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Detection of In Situ Hybridization to Human Chromosomes With the Atomic Force Microscope[1]

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Atomic force microscopy (AFM) permits one to generate a topographic representation of the sample under investigation with high spatial resolution. We assumed that cytochemical staining techniques, which yield reaction products which can be discriminated from the surrounding material on basis of their topographic properties, would be applicable in AFM. Here we show the validity of this assumption by employing an in situ hybridization technique in which the final label was the precipitated product of a peroxidase/diaminebenzidine reaction. After hybridization of the DNA probe pUC1.77 that recognizes the heterochromatic region of human chromosome 1 (1q12), the AFM clearly detects the sites of in situ hybridization. In situ hybridization with DNA probe p1-79 results in clear marking of the telomere region 1p36. The diameter of the probe p1-79 linked reaction product was 75–100 nm, indicating that resolution of 200 nm can readily be reached with this AFM approach of DNA mapping. This precision is directly linked with the amount of precipitated material. © 1993 Wiley-Liss, Inc.

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The atomic force microscope (AFM) [3] is a member of the growing family of scanning probe microscopes [9]. In contrast to traditional microscopy where radiation is used to visualize an object, e.g., light in the optical microscope or electrons in an electron microscope, the scanning probe microscope passes a probe in close proximity to the surface and collects spatially resolved information of various types of properties. In the AFM a stylus with a very sharp tip is mounted on a weak cantilever. Forces acting between the tip and the sample will deflect the cantilever. Detection of the tip displacements while the object is raster-scanned underneath the stylus, and displaying these in a two-dimensional matrix, gives a topographic representation of the sample.

Since its introduction, the AFM has been used to image a wide variety of samples, some of them revealing single atoms. Gap junctions [16], blood cells [7], chromosomes [12, 13, 25], and DNA molecules [6, 15] are some of the biological objects which have been imaged with the AFM. For good reviews on the use of AFM on biological samples, we refer to Engel [9] and Hoh and Hansma [17].

Chromosome structure and functioning is studied with all the microscopic techniques available so far. The knowledge on the packing of DNA inside chromosomes on the lower scale is quite complete. Starting with a double helix wound around nucleosomes (beads-on-a-string) the 30 nm chromatin fibers are formed [21]. Beyond this level of structural organization the gathering of information is difficult due to the low resolution of the optical microscope and elaborate sample preparation for the electron microscope.

For DNA mapping and to correlate DNA sequence information with chromosome structure, in situ hybridization techniques have been developed [4, 10, 18]. Radioactive labeled DNA probes and microautoradiography were originally used in such research for detection purposes. Owing to the track of the decaying particle in the photographic emulsion, however, full use of the resolution of the light microscope could not be made. For that and other reasons, new non-radioactive

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DNA labeling methods have been developed, which permit light microscopical detection of in situ hybridized DNA on the basis of fluorescence or enzyme generated dyes [for reviews see Lichter et al. (20) and Raap et al. (29)].

We hypothesized that if the final in situ hybridization label would generate typical morphological features that it should be detectable by the AFM on top of the normal topography of the object with a better resolution as compared to the light microscope. Enzyme cytochemical reaction products, such as the oxidized product of the peroxidase/diaminebenzidine (PO/DAB) reaction, are good candidate labels in this respect.

In this report we describe the first results on PO/DAB detection of in situ hybridized DNA obtained with the AFM. They show the validity of the hypothesis.

MATERIALS AND METHODS

Chromosomes

Metaphase chromosome spreads were produced from phytomagglutinin stimulated normal human peripheral blood lymphocyte cultures according to routine procedures. As substrates standard microscope slides (26×76 mm^2), cleaned with an alcohol/ether (1/1) solution, were used. Two DNA probes were used in this study: pUC1.77 and p1-79. Both hybridize to chromosome 1. In situ hybridization with biotinated pUC1.77 DNA was essentially according to Wiegant et al. [32]. It was detected with one layer of peroxidase conjugated to avidin followed by diaminebenzidine (DAB) reaction [11]. Probe pUC1.77 hybridizes to the heterochromatic region 1q12 [8] close to the centomere and p1-79 hybridizes to the telomere region of the short arms, 1p36 [5]. The DAB concentration and the duration of the amplification reaction are varied from 0.05% to 0.01%, respectively, 20 min to 2 min.

Atomic Force Microscope

A schematic representation of the atomic force microscope used [25], is shown in Figure 1. The deflection of the microfabricated cantilever with integrated pyramidal tip [1] (Park Scientific Instruments, Sunnyvale, CA) is detected with the optical beam deflection technique [22, 26]. A laser beam is focused on the reflective backside of the cantilever. The reflected beam is detected with a two-segment photodiode giving a difference signal when the laser beam moves over the photodiode due to a displacement of the tip. A feedback loop keeps the difference signal, and thus the applied force, at a preset value by moving the sample up and down with the piezo tube used for raster-scanning the sample. Data can be monitored in different modes of operation. In the height mode, the voltages applied to the z-electrode are displayed. In the error signal mode [27], movements of the cantilever, which cannot be compensated for by the feedback loop due to a limited bandwidth, are monitored. The resulting images have a shadowed appearance: bright regions correspond to the up slope of surface features and dark regions to the down slope. The images shown here are recorded in the error signal mode. When working in this mode, however, practical height information is lost. Height indications mentioned in the results section are obtained from images captured in the height mode (recorded simultaneously but not shown here).

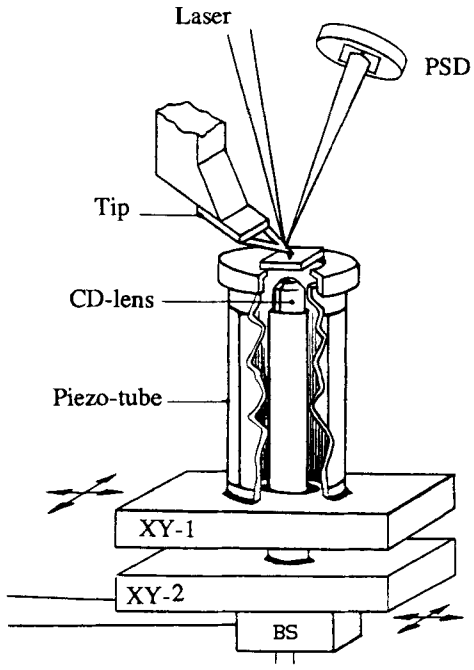

FIG. 1. Schematic representation of the AFM with integrated optical microscope. The sample is illuminated via a beam splitter (BS) and a compact disc lens (CD-lens) as an objective with light from a mercury arc lamp. The optical image, in reflection, is captured with a CCD-camera and shown on a video monitor (neither are shown). The AFM consists of a piezo tube for sample scanning and a cantilever with integrated pyramidal tip, whose displacements are detected with a laser beam reflected off the backside of the cantilever towards a photodiode (PSD). Both sample and lens can be moved using xy-stages. For more details we refer to Putman et al. [25].

The integrated optical microscope enables quick location and selection of objects. The object of interest can be positioned under the AFM tip and a high resolution image of the selected object can be obtained. The sample were imaged in air and the applied force was about 10^{-8} N, largely due to the water film on the surface [31]. The imaging time per picture (512×512 pixels) is 130 sec.

RESULTS AND DISCUSSION

Using the optical microscope, metaphase chromosomes were located and subsequently imaged with the AFM. From the metaphase spreads chromosome 1 was selected on the basis of its length and/or the visible presence (in the optical microscope) of the probe near the centromere. Figure 2A shows a human chromosome 1 which had been hybridized in situ with biotinated pUC1.77 DNA and detected with peroxidase-

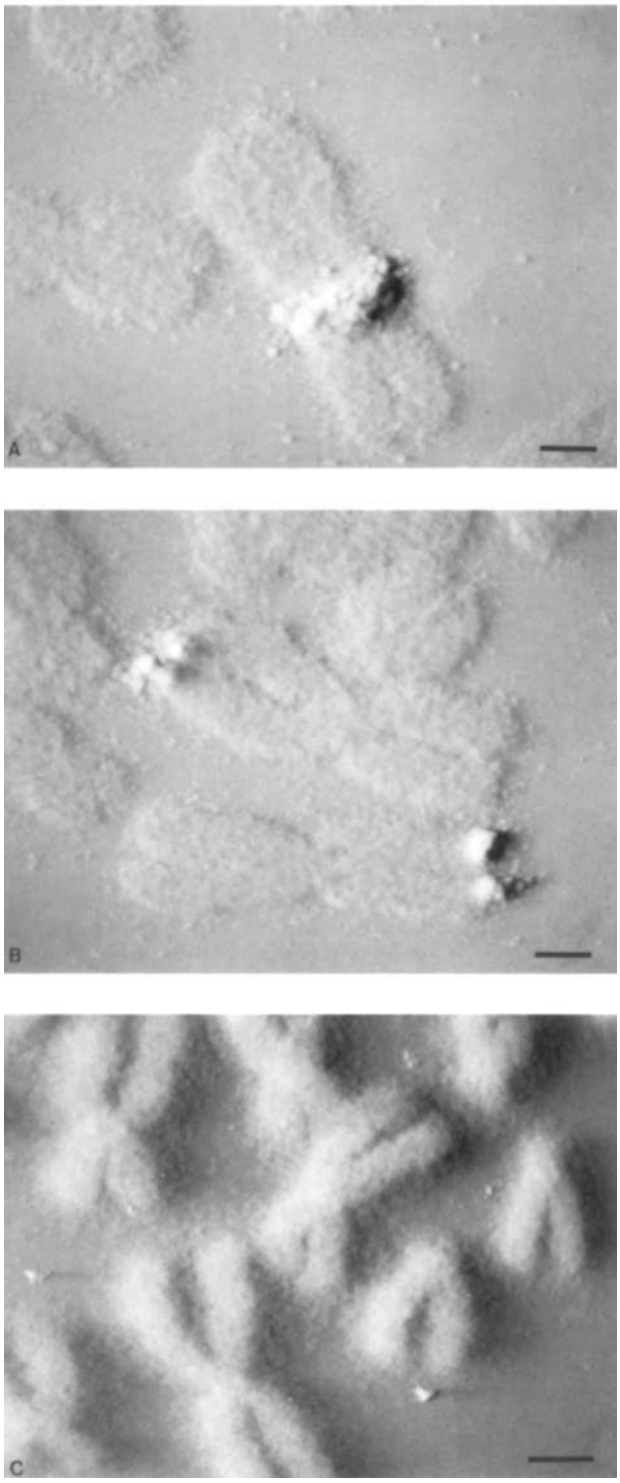

FIG. 2. AFM images of in situ hybridized human metaphase chromosomes 1 after immunocytochemical amplification with PO/DAB (DAB concentration 0.05%, duration 20 min). (**A**) DNA probe pUC1.77 near centromere, (**B**) DNA probe p1-79 at telomere of short arms, (**C**) chromosomes on a control sample. Image area: (a,b) 11 × 8 μm^2, bar: 1 μm, (c) 20 × 15 μm^2, bar: 2 μm.

conjugated avidin and the PO/DAB reaction. A clear topographic difference can be seen between the site of hybridization near the centromere and the chromosome arms. The height of the chromosome is surprisingly low: 75 nm. The corrugation on the chromosome is also small, namely, 10 nm. The difference in height between the probe linked signal and the arms of the chromosomes is 200 nm. An example from the sample hybridized with a probe for the telomere region of chromosome 1 is shown in Figure 2B, where the two homologues are incidentally present within the scanned area. The height difference between deposited material and chromosomes is 100 nm (top chromosome) and 150 nm (bottom chromosome). As a control an image of a chromosome spread is shown from a sample which has gone through the same preparation procedure as the other samples (Figure 2C), but no biotinated probe was added. No deposited material on any of these chromosomes can be seen.

Several examples of chromosomes in situ hybridized with both DNA probes are shown in Figure 3. They show large in situ hybridization signals close to the 1q12 region and smaller ones in the telomere region. In Figure 3A, a small chromosome lies adjacent to the centromere of chromosome 1. The average height difference between chromosome and the 1q12 signal is 400 nm and between chromosome and telomere signal 100 nm. The variation in the amount of deposited material can be considerable. The height at the centomere probe in Figure 3B is about 600 nm, but also heights of 300 nm have been observed (Fig. 2A). This agrees with observations by quantitative fluorescence microscopy by Nederlof et al., who showed that the fluorescence intensities of the signals could vary by a factor of 4 [23, 24]. It is reasonable to assume that this also holds for the variation in the amount of reaction product produced by the probe-linked PO/DAB reaction.

The deposited material in Figure 3B seems to have two parts with different morphology. The part indicated by the arrow seems to have a crystalline character. What one sees here, however, is not the morphology of the deposited material but of the tip. Instead that a sharp tip has imaged the sample surface, a sharp needle-like structure protruding upwards from the sample, with a smaller included angle than the pyramidal tip (72°), has imaged the tip [14]. The pyramidal shape can be clearly recognized.

In Figure 3C the diameter of the telomere signals is 200 nm. This does not imply, however, that the precision of location of the probe is limited to 200 nm; smaller features are visible. Supposing that the probe is at the center of the bump, the position of the probe can be determined with an accuracy better than 200 nm. Moreover, the precision of probe localization is directly coupled to the amount of material deposited during the PO/DAB amplification reaction, which is to some extent under experimental control.

For instance, decreasing the concentration of DAB and the duration of the amplification results in smaller

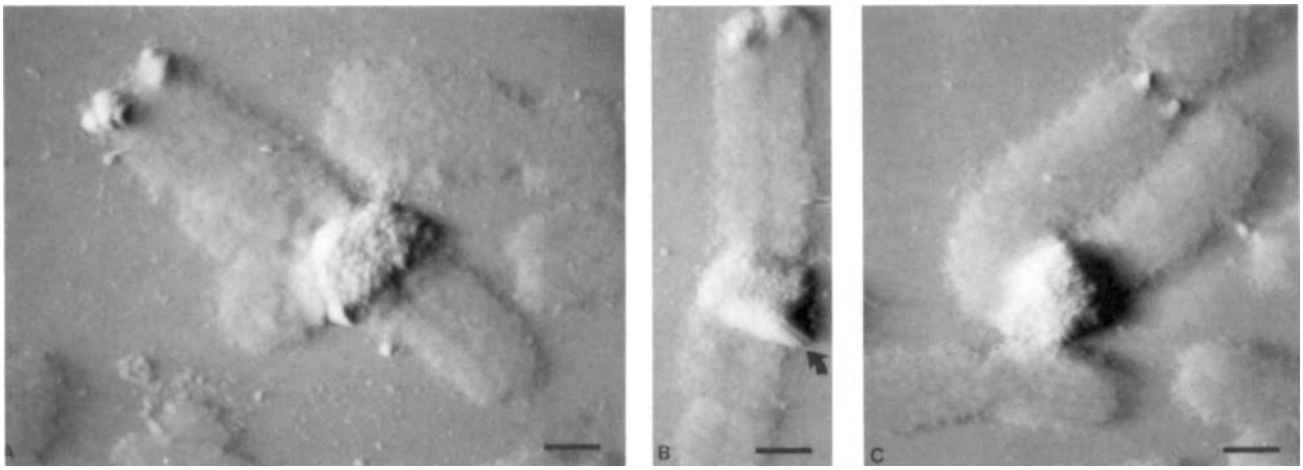

FIG. 3. AFM images of in situ hybridized human metaphase chromosomes after immunocytochemical amplification with PO/DAB (DAB concentration 0.05%, duration 20 min). Two probes have been used simultaneously: pUC1.77 and p1-79. Image area: (**A**) 11 × 8 μm^2, (**B**) 3.2 × 8 μm^2, (**C**) 7.7 × 8 μm^2, bar: 1 μm. Arrow in (B): see text.

hybridization signals. Figure 4 shows images of chromosome 1 from samples where the DAB concentration was decreased to 20% of its original value and the duration of the amplification reaction is one tenth of the time used before (Figs. 2 and 3). The arrows indicate the location of the telomere signals. The height difference between signal and chromosome varies from 50 nm (Fig. 4A) to 30 nm (Fig. 4D). The separation of the signals in Figure 4A, with diameters of 100 nm, respectively, 75 nm, is approximately 200 nm. The diameter of the right signal in Figure 4D is even smaller, 40 to 60 nm (with a height difference between probe and chromosome of less than 30 nm). Consequently, the precision of probe localization is in the same range as the diameter of the signal. It should be noted that the telomeric signals on the two chromatids do not appear to have a fixed position; a fact probably related to the chromosome preparation technique.

The results presented here clearly illustrate the potential of precise DNA probe localization with this AFM approach. It is most likely that other enzyme cytochemical detection principles are similarly applicable. The resolution obtained with the AFM is superior when compared to the resolution of light microscopy: features of 50 nm can be observed. Current lateral resolution limits on biological samples are in the 1–50 nm range, depending on the sample, the applied force and the sharpness of the AFM tip [17]. A sharper tip gives a smaller imaged diameter of the same object. In this study tips with a radius of 30 to 50 nm were used. Sharper tips with radii of 10 nm can be made by electron beam deposition [19] and new cantilevers with sharper tips and smaller included angles are becoming commercially available. In the z-direction the resolution of the AFM is far better, typically 0.1 nm. As shown, the height differences between the deposited material at the hybridization sites of p1-79 in the telomere region and the chromatids is strongly dependent on the concentration of the DAB and the duration of the amplification reaction. Bearing in mind that the corrugation on the arms of the chromosome is only 10 nm, a lower limit of 20 nm in height difference between signal and chromosome seems acceptable to discriminate the signal from the surrounding chromosomal material.

The signals in Figure 4C,D can hardly be discriminated from the surrounding chromosomal material. Only because we know, through the presence of the centomere probe and for its morphology as seen in the light microscope, the images are of chromosome 1, it seems plausible that the structures indicated by the arrows in Figure 4 are the actual probe-linked signals.

The presented results and arguments point into the direction of a signal detection with a precision of at least 200 nm and possibly 50 nm, giving a significant increase of the precision of probe localization over current optical techniques. The recent detection of immunogold labels (diameter 30 nm), with and without silver enhancement, on the surface of T-lymphocytes with the AFM [28], supports this prediction. Clearly, as probes get smaller, discrimination between probe and surrounding biological material becomes more difficult (see Fig. 4D). Owing to convolution between surface and tip the resulting AFM image may be dominated by the shape of the tip: sphere-like structures on the chromosome cannot be distinguished from the probes. The limitation of AFM for the detection of morphological signals linked to in situ hybridized DNAs is that the AFM (operated in the repulsive force mode used here) is essentially a surface technique. This may imply that no information can be obtained from regions within the chromosome as in fluorescence microscopy. It is a mat-

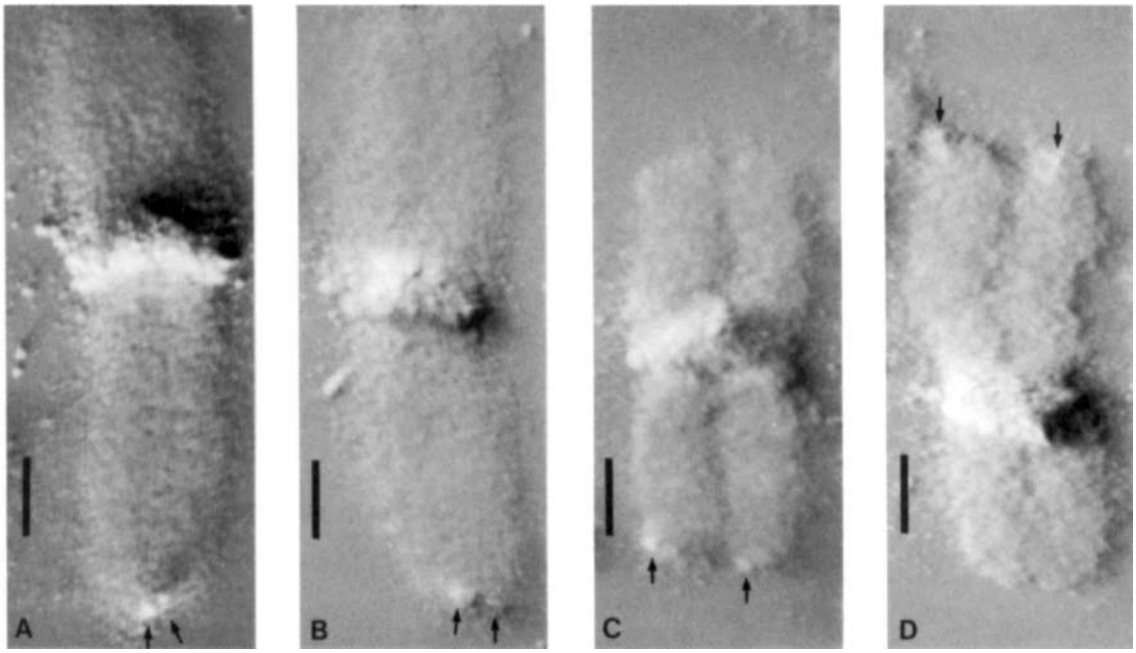

FIG. 4. AFM images of in situ hybridized human metaphase chromosomes prepared as in Figure 3 but the DAB concentration has been reduced to 0.01% and the duration of the amplification reaction limited to 2 min. Image area: (**A–D**) 3.2 × 8 μm^2, bar: 1 μm. Arrows indicate the position of the p1-79 probes at the telomere.

ter of further research in this field to analyze whether the AFM technique used here only reports surface hybridization. Also it is obvious that further research is necessary to see what value it has for chromosomal mapping of genomic DNA probes such as cosmids and yeast artificial chromosomes. Possibly, the method can be of value in lateral ordering of DNA on chromosomes [2]. Finally, it would be of considerable interest to apply combined LM/AFM to chromatin preparations of which DNA has been decondensed to the level of the Watson-Crick double helix [30, 33].

In conclusion, these first results indicate that AFM detection of DNA probes as presented here holds great promise for high resolution in situ hybridization.

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