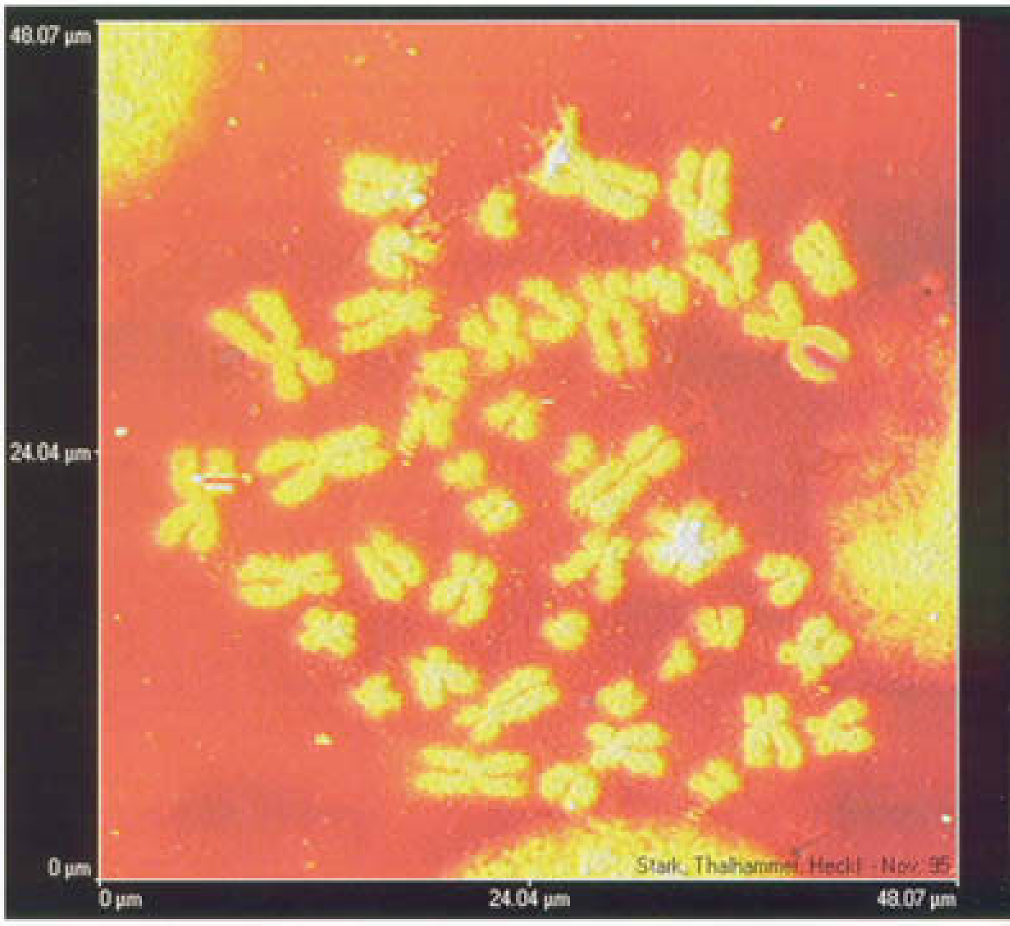
The Combination of AFM Nanodissection with PCR

A new tool for the generation of genetic probes

Wolfgang M. Heckl



Abstract

Scanning probe microscopes and especially the atomic force microscope (AFM) has proved to be a highly useful new technique for high resolution imaging of biological specimens [1, 2]. This is mainly due to its ease of use and small cost in comparison to electron microscopes as well as its effective integration into an optical microscope system, allowing for stepless magnification from the micrometer down to the molecular scale. Its biggest advantage, however, is the possibility to work under liquid (physiological) conditions providing a natural environment for the biomaterial under investigation. Nowadays not only topographic imaging but also friction and adhesion imaging as well as chemical imaging and force spectroscopy has been established.

Recently we have added a new dimension to the imaging capability of the AFM which was inherently there all the time and happened occasionally to every AFM user. When the AFM tip raster scans a surface in contact mode to record an image similar to the groping of a vinyl record by an old style record player needle, the tip exploits considerable forces (ranging from pN to µN) onto the specimen surface at a very localized region. A destructive interaction between tip and sample is usually a harmful disadvantage during imaging, but can be turned into an new application of the AFM, besides imaging. Any scanning probe tip can be used as a minute tool on a truly nanometric scale and be used for the manipulation of bio- and other material [3]. Thus we have exploited the function of an AFM tip to act as a "nanoscalpel" with the ability to dissect human chromosomes at a desired location. Due to the minute (in principle up to atomically sharp) tip established microdissection techniques can in fact be extended towards the submicron scale. The dissection and manipulation of biomaterial alone would not be very helpful. But the fact that the hydrophilic nature of the tip material (usually silicon or silicon nitride) tends to attract and retain biomolecules can profitably be used for what could be called a "nanoshuffle" besides the "nanoscalpel". Dissected chromosome material can thus be collected and extracted and used for further processing such as mass spectroscopy of minute material quantities (smaller than a femtogram) or biochemical processing. We have established a protocol which allows to PCR amplify the genetic mater-

KEYWORDS
atomic force microscopy, PCR, DNA, nanodissection

ial extracted by an AFM tip. If this technique can successfully be implemented in routine applications it may help to refine existing methods in cytogenetics and genome analysis.

Introduction

The chromosomal microdissection provides a direct approach for isolating DNA from any cytogenetically recognizable region. This dissected material can be used for numerous applications e.g. establishing region specific probes for fluorescence in situ hybridization (FISH), for the generation of chromosome band-specific libraries [4], for physical fine mapping or for cytogenetic analysis including prenatal diagnostics and evolutionary studies. These highly region-specific probes are extremely valuable for molecular cytogenetic studies, as well as for positional cloning projects and the search for disease genes as a medical application. If found the DNA base sequence is determined around that region. The search is completed if a DNA sequence is specifically only found in people with the disease in question. Positional cloning has so far been used to find over 50 disease genes including the gene for some type of breast cancer, duchenne muscular dystrophy and cystic fibrosis.

Since the invention of the atomic force microscope (AFM) [5] and its use in structural biology [6-9] an important area of research has focused on the use of the AFM as a micromanipulation tool. Various efforts have shown the possibility of the AFM to manipulate biological specimen like genetic material [10-13].

We have developed a new method of AFM-nanodissection in combination with highly sensitive PCR [14, 15]. These experiments were carried out by performing only one cut (one AFM linescan) to extract the region-specific genetic material which adhered to the AFM cantilever as shown in Figure 1.

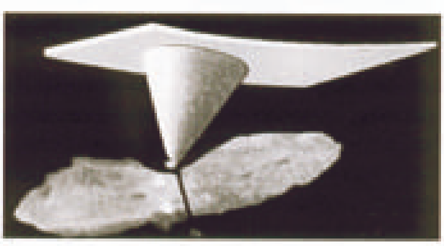


Fig. 1: AFM-cantilever tip with extracted DNA chromosome

Experiment

The experimental set-up is housed in a sterile flowhood (UV-C radiation) and consists of a combined inverted microscope (Zeiss) with an AFM (TopoMetrix) on top. This allows for the identification of fluorescently labeled chromosomes in a far field optical microscope prior to AFM non-contact imaging and dissection. Alternatively we have used AFM imaging of 30 nm gold beads linked to specific target sequences for the localization of the cut site.

by one line scan with the AFM, a "pre-set" in situ hybridization with chromosome specific DNA is performed.

The AFM operated in ambient air in non-contact mode using stiff cantilevers (Nanosensors kforce = 25...58 N/m, n+ Si).For dissection the force was increased to around 30 μN and one line scan to dissect the genetic material, was performed in contact mode. The DNA which adhered to the tip was transferred in a sterile 500 μl tube containing the "collection buffer". After that a new cantilever was used to visualize the nanoextraction site.

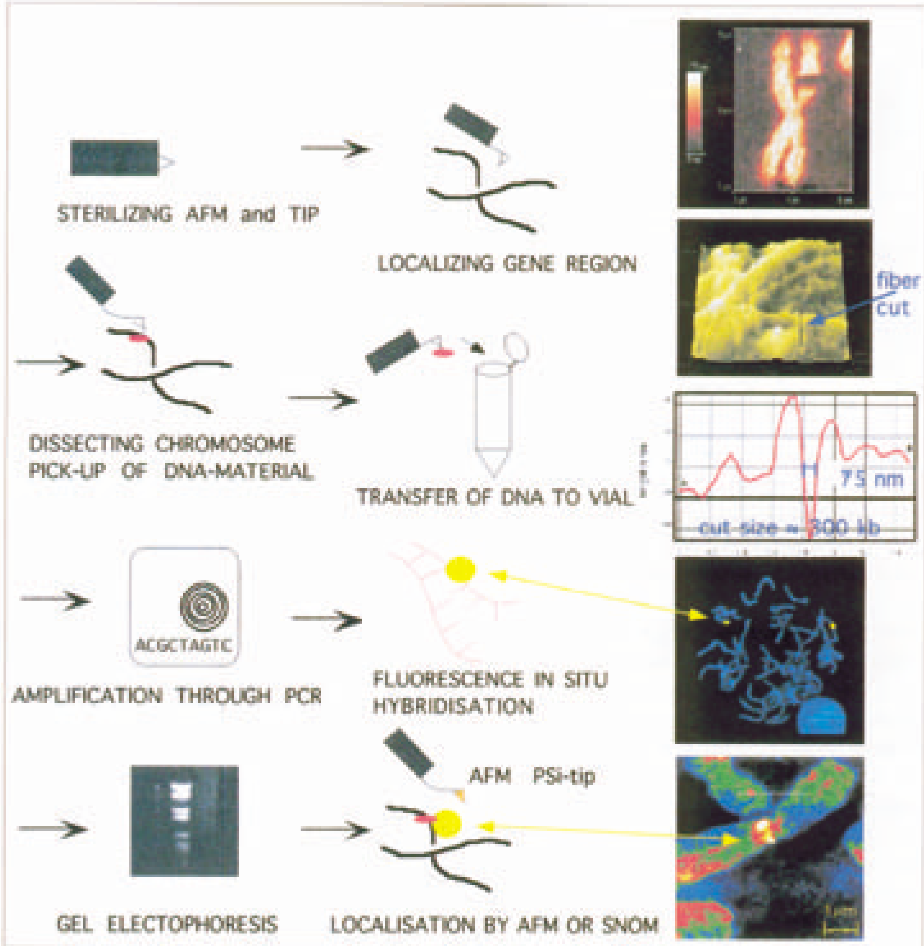


Fig. 2: Main preparation and experimental steps

The main preparation and experimental steps are outlined in the flow chart and Figure 2.

Prior to the experiment the whole equipment was sterilized to reduce the risk of contamination and kept under sterile conditions during the nanoextraction of the genetic material. Flow sorted chromosomes are prepared on a microscope cover slide and localized by a combination of optical and force microscopy in non contact mode.

To introduce primers for the subsequent PCR amplification and to double the amount of target DNA to be extracted

To "relax" the genetic material a TopoisomeraseI digestion was performed prior to a primary DOP-PCR.

For generating the probe the relaxed DNA was amplified with the primer 6MW (5'CCGACTCGAGNNNNNNATGTGG3') [16] using a modification of the DOP-PCR cycling conditions. Amplifications were carried out using a Personal Cycler (Biometra). This material was labeled for FISH by amplifying an amount of the primary PCR product in a secondary DOP-PCR reaction in which dTTP was reduced to an initial concentration of 100 μM and Biotin-16-dUTP (Boehringer

Flow sorting of chromosomes

↓

Degenerate Oligonucleotide Primed PCR (DOP-PCR) with low and high temperature cycles

↓

In situ hybridization of DOP-PCR product to human chromosomes

↓

optical (fluorescence) localization of the chromosome

↓

AFM imaging of chromosome in non contact mode

↓

nanodissection by AFM in contact mode
(one line scan = one cut)

↓

transfer of the AFM tip into vial

↓

amplification of dissected DNA by DOP-PCR with high temperature cycles

↓

labeling with hapten (biotin)

↓

FISH to validate nanodissected chromosome region

Mannheim) was added to 100 µM. Gel electrophoresis confirmed the amplification of the dissected product in comparison to the control of an used cantilever which was only operated in non-contact mode.

Fluorescence in situ hybridization was performed after standard protocols described elsewhere [17]. Biotinylated probes were detected with avidin coupled with fluoresceinisothiocyanate (FITC) (Vector), (Fig. 2).

Results and Discussion

Nanodissected chromosome fragments provide extraordinary small quantities of template DNA for PCR. This places unusually severe requirements on the prevention of PCR contamination. Therefore working in a clean room is desirable and sterilization of the equipment is necessary. Equally important is to account for the fact, that only one line scan was performed for extracting the genetic material, by increasing the DNA amount and introducing primers by a "pre-set" in situ hybridization.

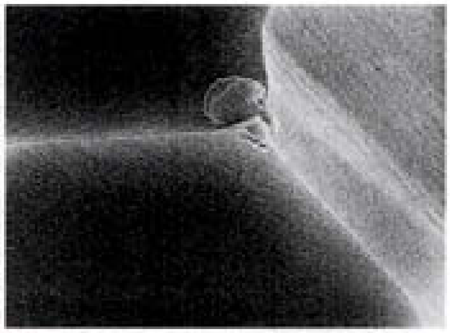


Fig 3: Electron micrograph of material pick up by the AFM nanoextraction technique

The main step is however to operate the AFM in a true non contact mode to prevent unwanted occasional pick up of DNA prior to dissection. As a control we always PCR process and validate a control consisting of a AFM tip which sits unused on the cantilever chip besides the one which is used for imaging and pick up.

The pick up of DNA is facilitated by the hydrophilic nature of the tip material. Fig. 3 shows an electron micrograph of around 200 fg of DNA as determined by its apparent size or the change in resonance frequency of the cantilever. This is an example that extraordinary small quantities can be handled with relative ease with applications far beyond the biochemical use as shown here. Time of Flight Secondary Ion Mass Spectroscopy may as well be used for the chemical identification of target material specifically extracted on a desired sample side after high resolution AFM imaging for its localization. Different types of AFM tip shapes (shuffle, forceps, knifes and so on) can be manufactured through ion beam deposition and milling/plasma etching techniques even better suitable for specific targets (Fig.3).

Another important experimental step is the use of the AFM in a "stamping" rather than a "scratching" mode during dissection and pick up of the biomaterial. This can be seen in Fig. 4 where the comparison of both modes clearly shows that stamping, i.e. vertical vibrating the tip (a) allows for fine cutting whereas in the case without vertical modulation (b) unusable

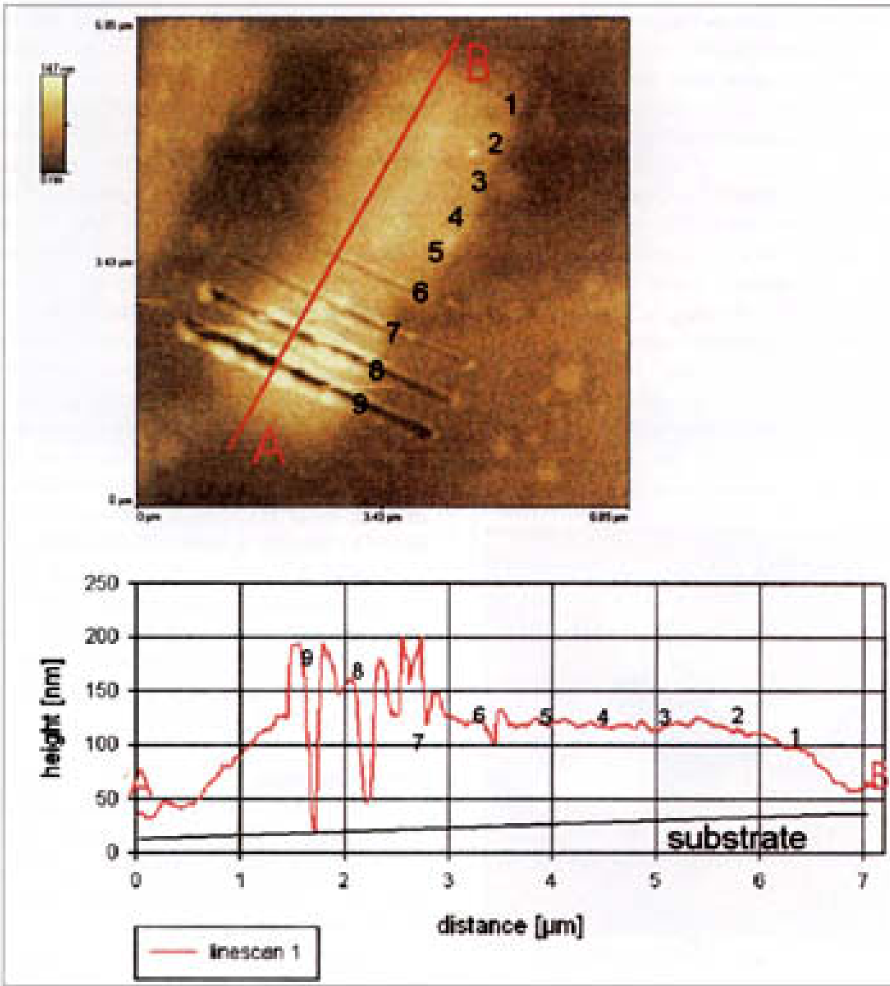


Fig. 4a: Using the AFM in a stamping mode with vertical vibration

debris is created during a series of cuts. Additionally it can be seen that the forces which are necessary for a precise AFM chromosome cut lay in a small range around 30 µN (cut number 9 in Fig. 4a, see line scan). This is due to the elastic behavior of the chromosome which depends on its preparation and especially its hydration state. We have measured typical elastic modules of a human metaphase chromosome of 0.2 to 0.3 MPa. The forces which have been applied are 2.8 µN (1), 8.4 µN (2), 11.2 µN (3), 14 µN (4), 16.8 µN (5), 19.6 µN (6), 22.4 µN (7), 25.2 µN (8), and 28 µN (9), (Fig. 4a, 4b).

Figure 4 shows the comparison between "nanostamping" i.e. cutting with 5 nm z-modulation @ 10 Hz during the cut with speed of 1 µm/sec and "nanoscratching", i.e. without z-modulation (from reference 15).

As a final step for the verification of the procedure fluorescence in-situ hybridization was performed. It showed a distinct signal at the position of the chromosome where the initial cut was performed and thus provided a proof for the protocol to work properly. Additional fluorescence intensity profiles based on images of the FISH chromosomes confirmed the result of the FISH experiment.

It is interesting to compare the range of different techniques for the collection of DNA-material. In the case of the glass needle technique the cut size is around 1–5 µm according to the limitation in producing glass micropipettes. Depending on the condensation state of the DNA this allows for the extraction of around 10-50 Megabases equal to around 20-100 fg of DNA. With this technique usually several copies of microdissected region are needed for PCR. UV-Laser cutting technique can aid in isolation of the desired chromosomal region which subsequently must be picked up by mechanical means of a glass needle or an AFM tip. Our experience shows that the destructive ablation with this technique allows for a minimal cut size around 1 µm due to the diffraction limited focusing of the laser beam [18]. Clearly its biggest disadvantage in comparison with the AFM technique is that the laser per se has no shuffle function. Therefore AFM with demonstrated cutting sizes down to around 50 nm with subsequent amplification and 5 nm (in the case of plasmid rings, yet without subsequent amplification) is a desirable addition to existing techniques which allows for the biochemical processing of around 2 Megabases for chromosomes and around 300 kilobases (less than 1 fg of DNA) for extended fiber chromatin.

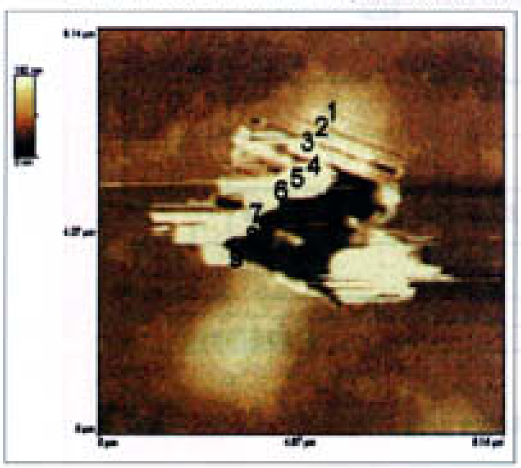


Fig. 4b: Dissection by AFM without vertical modulation

Conclusions

Based on its working principle the scanning probe microscopes cannot only be used for high resolution imaging of surface topography, but also at the same time they are perfect tools on the nanometric scale [3]. With this experiment we have demonstrated that it is possible to use the AFM tip like a mechanical nanoscalpel for nanodissection and as a nanoshuffle for subsequent nanoextraction and microcloning of genetic material from human chromosomes. In order to overcome the problems with minute amount of material to be processed at a time which is inherent for a sequential method like the scanning probe technique we combined the AFM-nanoextraction of a particular gene region with the biochemical polymerase chain reaction (PCR) technique for its amplification. This combination of nanomechanics and molecular cytogenetics may allow for the creation of much smaller genetic probes in comparison to standard microdissection techniques useful for high resolution mapping of the human genome. The next goal is to implement a near field optical microscope in order to identify a particular genomic region labeled with only few dye molecules for subsequent nanodissection. For this goal an AFM hybrid tip which is a tiny light emitter based on the electroluminescence of porous silicon as well has been constructed [19].

Acknowledgments

This article is based on the work of my two Ph.D. students Stefan Thalhammer and Robert Stark. Advise from Dr. Johannes Wienberg, Cambridge University, collaboration with Dr. Karin Schütze, PALM GmbH, Wolfratshausen and financial support from DFG (He1617-3/3), Bayerische Forschungsstiftung and Bayern Innovativ is greatefully acknowled.

Literature

[1] HECKL, W.M., A. ENGEL: Imaging Nucleic Acids with Scanning Probe Microscopes. Visualization of Nucleic Acids, ed. G.Morel, CRC Press: BocaRaton, Florida, 21–33 (1995).

[2] HECKL, W. M.: Scanning the Thread of Life, DNA under the microscope, in The Diagnostic Challenge, The Human Genome, E. P. Fischer and S. Klose, Editors., Piper Verlag München, 99–145, (1995)

[3] HECKL, W. M., ed. Visualization and Nanomanipulation of Molecules in the Scanning Tunneling Microscope. In Pioneering Ideas for the Physical and Chemical Sciences, eds. W. Fleischhacker and T. SchÃnfeld, Plenum Press: New York. 179, (1997).

[4] LÜDECKE, H. J., G. SENGER, U. CLAUSSEN, B. HORSTHEMKE, Nature, 338, 348, (1989)

[5] BINNIG, G., C. QUATE, C. GERBER: Physical Review Letters,56, 930, (1986)

[6] FRITSCHE W., S. A., JOVIN T. M.: Chromosoma, 103, 231, (1994)

[7] RASCH, P., U. WIEDEMANN, J. WIENBERG, W. M. HECKL: PNAS, 90, 2509, (1993)

[8] SCHAPER, A., L. I. PIETRANSANTA, T. M. JOVIN: Nucleic Acids Research, 25(25), 6004 (1993)

[9] VESENKA, J., M. C., S. SCHAUS, L. AMBROSIO, E. HENDERSON.: BioTechniques, 19, 2, 240, (1995)

[10] HAAF, T., D. C. WARD: Human Molecular Genetics, 3(5), 697, (1994)

[11] HANSMA, H. G., et al., Science, 256, 1180, (1992)

[12] HENDERSON, E., Nucleic Acids Research, 20(3), 445, (1992)

[13] JONDLE, D. M., L. AMBROSIO, J. VESENKA, E. HENDERSON: Chromosome Research, 3, 239, (1995)

[14] THALHAMMER, S., R. W. STARK, S. MÜLLER, J. WIENBERG, W.M. HECKL, J.: Struc. Biol., 119, 232, (1997)

[15] STARK, R. W., S. TAHLHAMMER, W. M. HECKL: Appl. Phys. Lett., A66, S579, (1998)

[16] TELENIUS, H., N. P. CARTERR, C. E. BEBB, M. NORDENSKJÄLD, B. A. J. PONDER, A. TUNNACLIFFE: Genomics, 13, 718, (1992)

[17] LICHTER, P., A. L. BOYLE, T. CREMER, D. C. WARD: Genet. Anal. Techn. Appl., 8, 24, (1991)

[18] THALHAMMER, S., R. STARK, K. SCHÜTZE, J. WIENBERG, W. M. HECKL: J. Biomed. Opt., 2, 115, (1997)

[19] GÖTTLICH, H., W. M. HECKL: Ultramicroscopy, 61, 145, (1995)

The Author

Prof. Dr. Wolfgang M. Heckl
Ludwig-Maximilians-Universität
Institut für Kristallographie und Angewandte Mineralogie
Theresienstr. 4, 80333 München, Germany
E-Mail: W.heckl@lrz.uni-muenchen.de
www.kri.physik.uni-muenchen.de/geo/crystal/stm