

Review

Atomic Force Microscopy as a Tool in Nanobiology Part I: Imaging and Manipulation in Cytogenetics

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Abstract.

Shortly after the physics Nobel prize award for the invention of the scanning tunneling microscope (STM), Gerd Binnig and Calvin Quate built the atomic force microscope (AFM) in order to avoid the limitations of the STM to image only conductive matter or thin layers of organics (1). Using the miniaturized record player principle similar to a stylus profilometer, it was then possible to image the surface of biological (non conducting) objects such as DNA and chromosomes down to the molecular scale (2-4). Most important in the development of the AFM to a universal instrument in bio-nanotechnology applications was the fact, that the tip of the cantilever used for imaging could also be used for measuring forces at the nanoscale and moreover as a nanoscale tool, down to the single atom level (5). Here we

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Key Words: AFM, high resolution imaging, micro-and nanodissection, nanoextraction, metaphase chromosome.

show how techniques like cutting, gripping and extracting biomaterial at the submicron scale under high-resolution image control have been developed, especially in cytogenetic studies. The combination of the nanomechanics tool box and modern biochemical techniques like PCR has immense potential for the future development of single molecule techniques, ranging from applications in DNA mechanics to cytogenetic studies and biochip development.

AFM as an imaging tool

Structural analysis with high resolution provides detailed information not only on high molecular complexes but can also be used for in vivo experiments of biological systems. Data can be recorded in real time. Besides structural information of biological systems, 3-dimensional topological data, micro mechanical behavior, dynamic processes and molecular interactions can be recorded. Table I shows a short comparison of AFM to other microscopic techniques, including the required sample preparation.

Cytogenetics is basically a visual science. Established microscopic techniques, such as light and electron microscopy, have been widely used for the study of chromosomes. After the invention of the atomic force microscope, it has been applied in different fields of genetics. Double-stranded DNA fixed on freshly cleaved mica was imaged in air by several groups (7,8). Hansma and coworkers successfully imaged plasmid-DNA fixed on mica in propanol (9). By adding new spreading chemicals, e.g. quaternary ammonium salts, it was possible to reduce surface impurities so that the surface density of the molecules could be reproducibly measured (10). After the introduction of the tapping mode, it became possible to image DNA with less potentially destructive shear forces during scanning, which resulted in a more detailed image of the macromolecule (11). In Figure 2a we have imaged a double-stranded plasmid, pUC19, in tapping mode in air. DNA in a higher condensation status in sperm cells was imaged by Allen and coworkers in air and in liquids (12).

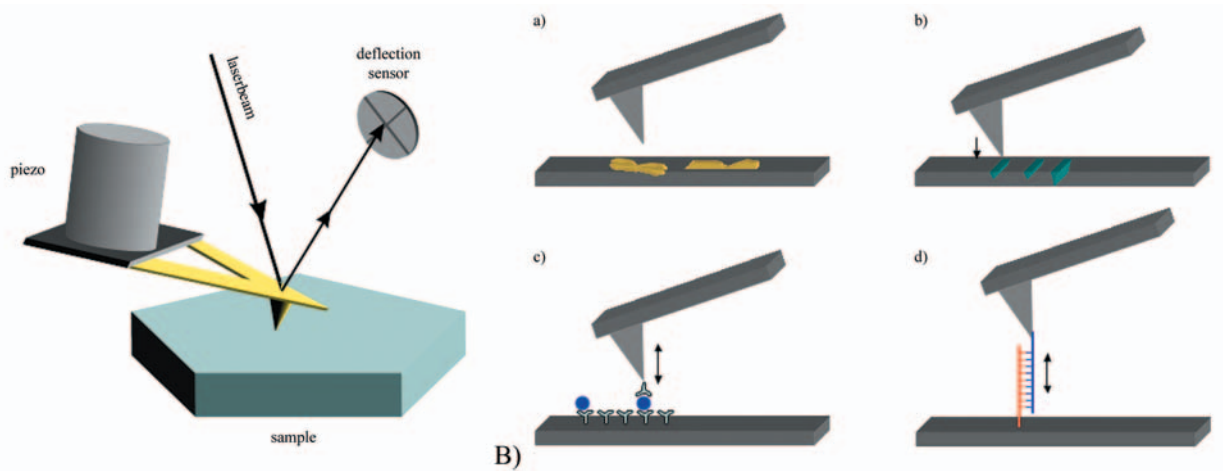


Figure 1. A) Set up of an Atomic Force Microscope (AFM), consisting of a piezo driven actuator and a laser beam deflection sensor and B) different methodical applications of an AFM in cytogenetics as: a) imaging tool, b) manipulation tool and c) –d) as a force sensor.

Table I. Comparison of different microscopic techniques to image the sample topography, e.g. metaphase chromosome (6)

	Conventional optical microscopy (SEM)	Scanning electron microscopy electron microscope (FEISEM)	Field emission in lens scanning (AFM)	Atomic force microscopy
Microscopic environment	ambient liquid vacuum	vacuum	liquid vacuum	ambient vacuum
Field depth	Small	high	medium	medium
Focus depth	medium	small	very small	small
Resolution				
x,y	100 nm	5 nm	0.7 nm	0.1-1.0 nm
z	n/a	n/a	n/a	0.01 nm
Magnification	1x-2*10 ³ x	10x-10 ⁶ x	9x-10 ⁵ x	5*10 ² x-10 ⁸ x
Necessary sample preparation	low	critical point drying or freeze-drying, coating	Critical point drying if necessary	low
necessary sample properties	samples do not have to be completely transparent	samples should not change and have to be vacuum compatible to visible light	vacuum compatibility	samples should not have excessive changes in height compared to tip geometry

Furthermore, Fritzsche and coworkers performed structural experiments on chromatin fibers (13), as well as volume determinations on metaphase chromosomes (14).

Structural examinations on metaphase chromosomes were performed by Heckl (2), where the comparison to electron microscopy was also made and later by de Grooth and Putman (15) and by Rasch *et al.* (16). Figures 2c and d show untreated human metaphase chromosomes. Using chemically and enzymatically untreated metaphase chromosomes, a GTG-like banding pattern, G-bands by trypsin using Giemsa, could be observed in the topographic images (17). Metaphase chromosomes imaged by AFM have revealed structures similar to those reported in light and electron microscopy.

Depending on the preparation technique, substructural details can be recorded in metaphase chromosomes (18, 19). After pepsin digestion of the metaphase chromosomes, a granular substructure was detected in contact mode. In this case not only the covering plasma layer but also scaffold stabilizing proteins were digested. The recorded details represent a nucleosomal structure, which was discussed by several authors (20,15,21,22). The recorded data are comparable to that generated by scanning electron microscopy (23). Metaphase chromosomes consist of 30 nm fibers folded in a tandem array of radial loops, which are packaged into a fiber with an overall diameter between 200-250 nm. High resolution AFM images of metaphase

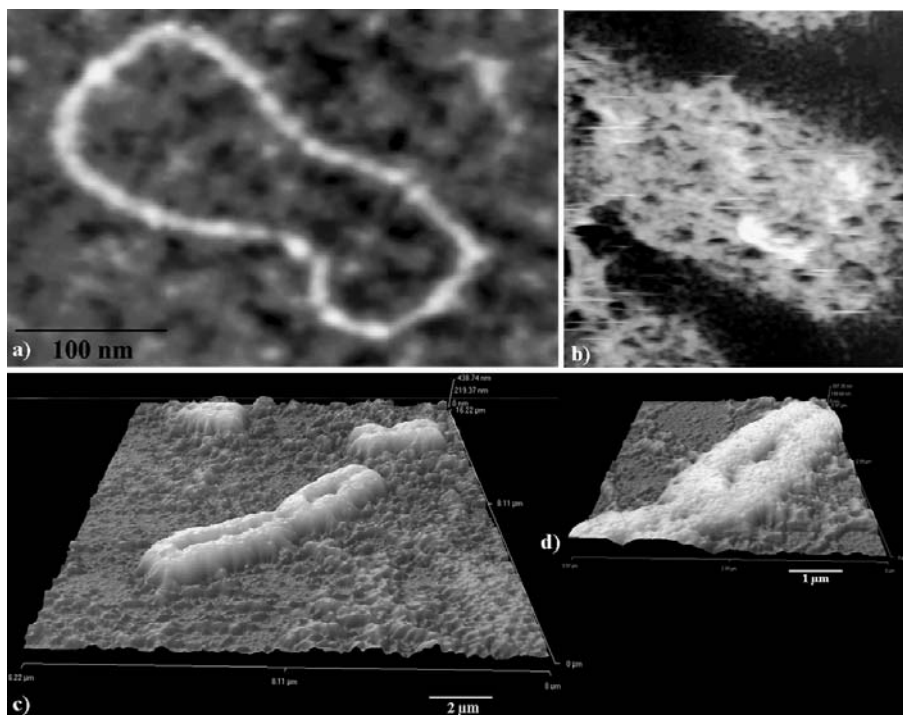


Figure 2. a) AFM image of plasmid pUC19 (~2.7 kbp) diluted in dH_2O ; deposited by spin stretching and imaged in tapping mode in air, bar: 100nm; b) metaphase chromosome after proteinase K digestion, fibrous network is clearly detectable; c) three-dimensional AFM image (recorded in contact mode) of untreated human chromosomes, bar: 2 μ m; d) AFM enhancement of the p-arm of the recorded chromosome, bar: 1 μ m.

chromosomes revealed structural features in the size range of 30-100 nm, which correspond to the loops of the 30 nm fiber (15). Other authors (20, 24) have reported features as small as 10-20 nm, which could correspond to individual nucleosomes. When scanning in contact mode the tip of the AFM can be contaminated by unwanted pick up of chromosomal material. The material adhering to the tip can limit the use of the tip for manipulation and microdissection experiments (see section AFM as a manipulator and dissecting tool). In non-contact mode the tip scans at a distance of a few hundred Angstrom over the chromosomal surface. The tip is not in contact with the sample surface and therefore does not get contaminated while scanning. The advantages and disadvantages of these two operating modes are combined in the tapping mode (25), in which the tip is in periodical contact with the chromosomal surface. In addition, lateral force microscopy was used to get a deeper look inside the chromosomal organization (26).

In conclusion, the described operation modes can be used for imaging chromosomal material, and to record substructure, depending on the sample preparation. The topography of the metaphase chromosomes are preserved and not deformed (15,17). Table II summarizes the methodical properties of contact-, non-contact- and tapping-mode for high resolution imaging and manipulation of metaphase chromosomes.

By using specific *in situ* hybridization techniques, distinct areas of hybridization can be detected in metaphase chromosomes. Biotinylated DNA probes were used for mapping and the specific sites were visualized by detecting the changes in topography induced by a peroxidase-diaminobenzidine reaction (27,16). Using the same detection technique, Kalle and coworkers were able to identify specific signals after RNA *in situ* hybridization (28). In studies on cereal chromosomes, a genome specific probe was used. By AFM imaging changes in height due to biotin-avidin-fluorescein isothiocyanate complexes, formed as a consequence of fluorescence *in situ* hybridization procedures, were detected (24). *In situ* hybridization with subsequent detection of the specific DNA probe was performed in our group. The specific hybridization signal was detected using 5 nm gold particles with subsequent silver enhancement (see Figure 3c)).

The AFM can also image genetic material in liquids. The viscoelastic properties of rehydrated chromosomes and volume determinations in liquids were recorded by AFM (29,22). In comparison to light- or electron microscopy, the AFM is able to operate in liquids and to perform local measurements at any point of the sample surface (30). Thus, data of the biophysical properties of the metaphase chromosome can be obtained.

Chromosome banding techniques have facilitated the precise identification of individual chromosomes. The GTG

Table II. Methodical properties of the different operation modes in AFM for high resolution imaging, manipulation and microdissection of metaphase chromosomes

operation mode	Contact mode	Non-contact mode	Tapping mode
tip loading force	low ° high	low	low
contact with sample surface	yes	no	periodical
manipulation of sample	yes	no	yes
contamination of AFM tip	yes	no	yes
microdissection	yes	no	no

banding obtained by digesting the chromosomes with proteolytic trypsin followed by Giemsa staining is the most widely used in routine chromosome analysis. The interpretation of the GTG-bands is still in progress. A direct role of the Giemsa stain in producing the GTG-bands was suggested (31). Several authors inferred that chromosomes contain a pre-existing structure, which is enhanced by GTG-banding. However, it is still unclear how this enhancement occurs (32,33). It is hypothesized that the differences between positive and negative GTG-bands may be induced by the spatial organization of chromosomal protein and DNA.

In atomic force microscopy no changes in color, as in light microscopy, can be detected. Differentiation can only occur by topographical information of the metaphase chromosome. The resulting image is not only the result of topographical changes in the chromosome surface, but also of the interaction of the tip with the viscoelastic properties of the chromosome. Figure 3a shows a topographic AFM image of a GTG-banded chromosome 7 homologue. The morphology of the chromosome is preserved; the banding pattern and the fibrous nature is detectable. Structural protrusions along the chromosome corresponding to the dark bands in Figure 3a are detectable. A linescan of the q-arm shows differences in height between dark and light bands of about 90 nm. The length of the ridges is about 540 nm. It is known from chromosomes imaged by scanning electron microscopy that the Giemsa light and dark bands differ in height (34). One must be aware that the AFM image not only represents the topology of the sample surface but also the compressibility of the sample, therefore height is partially expressed as topography. Figure 3b shows a human 2n=46, XX, female metaphase spread. The light and dark bands are clearly detectable and all chromosomes could be identified.

It is possible to identify features equivalent to G-banding pattern in untreated chromosomes and to use these for classification (17). As in light microscopy, dark and light bands can be correlated in GTG-banded chromosomes and can be classified accordingly (35). In former AFM studies, G-positive bands were detected to be areas with a higher surface relief (16).

After more than 20 years the discussion about the banding mechanism is still in progress. Not all related

biochemical and physical reactions are understood up to now. Chromosomal banding techniques produce a banding pattern in chromosomes to allow their specific classification. The comparison of unstained and Giemsa-stained chromosomes by phase contrast microscopy (36, 31) gave the basis for the hypothesis that staining techniques amplify a pre-existing structure of incomplete structural organization of chromatin. Electron microscopy supports this hypothesis (37,38). McMaster and coworkers suggested an influence of the stains on structure and morphology, based on their work on untreated metaphase chromosomes (39). In scanning electron microscopy light and dark bands in the R- and G-banding pattern can be differentiated by changes in height (34). This suggests that high resolution AFM has allowed an intrinsic banding pattern to be visualized which otherwise would have to be enhanced by accumulation of stains for viewing by light microscopy methods. The accuracy of chromosomal banding is strongly related to DNA organization and the associated proteins. AFM images in air and liquids of RNase, pepsin or trypsin-treated chromosomes suggest that the level of organization consists of a radial arrangement of chromatin loops, which are anchored to a folded fiber giving a pattern of bands differing in volume. Furthermore a model derived out of these data is proposed to link genome sequence, cytogenetics and chromosome structure (40,41).

The C-banding technique produces selective staining of constitutive heterochromatin. These bands are mostly located at the centromeric regions of chromosomes, hence they are known as C-bands. The original method described by Arrighi and Hsu (42) primarily involves treatment with an alkali, sodium hydroxide, to denature the chromosomal DNA, and then subsequent incubation in a salt solution. Another method, described by Sumner (43), utilizes a milder alkali, barium hydroxide. Both methods produce similar characteristic C-banding patterns. Figure 3c shows the AFM image, recorded in contact mode, of CGB-banded metaphase chromosomes. In comparison to light microscopic images, the stained centromeric regions are clearly detectable. C-banding for studying chromosome rearrangements near centromeres and for investigating polymorphisms was performed with AFM by Tan and coworkers (44).

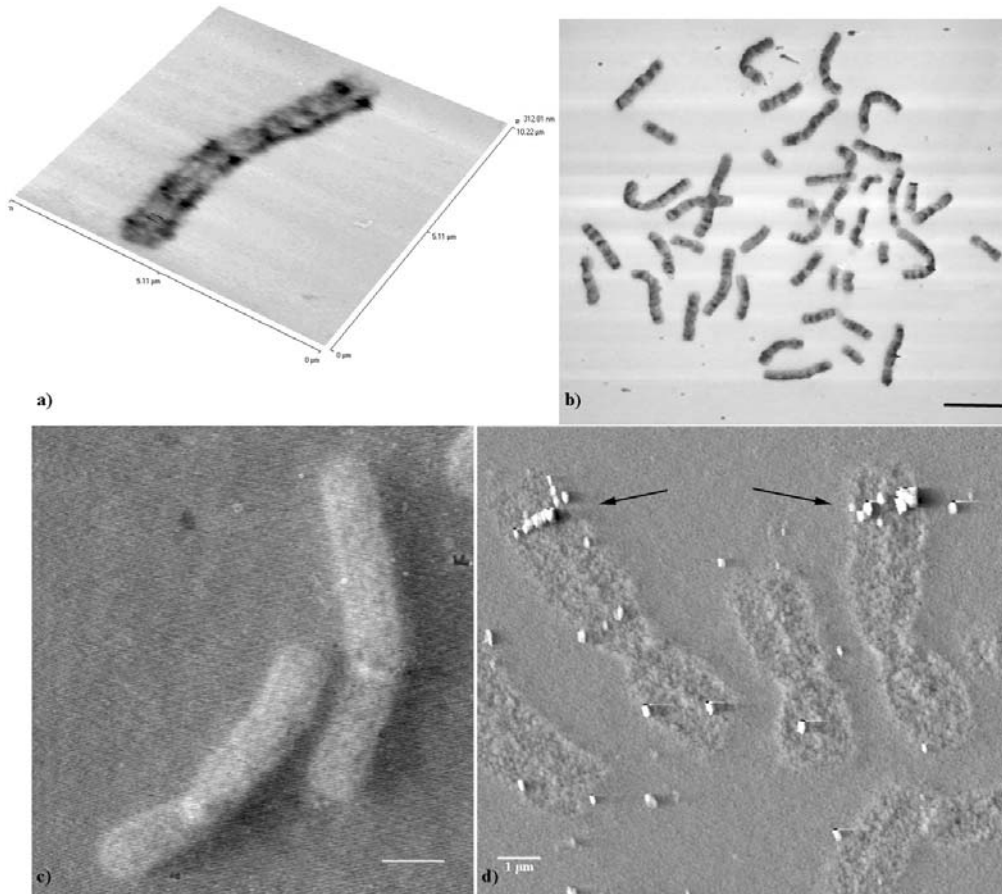


Figure 3. a) AFM image of a GTG-banded chromosome 7 homologue, topographic AFM image, gray scale inverted: the bright and dark banding pattern is detectable; b) AFM image of a $2n=46, XX$, female GTG-banded metaphase spread, bar $10 \mu\text{m}$; c) AFM image of CBG-banded metaphase chromosomes spread with highlighted centromeric region, bar: $2 \mu\text{m}$; d) AFM error signal image of in situ hybridized human metaphase chromosomes after detection using 5nm gold particles with subsequent silver enhancement. The probe used was MLL 11q23 (see arrows); bar: $1 \mu\text{m}$.

The detailed understanding of nuclear cell functions requires an accurate knowledge of the spatial organization of nuclear structures. The approach by scanning electron microscopy (SEM) provides higher resolution compared to light microscopy and permits surface analysis of the chromosomal structure, which cannot be adequately obtained from transmission electron microscopy (TEM). Nevertheless, in order to obtain high resolution in SEM observations, the use of a high electron accelerating voltage (up to 30 kV) is required (45-47). Under these experimental conditions, sputter-coating or conductive staining of the samples is generally required (48). Both procedures allow electron-charging dispersion from the sample but may obscure fine details and produce sample alterations (49). Today, only a few techniques are available for high resolution imaging of chromosomal material with reduced artifacts, such as the Field Emission In lens Scanning Electron Microscope (FEISEM) and the Atomic Force Microscope (AFM). The FEISEM represents a special kind of SEM, fitted with a cold cathode field emission electron

gun (50,51) that can operate at low accelerating voltage with reduced electron charging of the sample. In fact, the low voltage and low current electron beam of the FEISEM together with a liquid nitrogen anti-contamination device in correspondence to the specimen area and an "in lens" assembly of the electron-optic column allow high resolution imaging of the biological sample without any conductive staining or metal coating. Contamination of the specimen is greatly reduced compared to conventional SEM (50). The sample location between the objective pole pieces limits the dispersion of the secondary electrons collected by the magnetic field of the lens. In conclusion, these characteristics allow the observation of uncoated biological samples with a higher resolution than with conventional SEM (52-55).

FEISEM and AFM microscopy can be combined to observe the same metaphase chromosome samples obtained from standard cytogenetic preparations of human HL 60 cells. After cleaning the metaphase spreads with different procedures (52), the analysis of the same samples can be

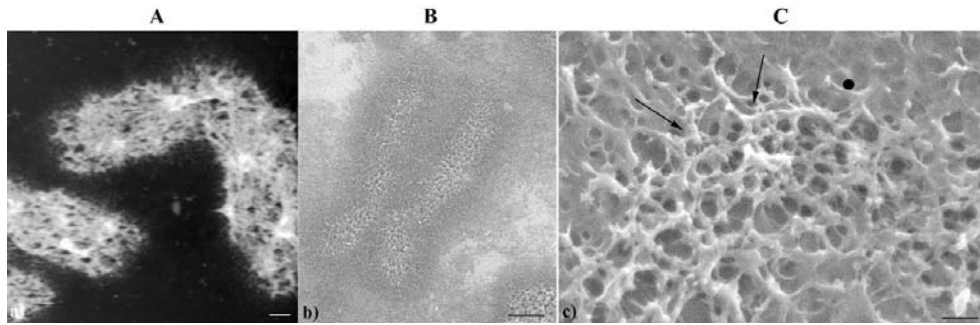


Figure 4. Human metaphase chromosomes after proteinase K treatment: a) AFM image, bar: 1 μm ; b) field emission inlens scanning electron microscopy image of a comparable chromosome. A dark halo surrounds the entire chromosome, bar: 1 μm ; c) increasing the magnification, the chromosomal surface appears to be constituted by a network. Some fibrillar structures parallel to the axis of the chromatid are detectable (arrows). The halo around the chromatid appears to be formed by a mix of fibers and homogeneous phase (dot), bar: 100 nm.

facilitated by the use of conductive glass (ITO glass) for the chromosome map preparation. These technical approaches show a high correlation of the respective morphological information, both in normal and treated samples. The high resolution potential of the FEISEM, together with the possibility of observing hydrated samples and/or nanomanipulating the specimen with the AFM, confirm morphological data and offer enhanced information on their biological significance (see Figure 4 a-c) (19).

AFM as a nanomanipulation and dissecting tool

By combining high structural resolution with the ability to control the image parameters at any place of the scan area, it is possible to use the AFM as a manipulation tool. In 1991, Hoh and coworkers demonstrated the possibility of using AFM as a microdissection device (56). They performed microdissection on junctions between cells. Controlled nanomanipulation of biomolecules was performed on genetic material (9). One hundred to 150 nm fragments were cut out of circular plasmid DNA rings. Isolated DNA adsorbed on a mica surface was dissected, in air (57-59) and in liquids, e.g. propanol (9), by increasing the applied force to about 5 nN at the AFM specimen. These experiments demonstrated the feasibility of microdissection in the nanometer range. Combining AFM imaging and microdissection, the organization of bovine sperm nuclei was observed and showed small protein and DNA-containing subunits of 50 to 100 nm in diameter (12). Tobacco mosaic viruses were dissected and placed on a graphite surface to record the mechanical properties of the virus binding (60).

Chromosomal dissection allows direct isolation from selected regions and can be used to build chromosome band libraries (61), mapping for cytogenetic analysis and for specific cloning projects. AFM microdissection of genetic material in different condensation status, like polytene

chromosomes of *Drosophila melanogaster*, was performed by the group of Henderson (62, 63). In thin chromosomal regions the cut size was 107 nm; in larger regions, depending on the AFM tip, the size increased to 170 nm. Also human metaphase chromosomes were microdissected and the extracted material was used for subsequent biochemical reactions (64-66). Manipulation of mouse chromosomes using modified tips and amplification of the collected material with subsequent southern hybridization of the extracted single-copy genomic DNA was described (65). AFM microdissection in a dynamic mode for the chemical and biological analysis of tiny chromosomal fragments was shown (66). In this approach the marker gene of the nucleolar organizing region (NOR) was amplified by designed primers for the 5.8S ribosomal DNA after performing a series of single-line scan microdissections. The dissected chromosomal fragments were collected in a second step by conventional microcapillary.

As illustrated in Figure 5 a-b, chromosomes can be dissected at selected regions by using non-contact imaging of the GTG-banded metaphase chromosomes and the microdissection process can be documented (64). Figure 5c shows an electron microscopic image of an AFM tip after microdissection. In this direct approach, the extracted genetic material, adhering to the tip, can be amplified by unspecific polymerase chain reaction and used as a probe for fluorescence *in situ* hybridization (FISH) (64). As previously described, AFM can also operate in a liquid environment. While performing microdissection in liquids, only uncontrolled dissections can be produced on rehydrated chromosomes (30).

The procedure of AFM nanoextraction

The methodical procedure of AFM-based micro- and nanodissection is described in references 64 and 67-69. All methodical steps are performed under sterile conditions to

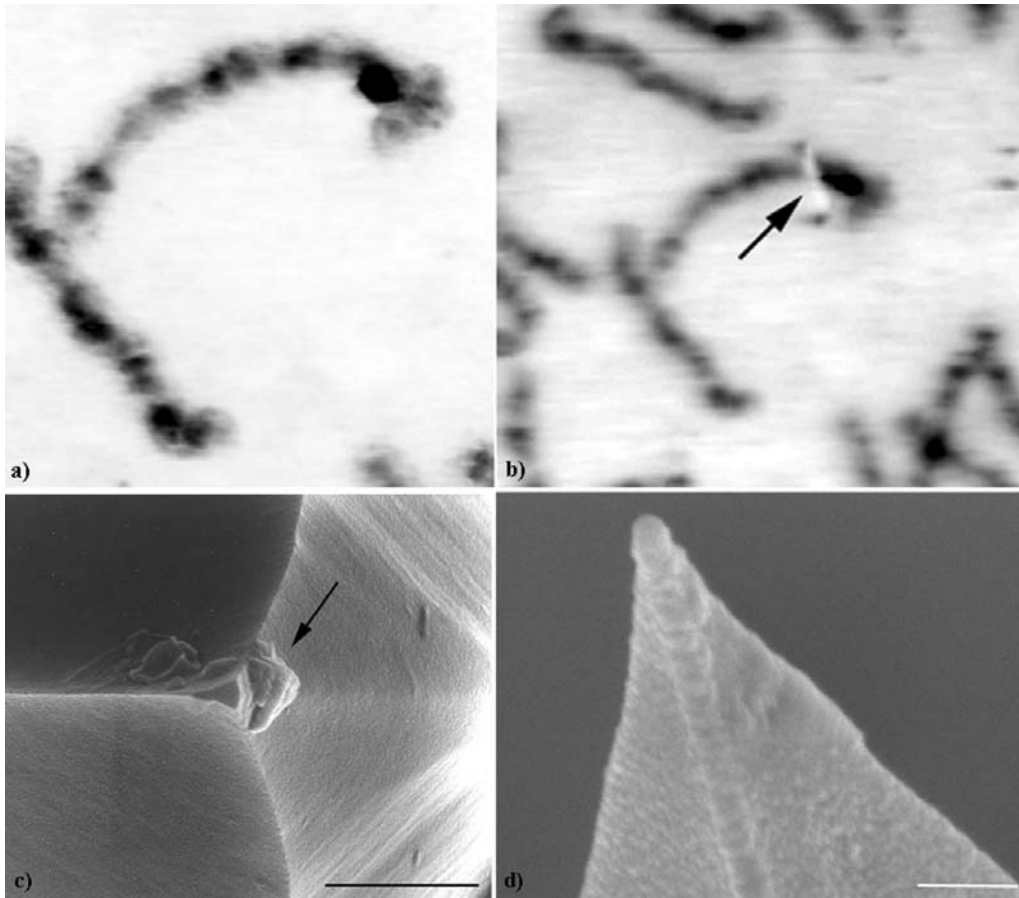


Figure 5. a) Non-contact AFM image of the GTG-banded human chromosome 7 before microdissection; b) AFM microdissection of the band 7q32 (see arrow); c) electron microscopic images of the AFM tip after microdissection. The arrow indicates the extracted DNA, bar: 1 μm ; d) electron microscopic image of an electron beam-deposited rough AFM tip to increase the extraction efficiency; bar: 200 nm.

avoid contamination. To identify the chromosomal region of interest and to minimize the contamination of the AFM tip, while scanning the area of interest, GTG-banded metaphase chromosomes are imaged in non-contact mode in ambient air. The identification can also be performed with a "pre-set" *in situ* hybridization of chromosome-specific painting probes. The chromosome can be identified *via* fluorescence microscopy (64) or AFM gold particle detection (Figure 3d). This "pre-set" hybridization also increases the amount of extracted genetic material. For microdissection, the chromosome is placed at a 90° angle to the scan direction and the chromosomal area is zoomed into. For distance control, amplitude detection is used and the damping level is set to 50% of the amplitude of free oscillation for imaging before extraction. After identification of the extraction site the scan is stopped and the feedback turned off. The loading force of the tip onto the sample is increased. Figure 6a-e shows the results of AFM microdissection by applying different loading forces to the specimen and by controlling the modulation of the z-piezo.

Depending on the applied loading force the micromanipulation of the metaphase chromosome results in a microindentation or microdissection (Figure 6 b)-c)).

To extract DNA, a single line scan at 1 $\mu\text{m}/\text{sec}$ is performed at this site. During dissection of the chromosome, the lateral forces play an important role. The tip performs a stick-slip movement and the forces between the tip and chromosome are reduced while operating with z modulation. The shear forces of the tip are reduced during dissection and reproducible cuts of 100 nm are possible, depending on the geometry of the tip. By changing the tip after each microdissection, serial cuts can also be performed (Figure 6d). During microdissection, not only the apex, but also the flank of the tip is in contact with the chromosome. Thus the loading area to the chromosome is increased and, under constant force, the loading force applied to the tip is decreased. The chromosomal material is not dissected in a first step but pushed like using a snowplough. Parts of the chromosomal material adhere *via* Van-der-Waals interaction and unspecific adsorption to the tip. Electron beam-deposited

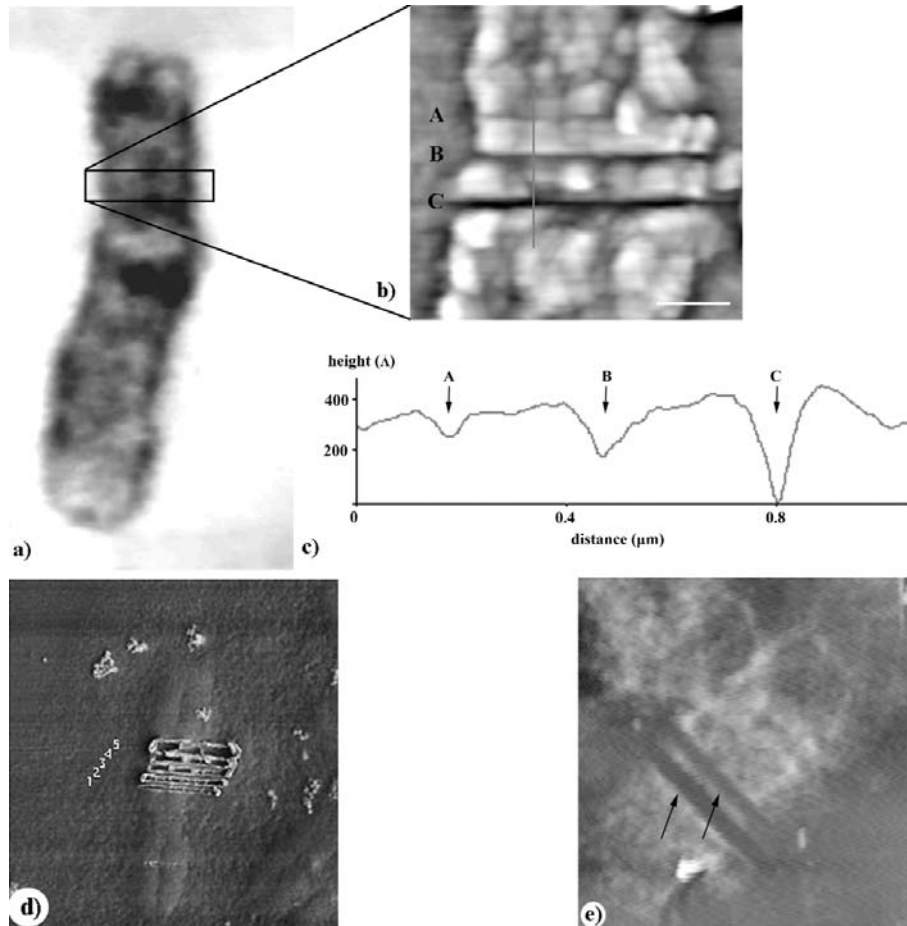


Figure 6. a) GTG-banded human chromosome 9 imaged in non-contact mode, frame marks the area of a series of microdissections; b) the chromosomal part was imaged by AFM in ambient conditions after a series of dissections made by AFM. For dissection, z-modulation ($\sim 5\text{nm}$) was used. The oscillation amplitude of the cantilever was smaller than 1% of the amplitude of free oscillation for all cuts. Each cut was performed by scanning one line scan at $1\ \mu\text{m/s}$ with a set up loading force: #A: $7\ \mu\text{N}$, #B: $9\ \mu\text{N}$, #C: $13\ \mu\text{N}$. bar: $500\ \text{nm}$ c) cross sectional analysis along the red line indicated in b. The applied forces result in an indentation (#A: $7\ \mu\text{N}$, #B: $9\ \mu\text{N}$ and total dissection of the metaphase chromosome; d) electron microscopic image of human metaphase chromosome after microdissection with vertical modulation of the z-piezo. Loading forces were #1: $16.8\ \mu\text{N}$, #2: $19.6\ \mu\text{N}$, #3: $22.4\ \mu\text{N}$, #4: $25.6\ \mu\text{N}$, #5: $> 27\ \mu\text{N}$; e) serial microdissections with the AFM, arrows mark the dissection.

tips (EBD) with a rough surface can be used to increase the extraction efficiency (Figure 5d) (Thalhammer *et al.* 2003 in preparation). The modified AFM tips can be used like a mechanical "nanoscalpell" and a "nanoshovel". As shown above, the influence of the physical parameters used for nanomechanical dissection is important for the result. Nanostamping by applying an oscillatory vertical movement of the tip, while cutting in a horizontal direction, is most important to avoid pure horizontal tearing of the chromosome instead of precise cutting and extraction (30). After the tip has been retracted from the sample surface, the cantilever is transferred into a reaction tube. A new cantilever is used to check the cut at the nanoextraction site on the chromosome. The reaction tube contains a collection buffer to stabilize the extracted genetic material. Enzymatic digestion of the chromosome stabilizing and covering proteins is performed to increase primer binding and

therefore the efficiency of the polymerase chain reaction (PCR). Unspecific amplification can be performed with PCR techniques using degenerated primers (64) or linker-adaptor PCR (Thalhammer *et al.*, 2003, in preparation). The generated genetic samples can be used for further cytogenetic studies, e.g. FISH (64) or amplification of specific target sequences (Thalhammer *et al.*, 2003, in preparation).

Microdissection has been performed with AFM in combination with laser cutting. In a specially designed experimental set up, the minimum cut sizes of human metaphase chromosome achieved by laser (in the order of $500\ \text{nm}$) have been compared to AFM-tip cuts (as small as about $100\ \text{nm}$) (64). This showed the advantage of the AFM technology with respect to precision towards single molecule manipulation (70). In addition the integrated set-up allowed for the *in situ* characterization of laser cuts far below the diffraction limit of light microscopy.

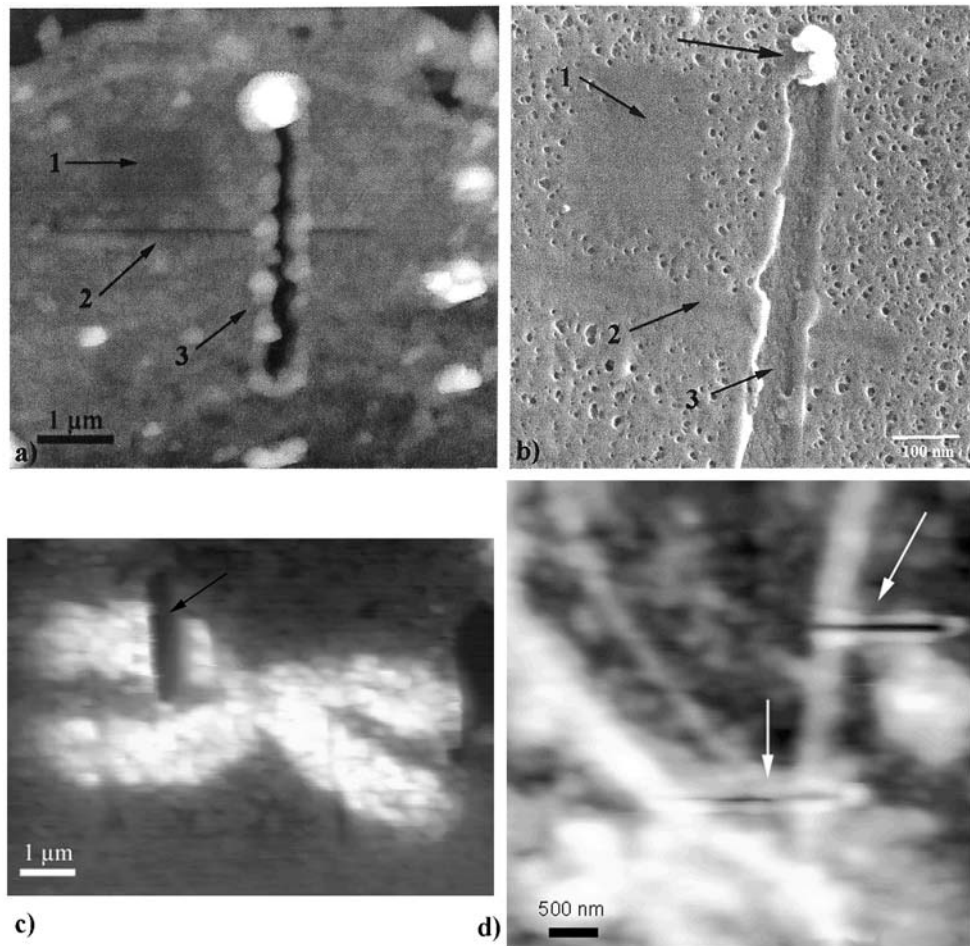


Figure 7. Atomic force microscopy microdissection of a) an interphase nucleus and b) corresponding electron microscopic image: increasing forces from 1 to 3 causes indentation or microdissection, big arrow indicates remaining compressed genetic material during extraction; c) microdissection of a single chromatid (see arrows) and d) microdissection of extended chromatin fibers (see arrows).

Conclusion and Outlook

More than ten years ago, at the advent of the AFM we described the ease of use of the AFM and how the possibility of investigating real time dynamics of biological objects in a liquid environment could make it a promising tool for new insights into biological mechanisms and structures in the future (4). This is true today, especially in the area of cytogenetics.

Based on the working principle the AFM cannot only be used for high resolution imaging of surface topography of genetic material, but also, at the same time, it is a perfect tool on the nanometric scale. In addition to high structural analysis and recording of the tip torsion while manipulating the surface structure (Figure 7), it is possible to record data of the 3-dimensional structure of genetic material, *e.g.* metaphase chromosomes or interphase nuclei. When AFM microdissection is applied to different genetic samples, such

as extended chromatin fibers (Figure 9), or single DNA plasmid molecules, it is possible to isolate the smallest cytogenetic samples (Thalhammer *et al.*, 2003, in preparation). Subsequently these samples can be by further processed by highly sensitive polymerase chain reactions and fluorescence *in situ* hybridization, for physical mapping of the genome, evolutionary studies or for diagnostic research. In the future, it will be interesting to implement a near field optical microscope in order to identify a particular genomic region labeled with only a few dye molecules for subsequent nanodissection or to use more sophisticated and smaller detection markers for localizing the gene region to be extracted.

Acknowledgements

We would like to thank M. Hennemeyer and G. Teti for contributing with parts of their work to this overview article. Also, the valuable contributions from G. Wanner, H. Lorenz, P. Gobbi

(Urbino, Italy) and M. Falconi and G. Mazzotti (Bologna, Italy) concerning electron microscopy studies are highly recognized. This work was supported by a Deutsche Forschungsgemeinschaft (DFG) SFB 486 grant, Germany.

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Received December 4, 2003
Accepted December 29, 2003

Review

Atomic Force Microscopy as a Tool in Nanobiology

Part II: Force Spectroscopy in Genomics and Proteomics

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Abstract.

Genomics have contributed greatly to the understanding of the molecular basis of disease and to the development of new therapies and drug design. In the post-genome era, proteomics aim in translating the nucleic acid information

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Key Words: AFM, force spectroscopy, protein-DNA complex.

archive into an understanding of how the cell actually works and how disease processes operate. The most relevant information archive to clinical applications and drug development involves the elucidation of the information flow of the cell: the program of protein pathway networks.

There are several reasons to use the atomic force microscope (AFM) in the field of proteomics. First the AFM provides a detailed look into the overall structure of proteins. Information on size, shape and subunit composition of the protein can be recorded, and on a functional base, it is possible to follow molecular interactions, e.g. bio-assembly processes (1,2). Second, protein surface interactions, the protein surface binding process and how it affects its biological functionality can be investigated by AFM techniques (3,4). The results can be used for the development of new biosensors and immunoassays.

Considering the recent advances in experimental genomics and proteomics, we emphasize the use of AFM as a force spectroscopy tool. When used as a sensor, AFM opens the possibility of enormous change in our ability to analyze and interpret complex biological processes, allowing, for example, the detection and elucidation of protein-DNA and protein-protein interactions.

AFM as a sensor

Understanding the force (derivative of energy with respect to distance) that drives specific molecular interactions is a challenging task in molecular and structural biology, because it is the most direct means of obtaining information about how the interaction energy between two different structures is distributed in space. Such specific interactions result in multiple weak, non-covalent bonds formed between defined portions of the interacting molecular partners.



Figure 1. Schematic set up of force spectroscopy experiments: By immobilizing a molecular partner either over AFM tip and substrate AFM can test a) intermolecular interactions between receptor and ligand; and b) complementary nucleic acid binding between DNA strands.

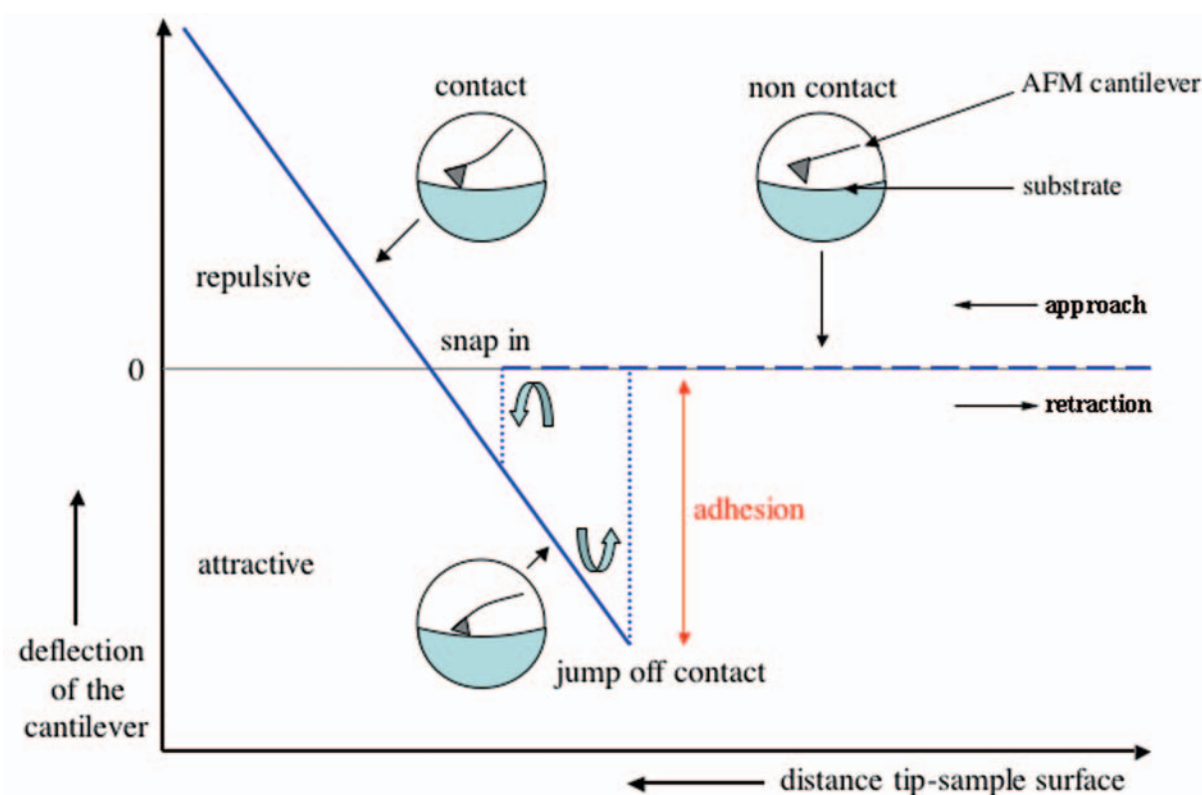


Figure 2. Force contact curve generated by an AFM: The deflection of the cantilever is shown as a function of the tip-sample surface distance. In the beginning of the approach cycle the AFM tip is not in contact with the surface; then, the AFM tip is being pushed into the surface resulting in a bending of the cantilever. In the retraction cycle the tip is being withdrawn from the surface, the tip adheres to the sample surface and, after the jump-off contact from the surface, the tip is brought to the non-contact position again.

The development of the Atomic Force Microscope (AFM), as a Scanning Probe Microscopy (SPM) family member, has opened new perspectives for the investigation of surfaces at high lateral and vertical resolution (5). During the last decade, AFM was proposed to study inter- and intra-molecular interactions forces in biological macromolecules (Figure 1), mainly due its precision and sensitivity to probe surfaces in physiological environments

with molecular resolution and with forces down to the piconewton range (5,6). Thus AFM is a useful tool to evaluate and to characterize mechanical properties of biological samples such as: topography, elasticity and adhesive properties. Furthermore, due to its ability to operate at least as well in liquid as in air, AFM opens the possibility of probing biological sample interactions in aqueous buffer *i.e.*, under conditions, close to their native environment.

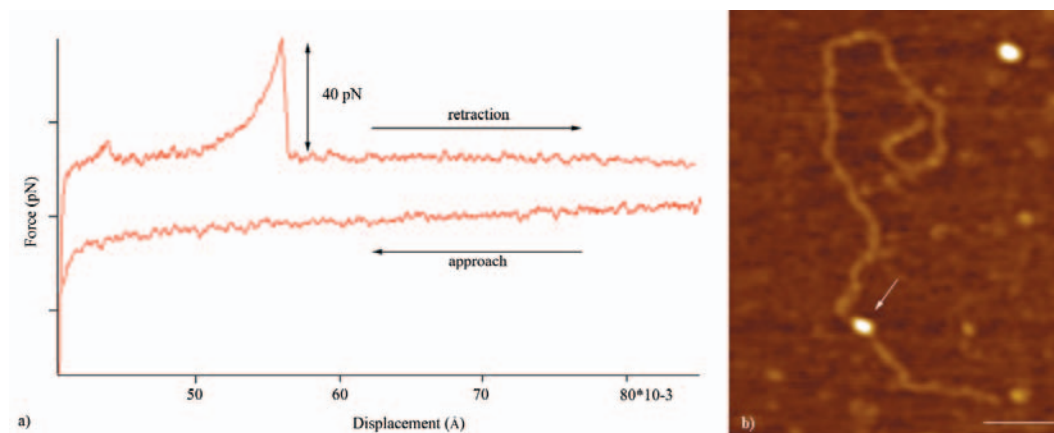


Figure 3. a) Force-distance curve: The protein LexA was covalently immobilized onto an amino group pre-treated glass slide via carboxymethylamylose and DNA (*recA* sequence) was covalently bonded to the AFM tip, also via carboxymethylamylose. An unbinding event could be identified at a certain distance from the point of contact due to the length of the polymer linker (carboxymethylamylose) and the stretching of this linker prior to the jump off contact. b) AFM images of LexA-DNA complex at a single molecule level. The specific binding of LexA to the DNA (see arrow) was achieved by incubating DNA (16 nM) with LexA (34 μ M) for 30 min at room temperature. The DNA fragment contained the *recA* operator. Imaging was performed in dynamic mode under ambient conditions; bar: 100 nm.

When recording a force curve the AFM tip starts far from the sample surface, where no interactions between either exist. As the tip-sample separation is reduced beyond a certain point, attractive dispersion and electrostatic forces between the two surfaces begin to interact, causing the flexible cantilever to bend towards the sample up to a certain point, where strong repulsive forces bend the cantilever outwards. At each distance, the cantilever bends until its elastic (restoring) force equals the tip-sample interaction force and the system is in equilibrium. The resultant plot of the cantilever deflection *versus* the separation between the tip and the sample is the force curve. The AFM combines high force sensitivity in the pico-newton range (7) with high lateral resolution, often better than a nanometer, which is in the molecular range. A typical force *versus* displacement curve generated by AFM is shown in Figure 2.

Inter- and intra-molecular interactions

For quantitative force adhesion measurements, the partners in the molecular recognition reaction have to be immobilized on both the substrate surface and the AFM tip (Figure 1a). Furthermore, the specific interactions must be compared to the background of non-specific probe/surface interactions, which may be as high as, or even higher, than the biological interactions. This is done by using non-functionalized probes or surfaces for comparison, by blocking the interactions between the immobilized molecular partners with free ligands in the medium, or by changing the pH or the salt concentration in the medium.

Concerning intermolecular interactions, biotin/streptavidin (or avidin) has been used as a model system for ligand-

receptor adhesion studies, because of its high affinity and the availability of structural and thermodynamic data (8,9). The unbinding forces of antibody-antigen complexes measured by means of AFM force spectroscopy correlate with the spontaneous association (k_{on}) and dissociation (k_{off}), and the dissociation constant ($K_D = k_{off}/k_{on}$), which describes the equilibrium behavior (10).

Furthermore, single molecule AFM experiments measuring coupling of stretch and twist movements, overstretching mechanics and base pairing forces provided an intimate look into the biophysical details of DNA. Direct measurements of the unbinding forces between single strands of DNA used covalently bound DNA oligonucleotides to the AFM tip and surface, respectively. The adhesive forces between complementary 20 base pair strands are in the nano-newton range, and the intra chain interaction resulting from the molecule's elasticity showed a long-range cohesive force behavior (11,12). Sequence-dependent mechanical properties of DNA measured by unzipping experiments showed that the transition from B- to S-DNA conformation occurs at significantly lower forces (65 piconewtons) in poly (dA-dT) compared to poly (dG-dC) oligonucleotides (13). Using individual double-stranded DNA molecules attached between an AFM tip and a gold surface, the mechanical stability of overstretched DNA was described (Figure 1b). In the case of Lambda phage a DNA B-S transition was observed followed by a second conformational transition, during which the DNA double helix melts into two single strands (14).

A major focus of current genomics research is the detection of single-nucleotide polymorphisms (SNPs) within known gene sequence, as well as in the whole genome (15). Such mismatched base pairing was detected using a gold-

coated AFM cantilever functionalized with thiolated 20- or 25-mer probe DNA oligonucleotides and exposed to target oligonucleotides of varying sequence in static and flow conditions, providing a distinct positive/negative signal for easy interpretation of oligonucleotides hybridization. The association of SNPs with disorders is expected to be particularly useful in identification of cancer and neural degenerative disorders such as Alzheimer's disease and is also related to the individual variations in drug responses (16).

Recently, dynamic studies of DNA-protein interactions came into the forefront of biophysical research. Single molecule force spectroscopy showed the specificity of the interaction between ExpG protein and the promoter regions in the galactoglucan biosynthesis (*exp*) gene cluster. Moreover the binding mechanism involved with respect to its thermal off-rate and additional molecular parameters, describing the energy landscape of the separation process, was characterized (17). Our group has shown the specific interactions between LexA repressor protein, derived from the SOS repair system of *Escherichia coli*, and two specific DNA binding motifs, *recA* and *yebG* (a topographic AFM image of the complex *recA*-LexA formation is shown in Figure 3b)). A typical force curve is shown in figure (Figure 3a)). This experiment showed a pronounced feature in the energy landscape. Along a force-driven pathway between the rupture force and the off rate (k_{off}) value for each operator (Costa *et al.*, 2003 in preparation).

Furthermore AFM force spectroscopy can be used to acquire detailed information in the process of DNA compaction. These studies are important to understand the mechanisms of histone movement for transcription, replication and repair of DNA. To determine the forces necessary to break the bonds between the DNA and histone octamers in the nucleosomal particles, AFM experiments were carried out by stretching single chromatin fibers bound to the AFM tip (18).

In the post-genomic area the understanding of protein folding plays an important role and has wide-ranging impact in structural biology. AFM also allows the study of intramolecular interactions like protein-folding mechanisms, especially unfolding of multi-domain protein molecules or individual protein domains. The unfolding and stretching of each domain creates an individual peak in the force curve, leading to a characteristic saw-tooth pattern. Single molecule force measurements of individual titin immunoglobulin domains showed an unfolding by forces in the range of 150 to 300 pN, and dependent on the pulling speed. After relaxation, refolding of immunoglobulin domains could be observed (19). Using recombinant constructs from different titin parts, the distribution of mechanical stability along more than 200 Ig and fibronectin III domains in titin could be demonstrated (20). It has been shown that the force required for the mechanical unfolding spectrin repeats is between 25

to 35 pN. In other words, the unfolding forces of the α -helical spectrin domains are five to ten times lower than those found in domains with β -fold, like in the immunoglobulin or fibronectin III domains, where the tertiary structure is stabilized by hydrogen bonds between adjacent strands. This demonstrates that the forces which stabilize the coiled-coil lead to a mechanically much weaker structure than multiple hydrogen-bonded β -sheets (21).

The combination of high resolution AFM imaging with force spectroscopy provides an insight into the interaction forces between the individual protomers of the hexagonally packed intermediate (HPI) layer of *Deinococcus radiodurans*. Individual protomers were sequentially stretched, unfolded and removed from a bacterial surface layer until an entire bacterial pore formed by six protomers was unzipped (22). Besides the possibility of single molecule force spectroscopy, the AFM can also be used as a manipulator. This was demonstrated with purple membrane patches, from *Halobacterium salinarum*, where individual molecules of the membrane protein bacteriorhodopsin could be localized, unfolded and extracted from the lipid membrane (23). Further, the influence of pH and local mutations on the stability of individual structural elements of bacteriorhodopsin against mechanical unfolding has been analyzed. The polypeptide loops act as a barrier for unfolding and contribute significantly to the structural stability of bacteriorhodopsin (24).

Cellular adhesion mediated by biological macromolecules and their respective ligands plays an essential role in a number of diverse biological phenomena including inflammation and cancer metastasis. How the adhesiveness of receptor-ligand interactions is controlled by the affinity of the individual receptors to single ligands is not well understood. Using single molecule force spectroscopy, the tensile strength and off-rate of single P-selectin molecules binding to single ligands on intact human polymorphonuclear leukocytes and metastatic colon carcinomas were probed *in situ* and compared to the overall adhesiveness of these cells for P-selectin substrates (25). Characterizing the biochemical and biophysical properties of functional P-selectin ligands on carcinomas will provide guidelines to engineer novel therapeutic agents that will selectively block ligand function and thus interfere with metastatic spreads.

Force-distance curves have been also utilized to identify cell partners that interact specifically in certain biological reactions. Cell-cell adhesion mediated by specific cell-surface molecules is essential for multi-cellular development. By functionalizing AFM tips with whole cells of a given type and studying their interaction with monolayers of other cell types, it was possible to identify the cell type in the uterine epithelium that interacts specifically with cells in the embryo during implantation (26). The cell-

cell adhesion mediated by specific cell-surface molecules has been investigated using a glycoprotein layer, contact site A (csA) as a prototype of cell-adhesion proteins. csA is expressed in aggregating cells of *D. discoideum*, which are engaged in development of a multicellular organism (27,28).

Viscoelastic properties of biomolecules

AFM has also become a powerful tool to measure the viscoelastic properties of biological structures and macromolecules. Whenever the effective stiffness of the cantilever and the biological sample on the surface are of comparable size and the AFM tip is pushed into the sample, the sample is indented. At the time when the stress (deformation force) and the strain (the amount of deformation) are linearly related, the deformation of the material is elastic, and the material will regain its original form upon relaxation. The depth of indentation can be used to perform local elasticity measurements of Young's elastic modulus (the mechanical resistance of a material while elongating or compressing). The capability of the AFM to provide information on the elastic properties of biological structures has been used to study different types of differentiated cells and organelles (29) and human chromosomes (30). The elasticity of human chromosomes was determined in neutral and alkaline pH but also in acetate buffers (30).

The behavior of soft biological samples is different to a hard surface, indicative in the force retraction curve. Whereas lift-off occurs quickly on hard surfaces, it may be considerably slowed down in the case of soft biological samples. For example, the lift-off speed has been used to estimate the viscosity of the sample and the elasticity of lysozyme adsorbed on mica (31).

Force mapping

Individual curves can be assembled into a force-volume providing a three-dimensional, laterally resolved description of the forces within the sample (29). A force-volume can be used to produce sample/surface maps reflecting different properties of the surface as adhesion, viscosity, elasticity, *etc.* Using this approach a map of antigenic sites on a surface by molecular recognition of an antigen by an antibody tethered to an AFM tip has been acquired (32).

The question as to whether cell division is driven by cortical relaxation outside the equatorial region or by cortical contractibility with the developing furrow alone has been approached by monitoring spatially-resolved changes in the cortical stiffness with time. Here force-mapping was used to track dynamic changes in the stiffness of the cortex of adherent cultured cells along a single scan-line during metaphase to cytokinesis (33).

Conclusion

The Atomic Force Microscope (AFM) as a force spectroscopy tool in genomics and proteomics opened new opportunities for studying the mechanical properties of biomolecules and their interactions in their native environment. Those tools have shown useful applications in genomics and proteomics studies by determining the binding affinity of DNA proteins dependent on the target DNA sequence for further correlative studies on physical affinity and biological relevance of the controlled gene. Further, force spectroscopy is a powerful analytical tool to investigate structural and functional features of biomolecules. AFM used in programmable DNA sensors (34) and protein biochips (35) opens promising applications in the new field of nanobiotechnology.

Acknowledgements

This work was supported by DAAD and Deutsche Forschungsgemeinschaft (DFG) SFB 486 grant, Germany.

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Received December 4, 2003
Accepted December 29, 2003