Subproject E2.4

Cell adhesion and migration on micro- and nanostructured substrates

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Introduction and Summary

The Young Scientist Research Group Nanobiology (E 2.4) was established at the CFN in October 2007. Work in the research group focuses on expanding the use of atomic force microscopy (AFM) for biological applications, in particular for studying cell adhesion and migration on micro- and nano-structured substrates. With AFM, samples can be imaged directly on the nanoscale without prior preparation steps such as staining or fixation. This makes AFM extremely suitable for imaging biological samples, since molecules or even living cells can be maintained under physiological conditions. In addition to its high-resolution imaging capability, AFM cantilever are ultrasoft springs and, when calibrated, can be used to measure the strength of inter- and intramolecular bonds and cellular adhesion forces.

The research group has obtained particular expertise in AFM-based single-cell force spectroscopy (SCFS). In SCFS, a living cell is gently attached to the AFM cantilever and pressed onto a substrate under defined conditions (contact force and time). With a force sensitivity spanning over four orders of magnitude, SCFS provides a unique opportunity to measure cellular adhesion forces from the single-molecule level to overall adhesion in the same experimental setup. AFM-based SCFS has provided important insight into molecular mechanisms involved in adhesion force generation, such as the transition from single-receptor mediated to cooperative receptor binding during the initial phase of cellular contact with an extracellular substrate. Furthermore, the adhesive properties of different surfaces can be accurately characterized. These studies are greatly aided by the incorporation of an AFM stage featuring an extended Z-piezo range of 100 µm (CellHesion module, JPK Instruments). A large retraction range ensures that even strongly adhering cells can be completely separated from an adhesive substrate during force spectroscopy.

A further field of interest within the research group is the development and fabrication of artificial cell adhesion substrates using microcontact-printing (µCP) and direct laser writing (DLW) With these techniques, two- and three-dimensional cell adhesion substrates can be decorated with micrometer-sized cell contact points, allowing us to study the influence of substrate geometry on cell behaviour. In some studies we also employ ultrathin collagen matrices in which individual collagen fibrils are patterned on the nanoscale. Such highly ordered matrices provide insight into fibril structure and mechanisms driving collagen fibril self-assembly. Furthermore, these matrices harbour great potential for the biofunctionalization of surfaces, can be used to direct cell migration and adhesion and provide structurally and chemically homogeneous surfaces for cell adhesion studies.

In the timeframe 2007-2010, subproject E2.4 has led to 7 publications and two book chapter contributions (“AFM-based Single-Cell Force Spectroscopy” in *Atomic Force Microscopy in Liquid*, Whiley VCH, in press, and “Studying Collagen Self-Assembly by Time-Lapse High-Resolution AFM” in *Atomic Force Microscopy: Its use in Biomedical Research*, Humana Press, in press.

Current research work and collaborations

1. Artificial heterofunctional collagen matrices

Collagen is the most abundant protein in animal tissues and constitutes the bulk material of the extracellular matrix. Its easy availability, low antigenicity and mechanical strength make collagen a prime material for surface biofunctionalization and building of artificial scaffolds for tissue engineering and bioengineering applications. In collaboration with the Faculty of Medicine at the University of Technology Dresden, we have engineered ultrathin collagen matrices as carriers for different retinal and corneal cell lines [E2.4:4], [E2.4:7]. In future, such thin collagen sheets could serve as transparent and biocompatible cell sheet carriers for transplantation purposes.

Successfully using tailored artificial collagen sheets in advanced biomedical applications requires that the matrix architecture can be adjusted with molecular precision. We have previously used high-resolution AFM imaging to study the effect of external factors (pH value, ion concentrations) on the self-assembly process of purified collagen monomers into collagen fibrils. In tissues, however, collagen assembly and fibril width are tightly regulated by additional macromolecules, in particular by members of the proteoglycan family such as lumican. In collaboration with Dr. Stephane Brezillon (University Champagne-Ardenne, Reims, France), we have recently shown that lumican directly modulates cell adhesion in addition to its proposed role as a regulator of collagen fibril morphology [E2.4:5]. In a current project we are investigating the effect of lumican on collagen fibrillogenesis on the molecular level. For these experiments highly-ordered collagen matrices are well-suited as a model system because the regular fibril arrangement makes alterations to the fibril structure readily visible by high-resolution AFM. We have been able to directly visualize the effect of lumican incorporation on collagen fibril architecture (fibril width and distance) with molecular resolution (Fig. 1). In parallel experiments, heterofunctional matrices consisting of both collagen and lumican are tested as cell adhesion substrates. Our results demonstrate a cooperative effect of lumican and collagen on cell adhesion via integrin alpha2-mediated adhesion. A manuscript describing our results is currently under preparation in our group.

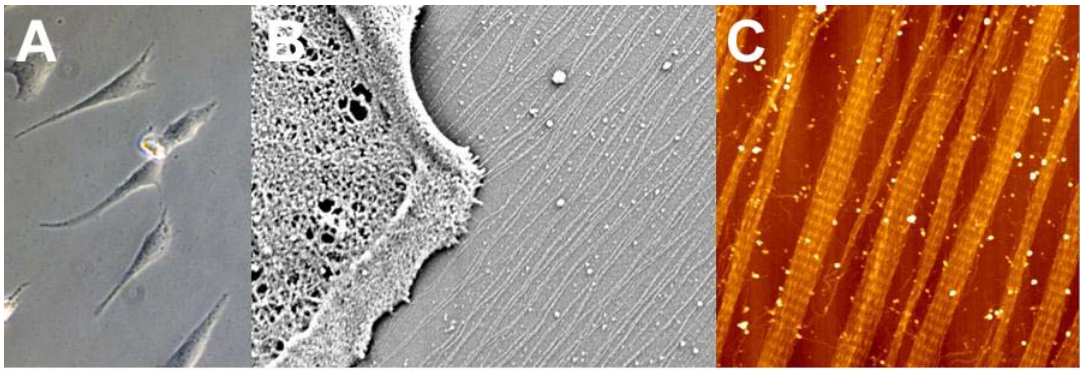

Fig. 1. Mixed collagen/lumican matrices to guide cell adhesion and migration. (A) Fibroblasts polarize and align in parallel on a bifunctional collagen/lumican layer. (B) Electron and (C) atomic force microscopy images reveal the effect of lumican on collagen fibril arrangement. The nanoscale fibril arrangement directs cell polarization and migration.

2. Developing novel surface functionalization protocols for structured cell adhesion substrates

Decorating non-biological surfaces with micro- and nanoscale biostructures is a fast expanding research area in bioengineering. However, successful surface functionalization requires that individual steps of the functionalization protocol can be accurately monitored. AFM features an unparalleled signal-to-noise ratio, allowing molecular and even atomic resolution. Thus, we routinely use the high spatial resolution of AFM to evaluate the quality of individual functionalization steps during different surface structuring and modification protocols, especially when these functionalization steps can not be monitored optically or by other microscopic techniques. For instance, we evaluated the integrity of thiol self-assembled monolayers (SAMs) (Fig. 2) applied to gold surfaces using a novel micro-contact printing protocol developed in the laboratory of Doris Wedlich (E2.2) [E2.4:3].

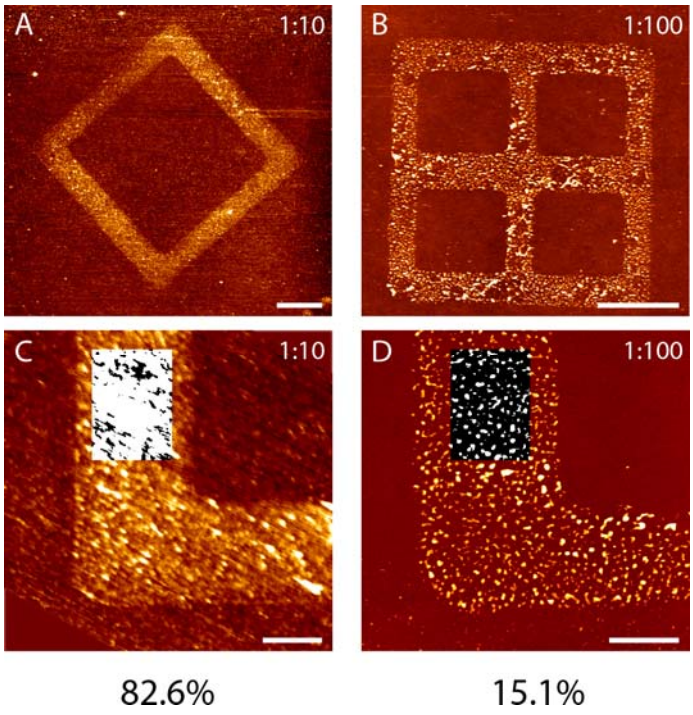

Fig. 2. (a) AFM height images of μCP SAM patterns with different benzylguanine thiol (BGT) to matrix thiol ratios after protein binding. Overview scans (top panels) and corresponding higher -resolution scans (bottom panels). The black and white insets in the lower panels represent areas in which protein densities were quantified by setting a height threshold of 3 nm above the sample surface. The ratio of protein-covered (white) to uncovered (black) areas is shown below the insets. The full range of the height scale corresponds to 13 nm. White bar: 5 μm. Images taken from [E2.4:3].

3. Bifunctional adhesion substrates for directly comparative single-cell force spectroscopy

Many established adhesion assays measure cell attachment to surfaces homogenously-coated with a single component of the extracellular matrix (ECM). In tissues, however, cells are usually exposed to a complex mixture of ECM molecules with which they interact in a spatially and temporally controlled manner. To directly compare the adhesion strength of an individual cell to two ubiquitous ECM components, we have used μCP to prepare bifunctional adhesion substrates featuring alternating stripes of laminin and collagen (Fig. 3).

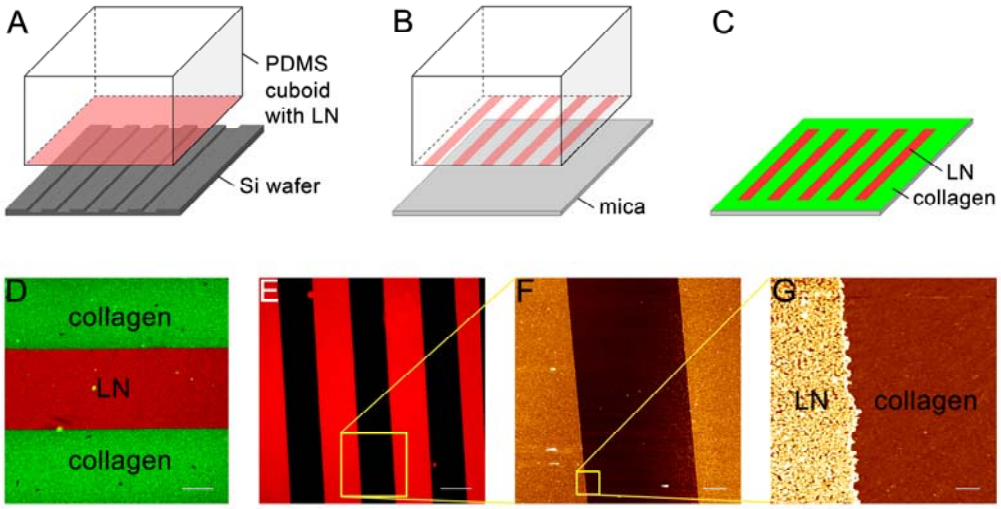

Fig. 3. Fabrication of laminin/collagen bifunctional substrates. (A) A laminin-coated PDMS cuboid is pressed onto a structured silicon wafer. Laminin adsorbs to ridge structures on the wafer. (B) When the PDMS cuboid is subsequently pressed onto a freshly cleaved mica disc, laminin stripes are transferred to the mica surface. (C) After adding collagen solution to the mica, the remaining surface is covered by collagen fibers. (D) Fluorescent image of laminin stripe (red) backfilled with collagen (green). Scale bar: 20 μm. Fluorescent (E) and AFM (E) images of the laminin/collagen bifunctional substrate. Scale bar: 50 μm. (F) AFM image of the square area indicated in (E). Height scale corresponds to 0-58 μm. Scale bar: 10 μm. (G) Higher resolution scan of the square area indicated in (F). Collagen fibers are visible at this resolution. Height scale corresponds to 0-37 μm. Scale bar: 1 μm.

Single living cells immobilized on an AFM cantilever are then alternatingly pressed on either stripe type and detachment forces are determined using single-cell force spectroscopy (SCFS). When tested alternatingly, cells display comparatively low adhesion to collagen, but strong adhesion to laminin (Fig. 4). When restricting the cell-substrate contact time to 10 sec, cells exhibited a consistent, surface-specific adhesion response over a large number of force cycle repetitions (>30), demonstrating that meaningful differential adhesion data can be acquired using SCFS and short contact time intervals. Testing many (n=30) cells on collagen- or laminin-functionalized surfaces yields a wide variation of adhesion forces across the cell population. In contrast, repeatedly testing the same cell (>30 force cycles) reveals a comparatively narrow adhesion force distribution. Thus, broad adhesion force distributions within cell populations originate from cell-to-cell variations

rather than from fluctuations in the adhesive response of individual cells. Performing SCFS on heterofunctional adhesion surfaces therefore provides quantitative and comparative information on the adhesion profile of individual cells not obtainable from bulk measurements on homogeneous adhesion substrates. A manuscript describing our results has been submitted.

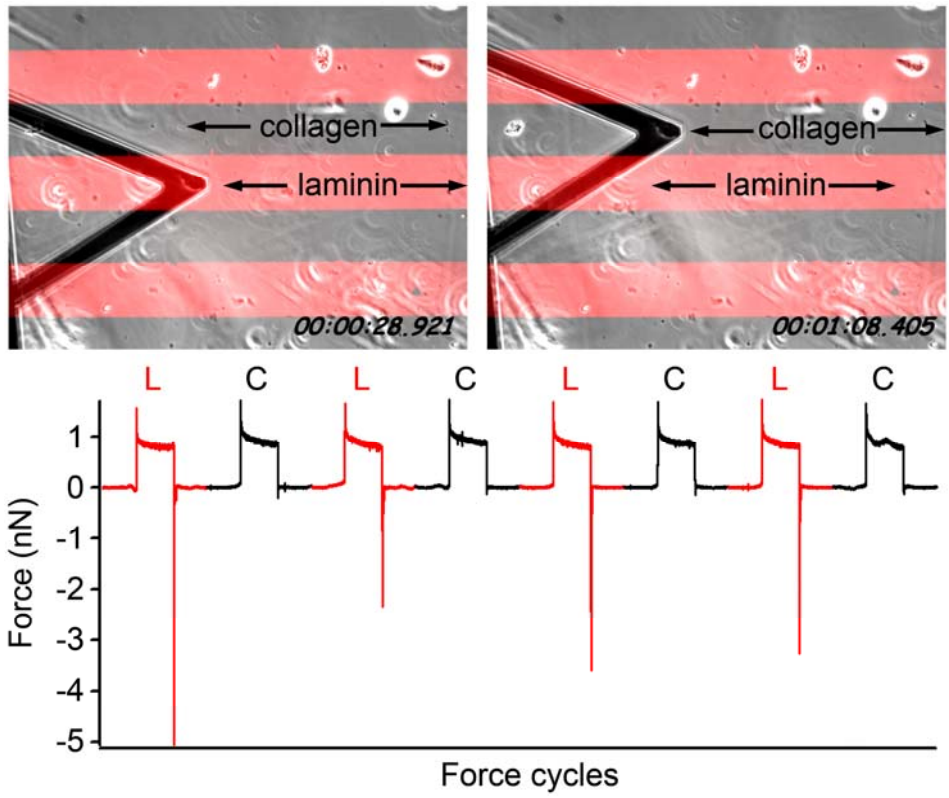

Fig. 4. Top panel: a single CHO cell attached to an AFM cantilever is alternatingly approached to laminin (L) and collagen (C) stripes. Systematic variation of the interaction position ensures that each substrate location is contacted only once. Bottom panel: sequence of 8 force curves generated in a pre-programmed sequence of force cycles alternating between collagen ("C", black) and laminin-coated ("L", red) areas.

4. Combining TIRF and SCFS

Initial contact of cells with an extracellular substrate is mediated by single-receptor binding events. Cell adhesion is then reinforced by clustering adhesion receptors into larger adhesive units. With the high force resolution of AFM, the dynamics of receptor clustering can be monitored on the force level. An experimental challenge has been to combine AFM with advanced light microscopy techniques, such as laser scanning confocal or total internal reflection (TIRF) microscopy. In this way, the clustering of receptors could be followed in living cells by optical time-lapse-microscopy and directly correlated to the adhesion force information. We have now successfully incorporated a TIRF setup into our single-cell force spectroscopy platform (Fig. 5). With this technique we are able to first observe the formation and rupture of macroscopic adhesion clusters by light microscopy and later to determine the rupture forces of these complexes during cell removal. Thus, unique quantitative information about the adhesion strength of individual adhesion clusters has become available for the first time.

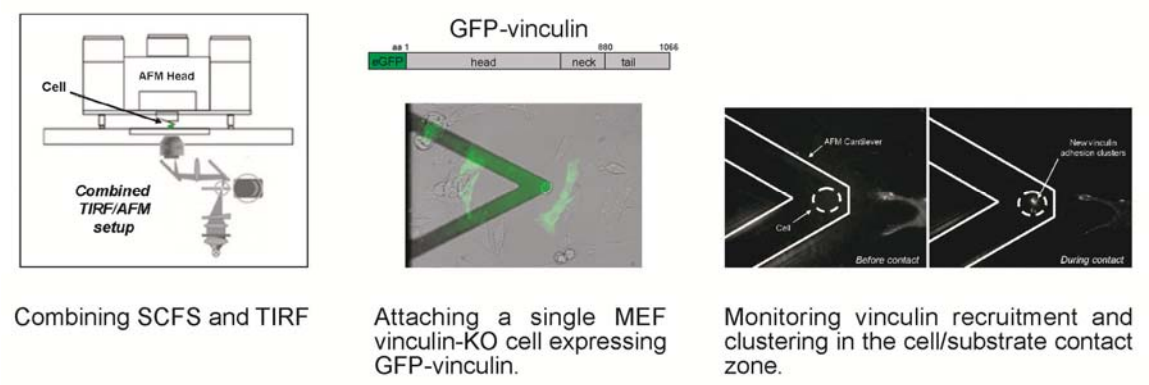

Fig. 5. Combined AFM and TIRF setup. Left panel: Schematic depiction of the AFM head scanner mounted onto an inverted optical microscope. A cell attached to an AFM cantilever is positioned so that the cell/substrate interface can be imaged by TIRF. Middle panel: by expressing a fluorescently-tagged protein, such as a vinculin-GFP fusion protein, nascent cellular adhesion sites can be visualized during SCFS. Right panel: early adhesion sites appearing within the cell/substrate adhesion site during SCFS.

5. Patterned 2D cell culture substrates for SCFS

AFM-based single-cell force spectroscopy (SCFS) is a sensitive method for measuring cell adhesion forces down to the single-molecule level. In SCFS measurements surfaces homogeneously coated with components of the extracellular matrix (ECM) are commonly used as adhesive substrates. In the natural environments of many cells, however, ECM molecules are arranged into complex scaffolds displaying a high degree of nano- and microscale structuring. Artificial micropatterned ECM substrates may mimic the cellular environment more closely than homogeneous substrates and ensure that results from SCFS experiments can be applied for understanding adhesive interactions between cells and ECM within a physiological setting.

In close collaboration with the group of Prof Martin Bastmeyer (E2.3), we have developed different methods for producing micropatterned substrates with low background adhesion. Firstly, using a thiol microcontact printing technique on gold surfaces, we have generated micrometer-sized ECM dots separated by low-adhesion areas passivated with polyethylenglycol (PEG) molecules (Fig. 6). In a complementary approach, we microcontact-printed ECM patches on muscovite mica surfaces and passivated the remaining areas with an ultrathin layer of collagen I. While an efficient adhesion promoter in many cell types, collagen arranged into an ordered monolayer provides an extremely low adhesion surface for cells not expressing collagen receptors of the integrin family, such as Chinese Hamster Ovary (CHO) cells.

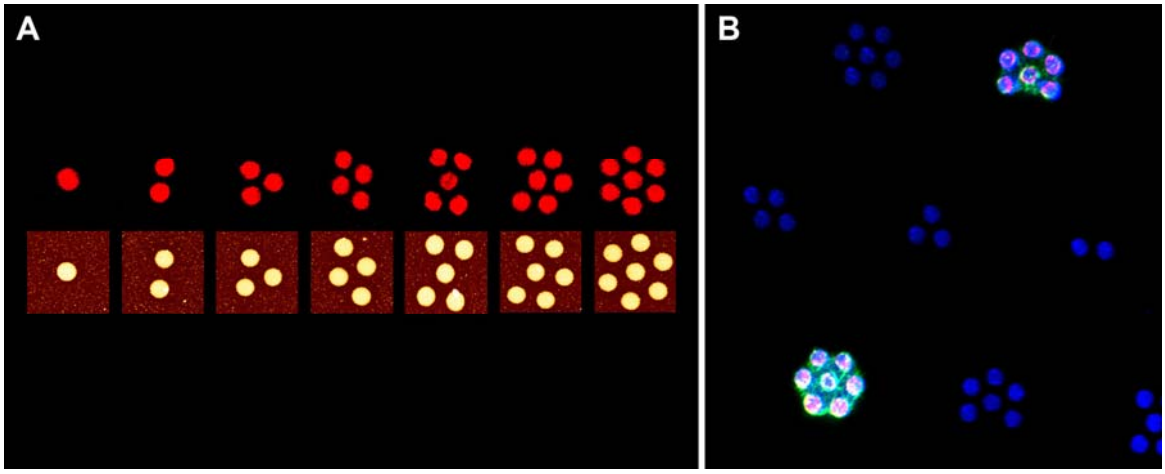

Fig. 6. Microcontact-printed laminin pattern. (A) Flurescence (top) and AFM (bottom) images of different adhesive dot patterns. (B) Fibroblasts cells adapt to the layout of the adhesive contacts presented by each individual pattern. Cell adhesion formation on these micropatterns are currently investigated by SCFS.

Furthermore, using direct laser writing we have produced narrow arrays of micrometer-sized columns, subsequently functionalized with ECM proteins. In this case the three-dimensionality of the created structures ensures zero background adhesion between the adhesive areas (top of the columns). Our results show that all three patterning methods produce substrates with highly specific adhesion patches separated by areas of low or zero non-specific adhesion. We are currently analysing the effect of different substrate patterns on the dynamics of cell adhesion formation, total adhesion strength and the shape of the AFM rupture force curves. First results indicate that specific rupture event patterns in the force curves correspond to the created pattern configurations.

6. Calibrating 3D cell culture substrates

Currently flat and rigid supports, such as glass or tissue culture plastic, are most commonly used for culturing eukaryotic cells. The natural environment of many cells, however, is formed by pliable, three-dimensional scaffolds. Recent studies suggest that the three-dimensional shape and flexibility of the extracellular environment governs important cellular processes, such as differentiation and growth. Understanding the complex mechanical interplay between cells and their environment therefore requires an accurate quantitation of cellular traction forces. To examine the scale of cellular traction forces, flexible three-dimensional cell culture substrates (Fig. 7) produced using direct laser writing were developed in the research groups of Prof Martin Wegner (A1.4) und Prof Martin Bastmeyer (E2.3). Light microscopy observations demonstrated that cellular traction forces are sufficient to deform these structures severely. However, quantitatively correlating scaffold pliability to cellular traction forces requires that the mechanical properties of the scaffold is known. Using the AFM cantilever as a microindentor, mechanical properties of the flexible structures (i.e. the E modulus) were determined and cellular traction forces could be approximated [E2.4:2], [E2.4:6].

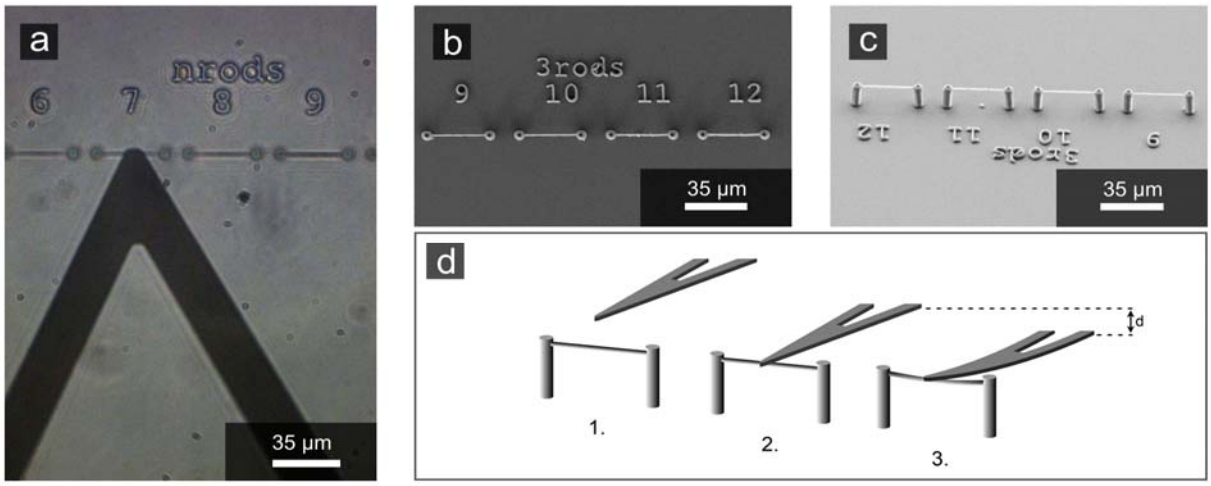

Fig 7: (a) Light microscopy image of an AFM cantilever in contact with a flexible structure produced by direct laser writing. (b) and (c) REM images of the test substrates. (d) Schematic depiction of a substrate calibration measurement. The cantilever is first positioned above a test structure (1). The piezo-driven cantilever is lowered until contact with the test substrate is established (2). The cantilever is lowered further until a preset contact force is reached (3). From the required piezo travel distance d, the substrate indentation can be deduced.

7. Additional collaborations with other CFN Projects

Incorporating a Bio-AFM setup into the CFN technology platform provides the opportunity to expand the scope of several other nanobiology projects. Synergies include the use of microstructured substrates for sensitive cell adhesion force measurements, the calibration of flexible cell culture substrates, the possibility to image fragile biological samples under physiological conditions and the monitoring of individual steps of biofunctionalization protocols. In particular, current cooperations within the CFN include:

- Studying hydration-dependent lipid bilayer spreading by high-resolution AFM scanning in liquid with S. Sekula (E3.2)
- Investigating the nanoroughness of optical resonators with H. Kalt (A5.4)
- Imaging DNA-Nanostructures by AFM with H. Puchta (C5.4)