



**Institut für  
Experimentelle Physik I**

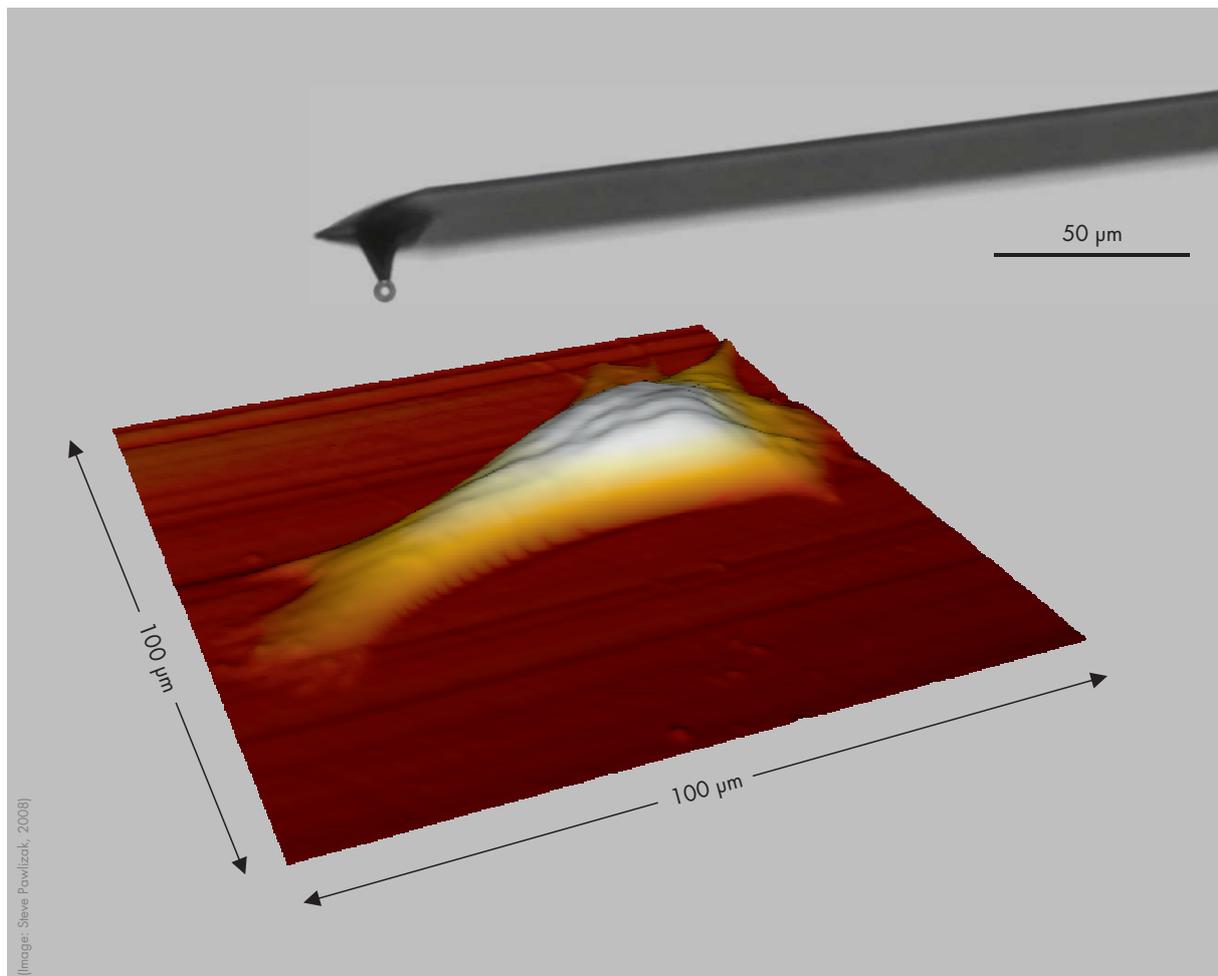
Abteilung:  
Physik der weichen Materie  
(Soft Matter Physics)  
Prof. Dr. Josef A. Käs

**UNIVERSITÄT LEIPZIG**

Fakultät für Physik und Geowissenschaften

Biophysics Lab Course WS 2012/13

# Scanning Force Microscopy: 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](http://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.

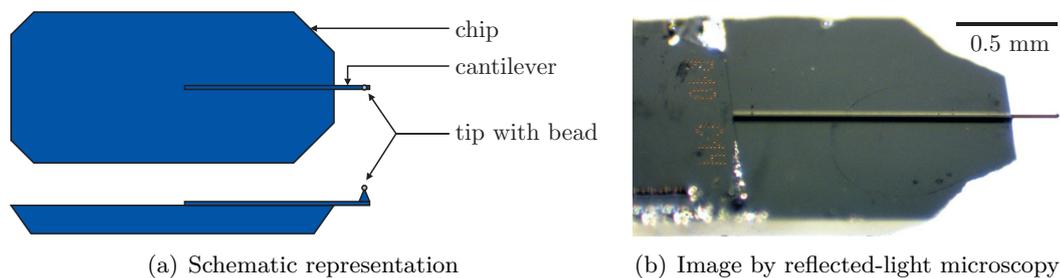
1. Determine the cantilever's material constants (sensitivity  $s$  and spring constant  $k$ ).
2. Image two cells using phase contrast microscopy and scanning force microscopy. Record height- and error-image. Create 3D views and cross-sections.
3. Determine the tilt angle of the substrate plane for 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, materials and method (experimental techniques), execution of measurements (summary of the experiments you have done), data analysis and 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 (PAULI-repulsion) can be described by the LENNARD-JONES potential. There are two main applications for scanning force microscopy:

- imaging samples with up to nanometer resolution (see [section 3](#))
- measuring forces in the piconewton regime (see [section 4](#))



**Figure 1:** Commercial cantilever, consisting of chip and leaf spring with tip, modified by gluing a 6  $\mu\text{m}$  polystyrene bead onto the tip. (Images taken from diploma thesis of Steve Pawlizak, 2009.)

## 2 Assembly (JPK NanoWizard® BioAFM)

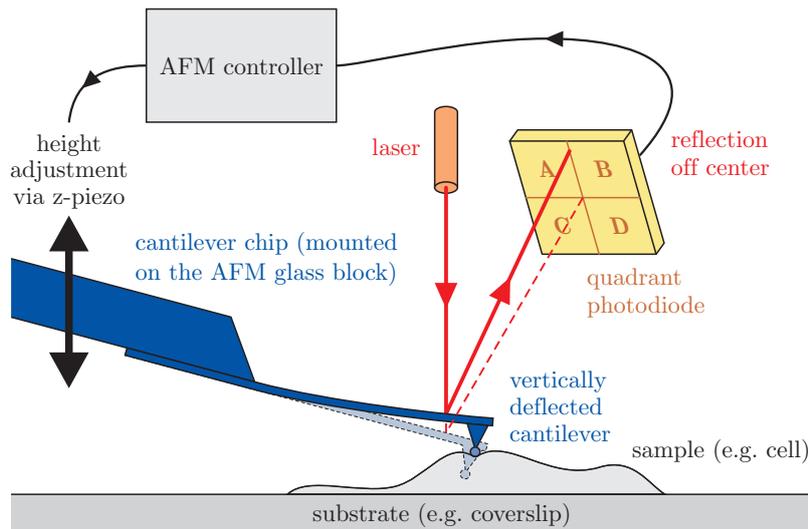
The core piece of an SFM is a soft elastic leaf spring, the *cantilever* (see front page). It extends from an approximately 4 mm long chip, shown in [Fig. 1](#). At its very end it has a sharp pyramidal tip. As soon as the cantilever comes very close to the sample or substrate, the leaf spring bends, i.e. the tip is moved upward or downward due to its interaction with the sample. This bending is detected with high precision by a *laser beam* which is directed onto the cantilever and reflected onto a position-sensitive *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 laser spot moves on the diode, which is measured as a change of the photo voltage called *deflection*  $u_C$  of the cantilever.

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 elements*<sup>1</sup>. 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.

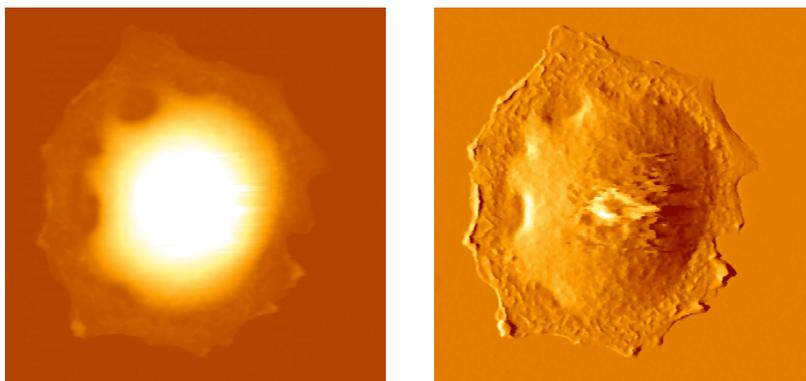
<sup>1</sup>A piezoelectric element is a device made from a material (crystals or ceramics) that expands or contracts in direct proportion to an applied electric field. They are used for precise movement and positioning.



**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 ( $u_C$ ). Subsequently, the z-piezo corrects the height  $z_S$  of the SFM head to which the cantilever is fixed. (Image taken from diploma thesis of Steve Pawlizak, 2009.)

### 3 Imaging

In *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 piezoelectric elements generate the scanning movement of cantilever, laser, and photodiode in x- and y-direction. While the tip of the cantilever is moved over the sample, the cantilever itself bends continuously with the surface topography as the tip is repelled by or attracted to the surface. The current cantilever deflection is detected by the diode as a voltage. Now there are two possible operational modes: In the so-called *constant force mode*, the signal from the diode 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. I.e. a feedback loop keeps the force acting on the cantilever more or less constant during the whole scan by continuously adjusting the z-piezo height. Plots of the laser deflection (error signal) and z-piezo height (height signal) vs. tip's x- and y-position over the sample surface provide topographic images 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.

In *constant height mode*, as the name implies, the height of the z-piezo is kept constant during the whole scan and only the changing cantilever deflection is recorded. However, if there is an obstacle too high, the cantilever may break because there is no feedback loop compensating the deflection.

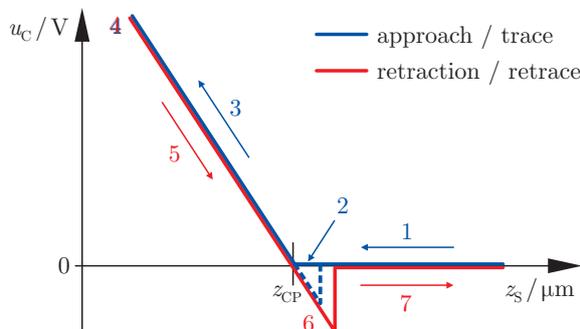
Instead of using the repulsive static *contact mode* described above, there are other dynamic imaging modes that are more suitable for gently scanning softer samples, such as *non-contact mode*, *intermittent contact mode (tapping mode)*, or *jumping mode*.

## 4 Force Measurement

### 4.1 Force-Distance-Curves

In *force spectroscopy mode*, the cantilever is pushed onto/into the sample or substrate at a certain point and then retracted again. During this procedure, the height  $z_S$  of the z-piezo and the deflection  $u_C$  of the cantilever are continuously recorded. The velocity of z-piezo and the setpoint of the maximum cantilever deflection (and, thus, the maximum applied force) can be controlled.

As a result, one gets a so-called *force-distance-curve* or *force-distance-plot* (even though the  $u_C$ -axis is not yet converted into the corresponding force  $F$ ). In Fig. 4 such a plot is shown for the case that the cantilever is pushed onto a hard substrate, such as a glass microscope slide or plastic petri dish. As one can see, such a plot consists of two parts, one showing the cantilever deflection while approaching the surface (*trace*, blue line), and the second while withdrawing the tip from the sample again (*retrace*, red line). 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 infinitely 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 deflection (7).

On soft viscoelastic samples like biological cells, a different behavior of the force-distance-curves has to be expected (e.g. different slope, non-linear curve) as the curve also contains the response of the sample due to the increasing and decreasing load. Hence, certain material properties of the sample can be derived from such a curve.

### 4.2 Force Calibration

The measured raw data itself provides just the scanner's z-piezo height  $z_S$  and the cantilever deflection  $u_C$  in volts. However, as a cantilever is essentially a linear-elastic leaf spring being bent under load, it is indeed possible to convert the deflection  $u_C$  into a corresponding force  $F$

using only two material constants.

Recording a force-distance-curve on a substrate that appears to be infinitely hard in comparison to the bending stiffness of the cantilever produces a characteristic graph, as shown in Fig. 4. This graph describes only the deflection behavior of the cantilever depending on the movement of the z-piezo, and it does not contain any information about the substrate itself. In fact, the linear relationship can be used to determine a material constant called the *sensitivity*  $s$  of the cantilever:

$$s := -\frac{1}{\text{slope}} = -\frac{\Delta z_S}{\Delta u_C} \quad [s] = \frac{\text{nm}}{\text{V}} \quad (1)$$

Knowing the sensitivity  $s$ , one can easily convert the cantilever deflection  $u_C$  into a corresponding length value:

$$z_C = s u_C \quad (2)$$

In the range of linear-elastic bending of the cantilever, HOOK's law is valid, relating the length  $z_C$  to the applied force  $F$  via the cantilever's *spring constant*  $k$ :

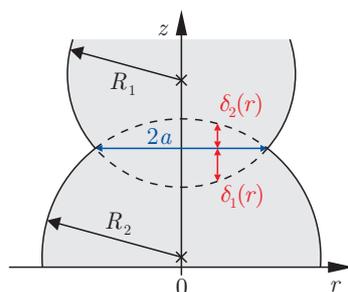
$$F = k z_C \quad [k] = \frac{\text{mN}}{\text{m}} \quad (3)$$

However, a reliable calibration of  $k$  is necessary to calculate  $F$ . There are several methods to do this. One approach is to calculate a theoretical spring constant based on the exact dimension of the leaf spring measured by electron microscopy and material properties. Another approach is to push the cantilever against very stiff substrate and then against a softer known reference spring. The unknown  $k$  is then calculated by rule of proportion from the ratio of the according sensitivity values. A third approach is based on adding masses to the cantilever and measure its deflection due to gravity.

Our method of choice is the so-called *thermal noise technique* which is already implemented into our SFM software and fully automated. In brief, the software measures the thermal fluctuations of the free cantilever over time. Fourier transformation of the noise data yields the power spectrum showing the resonance peak of the cantilever. This peak is fitted with a Lorenz curve. The area under this curve corresponds to  $\langle z^2 \rangle$  which is proportional to the energy  $E = \frac{1}{2}k \langle z^2 \rangle$  of a harmonic oscillator. According to the equipartition theorem, this energy is equal to the thermal energy  $E = \frac{1}{2}k_B T$ . With these two equations, the spring constant  $k$  can be calculated.

## 5 Analysis (Hertz Model)

In our case, the main reason for doing force measurements is to obtain information about the elastic (or viscoelastic) properties of biological cells. A measure for elasticity is the elastic modulus  $E$  (Young's modulus). The so-called HERTZ model is commonly used to derive  $E$  from SFM indentation experiments.



**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  $\delta_1, \delta_2$  depend on the distance  $r$  to the center (apex). (Image by Steve Pawlizak, 2009.)

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 LIFSHITZ<sup>2</sup>. The used equations all assume a static situation. Each of the two spheres has a defined radius  $R$ , Young's modulus  $E$  and POISSON ratio<sup>3</sup>  $\mu$ . 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.

$$p(r) \approx p_0 \sqrt{1 - \frac{r^2}{a^2}} \quad (a \ll R) \quad (4)$$

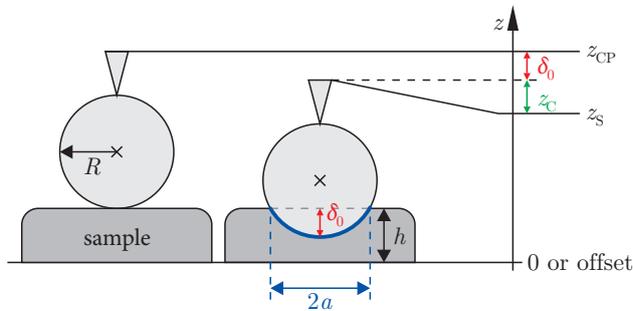
The equation above introduces the paraboloid approximation to a sphere. This assumption is generally good when the contact radius  $a$  is much less than the total radius of the sphere  $R$ . The maximum indentation  $\delta_0$  occurs obviously in the center ( $r = 0$ ). Because  $\delta_0$  is easily derived from the measured SFM data, it is advantageous to derive functions in terms of  $\delta_0$ . One can show that the total force  $F$  obeys to following relation:

$$F = \frac{4}{3} \frac{E_1 E_2}{E_2 (1 - \mu_1^2) - E_1 (1 - \mu_2^2)} \sqrt{\frac{R_1 R_2}{R_1 + R_2}} \delta_0^3 \quad (5)$$

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:

$$F = \frac{4}{3} \frac{E}{1 - \mu^2} \sqrt{R} \delta_0^3 \quad \Rightarrow \quad K := \frac{E}{1 - \mu^2} = \frac{3}{4} \frac{F}{\sqrt{R} \delta_0^3} \quad (6)$$

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



**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  $\delta_0$  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. (7). (Image taken from diploma thesis of Steve Pawlizak, 2009.)

The indentation  $\delta_0$  of the sample can be calculated accordingly Fig. 6.

$$\delta_0 = z_{CP} - z_S - z_C \quad (7)$$

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.

Considering eq. (3) and eq. (7), we can rewrite eq. (6) in the following form:

$$K = \frac{3}{4} \frac{k z_C}{\sqrt{R} (z_{CP} - z_S - z_C)^3} \quad (8)$$

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

<sup>3</sup>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. For biological materials, it is typically between 0.3 and 0.5.

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 constant  $K$  (or the elastic modulus  $E$  when the POISSON ratio  $\mu$  is known). Plots of the solutions 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. This can be due to the fact that the theory assumes the indented material to be perfectly elastic, isotropic, and homogeneous, which is clearly not the case for biological cells. Furthermore, the classical HERTZ model does not account for sample thickness and assumes infinitely thick samples (i.e. relatively large sample thickness in comparison to small indentations). For thin samples, however, substrate effects might not be neglected anymore.

Additionally, the basic HERTZ model does not describe a dynamic system having viscous properties. Thus, a large part of the cell response (cytoplasmic viscosity) is completely ignored. Nevertheless, the HERTZ model can be extended to accurately describe the viscoelastic response of cells to an oscillatory deformation with the frequency  $f$ . In this case, a frequency-dependent complex elastic modulus  $E^*(f) = E' + iE''$  is introduced, where  $E'$  is the storage modulus expressing the elastic response and  $E''$  is the loss modulus expressing the viscous response.

## References:

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## 6 Hints for Data Analysis

### 6.1 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  $\mu\text{m}$  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.

### 6.2 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.

### 6.3 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.

### 6.4 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  $\delta_0$  using [equ. \(7\)](#) and convert the vertical deflection into force  $F$  using [equ. \(3\)](#).
2. Plot  $F^{2/3}$  vs.  $\delta_0$  to see if you have a linear range where the HERTZ model applies.
3. To determine  $K$  either fit the linear range in  $F^{2/3}$ - $\delta_0$ -diagram or fit the HERTZ model to the curve in  $F$ - $\delta_0$ -diagram.
4. Additionally, you may plot  $K$  vs.  $\delta_0$  using [equ. \(6\)](#) 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.