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ARTICLE

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Atomic force microscopy and scanning near-field optical microscopy studies on the characterization of human metaphase chromosomes

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Abstract A better knowledge of biochemical and structural properties of human chromosomes is important for cytogenetic investigations and diagnostics. Fluorescence in situ hybridization (FISH) is a commonly used technique for the visualization of chromosomal details. Localizing specific gene probes by FISH combined with conventional fluorescence microscopy has reached its limit. Also, microdissecting DNA from G-banded human metaphase chromosomes by either a glass tip or by laser capture needs further improvement. By both atomic force microscopy (AFM) and scanning near-field optical microscopy (SNOM), local information from G-bands and chromosomal probes can be obtained. The final resolution allows a more precise localization compared to standard techniques, and the extraction of very small amounts of chromosomal DNA by the scanning probe is possible. Besides new strategies towards a better G-band and fluorescent probe detection, this study is focused on the combination of biochemical and nanomanipulation techniques which enable both nanodissection and nanoextraction of chromosomal DNA.

Keywords Atomic force microscopy · Fluorescence in situ hybridization · Metaphase chromosomes · Nanodissection · Scanning near-field optical microscopy

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Introduction

Since its introduction in 1969, in situ hybridization and in recent years especially fluorescence in situ hybridization (FISH) became very popular techniques in cyto- and molecular genetics research and diagnostics (John et al. 1969; Pardue and Gall 1969). FISH is used to detect certain nucleic acid molecules, for example RNA in the cytoplasm of a cell or DNA in the nucleus. This report is focused on the detection of chromosomal DNA in metaphase chromosome preparations, which is used for investigative but even more for diagnostic purposes.

In the standard FISH procedure, a nucleic acid probe is hybridized onto the target DNA of the chromosomes. By the use of fluorochrome-labelled probes, it is possible to selectively visualize whole chromosomes, chromosome parts, individual genes and unknown DNA fragments by using conventional optical fluorescence microscopy. However, this technique has some critical limitations, due to the diffraction limit of optical microscopy. The identification of large probes with a length of several kbp and more is a well-established procedure, but the determination of the distinct localization of smaller probes is still a serious challenge, because routinely applied steps of fluorescent probe detection will always completely cover the location of interest.

However, not only the commonly used techniques for the detection of chromosomal probes need further improvement. So does microdissection, a method used, for example, in combination with the polymerase chain reaction (PCR) to develop new chromosomal probes (Wesley et al. 1990). Chromosomal DNA isolated via microdissection using either glass tips or laser capture technologies is essential for several applications, like the generation of band-specific libraries, the development of chromosome maps, and cytogenetic analysis for diagnostic purposes. One fundamental problem of microdissection is the precise determination of the location of the extracted material. Thus efforts have been made to

establish new strategies for improvement in terms of nanodissection by using atomic force microscopy (AFM) and additionally by using scanning near-field optical microscopy (SNOM) (Pohl 1992).

AFM was introduced in 1986 (Binnig et al. 1986) and it was first established as a useful tool in materials science. AFM is able to measure forces between the sample surface and a suitable local probe. The probe usually consists of a pyramid-shaped silicon tip as part of a micromachined cantilever beam. The deflection of the cantilever is detected optically. The topography of the sample is usually imaged in the constant-force mode. A feedback circuit cares for a constant cantilever deflection by an adequate and locally varying vertical displacement of the probe during scanning. This displacement is a measure of the topography. Forces below 1 nN can be measured and deflections as small as 0.1 nm. The lateral resolution depends on the tip shape, on the loading force and on the sample elasticity. An enhancement in sensitivity is reached by oscillating the cantilever near its resonant frequency and by measuring the phase shift and the amplitude of the oscillations with respect to the driving signal. With this dynamic method, long-distance forces can be detected as well and the topography of the sample can be imaged in non-contact and intermediate-contact modes (Marti and Amrein 1993; Cohen and Lightbody 1998; Morris et al. 1999). Unlike some other scanning microscopes, AFM can be used on non-conductive materials, which makes it suitable especially for the characterization of biological samples, such as DNA and DNA–protein complexes (Moreno-Herrero et al. 2001; Umemura et al. 2001). Several groups were able to show that not only imaging but also manipulation of biological samples by AFM is possible. The manipulation of plasmid (Henderson 1992) as well as human chromosomal DNA (Thalhammer et al. 1997) were the main targets in this field.

SNOM, which has attracted much attention in recent years, yields topographical and optical images of samples simultaneously. Optical images are interesting in order to substitute traditional fluorescence optical microscopy and many other optical techniques used in traditional microscopy by SNOM-related methods (Betzig and Trautman 1992; Betzig et al. 1993; Monson et al. 1995). SNOM can be operated in various modes but one of the most widely used ones can briefly be described as follows. A tapered, sharp optical fiber probe scans the sample surface at close proximity by keeping the simultaneously measured shear-force constant (Pohl 1992). The varying vertical displacement is again used to reconstruct the sample topography. A small aperture, with a diameter which is usually less than 100 nm, is formed by Al coating of the tapered fiber. Light transmitted through the aperture hole is reflected by or transmitted through the sample and then collected by a far-field optical system. Various experiments and theories show that the optical resolution of SNOM is mainly determined by the size of the aperture when the probe–sample distance is in the near-field regime (Heinzelmann and Pohl 1994; Pohl et al. 1996). The result is a resolution far beyond the diffraction limit of traditional optical microscopy. This has been shown for a variety of biological samples (Ha et al. 1996; Van Hulst et al. 1997).

In the following, we introduce SNOM as a tool for the detection of chromosomal fluorescent DNA probes and we furthermore present some strategies that are essential for nanodissection at well-defined chromosome areas using either AFM or SNOM.

Materials and methods

Methods

Metaphase chromosome preparation

Chromosomes from lymphocytes were prepared according to the classical method. A volume of 0.5 mL of fresh blood was added to 8 mL PB-Max™ Karyotyping Medium (Life Technologies, Germany) and incubated at standard conditions (5% CO_2, 37 °C) for 72 h. After adding KaryoMAX-colcemid solution (Life Technologies, Germany) and incubating for another 90 min, the culture was transferred into a tube and centrifuged for 8 min at 1000 rpm. The supernatant was removed, followed by a hypotonic treatment (8 mL, 0.075 M KCl, 5 min, 37 °C). After centrifugation, the pellet was fixed by adding methanol/acetic acid (3:1; 8 °C). The latter steps were repeated until the pellet appeared to have a white color. The resuspended cells were dropped onto glass slides, which were previously washed with $EtOH_{abs}$. The samples were dried on a heating surface at 80 °C for 1 h and were stored at room temperature until usage. GTG banding was performed according to Seabright (1971).

Chromosome pretreatment

The chromosome slides were pretreated by a RNA digestion for 30 min at 37 °C in a humidified chamber (RNase, $c = 100$ mg/mL; Boehringer Mannheim, Germany). Alternatively, we additionally incubated the slides in solutions of trypsin or heparin, varying the concentrations and incubation times. Afterwards the slides were refixed in 4% paraformaldehyde in phosphate buffered saline (PBS).

Fluorescence in situ hybridization

We performed FISH using biotin-labeled DNA probes of the centromeric region of the chromosomes 1, 2, 7, or 8 (Oncor/Appligene, Germany) (Ermis et al. 1998). Aliquots of 10 μL of the hybridization mixture with a total amount of 50 ng of each probe were applied to the slide. Probe and target DNA were denatured simultaneously for 10 min at 80 °C. ISH was performed overnight at 37 °C in a humidified chamber. Afterwards, the slides were washed three times in 2×SSC (1×SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 60% formamide at 37 °C. Signals could be detected using streptavidin-fluorescein isothiocyanate (FITC) at a concentration of 4 mg/mL (Vector/Camon, Germany).

Detection and manipulation of chromosomes using AFM

A commercial AFM (Topometrix Explorer) was used in this study. AFM using the constant force mode is a standard method to obtain topographic information of weakly corrugated samples. Additionally, distinct structural manipulations on chromosomes can be

produced by applying different forces with cantilevers with a spring constant of 50 N/m (Nanosensors, Germany).

To perform straight cuts through a chromosome, the tip had to be moved precisely along the same line for several times. For each new line the force is increased to achieve a final value of 40 μN.

Detection and manipulation using SNOM

The detection of fluorescent labelled human metaphase chromosomes was carried out with an advanced commercial SNOM (Aurora, ThermoMicroscopes, USA) (see Fig. 6). It was operated in the illumination and transmission modes. Commercial single-mode optical fibers (F-SA, Newport, USA) were first sharpened by chemical etching and then coated by Al in a UHV chamber to form a sub-wavelength aperture hole. Common optical shear-force detection and feedback control were employed to control fiber motion and to obtain topographical information. A silicon avalanche photon diode (APD, C30902S, EG&G Optoelectronics, Canada), together with a homebuilt preamplifier, counting module, and D/A converter, were installed in order to reach a single-photon detection level. To reduce the dark counts of the detector, the APD was cooled down to just above 0 °C during operation. A 488 nm air-cooled Ar^+ laser (Uniphase, USA) was used as the fluorescence-exciting light source. A 0.7NA objective (60×, Nikon, Japan) was used to collect the fluorescent light in the photon detector. A 532 nm laser liner filter (10 nm FWHM, Newport, USA) was inserted between the detector and objective to discriminate the fluorescent light from the exciting laser and shear-force control laser. Each pixel was measured with a 10 ms integration time.

In order to adsorb genetic material onto the tip, the probe was moved towards the desired chromosomal area. Therefore the *z*-piezo voltage is raised at a fixed lateral position. Typically the piezo elongation is 200–400 nm. During the indentation process the phase of the fiber oscillations changes irregularly and no longer provides any information on the applied forces.

Results and discussion

The standard methods of imaging chromosomes from metaphase preparations are based on conventional light or fluorescence microscopy. Light microscopy is routinely used for chromosomal diagnostic purposes, where G-banded chromosomes are evaluated for karyotyping. The resolution of light microscopy is by far not good enough to analyze the fine structures of chromosomes. By using AFM, complete metaphase plates showing 46 chromosomes can easily be visualized in detail. A typical one is shown after GTG-banding in Fig. 1. The average height of the chromosomes is 80–100 nm, depending on the degree of condensation, which has already been shown previously (Musio et al. 1997). The width of the chromosomes is approximately 2 μm.

In general, highly resolved images exhibit resolutions of about 10 nm, which are comparable to non-contact measurements. The relationship between contact radius a, penetration depth h, and the radius R of a sphere, which is pressed towards a plane, is given by:

$$a = \sqrt{Rh} \tag{1}$$

This leads, assuming that we use a tip with a radius of 10 nm, to a penetration depth, which means a compression of the chromosomes, of 2.5 nm to 10 nm.

The visualization of the topography of chromosome 2 in terms of the linescan profile illustrates the differences

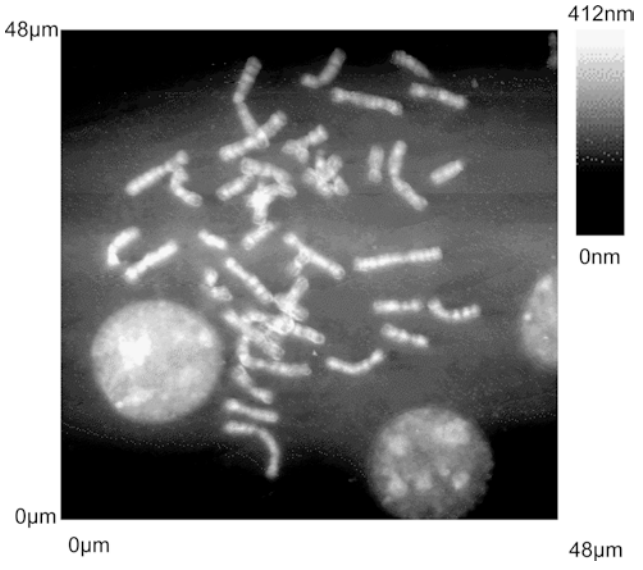

Fig. 1 GTG-banded human metaphase chromosomes of one cell and several nuclei visualized by AFM

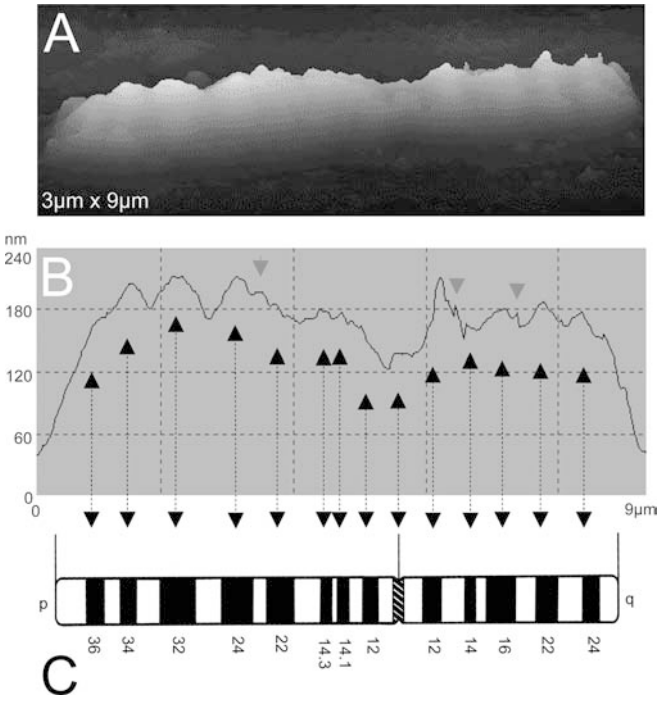

Fig. 2A–C Comparison of the topographic information of chromosome 2 with optical GTG-banding. **A** Topography of chromosome 2. **B** Linescan profile. **C** Banding according to ISCN. The highest regions in **B** exactly refer to the dark GTG-bands in **C**, as indicated by the *arrows*. *Triangles* in **B** show regions which were better resolved by AFM

in height of positively and negatively G-banded chromosomal areas (Fig. 2). Note that the lowest part within the chromosome represents the constriction at its centromeric region. Before comparing the topographical image with the optical ones, we had to determine the optical resolution for the given particular metaphase

spread. In the present case the resolution reaches the 400 bands haploid karyotype according to the International System for Human Cytogenetic Nomenclature (ISCN; Mitelman 1995).

The comparison of the conventional optical image and the topography produced by AFM yields more detailed structural information. The arrows in the linescan image (Fig. 2B) indicate peak areas of chromosome 2, which all precisely refer to positive G-bands. This could somehow be expected, since Ross and Gormley (1973) previously described "swollen" regions associated with G-bands. The main advantage of the topographic imaging using AFM is the superior resolution within positive G-bands: the bands p12, p16, and p22 exhibit several peaks (triangles in Fig. 2B). In contrast, the optical image, which is replaced here, due to its weak information content, by the schematic of chromosome 2 from the 400 bands haploid karyotype, only shows one dark band in these areas (Fig. 2C). The additional peaks, imaged by both contact and non-contact mode AFM using different tips, represent local highly condensed DNA regions, which seem to be insensitive to banding techniques. It is currently under investigation whether a reproducible visualization of these regions in chromosomes with the same length and consequently with the same number of bands is possible and whether these condensed regions are variant in different individuals.

A higher resolution within a certain band will offer the possibility to extract chromosomal material under better-defined conditions. Usually, complete chromosome bands are extracted by the method of conventional microdissection using piezo-driven manipulation tools and glass fibers. The extraction of small parts of chromosomal G-bands is not possible with this method.

AFM is a not only a useful tool to visualize well-defined chromosomal areas within bands, it can also be used to modify DNA with the intention to extract chromosomal DNA. By performing controlled tip movements, single DNA areas can be separated (Fig. 3A). It is thus possible to dissect chromosomal regions of variable size. In the given example, we separated a region with a size of 300 nm×300 nm and another region with a size of 500 nm×500 nm. The AFM tip was repeatedly moved back and forth along the respective lines. The applied force was increased from 2 μN for the first cut in steps of 4 μN to a finite value of 38 μN, applied as loading force in the last cut. The depth of the corrugation correlates with the loading force of the AFM, which in turn is highly dependent on the hydration of the chromosome. The minimum distance between two parallel cuts was as small as 200 nm. The trenches produced by two adjacent cuts in Fig. 3B are clearly separated, whereas cut 4 had obviously attached the trench produced by cut 3.

The purpose of the AFM-based method is to drastically decrease the amount of extracted DNA in comparison to the standard microdissection method (Xu and Ikai 1998). The extracted DNA is much more specific,

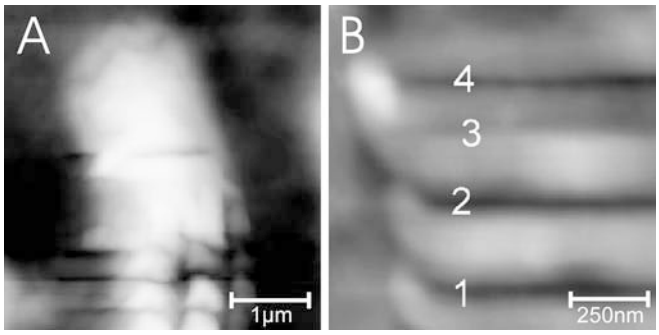

Fig. 3 **A** Separation of DNA blocks by AFM. **B** Corrugations in the chromosome surface introduced by AFM

which is an advantage in all downstream applications for diagnostic purposes, e.g. PCR and R-FISH.

Another goal of our investigations was to modify the chromosome structure by enzymatic and biochemical treatment to obtain a chromosome surface, which is more accessible to banding techniques and FISH in general and which afterwards offers a better resolution in chromosome imaging. This will also be of benefit for the extraction of more specific chromosome areas, because the amount of extracted DNA is further decreased.

Treatment of the fixed chromosomes with pepsin (10% in H_2O; Roche Molecular Biochemicals, Germany), which is a common procedure prior to a FISH experiment, resulted in a reduction of the chromosomal height (Tamayo and Miles 2000). So did the treatment with various solutions of trypsin (Biochrom, Germany), which acts similarly to pepsin as a protein-cleaving enzyme. Increasing cleavage of the chromosomal histones resulted in a reduction of the chromosomal height (Fig. 4A, B) to about 50 nm, with an additional effluence of the DNA (Fig. 4C). After extensive cleavage of the chromosomal histones, mainly the internal scaffold with its non-histone proteins is left (Adolph et al. 1977).

Incubation with the polyanion heparin ($c = 0.5$ mg/mL, $t = 5$ min; Roth, Germany), which strongly detracts both the positively charged histones and the largest part of the non-histone proteins from the DNA, also leads to a height reduction to about 40 nm and the effluence of the chromosomal DNA (Fig. 4D) (Courvallin et al. 1981). The remaining internal structure of the chromosome is likely to represent the DNA 30 nm chromatin fiber (Tamayo and Miles 2000). The DNA effluent is about 20 nm high (triangles in Fig. 4C, D), showing that the structure of the 30-nm fiber is influenced by highly concentrated trypsin as well as by heparin. Applying different banding techniques to these previously treated chromosomes should clarify which of them is the most suitable one to achieve the highest resolution.

The aforementioned modifications of the chromosomal structure also affect the tip force which is necessary to penetrate the chromosomal surface during AFM.

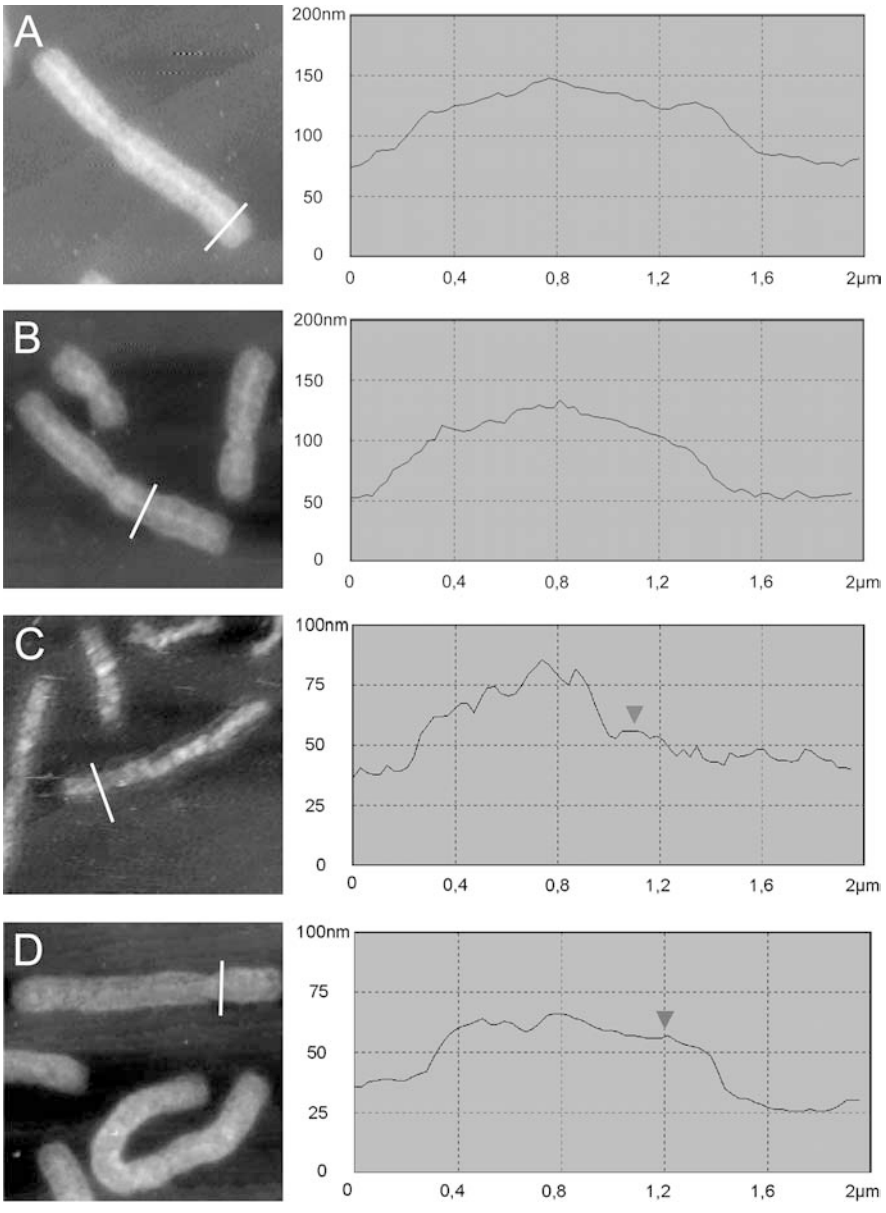

Fig. 4A–D Biochemical treatment of chromosomes and AFM measurements (10 µm×10 µm). **A** Trypsin; $c = 0.02\%$; incubation time = 1 min; resulting chromosomal height 80 nm. **B** Trypsin; $c = 0.1\%$; incubation time = 1 min; resulting chromosomal height 60 nm. **C** Trypsin; $c = 0.5\%$; incubation time = 3 min; resulting chromosomal height 50 nm. **D** Heparin; $c = 0.5\%$; incubation time = 3 min; resulting chromosomal height 40 nm

Figure 5A shows three approaches using the same type of tip with different tip forces. In general, lower tip forces are necessary using trypsinated chromosomes when compared to non-treated chromosomes (Fig. 3). Figure 5B indicates that the depth of the extraction area strongly depends on the tip force. The triangles mark DNA accumulations arising at the borders of the scanned area. Scanning with a tip force of 2.2 µN produces smaller accumulations at the borders, but results in a minor extraction depth. Based on these experiments, we suppose that at least a part of the missing DNA from the extraction area is permanently attached at the tip. However, the detection of DNA left on the tip still remains a methodical challenge. Until now, the direct visualization of the DNA on the tip by electron microscopy was not successful, owing to the detachment of the DNA from the tip during the preparation procedure. Also an indirect verification of DNA extraction by performing R-FISH experiments was not successful (data not shown). This was due to the small amount of DNA with an unknown sequence, which requires the usage of relatively unspecific DOP-PCR primers. Consequently, the extraction of small amounts of DNA by AFM or SNOM will only be useful in the future if suitable PCR protocols are developed at the same time to amplify very small amounts of extracted DNA.

There is a strong requirement for a better resolution in FISH experiments. Nowadays, it is a standard approach to visualize DNA probes on metaphase chromosomes, which have lengths of several kpb with a conventional optical fluorescence microscope. However, it still remains a challenge to visualize small probes (i.e. < 1 kpb) without several stages of signal amplification. The detection of the probe topography by AFM is not sufficient (De Grooth and Putman 1992; Putman et al. 1993; Rasch et al. 1993). Therefore, we used a dedicated

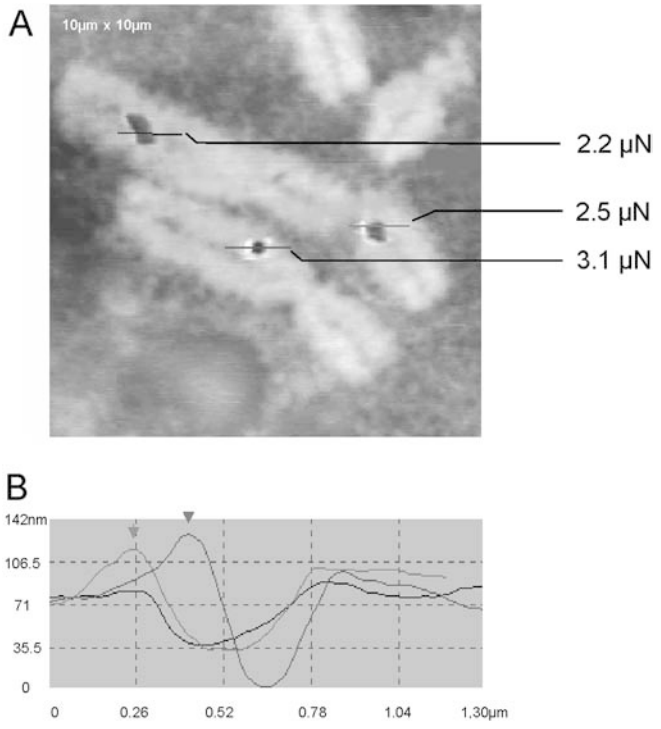

Fig. 5 **A** Extraction of DNA by AFM using different tip forces. **B** Referring linescan profiles

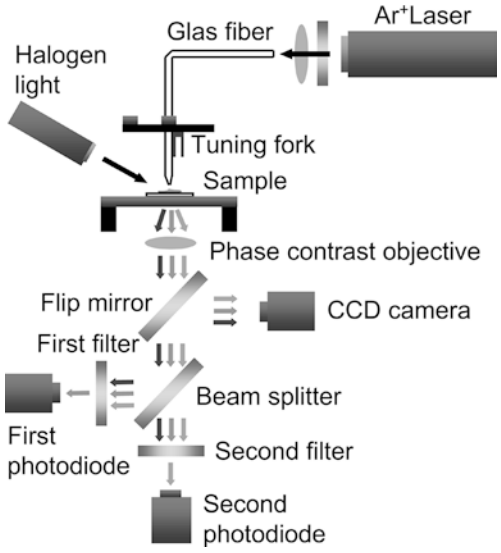

Fig. 6 Setup of the scanning near-field optical microscope (SNOM)

SNOM (Fig. 6) as a powerful tool for the visualization of DNA probes. It was already shown that this instrument is able to visualize very small structures of the order of about 30 nm and that even single protein molecules could be detected, due to the single-photon detection mode (Gao et al. 2001).

We hybridized fairly large DNA probes onto the chromosomes, using telomer (Fig. 7A) as well as centromer probes (Fig. 7B, C). Figure 7A shows an optical SNOM image of several chromosomes with a Cy2-labeled all-human telomer probe. All telomers should be marked by this probe, but actually this is never the case. Usually, signals are not intensive enough at all locations to ensure their detection by conventional fluorescence microscopy. The SNOM measurement clearly identifies all of the signals, including those with a very low intensity, by using its single-photon counting unit. The hybridization pattern of a FITC-labelled chromosome 1 centromere probe is shown in Figure 7. The corrugation of the topographical image is about 190 nm. The chromosome is easily identified and the FITC signal manifests itself as a hillock at the centromeric region (Fig. 7B). The corresponding FITC fluorescence signal is shown in Fig. 7C. Fluorescence signals not directly related to the centromeric region result from unspecifically bound DNA probes. These unspecific fluorescence signals could not be identified using conventional microscopy, again underlining the high potential of SNOM to detect small amounts of fluorescent dyes.

Our experiments have shown that SNOM is suitable for the high-resolution imaging of chromosomes in both the topographical and optical mode. Especially the detection of fluorescent signals from the chromosomes at high lateral resolution has many advantages in comparison to conventional microscopy in molecular cytogenetic research. By reduction of the amount of fluorescent markers and avoiding several stages of signal amplification, SNOM is able to visualize the hybridized DNA at a higher resolution than commonly used methods. The more precise local information will also be of benefit for downstream applications like the

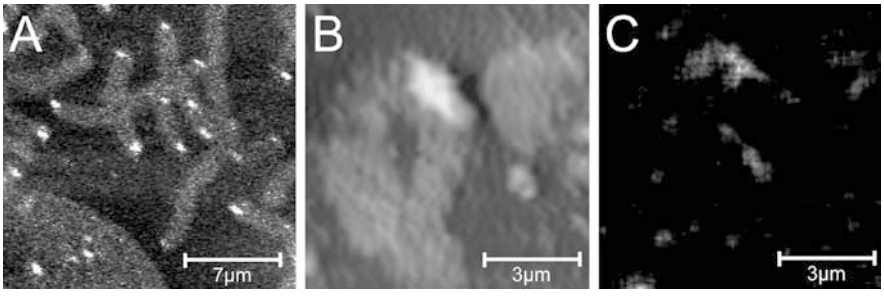

Fig. 7 **A** Fluorescence image of human metaphase chromosomes after in situ hybridization with a telomer probe labeled with Cy2. **B**, **C** Topographic and fluorescence image of the human metaphase chromosome 1 after in situ hybridization with a centromere probe labeled with FITC. **B** Shear-force image at a vertical scale of 190 nm; **C** Corresponding fluorescent signals

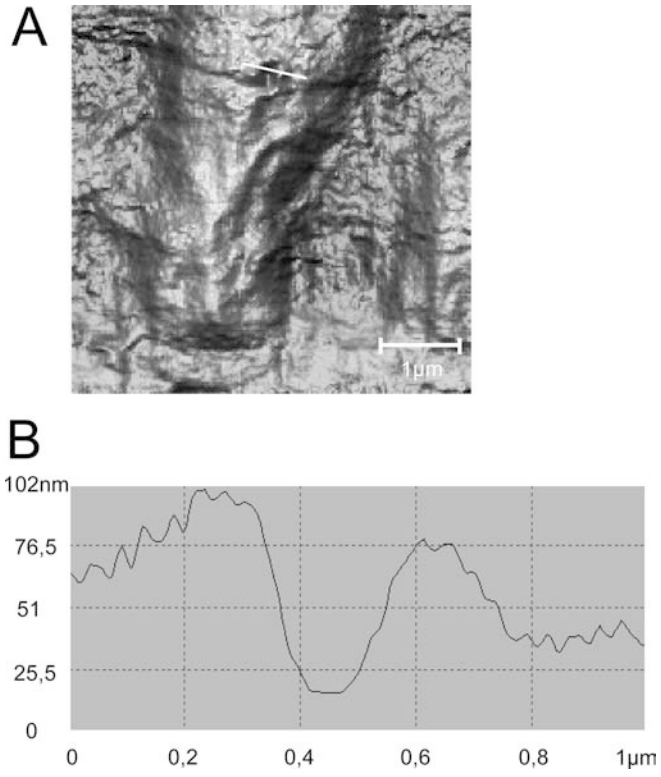

Fig. 8 **A** Topographical overview of the chromosomal surface after the indentation by the SNOM tip. The image was taken using the same SNOM tip again. **B** Linescan showing the depth of the indentation (50 nm×200 nm)

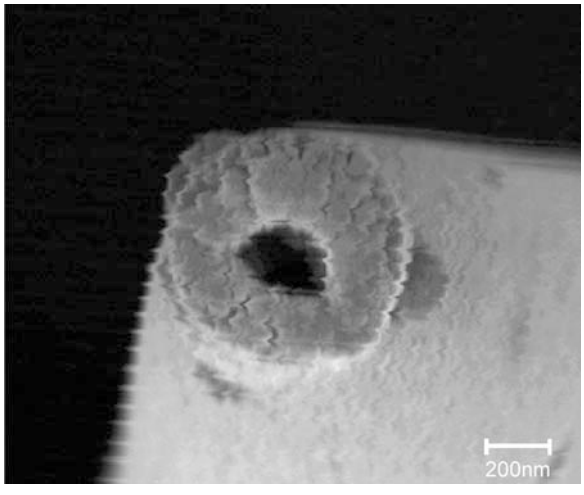

Fig. 9 Scanning electron micrograph of the die cutter end of a glass capillary

extraction of chromosome fragments. An extraction mode of SNOM differs from that of AFM, because a lateral movement of the tip using defined forces is not convenient in SNOM. The optical fiber is rather guided at a relatively large probe–sample distance (Fig. 8A). The indentation of the chromosome has a width of about 200 nm, related to the width of the tip, and a depth of about 50 nm (Fig. 8B).

Future effort will be concentrated on the optimization of the adhesion of chromosomal DNA to the tip during the extraction procedure. One attempt is focused on the development of new tip shapes. Figure 9 shows a glass capillary, designed as a die cutter at its end, which was produced by high-temperature pulling and subsequent etching in HF. The cavity of this tip has a diameter of less than 200 nm, so that it is suitable for topographical and optical imaging. Furthermore, the capillary can be used for the extraction of chromosomal DNA after being moved into the chromosome, because the DNA will be caught in the cavity of the capillary.

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