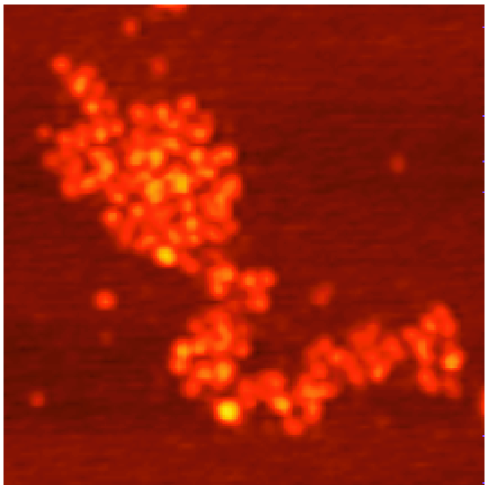
Atomic Force Microscopy on biological samples

Karls-Ruprecht Universität Heidelberg
Kirchhof-Institut für Physik - 2.208
Im Neuenheimer Feld 227
D-69120 Heidelberg

Betreuer: G.L. Heuvelman
KIP-2.19
Tel: 06221 54 9268
E-mail: Gerrit.Heuvelman@kip.uni-heidelberg.de
K. Rippe
KIP-3.19
Tel: 06221 54 9270
E-mail: Karsten.Rippe@kip.uni-heidelberg.de

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Technical support

Start-up

1. Turn on the computer and scanner by the power button behind the screen
2. Open the program 'nanoscope III 5.12r3' and turn the microscope button to start imaging, or the other button to open saved images
3. Under 'Microscope' -> 'Profile' search for 'TappingAFM' with file name 'afmprak'. All the parameter shown in this menu are described below.
4. Place the sample on the magnetic sample holder above the piezo.
5. Put the cantilever in the cantilever holder
6. Place the cantilever holder in the AFM head and fix it with adjustment I (as schematically shown in figure 1)
7. Move the sample to the area of interest with adjustments IIa and IIb using the optical microscope.
   (5, 6 and 8 up to 11 are only necessary using a new cantilever)
8. Locate the laser light on the cantilever tip. Before, verify that the mode switch on the multimode SPM's base is switched to 'AFM & LFM'. Align the laser spot on the cantilever by the laser position adjustments IIIa (X-axis) and IIIb (Y-axis). The maximized value for the SUM should be approximately 3.0-8.0 Volts.

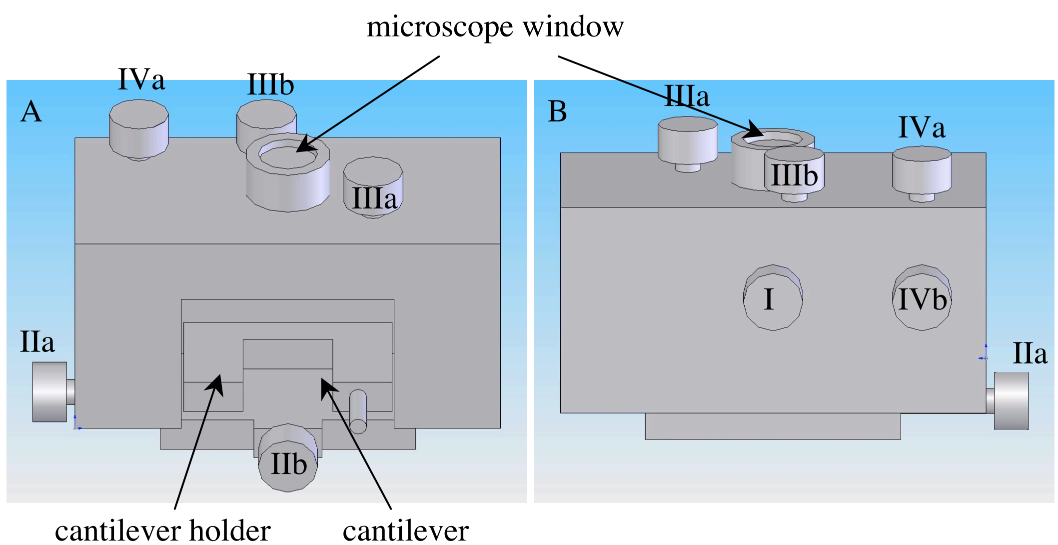


Figure 1 schematic image of an AFM head, (A) front and (B) rear view.

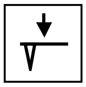
9. Adjust the photodiode positioner (adjusters IVa and Ivb) to set the reflected laser beam in the middle of the diode. The values A-B and C-D should be zero.
10. Put the mode switch on the multimode SPM's base back to 'TM AFM'.
11. Search for the resonant frequency by the 8th button in the upper screen (see below). 'Auto tune' will automatically search for this frequency and with 'back to image mode', this value will automatically set the value for 'drive frequency'.
12. Recheck all control panel parameters. The feedback gains and the scan rate are the

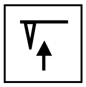
most important parameters. Start with the 'integral gain' set to 0.50 and the 'proportional gain' set to about 1.20. The scan rate should be set below 2 Hz. Use a scan size of 10 μm to get an overview.

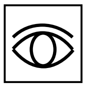
13. Focus with the optical microscope on the surface, e.g. an edge of the surface.
14. Use the coarse adjustment screws and the step motor to bring tip and surface closer together. The AFM head has to be totally vertical. If all goes well after re-engaging, a well-formed cantilever tip will begin to appear on the display monitor. Take care! If the sample is in focus, the tip has to be a little bit above the focus otherwise it will brake. Then use the 1st button in the upper screen (see below) to make the tip contacting the surface.
15. After the tip contacts the sample, the AFM will automatically start scanning the sample.
16. If the image doesn't look like it should be, you can try the following to make it better:

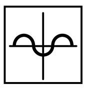
Buttons

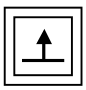
<u>Image mode</u>

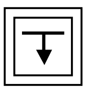
 approach surface automatically by step-motor (max 80 μm)

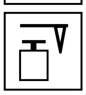
 retract tip from surface (5 μm per turn); retract about 25 μm to change scan area and much more to change sample.

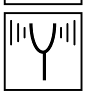
 change to image mode (normal)

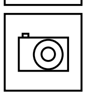
 single trace mode

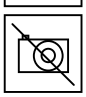
 start scanning at bottom of scan area

 start scanning on top of scan area

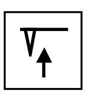
 force curve mode

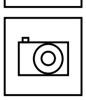
 search for resonant frequency mode

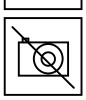
 capture image

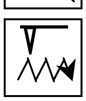
 exit capture command

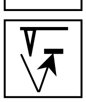
<u>Force mode</u>

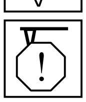
 retract tip from surface (5 μm per turn); retract about 25 μm to change scan area and much more to change sample.

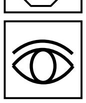
 capture image

 exit capture command

 the tip is continuously lowered and raised by distance equal to the Z scan size. This is the normal, default motion in the force mode

 approach and retract the surface once by a distance equal to the Z scan size.

 Halts all tip movements

 change to image mode (normal)

parameters for image mode

Scan controls:

scan size: Size of the scan along one side of the square. If the scan is non-square (as determined by the **aspect ratio** parameter), the value entered is the longer of the two sides. Maximum/minimum value: 500 nm - 10 μm.

aspect ratio: determines whether the scan is to be square (aspect ratio 1:1), of non-square. Default value 1:1.

X offset, **Y offset**: these controls allow adjustment of the lateral scanned area and the center of the scanned area. These values can be chosen between 0 and maximal 10 μm, depending on the scan size (piezo deflection in x- and y-direction is max. 10 μm).

scan angle: combines X-axis and Y-axis drive voltages, causing the piezo to scan the sample at varying X-Y angles. Value between 0 and 90 degrees.

scan rate: the number of lines scanned per second in the fast scan (X-axis on display monitor) direction. In general, the **scan rate** must be decreased as the **scan size** is decreased. Scan rates if 1.5-2.5 Hz should be used for larger scans on samples with tall features. High scan rates help to reduce drift, but they can only be used on very flat samples with small scan sizes. Maximum/minimum value: 0.1 Hz - 5 Hz.

tip velocity: the scanned distance per second in the fast scan direction (changes as the scan rate changes).

samples/line: number of imaged points per scanned line. Maximum/minimum value: 128 - 1024 (default value 512).

lines: number of scanned lines. This value is the same as **samples/line** for **aspect ratio** of 1:1. Maximum/minimum value: 128 – 1024; default value 512.

number of samples: sets the number of pixels displayed per line and the number of lines per scanned frame.

slow scan axis: starts and stops the slow scan (Y-axis on display monitor). This control is used to allow the user to check for lateral mechanical drift in the microscope or assist in tuning the feedback gains. Always set to ‘enable’ unless checking for drift or tuning gains. Default value: enable

Feedback controls:

SPM feedback: sets the signal used as feedback input. Possible signals are ‘Amplitude’ (default), ‘TM deflection’ and ‘Phase’.

integral gain and **proportional gain**: controls the response time of the feedback loop. The feedback loop tries to keep the output of the SPM equal to the setpoint reference chosen. It does this by moving the piezo in Z to keep the SPM’s output on track with the setpoint reference. Piezoelectric transducers have a characteristic response time to the feedback voltage applied. The gains are simply values that magnify the difference read at the A/D converter. This causes the computer to think that the SPM output is further away from the setpoint reference than it really is. The computer essentially overcompensates for this by sending a larger voltage to the Z piezo than it truly needed. This causes the piezo scanner to move faster in Z. This is done to compensate for the mechanical hysteresis of the piezo element. The effect is smoothed out due to the fact that the piezo is adjusted up to four times the rate of the display rate. Optimize the 'integral gain' and 'proportional gain' so that the height image shows the sharpest contrast and there are minimal variations in the amplitude image (the error signal). It may be helpful to optimize the 'scan rate' to get the sharpest image. Maximum/minimum value for integral gain: 0.1 – 4 and for proportional gain: 0.1 – 10. Default values between 2 and 3.

amplitude setpoint: The setpoint defines the desired voltage for the feedback loop. The setpoint voltage is constantly compared to the present RMS amplitude voltage to calculate the desired change in the piezo position. When the **SPM feedback** is set to amplitude, the Z piezo position changes to keep the amplitude voltage close to the setpoint; therefore, the vibration amplitude remains nearly constant. Changing the setpoint alters the response of the cantilever vibration and changes the amount of force applied to the sample. Maximum/minimum value: 1 – 8.

drive frequency: defines the frequency at which the cantilever is oscillated. This frequency should be very close to the resonant frequency of the cantilever. These value is around 300 kHz for the cantilevers used.

drive amplitude: defines the amplitude of the voltage applied to the piezo system that drives the cantilever vibration. It is possible to fracture the cantilever by using too high drive amplitude; therefore, it is safer to start with a small value and increase the value incrementally. If the amplitude calibration plot consist of a flat line all the way across, changing the value of this parameter should shift the level of the curve. If it does not, the tip is probably fully extended into the surface and the tip should be withdrawn before proceeding. Maximum/minimum value: 10 – 60, default 30 V.

<u>Channel 1, 2 and 3</u>

data type: height data corresponds to the change in piezo height needed to keep the vibrational amplitude of the cantilever constant. Amplitude data describes the change in the amplitude directly. Deflection data comes from the differential signal off of the top and bottom photodiode segments.

data scale: This parameter controls the vertical scale corresponding to the full height of the display and colorbar.

data center: Offsets centerline on the color scale by the amount entered.

line direction: Selects the direction of the fast scan during data collection. Only one-way scanning is possible.

Range of settings:

- Trace: Data is collected when the relative motion of the tip is left to right as viewed from the front of the microscope.
- Retrace: Data is collected when the relative tip motion is right to left as viewed from the front of the microscope.

scan line: The scan line controls whether data from the Main of Interleave scan line is displayed and captured.

realtime plane fit: Applies a software 'leveling plane' to each real-time image, thus removing up to first-order tilt. Five types of planefit are available to each real-time image shown on the display monitor.

Range of settings:

- None: only raw, unprocessed data is displayed.
- Offset: takes the Z-axis average of each scan line, then subtracts it from every data point.
- Line: takes the slope and Z-axis average of each scan line and subtracts it from each data point in that scan line. This is the default mode of operation, and should be used unless there is a specific reason to do otherwise.
- AC: takes the slope and Z-axis average of each scan line across one-half of that line, then subtracts it from each data point in that scan line.
- Frame: level the real-time image based on a best-fit plane calculated from the most recent real-time frame performed with the same frame direction (up or down).
- Captured: level the real-time image based on a best fit plane calculated from a plane captured with the capture plane command in.

off line plane fit: Applies a software 'leveling plane' to each off-line image for removing first-order tilt. Five types of plane-fit are available to each off-line image.

Range of settings:

- None: only raw, unprocessed data is displayed.
- Offset: captured images have a DC Z offset removed from them, but they are not fitted to a plane.
- Full: A best-fit plane that is derived from the data file is subtracted from the captured image.

highpass filter: This filter parameter invokes a digital, two-pole, highpass filter that removes low frequency effects, such as ripples caused by tortional forces on the cantilever when the scan reverses direction. It operates on the digital data stream regardless of scan direction. This parameter can be 'off' or set from 0 through 9. Settings of 1 through 9, successively, lower the cut-off frequency of the filter applied to the data stream. It is important to realize that in removing low frequency information from the image, the highpass filter distorts the height information in the image.

lowpass filter: This filter invokes a digital, one-pole, lowpass filter to remove high-frequency noise from the Real Time data. The filter operates on the collected digital data regardless of the scan direction. Settings for this item range from 'off' through '9'. Off implies no lowpass filtering of the data, while settings of 1 through 9, successively, lower the cut-off frequency of the filter applied to the data stream.

parameters for force mode

<u>Main control</u>

ramp channel: Defines the variable to be plotted along the X-axis of the scope trace. Default ‘Z’.

ramp size: This parameter defines the total travel distance of the piezo. Use caution when working in the force mode. This mode can potentially damage the tip and/or surface by too high values for the ramp size.

z scan start: This parameter sets the offset of the piezo travel (i.e., its starting point). It sets the maximum voltage applied to the Z electrodes of the piezo during the force plot operation. The triangular waveform is offset up and down in relation to the value of this parameter. Increasing the value of the ‘**z scan start**’ parameter moves the sample closer to the tip by extending the piezo tube.

scan rate: sets the rate with which the Z-piezo approach/retract the tip. Maximum/minimum value: 0.1 Hz - 5 Hz.

X offset and **Y offset**: controls the center position of the scan in the X- and Y- directions, respectively; same as in image mode. Range of settings: ±220 V.

number of samples: Defines the number of data points captured during each extension/retraction cycle of the Z-piezo. Maximum/minimum value: 128-1024; default value 512.

average count: sets the number of scans taken to average the display of the force plot. May be set between 1 and 1024. Usually it is set to 1 unless the user needs to reduce noise.

spring constant: This parameter relates the cantilever deflection signal to the Z travel of the piezo. It equals the slope of the deflection versus Z when the tip is in contact with the sample. For a proper force curve, the line has a negative slope with typical values of 10-50 mV/nm; however, 10-50 mV/nm; however, by convention, values are shown as positive in the menu.

display mode: The portion of a tip’s vertical motion to be plotted on the force graph. Range of settings:

- Extend: plots only the extension portion of the tip’s vertical travel.
- Retract: plots only the retraction portion of the tip’s vertical travel.
- Both: plots both the extension and retraction portion of the tip’s vertical travel

units: this item selects the units of the parameters, either ‘metric’ lengths or ‘volts’.

X-rotate: allows the user to move the tip laterally, in the X direction, during indentation. This is useful since the cantilever is at an angle relative to the surface. One purpose of X rotate is to prevent the cantilever from plowing the surface laterally, typically along the X direction, while it indents in the sample surface in the Z direction. Plowing can occur due to cantilever bending during indentation of due to X movement caused by coupling of the Z and X axes of the piezo scanner. When indenting in the Z direction, the X rotate parameter allows the user to add movement in the X direction. X rotate causes movement of the scanner opposite to the direction in which the cantilever points. Without X rotate control. The tip may be prone to pitch forward during indentation. It can be varied in a range of 0-90 degrees. Normally, it is set to about 22 degrees.

amplitude setpoint: same as in image mode.

<u>Channel 1, 2 and 3</u>

data type/data scale/data center: same as in image mode.

amplitude sens.: This item relates the vibrational amplitude of the cantilever to the Z travel of the piezo. It is calculated by measuring the slope of the RMS amplitude versus the Z voltage when the tip is in contact with the sample. The NanoScope system automatically calculates and enters the value from the graph after the operator uses the mouse to fit a line to the graph. This item must be properly calculated and entered before reliable deflection data in nanometers can be displayed. For a proper force curve, the line has a negative slope with typical values of 10-50 mV/nm; however, 10-50 mV/nm; however, by convention, values are shown as positive in the menu.

Trouble shooting

It happens rather often that one thinks to have checked all control panel parameters correctly and don't get an image. Here a list with the most common errors.

- Right input channel (see monitor). 'Height' signal for constant height and 'deflection' signal for constant deflection mode.
- Correct gains for 'proportional gain' and 'integral gain'. These parameters have to be between 0.1 and 5, depending on the softness/hardness of the sample. Start with the 'integral gain' set to 0.50 and the 'proportional gain' set to about 1.20.
- Is the height scale justified correctly for the different channels.
- What is the size of the scan area. The scan size can be varied between 100 nm and 10 μm. Use a scan size of 10 μm to get an overview.
- Is the piezo in its limit; between + of – 220V. This can be checked by the green/red setpoint line in the approach/retract bar. This green/red setpoint line should not be on the left or on the right site of the bar. This can be changed by the 'amplitude setpoint' parameter.
- Does the force used to image the probe, destroy the probe. Play around with the 'amplitude setpoint' parameter and the 'drive amplitude' parameter. The slope of the force curve shows the sensitivity of the TappingMode measurement. In general, higher sensitivities will give better image quality. The View -> Force Mode -> plot command plots the cantilever amplitude versus the scanner position (=force curve). The curve should show a mostly flat region where the cantilever has not yet reached the surface and the sloped region where the amplitude is being reduced by the tapping interaction. To protect the tip and sample, take care that the cantilever amplitude is never reduced to zero. Adjust the setpoint until the green setpoint line on the graph is just barely below the flat region of the force curve. This is the setpoint that applies the lowest force on the sample.

Poor image quality

Contaminated tip

Some types of samples may adhere to the cantilever and tip (e.g. certain proteins). This will reduce resolution giving fuzzy images. If the tip contamination is suspected to be a problem, it will be necessary to protect the tip against contamination.

Dull tip

AFM cantilever tips can become dull during use and some unused tips may be defective. If imaging resolution is poor, try changing to a new cantilever.

Multiple tip

AFM cantilevers can have multiple protrusions at the apex of the tip which result in image artifacts. In this case, features on the surface will appear two or more times in the image, usually separated by several nanometers (see figure 2A). If this occurs and this effect doesn't disappear after some time, change of clean the AFM tip.

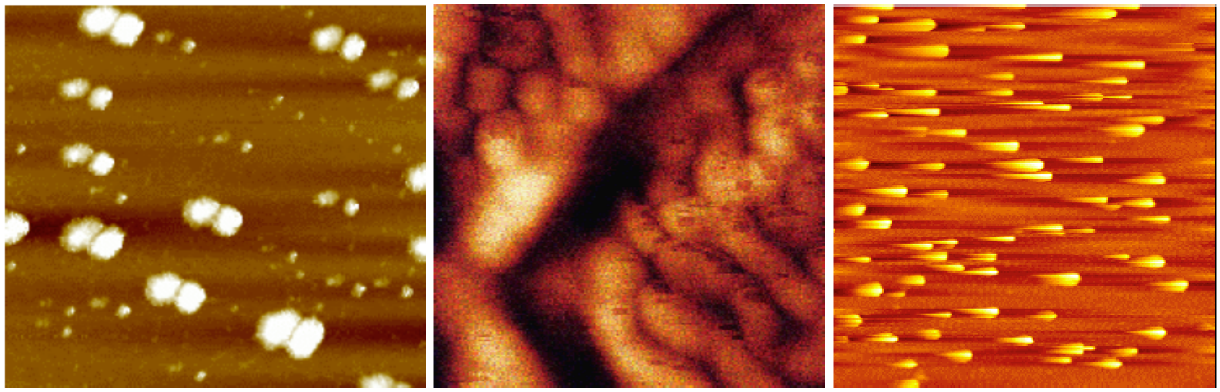


Figure 2 (A) multiple tip, (B) repeating patterns and (C) Silverfish structures due to high setpoint.

Repeating pattern

If a repeating pattern appears, more tip areas scan simultaneous (see figure 2B). Such a cantilever has to be changed.

To low setpoint

Some structure shows up that don't exist (e.g. circles). Change the 'amplitude setpoint' to a better value.

To high setpoint

If silverfish structures appear (see figure 2C), the adjustment of the integral and proportional gain can help, but if the setpoint is not adjusted correctly, increasing the gain will worse the noise of the image.

To high Drive frequency

The image surface looks destroyed; e.g. holes appear. These holes are artifacts and disappear when the drive frequency is lowered.

Other error sources

- Scanner beeps loud: decrease immediately the gains; the scanner is overdriven.
- The image seems only noise: Adjust 'amplitude setpoint'.
- Strong drift of signal: approach the tip from the surface with the 2nd button in the upper screen and retract again.
- During retraction of the tip, the scanner shows immediately contact with the surface, the image shows only noise: check the setpoint. Put the setpoint to zero and approach the surface again.

Theorie

Surface Measurement Parameters

From the measured surface images, it is possible to make an estimation of the smoothness of the surface. With the following image processing equations, one can quantify surfaces mathematical.

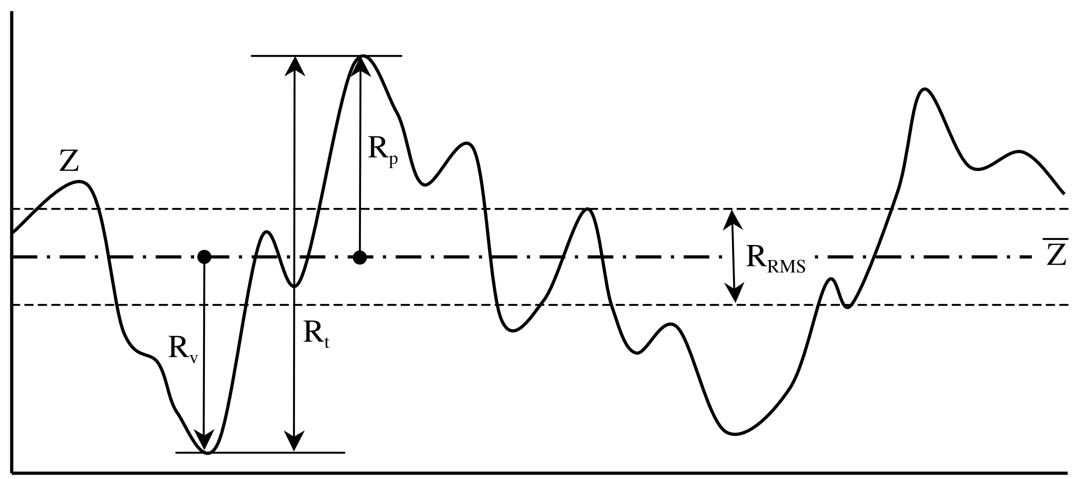


Figure 1. a schematic image of an image profile

Table I: surface measurement parameters

Term	Definition	Calculation	Use
Z	Mean Value	$\overline{Z} = \frac{1}{N}\sum_{i=1}^{N} Z_i$ where N is the number of data points, and Z is the surface height	
R_a	Roughness average is the main height as calculated over the entire measured length or area. It is quoted in micrometers.	Two-dimensional R_a: $R_a = \frac{1}{N}\sum_{i=1}^{N}\left\|Z_i - \overline{Z}\right\|$ Three-dimensional R_a: $R_a = \frac{1}{MN}\sum_{j=1}^{N}\sum_{i=1}^{M}\left\|Z_{ij} - \overline{Z}\right\|$ where M and N are the number of data points in X and Y, and Z is the surface height relative to the mean plane.	R_a is typically used to describe the roughness of machined surfaces. It is useful for detecting general variations in overall profile height characteristics and for monitoring an established manufacturing process.
R_{RMS}	The Root Mean Square (RMS) average between the height deviations and the mean line/surface, taken over the evaluation length/area.	Two-dimensional R_{RMS}: $R_{RMS} = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(Z_i - \overline{Z}\right)^2}$ Three-dimensional R_{RMS}: $R_{RMS} = \sqrt{\frac{1}{NM}\sum_{j=1}^{M}\sum_{i=1}^{N}\left(Z_{ij} - \overline{Z}\right)^2}$	RMS roughness describes the finish of optical surfaces. It represents the standard deviations of the profile heights.

R_p, R_v	Maximum profile peak height and maximum profile valley depth are the distances from the mean line/surface to the highest/lowest point in the evaluation length/area.	Measured	Peak height provides information about friction and wears on a part. Valley depth provides information about how a part might retain a lubricant.
R_t	Maximum height is the vertical distance between the highest and lowest points in the evaluation length/area.	$R_t = R_p + R_v$	Maximum height describes the overall roughness of a surface.
R_z	The average maximum profile of the ten greatest peak-to-valley separations in the evaluation area. Vision excludes an 11 × 11 region around each high (H) or low (L) point such that all peak or valley points won't emanate from one spike or hole.	$R_z = \frac{1}{10}\left[\sum_{i=1}^{10} H_i - \sum_{j=1}^{10} L_j\right]$	R_z is useful for evaluating surfaces texture on limited-access surfaces such as small valve seats and the floors and walls of grooves, particulary where the presence of high peaks or deep valleys is of functional significance.

Preparation tasks

What are the limits of the resolution of an optical microscope, according to Abbe's equation. See also: http://www.uiowa.edu/~cemrf/courses/light/light.htm. What limits the resolution of an Atomic Force Microscope.

What are piezo-materials and how can one use these in motion systems. See also: http://www.piezo.com/bendedu.html

Describe how a closed-loop (feed-back) control functionize. Discuss what happens with the measured image by changing the values for P and I (proportional and integration gain). See also:
http://www.netrino.com/Publications/Glossary/PID.html
http://www.engin.umich.edu/group/ctm/PID/PID.html#characteristics

Calculate the values for mean, R_a and R_{RMS}, for a line profile of a cosine-function with 5 periods within the measure length and for a cosine-function with 5.25 periods with in. Do this for a line profile with 200 points and for a line profile with 50 points.

Experiments

Goal of these experiments is to learn about the basic functioning and possibilities of the Atomic Force Microscope. The structure of a few surfaces will be observed and quantified. Also some biological samples will be imaged and researched.

Material

- AFM-cantilevers
- Calibration grid
- Metal plate
- Glass plate
- Mica plate
- Superglue
- Pair of tweezers
- Tesa-film
- Biological probes (ask for)

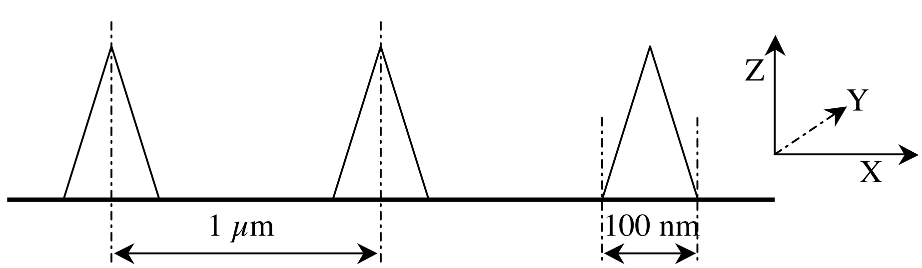
Introductory experiments

P and I values

1. Vary the values of the proportional gain P and the integral gain I, while imaging the surface of a calibration grid. Try to find optimal values for both parameters. Discuss the effect of high/low proportional and integral gain with respect to the image quality versus a reliable height measurement. (see webpages under 'preparation tasks' for a theoretical explanation of PD-controller)

Tip characterization

1. Make an image of a calibration grid to characterize the size of the AFM tip. The calibration grid has a rectangular shape in XY direction. The tickness of the grid wall is about 100 nm in average build on the surface with a triangular shape in XZ direction (see figure below). Calculate the AFM tip-radius using the recorded AFM image of the calibration grid.



Surface quantification

1. Make two AFM-images of a glass surface; one with a scan size of 500 nm and one with a scan size of 10 μm.
2. The same as point 1, but now with a mica-surface.
3. Use the surface parameters shown in table I to quantify the surfaces imaged under 1 and 2 for both the 500 nm and the 10 μm image. Describe and discuss the results. (height DNA is about 2 nm).

Force-Distance Curve

1. Use a mica-surface to make an approach and retract force-distance curve. Discuss the different parts of the curve as e.g. approach of the surface, position of the surface, peaks etc..

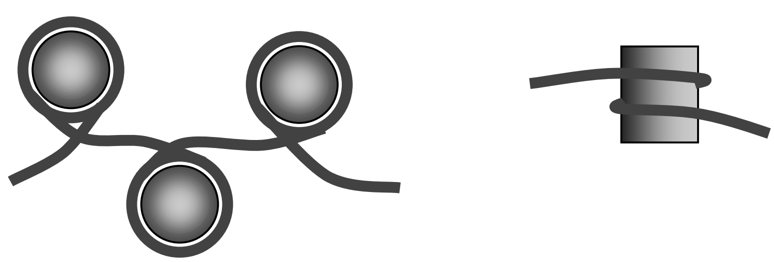
Amplitude/Phase imaging

1. Make an AFM image of the sticky side of a tesa-film. This imaging should include a height image, a phase image and an amplitude image.
2. Describe the differences between these three images (theoretical) and discuss the recorded data with respect to information output.

Biological probes

Chromatin fiber

Make an image of a chromatin fiber (scan size about 1 μm). Chromatin is a DNA fiber compacted by nucleosomes; the DNA is wrapped about 2 times around this protein (see figure below). Determine the size of a nucleosome (radius, height) and estimate the length of the DNA compacted in the recorded chromatin fiber. Discuss these measured sizes.



Human Hair

Make an image of a human hair and determine the height of the steps on it. The period of these steps are about 4 μm.

Cell wand

Make an image of the outside of a cell wand. Make also a force-distance curve on the cell wand and discuss the differences with the one recorded above on mica.

Chromosomes

Search for chromosomes with the optical microscope and make an AFM image of one. Show the height profile of one ‘leg’ of this recorded chromosome.

Own materials

Imaging of your own interesting materials. E.g. paper