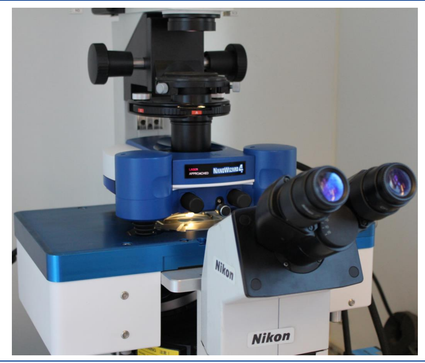
From single molecules to tissues - a new AFM toolkit for nanoscopic investigation of mechanics, structures and dynamic processes in life science

A. Dulebo, T. Müller, J. Barner, A. Koernig, T. Jähnke
JPK BioAFM Business, Bruker Nano GmbH, Am Studio 2D, 12489 Berlin, Germany, www.jpk.com

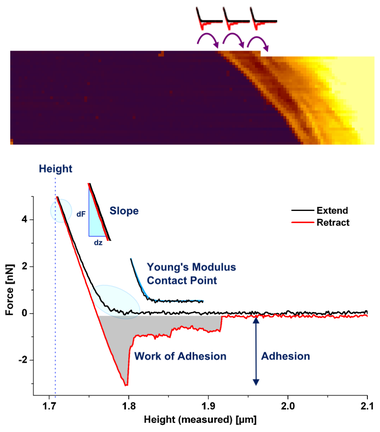
Introduction

Crucial parameters affecting cell adhesion, morphogenesis, cell differentiation and cancer invasion include the molecular interactions between cells and their extracellular matrix environment, their 3D topography and the corresponding mechanical properties. [1-3]. AFM (atomic force microscopy) is an advanced multi-parametric imaging technique which delivers 3D profiles of the surfaces of molecules and cells in the nm-range. It also enables the characterization of nanomechanical properties (adhesion, elasticity etc.) and the visualization of structural changes taking place at the molecular level. Mapping of tissue samples and thicker multi-cellular layers as well as the nanomechanical characterization of the extracellular matrix and its embedded cells requires a (semi)automated AFM approach for a millimeter range in x,y with nm–resolution. Large variations in the topography of tissue samples, combined with fluidity and stickiness of membrane and extracellular proteins, require a larger flexibility in the z-axis of the AFM [3]. To characterize non-elastic properties of life science samples we used: i) time-dependent creep compliance measurements and ii) micro-rheology [3].



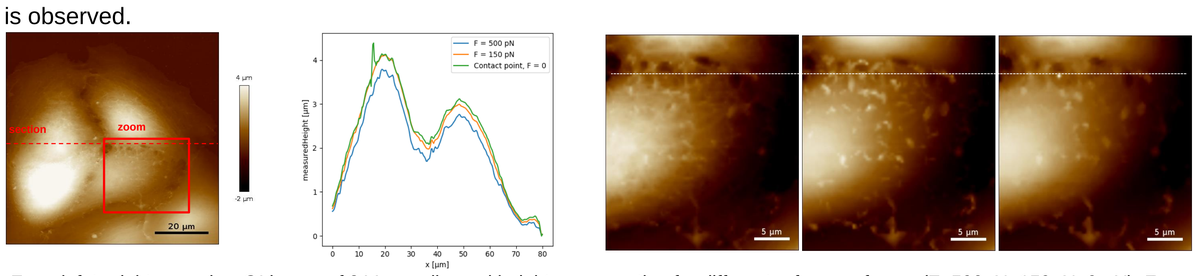
Quantitative Imaging (QI™)

- **Force spectroscopy-based** imaging mode providing real quantitative data by the acquisition of complete force distance curves at each image pixel
- Special QI™ tip movement algorithm prevents lateral forces and controls the vertical forces for gentle imaging of soft and fragile samples
- Any parameter can be presented as an image, such as:
  - **Adhesion** and **Work of Adhesion**
  - **Young's modulus** (Elastic modulus, stiffness)
  - **Contact point images** (sample topography at zero force reveals the native surface structure)
  - **Height images** at different interaction forces



Living cell imaging (Vero)

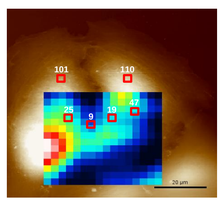
Vero cells were imaged using QI™ mode. Different forces resulting to different levels of deformation and visualizing different structures. At higher forces the image is dominated by the underlying cells cytoskeleton whereas at lower forces down to the contact point the undisturbed cell surface including membrane protuberances (microvilli) is observed.



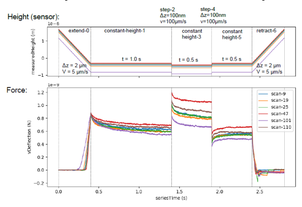
From left to right: overview QI image of 3 Vero cells, and height cross section for different reference forces (F=500pN, 150pN, 0 pN); Zoom - height image at F= 500pN, F= 150 pN and F= 0pN (contact point image); Bruker cell imaging probes (PFQNM-LC-A-CAL, k = 0.09 N/m) with a very well defined radius of curvature of R_{tip} = 70 nm and a tip length of 18 µm are used.

Step Response Measurements on Living VERO Cells

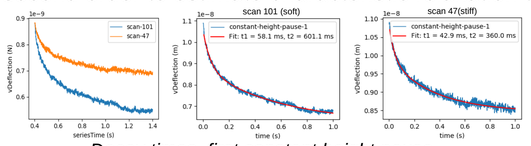
Step and creep experiments can not be performed in QI mode as a well defined but fixed motion protocol for the acquisition of force-distance data. Whereas in the "advanced force spectroscopy" software module the protocol for a cycle called "force ramp" can be built as a linear sequence of "segments" and can be operate as force map.



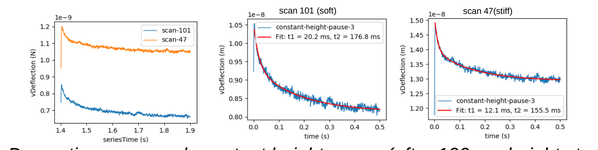
Overlay of topography derived from QI (F_{sp} = 500 pN) and force map (F_{sp} = 800 pN, 16 x 14 pixels). The force map topography is derived from the first extend segment as the z-position in which the force setpoint is reached. In each pixel of the shown force map a force-ramp is executed. Positions and corresponding indices of individual force curves discussed below are marked in red. As an example six force curves shown above are chosen by "eye" for evaluation of the visco-elastic relaxation times.



Data of two extrema (force-ramp 101 (stiff) and 47 (soft)) are shown in more detail below. For each of the two force-ramps, the first extend segment is used to calculate the apparent Young's modulus. The first (segment-1) and second (segment-3, after the 100 nm height step) constant height pauses are fitted by exponential decay second order to estimate characteristic relaxation times.



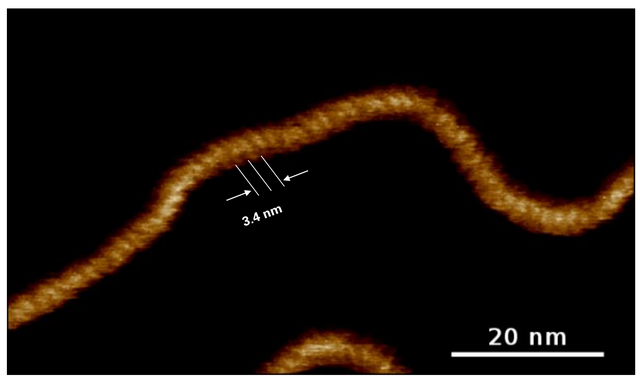
Decay times, first constant height pause

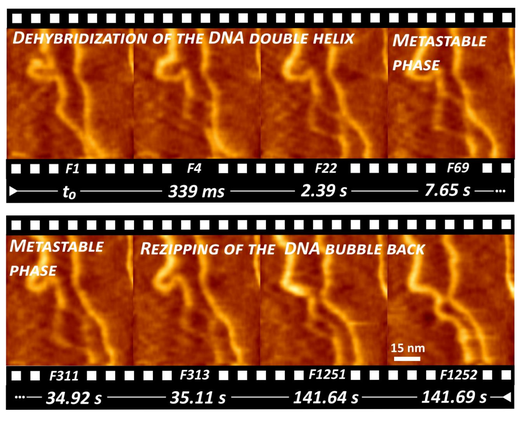


Decay times, second constant height pause (after 100 nm height step)

Reaching Submolecular Resolution and Observing DNA Dynamics

Biosamples, are quite often analysed at near-room or slightly elevated temperatures. Every AFM system is prone to physical phenomena such as different coefficients of thermal expansion of the AFM piezo-scanners, as well as being susceptible to external environmental heat dissipation. Unlike conventional configurations which require long scanning times of at least a few minutes, the application of fast scanning AFM mitigates this problem which is of particular importance in the case of very sensitive and quickly deteriorating samples.

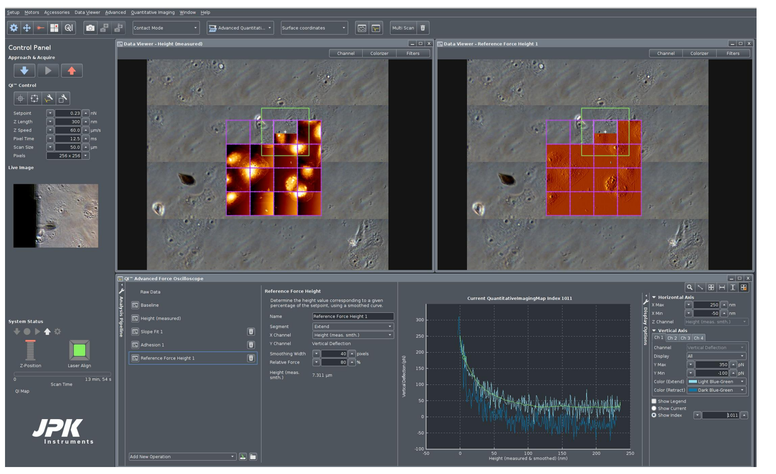




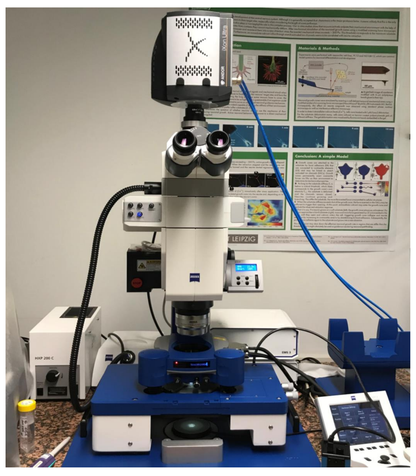
Application of high-speed AFM for studying the pUC19 DNA double helix (left), showing the double helical repeat of 3.4 nm

Correlative Microscopy - Optical Tiling and Multi-Region Imaging

Correlation between optical and AFM images can be done. In particular, advanced fluorescence methods such as confocal, spinning disc, FRET, TIRF, FLIM, PALM/STORM or STED can be used simultaneously with AFM thanks to our unique tip-scanning AFM design [4-7]. An example of multi-scan QI™ imaging of mammalian Vero cells on an inverted optical microscope is presented below. First, a DirectOverlay™ was performed to correlate the cantilever tip position with the corresponding optical image. Second, optical tiling with 4 x 4 images on 500 x 500 µm² was carried out to increase the region of interest. Third, a multi-scan range was selected. For this specific example, 4 x 4 QI™ scans with an individual map size of 50 µm were performed (frames are labeled in magenta). The green frame on position 14 indicates the current map position.



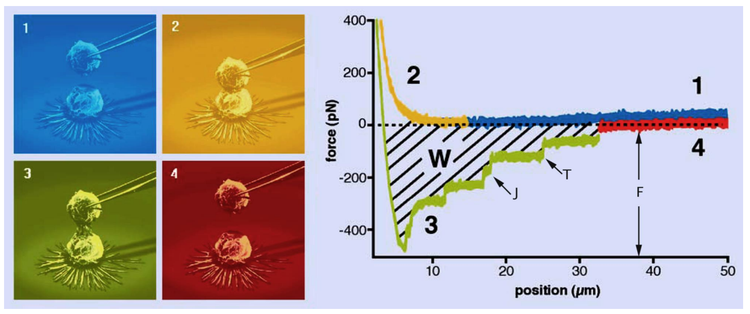
Screenshot of the NanoWizard HybridStage control software. Adherent Vero cells in phase contrast optical microscope, imaged under 37°C temperature control. Top: Optical tiling over 500 x 500 µm² consists of 4 x 4 optical images. The selected multi-scan region is indicated by the magenta colored frame: 4 x 4 maps with 50 x 50 µm² size. Top left: Height channel maps. Top right: Pixel difference map of the same region. Bottom: Advanced force oscilloscope and a force curve for a specific index position. QI™ mode settings: Force 0.23 nN, z- length 300 nm, 256 x 256 pixels. A PFQNM-LC-A cantilever with a tip height of approx. 19 µm and blunt tip (radius 70 nm) was used.



JPK HybridStage™ on a Zeiss upright AxioZoom V.16 fluorescence macroscope with an Andor EMCCD camera for force mapping experiments on non-transparent tissue samples. Image courtesy of Josef Käs and Thomas Fuhs, Uni Leipzig, Germany.

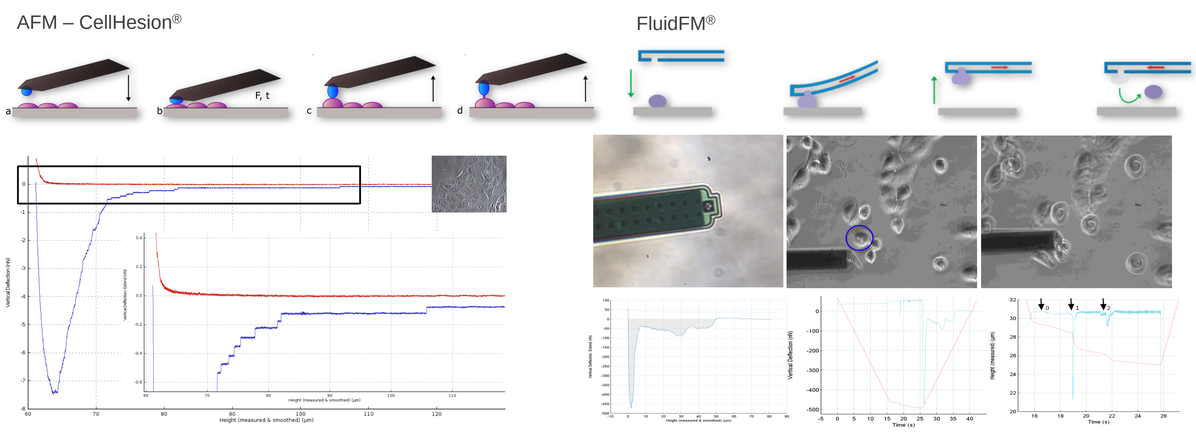
Single Cell Force Spectroscopy to study cell adhesion

AFM based Single Cell Force Spectroscopy (**SCFS**) allows for characterizing the cell adhesion process. To run a typical cell adhesion experiment 4 basic steps are involved:



1) A cell is chemically bound to a flexible cantilever probe
2) The cantilever-bound cell is approached and pressed onto the substrate until a preset force (~0.3-2 nN) is reached.
3) After a defined contact time, the cantilever-bound cell is elevated until...
4) ... the cell is completely separated from the substrate.

The flexible cantilever bends upwards/downwards as the cell is pressed towards/ separated from the surface. The cantilever probe can be thought of a spring which allows for converting the deformation into units of force. Consequently, adhesive interactions, such as maximum adhesion and single unbinding events can be quantified in the single cell range (pN-nN). Attachment can be done be functionalization or aspiration (FluidFM).



Force distance curve of a Vero cell (insert right: with an optical microscope image of Vero cells layer) in contact with a fibronectin coated tipless cantilever (50 µg/ml for 30 min; TL-2 from NanoWorld). Force settings: 3D piezo scanner 100 x 100 x 100 µm³, 1 nN, z-speed 10 µm/s, contact 10 sec. Lower right corner is the zoom of data out of the black box.

Top from left: FluidFM cantilever with fluid channel and aspiration aperture; target A549 cell - blue circle; After piezo retract with 100 µm, cell is detached, optical focus is adjusted; Bottom: Detachment force curve and detachment energy of a A549 cell; time axis in red – height , in blue – force with contact time of 10sec; Zoom in contact section - Pos 0 = starting contact under constant force condition, Pos 1 = Applying -500 mbar will induce a force peak and drop in height, Pos 2 = reaction of the cell.

References:
[1] Engler AJ. et al., Cell 126:677-689 (2006)
[2] Barriga EH. et al., Nature 554: 525-527 (2018)
[3] Rother et al. Open Biol. 4, 140046 (2014)
[4] Harke et al., Optical Nanoscopy 1: 3 (2012)
[5] Chacko et al., Cytoskeleton 70: 729-740 (2013)
[6] Chacko et al., PLoS ONE 8(6): e66608 (2013)
[7] Janel et al., Methods in Cell Biol. 140, 165-185, Chapter 9, (2017)
[8] JPK Application note on combination of AFM with STORM (2013)
[9] Gonnermann et al., Integr. Biology, pp. 1-8, (2015)