



**Institut für
Experimentelle Physik I**

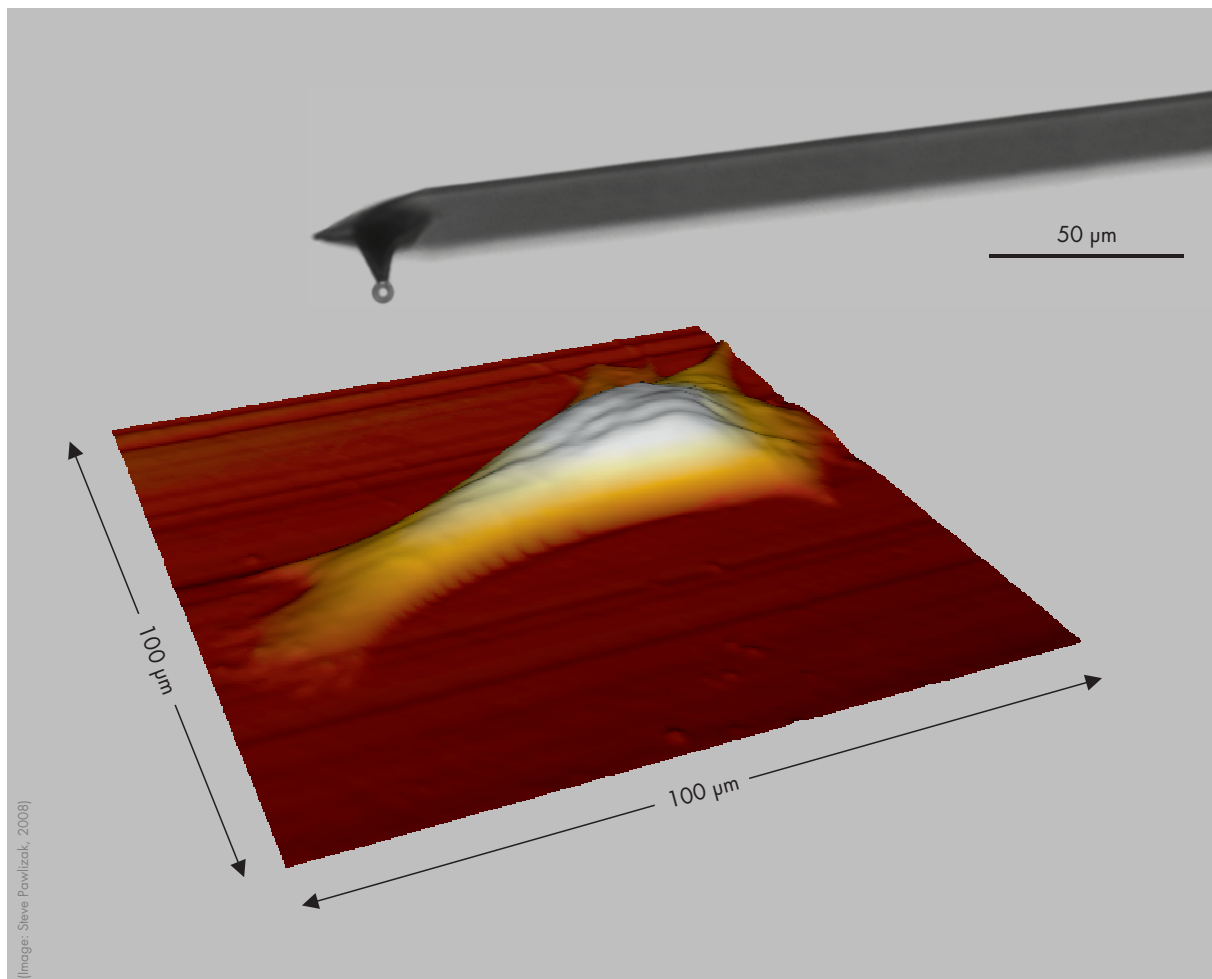
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Physik der weichen Materie
(Soft Matter Physics)
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UNIVERSITÄT LEIPZIG

Fakultät für Physik und Geowissenschaften

Biophysics Lab Course WS 2010/11

Scanning Force Microscopy I: Imaging & Cellular Elasticity



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This lab experiment gives an introduction into scanning force microscopy (SFM) and demonstrates one of its biophysical applications as a tool for studying elastic properties (rheology) of biological cells, especially of their cytoskeleton. The understanding of material properties of cells is an essential precondition to investigate the interplay between mechanics and biochemistry, which regulates cell functions and cell behavior.

Scanning force microscopy – known from surface analysis of solid state bodies – uses the cantilever, a microscopical leaf spring with a tip at its end, to probe a sample on nanometer scale mechanically.

In this lab experiment, the material constants of a cantilever are to be determined first. Subsequently, the local height and elasticity of cells shall be measured. The elasticity will be evaluated using the HERTZ model.

Preparation for this Experiment:

Before starting with the experiments, there will be a short pre-lab test to ensure that you are well prepared. That is why you should:

- read this tutorial thoroughly and additionally you may have a look at the given reference literature.
- inform yourself about cell biological background, especially about components of a cell and the cytoskeleton (e.g. biophysics lecture, www.softmatterphysics.com, Wikipedia).
- inform yourself about phase contrast microscopy.

Bring a USB stick for storing your data that you will collect during your measurements.

Experimental and Analysis Tasks:

Adjust the scanning unit (mounting the cantilever) and approach the cantilever onto the sample.

1. Determine the cantilever's material constants (sensitivity s and spring constant k).
2. Image two cells using phase contrast microscopy and scanning force microscopy. Record height- and error-image. Create 3D views and cross-sections.
3. Determine the tilt angle of the substrate plane for two cells with respect to the horizontal plane ($z = 0$).
4. Determine the local height of two cells at different positions on the cell surface (at least 5 points for each cell).
5. Determine the local elasticity K of two cells at different positions on the cell surface (at least 5 points for each cell). Compare this to the elasticity of the glass surface. If it is reasonable, you might calculate a mean elasticity \bar{K} .
6. Compare your trace and retrace curves. Compare your trace curves on glass to those on the cell surface. Describe the differences and explain where they come from.

To conclude this experiment and to get a grade, you are supposed to hand in a protocol, which should contain the well-known sections: background and theory, experimental techniques, execution of measurements (summary of the experiments you have done), analysis of data, discussion of results and error sources. Include the taken pictures and give detailed figure captions. Since there will not be any final oral examination, your grade will strongly depend on the quality of your protocol, but also on your preparation (pre-lab test) and experimental skills.

1 General Information

The precursor to the scanning force microscope (SFM), the scanning tunneling microscope, was developed by BINNIG and ROHRER in the early 1980s and earned them the Nobel Prize for Physics in 1986. The first SFM was invented by BINNIG, QUATE and GERBER in 1986. The SFM is also known as atomic force microscope (AFM) which refers to the interactions between probe and sample on the atomic level. The attractive Van-der-Waals-forces and repellent electric charges can be described by the LENNARD-JONES potential.

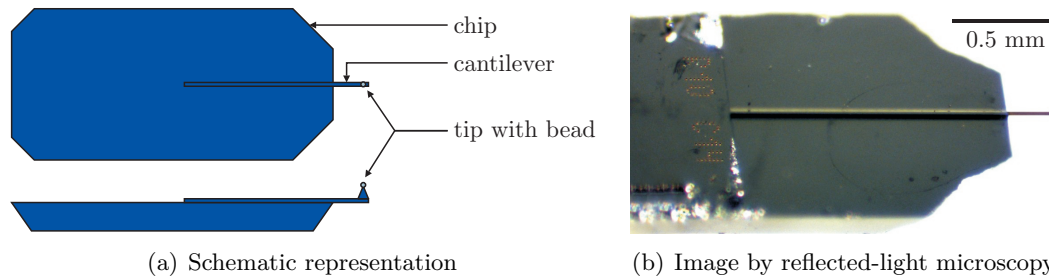


Figure 1: Commercial cantilever, consisting of chip and leaf spring with tip, modified by gluing a 6 μm polystyrene bead onto the tip. (Images taken from diploma thesis of Steve Pawlizak, 2009.)

2 Assembly (JPK NanoWizard® BioAFM)

The core piece of an SFM is a soft elastic leaf spring, the *cantilever* (see front page). It extends from an approximately 4 mm long chip, shown in Fig. 1. At its very end it has a sharp pyramidal tip. As soon as the cantilever comes very close to the sample surface the leaf spring bends. The deflection of the cantilever is detected with high precision by a laser beam which is directed onto the cantilever and reflected onto a four-quadrant photodiode (see Fig. 2). Initially, the laser spot is aligned to the center of the diode. When the cantilever bends, the reflection angle of the laser changes and the spot moves on the diode.

Cantilever, laser and photodiode are mounted on the *SFM scanning unit (SFM head)*. The SFM head is placed on the microscope table over the sample. It can be moved in three dimensions by a set of piezoelectrical elements¹. A *computer* is connected via a *controller box* to the SFM head for adjusting the piezo positions, data collection and analysis.

Our SFM setup, the JPK NanoWizard® BioAFM (JPK Instruments, Berlin), has the great advantage of permitting the simultaneous usage of a variety of light microscopy techniques (e.g. phase contrast) together with the SFM. This is especially helpful for biological research.

To avoid damaging living cells during the measurement and in order to have a well-defined geometrical shape of the probe for the calculation of the elastic moduli, we glue a polystyrene bead ($d = 6 \mu\text{m}$) to the tip of the cantilever.

3 Operation

The SFM operates by measuring attractive or repulsive forces between a tip and the sample. There are two main applications for scanning force microscopy:

- imaging samples with nanometer resolution
- measuring forces in the piconewton regime.

¹A piezoelectric element expands or contracts in direct proportion to an applied electric field.

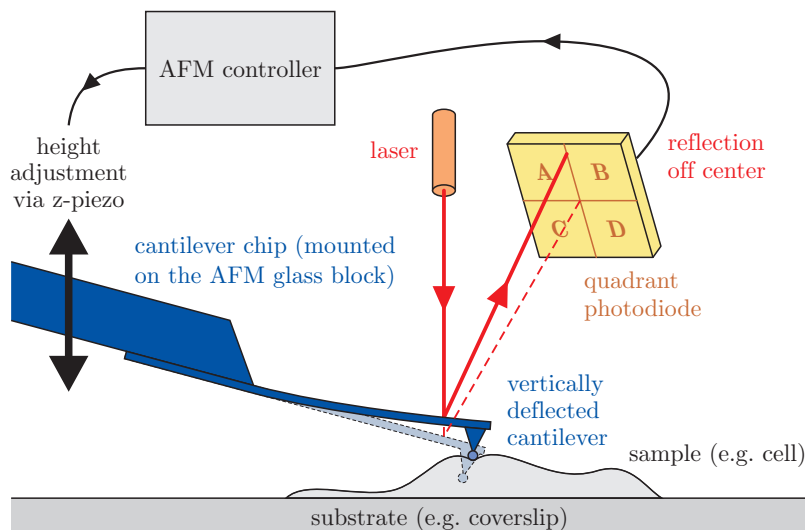


Figure 2: Schematic representation of measuring principle of an SFM: The bending of the cantilever is measured by a laser, whose deflection is detected by a four-quadrant photodiode. Subsequently, the z-piezo corrects the height z_C of the SFM head, which the cantilever is fixed to. (Image taken from diploma thesis of Steve Pawlizak, 2009.)

3.1 Imaging

In its repulsive *contact mode*, the cantilever tip slightly touches the sample (see Fig. 2). The scanning unit is moved as a raster-scan across the sample: two piezos generate the scanning movement of the cantilever, laser and photodiodes in x- and y-direction. While the tip of the cantilever moves over the sample, the cantilever itself bends consistently with the surface; i.e. as the tip is repelled by or attracted to the surface, the cantilever is deflected. The photodiodes register the resulting position changes of the laser reflection. The signal from the photodiodes is processed by a computer, which then sends a signal to the z-piezo, which moves the cantilever up or down to compensate the cantilever deflection. A plot of the laser deflection (error mode) and z-piezo position (height mode) vs. tip position on the sample surface provides the topography of the surface (see Fig. 3).

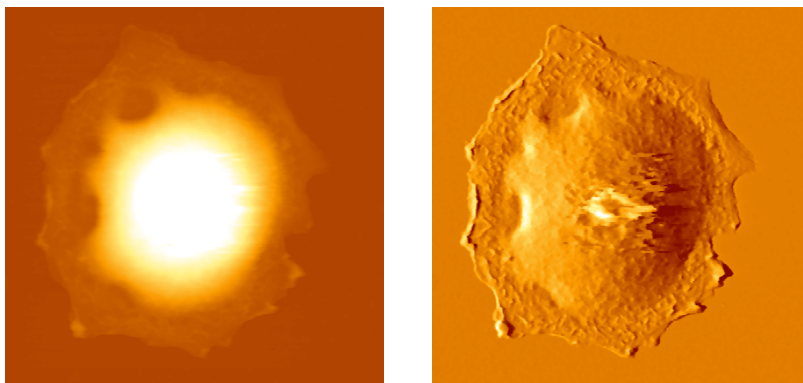


Figure 3: SFM image scan of a rat alveolar type II cell. The height signal (left image) represents the z-piezo position and the error signal (right image) represents the laser deflection.

It is also possible to use further modes for mapping of surfaces, e.g. *tapping mode* (*intermittent contact mode*), *jumping mode* or *force mapping*.

3.2 Force Measurement

In the *spectroscopy mode* the cantilever is pushed into the sample at a certain point and then retracted again. The velocity of the cantilever and the indentation depth can be controlled. During this procedure the height information of the z-piezo and the deflection of the cantilever are recorded. The results are two curves (see Fig. 4), one showing the tip-sample-distance versus the cantilever deflection while approaching the surface (trace, blue line), and the second giving the distance versus deflection while withdrawing the tip from the sample again (retrace, red

line). On a hard surface this gives a characteristic graph. To interpret this, the graph has to be read from right to left, due to the higher cantilever position at the beginning of the measurement.

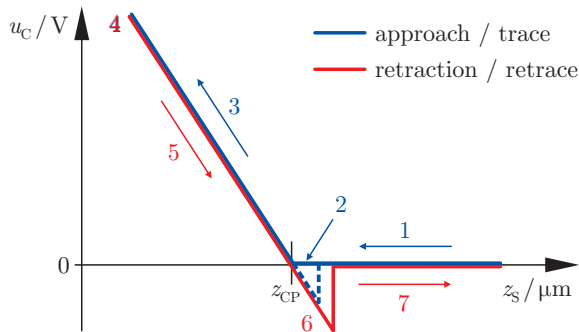


Figure 4: Diagram of an ideal force-distance-curve on a clean and infinite stiff surface. (Image taken from diploma thesis of Steve Pawlizak, 2009.)

Trace (extend): First the cantilever is moved down without touching the sample, i.e. no deflection but a decreasing distance is recorded (1). Very close to the surface the cantilever can suddenly be attracted by the sample due to attractive forces (e.g. electrostatic interaction), i.e. the cantilever flicks down the remaining distance and gives a small downward deflection (2). When the cantilever is moved further down, the cantilever is bent upwards in direct proportion to the z -piezo height (3). This characteristic linear slope can be used for calibration of the cantilever.

Retrace (retract): As soon as a defined setpoint of deflection is reached (4), the cantilever is withdrawn. While moving upwards again, the deflection of the cantilever decreases (5). Passing the contact point, the tip usually stays attached to the surface by adhesion, which causes the cantilever to bend in the opposite direction. At some point it suddenly loses contact and flicks back into its initial position (6). Further retraction causes no change of vertical deflection (7).

4 Analysis (Hertz Model)

The aim of these measurements is to obtain information about elastic or viscoelastic properties of biological cells. A measure for elasticity is the elastic modulus E (Young's modulus). For viscoelasticity storage E' and loss modulus E'' are calculated. To be able to calculate these material constants, a reliable calibration of the cantilever is necessary to calculate the applied force. A tool for determining the spring constant k of the cantilever is provided by the SFM software. The calculation of the moduli is based on a modified HERTZ model.

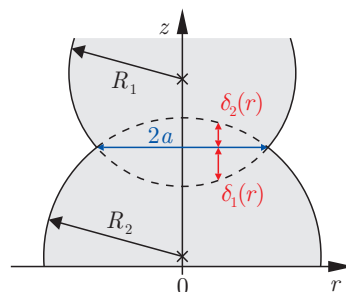


Figure 5: Schematic representation of two elastic spheres (radii R_1 , R_2) touching each other. The contact area has a diameter of $2a$. The local indentations δ_1 , δ_2 depend on the distance r to the center (apex).

The HERTZ model was originally developed to describe the elastic behavior of two spheres in contact (see Fig. 5), and is described in detail by LANDAU and LIFTSHITZ². The used equations

²L. D. Landau, E. M. Lifshitz: *Theory of Elasticity*, Pergamon Press, Oxford (1970).

all assume a static situation. Each sphere has a defined radius R , Young's modulus E and POISSON ratio μ . The POISSON ratio relates shear stress to compression stress. For an isotropic, incompressible medium the POISSON ratio is 0.5. Otherwise it is generally between 0 and 0.5. At the apex of the sphere a maximum pressure p_0 exists. The pressure distribution within the contact area of radius a is given by the following equation.

$$p(r) \approx p_0 \sqrt{1 - \frac{r^2}{a^2}} \quad (a \ll R) \quad (1)$$

The equation above introduces the paraboloid approximation to a sphere. This assumption is generally good when the contact radius a is much less than the total radius of the sphere R . The maximum indentation δ_0 occurs obviously in the center ($r = 0$). Because δ_0 is easily derived from the measured data, it is advantageous to derive functions in terms of δ_0 . One can show that the total force F obeys to following relation.

$$F = \frac{4}{3} \frac{E_1 E_2}{E_2 (1 - \mu_1^2) - E_1 (1 - \mu_2^2)} \sqrt{\frac{R_1 R_2}{R_1 + R_2}} \delta_0^3 \quad (2)$$

For our purposes, this can be simplified since the polystyrene bead has an extremely high elastic modulus E_1 in comparison to the force constant of the cantilever and in comparison to the elasticity $E_2 =: E$ of the sample. Thus, the elasticity E_1 of sphere 1 is then assumed to be infinite. Additionally, the cells are fairly flat meaning that radius R_2 is infinite in comparison to the radius $R_1 =: R$ of the polystyrene bead. Thus the equation relating the indentation to the measured force simplifies to the following:

$$F = \frac{4}{3} \frac{E}{1 - \mu^2} \sqrt{R} \delta_0^3 \quad \Rightarrow \quad K := \frac{E}{1 - \mu^2} = \frac{3}{4} \frac{F}{\sqrt{R} \delta_0^3} \quad (3)$$

A new elasticity constant K was defined, because both elastic modulus E and POISSON ratio μ represent material constants.

The measured data itself provides the scanner's z-piezo height z_S , and the cantilever deflection u_C in volts, which can easily converted into a length value z_C when the cantilever's sensitivity s is known:

$$z_C = s u_C \quad (4)$$

The cantilever deflection is the amount that the tip moves upward in response to the sample pressure.

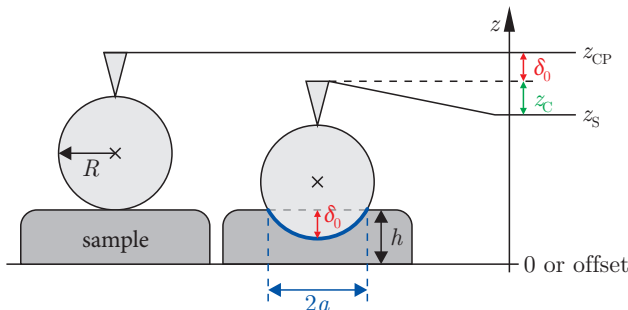


Figure 6: Schematic representation a spherical SFM probe first touching the sample only at one contact point (height of z-piezo z_{CP}) and then indenting the elastic sample. The indentation δ_0 can be calculated with the cantilever deflection z_C , the height z_{CP} of the contact point and the current height z_S of the z-piezo according to eq. (5). (Image taken from diploma thesis of Steve Pawlizak, 2009.)

The indentation δ_0 of the sample can be calculated accordingly fig. 6.

$$\delta_0 = z_{CP} - z_S - z_C \quad (5)$$

where z_{CP} is the contact point, which can be deduced from the data. It is generally defined as the z-piezo height where the slope of the data first changes from zero to some finite value.

According to HOOK's law, the force on the bead is product of the cantilever deflection z_C and the cantilever's spring constant k .

$$F = k z_C \quad (6)$$

Considering eq. (5) and eq. (6), we can rewrite eq. (3) in the following form:

$$K = \frac{3}{4} \frac{k z_C}{\sqrt{R}(z_{CP} - z_S - z_C)^3} \quad (7)$$

The elastic constant K can either be derived by fitting a force vs. indentation curve or by directly calculating the value from the known variables. This second solution provides an easy way to observe the indentation range over which the elastic constant, in fact, remains constant.

Uncertainties in any of the measured values will directly result in errors in the measurement of K . The sample position is measured using detectors on the scanner and thus is reasonably accurate (2–3%). The deflection of the cantilever is measured through a beam bounce method that is first calibrated on a hard surface and thus also results in fairly minor errors (2–3%). The crucial contact point z_{CP} is defined as the point at which the slope of the force curve makes its first abrupt change. This point is not as well-defined as the other two factors and will contribute the most significant error of approximately 5%. The errors in the elastic constant including uncertainties introduced by the calibration of the cantilever are between 15 and 25%.

The equations given above can be used to determine the elastic modulus E (Young's modulus). Plots of the solutions clearly show the range of indentation where the HERTZ model is valid. Occasionally, the HERTZ model is not appropriate and the elastic value predicted by this model changes throughout the whole indentation range. Additionally, the HERTZ model does not describe a dynamic system with viscous properties. Thus, a large part of the cell response (cytoplasmic viscosity) is completely ignored when using only this analysis. A first step is to extend the HERTZ model to accurately probe the viscous response of the cells as a function of the frequency. Furthermore, the effect of having thin samples must be considered as the HERTZ model assumes large spheres and small indentations.

References:

- JPK Instruments AG: *Tutorial for Scanning Probe Microscopy in Life Science*, http://www.jpk.com/tutorial/afm_principle.htm (2007)
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- E. Meyer, H. J. Hug, R. Bennewitz: *Scanning Probe Microscopy – The Lab on a Tip*, Springer, Berlin, Heidelberg, New York (2003).
- V. J. Morris, A. R. Kirby, A. P. Gunning: *Atomic force microscopy for biologists*, Imperial College Press, London (1999).

5 Hints for Data Analysis

5.1 Light Microscopy Images

Microscope images (*.jpg) taken by a CCD camera are meaningless without a scale bar which indicates the size of the shown objects. To include a scale bar, the actual magnification or resolution of the used imaging assembly has to be determined. This can be done by taking a reference image of a microscope slide with 10 μm scale (grating with 2 mm total length and 0.01 mm interval width) using the very same combination of camera, objective and camera mount. Afterwards, the corresponding number of pixels in the graphic file is counted with any image processing software.

5.2 SFM Image Scans

SFM image scans are saved in a proprietary JPK file format (*.jpk) and have to be post-processed to create common graphic files like JPEG (*.jpg). After your experiments you will have time to create different cross-sections and 3d views using the “JPK SPM Data Processing” software. If you want to edit your JPK files at home, you could use “Gwyddion”, which is an open source software for SPM data visualization and analysis. Windows- and Linux-versions can be downloaded for free from <http://gwyddion.net/>.

Do not forget to include some important scan parameters (IGain, PGain, setpoint, line rate, scan size, scan resolution in pixel) into your protocol.

5.3 Force-Distance-Curves

Force-distance-curves are saved in a proprietary JPK file format (*.jpk-force). Using the batch script *jpk-force-legacy-export*, they must be converted into plain ASCII text files (*.out) which can be imported into any scientific graphing and data analysis software you like (e.g. ORIGIN). Each text file has two sections, first one with the data of the trace curve and the second one with the data of the retrace curve. You can use the *splitforcefile* script to create separate text files for trace and retrace curves. For your analysis, the columns “height (measured & smoothed)” and “vertical deflection” of the trace curves are of interest only.

Every output file has a defined header containing some important parameters of the related force-distance-curve like relative setpoint, index and x - y -position. Especially, relative setpoint, z -length and scan time should be mentioned in your protocol. [Tab. 1](#) shows an example header.

5.4 Evaluation Steps

A. Find the contact points of the glass curves

1. Plot “vertical deflection” u_C vs. “height (measured & smoothed)” z_S .
2. Remove the offset in the vertical deflection u_C .
3. Plot offset-corrected “vertical deflection” u_C vs. “height (measured & smoothed)” z_S .
4. Fit the linear part of the curve and determine the point of intersection with the height-axis. This gives you the z -component of your position vector \vec{r}_i .
5. Determine the plane through three contact points and calculate its tilt angle towards the normal plane with $\vec{n} = (0, 0, 1)$.

B. Determine the local cell heights

1. Plot “vertical deflection” u_C vs. “height (measured & smoothed)” z_S .
2. Remove the offset in the vertical deflection u_C .
3. Plot offset-corrected “vertical deflection” u_C vs. “height (measured & smoothed)” z_S .
4. Read contact point from the graph. This gives you the z -component of your position vector \vec{r}_i .
5. Now you have the substrate plane and some cell surface vectors. Calculate the the distance between the cell surface and the plane.

C. Determine the local cell elasticities

1. Convert the height-axis into indentation δ_0 using [equ. \(5\)](#) and convert the vertical deflection into force F using [equ. \(6\)](#).
2. Plot $F^{2/3}$ vs. δ_0 to see if you have a linear range where the HERTZ model applies.
3. To determine K either fit the linear range in $F^{2/3}$ - δ_0 -diagram or fit the HERTZ model to the curve in F - δ_0 -diagram.
4. Additionally, you may plot K vs. δ_0 using [equ. \(3\)](#) to check in which range K actually remains constant during the indentation.

For each evaluation step, you should include the graphs with fittings and the equations for calculations into your protocol. It is not sufficient to state only the final results (tilt angle, height and elasticity).

```

# xPosition: 5.275310834813498E-6
# yPosition: -2.7797513321492007E-6
# index: 6
# approachID: 2008.05.28-14.04.45-00008
# forceSettings: relative
# zRelativeStart: 5.0E-7
# zRelativeEnd: 0.0
# setpoint: 2.0
# kLength: 1024
# zStartPauseOption: constantHeight
# zEndPauseOption: constantHeight
# tipsaverPauseOption: constantHeight
# feedbackMode: contact
# traceScanTime: 0.5
# retraceScanTime: 0.5
# pauseBeforeFirst: 0.0
# pauseAtStart: 0.0
# pauseAtEnd: 0.0
# pauseOnTipsaver: 0.0
# scanner: false
# date: Wed May 28 14:24:41 CEST 2008
#
# direction: trace
# columns: smoothedStrainGaugeHeight
vDeflection strainGaugeHeight height
hDeflection
# fancyNames: "Height (measured & smoothed)"
"Vertical deflection" "Height (measured)"
"Height" "Lateral deflection"
# heightMultiplier: 0.594612
# heightOffset: 0.0
# calibrationSlots: nominal volts nominal
nominal volts
# units: m V m m V
5.2247146E-6 -0.34344074 5.2249043E-6
4.499199E-6 0.7008103 9.539653
...

```

x-coordinate of the point where the curve was taken, in meters.

y-coordinate of the point where the curve was taken, in meters.

This index should correspond to the point no. in the SFM image scan.

Start–End gives the *z*-Length, which you set in the parameters, in meters.

Maximum vertical deflection, in volts. The cantilever bends until its vertical deflection reaches this setpoint you chose.

This is the approximate number of lines in your trace and retrace data tables.

Extend time in seconds. If you tried different trace scan times, you could investigate a correlation between indentation velocity and measured elasticity (optional).

Retract time in seconds.

Indicates that the following data table belongs to the trace-curve. The retrace data is the second part of the file and has an additional header. Use only the trace data for your evaluation.

These are the internal column names in a non-fancy way. Just ignore them. (The columns themselves are separated by spaces.)

These are the “real” column names. You need “height (measured & smoothed)” and “vertical deflection”, the first and second column, only. Note that the height decreases, because the *z*-piezo moves downwards. (The columns themselves are separated by spaces.)

These are the units of the columns. (The columns themselves are separated by spaces.)

End of header. The data of the trace-curve starts here. (The columns themselves are separated by spaces.)

Table 1: Example header of an output file of a force-distance-curve (*.out) with descriptions.