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The AFM as a tool for chromosomal dissection – the influence of physical parameters

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Abstract. Human metaphase chromosomes were dissected using an atomic force microscope (AFM) in ambient conditions and in buffer. Cutting with z-modulation in air yielded precise cuts at loading forces $F > 17\,\mu$N with a full width at maximum depth of 90 nm. After dissection, the chromosomal material adhered to the tip to be used for further biochemical processing. In liquids, we measured the effects of different types of buffer solution on swelling of the chromosomes and their elastic behaviour.

Chromosomal microdissection provides a direct approach for isolating DNA from cytogenetically recognizable regions. The dissected material can be used for various applications, including establishing probes for fluorescence in situ hybridization (FISH) [1], the generation of chromosome band-specific libraries [2] and physical mapping for cytogenetic analysis.

Imaging chromosomal material using an atomic force microscope (AFM) [3–5] has become a standard technique in biology since its invention in 1986 [6]. The AFM allows imaging of DNA in ambient as well as physiological conditions, and several experiments have also demonstrated the capability of the AFM to manipulate biological samples [7–11].

Recently, we demonstrated that it is possible to extract DNA from interesting regions of human metaphase chromosomes with higher precision than standard microdissection techniques [2, 12]. These could subsequently be used to generate genetic probes via polymerase chain reaction (PCR) amplification of this material [13].

Here, we focus on some parameters which are relevant for chromosomal AFM dissection and describe two different modes for dissection in ambient conditions. In the first mode, z-piezo modulation, "nanostamping" is used; in the second, direct contact mode without modulation, "nanoscratching" is used. Volume changes and the elastic behaviour of chromosomes are dependent on the buffer solution, which plays an important role in the precision of AFM dissection in liquids. In the present communication, both the dissection modes in air and the performance of dissection in liquids are evaluated.

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1 Materials and methods

1.1 Preparation of metaphase spreads

Human lymphocytes were cultivated in RMPI-media (1 μg/ml PHA, containing 10% foetal calf serum (FCS), 1% antibiotics mixture: Streptomycin/Penicillin) for 72 hours at 37 °C. Cells were arrested in metaphase with 0.05 μg/ml Colcemid 1 to 2 h before fixation. After centrifuging, hypotonic treatment (0.075 M KCl) and fixation of the cells were performed according to standard protocols [14]. Metaphases were prepared by drop fixation on microscope slides, air-dried, dehydrated with ethanol and stored in 70% ethanol at 4 °C until use.

1.2 Buffer solutions

TE-buffer: 9.9 mM Tris HCl; 1 mM EDTA; adjusted to pH 7,5. 1 × PBS: 1.37 M NaCl; 26.8 mM KCl; 80.9 mM $Na_2HPO_4 \times 2H_2O$; 17.6 mM KH_2PO_4; adjusted to pH 7.4 with 1N HCl.

1.3 Instrumentation

AFM Data were obtained by a hardware linearized microscope with 100 μm xy-scan range and a 10 μm z-scanner (Topometrix Explorer True Metrix). The AFM was mounted on top of an inverted microscope (Zeiss Axiovert 135). The experiments in buffer solutions were carried out in a liquid cell that consists of an O-ring fixed directly on the microscope slide. Three different types of cantilever were used for measurements: for dissection experiments in ambient conditions, stiff cantilevers (Nanosensors, $c = 45$ N/m, pyramidal

tip shape, cone half-angle $\alpha = 18°$, tip curvature radius $r = 10$ nm) were used. The sample was imaged in high-amplitude resonant mode (setpoint: 60% of free oscillation, scan rate 2 Hz). In liquids, soft cantilevers (Nanosensors, $c = 0.3$ N/m, pyramidal shape $\alpha = 18°$, $r = 10$ nm and Park Scientific $c = 0.02$ N/m, pyramidal shape, $\alpha = 35°$, $r = 25$ nm) were applied and the images were taken in contact mode at minimal force ($F = 2$ nN, scan rate 2 Hz). The force constants of the soft cantilevers were obtained by the built-in calibration function, which analyses thermal fluctuations [15], whereas the force constants of the stiff cantilevers were taken from the manufacturers' data. Layered imaging mode (i.e. force mapping [16], 200 layers, 50×50 pixels) was performed at a z-speed of 1 μm/s to minimize hydrodynamic drag during force curve acquisition.

1.4 Dissection in ambient conditions

High-amplitude resonant mode was used to image and select the chromosome of interest. The feedback system was then switched off and the z-piezo voltage was controlled manually. For dissection, a series of single-line scans was performed at defined loading forces at a speed of 1 μm/s. Two chromosomes of the same metaphase spread were dissected. The first experiment was performed with z-modulation (modulation amplitude of the z-piezo 5 nm at the cantilever's resonant frequency $f = 385$ kHz), the second without.

1.5 Experiments in buffer solution

A metaphase spread was selected with the inverted optical microscope. From this metaphase spread, one chromosome was selected for the experiments. All experiments in liquid were done with the same chromosome, as follows: the sample was initially immersed with PBS buffer and incubated for 15 min at room temperature. The chromosome was then imaged in constant force mode. Subsequently, the sample was imaged in layered imaging mode. This procedure was repeated after removing the PBS buffer by rapidly rinsing the liquid cell with distilled water and immersing the sample with TE buffer. Two different cantilevers (PSI and Nanosenors; see instrumentation) were used for comparison. After characterization, the chromosome was dissected.

The volume of the chromosomes was determined by the particle analysis tool of the Explorer software. In order to calculate the elasticity, eight force curves were analysed (only two curves per experiment are shown below).

1.6 Analysis of force curves

The force curves can be divided into two different regions. With the tip off the surface the cantilever deflection is constant. However, when the tip is in contact with the sample the force curve is sloped. The slope depends on the stiffness of the sample. For an infinitely hard sample and tip, the deflection δ of the cantilever is directly proportional to piezo position z and can be described by the expression

$$\delta = -(z - z_0)\,, \tag{1}$$

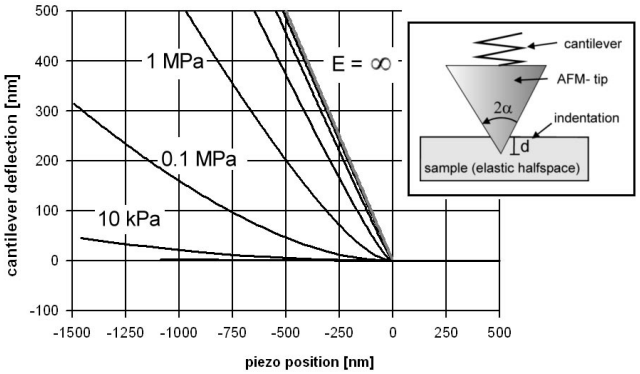

Fig. 1. Simulation of force curves on an elastic sample for different Young's moduli as calculated from the Hertz model. The scale of the ordinate (cantilever deflection from 0 to 500 nm) is transformed into the loading force by multiplication by the force constant c ($c = 0.3$ N/m; the force scale ranges from 0 to 150 nN). The small figure illustrates the configuration used for the simulation. The AFM tip is modelled as an infinitely hard cone with a half-angle a which indents (depth d) into an elastic half-space characterized by its elastic modulus (Young's modulus) E and its Poisson ratio ν. The tip is fixed to a spring with force constant c. Parameters used for the simulation characterize the Si tip used in the experiments: $\alpha = 18°$, $\nu = 0.5$, $c = 0.3$ N/m; the Young's modulus varies from $E = 10$ kPa to $E = 10^7$ kPa in steps by a factor of 10

where z_0 is the piezo position when the tip hits the sample (contact point). For elastic samples, the situation is more complicated because the indentation of the tip into the sample surface has to be taken into account [16–18]. In this case, force curves can be calculated from the Hertz model [19]. This theory describes two elastic surfaces in contact under load. For an infinitely hard cylindrical cone indenting into an elastic half-space (Fig. 1, small image) this leads to the expression for the indentation d:

$$d^2 = F \frac{2}{\pi \tan\alpha} \frac{1-\nu^2}{E}\,, \tag{2}$$

where F is the loading force $F = c\delta$, α is the cone half-angle, E the Young's modulus and ν the Poisson ratio of the elastic sample respectively. Equation (1) can now be written for soft samples in the contact regime as

$$z - z_0 = -\delta + \sqrt{\frac{2}{\pi} \frac{\left(1-\nu^2\right)}{E} \frac{1}{\tan\alpha} c\delta}\,. \tag{3}$$

Figure 1 illustrates the relation of the piezo position z, and the cantilever deflection δ as obtained from (3) for a cantilever used in the experiments.

To calibrate the sensor response, the linear parts of four force curves on the substrate (glass) were averaged. For an infinitely hard substrate, the slope of the force curve is 1 in the contact regime. The parameters E and z_0 of this model have been fitted to the experimental data using a least squares method.

2 Results

2.1 Dissection in ambient conditions

In Fig. 2a, a topographic AFM image of a human metaphase chromosome is shown after a series of AFM dissections. The

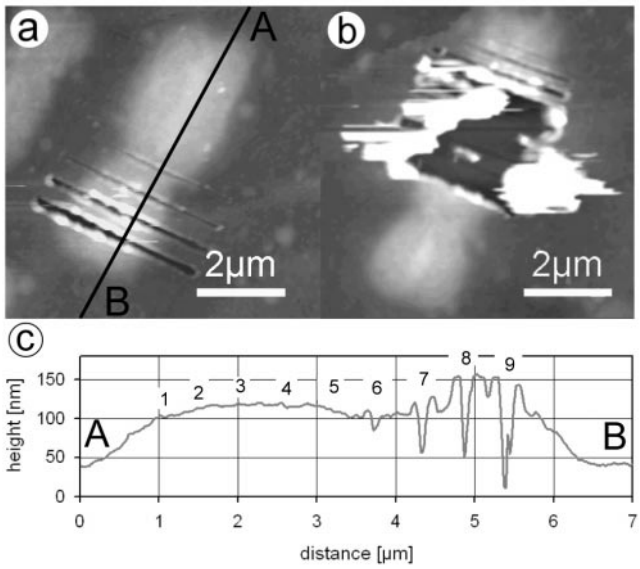

Fig. 2a–c. Comparison of two different AFM dissection modes in air (z-scale 0–180 nm). **a** The human metaphase chromosome was imaged by AFM in ambient conditions after a series of dissections made by AFM. For dissection, z-modulation (~ 5 nm) was used. The oscillation amplitude of the cantilever was smaller than 1% of the amplitude of free oscillation for all cuts. Each cut was performed by scanning one line at 1 µm/s at a certain loading force. No.1: 2.8 µN; No.2: 5.6 µN; No.3: 8.5 µN; No.4: 11.2 µN; No.5: 14.0 µN; No.6: 16.8 µN; No.7: 19.6 µN; No.8: 22.4 µN; No.9: 25.6 µN. **b** Human metaphase chromosome in the same metaphase spread as **a**. As before, a series of cuts has been made, but without z-modulation. To minimize effects of tip geometry, a chromosome oriented in the same direction as in **a** was selected and the same tip was used. **c** Cross-sectional analysis, as indicated in **a**. The positions of the different cuts are marked by the numbers

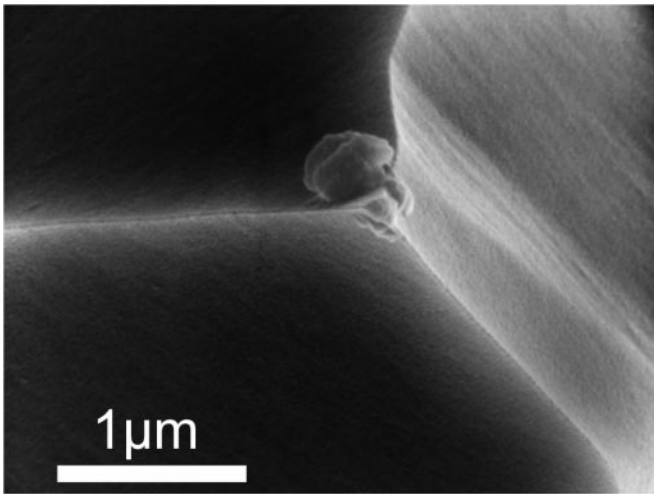

Fig. 3. Electron microscope image of an Si tip after a DNA-extraction experiment in air. Biological material adhering to the tip can be identified. At larger magnification (data not shown) it can be seen that a small area of the tip is broken off. The sample has been covered with carbon for SEM imaging

loading force of the tip was increased stepwise as described in the figure caption. For this experiment, z-modulation (~ 5 nm) was used to decrease the lateral forces and to avoid uncontrolled tip movement across the sample. The oscillation amplitude of the cantilever was completely dampened during dissection (damping $> 99\%$). The first cuts yielded very shallow (depth < 10 nm) scratches (No.1-5, Fig. 2c). Cut No.6, at a loading force of $F = 16.8\,\mu$N, reached a depth of 25 nm in chromosomal material and is the first cut in the series to affect the chromosomal material visibly. Above a loading force of $F = 22.4\,\mu$N (cut No.7, full width at half maximum depth (FWHMD): ~ 90 nm), the dissection of the chromosome was complete. Increasing the loading force from cut No.7 (22.4 µN) to cut No.9 (28.0 µN, FWHMD: 110 nm) leads to deeper and broader cuts. The last two cuts reached the surface of the glass slide. On the glass substrate there is a layer of biological material from the preparation process. In our experiments, this layer has a typical thickness of 20 nm to about 50 nm.

In the second set of experiments, a chromosome was dissected in direct contact mode (Fig. 2b). Already at cut No.1 (2.8 µN) the chromosome was visibly affected. Increasing the loading force led to deeper cuts. At $F = 8.6\,\mu$N (No.3) the chromosome was apparently cut through. A further increase of the loading force led to uncontrolled destruction of chromosomal material. Cross-sectional analysis of Nos. 5-10 (data not shown) revealed broad scratches (~ 300 nm) reaching down to the glass slide.

An electron microscope image of an AFM tip after a dissection experiment is shown in Fig. 3. Only one dissection was performed with this tip using z-modulation. Next to the tip apex, biological material from the cut chromosome can be identified which adheres to the tip. At larger magnification (data not shown), it can be seen that a small area of the tip has broken off. The successful amplification of this material by PCR shows that DNA material from the extraction site adheres to the tip [13].

2.2 Experiments in buffer solution

A series of experiments to determine volume change and elastic behaviour was carried out on one chromosome. In Fig. 4a, a topographic contact mode AFM image of the chromosome in PBS buffer is shown. The volume of the large chromosome in Fig. 4a was determined to be 6.0 μm^3 and the small chromosome 2.1 μm^3. The volume of the complete metaphase spread was 153 μm^3. Four force curves on the large chromosome and another four on the substrate have been selected for the calculation of the elastic modulus. The calculations yielded $E = 0.5\,\text{MPa} - 0.7\,\text{MPa}$, depending on the position on the chromosome.

The sample was then incubated in TE buffer and imaged by AFM (Fig. 4b). Cross-sectional analysis (Fig. 4c) illustrates the swelling of the chromosome. The volume of the large chromosome is 13.9 μm^3, the small one 5.0 μm^3 and of the whole metaphase 339 μm^3. Force curves in the approach direction obtained with different tips at positions indicated by the crosses in Fig. 4b are presented in Fig. 5. The force curves in the approach and withdrawal directions showed a small hysteresis due to viscoelastic effects but did not exhibit adhesion. The experimental force curve obtained on the chromosome is fitted for cantilever deflections ($\delta > 50$ nm (equivalent to indentations into the sample of roughly $d > 100$ nm) by a theoretical force curve with an elastic modulus of $E = 0.28$ MPa. Three other points on the chromosome showed E between 0.2 MPa and 0.3 MPa.

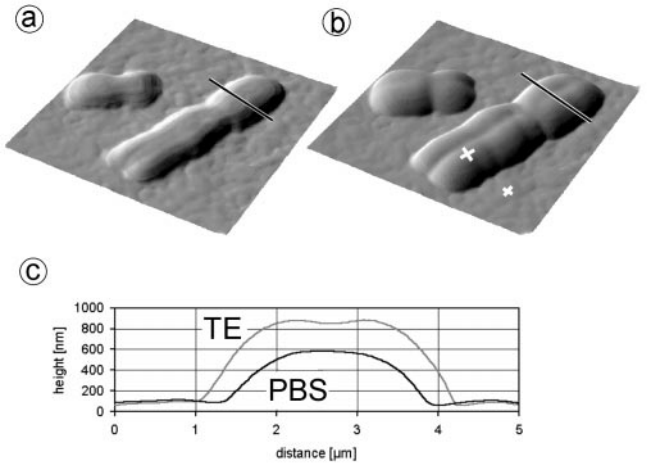

Fig. 4a–c. Swelling of chromosomes as observed in two different buffer solutions imaged by AFM in contact mode (image size: $10\,\mu m \times 10\,\mu m$). **a** Topographic AFM image taken in PBS ($F = 2$ nN, 2 Hz, Si_3N_4 tip, $\alpha = 35°$). **b** The identical chromosome was subsequently imaged in TE buffer with the same tip and parameters as before. The crosses indicate the positions where the force curves (Fig. 5) were selected from the layered images. **c** A cross-sectional analysis reveals height of 560 nm and a width (FWHMH) 1.9 µm in PBS, compared with a height of 900 nm and a width of 2.5 µm in TE buffer

The contact point was not easily determined because near the calculated contact point (in Figure 5a $z_0^{\mathrm{fit}} = 0$) the curvature of the force curve is lower than estimated. Analysing Fig. 5a manually, the contact point is at $z_0^{\mathrm{exp}} = 55$ nm.

The same chromosome was subsequently imaged using a sharp tip ($\alpha = 18°$, Fig. 6a) The contact AFM image exhibits distortions of the chromosome. These distortions could not be suppressed completely by slowing down scanning speed, increasing feedback sensitivity or decreasing loading force. The elastic modulus was calculated to be

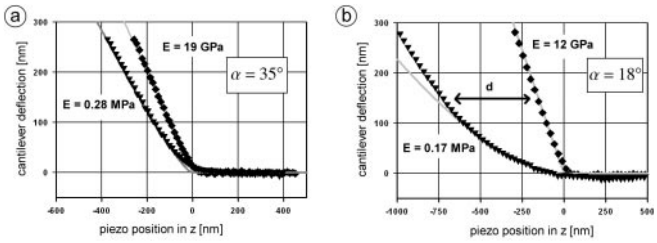

Fig. 5a,b. Force curves taken with two different tips in TE buffer at positions indicated in Fig. 4b. The triangles are experimental data measured on the chromosome in the approach direction, the continuous lines are the theoretical force curves for the Young's modulus as calculated from the fit. For comparison, force curves taken on the substrate are shown (squares). The offset of the z-position scale was chosen to yield the contact point from the fit as $z_0 = 0$. On the hard substrate, the calculations yield a high Young's modulus, as expected. **a** The Force curves were taken with an Si_3N_4 tip ($c = 0.02$ N/m, $\alpha = 35°$). The loading force calculated from the cantilever deflection ranges from 0 to 6 nN. It was not possible to obtain a good fit for small cantilever deflections. **b** Using an Si tip with a stiffer cantilever ($c = 0.3$ N/m, $\alpha = 18°$, loading force scale: 0 to 90 nN) yielded experimental data that is consistent with the Hertz model for cantilever deflections up to 100 nm ($F = 30$ nN). At large indentations (about 80% of the chromosome height) the apparent elastic modulus of the chromosome increases because the material is strongly compressed between the tip and the hard glass substrate [16, 23]. Even at forces $F < 5$ nN the tip penetrates the chromosome significantly (at 5 nN the indentation is $d \sim 80$ nm)

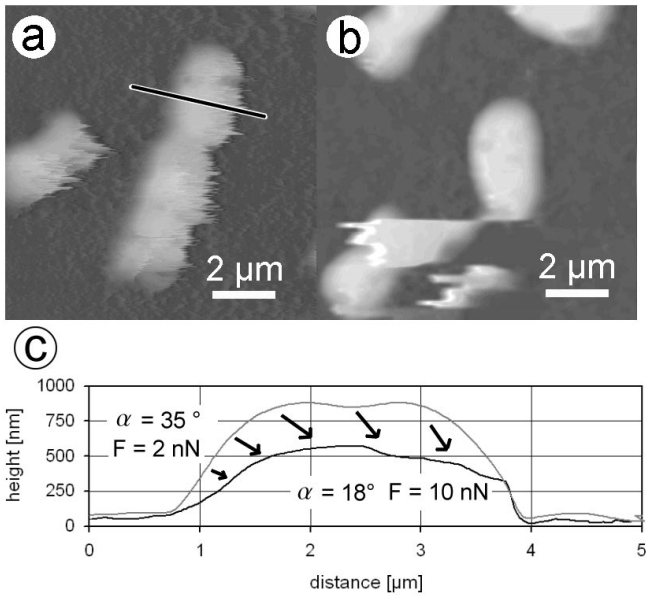

Fig. 6a–c. From scanning to dissection. The topographic AFM images of the same chromosome as in Fig. 5 were taken in contact mode in TE buffer (z-scale: 0–1000 nm, scan direction left to right). **a** This image was taken with a sharp tip ($c = 0.3$ N/m, $\alpha = 18°$). The image is blurred and stripes in the scan direction are visible. **b** The chromosome after a dissection experiment. For dissection the loading force was increased to 1 µN and a line scan at 1 µm/s was performed without z-modulation. A large part of the chromosome was pushed to the side in an uncontrolled manner. **c** A cross-sectional analysis of the blurred image compared with the cross-section of an undisturbed image (Fig. 4c) shows that the chromosomal structure in **a** is severely distorted by the scanning process

$E = 0.18$ MPa. Other points on the chromosome yielded $E = 0.16$ MPa to 0.21 MPa. After the elasticity measurements, several attempts were performed to dissect the chromosome. However, either the chromosome was not affected at all at small loading forces ($F < 1$ µN) or large parts with a size in the micron range were pushed around by the tip in an uncontrolled manner (Fig. 6b).

3 Discussion

3.1 Dissection in ambient conditions

The dissection experiments showed the dramatic effects of lateral forces on AFM dissection. With z-modulation controlled dissection, loading forces as high as $F = 28$ µN were possible. In contrast, without this modulation forces of about $F = 10$ µN dissection become difficult to control. This can be explained by the modulation reducing the friction on the glass slide even when the tip was in permanent contact with the glass [20]. Reducing the lateral forces on the glass when the tip entirely pierced the chromosome minimized the risk of breaking off large parts of the tip apex area. Thus the tip remained sharp and narrow cuts (FWHMD < 100 nm) were possible. The broad scratches (Fig. 2b) indicated that the end of the tip was broken off and that the contact area was therefore larger. The large area destroyed in the chromosome (Fig. 2b) was possibly a result of a part of the tip breaking off and the tip scratching around in an uncontrolled way. The

experiments showed that for precise AFM-dissection the *z*-modulation mode is more suitable than simple scratching, which affords minute adjustment of the loading forces to yield controlled cuts.

These experiments also illustrate that it is not primarily the pressure but the lateral forces which determine the quality of AFM dissection of chromosomes. Lateral forces are decreased by *z*-modulation, whereas the average pressure is not affected by the modulation.

3.2 Experiments in buffer solution

From the force curve (Fig. 5a) it can be estimated that at the imaging force of $F = 2$ nN the tip indented about 50–100 nm into the sample in TE buffer. In PBS the indentation was 20–50 nm. The uncertainty of the indentation is mainly caused by the uncertainty of the contact point. This leads to an underestimation of the true chromosomal volume by roughly 10%. On the other hand, the tip geometry induces an overestimation of the volume due to convolution effects. One can conclude that the estimated chromosomal value is at least of the right order of magnitude. The swelling of the chromosomal material in TE buffer as compared to PBS by a factor of ~ 2 can be explained by osmotic effects or conformational changes in the structure of chromatin induced by different ion concentrations [21]. In [22], the volume for an air-dried human metaphase chromosome was found to be between $0.18\,\mu m^3$ and $1.3\,\mu m^3$. For rehydration in PBS a 4–5.5-fold increase in volume was determined. Hence the volume of a large chromosome was about 5–7 μm^3. This result is confirmed by the chromosomal volume of $6\,\mu m^3$ in PBS in this communication. But our experiment also shows that not only rehydration but also composition of the buffer solution is an important factor determining the chromosomal volume.

In contrast with data obtained from sharp tips, the experimental data for blunt tips could not fit the Hertz model in a satisfactory way. Certainly, tip geometry is one important factor for the quality of the fit. The approximation of a pyramidal shaped tip as a radial symmetric cone with a half-angle $\alpha = 35°$ is too inaccurate. Additionally, a softer cantilever is more sensitive to electrostatic interaction. The electrostatic repulsion between the negatively charged Si_3N_4 and the negatively charged DNA can lead to a bending of the force curve in such a way that it is impossible to obtain a good fit of the data to the Hertz model, where only the elastic interaction is considered and the electrostatic interaction is neglected. Therefore, only data belonging to larger cantilever deflections ($\delta > 80$ nm; i.e. $F > 1.6$ nN) was used to fit the force curve, because then the elastic interaction between sample and tip can be expected to dominate. Accordingly, the Young's modulus of $E = 0.28$ MPa can only be considered as an estimate. For the Si tip, the experimental data and theoretical force curves were found to be consistent for cantilever deflections $\delta < 100$ nm (i.e. $F < 30$ nN; Fig. 5b). At larger forces the chromosomal material was compressed between tip and substrate and consequently the sample appears to become stiffer at large indentations [23]. For this force curve, an uncertainty of the contact point z_0 of about 50 nm remained as before. This again limits the accuracy of the numerical value for the Young's modulus ($E = 0.17$ MPa).

The influence of the elastic behaviour of the chromosome is illustrated by the distortion of the chromosomal structure (Fig. 6a). In the comparison of the cross-section of the undisturbed chromosome and the distorted structure (Fig. 6c), it is obvious that the tip is penetrating into the sample and is dragging parts of the chromosome into the scan direction. It was, however, necessary to increase the loading force further by a factor of 100 to dissect the chromosome entirely. The observation that dissection of a swollen chromosome is uncontrolled was expected. The chromosome increases in height from about 200 nm in air to nearly 1000 nm in TE buffer. Thus in the tip pushes the chromosome with the sidewall (the tip area in contact with the chromosome is about $0.3\,\mu m^2$) and exerts large lateral forces on the chromosome. Fixation of the chromosome to the glass substrate is reduced in buffer due to swelling. These factors lead to large parts of the chromosome being removed. This underlines the importance of the lateral forces for chromosomal dissection by AFM.

4 Summary and outlook

We have shown that AFM cutting of chromosomes with additional *z*-modulation in ambient conditions yields precise cuts at loading forces $F > 17\,\mu N$ with a full width at maximum depth of 90 nm. After dissection, the chromosomal material adhered to the tip. In liquid, we could demonstrate the sensitivity of the elastic behaviour and of the swelling of the chromosomes to the buffer (PBS versus TE buffer). The volume was increased by a factor of ~ 2 and the elastic modulus was reduced from $E = 0.6$ MPa to $E = 0.3$ MPa in TE buffer compared with PBS. Controlled dissection was not possible in buffer solution.

For biological application of the AFM in chromosomal dissection, precise, distinct and repositionable cuts are needed for investigation of chromosomal sub-bands. Serial cuts are required for high-resolution physical mapping of the genome. Thus an understanding of the mechanical cutting process and knowledge of the elastic parameters of the chromosomal material is essential. We have demonstrated that the AFM fulfils the requirements for precise dissection and can be used for further physical mapping experiments.

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