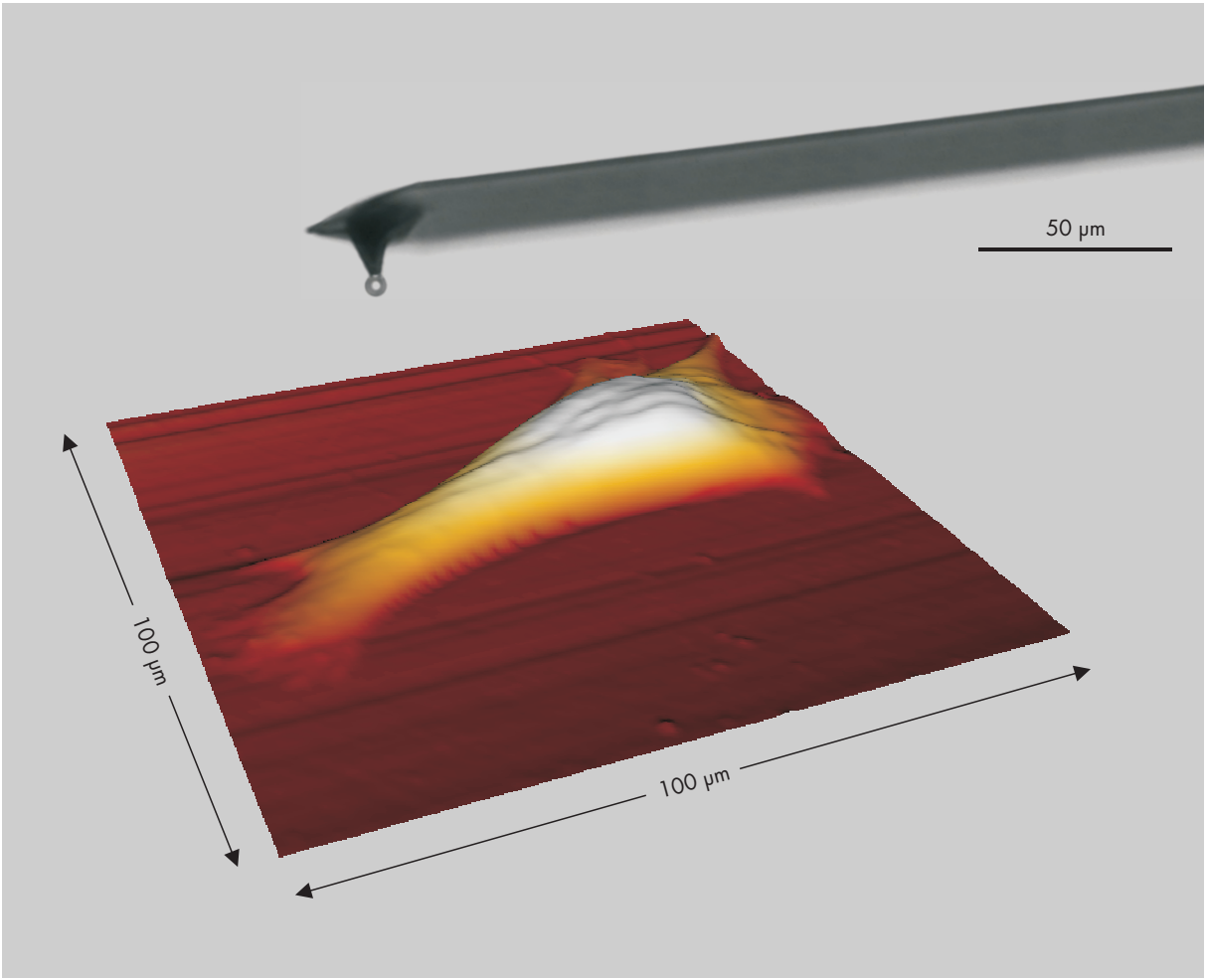
Institut für
Experimentelle Physik I
Abteilung:
Physik der weichen Materie
(Soft Matter Physics)
Prof. Dr. Josef A. Käs

UNIVERSITÄT LEIPZIG
Fakultät für Physik und Geowissenschaften

Biophysics Lab Course WS 2008/09

Atomic Force Microscopy (AFM) on Biological Samples



Institut für
Experimentelle Physik I
Linnéstraße 5
04103 Leipzig
Fax: (0341) 97 32479

Tutors:

Claudia Brunner
Zimmer: 327
Telefon: (0341) 97 32561
E-Mail: cbrun@physik.uni-leipzig.de

Steve Pawlizak
Zimmer: 132
Telefon: (0341) 97 32713
E-Mail: pawlizak@web.de

This lab experiment is intended to give an introduction into atomic force microscopy (AFM) and to demonstrate its biophysical application, in particular as a tool for examination of elastic properties (rheology) of biological cells, especially of the cytoskeleton. The understanding of material properties of cells is an essential precondition to eventually discover the interplay between mechanics and biochemistry, which regulates cell functions and cell behaviour.

Atomic force microscopy – known from surface analysis of solid state bodies – uses a microscopical leaf spring, the so called cantilever, with a sharp tip at its end to probe a sample on nanometer scale mechanically. For biophysical application we modify cantilevers by gluing a small polystyrene bead onto the tip to avoid damage of cells and in order to have a well defined stress profile.

In this lab experiment the material constants of a cantilever are to be measured first and later on the local height of cells in respect to their seating. Furthermore local elasticity of cells shall be determined 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 compartments of a cell and the cytoskeleton (e.g. biophysics lecture, www.softmatterphysics.com, Wikipedia).

Experimental tasks:

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

1. Image a cell using phase contrast microscopy and atomic force microscopy. Record height- and error-image and create a 3d image and cross sections.
2. Determine the cantilever's material constants (sensitivity s and spring constant k).
3. Determine the local height of a two cells at different positions on the cell surface.
4. Determine the local elasticity $\frac{E}{1-\mu^2}$ of two cells at different positions on the cell surface. Compare this to the elasticity of the glass surface.
5. Compare your trace and retrace curves on glass and cell surface. Discribe 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, analysis of data, discussion of results and error sources. It is sufficient that each group prepares one protocol.

1 Atomic Force Microscopy

The precursor to the atomic force microscope (AFM), the scanning tunneling microscope, was developed by Binnig and Rohrer in the early 1980s und earned them the Nobel Prize for Physics in 1986. The first AFM was invented by Binnig, Quate and Gerber in 1986. The AFM got its name from 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.

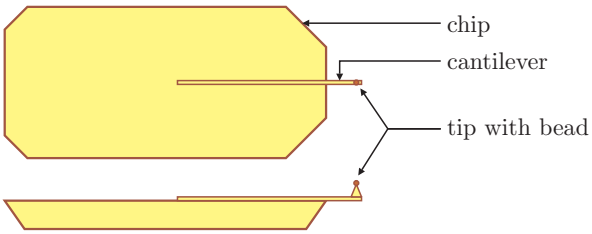


Figure 1: Schematic representation of commercial cantilever, consisting of chip and leaf spring with tip, modified by gluing a polystyrene bead ($d = 6\,\mu m$) onto the tip.

1.1 Assembly (JPK NanoWizard® AFM)

The core piece of an AFM is a soft elastic leaf spring, the cantilever. It is being held by an approximately 5 mm long chip, shown in Fig. 1. At its very end a pyramid shaped sharp tip is mounted perpendicular to the cantilever axis. To avoid damage of living cells during the measurement and in order to have a clearly defined geometrical shape of the probe for the following calculation of the moduli, we glue a polystyrene bead ($d = 6\,\mu m$) to the tip of the cantilever. The chip can be fixed to a glass block, which is part of the AFM scanning unit (AFM head), which makes it possible to use a variety of light microscopy techniques (e.g. phase contrast) together with the AFM-technique. In addition to the glass block, an infrared laser and a four-quadrant photodiode comprise the scanning unit (see Fig. 2).
The laser beam is reflected at an oblique angle from the very end of the cantilever to the position-sensitive photodiode. The head can be moved in three dimensions by a set of piezoelectrical elements[1]. A computer is connected via a signal access box to the photodiode and the piezos.

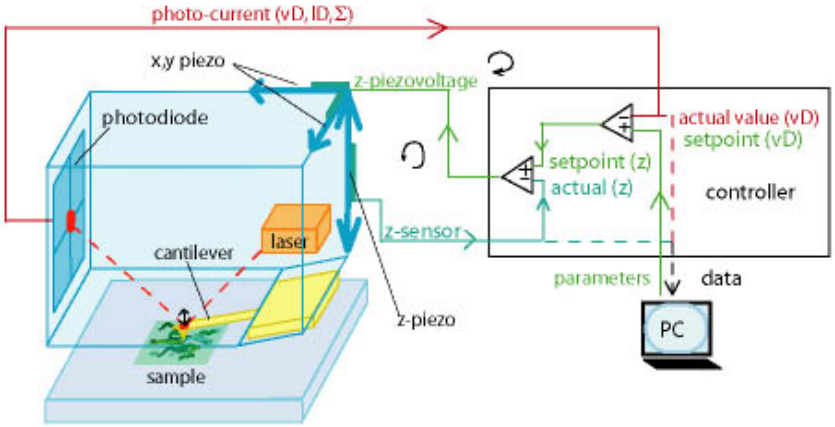


Figure 2: Schematic representation of our AFM setup (JPK NanoWizard® AFM), consisting of the scanning head, which is placed on the microscope table over the sample, a controller box for adjusting the piezo positions, and a PC for data collection and analysis.

1.2 Operation

The AFM operates by measuring attractive or repulsive forces between a tip and the sample. There are two main applications for Atomic Force Microscopy:

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

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

1.2.1 Imaging

In its repulsive *contact mode*, the cantilever tip slightly touches the sample. 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).

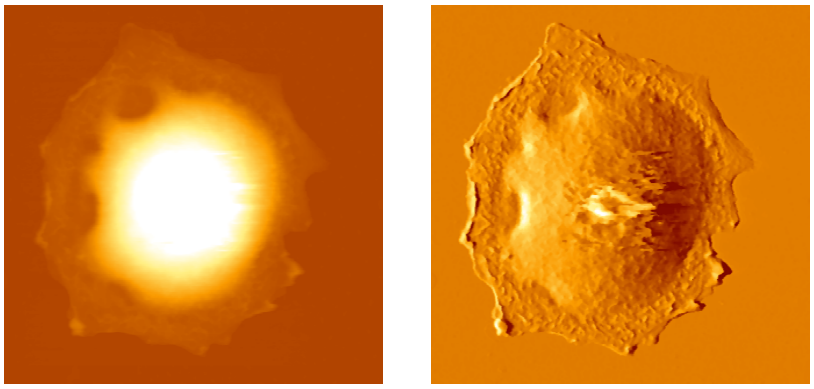


Figure 3: AFM image scan of a rat alveolar type II cell. The height signal (left image) represents the z-piezo position and the error signal (right image) represents the laser deflection.

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

1.2.2 Force Measurement

In the so-called *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.

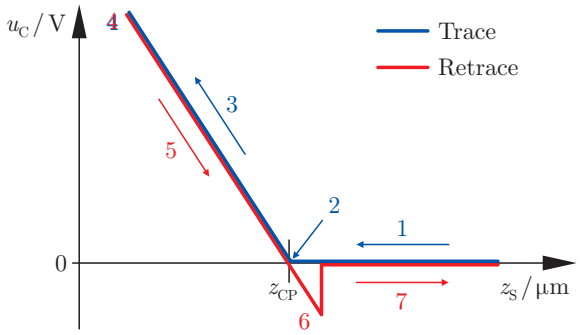


Figure 4: Diagram of an ideal force-distance-curve on a clean and infinite stiff surface.

Trace (extend): First the cantilever is moved down without touching the sample, i.e. no deflection but a declining distance is measured. Very close to the surface the cantilever suddenly is attracted by the sample, i.e. flicks down the remaining distance and gives a small downward deflection. When the cantilever is moved further down the cantilever is bent upwards.

Retrace (retract): First the cantilever is more and more unbent, while the cantilever is moved up again. Then the tip stays attracted to the surface by adhesion, which causes the cantilever to bend in the opposite direction until it suddenly loses contact and flicks up into a horizontal position. Further retraction no longer results in vertical deflection.

1.3 Analysis

The aim of these measurements is to figure out some 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 feature for determining the spring constant k of the cantilever is provided by the AFM software. The calculation of these moduli is based on a modified Hertz model (see section 2).

References:

- H. Hertz: *Über die Berührung fester elastischer Körper*, Journal für die reine und angewandte Mathematik 92, 156–171 (1881)
- R. E. Mahaffy, C. K. Shih, F. C. MacKintosh, J. Käs: *Scanning probe-based frequency-dependent microrheology of polymer gels and biological cells*, Physical Review Letters 85, 880–883 (2000)
- R. E. Mahaffy, S. Park, E. Gerde, J. Käs, C. K. Shih: *Quantitative analysis of the viscoelastic properties in thin regions of fibroblasts using AFM*, Biophysical Journal 86, 1777–1793 (2004)
- JPK Instruments AG: *Tutorial for Scanning Probe Microscopy in Life Science*, http://www.jpk.com/tutorial/afm_principle.htm (2007)
- G. Binnig, C. F. Quate, C. Gerber: *Atomic force microscope*, Physical Review Letters 56:930-933 (1986).
- 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).

2 Hertz Model

(taken from Thesis of Rachel Elaine Mahaffy, 2000, slightly modified)

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 Liftshitz[2]. This model was

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

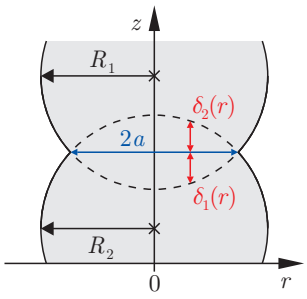


Figure 5: Schematic representation of two elastic spheres (radii R_1, R_2) touching eachother. The contact area has a diameter of $2a$. The local indentations δ_1, δ_2 depend on the distance r to the center (apex).

further refined to include other shapes by SNELDON[3]. The equations used 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 exists within the contact area of the two spheres that has a radius of a is given by the following equation.

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

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 total force F, a measured quantity, is the surface integral over the pressure distribution within the contact area.

$$F = \int_A p \, \mathrm{d}A = 2\pi \int_0^a p(r)\, r \, \mathrm{d}r \tag{2}$$

The indentation δ at a certain distance r is related to the surface integral of the pressure distribution with a constant prefactor related to the elastic modulus E and POISSON ratio μ.[4]

$$\delta(r) = \frac{1-\mu^2}{\pi\, E} \frac{F}{r}(s,\vartheta) = \frac{1-\mu^2}{\pi\, E} \int_A p(s,\vartheta)\, \mathrm{d}s\, \mathrm{d}\vartheta \tag{3}$$

$$p(s,\vartheta) = p_0 \sqrt{1 - \frac{r^2 + s^2 + 2\, r\, s \cos\vartheta}{a^2}}$$

The difficult integration is too long to be shown here in detail. The result is

$$\delta(r) = \frac{1-\mu^2}{E} \frac{\pi\, p_0}{4\, a} \left(2a^2 - r^2\right) \tag{4}$$

The maximum indentation δ_0 occurs where $r = 0$. Because δ_0 is easily derived from the data, it is advantageous to derive functions in terms of δ_0. The function δ can be used to determine the contact radius as a function of δ_0. The force in terms of indentation δ_0 at the center is given by the following equation.

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

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

[3] I. N. Sneddon: *The relation between load and penetration in the axisymmetric boussinesq problem for a punch of arbitrary profile*, International Journal of Engineering Science, Volume 3, Issue 1, 47–57 (1965).
[4] K. L. Johnson: *Contact Mechanics*, Cambridge University Press, Cambridge (1985).

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.

$$F = \frac{4}{3} \frac{E}{1-\mu^2} \sqrt{R\,\delta_0^3} \tag{6}$$

The measured data itself provides the scanner displacement z_S (z-piezo height), and the cantilever deflection u_C in Volts, which can easily converted into a length value $z_C = s\,u_C$ (s...cantilever's sensitivity). The contact point z_{CP} can be deduced from the data. It is generally defined as the point where the slope of the data first changes from zero to some finite value. The scanner displacement z_S is the distance that the sample moves upwards into the tip. The cantilever deflection z_C is the amount that the tip moves upward in response to the sample pressure. Thus, the indentation δ_0 of the sample is given by the following equation.

$$\delta_0 = z_S - z_C - z_{CP} \tag{7}$$

The force on the bead is product of the cantilever deflection z_C and the cantilever's spring constant k. Both of these values are calibrated for accuracy using the procedures discussed in the previous chapter.

$$F = k\,z_C \tag{8}$$

The elastic modulus E 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 distance over which the elastic constant, in fact, remains constant. Small adjustments in z_{CP} can lead to a more accurate constant value and the results of such a minor adjustment are quickly evident in the values of E.

$$\frac{E}{1-\mu^2} = \frac{3}{4} \frac{k\,z_C}{\sqrt{R\,(z_S - z_C - z_{CP})^3}} \tag{9}$$

Uncertainties in any of these values will directly result in errors in the measurement of E. 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 show clearly the range of indentation over which the Hertz model is valid, producing a constant. Occasionally, the Hertz model is not appropriate and the elastic value predicted by this model does not remain constant over all indentations. Additionally, the Hertz model does not accurately 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. The primary step is to extend the Hertz model to accurately probe the viscous response of the cells as a function of the frequency. Then, the effect of having thin samples must be investigated in order to ascertain the primary reasons for deviations from the Hertz model at large indentation depths for some regions of the cell.