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Biophysics Lab Course WS 2010/11

Scanning Force Microscopy II: Cellular Adhesion Forces

(Images taken from JPK CellHesion 200 Brochure, JPK Instruments AG, Berlin 2008)

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This lab experiment is based on the lab experiment entitled "Scanning Force Microscopy I: Imaging & Cellular Elasticity" and demonstrates further applications of force microscopy in the field of biophysics, in particular for studying cellular adhesion.
In this expermiment, a cantilever has to be functionalized with a certain protein that cells bind to. Then the material constants of the cantilever are to be determined. Subsequently, the maximum adhesion forces between two cells as well as between cells and the substrate shall be measured.

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:

- thoroughly read sections 1 to 3 of the tutorial for the experiment "Scanning Force Microscopy I: Imaging & Cellular Elasticity" for an introduction into the general working principle of scanning force microscopy.
- thoroughly read this tutorial 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:

1. Coat the cantilever with 10 $\frac{\text{mg}}{\text{ml}}$ fibronectin to allow cell attachment.

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

2. Determine the cantilever's material constants (sensitivity s and spring constant k).

3. Determine the maximum cell-cell-adhesion force F_{max} for different contact times and contact forces (at least for 5 cells). Determine the separation work W.

4. Determine the maximum cell-substrate-adhesion force F_{max} for different contact times and contact forces (at least 5 points on the substrate). Determine the separation work W.

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 Introduction

Adhesion of living cells – besides blood cells – to other cells, the extracellular matrix (ECM) or substrates is essential for proliferation and viability since suspended cells perform apoptosis. Nevertheless, interactions of cells with their environment in terms of mechanical effects and adhesion forces are poorly understood.

Almost 50 years ago Malcolm S. Steinberg made the observation that two different cell populations mixed together in a droplet of medium demix because of surface tension differences. Here the corresponding surface tensions were determined with a plate tensiometer which compresses a cellular spheroid and subsequently measures the contact angle of the "cell droplet" to the plates on top and bottom of the tensiometer. It could be observed that cells of the same kind preferentially adhere to each other: Cells will higher surface tension form a spheroid core surrounded by a shell of cells with lower surface tensions. However, there is a huge difference between the surface tension of a whole cellular spheroid and single cells!

The surface tension of single cells is among other things determined by their cadherin expression. Cadherins belong to the family of surface proteins which cause adhesion to other cells. For epithelial cells, the number of E-cadherins relates to interaction forces between such types of cells, whereas integrins are responsible for cell-substrate adhesion. During the last years the interaction of cells with other cells and substrates has come more and more into focus of current medical and biophysical research. Cell-substrate interaction for example is important for the application of implant materials such as hip replacement or drug delivery systems. The better cells adhere to such joint implant, the better the perspective for life-time application and bone preservation around the implant.

Furthermore, cell-cell interactions are a major determinant for cancer progression. Only from a reduction of cell-cell and cell-ECM adhesion cells gain the possibility to leave the primary tumor and travel around inside the body by entering the blood and lymph vessels. Recent studies showed that such reduction in interaction forces can be attributed to a loss of cadherin expression. Nevertheless, lots of open questions remain. Since cancer cells are usually softer (i.e. more deformable) than normal cells, malignant cells can form more contact sites to their environment. If such enhanced elasticity compensates the loss in E-cadherins, is still unknown because of the difficulties in measuring cell-cell interaction forces. A new CellHesion atomic force microscopy (AFM) technique allows tackling such problems. Thus, aim of this experiment is the measurement of cell-cell and cell-substrate adhesion forces of two different cell types as described in the following.

2 Assembly (JPK CellHesion® 200)

The JPK CellHesion® 200 (JPK Instruments AG, Berlin) is modified scanning force microscope[1] (SFM) specifically designed for cellular adhesion measurements. Its assembly is very similar to the JPK NanoWizard® BioAFM. It consists of a *scanning unit (scan head)*, a *controller box* and a *computer*. The former contains the fundamental SFM elements (in particular cantilever, laser, and photodiode) and it is placed on an inverted light microscope over the sample, permitting simultaneous usage of a variety of light microscopy techniques (e.g. phase contrast) together

[1] Note that the measurement principle of scanning force microscopy and its application for biological research have already been introduced in the tutorial for the experiment "Scanning Force Microscopy I: Imaging & Cellular Elasticity" using the example of the JPK NanoWizard® BioAFM (JPK Instruments AG, Berlin).

with SFM. However, there are some essential differences to the NanoWizard in the details.
The idea of an adhesion measurement is to stick a cell to the SFM cantilever and to use this cantilever-cell to probe other cells sitting on the substrate or the substrate itself. This requires special cantilevers without tips which would simply spear the cell. Additionally, the leaf spring has to be wide enough to provide a sufficient surface area for the cell to adhere to. (Of course such cantilevers are absolutely inappropriate for scanning and imaging.)
Common SFMs have a very limited travel range of the z-piezo element for moving the cantilever up and down. For instance, the JPK NanoWizard provides only 15 µm. On the other hand, epithelial cells typically have a diameter of 15 to 30 µm. Even though these cells flatten when they attach to a substrate such as glass, their height is still much too large to put two cells on top of each other and to separate them using a common SFM. For this reason, the CellHesion 200 has an extended z-piezo element with a maximum travel range of 100 µm. Unfortunately, the x- and y-piezo elements necessary for the SFM scanning/imaging mode have been completely removed. Hence, the CellHesion 200 literally is not a "scanning force microscope" anymore, but just a "force microscope"[2].

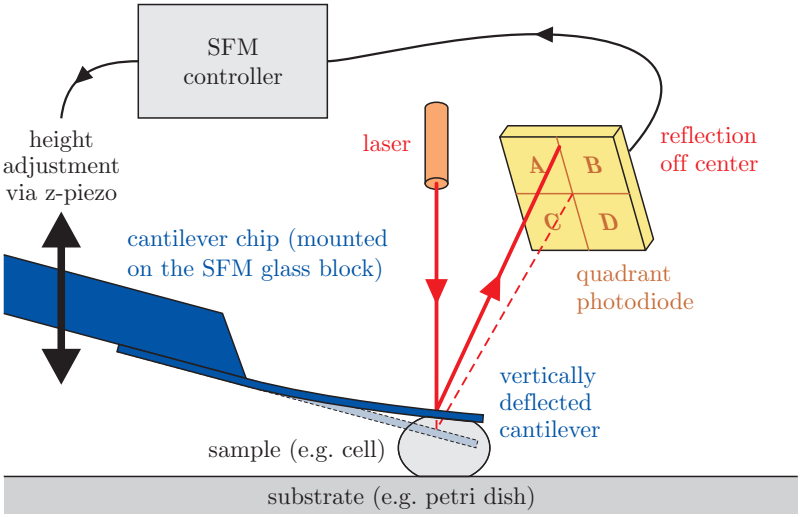

Figure 1: Schematic representation of measuring principle of an SFM: The bending of the cantilever is measured by a laser, whose deflection is detected by a four-quadrant photodiode. Subsequently, the z-piezo corrects the height z_C of the SFM head, which the cantilever is fixed to. (Image taken from diploma thesis of Steve Pawlizak, 2009, slightly modified 2011.)

3 Operation

There are two main applications for the JPK CellHesion 200:

- measuring adhesion forces between two cells and
- measuring adhesion forces between a cell and the substrate

both in the piconewton regime. Furthermore, elasticity measurements are also possible, but not important for this lab course experiment. The former two operating procedures are described in detail in the following:

3.1 Cell-Cell Adhesion

Cantilever functionalization: For cellular adhesion measurements using SFM, a probe cell has to be attached to the SFM cantilever. In order to make the cantilever's surface attractive for cells, the cantilever has to be coated with a certain adhesion protein such as *fibronectin*

[2]Nevertheless, we will still call it (modified) SFM due to its technical origin.

or *concanavalin A*. It is necessary that the cell-binding to the cantilever is stronger than the binding the cell will form with the surface to be probed (i.e. another cell or the substrate).

Cell capture: The functionalized cantilever is placed over the top of the target cell and gently lowered onto the cell. After a certain contact time the cantilever is retracted and the cell should be attached. To form a stronger contact, one will give the cell some time. This cell will be called *cantilever-cell*.

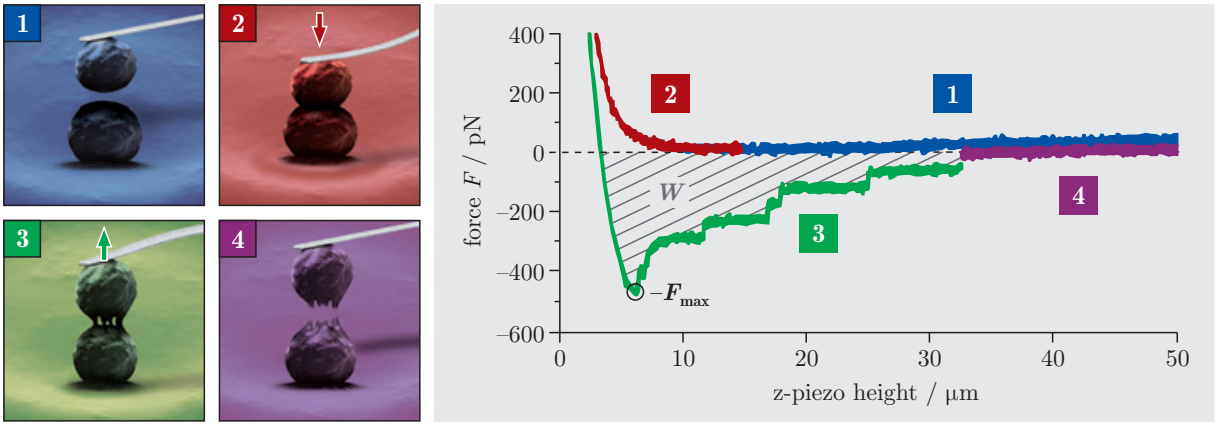

Figure 2: Illustration of the steps of a single cell-cell adhesion measurement cycle. The result is a force-distance curve, which allows to determine single molecule events, the "work of removal" W, tether formation, the maximum adhesion force $F_{\max}$ and viscoelastic parameters. (Image taken from JPK CellHesion 200 Brochure, JPK Instruments AG, Berlin 2008, slightly modified 2011.)

Probing a cell: During the whole process of probing a cell, the deflection/bending of the cantilever is recorded in dependence of the height of the cantilever, see Fig. 2. First the cantilever-cell is brought with a defined force into contact with another cell called *substrate-cell* which has to be well-adhered to the substrate (trace/extend part, 1 and 2 in Fig. 2). After a defined contact-time of the two cells, the cantilever is lifted up again (retrace/retract part, 3 and 4 in Fig. 2). The cells resists the attempt of separating them due to cellular adhesion. Therefore, the cantilever noticeably bends into the opposite direction. The force $F_{\max}$ necessary to separate both cells can be directly determined from the force-distance curve if the cantilever was correctly calibrated. This force corresponds to the *maximum adhesion force* between both cells for the applied set of measurement parameters.

3.2 Cell-Substrate Adhesion

Cell-substrate interaction can be analogously probed to cell-cell adhesion. Instead of pressing the cantilever-cell onto another cell, it is pressed onto the substrate, see Fig. 3. The resulting force-distance curve is qualitatively the same as for the cell-cell adhesion, see Fig. 2.

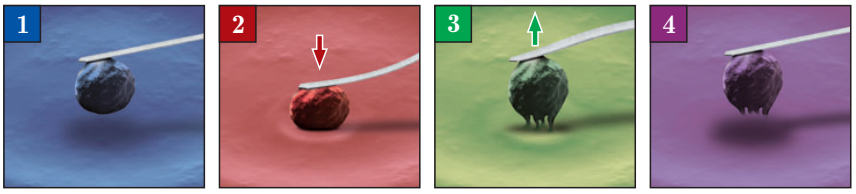

Figure 3: Illustration of the steps of a single cell-substrate adhesion measurement cycle. (Image taken from JPK CellHesion 200 Brochure, JPK Instruments AG, Berlin 2008.)

During adhesion of the cells to the cantilever and to other cells or the substrate, you can image the cells with the phase contrast microscope and take pictures or create movies. All experiments are repeated several times with the same cell and with different cells to gain statistically relevant information.

4 Calibration

The aim of these measurements is to obtain the maximum adhesion forces $F_{\max}$ between two cells or between cell and substrate.
The measured raw data itself provides the scanner's z-piezo height z_S and the cantilever deflection u_C in volts measured by the four-quadrant photodiode. The latter has to be converted into a force F which is acting on the cantilever. For this reason, the used cantilever has to be calibrated before each force measurement. In other words, the cantilever's material constants have to be determined. They depend on the current state of the cantilever (e.g. dirt on the cantilever arm) and the mounting angle of the cantilever on the glass block. A proper cantilever calibration is essential for the accuracy and reproducibility of force measurements. Unfortunately, the calibration is also the biggest source of error for SFM measurements.

Sensitivity: Since the four-quadrant photodiode detects the cantilever deflection u_C in Volts, a conversion factor is needed to get a useful height value z_C, which is comparable to the scanner's z-piezo height z_P. This is the so-called sensitivity s of the cantilever:

$$z_C = s\, u_C \tag{1}$$

To determine the sensitivity, a characteristic force-distance-curve on a quasi-infinite hard substrate (e.g. glass coverslip) is recorded. In this case the indentation of the substrate is negligible and the vertical deflection of the cantilever corresponds to the z-piezo height of the scanner. Then the sensitivity s is related to the constant slope in the repulsive range of the curve:

$$s = -\frac{1}{\text{slope}} = -\frac{\Delta z_P}{\Delta u_C} \tag{2}$$

Spring constant: For quantitative force measurements, the spring constant k of the cantilever is needed to convert the cantilever deflection z_C into force values. According to HOOKE's law, the force F exerted by the cantilever is the product of cantilever deflection and spring constant:

$$F = k\, z_C \tag{3}$$

The SFM software (JPK Instruments AG, Berlin) provides an automatic *thermal noise technique* to determine k for most contact mode cantilevers with resonance frequencies up to 70 kHz. The program measures the thermal vibrations of the cantilever deflection. The result is a frequency spectrum that shows a peak at the cantilever's resonance frequency. Fitting the area under the resonance peak, assuming that the cantilever behaves like a harmonic oscillator, and applying the equipartition theorem finally yields k.

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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 Force-Distance-Curves

Force-distance-curves are saved in a proprietary JPK file format (*.jpk-force) that can be opened and analysed with the JPK Data Processing software that also allows batch processing. Alternatively, you can convert those files into plain ASCII text files (*.out) using the batch script *jpk-force-legacy-export*. ASCII text files usually can be imported into any scientific graphing and data analysis software you like (e.g. ORIGIN). Each text file has several sections with the data for the extend, pause, and retract curves. Every section has a defined header containing some important parameters of the related force-distance-curve like relative setpoint or extend speed.