Combining atomic force microscopy with micropipette techniques for cell mechanical measurements

Introduction

Topography, roughness, and mechanical properties of biomaterials are crucial parameters affecting cell adhesion/motility, morphology and mechanics as well as the proliferation of stem/progenitor cells [1-4]. Nano-mechanical analysis of cells and tissue slices increasingly gains in importance in different fields of cell biology, like cancer research [5] and developmental biology [6]. Atomic force microscopy (AFM) is a powerful, multipurpose technology suitable not only for imaging a wide range of different samples with nanometer scale resolution under controlled environmental conditions, but also for mapping mechanical and adhesive properties of sample/cell systems and tissues.

Atomic force microscopy is not a high throughput technique as optical readout methods can be. However, the JPK NanoWizard® AFM can be seamlessly combined with methods such as fluorescence, confocal, TIRF, STED microscopy for high content analyses [e.g. 7, 8] showing that the JPK NanoWizard® AFM is versatile when combined with other single cell techniques. For a better understanding of how cells react on externally applied mechanical stimuli, some researchers have tried to connect fluorescence microscopy with AFM and micropipette related technologies like simple manipulation (e.g. [9]), aspiration, injection, and patch clamp for electric-physiological investigation. The simultaneous combination of different single cell technologies results to several technical challenges. In this report, we will describe how inverted microscopy can be equipped with micropipette aspiration and AFM indentation measurements on suspended mammalian cells.

Micropipette/patch clamp equipped with AFM – a short overview

In 1991, Häberle *et al*. [10] reported a non-simultaneous use of a patch pipette and AFM. The micropipette served to fix suspended cells to increase the AFM-image-resolution of the surface. Since 1994, the sequential use of AFM and patch clamp based on customized set ups was established ([10-15]). Typically, an upright microscope with dipping objective was used and the AFM scanner had to be adjusted separately to the objective lens and the detection sensor unit. However, these setups were only restrictedly usable for standard AFM in routine use operation mode (Langer et al., 2000 [16]). In 1995, Hörber *et al*. [12] described the first coupling of AFM and patch clamp technique for a simultaneous measurement of mechanical and electric properties of the cell membrane. In 1999, Iwamoto *et al*. [17] combined an arrangement of patch clamp and AFM to examine the formation of chloride ion channels in response to the VacA toxin. A year later, Bett and Sachs ([18]) used a force controlled cantilever to exert mechanical pressure on a cell while simultaneously recording the whole cell current. Using a planar patch clamp chip unit equipped with a customized AFM, Pamir *et al.* (2008, [19]) immobilized non-adherent Jurkat cells. They also were able to carry out mechanical manipulation under simultaneous electro-physiological characterization. A setup of inverted microscope, patch clamp and lab-designed AFM was used as described by Priel *et al*. (2007, [20]) to characterize the adhesion strength between the AFM tip and cell surface under parallel patch clamp recording for a better understanding of the Giga-seal formation. Beyder and Sachs (2009, [21]) published a similar setup to run force-clamp experiments in range of 50-500 pN to investigate the electro-mechanical coupling of the membrane of genetically modified adherent HEK-293 cells.

Integration of AFM, Fluorescence and Micropipette technique

a) AFM and invert microscopy

For a combination of AFM and epi-fluorescence microscopy, the methods should not disturb one another. The JPK NanoWizard® AFM is designed as a tip scanning system So the sample remains in optical focus while AFM scanning in x, y and z. The optical access design offers stability for imaging and, as the beam path is not disturbed, phase contrast and DIC imaging work perfectly (see sketch in Fig.1).

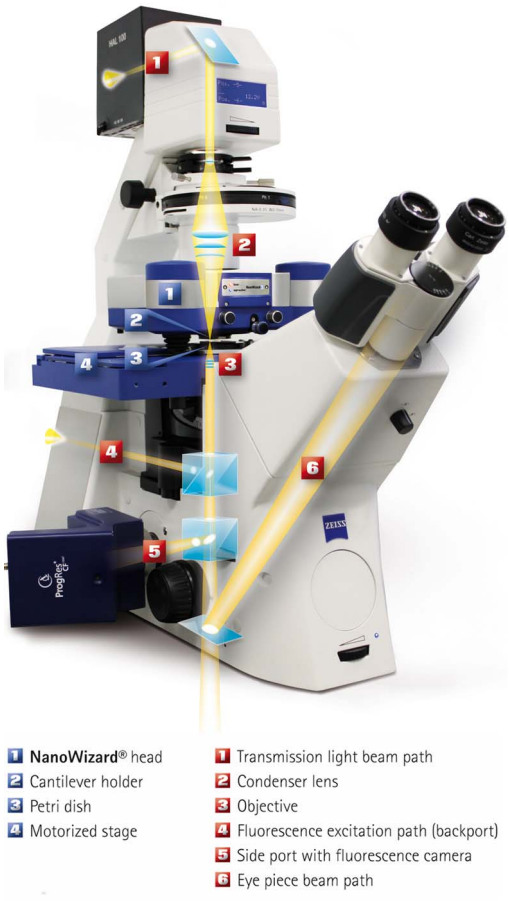

Fig. 1 Sketch of the epi-fluorescence and white light pathway of an AFM-inverted microscope setup.

b) System setup (Fig. 2)

The AFM setup with a JPK life science stage was installed on a LSM 510 Axiovert 200M (Zeiss, Jena) with a LD condenser (wd 70mm). An ImagingSource camera is under direct control by JPK software which is also used to adjust the laser beam on cantilever and simple optical overview. The AFM setup was driven by JPK's CellHesion® 200 software. Advanced optical images were captured with a objective lens LD Plan-Neofluar 63x/0,75 Korr Ph2 under Zeiss software control.

The micropipette system, TransferMan® NK2, and control box from Eppendorf was used. An adapter plate supports the installation of two pipette holders on both sides. A manual microinjector, CellTram® vario (Eppendorf), was used for simple pressure control, manual microinjection and liquid dispensing. The injection system was assembled with a pressure transmitter (CTE9N01GY0, First Sensor AG, Puchheim), micropipette, tubing, CellTram vario injector, pressure sensor and transmitter, signal receiver and signal analysis. A lab-written software program records and controls the pressure and signal analysis.

The micro-capillaries were produced with a micropipette puller from Sutter Instrument (Novato, CA, USA).

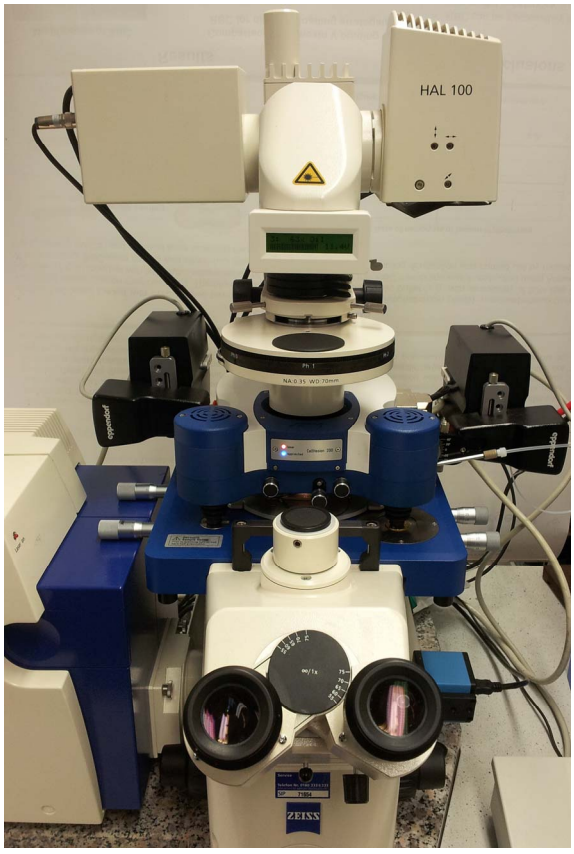

Fig. 2 The setup of a NanoWizard® AFM equipped with micropipette holders on an inverted microscope.

c) Technical details

AFM as well as micropipette techniques are sensitive to mechanical vibrations. If the AFM head is mounted on top of the sample, the free space for micropipette adjustment is rather limited. However, if the micropipette holder is too far away from the specimen, the manipulation experiments (e.g. aspiration) cannot usefully executed. JPK has

replaced the AFM head cables with 90° angular plugs, and modified the micropipette adapter plate (see Fig. 3).
Micropipette manipulation can easier executed if the setting angle between micropipette and cell surface is rather high. However, in combined setup with AFM it is only limited space available. In order to increase the distance between AFM head and sample, an extra-long glass cantilever holder equipped with a CoverSlipHolder electric (CSHe) was used (Fig. 4). The CSHe is intended to be compatible with cover slips (thickness of 170µm) for high resolution fluorescence microscopy. With this setup, the approach angle for the micropipette aspiration can be adjusted to about 15°.

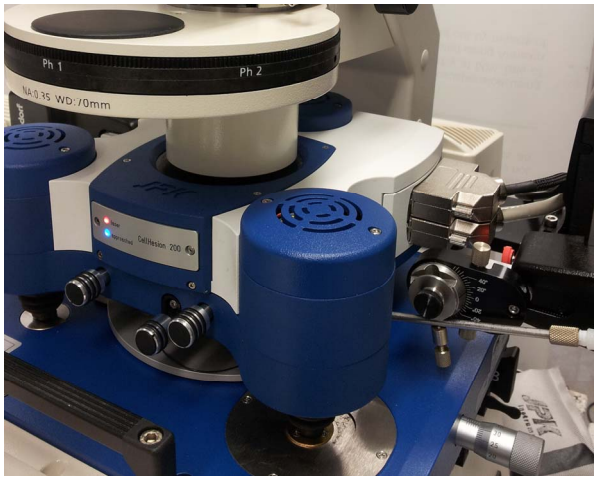

Fig. 3 90°-angular plug adapters enable a narrow distance between manipulator holder and AFM head.

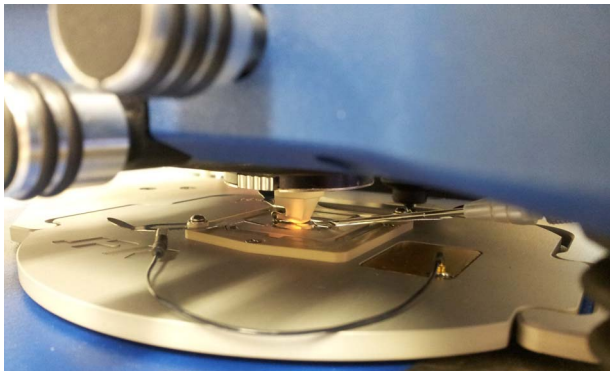

Fig. 4 Extra-long cantilever holder with cantilever is placed on a CoverSlipHolder electric (CSHe) with grounding cable. A silicone sealing stabilized with a PEEK frame allows the use of liquid for micropipette manipulation.

d) Micropipette aspiration with microscopy

In order to test the functionality of each of the individual single cell techniques, we first tested the combination micropipette technique with fluorescence microscopy (Fig.5). Various cell types need different micropipette tip-sizes. In Fig. 5, an example for red blood cell and tumor cell aspiration is given. Individual cells can be aspirated and the corresponding pressure changes can be analysed.

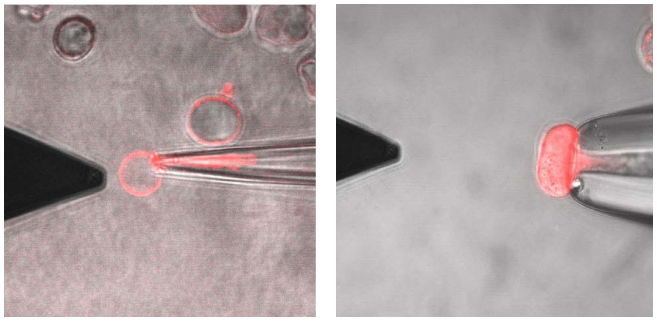

Fig. 5 CLSM-images of a micropipette aspirated cells (left: red blood cell, right: tumor cell T47D). The images are an overlay of fluorescence (labelled with Rhodamin G) and white light transmission.

e) Micropipette aspiration and AFM indentation measurements

MLCT cantilevers (Bruker AFM probes) with a nominal spring constant of 0.01 N/m were used. Cantilevers were mounted on the AFM and calibrated on the glass cover slide in buffer. To determine the spring constant of the cantilever the JPK supported thermal noise method was used.
As a next step, an aspirated cell was placed below a retracted cantilever tip. The indendation measurements were performed under JPK software control (see Fig. 6).

f) Data analysis

The JPK data processing software allows analysis of the recorded force distance curves to derive different features of the probe sample interaction such as the stiffness of the sample or probe-sample adhesion. The Young's modulus can be determined using the Hertz model fit (see Fig. 7). This feature provides elasticity fitting for all kinds of indenter geometries as well as variation of the fit parameters (either fixed values or fitted). All pre-

processing operations (e.g. Offset and Tilt Correction) should generally be applied to the Extend curve. Here it is not crucial to set the x- and y-offset since the baseline and contact point are variable fit parameters. It is important to apply the Tip-Sample Separation calculation.

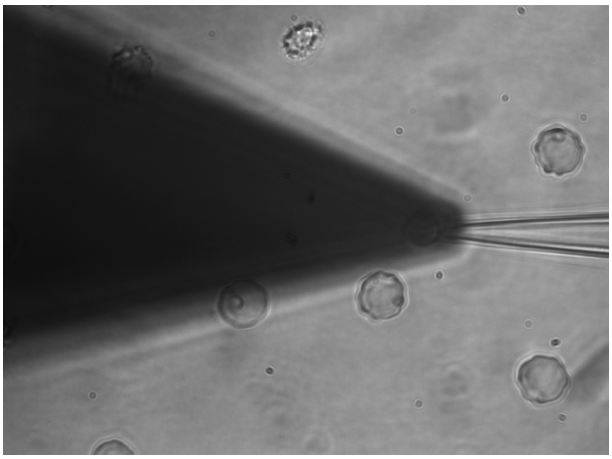

Fig. 6 Transmission image of a micropipette aspirated red blood cell below a retracted cantilever tip

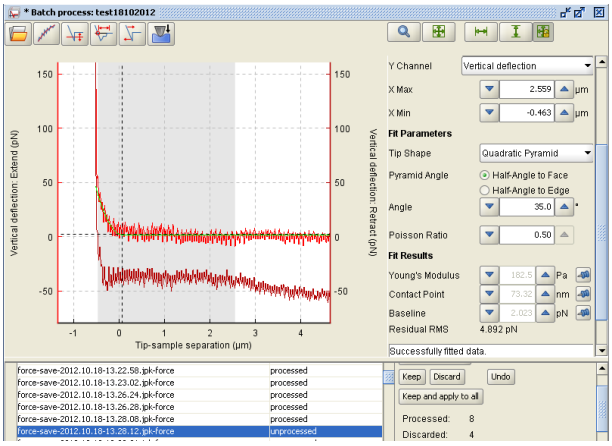

Fig. 7 Print screen of the JPK Data analysis software in batch processing.The batch processing feature allows the processing of large amounts of force curves in a convenient way. It allows automatic as well as manual adjustment of the processing functions. The results table is finally saved as a table text-file.
In this example, the calculated E value of the fitted curve results to 180 Pa.

Based on a pyramidal indenter geometry with an approach speed of 5µm/s and a maximum applied force of 500 pN , we calculated an average E-value of about 140 Pa. This range of the Young's modulus fits well when compared to those values reported in the literature [22,23].

Conclusions

The study of adhesion and cyto-mechanical properties of individual cells for the elucidation of fundamental processes in cell biology is becoming very popular in stem cell and cancer research [24]. This has caused a rise in the number of requests for combined measurements of fluorescence and force [25, 26].
The tip-scanning system of the JPK NanoWizard® equipped with the extra-long cantilever holder and the flat CoverSlipHolder allows the simultaneous use of micropipette aspiration while making AFM measurements.

Acknowledgements

We gratefully acknowledge Nadine Sternberg and Axel Steffen (Charité Berlin Germany) for experimental support. The work was supported by Bundesministerium für Wirtschaft und Technologie (BMWi) with grant number FKZ: 2606601FR0.

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Authors

PD. Dr. Hans Bäumler,
CAMPUS CHARITÉ MITTE
CharitéCentrum 14
Institut f.Transfusionsmedizin
Chariteplatz 1
10117 Berlin
Germany
hans.baeumler@charite.de

Dr. Torsten Müller
JPK Instruments AG
BouchéStr. 12
12435 Berlin
Germany
mueller@jpk.com