

# The Atomic Force Microscope as a New Microdissecting Tool for the Generation of Genetic Probes

S. Thalhammer and R. W. Stark

*Institut Für Kristallographie und Angewandte Mineralogie, Universität München; Theresienstr. 41, 80333 Munich, Germany*

S. Müller and J. Wienberg

*Department of Pathology, University of Cambridge, Cambridge, United Kingdom*

and

W. M. Heckl<sup>1</sup>

*Institut Für Kristallographie und Angewandte Mineralogie, Universität München; Theresienstr. 41, 80333 Munich, Germany*

Received February 3, 1997, and in revised form March 25, 1997

---

**The atomic force microscope (AFM) can be used to visualize and to manipulate biological material with relative ease and high resolution. This study was carried out to investigate whether probe sets, specific for subregions of the human genome and useful for the painting of chromosome bands, can be established by PCR amplification of AFM-dissected chromosome regions. Compared to standard microdissection techniques, the AFM can be used with much higher precision for the dissection of the region of interest and subsequent nanoextraction of DNA material. After scanning the area of interest in noncontact mode AFM, chromosome bands were cut by the AFM tip at high force. The genetic material of a single cut attached itself to the tip and was extracted and amplified using degenerate oligonucleotide-primed-PCR. Subsequent to hapten labeling, fluorescence *in situ* hybridization was performed and chromosome band-specific probes were visualized by standard fluorescence microscopy.** © 1997

Academic Press

---

## INTRODUCTION

Chromosomal microdissection provides a direct approach for isolating DNA from any cytogenetically recognizable region. The dissected material can be used for various applications including establishing probes for fluorescence *in situ* hybridization (FISH) (Lichter *et al.*, 1988), the generation of chromosome

band-specific libraries (Lüdecke *et al.*, 1989), and physical mapping for cytogenetic analysis. These highly region-specific probes are extremely valuable for molecular cytogenetic studies, as well as for positional cloning projects.

Since the invention of the AFM (Binnig, 1986) and its use in the structural biology of chromosomes (de Grooth *et al.*, 1992; Heckl, 1992; Rasch *et al.*, 1993; Fritsche *et al.*, 1994; Vesenka *et al.*, 1995) research has focused on the use of the AFM as a micromanipulation tool. Various efforts have shown the possibility of using the AFM to manipulate biological specimens including genetic material (Hansma *et al.*, 1992; Henderson, 1992; Mosher *et al.*, 1994; Jondle *et al.*, 1995).

We have gone a step further by developing a new method for AFM microdissection and combined this with highly sensitive PCR techniques. Minute amounts of material extracted by only single cuts (one AFM line scan in contact mode at high force) could be amplified to create region-specific probes of human chromosome 2.

## METHODS

### *Method Overview*

The main preparational and experimental steps are outlined in the flow chart of Fig. 1. In order to increase the specific amount of target DNA to be extracted by one line scan cut with the AFM, a "pre-set" *in situ* hybridization with chromosome-specific DNA is performed. Prior to the dissection and nanoextraction the chromosomal target area is localized by scanning the AFM tip in the noncontact mode in order to avoid contamination. For the dissection, one line scan was performed with increased force to drive the AFM tip into contact with the target area. DNA that adhered to

<sup>1</sup> To whom correspondence should be addressed.

### Microdissection of Chromosome Segments by Atomic Force Microscopy (AFM)

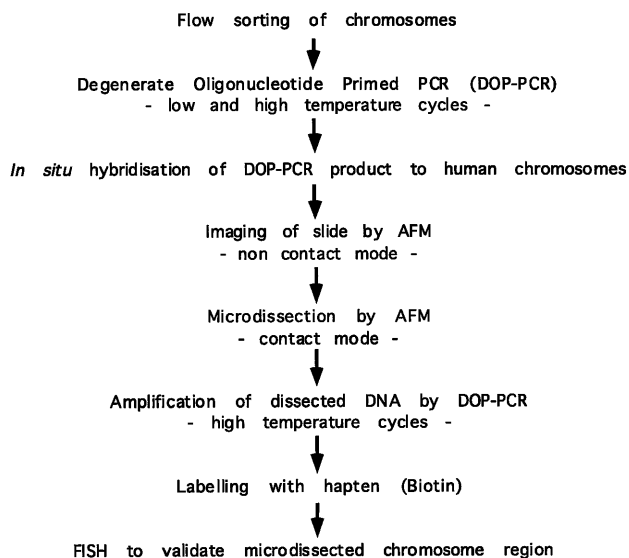


FIG. 1. Flow chart of the main preparational and experimental steps.

the tip was transferred in a sterile tube by incubation of the whole cantilever. DNA was relaxed by topoisomerase I digestion performed prior to a primary DOP-PCR (Telenius *et al.*, 1992). Secondary DOP-PCR was carried out to label the probe. Fluorescence *in situ* hybridization was performed according to standard protocols described elsewhere (Lichter *et al.*, 1991).

**"Preset" *in situ* hybridization.** An aliquot of flow-sorted chromosome 2-specific high-stringency DOP-PCR-amplified DNA was hybridized to metaphase chromosomes. This was performed without formamide. The denaturation was carried out in  $2\times$  SSC at  $75^{\circ}\text{C}$  for 90 sec. The chromosome 2-specific probe was treated like the probes described for FISH. After hybridization overnight at  $37^{\circ}\text{C}$  the slides were washed twice in  $2\times$  SSC/Tween at  $56^{\circ}\text{C}$  for 5 min, rinsed in  $\text{ddH}_2\text{O}$ , and dehydrated in an alcohol series (70, 90, 100%). The slides were stored in 70% ethanol at  $4^{\circ}\text{C}$  before dissection.

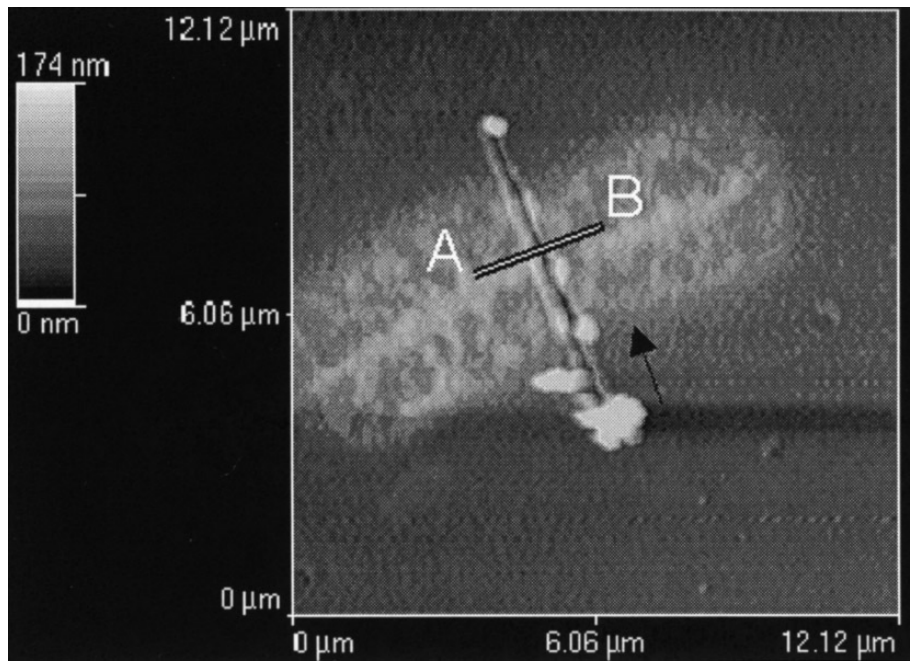
**AFM dissection and extraction.** For nanoextraction of the genetic material, a stand-alone AFM (TopoMetrix Explorer) was mounted onto an inverted optical microscope (Zeiss Axiovert 135). The experimental setup was kept under sterile conditions by UV-C irradiation. The AFM was operated in ambient air in noncontact mode using stiff cantilevers (nanosensors  $k_{\text{force}} = 25 \dots 58 \text{ N/m}$ ,  $n^+ \text{ Si}$ ). Experiments in solution are normally preferable for AFM experiments on biomaterial, but extracted DNA sticking on the tip could be washed off. For distance control, amplitude detection was used and the damping level was set to 30% of the amplitude of free oscillation for imaging before extraction. To perform the dissection and nanoextraction, a metaphase spread was selected with the inverted optical microscope. The sample was then scanned by the AFM and the scan range was reduced in multiple small steps to the size of chromosome 2 to avoid localization problems due to piezo hysteresis. After identification of the extraction site, the scan was stopped and the feedback turned off. The loading force of the tip onto the sample of about  $45 \mu\text{N}$  was applied using manual control of piezo  $z$  voltage. To extract DNA, one line scan was performed at this site. After the tip was retracted from the sample surface, the cantilever was transferred into a  $500\text{-}\mu\text{l}$  tube containing the collection buffer W1. A new

cantilever was used to control the cut at nanoextraction site on the chromosome.

**Topoisomerase I digestion.** To increase the effectivity of the amplification, a topoisomerase I digestion was performed. This DNA relaxing enzyme (Guan, 1993) was used to amplify primer binding. The collection buffer final reaction volume of  $25 \mu\text{l}$  consisted of  $25 \text{ mM}$  *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS),  $\text{pH } 9.3$ ,  $50 \text{ mM}$  KCL,  $1 \text{ mM}$  DTT,  $2 \text{ mM}$   $\text{MgCl}_2$ ,  $0.05\%$  (w/v) W1 detergent (Sigma). Topoisomerase I (Gibco/BRL) was added to a final concentration of 2 units and incubated for 30 min at  $37^{\circ}\text{C}$ . Inactivation of the topoisomerase I was performed in a final incubation of 10 min at  $94^{\circ}\text{C}$ . *Taq* polymerase (Perkin-Elmer) was then added (detailed information under DOP-PCR) and the primary DOP-PCR was carried out.

**DOP-PCR.** For generating the probe, the relaxed DNA was amplified with the primer 6MW (Telenius *et al.*, 1992) using a modification of the DOP-PCR cycling conditions. Each reaction was performed in a  $25\text{-}\mu\text{l}$  volume containing  $25 \text{ mM}$  TAPS,  $\text{pH } 9.3$ ,  $50 \text{ mM}$  KCL,  $1 \text{ mM}$  DTT,  $2 \text{ mM}$   $\text{MgCl}_2$ ,  $0.05\%$  w/v W1 detergent (Sigma), 5 units of *Taq* polymerase (Perkin-Elmer), and dNTPs, each at an initial  $200 \mu\text{M}$  concentration. The primer 6MW ( $5'\text{CCGACTCGAGNNNNNNATGTGG3}'$ ) was used at a  $4 \mu\text{M}$  final concentration. Amplifications were carried out using a Personal Cycler (Biometra). The primary cycling conditions consisted of an initial denaturation for 8 min at  $94^{\circ}\text{C}$  followed by eight low annealing temperature cycles each of  $94^{\circ}\text{C}$  for 60 sec,  $30^{\circ}\text{C}$  for 90 sec a ramp to  $72^{\circ}\text{C}$  over 180 sec, and  $72^{\circ}\text{C}$  for 180 sec. These cycles were followed immediately by 25 high-temperature annealing cycles of  $94^{\circ}\text{C}$  for 60 sec,  $62^{\circ}\text{C}$  for 60 sec, and  $72^{\circ}\text{C}$  for 90 sec, with a time increment of 10 sec. In the final cycle, the last step was extended to 8 min. This material was labeled for FISH by amplifying about 2 pg of the primary PCR product in a secondary DOP-PCR reaction. In these reactions dTTP was reduced to an initial concentration of  $100 \mu\text{M}$  and biotin-16-dUTP (Boehringer Mannheim) was added to  $100 \mu\text{M}$ . Secondary PCR cycling conditions were 5 min at  $94^{\circ}\text{C}$  followed immediately by 30 high annealing temperature cycles of  $94^{\circ}\text{C}$  for 60 sec,  $62^{\circ}\text{C}$  for 60 sec, and  $72^{\circ}\text{C}$  for 90 sec and in the final cycle this last step was extended to 8 min.

**FISH and fluorescence intensity profile.** After nonradioactive labeling of the probe, an aliquot was taken and coprecipitated with the same amount of cot-1 DNA ( $5 \mu\text{l}$  probe,  $5 \mu\text{g}$  cot-1 DNA), salmon sperm DNA,  $3 \text{ M}$  sodium acetate, and  $2.5 \text{ vol } \%$  of ethanol. The DNA was precipitated overnight at  $-20^{\circ}\text{C}$ . The pellet was resuspended in  $15 \mu\text{l}$  of hybridization buffer containing 50% deionized formamide, 10% dextran sulfate,  $0.5 \text{ M}$   $\text{Na}_2\text{H}_2\text{PO}_4$ ,  $0.5 \text{ M}$   $\text{Na}_2\text{HPO}_4$ ,  $2\times$  standard saline citrate (SSC), and  $\text{ddH}_2\text{O}$ . The labeled probes were denaturated at  $68^{\circ}\text{C}$  for 7 min, with 30 min preannealing at  $37^{\circ}\text{C}$ , and the target metaphase spreads were denaturated in a hybridization solution containing  $2\times$  SSC and 70% formamide at  $68^{\circ}\text{C}$  for 1 min. Probe and target were mixed together, covered with a coverslip, sealed with rubber cement, and hybridized in a moist chamber at  $37^{\circ}\text{C}$  overnight. The slides were then washed twice in  $2\times$  SSC, 50% formamide at  $45^{\circ}\text{C}$  and twice in  $2\times$  SSC at  $45^{\circ}\text{C}$ . To avoid nonspecific binding of the antibody, the slides were washed with  $4\times$  SSC/Tween solution at room temperature and incubated with 3% bovine serum albumin (BSA) dissolved in  $4\times$  SSC/Tween for 30 min at  $37^{\circ}\text{C}$ . Biotinylated probes were detected with avidin coupled with fluorescein isothiocyanate (FITC) (Vector). One hundred microliters of avidin FITC diluted 1:200 in  $4\times$  SSC/Tween/3%BSA solution was dropped on the slides and incubated for 45 min at  $37^{\circ}\text{C}$ . After three washes in  $4\times$  SSC/Tween at  $42^{\circ}\text{C}$  (5 min each), the chromosome preparations were counterstained with 4',6-diamidino-2-phenylindole,  $0.2 \text{ ng/ml}$  (DAPI) for 5 min and mounted in  $50 \mu\text{l}$  antifade (1 g *p*-phenyldiamine (Sigma) dissolved in 100 ml phosphate buffer ( $0.15 \text{ M}$  NaCl,  $0.01 \text{ M}$   $\text{KH}_2\text{PO}_4$ ,  $0.01 \text{ M}$   $\text{Na}_2\text{HPO}_4$ , adjusted to  $\text{pH}$



**FIG. 2.** Topographic AFM micrograph of human chromosome 2 after DNA extraction. For nanoextraction, one line scan with approximately 50  $\mu\text{N}$  contact force has been performed. The arrow indicates the dissecting direction of the tip. At the beginning of the cut, next to the arrow shaft, there is a particle visible. This particle can be explained as a part of the dissecting tip which broke off when the tip initially snapped onto the surface. The width of the cut is determined by the cross-section analysis shown in Fig. 3.

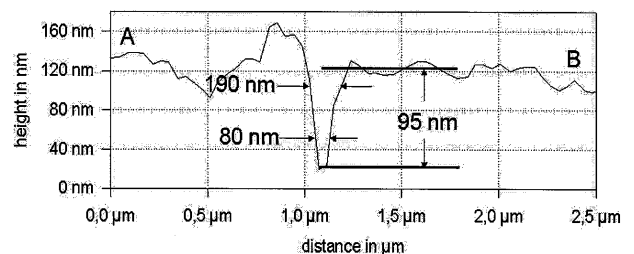
8.0 and diluted 1:10 with glycerol) to avoid bleaching of the hybridization signal.

Photographs were taken with a Zeiss Axiophot epifluorescence microscope. Digital images were obtained using a cooled CCD camera (Photometrics) coupled to the microscope. Camera control and digital image acquisition (8-bit gray scale) were performed on an Apple Macintosh computer. The DAPI and FITC images were merged as described by Ried *et al.* (1992). To validate the microdissected chromosome region a fluorescence intensity profile was measured.

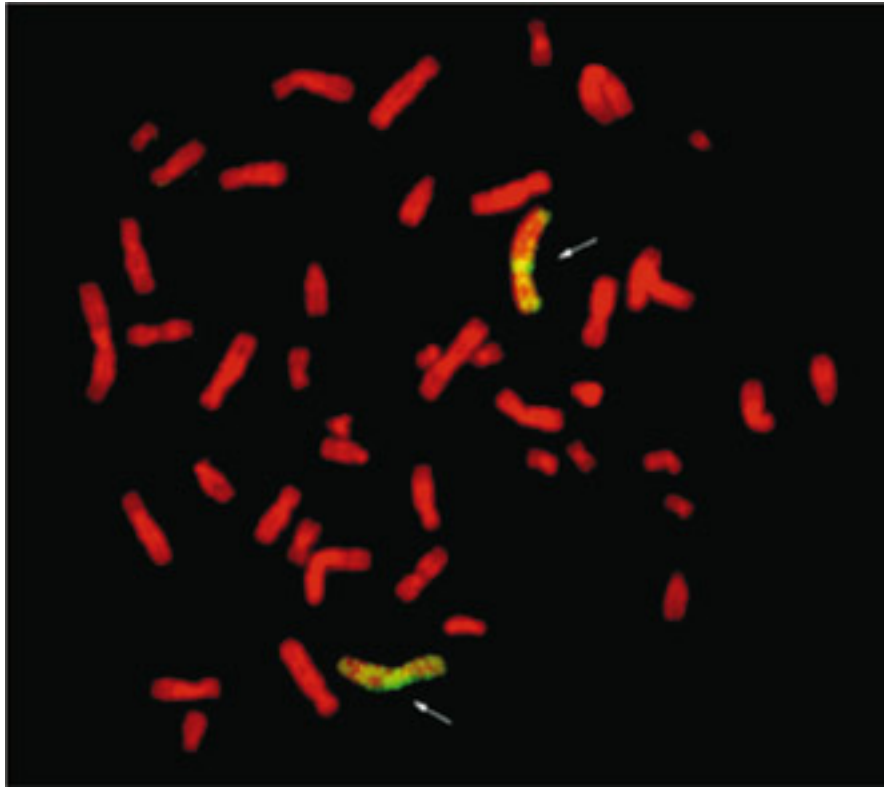
## RESULTS

The topographic AFM micrograph of human chromosome 2 with the extraction site, marked with an arrow, is shown in Fig. 2. The cut site is clearly visible. Additionally, there is also a particle with a diameter of about 1  $\mu\text{m}$  which is located on that point where the AFM tip first touched the surface at the onset of the cut. A cross-section analysis along line AB (Fig. 3) reveals a cut width of about 100 nm (full width at half-maximum height, FWHM). The DNA which sticks to the AFM tip due to hydrophilic interaction has been amplified by a modified DOP-PCR. The PCR product was checked by agarose gel electrophoresis (data not shown). Less than 1  $\mu\text{g}$  of dissected DNA could be amplified. An aliquot of the primary DOP-PCR product was also labeled by a secondary DOP-PCR to increase the amount of probe DNA for FISH. Fluorescence *in situ* hybridization showed a distinct signal at the cut site (Fig. 4). Some background signal was also visible on chromo-

some 2. This was a result of an imperfect noncontact imaging process prior to the dissection. Whenever the AFM tip touched the specimen surface due to disturbances in the noncontact mode, a minimum amount of genetic material may have stuck to the tip. To confirm the results of the FISH experiment a fluorescence intensity profile was measured (Fig. 5). It shows (a) the microdissected chromosome 2 with telomeric background, (b) a flow-sorted chromosome 2-specific paint, and (c) a negative control. The fluorescence signal of the dissected chromosome region is clearly visible in profile a, in comparison to the chromosome 2 paint. The fluorescence signal is relatively broad due to the fact that the displayed profiles show the mean of 10 measured metaphases. However, the maximum of the signal in 5a correlates with an area around the 2p12 band.



**FIG. 3.** Cross-sectional analysis of the cut site (Fig. 2). The profile reveals a full width at half-maximum height (FWHM) of around 100 nm.



**FIG. 4.** Fluorescence *in situ* hybridization of the dissected probe (labeled with FITC, hybridization signal marked with an arrow) and counterstaining of the metaphase chromosomes with DAPI proves the signal only at chromosome 2 and mainly in the dissected region.

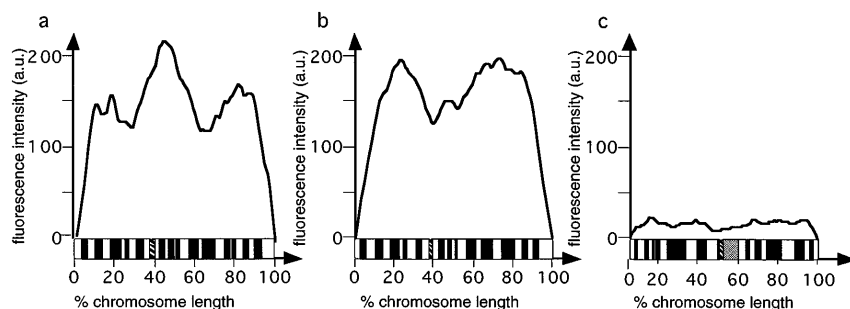
### DISCUSSION

This experiment showed clearly that DNA could be extracted by the AFM for subsequent use in molecular cytogenetics. However, only part of the dissected DNA could be extracted because there was still loose chromosomal material left along the cut site (Fig. 2). This situation could possibly be improved by using microfabricated cantilevers with distinct shaped tips, e.g., in the form of a “shuffle” where probe material can be collected and transported with a higher yield.

The particle observed at the onset of the cut site in Fig. 2 can be explained as debris from the dissecting tip which broke off when it came in first contact with

the surface. On one hand this leads to broader cuts but on the other hand the probability of contamination with DNA sticking on the tip as a result of the imaging process is reduced. The problem can be solved by avoiding the cantilever to snap into the sample surface by counterbalancing the adhesion forces between tip and sample through the use of appropriate cantilever spring constants.

To reduce problems which arise from unwanted arbitrary tip contacts on the specimen surface, much like in the AFM high-amplitude or tapping mode, real noncontact mode should be achieved in future experiments. From an experimental point of view



**FIG. 5.** Fluorescence intensity profiles of the dissected probe (a) in comparison with a chromosome 2 paint (b) and a negative control of unstained chromosome 1 (c).

the recovery of DNA molecules from different chromosome sites during standard noncontact imaging, as seen in the spread of markers in Fig. 3, is a measure of the performance of intermittent contact mode AFM. In the given context of the experiment, one way to improve the situation besides using stiffer cantilevers is to control (namely, to reduce) atmospheric humidity, which is partly responsible for the capillary force-driven tip specimen contact. For a discussion of various artifacts in noncontact mode AFM due to adsorbed moisture, see Dinte *et al.* (1996). This procedure will also reduce the compressibility of the biomaterial. (For analysis of sample elasticity see, e.g., Radmacher *et al.*, 1992). Because of the thin water film which covers the chromosome, the tip snaps back to the biomaterial and causes contamination of the tip prior to the dissection. Another approach is indicated by the particle visible at the beginning of the cut. This particle is interpreted as being a fractured part of the tip which broke off when the cantilever snapped into the surface. Unfortunately, only the very end broke off and not the entire contaminated part of the tip. However, it should be possible to discard the whole contaminated area of the tip immediately before the cut is performed by using tips with a defined point of fracture between the imaging and the extraction parts of the tip. This is much like a stinging nettle of certain plants. Electron-beam-deposited tips (Keller *et al.*, 1992; Wendel *et al.*, 1995) or tips made from carbon nanotubes (Dai *et al.*, 1996) seem to be adequate. This would help to increase the precision of extractions by avoiding contamination of the dissecting tip.

The developed modification of the DOP-PCR is able to amplify even less than a picogram of dissected DNA. This is mainly due to the time elongation for each cycle in the high-stringency part of the PCR to 10 sec, which increased the amount of DNA significantly in comparison to normal high-stringency cycles. Additionally, the amplified material was labeled with a high-stringency secondary DOP-PCR and fluorescence *in situ* hybridization was performed. As a result, the FISH image showed a distinct signal near the centromere (compare FISH and AFM images). Also, nonspecific background due to unwanted tip specimen contacts, as discussed above, was visible.

The fluorescence intensity profile showed the strong signal of the hybridized extracted probe, in comparison with a normal chromosome 2 paint and a negative control probe. The relatively high background at the telomeric sites was due to a contamination of the tip, as discussed above. The modified DOP-PCR nonspecifically amplified all DNA that stuck to the tip, including high repetitive sequence

parts, which bound additionally to the dissected DNA randomly on the metaphase chromosome. The high background staining in the centromeric region of chromosome 2 can also be due to specific tandem repetitive centromeric sequences, picked up while scanning the region of interest.

## SUMMARY AND OUTLOOK

With this experiment it has been demonstrated that it is possible to use the AFM tip like a mechanical "nanoscalpel" and a "nanoshuffle" as a new cytogenetic tool. Minute amounts of material extracted at particular chromosome sites can be processed by the use of a biochemical amplification technique. This opens the unique possibility of developing specific genetic probes from any discernable distinct region. One attractive application of the our goal "one cut-one probe" lies in physical fine mapping of chromosomes, especially for evolutionary studies. In comparison to standard microdissection techniques with glass needles or the newly developed laser cutting, AFM nanodissection has the potential to generate much smaller probes (Thalhammer *et al.*, 1997).

It is also worth to noting that the combination of nanoscale AFM experiments with PCR allows one to overcome a central problem inherent in serial techniques like scanning probe microscopy: their inefficiency compared to parallel processing techniques.

This work was supported through DFG Grants He1617/3-2 and He1617/3-3. We thank Dr. S. J. Sowerby for valuable discussion.

## REFERENCES

- Binnig, G., Quate, C. F., and Gerber, C. (1986) Atomic force microscopy, *Phys. Rev. Lett.* **56**, 930-933.
- Dai, H., Hafner, J. H., Rinzler, A. G., Colbert, D. T., and Smalley, R. E. (1996) Nanotubes as nanoprobe in scanning probe microscopy, *Nature* **384**, 147-150.
- deGroot, D. G., and Putman, C. A. J. (1992) High resolution imaging of chromosome related structures by atomic force microscopy, *J. Microsc.* **168**, 239-247.
- Dinte, B. P., Watson, G. S., Bobson, J. F., and Myhra, S. (1996) Artefacts in non-contact mode force microscopy: The role of adsorbed moisture, *Ultramicroscopy* **63**, 115-124.
- Fritsche, W., Schaper, A., and Jovin, T. M. (1994) Probing chromatin with the scanning force microscope; *Chromosoma* **103**, 231-236.
- Guan, X. Y., Trent, J. M., and Meltzer, P. S. (1993): Generation of band-specific painting probes from a single microdissected chromosome, *Human Molecular Genetics*, **22**, 1117-1121.
- Hansma, H., Vesenska, J., Siegerist, C., Kelderman, G., Morret, H., Sinsheimer, R. L., Elings, V., Bustamente, C., Hansma, P. K. (1992) Reproducible imaging and dissection of plamid DNA under liquid with the atomic force microscope, *Science* **256**, 1180-1184.
- Heckl, W. M. (1992) Scanning tunneling microscopy and atomic force microscopy on organic and biomolecules, *Thin Solid Films* **210/211**, 640-647.

- Henderson, E. (1992) Imaging and nanodissection of individual supercoiled plasmids by atomic force microscopy, *Nucleic Acids Res.* **20**(3), 445–447.
- Jondle, D. M., Ambrosio, L., Vesenka, J., and Henderson, E. (1995) Imaging and manipulation chromosomes with the atomic force microscopy, *Chromosome Res.* **3**, 239–244.
- Keller, D. J., and Chou, C. C. (1992) Imaging steep high structures by scanning force microscopy with electron beam deposited tips, *Surface Sci.* **268**(1–3), 333–339.
- Lichter, P., Cremer, T., Tang, C. C., Watkins, P. C., Manuelidis, L., and Ward, D. C. (1988) Rapid detection of human chromosome 21 aberrations by in situ hybridization, *Proc. Natl. Acad. Sci. USA* **85**, 9664–9668.
- Lichter, P., Boyle, A. L., Cremer, T., and Ward, D. C. (1991) Analysis of genes and chromosomes by non-isotopic in situ hybridization, *Genet. Anal. Techn. Appl.* **8**, 24–35.
- Lüdecke, H. J., Senger, G., Claussen, U., and Horsthemke, B. (1989) Cloning defined regions of the human genome by microdissection of banded chromosomes and enzymatic amplification, *Nature* **338**, 348–350.
- Mosher, C., Jondle, D., Ambrosio, L., Vesenka, J., and Henderson, E. (1994) Microdissection and measurement of polytene chromosomes using the atomic force microscope, *Scanning Microsc.* **8**(3), 491–497.
- Radmacher, M., Tillmann, R. W., Fritz, M., Gaub, H. E. (1992) From molecules to cells—Imaging soft samples with the atomic force microscope, *Science* **257**, 1900–1905.
- Rasch, P., Wiedemann, U., Wienberg, J., and W. M. Heckl, (1993) Analysis of banded human chromosomes and in situ hybridization patterns by scanning force microscopy, *Proc. Natl. Acad. Sci. USA* **90**, 2509–2511.
- Ried, T., Baldini, A., Rand, T. C., and Ward, D. C. (1992) Simultaneous visualization of seven different DNA probes by in situ hybridization using combinatorial fluorescence and digital microscopy, *Proc. Natl. Acad. Sci. USA* **89**, 1388–1392.
- Telenius, H., Pelmear, A. H., Tunnacliffe, A., Carter, N. P., Behmel, A., Ferguson-Smith, M. A., Nordednskjold, M., Pfragner, R., and Ponder, B. A. (1992) Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow sorted chromosomes, *Genes-Chromosomes-Cancer* **4**(3), 257–263.
- Thalhammer, S., Stark, R. W., Schütze, K., and Heckl, W. M. (1997) Laser microdissection of metaphase chromosomes and visualisation by scanning force microscopy, *Biomed. Optics* **2**, 115–119.
- Vesenka, J., Mosher, C., Schaus, S., Ambrosio, L., and Henderson, E. (1995) Combining optical and atomic force microscopy for life sciences research, *BioTechniques*, **19**(2), 240–249.
- Wendel, M., Lorenz, H., and Kotthaus, J. P. (1995) Sharpened electron-beam deposited tips for high-resolution atomic force microscope lithography and imaging, *Appl. Phys. Lett.* **67**(25), 3732–3734.