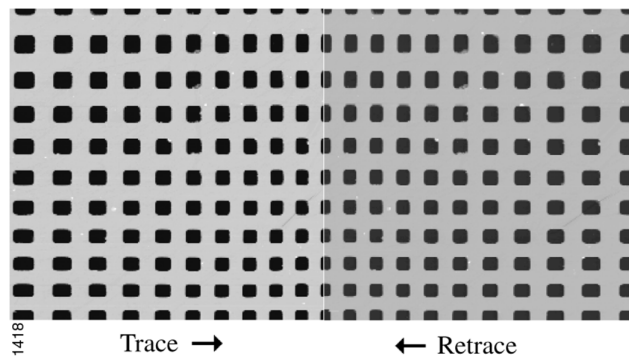


Figure 12: 100um x 100um scans in the forward (trace) and reverse (retrace) directions of a two-dimensional 10um pitch grating without linearity correction. Both scans are in the down direction. Notice the differences in the spacing, size, and shape of the pits between the bottom and the top of each image. The effect of the hysteresis loop on each scan direction is demonstrated.

1.3.3 Creep

Creep is the drift of the piezo displacement after a DC offset voltage is applied to the piezo, see Figure 11C. This may occur with large changes in X & Y offsets, and when using the frame up and frame down commands when the piezo travels over most of the scan area to restart the scan. When a large offset is performed, the scanner stops scanning and a DC voltage is applied to the scanner to move the requested offset distance. However, the scanner does not move the full offset distance all at once. It initially moves the majority of the offset distance quickly, and then slowly moves over the remainder. The scanning resumes after a majority of the offset distance has been moved although the scanner is still slowly moving in the direction of the offset. Creep is the result of this slow movement of the piezo over the remainder of the offset distance once scanning

has resumed. Creep appears in the image as an elongation and stretching of features in the direction of the offset for a short period of time after the offset.

2 PRACTICAL WORK

2.1 Preparation of Samples

2.1.1 Collagen

Material

Rattails, scissors, petri dish, mica on a metal disc, tweezers, scalpel, scotch tape

Method

Rattails are used as a source to extract collagen from. This structure is the elongation of the vertebral column and contains a lot of collagen fibers which can easily be isolated. The vertebral column comprises of a stack of bones with intervertebral discs in between forming a canal wherein neurons run from the brain to a specified location in the body. Different tendons containing a high fraction of collagen run along the vertebral column to stabilize it and these will be isolated.

First, the frozen tail needs to be thawed and 2 – 3 caudal vertebrae have to be cut off at the end of the tail that faces the rat body with scissors or pliers. At the intervertebral discs the vertebral column can easily be cut as there is only soft connective tissue at these sites and no bones present. This will expose bundles of collagen fibers running along the vertebrae. In the next step a short part of the skin at the end of the tail needs to be removed. Therefore the skin is cut with a scalpel and then simply stripped off in order to make the tendons accessible for extraction. White bundles should now be visible which can be isolated by wrapping them around tweezers and then slowly pulling them out of the tail. Make sure to immediately transfer the so isolated tendons into a petri dish containing some water in order to prevent them from drying.

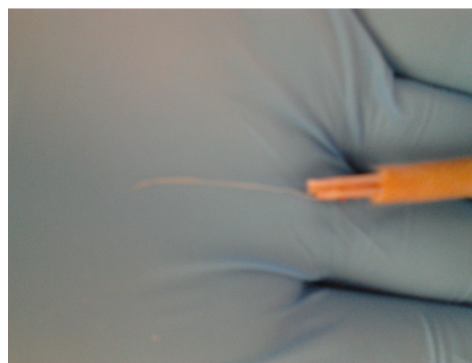


Figure 13: Skin of tail stripped, fiber bundle sticking out.

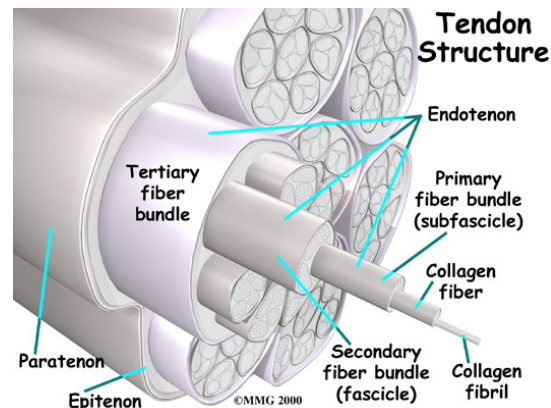


Figure 14: Schematic depiction of a tendon. For AFM imaging, we want to access the *collagen fiber*.

Now that the tendons are isolated the mica surface on top of which the collagen fibers will be imaged needs to be prepared. Therefore a disc of mica is glued on top of a metal disc first (already done for you by your TA). One of the characteristics of mica is a nearly perfect crystal, that can be cleaved due to its hexagonal sheet-like arrangement of its atoms. By using a piece of scotch-tape, one can cleave off the upper mica layers and get a very flat (atomically flat) surface.



Figure 15: Mica on a metal support is cleaved by removing scotch tape.

This done the mica substrate is placed into the lid of the petri dish and some water is added to the top. One bundle of the extracted tendons is placed across the mica disc such that the ends of the tendon are in contact with the plastic material of the petri dish and by drying out will be fixed to it easing the process of exposing the collagen fibers.

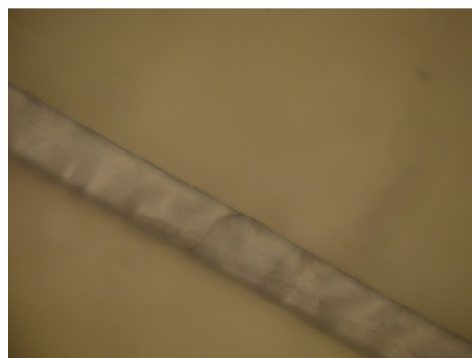


Figure 16: Fiber bundle as seen by upright microscope.

This bundle is now opened up thereby exposing a whole network of individual collagen fibers. With the help of two sharp tweezers the tendon can be pulled apart so that the individual fibers will be spread across the whole mica disc. This can be done under the light microscope where these small fibers will be visible. In order to dry the sample the water on the mica disc can be sucked away carefully at the brim with a paper towel and subsequently left for some time at room temperature.



Figure 17: Left: Fiber bundle is opened up under fluid by tweezers to expose individual collagen fibers. Right: Individual collagen fibers.

2.1.2 *E. coli*

Material

Centrifuge, microtubes, pipette, fresh *E.coli*, mica

Method

Bacteria will be taken from an *E.coli* culture with an ampicillin resistance grown overnight in Luria Broth medium supplemented with Ampicillin at 37°C in the shaker. 1 ml of bacteria solution are transferred into a microtube. Another microtube with an equal volume of water will be used as a counterweight in the microcentrifuge. The bacteria are pelleted at 13'000 rpm for 1 minute. After the supernatant is discarded the bacteria are resuspended in 1 ml Mili-Q and washed again. After the second washing step the bacteria are resuspended in 1 ml of Mili-Q. Depending on the turbidity of the solution between 100-200 µl will be evenly spread on the freshly cleaved mica disc. The mica disc can be dried by a nitrogen flow to remove the water layer on the sample.

2.2 Imaging

2.2.1 Setup: Loading and aligning the cantilever

The samples will be imaged with the Atomic Force Microscope *Multimode 8* from Bruker.

- Load cantilever into the cantilever assembly by pressing down on assembly (hits button on bottom to lift up the cantilever holder) and place cantilever into the groove. For tapping mode use the silicon tips (TAP300) (Figure 18). Once the cantilever is in, move the cantilever assembly into the head on the kinematic mount and tighten the cantilever assembly screw to hold assembly. (Figure 19) Make sure the sample is not higher than the kinematic mount (use UP and DOWN knob), otherwise the cantilever will brake when mounting the cantilever holder.



Figure 18: Cantilever holder with cantilever mounted.

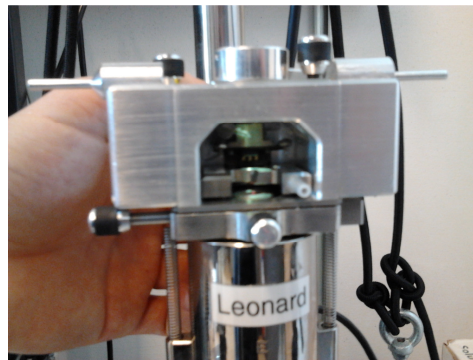


Figure 19: Tighten cantilever holder on kinematic mount.

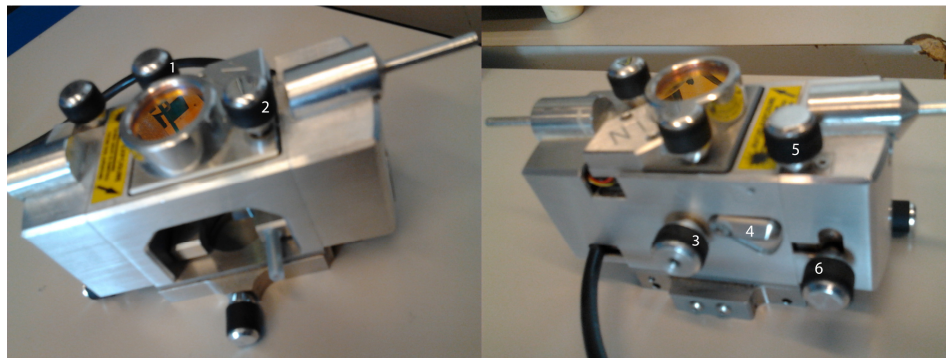


Figure 20: Cantilever head assembly: 1 & 2: Use to position laser onto cantilever. 3: Tighten cantilever assembly in head. 4: Adjust angle of mirror to direct reflection of laser onto photodiode. 5 & 6: Center photodiode on laserspot.

- Position the laser onto the cantilever and adjust the mirror angle to direct reflected laser on photodiode. Laser is adjusted by moving the laser on the chip of the cantilever such that it's not visible anymore on the sample surface. Back off the laser till it reappears, then translate along the chip till there is some sum signal on the display. Then, translate the laserspot along the cantilever and maximize the sum using the knobs indicated in Figure 20.

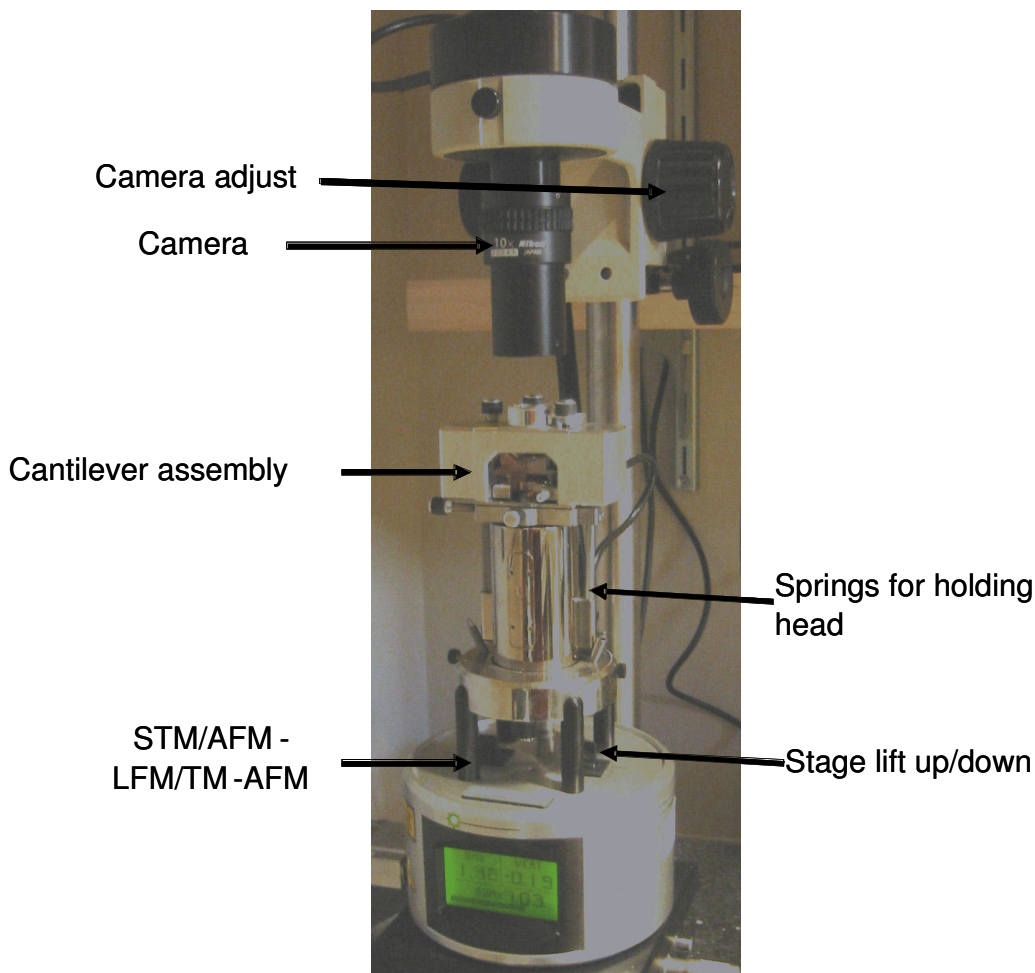


Figure 21: Multimode setup

- The camera can now be lowered in order to see the cantilever tip and/or surface of the sample (turn on monitor and adjust brightness of camera). If the tip is not visible, adjust the stage translators so that tip is visible on the screen.
- Use the mirror adjustment lever to maximize the reflected laser intensity on the four-quadrant detector, the sum should be about 6-7.2 (depending on the cantilever type, if it's lower don't worry) and is visible on the front display of the AFM base. At this time the vertical and horizontal should be adjusted to zero, adjust with knobs (Figure 20) until they are both close to zero.
- Manually move the stage lift up/down until the cantilever gets close to the surface (this can be monitored by the camera focus on sample, then defocus and adjust until the cantilever and sample are both close to in focus) or by eye (look at sample and cantilever, there should be two laser dots visible, one on the tip, another is a reflected dot off the sample, move the cantilever down until those dots are very close to coinciding. Be extra cautious since if the cantilever hits sample, it breaks.

- Start the software **Nanoscope 8.15**



Figure 22: Tapping Mode in air -> Load Experiment.

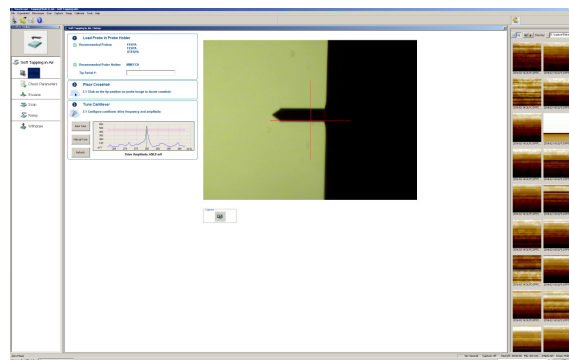


Figure 23: Press Manual Tune

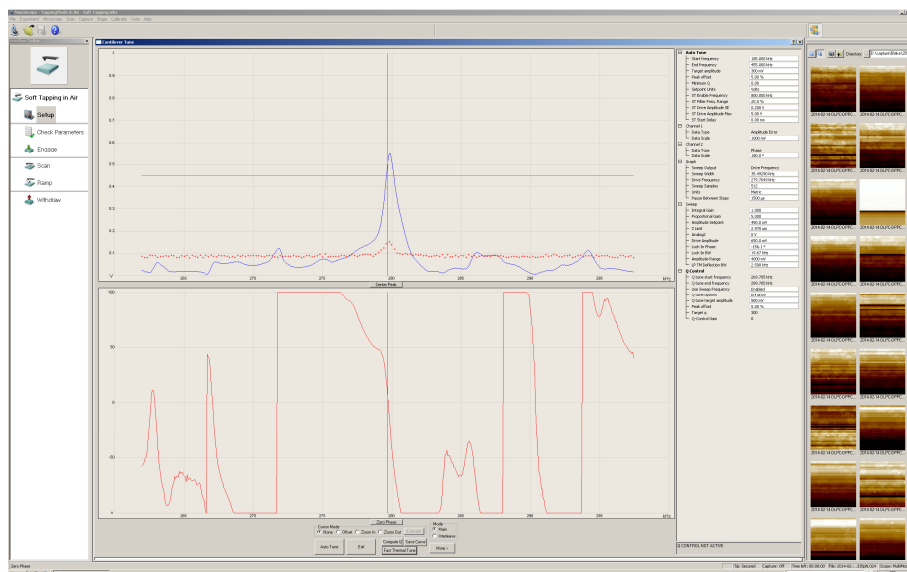


Figure 24: Set sweep width (100 kHz) and resonance frequency (300 kHz) according to cantilever specifications. Adjust Drive amplitude to get a peak maximum at around 0.5V. Green: chosen frequency. Pink: Amplitude setpoint (~80% of peak maximum). Press “Zero Phase” and “Fast thermal tune”.

- Make sure manual tune looks similar to Figure 24.

- Check Parameters and set as indicated in Figure 25, then engage.

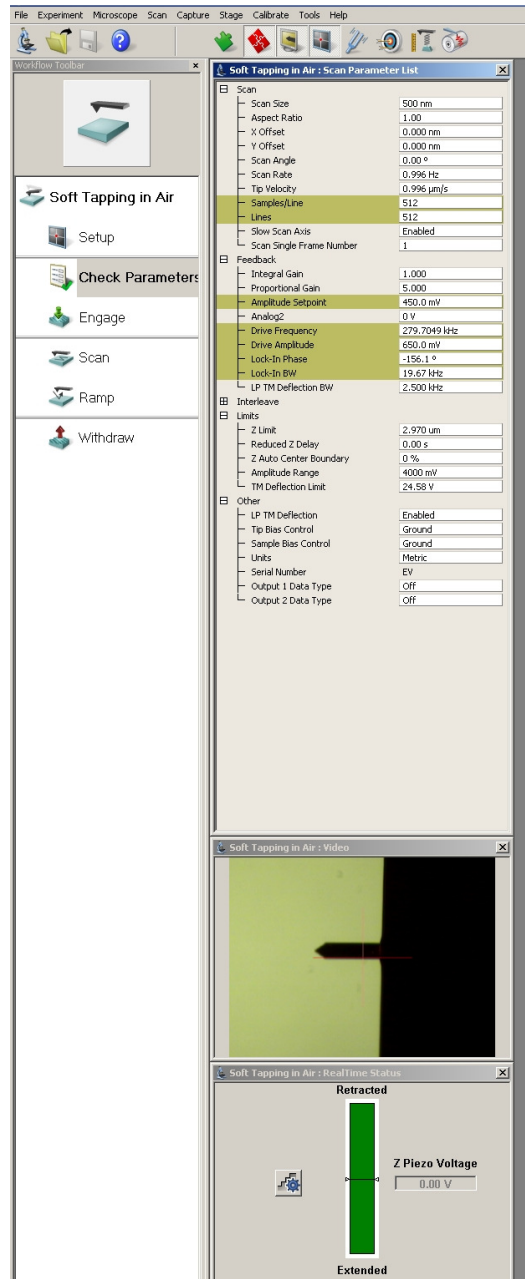


Figure 25: Check paramters.

As soon as cantilever touches surface, it starts scanning. There are all kinds of things that can be adjusted.

2.2.2 Optimizing Imaging parameters: tuning feedback

There are a lot of parameters and settings that can be adjusted in order to get a good resolution of the picture. The most important thing is to properly tune the amplitude setpoint and the feedback gains.

1. Increase the amplitude setpoint to a value where the tip is not anymore tracking the surface. This can be observed when the *trace* and *retrace* lines are not anymore superimposed and appear as straight lines.
2. Lower the amplitude setpoint gently, just until the tip is tracking again and the trace and retrace line overlap. Make sure that the AFM tracks everywhere. If the setpoint is not low enough, you will see areas where the tip never touches the surface. This is called “parachuting”.
3. Adjustment of the Integral and Proportional Gains will further improve the quality of the picture. First: increase the *integral gain* value in small steps until “*the feedback loop starts to ring*” which can be identified in the Amplitude window as an “increased source of noise”. Then lower the *integral gain* again to a value where it does not ring anymore. The same procedure can be done for the proportional gain.
4. Now, when the surface is being tracked well increase the scan size to several micrometers to get an overview of your sample.

2.3 Images to collect:

Collagen

- 30um x 30um scan at 1024 (samples per line) x 256 (lines)
- 10um x 10um scan at 1024 x 256
- 2-4um x 2-4um scan on single collagen fiber 1024 x 256

E.coli

- 30um x 30um scan at 1024 x 256
- 1 single *E.coli* ~5um x 5um at 1024x256

3 AFM IMAGE PROCESSING AND ANALYSIS

All measurements and measurement techniques are prone to artifacts. In AFM imaging, these artifacts are sometimes easy to spot and sometimes very difficult. Some artifacts can be easily avoided, if the user knows what to look for and knows the source of the artifact. A few artifacts are unavoidable, but knowing that they exist in an image helps to avoid misinterpreting them as genuine image features. This means that recognizing image artifacts is very important for the AFM user.

Therefore, some image processing is usually necessary before viewing or analyzing any AFM image. All processing is done with the aim of clarifying the data obtained during measurement. In other words, the purpose is to make it easier to measure and observe the features that have been measured.



Because image processing operations can also introduce artifacts in the image, they should only be used when necessary! Which processing operations will be used depends highly on the image and the properties we want to measure.

For the processing of the AFM images we will use **Gwyddion**, which is free and open source software for SPM (*Scanning Probe Microscopy*) data visualization and analysis. All information about this software can be found at <http://gwyddion.net>

3.1 Gwyddion file handling

3.1.1 Opening file:

Go to **File->Open** and then find the directory of the file you want to open. Once you click on the file, all the images that the file contains will be shown on the right.

Once the file is opened, go to **Meta->Show Data Browser** to open/close desired images (channels), i.e. images within the file that you wish to process and analyze. The images that are checked will be opened. But be aware that if you uncheck all of them – the opened file will close!

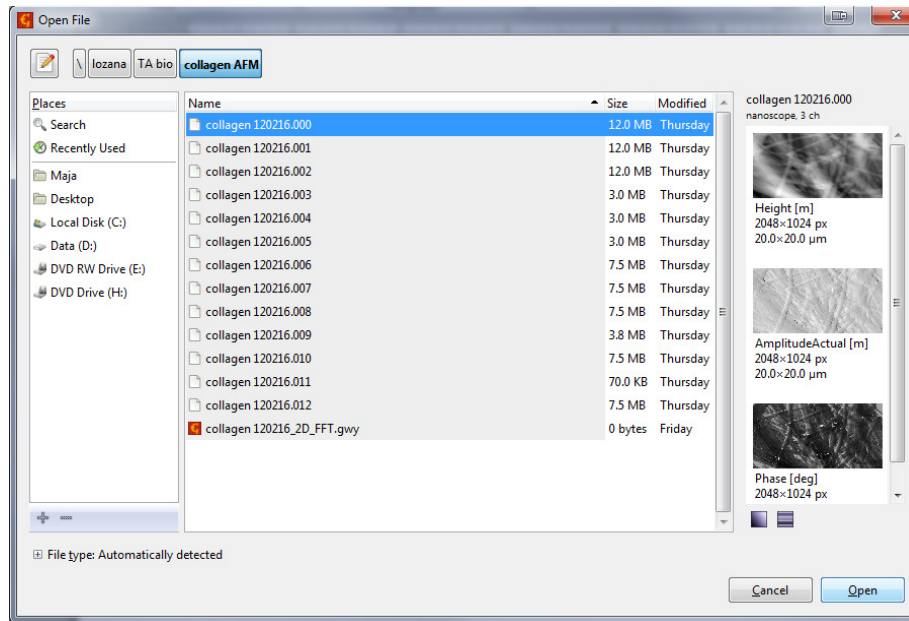


Figure 26: Opening a file in Gwyddion

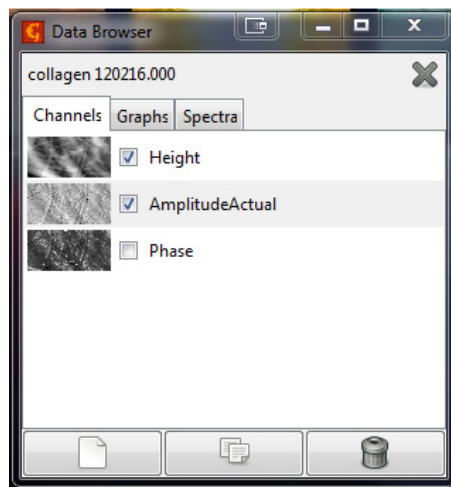


Figure 27: Opening images within the file in Gwyddion

3.1.2 Saving a file:

To save the file (containing all the images), go to **File->Save as**. Find the folder you wish to save your file in. Then, at the bottom of the window chose the proper extension of the file **.gwy**. On the top give the desired name to your file, but make sure to type it with the extension, e.g. **name_of_the_file.gwy**. Then click **Save**. You can also save just single image, by choosing one of the image formats, but also make sure to type the desired image name with the proper extension (e.g. **name_of_the_file.png**).

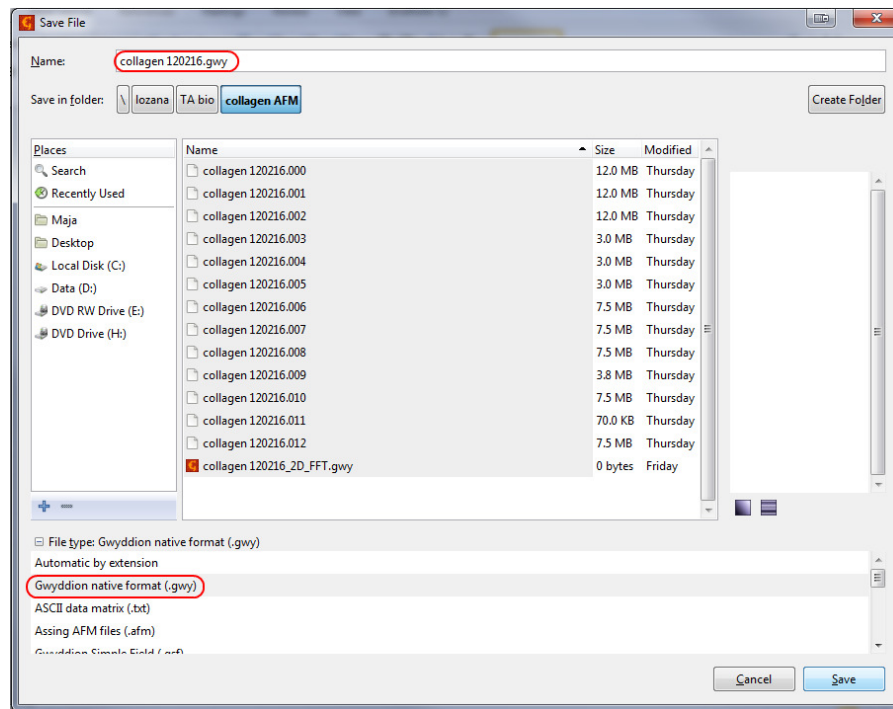


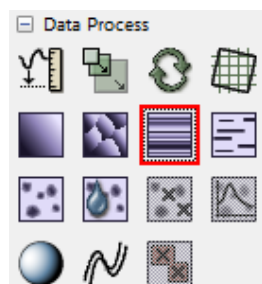
Figure 28: Saving a file in Gwyddion

3.2 Image corrections

Various types of image levelling operations are usually the first processing operations carried out on the AFM image data and often the only processing step used on AFM image data.

3.2.1 Line median matching

During imaging it can sometimes happen that cantilever change the level of interaction with sample or some mechanical disturbance can cause shift in the laser beam deflection. This will cause the jumps in the AFM height image on the slow scanning axis. That is why it is sometimes necessary to first do the line by line height median matching.

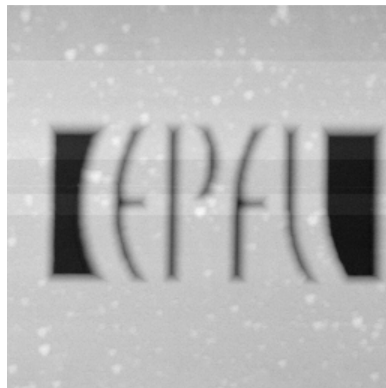


To do this in Gwyddion go to

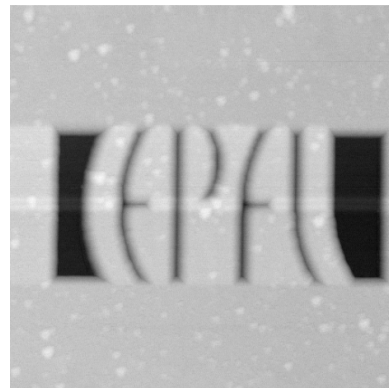
Data Process -> Correct lines by matching height median

This will do the height line median matching for all horizontal lines of the AFM image.

On the Figure 1 you can observe that, while this operation did some successful leveling on the upper part of the image, it introduced new leveling artifacts on the border areas of EPFL logo. So this operation should not be used on the images where there is a horizontal sample with large height difference with respect to the background. Misuse of this operation could also be explained on the example of vertically and horizontally imaged collagen fiber, Figure 30.

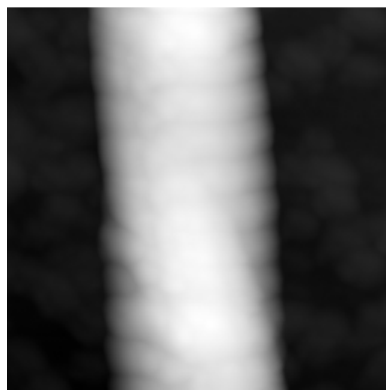


Before

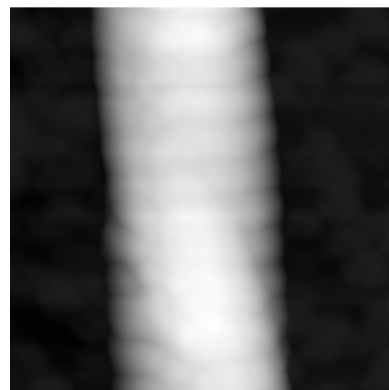


After

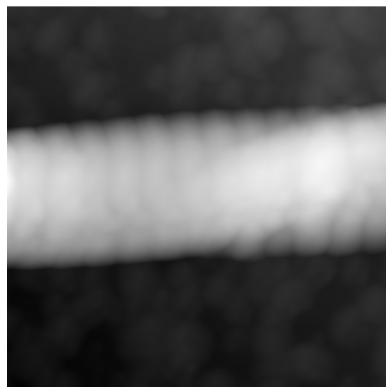
Figure 29: AFM image of the EPFL logo grating before and after the line by line height median subtraction operation



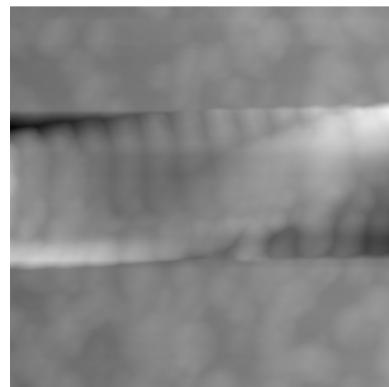
Collagen fiber, vertically imaged, before line median matching operation



Collagen fiber, vertically imaged, after line median matching operation ☺



Collagen fiber, horizontally imaged, before line median matching operation



Collagen fiber, horizontally imaged, after line median matching operation ☺

Figure 30: AFM images of vertically and horizontally imaged collagen fiber, before and after the line by line height median matching operation



In the case of horizontally imaged collagen fiber, we lost the height information of our collagen sample by doing the line median matching operation!

In the case of the artifacts on the EPFL logo image, these can be better removed by using the **Tools->Level rows using intersections with given lines**. With this operation we can actually select on which part of the image we want to apply the line median matching operation. This is shown in the images below.

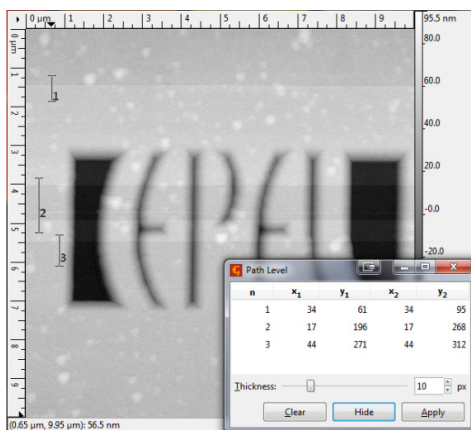


To do this in Gwyddion go to

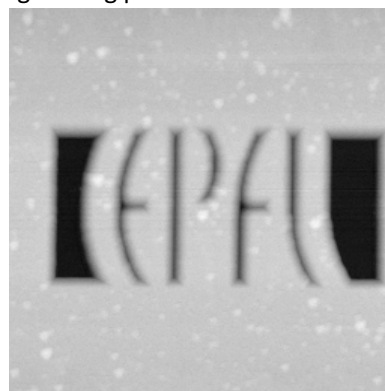
Tools->Level rows using intersection with given lines

Select the lines on which you would want to apply the line median subtraction and click **Apply**.

Notice that there is a thickness parameter. By setting it you can chose which amount of the line neighboring pixels will be used in leveling.



Before



After

Figure 31: AFM image of the EPFL logo grating before and after the line by line height median matching operation on specific areas

3.2.2 1st order plane leveling

If the background in the image (such as the substrate on which the sample was deposited) has considerable tilt in it, the change in height of the background will mask the changes in height associated with the sample. This effect is shown in the image below.

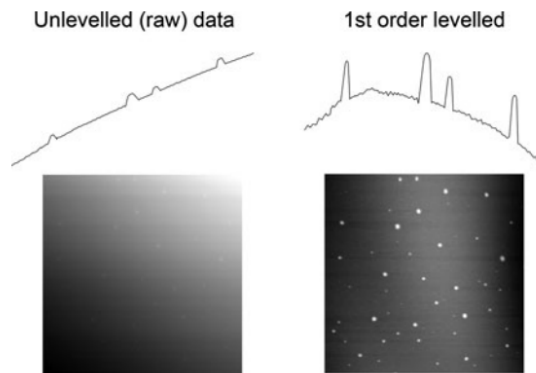
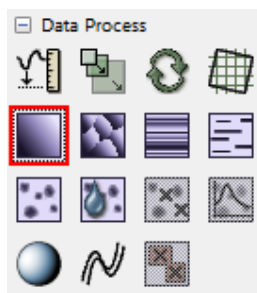


Figure 32: AFM image before and after background tilt removal

This effect can be avoided either by using a global plane subtraction, or by using a 3 point fitting (3 point plane subtraction).

3.2.3 Global plane subtraction



In order to do the plane subtraction, in Gwyddion go to **Data process->Level data by mean plane subtraction**. By clicking on it, Gwyddion will automatically calculate the background plane and subtract it from the image.



This operation should not be applied if the sample has large height difference with respect to the background because it will not flatten the background tilt properly!

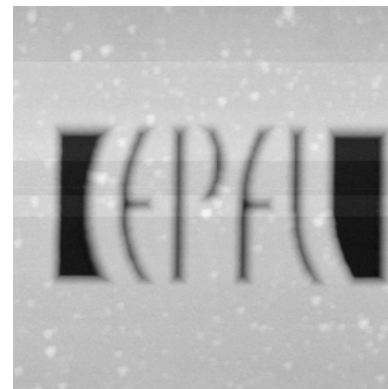
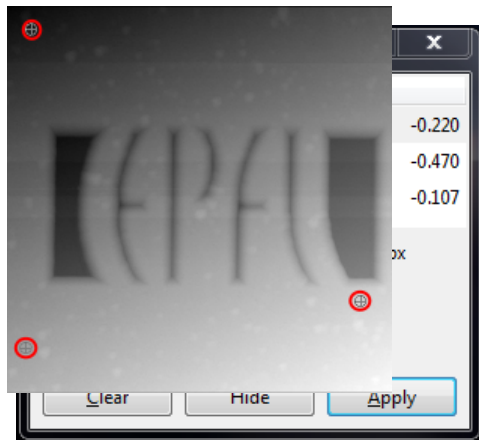
3.2.4 Three-point fitting

This procedure is similar to plane subtraction, but is a rather more 'manual' and often more accurate approach. In this method, the AFM user identifies three points on the image to define the plane to subtract.



In order to do the three-point fitting, in Gwyddion go to

Tools -> Level data by fitting the plane through a three points

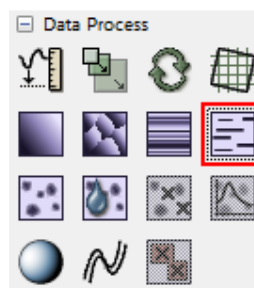


After

Figure 33: AFM image before and after three-points plane fitting

3.2.5 Scar correction

Scars (or stripes, strokes) are parts of the image that are corrupted by a very common scanning error: local fault of the closed loop. For instance, if a cantilever suddenly encounters a very high topography (e.g. like dirt present on the sample), it will take a while for the closed loop to return again to proper following of the sample topography. Line defects are usually parallel to the fast scanning axis in the image.



To remove scars in Gwyddion go to **Data Process->Correct horizontal scars (strokes)**. This function will automatically find and remove these scars, using neighboring lines to 'fill-in' the gaps.

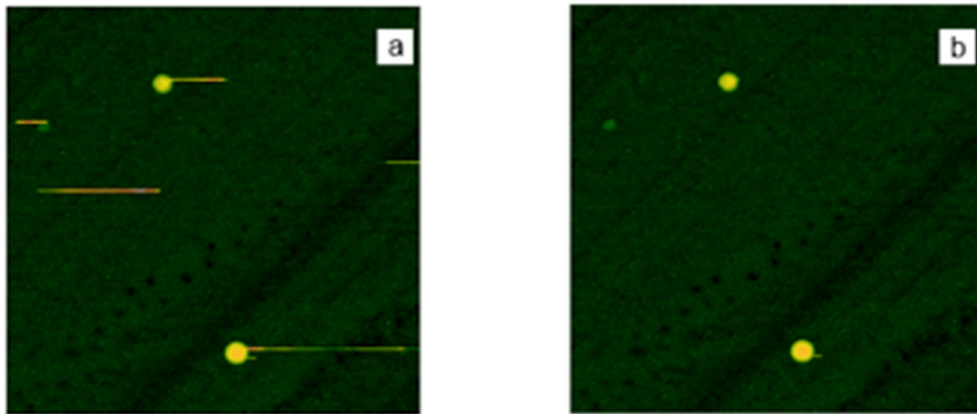


Figure 34: AFM image before and after scar correction

3.2.6 Higher order leveling

A common problem in AFM images is a scanner bow. It occurs mainly in instruments that use tube scanners, and is caused by a swinging motion of the free end of the scanner. This leads to introduction of a curve in the image plane, as shown in the image below.

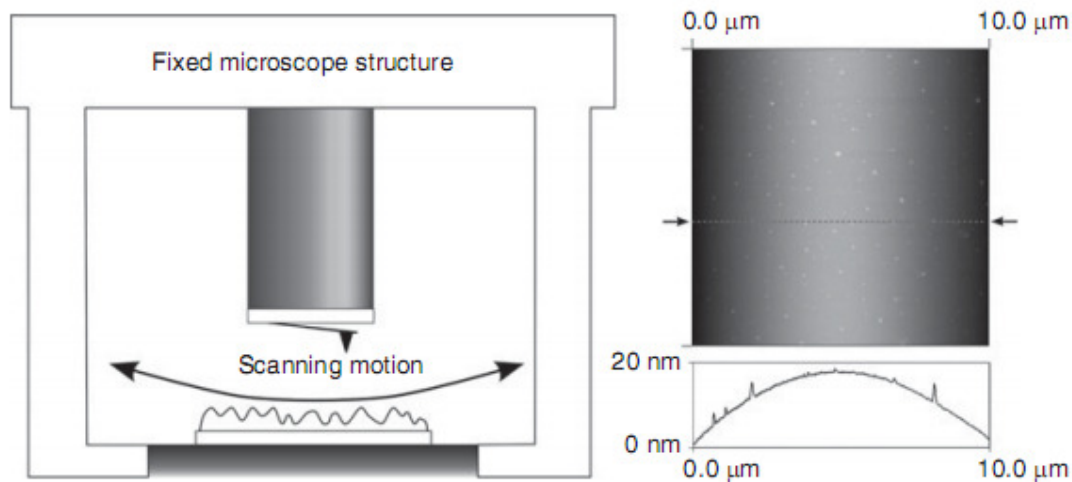
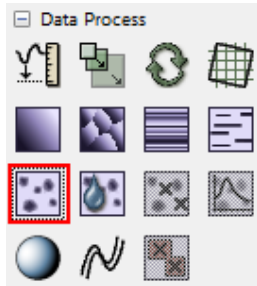


Figure 35: Effect of scanner bow on the AFM image

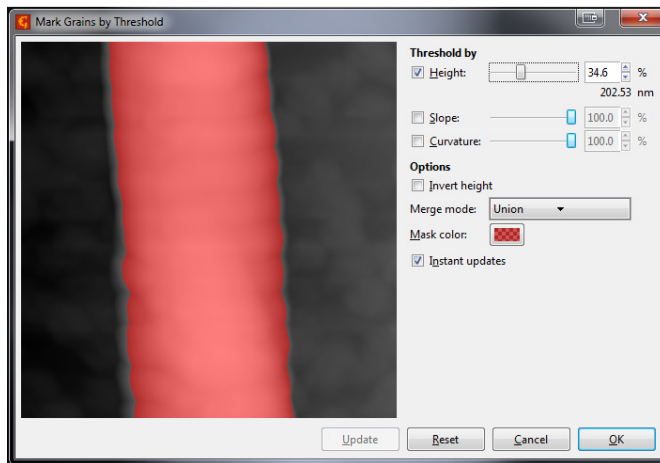
In order to remove this curve and similar image distortions that can arise from AFM system, we will use higher order polynomial two-dimensional curve fitting. This processing works well in the case where the background (on which the sample is deposited) is flat, and does not include any curvature. Typical examples of this case would include nanoparticles on a wafer, microorganisms on glass slides, or individual molecules on mica.

Note that if AFM microscope uses a flexure scanner instead of a tube scanner, scanner bow artifact will not be an issue.

Before doing this 2D curve fitting and removal operation, first we would need to select our sample to exclude it from the two-dimensional curve fitting, because we want to perform the 2D curve fitting only on the sample background.

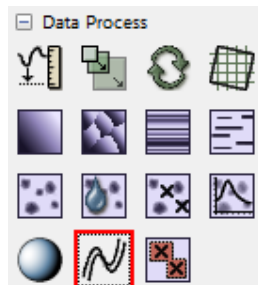


To do this in Gwyddion go to
Data Process -> Mark grains by threshold

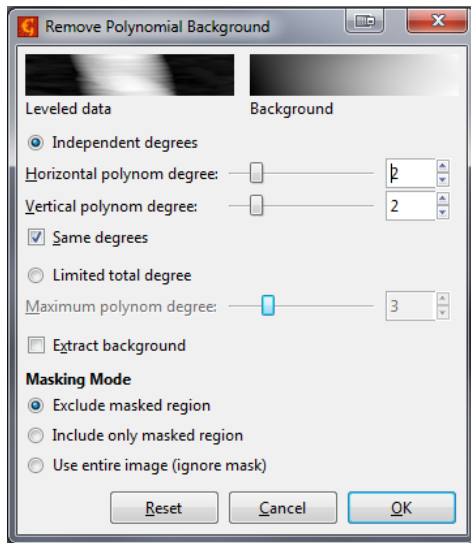


Check **Threshold by: Height** and change the percentage of the **Height** parameter until the entire sample is selected, then click **OK**

Now we will do the higher order polynomial two-dimensional curve fitting.



To do this in Gwyddion go to
Data Process -> Remove polynomial background



Set value for **Horizontal polynom degree** and **Vertical polynom degree** parameters (2-3).

In the **Masking Mode** part check the **Exclude masked region** option. This will exclude our previously selected sample from the 2D curve fitting.

Click **OK**.

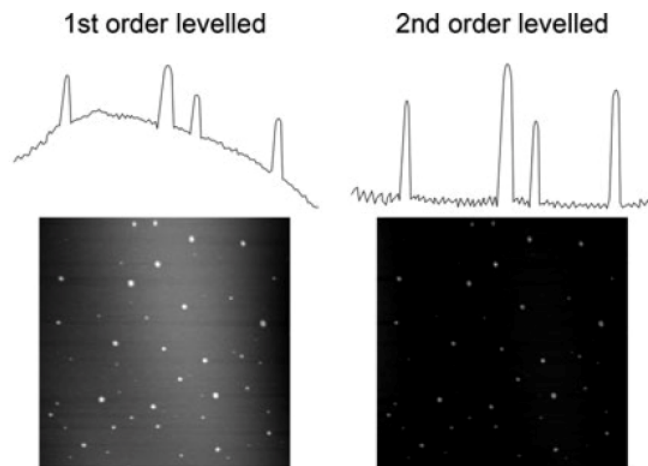


Image 8. AFM image before and after higher order 2D curve leveling

After this is done, to deselect the sample selection, again go to **Data Process -> Mark grains by threshold**, uncheck the **Threshold by: Height** and click **OK**.

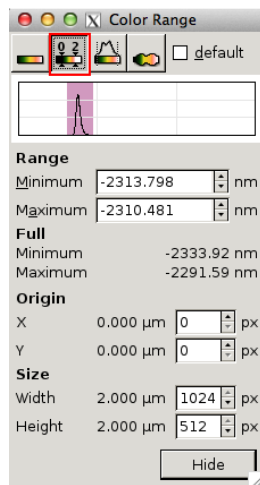
3.3 Image representation

3.3.1 Color Range

Gwyddion automatically sets the color range of the image to include all the topography in the image. If there are regions with high topography present in the image (e.g. dirt), the regions with the low height will be 'masked' by it and distinguishing the details in low topography regions will be hard. That is why it is sometimes needed to adjust the color range to the height range of interest.



To do this in Gwyddion go to
Tools -> Stretch color range to part of data



Window will open. Chose the second tab:



On the presented histogram select the desired range by dragging the mouse or by typing in **Minimum** and **Maximum** range values.

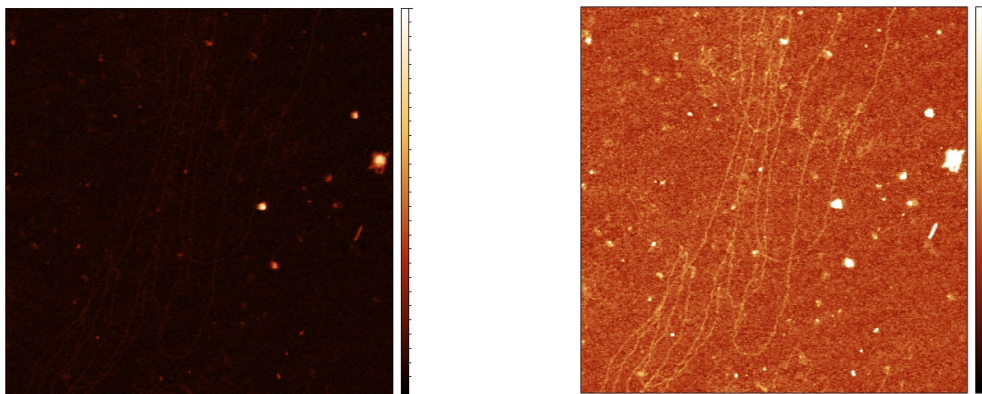
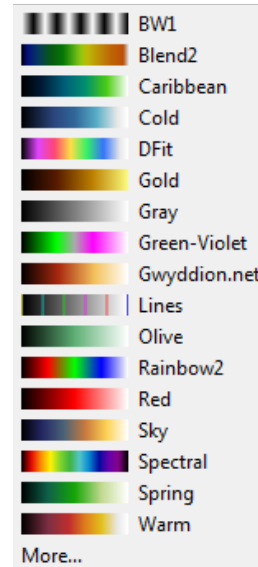


Figure 36: AFM image before and after the change of the color range

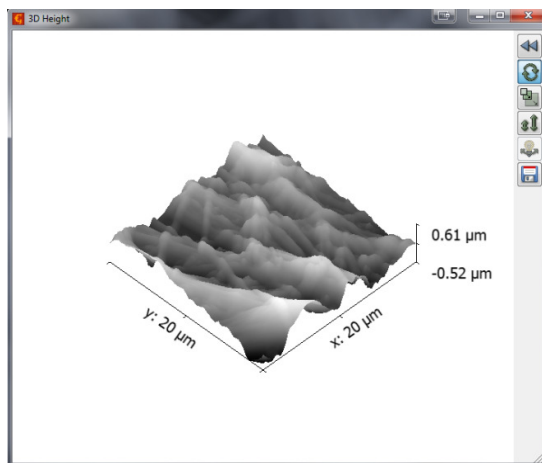
3.3.2 Color palettes

The color palette used to display an AFM image can be selected to make the image seem more visually compelling. In some cases, selecting a specialized color pallet can help with visualizing certain aspects of an image. In Gwyddion you can change the color palette by right clicking with a mouse on the color bar located on the right side of an AFM image.



3.3.3 3D Data representation

AFM height data is inherently three dimensional (3D). However, the standard method of rendering AFM data displays a two-dimensional (2D) image, using a color scale to represent height information. This is not a normal way for humans to see shapes, and can make interpretation difficult. In particular, for viewers unused to AFM data, it can be difficult to determine which features are higher than others, etc. One way to overcome this is to render the height information as a pseudo-three-dimensional image. The ability to display images that show what we want is vital, in order to make use of AFM data.



To present AFM image in 3D, in Gwyddion go to

View->Display a 3D view of data

This will present AFM image in 3D. On the right, there is a menu to process 3D image.



Use this button to enter advanced menu with possibilities such as choosing a color palette, choosing whether to show labels and axis, choosing type of lighting etc.



Use this button to rotate 3D AFM image.



If you selected the **Lighting** type of image presentation in advanced menu, use this button to change the spot of the light source.



Use this button to save 3D image.

3.4 Data analysis

3.4.1 Extracting profiles

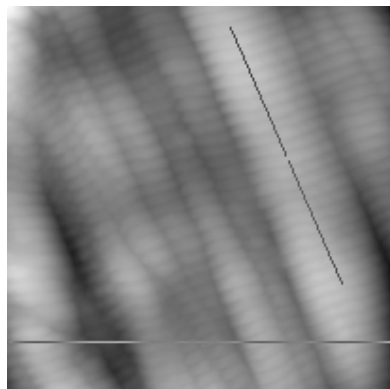
Many times it is necessary to extract line profiles from the image in order to examine certain sample characteristics. Since it is usually difficult to measure dimensions directly from AFM images, line profiles are usually extracted in order to measure dimensions from the AFM images. Gwyddion allows the user to arbitrarily define lines to be extracted, and these can be at any angle.



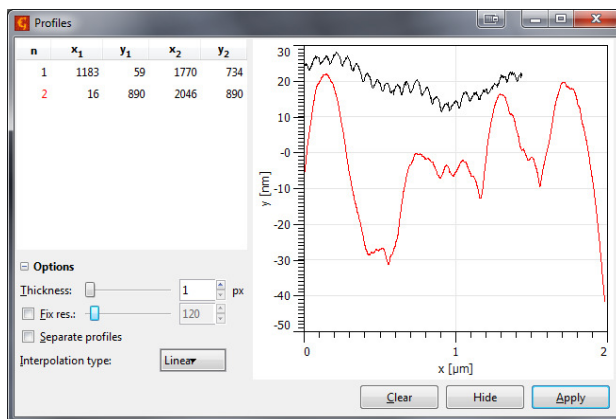
To do this in Gwyddion go to

Data Process -> Extract profiles

Clicking once on this button, external window will open to show line profiles.



Draw lines using mouse. If **SHIFT** button is held while doing this, lines will be drawn only under certain angles (use this to easily draw horizontal and vertical lines). You can draw more than one line.






In the external window you will get line profiles.

You can use mouse to draw several profiles in the image and they can be further moved and adjusted. The window includes a live profile graph preview.

Different 'thickness' can be set for profile lines. This means that more neighboring pixel data perpendicular to profile direction will be used for evaluation of one profile point of the profile line. This can be very useful for suppression of the image noise, when measuring objects in the image.

Click **Clear** button if you want to clear all selected line profiles. Click on the one of the profiles and press **Delete** button to delete that specific profile line. Click **Apply** button to continue further analysis of the line profiles. New window will open containing several options:

- | | |
|---|--|
|  | Use this marker button to measure distances in a graph, both horizontally and vertically. You can place many markers on a graph. |
|  | Use these buttons to zoom in on the desired area of the graph and to zoom out to full graph curve. |
|  | Use these buttons to logarithm x and y axes. |

3.4.2 2D FFT Analysis

In AFM image processing and analysis, a two-dimensional Fourier transform is an operation that converts the AFM image from the spatial domain, into the frequency, or more correctly, the wavelength domain. This is carried out by a mathematical operation known as a fast Fourier transform, so is sometimes also known as FFT analysis.

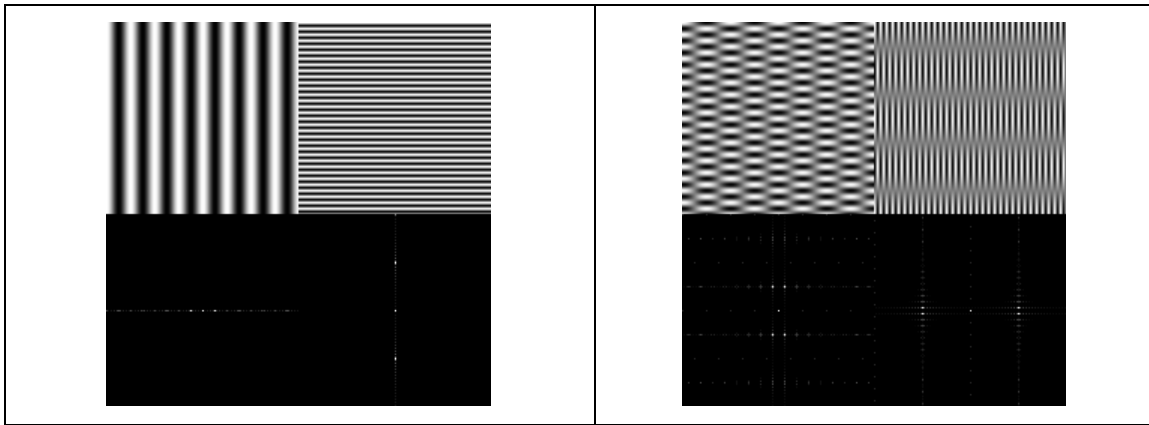
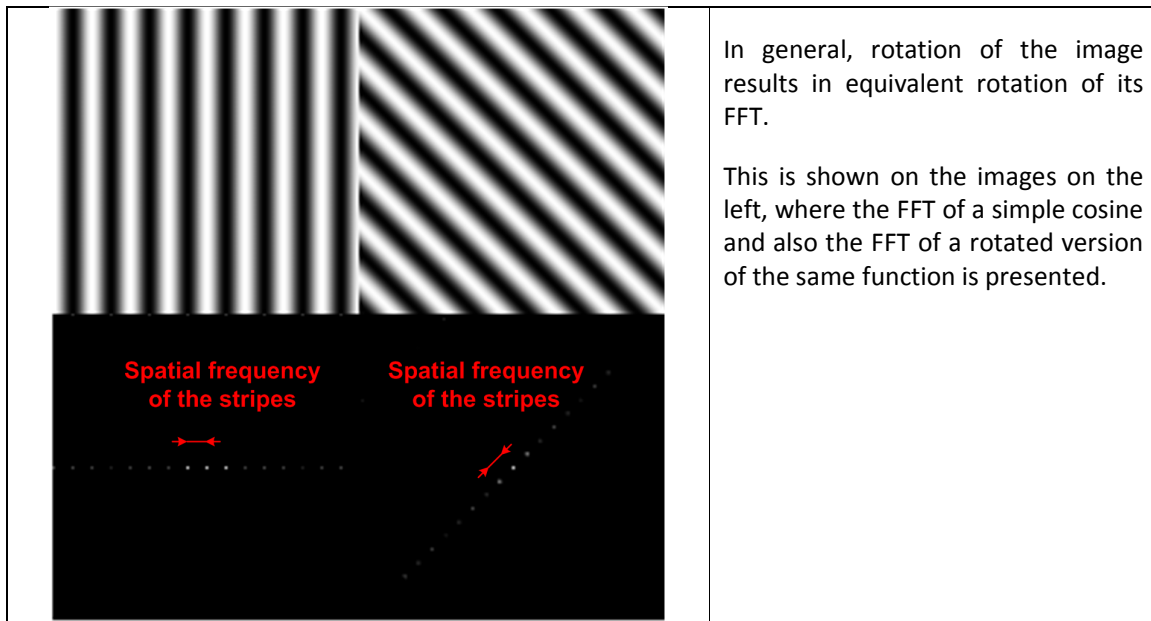



Figure 37: Several examples of 2D FFT of simple cosine-like images

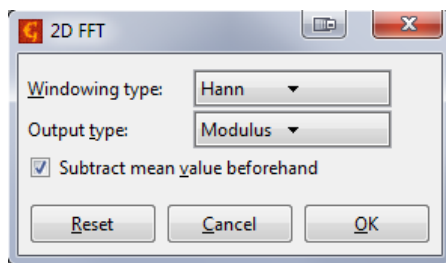
When transformed into Fourier space, the image will show features in terms of wavelength (or frequency). This is particularly useful to identify any repeating patterns in the image. For instance, 2D FFT can be used to calculate period of atomic lattice parameters, depth of corrugations on the collagen fibers etc. The Fourier transform can also be used to identify the frequency of noise in an image and to remove it.



In general, rotation of the image results in equivalent rotation of its FFT.

This is shown on the images on the left, where the FFT of a simple cosine and also the FFT of a rotated version of the same function is presented.

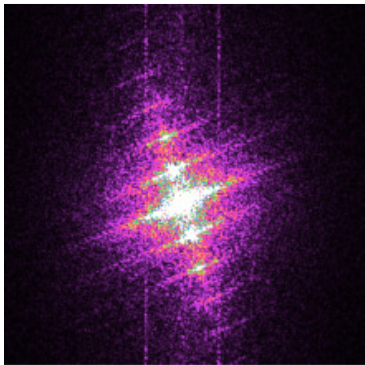
To do the 2D FFT in Gwyddion first open an image you want to apply it to. You might want to do the 2D FFT only on one part of the image. To do this go to **Tools->Crop data**  and select the desired image area. 2D FFT image will be much clearer if it is applied to the squared image (equal number of pixels on horizontal and vertical axis). So, to do this first go to **Data Process->Basic Operations->Square Samples**. Then do the 2D FFT by going to **Data Process->Integral Transforms->2D FFT**.



External window will be opened to set FFT parameters. You can leave default parameters and click **OK**.



You can click on the **View->Zoom in** button to zoom into 2D FFT image details.



Use on this image the **Extract profile** tool (which has already been explained), to determine spatial frequency and period of the measured pattern (e.g. period of the collagen corrugations).

3.4.3 Tip diameter estimation

Typically, cantilevers with blunt tips will lead to images with features larger than expected and with a flattened profile, due to the effect shown on the image below. Note that holes in a flat surface will show the opposite effect, appearing smaller with blunt probes than with sharp ones.

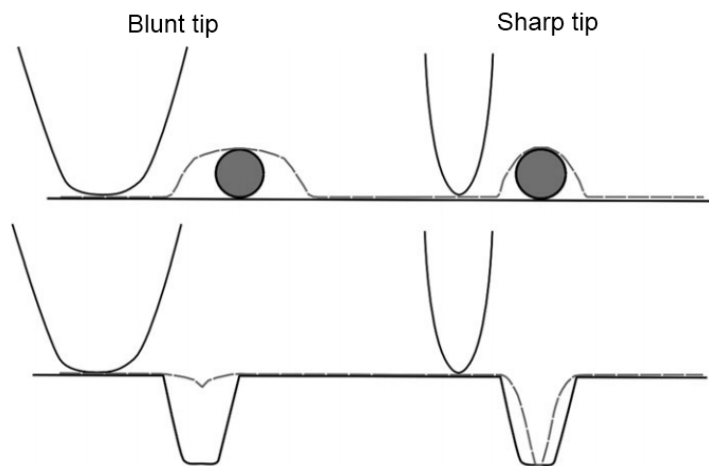
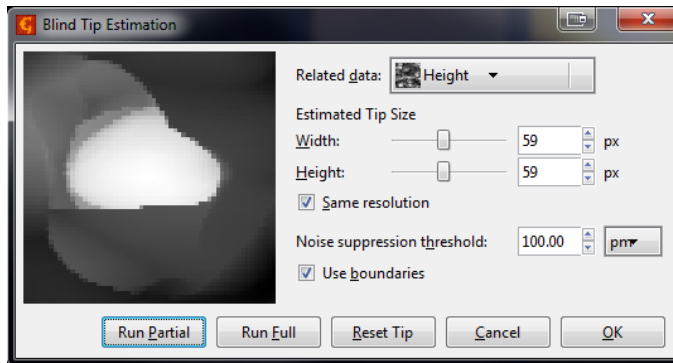


Figure 38: Illustration of the impact of the shape of a tip on the AFM image

In many cases this effect can be negligible, e.g. if the cantilever tip is much smaller than the features of the image being measured. However, sometimes it may significantly alter the apparent size of the features, and then it can noticeably change their appearance. Therefore, it is useful to know how to estimate diameter of the cantilever tip to properly evaluate the accuracy of measured data.

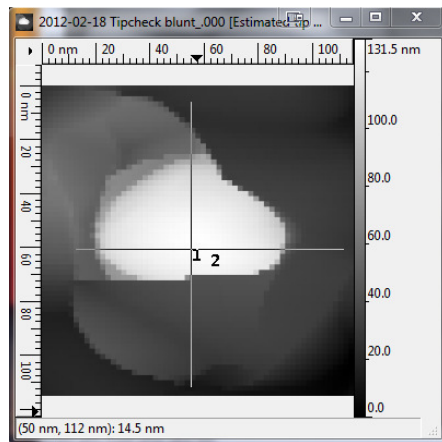
This estimation is usually done by first imaging a sample which has sharp and high edges in the topography (e.g. special “*Tipcheck AFM Tip Imaging Sample*”). Then, estimation of the tip topography can be done using the obtained image of the *Tipcheck* sample. This estimation is integrated in the most of the software for SPM image processing.

In Gwyddion, to do this estimation, first open the AFM image of the *Tipcheck* sample. Then go to **Data Process->Tip->Blind Estimation**.



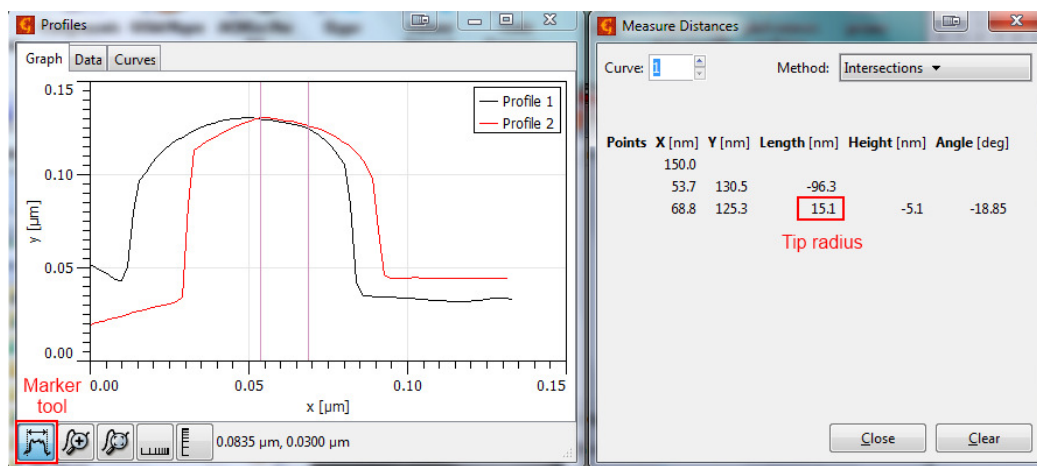
The window shown on the left will open. From the measured AFM image of the *Tipcheck* sample roughly estimate the width and the height of the tip (look at diameter of the grains in the sample image). Enter these estimates in the **Width** and the **Height** fields.

Click on the button **Run Partial**. Software will then calculate the topography of the tip and present it in the small window within. Click **OK** button.

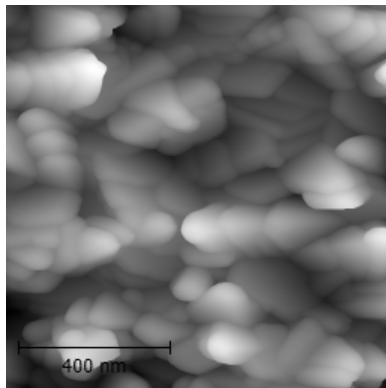


The image containing tip topography will then be opened. Use on this image the **Extract profile** tool (which has already been explained), to determine the diameter of the tip. Draw two perpendicular profile lines through the highest point of the tip (they don't need to be horizontal and vertical ones). Then in **Profiles** window click **Apply**.

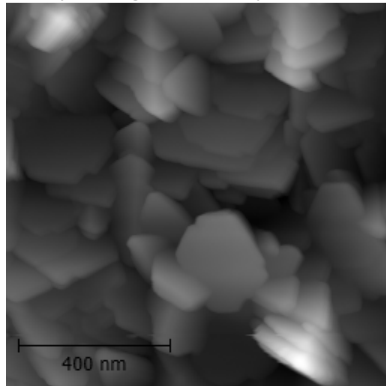
Click on the marker tool in new **Profiles** window (shown below) and find out the radius of the tip at 5 nm and 10 nm distance from the tip's highest point. Cantilever manufacturers typically cite this value as the tip radius.



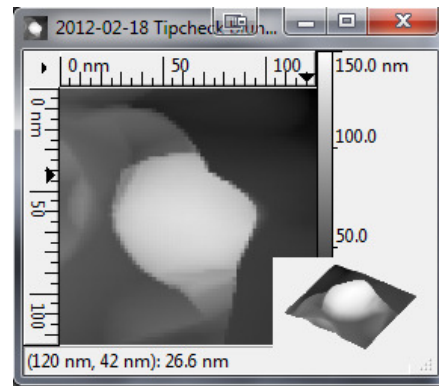
Tipcheck sample AFM images obtained with blunt and sharp tip cantilever are presented below, along with the tip topography estimates.



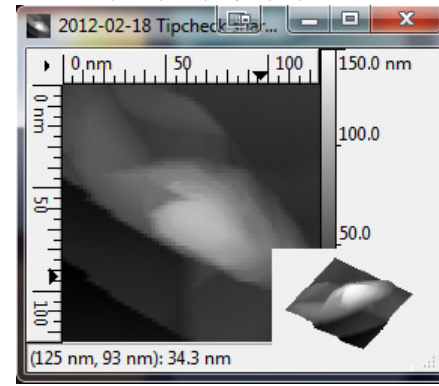
Blunt tip: Image of the Tipcheck sample



Sharp tip: Image of the Tipcheck sample



Blunt tip: Tip topography estimation



Sharp tip: Tip topography estimation

Figure 39: Sharp and blunt tip, AFM image comparison and tip topography estimation

Once tip topography is estimated, this can be used to reconstruct the more accurate image of the sample. In Gwyddion, this is done by going to **Data Process->Tip->Surface Reconstruction**. However, this usually successfully works only in the case when sharper tips are used for imaging.

4 ANALYSIS OF THE DATA OBTAINED IN THE LAB MODULE

4.1 Steps equal for all images

- Open AFM file in *Gwyddion* (see the chapter 3.1.1).
- Within the file, open the images (channels) you want to process. These could be for instance topography, amplitude or phase image (see the chapter 3.1.1).
- If needed rescale the image to set equal number of pixels in both axes (make image appear square). This is done by choosing **Data Process->Basic Operations->Square Samples**.
- Adjust the color range so that you have good contrast in the image and that you can inspect details of interest (see the chapter 3.3.1).

- Pick a color pallet you like (by right-clicking on the Z-scale and choosing the pallet, see chapter 3.3.2).
- Perform AFM image corrections such as background subtraction, line-by-line flattening and scar correction if necessary (see the whole chapter 3.2). **What is the correct order to do these operations?** Color range may change (reset) after each image correction applied, so readjust it if necessary.
- You should process all the images you obtained with AFM, print them and put them into your laboratory notebook. For each processed image you will need to write all the processing steps that you used and explain each briefly.

4.2 Analyzing AFM image of collagen fibrils

- Process AFM image of the collagen fibril to remove existing image artifacts (see the chapter 4.1).
- Determine the average depth and the period of the collagen fibril corrugations using **Extract profile** tool (see the chapter 3.4.1). Take several line profiles (10-20). For each line, note the length of the line and the number of periods. From this data, calculate the periodicity of the D banding period (corrugations period). **Estimate/calculate the error of your measurement. Should you report the standard error or the standard deviation, and why?**
- Determine the corrugation period of the collagen fibril again, this time by using 2D FFT analysis (see the chapter 3.4.2):
 - Crop the part of your AFM image where you have one single fibril with as many D-bands as possible. Try to make crop shape close to square.
 - Apply 2D FFT analysis
 - Extract the proper profile and measure the D-banding period (corrugation period) from the FFT (see the chapters 3.4.1 and 3.4.2)
 - Compare the values obtained by FFT with those obtained earlier.
- On your high resolution image (zoomed in collagen fibril) measure the depth of the corrugations, using **Extract profile** tool again (see the chapter 3.4.1). Calculate average corrugation depth and perform the appropriate error analysis. **On what cantilever property will the measured depth depend on?** (see the chapter 3.4.3)
- You can use **Excel** software to handle the measured data. However, you will need to input all the needed formulas by yourself (you are not allowed to use integrated **Excel** functions!). You can afterwards print the **Excel** sheet and put it into your laboratory notebook. You will have to write into your notebook all the formulas that you used to get the results!
- Write in the notebook answers to all asked questions.

4.3 Analyzing AFM image of E. Coli bacteria

- Process the E. Coli bacteria AFM image to remove existing image artifacts (see the chapter 4.1).
- From the high resolution image (zoomed in bacteria), make a 3D plot (see the chapter 3.3.3). Use the phase channel for color overlay. Adjust the scale, rotation and lighting to get the best displaying 3D image of your bacterium. Print this image and put it into your laboratory notebook.
- From the overview images, extract the lengths and heights of at least 50 bacteria (use **Extract profile** tool, see the chapter 3.4.1). Calculate the average length and the average height. **Estimate/calculate the error of your measurement. Should you report the standard error or the standard deviation, and why?**

- You can use **Excel** software to handle the measured data. However, you will need to input all the needed formulas by yourself (you are not allowed to use integrated **Excel** functions!). You can afterwards print the **Excel** sheet and put it into your laboratory notebook. You will have to write into your notebook all the formulas that you used to get the results!
- Write in the notebook answers to all asked questions.

4.4 Cantilever tip estimation

You will be given two AFM images of a *Tipcheck* sample, one taken with the sharp tip and one taken with the blunt tip (see the chapter 3.4.3).

- Perform the necessary AFM image processing to remove artifacts (see the chapter 4.1).
- Perform a **Blind Estimation** of the tip topography for each image (see the chapter 3.4.3).
- Measure the cone radius (tip radius) for each image at 5 nm and 10 nm from the tip apex. If the tip is not rotationally symmetric, calculate the maximum and minimum radius.
- Save the extracted profile lines of the estimated topographies for the sharp and the blunt tip and put them into your laboratory notebook and report the measured radii.

5 REFERENCES

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