Abstract. Atomic force microscopy (AFM) nowadays can be used not only as a high-resolution imaging tool for precise cytogenetic studies but, at the same time, for mechanical measurements and manipulation of genetic material. This combination allows, for the first time, identification of the sample area, microdissection and nanoextraction of genetic material for further biomedical and biochemical studies. In this review, we examine AFM techniques like cutting, gripping and extracting biomaterial at the submicron scale under high-resolution image control and potential applications in cytogenetics.

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 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 with 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). Furthermore, Fritzsch 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 (15, 20-22). 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 and 250 nm. High resolution AFM images of metaphase 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 peroxidasediaminobenzidine 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 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 the 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 have been 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 from 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).

<table>
<thead>
<tr>
<th>Operation mode</th>
<th>Contact mode</th>
<th>Non-contact mode</th>
<th>Tapping mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tip loading force</td>
<td>low → high</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>Contact with sample surface</td>
<td>yes</td>
<td>no</td>
<td>periodical</td>
</tr>
<tr>
<td>Manipulation of sample</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Contamination of AFM tip</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Microdissection</td>
<td>yes</td>
<td>no</td>
<td>no</td>
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Table II. Methodical properties of the different operation modes in AFM for high resolution imaging, manipulation and microdissection of metaphase chromosomes.
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 by different procedures (52), the analysis of the same samples can be facilitated by the use of AFM and FEISEM microscopy.
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 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 μm/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-Walls interaction and unspecific adsorption to the tip. Electron beam-deposited tips (EBD) with a rough surface can be used to increase the extraction efficiency (Figure 5d) (Thalhammer et al., 2003, in

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.
preparation). The modified AFM tips can be used like a mechanical "nanoscalpel" and a "nanoshovel". As shown above, the influence of the physical parameters used for nanomechnical 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 nm) have been compared to AFM-tip cuts (as small as about 100 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.
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 the 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 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.

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